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ORIGINAL PAPER

A major QTL for resistance to *Gibberella* stalk rot in maize

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Abstract *Fusarium graminearum* Schwabe, the conidial form of *Gibberella zeae*, is the causal fungal pathogen responsible for *Gibberella* stalk rot of maize. Using a BC_1F_1 backcross mapping population derived from a cross between '1145' (donor parent, completely resistant) and 'Y331' (recurrent parent, highly susceptible), two quantitative trait loci (QTLs), *qRfg1* and *qRfg2*, conferring resistance to *Gibberella* stalk rot have been detected. The major QTL *qRfg1* was further confirmed in the double haploid, F_2, BC_2F_1, and BC_3F_1 populations. Within a *qRfg1* confidence interval, single/low-copy bacterial artificial chromosome sequences, anchored expressed sequence tags, and insertion/deletion polymorphisms, were exploited to develop 59 markers to saturate the *qRfg1* region. A step by step narrowing-down strategy was adopted to pursue fine mapping of the *qRfg1* locus. Recombinants within the *qRfg1* region, screened from each backcross generation, were backcrossed to 'Y331' to produce the next backcross progenies. These progenies were individually genotyped and evaluated for resistance to *Gibberella* stalk rot. Significant (or no significant) difference in resistance reactions between homozygous and heterozygous genotypes in backcross progeny suggested presence (or absence) of *qRfg1* in '1145' donor fragments. The phenotypes were compared to sizes of donor fragments among recombinants to delimit the *qRfg1* region. Sequential fine mapping of BC_4F_1 to BC_6F_1 generations enabled us to progressively refine the *qRfg1* locus to a ~500-kb interval flanked by the markers SSR334 and SSR58. Meanwhile, resistance of *qRfg1* to *Gibberella* stalk rot was also investigated in BC_3F_1 to BC_6F_1 generations. Once introgressed into the 'Y331' genome, the *qRfg1* locus could steadily enhance the frequency of resistant plants by 32–43%. Hence, the *qRfg1* locus was capable of improving maize resistance to *Gibberella* stalk rot.

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Introduction

Stalk rot is one of the most devastating soil-borne diseases of maize, occurring in all continents of the world, including the Americas (Koehler 1960), Europe (Cook 1978; Ledencan et al. 2003), Africa (Chambers 1988), Asia (Younis et al. 1969; Lal and Singh 1984), and Australia (Francis and Burgess 1975). In the US State of Illinois, stalk rot reduces annual yield by at least 5%, and in certain years, it can reach 10–20% or more of the expected yield (University of Illinois 1995). Stalk rot has been detected in China back to the 1920s, and has recently become a major threat to maize production. It is reported that 10–20% of plants, and occasionally more than 60%, appear to be symptomatic in some areas, causing yield loss of up to 25% (Lu et al. 1995). Apart from yield loss, stalk rot induces lodging that further reduces yield, degrades grain quality, and causes problems during harvest (Cook 1978; Ledencan et al. 2003; University of Illinois 1995).

Worldwide increase of severity of stalk rot has prompted studies on all aspects of the disease, including etiology, epidemiology, germplasm evaluation, inheritance, and gene cloning. The pathogens causing stalk rot seem to be very

complicated, as different species of pathogens have been isolated from rotted stalk in different parts of the world (Chambers 1987). In China, *Fusarium graminearum*, *Fusarium verticillioides*, *Pythium graminicola*, and *Pythium inflatum* are reported as major causal pathogens of stalk rot (Mei 2003). In North America, *Diplodia maydis*, *Colletotrichum graminicola*, *Fusarium graminearum*, and *Fusarium moniliforme* are the major causal agents of stalk rot (Toman and White 1993; University of Illinois 1995). While *Erwinia* stalk rot is a devastating disease of maize grown in tropical countries, including India, Nepal, Bangladesh, Philippines, Thailand, Zimbabwe, Israel, Greece, and South Africa (Lal and Singh 1984).

F. graminearum (teleomorph *Gibberella zeae*) causes *Gibberella* stalk rot in maize. *F. graminearum* overwinters as saprophytic mycelia and chlamydospores on crop residues and attacks susceptible maize roots, resulting in greenish-wilt symptoms and rapid senescence during the kernel filling stage. Symptom development depends on several stress factors, including an excess or lack of moisture, heavy cloudiness, high planting density, foliar diseases, and corn borer infestation (Parry et al. 1995; Ledencan et al. 2003). Due to its soil-borne infection pathway, fungicidal control of *Gibberella* stalk rot is not effective. Alternatively, discovery/utilization of resistance gene(s) to improve maize tolerance to stalk rot is a cost-effective and environmentally friendly approach to reducing yield loss. Substantial numbers of maize germplasm have been evaluated for stalk rot resistance, and some have demonstrated high levels of resistance (Wang 2001; Ledencan et al. 2003; Afolabi et al. 2008). This would allow for identifying potential resistance genes/QTLs for resistance to stalk rot in maize by either genetic transformation or marker-assisted selection (MAS).

