Theor Appl Genet (2014) 127:633–645
DOI 10.1007/s00122-013-2247-x

ORIGINAL PAPER

Using near-isogenic barley lines to validate deoxynivalenol (DON) QTL previously identified through association analysis

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Received: 6 March 2013 / Accepted: 25 November 2013 / Published online: 18 December 2013

Abstract Fusarium head blight (FHB) and its associated mycotoxin, deoxynivalenol (DON), are the major biotic factors limiting cereal production in many parts of the world. A recent association mapping (AM) study of US six-row spring barley identified several modest effect quantitative trait loci (QTL) for DON and FHB. To date, few studies have attempted to verify the results of association analyses, particularly for complex traits such as DON and FHB resistance in barley. While AM methods use measures to control for the effects of population structure and multiple testing, false positive associations may still occur. A previous AM study used elite breeding germplasm to identify QTL for FHB and DON. To verify the results of that study, we evaluated the effects of the nine DON QTL using near-isogenic lines (NILs). We created families of contrasting homozygous haplotypes from lines in the original AM populations that were heterozygous for the DON QTL. Seventeen NIL families were evaluated for FHB and DON in three field experiments. Significant differences between contrasting NIL haplotypes were detected for three QTL across environments and/or genetic backgrounds, thereby confirming QTL from the original AM study. Several explanations for those QTL that were not confirmed are discussed, including the effect of genetic background and incomplete sampling of relevant haplotypes.

Communicated by M. J. Sillanpaa.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-013-2247-x) contains supplementary material, which is available to authorized users.

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Introduction

Genetic studies have identified many useful loci contributing to complex traits; unfortunately, many of them remain unutilized in plant breeding (Bernardo 2008). Validation is an important and often overlooked step between quantitative trait loci (QTL) identification and subsequent research in QTL cloning, genomics studies, or marker-assisted breeding. Efforts to conduct validation are often avoided due to the large numbers of QTL identified in mapping studies and the substantial amount of time and resources required to generate independent and appropriate testing populations. Despite these deterrents, validation studies have been conducted in many crops, including maize (Austin and Lee 1996; Landi et al. 2005), soybean (Fasoula et al. 2004), sunflower (Micic et al. 2005), and tomato (Foolad et al. 2001). Successful validation studies in barley have confirmed QTL with effects on agronomic, disease resistance, and quality-related traits, among others (Ahmad Naz et al. 2012; Canci et al. 2004; Spaner et al. 1999; Castro et al. 2003; Muñoz-Amatriaín et al. 2008; Romagosa et al. 1999; Yun et al. 2006). However, these validation efforts represent only a small fraction of the published QTL.

Association mapping (AM) is a powerful tool for utilizing genotype and phenotype data from diverse germplasm to detect marker-trait associations and has its own challenges in terms of validation (Zhu et al. 2008). In barley, AM studies have generally been successful in identifying causative QTL regions; however, results are difficult to reproduce across genetic backgrounds and experiments, and effect sizes are often small (Cockram et al. 2010; Kraakman et al. 2006; Massman et al. 2011; Rostoks et al. 2006; Roy et al. 2010; Stracke et al. 2009). Despite the expanded opportunities to discover QTL through AM, few of these QTL have been confirmed by independent

validation studies to enable subsequent genetic research or application in marker-based breeding. Verification of AM results is especially important due to the complex population structure that often exists in AM panels. Varying degrees of relatedness between individuals can result in spurious associations if not accounted for properly (Lander and Schork 1994). The diverse nature of AM panels and the potential for multiple segregating alleles at a QTL make it particularly important to validate effects in relevant germplasm. Furthermore, the power to detect QTL is contingent upon many factors that vary among studies including population size, marker density, linkage disequilibrium (LD) pattern, and effect size (Long and Langley 1999). Finally, the number of marker tests conducted in analyses along with phenotyping and/or genotyping errors can increase the potential for false positive associations.

Methods to validate QTL include, but are not limited to, testing of QTL with additional progeny from the original or independent mapping populations, confirming effects via marker-assisted selection (MAS), and contrasting allele comparison using near-isogenic lines (NILs). Using NILs, in particular, has been shown to be advantageous for a number of purposes beyond validation, including integrating molecular and genetic marker maps (Muehlbauer et al. 1988), identifying QTL (Kaeppler et al. 1993), and fine mapping (Brouwer and St. Clair 2004). When QTL are initially identified in wide crosses using exotic parents, NILs are attractive to breeders as they allow confirmation of QTL and quantification of allelic effects. Such application has supported QTL results for salt tolerance in soybeans (Hamwieh et al. 2011), Fusarium head blight (FHB) resistance and grain protein content in wheat (Pumphrey et al. 2007; Prasad et al. 2003; Singh et al. 2001), and disease resistance QTL in barley (Kongprakhon et al. 2009; Smith et al. 2004).

Past studies using recurrent selection strategies to introgress donor QTL alleles into recipient lines to construct NILs have been successful in confirming QTL effects, but these populations require additional time and resources to develop. In contrast, heterogeneous inbred families (HIFs) can be developed relatively easily from partially inbred lines after one generation of selfing and selection (Tuinstra et al. 1997). This approach has been tested in several crop species, including sorghum to characterize seed weight (Tuinstra et al. 1997) and drought resistance QTL (Tuinstra et al. 1998). Advanced inbred lines that are not completely homozygous at all loci provide a resource for simultaneous breeding and testing of effects in relevant backgrounds (Pumphrey et al. 2007).

Fusarium head blight, or "scab", is a disease of small grains which in the United States is caused primarily by the fungal pathogen *Fusarium graminearum* Schwabe [teleomorph: *Gibberella zeae* (Schwein)] and has been a major target for small grains breeding programs in the US (Bai and Shaner 2004; Buerstmayr et al. 2009; Steffenson and Smith 2006). Resistance to FHB and the accumulation of the mycotoxin produced by the pathogen, deoxynivalenol (DON), is both complex and quantitative, greatly affected by environmental factors, and therefore an appropriate candidate for MAS. No current barley varieties are immune, but varieties with improved resistance have been developed (Smith et al. 2013). Several previous bi-parental mapping studies have identified QTL for FHB resistance distributed across the barley genome (Dahleen et al. 2003; Mesfin et al. 2003; de la Pena et al. 1999; Zhu et al. 1999; Ma et al. 2000; Horsley et al. 2006). These studies have limited resolution (less than 10–20 cM; Holland 2007) and are often based on wide crosses using exotic sources of resistance. As a result, numerous other traits can potentially cosegregate with resistance, making interpretation of disease resistance difficult.

A previous AM study by Massman et al. (2011) identified QTL for FHB and DON accumulation using elite germplasm from four Midwest US barley breeding programs. In this study, we present a rare validation of QTL identified by association mapping in the Massman study using near-isogenic lines. Our specific objectives were to (1) validate DON QTL detected in the original AM study (2) compare allelic effects from the AM study to those from the NIL study (3) investigate haplotype diversity at DON QTL within the original AM panel.

Materials and methods

To validate DON QTL previously identified through association mapping, we developed sets of NILs for QTL regions using selected SNP markers informative for the regions under investigation. These markers were used to genotype progeny from the individual plants that were heterozygous at DON QTL identified in the prior AM study to identify near isogenic homozygote pairs at the QTL regions of interest.

