

Analysis of quantitative disease resistance to southern leaf blight and of multiple disease resistance in maize, using near-isogenic lines

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Abstract Maize inbred lines NC292 and NC330 were derived by repeated backcrossing of an elite source of southern leaf blight (SLB) resistance (NC250P) to the SLB-susceptible line B73, with selection for SLB resistance among and within backcross families at each generation. Consequently, while B73 is very SLB susceptible, its sister lines NC292 and NC330 are both SLB resistant. Previously, we identified the 12 introgressions from NC250P that differentiate NC292 and NC330 from B73.

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The goals of this study were to determine the effects of each introgression on resistance to SLB and to two other foliar fungal diseases of maize, northern leaf blight and gray leaf spot. This was achieved by generating and testing a set of near isogenic lines carry single or combinations of just two or three introgressions in a B73 background. Introgressions 3B, 6A, and 9B (bins 3.03–3.04, 6.01, and 9.02–9.03) all conferred significant levels of SLB resistance in the field. Introgression 6A was the only introgression that had a significant effect on juvenile plant resistance to SLB. Introgressions 6A and 9B conferred resistance to multiple diseases.

Introduction

Most disease resistance used in commercially grown maize (*Zea mays* L. ssp. *mays*) is quantitative rather than

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qualitative in nature. Qualitative disease resistance is usually controlled by one or a few genes with major effects (Bent and Mackey 2007) while quantitative disease resistance (QDR) is generally controlled by many genes that tend to have minor effects. Many qualitative resistance genes have been cloned and characterized and their mechanisms of action are reasonably well understood (Bent and Mackey 2007). Fewer genes for QDR have been identified (Kliebenstein and Rowe 2009) and correspondingly less is understood about the physiological or molecular genetic basis of QDR. A standard way to study genes or loci with quantitative effects is to construct near-isogenic lines (NILs) differing only for alleles of the gene or locus in question, which eliminates the effect of a segregating genetic background (Szalma et al. 2007). Any consistently detectable phenotypic differences between the NILs should then primarily be due to allelic differences at the locus being investigated.

Few, if any, crops are affected by only a single disease in a given agricultural region. The introgression of many separate resistance genes or quantitative trait loci (QTL) to develop lines resistant to all of the pathogens threatening the yield of a crop can be a lengthy, expensive process. Furthermore, some studies suggest that some resistance genes may confer a decrease in fitness in the absence of the disease to which they confer resistance (e.g., Brown 2003; Mitchell-Olds and Bradley 1996; Tian et al. 2003). A means to improve breeding efficiency would be to use single genes or loci conferring resistance to multiple pathogens, i.e., multiple disease resistance (MDR) genes or loci. There is some evidence for the existence of MDR genes/loci in the literature, including correlations between resistance ratings for multiple diseases across the lines of large populations (Mitchell-Olds et al. 1995) and co-localization of resistance genes and QTL for different diseases (Wisser et al. 2006; Wisser et al. 2005). There are several examples of naturally occurring MDR genes. For example, the qualitative resistance gene *Mi-1* confers resistance to both aphids and nematodes in tomato (Vos et al. 1998). The recently cloned quantitative but large-effect resistance gene *Lr34/Yr18* confers resistance to leaf

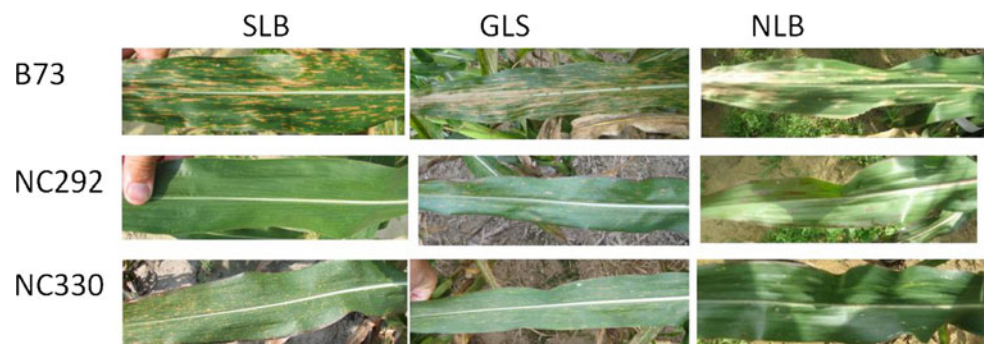
rust, stripe rust, stem rust, powdery mildew and various other diseases of wheat (Krattinger et al. 2009). Other genes conferring MDR in wheat include *Lr46/Yr29* (Rosewarne et al. 2008) and *Yr30* (Bariana et al. 2007; William et al. 2007).

Southern leaf blight (SLB), causal agent *Cochliobolus heterostrophus* (Drechs.) Drechs. [anamorph = *Bipolaris maydis* (Nisikado) Shoemaker], is a widespread disease with the potential to cause significant yield losses in hot, humid tropical and sub-tropical regions, such as the southeastern USA, parts of India, Africa, Latin America and Southern Europe. Prior to 1970 SLB had received little attention. However, in 1970 there was an SLB epidemic caused by *C. heterostrophus* race T on hybrids carrying Texas male-sterile cytoplasm (cms-T), which was widely used at the time in hybrid seed production (Ullstrup 1972). An estimated 15% drop in total maize production, at a loss of one billion dollars, was attributed to the SLB epidemic caused by race T. After the 1970 epidemic, cms-T maize was replaced by male-fertile, race T-resistant normal cytoplasm maize.

Currently, race O is the predominant cause of SLB in the United States (White 1999). Resistance to *C. heterostrophus* race O is quantitatively inherited with primarily additive or partially dominant gene action (Holley and Goodman 1989; Lim and Hooker 1976). Under experimental conditions, yield losses as high as 46% have been observed in maize inoculated with *C. heterostrophus* race O (Byrnes and Pataky 1989; Fisher et al. 1976). However, losses of this magnitude in commercial production are rare.

