



# Genomics Armed With Diversity Leads the Way in *Brassica* Improvement in a Changing Global Environment

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Technology, India

### \*Correspondence:

Jacqueline Batley  
jacqueline.batley@uwa.edu.au

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**Nur Shuhadah Mohd Saad, Anita A. Severn-Ellis, Aneeta Pradhan, David Edwards and Jacqueline Batley\***

School of Biological Sciences Western Australia and UWA Institute of Agriculture, University of Western Australia, Perth, WA, Australia

Meeting the needs of a growing world population in the face of imminent climate change is a challenge; breeding of vegetable and oilseed *Brassica* crops is part of the race in meeting these demands. Available genetic diversity constituting the foundation of breeding is essential in plant improvement. Elite varieties, land races, and crop wild species are important resources of useful variation and are available from existing gene pools or gene banks. Conservation of diversity in gene pools, gene banks, and even the wild is crucial in preventing the loss of variation for future breeding efforts. In addition, the identification of suitable parental lines and alleles is critical in ensuring the development of resilient *Brassica* crops. During the past two decades, an increasing number of high-quality nuclear and organellar *Brassica* genomes have been assembled. Whole-genome re-sequencing and the development of pan-genomes are overcoming the limitations of the single reference genome and provide the basis for further exploration. Genomic and complementary omic tools such as microarrays, transcriptomics, epigenetics, and reverse genetics facilitate the study of crop evolution, breeding histories, and the discovery of loci associated with highly sought-after agronomic traits. Furthermore, in genomic selection, predicted breeding values based on phenotype and genome-wide marker scores allow the preselection of promising genotypes, enhancing genetic gains and substantially quickening the breeding cycle. It is clear that genomics, armed with diversity, is set to lead the way in *Brassica* improvement; however, a multidisciplinary plant breeding approach that includes phenotype = genotype × environment × management interaction will ultimately ensure the selection of resilient *Brassica* varieties ready for climate change.

**Keywords:** *Brassica*, plant genomics, crop improvement, molecular breeding, food security

## INTRODUCTION

Predictions of exponential increases in the world population and climate change are forcing re-evaluation of efforts in addressing the demand for global food security. Plant crops account for more than 80% of the diet consumed by the human population, and the production of edible crops has thereby dominated almost half of the world's available land mass since the beginning of the twenty-first century (Leff et al., 2004; Herrera and Garcia-Bertrand, 2018). The Industrial and Green Revolutions have furthermore shaped the ways that these commodities are managed for efficiency and commercialization. The next revolution will require crop improvement not just to curb world hunger but also to address sustainability in the face of biotic and abiotic stresses triggered by the impending climate change. It is estimated that staple crop yield must improve by 70–110% to feed the predicted 10 billion population by 2050 (Saini et al., 2020). Crop projections and modeling studies have suggested that climate change may have already been responsible for a small yearly decrease in yield and calories in certain geographic regions (Ray et al., 2019). More recently, the coronavirus disease 2019 (COVID-19) pandemic has emphasized food security in terms of short-term and local supply (Cappelli and Cini, 2020). While food shortages are not a wide set concern as yet, a prolonged crisis could interfere with the current complex food supply network (de Paulo Farias and dos Santos Gomes, 2020; Siche, 2020). Globalized food distribution, though highly profitable, highlighted the critical gap in local production, closer to consumers and less likely affected by international restrictions (Cappelli and Cini, 2020). Meeting local population demand, while remaining sustainable in a shorter food supply chain, may yet have introduced another facet to food security in the post-COVID-19 era. Predicted environmental variation including rising temperatures and increases in carbon dioxide emissions could result in a drier atmosphere and an increase in evapotranspiration (Ficklin and Novick, 2017). In addition to water, most biological processes are temperature sensitive, and climate change is therefore undoubtedly going to affect all crop performance (Dusenge et al., 2019). As a result, resilience to abiotic stresses, such as heat, drought, and salinity, will become traits that are highly desirable in future crop improvement strategies. Reactive nitrogen plays an important role in plant growth, crop yield, and subsequently human nutrition (Dreccer et al., 2000). Alongside their benefits, agricultural practices such as nitrogen fertilizer application and nitrogen fixing crops have the potential to disturb the global nitrogen cycle and adversely affect human health (Townsend et al., 2003; Bodirsky et al., 2012). These practices, if poorly managed, can contribute significantly toward the release of nitrous oxide into atmosphere, which negatively affects the protective ozone layer and advances climate change (Crutzen and Ehhalt, 1977). In an effort to solve the global nitrogen challenge, Houlton et al. (2019) propose, amongst other things, the improvement of nitrogen-use efficiency in crop production. This could be achieved by altering fertilizers and fertilizer application practices, boosting soil health to promote nitrogen uptake, and developing improved crop varieties that efficiently utilize nitrogen (Houlton et al., 2019).

The genus *Brassica* consists of extensively agronomically diverse species. Oilseed canola include *Brassica rapa*, *Brassica napus*, and *Brassica juncea* varieties with internationally defined erucic acid and glucosinolate contents (Sharafi et al., 2015). Vegetable Brassicas include *B. rapa* ssp. *rapa* (turnip), ssp. *oleifera* (turnip rape), ssp. *chinensis* (pak choi/bok choy), and ssp. *pekinensis* (Chinese cabbage); *Brassica oleracea* ssp. *capitata* (cabbages), var. *italica* (broccoli), var. *botrytis* (cauliflower), ssp. *gemmifera* (Brussels sprouts), and ssp. *alboglara* (Chinese kale); and *B. napus* var. *napobrassica* (swede/rutabaga) (Cheng et al., 2016). Mustard *Brassica* include *Brassica nigra* (black mustard), *Brassica carinata* (Ethiopian mustard), and *B. juncea* (Indian mustard). The U triangle summarized the interspecific hybridization events between diploid progenitors *B. rapa* (AA), *B. nigra* (BB), and *B. oleracea* (CC) resulting in polyploidy, *B. juncea* (AABB), *B. napus* (AACC), and *B. carinata* (BBCC) (Nagaharu et al., 1935; Snowdon et al., 2002).

Brassicas are cultivated on a worldwide scale, and it is therefore almost a certainty that forecasted environmental changes would affect the crop (Francisco et al., 2017). Combined with the abiotic stresses, biotic stress is another challenge facing global *Brassica* production. Bebbler et al. (2013) combined observation data and mathematical equation to project pest distribution and proposed that pathogen and pests affecting global crops are moving polewards as the temperature rises. Pathogens of *Brassica* oilseed and vegetable crops, such as *Leptosphaeria maculans* (blackleg or stem canker), *Alternaria brassicae* (Alternaria blight), *Albugo candida* (white rust), *Pseudocercospora capsellae* (white leaf spot), *Plasmodiophora brassicae* (club root), and *Sclerotinia sclerotiorum* (Sclerotinia stem rot), extensively affect yield, seed quality, and crop development (Murray and Brennan, 2012). Breeding *Brassica* varieties that can withstand the pressures of a changing environment is perhaps the best strategy in ensuring sustainability.

Decreasing nutrient content of modern fruit and vegetable cultivars has raised concern in recent studies conducted in the USA and UK, predicting the future need for agricultural bio-fortification (Mayer, 1997; Davis et al., 2005; White and Broadley, 2005; Davis, 2009). Davis coined the term “genetic dilution effect” in 2005 after observing broccoli hybrids (*B. oleracea* var. *italica*) accumulating denser heads without the obvious proportional increase in nutrients (Davis et al., 2005). This is a result of breeding and selection of varieties based on yield and productivity, while overseeing the importance of nutrient content. The World Health Organization considers micronutrient deficiency as a major health challenge especially in developing and poor countries (Khush et al., 2012). Bio-fortification is a cost-effective and sustainable strategy in addressing malnutrition; however, this requires suitable genetic diversity within the genepool to be valuable in breeding (Garg et al., 2018; Kumar et al., 2020).

Available genetic diversity still constitutes the foundation of all breeding efforts. Elite varieties, land races, and crop wild species are important resources of useful variation that can be introgressed, re-introduced, or manipulated to obtain the required biotic and abiotic resilience in *Brassica* crops

(Dwivedi et al., 2017). The identification and exploitation of suitable variation are crucial for crop improvement (Hu et al., 2018) and can be elucidated at the genome scale (Varshney et al., 2018). Genomics can, in addition, contribute toward unraveling the genetic origin and molecular pathways involved in biotic and abiotic stress tolerance traits. Complete and accurate understanding of the ancestry of the *Brassica* species will assist in the tracking and exploitation of genetic inheritance of useful traits (Bancroft et al., 2011).

Crop improvement has always been a co-evolutionary process between humans and edible plants (Harlan, 1992); changes in plants brought about by cultivation allowed changes in human populations to take place. Plant breeding has largely relied on conventional breeding methods based on phenotypic selection. However, it is in doubt whether conventional breeding approaches alone would be adequate in addressing the impending challenges. During the course of the last three decades, genomics has become an integral part of all life sciences. Rapid advances in sequencing tools followed by cost reductions, as well as the development of high-throughput genotyping techniques, have led to advances in trait mapping, functional characterization, and ultimately crop improvement through genomic selection (GS) (Nepolean et al., 2018).

Without available genetic diversity, the introduction of genes may present a suitable solution. Weed and insect control using genetically modified (GM) crops has assisted farmers worldwide in attaining higher yields with fewer resources (Zhang et al., 2016). Cauliflower (Lu et al., 2006), mustard (Hong et al., 2002), and canola (Garg et al., 2018) have been subjected to transgenic bio-fortification efforts for beta-carotene, gamma-linolenic acid, and phytate degradation, respectively. Despite these efforts, so far, only Phytaseed canola has been released by BASF in the USA. Health concerns, consumer skepticism, and long and expensive regulatory processes are restricting the release of transgenic crops (Watanabe et al., 2005). Recent developments in genome editing (GE) provide an option to alter or introduce specific genes in order to obtain the desired trait expression. Oligo-directed mutagenesis (ODM), programmable sequence-specific nucleases (SSNs), and base-editing tools allow the precise creation of insertions/deletions (indels) or even the introduction of a complete sequence at a predetermined target location within the genome (Scheben et al., 2017).

To further address the pressing demands on crop improvement, an accelerated rate of genetic gain is required. The implementation of GS can fast-track the progress in crop breeding (Wang X. et al., 2018). In GS, predicted breeding values based on phenotype and genome-wide marker scores allow the preselection of promising genotypes, thereby substantially quickening the breeding cycle and enhancing genetic gains (Heffner et al., 2009). Further optimization of mating strategies is essential to prevent inbreeding and ensure long-term genetic gain (Allier et al., 2019).

Genomics armed with diversity is currently leading the way in crop improvement. Here, we review aspects of the *Brassica* crop improvement cycle (**Figure 1**) illustrating the importance of genetic diversity, creation of genomic resources, its exploitation in aid of trait discovery, and GE and GS of *Brassica*.

