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ORIGINAL PAPER

Marker-trait associations in Virginia Tech winter barley identified using genome-wide mapping

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Abstract Genome-wide association studies (GWAS) provide an opportunity to examine the genetic architecture of quantitatively inherited traits in breeding populations. The objectives of this study were to use GWAS to identify chromosome regions governing traits of importance in six-rowed winter barley (*Hordeum vulgare* L.) germplasm and to identify single-nucleotide polymorphisms (SNPs) markers that can be implemented in a marker-assisted breeding program. Advanced hulled and hulless lines (329 total) were screened using 3,072 SNPs as a part of the US. Barley Coordinated Agricultural Project (CAP). Phenotypic data collected over 4 years for agronomic and food quality traits and resistance to leaf rust (caused by *Puccinia hordei* G. Otth), powdery mildew [caused by *Blumeria graminis* (DC.) E.O. Speer f. sp. *hordei* Em. Marchal], net blotch (caused by *Pyrenophora teres*), and spot blotch [caused by *Cochliobolus sativus* (Ito and Kuribayashi) Drechsler ex Dastur] were analyzed with SNP genotypic data in a GWAS to determine marker-trait associations. Significant SNPs associated with previously described quantitative trait loci (QTL) or genes were identified for heading date on chromosome 3H, test weight on 2H, yield on 7H, grain protein on 5H, polyphenol oxidase activity on 2H and resistance to leaf rust on 2H and 3H, powdery mildew on 1H, 2H and 4H, net blotch on 5H, and spot blotch on 7H. Novel QTL also were identified for agronomic, quality, and disease resistance traits. These SNP-trait associations provide the opportunity to directly select for QTL contributing to multiple traits in breeding programs.

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Introduction

The use of genome-wide association mapping (GWAS) to identify marker-trait associations in plants has become a popular alternative to traditional bi-parental mapping studies. Association mapping has been used to study yield and yield stability and agronomic and morphological traits in barley (Kraakman et al. 2004; Inostroza et al. 2009). In barley, use of association genetics has been made possible by the availability of high parallel SNP assay platforms (Waugh et al. 2009). These platforms have allowed for sufficient marker densities for genome-wide scans and LD

gene identification (Waugh et al. 2009). In a panel of 190 barley cultivars, GWAS was used to identify SNP markers associated with row type (Ramsay et al. 2011), which provide an initial starting point for isolating the *INTERMEDIUM-C* gene. Steffenson et al. (2007) used GWAS to detect significant chromosome regions conferring resistance to stem rusts (*Puccinia graminis* Pers.: Pers. f. sp. *tritici* Eriks. and E. Henn and also Pers. f. sp. *secalis* Eriks. and E. Henn) in Sharon goatgrass (*Aegilops sharonensis*) and wild barley (*Hordeum vulgare* ssp. *spontaneum*).

GWAS have been used successfully in diverse crops to identify genomic regions contributing to numerous traits. Tian et al. (2011) used genome-wide nested association mapping to detect genes associated with leaf architecture traits including upper leaf angle, leaf length, and width in maize (*Zea mays* L.). Mamidi et al. (2011) identified loci involved in iron deficiency chlorosis tolerance in soybean (*Glycine max* L.). Yao et al. (2009) identified significant marker-trait associations for yield, thousand kernel weight, spikelets per spike, grains per spike, spikelet density, and plant height on chromosome 2A in wheat (*Triticum aestivum* L.). Adhikari et al. (2012) utilized GWAS to identify genomic regions associated with resistance to bacterial leaf streak (*Xanthomonas translucens* pv. *undulosa*) and spot blotch (*Cochliobolus sativus*) in spring wheat. Poland et al. (2011) used genome-wide nested association mapping to identify candidate genes of a quantitative nature related to plant defense in a study focusing on resistance to northern leaf blight (*Setosphaeria turcica*) in maize. These studies clearly demonstrate the effectiveness of GWAS to dissect complex genetic traits and identify markers associated with these traits.

The Barley CAP project focused on analysis of genotypic and phenotypic data for diverse barley germplasm in ten US breeding programs to identify marker-trait associations using GWAS (Blake et al. 2012; Waugh et al. 2009). Genotypic data were generated using two barley Oligo Pool Assays (OPAs) containing 3,072 SNPs with Illumina's Golden Gate assay (Close et al. 2009; Fan et al. 2006). The two barley OPAs were developed by Close et al. (2009) from barley expressed sequence tags (ESTs). Recently, a new consensus SNP map was developed by Munoz-Amatriaín et al. (2011) and contains 2,994 SNP loci in 1,163 marker Bins covering a genetic distance of 1,137 centimorgans (cM).

Germplasm from the ten US programs participating in the Barley CAP was comprised of two-rowed and six-rowed, winter and spring habit, and malt, feed, and food grade barley. Only the Virginia Tech and Oregon State University programs focus on winter barley. Unique structure of the programs allowed for the determination of LD within US barley programs as well as mapping of multiple traits through GWAS (Hamblin et al. 2010; Lorenz et al. 2010; Massman et al. 2010; Roy et al. 2010; Wang et al. 2012). Limited exchange between the Virginia Tech program and other US programs is likely due to differences in growth habit, adaptation, and end use (Hamblin et al. 2010). The Virginia Tech program had the lowest LD of any program (Hamblin et al. 2010). The Virginia Tech program is also unique in development of both hulled and hulless germplasm. Identification of QTL within the Virginia Tech program through GWAS is valuable for furthering marker assisted selection (MAS) within the program as well as dissemination of unique alleles to other breeding programs with similar breeding objectives.

GWAS has been used successfully to identify marker-trait associations within the barley CAP material. Using single SNP and haplotype analysis Lorenz et al. (2010) detected heading date QTL in 1,824 barley lines from CAP years 2006 and 2007. Wang et al. (2012) detected three major heading date QTL in a panel of 766 spring barley breeding lines and a subset of 384 lines. GWAS has been used to identify chromosome regions conferring resistance to Fusarium head blight [(*Fusarium graminearum* (Schwabe)] and spot blotch in barley (Massman et al. 2010; Roy et al. 2010). These studies highlight the potential of GWAS to identify markers associated with both novel and previously described QTL for agronomic traits and disease resistance.

The Virginia Tech barley breeding program is unique within the US in that its primary focus is on the development of winter hulled and hulless six-rowed varieties for use in feed and fuel industries. While several breeding programs focus on two- and six-rowed spring malting barley, only two programs (Virginia Tech and Oregon State University), with very unique and diverse germplasm, breed six-rowed winter feed barley (Hamblin et al. 2010). Greater diversity exists among the six-rowed winter feed barley germplasm, which is attributed to a more lax selection criteria for end-use quality than that required for malting barley (Hamblin et al. 2010). Currently, very few markers are being used in the Virginia Tech program for MAS and allele enrichment. The main markers currently being used for MAS are ones for selection of the *nud* locus which controls the hulless phenotype Taketa et al. (2008). Previous mapping studies pertaining to Virginia Tech barley have focused on development of markers for leaf rust (caused by *Puccinia hordei*) and net blotch resistance (caused by *Pyrenophora teres*) (Mammadov et al. 2007; O'Boyle 2009). Identification and validation of markers for additional agronomic, quality, and disease resistance traits in Virginia hulled and hulless barley germplasm would facilitate implementation of an effective marker-assisted variety development program. Other programs lacking resistance genes/QTL present in the Virginia Tech

germplasm could benefit from markers identified in this study for introgression of QTL into their breeding material. Effectiveness of GWAS to identify novel and previously described regions associated with traits of interest in barley has been well documented in previous studies (Lorenz et al. 2010; Massman et al. 2010; Roy et al. 2010; Wang et al. 2012). Validation of markers is essential to eliminate potential false-positive associations and to identify previously reported or novel sources of QTL/genes within a breeding program. Identification of markers mapped to previously reported regions containing QTL/genes give validation of true marker-trait associations, while markers identifying novel QTL need further validation. Concurrently, understanding the underlying genetic architecture helps to determine which alleles can be selected for without simultaneously selecting for deleterious alleles. The research findings presented here are the first large-scale attempt to identify markers for critical traits within the six-rowed hulled and hulless winter barley germplasm.

Yields of hulless barley are 10–30 % less than hulled barley on average (Choo et al. 2001; Liu et al. 1996). Thus, improving yield potential of hulless barley is an important priority. Higher starch and lower fiber and ash concentrations in hulless versus hulled barley make it desirable for use in food, feed, and biofuel production (Griffey et al. 2010). Concurrently, the hulless trait in barley has been associated with poor seedling emergence and low seed weight (Choo et al. 2001). Hulless lines yielded less than hulled lines even when adjusting for hull weight loss by adding 15–19 % to yield of hulless lines (Choo et al. 2001). The *nud* gene which controls the hulless phenotype contributed 47–57 % of the total variation for yield (Choo et al. 2001). Conversely, hulless lines have about 20 % greater test weight than hulled lines with the *nud* gene contributing 85–95 % of total variation for test weight (Choo et al. 2001). Significantly higher test weights and lower kernel weights for hulless lines have been reported for Virginia Tech barley (Griffey et al. 2010). It is hypothesized that the *nud* gene might have a pleiotropic effect on yield or might be linked to a yield QTL (Choo et al. 2001).

