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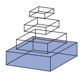

Allele mining in barley genetic resources reveals genes of race-non-specific powdery mildew resistance

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Race-non-specific, or quantitative, pathogen resistance is of high importance to plant breeders due to its expected durability. However, it is usually controlled by multiple quantitative trait loci (QTL) and therefore difficult to handle in practice. Knowing the genes that underlie race-non-specific resistance (NR) would allow its exploitation in a more targeted manner. Here, we performed an association-genetic study in a customized worldwide collection of spring barley accessions for candidate genes of race-NR to the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*) and combined data with results from QTL mapping as well as functional-genomics approaches. This led to the identification of 11 associated genes with converging evidence for an important role in race-NR in the presence of the *Mlo* gene for basal susceptibility. Outstanding in this respect was the gene encoding the transcription factor WRKY2. The results suggest that unlocking plant genetic resources and integrating functional-genomic with genetic approaches can accelerate the discovery of genes underlying race-NR in barley and other crop plants.

Keywords: *Hordeum vulgare, Blumeria graminis*

INTRODUCTION

Since approximately 10,000 years crop domestication has laid the basis for food-, feed-, fiber-, energy-, and health promoting-compound production for an ever increasing world population. However, while important domestication traits such as seed non-shattering have been fixed early on during this process, other potentially valuable alleles from wild crop relatives were lost due to genetic bottlenecks during domestication and breeding (Pourkheirandish and Komatsuda, 2007). This problem was further accentuated by modern variety breeding starting up in the early decades of the twentieth century, often returning to relatively narrow breeding pools aiming at efficient combination of the best varieties with the best alleles (Hyten et al., 2006; Haudry et al., 2007; Li et al., 2009). Nevertheless, plant genebanks have been established around the globe to save and maintain plant genetic resources in *ex situ* collections that include wild crop relatives as well as landrace material etc. These resources will have to be accessed and mined systematically in order to meet the challenge of increasing and securing crop yields worldwide in the near future under increasingly volatile climatic conditions (Feuillet et al., 2008).

One important aspect of yield security is the prevention of losses due to diseases and pests, which will have to be achieved by resistance breeding or gene technology rather than by chemical pest control in order to fulfill the requirement of sustainability. As far as resistance to microbial pathogens is concerned and within this group of organisms, especially to fungal pathogens two strategies are being followed: firstly, introduction of major resistance (*R*) genes usually conferring strong protection against specific fungal races that carry the matching avirulence genes. However, it has become clear that the efficacy of major *R* gene-mediated monogenic resistance, though relatively easy to introgress, is often broken down by new pathogen races after few years of use in the field – a phenomenon that is further accelerated by the planting of monogenically resistant cultivars on large acreage (Brown et al., 1993). The second strategy includes the accumulation of useful quantitative trait loci (QTL) of resistance donors in susceptible genotypes of a target crop species, which confers more durable and race-non-specific resistance (NR) but is, due to its polygenic mode of inheritance, more difficult to handle in breeding practice (Kou and Wang, 2010). In recent years advances in the understanding of the molecular biology of plant–pathogen interactions has allowed replacement of the operational term "avirulence" gene or protein by the mechanistic term "effector" designating pathogen-encoded proteins that are delivered to host cells in order to interact with host factors and support pathogen growth (Koeck et al., 2011). The co-evolutionary model for the non-durability of race-specific resistance versus the durability of NR was proposed by Jones and Dangl (2006) and has gained strong experimental support since then. According to this model NR, also often referred to as quantitative resistance, reflects pathogen-associated molecular pattern (PAMP)-triggered innate immunity (PTI) minus effector-triggered susceptibility (ETS). Plants posses a range of pathogen-recognition receptors of the receptor-like kinase type that bind

to and are activated by PAMPs. *R* Gene-mediated, race-specific resistance on the other hand reflects effector-triggered immunity (ETI) in plant genotypes carrying matching NB-LRR-type *R* genes for direct or indirect effector recognition. Although the rice receptor-like kinase Xa21 conferring resistance to *Xanthomonas oryzae* was initially referred to as *R* gene it has recently been re-classified as PAMP receptor (Thomma et al., 2011). Thus, both resistance mechanisms are triggered by different types of pathogen-derived signals and involve different signal perception and transduction components (Panstruga et al., 2009). They may, however, converge at some point resulting in overlapping sets of regulated genes and induced defense components such as reactive oxygen species or pathogenesis-related, antimicrobial proteins (Thomma et al., 2011).

In order to exploit NR in a more efficient manner for crop breeding knowledge about loci and – more importantly – underlying host genes are required. Prerequisites for such an approach will be the availability of extensive genetic and genomic resources and tools for high-resolution mapping, efficient back-crossing, and highly informed candidate-gene approaches. Barley (*Hordeum vulgare* ssp. *vulgare*) belonging to the Triticeae tribe of cereals, which includes wheat and rye, fulfills these requirements because well-characterized experimental populations and large collections of natural genetic variability (Rostoks et al., 2006; Waugh et al., 2009), high-density genetic maps (Close et al., 2009; Sato et al., 2009; Aghnoum et al., 2010), a physical genome map (Schulte et al., 2009), and extensive gene-expression data (Wise et al., 2007; Druka et al., 2010) have been made publicly available.

One of the major diseases of barley and wheat is powdery mildew caused by the obligate biotrophic ascomycete fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*). The interaction of barley with *Bgh* can be regarded as a model plant–pathogen interaction due to a large body of physiological, cellular, biochemical, and molecular information about changes in the host during compatible or resistant interactions (Collins et al., 2002; Huckelhoven, 2007; Wise et al., 2009) also taking advantage of high-throughput functional tools (Douchkov et al., 2005; Ihlow et al., 2008). Moreover, progress in identifying genes as well as effector proteins of *Bgh* has also been made recently (Bindschedler et al., 2009; Godfrey et al., 2010) and will be strongly supported by the availability of the genome sequence of the pathogen (Spanu et al., 2010). In summary, a better understanding of the barley–*Bgh* interaction – together with the *Arabidopsis thaliana*–*Golovinomyces orontii* interaction – will serve as important model for hundreds of powdery mildew–crop interactions, the clade of *Erysiphales* being extremely widespread and economically most important phylogenetic group of pathogens worldwide.

Here, we describe a candidate gene (CG) based approach in barley attacked by *Bgh* in order to identify potentially important host factors of NR. For this purpose, a trait-customized collection of barley *ex situ* genebank accessions was established that is enriched in lines exhibiting strong interaction phenotypes (strong NR versus high susceptibility). The collection was used for the re-sequencing of CGs derived either from previous functional-genomics approaches in barley or from *a priori* knowledge of genes relevant for plant innate immunity. As a result we generated combined genetic and functional-genomic evidence ("convergent evidence" CE throughout) for 11 barley genes to be involved either in host ETS or NR.

RESULTS

A TRAIT-CUSTOMIZED BARLEY COLLECTION

By referring to primary evaluation data of accessions from the IPK genebank over more than five decades, a list of 212 spring barley accessions was assembled that had been described as either resistant in a race-non-specific manner or as highly susceptible (Nover and Mansfeld, 1955; Nover and Lehmann, 1972, 1973). Re-evaluation of these accessions by detached leaf assays using two polyvirulent German *Bgh* isolates that together have overcome 44 major *R* genes representing almost all resistance specificities ever introduced into European barley germplasm (**Figure A1** in Appendix) resulted in the identification of 30 susceptible and 35 resistant accessions, most of them exhibiting strong phenotypes (**Figures 1A,B**). Because resistance segregated in some of the accessions, two single-seed descendant lines (referred to as "genotypes" throughout) were established from each of the 65 accessions and re-scored in the detached leaf assay. Field trials for *Bgh* resistance in Germany in 2007 (data not shown) and 2008 (**Figure 1C**) under high infection pressure derived from spreader rows revealed a strong correlation with resistance data from the detached leaf assay. NR in spring barley accessions could have been caused by known or unknown recessive loss-of-function alleles of the *Mlo* gene, a well-known susceptibility factor for powdery mildew in barley and other plant species (Buschges et al., 1997). We addressed this possibility in a transient complementation assay by bombarding a BAC clone carrying wildtype *Mlo* into epidermal cells of the resistant genotypes. As shown in **Figure 1D**, three lines (HOR 1457, HOR 1506, and HOR 3537) plus the positive control line Ingrid BC *mlo5* responded to BAC complementation with an increase in penetration efficiency of *Bgh* by a factor of at least five. Therefore, these genotypes were assumed to carry *mlo*-resistance alleles and excluded from the panel. Four out of five lines showing partial complementation by a factor of less than fivefold were not excluded because they might carry partially functional alleles of *Mlo* (highlighted by "?" below the bar chart) not completely masking the effect of other resistance QTL. Because *Mlo* was transiently expressed under the control of its own promoter, we expected not so observe hypersusceptibility in *Mlo*-containing genotypes as reported upon over-expression driven by a strong constitutive promoter (Elliott et al., 2002). Interestingly, the four partially *Mlo*-complementing lines exhibited HR-associated resistance, which is atypical for the *mlo*-resistance gene therefore suggesting indeed the effect of additional resistance loci. We also searched for the presence of the only known natural *mlo-11* allele, which was found in Ethiopian landraces and subsequently introgressed into European germplasm, by using a PCR-based DNA marker (**Figure A2** in Appendix; Piffanelli et al., 2004). Five lines including the three lines already excluded due to *mlo*-complementation were found to contain *mlo-11* and subsequently excluded from the collection. The two remaining *mlo-11* containing lines were partially (HOR 2543) or not (HOR 4408) complementing, which suggests presence of additional resistance loci effective against the *Bgh* isolate CH4.8 used here. The final trait-customized collection for race-non-specific, *mlo*-independent seedling resistance to

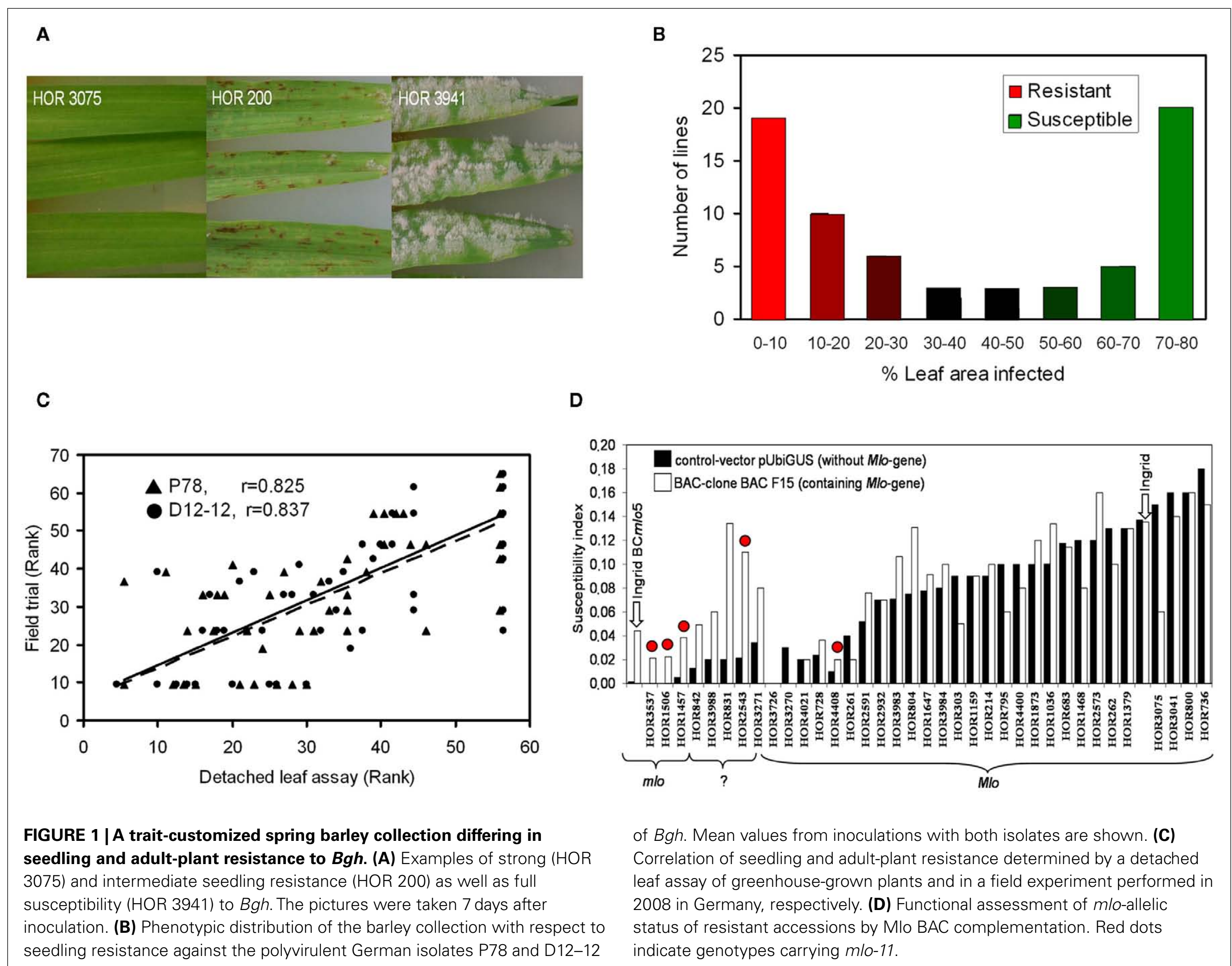

FIGURE 1 | A trait-customized spring barley collection differing in seedling and adult-plant resistance to *Bgh*. **(A)** Examples of strong (HOR 3075) and intermediate seedling resistance (HOR 200) as well as full susceptibility (HOR 3941) to *Bgh*. The pictures were taken 7 days after inoculation. **(B)** Phenotypic distribution of the barley collection with respect to seedling resistance against the polyvirulent German isolates P78 and D12–12 of *Bgh*. Mean values from inoculations with both isolates are shown. **(C)** Correlation of seedling and adult-plant resistance determined by a detached leaf assay of greenhouse-grown plants and in a field experiment performed in 2008 in Germany, respectively. **(D)** Functional assessment of *mlo*-allelic status of resistant accessions by Mlo BAC complementation. Red dots indicate genotypes carrying *mlo-11*.