Marker selection

A total of 28 candidate QTL were identified in the six-row panels from the original AM study spanning all seven chromosomes (Supplemental Table 1). We inspected SNP genotypes at those QTL for 463 six-row lines from the AM panels and selected those identified as heterozygotes. All lines were previously genotyped by two sets of SNP markers referred to as BOPA1 and BOPA2 as part of the barley Coordinated Agricultural Project (CAP; http://www.barleycap.org; Close et al. 2009). The SNP data for these lines are available in the Hordeum Toolbox

(http://thehordeumtoolbox.org; Blake et al. 2012). We also considered marker significance (*p* values) from the Massman et al. (2011) AM studies and distribution across the QTL region when making selections. Since the original study that used only BOPA1 markers was conducted, data for an additional 1,536 SNP markers (BOPA2) became available. Heterozygous markers within this pool were used to supplement the BOPA1 markers to span a QTL region. Based on these criteria, a total of forty-eight markers were selected to generate the NIL families for fifteen QTL regions, for which each region comprised of one to ten markers (Supplemental Table 2).

NIL development and genotyping

Markers and CAP lines used as parents for NIL development were selected simultaneously, considering both the number of heterozygous markers per line and the level of significance of the segregating markers spanning a QTL. Most parent lines were chosen to be heterozygous at only one DON QTL region. Twenty-four lines inbred to at least the F4 generation were ultimately identified to develop NIL families. Of those lines, 11 were developed from lines originating from the University of Minnesota (MN), 12 from North Dakota State University (ND), and 1 from Busch Agriculture Resources, Inc. (BA).

NIL families were generated by planting fifteen progeny seed from each of the 24 CAP lines in separate pots (one seed/pot) in a greenhouse. The seed of each CAP line was from a single plant that was genotyped with BOPA markers as part of the original AM study, thus we expected the progeny to segregate at a 1:2:1 ratio for the marker that was heterozygous in the CAP line parent. Tissue was harvested at the two-leaf stage from each plant and freeze dried for storage until genotyping. DNA extraction from the leaf tissue was carried out at the USDA-ARS Small Grains Genotyping Center in Fargo, ND using a modified wheat and barley extraction protocol (Pallotta et al. 2003). All lines were genotyped for the forty-eight SNP markers with a custom Veracode assay using Illumina's Bead Express Technology (Illumina Inc., CA). The Illumina Genome Studio software was used to score the marker genotypes. These data were used to assign lines into NIL classes of contrasting haplotypes. Seven of the initial twenty-four families were excluded from further analysis based on the following criteria: (1) segregation at more than two QTL (2) residual heterozygous loci in the NILs or (3) the genotypic class assignment was unclear based on Genome Studio clustering results. A total of 92 NILs were developed from 17 families that segregated for nine DON QTL regions (Table 1). The segregation of the 48 SNP markers for the 17 parents of the NIL families is shown in Supplemental Table 3.

Phenotypic evaluation of NILs

The 92 selected NILs and 17 NIL parent lines from the AM study were planted in the summer of 2010 in Stephen, MN to increase seed used to plant disease trials in the summer of 2011 at three locations: St. Paul MN, Crookston MN, and Osnabrock ND. All three locations were planted in a randomized complete block design, treating each NIL

Table 1 Seventeen barley Coordinated Agricultural Project (CAP) lines selected as parents for near isogenic line (NIL) progeny

CAP line	Parents of CAP line	Program[a]	CAP year	DON QTL[b]
FEG126-12	FEG66-31/M120	MN	2006	DON.17, DON.18
FEG132-63	FEG80-74/FEG67-12	MN	2006	DON.29
FEG148-40	FEG96-22/Rassmusson	MN	2006	DON.18
FEG149-18	ND20407/M118	MN	2006	DON.10
FEG168-09	Comp351/Rassmusson/M98-102	MN	2007	DON.17
M04-45	M001-71/M01-87	MN	2007	DON.13
ND23899	Drummond/ND17643	ND	2006	DON.18
ND25657	Stellar/ND20481	ND	2007	DON.31
ND25661	Stellar/ND20481	ND	2007	DON.13, DON.18[c]
ND25665	Stellar/ND20481	ND	2007	DON.10, DON.20
ND25681	Stellar/ND20481	ND	2007	DON.07
ND25684	Stellar/ND20481	ND	2007	DON.33
ND25691	Stellar/ND20481	ND	2007	DON.13
ND25694	Stellar/ND20481	ND	2007	DON.10, DON.31
ND25697	Stellar/ND20603	ND	2007	DON.10, DON.29, DON.30[c]
ND25728	ND19474/ND20477	ND	2007	DON.33
ND25732	ND19474/ND20477	ND	2007	DON.29

[a] CAP lines contributed by the University of Minnesota (MN) and North Dakota State University (ND) breeding programs

[b] DON QTL regions defined by Massman et al. (2011) in which at least one marker is heterozygous

[c] CAP parent heterozygous at multiple QTL, though progeny segregated for only one

family as a separate experiment. NILs and NIL parents were randomized within each family and families were randomized across five blocks. In each family, haplotypes were evaluated by at least one NIL for a minimum of two lines per family, though the total numbers varied between two and nine. For each entry, 4 g of seed was planted in 1.5 m single row spaced 0.3 m apart in St Paul (planted April 25) and Crookston (planted May 18). The Osnabrock disease nursery was planted on June 14 with approximately 15 kernels in 0.3 m single row plots.

Heading date (HD) was assessed as the number of days after planting in which 50 % of the heads in a plot had emerged half way or more from the boot. All entries for a block within a NIL family experiment were rated for disease on the same day. At the St. Paul and Crookston locations, ten arbitrarily selected spikes within each row were scored for FHB severity using the following scale corresponding to the percent of infected kernels on a spike: 0, 1, 3, 5, 10, 15, 25, 35, 50, 75, and 100 %. FHB severity and HD data from Osnabrock were not collected.

The St. Paul location was inoculated with a mixture of 50 *F. graminearum* isolates collected between 2005 and 2010 from Minnesota wheat and barley fields. Plots were inoculated twice with micro-conidia using CO_2-powered backpack sprayers, once just after heading when greater than 90 % of the spikes per row had emerged from the boot and again approximately 4 days later (Steffenson 2003). Due to the differential flowering times observed among families, over half the field (439 plots, including: all five reps of NIL families FEG149-18, ND25665, ND25694, FEG126-12, FEG148-40, ND25661, FEG132-63, ND25657, and ND25684; one rep of M04-45; two reps of ND25681; four reps of ND25732) was inoculated first on June 28 and again on July 1, while the second half (296 plots, including remaining NIL families and replications) was inoculated on July 1 and again July 5. All entries within a replication within a family were inoculated at the same time. Inoculum at Crookston and Osnabrock was applied as a *Fusarium*-colonized grain spawn at approximately 56 kg/ha at 2 weeks and 1 week before flowering (Horsley et al. 2006). All fields received mist-irrigation after inoculum was applied to facilitate disease development.

Plots were harvested in St. Paul and Crookston using a hand sickle and threshed on site with a custom Vogel thresher. Samples from Osnabrock were hand harvested by sickle, placed in paper bags, and later threshed in St. Paul. All grain samples were cleaned to remove excess chaff using a belt thresher. After cleaning, the grain was hand mixed and sub-sampled before grinding for toxin analysis. Approximately 20 g of grain from each plot was ground using a Cyclotec sample mill with a 1 mm mesh sieve and analyzed for DON using gas chromatography and mass spectrometry (Tacke and Casper 1996).

Analysis of NIL study haplotypes

Phenotypic data for each family were checked for departure from normality and homogeneity of variance by plotting quantiles as a QQ-plot and performing the Bartlett test, respectively. Observations from the phenotypic data sets were excluded from analysis if the values exceeded three standard deviations from the NIL family means. Phenotypic data for NILs (CAP parent line data were excluded) were used to estimate the variance components using the restricted maximum likelihood method of "PROC MIXED" implemented in SAS v 9.2 (SAS Institute 2008). A mixed model was fitted to a combined locations dataset on a family basis including haplotypes as a fixed effect and location, replication nested within location, line nested within haplotype, and line nested within haplotype by environment interaction as random effects. Inclusion of line nested within haplotype, and line nested within haplotype by environment interaction as random effect lowers the chance of false positive associations as demonstrated by Piepho (2005). The Kenward Roger method was implemented to approximate the appropriate denominator degrees of freedom. A QTL was considered validated if significant differences among haplotypes were observed below the $\alpha = 0.05$ threshold.