Quality traits of barley are important for brewing, food, feed, and fuel industries. Both positive and negative quality attributes are associated with hulled and hulless barley. Hulless barley has been shown to contain significantly higher starch concentrations than hulled and malting type barley (Griffey et al. 2010). Starch content of hulless lines ranged from 59.1 % in 2003 to 63.5 % in 2005, while that of hulled lines ranged from 53.5 % in 2003 to 59.2 % in 2002 (Griffey et al. 2010). Hulless barley has also been shown to contain higher levels of β-glucan which is desirable in food grade barley (Bhatty 1999; Griffey et al. 2010). Grain protein of hulled and hulless lines was reported to be similar and ranged from 7.98 % to 10.96 % in Virginia winter barley (Griffey et al. 2010). Barley grain contains higher levels of phenolic compounds than other cereal grains, which makes it more susceptible to discoloration by polyphenol oxidase (PPO) (Bendelow and LaBerge 1979; Baik et al. 1995). Oxidation of phenolic compounds by PPO has a negative effect on the esthetic properties of products made from barley (Baik et al. 1995; Quinde-Axtell et al. 2005). Gutierrez et al. (2011) used GWAS to detect genes and QTL for beer and malting quality.

Foliar fungal pathogens such as leaf rust, powdery mildew (caused by *Blumeria graminis* f. sp. *hordei*), and net blotch are prevalent diseases in the mid-Atlantic region of the US where winter feed barley is grown. They impact productivity by reducing green leaf area, photosynthesis, and transpiration which negatively affect yield and grain quality. Although many studies have focused on identification and mapping of resistance genes in spring type barley, few have focused on genes contributing to resistance within the Virginia Tech winter barley breeding material. Mammadov et al. (2007) developed and validated markers for leaf rust resistance genes *Rph5* and *Rph7*. O'Boyle et al. (2011) characterized net blotch resistance in the six-rowed winter barley cultivar Nomini and in two spring barley lines concluding that each carried single dominant resistance genes. Using a bi-parental population, O'Boyle (2009) mapped the resistance gene in Nomini near the centromere of chromosome 6H.

This study contributes to the growing use of GWAS to identify marker-trait associations in a variety of important agronomic plants. Use of improved methodology helps to limit false positives that can occur due to population structure. Currently, little marker information is available for genes or QTL contributing positively to agronomic, quality, and disease resistance traits in the six-row winter barley germplasm. Identification of markers through GWAS would greatly aid in MAS. The objectives of this study were to use GWAS to (1) identify chromosome regions governing traits of importance in six-rowed winter barley germplasm; (2) identify SNP markers that can be implemented in a MAS breeding program, and (3) identify the genetic architecture for five key traits.

Materials and methods

Field evaluations

A set of 329 hulled and hulless barley cultivars and advanced lines from the Virginia Tech program were evaluated for agronomic, disease resistance, and quality traits over a 4 year period. During each year of the study, barley lines in preliminary (F_7, F_8 and F_9 generations) and advanced tests

(F_9 and greater generations) were planted in a randomized complete block design with three replications at Warsaw and Blacksburg, VA. Plots were planted at standard seeding rates for hulled and hulless barley and managed according to standard practises recommended in the mid-Atlantic region (Thomason et al. 2009). Each experimental unit consisted of a seven-row yield plot, 2.7 m in length with 15.2 cm spacing between rows and a harvested plot area of 2.9 m^2. Common hulled (Callao, Price, Nomini, Thoroughbred, Wysor, VA92-42-46 and VA96-44-304) and hulless [Doyce, Eve (VA01H-68), Dan (VA03H-61) and VA01H-125] checks were used in each year of the study to provide consistent comparisons. These checks are used extensively within the program as parents and common checks in field trials. Agronomic data were collected for plant height, stem length, lodging resistance, grain yield, and test weight. Disease reaction under natural field infection was assessed using an ordinal 0–9 rating scale (0 = absence of disease symptoms and 9 = severe disease) related to percent disease severity for leaf rust, net blotch, and powdery mildew. All genotypic and phenotypic data for the Virginia Tech barley program used in this study are available at The Hordeum Toolbox (Blake et al. 2012).

Quality data

To measure PPO activity, de-hulled barley kernels were ground using a Cyclone sample mill (UDY Corporation, Fort Collins, CO) fitted with a screen having 0.5 mm openings. PPO activity was determined using a modified protocol of American Association of Cereal Chemists International (AACCI) Approved Method 22-85.01 (2010). Whole barley flour (0.20–0.22 g) was reacted with 1.5 ml of L-Dihydroxyphenylalanine (L-DOPA) for 1 h on a Labquake rotator (Barnstead Thermolyne, Dubuque, IA) at room temperature, followed by centrifugation at $5{,}000 \times g$ (times gravity) for 10 min. Absorbance was read at 475 nm with L-DOPA as the blank and used as the estimate of PPO activity.

Greenhouse screening

Lines were screened in the greenhouse each year for seedling reaction to leaf rust, powdery mildew, and spot blotch. Virulence/avirulence formulas for *P. hordei* isolates ND8702 (Race 8) and VA90-34 (Race 30) used in the study are described in Mammadov et al. (2007). Inoculation, screening, and rating methods are as described by Brooks et al. (2000). Scores from greenhouse ratings were transformed to a numeric scale to account for infection type (IT) in the association analysis (Steffenson, personal communication).

Bulk isolates of powdery mildew collected from infected volunteer barley plants in the field were used in greenhouse disease screening tests. Isolates were increased and maintained on the susceptible barley cultivars Thoroughbred and Dayton. Inoculation, screening and rating methods are as described by Moseman et al. (1984). Scores from greenhouse ratings were transformed to a numeric scale to account for IT in the association analysis (Steffenson, personal communication).

Seedling resistance to spot blotch was assessed using spot blotch isolate ND85F. Planting, inoculation, screening, and rating methods are as described by Roy et al. (2010).

Genotyping

Samples were genotyped at the USDA-ARS Biosciences Research Lab in Fargo, ND with two barley Oligo Pool Assays (OPAs) containing 3,072 SNPs using Illumina's Golden Gate assay (Fan et al. 2006). Data analysis of SNPs from the two barley OPAs was performed using Illumin's Bead Studio software with manual inspection. The two barley OPAs were developed by Close et al. (2009) from barley ESTs. The consensus SNP map developed by Munoz-Amatriaín et al. (2011) containing 2,994 SNP loci in 1,163 marker Bins covering a genetic distance of 1,137 centimorgans (cM) was used in the study to determine chromosome location of traits.

Statistical analysis

The Mean (Proc Mean) and GLM (Proc GLM) procedures in SAS version 9.2 (SAS Institute 2008) were used to analyze phenotypic data and to generate trait means and analyses of variance. Means within location and year, as well as across years and locations, were used in association analyses, which were conducted using TASSEL v. 3.0 (Bradbury et al. 2007). A total of 2,242 SNPs were initially investigated. Marker data were filtered for presence in a minimum of 246 (75 % of 329 lines) lines to be present in filtered data set. A minimum allele frequency (MAF) of 0.1 was used to further filter the data set. Thus, markers with a MAF of less than 10 % were removed from the data set prior to analysis. After filtering, a total of 1,213 SNPs were used in the association analysis. A mixed linear model (MLM) was used to determine marker trait associations (Yu et al. 2006). The MLM combines information for population structure (Q) and kinship (K) (Bradbury et al. 2007). Population structure (Q) was accounted for using a principle component analysis (PCA) (Price et al. 2006). The PCA was based on filtered SNP datasets generated in TASSEL. Kinship (K), which is the average relatedness between lines, was calculated in TASSEL using filtered SNP datasets. Prior knowledge of the breeding populations suggested that three major sub-populations exist in the

Virginia Tech barley breeding program. While the hulled barley breeding program has existed for over 60 years, the hulless barley breeding program was only initiated during the past 20 years. Crosses between hulled × hulless parents were made initially and to a lesser extent presently to develop superior hulless genotypes. Most of the lines evaluated in the current study were derived from hulled × hulled and hulless × hulless crosses, while fewer lines were derived from hulled × hulless crosses. Subpopulations arising from differences in hulled and hulless genotypes, and different elite parents used in development of hulled × hulless crosses account for the observed subpopulation divisions.

Post association analysis corrections are necessary to adjust for statistical confidence intervals based on the number of tests performed (Noble 2009). The Bonferroni (BON) option in Proc MULTTEST in SAS version 9.2 (SAS Institute 2008) was used to correct for multiple testing. Significant *P* values from the BON correction were used to identify significant marker-trait associations. Allele substitution effects presented in tables and text are the difference between the two genotypes for a given marker. Positive or negative signs for allele substitution effects designate which genotype was associated with a higher or lower value.

Linkage disequilibrium

LD (r^2) values were calculated from SNP datasets in HAPLOVIEW (Barrett et al. 2005. Only SNP alleles with a MAF >0.1 were used in calculations. LD values were plotted against the genetic distance (cM) to estimate LD decay and average distribution of r^2 values between SNP marker pairs in the full set of Virginia Tech barley germplasm. Additionally, LD was assessed between significant markers (after BON correction) to define boundaries of potential QTL identified in the association analysis.

Results

Analysis of phenotypic data

Phenotypic data for significant marker-trait associations are presented in Table 1. The full list of data analyzed in the GWAS is available in supplemental Table S1. Due to the larger number of traits and datasets analyzed, only those for which significant marker-trait associations were identified are presented herein. The number of genotypes (N) varied per analyzed dataset. Significant ($P \leq 0.05$) differences between genotypes existed for all analyzed traits. Mean values, standard deviations, minimum and maximum values show the variability of traits for lines analyzed in the study.