As several factors impact symptom development, such as pathogen population, varied environmental conditions, and plant growth status, proposed genetic models have been inconsistent. Both qualitative and quantitative genetic loci have been reported to confer resistance to stalk rot. Studies have indicated that resistance to stalk rot is quantitatively inherited and controlled by multiple genes with additive effects. After evaluating 150 $F_{2:3}$ families from a cross between the maize susceptible line 33-16 and resistant line B89, Pè et al. (1993) identified five resistance QTL loci to *Gibberella* stalk rot, located on chromosomes 1, 3, 4, 5, and 10. In another study, a major resistance QTL (*Rcg1*) against *Anthracnose* stalk rot, located on the long arm of chromosome 4, has been identified and cloned (Jung et al. 1994; Frey 2005). Whereas, a single resistance gene against *P. inflatum* has been mapped on chromosome 4 and located within a genetic distance of 5.7 cM flanked by simple sequence repeat (SSR) markers bnlg1937 and agrr286 (Yang et al. 2005). Likewise, a single resistance gene to *F. graminearm* is located on chromosome 6 at a genetic distance of 5.0 cM flanked by markers mmc0241 and bnl3.03 (Yang et al. 2004).

In this study, QTL analysis was performed to identify chromosomal regions for *Gibberella* stalk rot resistance, followed by sequential fine mapping of the major QTL, and subsequent evaluation of its genetic effect. Moreover, a high-resolution map of the *qRfg1* region was constructed to identify cosegregating or closely linked markers useful for a MAS breeding program to improve maize resistance to *Gibberella* stalk rot.

Materials and methods

Plant materials

Maize inbred lines '1145' (donor parent and completely resistant to *Gibberella* stalk rot) and 'Y331' (recurrent parent and highly susceptible to *Gibberella* stalk rot) were selected to develop various mapping populations, including multiple backcross populations (BC_1F_1, BC_2F_1, BC_3F_1, BC_4F_1, BC_5F_1, and BC_6F_1), self-pollinated populations (F_2 and BC_4F_2), and doubled haploid (DH) lines. All mapping populations were grown at the experimental farm of the China Agricultural University (Changping, Beijing), and artificially inoculated with *F. graminearum* Schwabe.

In 2004, 500 plants of the BC_1F_1 population were individually inoculated with *F. graminearum*. Highly resistant plants were selected and backcrossed to 'Y331' to generate $BC_{1:2}$ families. Molecular markers within the major QTL region were used to identify recombination events from $BC_{1:2}$ families and selected recombinants were subsequently backcrossed to 'Y331' to generate the next BC_3F_1 backcross population. This process was repeated during the years 2007–2009 to develop a series of advanced backcross populations, consisting of 2664 BC_4F_1, 2667 BC_4F_2, 4113 BC_5F_1, and 4035 BC_6F_1 plants for fine-mapping of the major QTL. These BC populations share almost the same 'Y331' genetic background with either heterozygous 'Y331'/'1145' or homozygous 'Y331'/'Y331' genotypes in the major QTL region, hence ensuring accurate assessment of maize resistance to *Gibberella* stalk rot.

Artificial inoculation in the field

The pathogen *F. graminearum*, derived from a single spore isolate, was kindly provided by Prof. Xiaoming Wang (Chinese Academy of Agricultural Sciences, Beijing). Conidia were cultured on the potato dextrose agar at 25°C for 5–7 days.

Maize kernels were dipped in water for ~20 h, and cooked in boiling water for 1 h. Thoroughly cooked kernel

were dried on a ventilated table, packed into plastic bags, and then autoclaved for 20 min at 121°C. To prepare inoculum for field evaluation, sterilized kernels were inoculated with a slab of *F. graminearum*, picked from the agar culture, and incubated at 25°C in complete darkness for 15 days.

Field inoculation of plants was conducted at the silking date. Prior to inoculation, all infected maize kernels were pooled and thoroughly mixed to ensure inoculum uniformity. A hole was dug in the ground, about 5–10 cm away from the stem, and approximately 70 g of maize kernels were placed, and then covered with soil. Following inoculation, the field was irrigated to increase soil humidity for fungal growth and infection.

Scoring of plants for symptoms

Plants were scored for stalk rot symptoms three times at weekly intervals, beginning one month following inoculation. The following typical symptoms of stalk rot were scored: browning reactions in lower internodes, spongy stem, wilting, lodging, and plant death. Following the final scoring, stalks of each plant were longitudinally cut to evaluate mycelial growth and root damage. Moreover, disease severity of *Gibberella* stalk rot was rated using a rating scale of 1–6. A rating scale of 6 corresponds to a highly susceptible reaction with severe symptoms detected at the first scoring; scale 5 corresponds to a susceptible reaction with visible symptoms observed at the second scoring; and scale 4 corresponds to an intermediate susceptible reaction with a few visible symptoms observed at the second scoring and symptoms detected at the last scoring. A rating scale of 3 corresponds to an intermediate resistant reaction with no symptom observed at the second scoring or some symptoms detected at the last scoring; a rating of 2 corresponds to a resistant reaction with a few symptoms observed at the last scoring; and a rating of 1 corresponds to a highly resistant reaction and without any observed symptoms at the last scoring.

Genotyping

Immature leaves were collected separately from each of the plants from all mapping populations. DNA extraction was performed according to the method described by Murray and Thompson (1980).

