Association Mapping of Septoria Speckled Leaf Blotch Resistance in U.S. Barley Breeding Germplasm

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ABSTRACT

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Septoria speckled leaf blotch (SSLB) is a sporadic but important disease of barley (*Hordeum vulgare*) in the Upper Midwest region of the United States and Prairie Provinces of Canada that is caused primarily by *Septoria passerinii*. Most of the widely grown cultivars in the region are susceptible to the disease. To identify and map SSLB resistance loci in U.S. barley breeding germplasm, we employed an association mapping approach using 3,840 breeding lines and cultivars and nearly 3,000 single-nucleotide polymorphism markers previously mapped to the seven barley

chromosomes. SSLB infection responses (IRs) were assayed on seedling plants in the greenhouse using a 0-to-5 scale. From the analysis of four yearly panels consisting of 960 lines each, four quantitative trait loci (QTL) for SSLB resistance were identified: one on chromosome 1H (*Rsp-qtl-1H_12_31144*), one on chromosome 3H (*Rsp-qtl-3H_12_31488*), and two on chromosome 6H (*Rsp-qtl-6H_11_21032* and *Rsp-qtl-6H_11_10064*). Individual resistance QTL reduced the mean IR from 9 to 38% compared with lines lacking any resistance alleles. However, the combination of all four resistance QTL together reduced the mean IR by 83%. The markers found associated with these QTL will be valuable for programs utilizing marker-assisted selection for SSLB resistance.

Septoria speckled leaf blotch (SSLB) is an important disease of barley (*Hordeum vulgare* L.) in the Upper Midwest region of the United States and the Prairie Provinces of Canada (28,51). Although severe and widespread epidemics have been reported in the region, they occur infrequently. The most common SSLB pathogen is *Septoria passerinii* Sacc., although *Stagonospora avenae* f. sp. *triticea* Bisset (teleomorph: *Phaeosphaeria avenaria* f. sp. *triticea* (G. F. Weber) O. E. Erikss. T. Johnson) is also frequently isolated from infected barley tissue (22). SSLB can cause yield losses of 20 to nearly 40% (17,18,50). In addition to reducing yield, the disease also can reduce the malting quality parameters of kernel size and extract level (17). The pathogen overwinters in infected residue and is mainly dispersed over short distances by rain-splashed pycnidiospores (28). The wide adoption of minimum or reduced tillage practices, resulting in more barley debris being left in the field, has led to increased inoculum levels (17,18). This factor, along with wet weather conditions that favor pathogen dispersal, spore germination, and infection, has contributed to the increased frequency and severity of SSLB epidemics in the Upper Midwest region of the United States, particularly during the 1990s (51).

There are only a few methods that can be used to manage SSLB in barley. These include removal or reduction of infested residue, application of fungicides, and planting of disease-resistant cultivars (31). Because barley is considered a low-input crop, residue reduction and fungicide application are not always cost effective. Therefore, the development of disease-resistant cultivars is the most attractive alternative because it is cost effective and environmentally friendly.

To identify SSLB resistance for breeding purposes, many *Hordeum* accessions have been evaluated for their reaction to *S. passerinii*. Most studies have been conducted with cultivated barley

(18,25,41,50,51). For example, Green and Dickson (18) tested 195 varieties and found that 53 were resistant, and Legge et al. (25) found that more than half of the 174 Turkish barley accessions they tested were resistant. From the screening of 250 accessions, Toubia-Rahme and Steffenson (51) identified 29 exhibiting resistance at both the seedling and adult plant stages and also found that all major cultivars from the Midwest region were highly susceptible. Resistance also has been identified in wild barley (*H. vulgare* subsp. *spontaneum*) and bulbous barley grass (*H. bulbosum*) (16,49,50,56). Toubia-Rahme and Steffenson (50) found that 17 of 24 wild barley accessions from the Middle East and Tibet were resistant. From the screening of 116 wild barley accessions from Jordan and Israel, Fetch et al. (16) identified 103 with SSLB resistance. Toubia-Rahme and Steffenson (49) tested 18 barley recombinant lines containing introgressions from *H. bulbosum* and found two that were highly resistant. This resistance was due to *H. bulbosum* chromatin, as revealed by cytogenetic analysis.

The genetic basis of SSLB resistance has not been studied as intensively as other diseases in barley, probably due to its relatively infrequent occurrence. To date, only four major genes (*Rsp1*, *Rsp2*, *Rsp3*, and *Rsp4*) from barley accessions CIho14300, CIho4780, and CIho10644 and breeding line Sep2-72, respectively, have been reported in cultivated barley (24,29,48,59). These genes were mapped using a standard biparental approach. The resistance locus *Rsp1* from CIho14300 was mapped to the short arm of chromosome 3H (24). Rasmusson and Rodgers (41) reported that *Rsp2* was closely linked with *Rsp3* at a recombination frequency of $3.9 \pm 1.3\%$. Zhong et al. (59) then confirmed the close linkage of *Rsp2* and *Rsp3* based on their association with the same amplified fragment length polymorphism (AFLP) marker, E-ACT/M-CAA-170, and mapped the two genes on the short arm of chromosome 1H. *Rsp4* was identified on the short arm of chromosome 6H in a mapping study using two elite barley breeding lines (48). In addition to these qualitative resistance genes, quantitative trait loci (QTL) for SSLB resistance also have been described in barley. Yu et al. (54)

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mapped two SSLB resistance QTL from a recombinant inbred line population developed from the cross of Zhenongda7/PI643302: one on the short arm of chromosome 1H that was coincident with *Rsp2* and the other on the distal region of the long arm of chromosome 2H. Luckert et al. (26) identified two major QTL from the Leger/CIho9831 doubled-haploid population which, together, explained 70% of the total variation: one mapped to the short arm of chromosome 3H and the other to the short arm of chromosome 6H near the *Rsp4* locus. Additionally, a few minor-effect QTL also were identified on chromosomes 1H, 2H, 4H, and 5H in that study.