Previously (Zwonitzer et al. 2009), we performed QTL mapping in an F_{2:3} population derived from a cross between the highly SLB-resistant line NC250A and the commonly used, SLB susceptible, maize line B73. We then examined two closely related sister lines, NC292 and NC330, which had been developed by backcrossing a close progenitor of NC250A (termed NC250P) with B73 for 3 and 4 generations respectively, followed by several generations of selfing. During this process, selection for SLB resistance was carried out in every generation. The resulting lines, NC292 and NC330, are B73 sister lines

Fig. 1 Different levels of SLB, GLS, NLB resistance in B73, NC292, and NC330. Photographs were taken from diseased ear leaves of plants grown in neighboring rows in the field



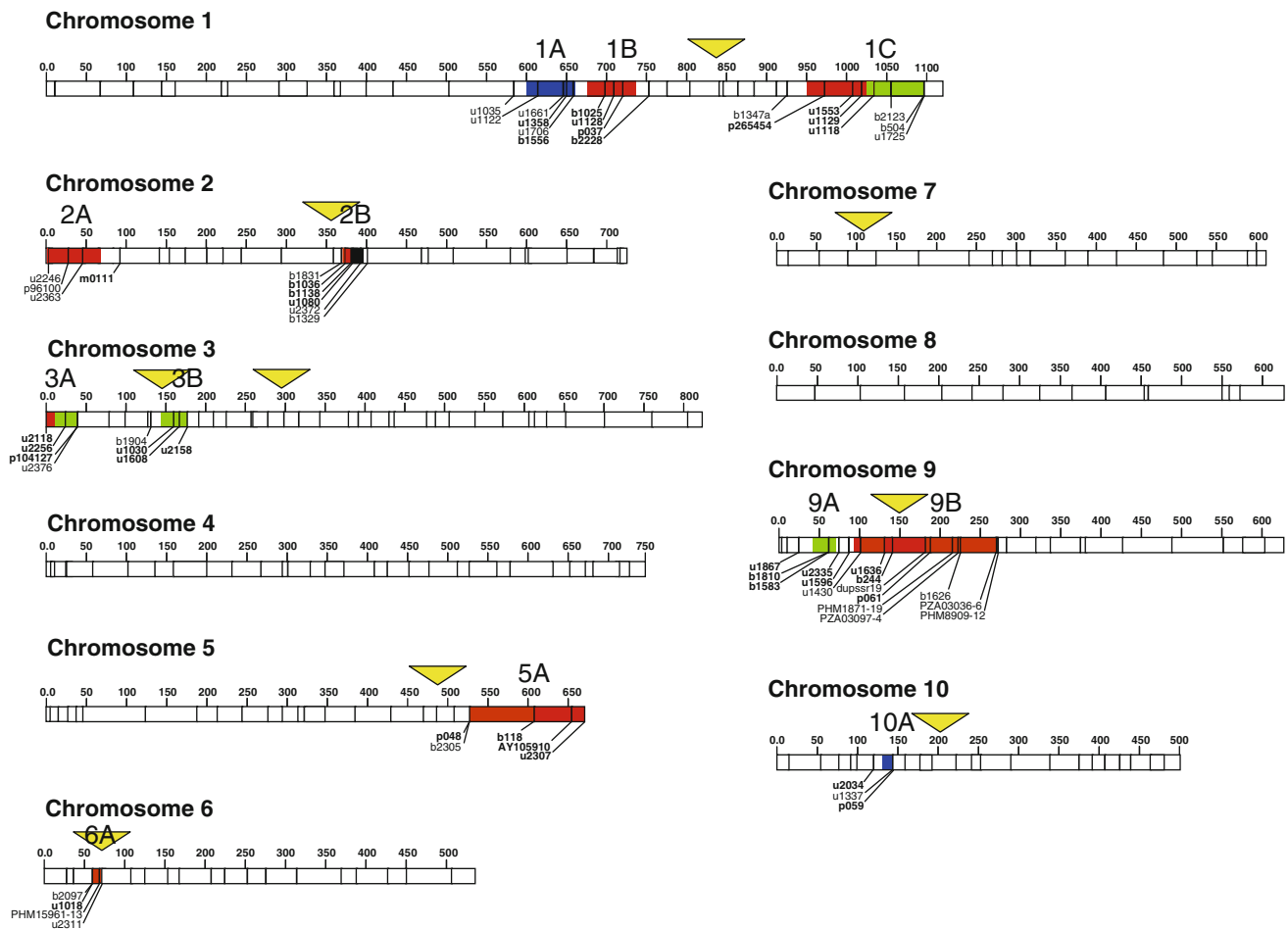


Fig. 2 Map of the 10 maize chromosomes showing NC250P introgressions and B73 background in the genomes of NC292 and NC330, adapted from Zwonitzer et al. (2009). The vertical lines on each chromosome represent marker positions. Numbers above the chromosomes indicate map positions according to the IBM2 2005 neighbors map (<http://www.maizegdb.org>). Marker names are only given for those within and flanking the introgressions. The white rectangles represent B73 background. Red rectangles show genomic

regions with NC250P introgressions in both NC292 and NC330. Green rectangles represent NC250P introgressions present only in NC292. The blue rectangles show NC250P introgressions that are present only in NC330. The yellow triangles above the chromosomes represent regions where QTL for resistance to SLB were detected in two F_{2:3} populations (B73rhm1 × NC250A and NC250A × B73). The NC250P introgressions were each given a number-letter identification code, which are indicated in the figure (color figure online)

which share ~90% of their genome with B73 but exhibit a high level of SLB resistance (Fig. 1). We identified the 12 genomic regions for which B73 differed from NC292 and NC330—eight of them common to both lines. Several of these regions colocalized with SLB resistance QTL identified from the NC250A × B73 F_{2:3} population (Fig. 2). NC292 and NC330 also show increased resistance to other diseases compared to B73 (Fig. 1). These include northern leaf blight (NLB), caused by *Setosphaeria turcica* (Luttrell) K.J. Leonard and E.G. Suggs (anamorph *Exserohilum turcicum* (Passerini) Leonard and Suggs), and gray leaf spot (GLS), caused by *Cercospora zea-maydis* (Tehon and E.Y. Daniels). SLB, GLS and NLB are all foliar, substantially necrotrophic, fungal pathogens of maize. All three pathogens are ascomycete fungi in the class Dothideomycetes and share some similar pathogenesis

characteristics (Beckman and Payne 1982; Jennings and Ullstrup 1957). For all these diseases, infection is initiated when spores land on the leaf surface, germinate, and penetrate either directly through the stomata or the leaf cuticle and epidermis. *S. turcica* grows intracellularly in the leaf while *C. heterostrophus* and *C. zea-maydis* grow intercellularly during initial infection. The latent periods (period of time from infection to sporulation) for the three fungi vary from a few days for *C. heterostrophus*, to approximately 2 weeks for *S. turcica*, (Carson 1995) and up to 3 weeks for *C. zea-maydis* (Beckman and Payne 1982). It seems likely that loci associated with regulating aspects of the parts of the pathogenesis process shared by two or more of these pathogens may be detected as MDR QTL. We have previously examined this hypothesis with equivocal results. While significant correlations between resistance to these three diseases were

observed in two independent RIL populations, very few colocalizing QTL for resistance to different disease were observed (Balint-Kurti et al. 2010; Zwonitzer et al. 2010). We have also found good evidence for the presence of MDR genes in the maize association mapping population (Wisser et al. 2011). Our present hypothesis is that genes and loci conferring MDR do exist but that most of them have relatively small effects, and are not therefore detected by QTL analysis while the larger effect QTL that are detected are disease-specific.