Maize SSR markers were retrieved from the Maize Genetics and Genomics Database (http://www.maizegdb.org/) and synthesized by Invitrogen (Beijing, China). SSR primers were initially used to screen parental lines to identify those polymorphic markers useful for linkage mapping and QTL analysis. PCR products were separated by electrophoresis on either 2% agarose gel or 6% polyacrylamide gel followed by sliver staining for visualization.

Linkage map construction and QTL detection

Linkage mapping with polymorphic SSRs was conducted using MAPMAKER 3.0b (Lincoln et al. 1992). Linkage groups were identified using the ‘Group’ command with a logarithm of odds (LOD) score of 3.0. Recombination frequency was converted into centiMorgans by using the Kosambi mapping function (Kosambi 1944).

The QTL cartographer (version 2.5 software package) was used to detect QTLs (Basten et al. 1997). A composite interval mapping (CIM) was performed to identify QTLs and to estimate their effects (Zeng 1994). A significant threshold for declaring a putative QTL was obtained from 1,000 permutations at $P < 0.05$ for each data set. A LOD score of 3.1 was used in the model to facilitate identification of putative QTLs that contribute to stalk rot resistance.

To confirm the major QTL, a tetrad grid χ^2 test (SAS 8.2 version) was used to test allelic frequencies for markers in the major QTL region in resistant and susceptible groups. If the percentage of heterozygous plants is significantly higher in the resistant group than that in the susceptible group, it indicates presence of a resistance QTL near this marker. Furthermore, a collection of 41 DH lines, 96 F_2 plants, 390 BC_2F_1, and 1406 BC_3F_1 individuals have been used to calculate the frequency of resistant plants in groups of different genotypes to confirm the identity of the resistance QTL.

Development of PCR-based markers

A major QTL has been detected in bins 10.03/4 in the initial QTL mapping population. The QTL confidence interval extends six BAC contigs according to the B73 BAC-based whole-genome physical map (http://www.maizesequence.org/). BACs, ESTs, and IDP sequences available in BAC contigs have been exploited to develop new polymorphic markers. Single or low-copy sequences have been detected by aligning retrieved sequences with high-throughput genomic sequences (HTGS) found in NCBI (http://www.ncbi.nlm.nih.gov/). All primers have been designed using the software PRIMER5.0 along with the following parameters: primer length is 20 nucleotides with a 40–60% GC content, and absence of secondary structure and consecutive tracts of a single nucleotide.

The microsatellite search tool, SSRHunter1.3 (Li and Wan 2005), was employed to mine SSR sequences present in BAC sequences, and flanking regions were used for primer design. Development of SSR markers was deemed successful when PCR products exhibited polymorphism

between parent lines '1145' and 'Y331' on either polyacrylamide or agarose gels.

To develop sequence-tagged sites (STS) and cleaved amplification polymorphic sequence (CAPS) markers, PCR products from the two parental lines were separately cloned into the pGEM-T vector. Three positive clones from each amplicon were sequenced to minimize possible mismatch nucleotides. Using CLUSTALX, multiple sequence alignments among sequenced amplicons and with their comparable B73 sequences were performed to ensure that the correct sequence was obtained. Thereafter, pairwise alignments were conducted to uncover all sequence divergences between the two parents. Insertion/deletions (InDels) were used to develop STS markers; while, single nucleotide polymorphism (SNP) was used to design CAPS with the stipulation that the SNP was related to a particular common restriction site. All developed SSRs, STS, and CAPS were validated and mapped onto the major QTL region by genotyping key recombinants and comparing them with the publicly available maize physical map (http://www.maizesequence.org/).

Fine mapping

Fine mapping of the major QTL for stalk rot resistance was performed as follows. First, high-density molecular markers within the QTL region were generated to resolve recombination events. Second, screening of new recombinants was conducted by genotyping all mapping populations using markers within the QTL region. Third, a progeny-testing strategy was adopted to obtain an accurate evaluation of a recombinant for resistance to stalk rot. As the major QTL could only increase frequency of resistant plants by ~40% and symptom development heavily depended on environmental factors and genetic backgrounds, an accurate evaluation of stalk rot resistance was critical for cloning of the resistance gene. Recombinants screened from all mapping populations were backcrossed to 'Y331' to generate backcross progenies. A backcross progeny, normally 100 or more plants, were grown in the field and scored for their disease reactions and to determine their genotypes. Those individuals with disease ratings of 1, 2, or 3 were considered resistant; while, those with ratings of 4, 5, or 6 were deemed susceptible. Two genotypes, presence or absence of '1145' donor region, were present in backcross progeny. If the donor region harbored the resistance QTL, plants with the donor region were expected to be more resistant than those lacking the donor region. On the contrary, if the donor region did not cover the resistance QTL, there would be no significant difference among plants with or without donor regions. For each recombinant-derived progeny, the frequency of resistant plants was separately calculated for two genotypes. Differences in resistance reactions between the two genotypes were tested using a two-way ANOVA, and followed by a *t*-test ($P < 0.05$). A $P < 0.05$ value indicated significant correlation between phenotype (resistance) and genotype (donor region) in the tested progeny, and its parental recombinant was deduced to harbor the resistance QTL in its donor region. Analysis of both resistance reaction and fragment size of the donor region for every recombinant allowed delimitation of the resistance QTL.