In addition to cultivated barley, the genetics of SSLB resistance also has been investigated in wild barley species. From a recombinant inbred line population derived from the wild barley accession OUH602, two SSLB resistance QTL (*Rsp-2H-7-11* and *Rsp-6H-10-14*) were identified (56). Toubia-Rahme et al. (49) found two resistance genes in introgression lines derived from bulbous barley grass: one on the long arm of chromosome 4H and the other on the short arm of chromosome 7H.

Although a number of molecular markers associated with SSLB resistance have been identified from mapping studies (24,48,54,56,59), very few have been utilized in marker-assisted breeding. Markers associated with *Rsp2*, *Rsp3*, and *Rsp4* were validated for their utility in marker-assisted selection (59) but only those associated with the latter gene are being used in practice (K. Smith, *personal communication*). One critical obstacle concerning the use of exotic resistance donors is that undesirable genes are often linked to the target genes under selection (57), a phenomenon known as linkage drag. Utilizing elite breeding germplasm is one means of reducing linkage drag because most undesirable alleles have been removed through repeated generations of selection. For example, agronomically advanced lines carrying *Rsp4* were identified in such a way (48). However, the disadvantages of working with biparental mapping populations of elite germplasm are that only a small portion of the total genetic variation available in barley is being examined and, thus, there are only a limited number of polymorphisms available for mapping. For example, only 8 and 25.7% of diversity arrays technology (DART) and simple sequence repeat markers, respectively, showed polymorphism in the study to map *Rsp4* (48). Therefore, it is possible that other resistance alleles remain undiscovered in this elite breeding germplasm.

Association mapping (AM) or linkage disequilibrium (LD) mapping is an alternative method to biparental mapping for identifying specific causal variants linked to phenotypic polymorphisms (42). AM can reduce linkage drag and uncover additional resistance loci in more diverse populations (33). Germplasm panels developed for AM are usually many generations removed from common ancestors. Recombination events occurring throughout the entire evolutionary history of a germplasm panel are used in AM (32,42) in contrast to only a few generations in the case of biparental mapping populations. Therefore, AM can achieve a higher mapping resolution of the causal variants than the biparental approach (33,39). However, AM has some dis-

advantages, most notably the requirement for controlling population structure within the mapping panel (55). Thus, a comprehensive analysis of population structure in the mapping panel is essential for AM. There are two methods widely used in population structure analysis: the Bayesian Markov Chain Monte Carlo (MCMC) approach, as implemented in the program STRUCTURE (14), and principle component analysis (PCA). These two methods often produce similar results in population subdivision (30,35, 46,60) but their effectiveness in accounting for structure in the AM of different panels of barley is still unknown.

To conduct the genome-wide AM of economically important traits in barley, the Barley Coordinated Agricultural Project (BCAP) (<http://www.barleycap.org>) was established in the United States. The complete BCAP germplasm for AM consists of 3,840 elite breeding lines and also cultivars from 10 different improvement programs (384 from each). This panel was genotyped with 3,072 single-nucleotide polymorphism (SNP) markers that were identified from barley expressed sequence tags (ESTs) and sequenced amplicons by Close et al. (8). Elite breeding lines were selected for BCAP in order to fully exploit agronomically pertinent germplasm and utilize beneficial alleles directly in subsequent breeding generations without the negative impact of linkage drag. A recent study revealed that all major Midwest barley cultivars are susceptible to SSLB (51). It is not known whether cultivars and elite breeding lines outside the Midwest carry any resistance. Information regarding the sources, chromosomal locations, and allelic effects of SSLB resistance loci in elite barley germplasm would be very valuable for programs breeding for resistance to this disease. Thus, the specific objectives of this study were to (i) determine the frequency of SSLB resistance in current U.S. barley breeding germplasm and (ii) identify SSLB resistance loci in this germplasm using a genome-wide AM approach.

MATERIALS AND METHODS

Plant materials. The barley germplasm panel used in this study came from 10 breeding programs in the United States, 8 based on spring barley and 2 based on winter or winter/facultative barley (Table 1). Details about the germplasm lines, as well as genotyping and phenotyping data used in this study, can be found in The Hordeum Toolbox website (<http://hordeumtoolbox.org/>). Aside from yield, quality, and agronomic traits, these breeding programs focus on different end uses for barley such as malting, feed, and food. All lines were inbred to at least the F₄ generation and selected to be representative of each program. Ninety-six lines were submitted from each of the 10 programs in each year of the project from 2006 to 2009. Thus, the total number of lines evaluated per year was 960, with the yearly panels designated as CAPI, CAPII, CAPIII, and CAPIV, respectively. The complete germplasm panel consisting of all 3,840 lines was designated as CAP.