The current study was undertaken to generate and evaluate NILs with different subsets of the NC250P introgressions found in NC292 and NC330 to verify previously identified SLB QTL and to determine directly the effects of specific introgressions on SLB, GLS and NLB resistance.

Materials and methods

Plant materials

B73 was developed at Iowa State University out of Cycle 5 of the *Iowa Stiff Stalk Synthetic* (BSSS: Russell 1972).

NC292, a yellow dent maize with SLB tolerance, was developed by crossing B73 and NC250P (Zwonitzer et al. 2009). The progeny were backcrossed to B73 three times. NC292 was subsequently derived by ear to row selfing. During the development of NC292, progeny were screened for SLB resistance among and within backcross families at each generation. The most resistant lines were used in the next cycle of backcrossing. The resulting NC292 inbred line exhibits a high level of resistance to SLB, has good standability, and reaches maturity 1–3 days earlier than the susceptible parent, B73.

NC330 descended from the same BC₃F₁ family as NC292. However, NC330 was backcrossed to B73 for one additional cycle and was again selected for SLB resistance. Although NC330 has good standability, it is slightly less SLB resistant than NC292.

Near-isogenic lines for this research were developed by crossing NC292 × B73 and NC330 × B73. The resulting F₁ progeny from each cross were backcrossed to B73 and then selfed for two generations to produce BC₁F₃ progeny. Leaf tissue was taken from individual BC₁F₁ plants and was genotyped to select families containing single or differing multiple NC250P introgressions. Genotyping was performed on 2,246 BC₁F₂ individuals to select for lines homozygous for specific NC250P introgression(s).

Field trials

All field trials were planted as augmented alpha lattices designed using the software AlphaGen (Scottish

Agricultural Services, Edinburgh, UK). Each complete replication for the SLB, NLB and GLS trials consisted of 17 incomplete blocks with 15 entries in each block (234 NILs used total), augmented with one plot per block of NC292 and one plot per block of B73. A complete list of all the lines used in each trial is shown in Table S1. The SLB trial was planted over two summer field seasons (2007 and 2008). In 2007, two replications of the experimental design were planted at each of two SLB locations; Clayton NC, and Tifton GA. One replication was planted in 2008, at the Clayton, NC location. These environments are here referred to as CL07, GA07, and CL08. The NLB trials included two replications at each of two locations in 2008: Clayton, NC and Aurora, NY (referred to as CL08 and NY08). The GLS trials each included two replications and were conducted in Andrews, NC in 2008 and 2010 (referred to as AND08 and AND10).

Plots in the SLB and NLB trials were planted as single rows 2 m long with 0.97 m between rows and 0.6 m alleys between ranges. GLS plots were planted to the same specifications as those in the other trials, but with 4 m plot lengths. The rows were not thinned. Ten seeds were planted in each row for the SLB and NLB trials. Fifteen were planted per row for the GLS trial.

Fungal growth and inoculation of field trials

Inoculum for the field SLB disease screening experiments was prepared as previously described by Carson et al. (2004). Rows were inoculated at the four- to six-leaf stage by placing approximately 20 grains of *C. heterostrophus* race O, isolate 2–16Bm sorghum grain culture in the leaf whorl (Carson 1998; Carson et al. 2004). Immediately after inoculation, overhead irrigation was applied to the field to provide free moisture to initiate fungal growth.

NLB inoculation in Clayton NC in 2008 was conducted using infected sorghum grain, prepared in a similar manner to the SLB inoculum (Carson et al. 2004). The NLB inoculum contained a mixture of isolates with various race specificities (*S. turcica* race 0, race 1, race 23, and race 23N). Plants were inoculated at the four- to six-leaf stage by placing approximately 20 grains of sorghum carrying NLB inoculum in the leaf whorl. The Aurora, NY NLB plots were inoculated with *E. turcicum* race 1. The NLB inocula used in Aurora included both sorghum grains and a spore suspension; these were prepared as described previously (Chung et al. 2010) and applied to the leaf whorl as in Clayton NC.

Rating of field trials

Days to anthesis (DTA), the number of days post-planting at which at least half the plants in a plot were shedding pollen

was rated for all of the field plots. The SLB and GLS trials were rated on a 1–9 scale with 1 being the most resistant and 9 being dead (Balint-Kurti et al. 2007). All scores were based on whole-plot averages. For SLB, plots were rated between 3 and 5 times at 10–14-day intervals starting around the time of anthesis. For GLS, plots were rated 2–3 times at 10–14-day intervals starting about 10 days after anthesis.

NLB was rated differently to GLS and SLB incubation period (IP), defined as the number of days between inoculation and the appearance of necrotic spots within lesions on at least half the plants in a plot, was assessed for all the NLB trials (but not for the SLB or GLS trials). The NLB trials were also rated for diseased leaf area (DLA), the estimated percentage of necrotic area out of the total leaf area of a plant, on five occasions at approximately 10-day intervals in Clayton, NC and three occasions at approximately 2-week intervals in Aurora, NY.

Screening juvenile plants for SLB resistance in growth chambers

A set of NILs and parental controls (see Table S1) were evaluated for juvenile plant SLB resistance in a growth chamber (GC) study. Two trials designated as T1 and T2 were conducted approximately 1 month apart in the fall of 2007 in the North Carolina State University Phytotron.

A $3 \times 3 \times 2.13$ m GC was used with a day length of 14 h per 24-h cycle (7a.m.–9p.m.) for the duration of both trials. Light intensity was constant during the daylight period, and plants were subjected to an irradiance of approximately 2.0 kilowatts per square meter (kW/m^2) from the fluorescent lamps and 0.8 kW/m^2 from the incandescent lamps.