Results

Maize resistance to *Gibberella* stalk rot in the field

Field evaluation of maize resistance to *Gibberella* stalk rot was conducted for a period of six continuous years in Beijing, China. Following artificial inoculation with *F. graminearum*, the inbred line '1145' showed complete resistance to *Gibberella* stalk rot and no symptoms were observed in the field; while, ~80% of 'Y331' plants were severely infected and exhibited severe stalk rot symptoms (Fig. 1a, b). All F_1 hybrids, regardless of their genetic identity, showed variable levels of resistance to *Gibberella* stalk rot, ranging from highly resistant (rating of 1, 83.6%), resistant (rating of 2, 13.7%), and intermediate resistant/susceptible (ratings of 3 and 4, 2.7%) (Fig. 1c). A total of 500 BC_1F_1 plants were evaluated for their reactions to stalk rot, and a skewed distribution of disease severity was observed. About two-thirds of BC_1F_1 plants were susceptible and one-third was resistant (Fig. 1d). The observed segregation ratio of 1 (resistant) to 2 (susceptible) was inconsistent with a single dominant gene segregating in the BC_1F_1 population. Based on this finding, it was proposed that resistance to *Gibberella* stalk rot was unlikely to be controlled by a single dominant gene (Yang et al. 2005), but was more likely to be a quantitatively inherited trait.

Progeny developed from a resistant recombinant could be divided into two subgroups with or without resistance QTL. All resistant recombinants selected within a period of 5 years (2005–2009) were evaluated, and frequencies of infected plants ranged from 18.24 to 48.90% for subgroups with resistance QTL and from 47.38 to 89.03% for subgroups without resistance QTL. Progeny derived from recombinant lacking the resistance QTL showed a similar frequency as that of the subgroup without resistance QTL (data not shown).

Construction of the linkage map

Of the 500 BC_1F_1 plants, 47 completely resistant and 47 highly susceptible plants were selected to form a BC_1F_1 mapping population. This selection of extreme phenotypes

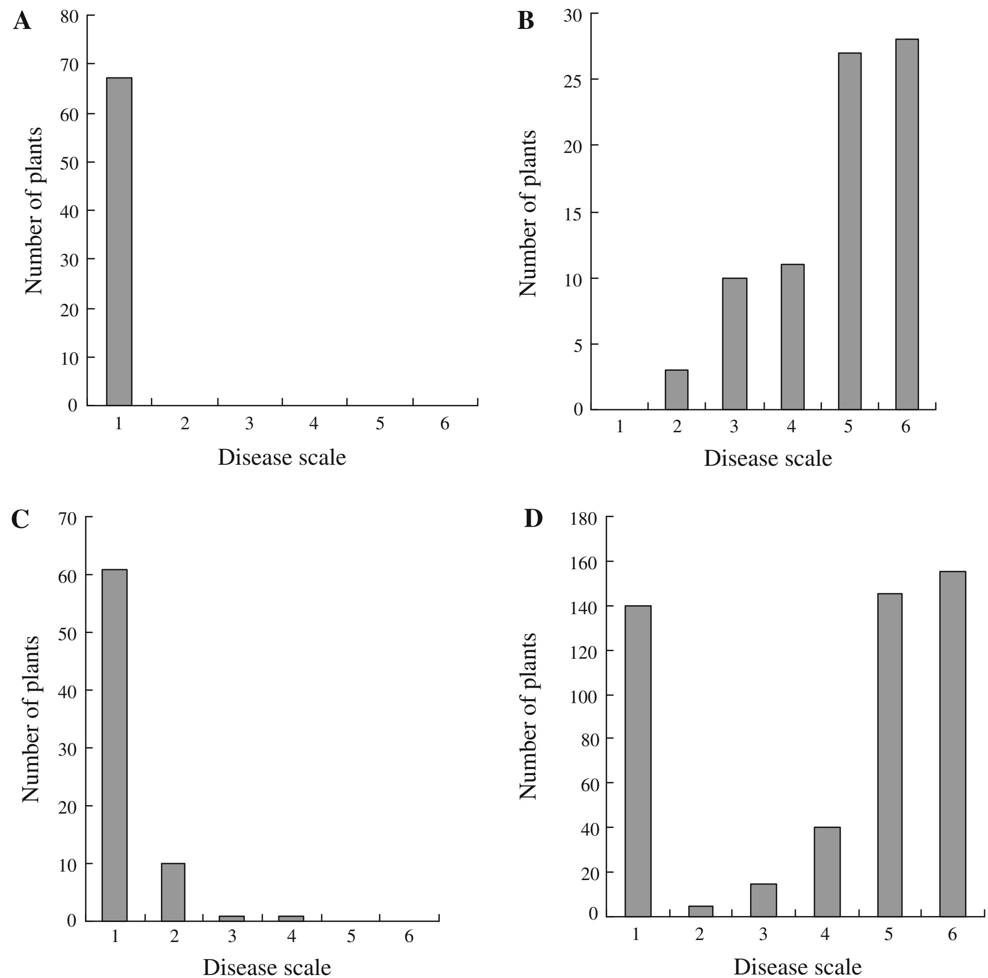


Fig. 1 Distribution of disease severity for the two parental lines, F_1 plants, and BC_1F_1 population. **a** The resistant parental line '1145'. **b** The susceptible parental line 'Y331'. **c** The '1145'/'Y331' F_1 hybrids. **d** The BC_1F_1 population

