Theor Appl Genet (2011) 123:307–326
DOI 10.1007/s00122-011-1585-9

ORIGINAL PAPER

Targeted discovery of quantitative trait loci for resistance to northern leaf blight and other diseases of maize

Chia-Lin Chung · Jesse Poland · Kristen Kump · Jacqueline Benson · Joy Longfellow · Ellie Walsh · Peter Balint-Kurti · Rebecca Nelson

Received: 1 August 2010 / Accepted: 24 March 2011 / Published online: 28 April 2011

Abstract To capture diverse alleles at a set of loci associated with disease resistance in maize, heterogeneous inbred family (HIF) analysis was applied for targeted QTL mapping and near-isogenic line (NIL) development. Tropical maize lines CML52 and DK888 were chosen as donors of alleles based on their known resistance to multiple diseases. Chromosomal regions ("bins"; $n = 39$) associated with multiple disease resistance (MDR) were targeted based on a consensus map of disease QTLs in maize. We generated HIFs segregating for the targeted loci but isogenic at ~97% of the genome. To test the hypothesis that CML52 and DK888 alleles at MDR hotspots condition broad-spectrum resistance, HIFs and derived NILs were tested for resistance to northern leaf blight (NLB), southern leaf blight (SLB), gray leaf spot (GLS), anthracnose leaf blight (ALB), anthracnose stalk rot (ASR), common rust, common smut, and Stewart's wilt. Four NLB QTLs, two ASR QTLs, and one Stewart's wilt QTL were identified. In parallel, a population of 196 recombinant inbred lines (RILs) derived from B73 × ;CML52 was evaluated for resistance to NLB, GLS, SLB, and ASR. The QTLs mapped (four for NLB, five for SLB, two for GLS, and two for ASR) mostly corresponded to those found using the NILs. Combining HIF- and RIL-based analyses, we discovered two disease QTLs at which CML52 alleles were favorable for more than one disease. A QTL in bin 1.06–1.07 conferred resistance to NLB and Stewart's wilt, and a QTL in 6.05 conferred resistance to NLB and ASR.

Introduction

Studies on the genetic architecture of disease resistance in a range of plant genomes have revealed that R-genes, resistance gene analogs (RGAs), defense response gene homologs (DRHs), and loci conditioning quantitative

Communicated by M. Bohn.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-011-1585-9) contains supplementary material, which is available to authorized users.

C.-L. Chung · J. Longfellow · E. Walsh · R. Nelson (✉)
Department of Plant Pathology and Plant-Microbe Biology, Cornell University, Ithaca, NY 14853, USA
e-mail: rjn7@cornell.edu

J. Poland · J. Benson · R. Nelson
Department of Plant Breeding and Genetics, Cornell University, Ithaca, NY 14853, USA

K. Kump
Department of Crop Science, North Carolina State University, Raleigh, NC 27695, USA

P. Balint-Kurti
U.S. Department of Agriculture, Agricultural Research Service, Department of Plant Pathology, North Carolina State University, Raleigh, NC 27695, USA

Present Address:
C.-L. Chung
Department of Plant Pathology and Microbiology, National Taiwan University, Taipei 10617, Taiwan

Present Address:
J. Poland
U.S. Department of Agriculture, Agricultural Research Service, Department of Agronomy, Kansas State University, Manhattan, KS 66506, USA

Present Address:
E. Walsh
Department of Plant Pathology, The Ohio State University, Wooster, OH 44691, USA

disease resistance (quantitative trait loci for disease, or disease QTLs) are not evenly distributed across the genome (Kanazin et al. 1996; Lopez et al. 2003; Wang et al. 2007a; Wang and Xiao 2002; Wisser et al. 2005, 2006). Major genes and QTLs for a given disease have frequently been co-localized to certain genomic regions [e.g., *rhm* and QTLs for southern leaf blight, and *Rp3* and QTLs for common rust in maize (Wisser et al. 2006)]. Different degrees of resistance may reflect allelic variants of identical gene(s) (Robertson 1989; Welz and Geiger 2000), differential performance of resistance in various genetic backgrounds or environmental conditions, or linkage of distinct genes affecting disease responses. Apparent clustering of QTLs for different diseases has been commonly observed (Williams 2003; Wisser et al. 2005, 2006), leading to the hypothesis that some chromosomal segments are associated with multiple disease resistance (MDR). Moreover, mapping of defense response gene homologs (DRHs) (Faris et al. 1999; Li et al. 1999; Ramalingam et al. 2003; Wang et al. 2007a) has revealed their co-localization with some disease QTLs in plants. This has led to the hypothesis that some disease QTLs may be controlled by DR genes (conserved defense machinery) (Faris et al. 1999) and therefore may contribute non-specific resistance.

Quantitative disease resistance is of agricultural importance, but current knowledge concerning its underlying mechanism(s) is limited. The possibility of broad-spectrum resistance is particularly intriguing (Poland et al. 2009), again for both practical and theoretical reasons. One line of evidence for the existence of MDR or broad-spectrum QTLs is based on co-localization of QTLs identified in a range of different mapping populations (Jo et al. 2008; Wisser et al. 2005, 2006; Yun et al. 2005). While these studies suggest that QTLs for multiple diseases co-localize, inferences from previous QTL reports have been limited by poor allelic sampling and low precision of QTL positions. In particular, the low resolution of most studies does not allow linkage to be distinguished from pleiotropy. The apparent MDR phenotype of a QTL is in some cases attributable to the linkage of R-genes against different pathogens. In tomato, for example, a region on the short arm of chromosome (Chr.) 6 carries the *Cf-2* and *Cf-5* genes for leaf mold resistance (Dixon et al. 1998), the *Mi* gene for root knot nematode resistance (Milligan et al. 1998), and a number of major gene loci for resistance to other diseases (Dickinson et al. 1993; Zhang et al. 2002). Broad-spectrum resistance has also been associated with pleiotropic genes involved in mechanisms different from typical R-genes. Several genes involved in non-host resistance, basal resistance, systemic acquired resistance, and defense signaling pathways are known to condition MDR [e.g., *mlo* in barley (Buschges et al. 1997); *RPW8.1* and *RPW8.2* in *Arabidopsis* (Wang et al. 2007b); *npr1* in Arabidopsis (Cao et al. 1998); *Lr34* in wheat (Krattinger et al. 2009)]. Whether MDR is conditioned by typical R-genes or non-R-genes has implications for the durability and performance of resistance.

To capture and characterize diverse alleles associated with MDR in maize, heterogeneous inbred family (HIF) analysis was explored as an alternative to conventional QTL mapping and near isogenic line (NIL) development. Classical studies of disease QTL mapping involve developing mapping population(s) from crosses between resistant and susceptible lines, evaluating DNA marker genotypes and phenotypes of interest and analyzing marker–trait association (Young 1996). Recombinant inbred lines (RILs) are widely used in QTL mapping and have been reported to provide the best statistical power for QTL detection (Kaeppler 1997). However, the development and genome-wide genotyping of the RIL population are time-consuming, and RIL populations are not suited for detailed phenotyping or genetic dissection. QTL characterization has usually been conducted using NILs, in which a given chromosomal segment is transferred from the donor genotype to the recurrent genotype through consecutive generations of backcrossing. HIF analysis has been demonstrated as a useful approach for developing NIL pairs that are isogenic at the majority of loci, but differ at specific QTLs, using intermediate materials easily achieved from general breeding programs (Tuinstra et al. 1997). It has been applied to validate the position and effect of QTLs (Balasubramanian et al. 2009; Borevitz and Chory 2004; Kobayashi et al. 2006; Loudet et al. 2005; Njiti et al. 1998; Pumphrey et al. 2007). To the authors' knowledge, however, the use of HIF analysis has not been reported for the analysis of allelic series for targeted loci.

This study was undertaken to investigate genomic regions associated with broad-spectrum resistance. Under the hypothesis that chromosomal regions where disease QTLs had been co-localized harbor gene(s) controlling MDR, alleles at loci associated with disease QTL clusters were extracted and characterized for response to eight diseases using HIF analysis. HIFs were derived from maize genotypes showing MDR phenotypes to generate genetic stocks suitable for uncovering the genetic basis of quantitative disease resistance and MDR. A parallel conventional QTL mapping study was conducted using a population of RILs derived for one of the resistance sources. The RIL-based QTL mapping allowed empirical assessment of the advantages and drawbacks of using HIFs in analyzing targeted QTL.

Materials and methods

Plant materials

Two maize genotypes were chosen as donors of multiple disease resistance for the development of genetic materials.

The first was CML52, a tropical inbred line developed by the International Maize and Wheat Improvement Center (CIMMYT); the second was DK888, a single-cross hybrid developed by Thailand Charoen Seeds Group in collaboration with US Dekalb Seeds (Ekasingh et al. 2001). The parental inbreds of DK888 are proprietary. For conventional QTL mapping, a population of recombinant inbred lines (RILs) derived from CML52 crossed to the B73 inbred was used. It is one of the 26 families that constitute the Nested Association Mapping (NAM) population developed by The Maize Diversity Project (Buckler et al. 2009; McMullen et al. 2009; Yu et al. 2008). The RIL population consisted of 196 F_6 lines. Lines were sib- or self-mated for two generations to increase seed for testing. The full set of 196 RILs was evaluated for SLB, and a subset of 159 and 147 RILs was evaluated for NLB and GLS, respectively. The same 159 RILs were phenotyped for ASR and for NLB, providing data for resistance to all three diseases on 147 RILs. All lines had been previously genotyped with 1,106 single nucleotide polymorphism (SNP) markers, of which 773 were polymorphic and used for QTL mapping. Further details on the RILs are publically available at http://www.panzea.org and http://www.maizegenetics.net.

For HIF (heterogeneous inbred family) analysis (Tuinstra et al. 1997), intermediate materials from the development of inbred lines were used. The overall strategy for HIF-based targeted QTL analysis is outlined in Fig. 1. The starting materials included 19 F_5 families from the cross of B73 × CML52 and 17 F_6 families from the cross of S11 × DK888 (S11 is a proprietary inbred), provided by The Maize Diversity Project and The USDA Germplasm Enhancement of Maize (GEM) Project (Balint-Kurti et al. 2006; Goodman 2005; Lee and Hardin 1997), respectively. Based on the maize disease QTL consensus map (Wisser et al. 2006), 39 bins associated with previously reported QTLs for resistance to NLB, SLB, GLS and other diseases were selected as candidate MDR regions. These regions of interest were targeted using 73 simple sequence repeat (SSR) markers (Supplementary Fig. 1). For the B73 × CML52 materials, a set of 94 individuals in 19 F_5 families (4–5 individuals per family) were initially tested for residual heterozygous loci, using 33 SSR markers covering 18 bins of interest. A subset of 43 $F_{5:6}$ HIFs from 13 F_5 families were generated and tested for 31 additional SSR markers covering 20 more bins. The other 51 F_5 lines were not advanced to F_6 because of poor agronomic performance. In total, 64 SSR markers targeting 38 bins associated with MDR were applied on 43 $F_{5:6}$ HIFs from the cross of B73 × CML52. For the S11 × DK888 materials, 46 $F_{6:7}$ HIFs were developed by selfing 1–4 individuals from each of the 17 F_6 families. Residual heterozygous loci were detected in the 46 $F_{6:7}$ HIFs with 17 SSR markers, focusing on 11 bins of interest. To check the levels of heterozygosity in the CML52 and DK888 derivatives, marker data from the F_5 families of B73 × CML52 and the F_6 families of S11 × DK888 were used to calculate (1) the average percentages of heterozygous loci per line (mean of 100% × number of heterozygous marker loci in a given line/total number of maker loci in a given line) and (2) the average percentages of heterozygous lines per locus (mean of 100% × number of lines heterozygous at a given locus/total number of lines being genotyped for a given locus). The subsequently derived NILs were generated by single-seed descent from eight selected B73 × CML52 HIFs and six selected S11 × DK888 HIFs segregating for candidate NLB QTLs or MDR hotspot regions. In this study, "NILs" refers to sets of HIF-derived $F_{5:7}$, $F_{5:8}$, $F_{6:7}$, $F_{6:8}$ or $F_{6:9}$ lines contrasting for genetic regions of interest but presumably isogenic at >98% of the genome (theoretical heterozygosity in the F_7 generation is 1.56%).

Disease evaluations

Plants were evaluated for disease resistance and for days to anthesis (DTA). DTA was scored on a row basis when >50% of the plants in a row started to shed pollen. The evaluation methods used for different diseases are described below.