Plants were grown in 600 ml Styrofoam cups in a 1:2 peat-lite and gravel mixture and watered overhead with a nutrient solution twice daily. Experimental units were a single cup containing one plant. Both trials were planted as an augmented incomplete block design with two replications per trial. This design was implemented to account for space availability, environmental variance, and inoculation variance within the GC. The susceptible parent, B73, was included six times per incomplete block and the resistant parents, NC292 and NC330, were represented once per incomplete block. A total of 42 different NILs were included in these trials (see Table S1).

During T1, the GC temperature was held at 22°C from planting until day 14, was then reduced to 19°C from days 14 to 20 and increased to 25°C upon inoculation on day 20, which is a more favorable temperature for fungal growth. The temperature was held constant at 25°C for the duration of T2.

Inoculation was carried out 20 and 14 days after planting for T1 and T2, respectively. The plants were spray-inoculated at the three- to four-leaf stage following a method based

on previous publications (Balint-Kurti and Carson 2006; Zhu et al. 1998). The inoculum consisted of spores suspended in a chilled solution of 0.05% agar and a 0.05% Tween 20 with a concentration of 5.4×10^3 spores/ml for T1. Due to the quick progression of infection in T1, the inoculum concentration was lowered to 1×10^3 spores/ml for T2. The third and fourth leaves were sprayed until runoff using a Nalgene hand-pump bottle containing the spore solution. The plants were allowed to dry for 30 min and then placed in clear plastic bags for approximately 16-h overnight, after which the bags were removed.

During the T1 trial, plants were rated daily from day 2 until day 5 after inoculation by visually estimating percentage of necrotic leaf area on a scale of 0–100%. Two days after inoculation both inoculated leaves were used to rate the percentage of necrotic leaf area. Subsequent ratings were only determined for the upper inoculated leaf due to the fact that the lower inoculated leaf had senesced too much to obtain accurate estimates of the percentage of necrotic leaf area. Trial T2 plants were rated daily from day 3 to day 7 after inoculation using the same procedure described previously. Ratings taken on the third and fourth days were collected on both inoculated leaves, while subsequent ratings were only recorded from the top inoculated leaf.

DNA extraction and molecular markers

For simple sequence repeat (SSR) analyses, DNA was extracted and PCR was performed as previously described (Zwonitzer et al. 2009).

Statistical analyses: field trials

Disease severity was calculated as weighted mean disease (WMD) for all plots in the SLB, NLB, and GLS trials as described previously (Balint-Kurti et al. 2006) and used as a dependent variable in the analyses of introgression effect estimates for these diseases. The data for each disease were modeled separately in SAS PROC MIXED (v.9.1; SAS Institute, Cary, NC). NIL least-squares means were calculated using SAS PROC MIXED. Mixed linear model fixed effects included DTA (SLB and NLB only) and genotype. Random effects included location, replication, incomplete block, and year (SLB and GLS only). Final models were obtained using forward selection via either Type III Tests for fixed effects or restricted likelihood ratio tests for random effects ($\alpha = 0.01$). Pearson correlation coefficients were obtained using PROC CORR.

Determination of introgression effect

The effect of an introgression was defined as the mean difference in disease expected with lines homozygous for

NC250P alleles in that genomic region relative to lines homozygous for B73 alleles. Two methods were used to calculate the effects of introgressions which we term here as the “Empirical” and the “Minimal-model” estimates.

Empirical model estimates

The empirical effect of each introgression was obtained by either (a) averaging the ratings (WMD or IP) for all single-introgression lines and subtracting the average rating of B73 or (b) for the introgression lines where single-introgression lines were not available, averaging over single-introgressions the differences of means between each class of double-introgression lines (introgression of interest and a secondary introgression) and the single-introgression lines of the secondary introgression in said class. ESTIMATE statements in PROC MIXED were used to obtain the “empirical” introgression effect estimates.

were not available for all the NILs; so including more than four or five of the 12 introgressions in a model might reduce the useful dataset significantly. Therefore, the minimal model used to estimate the disease resistance effect of a given introgression included no more than the minimal number of introgressions needed to obtain an accurate estimate of that effect. Table S2 shows the number of observations used in each minimal model.

Statistical analysis: growth chamber trials

To perform a combined analysis in GC trials T1 and T2, ratings from each experiment were adjusted separately based on the average response of the checks B73, NC292, and NC330 in each trial. An average disease score ratio was calculated as the average disease score for each NIL divided by the mean response for all checks in the particular trial. Trial, entry, and entry by trial were considered

$$\text{Empirical_Single-introgression_estimate} = \text{Mean_Rating_single_Introgression_NILs} - \text{Mean_Rating_B73}$$

$$\begin{aligned} \text{Empirical_NonSingle-introgression_estimate} \\ = \text{Average}_{\text{single-introgressions}} \left(\text{Mean_Rating_double-Introgression_NILs} - \text{Mean_Rating_Secondary_single-Introgression_NILs} \right) \end{aligned}$$

Minimal-model estimates

Minimal-model estimates for each disease were obtained using a separate linear model for each introgression in SAS PROC MIXED (v.9.1; SAS Institute, Cary, NC). For this purpose, a class variable was created for each introgression, with three class levels: NC250P allele-homozygous, heterozygous, or B73 allele-homozygous. Regressors in the minimal model for a particular introgression were one or more introgression class variables and all non-genotype factors/effects (DTA, year, location, etc.) included in the main model for genotype LSMEANS estimation. Inclusion of other introgression regressors than the one of interest would enable the model to account for the resistance or susceptibility conferred by those other introgressions in the NIL population.

As a first step in the minimal-model construction for a particular introgression, disease rating for NILs associated to this introgression was fitted in a model that includes genotype as regressor. Any genotype with an estimated contribution greater than 20% relative to B73 was deemed meaningful and included in the minimal set, participating in the posterior minimal modeling. Complete marker data

fixed-effect factors in calculating the GC average disease score ratio. Replication within trial and entry by replication within trial were considered as random effects. The empirical model approach (see above) was used to estimate introgression effects.

Results

Zwonitzer et al. (2009) reported eight shared NC250P introgressions in NC292 and NC330. NC292 and NC330 also had two introgressions that were unique to each line for a total of 12 introgressions. For the purposes of the present study, each introgression was given a unique identifier as shown in Fig. 2. The borders of the introgressions were estimated to be at a point half the distance between the outermost NC250P allelic marker of an NC250P introgression and the nearest B73 allelic marker.