A QTL haplotype was defined as the combination of marker genotypes for an individual for the set of markers selected to represent the QTL region. Haplotype effects for traits were calculated on a NIL family basis as the difference between haplotypes as a percentage of the family mean. The effect direction was determined by subtracting the numerically lower haplotype from the higher haplotype (i.e., haplotype 2 minus haplotype 1). Haplotype numbers were arbitrarily assigned.

Analysis of AM study haplotypes

To assess haplotype performance from the original AM data sets, we grouped CAPI and CAPII lines based on marker haplotype for the set of markers used to define each QTL region in the NIL study. The two haplotypes defined by each NIL family represent a subset of those that exist in the AM panel. Least squares means were calculated with data from four trials described in the Massman et al. (2011) study. Groups of lines in the AM panel representing the same haplotypes as those tested in the NIL study were used to determine effects. Effect sizes for DON, FHB, and HD were calculated based on the average performance of lines comprising a QTL haplotype as a percentage of the population mean. These calculations did not account for population structure and were based on phenotype data collected in 2006 and 2007 in the original study. A two-sided unpaired *t* test was used to determine whether the same

haplotype comparisons made in NIL trials were significant in the AM study. Finally, a Tukey's honest significance difference (HSD) means separation procedure was used to identify differences in mean performance among all the haplotypes observed in the AM panel.

Results

Disease pressure was sufficient at all three disease nurseries to assess phenotypic variation with average DON levels of 10.6, 11.5, and 32.7 ppm for Osnabrock, St. Paul, and Crookston, respectively. FHB severity was on average 5 % in St. Paul, 31 % in Crookston, and HD was on average about 10 days earlier in Crookston. Error variances were homogenous for all traits across all environments. The following sections describe the results from combined location analysis for the NIL families evaluated.

Single QTL NIL families

Fourteen NIL families segregating at a single QTL region were used to evaluate eight DON QTL (Table 2). In three instances, a single family was used to evaluate a region; in four cases two families were used, and in one case three families were used. Two haplotypes were defined by each NIL family and each haplotype within a family was represented by one to seven lines. Three of the fourteen NIL families segregating at a single QTL had significant differences between haplotypes for DON. Two NIL families were significant for HD, of which one was in common with a family significant for DON. Ten NIL families were not associated with any of the three traits. FHB severity was not significant between haplotypes in any family.

At QTL region DON.13, haplotype 2 conferred a decrease in DON concentration relative to haplotype 3 by 23 % in NIL family ND25691. HD was also significant in two of the NIL families with haplotype 2 conferring a 1.8 % (1 day) and 2.4 % (0.3 day) difference in flowering time compared to haplotypes 1 and 3, respectively. This haplotype consistently differs from the more susceptible, later maturing haplotypes 1 evaluated in M04-45, and the later maturing haplotype 3 for ND25691 at two markers less than 1 cM apart (Fig. 1a). This QTL was among the most significant for DON in four Massman mapping panels, including the CAPI [MN, North Dakota two-row (N2), N6, and BA lines from 2006; $n = 384$] CAPII (MN, N2, N6, and BA lines in 2007; $n = 384$), CAPI six-row (MN, N6, and BA six-row lines from 2006; $n = 224$), and CAPII six-row populations (MN, N6 and BA six-row lines from 2007; $n = 241$).

At QTL region DON.10 the effect of haplotype 4 decreased DON relative to haplotype 3 by 15 % (Table 2). These two haplotypes contrasted three markers spanning 1.6 cM of the 6.3 cM QTL region in the original mapping study (Fig. 1b). DON.10 was previously identified by several bi-parental mapping studies, and in the original AM study was significant in CAPI, CAPI six-row, and CAPII six-row mapping panels (de la Pena et al. 1999; Ma et al. 2000; Massman et al. 2011; Mesfin et al. 2003; Zhu et al. 1999).

Finally, QTL region DON.33 was evaluated using two NIL families with haplotypes defined by a single marker. The effect of haplotype 2 significantly decreased DON by 9 % relative to haplotype 1 in the ND25728 NIL family (Table 2).

Two QTL NIL families

NIL family FEG126-12 resulted in significant haplotype differences for FHB (Table 3). In this family, haplotype 2 of DON.17 is identical to haplotype 2 of single NIL family FEG168-09 which had no effect on DON (Fig. 2a; Table 2). The other haplotype, 3, is distinct from those evaluated in FEG168-09. The two haplotypes of DON.18, 5 and 6, are distinct from the other four haplotypes identified in single NIL families FEG148-40 and ND23899. Haplotype 3 at DON.17 and haplotype 5 at DON.18 occur together in two of the NILs and haplotype 2 at DON.17 and 6 at DON.18 occur together in the other NIL. The 3 and 5 haplotype combination is lower for FHB compared to the 2 and 6 haplotype combination. When these QTL were assessed individually in NILs, there was no effect on disease suggesting that the unique haplotypes segregating in the FEG126-12 are responsible for the effect on disease.

NIL families ND25665 and ND25694 were not significant for any trait (Table 3). ND25665 tested the effects of DON.10 and DON.20. In single QTL analysis, the DON.10 haplotype 4 resulted in a significant DON decrease, while haplotype 1 imparted no significant difference (Fig. 2b). DON.20 was not evaluated individually. The other two QTL line ND25694 segregated at DON.10 and DON.31 (Fig. 2c) and was not significant for any trait.

Overall in the NIL analysis, three QTL were validated for an effect on DON based on NIL haplotype comparisons and five were found to have no effect. One of the eight DON regions was found to have an effect on HD. NIL families segregating at multiple QTL suggest that haplotypes that were not segregating in the single QTL analysis could be responsible for the effect of those regions on DON or HD.

Haplotype effects in the association panel

There were additional QTL haplotypes in the AM panel (referred to as AM haplotypes) beyond those represented

Table 2 Significant haplotype effect differences from the near isogenic line (NIL) and association mapping (AM) studies for deoxynivalenol (DON), Fusarium head blight (FHB) and heading date (HD)

NIL family	# Lines per haplotype				DON QTL region								Haplotype effects[a]					
	1	2	3	4	DON.07	DON.10	DON.13	DON.17	DON.18	DON.29	DON.31	DON.33	NIL haplotype study[b]			AM haplotype study[c]		
													DON	FHB	HD	DON	FHB	HD
ND25681	2	1			X								–	–	–	−11 %**	–	−0.7 %**
FEG149-18	4	3				X							–	–	–	−20 %***	–	−0.8 %**
ND25697			1	1		X							−15 %**	–	–	−29 %***	–	–
M04-45	1	2					X						–	–	−1.8 %*	–	–	–
ND25691		2	4				X						14 %***	–	2.4 %***	17 %***	17 %**	–
ND25661			1	1			X						–	–	–	−17 %**	−29 %**	−0.8 %*
FEG168-09	3	4						X					–	–	–	27 %**	–	–
FEG148-40	7	2							X				–	–	–	44 %***	72 %**	1.1 %*
ND23899			2	4					X				–	–	–	–	–	–
FEG132-63	1	1								X			–	–	–	–	29 %*	−2 %***
ND25732	4		2							X			–	–	–	–	–	–
ND25657	1	2									X		–	–	–	–	−16 %***	–
ND25684	5	4										X	–	–	–	17.1 %***	21 %***	–
ND25728	1	1										X	−9 %**	–	–	17.1 %***	21 %***	−0.4 %*

*, **, *** Significant difference detected between haplotypes at p value > 0.05, 0.01, and 0.001, respectively