Northern leaf blight

The NLB trials were conducted in Aurora, New York in 2005–2009. Plants at the 5–6-leaf stage were inoculated with *Setosphaeria turcica* (anamorph *Exserohilum turcicum*) race 1 (isolate EtNY001) as previously described (Chung et al. 2010a). A number of disease components, including incubation period (IP), lesion number (LN), primary diseased leaf area (primary DLA), diseased leaf area (DLA), and area under the disease progress curve (AUDPC), were used to evaluate resistance to NLB. IP was rated for individual plants as the number of days after inoculation when a plant started showing wilted lesions. LN was rated for individual plants at 2–3 weeks after inoculation, as the total number of lesions on a plant. Primary DLA and DLA were rated for individual plants in segregating HIFs and on a row basis for derived NILs. Primary DLA was rated at 2–3 weeks after inoculation, as the percentage of infected leaf area of the inoculated leaves. DLA was rated as the percentage of infected leaf area of the entire plant, disregarding senescent bottom leaves. In each season, 3–4 DLA scores were taken at 10–14-day intervals, starting from 1–2 weeks after the onset of secondary infection. The DLA scores were used to

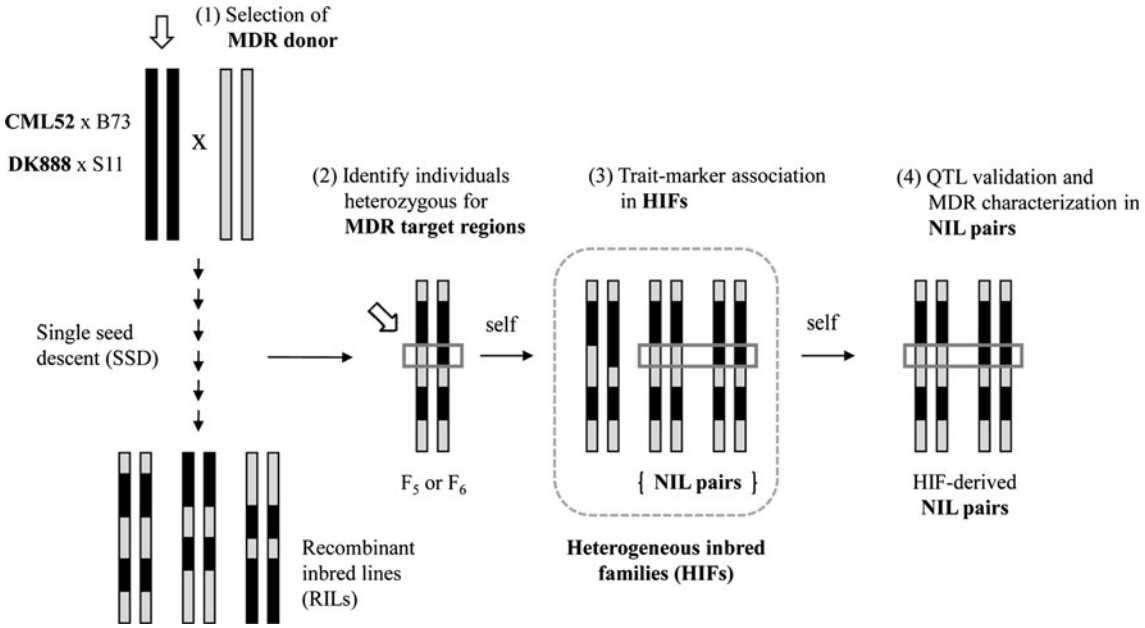

Fig. 1 Strategy for identifying loci associated with multiple disease resistance (*MDR*) using heterogeneous inbred families (*HIFs*). (*1*) Various maize genotypes were screened for a number of important maize diseases. Maize inbred line CML52 and hybrid DK888 showed good levels of resistance to multiple diseases and were thus chosen as MDR donors. (*2*) The nearly fixed F_5 and F_6 lines derived during the development of recombinant inbred line (*RIL*) populations were tested with markers covering the regions of MDR interest (the marker positions and the target regions are shown in Supplementary Fig. 1). (*3*) In each derived $F_{5:6}$ or $F_{6:7}$ HIF, loci conditioning traits can be identified if pairs of near-isogenic lines (NIL pairs) showed significantly different phenotypes. (*4*) Sets of NIL pairs in more isogenic backgrounds can be derived by advancing selected lines of the HIF. The specific QTLs isolated in NIL pairs were subsequently tested for their MDR effects

calculate $\text{AUDPC}_{\text{DLA}} = \sum_{i=1}^{n-1} \frac{(y_i+y_{i+1})(t_{i+1}-t_i)}{2}$, where $y_i =$ DLA at time i, $t_{i+1} - t_i =$ day interval between two ratings, $n =$ number of ratings (Wilcoxson et al. 1974).

Southern leaf blight

SLB trials were conducted in Clayton, North Carolina in 2007 and 2008, and Homestead, Florida in 2007. Plants at the 4–6-leaf stage were inoculated with *Cochliobolus heterostrophus* race O (isolate 2-16Bm) as previously described (Carson 1998; Carson et al. 2004). Disease severity was rated based on a 1–9 scale corresponding to the diseased leaf area on primarily the ear leaf. Four to six evaluations were taken at 5–10-day intervals from around 2 weeks after anthesis. The disease severity scores were used to calculate $\text{AUDPC}_{\text{Severity}} = \sum_{i=1}^{n-1} \frac{(y_i+y_{i+1})(t_{i+1}-t_i)}{2}$, where $y_i =$ disease severity at time i, $t_{i+1} - t_i =$ day interval between two ratings, $n =$ number of ratings.

Gray leaf spot

The GLS trials were conducted in Andrews, North Carolina in 2007–2008 for NILs, and Blacksburg, Virginia in 2008–2009 for RILs and in 2009 for NILs. Experiments were performed under natural disease pressure in non-tilled fields located in valleys with regular morning mists and heavy dews, which favor the development of GLS (caused by *Cercospora zeae-maydis* and/or *Cercospora zeina*). For the HIFs and NILs, disease severity was rated as described for SLB, with a minimum of three ratings per season. For the population of RILs, disease severity was scored based on a 1–5 scale with 0.25 increments, according to the disease progress on the ear leaf (Saghai Maroff et al. 1993). The evaluation was conducted four times at 7–8-day intervals starting around 2 weeks after anthesis. The $\text{AUDPC}_{\text{Severity}}$ was calculated as described above.

Anthracnose stalk rot

The ASR trials were conducted in Aurora, New York in 2007–2008. Plants were inoculated with *Colletotrichum graminicola* (teleomorph *Glomerella graminicola*) (isolate Cg151, obtained from Gary Bergstrom of Cornell University) at tasseling stage (Keller and Bergstrom 1988). Inoculations and disease evaluations were done as previously described (Chung et al. 2010a). Total diseased internode area (ASR %) was rated for individual plants, as the sum of the percentages of discolored area of individual internodes (Keller and Bergstrom 1988). For the HIFs and NILs, in 2007, six plants per row were inoculated, and eight consecutive internodes per plant were scored; in

2008, eight plants per row were inoculated, and six consecutive internodes per plant were scored. For the population of RILs, ASR inoculation was conducted in NLB plots in 2007 and 2008. For each row, four plants were inoculated, and five consecutive internodes per plant were scored. Data from all the scored internodes were summed for analysis, except for the B73 × CML52 RILs, for which a multivariate mixed model was used to calculate disease severity as described below.

Anthracnose leaf blight

The ALB trials were conducted in Aurora, New York in 2007. Plants were inoculated at the 5–6-leaf stage with *Colletotrichum graminicola* (teleomorph *Glomerella graminicola*). Inoculum was cultured on oatmeal agar for 2 weeks under fluorescent light at room temperature (Muimba-Kankolongo and Bergstrom 1990). Spore suspension (0.5 ml of 2×10^4 conidia per ml in 0.02% Tween 20) and colonized sorghum grains (1/4 teaspoon, ~ 1.25 ml) were placed in the whorl of each plant. The preparation of liquid and solid inoculum and the ratings of IP, DLA and $AUDPC_{DLA}$ were as described for NLB.

Common rust

Resistance to rust was evaluated in Aurora, New York in 2007–2008. In 2007, plants in NLB plots were evaluated for rust symptoms caused by natural infection. In 2008, plants at the 6–8-leaf stage were inoculated with *Puccinia sorghi* spores collected from naturally infected leaves harvested at the same location in 2007. Whorl inoculations were performed as previously described (Chung et al. 2010a). Disease severity was rated based on a 0–10 scale with 0.5 increments, corresponding to the percentage of infected leaf area of the entire plant. The severity scores were taken twice and averaged in 2007 and were evaluated three times at 9-day intervals from 4 weeks after inoculation in 2008. The $AUDPC_{Severity}$ was calculated for the 2008 data as described above.

Common smut

Resistance to smut was evaluated in Aurora, New York in 2007–2008. In 2007, plants in NLB plots were evaluated for naturally occurring smut galls. In 2008, plants were inoculated with six compatible strains of *Ustilago maydis* (UmNY001, UmNY002, UmNY003, UmNY004, UmNY008, and UmNY009), which were isolated from naturally infected smut galls collected at the same location in 2007. Inoculations were done as previously described (Chung et al. 2010a). The incidence and severity of the development of ear galls and stalk galls were rated at 4–5 weeks after anthesis. Severity scores were evaluated for individual plants on a 0–10 scale, corresponding to the number and size of galls and the disease severity of the entire plant.

Stewart's wilt

Resistance to Stewart's wilt was evaluated in the greenhouse at Cornell University in November–December 2007 and in the field at Aurora, New York in 2008–2009. Plants at the 5–6-leaf stage were inoculated with *Pantoea stewartii* (syn. *Erwinia stewartii*) strain PsNY003 (obtained from Helene Dillard of Cornell University) following a modified pin-prick method (Chung et al. 2010a). Primary DLA was rated as the percentage of infected area of the inoculated leaves. It was evaluated twice with 7-day interval starting 2 weeks after inoculation.

DNA extraction and genotyping

DNA extraction was conducted following a modified miniprep CTAB method (Doyle and Doyle 1987; Qiu et al. 2006) in 96-well plates (Chung et al. 2010a). Simple sequence repeat (SSR) markers were used for genotypic analysis. A modified single-reaction nested PCR method using the specific primer pair along with a fluorescently labeled universal primer (Schuelke 2000) was applied to incorporate fluorescent dye in PCR product. The PCR reaction, thermal cycling parameter, and multiplex fragment analysis were as described by Wisser et al. (2008), Schuelke (2000), and Chung et al. (2010a), respectively.

Experimental design

HIFs and NILs

For each HIF, 50–140 individuals were analyzed, according to the availability of seeds. Individual plants in a HIF were grown within a single block with two B73 rows as the borders. For QTL confirmation, NILs were evaluated with 2–3 replications per year, in 7-foot single-row plots with 8–10 plants per row. NILs of each set (NILs derived from a parental line) were randomized within a block, with one B73 and one CML52 row per block as controls.

RILs

The RILs were evaluated in Aurora, NY for NLB in 2007, 2008, and 2009, and for ASR in 2007 and 2008. Lines were planted in 7-foot single-row plots with 8–10 plants per row. The trial was conducted using a single replication with an augmented design, using each of the inbred parents (B73 and CML52) as repeated checks. Incomplete blocks

consisted of 18 RILs and each of the two parents. Resistance to GLS was evaluated in Blacksburg, VA in 2008 and 2009. The RILs were planted in 12-foot single-row plots with 12–14 plants per row. The trial was arranged in an augmented design with a single replication. Incomplete blocks consisted of 18 RILs and each of the two parents. SLB evaluations were conducted in Clayton, North Carolina in 2007 and 2008, and Homestead, Florida in 2007. In the Clayton and Homestead locations, lines were planted in 3.5-foot and 3-foot single-row plots, respectively, with approximately 6–8 plants per row. The same augmented design with a single replication was used. Incomplete blocks consisted of 20 RILs and the two parents.

Data analysis

QTL identification in HIFs

To identify QTLs in HIFs, lines of derived HIFs were individually genotyped for segregating marker loci, as well as phenotyped for DTA and different disease traits (details in the "Disease evaluations"). Analysis of variance (ANOVA) was conducted on an individual trait–marker basis in each of the segregating HIFs using JMP 7.0 (SAS Institute Inc., Cary, NC, USA). Markers were considered associated with disease traits when significant phenotypic differences were detected between homozygous genotypes carrying contrasting parental alleles, according to two-tailed Student's t test at $P < 0.05$. For the HIFs segregating for multiple markers, the Bonferroni correction was used to adjust the significance threshold levels. Stepwise regression, with a significance probability of $P < 0.05$ for each marker to enter/leave the model (van Dam et al. 2003), was also performed in JMP 7.0 to confirm the effects of identified markers in HIFs.