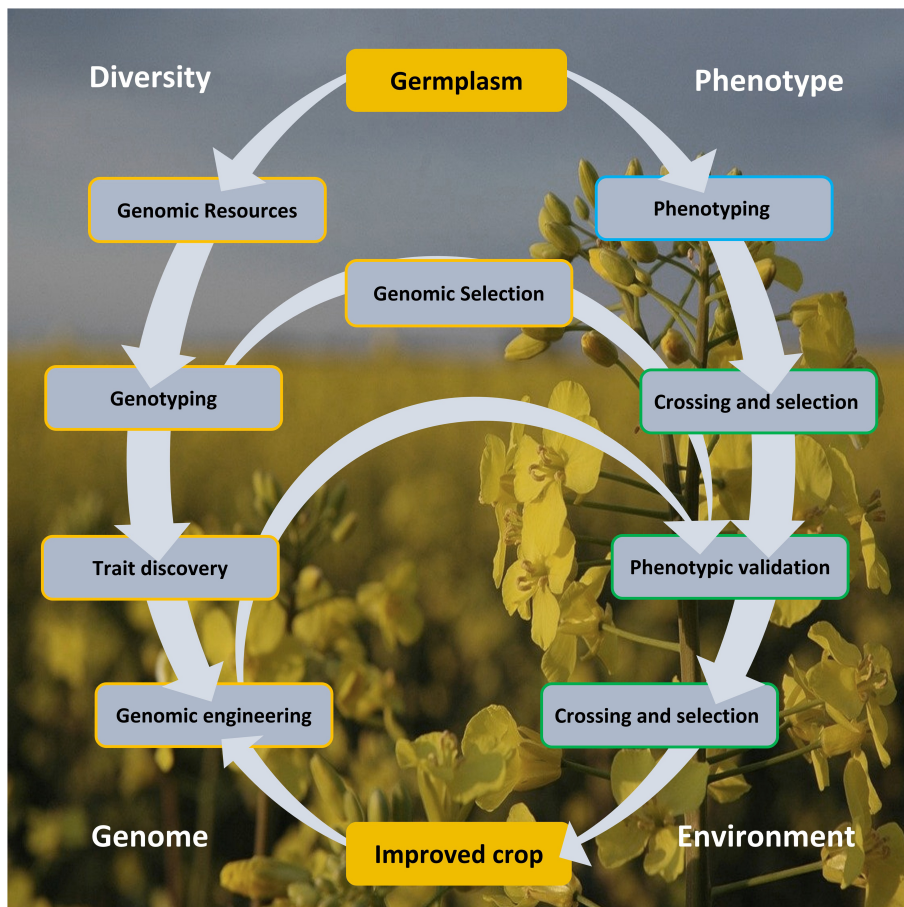
## ESTIMATION AND CONSERVATION OF DIVERSITY IN GERMPLASM

Crop breeding is required to be sustainable and to adapt promptly in the face of abiotic and biotic environmental changes (Zhang and Batley, 2020). Most modern crops were developed through repeated cycles of selection, “filtering out” varieties with desirable agronomic traits from ancestral wild species. However, potentially valuable genetic variation is often lost in the process, resulting in lowered trait heritability and increased genetic homogeneity (Rahman, 2013). Comparative population genomics can be used to identify these selective sweeps, or bottlenecks, and potential loci under selection (Slatkin, 2008). An example is the allotetraploid *Brassica napus* (AACC,  $2n = 38$ ), which originated from spontaneous interspecific hybridization events between *Brassica rapa* (AA,  $2n = 20$ ) and *Brassica oleracea* (CC,  $2n = 18$ ) (Chalhoub et al., 2014). Cultivated *B. napus* have been under intense selection over the past decades, which has led to a severe genetic bottleneck in the species (Becker et al., 1995). This is reflected by the extent of highly conserved regions found between the genomes of *B. napus* accessions found in almost all major gene pools (Werner et al., 2018). Accordingly, the decline of allelic variation and genetic diversity was reported in Canadian (Fu and Gugel, 2010) and Australian spring canola breeding initiatives (Cowling, 2007).

The key to sustainable crop improvement in the face of climate change, and increased pressure from pests and diseases, is maintaining this diversity. Diversity within germplasm provides breeders with valuable material to enable the selection of parental lines, exploitation of heterosis, and the expansion of breeding pools (Yousef et al., 2018). Heritable variation present within the crop germplasm is therefore essential for efficient breeding programs. Characterizing the genetic diversity allows breeders to select alleles at loci of interest and identify trait-associated markers suitable for introgression into new varieties. The assessment of germplasm diversity furthermore assists in the optimization of conservation strategies of germplasm collections (Rao and Hodgkin, 2002). This was highlighted in the study conducted by Yousef et al. (2018), in which cauliflower (*B. oleracea* var. *botrytis*) accessions originating from 26 countries were grouped into two major groups representing the two genebanks from which the accessions were obtained and not the country of origin. In this case, composition and accession type influenced the level of diversity and contributed toward the differentiation between the genebanks. Routine monitoring of genetic diversity in *ex situ* germplasm collections might therefore be essential to prevent potential loss of genetic diversity.

Wild species related to agricultural crops [crop wild relatives or (CWRs)] and landraces offer an attractive alternative source of variation (Dempewolf et al., 2017; Khan et al., 2020). Many of these unique resources are currently available from genebanks and seed repositories (Tanksley and McCouch, 1997). Introgression from CWRs or landraces can broaden the genetic base for modern breeding programs and can contribute to sought-after characteristics associated with relative tolerance to extreme environments and disease resistance. Despite the vast genetic potential locked up in these resources, their utilization





**FIGURE 1 |** The crop improvement cycle involves the exploitation of genomic diversity and phenotypic variability. These approaches offered a rapid and cost-effective lab-to-field and back loop, centered on arming the genomic arsenal by capitalizing on the variation in the germplasm resource, as defined in the genotype and from trait association. Genomic selection and engineering accelerated crop improvement, circumventing a more laborious and time-consuming conventional crossing and selection approach. Genomic engineering strategies also offer a more precise manipulation for improved crop development. The improved crop must be climate resilient and adaptive to the extreme environmental changes.

is hampered by inconsistent documentation, unintentional duplications, and a lack of available genetic information (Singh N. et al., 2019; Zhang and Batley, 2020). Combining available phenotypic and geographical descriptors with genomic sequence information, perhaps in the form of a universal molecular passport, could facilitate the selection of useful genetic variation and its use in breeding programs (Mascher et al., 2019; Singh N. et al., 2019).

Plant genetic resources, including CWRs in the wild, are considered under threat and in need of conservation (Dempewolf et al., 2017). Climate change, along with habitat fragmentation due to human activity, is predicted to result in drastic plant population declines and even result in extinction (Jump and Peñuelas, 2005). The creation of protected areas, efforts to reduce pollution and legal frameworks to protect endangered species, such as the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), may aid in the prevention of the decline or loss of biodiversity. Genetic approaches over time have contributed toward the

estimation of biodiversity and prioritization of conservation efforts. However, genome-scale data and associated high-density markers can improve estimations of genetic diversity and population structure. Transitioning from a genetics to conservation genomics approach is expected to have a positive impact on future conservation recommendations and policies (Supple and Shapiro, 2018).

## CREATION OF GENOMIC RESOURCES IN BRASSICA SPECIES

The smaller Brassicaceae genome from *Arabidopsis thaliana* was the first to be sequenced and benefited progress in *Brassica* sequencing given the high degree of genomic conservation between *A. thaliana* and *Brassica* species. In addition, DNA sequencing technology becomes more affordable with longer reads and higher throughput available at a fraction of the cost (Marri et al., 2018). These developments provided the

opportunity to improve *de novo* genome assembly, increase mapping certainty, and identify structural variants (SVs) (Amarasinghe et al., 2020). Furthermore, re-sequencing efforts have increased over time to resolve challenges and gaps in current studies, especially in complex and agronomically significant crops like *Brassica napus*.

As technology progresses, more advanced genomic tools become available, expanding the range of analysis and exploitation possibilities of germplasm resources. The PlabiPD database (<https://www.plabipd.de/>) maintains an updated list of sequenced plant species, including Brassicas, which can be visualized phylogenetically or temporally. Available genomic resources have played an important role in the advancement of breeding programs throughout the world in cereals, legumes, oilseeds, and even ornamental crops. Hence, here we examine the development and availability of nuclear and organellar genome resources generated in Brassicas.

## Nuclear Genome

Draft genomes and pangenome assemblies have been created for five domesticated and a wild *Brassica* species (Table 1). More broadly, 27 members of the Brassicaceae family have been sequenced, including three *Arabidopsis* (The Arabidopsis Genome Initiative, 2000; Hu T. T. et al., 2011; Akama et al., 2014; Briskine et al., 2017; Michael et al., 2018), three *Capsella* (Slotte et al., 2013; Kasianov et al., 2017), three *Eutrema* (Yang et al., 2013; Guo et al., 2018), and two *Raphanus* species (Kitashiba et al., 2014; Moghe et al., 2014; Shirasawa et al., 2020). The earlier *Brassica* genome assemblies focused on elite cultivars and combined Illumina paired-end reads with bacterial artificial chromosome (BAC)-end sequences to construct scaffolds and build high-quality assemblies. Markers from genetic maps were used, in addition, to merge assemblies and anchor scaffolds to pseudo-chromosomes. The genomes were either assembled *de novo* or based on a reference genome of the closest relative. The assembly of *B. napus* followed an earlier approach for allopolyploids by sequencing the diploid progenitor genome, similar to the methods used in the assembly of the strawberry and cotton genomes (Tennessen et al., 2014; Ming and Man Wai, 2015; Zhang et al., 2015).

Brozynska et al. (2016) explored the progress of CWR sequencing efforts and noted fewer efforts compared with domesticated relatives, a trend also observed in *Brassica*. Higher levels of heterozygosity in CWRs, which can result in greater assembly difficulties, might be contributing toward the trend (Brozynska et al., 2016). Recently, the genomes of two wild diploid perennial *Brassica* C-genome species were sequenced (Golicz et al., 2016; Kioukis et al., 2020). *Brassica macrocarpa* and *Brassica cretica*, both native to Greece, are potential wild progenitors of *Brassica oleracea*, which was thereby used as reference in the assembly of the two CWRs (Branca et al., 2012).

Next-generation sequencing (NGS) technologies, although extensively and successfully used in genome assembly, are limited by their relatively short read lengths. Shortcomings include misassembly and gaps in long repeat regions, difficulties in detecting larger SVs, transcript isoforms, and haplotype phasing (Van Dijk et al., 2018). Long-read third-generation

sequencing (TGS) technologies from Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT) allow less bias and more homologous coverage of the genome, thereby overcoming the challenges such as polyploidy and frequent repetitive elements. TGS and NGS are often combined with long-range mapping technologies (BioNano Genomics) and chromosome conformation capture (Hi-C) (Van Berkum et al., 2010), to enable the assembly of highly contiguous chromosome-level crop genome assemblies (Hu et al., 2018; Schreiber et al., 2018). Song et al. (2020) created eight high-quality *B. napus* reference genomes by integrating different combinations of Illumina, PacBio, Hi-C, and BioNano data. These high-quality *B. napus* reference genomes allowed the identification of SVs, including copy number variants (CNVs) and presence and absence variations (PAVs), and improve our understanding of the genome structure and genetic basis behind phenotype differentiation in *B. napus*.

Genomic comparison between two *B. napus* reference genomes by Bayer et al. (2017) highlighted the limitations of a single reference assembly, given the wealth of variation between individuals, concluding that the genetic diversity at a species level cannot be sufficiently captured by a single reference genome (Hurgobin and Edwards, 2017). The pangenome was “born” in an effort to capture the totality of diversity in a species or something broader (Vernikos, 2020). *B. oleracea* (Golicz et al., 2016) and *B. napus* (Hurgobin et al., 2018) were the first *Brassica* pangenomes to be published and investigated the diversity within the species. The second *B. napus* pangenome (Song et al., 2020) combined TGS technologies, including PacBio single-molecule real-time (SMRT) and ultralong nanopore sequencing. These pangenome assemblies demonstrated the prospect of uncovering and mining diversity within secondary crop gene pools for crop improvement (Voss-Fels and Snowden, 2016).

Affordability of sequencing is promoting a combination of *de novo* assembly and whole-genome re-sequencing (WGRS) efforts of wider gene pools, including close relatives and CWRs in an effort to identify and explore useful genetic variation (Brozynska et al., 2016). While the pangenome represents the genomic makeup of a species (Tettelin et al., 2005), the super-pangenome includes the CWR genetic variability that has been lost due to domestication and breeding selection bottlenecks (Khan et al., 2020). This was well-demonstrated by the discovery of an abundance of unique genes upon the inclusion of the wild *Brassica macrocarpa*, in comparison with the other eight domesticated varieties, in the *B. oleracea* pangenome (Golicz et al., 2016). These genes form part of the pangenome’s dispensable genome, and the findings yet again emphasize the value of the genetic variability captured within the wild *Brassica* spp. It is predicted that efforts to catalog and include more lines into pangenomes and super-pangenomes would probably never cease, thereby providing a constant contribution of valuable resources for crop improvement efforts in an ever-changing environment. Future exploration in genomic resources in *Brassica* will likely involve additional WGRS efforts and further pangenome studies to explore breeding histories and identify loci associated with important agronomic traits such as oil content and composition, seed quality, and disease resistance (Wang Y.