would highly increase the statistical power in detecting quantitative trait loci (QTLs) (Tanksley 1993). A total of 630 SSRs were screened for their polymorphisms between '1145' and 'Y331', and 118 polymorphic markers were selected to genotype the BC_1F_1 mapping population. Two markers, umc1800 and umc1257, showed severe segregation distortion and could not be placed on any chromosomal region. Thus, these two markers were excluded from the linkage mapping. The remaining 116 SSR markers were used to construct a linkage map using MAPMAKER 3.0b. The linkage map was comprised of 10 linkage groups and spanned a total of 1633 cM in length with an average of 14 cM per marker, and thereby was suitable for QTL detection.

Initial mapping of QTLs for resistance to *Gibberella* stalk rot

Resistance QTLs to *Gibberella* stalk rot were detected in bins 10.03/4 (named *qRfg1*) and bins 1.09/10 (named *qRfg2*) in the BC_1F_1 mapping population (Table 1). The QTLs *qRfg1* and *qRfg2* could explain 36.3 and 8.9% of the total phenotypic variation, respectively. Resistance alleles for both QTLs were derived from the resistant inbred line '1145'. To confirm the major QTL *qRfg1*, allele frequencies at the markers within the *qRfg1* region were tested using the whole BC_1F_1 population with a tetrad grid χ^2 method. A significant bias of allele frequency was observed between the resistant and susceptible BC_1F_1 subgroups for every anchored marker, thereby strongly suggesting presence of a valid resistance QTL in bins 10.03/4 (Table 2).

Additional evidence supporting presence of the QTL *qRfg1* in bins 10.03/04 was obtained from analysis of DH, F_2, BC_2F_1, and BC_3F_1 populations. The DH population consisted of 17 resistant and 24 susceptible lines, and each DH line was genotyped for the *qRfg1* region using the following seven SSRs, umc1246, umc1053, umc1453, umc2350, umc1115, umc1678, and umc1280. Of 24 susceptible DH lines, four lines had abnormal bands and were then discarded. The remaining 20 lines were of the same genotype as that of the susceptible parental line 'Y331', except for a single DH line. All 17 resistant DH lines shared the same genotype as that of the resistant parental line '1145'.

Table 1 Identification of resistance QTLs to *Gibberella* stalk rot using the selected BC_1F_1 mapping population

Name of QTL	Chromosome	Bins	LOD value	Genetic effect	Variance (R^2 %)	Source of resistance allele
qRfg1	10	10.03/04	13.1	0.6157	36.3	1145
qRfg2	1	1.09/10	3.8	0.3091	8.9	1145

Table 2 Correlation of genotype and resistance at markers in the *qRfg1* region

Bins	Marker	Percentage of heterozygote[a]		χ^2	*P* value
		In R group	In S group		
10.03	bnlg1079	72.92	25	22.0512	<0.0001
10.03	umc1336	70.83	29.17	16.6667	<0.0001
10.03	umc2349	77.08	25	26.053	<0.0001
10.03	umc2336	68.75	22.92	20.3077	<0.0001
10.04	umc1246	77.08	35.42	16.9312	<0.0001
10.04	phi062	66.67	27.08	15.1007	<0.0001
10.04	umc1053	68.75	22.92	20.3077	<0.0001
10.04	umc2350	72.92	27.08	20.1667	<0.0001
10.04	umc1115	72.92	25	22.0512	<0.0001

R group resistant group

S group susceptible group

P value probability of H0 hypothesis that is independent between genotype and trait

[a] The percentage of plants heterozygous for molecular markers within the *qRfg1* region in each group

Table 3 Resistance percentages under various genotypes in DH, F_2, BC_2F_1, and BC_3F_1 populations

	Genotype			Theoretical ratio	χ^2
	1/1	1/2	2/2		
DH lines					
Resistant plants	17	/	0		
Total plants	18	/	19	1:1	0.02
Resistance percentage	94.44	/	0		
F_2 population					
Resistant plants	19	32	5		
Total plants	22	57	17	1:2:1	3.895
Resistance percentage	86.36	56.14	29.41		
BC_2F_1 population					
Resistant plants	/	118	48		
Total plants	/	211	179	1:1	2.62
Resistance percentage	/	55.92	26.82		
BC_3F_1 population					
Resistant plants	/	372	176		
Total plants	/	728	678	1:1	1.78
Resistance percentage	/	51.1	25.96		

$\chi^2_{0.05,1} = 3.84$; $\chi^2_{0.01,1} = 6.63$; $\chi^2_{0.05,2} = 5.99$; $\chi^2_{0.01,2} = 9.21$

'1/1': homozygote, both alleles were derived from the resistant parent '1145'

'2/2': homozygote, both alleles were derived from the susceptible parent 'Y331'

'1/2': heterozygote, one allele was derived from '1145', and the other was derived from 'Y331'

The genotypic segregations of each population conformed to the expected ratios

Frequencies of resistant plants within the two genotypes in the *qRfg1* region were calculated (Table 3). Significant difference in frequencies of resistant plants between '1145' (94.44%) and 'Y331' (0%) genotypes indicated presence of a major resistance QTL in bins 10.03/04, and the resistant line '1145' contributed the resistance allele.