Creation of NILs

Of the 2,246 BC₁F₂ lines screened, 21 lines containing single NC250P introgressions were identified. Lines

containing single NC250P introgressions were identified for introgressions 1C, 2A, 2B, 3A, 3B, 9A, and 10A. Single-introgression lines carrying 1A, 1B, 5A, 6A, and 9B were not identified, but these introgressions were present in multiple double- and triple-introgression lines and in every case, several NIL pairs that differed only for these specific introgressions were identified (e.g., there was a line with the 3B, 2B, and 6A introgressions and a line with just the 3B and 2B introgressions—these lines differed just for the 6A introgression), therefore, the effects of these introgressions could be calculated (see “Materials and methods”). Table S1 shows a complete list of the lines used in these experiments and the introgressions they carried.

Growth chamber experiments: juvenile plant screening for SLB resistance

A combined analysis for both trials was performed (Table 1). The fixed-effect factors in the GC trials T1 and T2 were trial, entry, and entry by trial. The random effects were replication within trial and entry by replication within trial. Incomplete block within replication and trial were dropped from the model, since the variance component estimates were zero. The trial and entry by trial effects were not significant for the GC disease score ratio. Entry, replication within trial, and entry by replication within trial effects were highly significant. The 42 lines screened in the GC trials carried 30 unique introgression(s) or introgression combinations (Table S1).

Figure 3 shows the estimated effects (by the empirical model—see “Materials and methods”) of each of the

Table 1 *F*-test of the fixed-effect, and the variance component estimates and standard errors (SE) of the random effects in mixed-models analysis of juvenile plant resistance to SLB of 42 B73 NIL and checks (B73, NC292 and NC330)

GC Combined		
Fixed factor	<i>F</i> value	<i>p</i> value
Trial	0.19	0.71
Ent	0.04	<0.0001
Ent × trial	1.00	0.50
Random factor	Variance component estimate (SE)	<i>p</i> value
Rep (Trial)	0.01 (0.02)	<0.0001
Ent × Rep(Trial)	0.03 (0.01)	<0.0001
Within checks ^a	0.02 (0.003)	

Analysis was performed using an average disease score ratio (average SLB disease score for each NIL divided by the mean for all checks) for two growth chamber trials (GC Combined)

Ent Entry, *Rep* Replication

^a B73 was included 12 times per trial and NC292 and NC330 were included two times per trial

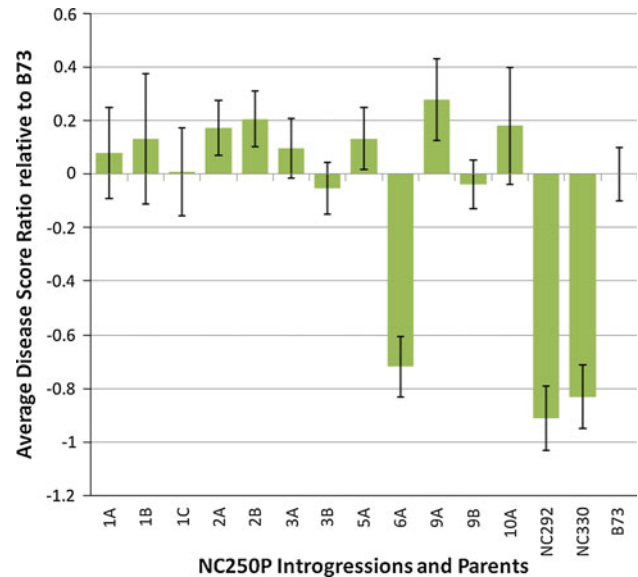


Fig. 3 Estimated effects on SLB resistance of each of the NC250P introgressions in a B73 background when assayed in juvenile plants in the growth chamber. Parental phenotypes (NC292, NC330 and B73) are also shown. B73 is represented as the baseline (value of zero). Effects are expressed as an average disease score ratio which was calculated as the average disease score for each near-isogenic line divided by the mean response for all checks (B73, NC292, and NC330) in a particular trial (see “Materials and methods”). Thus, a negative value indicates that the introgression confers resistance and a positive value that confers susceptibility. Standard errors are indicated for each estimate

introgressions in the growth chamber assay expressed as average disease score ratios using B73 as a baseline. It is clear that most of the juvenile resistance observed in the parental lines NC292 and NC330 was derived from the 6A introgression. None of the other introgressions had a comparable effect on resistance, in fact all of the introgressions with the exception of 3B, 6A and 9B had disease score ratios higher than B73, suggesting that they conferred a level of disease susceptibility (Fig. 3). However, none of these disease score ratios were significantly greater than B73 ($\alpha = 0.05$). It is noteworthy that the three introgressions with disease score ratios lower than B73 were the same introgressions that conferred significant SLB resistance effects in the field trials (see below).

Field experiments

Mixed-model analysis was carried out of the field trial data for all three diseases. Incomplete block was included as a random effect in the final model for all diseases. Location was included as a random effect in the SLB and NLB final models, and year and repetition were included as an additional random effect in the SLB and GLS models, respectively. Entry (genotype) was significant for all

Table 2 *F*-test of the fixed-effect, and the variance component estimates and standard errors (SE) of the random effects in mixed-models analysis of adult plant field resistance to SLB, GLS and NLB (using both WMD and IP to measure disease)

SLB		
Fixed factor	<i>F</i> value	<i>p</i> value
Ent	20.33	<0.0001
DTA	10.5	<0.0001
Random factor	Variance component estimate (SE)	
Location	0.62 (0.88)	
Year	0.64 (0.90)	
Incomplete block	0.02 (0.01)	
NLB (WMD)		
Fixed factor	<i>F</i> value	<i>p</i> value
Ent	5.14	<0.0001
DTA	2.49	0.002
Random factor	Variance component estimate (SE)	
Location	156.0 (221.0)	
Incomplete block	10.3 (2.1)	
NLB (IP)		
Fixed factor	<i>F</i> value	<i>p</i> value
Ent	2.76	<0.0001
DTA	4.48	<0.0001
Random factor	Variance component estimate (SE)	
Location	4.53 (6.43)	
Incomplete block	0.27 (0.11)	
GLS		
Fixed factor	<i>F</i> value	<i>p</i> value
Ent	7.81	<0.0001
Random factor	Variance component estimate (SE)	
Repetition	0.16 (0.13)	
Incomplete block	0.014 (0.0046)	

Random effects were selected for entry into each model based on restricted likelihood ratio tests ($\alpha = 0.05$)

Ent Entry

DTA Days to anthesis, a measurement representing number of days from planting until pollen shed

diseases ($p < 0.0001$, see Table 2). DTA was significant for SLB and both NLB traits.