Bgh consisted of 30 highly susceptible and 31 strongly to moderately resistant genotypes against two polyvirulent German *Bgh* isolates (**Table 1**). Fourteen of the resistant genotypes were also completely or partially resistant to four *Bgh* isolates from Israel that contained virulences for different *Mla* alleles present in wild barley (*H. vulgare* ssp. *spontaneum*; Dreiseitl and Dinoor, 2004). With the exception of HOR 3988, a landrace from India, no race-specific resistance pattern was observed with the Israeli isolates either. The customized collection represents only cultivated spring barley except for two genotypes of wild barley from Turkmenistan (HOR 2826) and from an unknown origin (HOR 1647).

INHERITANCE OF RESISTANCE TO *Bgh*

In order to characterize the mode of inheritance of *Bgh* resistance in the customized collection, three resistant lines were selected and crossed with the highly susceptible barley cv. Morex. The resistance donors were HOR 3271 (German cv "Steffi" possessing HR-associated resistance), HOR 3726 (North American cv "Nigrate" possessing papilla-associated resistance), and HOR 2932 (an Ethiopian landrace possessing HR-associated resistance). Phenotyping of F2 populations revealed quantitative trait segregation (**Figure 2**). Because major *R* genes can be inherited in a semi-dominant manner, a chi-square test for 1:2:1 segregation of strong (same phenotype as resistance donor) to intermediate resistance (all degrees of resistance between the parents) to susceptibility (same degree of susceptibility as cv. Morex) was performed. All three populations differed from the hypothetical 1:2:1 segregation pattern with $p = 0.002$ or lower suggesting that NR was based on several unlinked loci. QTL mapping was performed by using a panel of 384 highly polymorphic SNP markers. In each population 3–4 QTL were detected with profile LOD scores of composite interval mapping ranging from 3.0 to 18.8 (**Table 2**). Map positions of these QTL in the two populations showing HR-associated resistance were found to be in proximity of the race-specific resistance genes *Mlra*, *Mla*, *Mlk*, *MlLa*, and *mlt*, which opens up the question if resistance of the corresponding resistant parent was due to any of these *R* genes, especially because major *R* genes are known to mediate HR-associated resistance. This however appears unlikely for the following reasons: first, LOD scores of major *R* genes in populations of the size used here are usually higher (≥ 20) than the scores of the detected QTL. Second, each population possessed several additional weaker QTL with LOD scores between 2 and 3

Table 1 | Characterization of the customized barley collection for race-non-specific resistance to *Bgh*.

Accession[a]	Origin	Row number	Status of accession	% Inf. EU isol.[b]	% Inf. Israel isol.[c]	Resistance response[d]	*mlo-11*	Mlo compl.[e]
Ingrid BC mlo5	Denmark	2	Breeding line	**2.5**	n.a.	Papillae	NO	Full
HOR 2573[f]	Ethiopia	6	Landrace	**2.5**	**32.4**	HR	NO	NO
HOR 3270	Turkey	6	Breeding line	**2.5**	**5.8**	HR	NO	NO
HOR 3726[f]	USA	6	Cultivar	**2.5**	**11.4**	Papillae	NO	NO
HOR 1647[g]	Unknown	2	Wild	**2.9**	n.a.	HR	NO	NO
HOR 1036	Greece	6	Landrace	**3.3**	40.8	HR	NO	NO
HOR 2591	New Zealand	6	Cultivar	**3.3**	49.7	HR	NO	NO
HOR 4021	USA	6	Cultivar	**3.3**	**7.4**	HR	NO	NO
HOR 842	China	6	landrace	**3.6**	**14.5**	HR	NO	Partial
HOR 3075	Ethiopia	6	Landrace	**3.8**	**29.6**	HR	NO	NO
HOR 2932[f]	Ethiopia	6	Landrace	**4.5**	**32.4**	HR	NO	NO
HOR 3983	USA	6	Breeding line	**7.8**	42.7	HR	NO	NO
HOR 795	Ethiopia	2	Landrace	**8.1**	**26.8**	HR	NO	NO
HOR 728	Greece	6	Landrace	**9.7**	43.6	HR	NO	NO
HOR 1379	Greece	6	Landrace	**9.9**	52.1	HR	NO	NO
HOR 736	Albany	6	Landrace	**11.2**	54.4	HR	NO	NO
HOR 804	USA	6	Cultivar	**11.3**	46.0	HR	NO	NO
HOR 3984	USA	6	Breeding line	**12.0**	42.7	HR	NO	NO
HOR 261	USA	6	Cultivar	**13.3**	42.7	HR	NO	NO
HOR 683	Greece	6	Landrace	**13.4**	42.7	HR	NO	NO
HOR 3988	India	6	Landrace	**13.9**	**10.0**	HR	NO	Partial
HOR 1159	Greece	6	Landrace	**16.5**	**34.7**	HR	NO	NO
HOR 262	Unknown	6	Breeding line	**16.6**	41.3	HR	NO	NO
HOR 3041	India	2	Breeding line	**17.4**	**5.8**	HR	NO	NO
HOR 3271[f]	Germany	2	Cultivar	**19.1**	**2.5**	HR	NO	Partial
HOR 214	Japan	6	Landrace	**20.1**	45.0	HR	NO	NO
HOR 800	Yugoslavia	6	Breeding line	**20.5**	41.3	HR	NO	NO
HOR 1468	India	2	Breeding line	**21.3**	**4.1**	HR	NO	NO
HOR 4400	Germany	2	Cultivar	**22.4**	**2.5**	HR	NO	NO
HOR 1873	Greece	6	Landrace	**23.8**	48.3	HR	NO	NO
HOR 303	USA	2	Cultivar	**28.4**	38.9	HR	NO	NO
HOR 831	Unknown	2	Landrace	**33.8**	45.0	HR	NO	Partial
HOR 2826[g]	Turkmenistan	2	Wild	38.0	n.a.[h]		n.a	n.a.
HOR 844	Unknown	2	Landrace	38.0	n.a.		n.a	n.a.
HOR 3997	USA	2	Cultivar	42.7	n.a.		n.a	n.a.
HOR 4031	India	6	Landrace	54.4	n.a.		n.a	n.a.
Ingrid	Sweden	2	Cultivar	55.4	53		NO	NO
HOR 3275	China	6	Cultivar	59.1	n.a.		n.a	n.a.
BCC 3	Afghanistan	2	Landrace	61.4	n.a.		n.a	n.a.
BCC 1389	Ireland	2	Cultivar	61.4	n.a.		n.a	n.a.
BCC 190	Syria	2	Landrace	67.4	n.a.		n.a	n.a.
BCC 1376	Denmark	2	Cultivar	68.5	n.a.		n.a	n.a.
BCC 1408	UK	2	Cultivar	68.5	n.a.		n.a	n.a.
BCC 1405	UK	2	Cultivar	70.9	n.a.		n.a	n.a.
BCC 852	Canada	6	Cultivar	73.2	n.a.		n.a	n.a.
HOR 3941	Israel	2	Breeding line	73.2	n.a.		n.a	n.a.
HOR 4060	Israel	2	Cultivar	73.2	n.a.		n.a	n.a.
BCC 1431	Austria	2	Cultivar	75.5	n.a.		n.a	n.a.
BCC 888	Canada	6	Cultivar	75.5	n.a.		n.a	n.a.
BCC 903	Canada	2	Cultivar	75.5	n.a.		n.a	n.a.
BCC 423	China	6	Cultivar	75.5	n.a.		n.a	n.a.

(Continued)

Table 1 | Continued

Accession[a]	Origin	Row number	Status of accession	% Inf. EU isol.[b]	% Inf. Israel isol.[c]	Resistance response[d]	*mlo-11*	Mlo compl.[e]
BCC 1450	Finland	6	Cultivar	75.5	n.a.		n.a	n.a.
BCC 1430	France	2	Cultivar	75.5	n.a.		n.a	n.a.
HOR 2800	Iran	6	Landrace	75.5	n.a.		n.a	n.a.
BCC 1468	Kazakhstan	2	Cultivar	75.5	n.a.		n.a	n.a.
BCC 745	Nepal	6	Cultivar	75.5	n.a.		n.a	n.a.
BCC 1420	Netherlands	2	Cultivar	75.5	n.a.		n.a	n.a.
BCC 1452	Netherlands	6	Cultivar	75.5	n.a.		n.a	n.a.
BCC 1488	Russia	6	Landrace	75.5	n.a.		n.a	n.a.
BCC 1412	Sweden	2	Cultivar	75.5	n.a.		n.a	n.a.
BCC 1404	UK	2	Cultivar	75.5	n.a.		n.a	n.a.
BCC 893	USA	6	Cultivar	75.5	n.a.		n.a	n.a.
BCC 1498	Uzbekistan	6	Landrace	75.5	n.a.		n.a	n.a.

Scoring results below the median of class II (Altpeter et al., 2005) reflecting moderate to strong resistance are highlighted in bold.

[a] *Accession Nr. from IPK genebank.*

[b] *Mean resistance to two polyvirulent German isolates (total 44 virulences).*

[c] *Mean resistance to four Israeli isolates (total 18 virulences).*

[d] *Microscopic analysis of attacked epidermal cells.*

[e] *Complementation by bombarding epidermal cells with a BAC clone containing Mlo.*

[f] *Used as resistance donor for crosses with susceptible cv Morex to establish F2-mapping populations.*

[g] *H. vulgare ssp. Spontaneum.*

[h] *Not analyzed.*

(data not shown), in agreement with previous studies on quantitative resistance to *Bgh* that revealed a complex genetic setup of NR (e.g., Aghnoum et al., 2010). In summary, the QTL mapping of three selected barley genotypes exhibiting strong, race-non-specific seedling and adult-plant resistance to *Bgh* confirmed a polygenic mode of inheritance, as suggested by the phenotypic distribution of F2 progenies.

ASSOCIATION OF ALLELIC VARIANTS WITH RESPONSE TO *Bgh*

A total of 73 CGs were nominated based on previous transcript-profiling and transient-induced gene silencing (TIGS) data, a literature survey for genes that may be relevant for NR in barley, and genetic map information. Five additional genes were selected as negative controls because they were described to be involved in other traits such as the number of seed rows per spike ("row number" throughout) or seed development (Table S1 in Supplementary Material). Out of a total of 78 re-sequenced genes, 9 did not yield high-quality sequence data and 8 were monomorphic. The remaining 61 polymorphic genes were analyzed for SNP and deduced haplotypes (**Table 3**). Sequence diversity with 1 SNP per 48 bp was similar to what has been reported before in a worldwide collection of cultivated barley (Haseneyer et al., 2010b).

Population structure and kinship were determined by using 42 SSR markers evenly distributed over the barley genome (Tables S2 and S3 in Supplementary Material). Principal-component analysis (PCA) of kinship revealed no clear structuring of the population with respect to observed resistance or row number (**Figure 3A**; **Figure A3** in Appendix). The non-structuring of the data into two-rowed and six-rowed barley genotypes differs from other association-genetic studies in barley and probably reflects the high degree of genetic unrelatedness of members of the customized worldwide population (Haseneyer et al., 2010a). On the other hand, strong population structure was observed with respect to geographic origin (**Figure 3B**). Genotypes from USA, which all represent breeding lines or cultivars, were grouped together with genotypes from East Asia and the Balkans, suggesting that US breeders used such material rather frequently in their pedigree. The grouping of European cultivars with Ethiopian landraces suggests some preferred use of this exotic germplasm by European breeders, possibly for the introgression of major *R* genes.