QTL identification in NILs

The NILs were genotyped as well as phenotyped for DTA and different disease traits (details in the "Disease evaluations"). Each set of NILs was analyzed separately using JMP 7.0. Phenotypes of a set of NILs were first tested by fitting a linear least squares model with "genotype" and "replication" as fixed factors. In the cases of NILs being evaluated in multiple years, the variables "genotype", "year", and "replication within year" were used in the model (the insignificant variable "genotype × year" was removed from the model). When significant difference in least squares means of the NILs was detected within the set ($P < 0.05$), markers at which the NILs differed were analyzed for their associations with disease traits on an individual trait–marker basis. A linear least squares model was fit with "marker" and "replication" as fixed factors. Phenotypic difference between least squares means of contrasting homozygous genotypes at the target marker was determined by two-tailed Student's t test at $P < 0.05$. Markers significantly associated with traits were considered as candidate disease QTLs. The marker with greatest significance in a linked block was considered to reflect the most likely QTL position. For the sets of NILs segregating for more than one candidate QTL, potential correlation and/or interaction between the unlinked significant markers were analyzed following a series of statistical tests described by Szalma et al. (2007). Briefly, the NILs were grouped by the alleles at each of the significant marker loci. Trait–marker analysis was conducted for the NILs fixed for one marker but contrasting for the other marker(s). A QTL was declared if the lines contrasting for this locus were significantly different from each other ($P < 0.05$), having fixed the other potential QTLs in the genome.

QTL identification in RILs

For NLB, three disease severity ratings were taken each season, and a multivariate mixed model was used to obtain best linear unbiased predictions (BLUPs) of NLB disease severity at each time point. The trait distribution was skewed; hence, a square root transformation was used on the raw data for NLB prior to further analysis. The mixed model was: $D_t = Y_i + B_{j(i)} + L_k$, where D is the disease severity at time t, Y_i is the random effect of year i, B_j is the random effect of block j in year i, and L_k is the random effect of line k. Model solution provided BLUPs for NLB disease severity at each of the three time points. The BLUPs were used to calculate AUDPC as described above and were used for QTL analysis.

For ASR, four plants were inoculated and individually evaluated for disease severity at internodes 1–5. A similar multivariate mixed model was used for obtaining the best linear unbiased estimates (BLUEs) for ASR: $D_n = Y_i + B_{j(i)} + L_k$, where D is the disease severity for internode n, Y_i is the random effect of year i, B_j is the random effect of block j in year i, and L_k is the fixed effect of line k. The model solution provided BLUEs for ASR severity at each internode. The disease severity values for internodes 1–5 were summed and subsequently used as the ASR phenotype for QTL mapping.

For GLS evaluation, lines were evaluated for disease severity at three time points. The model for field effects is $D_t = Y_i + B_{j(i)} + L_k$, where D_t is the disease severity at time t, Y_i is the random effect of year i, $B_{j(i)}$ is the random effect of block j in year i, and L_k is the random effect of line k. AUDPC for GLS severity was calculated as described above.

Resistance to SLB was evaluated in three environments. In each trial, the same augmented design with a single

replication was used. Disease severity was scored on a row basis at two time points. A similar multivariate mixed model was used for obtaining the BLUPs for SLB: $D_t = E_i + B_{j(i)} + L_k$, where D is the disease severity at time t, E_i is the fixed effect of environment i, B_j is the random effect of block j in environment i, and L_k is the random effect of line k. The model solution provided BLUPs for SLB severity at the two time points. These values were averaged and subsequently used for QTL mapping.

To determine phenotypic correlation for different diseases in RILs, Pearson correlation coefficients were calculated using PROC CORR in SAS 9.1. For each of the traits, the BLUP values from each trait were used to determine correlations between resistance to the different diseases and relative maturity.

Three methods were employed for QTL mapping. Composite interval mapping (CIM) and inclusive composite interval mapping (ICIM) were conducted using QGene 4.2.3 (Joehanes and Nelson 2008). Baysian shrinkage regression (BSR; Xu 2003) was programed in R statistical software (R Development Team). To allow the identification of QTL that might have pleiotropic effects on disease resistance and relative maturity, DTA was not included as a covariate in the QTL models for disease resistance. The results of CIM and ICIM were very similar, and only the ICIM results are presented here. For ICIM, the default scan interval of 2 cM was used. Stepwise cofactor selection was used with a maximum of eight markers selected and a selection threshold of $F = 2$. For BSR, two or more highly correlated markers can absorb the effect of a single QTL; therefore, prior to further analysis, marker loci that were >0.90 correlated were removed. This left 338 markers for BSR analysis. Markov chain Monte Carlo (MCMC) sampling was run for 20,000 iterations with 10,000-iteration burn-in and thinning every ten iterations. The posterior means for additive allele effect at each maker locus were saved and reported.

Results

Selection of MDR genotypes

As a first step towards testing for MDR loci, genotypes with resistance to multiple diseases were identified (Fig. 1). MDR donors were selected based on a review of documented resistance performance of various maize genotypes (Supplementary Table 1) and confirmed through field evaluation (Supplementary Table 2). CML52 and DK888 were the best MDR genotypes for which suitable segregating materials were available. CML52 showed high levels of resistance to NLB, SLB, and GLS. It ranked among the top 5% of lines for MDR in a panel of 253 diverse lines (Wisser et al. 2011) and had a high level of resistance against ear rot among the 394 tropical lines developed by the International Maize and Wheat Improvement Center (CIMMYT; http://www.cimmyt.org/index.php?option=com_docman&task=doc_download&gid=84&lang=en). CML52 also showed strong resistance against the colonization and sporulation of *Aspergillus flavus*, as well as the accumulation of aflatoxin in a smaller set of diverse maize lines (Mideros et al. 2009; S. Mideros, personal communication). DK888 was evaluated as part of the Genetic Enhancement of Maize program of the USDA (Kraja et al. 2000) and found to be the overall best genotype carrying favorable alleles for resistance to NLB, SLB, GLS, northern leaf spot (NLS, caused by *Cochliobolus carbonum*, anamorph *Bipolaris zeicola*), and common rust.

The above lines of evidence led to the choice of CML52 and DK888 as desirable sources of MDR alleles. Data subsequently collected from B73, CML52, DK888 and derived HIFs and NILs further supported the superior resistance of CML52 and DK888 to various diseases (Supplementary Table 2). DK888 consistently showed outstanding performance for resistance to all the diseases tested though potential heterosis effects should be acknowledged. CML52 showed high levels of resistance to NLB, SLB, GLS, ALB, and rust, but was moderately resistant to Stewart's wilt, and was moderately susceptible to ASR. For ALB and rust, to which CML52 and DK888 were both resistant, DK888 was significantly more resistant than CML52. It is worth noting that B73 was moderately susceptible to most diseases, including NLB, SLB, GLS, ASR, ALB, and Stewart's wilt, but was resistant to common rust and common smut, both caused by obligate biotrophic fungi. For smut, no naturally occurring stalk galls were observed on B73. Even after inoculation with *U. maydis* sporidia through the silk channel, the incidence and severity of ear galls were as low as 8% (0.1 on a scale of 0–10, with 3.3 as the highest observed in this experiment).

Identifying individuals heterozygous for MDR target regions

To isolate disease QTL regions in NILs, 64 SSR markers were selected to target 38 bins (of the 100 bins of the maize genome, ~20 cM per bin) associated with identified QTLs for resistance to NLB, GLS, SLB, as well as other diseases [Supplementary Fig. 1; adapted from the study of Wisser et al. (2006)]. In the disease QTL consensus map in maize, NLB QTLs were identified in 41 bins on Chrs. 1–9, and GLS QTLs and SLB QTLs were found in 30 of these 41 bins. Among the 38 target bins, NLB QTLs, GLS QTLs, and SLB QTLs were reported in 25, 25, and 17 bins,

respectively. To enhance the likelihood of capturing MDR QTLs, markers were chosen to focus on the regions with greatest density of disease QTLs. Higher marker densities were used for genomic regions carrying large numbers of disease QTLs relative to gene density and the numbers of flowering time QTLs (Wisser et al. 2006). The majority of the target bins corresponded to QTLs for more than one of the three diseases (NLB, SLB, and GLS).

As expected, the initial sample sets consisting of 94 F_5 genotypes derived from B73 × CML52 and 46 F_6 genotypes derived from S11 × DK888 allowed successful identification of heterozygous lines for almost every locus of interest. The determination of sample sizes was based on the expected heterozygosity per locus (the probability that a given locus remains heterozygous) in the F_5 (6.25%) and F_6 (3.125%) generations. In the full set of CML52 derivatives, an average of 6.2% of the marker loci were found to be heterozygous per line, and an average of 6.2% of the lines were found to be heterozygous at a given locus. In the subset of 43 individuals in 13 F_5 families, the average percentages of "heterozygous loci per line" and "heterozygous lines per locus" were 7.3 and 7.4%, respectively. In the set of 46 individuals in 17 F_6 families derived from S11 × DK888, an average of 6.1% of the markers were heterozygous in an individual line, and an average of 4.6% of the lines were heterozygous for each marker locus. All the ratios conformed approximately to the expected heterogeneity in the F_5 and F_6 generations.

NLB QTLs identified in the HIFs derived from B73 × CML52 and S11 × DK888

The putative NLB QTLs identified in the HIFs are listed in Table 1. From 2005 to 2006, a total of 24 HIFs derived from B73 × CML52 (15 at F_6, 7 at F_7, and 2 at F_8) were evaluated for resistance to NLB using a range of phenotypic parameters, including IP, LN, Primary DLA, DLA, and AUDPC. Out of 27 bins investigated, significant phenotypic contrasts between CML52 and B73 homozygotes at the target loci were detected in five bins (1.06, 1.07–1.08, 5.03, 6.05, and 8.02–8.03; significance found within at least two families for each). By evaluating sets of NILs developed from selected lines in the HIFs, the resistance effects of CML52 alleles at bins 1.06 and 6.05 and B73 allele at bin 5.03 were validated. These QTLs coincided with NLB QTLs previously detected in two other maize populations (Freymark et al. 1993; Welz et al. 1999b). NLB resistance was detected for bins 1.07–1.08 and 8.02–8.03 in two HIFs of B73 × CML52. However, these two regions were not declared as NLB QTLs, as their effectiveness was not verified in derived NILs.

In 2006, three F_7 HIFs derived from S11 × DK888 were evaluated for NLB resistance. Out of five bins tested, the DK888 allele at bin 8.05–8.06 was found effective for delaying lesion formation by ~6 days in two HIFs. The resistance effect was further confirmed in a set of eight NILs for IP, DLA, and AUDPC (Table 1). Bin 8.05–8.06 has been described as a region associated with quantitative and qualitative resistance to NLB (Welz and Geiger 2000). The NLB QTLs at bin 8.05–8.06 were previously identified in other mapping populations (Schechert et al. 1999; Welz et al. 1999a, b). Two race-specific major genes for NLB resistance, *Ht2* and *Htn1*, were also mapped to this complex region (Simcox and Bennetzen 1993; Zaitlin et al. 1992).

To better localize the candidate NLB QTLs identified in the HIFs, 66 flanking SSR markers (6 for bin 1.06, 8 for bin 1.07–1.08, 14 for bin 5.03, 18 for bin 6.05, 5 for bin 8.02–8.03, and 15 for bin 8.06) were used to estimate the start and end points of heterozygous loci in F_5 and F_6 families of B73 × CML52 and S11 × DK888, respectively. The map interval of each QTL was assumed to extend halfway between two markers around each end of the QTL. The identified QTLs have an average size of 90 map units on the IBM2n map [~23 cM on an F_2 map (Lee et al. 2002)] and ~29 Mb on the physical map. The accuracy of the estimation was affected by the numbers of flanking markers used to determine the border of a QTL block.

Characterization of QTLs for multiple disease resistance

To uncover the resistance effects of MDR target regions, sets of $F_{6:7}$, $F_{6:8}$ or $F_{6:9}$ NILs contrasting for different target loci were developed from selected lines in HIFs. From 2007 to 2008, 15 sets of NILs differing at a total of 21 bins were evaluated for SLB, GLS, ALB, ASR, common rust, common smut, and Stewart's wilt. Only NILs targeting regions significantly associated with any traits of a given disease were phenotyped in the second year. However, to accurately determine the resistance spectra of the reliably expressed NLB QTLs, NILs contrasting for bins 1.06, 6.05, and 8.05–8.06 were evaluated for responses to different diseases in at least two environments. In the 2008 trials, ~36% of the previously detected trait–marker associations were not verified (e.g., ASR resistance conferred by the CML52 allele at bin 7.04 was seen in 2007 but not 2008). This implied that type II error was likely for the loci not included in the second-year trials.