[a] Haplotype effects significant in either NIL or AM haplotype analysis

[b, c] Effect of haplotype from the NIL or AM study, respectively

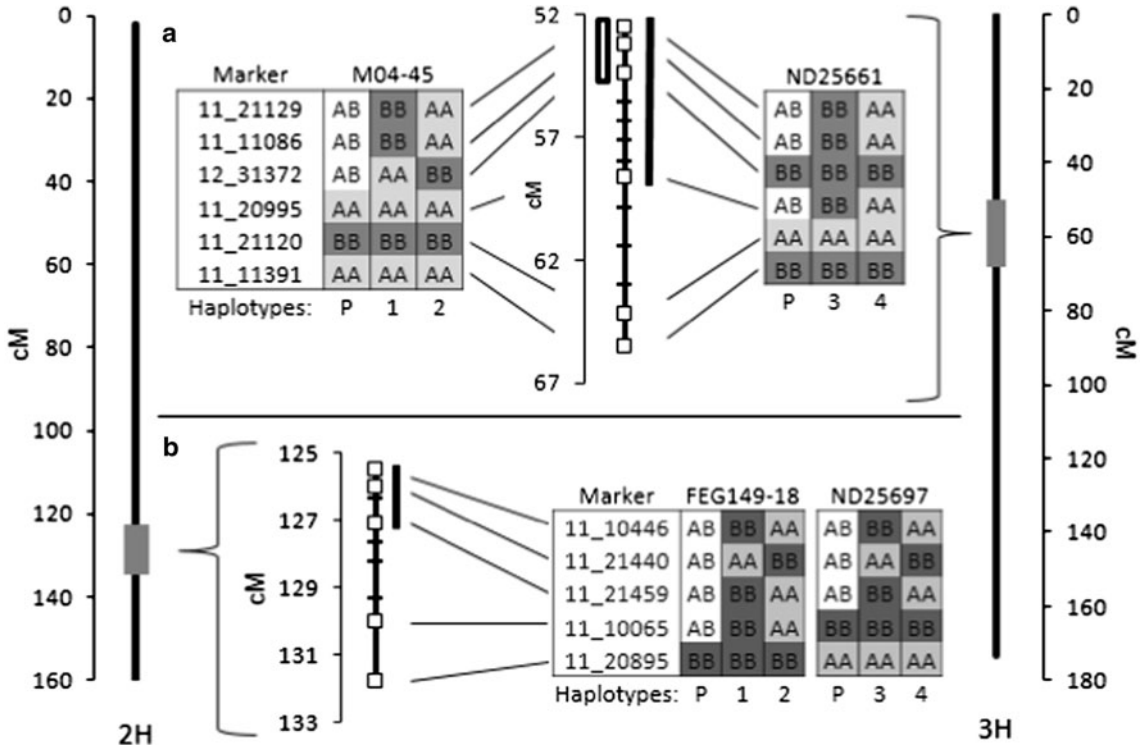

Fig. 1 QTL regions based on contrasting NIL family haplotypes for **a** DON.13 and **b** DON.10. For each QTL, the entire chromosome, the DON QTL region (including all BOPA markers within the region are shown; *square boxes* indicate selected markers, *dashes* represent non-informative intervening markers), and the NIL family haplotypes are shown within *brackets*. Each enclosed *block* indicates the marker haplotypes of the CAP parent (P) and NILs (numbers 1–4). AA and BB represent homozygous alleles, and AB represents heterozygotes. Narrowed regions are indicated by *thick black* (DON), and *black* and *white* (HD) *bars*

Table 3 Significant *p* value differences between near isogenic line (NIL) family haplotypes isolating two QTL across three families for deoxynivalenol (DON) concentration, Fusarium head blight (FHB) severity, and heading date (HD)

NIL family	Class[a]	DON QTL haplotypes evaluated[b]											Significant *p* values		
		DON.10			DON.17		DON.18		DON.20		DON.31				
		Hap 1	Hap 2	Hap 4	Hap 2	Hap 3	Hap 5	Hap 6	Hap 1	Hap 2	Hap 1	Hap 2	DON	FHB	HD
FEG126-12	1					X	X						–	0.013	–
	2				X			X							
ND25665	1			X					X				–	–	–
	2	X								X					
	3			X						X					
ND25694	1	X									X		–	–	–
	2		X									X			
	3		X								X				
	4	X										X			

[a] Haplotype classes in multiple QTL comparisons based on the combination of NIL haplotypes across two QTL

[b] DON QTL, as defined by Massman et al. (2011) and associated NIL haplotypes

in the NILs (Table 4). Additional AM haplotypes with greater than 1 % frequency within the population were identified in six of the eight QTL isolated by NIL families. The haplotypes observed in the NIL study were generally the most frequent haplotypes in the AM panel.

a

QTL	marker	NIL family FEG126-12														
DON.17	11_20422	AA	AA	AB	AB	AB	AB	AB	BB	AB	AB	BB	AA	AA	AB	BB
DON.17	11_20302	AA	AA	AB	AB	AB	AB	AB	BB	AB	AB	BB	AA	AA	AB	BB
DON.17	11_20777	BB	BB	AB	AB	AB	AB	AB	AA	AB	AB	AA	BB	BB	AB	AA
DON.17	11_21374	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
DON.17	11_21122	AA	AA	BB	AB	AB	AB	BB	BB	AB	AB	BB	AA	AA	AB	BB
DON.17	12_10860	BB	AB	AA	AB	AB	AB	AA	AA	AB	AB	AA	BB	BB	AB	AA
	haplotype:	**3**							**2**			**2**	**3**	**3**		**2**
DON.18	12_30328	BB	AB	AA	AB	AB	AB	AA	AA	AB	AB	AA	BB	AB	AB	AA
DON.18	11_10756	BB	AB	AA	AB	AB	AB	AA	AA	AB	AB	AA	BB	AB	AB	AA
DON.18	11_21073	AA	AB	BB	AB	AB	AB	BB	BB	AB	AB	BB	AA	AB	AB	BB
DON.18	11_20289	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
DON.18	11_11114	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
DON.18	11_20361	AA	AB	BB	AB	AB	AB	BB	BB	--	AA	BB	AA	AB	AB	BB
DON.18	11_21191	AA	AB	BB	--	AB	AB	BB	BB	AB	AA	BB	AA	AB	AB	BB
	haplotype:	**5**		**6**				**6**	**6**			**6**	**5**			**6**

b

QTL	marker	NIL family ND25665														
DON.10	11_10446	AA	AA	AB	AA	BB	AA	AB	AB	AB	AB	AB	AB	AB	AA	BB
DON.10	11_21440	BB	BB	AB	BB	AA	BB	AB	AB	AB	AB	AB	AB	AB	BB	AA
DON.10	11_21459	AA	AA	--	AA	BB	AA	--	--	--	--	--	--	--	AA	BB
DON.10	11_10065	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
DON.10	11_20895	AA	AA	AB	AA	BB	AA	BB	BB	AB	AB	AB	AB	BB	AA	BB
	haplotype:	**4**	**4**		**4**	**1**	**4**								**4**	**1**
DON.20	11_20838	AB	AB	BB	BB	AA	AB	AB	AB	AB	AB	AA	AB	BB	AA	AB
	haplotype:			**1**	**1**	**2**						**2**			**2**	

c

QTL	marker	NIL family ND25694														
DON.10	11_10446	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
DON.10	11_21440	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
DON.10	11_21459	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
DON.10	11_10065	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
DON.10	11_20895	BB	AA	AB	AA	AA	BB	AB	BB	AA	AB	AB	AB	AA	AA	AB
	haplotype:	**1**	**3**		**3**	**3**	**1**		**1**	**3**				**3**	**3**	
DON.31	11_20868	BB	AB	BB	AA	BB	AA	AB	AA	AB	BB	AA	AA	AA	AB	BB
	haplotype:	**1**		**1**	**2**	**1**	**2**		**2**		**1**	**2**	**2**	**2**		**1**