**TABLE 1** | List of domesticated and wild *Brassica* species nuclear genome assemblies.

Species	Current status	Assembly size (bp)	Number of protein-coding genes	Genomic tools	References	
<i>Brassica rapa</i> (2n = 10)	Chinese cabbage ssp. <i>pekinensis</i> cv. Chiifu 401–42, <i>de novo</i> assembly (version 1.5)	283.80 Mb	41,174	Illumina GAII short, medium, long insert libraries, BAC-end Sanger sequences, EST data, and genetic maps	Wang et al., 2011	
	Japanese turnip ssp. <i>rapa</i> cv. DH-VT117, reads mapped to <i>B. rapa</i> Wang et al. (2011) reference genome	282.93 Mb	40,708	Illumina HiSeq2000 short, medium, long insert libraries	Lin et al., 2014	
	Rapid cycling oil-like inbred line cv. RC-144, reads mapped to <i>B. rapa</i> Wang et al. (2011) reference genome	282.69 Mb	40,506			
	Chinese cabbage ssp. <i>pekinensis</i> cv. Chiifu 401–42, <i>de novo</i> assembly (version 2.0/2.5)	389.20 Mb	48,826	Illumina and PacBio sequencing, and genetic maps	Cai et al., 2017	
	Chinese cabbage ssp. <i>pekinensis</i> cv. Chiifu 401–42, <i>de novo</i> assembly (version 3.0)	353.14 Mb	45,985	Illumina HiSeq4000 short reads, PacBio SMRT long reads, BioNano optical mapping, Hi-C sequencing, and genetic map	Zhang L. et al., 2018	
	Yellow sarson ssp. <i>trilocularis</i> cv. Z1, <i>de novo</i> assembly	401.92 Mb	46,721	Illumina HiSeq2500 short, medium, long insert libraries, Oxford Nanopore long reads, BioNano optical mapping, and genetic map	Belser et al., 2018	
<i>Brassica oleracea</i> (2n = 18)	Chinese cabbage ssp. <i>chinensis</i> cv. NHCC001 (Suzhouqing), <i>de novo</i> assembly	405.33 Mb	48,158	Illumina short reads, PacBio Sequel SMRT long reads, and Hi-C sequencing	Li et al., 2020	
	Chinese kale ssp. <i>capitata</i> cv. TO1000DH3, <i>de novo</i> assembly	488.60 Mb	59,225	Illumina HiSeq2000, Roche 454 GS-FLX Titanium sequencing, and BAC-end sequences, and genetic map	Parkin et al., 2014	
	Chinese kale ssp. <i>capitata</i> cv. 02-12, <i>de novo</i> assembly	539.90 Mb	45,758	Illumina GAII shotgun sequencing combined with Roche 454 GS-FLX Titanium sequencing, BAC-end sequences, and genetic map	Liu et al., 2014	
	Pangenome of nine varieties including cabbages, kale, Brussels sprout, Kohlrabi, cauliflowers, broccoli, and wild <i>Brassica macrocarpa</i> , with draft genome by Parkin et al. (2014) as reference	587.00 Mb	61,379	Illumina HiSeq2000 and HiSeq2500 short reads	Golicz et al., 2016	
	Broccoli ssp. <i>botrytis italica</i> cv. HDEM, <i>de novo</i> assembly	554.97 Mb	61,279	Illumina HiSeq2500 short, medium, long insert libraries, Oxford Nanopore long reads, BioNano optical mapping, and genetic map	Belser et al., 2018	
	Cauliflower var. <i>botrytis</i> cv. C-8	584.60 Mb	47,772	Illumina HiSeq and X Ten short reads, and PacBio Sequel SMRT long reads	Sun et al., 2019	
	Cabbage var. <i>capitata</i> cv. D134 DH (commercially known as Zhonggan 18)	529.92 Mb	44,701	Illumina HiSeq2500 short reads, PacBio Sequel SMRT long reads sequencing, Illumina HiSeq X Ten and GemCode 10x Genomics short reads, and Hi-C sequencing	Lv et al., 2020	
	<i>Brassica napus</i> (2n = 38)	European winter-type cv. Darmor (version 4.1)	712.30 Mb	101,040	Roche 454-GS-FLX+ Titanium long reads, BAC-end sequences, and Illumina HiSeq short reads	Chalhoub et al., 2014
		European winter-type cv. Darmor (version 8.1)	616.70 Mb	80,382	Illumina HiSeq short, medium, and long insert libraries, and genetic map	Bayer et al., 2017
European spring-type cv. Tapidor, <i>de novo</i> assembly		625.90 Mb	70,162			
Asian semi-winter cv. ZS11, <i>de novo</i> assembly		854.98 Mb	90,731	Illumina HiSeq2500 short, medium, long insert libraries, BAC sequencing, and genetic maps	Sun et al., 2017	
Pangenome of 20 synthetic and 33 non-synthetic accessions based on Darmor v8.1 assembly		1,044 Mb	94,013	Illumina sequencing	Hurgobin et al., 2018	
Asian semi-winter cv. Ningyou 7, <i>de novo</i> assembly		994.00 Mb	104,179	Illumina, PacBio RS II, and Hi-C sequencing, and genetic map	Zou et al., 2019	
	Pangenome of eight varieties (Westar, No2127, Zheyu7, Gangan, Shengli, Tapidor, Quinta, ZS11) and <i>de novo</i> assemblies of each genome	1,001–1,033 Mb	94,586–100,919	Illumina short reads, PacBio SMRT long reads, Hi-C sequencing, and BioNano optical mapping	Song et al., 2020	
	European winter-type cv. Express 617	925.00 Mb	89,857	Illumina short reads, PacBio SMRT and Oxford Nanopore long reads, BioNano optical mapping, and genetic map	Lee et al., 2020	

(Continued)

TABLE 1 | Continued

Species	Current status	Assembly size (bp)	Number of protein-coding genes	Genomic tools	References
	European winter-type cv. Darmor (version 10)	924.00 Mb	108,190	Illumina HiSeq2500 short reads, PromethION Oxford Nanopore long reads, optical mapping, and genetic map	Rousseau-Gueutin et al., 2020
	Asian semi-winter cv. ZS11	1,160 Mb	106,059	Illumina short reads, PacBio SMRT long reads, Hi-C sequencing, and genetic map	Chen et al., 2020
<i>Brassica juncea</i> (2n = 36)	var. <i>tumida</i> cv. T84-66 inbred line, <i>de novo</i> assembly	949.60 Mb	80,050	Illumina HiSeq short reads, PacBio SMRT long reads, BioNano optical mapping, and genetic map	Yang et al., 2016a
	cv. Varuna	922.00 Mb	101,959	Illumina HiSeq1000 short reads, PacBio RS II SMRT long reads, BioNano optical mapping, and genetic map	Paritosh et al., 2020
<i>Brassica nigra</i> (2n = 16)	cv. YZ12151 DH, <i>de novo</i> assembly	396.90 Mb	49,826	Illumina HiSeq short reads, BAC-end sequences, and genetic map	Yang et al., 2016a
	cv. Sangam (line BnSDH-1)	515.40 Mb	46,227	Illumina HiSeq1000 and MiSeq short reads, Oxford Nanopore long-read sequencing and optical mapping, and genetic map	Paritosh et al., 2020
	cv. Ni100 (short and long reads)	447.00 Mb (SR), 506.00 Mb (LR)	56,331 (SR), 59,877 (LR)	Illumina and Roche 454 short reads, GridION Oxford Nanopore long reads, and genetic map	Perumal et al., 2020
	cv. CN115125 (C2) (long reads)	537.00 Mb	67,030	Illumina HiSeq2500 short reads, MinION Oxford Nanopore long reads, Hi-C sequencing, and genetic map	
<i>Brassica cretica</i> (2n = 18)	Four individuals from isolated Grecian population of subsp. <i>cretica</i> and subsp. <i>nivea</i> assembled based on related <i>B. oleracea</i> Liu et al. (2014) reference	40.02–412.52 Mb	30,360	Illumina HiSeq2500 short reads	Kioukis et al., 2020

et al., 2016; Lu et al., 2019; Dolatabadian et al., 2020; Gabur et al., 2020; Yan et al., 2020; Zhang et al., 2020).

## Organelle Genomes

In *Brassica*, the mitochondrial (mt) genome assembly predates that of the whole genome due to its significantly smaller size (~20 kbp) (Palmer and Herbon, 1988; Handa, 2003; Kode et al., 2005) and high copy number per cell (Lima et al., 2016) (Table 2). Due to its small size in comparison with other higher plants, the *Brassica* mt genome was used as an early model in the understanding the plant mt, structure, function, and content (Grewe et al., 2014). Comparative analysis of the mt genome can be used to study interspecific phylogenetic relationships (Darracq et al., 2011) and uncover the plant's evolutionary history (Xue et al., 2020). For example, the mt genomes of *Brassica rapa* subspecies with distinct morphologies were found to be highly conserved (Hatono et al., 2017). Furthermore, the rate of mutation for *Brassica* mt DNA was four times slower than that of the chloroplast (Cp) DNA (Palmer and Herbon, 1988). Xue et al. (2020) compared mt DNA of the six members of the U triangle (Nagaharu et al., 1935) and revealed that *B. oleracea* was undergoing the most mt genomic change, while the B-genomes containing *Brassica carinata* and *Brassica nigra* were identified as maternally more distantly related to the remaining *Brassica* accessions of the U triangle (Xue et al., 2020). It was

also suggested that *Sinapis arvensis* could have been misclassified based on both phylogenetic and mt genomic organization, placing it within the *Brassica* species and sister to the *B. nigra*–*B. carinata* lineage (Sang et al., 2020; Xue et al., 2020). Sang et al. (2020) also confirmed that the genome structure and evolutionary analysis of the *S. arvensis* organellar genomes were more similar to those of *B. nigra* and *B. carinata*. In addition, mt genes, encoding putative proteins with transmembrane domains, were discovered, which may explain the alloplasmic male sterility of novel cytoplasmic male sterility (CMS) derived from somatic cell hybridization between *B. napus* and *S. arvensis*.

Cp genomes of most land plants vary between 120 and 160 kbp in size (Wicke et al., 2011). Xiao-Ming et al. (2017) further established that cp gene lengths were proportionally to cp genome size, based on the analysis of 272 species including *B. napus* and 14 other members of the Brassicaceae family. Phylogenetic analysis of the cp genomes of *B. nigra* and *B. oleracea* with those of 10 reported species in the order Brassicales suggested that *B. oleracea* is closely related to *B. rapa* and *B. napus* while *B. nigra* was more diverse than the neighbor species *Raphanus sativus* (Seol et al., 2017). Li et al. (2017) completed the *de novo* assembly of cp genomes of 60 *Brassica* genotypes of the six U triangle species. Subsequent phylogenetic analyses divided the *Brassica* genus into four clades: *B. carinata* and *Brassica juncea*, in accordance with the U-triangle model, shared