Among the four NLB QTLs validated in the NILs, QTLs in bins 5.03 and 8.05–8.06 were not associated with resistance to any of the other diseases. Bins 1.06 and 6.05, on the other hand, were each associated with resistance to two diseases. The QTLs at bins 1.06 and 6.05 were identified from the HIFs and NILs segregating for both loci.

Table 1 QTLs for resistance to northern leaf blight (NLB) identified using heterogeneous inbred family (HIF) analysis

Cross of origin	QTL position		Mapping material		Resistance allele	NLB QTL(s) in the background	Allele effect (CML52 or DK888 homozygotes – B73 homozygotes)[c]							
	Maize bin[a]	Map-unit on IBM2n (Mb on physical)[b]	Source HIF (generation)	HIFs and NILs, sample size			R^2 [d]	IP (dpi)	LN (number)	DLA1 (%)	DLA2 (%)	DLA3 (%)	DLA4 (%)	AUDPC (%-day)
B73 × CML52	1.06[e]	516.2–545.6	1889_1 (F_5)[e]	F_6 HIF, $n = 47$	CML52	None		ns	ns	ns	–	–	–	–
		(182.6–189.8)		F_7 HIF, $n = 47$			0.28	1.5**	−14.5***	−7.2*	−8.7***	−11.4**	–	−244.1***
				8 F_8 and 10 F_9 NILs			0.66	ns	–	−7.5***	−10.3***	−9.5***	−11.0***	−282.0***
				3 F_7, 20 F_8 and 13 F_9 NILs			0.58	–	–	−5.1***	−11.3***	−13.8***	–	−480.8***
	6.05[e]	267.9–395.0	1889_1 (F_5)[e]	F_6 HIF, $n = 47$	CML52	None	0.16	3.1**	−14.6**	−4.5*	–	–	–	–
		(130.6–155.1)		F_7 HIF, $n = 47$			0.09	1.2**	−9.4*	−6.1*	−6.5*	−7.6*	–	−179.6*
				8 F_8 and 10 F_9 NILs			0.64	1.0***	–	−7.4**	−12.9***	−14.1***	−15.1***	−350.5***
				3 F_7, 20 F_8 and 13 F_9 NILs			0.53	–	–	−4.4***	−7.7***	−11.7***	–	−364.2***
			1889_3 (F_5)	F_6 HIF, $n = 98$		None	0.12	ns	−14.4***	−7.0***	−5.4***	−6.0**	–	−160.9***
				6 F_7 NILs			0.60	0.8*[§]	–	−11.5*	−20.5**	−17**	−16.3**	−464.5**
	5.03	130.9–221.0	1871_2 (F_5)	F_6 HIF, $n = 65$	B73	1.06	0.11	ns	8.2**	5.2*[§]	–	–	–	–
		(9.2–22.6)		F_7 HIF, $n = 58$		6.05	0.18	−0.4*[§]	2.7*	–	–	–	–	–
				8 F_8 NILs			0.32	ns	–	3.5**	4.8*[§]	4.6*[§]	6.0**	126.9*
			1871_3 (F_5)	F_6 HIF, $n = 38$			0.11	−2.9*[§]	ns	ns	–	–	–	–
	1.07–1.08[f]	697.1–748.5	1873_2 (F_5)	F_6 HIF, $n = 91$	CML52[f]	1.06	0.10	2.0*	−6.0*[§]	−2.5*[§]	–	–	–	–
		(222.3–232.3)		F_7 HIF, $n = 83$		5.03	0.07	ns	−10.7*[§]	−3.0*[§]	ns	−6.3***	–	−57.6*[§]
				4 F_8 NILs		6.05		ns	–	ns	ns	ns	ns	ns
	8.02–8.03[f]	123.9–299.9	1901_5 (F_5)	F_6 HIF, $n = 55$	CML52[f]	1.06	0.06	3.8**[§]	−3.3*[§]	–	–	–	–	–
		(12.2–108.4)		F_7 HIF, $n = 53$		6.05	0.07	8.4*	−10.7*[§]	–	–	–	–	–
				5 F_8 NILs		8.05–8.06		ns	ns	–	–	–	–	–
S11 × DK888	8.05–8.06	386.8–453.7	1851_1_2 (F_6)	F_7 HIF, $n = 53$	DK888	Unknown	0.23	5.7***	–	ns	–	–	–	–
		(143.2–163.4)		F_8 HIF, $n = 96$			0.61	6.5***	–	–	–	–	–	–
				2 F_8 and 6 F_9 NILs			0.93	−5.9***	–	−17.9***	−16.6***	−12.2***	–	−379.4***

[a] The maize genome is composed of 100 designated bins. Each bin is a chromosomal segment between two core RFLP markers

[b] The map position was based on the genetic map of the intermated B73 × Mo17 population (version IBM 2008 neighbors) and the B73 physical map. The map interval of each QTL was assumed to extend halfway between two markers around each end of the QTL. Marker names are provided for the QTLs mapped on a single marker basis (flanking markers were not tested)

[c] Different disease parameters, including incubation period (IP; days post inoculation, dpi), lesion number (LN), diseased leaf area (DLA), and area under the disease progress curve (AUDPC), were evaluated. Trait values shown are relative allele effects, which are the differences between the least squares means of CML52 homozygous genotypes and B73 homozygous genotypes, or between DK888 homozygous genotypes and S11 homozygous genotypes at the locus. The significance level was determined by pairwise two-tailed Student's t test on the least square difference (denoted as * $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$; [§]non-significant after the Bonferroni correction; *ns* non-significant; – the trait was not tested)

[d] Adjusted R-square, a ratio of mean squares instead of sum of squares (R-square), was used to account for different numbers of parameters in different models. The adjusted R-square for each QTL was retrieved from the model for AUDPC, or either LN or IP if AUDPC was not applicable

[e] Bins 1.06 and 6.05 were segregating in the same heterogeneous inbred families derived from the F_5 line 1889_1. The QTL effects of these two regions were verified in the NILs, with consideration of their correlation (details described in "Data analysis"). Their allelic effects shown are the phenotypic effects of a given locus, with the other locus segregating in the background

[f] The effects of candidate NLB QTLs at bins 1.07–1.08 and 8.02–8.03 were not validated in advanced NILs

The QTLs for NLB, ASR, and/or Stewart's wilt were declared with confidence as their effects were observed to be significant irrespective of whether the other locus was segregating or fixed (either homozygous for CML52 or B73 alleles) in the background. Epistatic interaction between NLB QTLs at bins 1.06 and 6.05 was not observed.

Bin 1.06 was significantly associated with resistance to NLB and Stewart's wilt. The CML52 allele was effective in decreasing DLA for NLB by 7–10%, and primary DLA caused by Stewart's wilt by ~28% (Table 2). The QTL position is in the vicinity of *Sw1*, a dominant major gene locus for Stewart's wilt previously found in inbred line Ki14 (Ming et al. 1999; Pataky et al. 2008). The effect of bin 1.06 for resistance to both NLB and Stewart's wilt has also been observed from the NILs derived from the inbred line Tx303 crossed to B73 (Chung et al. 2010b). In this case, the Tx303 allele at bin 1.06 contributed moderate resistance to NLB and strong resistance to Stewart's wilt.

Bin 6.05 was significantly associated with resistance to NLB and ASR. The CML52 allele effectively decreased DLA by 7–15% for NLB (Table 2) and decreased total diseased area caused by ASR in the stalk by ~25%. The ASR QTL in bin 6.05 is novel, perhaps not previously mapped because QTLs for ASR resistance have only been studied in two mapping populations (Jung et al. 1994; Weldekidan and Hawk 1993), from which an R gene underlying a major QTL at bin 4.07 has been cloned (Broglie et al. 2006). Two DK888-derived HIFs segregated for either *bnlg2249* or *bnlg1732* in bin 6.05. The locus *bnlg1732* was not associated with resistance to NLB, ASR, or five other diseases. The S11 allele at locus *bnlg2249*, on the other hand, was associated with ASR resistance, while its resistance to NLB was not evaluated (Table 2). Although its effect for NLB resistance is unknown, the S11 allele at *bnlg2249* in bin 6.05 was more effective than the CML52 allele (spanning *bnlg2249* and *bnlg1732*) in reducing ASR symptoms in maize stalks (reducing ~80 and ~40% of total discolored internode area in NILs and HIFs, respectively). An ASR-specific QTL at bin 5.06 was also detected in the DK888-derived HIF. The S11 allele at bin 5.06 conferred resistance by significantly reducing total discolored internode area by ~24% in NILs and by ~14% in HIFs.

Phenotypic correlation for different diseases in RILs

Phenotypic correlation among traits in a segregating population indicates linkage and/or pleiotropy. To examine this in the CML52 RIL populations, the Pearson correlation coefficients were determined among the disease resistance traits (Table 3). As expected, there were significant correlations between NLB, SLB, and GLS resistance and maturity. In general, later maturity results in less disease severity, and this was seen in the RIL population as a negative correlation between DTA and disease resistance. There was a significant positive correlation between NLB and GLS resistance, indicating linked or common QTLs, or possibly an indirect association based on shared effects of maturity. Distinct large-effect QTLs for NLB and GLS resistance were mapped to bins 8.05–8.06. A QTL for DTA was identified in the same region (Table 4). Surprisingly, there was a negative correlation between SLB and ASR resistance. This is probably largely attributable to a region of Chr. 3 (discussed in detail below) for which resistance to SLB was derived from CML52 while resistance to ASR was derived from B73, creating repulsion phase linkage in the RILs.

NLB, GLS, SLB, and ASR QTLs identified in CML52 RILs

The profile of likelihood of odds ratio (LOD) scores for ICIM is shown in Fig. 2. Permutation analysis for this data set provided the significance threshold of LOD = 9 for an experiment-wide alpha of 0.05. At this threshold, only one locus could be declared significant. The low LOD values reflected the small sample size. The CML52 RILs dataset was part of a larger analysis based on the nested association mapping population of which the CML52 RILs population is a subset. Thus, responses to NLB, GLS, and ASR were evaluated on a single replication per year. Because Type I error was not a great concern, a lower (arbitrary) threshold of LOD = 3 was used. At this low LOD level, the findings were quite consistent with the QTLs identified using the HIF strategy.

In bin 6.05, a small peak for ASR resistance (LOD = 2.6) co-localized with the QTL for NLB resistance. There was also limited support for ASR resistance based on BSR analysis, with a very minor effect of −0.05. The ASR QTL at bin 6.05 was considered credible as it was also identified in the CML52-derived HIFs/NILs. In bin 3.04, there were QTLs for SLB and ASR resistance. However, the resistance allele for these two QTLs came from opposite parents, and the QTL positions were not identical, reducing the likelihood of an MDR gene. There have been reports, however, of single genes conditioning resistance to one pathogen but increasing susceptibility to another (e.g., Mang et al. 2009). A QTL for NLB was located at the proximal end of bin 1.07 based on ICIM and BSR and was considered to be identical with the NLB QTL located to the distal margin of bin 1.06 based on HIF analysis (shown as bin 1.06–1.07 in Table 4).

