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ORIGINAL PAPER

Characterization and fine-mapping of a resistance locus for northern leaf blight in maize bin 8.06

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Abstract As part of a larger effort to capture diverse alleles at a set of loci associated with disease resistance in maize, DK888, a hybrid known to possess resistance to multiple diseases, was used as a donor in constructing near-isogenic lines (NILs). A NIL pair contrasting for resistance to northern leaf blight (NLB), caused by *Setosphaeria turcica*, was identified and associated with bin 8.06. This region of the maize genome had been associated in previous studies with both qualitative and quantitative resistance to NLB. In addition, bins 8.05–8.06 had been associated with quantitative resistance to several other diseases, as well as resistance gene analogs and defense response gene homologs. To test the hypothesis that the DK888 allele at bin 8.06 (designated $qNLB8.06_{DK888}$) conditions the broad-spectrum quantitative resistance characteristic of the donor, the NILs were evaluated with a range of maize pathogens and different races of *S. turcica*. The results revealed that $qNLB8.06_{DK888}$ confers race-specific resistance exclusively to NLB. Allelism analysis suggested that $qNLB8.06_{DK888}$ is identical, allelic, or closely linked and functionally related to *Ht2*. The resistance conditioned by *qNLB8.06* was incompletely dominant and varied in effectiveness depending upon allele and/or genetic background. High-resolution breakpoint analysis, using ~2,800 individuals in F_9/F_{10} heterogeneous inbred families and 98 F_{10}/F_{11} fixed lines carrying various recombinant events, delimited $qNLB8.06_{DK888}$ to a region of ~0.46 Mb, spanning 143.92–144.38 Mb on the B73 physical map. Three compelling candidate genes were identified in this region. Isolation of the gene(s) will contribute to better understanding of this complex locus.

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Introduction

Plants have evolved diverse mechanisms to combat pathogens. Some defense mechanisms condition complete resistance, while others provide intermediate forms of resistance. Mechanisms of complete resistance include R-genes and non-host resistance. R-gene-mediated resistance has often proven ephemeral, while quantitative resistance has generally been recognized as moderately effective, race non-specific and durable. Quantitative disease resistance (QDR) has, therefore, been more widely utilized in resistance breeding programs. QDR may, however, be conditioned by diverse mechanisms, and may vary in performance (Poland et al. 2009a). When QDR is conditioned by genes involved in the recognition of evolutionarily labile pathogen

effectors, it is likely to be both race-specific and non-durable. An understanding of the pathogen- and race-specificity of a locus is more likely to provide predictive power regarding the durability of resistance than its quantitative effect alone.

A range of mechanisms have been associated with QDR, some of which are broader in spectrum and more durable than others. Broad-spectrum resistance has commonly been divided into two classes: (1) resistance effective against all known variants of a given pathogen ("race non-specific resistance") and (2) resistance effective against more than one pathogen ("multiple disease resistance"). Some major resistance genes have been observed to confer moderate levels of either race-specific [e.g. *Rp1* in maize (Smith and Hulbert 2005)] or race-nonspecific resistance [e.g. *RB* in potato (Song et al. 2003)]. Despite the lower selection pressure caused by genes conditioning incomplete resistance, these genes could be overcome by evolving pathogen races [e.g. *R1* in potato (Trognitz and Trognitz 2007)]. Race-nonspecific QDR has also been shown to be associated with mechanisms other than R-genes. For example, the *Yr36* gene in wheat contains domains similar to the proteins involved in the signaling of non-R-gene-mediated defenses, including programmed cell death and innate immune response (Fu et al. 2009). In rice, the recessive allele of a susceptibility gene *Pi21*, encoding a proline-rich protein with putative heavy-metal binding and protein–protein interaction motifs, contributes resistance to blast disease (Fukuoka et al. 2009). The resistance of these non-R-genes has thus far been stable. Disease non-specific QDR have been found to be controlled by genes involved in basal resistance, systemic acquired resistance, and defense signaling pathways [e.g. *RPW8.1* and *RPW8.2* in *Arabidopsis* (Wang et al. 2007c); *npr1* in Arabidopsis (Cao et al. 1998)]. Agriculturally important genes of this type, including *Lr34* in wheat (Krattinger et al. 2009) and *mlo* in barley (Buschges et al. 1997) have been shown to confer durable resistance to a number of obligate pathogens. Available lines of evidence imply that durability of resistance is associated with non-specificity, as well as non-gene-for-gene recognition in mechanism and incomplete phenotype. Nevertheless, the ambiguity associated with the effectiveness and spectra of defense mechanisms complicates breeding for disease resistance.

A large number of studies have been conducted to map R-genes, resistance gene analogs (RGAs), and loci conditioning QDR (quantitative trait loci for disease, or disease QTL) in plants. Current knowledge in the genetic architecture of disease resistance, as inferred from overview of previous reports, may provide some insights on the types of resistance associated with different genetic loci, which may in turn have implications for the likely performance of genes at these loci. It has been widely noted that R-genes and disease QTL are not randomly distributed across the genome. Apparent clusters of QTL for different diseases have been observed in rice (Wisser et al. 2005), maize (Wisser et al. 2006), barley (Williams 2003) and other plants. The coincidence of defense-related genes and/or QTL for multiple pathogens in certain chromosomal segments has led to the hypothesis that these chromosomal regions are associated with broad-spectrum resistance that could be durable. Likewise, in a range of plant pathosystems, major genes and/or QTL affecting a given disease has been found to overlap. Association of major genes along with QTL [e.g. *rhm* and QTL for southern leaf blight, and *Rp3* and QTL for common rust in maize (Wisser et al. 2006)] may reflect the differential major and minor effects conferred by allelic variants of identical gene(s) (Robertson 1989; Welz and Geiger 2000), or the differential expression of resistance in various genetic backgrounds or environments. At a gene level, complex clustering of homologous or non-homologous R-genes [e.g. *Pi5* in rice (Lee et al. 2009) and *Rp1-D* in maize (Collins et al. 1999)] has been suggested as a genetic hallmark of rapid evolution of R-genes and race specificities (Hulbert et al. 2001; McDowell and Simon 2006).

Chromosomal regions associated with previously reported R-genes, RGAs, and disease QTL can be sources of genes conditioning diverse forms of resistance. However, due to the limitations of QTL analysis, such as low precision of QTL locations and allelic sampling in different studies (Wisser et al. 2006), the implication for resistance specificity should be used with caution. For a given allele at a disease QTL hotspot region, detailed evaluation will be required to clearly determine whether it confers broad spectrum or disease-specific resistance. Race-specificity of disease QTL, particularly for ones that co-localize with known R-genes, needs to be clarified prior to practical application. This is to prevent the deployment of disease QTL under the misleading assumption of QDR conferring non-specific and more durable protection for crops. Expectations for the long-term performance of a disease QTL can be more realistic if its underlying genetic basis is more fully explored. For instance, knowing whether a broad-spectrum phenotype is conditioned by a pleiotropic gene(s), a cluster of defense-related genes, or the linkage of diverse R-genes, is valuable in designing combinations of favorable alleles of resistance genes in crop-breeding programs.

In the maize genome, among the regions that may harbor genes involving diverse defense pathways, the fifth to sixth segment of chromosome 8 (bin 8.05–8.06) can be viewed as one of the most complex, important, and interesting. Bin 8.05–8.06 is known to be associated with QTL for resistance to various diseases, RGAs, and defense response gene homologs (DRHs). Co-localized QTL were mapped in different populations for resistance to northern leaf blight

(NLB) (Schechert et al. 1999; Welz et al. 1999a, b), southern leaf blight (SLB) (Bubeck 1992), gray leaf spot (GLS) (Bubeck et al. 1993; Clements et al. 2000; Maroof et al. 1996), common rust (Brown et al. 2001; Kerns et al. 1999), common smut (Luebberstedt et al. 1998), maize streak virus (Pernet et al. 1999), and aflatoxin accumulation in ears (Paul et al. 2003). In silico mapping anchored two RGAs, sharing conserved protein kinase (PK) domain with *Pto* in tomatoes and *Pbs1* in Arabidopsis, to bins 8.05 and 8.06 (Xiao et al. 2006, 2007). Several DRHs, including five members of the *S*-adenosyl methionine synthetase family involved in amino acid metabolism and an oxalate oxidase-like protein gene associated with hypersensitive responses, were mapped to the same region using genetic and in silico analysis (Wang et al. 2007a).

Bin 8.05–8.06 is also a locus accounting for a significant proportion of NLB resistance in maize germplasm. NLB, caused by *Setosphaeria turcica* (anamorph *Exserohilum turcicum*, syn. *Helminthosporium turcicum*), is a foliar disease of maize that causes periodic epidemics associated with significant yield losses (Perkins and Pedersen 1987; Raymundo and Hooker 1981; Ullstrup and Miles 1957) in most maize-growing regions of the world. In diverse biparental populations, a number of NLB QTL (Schechert et al. 1999; Welz et al. 1999a, b) as well as two major gene loci, *Ht2* (Yin et al. 2003; Zaitlin et al. 1992) and *Htn1* (Simcox and Bennetzen 1993), have been mapped to bin 8.05–8.06. Evaluation of a large multi-parental mapping population (known as the nested association mapping population) (McMullen et al. 2009; Yu et al. 2008), consisting of 5,000 recombinant inbred lines developed from 25 diverse maize lines, identified two largest effect NLB QTL in the same region (Poland et al. 2009b). In response to a recurrent selection program for NLB resistance (Ceballos et al. 1991), significant changes in allele frequencies provided evidence of selection acting at several loci in bin 8.05–8.06 (Wisser et al. 2008). To date, in the maize–*S. turcica* pathosystem, clustering of major genes and QTL has only been observed at bin 8.05–8.06 (Wisser et al. 2006).

As part of a larger attempt to capture diverse alleles at important resistance loci, we selected the maize hybrid DK888 as a source of potentially useful alleles. This genotype has been shown to harbor alleles for resistance to diverse diseases (Kraja et al. 2000) and derived lines have been produced. In the present study, we aimed to fine-map and characterize DK888 allele(s) in bin 8.05–8.06 and to determine their disease- and race-specificity. Identification of the genes underlying the QTL region will be an important basis for detailed mechanistic studies.

The "heterogeneous inbred family" (HIF) approach was utilized to rapidly generate near-isogenic lines (NILs) carrying contrasting alleles at bin 8.05–8.06 (Tuinstra et al. 1997). This approach involves extraction of NILs from nearly fixed lines, such as lines that have been produced by selfing segregating materials for several generations. Being isogenic at most of the genome, but contrasting for specific QTL of interest, HIF-derived NILs have been used to validate the position and effect of QTL (Borevitz and Chory 2004; Kobayashi et al. 2006; Loudet et al. 2005; Pumphrey et al. 2007). When compared with NILs generated by successive backcrossing, NILs derived from HIFs can be put to use in a shorter period of time (particularly if NILs are available, as they were in this case), and can possibly provide recombinant genetic backgrounds in which the QTL effects are well expressed (Tuinstra et al. 1997).

This study was undertaken to genetically dissect a complex genetic region associated with qualitative and quantitative resistance to NLB and a range of other diseases in maize. To identify, validate and characterize QTL, we isolated bin 8.05–8.06 of DK888, a maize hybrid carrying favorable alleles for multiple disease resistance (Kraja et al. 2000; C. Chung, unpublished) in NILs using HIF-based approach. We will hereafter identify this QTL with bin 8.06, as it was initially located to a region spanning the distal end of bin 8.05 to the distal end of bin 8.06 (mostly in bin 8.06), and was ultimately fine-mapped to bin 8.06. NILs differing for the specific region were investigated to gain insights into a series of questions, including the disease- and race-specificity of the QTL, the QTL in relation to the known co-localized major gene loci, and the gene action at the QTL. To further unravel the complex genetic architecture and defense mechanisms, high-resolution mapping was conducted using break-point analysis. Our study has laid the foundation for positional cloning of a *S. turcica* race-specific resistance gene(s) underlying bin 8.06 of maize. The markers closely linked to the major NLB QTL can also be used for practical resistance breeding.

Materials and methods

Plant materials

The initial plant materials for QTL identification were 17 F_6 heterogeneous inbred families (HIFs) from the cross of S11 × DK888, which were provided by The USDA Germplasm Enhancement of Maize (GEM) Project (Balint-Kurti et al. 2006; Goodman 2005; Lee and Hardin 1997). DK888 is a single-cross hybrid developed by Thailand Charoen Seeds Group in collaboration with US Dekalb Seeds. It was released in Thailand in 1991, and dominated the local Thailand hybrid maize seed market in 1990s (Ekasingh et al. 2001). DK888 is a maize genotype carrying favorable alleles for resistance to NLB, southern leaf blight, gray leaf spot, northern leaf spot and common

rust (Kraja et al. 2000). It also exhibited high levels of resistance to common smut and Stewart's wilt in our repeated field trials (C. Chung, unpublished). The subsequently derived HIFs and NILs were generated by single-seed descent from selected lines in the families segregating for bin 8.06. In this study, "NILs" refers to sets of HIF-derived F_8, F_9, F_{10} and F_{11} lines that contrasted for bin 8.06, but were presumably isogenic at >99.2% of the genome.