**TABLE 2** | List of domesticated *Brassica* species organelle genome sequences.

Genome type/species	Data accession	Size (bp)	Number of protein-coding gene	Genomic tools	References
<b>Mitochondrial genome</b>					
<i>Brassica rapa</i> (Chinese cabbage, turnip)	N/A	~218 kb	Mapped 12 genes (61 kb of genome was highly transcribed)	Physical map using <i>Pst</i> I, <i>Sal</i> I, <i>Bgl</i> I, <i>Kpn</i> I, and <i>Nru</i> I restriction endonucleases and cloning	Palmer and Shields, 1984; Makaroff and Palmer, 1987
<i>B. rapa</i> cv. Purple Top White Globe (turnip)	N/A	~218 kb	N/A	Physical map using <i>Sal</i> I and <i>Pst</i> I restriction endonucleases and cloning, and comparative mapping with 15 other enzymes	Palmer and Herbon, 1988
<i>B. rapa</i> cv. Suzhouqing	JF920285	219,747	34 (3 rRNA, 18 tRNA)	Roche 454 GS-FLX DNA pyrosequencing, PCR, and Sanger sequencing	Chang et al., 2011
<i>B. rapa</i> var. Oushou hakusai (Chinese cabbage)	AP017996	219,775	34 (3 rRNA, 18 tRNA)	Roche 454 GS-FLX DNA pyrosequencing, PCR, and Sanger sequencing	Hatono et al., 2017
<i>B. rapa</i> var. Chusei shiroguki sensuji kyomizuna (mizuna)	AP017997			Illumina MiSeq, PCR, and Sanger sequencing	
<i>Brassica napus</i> cv. American Purple Top (rutabaga)	N/A	~221 kb	N/A	Physical map using <i>Sal</i> I and <i>Pst</i> I restriction endonucleases and cloning, and comparative mapping with 15 other enzymes	Palmer and Herbon, 1988
<i>B. napus</i> cv. Westar	AP006444	221,853	34 (3 rRNA, 17 tRNA)	Long range PCR and sequencing	Handa, 2003
<i>B. napus</i> CMS plant (Polima)	FR715249	223,412	34 (3 rRNA, 18 tRNA)	Cycle sequencing, PCR, and Sanger sequencing	Chen et al., 2011
<i>Brassica oleracea</i> (cauliflower)	N/A	~217 kb	N/A	Physical map using <i>Sal</i> I, <i>Kpn</i> I, and <i>Bgl</i> I restriction endonucleases and cloning	Chétrit et al., 1984
<i>B. oleracea</i> cv. Brunswick (cabbage)	N/A	~219 kb	N/A	Physical map using <i>Sal</i> I and <i>Pst</i> I restriction endonucleases and cloning, and comparative mapping with 15 other enzymes	Palmer and Herbon, 1988
<i>B. oleracea</i> cv. 08C717	JF920286	360,271	56 (4 rRNA, 35 tRNA)	Roche 454 GS-FLX DNA pyrosequencing, PCR, and Sanger sequencing	Chang et al., 2011
<i>B. oleracea</i> var. botrytis (cauliflower)	N/A	219,962	33 (3 rRNA, 18 tRNA)	Illumina GAIIx, PCR, and Sanger sequencing	Grewe et al., 2014
<i>B. oleracea</i> var. Fujiwase (cabbage)	AP012988	219,952	34 (3 rRNA, 17tRNA)	Roche 454 GS-FLX DNA pyrosequencing, PCR, and Sanger sequencing	Tanaka et al., 2014
<i>B. oleracea</i> var. capitata cv. 4119 (cabbage)	KU831325	219,975	34 (3 rRNA, 19 tRNA)	Illumina HiSeq2000, PCR, and Sanger sequencing	Yang K. et al., 2018
<i>Brassica nigra</i> (black mustard)	N/A	~231 kb	N/A	Physical mapping using <i>Bst</i> EII- <i>Bgl</i> I and <i>Pst</i> I restriction endonucleases and cloning	Palmer and Herbon, 1986
<i>B. nigra</i> cv. Ni-138	AP012989	232,145	33 (3 rRNA, 17 tRNA)	Roche 454 GS-FLX DNA pyrosequencing, PCR, and Sanger sequencing	Yamagishi et al., 2014
<i>B. nigra</i> (black mustard)	KP030753	232,407	33 (3 rRNA, 19 tRNA)	Illumina HiSeq2500, PCR, and Sanger sequencing	Yang et al., 2016b
<i>Brassica juncea</i> cv. Jiangpu-yejiecai	JF920288	219,766	34 (3 rRNA, 18 tRNA)	Roche 454 GS-FLX DNA pyrosequencing, PCR, and Sanger sequencing	Chang et al., 2011
<i>Brassica carinata</i> cv. W29	JF920287	232,241	33 (3 rRNA, 17 tRNA)		
<b>Chloroplast genome</b>					
<i>B. rapa</i> cv. Chiifu-402-41, Z16 and FT	N/A	153,482	89 (4 rRNA, 37 tRNA)	Illumina Solexa sequencing, PCR, and Sanger sequencing	Wu et al., 2012
<i>B. rapa</i> cv. C1.3 (field mustard)	MG717286	153,483	N/A	Illumina HiSeq2500 sequencing	Ferreira de Carvalho et al., 2019
<i>B. rapa</i> cv. Z1 (field mustard)	MG717289	153,502			
<i>B. oleracea</i> cv. C1176	KR233156	153,366	80 (4 rRNA, 30 tRNA)	Illumina HiSeq short-read sequencing	Seol et al., 2017
<i>B. oleracea</i> cv. HDEM (wild cabbage)	MG717287	153,364	N/A	Illumina HiSeq2500 sequencing	Ferreira de Carvalho et al., 2019
<i>B. oleracea</i> cv. RC34 (wild cabbage)	MG717288	153,371			
<i>B. nigra</i> cv. IT119326	KT878383	153,633	80 (4 rRNA, 30 tRNA)	Illumina HiSeq short-read sequencing	Seol et al., 2017
<i>B. napus</i> cv. zy036	GQ861354	152,860	74 (4 rRNA, 30 tRNA)	Illumina Solexa sequencing, PCR and cycle sequencing	Hu Z.-Y. et al., 2011
<i>B. juncea</i>	KT581449	153,483	79 (4 rRNA, 30 tRNA)	Illumina HiSeq2500 sequencing, PCR, and Sanger sequencing	Prabhudas et al., 2016



their cp genome with hybridization donors *B. nigra* and *B. rapa*, respectively. Two types of cp genomes were discovered in *B. rapa*, while the presence of both *B. rapa* cp genomes in *B. napus* strongly suggests two independent hybridization events. These findings were consistent with mt genome findings (Palmer and Herbon, 1988).

Besides photosynthesis, a number of essential metabolic reactions are catalyzed in the cp. These include the biosynthesis of partial amino acids, lipids and fatty acids, vitamins, and isoprenoids, as well as the reduction of nitrites and sulfates (Chen et al., 2018). The cp is furthermore involved in the synthesis of defense-related hormones and signaling molecules metabolites associated with disease response and environment changes including heat and light (Lu and Yao, 2018). Genes present in the cp genomes could potentially be explored in *Brassica* to improve yield and resistance to biotic and abiotic stresses. It is clear that the composition and structure of the organellar genome not only hold potential in the elucidation of organellar genome evolution and phylogeny but also understanding thereof may be useful in the identification of breeding compatible germplasm resources and CMS and provide opportunities for the introducing of new agronomic and horticultural traits into *Brassica* crops (Daniell et al., 2016). Organellar genome sequences are therefore valuable assets in the future crop improvement efforts.

## FUNCTIONAL TRAIT DISCOVERY AND CHARACTERIZATION

The characterization and subsequent exploration of discovered genetic diversity can uncover useful genes linked to adaptation and resistance to abiotic and biotic constraints (Khan et al., 2020). Available *Brassica* reference genomes have provided the foundation to facilitate the fine-scale mapping and elucidation of functionally significant variations in *Brassica* accessions. In this section, we review genome-wide approaches utilized in the identification of alleles associated with desirable traits.

### Genome-Wide Single-Nucleotide Polymorphism Discovery Through Whole Genome Re-sequencing

Available reference genomes provide a valuable tool in the study and detection of genetic variation, which can be reliably integrated and reproduced between studies (Malmberg et al., 2018). Alignment and comparison of WGRS data to the reference genome allow the simultaneous detection of large numbers of unbiased genomic single-nucleotide polymorphisms (SNPs), indels, and SVs. WGRS therefore permits a more in-depth interrogation of the genome than complexity reduction methods, resulting in a significant increase in the number of SNP markers detected.

Annotated, high-quality SNPs and SVs set the stage for high-resolution genome-wide association studies (GWASs). Quantitative trait locus (QTL) discovery can lead to the identification of genetic loci and subsequently provide the basis for the functional validation of candidate genes controlling important traits (Lu et al., 2019). Subsequently, by identifying

these traits of interest, marker-assisted selection (MAS) could be introduced to advance breeding efforts for easily inherited traits (Harper et al., 2012). For example, in gene pyramiding, multiple genes or major QTLs are introgressed into a single genetic background (Pérez-de-Castro et al., 2012). This approach was recently implemented to introgress multiple genes conveying *Sclerotinia* resistance from wild *Brassica oleracea* into canola (Mei et al., 2020). Furthermore, as long-read sequencing technology advances and improves in accuracy, both PAVs and CNVs are likely to drive the latest source of rich adaptive variation including in crops subjected to biotic and abiotic stresses (Gabur et al., 2018).

In *Brassica* species, WGRS has facilitated the identification of intraspecific and interspecific genetic polymorphisms. Comparative analysis between different *Brassica rapa* morphotypes: Japanese turnip, rapid cycling, and Chinese cabbage cv. Chiifu, revealed, respectively 1,090, 1,118, and 1,464 unique genes in each of the genomes (Lin et al., 2014). Orthologous gene comparison suggested earlier divergence between the three varieties before a more recent domestication event. Gazave et al. (2016) used WGRS to survey the genetic diversity present in a worldwide collection of 782 accessions of *Brassica napus*. A total of 30,881 high-confidence SNP markers were identified, which upon analysis revealed distinct evolutionary histories for the A and C subgenomes. Wu et al. (2019) re-sequenced 991 spring, winter, and semi-winter *B. napus* germplasm accessions, originating from 39 countries. By mapping reads to the “Darmor-bzh” and “Tapidor” reference genomes, a total of 5.56 and 5.53 million SNPs, in addition to 1.86 and 1.92 million indels, were respectively identified. Comparison of SNPs using GWAS revealed a global pattern of genetic polymorphisms as well as paths of allelic drift within the main populations of *B. napus*. Selective sweeps disclosed the genetic basis of divergence between the ecotypes, while SNPs discovered in the promoter regions of FLOWERING LOCUS T and FLOWERING LOCUS C orthologs also corresponded with ecotype groups. Malmberg et al. (2018) furthermore utilized WGRS to develop genomic resources consisting of 4,029,750 high-confidence annotated SNPs with predicted effects, as well as SVs in the form of 10,976 deletions and 2,556 insertions. These valuable genomic resources have the potential to bring together global breeding efforts in the development of locally adapted *B. napus* varieties.

### Single-Nucleotide Polymorphism Arrays

Available sequencing data for *Brassica* crops allowed researchers to develop and use high-throughput molecular markers, such as SNPs, more efficiently (Clarke et al., 2013). These markers form an integral part of genomic diversity and, due to their abundance across the plant genome, have become an invaluable tool in crop improvement programs (Scheben et al., 2019). SNP screening can be carried out by WGRS, genotyping-by-sequencing (GBS), or alternatively using SNP arrays. High-density SNP arrays provide an alternative and reproducible genotyping platform, which has been widely used in the characterization of germplasm, GWAS, and QTL studies including the analysis of structural variation (You et al., 2018; Scheben et al., 2019).

A community-driven Brassica 60K (AC genomes) Illumina Infinium™ array (Clarke et al., 2016) was developed and more recently expanded to include the B-genome in the Brassica 90K Illumina Infinium™ array (Scheben et al., 2019). The usefulness of Brassica SNP arrays was demonstrated in the genotyping of resistance genes on chromosome A7 in *B. napus* (Dalton-Morgan et al., 2014), prediction of candidate genes for clubroot disease resistance in *B. napus* (Li et al., 2016), and the assessment of *de novo* homologous recombination events in *B. napus* (Higgins et al., 2018). In addition, several closely linked candidate genes were identified using the 60K Brassica SNP array in the development of functional haplotype markers for the improvement of the oleic acid content in rapeseed (Yao et al., 2020). Several yield associated traits have also been identified using the array platform including branching number (He et al., 2017), ovule numbers (Khan et al., 2019), seed quality (Gajardo et al., 2015), and stem strength (Li H. et al., 2018).

The creation of artificial Brassica allohexaploid could potentially result in the development of new oilseed and vegetable crops types with greater inter-subgenomic heterosis. These synthetic tri-genomic hexaploid Brassica species are potentially more vigorous and adaptable to a wider range of environmental conditions (Yan et al., 2009; Pradhan et al., 2010; Tian et al., 2010; Geng et al., 2013; Malek et al., 2013; Li et al., 2015; Gupta et al., 2016; Zhou et al., 2016; Mwathi et al., 2020). SNP genotyping was carried out by Gaebelein et al. (2019) using the 90K Brassica SNP array and GWAS to determine the relative impact of genome rearrangement events and inherited allelic variants on meiotic stability. A strong correlation between fertility and meiotic behavior in populations of Brassica allohexaploids segregating for alleles from parent allotetraploid species *B. napus*, *Brassica juncea*, and *Brassica carinata* was found. Potential genes of interest were subsequently identified for further investigation into meiotic regulation and future establishment of stable A, B, and C allohexaploids (Gaebelein et al., 2019).

Although SNP arrays can provide vital data to breeders and researchers, public accessibility to genotypes identified can be limited due to the lack of public repositories or databases designed to host crop SNP array data. To address this constraint, Scheben et al. (2019) established the CropSNP database (<http://snpdb.appliedbioinformatics.com.au>) for SNP array data generated on the Illumina Infinium™ Brassica 60 and 90K array platforms.

## Transcriptomics

One of the key challenges in genomics-based breeding remains the complex linking of genotype to phenotype across tissue types, developmental stages, and environmental conditions. Transcriptomics, although part of associated “omics,” have emerged as an exceptional tool in the functional inference of genetic variability (Wang et al., 2019) with technological innovation constantly advancing the field (Lowe et al., 2017; Wang et al., 2019).

RNA-Seq technology has been extensively used in the mapping of exon/intron boundaries, improvement of genome annotations, and the detection of rare transcripts and splicing variants (Pérez-de-Castro et al., 2012). For

example, the transcriptional regulation of anthocyanin biosynthesis in *B. juncea* was studied to identify differentially expressed genes between the purple and green leaves from a backcrossed BC3 segregation population. Genes associated with phenylpropanoid biosynthesis, phenylalanine metabolism, and flavonoid biosynthesis were differentially expressed, while genes involved with anthocyanin biosynthesis (*BjTT8* and *BjMYC2*) were up-regulated in the purple leaves. Understanding anthocyanin biosynthesis and its regulatory network in Brassica is a prerequisite in the development of health-promoting anthocyanin-rich vegetables (Heng et al., 2020).

The complex defense response between a *Sclerotinia sclerotiorum* resistant and susceptible line of *B. napus* was analyzed in a study by Wu et al. (2016). Dynamic transcriptome analyses uncovered differences between susceptibility and resistance associated with the magnitude of expression changes in genes involved in pathogen recognition, MAPK signaling cascade, WRKY transcription regulation, jasmonic acid/ethylene signaling pathways, and biosynthesis of defense-related protein and indolic glucosinolate. Valuable insights gained will assist in the development of effective strategies in *Sclerotinia*-resistance breeding (Lu et al., 2014; Wu et al., 2016).