There was general agreement between the ICIM and BSR QTL mapping analysis in the RIL populations. For SLB and NLB resistance, all of the QTL positions

Table 2 Resistance spectra of the QTLs identified using heterogeneous inbred family (HIF) analysis

Cross of origin	QTL position		Mapping materials		Resistance allele	Allele effect (CML52 or DK888 homozygotes − B73 homozygotes)[c]									
						NLB		Stewart's		ASR		SLB	GLS	ALB	Rust	Smut
	Maize bin[a]	Map-units on IBM2n (Mb on physical)[b]	Source HIF (generation)	HIFs and NILs, sample size		R^2 [d]	AUDPC (%-day)	R^2 [d]	Primary DLA (%)	R^2 [d]	Discolored internodes (total %)					
B73 × CML52	1.06[e]	516.2–545.6	1889_1 (F_5)	8 F_8 and 10 F_9 NILs	CML52	0.66	−282.0***	0.58	−28.2***		ns	ns	ns	ns	ns	ns
		(182.6–189.8)		3 F_7, 20 F_8 and 13 F_9 NILs		0.58	−480.8***	0..73	−15.1***		–	–	–	–	–	–
	6.05[e]	267.9–395.0	1889_1 (F_5)	8 F_8 and 10 F_9 NILs	CML52	0.64	−350.5***		ns	0.22	−25.3***	ns	ns	ns	ns	ns
		(130.6–155.1)		3 F_7, 20 F_8 and 13 F_9 NILs		0.53	−364.2***		ns		–	ns	ns	ns	ns	ns
			1889_3 (F_5)	6 F_7 NILs		0.60	−464.5**		ns		ns	ns	ns	ns	ns	ns
	5.03	130.9–221.0 (9.2–22.6)	1871_2 (F_5)	8 F_8 NILs	B73	0.32	126.9*		–		ns	ns	ns	ns	ns	ns
S11 × DK888	5.06	*umc2216*	1851_1_2 (F_6)	F_7 HIF, $n = 53$	S11		ns		–		–	–	–	–	–	–
		518.4 (202.0)		2 F_7 and 6 F_8 NILs			–		–	0.57	23.6***	ns	ns	ns	ns	ns
				F_8 HIF, $n = 139$			–		–	0.05	14.3**	–	–	–	–	–
	6.05	*bnlg2249*	1896_2_2 (F_6)	2 F_7 NILs	S11		–		–	0.42	80.9***	ns	ns	ns	ns	ns
		278.0 (134.7)		F_8 HIF, $n = 129$			–		–	0.04	41.4**	–	–	–	–	–
	6.05	*bnlg1732*	1896_1_3 (F_6)	F_7 HIF, $n = 74$	None		ns		–		–	–	–	–	–	–
		373.8 (151.9)		2 F_7 NILs			–		–		ns	ns	ns	ns	ns	ns
	8.05–8.06	386.8–453.7 (143.2–163.4)	1851_1_2 (F_6)	2 F_8 and 6 F_9 NILs	DK888	0.93	−379.4***		ns		ns	ns	ns	ns	ns	ns

[a] The maize genome is composed of 100 designated bins. Each bin is a chromosomal segment between two core RFLP markers

[b] The map position was based on the genetic map of the intermated B73 × Mo17 population (version IBM 2008 neighbors) and the B73 physical map. The map interval of each QTL was assumed to extend halfway between two markers around each end of the QTL. Marker names are provided for the QTLs mapped on a single marker basis (flanking markers were not tested)

[c] Effects of the targeted loci were determined by evaluating sets of the NILs for resistance to a range of important maize diseases, including northern leaf blight (NLB), Stewart's wilt, anthracnose stalk rot (ASR), southern leaf blight (SLB), gray leaf spot (GLS), anthracnose leaf blight (ALB), common rust (Rust), and common smut (Smut). Trait values shown are relative allele effects, which are the differences between the least squares means of CML52 homozygous genotypes and B73 homozygous genotypes, or between DK888 homozygous genotypes and S11 homozygous genotypes at the locus. The significance level was determined by pairwise two-tailed Student's *t* test on the least square difference (denoted as * $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$; *ns* non-significant; – not applicable because the trait was not tested)

[d] Adjusted *R*-square, a ratio of mean squares instead of sum of squares (*R*-square), was used to account for different numbers of parameters in different models

[e] Bins 1.06 and 6.05 were segregating in the same heterogeneous inbred families derived from the F_5 line 1889_1. The QTL effects of these two regions were verified in the NILs, with consideration of their correlation (details described in "Data analysis"). Their allelic effects shown are the phenotypic effects of a given locus, with the other locus segregating in the background

Table 3 Phenotypic correlations between the traits of disease resistance and plant maturity

	NLB	GLS	SLB	ASR	DTA
NLB		0.23			−0.19
GLS	0.23				−0.18
SLB				−0.17	−0.23
ASR			−0.17		
DTA	−0.19	−0.18	−0.23		

In the population of recombinant inbred lines (RILs; $n = 196$) derived from B73 × CML52, Pearson correlation coefficients were computed between the values of area under the disease progress curve (AUDPC) for northern leaf blight (NLB), AUDPC for gray leaf spot (GLS), AUDPC for southern leaf blight (SLB), total diseased internode area for anthracnose stalk rot (ASR), and days to anthesis (DTA). The significant correlation coefficients at $P < 0.05$ are shown

identified by BSR corresponded to peaks in the LOD profile for ICIM. There were additional QTLs identified by ICIM, on Chrs. 9 and 10 for SLB and on Chr. 4 for NLB, with peaks just above the (arbitrary) threshold of LOD = 3. BSR appeared to produce more robust results for GLS resistance, identifying all the QTLs from ICIM as well as three additional QTLs on Chrs. 4, 6 and 10. For ASR resistance, a single large-effect QTL was identified on Chr. 3 using both BSR and ICIM. However, there were several small-effect QTLs that were not consistently identified between the two methods. The mapping results for maturity were less consistent between ICIM and BSR than for the different disease resistances though the reason for this remains unclear.

The large-effect QTLs for each disease were consistently identified with both RIL-based mapping methods. Minor and potentially novel QTLs identified by ICIM or BSR were declared only if validated in the HIFs. Disease QTLs confirmed by at least two methods (both RIL-based methods or one RIL-based plus NILs) are listed in Table 4 with their genetic and physical map positions.

Pleiotropic QTLs for disease resistance and flowering time

The late-maturing properties of CML52 and DK888 led to the question of whether the effects of disease QTLs reflect indirect expression of flowering time QTLs. To address this, the NILs and RILs were scored for DTA on a row basis and analyzed for potential QTLs using ICIM and BSR. As previously noted, RIL-based mapping was conducted for each of diseases without accounting for variation in DTA as a model covariate to allow detection of QTL for DTA. Both ICIM and BSR detected several QTL peaks for DTA, with the majority showing minor effects (Fig. 2). DTA QTLs at bins 1.04, 1.06–1.07, 3.04, 8.05–8.06, and 9.04 were closely linked or identical to QTLs for GLS, NLB, SLB, NLB/GLS, and SLB, respectively (Table 4). The QTLs for SLB and DTA in bin 3.04 were identified by the same marker using BSR. Fine-mapping and further examination of the QTLs affecting disease resistance and flowering time will be needed to distinguish linkage from pleiotropy for these loci. For several of the disease QTLs, no association with maturity was detected.

In the HIF analysis, although variation for flowering time was sometimes observed among NILs within a set, none of the targeted loci were found to affect DTA, suggesting that the resistance conferred by the identified disease QTLs was not the result of a change in relative maturity. The results from CML52-derived NILs largely agreed with the results from RIL-based mapping, except for the identification of co-localized DTA and NLB QTL(s) at bin 1.06–1.07 in RILs but not NILs. No DTA effect was detected in the NILs segregating for bin 1.06–1.07, perhaps due to genetic background effect, or because the segregating region did not contain gene(s) affecting flowering time [linkage rather than pleiotropy for the DTA and NLB QTL(s) at bin 1.06–1.07].

Discussion

Disease QTLs identified using HIF- and RIL-based approaches

To discover maize loci conditioning resistance to single as well as multiple diseases, HIF- and RIL-based approaches were applied for QTL mapping using genetic materials derived from the broadly resistant maize lines CML52 and/or DK888. Each approach identified several QTLs, most of which were consistent for the two methods. The parallel studies provided cross-validation of the detected QTLs, particularly those with minor effects.

The HIF-based QTL approach aimed to capture and characterize resistance alleles at loci associated with previously identified major genes and disease QTLs. The selected CML52- and DK888-derived HIFs were among the advanced inbred populations available at the inception of the study. Seventy-three SSR markers were applied to target 39% of the maize genome, focusing on the regions associated with clusters of previously reported disease genes and QTLs. The strategy used here was to first identify QTLs for resistance to NLB (our primary disease of focus) in segregating HIFs, to generate sets of NILs contrasting for the candidate NLB QTLs, and to use the derived NILs for NLB QTL validation and characterization of resistance spectrum. Using HIF analysis, four NLB QTLs at bins 1.06, 5.03, 6.05 and 8.05–8.06, two ASR QTLs at bins 5.06 and 6.05, and one Stewart's wilt QTL at

Table 4 Disease QTLs identified using recombinant inbred line (RIL)-based QTL mapping

Maize bin[a]	Resistance or late-maturing allele	QTL identified in RILs using ICIM[b]						QTL identified in RILs using BSR[b]						Co-localized QTL identified in HIFs	
		QTL position[c]	Likelihood of odd (LOD)					QTL peak[c]	Standardized additive allele effect[d]					QTL position[e]	Resistance effect
		cM on NAM (Mb on physical)	NLB	SLB	GLS	ASR	DTA	cM on NAM (Mb on physical)	NLB	SLB	GLS	ASR	DTA	Map-unit on IBM2n (Mb on physical)	
1.04	CML52	78–82 (72.3–81.8)			3.2		6.4	86.6 (83.4)	−0.30		−0.40		0.46		
1.06–1.07	CML52	114–118 (200.4–205.8)	7.7				3.6	116.2 (203.0)	−0.58	−0.18			−0.19	516.2–545.6 (182.6–189.8)	NLB, Stewart's wilt
1.08	CML52	146–150 (248.5–255.6)		4.1				149.7 (~251.1)		−0.14				–	–
2.03	CML52	52.4–56.4 (21.4–24.3)		7.0				49.8 (19.8)		−0.54				–	–
3.04	CML52	30–48 (8.2–20.9)		4.6				50.2 (21.1)		−0.99			0.58	–	–
		52–56 (23.4–42.5)		13.6											
3.05	B73	62–66 (132.6–146.9)				7.7		65.2 (143.8)				0.66		–	–
5.03	B73	38–46 (12.0–15.5)	3.7					49.0 (18.2)	0.32					130.9–221.0 (9.2–22.6)	NLB
6.05	CML52	60.9–66.9 (148.5–152.3)	8.3			2.6		70.4 (154.5)	−0.46			−0.05		267.9–395.0 (130.6–155.1)	NLB, ASR
8.05	CML52	63.8–67.8 (117.9–128.0)			5.2		9.8[f]	67.4 (~128)			−0.54		0.58[f]		
8.05–8.06	CML52	73.8–83.8 (143.6–156.7)	14.6				9.8[f]	81.6[e] (154.7)	−0.74				0.58[f]	386.8–453.7 (143.2–163.4)	NLB (DK888 allele)
9.02	CML52	18–22 (11.1–12.7)		5.0				23.8 (13.7)		−0.60				–	–
9.04	CML52	48–58 (93.2–118.4)		3.8			16.5	53.1 (107.1)		−0.03			0.49	–	–

[a] The maize genome is composed of 100 designated bins. Each bin is a chromosomal segment between two core RFLP markers

[b] Loci for resistance to northern leaf blight (NLB), southern leaf blight (SLB), gray leaf spot (GLS), and anthracnose stalk rot (ASR) were identified in a recombinant inbred line (RIL) population derived from the cross of B73 × CML52. The RIL population consisted of 196 F_6 lines genotyped with 773 single nucleotide polymorphism (SNP) markers. QTLs declared here were detected (1) consistently from inclusive composite interval mapping (ICIM) and Bayesian shrinkage regression (BSR), or (2) from either ICIM or BSR but were validated using heterogeneous inbred family (HIF) analysis. Loci for days to anthesis (DTA) were also mapped in the RILs, but only the DTA QTLs co-localized with disease QTLs are shown

[c] For QTL identified in RILs, the map position was based on genetic map of the nested association mapping (NAM) population and the B73 physical map. In ICIM, the physical map interval of each QTL was estimated based on the physical position of the nearest flanking markers. The interval was considered as the ''two-LOD support interval'', which includes the QTL peak and its right and left loci with LOD scores dropping by two units. For BSR, markers showing a genetic contribution (effect >0.1 on standardized phenotype values) were identified as QTLs. Where two closely linked markers (<5 cM) both had an estimated effect size greater than 0.1, the combined estimated is given for that position

[d] For BSR, phenotypes for each trait were first standardized to allow better comparison of allele effect sizes across diseases. Therefore, the reported allele effect sizes represent the homozygous substitution effect in standard deviations

[e] For QTL identified in HIFs, the map position was based on the genetic map of the intermated B73 × Mo17 population (version IBM 2008 neighbors) and the B73 physical map. The map interval of each QTL was assumed to extend halfway between two markers around each end of the QTL

[f] Using both ICIM and BSR, the DTA QTL was mapped near (but not coinciding with) NLB and GLS QTL. The DTA QTL was located at 67.8–71.8 cM (128.0–140.5 Mb) for ICIM and at 66.9 cM (123.3 Mb) for BSR