Structured SNP-trait as well as haplotype–trait associations were calculated by using: a general-linear model; a general-linear model including row number as covariate; and a mixed-linear model (see Materials and Methods). Row number was included as a covariate into one model because of significant correlation between this trait and mean resistance to both *Bgh* isolates (Table S4 in Supplementary Material). The results of marker–trait associations from the general-linear model including population structure were highly similar to those from the mixed-linear model including both population structure and kinship, which indicates again a high degree of phylogenetic non-relatedness of members of the population (Johrde, 2010). Therefore, only results from the two general-linear models with or without row number as covariate are discussed here. Genes were assumed to be associated with a trait if Bonferroni-corrected p-values were smaller than 0.05. p-Values smaller than 0.01 were taken as sign of strong association with the trait. Bonferroni correction was based on the assumption that each polymorphic CG ($n = 87$ including some genes to be discussed elsewhere) represents o linkage block. For any set of randomly chosen markers and for any statistical model used in

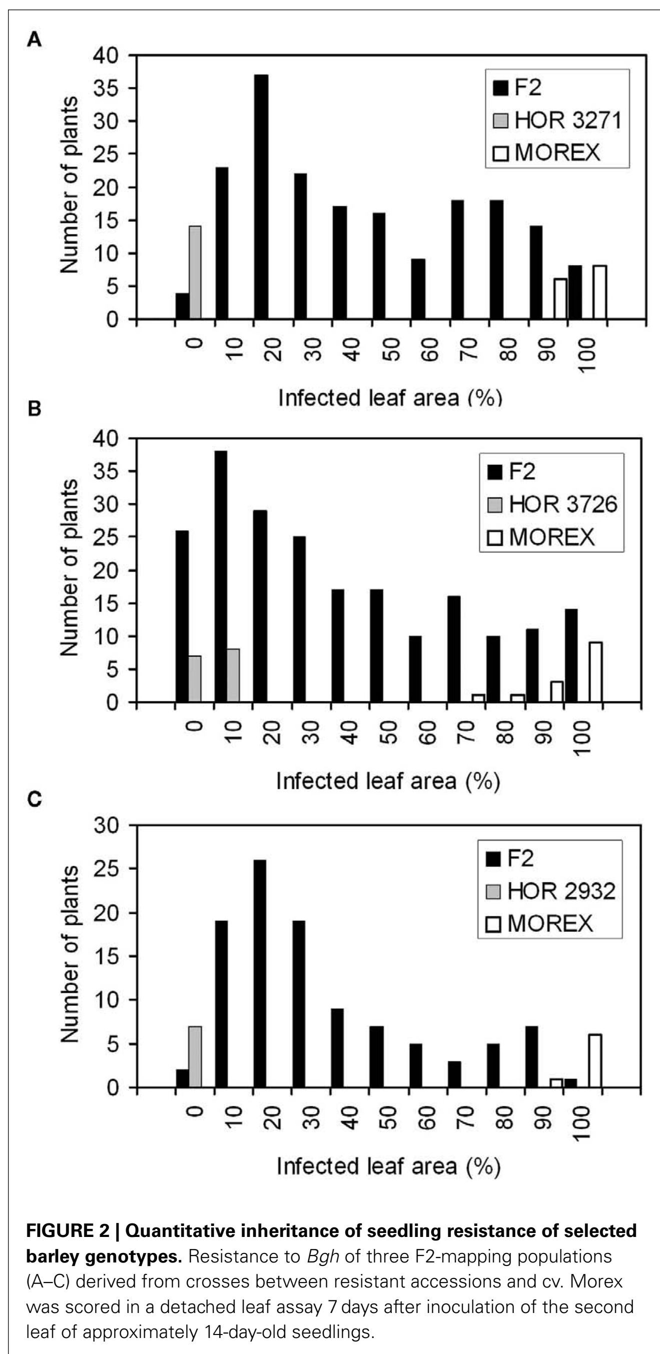

FIGURE 2 | Quantitative inheritance of seedling resistance of selected barley genotypes. Resistance to *Bgh* of three F2-mapping populations (A–C) derived from crosses between resistant accessions and cv. Morex was scored in a detached leaf assay 7 days after inoculation of the second leaf of approximately 14-day-old seedlings.

association genetics, the over-representation of spurious associations would result in a skewed distribution of *p*-values toward zero. In the perfect model for a given population, on the other hand, *p*-values would be expected to be randomly distributed between zero and one and lie on a diagonal when sorted. In order to test the accuracy of the selected models, we plotted sorted *p*-values of all SNP for traits "row number" and "*Bgh* interaction" (**Figure 4**). The distribution of *p*-values from both traits using the naive model was clearly skewed toward zero, as expected in the presence of population structure producing spurious associations. Using either the general- or mixed-linear models that take into account population structure resulted in an approximated random *p*-value distribution for "row number" but not for "*Bgh* interaction." This exactly meets the expectation because the CGs (and their SNP markers) were not selected at random but due to *a priori* knowledge about a function in NR or disease resistance in general. The same skewedness toward significant *p*-values has also been observed in sets of CGs of *A. thaliana* for 107 observed traits (Atwell et al., 2010).

Figure 5 shows the result from analysis of structured association of SNPs and haplotypes with the traits "*Bgh* interaction" and "row number." The fact that control gene *Vrs1*, a known factor determining row number in barley (Komatsuda et al., 2007) exhibited the strongest, significant association with "row number" indicates that the customized population, although relatively small, was suitable to detect marker–trait associations. A second CG (Contig5974_s_at) encoding HvWIR1a also showed highly significant association with row number. This may reflect genetic linkage with the *Vrs2* gene for row number (Pourkheirandish and Komatsuda, 2007), which was discovered in a mutant population of barley and mapped ca. three centimorgans away from *HvWIR1a* in the consensus map "Barley, Integrated, Marcel 2009"[1]. A total of 11 CGs were found to be associated with the *Bgh*-interaction phenotype at SNP or haplotype level (see also **Figure A4** in Appendix for graphical haplotypes). In most of the cases, association was also detected by using the general-linear model including row number as covariate. This indicates that the marker–trait associations were robust despite the fact that row number was correlated with resistance to *Bgh*.

The previously identified *Mla* gene for race-specific and *mlo* for race-NR of barley to *Bgh* were also included in the re-sequencing approach. In the case of *Mla*, a number of SNPs produced apparently heterozygotic calls, which most likely reflects PCR amplification of closely related *Mla*- and *RGH1* genes in several genotypes, as previously described (Wei et al., 2002). Therefore, only SNPs with homozygous calls derived from either gene were used for the calculation of association with the response to *Bgh*. Neither *Mla* nor *mlo* was associated with the response to *Bgh*, irrespective of the statistical model used for the analysis (Table S1 in Supplementary Material).

CO-LOCALIZATION OF GENES WITH RESISTANCE QTL

The 11 CGs that were significantly associated with the *Bgh*-interaction phenotype were genetically mapped, and map positions projected onto the "Barley, Integrated, Marcel 2009" map containing 6990 markers (see text footnote 1). This integrated map includes five biparental populations that have also been used for mapping of resistance QTL to *Bgh* (Aghnoum et al., 2010). Eight out of 11 CGs co-localized with resistance QTL to *Bgh* that have been described over the last 20 years (Schweizer and Stein, 2011) indicating a significant (χ^2, one-tailed $p = 0.0431$) over-representation (**Figure 6**). Thus, the associated CGs might be causally related to the QTL previously mapped to corresponding genome positions, or they reveal linkage to nearby causative genes within the QTL confidence intervals.

[1] http://wheat.pw.usda.gov/ggpages/map_shortlist.html

Table 2 | Mapping of resistance QTL against *Bgh* in three populations of resistant accessions x Morex.

F2-Population	Chr	cM	Left_marker	Right_marker	LOD[a]	Type[b]	Effect[c]	FreqCV[d]
Morex-1xHOR3726-1	4H	8	ge00201s01	ge00290s01	3.0	add	6.5	0.03
Morex-1xHOR3726-1	4H	113	ge00196s01	ge00212s01	4.5	add	−16.4	0.7
Morex-1xHOR3726-1	4H	113	ge00196s01	ge00212s01	4.5	dom	10.1	0.7
Morex-1xHOR3726-1	5H	96	ge00129s01	ge00374s01	4.9	add	−13.0	0.46
Morex-1xHOR3726-1	5H	96	ge00129s01	ge00374s01	4.9	dom	0.0	0.46
Morex-1xHOR3726-1	7H	17	ge00317s01	ge00180s01	7.7	add	−28.2	0.69
Morex-1xHOR3726-1	7H	17	ge00317s01	ge00180s01	7.7	dom	−13.4	0.69
Morex-2xHOR2932-2	1H	16	ge00346s01	ge00275s01	8.8	add	−19.7	0.94
Morex-2xHOR2932-2	1H	16	ge00346s01	ge00275s01	8.8	dom	0.0	0.94
Morex-2xHOR2932-2	1H	108	ge00033	ge00365s01	3.5	add	6.6	0.02
Morex-2xHOR2932-2	3H	155	ge00146	ge00227s01	3.1	add	6.2	0.05
Morex-2xHOR3271-2	2H	156	ge00372s01	ge00260s01	18.9	add	−21.7	0.91
Morex-2xHOR3271-2	2H	156	ge00372s01	ge00260s01	18.9	dom	−15.5	0.91
Morex-2xHOR3271-2	4H	111	ge00023	ge00212s01	5.3	add	−5.2	0.08
Morex-2xHOR3271-2	7H	22	ge00180s01	ge00384s01	4.0	add	−9.3	0.25
Morex-2xHOR3271-2	7H	22	ge00180s01	ge00384s01	4.0	dom	0.0	0.25

[a] *LOD, logarithm of odds, obtained by composite interval mapping.*

[b] *add, Additive QTL effect; dom, dominant QTL effect.*

[c] *QTL effect in percent of phenotypic variation.*

[d] *FreqCV, frequency in cross validation.*

Table 3 | Summary of polymorphic candidate genes used for association of allelic variants with traits by re-sequencing.

Selection criterium	Number of CG[a]	Number of SNP (bp/SNP)	Number of haplotypes
TIGS, OEX, Transcr. profiling	33	515 (42.2)	274
Evidence from literature	27	343 (60.3)	231
Negative control genes	1	7 (150.7)	8
Total	61	866	512
Average/gene		14.2 (47.8)	8.4

[a] *CG, candidate gene (only polymorphic sequences taken into account).*

TRANSCRIPT LEVELS OF ASSOCIATED CGs

The CGs significantly associated with *Bgh*-interaction phenotype are represented as oligonucleotides on a custom 44K barley transcript-profiling microarray (Chen et al., 2011) and were assessed for regulation of the corresponding transcripts in peeled barley epidermis attacked by either *Bgh* or the non-adapted wheat powdery mildew *B. graminis* f. sp. *tritici* (Bgt; **Figure 7**; Table S5 in Supplementary Material). Seven out of the 11 transcripts were either significantly up- or down-regulated upon pathogen attack, with no clear difference between host and non-host interaction. The strongest up-regulation was observed at 24 h.a.i., except for transcription factor *HvWRKY2* that exhibited a peak of mRNA abundance at 12 h.a.i.

TIGS OF ASSOCIATED CGs

Ten RNAi silencing (TIGS) or over-expression constructs were obtained for the CGs significantly associated with "*Bgh* interaction." These were used to test a phenotypic effect by TIGS or by transient over-expression in the barley–*Bgh* interaction. Because TIGS constructs were found to induce a weak but non-specific reduction of susceptibility of bombarded epidermal cells, data were tested for statistical significance using the median susceptibility index (normalized to corresponding empty-vector controls) of more than 1,000 constructs bombarded in different projects as more critical null-hypothetical value, rather than the value of the internal empty-vector control (**Figure A5** in Appendix). **Table 4** shows that TIGS of three candidates (*HvWRKY2, vacuolar targeting receptor* and an unknown protein) significantly increased resistance (reflected by reduced relative susceptibility index) whereas one (*chorismate synthase*) increased resistance upon transient over-expression. The rather low number of genes with a significant TIGS effect might be surprising because such an effect was one major initial selection criterion for our CGs. However, as discussed above, the initial set of candidates was compiled based on TIGS data that were tested with less stringent statistics. We cannot exclude, though, that the median-centered control caused false-negative results to some extent because (i) the obligate biotrophic pathogen *Bgh* might indeed be highly sensitive to