Limitations such as misassembly, associated with short read RNA-Seq, hampers the full-length assembly of transcripts from highly repetitive regions or analogous gene families. These difficulties are often more pronounced in polyploid plants. TGS technologies such as SMRT by PacBio and Oxford Nanopore single molecule structure sequencing (SMS-seq) provide an opportunity to construct full-length transcripts, with the possibility to capture structural variations, tertiary interactions, and the dynamics of riboswitch ligand binding (Bizuyehu et al., 2020). Full-length transcriptome sequencing (PacBio) was used by Tan et al. (2019) to explore the transcript and splice isoforms expressed during anther development in Chinese cabbage (*B. rapa* ssp. *pekinensis*). In addition to predicted fusion transcripts and poly-A sites, 53 key genes active during anther development were detected, of which eight annotated loci had alternatively spliced isoforms. The transcripts generated provided a valuable resource for the characterization of anther-specific gene expression and improved Chinese cabbage genome annotation (Tan et al., 2019).

An et al. (2019) conducted a comprehensive study comparing the genetic diversity of 183 *B. napus*, 112 *B. rapa*, and 42 *B. oleracea* accessions, along with 20 wild relatives (*Brassica hilarionis*, *Brassica villosa*, *Brassica montana*, *Brassica macrocarpa*, *Brassica rupestris*, *Brassica incana*, and *Brassica insularis*) and five other Brassicaceae species as outgroups in order to improve the understanding in the origin and diversification of *B. napus*. RNA-Seq reads generated from *B. rapa* accessions and *B. oleracea* and other wild C genome species were respectively mapped to the A and C genomes of the *B. napus* Darmor-bzh reference genome to identify SNPs. Six genetic clusters of *B. napus* were identified, which were shown to have undergone different selective pressures in accordance with known breeding histories. Although the multi-origin of *B. napus* remained elusive, the study contributed toward the identification of putative candidate genes to important agronomic traits, which,

along with high-quality SNPs identified, have the potential to facilitate rapeseed improvement and germplasm preservation.

In line with the pangenomics approach, He et al. (2015) assembled the first pan-transcriptome resources for the *Brassica* A and C genomes. The pan transcriptome was established using existing coding DNA sequence (CDS) gene models from the *B. oleracea* TO1000 and *B. napus* Darmor-bzh reference genomes in addition to preliminary CDS models from the *B. rapa* Chifu genome sequence assembly. The construction of the pan-transcriptome allows, in a similar fashion as the pangenome, the discovery of functional dispensable genes (Jin et al., 2016).

## Epi-Genomics

In addition to the identification of novel genes and useful haplotypes, epigenetic variation has the potential to contribute toward crop adaptation and productivity (Dwivedi et al., 2017). These epigenetic variations are plant developmental and adaptation responses to environmental constraints (Gallusci et al., 2017; Tirnaz and Batley, 2019b). Epigenomics encapsulates genotype  $\times$  environment interactions and their independent influence on the phenotype (Seymour and Becker, 2017) and may contribute as potential phenotypic resources for breeding.

Epigenetic regulation is independent of DNA sequence alteration and stably inherited during mitosis or meiosis (Weigel and Colot, 2012). DNA (de)methylation, histone modification, and chromatin remodeling involves regulatory reprogramming at transcriptional and post-transcriptional levels (Paszowski and Whitham, 2001; Tirnaz and Batley, 2019a). DNA methylation in plants includes *de novo* methylation, as well as maintenance and demethylation, as a means of regulatory check and balance in gene expression (Elhamamsy, 2016; Tirnaz and Batley, 2019a). Histone modification refers to the methylation or (de)acetylation of histone proteins at the N-terminal. Mono-, di-, or trimethylation of the lysine residue in the former results in various functional responses, while the histone acetylation and deacetylation are associated with gene activation and repression, respectively (Fuchs et al., 2006). Additionally, chromatin remodeling is influenced by histone octamer movement (Perrella and Kaiserli, 2016), ATP-dependent enzyme affecting nucleosome composition (Tariq and Paszowski, 2004) or histone variants (Rando and Ahmad, 2007) cause the DNA sequence to become inaccessible to transcriptional mechanisms, resulting in transcriptional silencing.

Epialleles, or epivariants, first coined in mammals by Rakyan et al. (2002), refer to genetically identical and stable alleles that are variably expressed due to epigenetic regulations and result in difference in phenotype (Richards, 2006; Dolinoy et al., 2010). Earlier studies on epialleles lack whole-genome information to decipher association with desirable traits (Seymour and Becker, 2017). While more studies have been conducted recently, epigenomics is still considered in its infancy compared with the characterization of genetic sequence- or structural-caused variation.

Epigenomic studies in *Brassica* crops have predominantly involved global or targeted methylation profiling for natural or experimentally induced epivariants as defined by Gallusci et al. (2017). Early *Brassica* methylation studies relied on a chemical

demethylation agent treatment using 5-azacytidine (5-AzaC) and on cytological (Solís et al., 2015) as well as targeted allele-specific and methylation-sensitive amplified polymorphism (MSAP) techniques to capture the global DNA methylation pattern (Shiba et al., 2006; Hauben et al., 2009) (refer **Table 3**). Hypomethylated populations created using 5-AzaC treatment could also be mined for epiallelic variation (Amoah et al., 2012). The forward and reverse screening of epigenetic variation was employed in functional and inheritance studies in *B. rapa* var. *trilocularis* and suggested to have potential as an intervention strategy for crop improvement (Amoah et al., 2012).

These techniques were superseded by whole-genome bisulfite sequencing (WGBS) first described in *Arabidopsis* (Cokus et al., 2008; Lister et al., 2008). With the use of the WGBS approach, DNA hypomethylation of the multiallelic *Bnams4* gene associated with male sterility was detected in young floral buds (Wang Z. et al., 2018). The male sterility trait promotes heterosis and hybrid development and hence is favored in crop improvement and breeding strategies (Saxena and Hingane, 2015).

Chromatin immunoprecipitation (ChIP) is another useful strategy for protein–gene interaction studies and, in the case of methylation, for investigating specific histone modifications (Das et al., 2004; Kawanabe et al., 2016). Kawanabe et al. (2016) developed positive and negative control primers to validate ChIP assays. The primers were targeted at histone modifications at H3K4me3 (trimethylation of the 4th lysine of H3), H3K9me2, H3K27me3, and H3K36me3 and used to study the response of 4 *FLC* paralogs to vernalization in *B. rapa* var. *pekinensis*. ChIP combined with WGBS further expanded the ability to investigate complex interactions between genetic and epigenetic factors (Li and Tollefsbol, 2011). These studies assisted in the identification of epigenetic markers, epigenetic QTL, and genes associated with floral and pollen development, self-incompatibility, salt and heat stress, vernalization, disease resistance, and male sterility and in the assessment of methylation profiles of introgression lines for crop improvement resource (**Table 3**). Nonetheless, due to the complexity of epigenetic interaction and its involvement in complex regulatory networks, these epigenomic approaches still require extensive investigation before their application in crop improvement can be implemented.

## Reverse Genetics

One of the most cost-effective and quick approaches used to identify genetic variation in crop populations is by Targeting Induced Local Lesions in Genomes (TILLING). This method combines chemical mutagenesis, to create lesions on the genome, and molecular techniques such as PCR and DNA pooling, to identify point mutations within the population. TILLING was first demonstrated in *Arabidopsis* using ethyl methanesulfonate (EMS) (McCallum et al., 2000), which results in base transitions by causing the G residues to alkylate and as a result pair with T instead of C (Rashid et al., 2011). Soon after, the technique was explored in *Brassica* species for various traits of interest including fatty acid content and shatter tolerance (**Table 4**). However, this method is less feasible and becomes more time-consuming if multiple genes are targeted, such as those involved in specific

**TABLE 3** | Exploration of epigenetic resources in *Brassica* for natural and experimentally induced epivariants.

Approach	<i>Brassica</i> species	Trait of interest	Source of epigenetic variation	References
Allele-specific bisulfite sequencing	<i>Brassica rapa</i>	Self-incompatibility	Natural	Shiba et al., 2006
Methylation-sensitive AFLP, cytosine extension assay	<i>Brassica napus</i>	Energy use efficiency	Experimental	Hauben et al., 2009
MSAP	<i>B. rapa</i> cv. <i>Osome</i>	Chromatin remodeling	Experimental	Sasaki et al., 2011
MSAP	<i>B. rapa</i> var. <i>trilocularis</i> cv. R-o-18	Hypomethylated epialleles	Experimental	Amoah et al., 2012
WGBS using NGS	<i>B. napus</i> var. <i>oleifera</i>	Salt stress	Natural	Marconi et al., 2013
WGBS using NGS	<i>Brassica oleracea</i> cv. T01000	Cytosine methylation	Natural	Parkin et al., 2014
Reduced representation bisulfite sequencing (RBBS)	<i>B. rapa</i> var. <i>oleifera</i> cv. 3H120	Hybridization and polyploidy	Natural	Chen et al., 2015
ChIP	<i>B. rapa</i> var. <i>pekinensis</i> cv. RJKB-T23 and RJKB-T24	Vernalization, high bolting	Natural	Kawanabe et al., 2016
MSAP	<i>B. oleracea</i> var. <i>botrytis</i> cv. Korso × <i>Brassica nigra</i> cv. G1/1 introgression lines	Methylation pattern and heredity	Natural	Wang G.-X. et al., 2016
WGBS using NGS	<i>B. oleracea</i> cv. 01-20S and 01-20F	Male sterility	Natural	Han et al., 2017
Methylated DNA immunoprecipitation sequencing (MeDIP-seq)	<i>B. rapa</i> ssp. <i>chinensis</i> cv. NHCC004	Heat tolerance	Experimental	Liu T. et al., 2017
WGBS using NGS	<i>B. rapa</i> cv. Suzhouqing (NHCC001)	Heat stress	Natural	Liu G. et al., 2018
Methylated DNA immunoprecipitation sequencing (MeDIP-seq)	<i>B. rapa</i> cv. RJKB-T23 and RJKB-T24	Fusarium disease resistance	Natural	Takahashi et al., 2018a
WGBS using NGS, small RNA-Seq, and ChIP	<i>B. rapa</i> var. <i>pekinensis</i> cv. RJKB-T24	DNA methylation and expression, and H3K9me2 modification and small RNA expression	Natural	Takahashi et al., 2018b
WGBS using NGS	<i>B. napus</i> cv. 7365A and 7365B	Male sterility	Natural	Wang Z. et al., 2018
ChIP sequencing (ChIP-seq) and RNA-Seq	<i>B. rapa</i> var. <i>pekinensis</i> RJKB-T23 and RJKB-T24 inbred lines	H3K27me3 in <i>FLC</i> regulation and vernalization	Natural	Akter et al., 2019
ChIP sequencing (ChIP-seq) and RNA-Seq	<i>B. rapa</i> ssp. <i>trilocularis</i> cv. R-o-18	H3K27me3 in epigenetic regulation	Natural	Payá-Milans et al., 2019
ChIP sequencing (ChIP-seq) and RNA-Seq	<i>B. rapa</i> ssp. <i>chinensis</i> cv. <i>Aijiaohuang</i>	Pollen wall formation	Experimental	Shen et al., 2019
Targeted enrichment using seq-cap epi	<i>B. napus</i> cv. Westar and Sturt	Resistant to <i>Leptosphaeria maculans</i>	Natural	Tirnaz et al., 2020
WGBS using ONT	<i>B. nigra</i> cv. C2 and Ni100	Active centromeres	Natural	Perumal et al., 2020

AFLP, amplification fragment length polymorphism; MSAP, methylation-sensitive amplified polymorphism; WGBS, whole-genome bisulfite sequencing; NGS, next-generation sequencing; ChIP, chromatin immunoprecipitation; ONT, Oxford Nanopore Technologies.

biopathways (Sashidhar et al., 2020). Examples of canola varieties developed that benefit from this approach include PodGuard trait for In Vigor R5520P and 1H51 RR varieties, which were commercially marketed by Bayer for their resistance to pod shattering (Raman et al., 2019).

TILLING has been further developed to include EcoTILLING (Ecotype) and ORG-EcoTILLING. EcoTILLING involves pooling DNA from only two individuals consisting of a reference and queried genotypes (Backes, 2013); therefore, instead of creating mutant populations, this more recent approach involves a study of allelic variation. EcoTILLING requires re-sequencing efforts to characterize and locate the genotypic polymorphism (Wang et al., 2010). Additionally, this approach is high-throughput and can associate natural variants with gene function, trait association, and phylogenetic relationships (Zeng et al., 2012). ORG-EcoTILLING was first utilized in Brassicas to explore the use of TILLING in organelle genomes by

combining CEL1 endonuclease, which cuts specific mismatches in heteroduplex DNA, and PCR, for three cp genes and one mt gene. ORG-EcoTILLING consistently confirmed *B. rapa* as *B. napus* maternal progenitors based on phylogenetic analysis. Additionally, it also uncovered the possibility of multiple origins and evolution throughout *B. napus* domestication with the identification of three additional divergences in the accessions.