Fig. 2 Two-QTL NIL families and associated haplotypes within each QTL regions with haplotypes defined as the contrasting allele combinations defined by markers chosen to span the region (Supplemental Table 2). **a** NIL family FEG126-12 at DON.17 and DON.18. **b** NIL family ND25665 at DON.10 and DON.20. **c** NIL family ND25694 at DON.10 and DON.31. Each NIL family is comprised of fifteen NIL lines. *Boxes* indicate NIL line classes composed of the combined haplotypes of two QTL. The genotypes AA and BB represent homozygous markers, AB represents heterozygotes

AM haplotype effects were significant in nine and seven of fourteen families for DON and FHB, respectively (Table 2, AM haplotype study). Effect sizes ranged from 11 to 44 % for DON and from 16 to 72 % for FHB. HD effects were small (less than 3 %) and significant in six families. HD and DON effects were in the same direction in four families, and opposite in one family. When a DON effect was detected in the NIL study, there was a similar effect in the AM study with one exception. In family ND25728, the direction of the effect was negative in the NIL study and positive in the AM study. In terms of the DON effect size, the two studies were in general agreement although the AM haplotype effects were generally larger. The detection of heading date was inconsistent between the AM and NIL studies.

Discussion

Validating QTL prior to further genetic investigation or implementing MAS in breeding is a prudent step to insure effective use of resources. This is particularly true for QTL discovered by AM since there is an increased risk of false positive discoveries due to the complex population structure

Table 4 Trait values for haplotypes comprised of more than one marker within the CAPI and CAPII association panel for eight deoxynivalenol (DON) QTL

Study	QTL	Haplotype	*n*	Trait[A]		
				DON (ppm)[B]	FHB (% severity)[C]	HD (DAP)[D]
NIL	DON.07	1	74	25.3[a]	10.2[a]	52.7[a]
NIL	DON.07	2	327	22.7[b]	11.0[a]	52.3[b]
AM	DON.07	3	54	20.9[b]	11.6[a]	52.6[a]
Total haplotypes: 5*			HSD**	2.4	1.7	0.3
NIL	DON.10	1	154	22.9[ab]	9.9[b]	52.7[a]
NIL	DON.10	2	118	18.7[b]	9.8[b]	52.3[a]
NIL	DON.10	3	119	26.4[a]	12.0[ab]	52.3[a]
NIL	DON.10	4	21	19.0[b]	10.2[b]	51.8[a]
NIL***	DON.10	5	27	26.0[a]	13.8[a]	52.4[a]
AM	DON.10	6	8	26.1[a]	15.2[a]	52.5[a]
Total haplotypes: 11			HSD	4.7	3.4	0.8
NIL	DON.13	1****	1	11.7	13.9	50.8
NIL	DON.13	2	289	21.7[b]	10.4[ab]	52.5[a]
NIL	DON.13	3	76	25.6[a]	12.2[a]	52.4[a]
NIL	DON.13	4	20	21.4[b]	9.0[b]	52.0[ab]
AM	DON.13	5	14	22.7[ab]	12.0[a]	51.7[b]
Total Haplotypes: 15			HSD	3.7	2.7	0.6
NIL	DON.17	1	7	17.4[b]	8.6[a]	52.1[a]
NIL	DON.17	2	379	23.9[a]	11.4[a]	52.4[a]
AM	DON.17	3	37	17.0[b]	7.7[a]	52.2[a]
AM	DON.17	4	22	19.2[ab]	7.8[a]	52.2[a]
Total haplotypes: 11			HSD	5.4	3.8	0.9
NIL	DON.18	1	13	16.9 [cd]	7.9 [cd]	52.1[a]
NIL	DON.18	2	9	26.0[a]	16.1[ab]	52.6[a]
NIL	DON.18	3	105	25.6[ab]	11.4[bc]	52.5[a]
NIL	DON.18	4	–	–	–	–
NIL***	DON.18	5	247	22.1[abc]	10.5 [cd]	52.4[a]
NIL***	DON.18	6	17	25.0[ab]	12.2[bc]	52.2[a]
AM	DON.18	7	8	11.4[d]	5.5[d]	50.5[b]
AM	DON.18	8	14	27.3[a]	18.0[a]	52.4[a]
AM	DON.18	9	8	18.2[bcd]	8.8 [cd]	52.4[a]
AM	DON.18	10	7	23.6[abc]	8.6 [cd]	52.6[a]
Total haplotypes: 23			HSD	7.7	5.3	1.3
NIL	DON.29	1	20	21.3[bc]	9.0[b]	52.8[ab]
NIL	DON.29	2	38	20.2[bc]	12.1[ab]	51.9[bc]
NIL	DON.29	3	85	23.6[abc]	9.8[b]	52.7[ab]
AM	DON.29	4	69	23.5[abc]	12.9[ab]	52.1[abc]
AM	DON.29	5	24	30.2[a]	16.8[a]	52.2[abc]
AM	DON.29	6	25	26.8[ab]	11.9[ab]	52.2[abc]
AM	DON.29	7	12	20.9[bc]	9.5[b]	52.8[ab]
AM	DON.29	8	16	25.4[ab]	12.1[ab]	52.5[ab]
AM	DON.29	9	20	22.1[abc]	8.4[b]	53.2[ab]
AM	DON.29	10	11	22.4[abc]	7.9[b]	52.3[abc]
AM	DON.29	11	12	19.5[bc]	9.5[b]	52.5[ab]
AM	DON.29	12	17	22.0[abc]	8.8[b]	52.3[abc]
AM	DON.29	13	14	22.4[abc]	8.5[b]	53.0[ab]
AM	DON.29	14	6	22.0[abc]	7.8[b]	52.6[ab]
AM	DON.29	15	9	14.9[c]	7.6[b]	51.0[c]
AM	DON.29	16	5	19.1[bc]	13.0[ab]	52.1[abc]
AM	DON.29	17	5	19.8[bc]	53.5[a]	16.3[a]
Total haplotypes: 60			HSD	8.8	5.9	1.4

NIL haplotype evaluated in NIL study, *AM* additional haplotype in association panel with >1 % frequency

[A] Average of all lines of the haplotype

[B] DON concentration in parts per million (ppm)

[C] Fusarium head blight severity (in % infected kernels)

[D] Heading date in days after planting (DAP)

* The number of unique haplotypes per QTL identified in the Massman et al. (2011) association panel

** Tukey's Honest Significant Difference (HSD) critical value with superscript letters indicating mean similarity between haplotypes at the $p > 0.05$ significance level

*** NILs from analysis of 2 QTL families

**** Haplotype class excluded from means separation procedure due to insufficient replication

that typically exists in AM panels. We were able to use NILs with contrasting marker haplotypes at QTL for DON detected by AM to validate and directly estimate haplotype effects. We validated both QTL that were consistently detected in the original AM study (DON.10 and DON.13) as well as one that was detected with less confidence (DON.33).

The FHB-related QTL we studied typically explained only 1–5 % of the observed variation in the AM study (Massman et al. 2011). In contrast, the *Fhb1* gene in wheat has been mapped repeatedly and accounts for 20–60 % of the variation observed in bi-parental mapping populations (Anderson et al. 2001; Buerstmayr et al. 2002; Buerstmayr et al. 2003; Waldron et al. 1999; Zhou et al. 2002). *Fhb1* NILs containing the resistant allele averaged 23 and 27 % less in disease severity and infected kernels, respectively, across several populations evaluated in the field (Pumphrey et al. 2007). Interestingly, only half of the NIL pairs for *Fhb1* studied showed a significant effect for disease spread as measured in a greenhouse assay; the phenotype that *Fhb1* directly impacts. In another study, Haberle et al. (2007) validated the effect size and direction of two FHB resistance-related QTL, which individually decreased severity by 27 % relative to the susceptible allele in a backcross population of winter wheat. Despite the fact that the QTL we studied explained a very small proportion of the genetic variation in the AM panel, the allelic effects of validated QTL ranged from a 9 to 15 % reduction in DON. This suggests validation efforts may be warranted even for associations that appear to have small effects when identified by AM.

