AEGILOPS: PROMISING GENESOURCES TO IMPROVE AGRONOMICAL AND QUALITY TRAITS OF WHEAT

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PUBLISHED IN: Frontiers in Plant Science

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88963-979-3
DOI 10.3389/978-2-88963-979-3

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AEGILOPS: PROMISING GENESOURCES TO IMPROVE AGRONOMICAL AND QUALITY TRAITS OF WHEAT

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Citation: Shewry, P., Rakszegi, M., Molnár, I., Darko, E., Tiwari, V. K., eds. (2020). Aegilops: Promising Genesources to Improve Agronomical and Quality Traits of Wheat. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-979-3

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frontiers in Plant Science

EDITORIAL
published: 14 July 2020
doi: 10.3389/fpls.2020.01060

Editorial: *Aegilops*: Promising Genesources to Improve Agronomical and Quality Traits of Wheat

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Keywords: ***Aegilops*** **sp., stress tolerance, quality traits, genome analysis, alien introgression**

Editorial on the Research Topic

Aegilops: Promising Genesources to Improve Agronomical and Quality Traits of Wheat

INTRODUCTION OF AEGILOPS

Aegilops species have contributed significantly to wheat improvement despite the challenges in exploiting wild species, such as crossability and incompatibility (Börner et al., 2015; Fedak, 2015). They have been used in particular as sources of genes conferring resistance to biotic stresses, but also for more complex traits such as abiotic stress and yield.

The genus *Aegilops* consists of 22 species with the C, D, M, N, S, T and U genomes, which have high allelic diversity relative to wheat. *Aegilops tauschii*, the D-genome donor of bread wheat, has been most widely used for wheat breeding, followed by *A. speltoides* and *A. ventricosa*. However, because most *Aegilops* species are in the secondary and tertiary gene pools of wheat they are difficult to utilize due to recombination barriers and useful variation from these species is only available in the form of translocation/introgression lines.

IDENTIFICATION OF DIVERSITY IN TRAITS FOR WHEAT IMPROVEMENT

As sources of tolerance to **biotic stresses**, 20% of the total number (over 75) resistance gene loci identified in cereals are present in *Aegilop*s species (Ponce-Molina et al., 2018). These include two thirds of the 54 loci for resistance to powdery mildew (Tang et al., 2018), and the 12 resistance loci for Cereal Cyst Nematodes (Ali et al., 2019). In the present topic, the addition of *A.markgrafii* chromosomes to wheat increased the resistance to 19 of 20 powdery mildew isolates in addition line AV(E) (Niu et al.).

New stem rust resistance genes have also been identified in *Aegilops*, such as *Sr46*, *Sr47*, *Sr51* and *Sr53* (in *A. tauschii*, *A. triuncialis*, *A. searsii* and *A. geniculata respectively;* Liu et al., 2011a; Liu et al., 2011b; Klindworth et al., 2012; Yu et al., 2015) and three additional genes in *A. tauschii* (Rouse et al., 2011), three genes in *Ae. sharonensis* (Singh et al., 2015; Yu et al., 2017) and one gene in *A. umbellulata* (Edae et al., 2016). In addition, it has been reported that 81% of accessions of *A.*

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *21 April 2020*
Accepted: *26 June 2020*
Published: *14 July 2020*

Citation:
Rakszegi M, Molnár I, Darkó É, Tiwari VK and Shewry P (2020) Editorial: Aegilops: Promising Genesources to Improve Agronomical and Quality Traits of Wheat. Front. Plant Sci. 11:1060. doi: 10.3389/fpls.2020.01060

longissima, 94% of *A. neglecta* and 88% of *A. cylindrica* (DDCC) and *A. peregrina* (SSUU) were resistant to the Ug99 race group of the stem rust pathogen (*Puccinia graminis* f. sp. tritici) (Huang et al., 2018; Olivera et al., 2018) (Kishii). In this topic Niu et al. reported that wheat/*A.markgrafii* addition lines AII(C) and AIII (D) were resistant to Ug99. Furthermore, *A. biuncialis, A. caudata, A. comosa, A. cylindrica, A. geniculata, A. neglecta, A. peregrina, A. triuncialis, and A. umbellulata* were evaluated for resistance to three highly virulent races (TTKSK, TRTTF and TTTTF) of *P. graminis* f. sp. tritici with 60–70% exhibiting low infection types. Association analyses showed that for a given species, the resistance genes are effective against multiple races (Olivera et al., 2018).

Brisco et al. (2017) identified several *A. tauschii* accessions showing resistance to Fusarium Head Blight and studies reported in this topic (Szabo-Hever et al.) have shown that *A. tauschii* accessions decreased disease severities by 18.3%, suggesting that either the D genome or the increased ploidy level could contribute to resistance in synthetic hexaploid lines.

Aegilops species are also a resource for novel genes and alleles providing tolerance to **abiotic stresses**. In this topic Suneja et al. provide a good example of the identification of several *A. tauschii* accessions as potential donors of adaptive plasticity to stress.

Aegilops species also serve as a resource for introducing useful genetic variation in **grain processing and nutritional quality** in wheat (*Triticum aestivum*). Seed storage proteins are the major determinants of end product quality and mainly consist of glutenins and gliadins. A large number of allelic forms of these proteins have been identified in *Aegilops* species and in some *Aegilops* species such as *A. searsii, A. geniculata* and *A. longissima* this variation have been linked with improved breadmaking quality. *Aegilops* species has also been explored for diversity in the grain texture-related proteins, called puroindolins (Pins) and grain softness proteins (GSP). In particular, studies carried out in a number of countries have identified almost 100 alleles of *Pin a, Pin b* and *GSP* across 200 lines/accessions. This allelic variation could be utilized in breeding programs to extend the textural characteristics of wheat (Kumar et al.).

Aegilops has attracted further attention in relation to increasing the grain mineral content of wheat. In particular, to produce biofortified wheat with higher the contents of iron and zinc in order to alleviate deficiencies in these minerals which currently affect more than 2 billion people worldwide (Cakmak, 2017; Black et al., 2013; Velu et al., 2018b). Some *Aegilops* species have been reported to contain three to four-fold higher concentrations of Zn and Fe grain content than wheat, including *A. longissima* (Sl), *A. kotschyi* (US), *A. peregrina* (US), *A. cylindrica* (CD), *A. ventricosa* (DN) and *A. geniculata* (UM) (Rawat et al., 2009). Amphiploid lines of durum wheat with *A. longissima*, partial amphiploids of bread wheat with *Ae. kotschyi* and addition/substitution lines of bread wheat with *A. kotschyi* also showed two to three times higher concentrations of Zn and Fe in grain than the wheat checks (Tiwari et al., 2008; Tiwari et al., 2010; Rawat et al., 2011), indicating that they are promising resources to improve wheat composition. Velu et al. developed translocation lines with rye and different *Aegilops* species in a wheat genetic background to increase the Zn content. Although the potential health benefits of *Aegilops* species by increased minerals in wheat have not yet been realized, they should have an impact in the future (Kishii).

ESTABLISHMENT AND EXPLOITATION OF GENOMIC RESOURCES IN *AEGILOPS* SPP.

A **high-throughput genotyping platform** has been specifically designed for screening species related to wheat and used to screen multiple accessions representing all species in the genus *Aegilops*. This application was useful for identifying diversity and determining the relationships within and between *Aegilops* species (Przewieslik-Allen et al.). Genome adaptability to environmental changes, especially to rapid climatic fluctuations, underlies the survival and **evolution of species**. In wild species, genetic and epigenetic changes are accompanied by significant alterations in the complex nuclear repetitive DNA fraction. Perpetual intra-organismal reshuffling of repetitive DNA mirrors the structural plasticity of the *A. speltoides* genome, which is related to genetic diversity through the distribution of the species in contrasting ecogeographical environments (Pollak et al.). Ruban and Badaeva proposed a model for the **evolution of the S-genome** of *A. speltoides*. The genomes of allopolyploid wheats have evolved by different species-specific chromosome translocations, sequence amplification, and elimination and re-patterning of repetitive DNA sequences. These events occurred independently in different wheat species and in *A. speltoides*. The 5S rDNA locus of chromosome 1S was probably lost in ancient *A. speltoides* prior to formation of cultivated *Triticum timopheevi* (AAGG genomes), but after the emergence of ancient emmer (AABB genomes). rDNA profiling and distribution was used to divide diploid *Aegilops* species into two groups corresponding to the Emarginata and Truncata sub-sections. It was found that the evolution of Emarginata species was associated with an increase of C-banding and heterochromatin, amplification of Spelt-52, re-pattering of the pAesp_SAT86, and a gradual decrease in the amount of the D-genome-specific repeats pAs1, pTa-535, and pTa-s53.

A. tauschii (2n = 2x = 14, genome DD), also known as Tausch's goatgrass, is the D genome donor of hexaploid bread wheat (*T. aestivum*, 2n = 2x = 42, AABBDD genome). It is a rich source for tolerance to biotic and abiotic stresses. A **TILLING** (Targeting Induced Local Lesions In Genomes) population of *A. tauschii* (TILL-D) was developed using ethyl methanesulphonate (EMS) as a mutagen which, together with the newly published *A. tauschii* reference genome sequence, will facilitate the discovery and validation of genes for agronomically important traits and their transfer into bread wheat (Rawat et al.). Population structure analysis based on high quality SNPs confirmed the differentiation of *A. tauschii* into two lineages (L1 and L2). A **MiniCore collection** consisting of 29 L1 and 11 L2 accessions was identified based on genotypic, phenotypic and geographical

data. This captures 84% of the total allelic diversity in the whole collection, showing that it is possible to reduce the number of accessions which need to be screened by 90% (Singh et al.). A **genome wide association study (GWAS)** of the grain Fe, Zn, Cu and Mn contents also indicated that *A. tauschii* lineage 2 had higher Fe and Cu concentration than lineage 1 (Arora et al.). The associations were related to genes encoding transcription factor regulators, mineral transporters and phytosiderophore synthesis.

The **stability of translocation or alien introgression** lines is always of concern. King et al. developed homozygous wheat/*A. muticum* dihaploid introgression lines and characterized their stability using genomic *in situ* hybridization and SNP analysis (King et al.). Zhang et al. studied the **efficiency of transferring** *A. tauschii* segments to wheat using a synthetic octaploid (AABBDDDD, 2n = 8x = 56) and used bridge crosses to mapped QTL for agronomically important traits.

Wheat/*A. markgrafii* **disomic addition lines** carrying the chromosomes B, C, D, E, F and G, respectively, were screened with SSR markers showing that they corresponded to wheat homoeologous groups 2, 5, 6, 7, 3, and 4, respectively. **Useful markers** were also identified for chromosome engineering of wheat (Niu et al.).

The papers brought together in this topic therefore illustrate the range of current research on the charcterisation of *Aegilops* species and identification of important traits for exploitation in wheat improvement.

AUTHOR CONTRIBUTIONS

MR, PS, VT, ÉD, and IM wrote this manuscript.

FUNDING

The topic is based upon the workplan of the COST Action 18101 SOURDOMICS—Sourdough biotechnology network towards novel, healthier and sustainable food and bioprocesses, where the members of the working group 1 were supported by COST (European Cooperation in Science and Technology). COST is a funding agency for research and innovation networks.

ACKNOWLEDGMENTS

We are grateful to all authors that contributed to this e-book.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 03 August 2018
doi: 10.3389/fpls.2018.01113

Development and Utilization of Introgression Lines Using Synthetic Octaploid Wheat (*Aegilops tauschii* × Hexaploid Wheat) as Donor

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Reviewed by:
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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *06 April 2018*
Accepted: *10 July 2018*
Published: *03 August 2018*

Citation:
Zhang D, Zhou Y, Zhao X, Lv L, Zhang C, Li J, Sun G, Li S and Song C (2018) Development and Utilization of Introgression Lines Using Synthetic Octaploid Wheat (Aegilops tauschii × Hexaploid Wheat) as Donor. Front. Plant Sci. 9:1113. doi: 10.3389/fpls.2018.01113

As the diploid progenitor of common wheat, *Aegilops tauschii* Cosson (DD, 2*n* = 2x = 14) is considered to be a promising genetic resource for the improvement of common wheat. In this work, we demonstrated that the efficiency of transferring *A. tauschii* segments to common wheat was clearly improved through the use of synthetic octaploid wheat (AABBDDDD, 2*n* = 8x = 56) as a "bridge." The synthetic octaploid was obtained by chromosome doubling of hybrid F_1 (*A. tauschii* T015 × common wheat Zhoumai 18). A set of introgression lines (BC_1F_8) containing 6016 *A. tauschii* segments was developed and displayed significant phenotype variance among lines. Twelve agronomic traits, including growth duration, panicle traits, grain traits, and plant height (PH), were evaluated. And transgressive segregation was identified in partial lines. Additionally, better agronomic traits could be observed in some lines, compared to the recurrent parent Zhoumai 18. To verify that the significant variance of those agronomic traits was supposedly controlled by *A. tauschii* segments, 14 quantitative trait loci (QTLs) for three important agronomic traits (thousand kernel weight, spike length, and PH) were further located in the two environments (Huixian and Zhongmou), indicating the introgression of favorable alleles from *A. tauschii* into common wheat. This study provides an ameliorated strategy to improve common wheat utilizing a single *A. tauschii* genome.

Keywords: wheat, *Aegilops tauschii*, quantitative trait loci, agronomic traits, introgression lines

INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most important cereal crops, accounting for 20% of the calories consumed by humans (Brenchley et al., 2012). Based on hybridization among varieties, many wheat varieties have now been bred through modern cultivation procedures and it should be noted that the process of wheat breeding has been greatly accelerated by the utilization of core collection in China. However, the genetic background of wheat varieties is becoming increasingly consistent, due to their derivation from only a few core collections (Tian et al., 2005; Hao et al., 2006; Xiao et al., 2012), which is currently leading to an increasingly severe risk of abiotic and biotic stress. It has long been realized that the exploration and utilization of desirable genes from wild relatives is an effective approach to improving the genetic background of common wheat

(Fu and Somers, 2009; Nevo, 2014). To date, this strategy has been used to transfer many alien genes/QTLs from wild relatives into fine cultivars, and 1BL/1RS is regarded as the most successful alien introgression in wheat-breeding programs (Lukaszewski, 1990, 2000; Jiang et al., 1993; Ren et al., 2009; Gill et al., 2011; Qi et al., 2011). The 1RS arm in translocation lines could not only compensate for the loss of the relevant wheat arms 1BS, but also confer positive heterotic effect to grain yield. In addition, many other wild relatives, including 6VS of *Dasypyrum villosum* (Chen et al., 2013), 2S of *Aegilops speltoides* (Klindworth et al., 2012), 7Ag of *Thinopyrum ponticum* (Niu et al., 2014), and 6P of *Agropyron cristatum* (Luan et al., 2010; Ye et al., 2015; Zhang et al., 2015), have also been further utilized for the improvement of common wheat.

Aegilops tauschii Cosson (DD, 2*n* = 2x = 14) is an annual, self-pollinated plant with a high level of genetic variability for disease resistance, productivity traits, and abiotic stress resistance (Singh et al., 2012). It is naturally distributed in central Eurasia, spreading from northern Syria and Turkey to western China. In China, it is mainly distributed in the Yili area of Xinjiang and the middle reaches of the Yellow River (including Shanxi and Henan provinces; Wei et al., 2008). Concerning its genetic background, *A. tauschii* can be subdivided into two phylogenetic lineages, designated as L1 and L2, which are broadly affiliated with *A. tauschii* ssp. *tauschii* and *A. tauschii* ssp. *strangulata*, respectively (Dvorak et al., 1998; Mizuno et al., 2010; Wang et al., 2013). Most of the exploited *A. tauschii* is generally derived from Transcaucasus and northern Iran, since it is believed that the *A. tauschii* in these regions (mainly from the L2 lineage) is involved in the origin of wheat D genome (Wang et al., 2013). By contrast, little is known about the genetic and phenotypic characteristics of *A. tauschii* (mainly L1 lineage) from the eastern and southern populations (i.e., those from Syria, Afghanistan, Pakistan, Central Asia, and China) (Matsuoka et al., 2009). Owing to the long genetic distance between L1 and L2, it is therefore believed that the genetic variation type of *A. tauschii* (L1 lineage) is more abundant than that of the wheat D genome (Lubbers et al., 1991; Dvorak et al., 1998, 2012; Wang et al., 2013). Therefore, like many wild crop progenitors, *A. tauschii* is considered to be a promising gene donor for the improvement of common wheat (Kilian et al., 2011).

As the diploid progenitor of common wheat, it is convenient to transfer *A. tauschii* genes into common wheat via recombination between homologous chromosomes. In addition, it is also possible that undesirable gene linkages can be easily broken by repeated backcrossing with common wheat (Gill and Raupp, 1987). To date, synthetic hexaploid wheat (tetraploid wheat × *A. tauschii*) has mainly been exploited as a "bridge" for transferring some superior genes of *A. tauschii* into common wheat (Miranda et al., 2007). Many previous researchers have identified and located numerous QTLs from synthetic hexaploid wheat with some of the QTLs being located on the D genome through advanced backcross population or introgression lines (ILs; Pestsova et al., 2006; Kunert et al., 2007; Naz et al., 2008; Yu et al., 2014). In addition, the desirable traits of *A. tauschii* may also be transferred to common wheat through direct crossing. Gill and Raupp (1987) proposed the first systematic direct gene transfer protocol. Wheat genomes A, B, and D could be improved concurrently through the hybridization of synthetic hexaploid wheat with common wheat. In comparison, unique advantages have been found in the hybridization of *A. tauschii* with common wheat, because this provides a strategy to transfer desired D genome regions (carrying target alleles) without disrupting adaptive allelic combinations (located in the A and B genomes). However, this method has drawn little attention (Fritz et al., 1995; Cox et al., 2006; Olson et al., 2013) due to the high sterility in the hybrid F_1 generation, caused by distant hybridization and extremely low ripening rates resulting from the backcross of the hybrid F_1 with the recurrent parent.

Fortunately, the above-mentioned challenge could be overcome through the use of the synthetic octaploid wheat (AABBDDDD, 2*n* = 8x = 56), obtained by chromosome doubling of hybrid F_1 (*A. tauschii* × hexaploid wheat), although this has seldom been reported in the literature. In addition, *A. tauschii* from the same region has been generally regarded as more suitable for hybridization with common wheat, compared to strains from other areas, due to its broad ecological adaptation to the native area (Matsuoka et al., 2009). In this work, a series of ILs (BC_1F_8) was developed through the media of synthetic octaploid wheat, obtained by direct crossing of common wheat and *A. tauschii* from the same region in China. Various agronomic traits of these ILs were extensively investigated and analyzed. In addition, 14 major QTLs for three important agronomic traits, which were derived from *A. tauschii*, were successfully identified in the two environments.

MATERIALS AND METHODS

Plant Materials

The diploid *A. tauschii* ssp. *tauschii* accession T015 (2*n* = 14, DD) was originally derived from Henan province. Zhoumai 18 (2*n* = 42, AABBDD), a type of control variety of cultivar registered in Henan province, was applied as the recurrent parent in this study.

Production of F_1 Hybrids Between Common Wheat and *A. tauschii*

Based on the traditional breeding method, *A. tauschii* accession T015 and Zhoumai 18 were directly crossed and the hybrid F_1 seeds were taken away 16 days after pollination. The method of embryo removal was reported by Sirkka and Immonen (1993). Seeds were surface sterilized for 8 min with 0.1% $HgCl_2$ and rinsed three times in 20 mL ddH_20. All handling of seeds and embryos was undertaken under sterile conditions in a laminar flow hood. Embryos were removed from the seeds and transferred to the endosperm of barley; the barley embryos were removed and the scutellums of the hybrid embryos were put in their place. An embryo culture media was used containing

a mixture of 4.1 g/L Murashige and Skoog salts (Murashige and Skoog, 1962) with 3% sucrose and no hormone at pH 5.8. The hybrid embryos were incubated in darkness at 25°C for 2 weeks and developed etiolated seedlings with roots, and then the hybrid seedlings were cultivated at 21°C in a 16 h photoperiod (50 $\mu mol/m^2 \cdot s^1$, fluorescent light) over the summer.

Chromosome Doubling Treatment and Population Construction

The method of chromosome doubling was reported by Taira et al. (1991). The hybrid F_1 seedlings were transferred to the greenhouse in September and were grown for 8 weeks at 21 ± 4°C with 10 h of supplemental light. The F_1 plantlets with well-formed tillers were uprooted from the soil and divided into two parts. One part was replanted as a control without treatment, and the other part was washed in running water. The roots of each plant were then cut back to a 4–5 cm length and immersed in beakers containing a 0.05% (*w/v*) colchicine solution of pH 7.0, supplemented with a 1.5% (*v/v*) solution of dimethyl sulfoxide (DMSO). Treatments were conducted for a 16 h period at room temperature. After the treatment, the roots were thoroughly washed in running water for 24 h. All the plants were transplanted into a greenhouse until flowering and seed formation.

The following year, emasculated florets of Zhoumai 18 were pollinated by synthetic octaploid wheat to produce 10 BC_1F_1 seeds. Afterward, the entire BC_1F_1 seeds were cultivated and self-fertilized to acquire BC_1F_2 generation. About 400 seeds of BC_1F_2 were randomly selected followed by further successive self-fertilization for six times to generate a BC_1F_8 population (**Figure 1**), in which 379 plants were randomly selected for genotyping and phenotyping in the present study. This population and Zhoumai 18 were cultivated in the 2015–2016 crop season, on the wheat breeding farms of the Huixian and Zhongmou, respectively. Seeds were sown at a distance of 10 cm between plants, and a 30 cm gap between rows, and were grown under consistent field conditions. The recurrent parent Zhoumai 18 was planted as a control.

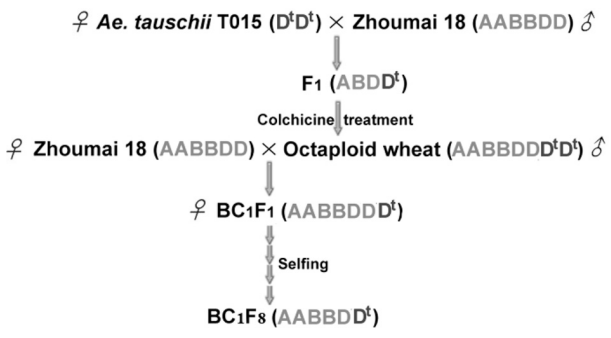

FIGURE 1 | Development of introgression lines by transferring chromosome segments from *A. tauschii* T015 into the wheat cultivar Zhoumai 18. D^t highlighted in blue designates the genome of *A. tauschii*.

Chromosome Karyotype and FISH of Synthetic Octaploid Wheat

The seeds of synthetic octaploid wheat were germinated at 25°C for 2–3 days. About 2 cm long root tips were treated for karyotyping chromosome preparation. Chromosome preparation and FISH were performed according to the method described by Andres and Kuraparthy (2013). The synthetic oligonucleotides pAs-1 and pSc119.2-1 were marked by 6-carboxytetramethylrhodamine (Tamra) and Alexa Fluor-488-dUTP, respectively (Tang et al., 2014). For sample examination, a drop of pre-mixed DAPI solution (Sangon Biotech, Shanghai, China) was deposited on each slide, and chromosomes were observed by an Olympus BX63 fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Investigation of Agronomic Traits

Twelve agronomic traits, including days to heading (DH), days to flowering (DF), plant height (PH), spike length (SL), spikelets (SPI), spikelet density (SD), grain number main spike (GNS), thousand kernel weight (TKW), grain length (GL), grain width (GW), grain perimeter (GP), and grain length/grain width (GL/GW), were scored by the method described in Li and Li (2006). PH was recorded just before harvest. DH and DF were noted in the field. After harvest, GNS, SL, and SPI were determined from three main spikes per line, while TGW, GL, GW, and GP were determined from three to five plants.

Map Construction and QTL Analysis

DNA was extracted from the fresh leaves of ILs and Zhoumai 18 in 2014 using the method described by Olson et al. (2013). The genetic map was constructed based on the physical positions of simple sequence repeat (SSR) markers from wheat D genome[1]. PCR reactions for SSR were performed using the method described by Röder et al. (1998). SSR markers were anchored and grouped into the seven *A. tauschii* chromosomes through sequence alignment between the primers and reference genome (AL8/78 accession; Zhao et al., 2017). The calculation of segment lengths and genome ratios followed the method described by Liu et al. (2006). The QTLs for agronomic traits were identified using QTL IciMapping Ver 4.0 (Meng et al., 2015). RSTEP-LRT-ADD mapping (stepwise regression-based likelihood ratio test for additive QTL) was adopted and a significant threshold of likelihood of odds (LOD) was estimated by running 1000 permutations with a type I error of 0.05.

Statistical Analysis

All statistical analyses were performed on IBM® statistics 19 (SPSS Inc.), including frequency distribution, correlation coefficient (Pearson correlation), and analysis of variance (ANOVA). ANOVA-general linear model (GLM) was performed to determine the significance of differences between the genotypes of the lines and environments. Genotype-by-environment (G × E) interactions were also analyzed using ANOVA-GLM.

[1]http://wheat.pw.usda.gov/cgi-bin/GG3/

RESULTS

Development of Introgression Lines Through Synthetic Octaploid Wheat

The ripening rates of reciprocal crosses exhibited significant differences utilizing *A. tauschii* T015 and Zhoumai 18 as parents (**Table 1**). Altogether 73 caryopses were obtained by pollinating 118 emasculated florets of *A. tauschii* T015, with a ripening rate of 61.9%. In contrast, no caryopses were obtained by pollinating 212 emasculated florets of Zhoumai 18. Caryopses collected 16 days after pollination were dissected, and not all of them were found to contain normal embryos (well-developed primordium and scutellum), and about 37.0% contained embryos. Moreover, the embryos were always found floating in a watery endosperm. The normal embryos on the endosperm of barley could germinate and grow into seedlings (**Figure 2A**). Some of the normally developed seedlings were backcrossed with Zhoumai 18 as the female parent, without obtaining any seed. The other seedlings were treated via colchicine to generate amphidiploid seeds (**Figure 2B**). Though these seeds were not full, they could grow normally, exhibiting a chromosome number of 56 in their root tip cells (**Figure 2C**). Except for the prominent characteristics of *A. tauschii* in glume color and hardness, the developed synthetic octaploid wheat showed an analogous phenotype with its male parent (**Figure 2D**). In total, 10 BC_1F_1 seeds were obtained through pollinating 16 emasculated florets of Zhoumai 18 with synthetic octaploid wheat as the male parent. Afterward, these BC_1F_1 plants successively self-fertilized for eight generations to generate 379 ILs (BC_1F_8), in which their phenotypic traits were stabilized after several generations, with no phenotype segregation found in each line, implying the cytogenetical stability of these lines. Furthermore, the chromosome karyotypes of the root tip cell were observed in four selected lines with good agricultural traits, and the number of chromosome in each line was determined to be 42 (**Supplementary Figure S1**).

Numbers and Positions of Introgressed *A. tauschii* Segments

To identify the distribution of chromosome segments from *A. tauschii* in the wheat D genome, 379 BC_1F_8 lines were successfully genotyped using SSR markers. Altogether 261 SSR markers were selected to construct a genetic map from the GrainGene 2.0 database. Polymorphism was detected in 130 SSR markers between *A. tauschii* T015 and Zhoumai 18, and 62 of these were established to be polymorphic in ILs, accounting for 47.7%. The numbers of polymorphic markers on each chromosome were found rather even, with an average value of 8.9 per chromosome. Excluding three unidentified markers, a physical map was constructed based on the 127 polymorphic SSR markers between parents, which displayed heterogeneous distribution on seven linkage groups of D genome, with a total length of 3954.48 Mb (**Figure 3**). The physical map illustrates that these polymorphic markers in different chromosomes, or different chromosome regions, exhibit uneven distribution. For example, some markers are concentrated in the same region with a minimum gap of only 0.11 Mb. However, huge distances were also found for some other markers. For instance, the distance between *Xgwm157* and *Xgwm30.1* on chromosome 2D was determined to be 307.9 Mb.

Since each line may contain more than one chromosome segment, altogether 6016 segments from *A. tauschii* were determined in ILs. Specifically, these ILs contained 5120 homozygous and 896 heterozygous segment (**Supplementary Table S4**), with an average of 13.51 homozygous and 2.37 heterozygous segments in each line. The number of segments ranged from 1 to 25 in each line, and only a single introgressed segment was observed in one line. Using the physical positions of the SSR markers, the size of each introgressed segment, the number of unique segment, and the ratios accounting for the whole donor genome were estimated (**Table 2**). The sizes of the introgressed segments ranged from 1.3 to 238.9 Mb, with an average size of 33.45 Mb in homozygous and 31.46 Mb in heterozygous segments. In addition, the distribution of chromosome segments from *A. tauschii* exhibited clear differences in the wheat D genome, and *A. tauschii* segments in each line were counted and graphed in **Supplementary Table S1** and **Supplementary Figure S2**. Typically, the introgression fragments from 1D of *A. tauschii* showed the least 651 fragments, only accounting for 10.8%, and those from 4D of *A. tauschii* possessed the most 1086 fragments, accounting for 18.5%. These results clearly reveal that the chromosome segments of *A. tauschii* have been transferred into common wheat by the "bridge" of synthetic octaploid wheat, which effectively broadens the genetic basis of common wheat.

TABLE 1 | Crossing/backcrossing outcomes for *A. tauschii*/SOW × *T. aestivum*.

	Cross patterns			
	T015 × Zhoumai18	**Zhoumai18 × T015**	**Backcross of hybrid F1 with Zhoumai18 (♂)**	**Backcross of SOW with Zhoumai18 (♀)**
No. of florets pollinated	118	212	224	16
No. of caryopses formed	73	0	–	–
No. of embryos formed	27	0	–	–
No. of crossed seeds formed	–	–	0	10

SOW, synthetic octaploid wheat.

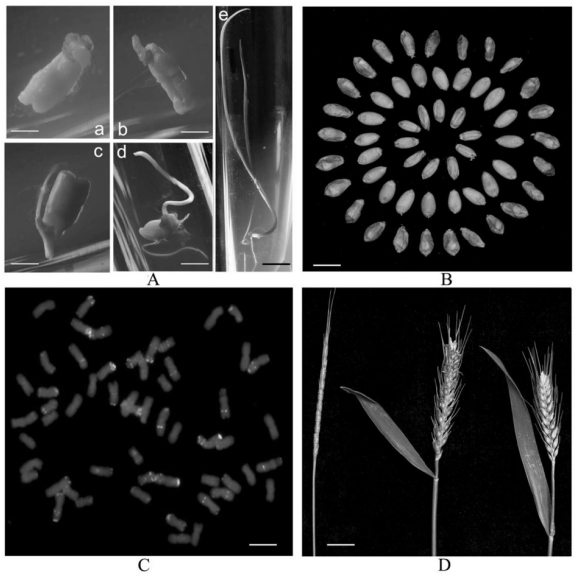

FIGURE 2 | The phenotype characteristics in the process of the development of hybrid F_1 and synthetic octaploid wheat. **(A)** Embryo culture, (a) to culture 2 days in darkness. Scale bars = 3 mm; (b) to culture 4 days in darkness. Scale bars = 3 mm; (c) to culture 8 days in darkness. Scale bars = 3 mm; (d) to culture 12 days in darkness. Scale bars = 3 mm; and (e) the hybrid seedling in light treatment. Scale bars = 2 cm. **(B)** Seeds of synthetic octaploid wheat and parents, outer cycle: seeds of synthetic octaploid wheat; middle cycle: Zhoumai 18; inner cycle: *A. tauschii* T015. Scale bars = 7 mm. **(C)** chromosome karyotype of synthetic octaploid wheat. Scale bars = 10 μm. **(D)** Spike characteristic of synthetic octaploid wheat and parents, left: *A. tauschii* T015; right: Zhoumai 18. Scale bars = 3 cm.

Phenotypic Variation of Introgression Lines

Some typical traits of *A. tauschii* could be observed in partial lines of ILs. For instance, the glume of some lines exhibited enhanced hardness and deepened color. Consequently, owing to the hardened glume, the spike threshing became difficult with enhanced pre-harvest sprouting resistance. As listed in **Table 3**, significant differences in many agronomic traits could be found among lines, including growth duration, panicle traits, grain traits, and PH (**Figure 4**). In addition, some lines showed apparent transgressive segregation. All the phenotype frequencies were normally distributed in the Huixian and Zhongmou environments (**Supplementary Figures S3, S4**), demonstrating a skewness range of −0.18∼0.72. PH showed the highest degree of variation in the ILs. The ranges of variation of PH in Huixian and Zhongmou were found to be 53.60–118.63 and 46.65–113.45 cm, with SD values of 11.76 and 11.89, respectively. TKW demonstrated the highest degree of variation in ILs, compared with other grain traits, and many lines with prominently increased TKW values appeared. For Huixian and Zhongmou, 34 and 24 lines presented more than 10% increased TKW than Zhoumai 18, respectively. The panicle traits, mainly consisting of SL, SPI, SD, and GNS, also exhibited significant differences among ILs, with the highest degree of variation found for the GNS. In the Huixian and Zhongmou observations, the variation regions of GNS were determined to be 32.30–73.50 and 34.75–78.00, respectively, with SD values of 6.39 and 7.29, respectively.

To detect the factors causing significant changes from the phenotypes described above, an ANOVA analysis of genotype, environment, and their interactions was conducted (**Table 4**). Significant differences between genotypes were found for all 12 traits investigated. The *F*-value ranged from 4.72 for GNS to 117.52 for DH. The environment had a large influence on all 12 traits. In particular, DH and DF were the traits most significantly influenced by environment since the cultivation time was not synchronized between the two environments. Significant differences of G × E interaction were observed for the other 11 traits, except for SD, indicating obvious interaction between genotypes and their environment.

Correlation Analysis Among Phenotypic Traits

Genetic correlations were calculated among lines for the agronomic traits in the population (**Supplementary Tables S2, S3**). In Huixian, the two traits of DH and DF showed significant positive correlation with each other ($r = 0.860$, $p < 0.01$), and were also positively correlated with SL and SPI. Meanwhile, a negative correlation was found between these two traits and PH, TKW, GNS, and SD. Among the panicle traits, SL and SPI displayed a positive correlation ($r = 0.158$, $p < 0.01$), and SD was observed to be negatively correlated with the former trait ($r = -0.843$, $p < 0.01$). As for the grain traits, TKW demonstrated a positive correlation with GL, GW, GP, and GL/GW. Concerning the trait of PH, it was found to be negatively correlated with GNS, SPI, and SD, but positively correlated with SL, TKW, GL, GW, GP, and GL/GW. Observations from the Zhongmou environment showed analogous correlations to those in Huixian, with the exception of a positive correlation between growth duration and GNS ($r = 0.114$, $p < 0.01$), and the negative correlation between TKW and GL/GW.

QTL Analysis of Partial Agronomic Traits in Introgression Lines

To elucidate the significant changes in the 12 traits mentioned above, supposedly controlled by *A. tauschii* segments, QTLs for three important agronomic traits (TKW, SL, and PH) of them were further identified (**Table 5**). The TKW is an important factor affecting yield. Three major QTLs for TKW, designated *QTKW.At-2D*, *QTKW.At-4D*, and *QTKW.At-6D*, were detected on the chromosomes 2D, 4D, and 6D, based on ICIM analysis, respectively (**Figure 5**), and the *QTKW.At-2D* could be detected in both the Huixian and Zhongmou areas. As clearly shown in **Table 5**, the positive alleles of additive effect were derived from *A. tauschii*, further revealing the huge value of genes from *A. tauschii* as a wild wheat resource (Singh et al., 2012). The *QTKW.At-2D* displayed the similar phenotypic variance values (PVEs) of 9.24 and 9.19% in Huixian and Zhongmou, corresponding to the additive effect of the values 1.22 and 1.35 g.

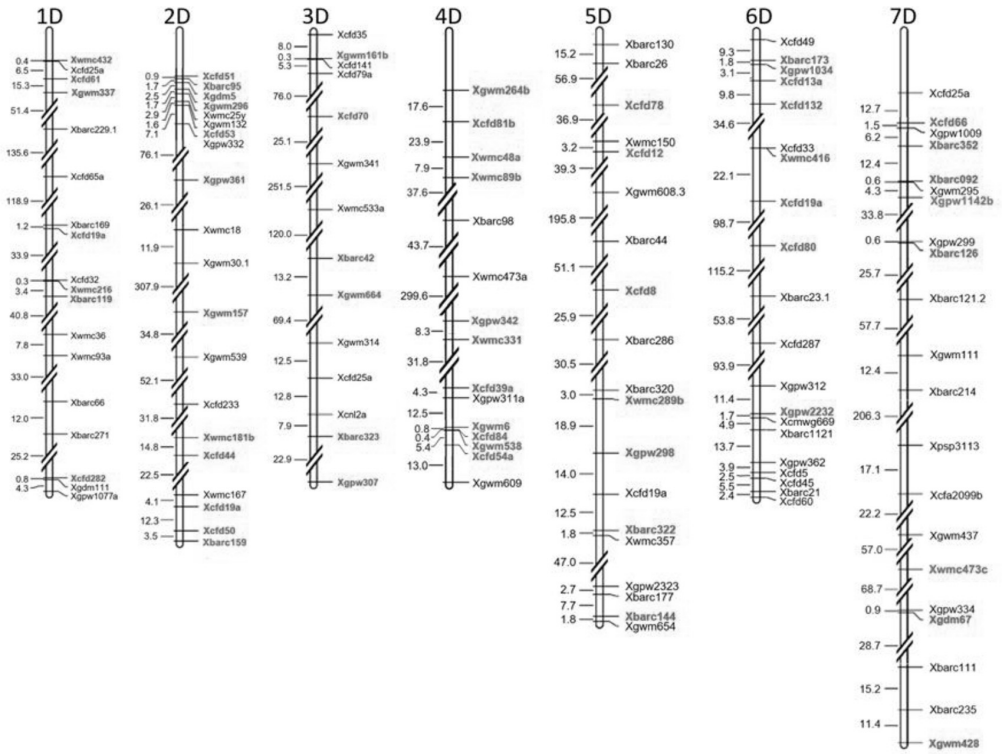

FIGURE 3 | Physical map constructed based on the 127 polymorphic SSR markers between parents. Polymorphic markers in the advanced backcross population are highlighted in red. The unit of distance is megabasepairs (Mb).

TABLE 2 | The size of introgressed segments detected in the ILs and cumulative proportion in the donor genome.

Chr.			Homozygous segments		Heterozygous segments		Maximum chromosome coverage (%)
	Polymorphic markers	Unique segments	No. of segments	Average length (Mb)	No. of segments	Average length (Mb)	
1D	7	9	981	17.53	105	12.48	20.44
2D	12	20	560	19.87	502	12.14	47.55
3D	7	11	665	42.16	49	27.89	30.38
4D	11	21	914	58.64	65	71.92	55.52
5D	8	8	733	20.11	64	28.62	27.91
6D	9	16	619	59.86	32	50.26	48.05
7D	8	9	648	15.98	79	16.93	23.75
Total	62	94	5120	33.45	896	31.46	36.23

Spike length is one of the significant spike traits for the improvement of common wheat. Altogether six major QTLs for SL, designated *QSL.At-2D.1*, *QSL.At-2D.2*, *QSL.At-3D*, *QSL.At-4D*, *QSL.At-5D*, and *QSL.At-7D* were detected in Huixian and Zhongmou (**Figure 5**), and *QSL.At-2D.1*, *QSL.At-2D.2*, *QSL.At-5D*, and *QSL.At-7D* were detected in both locations. *QSL.At-3D* was only detected in Zhongmou, whereas *QSL.At-4D* was observed in Huixian. Among these major QTLs, the PVEs of *QSL.At-2D.1* on chromosome 2D were the highest, and could explain 12.88 and 8.04% of the phenotypic variance in Huixian and Zhongmou corresponding to the additive effect of the values 0.35 and 0.30 cm.

The PH is also an important agronomic trait, and four major QTLs for PH, designated as *QPH.At-2D*, *QPH.At-3D*, *QPH.At-4D*, and *QPH.At-5D* hereafter (**Figure 5**), were observed in both Huixian and Zhongmou. The other QTL of *QPH.At-1D* was only detected in Huixian. Among them, the *QPH.At-4D* on chromosome 4D provided the highest explanation for the phenotypic variances in Huixian and Zhongmou, 27.55 and 17.22%, respectively. Moreover, the PVEs of *QPH.At-2D* and *QPH.At-5D* were also relatively high in both places, and could explain 13.95 and 8.92% of the mean phenotypic variance, corresponding to the mean additive effect of the values of 4.33 and 4.12 cm, respectively.

TABLE 3 | Twelve agronomic traits measured from the recurrent parents and the introgression lines in Huixian and Zhongmou.

Traits	Location	Parent	Introgression lines					
		Zhoumai 18	Mean	SD	Min–Max	C.V.(%)	Skewness	Kurtosis
DH	ZM	195.00	197.37	2.50	191.00–206.33	1.26	0.12	0.04
	HX	187.56	188.20	1.78	180.00–194.00	0.95	−0.15	1.32
DF	ZM	197.88	200.89	2.27	195.75–208.50	1.13	0.22	−0.12
	HX	192.72	193.94	1.82	189.00–199.00	0.94	0.22	−0.15
SL	ZM	9.27	9.98	1.06	6.95–13.58	10.60	0.15	0.44
	HX	8.47	9.77	0.97	7.10–12.87	9.91	0.26	0.35
SPI	ZM	23.25	21.95	1.25	18.50–26.00	5.70	0.17	0.51
	HX	21.02	21.56	1.20	18.00–25.33	5.55	0.07	0.37
GNS	ZM	59.33	54.40	7.29	34.75–78.00	13.40	0.19	−0.003
	HX	55.33	53.10	6.39	32.30–73.50	12.03	0.09	0.13
SD	ZM	24.73	22.24	2.65	15.84–32.37	11.93	0.54	0.71
	HX	26.14	22.26	2.38	16.26–33.02	10.71	0.54	0.93
PH	ZM	76.55	75.24	11.89	46.65–113.45	15.81	0.40	0.19
	HX	78.86	77.19	11.76	53.60–118.63	15.24	0.72	0.65
TKW	ZM	49.54	47.99	4.42	33.81–60.96	9.22	−0.13	0.005
	HX	48.63	48.27	4.01	37.48–59.02	8.31	0.08	−0.16
GL	ZM	5.96	6.58	0.38	5.51–7.55	5.82	−0.10	−0.21
	HX	5.98	6.44	0.40	5.46–7.41	6.27	−0.14	−0.48
GW	ZM	3.18	3.37	0.20	2.89–3.96	5.96	−0.004	−0.28
	HX	3.27	3.28	0.20	2.80–3.78	5.97	−0.05	−0.75
GP	ZM	15.25	16.85	0.97	14.30–19.53	5.74	−0.16	−0.32
	HX	15.46	16.44	1.03	13.98–18.73	6.29	−0.18	−0.71
GL/GW	ZM	1.89	1.97	0.09	1.71–2.25	4.62	0.11	0.09
	HX	1.84	1.98	0.09	1.70–2.28	4.71	0.19	0.49

DH, day to heading; DF, day to flowering; PH, plant height; SL, spike length; SPI, spikelets; SD, spikelet density; GNS, grain number main spike; TKW, thousand kernel weight; GL, grain length; GW, grain width; GP, grain perimeter; GL/GW, grain length/grain width; ZM, Zhongmou; HX, Huixian.

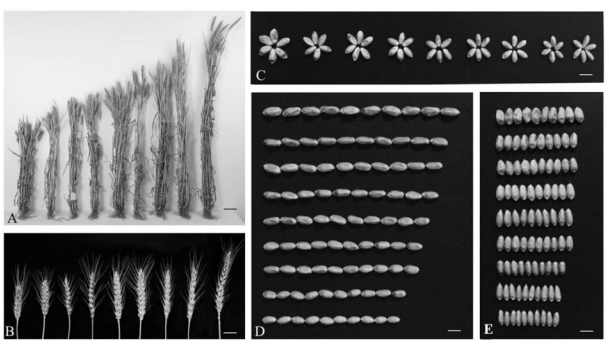

FIGURE 4 | The various phenotype traits from the introgression lines. **(A)** Plant height of partial strains. Scale bars = 8 cm. **(B)** Spike length of partial strains. Scale bars = 2 cm. **(C)** Thousand kernel weight of partial strains. Scale bars = 5 mm. **(D)** Grain length of partial strains. Scale bars = 5 mm. **(E)** Grain width of partial strains. Scale bars = 5 mm.

TABLE 4 | *F* values of ANOVA-GLM for genotype and environment as well as their interaction in the introgression lines.

Traits	Genotype (G)		Environment (E)		G × E interaction	
	df	*F*	*df*	*F*	*df*	*F*
DH	378	117.52**	1	28967.90**	378	21.36**
DF	378	82.00**	1	28120.59**	378	20.77**
PH	378	69.01**	1	148.59**	378	5.22**
SL	378	22.86**	1	132.51**	378	1.77**
SPI	378	6.53**	1	72.11**	378	1.44**
SD	378	14.69**	1	2.26*NS*	378	1.02*NS*
GNS	378	4.72**	1	16.68**	378	2.76**
GP	378	11.67**	1	338.57**	378	5.30**
GL/GW	378	15.45**	1	537.04**	378	6.05**
GL	378	11.08**	1	188.98**	378	4.87**
GW	378	14.54**	1	20.97**	378	7.15**
TKW	378	36.77**	1	38.03**	378	12.33**

*NS, not significant; **significant difference at $P < 0.01$.*

DISCUSSION

Direct introgression from diploid species into hexaploid wheat has been explored as a possible applied plant-breeding technique for the rapid introgression of useful traits. Gill and Raupp (1987) reported that a total of 219 hybrid embryos were obtained by the hybridization of hexaploid wheat "Wichita" or "Newton" with 3l accessions of *A. squarrosa* (2*n* = 14) as male parent, but only 24 F_1 hybrids were grown to maturity. Another work of direct crossing between *T. aestivum* and *A. tauschii* was reported by Sehgal et al. (2011). Their results showed that

TABLE 5 | Analysis of putative QTLs for partial agronomic traits in ILs.

Trait	QTL	Environment	Marker	Position (Mb)	LOD	PVE (%)	Add
TKW	*QTKW.At-2D*	Huixian	*Xcfd53*	2D (26.2)	7.05	9.24	1.22
		Zhongmou			7.02	9.19	1.35
		Combined			8.48	10.69	1.28
	QTKW.At-4D	Huixian	*Xwmc48a*	4D (71.1)	3.11	3.60	1.37
	QTKW.At-6D	Zhongmou	*Xcfd13a*	6D (16.6)	3.12	3.90	−0.88
PH	*QPH.At-2D*	Huixian	*Xgwm296*	2D (20.0)	17.89	12.61	4.25
		Zhongmou			18.61	13.29	4.41
		Combined			21.12	13.95	4.33
	QPH.At-3D	Huixian	*Xbarc323*	3D (602.1)	3.55	2.60	−2.01
		Zhongmou			5.17	3.82	−2.46
		Combined			5.35	3.63	−2.29
	QPH.At-4D	Huixian	*Xwmc48a*	4D (71.1)	34.72	27.55	11.09
		Zhongmou			22.37	17.22	8.87
		Combined			32.40	23.86	9.99
	QPH.At-5D	Huixian	*Xbarc144*	5D (562.8)	15.17	10.75	4.73
		Zhongmou			10.36	6.83	3.73
		Combined			14.10	8.92	4.12
	QPH.At-1D	Huixian	*Xwmc216*	1D (373.5)	2.66	2.06	1.72
SL	*QSL.At-2D.1*	Huixian	*Xcfd53*	2D (26.2)	14.19	12.88	0.35
		Zhongmou			10.48	8.04	0.30
		Combined			13.29	10.46	0.32
	QSL.At-2D.2	Huixian	*Xgwm296*	2D (20.0)	7.92	6.08	0.24
		Zhongmou			18.68	13.71	0.40
		Combined			14.25	9.81	0.31
	QSL.At-5D	Huixian	*Xbarc144*	5D (562.8)	4.49	3.47	0.22
		Zhongmou			5.27	3.59	0.24
		Combined			5.63	3.80	0.23
	QSL.At-7D	Huixian	*Xbarc126*	7D (91.3)	8.02	6.27	0.25
		Zhongmou			7.22	5.11	0.24
		Combined			7.61	5.11	0.22
	QSL.At-3D	Zhongmou	*Xgwm161b*	3D (8.1)	4.27	3.33	−0.19
	QSL.At-4D	Huixian	*Xgpw342*	4D (451.6)	3.67	3.71	−0.29

LOD, likelihood of odds; PVE, phenotypic variance explained by each QTL; Add, additive effect. Positive values of Add indicate the effects increasing trait values by A. tauschii alleles.

about 51.72% of the pollinated florets produced embryo-carrying caryopses and 6.80 plants for every 100 florets pollinated were obtained when *A. tauschii* was used as the female parent. However, only 0.09 plants for every 100 florets pollinated were obtained in the reciprocal. In this work, about 61.90% of the pollinated florets produced embryo-carrying caryopses, and 22.9% caryopses generated normal embryos with *A. tauschii* as the female parent. No embryo-carrying caryopses were obtained in the reciprocal. These results suggest that the hybrid F_1 was easily obtained when *A. tauschii* was used as the female parent rather than the male parent. In addition, a major bottleneck in direct gene transfer is the high sterility in the F_1 from distant hybridization and extremely low ripening rates by backcrosses of hybrid F_1 with the recurrent parent. In a study by Sehgal et al. (2011), self-seed was hardly expected in the hybrid F_1 from distant hybridization. Moreover, the untreated tillers produced an average of 0.47 backcross seeds per 100 florets, while the colchicine treated tillers could produce an average of 14.9 backcross seeds per 100 florets pollinated (with a range of 8.33–26.88 seeds). In this work, the backcross of synthetic octaploid wheat as male parent with the recurrent parent Zhoumai 18 resulted in a ripening rate of 62.5%. Therefore, only direct crosses with *A. tauschii* as the male parent were adopted for gene transfer (Cox et al., 2006), and using synthetic octaploid wheat as the male parent could obviously enhance backcross ripening rates with the recurrent parent. Specifically, the hybrid F_1 was obtained by *A. tauschii* as the female parent and was then doubled to generate the synthetic octaploid wheat. In addition, compared with single gene transfer, the development of ILs can incorporate more than one useful gene simultaneously into common wheat. Liu et al. (2006) cultivated an ILs containing Am3 chromosome segments, which included 162 homozygous and 166 heterozygous segments. In this work, the ILs containing 6016 *A. tauschii* segments were developed using synthetic octaploid wheat as a "bridge," and no phenotype segregation was found in each line, which indicates that these lines are cytogenetically stable, and could be utilized more easily through further breeding.

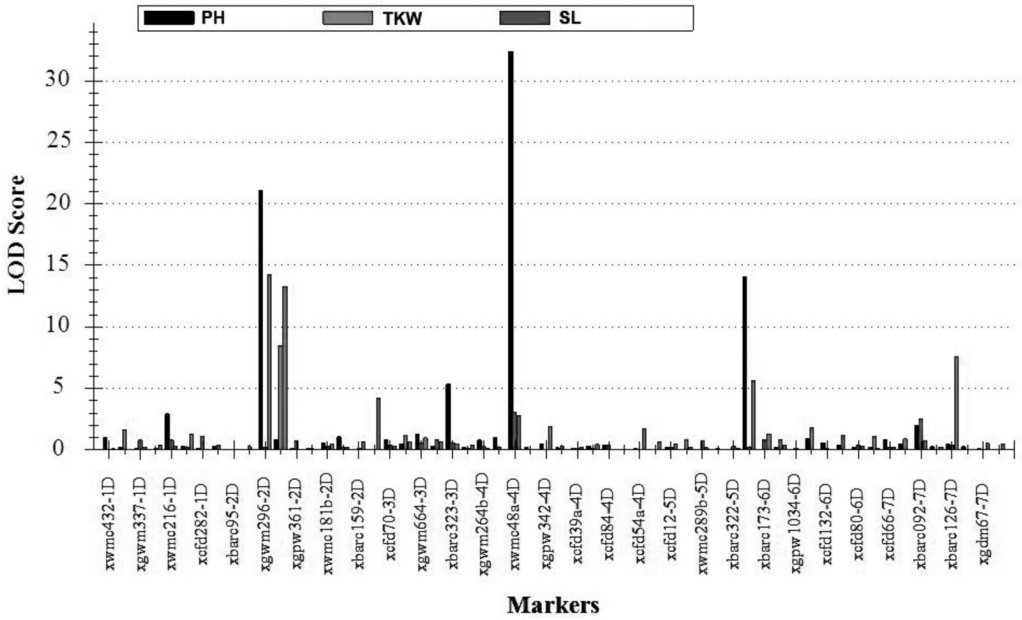

FIGURE 5 | The positions of putative QTLs of three agronomic traits detected in both Huixian and Zhongmou regions. PH, plant height; TKW, thousand kernel weight; SL, spike length.

It is well known that polyploids are more prone to receive portions of alien chromosomal introgression from related weedy species compared to diploids. Despite their overall inferior agronomic performance, wild and weedy species are likely to contain genetic factors that can increase the yield of modern varieties. In other words, quantitative traits of modern varieties may be improved using wild and weedy species (Frey et al., 1984). The 1RS arm in the translocation line 1BL/1RS wheat, for example, carries a battery of resistance traits and adaptation to abiotic stresses, as well as high-yield traits (Friebe et al., 1996; Sharma et al., 2011). In the process of improving common wheat by utilizing the desirable genes of *A. tauschii*, the yield, kernel weight, protein concentration, and kernel hardness were evaluated, based on 147 BC_2F_1-derived families from crossing between elite common wheat lines and *A. tauschii* (Fritz et al., 995). The results indicated that introgression of *A. tauschii* germplasm into the wheat genome had fewer effects on agronomic performance, compared to the extreme phenotypic differences between the two species. Variability for yield and protein was actually lower among strains carrying larger estimated amounts of *A. tauschii* segments. Thus, *A. tauschii* has been deemed to have a relatively neutral impact on the agronomic and quality traits of wheat but to serve as a source of important resistance genes. To date, many resistance genes of *A. tauschii* have been transferred into common wheat through the use of synthetic hexaploid wheat as a "bridge" (Naz et al., 2008; Dunckel et al., 2015; Wang et al., 2016). Through a doubled haploid (DH) population derived from synthetic-derived bread wheat line SYN1 and FHB-susceptible line Ocoroni, Zhu et al. (2016) identified a major QTL of Fusarium head blight (FHB) resistance on chromosome 2D, accounting for 25% of the phenotypic variation explained. Liu et al. (2006) investigated nine agronomic traits of 97 ILs containing Am3 chromosome segments, in which the Am3 was synthesized by the crossing of *Triticum carthlicum* with *A. tauschii*. The phenotype traits from ILs showed obvious change, and some strains displayed better agronomic traits than the recurrent parent. In this work, the agronomic traits among lines also showed significant variation. Although most of the strains were similar to the recurrent parent Zhoumai 18, some of them demonstrated apparent transgressive segregation (**Table 3**). In addition, 14 quantitative trait loci (QTLs) among three important agronomic traits (TKW, SL, and PH) were further located in the Huixian and Zhongmou, confirming the introgression of favorable alleles from *A. tauschii* into common wheat.

Genetic correlations between traits are due to linkage and/or pleiotropy and indicate the magnitude and direction of correlated response to selection, as well as the relative efficiency of indirect selection (Holland, 2006). When traits are highly correlated, plant breeders can select for the trait with higher heritability and simultaneously indirectly select for the other trait. The genetic correlation of agronomic traits of 188 recombinant inbred lines (RILs) from the spring wheat "Louise" × "Penawawa" were analyzed by Carter (2011), who found that flowering date and PH, as well as maturity date and PH, were moderately correlated. PH was positively correlated to grain yield, with taller plants having higher grain yield potential. Kumar et al. (2007) reported that grain yield was significantly correlated to SL in two mapping populations. In this work, PH was found to be negatively correlated with GNS, SPI, and SD, but positively correlated with SL, TKW, GL, GW, GP, and GL/GW. Similarly, TKW and SL showed significant positive correlation.

Plenty of studies have attempted to map QTL for grain yield and yield components of wheat under non-stress conditions (Kato et al., 2000; Börner et al., 2002; Huang et al., 2004, 2006; Mccartney et al., 2005; Marza et al., 2006; Narasimhamoorthy et al., 2006; Kuchel et al., 2007; Kumar et al., 2007; Cuthbert et al., 2008; Heidari et al., 2011). However, it is still necessary to confirm the role of important markers associated with grain yield across different genetic backgrounds and environments. Huang et al. (2003) reported detecting a major *QTgw.ipk-2D* on chromosome 2DL with a boundary from Xgwm539 to Xgdm6 in a BC_2F_2 population derived from a cross between the common wheat and the synthetic wheat. This QTL could explain 15.4% of the phenotypic variation. Crossa et al. (2007) used two linear mixed models to assess marker-trait associations. They identified significant associations between grain yield and the DArT markers wPt-4413 on chromosome 2D. Using association mapping, Edae et al. (2014) detected one stable QTL for grain yield on chromosome 2DS, under both irrigated and rain-fed conditions. The QTL associated with the DArT marker *wpt6531* is about 8 cM away from the *wpt4144* marker, which was associated with yield in the study of Crossa et al. (2007). Using two different RILs populations, Kumar et al. (2007) identified one QTL for grain yield on chromosome 2D with a boundary from *X*gwm261 to *Xcdo1379*. In addition, Narasimhamoorthy et al. (2006) detected a QTL for grain yield linked to Xgwm261. Interestingly, according to the linkage map of Crossa et al. (2007), the SSR markers (*Xgwm261*) were linked to the DArT marker wPt-4413, spanning 3.2 cM. Four QTLs for TKW (Huang et al., 2004, 2006; Cuthbert et al., 2008) were identified close (from 1.7 cM for *Xgwm296* to 7.9 cM for *Xwmc601*) to the DArT markerwPt-4413 on chromosome 2D, according to the linkage map of Crossa et al. (2007). Azadi et al. (2015) identified that two QTLs (*QTgw.abrii-2D1* and *QTgw.abrii-2D3*) were also close to the DArT marker wPt-4413. In the present study, one major QTL for TKW, designated *QTKW.At-2D*, was detected on the Xcfd53 of chromosome 2D in the Huixian and Zhongmou environments (**Table 3**). The QTL (*QTKW.At-2D*) was also close to the DArT marker wPt-4413 according to the linkage map of Crossa et al. (2007). Identification of this QTL for grain yield/ TKW at the same position suggests a possible pleiotropic QTL and also indicates that this region may play an important role in improving grain yield. When averaged across two environments, this QTL could explain 10.69% of the phenotypic variation, corresponding to the additive effect values of 1.28. The *Xcfd53* was associated with positive effects on TKW. Typically, the accession 150679, containing the above-mentioned marker, showed TKW values of 59.02 and 60.96 g in the two districts, providing high increments of 22.2% and 24.4% compared with Zhoumai 18, respectively. These results reveal that favorable alleles from *A. tauschii* can improve important agronomic traits of an elite wheat variety, even though *A. tauschii* itself is inferior to the cultivated variety in the phenotypic traits.

CONCLUSION

A set of ILs containing only *A. tauschii* segments was established by using synthetic octaploid wheat (AABBDDDD, $2n = 8x = 56$) as a "bridge." This bridge was obtained by the chromosome doubling of hybrid F_1 (*A. tauschii* T015 × common wheat Zhoumai 18). The agronomic traits among lines also showed significant phenotype variation. For every trait, some lines displayed better performance than the recurrent parent. In addition, 14 QTLs for three important agronomic traits (TKW, PH, and SL) were further located in Huixian and Zhongmou regions, respectively.

AUTHOR CONTRIBUTIONS

SL and CS conceived and designed the study. DZ, YZ, XZ, LL, CZ, JL, and GS generated the data and performed the analysis. DZ and YZ contributed reagents, materials, and analysis tools. DZ, YZ, SL, and CS wrote and revised the paper. All authors read and approved the final manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (Grant Nos. 31401379 and 31571649) and Project of Major Science and Technology in Henan Province (Grant No. 161100110400), and Project of Science and Technology Department of Henan Province (Grant No. 172102110004).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01113/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 08 November 2018
doi: 10.3389/fpls.2018.01616

Molecular and Cytogenetic Characterization of Six Wheat-*Aegilops markgrafii* Disomic Addition Lines and Their Resistance to Rusts and Powdery Mildew

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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: 23 August 2018
Accepted: 18 October 2018
Published: 08 November 2018

Citation:
Niu Z, Chao S, Cai X, Whetten RB, Breiland M, Cowger C, Chen X, Friebe B, Gill BS, Rasmussen JB, Klindworth DL and Xu SS (2018) Molecular and Cytogenetic Characterization of Six Wheat-Aegilops markgrafii Disomic Addition Lines and Their Resistance to Rusts and Powdery Mildew. Front. Plant Sci. 9:1616. doi: 10.3389/fpls.2018.01616

Aegilops markgrafii (Greuter) Hammer is an important source of genes for resistance to abiotic stresses and diseases in wheat (*Triticum aestivum* L.). A series of six wheat 'Alcedo'-*Ae. markgrafii* chromosome disomic addition lines, designated as AI(B), AII(C), AIII(D), AV(E), AIV(F), and AVIII(G) carrying the *Ae. markgrafii* chromosomes B, C, D, E, F, and G, respectively, were tested with SSR markers to establish homoeologous relationships to wheat and identify markers useful in chromosome engineering. The addition lines were evaluated for resistance to rust and powdery mildew diseases. The parents Alcedo and *Ae. markgrafii* accession 'S740-69' were tested with 1500 SSR primer pairs and 935 polymorphic markers were identified. After selecting for robust markers and confirming the polymorphisms on the addition lines, 132 markers were considered useful for engineering and establishing homoeologous relationships. Based on the marker analysis, we concluded that the chromosomes B, C, D, E, F, and G belong to wheat homoeologous groups 2, 5, 6, 7, 3, and 4, respectively. Also, we observed chromosomal rearrangements in several addition lines. When tested with 20 isolates of powdery mildew pathogen (*Blumeria graminis* f. sp. *tritici*) from five geographic regions of the United States, four addition lines [AIII(D), AV(E), AIV(F), and AVIII(G)] showed resistance to some isolates, with addition line AV(E) being resistant to 19 of 20 isolates. The addition lines were tested with two races (TDBJ and TNBJ) of the leaf rust pathogen (*Puccinia triticina*), and only addition line AI(B) exhibited resistance at a level comparable to the *Ae. markgrafii* parent. Addition lines AII(C) and AIII(D) had been previously identified as resistant to the Ug99 race group of the stem rust pathogen (*Puccinia graminis* f. sp. *tritici*). The addition lines were also tested for resistance to six United States races (PSTv-4, PSTv-14, PSTv-37, PSTv-40, PSTv-51, and PSTv-198)

of the stripe rust pathogen (*Puccinia striiformis* f. sp. *tritici*); we found no resistance either in Alcedo or any of the addition lines. The homoeologous relationships of the chromosomes in the addition lines, molecular markers located on each chromosome, and disease resistance associated with each chromosome will allow for chromosome engineering of the resistance genes.

Keywords: wheat, homoeology, chromosome engineering, molecular markers, alien introgression, stripe rust, leaf rust, powdery mildew

INTRODUCTION

Aegilops markgrafii (Greuter) Hammer (synonym *Ae. caudata* L., $2n = 2x = 14$, genome CC), is one of the most important diploid wild relatives of wheat (*Triticum aestivum* L., $2n = 6x = 42$, AABBDD genomes) because it carries resistance to powdery mildew [caused by *Blumeria graminis* f. sp. *tritici* (DC.) Speer], leaf rust (*Puccinia triticina* Erikss.), stem rust (*Puccinia graminis* Pers.: Pers. f. sp. *tritici* Eriks. and E. Henn.) and stripe rust (*Puccinia striiformis* Westend. f. sp. *tritici* Eriks.) (Valkoun et al., 1985; Dyck et al., 1990; Schubert and Blüthner, 1995; Xu et al., 2009; Weidner et al., 2012). A set of chromosome disomic addition lines carrying *Ae. markgrafii* accession 'S 740-69' chromosomes B, C, D, E, F, and G in wheat variety 'Alcedo' were developed by Schubert and Blüthner (1992, 1995). This set of disomic addition lines can serve as an alternate and direct genetic source for wheat germplasm enhancement. An addition line for chromosome A is absent from this set, and Niu et al. (2011) found that none of the six addition lines carried high-molecular-weight glutenins from *Ae. markgrafii*, suggesting that chromosome A may be homoeologous to group 1. Friebe et al. (1992) noted results from unpublished studies which support the conclusion that chromosome A belongs to group 1. Danilova et al. (2014, 2017) determined that chromosome A of *Ae. markgrafii* is homoeologous to the group 1 chromosomes of wheat by using fluorescence *in situ* hybridization (FISH) with cDNA probes.

Xu et al. (2009) identified two Alcedo-*Ae. markgrafii* S740-69 addition lines, AII(C) and AIII(D), that conditioned resistance to the Ug99 race group of the stem rust pathogen, the most virulent races appearing in Africa. To transfer these alien genes from the addition lines to wheat in a short period of time, detailed information concerning the homoeology between wheat and the added *Ae. markgrafii* chromosomes will be very useful. There are several ways to establish the homoeologous relationships between wheat and its wild relatives, including C-banding (Friebe et al., 1992), isozyme analysis (Schmidt et al., 1993), molecular marker analysis (Peil et al., 1998), sequential fluorescence *in situ* hybridization (FISH), and genomic *in situ* hybridization (GISH) (Xu et al., 2016). In addition, marker assisted selection has become a useful tool for the gene introgression process (Niu et al., 2011). Friebe et al. (1992) and Schmidt et al. (1993) used isozymes and the C-banding technique, respectively, to determine homoeologous relationships of the six addition lines, and determined that the chromosome in lines AII(C), AIII(D), and AIV(F) belonged to group 5, 6, and 3, respectively, but homoeologous relationships of chromosomes in lines AV(E), AI(B), and AVIII(G) were not clearly established. Peil et al. (1998) tested 88 SSR markers and identified only 20 that were useful to distinguish the *Ae. markgrafii* chromosomes; and because the marker number was less than 4 for each chromosome, the results did not indicate homoeology. In addition to homoeologous relationships of each added chromosome, knowledge of the Alcedo genetic background is needed. For example, Alcedo is a major donor of stripe rust resistance (Jagger et al., 2011), and in attempting to transfer stripe rust resistance from *Ae. markgrafii*, detailed information about Alcedo is important to ensure that the stripe rust resistance is from *Ae. markgrafii* and not Alcedo.

Simple sequence repeats (SSRs) have become very useful and desirable molecular markers because they are often codominant, highly reproducible, frequent in most eukaryotes, and have high allelic diversity (Mohan et al., 1997). With the development of sequencing technology, more and more SSRs (over 3000) are available for marker analysis in wheat, and many genetic maps featuring SSR markers are available for reference. Screening for polymorphisms between the parents using additional SSRs will help to determine the homoeologous relationships and the polymorphic markers can subsequently be used for marker-assisted gene introgression. Sequential FISH and GISH will produce additional chromosome constitution information for the addition lines. Our objectives in this study were to use additional SSR markers and sequential FISH and GISH to characterize Alcedo and its six *Ae. markgrafii* addition lines, determine the homoeologous relationships of the chromosomes, and develop useful SSR markers for marker assisted selection.

MATERIALS AND METHODS

Plant Material

Wheat cultivar 'Alcedo,' *Ae. markgrafii* (Greuter) Hammer (accession S740-69), the Alcedo-*Ae. markgrafii* amphiploid (W0492), and six Alcedo-*Ae. markgrafii* S740-69 disomic addition lines AI, AII, AIII, AV, AIV, and AVIII carrying the *Ae. markgrafii* chromosomes B, C, D, E, F, and G, respectively (Schmidt et al., 1993) were used for this study. A line carrying chromosome A was not available for this study. The original seed stocks of these lines were kindly provided by Dr. Richard R.-C. Wang, USDA-ARS Forage and Range Research Laboratory, Logan, UT, United States.

Fluorescence *in situ* Hybridization

Root-tips of plants were prepared for FISH following the procedure described by Xu et al. (2016). This included

pretreatment of root tips in ice water for 20–24 h, fixation in ethanol-acetic acid (3:1 ratio), pretreatment in 1% acetocarmine, and squashing on a slide using 45% acetic acid. Slides were examined to select samples with good preparations, and cover glasses were removed. Prepared slides were incubated in 100 μg/mL RNase in 2× saline sodium citrate (SSC) at 37°C for 1 h, then denatured in 70% formamide in 2× SSC at 72°C for 2 min followed by dehydration in a chilled graded ethanol series (70%, 95%, and 100%) at −20°C each for 5 min.

Multi-color FISH was carried out with two probes, pAS1 carrying about 1 kb of repeat sequence from *Ae. tauschii* Cosson (Rayburn and Gill, 1986) and labeled with digoxigenin-11-dUTP (Roche Diagnostics, Mannheim, Germany), and pSC119.2 carrying a highly repeated sequence from rye (*Secale cereale* L.) and labeled with biotin-16-dUTP (Enzo Life Sciences, Inc., Farmingdale, NY, United States). The two probes were equally mixed before hybridization and then added to the hybridization mix (15 μL formamide, 6 μL dextran sulfate, 3 μL 20× SSC, and 3 μL single stranded DNA). Fifteen microliters of hybridization mix were added to each slide and slides were covered with cover slips for incubation overnight. The slides were then washed as described by Xu et al. (2016). The fluorescent signals were detected with anti-digoxigenin-rhodamine (Roche Diagnostics) and fluorescein isothiocyanate-conjugated avidin (FITC-avidin) (Vector Laboratories, Inc., Burlingame, CA, United States) for both probes. The slides were mounted with VECTORSHIELD Antifade Mounting Medium (Vector Laboratories) containing 4′,6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO, United States). The slides were examined under a Zeiss Axioplan 2 Imaging Research Microscope (Carl Zeiss Light Microscopy, Germany). The GISH images were captured using an Axiocam HRm CCD (charge-coupled device) camera (Carl Zeiss Light Microscopy) and analyzed using imaging software AxioVision Release 4.5 (Carl Zeiss Light Microscopy).

Genomic *in situ* Hybridization

After FISH, the slides were washed in 0.1× SSC with 0.5% formamide three times each for 10 min at 42°C, then 2× SSC twice each for 10 min, then in 4× SSC overnight at room temperature. The slides were sequentially dehydrated in 70%, 95%, and 100% ethanol each for 5 min. Total genomic DNA from *Ae. markgrafii* was used as probe and labeled with biotin-16-dUTP by nick translation (Enzo Life Sciences, Inc.). Sheared genomic DNA from Chinese Spring was used for blocking. Detailed procedures of the chromosome preparation and hybridization were previously described by Xu et al. (2016). GISH signals were detected with FITC-avidin (Vector Laboratories). The slides were mounted with VECTORSHIELD Antifade Mounting Medium (Vector Laboratories) containing propidium iodide (PI) (Vector Laboratories) and were observed under the Zeiss Axioplan 2 Imaging Research Microscope for GISH analysis as described above. Photographs were captured using the Axiocam HRm CCD camera and analyzed using the imaging software AxioVision Release 4.5 for GISH analysis as described above.

Karyotype Analysis of *Ae. markgrafii* Chromosomes

The chromosome spreads from GISH and FISH analyses were used for karyotypic analysis of each of the *Ae. markgrafii* chromosomes in the six addition lines. Each of the *Ae. markgrafii* chromosomes was measured for lengths of short and long arms from at least 20 cells using the "Measure Length" tool in the imaging software AxioVision Release 4.5 (Carl Zeiss Light Microscopy). Total length of each *Ae. markgrafii* chromosome was calculated by adding the averages of long and short arms. The arm ratio (long arm/short arm) of each *Ae. markgrafii* chromosome was calculated from the lengths of short and long arms.

SSR Marker Analysis

DNA extraction from fresh leaves and SSR marker genotyping were carried out according to the procedure outlined by Niu et al. (2011). Markers studied included SSRs from the BARC (Song et al., 2005), GWM (Röder et al., 1998), WMC (Somers et al., 2004), CFA (Sourdille et al., 2003), GDM (Pestsova et al., 2000), CFD (Guyomarc'h et al., 2002), DuPw (Eujayl et al., 2002), KSM (Yu et al., 2004), CNL (Yu et al., 2004), and AC (Barkley et al., 2006) groups. Markers were assigned to chromosomes and chromosome groups based on locations reported in the citations or based on a search for the markers in the GrainGenes database[1]. DNA fragments were amplified by polymerase chain reaction (PCR) at an annealing temperature of 50°C and labeled with four different fluorescent dyes (6-FAM, VIC, NED, and PET). Amplified PCR products were separated by capillary electrophoresis using the ABI 3130xl Genetic Analyzer (Applied Biosystems, Forster City, CA, United States) according to the procedures of Chao et al. (2007). The genotype calls were analyzed using GeneMapper software v3.7 (Applied Biosystems).

Evaluation of *Ae. markgrafii* Addition Lines for Resistance to Leaf Rust, Stripe Rust, and Powdery Mildew

Wheat cultivar Alcedo, *Ae. markgrafii* accession S740-69, Alcedo-*Ae. markgrafii* amphiploid W0492, six disomic addition lines, and Chinese Spring were included in tests for resistance to leaf rust, stripe rust, and powdery mildew.

Leaf rust resistance was evaluated at North Dakota State University (Fargo, ND, United States) followed the procedures of Kertho et al. (2015). Two *P. triticina* races, TDBJ+*Lr21&Lr28* and TNBJ, were used to evaluate the genotypes. Race TDBJ+*Lr21&Lr28* produces a high infection type on *Lr21* and *Lr28*, while TNBJ has a high infection type on *Lr9*. The experiment was conducted using a randomized complete block design with two replicates, with the entire experiment being repeated for each race as described by Kertho et al. (2015). Approximately five seeds per genotype were planted in a greenhouse set at 22°C/18°C (day/night) with 16-h photoperiod. Ten-day-old seedlings were inoculated by spraying fresh urediniospores suspended in a light mineral oil (Soltrol-170,

[1]http://wheat.pw.usda.gov/cgi-bin/GG3/browse.cgi?class=marker

Phillips Petroleum, Bartlesville, OK, United States). Following inoculation, plants were placed into a darkened dew chamber maintained at 20°C for 16–24 h. Following the incubation period, the plants were removed to a greenhouse maintained at 20°C with a normal 16/8 h day/night photoperiod. Genotypes were scored for infection types (ITs) at 12–14 days post inoculation using the 0–4 scale (McIntosh et al., 1995). Infection types of 2 or lower were considered resistant, and ITs 3 or higher were considered susceptible.

Resistance to stripe rust was evaluated at USDA-ARS, Wheat Health, Genetics, and Quality Research Unit, Pullman, WA, United States, using six *P. striiformis* f. sp. *tritici* races, PSTv-4, PSTv-14, PSTv-37, PSTv-40, PSTv-51, and PSTV-198 (Wan and Chen, 2014; Wan et al., 2016). For each race test, 5–10 seeds per line were planted and seedlings at the two-leaf stage were uniformly inoculated with a mixture of urediniospores with talc at a 1:20 ratio and kept in a dew chamber for 24 h at 10°C and 100% relative humidity without light. The inoculated plants were them moved to a growth chamber at a diurnal temperature cycle gradually changing from 4°C at 2:00 am to 20°C at 2:00 pm and a diurnal cycle of 8 h dark/16 light corresponding to the low/high temperature cycle (Wan and Chen, 2014). Plants were scored 20 days post inoculation using the 0–9 scale of Line and Qayoum (1992).

Powdery mildew tests were conducted at the USDA-ARS, Plant Science Research Unit, Raleigh, NC, using the detached-leaf method as described by Worthington et al. (2014). Twenty isolates representing differing United States regional virulence profiles were used for tests. These isolates originated from nine US states of the Southeast, Mid-Atlantic, Great Lakes, and Great Plains wheat growing regions. To simplify presentation, two Montana isolates are included as "Great Plains" isolates despite originating west of the Great Plains because they exhibit a similar virulence profile to isolates from the Great Plains (Cowger et al., 2018). Inoculations were performed following Worthington et al. (2014). In brief, two 1.5-cm detached leaf segments from each genotype were floated on 0.5% water agar containing 50 mg L^{-1} benzimidazole in a Petri plate. Each plate also contained four replicate leaf segments of a susceptible wheat cultivar as a positive control. Four replicate Petri plates per host genotype were inoculated with each isolate of *B. graminis* f. sp. *tritici*. The plates were then placed in a growth chamber set to 18°C with an 11-h photoperiod. Disease reactions were scored 10 days post-inoculation using the 0–9 scale of Leath and Heun (1990). Reactions were then classified as resistant (R), intermediate (I), or susceptible (S) based on whether the predominant reaction among the replicate plates was <4, 4 to 6, or >6, respectively.

RESULTS

GISH and FISH Analysis of Six Alcedo-*Ae. markgrafii* Disomic Addition Lines

The six Alcedo-*Ae. markgrafii* disomic addition lines, AI(B) through AVIII(G), were examined for differences in spike morphologies (**Figure 1**). We observed that AI(B) is unique in its non-free threshing spikes, AII(C) has large glumes, AIII(D) and AV(E) have awns, and AIV(F) has club spikes with brittle rachis. Lines AV(E) and AVIII(G) have sterile spikelets on the top and in the upper half portion of the spikes, respectively (**Table 1**).

The GISH analysis (**Figure 2**) showed that all six lines had a mitotic chromosome number of $2n = 44$, and in each case, only one pair of chromosomes showed a distinct green coloration (arrows) compared to the red coloration of the remaining 42 chromosomes. No structural abnormalities were observed on any of the chromosomes. These results indicated that each addition line carried only one intact chromosome pair from *Ae. markgrafii*. Karyotypic characteristics of six *Ae. markgrafii* chromosomes are listed in **Table 1**. The long arm to short arm ratios of the *Ae. markgrafii* chromosomes B, C, D, E, F, and G were 3.29, 1.85, 2.31, 3.79, 4.24, and 4.63, respectively (**Table 1**). The FISH results showed that the *Ae. markgrafii* chromosomes (arrows) in all the additions had the pSC119.2 hybridization signals in the telomeric regions in either one or both arms (**Figure 2**). The general morphologies of the six *Ae. markgrafii* chromosomes from GISH/FISH analysis are consistent with those from the N- and C-banded karyotypes reported by Schubert et al. (1987) and Friebe et al. (1992), respectively. By comparing to the reference karyotype developed based on *Ae. markgrafii* accession MvGB428 (Molnár et al., 2015), we found that only chromosomes C and F had the identical pSC119.2 band patterns as chromosomes 5C and 3C, respectively.

Identification of SSR Markers Associated to *Ae. markgrafii* Chromosomes

In this study, 1,500 SSR primer pairs were used to detect polymorphism between the parents, Alcedo and *Ae. markgrafii* accession S740-69. Nine hundred and thirty-five pairs of SSRs (62.3%) amplified polymorphic bands. From those polymorphic primer pairs, SSRs located on group 1 chromosomes, the majority of the SSRs that produced dominant bands and SSRs that produced weak bands were eliminated from further analyses. As a result, only 234 robust SSRs were selected for analysis of the Alcedo-*Ae. markgrafii* addition lines. These SSRs were comprised of 27 BARCs, 58 GWMs, 72 WMCs, 14 CFAs, 16 GDMs, 34 CFDs, 5 DuPws, 4 KSMs, 3 CNL, and 1 AC. These SSRs belonged to six homoeologous groups, 52 to group 2, 45 to group 3, 35 to group 4, 47 to group 5, 24 group to 6, and 31 to group 7. Analysis of the *Ae. markgrafii* addition lines resulted in the elimination of additional SSRs. As a result, only 132 SSRs were polymorphic between the addition lines and Alcedo (**Supplementary Table 1**). However, many of these SSRs mapped to multiple groups (**Supplementary Table 1**), and therefore, in the summarized distribution of the SSRs to chromosome groups, it appears that there are more than 132 polymorphic SSRs (**Table 2**).

The assignment of *Ae. markgrafii* chromosomes to homoeologous groups was determined based on the distribution of the polymorphic SSR markers among the addition lines. Of the 28 polymorphic markers identified for addition line AI(B) (**Table 2**), 17 (61%) mapped to group 2 chromosomes, suggesting that the alien chromosome in the AI(B) addition line belongs to

FIGURE 1 | The morphology of the spikes of Alcedo, the amphiploid between Alcedo and *Aegilops markgrafii*, and six wheat addition lines carrying the alien chromosomes from *Ae. markgrafii.* 1, amphiploid, 2, AI(B); 3, AII(C); 4, AIII(D); 5, AV(E); 6, AIV(F); 7, AVIII(G); 8, Alcedo.

TABLE 1 | Karyotypic characteristics of *Aegilops markgrafii* chromosomes and the spike agronomic traits of the six Alcedo-*Ae. markgrafii* S740-69 disomic addition lines.

Addition lines	Length (μm) of *Ae. markgrafii* chromosome			Arm ratio (L:S)	Spike traits
	Long arm (L)	Short arm (S)	Total		
AI(B)	6.73	2.05	8.78	3.29	Non-free threshing
AII(C)	6.63	3.58	10.21	1.85	Large glumes
AIII(D)	5.94	2.57	8.51	2.31	Awned
AV(E)	6.32	1.67	7.99	3.79	Top of spike is sterile, awned
AIV(F)	6.36	1.50	7.86	4.24	Club spikes, brittle rachis
AVIII(G)	6.08	1.31	7.39	4.63	Top half of spike is sterile

Non-free threshing QTLs were mapped to group 2 (Sood et al., 2009), brittle rachis QTLs located on group 3 chromosome (Nalam et al., 2006; Watanabe et al., 2006).

group 2. Similar comparisons for the other five addition lines clearly indicate that the *Ae. markgrafii* chromosomes in the lines AII(C), AIII(D), and AV(F) belongs to groups 5, 6, and 3, respectively. The chromosome in line AV(E) might belong to group 7 or group 3, and the G addition chromosome might belong to group 2, 3, or 4 (**Table 3**).

Reactions of Alcedo-*Ae. markgrafii* Addition Lines to Leaf Rust, Stripe Rust, and Powdery Mildew

The addition lines were tested with two races (TDBJ and TNBJ) of the leaf rust pathogen (**Table 4**). As expected, Chinese Spring was susceptible to both races. Alcedo had an intermediate (2^+3) or resistant (2) IT to TDBJ and TNBJ, respectively; indicating that it carries at least one leaf rust resistance gene. The *Ae. markgrafii* parent (S740-69) was highly resistant to both races, with an immune response. For the six disomic addition lines, only AI(B) exhibited resistance, with a level of resistance similar to S740-69. The B chromosome appears to be a good source of leaf rust resistance.

The addition lines and parents were tested with six races of the stripe rust pathogen (**Table 4**). Although Alcedo had been reported to carry two genes for stripe rust resistance, Alcedo was observed to be highly susceptible (IT 8) to all six US races. For the remaining parents and addition lines, the *Ae. markgrafii* parent (S740-69) was immune, but all other lines were highly susceptible. The amphidiploid W0492 was included in the stripe rust tests, and it expressed an IT similar to Alcedo and all addition lines; and this indicated that the resistance in S740-69 could not be confirmed to any of the seven *Ae. markgrafii* chromosomes.

When tested with powdery mildew (**Table 5**), Chinese Spring was susceptible to all 20 isolates, while Alcedo was susceptible to 19 isolates and had an intermediate reaction to isolate *MTG1-1a*. In contrast, S740-69 was resistant to all 20 isolates, indicating *Ae. markgrafii* was an excellent source of powdery mildew resistance. Among the addition lines, AI(B) and AII(C) were susceptible to almost all isolates, indicating that resistance was not contributed by the B and C chromosomes. The remaining four addition lines had varying levels of resistance. Line AV(E) had resistance to 19 of 20 isolates and an intermediate reaction to isolate *MSG-D-1-5*. Line AIII(D) was resistant to all eight Great Plains isolates,

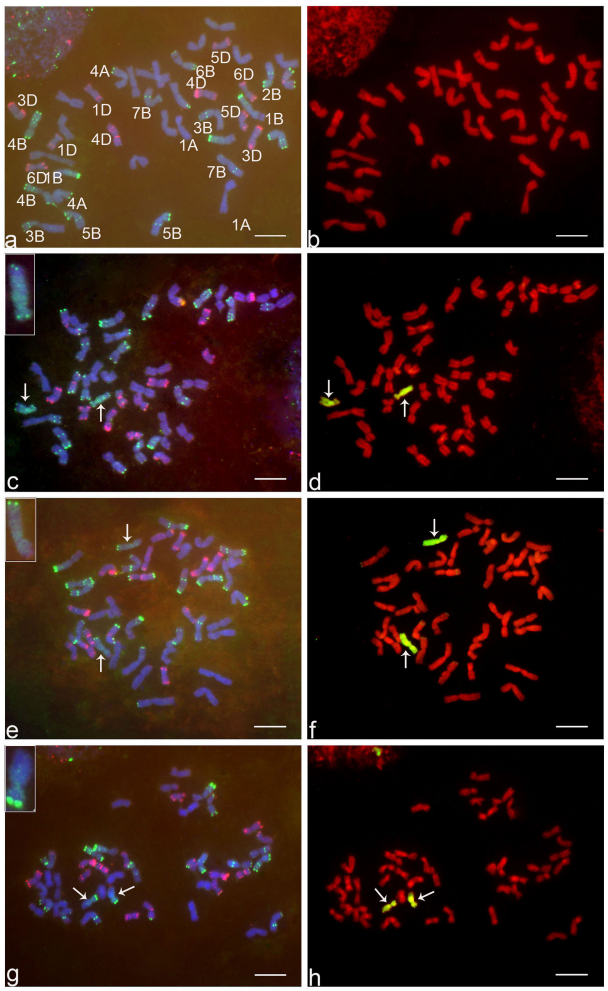

FIGURE 2 | Continued

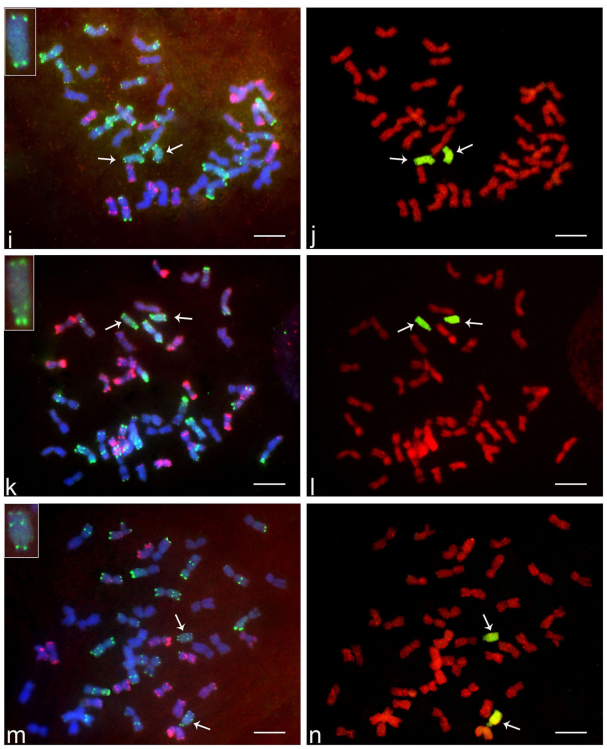

FIGURE 2 | FISH and GISH on the somatic metaphase chromosomes of six addition lines and their wheat parent 'Alcedo.' The left side of figure **(a,c,e,g,i,k,m)** are FISH results, where red indicates pAs1 hybridization sites detected by rhodamine fluorescence and green indicates pSc119.2 hybridization sites detected by FITC fluorescence. The right sides of figure **(b,d,f,h,j,l,n)** are GISH results, where green indicates *Aegilops markgrafii* chromatin detected by FITC fluorescence. **a** and **b**, Alcedo; **c** and **d**, AI(B); **e** and **f**, AII(C); **g** and **h**, AIII(D); **i** and **j**, AV(E); **k** and **l**, AIV(F); **m** and **n**, AVIII(G). Arrows indicate the alien chromosomes. Bar represents 10 μm.

but had a mixture of R, I, and S reactions to isolates from the other geographical regions. Line AIV(F) also had resistance to all Great Plains isolates, but AIV(F) had a different mixture of R, I, and S reactions to other isolates as compared to AIII(D). AVIII(G) had a mixture of reactions without regard to the region of origin and had more intermediate reactions than the other addition lines. In summary, the E chromosome conditioned resistance to nearly all isolates, the D and F chromosomes conditioned resistance to the Great Plains isolates and some isolates from other regions, and the G chromosome conditioned resistance to some isolates without regard to the region of origin.

DISCUSSION

In assigning SSRs to specific *Ae. markgrafii* chromosomes, the addition lines must exhibit a high level of homogeneity relative to Alcedo to exclude detection of polymorphisms on the wheat chromosomes. Friebe et al. (1992) concluded from C-banding results that the Alcedo-*Ae. markgrafii* addition lines were not in a pure Alcedo background. This is supported by the results of Niu et al. (2011), who studied HMW glutenin subunits in the six addition lines. They found addition line AIII(D) differed from Alcedo at all three *Glu-1* loci, which indicated an additional wheat genotype in the parentage of AIII(D) rather than the presence of biotypes in Alcedo. Variability in the wheat background of the addition lines complicates determination of the origin of the observed polymorphisms. For example, in AIII(D) the evidence suggests that the *Ae. markgrafii* chromosome is homoeologous to group 6, but additional markers also mapped to all the other chromosome groups.

The observed variability in the assignment of molecular markers to chromosomes indicates the presence of chromosomal rearrangements. Studies by Danilova et al. (2017) and Gong et al. (2017) found that the Alcedo-*Ae. markgrafii* additions lines carried several inversions and translocations. While both studies found a high level of rearrangement, the two studies did not agree on the rearrangements carried by each chromosome (**Table 3**). For example, Danilova et al. (2017) concluded that

TABLE 2 | The distribution of the polymorphic SSR marker belonging to different homoeologous groups in six Alcedo-*Aegilops markgrafii* disomic addition lines.

Addition lines	Number of SSR markers belonging to homoeologous group						Total
	2	3	4	5	6	7	
AI(B)	17	3	5	3	6	4	28
AII(C)	7	7	3	29	2	3	36
AIII(D)	4	6	3	6	12	8	29
AV(E)	4	7	3	0	3	8	19
AIV(F)	3	14	0	3	2	2	20
AVIII(G)	7	11	5	1	1	2	25
Total	42	48	19	42	26	27	132

The total number of polymorphic markers was less than the sum of markers for the six groups because several markers mapped to more than one homoeologous group.

TABLE 3 | Assignment of homoeologous groups of six *Aegilops markgrafii* chromosomes derived from six Alcedo-*Ae. markgrafii* disomic addition lines.

Addition line	Homoeologous groups assigned in this study			Homoeologous groups assigned by		
	SSR markers	Karyotype and pSC119.2	Spike traits	Schmidt et al. (1993)	Gong et al. (2017)	Danilova et al. (2017)
AI(B)	2	–	2	4/5	1/2/3/5	2/4
AII(C)	5	5	–	5	2/5	5
AIII(D)	6	–	–	6	2/5/6	6/7
AV(E)	7/3	–	–	–	1/2/7	7
AIV(F)	3	3	2/3	3	2/3	3
AVIII(G)	3/2/4	–	–	4/3	1/2/3/4	4/2/3

Assignment of homoeologous groups using karyotype and pSC119.2 probe was based on the Ae. markgrafii reference karyotype (Molnár et al., 2015).

chromosome D was mainly a group 6 chromosome with the long arm telomere composed of a translocated 7CL telomeric region. In contrast, Gong et al. (2017) concluded that the rearrangements in chromosome D involved chromosomes 2C, 5C, and 6C.

For each addition line, we observed markers that were not associated with the homoeologous group identified for that line. For example, of the 28 polymorphic markers associated with addition line AI(B), 17 were group 2 markers, and 11 markers were therefore not associated with group 2. There is more than one possible explanation for the markers that do not fit with the alien chromosome. Some of these markers may represent polymorphisms present in the addition lines that were not eliminated during backcrossing to Alcedo, and therefore these markers would not be associated with the alien chromosome. Some may actually be associated with the alien chromosome but have simply not been previously identified to that homoeologous group. Finally, some may be associated with the alien chromosome, but the rearrangements present results in markers being identified with multiple homoeologous groups. For example, Danilova et al. (2017) concluded that the *Ae. markgrafii* chromosome D carried a group 6/7 rearrangement, and 19 of the 29 markers we observed for AIII(D) would fit with this rearrangement. Similarly, Danilova et al. (2017) concluded that *Ae. markgrafii* chromosome G carried a 4/2/3 rearrangement, and 21 of the 25 markers we observed fit this rearrangement. Therefore, our results seem to fit well with the conclusions of Danilova et al. (2017). However, considering the high levels of rearrangements in the *Ae. markgrafii* genome, it is possible that the differences in the present study from Danilova et al. (2017) and Gong et al. (2017) may represent observational differences, with additional rearrangements yet to be discovered. The GISH and FISH analysis showed that the *Ae. markgrafii* chromosomes in AII(C) and AIV(F) (**Figure 2**) are morphologically most like chromosomes 5C and 3C of the reference karyotype (Molnár et al., 2015), respectively. Taken together, the spike traits (**Table 1**), molecular marker data (**Table 2**), and FISH and GISH analyses (**Figure 2**) indicated that *Ae. markgrafii*

TABLE 4 | Infection types (IT) observed on *Aegilops markgrafii* addition lines when tested with two races of the leaf rust pathogen (*Puccinia tritcina*, *Pr*) and one race of stripe rust pathogen (*Puccinia striiformis* f. sp. *tritici*, *Pst*).

Line or genotype	IT to *Pr* race		IT to *Pst* race
	TDBJ	TNBJ	PSTv-14
Chinese Spring	32	32	8
Alcedo	$2^{+}3$	2	8
S740-69 (*Ae. markgrafii*)	0	0	0
W0492 (amphidiploid)	–	–	8
AI(B)	0;	;	8
AII(C)	3	3	8
AIII(D)	2	3	8
AV(E)	3	3	8
AIV(G)	3	3	8
AVIII(G)	3	3^{-}	8

The value reported is the higher of the two reps.
Five other stripe rust races tested on the lines produced identical results.

TABLE 5 | Reactions of six Alcedo-*Aegilops markgrafii* addition lines, their parents, and the Chinese Spring check when tested with 20 isolates of powdery mildew pathogen collected from nine states and four regions of the United States.

Isolate	State	Region	Line or genotype								
			Chinese Spring	Alcedo	S740-69	AI(B)	AII(C)	AIII(D)	AV(E)	AIV(F)	AVIII(G)
GAP-A-2-3	GA	Southeast	S	S	R	S	S	S/I	R	I	R
GAP-B-2-2	GA	Southeast	S	S	R	S/I	S	S	R	S	R
MSG-A-3-1	MS	Southeast	S	S	R	S	S	R	R	R	I
MSG-D-1-5	MS	Southeast	S	S	R	S	S	S	I	S	R/I
NCF-D-1-1	NC	Mid-Atlantic	S	S	R	S	S	R	R	I	I
NCC-B-1-3	NC	Mid-Atlantic	S	S	R	S	S	I	R	S	R
NYA-E-3-3	NY	Great Lakes	S	S	R	S	S	-	R	S	R
NYB-E-1-2	NY	Great Lakes	S	S	R	S	S	S	R	S	S
PAF(14)-D-1-2	PA	Great Lakes	S	S	R	S	S	S	R	S	R
PAF-E-2-2	PA	Great Lakes	S	S	R	S	S	R	R	R	I
MIR(14)-D-3-3	MI	Great Lakes	S	S	R	S	S	R	R	I	R
MIR(14)-E-1-3	MI	Great Lakes	S	S	R	S	S	R	R	I	R
MTG1-3a	MT	Great Plains	S	S	R	S	S/I	R	R	R	S/I
MTG1-1a	MT	Great Plains	S	I	R	S	S	R	R	R	R
OKH-A-2-3	OK	Great Plains	S	S	R	S	S	R	R	R	R/I
OKS-A-2-2	OK	Great Plains	S	S	R	S	S	R	R	R	I
OKS-B-2-2	OK	Great Plains	S	S	R	S	S	R	R	R	I
NEI3-1	NE	Great Plains	S	S	R	S	S	R	R	R	I
NEI1-3	NE	Great Plains	S	S	R	S	S	R	R	R	I
NEI5-5	NE	Great Plains	S	S	R	S	S/I	R	R	R	I

Average scores were less than 4, between 4 and 6, and higher than 6 for resistant (R), intermediate (I), and susceptible (S), respectively.

chromosomes in AI(B), AII(C), AIII(D), AV(E), AIV(F), and AVIII(G) belong to groups 2, 5, 6, 7, 3, and 4, respectively.

Spike traits were recorded for each *Ae. markgrafii* addition line, and in two instances, the observed trait (**Table 1**) corresponded with the molecular marker data. In AI(B), spikes were non-free threshing. Genes for tenacious glume (*Tg*) have been identified on group 2 chromosomes (Simonetti et al., 1999; Jantasuriyarat et al., 2004; Sood et al., 2009; Faris et al., 2014; Katkout et al., 2014). Genes for brittle rachis have been located to group 3 chromosomes (Nalam et al., 2006; Watanabe et al., 2006). These observations agree with the conclusion that *Ae. markgrafii* chromosomes B and F are homoeologous to group 2 and 3, respectively. Other spike traits did not yield useful homoeology information. For example, large glumes and club spikes were associated with chromosomes C (group 5) and F (group 3), respectively. The large glume trait of *T. polonicum* has been mapped to group 7 chromosomes (Watanabe, 1999), while the club spike trait is a group 2 characteristic (Johnson et al., 2008). The failure to observe corresponding results between the molecular data and the morphological traits may represent either incomplete knowledge of the trait, impurity of the Alcedo background, or may indicate that the chromosomes in question carry chromosomal rearrangements.

We observed resistance to leaf rust conditioned by chromosome B of *Ae. markgrafii*. In contrast, Gong et al. (2017) found that chromosome D conditioned resistance to leaf rust, while chromosome B provided no leaf rust resistance. It is possible that the differences in these two studies may represent differential response to races. However, Iqbal et al. (2007) transferred leaf rust resistance from *Ae. markgrafii* to wheat chromosome arm 2AS, and they noted that chromosome B was the likely source of this gene.

Alcedo has been reported to carry two major and two minor genes conferring adult-plant resistance to stripe rust (Jagger et al., 2011). When we tested the addition lines for resistance to six US races of *P. striiformis* f. sp. *tritici* in the seedling stage, we observed a susceptible reaction on Alcedo, all addition lines, and the amphidiploid. Our results were consistent with Jagger et al. (2011) that Alcedo was susceptible in the seedling stages to the United Kingdom isolates used in the field tests. However, the seedling tests could not detect the adult-plant resistance in Alcedo. Nevertheless, the seedling data showed that the addition lines did not get any genes from *Ae. markgrafii* for all-stage resistance against the current predominant and most virulent races in the United States. Further tests of the lines with the races at the adult-plant stage or in the field are needed to determine if the addition lines inherited any adult-plant resistance genes from Alcedo and/or from *Ae. markgrafii*.

For resistance to powdery mildew, Gong et al. (2017) tested the six addition lines and parents using mixed races of the pathogen in China. They identified only line AV(E) as carrying resistance from *Ae. markgrafii*. However, we found that four addition lines, AIII(D), AV(E), AIV(F), and AVIII(G), carried powdery mildew resistance and *Ae. markgrafii* accession S740-69 was resistant to all 20 isolates tested in our study. The resistance conferred by chromosomes D, F, and G was generally confined to isolates originating from a geographical region and thus restricts their

adaptability. The E chromosome conferred resistance to 19 of the 20 powdery mildew isolates in the test, making it particularly attractive for alien gene introgression.

We report here tests for powdery mildew, stripe rust, and leaf rust resistance. The previous study of Xu et al. (2009) identified chromosomes C and D as carrying resistance to the Ug99 race group of the stem rust pathogen. Therefore, each of the six addition lines carries resistance to at least one fungal disease, making this a rich resource for gene introgression. Alien gene introgression is very valuable for introduction of new traits into wheat. Historically these introgressions were the product of homoeologous recombination or radiation induced chromosomal breakage which usually required standard cytogenetic techniques. With the incorporation of molecular markers as a tool to select recombinants, induced homoeologous recombination is much more effective than techniques that relied on cytogenetic observation. This study therefore identifies not only which lines carry disease resistance genes, but also identifies markers that can be used to detect recombination. By using the SSR markers associated with *Ae. markgrafii* chromosome D, we recently introgressed a new gene for Ug99 resistance from AIII(D) into common wheat (Xu et al., 2017).

AUTHOR CONTRIBUTIONS

SX conceived and planned this study. ZN, DK, and SC conducted marker analysis. ZN, XWC, BF, BG, and SX conducted molecular and cytogenetic analysis on alien chromosomes. RW and CC performed assay for powdery mildew resistance. MB and JR conducted leaf rust test. XMC conducted stripe rust test. ZN, DK, and SX wrote the manuscript. All authors reviewed and edited the manuscript.

FUNDING

This research was supported in part by funds to SX provided through a grant from the Bill & Melinda Gates Foundation and United Kingdom Department for International Development to Cornell University for the Borlaug Global Rust Initiative (BGRI) Durable Rust Resistance in Wheat (DRRW) Project and the USDA-ARS CRIS Project No. 5442-22000-080-037-00D.

ACKNOWLEDGMENTS

Authors thank Mary Osenga for technical support in SSR marker analysis. All opinions expressed in this paper are the author's and do not necessarily reflect the policies and views of USDA. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the United States Department of Agriculture. USDA is an equal opportunity provider and employer.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01616/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 14 November 2018
doi: 10.3389/fpls.2018.01665

TILL-D: An *Aegilops tauschii* TILLING Resource for Wheat Improvement

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Aegilops tauschii (2n = 2x = 14, genome DD), also known as Tausch's goatgrass, is the D genome donor of bread or hexaploid wheat *Triticum aestivum* (2n = 2x = 42, AABBDD genome). It is a rich reservoir of useful genes for biotic and abiotic stress tolerance for wheat improvement. We developed a TILLING (Targeting Induced Local Lesions In Genomes) resource for *Ae. tauschii* for discovery and validation of useful genes in the D genome of wheat. The population, referred to as TILL-D, was developed with ethyl methanesulfonate (EMS) mutagen. The survival rate in M_1 generation was 73%, out of which 22% plants were sterile. In the M_2 generation 25% of the planted seeds showed phenotypic mutations such as albinos, chlorinas, no germination, variegated, sterile and partially fertile events, and 2,656 produced fertile M_2 plants. The *waxy* gene was used to calculate the mutation frequency (1/70 kb) of the developed population, which was found to be higher than known mutation frequencies for diploid plants (1/89–1/1000 kb), but lower than that for a polyploid species (1/24–1/51 kb). The TILL-D resource, together with the newly published *Ae. tauschii* reference genome sequence, will facilitate gene discoveries and validations of agronomically important traits and their eventual fine transfer in bread wheat.

Keywords: *Aegilops tauschii*, D genome donor, bread wheat, TILLING, mutation frequency, genes

OPEN ACCESS

Edited by:
Hikmet Budak, Montana State University, United States

Reviewed by:
Shun Sakuma, Tottori University, Japan
Robert VanBuren, Michigan State University, United States

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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *30 August 2018*
Accepted: *26 October 2018*
Published: *14 November 2018*

Citation:
Rawat N, Schoen A, Singh L, Mahlandt A, Wilson DL, Liu S, Lin G, Gill BS and Tiwari VK (2018) TILL-D: An Aegilops tauschii TILLING Resource for Wheat Improvement. Front. Plant Sci. 9:1665. doi: 10.3389/fpls.2018.01665

INTRODUCTION

Hexaploid bread wheat *Triticum aestivum* (2n = 6x = 42, AABBDD) arose by a hybridization event between cultivated emmer wheat *T. turgidum* (2n = 4x = 28, AABB) and *Aegilops tauschii* (2n = 2x = 14, DD) in the south of the Caspian Sea around 8,000 years ago (McFadden and Sears, 1946; Kihara et al., 1965; Wang et al., 2013). *T. aestivum* is a comparatively young member of the Triticeae tribe having a narrow genetic base (Dvorak et al., 1998). Enhancing the genetic diversity of wheat will be essential to cope with rapid evolution of pathogen races, changing climatic conditions, and demand for increasing crop production (Mba et al., 2012; Mondal et al., 2016). *Ae. tauschii* is an excellent source of useful genes against diseases and abiotic stresses (Gill et al., 1986b; Dhaliwal et al., 1991; Assefa and Fehrmann, 2004; Kalia et al., 2016). Genetic closeness to wheat, easy crossability and rich diversity of useful genes and alleles make it simple and convenient to use *Ae. tauschii* for wheat improvement (Gill et al., 2006). Transfer of useful genes from *Ae. tauschii* to wheat can be done either by direct hybridization or as synthetic hexaploid wheat (McFadden and Sears, 1946; Gill and Raupp, 1987). Both of these approaches have

been used to transfer resistance against pathogens (Dhaliwal et al., 1991; Cox et al., 1994; Murphy et al., 1998; Singh et al., 2000), pests (Gill et al., 1986a; Xu et al., 2006), abiotic stresses (Ryan et al., 2010; Pradhan et al., 2012; Ogbonnaya et al., 2013), for quality traits (Lagudah et al., 1987; Cox et al., 1997; Brown-Guedira et al., 2005) as well as yield parameters (Mujeeb-Kazi et al., 2008; Okamoto et al., 2013).

Bread wheat is one of the most important staple food crops of the world. Due to its large genome size ($\sim 16 \times 10^9$ bp/1C, Arumuganathan and Earle, 1991; International Wheat Genome Sequencing Consortium [IWGSC], 2018), high repeat content (more than 85%, Kam-Morgan et al., 1989; International Wheat Genome Sequencing Consortium [IWGSC], 2018), and the three constituent homologous genomes, gene-discovery and map-based cloning has lagged behind other crops such as maize and rice (Rasheed et al., 2018). Diploid progenitors such as the D genome donor of wheat – *Ae. tauschii* provide a practical alternative for gene identification and cloning in wheat (Huang et al., 2003; Ling et al., 2004). The recent availability of reference-quality genome sequence of *Ae. tauschii* will speed up gene cloning in wheat, especially those coming from the D genome (Luo et al., 2017; Rasheed et al., 2018). This will open tremendous opportunities to transfer more useful genes from *Ae. tauschii* as well as their identification and molecular characterization. Genetic and reverse-genetic populations of *Ae. tauschii* will provide resources for identifying and validating useful genes with the help of its high-quality reference genome sequence. In the present work, we report the development and characterization of a TILLING (Targeting Induced Local Lesions In Genomes) resource of *Ae. tauschii*. Establishing phenotype to genotype relations and allocating function to variant alleles/ genes is comparatively easier working directly with the 'D' genome TILLING resource.

TILLING offers several advantages over other reverse genetics approaches because it can successfully be applied to any plant species or variety (Greene et al., 2003). Gene validation strategies such as genome-editing or RNAi-induced gene silencing are promising but limited because of transformation bottlenecks. At present, transformation efficiency of most of the wheat varieties is very low (Bhalla, 2006; Harwood, 2012). Additionally, TILLING populations provide immortal collections of variants for any gene, unlike transformation-based approaches, where every gene has to be targeted specifically (Uauy et al., 2009). TILLING has been used extensively to validate gene functions in wheat gene cloning projects (Krattinger et al., 2009; Periyannan et al., 2013; Saintenac et al., 2013; Moore et al., 2015; Rawat et al., 2016). TILLING in a number of genotypes of hexaploid and tetraploid wheat has been reported by several researchers (Slade et al., 2005; Xin et al., 2008; Dong et al., 2009; Uauy et al., 2009). Rawat et al. (2012) developed a TILLING resource in 'A' genome wheat *T. monococcum* as a diploid model to investigate gene functions in bread wheat. In addition to their use as reverse genetic resources for gene validation, mutagenized populations have been used for rapid cloning of disease resistance genes in plants using MutRenSeq approach (Steuernagel et al., 2016). TILLING populations also serve as resources for forward genetic screens for useful novel mutations in genes (Caldwell et al., 2004; Parry et al., 2009; Kurowska et al., 2011). In the present work, we report the development and characterization of a TILLING resource of *Ae. tauschii* as a tool for gene discovery and validation for D genome of bread wheat.

MATERIALS AND METHODS

Plant Material and EMS Mutagenesis

Aegilops tauschii subsp. *strangulata* (WGRC accession number TA 2450) was used to develop the TILL-D TILLING population. **Figure 1** shows some pictures of wild type *Ae. tauschii* subsp. *strangulata* plants and spikes. Seeds were manually peeled from the tough spikes of *Ae. tauschii*. Accession TA 2450 is a winter-type genotype originally collected from Iran, 5 km west of Behshahr (36.692373 latitude, 53.475609 longitude, 8 m altitude). Subsequently seeds were maintained and increased at the Wheat Genetics Resource Center (WGRC) at Kansas State University, Manhattan, KS, United States. All the plants were grown in a 1:1 vermiculite:soil mixture in cone of 2 inch diameter. The seedlings at two leaf-stage were vernalized at 4°C for 6 weeks in growth chambers, after which they were grown in greenhouse at 20–25°C with a light period of 16 h. Since *Ae. tauschii* shatters at maturity, all the plants were individually enclosed in plastic covers before the onset of flowering to avoid loss of seeds.

To chemically mutagenize the seeds, Ethyl methanesulfonate (EMS) from Sigma Aldrich (Cat No. M0880-25G) was used as the mutagen. To determine the appropriate concentration of EMS, two rounds of tests were made. The target of first round was to optimize a dose of the EMS needed to achieve 40–60% survival among the M_1 plants. Based on our prior experience with a diploid 'A' genome *Triticum monococcum* TILLING population (Rawat et al., 2012), first round of treatments included low concentrations of EMS solutions (0.15, 0.2, 0.24, and 0.27%). The protocol for mutagenesis was same as of Rawat et al. (2012). Briefly, 100 seeds of TA 2450 were soaked in 50 ml water in 250 ml glass flasks for 8 h of imbibition on a shaker at 100 rpm and then treated with five different doses (0, 0.15, 0.2, 0.24, and 0.27%) of EMS for 16 h on shaker at 75 rpm. The treated seeds were washed under running water for 8 h and then transplanted individually into cones. Observations were made 15 days after transplanting to estimate the survival frequency. However, the LD50 of these low treatments were found to be much lower than desirable. So, another round of dosage optimization was made with much higher concentrations this time (0.3, 0.4, 0.5, 0.6, and 0.7%) using the same method. Untreated controls were included in all the experiments to make valid comparisons of all the treatment dosages. EMS dose of 0.6% was found to provide 60% survival and was selected for treating a total of 5,300 TA-2450 seeds.