Likewise, 96 F_2 individuals were individually genotyped for the *qRfg1* region using the following three markers, STS01, phi062, and umc1453. These three markers showed no distortion from the expected segregation ratio of 1:2:1 (Table 3). Dosage effects were also observed wherein the numbers of resistance alleles were related to frequencies of resistant plants. Homozygous '1145'/'1145' alleles (1/1) had the highest frequencies of resistant plants (86.36%), followed by heterozygous '1145'/'Y331' alleles (1/2) exhibiting an intermediate frequency of resistant plants (56.14%), and finally homozygous 'Y331'/'Y331' alleles (2/2) exhibiting the lowest frequency of resistant plants (29.41%). These results not only further confirmed presence of a major resistance QTL, but also suggested existence of a particular genetic mode for resistance to *Gibberella* stalk rot.

A total of 390 BC_2F_1 individuals derived from recombinants carrying the resistance QTL were genotyped for the *qRfg1* region using three markers (umc2349, unc1246 and umc1453). The frequency of resistant plants was 55.92% for heterozygous (1/2) and 26.82% for homozygous (2/2) alleles. Furthermore, analysis of 1406 BC_3F_1 individuals indicated that frequency of resistant plants was 51.1% for heterozygous (1/2) and 25.96% for homozygous (2/2) alleles. Collectively, all these data confirmed presence of a major QTL, *qRfg1,* in the resistant parent '1145' that conferred resistance to *Gibberella* stalk rot.

Development of PCR-based markers in the *qRfg1* region

The major QTL covers a large chromosomal region in the initial QTL analysis, owing to a limited population size and

variability in resistance evaluation. Thereby, fine mapping is necessary to narrow down the QTL region to allow for cloning of the resistance gene as well as for its use for MAS.

As the first step towards fine mapping, the *qRfg1* region must be saturated with high-density molecular markers. Accordingly, single/low-copy BAC sequences available in bins 10.03/4 were retrieved from the maize database and used to mine SSR sequences. A total of 320 sequences containing SSRs were obtained from 30 anchored BACs covering the whole *qRfg1* region. Following BLASTn comparison against the maize HTGS database, 180 of 320 SSR-containing sequences were found to be low-copy sequences and suitable for designing SSR primers. Of 180 primer pairs, 41 yielded polymorphic bands between '1145' and 'Y331', and were then converted into SSR markers (Table 4). Thus, development of SSR markers depended on both appropriate SSR-flanking regions and polymorphisms between parental lines.

Single/low-copy BAC sequences and anchored ESTs/IDPs were used to develop PCR-based STS and CAPS markers. Following BLASTn comparisons against the HTGS database of NCBI, single/low-copy BAC sequences were used to design primers to amplify parental lines. For anchored ESTs, sequences flanking introns were used to design primers to exploit sequence variations associated with non-coding regions. For anchored IDPs, primers were designed on end sequences to amplify fragments as long as possible. Sequence divergences from either BACs or ESTs or IDPs were used to develop either STS or CAPS markers. In total, 74 unique primer pairs were designed of which 12 co-dominant STS, a single dominant STS, and five CAPS markers were developed and demonstrated presence of distinct polymorphic bands between the two parental lines (Table 4). As the dominant STS marker cannot distinguish heterozygotes from homozygotes, it was excluded in the present study.

For those SSR markers that are closely linked to the major QTL but failed to yield perfect PCR amplification, primers were re-designed to optimize the utility of these SSRs. For example, the SSR marker umc1053, corresponding to the gene coding cell wall invertase (AF043346), is located in the middle of the major QTL region, new primers were designed within the umc1053 sequence and were then combined with the original umc1053 primers to form various primer combinations. Each primer pair was used to amplify the two parents, and the resultant PCR products were simultaneously separated on 2% agarose to select for the best primer combination. Fortunately, the primer pair umc1053RP/AF043346LP was found to yield distinct and polymorphic bands between the two parents. These optimized SSRs were more efficient and reliable in PCR reactions, thus contributing to an efficient and cost-effective genotyping.