The TILLING approach is considerably more accessible in terms of cost and time than other specialized reverse genetics approaches like RNA interference (RNAi) or gene silencing, and virus-induced gene silencing (VIGS). RNAi gained traction with the ability to knock down gene functions mediated by small interfering RNA (siRNA) or microRNA (miRNA) (Pe'ery et al., 2003; Limeria et al., 2017). Traits introduced using RNAi, such as self-compatibility in *B. rapa*, have been found to be stable even in crosses with commercial variety (Jung et al., 2012). This will help improve likelihood for the seeds to be used in



**TABLE 4** | Reverse genetic resource in *Brassica* over the last 10 years.

Approach/Species	Trait of interest (target gene(s))	References
<b>RNAi</b>		
<i>B. rapa</i> cv. Osome	Self-compatibility S-locus ( <i>SRK</i> , <i>SP11/SCR</i> )	Jung et al., 2012
<i>B. juncea</i> cv. Varuna	Glucosinolate transcriptional regulator ( <i>MYB28</i> )	Augustine et al., 2013
<b>TILLING</b>		
<i>B. napus</i> cv. Ningyou 7	Seed erucic acid content ( <i>FAE1</i> )	Wang et al., 2008
<i>B. napus</i> cv. YN01-429 x Express 617	Sinapine synthesis ( <i>SGT</i> , <i>REF1</i> )	Harloff et al., 2012
<i>B. napus</i> cv. DH12075	Demonstrate mutation density (39 genes)	Gilchrist et al., 2013
<i>B. napus</i> cv. Express 617	Phytic acid (six gene families: <i>MIPS</i> , <i>MIK</i> , <i>2-PGK</i> , <i>IPK1</i> , <i>IPK2</i> , and <i>MRP5</i> )	Sashidhar et al., 2019
Three <i>B. rapa</i> ssp. <i>trilocularis</i> cv. R-o-18 mutants	CAX1 transporter for Calcium homeostasis ( <i>BraA.cax1a</i> )	Navarro-León et al., 2018
<i>B. oleracea</i> cv. TO1000	Abiotic stress response (15 genes)	Himelblau et al., 2009
<i>B. rapa</i> ssp. <i>trilocularis</i> cv. R-o-18	Redundant genes function (three <i>REPLUMLESS</i> , one <i>INDEHISCENT</i> , two <i>METHYLTRANSFERASE1</i> )	Stephenson et al., 2010
<b>EcoTILLING</b>		
101 <i>B. napus</i> , nine <i>B. oleracea</i> and seven <i>B. rapa</i> accessions	Seed erucic acid content ( <i>FAE1</i> )	Wang et al., 2010
<b>ORG-EcoTILLING</b>		
90 <i>B. napus</i> , three <i>B. rapa</i> , and three <i>B. oleracea</i> accessions	Breeding history and maternal inheritance ( <i>accD</i> , <i>matK</i> , <i>rbcL</i> )	Zeng et al., 2012
<b>VIGS</b>		
<i>B. napus</i> (winter varieties)	Flowering ( <i>FLC</i> )	Álvarez-Venegas et al., 2011
<i>B. rapa</i> ssp. <i>Chinensis</i> cv. Wuyueman and 49 Caixin	Flowering ( <i>MAF</i> )	Huang et al., 2018
<i>B. rapa</i> cv. Sijjucaixin	Carotenoid biosynthetic pathway ( <i>PDS</i> )	Yu et al., 2018

commercial cultivation. Similar to RNAi, VIGS also involves introduction of dsRNA molecules. A viral vector genome such as *Cabbage Leaf Curl Virus* (CaLCuV) is modified to include the plant target gene fragment (150–800 bp) and to remove the viral inducing host gene, thus forming a recombinant virus (Lu et al., 2003; Ramegowda et al., 2014; Bekele et al., 2019). The recombinant vector introduces infection in the plant, is amplified, and generates dsRNA molecules. These dsRNA molecules triggered post transcriptional gene silencing, once detected by the host plant, causing it to be cleaved into siRNA. The RNAi silencing complex and antisense siRNA strands associates together and begin to target RNAs, which complemented the siRNAs. These target-specific RNAs were screened and destroyed, which subsequently caused the target gene to be silenced. VIGS can also be utilized for tissue-specific gene silencing, helpful in screening stress responses, and induced transcriptional gene silencing by targeting the gene promoter (Kanazawa et al., 2011; Senthil-Kumar and Mysore, 2011; Bekele et al., 2019). VIGS research in *Brassica* includes interest in understanding the vernalization pathway in Brassicas as a means to improve flowering and reproductive development by silencing genes associated with late flowering (Álvarez-Venegas et al., 2011) or floral organ transition (Huang et al., 2018).

## GENOME MANIPULATION

Traditional breeding approaches rely on the diversity found in local land races, mutation panels, or even germplasm from related species to introduce desired traits or elite alleles through

costly and time-consuming backcrossing programs (Dwivedi et al., 2017). The absence of natural genetic diversity and potential linkage drag introducing closely linked unwanted agronomic characteristics has plagued crop improvement efforts (Holme et al., 2013). Increased availability of genomic resources, identified and well-characterized genes, as well as a deeper understanding of underlying molecular mechanisms has led the way for the introduction of innovative approaches to overcome these limitations and fast-track crop breeding (Scheben et al., 2016; Hickey et al., 2019).

## Gene Transformation

Genetic modification through the introduction of transgenes was developed in an attempt to expand the available genepool (Kamthan et al., 2016). *Agrobacterium tumefaciens* and biolistic techniques are widely and efficiently employed to mediate the transfer of selected exogenous genes or regulatory elements from an unrelated species or even non-plant organism (Moloney et al., 1989; Altpeter et al., 2016). Numerous transgenic *Brassica* spp. have been developed in an attempt to introduce traits such as salt tolerance (Kim et al., 2016), disease resistance (Grison et al., 1996; Aghazadeh et al., 2016; Zarinpanjeh et al., 2016), reduced sinapine content (Wolfram et al., 2010; Harloff et al., 2012), and herbicide tolerance (Beversdorf et al., 1988; De Block et al., 1989; Cuthbert et al., 2001). The potential of transgenics in advanced plant metabolic engineering is however best demonstrated in the development of transgenic omega-3 *Brassica napus* varieties by BASF and Cargill, and Nuseed, CSIRO and GRDC (Napier et al., 2019) respectively, as well as *Brassica juncea* by Wu et al. (2005).

Transgenic *B. napus* accumulating long-chain polyunsaturated fatty acids (LC-PUFAs) were engineered by each of the initiatives through the introduction of large multi-transgene cassettes. The BASF cassette (~44 kbp) contained 12 genes, while the Nuseed initiative's cassette (~23 kbp) contained six omega-3 LC-PUFA biosynthetic genes. Each gene was under the regulation of a seed-specific promoter. Each cassette also contained a gene for herbicide resistance (Connelly and MacIntosh, 2018; Sottosanto et al., 2018). The LC-PUFA profile produced by the two omega-3 transgenic canola varieties varied and respectively contained ~7% eicosapentaenoic acid (EPA), ~3% docosapentaenoic acid (DPA), and ~1% docosahexaenoic acid (DHA) (LBFLFK); and <0.5% EPA, ~1% DPA, and ~10% DHA (NS-B50027-4) respectively (Napier et al., 2019). Very-long-chain (VLC) PUFA accumulating *B. juncea* was on the other hand engineered in a stepwise approach through a series of transformations with increasing numbers of transgenes. The resulting transgenic *B. juncea* yielded up to ~15% EPA, ~4% DPA, and ~1.5% DHA (Wu et al., 2005).

In addition to the transformation of the nuclear genome, modification of the cp genome has also been established. The introduction of foreign genes into the cp can address nuclear transgenic limitations such as low-level transgene expression (Jin and Daniell, 2015) and the potential transgene escape via pollen (Daniell, 2007). The stable integration and expression of more than 40 cp-based transgenes has been reported by Daniell et al. (2016), most of which were aimed at potentially enhancing biotic stress tolerance and consequently yield. An example is the development of a cabbage-plastid transformation system for introduction of the insecticidal *cry1Ab* gene. With the use of a species-specific vector, the expression of the BT-toxin facilitated the control of the diamond moth, an economically important *Brassica* pest (Liu C. W. et al., 2008). Introduced insect resistance using *cry* genes was also reported for rapeseed (Schuler et al., 2004) and collards (Cao et al., 2005).

Improved weed and insect control using GM crops has assisted farmers in attaining higher yields with fewer resources. The analysis by Brookes and Barfoot (2018) estimated that the adoption of commercial herbicide-tolerant GM canola and sugar beet led to a total global income gain of \$559 million in 2016 and \$6.44 billion cumulatively since 1996. Despite the commercial benefits, growing consumer skepticism (Frewer et al., 2013), as well as potential risks to human health and the environment (Zhang et al., 2016), has encouraged the development of alternative genomic modification technologies.

Cisgenesis and intragenesis, based on the same gene transfer technologies as transgenesis, were consequently developed. Genetic crop modification thereby involved the introduction of target DNA from the same plant species, or a sexually compatible species, for crop improvement. Resulting crop plants are free from any foreign DNA mitigating associated risk to some extent (Espinoza et al., 2013; Holme et al., 2013). Alternative approaches such as reverse breeding exploit the use of transgenes to accelerate initial breeding. The unwanted transgene is eliminated through Mendelian segregation during the later stages of the breeding process (Dirks et al., 2009; Basso et al., 2020).

## Genome Editing

Recently, GE technologies have come to the foreground, allowing the precise and permanent modification of specific genes or genomic regions. ODM, programmable SSNs, and base editing provide an opportunity to study gene function and alter crop traits through the mutation of specific genes, reprogramming of epigenetic markers, and the generation of site-specific sequence modifications (Voytas and Gao, 2014; Ran et al., 2017; Jansing et al., 2019).

### Oligo-Directed Mutagenesis

Traditional mutation breeding using chemical or irradiation results in random mutations in the genome. The movement toward a more desired and controlled site-specific targeted mutagenesis took shape in the 1970s (Lusser and Davies, 2013). ODM, also known as targeted gene repair, oligonucleotide-directed gene targeting, genoplasty, and chimeraplasty, makes use of 20–100 bp of DNA or RNA oligonucleotides to introduce mutations at the target site. The synthesized oligonucleotides are designed to be homologous to the target site with the exception of 1–4 bp (Lusser et al., 2012). Upon transfection, the oligonucleotides associate with the target site, prompting DNA repair at the sequence mismatch sites resulting in base pair mutations, deletions, or reversal of mutations (Lusser et al., 2012; Lusser and Davies, 2013). With a difference of often just a few nucleotides underlying important traits in plants, the application of ODM held potential as a non-GM organism (GMO) base pair-specific oligonucleotide-directed gene editing platform to augment the genetic diversity of a specific genotype. The use of ODM was furthered by Cibus as part of the commercial Rapid Trait Development System (RTDS™) introducing novel and commercially valuable traits such as herbicide resistance into a variety of crops including oilseed rape (Gocal et al., 2015). Ruiter et al. (2003) found that spontaneous mutation in plants obscured the intended sequence modifications in *B. napus* mediated through self-complementary RNA–DNA chimeric oligonucleotides or chimeraplasty. Studies by Sauer et al. (2016) confirmed that significant precise gene-editing events in plants could be realized by ODM alone and suggested that ODM efficiency could be further improved in combination with reagents that cause DNA double-stranded breaks (DSBs).

### Programmable Sequence-Specific Nuclease

GE using programmable SSNs is generally achieved through the induction of a controlled DSB at a target locus using SSNs. The DBS activates the intracellular DNA-repair pathways and is repaired through either non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Gaj et al., 2013). The imprecise re-joining of the DBS through NHEJ leads to the introduction of indels at the target loci and disruption of gene function. On the contrary, HDR entails the use of an exogenous DNA-repair template to bridge the DSB site. The repair template, a double-stranded DNA vector or a single-stranded DNA oligonucleotide, enables the introduction of a precise mutation or insertion to alter gene function (Zhang et al., 2013). Several engineered nuclease systems have been developed including meganucleases (MN), zinc finger nucleases (ZFNs), transcription activator-like

effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR) coupled with a CRISPR-associated protein (Cas).