A total of 3,887 M_1 plants were derived from the M_0 EMS-treated seed and were allowed to self. Two thousand nine hundred and seventy M_1 plants were fertile. A single M_2 plant was grown from every M_1 plant to prevent genetic redundancy. Tissue was collected, and the spikes cataloged at maturity for all fertile 2,656 M_2 individuals of the TILLING population.

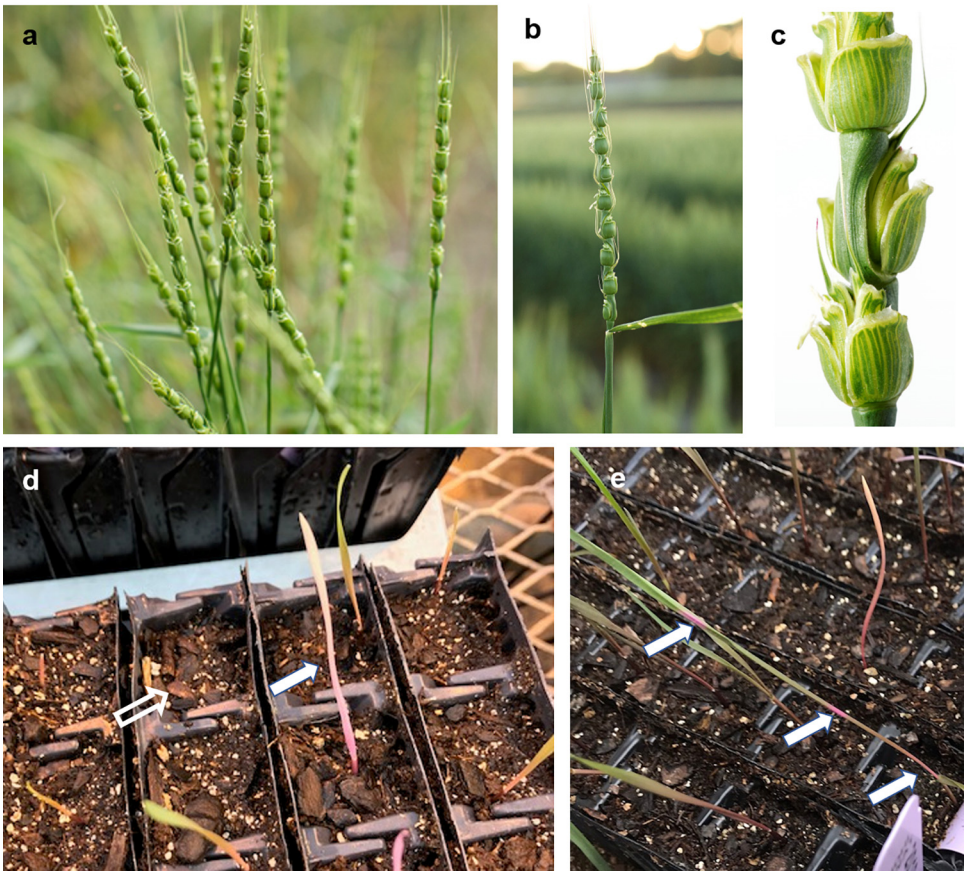

FIGURE 1 | Spikes of wild type *Aegilops tauschii* subsp. *strangulata* and some phenotypic mutant seedlings. **(a,b)** Spikes of *Ae. tauschii* subsp. *strangulata* wild type plants, **(c)** close-up of a some spikelets of a wild type spike, **(d)** an albino mutant (marked with a solid arrow), and no germination (marked with a hollow arrow), **(e)** a variegated mutant seedling showing bands of pink coloration (indicated with arrows) on the leaf.

Development of DNA Pools

Leaf tissue from all M_2 individuals was collected at the four-leaf stage in 96-well blocks. The tissue was lyophilized and stored in −80°C until use. A set of 1,180 M_2 plants was used for DNA extraction and subsequent characterization of the TILLING population. DNA was isolated using a Kingfisher Flex DNA extraction robot with Biosprint 96 plant DNA extraction kit (Qiagen, Valencia, CA, United States) according to the manufacturer's instructions. DNA was quantified on a Nano-drop and normalized to 25 ng/μl in 96-well blocks. Subsequently, 4x pooling was done, combining four plates of DNA into one pool plate retaining the row and column identity of the samples. Two hundred microliters of normalized DNA from each pool member was combined. The mutants were cataloged, and their DNA was identified with a unique ID as Pool-Plate-Row-Column.

TILLING for Genes of Interest

Waxy gene, that encodes a granule-bound starch synthase protein required for the synthesis of amylose in starchy endosperms was used for characterizing mutation frequency of our TILLING population (Yamamori et al., 1994). Two primer pairs were used that covered Exons 2–5 and Exons 5–7. **Figure 2** shows the position of primers used for the *waxy* gene. One more gene, *4-coumarate-CoA ligase 1* (*4CL1*), from lignin biosynthesis pathway, previously used in characterizing a diploid 'A' genome TILLING population (Rawat et al., 2012) was also used for the sake of making comparison with another diploid wheat species. List of primers used has been provided in **Table 1**.

PCR, Cel-I Assays and Mutant Detection

The primers were used on pooled DNA for PCR amplification using Bioline MyTaq PCR kits (Bioline, Tauton, MA, United States) in 25 ul volume, using BioRad 100 thermocycler (BioRad, Hercules, CA, United States). All 1,180 pooled M_2 individuals were screened for mutations in the waxy gene and 4CL1 gene. A touch down PCR profile (95°C–5 min, seven cycles of 95°C–1 min, 67–60°C-min with a decrease of 1°C per cycle, 72°C–2 min, followed by 30 cycles of 95°C–1 min, 60°C–1 min, 72°C–2 min, and a final extension of 72°C–7 min) was used. PCR products were subsequently denatured and slowly reannealed to form heteroduplexes between mismatched DNA (95°C–2 min, five cycles of 95°C–01 s, 95–85°C-1 min with a decrease of 2°C per cycle, and 60 cycles of 85–25°C–10 s. Cel-1 endonuclease was extracted from celery stalks following the

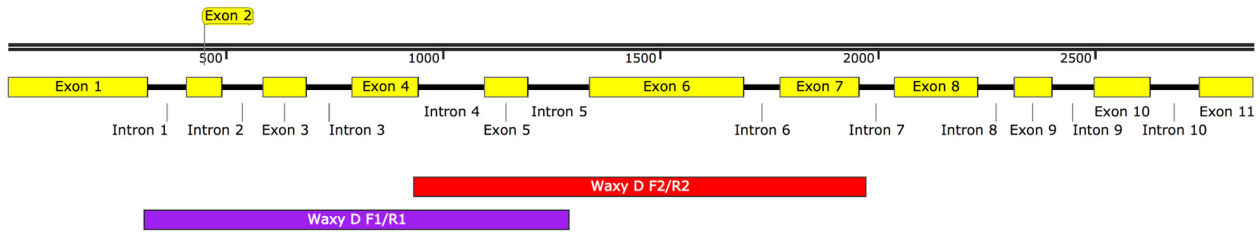

FIGURE 2 | Position of primers used for TILLING *waxy* gene in the TILL-D resource.

TABLE 1 | Primer sequences and product sizes of the primers used for TILLING.

Primer name	Gene	Sequence 5′–3′	Product size
Waxy_D_F1	*Waxy*	CCATGGCCGTAAGCTAGAC	978
Waxy_D_R1		CGCAAAATTGATATGCCTGTT	
Waxy_D_F2	*Waxy*	TGGGCCCTACGGTAAGATC	1039
Waxy_D_R2		GGGCTCGATGATGTACCAGG	
4CL1_CF	*4CL1*	AGAGTCCACCAAGAACACCATC	782
4CL1_CR		CTGGCTCTCAAGTCCTTCCTC	

protocol of Till et al. (2006) The homemade Cel-1 was tested for optimum activity with known mutants characterized previously. Two and a half μl of Cel-I was added to the heteroduplexed products and incubated at 45°C for 45 min. Reactions were stopped using 2.5 μl 0.5 M EDTA.

The digested products were visualized on 2.5% agarose gels. Mutants could be identified as the products showing cleaved bands in addition to the full-length, uncleaved product (**Figure 3**). The total number of bases scanned was calculated by subtracting 20% of the product size, to take into account the primer base pairs and terminal regions that escape detection as has been done previously on various detection platforms (Slade et al., 2005; Dong et al., 2009; Rawat et al., 2012).

Deconvolution and Sequencing of Mutants

Pools identified to carry mutation were deconvoluted using the same procedure as described above on the individual members of the pools. To identify the homo/heterozygosity of the mutant plants, PCR was performed with each constituent pool member in two copies, one with wild type *Ae. tauschii* DNA added, and another without it. After identifying the individual carrying the mutation, Sanger sequencing of the PCR product was done on an ABI3739xl (Applied Biosystems, Foster City, CA, United States) as per the manufacturer's instructions. Provean scores were calculated for mis-sense mutations using PROVEAN protein webtool at http://provean.jcvi.org.

RESULTS

Development of the TILL-D Population

Two rounds of dosage optimization experiments were conducted for developing the TILL-D population. The initial experiment was done with EMS concentrations of 0.15, 0.2, 0.24, 0.27, and 0.3%. However, the kill-rate, an indicator of the effectiveness of mutagenesis, was very low (**Figure 4**) with these concentrations. Next round of treatments included much higher concentrations (0.3, 0.4, 0.5, 0.6, and 0.7%) of EMS (**Figure 4**). Treatment with 0.6% EMS solution was found to provide an optimal kill-rate of 40% and was selected for the treatment of the entire batch of 5,300 seeds of *Ae. tauschii* accession TA2450. A total of 3,887 M_1 plants were produced from the treatment. Out of these 2,970 plants set M_2 seeds and were planted to produce the M_2 population. However, 153 M_2 seeds did not germinate, so leaf tissue could not be collected for these. One hundred and sixty-one M_2 plants were sterile. Seeds could not be retrieved in M_3 generation for both these types of mutants. The final size of the fertile TILL-D population that was cataloged at M_3 generation was 2,656 individuals.

The M_2 population showed phenotypic mutants such as albinos, chlorinas, very short, variegated, grass-like, and male sterile (**Figure 1**). Some M_2 seeds did not germinate at all. **Figure 5** shows the range of phenotypic mutants observed for the TILLING population. A total of 24.7% of the planted population showed phenotypic mutants.

TILLING for Identifying Mutants

Since the goal of this work was to characterize the mutation frequency of the TILL-D resource, we selected the *waxy* gene that has been extensively used for characterizing various TILLING populations. Mutation frequencies for the *waxy* gene can be compared with known diploid, tetraploid, and hexaploid wheat TILLING populations (Slade et al., 2005; Dong et al., 2009; Rawat et al., 2012). Cel-1 based mutation detection for *waxy* gene was done with two primer pairs, waxy_D_F1+R1 and waxy_D_F2+R2, giving product sizes of 978 and 1039 bp, respectively. A total of seven mutants, four for waxy_D_F1+R1, and three for waxy_D_F2+R2 were identified after screening 230 and 245 kb, respectively. The mutation

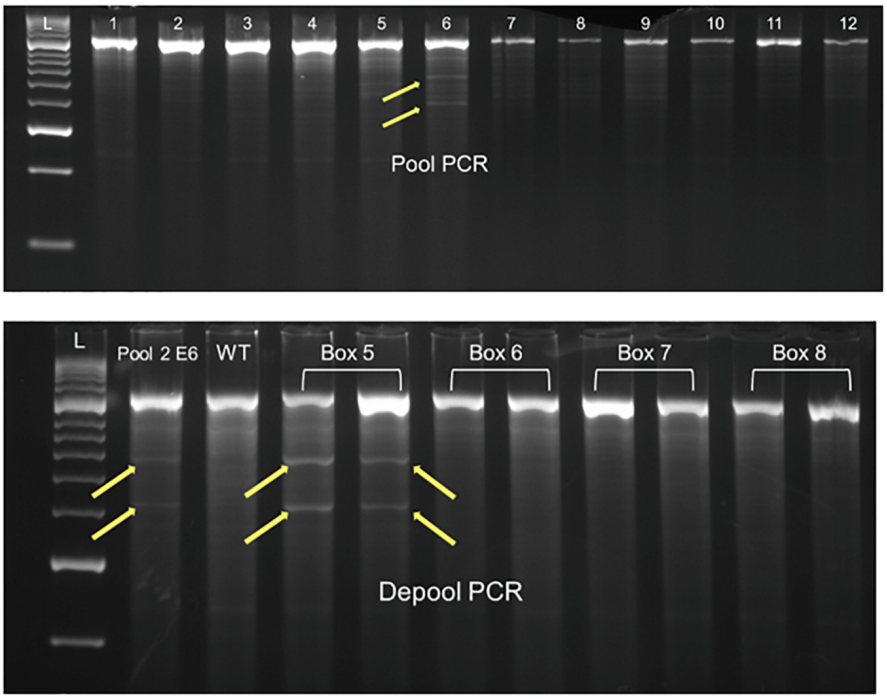

FIGURE 3 | Mutant identification using Cel-1 assay and agarose-gel based platform.

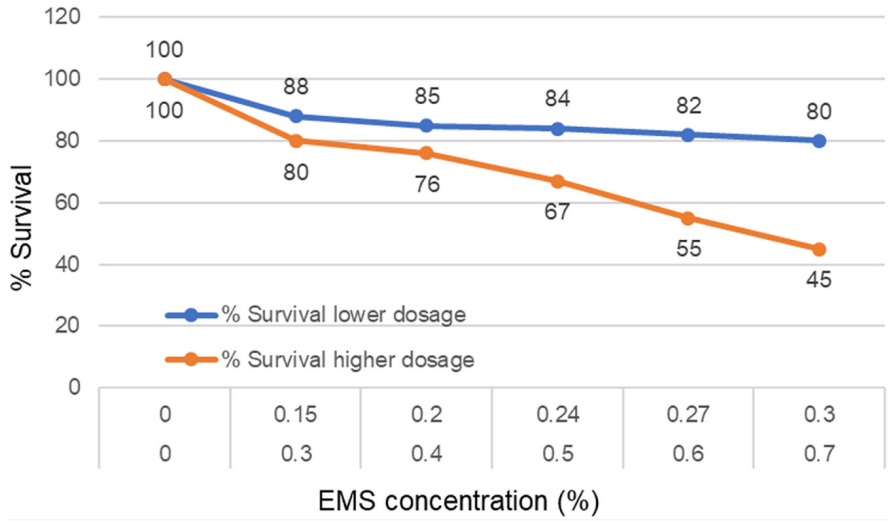

FIGURE 4 | Dosage optimization with various EMS concentrations to find appropriate concentration providing optimum survival of mutagenized individuals.

frequency of the TILL-D population using the *waxy* gene was found to be 1/70 kb. All the mutants identified for *waxy* were heterozygous.

TILLING was also performed on the gene *4CL1* using 4CL1_CF+CR primer pair, generating a product size of 782 bp. Two mutants were found after scanning a total of 185 kb, providing a mutation frequency of 1/92 kb for this gene. One of the *4CL1* mutants was homozygous, and the other had a heterozygous mutation.

The overall mutation frequency of the TILL-D population scanning a total of 661 kb using all the three primers was found to be 1/77 kb.

Sequencing of the Mutants

All the mutants identified were G > A or C > T transitions, characteristic mutations produced by EMS treatments (**Table 2**). Out of the seven mutants identified for the *waxy* gene, three lied in the introns. Out of the remaining four, two were silent

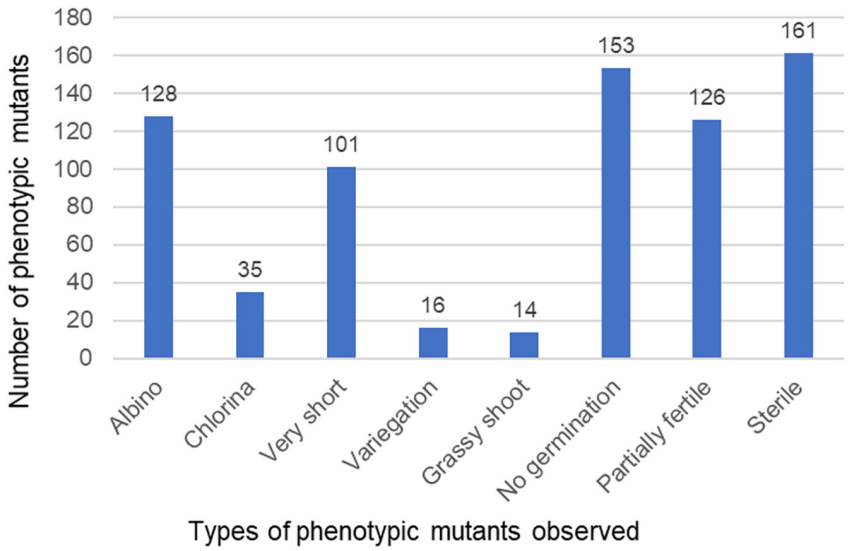

FIGURE 5 | Number of phenotypic mutants observed in the TILLING population.

TABLE 2 | Details of the sequence variations found in the mutant individuals of the TILL-D population.

Primer set forward/reverse	Plant ID (Pool-Box-RowColumn)	Base pair change	Homo/ heterozygous	Type of mutation	Amino acid change	Location of mutation	Provean score	Prediction
Waxy_D_F1+R1	2-8-E5	C > T	Hetero	Intronic	–	Intron 3	–	–
	2-5-E6	G > A	Hetero	Silent	Silent	Exon 4	–	–
	2-6-E2	C > T	Hetero	Intronic	–	Intron 3	–	–
	3-9-B5	C > T	Hetero	Silent	Silent	Exon 4	–	–
Waxy_D_F2+R2	3-9-H4	C > T	Hetero	Intronic	–	Intron 6	–	–
	2-7-D2	G > A	Hetero	Mis-sense	G348D	Exon 6	−6.098	Deleterious
	2-7-G9	C > T	Hetero	Mis-sense	A376T	Exon 7	−1.230	Neutral
4CL1_CR+CF	3-10-E10	C > T	Homo	Intronic	–	Inton 1	–	–
	1-4-F12	A > G	Hetero	Intronic	–	Intron 2	–	–

mutations that did not have any amino acid change, and the other two were mis-sense mutations that changed amino acid sequences. PROVEAN scores were calculated for the two mis-sense mutations and one was found to be deleterious (**Table 2**). The two mutations identified for the *4CL1* gene were both intronic.

DISCUSSION

Aegilops tauschii, the diploid D genome donor of hexaploid wheat, has been extensively used as a rich source of useful genes of biotic and abiotic stress tolerance, quality improvement and yield parameters for wheat improvement (Gill et al., 1986b; Dhaliwal et al., 1991; Rasheed et al., 2018). With the genome sequence availability of *Ae. tauschii* (Luo et al., 2017), it will be comparatively easier now to map the genes of interest derived from it. The TILL-D population provides an array of allelic variants that will be useful for both forward and reverse genetics of desirable traits. Nevertheless, developing mutagenized populations in diploid plants is a delicate exercise, as too low concentration of the mutagen is not very effective in creating sufficient mutations in the genome, and a high concentration treatment with the mutagen is detrimental to the survival of the plants. Polyploids such as durum and bread wheat, however, can tolerate higher doses of mutations because of genome buffering (Comai, 2005; Feldman and Levy, 2012). EMS concentrations ranging from 0.6 to 1% have been used in developing polyploid wheat TILLING populations (Sestili et al., 2009; Uauy et al., 2009; Chen et al., 2012; Rawat et al., 2016). On the other hand, Rawat et al. (2012) developed a diploid 'A' genome wheat *Triticum monococcum* TILLING population using a concentration of 0.25% EMS, achieving the mutation rate of 1/92 kb. In this work, however, *Ae. tauschii* apparently tolerated a much higher concentration of mutagen (0.6% EMS) as compared with *T. monococcum*. The tolerance of *Ae. tauschii* to higher mutagen concentration than *T. monococcum* is interesting and the exact reason for this observation is not known, as no prior documented report on mutagenesis of wild relatives of crop plants is available. *Ae. tauschii*, being a wild relative may be

hardier than cultivated einkorn wheat to tolerate mutations, that is also supported by the high mutation frequency of the TILL-D population.

Mutation frequency of the TILL-D population was found to be quite high (1/77 kb) for a diploid plant. Diploid TILLING populations of Arabidopsis (1/300 kb, Greene et al., 2003; 1/170 kb Till et al., 2003), sorghum (1/526 kb, Xin et al., 2008), rice (1/294 kb, Till et al., 2007; 1/135 kb, Suzuki et al., 2008), barley (1/1000 kb, Caldwell et al., 2004; 1/374 kb, Talame et al., 2008), and einkorn wheat (1/92 kb Rawat et al., 2012) have been reported to have much lower mutation frequencies. Massa et al. (2011) studied the gene space dynamics during the evolution of diploid *Ae. tauschii, Brachypodium distachyon,* rice, and sorghum, and found that due to widespread gene duplication and very low gene deletion events in *Ae. tauschii,* the overall gene number increased by 7,813 genes from the common ancestor. The rate of gene duplications and insertions over the past 45–60 million years was the highest and rate of gene deletions was the lowest in *Ae. tauschii* among the four diploid genomes relative to the common ancestor. This gene redundancy and higher gene content may be involved in making *Ae. tauschii* more tolerant of mutagenesis events in its genome. However, an exact explanation of this observation should be investigated further.

Different mutation frequencies are reported for different genes in wheat (Slade et al., 2005; Uauy et al., 2009). Therefore, we selected *waxy* gene for calculating the mutation frequency of *Ae. tauschii* TILLING population, to make valid comparisons with other diploid, tetraploid, and hexaploid wheat TILLING populations. A mutation frequency of 1/70 kb was observed for *waxy* gene in our TILL-D population, whereas tetraploid and hexaploid wheat TILLING populations have been reported to have much higher mutation frequencies for the same gene. Slade et al. (2005) reported mutation frequencies of 1/24 and 1/40 kb for *waxy* gene in TILLING populations of hexaploid wheat variety 'Express' and tetraploid wheat variety 'Kronos,' respectively. TILLING populations of hexaploid wheat varieties QAL2000 and Ventura were found to have mutation frequencies of 1/23 and 1/36 kb, respectively (Dong et al., 2009). The order of mutation frequencies of diploid < tetraploid < hexaploid with the same gene is as per expectations because of genome buffering that allows polyploids to tolerate higher number of variations per genome. It is an evolutionary advantage for the plants, but at the same time, makes it difficult to relate functions with genes (Comai, 2005; Dubcovsky and Dvorak, 2007; Rawat et al., 2012). Therefore, diploid genotypes provide resources for straightforward gene validation studies, allele mining, and quick gene discoveries.

CONCLUSION

The *Ae. tauschii* TILLING population developed will be a useful genetic resource for wheat improvement. Coupled with the *Ae. tauschii* genome sequence, it will provide a platform for allele mining and gene discovery in wheat. For gene cloning experiments of 'D' genome mapped genes it provides a permanent reverse genetic resource for gene function validation. With a high mutation frequency of 1 mutation every 77 kb, it is a rich permanent collection of variant alleles that can be exploited for either reverse genetics strategies or forward genetic screens to sift useful traits. Hexaploid TILLING populations in wheat have been found to have much higher mutation frequencies, but to see a phenotype due to a variant allele it is important to create mutation on all functional homoeologous (A, B, and D genome) copies of the gene. Having a mutant allele in a diploid will express the phenotype readily, leading to quick gene discovery. Such information can be used to generate mutants in bread wheat using hexaploid TILLING populations or gene editing approaches. Seed of the TILL-D population are being increased and will be made available after the M_4 generation to users upon request.

AUTHOR CONTRIBUTIONS

NR and VT conceived the study, planned the experiments, and were primarily responsible for drafting and revising the manuscript with contributions from co-authors. AS, LS, and AM performed DNA extraction, pooling, PCRs, and *Cel*-I extraction and sequence analysis. NR, GL, DW, BG, and SL developed the mutant population. DW phenotyped the population, collected and archived the tissue and seeds stocks. All authors read and approved the final manuscript.

FUNDING

The work at University of Maryland was supported by Maryland Agriculture Experiment Station grant to NR (MAES Grant No. 2956952). The work at Kansas State University was supported by NSF-IUCRC (Grant No. IIP-1338897).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 22 November 2018
doi: 10.3389/fpls.2018.01719

Identification of New Sources of Resistance to Wheat Stem Rust in *Aegilops* spp. in the Tertiary Genepool of Wheat

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Recent stem rust epidemics in eastern Africa and elsewhere demonstrated that wheat stem rust is a re-emerging disease posing a threat to wheat production worldwide. The cultivated wheat gene pool has a narrow genetic base for resistance to virulent races, such as races in the Ug99 race group. Wild relatives of wheat are a tractable source of stem rust resistance genes. *Aegilops* species in the tertiary genepool have not been exploited to any great extent as a source of stem rust resistance. We evaluated 1,422 accessions of *Aegilops* spp. for resistance to three highly virulent races (TTKSK, TRTTF, and TTTTF) of *Puccinia graminis* f. sp. *tritici*. Species studied include *Ae. biuncialis*, *Ae. caudata*, *Ae. comosa*, *Ae. cylindrica*, *Ae. geniculata*, *Ae. neglecta*, *Ae. peregrina*, *Ae. triuncialis*, and *Ae. umbellulata* that do not share common genomes with cultivated wheat. High frequencies of resistance were observed as 977 (68.8%), 927 (65.2%), and 850 (59.8%) accessions exhibited low infection types to races TTKSK, TTTTF, and TRTTF, respectively. Contingency table analyses showed strong association for resistance to different races in several *Aegilop*s spp., indicating that for a given species, the resistance genes effective against multiple races. Inheritance studies in selected accessions showed that resistance to race TTKSK is simply inherited.

Keywords: wild wheats, disease resistance, Ug99, genetic resources, tertiary genepool

OPEN ACCESS

Edited by:
István Molnár,
Centre for Agricultural Research (MTA), Hungary

Reviewed by:
Peter Michael Dracatos, University of Sydney, Australia
Hakan Ozkan, Çukurova University, Turkey

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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *26 August 2018*
Accepted: *05 November 2018*
Published: *22 November 2018*

Citation:
Olivera PD, Rouse MN and Jin Y (2018) Identification of New Sources of Resistance to Wheat Stem Rust in Aegilops spp. in the Tertiary Genepool of Wheat. Front. Plant Sci. 9:1719. doi: 10.3389/fpls.2018.01719

INTRODUCTION

Wheat stem rust, caused by *Puccinia graminis* Pers.:Pers. f. sp. *tritici* Eriks. & E. Henn. (*Pgt*), is a devastating disease of durum wheat (*Triticum turgidum* L. ssp. *durum*) and common or bread wheat (*T. aestivum* L.). Severe epidemics have been reported in all major wheat growing areas in the world (Roelfs, 1985; Saari and Prescott, 1985). For decades, stem rust has been under effective control through the use of genetic resistance. The occurrence and spread of *Sr31*-virulence races in the Ug99 race group in East Africa and other virulent races causing epidemics and localized outbreaks in Ethiopia (Olivera et al., 2015), Europe (Bhattacharya, 2017; Olivera Firpo et al., 2017; Lewis et al., 2018) and Central Asia (Shamanin et al., 2018), indicates that the disease is re-emerging as a threat to wheat production. Races in the Ug99 group have been detected across South, East and northern Africa, and the Middle East (Pretorius et al., 2000; Singh et al., 2015; Newcomb et al., 2016), and have the potential to reach critical wheat growing regions in the world (Park et al., 2011). The Ug99

race group has been rapidly evolving, producing variants with virulence to stem rust resistance genes including *Sr24* (Jin et al., 2008), *Sr36* (Jin et al., 2009), and *SrTmp* (Newcomb et al., 2016) that are important in stem rust resistance breeding (Singh et al., 2015).

The cultivated wheat gene pool has a narrow genetic base for resistance to the contemporary virulent races, such as TTKSK (Jin and Singh, 2006; Singh et al., 2006; Newcomb et al., 2016), TRTTF (Olivera et al., 2012); and TKTTF (Olivera Firpo et al., 2017). In order to broaden the basis of stem rust resistance in wheat breeding programs, it is necessary to identify and introgress effective genes from all genepools of wheat. Wild relatives of wheat are a tractable source of stem rust resistance genes. Indeed, a number of resistance genes derived from wild relatives of wheat appeared to be more effective against the races in the Ug99 group than *Sr* genes of wheat origin (Singh et al., 2006; Jin et al., 2007). *Aegilops* is the most closely related genus to *Triticum* (Kimber and Feldman, 1987; Jiang et al., 1994) and comprises 23 species that include diploid, tetraploid, and hexaploid genomes (van Slageren, 1994). *Aegilops* species are known to be a rich source of stem rust resistance, and several stem rust resistance genes have been transferred into cultivated wheat (Friebe et al., 1996; Schneider et al., 2008; Liu et al., 2011a,b; Olson et al., 2013a,b).

Ease of hybridization and reduced linkage drag make introgression from species in the primary gene pool preferred by wheat breeders to incorporate new alleles in their breeding programs (Feuillet et al., 2008). However, species in the secondary and tertiary gene pools constitute an important reservoir of genetic variability (Qi et al., 2007). *Aegilops* species in the tertiary genepool have not been exploited to any great extent for wheat improvement, and for resistance to TTKSK and other virulent *Pgt* races in particular. The objective of this study was to evaluate a collection of nine *Aegilops* species in the tertiary gene pool of wheat for resistance to race TTKSK and other *Pgt* races.

MATERIALS AND METHODS

Germplasm

A total of 1,422 accessions of nine *Aegilops* species (three diploid and six tetraploid) deposited at the USDA-ARS, National Small Grain Collection (NSGC), Aberdeen, ID, were evaluated in this study. Species, the number of accessions and country of origin of each *Aegilops* species are given in **Table 1**.

Inoculation, Incubation, and Disease Assessment

With the objective of identifying multiple and diverse resistance genes in individual accessions, we evaluated this *Aegilops* collection against multiple races with different virulence spectrum and origin. All accessions were characterized for reaction to three virulent *Pgt* races: TTKSK (Kenya), TRTTF (Yemen), and TTTTF (United States). Accessions resistant to the three races were further evaluated for their reaction to four additional US races (TPMKC, RKRQC, QTHJC, and QFCSC). The race designations are based on the letter code nomenclature system (Roelfs and Martens, 1988; Roelfs et al., 1993; Jin et al., 2008). Avirulence/virulence profile of the *Pgt* isolates used in the disease assessments is summarized in **Table 2**. Disease evaluations were conducted in two independent experiments. In each experiment, five seedlings per accession were inoculated with each race on fully expanded primary leaves 8–9 days after planting. Details on inoculation procedures and disease assessment were described by Jin et al. (2007). Disease reactions were classified according to Stakman et al. (1962). Infection types (ITs) 0, 1, and 2 were considered as resistant reactions and ITs 3 and 4 were considered as susceptible. Wheat cultivar McNair 701 (Cltr 15288) was included as susceptible check. Analyses of association via contingency tables were conducted to assess potential relationships of resistance to different *Pgt* races.

Inheritance Study

Bi-parental crosses between selected resistant accessions and a susceptible accession in five *Aegilops* species were made and F_2 progeny were produced by selfing F_1 plants. Seventeen F_2 populations (four from *Ae. cylindrica*, four from *Ae. peregrina*, six from *Ae. triuncialis*, two from *Ae. umbellulata*, and one from *Ae. comosa*) were evaluated for reaction to race TTKSK to determine the inheritance of resistance based on phenotypic ratios. Chi-square (χ^2) test was used to determine the goodness of fit to expected genetic ratios in the F_2 generation.

RESULTS

A wide array of infection types was observed across the *Aegilops* spp. and ranged from highly resistant (IT 0) to highly susceptible (ITs 3+ and 4). Low ITs (; or ;1−) were frequently observed in *Ae. caudata, Ae. cylindrica, Ae. neglecta, Ae. peregrina* and *Ae. triuncialis*, whereas ITs 2- and 2-; were predominant in *Ae. biuncialis* and *Ae. geniculata*. We observed a high percentage of resistance in this *Aegilops* collection as 977 (68.8%), 927 (65.2%), and 850 (59.8%) accessions produced low infection types to races TTKSK, TTTTF, and TRTTF, respectively (**Table 3**). Five hundred and fifty one (38.8%) accessions were resistant to the three races evaluated. The frequencies of accessions resistant to race TTKSK varied among the species: over 80% in six *Aegilops* species (*Ae. caudata, Ae. cylindrica, Ae. geniculata, Ae. neglecta, Ae. peregrina,* and *Ae. triuncialis*), and below 30% in three species (*Ae. biuncialis, Ae. comosa,* and *Ae. umbellulata*) (**Table 3**).

Pairwise association for resistance to races TTKSK, TRTTF, and TTTTF exhibited variation among species and pathogen races. Over 75% of the accessions of *Ae. geniculata* and *Ae. neglecta* were resistant to races TTKSK, TRTTF, and TTTTF (**Table 3**). Resistance to pairs of the three *Pgt* races in *Ae. geniculata* and *Ae. neglecta* were highly associated (**Table 4**), suggesting that accessions resistant to race TTKSK are likely to be resistant to races TRTTF and TTTTF. Association for the reaction to races TTSKS, TRTTF, and TTTTF was also observed

TABLE 1 | Number of accessions and country of origin of *Aegilops* species used in this study.

	geniculata	*cylindrica*	*biuncialis*	*triuncialis*	*comosa*	*caudata*	*neglecta*	*peregrina*	*umbellulata*	TOTAL
Turkey	79	87	82	148	1	33	125	3	66	624
Greece	34	2	85	73	58	28	27	0	0	307
Macedonia	3	5	11	21	0	0	13	0	0	53
Israel	0	1	0	0	0	0	0	48	0	49
Syria	11	0	15	9	0	0	0	7	0	42
Serbia	1	9	12	6	0	0	1	0	1	30
Cyprus	6	0	13	4	0	0	0	6	0	29
Ukraine	2	12	6	0	0	0	0	0	0	20
France	10	0	0	7	0	0	1	0	0	18
Azerbaijan	0	2	4	5	0	0	3	0	1	15
Iraq	0	1	1	4	0	1	7	0	1	15
Iran	0	0	0	10	0	0	1	0	2	13
Montenegro	4	0	0	1	0	0	7	0	0	12
Afghanistan	0	2	0	8	0	0	0	0	0	10
Others	15	24	4	9	0	1	9	5	0	67
Unknown	18	6	29	48	1	2	8	4	2	118
TOTAL	183	151	262	353	60	65	202	73	73	1422

TABLE 2 | Isolate designation, origin, and virulence phenotype of *Puccinia graminis* f. sp. *tritici* races used to evaluate resistance in *Aegilops* spp.

Race	Isolate	Origin	Virulence / avirulence formula
TTKSK[1]	04KEN156/04	Kenya	*Sr5 6 7b 8a 9a 9b 9d 9e 9g 10 11 17 21 30 31 38 McN / Sr24 36 Tmp*
TRTTF	06YEM34-1	Yemen	*Sr5 6 7b 9a 9b 9d 9e 9g 10 11 17 21 30 36 38 McN Tmp / Sr8a 24 31*
TTTTF	01MN84A-1-2	United States	*Sr5 6 7b 8a 9a 9b 9d 9e 9g 10 11 17 21 30 36 38 McN Tmp / Sr24 31*
TPMKC	74MN1409	United States	*Sr5 7b 8a 9d 9e 9g 10 11 17 21 36 McN Tmp / Sr6 9a 9b 24 30 31 38*
RKRQC	99KS76A-1	United States	*Sr5 6 7b 8a 9a 9b 9d 9g 17 21 36 McN / 9e 10 11 24 30 31 38 Tmp*
QTHJC	75ND717C	United States	*Sr5 6 8a 9b 9d 9g 10 11 17 21 McN / 7b 9a 9e 24 30 31 38 36 Tmp*
QFCSC	06ND76C	United States	*Sr 5 8a 9a 9d 9g 10 17 21 McN / Sr6 7b 9b 9e 11 24 30 31 36 38 Tmp*

[1] *Race nomenclature was based on Roelfs and Martens (1988) and Jin et al. (2008).*

TABLE 3 | Number and frequency of *Aegilops* accessions resistant to *Puccinia graminis* f. sp. *tritici* races TTKSK, TRTTF, and TTTTF at the seedling stage.

Species	Genome	Accessions evaluated	TTKSK		TRTTF		TTTTF		Resistant to 3 races	
			Number	Frequency	Number	Frequency	Number	Frequency	Number	Frequency
Ae. biuncialis	UUMM	262	75	0.27	179	0.68	82	0.31	34	0.13
Ae. caudata	CC	65	54	0.83	40	0.62	50	0.77	32	0.49
Ae. comosa	MM	60	10	0.17	10	0.17	11	0.19	3	0.05
Ae. cylindrica	DDCC	151	133	0.88	1	0.01	102	0.68	1	0.01
Ae. geniculata	UUMM	183	145	0.80	159	0.87	156	0.86	136	0.75
Ae. neglecta	UUMM	202	189	0.94	183	0.91	170	0.84	158	0.78
Ae. peregrina	SSUU	73	64	0.88	47	0.64	24	0.33	14	0.19
Ae. triuncialis	UUCC	353	290	0.82	198	0.56	315	0.98	166	0.47
Ae. umbellulata	UU	73	17	0.23	33	0.45	17	0.23	7	0.10
TOTAL		1,422	977	0.69	850	0.60	927	0.65	551	0.39

in *Ae. triuncialis* (**Table 4**). Resistance with race specificity was observed in accessions of the remaining species, most noticeably in *Ae. cylindrica*, where only one accession exhibited resistance to race TRTTF.

A group of 408 accessions resistant to races TTKSK, TRTTF, and TTTTF, were evaluated against US races TPMKC, RKRQC, QTHJC, and QFCSC. Three hundred ninety-six accessions remained resistant to all the races evaluated (**Supplementary Table 1**), indicating these accessions possess genes with broad spectrum resistance.

Sixty-five percent of the accessions evaluated in this study are native to Turkey or Greece. The frequencies of accessions resistant to all races from both countries were similar (30.6%) (**Table 5**). Higher frequencies of resistance were

TABLE 4 | Probability from contingency tables for association analysis of the reactions of accessions of *Aegilops* ssp. to races TTKSK, TRTTF, and TTTTF of *Puccinia graminis* f. sp. *tritici*.

	TTKSK vs. TTTTF		TTKSK vs. TRTTF		TRTTF vs. TTTTF	
	***P*-value**	**Association[a]**	***P*-value**	**Association**	***P*-value**	**Association**
Ae. biuncialis	< 0.001	Highly associated	0.648	Independent	0.989	Independent
Ae. caudata	0.608	Independent	< 0.001	Highly associated	0.740	Independent
Ae. comosa	0.014	Associated	0.158	Independent	0.038	Associated
Ae. cylindrica	0.037	Associated	0.756	Independent	0.545	Independent
Ae. geniculata	< 0.001	Highly associated	< 0.001	Highly associated	< 0.001	Highly associated
Ae. neglecta	< 0.001	Highly associated	0.011	Associated	< 0.001	Highly associated
Ae. peregrina	0.615	Independent	< 0.001	Highly associated	0.944	Independent
Ae. triuncialis	< 0.001	Highly associated	< 0.001	Highly associated	< 0.001	Highly associated
Ae. umbellulata	< 0.001	Highly associated	0.629	Independent	0.277	Independent

[a]*Based on P < 0.05.*

TABLE 5 | Number and percentage of resistant *Aegilops* species accessions according to country of origin.

Country of origin	Number of accessions evaluated	Number of resistant accessions[1]	Percentage (%) resistant accessions
Turkey	624	191	30.6
Greece	307	94	30.6
Macedonia	53	19	35.8
Israel	49	7	14.3
Syria	42	9	21.4
Serbia	30	3	10.0
Cyprus	29	4	13.8
Ukraine	20	1	5.0
France	18	7	38.9
Azerbaijan	15	2	13.3
Iraq	15	7	46.7
Iran	13	2	15.4
Montenegro	12	9	75.0
Afghanistan	10	2	20.0
Others	67	15	22.4
Unknown	118	36	30.5

[1]*Accessions resistant against all Pgt races (TTKSK, TRTTF, and TTTTF) evaluated.*

obtained in accessions from Macedonia (35.8%), France (38.9%), Iraq (46.7%), and Montenegro (75.0%), but the numbers of accessions evaluated from these countries were significantly smaller.

Segregation ratios of the F_2 progeny from biparental crosses between resistant and susceptible accessions indicated that resistance to race TTKSK in selected accessions is mostly conferred by single genes (**Table 6**). Eight resistant *Aegilops* accessions carry a single gene with dominant effect, whereas two resistant accessions carry a single gene with recessive effect. Two genes conferring resistance to race TTKSK were observed in three accessions of *Ae. triuncialis*. Inheritance with epistatic effect between two genes was also observed in four resistant parents. Segregation ratios of the F_2 progeny of one *Ae. triuncialis* and one *Ae. umbellulata* resistant parent fit to a 9:7 ratio indicating the presence of a complementary gene action with duplicate recessive epistasis. Epistatic effect between two dominant genes was also observed in two *Ae. peregrina* resistant parents (**Table 6**), where the F_2 progenies fit to a 11:5 ratio.

DISCUSSION

Races of *P. graminis* f. sp. *tritici*, such as the Ug99 race group, TKTTF and others detected from the contemporary *Pgt* populations worldwide, are a serious threat to bread and durum wheat production worldwide because of their broad virulence to many cultivars and rapid geographic spread. The limited number of stem rust resistance genes effective against these virulent races requires the identification of new sources of resistance. Different *Aegilops* species have contributed several stem rust resistance genes effective against race TTKSK including *Sr32, 33, 45, 46, 47, 51, 53*, *SrTA10187* and *SrTA10171* (Friebe et al., 1996; Schneider et al., 2008; Liu et al., 2011a,b; Olson et al., 2013a,b). However, only one gene, *Sr53,* is derived from *Ae. geniculata* in the tertiary genepool. Results from this study demonstrated that *Aegilops* species in the tertiary genepool of wheat are a rich source of resistance to race TTKSK and other *Pgt* races with broad virulence.

Although the overall frequency of resistant accessions in the entire *Aegilops* collection evaluated against races TTKSK, TRTTF, and TTTTF in this study was over 60%, we observed significant variation among species. Only two species (*Ae. geniculata* and *Ae. neglecta*) exhibited a high frequency of resistance (over 80%) against the three races. Interestingly in *Ae. biuncialis*, a species that also shares the same genome constitution as *Ae. geniculata* and *Ae. neglecta* (UUMM), the frequencies of resistance varied, exhibiting a high level of race specificity. Differences in the frequencies of resistance to stem, stripe, and leaf rust in species carrying the same genome have been also reported in the Section Sitopsis (SS genome) of *Aegilops* (Anikster et al., 2005; Scott et al., 2014). In species such as *Ae. geniculata* and *Ae. neglecta* where there is a high degree of association of the reactions to races

TABLE 6 | Segregation of F_2 populations of bi-parental crosses of *Aegilops* spp. to race TTKSK of *P. graminis* f. sp. *tritici*.

Species	Cross[a]	F_2 plants				
		Resistant	Susceptible	Ratio tested (R:S)	X^2	*P*-value
Aegilops comosa	PI 551049 (S) x PI 551054 (R)	28	108	1:3	1.412	0.235
Aegilops cylindrica	PI 554216 (S) x PI 254864 (R)	139	47	3:1	0.007	0.933
Aegilops cylindrica	PI 554216 (S) x PI 374345 (R)	140	32	3:1	3.752	0.053
Aegilops cylindrica	PI 554216 (S) x PI 568161 (R)	109	37	3:1	0.009	0.924
Aegilops cylindrica	PI 554216 (S) x PI 573369 (R)	104	27	3:1	1.346	0.246
Aegilops peregrina	PI 487274 (S) x PI 487278 (R)	24	69	1:3	0.032	0.858
Aegilops peregrina	PI 483010 (S) x PI 603931 (R)	107	55	11:5	0.550	0.458
Aegilops peregrina	PI 483010 (S) x PI 604185 (R)	127	57	11:5	0.002	0.937
Aegilops peregrina	PI 483010 (S) x PI 604193 (R)	135	49	3:1	0.261	0.610
Aegilops triuncialis	PI 173615 (S) x PI 219868 (R)	110	35	3:1	0.057	0.811
Aegilops triuncialis	PI 173615 (S) x PI 221899 (R)	59	41	9:7	0.307	0.579
Aegilops triuncialis	PI 173615 (S) x PI 254860 (R)	82	7	15:1	0.396	0.529
Aegilops triuncialis	PI 330492 (S) x PI 254861 (R)	71	25	3:1	0.056	0.814
Aegilops triuncialis	PI 330492 (S) x PI 374357 (R)	175	15	15:1	0.877	0.349
Aegilops triuncialis	PI 173615 (S) x PI 491436 (R)	150	11	15:1	0.093	0.760
Aegilops umbellulata	PI 542369 (S) x PI 298905 (R)	147	53	3:1	0.240	0.624
Aegilops umbellulata	PI 554395 (S) x PI 542375 (R)	90	64	9 :7	0.301	0.584

[a] *Female parent/Male parent; (R) and (S) indicate the resistant and susceptible parent, respectively.*

TTKSK, TRTTF, and TTTTF, it is highly likely that the genes that confer resistance to one race is also effective against the other races. The progeny populations via bi-parental crosses initiated through this study will be further developed and analyzed to understand the genetic relationships for resistance to different races in these selected accessions.

Race specificity was a common feature observed in this *Aegilops* collection, as five species exhibited a percentage of accessions resistant to all three races TTKSK, TRTTF, and TTTTF below 20% (**Table 3**), and have no association of the reaction of two out of three races. Previous studies also report race specificity in *Aegilops* species (Olivera et al., 2007; Scott et al., 2014). Since gene introgression from *Aegilops* species in the tertiary genepool is a long and laborious process, it is preferable to use accessions that carry stem rust resistance that is effective against multiple races. About 30% (396 accessions) were resistant against all the races evaluated, indicating the availability of potential sources of new and diverse stem rust resistance genes that could be very useful in wheat breeding programs. Most of these resistant accessions (84%) were from the tetraploid species *Ae. geniculata, Ae. neglecta*, and *Ae. triuncialis*. Additional studies are required to assess the diversity in these resistant accessions to allow the identification of donor accessions that are likely to contribute non-redundant stem rust resistance genes. Choosing resistant accessions from geographically diverse countries of origin and exhibiting different infection types for gene introgression is a first step to maximize the chances of capturing new and unique resistance genes (Anikster et al., 2005).

Sixty-five percent of the accessions evaluated in this study originated from Turkey or Greece, two countries having the largest numbers of *Aegilops* species. Turkey is known to be the center of diversity for *Aegilop*s (Eig, 1929), and 17 out of the 23 *Aegilops* species have been identified in its territory (van Slageren, 1994). The nine species evaluated in this study are present in Turkey. The number and frequency of resistant accessions from Turkey and Greece (n = 285, 30.6%) from this study demonstrated that valuable sources of new genetic variation for stem rust resistance are present in these countries.

A prior knowledge on the inheritance of resistance in wild wheat relatives will facilitate alien gene introgression into wheat. We produced 17 biparental crosses to investigate the inheritance of TTKSK resistance. These populations will be further developed to map resistance genes and to develop closely linked markers within the wild species. Simple inheritance of stem rust resistance was found in most selected resistant accessions. Our result of a single dominant gene segregating in the *Ae. umbellulata* biparental F_2 population from a cross between PI 542369 and PI 298905 was confirmed in an F_3 population and mapped to chromosome 2U (Edae et al., 2016). A similar approach will be followed to characterize the resistance identified in this study. Two stem rust resistance genes were identified in three *Ae. triuncialis* resistant parents. Further studies are needed to characterize the effectiveness of each resistance gene. Multiple stem rust resistance genes with different resistance profile were reported in *Ae. sharonensis* (Olivera et al., 2008; Yu et al., 2017). A more complex inheritance of stem rust resistance with genes exhibiting epistatic effects was also observed in three *Aegilops* species. These results highlight the value of studying the genetics of stem rust resistance in the wild relative before attempting wide crosses for gene transferring.

Aegilops species in the tertiary genepool do not possess genome(s) homologous to the cultivated forms, and gene transfer through homologous recombination cannot be achieved with

these species (Harlan and de Wet, 1971). Cytogenetic techniques such as irradiation and chemical treatments, production of synthetic amphiploids, use of gametocidal chromosomes, or *Ph1* gene mutants may be required for gene introgression into the cultivated forms (Friebe et al., 1996; Zaharieva and Monneveux, 2006). However, the introgression of alien chromatin to substitute for homoeologous chromosome segments has the potential of a simultaneous introduction of deleterious DNA that can affect agronomic and quality traits of wheat (Feuillet et al., 2008; Wulff and Moscou, 2014). New sequencing technologies, like Genotyping-By-Sequencing, have allowed the development of genetic linkage maps in wild relatives of wheat with non-previous available markers, and the identification of closely linked markers that can facilitate the gene transfer process by reducing the introgressed alien chromatin segment into elite materials (Edae et al., 2016, 2017). The sources of resistance identified from the tertiary genepool will also serve as targets for resistance gene cloning. Cloned genes and their delivery as transgenes in single or multiple resistance gene cassettes will completely resolve the linkage drag problem and ensure the effectiveness and durability of genes derived from more distant relatives of wheat (Wulff and Moscou, 2014). Today, new cloning techniques like mutational genomics (MutRenSeq) (Steuernagel et al., 2016) and association genetics with R gene enrichment sequencing (AgRenSeq) (Arora et al., 2018) allow a rapid and cheaper discovery and cloning of resistance genes. These technologies are opening new doors for fully exploiting the richness and diversity of wild relatives for wheat improvement.

AUTHOR CONTRIBUTIONS

PO and YJ were involved in the experimental design and manuscript preparation. PO performed the experiments and completed the data analysis. MR was involved in manuscript preparation and revision.

FUNDING

This research was funded by USDA-ARS and the Durable Rust Resistance in Wheat (OPP1144199) project administrated by Cornell University and funded by the Bill and Melinda Gates Foundation and the United Kingdom Department for International Development.

ACKNOWLEDGMENTS

We thank H. Bockleman, United States Department of Agriculture-Agricultural Research Service, National Small Grains Collection, Aberdeen, ID, for providing *Aegilops* spp. seeds.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01719/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 04 December 2018
doi: 10.3389/fpls.2018.01756

Evolution of the S-Genomes in *Triticum-Aegilops* Alliance: Evidences From Chromosome Analysis

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Five diploid *Aegilops* species of the *Sitopsis* section: *Ae. speltoides, Ae. longissima, Ae. sharonensis, Ae. searsii*, and *Ae. bicornis*, two tetraploid species *Ae. peregrina* (=*Ae. variabilis*) and *Ae. kotschyi* (*Aegilops* section) and hexaploid *Ae. vavilovii* (*Vertebrata* section) carry the S-genomes. The B- and G-genomes of polyploid wheat are also the derivatives of the S-genome. Evolution of the S-genome species was studied using Giemsa C-banding and fluorescence *in situ* hybridization (FISH) with DNA probes representing 5S (pTa794) and 18S-5.8S-26S (pTa71) rDNAs as well as nine tandem repeats: pSc119.2, pAesp_SAT86, Spelt-1, Spelt-52, pAs1, pTa-535, and pTa-s53. To correlate the C-banding and FISH patterns we used the microsatellites $(CTT)_{10}$ and $(GTT)_9$, which are major components of the C-banding positive heterochromatin in wheat. According to the results obtained, diploid species split into two groups corresponding to *Emarginata* and *Truncata* sub-sections, which differ in the C-banding patterns, distribution of rDNA and other repeats. The B- and G-genomes of polyploid wheat are most closely related to the S-genome of *Ae. speltoides*. The genomes of allopolyploid wheat have been evolved as a result of different species-specific chromosome translocations, sequence amplification, elimination and re-patterning of repetitive DNA sequences. These events occurred independently in different wheat species and in *Ae. speltoides*. The 5S rDNA locus of chromosome 1S was probably lost in ancient *Ae. speltoides* prior to formation of Timopheevii wheat, but after the emergence of ancient emmer. Evolution of *Emarginata* species was associated with an increase of C-banding and $(CTT)_{10}$-positive heterochromatin, amplification of Spelt-52, re-pattering of the pAesp_SAT86, and a gradual decrease in the amount of the D-genome-specific repeats pAs1, pTa-535, and pTa-s53. The emergence of *Ae. peregrina* and *Ae. kotschyi* did not lead to significant changes of the S*-genomes. However, partial elimination of 45S rDNA repeats from 5S* and 6S* chromosomes and alterations of C-banding and FISH-patterns have been detected. Similarity of the S^v-genome of *Ae. vavilovii* with the S^s genome of diploid *Ae. searsii* confirmed the origin of this hexaploid. A model of the S-genome evolution is suggested.

Keywords: wheat, *Aegilops*, S-genome of *Ae. speltoides*, S*-genome of other *Aegilops* species, chromosome, karyotype evolution, C-banding, FISH

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *30 July 2018*
Accepted: *12 November 2018*
Published: *04 December 2018*

Citation:
Ruban AS and Badaeva ED (2018) Evolution of the S-Genomes in Triticum-Aegilops Alliance: Evidences From Chromosome Analysis. Front. Plant Sci. 9:1756. doi: 10.3389/fpls.2018.01756

INTRODUCTION

Evolutionary goat grasses, or *Aegilops* are closely related to wheat and contributed two of the three subgenomes of hexaploid bread wheat (Sears, 1969; Kihara, 1975; Feldman, 2001). The natural distribution area of the genus *Aegilops* L. covers the Mediterranean basin, southwestern and central Asia (Witcombe, 1983; Kimber and Feldman, 1987; Van Slageren, 1994; Kilian et al., 2011). Their center of origin is thought to be located in Transcaucasia (Hammer, 1980; Van Slageren, 1994), or in the Fertile Crescent (Kimber and Feldman, 1987). These regions contain the highest concentration of *Aegilops* species. Goat grasses inhabit a broad range of environments and are characterized by very wide adaptation. Owing to this, many goat grasses exhibit good resistance to fungal diseases and pests (Hammer, 1980; Gill et al., 1985; Makkouk et al., 1994; El Bouhssini et al., 1998; Monneveux et al., 2000; Schneider et al., 2008; Zhao et al., 2016), heat, drought or frost tolerance and cold hardiness (Limin and Fowler, 1985; Damania et al., 1992; Monneveux et al., 2000; Pradhan et al., 2012). Some *Aegilops* accessions are characterized by high grain quality and increased micronutrient content (Rawat et al., 2011; Farkas et al., 2014; Rakszegi et al., 2017) that can be used for wheat improvement. Although many agronomically useful genes have already been transferred from *Aegilops* to common wheat varieties or breeding lines (Knott and Dvorák, 1976; Schneider et al., 2008; Rawat et al., 2011; McIntosh et al., 2013; Zhang et al., 2015), their genetic potential in broadening genetic diversity of wheat is not fully exploited. Utilization of gene pool of *Aegilops* requires good knowledge of genetics and genomics of these species, including their karyotypes and chromosomal structures.

In addition to the great potential for wheat breeding, goat grasses can also be an attractive model for studying mechanisms of reticulate evolution. Depending on taxonomical system, the genus *Aegilops* is classified into 20 (Kihara, 1954), 22 (Zhukovsky, 1928; Eig, 1929; Van Slageren, 1994), 24 (Kimber and Feldman, 1987), 25 (Chennaveeraiah, 1960), or 26 species (Witcombe, 1983). These species are split into sections based on morphological criteria or genome composition. At present, the system suggested by Van Slageren (1994) is commonly accepted; therefore, we will follow this nomenclature. According to it, 10 *Aegilops* species are diploid and 12 – polyploid, that were formed as a result of hybridization of different diploid progenitors.

Based on a series of pioneering works of the famous Japanese geneticist (Kihara, 1937, 1949, 1954, 1957, 1963; Lilienfeld, 1951; Kihara et al., 1959), diploid *Aegilops* were divided into three major genomic groups, C, D, and S. The C-genome group included two species; the D-genome group included four species; and the S-genome group consisted of three species of the *Sitopsis* (Jaub. & Spach) Zhuk. section: *Ae. longissima* Schweinf. & Muschl. (including *Ae. sharonensis* Eig), *Ae. bicornis* (Forssk.) Jaub. & Spach, and *Ae. speltoides* Tausch (Kihara, 1937, 1949; Lilienfeld, 1951). A new diploid species of the *Sitopsis* section—*Ae. searsii* Feldman and Kislev ex Hammer, has been discovered later by Feldman and Kislev (1977). Analysis of the karyotype, meiotic chromosome pairing, pollen fertility and seed set in *Ae. longissima x Ae. searsii* hybrids showed that *Ae. searsii* possesses the S*-genome (Feldman et al., 1979; Yen and Kimber, 1990a).

Thus, current taxonomy recognizes five diploid species carrying the S-genome: *Ae. speltoides* including ssp. *ligustica* (Savign.) Fiori (SS) and ssp. *speltoides* Boiss., *Ae. bicornis* (S^bS^b), *Ae. searsii* (S^sS^s), *Ae. sharonensis* ($S^{sh}S^{sh}$), and *Ae. longissima* (S^lS^l) (Van Slageren, 1994; Kilian et al., 2011; Feldman and Levy, 2015). These species are morphologically similar, but can be easily distinguished by their habitat, climatic adaptation, and distribution areas. Based on differences in spike morphology, Eig (1929) divided the *Sitopsis* group into two sub-sections, *Truncata* and *Emarginata*. Subsection *Truncata* includes only one species–*Ae. speltoides* (SS), which grows in central, eastern, and northern part of the *Sitopsis* area. This species consists of two forms, *ligustica* and *auscheri,* which differ in their fruiting spike and the mode of seed dispersal (Eig, 1929; Zohary and Imber, 1963), but are similar in karyotype structure (Chennaveeraiah, 1960). Their hybrids are fully fertile and show complete meiotic chromosome pairing (Zohary and Imber, 1963). *Ae. speltoides* has the lowest nuclear DNA content (1C = 5.81 ± 0.123 pg) within the *Sitosis* group (Eilam et al., 2007) and differs significantly from *Emarginata* species in its chromosome morphology (Chennaveeraiah, 1960), Giemsa C-banding (Teoh and Hutchinson, 1983; Friebe and Gill, 1996; Friebe et al., 2000) and FISH patterns (Yamamoto, 1992a,b; Jiang and Gill, 1994b; Badaeva et al., 1996a,b; Salina et al., 2006b; Raskina et al., 2011; Belyayev and Raskina, 2013).

The subsection *Emarginata* includes four species: *Ae. bicornis, Ae. searsii, Ae. sharonensis,* and *Ae. longissima,* which grow in the central and southern part of the *Sitopsis* section habitat (Feldman and Kislev, 1977). Study of the chromosome pairing of intraspecific hybrids (Kihara, 1954, 1963; Feldman et al., 1979; Yen and Kimber, 1989, 1990a,b,c), similarity of karyotype structure (Riley et al., 1958; Chennaveeraiah, 1960), the number and distribution of 5S and 45S rDNA loci (Yamamoto, 1992a,b; Badaeva et al., 1996b), and the distribution of pSc119.2 sequence (Badaeva et al., 1996a) suggest a close relationship of *Emarginata* species, although they differ from each other in genome size (Eilam et al., 2007) and C-banding patterns (Friebe and Gill, 1996).

Morphologically, *Ae. bicornis* is the most primitive species in this group (Eig, 1929). It is more difficult to produce hybrids with *Ae. bicornis* than with other *Aegilops* of the S-genome group (Kimber and Feldman, 1987). Genome size of *Ae. bicornis* (1C = 6.84 ± 0.097 pg) is only little larger than that of *Ae. searsii* (1C = 6.65 ± 0.091 pg), and is lower than of *Ae. longissima* (1C = 7.48 ± 0.082 pg) or *Ae. sharonensis* (1C = 7.52 ± 1.000 pg) (Eilam et al., 2007). Morphologically *Ae. searsii* resembles *Ae. longissima,* but differs from it in a number of morphological traits which are considered as evolutionary advanced (Feldman and Kislev, 1977). *Ae. longissima* x *Ae. searsii* hybrids exhibit meiotic irregularities and are highly sterile (Feldman et al., 1979). By contrast, the F_1 hybrids *Ae. longissima* x *Ae. sharonensis* are fertile and show complete chromosome pairing in meiosis. Isolation of these species is caused by different ecological requirements (Feldman and Levy, 2015). According to other hypothesis (Waines and Johnson, 1972), *Ae. sharonensis* could be a hybrid between

Ae. longissima and *Ae. bicornis*. *Ae. longissima* carries a species-specific 4S*/7S* translocation (Tanaka, 1955; Yen and Kimber, 1990b; Friebe et al., 1993; Naranjo, 1995), while no structural rearrangements have been identified in other species of this group (Yen and Kimber, 1989, 1990a,b,c; Maestra and Naranjo, 1997, 1998; Luo et al., 2005; Dobrovolskaya et al., 2011).

The similarity of *Emarginata* species and separate position of *Ae. speltoides* within the *Sitopsis* section was confirmed by molecular analyses of nuclear and cytoplasmic DNA. Based on the variation of repeated nucleotide sequences (RNS) Dvorák and Zhang (1992) showed that the *Sitopsis* species are phylogenetically similar, but *Ae. speltoides* is clearly separated from species of the *Emarginata* group. RAPD- and AFLP analyses revealed that *Ae. speltoides* forms a cluster with polyploid wheats, which is separated from other *Sitopsis* species (Kilian et al., 2007, 2011; Goryunova et al., 2008). Study of organellar DNAs by PCR-single-strand conformational polymorphism (PCR-SSCP) revealed high similarity of *Ae. bicornis* - *Ae. sharonensis* - *Ae. longissima* plasmons and their distinctness from plasmon of *Ae. speltoides* (Wang et al., 1997).

Comparative sequence analysis provided further insights into the evolution of *Triticum* and *Aegilops* and allowed the estimation of divergence time of different genomic groups. Comparison of chloroplast (Yamane and Kawahara, 2005; Golovnina et al., 2007; Gornicki et al., 2014; Middleton et al., 2014; Bernhardt et al., 2017) and nuclear DNA sequences (Petersen et al., 2006; Salse et al., 2008; Marcussen et al., 2014) strongly suggest that *Ae. speltoides* occupies a basal position on the phylogenetic tree of *Aegilops/Triticum* (Petersen et al., 2006; Kawahara, 2009). Probably *Ae. speltoides* diverged from the progenitor of the Triticeae much earlier than diploid wheat and *Aegilops* species (Yamane and Kawahara, 2005; Salse et al., 2008; Gornicki et al., 2014; Middleton et al., 2014; Bernhardt et al., 2017). Estimates obtained from the analyses of nuclear DNA sequences placed the possible divergence time within the period from ∼7 MYA (Marcussen et al., 2014) to 3.5–2.7 MYA (Dvorák and Akhunov, 2005; Salse et al., 2008). Estimates obtained from chloroplast DNA favored a more recent origin of *Ae. speltoides* – 4.1–3.6 MYA (Bernhardt et al., 2017) to 2.67 ± 1.1. MYA (Middleton et al., 2014). Marcussen et al. (2014) supposed that the D-genome lineage (which indeed included D, M, and S* genome species, Sandve et al., 2015) emerged through ancient homoploid hybridization between A and S genomes. The members of *Emarginata* group are thought to radiate from common ancestor approximately 1.0–2.0 MYA (*Ae. searsii*) – 1.4 MYA (*Ae. bicornis*) – to 0.4 MYA (*Ae. sharonensis*) (Marcussen et al., 2014; Feldman and Levy, 2015).

Hypothesis that the B and G genomes of polyploid wheats originated from a diploid S-genome *Aegilops* species was put forward in the middle XXth (Sears, 1956; Riley et al., 1958). Different taxa were suggested as potential progenitors of polyploid wheat (Haider, 2013). All species of the *Sitopsis* section have been considered as the B-genome donors: *Ae. speltoides* (Sarkar and Stebbins, 1956; Tanaka et al., 1979; Bahrman et al., 1988; Kerby et al., 1990; Daud and Gustafson, 1996; Maestra and Naranjo, 1998; Yan et al., 1998; Blake et al., 1999; Rodríguez et al., 2000a; Haider, 2013), *Ae. bicornis* (Sears, 1956), *Ae. longissima* (Tanaka, 1956; Konarev et al., 1976; Konarev, 1980; Peacock et al., 1981), *Ae. searsii* (Feldman and Kislev, 1977; Nath et al., 1983, 1984; Kerby et al., 1990; Liu et al., 2003), *Ae. sharonensis* (Kushnir and Halloran, 1981) or yet unknown species of the *Emarginata* group (Kerby et al., 1990). Molecular analyses of common wheat genome and genomes of related species confirmed the ancestry of wheat B- genome from *Ae. speltoides* or the species close to it (Talbert et al., 1991; Petersen et al., 2006; Goryunova et al., 2008; Salse et al., 2008; Marcussen et al., 2014). Based on the analysis of nuclear or plastid DNA, ancient tetraploid emmer could emerge 0.4–0.8 MYA (Huang et al., 2002; Dvorák and Akhunov, 2005; Yamane and Kawahara, 2005; Golovnina et al., 2007; Gornicki et al., 2014; Marcussen et al., 2014; Middleton et al., 2014; Bernhardt et al., 2017).

The origin of the G-genome of *Triticum timopheevii* Zhuk. from the S-genome of *Ae. speltoides* was first hypothesized by Giorgi and Bozzini (1969) based on comparison of chromosome morphologies and was later confirmed by numerous studies including chromosome pairing analysis of intraspecific hybrids (Shands and Kimber, 1973; Tanaka et al., 1979; Maestra and Naranjo, 1999; Rodríguez et al., 2000a), comparison of C-banding (Badaeva et al., 1996a) and ISH patterns (Jiang and Gill, 1994a,b; Salina et al., 2006b), isozyme profiles (Konarev et al., 1976; Nakai, 1978; Jaaska, 1980), AFLP- (Kilian et al., 2007, 2011) and RFLP-analyses (Dvorák and Zhang, 1990; Talbert et al., 1991; Dvorák, 1998), sequencing of nuclear (Huang et al., 2002) and cytoplasmic DNA (Sasanuma et al., 1996; Yamane and Kawahara, 2005; Golovnina et al., 2007; Gornicki et al., 2014). These studies revealed that *Ae. speltoides* is more closely related to the G genome of *T. timopheevii* than to the B-genome of common wheat and suggested that ancient *T. timopheevii* could emerge approximately 0.4 MYA (Huang et al., 2002; Gornicki et al., 2014).

The S*-genome is identified in two tetraploid *Aegilops* species belonging to the section *Aegilops* L.: *Ae. peregrina* (Hach. in Fraser) Maire & Weiller (=*Ae. variabilis* Eig, $U^pU^pS^pS^p$) and *Ae. kotschyi* Boiss. ($U^kU^kS^kS^k$). Based on the "analyzer" method H. Kihara (1954) proposed that *Ae. peregrina* is a hybrid between *Ae. umbellulata* Zhuk. and a diploid species of the *Sitopsis* group (Lilienfeld, 1951), although conventional chromosome staining did not reveal the S*-genome in these species (Chennaveeraiah, 1960). Cytoplasmic genomes of *Ae. peregrina* and *Ae. kotschyi* are most closely related to the cytoplasmic genome of *Ae. searsii* (Ogihara and Tsunewaki, 1988; Siregar et al., 1988). However, meiotic analysis of the F_1 hybrids between *Ae. kotschyi* and induced autotetraploid of three *Sitopsis* species showed that *Ae. kotschyi* shared the S* genome with *Ae. longissima* (Yen and Kimber, 1990d). Yu and Jahier (1992) come to the same conclusion based on chromosome pairing analysis in hybrids of *Ae. variabilis* (=*Ae. peregrina*) with different *Sitopsis* species. RFLP profiles of RNS suggested that the S* genome of *Ae. peregrina* and *Ae. kotschyi* could have originated from *Ae. longissima* or *Ae. sharonensis* or the species immediately preceding the divergence of these diploids (Zhang et al., 1992). C-banding and FISH analyses confirmed highest similarity of the S*-genome of these tetraploids with *Ae. longissima* or *Ae.*

sharonensis (Jewell, 1979; Jewell and Driscoll, 1983; Friebe et al., 1996; Badaeva et al., 2004; Zhao et al., 2016).

Ae. vavilovii (Zhuk.) Chennav. ($D^1D^1X^{cr}X^{cr}S^vS^v$) is a hexaploid taxa belonging to section *Vertebrata* Zhuk. emend Kihara, complex Crassa. *Ae. vavilovii* originated from hybridization of tetraploid *Ae. crassa* Boiss. with a species of *Emarginata* group, possibly *Ae. longissima* (Kihara, 1963; Kihara and Tanaka, 1970). Originally *Ae. vavilovii* was treated as a subspecies of hexaploid *Ae. crassa,* and its taxonomic rank was raised to independent biological species by Chennaveeraiah (1960). Although this author was unable to determine genome constitution of *Ae. vavilovii,* he noticed a pairwise similarity of the satellite chromosomes in karyotype of this species.

Yen and Kimber (1992) failed to identify the exact donor of the S^v-genome of *Ae. vavilovii* based on analysis of chromosome pairing in the F_1 hybrids of *Ae. vavilovii* with induced autotetraploids of the *Sitopsis* species and proposed that the S^v-genome is substantially modified. By using molecular markers (Talbert et al., 1991) showed that the S^v-genome of *Ae. vavilovii* is related to the S^*-genome of *Emarginata* group. Data collected by molecular methods (Zhang and Dvorák, 1992), C-banding and FISH analyses (Badaeva et al., 2002; Zhang et al., 2002) confirmed, that *Ae. vavilovii* contains the S^v-genome that could probably derive from *Ae. searsii* (Badaeva et al., 2002).

Because of the genetic relatedness of the S-genome *Aegilops* species and polyploid wheats as well as of their potential for wheat improvement, they have been attracting the attention of researchers over the past century. Numerous intraspecific hybrids have been created to transfer desired genes from *Aegilops* to wheat (Schneider et al., 2008). Sets of addition, substitution or translocation wheat-*Aegilops* lines, including *Ae. speltoides* (Friebe et al., 2000; Liu et al., 2016), *Ae. searsii* (Pietro et al., 1988; Friebe et al., 1995), *Ae. sharonensis* (Olivera et al., 2013), *Ae. longissima* (Friebe et al., 1993), and polyploid *Ae. peregrina,* (Jewell and Driscoll, 1983; Friebe et al., 1996; Yang et al., 1996) and *Ae. kotschyi* (Rawat et al., 2011) were obtained and characterized using a combination of C-banding and analyses with the group-specific molecular or isozyme markers. As a result of these studies, the genetic classifications were developed for C-banded chromosomes of several S-genome species (Friebe and Gill, 1996).

From another side, the S-genomes were extensively examined by FISH with various DNA probes (Yamamoto, 1992a; Badaeva et al., 1996a,b, 2002, 2004; Belyayev et al., 2001; Zhang et al., 2002; Giorgi et al., 2003; Salina et al., 2006b, 2009; Raskina et al., 2011; Ruban et al., 2014; Molnár et al., 2016; Zhao et al., 2016). Probe pSc119.2 was used most frequently (Badaeva et al., 1996a, 2002, 2004; Molnár et al., 2016; Zhao et al., 2016), however, the pSc119.2 signals are located predominantly in subtelomeric chromosome regions, thus hindering unequivocal chromosome identification. Probe pAs1, which proves to be highly informative for many *Aegilops* species, is not very useful for the S-genome analysis owing to a small number of detected sites (Badaeva et al., 1996a). In most papers FISH-labeled *Aegilops* chromosomes were classified based on their morphology, which is not sufficient to determine their correspondence to the genetic nomenclature of C-banded chromosomes. Owing to this, it was necessary to find FISH markers for the precise identification of all S-genome chromosomes and coordination of classification systems.

Recently, Komuro et al. (2013) isolated and characterized a number of repetitive DNAs from the wheat genome, which can potentially be used for molecular-cytogenetic analysis of wheat and *Aegilops* species. Several new sequences have been described in other papers (Salina et al., 1998, 2009; Adonina et al., 2015; Badaeva et al., 2015; Zhao et al., 2016). In this study we characterized the S genomes of diploid and polyploid *Triticum* and *Aegilops* species using C-banding and FISH with a set of "classical" [pSc119.2, pAs1, pTa71, pTa794, Spelt-1, Spelt-52] and novel [pAesp_SAT86, $(CTT)_n$, $(GTT)_n$, pTa-535, pTa-s53] probes in order to assess evolutionary changes in the *Triticum-Aegilops* alliance.

MATERIALS AND METHODS

Plant Material

Five diploid (*Aegilops speltoides, Ae. longissima, Ae. sharonensis, Ae. searsii, Ae. bicornis*), two tetraploid (*Ae. peregrina* and *Ae. kotschyi*) and one hexaploid (*Ae. vavilovii*) *Aegilops* species carrying the S-genome have been examined in comparison with two tetraploid wheats, *T. timopheevii* and *T. dicoccoides*. The list of accessions, their ploidy level, genome constitution and the origin are given in **Table S1**.

DNA Probes

Following probes were used for FISH:

Plasmid clones pTa71 - a 9 kb long sequence of common wheat encoding 18S, 5.8S and 26S rRNA genes including spacers (Gerlach and Bedbrook, 1979), pTa794 – a 420 bp long sequence of wheat containing the 5S rRNA gene and intergenic spacer (Gerlach and Dyer, 1980), pAs1 - a 1 kb fragment derived from *Ae. tauschii* and belonging to *Afa* family (Rayburn and Gill, 1986), pSc119.2 – a 120 bp long sequence isolated from rye (Bedbrook et al., 1980), pTa-s53 – a 587 bp DNA fragment isolated from common wheat (Komuro et al., 2013), Spelt-1 – a 150 bp fragment isolated from *Ae. speltoides* (Salina et al., 1997), Spelt-52 (homolog of pAesKB52) – a 276 bp long DNA fragment isolated from *Ae. speltoides* (Salina et al., 2004a), and pAesp_SAT86 - a new satellite family with a monomer length of 86 bp isolated from Ae. *speltoides* genomic DNA (Badaeva et al., 2015) and showing 91-94% similarity to wheat repeat pTa-713 described in Komuro et al. (2013) were labeled with dUTP-ATTO-488, dUTP-ATTO-550, dUTP-ATTO-647N by nick-translation using an Atto NT Labeling Kit (Jena Bioscience, Germany) or with FITC (fluorescein-12-dUTP, Roche, Germany) or biotin (biotin-16-dUTP, Roche, Germany) by nick-translation using the Nick Translation Mix (Roche, Germany) according to manufacturers' instruction.

Probe pTa535-1 was used as 5′ 6-carboxyfluorescein (6-FAM) or 6-carboxytetramethylrhodamine (TAMRA) end-labeled (MWG, Germany) oligo probe (5′-AAA AAC TTG ACG CAC GTC ACG TAC AAA TTG GAC AAA CTC TTT CGG AGT ATC AGG GTT TC-3′) (Komuro et al., 2013; Tang et al., 2014).

The oligo-$(CTT)_{10}$ or complementary oligo-$(GAA)_{10}$ probes [thereafter $(CTT)_n$] were labeled with 5/6-Sulforhodamine 101-PEG3-Azide or 6-Carboxyfluorescein Azide by click chemistry (Baseclick, Germany).

The oligo-$(GTT)_9$ probe labeled at the 3′-end with fluorescein-12-dUTP was synthesized in the Laboratory of Biological Microchips at the Engelhardt Institute of Molecular Biology, Moscow, Russia.

Giemsa C-Banding Method

The Giemsa C-banding method described in Badaeva et al. (1994) was used for analysis. Seeds were soaked in water for 24 h at room temperature and then kept at 4°C overnight on wet filter paper in Petri dishes. For the next 24 h Petri dishes were placed at 24°C. Roots were cut and treated with 0.05% colchicine for 3 h. Further, roots were fixed in 45% acetic acid for 4 h, washed with distilled water and treated with 0.2 N HCl for 15 min at 4°C and for 5 min at 60°C. After overnight treatment with a 4 mg/ml Cellulysine (Fluka, Switzerland) solution at 24°C root meristems were squashed in drop of 45% acetic acid. Slides were frozen in liquid nitrogen and coverslips were removed. After that slides were placed into 96% ethanol at room temperature. Chromosomes of wheat were classified according to nomenclature suggested in Gill et al. (1991), Badaeva et al. (2016); chromosomes of *Aegilops* species were classified according to the nomenclature of Friebe et al. (1993, 1995, 1996, 2000), Friebe and Gill (1996). Karyotype of one typical accession per each species was taken as standard for alignment of C-banding and FISH patterns.

Fluorescence *in situ* Hybridization

Detailed protocols of the pretreatment of the materials, fixation and chromosomal preparation are given in Badaeva et al. (2017). Briefly, seeds were germinated in Petri dishes on wet filter paper at 24°C in dark. Roots were excised when 2 cm long, treated with ice-cold water for 24 h, and fixed with ethanol:acetic acid (3:1) fixative for at least 4 days at room temperature. Before slide preparation roots were stained in 2% acetocarmine for 15 min. Meristems were cut off and squashed in a drop of 45% acetic acid. Slides were frozen in liquid nitrogen and coverslips were removed with a razor blade. The slides were kept in 96% ethanol in a freezer.

Hybridization mixture contained 1 g dextran sulfate dissolved in 1 ml of distilled water, 5 ml deionized formamide, 1 ml of 20x SSC, 1 ml Herring sperm DNA (10 mg/ml, Promega, USA). Per slide 40–60 ng of each labeled probe were added to 18 μl hybridization mixture. Post hybridization washes were carried out as follows: for probes labeled with biotin or fluorescein the slides were washed in 0.1x SSC 2 × 10 min, then in 2x SSC 2 × 10 min at 42°C. Slides hybridized with directly labeled probes were washed at 58°C in 2x SSC for 20 min. The probes labeled with fluorescein were detected using anti-fluorescein/Oregon green®, rabbit IgG fraction, Alexa Fluor® 488 conjugated antibody (Molecular Probes, USA). Biotin was detected with sptreptavidin-Cy3 (Amersham Pharmacia Biotech, USA). The slides were counter-stained with DAPI (4′,6-diamidino-2-phenylindole) in Vectashield mounting media (Vector laboratories, Peterborough, UK) and examined with a Zeiss Imager D-1 microscope. Selected metaphase cells were captured with an AxioCam HRm digital camera using software AxioVision, version 4.6. Images were processed in Adobe Photoshop®, version CS5 (Adobe Systems, Edinburgh, UK). For classification, chromosomes were aligned with the C-banding patterns based on the hybridization patterns of labeled CTT- and GTT-satellite sequences.

RESULTS

Analysis of Diploid Species

According to the C-banding and FISH patterns of nine probes, five diploid species of the *Sitopsis* section split into two groups corresponding to taxonomically recognized sub-sections *Truncata* (*Ae. speltoides*) and *Emarginata* (*Ae. longissima, Ae. sharonensis, Ae. searsii, Ae. bicornis*).

Sub-section *Truncata: Ae. speltoides*

The karyotype of *Ae. speltoides* consists of metacentric or submetacentric chromosomes; the chromosome pairs 1S and 6S carry large satellites in their short arms (**Figure S1**). All chromosomes contain large Giemsa-positive pericentromic heterochromatin, prominent subtelomeric C-bands, and some small or medium sized interstitial bands. Giemsa-patterns allowed the identification of all *Ae. speltoides* chromosomes. We observed significant variations of Giemsa bands between plants within and between accessions. Heteromorphism of homologous chromosome has been recorded in all studied genotypes (**Figure S1**).

The $(CTT)_{10}$ clusters (**Figure 1**, CTT) are located in proximal and interstitial chromosome regions, overlapping with Giemsa N-bands (Jiang and Gill, 1994b). No $(CTT)_{10}$ signals were found in the sub-telomeric parts of the chromosomes possessing C-bands. The $(GTT)_9$ probe forms prominent proximal clusters (**Figure 1**, GTT, **Figures 2**, **3f**), often exceeding the size of $(CTT)_n$-signals. The abundance of the GTT-microsatellite is an important diagnostic feature of *Ae. speltoides* chromosomes.

The pSc119.2 labeling patterns are represented by subtelomeric and interstitial signals allowing the discrimination of all *Ae. speltoides* chromosomes. Some hybridization sites are found in all genotypes, whereas other vary in the presence and signal size (**Figure 1**). Based on dual-color FISH with $(CTT)_{10}$ and pSc119.2 probes we corrected previously published classification of pSc119.2-labeled chromosomes (Badaeva et al., 1996a) according to the genetic nomenclature (Friebe et al., 2000). In particular, the chromosomes 2S and 3S have been renamed.

Major NORs are detected on chromosomes 1S and 6S, and one pair of 5S rDNA loci are mapped on the chromosome 5S (**Figures 1**, **2**). In addition, accession TA1873 shows one minor site on the long arm of one 5S chromosome.

Repeat pAesp_SAT86 exhibits significant variation of labeling patterns between *Ae. speltoides* genotypes (**Figure 1**). Two sites located in the short arm of 4S and pericentromeric region of 7SL are permanent (**Figure 1**, yellow arrows). In genotypeTS89 this repeat is transferred to the long arm of 4S, probably due to a

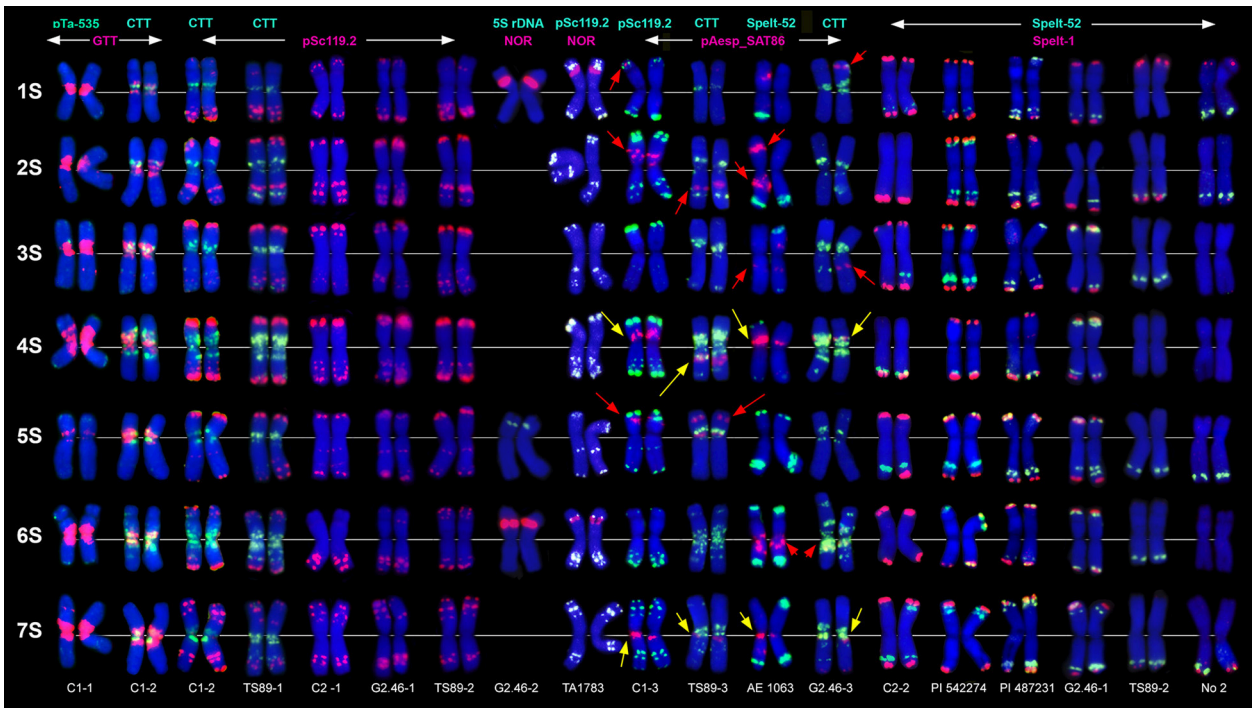

FIGURE 1 | Localization of different DNA sequences on chromosomes of *Ae. speltoides*. Probe combinations are shown on the top; signal color corresponds to probe name. Accessions numbers are indicated in the bottom: C1-1–C1-3 genotypes from Technion park, Haifa, Israel; TS89-1–TS89-3–genotypes from Katzir, Israel; C2-1–C2-2–genotypes from Nahal Mearot, Israel; G2.46-1–G2.46-3–genotypes from Ramat haNadiv, Israel. Permanent pAesp_SAT86 loci are indicated with yellow arrows; polymorphic sites are shown with red arrows.

pericentric inversion. Several facultative pAesp_SAT86 sites were found in more than one genotype (**Figure 1**, red arrows), while some signals were detected in single genotypes on either one or both homologous chromosomes.

The labeling patterns of Spelt-1 and Spelt-52 probes are highly polymorphic (**Figure 1**). The Spelt-1 sequence is located in subtelomeric regions of either one or two chromosome arms. The number of loci per diploid genome varied from six (TS89 Katzir and No2 from Turkey) to 27 (PI 542274 from Turkey). Genotypes differ from each other in the size and chromosome location of the Spelt-1 clusters. The Spelt-52 signals of variable size are located in distal chromosome regions, proximally to Spelt-1. The number of Spelt-52 clusters per diploid genome varied from eight to 22 (**Figure 1**), the size and chromosomal distribution are highly polymorphic. Genotypes differ from each other in a ratio of Spelt-1/Spelt-52 repeats. Thus, the Spelt-1 could significantly prevail over Spelt-52, or the Spelt-52 could be more abundant (**Figure 1**).

Only few inconsistent, dot-like pTa-535 signals have been detected in *Ae. speltoides* (**Figure 1**). No hybridization was found with pAs1 and pTa-s53 probes.

Sub-section Emarginata

Four species of the *Emarginata* sub-section have a similar karyotype, which is distinct from that of *Ae. speltoides* (**Figures S1**, **S2**). Chromosome pairs 5S* and 6S* carry unequal satellites: large on 6S* and small on 5S* chromosomes (**Figure S2**). Most *Ae. sharonensis* genotypes collected in Keshon (Israel) are heterozygotes (**Figures S2c1,c2**) indicating that open pollination is common in this population.

The karyotypes of *Emarginata* species differ in heterochromatin content detected by Giemsa staining. *Ae. bicornis* and *Ae. searsii* showed small-to-medium C-bands located in interstitial chromosome regions (**Figures S2a1–b4**). *Ae. sharonensis* and *Ae. longissima* exhibit prominent pericentromeric and subtelomeric and many interstitial C-bands (**Figures S2c1–d10**). C-banding patterns allowed the chromosome identification in all *Emarginata* species. A species-specific translocation between 4S* and 7S* is found in all *Ae. longissima* accessions.

The $(CTT)_{10}$-hybridization pattern (**Figures 3d,e,i,j**, **4**, **5**) corresponds to the C-banding pattern. As expected, *Ae. bicornis* and *Ae. searsii* carry predominantly small CTT-signals (**Figures 3e,j**), while *Ae. sharonensis* and *Ae. longissima* possess prominent pericentromeric and distinct interstitial CTT-clusters. In contrast to *Ae. speltoides*, the $(GTT)_9$ probe hybridizes poorly on the chromosomes of *Emarginata* species. Probably, accumulation of heterochromatin in this evolutionary lineage was mainly due to amplification of CTT-repeat, contributing to an increase of nuclear DNA content in *Ae. sharonensis*/*Ae. longissima* genomes as compared to *Ae. bicornis*/*Ae. searsii* (Eilam et al., 2007).

Emarginata species display similar pSc119.2 hybridization patterns consisting of subtelomeric signals of variable size in one or both chromosome arms. Interstitial loci were rarely found

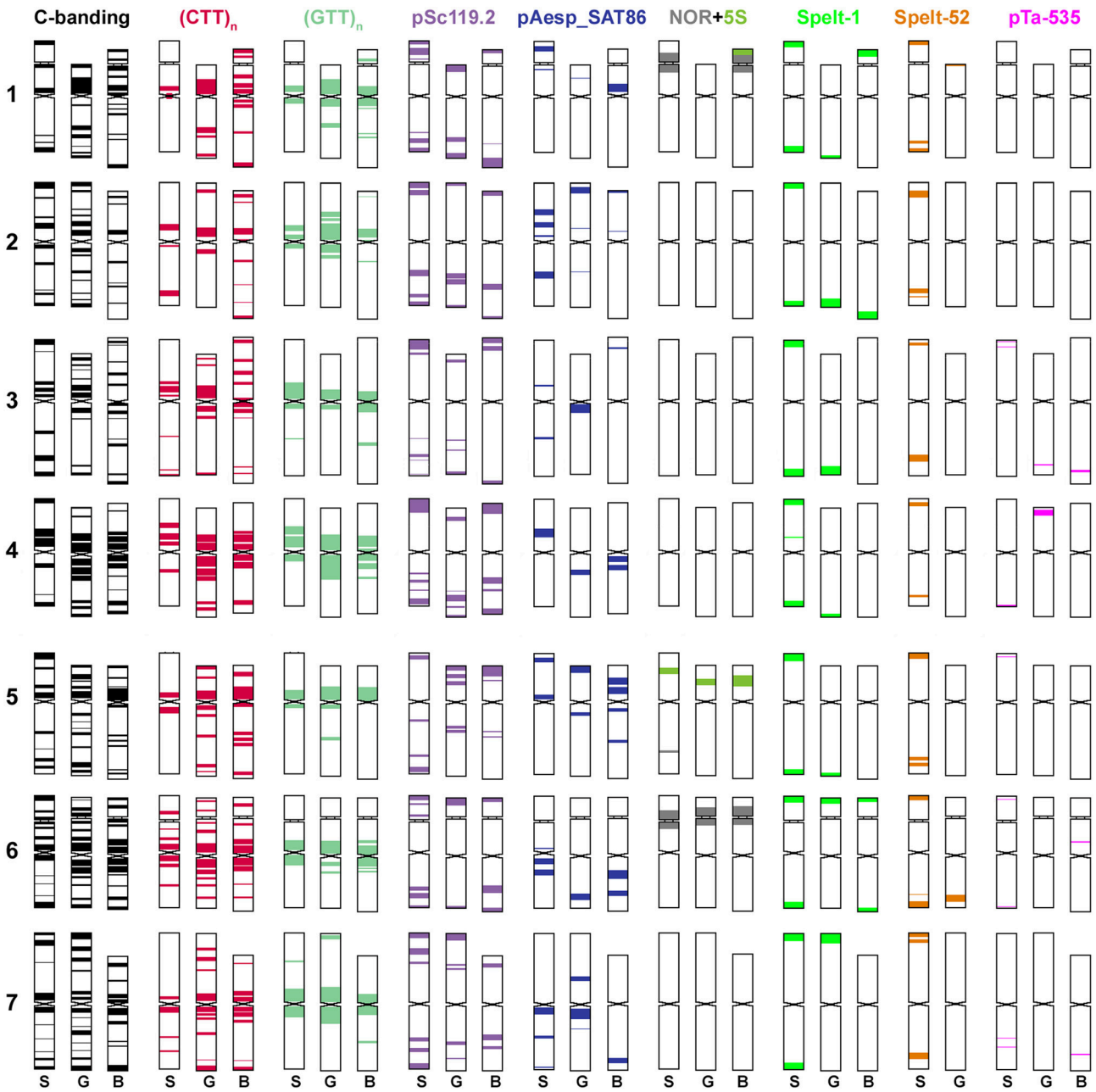

FIGURE 2 | Idiogram showing relative positions of C-bands and nine DNA probes (probe names are given on the top) on chromosomes of *Ae. speltoides* (S), the B-genome (B) of common wheat and G-genome (G) of *T. timopheevii*.

(**Figures 4**, **5**). Permanent interstitial sites are found on 2S*S (*Ae. sharonensis* and *Ae. bicornis*), 4S^{l}L (*Ae. longissima*), and 7S^{s} (*Ae. searsii*) only. The pSc119.2 cluster in the middle of 5S*S is present in all *Ae. searsii* accessions and some *Ae. longissima* and *Ae. sharonensis* lines (**Figure 4**). One or two polymorphic pSc119.2 sites were rarely observed on 1S^{b}L and 4S^{b}L of *Ae. bicornis*.

The number and location of 5S and 45S rDNA loci in *Emarginata* species is similar and differ from that in *Ae. speltoides* (**Figures 1**, **4**). Major NORs are located on 5S*S and 6S*S and permanent minor NORs are found on 1S*S (**Figure 4**). Additional minor sites were detected in the terminal region of 6S*L of all *Ae. searsii* accessions and some lines of *Ae. bicornis* and *Ae. longissima*. All species possess two 5S rDNA loci located in the short arms of chromosome 1S* and 5S*, distally (1S*) or proximally (5S*) to the 45S rDNA loci.

The distribution of pAesp_SAT86 signals is species- and chromosome-specific. An intraspecific polymorphism was

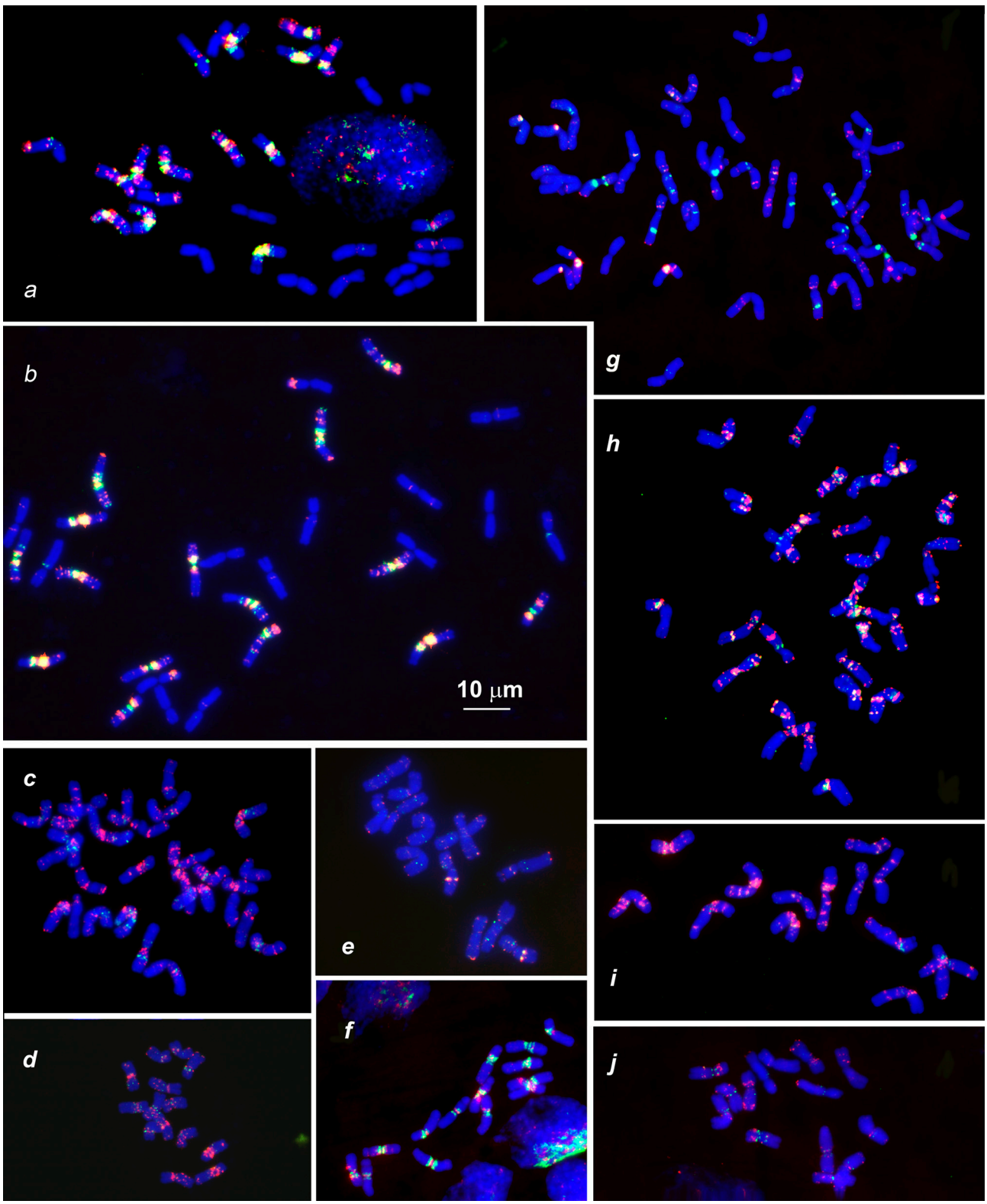

FIGURE 3 | Distribution of $(CTT)_{10}$ and $(GTT)_9$ probes (red and green colors respectively) on chromosomes of wheat and *Aegilops* species: **(a)**, *Triticum dicoccoides* (IG 46396); **(b)**, *T. araraticum* (IG 116164); **(c)**, *Ae. kotschyi* (TA2206); **(d)**, *Ae. longissmia* (AE 904); **(e)**, *Ae. searsii* (AE 1071); **(f)**, *Ae. speltoides* (C1, Technion park, Israel); **(g)**, *Ae. vavilovii* (K-3637); **(h)**, *Ae. peregrina* (C11, Nahal Mearot, Israel); **(i)**, *Ae. sharonensis* (C6, Keshon, Israel); **(j)**, *Ae. bicornis* (K-666). Scale bar, 10 μm.

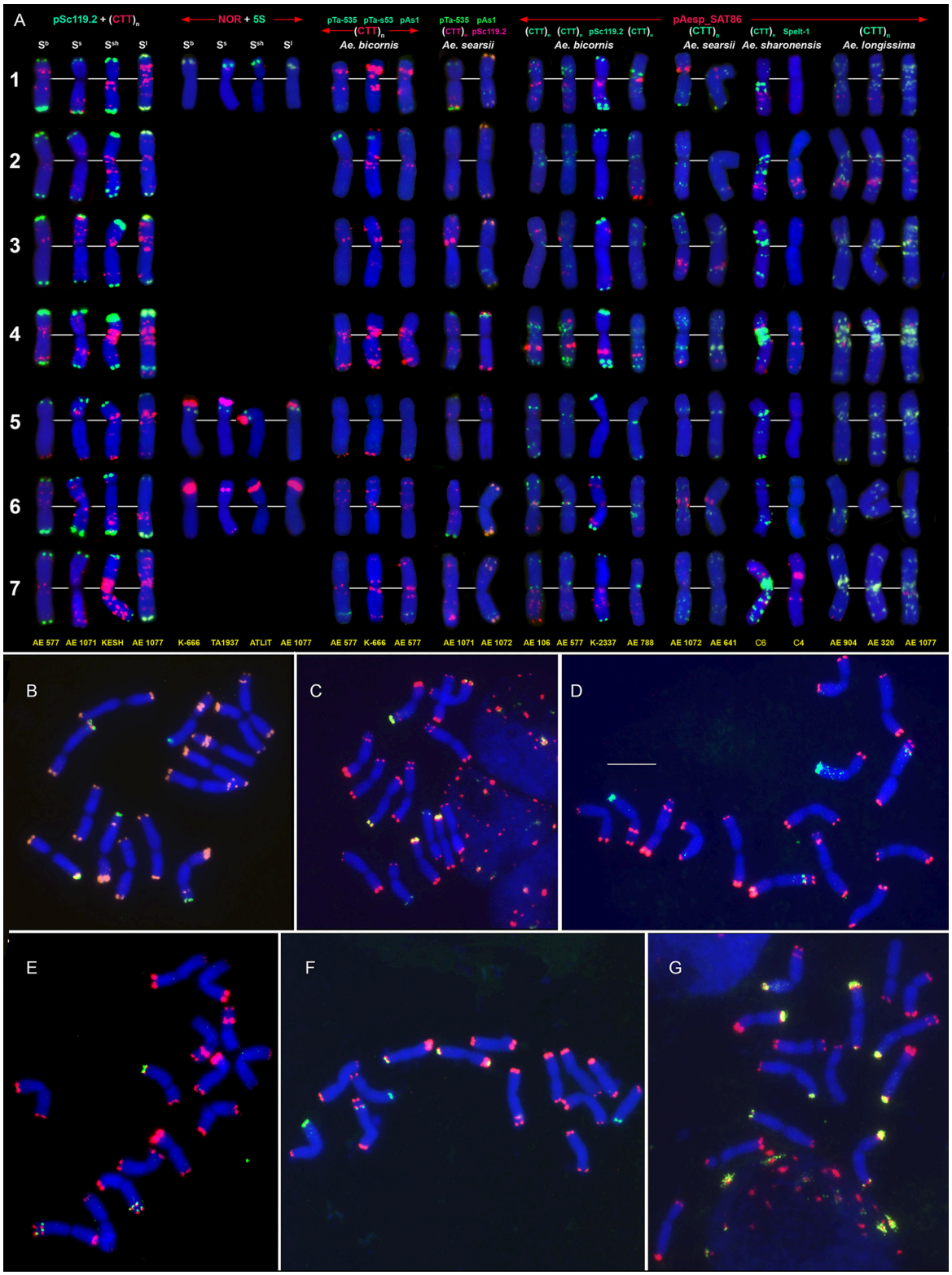

FIGURE 4 | Distribution of repeated DNA sequences on chromosomes of four species of *Emarginata* group **(A)**, *Ae. bicornis* (S^b), *Ae. searsii* (S^s), *Ae. sharonensis* (S^{sh}), and *Ae. longissima* (S^l). **(A)** Probe combinations are given on the top, accession names are shown below karyograms. Signal color corresponds to probe name. 1–7 – homoeologous groups. Polymorphisms of Spelt-52 patterns on *Ae. longissima* **(B–D)** and *Ae. sharonensis* **(E–G)** chromosomes: **(B)**, K-905; **(C)**, K-907; **(D)**, C3 (HaBonim); **(E)**, C6 (Keshon); **(F)**, C7 (HaBonim); **(G)**, i-570030. The pSc119.2 signals are shown in red, Spelt-52–in green. Scale bar, 10 μm.

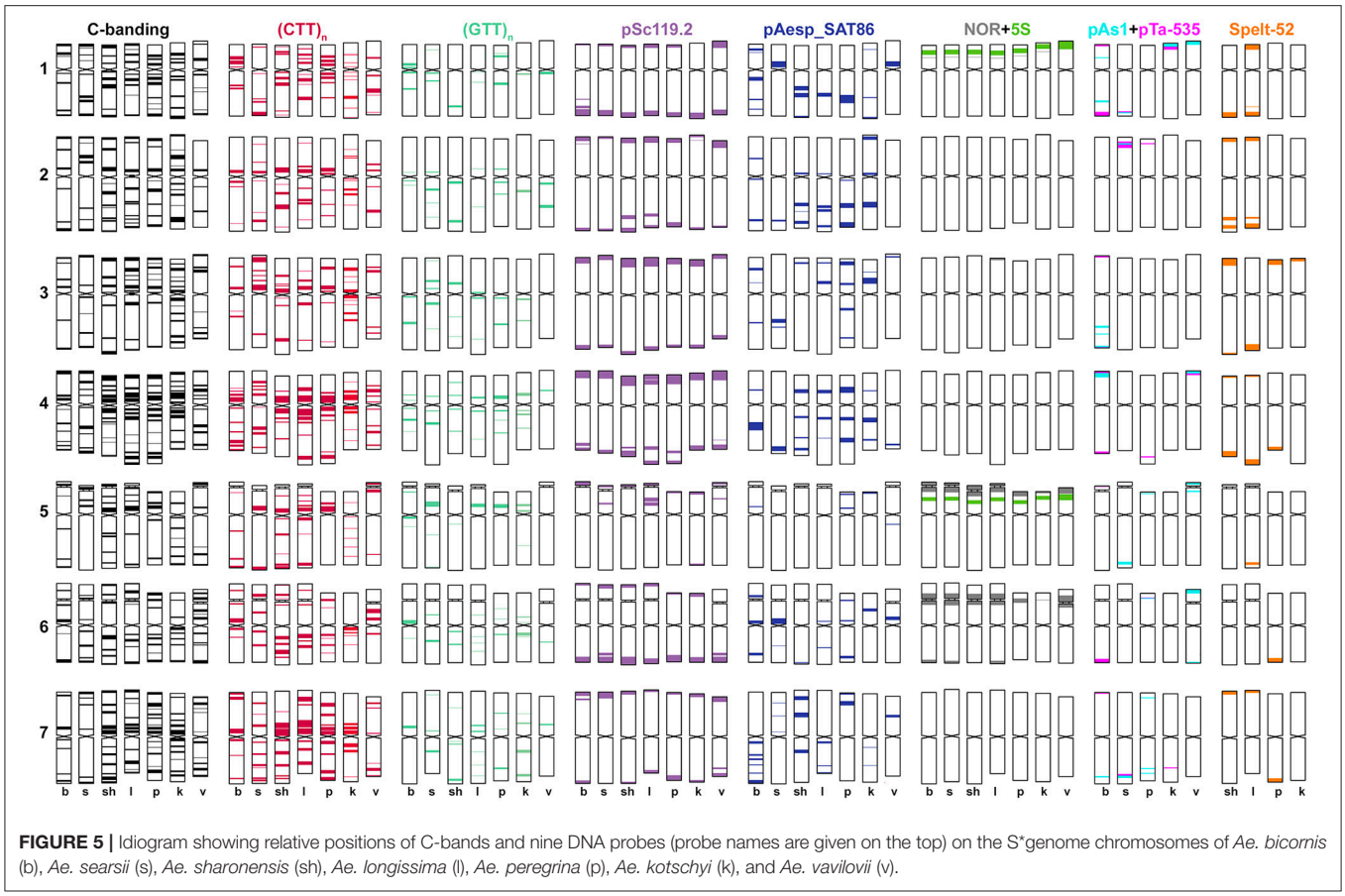

FIGURE 5 | Idiogram showing relative positions of C-bands and nine DNA probes (probe names are given on the top) on the S*genome chromosomes of *Ae. bicornis* (b), *Ae. searsii* (s), *Ae. sharonensis* (sh), *Ae. longissima* (l), *Ae. peregrina* (p), *Ae. kotschyi* (k), and *Ae. vavilovii* (v).

detected in *Ae. bicornis* and *Ae. longissima,* labeling patterns are virtually invariable in *Ae. searsii* (**Figures 4**, **5**). Distribution of pAesp_SAT86 clusters on *Ae. sharonensis* and *Ae. longissima* chromosomes is similar and differs from *Ae. bicornis* and *Ae. searsii,* which, in turn, are clearly distinct from each other. No similarity between homoeologous chromosomes of different species has been observed, though the chromosome $3S^s$ (*Ae. searsii*) shows almost the same distribution of pAesp_SAT86 sequence as the chromosomes 2S* of *Ae. sharonensis* and *Ae. longissima.*

The Spelt-1 repeat was not found in any *Emarginata* species, and Spelt-52 is detected in *Ae. sharonensis* and *Ae. longissima* only (**Figures 4B–G**). Signals of variable size are located in terminal regions of either one or both arms of all chromosomes except 6S*. Only two interstitial loci are found in the long arms of 2S* and 4S*. Distribution of Spelt-52 is highly diverse and polymorphisms are often observed even between homologous chromosomes. Depending on genotype, the number of signals ranges from 0 to 14. Most *Ae. longissima* accessions carry a Spelt-52 site in the long arm of 5S*, while it is absent in six out of 8 *Ae. sharonensis* accessions (**Table S2**). No other differences in labeling patterns were found between these species.

Distinct signals of the D-genome specific probes pAs1, pTa-535 or pTa-s53 are revealed in *Ae. bicornis* and *Ae. searsii* only (**Figures 4A**, **5**). Two small pTa-535 sites are found in the distal parts of $2S^bS$ and $7S^bL$ chromosomes of *Ae. bicornis;* the first one overlaps with pTa-s53, and the second—with pAs1 sites. The pTa-535 probe hybridizes to subterminal regions of five pairs of *Ae. searsii* chromosomes, $1S^sL$ and $6S^sL$ exhibiting the largest signals. A relatively intense pAs1 signal is detected in a terminus of $4S^sS$ and few very weak interstitial signals are observed on $1S^sL$, $3S^sL$, and $7S^sL$. Faint, dispersed, non-specific pAs1 signals are distributed in distal halves of *Ae. sharonensis* and *Ae. longissima* chromosomes, while pTa-s53 and pTa-535 did not hybridize to the chromosomes of these species.

Analysis of Polyploid Species: Wheats

Differences between emmer and Timopheevii wheat are mainly due to species-specific translocations identified in both evolutionary lineages (Naranjo et al., 1987; Liu et al., 1992; Jiang and Gill, 1994a; Maestra and Naranjo, 1999; Salina et al., 2006a). The $(CTT)_{10}$ signals on *T. araraticum* and *T. dicoccoides* chromosomes (**Figures 3a,b**, **6a,c,h**) mainly correspond to the C-bands, whereas $(GTT)_9$ forms large clusters in proximal regions of all B- and G-genome chromosomes (**Figure 2**, G,B; **Figures 3a,b**); their positions mainly overlapped with the location of $(CTT)_{10}$ clusters. A similar pattern is also observed in *Ae. speltoides* (**Figure 3f**).