Two sets of isolines with and without the *Ht* major genes were obtained from Peter Balint-Kurti of the USDA-ARS unit at North Carolina State University (a total of 6 differential lines: Pa91, Pa91*Ht1*, Pa91*Ht2*, Pa91*Ht3*, B68, and B68*Htn1*). *Ht1*, *Ht2*, and *Htn1* were derived from maize lines GE440, NN14B and Peptilla, respectively, while *Ht3* was derived from *Tripsacum floridanum* (Welz and Geiger 2000; M. Carson, pers. comm.). Several F_1 and F_2 populations were developed by crossing the differential lines with the F_9 NILs carrying DK888 or S11 alleles at the QTL region. The differential lines were also used to provide reference phenotypes of major gene resistance to *S. turcica*.

Disease evaluations

Northern leaf blight

Resistance to NLB was evaluated with *S. turcica* race 1 (isolate EtNY001) in a greenhouse at Cornell University, and at Cornell's Robert Musgrave Research Farm in Aurora, NY from 2006 to 2009. The isolate EtNY001, originally collected from an infected leaf collected in Freeville NY in 1983, is compatible on Pa91*Ht1*, and incompatible on Pa91*Ht2*, Pa91*Ht3*, and B68*Htn1* (Supplementary Table 1) under the standard greenhouse conditions established for NLB assays (Leonard et al. 1989). Another four *S. turcica* isolates representing different races, including Et10a (race 0), Et1001A (race 1), Et86A (race 23), and Et28A (race 23N), were obtained from P. Balint-Kurti, and used exclusively for the race-specificity tests in the greenhouse. In the greenhouse, plants at the 5–6-leaf stage were inoculated with 0.5 ml of spore suspension (4×10^3 conidia per ml in 0.02% Tween 20) in the whorl, and kept in a mist chamber at >85% RH overnight. In the field, plants at the same stage were inoculated with spore suspension along with colonized sorghum grains (1/4 teaspoon, ~1.25 ml) in the whorl. The use of both liquid and solid inoculum was intended to ensure the viability of inoculum under dry weather conditions. *S. turcica* was cultured on lactose–casein hydrolysate agar (LCA) for 2–3 weeks, under a 12 h/12 h normal light–dark cycle at room temperature. Liquid inoculum was prepared by dislodging the conidia from the plates with sterilized ddH_2O, filtering the suspension through four layers of cheesecloth, and adjusting the concentration with the aid of a haemocytometer. The substrate for solid inoculum consisted of sterilized sorghum grains in 1-gallon milk jugs. For each jug, 900 ml of sorghum grains was soaked overnight in 600 ml of dH_2O and then autoclaved twice. The spore suspension from each heavily colonized LCA plate was distributed between 3–5 jugs of sterilized sorghum grains. The inoculated jugs were shaken every day until use to prevent caking and to accelerate fungal colonization. Incubation period (IP) was rated as the number of days post inoculation (dpi) when observing the appearance of first wilted lesion on a plant. IP was checked every day until 25 dpi. The 50% IP was recorded on a row basis when >50% of the plants in a row started showing the lesions. Primary diseased leaf area (PrimDLA) was rated as the percentage of infected leaf area of the inoculated leaves for individual plants at 2–3 weeks after inoculation. Diseased leaf area (DLA) was rated as the percentage of infected leaf area of the entire plant, disregarding decayed bottom leaves for individual plants or on a row basis for fixed lines. DLA was recorded ~10 days before anthesis (2–3 weeks after the onset of secondary infection).

Southern leaf blight

Resistance to SLB was evaluated with *Cochliobolus heterostrophus* race O in the greenhouse in September 2007 and in Clayton, North Carolina in 2008. In the greenhouse trial, plants at the 5–6-leaf stage were inoculated with the isolate C5 (ATCC 48332) obtained from G. Turgeon at Cornell University. Inoculum was cultured on complete medium with xylose (CMX) under continuous fluorescent light for 7–10 days, and spore suspension was prepared as described for NLB. About 0.5 ml of spore suspension (5×10^4 conidia per ml, 0.02% Tween 20) was evenly sprayed on the first fully expanded leaf with an airbrush (Badger® Model 150) at 20 psi. After inoculation, the plants were kept in a mist chamber at >85% RH overnight. Lesion length was measured at 4 dpi from 20 randomly chosen lesions on each plant. Primary DLA was rated at 6 dpi as the percentage of infected leaf area of the inoculated leaf. In the field trial, plants at the 4–6-leaf stage were inoculated 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. Disease was evaluated for three times with 10–12-day interval from around 2 weeks after anthesis. The disease severity scores were used to calculate area under the disease progress curve $\mathrm{AUDPC} = \sum_{i=1}^{n-1} \frac{(y_i+y_{i+1})(t_{i+1}-t_i)}{2}$, where y_i, disease severity at time i, $t_{i+1} - t_i$, day interval between two ratings; n, number of ratings (Wilcoxson et al. 1974).

Gray leaf spot

Resistance to naturally occurring GLS (caused by *Cercospora zeae-maydis* and/or *Cercospora zeina*) was evaluated in Blacksburg, Virginia in 2008. The non-tillage field was located in a valley with regular morning mists and heavy dews, conditions that favor GLS development. Disease severity was scored based on a 1–10 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 with a 7–8-day interval from about 2 weeks after anthesis. The AUDPC was calculated as described above.

Anthracnose leaf blight

Resistance to anthracnose leaf blight (ALB) was evaluated in the greenhouse in September 2007 and 2008, with *Colletotrichum graminicola* (teleomorph: *Glomerella graminicola*) isolate Cg151 (obtained from G. Bergstrom of Cornell University). Inoculum was cultured on oatmeal agar for 2 weeks under continuous fluorescent light at room temperature (Muimba-Kankolongo and Bergstrom 1990). Each plant at the 5–6-leaf stage was inoculated in the whorl with 0.5 ml of spore suspension (2×10^4 conidia per ml, 0.02% Tween 20, prepared as described above), then kept in a mist chamber at >85% RH overnight. Individual plants were rated for IP, latent period and PrimDLA. LP was rated as the number of dpi when observing the first appearance of black acervuli on the lesions. The ratings of IP and PrimDLA were as described for NLB.

Anthracnose stalk rot

Resistance to anthracnose stalk rot (ASR) was evaluated with *C. graminicola* isolate Cg151 in the greenhouse in December 2007 and in Aurora NY in 2008. For each plant at tasseling stage (Keller and Bergstrom 1988), the first internode above the brace root was punctured with an ice pick, a 1 ml pipette tip was inserted, and the plant was inoculated with 1 ml of spore suspension (10^6 conidia per ml, 0.02% Tween 20, prepared as described for ALB) through the tip. At 4 weeks post inoculation, the stalk of each individual plant was split longitudinally, and the percentages of discolored area were rated for 8 (the trial in 2007) or 6 (2008) consecutive internodes (Keller and Bergstrom 1988). Data from all the scored internodes were summed for analysis.

Common rust

Resistance to rust was evaluated in the greenhouse in September 2007 and in Aurora, New York in 2008, with urediniospores of *Puccinia sorghi* collected from naturally infected leaves at Aurora NY in 2007. In the greenhouse trial, about 200–300 mg of stock urediniospores (preserved at −80°C) were suspended in 100 ml of Sortrol oil (Chevron Phillips Chemical Company, Phillips, TX, USA) (Webb et al. 2002). About 1 ml of suspension was evenly applied on the first two fully expanded leaves of each plant with a spray gun (Preval, Yonkers, NY, USA). Plants were kept in a mist chamber at >85% RH overnight. Individual plants were observed daily and rated for first pustule appearance, which is the number of dpi when the first pustule on a plant is observed. Pustules on the inoculated leaves were counted at 10 dpi. PrimDLA was rated at 14 dpi as described above. For the field trial, inoculum was increased on 3–4-leaf stage seedlings of susceptible sweet corn inoculated in the greenhouse. The urediniospores were collected by agitating infected leaves with matured rust pustules in distilled water, and filtering the spores through four layers of cheesecloth. Field plants at 6–8-leaf stage were inoculated with 1 ml of spore suspension (2×10^5 urediniospores per ml, 0.02% Tween 20) in the whorl (Pataky and Campana 2007). Disease severity was rated on a row basis using a 0–10 scale with 0.5 increments, corresponding to the percentage of infected leaf area of the entire plant (0 = no disease, 1 = 10%, 10 = 100%). The AUDPC was calculated as described above, from three severity scores evaluated with 9-day interval from 4 weeks after inoculation.

Common smut

Resistance to smut was evaluated in the greenhouse in November 2007, and in Aurora, NY in 2008, with six compatible strains of *Ustilago maydis* (UmNY001, UmNY002, UmNY003, UmNY004, UmNY008 and UmNY009) which were isolated from naturally infected smut galls collected in Aurora, NY in 2007 [isolation procedure: (Thakur et al. 1989b); compatibility test: (Puhalla 1968)]. The first ear of each plant was shoot bagged, and injected with 2 ml of sporidial suspension (10^6 sporidia per ml in 0.02% Tween 20) through the silk channel, when the silk had emerged 1–5 cm. Inoculum was prepared by culturing the isolates separately in potato-dextrose broth (PDB) on a shaker at 100 rpm at room temperature for 1 day, adjusting the sporidial concentrations with sterilized ddH_2O, and mixing equal amounts of compatible strains right before inoculation (du Toit and Pataky 1999). In the greenhouse trial, the volume (length × width × height) and weight of ear galls were measured. In the field trial, the incidence and severity of ear galls and naturally occurring stalk galls were rated at 4–5 weeks post-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 with *Pantoea stewartii* (syn. *Erwinia stewartii*) strain PsNY003 (obtained from H. Dillard of Cornell University) in Aurora, NY in 2008. Plants at the 5–6-leaf stage were inoculated following a modified pinprick method (Blanco et al. 1977; Chang et al. 1977). Whorl leaves of each plant were pierced twice with a specialized inoculator pre-dipped in bacterial suspension [10^7 colony forming units per ml in sterilized 0.1 M NaCl solution, prepared as described by Suparyono and Pataky (1989)]. Multiple-pin inoculators was made with 30 T-pins (1.5-inch long), pieces of 5.5 × 6.5 cm sponge, and cork board (3/8-inch thick) fastened on two arms of a tong with rubber bands. PrimDLA (as described for NLB) was rated on a row basis at 2 and 3 weeks after inoculation.

Genotyping assays

DNA extraction

Plant genomic DNA was extracted following a modified mini-prep CTAB method (Doyle and Doyle 1987; Qiu et al. 2006). The high-throughput extraction was conducted using 96-well plates (Corning® Costar 96 Well Polypropylene Cluster Tubes). For each sample, about 0.1 g of leaf tissue was frozen and ground with a stainless steel ball (5/32-inch diameter, OPS Diagnostics, NJ, USA), at 450 strokes per min for 50–120 s using Genogrinder 2000 (SPEX CertiPrep Inc., Metuchen, NJ, USA). Pulverized sample was suspended in 500 µl of CTAB extraction buffer [2% (w/v) hexadecyltrimethylammonium bromide, 1.4 M NaCl, 100 mM Tris–HCl (pH 8.0), 20 mM EDTA (pH 8.0), 0.2% (v/v) of 2-mercaptoethanol; 2-mercaptoethanol was added prior to use], and incubated at 65°C for 30–50 min. The CTAB suspension was mixed thoroughly with 400 µl of chloroform/isoamyl alcohol (24:1, v/v) for 3 min, then centrifuged at 5,200 rpm for 15 min. The supernatant was transferred to a new tube, mixed with 300 µl of isopropanol, and incubated at −20°C overnight. DNA was precipitated by centrifuging the sample at 5,200 rpm at 4°C for 12 min, and recovered by repeatedly discarding the supernatant and rinsing with 70% then 100% ethanol. The air-dried DNA pellet was dissolved in 100–150 µl of Tris–EDTA buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0).