### Zinc Finger Nucleases

ZFN, first described by Kim et al. (1996), employs novel hybrid site-specific endonucleases created by the linking of two different zinc finger proteins (ZFPs) to the cleavage domain of the bacterial FokI endonuclease. The zinc finger domains are designed to each recognize and bind to a unique 3- to 4-bp DNA sequence adjacent to the target site. The tandem repeats can be constructed to recognize an extended 9- to 18-bp DNA sequence (Lin and Musunuru, 2016). In an attempt to modify seed oil composition in *B. napus*, Gupta et al. (2012) engineered ZFP transcription factors (TFs) to fuse to a conserved region downstream of the transcription start site of two canola KASII genes. The modification resulted in the escalated expression of the KASII mRNA, increasing C18 and lowering palmitic acid levels well as the overall saturated fatty acid content in the seed. Canola oil with a lower saturated fat content is more desirable and potentially poses health benefits (Hyseni et al., 2017). Despite the progress in technology, the engineering of the desired ZFN-binding domain remains challenging and time-consuming, with further limitations presenting in selection of the target site (Cox et al., 2015).

### Transcription Activator-Like Effector Nucleases

TALENs are similar in structure to ZFNs, composed of dimeric DNA-binding proteins fused to the nuclease domain FokI (Cermak et al., 2011). The central domain of the TAL effector consists of a polymorphic repeat of ~34 amino acids with hypervariable di-amino acids at positions 12 and 13. These so-called repeat variable di-residues (RVDs) associate and recognize a corresponding C, T, A, or G nucleotide (Scholze and Boch, 2010). The longer DNA recognition sites promote specificity and reduce potential off-target effects (Li et al., 2012). The TAL effector DNA binding domain is more flexible and can be customized, which broadens its potential application.

Sun et al. (2013) demonstrated the suitability of the TALEN construct to alter the endogenous FRIGIDA (vernalization determinant) gene in *Brassica oleracea* var. *capitata*, further suggesting that the method could be applied to related *Brassica* spp. TALENs with mt localization signals (mitoTALENs) were designed by Kazama et al. (2019) to knock out CMS-associated genes at orf79 and orf125, respectively, of CMS varieties of rice and *B. napus* (SW18). Induced mt modifications restored fertility without causing noticeable phenotypic changes and were found to be stable and maternally inherited. The successful modifications of the mt genome pose the prospect of “mitochondrial breeding” in plants (Kazama et al., 2019), which can play an important role in the study and future conditioning of plant responses toward climate change (Budar and Roux, 2011; Sweetman et al., 2019; Florez-Sarasa et al., 2020). TALENs similar to ZFNs are time-consuming genome manipulation techniques (Razzaq et al., 2019) and require the extensive screening of large numbers of manipulated individuals.

### RNA-Guided Nucleases

The second-generation CRISPR/Cas9 system provides an alternative approach in targeted nucleases. In contrast to the ZFN's and TALEN's engineered protein associated DNA-binding systems, the CRISPR/Cas9 system has a single-guide RNA (sgRNA) bound to the Cas9 endonuclease that directs the complex to a specific site in the genome (Jinek et al., 2012; Cong et al., 2013). Recognition is attained through base pairing between the programmable 20-bp-long spacer region at the 5'-end leading sequence of the gRNA and specific DNA target. The Cas9 nuclease uses the CRISPR gRNA–DNA pairing as guide in combination with adjacent the DNA protospacer-adjacent motif (PAM) to cleave the DNA. This simplicity and flexibility in the programming of the CRISPR/Cas9 system have facilitated its adoption and exploitation in plants including *Brassica* (Zhang et al., 2018b). Lawrenson et al. (2015) demonstrated the efficiency of the CRISPR/Cas9 GE tool for the first time in the knockout of target genes in *B. oleracea*. Introduced mutations were stably inherited and transgene-free plants obtained through segregation.

Due to the allotetraploid nature of some *Brassica* species, the observed effect of single gene modification is often limited by its potential redundant function. Modification of all homologous genes is hence required to obtain a reliable altered genotype and phenotype (Sashidhar et al., 2019). Multiple guide sequences can be encoded into a single CRISPR array, allowing the simultaneous editing of several sites (Cong et al., 2013), making the CRISPR/Cas9 system a valuable tool in the knockout of redundant genes or parallel pathways in polyploids. Sashidhar et al. (2019) illustrate a case in point with the CRISPR/Cas9-mediated knockout of multiple paralogs of the key enzyme inositol tetrakisphosphate kinase (ITPK) involved in the synthesis of phytic acid in *B. napus* seed. A noticeable change in the phytic acid content of *B. napus* seed was observed only in triple mutants of the essential *BnITPK* genes.

The CRISPR/Cas9 GE tool has been widely adopted in the manipulation and study of a variety of genes underlying agronomical important traits in *Brassica*. These include traits such as pod shatter resistance (Braatz et al., 2017; Zhai et al., 2019), multi-ocular silique (Yang Y. et al., 2018), increase in oleic acid content in seed (Okuzaki et al., 2018), and seed coat color (Zhai et al., 2020) (Table 5). Even though the use of the CRISPR/Cas9 system is popular in gene knockout or knock-in studies, it is limited by the introduction of random indels at the target site in addition to the possibility off-target mutations.

### Targeted Base Editing

Targeted base editing is one of the newest additions to GE. The technique is based on the CRISPR/Cas9 system and enables the direct and irremediable conversion of a selected target base without the induction and repair of a DSB (Komor et al., 2016). In the base-editing system, a cytosine or adenosine deaminase domain is fused to the N-terminus of a deactivated Cas9 (dCas9) of Cas9 nickase (nCas9). Although the Cas9 retains the ability to be guided by the gRNA, it instead mediates the direct conversion of cytosine (C) to uridine (U) resulting in a C-to-T or G-to-A substitution resulting in a single controlled point mutation

**TABLE 5** | Application of programmable sequence-specific nucleases and targeted base editing tools in genome editing of *Brassica*.

<i>Brassica</i> species	Target gene/s and modification	Editing system	Modified/introduced trait	References
<b>Programmable sequence-specific nucleases</b>				
<i>Brassica napus</i>	Introduction of ZFP-transcriptional activators (TF) to activate two canola $\beta$ -ketoacyl-ACP Synthase II designed ( <i>KASII</i> ) genes	ZFN	Elevated <i>KASII</i> transcript levels, decrease in palmitic acid, increased in total C18, and reduced total saturated fatty acid content	Gupta et al., 2012
<i>Brassica oleracea</i> var. <i>capitata</i>	Targeted cleaving of the endogenous <i>FRIGIDA</i> ( <i>FRI</i> ) gene	TALENs	Earlier flowering	Sun et al., 2013
<i>B. napus</i>	Knockout of CMS-associated genes ( <i>orf125</i> )	mitoTALENs	Restored male fertility	Kazama et al., 2019
<i>B. oleracea</i>	Indel mutations of the <i>BoIC.GA4a</i>	CRISPR/Cas9	Dwarf phenotype and alterations in pod valve margins	Lawrenson et al., 2015
<i>B. napus</i>	Knockout mutations of <i>BnaRGA</i> , <i>BnaDA2</i> , and <i>BnaFUL</i>	CRISPR/Cas9	Demonstrate CRISPR/Cas9 efficiency in creating targeted genome modifications at multiple loci that are stable and inheritable	Yang et al., 2017
<i>B. napus</i>	Induced mutation in <i>BnWRKY11</i> and <i>BnWRKY70</i>	CRISPR/Cas9	<i>BnWRKY70</i> mutants exhibited enhanced resistance to <i>Sclerotinia</i> , while <i>BnWRKY11</i> mutants showed no significant difference in <i>Sclerotinia</i> resistance	Sun et al., 2018
<i>B. napus</i>	Modification of a fatty acid desaturase 2 gene ( <i>FAD2</i> )	CRISPR/Cas9	Increase of oleic acid content seed	Okuzaki et al., 2018
<i>B. napus</i>	Knockout of rapeseed homologs of <i>CLAVATA3</i> ( <i>CLV3</i> )	CRISPR/Cas9	Produced more leaves, multilocular siliques, and higher number of seed	Yang Y. et al., 2018
<i>B. napus</i>	Knockout of <i>INDEHISCENT</i> ( <i>IND</i> ) and <i>ALCATRAZ</i> ( <i>ALC</i> ) gene homologs	CRISPR/Cas9	Association of the <i>BnIND</i> gene with pod shatter resistance, whereas the <i>BnALC</i> gene appears to have limited potential for rapeseed shatter resistance	Tang et al., 2018
<i>B. napus</i>	Knockout of SPL3 homologous gene copies, <i>BnSPL3-A5/BnSPL3-A4/BnSPL3-C3/BnSPL3-C4/BnSPL3-Cnn</i>	CRISPR/Cas9	Developmental delay	Li C. et al., 2018
<i>B. napus</i>	Knockout mutations of the histone lysine methyltransferases <i>BnaSDG8.A</i> and <i>BnaSDG8.C</i>	CRISPR/Cas9	<i>BnaSDG8.A</i> and <i>BnaSDG8.C</i> help control the <i>B. napus</i> floral transition by directly altering the H3K36m2/3 levels at the <i>BnaFLC</i> chromatin loci	Jiang et al., 2018
<i>B. oleracea</i> var. <i>capitata</i>	Induced mutations in phytoene desaturase gene <i>BoPDS</i> , S-receptor kinase gene <i>BoSRK</i> , male-sterility-associated gene <i>BoMS1</i>	CRISPR/Cas9 based on endogenous tRNA processing	<i>BoSRK3</i> gene mutation suppressed self-incompatibility completely <i>BoMS1</i> gene mutation produced a completely male-sterile mutant	Ma et al., 2019
<i>B. napus</i>	Induced mutation in <i>INDEHISCENT</i> ( <i>IND</i> ) and <i>ALCATRAZ</i> ( <i>ALC</i> )	CRISPR/Cas9	Establishes that <i>BnIND</i> gene is essential for pod shatter and highly conserved in <i>Brassica</i>	Zhai et al., 2019
<i>B. napus</i>	Knockout of seven <i>BnLPAT2</i> homologous genes and four <i>BnLPAT5</i> homologous genes	CRISPR/Cas9	Enlarged oil bodies, disrupted distribution of protein bodies and increased accumulation of starch confirming the role of <i>BnLPAT2</i> and <i>BnLPAT5</i> in oil biosynthesis	Zhang et al., 2019
<i>B. napus</i>	Knockout of five homoeologs ( <i>BnJAG.A02</i> , <i>BnJAG.C02</i> , <i>BnJAG.C06</i> , <i>BnJAG.A07</i> , and <i>BnJAG.A08</i> ) of the <i>JAGGED</i> ( <i>JAG</i> ) gene	CRISPR/Cas9	Affected development of the lateral organs in organizing pod shape and size as well as viable seed. <i>BnJAG.A08-NUB</i> gene caused significant changes in the pod dehiscence zone	Zaman et al., 2019
<i>B. napus</i>	Knockout mutations of <i>BnaMAX1</i> homologs	CRISPR/Cas9	Semi-dwarf and increased branching phenotypes with more siliques, contributing to increased yield per plant.	Zheng et al., 2020
<i>B. napus</i>	Mutation of the M-locus protein kinase (MLPK) <i>BnaMLPK</i> genes	CRISPR/Cas9	Revealed that MLPK is a positive regulator of the self-incompatibility response	Chen et al., 2019
<i>B. napus</i>	Knockout mutations of the <i>BnA10.LMI1</i> gene	CRISPR/Cas9	Demonstrated <i>BnLLA10</i> regulates the development of leaf lobes	Hu et al., 2018
<i>Brassica carinata</i>	Mutation of the <i>FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 1</i> ( <i>BcFLA1</i> ) gene	CRISPR/Cas9	Down regulation of <i>BcFLA1</i> decreased root growth under Pi-deficient conditions predicting role	Kirchner et al., 2018
<i>B. napus</i>	Knockout mutation of the <i>APETALA2</i> ( <i>BnAP2</i> ) genes	CRISPR/Cas9	Loss of <i>AP2</i> function severely affects sepal and petal development and generates sepal carpeloid and apetalous mutants	Zhang et al., 2018a
<i>Brassica campestris</i>	Induced <i>BcPME37c</i> mutant	CRISPR/Cas9	<i>BcPME37c</i> mutation led to the abnormal thickening of the pollen intine	Xiong et al., 2019

(Continued)