Although the pSc119.2 hybridization patterns in these two wheat species are distinct and species-specific, they share some

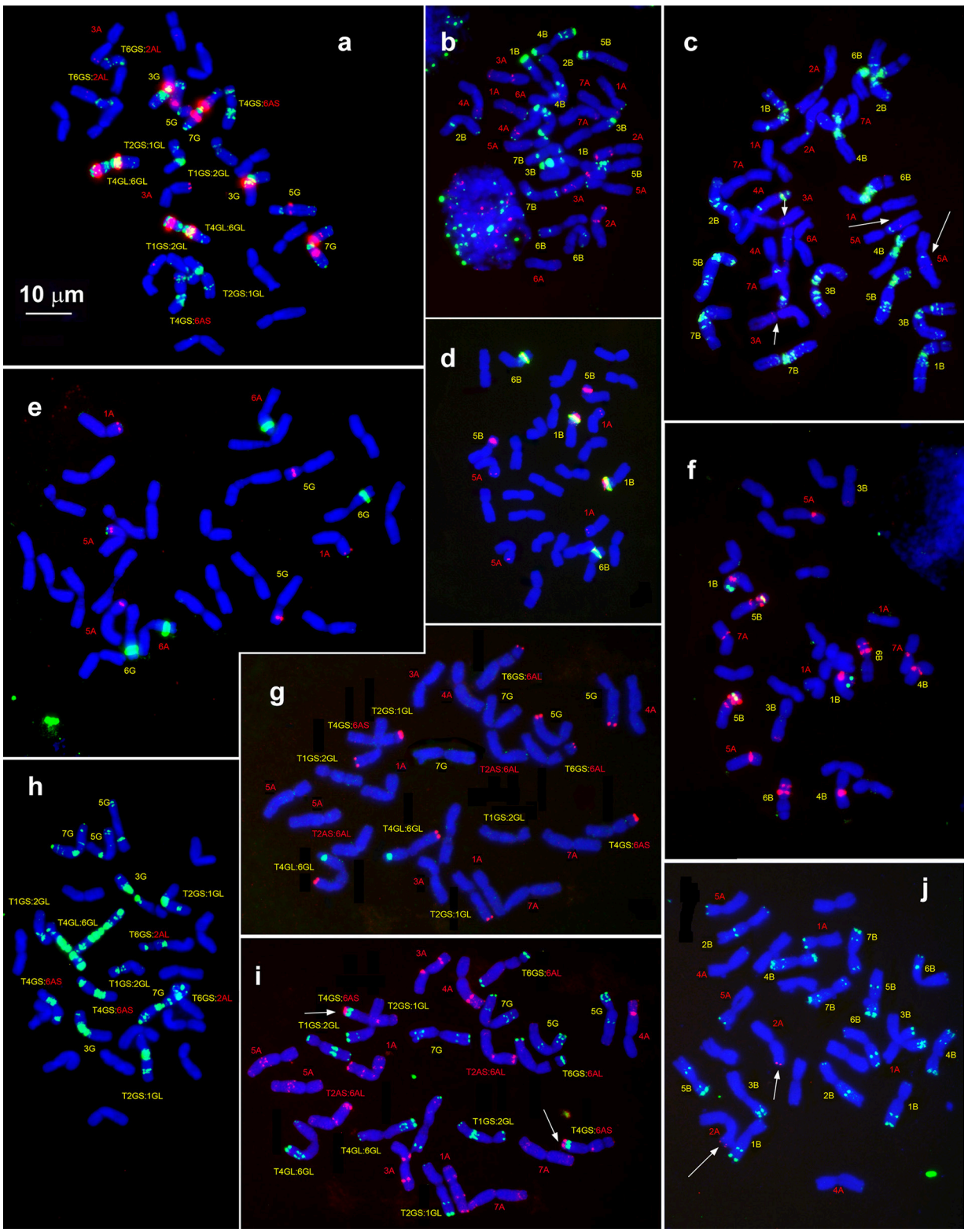

FIGURE 6 | Hybridization patterns of $(CTT)_{10}$ (**a,c,h**, green), pSc119.2 (**b,i,j**, green), pTa-535 (**c,i**, red), pTa-s53 (**b,h**, red), pAesp_SAT86 (**f,h**, red), NORs (**d,e**, green), and 5S rDNA (**d,e**, red), 5S rDNA (**f**, green); Spelt-1 (**j,g**, red) and Spelt-52 (**g**, green) on metaphase chromosomes of *T. dicoccoides,* IG 46396 **(b–d,f,j)** and *T. araraticum,* K-59940 **(a,e,h,g,i)**. Position of pTa-s53 hybridization sites on *T. dicoccoides* chromosomes **(c)**, huge cluster of pTa-535 sequence on the chromosome 4GS **(i)** and Spelt-1 site on *T. dicoccoides* chromosome 2AL **(j)** are indicated with arrows. Scale bar, 10 μm.

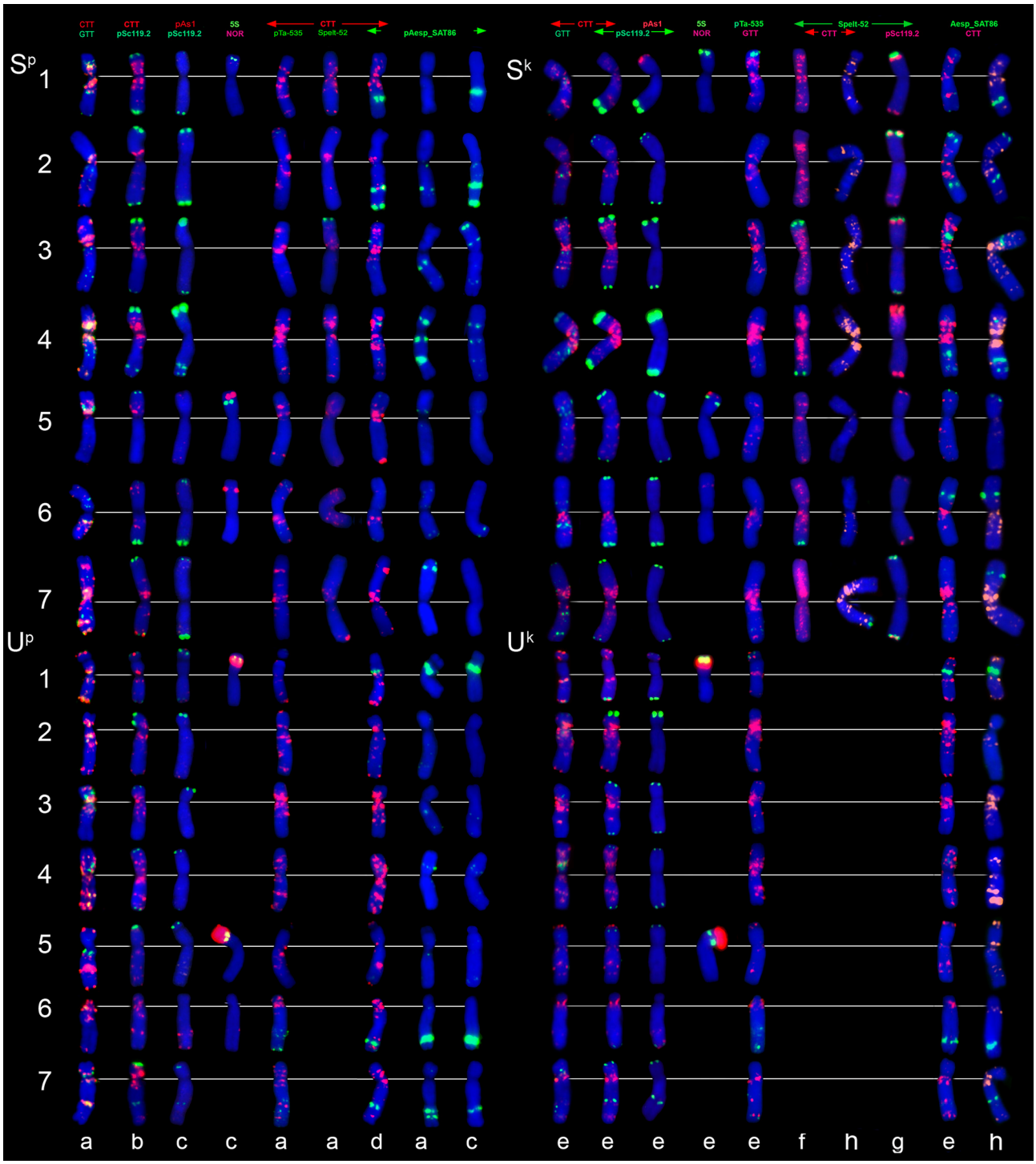

FIGURE 7 | Distribution of repetitive DNA families on chromosomes of *Ae. peregrina* left side and *Ae. kotschyi* (right side of the figure): **(a)**, C11 (Nahal Mearot, Israel); **(b)**, C8 (Haifa, Carmel, Israel); **(c)**, K-61; **(d)**, C9 (Keshon, Israel); **(e)**, TA2206; **(f)**, K-91; **(g)**, hybrid *Ae. umbellulata* TU04 × *Ae. sharonensis* TH02; **(h)**, K-2905. Probe combinations are given on the top; signal color corresponds to probe name. The S-genome chromosomes are shown on the top, the U-genome–on the bottom part of the figure.

similar features. As in *Ae. speltoides,* pSc119.2 signals are located in interstitial and subtelomeric regions of orthologous chromosome allowing a complete chromosome identification.

Two chromosome pairs of *T. araraticum* and *T. dicoccoides* carry major NORs (**Figures 6d,e**). These are 1B and 6B in *T. dicoccoides* and 6G and $6A^t$ in *T. araraticum* (transfer of NORs

from 1G to $6A^t$ is due to species-specific translocation 1G-4G-$6A^t$ in Timopheevii lineage, Jiang and Gill, 1993). Group 1 and 5 chromosomes of *T. dicoccoides* display eight 5S rDNA signals (**Figure 6d**), but only six - in *T. araraticum* (chromosomes $1A^t$, $5A^t$, and 5G, see **Figure 6e**). Chromosome 5S of *Ae. speltoides* shows one 5S rDNA locus, therefore 1S likely lost the 5S rDNA locus in the progenitor of *Ae. speltoides* after the formation of ancient emmer, but prior to the divergence of Timopheevii wheat.

The pAesp_SAT86 clusters are found on both A and B/G genome chromosomes (**Figures 6a,f**), *T. dicoccoides* and *T. araraticum* show different labeling patterns and both exhibit broad intraspecific polymorphisms (Badaeva, unpublished). A large pAesp_SAT86 signal is found on 1BS of all emmer (**Figure 6f**) and common wheat (Komuro et al., 2013), but it is absent on 1G of *T. timopheevii* (**Figure 6a**). By contrast, huge 3GL- and 7GL-located pericentromeric pAesp_SAT86 clusters are missing on the homoeologous chromosomes of emmer wheat. At the same time, similar labeling patterns were observed on 4B/4G, 5B/5G, and 6B/6G of these species.

Very weak Spelt-52 signals were seen on 1GS and large on 6GL of *T. araraticum*. The same sequence was not detectable in wild emmer. Two faint Spelt-1 signals were revealed on the chromosome pair 2A of *T. dicoccoides* (**Figure 6j**), whereas ten clear signals were observed on chromosomes $6A^t$S, 1GL, 4GL, 5GL, and 6GS of *T. araraticum* (**Figure 6g**).

Probe pTa-535 hybridized predominantly on the A-genome chromosomes of both wheat species (**Figures 6b,i**, red color). A large pTa-535 cluster was found on the short arm of 4G of *T. araraticum* (**Figure 6i**, indicated with arrows). Overlapping, small pAs1/ pTa-535 signals are detected in distal halves of 3GL and 3BL. In addition, faint pAs1 signals were found in the satellite of 1B, in the middle of 6BS and 7BL of wild emmer. *T. araraticum* carries small pAs1 loci on 5GL and 7GL and in the satellite of $6A^t$ (data not shown). Only weak pTa-s53 signals were observed on chromosomes 3AS and 5AL of *T. dicoccoides* (**Figure 6c**), and no hybridization was found on *T. araraticum* (**Figure 6h**).

Polyploid *Aegilops*: *Ae. peregrina and Ae. kotschyi*

Ae. peregrina and *Ae. kotschyi* are both tetraploids with the same genome constitutions UUS*S*. Their C-banding patterns are generally similar, however, some differences in morphology and heterochromatin distribution on chromosomes 2S*, 4S*, and 7S* are observed (**Figure S3**). According to C-banding patterns, *Ae. peregrina* carries 4S-7S* translocation and therefore the S^p-genome is originated from *Ae. longissima*. The S^k genome of *Ae. kotschyi* is more diverged from the S*-genomes of *Emarginata* species, but shares similar structure and C-banding pattern of chromosome 4S* with *Ae. sharonensis* (**Figures S2**, **S3**).

FISH with $(CTT)_{10}$ and $(GTT)_9$ probes reveals large CTT-clusters on all chromosomes, but only few weak GTT-signals on some U and S*-genome chromosomes of both species (**Figures 3c,h**). Distribution of $(CTT)_{10}$ probe corresponds to the C-banding patterns (**Figure 7**, **Figure S3**), and dual-color FISH allows aligning of the CTT/C-banding and pSc119.2-FISH patterns (**Figure 5**). Positions of pSc119.2 clusters on chromosomes of the two species are similar except for 4S*, which carries two prominent subtelomeric signals in *Ae. kotschyi,* but one huge cluster in the short and two smaller sites in the long arm in *Ae. peregrina* (**Figure 7**). Labeling patterns varies between the accessions. Owing to subterminal location of pSc119.2 sites and polymorphism of labeling patterns, applicability of the pSc119.2 probe for chromosome identification of *Ae. peregrina* and *Ae. kotschyi* is limited.

Distribution of 45S and 5S rDNA loci on *Ae. peregrina* and *Ae. kotschyi* chromosomes is similar **Figure 7**, (NOR+5S) and corresponds to that in the parental species. Signal size of pTa71 probe (45S rDNA) on $5S^k$ and especially $6S^k$ chromosomes of *Ae. kotschyi* is significantly smaller than on the orthologous chromosomes of *Ae. peregrina,* which can be an indicative of more extensive gene loss at the respective loci.

FISH reveals similar hybridization patterns of pAesp_SAT86 probe on chromosomes of *Ae. peregrina* and *Ae. kotschyi*. According to dual-color FISH, the largest pAesp_SAT86 signals are located on chromosomes 1S*L (polymorphic), 2S*L, 1US, 6UL, and 7UL. Chromosomes 3S* and 4S* carry medium and 5S*S, 6S*L, and 4US – faint signals (**Figure 7**). Labeling patterns of chromosomes 3S*, 4S*, 6S*, and 7S* are polymorphic. In contrast to *Ae. peregrina* and diploid *Emarginata* species, the chromosome $6S^k$ of *Ae. kotschyi* carries large pAesp_SAT86 cluster in the short arm.

The Spelt-1 repeat is not found in these tetraploid species, while Spelt-52 is revealed only in few accessions of *Ae. kotschyi* and *Ae. peregrina*. Small Spelt-52 clusters are observed on four out of seven S*-genome chromosome: 3S*S, 4S*L, 6S*L, and 7S*L. Number of signals varies from two to six (**Figure 7**), nearly half of genotypes we examined do not exhibit any hybridization. This is strictly different from what is observed in a newly synthesized hybrid *Ae. umbellulata x Ae. sharonensis,* in which 12 distinct Spelt-52 signals are observed in either one or both arms of chromosome pairs 1S*, 2S*, 3S*, and 7S* (**Figure 7g**).

Very few weak pAs1 and pTa-535 signals are located predominantly on the U-genome chromosomes of *Ae. kotschyi* and *Ae. peregrina* (**Figure 7**), while the pTa-s53 sequence is totally absent.

Polyploid *Aegilops*: *Ae. vavilovii*

The hexaploid species *Ae. vavilovii* with the genome constitution $D^1D^1X^{cr}X^{cr}S^vS^v$ is characterized by a medium amount of Giemsa bands. Small and medium sized bands are distributed predominantly in interstitial chromosome regions (**Figure 8**). Two chromosome pairs are submetacentrics with small satellites, which morphologically correspond to 5S*. Two other pairs are metacentrics with large satellites, which is typical for chromosome 6S*. The C-banding pattern of *Ae. vavilovii* is similar to the parental species: *Ae. crassa* (Badaeva et al., 1998, 2002) and *Ae. searsii* (Friebe et al., 1995; Friebe and Gill, 1996). Intraspecific variations due to chromosomal rearrangements were identified in two of the three accessions of *Ae. vavilovii*.

The distribution of $(CTT)_{10}$ signals is generally similar to the observed C-banding patterns (**Figure 8**). The $(GTT)_9$ probe results in distinct signals on five pairs of the S^v genome

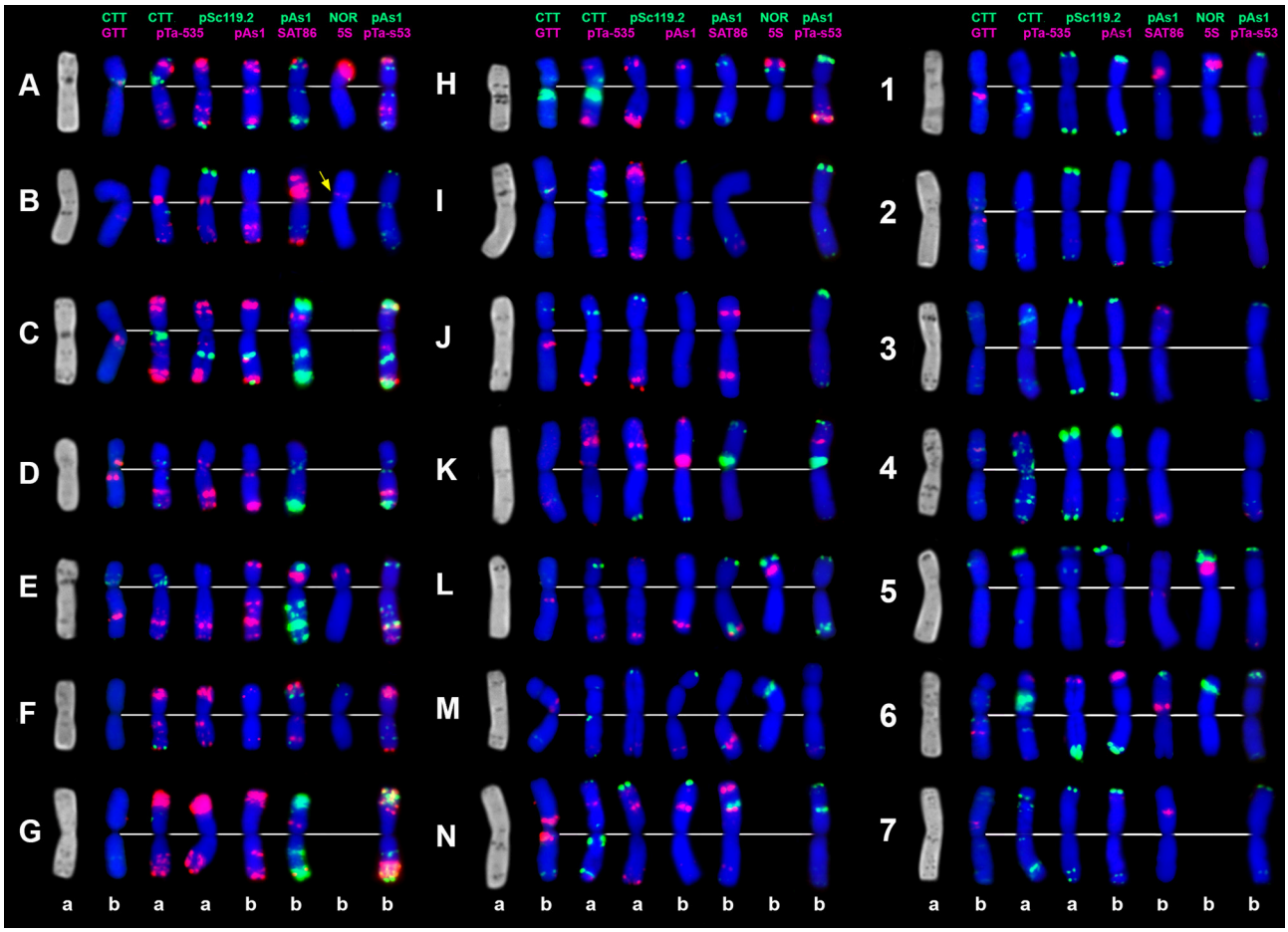

FIGURE 8 | C-banding (left) and FISH patterns of *Ae. vavilovii* chromosomes: a – K-3635; b – K-3637. Chromosomes are assigned to genome D^1 (left), X^{cr} (middle), and to the S^v genome (right) according to similarity with chromosomes of *Ae. crassa* (Badaeva et al., 1998) and *Ae. searsii* (Friebe et al., 1995). Probe combinations are shown on the top, signal color corresponds to probe name. Chromosomes derived from *Ae. crassa* are designated with letters (A–N); the S^v genome chromosomes are numbered 1–7 according to genetic nomenclature (Friebe et al., 1995).

chromosomes and small to medium clusters on seven pairs of the D^1 and X^{cr} genome chromosome. $(GTT)_9$ signals only partially overlap with $(CTT)_{10}$ loci (**Figure 3g**).

Probe pSc119.2 hybridized with all S^v and some X^{cr} genome chromosomes. Signals are located in subterminal chromosome regions; interstitial sites were found in the middle of $5S^vS$ and in the distal region of $4S^vL$ and $7S^vL$ (**Figure 8**).

Probe pTa71 revealed eight major and eight minor NOR sites in *Ae. vavilovii*. The major NORs are located on group 5 and 6 chromosomes belonging to S^v and X^{cr} genomes. Minor NORs mapped on all three pairs of group 1 chromosomes and, surprisingly on $6D^1S$. Six of 5S rDNA sites are located on group 1 and 5 chromosomes. An additional, minor 5S rDNA locus is detected in the proximal region of an unknown small metacentric chromosome (**Figure 8**, shown with arrow).

The pAesp_SAT86 signals of different sizes were detected on many *Ae. vavilovii* chromosomes; the number of loci varies from one to three per chromosome (**Figure 8**). Distribution of pAesp_SAT86 sites on $2S^v$, $3S^v$, and $7S^v$ is different from *Ae. searsii*, while the remaining chromosomes of these genomes show similar labeling patters.

The A/D-genome-specific probes pAs1, pTa-535, and pTa-s53 hybridize mainly to the D^1-genome and partially to X^{cr} genome chromosomes of *Ae. vavilovii* (**Figure 8**). The S^v genome possesses the lowest amount of these sequences. Small pAs1 signals were observed only in terminal regions of $4S^vS$ and $6S^vS$. Neither Spelt-1, nor Spelt-52 hybridization sites were detected in *Ae. vavilovii.*

DISCUSSION

Karyotype analysis as a tool for studying evolutionary processes must be based on an unified chromosome nomenclature. The first classification of chromosomes according to their homoeologous relationships and genome affinities was developed for common wheat by Sears (1954), and since then it is used as standard in genetic and cytogenetic studies of the *Triticeae.* Although the nomenclature of Giemsa C-banded chromosomes is now

available for many *Aegilops* species, including *Ae. speltoides, Ae. searsii, Ae. longissima,* and *Ae. peregrina* (Friebe and Gill, 1996), their correspondence to the distribution of FISH probes is not known.

In order to link C-banding and FISH patterns (Jiang and Gill, 1993) developed a method of sequential C-banding and *in situ* hybridization analysis. An alternative approach was suggested by Pedersen and Langridge (1997), who used the barley probe pHvG38 containing a GAA-satellite sequence for the identification of wheat chromosomes. Later this sequence was successfully used for the FISH analyses of wheat, barley, rye and some other cereal chromosomes (Pedersen et al., 1996; Cuadrado et al., 2000; Vrána et al., 2000; Cuadrado and Jouve, 2002; Kubaláková et al., 2005; Kato, 2011; Komuro et al., 2013; Adonina et al., 2015; Badaeva et al., 2016), however it was rarely applied for *Aegilops* species (Molnár et al., 2005, 2016; Mirzaghaderi et al., 2014).

The CTT–labeling patterns of *Aegilops* chromosomes obtained in our study basically correspond to their C-banding patterns. Therefore, we used the CTT-signals as landmark to identify chromosomes according to the genetic nomenclature. This allowed us to compare karyotypes based on chromosome homoeology and to trace chromosomal changes that could have occurred over the course of species evolution.

Ae. speltoides and Polyploid Wheats Are Cytogenetically Distinct From the S*-Genome of Other Diploid and Polyploid *Aegilops* Species

Based on C-banding and FISH patterns it is possible to divide the S-genome chromosomes of diploid and polyploid wheat and *Aegilops* species into two distinct groups. The first one includes *Ae. speltoides* and polyploid wheat. The second contains four diploid species of the *Emarginata* sub-section and three polyploid *Aegilops,* in agreement with molecular phylogenetic analyses (Yamane and Kawahara, 2005; Golovnina et al., 2007; Goryunova et al., 2008; Salse et al., 2008; Gornicki et al., 2014; Marcussen et al., 2014; Middleton et al., 2014; Feldman and Levy, 2015). The main diagnostic features of these groups can be described as follows.

1. The satellite chromosomes of the S-genome of *Ae. speltoides* and B/G-genomes of polyploid wheats belong to homoeologous groups 1 and 6 (Dvorák et al., 1984; Friebe et al., 2000). The satellites are large and nearly equal in size (Chennaveeraiah, 1960). The satellite of *T. timopheevii* chromosome 1G is transferred to $6A^t$ as a result of a species-specific translocation (Jiang and Gill, 1994a; Rodríguez et al., 2000b; Dobrovolskaya et al., 2011). Major 45S rDNA sites are located on the short arms of group 1 and 6 chromosomes (**Figure 2**) (Yamamoto, 1992a,b; Jiang and Gill, 1994b; Badaeva et al., 1996b; Raskina et al., 2011; Belyayev and Raskina, 2013; Molnár et al., 2016). In addition to major NORs, Jiang and Gill (1994b) revealed minor 45S rDNA loci in the long arm of chromosome 1B of common and durum wheat, 1G of *T. timopheevii* and 1S of *Ae. speltoides*, which were never observed in other S*-genome *Aegilops* species. Diploid *Emarginata* species possess two pairs of satellite chromosomes assigned to genetic groups 5 and 6 (Friebe et al., 1993, 1995; Friebe and Gill, 1996); satellites significantly differ in size (Chennaveeraiah, 1960). The secondary constrictions of 5S* and 6S* are suppressed in polyploid *Ae. peregrina* and *Ae. kotschyi,* but are extended in hexaploid *Ae. vavilovii.* FISH with the probe pTa71 revealed major 45S rDNA sites on 5S* and 6S* chromosomes of diploid and polyploid *Aegilops* species, but signal sizes were significantly reduced in tetraploid *Ae. peregrina* and *Ae. kotschyi* (**Figures 4**, **7**). Permanent minor 45S rDNA loci were present on chromosome 1S*, and additional minor site was detected in the terminus of 6S*L of all *Ae. searsii* and some *Ae. bicornis* and *Ae. longissima* accessions (**Figure 5**). Earlier we also found minor 45S rDNA locus in a terminus of the short arm of an unknown chromosome, probably $3S^l$, of *Ae. longissima,* accession TA1912 (Badaeva et al., 1996b). These observations are in agreement with previously published results (Yamamoto, 1992a,b; Friebe et al., 1993; Badaeva et al., 1996b, 2002, 2004).
2. The S, B, and G genomes are enriched in GTT-repeats (**Figures 1**–**3**). This microsatellite is especially abundant in proximal chromosome regions, but rarely appears in interstitial locations. The GTT-sites do not always overlap with the CTT-clusters, and proximal GTT-signals could be observed in chromosome regions lacking Giemsa C-bands. By contrast, the S*-genome chromosomes of *Aegilops* species show poor labeling with the $(GTT)_9$ probe (**Figures 3**, **5**). The GTT-interstitial signals mainly overlap with the $(CTT)_n$ clusters (**Figures 3c–e**, **h–j**).
3. The distribution of pSc119.2 repeat in *Ae. speltoides* and the B/G genomes of wheat observed in our study (**Figures 1**, **2**) is similar to what was reported before (Jiang and Gill, 1994a; Badaeva et al., 1996a; Schneider et al., 2003; Kubaláková et al., 2005; Salina et al., 2006b; Komuro et al., 2013) and is distinct from the S*-genome chromosomes of other *Aegilops* species in preferentially interstitial signal location.
4. The Spelt-1 sequence is present in the S-genome of *Ae. speltoides* (Salina et al., 1997, 2006b; Raskina et al., 2011; Belyayev and Raskina, 2013) and the B/G genomes of polyploid wheats (Salina, 2006; Salina et al., 2006b; Zoshchuk et al., 2007, 2009), but it is absent from the S*-genome of other diploid and polyploid *Aegilops* species.

Different Families of Repetitive DNA Show Different Evolutionary Rates

Our data and previous findings imply that the evolutionary rate varies between different families of repetitive DNAs. Despite distinct differences between *Ae. speltoides*/polyploid wheats and other S*-genome *Aegilops* species in the distribution of rDNA probes, the patterns of 45S and 5S rDNA loci was highly conserved within each group. Only minor intra-and inter-specific variations were observed,

(1) Regarding the appearance of minor NORs, which occur at similar positions on the orthologous chromosomes (Yamamoto, 1992a,b; Badaeva et al., 1996b), and

(2) The decrease of signal size on the S*-genome chromosomes of tetraploid *Aegilops* species (Yamamoto, 1992a,b; Badaeva et al., 2004). Such signal reduction could be explained by uniparental elimination of genes (Shcherban et al., 2008).

The distribution of the rye-derived pSc119.2 repeat is also found to be relatively conserved within each of the two S-genome groups. This sequence with a 120 bp-long repeat unit is broadly distributed in the *Triticaea* and some *Aveneae* species and constitutes large and evolutionary old component of their genomes (Contento et al., 2005). The repeat units isolated from wheat, rye, barley and *Aegilops* species showed 70-100% similarity to each other. Nucleotide sequences of pSc119.2 repeat units are not species-specific, and one site may contain diverse members of the family (Contento et al., 2005). The authors proposed that these individual pSc119.2 sites are transferred as blocks and can be translocated within the genome resulting in position variation and site numbers. Similar was observed in our material. Most cereals, including barley (Taketa et al., 2000; Zhao et al., 2018), *Aegilops* (Badaeva et al., 1996a, 2002, 2004; Linc et al., 1999; Molnár et al., 2005, 2016), *Agropyron* (Brasileiro-Vidal et al., 2003; Li et al., 2018; Said et al., 2018), *Elytrigia* (Linc et al., 2012), *Haynaldia* (Zhang et al., 2013), possess predominantly subtelomeric pSc119.2 clusters. Therefore, a terminal location of pSc119.2 satellite family is probably a more primitive character compared to interstitial locations. Intercalary pSc119.2 sites are typical for *Ae. speltoides* (Badaeva et al., 1996a; Molnár et al., 2016), B- and G-genomes of polyploid wheats (Jiang and Gill, 1994a; Schneider et al., 2003), and rye (Cuadrado and Jouve, 2002); the rye genome being highly rearranged relative to wheat (Liu et al., 1992). Strong differences in the distribution of pSc119.2 sites in the R and S genome chromosomes suggest that transposition of this repeat proceeded in genomes of rye and *Ae. speltoides* independently, likely, after their radiation from the ancestral form.

Comparison of C-banding patterns with the distribution CTT+ GTT-microsatellite sequences shows that heterochromatin blocks detected by Giemsa staining in different *Triticum* and *Aegilops* species could have different sequence composition. Thus, *Ae. speltoides* chromosomes carry prominent proximal and telomeric C-bands and only few intercalary bands, which is considered as primitive karyotype structure (Stebbins, 1971). Only proximal bands overlap with both $(CTT)_{10}$ and $(GTT)_9$ clusters. The GTT-repeat is more abundant in these chromosomal regions. Intercalary C-bands correspond to CTT-signals, and probably they are composed by this microsatellite mainly. Neither $(CTT)_{10}$, nor $(GTT)_9$ signals were detected in telomeric heterochromatin, which is enriched in Spelt-1 and Spelt-2 repeats.

The C-banding patterns of the S*-genome *Emarginata* species and their polyploid derivatives are very similar to their CTT-hybridization patterns indicating that this sequence is a major component of Giemsa-positive heterochromatin. The GTT-microsatellite is present in much lower quantities, and only few C-bands contain this sequence solely. Species of this genomic group exhibit drastic differences in the content of C-positive heterochromatin. Diploid *Ae. bicornis, Ae. searsii,* and hexaploid *Ae. vavilovii* are low heterochromatic; the $(CTT)_{10}$-signals are small and located mainly in the intercalary chromosome regions. Karyotypically *Ae. searsii* is distinct from other diploid species and its divergence was accompanied mainly by heterochromatin re-pattering visualized by Giemsa-staining and FISH with the CTT-microsatellite probe. The genomes of *Ae. sharonensis, Ae. longissima, Ae. kotschyi,* and *Ae. peregrina* are highly heterochromatic; prominent C-bands and CTT-signals are distributed in proximal and intercalary chromosome regions (**Figure 5**). Thus, massive amplification of the CTT-repeat occurred at the stage of radiation of *Ae. sharonensis* and *Ae. longissima*, resulting in an increase of nuclear DNA (Eilam et al., 2007) and the amount of heterochomatin.

Three tandemly repeated DNA families, pAesp_SAT86, Spelt-1, and Spelt-52 show the highest rate of evolution in the *Triticum-Aegilops* group. pAesp_SAT86 sequence is detected in all S-genome species (**Figures 1**, **3**, **7**, **8**) and the B/G genomes of polyploid wheat (**Figure 6**). The labeling patterns are extremely variable in *Ae. speltoides* (**Figure 1**) and differ from polyploid wheat species which, in turn, are distinct from each other (Komuro et al., 2013; Badaeva et al., 2016). Diploid *Emarginata* species and their polyploid derivatives display species-specific patterns of pAesp_SAT86 probe (**Figures 4–8**). *Ae. bicornis* shows the highest degree of intraspecific pAesp_SAT86-polymorphism, while little variation has been observed in *Ae. searsii, Ae. sharonensis* (**Figure 4**), *Ae. kotschyi* (**Figure 7**), and *Ae. vavilovii* (data not shown). *Ae. bicornis* and *Ae. searsii* differ from each other and from other species of this group (**Figure 5**). *Ae. sharonensis* is more similar with *Ae. longissima* and *Ae. peregrina* in the distribution of pAesp_SAT86 clusters and only slightly different from *Ae. kotschyi*. The pTa-713 (homolog of pAesp_SAT86) hybridization patterns of *Ae. peregrina* reported by Zhao et al. (2016) is consistent with our results, though there are some discrepancies in chromosome designations.

The Spelt-1 repeat is found in *Ae. speltoides* and the B/G genomes of polyploid wheats. In *Ae. speltoides* it comprises nearly 2% of the nuclear genome (10^5–10^6 copies). The copy number of constituent sequence related to Spelt-1 is ∼40–60 reduced in genomes of tetraploid wheats, and ∼1200–2400 times reduced in genomes of other *Triticeae* (Pestsova et al., 1998; Salina et al., 1998; Salina, 2006). Minor amounts of Spelt-1 exist in genomes of rye, cultivated barley, most diploid and polyploid wheat as well as *Aegilops* species indicates that this sequence was already present in minor quantities in the common ancestor of the *Triticeae* (Salina et al., 1998). High homology (97–100%) of individual repetitive units implies that massive amplification of Spelt-1 repeat occurred in ancient *Ae. speltoides* after radiation from the common ancestor of the *Triticeae* (Salina et al., 1998; Salina, 2006). Spelt52 is homologous to the pAesKB52 repeat isolated earlier from *Ae. speltoides* by Anamthawat-Jonsson and Heslop-Harrison (1993). This repeat consists of monomers of two types, Spelt52.1 and Spelt52.2, which share a homologous stretch of 280 bp and have two regions without sequence similarity of 96 and 110 bp, respectively. *Ae. speltoides* displays intraspecific variation in the occurrence of Spelt52 monomer types, whereas *Ae. longissima, Ae. sharonensis,* and *Ae. bicornis* showed no

interspecific variation (Salina et al., 2004a). The Spelt-52 is abundant in *Ae. speltoides* accounting for approximately 1% of nuclear genome (Anamthawat-Jonsson and Heslop-Harrison, 1993; Salina, 2006) and is also highly represented (1.0×10^4 – 2.5×10^5 copies) in *Ae. longissima* and *Ae. sharonensis*, but it present in minor quantities in *Ae. bicornis* and *Ae. searsii* (Salina, 2006).

Coincidently with previous findings (Salina et al., 2006b; Raskina et al., 2011; Belyayev and Raskina, 2013), we observed significant intraspecific variation of Spelt-1 and Spelt-52 labeling patterns (**Figure 2**). Strict differences in a ratio of Spelt-1/ Spelt-52 repeats detected between genotypes can be due to geographical origin of the material. Earlier, Raskina et al. (2011) found that the amount of Spelt-1 and, in lower extent, the Spelt-52 repeat decreases in marginal populations of *Ae. speltoides*.

Although pAs1 and pTa-535 repeats are abundant in some cereal genomes (Rayburn and Gill, 1986; Badaeva et al., 1996a; Taketa et al., 2000; Komuro et al., 2013), they are poorly represented in the S genomes of *Triticum* and *Aegilops*. Thus, we failed to detect any pAs1signals in *Ae. speltoides*, but Molnár et al. (2016) revealed small pAs1 signals on the chromosome 3S. Wheat chromosomes 3BL - 3GL and 7BL - 7GL possess pAs1 and pTa-535 clusters in similar positions (Schneider et al., 2003; Badaeva et al., 2016), although they are not detected in *Ae. speltoides*. Probably, these loci were present in the genome of ancient *Ae. speltoides,* but they were eliminated after radiation of polyploid wheats. The pAs1 and pTa-535 repeats are also poorly represented in genomes of *Emarginata* species. Two distinct interstitial pAs1 sites overlapping with either pTa-535, or with pTa-s53 loci are found in *Ae. bicornis.* pAs1 and pTa-535 are less abundant in *Ae. searsii, Ae. sharonensis* and *Ae. longissima*. Only chromosome $1S^k$ of tetraploid *Ae. kotschyi* contains a distinct pAs1/pTa-535 cluster, and these sequences are absent from the S* genomes of *Ae. peregrina* and *Ae. vavilovii.*

Evolution of the S-Genome

Summarizing our data and the results of other authors (Kihara, 1954; Chennaveeraiah, 1960; Kihara and Tanaka, 1970; Yen and Kimber, 1990b; Zhang and Dvorák, 1992; Zhang et al., 1992; Dvorák, 1998; Feldman and Levy, 2015), the following scenario of the S-genome evolution can be suggested (**Figure 9**).

According to molecular phylogeny, *Ae. speltoides* is the most distinct diploid *Aegilops,* which diverged from the common ancestor very early, prior to the split of diploid wheat and *Aegilops* species (Salse et al., 2008; Gornicki et al., 2014; Marcussen et al., 2014; Feldman and Levy, 2015). Divergence of *Ae. speltoides* from an ancestral form was not associated with major translocations, because neither meiotic analysis (Rodríguez et al., 2000a), nor microsatellite mapping (Dobrovolskaya et al., 2011) detected structural chromosmal rearrangements in the S-genome. However some genomic changes not causing linkage group perturbations did probably occur at the early stages of *Ae. speltoides* speciation. As was shown earlier, major NORs in *Triticum* and *Aegilops* species are located on group 1, 5, and 6 chromosomes (Appels et al., 1980; Appels and Honeucutt, 1986), while 5S rDNA loci are located separately from NORs in the short arms of group 1 and 5 chromosomes (Appels et al., 1980; Dvorák et al., 1989). The chromosome 5S of *Ae. speltoides* and B/G genome of polyploid wheats does not contain 45S rDNA loci, therefore the loss of respective NOR probably occurred prior to formation of ancient emmer. Other early genomic changes of ancient *Ae. speltoides* included the transposition of the pSc119.2 repeat from subtelomeric to interstitial chromosome regions and also the amplification of Spelt-1 repeat.

As mentioned above, *Ae. speltoides* and the B/G genomes of polyploid wheats are characterized by the abundance of GTT-microsatellite (Cuadrado et al., 2000; Badaeva et al., 2016), which is poorly represented in diploid wheats (Badaeva et al., 2015) and most *Aegilops* species (**Figure 3**). This difference can be caused by massive amplification of GTT-repeat in the ancient *Ae. speltoides.* Alternatively, this repeat could be eliminated from the progenitor of wheat and *Aegilops* species. Taking into consideration the abundance of GTT-repeat in rye and *Hordeum* (Cuadrado and Jouve, 2002, 2007; Dou et al., 2016), the second scenario seems to be more likely. The progenitor of *Ae. speltoides* probably possessed minor amounts of Spelt-52 and the D-genome specific repeats pTa-535, or pAs1, as they are still present in *Ae. speltoides* and the B/G genomes of polyploid wheats (Schneider et al., 2003; Badaeva et al., 2016; Molnár et al., 2016). However, these sequences could be of the A-genome origin, which spread to the S-genome following allopolyploidization.

The emergence of tetraploid emmer was accompanied by the species-specific translocation involving the chromosomes 4A-5A-7B (Naranjo et al., 1987; Liu et al., 1992; Maestra and Naranjo, 1999). In addition to structural chromosome rearrangements, other genetic and epigenetic changes occurred in a newly formed polyploid, including inactivation of the 45S rDNA loci on the A-genome chromosomes, re-distribution of Giemsa C-bands and repetitive DNA families on both A and B-genome chromosomes. Evolution of polyploid wheat resulted in polymorphisms of various DNA sequences and heterochromatin patterns that were described in many publications (Friebe and Gill, 1996; Schneider et al., 2003; Badaeva et al., 2016).

Subsequent evolution of *Ae. speltoides* occurred independently of polyploid emmer and was accompanied by several transposon insertions (Salse et al., 2008) and the loss of the 5S rDNA locus on the chromosome 1S, which is present in emmer and common wheat (Mukai et al., 1990), but absent in *T. timopheevii* (Badaeva et al., 2016) and modern *Ae. speltoides.* Although *T. timopheevii* derived from the same parental species as emmer, different parental genotypes were involved in the origin of these two lineages (Golovnina et al., 2007). Timopheevii wheat emerged much later, than ancient emmer - nearly 0.4 MYA (Gornicki et al., 2014) and its formation was accompanied by different species-specific translocation involving the chromosomes 1G-4G-$6A^t$ + $3A^t$-$4A^t$ (Jiang and Gill, 1994a; Maestra and Naranjo, 1999; Rodríguez et al., 2000b; Dobrovolskaya et al., 2011). As a result, a major NOR was translocated from chromosome 1G to $6A^t$, and a massive cluster of the A/ D-genome specific repeat pTa-535 appeared on the short arm of chromosome 4G (**Figure 6g**). Existence of Spelt-52 sites and a spread of Spelt-1 to most *T. timopheevii* chromosomes (Salina et al., 2006b; Zoshchuk et al., 2007; Badaeva et al., 2016) suggests a massive amplification of these sequences in *Ae. speltoides* prior to emergence of ancient *T. timopheevii*.

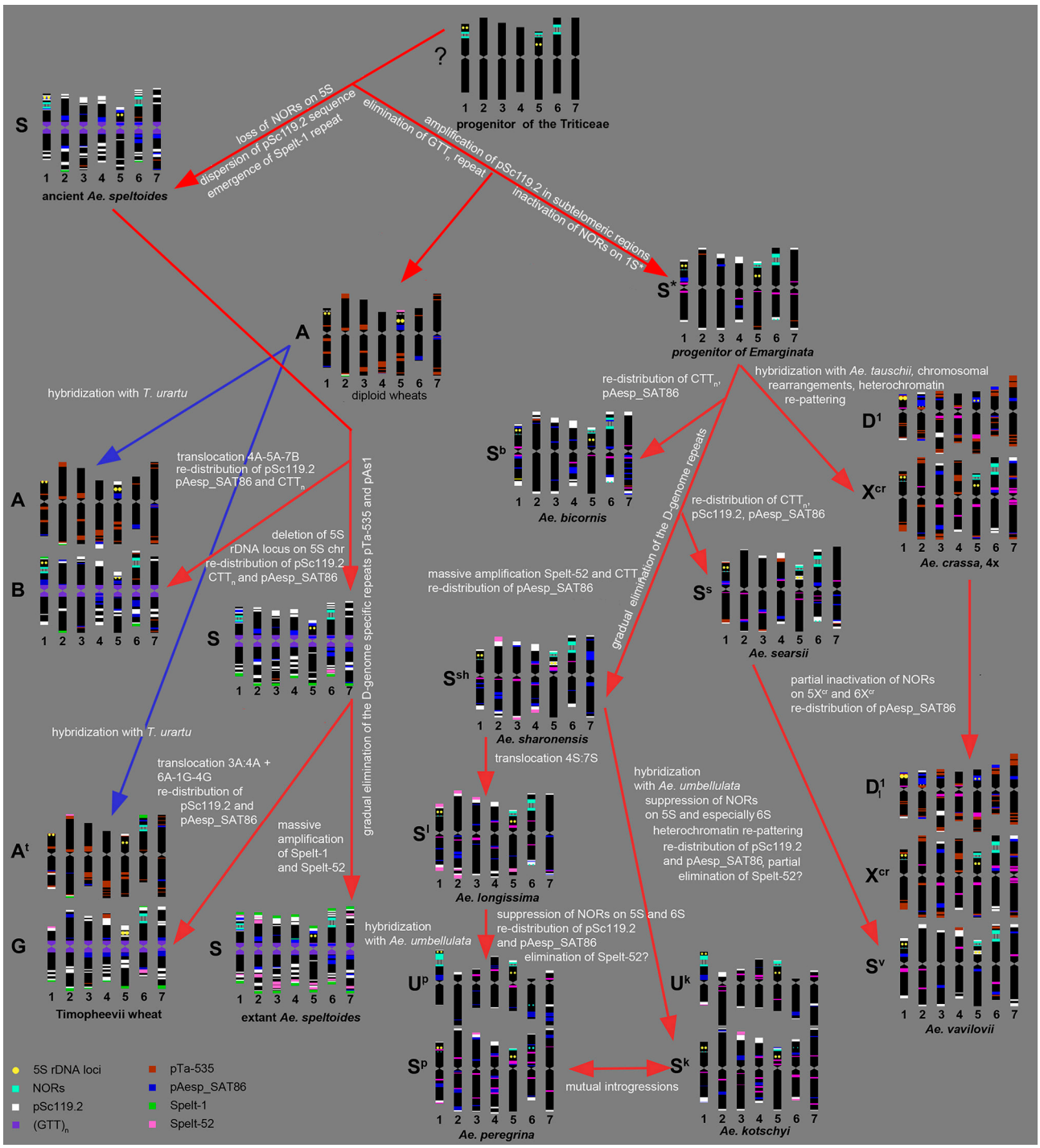

FIGURE 9 | Hypothetic scheme of the S-genome evolution deduced from the results of molecular-cytogenetic analysis.

Results of molecular cytogenetic analysis suggest that genome re-structuring process is still ongoing in natural populations of *Ae. speltoides*. This is exemplified by intraspecific C-banding polymorphisms and diversity of labeling patterns of pAesp_SAT86, Spelt-1 and Spelt-52 probes observed in this and other studies (Belyayev and Raskina, 2011, 2013; Raskina et al., 2011), fluctuation of copy number of retrotransposons and tandem repeats, and high number of chromosomal rearrangements (Belyayev and Raskina, 2013; Shams and Raskina, 2018).

The species of the *Emarginata* group are closely related to each other (Eig, 1929; Kihara, 1954; Friebe and Gill, 1996; Kilian et al., 2011; Gornicki et al., 2014; Feldman and Levy, 2015), which is supported by their similar karyotypes (Chennaveeraiah, 1960), C-banding and pSc119.2-labeling patterns (Badaeva et al., 1996a), distribution of rDNA probes (Yamamoto, 1992a,b; Badaeva et al., 1996b). Separation of *Emarginata* species from a common ancestor was associated with inactivation of major NORs on chromosome 1S* accompanied with the significant loss of 45S rDNA repeat copies. Despite similarity of pSc119.2 labeling patterns, there are obvious, but discontinuous changes in the patterns of other sequences. Our data show that most drastic changes occurred probably at the stage of radiation of *Ae. sharonensis-Ae. longissima*. These are massive amplification of Spelt-52 and CTT-repeats resulting in the gain of heterochromatin in *Ae. sharonensis* and *Ae. longissima*, leading to an approximately 12% increase of nuclear DNA content in *Ae. sharonensis/ Ae. longissima* as compared to *Ae. searsii/ Ae. bicornis* (Eilam et al., 2007). By contrast, the amount of the D-genome repeats pTa-535, pAs1 and especially pTa-s53 gradually decreased, and these sequences nearly disappeared in genomes of *Ae. sharonensis* and *Ae. longissima.* Spelt-52 patterns of *Ae. sharonensis* and *Ae. longissima* chromosomes are highly polymorphic. The similar distribution of all analyzed DNA sequences on chromosomes of *Ae. sharonensis* and *Ae. longissima* (**Figures 3**, **4**) point to a rather recent divergence of these species, which was accompanied by the species-specific translocation 4S*-7S* in *Ae. longissima*.

Formation of tetraploid *Ae. peregrina* and *Ae. kotschyi* did not cause significant alterations of the parental genomes. Considering the structure of chromosome 4S*, the S^p-genome of *Ae. peregrina* was donated by *Ae. longissima,* while *Ae. sharonensis* or the form preceding the split of these diploids could be the source of the S*-genome of *Ae. kotschyi.* These data are consistent with observations of other authors (Yu and Jahier, 1992; Zhang et al., 1992; Friebe et al., 1996), however they contradict the hypothesis about the possible ancestry of *Ae. searsii* in the origin of *Ae. peregrina* (Siregar et al., 1988). Merging of U and S* genomes in the tetraploid *Ae. peregrina* and *Ae. kotschyi* led to inactivation of 45S rDNA loci on the S*-genome chromosomes (**Figure 7**). Similar was also recorded in the artificial allopolyploid *Ae. umbellulata* × *Ae. sharonensis* (Shcherban et al., 2008). Significantly smaller 45S rDNA sites on *Ae. kotschyi* chromosome $6S^k$ compared to the $6S^p$ of *Ae. peregrina* evidences in favor of a higher extent of gene loss at the respective locus, which can be due to earlier origin of *Ae. kotschyi.* The assumption that *Ae. kotschyi* is an older species is also supported by higher divergence of C-banding patterns relative to the parental species.

Interestingly, *Ae. peregrina* and *Ae. kotschyi* both possess only minor quantities of the Spelt-52 repeat, which is abundant in their diploid parents. According to the analyses of artificial wheat-*Aegilops* or *Aegilops-Aegilops* hybrids, the Spelt-52 was either amplified or retained at the same level upon polyploidization (Salina et al., 2004b). Considering these results we can expect massive amplification of the Spelt-52 sequence in *Ae. peregrina* and *Ae. kotschyi* genomes. However, this is not the case. Low amount of Spelt-52 in these species can be caused by the so-called "originator effect," if they obtained their S*genomes from genotype depleted with this repeat, or it can be caused by sequence elimination after formation of tetraploids.

The S^v-genome chromosomes of *Ae. vavilovii* are very similar to the S^s-genome chromosomes of *Ae. searsii,* which further supports their close relationships (Zhang and Dvorák, 1992; Dubkovsky and Dvorák, 1995). Our results strongly suggest that the X^{cr} genome of *Ae. vavilovii* is also the derivative of the S* genome of an unknown *Emarginata* species, but not of *Ae. speltoides* as proposed by Dubkovsky and Dvorák (1995); Edet et al. (2018). Significant differences between the X^{cr} and S^s genomes, as well between X^{cr} and S*-genomes of all diploid *Emarginata* species in the C-banding and labeling patterns demonstrate that the X^{cr} genome was significantly modified during speciation.

CONCLUSIONS

Analysis of the S-genomes of diploid and polyploid *Triticum* and *Aegilops* species using FISH with nine DNA probes, including 5S and 45S rDNA, two microsatellites and five tandem repeats showed an isolated position of *Ae. speltoides* among other *Aegilops* species. In addition, close relationships with the B and G genomes of polyploid wheats were observed, thus confirming previous molecular-phylogenetic data (Yamane and Kawahara, 2005; Petersen et al., 2006; Golovnina et al., 2007; Salse et al., 2008; Gornicki et al., 2014; Marcussen et al., 2014; Middleton et al., 2014; Bernhardt et al., 2017). The evolution of polyploid wheats was associated with different species-specific chromosome translocations and the amplification/ elimination of repeats, re-pattering or, possibly with an exchange of repetitive DNA families with the A-genome chromosomes. Evolutionary changes in the *Ae. speltoides* genome occurred independently from polyploid wheats.

Diploid *Aegilops* species of *Emarginata* group are similar, but are substantially different from *Ae. speltoides* based on C-banding and FISH patterns. The genome evolution in this group was mainly associated with an increase of high copy DNA fraction due to amplification of CTT-repeat, re-distribution of C-bands, $(CTT)_n$-, $(GTT)_n$-, and pAesp_SAT86-clusters, massive amplification of Spelt-52 and gradual elimination of the D-genome-specific sequences pAs1, pTa-535 and pTa-s53. These changes were more profound at the stage of divergence of *Ae. sharonensis/Ae. longissima*. Tetraploid *Ae. peregrina* and *Ae. kotschyi* originated independently from hybridization of *Ae. umbellulata* with *Ae. longissima* (*Ae. peregrina*) or *Ae. sharonensis* or its immediate precursor (*Ae. kotschyi*). The S*-genomes of both tetraploids show little differences to the parental species. The S^k-genome is characterized by more modifications than the S^p-genome, suggesting that *Ae. kotschyi* is older than *Ae. peregrina.* Chromosome introgressions recorded in some accessions of both species (Badaeva et al., 2004) can be explained by gene flow between *Ae. peregrina* and *Ae. kotschyi.*

Our study confirmed that *Ae. vavilovii* is a natural hybrid between tetraploid *Ae. crassa* and *Ae. searsii*. The similarity of C-banding and FISH patterns of *Ae. vavilovii* and corresponding parental species points to rather recent origin of this hexaploid. The assumption that the X^{cr} genome is an additional derivative of the S*genome obtained from an unknown or extinct species of the *Emarginata* group, which was substantially modified over the course of evolution is supported.

AUTHOR CONTRIBUTIONS

EB planned the research, performed and coordinate the analysis. EB and AR performed the research and wrote the paper.

ACKNOWLEDGMENTS

The authors thank Drs. O.M. Raskina and A.A. Belyayev (Institute of Evolution, University of Haifa, Israel) for providing seeds and for the opportunity to collect seed materials from their natural habitat. Additional material was obtained from gene banks of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK, Gatersleben, Germany); VIR Collection of Plant Genetic Resources (Saint Petersburg, Russia); Institute of Evolution, University of Haifa (Haifa, Israel); Weizmann Institute of Science (Rehovot, Israel); USDA-ARS (Aberdeen, Idaho, USA); Wheat Genetics and Genomic Resource Center, Kansas State University (Manhattan, Kansas, USA); ICARDA (Aleppo, Syria). We thank Dr. A. Houben [Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany] for critical reading and valuable comments. We thank Dr. S. Surzhikov (Engehardt Institute of Molecular Biology, Russian Academy of Sciences) for the synthesis of oligo-probes for analysis. This work was supported by grant from Russian State Foundation of Basic Research (project No 17-04-00087). The publication of this article was funded by the Open Access Fund of the Leibniz Association.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01756/full#supplementary-material

Figure S1 | Diversity of the C-banding patterns in *Aegilops speltoides* accessions: **(a)**, No1, from Turkey (provided by Dr. N. Aminov); **(b–f)**, genotypes collected from Israeli populations; **(b,c)**, C1, Technion park, Haifa; **(d)**, C2, Nahal Mearot; **(e)**, G2.46, Ramat haNadiv; **(f)**, C14, Keshon; **(g)**, no 2734 (unknown provided by Dr. B. Kilian); **(h)**, No 2 from Iran (provided by Dr. N. Aminov); **(i)**, i-570060; **(j)**, TS89, Katzir, Israel; **(k)**, PI 487233, and **(l)**, PI 487231 (from Syria); **(m)**, PI 542269 (Turkey). Arrows show unbalanced chromosome modifications.

Figure S2 | Interspecific and intraspecific variation of the C-banding patterns in *Ae. bicornis* **(a1–a3)**, *Ae. searsii* **(b1–b4)**, *Ae. sharonensis* **(c1–c8)**, *and Ae. longissima* **(d1–d10)**. Accession codes: **(a1)**, TA1942; **(a2)**, TB04-3; **(a3)**, TB10-2; **(b1)**, G.7.15; **(b2)**, TE01-1; **(b3)**, G7.12; **(b4)**, IG 47619; **(c1,c2)**, C6, Keshon; **(c3)**, C5, Caesaria; **(c4)**, TH04; **(c5)**, TH01; **(c6)**, TH02; **(c7)**, C4, Atlit; **(c8)**, C7, HaBonim; **(d1)**, TL06; **(d2)**, TL01; **(d3)**, G6.77 (Sa'ad); **(d4)**, G6.58 (Tel Akko); **(d5)**, TL03; **(d6)**, C3 (HaBonim); **(d7)**, G6.32 (Nizzanim); **(d8)**, G6.55 (Zomet Shoked); **(d9)**, G17-3; **(d10)**, TL05.

Figure S3 | C-banding polymorphism of *Ae. peregrina* **(a–f)** and *Ae. kotschyi* **(g–j)** chromosomes: **(a)**, TA1888; **(b)**, C11 (Nahal Mearot, Israel); **(c)**, K-61; **(d)**, C12 (Caesaria, Israel); **(e)**, C13 (Natufia, Israel); **(f)**, i-570632; **(g)**, PI 487279; **(h)**, K-91; **(i)**, TA2206; **(j)**, K-201; **(k)** K-2905. 1–7, homoeologous groups.

Table S1 | List of material and their origins.

Table S2 | Distribution of Spelt-52 probe on chromosomes of *Ae. longissima* and *Ae. sharonensis*.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 04 December 2018
doi: 10.3389/fpls.2018.01779

Repetitive DNA in the Architecture, Repatterning, and Diversification of the Genome of *Aegilops speltoides* Tausch (Poaceae, Triticeae)

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The genome's adaptability to environmental changes, especially during rapid climatic fluctuations, underlies the existence and evolution of species. In the wild, genetic and epigenetic genomic changes are accompanied by significant alterations in the complex nuclear repetitive DNA fraction. Current intraspecific polymorphism of repetitive DNA is closely related to ongoing chromosomal rearrangements, which typically result from erroneous DNA repair and recombination. In this study, we addressed tandem repeat patterns and interaction/reshuffling both in pollen mother cell (PMC) development and somatogenesis in the wild diploid cereal *Aegilops speltoides*, with a focus on genome repatterning and stabilization. Individual contrasting genotypes were investigated using the fluorescent *in situ* hybridization (FISH) approach by applying correlative fluorescence and electron microscopy. Species-specific Spelt1 and tribe-specific Spelt52 tandem repeats were used as the markers for monitoring somatic and meiotic chromosomal interactions and dynamics in somatic interphase nuclei. We found that, the number of tandem repeat clusters in nuclei is usually lower than the number on chromosomes due to the associations of clusters of the same type in common blocks. In addition, tightly associated Spelt1–Spelt52 clusters were revealed in different genotypes. The frequencies of nonhomologous/ectopic associations between tandem repeat clusters were revealed in a genotype-/population-specific manner. An increase in the number of tandem repeat clusters in the genome causes an increase in the frequencies of their associations. The distal/terminal regions of homologous chromosomes are separated in nuclear space, and nonhomologous chromosomes are often involved in somatic recombination as seen by frequently formed interchromosomal chromatin bridges. In both microgametogenesis and somatogenesis, inter- and intrachromosomal associations are likely to lead to DNA breaks during chromosome disjunction in the anaphase stage. Uncondensed/improperly packed DNA fibers, mainly in heterochromatic regions, were revealed in both the meiotic and somatic prophases that might be a result of broken associations. Altogether, the data obtained showed that intraorganismal dynamics of repetitive DNA under the conditions of natural

OPEN ACCESS

Edited by:
Eva Darko,
Hungarian Academy of Sciences (MTA), Hungary

Reviewed by:
Adél Sepsi,
Hungarian Academy of Sciences (MTA), Hungary
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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *29 August 2018*
Accepted: *15 November 2018*
Published: *04 December 2018*

Citation:
Pollak Y, Zelinger E and Raskina O (2018) Repetitive DNA in the Architecture, Repatterning, and Diversification of the Genome of Aegilops speltoides Tausch (Poaceae, Triticeae). Front. Plant Sci. 9:1779. doi: 10.3389/fpls.2018.01779

out-crossing and artificial intraspecific hybridization mirrors the structural plasticity of the *Ae. speltoides* genome, which is interlinked with genetic diversity through the species distribution area in contrasting ecogeographical environments in and around the Fertile Crescent.

Keywords: *Aegilops speltoides*, interphase nuclei, nonhomologous recombination, repetitive DNA, somatic recombination, tandem repeats

INTRODUCTION

In the wild, the genome's ability to adapt to changing environments, especially in a period of significant climatic changes, underlies the existence of the species and its evolution (Grant, 1981; Tchernov, 1988). In plant populations, genetic changes and epigenetic modifications are accompanied by significant diversification in the abundances and patterns of repetitive DNA, which is the prevailing genomic fraction in cereals (Bennetzen, 1996; Feschotte et al., 2002). Transposable elements (TEs) and tandem repeats compose the largest portion of the genome of wild diploid grass species *Aegilops speltoides* (sect. Sitopsis; 2n = 2x = 14) (Middleton et al., 2013). *Ae. speltoides* is a dimorphic species; the differences in spike morphology are controlled through a block of closely linked genes encoding dominant ssp. *ligustica* and recessive ssp. *aucheri* morphotypes, which coexist in mixed panmictic populations with different ratios (Zohary and Imber, 1963; Kimber and Feldman, 1987). Plants with intermediate *ligustica/aucheri* phenotypes have also been observed in the wild, suggesting genetic changes in the linked group of genes (Belyayev and Raskina, 2013). *Ae. speltoides* is only out-crossing species among the five diploid species of the sect. Sitopsis; however, under a changing environment, specifically, in drought conditions, *Ae. speltoides* transits to self-pollination, which is an extremely rare phenomenon in the plant kingdom (Zohary and Imber, 1963). The plasticity of the genome underlies a wide range of *Ae. speltoides* distribution and adaptability to contrasting ecogeographical environments in and around the Fertile Crescent (Zohary and Imber, 1963; Kimber and Feldman, 1987). Thus, at the northern periphery of the species distribution area, Turkish winter-type populations have a long vegetative cycle and exhibit a specific morphology, which significantly distinguishes them from the peripheral/marginal southern Israeli populations (Belyayev and Raskina, 2013). In parallel, the *Ae. speltoides* genome is characterized by high intraspecific polymorphism in abundance and patterns of different types of repetitive DNA, specifically, TEs (Middleton et al., 2013; Yaakov et al., 2013) and tandem repeats (Badaeva et al., 1996; Raskina et al., 2011; Molnár et al., 2014; Raskina, 2017), which underlie permanent intraorganismal and intraspecific genome reshuffling (Belyayev et al., 2010; Shams and Raskina, 2018). The current intraspecific polymorphism and intraorganismal dynamics of the highly repetitive DNA fraction in the genome of *Ae. speltoides* is largely caused by ongoing chromosomal rearrangements, which are typical results of erroneous DNA repair and recombination (Andersen and Sekelsky, 2010; Knoll et al., 2014; Zeman and Cimprich, 2015).

In the present research, we addressed repetitive DNA dynamics in the *Ae. speltoides* genome, both during pollen mother cell (PMC) development and in somatogenesis, with a focus on genome repatterning and stabilization. We traced tandem repeats' reshuffling/interactions during the cell cycle using fluorescent *in situ* hybridization (FISH), applying correlative fluorescence and electron microscopy. Species-specific Spelt1 (Salina et al., 1998) and tribe-specific Spelt52 (Anamthawat-Jónsson and Heslop-Harrison, 1993) tandem repeats were used as the markers for monitoring somatic and meiotic chromosomal interactions and dynamics in somatic interphase nuclei. We found that Spelt1 and Spelt52 demonstrated sequence-specific and genotype-/population-specific abundances and dynamics in interphase nuclei. The number of tandem repeat clusters in nuclei is usually lower than the number on chromosomes due to the associations of clusters of the same type in common blocks. In addition, tightly associated Spelt1–Spelt52 clusters were revealed in different genotypes. An increase in the number of tandem repeat clusters in the genome causes an increase in the frequencies of their associations in common blocks in interphase nuclei. Frequent cell-specific interchromosomal somatic associations and nonhomologous recombination in microsporogenesis were revealed. It is speculated that significant number of nonhomologous chromosomal associations detected in microgametogenesis might be the consequences of cell-specific ectopic recombination events that occurred in premeiotic cell lineages.

MATERIALS AND METHODS

Plant Material

Original plants of *Ae. speltoides* from contrasting allopatric populations Cankiri (Turkey; PI 573448, USDA), Ankara (Turkey; PI 573452, USDA), Katzir (Israel; 2.93, Institute of Evolution University of Haifa), Ramat haNadiv (Israel; 2.46, Institute of Evolution University of Haifa), and artificial F_1–F_2 intraspecific hybrids (Raskina, 2017), were analyzed.

Preparation of Chromosomal Spreads

Anthers containing PMC cells at the pachytene-diakinesis stages and seedling shoot apical meristems of individual plants were used for meiotic and mitotic chromosome spreads, respectively. Seeds were germinated on moist filter paper at 24°C in the dark. Seedlings 5–7 mm length were transferred to ice water for 24–26 h to accumulate metaphases and then fixed in 3:1 (v/v) 100% ethanol:acetic acid. The procedure of chromosome

spread preparation has previously been described (Raskina et al., 2004). Specifically, the fixed seedlings and anthers were washed (3 × 5 min) in water and then incubated in an enzyme buffer (10 mM citrate buffer at pH 4.6) and partially digested (meristem–for 50 min, anthers–for 30 min) in 6% pectinase plus 0.5% cellulase (NBC Biomedicals, United Kingdom) plus 5% cellulase "Onozuka" R-10 (Yakult Honsha Co., Ltd.) followed by washes in enzyme buffer (3 × 5 min) and distilled water (3 × 5 min). The material, in a drops of water, was transferred onto a grease-free microscope slide, and the cells were spread with a metal stainless needle in the drop of 60% acetic acid at 45–47°C on the hot plate, then fixed in 3:1 (v/v) 100% ethanol : acetic acid, and then immersed in absolute ethanol for 3–5 s. Dry chromosome spreads were used for *in situ* hybridization.

In situ Hybridization Procedures

For the FISH experiments, cytological slides of individual anthers and seedling shoot apical meristems containing well-spread chromosomal plates were used. The FISH procedures were conducted as previously described (Shams and Raskina, 2018).

Cells were treated with 1 μg/mL of DNase-free RNase A in 2 × SSC (0.3 M NaCl plus 30 mM trisodium citrate) for 60 min at 37°C followed by 3 × 3 min washes with 2 × SSC at 37°C. The preparations were then dehydrated in an ethanol series (70, 90, and 100%, 3 min each) at room temperature, washed 2 × 2 min with 2 × SSC, and then allowed to air-dry. The hybridization mixture (20 μL per slide under the glass coverslip 22 mm × 22 mm) contained 10% dextran sulfate, 2 × SSC, and 50 ng each of DNA probe. DNA probes and chromosome spreads were simultaneously denatured at 95°C for 3 min and hybridized using ThermoBrite StatSpin System (Abbott, United States). Hybridization was carried out at 63°C for 2 h. After removal of the coverslips in 2 × SSC at 63°C, the slides were washed for 2 × 5 min in 2 × SSC at 63°C, additionally once in 0.1 × SSC for 5 min at 63°C to increase stringency, then cooled to 37°C and washed for 2 × 5 min in 0.1 × SSC; cooled to room temperature, washed in distilled water for 1 min, allowed to air-dry for 20 min, and mounted in VECTASHIELD antifade mounting medium (Vector Laboratories).

Tandem repeats Spelt1 (Salina et al., 1998), Spelt52 (Anamthawat-Jónsson and Heslop-Harrison, 1993), pSc119.2 (Bedbrook et al., 1980), pTa71 (for the localization of 45S rDNA) (Taketa et al., 2000), and As5SDNAE (for the localization of 5S rDNA) (Baum and Bailey, 2001) were used as the DNA probes for FISH. The DNA probes were directly labeled with Cy-3, Fluorescein-12-dUTP, and ATTO-425 (Jena Bioscience, Germany). AT-specific 4′,6-diamidino-2-phenylindole (DAPI) fluorochrome was used for differential staining.

Epi-Fluorescence Imaging

The slides were examined on a Leica DMR fluorescent microscope equipped with a DFC300 FX CCD color camera using following filter sets: A for DAPI, I3 for Fluorescein-12-dUTP, N2.1 for Cy3, FI/RH for Fluorescein-12-dUTP/Cy3, and B/G/R for blue/green/red fluorescence.

Confocal Image Acquisition and Analysis

Confocal imaging was done using a LEICA SP8 (CTR6000) microscope with a Leica HC PL APO CS2 × 63 N.A 1.4 oil objective. Lasers used: EX 405 EM 430–470 for DAPI, EX 488 EM 500–540 for Fluorescein-12-dUTP, and EX 552 EM 560–590 for Cy3. All images were collected with the HyD (hybrid detector). Image z-stacks of 15–25 × 0.2 μm slices per specimen were acquired and reconstructed by the 3D built-in module of LEICA SP8 in the LAS AF software (**Supplementary Figures S1–S3**). 3D Movies were created from the z-stacks by the LEICA SP8 3D module (**Supplementary Movies S1–S4**). For statistical analysis and 3D reconstruction the ImageJ software[1] was used (**Supplementary Figure S3B**).

For 3D reconstruction modeling, the images were processed using the Imaris surface reconstruction tool with module "Imaris cell" (Bitplane Scientific Software, Zurich, Switzerland) (**Supplementary Figure S4**).

Scanning Electron Microscopy

The scanning electron microscopy (SEM) images were recorded using Field emission scanning electron microscopes (i) Sigma-HD, ZEISS, and (ii) JEOL, JSM-7800F, accelerating voltage of 1.5–2.0 kV. Slides were carbon coated using Quorum Q150T.

RESULTS

Patterns of Tandem Repeats on Chromosomes and in Somatic Interphase Nuclei in Plants From the Cankiri Population

There are nine Spelt52 clusters of different sizes and fluorescence intensity, two large Spelt1 clusters on the long arms of the both homologs of chromosome 7, and two small Spelt1 clusters on the long arms of both homologs of chromosomes 5 in the diploid genome(s) of plant(s) from the Cankiri population (**Figure 1A**). The number of Spelt52 clusters in the interphase nuclei varied between six and nine. Among 300 interphase nuclei of different sizes and varying degrees of chromatin compactness analyzed on two slides from the seedling shoot apical meristems, two large Spelt1 clusters were observed in most cases (**Figures 1A,B**) and only in eight nuclei (2.7%) was a single Spelt1 cluster revealed (**Figure 1C**). Thus, these data point to separation of the homologous chromosomes in nuclear space; associations of the distal/terminal chromosomal regions and the formation of complex tandem repeat clusters rarely occurred.

Patterns of Tandem Repeats on Chromosomes and in Somatic Interphase Nuclei in Plants From the Katzir Population

In contrast to the genotypes from the Cankiri population, plants from the Katzir population are enriched with Spelt1

[1]https://imagej.net/Fiji

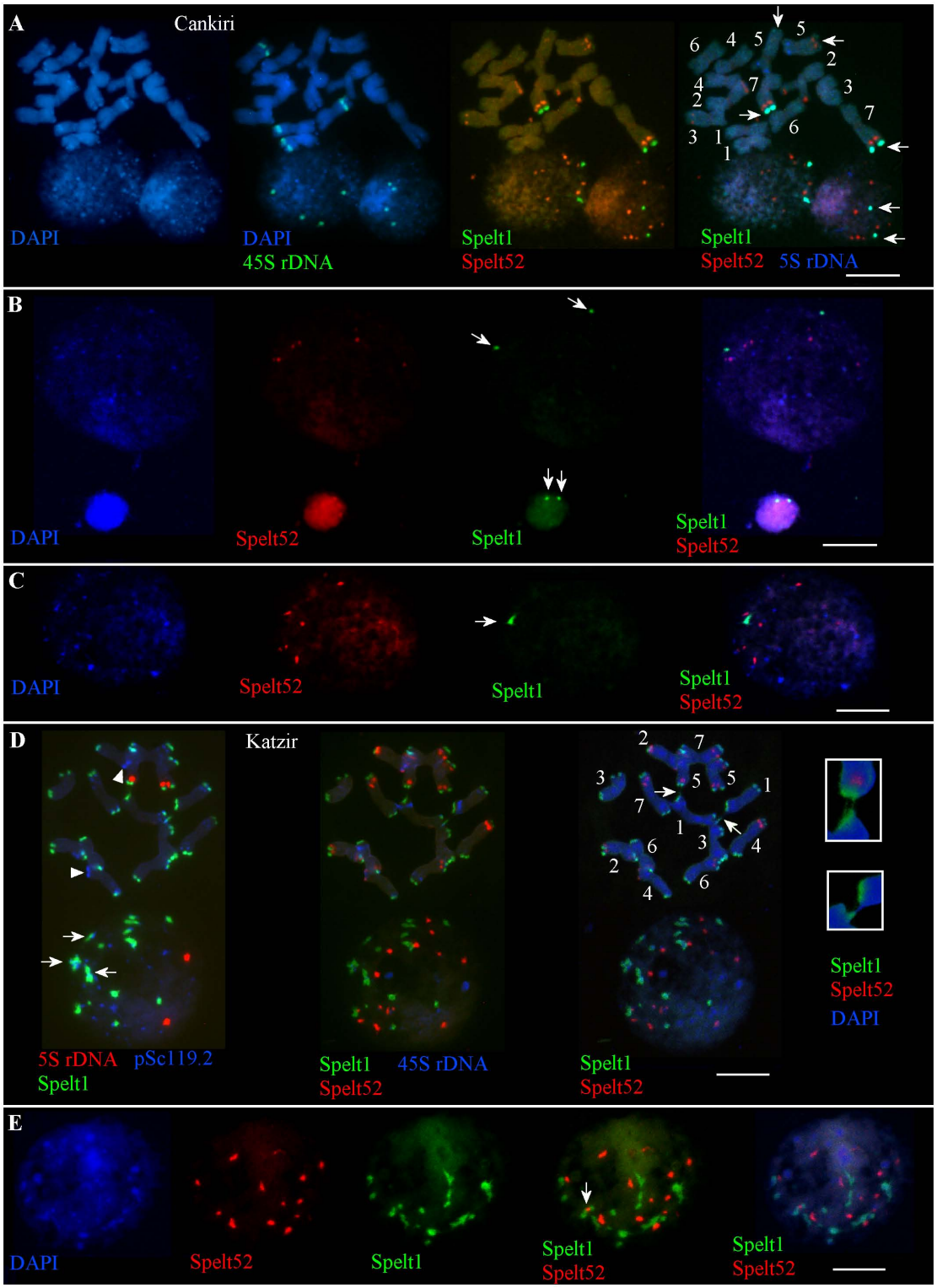

FIGURE 1 | Patterns of tandem repeats in the *Ae. speltoides* contrasting genotypes. Fluorescent *in situ* hybridization (FISH) on somatic chromosomes and interphase nuclei from the seedling shoot apical meristems of the plants from Cankiri **(A–C)** and Katzir **(D,E)** populations. DNA probes for FISH: Spelt1 (in green), Spelt52 (in red), pSc119.2 (in blue), 5S rDNA (in blue, red), and 45S rDNA (in green). **(A)** Somatic chromosomes **(top)** and interphase nuclei **(bottom)** of the plant from the Cankiri population. Two large terminal Spelt1 clusters on the long arms of homologous chromosome 7 and two small terminal clusters on the long arms of homologous chromosome 5 were revealed (arrows). Nine Spelt52 clusters were detected in the diploid genome. In the interphase nuclei, Spelt52 and two large

(Continued)

FIGURE 1 | Continued
Spelt1 (arrows) clusters are separated from each other. Four separated 45S rDNA clusters are revealed in the nuclei. **(B)** Two large Spelt1 clusters in the interphase nuclei of different sizes are indicated with arrows. **(C)** Single Spelt1 cluster (arrow) and separated Spelt52 clusters are observed in the interphase nucleus of Cankiri plant. **(D)** Tandem repeats Spelt1 and pSc119.2 form complex clusters in chromosome termini **(top left)**. There are 26 Spelt1 clusters in the diploid genome; 12 chromosomes carry the Spelt1 cluster in both arms and two chromosomes carry Spelt1 in one arm, with terminal pSc119.2 clusters shown in the other arm with arrowheads. In the interphase nucleus **(bottom)**, 14 Spelt1 clusters are revealed due to the association of individual clusters. In addition, Spelt1 and pSc119.2 tandem repeats compose common clusters (arrows). Two 5S rDNA clusters are separated in the nuclear space. Re-probing with Spelt52 and 45S rDNA **(middle)** revealed 14 Spelt52 clusters on the chromosomes and in the nucleus. Ectopic chromatin fibers between nonhomologous chromosomes (arrows; enlargement in the small boxes) are indicated with arrows **(right)**. **(E)** Interphase nucleus of the Katzir genotype. Spelt52 clusters are separated in the nuclear space, while the number of Spelt1 clusters is almost half that on the chromosomes. Tightly associated Spelt1 and Spelt52 clusters are indicated with arrow. Scale bar = 10 μm.

tandem repeats (**Figures 1D,E**). There are 14 Spelt52 and 26 large terminal Spelt1 clusters in the diploid genome; only two chromosome arms do not carry Spelt1 clusters (**Figure 1D**, top); instead, clusters of the pSc119.2 tandem repeat are detected in these positions. The genome of *Ae. speltoides* is enriched with the pSc119.2 tandem repeat, which forms numerous clusters in the distal/terminal and intercalary chromosomal regions (Badaeva et al., 1996; Molnár et al., 2014). In the interphase nuclei, the Spelt1 and pSc119.2 tandem repeats form common clusters (**Figure 1D**). The number of Spelt1 clusters is reduced almost by half due to associations, while the number of Spelt52 clusters equals the number of clusters on the chromosomes, and 5S rDNA clusters are separated in the nuclear space. Among the 130 nuclei from the seedling shoot apical meristems analyzed on this cytological slide, the number of Spelt1 clusters was reduced in most cases, and one to three tightly associated Spelt1–Spelt52 clusters were revealed in 123 (94.6%, n = 130) nuclei (**Figures 1D,E**); and in other genotype, colocalization of the Spelt1–Spelt52 clusters was revealed in most cases (78.5%, n = 250).

Patterns of Tandem Repeats on Chromosomes and in Somatic Interphase Nuclei in Plants From the Ramat haNadiv Population

Meiotic chromosomes and interphase nuclei from the somatic anther tissues were analyzed on the same slide(s) for different genotypes from the Ramat haNadiv population (**Figure 2**). At the early stages of anther' development, specifically, at the pachytene–diakinesis–metaphase I stages, the following cell layers, namely epidermis, endothecium, middle layer, tapetum, and connective tissues are distinguished (Browne et al., 2018). The nuclei of different sizes, shapes, and chromatin compactness at different stages of the interphase from the anther's somatic tissues vary significantly in the numbers of Spelt1 and Spelt52 clusters (**Figures 2A–A8**, **Supplementary Figures S1,S2,** and **Supplementary Movies S1,S2**). Colocalization of the tandem repeat clusters of the same type is observed in a cell-specific manner; in addition, Spelt1 and Spelt52 tandem repeats form tightly associated/common clusters (100%; n = 400; three slides/genotypes), which are condensed or partially decondensed at different interphase stages and in different anther somatic tissues. Numerous polyploid (**Figure 2A3**) and amitotically dividing tapetal nuclei (**Figure 2A6**) are revealed on the same cytological slide(s). Typically, partial decondensation of tandem repeat clusters and the occurrence of large common clusters are observed in small compact nuclei (**Figures 2A4–A6,C,E, Supplementary Figures S3,S4,** and **Supplementary Movies S3,S4**). Somatic recombination in the interphase nucleus was documented using a SEM (**Figure 2D**). Specifically, two chromatin forks/two different chromosomes are recombined in their terminal regions, which form continuous chromatin fiber. At the end of the interphase/in the early somatic prophase stage, as condensed chromatin fibers become visible, numerous associations between clusters of the same type and between Spelt1 and Spelt52 clusters are still observed (**Figures 2A7,A8**, **G**,**H**).

Nonhomologous Chromosome Associations in Microsporogenesis in Different Genotypes From the Ramat haNadiv Population

Along with somatic interphase nuclei from the anther tissues, meiotic cells were analyzed on the same slide(s). A wide spectrum of cell-specific structural alterations and chromosomal rearrangements at the meiosis I stages were revealed in different genotypes (**Figures 2I–K**). Thus, alterations in Spelt1 and Spelt52 clusters' condensation and the appearance of tightly associated clusters were observed in late meiotic prophase I (**Figure 2I**); chromatin fibers appear damaged and uncondensed. At the stages of diakinesis–metaphase I, cell-specific chromosomal rearrangements and numerous nonhomologous associations were revealed in all plants (**Figures 2J,K**). Interchromosomal fibers were documented using both fluorescent and scanning electron microscopy.

Tandem Repeat Cluster Associations in Somatic Interphase Nuclei in Parental Genotype From the Katzir Population and Intraspecific Hybrids of *Aegilops speltoides*

In the maternal genotype from the population Katzir, there are 12 Spelt52 and 22 Spelt1 clusters on 14 individual chromosomes, or 14 Spelt1 and 7 Spelt52 clusters per seven bivalents, that is, when homologs are paired (**Figure 3A**, on the left). In somatic nuclei (**Figure 3A**, on the right), the number of both types of clusters vary and often is smaller than the number on 14 chromosomes (statistical analysis was not performed).

In the F1 hybrid genotype (**Figure 3B**; Raskina, 2017), maternal and paternal chromosomes are identified according

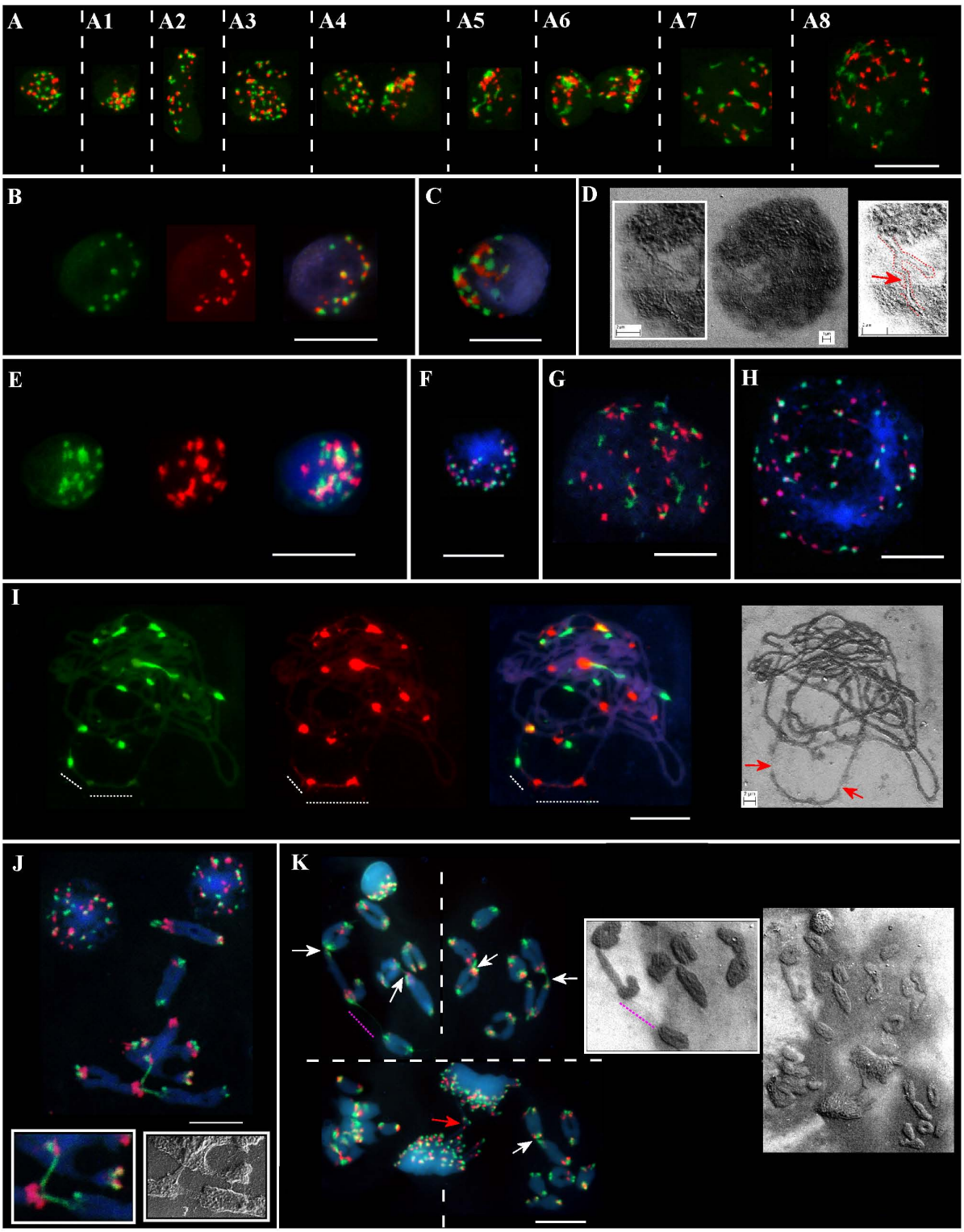

FIGURE 2 | Patterning of Spelt1 and Spelt52 tandem repeats on meiotic chromosomes and in interphase nuclei from the anther' somatic tissues in individual genotypes of *Ae. speltoides* from the Ramat haNadiv population. DNA probes for FISH: Spelt1 (in green) and Spelt52 (in red); counterstaining with DAPI (in blue). **(A)** Interphase nuclei at different interphase stages belonging to different anther somatic tissues from the same cytological slide. **(A–A8)** (**Supplementary Figures S1,S2** and **Supplementary Movies S1,S2**) In nuclei of different sizes and shapes, various numbers of Spelt1 and Spelt52 clusters are observed. The nuclei differ in number and sizes of associated clusters of the same types and Spelt1–Spelt52 colocalized clusters. Condensed and undercondensed single

(Continued)

FIGURE 2 | Continued
and associated Spelt1 and Spelt52 tandem repeat clusters are revealed in different nuclei. A polyploid nucleus is shown in **A3**. **(A6)** Amitotically dividing nuclei. **(A7,A8)** Nuclei at the late interphase–early prophase stages. Spelt1 and Spelt52 clusters are partially decondensed and interconnected with extended fibers; due to associations, the cluster number is less than on meiotic chromosomes **(J,K)**. **(B–D)** Nuclei from the same cytological slide. **(B)** Number of condensed Spelt1 and Spelt52 clusters of different sizes and fluorescent intensities are 11 and 17, respectively. Most Spelt1 and Spelt52 clusters associate in common clusters. **(C)** Large Spelt1 and Spelt52 clusters aggregate in complex blocks. **(D)** In the image of the interphase nucleus obtained using scanning electron microscopy, two interconnected Y-shaped chromatin structures are revealed. Chromatids of two different chromosomes form a double-strand chain (red dashed line and arrow in the scheme on the right). **(E–H)** Nuclei of the other genotype from the same cytological slide. **(E)** (**Supplementary Figures S3,S4** and **Supplementary Movies S3,S4**) Large Spelt1 and Spelt52 partially decondensed clusters aggregate in complex blocks. **(F)** Highly condensed clusters of Spelt1 and Spelt52 form common blocks. **(G,H)** Large nuclei at the late interphase–early prophase stages; condensed chromatid fibers are revealed. Spelt1 and Spelt52 clusters interconnected with extended fibers. **(I)** Meiotic prophase I, pachytene stage: homologous synapsis is completed. Associated Spelt1 and Spelt52 clusters form common blocks, and extended fibers between clusters are observed; improper condensation and damaged chromatin fibers are indicated with dashed lines and arrows on the image obtained via scanning electron microscopy **(right)**. **(J,K)** Meiotic chromosomes and nuclei of different genotypes. Numbers of Spelt52 vary from 11 to 12 clusters and Spelt1 from 13 to 14 clusters per seven bivalents. **(J)** Three bivalents are involved in nonhomologous recombination. Extended Spelt1 fibers connect two homologs of one bivalent with single chromosomes of two other bivalents. In two **small boxes**, enlargement of this region obtained with confocal fluorescent **(left)** and scanning electron microscopy **(right)**, respectively, is shown. **(K)** Four meiotic chromosomal plates at the diakinesis–metaphase I stages and dividing polyploid cell of tapetum at the anaphase stage with chromosomal bridges (red arrow). Images were taken consistently using fluorescent and scanning electron microscopes. Nonhomologous chromosome associations are indicated with arrows; the extended thin Spelt1 fiber between two bivalents is indicated with a pink dashed line. Scale bar = 10 μm.

to contrasting Spelt1 and Spelt52 patterning, as detailed in the small box. Maternal chromosomes carry 12 Spelt1 and 2 Spelt52 clusters; paternal chromosomes carry 3 Spelt1 and 5 Spelt52 clusters. In terms of the seven bivalents, there are 13 Spelt1 and 5 Spelt52 clusters in the genome. Somatic interphase nuclei from the anther tissues on the same cytological slide differ in the number of Spelt1 and Spelt52 clusters. Two nuclei (on the left and in the middle) are similar and contain 12 Spelt1 and 8 Spelt52 clusters, while in the third nucleus (on the right), the numbers of clusters are reduced due to associations.

In the F2 hybrid genotype (**Figure 3C**), the Spelt1 fibers between the homologous chromosomes at the stage of anaphase I are the consequences of meiotic recombination in the terminal chromosome regions; interchromosomal connections will be broken, and homologs will move to opposite pools with altered termini.

Ectopic Associations of Somatic Chromosomes in Different Genotypes

Frequent cell-specific interchromosomal somatic associations in apical shoot meristem were revealed in plants from different populations. Thus, in the Katzir genotype, among 32 metaphase plates analyzed on the same cytological slide, in 21 cases (65.6%), ectopic associations between chromosomes were detected in a cell-specific manner, regardless of the presence or absence of any tandem repeat clusters in the points of chromosomal connections (**Figure 1D**). Interchromatid somatic associations between three chromosomes were documented for the other genotype from the Katzir population (**Figure 4A**). In this instance, two chromatids of one chromosome are connected with single chromatids of two other chromosomes. Clusters of Spelt1 and Spelt52 are involved in one association and form interchromosomal fibers; in other association, the fiber between chromatids of two different chromosomes was revealed with DAPI staining.

No associations between the Spelt1 clusters were observed for homologous chromosomes 7 in the Cankiri plants. Partially decondensed Spelt1 clusters were documented in somatic nuclei (**Figure 4B**). Cell-specific alterations in Spelt1 clusters' condensation/decondensation was observed in somatic prophase cells (**Figure 4C**).

In the genotype from the Ramat haNadiv population, intrachromosomal ectopic recombination was revealed (**Figure 4D**). Here, the Spelt1 fiber is observed as a dotted line, which may be an indication of intercalation/alternation of Spelt1 subclusters with some other type of uncolored DNA sequences or decondensation of uncolored regions. Ectopic associations between chromosomes 1 and 6, which carry 45S rDNA clusters, and chromosome 5, carrying 5S rDNA clusters, were documented for the genotype from the Ankara population (**Figure 4E**). Ectopic interchromatid fibers, which are revealed with DAPI staining, connect the short and/or long arms of somatic chromosomes, and the rDNA clusters appear intact.

In the F2 hybrid (♀Cankiri × ♂Katzir) genotype, at different interphase stages, 12 Spelt1 clusters were typically interconnected when decondensed (**Figure 4F**), and they apparently formed two groups of six clusters each when condensed, in the diploid nucleus (**Figure 4G**). On the prometaphase chromosomes (**Figure 4H**), partial decondensation and interconnections between five Spelt1 clusters were observed. In this genotype, two chromosomes carried Spelt1 clusters on both the short and long arms (**Figure 4I**). Therefore, the fibers between the four clusters in the prometaphase could be consequences of cell-specific ectopic associations between the short and long arms in the interphase, with the fifth cluster belonging to some other chromosome; alternatively, all five clusters could belong to different/nonhomologous chromosomes. However, in the other F2 hybrid genotype (**Figures 4J–L**) containing 10 Spelt1 clusters in the diploid genome, all chromosomes carry single Spelt1 clusters in one arm; while in the nuclei, associations of two and three clusters were revealed (**Figures 4J,L**). Two clusters may have belonged to homologous chromosomes, while three undoubtedly pointed to nonhomologous associations. In addition, ectopic chromatin fibers were detected between the chromosomal regions, and these did not carry tandem repeat

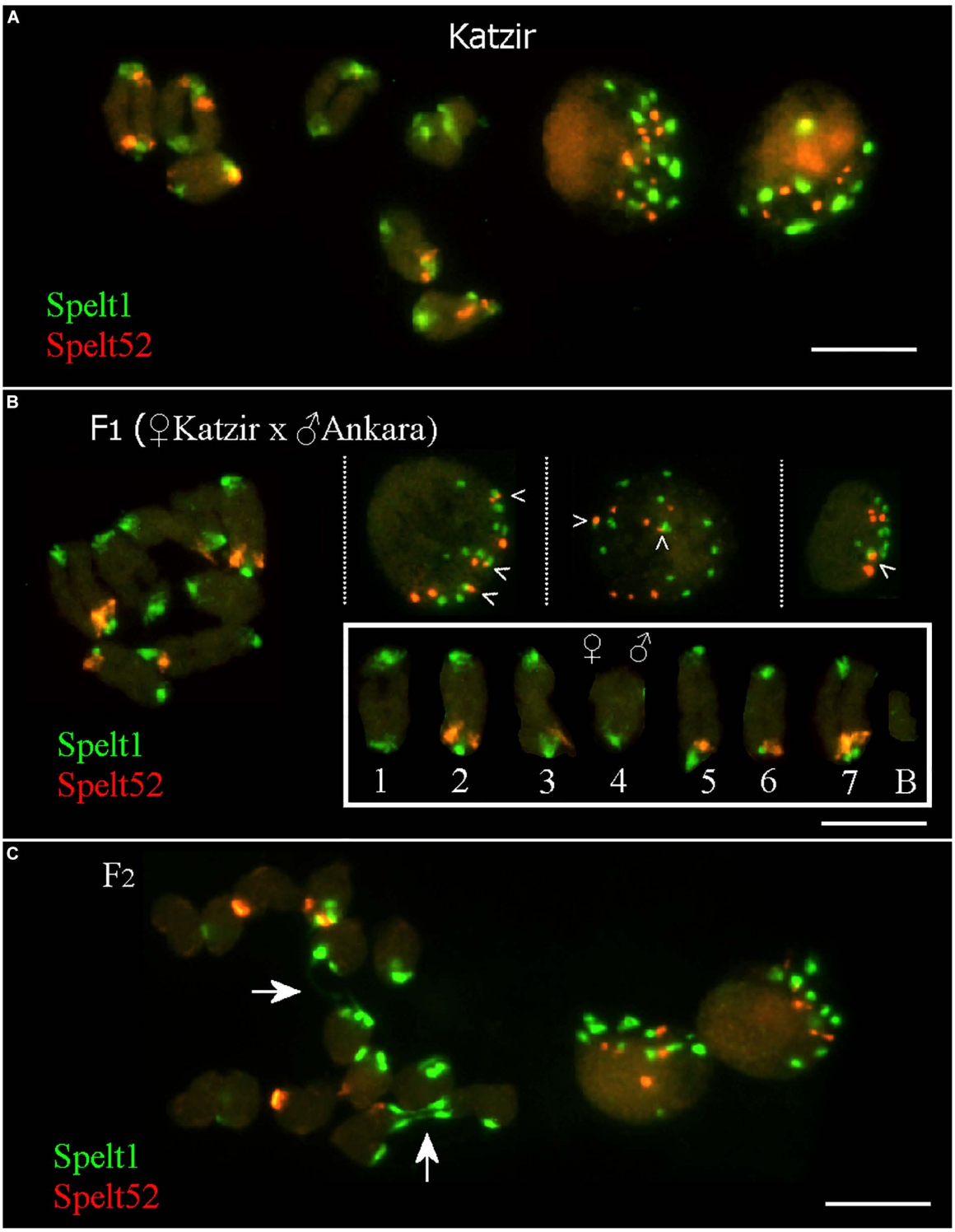

FIGURE 3 | Fluorescent *in situ* hybridization (FISH) with Spelt1 and Spelt52 tandem repeats on meiotic chromosomes and interphase nuclei from the anther' somatic tissues of individual genotypes of *Ae. speltoides*. **(A)** In the genotype from the population Katzir, there are 22 Spelt1 clusters on 14 individual chromosomes and 14 clusters per 7 bivalents. There are 12 Spelt52 clusters on individual chromosomes, and seven clusters per seven bivalents. Two interphase nuclei differ in the abundance of tandem repeats clusters: there are 13 Spelt1 and 10 Spelt52 clusters in one nucleus **(left)**, whereas 8 clusters of Spelt1 and 5 clusters of Spelt52 are revealed in the other nucleus **(right). (B)** In the hybrid F1 genotype (2n = 2x = 14 + B) obtained in the crosses between plants from the Katzir and Ankara populations, there are 15 Spelt1 clusters (12 from the maternal genome and 3 from the paternal genome) and 7 Spelt52 clusters (two from Katzir genotype and five

(Continued)

FIGURE 3 | Continued
from Ankara genotype) on 14 individual chromosomes (**small box;** the orientation of bivalents corresponds to the position of the maternal chromosomes on the left and paternal chromosomes on the right). In terms of the number of clusters per seven bivalents, there are 13 Spelt1 (both homologous chromosomes 4 do not carry Spelt1 in the short arms) and 5 Spelt52 (in the long arms of one or two conjugated homologous chromosomes 2, 3, 5, 6, and 7) clusters. In three different interphase nuclei from the same cytological slide **(top)**, there are 12 Spelt1 and 8 Spelt52 clusters in one nucleus **(left)**; the same numbers are revealed in the second nucleus **(middle)**, and 8 Spelt1 and 5 Spelt52 clusters were detected in the third nucleus **(right)**. Tightly associated Spelt1 and Spelt52 clusters are indicated with checkmarks. **(C)** Meiotic chromosomes at the anaphase I stage and interphase nuclei from the same cytological slide of the hybrid F2 genotype. Stretched Spelt1 fibers between homologous chromosomes are indicated with arrows. Scale bar = 10 μm.

clusters (**Figure 4L**). Altogether, the data obtained show that different chromosomes can be randomly involved in somatic associations.

Thus, tandem repeat cluster associations were revealed in nuclei at different interphase stages in both shoot apical meristems and anther' somatic tissues. Ectopic associations between somatic chromosomes were documented in different original and hybrid genotypes. In microsporogenesis, nonhomologous/ectopic recombination was documented in different genotypes. As a possible result of broken associations, uncondensed/improperly packed DNA fibers, mainly in heterochromatic regions, were revealed in both the meiotic and somatic prophases-metaphases, especially in distal/terminal chromosomal regions enriched with different types of highly repetitive DNA.

DISCUSSION

The data obtained evidence on dynamic ectopic chromosomal interactions during somatic cell proliferation and differentiation and nonhomologous recombination in microsporogenesis of *Ae. speltoides*.

Sequence- and Genotype-Specific Tandem Repeat Patterns and Dynamics Evidence on Homologous Chromosome Separation in Interphase Nuclei

The patterning of two types of tandem repeats, Spelt1 and Spelt52, changed dynamically in somatic interphase nuclei. In the nuclei at different interphase stages in various somatic tissues, the numbers of Spelt1 and Spelt52 clusters varied significantly, and they were mostly less than the numbers of clusters on the somatic and meiotic chromosomes. Associations of the clusters of the same type cause a reduction in their number; moreover, Spelt1 and Spelt52 tandem repeats formed joint clusters in nuclei. The reduction of the tandem repeat cluster number in the somatic nuclei may be the result of homologous and/or nonhomologous associations; in addition, intrachromosomal ectopic recombination could reduce the number of clusters. Individual genotypes from contrasting populations and intraspecific hybrids differed in the total abundance and chromosomal patterning of Spelt1 and Spelt52 and cluster dynamics in the interphase stage. In the Cankiri plants, distal regions of homologous chromosomes 7 generally separated in the nuclear space during the interphase. In parallel, in most cases, two 5S rDNA (located on chromosome 5) and four 45S rDNA (located on chromosomes 1 and 6) clusters also were separated in the nuclei. These data point to the separation of the homologous chromosomes of two subgenomes in the nucleus during the interphase stages, and they are consistent with data obtained in studies of other plant species, which have revealed largely random homologous and nonhomologous chromosome arrangements and a low frequency of pairing/associations in interphase nuclei (Pecinka et al., 2004; Schubert et al., 2007, 2014).

The Dynamics of Spelt1 and Spelt52 Tandem Repeats Differ in the Interphase Nuclei

Associations of Spelt52 clusters were observed with a lower frequency than associations of Spelt1 clusters. The number of Spelt52 clusters in interphase nuclei mainly corresponded to the cluster number on the chromosomes. In the nuclei, both types of tandem repeat are usually separated due to decondensation of interspersed DNA sequences of other types. However, in this study, associated complex Spelt1–Spelt52 clusters were revealed in interphase nuclei in different genotypes. The highest frequency of cluster fusions was found in the Ramat haNadiv population, in which the abundances of both types of tandem repeats were highest in comparison with plants from the Cankiri and Katzir populations and hybrid genotypes (Raskina et al., 2011). The association of the Spelt1–Spelt52 clusters most likely indicated the elimination of intercluster sequences due to typically high frequencies of rearrangements in heterochromatic regions, specifically in the *Ae. speltoides* genome. However, we were unable to discriminate intrachromosomal associations and recombination events in nuclei, except when the number of colocalized clusters exceeded the number on the homologous chromosomes (**Figures 4F,J**).

An Increase in the Number of Tandem Repeat Clusters in the Genome Causes an Increase in the Probability of Their Interactions

A comparison of contrasting genotypes indicated that, as the abundance of Spelt1 and Spelt52 tandem repeats increases in the genome, the frequency of cluster associations in the interphase also rises. In the Cankiri genotypes, a single Spelt1 cluster was found in approximately 3% of the nuclei; however, Spelt1 clusters associations was a common phenomenon in plants from Katzir and Ramat haNadiv. The associations of Spelt1 clusters exceeded the frequency of associations of Spelt52 clusters. At the same

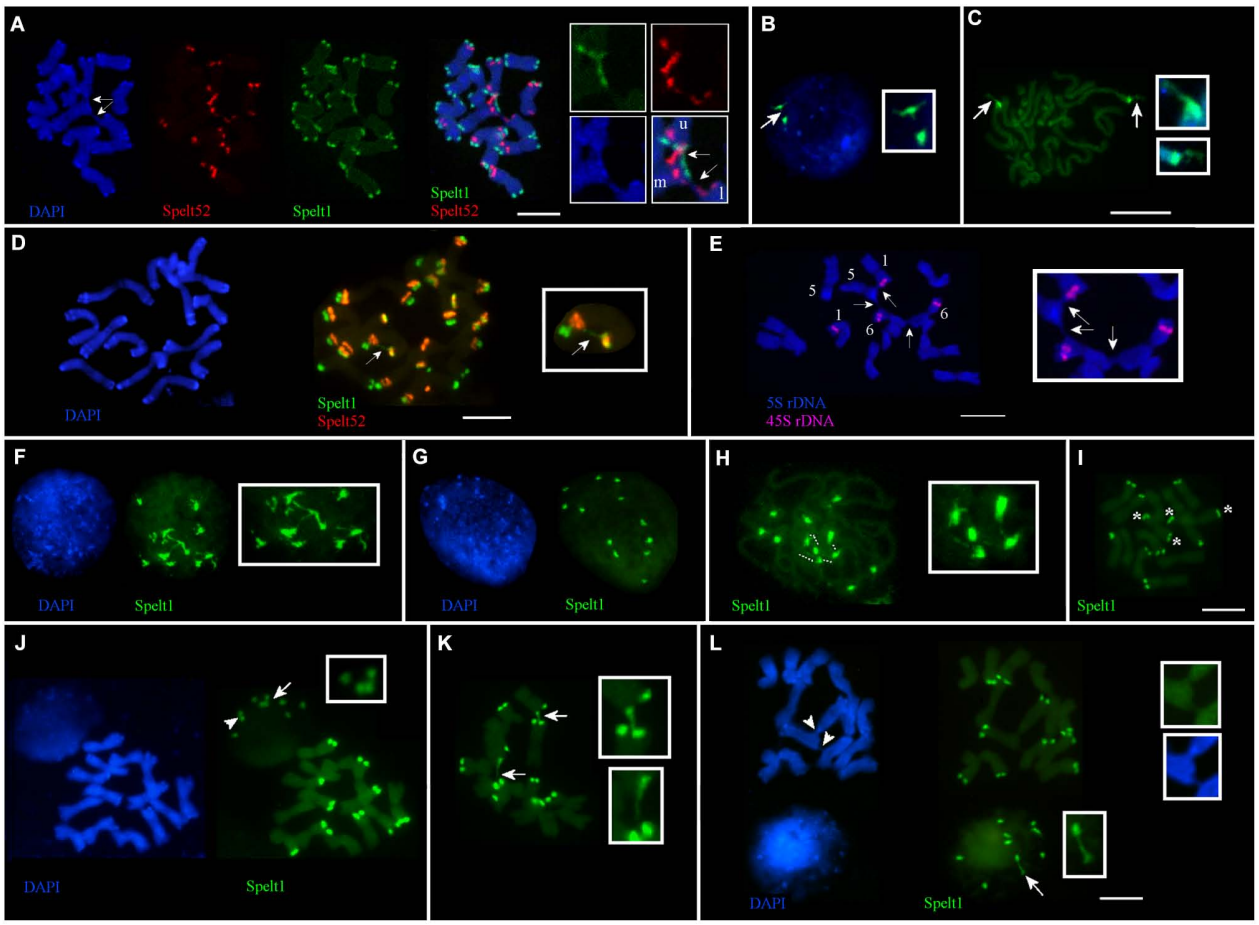

FIGURE 4 | Cell-specific interchromosomal and intrachromosomal ectopic associations in different genotypes of *Ae. speltoides*. The chromosomes and nuclei from the seedling shoot apical meristems. **(A)** Genotype from Katzir population. Three chromosomes are involved in ectopic associations (arrows; enlargement in the small boxes). Specifically, Spelt1 and Spelt52 clusters of one of the two chromatids of the upper chromosome (this chromosome is indicated by the letter "u" in the small box) form ectopic fibers with similar clusters located on the one of two chromatids of the second chromosome (marked "m" in the small box), which is located in the middle. One chromatid of the third lower chromosome (marked "l" in the small box), which carries the Spelt52 cluster, forms ectopic fiber (visualized by DAPI staining) with the second chromatid of the "m" chromosome, and in this case, unknown sequences are involved in the ectopic association. **(B)** Interphase nucleus and **(C)** somatic prophase chromosomes of the Cankiri genotype. In the nucleus, one Spelt1 cluster is partially decondensed (arrow; enlargement in the small box). Both Spelt1 clusters on the prophase chromosomes are partially decondensed (arrows; enlargement in the small boxes). **(D)** Intrachromosomal ectopic association in the genotype from the Ramat haNadiv population. Ectopic Spelt1 fiber was revealed as a dotted line between the Spelt1 cluster of the short arm and Spelt52 cluster of the long chromosome arm (arrow; enlargement in the small box). This fiber consists of interspersed small green Spelt1 clusters and uncolored regions of unknown sequences. **(E)** Ectopic associations between somatic chromosomes bearing 5S rDNA and 45S rDNA clusters of plant from the Ankara population. One homolog of chromosome 5, one homolog of chromosome 1, and both homologs of chromosome 6 are involved in somatic associations (arrows; enlargement in the small box). Clusters of 5S rDNA and 45S rDNA appear intact; ectopic fibers are formed by some other sequences and revealed by DAPI staining. **(F–I)** Interphase nuclei and chromosomes from the same cytological slide of the hybrid genotype F2 (♀Cankiri × ♂Katzir). **(F)** Decondensed Spelt1 clusters interconnected by extended fibers. **(G)** Condensed clusters of Spelt1 form two groups of six clusters each in the nucleus. **(H)** Spelt1 clusters on prophase chromosomes are partially decondensed and interconnected by extended fibers (dashed lines; enlargement in the small box). **(I)** Metaphase plate: there are 12 Spelt1 clusters in the diploid genome. Two chromosomes carry Spelt1 clusters in both arms (asterisk), and 12 carry the cluster in the one arm. **(J–L)** Interphase nuclei and chromosomes from the same cytological slide of another hybrid genotype F2. **(J)** There are 10 Spelt1 clusters in diploid genome; none of the chromosomes contains clusters in both arms. In the interphase nucleus, associations of three (arrow; enlargement in the small box) and two clusters (arrowhead) are observed. **(K)** Ectopic associations between Spelt1 clusters of different chromosomes (arrows; enlargement in the small boxes) are revealed in a cell-specific manner. **(L)** Two Spelt1 clusters are connected by stretched fiber in the interphase nucleus (arrow, enlargement in the small box); ectopic associations between chromosomes are indicated with arrowheads (enlargement in two small boxes). Scale bar = 10 μm.

time, the two types of tandem repeats formed tightly associated complex clusters at different interphase stages.

Along with the dynamic change in the number of clusters as a result of their associations, partial decondensation of clusters in nuclei of different types was revealed. In the genome of *Ae. speltoides*, both types of tandem repeats are an integral part of heterochromatin, which is known to be condensed throughout the cell cycle, except for the time of replication in the late S-phase (Grewal and Moazed, 2003). The highest degree of associations of clusters of both types, with a decrease

in their number and simultaneous decondensation have been observed in relatively small, dense and brightly DAPI-fluorescent nuclei (**Figures 2C,E**). Probably, these nuclei are at the stage of heterochromatin replication; however, this question remains open and requires additional study.

The decondensation of Spelt1 and Spelt52 clusters was also found in somatic nuclei of other types, which, according to their sizes and chromatin fiber compactness, were at the late interphase–early prophase stages (**Figures 2A7,A8**, **G**,**H**). Extended fibers between clusters of the same type and between Spelt1–Spelt52 clusters was most likely caused by ectopic interactions in the earlier interphase, and in turn, provide the proof for such events. At the metaphase stage, cell-specific ectopic associations between somatic chromosomes were revealed in all the investigated genotypes.

Repetitive DNA Reshuffling Is Required for Genome Stabilization

In the interphase nuclei of hybrid genotypes, an increase in the number of Spelt1 clusters does not allow the discrimination of homologous chromosomes. Nevertheless, condensed Spelt1 clusters often form two spatially separated groups, which mirror the spatial arrangements of the terminal chromosomal regions of two subgenomes. In contrast, at the interphase stages, when the Spelt1 clusters are decondensed, they are usually interconnected by extended fibers, and the number of interconnected clusters evidences frequent nonhomological associations. It was shown for allopolyploid wheat that, in the somatic nuclei, homologous and nonhomologous chromosomes display non-random arrangement; different subgenomes occupy different territories, and homologous chromosomal sets are associated (Avivi and Feldman, 1980; Avivi et al., 1982).