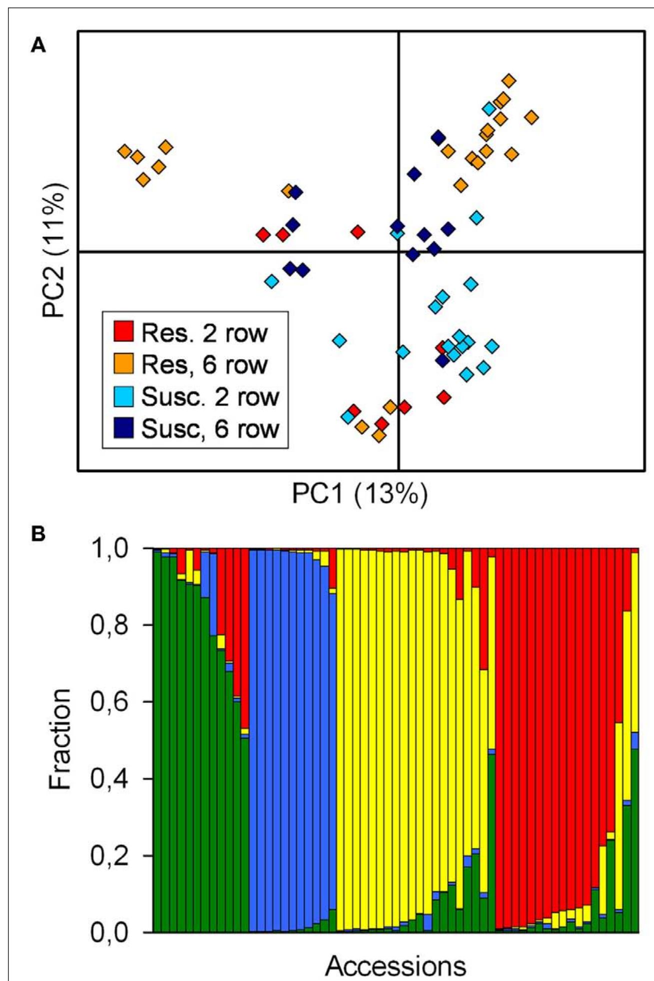

FIGURE 3 | Population stratification within the customized barley collection used for association of allelic variants with traits. **(A)** Principal component analysis (PCA) was performed by using 42 SSR markers. Minor SSR alleles (frequency < 0.05) were eliminated prior to analysis. For display of PC3 and 4 see **Figure A3** in Appendix. **(B)** Population subgroups were determined as proposed by Evanno et al. (2005) by using the software STRUCTURE (Pritchard et al., 2000) and the same 42 SSR markers as for PCA. Green, accessions mostly from Near East and Central Asia; blue, accessions mostly from East Asia and USA; yellow, accessions mostly from Balkans and USA; red, accessions mostly from Europe and Ethiopia.

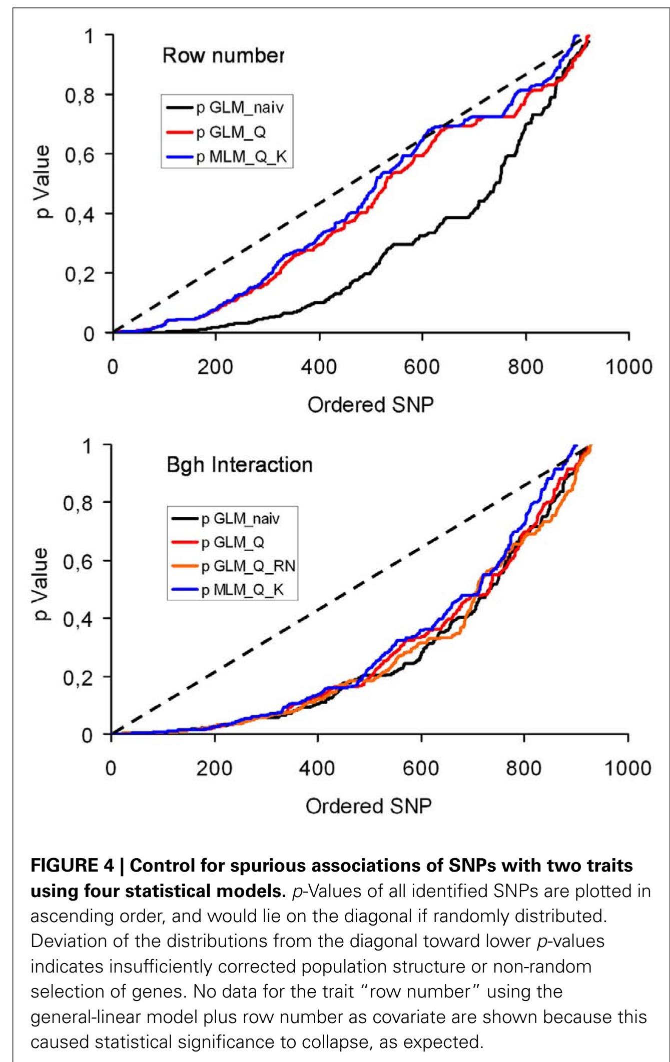

FIGURE 4 | Control for spurious associations of SNPs with two traits using four statistical models. *p*-Values of all identified SNPs are plotted in ascending order, and would lie on the diagonal if randomly distributed. Deviation of the distributions from the diagonal toward lower *p*-values indicates insufficiently corrected population structure or non-random selection of genes. No data for the trait "row number" using the general-linear model plus row number as covariate are shown because this caused statistical significance to collapse, as expected.

any RNAi-mediated disturbance of host–cell metabolism, homeostasis, or effector responsiveness, and because (ii) many of the candidates selected based on the preliminary TIGS data finally did obtain a CE score of three (out of a maximum of four) suggesting relevance for barley–*Bgh* interaction (see below).

CE SCORE FOR GENES AFFECTING *Bgh* INTERACTION

We assigned a CE score to the genes significantly associated with the *Bgh* interaction phenotype by assessing their localization inside QTL confidence intervals, their transcript regulation upon *Bgh* attack, and TIGS- or over-expression effects on resistance to *Bgh* (**Table 5**). This revealed one gene encoding HvWRKY2 (Contig4387_at) that obtained the maximum CE score of 4 and thus represents a prime candidate for future validation. Eight additional candidates were assigned a CE score of 3 and will also deserve further attention. The nine CGs with strong CE for a role in NR of barley to *Bgh* include five with a putative role in signal perception or defense- and cell-death regulation (*HvWRKY2, Calreticulin, Cys-rich RLK, OPD-reductase, alpha/beta hydrolase*) and one of each implicated in primary metabolism (*chorismate synthase*), transport (*vacuolar targeting receptor*), and stress response (*HvWIR1a*).

DISCUSSION

In this study we mined barley genetic resources for genes and alleles that may be relevant for NR to *Bgh*. Subsequently converging evidence for the identified genes was generated by integrating forward- and reverse-genetic datasets. As a result, 11 CGs were identified that exhibited significant SNP or haplotype associations with the *Bgh*-interaction phenotype being either associated with strong, race-non-specific seedling resistance or high susceptibility in a trait-customized worldwide collection of spring barley.

Several observations suggest that the seedling resistance to *Bgh* we selected for during the phenotypic screening of an initial set

Probe-set Barley1	AM ID	Ref.	Annotation (BlastX)	***Bgh* interaction**				**Row number**	
				SNP		**Hapl.**		**SNP**	**Hapl**
				GLM_Q	**GLM_Q_RN**	**GLM_Q**	**GLM_Q_RN**	**GLM_Q**	**GLM_Q**
X56136	AM_IPK_PM01	Runeberg-Roos and Saarma (1998)	Aspartate protease	6,3E-04	4,6E-04	1,3E-03	1,1E-03	7,9E-04	4,0E-04
Contig12036_at	AM_IPK_PM02	Vitale and Hinz (2005)	Vacuolar targeting receptor	2,8E-05	7,4E-05	4,6E-04	1,4E-03	2,6E-02	2,6E-02
Contig5146_at	AM_IPK_PM03	Li et al. (2009)	OPD-reductase	1,1E-03	7,3E-04	2,4E-04	2,3E-04	2,1E-03	9,5E-04
AB259783	AM_IPK_Vrs1	Komatsuda et al. (2007)	Vrs1	7,7E-03	9,2E-03	7,7E-03	9,2E-03	4,3E-05	9,3E-06
Contig4636_at	AM_IPK_PM04	n.a.	Unknown protein	6,1E-06	7,1E-05	n.a.	n.a.	1,3E-01	n.a.
Contig1903_at	AM_IPK_PM05	Saijo et al. (2009)	Calreticulin	3,7E-04	4,3E-04	1,7E-03	2,1E-03	1,6E-03	4,0E-03
Contig5108_s_at	AM_IPK_PM06	Hu et al. (2009)	Chorismate synthase	1,5E-04	9,4E-06	3,1E-03	1,3E-03	1,5E-01	6,9E-02
Contig2169_at	AM_IPK_PM07	Waspi et al.(1998)	Alpha/beta hydrolase	2,1E-04	3,0E-04	2,1E-04	5,0E-04	8,3E-01	8,3E-01
Contig5974_s_at	AM_IPK_PM08	Douchkov et al. (2010)	HvWIR1a	1,5E-02	1,2E-02	3,4E-04	1,8E-03	9,4E-03	6,4E-05
Contig24190_at	AM_IPK_PM09	Acharya et al.(2007)	Cys-rich receptor-like kinase	5,2E-04	1,3E-03	5,2E-04	3,4E-04	2,2E-03	2,2E-03
Contig6454_at	AM_IPK_PM10	Keisa et al. (2008)	HvLSD1b	1,4E-04	5,1E-03	3,6E-03	5,1E-03	7,8E-02	9,8E-02
Contig4387_at	AM_IPK_PM11	Shen et al. (2007)	HvWRKY2	2,4E-04	2,3E-04	2,4E-04	2,3E-04	9,5E-04	9,5E-04

FIGURE 5 | Candidate genes significantly associated with resistance to *Bgh*. Association of SNP and gene haplotypes was calculated for traits "*Bgh* interaction" and "row number" by using general-linear models that included population structure (Q-matrix, both traits) or population structure plus row number (RN) as covariate (only *Bgh*). The *Vrs1* gene (highlighted in green) was included as positive control for association with row number. Genes highlighted by gray shading are located within confidence intervals of QTLs for resistance to *Bgh*. Please note that *HvWIR1a* showing also significant association with RN mapped close to RN gene *Vrs2* (Pourkheirandish and Komatsuda, 2007). Yellow and orange shading indicates significant association with Bonferroni-corrected $p < 0.05$ and $p < 0.01$, respectively. Only the most significant SNP and haplotype per candidate gene and model is shown.

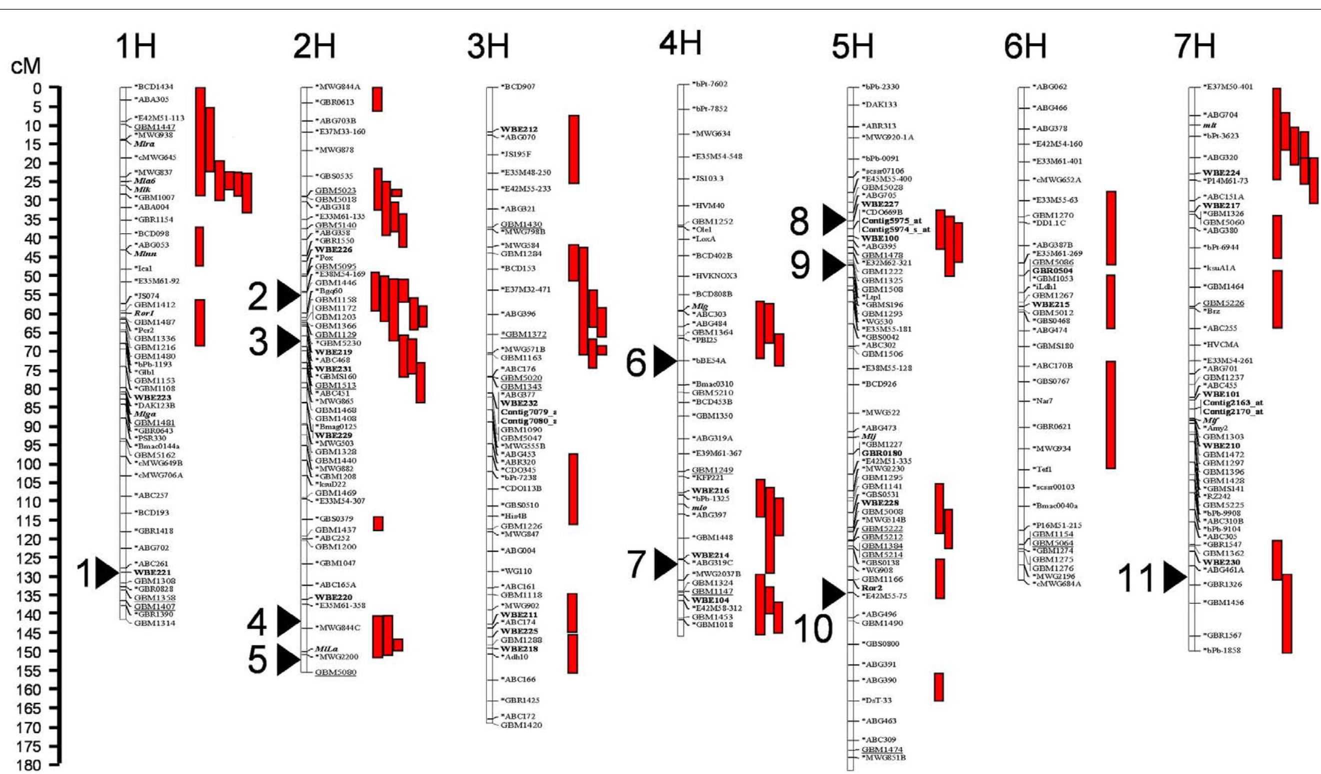

FIGURE 6 | Co-localization of associated genes with QTL for resistance to *Bgh*. QTL positions projected onto the "Marcel et al., integrated, 2009" map of barley are indicated by bars (Schweizer and Stein, 2011). They either span the distance between QTL flanking markers or indicate peak marker positions ±5 cM. QTL longer than 30 cM were excluded from the analysis. Numbers 1–11 indicate AM_IPK_PM01–11 (see also **Figure 5**).