SNP genotyping and genome map construction. Lyophilized leaf tissue from a single plant selection of each barley line was

TABLE 1. Breeding programs of the Barley Coordinated Agricultural Project contributing germplasm to the association mapping panel for identifying Septoria speckled leaf blotch resistance

Breeding program	Location	Breeder	Growth habit	Row type	Primary use
University of Minnesota (MN)	St. Paul, MN	Kevin Smith	Spring	Six	Malting
North Dakota State University (N6)	Fargo, ND	Richard Horsley	Spring	Six	Malting
USDA-ARS Aberdeen (AB)	Aberdeen, ID	Don Obert	Spring	Six/two	Malting/feed
Utah State University (UT)	Logan, UT	David Hole	Spring	Six/two	Feed
Busch Agricultural Resources Inc. (BA)	Ft. Collins, CO	Blake Cooper	Spring	Six/two	Malting
North Dakota State University (N2)	Fargo, ND	Richard Horsley	Spring	Two	Malting
Washington State University (WA)	Pullman, WA	Steve Ullrich	Spring	Six/two	Malting/feed/food
Montana State University (MT)	Bozeman, MT	Tom Blake	Spring	Two	Malting/feed/food
Oregon State University (OR)	Corvallis, OR	Patrick Hayes	Winter/facultative	Six/two	Malting/feed/food
Virginia Polytechnic Inst. & State Univ. (VT)	Blacksburg, VA	Carl Griffey	Winter	Six	Feed

sent to the United States Department of Agriculture–Agricultural Research Service (USDA-ARS) Biosciences Research Laboratory in Fargo, ND for DNA extraction and genotyping. DNA was isolated according to standard procedures (34). Following the protocols of Illumina’s GoldenGate Bead Array Technology (Illumina, San Diego, CA) (15), two barley oligonucleotide pool assays (BOPA1 and BOPA2) (8) containing allele-specific oligos for a set of 3,072 SNPs were used to genotype the barley lines. All lines were genotyped by Illumina SNP technology (19) on the Illumina BeadStation 500G. Based on the consensus map developed by Close et al. (8), 2,943 of 3,072 SNPs (BOPA1 and BOPA2) were mapped to the seven barley chromosomes and spanned 1,099 centimorgans (cM). Of these, SNP markers with >20% missing data and <5% minor allele frequency (MAF) in each mapping panel were removed. For the complete mapping panel (CAP), the average density of SNP markers was 1.9 per centimorgan (60). Gaps between SNP markers were 0 to 10 cM with a mean of 0.5 cM. Only 10 gaps >5 cM were identified: 1 each on chromosomes 1H, 3H, and 6H, 4 on 5H, and 3 on 7H (60).

Plant growth conditions, pathogen isolate, inoculation, and incubation conditions. All phenotyping experiments were conducted at the Plant Growth Facility on the St. Paul campus of the University of Minnesota. Four seeds of each line were sown in the corners of square plastic pots (10 by 10 cm) filled with 50% native soil and 50% potting mix (Sunshine MVP; Green Island Distributors, Inc., Riverhead, NY) and grown at $20 \pm 3^\circ\text{C}$ with a 14-h photoperiod (230 to $270 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ provided by 1,000-W sodium vapor lamps). To monitor the infection level and virulence of the *S. passerinii* isolate, a single seed of the susceptible line ND5883 (PI 643237) also was sown in the center of each pot. Controlled release (Osmocote 14-14-14; Scott’s Company, Marysville, OH) (1.4 g/pot) and water-soluble (Peters Dark Weather 15-0-15; Scott’s Company) (0.1 g/pot) fertilizer formulations were applied at planting and then every fortnight with Peters 20-10-20 until completion of the experiment. The experiment was conducted using a completely randomized design and was repeated once over time. If means of a line across the two experiments differed by two units or more, the line was tested again in a third experiment.

Fig. 1. Boxplots of Septoria speckled leaf blotch infection response (IR) data in U.S. barley breeding germplasm. Five statistics (bars) are represented in each boxplot from bottom to top: the smallest observation, lower quartile, median, upper quartile, and largest observation, respectively. Data points positioned outside this range are extreme values and are represented as circles. Boxplots of IR data in **A**, each yearly panel and **B**, each breeding program. The thin red horizontal line indicates the threshold between resistance and susceptibility at $\text{IR} = 2$. Numbers below the red line are the frequency of resistant lines in each breeding program. Breeding programs: University of Minnesota (MN), North Dakota State University (N6), USDA-ARS Aberdeen (AB), Utah State University (UT), Busch Agricultural Resources Inc. (BA), North Dakota State University (N2), Washington State University (WA), Montana State University (MT), Oregon State University (OR), and Virginia Polytechnic Institute and State University (VT).

Isolate sp97-15 of *S. passerinii* was used in all experiments and was originally isolated from a barley plant collected in a field in North Dakota in 1997. Pycnidiospores of isolate sp97-15 were stored in tubes containing 15% sterile glycerol at -80°C . Ten days before inoculation, a 0.2-ml aliquot of the pycnidiospore-glycerol suspension was plated onto yeast maltose agar media (13) and grown in an incubator at 21°C and a 12-h photoperiod (32-W cool fluorescent lamps emitting 150 to $270\ \mu\text{mol photon m}^{-2}\text{ s}^{-1}$) for 1 week. Pycnidiospores were harvested from the resulting pycnidia by adding double-distilled water to the plate and gently scraping the surface of the culture medium with a sterile rubber spatula. The spore suspension was then homogenized for 30 s in a blender (20,000 rpm) and filtered through four layers of cheesecloth. The filtered inoculum was adjusted to a concentration of 2.5×10^6 spores/ml by adding sterile distilled water. Finally, 10 μl of Tween 20 (Fisher Scientific, Pittsburgh) per 100 ml of inoculum suspension was added to facilitate even distribution and adherence of pycnidiospores on the leaf surface. Inoculum was applied to 14-day-old plants (two-and-a-half-leaf stage) at a rate of 0.31 ml/plant using an atomizer (Paasche Airbrush Company, Harwood