Intraspecific hybrids, which were investigated in this work, were obtained by crossing genotypes with contrasting Spelt1 contents (Raskina, 2017). In the F1, homologous chromosomes preserved the parental patterns of tandem repeats. In contrast, the Spelt1 and Spelt52 clusters were almost evenly distributed between homologous chromosomes in F2 descendants (**Figure 3**). For cross-pollinated *Ae. speltoides*, heterozygosity for chromosomal rearrangements in distal/terminal regions is an inherent feature of individual plants in wild populations; however, homologous chromosomes demonstrate significant similarity in their heterochromatin patterns (Raskina et al., 2011). Presumably, an artificial asymmetry in tandem repeat patterns in the F1 intraspecific plant(s) could negatively affect the hybrid genomes' stability, specifically, by altering the chromosome pairing and segregation. In addition, the subtelomeric location of Spelt1 may be tightly connected with telomere functioning and dynamics during both mitosis and meiosis (Bozza and Pawlowski, 2008; Tiang et al., 2012; Mainiero and Pawlowski, 2014). In the early meiotic prophase, homolog pairing is accompanied by repositioning of chromosomes in the nuclear space and "telomere bouquet" formation; and clustering of telomeres on the nuclear envelope may continue until early pachytene. Premeiotic and meiotic processes of telomere clustering involve subtelomeric DNA sequences (Bozza and Pawlowski, 2008), and the pattern and dynamics of Spelt1 in interphase nuclei may be associated with telomere dynamics and homologous recognition (Calderón et al., 2014; Sepsi et al., 2017).

Genome stabilization implies rearrangement/repatterning of heterochromatic clusters toward increasing similarity between homologs. Indeed, extended Spelt1 fibers between somatic and meiotic chromosomes is a common phenomenon for all the investigated genotypes. It can be assumed that the homogenization and stabilization of the genome, especially in the context of intraspecific hybrids, could be achieved gradually through mitotic cell proliferation and meiotic recombination, leading to heterochromatic cluster rearrangements in distal/terminal chromosomal regions. It may be proposed that cell-specific ectopic recombination events that occurred in premeiotic cell lineages result significant numbers of nonhomologous chromosomal associations detected in microgametogenesis in *Ae. speltoides*.

Nonhomologous Meiotic Chromosome Associations May Be Consequences of Erroneous DNA Replication and Repair in Premeiotic Somatic Cell Lineages and/or Ectopic Meiotic Recombination

The spatial distributions of individual chromosome territories in interphase nuclei is tightly related with processes of DNA transcription, repair and recombination (Fransz and De Jong, 2011; Schubert and Shaw, 2011; Hübner et al., 2013). Chromosomal rearrangements and alterations in chromosome structure are the direct consequences of errors occurring in the processes of DNA replication and repair (Andersen and Sekelsky, 2010; Lambert et al., 2010; Borde and de Massy, 2013). Specifically, in the cases of DNA double-strand breaks (DSBs) when homologous DNA strands are unavailable, the same or similar ectopic sequences may serve as the source for DNA repair (Puchta, 2005; Wicker et al., 2010; Anand et al., 2014; Knoll et al., 2014). Homologous and nonhomologous/ ectopic associations may occur between mitotic chromosomes (Godin and Stack, 1976; Klasterska, 1978; Lavania and Sharma, 1978; Pedrosa et al., 2001; Ghosh, 2003) in interphase plant nuclei between spatially distant regions (Schubert et al., 2014). As homologs are separated in the nucleus, nonhomologous chromosomes appear in closer proximity to each other in the subgenome, and ectopic sequences serve as templates in the replication/reparation processes.

In large genomes of cereals, chromosomes display a Rabl orientation in the interphase nuclei, when telomeres and centromeres cluster in opposite nucleus pools. It is assumed that this arrangement is a consequence of the preceding anaphase (Scherthan, 2001). The karyotype of *Ae. speltoides* is composed of metacentric/slightly submetacentric chromosomes that are similar in size, and the terminal regions of short and long chromosome arms appear in close proximity in the anaphase stage. Seemingly, in *Ae. speltoides*, in each of the subgenomes and between the subgenomes, ectopic recombination may often occur randomly, as was shown for *Arabidopsis* (Schubert et al., 2007, 2012, 2014). This is evidenced by the low frequency

of homologous chromosome associations in Cankiri plants. In addition, intrachromosomal recombination may be the reason for the tandem repeat cluster association/intercalation, along with the interchromosomal ectopic associations in mitosis.

Ectopic chromatin fibrils were revealed not only between Spelt1 and Spelt52 clusters, but also between distal/terminal and intercalary chromosomal regions. Plant genomes are enriched with various types of repetitive DNA, and TEs are the prevailing genomic fraction in *Ae. speltoides* (Middleton et al., 2013). The ubiquitous distribution of TEs determines the probability of ectopic recombination between almost any parts of the plant chromosome, and nonhomologous meiotic recombination has been shown for *Ae. speltoides* previously (Raskina, 2017). Often, illegitimate recombination is observed in the heterochromatin regions, comprising various repetitive DNA types, primarily tandem repeats and TEs.

During the interphase stage, the spatial distribution of chromosomes in the nucleus changes (Avivi and Feldman, 1980; Schubert et al., 2014). The movements in the nucleus and simultaneous condensation of the chromosomes toward the metaphase stage appear to be accompanied by breaks of numerous ectopic associations. In the early meiotic prophase in different species, heterochromatic clusters are dynamically associated and dissociated (Bozza and Pawlowski, 2008), and chromosome movements may be the mechanisms involved in the rupturing of interchromosomal associations and entanglements (Koszul and Kleckner, 2009). However, numerous intra- and interchromosomal fibers are detected at the stages of somatic metaphase and until late meiosis in *Ae. speltoides*. When these links are broken, at all stages of cell proliferation and differentiation, significant portions of chromatin—especially in distal/terminal heterochromatic chromosomal regions—appear to be damaged and improperly packed/decondensed. This could provoke chromosomal aberrations in the following cell cycle. However, chromosome rearrangements contribute to heterochromatin cluster repatterning and genome stabilization, as was discussed above for intraspecific hybrids. In the ontogenesis of contrasting genotypes, the overall abundance/copy numbers and structural integrity of certain DNA sequences changed tissue-specifically (Shams and Raskina, 2018), and significant differences between individual plants of *Ae. speltoides* were documented.

Altogether, the obtained data showed that perpetual intraorganismal reshuffling of repetitive DNA mirrors the structural plasticity of the *Ae. speltoides* genome, which is interlinked with genetic diversity through the species distribution area in contrasting ecogeographical environments in and around the Fertile Crescent.

AUTHOR CONTRIBUTIONS

EZ, YP, and OR conducted confocal and electron microscope imaging and analyzed the data. OR conducted the experiments and drafted the manuscript. All of the authors read and approved the manuscript.

FUNDING

This work was financially supported by the Israel Science Foundation under grant number 723/07 for OR.

ACKNOWLEDGMENTS

We are most grateful to the reviewers for their helpful comments. We are very grateful to Dr. Orit Gal Gerber for the opportunity to conduct this research in The Interdepartmental Equipment Unit, Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01779/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 11 December 2018
doi: 10.3389/fpls.2018.01829

Genetic Diversity and Resistance to Fusarium Head Blight in Synthetic Hexaploid Wheat Derived From *Aegilops tauschii* and Diverse *Triticum turgidum* Subspecies

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OPEN ACCESS

Edited by:
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Hungarian Academy of Sciences (MTA), Hungary

Reviewed by:
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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: 31 August 2018
Accepted: 26 November 2018
Published: 11 December 2018

Citation:
Szabo-Hever A, Zhang Q, Friesen TL, Zhong S, Elias EM, Cai X, Jin Y, Faris JD, Chao S and Xu SS (2018) Genetic Diversity and Resistance to Fusarium Head Blight in Synthetic Hexaploid Wheat Derived From Aegilops tauschii and Diverse Triticum turgidum Subspecies. Front. Plant Sci. 9:1829. doi: 10.3389/fpls.2018.01829

Synthetic hexaploid wheat (SHW) can serve as a bridge for the transfer of useful genes from *Aegilops tauschii* and tetraploid wheat (*Triticum turgidum*) into common wheat (*T. aestivum*). The objective of this study was to evaluate 149 SHW lines and their 74 tetraploid parents for their genetic diversity, breeding values and inter-genomic interactions for resistance to Fusarium head blight (FHB). The genetic diversity analysis was performed based on the population structure established using 4,674 and 3,330 polymorphic SNP markers among the SHW lines and tetraploid parents, respectively. The results showed that all *T. carthlicum* and most *T. dicoccum* accessions formed different clusters and subpopulations, respectively, whereas all the *T. durum*, *T. polonicum*, *T. turgidum*, and *T. turanicum* accessions were clustered together, suggesting that *T. durum* was more closely related to *T. polonicum*, *T. turgidum*, and *T. turanicum* than to *T. dicoccum*. The genetic diversity of the SHW lines mainly reflected that of the tetraploid parents. The SHW lines and their tetraploid parents were evaluated for reactions to FHB in two greenhouse seasons and at two field nurseries for 2 years. As expected, most of the SHW lines were more resistant than their tetraploid parents in all environments. The FHB severities of the SHW lines varied greatly depending on the *Ae. tauschii* and tetraploid genotypes involved. Most of the SHW lines with a high level of FHB resistance were generally derived from the tetraploid accessions with a high level of FHB resistance. Among the 149 SHW lines, 140 were developed by using three *Ae. tauschii* accessions Clae 26, PI 268210, and RL 5286. These SHW lines showed FHB severities reduced by 21.7%, 17.3%, and 11.5%, respectively, with an average reduction of 18.3%, as compared to the tetraploid parents, suggesting that the D genome may play a major role in reducing disease severity in the SHW lines. Thirteen SHW lines

consistently showed a high level of FHB resistance compared to the resistant check, Sumai 3, in each environment. These SHW lines will be useful for the development of FHB-resistant wheat germplasm and populations for discovery of novel FHB resistance genes.

Keywords: wheat, synthetic hexaploid wheat, *Aegilops tauschii*, tetraploid wheat, *Triticum turgidum*, Fusarium head blight, genetic diversity

INTRODUCTION

Fusarium head blight (FHB), also known as scab, is a destructive disease of durum wheat [*Triticum turgidum* L. ssp. *durum* (Desf.) Husn., $2n = 4x = 28$, AABB] and common wheat (*T. aestivum* L. em Thell., $2n = 6x = 42$, AABBDD) in the humid and semi-humid wheat-growing areas of the world (Schroeder and Christensen, 1963). This disease, mainly caused by fungal pathogen *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schw.) Petch.] in North America, can lead to severe losses not only in grain yield but also in quality. Mycotoxins, the secondary metabolites of this pathogen, make the harvested grain unsuitable for consumption as food or feed (Gilbert and Tekauz, 2000). Since the early 1990s, FHB has become a serious threat to wheat production globally due to its frequent outbreaks in many wheat-growing regions including the United States, Canada, Europe, and China (see review by McMullen et al., 1997, 2012; Zhang et al., 2012). The severe epidemics of this disease in North America in the early 1990s resulted in an estimated loss of at least 100 million bushels annually for the years 1991, 1993, and 1994 (McMullen et al., 1997). A recent estimate for the value of yield loss for wheat in the United States was $1.176 billion in 2015 and 2016 (Wilson et al., 2018). To confine this threat, an emphasis has been placed on FHB resistance breeding in wheat. Tremendous work had been put into finding new resistance sources with a focus mainly on the resistance present in the exotic wheat germplasm from China and various gene banks. As a result, more than 50 FHB resistance quantitative trait loci (QTL) have been identified, and the most notable QTL were mapped on chromosome arms 3BS (*Fhb1*), 5AS (*Qfhs.ifa-5A*), 5AL (*Qfhb.rwg-5A.2*), and 6BS (*Fhb2*) from common wheat 'Sumai 3' and PI 277012 (see reviewed by Buerstmayr et al., 2009; Chu et al., 2011; Zhao et al., 2018b).

To widen the genetic resources of FHB resistance, it is necessary to identify and transfer novel resistance QTL from the germplasms of wheat and its related species that have not been tapped for FHB. Hexaploid wheat is known to originate as a result of hybridization between an AB genome-containing tetraploid wheat (*T. turgidum* spp., $2n = 4x = 28$, AABB) and the diploid goatgrass *Aegilops tauschii* Cosson ($2n = 2x = 14$, DD), which contributed the D genome (Kihara, 1944; McFadden and Sears, 1946). Therefore, the world core collections of eight tetraploid wheat subspecies [*T. turgidum* ssp. *carthlicum* (Nevski) Á. Löve & D. Löve, *T. turgidum* ssp. *dicoccoides* (Körn. ex Asch. & Graebner) Thell., *T. turgidum* ssp. *dicoccum* (Schrank ex Schübler) Thell., *T. turgidum* ssp. *durum*, *T. turgidum* ssp. *polonicum* (L.) Thell., *T. turgidum* ssp. *turanicum* (Jakubz.) Á. Löve & D. Löve, *T. turgidum* ssp. *paleocolchicum* (Menabde) Á. Löve & D. Löve, and *T. turgidum* ssp. *turgidum*, which are abbreviated as *T. carthlicum*, *T. dicoccoides*, *T. dicoccum*, *T. durum*, *T. polonicum*, *T. turanicum*, *T. paleocolchicum*, and *T. turgidum*, respectively] and *Ae. tauschii* have been considered as invaluable genetic resources for wheat improvement (Börner et al., 2015; Fedak, 2015; Arora et al., 2018). Many unique genes for resistance to several major wheat diseases and insects, such as rusts, powdery mildew, Hessian fly, and greenbug, have been transferred from tetraploid wheat and/or *Ae. tauschii* into common wheat and extensively used in wheat breeding and production globally (see reviews by Ogbonnaya et al., 2013; Börner et al., 2015; Fedak, 2015).

Tetraploid wheat and *Ae. tauschii* have been used less as sources of FHB resistance because tetraploid wheat is generally more susceptible to FHB than hexaploid wheat, and *Ae. tauschii* plants are not suitable for direct evaluation for FHB resistance in field conditions because of their premature seed shattering nature. Buerstmayr et al. (2012) suggested that tetraploid durum wheat, which is known to be highly susceptible to FHB, does not necessarily lack FHB resistance alleles. Their findings that a resistance QTL introgressed from hexaploid wheat into durum improved resistance in only a few cases supported the hypothesis that either most durum wheat genotypes possess suppressors that silence or reduce the effect of resistance QTL (Stack et al., 2002; Garvin et al., 2009), or the D genome contributes resistance-inducing genes that are absent in durum wheat (Fakhfakh et al., 2011). Actually, a number of accessions of *T. dicoccoides* (Miller et al., 1998; Buerstmayr et al., 2003; Oliver et al., 2007), *T. dicoccum* (Oliver et al., 2008), *T. polonicum* (Wiwart et al., 2013), and *T. carthlicum* (Oliver et al., 2008) were identified to have moderate to high levels of FHB resistance. Several FHB resistance QTL were identified in durum wheat (Somers et al., 2006; Zhang et al., 2014; Zhao et al., 2018a), *T. dicoccoides* (Otto et al., 2002; Stack and Faris, 2006; Chen et al., 2007; Gladysz et al., 2007; Kumar et al., 2007; Buerstmayr et al., 2013), *T. dicoccum* (Buerstmayr et al., 2012; Zhang et al., 2014), and *T. carthlicum* (Somers et al., 2006). Brisco et al. (2017) identified five and seven *Ae. tauschii* accessions showing resistance and moderate resistance, respectively, suggesting that *Ae. tauschii* can be a potential source of novel FHB resistance.

One way to bring potential new resistance genes from tetraploid wheat and *Ae. tauschii* germplasm into wheat breeding programs is the development of synthetic hexaploid wheat (SHW) (*xAegilotriticum* spp., $2n = 6x = 42$, AABBDD). Since the 1940s, over 1,500 SHW lines have been developed and a large number of the SHW lines have been identified to exhibit resistance to major wheat diseases (rusts, Septoria, barley yellow dwarf virus, crown rot, tan spot, spot blotch, nematodes, powdery

mildew, FHB, etc.) and insects (Hessian fly and greenbug) and tolerance to abiotic stresses (drought, heat, salinity/sodicity, and waterlogging) as well as novel grain yield and quality traits (see review by Ogbonnaya et al., 2013). A large number of adapted wheat germplasms and populations have been developed from elite SHW lines (Lazar et al., 1996; Yang et al., 2006, 2009; Dreisigacker et al., 2008; Lage and Trethowan, 2008; Jafarzadeh et al., 2016) and some of SHW-derived germplasms have been successfully utilized to develop common wheat varieties, such as the highly yielding variety 'Chuanmai 42' (Yang et al., 2009) and greenbug-resistant varieties 'TAM 110' (Lazar et al., 1997) and 'TAM 112' (Rudd et al., 2014). Previous efforts to develop SHW germplasm for wheat improvement have mostly targeted the genetic diversity of the D genome present in world core collections of *Ae. tauschii*. Noticeably, most of the SHW lines that are currently available were developed from the crosses between durum wheat and diverse *Ae. tauschii* accessions by L. R. Joppa at USDA-ARS (Fargo, ND, United States; Xu et al., 2010) and Mujeeb-Kazi and Delgado (2001) and Mujeeb-Kazi (2003) at the International Maize and Wheat Improvement Center, Mexico (CIMMYT). Therefore, most of the tetraploid wheat germplasm resources other than durum have not been intentionally utilized for SHW production for wheat breeding programs except for a small number of *T. dicoccoides*, *T. carthlicum*, and *T. dicoccum* accessions that were sporadically used (Lange and Jochemsen, 1992; Xu and Dong, 1992; Lage et al., 2006).

To incorporate the genetic diversity from under-exploited tetraploids into SHW germplasm resource, we recently developed 200 new SHW lines, with 178 lines being developed using six tetraploid subspecies *T. carthlicum*, *T. dicoccum*, *T. dicoccoides*, *T. polonicum*, *T. turgidum*, and *T. turanicum*. These new SHW lines plus durum Langdon-derived SHW lines previously developed by L. R. Joppa (Xu et al., 2010) represent a unique resource for wheat improvement and for investigating polyploidization and intergenomic interactions in wheat. The objectives of this study were to identify FHB resistant SHW lines and to investigate the effect of the D-genome chromosomes derived from various *Ae. tauschii* accessions on FHB resistance by evaluating the genetic diversity and FHB resistance in a subset of 149 SHW lines and their 74 tetraploid parents.

MATERIALS AND METHODS

Plant Materials

A total of 149 SHW lines and their tetraploid wheat (*T. turgidum* L.) parents were used in genetic diversity analysis and evaluation for resistance to FHB. These SHW lines were developed from crossing 10 *Ae. tauschii* accessions to 74 tetraploid wheat accessions belonging to durum wheat and five other tetraploid wheat subspecies (*T. carthlicum*, *T. dicoccum*, *T. polonicum*, *T. turgidum*, and *T. turanicum*). The accession or line numbers and sources of the tetraploid wheat and *Ae. tauschii* accessions are listed in **Supplementary Table S1**, and the line numbers and pedigrees of the SHW lines are listed in **Supplementary Table S2**. Of the 10 *Ae. tauschii* accessions, four (CIae 17, PI 268210, RL 5286, and TA 2377) and six (CIae 19, CIae 22, CIae 25, CIae 26, PI 476874, and TA 1675) were classified as subspecies *strangulata* and *tauschii*, respectively. Three (CIae 26, PI 268210, and RL 5286) of the *Ae. tauschii* accessions were used as the parents of 140 (94%) SHW lines. Except for seven durum 'Langdon'-derived SHW lines (SW7, SW8, SW9, SW25, SW52, SW53, and SW59) developed by Dr. L. R. Joppa (Xu et al., 2010), all other lines were recently produced by crossing seven *Ae. tauschii* accessions (CI 22, CIae 26, PI 268210, RL 5286, PI 476874, TA 1675, TA 2377) with the 74 tetraploid wheat accessions.

Genetic Diversity Analysis on the SHW Lines and Their Tetraploid Wheat Parents

The SHW lines and their tetraploid parents were genotyped with the Illumina iSelect wheat 9K array containing 9,000 gene-derived SNPs (Cavanagh et al., 2013) using Illumina's Infinium method following the manufacturer's protocols (Illumina Inc., San Diego, CA, United States). The SNP genotype calls were performed using the genotyping module implemented in the Illumina's GenomeStudio software v.2011.1. Genotype data were manually inspected for call accuracy before exporting the SNP data file. Heterozygote calls were converted into missing data, markers with poorly separated clusters were excluded, and SNPs with a missing data rate of 10% or higher as well as those with minor allele frequency (MAF) of 0.05 or lower were filtered out. The high-density SNP-based consensus map developed by Maccaferri et al. (2015) for tetraploid wheat and the consensus map previously produced by Cavanagh et al. (2013) for hexaploid wheat were used to remove SNPs with no map information. The final SNP data set for molecular analysis consisted of 3,330 and 4,674 markers for tetraploid parents and SHW lines, respectively.

Polymorphic information content (PIC) was applied to assess genetic diversity and was calculated for single loci as

$$PIC = 1 - \sum_{i=1}^{k} P_i^2$$

where k is the total number of alleles detected for a given marker locus and P_i is the frequency of the i-th allele in the set of genotypes investigated (Anderson et al., 1993). In our analysis the PIC = $1 - (p^2 + q^2)$ formula was used, where p and q denote the frequencies of the two alleles (Ghislain et al., 1999).

Genetic diversity present among SHW lines and their tetraploid parents was evaluated using both principle component analysis (PCA) in TASSEL4 (Bradbury et al., 2007) and cluster analysis in the R program[1].

FHB Resistance Evaluation

Evaluation experiments were performed for evaluating Type II resistance (resistance to spread in the spike) in both greenhouse condition and field nurseries based on the well-established procedures for plant culture, inoculation, and disease scoring as described by Chu et al. (2011) and Zhang et al. (2014). Out of the 74 tetraploid parents, only one accession (*T. dicoccum* PI 272572) was not evaluated because of the low germination rate.

[1]https://www.r-project.org/

In the field and greenhouse evaluation experiments, common wheat varieties 'Sumai 3' and 'Grandin' were used as resistant and susceptible checks, respectively. In greenhouse experiments, a total of 224 genotypes (149 SHW lines, 73 tetraploid parents, and two checks) were evaluated in two seasons in winter 2015 and 2016, respectively, using a randomized complete block design (RCBD) with three replicates. Each genotype was planted in a plastic pot (16.2 × 18.4 cm) with four seeds for each genotype per replicate. Therefore, a total of 12 plants per genotype were evaluated for most of the lines in each greenhouse experiment. The greenhouse settings for photoperiod and temperature were 16 h and 22°C, respectively. The inoculum was prepared at a concentration of 50,000 spores mL^{-1} from three strains of pathogenic *F. graminearum*. For inoculation, 10 μL of inoculum was injected into a single central spikelet near the center of each spike at anthesis as described by Stack et al. (2002). Each inoculated spike was misted and then covered with a misted plastic bag for 72 h. For each genotype, about 10 spikes in each replicate were inoculated. Disease scoring was performed by counting infected spikelets and total spikelets on each spike at 21 days post-inoculation, and disease severity for each line was calculated as the percentage of total infected spikelets in total spikelets from all the scored spikes.

In the field experiments, the plant materials were planted in mist-irrigated nurseries using a RCBD with three replicates at two locations (Fargo and Prosper, ND, United States) in the summers of 2015 and 2016. Each genotype was planted in a hill plot with 15 seeds. Inoculum was prepared using the grain spawn inoculation method, in which autoclaved corn seeds were infected with a mixture of spores produced separately from 20 *F. graminearum* strains, including 10 3ADON (3-acetyl-deoxynivalenol) producers and 10 15ADON (15-acetyl-deoxynivalenol) producers, collected from the field in North Dakota (Puri and Zhong, 2010). At the boot stage of the earliest lines, inoculum was evenly applied among plots at a rate of 35.6 g m^{-2}. The nursery was misted for 2 min in 1-h intervals for 12 h daily (4:00 p.m. to 4:00 a.m.), until about 14 days after anthesis of most the genotypes. Ten spikes for each line were individually examined at 21 days post anthesis as the number of infected spikelets per spike using a visual scale: 0 = no spikelets infected, 100 = all spikelets infected based on the method of Stack and McMullen (1998).

Plant height (PH) data were collected in the field experiments, and days to flowering (DTF) data were collected in the field and greenhouse experiments to determine the correlation of these traits with FHB disease severity. PH was measured from the ground surface to the top of the spike excluding the awns. DTF was calculated from January 1 in the greenhouse experiments, and in the field experiments it was calculated from July 1 in 2015 and from June 20 in 2016 when 50% of spikes in a hill were flowering.

Statistical Analyses

All the statistical analyses were performed separately using evaluation data from hexaploid entries (SHW lines and checks), the tetraploid wheat parents, and the two groups combined. Descriptive statistics were calculated using the software JMP Genomics 7 (SAS Institute, Cary, NC, United States). A normality test for distribution of disease severity was performed using Shapiro-Wilk under the "Goodness of Fit" option using the same program. Bartlett's test under the general linear model (GLM) procedure was used to test homogeneity of disease severity variances among the experiments using SAS program version 9.4 (SAS Institute). The reduction in FHB severity in the SHW lines was calculated as the difference in FHB severity between the SHW lines and their respective tetraploid parents. To determine the significance of the reduction, the least significant difference (LSD) value was used. Correlation coefficients between disease severity and PH or DTF were calculated using the PROC CORR procedure (SAS Institute). The same procedure was used to calculate the correlation coefficients between the disease severity of SHW lines and their tetraploid parents. To test homogeneity of PH and DTF data, Levene's test was used under the GLM procedure (SAS Institute). Broad sense heritability was estimated across environments according to Nyquist (1991) with the following formula: H^2 across environments = $1 - (MS_{G \times E}/MS_G)$, where $MS_{G \times E}$ was mean square genotype × environment; and MS_G was mean square genotype.

RESULTS

Genetic Diversity of the SHW Lines and Their Tetraploid Wheat Parents

A total of 4,674 SNP markers that were polymorphic among the SHW lines were mapped based on the consensus map previously produced by Cavanagh et al. (2013) resulting in an average of 223 markers per chromosome. The markers spanned a genetic distance of 3,445 cM with an average density of 0.7 cM per marker. However, the average marker density for the D genome was poor at 24 markers per chromosome. The number of markers ranged between 3 (chromosome 4D) and 504 (chromosome 2B). The PIC value was between 0.1 and 0.5 with an average of 0.38 (data not shown). The consensus map developed by Maccaferri et al. (2015) was used to assign the map positions for 3,330 SNP markers for the tetraploid parents resulting in an average of 238 markers per chromosome. The total genetic distance was 2,532.8 cM with an average density of 0.8 cM per marker. The number of markers ranged between 95 (chromosome 4B) and 386 (chromosome 2B). The PIC value varied between 0.1 and 0.5 with an average of 0.39 (data not shown).

To evaluate genetic similarity, results from PCA indicated that three subpopulations were likely present in both the SHW lines and the tetraploid parents. Results from cluster analysis further confirmed the presence of three major clusters separating 73 tetraploid parents (**Figure 1**) and 149 SHW lines (**Figure 2**). The cluster 1 in tetraploid samples (**Figure 1**, black) consisted of one *T. dicoccum* accession (PI 352548-1) and all 21 *T. carthlicum* accessions. Cluster 2 consisted of 50 individuals (**Figure 1**, blue) which were further grouped into two subpopulations with one consisting of only

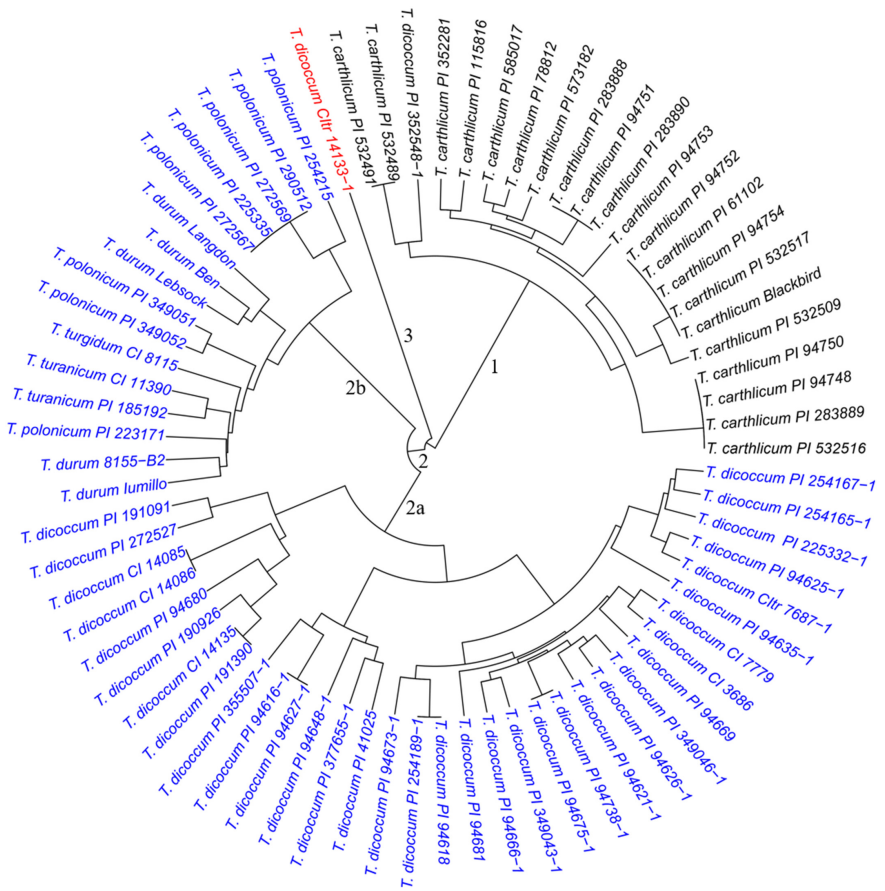

FIGURE 1 | Dendrogram of the 73 tetraploid parents, forming three major clusters. Cluster 1 incluides one *T. dicoccum* and 21 *T. cathlicum* accesions. Cluster 2a consists of 34 *T. dicoccum* accesions. Cluster 2b contains all durum, *T. polonicum*, *T. turgidum*, and *T. turanicum* accessions. Accession *T. dicoccum* CItr 14133-1 is an outlier.

T. dicoccum accessions (**Figure 1**, subpopulation 2a) and another containing all durum, *T. polonicum*, *T. turgidum*, and *T. turanicum* accessions (**Figure 1**, subpopulation 2b). Interestingly, *T. dicoccum* accession CItr 14133-1 (Entry 21) may be an outlier, as it did not share similarity with any of the tetraploid lines (**Figure 1**, red).

The three clusters separating 149 SHW lines (**Figure 2**) generally corresponded well with the clustering of the tetraploid parents (**Figure 1**). Cluster 1 (**Figure 2**, black) consisted of the 43 SHW lines derived from all the accessions in the tetraploid wheat cluster 1. Cluster 2 (**Figure 2**, red) contained 30 SHW lines mainly derived from the accessions belonging to tetraploid wheat cluster 2b. Cluster 3 (**Figure 2**, blue) consisted of 76 SHW lines derived from all the *T. dicoccum* accessions belonging to tetraploid wheat cluster 2a. Although *T. dicoccum* CItr 14133-1 was separated alone from all other tetraploid parents, its three SHW lines (SW91, SW92, and SW93) were grouped into the SHW cluster 2 (**Figure 2**, red) with the SHW lines derived from tetraploid wheat cluster 2b. These analyses showed that the genetic diversity of this set of SHW lines obviously reflected that of the tetraploid wheat accessions. The results also clearly indicated that among the tetraploid accessions used, including *T. dicoccum*, *T. turgidum*, *T. turanicum*, *T. durum,* and *T. polonicum* lines, *T. durum* was genetically more similar with *T. polonicum* than with *T. dicoccum*.

Reactions to FHB of SHW Lines and Their Tetraploid Wheat Parents

The 149 SHW lines and their 73 tetraploid parents, together with the two hexaploid checks (Sumai 3 and Grandin), were evaluated for reactions to FHB in two greenhouse seasons and two field nurseries (Fargo and Prosper) in 2 years (**Supplementary Table S2**). However, two SHW lines, SW9 and SW52 (Entries 4 and 6), and *T. polonicum* accession PI 272567 (Entry 204) were not evaluated in the field experiments in 2015 due to low germination rate. The Bartlett's test for disease severity variances showed heterogeneity across the two greenhouse seasons and the 2 years of field experiment at two locations ($\chi^2_{df=5} = 205.6$, $P < 0.0001$). However, the data from the field tests showed homogeneity between the two locations in each year (2015: $\chi^2_{df=1} = 2.88$, $P = 0.0896$; 2016: $\chi^2_{df=1} = 2.31$, $P = 0.1284$), as well as between the two greenhouse experiments ($\chi^2_{df=1} = 2.88$, $P = 0.0895$). Therefore, the disease severity data from the two greenhouse experiments (FHBGH) as well as from the

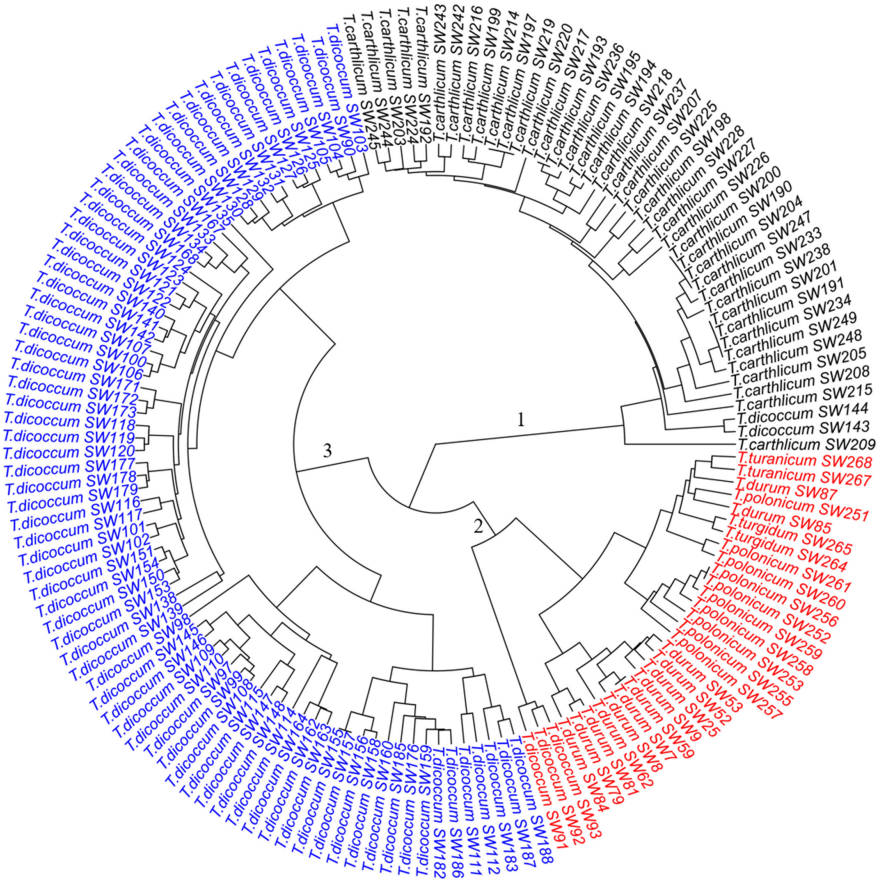

FIGURE 2 | Dendrogram of the 149 SHW lines, forming three major clusters. Cluster 1 consists of 43 SHW lines derived from the accessions in the tetraploid wheat cluster 1. Cluster 2 contains 30 SHW lines corresponding to tetraploid wheat cluster 2b and the outlier *T. dicoccum* CItr 14133-1. Cluster 3 contains 76 SHW lines belonging to the tetraploid wheat cluster 2a.

field experiments within each year (FHB15 and FHB16) were combined. Thus, these three sets of FHB severity data were used in the subsequent statistical analyses. In addition, the overall mean of disease severity from all the experiments is presented in **Supplementary Table S2** to provide general information about the resistance level of each genotype.

The resistant check Sumai 3 had the expected level of FHB resistance in all environments (FHB severity: 8.6–17.1%) (**Table 1** and **Supplementary Figure S1**). The susceptible check Grandin had the expected level of susceptibility only in the greenhouse experiments (55.3%), but it did not exhibit the expected level of susceptibility in the field environments (26.4% in FHB15 and 24.7% in FHB16) (**Table 1** and **Supplementary Figure S1**). Such low FHB severities were probably caused by early flowering dates of Grandin plants. We observed that Grandin was always among a few lines that had the earliest flowering dates, and it flowered at 9.9, 6.2, 14.7, and 12.4 days earlier than the population average in Prosper and Fargo nurseries in 2015 and 2016, respectively. At the early stage of the experiments, the inoculum pressure was likely not adequately built up. The FHB severity of the SHW lines and tetraploid parents, as expected, was highly variable among different environments. The average FHB severities of the entire population (SHW lines, tetraploid parents, and checks) were 29.9%, 49.8%, and 41.9% in FHB15, FHB16, and FHBGH, respectively (**Table 1** and **Supplementary Figure S1**). The tetraploid parents (average FHB severities: FHB15 = 33.8%, FHB16 = 71.3%, FHBGH = 56.9%) had more variable expressions of FHB than SHW lines (FHB15 = 28.2%, FHB16 = 39.7%, FHBGH = 34.6%) in different experiments (**Figures 3**, **4**). For FHB severity, the heritability (H^2) values were 0.70, 0.85 and 0.64 among experiments in FHB15, FHB16 and FHBGH, respectively (**Table 1**), indicating good reproducibility of the experiments.

Several resistant genotypes were identified in the field as well as in the greenhouse. Among the 25 most FHB-resistant SHW lines listed in **Table 2**, 13 lines (SW53, SW87, SW91, SW92, SW93, SW157, SW159, SW162, SW188, SW203, SW252, SW253, and SW261) showed a high level of FHB resistance. Their FHB severities were not significantly different ($P = 0.05$) from Sumai 3 in all three environments in which they were successfully evaluated. Among these SHW lines, eight (61.5%) were derived from the crosses involving *Ae. tauschii* CIae 26, suggesting that *Ae. tauschii* CIae 26 may carry FHB resistance QTL. Three resistant SHW lines SW91, SW92, and SW93 were derived from *T. dicoccum* CItr 14133-1 crossed with three *Ae.*

TABLE 1 | Statistical analysis on Fusarium head blight (FHB) severity, days to flowering, and plant height data of a panel of 149 synthetic hexaploid wheat (SHW) lines and their tetraploid wheat parents evaluated in the field and greenhouse experiments.

Data set	Mean	SD	Median	Range	Sumai 3	Grandin	LSD	H^2
FHB15	29.9	13.5	29.0	3.4–92.8	8.6	26.4	15.8	0.70
FHB16	49.8	22.6	43.5	7.1–98.8	9.7	24.7	19.4	0.85
FHBGH	41.9	17.5	38.1	8.5–100.0	17.1	55.3	13.7	0.64
PH	89.7	8.5	89.5	67.5–130.0	85.4	80.4	5.8	0.90
DTF GH	31.2	6.6	30.8	18.3–60.5	24.8	18.3	4.7	0.86
DTF15 Pro	23.2	5.8	22.0	13.0–37.0	19.7	13.3	4.2	
DTF15 Far	11.9	4.9	11.0	3.0–29.0	5.0	5.7	5.0	
DTF16 Pro	25.7	6.8	24.0	11.0–45.0	24.7	11.0	4.6	
DTF16 Far	25.1	7.5	24.0	10.0–45.0	11.3	12.7	14.7	

Data set: FHB15 and FHB16 are combined FHB severity data from to field locations (Fargo, Prosper) in 2015 and 2016, respectively; FHBGH is combined FHB severity data from the two greenhouse experiments; PH is combined plant height data from all field experiments (2015, 2016); DTF GH is combined days to flowering data from the two greenhouse experiments; DTF15 Pro and DTF16 Pro are days to flowering data in Prosper in 2015 and 2016, respectively; DTF15 Far and DTF16 Far are days to flowering data in Fargo in 2015 and 2016, respectively. SD, standard deviation; LSD, least significant differences ($\alpha < 0.05$); H^2, broad-sense heritability.

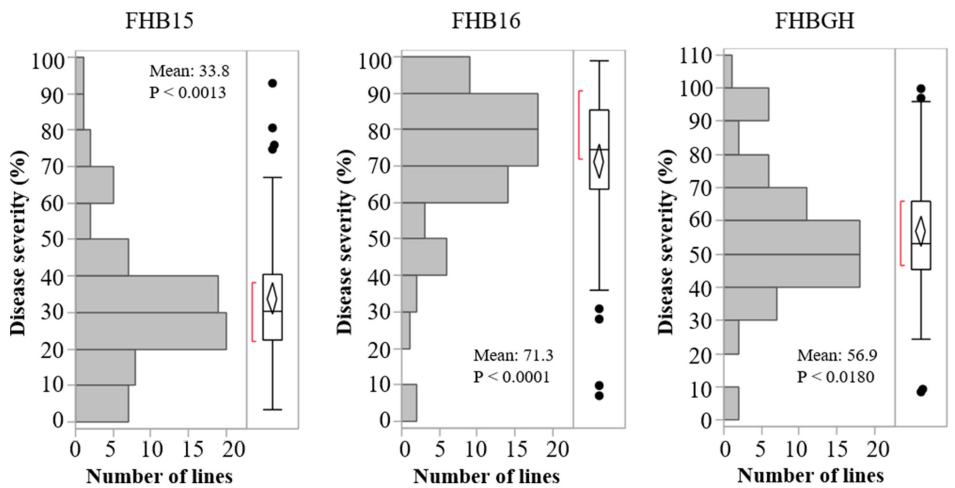

FIGURE 3 | Distribution of Fusarium head blight (FHB) disease severities among the tetraploid wheat lines in the three environments (FHB15, FHB16, and FHBGH). Letter "P" represents probability from normality test for distribution of disease severity.

tauschii accessions, indicating that CItr 14133-1 may carry FHB resistance QTL. In fact, CItr 14133-1 showed a high level of FHB resistance in the field conditions with 8.1% and 7.1% disease severities in FHB15 and FHB16, respectively. Similarly, two *T. dicoccum* accessions PI 191091 and *T. dicoccum* PI 272527 showed a high level of resistance comparable to Sumai 3 in all the environments. In particular, PI 272527 had the highest level of FHB resistance among all the tetraploid accessions and SHW lines evaluated in this study, with disease severities being 3.4%, 9.8%, and 8.5% in FHB15, FHB16, and FHBGH, respectively. The two SHW lines SW187 and SW188 derived from PI 272527 also had low disease severities.

Effects of Plant Height and Days to Flowering on FHB Severity of SHW Lines and Their Tetraploid Parents

The Levene's test for PH showed homogeneity of error variances across the field experiments ($P < 0.1437$, $df = 3$), therefore all the experiments were combined for further analyses. The tetraploid parents showed a wide variation in PH, ranging from 67.5 to 130.0 cm, whereas the SHW lines ranged from 73.8 to 108.9 cm (**Table 1** and **Supplementary Table S3**). The DTF data from the greenhouse experiments were combined based on the homogeneity test result ($P < 0.3242$, $df = 1$), whereas the DTF data from field experiments were heterogeneous ($P < 0.0001$, $df = 3$) and were analyzed separately in the further analyses. The plants started flowering early in 2016 (30th of June in Fargo, 1st of July in Prosper) due to the warm weather in May and June. However, the flowering period was longer in 2016 (35 days in Fargo, 34 days in Prosper) than in 2015, when the flowering started later (3rd of July in Fargo, 13th of July in Prosper) and took a shorter period (26 in Fargo and 24 days in Prosper) (**Table 1** and **Supplementary Table S3**). Analyzing the DTF data separately for hexaploid lines and tetraploid parents showed that the two groups started flowering about the same time and flowering lasted for about the same period in both years at both locations (data not shown).

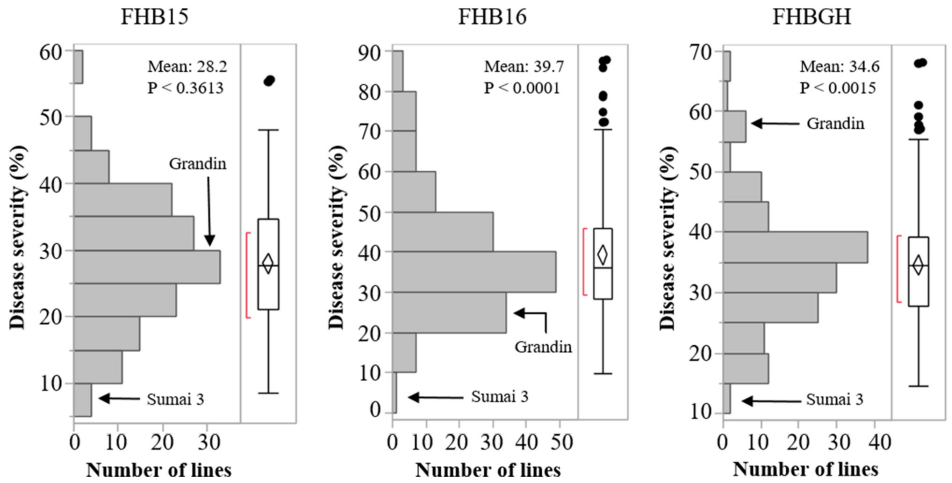

FIGURE 4 | Distribution of Fusarium head blight (FHB) disease severities among the SHW lines in the three environments (FHB15, FHB16, and FHBGH). The two checks, Sumai 3 and Grandin, were included in the data set. Letter "P" represents probability from normality test for distribution of disease severity.

The Pearson's correlation coefficients between FHB severity and PH were significant in the field experiments ($r = -0.273$ and -0.226, $P < 0.001$), indicating that the shorter plants had higher disease severities (**Supplementary Table S4**). However, no significant correlation was detected between the field PH data and the greenhouse FHB data ($r = 0.072$; $P = 0.282$). Also, the plants that flowered later showed lower disease severity in the field experiments, except in the experiment at the Fargo location in 2016. The PH and DTF did not influence the FHB severity in the greenhouse experiments. Significant correlations were detected among DTF and FHB data collected in various environmental conditions (**Supplementary Table S4**).

Decreases of FHB Disease Severities in SHW Lines Compared With Their Tetraploid Parents

Correlation analysis of the FHB severities between SHW lines and their tetraploid parents showed that both the overall data set and the 2016 field data of tetraploids were significantly correlated with the FHB severity data of their SHW lines in all environments ($P < 0.05$) (**Table 3**). The FHB severity of SHW lines in the greenhouse (SHWGH) was significantly correlated with the data of tetraploids in all environments except in 2015 (Tetr15), suggesting that FHB resistance in the tetraploid parents can be expressed at the hexaploid level.

A comparison of FHB severities between individual SHW lines and their respective tetraploid parents showed that most SHW lines had lower FHB disease severities than their tetraploid wheat parents, especially under environments with high disease pressures (**Supplementary Table S2**). The total numbers of the SHW lines with lower FHB disease severities than their tetraploid wheat parents were 80 (55.2%), 135 (91.2%), and 134 (90.5%) in FHB15, FHB16, and FHBGH, respectively. The total numbers of the SHW lines with significant FHB reduction ($P < 0.05$) over their tetraploid wheat parents were 24 (16.6%), 108 (73.0%), and 98 (66.2%) in FHB15, FHB16, and FHBGH, respectively. On the contrary, there were only 14 (9.7%), 1 (0.7%), and 1 (0.7%) SHW lines having significant increases ($P < 0.05$) of FHB severities over their tetraploid parents in FHB15, FHB16, and FHBGH, respectively. Because most of these SHW lines were derived from FHB-susceptible tetraploid parents, the significantly higher FHB severities of these SHW lines were mainly caused by unusually low FHB severities of their tetraploid parents. For example, *T. carthlicum* PI 94751 had FHB severities 72.6% in FHB16 and 56.8% in FHBGH, however, it had FHB severity only 13.7% in FHB15. An FHB-susceptible genotype can occasionally exhibit a resistant reaction with low FHB severity, which might result from unfavorable environmental conditions for disease development or escape of inoculation. This phenomenon commonly occurs in the field FHB evaluation, especially in highly variable weather conditions.

To analyze the effects of the tetraploid subspecies and *Ae. tauschii* genotypes on the FHB resistance of the SHW lines, the percentages of FHB severity reductions in the 140 SHW lines derived from three *Ae. tauschii* accessions (CIae 26, PI 268210, and RL 5286) were grouped by their tetraploid subspecies and *Ae. tauschii* accessions (**Table 4**). The high levels of FHB severity reductions were largely observed in the SHW lines derived from *T. durum* (27.6%), *T. polonicum* (55.5%), *T. turgidum* (45.2%), and *T. turanicum* (51.4%), whereas low levels of FHB severity reductions were observed in the SHW lines derived from *T. dicoccum* (16.0%) and *T. carthlicum* (11.0%). Similarly, different *Ae. tauschii* genotypes also affected the FHB severities of the SHW lines. Across the six tetraploid subspecies, the three *Ae. tauschii* accessions CIae 26, PI 268210, and RL 5286 resulted in 21.7%, 17.3%, and 11.5% of the FHB severity reduction in their SHW lines, respectively (**Table 4**). We observed that the highest levels of FHB severity reduction occurred in the SHW lines from the hybrids of *T. turanicum* accessions crossed with *Ae. tauschii*

TABLE 2 | Fusarium head blight (FHB) severity of most resistant synthetic hexaploid wheat (SHW) lines and tetraploid wheat lines.

Entry No.	Line	Materials/Pedigree	Average FHB severity			
			2015	2016	GH	Overall
23	SW92	Cltr 14133-1/RL 5286	12.3	10.3	17.1***	13.4
22	SW91	Cltr 14133-1/Clae 26	9.9	20.0	14.7***	14.9
24	SW93	Cltr 14133-1/PI 268210	10.5	25.3	14.6***	16.8
4	SW9	Langdon/Clae 26	*n.d*	15.0***	19.9***	18.3***
134	SW187	PI 272527/Clae 26	9.5	29.4*	17.8	19.6**
216	SW261	PI 349052/Clae 26	15.0*	27.7***	18.0***	20.1***
7	SW53	Langdon/PI 268210	11.4	18.6***	25.5***	20.1***
110	SW162	PI 41025/Clae 26	21.3***	23.5***	16.2*	20.3***
127	SW182	PI 190926/Clae 26	13.6	33.2	17.1**	20.8*
203	SW253	PI 254215/Clae 26	19.6***	20.0***	23.0***	20.9***
131	SW185	PI 191390/Clae 26	15.5	35.1	19.7**	21.5
132	SW186	PI 191390/PI 268210	15.4	32.6	20.9**	21.8
104	SW157	CI 14086/Clae 26	20.9	18.5***	25.8***	21.9***
107	SW159	CI 14135/Clae 26	21.5	23.3	21.5	22.1
154	SW203	PI 94753/PI 268210	10.0	27.6*	30.5	22.4*
102	SW156	CI 14085/PI 268210	25.6	18.8***	23.4***	22.6***
16	SW87	8155-B2/Clae 26	20.3***	20.8***	25.9***	22.7***
129	SW183	PI 191091/Clae 26	13.1	38.7	15.2	22.9
223	SW268	PI 185192/PI 268210	14.5***	44.0***	18.2***	23.3***
111	SW163	PI 41025/PI 268210	19.9***	32.6***	19.7	24.5***
86	SW143	PI 352548-1/Clae 26	26.6**	25.4	22.7*	24.9**
105	SW158	CI 14086/PI 268210	25.3	23.0***	26.6***	24.9***
201	SW252	PI 225335/Clae 26	15.8***	28.3***	30.2***	25.3***
18	SW85	Iumillo/Clae 26	25.2	25.6***	25.2***	25.3***
135	SW188	PI 272527/RL 5286	20.5*	26.5	30.3**	25.7***
133	PI 272527	*T. dicoccum* PI 272527	3.4	9.8	8.5	7.2
21	Cltr 14133-1	*T. dicoccum* Cltr 14133-1	8.1	7.1	42.5	20.6
128	PI 191091	*T. dicoccum* PI 191091	22.5	28.1	9.3	22.0
106	CI 14135	*T. dicoccum* CI 14135	8.5	36.0	24.4	22.9
130	PI 191390	*T. dicoccum* PI 191390	4.9	41.3	40.3	28.2
	Sumai 3	*T. aestivum*	8.6	9.7	17.1	11.8
	Grandin	*T. aestivum*	26.4	24.7	55.3	35.5

, **, and * indicate that the SHW lines were significantly different from their respective tetraploid parents at the 0.05, 0.01, and 0.001 probability levels, respectively (LSD test). Line number followed by "−1" (e.g., Cltr 14133-1) indicated a single plant selection from the original seed stock.*

TABLE 3 | Pair-wise correlation coefficients between synthetic hexaploid wheat (SHW) lines and their tetraploid parents for Fusarium head blight (FHB) severity.

Data set	SHW15	SHW16	SHWGH	SHWALL	Tetr15	Tetr16	TetrGH
SHW16	0.138						
SHWGH	0.383***	0.363***					
SHWALL	0.605***	0.795***	0.758***				
Tetr15	0.098	0.060	0.103	0.113			
Tetr16	0.289***	0.412***	0.348***	0.490***	0.547***		
TetrGH	0.032	−0.039	0.263**	0.092	0.505***	0.507***	
TetrALL	0.183*	0.180*	0.290***	0.290***	0.815***	0.842***	0.815***

, **, and * indicate significant at 0.05, 0.01, and 0.001 probability levels, respectively. Data set: SHW15 and SHW16 are combined FHB severity data of SHW lines from both experimental locations (Fargo, Prosper) in 2015 and 2016, respectively; SHWGH and TetrGH are combined FHB severity data of SHW and tetraploid lines, respectively, from the two greenhouse experiments; SHWALL and TetrALL are combined overall FHB severity data of SHW and tetraploid lines, respectively; Tetr15 and Tetr16 are combined FHB severity data of tetraploid lines from both experimental locations (Fargo, Prosper) in 2015 and 2016, respectively.*

TABLE 4 | Average reductions in Fusarium head blight (FHB) severity calculated from 140 synthetic hexaploid wheat (SHW) lines derived from crosses of six tetraploid wheat subspecies (*T. turgidum* ssp.) with three *Ae. tauschii* accessions (CIae 26, PI 268210, and RL 5286).

Tetraploid subspecies	CIae 26				PI 268210				RL 5286				Overall Avg
	2015	2016	GH	Avg	2015	2016	GH	Avg	2015	2016	GH	Avg	
T. durum	−29.7	−56.5	−56.0	−49.6	−3.8	−34.4	−44.6	−25.9	7.0	−21.6	−9.8	−8.2	−27.6
T. dicoccum	−3.5	−25.0	−20.5	−16.3	−5.4	−31.6	−17.9	−18.3	−0.9	−25.5	−10.3	−12.2	−16.0
T. carthlicum	6.0	−24.0	−15.9	−11.3	7.2	−23.8	−15.8	−10.8	13.7	−37.3	−9.3	−10.9	−11.0
T. polonicum	−47.9	−58.0	−59.6	−55.5									−55.5
T. turgidum	−14.5	−50.1	−64.1	−41.3	−24.9	−59.8	−57.7	−44.5					−45.2
T. turanicum	−39.0	−63.3	−57.5	−59.2	−49.2	−37.9	−61.4	−51.8					−51.4
Avg	−6.9	−31.1	−26.7	−21.7	−2.6	−29.9	−19.6	−17.3	2.3	−26.9	−10.1	−11.5	−18.3

2015: Average FHB severity decrease in field experiments (Fargo and Prosper, ND, United States) in 2015. 2016: Average FHB severity decrease in field experiments (Fargo and Prosper, ND, United States) in 2016. GH: Average FHB severity decrease in two greenhouse experiments. Avg: Data were calculated from the average FHB severity data of the individual lines in each environment.

PI 268210 (69.0%) and CIae 26 (67.4%), and *T. polonicum* (66.7%) and *T. durum* (65.1%) accessions crossed with *Ae. tauschii* CIae 26 (data not shown). For all the SHW lines, there was an overall average of 18.3% FHB severity reduction compared with their tetraploid wheat parents (**Table 4**), indicating that the D genome may play an important role in FHB resistance in wheat.

DISCUSSION

Synthetic hexaploid wheat has been considered as a valuable germplasm resource for introducing unique genes of agronomically important traits into bread wheat from its closely related or progenitor species in the primary gene pool (Ogbonnaya et al., 2013). For resistance to FHB, Mujeeb-Kazi et al. (2001a,b) evaluated a large number of the SHW lines targeting *Ae. tauschii* genetic diversity developed at CIMMYT and identified 16 SHW lines having a level of resistance as good as the resistant check Sumai 3. Mujeeb-Kazi et al. (2001a) incorporated the FHB-resistant SHW lines into wheat breeding at CIMMYT. Our present study reveals that the SHW lines we recently developed and investigated in our program are also good sources of FHB resistance. Among 149 SHW lines evaluated, many lines showed a high level of FHB resistance in different experiments with 13 lines, namely SW53, SW87, SW91, SW92, SW93, SW157, SW159, SW162, SW188, SW203, SW252, SW253, and SW261, showing FHB severity comparable to the level of Sumai 3 in all experiments. Some of these lines should serve as useful genetic stocks that can be used for development of adapted wheat germplasm and varieties in breeding programs.

It is well known that cultivated tetraploid wheat is more susceptible to FHB than hexaploid wheat (Stack et al., 2002; Oliver et al., 2008; Zhang et al., 2014). As expected, most of the SHW lines evaluated in our study were more resistant than their tetraploid wheat parents in all environments (**Supplementary Table S2**). On average, 140 SHW lines derived from three *Ae. tauschii* accessions (CIae 26, PI 268210, and RL 5286) decreased their disease severities by 18.3%, suggesting that either the D genome or the increased ploidy level reduced the disease severity in the SHW lines. The data from our experiment provide some evidence to support the hypothesis (Fakhfakh et al., 2011) that the D genome may play an important role in FHB resistance. Conceivably, the D genome may be necessary for expression or increased expression of some FHB resistance QTL located on the A- and/or B-genome chromosomes. It is also possible that the silencing of suppressors present on the A and/or B genome of tetraploids by D genome may lead to expression of resistance.

The evaluation data showed that the FHB severities of the SHW lines varied greatly with different *Ae. tauschii* and tetraploid wheat genotypes involved. The three *Ae. tauschii* accessions, CIae 26, PI 268210, and RL 5286, resulted in 21.7%, 17.3%, and 11.5% of the FHB severity reduction in their SHW lines, respectively (**Table 4**). Because *Ae. tauschii* CIae 26 and PI 268210 caused the large reduction of FHB severities, they may carry FHB resistance QTL. Therefore, we inferred that the increased FHB resistance in the SHW lines derived from CIae 26 and PI 268210 might be the result of mutual or additive effects from the D genome and its FHB resistance QTL. The two *Ae. tauschii* accessions may have different QTL because they had different effects on the FHB severity reduction in their SHW lines. Brisco et al. (2017) recently evaluated 109 *Ae. tauschii* accessions in the greenhouse and detected significant variation in FHB severity. Among the 10 *Ae. tauschii* accessions in the present study, two accessions, namely CIae 25 (TA1703) and TA 2377, were evaluated for FHB resistance by Brisco et al. (2017) and they were identified as moderately susceptible-moderately resistant and susceptible, respectively. However, in our experiment, their SHW lines (SW8 and SW62) had significant reductions in FHB severities over their durum parent Langdon in two (FHB16 and FHBGH) and three environments, respectively. Because the *Ae. tauschii* parents were not evaluated in our study, we cannot determine if the FHB severities of the SHW lines were associated with those of their *Ae. tauschii* parents. Therefore, further studies are needed to elucidate the relationships of FHB resistance between SHW lines and their *Ae. tauschii* parents by evaluating the SHW lines along with their *Ae. tauschii* parents.

Regarding the effect of tetraploid wheat genotypes on the FHB resistance of the SHW lines, we found that there were

positive correlations between the tetraploids and their SHW lines under the environments with high FHB disease pressures in the field nurseries in 2016 ($r = 0.412$, $p < 0.001$) and greenhouse ($r = 0.263$, $p < 0.01$) (**Table 3**). Most of the SHW lines with a high level of FHB resistance were derived from tetraploid wheat accessions with a high level of FHB resistance. For example, most of *T. polonicum*, *T. turgidum*, and *T. turanicum* accessions evaluated in our study had high disease severities 83.2%, 78.3%, 81.5%, respectively, whereas most *T. dicoccum* and *T. carthlicum* had relatively low disease severities (**Supplementary Table S2**). The SHW lines derived from these *T. polonicum*, *T. turgidum* and *T. turanicum* accessions had high levels of reductions in FHB severity (55.5%, 45.2%, 51.4%, respectively), whereas those SHW lines derived from the *T. dicoccum* and *T. carthlicum* had low levels of reductions in FHB severity (16.0% and 11.0%, respectively) (**Table 4**). Noticeably, two *T. dicoccum* accessions PI 272527 and PI 191091 exhibited high levels of FHB resistance in all the environments, suggesting that they may carry major FHB resistance QTL (**Table 2**). The SHW lines derived from the crosses between these FHB-resistant *T. dicoccum* accessions and different *Ae. tauschii* accessions also consistently showed high levels of FHB resistance across different environments. The lower levels of reductions in FHB severity in the SHW lines involving *T. dicoccum* and *T. carthlicum* is supported by the previous findings that some *T. dicoccum* and *T. carthlicum* accessions have FHB resistance QTL (Gagkaeva, 2003; Clarke et al., 2004; Gladysz et al., 2004; Somers et al., 2006; Buerstmayr et al., 2012). We previously conducted QTL analysis on FHB resistance in two *T. dicoccum* accessions, PI 41025 and PI 272527, and identified two QTL on chromosomes 3A and 5A from PI 41025 and four QTL on chromosomes 1A, 3A, 5A, and 7B, derived from PI 272527 (Zhang et al., 2014, 2017).

In addition to *Ae. tauschii* and tetraploid wheat genotypes, the levels of FHB severity decrease in the SHW lines varied among the environments. On average, there were 29.8% and 21.0% reductions under the environments with high FHB pressures in the field nurseries in 2016 (FHB16) and greenhouse (FHBGH), respectively. However, only a 3.3% reduction was observed under low FHB pressure in the field nurseries in 2015 (FHB15), mostly because the low FHB severities in the field conditions in 2015 reduced the differences between the tetraploids and the SHW lines. This observation is in line with the fact that modern durum crop is more susceptible than bread wheat under the environments with high FHB pressures.

Li et al. (2006) investigated the genetic diversity and genetic relationships among 48 tetraploid wheat accessions belonging to *T. turgidum, T. durum*, *T. carthlicum*, *T. paleocolchicum*, *T. turanicum,* and *T. polonicum* using simple sequence repeat (SSR) markers and grouped *T. durum*, *T. turgidum* and *T. polonicum* into the same cluster in their experiment. Dreisigacker et al. (2008) genotyped a set of 348 accessions from five different tetraploid subspecies using 21 SSR markers and separated *T. dicoccum* accessions from *T. durum* accessions by principal coordinate analyses. In our study, we found that accessions from *T. durum*, *T. polonicum*, *T. turanicum*, and *T. turgidum* formed a subpopulation, whereas all *T. carthlicum* and most *T. dicoccum* accessions formed two different clusters. Genetic diversity of the SHW lines in our study clearly reflected that of the tetraploid wheat parents. Therefore, these SHW lines represent a unique genetic resource by preserving the high level of genetic diversity from their tetraploid parents.

Lage et al. (2003) analyzed 54 SHW lines derived from 21 *T. dicoccum* and 15 *Ae. tauschii* parental accessions using amplified fragment length polymorphism (AFLP) markers. They also found that the genetic diversity of the SHW lines was associated with the *T. dicoccum* parents rather than their *Ae. tauschii* parents. Dreisigacker et al. (2008) suggested that "SHW diversity would be expected to preferably reflect the diversity of the tetraploid parent" because the tetraploid wheat parent contributed two-thirds of the SHW genome. When genotyping a set of 56 SHW lines derived from durum wheat with only D-genome SSR markers, Dreisigacker et al. (2008) found that the genetic diversity of the SHW lines was closely associated with the subspecies and geographic origin of the *Ae. tauschii* parents. Among the 10 *Ae. tauschii* accessions used in our study, four and six belong to the subspecies *strangulata* and *tauschii*, respectively. The fact that genetic diversity of the SHW lines was not related to the *Ae. tauschii* parents in our study is likely due to the paucity of molecular markers on the D genome because there are approximately ninefold less markers for the D genome than for the A and B genomes.

Use of association mapping analysis to identify FHB resistance genes/QTL in both SHW and tetraploid populations was attempted. However, no associations of markers with significant effects on FHB resistance were detected in either the SHW population or the tetraploid genotypes. This is likely due to the low number of SHW lines used and/or the low frequency of resistance genes and alleles present in the populations. Nonetheless, the results from this study might provide guidance in selecting SHW lines for development of mapping populations to identify FHB resistance genes/QTL using linkage analysis. The SHW lines showing high levels of resistance in all environments, such as SW87, SW162, SW252, SW253, and SW261, might be suitable parents for future development of mapping populations for QTL analysis of FHB resistance.

AUTHOR CONTRIBUTIONS

SX and SC initiated and planned this study. AS-H, SC, SX, QZ, SZ, TF, and EE conducted the FHB evaluations in field nurseries and greenhouse. AS-H and SC conducted marker analysis and association mapping. AS-H and SX analyzed the FHB data. SX and YJ conceived and planned the research on development of the SHW lines. QZ, SX, YJ, XC, and JF developed the SHW lines. AS-H, SX, and SC wrote the manuscript. All authors reviewed and edited the manuscript.

FUNDING

This material was based upon work supported by the United States Department of Agriculture, under Agreement

No. FY16-SP-007 and the CRIS Project No. 5442-22000-080-037-00D. This is a cooperative project with the US Wheat and Barley Scab Initiative. This research was supported in part by an appointment to the Agricultural Research Service (ARS) Research Participation Program administered by the Oak Ridge Institute for Science and Education (ORISE) through an interagency agreement between the United States Department of Energy (DOE) and the United States Department of Agriculture (USDA). ORISE is managed by Oak Ridge Associated Universities (ORAU) under DOE contract number DE-SC0014664.

ACKNOWLEDGMENTS

We thank Terrance Peterson, Mary Osenga, and Joseph Mullins for assistance with field disease evaluations, Danielle Holmes and Joseph Mullins for preparing inoculum for greenhouse and field disease evaluations, respectively. All opinions expressed in this paper are the author's and do not necessarily reflect the policies and views of USDA, ARS, DOE, or ORAU/ORISE. Mention of trade names or commercial products in this article was solely for the purpose of providing specific information and does not imply recommendation or endorsement by the United States Department of Agriculture. USDA was an equal opportunity provider and employer.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01829/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 10 January 2019
doi: 10.3389/fpls.2018.01971

Assessing Genetic Diversity to Breed Competitive Biofortified Wheat With Enhanced Grain Zn and Fe Concentrations

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Breeding wheat with enhanced levels of grain zinc (Zn) and iron (Fe) is a cost-effective, sustainable solution to malnutrition problems. Modern wheat varieties have limited variation in grain Zn and Fe, but large-scale screening has identified high levels of Zn and Fe in wild relatives and progenitors of cultivated wheat. The most promising sources of high Zn and Fe are einkorn (*Triticum monococcum*), wild emmer (*T. dicoccoides*), diploid progenitors of hexaploid wheat (such as *Aegilops tauschii)*, *T. spelta*, *T. polonicum*, and landraces of *T. aestivum*. This study evaluate the effects of translocations from rye and different *Aegilops* species in a "Pavon-76" wheat genetic background and utilized in the wheat biofortification breeding program at CIMMYT that uses diverse genetic resources, including landraces, recreated synthetic hexaploids, *T. spelta* and pre-breeding lines. Four translocations were identified that resulted significantly higher Zn content in "Pavon 76" genetic background than the check varieties, and they had increased levels of grain Fe as well-compared to "Pavon 76." These lines were also included in the breeding program aimed to develop advanced high Zn breeding lines. Advanced lines derived from diverse crosses were screened under Zn-enriched soil conditions in Mexico during the 2017 and 2018 seasons. The Zn content of the grain was ranging from 35 to 69 mg/kg during 2017 and 38 to 72 mg/kg during 2018. Meanwhile grain Fe ranged from 30 to 43 mg/kg during 2017 and 32 to 52 mg/kg during 2018. A highly significant positive correlation was found between Zn and Fe ($r = 0.54$; $P < 0.001$) content of the breeding lines, therefore it was possible to breed for both properties in parallel. Yield testing of the advanced lines showed that 15% (2017) and 24% (2018) of the lines achieved 95–110% yield potential of the commercial checks and also had 12 mg/kg advantage in the Zn content suggesting that greater genetic gains and farmer-preferred wheat varieties were developed and deployed. A decade of research and breeding efforts led to the selection of "best-bet" breeding lines and the release of eight biofortified wheat varieties in target regions of South Asia and in Mexico.

Keywords: biofortification, iron, rye, translocation, wild relatives, wheat, zinc

OPEN ACCESS

Edited by:
Marianna Rakszegi, Centre for Agricultural Research (MTA), Hungary

Reviewed by:
Soren K. Rasmussen, University of Copenhagen, Denmark
Heinrich Grausgruber, University of Natural Resources and Life Sciences Vienna, Austria

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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *13 July 2018*
Accepted: *19 December 2018*
Published: *10 January 2019*

Citation:
Velu G, Crespo Herrera L, Guzman C, Huerta J, Payne T and Singh RP (2019) Assessing Genetic Diversity to Breed Competitive Biofortified Wheat With Enhanced Grain Zn and Fe Concentrations. Front. Plant Sci. 9:1971. doi: 10.3389/fpls.2018.01971

INTRODUCTION

Micronutrient deficiency, also known as hidden hunger, is one of the most important challenges facing humanity today. It is caused by a lack of essential vitamins and minerals (primarily vitamin A, iron, and zinc) in the diet and currently affects more than two billion people worldwide (White and Broadley, 2009; WHO, 2017). Pregnant women and young children are particularly prone to acute micronutrient deficiency, which can impair the physical and mental development of children under 5 years of age (Black et al., 2013). Globally, undernutrition contributes to 45 percent of child deaths each year (WHO, 2017), while in low- and middle-income countries it also causes gross domestic product losses of up to 8 percent. Biofortification offers a sustainable solution to increase food and nutritional security for millions of resource-poor consumers where major staples provide most of their dietary energy (Bouis et al., 2011). The wheat (*Triticum aestivum* L.) biofortification program at CIMMYT leading the partnership based global effort to breed competitive wheat varieties with 40% higher Zn concentration (+ 12 mg/kg) over the commercial varieties in the target regions of South Asia (Velu et al., 2011; Singh and Velu, 2017). The primary target nutrient for wheat is zinc (Zn), as millions of resource-poor wheat consumers in South Asia and Africa are prone to Zn deficiency (Stein, 2010).

Wheat is a major staple crop that provides more than 20% of dietary energy and protein consumption worldwide (Braun et al., 2010). Varieties with improved nutritional quality, protein content, high grain yield, and desirable processing quality in adapted elite genetic backgrounds with tolerance to stresses and diseases can help alleviate nutrient deficiencies. Breeding biofortified wheat with enhanced micronutrient concentrations has emerged as a long-term, sustainable solution for micronutrient deficiency (Pfeiffer and McClafferty, 2007). In combination with other strategies, such as supplementation or fortification, biofortification aims to reach micronutrient-deficient rural people who have limited access to formal markets and health care systems. To assure nutritional and food security, it is therefore paramount that suitable biofortified wheat varieties are developed, released, and disseminated for widespread adoption. Since grain nutrition is a non-visible trait, it is essential that new cultivars are not only rich in grain Zn, but that they have a higher yield than current cultivars. This will reduce poverty due to increased incomes and decrease childhood stunting and malnutrition.

Bread wheat (*Triticum aestivum*) is derived from a fertile hexaploid hybrid cross between wild emmer (*T. dicoccoides)* and goat grass (*Ae. tauschii)*. While bread wheat may have evolved several times, it is quite unlikely that its genetic variation is a representative sample of the genetic variation in its progenitors. Recent trait discoveries *in Ae. tauschii* have identified agronomically useful traits that may not be present in bread wheat (Mondal et al., 2016; Vikram et al., 2016). It is recommended that considerable emphasis be placed on exploiting the three species that contributed the wheat A, B, and D genomes due to their long evolutionary history and adaptation to diverse environmental conditions for stress tolerance and potential diversity for nutritional quality traits (Dubcovsky et al., 1998).

The substantial genetic diversity in primary, secondary, and tertiary wheat gene pools serve as raw material for the development of nutritious wheat varieties through breeding (Graham et al., 1999; Monasterio and Graham, 2000; Velu et al., 2014). However, the range of genetic variation for Zn and Fe is considerably lower in improved materials than in landraces and progenitor species. In the case of Zn, the range of variation, particularly among the unadapted species, is sufficient to have a positive impact on human nutrition. However, evidence suggests that mineral concentration is diluted as yield potential rises, increasing the difficulty of using unadapted mineral-rich sources, such as wild relatives to improve adapted wheat (Ortiz-Monasterio et al., 2007). Significant efforts have therefore been made to screen landraces, which tend to have a better agronomic type than wild relatives. Some landraces look very promising, and some have high grain concentrations of Zn and Fe.

Targeted utilization of alien chromosomes through translocations offers an alternative approach for improving nutritional quality along with essential core traits of high yield, durable disease resistance, and end-use quality for making products, such as leavened bread and flat bread, such as *chapattis*. One example is the 1BL.1RS translocation, in which 1RS chromosome from rye has been widely introduced into wheat, where it has replaced long arm of chromosome 1B. This has introduced a new source of leaf rust, stem rust, yellow rust and powdery mildew resistance present on the 1R chromosome. Approximately 60% of bread wheat material from the International Maize and Wheat Improvement Center (CIMMYT) has had this 1BL.1RS translocation at some stage (Rajaram et al., 2002), however, frequency of lines with this translocations has gone down significantly due to new virulent strains of rust fungus and the negative effect of 1RS translocation on end-use quality. Though the effect of this translocation on grain Zn concentration has not yet been evaluated.

Synthetic hexaploids (using *T. durum* or *T. dicoccum* and diverse sources of *Ae. tauschii*) offer large variability for agronomic and nutritional quality traits. There have been successful introgressions of quantitative traits from synthetic hexaploids into adapted germplasm, a process that involves limited backcross populations, which are then evaluated for agronomic traits and grain Zn and Fe concentrations (Velu et al., 2016). These introgressions have been utilized in released varieties, such as "WB02," and "Zinc-Shakti," which have Zn levels 20–40% higher than local varieties (Singh and Velu, 2017). Capturing genetic variation from wild relatives and landraces through targeted crosses and early generation selection for agronomic and disease resistance and later generations for yield and yield stability and Zn concentrations showed large number of lines combine high yield and high Zn.

CIMMYT's biofortification program exploits diverse genetic resources and utilizes new wheat varieties that are high-yielding,

Abbreviations: CV, coefficient of variation; Fe, iron; H^2, broad sense heritability; LSD, least significant difference; PSI, particle size index; SD, standard deviation; TKW, thousand kernel weight; Zn, zinc.

more heat and drought tolerant, and have better end-use quality. Thus, superior agronomic traits are inherent in the biofortified wheat varieties, along with nutrition. This study aimed to test the effect of translocations from different rye and *Aegilops* species on grain Zn content in "Pavon 76," wheat genetic background across 2 years in Mexico (Set I). Advanced lines derived from crosses with diverse progenitors having enhanced Zn and Fe contents were also evaluated and selection was carried out to achieve the dissemination of agronomically superior wheat varieties with significantly increased Zn and Fe concentrations (Set II).

MATERIALS AND METHODS

Experimental Site

Field experiments were conducted from 2016 to 2018 at the Norman E. Borlaug Experimental Station (CENEB) in Ciudad Obregon, Mexico (27°20′N; 109°54′ W; 38 masl). Irrigation was supplied five times during the growing season to avoid water stress.

Experimental Design and Crop Management

Set I

The historic spring bread wheat variety "Pavon 76," and the 62 translocated lines were sown in a randomized block design with two commercial checks ("Kachu" and "PBW 343") in 2 years (2017, 2018). "Kachu" is a "Kauz" derived high-yielding variety grown more widely in India; "PBW 343" (Attila) is a historical variety still grown by farmers in South Asia. Each genotype was planted in a double row of 1 m length with a bed to bed distance of 80 cm. All recommended agronomic practices were followed. The commercial form of $ZnSO_4 \cdot 7H_2O$ was applied as basal fertilizer, along with 50% of the recommended 200 kg/ha nitrogen and 100% of the 50 kg/ha phosphorus fertilizers. The remaining 50% or 100 kg/ha N was applied as top dressing during the second irrigation, or 30 days after sowing. At maturity, whole plots were harvested and 30 g grain samples from each plot were used for micronutrient analysis. We also measured thousand kernel weight (TKW).

Set II

Advanced lines were derived from diverse crosses of CIMMYT elite breeding lines with high Zn synthetic hexaploids, landraces, and other sources. The simple and top cross (three-way) derivatives were advanced to large F2 populations (>2,000 plants/cross) and F3, F4, F5 bulks of about 400–800 plants were grown and plants resistant to yellow rust and leaf rust were bulked separately in Toluca and at CENEB. From the F4 and F5 grown in Toluca, yellow rust resistance and *Septoria tritici* blotch resistant plants were harvested and individual head-rows were sown. After visual selection of individual heads based on plump and bold grains seed were sown at CENEB as head-rows. Head-rows were evaluated for agronomic characteristics and resistance to leaf and stem rusts compared to repeated checks. The best performing F6 head-rows exhibiting resistance to rusts, and having superior agronomic performance, bold and plump grain types were analyzed for Zn and Fe concentrations. Grain protein content and grain hardness were also measured using a Near Infra-Red Spectroscopy (NIRS) assay at the CIMMYT Wheat Quality Laboratory on advanced selected lines.

F6 lines were advanced to the first yield trials, grown in an alpha-lattice-Latinized design with three replicates both in the 2017 and the 2018 crop seasons. Each trial comprised of two checks and 28 entries in both seasons. Trials were planted in November and harvested in late April and five times surface irrigation (>500 mm) was provided to avoid water stress. For weed control, 20.6% flucarbazone-sodium was applied at the rate of 0.5 L/ha to control narrow leaf weeds and a mix of Starane (fluroxypyr-meptyl, 45.52%) and Buctril (bromoxynil octanoate, 31.7%), was applied at the rate of 0.4 and 0.3 L/ha, respectively for broad leaf weeds just before sowing. For insect control, Admire (imidacloprid, 30.2%) was applied at the rate of 0.75 L/ha during tillering/booting stage of the crop. Approximately 200 kg/ha N was applied. The commercial form of Zn fertilizer ($ZnSO_4 \cdot 7H_2O$) was applied to optimize and homogenize available soil Zn in order to reduce soil Zn heterogeneity at CENEB.

Whole plots were harvested after physiological maturity and grain yield was measured. Quality traits were analyzed using 50 g seed from each advanced selected wheat lines. TKW, grain Zn, and Fe concentrations scores were measured for all entries.

Micronutrient Analysis

Grain samples weighing ∼30 g and free from dust particles, chaff, glumes, and other plant material were prepared for determining micronutrient concentration and thousand kernel weight. Thousand kernel weight (TKW) was measured with a SeedCount digital imaging system (model SC5000, Next Instruments Pty Ltd), New South Wales, Australia. Grain Zn and Fe concentration (in mg/kg) were measured by a bench-top XRF machine (Oxford instruments, UK) (Paltridge et al., 2012). This Energy-dispersive X-Ray Fluorescence spectrometry (EDXRF) technique have been standardized to perform non-destructive elemental analysis of whole grain wheat samples for Zn and Fe testing at CIMMYT. The EDXRF methods was developed using a large set of randomly selected wheat samples with variable seed properties (30–70 mg/kg Zn and 30–50 mg/kg Fe contents) (Paltridge et al., 2012). As a highly significant positive correlation was observed in a preliminary analysis between the EDXRF and the Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) measurements when used for Zn and Fe ($r = 0.9$ and 0.9, respectively; $P < 0.001$) analysis, with lower than 5% coefficient of variation (CV), the EDXRF found to be a rapid, economical and near accurate measurement of grain Zn and Fe in whole grain wheat samples.

End Use Quality Analysis

Competitive high yielding, high Zn candidate lines (770 lines from the F6 generation) were analyzed for processing and end-use quality parameters. Dough deformation work (W), and dough strength vs. extensibility (P/L) were measured using Chopin Alveograph (Chopin Technologies, France). To assess the end-use quality of yeast-leavened bread, pup loaves were baked as pan bread using AACC method 10–09 (AACC, 2000)

using the (Guzman et al., 2015) method for the adjustment of the optimal water absorption. Bread loaf volume was measured by rapeseed displacement in accordance with AACC method 10–05.01 (AACC, 2000). Wheat kernel hardness was measured based on particle size index measurement according to AACC Method 55–30.

Statistical Analysis

Statistical analyses was carried out using PROC MIXED in SAS 9.2 (SAS Institute, Cary, NC, USA) software. Mean comparisons between the original line "Pavon 76" and its translocated lines (Set I) were made for all four traits (grain yield, TKW, Zn, Fe) in the study using Tukey's test. Broad-sense heritability (H^2) was estimated across environments for Zn and Fe content using the breeding lines (Set II), using the formula $H^2 = \sigma_g^2/(\sigma_g^2 + \sigma_{gl}^2/l + \sigma_e^2/rl)$, where σ_g^2 is genotypic variance, σ_{gl}^2 is the genotype × location variance, and σ_e^2 is the residual error variance for r replicates and l locations. Genotypic values (i.e., line means of Set II) were estimated as Best Linear Unbiased Estimators with a random effect for replicates nested within each environment.

RESULTS

Identification of High Zn Wheat Lines With Rye Chromosome Translocations

The sixty-two "Pavon 76" translocation lines (Set I), which showed high variation in their morphological properties (heading date and plant height, data not shown) also showed high variation in both Zn and Fe content, but there was also a significant variation in the thousand kernel weight (TKW) of the samples as well (**Table 1**). Grain Zn content varied from 38.6 to 57.6 mg/kg (mean = 47.1 mg/kg), whereas Fe content ranged from 32.0 to 53.0 mg/kg (mean = 36.6 mg/kg), next to the TKW ranges from 31.5 to 48.0 g (mean = 37.6 g). Tukeys' test showed that four translocation lines had significantly outstanding Zn content (LSD5% = 3.6) with more than 10 mg/kg Zn advantage over trial mean.

Grain Zn concentration of entry GID: 7615628 disomic line with translocation from 1Rr rye chromosome showed the highest grain Zn with 9.6 mg/kg Zn concentration over the average Zn content of the checks, but had 18.1 mg/kg higher Zn content than the "Pavon 76." This was followed by entry GID: 7615697 containing chromosome 2Ds from rye with 8.1 mg/kg improvement in Zn content compared to the average of the controls, but have 16.6 mg/kg higher Zn content than "Pavon 76." Lines GID: 7615636 and 7615732 also had outstanding Zn content having MA1S.1RLe and 1RS.1AL translocations, respectively. These lines were selected to use in breeding programs aiming to increase the mineral content of wheat. Compared to "Pavon 76," the Fe content of these four lines also increased by 4.3–8.5 mg/kg as a result of translocation, but they had lower Fe content than the check line, "PBW343."

The size and weight of the kernel was expected to influence the Zn and the Fe concentration in the seed, so a correlation analysis was carried out. Data showed that there was a significant negative correlation between the TKW and the Zn content of the kernel

TABLE 1 | Summary of mean values for Fe and Zn concentration and TKW among Pavon 76 translocation lines (Set I) (2017, 2018).

	Genotype identifier (GID)	Cross	Fe (mg/kg)	Zn (mg/kg)	TKW (g)
1	7615612	Pavon 76, + 1R(1B)	33.7	47.7	36.5
2	7615614	Pavon 76, + 1R(1A)	34.5	53.0	36.5
3	7615616	Pavon 76, + 1R(1D)	35.9	50.4	36.0
4	7615618	Pavon 76, + 1R.1D5+10-2(1D)	34.4	49.8	34.5
5	7615620	Pavon 76, + 1Rinv(1A)	35.5	47.5	34.5
6	7615622	Pavon 76, + 1R(1D), PAVON7	33.4	49.1	35.0
7	7615624	Pavon 76, + 1Rr(1A), PAVON8	49.1	49.5	35.0
8	7615626	Pavon 76, + 1Rr(1B), PAVON9	32.4	45.2	37.0
9	7615628	Pavon 76,' + 1Rr(1D), PAVON 10	37.8	57.6	34.5
10	7615630	Pavon 76, + 1Ri(1B)	35.7	52.5	36.0
11	7615632	Pavon 76, + MA1S.1RLe(1A)	36.0	51.1	35.0
12	7615634	Pavon 76, + MA1S.1RLe(1B)	33.7	49.0	39.0
13	7615636	Pavon 76, + MA1S.1RLe(1D)	37.3	55.7	39.5
14	7615638	Pavon 76, + 2Rrec(2B)	36.9	48.9	35.5
15	7615640	Pavon 76, + 4Aril	32.7	45.9	39.5
16	7615644	Pavon 76, + 1RS.1AL	35.8	43.2	37.0
17	7615646	Pavon 76, + 1RS.1BLcim	34.3	44.5	38.5
18	7615648	Pavon 76, + 1RS.1BL gnr	35.8	48.1	38.0
19	7615650	Pavon 76, + 1RS.1DLbb	36.3	47.7	42.0
20	7615652	Pavon 76, + 1RS.1DLw	32.1	43.6	37.5
21	7615654	Pavon 76, + 1RS.1ALrh	41.7	42.9	37.5
22	7615656	Pavon 76, + 1RSe.1AL	53.0	44.0	39.5
23	7615658	Pavon 76, + 1RSe.1BL	36.5	43.1	40.0
24	7615660	Pavon 76, + 1RSe.1DL	35.6	43.8	40.5
25	7615662	Pavon 76, + 1RSv.1AL	35.9	44.4	38.5
26	7615664	Pavon 76, + 1RSv.1BL	34.7	46.8	38.5
27	7615666	Pavon 76, + 1RSv.1DL	33.9	44.8	39.0
28	7615668	Pavon 76, + 1RSi.1BL	36.8	47.5	39.5
29	7615670	Pavon 76, + MA1	36.2	47.5	39.0
30	7615672	Pavon 76, + MA2	37.5	51.9	38.5
31	7615674	Pavon 76, + Te1	35.4	47.4	38.0
32	7615676	Pavon 76, + Te2	34.8	46.9	36.5
33	7615678	Pavon 76, + 1RSe.1BLv	35.5	47.0	35.5
34	7615680	Pavon 76, + 1AS.1RLe	35.7	47.8	34.0
35	7615682	Pavon 76, + 1BS.1RLe	34.5	47.7	35.0
36	7615684	Pavon 76, + 1DS.1RLe	44.2	48.1	35.5
37	7615687	Pavon 76, + 1DS.1RLbb	36.2	52.9	35.5
38	7615688	Pavon 76, + 2RS.2BLcs	35.1	49.6	39.0
39	7615690	Pavon 76, + 2BS.2RLcs	36.1	45.3	35.0
40	7615692	Pavon 76, + 2BSp.2RLbl	32.0	44.2	36.0
41	7615694	Pavon 76, + 2D(s) + 2"	36.3	44.1	36.5
42	7615697	Pavon 76, + 2D(s) + 4"	40.5	56.1	32.5
43	7615699	Pavon 76, + 3RS.3DLrh	34.0	45.0	39.5
44	7615700	Pavon 76, + 3RS.3DLcs	32.3	44.4	37.5
45	7615702	Pavon 76, + 3DS.3RLcs	36.7	44.0	38.0
46	7615704	Pavon 76, + 7DS.4RLm	33.6	45.4	37.5
47	7615706	Pavon 76, + 5RS.5ALcs	34.0	42.8	36.5

(Continued)

TABLE 1 | Continued

	Genotype identifier (GID)	Cross	Fe (mg/kg)	Zn (mg/kg)	TKW (g)
48	7615708	Pavon 76, + 5RS.5BLe	35.2	43.9	38.0
49	7615710	Pavon 76, + 5D.5R-1"	40.3	44.3	39.0
50	7615712	Pavon 76, + 5RS.5DLrh	32.7	42.3	37.5
51	7615714	Pavon 76, + 6BS.6RLbb	34.9	48.1	41.0
52	7615716	Pavon 76, + 7A.7S-S3	36.7	38.6	40.0
53	7615718	Pavon 76, + 7A.7S-L7	33.8	43.8	41.5
54	7615720	Pavon 76, + 7A.7S-L5	39.3	44.8	42.0
55	7615722	Pavon 76, ' + 7A.7S-Gb5	50.5	43.1	42.0
56	7615728	Pavon 76, 1AS.#2L	34.4	52.1	31.5
57	7615730	Pavon 76, 1RSi.1BL	47.2	44.6	38.5
58	7615732	Pavon 76, 1RS.1AL" 1RS.1DL"	36.3	54.0	43.0
59	7615734	Pavon 76, + 2BS.2RLcs, PAVON85	35.4	43.9	36.5
60	7615736	Pavon 76, + 2R.2B"	35.7	50.4	35.0
61	7615739	Pavon 76, + 1D+9"	33.4	44.7	35.5
62	7615740	Pavon 76, + 2AS.2RLcs	34.8	48.1	32.5
parent	7615724	Pavon 76	32.0	39.5	38.5
check1	2430154	PBW343 (Check)	47.4	48.5	45.0
check2	4755014	Kachu #1 (Check)	36.5	47.5	44.0
		Mean	36.6	47.1	37.6
		Minimum	32.0	38.6	31.5
		Maximum	53.0	57.6	48.0
		SD±	4.4	3.8	2.8
		$LSD_{5\%}$	1.6	3.6	2.9
		H^2	0.52	0.80	0.90

H^2, broad sense heritability; LSD, least significant difference; SD, standard deviation; TKW, thousand kernel weight.

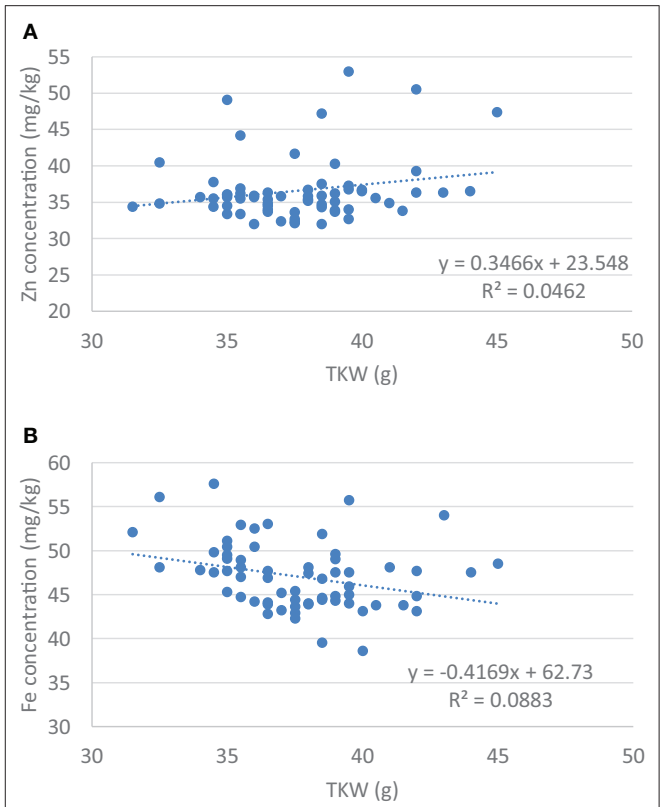

FIGURE 1 | Regression analysis between **(A)** Grain Zn concentration and the thousand kernel weight (TKW) and **(B)** Grain Fe concentration and thousand kernel weight (TKW).

($r_{5\%} = -0.297$, $r_{5\%krit} = 0.250$, $n = 62$), but no correlation was found in case of the Fe content ($r_{5\%} = 0.215$, $r_{5\%krit} = 0.250$, $n = 62$). At the same time regression analysis did not show any association between the size of the kernel and its Zn or Fe content (**Figure 1**). This may refer to the possibility to select lines with stably high Zn and/or Fe content without dilution effects of the seed size. There was no correlation between the Zn and Fe content in case of the wheat/rye translocation lines ($r_{5\%} = 0.043$).

Stability, Heritability of Zn and Fe Concentration

Combined analysis across years for Pavon 76 translocation lines (Set I) showed significant year effects on grain Zn and TKW ($P < 0.001$). Broad sense heritability was high for Zn and TKW ($H^2 = 0.80$ and 0.90, respectively) while it was intermediate for Fe content ($H^2 = 0.52$) (**Table 1**), with a coefficient of variation below 10%, referring to good management of trials across years.

Analysis of Set II lines showed similar results with high heritability values for Zn (0.79 and 0.83 in 2017 and 2018, respectively) and TKW (0.85 in 2017) and medium level for Fe content (0.67 and 0.66 in 2017 and 2018, respectively) (**Table 2**). However, highly significant positive correlation has been observed between Fe and Zn concentrations during 2017 ($R^2 = 0.25$; $P < 0.05$) an 2018 ($R^2 = 0.21$; $P < 0.05$) crop seasons (**Figure 2**).

Next to Zn and Fe content, the heritability of other main seed components and properties were also studied. Grain yield and physical properties of the seed were highly heritable with 0.82, 0.79 (2017, 2018) heritability values for grain yield, 0.75 and 0.71 for test weight (2017, 2018), 0.75 and 0.65 for grain hardness (2017, 2018), 0.6 and 0.7 for protein content (2017, 2018), and 0.7 and 0.74 for loaf volume (2017, 2018).

Breeding for High Zn Wheat Genotypes

In Set II experiment, where breeding lines were evaluated, highly significant differences were observed between genotypes during the 2017 and 2018 crop seasons. The average yield potential during the 2017 season was 6.2 t/ha, with a range of 5.8–8.1 t/ha, whereas it was 6.8 t/ha mean with the range of 4.3 to 8.4 t/ha in 2018 (**Table 2**; **Figure 3**). About 15 percent of the advanced lines achieved 95–110 percent of the yield potentials of the two check varieties ("Kachu" and "Borlaug,") whereas in 2018, 24% of the lines showed 95–110% grain yield potential compared to the two checks (which were "Mayil" and "Borlaug 100" in 2018).

Grain samples yielded similarly or better than checks were analyzed for grain Zn and Fe content with XRF method. Results

TABLE 2 | Summary of mean, maximum, and minimum values for Fe and Zn concentration and grain yield among advanced high Zn lines (selection from Set II), 2017 and 2018.

Statistics	Grain Zn (mg/kg)	Grain Fe (mg/kg)	Grain yield (t/ha)	TKW (g)	Test weight (kg/hL)	Grain protein (%)	Grain hardness (PSI)	Loaf volume (cm^3)
2017, *N* = 416 Lines								
Trial mean	52	35	6.2	47.8	79.3	13	71	724
Range	35–69	30–43	5.8–8.1	40.8–61.0	73.8–82.3	11.2–15.5	23–86	500–935
H^2	0.79	0.67	0.82	0.85	0.75	0.6	0.75	0.7
CV (%)	7.8	9.8	5.1	8	9	8.5	10	12
LSD	4	3.2	0.64	3.3	1.4	0.68	6	67.13
2018, *N* = 354 Lines								
Trial mean	53	40	6.8	50.8	80.1	13.2	63	720
Range	38–72	32–52	4.3–8.4	41.3–59.7	76.4–83.3	11.5–15.3	49–79	555–855
H^2	0.83	0.66	0.79	0.86	0.71	0.7	0.65	0.74
CV (%)	8.8	10.2	4.61	5.52	8.7	6.8	9.8	10.4
LSD	5.3	3	0.63	4.1	2.2	0.87	5.4	58.7

CV, coefficient of variation; H^2, broad sense heritability; LSD, least significant difference; PSI, particle size index; SD, standard deviation; TKW, thousand kernel weight.

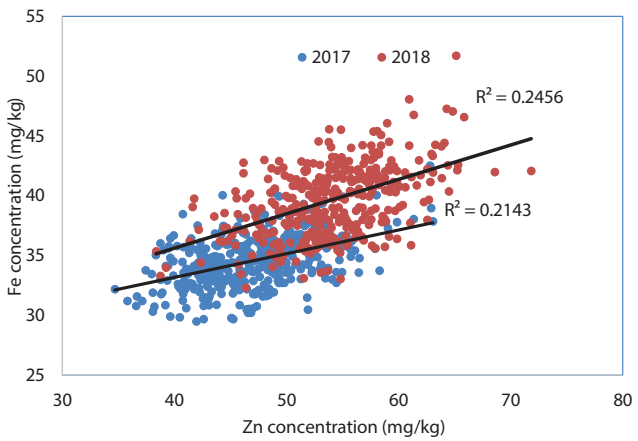

FIGURE 2 | Association between grain Zn and Fe concentrations using selected lines of Set II, 2017 and 2018.

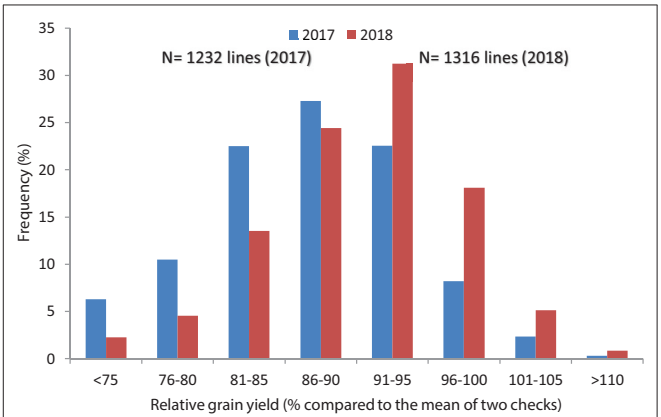

FIGURE 3 | Frequency distribution of relative grain yield potential of high Zn lines compared to checks in yield trials (Set II) of 2017 and 2018.

showed about 4.8 and 5.4 mg/kg average Zn increase in 2017 and 2018, respectively for the selected lines compared to the checks average, while the changes in individual lines could go up to 21–23 mg/kg Zn increase above the checks average (**Table 2**, **Figure 4**).

Grain Zn varied from 35 to 69 mg/kg (mean = 52 mg/kg) and 38 to 72 mg/kg (mean = 53 mg/kg) during the 2017 and 2018 seasons, respectively, whereas grain Fe ranged from 30 to 43 mg/kg (mean = 35 mg/kg) during 2017 and 32 to 52 mg/kg (mean = 40 mg/kg) during 2018 (**Table 2**).

In terms of end use quality most of the lines expressed better processing quality and semi-hard to hard-grain texture, except for six entries that had tenacious gluten property. TKW varied from 40.8 to 61.0 g (mean = 47.8 g) in 2017 and 41.3 to 59.7 g (mean = 50.8 g) during 2018, while test weight ranged from 73.8 to 82.3 kg/hl (mean = 79.3 kg/hl) in 2017 and 76 to 83 (80 kg/hL) during 2018 crop season, indicating potentially good milling quality (**Table 2**). Meanwhile loaf volume ranged from 500 to 935 cm^3/100 g of flour (mean = 724 cm^3) in 2017 and from 555 to 855 cm^3 (mean of 720 cm^3) in 2018. The protein content of these lines was also variable changing between appr. 11.5 and 15.5% in both years. These results indicate that high Zn candidate lines could possess desirable end-use and processing qualities for making various types of flat and yeast leavened breads.

About twenty best breeding lines were identified and selected for further testing in different environments as they had 8.3–24 mg/kg advantage in their Zn concentration and 0–6% yield superiority for grain yield compared to the controls (**Table 3**). These lines had test weight ranging from 78.2 to 82.5 kg/hl, TKW from 44.3 to 57.2 g, PSI form 52 to 14.4, grain protein content from 12.2 to 14.4%, Fe content from 33.1 to 57.2 mg/kg, Alveograph W from 112 to 358 ($*10^{-4}$J), Alveograph P/L value from 0.9 to 2.9 and loaf volume from 610 to 780 cm^3. This

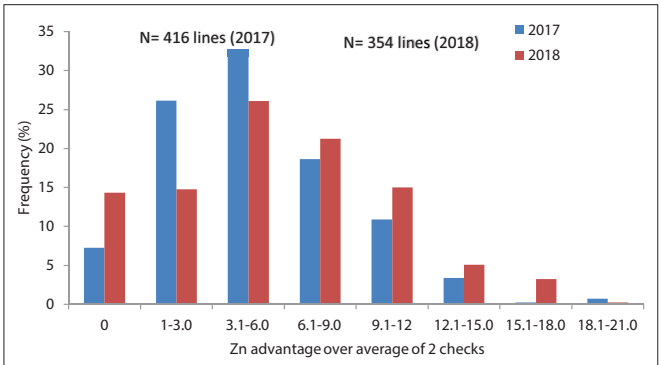

FIGURE 4 | Frequency distribution of grain Zn concentration changes related to check average in advanced high Zn lines (selection from Set II), in 2017 and 2018.

means that selected high Zn lines still have variable physical and compositional properties, but their breadmaking quality is expected to be excellent for most lines.

DISCUSSION

CIMMYT's biofortification breeding program has made significant progress in developing competitive, high zinc wheat lines using landraces and wild relatives (Velu et al., 2014; Singh and Velu, 2017). The transfer of traits from wild relatives often requires considerable cytological manipulation and the incorporation of an alien chromosomal segment in the elite breeding material that will not recombine. CIMMYT has historically used translocation lines, such as 1BL.1RS (Rajaram et al., 1983; Villareal et al., 1991, 1994a). Most recently, the 2NS segment from *Ae. ventricosa* has offered novel traits, such as the *Yr17* gene for yellow rust resistance and a pleiotropic effect on wheat blast resistance.

In the 1990s, CIMMYT increased the genetic diversity of its wheat breeding program by developing synthetic wheats and crossing these with elite breeding lines (Villareal et al., 1994b). Synthetic wheats are developed by crossing the A and B genome donor (*T. dicoccum* or *T. durum*) of bread wheat with the D genome donor, *Ae. tauschii*. It is reasonable to speculate that this expansion of genetic variability may have contributed to the increase in the rate of improvement for different traits, including stress tolerance (Mondal et al., 2016), nutritional quality (Crespo-Hererra et al., 2017), industrial quality (Burnett et al., 1995) and disease resistance (Singh et al., 1998; Shumny et al., 2016).

This study describes the successful integration of novel alleles for Fe and Zn from wild relatives of wheat, by using synthetic hexaploid derivatives from tetraploid *T. dicoccum* with diploid *Ae. tauschii*. It is noteworthy that the first high Zn wheat (Zinc-Shakti = Croc_1/*Ae.squarrosa*(210)//Inqalab91*2/Kukuna/3/PBW343*2/Kukuna), which had a significantly high grain Zn concentration, was inherited from *Ae. tauschii* via synthetic wheat developed from *T. durum*× *Ae. tauschii* parents. This statement was based on the assumption that "Croc," the (durum) parent did not have high Zn contributing alleles. In the case of "Mayil" ("WB-02") variety, the high Zn alleles derived from both parents *T. dicoccum* and *Ae. tauschii*.

In the case of breeding for higher grain Zn, much larger segregating populations were grown to enable selection of good agronomic type and disease resistance before selecting for Fe and Zn. The resulting advanced lines were tested at CENEB, Cd. Obregon, Mexico in an Alpha lattice design with three replications yield trial, following which the high-yielding and high Zn lines were tested in second year with six artificially manipulated environments in Obregon ranging from early-sown to late-sown (heat stress) and severe drought to moderate stress by restricted irrigation systems (Velu et al., 2016) and parallel screening in multiple sites in target countries of India and Pakistan, one of the target environments for these nutrient-enriched wheat cultivars (Velu et al., 2012, 2016, 2018). Genotypes were identified that had significantly higher grain yield and grain Zn concentration across locations. In some locations, the improvement in Zn concentration was up to 30–50% higher than that of the recurrent parent. These materials represent a significant stepping stone to achieve the ultimate goal of micronutrient-enriched wheat. Competitive high Zn wheat varieties have been tested broadly for adaptation and stability in target locations and released by national programs in some developing countries (Velu et al., 2012, 2015; Baloch et al., 2015). Some of these, such as the "Zinc-Shakti," "WB-02," "HPBW-01," and "Ankur Shiva" wheat varieties released in India by public and private partners and, more recently, "Nohely-F2018" released in Mexico for the Mexicali valley of northern Sonora region. Interestingly "BARI Gom 33" (="Kachu"/"Solala") released in Bangladesh during 2017 showed 7–8 mg/kg Zn advantage, and also offer resistance to wheat blast which is caused by *Magnaporthe oryzae*.

Another approach to increase micronutrient concentration is to use introgression segments or translocation of chromosomes from more distantly related species or unrelated species that carry the genetic code for high Fe and Zn, such as rye translocations in a Pavon wheat background. Rye and wild relatives are efficient in nutrient uptake and show adaptation in Zn deficient environments (Graham et al., 1999). In this study, some of the translocation lines showed significantly higher Zn concentration than the recurrent parent. The fact that rye is a nutrient use efficient crop suggests that some genes associated with high grain Zn and Fe might be present in the 1R chromosome (Monasterio and Graham, 2000). Genetic introgression with the short arm of rye chromosome 1 (1RS) have also generated improvements in wheat root traits (Kim et al., 2004), in addition to improved resistance to leaf rusts and powdery mildews (Villareal et al., 1994a; Ehdaie et al., 2003; Singh et al., 2011) which could contribute to better nutrient uptake. Wheat genotypes containing the rye chromosome arm 1RS are also reported to have enhanced grain yields, speculatively attributed to a superior rooting system (Villareal et al., 1991; Moreno-Sevilla et al., 1995). Bread wheat genotypes with the 1RS translocation were found to have higher root mass, thinner roots, and larger root length density in pot experiments under controlled environment conditions (Ehdaie et al., 2003). There are also reports showing differential

TABLE 3 | Characterization of the best genotypes from most recent high Zn wheat lines (2018).

No	GID	Cross name	Grain yield (t/ha)	Grain yield % of Borlaug 100	Zn (mg/kg)	Znchange (mg/kg)	Fe (mg/kg)	TWkg/hl	TKWg	PSI	Grain protein %	W $*10^{-4}$ J	P/L	Loaf volume cm^3
1	8233108	VALI/3/2*QUAIU/BECARD//BECARD	7.74	106	51.7	10.7	35.6	78.9	47.3	69	12.4	230	1.4	660
2	8231520	NG8201/KAUZ/4/SHA7//PRL/VEE#6/3/FASAN/5/MILAN/KAUZ/6/ACHYUTA/7/PBW343*2/KUKUNA/8/IWA 8600211//2*PBW343*2/KUKUNA/9/KACHU*2/5/WBLL1*2/TUKURU/3/T.DICOCCON PI94624/AE.SQUARROSA (409)//BCN/4/WBLL1*2/TUKURU	7.83	106	52.7	11.7	40.2	81.3	49.0	63	12.2	165	1.1	685
3	8232992	SHAKTI/7/2*TRAP#1/BOW/3/VEE/PJN//2*TUI/4/BAV92/RAYON/5/KACHU #1/6/TOBA97/PASTOR/3/T.DICOCCON PI94624/AE.SQUARROSA (409)//BCN/4/BL 1496/MILAN/3/CROC_1/AE.SQUARROSA (205)//KAUZ	7.51	105	53.9	12.9	36.8	81.1	50.6	65	13.7	308	1.1	750
4	8233528	FRET2/KUKUNA//FRET2/3/WHEAR/4/IWA 8600211//2*PBW343*2/KUKUNA/5/KACHU/BECARD//WBLL1*2/BRAMBLING/6/BOKOTA	7.62	105	49.3	8.3	42.9	80.6	52.3	66	14.0	247	1.1	765
5	8233114	VALI/3/2*QUAIU/BECARD//BECARD	7.89	104	52.2	11.2	37.0	82.5	49.9	68	12.4	245	2.9	610
6	8232346	THB/KEA//PF85487/3/DUCULA/4/WBLL1*2/TUKURU/5/IWA 8600211//2*PBW343*2/KUKUNA/6/MUTUS/AKURI #1//MUTUS/7/MUCUY	7.85	103	57.0	16.0	41.6	79.2	48.4	60	12.8	282	1.4	705
7	8233095	VALI*2/7/TRAP#1/BOW/3/VEE/PJN//2*TUI/4/BAV92/RAYON/5/KACHU #1/6/TOBA97/PASTOR/3/T.DICOCCON PI94624/AE.SQUARROSA (409)//BCN/4/BL 1496/MILAN/3/CROC_1/AE.SQUARROSA (205)//KAUZ	7.67	103	58.0	17.0	42.1	80.1	53.5	59	14.2	294	2.3	755
8	8232996	SHAKTI/7/2*TRAP#1/BOW/3/VEE/PJN//2*TUI/4/BAV92/RAYON/5/KACHU #1/6/TOBA97/PASTOR/3/T.DICOCCON PI94624/AE.SQUARROSA (409)//BCN/4/BL 1496/MILAN/3/CROC_1/AE.SQUARROSA (205)//KAUZ	7.52	102	53.7	12.7	33.7	80.3	46.4	71	13.0	256	1.8	725
9	8231667	DANPHE #1*2/3/T.DICOCCON PI94625/AE.SQUARROSA (372)//SHA4/CHIL/4/WBLL1*2/KURUKU//KRONSTAD F2004/3/WBLL1*2/BRAMBLING/5/MUTUS*2/HARIL #1	8.19	102	52.0	11.0	38.1	79.6	50.7	64	12.6	299	2.3	730
10	8233575	BABAX/KS93U76//BABAX/3/ATTILA/3*BCN//TOBA97/4/WBLL1*2/KURUKU/5/IWA 8600211//2*PBW343*2/KUKUNA/6/DANPHE #1*2/SOLALA/7/SUP152/BLOUK #1	7.41	102	57.5	16.5	42.1	80.2	52.2	65	13.0	358	1.0	770
11	8233526	FRET2/KUKUNA//FRET2/3/WHEAR/4/IWA 8600211//2*PBW343*2/KUKUNA/5/KACHU/BECARD//WBLL1*2/BRAMBLING/6/BOKOTA	7.36	102	54.3	13.3	43.2	81.6	53.3	64	13.8	219	1.3	755

(Continued)

TABLE 3 | Continued

No	GID	Cross name	Grain yield (t/ha)	Grain yield % of Borlaug 100	Zn (mg/kg)	Znchange (mg/kg)	Fe (mg/kg)	TWkg/hl	TKWg	PSI	Grain protein %	W $*10^{-4}$ J	P/L	Loaf volume cm^3
12	8232892	TRAP#1/BOW/3/VEE/PJN//2*TUI/4/BAV92/RAYON/5/KACHU #1/6/TOBA97/PASTOR/3/T.DICOCCON PI94624/AE.SQUARROSA (409)//BCN/4/BL 1496/MILAN/3/CROC_1/AE.SQUARROSA (205)//KAUZ/7/NADI	7.36	101	52.8	11.8	36.9	79.5	53.4	56	13.7	245	1.6	780
13	8232875	C80.1/3*BATAVIA//2*WBLL1/3/ATTILA/3*BCN*2//BAV92/ 4/WBLL1*2 /KURUKU/5/IWA 8600211//2*PBW343*2/KUKUNA/6 /KACHU/SAUAL/4/ATTILA*2/PBW65//PIHA/3/ATTILA/2*PASTOR	7.13	100	53.0	12.0	33.6	79.4	49.0	67	13.1	191	1.5	670
14	8232914	KIRITATI//HUW234+LR34/PRINIA/3/GEN/OPATA/8/NG8201/KAUZ/4/SHA7//PRL/VEE#6/3/FASAN/5/MILAN/KAUZ/6/ACHYUTA/7/PBW343*2/KUKUNA/9/SUP152/BAJ #1	7.24	99	55.6	14.6	37.6	80.5	47.1	59	14.0	176	0.7	735
15	8232919	PAURAQ/4/SLM//AG/6*INIA66/3/SLM/5/PAURAQUE #1/6/BECARD #1/5/KIRITATI/4/2*SERI.1B*2/3/KAUZ*2/BOW//KAUZ	7.24	99	51.5	10.5	33.1	78.2	49.7	65	12.7	112	0.9	680
16	8231590	TRAP#1/BOW/3/VEE/PJN//2*TUI/4/BAV92/RAYON/5/KACHU #1/6/TOBA97/PASTOR/3/T.DICOCCON PI94624/AE.SQUARROSA (409)//BCN/4/BL 1496/MILAN/3/CROC_1/AE.SQUARROSA (205)//KAUZ/7/FRNCLN/DANPHE	8.09	99	50.8	9.8	41.4	79.6	46.5	67	12.5	141	1.4	690
17	8231560	TRAP#1/BOW/3/VEE/PJN//2*TUI/4/BAV92/RAYON/5/KACHU #1/6/TOBA97/PASTOR/3/T.DICOCCON PI94624/AE.SQUARROSA (409)//BCN/4/BL 1496/MILAN/3/CROC_1/AE.SQUARROSA (205)//KAUZ/7/MUCUY	7.25	99	60.9	19.9	48.1	79.5	57.2	52	14.4	150	0.8	755
18	8232123	PASTOR/2*SITTA//PBW343*2/KUKUNA/4/CMH80.397//RL6010/5*SKA/3/CMH80.397/5/NELOKI/7/2*TRAP#1/BOW/3/VEE/PJN//2*TUI/4/BAV92/RAYON/5/KACHU #1/6/TOBA97/PASTOR/3/T.DICOCCON PI94624/AE.SQUARROSA (409)//BCN/4/BL 1496/MILAN/3/CROC_1/AE.SQUARROSA (205)//KAUZ	7.65	98	65.0	24.0	42.1	80.0	52.2	53	13.7	213	1.2	775
19	8233054	DANPHE #1*2/SOLALA//BORL14/3/BOKOTA	7.36	98	55.7	14.7	40.3	79.4	44.3	67	12.9	291	1.9	710
20	8233160	VILLA JUAREZ F2009/3/T.DICOCCON PI94625/AE.SQUARROSA (372)//3*PASTOR/4/WBLL1*2/BRAMBLING/8/PSN/BOW//SERI/3/MILAN/4/ATTILA/5/KAUZ*2/CHEN//BCN/3/MILAN/6/WBLL1*2/SHAMA/7/IWA 8600211//2*PBW343*2/KUKUNA/9/SUP152/BLOUK #1	7.50	98	54.6	13.6	42.6	82.5	52.2	63	12.5	255	2.0	725

TW, test weight; TKW, thousand kernel weight; PSI, particle size index; W, Alveograph deformation work; P/L, Alveograph ratio of dough strength and extensibility.

performance for milling and processing quality (Graybosch et al., 1993; Fenn et al., 1994; Dimitrijevic et al., 2008; Zhao et al., 2012).