of 212 accessions was due to NR and distinct from race-specific resistance mediated by *major R* gene or from *mlo*-mediated race-NR: firstly, accessions likely to carry *mlo*-resistance alleles were excluded from the panel based on genotyping for *mlo-11* and complementation by *Mlo* in a transient expression assay. Secondly, *Mlo* was extensively re-sequenced in the final population (almost the entire length of mRNA) without significant association to the "*Bgh* interaction" trait (Table S1 in Supplementary Material). Thirdly, re-sequencing of *Mla* did also not reveal significant association with "*Bgh* interaction," indicating that resistance was probably not

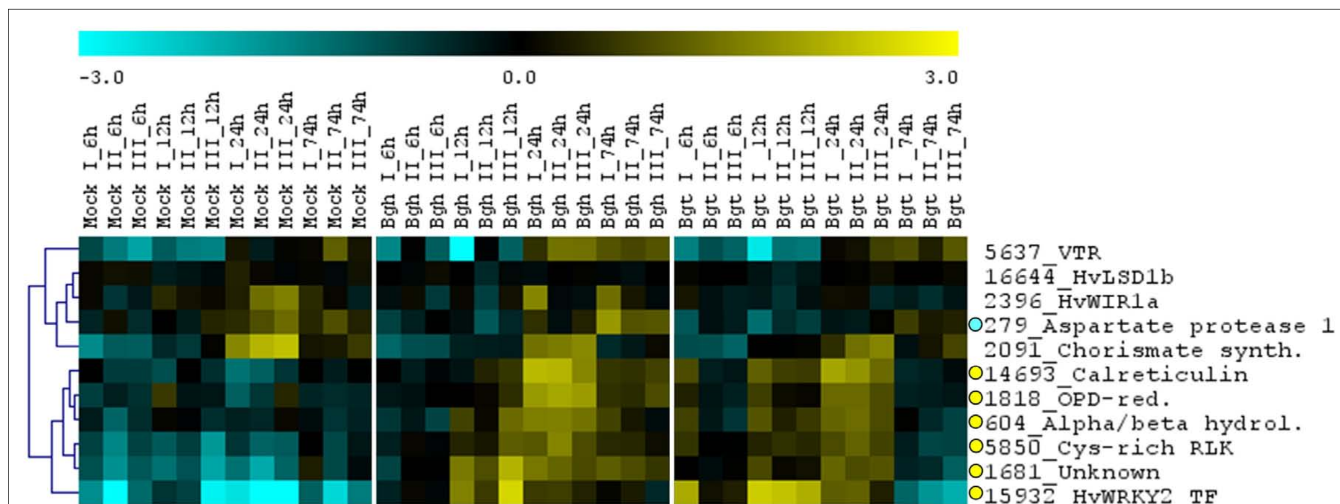

FIGURE 7 | Transcript regulation of associated genes in *Bgh*-attacked barley epidermis. Hierarchical clustering of normalized signal intensities after log(2)-transformation and median centering per transcript. RNA was derived from peeled epidermis after inoculation with *Bgh* or the wheat powdery mildew (Bgt). Data from three independent biological replicates are shown (I–III). Significantly ($p < 0.05$; FDR < 0.05) regulated genes are highlighted by yellow (upregulated) or blue (down-regulated) dots. Numbers indicate HarvEST_assembly_U35 unigene IDs. For the complete dataset see Table S5 in Supplementary Material.

Table 4 | TIGS of CGs significantly associated with resistance to *Bgh*.

Probe-set Barley1	Annotation (BlastX)	Relative SI (%)[a]	*p* (*t*-Test)[b]
Contig1903_at	Calreticulin	n.a.	n.a.
Contig4387_at	**HvWRKY2**	**44.6**	**0.04035**
Contig12036_at	**Vacuolar targeting receptor**	**57.6**	**0.03399**
Contig4636_at	**Unknown protein**	**66.4**	**0.0416**
Contig5146_at	OPD-reductase	68.8	0.18559
Contig6454_at	HvLSD1b	71.7	0.19973
Contig5108_s_at	**Chorismate Synthase**	**72.0**[c]	**0.0067**[c]
Contig2169_at	Alpha/beta hydrolase	72.2	0.18728
Contig5974_s_at	HvWIR1a	72.7	0.12130
Contig24190_at	Cys-rich receptor-like kinase	78.2	0.36246
Contig2354_at	Aspartate protease gene	91.7	0.44733

[a] *Relative SI, susceptibility index relative to the empty-vector control pIPKTA30; Mean value of at least five independent bombardment experiments.*

[b] *One-sample t-test of the log(2)-transformed relative SI against the hypothetical value "−0.355" corresponding to the median value of first-round TIGS results of 1084 constructs. This hypothetical value is assumed to represent a non-specific effect of any RNAi construct. Candidate genes with significant TIGS or over-expression effect are highlighted in bold.*

[c] *No TIGS data available. Instead the gene was transiently overexpressed. Statistical significance was calculated by one-sample t-test of the log(2)-transformed relative SI against the hypothetical value "0" corresponding to pIPKTA30 internal empty-vector control.*

caused by unexploited *Mla* alleles such as *Mla16–Mla20* derived from *H. vulgare* ssp. *spontaneum*, against which virulence in European *Bgh* isolates might still be absent. Fourthly, testing of resistant accessions with four *Bgh* isolates from Israel collected near the center of diversity of *H. vulgare* ssp. *spontaneum* and differentiating between *Mla* alleles of wild barley, identified 14 genotypes to be at least partially resistant in a race-non-specific manner (Dreiseitl and Dinoor, 2004). Finally, resistance of three F2 populations derived from crosses of resistant accessions (including two accessions exhibiting HR-type resistance) with cv Morex segregated in a quantitative manner indicating the action of several unliked resistance QTL rather than major *R* genes. Indeed QTL mapping revealed 3–4 QTL in each of these populations. The sum of the additive QTL effects explained between 32.5 and 64.1% of the phenotypic variation in the individual populations suggesting a complex genetic setup of NR with additional loci of minor effect not detected here. Two of the resistance donors are cultivars (HOR 3726, cv "Nigrate" and HOR 3271, cv "Steffi") and thus preferable for resistance-breeding purposes because they would introduce less linkage drag. Especially interesting in this respect would be the strong QTLs on the bottom of chromosome 2H and on the top of 7H explaining together 50% of phenotypic variation. Remarkably,

Table 5 | Converging evidence for CGs associated with resistance to *Bgh*.

Probe-set Barley1	Annotation (BlastX)	Chr	cM	Mapped[a]	Sign AM	In QTL	Regul.[b]	TIGS effect[c]	CE score[d]
Contig4387_at	**HvWRKY2**	7H	136.6	ZIPPER_stringent	SNP&Hapl	YES	UP	YES	**4.0**
Contig5108_s_at	**Chorismate synthase**	4H	62.1	ZIPPER_stringent	SNP	YES	NS	YES (OEX)	**3.0**
Contig1903_at	**Calreticulin**	2H	151.4	BOPA1_2	SNP	YES	UP	Missing	**3.0**
Contig2169_at	**Alpha/beta hydrolase**	4H	126.1	Marcel_09_integr.	SNP&Hapl	YES	UP	NS	**3.0**
Contig24190_at	**Cys-rich receptor-like kinase**	5H	46.2	BOPA1_2	SNP&Hapl	YES	UP	NS	**3.0**
Contig5974_s_at	**HvWIR1a**	5H	35.5	Marcel_09_integr.	Hapl	YES	UP	NS	**3.0**
Contig12036_at	**Vacuolar targeting receptor**	2H	46.2	Marcel_09_integr.	SNP&Hapl	YES	NS	YES	**3.0**
Contig5146_at	**OPD-reductase**	2H	64.2	BOPA1_2	Hapl	YES	UP	NS	**3.0**
Contig4636_at	**Unknown protein**	2H	136.2	Marcel_09_integr.	SNP	NO	UP	YES	**3.0**
Contig6454_at	HvLSD1b	5H	134.6	Marcel_09_integr.	SNP	YES	NS	NS	2.0
Contig2354_at	Aspartate protease	1H	128.1	BOPA1_2	SNP	NO	DOWN	NS	2.0

[a] *BOPA1_2, Consensus map "BOPA1 and BOPA2 Consensus" based on barley oligo pool arrays (OPA) 1 and 2(Close et al., 2009); Marcel_09_integr, consensus map "Barley, Integrated, Marcel 2009" as described by Aghnoum et al., 2010; ZIPPER_stringent, in silico mapping by synteny-based gene-order prediction in grasses (Mayer et al., 2011).*

[b] *Transcript regulation in Bgh-attacked epidermal peels (see also **Figure 7**).*

[c] *OEX, transient over-expression; NS, not significant.*

[d] *CE, convergent evidence (see Materials and Methods). Candidate genes with CE score of 3–4 are highlighted in bold.*

one of the CGs with a CE score of 3 encoding a calreticulin protein maps close to the QTL on the long arm of 2H. The donor of this QTL (HOR 3726) also carries the resistance-associated SNP12 in the calreticulin gene. Taken together, our results suggest the existence in cultivated barley of strong NR despite the presence of *Mlo* susceptibility alleles, which appears to be brought about by efficient allele combinations of defense- or susceptibility-related host genes.

Unlike proposals by other studies of NR or *mlo*-mediated, race-NR of barley (Schulze-Lefert, 2004), most of the resistant genotypes did not exhibit papilla-associated penetration resistance but responded to *Bgh* isolates by triggering HR at early or later stages during the interaction. In the latter case, macroscopically visible, darkly pigmented leaf flecks became visible on *Bgh*-attacked leaves. The absence of rapidly acting (penetration) resistance is also reflected by substantial haustorium formation in GUS-expressing transformed cells of the majority of resistant lines. Strong support for the absence of a tight link between HR and race-specificity of resistance comes from the characterization of *Pm21*-mediated-NR in wheat, which is associated with HR (Cao et al., 2011). Four out of 11 genes associated with the *Bgh*-interaction phenotype encode proteins that were proposed to be relevant for cell-death responses in barley or other plants: the identical *aspartate protease* gene (Contig2354_at) was highly expressed during autolysis of barley sieve cells (Runeberg-Roos and Saarma, 1998); OPD-reductase (Contig5146_at) is a key enzyme of the jasmonic-acid biosynthetic pathway, which is implicated in senescence- and cell-death regulation via the EDS1/PAD4/SAG101 protein complex (Reinbothe et al., 2009); Lsd1 proteins (Contig6454_at) were described as negative regulators of oxidative-stress-triggered runaway cell death in *A. thaliana* (Dietrich et al., 1997); *HvWRKY2* (Contig4387_at) has been implicated both in *Mla10*-mediated cell death and PTI in *Bgh*-attacked barley (Shen et al., 2007). It therefore appears possible that allelic diversity in genes regulating cell-death responses contribute to NR in the population used here.

We also tested further components of cell-death pathways such as *bax-inhibitor 1* or *EDS1* (Falk et al., 1999; Huckelhoven et al., 2003). Whereas *EDS1* turned out to be monomorphic in the customized collection, *Bax-inhibitor 1* might be also be associated with *Bgh*-interaction phenotype although the most strongly associated SNP $[-\text{Log}(10)p = 2.9]$ was not significant after Bonferroni correction ($p = 0.105$).

Several of the CGs associated with the *Bgh*-interaction phenotype or close paralogs had previously been discussed with respect to plant–pathogen interactions: the identical *chorismate synthase* gene (Contig5108_s_at) was found to be upregulated at the transcript level in *Bgh*-attacked barley leaves and to affect NR in transient over-expression and silencing experiments (Hu et al., 2009). The enzyme occupies an important branch point downstream of the shikimate pathway leading to the synthesis of the aromatic amino acids phenylalanine and tryptophan, precursors of lignin-like materials accumulating in attacked cell walls and indole alkaloids such as gramine exhibiting antimicrobial activity in barley, respectively (Matsuo et al., 2001). Another candidate, *calreticulin* (Contig1903_at), is the closest and second closest homolog to CRT1 and CRT3 of *A. thaliana*, respectively, that were reported as important components of defense-signaling during ETI and receptor-quality control during PTI (Saijo et al., 2009; Kang et al., 2010). Finally, a RLK containing a N-terminal cysteine-rich domain distinct from the DUF26-motif might be involved in PAMP perception (Jones and Dangl, 2006).