Heights, IL) pressurized (20 kPa) by CO_2 . After inoculation, plants were moved into chambers and misted by household ultrasonic humidifiers for 30 min to establish a thin layer of free moisture on the leaf surfaces. Thereafter, the inoculated seedlings were maintained under near-saturated conditions by periodic misting from the humidifiers for 72 h. The first 40 h of the infection period were in darkness at $21 \pm 2^{\circ}\text{C}$ followed by a 5-h photoperiod (150 to $250\ \mu\text{mol photon m}^{-2}\text{ s}^{-1}$ provided by 400-W sodium vapor lamps) for each of the next 2 days, when the temperature was allowed to rise to 26°C . After the infection period, plants were allowed to dry slowly before being returned to the greenhouse under the same conditions described above. In the greenhouse, plants were misted twice a day to create a more humid environment during the disease incubation period. Infection response (IR) assessments were made on the second leaves of plants 3 weeks after inoculation using the 0-to-5 rating scale of Rosielle (44) as modified by Toubia-Rahme and Steffenson (51). In this scale, 0 = immune (no visible symptoms), 1 = highly resistant (presence of hypersensitive flecks), 2 = resistant (no or very sparse pycnidial production and coalescing of lesions,

Fig. 2. Genome-wide association of Septoria speckled leaf blotch resistance in the complete panel of U.S. barley breeding germplasm. **A**, Quantile-quantile plot of naïve (in blue) and mixed linear model (MLM) corrected association P values (MLM with P matrix shown in blue and Q matrix in red). Genome-wide association of Septoria speckled leaf blotch resistance using the MLM with **B**, the Q matrix and **C**, the P matrix. The thin red horizontal line indicates the threshold false discovery rate = 0.05. Markers most significantly associated with resistance in a chromosomal region are labeled. Other significantly associated markers are listed in Table 2.

mainly towards the leaf tip and edges), 3 = intermediate (light pycnidial production and moderate coalescing of lesions, mostly towards the leaf tip but also elsewhere on the leaf blade), 4 = susceptible (moderate pycnidial production, with lesions coalescing considerably across the leaf blade), and 5 = highly susceptible (large and abundant pycnidia with lesions coalescing extensively across the leaf blade). All plants of each accession were rated as described above, and the IR means of the two experiments were used in the data analysis.

Population structure. Two different methods were used to control population structure within the mapping panels. The first was a Bayesian MCMC approach implemented in the program STRUCTURE (version 2.1) (14,37,38). STRUCTURE was used to estimate the membership probability of each barley line to a number of hypothetical subpopulations (K). To avoid overestimation of subpopulation divergence caused by tightly linked SNP markers (14), a subset of 205 SNP markers with MAF > 0.05 was used to calculate the final subpopulation membership matrix (Q) within the CAP using the predetermined optimal K number of 9 (60). The second method used for controlling population structure was PCA (35), a classical nonparametric linear dimensionality reduction technique (20), which is based on the simple matching of SNP alleles and implemented in TASSEL (version 3.0) (<http://www.maizogenetics.net>). SNP markers with MAF > 0.05 were used in PCA. A matrix P, composed of the first three principle component vectors accounting for population structure, was generated. PCA subdivided the population in a very similar way to STRUCTURE and confirmed that K = 9 was optimal (60).

LD. LD extent was previously estimated by pairwise marker associations using an efficient mixed linear model (MLM) approach (9,21) in the four individual panels of CAPI, CAPII, CAPIII, and CAPIV (60). The pairwise marker associations were not estimated in the CAP due to the very high computational requirements.

Associations extending up to the genetic length of each chromosome were found before MLM correction but they declined to within 50 cM after correction (60). The four panels had very similar patterns of distribution for significant associations. The mean values of significant pairwise marker associations were 6.9, 6.3, 6.6, and 7.5 cM for CAPI, CAPII, CAPIII, and CAPIV, respectively. More than half (58.0, 61.1, 57.5, and 54.6% in CAPI, CAPII, CAPIII, and CAPIV, respectively) of the significant associations were found between markers within 5 cM and <5% were found between markers >25 cM apart (60).

In cases where multiple, closely linked SNP markers were found significantly associated with SSLB resistance, LD (as measured by D') among the linked and neighboring markers was calculated in the program Haploview 4.2 (4) and represented as a "heat" map.

Association mapping. An MLM approach implemented in the software TASSEL (version 3.0) was used to detect associations between SNP markers and seedling SSLB resistance in U.S. barley breeding germplasm. The formula used was $Y = X\beta + Mv + u + e$, where Y is the vector of phenotypic values, X is the vector of SNP marker genotypes, M is the population structure matrix Q (from STRUCTURE) or P (from PCA) and other covariate vectors, such as "year," β is the coefficient of the marker effect being estimated, v is the coefficient of the population structure, u is the vector of random effects, and e is the vector of residuals. In addition, the variances of u and e are: $\text{Var}(u) = 2KV_g$ and $\text{Var}(e) = V_R$, where K is the kinship matrix inferred from genotypes based on the proportion of shared allele values, V_g is the genetic variance, and V_R is the residual variance. Each SNP marker was then fitted individually into the MLM, and a P value was generated. Concurrently, a naive model (where $y = X\beta$) was used to demonstrate the effectiveness of controlling structure with the MLM. The Benjamini-Hochberg false discovery rate of q value = 0.05 was used to correct for multiple comparisons using pro-

gram QVALUE (47) for each individual association test. AM analyses for SSLB resistance were conducted in the CAP as well as the four individual yearly panels (CAPI, CAPII, CAPIII, and CAPIV).

Candidate gene scan. Possible candidate genes associated with SSLB resistance were investigated by searching the putative homologue of significant SNP sequences in the rice (<http://rice.plantbiology.msu.edu/>) and *Brachypodium* (<http://www.phytozome.net/>) genomes. Candidate plant disease-related genes were scanned in a 200-kbp window (i.e., 100 kbp in both directions from each significant SNP).