Population structure and LD within Virginia Tech barley germplasm

Accounting for population structure prior to an association analysis is important to account for subdivisions that may lead to spurious marker-trait associations. Population substructure was accounted for using PCA (Fig. 1). Prior knowledge of the breeding program's composition suggested the existence of three sub-populations consisting of hulled, hulless, and hulled × hulless genotypes. A screen plot indicated that three principle components (PC) accounted for 31 % of total observed variation (data not shown). PC1 accounted for 16 % of the variation and separated hulled lines from the remainder of lines in the study. PC2 accounted for 11 % of the variation and separated hulless genotypes derived primarily from crosses among hulled and hulless parents. PC3 accounted for 4 % of the total observed variation and further subdivided hulless genotypes derived from hulled × hulless crosses. Wang et al. (2012) demonstrated the effectiveness of PCA to account for population structure prior to GWAS.

LD (r^2) was assessed in the full set of Virginia Tech lines using 2,242 SNPs. LD was found to decrease below the basal level ($r^2 < 0.1$) over a distance of 5–10 cM (Fig. 2). At 0 cM average LD among the 2,243 SNPs was $r^2 = 0.35$. Concurrently, 49.9 % of SNP pairs had an average LD below the basal level ($r^2 < 0.1$). At 50 cM marker pairs are not considered to be linked. Average LD among SNP pairs at 50 cM was $r^2 = 0.04$. A high portion of SNP pairs (8.9 %) have an LD greater than $r^2 > 0.1$.

GWAS for QTL contributing to agronomic traits

GWAS was conducted on yield, test weight, heading date, plant height, lodging, and stem length. Marker-trait associations for QTL contributing to heading date, plant height, test weight, and yield were identified on chromosomes 1H, 2H, 3H, 5H, 6H, and 7H (Table 2). Marker-trait associations were mapped to regions of the barley genome previously described in the literature as containing QTL for these traits as well as to previously unreported regions that were classified as novel QTL.

QTL *QHd-3H.102* for heading date was identified on chromosome 3H by marker 1_0583 which explained 6 % of the variation. The mean difference in heading date between genotypes having different marker alleles was 2.5 days. This region corresponds to a previously mapped QTL for heading date *QHd.StMo-3H.2* described by Hayes et al. (1993) and *QHd3H.100* described by Wang et al. (2012). A single significant novel QTL (*QPh-1H.30*) for plant height was identified on chromosome 1H by marker 1_0238 which explained 4 % of the variation. There was a 4.7-cm mean

Table 1 Means of phenotypic data for barley traits having significant marker-trait associations

Source[a]	Trait[b]	N[c]	Mean[d]	Std.Dev.[e]	Min.[f]	Max.[g]	*P*[h, i]
Across Years and Envs.	HD	654	110.27	5.0	99.3	123	<0.0001
06 Across Envs.	PH	192	82.7	7.8	57.7	103	0.0214
Across Years and Envs.	TWT	752	651.2	76.39	452.17	804.14	<0.0001
06 Across Envs.	TWT	192	672.6	69.8	514.8	804.1	<0.0001
Warsaw Across Years	TWT	376	661.4	71.8	524.2	787.2	<0.0001
Warsaw Across Years	Yield	376	5,026	1587	2,627	9,221	<0.0001
Across Years and Envs	Yield	752	5,036	1,768	1,784	9,221	<0.0001
09 Across Envs.	GP	178	11.33	1.2	4	13.45	<0.0001
Across Years	PPO	376	0.46	0.29	0.13	1.52	0.05
08 GH	LR Race 8	96	1.17	1.04	0	3.6	<0.01
08 GH	LR Race 30	82	1.73	0.84	0	2.6	<0.01
Across Years GH	PM	368	2.1	1.3	0.5	5	<0.0001
09 Across Envs.	PM	184	1.3	1.8	0	8.3	<0.0001
08 Warsaw	PM	93	1.6	2	0	8	<0.0001
Across Years and Envs	NB	667	3.4	1.6	0.3	9	<0.0001
Across Years	SB	373	5	1.3	1.5	8	0.0012

[a] Source of data analyzed; 2006 (06), 2008 (08), 2009 (09), Environments (Envs.), Greenhouse (GH), Across Years and Envs. (Data from 2006 to 2009 for Blacksburg and Warsaw, VA), Warsaw Across Years (Data from 2006 to 2009 for Warsaw, VA), Across Envs. (Data from Blacksburg and Warsaw, VA for a given year), Across Years GH (Data from 2006 to 2009 GH screenings)

[b] Trait analyzed and units: heading date (HD, days to heading), plant height (PH, cm), test weight (TWT g l^{-1}), yield (kg ha^{-1}), grain protein (GP; %), polyphenol oxidase activity (PPO; absorbance, dry weight basis), leaf rust race 8 (LR Race8; 0–3.6), leaf rust race 30 (LR Race 30; 0–3.6), powdery mildew GH (PM; 0–5), powdery mildew field (PM; 0–9), net blotch (NB; 0–9), spot blotch (SB; 1–10)

[c] Number of individuals analyzed in the dataset

[d] Mean value for trait analyzed

[e] Standard deviation (SD) for trait analyzed

[f] Minimum value observed for trait analyzed

[g] Maximum value observed for trait analyzed

[h] *P* value as determined from analysis of variance for each dataset

[i] *P* values for leaf rust race 8 and race 30 datasets based on repeated checks throughout years

difference in plant height between genotypes having different marker alleles.

The test weight QTL *QTwt-2H.164* identified by marker 2_1436 mapped to the same region containing *QTw.IgDa-2H* described by Backes et al. (1995). This marker explained 6 % of the variation, with the favorable allele being associated with an increased test weight of 18.0 g l^{-1} relative to the alternative allele in the Warsaw across year's dataset. Markers on chromosomes 2H, 5H, and 7H were associated with potentially novel QTL that explained 4–10 % of the variation in test weight. A total of three markers (3_0216, 1_0138 and 1_1307) associated with the novel QTL *QTwt-2H.107-109* on chromosome 2H explained 7–10 % of the variation in the Warsaw across year's dataset with allele substitution effects ranging from 21.1 to 24.0 g l^{-1}. The novel QTL *QTwt-7H.91-94* on chromosome 7H explained 4–7 % of the variation in test weight when data were analyzed across years and environments. Unfavorable alleles were associated with a decrease in test weight of 51.9 and 104.2 g l^{-1} which was noted for markers 1_0143 and 2_0685 associated with this QTL.

The yield QTL *QYld-7H.94* identified on chromosome 7H was identified by marker 2_0685 and explained 5 % of variation for yield in the Warsaw across year's dataset. The favorable allele was associated with an increase in yield of 1693.3 kg ha^{-1} for marker 2_0685. The region containing 2_0685 corresponds to the region containing the previously described yield QTL *QYld.HaTR-7H* (Tinker et al. 1996; Rostoks et al. 2005). A novel QTL *QYld-6H.7* was identified on chromosome 6H by two markers (3_0651 and 2_1204). The markers explained 3 % of variation with allele substitution effects ranging from 539.0 to 580.2 kg ha^{-1} for yield when data were analyzed across years and environments.

GWAS for QTL contributing to grain quality traits

Association mapping was conducted for amylose, beta glucan and grain protein concentration, grain weight, grain

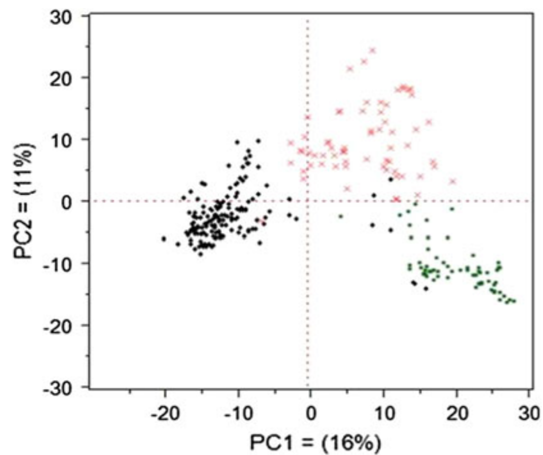

Fig. 1 Principal component analysis (*PCA*) of 361 hulled and hulless barley lines from the Virginia Tech program. Principle component 1 (PC1) explained 16 % of variation and separated hulled (*black*) *lines* from the remainder of the lines in the study. *PC2*, accounted for 11 % of the variation and separated hulless lines (*red and green*) in the study. PC3 (not shown), accounted for 4 % of the total observed variation and provided further subdivision between lines derived from hulled × hulless crosses (*red and green*)

hardness (SKCS), hull proportion, phenolic compound content, and PPO activity. Significant QTL for grain protein and PPO activity were identified on chromosomes 2H, 5H, and 6H (Table 3). QTL were not detected for amylose, beta glucan concentration, grain weight hardness (SKCS), hull proportion, or phenolic compound content. Lack of QTL identified for these traits is due to little phenotypic variation among lines or lack of significant associations.

A single marker (3_1417) identified QTL *QGp-5H.96* on chromosome 5H. This marker mapped to a region that contained the previously described QTL *QGpc.HaMo-5H* for grain protein described by Marquez-Cedillo et al. (2000). Marker 3_1417 described 15 % of the variation for grain protein across environments in 2009. The favorable allele at marker 3_1417 was associated with an increase in grain protein of 2.55 % relative to the alternate allele. A novel QTL *QGp-6H.142* for grain protein was identified on chromosome 6H by marker 2_0537 and described 13 % of the variation for grain protein across environments in 2009. The unfavorable allele associated with *QGp-6H.142* was associated with a 2.03 % decrease in grain protein.