Two genes (*HvWIR1a* and *Cys-rich RLK*) were found to co-localize inside the meta-QTL for resistance to *Bgh* on chromosome 5HS and both were significantly associated with the *Bgh*-interaction phenotype. This can be explained either by a complex genetic setup of the QTL with several underlying genes or by LD between the associated candidates and a nearby causative gene. We found that the *Cys-rich RLK* gene was not polymorphic between Dom and Rec, the parents of the OWB population that contributed one of the QTL to the meta-QTL and

thus probably not responsible for the resistance QTL, at least not in this population. By contrast, different (rare) alleles of *HvWIR1a* are present in Dom and Rec leaving *HvWIR1a* as the more likely candidate for the causative gene for NR, as previously suggested (Douchkov et al., 2011). However, we neither found a significant TIGS effect of *HvWIR1a* nor of the *Cys-rich RLK*. The negative result of *HvWIR1a* silencing differs from a previously reported result (Douchkov et al., 2011) and can be attributed to the fact that we applied a more stringent statistical analysis here, as described above. In conclusion it appears quite possible that another, yet undiscovered gene located in the region is responsible for the observed, robust meta-QTL effect against *Bgh*. Unlike the initial assumption that association-genetic analyses result in narrower peaks of significant markers compared to biparental QTL analysis, due to many more meiosis events having broken down genetic linkage, genomic regions of markers significantly associated with a given trait can be wide and blurry, sometimes even without a clear maximum. This phenomenon has been discussed in terms of two factors confounding the analysis and sometimes prohibiting the identification of the causative genes: firstly, co-selection between nearby genes can occur if they together influence a given trait and secondly, population structure can cause "fixed" loci of low genetic diversity (Atwell et al., 2010). Therefore, saturating the resistance QTL confidence interval on barley chromosome 5HS with genes and performing systematic functional analysis to provide CE might be a more promising approach to identify the causative gene(s).

HvWRKY2, which was the only gene with maximum CE score in this study, was proposed earlier as an important factor of ETS as well as *Mla10*-mediated resistance (Shen et al., 2007). According to the current model HvWRKY2 is a transcriptional repressor of defense-related genes, and sequestration of the repressor by a Mla10/Avra10 complex results in the triggering of hypersensitive cell death (Shen et al., 2007). It remains open whether HvWRKY2 is directly targeted by an effector protein (e.g., AvrMla10) of *Bgh* triggering either its transcriptional up-regulation or its protein stabilization or activation. Here we report on natural allelic diversity of *HvWRKY2* that was associated with *Bgh*-interaction phenotypes by unlocking worldwide barley genetic resources. This result lends the first direct genetic evidence that *HvWRKY2* is indeed participating in plant–pathogen co-evolution, at least in the barley–*Bgh* system, and that valuable alleles might be identified and exploited for breeding. Interestingly, *HvWRKY2* was one of the few examples where we could identify a resistance- (and not susceptibility-) associated haplotype that was defined by a non-synonymous exonic SNP (**Figure A4** in Appendix).

To conclude, by searching for natural allelic diversity associated either with susceptibility or NR to *Bgh* in a trait-customized collection of barley genotypes we identified 11 genes that were further analyzed in an approach of converging evidence. This produced robust information for a number of candidates likely to explain at least part of the phenotypic variation observed in barley interacting with *Bgh*. Five out of nine top candidates with CE score of 3–4 (*Cys-rich RLK, Calreticulin, OPD-reductase, HvWRKY2, chorismate synthase*) have been implicated in PTI supporting the notion that the observed NR is based on PTI, despite the fact that resistance was primarily associated with cell-death responses and not papilla formation. We are currently using several of these candidates for marker-assisted introgression and outcrossing of favorable and unfavorable alleles, respectively, in elite spring barley lines suffering from susceptibility to the disease. This should allow conclusions about their value for improving durable and broadly acting resistance against *Bgh* – and maybe one or several other fungal diseases – by a knowledge-based approach.

MATERIALS AND METHODS

POWDERY MILDEW RESISTANCE SCORING

Barley plants used for association of allelic variants with traits were grown in 7 × 11 multipot trays containing compost soil from IPK nursery without fertilization in a greenhouse at 17–20˚C with supplemental light from sodium halogen lamps to reach a photoperiod of 16 h. Hypervirulent *B. graminis* (DC.) E. O. Speer f. sp. *hordei*, isolates 78P and D12–12 were used for resistant screening and cultivated by weekly inoculation of 7-day-old seedlings of barley cv "Golden Promise." Second leaves of 2-week-old seedlings were inoculated with *Bgh* 78P or D12–12 in a detached leaf assay, and *Bgh* symptoms were scored 7 days after inoculation as described (Altpeter et al., 2005). For resistance scoring of segregating F2 populations, isolate CH4.8 was used, and disease severity on second leaves was determined by estimating the percentage of the area covered by pustules (rounded to the nearest multiple of 10, e.g., 30 or 70%). Additional inoculation experiments in the detached leaf assay were performed with four powdery mildew isolates (H-148, H-289, Y-035, and Y-069) from Israel described as virulent against different *Mla* alleles present in wild barley *H. vulgare* ssp. *spontaneum* (Dreiseitl and Dinoor, 2004). Powdery mildew symptoms were also rated on plants grown under high infection pressure in double rows in the field at one location (KWS LOCHOW GmbH, Wohlde, Germany) in years 2007 and 2008.

SCREENING FOR *mlo*-ALLELES

Segments of second leaves of 14-day-old powdery mildew-resistant accessions were co-bombarded with a mixture of GUS-reporter plasmid pUbiGUS (Schweizer et al., 1999) and BAC F15, carrying wildtype *Mlo* (Buschges et al., 1997). Two days after the bombardment leaf segments were inoculated with *Bgh* isolate D12–12 at a density of approx. 60 conidia mm^{-2}, and haustoria in GUS-stained (transformed) epidermal cells were counted 48 h after infection. The susceptibility index was calculated as (ΣGUS-stained epidermal cells containing at least one haustorium/ΣGUS-stained epidermal cells). Each accession was bombarded with or without BAC F15, and complementation of susceptibility by a factor of at least five was taken as indication of *mlo*-mediated resistance. For genotyping of *mlo-11* isolated DNA of powdery mildew-resistant accessions was used for PCR-based screening as described by (Piffanelli et al., 2004).

INHERITANCE OF RESISTANCE – F2-ANALYSIS

Crosses between resistant accession and susceptible cultivar Morex were performed using Morex as female parent. Ten-50 single F1 plants were self pollinated to establish F2 populations. One to two F2 populations per cross, each containing 108 F2 individuals, were

used for phenotyping, and 92 individuals per population were used for QTL mapping.

QTL MAPPING

Genetic linkage maps were generated using JoinMap 3.0 (Van Ooijen and Voorrips, 2001) assuming Haldane's mapping function (Haldane, 1919). Markers were assigned to linkage groups at logarithm of odds (LOD) ≥ 3.0 with a maximum recombination fraction of 0.4. The maps in the respectively mapping populations were developed based on the "barley VeraCode (Illumina) 1" set of 384 highly polymorphic SNPs derived from barley OPA1 and OPA2 marker sets (Close et al., 2009).

Quantitative trait loci analysis was performed using composite interval mapping (CIM) with the program package PLABMQTL QTL (Utz and Melchinger, 1996). For detection of QTL LOD threshold was set to 3.0. After QTL detection, critical LOD scores were determined for all traits in all populations based on 1,000 permutations ($\alpha = 10\%$) as recommended by Churchill and Doerge (1994).

CG Re-SEQUENCING AND DETERMINATION OF POLYMORPHISMS

Genomic DNA isolation was done using the "DNeasy 96 Plant Kit" (Qiagen, Hilden, Germany) in a collection of 61 spring barley genotypes that differed strongly in susceptibility or resistance to *Bgh.* DNA Fragments of CGs were amplified by PCR from genomic DNA. PCR reactions contained (in a volume of 10 µl) 30 ng DNA template, 1 µM each of forward and reverse primer (Table S6 in Supplementary Material), 5 µl Taq PCR master mix (Qiagen, Hilden, Germany), and were used for 40 cycles at 94/N/72˚C (30 s at each temperature; N, annealing temp.), followed by a final elongation step at 72˚C for 7 min. PCR Fragments were purified by using PCR *clean-up* NucleoFast® 96PCR plates (Macherey-Nagel, Düren, Germany) before Sanger sequencing on an ABI 3730 instrument. Lasergene7/SeqMan (DNAstar Inc., Madison, USA) software was used for sequence analysis. SCF Trace files of sequences were aligned and SNPs were identified. All identified SNP and the corresponding haplotypes were exported to Excel tables.

POPULATION STRUCTURE AND KINSHIP MATRIX

For the detection of population structure, 42 SSR markers evenly distributed over the seven barley chromosomes were used (Haseneyer et al., 2010a). The resulting SSR allelic patterns were used to calculate the Q-matrix for population stratification by STRUCTURE 2.1 software (Pritchard et al., 2000). The admixture model was used and burn in of 1,000,000 followed by 5,000,000 iterations. The most likely number (K) of subgroups was chosen on the basis of second-order-rate change of the likelihood function as described by Evanno et al. (2005). K-matrix for kinship was determined by using the software package SPAGeDi1.2 (Hardy and Vekemans, 2002). Negative kinship values were set to zero following Yu et al. (2006).

ASSOCIATION OF ALLELIC VARIANTS WITH TRAITS

Association of SNP and haplotypes with traits "*Bgh* interaction" and "row number" was calculated by using the TASSEL 2.0.1 software[2] and two general-linear models (GLM1 = genotype + Q + trait; GLM2 = genotype + Q + row_number + trait). GLM2 was not used for trait "row number." In addition, a mixed-linear model was calculated (MLM = genotype + Q + K + trait) but data are not shown here because they deviated only very slightly from GLM1.

CALCULATION OF LD

Linkage disequilibrium between loci was calculated using the TASSEL 2.0.1 software with 1000 permutations. For LD-description, significance level (p-value) and correlation coefficient (R^2-value) were chosen.

GENETIC MAPPING OF CGs

Genetic map positions were derived from the single "QSM" population of Q21861 × SM89010 (Moscou et al., 2011), or from consensus maps "Barley, OPA123-2008, Consensus" (Close et al., 2009), "Barley, Integrated, Marcel 2009" (Aghnoum et al., 2010), and *in silico* mapping by synteny-based gene-order prediction in grasses (Mayer et al., 2011). All map positions of CGs were projected onto the "Barley, Integrated, Marcel 2009" map as described (Schweizer and Stein, 2011), except for positions derived from the "QSM" population: here local offset of approximately 4.5 cM was manually corrected by using a number of common markers with "Barley, Integrated, Marcel 2009" at the QTL region on chromosome 5HS.

TRANSIENT-INDUCED GENE SILENCING

cDNA Fragments of approximately 500 bp were PCR amplified from barley EST clones by using primers 5′-GCG GCA CTC GTC TCC GCG and 5′-TGA ATT AAA ATT TCT TTT CTG AAC C (Contig4387_at); 5′-CTC ACT AAA GGG AAC AAA AGC TGG AG and 5′-CCC GAA CAT ATC GCT CGT A (Contig12036_at); 5′-CTC ACT AAA GGG AAC AAA AGC TGG AG and 5′-CGC CAG GTA CAT AAC GAA CG (Contig4636_at); 5′-GAT TGA TCA GTT CTT AAA GG and ATC GGA GAG GTA GAA AGT AT (Contig5146_at); 5′-TTG TCA GAC CAG TCA ATA AT and 5′-ACA TCT TCA GAA TAC AGC TT (Contig6454_at); 5′-AGA GCC AAG CCC ACG AGG AAC-3′ and 5′-GGA ATT CGA TTG GTG GGG TGG-3′ (Contig5108_s_at); 5′-CTC ACT AAA GGG AAC AAA AGC TGG AG and 5′-TCT TGC TTC CAA GGA TCA ACA G (Contig2169_at); 5′-CTC ACT AAA GGG AAC AAA AGC TGG AG and 5′-ATC TAT AGG CCG CCC GTT C (Contig5974_s_at); 5′-GAG GGA GCA GTT ATC CAT CT and 5′-ACT TGG AAA TTT CTG AAA CGA (Contig24190_at); 5′-TGC TCT ATC ATT TTA TAC CAC ACA GG and TTC GAT TGC TGG ATC TTT ACA A (Contig2354_at). The resulting DNA fragments were used for the generation of RNAi constructs, and TIGS experiments were performed by particle bombardment as described (Douchkov et al., 2005). Three days after the bombardment leaf segments were inoculated with *Bgh* isolate CH4.8 at a density of 150–200 conidia mm^{-2}, and GUS-stained (transformed) epidermal cells as well as haustoria-containing transformed (susceptible) cells were counted 48 h after inoculation. The susceptibility index was calculated relative to the empty-vector control pIPKTA30, and values

[2]http://www.maizegenetics.net/

were log(2)-transformed in order to normalize their distribution for statistical analysis by a one-sample t-test. This test was performed against the hypothetical relative susceptibility-index value "−0.355" corresponding to the observed median of more than 1,000 RNAi constructs and reflecting a non-specific TIGS effect.