Simple sequence repeat markers

Simple sequence repeat (SSR) primers were chosen from the Maize Genetics and Genomics Database (MaizeGDB) (http://www.maizegdb.org/). To integrate the fluorescent dye in the PCR product, the specific primer pair and a fluorescently labeled universal primer were used in a single-reaction nested PCR (Schuelke 2000). Each PCR reaction was performed as described by Wisser et al. (2008) in a total volume of 13 µl, with the same thermal cycling parameters as described by Schuelke (2000). The resulting amplicons labeled with different dyes were multiplexed (up to four PCR reactions were combined) and analyzed with the Applied BioSystems 3730xl DNA Analyzer at Biotechnology Resource Center at Cornell University. Each sample consisted of 0.7 µl PCR product per primer pair, 0.05–0.1 µl GeneScan-500 LIZ size standard, and 9 µl formamide (Applied Biosystems, Foster City, CA, USA). The sizes of amplicons were scored using GeneMapper v. 3.0 (Applied Biosystems).

Single-nucleotide polymorphism (SNP) and cleaved amplified polymorphic site (CAPS) markers

The B73 genomic sequences were used as a reference map for identifying polymorphisms between DK888 and S11. Various genes across the QTL region were chosen as the templates for marker design. Gene sequences were obtained from the database of the Maize Genome Sequencing Project (the Maize Sequence Database, http://www.maizesequence.org; same annotated genes currently available at http://archive.maizesequence.org), and the specific primers for each gene were designed using Primer 3 (Rozen and Skaletsky 2000). Each PCR reaction was performed in a total volume of 16 µl, containing final concentrations of 1× PCR buffer [10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.1% (v/v) Triton X-100], 1.5 mM $MgCl_2$, 1 µM forward-specific primer, 1 µM reverse-specific primer, 1–3 units *Taq* polymerase, and 20–50 ng template DNA. The thermal cycling parameters for different sets of primers can be found in Supplementary Table 2. PCR products amplified from DK888 and S11 homozygotes were purified with exonuclease I and shrimp alkaline phosphatase (New England Biolabs, Ipswich, MA, USA), and sequenced at Biotechnology Resource Center at Cornell University. The DNA sequencing was performed using BigDye Terminator and AmpliTaq-FS DNA Polymerase, and analyzed on the Applied BioSystems 3730×1 DNA Analyzer (Applied Biosystems). The sequencing results were then aligned and analyzed for SNPs, small indels (insertions/deletions), and restriction sites using BioLign version 2.0.9 (developed by T. Hall, http://en.bio-soft.net/dna/BioLign.html). CAPS markers were developed if restriction-site polymorphisms were detected. For CAPS markers, PCR products were completely digested with specific restriction endonucleases (New England Biolabs), and the resulting polymorphic fragments were revealed using standard agarose gel

electrophoresis followed by ethidium bromide staining. The SNP and CAPS markers are listed in Supplementary Table 2.

Genetic map

A genetic map of 11 SSR markers spanning the $qNLB8.06_{DK888}$ region was constructed using genotypic data from segregating F_9 families. The map distances between SSR markers were estimated using MapDisto 1.7.0 (Lorieux 2007) based on Kosambi's mapping function (Kosambi 1944). The relative genetic distances between the 12 and 7 newly developed SNP markers in the intervals of *umc2199–umc2210* and *umc2210–umc1287*, respectively, were calculated by the proportion of identified crossover events between SSR markers. Corresponding physical positions of the markers were obtained from the physical map of the inbred line B73, based on the Maize Sequence Database.

QTL analysis

Single marker analysis and interval mapping (Lander and Botstein 1989) were performed using Windows QTL Cartographer 2.5 (Wang et al. 2007b) to analyze QTL position in segregating heterogeneous inbred families. In interval mapping, QTL were scanned at a walk speed of 0.5 cM. The threshold values were based on the likelihood of odds ratio (LOD) scores from 1,000 permutations of the original at a significance level of $P = 0.01$ (Churchill and Doerge 1994). The LOD threshold used in the study was averaged from the threshold value calculated for each trait. For the marker locus closest to the QTL peak, the additive effect and the proportion of phenotypic variance explained by the QTL (R^2) were obtained using the Windows QTL Cartographer. The R^2 values for single marker analysis were from the analysis of variance (ANOVA) conducted in JMP 7.0 (SAS Institute Inc., Cary, NC, USA). The allele effect was designated as the mean difference between DK888 homozygotes and S11 homozygotes at a locus. The 95% confidence interval for the QTL was estimated according to the "1-LOD support interval", which includes the QTL peak and its right and left loci with LOD scores dropping within 1 (Lander and Botstein 1989).

Experimental design and statistical analysis

HIFs for the identification and fine-mapping of the QTL

From 2006 to 2008, individual plants in each heterogeneous inbred family were genotyped with segregating markers, and phenotyped for resistance to NLB in a controlled greenhouse at Cornell University, or in Aurora, NY (Table 1). To control environmental variations, plants in a family were grown within a single block. Data were analyzed using Windows QTL Cartographer 2.5 as described in "QTL analysis". The analysis of variance was also carried out on an individual trait-marker basis using JMP 7.0. The phenotypic differences among different genotypes were determined by pairwise two-tailed Student's t test at $P < 0.05$.

F_8 and F_9 NILs for the characterization of the QTL

In 2007 and 2008, evaluations for seven different diseases were conducted independently in the field and/or greenhouse using a pair of F_8 NILs, B73 and DK888. Plants were grown in a randomized complete block design (RCBD), with two replications and 10 kernels per genotype (row) per replication in the field, and two replications and 6–8 plants per genotype per replication in the greenhouse. Data were analyzed using JMP 7.0 by fitting linear least squares models with "genotypes", "environments" and "replications within environments" as independent variables. Differences between the least squares means of the NILs were determined by two-tailed Student's t test at $P < 0.05$.

Race-specificity tests were carried out in the greenhouse using a pair of F_8 NILs in September 2007, and a set of six F_9 NILs (4 NILs with $qNLB8.06_{DK888}$, and 2 NILs with $qNLB8.06_{S11}$) in September 2008. This was a split plot design (RCBD on the whole plots) with "*S. turcica* isolates" as the whole plot treatments and "the alleles at *qNLB8.06*" as the split plot treatments. In each environment, the trial consisted of two replications, 6–8 plants per NIL per isolate per replication. The maize differential lines Pa91, Pa91*Ht1*, Pa91*Ht2*, Pa91*Ht3*, B68, and B68*Htn1* were included as control in 2008. Data were analyzed as described above, with "the alleles at *qNLB8.06*", "*S. turcica* isolates", "alleles by isolates", "environments", and "replications within environments" as variables. Differences among the "alleles by isolates" were determined by Tukey–Kramer HSD (honestly significant difference) test at $P < 0.05$.

F_{10} and F_{11} NILs for high-resolution mapping of the QTL

A total of 13 F_{10} and 85 F_{11} NILs were evaluated at Aurora NY in 2008 (for IP and DLA) and 2009 (for IP only), respectively. The NILs were put in rows (10 kernels per row) with two replications per year. In 2008, the 13 F_{10} NILs were randomized within each replication, with DK888 and B73 rows as control. In 2009, the 85 F_{11} NILs originated from 11 F_9 families were grown in 11 randomized blocks, according to their parental families (NILs from the same F_9 line were randomized in one block). Two extra control rows, originating from the corresponding F_9 lines, were grown on one side of each block. The resistant and susceptible control rows were two F_{10} NILs

Table 1 Summary of QTL analysis for *qNLB8.06*$_{DK888}$ in segregating heterogeneous inbred families (HIFs) derived from S11 × DK888

Mapping population (numbers of HIFs)	Sample size	Phenotyped sample size	Environment	Trait	Single marker analysis					Interval mapping				
					Nearest marker	QTL position (cM)[a]	LOD	Allele effect[b]	R^2 [c]	Marker interval	QTL interval (cM)[a]	LOD	Allele effect[b]	R^2
1 F_7	53	53	Aurora NY, 06	IP	*umc1149*	38.33	2.7	5.9 days	0.21	–	–	–	–	–
1 F_8	96	96	GH, Apr–Jun 07	IP	*umc1287*	22.40	15.0	6.8 days	0.62	–	–	–	–	–
				PrimDLA			9.5	−22.4%	0.38	–	–	–	–	–
12 F_9	571	225[d]	GH, Oct–Dec 07[e]	IP	*umc1287*	22.40	77.6	2.6 days	0.32	*umc2199–umc1287*[e]	0–25.40[e]	29.4	2.9 days	0.59
				PrimDLA			31.2	−11.7%	0.14			7.9	−16.1%	0.19
13 F_{10}	1,191	745[d]	GH, Apr–Jun 08	IP	*ctg358-20*	10.20	86.2	4.7 days	0.45	*ctg358-18–ctg358-44*	9.86–11.20	97.6	5.7 days	0.47
14 F_9	1,056	1,056	Aurora NY, 08	IP	*ctg358-20*	10.20	77.8	4.0 days	0.35	*ctg358-18–ctg358-44*	9.86–11.20	96.3	5.2 days	0.35
				DLA	*ctg358-05* *ctg358-37*	10.28	172.6	−14.9%	0.60			210.8	−18.6%	0.60

Resistance to northern leaf blight (NLB) was evaluated in the greenhouse or field with disease components including: incubation period (IP), primary diseased leaf area on inoculated leaves, and/or diseased leaf area on entire plants

In single marker analysis, the marker closest to the QTL peak, and its corresponding likelihood of odds ratio (LOD), allele effect, and proportion of phenotypic variance explained by QTL (R^2), are reported

In interval mapping, the marker interval covering the 95% confidence interval for QTL position (1-LOD support interval) is reported

The LOD, allele effect and R^2 were from the marker closest to QTL peak

[a] The map position is based on the genetic map constructed using F_9 families derived from S11 × DK888. The genetic map and the likelihood of the presence of QTL are shown in Fig. 5

[b] The allele effect is the difference between DK888 homozygotes and S11 homozygotes at the marker closest to the QTL peak

[c] The R^2 values for single marker analysis were calculated from the analysis of variance (ANOVA) performed in JMP 7.0 and all the other data were retrieved from the output of Windows QTL Cartographer 2.5

[d] In space-limited greenhouse, recombinant individuals for target region were selected for phenotyping

[e] The resistance was not as effective in this environmental condition. The QTL interval was estimated conservatively (not based on the 1-LOD support interval)

homozygous for DK888 and S11 alleles for the entire QTL region (*umc2199–umc1287*). In addition to the IP and DLA ratings, the NILs were classified as "resistant" or "susceptible" based on the comparisons with the control lines in the same block. Using JMP 7.0, data from 2008 to 2009 were analyzed separately by fitting a mixed model with each "marker" as a fixed factor, and "replications" and "blocks within replications" as random effects. The analyses were performed on an individual marker–trait basis. Significance levels of marker–QTL associations were represented by the negative logarithm P values ($-\log P$) calculated from the resulting F statistics.

F_1 and F_2 populations for allelic analysis

In 2009, the allelic relationships between $qNLB8.06_{DK888}$ and *Ht2*, and between $qNLB8.06_{DK888}$ and *Htn1*, were evaluated in the greenhouse and in the field at Aurora, NY, respectively. The F_1 and F_2 progenies derived from different pairs of Pa91, Pa91*Ht2*, and B68*Htn1* crossed with the NIL carrying DK888 or S11 allele at *qNLB8.06*, were individually phenotyped for IP, PrimDLA (scored at 18 days after inoculation, greenhouse only), and lesion types. Two F_{10} NILs contrasting for *qNLB8.06*, Pa91, Pa91*Ht2*, B68, and B68*Htn1* were used as control. In the greenhouse, different genotypes were arranged following a RCBD with two replications, four blocks per replication. Each block consisted of 5–6 plants per F_1 population and control genotypes, and 10–12 plants per F_2 population. Data from F_1 progenies and control genotypes were analyzed using JMP 7.0 by fitting a linear least squares model with "genotypes", "replications" and "blocks within replications" as independent variables. Differences among the least squares means of genotypes were determined by Tukey–Kramer HSD test at $P < 0.05$. In the field, plants were put in rows with 10 kernels per row (average germination rate was 38%). Plants in each population (10 rows per F_1 population, and 24 rows per F_2 population) were grown in one block, with each population bordered by control rows. The F_1 progenies were compared with the control genotypes in the same block. Data were analyzed as described above, with "genotypes" as the variable.