NIL least-squares means for disease severity were significantly correlated between the three field environments for all three diseases (Pearson Correlation Coefficients 0.56–0.74 for SLB, 0.65 for NLB-WMD, 0.69 for GLS,

Table 3 Pearson correlation coefficients between NIL least-squares means estimates for each disease rating; SLB weighted mean disease (SLB-WMD), Northern leaf blight weighted mean disease (NLB-WMD), Northern leaf blight incubation period (NLB-IP) and GLS weighted mean disease (GLS-WMD)

	NLB-WMD	NLB-IP	GLS-WMD
SLB-WMD	0.23*	−0.10	0.09
NLB-WMD		−0.44**	0.38**
NLB-IP			−0.30*

* $p < 0.001$

** $p < 0.0001$

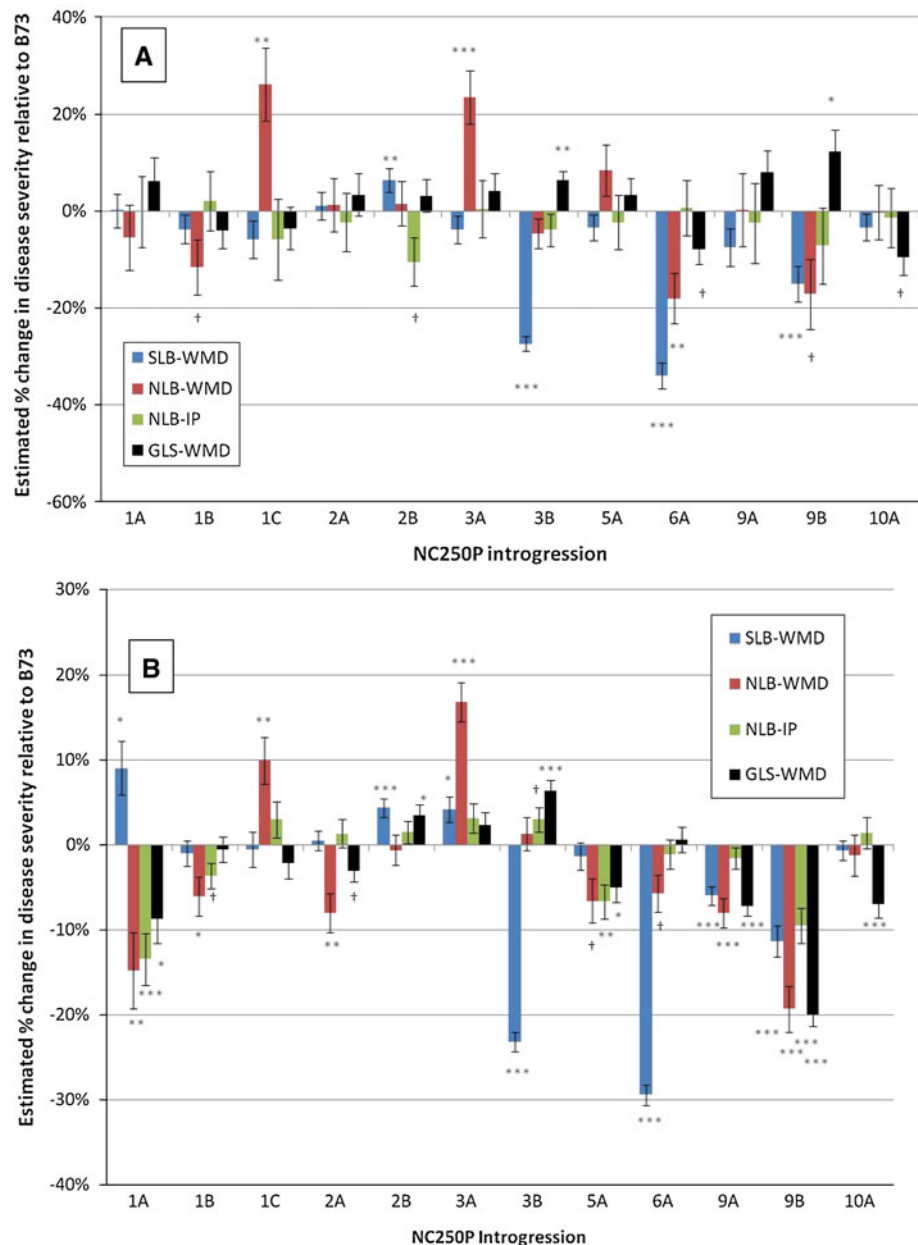
$p < 0.0001$ in all cases). Pearson correlation coefficients between NIL least-squares means for all four disease ratings, SLB-WMD, NLB-WMD, NLB-IP and GLS-WMD are shown in Table 3. GLS-WMD was significantly correlated with both NLB measurements. SLB-WMD was moderately correlated with only NLB-WMD but not with the other traits. It should be noted that NLB-IP is rated on an opposite scale to the other traits, i.e. a higher number for NLB-IP denotes a higher level of resistance, while the opposite is true of the other traits. This explains why the correlations between NLB-IP and the other traits are negative.

Field experiments: introgression effect estimates

The effects calculated by the empirical method were treated as the primary estimates because using the direct comparison of single- and double-introgression lines to estimate the effects of each introgression was considered as a more direct (and therefore more accurate) approach. The empirical estimates for the 12 NC250P introgressions were calculated using a maximum combined total of 32 single- and double-introgression NILs per introgression and often this number was substantially smaller (see Table S1 for details). Empirical estimates were sometimes therefore derived from only a few direct comparisons between lines. This meant that individual observations might have large effects on the estimates made. The minimal-model estimates were calculated as a check in case some of the empirical model effects might have had been artificially skewed because of the small number of observations and comparisons used in their calculation. During the forward selection process for each minimal model, adding additional introgressions (selected from introgressions whose empirical estimates were significant) reduced the full dataset by at least 30% in every case, i.e. to fewer than 163 NILs). This was because lines without complete marker data for the introgressions included in the model were excluded from the analysis.

The results of the empirical and minimal-model analyses are shown in Fig. 4. Overall, the two model estimates gave

Fig. 4 “Empirical” (a) and “Minimal Model” (b) estimates of introgression effects against all seven disease ratings investigated for the 12 NC250P introgressions that differentiate the NILs. The introgression effects for each disease were normalized as the percentage increase or decrease in disease that each introgression conferred relative to the least-squares mean disease rating of the susceptible parent line B73 for four foliar disease ratings: SLB-WMD, NLB-WMD, NLB-IP, and GLS-WMD. Bars below the 0% line indicate that the introgression conferred a level of resistance relative to B73 and vice versa. † $p < 0.05$, * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$



broadly similar results. For SLB, introgressions 3B and 6A provided high levels of resistance and were highly significant by both analysis methods (Fig. 4). Introgression 9B also provided significant levels of SLB resistance and introgression 2B conferred modest but significant levels of SLB susceptibility by both analysis methods.