Fine mapping of *qRfg1*

Initial QTL analysis suggested that the major resistance QTL *qRfg1* resided between bnlg1079 and umc1115 in bins 10.03/4. To fine map *qRfg1*, the flanking markers bnlg1079 and umc1115 were used to screen recombinants from all BC_2F_1 plants. A total of 19 BC_2F_1 selected recombinants were backcrossed to 'Y331' to develop corresponding BC_3F_1 populations. From these 19 BC_3F_1 populations (a total of 2182 BC_3F_1 individuals), 24 recombinants were screened for the *qRfg1* region by using the flanking markers bnlg1079 and umc1115, and their genotypes were investigated using 11 markers (umc2349, CAPS01, STS01, STS02, umc1246, phi062, umc1053, STS06, umc1453, umc2350, and bnlg2336) within the bnlg1079/umc1115 interval (Fig. 2). Each BC_3F_1 recombinant was backcrossed to 'Y331' to generate the subsequent BC_4F_1 progeny. QTL analysis of 24 BC_3F_1 recombinants and their BC_4F_1 progeny narrowed down the *qRfg1* region into the confidence interval between umc2349 and phi062 (Fig. 2a). The QTL *qRfg1* could explain 50.83% of the total phenotypic variation and exerted its genetic effect to increase the frequency of resistant plants by 34.02%. As shown, this fine mapping step not only narrowed down the *qRfg1* region, but also increased the contribution of *qRfg1* to the total phenotypic variation. Similarly, based on 34 BC_4F_1 recombinants and their 2683 BC_4F_2 plants, a QTL peak was also detected in the same interval of umc2349/phi062 and, in this case, the QTL *qRfg1* could explain 65.47% of the total phenotypic variation and increased the frequency of resistant plants by 31.62% (Fig. 2).

In an attempt to delimit *qRfg1* to a single or a few candidates, recombinant events within or very close to the *qRfg1* locus must be occur and ought to be identified. Thus, BC_4F_1 populations were individually genotyped at four *qRfg1*-associated markers, including umc2349, STS01, umc1246, and phi062, and 36 BC_4F_1 recombinants were identified. These 36 BC_4F_1 recombinants were further investigated at 12 newly developed markers (SSR118, SSR239, SSR243, STS434, SSR248, SSR106, SSR105, STS02, SSR179, SSR261, SSR156, and SSR164) to reveal detailed structures of their donor regions (Fig. 2). Meanwhile, each BC_4F_1 recombinant was backcrossed to 'Y331' to produce a corresponding BC_5F_1 progeny. All 4325 BC_5F_1 plants were individually investigated for their genotypes and phenotypes to deduce phenotypes of all 36 BC_4F_1 recombinants. QTL analysis of these BC_4F_1 recombinants and their BC_5F_1 progeny narrowed down the *qRfg1* locus to the STS01/SSR106 interval with a higher LOD score of 11.93 and a higher genetic contribution (76.07%) to the total phenotypic variation (Fig. 2). Whereas, the genetic effect of *qRfg1* to *Gibberella* stalk rot remained fairly stable (35.15%). For the BC_5F_1 population, 41 new