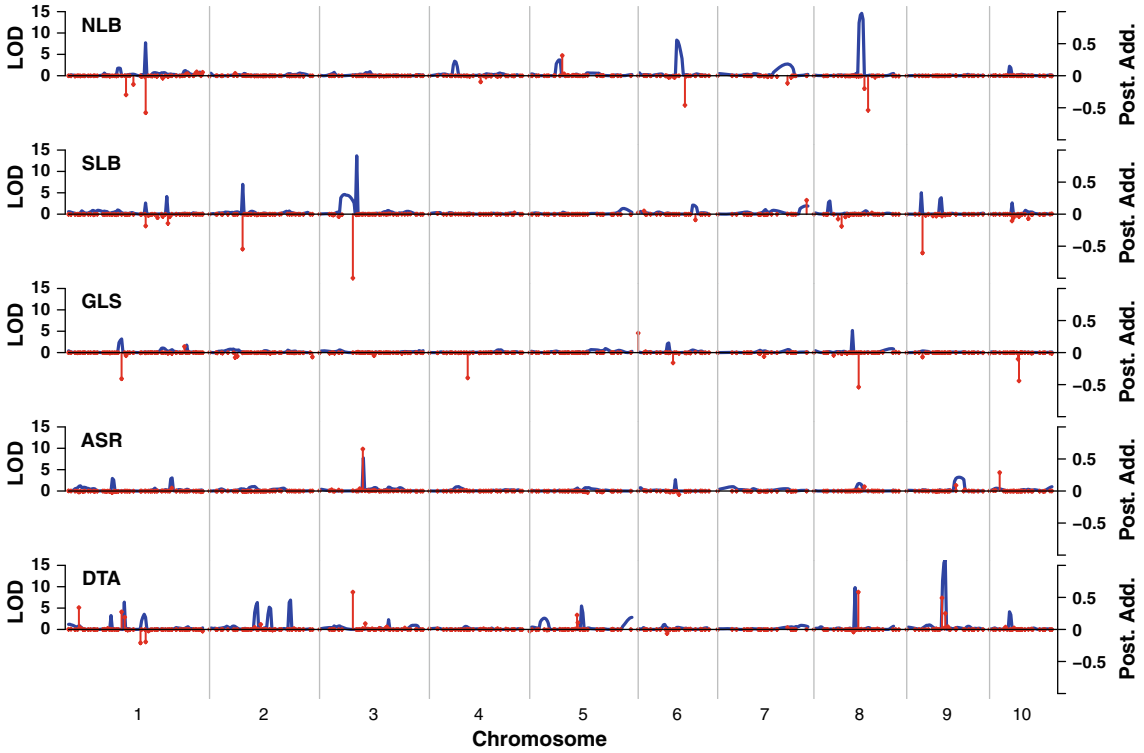

Fig. 2 Quantitative trait loci (QTLs) detected by inclusive composite interval mapping (ICIM; in *blue*) and Bayesian shrinkage regression (BSR; in *red*) in the population of recombinant inbred lines (RILs) derived from the B73 × CML52 cross. The likelihood of odds ratio (*LOD*) scores detected by ICIM are shown on the *left axis*; the mean posterior additive effects (*Post. Add.*) detected by BSR are shown on the *right axis*. Loci conditioning resistance to northern leaf blight (*NLB*), southern leaf blight (*SLB*), gray leaf spot (*GLS*), and anthracnose stalk rot (*ASR*), as well as loci affecting days to anthesis (*DTA*) are shown on the respective plots. Individual markers are shown by *diamonds* at their genetic position on each chromosome. Chromosomes are separated by *vertical gray lines* with chromosome numbers designated below the plots. For BSR, negative allele effects for NLB, SLB, GLS, and ASR indicate resistance comes from CML52. For DTA, positive allele effects indicate that later flowering comes from CML52. To provide comparison of effect sizes for BSR, each trait was standardized (i.e., mean = 0 and standard deviation = 1) prior to analysis

bin 1.06 were detected and validated. The average size of the identified QTLs was 90 map-units on the IBM2n map, which is slightly smaller than the average disease QTL size in previous mapping studies [107 map-units on the IBM2n map (Wisser et al. 2006)]. The precision of QTL locations was improved with increased marker density (66 additional markers) surrounding the QTL regions.

Using the RIL-based approach, QTLs were identified for NLB at bins 1.06–1.07, 5.03, 6.05 and 8.05–8.06, for SLB at bins 1.08, 2.03, 3.04, 9.02 and 9.04, for GLS at bins 1.04 and 8.05, and for ASR at bin 3.05 and 6.05. Different statistical methods for QTL mapping have been developed to improve the genome-wide genetic analysis of complex traits. While ICIM and BSR take different statistical approaches to identification of QTLs, it is expected that there should be concordance in the results for 'real' QTLs. This was generally the case in the present study, especially for QTLs that were validated in the HIFs. Since the RILs utilized in the study were genotyped with a high density of SNP markers, the QTL resolution was significantly increased. The average size of the identified QTLs was ~6.5 cM on the NAM map and ~10 Mb on the physical map. The high-precision mapping successfully distinguished the SLB and ASR QTLs on Chr. 3.

Mapping results from the parallel HIF- and RIL-based studies were used for cross-validation. QTLs identified in HIFs/NILs largely conformed to the QTLs mapped in RILs. Most of the genetic regions investigated in the CML52 HIFs/NILs (27 bins as a total) did not show significant effects for resistance to NLB, SLB, GLS, or ASR, which was consistent with their lack of phenotypic effects in the RILs. HIF- and RIL-based approaches detected co-localized NLB QTLs at bins 1.06–1.07, 5.03 and 6.05, and co-localized ASR QTLs at bin 6.05 (QTLs were considered co-localized if they are overlapping or nearly overlapping).

Five large-effect QTLs for resistance, including a NLB QTL at bin 8.05–8.06, SLB QTLs at bins 2.03, 3.04 and 9.02, and an ASR QTL at bin 3.05, as well as one minor-effect SLB QTL at bin 1.08, were mapped in RILs but not HIFs/NILs. These genetic regions were not targeted by the markers utilized, were fixed in all the available HIFs, or were not evaluated due to poor agronomic performance of

the HIFs. (Note that the region of bin 1.08 tested in CML52 HIFs was ~20 Mb distant from the SLB QTL identified at bin 1.08 in the RILs. As a result, the SLB QTL at bin 1.08 was not captured and evaluated in HIFs.)

HIF-based QTL analysis: considerations and lessons learned

The use of RILs is an efficient way of getting a whole-genome QTL map at reasonable resolution, especially when populations with high-density marker data are available. In the present study, RIL-based mapping detected all the QTLs identified in HIFs, as well as several additional QTLs. The objective of this study, however, was not to identify all QTLs in a given genotype, but rather to test the hypothesis that selected loci from selected genotypes condition MDR. Relevant RIL populations were not available at the inception of this study, whereas we had access to intermediate inbred material suitable for the extraction of NIL pairs. This study demonstrates that HIF-derived NILs can be used for QTL mapping, particularly when, as in this study, partially inbred materials are available and certain loci are to be targeted.

The efficiency of HIFs in this study was the use for analysis of multiple diseases at specific loci associated with MDR. The HIF strategy presented here, as modified from the methodology of Tuinstra et al. (1997), involved selecting QTLs for the primary focus of disease (NLB), generating NILs for those QTLs, and testing them for response to other diseases. To be able to focus on the regions for resistance to NLB, individual trait–marker analysis was conducted in earlier generations of HIFs, which may cause higher probability of type II error due to the evaluation of individual plants with heterogeneous genetic backgrounds. This problem was addressed by confirming the candidate QTLs in another HIF(s) and/or advanced NILs. Along the way, selfed seeds were obtained from a large proportion of genotyped lines within selected HIFs, which became useful as sources of NILs and segregating populations suitable for subsequent QTL characterization and fine-mapping. As a result, the QTLs at bins 1.06 and 6.05 were confirmed to confer broader-spectrum resistance, and the NLB-specific QTL at bin 8.05–8.06 was delimited to a region of 460 kb (Chung et al. 2010a).

While a HIF-based approach was successfully utilized, certain weaknesses should be acknowledged. The outcome of HIF-based analysis is determined by the genetic structure of the starting materials. Starting with limited number of F_5 or F_6 families, HIFs segregating for a given locus may or may not be available. While the large-effect QTLs in targeted regions were clearly identified using HIFs, some QTLs were identified only in the RILs either because they were not targeted or because of the nature of the starting materials: they were fixed in the selected HIFs or were captured in NIL pairs with resistant backgrounds or unsuitable agronomic performance. Alternatively, the QTLs not identified in the NILs could have been false positives in the RIL-based analysis as we were unable to do a suitable determination of type I error.

In this study, it is difficult to determine the relative detection power of HIF- and RIL-based mapping by comparing allelic effects of a given QTL resulting from the two approaches. Evaluations of HIFs and RILs were conducted by different researchers in different environments. The magnitudes of QTL effects have proven to be vulnerable to different people's ratings though the proportion of phenotypic variation explained was relatively constant between individuals (Poland and Nelson, 2011). However, the proportion of phenotypic variance that a QTL will account for is determined both by the QTL and other QTLs segregating in the population. While many other QTLs were segregating in the RIL population, the NIL backgrounds were largely fixed, making a comparison of allele effects not meaningful. Using simulated data, Kaeppler (1997) showed that QTL mapping using RILs generally (and sometimes substantially) provided better power over the use of backcross-derived NILs despite the higher precision phenotyping in the NILs. Since the HIF-based approach is conducted on the basis of contrasting effects of pairs of NILs, it may provide similar power as the conventional NIL-based approach and therefore less power than RIL-based approach.

The major weakness of the HIF-based approach to NIL development is the effect of diverse backgrounds in selected HIFs and derived NIL pairs. In contrast to backcross-derived NILs, which are in the background of the (susceptible) recurrent parent, HIF-derived NILs have genetic constitutions recombined from two parental genomes. The mosaic genome of HIF-derived NILs may or may not be appropriate for QTL expression. The observed effectiveness of a QTL can be masked by major-effect QTLs (Eshed and Zamir 1995; Keurentjes et al. 2007) or affected by epistatic QTLs (Njiti et al. 1998; Szalma et al. 2005) in the unlinked region(s) of the genome. In this study, QTL effects were masked by resistant genetic backgrounds in two NIL pairs. All the QTLs detected using the HIF-based approach were identified in HIFs/NILs exhibiting low to moderate levels of resistance. In contrast, the minor-effect NLB QTL at bin 5.03 was not found to be effective in a HIF carrying resistance alleles at three larger-effect NLB QTLs. The masking or epistatic effect caused by genetic background has been observed in other studies, in which some candidate QTLs were validated in the NILs derived from some but not all the chosen HIFs (Pumphrey et al. 2007; Tuinstra et al. 1997).

The observation that disease QTL effects are dependent on genetic background, and that minor QTL effects can be masked by major QTL effects, has implications for the value of marker-assisted selection (MAS) in resistance breeding programs. Use of MAS has been proposed (and demonstrated in some cases) to be more efficient than conventional phenotypic selection for traits that are difficult to manage (Flint-Garcia et al. 2003; Ribaut and Ragot 2007; Xu and Crouch 2008; Yousef and Juvik 2001). This refers to the penetrance of the target loci and the costs associated with phenotyping. As a complex trait, disease resistance is controlled by multiple loci affecting diverse defense mechanisms. Pyramiding favorable alleles for multiple QTLs conditioning resistance to a single disease and/or various diseases is expected to result in resistance that is more durable. However, in practice, phenotypic selection alone may not be effective for combining desirable alleles for multiple QTLs as some QTL effects would be undetectable in certain working backgrounds. MAS, on the other hand, is relatively attractive because of its potential of tracking and pyramiding favorable chromosomal segments regardless of the manifested phenotypes.

Association and non-association between plant maturity and disease resistance

Plant maturity has been found to be negatively correlated with disease resistance for necrotrophic diseases in maize (Bubeck et al. 1993; Carson 1999; Keller and Bergstrom 1988; Leonard and Thompson 1976). Late-flowering maize lines tend to be more resistant to NLB, SLB, and GLS. In the maize association panel, 48, 45, and 52% of resistance variation to NLB, SLB, and GLS, respectively, were explained by variation in days to anthesis (Wisser et al. 2011). While both CML52 and DK888 are late-maturing (Supplementary Table 2), we provided evidence that part of their resistance is the result of defense mechanisms rather than developmental effects. We evaluated juvenile plants of CML52 and DK888 in repeated greenhouse and field trials for NLB, the primary emphasis of disease at the inception of this study. Compared to B73, the formation of NLB lesions in juvenile plants of CML52 and DK888 was delayed by approximately 4 and 5 days in the greenhouse, and 4 and 9 days in the field, respectively (data not shown). The results suggested that NLB resistance in both genotypes is effective regardless of their maturity stage or growing environment (greenhouse vs. field). For resistances to NLB, GLS, and SLB, maturity and resistance effects were co-localized at some chromosomal segments but not at others in the RIL-based QTL analysis. Genetic dissection and detailed investigation will be needed to uncover the complex relationship between resistance and physiological mechanisms in plants.