A QTL *QPPO-2H.119-125* for PPO activity was identified on chromosome 2H. This QTL mapped to the region described by Taketa et al. (2010) as containing duplicate *PPO* genes. Markers associated with the region explained 6–15 % of the variation observed for PPO activity in the across years dataset. Allele substitution effect for absorbance ranged from −0.15 to −0.27 for favorable alleles associated with the PPO gene containing region. Markers 2_0099, 2_1184, and 20064 were the most significant markers associated with polyphenol oxidase activity explaining the greatest amount of variation (15 %). Reductions in absorbance are indicative of reductions in discoloration in products made from barley grain.

GWAS for QTL contributing to disease resistance

Identification of regions of the genome contributing to disease resistance is important for development of cultivars resistant to multiple pathogens. Association mapping was conducted for resistance to leaf rust, powdery mildew, net blotch, and spot blotch. Significant ($P \leq 0.05$) marker-trait associations were identified on chromosomes 2H, 3H, 6H, and 7H for leaf rust resistance (Table 4), 1H, 2H, and 4H for powdery mildew resistance (Table 5), 5H for net blotch, and 7H for spot blotch (Table 6).

Seedling resistance to leaf rust was assessed for two *P. hordei* races (8 and 30). Significant ($P \leq 0.05$) marker-trait associations were identified for chromosome regions containing previously described *Rph* genes which confer resistance to *P. hordei* race 8, race 30, and races contributing to natural field infections (Table 4). Markers 2_1398 and 3_0297 identified QTL *QRph-3H.11* which confers resistance to *P. hordei* race 8. These markers map to the 3H chromosome region containing gene *Rph7* (Parlevliet, 1976). Three markers on chromosomes 2H and 3H were

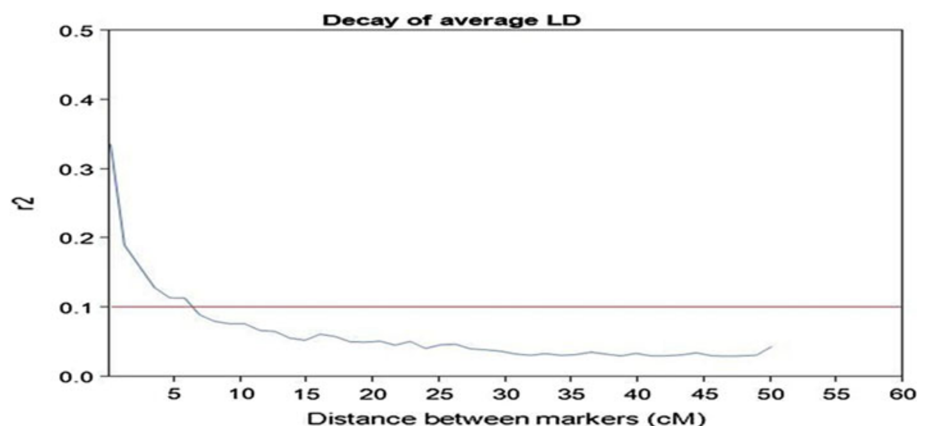

Fig. 2 Linkage Disequilibrium (LD) decay in Virginia Tech barley

Table 2 Significant marker-trait associations for quantitative trait loci (QTL) associated with agronomic traits in the Virginia Tech barley germplasm

Data[a]	Trait[b]	Marker[c]	Chr.[d]	cM[e]	Bin[f]	r^2[g]	BON[h]	QTL[i]	Effect[j]	Type[k]	References[l]
Across Years and Envs.	HD	1_0583	3H	102.66	101	0.06	0.0061	*QHd-3H.102*	−2.5	*QHd.StMo-3H.; QHd3H.100*	Hayes et al. (1993); Wang et al. (2012)
06 Across Envs.	PH	1_0238	1H	30.15	34	0.04	0.0500	*QPh-1H.30*	4.7	Novel	Novel
Across Years and Envs.	TWT	3_1284	2H	15.98	18	0.03	0.0062	*QTwt-2H.15*	−42.8	Novel	Novel
06 Across Envs.	TWT	1_0196	2H	89.68	106	0.08	0.0026	*QTwt-2H.89*	−61.7	*QTw.nab-2H*	Marquez-Cedillo et al. (2000)
Warsaw Across Years	TWT	3_0216	2H	107.92	123	0.07	0.0089	*QTwt-2H.107-109*	21.1	Novel	Novel
Warsaw Across Years	TWT	1_0138	2H	108.58	124	0.10	<0.0001	*QTwt-2H.107-109*	24.0	Novel	Novel
Warsaw Across Years	TWT	1_1307	2H	109.29	125	0.09	0.0003	*QTwt-2H.107-109*	23.0	Novel	Novel
Warsaw Across Years	TWT	2_1436	2H	164.35	181	0.06	0.0210	*QTwt-2H.164*	18.0	*QTw.IgDa-2H*	Backes et al. (1995)
Warsaw Across Years	TWT	1_1448	5H	131.64	131	0.06	0.0322	*QTwt-5H.131*	−16.3	Novel	Novel
Across Years and Envs.	YLD	3_0651	6H	7.87	8	0.03	0.0039	*QYld-6H.7-8*	580.2	Novel	Novel
Across Years and Envs.	YLD	2_1204	6H	8.74	8	0.03	0.0082	*QYld-6H.7-8*	539.0	Novel	Novel
Warsaw Across Years	TWT	1_1014	7H	54.32	63	0.06	0.0127	*QTwt-7H.54*	17.7	Novel	Novel
Across Years and Envs.	TWT	1_0143	7H	91.12	99	0.04	0.0002	*QTwt-7H.91-94*	−56.5	Novel	Novel
Across Years and Envs., Warsaw Across Years	TWT	2_0685	7H	94.34	101	0.07, 0.19	<0.0001, <0.0001	*QTwt-7H.91-94*	−102.9, −121.6	Novel	Novel
Warsaw Across Years	YLD	2_0685	7H	94.34	101	0.05	0.0500	*QYld-7H.94*	1693.3	*Qyld.HaTR-7H*	Tinker et al. (1996); Rostoks et al. (2005)

[a] 2006 (06) and Environments (Envs.); Data set where significant marker-trait association for QTL were found

[b] Heading date (HD), plant height (PH), test weight (TWT), and yield (YLD); traits for which significant marker-trait association exists

[c] Marker showing significant association with QTL for agronomic traits

[d] Chromosome where QTL was located

[e] Position on chromosome in (cM) where significant markers were located according to Munoz-Amatriaín et al. (2011)

[f] Bin locations of barley chromosome according to Munoz-Amatriaín et al. (2011)

[g] Percent of phenotypic variation explained by the marker identified as r^2

[h] Corrected P value after Bonferroni correction

[i] QTL identified in association analysis. QTL name consist of trait, chromosome and region identified

[j] Allele substitution effect relative to alternate allele present at the locus detected in association mapping on HD (days to heading), PH (cm), TWT (g l^{-1}) and YLD (kg ha^{-1})

[k] Previous identification for QTL/gene in a region

[l] QTL previously identified in region are identified by their reference. QTL identified but not previously described in literature are identified as novel

Table 3 Significant marker-trait associations for quantitative trait loci (QTL)/genes associated with quality traits in Virginia Tech barley germplasm

Data[a]	Trait[b]	Marker[c]	Chr.[d]	cM[e]	Bin[f]	r^2[g]	BON[h]	QTL[i]	Effect[j]	Type[k]	References[l]
09 Across Envs.	GP	3_1417	5H	96.12	97	0.15	0.0023	*QGp-5H.96*	2.55	*QGpc.HaMo-5H*	Marquez-Cedillo et al. (2000)
09 Across Envs.	GP	2_0537	6H	142.2	126	0.13	0.0016	*QGp-6H.142*	−2.03	Novel	Novel
Across Years	PPO	1_1094	2H	119.72	135	0.06	0.0296	*QPPO-2H.119-125*	−0.15	*PPO*	Taketa et al. (2010)
Across Years	PPO	2_0099	2H	124.29	136	0.15	<0.0001	*QPPO-2H.119-125*	−0.25	*PPO*	Taketa et al. (2010)
Across Years	PPO	2_1184	2H	124.98	138	0.15	<0.0001	*QPPO-2H.119-125*	−0.26	*PPO*	Taketa et al. (2010)
Across Years	PPO	2_0064	2H	124.98	138	0.15	<0.0001	*QPPO-2H.119-125*	−0.27	*PPO*	Taketa et al. (2010)
Across Years	PPO	1_0988	2H	124.98	138	0.06	0.0276	*QPPO-2H.119-125*	−0.22	*PPO*	Taketa et al. (2010)
Across Years	PPO	1_0128	2H	124.98	138	0.06	0.0465	*QPPO-2H.119-125*	−0.15	*PPO*	Taketa et al. (2010)
Across Years	PPO	1_0989	2H	125.97	139	0.06	0.0276	*QPPO-2H.119-125*	−0.22	*PPO*	Taketa et al. (2010)

[a] 2009 (09) and Environments (Envs.); Data set where significant marker-trait association for QTL were found

[b] Grain protein (GP) and Polyphenol oxidase activity (PPO); Traits for which significant marker-trait association exists

[c] Marker showing significant association with QTL/qene for quality traits

[d] Chromosome where QTL/gene was located

[e] Position on chromosome in (cM) where significant markers were located according to Munoz-Amatriaín et al. (2011)

[f] Bin locations of barley chromosome according to Munoz-Amatriaín et al. (2011)

[g] Percent of phenotypic variation explained by the marker identified as r^2

[h] Corrected *P* value after Bonferroni correction

[i] QTL/gene identified in association analysis. QTL name consist of trait, chromosome and region identified

[j] Allele substitution effect relative to alternate allele present at the locus detected in association mapping on grain protein (%) and polyphenol oxidase activity (absorbance, dry weight basis)

[k] Previous identification for QTL/gene in a region

[l] QTL/gene previously identified in region are identified by their reference. QTL identified not previously described in literature are identified as novel

associated with regions containing previously reported genes conferring resistance to *P. hordei* race 30. The unfavorable allele for 2_0159 identifying QTL *QRph-3H.6* was associated with an increase in leaf rust scores by 1.83 relative to the alternative allele. Markers 1_0376 and 1_1227 identified the QTL *QRph-2H.149* on chromosome 2H and both explained 23 % of the variation with favorable alleles being associated with a reduction in leaf rust scores of 0.61 relative to the unfavorable alleles. These markers mapped to the region containing *Rph15* (Weerasena et al., 2004). Novel QTL for seedling leaf rust resistance were found on chromosomes 7H for race 8 and on 6H for race 30. A single marker (1_0713) was associated with the novel QTL *QRph-7H.81* for seedling resistance to race 8, while marker 3_1274 was associated with *QRph-6H.49*, which contributes to race 30 resistance.