Identification of candidate genes

Putative genes in the B73 genomic sequences have been predicted by the Maize Genome Sequencing Project using the Gramene pipeline (Liang et al. 2009) (data available at http://www.maizesequence.org/ and http://archive.maizesequence.org/). The evidence-based gene prediction was conducted by aligning the sequences of known proteins, full-length cDNAs, and expressed sequence tags (ESTs) from maize as well as cross-species libraries to the bacterial artificial chromosome (BAC) contigs of B73. We surveyed existing predicted genes spanning the fine-mapped QTL interval. The potential identities of the predicted coding sequences were subsequently determined by performing basic local alignment search tool (BLAST) searches at the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Identification of an incompletely dominant NLB QTL ($qNLB8.06_{DK888}$) by HIF analysis

Following the HIF methodology described by Tuinstra et al. (1997), the study's first step was to detect residual heterozygosity at potential disease QTL regions in the HIFs derived from S11 × DK888. Forty-six individuals of 17 F_6 families (1–4 individuals per family) were analyzed with 17 markers covering 12 bins. The marker targeting bin 8.06 was *umc1149*. An individual heterozygous for *umc1149* and another marker at bin 5.06 (*umc2216*) was identified, and was used to generate the genetic materials for subsequent QTL analysis.

In 2006, a F_7 family consisting of 53 individuals was evaluated for resistance to NLB (Table 1). The F_7 progeny were segregating for *umc1149* and *umc2216*, but isogenic at ~98.4% of the genome. Variation in disease response co-segregated with *umc1149* (DK888 allele for resistance; allele effect in IP = 5.9 dpi, LOD = 2.7, $R^2 = 0.21$), not *umc2216*, indicating the existence of a candidate NLB QTL at bin 8.06. In 2007, the finding was further validated in a F_8 family (96 individuals) segregating for *umc1149*, but fixed at *umc2216*. Consistently, the QTL contributed strong effects on reducing disease. As much as 62 and 38% of phenotypic variation in IP and PrimDLA, respectively, were explained by the QTL (Table 1).

To more precisely localize the identified NLB QTL, an additional 15 SSR markers across bins 8.05–8.06 were used to estimate the start and end points of heterozygous loci in the HIFs. Assuming that each end of the QTL segment lies halfway between the last marker for the introgression and the first marker outside it, the QTL was determined to reside in the interval of 386.8–453.7 cM on the IBM 2008 neighbors map, and between 136.2 and 156.0 Mb on the B73 physical map. This is a region spanning bins 8.05 and 8.06, but located mostly in bin 8.06. Among the nine markers analyzed in the F_8 family (*umc1287*, *umc1828*, *umc2356*, *umc1149*, *bnlg240*, *umc1997*, *umc1728*, *umc2361*, *umc2395*), the QTL was closest to *umc1287*.

The identified QTL locating mostly in bin 8.06, designated as *qNLB8.06*, showed incompletely dominant resistance (Fig. 1). It was observed that the level of resistance in

DK888 homozygotes was much greater than in the heterozygotes or in the S11 homozygotes. The magnitudes and significance levels of the differences among the three genetic classes (PrimDLA: DK888/DK888 − S11/S11 = −22.0%, $P < 0.0001$; DK888/DK888 − heterozygotes = −14.1%, $P < 0.0001$; heterozygotes − S11/S11 = −7.9%, $P = 0.005$) suggested that the resistance performance in heterozygotes is more similar to S11 homozygotes. The same type of gene action was consistently seen in the subsequent mapping populations.

qNLB8.06$_{DK888}$ is not effective for multiple disease resistance

To understand the resistance spectrum of *qNLB8.06*$_{DK888}$, a pair of F_8 NILs was characterized for resistance to GLS, SLB, ALB, ASR, common rust, common smut and Stewart's wilt (Table 2). The NILs derived from a single F_7 line were contrasting for the QTL region but isogenic at ~99.2% of the genome, according to the theoretical level of heterozygosity in F_8 progeny. Based on the trials conducted in 2007–2008 in the field and/or controlled greenhouse, no significant differences were found between the NIL pairs for response to any of the seven diseases. The result suggested that although DK888 harbors multiple disease resistance, the resistance conferred by *qNLB8.06*$_{DK888}$ is NLB specific.

qNLB8.06$_{DK888}$ conditions race-specific resistance

Race-specific responses in IP, PrimDLA and lesion type were observed for the NILs (F_8 and F_9) carrying DK888 allele(s) at *qNLB8.06*. As shown in Fig. 2, races 0 and 1 were avirulent to *qNLB8.06*$_{DK888}$, while races 23 and 23N were highly virulent to it. Typical resistance symptoms

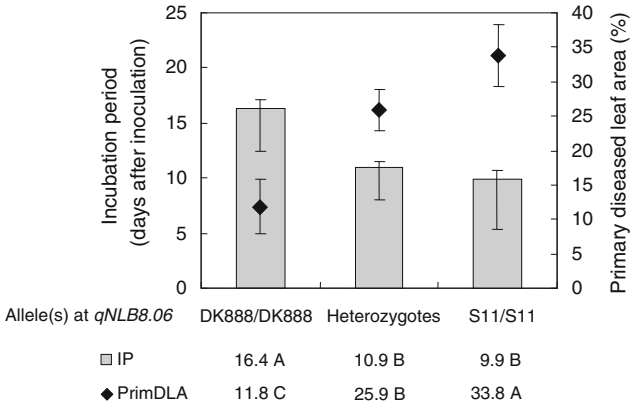

Fig. 1 Gene action at *qNLB8.06*. DK888 homozygotes showed greater resistance than the heterozygotes and S11 homozygotes, suggesting that resistance conferred by the DK888 allele(s) is incompletely dominant. The phenotypic differences among the three genetic classes were tested in an F_8 family by ANOVA, followed by pairwise Student's *t* test at $P < 0.05$

caused by the incompatible interactions between *qNLB8.06*$_{DK888}$ and race 0/race 1 were characterized by prolonged IP, decreased DLA and resistant-type lesions. The resistant-type lesions were slightly chlorotic and more restricted, in contrast to the susceptible-type lesions that extended greatly after the first appearance. The chlorosis, likely induced by the hypersensitive response surrounding the infection sites, was more distinct in early stages of lesion development. Once the pathogen grew out from the localized primary-infected region, the resistant or susceptible reactions were differentiable by size rather than the type of mature lesion.

The defense mechanism conferred by *qNLB8.06*$_{DK888}$ was ineffective when inoculated with races 23 and 23N. The observed race-specificity suggested that the QTL could coincide with the major genes *Ht2*, *Ht3*, and/or *Htn1*. While *Ht3* locus has not been mapped, *Ht2* and *Htn1* loci have been mapped to bin 8.05–8.06, which suggests that *qNLB8.06*$_{DK888}$ may encompass *Ht2* and/or *Htn1*, or some novel modulator(s) conditioning the expression of *Ht2* and/or *Htn1*. *Ht2* is a creditable candidate for *qNLB8.06*$_{DK888}$ based on the compatibility of race 23. The relationship between *qNLB8.06*$_{DK888}$ and *Htn1* is ambiguous, as the compatibility of race 23N may have been caused by *Ht2* and *Htn1*, or *Ht2* alone. However, there is no naturally occurring race N isolate available for further resolving the question. It is also worth noting that the resistance reactions of *qNLB8.06*$_{DK888}$ did not fully resemble those on the maize differential lines Pa91*Ht2* or B68*Htn1*. As illustrated by the control trials (Supplementary Table 1), the lesions on Pa91*Ht2* were more chlorotic associated with accumulated reddish pigmentation, and the lesions on B68*Htn1* were of the susceptible type, consistent with previously reported lesion types of *Ht2* and *Htn1* (Welz and Geiger 2000). In contrast to *qNLB8.06*$_{DK888}$, *Ht2* and *Htn1* were effective in delaying lesion formation by 2–3 days and 2–9 days, respectively.

Allelism with known major genes at *qNLB8.06*

qNLB8.06 in relation to Ht2

To understand the allelism and interactions between *qNLB8.06*$_{DK888}$ and *Ht2*, the F_1 and F_2 progenies of *qNLB8.06*$_{DK888}$ × *Ht2*$_{NN14B}$, *qNLB8.06*$_{DK888}$ × *Ht2*$_{Pa91}$, *qNLB8.06*$_{S11}$ × *Ht2*$_{NN14B}$, and *qNLB8.06*$_{S11}$ × *Ht2*$_{Pa91}$ were evaluated in the greenhouse (Fig. 3). *Ht2*$_{NN14B}$ represented the resistance allele (from the donor line NN14B) at the *Ht2* locus in the isoline Pa91*Ht2*, and *Ht2*$_{Pa91}$ represented the susceptible allele in the recurrent line Pa91. As expected, all the F_1 and F_2 individuals of *qNLB8.06*$_{S11}$ (S) × *Ht2*$_{Pa91}$ (S) were susceptible. In contrast, no susceptible plants were found in either F_1 or F_2 individuals of

Table 2 Resistance spectrum of *qNLB8.06*$_{DK888}$

Disease	Parameter (unit)	Allele(s) at *qNLB8.06* in the NIL		P^b
		DK888[a]	S11[a]	
GLS	AUDPC (area unit)[c]	55.7 ± 2.1	57.1 ± 2.1	0.47
SLB	Lesion length (mm)[d]	1.2 ± 0.05	1.2 ± 0.05	0.72
	Primary diseased leaf area (%)[d]	29.5 ± 1.2	30.0 ± 1.2	0.58
	AUDPC (area unit)[c]	27.8 ± 3.7	23.7 ± 2.6	0.33
ALB	Incubation period (days after inoculation)[d]	7.9 ± 0.2	7.9 ± 0.2	0.80
	Latent period (days after inoculation)[d]	9.8 ± 0.3	9.8 ± 0.3	0.99
	Primary diseased leaf area (%)[d]	44.0 ± 6.7	46.7 ± 6.7	0.57
ASR	Discolored internode area (total% of internode)[c,d]	102.5 ± 10.1	105.8 ± 9.4	0.63
Common rust	First pustule appearance (days after inoculation)[d]	7.5 ± 0	7.5 ± 0	0.99
	Number of pustules (number of pustules)[d]	163.9 ± 51.7	149.5 ± 46.8	0.71
	Primary diseased leaf area (%)[d]	14.4 ± 3.1	15.0 ± 2.7	0.79
	AUDPC (area unit)[c]	46.1 ± 2.2	46.1 ± 2.2	0.99
Common smut	Volume of ear gall (cm^3)[d]	273.8 ± 129.3	167.5 ± 123.3	0.26
	Weight of ear gall (g)[d]	127.4 ± 57.6	78.9 ± 54.9	0.25
	Incidence of ear gall (%)[c]	29.0 ± 10.0	23.0 ± 10.0	0.49
	Severity of ear gall (scale)[c]	1.8 ± 0.6	1.0 ± 0.6	0.19
	Incidence of stalk gall (%)[c]	0.0 ± 0.0	0.0 ± 0.0	0.99
	Severity of stalk gall (scale)[c]	0.0 ± 0.0	0.0 ± 0.0	0.99
Stewart's wilt	Primary diseased leaf area (%)[c]	72.5 ± 4.9	72.5 ± 4.9	0.99

The near isogenic lines (NILs) carrying DK888 or S11 alleles at bin 8.06 were evaluated for resistance to a range of important diseases in maize, including gray leaf spot (GLS), southern leaf blight (SLB), anthracnose leaf blight (ALB), anthracnose stalk rot (ASR), common rust, common smut, and Stewart's wilt

Different disease components were evaluated in the field and greenhouse

No significant contrasts were observed between the NIL pairs, indicating *qNLB8.06*$_{DK888}$ is not effective for any of the diseases

[a] Trait values are 95% confidence intervals of least squares means, calculated from the linear least squares model with "genotypes", "environments" and "replications within environments" as independent variables

[b] Two-tailed Student's *t* test was conducted on the least squares difference between the NIL pairs

[c] Disease parameters evaluated in the field

[d] Disease parameters evaluated in the greenhouse

qNLB8.06$_{DK888}$ (R) × *Ht2*$_{NN14B}$ (R) (Fig. 3b). Distinct chlorotic–necrotic lesions were observed in almost all the plants derived from *qNLB8.06*$_{DK888}$ (R) × *Ht2*$_{NN14B}$ (R). No susceptible individuals were observed, though 3 out of 35 F_1 individuals and 6 out of 72 F_2 individuals showed an intermediate phenotype on lower leaves, which is possibly caused by incomplete expression of resistance under low light intensity (Reuveni et al. 1993; Thakur et al. 1989a). Complementation of the DK888 and NN14B alleles in resistance phenotypes suggests that *qNLB8.06*$_{DK888}$ is likely to be identical, allelic, or closely linked to the *Ht2* locus. A larger F_2 population will be required for differentiating allelism from close linkage.