Implications of MDR

To study the phenomenon of MDR, a diverse set of pathogen species was evaluated on a small set of maize genotypes reputed to possess MDR, as well as on a large set of their near-isogenic derivatives, targeting chromosomal regions previously shown to be associated with MDR. MDR was observed in the study at the level of genotype and co-localized QTL. Among the genotypes used as sources of alleles, the hybrid DK888 exhibited superior resistance to all the eight diseases tested; the tropical inbred line CML52 showed superior to moderate levels of resistance to all the tested fungal leaf diseases, including NLB, SLB, GLS, ALB, and common rust, and the bacterial disease Stewart's wilt; the elite inbred line B73 showed resistance only to common rust and common smut, which are caused by biotrophic fungi. Based on HIF- and/or RIL-based QTL analyses, the CML52 allele at bin 1.06–1.07 was associated with co-localized NLB QTL and Stewart's wilt QTL, and the CML52 allele at bin 6.05 was associated with co-localized NLB QTL and ASR QTL. Identification of these QTLs confirmed the hypothesized existence of chromosomal segments conditioning MDR (albeit for two diseases per locus) in these broadly resistant genotypes.

Genotypes with MDR characteristics and genomic regions contributing MDR have been recognized for a range of plants. MDR phenotype could be due to pyramiding or linkage of genes with disease-specific effects, or to the presence of gene(s) with broad-spectrum effects. In general, race-specific (and hence pathogen-specific) resistance is controlled by resistance (R) genes, which encode proteins that can recognize specific pathogen effectors and trigger rapid induction of the hypersensitive response and a series of defense reactions (Jones and Dangl 2006). Genes with pleiotropic resistance effects, on the other hand, may be key regulatory genes controlling the recognition or signaling of non-host resistance, basal resistance, and systemic acquired resistance [e.g., *mlo* (Buschges et al. 1997), *npr1* (Cao et al. 1998), and *Lr34* (Krattinger et al. 2009)]. They may also be defense response (DR) genes functioning as the downstream components of a variety of defense mechanisms [e.g., pathogenesis-related (PR) genes (Edreva 2005)]. In addition, the specificity of resistance may be associated with temporal and spatial induction of antimicrobial structures or compounds and the spectrum of these defense responses. This type of broad-spectrum resistance, controlled by a single pleiotropic gene or multiple genes, could affect pathogens with similar mode of pathogenesis.

The eight pathogens evaluated here have diverse lifestyles (Supplementary Table 2). These pathogens can be classified based on the plant tissues that they colonize. Bin 1.06 was associated with resistance to the two pathogens

that cause wilting lesions by growth within the xylem: *S. turcica* (the fungus causing NLB) and *P. stewartii* (the bacterial pathogen causing Stewart's wilt). This suggests the possibility that both might be affected by a defense mechanism that either prevents pathogen invasion or reduces pathogen growth and dissemination in the xylem lumen. In rice, a peroxidase has been implicated in the vascular defense response against the bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae*. Induction and accumulation of the peroxidase, PO-C1, in the xylem parenchyma, vessel walls, and lumen is associated with the rapid thickening of the xylem secondary wall and the generation of antimicrobial reactive oxygen species (Hilaire et al. 2001). This and/or other defense mechanisms could function in the maize vascular system. A large number of peroxidases as well as other defense-related proteins, such as chitinase, beta-glucanase, and polygalacturonase inhibitor (PGIP), have been characterized in the maize xylem sap. The antifungal activity of the sap proteins has also been confirmed (Alvarez et al. 2006).

The CML52 allele(s) at bin 6.05 was associated with resistance to NLB and ASR, while the same chromosomal segment from S11 was associated with resistance to ASR but not NLB or other diseases. NLB and ASR occur in distinct tissues (leaf vs. stalk tissues). The two causal organisms, *S. turcica* and *C. graminicola*, both hemibiotrophic fungi, share similar modes of initial colonization: their early development involves biotrophic interactions with primary infected epidermal cells and the subsequent intercellular hyphal growth and colonization of chlorenchyma or parenchyma cells. Subsequently, different means of progression are used by *S. turcica* and *C. graminicola*: the former grows aggressively in the xylem vessels then spreads into the neighboring chlorenchyma cells, while the latter colonizes the mostly nonliving fiber cells associated with the vascular bundles and rind, then breaks into the adjacent parenchyma cells (Venard and Vaillancourt 2007a). For a single defense mechanism to work against both pathogens, it would have to function in both leaf chlorenchyma and stalk parenchyma, before the pathogen enters into the respective tube structures, or after the pathogen emerges to consume the neighboring tissues. There is, however, little reason to expect that resistance expressed in the leaf and stalk tissues would be controlled by the same gene(s). Leaf and stalk resistance to the same or closely-related pathogen species have been found to be non-correlated (Lim and White 1978; Venard and Vaillancourt 2007b; Zuber et al. 1981). Similarly, no correlation was observed between resistance to ASR and ALB (both caused by *C. graminicola*) among the HIFs in the present study, and none of our identified ASR QTLs showed ALB effects. It is thus suspected that resistance to NLB and ASR are conditioned by linked genes in bin 6.05.

Conclusion

Several disease-specific QTLs and two MDR QTLs were identified and validated using HIF-based targeted QTL analysis and classical RIL-based QTL mapping. While the value of the disease QTL consensus map to MDR QTL prediction was not rigorously assessed, detection of chromosomal segments conditioning resistance to more than one disease reflected the generally observed clustering of disease QTLs in plants. Overall, the study allowed empirical comparisons for the advantages and limitations of using HIFs and RILs for distinct and complementary purposes. Evaluations of a range of pathogens causing important diseases revealed chromosomal segments conditioning pleiotropic resistance. While the specific combinations of resistance provoke speculation on the underlying mechanisms, further studies are needed to shed light on these mechanisms. QTL resolution in this study was higher than in conventional QTL mapping but is not sufficient for identifying candidate genes affecting resistance. Fine-mapping and positional cloning is underway to resolve the complex genetic mechanisms.

Acknowledgments We thank Randall Wisser for integrating the markers used in the HIF analysis onto the disease QTL consensus map, Erik Stromberg for his help with field trials in Virginia, Kent Loeffler for photography, and Pioneer Hi-Bred International Inc. for the supply of sorghum grains used in production of inoculum. Seed for the F_5 families and the RIL population from B73 × CML52 were generously provided by Edward Buckler. Seed for the F_6 families from S11 × DK888 were kindly provided by Major Goodman. We appreciate help from Oliver Ott, Kristen Kennedy, Zura Kerdieh, and Donna Stephens regarding research aspects. The work was funded by The CGIAR Generation Challenge Program, The McKnight Foundation, USDA-ARS, The Bill & Melinda Gates Foundation, and Ministry of Education, Taiwan.

References

Alvarez S, Goodger JQD, Marsh EL, Chen S, Asirvatham VS, Schachtman DP (2006) Characterization of the maize xylem sap proteome. J Proteome Res 5:963–972

Balasubramanian S, Schwartz C, Singh A, Warthmann N, Kim MC, Maloof JN, Loudet O, Trainer GT, Dabi T, Borevitz JO, Chory J, Weigel D (2009) QTL mapping in new *Arabidopsis thaliana* advanced intercross-recombinant inbred lines. PLoS One 4:e4318

Balint-Kurti PJ, Blanco M, Millard M, Duvick S, Holland J, Clements M, Holley R, Carson ML, Goodman MM (2006) Registration of 20 GEM maize breeding germplasm lines adapted to the southern USA. Crop Sci 46:996–998

Borevitz JO, Chory J (2004) Genomics tools for QTL analysis and gene discovery. Curr Opin Plant Biol 7:132–136

Broglie KE, Butler KH, Butruille MG, da Silva Conceicao A, Frey TJ, Hawk JA, Jaqueth JS, Jones ES, Multani DS, Wolters PJCC, E.I. du Pont de Nemours and Company, Pioneer Hi-Bred International, Inc., University of Delaware United States (2006)

Polynucleotides and methods for making plants resistant to fungal pathogens. United States Patent 20060223102

Bubeck DM, Goodman MM, Beavis WD, Grant D (1993) Quantitative trait loci controlling resistance to gray leaf spot in maize. Crop Sci 33:838–847

Buckler ES, Holland JB, Bradbury PJ, Acharya CB, Brown PJ, Browne C, Ersoz E, Flint-Garcia S, Garcia A, Glaubitz JC, Goodman MM, Harjes C, Guill K, Kroon DE, Larsson S, Lepak NK, Li H, Mitchell SE, Pressoir G, Peiffer JA, Rosas MO, Rocheford TR, Cinta Romay M, Romero S, Salvo S, Sanchez Villeda H, da Silva HS, Sun Q, Tian F, Upadyayula N, Ware D, Yates H, Yu J, Zhang Z, Kresovich S, McMullen MD (2009) The genetic architecture of maize flowering time. Science 325:714–718

Buschges R, Hollricher K, Panstruga R, Simons G, Wolter M, Frijters A, Van Daelen R, Van der Lee T, Diergarde P, Groenendijk J, Topsch S, Vos P, Salamini F, Schulze-Lefert P (1997) The barley *Mlo* gene: a novel control element of plant pathogen resistance. Cell 88:695–705

Cao H, Li X, Dong X (1998) Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. PNAS 95:6531–6536

Carson ML (1998) Aggressiveness and perennation of isolates of *Cochliobolus heterostrophus* from North Carolina. Plant Dis 82:1043–1047

Carson ML (1999) Helminthosporium leaf spots and blights. In: White DG (ed) Compendium of corn diseases, 3rd edn. The American Phytopathology Society, St. Paul, MN, pp 15–24

Carson ML, Stuber CW, Senior ML (2004) Identification and mapping of quantitative trait loci conditioning resistance to southern leaf blight of maize caused by *Cochliobolus heterostrophus* race O. Phytopathology 94:862–867

Chung C, Jamann T, Longfellow J, Nelson R (2010a) Characterization and fine-mapping of a resistance locus for northern leaf blight in maize bin 8.06. Theor Appl Genet 121:205–227

Chung C, Longfellow J, Walsh E, Kerdieh Z, Van Esbroeck G, Balint-Kurti PRN (2010b) Resistance loci affecting distinct stages of fungal pathogenesis: use of introgression lines for QTL mapping and characterization in the maize—*Setosphaeria turcica* pathosystem. BMC Plant Biol 10:103

Dickinson MJ, Jones DA, Jones JDG (1993) Close linkage between the *Cf-2*/*Cf-5* and *Mi* resistance loci in tomato. Mol Plant Microbe Interact 6:341–347

Dixon MS, Hatzixanthis K, Jones DA, Harrison K, Jones JDG (1998) The tomato *Cf-5* disease resistance gene and six homologs show pronounced allelic variation in leucine-rich repeat copy number. Plant Cell 10:1915–1925

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Edreva A (2005) Pathogenesis-related proteins: research progress in the last 15 years. Gen Appl Plant Physiol 31:105–124

Ekasingh B, Gypmantasiri P, Thong-Ngam K (2001) Impact of maize breeding research in Thailand: public- and private-sector collaboration. In: Gerpacio RV (ed) Impact of public- and private-sector maize breeding research in Asia, 1966–1997/98. International Maize and Wheat Improvement Center (CIMMYT), pp 95–104

Eshed Y, Zamir D (1995) An introgression line population of *Lycopersicon pennellii* in the cultivated tomato enables the identification and fine mapping of yield-associated QTL. Genetics 141:1147–1162

Faris JD, Li WL, Liu DJ, Chen PD, Gill BS (1999) Candidate gene analysis of quantitative disease resistance in wheat. Theor Appl Genet 98:219–225

Flint-Garcia SA, Darrah LL, McMullen MD, Hibbard BE (2003) Phenotypic versus marker-assisted selection for stalk strength and second-generation European corn borer resistance in maize. Theor Appl Genet 107:1331–1336

Freymark PJ, Lee M, Woodman WL, Martinson CA (1993) Quantitative and qualitative trait loci affecting host-plant response to *Exserohilum turcicum* in maize (*Zea mays* L.). Theor Appl Genet 87:537–544

Goodman MM (2005) Broadening the U.S. maize germplasm base. Maydica 50:203–214

Hilaire E, Young SA, Willard LH, McGee JD, Sweat T, Chittoor JM, Guikema JA, Leach JE (2001) Vascular defense responses in rice: peroxidase accumulation in xylem parenchyma cells and xylem wall thickening. Mol Plant Microbe Interact 14:1411–1419

Jo Y-K, Barker R, Pfender W, Warnke S, Sim S-C, Jung G (2008) Comparative analysis of multiple disease resistance in ryegrass and cereal crops. Theor Appl Genet 117:531–543

Joehanes R, Nelson JC (2008) QGene 4.0, an extensible Java QTL-analysis platform. Bioinformatics 24:2788–2789