QTL were identified for seedling and field resistance to powdery mildew (Table 5). The QTL *QPm-1H.8-11* was identified by four markers (2_1174, 2_1226, 3_0950 and 3_0951) on chromosome 1H. Markers identifying *QPm-1H.8-11* explained 6–8 % of the variation for powdery mildew resistance and were associated with allele substitution effects ranging from −0.95 to −1.06 across years in the greenhouse. These markers map to the region containing the multi-allelic *Mla* resistance gene cluster (Wei et al. 1999). Marker 1_1050 mapped to the region containing the previously described resistance gene *MlLa* (Giese et al., 1993). Markers 3_0142, 1_0785 and 3_1139, associated with the QTL *QPm-4H.100-114*, were the only markers associated with field resistance to powdery mildew. Favorable alleles for marker 3_0142 and 1_0785 were associated with a consistent reduction in powdery mildew scores of 3.17–3.39 relative to the alternative alleles for these markers and explained 23–26 % of the variation for powdery mildew resistance in the Warsaw 2008 dataset.

Table 4 Significant marker-trait associations for quantitative trait loci (QTL)/genes associated with leaf rust resistance in Virginia Tech barley germplasm

Data[a]	Race[b]	Marker[c]	Chr.[d]	cM[e]	Bin[f]	r^{2g}	BON[h]	QTL[i]	Effect[j]	Type[k]	References[l]
08 GH	Race 30	1_0376	2H	149.27	164	0.23	0.0468	*QRph-2H.149*	−0.61	*Rph15*	Weerasena et al. (2004).
08 GH	Race 30	1_1227	2H	149.27	164	0.23	0.0500	*QRph-2H.149*	−0.61	*Rph15*	Weerasena et al. (2004).
08 GH	Race 30	2_0159	3H	6.31	4	0.31	0.0038	*QRph-3H.6*	1.83	*Rph5*	Roane and Starling (1967)
08 GH	Race 8	2_1398	3H	11.01	8	0.27	0.002	*QRph-3H.11*	1.65	*Rph7*	Parlevliet (1976)
08 GH	Race 8	3_0297	3H	11.01	8	0.27	0.002	*QRph-3H.11*	1.65	*Rph7*	Parlevliet (1976)
08 GH	Race 30	3_1274	6H	52.85	40	0.25	0.002	*QRph-6H.49*	−0.69	Novel	Novel
08 GH	Race 8	1_0713	7H	81.78	88	0.31	0.0035	*QRph-7H.81*	0.43	Novel	Novel

[a] 2008 (08) and Greenhouse (GH); Data set where significant marker-trait association for QTL were found

[b] *P. hordei* isolates ND8702 (Race 8) and VA90-34 (Race 30) were used in greenhouse screenings; Traits for which significant marker-trait association exists

[c] Marker showing significant association with QTL/qene for leaf rust resistance

[d] Chromosome where QTL/gene was located

[e] Position on chromosome in (cM) where significant markers were located according to Munoz-Amatriaín et al. (2011)

[f] Bin locations of barley chromosome according to Munoz-Amatriaín et al. (2011)

[g] Percent of phenotypic variation explained by the marker identified as r^2

[h] Corrected *P* value after Bonferroni correction

[i] QTL/gene identified in association analysis. QTL name consist of trait, chromosome and region identified

[j] Allele substitution effect relative to alternate allele present at the locus detected in association mapping on LR (Race 8 and Race 30) scores (0–3.6)

[k] Previous identification for QTL/gene in a region

[l] QTL/gene previously identified in region are identified by their reference. QTL identified not previously described in literature are identified as novel

Markers identifying *QPm-4H.100-114* mapped to the region containing the previously described *mlo* resistance gene (Buschges et al., 1997). The unfavorable allele for marker 3_1139 identifying the novel QTL QPm-4H.114 resulted in an increase of 2.94 in powdery mildew scores across environments in 2009 relative to the alternative allele. Marker 1_1050 was associated with QTL *QPm-2H.169* on chromosome 2H and explained 6 % of the variation for powdery mildew resistance across years in the greenhouse. Presence of the unfavorable allele in barley genotypes was associated with an increase of 1.05 in powdery mildew scores relative to the alternative allele; thus the deleterious effect of *QPm-2H.169* on powdery mildew resistance needs to be evaluated further.

Marker-trait associations ($P \leq 0.05$) for field resistance to net blotch and seedling resistance to spot blotch were identified on chromosomes 5H and 7H, respectively (Table 6). Marker 2_0987 identified the QTL *QNb-5H.38* for net blotch resistance and explained 8 % of the variation across environments with the favorable allele being associated with a reduction in net blotch score by 3.69. Marker 2_0987 mapped to the region containing a previously identified QTL *QNb.StMo-5H* (Steffenson et al. 1996) for net blotch resistance. A novel QTL *QNb-5H.60* associated with net blotch resistance was identified on chromosome 5H by marker 3_0007, which explained 9 % of the variation for net blotch resistance across environments. The unfavorable allele for this marker was associated with an increase in net blotch scores of 1.97 relative to the alternative allele.

Markers 2_0495 and 1_0451 identified the QTL *QSb-7H.18-27* associated with seedling resistance to spot blotch on chromosome 7H. These markers explained 6–7 % of variation for spot blotch resistance. Unfavorable alleles for these markers were associated with increases in disease scores of 0.77 and 0.93, respectively. These markers mapped to a region containing a previously described QTL associated with spot blotch resistance (Steffenson et al. 1996; Roy et al. 2010).

LD pattern for QTL in hulled and hulless germplasm

The pattern of LD (R^2) between markers identifying regions containing QTL detected in the association analysis was investigated between all pair-wise combinations of markers. Regions of interest included the multiple markers identifying the test weight QTL QTwt-2H.107-109 on chromosome 2H, yield and test weight QTL (QYld-7H.94 and QTwt-7H.91-94) on chromosome 7H, PPO activity QTL *QPPO-2H.119-125* on chromosome 2H, leaf rust resistance QTL (*QRph-3H.6* and *QRph-3H.11*) on chromosome 3H, and powdery mildew resistance QTL *QPm-1H.8-11* on

Table 5 Significant marker-trait associations for quantitative trait loci (QTL)/genes associated with powdery mildew resistance in Virginia Tech barley germplasm

Data[a]	Marker[b]	Chr.[c]	cM[d]	Bin[e]	r^2[f]	BON[g]	QTL[h]	Effect[i]	Type[j]	References[k]
Across years GH	2_1174	1H	8.96	14	0.07	0.009	*QPm-1H.8-11*	−1.00	*Mla* Resistance cluster	Wei et al. (1999)
Across years GH	2_1226	1H	9.37	15	0.08	0.0006	*QPm-1H.8-11*	−1.03	*Mla* Resistance cluster	Wei et al. (1999)
Across years GH	3_0950	1H	11.35	17	0.06	0.0168	*QPm-1H.8-11*	−1.06	*Mla* Resistance cluster	Wei et al. (1999)
Across years GH	3_0951	1H	11.35	17	0.06	0.0321	*QPm-1H.8-11*	−0.95	*Mla* Resistance cluster	Wei et al. (1999)
Across years GH	1_1050	2H	169.66	186	0.06	0.0281	*QPm-2H.169*	1.05	*MlLa*	Giese et al., (1993)
08 Warsaw	3_0142	4H	100.97	117	0.23	0.0179	*QPm-4H.100-102*	−3.17	*mlo*	Buschges et al. (1997)
08 Warsaw	1_0785	4H	102.93	119	0.26	0.0103	*QPm-4H.100-102*	−3.39	*mlo*	Buschges et al. (1997)
09 Across Envs.	3_1139	4H	114.61	133	0.16	0.001	*QPm-4H.114*	2.94	Novel	Novel

[a] 2008 (08), 2009 (09), Environments (Envs.) and Greenhouse (GH); Data set where significant marker-trait association for QTL were found

[b] Marker showing significant association with QTL/qene for powdery mildew resistance

[c] Chromosome where QTL/gene was located

[d] Position on chromosome in (cM) where significant markers were located according to Munoz-Amatriaín et al. (2011)

[e] Bin locations of barley chromosome according to Munoz-Amatriaín et al. (2011)