RESULTS

Disease phenotype data. The susceptible control ND5883 was infected in >99.5% of the individual pots tested across experiments. Moreover, in >90.4% of these cases, it exhibited a susceptible IR of 3 to 5. Thus, the infection levels and virulence phenotype of isolate sp97-15 were as expected based on many previous studies (49,50,51,59), and the probability of disease escape was nearly nil. All of the panels (CAPI to CAPIV) exhibited the full range of IRs of 0 to 5 (Fig. 1A). The mean and median IRs were highest in CAPI (3.5 and 4.3, respectively) and lowest in CAPIV (2.3 and 2.5). Values for CAPII (3.0 and 3.3) and CAPIII (2.5 and 3.0) were intermediate to these two panels. A moderate to high level of phenotypic variation was observed in all panels. The interquartile range (IQR) (i.e., the distance between upper and lower quartile values, a measure of variability in the data) indicated that variation for IR was greatest in CAPI (IQR = 2.6) and least in CAPII (IQR = 1.8) (Fig. 1A).

Marked differences were observed among individual breeding programs with respect to the frequency of resistant (IRs ≤ 2) lines and their IR range (Fig. 1B). Virginia Polytechnic Institute and State University (VT) had the highest frequency of resistant lines at 69.0%, whereas University of Minnesota (MN) and Busch Agricultural Resources Inc. (BA) had the lowest frequency at 7.3 and 6.8%, respectively. The other programs had frequencies intermediate to these extremes. With respect to the range of variation, the five programs with the lowest frequency of resistance (MN, North Dakota State University [NDSU] six-rowed program [N6], USDA-ARS Aberdeen [AB], BA, and Montana State University [MT]) exhibited a much narrower IQR range (1.1 to 1.6) than the five programs (Utah State University, NDSU two-rowed program [N2], Washington State University, Oregon State University, and VT) (2.0 to 3.0) with a higher frequency of resistance.

AM. AM with the Q (from STRUCTURE) and P (from PCA) matrices were performed in the CAP, and the results were compared. Under the MLM assumption, if there is no association between markers and the observed phenotypes, the P values from the association test should follow a uniform distribution (55,58). The quantile-quantile plot of cumulative (expected) and observed P values in the negative $\log_{10}$ scale showed that observed P values from the naive model, without considering structure and relatedness effects, were much smaller than the expected P values and, therefore, highly skewed from the straight line $y = x$ (i.e., uniform distribution) (Fig. 2A). In contrast, observed P values from the MLM considering structure (Q matrix from STRUCTURE or P matrix from PCA) were almost uniformly distributed and, thus, only a few P values from truly associated markers were biased from the straight line $y = x$ (Fig. 2A).

The MLM employing either the Q or P matrix in the CAP yielded very similar AM results, except that SNP marker 11_21226 was only significantly associated in the analysis with the Q matrix (Table 2; Fig. 2B and C). Common markers significantly associated with SSLB resistance were found on three chromosomes: the short arm of chromosome 1H with SNP markers 11_10419, 12_31144, 12_10636, 12_30933, and 12_11011; the short arm of chromosome 3H with 12_31448; and the short arm

of chromosome 6H with 11_21032, 11_10064, and 11_10023 (Table 2).

AM for SSLB resistance was then conducted using the P matrix in the individual yearly panels (i.e., CAPI to CAPIV). The P matrix was chosen over the Q matrix because it is computationally less intensive to perform and confers similar results (Table 2). On chromosome 1H, two SNP markers, 11_10419 and 12_31144, were found significantly associated in all four panels. Two additional markers (12_10636 and 12_30933) also were found associated on chromosome 1H but only in two of the four yearly panels. Additionally, SNP marker 11_21226 was found significantly associated only in CAPI, whereas markers 12_11011 and 11_10332 were found significantly associated only in CAPII. On chromosome 3H, SNP marker 12_31488 was found significantly associated with SSLB resistance in CAPI and 11_21294 in CAPII. On chromosome 6H, SNP markers 11_21032 and 11_10064 were significantly associated with resistance in at least two of the four yearly panels, and SNP markers 11_20294 and 11_10023 were associated in only one yearly panel. The results from the individual panels confirmed the most significant marker associations found in the CAP (Table 2). Allele effects of each significant SNP marker were calculated for CAP and for each yearly panel. All significant markers contributed small effects (R^2 of 0.04 to 6% (Table 2).

In summary, 13 markers were found significantly associated with SSLB resistance at the seedling stage: 7 on chromosome 1H, 2 on 3H, and 4 on 6H. To avoid false-positive associations, only those markers found associated in at least two panels (i.e., CAP [Q], CAP [P], and CAPI to CAPIV) were investigated further. Using this criterion, six SNP markers (11_10419, 12_31144, 12_10636, 12_30933, 12_11011, and 11_21226) within a 5-cM region on chromosome 1H were confirmed for their association with SSLB resistance. LD analysis of markers was performed in this region to determine whether these six SNP markers might be detecting the same QTL. All six markers were in high LD with each other because most D' values were >0.72 and often much higher (>0.88) (Fig. 3A). This result suggests that the markers are detecting the same QTL. Among these significant markers, 12_31144 had the lowest P value in the association test of the CAP; therefore, the resistance QTL associated with the marker was designated as *Rsp-qtl-1H_12_31144*. On chromosome 3H, only one SNP marker (12_31488) was confirmed in more than one panel; thus, the resistance QTL associated with this marker was designated *Rsp-qtl-3H_12_31488*. On chromosome 6H, three SNP markers (11_21032 11_10064 and 11_10023) were found significantly associated with resistance in more than one panel. LD analysis among these three markers revealed high LD be-

tween 11_10064 and 11_10023 ($D' = 0.92$) only. Given the high LD and very close linkage of both markers, it is highly probable that they are detecting the same QTL (Fig. 3B). Because marker 11_21032 is distant from the other two associated markers (12.6 cM from 11_10064 and 13.3 cM from 11_10023) and is in low LD with them ($D' = 0.56$ and 0.49 , respectively), it is likely detecting a separate resistance QTL. Thus, the two QTL identified on chromosome 6H were designated *Rsp-qtl-6H_11_21032* and *Rsp-qtl-6H_11_10064*.