The development of less expensive, easier to use colorimetric assays or near infrared spectroscopy methods is essential in order to replace atomic absorption or inductively coupled plasma analysis for measuring the Zn and Fe content of the grain during breeding programs. Recently Cardoso et al. (2018) showed μ-XRF based imaging technique for localization of grain Zn and Fe in wheat. These techniques will allow breeders to test for nutrient expression in more than one environment as early as possible in the breeding process. Once advanced or pure line progeny with good levels of Fe and Zn have been identified, they must be tested across a range of locations in different years to establish the stability of nutrient expression. Significant genotype × location interaction can be expected (Ortiz-Monasterio et al., 2007; Velu et al., 2014), and the identification of genotypes with stable expression across environments is essential.

CONCLUSION

About 15 and 24 percent of the breeding lines showed high yield and 12 mg/kg increase in Zn content parallelly which refers to the feasibility of developing competitive biofortified varieties with good agronomical traits and adaptability to local target environments. The positive shift in grain yield potential from 2017 to 2018 (about 0.6 t/ha) (**Figure 2**, **Table 2**) indicates great progress in terms of achieving higher genetic gain for grain yield potential of high Zn lines. Fe content was found to have high significant positive correlation with the Zn content in this experiment during 2017 ($r = 0.46$; $P < 0.01$) and 2018 ($r = 0.50$; $P < 0.01$) suggesting that simultaneous improvement of both Zn and Fe is feasible (**Figure 4**).

Altogether these results demonstrate that large genetic diversity is available in translocation lines for improving the nutritional content of wheat with enhanced grain Zn concentration. The wheat breeding program could effectively utilize these diverse genetic resources to improve nutritional quality of wheat, but improvement was also achieved in yield potential and wheat processing quality. Furthermore, these genetic resources are expected to improve the stress tolerance and disease resistance of the plants. Gene discovery and mapping studies would enhance breeding efficiency for high Zn content in the future.

AUTHOR CONTRIBUTIONS

GV conducted the field experiments and drafted the manuscript. LC, JH, RS, and TP helped with the field phenotyping and provided germplasm. CG conducted grain quality analysis.

FUNDING

We greatly acknowledge funding support from HarvestPlus challenge program (partly funded by Bill and Melinda Gates Foundation [OPP1019962]), the United Kingdom Department for International Development (DFID) and the CGIAR Research Program on Agriculture for Nutrition and Health (CRP-A4NH) for financial support.

ACKNOWLEDGMENTS

Special thanks to reviewer for the meaningful comments to improve quality of this manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 24 January 2019
doi: 10.3389/fpls.2018.01993

Developing a High-Throughput SNP-Based Marker System to Facilitate the Introgression of Traits From *Aegilops* Species Into Bread Wheat (*Triticum aestivum*)

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OPEN ACCESS

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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: 11 October 2018
Accepted: 21 December 2018
Published: 24 January 2019

Citation:
Przewieslik-Allen AM, Burridge AJ, Wilkinson PA, Winfield MO, Shaw DS, McAusland L, King J, King IP, Edwards KJ and Barker GLA (2019) Developing a High-Throughput SNP-Based Marker System to Facilitate the Introgression of Traits From Aegilops Species Into Bread Wheat (Triticum aestivum). Front. Plant Sci. 9:1993. doi: 10.3389/fpls.2018.01993

The genus *Aegilops* contains a diverse collection of wild species exhibiting variation in geographical distribution, ecological adaptation, ploidy and genome organization. *Aegilops* is the most closely related genus to *Triticum* which includes cultivated wheat, a globally important crop that has a limited gene pool for modern breeding. *Aegilops* species are a potential future resource for wheat breeding for traits, such as adaptation to different ecological conditions and pest and disease resistance. This study describes the development and application of the first high-throughput genotyping platform specifically designed for screening wheat relative species. The platform was used to screen multiple accessions representing all species in the genus *Aegilops*. Firstly, the data was demonstrated to be useful for screening diversity and examining relationships within and between *Aegilops* species. Secondly, markers able to characterize and track introgressions from *Aegilops* species in hexaploid wheat were identified and validated using two different approaches.

Keywords: *Aegilops*, wheat, genotyping array, single nucleotide polymorphism (SNP), introgression, wheat relative

INTRODUCTION

Aegilops is a genus of Eurasian annual grasses in the Poaceae known as the goatgrasses. There are 23 species within *Aegilops*; these species represent six different genome types (D, S, U, C, N, and M) and three different ploidy levels (diploid, tetraploid, and hexaploid) (The Plant List, 2013; Molnár et al., 2016; **Figure 1**). The genus *Aegilops* is the most closely related to the genus *Triticum*, which contains *Triticum aestivum* (bread wheat) and other domesticated wheats. Researchers have suggested that *Aegilops* and *Triticum* should be combined into a single evolutionary complex or even the same genus (Yamane and Kawahara, 2005). The close genetic relationship is evidenced by the numerous hybridisations that occur between members of both genera and by the presence of *Aegilops* in the evolutionary history of many *Triticum* species. Where geographic distributions are similar, gene flow has occurred between species; some species, such as *Aegilops cylindrica* have spread with wheat and have become uncontrollable weeds in wheat (Donal and Ogg, 1991). If treated separately, *Aegilops* appears to be basal to *Triticum*, with evidence indicating the genus' *Triticum* and *Aegilops* diverged an estimated 4 million years ago (Huang et al., 2002).

Aegilops has been divided into six sections based on morphological and genetic analysis. These are *Sitopsis* (Jaubert and Spach, 1850–1853) Zhuk., *Amblyopyrum* (Jaubert and Spach, 1850–1853) Eig, *Polyeides* Zhuk., *Cylindropyum* (Jaubert and Spach, 1850–1853) Zhuk., *Comopyrum* (Jaubert and Spach, 1850–1853) Zhuk., *Vertebrata* Zhuk (**Table 1**; Zhukovsky, 1928; Eig, 1929; Yamane and Kawahara, 2005; Schneider et al., 2008; Wang et al., 2013). *Aegilops mutica (syn. Amblyopyrum muticum)* has been separated by some researchers and placed into a monospecific genus called *Amblyopyrum* (Van Slageren, 1994) but for the purposes of this study has been included with other members of *Aegilops* for analysis. *Ae. speltoides* is thought to be the closest relative to the wheat B-genome and is also the donor of the G-genome of *Triticum timopheevii* (Dvorak et al., 1998a; Feldman, 2001). *Ae. tauschii* is the progenitor of the wheat D-genome, hybridizing with the AB-genome progenitor ∼10,000 years ago to produce hexaploid bread wheat (McFadden and Sears, 1946). This rare hybridization event is thought to have only occurred once or a small number of times resulting in a severe genetic bottleneck (Charmet, 2011). Further inbreeding and domestication pressures have resulted in a narrow gene pool for modern bread wheat breeding.

The genus *Aegilops* promises to be an important resource for wheat breeding as it harbors a high level of genetic diversity, particularly with relation to adaptation to different ecological conditions and pest and disease resistance. All *Aegilops* species are undomesticated and have wide geographic distributions and natural variation (Ostrowski et al., 2016). *Aegilops* contains species belonging to the secondary gene pool of wheat, meaning they have a genome homologous with wheat and conventional crossing may be used to transfer genes to wheat (*Ae. tauschii* and *Ae. speltoides*). Other more distantly related members of the genus belong to the tertiary gene pool of wheat and may need specific breeding techniques for gene transfers to wheat, although crosses between the two genera have been reported to occur naturally (Popova, 1923; Leighty and Taylor, 1927; Schneider et al., 2008). Interspecific hybridization between bread wheat and members of the genus *Aegilops* has been used historically in wheat breeding to confer beneficial traits from *Aegilops* into bread wheat. These include resistance to rusts, powdery mildew, eyespot, nematodes, hessian fly and wheat aphid (see Schneider et al., 2008 for a full review). The genus *Aegilops* is a potential source of further genes conferring agronomically valuable traits, such as drought tolerance, salt tolerance, heat tolerance, tolerance to toxicity and nutritional and bread-making quality traits of potential use in plant breeding (Molnár et al., 2004; Colmer et al., 2006; Schneider et al., 2008; Kilian et al., 2011).

Advances in genome sequencing over the last decade have had huge impacts on our knowledge of the large and complex hexaploid wheat genome and our ability to develop molecular markers (Uauy, 2017; The International Wheat Genome Sequencing Consortium (IWGSC), 2018). The knock-on effects of these developments have been seen in the breeding lines developed, the widespread adoption of molecular markers in breeding programmes and the development of new breeding techniques, such as genomic selection (Schneider et al., 2008; Bassi et al., 2016). At the same time there has been recognition of the importance of pre-breeding programmes specifically targeted at introducing genetic diversity from exotic sources, such as landraces and wheat relatives (Moore, 2015). The introduction of such diverse material has necessitated the development of specific molecular markers that are able to identify and characterize wheat relative DNA in the wheat genome (Winfield et al., 2016).

With the development of genomic tools and technologies enabling precise and efficient breeding *Aegilops* promises to be an increasingly important resource of genetic diversity in future wheat breeding. A potential drawback of utilizing wide crosses to introduce diversity in this way is the inclusion of large non-recombining blocks from a relative into the wheat genome. However, with the development of genomic technologies, improved crossing techniques and gene editing technologies it is becoming possible to target genomic regions with increased precision. To enable these techniques to be employed successfully there is a requirement for increasingly dense and precise molecular markers, which can be utilized in a high-throughput manner. A key challenge is to develop markers to track introgressed DNA in the wheat genetic background. This study describes the identification, validation and use of markers systems for facilitating the introgression of *Aegilops* species into hexaploid wheat. The wide range of species used in the study represent the three different ploidy levels and six genome types found within the genus. The markers developed have enabled the detection of *Aegilops* introgressions in newly developed lines with examples of how these markers have been deployed in different introgression projects.

MATERIALS AND METHODS

Germplasm

The accessions grown for DNA extraction (listed in **Supplementary File 1**) were grown in peat-based soil in pots and maintained in a glasshouse at 15–25°C with 14-h light, 8-h dark. Leaf tissue was harvested 4 weeks after germination, frozen in liquid nitrogen and stored at −20°C prior to nucleic acid extraction. Genomic DNA was prepared using a phenol–chloroform extraction method (Burridge et al., 2017), treated with RNase-A (QIAGEN Ltd., Manchester, UK) according to the manufacturer's instructions and purified using the QiaQuick PCR purification kit (QIAGEN Ltd).

Genotyping

The original SNP collection consisted of 819,571 SNPs obtained from genic sequences derived via targeted capture re-sequencing of numerous wheat lines and validated on the Axiom® HD Wheat Genotyping Array (Winfield et al., 2016; Affymetrix UK Ltd, High Wycombe, UK; EVA accession PRJEB29561). The most informative 36,711 SNPs were selected for inclusion on The Axiom® Wheat-Relative Genotyping Array, based on data from screening ten wild relative species (*Ae. mutica, Ae. speltoides, Aegilops. tauschii, Triticum timopheevii, T. urartu, Secale cereale, Thinopyrum bessarabicum, Th. elongatum, Th. Intermedium,* and *Th. Ponticum,* **Supplementary File 2**; http://www.cerealsdb.uk.net/cerealgenomics/CerealsDB/axiom_download.php).

Markers were selected to maximize polymorphism between

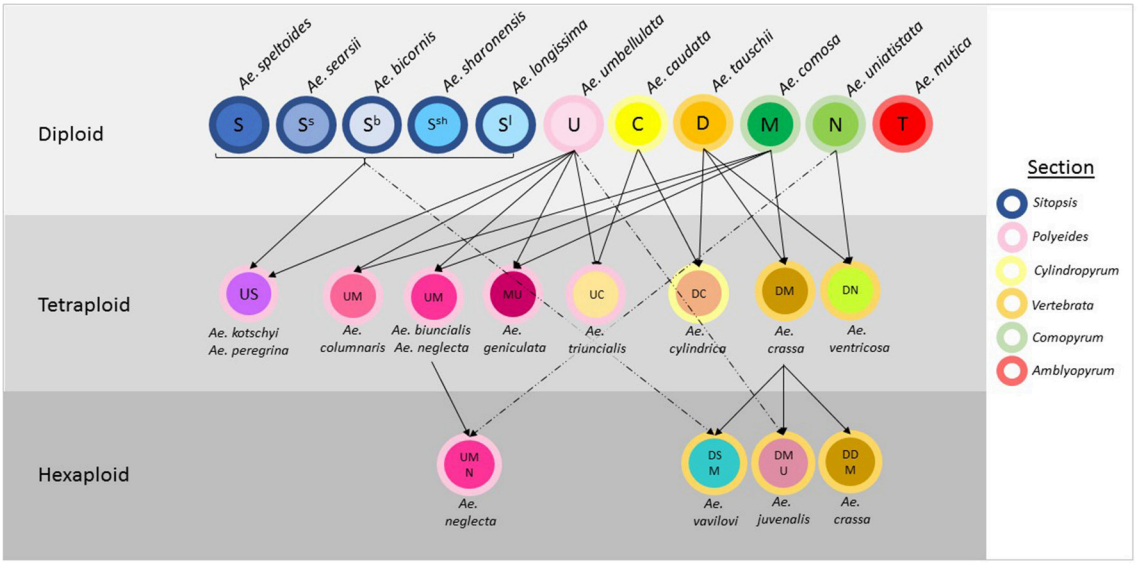

FIGURE 1 | Relationships between species in the genus Aegilops. The genome classification of each species is indicated within circles representing the species and arrows designate hybridisations between species. The color of the outline of each circle represents the section the species is allocated to.

the relative species' and wheat. Markers chosen behaved in a co-dominant manner making them potentially useful for identifying heterozygous calls in wheat-relative crosses (Allen et al., 2012). The Axiom® Wheat-Relative Genotyping Array was used to screen 278 *Aegilops* accessions using the Affymetrix GeneTitan™ system according to the procedure described by Affymetrix (Axiom®2.0 Assay Manual Workflow User Guide Rev3). Allele calling was carried out using the Affymetrix proprietary software packages Axiom Analysis Suite™. A variant Dish QC threshold of 0.75 was used instead of the default value (0.8) to account for the lower call rates typically obtained from hybridizing wheat relatives and progenitors to the array (Winfield et al., 2016).

The probes on the array are biallelic; for each locus there is a maximum of three calls possible (AA, AB, BB or 0, 1, 2) possible. The clustering pattern for each locus will depend upon the other lines that have been screened. For diploid species the clustering and genotype calling is straightforward, however screening polyploid lines is more complex. Although the intended application of the array is for specific intraspecific crosses where discrete populations are analyzed separately it is also possible to use the array to screen more diverse collections. For more complex populations or collections, a recommended approach would be to focus on particular loci of interest that produce clear clustering patterns. If necessary, it would be possible to examine the behavior of this subset of probes on the array and relate these to known genotypes. An important factor to note is whether the probe is "co-dominant" and interacts with just the genome of interest or if homeologous genomes also hybridize which will complicate the clustering pattern (Allen et al., 2012). Generally, probes that preferentially or specifically hybridize to a single genome will give higher quality clustering patterns even when screening diverse lines and users could preferentially choose these for further analysis.

For example, the accessions of DDMMUU genome have the genotype ABAAAA and BBAAAA and an accession of the DD genome has the genotype AB. For a dominant probe you would get the following calling pattern: 0 = ABAAAA, 1 = BBAAAA, 2 = AB (assuming the interaction of the MM and UU genomes also). For a co-dominant, D-genome specific probe you would get 0 = BB, 1 = AB. Introducing more different lines with different genome compositions could further complicate the clustering pattern.

Assignment of a physical map position to the SNP markers was achieved by BLAST searching the probe sequences to the International Wheat Genome Sequencing Consortium (IWGSC) whole genome assembly v1.0. For further analysis (see below) data were screened for quality using the monomorphic and call rate filtering of Axiom Analysis Suite. High quality probes taken forward for PCoA and phylogenetic analysis had a call rate of 80% or higher.

Dimensionality Reduction

A distance matrix was generated from the genotype scores using R package SNPRelate (Zheng et al., 2012). The proportion of variance for the first six eigenvalues was as follows: 26.45, 15.93, 7.21, 4.60, 4.42, 3.97. The first two eigenvalues with over 42% of the variance were plotted as a PCA plot.

Phylogenetic Analysis

Evolutionary relationships between Aegilops varieties were investigated using the genotype calls from the wheat relative genotyping array. The SNPhylo pipeline (version 20160204; Lee et al., 2014) was used to construct phylogenetic trees based on a haplotype map of 278 Aegilops varieties that was derived from

TABLE 1 | Details of germplasm used in study.

Species	Section	Genome	Ploidy	Number of accessions	Number of SNPs within species	Number of SNPs compared to *Triticum aestivum*
Aegilops mutica	*Amblyopyrum*	TT	2	12	15,617	33,816
Aegilops comosa	*Comopyrum*	MM	2	14	16,103	33,305
Aegilops uniaristata	*Comopyrum*	NN	2	6	9,190	33,120
Aegilops caudata	*Cylindropyrum*	CC	2	7	11,668	33,433
Aegilops cylindrica	*Cylindropyrum*	DDCC	4	7	13,147	30,052
Aegilops biuncialis	*Polyeides*	UUMM	4	8	13,040	33,129
Aegilops columnaris	*Polyeides*	UUMM	4	7	13,953	33,419
Aegilops geniculata	*Polyeides*	MMUU	4	5	12,412	33,772
Aegilops kotschyii	*Polyeides*	SSUU	4	8	11,658	33,024
Aegilops neglecta	*Polyeides*	UUMM/UUMMNN	4 or 6	6	17,373	33,306
Aegilops peregrina	*Polyeides*	UUSS	4	9	16,428	33,127
Aegilops triuncialis	*Polyeides*	UUCC	4	17	17,265	33,378
Aegilops umbellulata	*Polyeides*	UU	2	15	15,254	33,365
Aegilops bicornis	*Sitopsis*	SS	2	5	17,817	34,085
Aegilops longissima	*Sitopsis*	SS	2	10	15,835	33,093
Aegilops searsii	*Sitopsis*	SS	2	13	10,575	32,804
Aegilops sharonensis	*Sitopsis*	SS	2	13	18,220	33,339
Aegilops speltoides	*Sitopsis*	SS	2	38	24,524	32,401
Aegilops crassa	*Vertebrata*	DDMM/DDMMMM	4 or 6	7	11,798	30,103
Aegilops juvenalis	*Vertebrata*	DDMMUU	6	5	12,990	31,120
Aegilops tauschii	*Vertebrata*	DD	2	22	21,867	31,212
Aegilops vavilovii	*Vertebrata*	DDSSMM	6	5	13,893	30,969
Aegilops ventricosa	*Vertebrata*	DDNN	4	11	14,631	30,211

the genotype calls. The SNPhylo pipeline removed 13,253 lines of low quality data from the hapmap file, using the software's default parameters. Low quality data was defined as monomorphic, a MAF score of <0.1 or where 10% or more of the varieties had missing data. *Aegilops mutica* was assigned as an outgroup to root the tree based upon PCA results and putative reclassification to a separate genus from *Aegilops*. The hapmap file was then submitted to the SNPhylo pipeline, and a maximum likelihood tree was generated with a bootstrapping value set to 10,000. The Newick strings generated by SNPhylo were imported into the R package ggtree (version 1.2.17; Yu et al., 2017) which was used to construct a circular dendrogram.

Introgression Detection

The identification of putative introgressions was performed by comparing the genotype calls of hexaploid lines to Aegilops accessions over a 10 SNP window and calculating a percentage match. Analysis of control introgression lines indicated that a match of 40% or higher within the 10 SNP window was indicative of an introgression in the wheat background. This threshold was chosen based on the screening of known introgressions, such as 1B/1RS.

RESULTS

Diversity Within the Genus *Aegilops*

Of the 36,711 SNPs on the wheat relative array 34,602 (94.3%) were polymorphic in the entire collection of *Aegilops* accessions used in the study (**Supplementary File 2**). The SNPs fell into the following classification categories: Poly High Resolution, 18.8%; No Minor Homozygote, 10.3%; Off Target Variant, 19.3%; Mono High Resolution, 5.6%; Call Rate Below Threshold, 5.6%; Other, 40.3%. The number polymorphic within each species ranged from 9,190 (25.0%; *Ae. uniaristata*) to 24,524 (66.9%; *Ae. speltoides*) and was related to the number of accessions genotyped ($R^2 = 0.56$) (**Table 1**). This was explored further by selecting four random subsets of five accessions for each of *Ae. speltoides* and *Ae. tauschii*. The number of polymorphisms detected in the *Ae. speltoides* subsets ranged from 10,842 to 12,366 and for *Ae. tauschii* ranged from 6,555 to 11,866. These figures are comparable to the other species sampled and suggest that the number of accessions has a clear effect on the number of polymorphisms detected rather than the species in question. The number of SNPs polymorphic between each species and the wheat samples genotyped ranged from 30,052 (81.9%; *Ae. cylindrica*) to 34,085 (92.8%; *Ae. bicornis*) and there appeared to be no relationship ($R^2 = 0$) between the number of accessions genotyped and the number of polymorphisms detected when compared with wheat. There was no significant relationship between the number of polymorphic SNPs within a species compared to between the species and wheat ($R^2 = 0.0047$).

A principal component (PCoA) analysis was used to visualize the relationship between genotyped accessions (**Figure 2**; **Supplementary File 3**). In general species containing a D-genome (section Vertebrata) were distributed in discrete clusters along the PC2 axis. The diploid *Ae. tauschii* clusters

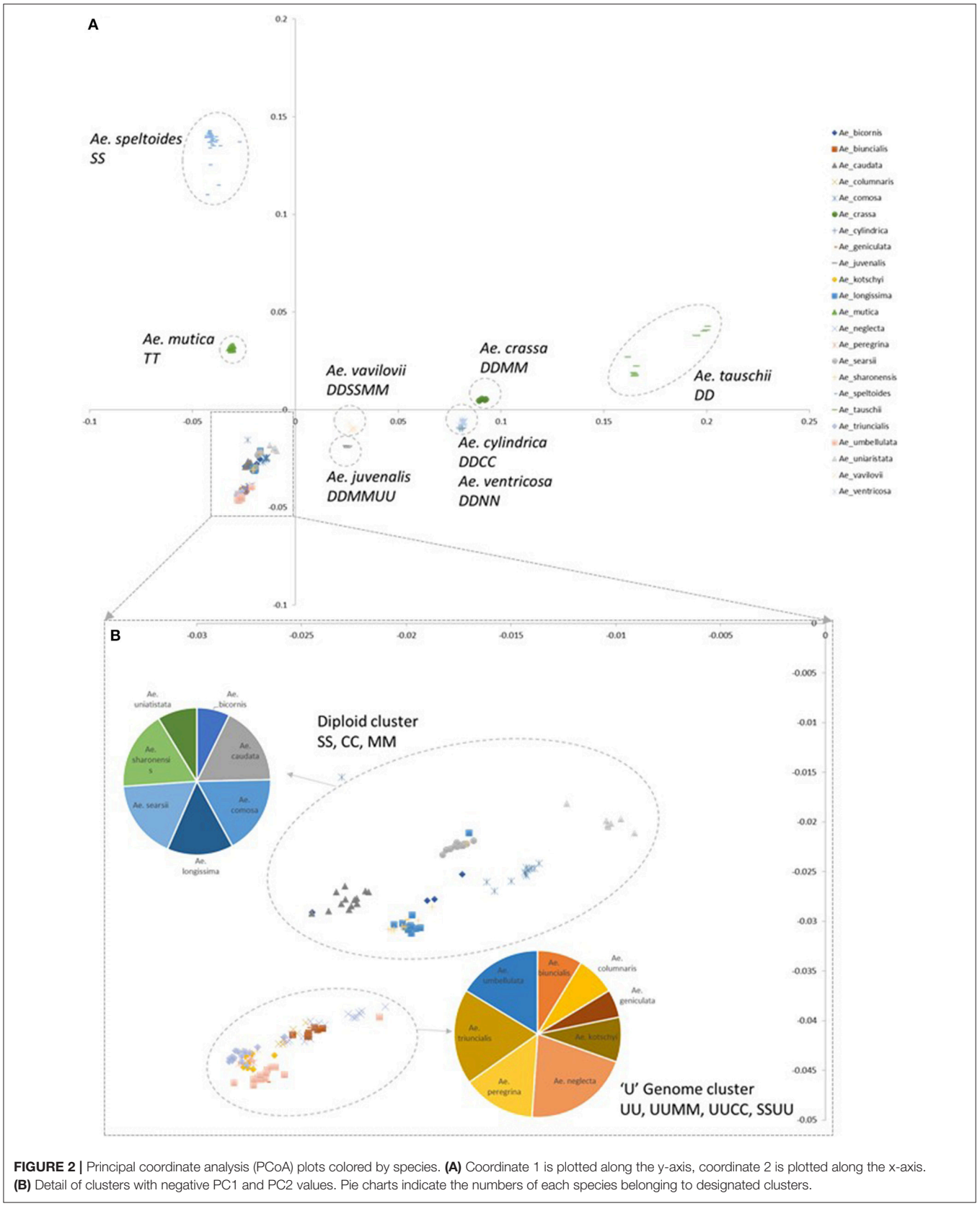

FIGURE 2 | Principal coordinate analysis (PCoA) plots colored by species. **(A)** Coordinate 1 is plotted along the y-axis, coordinate 2 is plotted along the x-axis. **(B)** Detail of clusters with negative PC1 and PC2 values. Pie charts indicate the numbers of each species belonging to designated clusters.

TABLE 2 | Genetic differentiation measured as Nei's standard genetic distance (above diagonal) and Fst measures (below diagonal) between species.

	Ae ventricosa	*Ae speltoides*	*Ae bicornis*	*Ae columnaris*	*Ae mutica*	*Ae neglecta*	*Ae biuncialis*	*Ae juvenalis*	*Ae triuncialis*	*Ae tauschii*	*Ae caudata*	*Ae umbellulata*	*Ae longissima*	*Ae geniculata*	*Ae searsii*	*Ae crassa*	*Ae uniaristata*	*Ae kotschyi*	*Ae peregrina*	*Ae comosa*	*Ae cylindrica*	*Ae vavilovii*	*Ae sharonensis*
Ae ventricosa	0	0.33	0.22	0.22	0.28	0.19	0.2	0.16	0.22	0.13	0.25	0.25	0.25	0.21	0.27	0.12	0.18	0.23	0.23	0.2	0.12	0.17	0.25
Ae speltoides	0.58	0	0.2	0.22	0.17	0.2	0.22	0.25	0.21	0.48	0.23	0.24	0.22	0.22	0.24	0.35	0.25	0.22	0.21	0.23	0.34	0.25	0.22
Ae bicornis	0.5	0.44	0	0.12	0.17	0.11	0.13	0.16	0.1	0.36	0.11	0.15	0.04	0.13	0.07	0.25	0.17	0.07	0.07	0.15	0.21	0.13	0.04
Ae columnaris	0.54	0.5	0.38	0	0.16	0.02	0.06	0.13	0.03	0.38	0.1	0.05	0.16	0.05	0.18	0.26	0.17	0.08	0.07	0.14	0.21	0.18	0.15
Ae mutica	0.58	0.44	0.44	0.48	0	0.15	0.16	0.21	0.15	0.45	0.17	0.19	0.2	0.17	0.22	0.31	0.21	0.18	0.17	0.18	0.28	0.22	0.2
Ae neglecta	0.48	0.49	0.34	0.1	0.44	0	0.03	0.11	0.04	0.37	0.11	0.05	0.15	0.03	0.17	0.24	0.11	0.07	0.06	0.1	0.2	0.16	0.14
Ae biuncialis	0.52	0.51	0.4	0.27	0.49	0.14	0	0.13	0.05	0.38	0.12	0.06	0.17	0.02	0.18	0.26	0.13	0.08	0.07	0.07	0.22	0.18	0.16
Ae juvenalis	0.44	0.52	0.42	0.4	0.51	0.36	0.41	0	0.12	0.25	0.18	0.13	0.2	0.12	0.21	0.09	0.21	0.13	0.12	0.19	0.16	0.06	0.19
Ae triuncialis	0.54	0.51	0.35	0.16	0.47	0.18	0.25	0.4	0	0.39	0.07	0.04	0.15	0.05	0.17	0.26	0.16	0.06	0.05	0.14	0.18	0.17	0.14
Ae tauschii	0.41	0.67	0.63	0.67	0.7	0.65	0.67	0.56	0.67	0	0.4	0.4	0.39	0.38	0.4	0.11	0.4	0.39	0.39	0.38	0.11	0.25	0.38
Ae caudata	0.57	0.52	0.36	0.4	0.5	0.37	0.44	0.5	0.3	0.69	0	0.14	0.18	0.13	0.19	0.29	0.18	0.14	0.13	0.15	0.16	0.2	0.17
Ae umbellulata	0.58	0.54	0.46	0.24	0.54	0.21	0.27	0.44	0.21	0.7	0.49	0	0.19	0.05	0.21	0.29	0.19	0.08	0.07	0.16	0.24	0.21	0.18
Ae longissima	0.56	0.5	0.15	0.49	0.53	0.44	0.5	0.51	0.47	0.67	0.52	0.56	0	0.17	0.1	0.27	0.21	0.08	0.07	0.18	0.26	0.16	0.01
Ae geniculata	0.52	0.5	0.39	0.25	0.49	0.12	0.11	0.4	0.23	0.67	0.45	0.23	0.5	0	0.18	0.26	0.14	0.08	0.07	0.09	0.22	0.18	0.16
Ae searsii	0.6	0.54	0.3	0.56	0.59	0.49	0.56	0.56	0.52	0.71	0.58	0.61	0.41	0.57	0	0.29	0.22	0.14	0.13	0.2	0.27	0.13	0.1
Ae crassa	0.38	0.59	0.52	0.59	0.61	0.54	0.59	0.32	0.58	0.37	0.62	0.64	0.6	0.59	0.65	0	0.29	0.27	0.26	0.27	0.11	0.09	0.27
Ae uniaristata	0.49	0.54	0.49	0.53	0.56	0.39	0.47	0.54	0.5	0.7	0.56	0.58	0.58	0.5	0.64	0.65	0	0.18	0.18	0.12	0.26	0.22	0.2
Ae kotschyi	0.56	0.51	0.28	0.33	0.52	0.28	0.35	0.43	0.28	0.69	0.49	0.35	0.33	0.34	0.51	0.61	0.57	0	0.02	0.16	0.23	0.16	0.07
Ae peregrina	0.54	0.5	0.26	0.28	0.49	0.25	0.3	0.4	0.23	0.67	0.44	0.29	0.29	0.29	0.47	0.58	0.52	0.09	0	0.15	0.22	0.15	0.06
Ae comosa	0.51	0.51	0.41	0.45	0.51	0.34	0.28	0.49	0.44	0.67	0.48	0.51	0.51	0.34	0.57	0.59	0.43	0.5	0.46	0	0.24	0.19	0.18
Ae cylindrica	0.37	0.58	0.48	0.52	0.58	0.49	0.54	0.43	0.5	0.38	0.48	0.58	0.57	0.54	0.62	0.36	0.6	0.57	0.54	0.55	0	0.17	0.25
Ae vavilovii	0.45	0.51	0.36	0.48	0.52	0.44	0.49	0.23	0.48	0.56	0.52	0.54	0.45	0.48	0.44	0.31	0.55	0.47	0.45	0.5	0.45	0	0.15
Ae sharonensis	0.56	0.5	0.14	0.48	0.53	0.43	0.49	0.5	0.45	0.67	0.52	0.54	0.04	0.49	0.41	0.59	0.57	0.31	0.27	0.51	0.57	0.45	0

are located furthest from other *Aegilops* species, the tetraploid D-genome species *Ae. crassa, Ae. cylindrica*, and *Ae. ventricosa* mid-way along the axis and the hexaploid *Ae. vavilovii* and *Ae. juvenalis* closest to other *Aegilops* species. *Aegilops tauschii* formed two discrete clusters reflecting the two separate gene pools sampled in the accessions (Dvorak et al., 1998b; Mizuno et al., 2010; Jones et al., 2013). Along the PC1 axis the bread wheat B-genome progenitor species *Ae. speltoides* clustered furthest from other *Aegilops* species and the other samples clustering with positive PC1 values belong to *Ae. mutica*. The remaining samples cluster more closely with negative PC1 and PC2 values. They are split into two locations; one consisting of diploid S, C, M, and N genome species; the other of diploid and tetraploid U genome containing species (section *Polyeides*).

A comparison of genetic differentiation and Fst between species (**Table 2**) revealed relationships between species with *Ae. tauschii* in particular showing a high degree of differentiation to most other species (except the related *Ae. crassa, Ae. ventricosa,* and *Ae. cylindrica*). Diploid species of different genome classifications showed a high degree of differentiation to each other but more similarity to polyploid species from the same section (e.g., *Ae. umbellulata* and polyploid species in section *Polyeides*) or containing a common genome (e.g., U-genome containing tetraploids). Other relationships are also revealed; the *Sitopsis* species showing greatest similarity to the

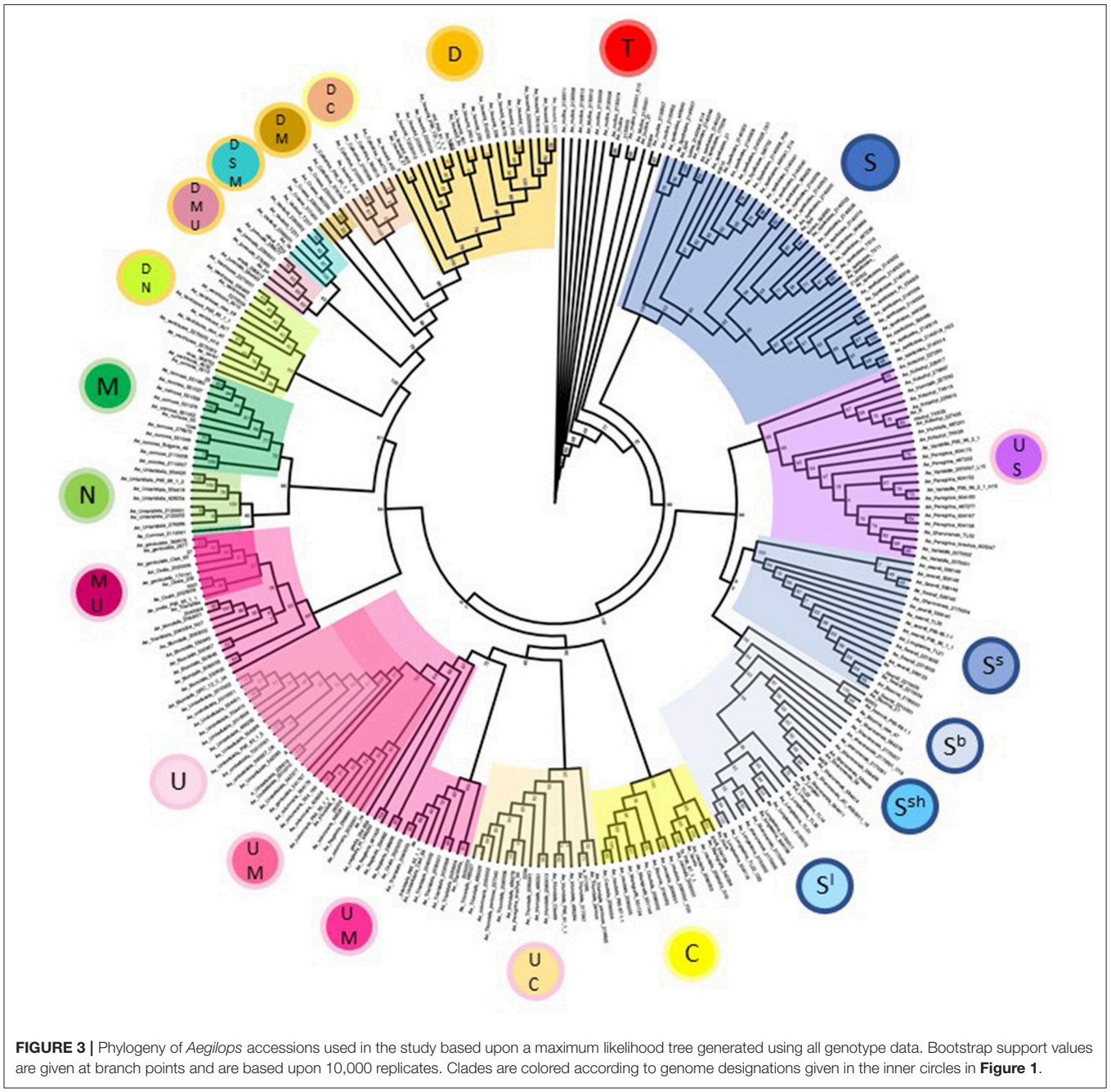

FIGURE 3 | Phylogeny of *Aegilops* accessions used in the study based upon a maximum likelihood tree generated using all genotype data. Bootstrap support values are given at branch points and are based upon 10,000 replicates. Clades are colored according to genome designations given in the inner circles in **Figure 1**.

S-genome of *Ae. kotschyi* and *Ae. peregrina* is *Ae. sharonensis*, while the UUMM tetraploid showing greatest similarity (Fst) to *Ae. juvenalis* (DDMMUU) is *Ae. geniculata* suggesting evolutionary relationships between these species.

Phylogenetic Relationships Within the Genus *Aegilops*

Phylogenetic analysis of *Aegilops* species used in the study broadly reflects the picture seen in the principal component analysis but allowed us to examine relationships between species and accessions in greater detail (**Figure 3**). All major branches had good bootstrap support with values over 80%. *Aegilops speltoides* accessions formed a separate clade to other S-genome species in the study at the base of the tree. Other S-genome containing species grouped together with tetraploids *Ae. kotschyi* and *Ae. peregrina* (UUSS) forming a separate clade to diploids *Ae. searsii, Ae. sharonensis, Ae. bicornis,* and *Ae. longissima.* The C-genome containing species *Ae. caudata* (diploid, CC) and *Ae. triuncialis* (tetraploid, UUCC) form two neighboring subclades of the clade also containing the U-genome containing diploid *Ae. umbellulata* (UU) and tetraploids *Ae. columnaris* and *Ae. neglecta* (tetraploid, UUMM). Interestingly, other UUMM tetraploids *Ae. geniculata, Ae. biuncialis,* and *Ae. ovata (syn. Ae. neglecta*, The Plant List) formed a separate clade to these,

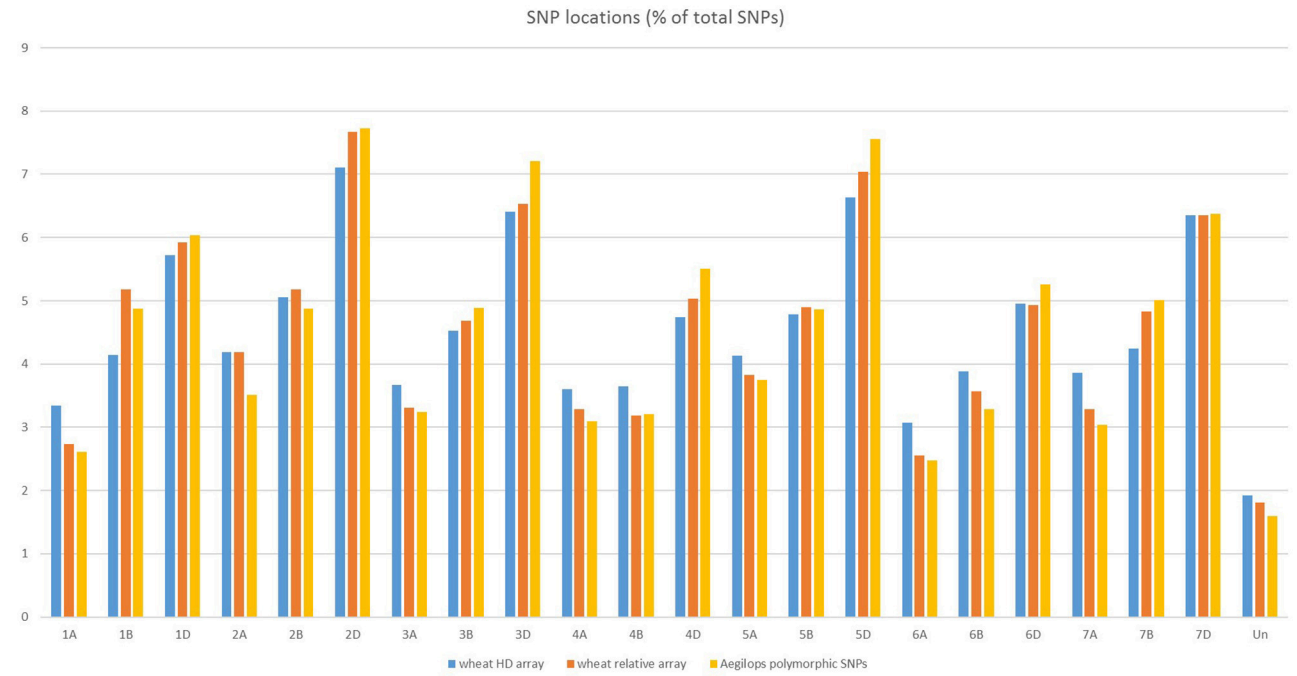

FIGURE 4 | Chromosome locations of SNPs on the HD array, the wheat relative array and SNPs that are polymorphic between members of the genus *Aegilops* and *Triticum aestivum* from the wheat relative array.

indicating diversity present in this group both within and between species. The diploid species *Ae. comosa* (MM) and *Ae. uniaristata* (NN) form neighboring subclades reflecting genetic similarity between these species. Finally, D-genome containing species were organized in a series of distinct subclades, including hexaploids *Ae. vavilovii* (DDSSMM) and *Ae. juvenalis* (DDMMUU); tetraploids *Ae. crassa* (DDMM), *Ae. cylindrica* (DDMM), and *Ae. ventricosa* (DDNN) and the diploid *Ae. tauschii* (DD). The *Ae. tauschii* subclade splits into the two distinct clusters also seen in the principal component analysis and relates to the geographic origin of the accessions selected (Dvorak et al., 1998b; Mizuno et al., 2010).

Detecting *Aegilops* Introgressions in Wheat

The SNPs on the wheat relative array were assigned a putative chromosomal map location in wheat based upon BLAST alignment of the probe sequence surrounding each SNP against the Chinese Spring v 1.0 genome assembly (Winfield et al., 2016; The International Wheat Genome Sequencing Consortium (IWGSC), 2018). Over 98% of SNPs (36,009) on the wheat relative array were assigned a chromosome location (**Supplementary File 4**). The proportion of chromosome assignments were compared to the larger wheat HD array and calculated separately for SNPs that were polymorphic between the *Aegilops* and wheat on the wheat array (**Figure 4**). All three datasets had the highest proportion of SNPs located in the D-genome, then B genome, with the lowest proportions assigned to the A-genome. This was seen most dramatically in the set which were polymorphic between *Aegilops* and wheat (A genome 21.7%; B genome 31.0%; D genome 45.7%). The distribution of SNPs along chromosomes (**Figure 5**) demonstrated the higher proportion of SNPs in the D genome and also revealed a bias of SNP distribution toward the telomeres, as has been previously reported for exome based SNPs.

The SNP collection on the wheat relative array was employed in two different projects to identify introgressed segments of *Aegilops sp.* in a hexaploid wheat background. These projects took two different approaches to introducing the introgressions and in identifying the markers to track the introgressions. The first study used a specific accession of *Ae. sharonensis* to produce recombinant plants resistant to African Stem Rust. This project used the Axiom wheat HD array to identify and track introgressed regions by comparing the recombinant line and the *Aegilops sharonensis* genotype over 10 SNP windows (Millet et al., 2017). In this study we have shown that this analysis can be repeated using the subset of SNPs on the wheat relative array (**Figure 6**). The second project introgressed *Ae. speltoides* into a hexaploid wheat background with the aim of generating a population where individuals contained specific introgressed segments, which together represent the majority of the *Ae. speltoides* genome (King et al., 2017a). The approach taken to achieve this was to generate a genetic map containing 22,258 polymorphic SNPs (60.6% of total SNPs on the array) and refined this to 544 high quality framework markers. This map was used to inform and identify introgressed segments across individuals from five backcrossed populations, which were then confirmed by genomic *in situ* hybridization (GISH). A high frequency of introgressions were identified and it was possible to track these

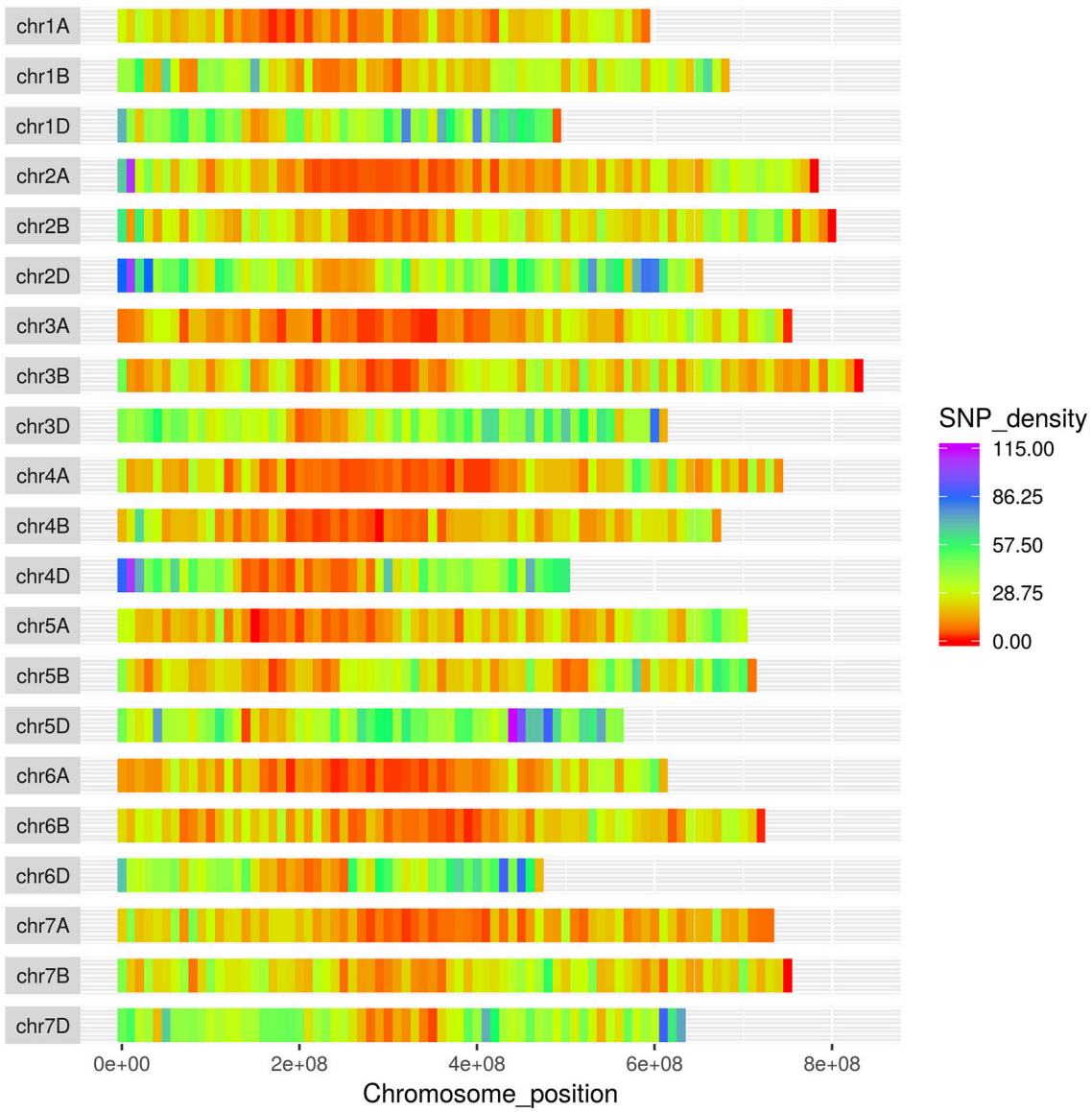

FIGURE 5 | Distribution of SNPs from the Axiom® Wheat-Relative Genotyping Array in the wheat genome. The x-axis represents the physical distance along each chromosome, split into 10 Mbp windows.

through the back-crossing process using markers from the wheat relative array.

DISCUSSION

This study describes the development and application of the first high-throughput genotyping platform specifically designed for screening wheat relative species. The Axiom® Wheat-Relative Genotyping Array contains a framework of over 36,000 SNP markers selected to be useful when screening a diverse range of species with a variety of genome structure and ploidy levels. The platform was used in this study to perform the largest screen of the genus *Aegilops* to date with multiple accessions representing all species in the genus. The array data was demonstrated to be useful for screening diversity within and between *Aegilops* species and was able to be used for examining relationships within the genus. Furthermore, the data was used to identify and track introgressions from *Aegilops* species in hexaploid wheat using two different approaches.

Over 94% of the SNPs on the array detected a polymorphism between *Aegilops* species. The average number of polymorphic SNPs within a species was 14,231 (36.8%) although the data suggested that when higher numbers of accessions were screened the number of polymorphic SNPs also increased. In the cases of *Ae. tauschii* (22) and *Ae. speltoides* (38) the number of polymorphisms detected doubled compared to random sets of five accessions. This suggests there is a high level of genetic diversity present in these species and careful selection of

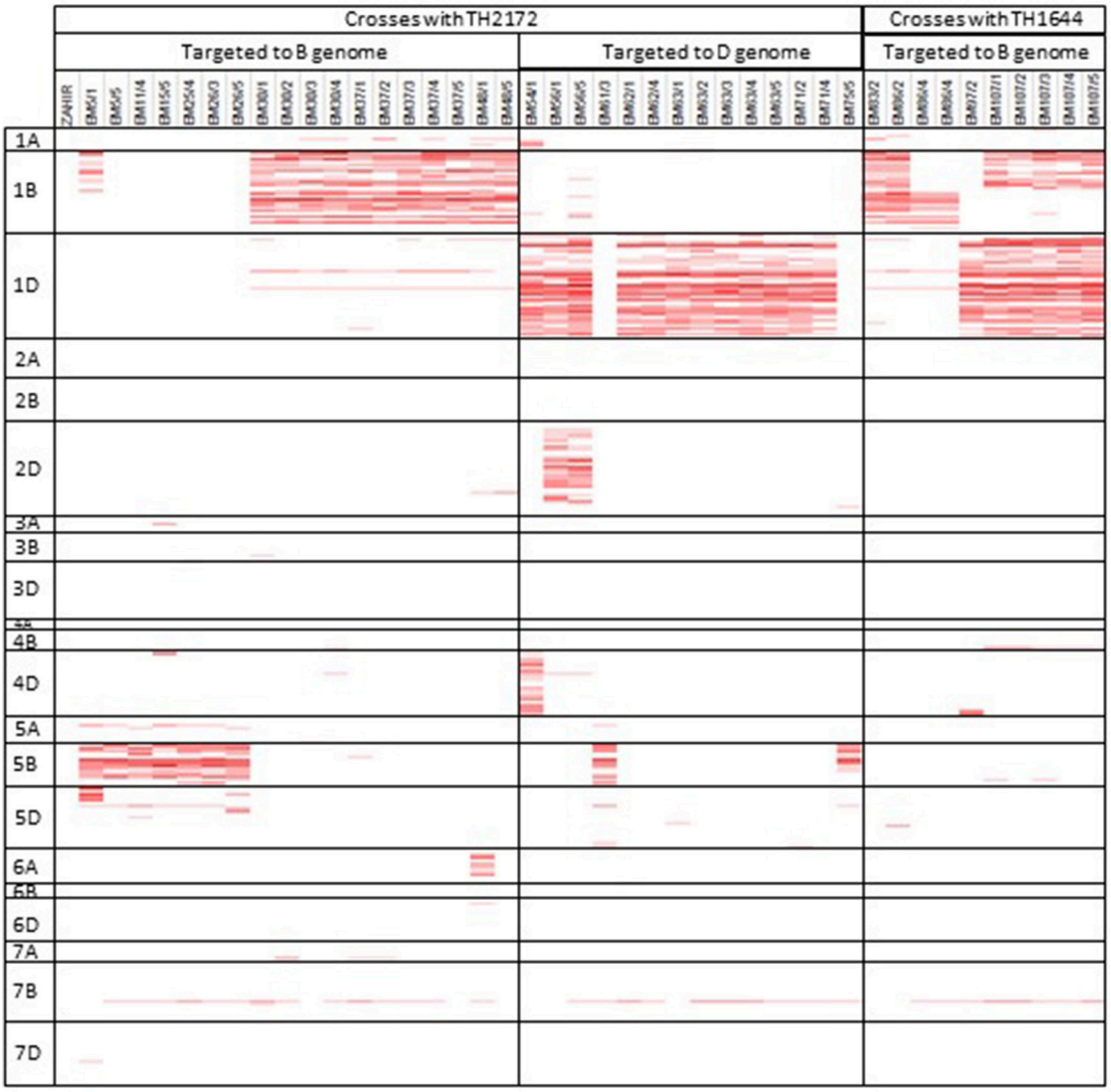

FIGURE 6 | Identification of *Aegilops sharonensis* introgression segments in 41 recombinant wheat lines resistant to African *Puccinia graminis* f. sp. *Tritici*. Genotype calls from the array were compared to *Ae. sharonensis* over a 10 SNP window and a score of over 40% is considered indicative of introgressed material and is highlighted in red.

accessions will aid in sampling the variation present. The data revealed phylogenetic relationships between species in the genus *Aegilops*. As has been previously reported these data supported *Ae. speltoides* as the most basal species in the genus (Yamane and Kawahara, 2005). *Ae. speltoides* clustered separately to all other members of the Sitopsis section in both the PCA and phylogenetic analysis where it consistently was located at the base of the tree (99% bootstrap value).

The tetraploid U-genome containing species clustered closely on the PCA plot (**Figure 2**) and showed genetic similarity (**Table 2**). However, these species were clearly separated in the phylogenetic analysis where UUSS species *Ae. kotschyi* and *Ae. peregrina* are in a clade also containing S-genome diploids, whilst UUMM tetraploids located to the same clade as U-genome parent *Ae. umbellulata*. *Ae. triuncialis* (UUCC) located to a subclade between its two parental species *Ae. caudata* and *Ae. umbellulata*. Previous studies of the origin and evolution of polyploid *Aegilops* showed that the genomes of some species are very similar to those of the diploid progenitors, while other species are more modified (Kihara, 1954; Molnár et al., 2016). One theory of the process behind intraspecific divergence is that extinct species were the source of modified genomes or alternatively they were significantly rearranged during evolution. A third hypothesis is that the rate of parental genome modification in polyploids is different with one genome remaining similar to the parental genome (pivotal genome) and the second (differential genome) undergoing modification by complete or segmental chromosome substitutions (Zohary and Feldman, 1962; Kimber and Feldman, 1987; Badaeva et al., 2004). Two pivotal genomes (D and U) have been identified in

Aegilops where genomes in all related species were similar to each other and to that of the parental diploid species, whereas the second genomes were modified compared to the original. Our data suggest the pivotal genome in polyploid UUMM species' *Ae. columnaris, Ae. biuncialis, Ae. neglecta* and *Ae. geniculata* is the U-genome, while *Ae. triuncialis* is found to be similar to both of its parental species, suggesting the C-genome has not diverged significantly as has been previously observed (Badaeva et al., 2004). However, *Ae. kotschyi* and *Ae. peregrina* cluster more closely with S-genome species suggesting that the U-genome in this instance is not pivotal.

Within the UUMM polyploid clade the four related tetraploid species are not organized into discrete sub-clades with and some mixing of species is seen within this clade and, where branching occurs, bootstrap support is not always high. This may suggest a polyphyletic origin of these species with different sources of parental genomes (one accession of *Ae. umbellulata* falls into a subclade of *Ae. neglecta*). Alternatively, this may indicate introgressive hybridization and gene flow between these genetically similar species. With multiple possible sources of the S-genome in *Ae. kotschyi* and *Ae. peregrina* the genetic diversity scores were compared and revealed the closest species to both were *Ae. sharonensis* and *Ae. longissima* as has previously been observed (Badaeva et al., 2004). When observed in the tree this result is explained by an *Ae. sharonensis* accession falling into the *Ae. peregrina* clade, a result which requires further investigation. Map locations of the SNPs on the Wheat-Relative array revealed a greater proportion were located on the D-genome than the A- or B-genomes. This contrasts with the usual results obtained when mapping polymorphisms in bread wheat, which are usually lacking in D-genome markers and reflects untapped diversity in D-genome relatives (Allen et al., 2016). The high level of intraspecific diversity present within *Ae. tauschii* and between this species and all others in the genus (except close relatives *Ae. crassa* and *Ae.* cylindrica) was observed in the PCoA analysis and high genetic diversity measures obtained (**Figure 2**; **Table 2**).

All *Aegilops* species screened had a high proportion of polymorphic SNPs when compared to bread wheat (average 88.8%) indicating a high level of potentially useful markers for detecting introgressions in a hexaploid wheat background. The specific number of polymorphic SNPs for a cross would depend upon the accession chosen and the wheat cultivar used. Although the array has been demonstrated to be useful in detecting diversity and relationships between a large collection of species, the real power and intended application of this tool is for specific intraspecific crosses where discrete populations are analyzed separately. In two specific case studies we have shown that the array may be applied to detecting and tracking *Aegilops* introgressions by two different strategies; in both cases multiple introgressions were detected. In the *Aegilops speltoides* project a high number of introgressions were detected and confirmed via GISH and additionally a gametocidal gene was detected (King et al., 2017a). In the *Ae. sharonensis* project recombinant chromosomes were identified and the gene conferring resistance to Ug99 group races was located on chromosome 1B, between 280 and 650 Mbp (Millet et al., 2017). The wheat-relative array has also been used in a third introgression project to screen wheat /*Am. muticum* (*syn. Ae. mutica*) recombinant chromosomes. The array enabled the identification and characterization of genome wide introgressions of various sizes (from large to very small) (King et al., 2017b).

The utility of the wheat relative genotyping array has been demonstrated to be effective in detecting both intra- and inter-specific diversity with insights into the structure and relationships within the genus *Aegilops*. The array data has been shown to be effective in identifying introgressed regions of *Aegilops* in a hexaploid bread wheat background. The application of the array extends beyond *Aegilops* and *Triticum,* having been designed for a wide range of species including those outside of these two genera. By linking with additional resources, such as the annotated genome sequence for wheat (The International Wheat Genome Sequencing Consortium (IWGSC), 2018) and with the development of technologies that enable targeted and precise introgression the potential gain from introducing selected beneficial genes from wild relatives of wheat is an exciting and increasingly feasible prospect.

AUTHOR CONTRIBUTIONS

AP-A analyzed the data and wrote the manuscript. AB, DS, and LM prepared samples. AB and DS performed genotyping. AB performed PCoA. PW performed phylogenetic analysis. IK, JK, KE, and GB developed the genotyping platform. All authors discussed the results and contributed to the final manuscript.

ACKNOWLEDGMENTS

We are grateful to the Germplasm Resources Unit (GRU) for providing many of the accessions used in this paper and to the Biotechnology and Biological Sciences Research Council, UK for funding this work (award BB/N021061/1, BBS/E/J/000PR9781).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01993/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 25 January 2019
doi: 10.3389/fpls.2019.00009

Genomic Analysis Confirms Population Structure and Identifies Inter-Lineage Hybrids in *Aegilops tauschii*

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Aegilops tauschii, the D-genome donor of bread wheat, *Triticum aestivum*, is a storehouse of genetic diversity, and an important resource for future wheat improvement. Genomic and population analysis of 549 *Ae. tauschii* and 103 wheat accessions was performed by using 13,135 high quality SNPs. Population structure, principal component, and cluster analysis confirmed the differentiation of *Ae. tauschii* into two lineages; lineage 1 (L1) and lineage 2 (L2), the latter being the wheat D-genome donor. Lineage L1 contributes only 2.7% of the total introgression from *Ae. tauschii* for a set of United States winter wheat lines, confirming the great amount of untapped genetic diversity in L1. Lineage L2 accessions had overall greater allelic diversity and wheat accessions had the least allelic diversity. Both lineages also showed intra-lineage differentiation with L1 being driven by longitudinal gradient and L2 differentiated by altitude. There has previously been little reported on natural hybridization between L1 and L2. We found nine putative inter-lineage hybrids in the population structure analysis, each containing numerous lineage-specific private alleles from both lineages. One hybrid was confirmed as a recombinant inbred between the two lineages, likely artificially post collection. Of the remaining eight putative hybrids, a group of seven from Georgia carry 713 SNPs with private alleles, which points to the possibility of a novel L1–L2 hybrid lineage. To facilitate the use of *Ae. tauschii* in wheat improvement, a MiniCore consisting of 29 L1 and 11 L2 accessions, has been developed based on genotypic, phenotypic and geographical data. MiniCore reduces the collection size by over 10-fold and captures 84% of the total allelic diversity in the whole collection.

Keywords: ***Aegilops tauschii*, genotyping-by-sequencing, inter-lineage hybrid, population structure, single nucleotide polymorphism, *Triticum aestivum***

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *30 August 2018*
Accepted: *07 January 2019*
Published: *25 January 2019*

Citation:
Singh N, Wu S, Tiwari V, Sehgal S, Raupp J, Wilson D, Abbasov M, Gill B and Poland J (2019) Genomic Analysis Confirms Population Structure and Identifies Inter-Lineage Hybrids in Aegilops tauschii. Front. Plant Sci. 10:9. doi: 10.3389/fpls.2019.00009

INTRODUCTION

World population is projected to reach 9.7 billion by 2050, increasing pressure on the food system and challenging food security (Fao et al., 2014). Wheat, among other major food crops, is currently at an estimated genetic gain of 1% per year. This must more than double to achieve the estimated 2.4% per year to meet the projected production levels needed to provide enough calories and

protein to the billions around the world in the coming decades (Ray et al., 2013). However, limited genetic diversity present in the elite wheat cultivars pose a serious threat to this goal (Akhunov et al., 2010). To mitigate this genetic diversity problem, use of crop wild relatives and progenitors, such as goat grass (*Ae. tauschii* Coss.), presents a promising solution and the best resource.

Aegilops tauschii originated as the result of hybridization between diploid A and B genome progenitors (Marcussen et al., 2014), and became the diploid D-genome donor of bread wheat (*Triticum aestivum* L.). *Ae. tauschii* is native throughout the Caspian Sea region and into central Asia and China. Natural hybridization of tetraploid wheat and *Ae. tauschii* about 8,000–10,000 years ago (Renfrew, 1973; Bell, 1987) led to the formation of hexaploid wheat with *Ae. tauschii* contributing many genes that expanded the climatic adaption and improved bread making quality (Kihara, 1944; McFadden and Sears, 1946; Yamashita et al., 1957; Kerber and Tipples, 1969; Lagudah et al., 1991). However, during bread wheat evolution, only a handful of *Ae. tauschii* accessions from a small region hybridized with wheat leading to a narrow genetic base of the wheat D genome (Lagudah et al., 1991). Multiple studies have corroborated this, showing that the D-genome of wheat has the least genetic diversity as compared to its counterparts, A and B genomes (Kam-Morgan et al., 1989; Lubbers et al., 1991; Akhunov et al., 2010). However, much greater genetic diversity is present in this wild donor of the D-genome (Naghavi et al., 2009).

With a pressing need to develop better yielding wheat varieties to feed a growing population and adapt to a changing climate, *Ae. tauschii* is a valuable source of novel alleles for wheat improvement (Kihara, 1944; Lagudah et al., 1991). *Aegilops tauschii* harbors considerable genetic diversity for diseases and abiotic factors relative to the wheat D-genome, and is split into two subspecies known as *Ae. tauschii* ssp. *tauschii* (Lineage 1; L1) and ssp. *strangulata* (Lineage 2; L2). The L2 ssp. *strangulata* is known to be the D-genome donor (Jaaska, 1978; Nakai, 1979; Nishikawa et al., 1980; Jaaska, 1981). Ssp. *tauschii* is further split into three varieties- *typica*, *anathera,* and *meyeri*, whereas ssp. *strangulata* is monotypic. Phenotypic classification of these subspecies, especially to varieties, is challenging. Therefore phenotypic data often poorly correlate with genetic classification (Lubbers et al., 1991; Dvorak et al., 1998).

Genetic diversity present in *Ae. tauschii* has been utilized via synthetic hybridization of tetraploid wheat and wild *Ae. tauschii* (McFadden and Sears, 1945; Kihara and Lilienfeld, 1949), and introgressed to bread wheat through direct crossing (Gill and Raupp, 1987). However, considerable amounts of untapped genetic diversity remain present in this species. In this study, we characterized the full *Ae. tauschii* collection held at Wheat Genetics Resource Center at Kansas State University in Manhattan, KS, United States with the main objectives to genetically characterize the *Ae. tauschii* collection, study the population structure within *Ae. tauschii*, and develop a genetically diverse MiniCore set to facilitate the use of *Ae. tauschii* for wheat improvement. In conclusion, we present a strategy to utilize the genetic diversity from *Ae. tauschii* to broaden the genetic base of D-genome of hexaploid wheat.

MATERIALS AND METHODS

Plant Material

This study included 569 *Ae. tauschii* accessions from Wheat Genetics Resource Center (WGRC) at Kansas State University (K-State) in Manhattan, KS, United States. Most of the *Ae. tauschii* accessions were collected in 1950s and 1960s from 15 different countries by several explorers, however, a recent exploration was carried out by WGRC scientists in 2012 in Azerbaijan to fill the geographical gaps in the collection and sample more genetic diversity (**Supplementary Figure S1** and **Supplementary Table S1**). Passport data, including longitude and latitude of the collection site, were available for most of the accessions and were plotted on the map to visualize the distribution (**Figure 1**). To study the relationship between *Ae. tauschii* and hexaploid wheat (*T. aestivum* L.), 103 wheat varieties from a panel of diverse United States winter wheat accessions were also included in the study (Grogan et al., 2016) (**Supplementary Table S1**).

Plant Tissue Collection and Genotyping-by-Sequencing

A single plant for each accession was grown in $2'' \times 2''$ pots in the greenhouse. About five centimeter of leaf tissue from single 2–3 weeks old seedlings were collected in 96-well tissue collection box and stored at −80°C until DNA extraction. Tissues were lyophilized in the lab for 24–36 h, followed by genomic DNA extraction using Qiagen BioSprint 96 DNA Plant Kit (QIAGEN, Hilden, Germany). Extracted DNA was quantified with Quant-iT^{TM} PicoGreen® dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, United States). One random well per plate was left blank for quality control and library integrity. DNA samples were genotyping using genotyping-by-sequencing (GBS) (Poland et al., 2012a). GBS libraries were prepared in 96 plexing using two restriction enzymes—a rare cutter *PstI* (5′-CTGCAG-3′), and a frequent cutter *MspI* (5′-CCGG-3′) with a common reverse adapter ligated. Full protocol is available at the KSU Wheat Genetics website[1]. GBS libraries were sequenced on 10 lanes on Illumina HiSeq2000 (Illumina, San Diego, CA, United States) platform at University of Missouri (UMC; Columbia, Missouri) or McGill University-Génome Quebec Innovation Centre (Montreal, Canada) facility.

SNP Genotyping and Data Filtering

Single nucleotide polymorphisms (SNPs) discovery and genotyping was performed in single step with Tassel 5 GBSv2 pipeline (Glaubitz et al., 2014), using *Ae. tauschii* genome assembly (Aet v4.0; NCBI BioProject PRJNA341983) as the reference. Tassel was run with *bowtie2* aligner for tags mapping in Linux HPC environment via shell script. Genotypic data were processed in R statistical programming language (R Core Team, 2015) using custom R scripts. Population level SNP filtering was performed and SNPs with minor allele frequency (MAF)

[1]http://wheatgenetics.org/download/send/3-protocols/74-gbs-protocol

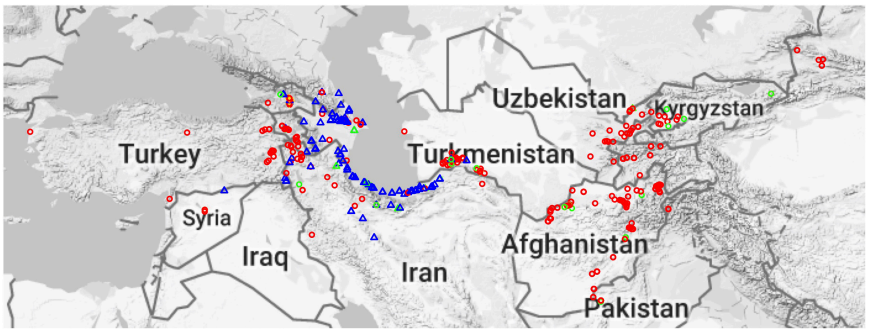

FIGURE 1 | Geographical distribution of *Aegilops tauschii* accessions. Red circles represent Lineage 1 (L1), blue triangles Lineage 2 (L2), and gold plus sign (+) are putative hybrids. Green circles and triangles represent MiniCore accessions, and their shapes represent their lineage.

less than 0.01 and missing data more than 20% were removed. Further, SNPs with heterozygosity greater than 5% were removed because *Ae. tauschii* accessions are highly inbred. Fisher's exact test at alpha 0.001 with Bonferroni correction was performed to determine if the putative SNPs were from allelic tags as described in Poland et al. (2012b). Individual samples with more than 80% missing SNP calls and more than 5% heterozygosity were also removed. Retained markers and samples were used for further analyses.

Population Structure and Ancestry Analysis

Population structure and ancestry analysis was performed with fastSTRUCTURE software (Raj et al., 2014), cluster analysis, and principal component analysis (PCA). Initially, fastSTRUCTURE was run with all filtered SNPs at $K = 2$ using 'simple' prior to partition all *Ae. tauschii* accessions into L1 and L2 lineages. Per the developer recommendation for computational efficiency, fastSTRUCTURE was run with 'simple' prior and random seed for $K = 2$ to $K = 8$ with three replications each to detect the optimum values of K. Once the optimum K was determined, final fastSTRUCTURE analysis was performed using 'logistic' prior with all the SNPs. Only those accessions with available passport information were used in this analysis, and passport information was used to group and order accessions. To ensure the label collinearity for multiple iterations of each K run, fastSTRUCTURE results were processed using *CLUMPAK* package (Jakobsson and Rosenberg, 2007; Kopelman et al., 2015) and plotted using *Distruct* program (Rosenberg, 2004). Optimal K-value was determined using 'chooseK' utility provided with fastSTRUCTURE.

Phylogenetic cluster analysis was performed in R language. Genetic distances were computed using 'dist' function with Euclidean method. Distance matrix was converted to a phylo object using 'ape' package (Paradis et al., 2004). Using 'phyclust' package (Chen, 2011), a neighbor joining unrooted tree was plotted to indicate subpopulation clusters and identify tentative cryptic outliers that were not identified phenotypically. Cluster analysis was performed using default parameters in 'dist,' 'ape,' and 'phyclust.'

Principal component analysis was performed in R language. Eigenvalues and eigenvectors were computed with 'e' function using 'A' matrix output of rrBLUP package (Endelman, 2011). First three eigenvectors were plotted as three principal components to observe clustering. All analyses were performed separately for *Ae. tauschii* only to detect subpopulation, and with wheat to study the wheat-*Ae. tauschii* relationship. L1 and L2 accessions were identified from fastSTRUCTURE partitioning of two lineages at $K = 2$ and projected onto the PCA. To find the best variables explaining the differentiation within lineages, correlation coefficients were computed for PC2 and PC3 vs. longitude, latitude and altitude.

Genetic Diversity Analysis

As a measure of average heterozygosity over multiple SNPs in a given population, Nei's diversity index (Nei, 1973) was computed for the whole population, and separately for L1, L2, wheat, and combined for L1 and L2. Additionally, pairwise F_{ST} between subpopulations, and lineage wise minor allele frequency (MAF) were computed and plotted using custom R scripts. Pairwise F_{ST} were computed among L1, L2, and wheat in all combinations. MAF plots were plotted separately for L1 and L2.

Lineage-Specific Allelic Contribution to Putative L1–L2 Hybrids and Wheat D-Genome

Lineage specific private alleles are the ones that are segregating in one lineage but fixed in the other. To determine a lineage specific allele at a SNP site, dataset was split into L1 and L2 accessions. SNP sites where MAF was zero in one lineage but greater than zero in the other lineage, were filtered and the segregating lineage specific allele identified. L1 and L2 private alleles were assigned different colors and plotted for each putative hybrid separately. For each hybrid, lineage specific contribution was determined as percentage of alleles contributed by specific lineage. Using private allele SNPs, allele matching was performed as described in Singh et al. (2019) to find the putative parents of each hybrid from both L1 and L2. For wheat D-genome, a consensus of lineage specific alleles was determined, and lineage specific alleles were plotted across all wheat D-genome chromosomes. For those SNP sites,

where more than one wheat lines had L1 specific allele, it was considered as a putative introgression from L1. Lineage specific contribution was determined as percentage of alleles contributed by specific lineage across the consensus.

Genetically Diverse Representative Core-Set Selection

All SNPs were used to select a representative core-set from the *Ae. tauschii* collection. The core-set was selected in two steps. First, software package PowerCore was used with default settings (Kim et al., 2007), which selects the lines to retain most diverse alleles by implementing advanced M (maximization) strategy. Then the number of selected accessions was further reduced by phenotypically guided selection using the available phenotypic data for Leaf rust composite, Stem rust race TTKSK (Rouse et al., 2011) and Hessian fly biotype D resistance. The diversity captured by the MiniCore was assessed by the percent segregating SNPs present in the selected accessions relative to the whole collection.

RESULTS

Geographical Distribution of *Ae. tauschii*

Aegilops tauschii is mainly found around the Caspian Sea and in central Asia but is found as far West as Turkey (Lon: 26.327362, Lat: 40.009735) and as far East as eastern China (Lon: 111.048058, Lat: 34.059486). Geographical origin data was known for most of the accessions (**Figure 1**). The majority of the accessions come from Afghanistan, Iran and Azerbaijan (**Supplementary Figure S2**). L1 is spread across the entire *Ae. tauschii* geographical range, whereas L2 is only present in Transcaucasia and around the Caspian Sea region (**Figure 1**). However, we did find one L2 accession in Uzbekistan, which is the first report of an L2 accession out of their natural habitat.

Genomic Profiling

Genotyping-by-sequencing (GBS) generated 318,639 putative single nucleotide polymorphisms (SNPs) from a total of 672 samples consisting of 569 *Ae. tauschii* and 103 wheat lines. Filtering the SNPs based on missing data, MAF, heterozygosity, and Fisher's exact test resulted in 13,582 SNPs. Additionally, poor samples were removed based on the amount of missing data and heterozygosity. Twenty *Ae. tauschii* samples with more than 80% missing SNP calls and 5% heterozygosity were removed, which resulted in a dataset of 13,582 SNPs for 652 samples consisting of 549 *Ae. tauschii* and 103 wheat samples. Finally, after removing 447 SNPs that were private to wheat, a total of 13,135 high quality SNPs were retained and used for further analyses.

Population Structure Analysis

All SNPs were used to infer the ancestry of filtered samples using variational Bayesian inference algorithm fastSTRUCTURE. Global analysis was run for *Ae. tauschii* and wheat together for K ranging from two to eight with three iterations for each K (**Figure 2**). Samples were pre-assigned labels based on their geographical origin, and this information was used for plotting the membership coefficients. At $K = 2$, L1 and L2 split from each other within *Ae. tauschii* and wheat remained clustered with L2 of *Ae. tauschii*. Nine accessions showed a very distinct structural differentiation as admixture of L1 and L2 (**Figure 2**; group 15). These nine accessions were hypothesized as the possible hybrids between L1 and L2 and were analyzed separately. Using "chooseK" utility provided with fastSTRUCTURE $K = 6$ was determined to be the optimal, where marginal likelihood of the data was maximized. For this study, we also found that K-values ranging from 2 to 6 were optimal and gave biological and geographic inference. At $K = 3$ L1, L2 and wheat were completely separated, with over half of the Iranian and few Azerbaijan accessions from lower altitudes showing admixture. At $K = 4$, L1 showed sub-population differentiation where accessions from Armenia, Azerbaijan, Georgia, Russia, Syria, and Turkey clustered separately from accessions originated in Afghanistan, China, Kyrgyzstan, Pakistan, Tajikistan, Turkmenistan, and Uzbekistan. Accessions from Iran showed mixture of accessions from these two groups. Putative hybrids showed clear similarity with accessions from the western side of Caspian Sea in L1 and Iranian accessions in L2. At $K = 5$, L2 accessions showed some differentiation where more than half of the accessions from Iran occurring at lower altitudes differentiated from Armenia, Azerbaijan, Georgia, and Turkey. For $K > 5$ no further information was provided by the population structure analysis in terms of population differentiation within L1 and L2, however, putative hybrids formed their own cluster. Wheat showed no sub-population differentiation at all. Therefore, we determined $K = 5$ to be a secondarily optimal stratification level after the optimal $K = 3$.

Population structure analysis was also run only on *Ae. tauschii* to determine the impact of the wheat outgroup on the pattern of *Ae. tauschii* grouping (**Supplementary Figure S3**). Marginal likelihood of the data was maximized at $K = 5$. At $K = 2$, L1 and L2 differentiated strongly, and the same group of nine accessions as possible hybrid was evidenced as admixture of L1 and L2. At $K = 3$, L1 showed the same population differentiation as the global analysis. Accessions from the eastern side of Caspian Sea differentiated from the western side. At $K = 4$, L2 Iranian accessions showed admixture and differentiate from other accessions. At $K = 5$, putative hybrids differentiated to form their own cluster. At $K > 5$ no more useful information was provided by the population structure analysis.

Principal Component and Cluster Analysis

Principal component analysis was run as a second approach to cluster accessions and detect subpopulations. The same set of 13,135 *Ae. tauschii* specific SNPs were used for PCA. The inferred lineages for *Ae. tauschii* individuals by population structure analysis were used to color the accessions in PCA (**Supplementary Figure S4**) and phylogenetic cluster analysis (**Figure 3**). Principal component analysis was performed separately for two datasets- *Ae. tauschii* with wheat, and *Ae. tauschii* only. As expected, the population differentiation

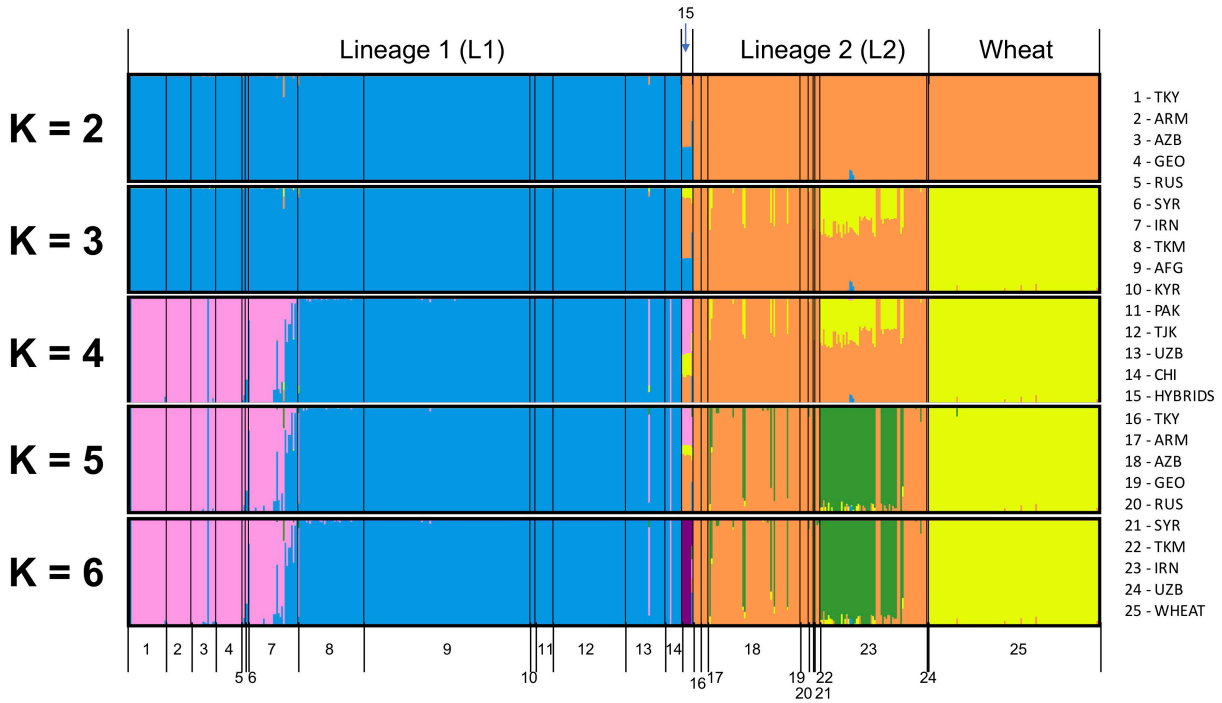

FIGURE 2 | Global population structure analysis for *Ae. tauschii* L1, L2, putative hybrids and wheat for $K = 2$ to $K = 6$. An additional color is added with each increase in the value of K. Each vertical bar represents an individual, where the proportion of the color bar representing membership coefficient for each subpopulation. A bar with only a single color represents its ancestry to a single population, and a mixture of colors represents admixture from different populations.

observed by fastSTRUCTURE was confirmed with PCA as three distinct groups–L1, L2 and wheat–were observed in the first two components of the PCA (**Supplementary Figure S4**). PC1 explained 55% of the variation separating L1 and L2. PC2 explained 7% of the variation and separates out wheat from L2 of *Ae. tauschii*. Corroborating previous reports, the wheat was observed to be more closely related to L2 accessions.

Principal component analysis with only the *Ae. tauschii* accessions, also confirmed the strong population differentiation between two *Ae. tauschii* lineages, L1 and L2. In this analysis, PC1 explained 53% the variation in the dataset (**Figure 4** and **Supplementary Figure S4**). When analyzed in the absence of wheat, L1 shows a strong within lineage differentiation on the second principal component explaining 4% of the variation, and L2 on the third principal component explaining 4% of the variation (**Figure 4**). To find the variables explaining the most variation within lineages along PC2 and PC3, the correlation coefficients were computed to agroclimatic variables. Correlation analysis showed that the L1 differentiation was strongly correlated with the longitudinal gradient of accessions with an east-west gradient relative to the Caspian Sea, and L2 with altitude relative to sea level (**Supplementary Figure S5**). After removing the outlier accessions, when the longitudes of L1 accessions are plotted against PC2, it clearly separated the accessions in east and west of Tehran, Iran (**Figure 5**). On the third principal component, population differentiation was also observed, which corresponded to the altitude of origin of the L2 accessions in reference to the sea level ($r = 0.61$). PC3 vs. altitude plot also shows a clear trend with PC3 separating the accessions according to their altitude, however, there are few outliers present on the both ends (**Supplementary Figure S6**). Generally, lower altitude accessions clustered together separately from the higher altitude accessions. We found that the strongest differentiation between L2 clusters was at around 150m above sea level. Overall the PCA results were in strong agreement with the population differentiation observed with fastSTRUCTURE.

As a final assessment of population structure, Cluster analysis was performed by computing genetic distances among accession using Euclidean method. An unrooted tree in this cluster analysis splits samples into three distinct clades- L1, L2 and wheat (**Figure 3**). Wheat and L2 were more closely related than wheat and L1, and L1 and L2. L1 and L2 further shows two clades within that could again be attributed to longitudinal variation from the Caspian Sea and altitude, respectively. Wheat essentially did not show any differentiation within.

Admixed *Ae. tauschii* Accessions Are L1–L2 Hybrids, or Possibly a New Lineage

Nine accessions showed up in STRUCTURE, PCA and cluster analysis as admixture of *Ae. tauschii* lineages L1 and L2. To test their origin as hybrids between L1 and L2 accessions, private alleles in both lineages were filtered and tested in the hybrid samples. A total of 4,711 L1 and 4,700 L2 private alleles were identified in the whole collection. Based on the total number

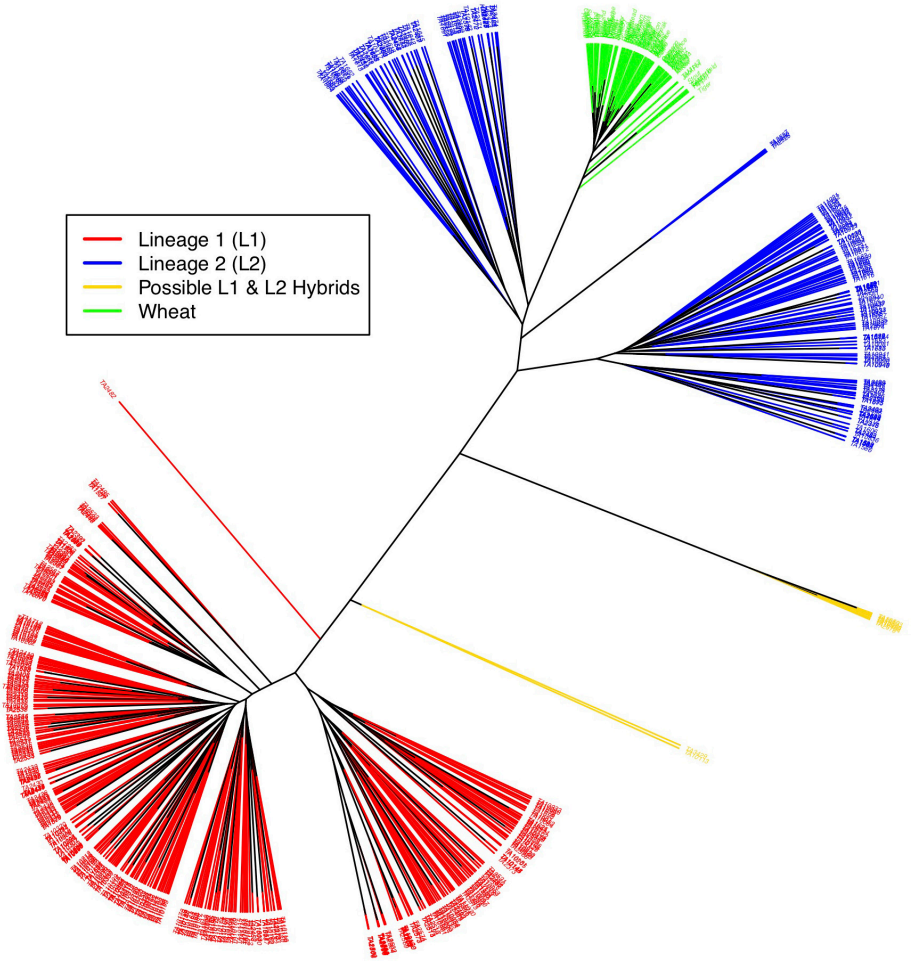

FIGURE 3 | Neighbor-joining tree showing relationship between L1, L2, possible L1–L2 hybrids and wheat. Red branches represent L1 accessions, blue L2, gold L1–L2 hybrids, and green wheat. Wheat is closely related to L2 of *Ae. tauschii*. Putative hybrids cluster out separately and appear in between the two lineages.

of SNPs assayed in putative hybrids, lineage specific alleles contributed by L1 ranged from 48 to 70%, and L2 ranged from 30 to 52%. Out of nine putative hybrid samples, only TA3429 was confirmed as a typical bi-parental recombinant inbred line between L1 and L2 accession(s), in which the chromosomal segments from L1 and L2 were clearly demarcated without any overlap (**Figure 6**). The other eight putative hybrids showed no such clear pattern but ambiguous distribution of private alleles (**Supplementary Figure S7**). Private alleles were visualized for one randomly selected accession from each L1 and L2, which showed no contribution from the other lineage (top row, **Supplementary Figure S7**).

Seven out of the eight unclear putative hybrids originated in Georgia and one in Turkmenistan. A total of 2,098 SNPs with private L1 or L2 alleles were assayed in these hybrids. Exploring further, we found that 1,988 SNPs were fixed (private alleles contributed by L1 or L2). Only 110 SNPs were segregating mostly from the one accession from Turkmenistan (**Supplementary Figure S8**). Removing that accession left only six segregating SNPs and 1,768 fixed SNPs in seven putative hybrids from Georgia. Failing to construct their expected hybrid haplotypes, we hypothesized that these putative hybrids from Georgia are an isolated lineage that probably resulted from a single hybridization event between an L1 and L2 accession. To determine this, we filtered out the SNPs to find if there were any alleles private to these hybrids, and we found 713 SNPs with alleles private to these hybrids from Georgia. Of these 713 SNPs, only 29 were segregating within these hybrids, and the remaining were fixed.

To find potential L1 and L2 parents of each putative hybrid, allele matching was performed. SNPs with lineage specific private alleles were used to find the closest accession from each lineage. Lowest and highest percent identity was found to be 76.96 and 85.02%, respectively, between a pair of hybrid and L1 accessions. Similarly, the lowest and highest percent identity between any pair of hybrid and L2 accessions was found to be 74.2 and 77.62%, respectively. These lower identity coefficients confirm that the potential parental accessions of these putative hybrids were not found in this collection. List of putative hybrids with highest matching accessions is summarized in **Table 1**.

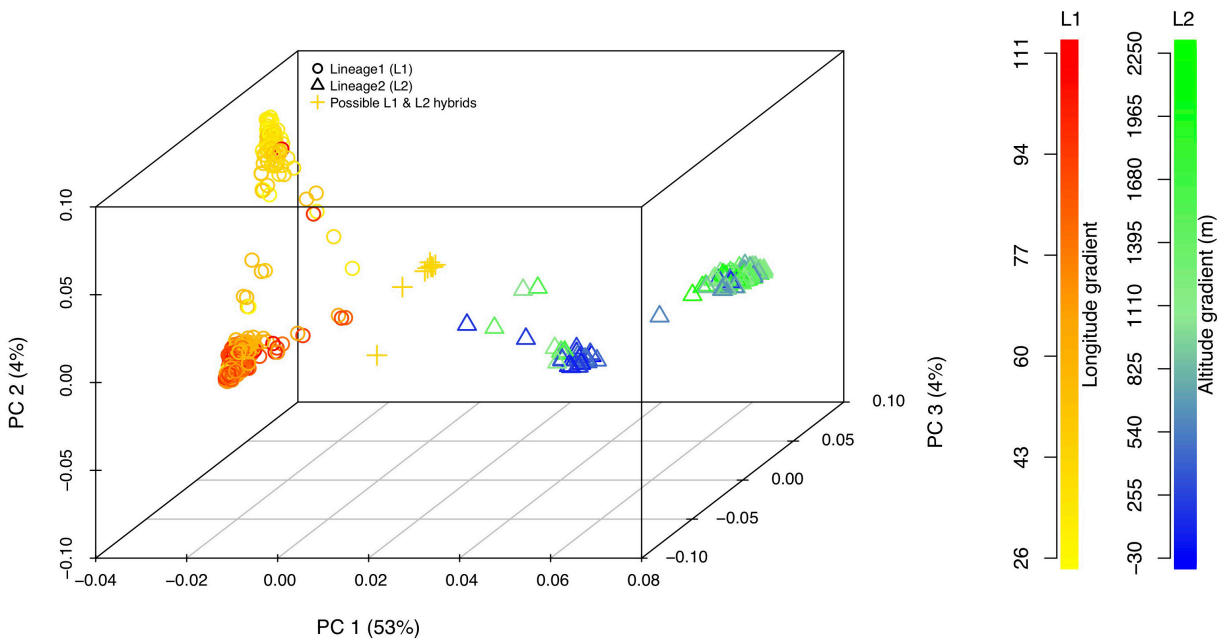

FIGURE 4 | 3-D principal component analysis plot for L1, L2, and possible L1–L2 hybrids. Lineage1 (L1) is colored based on the longitudinal gradient and Lineage2 (L2) is colored with altitudinal gradient with reference to the sea level. Empty circles represent L1 (yellow–red gradient) and empty triangles represent L2 (blue–green gradient). Gold plus sign (+) represent putative L1–L2 hybrids. Legends for the color gradient are shown on the right-hand side.

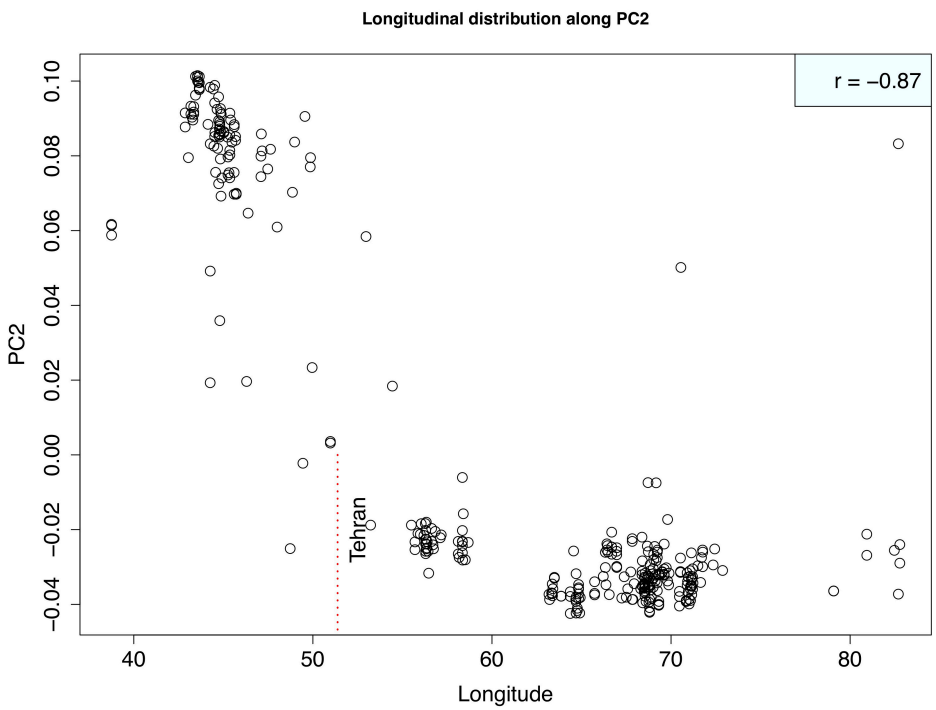

FIGURE 5 | Scatterplot showing the relationship between Longitude of Lineage1 accessions and second principal component. Strong correlation between two variables is evident suggesting that PC2 is separating out western (lower longitudes) from eastern (higher longitudes) accessions. Correlation coefficient is shown at the top right corner. Vertical red dotted line marks the longitude of Tehran, Iran that demarcates the eastern vs. western accessions.

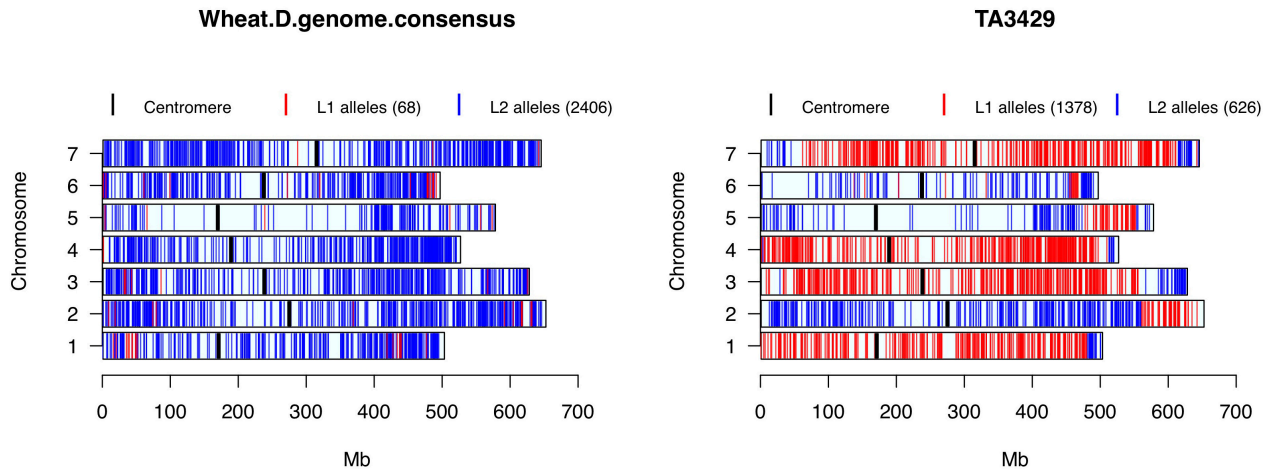

FIGURE 6 | Wheat D-genome consensus and putative hybrid TA3429 chromosomes showing the distribution of L1 and L2 specific alleles. Red color represents L1 specific alleles, and blue represents L2 specific alleles. Centromeres are marked with thick black bars. Numbers in parentheses in the legend represent the number of lineage specific alleles.

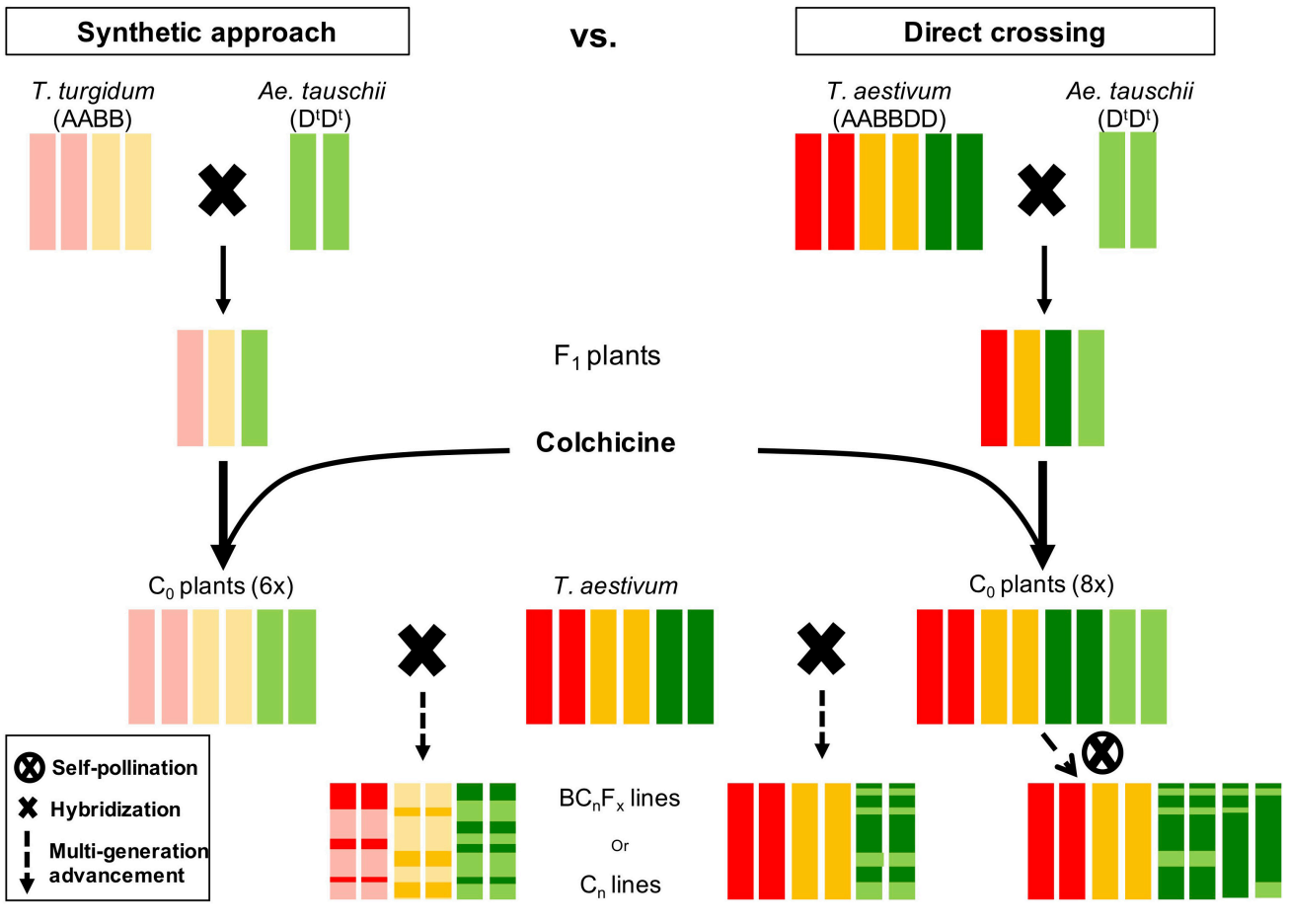

FIGURE 7 | Side-by-side comparison of two strategies to introgress genetic diversity from *Ae. tauschii* to wheat. Red bars represent A-genome, gold B-genome, dark green wheat D-genome, and light green *Ae. tauschii* D-genome. In synthetic approach the resulting line is a mosaic of all three genomes whereas in direct crossing only D-genome is shuffled.

TABLE 1 | Putative hybrids and their tentative L1 and L2 parents.

Putative hybrid	Origin	Putative L1 parent	Putative L2 parent
TA10103	Georgia	TA10181 (76.96%)	TA2527 (76.78%)
TA10104	Georgia	TA10181 (77.3%)	TA2527 (77.13%)
TA10113	Turkmenistan	TA10181 (83.65%)	TA2527 (73.64%)
TA10928	Georgia	TA10181 (77.43%)	TA2527 (77.62%)
TA10929	Georgia	TA10181 (77.2%)	TA2527 (77.14%)
TA2576	Georgia	TA10181 (77.24%)	TA2527 (77.49%)
TA2580	Georgia	TA10181 (77.17%)	TA2527 (76.93%)
TA2582	Georgia	TA10181 (77.47%)	TA2527 (76.92%)
TA3429	Unknown	TA1634 (85.02%)	TA2377 (74.2%)

Numbers in parenthesis represent percent identity of the hybrid with the parent.

Lineage-Specific Private Allelic Contribution to Wheat D-Genome

All wheat lines had similar distribution of lineage specific alleles across all chromosomes with minor differences (data not shown), therefore, to determine the lineage-specific contribution of *Ae. tauschii* to wheat D-genome, a consensus of private alleles distribution was determined. As it has been shown that the L2 of *Ae. tauschii* contributed the D-genome of wheat, all private alleles were assumed to be contributed by the L2. However, if at least two different wheat lines carried the same private allele from L1 at a given SNP site, it was considered a putative introgression from L1 in the consensus. We observed that the D-genome consensus carried only 68 (2.7%) alleles from L1 and 2,406 (97.3%) alleles from L2. Two chromosomes, 1D and 6D, carried 54% of the total L1 alleles with majority of the introgressions present in the distal regions (**Figure 6**).

Genetic Diversity

Nei's diversity index was computed using all SNPs separately for *Ae. tauschii* L1, L2, possible hybrids, wheat and *Ae. tauschii* collection combined. Highest Nei's diversity index was observed for L2 = 0.1326 followed by L1 = 0.0872, and wheat of 0.0158. Higher values of the Nei's index indicates greater allelic diversity in a given population. Combined Nei's index for *Ae. tauschii* was 0.2382 and the whole dataset including wheat was 0.2597.

To evaluate population differentiation between the different pairs of *Ae. tauschii* lineages and wheat, pairwise F_{ST} statistics were computed. Highest F_{ST} were observed between L1 and wheat, followed by wheat and L1–L2 hybrids, and wheat and L2 (**Table 2**). The population differentiation between L1 and wheat also supports the large number of novel of alleles found in this lineage that are absent from the wheat pool.

TABLE 2 | Pairwise F_{ST} coefficients among L1, L2, L1–L2 hybrids, and Wheat.

	L2	L1–L2	Wheat
L1	0.5635	0.0928	0.6261
L2	–	0.1183	0.3046
L1–L2	–	–	0.5262

Higher values represent stronger population differentiation.

Minor allele frequency was computed and plotted separately for L1, L2 and jointly for both lineages (**Supplementary Figures S9, S10**). Individually, MAF spectrum for L1 and L2 showed an expected distribution with majority of alleles present at very low frequency (**Supplementary Figures S9A,B**). Joint distribution of L1 and L2 MAF revealed that majority of the alleles segregating in one lineage were close to fixation in the other lineage (**Supplementary Figure S9C**). Chromosome-wise map for MAF, revealed that majority of the polymorphic markers were present on the distal ends of the chromosomes (**Supplementary Figure S10**), and L2 has higher proportion of polymorphic markers as indicated by the density and height of L2 bars.

Core-Set Selection

Genetically diverse core-set was selected using software package PowerCore that implements advanced M (maximization) strategy to select diverse accession by reducing allelic redundancy and keeping the allele frequency spectrum similar. Initially 107 *Ae. tauschii* accessions were selected using advanced M strategy implemented in PowerCore (**Supplementary Table S2**). These accessions were then plotted on a phylogenetic tree and selected using known phenotypic information on disease and insect resistance to get the size of this core to a manageable number. This selection was guided by phenotypic data for resistance to Leaf rust composite, Stem rust TTKSK race and Hessian fly biotype D. Other factors, such as the available geographical origin and the history of their previous use in genetic mapping, were also taken into account to pick the representative accessions. Finally, 40 accessions were selected to comprise a MiniCore that is distributed uniformly across the WGRC *Ae. tauschii* collection (**Supplementary Figure S11**). Nei's diversity index computed for the MiniCore (0.2235) compared to the whole collection (0.2382) suggests allelic richness in the MiniCore. Also, in the MiniCore, we were able to retain the 11,041 segregating SNPs out of 13,135 from the whole *Ae. tauschii* collection. By reducing the collection size by over 10-fold, we were still able to capture ∼84% of the segregating alleles present in the whole WGRC collection. MiniCore consists of 29 accessions from L1 and 11 accessions from L2 of *Ae. tauschii*.

DISCUSSION

Geographical Distribution of *Ae. tauschii*

Caspian Sea region is thought to be the center of origin of *Ae. tauschii*. Most of the accessions in our collection were also sampled from this region (**Figure 1**). Consistent with the current literature, we observed in our study that L2 of *Ae. tauschii* is spread on a narrow longitudinal range from northeastern Syria to northeastern Iran spanning a distance of 1625 km, whereas L1 is found from southern Turkey to northwestern China, spanning over 4000 km. However, we did find one L2 accession TA10124 originated in Uzbekistan. It is possible that the passport data for this accession was recorded wrong, but if true, it might point to the possibility of L2 migrating out of its natural habitat and extending eastbound. However, more sampling is required to make any further claims. Most of the accessions were acquired

from other genebanks, however, to fill up the geographical gaps, a recent exploration was conducted in 2012 by WGRC researchers (blue dots, **Supplementary Figure S1**). Multiple accessions from both lineages are found to overlap at similar altitudes, with L1 accessions generally inhabiting higher altitudes than L2 (**Supplementary Figure S12A**). Majority of L1 and L2 accessions fall in the similar latitude distribution, but some L1 accessions were widely spread (**Supplementary Figure S12C**).