[f] Percent of phenotypic variation explained by the marker identified as r^2

[g] Corrected *P*-value after Bonferroni correction

[h] QTL/gene identified in association analysis. QTL name consist of trait, chromosome and region identified

[i] Allele substitution effect relative to alternate allele present at the locus detected in association mapping on powdery mildew GH (0–5) and powdery mildew field (0–9)

[j] Previous identification for QTL/gene in a region

[k] QTL/gene previously identified in region are identified by their reference. QTL identified not previously described in literature are identified as novel

chromosome 1H (Fig. 3a–e). Investigating LD within the regions allowed for the identification of boundaries for QTL and detection of other markers in LD with significant markers identified in the association analysis. Markers in LD ($R^2 > 0.2$) were identified for these regions of interest. A novel QTL for test weight (*QTwt-2H.107-109*) was identified by markers 3_0216, 1_0138, and 1_1307 on chromosome 2H. A high degree of LD ($R^2 = 0.37$–0.95) existed between these markers, and they are considered to identify the same test weight QTL. These markers also share varying degrees of LD with flanking markers. However, no other markers in the region were significant for test weight in the association analysis. A novel QTL for test weight (*QTwt-7H.91-94*) was identified by two markers (1_0143 and 2_0685) that were in high LD ($R^2 = 0.89$) and considered to detect the same QTL. Additionally marker 2_0685 identified the yield QTL (*QYld-7H.94*), suggesting that the test weight and yield QTL were coincidental. Markers 1_10143 and 2_0685 were in moderate to high LD ($R^2 = 0.25$–0.63) with seven other markers; however, these markers were not significantly associated with yield or test weight in the association analysis. A total of seven markers identified the QTL *QPPO-2H.119-125* for PPO activity on chromosome 2H. Markers in this region showed moderate to high LD ($R^2 = 0.23$–1.0) and are considered to identify the same QTL. Varying degrees of LD were shared between these markers and additional markers in the area that were not significant for PPO activity. Two QTL (*QRph-3H.6* and *QRph-3H.11*) for resistance to *P. hordei* races 30 and 8, respectively, were identified 5 cM apart on chromosome 3H. Mammadov et al. (2007) estimated the distance between *Rph5* and *Rph7* to be around 5 cM. A single marker 2_0159 identified *QRph-3H.6* (same region as gene *Rph5*) while two markers 2_1398 and 3_0297 identified *QRph-3H.11* (same region as *Rph7*). Markers identifying *QRph-3H.6* and *QRph-3H.11* are not in LD ($R^2 = 0.03$–0.04) and these QTL are considered to be independent. Four markers identified the QTL *QPm-1H.8-11*, which mapped to the same region as the previously described *Mla* resistance cluster (Wei et al. 1999). Markers 2_1174, 2_1226, and 3_0950 exhibited moderate to high LD ($R^2 = 0.27$–0.63), while marker 3_0951 exhibited high LD ($r^2 = 0.36$) with 3_0950 but was not in LD ($R^2 = 0.04$–0.06) with markers 2_1174 and 2_1226. Markers 2_1174 and 2_1226 share a high degree of LD with multiple markers on chromosome 4H. However, these markers were not significant for powdery mildew resistance.

Table 6 Significant marker-trait associations for quantitative trait loci (QTL)/genes associated with net blotch and spot blotch resistance in Virginia Tech barley germplasm

Data[a]	Trait[b]	Marker[c]	Chr.[d]	cM[e]	Bin[f]	r^{2g}	BON[h]	QTL[i]	Effect[j]	Type[k]	References[l]
Across Years and Envs.	NB	2_0987	5H	38.78	34	0.08	0.0464	*QNb-5H.38*	−3.69	*QNb.StMo-5H*	Steffenson et al. (1996)
Across Years and Envs.	NB	3_0007	5H	60.21	62	0.09	0.0330	*QNb-5H.60*	1.97	Novel	Novel
Across Years	SB	2_0495	7H	18.73	25	0.06	0.0433	*QSb-7H.18-27*	0.77	*QSb.StMo-7H; Rcs-qtl-7H-bPb-4584*	Steffenson et al. (1996); Roy et al. (2010)
Across Years	SB	1_0451	7H	27.09	31	0.07	0.0088	*QSb-7H.18-27*	0.93	*QSb.StMo-7H; Rcs-qtl-7H-bPb-4584*	Steffenson et al. (1996); Roy et al. (2010)

[a] Environments (Envs.); Data set where significant marker-trait association for QTL were found

[b] Disease for which significant marker-trait association were found; net blotch (NB) and spot blotch (SB)

[c] Marker showing significant association with QTL/qene for net blotch and spot blotch resistance

[d] Chromosome where QTL/gene was located

[e] Position on chromosome in (cM) where significant markers were located according to Munoz-Amatriaín et al. (2011)

[f] Bin locations of barley chromosome according to Munoz-Amatriaín et al. (2011)

[g] Percent of phenotypic variation explained by the marker identified as r^2

[h] Corrected *P*-value after Bonferroni correction

[i] QTL/gene identified in association analysis. QTL name consist of trait, chromosome and region identified

[j] Allele substitution effect relative to alternate allele present at the locus detected in association mapping on net blotch (0–9) and spot blotch (1–10) scores

[k] Previous identification for QTL/gene in a region

[l] QTL/gene previously identified in region are identified by their reference. QTL identified not previously described in literature are identified as novel

Discussion

Barley CAP

The purpose of barley CAP was to develop SNP markers for identification of QTL in breeding programs to be utilized for MAS. A total of 96 lines were submitted during all 4 years of the study by the Virginia Tech winter barley program. Although it was possible to genotype 384 unique individuals, common checks and individuals from a separate mapping study were submitted for genotyping. Therefore, a total of 329 lines were used for the GWAS. A total of 318 experimental lines represented promising breeding material being evaluated for agronomic performance, quality traits, and disease resistance. Genotyping of common checks and experimental lines allowed for the determination of LD within the breeding program and detection of QTL through GWAS. A total of 23 traits (supplemental Table S1) were investigated in the current study.

GWAS of agronomic traits

GWAS of agronomic traits in the Virginia Tech breeding program revealed common and novel QTL for heading date, plant height, test weight, and yield. Of the markers identified, 15 were mapped to previously described QTL for heading date, test weight, and yield. The QTL *QHd.3H-102* for heading date mapped to the same region as the previously reported QTL *QHd.StMo-3H.2* (Hayes et al. 1993). Restriction fragment length polymorphism (RFLP) markers ABG453 and ABC307B were the initial significant flanking markers identifying *QHd.StMo-3H.2* which spanned an 8.5-cM region on the Barley consensus SNP map (Rostoks et al. 2005). Marker 1_0583 associated with the region containing *QHd.StMo-3H.2* mapped less than 1 cM from ABC453 on the 2009 SNP consensus map (Close et al. 2009). The favorable allele was associated with a reduction in heading dates of 2.5 days relative to the alternative allele. The marker identifying *QHd.3H-102* would be useful in development of earlier heading varieties.

Of the ten markers associated with test weight QTL, two mapped to regions containing previously described QTL *QTw.nab-2H* (Marquez-Cedillo et al. 2000) and *QTw.IgDa-2H* (Backes et al. 1995) on chromosome 2H. Marquez-Cedillo et al. (2000) mapped *QTw.nab-2H* to a region on chromosome 2H flanked by markers *vrs1* and MWG503. In addition to *QTw.nab-2H*, QTL for kernel