Allele effect. Allele effects of the four QTL identified (*Rsp-qtl-1H_12_31144*, *Rsp-qtl-3H_12_31488*, *Rsp-qtl-6H_11_21032*, and *Rsp-qtl-6H_11_10064*) in this study were investigated by examining their ability for reducing the mean IR. Given two alleles (A and B) for each SNP, the resistance allele for each QTL was “A” for *Rsp-qtl-1H_12_31144*, “A” for *Rsp-qtl-3H_12_31488*, “B” for *Rsp-qtl-6H_11_21032*, and “B” for *Rsp-qtl-6H_11_10064*. Each individual QTL reduced just a small to moderate proportion of the mean IR (9 to 38%) compared with lines that lack any resistance alleles; however, the combination of all four QTL reduced the mean IR by 83% (Table 3). In general, the mean IR had a strong negative correlation ($r = -0.9$) with QTL number but there was one case where lines carrying just one QTL (“BBAB”) had a lower IR than lines carrying two QTL (“BAAB”).

Candidate gene scan. Markers associated with the resistance QTL on chromosome 1H were all in the same LD block. Thus, the marker with the greatest significance level (12_31144) was examined by BLAST search of the EST sequence from which the SNP was derived in a 200-kbp window of the rice (<http://rice.plantbiology.msu.edu>) and *Brachypodium* (<http://www.phytozome.net>) genomes. However, no homolog was found in either genome. The next most significant marker (11_10419) was subsequently examined. A possible candidate gene identified from the rice genome was C2H2, a zinc-finger protein. This protein is known to function as a key transcriptional repressor involved in pathogen defense of plants (6). Markers associated with resistance on chromosome 3H also were examined in both model genomes but no genes with obvious pathogen defense functions were found. Finally, the significantly associated markers on chromosome 6H (11_21032 and 11_10064) were examined for homologs in both genomes. No candidate gene was found for 11_21032 but an interesting gene coding for a C2H2 zinc-finger protein was identified for 11_10064 from the rice genome.

DISCUSSION

All of the most widely grown barley cultivars in the Upper Midwest region are susceptible to SSLB and, therefore, vulnerable

TABLE 2. Single-nucleotide polymorphism (SNP) markers found significantly associated with Septoria speckled leaf blotch resistance in different panels of US barley breeding germplasm

SNP marker	Chromosome	Position (cM)	Germplasm panels (%) ^a					
			CAP (Q)	CAP (P)	CAPI	CAPII	CAPIII	CAPIV
11_10419	1H	3.8	4×10^{-17} (2.0)	2×10^{-14} (2.0)	8×10^{-10} (6.0)	7×10^{-9} (5.0)	2×10^{-7} (5.0)	2×10^{-7} (6.0)
12_31144	1H	3.8	1×10^{-19} (3.0)	8×10^{-16} (2.0)	7×10^{-11} (6.0)	2×10^{-5} (3.0)	2×10^{-7} (5.0)	2×10^{-3} (3.0)
12_10636	1H	4.5	5×10^{-9} (1.0)	1×10^{-8} (1.0)	...	1×10^{-3} (2.0)	2×10^{-2} (2.0)	...
12_30933	1H	6.0	3×10^{-5} (0.7)	3×10^{-3} (0.5)	2×10^{-3} (2.0)	...	1×10^{-2} (2.0)	...
12_11011	1H	6.0	3×10^{-5} (0.7)	3×10^{-4} (0.04)	...	1×10^{-2} (2.0)	...	...
11_21226	1H	8.8	3×10^{-2} (0.3)	...	9×10^{-6} (4.0)	...	...	...
11_10332	1H	15.4	...	...	...	6×10^{-3} (2.0)	...	...
12_31488	3H	2.9	5×10^{-2} (0.2)	3×10^{-2} (0.4)	2×10^{-2} (2.0)	...	...	...
11_21294	3H	88.8	...	...	...	6×10^{-3} (2.0)	...	...
11_20294	6H	5.4	...	...	...	4×10^{-2} (2.0)	...	...
11_21032	6H	9.1	3×10^{-7} (1.0)	6×10^{-6} (0.9)	...	6×10^{-3} (2.0)	3×10^{-3} (2.0)	...
11_10064	6H	21.7	5×10^{-20} (3.0)	8×10^{-19} (3.0)	1×10^{-5} (4.0)	1×10^{-10} (6.0)	2×10^{-7} (4.0)	...
11_10023	6H	22.4	2×10^{-3} (0.6)	3×10^{-3} (0.5)	...	...	2×10^{-3} (2.0)	...