SNP Discovery and Ascertainment Bias

Using *Ae. tauschii* genome assembly Aet v4.0 as the reference, GBS produced 13,135 high quality SNP markers useful to assess genetic diversity in the collection. We expected some bias in the two lineages because the reference genome (Aet v4.0) represents *Ae. tauschii* ssp. *strangulata*. However, as we did not use any prior SNP information to call SNPs, we expect the ascertainment bias be minimal. Splitting two lineages and computing MAF separately revealed that both lineages had about similar distribution of MAF (**Supplementary Figure S9**), but with elevated MAF in L2 (**Supplementary Figure S10**). Because the goal of this project was not to assess any specific genomic region, using Aet v4.0 reference genome should not pose a problem.

Population Structure Analysis

Global population structure analysis showed the expected *Ae. tauschii* subpopulations (L1 and L2) with each having two subgroups, and wheat D-genome forming a third group which was most closely clustered to L2. These findings are largely in agreement with known population structure of *Ae. tauschii*, confirming the utility of our genotyping approach. In addition to these five groups, we unexpectedly found putative hybrids clustered together (**Figure 2**). This small group of nine accessions showed up as admixture of L1 and L2. At $K = 3$ wheat split from L2 sharing ancestry with most of Iranian and few Azerbaijan accessions. One common feature of these accessions is that they all occur at lower altitudes from the sea level. This points to the possibility that these accessions or their ancestors could have been involved in the origin of wheat.

Aegilops tauschii L1 showed intra-lineage population differentiation in accordance with relative position of East or West of the Caspian Sea. This was also clear in the principal component analysis where L1 was differentiated by PC2 along longitudinal gradients (**Figure 4**). Iranian accessions did not show clear population differentiation by falling into the eastern or western group but rather show admixture. Iran is at the center of origin for *Ae. tauschii* and could be seen as a transition region for the East and West clades of L1. The majority of the L2 accessions occur in Azerbaijan and Iran, both of which are on one side of the Caspian Sea with Iran expanding to the eastern side, therefore longitudinal gradient did not explain much of the weak population structure within L2 at $K = 5$. However, we found that this population differentiation could be attributed to the altitude of the origin of L2 accessions where accessions originating at less than 150 m above sea level cluster separately from the accessions from more than 150 m above sea level (**Supplementary Figure S6**).

The accessions that were admixture clustered separately from all other accessions and did show unique ancestry. These admixed putative hybrids were observed to have shared ancestry with L1 accessions from Turkey and Transcaucasia, and L2 accessions from Iranian and Azerbaijan accessions occurring at lower altitudes. This could possibly mean that their original parents belong to these geographical regions.

Inter-Lineage Hybridization and the Origin of a New Lineage

Aegilops tauschii is a highly self-pollinated species, therefore natural hybrids between L1 and L2 are rare and have been the subject of limited reports. Wang et al. (2013) reviewed that collectively, only 1.4% accessions have been classified L1–L2 intermediates in several studies. They also found two intermediate accessions falling in between L1 and L2. Based on haplotype distribution similarity and close geographical proximity of origin, they speculated that these two accessions could have originated from the hybridization of a single L2 plant with an L1 plant.

In the present study, we found nine such intermediate accessions that fall in between the L1 and L2 in the fastSTRUCTURE, PCA and cluster analyses. Using the SNPs with private alleles, the allele matching of putative hybrids with L1 and L2 accessions did not result in a perfect match, which suggests that the real parental accessions could be missing in our collection. This suggests that the natural hybridization of L1 and L2 accessions is indeed rare, and these hybrids possibly originated from one or few of these rare events. These findings are in alignment with Wang et al. (2013), where they suggested a single hybridization event could have resulted in the two intermediate individuals in their data. Seven of the hybrids identified in our study were found in Georgia, one in Turkmenistan, and one with missing passport data. Both lineages co-exist in Georgia and Turkmenistan, therefore they are not isolated by distance. It is possible that they are reproductively isolated given their inbreeding nature. Similar pattern of reproductive isolation and rare hybridization was reported in rice landraces (Huang et al., 2010), and switchgrass (Mizuno et al., 2010; Sohail et al., 2012; Grabowski et al., 2014).

The distribution of L1 and L2 private alleles in these admixed accessions supports our hypothesis that these accessions could have arisen from L1 to L2 hybrids (**Figure 6** and **Supplementary Figure S7**). One hybrid, TA3429, showed a typical recombinant inbred pattern, which was different than other hybrids. This accession was actually received from a Japanese collection with few other germplasm lines, and was labeled as 4× (tetraploid). However, when tested phenotypically and cytologically, it was diploid like normal *Ae. tauschii*. Therefore, it is possible that this accession was in fact an artificially created hybrid between an L1 and L2 accession as a diploid.

All other admixed accessions appear to be derived from a rare hybridization event between an L1 and L2 accession followed by isolation and possibly multiple intercrossing events. We found that the majority of the L1, L2 private SNP alleles assayed in these putative hybrids were fixed, and only 110 were

segregating with majority of the hybrids carrying same private alleles. Exploring further, we identified 713 SNPs with alleles private to the admixed accessions from Georgia. Of these 713 SNPs, only 29 were segregating among these hybrids. Together this supports the possibility that these accessions resulted from single hybridization event. Though a limited sample, this points to the possibility of development of an unreported lineage as a result of rare hybridization event between an L1 and L2 accession, however, more samples are needed from these areas to shed new light on the nature of hybridizations among both lineages.

Genetic Diversity

Wheat had the lowest Nei's index, which is expected because of its domestication and polyploidization, compared to its wild progenitor, *Ae. tauschii*. Reduction in genetic diversity has also been reported in cotton as a result of change in ploidy level (Iqbal et al., 2001). Wheat lines in our study also represent a relatively narrow collection of United States winter wheat, leading to the lowest Nei's index. Highest Nei's index was observed for L2, followed by L1. This can be attributed to the differences in distribution of L1 and L2 across their natural habitat. L1 is distributed across the longitudinal gradient, whereas L2 is distributed across the altitudinal gradient. Latitude is known to affect the weather temperature with cooler temperatures away from the equator (Rind, 1998), but the latitude distribution for L1 and L2 was similar for the majority of accessions except few outliers (**Supplementary Figure S12C**). Therefore, the expected effect of latitude should be minimal. Longitude distribution for L1 was more extensive as compared to L2 (**Supplementary Figure S12B**). As shown in **Figure 1**, the majority of the L2 accessions are distributed around the Caspian Sea as compared to very few L1 accessions. Therefore, the longitude effect is more pronounced in L1 than L2. Moreover, the altitude distributions for L1 and L2 were also different (**Supplementary Figure S12A**), with more L2 accessions growing at lower altitude. Altitude is known to have an effect on the temperature (Körner, 2007). Therefore, L2 accessions might have selected alleles to survive in different temperatures. Combined *Ae. tauschii* had higher Nei's index as compared to any single lineage, which is expected because all the allelic diversity is assayed in the whole collection.

Ae. tauschii Contribution to the Wheat D-Genome

We assayed wheat D-genome chromosomes for lineage specific introgressions from *Ae. tauschii*. A majority of the introgressions mapped to L2, which is consistent with the current and past literature. Calculating the percentage of lineage specific alleles, we observed that L1 had only contributed 2.7% of the total *Ae. tauschii* introgressions in comparison to 97.3% by L2. This supports previous reports and points to the need to use L1 accessions for broadening the genetic base of hexaploid wheat and harness the untapped genetic diversity present in *Ae. tauschii* L1. With this goal, we developed a small set of *Ae. tauschii* (MiniCore) consisting of 29 L1 and 11 L2 accessions to facilitate wheat improvement.

Genetically Diverse Representative MiniCore

Accessing the genetic diversity present in wild relatives can be a challenging task for breeders due to the large number of accessions and confounding physiology of the wild plants. Wild accessions with overall poor phenotype could be the source of agronomically important alleles. Efficient use of germplasm collections can often be facilitated through a targeted subset of the total accession that is optimized to capture a maximum amount of the total diversity in a minimum number of accessions. To facilitate the use of *Ae. tauschii* accessions in wheat breeding, we selected only 40 accessions to develop a small MiniCore set that captures 84% of the segregating alleles from the whole collection. MiniCore was carefully selected from both the lineages of *Ae. tauschii* but the main focus was to target more from L1. This is because L1 is a reservoir of untapped genetic diversity that has not been leveraged by the breeders. L2 accessions were chosen because this lineage is the source for many of the diseases and insect resistance. These accessions can be utilized to bring in novel genetic variation for wheat rusts, insect resistance, heat and drought tolerance to produce climate resilient wheat varieties. This MiniCore consisting of genetically diverse accessions was selected with an objective to broaden the genetic base of wheat D-genome. However, in future, the selection can be optimized based on the recombination rate and the distribution of *Ae. tauschii* regions that are already introgressed in the wheat D-genome.

Future Work and Strategy to Utilize Genetic Diversity in *Ae. tauschii*

Untapped genetic diversity in *Ae. tauschii* is of great interest to breeders and geneticists for wheat improvement and broadening the narrow D-genome (Kihara, 1944; Lagudah et al., 1991; Lubbers et al., 1991; Akhunov et al., 2010). *Aegilops tauschii* has been utilized via synthetic bridge crossing and direct crossing (McFadden and Sears, 1945; Kihara and Lilienfeld, 1949; Gill and Raupp, 1987), however, both of these strategies have drawbacks. Synthetic bridge crossing involves a tetraploid parent that ultimately brings the genetic diversity in A and B genomes, which makes it difficult and time-consuming process to get rid of undesirable diversity from A and B genomes (**Figure 7**). Whereas, direct crossing of *Ae. tauschii* with wheat generally result in high F_1 sterility rendering it less lucrative to researchers (Olson et al., 2013; Cox et al., 2017). However, another novel strategy, which adopts beneficial steps from both these strategies, is "octo-amphiploid bridge" mediated direct genetic transfer, which hasn't been reported in literature much. Using this strategy, Zhang et al. (2018) recently identified 18 QTLs for three agronomic traits, i.e., thousand kernel weight, spike length, and plant height. Briefly, this strategy involves crossing *Ae. tauschii* directly with wheat producing a haploid F_1 (n = 28; ABDD^t; **Supplementary Figure S13**), followed by colchicine doubling resulting in an octo-amphiploid ($2n = 8x = 56$; AABBDDD^{t}D^t) (**Figure 7**). This octoploid can then be either self-fertilized for several generations to develop recombinant inbred lines (RIL) population or backcrossed with hexaploid wheat to develop near

isogenic lines for genetic mapping. Since there are four copies of D-genome chromosomes, the progeny will follow tetrasomic inheritance for any given trait with five expected genotypes; nulliplex, simplex, duplex, triplex and quadriplex. Presence of range of genotypes with a single allele differences present an opportunity to study the dosage effect in addition of the genetic mapping. Extending the disomic inheritance model to this octoploid, typical RIL like 96% homozygosity should be achieved after 20 generations of selfing compared to six generations for disomic inheritance (**Supplementary Figure S14**). However, theoretically moderate frequencies for homozygous individuals for each allele (nulliplex and quadriplex) are achieved at F_5 or F_6 that can be used for genetic mapping. Once an associated genetic marker is identified for a trait, it can be used to identify a homozygous line for a given trait and backcrossed with wheat recover euploid wheat ($2n = 6x = 42$) with a desired gene introgressed in it (**Supplementary Figure S15**). Our initial results indicate that depending on the hexaploid wheat used, euploidy can be achieved as soon as after one or two backcrosses.

CONCLUSION

Studying genetic diversity in *Ae. tauschii* is very important to wheat improvement in the wake of unpredictable climate and evolving biotic stresses. In this study, we confirmed that *Ae. tauschii* L1 has immense amount of untapped genetic diversity that can be used for wheat improvement. We also provided the evidence of natural *Ae. tauschii* L1–L2 hybrids, which opens the door to the possibility of new genetic variation. Finally, selection of forty genetically diverse accessions will facilitate the use of *Ae. tauschii* for wheat improvement for abiotic and biotic stresses via octo-amphiploid mediated bridge crossing, which will ultimately result in higher genetic gains and faster wheat improvement.

DATA AVAILABILITY

Sequence reads generated using genotyping-by-sequencing are available from NCBI SRA under accession SRP141206. R-code and other scripts are available at GitHub repository https://github.com/nsinghs/Code_Ae_tauschiiDiversity.

AUTHOR CONTRIBUTIONS

NS analyzed the data and wrote the manuscript. SW performed the GBS. VT and SS developed and contributed to the idea. JR acquired, managed, and provided *Ae. tauschii* accessions. DW collected and provided phenotypic data. MA provided *Ae. tauschii* accessions. BG and JP conceived and developed the idea. JP wrote the manuscript.

FUNDING

This is contribution #19-042-J from the Kansas Agricultural Experiment Station, Manhattan. This study was conducted under the auspices of the Wheat Genetics Resource Center (WGRC) and Industry/University Collaborative Research Center (I/UCRC) through support of industry partners and partially funded by NSF grant contract (IIP-1338897).

ACKNOWLEDGMENTS

We would like to acknowledge the contribution of Industry Advisory Board to improve this manuscript with their valuable comments and suggestions. A special thanks to Drs. Dal-Hoe Koo and Bernd Friebe for performing chromosome counts on the octo-amphiploid F_1 and several backcross progenies.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00009/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 31 January 2019
doi: 10.3389/fpls.2019.00034

Development of Stable Homozygous Wheat/*Amblyopyrum muticum* (*Aegilops mutica*) Introgression Lines and Their Cytogenetic and Molecular Characterization

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OPEN ACCESS

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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: 17 September 2018
Accepted: 10 January 2019
Published: 31 January 2019

Citation:
King J, Newell C, Grewal S, Hubbart-Edwards S, Yang C-y, Scholefield D, Ashling S, Stride A and King IP (2019) Development of Stable Homozygous Wheat/Amblyopyrum muticum (Aegilops mutica) Introgression Lines and Their Cytogenetic and Molecular Characterization. Front. Plant Sci. 10:34. doi: 10.3389/fpls.2019.00034

Wheat is one of the world's most important sources of food. However, due to its evolution its genetic base has narrowed, which is severely limiting the ability of breeders to develop new higher yielding varieties that can adapt to the changing environment. In contrast to wheat, its wild relatives provide a vast reservoir of genetic variability for most, if not all, agronomically important traits. Genetic variation has previously been transferred to wheat from one of its wild relatives, *Ambylopyrum muticum* (previously known as *Aegilops mutica*). However, before the genetic variation available in this species can be assessed and exploited in breeding and for research, the transmission of the chromosome segments introgressed into wheat must first be stabilized. In this paper we describe the generation of 66 stably inherited homozygous wheat/*Am. muticum* introgression lines using a doubled haploid procedure. The characterisation and stability of each of these lines was determined via genomic *in situ* hybridization and SNP analysis. While most of the doubled haploid lines were found to carry only single introgressions, six lines carried two. Three lines carried only complete *Am. muticum* chromosomes, 43 carried only small or very small introgressions and the remainder carried either only large introgressions or a large plus a small introgression. The strategy that we are employing for the distribution and exploitation of the genetic variation from *Am. muticum* and a range of other species is discussed.

Keywords: wheat, introgression, *Amblyopyrum muticum*, doubled haploid, SNP markers, genomic *in situ* hybridization

INTRODUCTION

Wheat is one of the world's leading sources of food providing circa 20% of the world's daily intake (Reynolds et al., 2012). Following a history of continued yield improvements by breeders, wheat yields are now plateauing at a time when the world's population is rapidly increasing (Charmet, 2011). The reason for this plateauing is a lack of genetic variation within modern day wheat varieties compounded by environmental change, i.e., hexaploid wheat only evolved once or twice circa 10,000 years ago and thus it has been through a significant genetic bottle-neck. In contrast to

wheat its wild relatives provide a vast reservoir of genetic variation for potentially most, if not all, traits of agronomic importance. In the past there have been several examples of the exploitation of genetic variation from wild relatives for wheat improvement. For example, the transfer of a segment of *Aegilops umbellulata* to wheat conferring resistance to leaf rust (Sears, 1955), the transfer of a segment of *Aegilops ventricosa* carrying resistance to eyespot (Doussinault et al., 1983) and its subsequent release as the variety Rendevouz.

Even though there have been a number of successes in the past, the genetic variation available within the wild relatives remains largely untapped with regard to its exploitation in breeding programs. The main reason for this has been the lack of high throughput technological screens to identify when genetic variation has been introgressed into wheat. A direct result is that where in the 1970s and 1980s there were many hundreds of scientists working in the field there are now very few. However, the advances in technology, e.g., gene and genome sequencing, comparative mapping, molecular marker development etc., over the last 10–15 years has now resulted in the development of systems that can be utilized for the high throughput detection and high-resolution characterisation of wheat/wild relative introgressions. King et al. (2017, 2018) and Iefimenko et al. (2015) used an Axiom array in combination with a specific crossing strategy to generate and identify introgressions from *Ambylopyrum muticum*, *Aegilops speltoides* and *Thinopyrum bessarabicum*. Many hundreds of new introgressions were generated and detected in these works. The frequency of introgression between wheat and *Am. muticum* and *Ae. speltoides* was high enough to generate linkage maps of these species, with over 500 new introgressions developed from *Am. muticum* and *Ae. speltoides* (King et al., 2017, 2018).

In the past much of the work undertaken had been aimed at transferring genetic variation from a wild relative to wheat for a single trait. This strategy normally required the production of an interspecific hybrid followed by the generation of wheat/wild relative addition and substitution lines (King et al., 2016). A chromosome manipulation program was then undertaken to introgress a small chromosome segment, from the chromosome of the wild relative (that carried the gene(s) controlling the target trait) into wheat. The work undertaken by King et al. (2017, 2018) and Iefimenko et al. (2015) used a different strategy. Although *Am. muticum Ae. speltoides* and *Th. bessarabicum* all carry genetic variation for a range of traits such as disease resistance, salt tolerance, etc., the main aim of these works was to introgress the entire genome of these species into wheat in small chromosome segments, i.e., transfer all of the genetic variation in these species into wheat. In the future each of the introgression lines carrying a chromosome segment from these three wild relatives will be screened phenotypically for a range of traits. This strategy will allow the phenotypic analysis of the entire genomes of each of the wild relatives for a wide range of traits (the limiting factor being the number of traits screened for) rather than a single trait.

In order for each of the introgression lines to be analyzed phenotypically they need to be multiplied and stably inherited. All of the introgressions initially produced by King et al. (2017, 2018) and Iefimenko et al. (2015) are in the heterozygous state with the result that the progeny produced from plants carrying them will segregate for lines with and without the introgression. In contrast, lines homozygous for introgressions are expected to be stably inherited and thus can be multiplied and distributed for large scale trait analysis.

In this work, however, we focused on the development of homozygous *Am. muticum* introgression lines a species that has been shown with limited previous trait analysis to contain genetic variation for environmental stresses (Iefimenko et al., 2015) and powdery mildew (Eser, 1998) and their characterisation via SNP analysis and genomic *in situ* hybridization (GISH). The strategy for exploitation of introgressions is discussed, i.e., all stable homozygous introgressions that are generated will be subjected to a wide range of trait analyses via our collaborators both in the United Kingdom and globally, in order to determine the agronomic and scientifically important genetic variation carried by the *Am. muticum* introgressions.

MATERIALS AND METHODS

Plant Material

Wheat/*Am. muticum* introgressions were generated as described by King et al. (2017). In summary *T. aestivum,* vars. Chinese Spring and Pavon 76, were pollinated with *Am. muticum* (which carries suppressors of *Ph1*/promoters of homoeologous recombination). Accessions 2130004 and 2130012 of *Am. muticum* were obtained from the Germplasm Resource Unit at the JIC, United Kingdom. The F_1 interspecific hybrids produced were then backcrossed to *T. aestivum* vars. Paragon or Pavon 76 to recover introgressions in a wheat background. The BC_1 population and its subsequent progeny were also backcrossed to Paragon to produce a BC_3 populations which were themselves crossed to maize to initiate doubled haploid (DH) production (**Figure 1**). When the work described in this paper was initially undertaken the Axiom® Wheat-Relative Genotyping Array described by King et al., 2017 was not available. Thus, selection of BC_3 plants for DH production was based upon the identification of plants carrying introgressions in the BC_2 individuals via GISH analysis. However, leaf material was taken from each of the BC_3 plants used for DH production for SNP analysis when the genotyping array became available.

Doubled Haploid Production

The DH production procedure used was as described by Laurie and Reymondie (1991). In summary 1 day after pollination with maize (cultivars Northern Extra Sweet, Prelude and Sundance), internodes below pollinated spikes were filled with 10 mg l^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D solution) with a syringe and the holes sealed with petroleum jelly. The 2,4-D solution was also injected into each floret. After 14–21 days embryos were excised and cultured. Colchicine treatment was carried out as described in Nemeth et al. (2015).

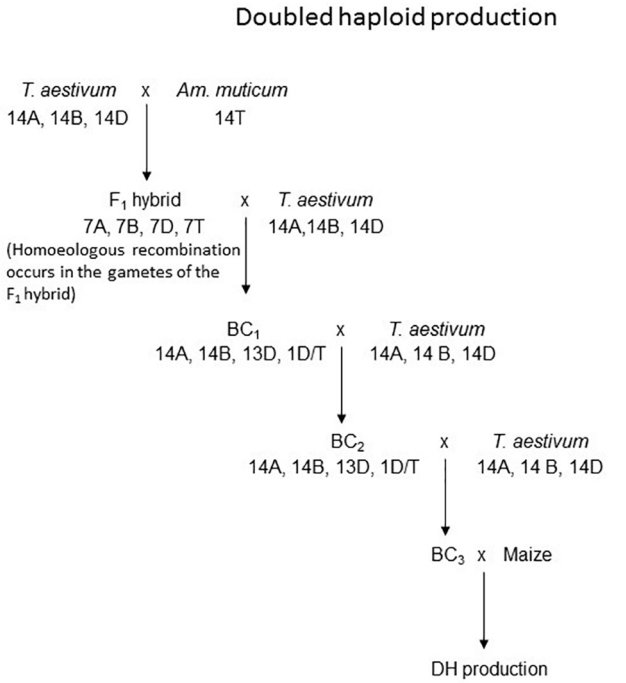

FIGURE 1 | Derivation of the material used to develop DH homozygous introgression lines. This example shows an ideogram of an *Am. muticum*/*T. aestivum* D genome recombinant and the subsequent development of a DH line from it.

Detection of Wheat/*Am. muticum* Introgressions

Marker Analysis

A 35K Axiom® Wheat-Relative Genotyping Array (Affymetrix, Santa Clara, CA, United States) was used to genotype a set of BC_1, BC_2 and BC_3 wheat/*Am. muticum* introgression lines by King et al. (2017). The genetic map generated for *Am. muticum* by King et al. (2017) was used in conjunction with the 35K Axiom® Wheat-Relative Genotyping Array to detect and characterize *Am. muticum* segments in the DH lines and in the BC_3 lines they originated from as described in King et al. (2017). [All the SNPs incorporated in the array formed part of the Axiom® 820K SNP array (Winfield et al., 2016) with the dataset for the Axiom® 820K SNP Array available at www.cerealsdb.uk.net (Wilkinson et al., 2012, 2016)]. The SNPs used were polymorphic between *Am. muticum* and the three wheat cultivars used in the generation of the DH lines (Chinese Spring, Paragon and Pavon 76). Also, most SNPs were not genome-specific in wheat, i.e., they had copies on more than one genome of wheat and thus, were unable to distinguish between a heterozygous and a homozygous segment since presence of either type of segment produced a heterozygous call.

Cytogenetic Analysis

The protocol for genomic *in situ* hybridization (GISH) was as described in Zhang et al. (2013); Kato et al. (2004), and King et al. (2017). Genomic DNAs was isolated from *Am. muticum* and the three putative diploid progenitors of bread wheat, i.e., *T. urartu* (A genome), *Ae. speltoides* (B genome) and *Ae. tauschii* (D genome). Genomic DNAs of *Am. muticum*, *T. urartu* and *Ae tauschii* were labeled by nick translation with ChromaTide Alexa Fluor 546-14-dUTP, ChromaTide Alexa Fluor 488-5-dUTP [Thermo Fisher Scientific (Invitrogen), Waltham, MA, United States] and Alexa Fluor 594-5-dUTP [Thermo Fisher Scientific (Invitrogen), Waltham, MA, United States], respectively. Genomic DNA of *Ae. speltoides* was fragmented to 300–500 bp at 100°C.

Preparation of chromosome spreads was as described in Kato et al. (2004) and King et al. (2017). Slides were probed using labeled genomic DNAs of *Am. muticum* (100 ng), *T. urartu* (100 ng), *Ae. tauschii* (200 ng) and fragmented genomic DNA of *Ae. speltoides* (5000 ng) as blocker in a ratio of 1:1:2:50 per slide to detect the *Am. muticum* introgressions and the AABBDD genomes of wheat. Slides were counterstained with Vectashield mounting medium with 4′-6-diamidino-2-phenylindole,dihydrochloride (DAPI) and analyzed using a Zeiss Axio ImagerZ2 upright epifluorescence microscope (Carl Zeiss Ltd, Oberkochen, Germany) with filters for DAPI (Ex/Em 358/461 nm, blue), Alexa Fluor 488 (Ex/Em 490/520 nm, green), Alexa Fluor 594 (Ex/Em 590/615 nm, red) and Alexa Fluor 546 (Ex/Em 555/570 nm, yellow). Photographs were taken using a MetaSystems Coolcube 1 m CCD camera. Further slide analysis was carried out using Meta Systems ISIS and Metafer software (Metasystems GmbH, Altlussheim, Germany).

RESULTS

Sixty-nine BC_3 plants derived from BC_2 lines (characterized by GISH and identified as carrying *Am. muticum* chromosomes and introgressions) were pollinated with maize in order to generate DH lines. Subsequent SNP analysis of the 69 BC_3 plants using the newly developed Axiom® Wheat-Relative Genotyping Array indicated that 57 of the 69 BC_3 individuals selected carried *Am. muticum* chromosomes and/or wheat/*Am. muticum* introgressions. Of the 12 BC_3 plants that did not carry *Am. muticum* chromosomes and wheat/*Am. muticum* introgressions 11 (92%) produced DHs (**Supplementary Table S1**).

Of the 57 BC_3 plants carrying *Am. muticum* chromosomes and/or wheat/*Am. muticum* introgressions 32 (56%) produced DHs. In total 220 DH plants were produced of which 161 (73%) grew and produced seed. The remaining 59 (27%) DHs either died or were sterile. SNP analysis indicated that of the 161 DH plants that set seed, 93 (58%) did not carry any *Am. muticum* chromosomes and/or wheat/*Am. muticum* introgressions (**Supplementary Table S1**). SNP analysis revealed that the remaining 68 DH plants that set seed all carried one or two wheat/*Am. muticum* introgressions or chromosomes (**Table 1** and **Supplementary Table S1**). **Table 1** gives the genome information for one DH plant for each different segment - each of these selected plants is also shown with GISH in **Figure 2**. Full genome information for all BC3 plants used and all DH plants produced is given in **Supplementary Table S1**. DH-4 was subsequently lost due to very low seed set and germination.

TABLE 1 | Genome information for BC_3 and DH plants showing the number, linkage groups and size of *Am. muticum* segments present, the wheat genome involved in the recombination (where known) and the number of A, B, and D genome chromosomes.

BC_3 code	No. of segments in BC3	No. of DH plants produced with segment(s)	DH plant with GISH validation	No. of segments in DH plants	Linkage group of DH segments	Segment size	Wheat genome recombined with	No. of A chromosomes	No. of B chromosomes	No. of D chromosomes	Total No. of chromosomes
165D	2	1	1	1	6	Whole		14/16	14	12	42/44
174D	1	7	8	1	2	Very small	D	14	14	14	42
177A	2	4	15	2	2, 4	Large, Large	B, B	12	14	14	42
			17	1	4	Large	B	14	12	14	42
177B	2	3	19	2	4, 7	Large, Large	B, B	14	12	14	42
			21	1	4	Large	B	14	12	14	42
182B	2	2	28	1	6	Telo		14/15	13/14	14	41/42/43 + /−T
			29	1	7	Whole		14	14	11	41
185A	1	11	62	1	4	Small	D	14	14	13/14	41/42
186A	4	7	84	1	5	Very small	D	14	14	14	42
			86	1	2	Very small	D	12	14	16	42
			89	2	2, 5	Very small, Very small	D, D	12	14	16	42
187A	4	2	93	1	1/6cf	Whole	Centric fusion	12	14	12	40
			94	1	2	Small	D	12	14/15	16	42/43
187B	3	2	96	1	4	Small	D	14	14	14	42
189B	2	3	121	2	4, 7	Large, Large	D, D	14	14	10	42
			122	1	7	Large	D	14	14	12	42
190A	1	15	355	1	1	Very small	A	16	12	14	42
194A	2	1	161	1	1	Large	B	12	14	12	40
197B	1	8	191	1	7	Large	D	14	14	12	42
197E	2	1	203	1	7	Very small, Large	D, D	14	14	12	42

Of the 68 DH lines with segment(s) only GISH validated DH lines (represented in ***Figure 2****) have detailed information shown above. For complete genome information on all DH lines produced, including those with no segments, see supplemental data (****Supplementary Table S1****).*

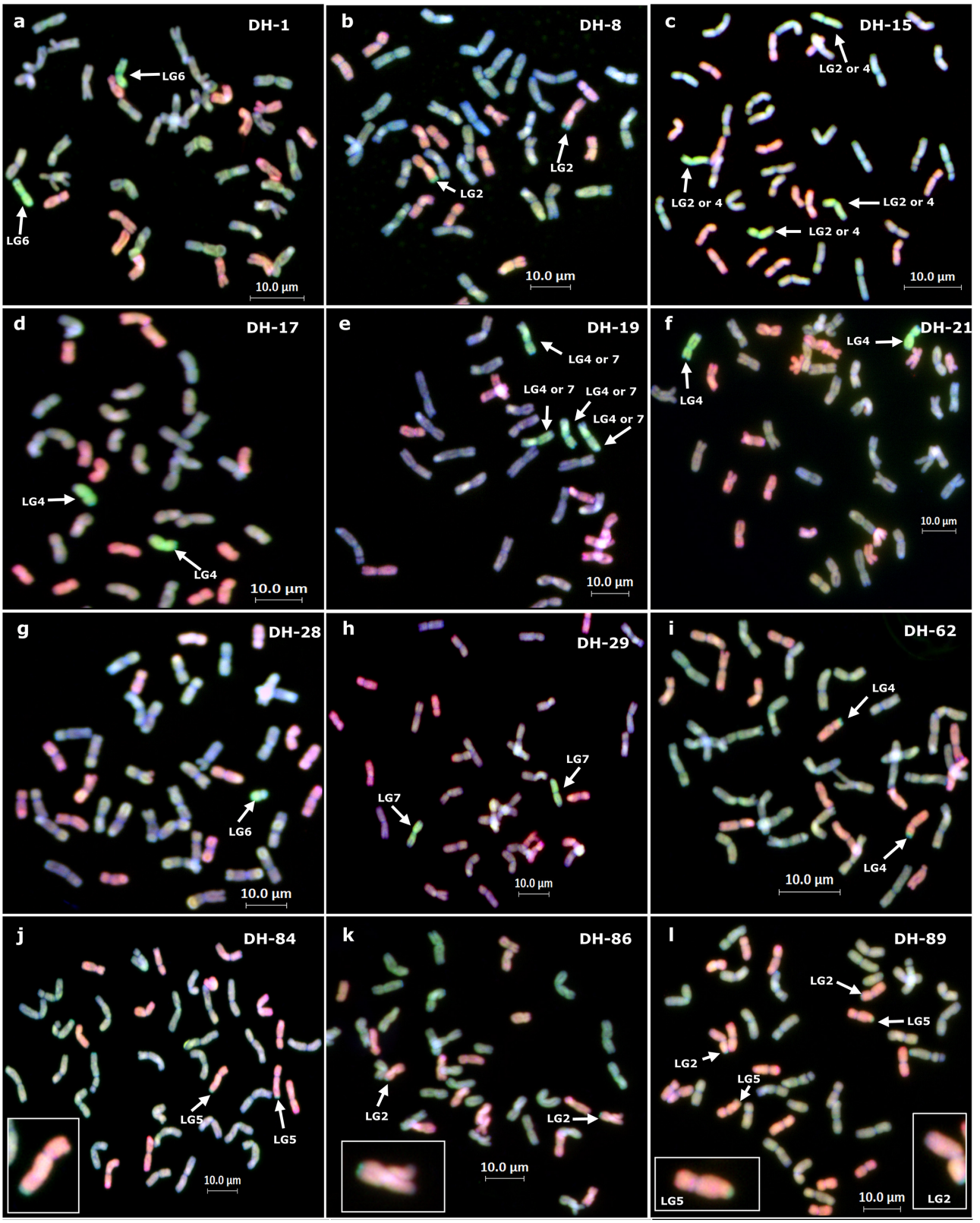

FIGURE 2 | Continued

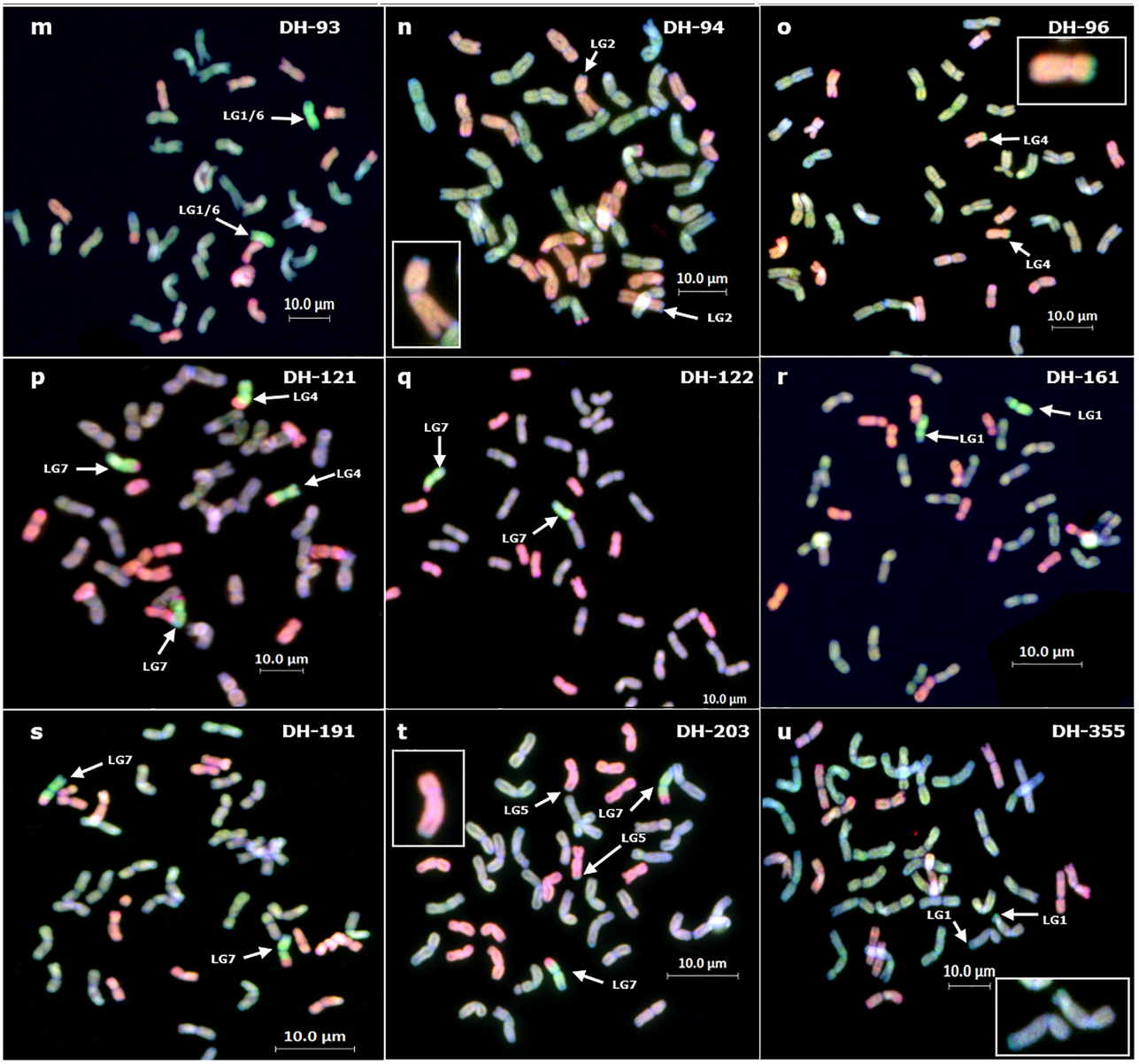

FIGURE 2 | GISH analysis of DH lines showing the different segments present. **(a)** DH-1 **(b)** DH-8 **(c)** DH-15 **(d)** DH-17 **(e)** DH-19 **(f)** DH-21 **(g)** DH-28 **(h)** DH-29 **(i)** DH-62 **(j)** DH-84 **(k)** DH-86 **(l)** DH-89 **(m)** DH-93 **(n)** DH-94 **(o)** DH-96 **(p)** DH-121 **(q)** DH-122 **(r)** DH-161 **(s)** DH-191 **(t)** DH-203 **(u)** DH-355. All GISH was carried out using four colors as indicated in Materials and Methods, but all photos shown were taken using three colors with a green filter for Alexa Fluor 546 for best visualization of the *Am. muticum* segments (bright green segment indicated with white arrows). The A and B genome chromosomes are colored the same (blue/light green) under this color capture. The D genome is shown in red. Small segments are also shown as enlargements.

Fifty seven of the lines were analyzed using multi-color GISH (mcGISH; **Figure 2** shows the GISH for one DH plant for each different segment) and the linkage group of the *Am. muticum* and/or wheat/*Am. muticum* introgression that each DH derived line was determined via SNP analysis (**Table 1**). SNP analysis revealed that one of the lines, DH-93, carried linkage group 1L and linkage group 6S markers. However, cytogenetic analysis indicated the presence of a single pair of chromosomes. Since previous work has shown that *Am. muticum* linkage group 6 and group 1 chromosomes are not translocated relative to wheat (King et al., 2017) this observation indicates the presence of a translocated *Am. muticum* 6S.1L chromosome potentially derived from mis-division of complete chromosomes followed by centric fusion.

McGISH analysis of the progeny derived from the 67 fertile DH individuals indicated that the wheat/*Am. muticum* introgressions and complete chromosomes were stably transmitted to the next generation with one exception. DH-28 was found to be heterozygous for a telosome derived from *Am. muticum* linkage group 6 (**Figure 2g**). As a result, the progeny

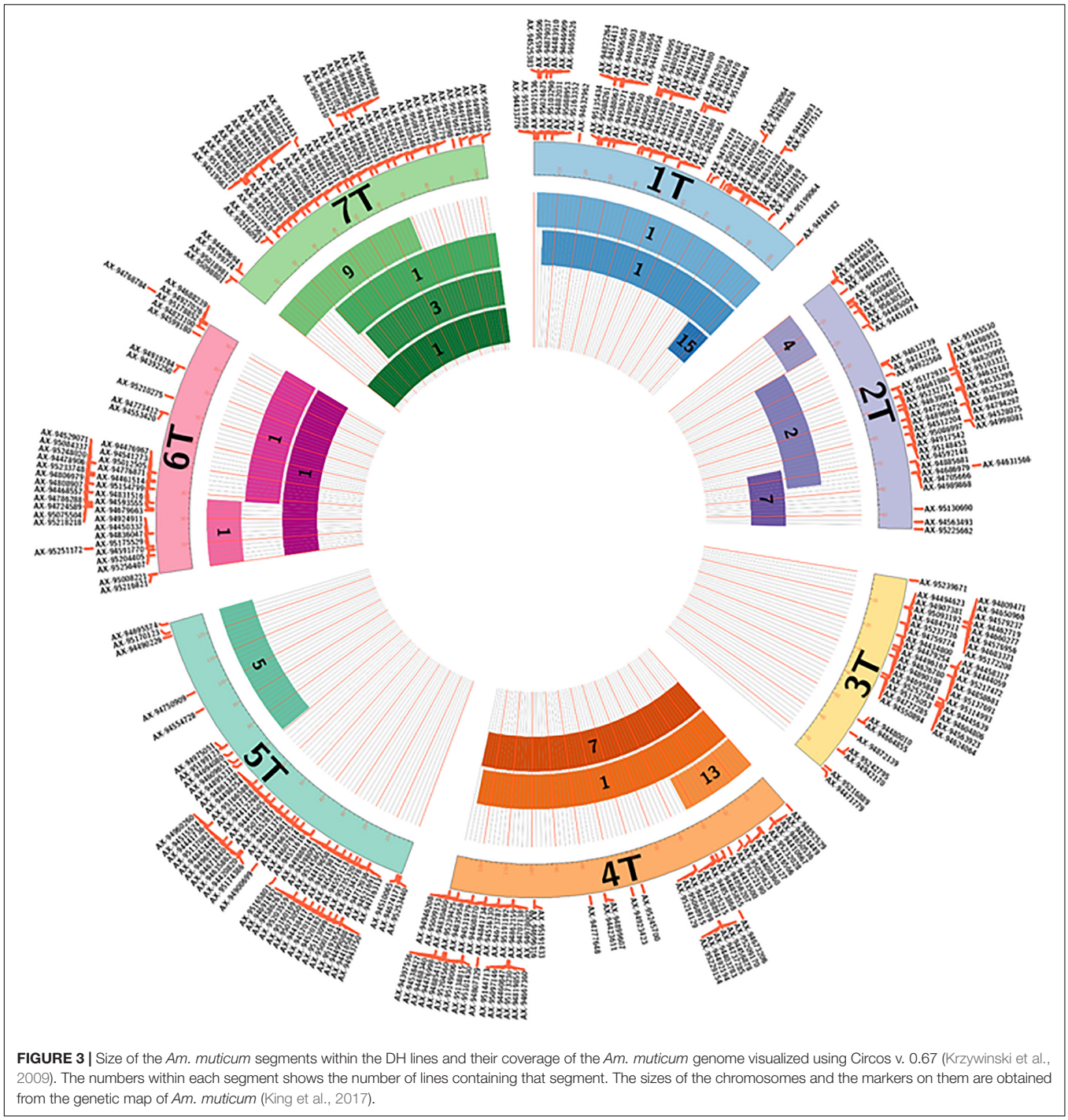

FIGURE 3 | Size of the *Am. muticum* segments within the DH lines and their coverage of the *Am. muticum* genome visualized using Circos v. 0.67 (Krzywinski et al., 2009). The numbers within each segment shows the number of lines containing that segment. The sizes of the chromosomes and the markers on them are obtained from the genetic map of *Am. muticum* (King et al., 2017).

derived from this DH segregated for the presence or absence of this chromosome.

The DH plants produced carried segments from linkage groups 1, 2, 4, 5, 6, and 7 (**Figure 3**) although the introgressed segments did not cover the whole of these linkage groups. Only one DH plant (DH-4) was found to contain a segment from linkage group 3. However, this plant was subsequently lost as the pollen fertility was very low and thus the line produced very few seed which were shriveled and failed to germinate.

McGISH also revealed that while the introgressions/chromosomes were largely stably inherited, the number of chromosomes of each wheat genome varied in some of the DH lines (**Table 1**, **Supplementary Table S1** and **Figure 2**). For example, DH-1 carried a pair of *Am. muticum* group 6 chromosomes but the number of A genome chromosomes varied, i.e., two plants carried 14 A genome chromosomes, 14 B chromosomes and 12 D chromosomes, while a third plant carried 16 A genome chromosomes, 14 B genome

chromosomes and 12 D genome chromosomes. In addition to wheat/*Am. muticum* introgressions, several lines also carried intergenomic wheat recombinants, e.g., A/B, A/D recombinants.

DISCUSSION

In the past, attempts to introduce genetic variation from a wild relative have generally focused on introgressing a single chromosome segment carrying genetic variation for a single trait [frequently using substitution lines/addition lines as a starting point (King et al., 2016)]. In contrast the objective of the work described here is not focused on just single traits, i.e., we are attempting to identify useful genetic variation for a wide range of traits from *Am. muticum* for future exploitation. In order to do this, we aim to generate very large numbers of introgressions in wheat from *Am. muticum* (ideally, we would like to introgress the entire genome of *Am. muticum* into wheat). In order to identify as much genetic variation as possible, a wide range of trait analyses will be performed on each introgression line generated (by ourselves and collaborators in both the public and private sectors globally). In addition, all lines derived from the (BBSRC funded) Wheat Research Centre at the University of Nottingham will be made available upon request (subject to handling charges, e.g., phytosanitary certificates).

A key factor in this strategy is the bulking and distribution of seed for trait analysis. However, before seed can be bulked each individual introgression must first be in a homozygous state to ensure that it is stably inherited to the next generation (all the introgressions we generate are initially in a heterozygous state and thus, any progeny derived from them will segregate for their presence and absence). The generation of DH lines in the work described in this paper represents one of the methods we are employing to generate homozygous introgression lines. In this work, 56% (32) of BC_3 plants carrying an *Am. muticum* chromosome or introgression produced DHs as compared to 92% (11) of BC_3 plants which did not carry *Am. muticum* chromosomes or introgressions. From the 32 BC_3 plants a total of 220 DH plants were produced, but only 68 of those that produced seed carried *Am. muticum* introgressions or chromosomes (with one of these lines being subsequently lost). These results indicate that the DH technique has resulted in the successful generation of homozygous introgressions albeit at a relatively low frequency. However, further work is required to optimize the protocols used to increase the frequency of DH generation from lines carrying introgressions and chromosome segments from the wild relatives of wheat, e.g., 2,4-D concentration, timing of embryo excision, colchicine concentration, etc.

One of the key objectives of the work outlined above is to introgress the entire genome of *Am. muticum* into wheat. The lines described here do not cover the entire genome as shown in **Figure 3**. In particular, the stable lines produced do not contain any segments from linkage group 3 of *Am. muticum*. However, it is difficult to establish at this stage if the regions not represented point to regions of the genome that are recalcitrant to transmission or are simply not represented due to the relatively small sample size. We also did not observe any examples of where an introgression was detected by SNP analysis that was not detected by GISH analysis (cryptic introgressions). However, again due to the relatively small sample size, it was not possible to determine if cryptic introgressions do or do not occur.

Initially, lines homozygous for large introgressions are being generated, distributed, e.g., Australia, United States, commercial breeding companies, and are being used for preliminary trait analyses. This initial analysis will enable the determination of which regions of the genome of *Am. muticum* carry genetic variation for target traits. The second stage of analysis will focus on the analysis of small introgressions derived from the large regions that have been found to carry target genetic variation (homozygous lines will need to be generated for each of the small introgression lines prior to the distribution for trait analysis). In this way we will identify the smallest introgression that carries the gene(s) controlling the target trait (the smaller the introgression the less likely it will be that it will carry deleterious genes in addition to the target gene). If small introgressions are not available, then overlapping introgressions will be intercrossed to produce smaller ones as described by Sears (1955) or further introgressions will be generated.

A further requirement of the strategy being undertaken is that once homozygous, each introgression must be stably inherited. Out of the viable 67 DH lines generated only one, DH-28 (1.5%), was not stably inherited. The remaining 66 DH (98.5%) were found to be stably inherited.

A number of abnormalities were observed within the wheat genome, e.g., the number of chromosomes of the three wheat genomes was occasionally found to vary from the euploid condition (i.e., 14 A, 14 B and 14D chromosomes). In addition, intergenomic recombinants were observed between the three genomes of wheat. The reason for these abnormalities may result from the strategy that was employed to generate introgressions, i.e., euploid wheat was pollinated with *Am. muticum* to produce an interspecific F_1 hybrid which was then backcrossed to euploid wheat to produce a BC_1 population. The F_1 hybrids generated were haploid for each of the three wheat genomes and the *Am. muticum* genome and thus the only recombination that could occur was between homoeologous chromosomes (King et al., 2017, 2018). However, while this strategy resulted in the generation of a high frequency of wheat/*Am. muticum* recombination and hence introgressions it also appears to have led to the generation of homoeologous recombination between the three genomes of wheat.

The variable aneuploid number of A, B and D genome chromosomes was probably also derived from the interspecific F_1s, i.e., the haploid genome complement of the F_1 would have resulted in the production of unbalanced gametes and thus variable numbers of A, B and D genome chromosomes in the BC_1 generation. In order to restore the diploid chromosome complement of the wheat genome and to remove any wheat/wheat intergenomic recombinants, further backcrossing will be required.

Of the 66 stable DHs generated, 23 that carry large segments, and upon request from our collaborators a further five carrying small introgressions, have now been released. The remaining DHs will be made available in the near future. In this program, we have demonstrated that DH procedures can be used to generate homozygous introgression lines. However, in addition to using DH procedures we are also generating homozygous introgression lines via self-fertilization of heterozygous lines and progeny testing. To assist us in identifying homozygous introgression lines (produced either by DH technology or by self-fertilization) we are developing circa 1000 KASP markers to facilitate selection.

In this paper, we have only described work on one wild relative, i.e., *Am. muticum*. However, we are working on a number of other species and we aim to use DH techniques and self-fertilization to initially produce large homozygous introgressions that span the genomes of these relatives (and smaller homozygous introgressions as required). Thus, over the coming years, many hundreds of homozygous introgression lines will be made available for trait analysis. In this way we intend to facilitate the large-scale exploitation of genetic variation from the wild relatives of wheat for wheat improvement.

In the past, there has been some reticence in using genetic variation from the wild relatives of wheat, mainly stemming from the fact that target genes may also be associated with deleterious genes. However, the development of new technologies provides the means by which this problem can now be overcome. We believe the biggest threat to the exploitation of genetic variation from wheat's wild relatives, lies in the fact that whereas there were hundreds of active scientists in the field in the 1970s and 1980s, very few with the requisite expertise now remain.

AUTHOR CONTRIBUTIONS

JK, SG, C-yY, SH-E, DS, SA, and IK carried out the crossing program. CN and AS carried out the doubled haploid production. SG analyzed the genotyping data. C-yY carried out the genomic *in situ* hybridization. IK wrote the manuscript with assistance from JK. All authors have read and approved the final manuscript.

FUNDING

This work was supported by the Biotechnology and Biological Sciences Research Council [grant number BB/P016855/1] as part of the Designing Future Wheat Programme (DFW). The funding body played no role in the design of the study and collection, analysis, and interpretation of data, and in writing the manuscript.

ACKNOWLEDGMENTS

We thank Prof. Keith Edwards, Dr. Alexandra Allen, and Dr. Amanda Burridge for their technical assistance in the genotyping.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00034/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 07 February 2019
doi: 10.3389/fpls.2019.00054

Genome-Wide Association Mapping of Grain Micronutrients Concentration in *Aegilops tauschii*

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Bread wheat is an important and the most consumed cereal worldwide. However, people with predominantly cereal-based diets are increasingly affected by micronutrient deficiencies, suggesting the need for biofortified wheat varieties. The limited genetic diversity in hexaploid wheat warrants exploring the wider variation present in wheat wild relatives, among these *Aegilops tauschii*, the wild progenitor of the bread wheat D genome. In this study, a panel of 167 *Ae. tauschii* accessions was phenotyped for grain Fe, Zn, Cu, and Mn concentrations for 3 years and was found to have wide variation for these micronutrients. Comparisons between the two genetic subpopulations of *Ae. tauschii* revealed that lineage 2 had higher mean values for Fe and Cu concentration than lineage 1. To identify potentially new genetic sources for improving grain micronutrient concentration, we performed a genome-wide association study (GWAS) on 114 non-redundant *Ae. tauschii* accessions using 5,249 genotyping-by-sequencing (GBS) markers. Best linear unbiased predictor (BLUP) values were calculated for all traits across the three growing seasons. A total of 19 SNP marker trait associations (MTAs) were detected for all traits after applying Bonferroni corrected threshold of $-\log_{10}(P\text{-value}) \geq 4.68$. These MTAs were found on all seven chromosomes. For grain Fe, Zn, Cu, and Mn concentrations, five, four, three, and seven significant associations were detected, respectively. The associations were linked to the genes encoding transcription factor regulators, transporters, and phytosiderophore synthesis. The results demonstrate the utility of GWAS for understanding the genetic architecture of micronutrient accumulation in *Ae. tauschii*, and further efforts to validate these loci will aid in using them to diversify the D-genome of hexaploid wheat.

Keywords: wild progenitors, *Aegilops tauschii*, micronutrients, GWAS, biofortification

OPEN ACCESS

Edited by:
Vijay Kumar Tiwari, University of Maryland, College Park, United States

Reviewed by:
Amita Mohan, Washington State University, United States
Igor Pacheco, Universidad de Chile, Chile
Raju Datla, National Research Council Canada (NRC-CNRC), Canada

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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *30 August 2018*
Accepted: *16 January 2019*
Published: *07 February 2019*

Citation:
Arora S, Cheema J, Poland J, Uauy C and Chhuneja P (2019) Genome-Wide Association Mapping of Grain Micronutrients Concentration in Aegilops tauschii. Front. Plant Sci. 10:54. doi: 10.3389/fpls.2019.00054

INTRODUCTION

The global population is anticipated to cross the mark of 9.7 billion by 2050. Ensuring food and nutritional security to this population poses a huge challenge especially under impending climatic variability and resource scarcity. Adequate intake of nutritious food enriched with essential micronutrients is a prerequisite for humans to meet their metabolic needs and maintain good

health. The term "micronutrients" refers to a broad list of minerals and vitamins that the body needs in adequate proportions to function properly. They play an important role in cell physiology as cofactors for proteins that carry out the fundamental biological functions (Tapiero et al., 2003). Some of the micronutrients are relatively scarce in common food sources, which can lead to their deficiencies in humans. People living in developing countries and who tend to rely heavily on cereal-based diets are particularly prone to suffer from micronutrient deficiencies, a phenomenon often termed as "hidden hunger" (Khush et al., 2012). Iron (Fe) deficiency is the most prevalent nutritional disorder in the world affecting 2 billion people worldwide and suboptimal zinc (Zn) nutrition is more common than previously believed (Stoltzfus and Dreyfuss, 1998; World Health Organiztion, 2006). These deficiencies may cause several physiological disorders, including impaired mental and physical development, anemia, tissue hypoxia, stunting, and blindness (Stevens et al., 2013).

Several strategies including food fortification, supplementation, and dietary diversification have been implemented to fight these deficiencies. However, the need to have a more sustainable and cost-effective solution continues to be pursued globally. Biofortification of existing crops, that is, the development of nutritionally enriched crop varieties, is one of the most powerful tools to address micronutrient malnutrition. It uses conventional breeding and/or biotechnology approaches to increase the micronutrient content in the edible part of staple crops. Wheat is one of the most important cereal crops serving as a staple food source for 30% of the human population. It provides up to 60% of the daily calories intake especially for people living in developing countries. Therefore, the nutritional quality of wheat has a significant impact on overall human health worldwide. Cultivated wheat, however, contains sub-optimal quantities of micronutrients with the majority of Fe and Zn localized to the seed aleurone and embryo, which are removed during milling. In different studies, the range of these micronutrients in wheat was reported between 28.8–50.8 mg/kg for Fe, 13.5–34.5 mg/kg for Zn (Zhao et al., 2009), 24–28 mg/kg for Mn, and 3.5–4.4 mg/kg for Cu (Suchowilska et al., 2012), while the HarvestPlus has established target levels of 52 and 33 mg/kg for Fe and Zn (Bouis and Welch, 2010), which is higher than or closer to the upper range of the aforementioned values.

Genetic biofortification of wheat varieties using both classical breeding approaches to characterize germplasm for mineral variability and marker-assisted selection (MAS) using gene-based markers can enhance the micronutrient content of the edible part as well as their bioavailability (Khush et al., 2012). However, a major bottleneck for wheat biofortification is the genetic erosion during domestication which limited the genetic variability for Fe and Zn in the cultivated wheat gene pool. The genetic variation of these micronutrients in wild wheat progenitors offers a potentially rich resource for the future genetic improvement of wheat nutritional value. The wild relatives of hexaploid wheat include *Aegilops tauschii*, *Triticum boeoticum*, *Triticum monococcum*, *Triticum dicoccoides*, *Aegilops kotschyi*, *Aegilops longissima*, and *Aegilops speltoides*, and have been reported among the most promising sources of high Fe and Zn grain concentration (Cakmak et al., 2000; Chhuneja et al., 2006; Rawat et al., 2009).

Ae. tauschii is an attractive resource for improving the genetic variability of micronutrients in cultivated wheat as it can recombine with the D-genome of hexaploid wheat. *Ae. tauschii* is a diploid ($2n = 14$, DD), self-pollinating (cleistogamic) goatgrass species in the Triticeae tribe of the grass family. It consists of two phylogenetic lineages, designated as L1 and L2, broadly associated with ssp. *tauschii* and ssp. *strangulata*, respectively (Wang et al., 2013). Using *Ae. tauschii* for biofortification requires an understanding of the genetic architecture of mineral nutrient accumulation in the grains. Mineral accumulation is a complex quantitative trait controlled by multiple genes and greatly affected by genetic × environment interactions. Therefore, it is important to dissect the genetic basis of variability governing Fe and Zn concentrations in the grains in order to exploit this variability in the development of micronutrient enriched cultivars.

Most genetics studies undertaken in wheat have used linkage mapping to study the genetic basis of micronutrient accumulation. This involves establishing linkage disequilibrium (LD) in populations derived from bi-parental crosses to identify genes/QTLs associated with the trait of interest. However, due to restricted number and position of meiotic events, the resolution of QTL mapping is often confined to 10–30 cM and it can analyze only a small fraction of total possible alleles that exist in the population from which the parents originated (Zhu et al., 2008). In contrast, association mapping (AM) offers an alternative to linkage mapping and can help identify alleles represented in a broader set of germplasm (Yu and Buckler, 2006). In this study, we report the investigation of the loci controlling accumulation of four micronutrients (Fe, Zn, Cu, and Mn) in *Ae. tauschii* germplasm through genome wide association studies (GWASs).

MATERIALS AND METHODS

Plant Material

A set of 167 *Ae. tauschii* accessions maintained at the Wheat Germplasm Collection, Punjab Agricultural University (PAU), Ludhiana (30° 52′N, 75° 56′E), were used in this study and the detailed information of these accessions was provided in Arora et al. (2017). Two bread wheat cultivars, PBW343 and WL711, were included in the study as reference checks for phenotypic variation observed in the *Ae. tauschii*.

Grain Digestion and Micronutrient Evaluation

During the normal cropping season, *Ae. tauschii* accessions were grown at PAU, Ludhiana, for three consecutive seasons with recommended agronomic practices. Each accession was planted in a single row of 2 m length with 0.7 m spacing between the rows. The spikes were harvested at maturity and stored in glassine bags. Precautions were taken to avoid any metallic or dust contamination of grains while harvesting and analyzing. For each accession the grains were divided into three parts and analyzed as three replicates for Fe, Zn, Cu, and Mn concentrations using simultaneous multi-element inductively coupled plasma–optical

emission spectrometer (ICP-OES, Perkin Elmer). Briefly, the whole grain samples were quickly washed with distilled water to remove any surface contamination and dried in hot air oven at 50°C for 24 h. The samples (0.5 g) along with operational blanks and standard solution of known concentrations were digested in 5 ml of distilled nitric acid (Analytical Reagent Grade, Merck) at 140°C for 45 min in a Microwave Digestion System (Perkin Elmer) to obtain clear digests. Following digestion, the volume of each sample was made up to 25 ml using Milli-Q water and elemental determination was performed by ICP-OES. For calculating the grain micronutrient concentration, the mean of element specific blank concentration was subtracted from each data point. The data were then multiplied by initial sample volume, divided by initial weight of grains, and expressed as μg element g^{-1} dry grain material (ppm) (Khokhar et al., 2018).

Statistical Analysis

The statistical parameters including mean, standard deviation, coefficient of variation (CV), frequency distribution, and analysis of variance (ANOVA) for the grain micronutrient concentrations were calculated in the R statistical package. The broad heritability [H^2 = VG/(VG + VE)] for each trait was estimated individually by considering genetic (VG), environmental (*VE*), and error variance (VE). Variance components for all traits were analyzed using general linear model to detect the effect of genotypes and years using one-way ANOVA. Phenotypic best linear unbiased predictor (BLUP) was estimated for each accession and trait using the lme4 package in R (Bates et al., 2014) and these values were used for correlation analysis between grain size and micronutrients concentration.

Genotyping and Marker Trait Association Analysis

Ae. tauschii accessions were genotyped using the genotyping-by-sequencing (GBS) method as described in Poland et al. (2012). Briefly, the raw Illumina data were trimmed to 64 bp tags and unique tags were internally aligned to find putative SNPs. The Fisher exact test was used to determine if the two alleles were independent SNP markers. The SNPs with minor allele frequency above 5% and missing data less than 70% were positioned in the Synthetic × Opata reference genome map (Chapman et al., 2015). Detailed information on SNP genotyping and population structure of these *Ae. tauschii* accessions has been described previously (Arora et al., 2017).

Genotyping-by-sequencing-based SNP markers were used to find the genetic identity between the accessions. From the group of accessions that had >99% genetic identity and high phenotypic similarity, only single accessions were retained for further marker trait association (MTA) studies. The AM was conducted for 114 non-redundant accessions using 5,249 SNP markers on the BLUP values of each phenotype. For conducting MTA, a R GWAS package called FarmCPU (Fixed and random model Circulating Probability Unification) (Liu et al., 2016) was used. It used first three components of PCA as covariate in the regression model and calculated the *p*-value threshold for each trait by using 1,000 permutations. The *p*-value distribution for four micronutrients was shown in quantile–quantile (Q-Q) plot. To search for the putative candidate genes associated with these markers, we determined the LD decay for both the lineages. The tags were mapped to the *Ae. tauschii* reference genome (Luo et al., 2017) to get their physical coordinates and LD estimates between marker pairs were obtained using TASSEL v5 for both the lineages. We took the 95th percentile of r^2 values as the estimator of short-range LD, and the distance at which this short-range LD is halved as the estimator of LD distance.

RESULTS

Phenotypic Variation

A wide range of variation for all four grain micronutrients was observed in the *Ae. tauschii* panel from the seed harvested in three consecutive years 2011–2013 (**Figure 1**). The variation ranged from 30.33 to 69.44 ppm (mean ± SD = 47.26 ± 7.58 ppm) for grain Fe, 17.54 to 49.78 ppm (30.73 ± 5.88 ppm) for grain Zn, and 1.02 to 6.50 ppm (3.62 ± 1.02 ppm) for grain Cu and 15.02 to 59.10 ppm (33.58 ± 7.7 ppm) for grain Mn concentration. A total of 2.28-, 2.83-, 6.37-, and 3.93-fold variation for Fe, Zn, Cu, and Mn, respectively, was observed among the 167 *Ae. tauschii* accessions. Grain Mn concentration had the highest heritability (0.67), while Zn had the lowest heritability (0.37). For Fe and Cu, the heritability estimates were 0.42 and 0.53, respectively (**Table 1**). Both Fe and Zn concentrations were slightly higher in 2011 than 2012 and 2013 whereas Cu concentration was higher for 2013 (**Supplementary Figure S1**). This variation can be attributed to environmental effects. ANOVA showed significant effects of the genotypes and the year on micronutrient concentration in grains. Compared with the two bread wheat cultivars used as check in the study, PBW343 and WL711, the concentration of all four micronutrients was significantly higher in the *Ae. tauschii* germplasm (**Supplementary Table S1**). Both these wheat lines are widely grown cultivars in India, especially PBW343 which has the 1BL/1RS translocation.

Relationship Between Grain Micronutrients and Grain Size

The phenotypic values for the 3 years were converted into BLUP values to get unbiased mean estimates. A strong positive linear relationship was found between BLUPs and mean values with the shrinkage of BLUPs toward the population average. The BLUP values depicted a normal distribution for grain Fe, Zn, and Cu concentration (**Supplementary Figure S2**). Significant positive correlations between grain Fe, Zn, and Cu concentrations were observed, while Mn did not show any significant correlation with other minerals (**Figure 2**). As micronutrient concentrations are highly influenced by the environment, correlations of the four micronutrients were also assessed across years. A strong positive correlation was observed between years for grain Mn concentration (r = 0.65–0.70), whereas for grain Cu (r = 0.49–0.56), Fe (r = 0.37–0.45), and Zn (r = 0.26–0.44), moderate positive correlations were found between the years (**Supplementary Figure S3**). The high correlation observed for

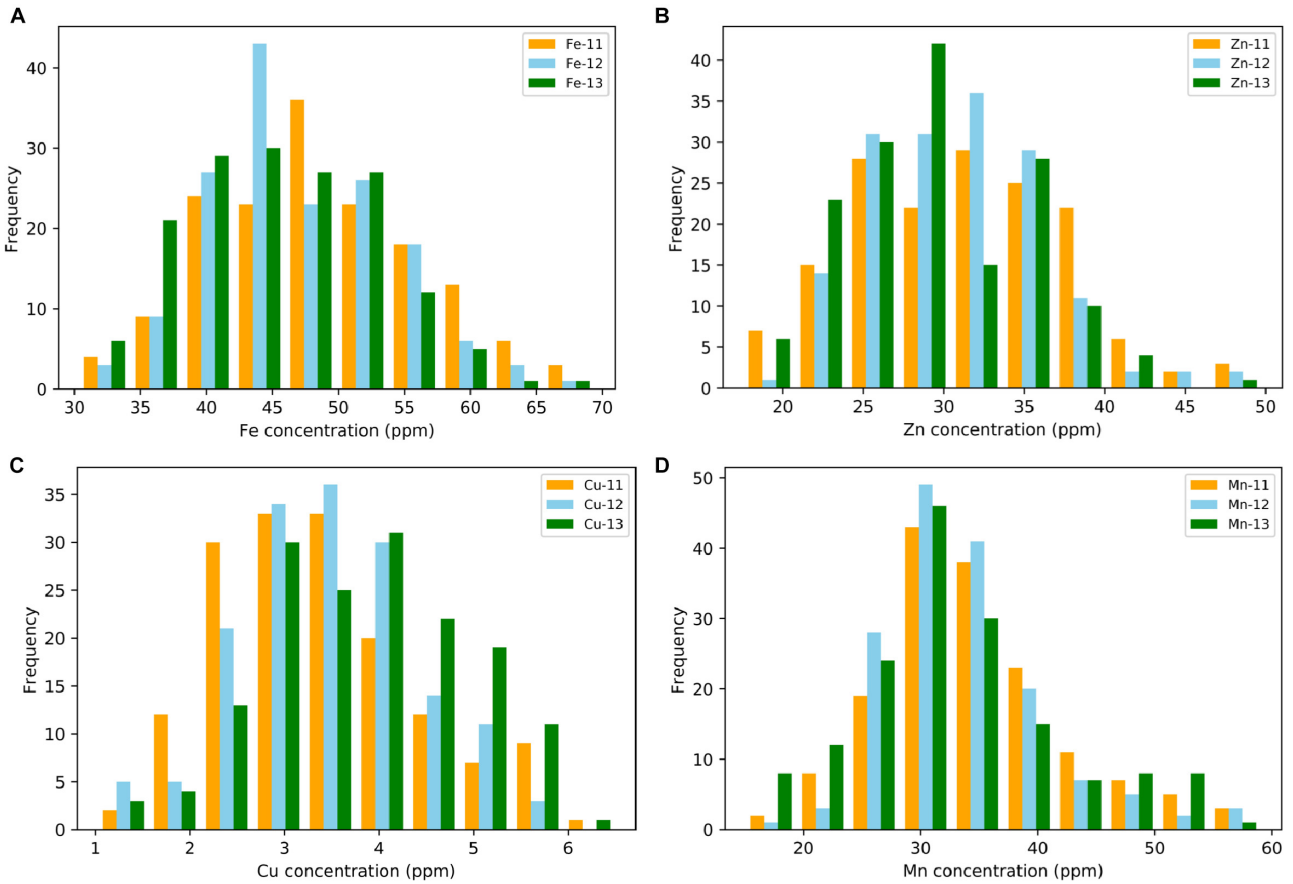

FIGURE 1 | Phenotypic distribution for grain **(A)** iron—Fe, **(B)** zinc—Zn, **(C)** copper—Cu, and **(D)** manganese—Mn in year 2011, 2012, and 2013.

TABLE 1 | Descriptive statistics, broad sense heritability (H^2), and *F*-value from analysis of variance for the grain micronutrients concentration in year 2011, 2012, and 2013.

Micronutrient	Year	Mean ± SD (ppm)	CV%	Range		H^2	*F*-values from ANOVA	
				Min	Max		Year	Genotype
Iron	2011	48.55 ± 8.23	16.9	31.10	69.44			
	2012	47.36 ± 6.98	14.7	30.33	65.59			
	2013	45.88 ± 7.28	15.8	30.82	69.39			
	2011–2013	47.26 ± 7.58	16.0	30.33	69.44	0.42	8.07***	3.12***
Zinc	2011	31.48 ± 6.21	19.7	17.54	47.06			
	2012	30.85 ± 5.55	17.9	19.90	49.78			
	2013	29.86 ± 5.81	19.4	18.14	46.68			
	2011–2013	30.73 ± 5.88	19.1	17.54	49.78	0.37	4.87**	3.03***
Copper	2011	3.38 ± 1.05	30.3	1.02	6.50			
	2012	3.46 ± 0.91	26.0	1.35	5.88			
	2013	3.82 ± 1.05	26.9	1.20	6.27			
	2011–2013	3.62 ± 1.02	28.4	1.02	6.50	0.53	18.6***	4.14***
Manganese	2011	34.42 ± 7.95	24.6	16.07	59.10			
	2012	33.41 ± 6.83	21.0	16.25	57.62			
	2013	32.91 ± 8.53	27.0	15.02	55.28			
	2011–2013	33.58 ± 7.7	24.3	15.02	59.10	0.67	4.13*	7.71***

*ns: not significant; ***, **, and *, significant at $P < 0.001$, $P < 0.01$, and $P < 0.05$, respectively.*

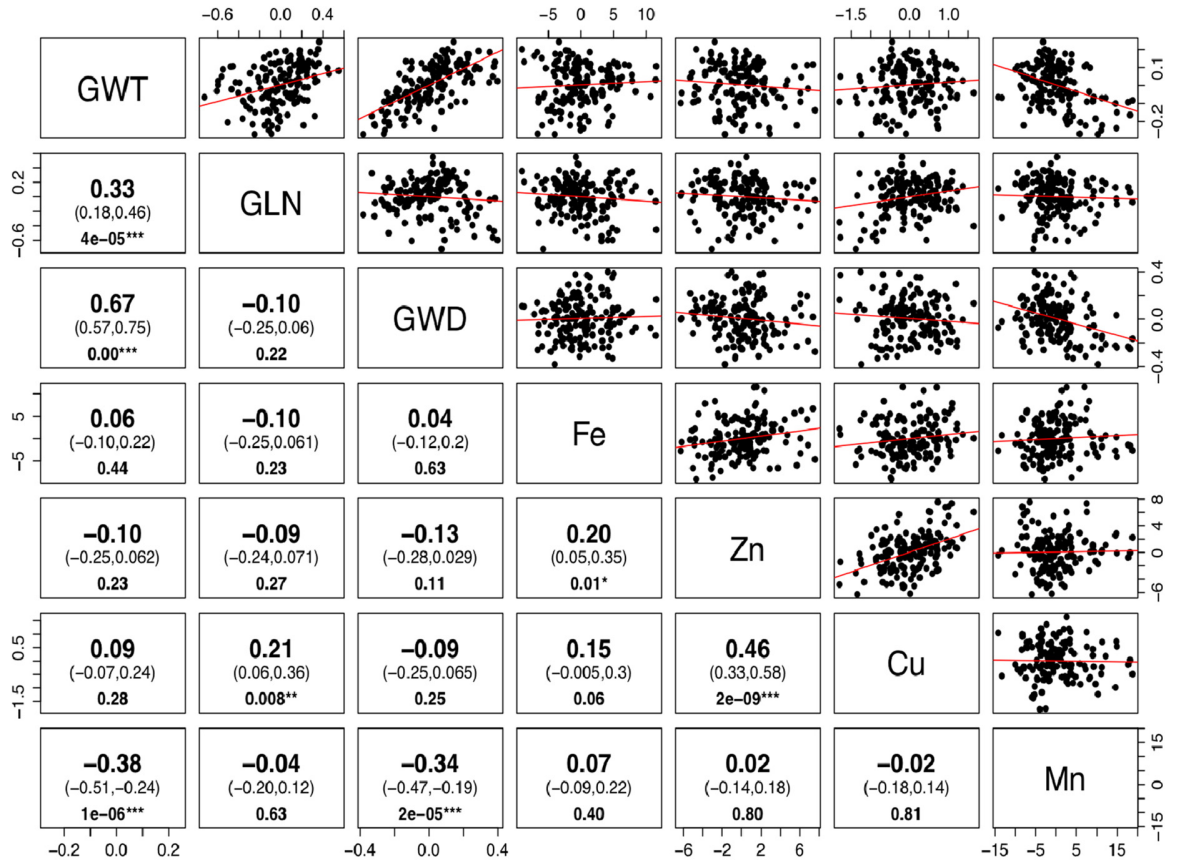

FIGURE 2 | Correlation for grain size and micronutrients concentration in *Ae. tauschii* accessions. Phenotypic correlations between 50-grain weight (GWT), grain length (GLN), grain width (GWD), iron (Fe), zinc (Zn), copper (Cu), and manganese (Mn) concentrations. The upper and lower 95% confidence intervals are included in parenthesis below the correlation value. *P*-value for significant correlations is shown at the bottom. (Note: ***, significant at $P < 0.001$; **, significant at $P < 0.01$; *, significant at $P < 0.05$.)

Mn between the years also explains its high heritability value compared to other micronutrients.

There is a perception that higher micronutrient concentration in wild species is a result of concentration effects due to smaller seeds. To determine whether seed size has any significant effect on micronutrient concentrations, we estimated correlations between the BLUP values for grain micronutrient concentrations and grain weight of these accessions. Very weak to almost no correlation was observed between grain weight and grain Fe, Zn, and Cu concentrations with Pearson correlation coefficient of 0.06, −0.10, and 0.09, respectively. Grain Mn concentration, however, had significant but negative correlation with grain weight (**Figure 2**). Detailed dissection of grain architecture has been reported in Arora et al. (2017).

Variation Between Lineages

Ae. tauschii is genetically divided into two lineages which are referred to as L1 and lineage 2 (L2). L1 predominantly encompasses accessions belonging to subspecies *tauschii* and L2 accessions belonging to ssp. *strangulata*. Significant differences ($p > 0.05$) in the mean values of grain Fe, Cu, and Mn concentrations were detected between the two lineages; however, no significant difference was observed for zinc concentration (**Supplementary Table S2**). Overall, L2 had significantly higher concentrations of grain Fe and Cu than L1, whereas L1 had higher Mn concentration than L2 as depicted by box plots in **Figure 3**.

In this study, we have identified several accessions with high mean value for Fe, Zn, and Cu concentrations. Three accessions pauT14334, pauAT14145, and pauAT3751 had high iron concentration with an average of 63.78, 63.67, and 62.58 ppm, respectively. Four accessions pauAT14360, pauAT14136, pauAT14158, and pauAT14139 accumulated the highest concentration for both iron and zinc with an average of 56.73, 53.25, 55.28, and 56.13 ppm iron and 41.70, 40.79, 38.74, and 39.80 ppm zinc, respectively. Accession pauAT14162 was found to have high concentrations of both Zn and Cu with mean values of 46.45 and 5.25 ppm, respectively. The accessions reported for higher Mn concentration were moderate for other micronutrients. Most accessions with higher concentration of Fe, Zn, and Cu belonged to L2; however, accessions with higher Mn concentration belonged to L1 (**Table 2**). This was also observed when the two lineages were compared for all micronutrients (**Figure 3**).

TABLE 2 | List of selected accessions of *Ae. tauschii* with high grain iron (Fe), zinc (Zn), copper (Cu), and manganese (Mn) concentrations.

Accession	Fe (ppm)	Accession	Zn (ppm)	Accession	Cu (ppm)	Accession	Mn (ppm)
pau14334^{L2}	63.78	pau14162 ^{L1}	46.45	pau14209^{L2}	5.67	pau3826^{L1}	55.52
pau14145^{L2}	63.67	pau14118^{L2}	42.02	pau14181^{L2}	5.31	pau14354^{L1}	54.83
pau3751^{L2}	62.58	pau14360 ^{L2}	41.70	pau14162 ^{L1}	5.25	pau3759^{L1}	54.32
pau14252^{L1}	59.18	pau14096^{L2}	40.94				
pau14962^{L1}	58.99	pau14136 ^{L2}	40.79				
pau14206^{L2}	58.48	pau9822^{L1}	39.67				
pau3544^{L2}	58.42	pau14111^{L1}	39.11				
pau14159^{L2}	57.74	pau14158 ^{L2}	38.74				
pau14360 ^{L2}	56.73	pau14139 ^{L2}	39.8				
pau14139 ^{L2}	56.13						
pau14200^{L2}	55.65						
pau14165^{L2}	55.52						
pau14158 ^{L2}	55.28						
pau17^{L2}	55.04						
pau3769^{L2}	55.02						
pau14136 ^{L2}	53.25						

The lineage is mentioned for each accession as superscript. Accessions with both high Fe and Zn are colored gray and those with high Zn and Cu are colored turquoise green.

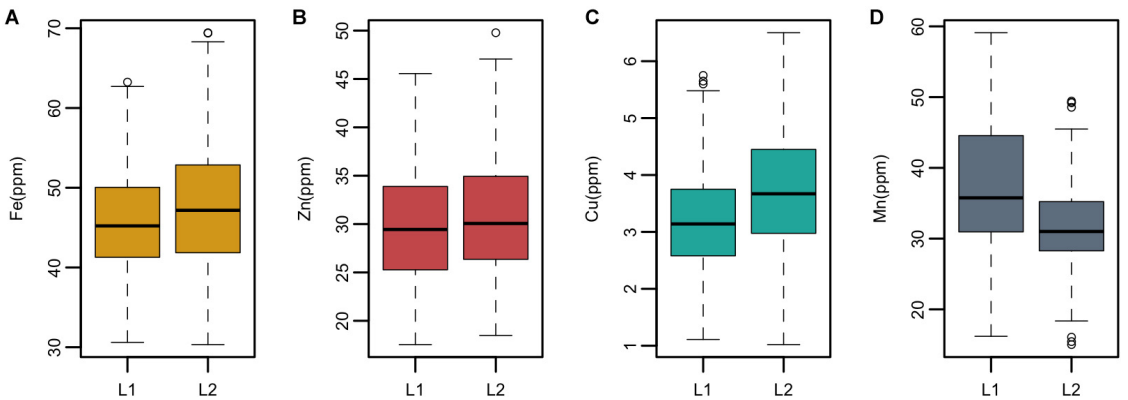

FIGURE 3 | Boxplots showing mean, median, and range of phenotypic variation between the two lineages L1 and L2 of *Ae. tauschii* for grain **(A)** iron—Fe, **(B)** zinc—Zn **(C)**, copper—Cu, and **(D)** manganese—Mn.

Detection of Marker Trait Associations

The genetic basis of accumulation of Fe, Zn, Cu, and Mn in the grains of *Ae. tauschii* was studied using genome wide AM. GWAS analysis was performed using FarmCPU for 114 non-redundant *Ae. tauschii* accessions with 5,249 SNP markers. Population structure for this panel was inferred by principal component analysis (PCA) in our previous study (Arora et al., 2017). The accessions were divided into two major clusters, L1 and L2, with some intermediate accessions represented as admixture (**Supplementary Figure S4**). The FarmCPU used first three components of PCA as covariate in association analysis. In FarmCPU, the default *p*-value threshold is the Bonferroni-corrected threshold (indicated by the green line in Manhattan plots). As the Bonferroni-corrected threshold is overly strict, it allows to calculate threshold using the "p.threshold" function which permutes the phenotypes to break the relationship with the genotypes. We permuted the phenotypes 1,000 times, a vector of minimum p value of each experiment was outputted and the 95% quantile value of the vector was used as p.threshold in this study. This method gave −log(*p*-value) of 4.68 which was used as a cut-off to define significant associations. There were 19 MTAs above the threshold −log(P) score of 4.68, distributed on all the seven *Ae. tauschii* chromosomes. The details of these MTAs are summarized in **Table 3** and depicted as Manhattan plots in **Figures 4A–D**. The Q-Q plots illustrating observed associations between SNPs and grain micronutrient concentrations compared to expected associations after accounting for population structure are presented in **Figures 4E–H**.

A total of five, four, three, and seven MTAs were detected for Fe, Zn, Cu, and Mn, respectively, with log(*p*-value) of ≥4.68. For Fe, the most significant MTA was detected on chromosome 4D followed by chromosomes 2D, 1D, 7D, and 3D (**Figure 5**). For Zn, the significant MTAs were detected on chromosomes 2D, 4D, 6D, and 7D. Fe and Zn MTAs on 4D were located in

TABLE 3 | List of significant marker loci associated with BLUP values of grain micronutrient (Fe, Z, Cu, Mn) concentration.

Trait	SNP ID	Chromosome	Position [#](cM)	*p*-value	MAF	Effect*	−log(*p*-value)
Fe	AT68157	4D	66.6	2.16E-07	0.20	3.45	6.67
	AT76904	2D	89.9	2.35E-06	0.23	2.38	5.63
	AT45556	1D	143.5	4.45E-06	0.22	−2.98	5.35
	AT2276	7D	51.6	5.80E-06	0.40	4.03	5.24
	AT88633	3D	120.8	2.07E-05	0.26	1.47	4.68
Zn	AT2707	2D	19.7	1.08E-09	0.21	3.39	8.97
	AT65894	4D	65.5	1.61E-05	0.12	2.80	4.79
	AT77346	6D	29.8	1.63E-05	0.18	−2.16	4.79
	AT92754	7D	1.1	1.98E-05	0.33	2.59	4.70
Cu	AT75576	5D	151.8	1.03E-07	0.28	−0.76	6.99
	AT62347	1D	55.9	3.86E-06	0.07	0.49	5.41
	AT37896	6D	58.6	2.04E-05	0.21	0.31	4.69
Mn	AT105092	6D	144.0	1.55E-07	0.49	−5.74	6.81
	AT102954	4D	1.0	1.61E-07	0.19	−5.96	6.79
	AT33443	5D	27.6	8.56E-07	0.23	2.39	6.07
	AT359	5D	89.6	1.60E-06	0.14	2.68	5.80
	AT78733	7D	117.5	2.29E-06	0.11	3.16	5.64
	AT4038	7D	71.6	6.51E-06	0.41	2.17	5.19
	AT102015	2D	64.6	1.31E-05	0.11	−2.34	4.88

MAF, minor allele frequency.

[#]Position of the SNPs is according to POPSEQ data (Chapman et al., 2015; Edae et al., 2015).

An allelic effect size is the magnitude of the effect of an allele on a phenotype. Sign of the allelic effect estimate is with respect to the nucleotide that is second in alphabetical order. For associated SNP markers, where positive effects have been reported second allele in alphabetical order is favorable, and where negative effects have been reported, the first allele is favorable. This is illustrated by allele specific boxplots in* ***Figure 6**.*

the mapping bins 1.1 cM apart. MTAs for Cu were found on chromosomes 5D, 1D, and 6D while Mn MTAs were mapped on chromosomes 6D, 4D, 5D, 7D, and 2D. The allelic effects of the significant linked SNP markers were determined by calculating mean grain micronutrient concentrations for both the SNP alleles individually and represented as box plots in **Figure 6**.

Candidate Genes

To define the search space for putative candidate genes in the vicinity of associated markers, the LD decay distance was determined for both the lineages. The decay for L1 and L2 was at 98 and 177 kb, respectively (**Supplementary Figure S5**). We first used the Wheat reference Chinese Spring (CS) RefSeq v1.0 genome (Internatioanl Wheat Genome Sequence Consortium, 2018) to search for genes, since it has been well-annotated compared to *Ae. tauschii* reference, and took the L2 LD block (177 kb) to define the gene search space around the marker because of the proximity of CS D-genome to the L2 (Wang et al., 2013). We mapped the SNP markers to CS RefSeq v1.0 genome (Ref) and fetched the annotated genes in 177-kb region around the marker[1]. For these candidates, we looked for gene networks on KnetMiner[2] and reported the candidates that were associated with micronutrient accumulation (**Table 4**).

Based on these search criteria, we found some candidate genes in the vicinity of these markers that were associated with vesicle transport, development, and transcription regulation. The Fe MTA AT45556 on chromosome 1D mapped in close proximity of the gene ADP- ribosylation factor (ARF), important in vesicle transport and involved in the diurnal changes in mugineic acid family phytosiderophores (MAs) secretion (Nozoye et al., 2004; D'Souza-Schorey and Chavrier, 2006). Another candidate gene for Fe concentration underlying marker AT2276 on chromosome 7D encodes an AT-hook motif nuclear localized protein which functions in the regulation of gene expression.

The Zn MTA AT65984 on chromosome 4D mapped adjacent to an abscisic acid-induced protein, HVA22 which inhibits gibberellin (GA)-mediated programmed cell death in cereal aleurone cells and acts as a positive factor for metal accumulation under stress conditions (Singh et al., 2014). AT2707, associated with Zn concentration on chromosome 2D, lies close to the predicted Scarecrow-like 3 (SCL3) GRAS transcription regulator. It functions as a positive regulator to integrate and maintain a functional GA pathway (Zhang et al., 2011). An interesting candidate gene called ABC transporter is also associated with AT2707, and is involved in the export or import of a wide variety of substrates ranging from small ions to macromolecules. Another Zn MTA (AT77346 on 6D) showed association with a Malonyl-coenzyme A: anthocyanin 3-*O*-glucoside-6″-*O*-malonyltransferase gene.

Cu MTA AT75576 on 5D mapped to a guanylate-binding protein which has a critical role in the regulation of a range of cellular processes including growth, differentiation, and intracellular transportation. The region around marker AT105092 on 6D for Mn was associated with a gene coding for TCP transcription factors. It constitutes a plant-specific

[1]https://urgi.versailles.inra.fr/WheatMine/genomicRegionSearch.do

[2]http://knetminer.rothamsted.ac.uk

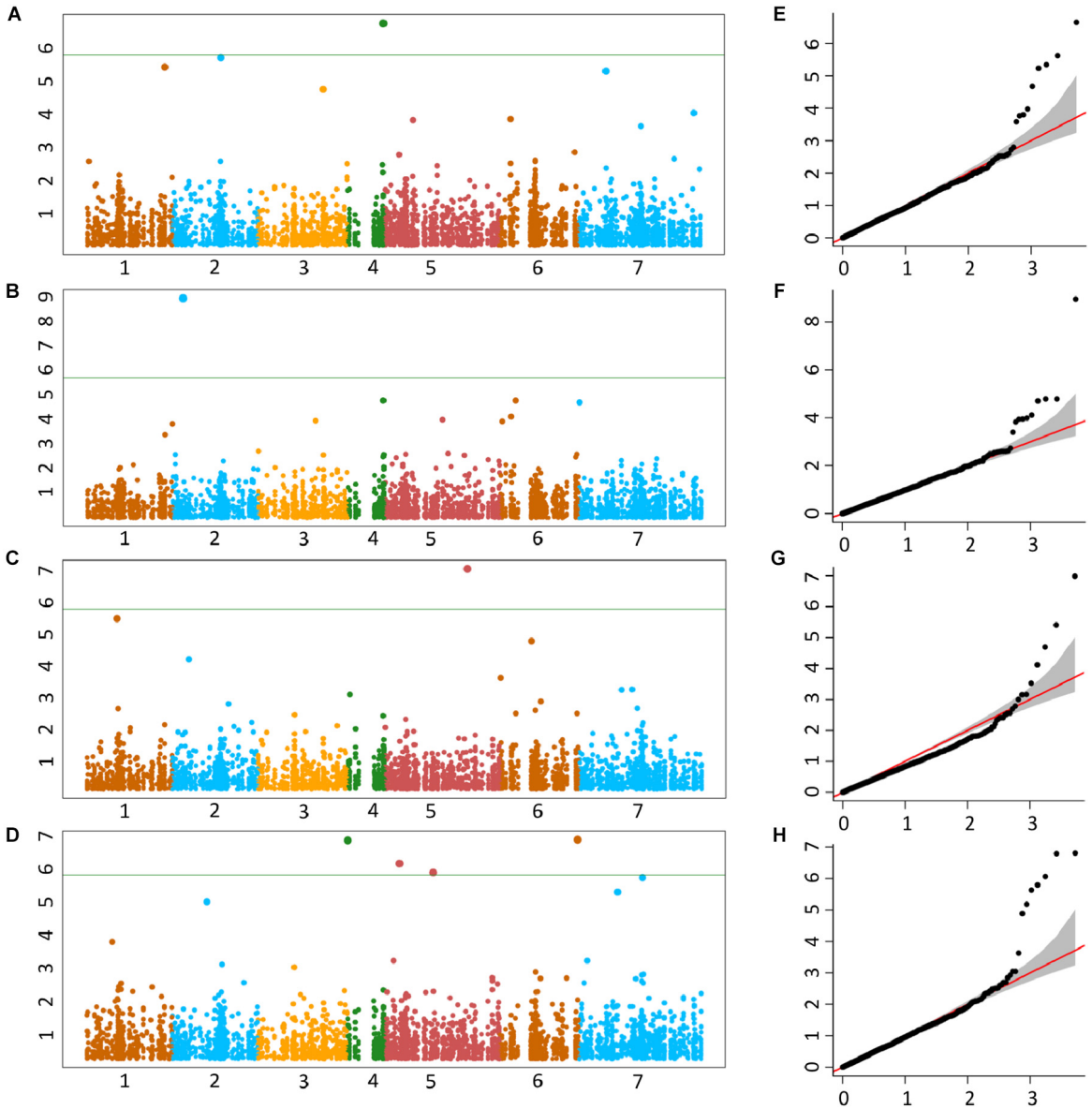

FIGURE 4 | Manhattan plots representing seven chromosomes carrying the significant markers detected by MLM models using BLUP values for grain **(A)** Fe, **(B)** Zn, **(C)** Cu, and **(D)** Mn. Quantile–quantile (Q-Q) plots for grain Fe, Zn, Cu, and Mn **(E–H)** showing expected null distribution of *p*-values, assuming no associations, represented as red solid line; distribution of *p*-values observed using mixed linear model (MLM) represented as a black dots.

family of developmental regulators and shares a conserved region that is predicted to form a non-canonical basic helix-loop-helix DNA-binding domain called the TCP domain (Cubas et al., 1999). 5D marker AT359 associated with grain Mn showed sequence similarity to auxin-responsive protein AtMHX, which regulates metal homeostasis mainly in tissues with photosynthetic potential (David-Assael et al., 2006). Mn marker AT102015 on 2D mapped to a gene coding for F-box domain containing protein. The extensive list of all the candidate genes associated with markers is provided in **Table 4** and further investigation is required to understand the role of these candidate genes in grain micronutrients concentration.

DISCUSSION

Micronutrient malnutrition affects more than 2 billion people in the world, with Fe and Zn among the essential minerals that are often lacking in human diets (White and Broadley, 2009). Fe is important for oxygen transportation and hemoglobin formation, whereas Zn plays a central role in growth, development, and in the immune system (Roohani et al., 2013; Abbaspour et al., 2014). WHO data estimate that Fe-deficiency anemia in children and adults results in 19.7 million DALYs (disability-adjusted life years), or 1.3% of global total DALYs (World Health Organiztion, 2009). Therefore, increasing Fe and Zn in human diets, especially

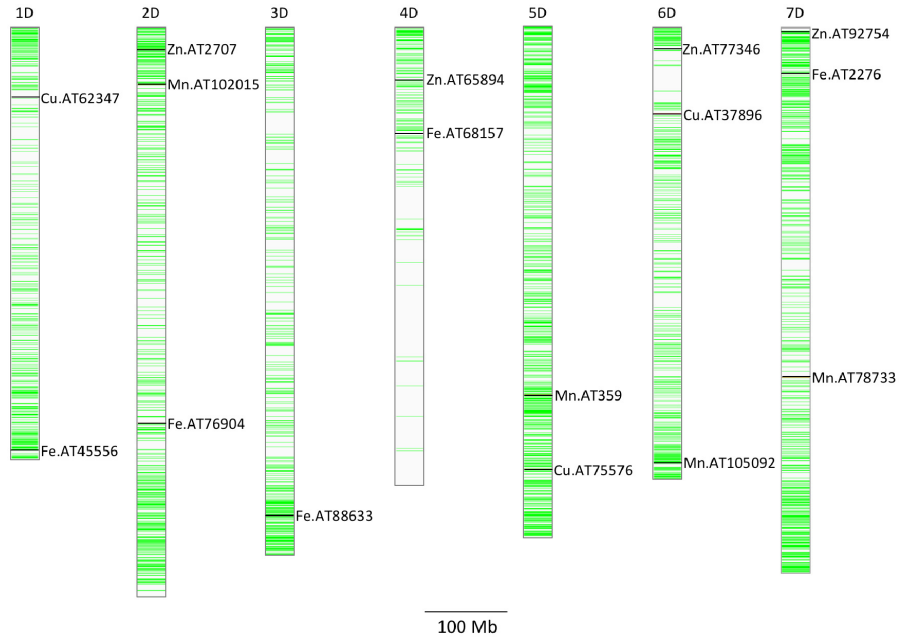

FIGURE 5 | Distribution of GBS SNP markers across the seven *Ae. tauschii* chromosome arms. The horizontal line color bars indicate the chromosomal position of the markers. The associated markers for micronutrients concentration reported in this study are indicated by black bars. Associated SNPs are indicated by name of the micronutrient followed by AT indicating *Ae. tauschii* and SNP number.

in developing countries which rely almost exclusively on cereal based diets, assumes tremendous significance.

Many studies have reported that there is a wide variation in grain Fe and Zn concentrations in wheat wild relatives. These levels of variation are significantly higher than those observed in elite wheat cultivars (Cakmak et al., 2000; Monasterio and Graham, 2000). The present investigation focused on elucidating the variation of four micronutrients in *Ae. tauschii*, the D genome donor of bread wheat. Free recombination between *Ae. tauschii* and D-genome chromosomes of bread wheat and the availability of its genome sequence makes it an attractive resource for wheat biofortification.

Ae. tauschii: Potential Source for Wheat Grain Micronutrients Enrichment

In the *Ae. tauschii* accessions, almost twofold genetic variation was observed for Fe and 2.8-fold for Zn followed by Mn (3.9-fold) and Cu (6.3-fold). Even the mean concentrations of Fe, Zn, and Cu in *Ae. tauschii* panel were 1.84, 1.43, and 1.72 times to that observed in the bread wheat checks planted and analyzed along with this germplasm set. In different studies, the concentrations of Fe and Zn in elite cultivars have been reported to vary between 25–56 and 13.5–39 mg/kg, respectively (Morgounov et al., 2007; Zhao et al., 2009). In contrast, in the *Ae. tauschii* accessions studied here, Fe was as high as 69 mg/kg and Zn up to 50 mg/kg. CIMMYT and Harvest Plus have used *Ae. tauschii* for developing synthetic hexaploid wheat which were found to have better accumulation of Fe and Zn in grains than *T. aestivum* (Calderini and Ortiz-Monasterio, 2003). These studies support our assertion that the D-genome is a promising source of high micronutrient concentrations. The accessions reported in this study with higher Fe and Zn can serve as a useful source for developing synthetic hexaploid wheat.

Grain micronutrient concentrations are quantitatively inherited traits, as shown by the continuous distribution. Genetic variance was low to moderate (range, 0.16–0.58), indicating high environmental influence on trait expression and/or complex genetic architecture. High genotype × environment interactions for grain nutrient concentrations have been reported for both wheat and wild emmer wheat (Oury et al., 2006; Morgounov et al., 2007; Chatzav et al., 2010). These studies suggested that genotype × environment interactions are non-cross over interactions, and therefore reasonable advances in selection and breeding can be expected.

Ae. tauschii is genetically divided into two diverse lineages which are referred to as L1 and L2. L1 consists of subsp. *tauschii var. typica* and *anathera* and L2 consists of subsp. *stangulata* and subsp. *tauschii var. meyeri* (Kihara and Tanaka, 1958). A very conspicuous observation was the differential accumulation of micronutrients in the two *Ae. tauschii* subspecies. The Student's *t*-test revealed significant ($p > 0.05$) difference in the mean values of grain Fe, Cu, and Mn concentrations between the two lineages; however, no significant difference was observed for Zn concentration. L2 accessions had the highest mean values for all traits studied except grain Mn which was highest in L1. Mansouri et al. (2013) evaluated 15 morphological characters of *Ae. tauschii* and concluded that ssp. *strangulata* has higher mean values for most of the traits including 100-grain weight. Research conducted

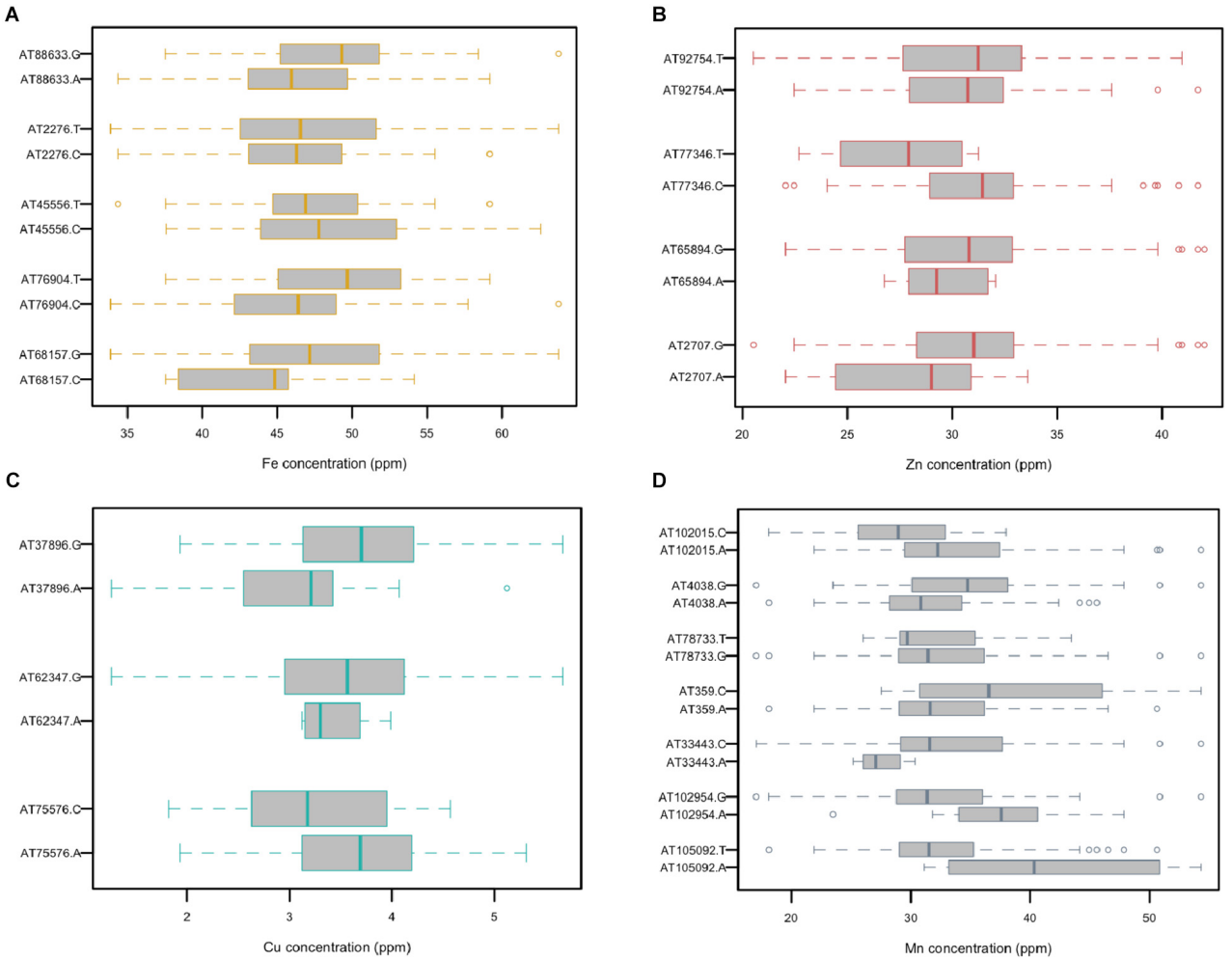

FIGURE 6 | Comparison of the allelic effects for the SNP markers associated with grain **(A)** Fe, **(B)** Zn, **(C)** Cu, and **(D)** Mn concentration.

by Chatzav et al. (2010) in wild emmer found significant differences in grain nutrient concentrations between the two groups (northern and southern), albeit of negligible magnitude. To our knowledge, these findings are the first study to report significant micronutrient differences between the two lineages of *Ae. tauschii*. So far, numerous genetic studies were conducted based on molecular markers to differentiate these lineages.

A significantly positive relationship was observed between grain Fe and Zn concentration (r^2 = 0.20) and Zn–Cu concentration (r^2 = 0.46). The positive correlation suggests that there could be common genetic factors affecting the accumulation of these micronutrients in grains. The existence of positive correlations between grain iron and zinc has been reported repeatedly in bread wheat (Zhao et al., 2009; Xu et al., 2012; Srinivasa et al., 2014), wild emmer (Cakmak et al., 2004; Peleg et al., 2008), and triticale (Feil and Fossati, 1995). However, the co-localization of QTL for grain Fe and Zn has been reported in tetraploid wheat (Uauy et al., 2006; Peleg et al., 2009).

Correlation coefficients between grain weight and Fe, Zn, and Cu concentrations were very low (r = 0.06, −0.10, and 0.09, respectively) and non-significant. The hypothesis that grain weight may affect grain micronutrients concentration was not supported by the data in this study, as no significant correlations were observed between grain size and Fe, Zn, and Cu accumulation. Identification of some of the *Ae. tauschii* accessions with larger seeds and higher micronutrient concentration (pau14360, pau14159, pau14139, pau14158, pau14334, pau14136) contradict this concept that higher micronutrient concentration in wild species is a result of concentration effect due to smaller seeds. Similar results showing no concentration effect in wild species were reported in A-genome diploid wheat (Tiwari et al., 2009), durum wheat (Ficco et al., 2009), *T. dicoccoides* accessions (Cakmak et al., 2004), and wheat cultivars (Morgounov et al., 2007). On the other side, Mn concentration was significantly and negatively correlated with grain weight and width (r = 0.38, r = 0.34, respectively). An interesting finding of this study is that L1 was found to have accessions with smaller grain size and higher Mn concentration. Of the four micronutrients studied,

TABLE 4 | Candidate gene predicted in genomic regions harboring grain micronutrient marker trait associations.

SNP ID	Candidate genes	Function
Fe/AT45556	Putative ADP-ribosylation factor	Vesicle transport
Fe/AT2276	AT-hook motif nuclear-localized protein	DNA binding motif
	FAD/NAD(P)-binding domain	Oxidation–reduction process
	Kinesin motor domain	Microtubule motor activity; organelle transport
	YEATS	Regulation of transcription
Fe/AT_68157	Pentatricopeptide repeat	RNA-binding proteins
	Glycosyl transferase, family 1	Acetylglucosaminyltransferase activity
	Response regulator receiver domain	Signal transduction response regulator
Fe/AT76904	FAD/NAD(P)-binding domain	Oxidation–reduction process
	Cytochrome oxidase assembly protein 1	Mitochondrial membrane protein
	WRKY domain	Transcription factor activity
	VHS and GAT domain	Vesicular trafficking
Zn/AT65894	HVA22-like protein with RNA recognition motif	Development
	WD40/YVTN repeat-like-containing domain	Protein binding
	NAC domain	Regulation of transcription
Zn/AT2707	Scarecrow-like 3 (SCL3)	GRAS transcription regulator
	UDP-glucuronosyl/UDP-glucosyltransferase	Regulation of ion transmembrane
	ABC transporter	ATPase activity coupled with transmembrane movement of substances
Zn/AT77346	Malonyl-coenzyme A: anthocyanin 3-*O*-glucoside-6′′-*O*-malonyltransferase	Development
	Ribosome-inactivating protein	rRNA N-glycosylase activity
	Catalase immune-responsive domain (CAT3)	Catalase activity
	Bifunctional inhibitor	Plant lipid transfer protein
Zn/AT92754	Zinc-binding in reverse transcriptase with zf-RVT domain	Not known
	Zinc finger, PMZ-type	Zinc ion binding
	Kelch-type beta propeller	Protein binding
	NB-ARC and LRR	ADP binding
Cu/AT75576	FAD/NAD(P)-binding domain; GDP dissociation inhibitor; Guanylate-binding protein	Oxidoreductase activity; protein transport
	BTB/POZ domain; MATH/TRAF domain	Protein binding
	Ulp1 protease family, C-terminal catalytic domain	Cysteine-type peptidase activity
Cu/AT62347	Reverse transcriptase zinc-binding domain	Zinc-binding in reverse transcriptase
Cu/AT37896	F-box domain	Protein binding
	EF-hand binding site	Protein binding
Mn/AT105092	TCP21-like	Transcription factor
	F-box domain; Phloem protein 2-like	Protein binding
Mn/AT359	Auxin-responsive protein AtMHX	Metal homeostasis
Mn/AT102015	F-box domain containing protein	Protein binding
	NADH-ubiquinone reductase complex 1	ATP generation
	K+ potassium transporter	Potassium ion transmembrane transporter activity
Mn/AT102954	Serine-threonine/tyrosine-protein kinase	Protein kinase activity
Mn/AT33443	Kinesin motor domain	ATP binding; microtubule motor activity
Mn/AT78733	Glycosyl transferase	Acetylglucosaminyltransferase activity

only Mn accumulation in grains was affected by smaller grain size (concentration effect).

Various studies had been conducted in wheat to map the QTLs responsible for Fe and Zn concentration. GWAS in *Ae. tauschii* identified QTL for grain micronutrient concentrations on all seven chromosomes with each chromosome harboring QTL for more than one micronutrient. Tiwari et al. (2009) mapped QTL for grain Fe and Zn concentration in a RIL population of diploid A genome wheat *T. monococcum* and *T. boeoticum*. The significant QTLs for grain Fe were located on chromosomes 2A and 7A and for Zn on chromosome 7A. *Ae. tauschii* chromosomes 2D and 7D also located one QTL each for grain Fe, Zn, and Mn though the locations of these QTL were different indicating independent genetic elements controlling these three traits. Another work by Shi et al. (2008) detected as many as four QTLs for grain Zn concentration and seven for grain Zn content. The QTL detected on chromosome 7A explained the highest level of phenotypic variation. Chromosome 5D and 6D did not map

any loci for grain Fe in the present study and 1D did not have any association with grain Zn and Mn.

Annotation of the 177-kb genomic regions in CS genome on either side of the SNPs associated with micronutrient grain content identified some genes hypothesized to be directly involved in micronutrient acquisition and translocation. The release of phytosiderophores (PSs) by grass species is considered a highly efficient Fe acquisition mechanism. These low-molecular-weight, nonproteinogenic amino acids form soluble complexes with Fe(III) that are taken up as the intact PS–metal complex, with Fe remaining in its oxidized form, Fe(III) (Oburger et al., 2014). MTA AT45556 annotated a putative ADP-ribosylation factor involved in vesicle transport and has been reported to contribute to diurnal changes in the expression of genes that participate in PS synthesis in rice (Nozoye et al., 2004). ADP-ribosylation factor might also be regulating synthesis of phytosedirophores from *Ae. tauschii* roots. *Aegilops* species have been reported to release two to four times higher PS than cultivated wheats (Kumari et al., 2010). The associated markers Mn/AT102015 and Zn/AT65984 were mapped close to the genes coding for abscisic acid-induced protein, HVA22, and F-box domain containing protein, respectively. Both these proteins were found to exhibit upregulation of transcripts in grains of the high mineral wheat variety compared to a low mineral variety. The proteins increased tolerance to stress during grain filling, which was suggested as a positive factor for metal accumulation (Singh et al., 2014). The SNP marker AT359 associated with auxin-responsive protein AtMHX is an auxin regulated vacuolar transporter functions in metal homeostasis. It exchanges protons with Mg2+, Zn2+, and Fe2+ ions mainly in tissues with photosynthetic potential (David-Assael et al., 2006). However, further functional validation of these genes and their role in micronutrient uptake in *Ae. tauschii* grains is still needed.

CONCLUSION

To our knowledge, this is the first study to report GWAS for Fe, Zn, Cu, and Mn concentration in *Ae. tauschii* and further genetic and functional analysis of the associated genomic regions may shed light on the impact of these loci for improving micronutrient concentration of wheat. Overall, a number of accessions with high level of grain micronutrients have been identified especially for Fe and Zn which play an important role in tackling micronutrient deficiencies or hidden hunger. Bio-enriched *Ae. tauschii* accessions and genomic regions harboring grain Fe/Zn QTL provide a jumping board for developing biofortified wheat varieties.

AUTHOR CONTRIBUTIONS

SA carried out the phenotyping of the germplasm, analyzed both genotype and phenotype data, and wrote the draft of the manuscript. JC helped in LD analysis. CU contributed to the genome-wide association mapping, manuscript preparation, and candidate gene search. JP supervised genotyping by sequencing of the *Ae. tauschii* mapping panel, diversity analysis, and genome-wide association mapping. PC conceived the idea, designed and supervised the study, prepared the draft of the manuscript, and submitted it. All the authors have read and approved the manuscript.

FUNDING

Financial assistance was provided by the Department of Biotechnology, Ministry of Science and Technology, Government of India, New Delhi, in the form of grants BT/IN/UK/08/PC/2012 and 102/IFD/SAN/5170/2011-2012.

ACKNOWLEDGMENTS

The supply of the *Aegilops tauschii* germplasm by Wheat Genetics Resource Center (Kansas State University), University of Missouri, and IPK Gatersleben is also gratefully acknowledged. The help provided by Dr. Inderjit Singh Yadav, Assistant Professor of Bioinformatics, in the analysis is gratefully acknowledged.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00054/full#supplementary-material

FIGURE S1 | Boxplots representing variation in *Ae. tauschii* grain micronutrient concentrations for the three crop seasons—2011, 2012, 2013. The variation for grain **(A)** iron, **(B)** zinc, **(C)** copper, and **(D)** manganese concentrations observed between the years.

FIGURE S2 | Distribution of best linear unbiased predictions (BLUP) values with normality curve for grain **(A)** iron, **(B)** zinc, **(C)** copper, and **(D)** manganese.

FIGURE S3 | Correlation matrix for grain micronutrient concentrations in *Ae. tauschii* for year 2011–2013. The values in the column are correlation coefficient at the top and *p*-value for significant correlations is shown at the bottom. The symbols ***, **, and *, indicate significant at $p < 0.001$, $p < 0.01$, and $p < 0.05$, respectively. The colors represent strength of correlation from strongly positive (strong red) to negative (regent blue).