Our primary objective was to validate DON QTL, but we also observed associations between DON and heading date that have been previously noted (de la Pena et al. 1999; Ma et al. 2000). In the AM study, three of the five DON QTL investigated (DON.10, DON.13, DON.29) were associated with heading date (Massman et al. 2011). It is often speculated that later heading results in lower disease as a result of disease avoidance rather than disease resistance *per se*, but there is some evidence supporting tight linkage of HD and resistance genes (Massman et al. 2011; Nduulu et al. 2007). From our study of DON.13, the differential association between DON and HD among two NIL families indicates that linkage between the traits has likely been broken.

Failure to validate QTL

NILs provided a relatively simple means to validate QTL. No difference among NILs would suggest that the QTL was a false positive. Not surprisingly and consistent with past studies of FHB and DON, not all regions were confirmed in our study; however, several potential explanations could explain the failure to validate a QTL.

First, the effect of genetic background may have played a role in detecting differences among NILs. Evidence for a background effect is given by the two DON.33 NIL families that were both from the ND breeding program but have different pedigrees. Both tested the effects of identical haplotypes in the region using a single marker, but only one family was significant for DON. Pumphrey et al. (2007) found evidence for a background effect in the validation of *Fhb1*. The authors hypothesized that higher background resistance in some NIL families might make it more difficult to see a difference between NILs compared to NILs from families with lower base levels of resistance. Greater background resistance may also explain what we observed at DON.13. The ND family ND25691 resulted in higher overall DON levels than the MN family M04-45 (data not shown); however, the lower DON in M04-45 may be due to the fact that it is hulless and lower DON may result from the loss of the hull during harvest (Legzdina and Buerstmayr 2004; Clear et al. 1997).

Another possible explanation for lack of validation is that the haplotypes conferring an effect in the AM panel were not those that were contrasted in the NILs that we developed. This could explain why at DON.13 only one of the three NIL families showed a difference in DON. A total of four haplotypes were evaluated, but only the family that contrasted haplotypes 2 and 3 was significantly different for DON. Haplotype effect estimates from AM population indicated that haplotype 3 conferred the least resistance which is consistent with the effect directions observed in the NIL study.

The complications of validation using NILs when multiple haplotypes for a QTL are segregating is apparent when we look at the large number of haplotypes present at some QTL regions (Table 4). Since any NIL family will compare only two haplotypes, it is possible that our sample may miss the most informative haplotypes. This may indeed be the case for DON.29 where haplotypes 1, 2, and 3 did not differ from each other when tested as NILs. However, the AM results indicate that haplotype 5 would have shown an effect on DON if contrasted with haplotypes 1, 2, or 3. We also note there are cases where a difference in performance predicted by the AM study was not observed in the NILs as occurred with comparisons of haplotypes 1 and 2 at DON.18 (Tables 2, 4). Consideration of multiple haplotypes in the context of NIL validation also suggests that conducting AM by haplotype rather than by SNP could increase power to detect associations (Hamblin and Jannink 2011; Lorenz et al. 2010).

Finally, if a QTL was not validated it is possible that even among near-isogenic lines there could be other loci for disease resistance still segregating, masking the effect for which the NILs were designed. We attempted to account for this by genotyping all NILs with a set of 48 markers that mapped to known QTL. In fact, several NIL parents were segregating at more than two DON QTL and were excluded from our study because of the anticipated complexity in

interpreting those results. Given the large number of loci that likely contribute to DON and the level of residual heterozygosity present in the CAP line parents, SNPs associated with DON that were not included in our 48 SNP screening panel could still be segregating among NILs.

Advantages of using NIL validation

The choice of QTL validation method is a function of the trait of interest and the organism under study. The vast majority of AM studies that have been conducted have been done in humans for disease-related traits (http://www.genome.gov/gwastudies) and approaches for validation are limited to conducting subsequent association studies in panels that are distinct from the original discovery panel. Therefore, the factors that could potentially limit power and increase bias in the original study (i.e., rare variants and population structure) are still relevant in the validation study.

The ability to easily generate NILs in plant systems offers substantial advantages with regard to validation and characterization of QTL. Measuring allelic effects in near-isogenic backgrounds eliminates the factors present in AM studies that can limit detection, such as population structure, varying allele frequency, and extent of LD. Using NILs, each allele or haplotype is evaluated at a designed frequency determined by the number of NILs generated, creating a situation with optimum power to detect an association. This reduces the confounding relationships of both differential allele frequencies and background effects. NIL-based analysis is not subject to bias caused by population structure because QTL are tested in a fixed genetic background. However, analysis of identical NIL haplotypes developed across different populations provides an opportunity to investigate background effects on isolated QTL.

This NIL approach compared haplotypes on a family-wise basis without the disadvantage of confounding background effects. Identical QTL haplotypes among families were identified in several instances suggesting that a multi-family analyses may increase statistical power to detect effect differences. To test this hypothesis, additional field testing would be required to evaluate all relevant haplotypes together in the same experiment; however, the increase in power due to greater haplotype representation may be negated by additional segregation across families. For this reason, we chose an experimental design that limited our analyses to individual NIL families for the most direct comparisons possible.

In addition, the NIL validation approach offered the possibility to refine the QTL interval and increase the map resolution of QTL. At DON.13 and DON.10, we were able to significantly reduce the size of the QTL region defined in the original study (Fig. 1a, b). We developed NILs by screening 15 progeny from each heterozygous parent and selecting the contrasting homozygotes. To further increase mapping resolution, one could easily select the heterozygotes, allow them to self-pollinate to generate large numbers of progeny, screen them with appropriate flanking markers, and obtain more recombinant NILs.

Finally, because NILs were derived from current breeding material instead of mapping populations, we can achieve the simultaneous benefits of validation and germplasm improvement (Pumphrey et al. 2007). Breeding for FHB resistance and lower DON has been difficult due to the complex nature of the trait and unfavorable linkages between resistance and other traits (Mesfin et al. 2003; Nduulu et al. 2007). The major resistance QTL identified in bi-parental mapping populations have been linked to tall plant height and late heading which are both undesirable from an agronomic perspective. AM and subsequent validation in elite breeding material have identified QTL that can reduce DON by measurable amounts without negatively affecting other traits. We were able to validate QTL with larger effects that could be exploited by traditional MAS approaches. However, it is likely that much of the variation for FHB resistance and lower DON is explained by loci with relatively small effects. Recently, genomic selection approaches have been shown to be effective in predicting DON with a level of accuracy that should accelerate gain from selection (Lorenz et al. 2012). Ultimately, the cost and format of the available marker genotyping technology will determine which approach is most promising. Taken as a whole, our results suggest a combination of MAS for QTL regions such as DON.10 and DON.13 and genomic selection may best serve breeding objectives for the reduction of DON in barley.

Acknowledgments We thank Ed Schiefelbein, Guillermo Velasquez, Karen Beaubien, Celeste Falcon, Leticia Kumar, Vikas Vikram, Ahmad Sallam, Richard Horsley, the University of Minnesota—Crookston field crews, and the University of Minnesota Small Grains Pathology lab for their contributions to phenotyping and field logistics. In addition, we thank Shiaoman Chao for SNP genotyping, Yanhong Dong for toxin analysis, and an anonymous reviewer for constructive suggestions on a prior version of this manuscript. Funding for this work was supported by grants from the National Institute of Food and Agriculture U.S. Dept. of Agriculture Agreement No. 2006-55606-16722 "Barley Coordinated Agricultural Project: Leveraging Genomics, Genetics, and Breeding for Gene Discovery and Barley Improvement"; the U.S. Wheat and Barley Scab Initiative U.S. Dept. of Agriculture ARS Agreement No. 59-0206-9-072, and the Rahr Foundation. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the U.S. Department of Agriculture.