For NLB-WMD, introgressions 1C and 3A provided significant levels of susceptibility and introgression 9B significant levels of resistance according to both analysis methods. For GLS, introgressions 3B and 10A conferred significant levels of susceptibility and resistance, respectively, by both analysis methods (Fig. 4).

Of the 12 NC250P introgressions present in the NILs, only two conferred statistically significant levels of

resistance to more than one disease by the empirical estimation method (Fig. 4a). Introgression 6A conferred resistance to SLB, NLB and GLS and introgression 9B conferred resistance to SLB and NLB. However, it should be noted that in the case of the GLS resistance conferred by 6A, the minimal model did not detect this effect. Using the minimal-model analysis, six introgressions were identified as conferring statistically significant levels of resistance to more than one disease; 1A, 2A and 5A to NLB and GLS, 6A to SLB and NLB and 9A and 9B to SLB, NLB and GLS. Both models suggest therefore that 6A and 9B confer MDR.

Figure 5 shows the actual resistance levels of the NC292 and NC330 compared to their expected levels

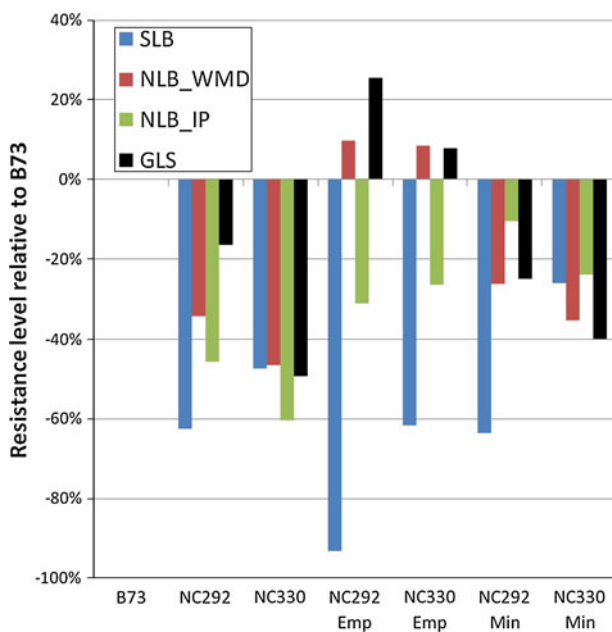


Fig. 5 The experimentally observed levels of resistance in B73, NC292 and NC330 to SLB, NLB-WMD, NLB-IP and GLS of compared with the sum of the effects of all the introgressions carried by NC292 and NC330 as calculated by the empirical model (“Emp”) and the minimal model (“Min”). The introgression effects for each disease were normalized as the percentage increase or decrease that each introgression conferred relative to the least-squares mean disease rating of the susceptible parent line B73. The vertical axis applies to estimates for effects against the four foliar disease ratings (SLB, NLB-WMD, NLB-IP, and GLS)

based on the sum of all the calculated effects of all the introgressions they carry. For SLB the expected levels of resistance are fairly close to the observed, but for both GLS and NLB the expected levels of resistance, particularly as calculated by the empirical model, appear to be substantial underestimates.

Discussion

Previously (Zwonitzer et al. 2009), we had identified QTL for SLB resistance in a population derived from a cross between the SLB susceptible line B73 and the resistant line NC250A. We identified several QTL, including, in order of decreasing R^2 value, QTL in bins 6.01, 9.02, 1.09, and 3.03 and 2.05/06. We then examined two closely related sister lines NC292 and NC330 which had been developed by backcrossing a direct progenitor of NC250A (termed NC250P) with B73 for three and four generations, respectively, followed by several generations of selfing. During this process, selection for SLB resistance was carried out in every generation. The resulting lines, NC292 and NC330 are therefore B73 sister lines that exhibit a high level of SLB resistance (Fig. 1). We identified the 12

genomic regions where B73 differed from NC292 and NC330. These were the regions where NC250P introgressions had been retained in the NC292 and NC330 genomes, either because of selection or by chance.

As expected, several of these introgressions colocalized with SLB resistance QTL identified from the NC250A × B73 population (Fig. 2). The presumption for these was that they had likely been retained because of selection rather than by chance. All of the strongest SLB resistance, QTL identified in the NC250A × B73 population had been mapped at or near to introgressions found in NC292 and NC330. The QTL in bins 6.01 and 9.02 co-localized precisely with introgressions that had been retained in both NC292 and NC330 (introgressions 6A and 9B). The QTL in bin 3.03 colocalized precisely with introgression 3B which was found in NC292 but not in NC330. The 2.05/6 QTL mapped about 20 Imu (IBM map units, sensu Balint-Kurti et al. 2007) from introgression 2B [4 Imu is approximately equal to 1 centiMorgan (Lee et al. 2002)]. The QTL in bin 1.09 mapped equidistant between introgressions 1B and 1C, about 120 Imu (~30 cM) from each. We previously speculated that the QTL identified in bin 1.09 represented the combined resistance effects of introgressions 1B and 1C and that the mapping population was too small to separate their effects.

In this paper, we performed experiments to directly validate the effects of all the introgressions. This was achieved by creating a set of B73 NILs carrying single and various combinations of 2 and 3 introgressions, such that by comparing the resistance levels of lines differing only for single introgressions, their effects could be directly calculated. Effects calculated in this way were termed the ‘empirical’ estimates. This was the primary effect estimation method. Another estimation method, termed as the “minimal-model” method was also used to estimate the effects of the introgressions. This was because the empirical method used a relatively small number of comparisons and observations to calculate effects and thus was susceptible to being skewed by one or two anomalous observations. The minimal-model method used many more comparisons/observations to calculate each estimate, but was a more indirect method of estimating the introgressions effect and was itself susceptible to being skewed by the non-random distribution of other introgressions within the population. The estimates derived from both methods were relatively consistent (compare Fig. 4a, b). Of the 14 effects that were significant at $p < 0.05$ by the empirical model, 11 were also significant by the minimal model while three—the GLS resistance conferred by introgression 6A, the GLS susceptibility conferred by introgression 9B and the NLB-IP resistance conferred by introgression 2B—were not significant in the same direction by the minimal-model method. In this discussion, we will largely confine

ourselves to discussion of the 11 effects that were confirmed by both estimation methods.