Significantly different levels of NLB resistance were observed in the four F_1 progenies with the same hybrid background: *qNLB8.06*$_{DK888}$ × *Ht2*$_{NN14B}$ > *qNLB8.06*$_{DK888}$ × *Ht2*$_{Pa91}$ > *qNLB8.06*$_{S11}$ × *Ht2*$_{NN14B}$ > *qNLB8.06*$_{S11}$ × *Ht2*$_{Pa91}$ (Fig. 3e). Although they showed some levels of resistance, the F_1 progenies of neither *qNLB8.06*$_{DK888}$ × *Ht2*$_{Pa91}$ (Fig. 3c) nor *qNLB8.06*$_{S11}$ × *Ht2*$_{NN14B}$ (Fig. 3d) showed typical resistant chlorotic–necrotic lesions, indicating incomplete dominance of the *qNLB8.06*$_{DK888}$ and *Ht2*$_{NN14B}$ alleles. The quantitative difference between the F_1 progenies of *qNLB8.06*$_{DK888}$ × *Ht2*$_{Pa91}$ and *qNLB8.06*$_{S11}$ × *Ht2*$_{NN14B}$ also suggested differential allelic effects, though the effectiveness of individual alleles could not be determined.

As expected, marked segregation of resistant, intermediate and susceptible phenotypes was observed in the F_2 populations from the crosses of *qNLB8.06*$_{DK888}$ (R) × *Ht2*$_{Pa91}$ (S) and *qNLB8.06*$_{S11}$ (S) × *Ht2*$_{NN14B}$ (R) (Fig. 3c, d). The intermediate phenotypes in heterozygotes complicated the classification of resistant and susceptible plants. We decided not to pursue Mendelian segregation ratio test because we did not manage to generate F_3 progenies for confirmation, and Mendelian analysis on F_2 individuals would provide meaningful results only if based on

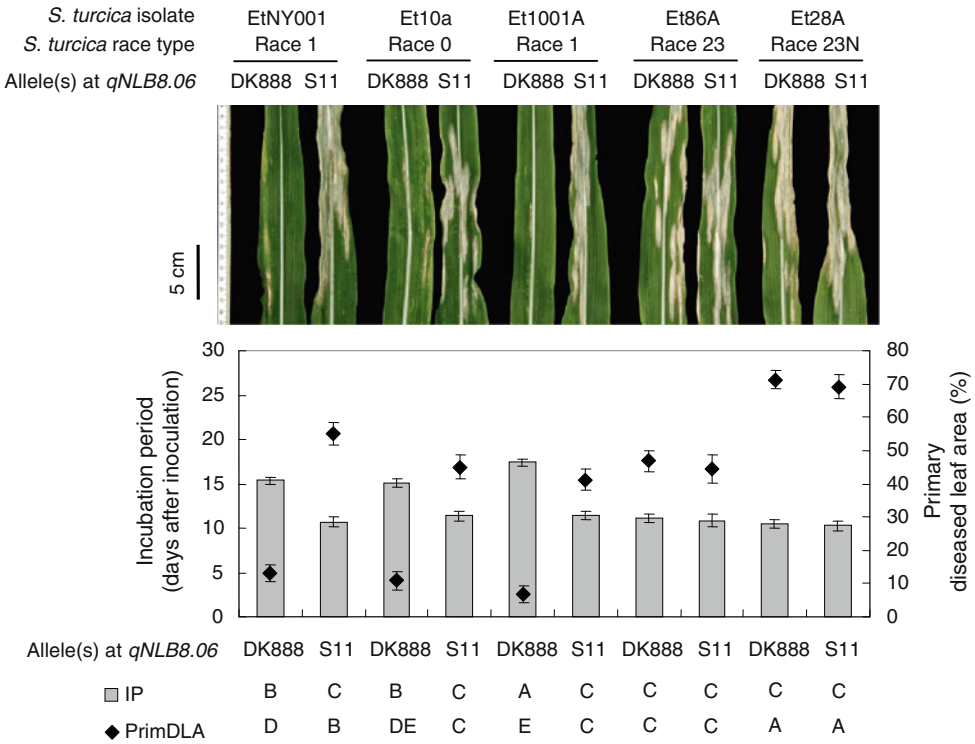

Fig. 2 Race-specificity of $qNLB8.06_{DK888}$. The F_8 and F_9 NILs carrying DK888 or S11 alleles at bin 8.06 were evaluated for resistance to different races of *S. turcica*. Photographs were taken 20 days after inoculation. The *error bars* represent the 95% confidence interval of the least squares means; determined by Tukey–Kramer HSD (honestly significant difference) test at $P < 0.05$, significant differences for incubation period (IP, *bars*) and primary diseased leaf area (PrimDLA, *dots*; scored at 17 days after inoculation) were indicated as *different letters* below the graph. The results provided evidence that $qNLB8.06_{DK888}$ conditions resistance to race 0 and race 1, but not race 23 and race 23N of *S. turcica*

complete dominant or recessive genes with high penetrance. Nevertheless, careful observation and ratings were still conducted, from which the incomplete dominance of the $qNLB8.06_{DK888}$ and $Ht2_{NN14B}$ alleles, and the likely differential allelic effects were confirmed.

Induced accumulation of reddish pigmentation surrounding chlorotic–necrotic lesions was associated with the $Ht2_{NN14B}$ allele and/or Pa91 genetic background. Extensive reddish pigmentation was consistently observed on diseased leaves of Pa91*Ht2* (Fig. 3a). In contrast, the pigmentation was never seen on the NILs carrying $qNLB8.06_{DK888}$ or $qNLB8.06_{S11}$, or their derived lines. All the F_1 and F_2 progenies used in this allelism test, however, showed different degrees of accumulated pigmentation. Relative to the F_2 progeny derived from $qNLB8.06_{DK888} \times Ht2_{Pa91}$, more individuals with higher degrees of reddish pigmentation were seen in the F_2 populations of $qNLB8.06_{DK888} \times Ht2_{NN14B}$ and $qNLB8.06_{S11} \times Ht2_{NN14B}$. Variation in the pigmentation was also seen in the F_2 population of $qNLB8.06_{DK888} \times Ht2_{NN14B}$. The variation implies the involvement of the gene(s) controlling the biosynthesis of anthocyanins. However, these results did not clearly differentiate between an influence of the *qNLB8.06*(*Ht2*) locus or the genetic background.

qNLB8.06 in relation to Htn1

To understand the allelism and interactions between $qNLB8.06_{DK888}$ and *Htn1*, the F_1 and F_2 progenies of $qNLB8.06_{DK888} \times Htn1_{Peptilla}$ and $qNLB8.06_{S11} \times Htn1_{Peptilla}$ were evaluated in the field (Fig. 4). $qNLB8.06_{DK888} \times Htn1_{B68}$ and $qNLB8.06_{S11} \times Htn1_{B68}$ were not included due to unavailability of seed. $Htn1_{Peptilla}$ represented the resistance allele (from the donor line Peptilla) at the *Htn1* locus in the isoline B68*Htn1*, and $Htn1_{B68}$ represented the susceptible allele in the recurrent line B68. Similar to $qNLB8.06_{DK888}$ and $Ht2_{NN14B}$, $Htn1_{Peptilla}$ was much less effective in the heterozygous than homozygous state. Homozygous $Htn1_{Peptilla}$ in B68*Htn1* (average IP = 23.8 dpi) increased IP by 10.5 days relative to B68 (average IP = 13.3 dpi). Heterozygous $Htn1_{Peptilla}$ in the F_1 progeny of $qNLB8.06_{S11} \times Htn1_{Peptilla}$ (average IP = 15.9 dpi, Fig. 4c), however, only increased IP by 2.6 ($P = 0.044$) and 2.5 days ($P = 0.033$) relative to B68 and the NIL carrying $qNLB8.06_{S11}$, respectively. The incomplete dominance of *Htn1* has been described (Raymundo et al. 1981). It was also observed that when $Htn1_{Peptilla}$ and $qNLB8.06_{DK888}$ were both heterozygous, the plants displayed an intermediate resistant phenotype that was characterized by slightly chlorotic–necrotic lesions (Fig. 4b) and moderately increased IP [average IP = 18.8 dpi, significantly different from and in between of homozygous $Htn1_{Peptilla}$ (average IP = 24.3 dpi) and homozygous $qNLB8.06_{DK888}$ (average IP = 16.4 dpi)]. The intermediate phenotype conforms to previously reported phenotype resulting when heterozygous *Htn1* interacts with heterozygous *Ht2* (Simcox and Bennetzen 1993). This implies some functional similarity of $qNLB8.06_{DK888}$ and *Ht2*.