^a For the association mapping, Q indicates Q matrix from Bayesian Markov Chain Monte Carlo as implemented by the STRUCTURE program (14) and P indicates P matrix from principle component analysis. Shown are the q values (multiple testing corrected P value) and, in parentheses, percent phenotypic variation explained by the individual SNP marker.

to severe yield loss during epidemics. Thus, it is important to incorporate SSLB resistance in these barley breeding programs. To determine whether SSLB resistance exists in U.S. breeding germplasm, 3,840 advanced lines and cultivars from 10 programs were evaluated for *S. passerinii* at the seedling stage (Fig. 1B). Unfortunately, most breeding germplasm exhibited a high level of susceptibility. Still, resistant lines were identified in all 10 programs and offer a diversity of parental material for use in breeding for SSLB resistance. The VT program had the highest frequency of resistant lines at 69% (Fig. 1B). This resistance is likely derived from cultivar Thoroughbred, which is known to carry SSLB resistance (5). The Upper Midwest region of the United States is SSLB prone, yet the regional six-rowed breeding programs of MN and N6 had a relatively low frequency of resistance at 7.3 and 12.5%, respectively. Due to the SSLB epidemics of the late 1990s, breeders in the Midwest region have been incorporating *Rsp* genes into their germplasm but many of the derived lines have not yet made it into advanced testing (K. Smith and R. Horsley, *personal communications*). In contrast, the

two-rowed Midwest breeding program of N2 had a moderately high frequency of resistant lines at 48.1%. Toubia-Rahme and Steffenson (51) also identified a few SSLB-resistant lines from this breeding program. This resistance may be derived from Gloria-sib/Copal-sib, a series of lines developed by the ICARDA/CIMMYT barley breeding program in Mexico (51).

In addition to evaluating U.S. breeding germplasm for SSLB, QTL contributing to resistance were identified using an AM approach. Four QTL conferring seedling resistance were identified on chromosomes 1H (i.e., *Rsp-qtl-1H_12_31144*), 3H (*Rsp-qtl-3H_12_31488*), and 6H (*Rsp-qtl-6H_11_21032* and *Rsp-qtl-6H_11_10064*). SNP markers associated with *Rsp-qtl-1H_12_31144* (Table 2; Fig. 2) were mapped to a 5.0-cM region on the short arm of chromosome 1H in the map of Close et al. (8). This region is near the mapping position of *Rsp2/Rsp3* reported by other workers (24,59). *Rsp2* is closely linked with *Rsp3* because no susceptible recombinants were found among 458 F₂ progeny from the cross Clho 4780/Clho10644 (59). This result also is in agreement with an earlier study by Rasmusson and Rogers (41). Moreover, five DArT markers tightly linked with *Rsp3* were mapped to the 2.3- to 5.8-cM interval on the short arm of chromosome 1H (24). Therefore, *Rsp-qtl-1H_12_31144* appears to correspond with the SSLB resistance locus *Rsp2/Rsp3*. Yu et al. (54) also found a major QTL at this location on their DArT map. The chromosome 3H QTL (*Rsp-qtl-3H_12_31488*) identified in this study was mapped to the 2.9-cM position on the short arm of chromosome 3H (8) (Table 2). Lee and Neate (24) flanked *Rsp1* with two DArT markers on the short arm of 3H at an estimated map position of 31.8 cM on the DArT marker consensus map (53). In addition, Luckert et al. (26) mapped a QTL from Clho9831 in the 1.0- to 10.2-cM interval of chromosome 3H of the DArT consensus map. Therefore, *Rsp-qtl-3H_12_31488* may lie at the same position as the QTL mapped by Luckert et al. (26) but is definitely not *Rsp1*. On the short arm of chromosome 6H, two QTL were identified in this study. *Rsp-qtl-6H_11_21032* was mapped at 9.1 cM on the chromosome and *Rsp-qtl-6H_11_10064* in the 21.7- to 22.4-cM interval (Table 2) on the SNP map (8). *Rsp4* was recently mapped to bins 3 to 4 (i.e., 26.4 to 46.0 cM) (<http://barleygenomics.wsu.edu>) on chromosome 6H in a recombinant inbred line population (48). This location is very close to the map position of *Rsp-qtl-6H_11_10064*

Fig. 3. Heat map of linkage disequilibrium (LD) for single-nucleotide polymorphism (SNP) markers significantly associated with seedling Septoria speckled leaf blotch resistance on barley chromosome **A**, 1H and **B**, 6H. Number in each cell is the pairwise LD calculated in $D' \times 100$. SNP markers significantly associated with resistance were underlined and the most significantly associated markers are labeled with an asterisk.

TABLE 3. Effect of haplotypes contributing to Septoria speckled leaf blotch (SSLB) resistance in the complete panel of U.S. barley breeding germplasm

Haplotype ^a	Line count ^b	Mean IR ^c	QTL count ^d	Disease reduction (%) ^e
AABB	3	0.7	4	83
ABBB	70	1.0	3	75
AAAB	103	1.1	3	74
ABBA	22	1.3	2	69
AAAA	93	1.5	2	64
BBBB	41	1.6	2	62
ABAB	209	2.1	2	51
BBAB	127	2.6	1	38
BAAB	94	3.0	2	30
BBBA	79	2.9	1	31
ABAA	1,536	3.7	1	13
BAAA	47	3.9	1	9
BBAA	1,206	4.2	0	0

^a Allele haplotypes at the loci of *Rsp-qtl-1H_12_31144*, *Rsp-qtl-3H_12_31488*, *Rsp-qtl-6H_11_10064*, and *Rsp-qtl-6H_11_21032*, which gave different levels of SSLB resistance in the association mapping analysis.

^b Only count numbers of each haplotype >10 were considered, except haplotype AABB, which was added manually.

^c Infection response (IR) to SSLB.

^d Number of quantitative trait loci (QTL) based on genotypes of single-nucleotide polymorphism markers 12_31144, 12_31488, 11_10064, and 11_21032 within each particular haplotype.

^e Disease reduction rate for each haplotype as assessed by comparing to barley lines lacking any resistance QTL (i.e., BBAA).

found in this study. Therefore, *Rsp-qt1-6H_11_10064* is likely coincident with *Rsp4*. Luckert et al. (26) also found a QTL from CIho9831 closely linked with AFLP marker E35616 at this location. The other QTL (*Rsp-qt1-6H_11_21032*) identified on chromosome 6H was not in LD with *Rsp-qt1-6H_11_10064* and mapped ≈ 12 cM distant from it (Fig. 3B); thus, it may be a novel QTL for SSLB resistance.