TRANSCRIPT-PROFILING

Seven-day-old barley plants of cv. Vada were inoculated with *Bgh* (isolate CH4.8) and Bgt (Swiss field isolate FAL92315), and the abaxial epidermis of inoculated primary leaves or from non-inoculated control leaves was peeled at 6–74 h after inoculation, as described (Zellerhoff et al., 2010). Total RNA was extracted by using the RNeasy plant mini kit (Qiagen, Hilden, Germany), checked for quantity and quality by using an Agilent Bioanalyser 2100 (Agilent Technologies Inc.), and hybridized to a 44K Agilent oligonucleotide array as described (Chen et al., 2011). Single-channel array processing was utilized followed by data normalization with default parameters, and significant transcript-regulation events were determined by using GeneSpring GX (v11.5.1) software (Agilent technologies Inc.). Transcripts were assumed to be significantly regulated if p-values corrected for false-positive rate (FDR, Benjamini–Hochberg method) were smaller than 0.05. All primary data of the analyzed CGs are shown in Table S5 in Supplementary Material.

CE SCORE

The CE score of selected CGs was obtained by assigning each of the following statistically significant results one point, and by adding up points: SNP or haplotype association with the trait "*Bgh* response"; localization inside QTL confidence interval for resistance to *Bgh*; transcript regulation in epidermal peels; and altered *Bgh* interaction upon TIGS or transient overexpression.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Plant-Microbe_Interaction/10.3389/fpls.2011.00113/abstract

Table S1 | Summary of association of allelic variants of polymorphic CGs with the *Bgh*-interaction phenotype and with row number.

Table S2 | Kinship (K) matrix of the customized barley collection based on 42 SSR markers. Software SPAGeDi1.2 (Hardy and Vekemans, 2002) was used to calculate kinship.

Table S3 | Structure (Q) matrix of the customized barley collection based on 42 SSR markers. Software STRUCTURE (Pritchard et al., 2000) was used to calculate population structure.

Table S4 | Correlation between row number of barley genotypes and resistance to *Bgh*.

Table S5 | Normalized and log(2) transformed signal intensity data (single-channel analysis) of selected transcripts corresponding to genes associated with resistance to *Bgh*. RNA was extracted from epidermal peels of barley after inoculation with either *Bgh* or the non-host pathogen *B. graminis* f. sp. *tritici* (Bgt).

Table S6 | Primers used for the re-sequencing of CGs. Please note that the lower case letters correspond to adapters containing T3 or T7 primer binding sites used for Sanger sequencing of amplified DNA fragments on an ABI 3730 sequencer.

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APPENDIX

Barley differential	Resistence gene(s)	Disease rating	
		P78	D12/12
Hord. Spontaneum	Mla16	0	0
Hord. Spontaneum	Mla17	1-2	2
Hord. Spontaneum	Mla18	0	0
Hord. Spontaneum	Mla19	0	0
Hord. Spontaneum	Mla20	1-2	0
P13	Mla23	2	2
Hord. Spontaneum	Mla26	1-2	0
Hord. Spontaneum	Mlf	2	1
Apex	mlo11, Mlg, Ml(CP)	0	0
P22	mlo5	0	0
Alexis	mlo9	0	0
Aura	Mla6, Mla14, Mlg, Ml(CP)	3	0
Meltan	U, Mla13, Ml(Im9), Ml(Hu4)	3	0
P12	Mla22	0	3
Gunnar	Mla3, Ml(Tu2)	0	2-3
Hord. Spontaneum	Mla28	2-3	0
Hord. Spontaneum	Mla27	0	4
Olga	U	4	0
P03	Mla6, Mla14	4	0
P06	Mla7, Ml(LG2)	4	0
P08B	Mla9	4	0
P09	Mla10, Ml(Du2)	4	0
P11	Mla13, Ml(Ru3)	4	0
Teo	Mla7, Mlg, Ml(CP), Mlk, MLa	3	1
Goldi	U, Mla12, MlLa	3-4	0
Lerche	Mlg, Ml(CP), Mla7	3	2
P01	Mla1	1-2	3
P02	Mla3	1-2	3
Borwina	Ml(BW1,2)	2-3	2
Amazone	Mlg, U	2	4
Camilla	U	2	4
P21	Mlg, Ml(CP)	3	3
Maresi	Mla12, Ml(AB), Mlg, Ml(CP)	2-3	3
P15	Ml(Ru2)	2-3	3
P20	Mlat	2	3-4
Thuringia	Ml(St1,2), U	2	3-4
Hord. Spontaneum	Mlj	2-3	2-3
Steffi	Ml(St1,2)	1-2	3-4
Katharina	U	3	4
P18	Mlnn	4	3
P23	Ml(La)	4	3
Kredit	Ml(Kr)	4	2-3
Marinka	Mlg, Ml(CP), Mla7	4	2-3
P17	Mlk	4	2-3
Lotta	Ml(Ab)	3-4	3
P19	Mlp	2-3	3-4
Banteng	Ml(Ba)	4	4
P04 B	Mla7, U	4	4
P10	Mla12	4	4
Dura	Ml(Dr)	4	3-4
Hellas	He	4	3-4
P14	Mlra	4	3-4
P24	MlH	4	3-4
Sissy	Mla12, Mlg, Ml(CP)	4	3-4
Trumpf	Mla7, Ml(AB)	4	3-4

FIGURE A1 | Virulence spectrum of two polyvirulent German *Bgh* isolates. Disease symptoms were scored from 0 (resistant) to 4 (fully susceptible) using the scoring system as described (Jensen et al., 1992).

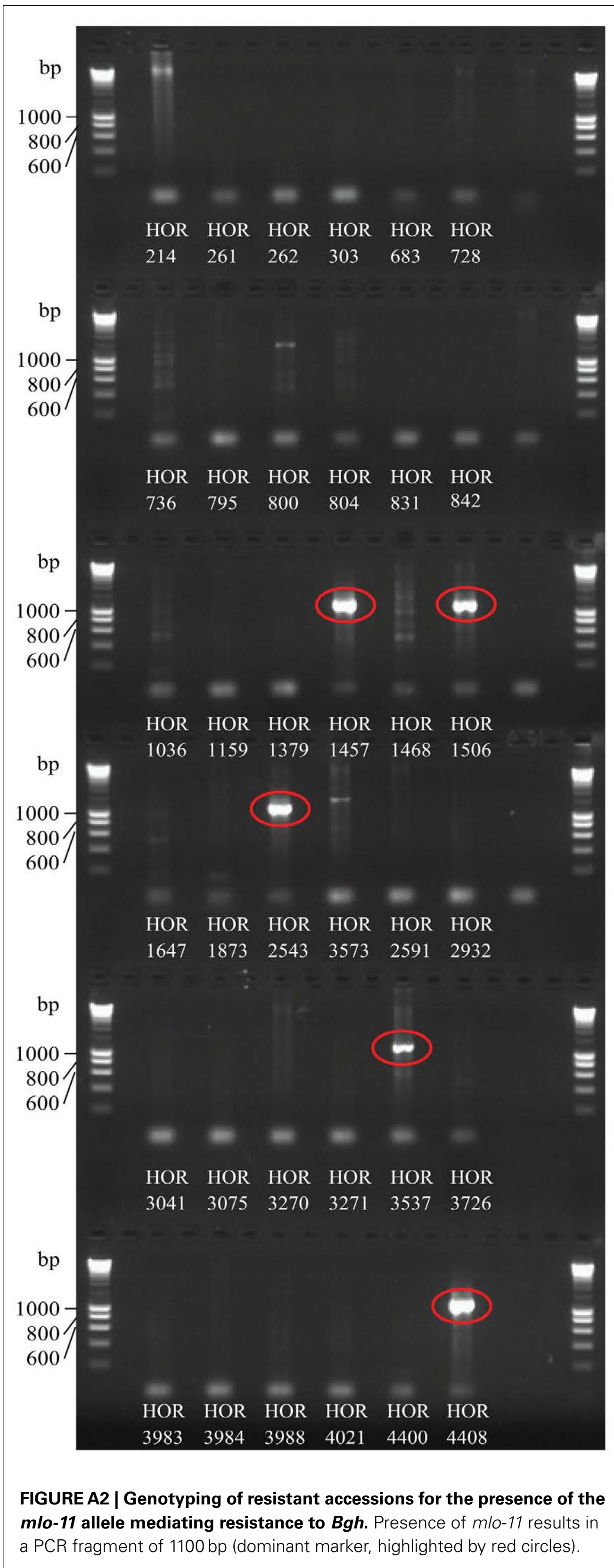


FIGURE A2 | Genotyping of resistant accessions for the presence of the *mlo-11* allele mediating resistance to *Bgh*. Presence of *mlo-11* results in a PCR fragment of 1100 bp (dominant marker, highlighted by red circles).

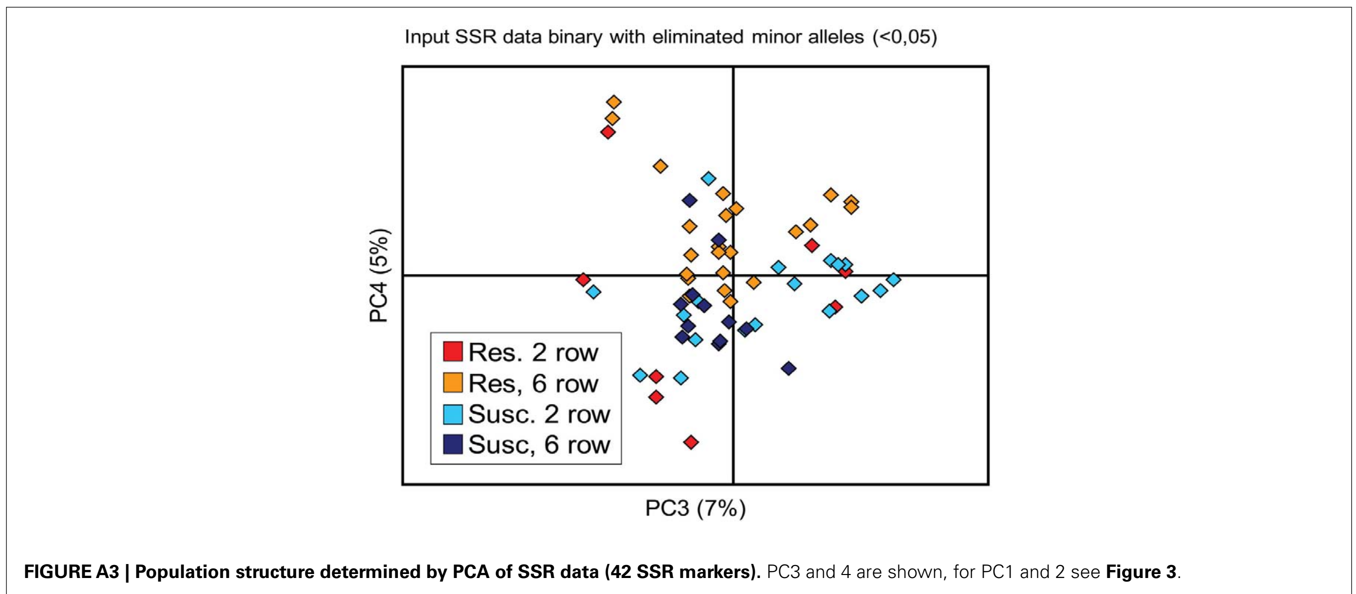
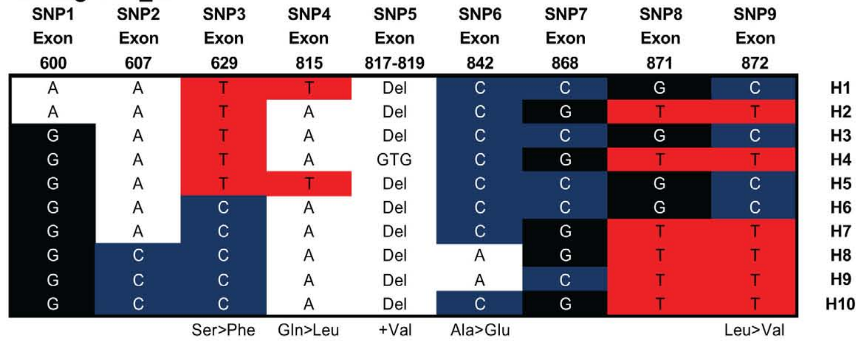
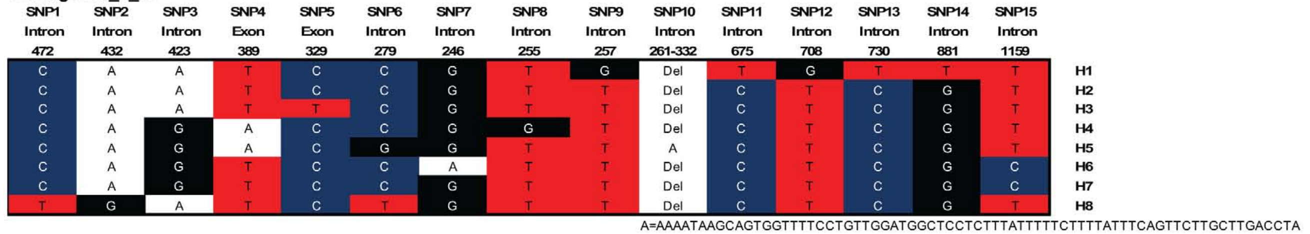
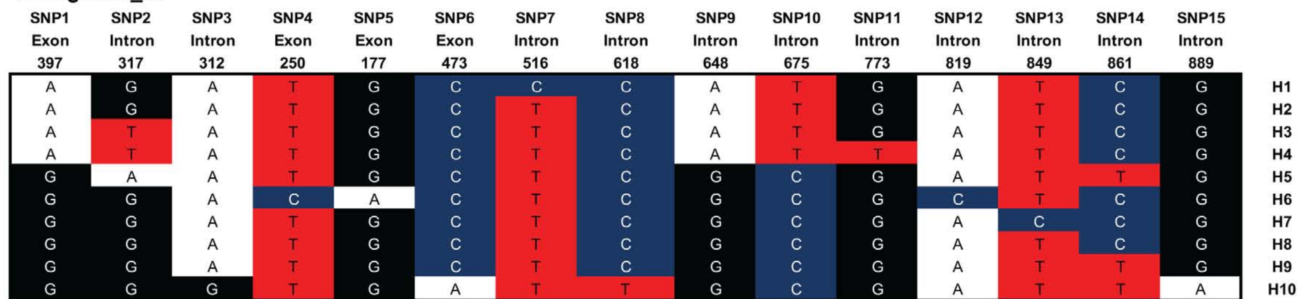
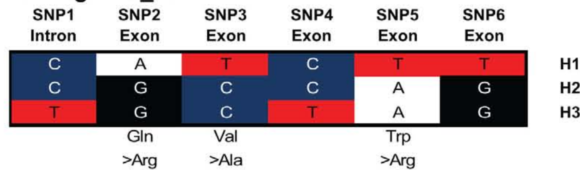