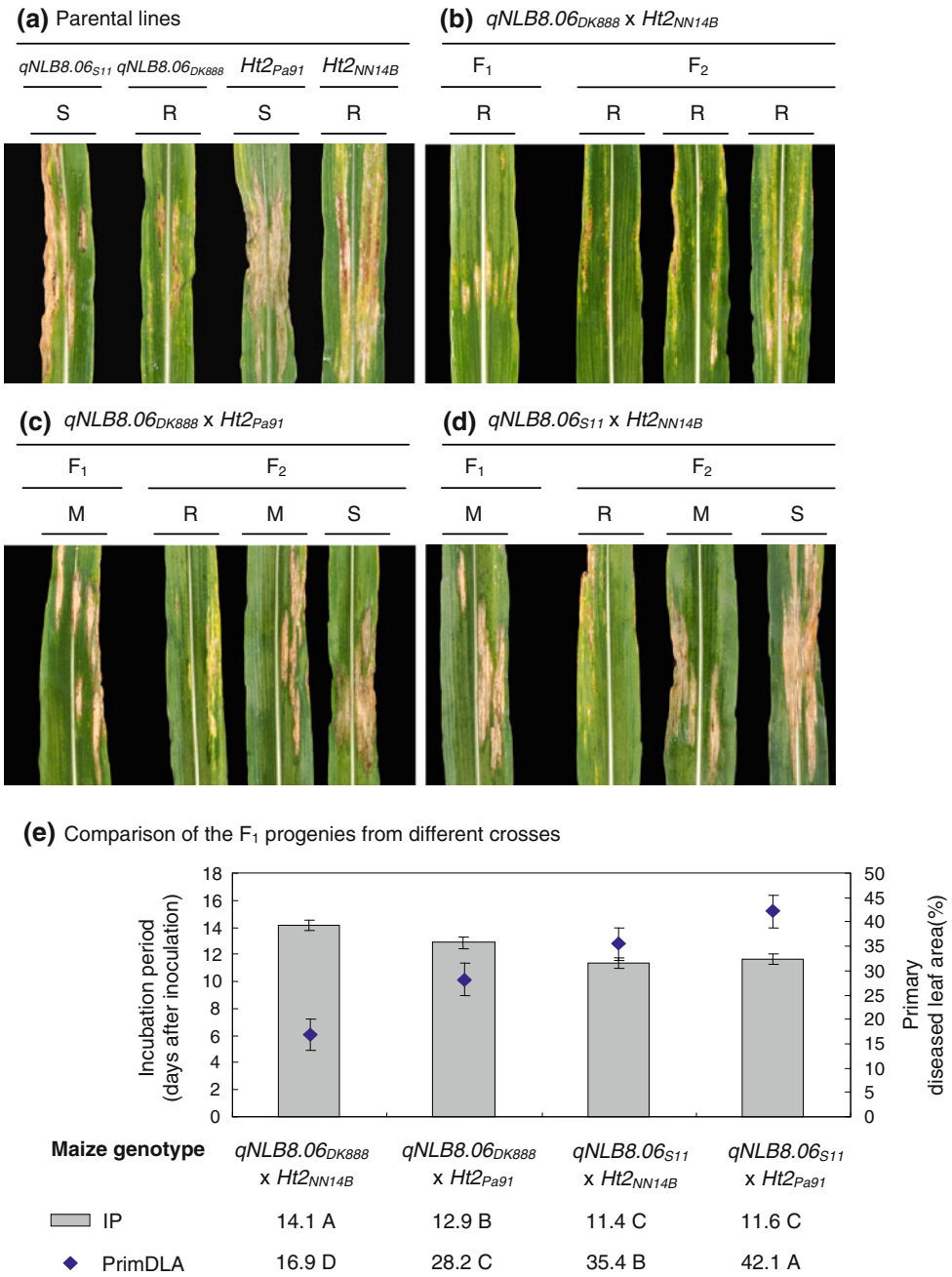

Fig. 3 Analysis of allelism between $qNLB8.06_{DK888}$ and *Ht2*. **a** Crosses were made between the near-isogenic lines (NILs) contrasting for bin 8.06 (alleles designated $qNLB8.06_{S11}$ and $qNLB8.06_{DK888}$), Pa91 (allele designated $Ht2_{Pa91}$), and Pa91*Ht2* (allele designated $Ht2_{NN14B}$). Plants carrying homozygous $qNLB8.06_{DK888}$ showed chlorotic–necrotic resistance lesions, and plants carrying homozygous $Ht2_{NN14B}$ showed chlorotic–necrotic resistance lesions with accumulated *reddish* pigmentation. The F_1 and F_2 progenies of $qNLB8.06_{DK888} \times Ht2_{NN14B}$ (**b**), $qNLB8.06_{DK888} \times Ht2_{Pa91}$ (**c**), $qNLB8.06_{S11} \times Ht2_{NN14B}$ (**d**), and $qNLB8.06_{S11} \times Ht2_{Pa91}$ (not shown) were evaluated for resistance to race 1 of *S. turcica* (EtNY001) in the greenhouse. **b** Complementation between the $qNLB8.06_{DK888}$ and $Ht2_{NN14B}$ alleles in resistance phenotypes was observed. **c**, **d** Intermediate phenotype (less susceptible-type lesions) was observed in all the F_1 individuals and a considerable proportion of the F_2 individuals, suggesting that the resistance conditioned by either $qNLB8.06_{DK888}$ or $Ht2_{NN14B}$ was incompletely dominant. **e** Significant differences in incubation period (IP, *bars*) and primary diseased leaf area (PrimDLA, *dots*; scored at 18 days after inoculation) were observed among the four F_1 progenies. The F_1 individuals were comparable, as they differed at bin 8.06 and *Ht2* but isogenic for the rest of the genome. Trait values are least squares means calculated from the linear least squares model with "genotypes", "replications" and "blocks within replications" as independent variables. Differences were determined by Tukey–Kramer HSD (honestly significant difference) test at $P < 0.05$, and indicated as *different letters* below the graph. The result confirmed the incomplete dominance of $qNLB8.06_{DK888}$ and $Ht2_{NN14B}$, and implicated the potential existence of different alleles at bin 8.06. Photographs were taken on the sixth leaves at 19 days after inoculation. Disease phenotypes are denoted as *R* resistant, *M* intermediate, and *S* susceptible

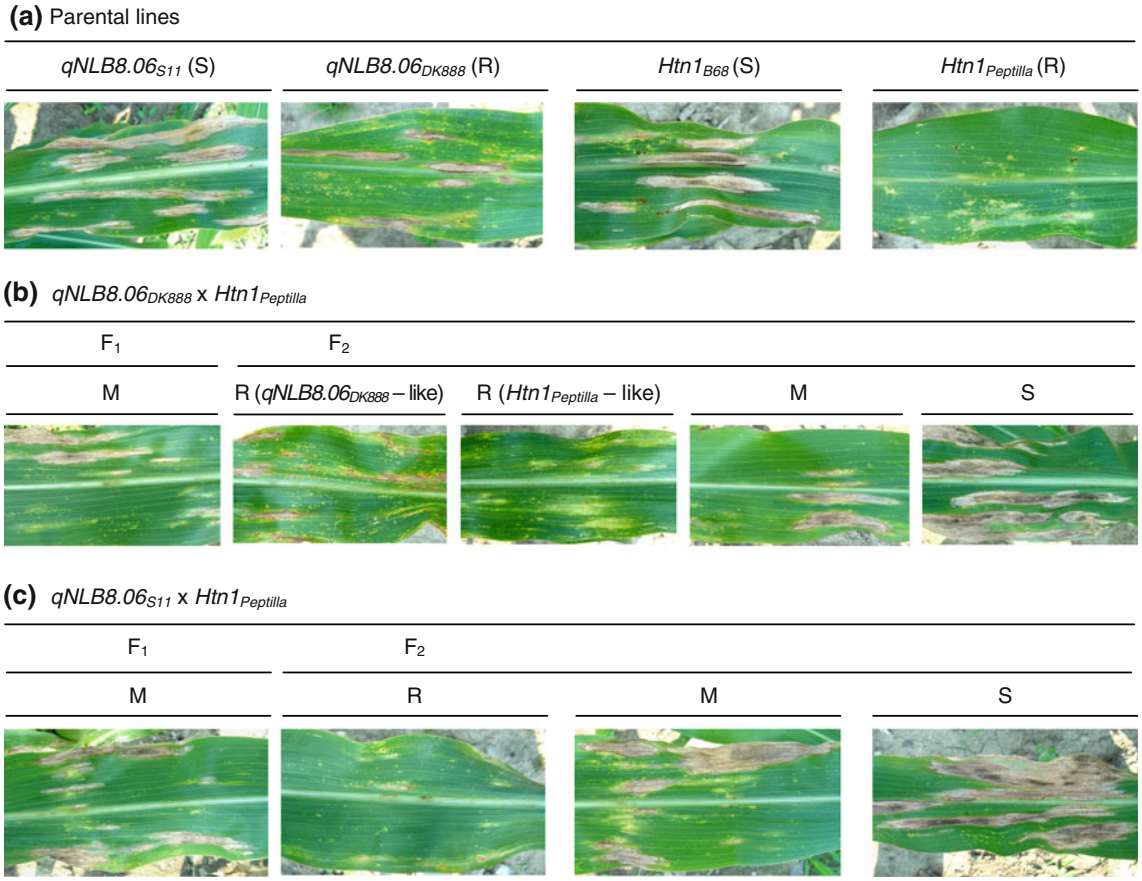

Fig. 4 Analysis of allelism between *qNLB8.06*$_{DK888}$ and *Htn1*. **a** Crosses were made between the near-isogenic lines (NILs) contrasting for bin 8.06 (alleles designated *qNLB8.06*$_{S11}$ and *qNLB8.06*$_{DK888}$), and B68*Htn1* (allele designated *Htn1*$_{Peptilla}$). The line B68 (allele designated *Htn1*$_{B68}$) was used as control. Plants carrying homozygous *qNLB8.06*$_{DK888}$ showed chlorotic–necrotic resistance lesions, and plants carrying homozygous *Htn1*$_{Peptilla}$ showed extraordinarily delayed formation of lesions (until 25 days after inoculation, only a few lesions were observed on B68*Htn1*). The F_1 and F_2 progenies of *qNLB8.06*$_{DK888}$ × *Htn1*$_{Peptilla}$ (**b**) and *qNLB8.06*$_{S11}$ × *Htn1*$_{Peptilla}$ (**c**) were evaluated for resistance to race 1 of *S. turcica* (EtNY001) in the field. **b** *qNLB8.06*$_{DK888}$ and *Htn1*$_{Peptilla}$ appeared to be non-allelic, based on the segregation of plants exhibiting chlorotic–necrotic lesions, delayed lesion formation, intermediate phenotypes, and susceptible lesions in their F_2 progeny. **c** The resistance conditioned by *Htn1*$_{Peptilla}$ was incompletely dominant. Photographs were taken on the seventh leaves at 25 days after inoculation. Disease phenotypes are denoted as *R* resistant, *M* intermediate, and *S* susceptible

Unlike the complementation of *qNLB8.06*$_{DK888}$ and *Ht2*$_{NN14B}$, resistance phenotypes of homozygous *qNLB8.06*$_{DK888}$ (chlorotic–necrotic), homozygous *Htn1*$_{Peptilla}$ (extremely prolonged IP), and heterozygous *qNLB8.06*$_{DK888}$ in combination with heterozygous *Htn1*$_{Peptilla}$ (intermediate) segregated in the F_2 progeny of *qNLB8.06*$_{DK888}$ × *Htn1*$_{Peptilla}$ (Fig. 4b). The Mendelian segregation ratio test was not employed because of the ambiguity in phenotypic classification. Nonetheless, it was clearly observed that 4 out of 82 F_2 individuals showed a susceptible phenotype (shorter IP with extended long lesions), which is presumably associated with recombination events between *qNLB8.06* and *Htn1*. The result indicates that *qNLB8.06* and *Htn1* are non-allelic. Considering the results of the allelism analysis, the locus designation was modified to *qNLB8.06(Ht2)*.

Fine-mapping of *qNLB8.06(Ht2)*

Breakpoint analysis was conducted to refine *qNLB8.06(Ht2)*$_{DK888}$. Around 2,800 individuals (from 26 F_9 families and 13 F_{10} families) segregating for bin 8.06 were used for QTL analysis. Disease evaluations were carried out in three environmental conditions: Oct–Dec in the greenhouse, Apr–Jun in the greenhouse, and May–Aug in the field (Table 1). In the space-limited greenhouse, plants were initially all genotyped for flanking markers of the target QTL interval. Subsequently, only the identified recombinant individuals were kept for disease evaluations. The mapping results from single marker analysis and interval mapping are summarized in Table 1, and displayed in the QTL likelihood map in Fig. 5.

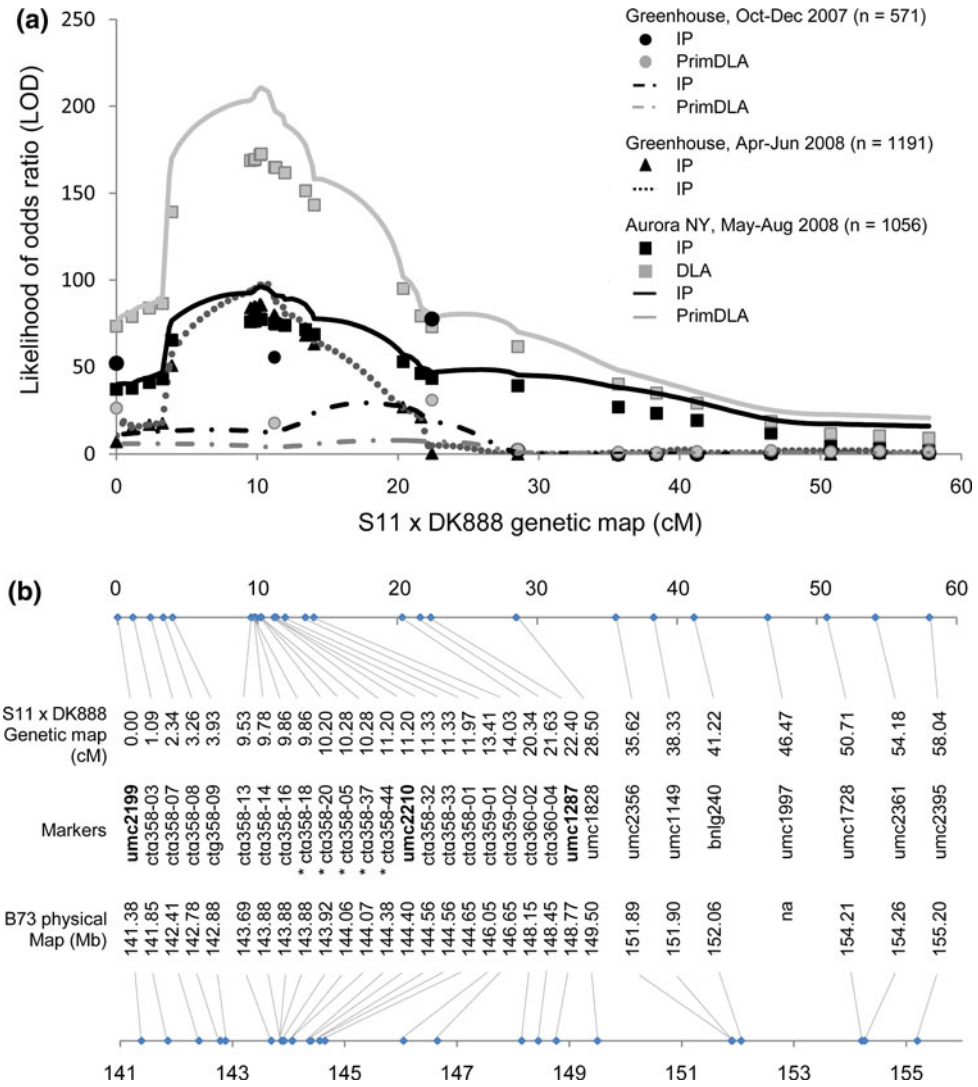

Fig. 5 Likelihood map of *qNLB8.06(Ht2)*$_{DK888}$. **a** The likelihood that the loci in bin 8.06 are associated with NLB resistance was analyzed by single marker analysis (*circle*, *triangle*, and *square dots*) and interval mapping (*solid* and *dashed lines*). The likelihood of odds ratio (LOD) lines and dots for incubation period (IP) are shown in *black*, and the LOD lines and dots for primary diseased leaf area (PrimDLA) and DLA are shown in *gray*. The average LOD threshold for all traits, based on 1,000 permutations at $P = 0.01$, is 3.3 (not shown in the figure). The resistance of *qNLB8.06(Ht2)*$_{DK888}$ was not as effective in the greenhouse trial conducted in Oct–Dec 2007. The most likely QTL position, based on the data obtained from the evaluations in the greenhouse in Apr–Jun 2008 and in the field in May–Aug 2008, was located between *ctg358-18* to *ctg358-44*. This interval corresponds to 9.86–11.20 cM on the S11 × DK888 genetic map, and 143.88–144.38 Mb on B73 physical map. **b** The genetic and physical positions of the markers are shown

In a population consisting of 571 F_9 individuals, *qNLB8.06(Ht2)*$_{DK888}$ was found to be likely located between *umc2199–umc1287*. The LOD scores at this interval were >10 for IP and >5 for PrimDLA, whereas the LOD values dropped to <3 from around 27–58 cM (between *umc1287* and *umc1828*). A high-resolution map was constructed with 19 newly developed SNP and CAPS markers around *umc2199–umc1287* (Fig. 5). Initially, two and five SNP/CAPS markers between *umc2199–umc2210* and *umc2210–umc1287*, respectively, were designed to cover the QTL region at low density. By testing the co-segregation of markers and traits, the interval of *ctg358-07–ctg358-01* was found to be the most significantly associated with resistance. An additional 12 SNP/CAPS markers were then developed to saturate this region. Marker segregation data showed that the order of the SSR, SNP and CAPS markers used in the study agree with their physical positions in the genome sequence of B73.