From the discussion above, it is evident that AM is more efficient for mapping SSLB resistance loci than constructing a series of different biparental mapping populations from individual resistance donors. In this study, we validated three previously reported resistance loci (two *Rsp* genes and one QTL) and identified one new QTL from a large AM panel consisting of advanced U.S. barley breeding germplasm. Although additional research will be required to validate whether the loci found in this study actually lie at the exact positions of previously described loci, AM provided an efficient means for detecting and mapping SSLB resistance loci. The efficiency and resolution of AM mapping will continue to increase as thousands of additional SNP markers are generated for barley.

The identification of SNP markers associated with resistance loci offers great opportunities for using marker-assisted selection in breeding. One critical obstacle concerning the use of exotic resistance donors is linkage drag. Utilizing elite breeding germplasm is one means of reducing linkage drag because most undesirable alleles have been removed through repeated generations of selection. In this study, all of the lines were inbred to at least the F_4 generation and have undergone some selection for various agronomic traits; thus, the amount of linkage drag associated with these resistance loci will be reduced, and the markers found associated with them can be directly used in breeding resistant cultivars. However, there are challenges in applying genome-wide QTL mapping in elite breeding populations due to the low numbers of polymorphic markers, which can result in low-resolution QTL mapping. St. Pierre et al. (48) tested 2,144 molecular markers in order to map loci for SSLB and net blotch resistance in a recombinant inbred line population but only 192 (9%) were polymorphic. This resulted in a low-resolution map that could only be defined by bins. In this study, a large collection of breeding lines ($n = 3,840$) from 10 different breeding programs was utilized. Although CAP contained many elite breeding lines, it was still sufficiently diverse at other loci, allowing for the successful mapping of SSLB resistance through an AM approach. In a previous study of the BCAP germplasm, 2,099 of 3,072 (68%) SNP markers were polymorphic (60). Based on an AM simulation study, more than half of the significant associations were located within 5 cM and 95% were within 25 cM (60). In addition, by using the same set of SNP markers, Ramsay et al. (40) and Cockram et al. (9) were able to achieve sufficiently high-resolution mapping for candidate gene identification. In this study, we found two resistance gene candidates, which happen to belong to the same gene family: the C2H2 zinc-finger protein. These proteins constitute one of the largest families of transcriptional regulators and are known to play a key role in the defense and stress response pathways in *Arabidopsis* and rice (1,6,11). Recent genetic studies also showed the possible interaction between different zinc-finger proteins during stress (7,43).

In this study, all individual QTL conferring SSLB resistance explained only a small portion of the phenotypic variation because R^2 values were 0.04 to 6% (Table 2). Together, the four identified QTL of *Rsp-qt1-1H_12_31144*, *Rsp-qt1-3H_12_31488*, *Rsp-qt1-6H_11_10064*, and *Rsp-qt1-6H_11_21032* explained only 6.3% ($R^2 = 2\% + 0.4\% + 0.9\% + 3\% = 6.3\%$) of the phenotypic variation in CAP (with P matrix in MLM) (Table 2). The low proportion of phenotypic variance explained by the identified QTL in this study was similar to that reported in other genome-wide association efforts (2,27,45,61) and can be attributed to the common problem in population genetics known as “missing

heritability” (27). The possible underlying reasons for this include (i) unidentified alleles, (ii) underestimation of single QTL effects due to incomplete LD between SNP markers and the causal gene, and (iii) failure to consider the existence of gene–gene interactions (i.e., epistasis) because only single marker effects were investigated in this study (27). One way to increase the amount of phenotypic variation explained is to increase the density of markers. In *Arabidopsis*, the amount of phenotypic variation accounted for in 107 traits was moderately high (at least one significant SNP marker with $MAF \geq 15\%$ was found to explain at least 20% of phenotypic variation), because 250,000 SNP markers were used in the analysis (3).

Population structure is a critical factor in AM. When population structure is present, spurious associations may be found between causal variants and unlinked markers (12). Two widely used methods that account for population structure are Bayesian MCMC implemented in the STRUCTURE program (37) and PCA (35,36). Both methods were used in AM and yielded very similar results (Table 2; Fig. 2). However, PCA may be the preferred method for AM in CAP because (i) PCA generally performs as well as STRUCTURE (Fig. 2) (23,35), (ii) STRUCTURE tends to overestimate the subpopulation number (23), (iii) STRUCTURE may fail to reveal subpopulations due to its qualitative classification nature (35,52), (iv) AM with PCA was successfully used for identifying loci controlling disease resistance and also agronomic traits in other studies (10,61), and (v) running PCA is fast and only requires limited computational resources for large datasets such as CAP (23,35).

Although small allele effects were found for all four individual QTL identified in this study, their cumulative effect together resulted in a marked decrease of the seedling IR (Table 3). Therefore, breeders can achieve a high level of SSLB resistance by selecting all four QTL in one genotype. The pyramiding of multiple resistance loci may also contribute to longer-lasting resistance, as demonstrated in other pathosystems (61). One very critical investigation that must be made, however, is to determine whether the resistance QTL identified at the seedling stage will also be effective at the adult plant stage—the time when SSLB starts to infect the crop. Adult plant testing was not done for the BCAP germplasm due to difficulties in establishing reliable field screening nurseries. However, based on several previous studies, it appears that some of the SSLB resistance loci effective at the seedling stage also confer resistance at the adult plant stage (51).

In summary, 3,840 advanced lines from 10 breeding programs in the United States were evaluated for *S. passerinii* at the seedling stage and four QTL for SSLB resistance were identified through an AM approach. The markers found associated with these QTL will be valuable for programs utilizing marker-assisted selection for SSLB resistance.

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