References

Ahmad Naz A, Ehl A, Pillen K, Léon J (2012) Validation for root-related quantitative trait locus effects of wild origin in the cultivated background of barley (*Hordeum vulgare* L.). Plant Breed 3:392–398

Anderson JA, Stack RW, Liu S, Waldron BL, Fjeld AD, Coyne C, Moreno-Sevilla B, Mitchell Fetch J, Song QJ, Cregan PB, Frohlberg RC (2001) DNA markers for fusarium head blight resistance QTLs in two wheat populations. Theor Appl Genet 102:1164–1168

Austin DF, Lee M (1996) Genetic resolution and verification of quantitative trait loci for flowering and plant height with recombinant inbred lines of maize. Genome 39:957–968

Bai G, Shaner G (2004) Management and resistance in wheat and barley to Fusarium head blight. Ann Rev Phytopathol 42:135–161

Bernardo R (2008) Molecular markers and selection for complex traits in plants: learning from the last 20 years. Crop Sci 48:1649–1664

Blake VC, Kling JG, Hayes PM, Jannink JL, Jillella SR, Lee J, Matthews DE, Shao S, Close TJ, Muehlbauer GJ, Smith KP, Wise RP, Dickerson JA (2012) The Hordeum toolbox: the barley CAP genotype and phenotype resource. Plant Genome 5:81–91

Brouwer DJ, St. Clair DA (2004) Fine mapping of three quantitative trait loci for late blight resistance in tomato using near isogenic lines (NILs) and sub-NILs. Theor Appl Genet 108:628–638

Buerstmayr H, Lemmens M, Hartl L, Doldi L, Steiner B, Stierschneider M, Ruckenbauer P (2002) Molecular mapping of QTLs for Fusarium head blight resistance in spring wheat. I. Resistance to fungal spread (Type II resistance). Theor Appl Genet 104:84–91

Buerstmayr H, Steiner B, Hartl L, Griesser M, Angerer N, Lengauer D, Miedaner T, Schneider B, Lemmens M (2003) Molecular mapping of QTLs for Fusarium head blight resistance in spring wheat. II. Resistance to fungal penetration and spread. Theor Appl Genet 107:503–508

Buerstmayr H, Ban T, Anderson JA (2009) QTL mapping and marker-assisted selection for Fusarium head blight resistance in wheat: a review. Plant Breed 128:1–26

Canci PC, Nduulu LM, Muehlbauer GJ, Dill-Macky R, Rasmusson DC, Smith KP (2004) Validation of quantitative trait loci for Fusarium head blight and kernel discoloration in barley. Mol Breed 14:91–104

Castro AJ, Chen X, Corey A, Filichkina T, Hayes PM, Mundt C, Richardson K, Sandoval-Islas S, Vivar H (2003) Pyramiding and validation of quantitative trait locus (QTL) alleles determining resistance to barley stripe rust. Crop Sci 43:2234–2239

Clear RM, Patrick SK, Nowicki T, Gaba D, Edney M, Babb JC (1997) The effect of hull removal and pearling on Fusarium species and trichothecenes in hulless barley. Can J Plant Sci 77:161–166

Close TJ, Bhat PR, Lonardi S, Wu Y, Rostoks N, Ramsay L, Druka A, Stein N, Svensson JT, Wanamaker S, Bozdag S, Roose ML, Moscou MJ, Chao S, Varshney RK, Szucs P, Sato K, Hayes PM, Matthes DE, Kleinhofs A, Muehlbauer GJ, DeYoung J, Marshall DF, Madishetty K, Fenton RD, Condamine P, Graner A, Waugh R (2009) Development and implementation of high-throughput SNP genotyping in barley. BMC Genomics 10:582

Cockram J, White J, Zuluaga DL, Smith D, Comadran J, Macaulay M, Luo Z, Kearsey MJ, Werner P, Harrap D, Tapsell C, Liu H, Hedley PE, Stein N, Schulte D, Steuernagel B, Marshall DF, Thomas WTB, Ramsay L, Mackay I, Balding DJ, Waugh R, O'Sullivan DM, AGOUEB Consortium (2010) Genome-wide association mapping to candidate polymorphism resolution in the unsequenced barley genome. Proc Natl Acad Sci USA 107:21611–21616

Dahleen LS, Agrama HA, Horsley RD, Steffenson BJ, Schwarz PB, Mesfin A, Franckowiak JD (2003) Identification of QTLs associated with Fusarium head blight resistance in Zhedar 2 barley. Theor Appl Genet 108:95–104

de la Pena RC, Smith KP, Capettini F, Muehlbauer GJ, Gallo-Meagher M, Dill-Macky R, Somers DA, Rassmuson DC (1999) Quantitative trait loci associated with resistance to Fusarium head blight and kernel discoloration in barley. Theor Appl Genet 99:561–569

Fasoula VA, Harris DK, Boerma HR (2004) Validation and designation of quantitative trait loci for seed protein, seed oil, and seed weight from two soybean populations. Crop Sci 44:1218–1225

Foolad MR, Zhang LP, Lin GY (2001) Identification and validation of QTLs for salt tolerance during vegetative growth in tomato by selective genotyping. Genome 44:444–454

Häberle J, Schmolke M, Schweizer G, Korzun V, Ebmeyer E, Zimmermann G, Hartl L (2007) Effects of two major Fusarium head blight resistance QTL verified in a winter wheat backcross population. Crop Sci 47:1823–1831

Hamblin MT, Jannink JL (2011) Factors affecting the power of haplotype markers in association studies. Plant Genome 4(2):145–153

Hamwieh A, Tuyen DD, Cong H, Benitez ER, Takahashi R, Xu DH (2011) Identification and validation of a major QTL for salt tolerance in soybean. Euphytica 179:451–459

Holland JB (2007) Genetic architecture of complex traits in plants. Curr Opin Plant Biol 10:156–161

Horsley RD, Schmierer D, Maier C, Kudrna D, Urrea CA, Steffenson BJ, Schwarz PB, Franckowiak JD, Green MJ, Zhang B, Kleinhofs A (2006) Identification of QTLs associated with Fusarium head blight resistance in barley accession CIho 4196. Crop Sci 46:145–156

Kaeppler SM, Phillips RL, Kim TS (1993) Use of near-isogenic lines derived by backcrossing or selfing to map qualitative traits. Theor Appl Genet 87:233–237

Kongprakhon P, Cuesta-Marcos A, Hayes PM, Richardson KL, Sirithunya P, Sato K, Steffenson B, Toojinda T (2009) Validation of rice blast resistance genes in barley using a QTL mapping population and near-isolines. Breeding Sci 59:341–349

Kraakman ATW, Martínez F, Mussiraliev B, Van Eeuwijk FA, Niks RE (2006) Linkage disequilibrium mapping of morphological, resistance, and other agronomically relevant traits in modern spring barley cultivars. Mol Breed 17:41–58

Lander ES, Schork NJ (1994) Genetic dissection of complex traits. Science 265:2037–2048

Landi P, Sanguineti MC, Salvi S, Giuliani S, Bellotti M, Maccaferri M, Conti S, Tuberosa R (2005) Validation and characterization of a major QTL affecting leaf ABA concentration in maize. Mol Breed 15:291–303

Legzdina L, Buerstmayr H (2004) Comparison of infection with Fusarium head blight and accumulation of mycotoxins in grain of hulless and covered barley. J Cereal Sci 40:61–67