Jones JDG, Dangl JL (2006) The plant immune system. Nature 444:323–329

Jung M, Weldekidan T, Schaff D, Paterson A, Tingey S, Hawk J (1994) Generation-means analysis and quantitative trait locus mapping of anthracnose stalk rot genes in maize. TAG 89:413–418

Kaeppler SM (1997) Quantitative trait locus mapping using sets of near-isogenic lines: relative power comparisons and technical considerations. Theor Appl Genet 95:384–392

Kanazin V, Marek LF, Shoemaker RC (1996) Resistance gene analogs are conserved and clustered in soybean. PNAS 93:11746–11750

Keller NP, Bergstrom GC (1988) Development predisposition of maize to anthracnose stalk rot. Plant Dis 72:977–980

Keurentjes JJB, Bentsink L, Alonso-Blanco C, Hanhart CJ, Vries HB-D, Effgen S, Vreugdenhil D, Koornneef M (2007) Development of a near-isogenic line population of *Arabidopsis thaliana* and comparison of mapping power with a recombinant inbred line population. Genetics 175:891–905

Kobayashi S, Araki E, Osaki M, Khush GS, Fukuta Y (2006) Localization, validation and characterization of plant-type QTLs on chromosomes 4 and 6 in rice (*Oryza sativa* L.). Field Crop Res 96:106–112

Kraja A, Dudley JW, White DG (2000) Identification of tropical and temperate maize populations having favorable alleles for disease resistance. Crop Sci 40:948–954

Krattinger SG, Lagudah ES, Spielmeyer W, Singh RP, Huerta-Espino J, McFadden H, Bossolini E, Selter LL, Keller B (2009) A putative ABC transporter confers durable resistance to multiple fungal pathogens in wheat. Science 323:1360–1363

Lee J, Hardin B (1997) GEM searches for treasures in exotic maize. Agric Res 45:4–6

Lee M, Sharopova N, Beavis WD, Grant D, Katt M, Blair D, Hallauer A (2002) Expanding the genetic map of maize with the intermated B73 × Mo17 (IBM) population. Plant Mol Biol 48:453–461

Leonard KJ, Thompson DL (1976) Effects of temperature and host maturity on lesion development of *Colletotrichum graminicola* on corn. Phytopathology 66:635–639

Li WL, Faris JD, Chittoor JM, Leach JE, Hulbert SH, Liu DJ, Chen PD, Gill BS (1999) Genomic mapping of defense response genes in wheat. Theor Appl Genet 98:226–233

Lim SM, White DG (1978) Estimates of heterosis and combining ability for resistance of maize to *Colletotrichum graminicola*. Phytopathology 68:1336–1342

Lopez CE, Acosta IF, Jara C, Pedraza F, Gaitan-Solis E, Gallego G, Beebe S, Tohme J (2003) Identifying resistance gene analogs

associated with resistances to different pathogens in common bean. Phytopathology 93:88–95

Loudet O, Gaudon V, Trubuil A, Daniel-Vedele F (2005) Quantitative trait loci controlling root growth and architecture in *Arabidopsis thaliana* confirmed by heterogeneous inbred family. Theor Appl Genet 110:742–753

Mang HG, Laluk KA, Parsons EP, Kosma DK, Cooper BR, Park HC, AbuQamar S, Boccongelli C, Miyazaki S, Consiglio F, Chilosi G, Bohnert HJ, Bressan RA, Mengiste T, Jenks MA (2009) The Arabidopsis *RESURRECTION1* gene regulates a novel antagonistic interaction in plant defense to biotrophs and necrotrophs. Plant Physiol 151:290–305

McMullen MD, Kresovich S, Villeda HS, Bradbury P, Li H, Sun Q, Flint-Garcia S, Thornsberry J, Acharya C, Bottoms C, Brown P, Browne C, Eller M, Guill K, Harjes C, Kroon D, Lepak N, Mitchell SE, Peterson B, Pressoir G, Romero S, Rosas MO, Salvo S, Yates H, Hanson M, Jones E, Smith S, Glaubitz JC, Goodman M, Ware D, Holland JB, Buckler ES (2009) Genetic properties of the maize nested association mapping population. Science 325:737–740

Mideros SX, Windham GL, Williams WP, Nelson RJ (2009) *Aspergillus flavus* biomass in maize estimated by quantitative real-time polymerase chain reaction is strongly correlated with aflatoxin concentration. Plant Dis 93:1163–1170

Milligan SB, Bodeau J, Yaghoobi J, Kaloshian I, Zabel P, Williamson VM (1998) The root knot nematode resistance gene *Mi* from tomato is a member of the leucine zipper, nucleotide binding, leucine-rich repeat family of plant genes. Plant Cell 10:1307–1319

Ming R, Brewbaker JL, Moon HG, Musket TA, Holley RN, Pataky JK, McMullen MD (1999) Identification of RFLP makers linked to a major gene, *sw1*, conferring resistance to Stewart's wilt in maize. Maydica 44:319–323

Muimba-Kankolongo A, Bergstrom GC (1990) Transitory wound predisposition of maize to Anthracnose stalk rot. Can J Plant Pathol 12:1–10

Njiti VN, Doubler TW, Suttner RJ, Gray LE, Gibson PT, Lightfoot DA (1998) Resistance to soybean sudden death syndrome and root colonization by *Fusarium solani* f. sp. *glycine* in near-isogenic lines. Crop Sci 38:472–477

Pataky JK, Bohn MO, Lutz JD, Richter PM (2008) Selection for quantitative trait loci associated with resistance to Stewart's wilt in sweet corn. Phytopathology 98:469–474

Poland JA, Nelson RJ (2011) In the eye of the beholder: the effect of rater variability and different rating scales on QTL mapping. Phytopathology 101:290–298

Poland JA, Balint-Kurti PJ, Wisser RJ, Pratt RC, Nelson RJ (2009) Shades of gray: the world of quantitative disease resistance. Trends Plant Sci 14:21–29

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

Qiu F, Wang H, Chen J, Zhuang J, Hei L, Cheng S, Wu J (2006) A rapid DNA mini-prep method for large-scale rice mutant screening. Rice Sci 13:299–302

Ramalingam J, Cruz CMV, Kukreja K, Chittoor JM, Wu JL, Lee SW, Baraoidan M, George ML, Cohen MB, Hulbert SH, Leach JE, Leung H (2003) Candidate defense genes from rice, barley, and maize and their association with qualitative and quantitative resistance in rice. Mol Plant Microbe Interact 16:14–24

Ribaut J-M, Ragot M (2007) Marker-assisted selection to improve drought adaptation in maize: the backcross approach, perspectives, limitations, and alternatives. J Exp Bot 58:351–360

Robertson DS (1989) Understanding the relationship between qualitative and quantitative genetics. In: Helentjaris T, Burr VAB (eds) Development and application of molecular markers to problems in plant genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp 81–87

Saghai Maroff MA, Van Scoyoc SW, Yu YG, Stromberg EL (1993) Gray leaf spot disease of maize: rating methodology and inbred line evaluation. Plant Dis 77:583–587

Schechert AW, Welz HG, Geiger HH (1999) QTL for resistance to *Setosphaeria turcica* in tropical African maize. Crop Sci 39:514–523

Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. Nat Biotechnol 18:233–234

Simcox KD, Bennetzen JL (1993) The use of molecular markers to study *Setosphaeria turcica* resistance in maize. Phytopathology 83:1326–1330

Szalma SJ, Buckler ES, Snook ME, McMullen MD (2005) Association analysis of candidate genes for maysin and chlorogenic acid accumulation in maize silks. Theor Appl Genet 110:1324–1333

Szalma SJ, Hostert BM, LeDeaux JR, Stuber CW, Holland JB (2007) QTL mapping with near-isogenic lines in maize. Theor Appl Genet 114:1211–1228

Tuinstra MR, Ejeta G, Goldsbrough PB (1997) Heterogenous inbred family (HIF) analysis: a method for developing near-isogenic lines that differ at quantitative trait loci. Theor Appl Genet 95:1005–1011

van Dam J, Levin I, Struik PC, Levy D (2003) Identification of epistatic interaction affecting glycoalkaloid content in tubers of tetraploid potato (*Solanum tuberosum* L.). Euphytica 134:353–360

Venard C, Vaillancourt L (2007a) Colonization of fiber cells by *Colletotrichum graminicola* in wounded maize stalks. Phytopathology 97:438–447

Venard C, Vaillancourt L (2007b) Penetration and colonization of unwounded maize tissues by the maize anthracnose pathogen *Colletotrichum graminicola* and the related nonpathogen *C. sublineolum*. Mycologia 99:368–377

Wang S, Xiao X (2002) Isolation and linkage mapping of disease-resistance-like sequences from various rice cultivars, containing different recognition specificities. Plant Breed 121:203–209

Wang G-X, Chen Y, Zhao J-R, Li L, Korban SS, Wang F-G, Li J-S, Dai J-R, Xu M-L (2007a) Mapping of defense response gene homologs and their association with resistance loci in maize. J Integr Plant Biol 49:1580–1598

Wang W, Devoto A, Turner JG, Xiao S (2007b) Expression of the membrane-associated resistance protein *RPW8* enhances basal defense against biotrophic pathogens. Mol Plant Microbe Interact 20:966–976

Weldekidan T, Hawk JA (1993) Inheritance of anthracnose stalk rot resistance in maize. Maydica 38:189–192

Welz HG, Geiger HH (2000) Genes for resistance to northern corn leaf blight in diverse maize populations. Plant Breed 119:1–14

Welz HG, Schechert AW, Geiger HH (1999a) Dynamic gene action at QTLs for resistance to *Setosphaeria turcica* in maize. Theor Appl Genet 98:1036–1045

Welz HG, Xia XC, Bassetti P, Melchinger AE, Luebberstedt T (1999b) QTLs for resistance to *Setosphaeria turcica* in an early maturing Dent × Flint maize population. Theor Appl Genet 99:649–655

Wilcoxson RD, Atif AH, Skovmand B (1974) Slow rusting of wheat varieties in the field correlated with stem rust severity on detached leaves in the greenhouse. Plant Dis Rep 58:1085–1087

Williams KJ (2003) The molecular genetics of disease resistance in barley. Aust J Agric Res 54:1065–1079

Wisser RJ, Sun Q, Hulbert SH, Kresovich S, Nelson RJ (2005) Identification and characterization of regions of the rice genome associated with broad-spectrum, quantitative disease resistance. Genetics 169:2277–2293

Wisser RJ, Balint-Kurti PJ, Nelson RJ (2006) The genetic architecture of disease resistance in maize: a synthesis of published studies. Phytopathology 96:120–129

Wisser RJ, Murray SC, Kolkman JM, Ceballos H, Nelson RJ (2008) Selection mapping of loci for quantitative disease resistance in a diverse maize population. Genetics 180:583–599

Wisser RJ, Kolkman JM, Patzoldt ME, Holland JB, Yu J, Krakowsky M, Nelson RJ, Balint-Kurti PJ (2011) Multivariate analysis of maize disease resistances suggests a pleiotropic genetic basis and implicates a *GST* gene. PNAS. doi:10.1073/pnas.1011739108

Xu S (2003) Estimating polygenic effects using markers of the entire genome. Genetics 163:789–801

Xu Y, Crouch JH (2008) Marker-assisted selection in plant breeding: from publications to practice. Crop Sci 48:391–407

Young ND (1996) QTL mapping and quantitative disease resistance in plants. Annu Rev Phytopathol 34:479–501

Yousef GG, Juvik JA (2001) Comparison of phenotypic and marker-assisted selection for quantitative traits in sweet corn. Crop Sci 41:645–655

Yu J, Holland JB, McMullen MD, Buckler ES (2008) Genetic design and statistical power of nested association mapping in maize. Genetics 178:539–551

Yun SJ, Gyenis L, Hayes PM, Matus I, Smith KP, Steffenson BJ, Muehlbauer GJ (2005) Quantitative trait loci for multiple disease resistance in wild barley. Crop Sci 45:2563–2572

Zaitlin D, DeMars SJ, Gupta M (1992) Linkage of a second gene for NCLB resistance to molecular markers in maize. Maize Genet Coop Newslett 66:69–70

Zhang LP, Khan A, Nino-Liu D, Foolad MR (2002) A molecular linkage map of tomato displaying chromosomal locations of resistance gene analogs based on a *Lycopersicon esculentum* × *Lycopersicon hirsutum* cross. Genome 45:133–146

Zuber MS, Ainsworth TC, Blanco MH, Darrah LL (1981) Effect of anthracnose leaf blight on stalk rind strength and yield in F_1 single crosses in maize. Plant Dis 65:719–722