Only the three introgressions which precisely colocalized with SLB resistance QTL, introgressions 3B, 6A and 9B, conferred significant levels of SLB resistance by both estimation methods. Somewhat surprisingly, introgressions 1B, 1C and 2B did not provide significant levels of SLB resistance despite *almost* colocalizing with the previously detected SLB resistance QTL. It seems therefore that the extremely high SLB resistance exhibited by NC292 is based on only three introgressions from NC250P and the somewhat lower (but still rather high) SLB resistance exhibited by NC330 is based on only two NC250P introgressions (NC330 does not carry the 3B introgression). It also appears that the other introgressions that did not confer significant levels of SLB resistance may have been retained in the NC292/NC330 genomes simply by chance rather than due to selection. It is also possible that the modest, though not statistically significant levels of SLB resistance conferred by some of the introgressions (1B, 1C, 3A, 5A, 9A, 10A—Fig. 4a) may have been sufficient to allow them to be selected.

Another possibility is that there were epistatic interactions present in NC250P that were broken up during the development of the NILs, so that while certain loci were selected together during the development of NC330 and NC292, their effects were not detectable in isolation from one another in the NILs. Minor epistatic interactions were detected in the previous study (Zwonitzer et al. 2009). The SLB resistance QTL in bin 10.03 which is close to introgression 10A was shown to have an interaction effect with the QTL in bin 9.02 which colocalizes with the 9B introgression. When the calculated effects of all the introgressions present in NC292 and NC330 were summed, the “expected” levels of SLB resistance were fairly close to the observed levels (Fig. 5) again arguing against a major role for epistasis in SLB resistance in these lines. A lack of epistasis between SLB resistance QTL has commonly been observed, most recently in a study of the 5000-line NAM population (Kump et al. 2011).

The average effect of the 6A introgression in the SLB GC trials was highly significant. Thus, 6A conferred high levels of SLB resistance in both juvenile and adult plants. The 6A introgression is located in approximately the same genomic regions as the *rhml* gene, which has been reported to confer juvenile resistance to SLB (Thompson and Bergquist 1984; Zaitlin et al. 1993). In a B73 × Mo17 RIL population, Balint-Kurti and Carson (2006) identified a Mo17-derived SLB QTL in bin 6.00 (near the location of 6A) for juvenile but not adult plant resistance. The other two introgressions that conferred significant levels of adult plant SLB resistance, 3B and 9B, did not confer statistically significant levels of SLB resistance at the juvenile stage although it is noteworthy that of all the other

introgressions, these were the two that conferred the highest level of SLB resistance in juvenile plants. So it is likely that there were conferring some level of juvenile plant resistance but that this effect did not rise to the level of significance in these studies.

NC292 and NC330 are substantially more resistant than B73 to two other foliar diseases; NLB and GLS (Figs. 1, 5), suggesting the hypothesis that some of the introgressions confer MDR. The inter-disease correlations amongst the NILs were low (Table 3) but in two cases (SLB-NLB and GLS-NLB) they were highly statistically significant which support this hypothesis. When the effects of individual introgressions were calculated (Fig. 4), it was found that two introgressions conferred statistically significant levels of resistance to two diseases under both methods of analysis: 6A conferred resistance to SLB and NLB and introgression 9B conferred resistance to SLB and NLB.

We had previously investigated MDR to SLB, NLB and GLS in two RIL populations (Balint-Kurti et al. 2010; Zwonitzer et al. 2010). Although we observed significant levels of correlation between resistances to the three diseases in both populations, only one QTL in bin 2.04 conferred MDR for SLB and GLS. This QTL is located about 30 cM from the 2B introgression. We have also recently reported strong evidence for MDR loci in the 300 line maize association mapping population (Wisser et al. 2011). Arguably, the data presented here provide some support to the notion discussed elsewhere (Mitchell-Olds et al. 1995) that selection for resistance to one disease will likely also select indirectly for resistance to other diseases, but the situation is not clear cut.

While DTA was found to be a significant factor for both SLB and NLB (Table 2), this appears to have had an environmental rather than genotypic basis. None of the introgressions causing significant levels of SLB resistance (3B, 6A, 9B) caused significant changes to DTA. In a previous study (Zwonitzer et al. 2009), there was no significant correlation between DTA and SLB resistance in an F_{2:3} population derived from a NC250A × B73 cross. However, since DTA was a significant factor for SLB and NLB it was included as a fixed effect in our statistical models for these diseases.

Interestingly, in a couple of instances NC250P introgressions appeared to confer increased levels of susceptibility to GLS and NLB. In particular, introgressions 1C and 3A conferred increased levels of susceptibility to NLB and 3B to GLS (Fig. 4). In the case of introgression 3B, the same introgression is conferring increased resistance to SLB and increased susceptibility to GLS. It is interesting to note that 9B also appears to confer resistance to SLB and susceptibility to GLS (at least by the empirical model Fig. 4a).

NC330 is substantially more GLS resistant than NC292 (Fig. 5). This difference is particularly evident late in the

season (later than the point at which the pictures in Fig. 1 were taken). This is presumably due to the combined effects of several introgressions that are not shared between NC330 and NC292: 10A, which increases GLS resistance, is only found in NC330 and 3B, which increases GLS susceptibility, is only found in NC292.

In summary, in this study, we have generated a set of NILs with which we have validated several previously identified QTL. We have shown that the extremely high level of SLB resistance, almost immunity, displayed by the line NC292 is based in large part on the combined effects of just three QTL carried on introgressions 3B, 6A and 9B. This would appear to contradict the dogma that QDR is usually based on the combined effects of multiple genes of small effect. In fact we would argue that the case reported here is an anomaly. Many recent papers confirm the dogma, most recently in a study of the 5000-line NAM population (Kump et al. 2011) in which 32 QTL were identified which were predominantly additive in effect and all of which had effects smaller than 5%. At least two of these QTL confer resistance to other diseases, 6A to NLB and 9B to NLB. Additionally, 3B and 9B both confer susceptibility to GLS. We are currently fine mapping the genes underlying the SLB resistance conferred by both 3B and 6A (Kump et al. 2010). Once they are identified it will be important to understand their mechanisms and whether the differential effects on multiple diseases are conferred by single or sets of closely linked genes.

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