Long AD, Langley CH (1999) The power of association studies to detect the contribution of candidate genetic loci to variation in complex traits. Genome Res 9:720–731

Lorenz AJ, Hamblin MT, Jannink JL (2010) Performance of single nucleotide polymorphisms versus haplotypes for genome-wide association analysis in barley. PLoS ONE 5(11):e14079

Lorenz AJ, Smith KP, Jannink JL (2012) Potential and optimization of genomic selection for Fusarium head blight resistance in six-row barley. Crop Sci 52:1609–1621

Ma Z, Steffenson BJ, Prom LK, Lapitan NLV (2000) Mapping of quantitative trait loci for Fusarium head blight resistance in barley. Phytopathology 90:1079–1088

Massman J, Cooper B, Horsley R, Neate S, Dill-Macky R, Chao S, Dong Y, Schwarz P, Muehlbauer GJ, Smith KP (2011) Genome-wide association mapping of Fusarium head blight resistance in contemporary barley breeding germplasm. Mol Breed 27:439–454

Mesfin A, Smith KP, Dill-Macky R, Evans CK, Waugh R, Gustus CD, Muehlbauer GJ (2003) Quantitative trait loci for Fusarium head blight resistance in barley detected in a two-rowed by six-rowed population. Crop Sci 43:307–318

Micic Z, Hahn V, Bauer E, Melchinger AE, Knapp SJ, Tang S, Schön CC (2005) Identification and validation of QTL for *Sclerotinia*

midstalk rot resistance in sunflower by selective genotyping. Theor Appl Genet 111:233–242
Muehlbauer GJ, Specht JE, Thomas-Compton MA, Staswick PE, Bernard RL (1988) Near-isogenic lines-A potential resource in the integration of conventional and molecular marker linkage maps. Crop Sci 28:729–735
Muñoz-Amatriaín M, Castillo AM, Chen XW, Cistué L, Vallés MP (2008) Identification and validation of QTLs for green plant percentage in barley (*Hordeum vulgare* L.) anther culture. Mol Breed 22:119–129
Nduulu LM, Mesfin A, Muehlbauer GJ, Smith KP (2007) Analysis of the chromosome 2 (2H) region of barley associated with the correlated traits Fusarium head blight resistance and heading date. Theor Appl Genet 115:561–570
Pallotta MA, Warner P, Fox RL, Kuchel H, Jefferies SJ, Langridge P (2003) Marker assisted wheat breeding in the southern region of Australia. In: Pogna NE, Romano M, Pogna EA, Galerio G (eds) Proceedings of the tenth international wheat genetics symposium. Institutio Sperimentale per la Cerealcoltura, Rome, pp 789–791
Piepho HP (2005) Statistical tests for QTL and QTL-by-environment effects in segregating populations derived from line crosses. Theor Appl Genet 110:561–566
Prasad M, Kumar N, Kulwal PL, Röder MS, Balyan HS, Dhaliwal HS, Gupta PK (2003) QTL analysis for grain protein content using SSR markers and validation studies using NILs in bread wheat. Theor Appl Genet 106:659–667
Pumphrey MO, Bernardo R, Anderson JA (2007) Validating the *Fhb1* QTL for Fusarium head blight resistance in near-isogenic wheat lines developed from breeding populations. Crop Sci 47:200–206
Romagosa I, Han F, Ullrich SE, Hayes PM, Wesenberg DM (1999) Verification of yield QTL through realized molecular marker-assisted selection responses in a barley cross. Mol Breed 5:143–152
Rostoks N, Ramsay L, MacKenzie K, Cardle L, Bhat PR, Roose ML, Svensson JT, Stein N, Varshney RK, Marshall DF, Graner A, Close TJ, Waugh R (2006) Recent history of artificial outcrossing facilitates whole-genome association mapping in elite inbred crop varieties. Proc Natl Acad Sci USA 103:18656–18661
Roy JK, Smith KP, Muehlbauer GJ, Chao S, Close TJ, Steffenson BJ (2010) Association mapping of spot blotch resistance in wild barley. Mol Breeding 26:243–256
SAS Institute (2008) SAS-statistical analysis software for windows, 9.2. SAS Institute, Cary, NC
Singh H, Prasad M, Varshney RK, Roy JK, Balyan HS, Dhaliwal HS, Gupta PK (2001) STMS markers for grain protein content and their validation using near-isogenic lines in bread wheat. Plant Breed 120:273–278
Smith KP, Evans CK, Dill-Macky R, Gustus C, Xie W, Dong Y (2004) Host genetic effect on deoxynivalenol accumulation in Fusarium head blight of barley. Phytopathology 94:766–771
Smith KP, Budde A, Dill-Macky R, Rasmusson DC, Schiefelbein E, Steffenson B, Wiersma JJ, Wiersma JV, Zhang B (2013) Registration of 'Quest' spring malting barley with improved resistance to Fusarium head blight. J Plant Regist 7:1–5. doi:10.3198/jpr2012.03.0200crc
Spaner D, Rossnagel BG, Legge WG, Scoles GJ, Eckstein PE, Penner GA, Tinker NA, Briggs KG, Falk DE, Afele JC, Hayes PM, Mather DE (1999) Verification of a quantitative trait locus affecting agronomic traits in two-row barley. Crop Sci 39:248–252
Steffenson BJ (2003) Fusarium head blight of barley: Impact, epidemics, management, and strategies for identifying and utilizing genetic resistance. In: Leonard KL, Bushnell WT (eds) Fusarium head blight of wheat and barley. The American Phytopathological Society, St. Paul, pp 241–295
Steffenson BJ, Smith KP (2006) Breeding barley for multiple disease resistance in the upper Midwest region of the USA. Czech J Genet Plant Breed 42:79–85
Stracke S, Haseneyer G, Veyrieras JB, Geiger HH, Sauer S, Graner A, Piepho HP (2009) Association mapping reveals gene action and interactions in the determination of flowering time in barley. Theor Appl Genet 118:259–273
Tacke BK, Casper HH (1996) Determination of deoxynivalenol in wheat, barley, and malt by column cleanup and gas chromatography with electron capture detection. J AOAC Int 79:472–475
Tuinstra MR, Ejeta G, Goldsbrough PB (1997) Heterogeneous inbred family (HIF) analysis: a method for developing near-isogenic lines that differ at quantitative trait loci. Theor Appl Genet 95:1005–1011
Tuinstra MR, Ejeta G, Goldsbrough PB (1998) Evaluation of near-isogenic sorghum lines contrasting for QTL markers associated with drought tolerance. Crop Sci 38:835–842
Waldron BL, Moreno-Sevilla B, Anderson JA, Stack RW, Frohberg RC (1999) RFLP mapping of QTL for Fusarium head blight resistance in wheat. Crop Sci 39:805–811
Yun SJ, Gyenis L, Bossolini E, Hayes PM, Matus I, Smith KP, Steffenson BJ, Tuberosa R, Muelbauer GJ (2006) Validation of quantitative trait loci for multiple disease resistance in barley using advanced backcross lines developed with a wild barley. Crop Sci 46:1179–1186
Zhou W, Kolb FL, Bai G, Shaner G, Domier LL (2002) Genetic analysis of scab resistance QTL in wheat with microsatellite and AFLP markers. Genome 45:719–727
Zhu H, Gilchrist L, Hayes P, Kleinhofs A, Kudrna D, Liu Z, Prom L, Steffenson B, Toojinda T, Vivar H (1999) Does function follow form? Principal QTLs for Fusarium head blight (FHB) resistance are coincident with QTLs for inflorescence traits and plant height in a doubled-haploid population of barley. Theor Appl Genet 99:1221–1232
Zhu C, Gore M, Buckler ES, Yu J (2008) Status and prospects of association mapping in plants. Plant Genome 1:5–20