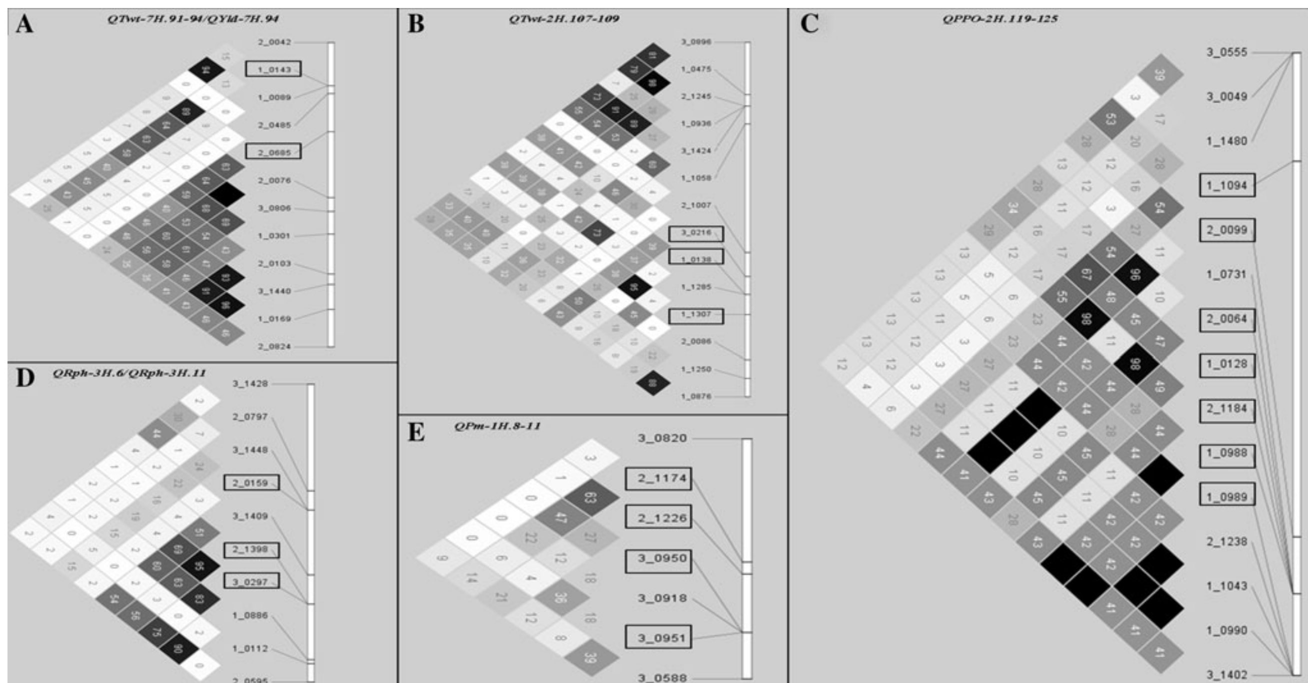

Fig. 3 Linkage Disequilibrium (LD) pattern for markers identifying QTL:(*A*) *QTwt-7H.91-94* and *QYld-7H.94* on chromosome 7H; (*B*) *QTwt-2H.107-109* on chromosome 2H; (*C*) *QPPO-2H.119-125* on chromosome 2H; (*D*) *QRph-3H.6* and *QRph-3H.11* on chromosome 3H; and (*E*) *QPm-1H.8-11* on chromosome 1H. Pairwise r^2 values are shown in *shaded boxes* with solid *black boxes* indicating $R^2 = 1.0$. Significant markers for each QTL are outlined in *black*

plumpness, grain protein concentration, soluble protein to total protein (S/T) ratio and diastatic power, which is the joint action of α-amylase and β-amylase and any other carbohydrate-degrading enzymes, were also mapped to the region flanked by *vrs1* and MWG503. These QTL are thought to be coincidental to the *vrs1* locus (Marquez-Cedillo et al. 2000). The *vrs1* locus is known to control the six-row phenotype in barley (Ubisch, 1916; Lundqvist et al. 1997). Marquez-Cedillo et al. (2000) hypothesized that inflorescence architecture controlled by *vrs1* could determine grain size and partitioning of proteins and enzymes to kernels and that grain protein concentration, S/T protein ratio and diastatic power could be controlled by a gene or genes linked to *vrs1*. Komatsuda et al. (2007) determined that *Vrs1* (two-rowed phenotype) encodes for a member of the homeodomain-leucine zipper (HD-ZIP) class of transcription factors. Concurrently, it was shown that the six-rowed phenotype controlled by *vrs1* arose due to a mutation in the HD-ZIP I-class homeobox gene (Komatsuda et al. 2007). We identified markers in the *vrs1* region associated with *QTw.nab-2H* for test weight. Interestingly, the Virginia Tech program is entirely of six-row (*vrs1*) morphology. Marker 1_0196 mapped 17.56 cM proximal of *vrs1* (Szucs et al. 2009). For marker 1_0196 two alleles are present in the breeding materials, with neither being fixed within the hulled or hulless subpopulations. Thus, either there are two or more *vrs1* alleles segregating in the breeding materials with one of them contributing directly to increased test weight. Alternatively, it is possible that the same *vrs1* allele is present in all breeding lines but it was from different sources that carry closely linked alternative alleles for the test weight QTL. Backes et al. (1995) mapped *QTw.IgDa-2H* to a region on chromosome 2H flanked by RFLP markers MWG866 and MWG829. Variation explained for this QTL was low (Backes et al. 1995). Of the two markers identifying previously described QTL for test weight, marker 2_1436 was significant in the Warsaw dataset across years suggesting that it may be useful in selecting for improved test weight in this environment.

The QTL *QYld-7H.94* for yield mapped to the same position as the previously described QTL *QYld.HaTR-7H* (Tinker et al. 1996; Rostoks et al. 2005). Tinker et al. (1996) mapped *QYld.HaTR-7H* to a region on chromosome 7H flanked by RFLP markers MWG626 and MWG571D. These markers flank Amy2 and ABC154 on the 2nd Barley consensus map (Qi et al. 1996). Marker ABG701 mapped 8.3 cM proximal to the QTL on the short arm of chromosome 7H, ABC154B mapped to the same position as *QYld.HaTR-7H* and AMY2 mapped 3.8 cM distal from the QTL on the agronomic QTL consensus map (Rostoks et al. 2005). Additionally, QTL for dormancy and spikes per unit

area map to the same region as *QYld.HaTR-7H* on the consensus map (Rostoks et al. 2005). The SNP marker 2_0685 identifying the region containing *QYld.HaTR-7H* in the current study maps 2 cM from ABG701 and 34 cM from Amy2 on the 2009 consensus map (Close et al. 2009). Therefore, the SNP marker identifying *Qyld-7H.94* falls within a 10–30 cM region of the markers identifying *QYld.HaTR-7H*. The favorable allele for the marker identifying *QYld-7H.94* was associated with a 1693.3-kg ha^{-1} yield increase in Warsaw across years. Hulless barley lines yield 10–30 % less than hulled cultivars on average (Liu et al. 1996; Choo et al. 2001) and constitute a large portion of the Virginia Tech barley program. Conversely, test weights of hulless lines are significantly higher than those of hulled lines (Griffey et al. 2010). Due to the inverse relationship between yield and test weight and the presence of hulled and hulless lines in the Virginia Tech program, it can be hypothesized that *QTwt-7H.91-94* is a coincident QTL with *QYld.HaTR-7H*. Adversely, the unfavorable allele for marker 2_0685 associated with the yield QTL is fixed within the hulless population. The true effect of the *nud* locus on this QTL is not known. Rostoks et al. (2005) positioned *QYld.HaTR-7H* at 91 cM which is approximately 3.8 cM from Amy2 and 13 cM proximal of the *nud* locus (104 cM). Szucs et al. (2009) positioned 2_0685 marker 1.09 cM distal of the nud locus. Based on the position of 2_0685 relative to the nud locus it is likely that recombination between marker 2_0585 and the nud locus is unlikely. Therefore, breakup of any negative linkages that exist between the *nud* locus and *QYld.7H.94* within the hulless subpopulation would be difficult. Screening for *QYld-7H.94* within the hulled subpopulation would be beneficial for continued yield improvement. Markers 2_1204 and 3_0651 identified a novel QTL *QYld-6H.7-8* for yield on chromosome 6H in the across environments dataset. Favorable alleles for markers associated with *QYld-6H.7-8* were associated with an increase in yield of 539.0 and 580.2 kg ha^{-1} relative to the alternative allele for these markers. Unlike alleles for marker 2_0685 identifying *QYld-7H.94*, the favorable alleles for markers 2_1204 and 3_0651 identifying *QYld-6H.7-8* are not fixed within the hulled or hulless subpopulations. These alleles would be useful for selecting improved yields in both subpopulations. Identification of parents within the hulled and hulless subpopulations possessing favorable alleles prior to crossing would be of great benefit in development of improved germplasm and varieties. In hulled × hulless populations, selection of parents from the hulled and hulless subpopulations containing multiple favorable alleles for yield would aid in the development of both hulled and hulless lines possessing superior yield potential. Use of these marker alleles would be beneficial in selecting for improved yield in both subpopulations.

GWAS for quality traits

GWAS of quality traits in the Virginia Tech breeding program revealed common and novel QTL for grain protein and PPO activity. Hulled and hulless barley genotypes have been reported to contain similar levels of grain protein (Griffey et al. 2010). Improvement of grain protein in hulled and hulless genotypes would greatly improve their marketability for use in health beneficial foods and ethanol production (Griffey et al. 2010). Markers associated with grain quality QTL were identified on chromosomes 2H, 5H, and 6H. The marker 3_1417 identified *QGp-5H.96* on chromosome 5H mapped to the region containing the previously described QTL *QGpc.HaMo-5H* (Marquez-Cedillo et al. 2000). Marquez-Cedillo et al. (2000) mapped *QGpc.HaMo-5H* to a region on chromosome 5H flanked by RFLP markers ABC302A and MWG635D. Subsequently, *QGpc.HaMo-5H* was positioned between SNP markers 2_0818 and 1_0183 at 91 cM on the Oregon Wolfe Barley 2008 consensus map (Szucs et al. 2009). The SNP marker 3_1417 which identified *QGp-5H.96* in the current study maps between 20 and 30 cM from the SNP markers flanking *QGpc.HaMo-5H* as positioned by Szucs et al. (2009) on the current SNP consensus map (Munoz-Amatriaín et al. 2011). Although markers identifying these QTL map to similar positions, further evaluation is needed to determine if the QTL identified within this region are unique. A novel QTL was indentified on chromosome 6H by marker 2_0537. The unfavorable allele for marker 2_0537 was associated with a reduction in grain protein of 2.03 %. Selection of this QTL may be beneficial in malt barley breeding programs as lower protein concentration is desirable. Within the Virginia Tech germplasm the allele for decreased grain protein is found in a high proportion of both hulled and hulless genotypes suggesting that selection for the allele may be useful in reduction of grain protein when developing malt barley genotypes. However, for genotypes developed for feed and fuel production, decreased grain protein is undesirable. QTL for grain protein have been previously identified on chromosome 6H by See et al. (2002) and Canci et al. (2003). The QTL identified by See et al. (2002) is near marker hvm74, while the QTL identified by Canci et al. 2003 is flanked by markers MWG916–Bmag0807. Comparison of positions for these markers relative to 2_0357 suggests that *QGp-6H.142* is a novel QTL.