To increase efficiency, individuals in mapping populations were selectively genotyped for SNP/CAPS markers, based on their genotypes at the flanking markers of the target interval. Considering the incompletely dominant gene action of *qNLB8.06(Ht2)*$_{DK888}$, the genotyping strategy aimed to capture the homozygous DK888 segment(s), which provided informative resistance phenotype for QTL analysis. Therefore, all the recombinant individuals that were homozygous for the DK888 allele at either one of the flanking markers were genotyped for intermediate SNP/CAPS markers. Individuals that were homozygous for identical alleles at the two flanking markers were assumed homozygous for the entire interval.

In a population consisting of 1,191 F_{10} individuals and a population consisting of 1,056 F_9 individuals, *qNLB8.06 (Ht2)*$_{DK888}$ was delimited to a region of ~1.34 cM (~0.5 Mb) between *ctg358-18–ctg358-44* (Table 1; Fig. 5). In the two experimental environments, the resistance of

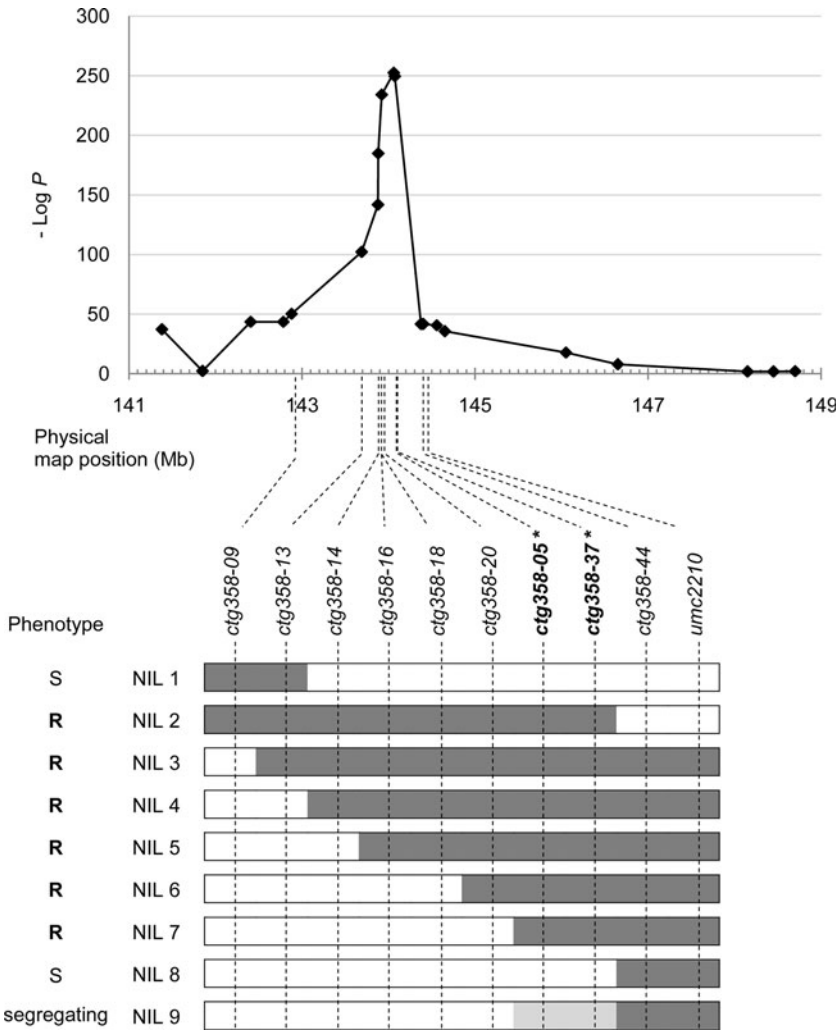

Fig. 6 Validation of $qNLB8.06(Ht2)_{DK888}$ position. F_{10} and F_{11} near-isogenic lines (NILs) capturing various recombination events at bin 8.06 were evaluated for resistance to northern leaf blight (NLB). The likelihood of each locus being associated with incubation period is represented by negative logarithm *P* values (−log *P*) derived from a mixed model analysis. Genotypic compositions and disease phenotypes (*R* resistant, *S* susceptible, segregating *R* and *S* plants segregating in a row) of nine representative NILs are shown. The *solid bars* and *open bars* represent the loci homozygous for DK888 alleles and S11 alleles, respectively. The *gray bar* represents heterozygous loci. $qNLB8.06(Ht2)_{DK888}$ was delimited to a map interval between *ctg358-20* and *ctg358-44* (10.20–11.20 cM on the S11 × DK888 genetic map, and 143.92–144.38 Mb on B73 physical map)

$qNLB8.06(Ht2)_{DK888}$ was well expressed, allowing accurate linkage analysis on the basis of distinct phenotypes. Averaged from the effects estimated from single marker analysis and interval mapping, the DK888 allele increased IP by ∼5 days and decreased DLA by ∼17%. About 35–47% and 60% of the variance in IP and DLA, respectively, were explained by $qNLB8.06(Ht2)_{DK888}$. Significant evidence of QTL (LOD > 3.3, the average threshold for all traits) was consistently found between *umc2199–umc1287* (0–22.4 cM). In this interval, QTL peaks were detected at approximately the same map position (∼10.2 cM) for IP and DLA (highest LOD scores: ∼97 for IP, and ∼210 for DLA). If adopting the 1-LOD drop method, the most likely QTL position can be predicted to a tight region between *ctg358-18–ctg358-44* (Fig. 5; 9.86–11.20 cM on the S11 × DK888 genetic map, and 143.88–144.38 Mb on B73 physical map).

To further confirm the location of $qNLB8.06(Ht2)_{DK888}$, a total of 13 F_{10} and 85 F_{11} NILs were evaluated for IP, DLA (only in the 2008 trial) and lesion types at Aurora NY in 2008 and 2009, respectively. The NILs were derived from selected lines covering different breakpoints around *umc2199–umc1287*. The result of single marker–trait analysis and the genotypic compositions of nine representative fixed NILs are shown in Fig. 6. Evaluations conducted in 2008 and 2009 led to the same results. Because F_{11} NILs captured more recombination events in more homogeneous backgrounds, the data from the 2009 trial was shown to represent the overall result. Markers *ctg358-20*, *ctg358-05*, *and ctg358-37* were found to be the most significantly associated with disease traits (−log of $P > 200$, Fig. 6). Among the three markers, *ctg358-20* is likely to reside outside of the QTL region, based on the "resistant" phenotype of NIL7 (S11/S11 at *ctg358-20*, DK888/DK888 at *ctg358-05* and *ctg358-37*). Evidence of the QTL tightly linked to *ctg358-05* and *ctg358-37* was also found in the rows of NIL9 (Fig. 6; heterozygous at *ctg358-05* and *ctg358-37*), where individual plants segregated for resistance. The number of resistant: intermediate/susceptible plants were 4:21, which does not deviate from

the expected 1:3 segregation ratio ($\chi^2 = 1.2$, $P = 0.3$) of a single incompletely dominant gene. *qNLB8.06(Ht2)*$_{DK888}$ was thus validated to locate between (but not overlapping with) *ctg358-20* and *ctg358-44* in bin 8.06 (10.20–11.20 cM on the S11 × DK888 genetic map, and 143.92–144.38 Mb on B73 physical map). There is some ambiguity regarding the precise boundary between bins 8.05 and 8.06. The region 143.92–144.38 Mb was located to bin 8.06 in MaizeGDB, while it was located to a gap between bins 8.05–8.06 in the Maize Sequence Database.

Candidate genes underlying *qNLB8.06*$_{DK888}$

On the basis of the annotation of the Maize Genome Sequencing Project (as of August 2009, available at http://archive.maizesequence.org/), the genomic region between *ctg358-20* and *ctg358-44*, spanning 0.46 Mb, harbors a large number of transposable elements (TEs) and 12 putative genes (GRMZM2G135202, GRMZM2G164612, GRMZM2G164640, GRMZM2G 091973, GRMZM2G092018, GRMZM2G119720, GRMZM2G018260, GRMZM2G122912, GRMZM2G 006188, GRMZM2G042017, GRMZM2G077187 and GRMZM2G065538). The abundance of TEs has been generally observed in the entire maize genome (Wei et al. 2007). Of the 12 non-TE genes, eight genes encode putative proteins with similarities to known protein domains or motifs in the InterPro databases. Putative genes that can be associated with previously reported R-genes or defense-related genes include two protein kinase-like genes (GRMZM2G135202 and GRMZM2G164612 with conserved domains IPR017441, IPR002290, IPR001245, IPR017442, IPR011009, IPR008271) and one serine–threonine-specific protein phosphatase-like gene (GRMZM2G119720 with conserved domain IPR006186). The two protein kinase-like genes are closely linked (2,632 bp apart) and highly homologous to each other (78% genomic sequence identity; 97% putative transcript identity; putative proteins different for 1 out of 290 amino acid residues).

Discussion

Production of NILs for a complex resistance locus using HIFs

Near-isogenic lines carrying contrasting alleles at maize bin 8.06 were successfully generated, and the region was characterized and dissected using HIF analysis. The HIF-based QTL approach was conducted as part of a larger effort to capture diverse alleles at the loci associated with complex types of disease resistance. To increase the probability of finding alleles conditioning broad-spectrum resistance, maize lines possessing multiple disease resistance were used as donors. In the present study, the broadly resistant maize hybrid DK888 was used as a source of alleles. Considering the importance of bins 8.05–8.06 in NLB resistance (2 major genes and many co-localized QTL have been mapped to the region), the effect of DK888 allele(s) at bin 8.06 was first tested for response to NLB.

We detected, validated and localized an NLB QTL at bin 8.06 (designated *qNLB8.06*) using initially 1 single SSR marker and subsequently 15 additional markers. The F_7 and F_8 families in which *qNLB8.06*$_{DK888}$ was identified were expected to segregate for <1.6% of the genome. In these HIFs, *qNLB8.06*$_{DK888}$ appears to be a major QTL explaining a large proportion (14–62%) of phenotypic variations in NLB resistance. *qNLB8.06*$_{DK888}$ consistently conferred resistance in juvenile and adult plants across greenhouse and field environments. Relative to S11 allele(s), DK888 allele(s) at bin 8.06 was effective for delaying lesion formation by about 2.6–6.8 days, and reducing diseased leaf area by about 12–22% of the primarily inoculated leaves and about 15% of the entire plant. Overall, HIF analysis proved to be an efficient way to extract targeted QTL from the nearly fixed recombinant inbred lines (Tuinstra et al. 1997). Genetic stocks derived during the procedure were readily applicable for subsequent work of characterizing and fine-mapping QTL. Clear expression of the disease phenotypes in the NILs indicated that the QTL was transferred to an appropriate genetic background for QTL examination.

qNLB8.06 conditions race-specific resistance to NLB

The hypothesis that DK888 allele(s) at bin 8.06 conditions disease- and race-nonspecific resistance was tested. The DK888 allele(s) at bin 8.06 conferred resistance only to NLB among the several diseases tested. The resistance was also characterized by its specificity to race 0 and race 1, but not to race 23 and race 23N of *S. turcica*. The compatibility with race 23 and race 23N led to the question of whether *qNLB8.06*$_{DK888}$ is the same or different from the known major genes *Ht2* and *Htn1*. We found that *qNLB8.06*$_{DK888}$ is likely to be identical, allelic, or very closely linked (and functionally related) to *Ht2*$_{NN14B}$, on the basis of their overlapping map locations, their similarities in race-specificity and resistance phenotypes, and their complementation for resistance in the F_1 and F_2 test progenies. *qNLB8.06*$_{DK888}$ and *Htn1* appear to be linked and functionally dissimilar genes, according to the intermediate resistant phenotype in their F_1 progeny, and the segregation of F_2 individuals showing chlorotic–necrotic lesion type (typical *Ht2* phenotype), intermediate lesion type, or delayed formation of lesions (typical *Htn1* phenotype).