FIGURE A3 | Population structure determined by PCA of SSR data (42 SSR markers). PC3 and 4 are shown, for PC1 and 2 see **Figure 3**.









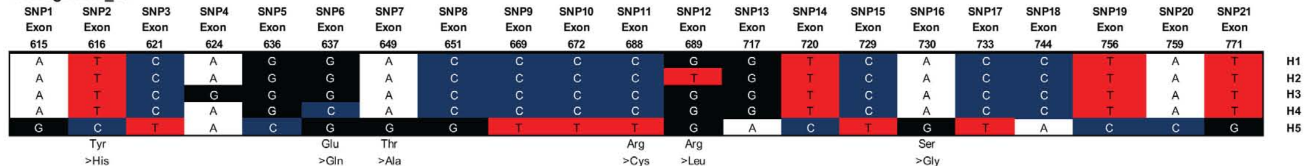
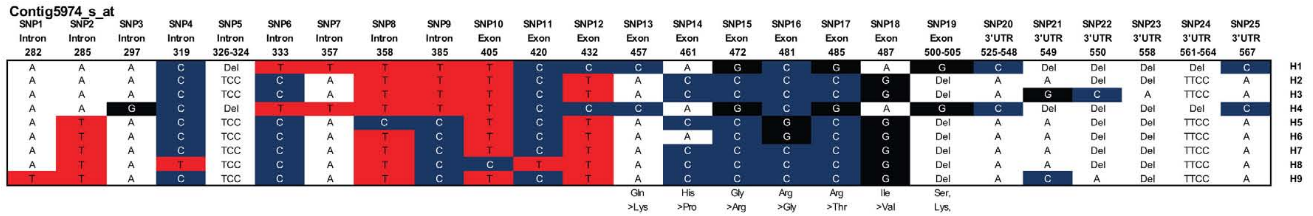
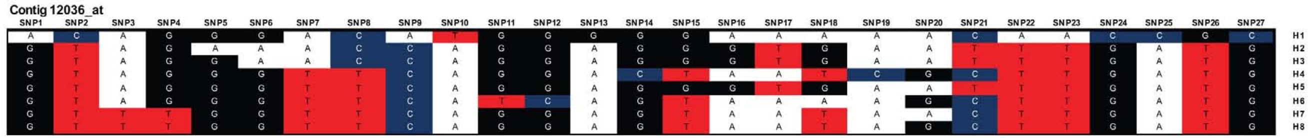
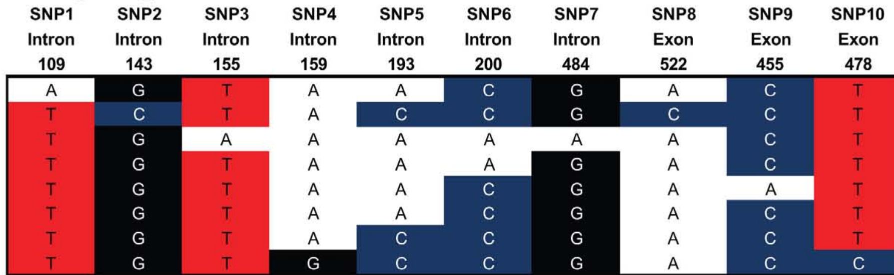
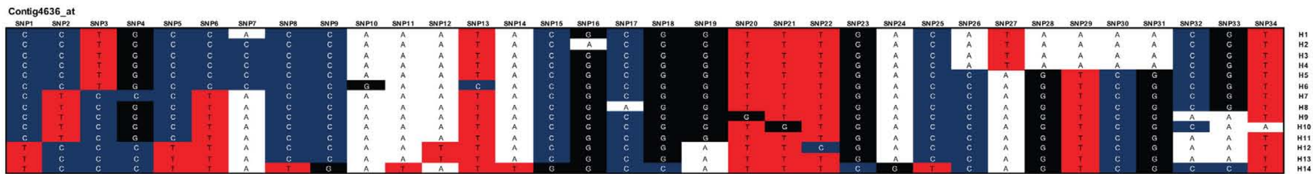


FIGURE A4 | Continued









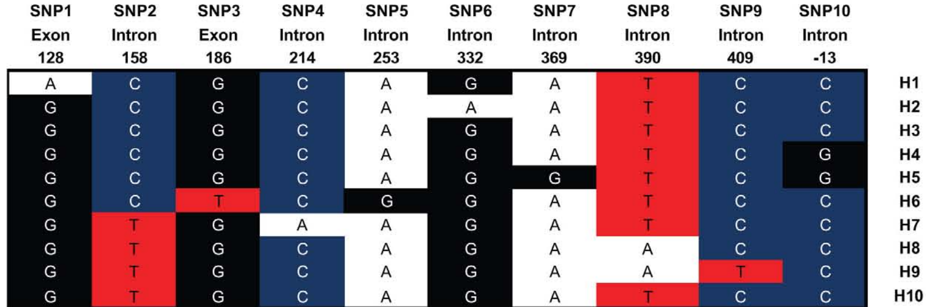


FIGURE A4 | Continued

Contig2354_at

SNP1 Intron 827-828	SNP2 Intron 840	SNP3 Intron 860	SNP4 Intron 964	SNP5 Intron 973	SNP6 Intron 978	SNP7 Intron 983	SNP8 Intron 1007	SNP9 Exon 975	SNP10 Exon 993	SNP11 Intron 1035	SNP12 Intron 1097	SNP13 Intron 1119	SNP14 Intron 1216	SNP15 Intron 1228	SNP16 Intron 1243	SNP17 Intron 1249	SNP18 Intron 1272	SNP19 Intron 1313	SNP20 Intron 1378	
AT	C	G	T	G	T	T	T	G	C	A	C	C	A	A	C	T	T	T	T	H1
AT	T	G	T	G	T	T	T	G	C	A	C	T	A	A	C	T	T	T	T	H2
AT	T	G	T	G	T	T	T	G	C	A	C	T	T	A	C	T	T	T	T	H3
Del	C	C	A	T	C	C	T	A	C	A	C	T	T	G	C	Del	T	T	T	H4
Del	C	C	A	T	T	C	C	A	C	A	T	T	T	G	T	Del	T	T	T	H5
Del	C	C	A	T	T	C	T	A	C	A	T	T	T	G	T	Del	T	T	T	H6
Del	C	C	A	T	T	C	T	A	C	A	C	T	T	G	C	Del	T	T	T	H7
Del	C	C	A	T	T	C	T	A	C	A	T	T	T	G	T	Del	T	T	T	H8
Del	C	C	A	T	T	C	T	A	T	A	C	T	T	G	C	Del	T	T	T	H9
Del	C	C	A	T	T	C	T	A	C	A	C	T	T	G	C	Del	C	A	T	H10
Del	C	C	A	T	T	C	T	A	C	G	C	T	T	G	C	Del	T	T	T	H11
Del	C	C	A	T	T	C	T	A	C	A	C	T	T	G	C	Del	T	T	C	H12
Del	C	C	A	T	T	C	T	A	C	A	C	T	T	G	C	Del	C	A	T	H13
Del	C	C	A	T	T	C	T	A	C	A	T	T	T	G	T	Del	T	T	T	H14

Vrs1 (AB259783)

SNP1 Exon 225	SNP2 Intron	SNP3 Exon ***456***	SNP4 Exon 550	SNP5 Exon ***693***	SNP6 Exon 772	SNP7 Exon 905	
C	C	DEL	A	T	G	C	H1
C	C	G	A	C	G	C	H2
C	C	G	A	C	G	T	H3
C	C	G	A	C	T	C	H4
C	C	G	A	T	G	C	H5
C	T	G	A	C	T	C	H6
C	T	G	G	C	T	C	H7
G	C	G	A	C	G	C	H8

Frame shift

FIGURE A4 | Graphical SNP- and haplotype tables of genes significantly associated with resistance to *Bgh*. Amino-acid changes caused by non-synonymous SNP or in/dels located within coding regions of CGs are shown below tables. *Vrs1* Is shown as example of the gene most significantly associated with row number. H1–H14, haplotypes Nr. 1–14 per CG.

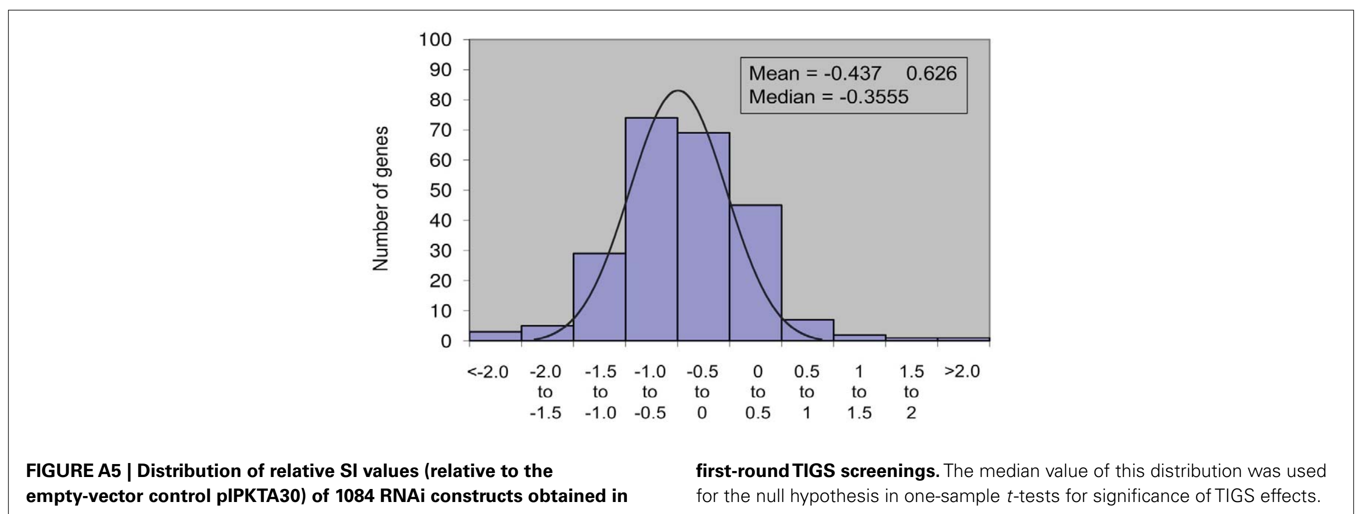


FIGURE A5 | Distribution of relative SI values (relative to the empty-vector control pIPKTA30) of 1084 RNAi constructs obtained in first-round TIGS screenings. The median value of this distribution was used for the null hypothesis in one-sample *t*-tests for significance of TIGS effects.