Polyphenol oxidase activity is associated with discoloration of products made from barley grain (Jerumanis et al. 1976; Baik et al. 1995; Quinde et al. 2004; Quinde-Axtell et al. 2006). Quinde et al. (2004) reported that variation exists among barley genotypes for PPO activity. In the current study, seven markers identified a single QTL *QPPO-2H.119-125* for PPO activity on chromosome 2H,

which mapped to the region of previously reported duplicate *PPO* genes (Taketa et al. 2010). These duplicate genes were mapped to the long arm of chromosome 2H and were flanked by the RFLP marker MWG882 and microsatellite marker Bmac0415 (Taketa et al. 2010). Taketa et al. (2010) determined that *PP01* and *PPO2* are physically separated on chromosome 2H. *PPO1* controlled phenol reactions in the hull, caryopses and rachis, and *PPO2* controlled phenol reaction in the crease of the caryopses (Taketa et al. 2010). A high degree of LD was observed for all markers associated with *QPPO-2H.119-125* in the current study. No other traits were in LD with markers identifying *QPPO-2H.119-125*; thus, selection for this trait alone will be possible. Favorable alleles for selection of reduced PPO activity exist in both the hulled and hulless subpopulations. Selection for reduced values in both subpopulations should aid in the development in grain with improved quality.

GWAS for disease resistance

Markers associated with resistance to leaf rust, powdery mildew, net blotch, and spot blotch are of extreme importance to the Virginia Tech program. GWAS identified both common and novel QTL for disease resistance. Currently, 19 *Rph* genes conferring resistance to leaf rust have been identified in barley. In Virginia, two primary *P. hordei* races (8 and 30) are used for screening material for leaf rust resistance (Brooks et al. 2000). Prior work focusing on identification of leaf rust resistance genes has provided knowledge of genes within the program. Mammadov et al. (2007) validated markers for *Rph5* and *Rph7* within the Virginia Tech program; however, a recent race change rendered *Rph7* ineffective. In the current study seven markers identified QTL for resistance to *P. hordei* races 8 and 30. Markers identifying common QTL in the current study mapped to regions containing resistance genes *Rph5*, *Rph7* and *Rph15* (Parlevliet, 1976; Roane and Starling, 1967; Weerasena et al. 2004). Mammadov et al. (2007) mapped Rph5 to the short arm of chromosome 2H with markers ABG70 and TC2863-12.4 and Rph7 with AY6242926-C11. The distance between *Rph5* and *Rph7* was estimated to be around 5 cM (Mammadov et al. 2007) which is consistent with our results for *QRph-3H.6* and *QRph-3H.11*. Furthermore, these genes were considered to be linked in repulsion in a majority of Virginia Tech barley lines screened by Mammadov et al. (2007). LD was extremely low ($R^2 = 0.03–0.04$) between markers associated with *Rph5* and *Rph7*, further suggesting they are inherited independently. Incorporating both *Rph5* and *Rph7* into a single genotype is tedious without the use of molecular markers. Although LD is low between *Rph5* and *Rph7*, use of SNP markers to pyramid these resistance genes into a single genotype will improve the ability to successfully identify genotypes carrying both genes. Gene *Rph7* confers resistance to race 8, while genes *Rph5* and *Rph15* confer resistance to race 8 and race 30. Unfavorable alleles for markers identifying QTL which mapped to the regions containing *Rph5* and *Rph7* were associated with increases in leaf rust scores. These unfavorable alleles associated with regions containing *Rph5* and *Rph7* were present in a large number (~80 %) of lines included within the study suggesting that favorable alleles are in low frequency within the Virginia Tech program. Favorable alleles for markers identifying the region containing *Rph15* are present in 10 % of lines for 1_0376 and 60 % of lines for 1_1227. The effectiveness of markers identifying the region containing *Rph15* needs to be further validated to determine their usefulness in MAS. Identification of these QTL also confirms the possibility to detect multiple loci in a relatively small region within the genome using association mapping.

Relatively little is known about powdery mildew resistance in the Virginia Tech barley program. Identification of markers allowing for selection of powdery mildew resistance is of great interest. A total of eight markers identified QTL conferring resistance to powdery mildew. Markers identifying common QTL in the current study mapped to regions containing the *Mla* resistance gene cluster, the *MlLa* resistance gene, and the *mlo* resistance gene (Buschges et al. 1997; Giese et al. 1993; Wei et al. 1999). Wei et al. (1999) mapped alleles *Mla6*, *Mla14*, *Mla13,* and *Ml-Ru3* to the region flanked by Hor1 and Hor2 on chromosome 1H. The 2005 SNP consensus map has markers Hor5 (13.89 cM), MWG938 (14.58 cM), Hor2 (14.76 cM), and Hor1 (26.8 cM) on the short arm of chromosome 1H (Rostoks et al. 2005). Markers Hor5 (2.08 cM) and MWG938 (6.37 cM) mapped to the same region as the SNP markers 2_1174, 2_1226, 3_0950, and 3_0951 which identified the *Mla* region in the current study (Close et al. 2009). With as many as 32 resistance genes/alleles being identified in the *Mla* gene cluster (Jorgensen, 1992, 1994; Kintzios et al. 1995; Wei et al. 1999), it is possible that individual markers displaying low LD values may be associated with different resistance genes at this locus. Favorable alleles associated with markers identifying the region containing the *Mla* resistance gene cluster were found in 38–64 % of lines in the study. Wei et al. (1999) concluded that the *Mla* region investigated in their study contained 11 copies of nucleotide-binding site of leucine-rich repeat (NBS-LRR) resistance-gene homologs (*RGHs*) which are present in three distinct families. Of particular interest to the barley breeding community are markers mapping to the region known to contain the *mlo* resistance gene. The *mlo* resistance gene is resistant to all known races of powdery mildew which makes it of great value to programs in areas affected by this disease (Jorgensen

1992). LD between markers identifying *QPm-4H.100-102* (*mlo*) was high ($R^2 = 0.98$), while 3_1139 identifying *QPm-4H.114* was not in LD ($R^2 = 0.01$) with markers 3_0142 and 1_0785. The favorable alleles associated with marker identifying the region containing *mlo* were found in 66 % of lines within the study and were associated with a reduction in powdery mildew ratings of 3.17 and 3.39 relative to the alternative alleles, respectively. Among the markers associated with powdery mildew in the current study, the unfavorable allele of marker 3_1139 associated with novel QTL *QPm-4H.114* was associated with an increase in powdery mildew scores relative to the alternative allele. The unfavorable allele for marker 3_1139 was found in 22 % of the lines in this study. *QPm-4H.100-102* and *QPm-4H.114* are considered to be independent QTL for powdery mildew resistance. Thus, simultaneous selection for *QPm-4H.100* and selection against the unfavorable allele for the marker 1_1050 associated with *QPm-4H.114* should be possible.

O'Boyle et al. (2011) determined that the hulled cultivar Nomini carries a single dominant gene for net blotch resistance. In a bi-parental mapping study O'Boyle (2009) mapped the resistance gene in Nomini to the centromeric region of chromosome 6H. The study by O'Boyle (2009) was the first effort to map net blotch resistance in Virginia Tech winter barley. In the current study QTL for resistance to net blotch was identified on chromosome 5H. A single marker 2_0987 identified *QNb-5H.38* which mapped to the region containing the previously described net blotch QTL *QNb.StMo-5H* (Steffenson et al. 1996). Presence of the unfavorable allele for marker 3_0007 identifying QTL *QNb-5H.60* was associated with an increase in net blotch scores by 1.97 relative to the alternative allele. Thus further evaluation of the presence and effects of this QTL on barley germplasm likely is needed to eliminate its adverse effect on resistance.

A QTL *QSb-7H.18-27* for seedling resistance to spot blotch was identified on chromosome 7H with a high degree of LD ($R^2 = 0.59$) between markers 2_0495 and 1_0451 (Data not shown). These markers mapped to a region containing previously described QTL for spot blotch resistance (Steffenson et al. 1996; Roy et al. 2010). Roy et al. (2010) identified QTL *Rcs-qtl-7H-bPb-4584* for spot blotch resistance using a DArT marker on chromosome 7H through association analysis. This QTL is located at the putative *Rcs5* locus on chromosome 7H (Roy et al. 2010). The *Rcs5* gene is considered to be a major effect locus which confers both seedling and adult plant resistance to spot blotch, while Roy et al. (2010) reported *Rcs-qtl-7H-bPb-4584* to be a low-effect QTL explaining only 3.1 % of the variation. In the current study, *QSb-7H.18-27* has a slightly larger effect explaining 6 % to 7 % of the variation for spot blotch resistance. Roy et al. (2010) hypothesize that smaller effects reported in association mapping studies versus those reported in previous mapping studies may be due to their smaller population sizes which overestimated QTL effects or the presence of a resistance allele at more than one QTL which could affect QTL effect estimates.

Conclusions

Rapid advances in molecular and statistical techniques have paved the way for new mapping techniques such as association mapping. The current study highlights the usefulness of association mapping in identify marker-trait associations for numerous agronomic, quality, and disease resistance traits in a breeding program. Furthermore, it serves as a valuable reference for researchers interested in the application of association genetic techniques. Concurrently, this is the first large-scale mapping project to identify markers associated with regions where novel QTL and previously described QTL/genes reside in Virginia Tech hulled and hulless winter barley germplasm. Of particular interest are markers associated with known QTL/genes which will allow for selection of important agronomic, quality, and disease resistance traits. Further validation of novel QTL and use of diagnostic markers for previously identified genes and QTL will facilitate improvement of these traits in a marker-assisted selection breeding program.

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