FIGURE S4 | The heat map for non-redundant *Ae. tauschii* accessions created using GAPIT. The panel is genetically divided into two diverse lineages which are referred to as lineage 1 (L1) and lineage 2 (L1).

FIGURE S5 | The LD decay plot for lineage 1 and 2 of *Ae. tauschii*.

TABLE S1 | Comparison of micronutrient concentration between hexaploid wheat cultivars (WL711 and PBW343) and *Aegilops tauschii* germplasm.

TABLE S2 | Descriptive statistics of *Ae. tauschii* accessions for micronutrients concentration based on two lineages (L1, L2).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a past co-authorship with the authors CU and JP.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 25 February 2019
doi: 10.3389/fpls.2019.00211

Stress Adaptive Plasticity: *Aegilops tauschii* and *Triticum dicoccoides* as Potential Donors of Drought Associated Morpho-Physiological Traits in Wheat

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The inconsistent prevalence of abiotic stress in most of the agroecosystems can be addressed through deployment of plant material with stress adaptive plasticity. The present study explores water stress induced plasticity for early root-shoot development, proline induction and cell membrane injury in 57 accessions of *Aegilops tauschii* (DD-genome) and 26 accessions of *Triticum dicoccoides* (AABB-genome) along with durum and bread wheat cultivars. Thirty three *Ae. tauschii* accessions and 18 *T. dicoccoides* accessions showed an increase in root dry weight (ranging from 1.8 to 294.75%) under water stress. Shoot parameters- length and biomass, by and large were suppressed by water stress, but genotypes with stress adaptive plasticity leading to improvement of shoot traits (e.g., *Ae tauschii* accession 14191 and *T. dicoccoides* accession 7130) could be identified. Water stress induced active responses, rather than passive repartitioning of biomass was indicated by better shoot growth in seedlings of genotypes with enhanced root growth under stress. Membrane injury seemed to work as a trigger to activate water stress adaptive cellular machinery and was found positively correlated with several root-shoot based adaptive responses in seedlings. Stress induced proline accumulation in leaf tissue showed marked inter- and intra-specific genetic variation but hardly any association with stress adaptive plasticity. Genotypic variation for early stage plasticity traits viz., change in root dry weight, shoot length, shoot fresh weight, shoot dry weight and membrane injury positively correlated with grain weight based stress tolerance index (r = 0.267, r = 0.404, r = 0.299, r = 0.526, and r = 0.359, respectively). In another such trend, adaptive seedling plasticity correlated positively with resistance to early flowering under stress (r = 0.372 with membrane injury, r = 0.286 with change in root length, r = 0.352 with change in shoot length, r = 0.268 with change in shoot dry weight). Overall, *Ae. tauschii* accessions 9816, 14109, 14128, and *T. dicoccoides* accessions 5259 and 7130 were identified as potential donors of stress adaptive plasticity. The prospect of the study for molecular marker tagging, cloning of plasticity genes and creation of elite synthetic hexaploid donors is discussed.

Keywords: *Aegilops tauschii*, *Triticum dicoccoides*, water stress, genetic variation, stress adaptive plasticity, root-shoot development, proline induction, membrane injury

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *27 September 2018*
Accepted: *07 February 2019*
Published: *25 February 2019*

Citation:
Suneja Y, Gupta AK and Bains NS (2019) Stress Adaptive Plasticity: Aegilops tauschii and Triticum dicoccoides as Potential Donors of Drought Associated Morpho-Physiological Traits in Wheat. Front. Plant Sci. 10:211. doi: 10.3389/fpls.2019.00211

INTRODUCTION

Alternate morpho-physiological manifestations of genes in response to specific environmental cues represent a key adaptation and survival strategy among plants, offsetting their limited capability to change the growth environment. This strategic property often referred to as plasticity has been defined as the ability of a single genotype to produce more than one phenotype in response to environment (Bradshaw, 1965). Plant phenotypic plasticity can be either a passive consequence of resource availability, physical conditions etc, or an active (adaptive) response to these conditions (Des Marais and Juenger, 2010). The latter generally implies specific development, physiological and reproductive adjustments that are thought to optimize plant fitness (Pacheco-Villalobos and Hardtke, 2012). Extensive above ground architectural changes in response to biotic and abiotic factors (Tomlinson and O'Connor, 2004), shifts in patterns of root development in search of nutrients and moisture (Lloret et al., 1999; Hodge, 2004), exudation of metabolites by roots for nutrient acquisition (Metlen et al., 2009), accumulation of osmolytes (Seki et al., 2007), modulation of stomatal density (Hetherington and Woodward, 2003), changes in leaf pigmentation (Chalker-Scott, 1999) are some of the well recognized examples of adaptive plasticity in plants. In study of natural plant populations, phenotypic plasticity is no longer seen as a source of noise (Nicotra and Davidson, 2010) in fact, it is receiving increasing recognition as a feature of ecological and evolutionary significance (de Jong, 2005; Bradshaw, 2006; Lande, 2009; Nicotra et al., 2010; Des Marais et al., 2013; Aspinwall et al., 2014; Bloomfield et al., 2014; Matesanz and Milla, 2018).

Genetic variation in plasticity in response to abiotic stress, particularly in model plant species like *Arabidopsis thaliana* (Mouchel et al., 2004; Kesari et al., 2012) and *Brachyipodium distachyon* (Luo et al., 2011; Pacheco-Villalobos and Hardtke, 2012) is well indicated. Genetic variation has been identified in natural accessions of Arabidopsis for *Bravix Radix (BRX)* locus, a transcription factor responsible for controlling root proliferation and its elongation (Mouchel et al., 2004; Beuchat et al., 2010). Using Recombinant inbred lines (RILs), two robust QTLs, *EDG1,* and *EDG2* (elicitors of drought growth) contributing to plasticity in root system size under mild osmotic stress were identified in Arabidopsis (Fitz Gerald et al., 2006). Also, Pajoro et al. (2017) envisaged the contribution of transposons and alternative splicing toward thermoplasticity in flower development in natural accessions of Arabidopsis. Crop scientists are just beginning to embrace the plasticity concept (Sardas et al., 2009; Melino et al., 2015). With respect to plasticity in crop species Nicotra et al. (2010) have raised two outstanding questions. First seeks to understand if crop breeding has led to reduction in adaptive plasticity. When the impact of breeding on phenotypic plasticity of oat's varieties was examined, modern varieties (as compared to the older ones) showed least plasticity in stem elongation in response to variation in light conditions (Semchenko and Zobel, 2005). Information so far is, however, insufficient for a consensus to be reached on this issue. The second question seeks to know if we can breed crops for plasticity in key traits with the ultimate goal of improving yield stability under climate perturbations. Key functional traits such as leaf mass per unit area, stomatal size and density, plant height at maturity, flowering time, seed size, water use efficiency, leaf morphology, root to shoot ratio, plant chemical defenses etc. have been recommended for investigation of adaptive phenotypic plasticity. Several studies are now addressing these and other related questions. Ehdaie et al. (2012) for instance, evaluated a set of near isogenic wheat-rye translocation lines for root allocation and plasticity under well watered and drought conditions and found adaptive phenotypic plasticity of root system components to reduce negative impact of drought stress on grain yield. Integrating these researches into practical cereal breeding is likely to emerge as a major future requirement.

Wheat, as a crop, epitomizes the effectiveness of the genetic strategy in food-securing the world in the face of increasing population and rising per capita consumption. In wheat, as in other green revolution crops, enhanced productivity was largely achieved through selection for performance in a specific environmental situation represented by assured and high input use. This strategy, however, proved less effective for the inherently variable drought stress environments which represent a substantial proportion of wheat growing regions of the world (Ludlow and Muchow, 1990). Presently, besides tolerance to natural stress, there is also a need to develop genotypes adapted to low input use in the view of environmental and resource depletion concerns. Trait plasticity may prove useful to buffer productivity in the face of unmanageable spatial and temporal variations in production conditions. Trait plasticity is thus an attractive prospect in the light of sustainable agriculture but donor options in the cultivated germplasm are likely to be constrained owing to the selection regimes historically employed. In case of wheat, severe genetic bottlenecks were imposed by rare interspecific hybridization and polyploidization events accompanying bread wheat domestication (Cox, 1997). As a result, lower levels of polymorphism are observed for many traits in common wheat in comparison with its progenitor species (Kam-Morgan et al., 1989). The three wheat genomes (A-, B-, and D-) of cultivated wheat have their ancestral complements enshrined in two immediate progenitor species, *Aegilops tauschii* (DD-genome donor) and *T. dicoccoides* (AABB-genome donor). Since the potential for recombination based gene transfers from progenitor species is enormous (as compared to non-progenitor donors), incorporation of adaptive plasticity traits from these wheat progenitors could greatly expand the available domesticated gene pool and enrich the possibilities of combining resilience and productivity of wheat varieties making them perform better over a range of predictable and unpredictable environment regimes.

With these points in mind, the primary objective of the present study was to identify genetic variation for "water stress adaptive plasticity" in a set of accessions belonging to two species which are the immediate wild progenitors of hexaploid wheat. Productivity/yield oriented indices generally employed as a measure of stress adaptation in cultivated wheats would not be relevant for this set. Considering the nature of

target traits as well as the plant material, lab based assays were seen to be more appropriate. Accordingly seedling traits (length and biomass of both root and shoot) formed the core of the experiment for studying stress induced plasticity. In a second experiment, two characters based on leaf tissue (proline content and cell membrane injury) were assayed at vegetative stage from field grown plants. Leaf tissue could be conveniently sampled, irrespective of species differences and field stress provided the required induction of tolerance mechanisms. A third experiment dealt with field observations on flowering time, plant growth (height) and yield components (spike length and grain weight). This experiment was aimed at relating stress adaptive changes in seedling and early/vegetative stage with one or more productivity based indices of stress tolerance. In all the experiments, "change in trait value" across well watered and water stress conditions rather than the absolute values formed the basis of analysis. This helped us to focus on "stress adaptive plasticity" and also make comparisons across species. Further, as the absolute values of these parameters vary greatly across the three species employed here, comparable observations were generated in the form of "change in trait value" across well watered (WW) and water stress (WS) conditions. The study reports wide variation both within and between the species, trait interactions and trade-offs, demarcation of potential donors for use in breeding program and considerations for a wheat improvement strategy.

MATERIALS AND METHODS

Choice of Germplasm

The wild progenitor species germplasm used in the present study consisted of 57 accessions of *Aegilops tauschii* and 26 accessions of *Triticum dicoccoides*. The two germplasm sets are listed in **Supplementary Tables S1a,b** with respect to their pau gene bank accession numbers. To refer to a particular accession in the text, numeral component of the designation is used. *Aegilops tauschii* and *Triticum dicoccoides* germplasm maintained at Punjab Agricultural University was received from different sources (University of Missouri and Kansas State University, United States; IPK, Gatersleben, Germany; Centre for Plant Breeding Research, Wageningen, Netherlands and National Bureau of Plant Genetic Resources, New Delhi, India) over a period of time (**Supplementary Tables S1a,b**). Subsets of this material have been subjected to screening for genetic variation for acquired thermotolerance with respect to cell membrane stability and TTC based cell viability (Gupta et al., 2010), disease resistance and high molecular weight glutenin subunits (Chhuneja et al., 2010), alleles of vernalization genes at *VRN-A1* and *VRN-B1* loci (Chhuneja et al., 2015) and detection of SNPs for grain size variation (Arora et al., 2017) in earlier studies at our center.

Cultivars of bread wheat- PBW-343, PBW-550, PBW-621, C-306, and durum wheat- PDW-291, PDW-314, and WHD-943 were included in present study as reference material (**Supplementary Table S1c**), with which attributes of wild accessions were compared. As the number of cultivated lines was considerably smaller, their comparison with the progenitor sets (in relation to spectrum of variation) may not be fully justified though some reprieve was provided by the deliberate inclusion of tall, traditional rain-fed cultivar (C-306) along with modern day high productivity varieties (PBW-343, PBW-550, and PBW-621) recommended for cultivation under irrigated conditions. Similarly, inclusion of durums (PDW-291, PDW-314, and WHD-943) along with bread wheat cultivars added an element of variation which might have taken a much larger random set of cultivated wheats to encompass.

Evaluation of Stress Adaptive Plasticity of Different Accessions

Seedling Assays

For evaluating plasticity in root-shoot development under water stress conditions, a preliminary experiment was carried out to optimize the concentration (10, 15, 20, and 25%) of polyethylene glycol (PEG) solution for the current study. A parallel set up involving different concentrations of mannitol was also used (2, 3, 4, 5, and 6%). Twenty five per cent PEG was found to cause about 50% reduction in growth and thereafter, this concentration was used for the complete study. Responses of 14 day old seedlings were observed in terms of length and weight of both roots and shoots under well watered and water stress conditions. Before deciding on the use of propagation trays, a subset of 23 accessions (twelve *Ae. tauschii*, seven *T. dicoccoides*, two bread wheat and two durum wheat cultivars) were grown in three types of containers, namely, propagation trays, small cups and root trainer trays. The genotypic values for root and shoot growth under both well watered and water stress conditions correlated well across container systems (**Supplementary Table S2**). As the growth studies in propagation tray involved shorter time frame and required lesser space, further study on complete set was carried out in this mode. This set up would favor use of seedling assay, if need be, on a breeding scale.

For the present study, seven seeds (each) of *Ae. tauschii* and *T. dicoccoides* accessions along with check wheat cultivars were sown in triplicate (a total of 21 seeds per accession in each water regime) in two sets of propagation trays (with adequate size to support root-shoot growth for about 2 weeks) where one set served as control (well-watered) and the other set was subjected to polyethylene glycol (PEG) based water stress. Three replicates (where seven seedlings constituted one biological replicate) were sown in two sets of propagation trays in completely randomized design (CRD). These trays were placed in a Conviron growth chamber PGR15 maintained at a temperature of 25 $\pm$ 2°C and a light intensity of 400 μmol m^{-2} s^{-1}. Both the sets were watered with one-fourth strength Murashige and Skoog (MS) salt solution for first week (normal irrigation). From 8th day onwards (when seedlings were 5–7 cm long), 25% poly ethylene glycol (PEG-6000) in 0.25 $\times$ MS solution was used as the moisture stress inducing medium (-0.95 MPa) (modified from Blum et al., 1980). Thereafter, every alternate day, two sets were watered with 0.25 $\times$ MS medium (well watered) and 25% PEG solution in 0.25 $\times$ MS medium (water stress), respectively, till

next 7 days when the tissue was harvested and the data was eventually recorded for root length, root biomass (fresh and dry weight), shoot length and shoot biomass (fresh and dry weight) under both well watered and water stress conditions. One of the *T. dicoccoides* accessions 14004-2 (**Supplementary Table S1b**), however, did not germinate and was excluded from the seedling based assay.

Field Study

With regard to field based investigation, 30 seeds of *Ae. tauschii* accessions were initially sown in propagation trays in first week of September (10-09-2010 and 08-09-2011) and kept for vernalization in cold chambers maintained at 4°C with 8/16 h light/dark regime for 7 weeks (Suneja et al., 2015b, 2017). After vernalization treatment, plants were conditioned at 15°C and 8/16 h light /dark for 1 week. Vernalized seedlings of *Ae. tauschii* and germinated seedlings of *T. dicoccoides* were then transplanted in the experimental fields of Department of Plant Breeding and Genetics, P.A.U. Ludhiana (30°54′N and 75°48′E) in two sets (later demarcated as irrigated/well watered and rain-fed/water stress) in the first week of November (05-11-2010 and 03-11-2011). The soil type of the experimental area is sandy loam and soil is non-saline with slightly alkaline pH of 8.0 and organic carbon content of 0.4%.

Two ridges with 3 m wide buffer zone were maintained between different irrigation treatments. Further, non-experiment border rows were planted (at margins) to take care of any seepage effect that might have arisen. A total of five plants per accession (in each replicate) were transplanted to constitute one plot. Therefore, a total of 30 germinated seedlings of each accession were transplanted (15 irrigated and 15 rain-fed) in randomized complete block design (RBD). After 45 days of transplanting, natural day length was supplemented with arrangement of halogen incandescent lamps and fluoroscent lights in field at regular spacing. Lights were switched on prior to sunset till late night to provide about 16 h of continuous light per day. This extension of light hours during short day winters of North India allowed wild species to flower normally. Cultivated wheat lines were incorporated in this set through seeding as practiced conventionally. Unlike the wild wheat progenitors, particularly *Aegilops tauschii,* that are typically adapted to temperate environment and have "winter" growth habit, cultivated wheats grown in tropical and sub-tropical regions of India are "spring" wheats. Therefore, wild and cultivated spring type accessions were handled differently with respect to crop raising practice.

Standard agronomic practices as followed for irrigated wheat in the region formed the basis of irrigation to non-stressed plots throughout the crop season. Briefly, after one round of heavy, pre-sowing irrigation (10 cm), four more rounds of irrigation (7.5 cm each) were given to the crop at 4–5 weeks interval depending upon the rainfall. The water stressed rain-fed set, on the other hand, received water only from rain as all the irrigation (except pre-sowing irrigation) was completely withheld throughout the season. During each crop season, the per cent moisture content in soil was determined gravimetrically at maximum tillering stage (about 60 days after sowing) from 6 different field locations (one from each replicate in each treatment) at four different depths- 0–30 cm, 30–60 cm, 60–90 cm, and 90–120 cm (**Supplementary Table S3**). At maximum tillering stage, a 36.7% difference in soil moisture content between well watered and water stress replicates during 2010–11 and a 66.4% difference in soil moisture content in 2011–12 was recorded.

Overall, the crop season 2010–11 received a total rainfall of 128.8 mm, while 108.8 mm rainfall was recorded in 2011–12. Month-wise distribution of rainfall during two crop seasons has been indicated in **Figure 1**. For two leaf tissue based traits which were sampled at Zadok GS30 stage (about 60 days after transplanting), there were two rainfall episodes just ahead of sampling during 2010–11 while in 2011–12, almost a month long rain-free period was available prior to sampling, as seen in **Figure 1**. For these traits, observations from 2011 to 2012 were used for exploring genotypic variation for stress adaptive plasticity. For all other field based traits, observations from both years were used for analysis. The crop was harvested in the first week of May during both the years. As maturity in wild accessions is staggered and that seeds shatter on maturity, net bags were put on spikes 20 days prior to harvesting.

(i) Estimation of proline

For determination of proline content, five fully expanded penultimate leaves (second leaf from the top) were collected from field during vegetative stage (60 days after transplanting) corresponding to Zadoks growth stage GS30 (Zadoks et al., 1974). Briefly, 100 mg of the leaf tissue was weighed, homogenized in 3% aqueous sulfosalicyclic acid and the content of proline was estimated using Ninhydrin reagent assay (Bates et al., 1973). Leaf proline content was estimated under well watered and water stress conditions and degree of proline induction under water stress was calculated to provide a measure of metabolic plasticity.

(ii) Estimation of Membrane Injury

The assay for percent membrane damage was performed as mentioned in Suneja et al. (2017). For this, four fully expanded penultimate leaves (5 cm long) per accession (randomly from five plants of each plot) from rain-fed replications (Zadok growth GS30 corresponding to 60 days after transplanting) were distributed into two sets, i.e., three replicates each of control (deionised water) and *in vitro* stress treatment (40% PEG). After 24 h of PEG treatment, conductivity (μ siemens) was recorded, respectively, for control and stressed samples using a digital conductivity meter. Membrane injury index, as an indicator of cell membrane instability was worked out and membrane damage was expressed in per cent units as:

$$\text{Membrane injury index} = 1 - \frac{1 - \left(\frac{T1}{T2}\right)}{1 - \left(\frac{C1}{C2}\right)} \times 100$$

T_1, T_2 = Mean conductivity of stressed sample before and after autoclaving, respectively.
C_1, C_2 = Mean conductivity of control sample before and after autoclaving, respectively.

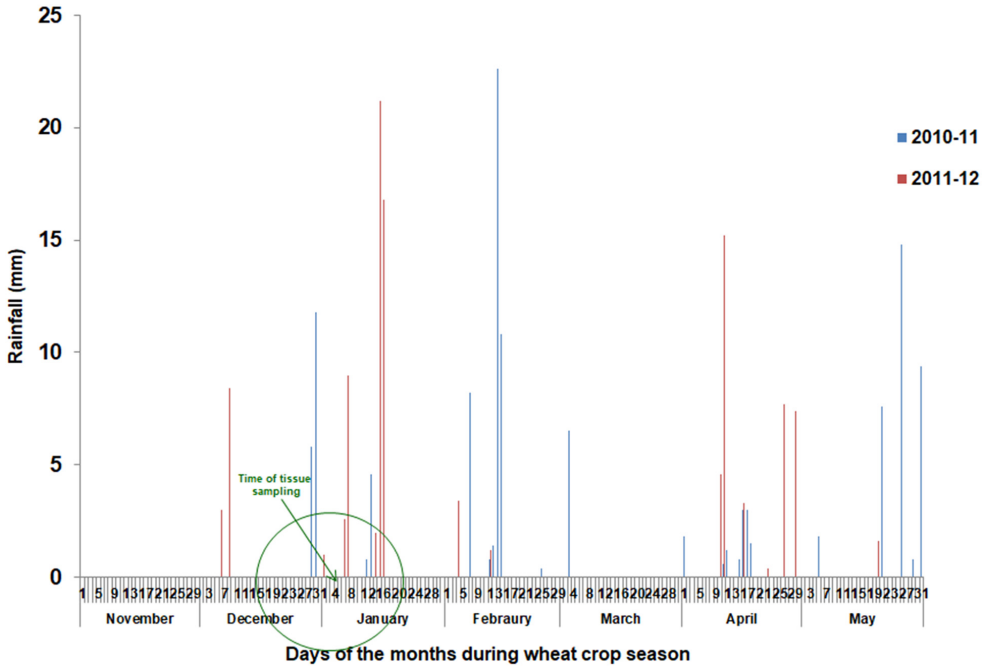

FIGURE 1 | Rainfall pattern during period of field experimentation.

Hence, stress adaptive plasticity measures of all accessions belonging to wild and cultivated wheat species were estimated on the basis of observations recorded under well watered and water stress conditions (both in case of seedling based lab assays and field based screening of accessions). The estimate of stress adaptive plasticity was obtained by expressing the trait value under stress as percentage of non-stress value for the same accession. No change due to water stress was given a value of 0. Reduction in trait value under stress would lead to a plasticity measure of less than 0 (negative value). A truly stress adaptive response, say in case of root growth would be indicated by an increase in root size under water stress and consequently a plasticity value of greater than 0 (positive change) was assigned to it. The criteria thus used, favored stress adaptive changes to be elucidated rather than changes irrespective of direction. Further, using non-stress (for well-watered conditions) trait value from the same accession as a benchmark and employing change in trait value rather than absolute value as a measure for analysis made comparison across species (which vary in size and morphology) possible.

Observations on Productivity Associated Traits and Derivation of Water Stress Tolerance Indices

Observations were recorded for days to flowering, plant height and length of the spike in field grown crop under both well watered and water stress conditions. Days to flowering was recorded as the number of days taken for 50% ear emergence from the date of transplanting. After the completion of flowering, plant height (in cm) was measured from base of the plant to the tip of the spike excluding awns and recorded as average height of five plants per accession for each replication. Spike length (in cm) was measured from neck node to the tip of the uppermost spikelet excluding awns and recorded for five spikes of each accession for each replication. For measurement of grain weight, the seeds were dehulled from spikelets of *Ae.tauschii* and *T. dicoccoides* and threshed out from ears of cultivated wheats to obtain 100-grain weight. Morphological tolerance indices were calculated on the basis of change in days to flowering (Tolerance Index 1), change in spike length (Tolerance index 2), change in plant height (Tolerance index 3) and change in grain weight (Tolerance index 4) from trait values under well watered and water stress conditions.

Statistical Analysis

Analysis of variance (ANOVA) was performed to determine the role of genotypes (G), water stress regimes (W) and G x W interactions on variability in the expression of different drought-adaptive morpho-physiological traits ($P \leq 0.05$, DSASTAT software 1.101). With respect to responsiveness under stress, boxplots and frequency distribution histograms were developed (using R statistical package and MS Excel 2007) for each trait to determine the extent of natural genetic variation both within and between the three groups of germplasm- *Ae. tauschii*, *T. dicoccoides* and check wheat cultivars. Using R version 3.5.1, best linear unbiased predictors (BLUPs) were obtained. When estimating BLUPs using random effect "ranef" command in lme4 package in R, variance components for all traits were analyzed using general linear mixed model to determine the effect of genotype, year and genotype

× year interaction separately for well watered and water stress conditions.

The estimated phenotypic BLUP values were further used to perform correlation analysis and hierarchical cluster analysis (HCA). Correlation coefficients for the complete set (excluding three genotypes as data for a few traits was missing) were computed for associating tolerance behavior with early stage adaptive plasticity (change in trait expression) under water stress using SPSS 16.0 (IBM Corp., Armonk, NY, United States). For multivariate analysis and for overall assessment of accession-specific response to each water stress adaptive trait, a heat map was generated (using JMP14) that allowed hierarchical clustering (following Ward's Method) of all the wild accessions and check wheat cultivars.

RESULTS

The germplasm set consisting of diploid (*Ae. tauschii*) and tetraploid (*T. dicoccoides*) progenitor species along with check wheat cultivars was subjected to evaluation of plastic responses in relation to water stress. A laboratory based experiment was aimed at recording adaptive changes in root and shoot growth (length and biomass) in response to stress. A second set of observations evaluated membrane injury and degree of proline induction at vegetative stage (about 60 days after transplanting) under field conditions. A third set of observations were recorded on flowering time, plant height, spike length and grain weight under irrigated and rain-fed conditions to assess variation in genotypic responses measured as difference in trait value under stress and non-stress conditions. While the first two sets of traits aimed at uncovering active adaptive responses, the third including time to flower, plant height and yield components represented impact of these responses in terms of tolerance to stress. The overall aim was to establish genetic variation for stress adaptive plasticity using low environmental noise, easy to observe traits. A further premise was that this early stage adaptive plasticity might reflect in the performance based tolerance indices.

As per the analysis of variance (**Supplementary Table S4**), the genotypes (G) constituting the germplasm set varied significantly for all seedling based growth traits. Trait expression for length and biomass attributes was significantly affected by water stress regimes (W) reflecting sufficient contrast maintained across the two treatments. A significant G X W interaction was observed for all evaluated traits indicating differential response of genotypes to the two water regimes. **Tables 1**, **2** summarizes information on mean values and range of response of the two wild species along with check wheat cultivars for lab based seedling growth assays and field based physio-biochemical evaluations, respectively.

Stress Adaptive Plasticity in Seedling and Tissue Based Assays

Root Length (RL)

Across both well-watered and water-stress regimes, root length tends to increase with an increase in ploidy level, i.e., from diploid (*Ae. tauschii*) to tetraploid (*T. dicoccoides*) accessions and further on to hexaploid wheats (**Figures 2Ai,ii**). Under well-watered conditions, large number of accessions across the groups clustered in the length range of 4–6 cm when measured on 14 days old seedlings. Average root length for both wild species decreased under water stress (3.82 cm to 3.66 cm for *Ae. tauschii* and from 5.40 to 5.26 cm for *T. dicoccoides*) (**Table 1** and **Figures 2Ai,ii**), yet several accessions showed root elongation (**Figure 2Aiii**). *Ae. tauschii* accessions 9803, 9814, 14191, 14128, 14226, 14109 and *T. dicoccoides* accessions 5364 and 7130 presented greater than 50% increase in root length under water stress (**Table 3**). Diversity was wider for the increase than the decrease in root length (**Figure 3A**). Under water stress conditions, maximum reduction in root length (60%) was seen in a *T. dicoccoides* accession (7120, **Supplementary**

TABLE 1 | Genotypic variation in wheat germplasm set for lab based seedling growth traits recorded under well watered and water stress conditions induced by 25% PEG treatment.

Traits	Water stress regimes	*Aegilops tauschii*		*Triticum dicoccoides*		Check wheat cultivars		Full set	
		Mean	Range	Mean	Range	Mean	Range	Grand mean	LSD (0.05)
Root length (cm)	WW	3.82	1.13–6.20	5.40	2.23–8.02	5.66	4.97–7.28	4.41	1.12
	WS	3.66	1.74–6.17	5.26	2.23–7.03	7.27	5.34–9.63	4.40	1.05
Root fresh weight (mg)	WW	329.81	50.00–890.00	221.45	55.00–707.00	1944.76	1110.00–2586.67	426.39	186.23
	WS	296.03	128.00–533.33	272.97	49.00–840.60	1157.62	731.67–1493.33	357.32	163.74
Root dry weight (mg)	WW	47.77	19.05–112.50	42.44	15.00–109.00	149.76	98.33–221.67	54.30	31.86
	WS	56.59	30.00–122.50	56.49	22.33–103.25	135.00	113.33–151.67	62.73	30.41
Shoot length (cm)	WW	19.59	9.90–27.72	19.47	12.35–23.57	15.04	10.92–22.97	19.20	3.48
	WS	11.12	7.17–16.75	13.90	9.23–16.43	12.38	10.18–15.37	12.00	1.54
Shoot fresh weight (mg)	WW	1828.27	460.00–3790.00	1307.31	573.33–2343.07	2482.62	1476.67–3110.00	1733.39	345.80
	WS	666.24	235.00–1225.00	830.46	480.00–1836.67	766.19	585.00–1043.33	720.23	169.37
Shoot dry weight (mg)	WW	274.10	80.00–920.00	143.21	50.00–287.29	279.52	215.00–355.00	237.76	85.90
	WS	185.35	70.00–370.00	144.58	33.33–360.00	175.24	148.33–211.67	173.10	54.48

WW, well watered; WS, water stress.

TABLE 2 | Genotypic variation in wheat germplasm set for leaf tissue based traits (stress developed under field conditions) recorded under well waterd and water stress conditions.

Traits	Water stress regimes	*Aegilops tauschii*		*Triticum dicoccoides*		Check wheat cultivars		Full set		
		Mean	Range	Mean	Range	Mean	Range	Grand mean	LSD (0.05)	h^2
Proline content (mg g^{-1} FW)	WW	0.52	0.12–1.01	0.13	0.04–0.68	0.11	0.06–0.16	0.38	0.38	0.69
	WS	1.12	0.25–2.53	0.33	0.16–0.53	0.44	0.40–0.56	0.85	0.85	0.56
Membrane Injury (%)	*In vitro* stress	51.74	21.43–66.86	73.00	43.46–87.19	76.25	58.27–89.38	59.94	14.62	0.72

h^2, Repeatability calculated over two crop seasons 2010–11 and 2011–12. WW, well watered; WS, water stress.

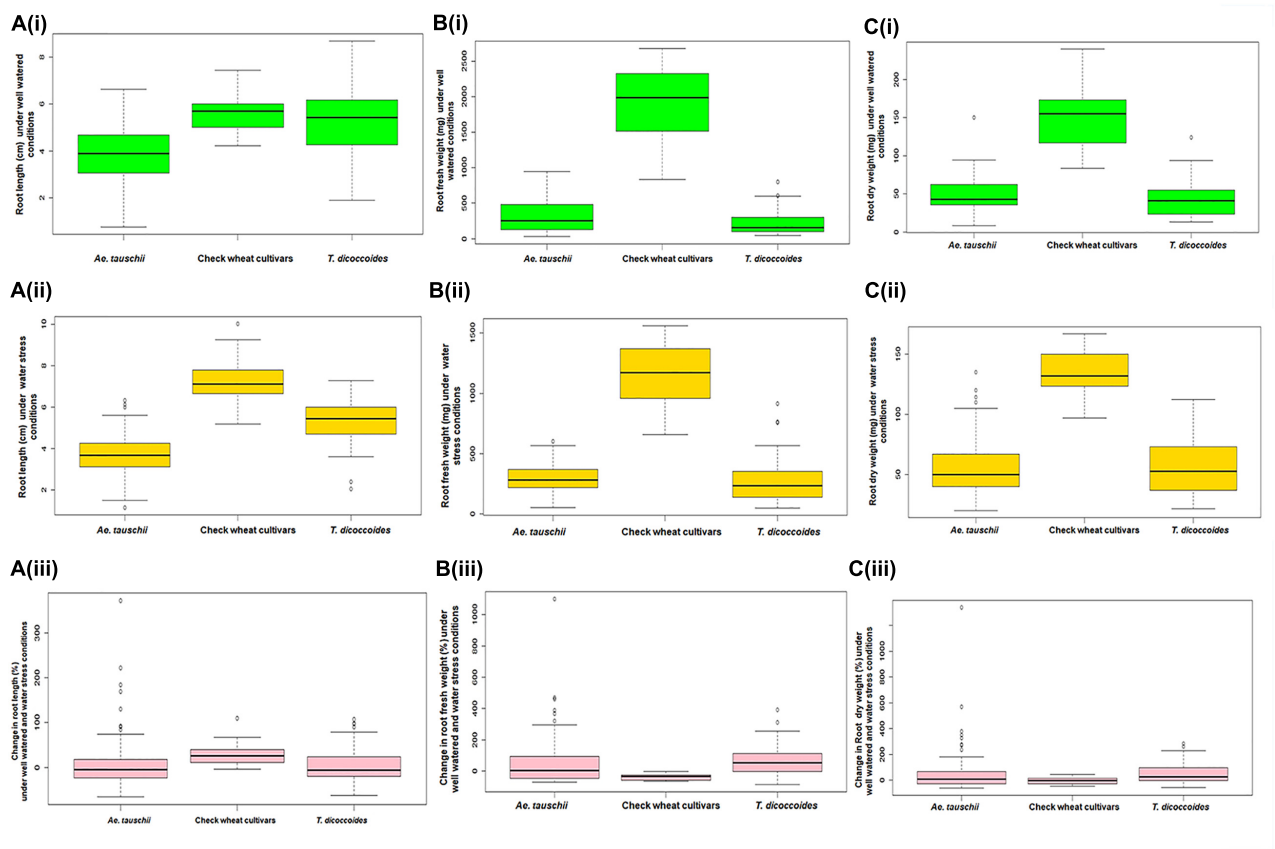

FIGURE 2 | Boxplot representation of root traits of accessions of *Ae. tauschii*, *T. dicoccoides* and check wheat cultivars under well watered and water stress conditions. **(A)** Root length under **(Ai)** well watered, **(Aii)** water stress conditions, and **(Aiii)** change in root length under well watered and water stress conditions. **(B)** Root fresh weight under **(Bi)** well watered, **(Bii)** water stress conditions, and **(Biii)** change in root fresh weight under well watered and water stress conditions. **(C)** Root dry weight under **(Ci)** well watered, **(Cii)** water stress conditions, and **(Ciii)** change in root dry weight under well watered and water stress conditions.

Table S5), whereas highest water stress-mediated induction (197.06%) was observed in an *Ae. tauschii* accession (9803) (**Table 3** and **Supplementary Table S5**). In case of *T. dicoccoides*, the maximum increase in root length (97.47% in accession 5364) was higher than the greatest increase for this trait observed in cultivated set (85% in PDW-314) (**Figure 3A**).

Root Fresh Weight (RFW)

The spectrum of variation for root biomass (fresh and dry weight) distribution under well-watered and water stress conditions was much broader than that observed for root length. Wide genotypic variability could be identified for root fresh weight both within and between the three groups of germplasm (**Figure 2B**). Under both well watered and water stress conditions, demarcation between cultivated wheats and wild species was quite distinct as check wheat cultivars (both bread wheat and durum wheats) developed very high root biomass (1110–2587 mg) relative to both the species of its wild progenitors. With regard to wild accessions, *Ae. tauschii* dominated the upper limits of root biomass development

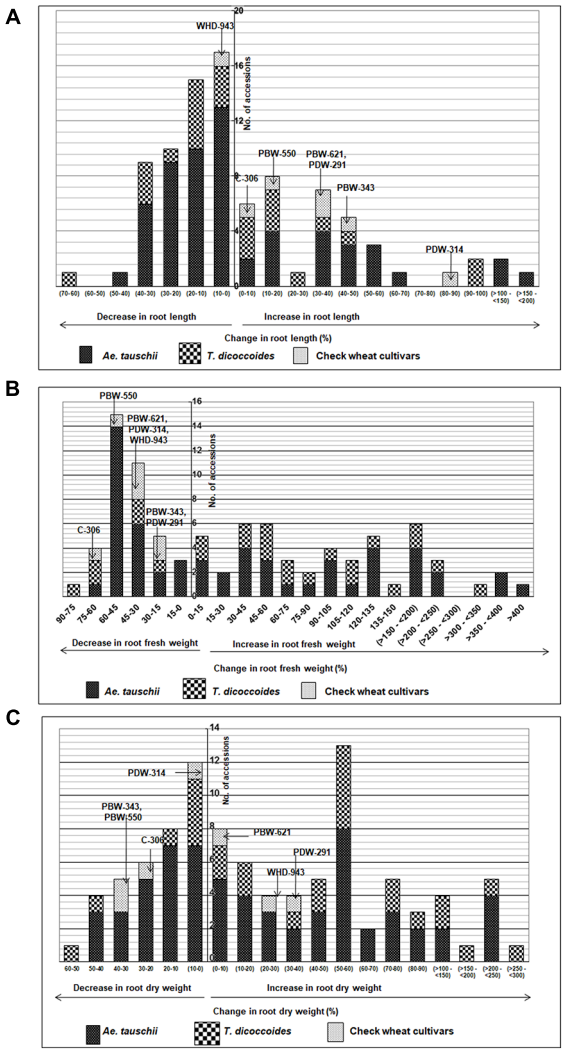

FIGURE 3 | Distribution of accessions of *Ae. tauschii*, *T. dicoccoides* and check wheat cultivars with regard to change in **(A)** root length, **(B)** root fresh weight, and **(C)** root dry weight under well watered and water stress conditions.

(50–890 mg) under well watered conditions (**Figure 2Bi**). However, under PEG-induced decreased water availability, *T. dicoccoides* accessions occupied the higher range of 49–841 mg (**Figure 2Bii**).

Although cultivated wheats (as a group) developed better average fresh root biomass than the wild species under both well watered and water stress regimes (**Figures 2Bi,ii**), an overall reduction (15–75%) in root fresh weight (RFW) was noticed in them (**Figure 2Biii**). No cultivated wheat showed an increase in root fresh weight under water stress. Wild species, on the other hand, demonstrated wide variation with respect to this developmental plasticity. Thirty one *Ae. tauschii* accessions and 19 *T. dicoccoides* accessions showed increase in fresh root biomass (ranging from 3 to 483%) under water stress (**Figure 3B**), the highest value for *Ae. tauschii* and *T. dicoccoides* being recorded by accession 14128 and 4667, respectively (**Table 3**).

Root Dry Weight (RDW)

As with root fresh weight, bread wheat and durum wheat cultivars maintained greater root dry mass under both well watered and water stress conditions. Whereas the upper limit for root dry matter accumulation was 123 mg for wild accessions (**Table 1** and **Figure 2Ci**), it was almost double for the cultivated set (222 mg). Better part of the wild accessions (nearly 80% of both *Ae. tauschii* and *T. dicoccoides*) accumulated root dry biomass in the range of 25–75 mg under both well watered and water stress conditions (**Figures 2Ci,ii**). Relative to the changes observed in root fresh weight where data for check wheat cultivars revealed an overall reduction under water stress (**Figure 2Ciii**), root dry matter exhibited a mixed trend toward induced and/or retarded dry matter accumulation (**Figure 3C**). Within the wild germplasm pool, the range of positive changes in root dry matter accumulation was wider (up to 300%) than that in which decrease in root dry mass was observed (up to 60%) (**Figure 3C**). Some of the cultivated wheats registered an increase in root dry weight under stress, but the percentage increase (34%) was lower than that observed in wild wheats (295%) (**Figure 3C**). Among the 25 evaluated accessions of *T. dicoccoides*, only seven witnessed curtailment in root dry matter under water stress. Rest 18 experienced a net increase in root dry weight. Within *Ae. tauschii* group, accessions were more or less uniformly distributed in the respective lots (25 experiencing net reduction and 33 net gain in root dry weight under stress). Greater than 200% increase in root dry weight was found in *Ae. tauschii* accessions 9809, 9810, 9814, 9816, 14109 and *T. dicoccoides* accession 7130, 13992, and 14004 (**Table 3**). For the germplasm set used in the present study, water stress seemed to induce a wide range of re-partitioning of resources allocated to different plant parts. The pattern varied markedly for cultivated and wild wheats.

Shoot Length (SL)

Within the present germplasm set, wider distribution range for shoot length was observed in the well watered (**Figure 4Ai**) than the water stressed set (**Figure 4Aii**). In well watered set, shoot length varied from 9.9 to 27.72 cm in *Ae. tauschii* and 12.35–23.57 cm for *T. dicoccoides* (**Table 1** and **Figure 4Ai**). However, in water stress set, shoot length varied from 7.17 to 16.75 cm in *Ae. tauschii* and 9.23 to 16.43 cm for *T. dicoccoides* (**Table 1** and **Figure 4Aii**). Where cultivated wheats exhibited root elongation under water stress, shoot length, by and large was suppressed in this group (**Figures 4Aii,iii**). Low water potential developed due to PEG treatment resulted in up to 60% reduction in shoot length (**Figure 5A**). Under water stress, a substantial increase in shoot length of the order of 20.20% (*Ae. tauschii* 14191) and 29.55% (*T. dicoccoides* 7130) was observed in progenitor species (**Figure 5A**). These accessions had also shown a 101.50 and 91.53% increase in root length, respectively (**Table 3**).

Shoot Fresh Weight (SFW)

Among the wilds, *Ae. tauschii* accessions acquired very high shoot fresh weight under well watered conditions that ranged

TABLE 3 | Genotypes showing high levels of plasticity for different morpho-physiological traits across well watered and water stress conditions.

Traits	*Ae. tauschii*	*T. dicoccoides*	Cultivars
Root length (cm)	14122* (36.82%), 14119* (46.32%), 14109 (53.42%), 14226* (55.72%), 14128 (57.85%), 14191 (101.50%), 9814 (112.54%), 9803 (197.06%)	4667 (63.43%), 7130 (91.53%), 5364* (97.47%)	PDW-314* (85%)
Root fresh weight (mg)	9816 (364.42%), 14109* (374.85%), 14128 (483.33%)	7056* (148.66%), 4657 (163.01%), 5364* (198.22%), 5259 (215.25%), 4667 (311.36%)	–
Root dry weight (mg)	9809 (200%), 9810* (206.67%), 9816 (246%), 9814* (250.40%), 14109* (294.75%)	7079* (46.02%), 7108* (75.58%), 13992 (167%), 7130 (205%), 14004 (254%)	PDW-291 (34%)
Shoot length (cm)	14191 (20.20%)	7130 (29.55%),	PDW-291 (0.96%)
Shoot fresh weight (mg)	14191 (18.6%)	–	–
Shoot dry weight (mg)	9809 (43.75%), 9803 (94.74%)	7108* (14.66%), 7056* (47.70%), 7054 (59.18%), 7079* (64.89%), 7130 (246.67%)	–
Proline content (mg g^{-1} FW)	14122 (202.21%), 14128 (205.53%), 14096 (230.66%), 14170* (337.79%), 14113* (366.61%), 14169* (398.31%), 14173* (418.09%), 14119* (430.70%)	7130 (211.76%), 5259* (255.07%), 4654 (329.84%), 4655 (468.06%), 5364 (477.19%), 4656 (835.14%)	C-306* (606.25%)
Membrane injury (%)[#]	14178* (21.43%), 9799* (24.66%), 14240* (29.30%), 3491* (30.32%), 3769* (34.73%), 3761 (37.31%), 14170 (37.33%)	7079* (43.46%), 4655* (49.49%), 14004-2* (54.13%)	C-306* (58.27%)

[#]*Recorded only under water stress conditions. *Combines high plasticity and high absolute value (present in top non-significant group with respect to absolute values under water stress conditions, please see details in* ***Supplementary Table S5****).*

between 460 and 3790 mg (**Figure 4Bi**). In water stressed set, this upper limit of shoot biomass, however, came down for all the three groups of germplasm (**Figure 4Bii**), to almost 50% of that observed under well-watered conditions (3790 mg). Maximum number of accessions lay in the range of 500–750 mg shoot biomass within the water stress set (**Figure 4Bii**). Where *Ae. tauschii* and check wheat cultivars exhibited maximum shoot fresh weight up to 1250 mg, a *T. dicoccoides* accession stood as an outlier as it developed shoot fresh weight of 1837 mg under water stress. Bread wheat and durum wheat cultivars experienced 45–90% reduction in shoot fresh weight (**Figure 4Biii**). Interestingly, 25 wild accessions showed less than 45% fresh weight reduction under water stress (**Figure 5B**). An *Ae. tauschii* accession 14191 was the only accession in the present set to have exhibited an increase in shoot fresh weight (18.6%). It had also shown an increase in shoot and root length under water stress.

Shoot Dry Weight (SDW)

Ae. tauschii exhibited greatest variability in shoot dry matter accumulation within well watered set, evident from the extremely high value of shoot dry weight of a few outlier *Ae. tauschii* accessions (**Figure 4Ci**). Within the water stressed set, majority of the germplasm lines had shoot dry weight in the range of 100–200 mg (**Figure 4Cii**). Several wild accessions (*Ae. tauschii* and *T. dicoccoides*) accumulated shoot dry matter more than the maximum shoot dry matter accumulated by wheat cultivars under water stress. Further, though an overall reduction in shoot dry matter was evident, abundant genotypic variation existed among the wild species with respect to change in shoot dry weight under stress (**Figure 4Ciii**). An average reduction in shoot dry weight within the cultivated pool spanned a range of 15–60% (**Figure 5C**). Wide genotypic variation existed between wild species accessions with respect to change in shoot dry weight under water stress. An increase in shoot dry weight was observed in two accessions of *Ae. tauschii* 9803, 9809 and in 14 of the 25 accessions of *T. dicoccoides* (up to 240%) (**Figure 5C** and **Table 3**). These and other genotypes where stress induced increase in root length or biomass helped minimize reduction in (even if not improve) shoot growth represent true adaptive plasticity.

Proline Accumulation in Leaf Tissues

Inter- and intra-specific variation in the extent of proline accumulation, a well known water stress responsive metabolite was analyzed under field conditions. Under well-watered conditions, all the cultivated wheats and majority of the *T. dicoccoides* accessions maintained a relatively lower basal levels of proline (less than 0.25 mg g^{-1} FW) in their leaf tissue (**Figure 6A**). *Ae. tauschii* accessions seem to go far beyond this range, as is evident from their higher values of proline accumulation that varied from 0.25 to 1.25 mg g^{-1} FW under well watered conditions (**Table 2**). Effect of differential water regimes was quite dramatic as an increase in proline content under water stress conditions emerged as a common feature across all the three groups of germplasm (**Figure 6B**). The mean proline content in the *T. dicoccoides* rose from 0.13 to 0.33 mg g^{-1} FW under water stress (**Table 2**). The average value of proline accumulated by *Ae. tauschii* accessions under water stress conditions (1.12 mg g^{-1} FW) almost doubled as compared to its content under well-watered conditions (0.5 mg g^{-1} FW).

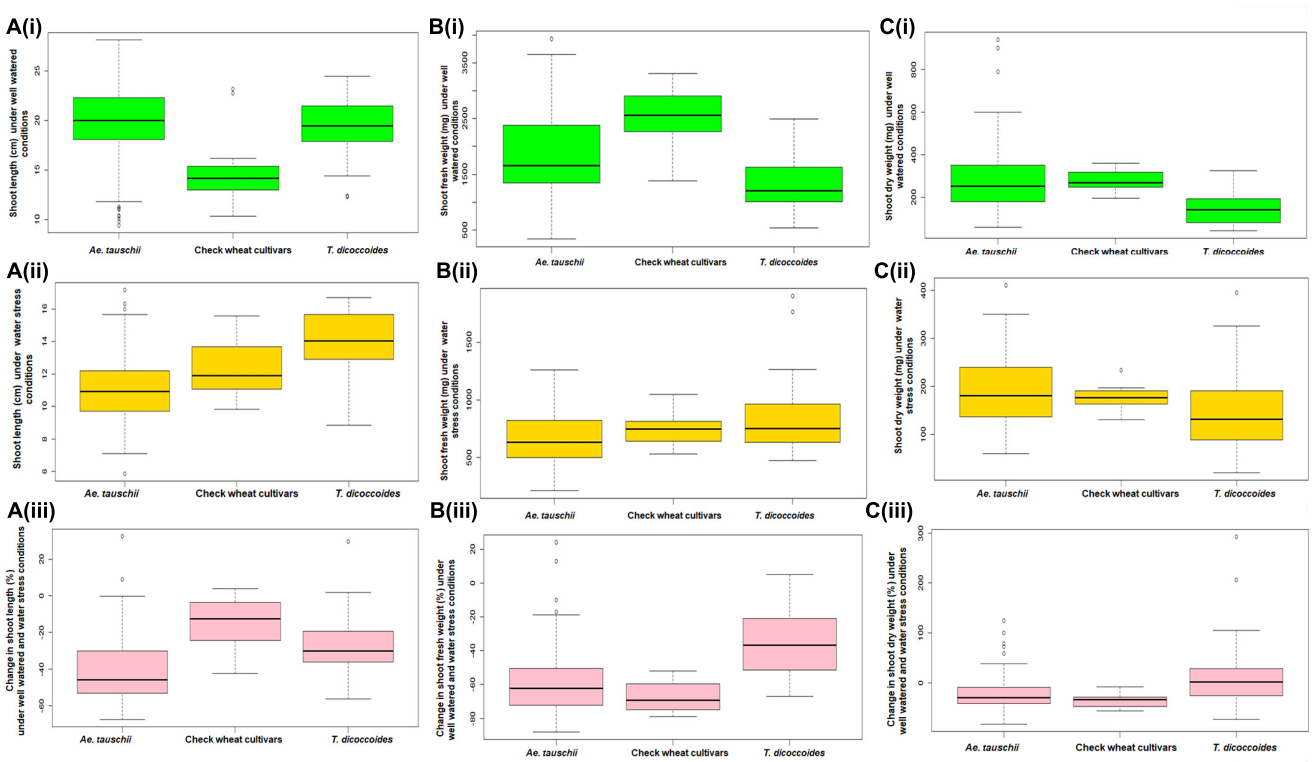

FIGURE 4 | Boxplot representation of shoot traits of accessions of *Ae. tauschii*, *T. dicoccoides* and check wheat cultivars under well watered and water stress conditions. **(A)** Shoot length under **(Ai)** well watered, **(Aii)** water stress, and **(Aiii)** change in shoot length under well watered and water stress conditions. **(B)** Shoot fresh weight under **(Bi)** well watered, **(Bii)** water stress, and **(Biii)** change in shoot fresh weight under well watered and water stress conditions. **(C)** Shoot dry weight under **(Ci)** well watered, **(Cii)** water stress, and **(Ciii)** change in shoot dry weight under well watered and water stress conditions.

More than 50% of the *Ae. tauschii* accessions accumulated amount of proline more than the highest levels of proline accumulated by *T. dicoccoides* and check wheat cultivars under stress (**Figure 6B**).

With respect to degree of proline induction under water stress, wide genotypic variability could be identified both within and between the three species of wheat (**Figure 6C**). C-306, a rain-fed bread wheat cultivar of pre-green revolution era demonstrated a 606.25% rise in proline content under stress (**Table 3**). Higher levels of proline inducibility were observed in the accessions of *T. dicoccoides* that varied from 50 to 850% (**Figure 6C**). The highest level of proline induction was found in *T. dicoccoides* accession 4656 which exhibited an increase of 835.14% under water stress (**Supplementary Table S6**). While both constitutive (un-induced) and elevated (induced) levels of proline were higher in *Ae. tauschii*, comparatively lower degree of proline induction (up to 450%) was observed in them. Nine of the 26 *T. dicoccoides* accessions and seven of the 57 *Ae. tauschii* accessions registered greater than 250% increase in proline content under water stress (**Supplementary Table S6**).

Membrane Injury in Leaf Cells

The screening of field grown wild accessions for membrane injury using PEG-6000 revealed ample genetic variability both within and between the three groups of germplasm- *Ae. tauschii*, *T. dicoccoides*, and cultivated wheats. Per cent membrane injury ranged from 21.43 to 89.38% (**Table 2**). Among cultivated wheats, drought adapted variety C-306 showed minimum membrane injury of 58.27% **(Table 3)** as against WHD-943, which suffered a damage of 89.38% (**Supplementary Table S6**). Among the accessions of *Ae. tauschii*, membrane injury levels ranged from 21.43 to 66.86% (**Figure 7**). *Ae. tauschii* accessions 9799, 14178, 14240 experienced membrane damage between 20 and 30% (**Table 3**), thus qualifying as accessions maintaining maximum cell membrane stability under water stress. Eight additional lines revealed membrane injury up to 40%. It could be seen that within a set of 57 *Ae. tauschii* accessions, 20 suffered membrane damage lower than the minimum injury seen in a check wheat cultivar, i.e., C-306 (58.27%). As could be seen from the boxplot representation (**Figure 7**), the lower membrane injury spectrum was primarily occupied by *Ae. tauschii*, whereas higher injury range was populated by *T. dicoccoides* and checks. Compared to *Ae. tauschii*, *T. dicoccoides* displayed relatively higher membrane injury levels, as all accessions lay in the range of 43.46–87.19%. *T. dicoccoides* suffered an average membrane damage of 73% (**Table 2**), which was higher than the maximum membrane injury noted in *Ae. tauschii* (66.86% in 14211). Nevertheless, membrane injury levels lower than C-306, i.e., 49.49% (accession 4655) and 43.46% (accession 7079) could be identified in *T. dicoccoides*. Later analysis revealed that higher cell membrane injury may be

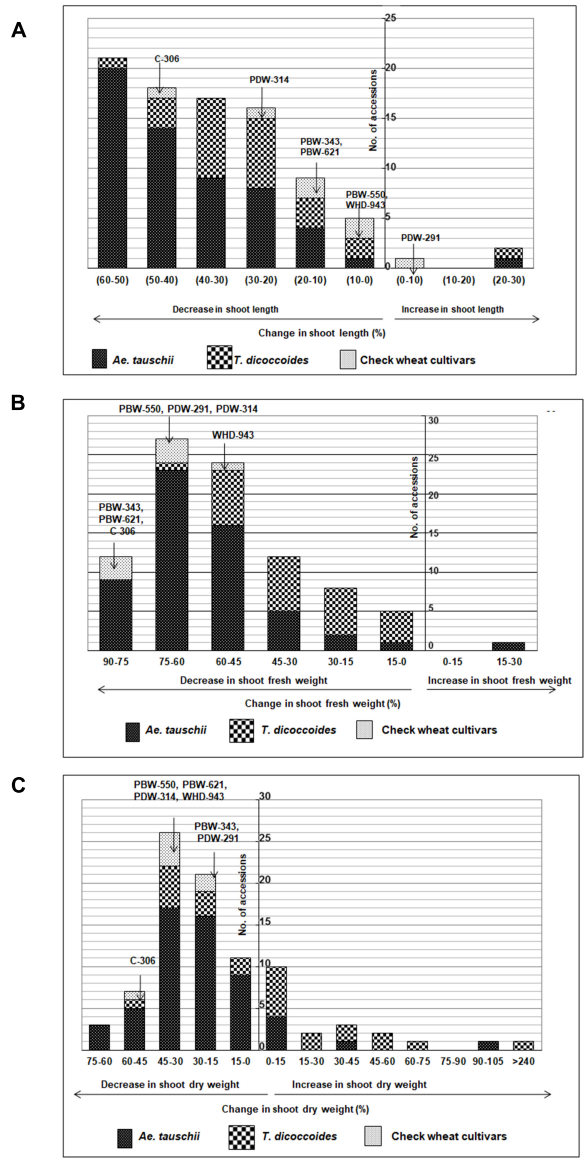

FIGURE 5 | Distribution of accessions of *Ae. tauschii*, *T. dicoccoides* and check wheat cultivars with regard to change in **(A)** shoot length, **(B)** shoot fresh weight, and **(C)** shoot dry weight under well watered and water stress conditions.

desirable for inducing stress adaptive plasticity in other morpho-physiological traits.

Change in Agronomic and Productivity Related Traits Due to Water Stress: Use as Tolerance Indices

Plasticity assays presented above were confined to easily quantifiable, vegetative stage traits. The possibility of early-stage stress adaptive plasticity translating into improved stress tolerance needed to be probed. In other words, if a genotype records less or no reduction under water stress in a seedling assay (i.e., high adaptive plasticity) do we expect greater resilience or lesser reduction in an advanced stage productivity related trait? Four prospective tolerance indices based on changes in trait value for days to flowering (tolerance index 1), plant height (tolerance index 2), spike length (tolerance index 3), and grain weight (tolerance index 4) have been employed in the present study (**Supplementary Table S7**). Variation in these traits is presented and subsequently used to determine correlation with stress adaptive plasticity for seedling and leaf tissue based traits.

Analysis of variance carried out for phenotypic traits evaluated over two crop seasons 2010–11 and 2011–12 on the present wheat germplasm set revealed significant effect of genotype, water stress regime, year and their respective interactions (**Table 4**). BLUP values for these traits were estimated across the years and used to conduct further analysis in the form of correlations and hierarchical clustering. Different accessions of *Ae. tauschii* took 107–127 days to flower under well watered conditions, which reduced to a period between 99 and 124 days under water stress conditions. *T. dicoccoides* accessions, on the other hand, took 105–124 days for heading under well-watered and 103–122 days to flower under water stress conditions. Cultivated wheats flowered earlier, i.e., between 88 and 109 days under well watered and 85–104 days under water stress conditions. Plant height, in case of *Aegilops tauschii*, ranged from 65.00 to 106.00 cm under well-watered and 44.33–85.00 cm under water stress conditions (**Figures 8Ai–iii**). Several *Ae. tauschii* accessions suffered minimum decline and maintained their plant height even under water stress conditions. Compared to *Ae. tauschii* and check wheat cultivars used, the present set of *T. dicoccoides* seemed to encompass an upward shifted spectrum of genetic variation for plant height-89.33–170 cm under well watered and 76.33–147.33 cm under water stress conditions (**Figures 8Ai,ii**). *Triticum dicoccoides* faced an overall reduction in average plant height under water stress conditions (average height of 128.20 and 115.45 cm, respectively, in well watered and water stress set), i.e., maximum 41% reduction in plant height was observed in wild species as compared to 8% reduction observed in check wheat cultivars (**Figure 8Aiii**).

With respect to spike length, range varied from 10 to 18 cm in *Ae. tauschii* and 15.6–25 cm in *T. dicoccoides* under well watered conditions (**Figure 8Bi**). The spike length range shifted to 9.6–14.8 cm in *Ae. tauschii* and 11.33–21 cm in *T. dicoccoides* under water stress conditions (**Figure 8Bii**). As far as grain weight is concerned, with increase in ploidy levels, grain weight was found to increase for three groups of species under both water regimes (**Figure 8C**). The grain weight varied from 1140 to 2145 mg (well watered) to 1155–2248 mg (water stress) for diploid progenitor *Ae. tauschii*. In case of tetraploid *T. dicoccoides*, this range lay between 1134 and 4315 mg (well watered) to 1785–4248 mg (water stress). The variation in present day check wheat cultivars extended beyond this limit as they developed heavier grains to the extent of 3925–6068 mg under well-watered and 4413–5805 mg under water stress conditions. However, with regard to change in grain weight under water stress, an increase in grain weight was observed in several accessions (**Figure 8Ciii**).

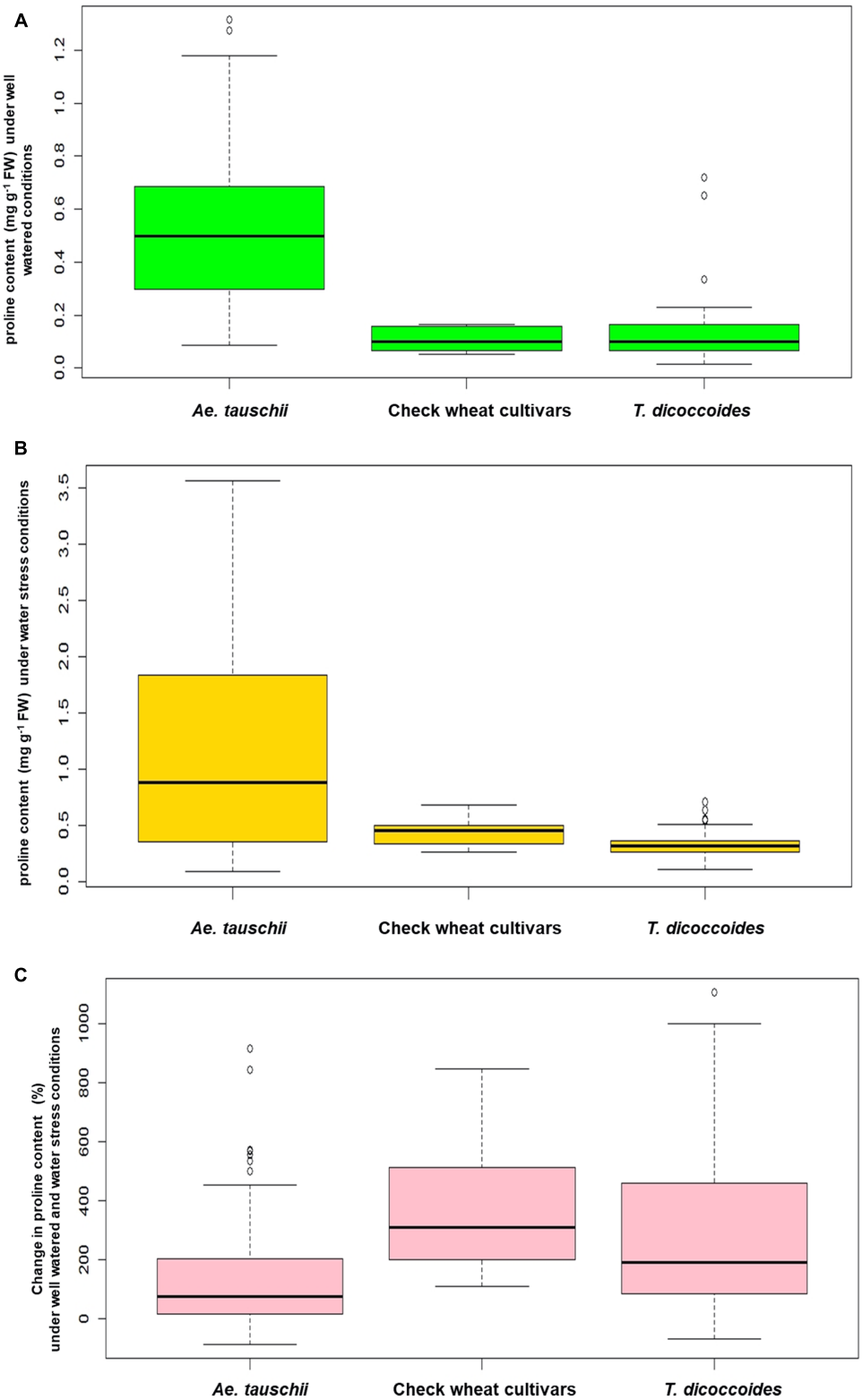

FIGURE 6 | Boxplot representation of proline content in accessions of *Ae. tauschii*, *T. dicoccoides* and check wheat cultivars **(A)** under well watered, **(B)** water stress and **(C)** change in proline content under well watered and water stress conditions.

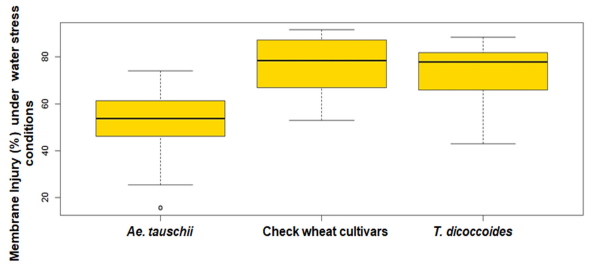

FIGURE 7 | Boxplots representation of per cent membrane injury in accessions of *Ae. tauschii*, *T. dicoccoides* and check wheat cultivars under water stress conditions.

Associations Within and Between Early Stage Adaptive Plasticity and Tolerance Indices

Correlations between length and biomass observations recorded on the same morphological trait (e.g., root length) were observed as per expectation. However, remarkably strong positive correlations emerged between root and shoot trait based plasticities (**Table 5**). For instance, positive associations could be seen between changes in root and shoot length ($r = 0.447$, $n = 87$), change in root length and change in shoot dry weight ($r = 0.470$). Change in root dry weight revealed slight good correspondence with both change in shoot fresh weight ($r = 0.251$) as well as change in shoot dry weight ($r = 0.369$). This was unexpected with respect to balanced growth hypothesis where root adaptive responses to stress are often at cost of shoot growth. These positive correlations indicated that root responses were truly adaptive in nature. In genotypes which responded to water stress by increasing root growth, the reduction in shoot length was less severe, compared to genotypes which could not respond by an increase in their root growth.

Membrane injury seems to work as an excellent stress adaptive plasticity induction mechanism as indicated by significant positive association with all the three shoot characters i.e., change in shoot length ($r = 0.332$), change in shoot fresh weight ($r = 0.296$) and change in shoot dry weight ($r = 0.276$). Growth induction for shoot based characters reflected their better stress adaptive plasticity of shoots than roots. Further, membrane injury was positively correlated with change in the content of proline ($r = 0.227$) and change in grain weight under water stress ($r = 0.359$). Genotypes showing higher membrane injury under water stress suffered lower reduction in root and shoot parameters as well as grain weight. This may not be plausible if we regard membrane injury as a stress induced damage, but can be explained to some extent if membrane injury serves as a stress signal for activating adaptive responses. Out of the four tolerance indices, tolerance index based on plant height and spike length correlated weakly with plasticity indices (**Table 5**). Tolerance based on days to flowering correlated well with several plasticity indices ($r = 0.372$ with membrane injury, $r = 0.286$ with change in root length, $r = 0.352$ with change in shoot length, $r = 0.268$ with change in shoot dry weight). Strongest impact of early stage plasticity indices was, however, observed on grain weight based tolerance index which was positively associated with change in root dry weight under water stress ($r = 0.267$), change in shoot fresh weight ($r = 0.299$), change in shoot length ($r = 0.404$) and change in shoot dry weight ($r = 0.526$). These correlations furnish a link between field and seedling based plasticities.

Based on Best linear unbiased predictor (BLUP) values of plasticity scores and tolerance indices, HCA, employing Ward's Method was performed using squared euclidean distance matrix to elucidate genotypic variation within the present germplasm for responsiveness to water stress. The overall stress adaptive response of three groups of species with respect to various water stress plastic traits is presented as a heat map (**Figure 9**). The heat map is based on change in trait values observed across well watered and water-stress conditions. Stress adaptive plasticity seemed to have a strong genotypic basis rather than an exclusive property of a species or a group. Nevertheless, strong species based trends were also visible. The genotypic clustering formed two major groups- the smaller group (cluster B) represented by resilient genotypes which showed either less of the unfavorable change or in some cases positive change under water stress conditions. This group consisted of nine *Ae. tauschii* and 13 *T. dicoccoides* accessions. Together, these constitute the group of genotypes possessing greater stress adaptive plasticity. Among these, three *Ae. tauschii* accessions 9816, 14109, 14128 and two *T. dicoccoides* accessions 5259 and 7130 emerged as the five most stress adaptive genotypes considering all the traits. Out of these, accessions 14109 and 7130 have been marked for showing higher stress adaptive plasticity for root elongation and dry matter accumulation and accession 5259 for greater root fresh weight

TABLE 4 | Genotypic variation in wheat germplasm set for field based traits recorded under well watered and water stress conditions.

Traits/Sources of variation	df	Mean square values			
		Days to flowering (DTF)	Plant height (PLT)	Spike length (SPK)	Grain weight (GW)
Genotype (G)	88	497.85*	6859.76*	90.28*	288076.65*
Water stress regime (W)	1	3270.75*	29847.46*	654.3*	43056.58*
Year (Y)	1	530.91*	64569.69*	2553.26*	670422.48*
G X W	88	48.61*	488.08*	11.61*	22011.78*
G X Y	88	32.12*	91.74*	2.51*	1884.63*

*Significant at 0.05 level.

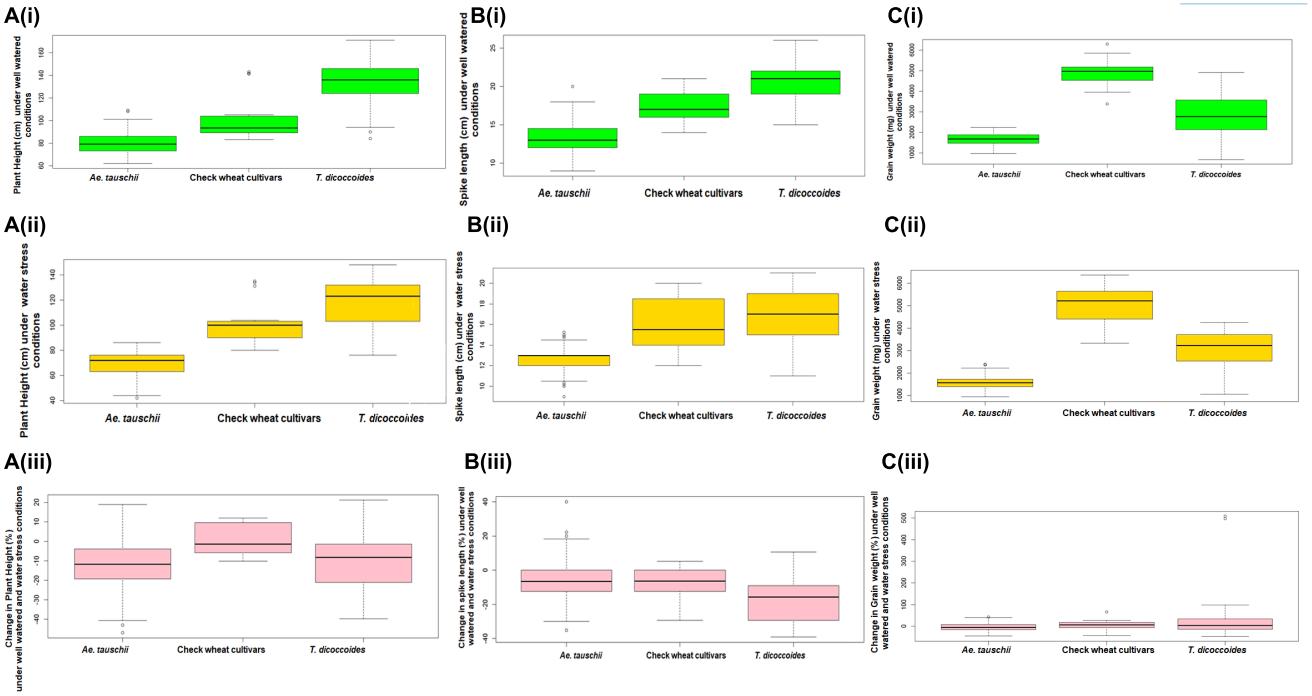

FIGURE 8 | Boxplots representation of phenotypic traits of accessions of *Ae. tauschii*, *T. dicoccoides* and check wheat cultivars under well watered and water stress conditions. **(A)** Plant height under **(Ai)** well watered, **(Aii)** water stress, and **(Aiii)** change in plant height under well watered and water stress conditions. **(B)** Spike length under **(Bi)** well watered, **(Bii)** water stress, and **(Biii)** change in spike length under well watered and water stress conditions. **(C)** Grain weight under **(Ci)** well watered, **(Cii)** water stress, and **(Ciii)** change in grain weight under well watered and water stress conditions.

TABLE 5 | Correlations between changes in trait values used for the study of stress adaptive plasticity and BLUP values based tolerance indices under water stress conditions.

	ΔProline	ΔRL	ΔRFW	ΔRDW	ΔSL	ΔSFW	ΔSDW	ΔDTF	ΔPLT	ΔSPK	ΔGW
MI	0.227*	0.135	0.149	0.123	0.332**	0.296**	0.276**	0.372**	0.093	0.004	0.359**
ΔProline		0.108	0.053	0.03	0.033	−0.058	0.007	0.065	0.225*	0.016	−0.06
ΔRL			0.351**	0.440**	0.447**	0.301**	0.470**	0.286**	0.089	0.006	0.134
ΔRFW				0.546**	0.09	0.172	0.101	−0.008	−0.006	0.214*	0.02
ΔRDW					0.187	0.251*	0.369**	0.202	0.09	0.092	0.267*
ΔSL						0.586**	0.478**	0.352**	−0.057	−0.07	0.404**
ΔSFW							0.603**	0.174	−0.074	−0.11	0.299**
ΔSDW								0.268*	0.057	−0.058	0.526**
ΔDTF									0.053	−0.023	0.279**
ΔPLT										0.219*	−0.1
ΔSPK											−0.202

**Correlation significant at the 0.05 level; **Correlation significant at the 0.01 level. Δ Change under water stress. MI, Membrane injury; RL, root length; RFW, root fresh weight; RDW, root dry weight; SL, shoot length; SFW, shoot fresh weight; SDW, shoot dry weight; DTF, days to flowering; PLT, plant height; SPK, spike length; GW, grain weight.*

acquisition and higher proline induction under water stress conditions. These progenitor accessions have been used in wheat breeding programme at our center to develop synthetic wheats. Cultivated wheats, on the heat map, were placed in the larger group (cluster A) representing moderate to low stress adaptive behavior. Notably, the cultivated types formed a close cluster and represented moderate levels of plasticity. Apparently, the wild species had a larger spectrum of variation and some of them constituted the group representing the least adaptive behavior.

DISCUSSION

Wild progenitors have been widely used as donors of resistance to biotic stresses such as powdery mildew (Rong et al., 2000), yellow rust (Gill and Raupp, 1987; Goodman et al., 1987; Cox et al., 1990), and karnal bunt (Villareal et al., 1995). Similarly, genes for productivity traits from *Ae. tauschii* (Gororo et al., 2002) and for higher grain weight and protein content from *T. turgidum* var *dicoccoides* have also been transferred to *T. aestivum*

(Kushnir and Halloran, 1984; Mesfin et al., 2000). Our center has successfully used marker based strategy to tag and transfer several disease resistance genes (e.g., *Lr57*/*Yr40*, *Lr76*/*Yr70*, *Lr58*) from wild species to the wheat breeding pipeline. The wild progenitors have been used less extensively as donors of abiotic stress tolerance though *T. dicoccoides* (Peleg et al., 2005) and *Ae. tauschii* (Kurahashi et al., 2009) have been targeted for drought (Peleg et al., 2005), high temperature (Pradhan et al., 2012), or salinity stress (Siasho et al., 2016). Trait plasticity as a component of productivity and stress tolerance remains a future goal, even though wild species are known to grow and thrive in alternatively harsh and favorable conditions.

The set of wild accessions on which this study is based have also been a part of other reports in literature. For instance, Shah et al. (2000) found *Ae. tauschii* accessions 3733 and 3734 to be totally immune to leaf rust. A new source of greenbug resistance has been derived from *Ae. tauschii* accession 9783 (Weng et al., 2005). Likewise, *T. dicoccoides* accessions 4657 and 4675 exhibited an intermediate fusarium head blight (FHB) reaction, whereas accession 5259 was found susceptible to FHB reaction (Oliver et al., 2007). Besides these reports, entire set of lines used in the study has also been characterized for various traits of economic interest at our center (Chhuneja et al., 2010, 2015; Gupta et al., 2010; Suneja, 2014; Suneja et al., 2015a,b, 2017; Arora et al., 2017). A direct hybridization approach to gene transfer from *Ae. tauschii* Coss. to *Triticum aestivum* has also been developed (Seghal et al., 2011). This cross referencing of *Ae. tauschii* and *T. dicoccoides* accessions to other studies indicates all round worth of the lines and is likely to promote their judicious use in future wheat breeding programmes.

With accessions of diploid (*Ae. tauschii*) and tetraploid (*T. dicoccoides*) progenitor species as the core genetic material, the present study was aimed to decipher how plasticity indices based on seedling and vegetative traits are able to correlate with agronomic and productivity related tolerance indices. Between these two sets of traits, there are several developmental steps which are likely to dimnish the influence of early stage stress adaptive behavior. There is a possibility that these simple early stage stress adaptive plasticity indices may represent a broader based plasticity mechanisms operating in these genotypes. The strategy of using changes in trait values as we shift from non-stress to stress conditions rather than *per se* trait values under stress allowed a delineation of stress adaptive plasticity. The association of early stage and more likely to be adaptive responses with later stage productivity based tolerance indices emerged as an important finding. Having established a strong genetic basis for the plasticity phenomenon, in this (by design) broad spectrum germplasm set, the identification of donors open up several follow up avenues.

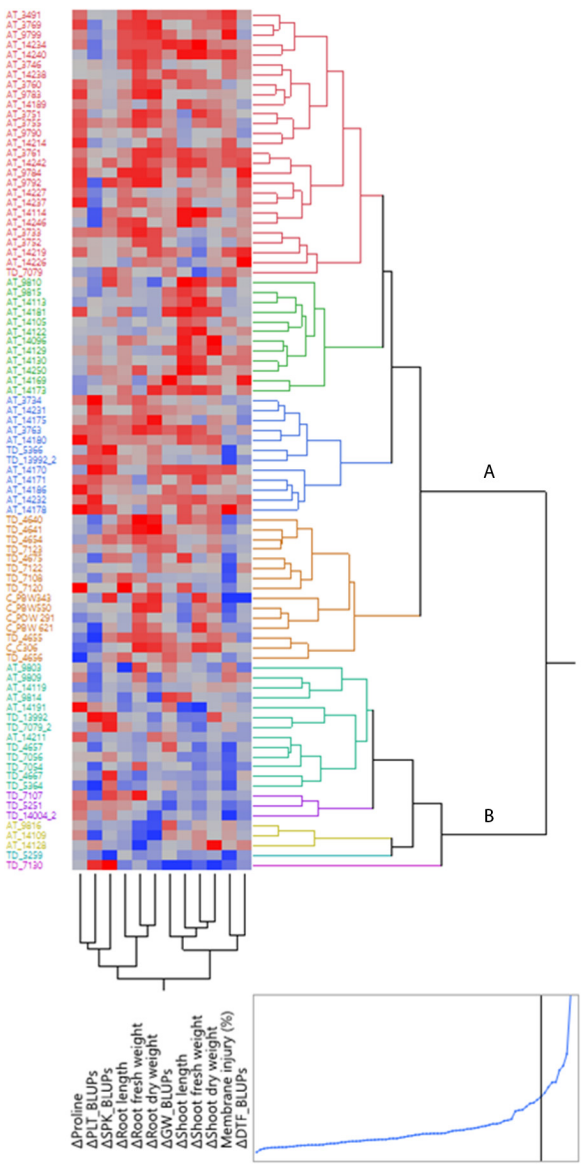

FIGURE 9 | Hierarchical cluster analysis (HCA) of early stage adaptive plasticity traits and BLUP derived tolerance indices in wheat germplasm set. AT, TD, and C correspond to accessions of *Aegilops tauschii*, *Triticum dicoccoides* and check wheat cultivars, respectively. Each accession is visualized in a single row and each trait value is represented by a single column. Red indicates unfavorable change or reduction in trait value, whereas less of the unfavorable change or increase in trait value is depicted in blue.

Genotypic Variation for Stress Adaptive Plasticity in Root-Shoot Traits

In this study, marked induction behavior in terms of root development (increase in root length and dry weight) came to light in some of the wild species accessions. Kadam et al. (2015) found root length of wheat increased in response to water deficit stress and reported plasticity in root length, thickness, root weight density, xylem diameter and vessel number along the length of the root. An increase in root: shoot ratio and absolute root mass in response to moisture stress has been previously reported in wheat by Blum et al. (1983) and Reynolds et al. (2007). Balanced growth hypothesis (Bloom et al., 1985) suggests that some plants respond to drought by stimulating or maintaining root growth while reducing shoot growth. Using Lockhart's equation, Hsiao and Xu (2000) elucidated that the underlying mechanism behind

shift in allometry are the differences in the sensitivity of root and shoot growth to water stress.

At the level of stress administered in the present study, the responses went beyond redistribution of resources between root and shoot to stress adaptive plasticity as revealed by positive correlation between root and shoot based plasticity. This resulted from genotypes which responded to water stress by increasing root growth and consequently maintained better shoot length, compared to genotypes which could not respond by an increase in their root growth. While this phenomenon has been mentioned in results, genotypes going even one step further in their responses need to be mentioned. Greater adaptation to water stress than well-watered conditions was observed in *Ae. tauschii* accession 14191 and *T. dicoccoides* accession 7130, where in addition to increase in the relative size of root (adaptive plasticity of 101.50 and 91.53%, respectively), shoot growth displayed a stress adaptive plasticity of 20.20 and 29.55%, respectively. Similarly, *Ae. tauschii* accession 9803 expressed stress adaptive plasticity of the order of 94.74% in shoot dry weight and 34% in root dry weight. These lines proved to be notable exceptions to the norm as an increase in both root- and shoot length was evident under water stress. Greater adaptation to water stress could be found in some wild accessions where root length/weight is relatively low under well watered conditions. These accessions seem to have greater adaptation to water stress than well watered conditions. Such remarkable responses warrant a strong genetic basis.

Proline: High Inducibility but Complex Role

Lower basal levels of proline in *T. dicoccoides* (in comparison to *Ae. tauschii*) under well watered conditions may hint toward their local adaptation to arid climates of Israel and regions of North Crescent where *T. dicoccoides* originated. Abundant genetic variation in water stress induced proline accumulation was identified in accessions of *Arabidopsis thaliana* (Verslues and Juenger, 2011; Kesari et al., 2012; Verslues et al., 2014). Accessions from generally drier regions have lower proline accumulation. Accessions that habitually face drought may have other metabolic adjustments such that higher levels of proline may not be needed as long as a particular threshold level of osmotic potential is maintained in the cell (Kesari et al., 2012). *T. dicoccoides* evolved in a relatively restricted geographic region, i.e., eastern Mediterranean region, characterized by a long, hot dry summer and a short, mild wet winter with fluctuating amounts and distribution of rainfall (Loss and Siddique, 1994; Peleg et al., 2005). Stress responsive higher proline induction in *T. dicoccoides* may be aligned to dynamic up-regulation of *P5CS1* (proline biosynthesis) or down-regulation of *ProDH* (proline degradation). *Ae. tauschii* that showed higher constitutive but lower levels of proline induction, is known to be adapted to a more continental climate of Central Asia. However, further investigations are necessary to reveal the background of high proline content in *Ae. tauschii* accessions. AABB-genome through its metabolic plasticity and DD-genome through heightened basal expression together might have contributed in enhancing the fitness of natural hexaploid wheat across diverse eco-geographical environments. A similar observation has also been reported in a study where the expression pattern of *HKT1;5* was studied in 2x (diploid), 4x (tetraploid), nat-6x (natural hexaploid) and neo-6x (synthetic hexaploid) genomes of wheat in response to salt stress (Yang et al., 2014).

Inter-Trait Associations: Uncovering Network of Plasticities

Three remarkable observations with respect to network of plasticities emerged and may serve as lead for further studies. First is the positive correlation between root and shoot based stress adaptive plasticities which deviated from the generally observed resource allocation to roots at the cost of shoots under water stress (Hsiao and Xu, 2000; Weiner, 2004; Gargallo-Garriga et al., 2014). Second is concerning membrane injury serving as a signal or trigger for stress adaptive plasticity and thus showing positive association with various morpho-physiological attributes. Third important observation related to the marked inducibility in proline accumulation in leaf tissue, but its largely negative connotations for stress adaptive plasticity. Finally, the association of early stage and more likely to be adaptive responses with later stage productivity based tolerance indices emerged as an important finding. The associations observed in this study point toward the larger perspective that wild species are able to capitalize on plasticity to ensure fitness in variable environments (Vilela and Gonzalez-Paleo, 2015).

Opportunities for Genetic Analysis and Molecular Marker Tagging

Identifying genes responsible for drought response has been challenging because of polygenic nature as well as issues concerning easy evaluation of these abiotic stress responsive traits. Inducible traits would be even harder to pursue in breeding programmes, but molecular marker assisted selection may prove to be a powerful tool as demonstrated by the success in transfer of submergence tolerance gene in rice (Bailey-Serres et al., 2010). Root growth angle as a trait was not targeted in the present study, however, this trait is receiving increased attention due to cloning of *DRO1* (Deep Rooting) locus in rice (Uga et al., 2013). Recent reviews foresee optimization of root system architecture (RSA) as the basis of second green revolution (Meister et al., 2014). Attempts to identify a gene or a set of genes that control the switch for shift in root-shoot allometry under water stress are at present largely lacking. Genetic and molecular marker analysis for induction of root growth under water stress at the diploid level using contrasting *Ae. tauschii* parents (accessions 9803, 9814, 14109, and 3769) offers itself as a feasible prospect. Inducible traits represent the best option in the face of expected variations in stress over space and time.

Identification of Potential Donors

On the whole, many wild accessions could be identified as suitable donors for a suite of water stress responsive traits. *Aegilops tauschii* accessions 9816 and 14109 revealed higher stress adaptive plasticity in terms of increased root biomass (fresh and dry weight) under stress. *Aegilops tauschii* accession

14128 exhibited root elongation and higher proline induction under water stress. *Aegilops tauschii* accession 9809 increased root and shoot dry weight under stress. This accession also displayed physiological plastic responses in terms of increased activity of ROS scavenging enzymes under drought stress (Suneja et al., 2017). Within *T. dicoccoides* group, accession 7130 displayed root and shoot elongation, increased root and shoot dry matter accumulation and higher proline induction under stress. *T. dicoccoides* accession 5259 too accumulated more proline and acquired higher root fresh weight under conditions of decreased water availability. These genotypes represent a situation where well watered condition seems to be more stressful than the water stress (at the level of stress administered in this study). This accession-specific behavior invites opportunities for molecular genetic analysis of inducibility under stress as a trait, associated pleiotropic effects, if any, for eventual introgression into elite wheat cultivars. Crosses have been conducted between *T. dicoccoides* accession 5259, 7130 and *Ae. tauschii* accessions 9816, 14109, and 14128 to develop synthetic hexaploid wheats that might combine favorable drought responsive traits from AABB- and DD-genome of wild progenitors of wheat, leading to enhanced trait expression due to gene interaction. Subsequent crosses with high yielding wheat cultivars may help tailor their genetic makeup that enables them to thrive and perform well under conditions of unanticipated or variable environmental stress. Ideally, a winning combination of root and shoot traits along with appropriate metabolic switches may be successfully met to enhance water stress resilience of present day wheat cultivars.

AUTHOR CONTRIBUTIONS

YS conducted the experiments, generated data, carried out the analysis, and prepared the draft of the manuscript. AG and NB conceived the idea, designed and supervised the study, interpreted results, revised, and finalized the manuscript.

FUNDING

This work was supported by the financial grant received from Innovation in Science Pursuit for Inspired Research (INSPIRE) Programme, Department of Science and Technology, Government of India (Grant No. DST/INSPIRE Fellowship/2010 (162)).

ACKNOWLEDGMENTS

We thank School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana for sharing the seed of *Aegilops tauschii* and *Triticum dicoccoides* accessions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00211/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 18 March 2019
doi: 10.3389/fpls.2019.00308

Potential of *Aegilops* sp. for Improvement of Grain Processing and Nutritional Quality in Wheat (*Triticum aestivum*)

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OPEN ACCESS

Edited by:
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Rothamsted Research (BBSRC),
United Kingdom

Reviewed by:
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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *30 August 2018*
Accepted: *26 February 2019*
Published: *18 March 2019*

Citation:
Kumar A, Kapoor P, Chunduri V, Sharma S and Garg M (2019) Potential of Aegilops sp. for Improvement of Grain Processing and Nutritional Quality in Wheat (Triticum aestivum). Front. Plant Sci. 10:308. doi: 10.3389/fpls.2019.00308

Wheat is one of the most important staple crops in the world and good source of calories and nutrition. Its flour and dough have unique physical properties and can be processed to make unique products like bread, cakes, biscuits, pasta, noodles etc., which is not possible from other staple crops. Due to domestication, the genetic variability of the genes coding for different economically important traits in wheat is narrow. This genetic variability can be increased by utilizing its wild relatives. Its closest relative, genus *Aegilops* can be an important source of new alleles. *Aegilops* has played a very important role in evolution of tetraploid and hexaploid wheat. It consists of 22 species with C, D, M, N, S, T and U genomes with high allelic diversity relative to wheat. Its utilization for wheat improvement for various abiotic and biotic stresses has been reported by various scientific publications. Here in, for the first time, we review the potential of *Aegilops* for improvement of processing and nutritional traits in wheat. Among processing quality related gluten proteins; high molecular weight glutenins (HMW GS), being easiest to study have been explored in highest number of accessions or lines i.e., 681 belonging to 13 species and selected ones like *Ae. searsii*, *Ae. geniculata* and *Ae. longissima* have been linked with improved bread making quality of wheat. Gliadins and low molecular weight glutenins (LMW GS) have also been extensively explored for wheat improvement and *Ae. umbellulata* specific LMW GS have been linked with wheat bread making quality improvement. *Aegilops* has been explored for seed texture diversity and proteins like puroindolins (*Pin*) and grain softness proteins (*GSP*). For nutrition quality improvement, it has been screened for essential micronutrients like Fe, Zn, phytochemicals like carotenoids and dietary fibers like arabinoxylan and β-glucan. *Ae. kotschyi* and *Ae. biuncialis* transfer in wheat have been associated with higher Fe, Zn content. In this article we have tried to compile information available on exploration of nutritional and processing quality related traits in *Aegilops* section and their utilization for wheat improvement by different approaches.

Keywords: *Aegilops*, grain micronutrients, puroindolins, gliadins, dietary fiber, glutenins, phytochemicals

INTRODUCTION

Some of the most important cereal crops in the world are the members of the grass (Poaceae) family and belong to three major subfamilies – Pooideae, Oryzoideae and Panicoideae. These subfamilies diverged from a common ancestor around 50–70 million years ago (Bolot et al., 2009) (**Figure 1A**). Genus *Aegilops* is the closest relative of wheat followed by rye, barley, oats and brome in the Pooideae subfamily, rice in Oryzoideae, millets, sorghum and maize in Panicoideae (**Figure 1A**). Among the Pooideae, wheat (*Triticum* L.) is one of the major staple foods in the world. Due to its unique flour composition and viscoelastic properties, wheat is more suitable for industrialized food production than any other crop. Recently, demand for wheat based convenience foods (fast, ready to eat, frozen etc.) have increased due to the rise in urban population and changing lifestyles. Therefore, the end product quality of wheat has become important. With an increasing concern for texture and taste, there have been a lot of challenges for breeders to develop cultivars that satisfy specific end product requirements. Nutrition is another important aspect of wheat research. There are approximately two billion people in the world that suffer from nutrient deficiency also known as hidden hunger (World Health Organization, 2006). Since wheat provides around one fifth of calorific input to people across the world (Food and Agriculture Organization of United Nations [FAO], 2014), enhancing its nutritive value becomes of great importance.

A lot of breeding programs have been initiated to select or develop varieties with improved nutrient content and specific end product quality. The existing germplasm of wheat have been extensively explored for traits related to end product quality and nutrition. The Green revolution has resulted in the development of high yielding and disease resistant varieties and most of the varieties grown today consist of an assembly of genes pyramided by breeders (Lopes et al., 2015). The breeding programs thus have relied on limited number of parent lines for development of wheat germplasm. A report has suggested that due to this genetic bottleneck the population size of wheat has been reduced by 6% (Cavanagh et al., 2013). This narrow genetic diversity often limits the improvement of many traits in wheat. Therefore, the need to explore secondary and tertiary gene pools of wheat has grown. Secondary and tertiary gene pools of wheat mainly consist of wild varieties that are outstanding sources of genetic variability. The secondary gene pool of wheat mainly consists of polyploid *Triticum* and some of *Aegilops* species that share at least one of the A, B and D genomes of hexaploid wheat. The tertiary gene pool consists of wild species with genomes other than A, B and D of wheat. The relationship within and between *Aegilops* and *Triticum* has been a matter of debate and many classification systems exist (Kilian et al., 2011). The latest monograph of Van Slageren (1994) which is based on morphological studies is mostly followed for classification and nomenclature of *Aegilops* and same has been followed in this review article. For wheat the classification system by Dorofeev et al. (1979) is mainly followed. The *Aegilops* genus consists of 11 diploid, 10 tetraploid and 2 hexaploid species (**Figure 1B**). Species of *Aegilops* occur in Eurasia and North America, but most species are found near the center of origin, the Fertile Crescent in the Middle East, and around the Mediterranean Sea (**Figure 2**). These species consist of C, D, M, N, S, T and U genomes which have evolved from a common ancestor (**Figure 1B**) and can be used to incorporate genetic material from the wider gene pool into newly developed cultivars of wheat, thus increasing its genetic diversity.

There have been many reports of species of *Aegilops* being utilized for the improvement of agronomic traits such as rust resistance, powdery mildew resistance and tolerance against other abiotic stresses. More than 41 resistance genes for various biotic and abiotic stresses have been transferred from *Aegilops* to wheat via chromosome translocations or homoeologous recombination (Zhang et al., 2015) and many of these genes have been fairly successful in many breeding programs (Jahier et al., 1989; Ambrozkova et al., 2002; Zhang et al., 2015). This review summarizes the potential of *Aegilops* species for utilization in improvement of end product and nutritional quality of wheat.

UTILIZATION OF *Aegilops* FOR IMPROVEMENT OF END PRODUCT QUALITY OF WHEAT

The end product quality of wheat is affected by a number of factors such as: total protein content, grain texture and seed storage proteins composition. Seed storage proteins are the major determinants of end product quality and mainly consist of glutenins and gliadins. A large number of alleles of glutenins and gliadins have been explored in *Aegilops* species with their implications on end product quality. Grain texture related puroindolins, grain softness protein (*GSP*) and many other grain quality related genes have also been reported from *Aegilops*.

High Molecular Weight Glutenins (HMW GS)

High molecular weight glutenins are the major determinants of bread making quality of wheat. Their importance can be attributed to the fact that though they constitute only about 12% of total seed storage proteins, up to 60% of alterations in baking parameters are affected by them (Payne et al., 1987). HMW GS are coded by *Glu1* loci present on the long arms of homoeologous group 1 chromosomes (1A, 1B and 1D) named as *Glu A1*, *Glu B1* and *Glu D1*, respectively. Each locus produces two subunits of different size; called x-type (larger) and y-type (smaller) subunits i.e., $1A_x$, $1A_y$; $1B_x$, $1B_y$ and $1D_x$, $1D_y$. Subunits $1B_x$, $1D_x$ and $1D_y$ are expressed in most of the bread wheat cultivars while $1B_y$ and $1A_x$ are expressed in some wheat cultivars. The gene coding $1A_y$ generally remains silent in most of bread wheat cultivars (Halford et al., 1989). Only 21 alleles have been reported for *Glu A1* locus, while for *Glu B1* more than 69 alleles and for *Glu D1* only 29 alleles have been documented in bread wheat germplasm (McIntosh et al., 2013).

Due to this limited genetic diversity, high levels of allelic variations at *Glu 1* loci are required in the quality wheat breeding practice. These are easiest to study as they can be conveniently resolved and identified by electrophoresis. Among

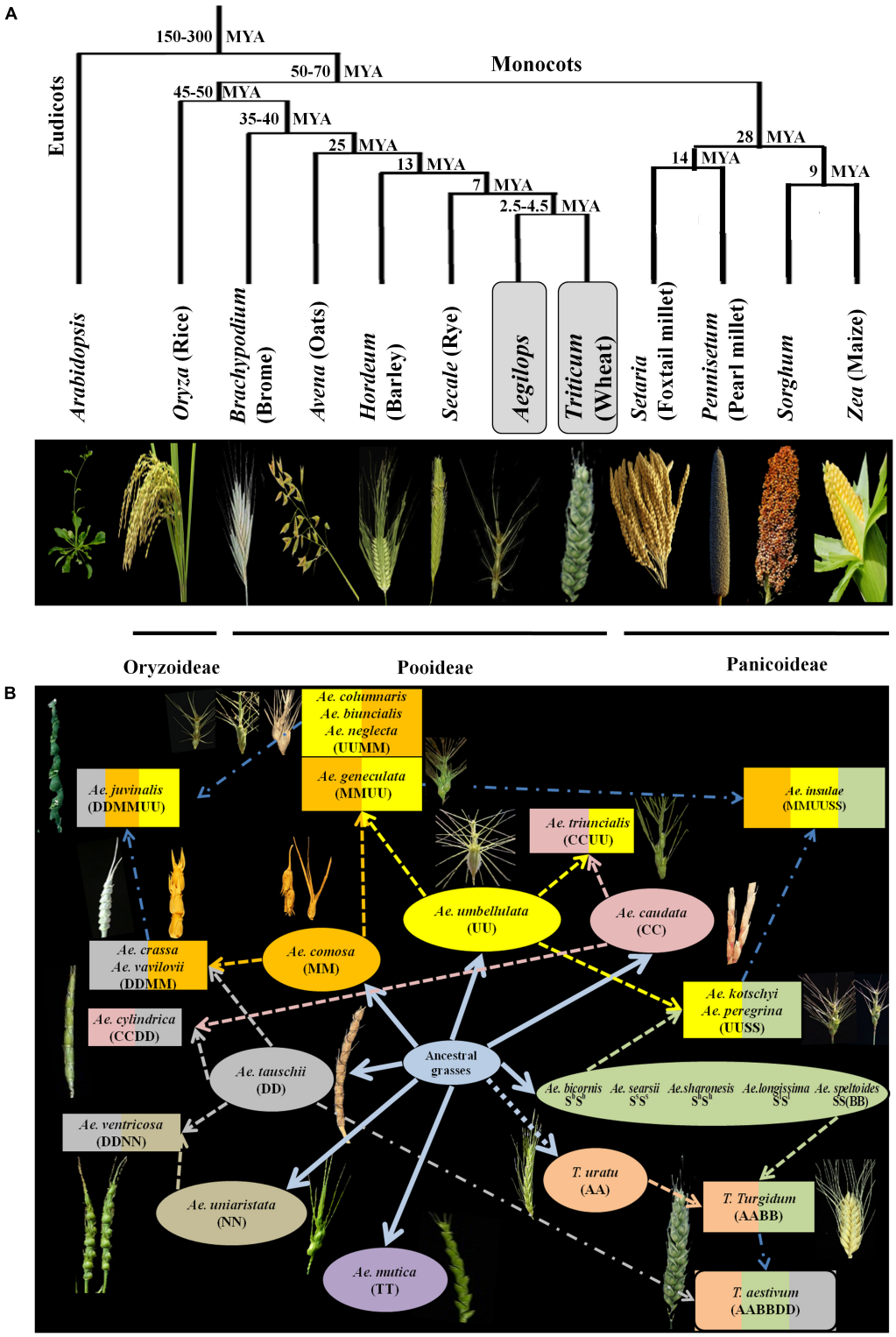

FIGURE 1 | (A) Evolutionary relationship among different cereals. *Aegilops* is the closest relative of wheat. Divergence times from a common ancestor are indicated on the branches of the phylogenetic tree in million years (MYA). Modified from Bolot et al. (2009). **(B)** Hypothesized evolution of wheat and species of *Aegilops*. Seven different genomes of *Aegilops* evolved from common ancestor (color coded). Colored dash arrows indicate the involvement of species for formation of other species of *Aegilops*. Blue colored dash dot arrows indicate hypothetical involvement of species. Hypothetical wheat evolution is also explained, cross between *Triticum urartu* and *Ae. speltoides* led to formation of *Triticum turgidum* which further hybridized with *Ae. tauschii* to form cultivated *Triticum aestivum.* Modified from Meimberg et al. (2009).

FIGURE 2 | World wide distribution of *Aegilops* species. Species of *Aegilops* are mainly distributed in Eurasia and North America with highest density of occurrence in Fertile Crescent near Middle East (Colors of pins indicate number of species of *Aegilops* found in that area). Data taken from Kew (RBG Grassbase) database (Clayton et al., 2006).

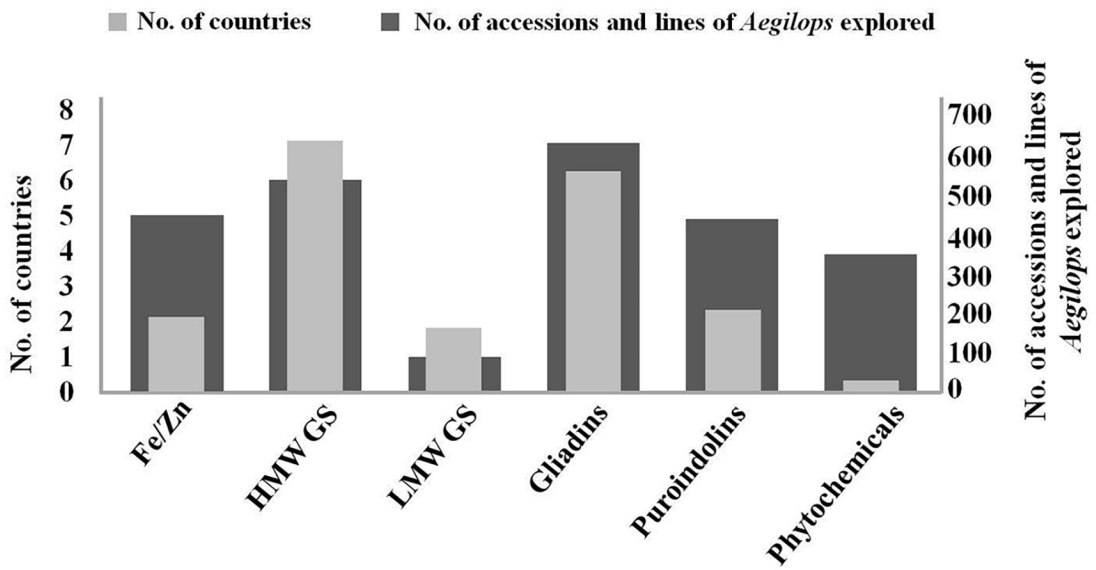

FIGURE 3 | Number of accessions/lines of *Aegilops* explored along with number of countries involved in their exploration for improvement of quality and nutritional traits in wheat. HMW GS are most explored, while phytochemicals are least explored among different groups across the world. Gliadin exploration is being carried out by highest number of countries with LMW and phytochemicals being in the lowest category.

the traits explored here, more than 600 lines and accessions of *Aegilops* have been studied across the world for their rich genetic diversity for HMW GS (**Figure 3**). Fairly large numbers of countries are involved in the exploration of HMW GS and their distribution across countries is also uniform (**Figure 4**). Primary structures of most of the *Aegilops* specific HMW GS are similar to wheat subunits. They contain conserved N-, C-terminals and a central variable repetitive region (Mackie et al., 1996;

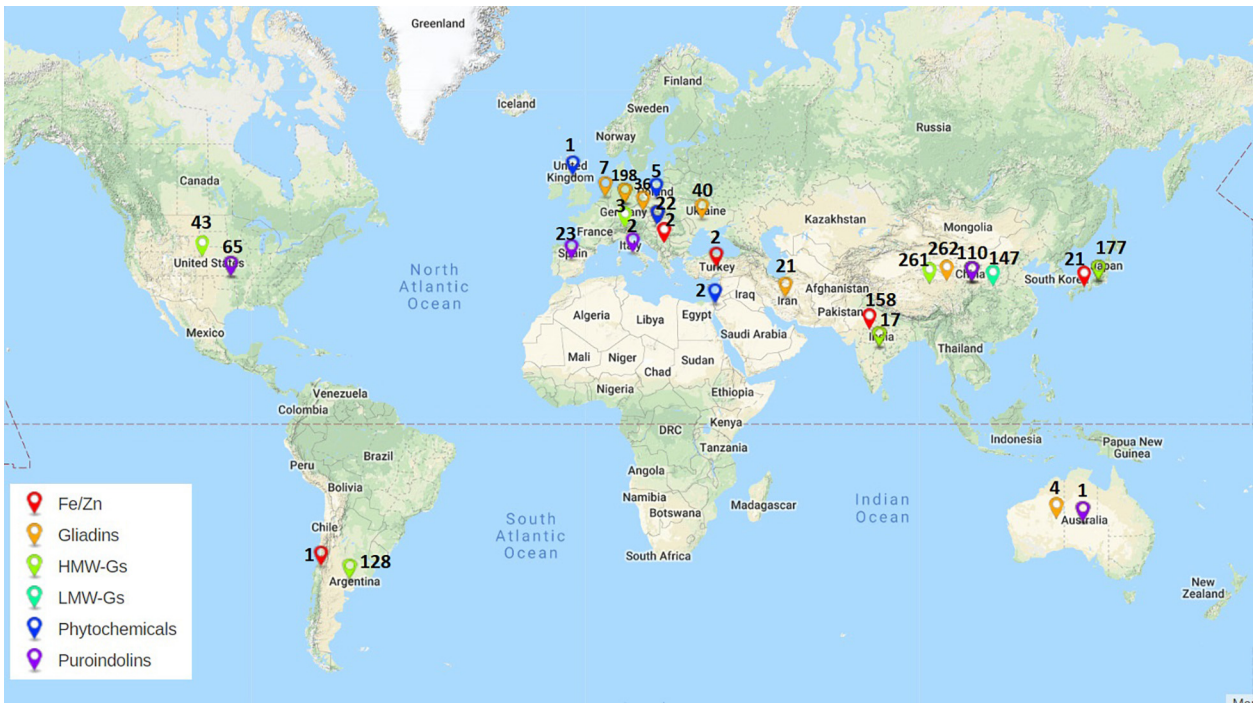

FIGURE 4 | Work done across the world on *Aegilops* for improvement of quality and nutrition. Colors of pins indicate different traits. Numbers along pins indicate total number of accessions/lines explored.

Wan et al., 2000; Xie et al., 2001). More than 30 subunits of HMW GS from *Ae. bicornis*, *Ae. longissima*, *Ae. sharonensis*, *Ae. searsii, Ae. cylindrica*, *Ae. umbellulata, Ae. caudata*, *Ae. juvenalis*, *Ae. kotschyi, Ae. comosa, Ae. uniaristata, Ae. crassa, Ae. ventricosa* and *Ae. speltoides* have been reported and studied (**Table 1**) (Wan et al., 2000, 2002; Xie et al., 2001; Liu et al., 2003; Sun et al., 2006; Farkhari et al., 2007; Jiang et al., 2012; Ma et al., 2013). Many of these HMW GS have been cloned and their sequence information is available.

Aegilops tauschii is regarded as D genome donor of wheat and its many accessions for HMW GS have been explored. For HMW GS, extensive studies have been done on the *Glu D1* loci from *Ae. tauschii* as variation in this locus is very important in determining dough strength and other end product qualities. More than 40 HMW GS allelic variants have been reported from multiple accessions of *Ae. tauschii* (Yan et al., 2002, 2003, 2004; Wan et al., 2005; Zhang et al., 2008; An et al., 2009; Wang K. et al., 2012). Many D genome synthetic hexaploids have been generated by crossing tetraploid durum wheat with *Ae. tauschii* and thus HMW GS alleles 2.1*D, 2.1D, 1.5D, 2D, 3D, 4D, 5D, 10D, 10.5D, 12D, 12*D, T2 (Pflüger et al., 2001), 2-1D, 2-2D, 2-3D, 1.5-1D, 2.1-1D, 10-1D, and 12-1D (Xu et al., 2010) have been transferred to wheat. D genome specific subunits of $5D_x+10D_y$ have been reported to be most important for bread making quality of wheat (Branlard and Dardevet, 1985b). Attempts have been made to replace null *Glu A1* allele of wheat with *Glu D1* allele carrying $5D_x+10D_y$ subunits (Ceoloni et al., 1996; Ammar et al., 1997). Substitution of chromosome 1A with 1D has shown improvement in dough strength (Liu and Shepherd, 1995; Garg et al., 2007). A chromosomal translocation line 1AS.1AL-1DL carrying *Glu D1d* alleles ($5D_x+10D_y$) was generated in durum wheat background and was reported to possess improved mixing properties (Klindworth et al., 2005). Transfer of *Glu D1* locus to chromosome 1R and 1A of *Triticale* has also been shown to improve bread making properties (Lukaszewski, 2006).

Implications of many HMW GS from *Aegilops* species on product quality have been studied. Subunits 1.1C and 9C from *Ae. caudata* led to increased gluten strength (Du and Zhang, 2017) while 2D+T1+T2 subunits from *Ae. tauschii* are associated with low gluten index and gluten resistance (Hsam et al., 2001). Disomic addition lines (DALs) from *Ae. searsii* have been used to transfer HMW GS subunits $1S^s{}_x1$, $1S^s{}_x2$, $1S^s{}_y1$and $1S^s{}_y2$ into wheat (Garg et al., 2009). These addition lines showed improved specific sedimentation, mixing properties and polymeric protein content. Similarly, DAL-$1U^b$ of *Ae. biuncialis* (Zhou et al., 2014) were generated to transfer $1U^b{}_x$ and $1U^b{}_y$ subunits to wheat and these lines showed increased protein content, Zeleny sedimentation value, wet gluten content, and grain hardness. Addition lines of *Ae. umbellulata* showed negative impact of its HMW GS on dough strength (Garg et al., 2009). Addition of $1U^g$ chromosome to transfer $1U^g{}_x$ and $1U^g{}_y$ subunits from *Ae. geniculata* led to reduced dough strength (Garg et al., 2016). Addition of $1M^g$ chromosome from *Ae. geniculata* to Chinese Spring background of wheat improved dough strength significantly (Garg et al., 2016). Many disomic

TABLE 1 | *Aegilops* species explored for high molecular weight glutenins.