These observations conformed to previously reported non-allelism of *Ht2* and *Htn1* (Simcox and Bennetzen 1993). *Htn1* was mapped to ~10 cM distal to *Ht2* in the F_2 progeny of W22*Htn1* × A619*Ht2*. In our group, concurrent work of fine-mapping *Htn1* using a population consisting of ~2,600 F_2 individuals derived from B68 × B68*Htn1* is underway (J. Kolkman, pers. comm.). The map distance between *qNLB8.06(Ht2)* and *Htn1* will be further clarified.

qNLB8.06(Ht2) shows incomplete dominance

Available evidence on gene action at *Ht2* and *Htn1* from previous studies is ambiguous. For *Ht2*, both complete dominance (Yin et al. 2003; Zaitlin et al. 1992) and incomplete dominance (Ceballos and Gracen 1989; Hooker 1977) have been observed in different genetic materials. Reduced resistance in the heterozygotes (incomplete dominance) and the variable expression of resistance in different genetic backgrounds have also been reported for *Htn1* (Raymundo et al. 1981). The effects of *Ht2* and *Htn1* have been found to be highly sensitive to environmental conditions in others' experiments (Reuveni et al. 1993; Thakur et al. 1989a) and our repeated greenhouse and field trials (data not shown). In the present study, both *DK888* and *NN14B* alleles at *qNLB8.06(Ht2)* conditioned incomplete dominance and race-specific resistance to *S. turcica* (NN14B is the resistance donor line used to derive Pa91*Ht2* isoline). High levels of resistance and the distinct chlorotic–necrotic lesions were only seen on the plants containing two copies of resistance alleles (*DK888*/*DK888*, *NN14B*/*DK888* or *DK888*/*NN14B*) at the locus. One copy of the resistance allele along with one copy of a susceptible allele resulted in differential intermediate degrees of disease and susceptible-type lesions.

Incomplete dominance has been widely observed for diverse resistance genes. Examples include the R-genes *Cf* genes in tomato lines (Hammond-Kosack and Jones 1994), the susceptibility-conferring R-gene *LOV1* in *Arabidopsis* (Lorang et al. 2007), and the detoxification gene *Hm2* in maize (Chintamanani et al. 2008). Incomplete dominance is generally associated with a gene dosage effect. Higher expression of resistance gene product in homozygous individuals may lead to more effective perception of pathogen invasion, activation of defensive responses, or elimination of cell damage. The dosage-dependent hypothesis has been tested, to a limited extent, for a few resistance genes. Tomato *Cf* genes (encoding proteins with extracellular leucine rich repeats and transmembrane domain) against leaf mold caused by *Cladosporium fulvum* displayed weakened resistance in heterozygous states (Vidhyasekaran 2007). Homozygous *Cf* lines were capable of responding to a twofold lower concentration of race-specific elicitors than heterozygous lines (Hammond-Kosack and Jones 1994). In the case of the maize *Hm2* gene (encoding HC-toxin reductase) against the leaf spot and ear mold caused by *Cochliobolus carbonum* race 1, intermediate resistance in heterozygotes has been associated with lower abundance of *Hm2* transcripts (Chintamanani et al. 2008). Although the underlying genes are currently unknown, the resistance phenotypes conferred by *qNLB8.06(Ht2)*$_{DK888}$ as well as other *Ht* major genes are expressed in a similar dosage-dependent manner. This is consistent with the observations that triploid (*Ht1 Ht1 Ht1*) and tetraploid (*Ht1 Ht1 Ht1 Ht1*) maize seedlings displayed a higher level of resistance to NLB than monoploid (*Ht1*) and diploid (*Ht1 Ht1*) seedlings (Dunn and Namm 1970). The dosage-dependent hypothesis and resistance response kinetics can be further characterized by manipulating the isolated resistance gene(s) and its corresponding *S. turcica* effector(s) in follow-up studies.

Allele- and genetic background-dependent expression of *qNLB8.06(Ht2)*

The resistance conditioned by *qNLB8.06(Ht2)* varied depending on allele variants and/or genetic backgrounds. The genetic background effect has been previously reported: Ceballos and Gracen (1989) showed that the expression of *Ht2* can be inhibited by a dominant suppressor gene *Sht1* in B14-related inbred lines. In this study, the differential performance of *DK888/Pa91* and *S11/NN14B* at *qNLB8.06(Ht2)* in the same genetic background (F_1 hybrid of the Pa91 and the DK888 × S11 NIL) suggested functional allelic diversity. The existence of allelic series for resistance gene(s) at *qNLB8.06(Ht2)* can also be inferred from other studies, in which the *NN14B* allele was more resistant than *Oh43* allele, and the *Oh43* allele was more resistant than *B73* allele at *qNLB8.06(Ht2)* (Ceballos and Gracen 1989; Dong et al. 2008; Moghaddam and Pataky 1994; Poland et al. 2009b; Zaitlin et al. 1992; Zhang et al. 2007).

While different alleles at bin 8.06 appeared to contribute varying degrees of resistance to NLB, it remained unclear whether the differential expression was conditioned by a single gene or multiple linked genes. Our observation implied the involvement of at least one linked gene in modulating anthocyanin biosynthesis induced in the incompatible reaction of *qNLB8.06(Ht2)*. Anthocyanins are antioxidants that can protect plant cells against the high levels of oxidative stresses in defense reactions (Hammerschmidt 2005). In the maize–*Cochliobolus heterostrophus* pathosystem, accumulation of anthocyanin has been reported to occur in the uninfected epidermal cells surrounding the lesions (Hipskind et al. 1996). In our allelism analysis, the accumulation of anthocyanins on

diseased tissues was associated with the $Ht2_{NN14B}$ allele and/or the Pa91 genetic background. Genes controlling anthocyanin biosynthesis in maize have been isolated and mapped to several loci on different chromosomes (Bernhardt et al. 1998), including an *a4* locus (*dihydroflavonol 4-reductase*) residing between *umc2210* and *umc1287* at bin 8.06 (the map location indicated on MaizeGDB). Since the reddish pigmentation was never observed on the resistant plants carrying *qNLB8.06* $(Ht2)_{DK888}$ in the DK888 × S11 background, the key resistant gene(s) are apparently not anthocyanin-related. Nevertheless, with certain alleles, the anthocyanin-related gene(s) at bin 8.06 and/or other unlinked loci may contribute additive effects to the resistance of *qNLB8.06(Ht2)*.

Map location of *qNLB8.06(Ht2)*

Several major genes and QTL have been isolated by map-based positional cloning [eg. *Pi5-1* and *Pi5-2* against rice blast (Lee et al. 2009), *Rcg1* against ASR of maize (Broglie et al. 2006), and *Yr36* against wheat stripe rust (Fu et al. 2009)]. In the present study, we used ~2,800 individuals in 39 F_9 or F_{10} heterogeneous inbred families and 98 F_{10}/F_{11} fixed lines to localize $qNLB8.06(Ht2)_{DK888}$ from a region of ~19.8 to 0.46 Mb (1 cM). Within the 1 cM interval delimited by *ctg358-20* and *ctg358-44*, *ctg358-20*, *ctg358-5* and *ctg358-37* are closely linked to each other in a 0.08 cM region, whereas *ctg358-44* was located at a distance of 0.92 cM. Although there were 34 out of 98 fixed lines capturing recombination events between *ctg358-37* and *ctg358-44*, we have not succeeded in developing polymorphic markers for this region. *qNLB8.06(Ht2)* can be further delimited by genotyping the 34 lines with more newly developed markers.

The 0.46 Mb region resides within the intervals of *Ht2* previously estimated from the F_2 populations of A619*Ht2* × W64A (Zaitlin et al. 1992) and W22*Htn1* × A619*Ht2* (Simcox and Bennetzen 1993). It also resides within the map intervals of the NLB QTL identified in the $F_{2:3}$ lines derived from Lo951 × CML202 (Schechert et al. 1999; Welz et al. 1999a), and the NLB QTL identified across the NAM population consisting of RILs derived from 25 diverse maize lines crossed with B73 (Poland et al. 2009b) (J. Poland, pers. comm.). However, some discrepancies were found in previous fine-mapping study using 890 F_2 individuals from the cross of 77*Ht2* and Huobai (Yin et al. 2003). The inconsistent *Ht2* positions as well as the converse order of linked markers observed in the 77*Ht2* × Huobai population suggest that the *qNLB8.06*(*Ht2*) locus may be divergent among some maize lines.

It has been recognized that the recombination rate for a given resistance locus can vary depending on the similarity of the haplotypes that are paired [e.g. the maize *Rp1* locus (Ramakrishna et al. 2002)]. Lower recombination rate flanking the rice *Pi5-1* and *Pi5-2* genes was observed in a population derived from the *RIL260* and *Nipponbare* cultivars, for which the resistant and susceptible alleles from the two cultivars are significantly divergent (Lee et al. 2009). Conversely, R-gene clusters have been widely associated with high recombination frequencies (Bakker et al. 2006; Meyers et al. 2005). In the S11 × DK888 mapping population, the ratio of physical to genetic distance in the ~7.4 Mb region between *umc2199* and *umc1287* at bin 8.06 was ~330 kb/cM. A higher physical to genetic ratio (460 kb/cM) was observed for the 0.46 Mb region of *qNLB8.06(Ht2)*, indicating a lower recombination frequency flanking the resistance gene(s). This implies the possibility of low similarity between the DK888 and S11 alleles at *qNLB8.06(Ht2)*, and the absence of clustering of homologous resistance genes (which facilitates crossovers) in both alleles. More insights on the evolution of *qNLB8.06(Ht2)* will be gained by detailed investigation of the natural allelic diversity in maize germplasm.

Candidate genes underlying *qNLB8.06(Ht2)*

Three compelling candidate genes, including two tandem protein kinase (PK)-like genes and one protein phosphatase (PP)-like gene, were identified within the delimited 0.46 Mb interval of $qNLB8.06_{DK888}$. The two tandem PK-like genes contain the conserved kinase catalytic domain of serine/threonine-specific and tyrosine-specific protein kinases. The PK domain is one of a few conserved domains or motifs shared among R-genes (Xiao et al. 2007). The PP-like gene, on the other hand, has the conserved domain of serine/threonine-specific protein phosphatases, which have been associated with negative regulation of R-gene and non-R-gene-mediated defense signaling in rice, *Arabidopsis*, and tobacco (He et al. 2004; Park et al. 2008; Schweighofer et al. 2007). Overall, our preliminary analysis suggested that an R-gene(s) equipped with PK domain and/or a serine/threonine-specific PP gene may underlie $qNLB8.06(Ht2)_{DK888}$. Given the high degree of gene non-colinearity among maize lines (Buckler et al. 2006; Fengler et al. 2007; Fu and Dooner 2002), it is possible that the resistance gene(s) or regulatory sequence(s) does not exist in B73 genotype.

Conclusion

Using a HIF-based QTL approach to target a complex genetic region, we identified, characterized and fine-mapped an NLB QTL likely to be identical, allelic, or closely linked to the known major gene *Ht2*. We provided potentially useful information regarding the resistance spectrum

and closely linked markers of the locus. The knowledge will benefit its appropriate deployment in resistance breeding programs. To further delimit *qNLB8.06(Ht2)*$_{DK888}$ and finally isolate the underlying genetic determinant(s), more lines capturing recombination events between flanking markers *ctg358-20* and *ctg358-44* will be screened, and more polymorphic markers will be developed to saturate the interval. Association analysis based on the three identified candidate genes will be tested in a set of $\sim$300 diverse maize lines, which has been evaluated in our group over three years for resistance to NLB (J. Kolkman, pers. comm.). In light of the potential non-homologies between DK888 and B73 alleles at *qNLB8.06*, alternatives to candidate gene analysis, such as chromosome walking or construction of a BAC library of *qNLB8.06*$_{DK888}$, may be required. Once the genetic determinant(s) underlying *qNLB8.06* is elucidated, more intriguing hypotheses about the complex genetic architecture, the evolution of resistance gene(s), gene functions and regulations in response to pathogen attack under different environmental conditions can then be addressed.

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