TABLE 5 | Continued

Brassica species	Target gene/s and modification	Editing system	Modified/introduced trait	References
<i>B. napus</i>	Targeted knockout of <i>TRANSPARENT TESTA 2</i> ( <i>BnTT2</i> ) homologs	CRISPR/Cas9	<i>BnTT2</i> homologs had conserved but redundant functions in regulating seed color. Homozygous mutants of <i>BnTT2</i> homologs increased oil content and improved fatty acid composition with higher linoleic acid (C18:2) and linolenic acid (C18:3)	Xie et al., 2020
<i>B. napus</i>	Knockout lines of <i>BnaA9.WRKY47</i>	CRISPR/Cas9	<i>BnaA9.WRKY47</i> contributed to adaptation to B deficiency by up-regulating <i>BnaA3.NIP5;1</i> expression to facilitate efficient B uptake. Suggested <i>BnaWRKY</i> involvement in adaptation to low B	Feng et al., 2020
<i>B. napus</i>	Knockout mutations of all of <i>BnaFAD2</i> homologs	CRISPR/Cas9	Mutation type of <i>BnaFAD2</i> affected oleic levels varied, suggesting that fatty acid levels might be manipulated through precise editing of specific regions of a gene	Huang et al., 2020
<i>B. napus</i>	Generated diverse mutants of the male-sterility allele MS5a and MS5c	CRISPR/Cas9	Male fertility dependent on expression levels and protein sequences of MS5a and MS5c	Xin et al., 2020
<i>B. napus</i>	Created gain-of-function mutant of <i>BnaA6.RGA</i> , <i>bnaa6.rga-D</i> , and the loss-of-function quadruple mutant, <i>bnarga</i>	CRISPR/Cas9	Quadruple mutant <i>bnarga</i> significantly decreased the sensitivity of stomatal closure, suggesting that <i>BnaRGA</i> proteins play important roles in plant adaptation to water-deficit stress	Wu et al., 2020b
<i>B. napus</i>	Knockout members of the <i>BnSFAR4</i> and <i>BnSFAR5</i> gene families	CRISPR/Cas9	Significant increase of seed oil content	Karunaratna et al., 2020
<i>B. napus</i>	Induced calreticulin ( <i>BnCRT1a</i> ) mutants	CRISPR/Cas9	Loss of function of <i>CRT1a</i> results in activation of the ethylene signaling pathway. Observed reduced susceptibility to <i>Verticillium longisporum</i>	Pröbsting et al., 2020
<i>B. napus</i>	Knockout three functional paralogs of <i>BnITPK</i>	CRISPR/Cas9	Reduction of phytic acid and increase of free phosphorus in seed	Sashidhar et al., 2020
<i>B. napus</i>	Mutation of the <i>BnTT8</i> genes	CRISPR/Cas9	Yellow seed coat, altered oil content, and fatty acid composition in seeds	Zhai et al., 2020
<b>Targeted base editing</b>				
<i>B. napus</i>	C-to-T substitution at the P197 position of <i>BnALS 1</i>	Base editing	Tribenuron-methyl herbicide resistance	Wu et al., 2020a
<i>B. napus</i>	A-to-G substitutions in <i>BnALS</i> and <i>BnPDS</i>	Base editing	Single amino acid change in the FT protein or mis-splicing of the PDS3 RNA transcript resulted in late-flowering and albino phenotypes	Kang et al., 2018
<i>B. napus</i>	A-to-G substitutions in <i>ALS</i> , <i>RGA</i> , and <i>IAA7</i> genes	Base editing system	Base-edited plants of <i>ALS</i> conferred high herbicide resistance, while base-edited <i>RGA</i> or <i>IAA7</i> plants exhibited decreased plant height	Cheng et al., 2020

ZFP, zinc finger protein; TF, transcription factor; ZFN, zinc finger nuclease; CMS, cytoplasmic male sterility.

or base correction rather than a random gene disruption. The precise mutations can lead to the introduction of stop codons, changes in amino acids, and regulatory site modification, thereby improving the resolution in the functional analysis of genes and proteins to a single nucleotide or amino acid (Komor et al., 2016). Kang et al. (2018) established an adenosine base editing (ABE) system in *B. napus*, demonstrating the efficiency of ABE in generating A-to-G substitutions at the target *BnALS* and *BnPDS* loci. The substitution resulted in a single amino acid change in the FT protein or mis-splicing of the PDS3 RNA transcript generating germline transmissible transgenic *Brassica* plants with late-flowering and albino phenotypes. The nCas9 cytosine base-editing system was employed by Wu et al. (2020a) to introduce a C-to-T conversion at the P197 position of the *BnALS 1* gene in *B. napus*. The P197S substitution conferred tribenuron-methyl resistance generating transgene-free homozygous mutants. Herbicide resistance and dwarfed plant architecture, both important traits in the commercial cultivation of oilseed rape, were reportedly introduced by Cheng

et al. (2020) using the A3A-PBE base-editing system. The A3A-PBE base-editing system enabled the substitution of C to T with increased efficiency (>20%) and wider editing window.

Modifications brought upon by genome-editing technologies pose a significantly lower risk than those associated with transgenics. In general, only a few selected nucleotides are altered, rendering changes similar to those that occur in natural populations (Voytas and Gao, 2014). After the genomic-editing agents were segregated out, it is not possible to differentiate between a naturally occurring mutation and the gene edit. GE is therefore a valuable tool to establish rapid and precise changes to aid crop improvement (Zhang et al., 2018b).

## GENOMIC SELECTION IN CROP IMPROVEMENT

Plant breeding is founded on the principles of collection, induction, and rearrangement of genetic diversity followed

by phenotypic-driven selection. Conventional breeding success was achieved through the exploitation of natural or mutation-induced variation followed by efficient selection of desirable traits largely based on phenotypic observation (Pérez-de-Castro et al., 2012). This approach has several limitations including long periods required (5–12 years) to develop a crop variety, high environmental noise, and being less effective in the improvement of complex and low heritable traits (Tuberosa, 2012).

Desirable traits often include characteristics such as increased yield, plant architecture, tolerance to environmental stresses, and resistance against pests and diseases. An interlinking network of multiple “minor” genes regulates the expression of these agronomically important features. Phenotypic expression is further shaped by non-genetic factors including genotype–environment interactions (Werner et al., 2018). It is not possible to accurately access these intricate and dynamic interdependencies based on phenotypic observation alone in conventional breeding. Crop development is therefore limited by extended periods of selections of up to 10 years, environmental noise, and low heredity of complex traits in conventional breeding approaches.

With the onset of the genomics era (Nepolean et al., 2018), marker-assisted breeding (MAS) was developed to address the limitations posed by conventional breeding (Collard and Mackill, 2008). In MAS, functional markers linked to QTL are used to detect important traits through linkage mapping or GWAS. As only statistically significant marker–trait associations are retained (Arruda et al., 2016), MAS is restricted to the detection of traits controlled by a limited number of QTL with large contributions to phenotypic variation. MAS has therefore limited value in the selection of traits under complex genetic control and is, as such, outperformed by traditional phenotypic selection (Bernardo, 2001, 2016; Zhao et al., 2014).

Fast-evolving genomic tools and vast amounts of available genomic sources are permitting the establishment of genotype–phenotype relationships, in particular for complex multi-genic traits (Pérez-de-Castro et al., 2012). Genome-wide selection or GS (Meuwissen et al., 2001), contrary to MAS, includes all marker information in the prediction model, reducing marker bias and allowing the potential to explain variance even with small-effect QTL. Predicted marker effects based on phenotype and high-density marker scores are then used to estimate the breeding value of untested genotypes (Zhao et al., 2015). This estimate, applied in the preselection of promising genotypes, can accelerate progress in crop breeding and reduce cost in comparison with conventional breeding (Wang X. et al., 2018).

Hybrid breeding has been widely used in the improvement of crop performance through the exploitation of heterosis (Liu et al., 2020). In heterosis, hybrid offspring created have the potential to outclass agronomic characteristics of the parents. The selection of suitable parental combinations is therefore of essence and can pose a major challenge in the development of hybrids. However, GS has shown the potential to predict hybrid performance (Zhao et al., 2015); resulting hybrid genotypes can be inferred from their inbred parents and potentially reduce genotyping cost and

generation interval (Wang X. et al., 2018). GS methods for hybrid canola breeding was evaluated by Jan et al. (2016); genome-wide SNP profiles were used to evaluate the prediction of the best possible parental combination of pollinators crossed with the two tester lines in a testcross performance for a number of important traits in spring canola. Based on genome-wide SNP markers, it was determined that testcross performance prediction in canola breeding could be an effective and efficient method to preselect promising pollinators for combinations with available male-sterile maternal lines, thereby promoting the efficient allocation of breeding resources (Jan et al., 2016).

Würschum et al. (2014) investigated the potential of GS in rapeseed breeding reporting medium-to-high prediction accuracies for several morphological-, quality-, and yield-related traits. Despite lower accuracy in the prediction of some novel families, it was concluded that with increased marker availability, GS will provide a valuable genomic tool in knowledge-based rapeseed breeding. GS has further been applied in winter-type oilseed rape (Werner et al., 2017), spring-sown canola (Jan et al., 2016), and a biparental population based on a cross between a European winter cultivar and a Chinese semi-winter cultivar (Zou et al., 2016; Liu P. et al., 2017). Werner et al. (2018) investigated the value of marker selection approaches in Asian rapeseed and illustrated that high prediction accuracies for polygenic traits are achievable with low marker density, given that the representative markers were selected with regard to the genome-wide linkage disequilibrium (LD) structure in a population.

Increased phenotypic heritability has been shown to have a greater impact on whole-genome prediction accuracies, more so than training set population size and marker density (Zhang et al., 2017). Fikere et al. (2020) reported moderate-to-high genomic prediction accuracies using genomic best linear unbiased prediction (GBLUP) models upon evaluating genetic correlations and genomic prediction accuracies for several agronomic, disease, and seed quality traits in canola. The inclusion of genotype-by-environment interaction in the GBLUP model resulted in further, though slight, improvements in predictions. Koscielny et al. (2020) confirmed these findings, demonstrating higher accuracy in whole-genome predictions within the stress treatment than within the control treatment for the majority of traits evaluated. It is therefore important, even in the genomics era, to link selected phenotypic or demographic models with the underlying processes of genomic variation. As demonstrated in the CWR *Brassica cretica*, if variation is largely selectively neutral, it is not possible to assume that a diverse population will inescapably display the wide-ranging adaptive diversity required for further crop improvement (Kioukias et al., 2020).

## FUTURE PROSPECTS

Mechanisms of interaction for stress responses involve complex interactions and traits and are therefore more difficult to investigate than direct interaction (Werner et al., 2018). While genomic advances have exponentially increased during the past

decades, high-throughput phenotyping has not caught up yet. To accelerate plant breeding and improve our understanding of genotype underlying expressed phenotype, dedicated high-throughput phenotyping approaches are required (Singh D. et al., 2019). It is therefore not surprising that high-throughput phenomics has increased in popularity, especially for the management and data collection of *Brassica* oilseed and vegetable crops. Improvements in sensor, drone, and remote sensing technology, as well as high throughput phenotyping techniques, are simplifying and enabling the quantification of complex phenotypic traits without the necessity of destructive sampling (Parmley et al., 2019). *Brassica* physiological studies, for example, plant height and biomass data (Moeckel et al., 2018), flower number (Wan et al., 2018), vegetation and flower fraction (Fang et al., 2016), and nitrogen nutrient studies (Graeff et al., 2008; Liu S. et al., 2018), have been generated using unmanned aerial vehicles. Assimilating large amounts of phenotypic data with the capabilities of machine learning will provide breeders with the analytical tools to optimize cultivar development in relation to target environment and accelerate the rate of genetic gain (Parmley et al., 2019).

Besides phenotypic characteristics, crop breeding requirements are also dictated by an assortment of additional major and minor variables such as environment, cultivation, and management practices and fluctuating consumer needs (Araus et al., 2018). Aligning breeding objectives to an increasing number of critical factors will require cross-disciplinary approaches driven by breeding teams, climate specialist, bioinformaticians, and crop modelers (Beveridge et al., 2019; Stöckle and Kemanian, 2020). Crop modeling can assist breeders in comprehending the influence and interaction of variable factors in the selection of desirable varieties (Stöckle and Kemanian, 2020).

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## CONCLUSION

Crop breeding has benefitted from the advancement of genomic tools and associated analysis pipelines. Available genomic resources and lower cost of high-throughput sequencing have contributed toward the increase in WGRS efforts. The vast amount of genomic information created and advances in genomic tools developed will significantly improve capturing the range of genetic diversity estimation and enhance the capturing and exploitation of diversity in *Brassica* germplasm profiles. The genetic libraries of CWRs should be further explored, as quality of available references and assembly methods has improved. The availability of GE tools has improved in precision and specificity; these systems are highly customizable and can be advantageously exploited to fast-track crop improvement. Although genomics is currently taking the center stage, a multidisciplinary plant breeding approach that includes phenotype = genotype × environment × management interaction backed by big data capabilities will ultimately ensure the selection of future-proof *Brassica* crops.

## AUTHOR CONTRIBUTIONS

AS-E, NM, and JB conceptualized the manuscript. NM, AS-E, and AP wrote the manuscript, with additions and edits from JB and DE. The tables were prepared by NM and AS-E. AS-E illustrated the figure. All authors read and approved the final manuscript.

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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.

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