S.No.	Species	Lines/accessions	Subunits	Reference
1.	*Ae. caudata* (CC)	Y588	$1C_x$, $1C_y$	Liu et al., 2003
2.	*Ae. caudata* (CC)	Y46	1.1C, 9.1C Increased gluten strength	Du and Zhang, 2017
3.	*Ae. tauschii* (DD)	TD12, TD26, and TD190	DT^1, DT^2 Low gluten index, gluten resistance	Hsam et al., 2001
4.	*Ae. tauschii* (DD)	SHW line	2.1*D, 2.1D, 1.5D, 2D, 3D, 4D, 5D, 10D, 10.5D, 12D, 12*D, DT^2	Pflüger et al., 2001
5.	*Ae. tauschii* (DD)	As2396	13D	Yan et al., 2002
6.	*Ae. tauschii* (DD)	TD159	12.1D	Yan et al., 2004
7.	*Ae. tauschii* (DD)	Multiple accessions	2.1D, 1.5D, 1.5*D, 2D, 3D, 4D, 5.1D, 5D, 5*D 10D, 10.1D, 10.2D, 10.3*D, 10.4D, 11D, 12D, 12.1*D, 12.2*D, DT^2, 12.3D, 12.4*D, 12.5D	Yan et al., 2003
8.	*Ae. tauschii* (DD)	RM0198, AS2388	2D, 2.1D, 12D	Wan et al., 2005
9.	*Ae. tauschii* (DD)	TD81, TD130	5.1*D, 5*D, 12.1*D, 10.1D	Zhang et al., 2008
10.	*Ae. tauschii* (DD)	TD16	1.6D	An et al., 2009
11.	*Ae. tauschii* (DD)	TD87, TD130, TD151	12.1*D, 12.2D	Zhang et al., 2009
12.	*Ae. tauschii* (DD)	SHW line	2-1D, 2-2D, 2-3D, 1.5-1D,2.1-1D, 10-1D, 12-1D	Xu et al., 2010
13	*Ae. tauschii* (DD)	T67 and T132	3D, 4D	Wang K. et al., 2012
14.	*Ae. bicornis* (S^bS^b)	Clae 70	$2.9S^b$, $2.3S^b$	Jiang et al., 2012
15.	*Ae. longissima* (S^lS^l)	PI 604122	$2.9S^l$, $2.3S^l$	Jiang et al., 2012
16.	*Ae. longissima* (S^lS^l)	DSL -$1S^l$(1B)	$2.3*S^l$, $16*S^l$ Improved dough strength and baking quality	Wang S. et al., 2013
17.	*Ae. longissima* (S^lS^l)	DSL -$1S^l$(1A)	$1S^l_x$, $1S^l_y$ Higher dough strength, farinograph development time, stability time, gluten index, bread loaf volume, and bread quality score	Garg et al., 2014
18.	*Ae. searsii* (S^sS^s)	Multiple accessions	$48586S^s$, $48586S^s$, $49077S^s$, $49077S^s$	Sun et al., 2006
19.	*Ae. searsii* (S^sS^s)	Multiple DALs	$1S^s_x$, $2S^s_x$, $1S^s_y$, $2S^s_y$ Improved specific sedimentation, mixing properties and polymeric protein content	Garg et al., 2009
20.	*Ae. searsii* (S^sS^s)	DSL- GL1402 1B($1S^s$)	$2114S^s$, $2114S^s$ Better dough strength and mixing properties	Du et al., 2018
21.	*Ae. sharonensis* ($S^{sh}S^{sh}$)	PI 584388	$2.9S^{sh}$, $2.3S^{sh}$	Jiang et al., 2012
22.	*Ae. speltoides* (SS)	Multiple accessions	$15*S_x$, $15*S_y$	Ma et al., 2013
23.	*Ae. umbellulata* (UU)	IG46953, Y39, Y137, and Y139	$1U_x$, $1U_y$	Liu et al., 2003
24.	*Ae. cylindrica* (CCDD)	Multiple accessions	$1C_x$, $1C_y$	Wan et al., 2000
25.	*Ae. biuncialis* ($U^bU^bM^bM^b$)	DAL1U^b	$1U_x$, $1U_y$ Increased protein content, Zeleny sedimentation value, wet gluten content, and grain hardness	Zhou et al., 2014
26.	*Ae. geniculata* (MMUU)	Multiple DALs DSLs- $1M^g$(1A), $1M^g$(1B), $1M^g$(1D)	$1U^g_x$, $1U^g_y$ $1M^g_x$, $1M^g_y$	Garg et al., 2016
27.	*Ae. kotschyi* (UUSS)	Multiple accessions	$2.3U/S_x$, $1*U/S_x$, $3*U/S_x$, $20*U/S_y$, $8*U/S_y$	Ma et al., 2013
28.	*Ae. kotschyi* (UUSS)	Wheat- *Ae. kotschyi* acc. 396 derivative 49-1-73-10	$1U_x$, $1U_y$	Singh et al., 2016
29.	*Ae. juvenalis* (DDMMUU)	Not mentioned	$1J_x$,$2J_x$,$1J_y$,$2J_y$	Xie et al., 2001

SHW – Synthetic hexaploid wheat, DALs – Disomic addition lines, DSLs – Disomic substitution lines.

substitution lines (DSLs) have also been generated from DALs. Addition line of $1M^g$ chromosome from *Ae. geniculata* was used to generate chromosome specific DSLs- $1M^g$(1A), $1M^g$(1B) and $1M^g$(1D). DSLs- $1M^g$(1A) and $1M^g$(1B) showed improved dough strength and mixing properties but $1M^g$(1D) showed reduced dough strength (Garg et al., 2016). Substitution of chromosome $1S^l$ from *Ae. longissima* with chromosomes 1A (Garg et al., 2014) and 1B (Wang S. et al., 2013) significantly improved bread making qualities of wheat. Similarly substituting chromosome $1S^s$ from *Ae. searsii* with 1B led to better dough strength and mixing properties (Du et al., 2018). All these addition and substitution lines that improved dough strength can be utilized to

transfer HMW GS alleles into wheat in form of fine translocations with least linkage drag.

Low Molecular Weight Glutenins (LMW GS)

Low molecular weight glutenins account for 60% of total glutenins and one third of seed storage proteins. Genes that code for LMW GS (*Glu A3*, *Glu B3* and *Glu D3)* are present on the short arms of group 1 homoeologous chromosomes (Singh and Shepherd, 1988; Sreeramulu and Singh, 1997). Only six alleles at *Glu A3*, nine at *Glu B3* and five at *Glu D3* have been reported in wheat germplasm (McIntosh et al., 2013). There are additional three loci (*Glu 2*, *Glu 4* and *Glu 5)* present on chromosomes *1B*, *1D* and *7D* (Jackson et al., 1985; Liu and Shepherd, 1995; Sreeramulu and Singh, 1997). On the basis of SDS PAGE mobility LMW GS can be classified into B, C and D types (Jackson et al., 1983). B type LMW GS are further classified into m, s and i type on the basis of first amino acid methionine, serine and isoleucine, respectively (Muccilli et al., 2010). Besides these three types, a novel LMW GS, l type was identified specifically in *Aegilops* with first amino acid being leucine (Wang K. et al., 2011).

Low molecular weight glutenins provide viscoelastic properties to the dough and some of their alleles have been reported to be associated with good bread making quality. *Aegilops* species serve as rich source of genetic diversity of LMW GS. More than 13 alleles of LMW GS from *Ae. tauschii* (Pei et al., 2007; Zhao et al., 2008; Cao et al., 2018), 12 alleles from *Ae. longissima* (Jiang et al., 2008; Huang et al., 2010a), 11 alleles from *Ae. comosa* (Wang K. et al., 2011), 4 alleles from *Ae. neglecta* (Li X. et al., 2008), 3 alleles from *Ae. umbellulata* and one from *Ae. kotschyi* (Li X. et al., 2008), *Ae. uniaristata, Ae. caudata* and *Ae. speltoides* each (**Table 2**) (Li et al., 2010) have been identified and characterized (**Table 2**). Most of these LMW GS genes have been cloned and their sequence information is available in NCBI. There is large amount of variability present in *Aegilops* specific LMW GS. *Ae. tauschii* exhibits even greater variation in LMW GS sequences than wheat (Rehman et al., 2008). There have been reports of novel LMW GS genes *Glu U3a* and *Glu U3b* from wheat-*Ae. umbellulata* 1U(1B) substitution line showing improved bread making and mixing properties. This substitution line was used to transfer the LMW GS genes to wheat. The line thus developed showed improvement in dough development time, stability time, farinograph quality number, gluten index, loaf size and inner structure (Wang et al., 2017). The variability in LMW GS genes found in *Aegilops* species indicates a large potential for their utilization in improvement of end product qualities of wheat. In comparison to HMW GS, works on transfer of LMW GS alleles from *Aegilops* species to wheat cultivars have been limited. As per literature only 147 accessions/lines have been explored for LMW GS, which too mainly in China (**Figures 3**, **4**) and further exploration is needed.

Gliadins

Gliadins account for 40–50% of total seed storage proteins. They have impacts on both processing and nutritional quality. Gliadins can be separated into α-/β-, γ-, and ω-gliadins based on differences in their mobility on SDS PAGE gel. *Gli 1* loci present on short arms of homoeologous group 1 chromosomes code for all ω- and most of γ-gliadins, while, *Gli 2* loci on the short arms of homoeologous group 6 chromosomes code for all α-, most of the β-, and some of the γ-gliadins (Payne, 1987; Metakovsky et al., 1990; Metakovsky, 1991). The effect of gliadins on rheological properties of dough has been studied (Branlard and Dardevet, 1985a). Due to lack of free cysteine residues in most of the gliadins, they are unable to form intermolecular S-S linkages. Hence, their overall impact on processing quality is small as compared to glutenins (Qi et al., 2009). Gliadins may act as chain terminators for gluten polymer. They therefore might limit the size of gluten complex and hence affect end product quality (Muccilli et al., 2005). However, many gliadins with odd number of cysteins also exist (Anderson et al., 2001; Goryunova et al., 2012). So some gliadins might also participate in gluten polymerization. It has been hypothesized that gliadins proteins contribute mostly toward dough cohesiveness (Uthayakumaran et al., 2000) and viscosity (Pistón et al., 2011) rather than resistance and extension. Studies on effect of *Aegilops* specific gliadins on product quality are limited. Multiple accessions of *Ae. biuncialis* and *Ae. umbellulata* have been reported to possess high gluten quality indices due to gliadins (Ahmadpoor et al., 2014) (**Table 3**). Gliadins from *Ae. cylindrica* (Khabiri et al., 2013), *Ae. biuncialis* (Kozub et al., 2012) and *Ae. geniculata* (Medouri et al., 2015) have been characterized on the basis of mobility on SDS PAGE (**Table 3**). Many ω-gliadins have been sequenced and characterized from *Ae. tauschii* (Yan et al., 2003; Hassani et al., 2009). γ-gliadins have been characterized from *Ae. caudata, Ae. uniaristata, Ae. mutica, Ae. umbellulata* (Goryunova et al., 2012), *Ae. bicornis, Ae. searsii, Ae. sharonensis* (Qi et al., 2009; Huang et al., 2010b), *Ae. longissima* (Qi et al., 2009), *Ae. tauschii* (Qi et al., 2009; Goryunova et al., 2012; Wang S. et al., 2012), *Ae. speltoides* (Huang et al., 2010b; Goryunova et al., 2012), *Ae. markgrafii* (Li M. et al., 2017) and *Ae. cylindrica* (Wang S. et al., 2012) (**Table 3**).

Although fairly large number of lines and accessions (more than 400) of *Aegilops* have been explored for gliadins (**Figure 3**) and their exploration is quite distributed across several countries of the world (**Figure 4**), most of the research conducted on gliadins of *Aegilops* is related to identification and characterization of allergic epitopes of celiac disease (Juhász et al., 2018). α-Gliadins are considered to be most allergic and are mostly responsible for inflammatory responses to celiac disease. α-Gliadins from *Ae. speltoides* (Spaenij-Dekking et al., 2004) and *Ae. tauschii* (Xie et al., 2010; Li et al., 2012, 2013) have been reported to be less allergic than corresponding wheat alleles. Novel α-gliadins have been reported from *Ae. bicornis, Ae. searsii, Ae. sharonensis* (Huang et al., 2010c, 2016), *Ae tauschii* (Xie et al., 2010; Li Y.G. et al., 2017), *Ae. comosa, Ae. umbellulata, Ae. markgrafii* and *Ae. uniaristata* (Li et al., 2012) (**Table 3**). These gliadins could contain useful variation and can be replaced from more allergic gliadins in wheat.

Puroindolins and Grain Softness Protein

Grain texture plays important role in determining end product quality of wheat. Soft textured wheat is mostly used for pastries

TABLE 2 | *Aegilops* species explored for low molecular weight glutenins.

S.No.	Species	Lines/accessions	Characteristics	Reference
1	*Ae. caudata* (CC)	PI254863	*AmLMW-m1*	Li et al., 2010
2	*Ae. tauschii* (DD)	T121, T128, T132	*LMW-T121*, *LMW-T128*, *LMW-T132*	Pei et al., 2007
3	*Ae. tauschii* (DD)	Multiple accessions	*GluD^{t}3-3*, *GluD^{t}3-6*	Zhao et al., 2008
4	*Ae. tauschii* (DD)	Multiple accessions	*TaALPb7D-(A–M)*	Cao et al., 2018
5	*Ae. comosa* (MM)	PI551017	*AcLMW-m1*	Li et al., 2010
6	*Ae. comosa* (MM)	PI 551017, PI 551019	*AcLMW-L1*, *AcLMW-L2*, *AcLMW-L3*, *AcLMW-L4*, *AcLMW-I1*, *AcLMW-I2*, *AcLMW-I3*, *AcLMW-M1*, *AcLMW-M2*, *AcLMW-M3*	Wang K. et al., 2011
7	*Ae. uniaristata* (NN)	PI554419	*AuLMW-m1*	Li et al., 2010
8	*Ae. speltoides* (SS)	PI170204	*AsLMW-m1*	Li et al., 2010
	Ae. longissima (S^lS^l)	PI604108, PI604110	*TzLMW-m1*, *TzLMW-m2*, *TdLMW-m1* *AlLMW-m2*	Jiang et al., 2008
9	*Ae. longissima* (S^lS^l)	PI604103, PI604124, PI604126, PI604129	*SL124-1*, *SL126-1*, *SL129-1*, *SL129-2*, *SL129-3*, *SL129-4*, *SL103-1*, *SL103-2*	Huang et al., 2010a
11	*Ae. umbellulata* (UU)	PI222762	*AumLMW-m1*	Li et al., 2010
12	*Ae. umbellulata* (UU)	DSL -1U(1B)	*Glu-U3a, Glu-U3b* Improved dough development time, stability time, farinograph quality number, gluten index, loaf size and inner structure	Wang et al., 2017
13	*Ae. umbellulata* (UU)	CNU609 [CS- DSL 1U(1B) derivative]	*Glu-U3a, Glu-U3b* Improved dough development time, stability time, farinograph quality number, gluten index, loaf size and inner structure	Wang et al., 2017
14	*Ae. neglecta* (UUMM)	PI298897	*AnLMW-m1*, *AnLMW-m2*, *AnLMW-m3*, *AnLMW-m4*	Li X. et al., 2008
15	*Ae.kotschyi* (UUSS) *Ae. juvenalis* (DDMMUU)	PI226615, PI330485	*AjkLMW-I*	Li X. et al., 2008

DSL – Disomic substitution line, ALP – Avenin like protein.

and biscuits, while hard textured wheat is used in making bread, pasta and noodles (Morris and Rose, 1996). Grain texture is determined by the hardness (*Ha*) locus present on the telomeric region of short arm of chromosome 5D of wheat which contains ten tightly linked genes (Chantret et al., 2005). Among them, three genes- *puroindolin a* (*Pin a*), *puroindolin b* (*Pin b*) and *grain softness protein-1* (*GSP*) play major role in determining seed texture. These three genes code for the proteins which constitute a 15 kDa complex- friabilin, with *Pin a*, *Pin b* as major components and *GSP-1* as minor component (Cuesta et al., 2015). This protein complex is found abundantly on the surface of starch granules of soft textured wheat and in very small amounts in hard textured wheat (Chen et al., 2005). Presence of this complex results in prevention of adhesion between starch granules and gluten matrix and hence soft texture (Greenwell and Schofield, 1986). *Pin a* and *Pin b* genes have also been associated with antimicrobial properties conferring protection to seed (Dubreil et al., 1998; Miao et al., 2012). *Pin a* especially has been hypothesized to have evolved in response to plant pathogens to enhance plant fitness (Massa and Morris, 2006). Soft seed

TABLE 3 | *Aegilops* species explored for gliadins.

S. No.	Species	Lines/accessions	Characteristics	Reference
1	*Ae. caudata* (CC)	κ-2255	γ-gliadins	Goryunova et al., 2012
2	*Ae. caudata* (CC)	PI573416, PI551119, PI298889, PI564196	α-gliadins	Li et al., 2012
3	*Ae. caudata* (CC)	Y46	γ-gliadins	Li M. et al., 2017
4	*Ae. tauschii* (DD)	Multiple accessions	ω-Gliadins	Yan et al., 2003
5	*Ae. tauschii* (DD)	AUS18913, CPI110856	ω-gliadin γ-gliadin	Hassani et al., 2009
6	*Ae. tauschii* (DD)	AS60	γ-gliadins	Qi et al., 2009
7	*Ae. tauschii* (DD)	AUS18913, CPI110856	ω-gliadin	Hassani et al., 2009
8	*Ae. tauschii* (DD)	T15, T43, T26	α-gliadins	Xie et al., 2010
9	*Ae. tauschii* (DD)	κ-1368	γ-gliadins	Goryunova et al., 2012
10	*Ae. tauschii* (DD)	AT9, AT9.1, AT25, AT48, AT176	γ-gliadins	Wang S. et al., 2012
11	*Ae. tauschii* (DD)	T006	α-gliadins	Li Y.G. et al., 2017
12	*Ae. comosa* (MM)	PI551020	α-gliadins	Li et al., 2012
13	*Ae. uniaristata* (NN)	κ-650	γ-gliadins	Goryunova et al., 2012
14	*Ae. uniaristata* (NN)	PI276996, PI276996, PI554420, PI554418	α-gliadins	Li et al., 2012
15	*Ae. bicornis* (S^bS^b)	Clae 47	γ-gliadins	Qi et al., 2009
16	*Ae. bicornis* (S^bS^b)	Clae 47, Clae 70	γ-gliadins	Huang et al., 2010c
17	*Ae. bicornis* (S^bS^b)	Clae 47	α-gliadins	Huang et al., 2016
18	*Ae. longissima* (S^lS^l)	PI 604104	γ-gliadins	Qi et al., 2009
19	*Ae. longissima* (S^lS^l)	PI 604104, PI604129, PI604130, PI604131, PI604133	γ-gliadins	Huang et al., 2010c
20	*Ae. searsii* (S^sS^s)	PI 599123	γ-gliadins	Qi et al., 2009
21	*Ae. searsii* (S^sS^s)	PI 599122, PI599124, PI599138, PI599139, PI599150	γ-gliadins	Huang et al., 2010c
22	*Ae. searsii* (S^sS^s)	Multiple accessions	α-gliadins	Huang et al., 2016
23	*Ae. sharonesis* ($S^{sh}S^{sh}$)	Clae 32	γ-gliadins	Qi et al., 2009
24	*Ae. sharonensis* ($S^{sh}S^{sh}$)	PI584350	α-gliadins	Huang et al., 2010b
25	*Ae. sharonensis* ($S^{sh}S^{sh}$)	Clae 32, PI 584345, PI 584349, PI584350, PI584357, PI584391	γ-gliadins	Huang et al., 2010c
26	*Ae. sharonensis* ($S^{sh}S^{sh}$)	Multiple accessions	α-gliadins	Huang et al., 2016
27	*Ae. speltoides* (SS)	PI 584391, PI554305, PI560527	γ-gliadins	Huang et al., 2010c
28	*Ae. speltoides* (SS)	CGN10682, CGN10684	γ-gliadins	Goryunova et al., 2012
29	*Ae. umbellulata* (UU)	κ-1588	γ-gliadins	Goryunova et al., 2012
30	*Ae. umbellulata* (UU)	PI298906, PI542364, PI573516	α-gliadins	Li et al., 2012
31	*Ae. mutica* (TT)	κ-1581	γ-gliadins	Goryunova et al., 2012

(Continued)

TABLE 3 | Continued

S. No.	Species	Lines/accessions	Characteristics	Reference
32	*Ae. cylindrica* (CCDD)	PI256029	γ-gliadins	Wang S. et al., 2012
32	*Ae. cylindrica* (CCDD)	Multiple accessions	Gliadins	Khabiri et al., 2013
34	*Ae. geniculata* (MMUU)	Multiple accessions	Gliadins	Medouri et al., 2015
35	*Ae. biuncialis* ($U^bU^bM^bM^b$)	Multiple accessions	Gliadins	Kozub et al., 2012

texture is associated with wild type alleles of *Pin a* and *Pin b* (*Pina-D1a* and *Pinb-D1a*) and many mutations in those alleles have been linked with hard texture (Giroux and Morris, 1998). *Pin a* and *Pin b* genes are not present on A and B genome specific chromosomes (Li W. et al., 2008) and diploid species with A and B genomes as well as tetraploid durum wheat lack them, as a result of which durum has a very hard kernel texture (Chen et al., 2005). This also indicates *Ae. tauschii* as the donor of *Pin* genes in hexaploid wheat. Species of *Aegilops* have been explored for presence of different *Pin* alleles. Many novel *Pin* alleles have been reported from multiple accessions of *Ae. tauschii* (**Table 4**) (Massa et al., 2004; Gazza et al., 2006; Simeone et al., 2006; Liu et al., 2016). Many accessions of *Ae. tauschii* have been crossed with tetraploid durum wheat to produce synthetic wheat lines with different textures (Reynolds et al., 2010; Li et al., 2007). Many other *Aegilops* species have also been explored for variability in *Pin a* and *Pin b* gene alleles.19 alleles of puroindolins from *Ae. speltoides*, 9 alleles from *Ae. searsii*, 8 alleles from *Ae. comosa*, 7 from *Ae. caudata* and *Ae. umbellulata* each, 4 from *Ae. longissima, Ae. ventricosa* and *Ae. bicornis* each and 2 from *Ae. sharonensis* have been reported (**Table 4**) (Gazza et al., 2006; Simeone et al., 2006; Cuesta et al., 2013, 2015).

Unlike *Pin a* and *Pin b, GSP* genes are present on A and B genome specific chromosomes (5A, 5B). However, their deletion does not impact the grain texture (Chen et al., 2005). *GSP* genes have been characterized in many species of *Aegilops.* Many novel *GSP* alleles in *Ae. tauschii*, *Ae. comosa, Ae. caudata, Ae. searsii, Ae. speltoides* and *Ae. umbellulata* have been reported and characterized (Massa et al., 2004; Cuesta et al., 2015). Almost 100 alleles of *Pin a*, *Pin b* and *GSP* have been identified across 200 lines/accessions of *Aegilops* (**Figure 3**). Their exploration is quite uniform across different countries in the world (**Figure 4**). All these alleles can serve as useful source of variation and need to be evaluated and utilized in breeding programs for extending the textural characteristics of wheat.

UTILIZATION OF *Aegilops* FOR IMPROVEMENT OF NUTRITIONAL QUALITY OF WHEAT

Improvement of nutrition is a very important aspect of wheat research as there are over two billion people worldwide, suffering from deficiencies in proteins and micronutrients (World Health Organization, 2006). Nutritive value of wheat can be enhanced by increasing micronutrients like Fe and Zn, protein content, dietary fibers and many other phytochemicals such as carotenoids, vitamins etc. *Aegilops* genus can serve as important source for enhancing nutrition in wheat due to its high genetic variability.

Improvement of Grain Micronutrients Concentration

Micronutrients play very important role as health promoting factors. Since most of the world's population especially developing nations depend on cereal based diet to fulfill their micronutrients requirements, it becomes very important to develop the varieties with improved micronutrients content. Iron and zinc are the most important components among micronutrients. Most varieties of wheat lack sufficient levels of iron and zinc due to low genetic variability. To overcome this limited genetic variability more than 180 lines/accessions of *Aegilops* have been explored (**Figures 2**, **3**). Many accessions of *Ae. kotschyi* (Chhuneja et al., 2006; Rawat et al., 2009a,b, 2011), *Ae. longissima* (Kumari et al., 2013), *Ae. tauschii, Ae. peregrina*, *Ae. cylindrica*, *Ae. ventricosa* and *Ae. geniculata* (Rawat et al., 2009b) have been reported to have higher contents of iron and zinc in seeds (**Table 5**). These accessions can be exploited for increasing grain iron and zinc content. Amphiploids (Tiwari et al., 2010) and partial amphiploids (Rawat et al., 2009b) generated by crossing *Ae. kotschyi* accessions with wheat have been reported to have higher grain iron and zinc content. Many disomic and monosomic addition lines specific to various *Aegilops* species have been explored for higher micronutrient content. Fair exploration of grain micronutrient content has been carried out in many countries (**Figure 3**). Major exploration of *Aegilops* for Fe/Zn is from India (158 lines and accessions) as compared to other countries (**Figure 4**). Many disomic and monosomic addition lines of *Ae. peregrina*, *Ae. longissima* and *Ae. umbellulata*, in wheat have been explored for grain iron and zinc concentrations (Kumari et al., 2012). Addition of chromosome pairs $1S^l$ (Wang S. et al., 2011), $2S^l$ (Wang S. et al., 2011; Kumari et al., 2012) and $7S^l$ (Wang S. et al., 2011) of *Ae. longissima* into wheat showed increase in grain iron and zinc content. Similarly, DALs of chromosomes $2S^v$, $2U^v$, $7U^v$ (Kumari et al., 2012) and $4S^v$ (Wang S. et al., 2011) of *Ae. peregrina*, 2U (Kumari et al., 2012) and 6U (Wang S. et al., 2011; Kumari et al., 2012) of *Ae. umbellulata*, $1S^s$ and $2S^s$ of *Ae. searsii* (Wang S. et al., 2011), $5M^g$ of *Ae. geniculata* (Wang S. et al., 2011) and B chromosome additions from *Ae. caudata* (Wang S. et al., 2011) have been reported to increase the iron and zinc content in grains (**Table 5**). The addition lines can be used to produce DSLs which are better materials to study the compensation effect of alien chromosomes into wheat. Substitution of 4B chromosome of wheat with $3M^b$ chromosome

TABLE 4 | *Aegilops* species explored for puroindolins and grain softness proteins.

S.No.	Species	Source	*Pin a* Alleles	*Pinb* Alleles	*GSP* Alleles	Reference
1	*Ae. caudata* (CC)	Multiple accessions	*Pina-C1-I*, *Pina-C1-II*, *Pina-C1-III*	*Pinb-C1-I*, *Pinb-C1-II*, *Pinb-C1-III*, *Pinb-C1-IV*		Cuesta et al., 2013
2	*Ae. caudata* (CC)	Multiple accessions			*GSP-C1-I*, *GSP-C1-II*, *GSP-C1-III*, *GSP-C1-IV*	Cuesta et al., 2015
3	*Ae. tauschii* (DD)	CPI110799	*Pina*	*Pinb*	*GSP*	Turnbull et al., 2003
4	*Ae. tauschii* (DD)	Multiple accessions	*Pina-D1g*, *Pina-D1a*, *Pina-D1c*, *Pina-D1d*, *Pina-D1e*, *Pina-D1f*	*Pinb-D1i*, *Pinb-D1j*, *Pinb-D1h*, *Pinb-D1a*	*GSP-D1g*, *GSP-D1h*, *GSP-D1c*, *GSP-D1e*, *GSP-D1d*, *GSP-D1f*, *GSP-D1b*	Massa et al., 2004
5	*Ae. tauschii* (DD)	TA1704, TA1691, TA2381, TA10	*Pina-D1d*, *Pina-D1a*, *Pina-D1c*	*Pinb-D1i*, *Pinb-D1j*, *Pinb-D1h*		Simeone et al., 2006
6	*Ae. tauschii* (DD)	L35	*Pina-D1d*	*Pinb-D1i*		Gazza et al., 2006
7	*Ae. tauschii* (DD)	SHW	*Pina-D1a*, *Pina-D1c*	*Pinb-D1h*, *Pinb-D1j*		Li et al., 2007
8	*Ae. tauschii* (DD)	SHW	*Pina-D1c*	*Pinb-D1h*		Reynolds et al., 2010
9	*Ae. tauschii* (DD)	Multiple accessions	*Pina-D1o*	*Pinb-D1dt*, *Pinb-D1it*		Liu et al., 2016
10	*Ae. comosa* (MM)	Multiple accessions	*Pina-M1-I*, *Pina-M1-II*, *Pina-M1-III*	*Pinb-M1-I*, *Pinb-M1-II*, *Pinb-M1-III*, *Pinb-M1-IV*, *Pinb-M1-V*		Cuesta et al., 2013
11	*Ae. comosa* (MM)	Multiple accessions			*GSP-M1-I*, *GSP-M1-II*	Cuesta et al., 2015
12	*Ae. speltoides* (SS)	TA2368, TA1789, TA1777	*Pina-S1c*, *Pina-S1d*, *Pina-S1e*	*Pinb-S1c*, *Pinb-S1d*, *Pinb-S1e*		Simeone et al., 2006
13	*Ae. speltoides* (SS)	Multiple accessions	*Pina-S^1-I*, *Pina-S^1-II*, *Pina-S^1-III*, *Pina-S^1-IV*	*Pinb-S^1-I*, *Pinb-S^1-II*, *Pinb-S^1-III*, *Pinb-S^1-IV*, *Pinb-S^1-V*, *Pinb-S^1-VI*, *Pinb-S^1-VII*, *Pinb-S^1-VIII*, *Pinb-S^1-IX*		Cuesta et al., 2013
14	*Ae. speltoides* (SS)	Multiple accessions			*GSP-S1-I*, *GSP-S1-II*, *GSP-S1-III*, *GSP-S1-IV*, *GSP-S1-V*, *GSP-S1-VI* *GSP-S1-VII*	Cuesta et al., 2015
15	*Ae. searsii* (S^sS^s)	TA1837, TA2355	*Pina-S^{s}1a*, *Pina-S^{s}1b*	*Pinb-S^{s}1b*, *Pinb-S^{s}1a*		Simeone et al., 2006
16	*Ae. searsii* (S^sS^s)	Multiple accessions	*Pina-S^{s}1-I*, *Pina-S^{s}1-II*	*Pinb-S^{s}1-I*, *Pinb-S^{s}1-II*, *Pinb-S^{s}1-III*		Cuesta et al., 2013
17	*Ae. searsii* (S^sS^s)	Multiple accessions			*GSP-S^{s}1-I*, *GSP-S^{s}1-II*	Cuesta et al., 2015
18	*Ae. longissima* (S^lS^l)	TA1912, TA1921,	*Pina-S^{l}1a*, *Pina-S^{l}1b*	*Pinb-S^{l}1a*, *Pinb-S^{l}1b*		Simeone et al., 2006

(Continued)

TABLE 4 | Continued

S.No.	Species	Source	*Pin a* Alleles	*Pinb* Alleles	*GSP* Alleles	Reference
19	*Ae. bicornis* (S^bS^b)	TA1954, TA1942	*Pina-S^b1a*, *Pina-S^b1b*	*Pinb-S^b1a*, *Pinb-S^b1b*		Simeone et al., 2006
20	*Ae. sharonensis* (S^hS^h)	TA1999	*Pina-S^{sh}1a*	*Pinb-S^{sh}1a*		Simeone et al., 2006
21	*Ae. umbellulata* (UU)	Multiple accessions	*Pina-U1-I*, *Pina-U1-II*, *Pina-U1-III*, *Pina-U1-IV*	*Pinb-U1-I*, *Pinb-U1-II*, *Pinb-U1-III*		Cuesta et al., 2013
22	*Ae. umbellulata* (UU)	Multiple accessions			*GSP-U1-I*, *GSP-U1-II*, *GSP-U1-III*, *GSP-U1-IV*	Cuesta et al., 2015
23	*Ae. ventricosa* (DDNN)	L36	*Pina-D1a*, *Pina-N1a*	*Pinb-D1h, and Pinb-N1a.*		Gazza et al., 2006

SHW – Synthetic hexaploid wheat.

of *Ae. biuncialis* (Farkas et al., 2014) also lead to increased iron and zinc content. Similarly, 2S(2A), 7U(7A) substitutions specific to *Ae. kotschyi* (Tiwari et al., 2010) have been reported with increased grain iron and zinc content.

Disomic addition/substitution lines can be utilized to introgress useful variability of high grain Fe and Zn from *Aegilops* into wheat in form of short arm or fine chromosomal translocations through induced homoeologous pairing. Interspecific hybrids of *Ae. longissima* with *T. turgidum* (Tiwari et al., 2008) and *Ae. kotschyi* (Sheikh et al., 2018) produced after crossing addition /substitution lines with tetraploid and hexaploid wheat also showed elevated levels of grain iron and zinc content. *Ae. biuncialis* specific translocation line 3M^b.4BS (Farkas et al., 2014) and many U/S chromosome specific fine translocations of *Ae. kotschyi* in wheat (Verma et al., 2016a,b) have been produced with least linkage drag effect. These lines also showed significant increase in grain iron and zinc content.

Improvement in Phytochemicals Concentration

Studies on phytochemical contents of *Aegilops* species have been limited (**Figure 3**) with their work mainly being carried out in Europe (**Figure 4**). But given the rich genetic diversity of *Aegilops*, many phytochemicals such as phenolic acids, carotenoids, tocopherols, alkylresorcinols, benzoxazinoids, phytosterols and lignans can be explored in *Aegilops* species. Many phenolic diglycerides have been detected in *Ae. geniculata* (Cooper et al., 1978) (**Table 6**). p-hydroxybenzaldehyde, vanillin and mono-epoxylignanolide (MEL) have been detected in *Ae. geniculata* (Cooper et al., 1994). Alloplasmic lines derived from wheat and *Ae. squarossa* have been shown to increase the lutein content (Atienza et al., 2008). Synthetic hexaploid wheat (SHW) lines generated by crossing tetraploid durum wheat and *Ae. tauschii* also showed increased yellow pigment content and might be useful source for increasing carotenoids content in wheat (Li et al., 2015). DALs of *Ae. geniculata* and *Ae. biuncalis* showed increase in total protein content and polymeric proteins (Rakszegi et al., 2017) hence enhancing the nutritive value (**Table 6**).

Improvement in Dietary Fibers Concentration

Dietary fib**e**rs are important components of wheat which impact processing quality and have many health benefits. The major components of dietary **fibers** in wheat grain are cell wall polysaccharides, arabinoxylan (AX) and (1-3)(1-4)- β-D-glucan (β-glucan). Both of these occur in soluble and insoluble forms with different health benefits such as reduced risks of type II diabetes, coronary heart diseases and prevention of colon cancer. Soluble forms of dietary **fibers** also include FODMAPs (Fermentable oligosaccharides, disaccharides, monosaccharides and polyols) which are a group short chain carbohydrates. A diet rich in FODMAPs is often associated with diseases like Crohn disease and irritable bowel syndrome (IBS), which is a chronic gastrointestinal disease (Khan et al., 2015). Dietary **fiber** components have been reported to affect processing quality of wheat in terms of bread making and starch gluten separation. Arabinoxylan have effects on water absorption and development time of dough (Courtin and Delcour, 1998). β-glucan confers high viscosity, higher water absorption, lower loaf volume, height and stiffer dough (Symons and Brennan, 2004; Cleary et al., 2007; Skendi et al., 2009). From nutrition point of view higher levels of β-glucan are sought in food products as they lower serum cholesterol levels and regulate glucose levels in blood (McIntosh et al., 1991; Cavallero et al., 2002). Variability and composition of dietary **fiber**s have been extensively studied in wheat and related cereal grains. Wheat primary gene pool has been explored in the European HEALTHGRAIN cereal diversity screening project[1] for dietary **fiber**s and other phytochemicals. However, such studies in wild species of wheat have been limited. There have been reports of recombinants of *Triticale* with *Ae. crassa* and *Ae. juvenalis* showing higher dietary **fiber** content along with increased values of total protein content, thousand kernel weight and volume weight (Boros et al., 2010) (**Table 6**). Both the

[1]https://healthgrain.org

TABLE 5 | *Aegilops* species explored for grain micronutrient content.

S.No.	*Aegilops* sp.	Lines/Accessions	Trait	Reference
1.	*Ae. caudata* (CC)	DALs	Iron, Zinc	Wang S. et al., 2011
2.	*Ae. tauschii* (DD)	SHW	Zn uptake	Cakmak et al., 1999
3.	*Ae. tauschii* (DD)	SHW	Iron, Manganese, Zinc, Calcium, Uptake of Iron, Manganese, Potassium, Phosphorus	Calderini and Ortiz-Monasterio, 2003
4.	*Ae. tauschii* (DD)	SHW	Iron, Zinc	Chhuneja et al., 2006
5.	*Ae. longissima* (S^lS^l)	DALs $1S^l$, $2S^l$	Iron, Zinc	Wang S. et al., 2011
6.	*Ae. longissima* (S^lS^l)	$2S^l$, $7S^l$	Iron, Zinc	Kumari et al., 2012
7.	*Ae. longissima* (S^lS^l)	DALs	Iron, Zinc, Copper, Manganese, Calcium, Magnesium, Potassium	Kumari et al., 2012
8.	*Ae. longissima* (S^lS^l)	Wheat – *Ae. longissima* derivatives	Iron, Zinc	Sharma et al., 2014
9.	*Ae. longissima* (S^lS^l)	Hybrids	Iron, Zinc	Tiwari et al., 2008
10.	*Ae. searsii* (S^sS^s)	DALs $1S^s$, $2S^s$	Iron, Zinc	Wang S. et al., 2011
11.	*Ae. umbellulata* (UU)	DALs 2U, 6U	Iron, Zinc	Wang S. et al., 2011
12.	*Ae. umbellulata* (UU)	DAL 2U	Iron, Zinc	Kumari et al., 2012
13.	*Ae. cylindrica* (CCDD)	DALs	Iron, Zinc	Rawat et al., 2009a
14.	*Ae. cylindrica* (CCDD)	Accessions and interspecific hybrids with *Triticum aestivum*	Iron, Zinc	Rawat et al., 2009a
15.	*Ae. ventricosa* (DDNN)	DALs	Iron, Zinc	Rawat et al., 2009b
16.	*Ae. ventricosa* (DDNN)	Accessions and interspecific hybrids with *Triticum aestivum*	Iron, Zinc	Rawat et al., 2009a
17.	*Ae. geniculata* (MMUU)	Accessions and interspecific hybrids with *Triticum aestivum*	Iron, Zinc	Rawat et al., 2009a
18.	*Ae. geniculata* (MMUU)	DAL 5 M^g	Iron, Zinc	Wang S. et al., 2011
19.	*Ae. biuncialis* ($U^bU^bM^bM^b$)	DSLs $3M^b$(4B), Translocation line $3M^b$.4BS	Potassium, Zinc, Iron, Manganese	Farkas et al., 2014
20.	*Ae. kotschyi* (UUSS)	Not mentioned	Iron, Zinc	Chhuneja et al., 2006
21.	*Ae. kotschyi* (UUSS)	DALs	Iron, Zinc	Rawat et al., 2009a
22.	*Ae. kotschyi* (UUSS)	Accessions and interspecific hybrids with *Triticum aestivum*	Iron, Zinc	Rawat et al., 2009a
23.	*Ae. kotschyi* (UUSS)	Amphiploids	Iron, Zinc	Rawat et al., 2009b
24.	*Ae. kotschyi* (UUSS)	Amphiploids (AABBDDUkUkSkSk)	Macronutrients, Micronutrients	Tiwari et al., 2010
25.	*Ae. kotschyi* (UUSS)	DSLs 2S, 7U	Iron, Zinc	Tiwari et al., 2010
26.	*Ae. kotschyi* (UUSS)	DALs, DSL	Iron, Zinc	Rawat et al., 2011
27.	*Ae. kotschyi* (UUSS)	Hybrids	Iron, Zinc	Sheikh et al., 2018
28.	*Ae. kotschyi* (UUSS)	Hybrids with small alien introgression	Iron, Zinc	Verma et al., 2016a
29.	*Ae. kotschyi* (UUSS)	U/S introgression	Iron, Zinc	Verma et al., 2016b
30.	*Ae. kotschyi* (UUSS)	DSLs	Iron, Zinc	Sharma et al., 2018
31.	*Ae. kotschyi* (UUSS)	Hybrids	Iron, Zinc	Sharma et al., 2018
32.	*Ae. kotschyi* (UUSS)	Derivatives	Iron, Zinc	Sheikh et al., 2018
33.	*Ae. kotschyi*	Fine translocation line U/S	Iron, Zinc	Verma et al., 2016b
34.	*Ae. peregrina* (UUSS)	DALs	Iron, Zinc	Rawat et al., 2009a
35.	*Ae. peregrina* (UUSS)	Accessions and interspecific hybrids with *Triticum aestivum*	Iron, Zinc	Rawat et al., 2009a
36.	*Ae. peregrina* (UUSS)	DAL $4S^v$	Iron, Zinc	Wang S. et al., 2011
37.	*Ae. peregrina* (UUSS)	DALs $2S^v$, $2U^v$, $7U^v$	Iron, Zinc	Kumari et al., 2012
38.	*Ae. peregrina* (UUSS)	DSLs	Iron, Zinc	Sharma et al., 2018
39.	*Ae. peregrina* (UUSS)	Derivatives	Iron, Zinc	Sheikh et al., 2018
40.	*Ae. peregrina* (UUSS)	Hybrids	Iron, Zinc	Sharma et al., 2018

DALs – Disomic addition lines, DSLs – Disomic substitution lines, SHW – synthetic hexaploid wheat.

TABLE 6 | *Aegilops* species explored for phytochemicals and dietary fibers.

S.No.	Species	Source	Traits	Reference
1	*Ae. speltoides* (SS)	2140008	DIMBOA-glucoside	Elek et al., 2014
2	*Ae. crassa* (DDMM)	Recombinants of *Triticale* with *Ae. crassa*	Protein, dietary fiber, thousand kernel weight, volume weight	Boros et al., 2010
3	*Ae. geniculata* (MMUU)		Tricin and flavo-lignan	Cooper et al., 1977
4	*Ae. geniculata* (MMUU)		Scopoletin and p-coumaric acid	Cooper et al., 1978
5	*Ae. geniculata* (MMUU)	$2U^g$, $4U^g$, $5U^g$, $7U^g$, $2M^g$, $5M^g$, $7M^g$ DALs	Protein content	Rakszegi et al., 2017
6	*Ae. geniculata* (MMUU)	$1U^g$, $1M^g$ DALs	Polymeric glutenin proteins	Rakszegi et al., 2017
7	*Ae. geniculata* (MMUU)	$5U^g$, $7U^g$ DALs	Arabinoxylan	Rakszegi et al., 2017
8	*Ae. biuncialis* (UUMM)	$1U^b$ DAL	Arabinoxylan	Rakszegi et al., 2017
9	*Ae. geniculata* (MMUU)	$5U^g$, $5M^g$,$7M^g$ DALs	β-glucan	Rakszegi et al., 2017
10	*Ae. biuncialis* (UUMM)	$3U^b$, $2M^b$, $3M^b$, and $7M^b$ DALs	Protein	Rakszegi et al., 2017
11	*Ae. biuncialis* (UUMM)	$5U^b$, $5M^b$, $7M^b$ DALs	β -glucan	Rakszegi et al., 2017
12	*Ae. juvenalis* (DDMMUU)	Recombinants of *Triticale* with *Ae. juvenalis*	Protein, dietary fiber, thousand kernel weight, volume weight	Boros et al., 2010

DALs – Disomic addition lines, DIMBOA – 2,4-dihidroxy-7-methoxy-1,4-benzoxazin-3-one.

species can be utilized for improving the nutrition value of wheat. Addition of $5U^g$, $7U^g$ chromosome pairs of *Ae. geniculata* and $1U^b$ of *Ae. biuncialis* into wheat have resulted in increased arabinoxylan content (Rakszegi et al., 2017). Similarly, addition of $5U^g$, $5M^g$, and $7M^g$ chromosome pairs from *Ae. geniculata* and $5U^b$, $5M^b$, and $7M^b$ chromosomes from *Ae. biuncialis* have been reported to result in elevated levels of β-glucan content in wheat (Rakszegi et al., 2017). Since there is a large genetic diversity available in *Aegilops* species, they need to be explored for dietary **fiber**s content and their potential use for enhancing nutritional value of wheat.

CONCLUSION

Quality and nutrition are two very important aspects of wheat research. Over the past few years, a lot of emphasis has been given by breeders worldwide to improve the end product quality of wheat and to develop varieties that meet specific end product and nutritional requirements. New sources of genetic variations in wheat are always sought after because of the narrow genetic diversity. Wild species of wheat can serve as excellent source of new variations that can be incorporated into wheat. Close relatedness to wheat makes *Aegilops* the most favorable genetic resource for wheat improvement through alien gene introgression. The basic approach for alien gene transfer is to cross the wild relative with wheat to generate interspecific hybrids followed by embryo rescue and colchicine treatment to double chromosomes. The amphiploids generated are then backcrossed multiple times with wheat to generate addition/substitution lines (Friebe et al., 1995, 1996, 1999). A large number of wheat-*Aegilops* amphiploids and chromosome addition/substitutions lines are available (Schneider et al., 2008). But these addition/substitution lines and amphiploids have no practical application in agriculture as the *Aegilops* chromosome segment carrying the gene of interest must be transferred to the wheat chromosome as translocation. The *Ph1* locus, present at the long arm of chromosome 5B regulates chromosome pairing in wheat and ensures that only homologous chromosomes pair at metaphase. To generate translocations between wheat chromosome and alien chromosome, *Ph1* mutants or *Ph1* suppressors can be used to bypass the *Ph1* control mechanism of homologous pairing. Translocations can also be generated via radiation induced chromosome breaks followed by random recombination. The recombinants generated then need to be screened using chromosome pairing, C banding pattern and *in situ* hybridization. Thus, the whole process of alien gene transfer is laborious and time consuming. However, with technological advancements and development of new high throughput marker technologies it is now possible to identify desirable recombinants from a large population with great precision and efficiency (Niu et al., 2011; Tiwari et al., 2014).

A large number of countries throughout the world are participating in the exploration of *Aegilops*. HMW GS are most explored, while phytochemicals are least explored among different research groups across the world. Gliadins have been explored by highest number of countries while, LMW GS and phytochemicals are least explored around the world (**Figure 3**). Based on this review we are aware that more than 95 subunits of HMW GS, 51 novel alleles of LMW GS, 34 alleles for *Pin a*, 40 alleles for *Pin b* and 26 alleles for *GSP* in *Aegilops* have been reported across multiple accessions, synthetic lines, addition/substitution lines and translocation lines (**Figure 3**). These can serve as excellent genetic sources of variation for wheat quality improvement. Large numbers of publications have arisen for *Aegilops* exploration for improvement of nutrition and processing quality. Highest exploration has been carried out in China and Europe followed by Japan and India (**Figure 4**). Major

work on LMW GS has been carried out in China, Fe/Zn in India, others having good distribution across countries (**Figure 4**). More than 14 species of *Aegilops* have been proven to be excellent sources for the improvement of grain micronutrient content, protein content, dietary fiber content and phytochemical content. Many *Aegilops* species have already been incorporated in various breeding programs across the world. Still there is further need to explore *Aegilops* species to identify new variations. Though a large number of accessions are available in gene banks, many accessions of *Aegilops* species still remain unexploited. The real bottleneck for introgressing useful genes into wheat from *Aegilops*, however, is the generation of fine translocation lines containing the smallest possible segment of alien chromosome with the gene of interest. Although a lot of scientific exploration has been carried out, practically we still are nowhere in terms of introgressing and utilizing genes related to quality and nutrition from *Aegilops* species. There is still a long way to go. It is anticipated that the availability of the newly annotated wheat genome sequence (International Wheat Genome Sequencing Consortium, Appels et al., 2018) along with new genomic tools and genetic resources will aid the further exploration and exploitation of *Aegilops* species and the transfer of useful traits into wheat.

AUTHOR CONTRIBUTIONS

AK and MG built the layout of article. AK, MG, and PK collected the literature. AK wrote the article. MG, VC, SS, and PK helped in manuscript editing. PK did the reference management. All authors prepared images and tables.

ACKNOWLEDGMENTS

We are thankful to Department of Biotechnology, Government of India for the fellowship provided to AK. Contribution of Ms. Simran Bhatia, Ms. Neetu, and Ms. Anita Kumari in manuscript editing is truly acknowledged.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

HYPOTHESIS AND THEORY
published: 09 May 2019
doi: 10.3389/fpls.2019.00585

An Update of Recent Use of *Aegilops* Species in Wheat Breeding

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Aegilops species have significantly contributed to wheat breeding despite the difficulties involved in the handling of wild species, such as crossability and incompatibility. A number of biotic resistance genes have been identified and incorporated into wheat varieties from *Aegilops* species, and this genus is also contributing toward improvement of complex traits such as yield and abiotic tolerance for drought and heat. The D genome diploid species of *Aegilops tauschii* has been utilized most often in wheat breeding programs. Other *Aegilops* species are more difficult to utilize in the breeding because of lower meiotic recombination frequencies; generally they can be utilized only after extensive and time-consuming procedures in the form of translocation/introgression lines. After the emergence of Ug99 stem rust and wheat blast threats, *Aegilops* species gathered more attention as a form of new resistance sources. This article aims to update recent progress on *Aegilops* species, as well as to cover new topics around their use in wheat breeding.

Keywords: *Aegilops*, introgression, translocation, wheat breeding, stress tolerance, wild species, genetic resources

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Edited by:
Peter Shewry,
Rothamsted Research (BBSRC),
United Kingdom

Reviewed by:
Matthew Rouse,
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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *20 February 2019*
Accepted: *18 April 2019*
Published: *09 May 2019*

Citation:
Kishii M (2019) An Update of Recent Use of Aegilops Species in Wheat Breeding. Front. Plant Sci. 10:585. doi: 10.3389/fpls.2019.00585

INTRODUCTION

According to the latest revision of *Aegilops* L. taxonomy, (van Slageren system) which is used by most researchers (including this article), *Aegilops* consists of 23 species, having the D, S, U, C, N, and M genomes (van Slageren, 1994). Since the taxonomy has frequently changed (Kihara, 1954; Hammer, 1980; Witcombe, 1983; Kimber and Sears, 1987; van Slageren, 1994) this has led to some confusion about species names, and so a list of *Aegilops* species is provided below (**Table 1**). The biggest change from the previous taxonomy system is that *Ae. mutica* Boiss. has been removed and assigned a new species name: *Ambylopyrum muticum* (Boiss.) Eig. In the future, it will also be possible to make further modifications to reflect molecular findings (Edet et al., 2018).

One of the most important aspects of *Aegilops* is that it is closely related to bread wheat *Triticum aestivum* L. (AABBDD), which is one of the most important calorie sources for human nutrition. The D genome originated from the diploid species of *Aegilops tauschii* Coss. (= *Ae. squarrosa* L.) (Kihara, 1946; McFadden and Sears, 1946), and the B genome was derived from a closely related species to *Ae. speltoides* Tausch (Riley et al., 1958; Sasanuma et al., 1996; Petersen et al., 2006; Kilian et al., 2007; Zhang W. et al., 2018) which has the S genome. *Aegilops* species are distributed from Europe to western China in a species-specific manner (van Slageren, 1994), adapted to many different climatic zones including drought/heat environments, different disease hot spots and nutrient-poor areas. It has been reported that *Aegilops* possesses useful traits

Abbreviations: BC_1, backcrossed generation 1; CIMMYT, International Maize and Wheat Improvement Center; SHW, synthetic hexaploid wheat.

TABLE 1 | Taxonomy and genomic constitution of *Aegilops* species.

Genome*	Taxonomy system			
	van Slageren, 1994	Witcombe, 1983	Hammer, 1980	Kimber and Sears, 1987
D	*Ae. tauschii*	*Ae. squarrosa*	✓	*Triticum tauschii*
S	*Ae. speltoides*	✓	✓	*T. speltoides*
"	"	*Ae. ligustica*	✓	*T. speltoides*
S^b	*Ae. bicornis*	✓	✓	*T. bicorne*
S^l	*Ae. longissima*	✓	✓	*T. longissimum*
S^{sh}	*Ae. sharonensis*	✓	*Ae. longissima*	*T. sharonense*
S^s	*Ae. searsii*	✓	✓	*T. searsii*
C	*Ae. caudata*	✓	*Ae. markgrafii*	*T. dichasians*
M	*Ae. comosa*	✓	✓	*T. comosum*
N	*Ae. uniaristata*	✓	✓	*T. uniaristatum*
U	*Ae. umbellulata*	✓	✓	*T. umbellulatum*
CD	*Ae. cylindrica*	✓	*Ae. cylindrica*	*T. cylindricum*
DN	*Ae. ventricosa*	✓	✓	*T. ventricosum*
DM	*Ae. crassa*	✓	✓	*T. crassum*
DDM	"	✓	✓	*T. crassum*
DMS	*Ae. vavilovii*	✓	*Ae. crassa*	*T. syriacum*
DMU	*Ae. juvenalis*	✓	✓	*T. juvenale*
US	*Ae. peregrina*	✓	✓	*T. peregrinum*
US	*Ae. kotschyi*	✓	✓	*T. kotschyi*
UC	*Ae. triuncialis*	✓	✓	*T. triunciale*
UM	*Ae. biuncialis*	*Ae. lorentii*	*Ae. lorentii*	*T. macrochaetum*
UM	*Ae. columnaris*	✓	✓	*T. columnare*
UM	*Ae. geniculata*	*Ae. ovata*	✓	*T. ovatum*
UM	*Ae. neglecta*	*Ae. triaristata*	✓	*T. neglectum*
UMN	"	*Ae. triaristata*	✓	*T. rectum*
T	*Amblyopyrum mutica*	*Ae. mutica*	*Ae. mutica*	*T. tripsacoides*
	–	–	*Ae. turcomanica*	–

*Genome symbols follow to Waines and Barnhart (1992). ", same as the above; ✓, same species name to van Slageren (1994).

for wheat breeding (For review to see; Kilian et al., 2011) including drought tolerance (Damania et al., 1992; Waines et al., 1993; Rekika et al., 1998; Monneveux et al., 2000; Farooq and Azam, 2001), heat tolerance (Waines, 1994), salinity (Colmer et al., 2006), aluminum toxicity tolerance (Miller et al., 1995) and resistance to several pests and diseases such as rust (Mihova, 1988; Anikster et al., 2005; Liu et al., 2010; Rouse et al., 2011; Vikas et al., 2014; Huang S. et al., 2018; Olivera et al., 2018), powdery mildew (Lutz et al., 1994; Buloichik et al., 2008), Hessian fly (El Bouhssini et al., 2008), cereal aphid (Holubec and Havlıckova, 1994) and barley yellow dwarf virus (BYDV) (Makkouk et al., 1994). In addition, the species can adapt to low phosphorous environments (Liu et al., 2015) and can contribute to higher iron and zinc content in wheat grain (Rawat et al., 2009).

In order to effectively exploit these useful traits in wheat, it is necessary to overcome extra difficulties with the introgression process, including a hybridization barriers, incompatibilities/hybrid abnormalities, sterility of F_1s and, reduced meiotic chromosome pairings. Despite these obstacles, many *Aegilops* genes have been transferred to wheat and have been heavily utilized over the last 60 years (For review to see; Schneider et al., 2008; Kilian et al., 2011). *Aegilops* is also contributing to abate two recent threats to the global wheat production: Ug99 stem rust race derivatives and wheat blast (*Magnaporthe oryzae Triticum*). When Ug99 (original pathotype TTKSK) appeared in the early 2000s (Pretorius et al., 2000), more than 80% of wheat varieties did not have resistance against the race (Pretorius et al., 2000) and as such, wheat breeders sought resistance traits in *Aegilops*. When Wheat blast disease emerged in Bangladesh in 2016 (Ceresini et al., 2018), resistant wheat varieties were non-existent in the country, as well as neighboring India. Yet, a resistant variety was released within 2 years because of a resistance gene from *Aegilops* that was previously introgressed and ready for use (Cruz et al., 2016; Velu et al., 2018a; Mahmud, 2019).

In this paper, I will first review some difficulties relating to the use of *Aegilops* species (**Supplementary Figure S1**). Then, I will provide information on the contribution of *Aegilops* to wheat breeding in terms of identified genes in *Aegilops*, as well as some recent information on how *Aegilops* has contributed to the crisis prevention of Ug99 stem rust and wheat blast disease, which may change perspectives of *Aegilops* species as important sources for wheat breeding.

HYBRIDIZATION BARRIERS BETWEEN WHEAT AND *AEGILOPS* SPECIES AND CROSSABILITY GENES

To utilize the genetic resources in the *Aegilops* genus, it is necessary to first produce hybrids between wheat and *Aegilops* species. Wheat can be either a female or male parent of the F_1s, depending on species and specific cross combinations.

In wheat × *Aegilops* crosses, crossability genes on the wheat side have been highlighted for their significant role on the success rate of obtaining F_1 hybrids with *Aegilops* species (**Figure 1**). This is a key point considering it is very difficult to produce F_1s using low crossable wheat parents. While East Asian wheat landraces generally have higher crossability success rates with *Aegilops* and other alien species (e.g., rye), European ones have lower rates of success (Zeven, 1987), presumably because European wheat has had greater chances to cross-pollinate with rye histroically. Even though crossability is a QTL trait and controlled by several genes (Alfares et al., 2009), two dominant genes *Kr1* (5BL) and *Kr2* (5AL) were two major genes (Lein, 1943) affecting pollen tube growth (Riley and Chapman, 1967). These two genes have effects across different species including *Hordeum* and *Aegilops* (Snape et al., 1979; Koba and Shimada, 1993). *Kr1* has a stronger effect than *Kr2*, and dominant alleles (*Kr1* and *Kr2*) have inhibition effects. Plants with *Kr1Kr2* show less than 10% crossability, *Kr1kr2* showed between 10 and 25% crossability, *kr1Kr2* between 25 and 50% and plants with the *kr1kr2* genotype more than 50% crossability (Lein, 1943). Additionally, crossability genes were also reported as *Kr3* on 5D (homoeologous of *Kr1* and *Kr2*) and *Kr4* on 1A (Krolow, 1970; Zheng et al., 1992). More recently, *SKr* on 5BS was reported to have a stronger crossable effect than *Kr1* (Tixier et al., 1998; Alfares et al., 2009; Mishina et al., 2009).

FIGURE 1 | Effects of crossability on seed setting. **(A)** A high crossable durum line. **(B)** A low crossable durum line. The durum spikes show seed setting 2 weeks after pollination with *Ae. tasuchii*. The high crossable line **(A)** sets six grains, while the low crossable line **(B)** sets zero grains.

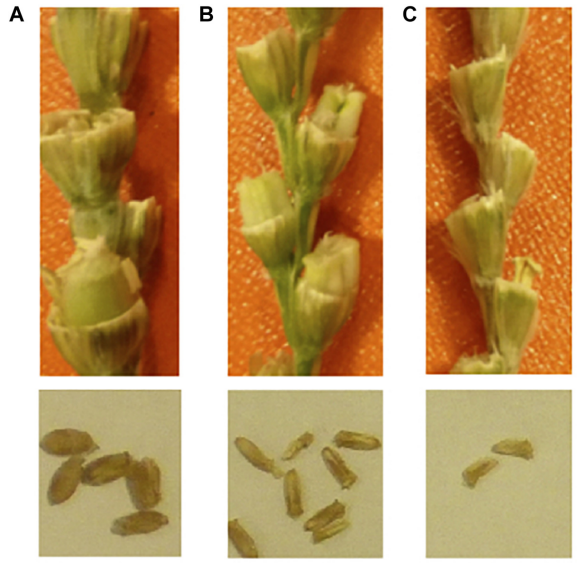

FIGURE 2 | Endosperm development deficiency in F_1 grains. **(A)** normal selfed-seeds of durum wheat, **(B)** durum wheat cv. LANGDON × *Ae. tauschii*; **(C)** durum wheat cv. CIRNO × *Ae. tauschii*. The seed sizes are smaller in durum × *Ae. tauschii*. The panel **(B)** has some amount of endosperm, and the seeds can germinate. The panel **(C)** has no endosperm, and the seeds will not germinate. Embryo rescue is necessary on the right.

If it is too difficult to produce F_1 hybrids in wheat × certain *Aegilops* species (wheat as females), pollination in the opposite cross direction (*Aegilops* as females) may be more successful. Dale et al. (2017) reported 0% seed setting in bread wheat × *Ae. tauschii* crosses (probably due to a crossability problem of the bread wheat parents), while it was 30% in *Ae. tauschii* × bread wheat. The seed-setting rate with *Aegilops* as female parents is variable across these species. Yuan et al. (2017) reported the rate was about 0.2% in *Ae. speltoides* × bread wheat, 2–9% in *Ae. cylindrica*, 12–15% in *Ae. ovata* and 22–47% in *Ae. tauschii*. It must be cautioned that the seed setting does not always mean success in obtaining F_1 plants.

ENDOSPERM AND EMBRYO DEVELOPMENT DEFICIENCY AND EMBRYO RESCUE

Gill et al. (1981) observed endosperm abortion and embryo lethality or semi-lethality and seedling death in crosses between *Ae. tauschii* and three diploid *Triticum* species. While the reaction types were different in each three *Triticum* species, the same thing is common in *Ae. tauschii* × bread wheat crosses. Even though the initial seed-setting rate was a 47% (Yuan et al., 2017), the seedling formation rate dropped to 1%. Sehgal et al. (2011) reported that an average of 35% initial embryo formation ended in an average of 7% F_1 plants.

The degree of endosperm development deficiency is cross-combination specific. However, high polyploidy *Aegilops* tend to set endosperm more when crossed with wheat, while diploid *Aegilops* species set less (data not shown). To overcome endosperm abortion, embryo rescue is necessary to recover hybrid seedlings. In this procedure, embryos are dissected from developing grains and transferred to an agar medium with nutrients such as sugar and salts for proper development (Miller et al., 1987). While some wheat lines such as Langdon (durum wheat) or various East Asian landrace lines tend to develop enough endosperm for the embryo to form seeds (Koba and Shimada, 1993), the amount of endosperm sometimes will be lower than normal "wheat × wheat crosses" (**Figure 2**). It is possible to skip embryo rescue if using these lines. The genetic background of forming unreduced gametes in wheat is not known yet.

OVERCOMING STERILITY OF F_1S AND UNREDUCED GAMETES

The genome of F_1s between wheat and *Aegilops* in haploids causes sterility until doubling the chromosome numbers. One option

is to conduct direct backcrossing of F_1s with wheat as a pollen donor. Even though the rate of seed set is extremely low, it is possible to obtain BC_1 plants (Cox et al., 1990; Fritz et al., 1995; Zemetra et al., 1998; Olson et al., 2013). The alternative is through chemical treatments such as colchicine (Blakeslee and Avery, 1937; Tang and Loo, 1940; Bennett and Smith, 1979) and N_2O gas (Hansen et al., 1988). Some wheat lines such as Langdon produce unreduced gametes, which is a gamete with a 2n nucleus resulting from abnormal meiosis (Fukuda and Sakamoto, 1992; Cai et al., 2010) that leads to spontaneous amphidiploid formation. The formation of unreduced gametes have been reported in durum × *Ae. tauschii*, *Ae. speltoides*, *Ae. longissima*, *Ae. umbellulata*, *Ae. comosa*, *Ae. ovata*, (= *Ae. geniculata*), *Ae. ventricosa*, *Ae. crassa* and *Ae. triuncialis* (Xu and Dong, 1992; Matsuoka and Nasuda, 2004; Tiwari et al., 2008; Fakhri et al., 2016). The rate of formation is different among *Aegilops* species and prevented by the presence of a shared homologous subgenomes (Fakhri et al., 2016). Additionally, it depends on the genotype of the *Aegilops* parents (Matsuoka and Nasuda, 2004; Fakhri et al., 2016).

HYBRID NECROSIS/WEAKNESS ABNORMALITY

Hybrid necrosis, chlorosis and bushy plant formation is very common in "normal" wheat × wheat cross (Hermsen, 1963; Hermsen and Waninge, 1972; Pukhalskiy et al., 2000; Chu et al., 2006). The *Ne1-Ne2* necrosis system is the best known hybrid necrosis system in wheat, which is caused when two complementary genes of *Ne1* (5BL) and *Ne2* (2BS) are found in the same plant (Tsunewaki, 1960; Nishikawa et al., 1974; Chu et al., 2006). However, this phenomenon is more frequent and complex in wheat × *Aegilops* crosses. Necrosis in *T. turgidum* × *Ae. tauschii* was first reported in the 1960s (Nishikawa, 1960, 1962a,b; **Figure 3**). Mizuno et al. (2010) did further analysis using a set of synthetic wheat lines that had one common durum wheat parent "Langdon" and different *Ae. tauschii* accessions. They found four different types of hybrid abnormality and responsible genes *Net1* (7DS), *Net2* (2DS), and Hybrid chlorosis1 (*Hch1*; 7DS) in *Ae. tauschii* (Mizuno et al., 2010, 2011; Nakano et al., 2015). The mode of action of these genes should be complementary with genes on the durum side, because the hybrid abnormalities take place only when *Ae. tauschii* is crossed with durum wheat. Hypersensitive response-like reactions were observed for *Net1* necrosis, indicating that it is a kind of disease response reaction (Jeuken et al., 2009; Mizuno et al., 2010). Okada et al. (2017) also reported growth abortion and grass-clump dwarf phenotype in durum × *Ae. umbellulata*. They also showed a repressed expression of the shoot meristem maintenance-related and cell cycle-related genes in the plants with the grass-clump dwarf phenotype. To avoid a problem with hybrid seedling death, Dhaliwal et al. (1986) reported the suppression of *Ne1-Ne2* necrosis at high temperatures. The author also confirmed that incubation at 28°C suppressed necrosis in F_1s between emmer × *Ae. tauschii* (**Supplementary Figure S2**). However, the high temperature causes pollen sterility.

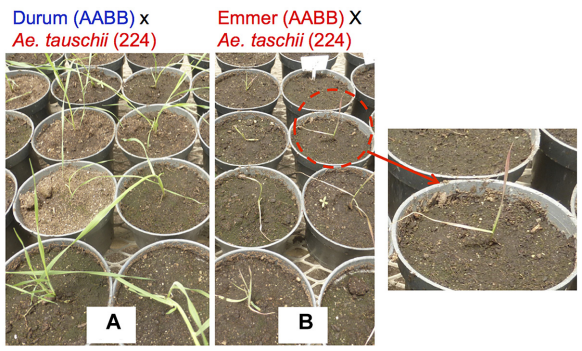

FIGURE 3 | Hybrid necrosis observed in tetraploid wheat × *Ae. tauschii*. **(A)** F_1 from *Triticum turgidum* ssp. durum cv. CIRNO C 2008 × *Ae. tauschii* WX (224); all plants of this group are growing normally. **(B)** F_1 from *Triticum* turgidum ssp. dicoccum (PI 233433) × *Ae. tauschii* WX (224); all plants are showing a necrosis symptom of reddish leaf color.

GAMETOCIDAL GENES

A group of gametocidal genes (*Gc*), sometimes considered as selfish genes, is another type of obstacle in which the genes cause chromosome breakages in gametes without *Gc* (Endo and Tsunewaki, 1975; Maan, 1975; Endo and Katayama, 1978). This happens when a plant becomes heterozygous in *Gc* —half of the gametes will have *Gc* and the other half will have no *Gc*. Gametes without *Gc* show reduce fitness, which is to the advantage of gametes with *Gc* for the transmission to the next generation (For review, see Tsujimoto, 2005; Endo, 2007; Niranjana, 2017). *Gc* genes have been identified in accessions of certain species that have C, S, S^l, or M genomes and mostly confined to three different homoeologous groups: 2, 3, and 4 (Endo, 2007). The identified genes include chromosome 3C of *Ae. markgrafii* (= *Ae. caudata*) and *Ae. triuncialis* (Endo and Tsunewaki, 1975), 2C of *Ae cylindrica* (Endo, 1979), $2S^l$ and $4S^l$ of *Ae. longissima*, $2S^{sh}$ and $4S^{sh}$ of *Ae. sharonensis* (Maan, 1975; Endo, 1985), 2S and 6S of *Ae. speltoides* (Tsujimoto and Tsunewaki, 1984, 1988; Kota and Dvorak, 1988) and 4M of *Ae. geniculata* (Kynast et al., 2000). The effects of *Gc* genes are variable; some cause lethality to gametes, while others are mild, allowing incorporation of the gamete into progenies. King et al. (2018) reported the presence of a 2S chromosome segment in all of the developed wheat-*Ae. speltoides* introgression lines due to the gametocidal effect. When researchers use these species, it is better to keep in mind that extra difficulties may arise from *Gc* genes. The suppression of *Gc* genes was reported in Norin 26, which inhibits *Ae. triuncialis Gc3-C1* action and is designated as *Igc1* (Tsujimoto and Tsunewaki, 1985). The presence of additional suppressor genes can also be predicted because the effect of a *Gc* gene is different in various wheat backgrounds. The *Gc* of chromosome 3C is usually lethal but when found in "Chinese Spring" background, it is mild. In addition, Friebe et al. (2003) produced a mutant of the *Ae. sharonensis Gc2* gene (designated as *Gc2mut*) which has a suppression effect on *Gc2*, which will be useful to reduce problems of *Gc* genes in wheat breeding scheme.

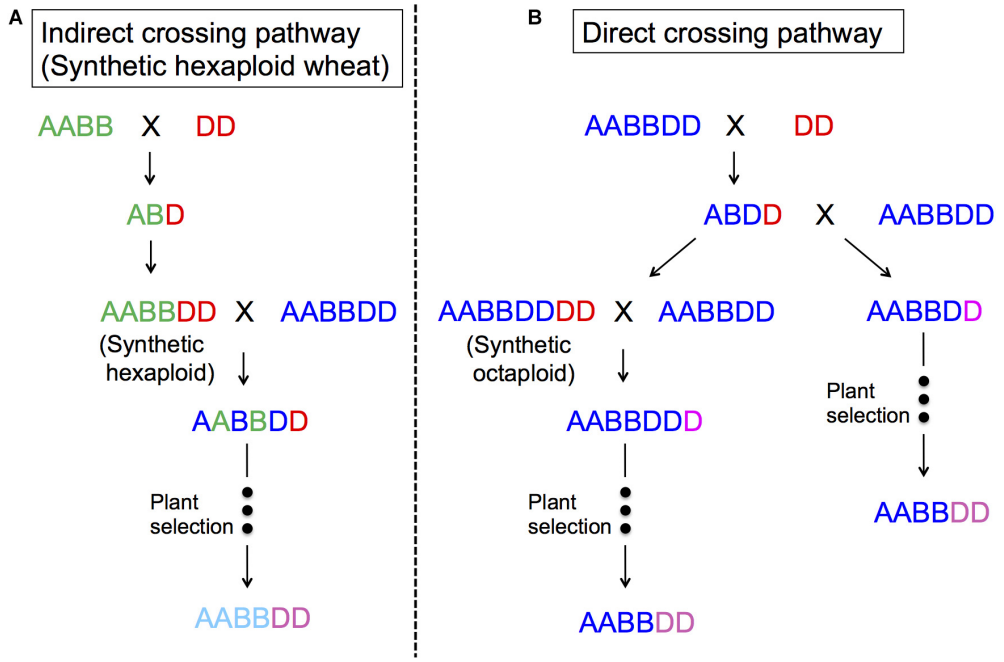

FIGURE 4 | The use of *Ae. tauschii* in wheat breeding. **(A)** indirect crossing pathway (synthetic hexaploid pathway) through durum × *Ae. tauschii* crosses; **(B)** direct crossing pathway through bread wheat × *Ae. tauschii* crosses.

THE USE OF *Ae. tauschii* FOR WHEAT BREEDING

Aegilops tauschii is the easiest species in this genus to utilize in wheat breeding, because there is little to no inhibition to meiotic chromosome pairing with the D genome chromosomes of bread wheat. According to several sources, bread wheat originated about 10,000 years ago (Wang et al., 2013; Matsuoka and Takumi, 2017), which is relatively recent and not long enough for genomic differentiation. Furthermore, *Ae. tauschii* contrasts with diploid A genome ancestors. Luo et al. (2000) reported about a 1/6 recombination-rate reduction between *Triticum monococcum* 5A and bread wheat 5A chromosomes when compared to the recombination rate between two *T. monococcum* 5A homologous chromosomes. Even though the A genome of bread wheat and that of the diploid ancestor can form perfect bivalents during meiosis in the F_1s of AAB (Gill et al., 1988), there are likely to be significant differences in base sequences and chromosome structures (such as inversion, translocations, deletion/duplications, or heterochromatin structures) after the tetraploid wheat formation – i.e., 100,000–500,000 years ago (Huang et al., 2002).

The spontaneous formation of bread wheat in nature was a rare event during which only a very limited number of *Ae. tauschii* plants were involved, based on molecular data and field observations (Dvorak et al., 1998; Matsuoka, 2011; Wang et al., 2013). The genetic diversity of *Ae. tauschii* is far greater in comparison to bread wheat's D genome diversity (Dvorak et al., 1998; Wang et al., 2013). Matsuoka et al. (2013) proposed sub-dividing *Ae. tauschii* into three groups, TauL1, L2, and L3, and found that bread wheat is close to TauL2 but distinct from TuL1. Even though it is not obvious as in the case of *T. monococcum*, crosses of bread wheat with *Ae. tauschii* accessions of TauL1 may show a reduction in chromosome recombination rates of the A-genome chromosomes.

Figure 4 represents two ways to utilize *Ae. tauschii* in wheat breeding, either through direct crossing or indirect crossing (synthetic wheat). With indirect crossing, tetraploid wheat (AABB) will be crossed with *Ae. tauschii* (DD) to produce an F_1 (ABD), and subsequently this F_1 will have its chromosome number doubled naturally or artificially to produce so-called synthetic wheat (AABBDD). Synthetic wheat can then be used in wheat breeding by crossing with bread wheat. Synthetic wheat lines were first developed in the United States and Japan in 1940s (Kihara, 1944, 1946; McFadden and Sears, 1944). During the next few decades, a number of synthetic wheat lines were developed by various groups (Kihara and Lilienfeld, 1949; Tanaka, 1961; Dyck and Kerber, 1970; Kerber and Dyck, 1979; Hatchett et al., 1981; Chèvre et al., 1989; Valkoun et al., 1990; Lange and Jochemsen, 1992; Lutz et al., 1994; Wang et al., 2006). Later in the 1980s, CIMMYT started a large-scale production of synthetic wheat, developing more than 1,000 lines (Das et al., 2016; Li et al., 2018). Matsuoka et al. (2007) also reported another set of "Langdon" synthetic wheat lines, and Zeng et al. (2016) produced synthetic wheat using local Chinese land races that were more adaptable to China.

In the direct crossing pathway, *Ae. tauschii* (DD) is crossed with bread wheat (AABBDD) to make an F_1 (ABDD). These F_1s are then backcrossed with the same bread wheat (AABBDD) to generate BC_1, where the plant selection process begins.

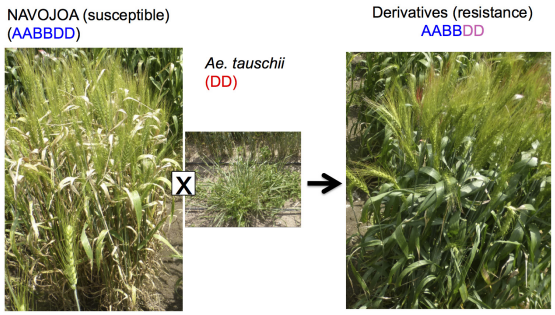

FIGURE 5 | Improvement of Septoria disease resistance through synthetic octaploid wheat. The derivative shows resistance while wheat parent line "NAVOJOA" is susceptible.

Gill and Raupp (1987) and Cox et al. (1992) reported this method as successful for transferring Hessian fly and rust resistance. The merit of this method is that it will only change the D genome, making it easy to perform some analyses, as well as directly improving the "best" line without contribution from durum wheat. One of disadvantage of this method may be sterility of the F_1 plants even as females, and as such, it is necessary to backcross a large number of spikes to have enough BC_1 seeds to introgress the whole genome (Cox et al., 1990; Fritz et al., 1995; Olson et al., 2013). It is important to note that the seed setting rates in F_1 plants also depend on *Ae. tauschii* accessions (Matsuoka and Takumi, 2017).

Octaploid synthetic wheat is another way to utilize *Ae. tauschii* in wheat breeding, in which an F_1 (ABDD) from bread wheat (AABBDD) × *Ae. tauschii* (DD) has its chromosome number doubled to produce an octaploid synthetic wheat (AABBDDDD) (Chèvre et al., 1989). Sehgal et al. (2011) and Zhang D. et al. (2018) reported the production of five and one AABBDDDD lines, respectively. CIMMYT has also produced a few hundred octaploid synthetic wheat lines (**Supplementary Figure S3**). This research resulted in the successful transfer of a dormancy QTL (Dale et al., 2017) and Septoria tritici Blotch resistance (**Figure 5**).

Table 2 summarizes the resistance genes identified and/or transferred into wheat, including leaf rust, stem rust, stripe rust, powdery mildew, and Hessian fly resistance. It is difficult to identify genes related to abiotic stress (drought and heat) and yield potential, as these traits are not obvious by sight. However, synthetically derived lines have shown up to a 30% yield increase under rain-fed conditions, and a 45% yield increase under drought condition over their wheat parents (Narasimhamoorthy et al., 2006; Dreccer et al., 2007; Trethowan and Mujeeb-Kazi, 2008; Li et al., 2014) and better performance under heat (Iehisa and Takumi, 2012; Jafarzadeh et al., 2016). The percentage of synthetic derivative lines (SDLs) in the Semiarid Wheat Yield Trial reached 52% in 2010, with a five-year average (2010–2015) of 35%. At least, 62 wheat varieties were released using CIMMYT synthetic wheat in their pedigree around the world since 2003 (Li et al., 2018). Elbashir et al. (2017) reported that synthetic derivative lines are promissive for improving heat tolerance in the analysis of multiple synthetic derivative (MSD) lines that cover the whole diversity of *Ae. tauschii*.

TABLE 2 | Identified or transferred biotic resistance genes of *Ae. tauschii* into wheat.

Disease/ pest	Gene (s)	Method	References
Leaf rust	*Lr21*	SHW	Dyck and Kerber, 1970
	Lr22a	SHW	Rowland and Kerber, 1974
	Lr32	SHW	Kerber, 1987
	Lr41	Direct	Cox et al., 1994
	Lr42	Direct	Cox et al., 1994
Stem rust	*Sr33*	SHW	Dyck and Kerber, 1970
	Sr45	SHW	Marais et al., 1998
	Sr46	SHW	Yu et al., 2015
Stripe rust	*Yr28*	SHW	Singh et al., 2000
Powdery mildew	*Pm2a*	SHW	Lutz et al., 1995
	Pm19	SHW	Lutz et al., 1995
	Pm34	Direct	Miranda et al., 2006
	Pm35	Direct	Miranda et al., 2007
	Pm58	Direct	Wiersma et al., 2017
Septoria tritici	*Stb5*	SHW	Arraiano et al., 2001
	Stb16	SHW	Ghaffary et al., 2012
Septoria nodorum	*Snb3*	SHW	McIntosh et al., 2008
Tan spot	*Tsr3* (= *tsn3*)	SHW	Tadesse et al., 2006
Cyst nematode	*Cre3*	SHW	Eastwood et al., 1991
	Cre4	SHW	Eastwood et al., 1994
Root knot nematode	*Rkn1*	SHW	Kaloshian et al., 1990
Hessian fly	*H13*	SHW	Gill et al., 1987
	H22	Direct (D)	Raupp et al., 1993
	H23	Direct (D)	Raupp et al., 1993
	H24	Direct (D)	Raupp et al., 1993
	H26	SHW	Cox and Hatchett, 1994
Greenbug	*Gb3*	SHW	Hollenhorst and Joppa, 1983
	Gb4	SHW	Martin et al., 1982
	Gb7	SHW	Weng et al., 2005
	(*Gba*, *Gbb*, *Gbc*,	SHW	Zhu et al., 2005
	Gbd, *Gbx2*,	SHW	"
	Gbx1, *Gbz*)*	Direct	"
Russian wheat aphid	*Dn3*	SHW (D)	Nkongolo et al., 1991
Wheat curl mite	*Cmc1*	Direct (D)	Thomas and Conner, 1986
	Cmc4	Direct	Malik et al., 2003
Soil-Borne Cereal Mosaic Virus	*SBWMV* (= allelic of *Sbm1*?)	Direct	Hall et al., 2009

**It may be allelic of Gb3. SHW, synthetic hexaploid wheat; Direct, direct crossing; (D), Ae. tauschii as female parents. The species name of disease/pest are following: Leaf rust (Puccinia recondita), Stem rust (Puccinia graminis), Stripe rust (Puccinia striiformis), Powdery mildew (Erysiphe graminis), Septoria tritici (Mycosphaerella graminicola), Septoria nodorum (Mycosphaerella graminicola), Tan spot (Pyrenophora tritici-repentis), Cyst nematode (Heterodera avenae), Root knot nematode (Meloidogyne spp.), Hessian fly (Mayetiola destructor), Green bug (Schizaphis graminum), Russian wheat aphid (Diuraphis noxia), Wheat curl mite (Eriophyes tulipae), Root knot nematode (Meloidogyne spp.) and Soil-Borne Cereal Mosaic Virus.*

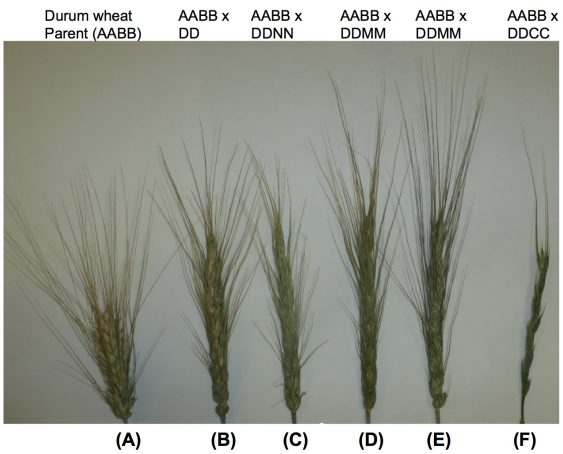

FIGURE 6 | Amphidiploid lines derived from between durum and diploid or tetraploid *Aegilops* with a D genome. **(A)** durum wheat cv. CIRNO C 2008, (AABB); **(B)** durum cv. CIRNO C 2008 × *Ae. tauchii* (895), (AABBDD); **(C)** durum cv. CIRNO C 2008 × *Ae. ventricosa* (PI 542385), (AABBDDNN); **(D)** durum cv. ACONCHI 89 × *Ae. crassa* (PI 542385), (AABBDDMM); **(E)** durum cv. CIRNO C 2008 × *Ae. crassa* (PI 542385), (AABBDDMM); **(F)** durum cv. CIRNO C 2008 × *Ae. cylindrica* (PI 639294), (AABBDDCC). All amphidiploids were produced and maintained at CIMMYT.

THE USE OF TETRAPLOID/HEXAPLOID *Aegilops* SPECIES WITH A D GENOME

Hybrids between tetraploid *Aegilops* species with the D genome can show meiotic pairing with the D genome chromosomes of bread wheat (Kimber and Zhao, 1983). These species include *Ae. cylindrica*, *Ae. crassa*, and *Ae. ventricosa*, *Ae. juvenalis* and *Ae. vavilovii*. Amphiploids of wheat with *Ae. crassa* have been reported (Jovkova et al., 1977; Xu and Dong, 1992), and a number of amphiploids of *Ae. ventricosa* × durum wheat were produced (Delibes et al., 1987). CIMMYT has also developed 20 amphiploid lines of these species using durum and bread wheat (**Figure 6**) for bread wheat D genome improvement (**Supplementary Figure S4**).

The eye spot resistance gene *Pch1* (one of two strong seedling resistance genes) was transferred from an amphiploid (AABBDDNN) between tetraploid wheat (AABB) × *Ae. ventricosa* (DDNN) (**Table 2**). This amphiploid was crossed with bread wheat (AABBDD) to have a derivative line named "VPM1" (Maia, 1967; Doussinault et al., 1983). It was later determined that the location of the transferred *Pch1* is chromosome 7D (Mena et al., 1992).

THE USE OF OTHER *Aegilops* SPECIES AND CHROMOSOME PAIRING

For the use of other *Aegilops* species, a reduced chromosome pairing frequency is more problematic. *Ae. cylindrica*, *Ae. crassa*, *Ae. ventricosa*, *Ae. Juvenalis,* and *Ae. vavilovii* are also categorized in this group due to the presence of non-D genomes.

Because of the lack of recombination, it is common to produce so-called alien chromosome addition or substitution lines in which one pair of *Aegilops* (or alien) chromosomes is added to or substituted for a pair of wheat chromosomes, respectively (**Supplementary Figure S5**). A number of addition lines have been produced from 14 different *Aegilops* species (For a review see; Schneider et al., 2008; Kilian et al., 2011). These addition lines are very useful for analysis and locating useful genes at the chromosomal level. However, addition lines have less breeding value, because they have many negative factors and the presence of an extra alien chromosome disrupts the genetic harmony of a genome. To be more appropriate for breeding, it is necessary to produce introgression lines (small *Aegilops* chromosome segment transfers) or Robertsonian/centromeric translocation lines (Robertson, 1916), in which one of the *Aegilops* chromosome arms is translocated to a wheat chromosome, replacing an arm of that wheat chromosome (**Supplementary Figure S5**). These translocations can be obtained spontaneously from addition/substitution lines or amphiploids in backcrossing populations, or using wheat monosomic lines ($2n$ = 41; with one of the homoeologous chromosomes is missing). All of these can lead to the occurrence of univalent chromosomes during meiosis. Then, the meiotic spindle fiber will attach to the both sides of univalent chromosomes, which then causes chromosome breakages through the centromeric regions at high frequency. The broken chromosome arms are sticky and may fuse to other broken chromosomes to produce centromeric translocations (**Supplementary Figure S6**).

Homoeologous meiotic pairing between chromosomes of wheat and *Aegilops* species is inhibited mostly by the *Ph1* gene (5BL) (Okamoto, 1957; Riley and Chapman, 1958; Sears and Okamoto, 1958; Riley, 1960). Therefore, the meiotic barrier can be overcome by suppressing of *Ph1* activity. Sears (1977) produced the *ph1b* mutant in which the *Ph1* locus is missing and this is the most widely used *Ph1* gene mutant in wheat breeding. Another gene which affects homoeologous chromosome pairing was identified as *Ph2* (3D) (Mello-Sampayo, 1971) and has a mild inhibition effect on *Ph1* (Sears, 1982). Additional mutants, *ph1c* (Giorgi, 1983) and *ph2* (Sears, 1982) are also available, even though they have been rarely used in the breeding. It is also known that the presence of *Ph1* suppressors or promotors of homoeologous chromosome paring are present in some accessions of *Aegilops* species: *Ae. speltoides* (Feldman and Mello-Sampayo, 1967; Dover and Riley, 1977; Dvorak et al., 2006), *Ae. longissima* (7), *Ae. mutica* (Dover and Riley, 1972), *Ae. umbellulata* (Riley et al., 1973), *Ae. Peregrina,* and *Ae. kotschyi* (Fernandez-Calvin and Orellana, 1991) and *Ae. geniculata* (Koo et al., 2017). Therefore, the transfer of traits may be easier in accessions that have the suppressive effects. The *Ae. speltoides* genes are considered to be suppressants, because they can promote more meiotic pairing in the presence of the *Ph1* gene (Dover and Riley, 1972). A couple of suppressor genes of *Ae. speltoides* has been transferred into wheat and designated as *PhI* (Chen et al., 1994) and *Su1-Ph1* (7S) and *Su2-Ph1* (3S) (Dvorak et al., 2006; Li et al., 2017). Since these genes are dominant, they can be faster and easier to utilize in breeding. Yet the effects of *PhI* have been shown to be lower than that of *ph1b* (Aghaee-Sarbarzeh et al., 2000).

Sometimes it is difficult to induce homoeologous recombination due to different homoeologous co-linearity between

wheat and *Aegilops* chromosomes (Molnár et al., 2013, 2016). It is also known that centromeric and other chromosomal regions may have very low recombination rates, even in wheat × wheat crosses (Saintenac et al., 2009). In these cases, other methodologies become an alternatives. Sears (1956) demonstrated a successful transfer of *Ae. umbellulata Lr9* gene into wheat using irradiation. Yet this is the only success story using irradiation for introgression of *Aegilops* chromatin for wheat breeding until recently. Singh et al. (2016) and Verma et al. (2016) recently reported the production of a translocation by irradiation of *Ae. kotschyi* hybrids. Mild effect *Gc* genes and some chemicals can also induce random translocations, much like irradiation. Even though it is not for breeding purposes, the *Gc* system has been used for producing translocations of wheat-rye and wheat-barley (Joshi et al., 2013; Li et al., 2013; Ishihara et al., 2014).

USEFUL GENES OF *Aegilops* TRANSFERRED TO WHEAT

Through the use of the various techniques described above, a number of genes have been transferred from *Aegilops* (including *Ae. tauschii*) to wheat (**Tables 3**, **4**). In terms of total number, leaf rust resistance genes are the most numerous (20), followed by powdery mildew (15), and green bug (12). Since more than 75 resistance gene loci have been identified and permanently designated as resistance genes by 2018 (Ponce-Molina et al., 2018). *Aegilops* provided more than 20% of them. For powdery mildew, 54 resistance loci were found by 2018 (Tang et al., 2018), and *Aegilops* contributed about 20%. For Cereal Cyst Nematodes (CCN) resistance genes, a total of 12 genes have been identified, including *Cre1-8*, *CreR*, *CreV*, *CreX*, *CreY* (Ali et al., 2019). Of them, two (*Cre1* and *Cre8*) are indigenous to the wheat genepool. The others are from *Ae. tasuchii* (*Cre3* and *Cre4*), *Ae. ventricosa* (Zhuk.) (*Cre2*, *Cre5,* and *Cre6*), *Ae. triuncialis* L. (*Cre7*); *Ae. peregrina* (*CreX* and *CreY*), *Secale cereale* (*CreR*) and *Dasypyrum villosum* (*CreV*) (Zhang et al., 2016), showing that two thirds of them are from *Aegilops*. In terms of actual species of origin, *Ae. tauschii* has provided the most number of genes, followed by *Ae. speltoides* and then *Ae. ventricosa*. It is worth noting that most of the disease resistances from *Ae. ventricosa* are provided by a single 2NS-2AS translocation, including *Lr37*, *Sr38*, *Yr17*, *Cre5*, *Rkn3* (Bariana and McIntosh, 1993, 1994; Jahier et al., 1996, 2001; Helguera et al., 2003; Tanguy et al., 2005; Williamson et al., 2013); this translocation has originated from VPM1 (Maia, 1967) that also has *Pch1* resistance on 7D (Mena et al., 1992).

Recently, *Aegilops* has gathered more attention for improving micro-nutrient content (such as Fe and Zn) in wheat grains. Zn deficiency affects 17.3% of the world's population across Asia and Africa, leading to the deaths of more than 400,000 children each year (Cakmak, 2007; Black et al., 2013; Velu et al., 2018b). Micro-nutrient rich wheat, i.e., bio-fortified wheat, can improve the lives of these people. It is difficult to find high Zn and Fe content germplasm in the wheat genepool (Cakmak et al., 2010), even though some *Aegilops* species show three to four-fold higher Zn and Fe grain content, including *Ae. longissima* (S^l),

TABLE 3 | Identified or transferred biotic resistance genes in *Aegilops* (other than from *Ae. tauschii*) into wheat.

Disease/ pest		Genome	Gene	References
Eyespot	*Ae. ventricosa*	DN	*Pch1*	Doussinault et al., 1983
		(recombination between two D genomes)		
Leaf rust	*Ae. umbellulata*	U	*Lr9*	Sears, 1956
			Lr76	Bansal et al., 2017
	Ae. speltoides	S	*Lr28*	McIntosh et al., 1982
			Lr35	Kerber and Dyck, 1990
			Lr36	Dvorak and Knott, 1990
			Lr37	Bariana and McIntosh, 1993
			Lr47	Helguera et al., 2000
			Lr51	Helguera et al., 2005
			Lr66	Marais et al., 2009a
	Ae. kotschyi	US	*Lr54*	Marais et al., 2005
	Ae. sharonensis	S^{sh}	*Lr56*	Marais et al., 2010
	Ae. geniculata	UM	*Lr57*	Kuraparthy et al., 2007
	Ae. triuncialis	UC	*Lr58*	Kuraparthy et al., 2011
	Ae. peregrina	US	*Lr59*	Marais et al., 2008
	Ae. neglecta	UM	*Lr62*	Marais et al., 2009b
Stem rust	*Ae. speltoides*	S	*Sr32*	McIntosh, 1988
			Sr39	Kerber and Dyck, 1990
			Sr47	Klindworth et al., 2012
	Ae. comosa	M	*Sr34*	McIntosh et al., 1982
	Ae. ventricosa	DN	*Sr38*	Bariana and McIntosh, 1993
	Ae. searsii	S^s	*Sr51*	Liu et al., 2011a
	Ae. geniculata	UM	*Sr53*	Liu et al., 2011b
Stripe rust	*Ae. comosa*	M	*Yr8*	Riley et al., 1968
	Ae. ventricosa	DM	*Yr17*	Bariana and McIntosh, 1993
	Ae. kotschyi	US	*Yr37*	Marais et al., 2005
	Ae. sharonensis	S^{sh}	*Yr38*	Marais et al., 2010
	Ae. geniculata	UM	*Yr40*	Kuraparthy et al., 2007
	Ae. neglecta	UM	*Yr42*	Marais et al., 2009b
	Ae. umbellulata	U	*Yr70*	Bansal et al., 2017
Powdery mildew	*Ae. speltoides*	S	*Pm1d*	Hsam et al., 1998
			Pm12	Jia et al., 1996
			Pm32	Hsam et al., 2003
			Pm53	Petersen et al., 2015
	Ae. longissima	S^l	*Pm13*	Donini et al., 1995
	Ae. geniculata	UM	*Pm29*	Zeller et al., 2002
	Ae. umbellulata	U	*Pm57*	Liu et al., 2017
Cyst nematode	*Ae. ventricosa*	DN	*Cre2*	Delibes et al., 1993
			Cre5	Jahier et al., 1996
			Cre6	Ogbonnaya et al., 2001
	Ae. triuncialis	UC	*Cre7*	Romero et al., 1998
	Ae. peregrina	US	(*CreX*)	Barloy et al., 2007
			(*CreY*)	Barloy et al., 2007
Root knot nematode	*Ae. peregrina*	US	*Rkn2*	Yu et al., 1990
	Ae. ventricosa	DN	*Rkn3*	Williamson et al., 2013
Hessian fly	*Ae. ventricosa*	DN	*H27*	Delibes et al., 1997
	Ae. triuncialis	UC	*H30*	Martin-Sanchez et al., 2003
Green bug	*Ae. speltoides*	S	*Gb5*	Friebe et al., 1991

The species name of disease/pest are following: Eyespot (Tapesia yallundae), Leaf rust (Puccinia recondita), Stem rust (Puccinia graminis), Stripe rust (Puccinia striiformis), Powdery mildew (Erysiphe graminis), Cyst nematode (Heterodera avenae), Root knot nematode (Meloidogyne spp.), Root knot nematode (Meloidogyne spp.), Hessian fly (Mayetiola destructor) and Green bug (Schizaphis graminum).

TABLE 4 | List of resistance gene against stem rust Ug99 race.

Origin of Sr genes	Effective Sr genes				
Triticum aestivum	*Sr9h*	*Sr15*[*1]	*Sr28*	*Sr42*	+ 2 temporal*[2]
(Partial; APR*[a])	*Sr55*	*Sr56*	*Sr57*	*Sr58*	
Triticum dicoccum	*Sr2*	*Sr13*			
Triticum timopheevi	*Sr36*	*Sr37*			
Triticum araraticum	*Sr40*				
Triticum monococcum	*Sr21*	*Sr22*	*Sr35*	*Sr60*	+ 2 temporal*[3]
Aegilops tauschii	*Sr33*	*Sr45*	*Sr46*		+ 3 temporal*[4]
Aegilops speltoides	*Sr32*	*Sr39*			
Ae. sharonensis					+ 3 temporal*[5]
Aegilops searsii	*Sr51*				
Aegilops triuncialis	*Sr47*				
Aegilops geniculata	*Sr53*				
Aegilops umbellulata					+ 1 temporal*[6]
Thinopyron ponticum	*Sr24*	*Sr25*	*Sr26*	*Sr43*	
Thinopyrum intermedium	*Sr44*				
Secale cereale	*Sr27*	*Sr50*	*Sr59*		+ 1 temporal*[7]
Dasypyrum villosum	*Sr52*				

*The table was constructed according to Kielsmeier-Cook et al. (2015) and Randhawa et al. (2018) with updates by the author. *[a]Partial resistance genes; APR, adult plant resistance gene; *[1]Data from multiple research groups are not consistent (Singh et al., 2015); *[2]3SrND643 (Basnet et al., 2015), SrTmp, SrCad (cosegregating with Sr42; Hiebert et al., 2016); *[3]Sr60 (Chen et al., 2018), SrTm4 (Briggs et al., 2015), SrTm5 (Chen et al., 2018); *[4]SrTA10171, SrTA10187, SrTA1662; *[5]SrSha7 (Singh et al., 2015); Sr-1644-1S^{sh} and Sr-1644-5S^{sh} (Yu et al., 2017); *[6]2U chromosome (Edae et al., 2016); *[7]Sr59 (Rahmatov et al., 2016), Sr1RSAmigo.*

Ae. kotschyi (US), *Ae. peregrina* (US), *Ae. cylindrica* (CD), *Ae. ventricosa* (DN), *Ae. geniculata* (UM) (Rawat et al., 2009). Amphiploid durum- *Ae. longissima* and partial amphiploids of wheat – *Ae. kotschyi* show two to three times higher levels of Zn and Fe grain content than the parental wheat line (Tiwari et al., 2008, 2010). Rawat et al. (2011) further reported Zn grain content three times higher in wheat- *Ae. kotschyi* addition/substitution lines than the wheat parent.

In addition to the benefit for wheat breeding mentioned above, it is also important to highlight that *Aegilops* introgression lines have a level of diversity and unique traits that wheat lacks. Even though these are of no immediate benefit at this moment, their value could be seen in the future, as exemplified by two recent global wheat production threats.

A STORY OF *Aegilops* TRANSLOCATIONS ON STEM RUST UG99 RACE

A serious threat to global wheat production is the emergence of stem rust Ug99 race, which was recognized in Uganda in 1999 (Pretorius et al., 2000). This disease had the potential to develop into a global catastrophe, as more than 70% of wheat varieties around the world did not have resistance against Ug99 in the early 2000s (Singh et al., 2015). Many wheat breeders and pathologists, who had thought stem rust was no longer a problem, were caught unprepared and were then spurred to search for new resistant sources. The researchers realized that while the bread and durum wheat gene pools do not have many resistant sources, resistance is available outside the genepool from ancestral and alien species including many in *Aegilops* (**Table 4**, based on Yu et al., 2014; Kielsmeier-Cook et al., 2015; Randhawa et al., 2018 with updates by the author). This has also promoted various studies to identify new stem rust resistance genes, which led the identification of *Sr46* (*Ae. tauschii*; Yu et al., 2015), *Sr47* (*Ae. triuncialis*; Klindworth et al., 2012), *Sr51* (*Ae. searsii*; Liu et al., 2011a), *Sr53* (*Ae. geniculate*; Liu et al., 2011b) and three additional genes in *Ae. tauschii* (Rouse et al., 2011), three genes in *Ae. sharonensis* (Singh et al., 2015; Yu et al., 2017) and one gene in *Ae. umbellulata* (Edae et al., 2016). In addition, it has been reported that 81% of *Ae. longissima* (out of 394 accessions), 94% of *Ae. neglecta* (189 out of 202 accessions tested), 88% of *Ae. cylindrica* (DDCC) and *Ae. peregrina* (SSUU) were Ug99 resistant (Huang S. et al., 2018; Olivera et al., 2018).

Even though introgression lines of two Ug99 resistance genes (*Sr32* and *Sr39*) from *Ae. speltoides* were available, they were not used in wheat breeding program due to the presence of large *Ae. speltoides* segments and associated negative factors on agronomy (Friebe et al., 1996). Fortunately, researchers in Australia and the United States started preparing for the possible appearance of dangerous new stem rust pathogen races back in the early 1990s and the reports of Ug99 just confirmed their expectations. Based on that work, shortened introgressions of chromosome 2S segments with *Sr32* and *Sr39* were already developed using the *ph1b* mutant and have been quickly distributed around the world (Mago et al., 2009, 2013; Niu et al., 2011).

It is notable that it has eight resistance genes (+ three temporary assigned genes) in the bread wheat gene pool are effective to Ug99, but four of them (*Sr55*, *Sr56*, *Sr57*, and *Sr58*) are partial or adult plant resistance genes (APR), so it is necessary to combine them with other genes to exert a higher level of resistance (Gustafson and Shaner, 1982; McIntosh et al., 1995, 1998, 2012).

A STORY OF THE 2NS TRANSLOCATION IN RELATION TO WHEAT BLAST DISEASE

Wheat blast caused by *Pyricularia oryzae* (*Magnaporthe oryzae*) is an emerging disease that was first recognized in Brazil in the 1980s (Igarashi et al., 1986). The pathogen gained an ability to infect the new host plant wheat through a mutation of an avirulence gene (Inoue et al., 2017). Since then, it has been a serious obstacle for wheat production in central and south Brazil, south-east Paraguay and eastern Argentina, affecting 300 million ha of wheat fields and reducing the yield of infected areas 100–10% (Kohli et al., 2011; Perello et al., 2015; Duveiller et al., 2016). The disease jumped to Bangladesh in 2016 and spread to 15,000 ha (Malaker et al., 2016). Because of this serious threat to the wheat production of South Asia, quick remedial action was required to prevent a devastating epiphytotic (Mottaleb et al., 2018). Eight different resistance genes against wheat blast (*Rmg1-8*) have been reported, and

only two of them (*Rmg7* and *Rmg8*) are effective in the field in Bangladesh (Anh et al., 2017). Since *Rmg7* and *Rmg8* recognize the same avirulence gene peptide of the pathogen, both resistance genes are functionally equivalent to a single gene for resistance (Anh et al., 2017). Despite of lacking resistance sources, a new resistance wheat variety, "BARI com" was released in Bangladesh within 2 years in 2018. This happened because of the existence of the 2NS-2AS translocation (Cruz et al., 2016; Velu et al., 2018a; Mahmud, 2019). This translocation has been utilized in wheat breeding programs because of rust resistances (Juliana et al., 2017), but it also happens to have a strong wheat blast resistance. If 2NS-2AS had not have been produced, the wheat blast issue would have been a much more serious problem in the last few years. Another amazing finding with the 2NS-2AS translocation is that nearly 90% of advanced lines of the CIMMYT bread wheat program have this translocation (Juliana et al., 2017; Philomin Juliana, Personal communication). As in the case of the T1BL.1RS translocation that dominated wheat cultivars for decades, a beneficial translocation can have a huge impact on wheat breeding and production.

THE USE OF *Aegilops* IN THE GENOMIC AGE FOR BREEDING AND PRE-BREEDING

During the last several decades, cytogenetic methods not only have been essential tools for screening and understanding the nature of translocations and alien introgressions from a number of progenies, but also possess the major constraint in handling large numbers of samples. But new cytogenetic FISH/GISH technology using oligo probes expands the capacity, proving a valuable tool in cytogenetics (Du et al., 2017; Huang X. et al., 2018). More importantly, recent progress in high through-put genotyping technology and availability of molecular methods makes it possible to detect alien segments very easily, which has been promoting the production of alien segment introgressions. Niu et al. (2011) screened about 1,000 plants and found 40 smaller alien recombinants of *Ae. speltoides* 2S chromosome using the *ph1b* mutant. The development of translocations which cover a whole genome have been demonstrated in *Ambylopyrum mutica* (= *Ae. mutica*) (King et al., 2017) and *Ae. speltoides* (King et al., 2018). The number of estimated introgression segments obtained are about 200 of *Am. mutica* (King et al., 2017), and a map of about 600 cM was made with 540 plants in the case of *Ae. speltoides* (King et al., 2018), which allowed the construction of linkage maps even using wheat- *Aegilops* introgression lines and the Axiom 35K SNP array that was constructed on a wheat sequence based Axiom 820K SNP array by optimizing for finding polymorphism between wheat and *Aegilops* species. An increased number of whole genome linkage or physical maps in *Aegilops* species have been available (**Table 5**). A 4-gigabase physical map based on BAC clones of *Ae. tauschii* led the construction of a 10 K *Ae. tauschii* Infinium SNP array (Luo et al., 2013). Moreover, the draft sequence of *Ae. tauschii* has been recently reported (Luo et al., 2017), and a TILLING population of *Ae. tauschii* was reported (Rawat et al., 2018). It will be possible to have additional physical maps and draft sequences in another *Aegilops* species in near future that will facilitate their use in wheat breeding and gene identifications.

Yet the biggest limitation and challenge for the use of *Aegilops* is still reduced recombination rates between wheat and *Aegilops* chromosomes that is sometimes prohibitive in producing an *Aegilops* introgression segment. The new technologies such as

TABLE 5 | List of whole or semi-whole genome genetic or physical maps in *Aegilops* species.

Species name	Type of markers	Type of populations	References
Aegilops tauschii	RFLP*[1]	F2 of *Ae. tauschii*	Gill et al., 1991
	RFLP; SSR*[2]	F2 and F3 of *Ae. tauschii*	Boyko et al., 2002
	SSR	F2 of *Ae. tauschii*	Okamoto et al., 2013
	10K SNP array of *Ae. tauschii*	F2 of *Ae. tauschii*	Luo et al., 2013
	EST*[3]; SSR; RJM*[4]	RH*[10] of synthetic wheat	Kumar et al., 2012
	DArT*[5]; SSR	RH of *Ae. tauschii*	Kumar et al., 2015
	SSR	F2 of *Ae. tauschii*	Nishijima et al., 2018
Aegilops speltoides	RFLP	F2 of *Ae. speltoides*	Dvorak et al., 2006
	Axiom 35K SNP array	Wheat/*Ae. speltoides* introgressions	King et al., 2018
Aegilops longissima	RFLP	F2 of *Ae. longissima*; CS/*Ae. longissima* addition	Zhang et al., 2001
	SSR	RIL*[11] of *Ae. longissima*	Sheng et al., 2012
	RNA-seq*[6]	CS/*Ae. longissima* addition	Wang et al., 2018
Aegilops sharonensis	DArT; SSR	F2 of *Ae. sharonensis*	Olivera et al., 2013
	OPA*[7]	RIL and F2 of *Ae. sharonensis*	Yu et al., 2017
Aegilops umbellulata	RFLP	CS/*Ae. umbellulata* addition	Zhang et al., 1998
	GBS*[8]	F2 of *Ae. umbellulata*	Edae et al., 2016
	GBS	F2 of *Ae. umbellulata*	Edae et al., 2017
Aegilops caudata	SSR	CS/*Ae. caudata* addition	Niu et al., 2018
Aegilops comosa	PAUG*[9]	CS/*Ae. comosa* addition	Liu et al., 2019
Amblyopyrum mutica	Axiom 35K SNP array	Wheat/*Am. mutica* introgressions	King et al., 2017

*[1]*RFLP, restriction fragment length polymorphism;* *[2]*SSR, simple sequence repeat;* *[3]*EST, expression sequence tag;* *[4]*RJM, repeat DNA junction marker;* *[5]*DArT, diversity arrays technology;* *[6]*RNA-seq, RNA sequencing;* *[7]*OPA, oligo pool assay;* *[8]*GBS, genotyping-by-sequence;* *[9]*PAUG, PCR-based landmark unique gene;* *[10]*RH, radiation hybrid;* *[11]*RIL, recombinant inbred line.*

MutChromSeq (mutant chromosome sequencing), MutRenSeq (Mutagenesis Resistance gene enrichment and sequencing) and AgRenSeq (Association Genetics R gene enrichment Sequencing) may provide an alternative to overcome gene identification obstacles. These techniques allow a rapid isolation of mutated genes with mutagenesis by sequencing sorted chromosomes (MutChromSeq) or enriching target gene families by exome capture (MutRenSeq) or a rapid isolation of natural variants by enriching target gene family (AgRenSeq) and sequencing for resistance gene homologs. Steuernagel et al. (2016) reported the cloning of *Sr22* and *Sr45* from bread wheat using MutRenSeq, Sánchez-Martín et al. (2016) reported the cloning of *Pm2* using MutChromSeq, and, Arora et al. (2019) demonstrated the discovering and cloning of *Sr33*, *Sr45*, *Sr46*, and *SrTA1662* from a panel of about 200 *Ae. tauschii* accessions using AgRenSeq. Development of new methodologies which can compensate the reduced recombination rate may overcome the biggest constrains of the use of *Aegilops*. Alternatively, we may be able to find new variations or genes to increase the recombination rate from *Aegilops* like *PhI* genes (Chen et al., 1994; Dvorak et al., 2006; Li et al., 2017).

As we can see from the stories of Ug99 and wheat blast, *Aegilops* species are important not only for pre-breeding but also for a proactive main-stream breeding. It is still necessary to induce a certain level of recombination between wheat and alien chromosomes for the use of *Aegilops*, but the new technologies are opening up a new era of *Aegilops* for wheat breeding.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

FUNDING

This work was supported by the CGIAR Research Program on Wheat (CRP-Wheat) and Japan ODA from Ministry of Foreign Affairs.

ACKNOWLEDGMENTS

Special thanks to reviewers for the meaningful comments to improve the quality of this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00585/full#supplementary-material

FIGURE S1 | Various constrains in obtaining F_1/amphiploids between wheat and *Aegilops* species. In this case, the cross is between bread wheat (AABBDD) and *Ae. speltoides* (SS). Measures corresponds to the constraints of the left.

FIGURE S2 | Suppression of hybrid necrosis in F1s between emmer x *Ae. tauschii* by incubation at 28°C. **(A)**: incubation at 28°C; **(B)**: incubation at 22°C.

FIGURE S3 | Synthetic octaploid and hexaploid wheat lines. **(A)** bread wheat cv. BORLAUG 100 × *Ae. tauchii* (WX 700), (AABBDDDD); **(B)** bread wheat cv. BORLAUG 100 × *Ae. tauchii* (WX 1195), (AABBDDDD); **(C)**: bread wheat cv. BORLAUG 100 × *Ae. tauchii* (KU 2096), (AABBDDDD); **(D)**: bread wheat cv. BORLAUG 100 × *Ae. tauchii* (KU 2811), (AABBDDDD); **(E)**: durum cv. ACONCHI 89 × *Ae. tauchii* (KU 2811), (AABBDD); **(F)**: durum cv. CIRNO C 2008 × *Ae. tauchii* (KU 2811), (AABBDD). DD, *Ae. tauschii*. All amphiploids were produced and maintained at CIMMYT.

FIGURE S4 | The use of the D genome in tetraploid *Aegilops* species.

FIGURE S5 | The use of *Aegilops* species (except *Ae. tauschii*) for wheat breeding. Introgression lines can be produced from any part of F_1 haploid, amphiploid, addition/substitution lines, and centromeric translocation line.

FIGURE S6 | The mechanism of forming Robertsonian (centromeric) translocation. During meiosis, spindle fibers will attach to the both side of univalent chromosomes, which leads chromosome breakage at the centromeric region at high frequency (the right one). Broken chromosomes may fuse with other broken chromosome arm, forming centromeric translocation. The 3A, 3B, and 3U are 3A, 3B, and 3U chromosomes. S, short arm; L, long arm.

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