Table 4 The newly developed molecular markers for fine-mapping of *qRfg1*

Contig	Location (Mb)	BAC	Marker	Forward primer (5′–3′)	Reverse primer (5′–3′)	Type	Annealing temperature (°C)	Predicted size(bp)	Restriction enzymes
409	94.3	AC207764.1	CAPS372	CATCGTCAGGTAGGAGTCGT	GAGCGTGCGGAGAGAATAAG	CAPS	60	697	PvuII
409	94.5	AC199598.3	CAPS353	CGCTAAGTCGGACCAGTAAT	CCTGAGGCACGTAACGGTAT	CAPS	60	435	CfrI
409	94.7	AC199190.2	CAPS401	GGCATAAGTTCAGAGAGGTT	CAAGACATCTCAAGGCTCAA	CAPS	60	541	BglI
409	94.7	AC199190.2	CAPS402	TCGCTCTTGAGCCTTGAGAT	GCAACGCATGGAGTATCAAC	CAPS	62	719	MnlI
409	94.8	AC208625.2	CAPS429	TGATACAAGCTGCACTTCAT	GAATCCTCATCGCTCACTTC	CAPS	60	531	AvaII
409	89.3	AC203357.2	SSR35	CTAGAAGACGAACGACGCAC	CCGTAGCAAGAACTCAGCAG	SSR	60	150	
408	89.3	AC203357.2	SSR36	CCTTGCCTTCATCACATCAC	GCCACCTGAGGAGGAGAATA	SSR	60	100	
408	89.2	AC199910.3	SSR44	CAGACACAGTCATGGCATTG	GATGGCTGCATCGACAGGTA	SSR	62	230	
408	89.2	AC199910.3	SSR46	GTGCCTTGCCTTCATCACAT	GAATAGTAGTGCGGCAGCAG	SSR	60	120	
408	89.2	AC199910.3	SSR47	CACACACATCCACAAGACCT	TCCAAGACAAGAGCTTCAGC	SSR	62	260	
408	89.8	AC199550.2	SSR77	GCATCGCAAGCACCTCTCTT	TCCTTCGTATGAGCCGTTAC	SSR	58	230	
408	91.2	AC199220.2	SSR85	GGATCTTCGTTGCAGTTCTT	CATCAGTGATCCTCCACCAT	SSR	58	150	
408	91.2	AC199220.2	SSR87	CGATGCAGCAGATTCCTCGT	CATGCACATGATTGGTTGGT	SSR	58	100	
408	91.2	AC199220.2	SSR90	GGTAAGTATCGGACATTCCT	CCGGGAGCATGTATCTGTAT	SSR	58	100	
408	91.2	AC199220.2	SSR93	CGCCGTACAGACTGCTATGA	CACATGCTACGACTGCGATG	SSR	60	230	
408	91.7	AC188000.3	SSR100	CGTGCGACTAAGGATAGCTG	CGACCACGATACAACCTCAT	SSR	58	100	
409	92.7	AC203310.3	SSR198	GCCTCCACTTCAGCATACCA	CCATCTTCATTCCATCCACC	SSR	60	166	
409	93.4	AC188611.2	SSR114	CTCGTGCTTATACCATCTCC	TAGAAGACCTCGTGGCATGT	SSR	57	240	
409	93.7	AC200226.2	SSR118	TACGCATCGTCATCGTCGTC	CCTCCATCGCTTGTCGTGTT	SSR	60	225	
409	93.7	AC200226.2	SSR120	GATTAGCGGATAACGGACAG	TCCAATCCAATCCAATCCAG	SSR	60	254	
409	93.8	AC217945.4	SSR334	TTCGAGCATGCCAAAGAAGT	GGTGCACACAGACATGGAAT	SSR	58	301	
409	93.8	AC217945.4	SSR337	CACCAGCTTAATTGTCCTGT	CCACCGTAACAACTCGTACT	SSR	58	96	
409	93.9	AC199625.3	SSR343	CTATCCCACCGTTGCTTCCT	CTGAGAGATCGAGCGAGGAT	SSR	58	244	
409	93.9	AC199625.3	SSR344	GCATGGCTCATCCCTTACTT	TGAGAGATCGAGCGAGGATA	SSR	58	164	
409	94.3	AC207764.1	SSR58	GACGCTGCACAATAGGTTCT	TCATATACACCGACGACCTG	SSR	58	120	
409	94.5	AC199598.3	SSR239	GGACTGCTAGATGCCATGTT	CTACAAGCCAAGCCTGGATT	SSR	60	188	
409	94.7	AC199190.2	SSR243	TAGAGGACGTTGTTGGAGAG	CTGATCGAGAGTGTCGTGAG	SSR	60	294	
409	94.8	AC208625.2	SSR248	TTCAAGTAGCAGCATGCATC	GACGAGATACGCGACTACGA	SSR	60	253	
409	94.9	AC210829.2	SSR255	TCGACGAGATACGCGACTAC	CAGTACAAAGCCGATCCAAG	SSR	60	207	
409	94.9	AC210829.2	SSR256	GCCAAGAGTTCTAAGCACTG	TTCAAGTAGCAGCATGCATC	SSR	60	218	
409	95.2	AC199413.2	SSR106	TTGAAGTCAGCAGGAGTTGG	CTTGCTTGCTCTTGGTCCAC	SSR	60	110	
409	95.4	AC199353.2	SSR105	GTTCATCCTGATTCCCATCC	CAGCCTTGCTTCTACACCAC	SSR	60	110	
409	95.5	AC208863.3	SSR300	TGCCTCACCTGCGTAATGTG	CTGCTGCCACTGCCATCTAC	SSR	58	215	

Table 4 continued

Contig	Location (Mb)	BAC	Marker	Forward primer (5′–3′)	Reverse primer (5′–3′)	Type	Annealing temperature (°C)	Predicted size(bp)	Restriction enzymes
409	95.6	AC197328.3	SSR301	CCATCTCTGTTGTCTTGGAT	GTCGAGGTACAGTCTTGCAT	SSR	58	210	
409	95.7	AC191701.2	SSR302	TAATGGCAGACCGAGTCTTC	CTCGCTCTTAACTGCTACGC	SSR	58	202	
409	96.1	AC203323.2	SSR12	CATCATCGTCGTCATCGGTC	TCATCCATGTTATGCCTGCC	SSR	60	236	
409	96.1	AC203323.2	SSR14	CTGGACTCCACAACCTCATC	CCGGCACTGTAAGTACATTG	SSR	60	210	
409	96.4	AC197223.2	SSR285	GAGACATAGCGGCTTATGGT	CGCTTATTGTGAGCTGCTCT	SSR	60	370	
409	97.1	AC197035.3	SSR179	GTAAGCAACATACCCTCTGT	CGAAAAACTATGAGTACGGA	SSR	57	270	
409	97.6	AC207633.3	SSR261	GGAGTATCAATCTTCGAGGC	GTGGTCAATGCAATTCAGAG	SSR	60	173	
411	99.9	AC199934.3	SSR152	GTATATGTAGCCAGGCATCC	CTCTTCCTCTCGCAATGTTG	SSR	58	150	
411	101.5	AC190767.1	SSR147	TTGTGTCAACACCTCCAGAT	GCGCCTAGGAAGATTAAGTG	SSR	58	100	
411	102.4	AC190929.3	SSR156	GGTGTATGAAGTGCTTGGTA	GGGTTAGGGTCCTGTAAGTA	SSR	58	250	
411	105.1	AC202104.3	SSR164	CACGCAGTCATGTGAGGTCC	GGAGGCAGACTCTTGGCGAT	SSR	60	250	
411	112	AC198934.2	SSR172	CCAATGTGGTCTCAGAAACG	CCGAAAATGATGCAGAATGT	SSR	58	260	
411	112	AC198934.2	SSR173	GACGTGGTAGGACCGTTGAA	CATGCGAGACCCGTTGAAAT	SSR	58	250	
409	92.7	AC203310.3	STS01	CCTCCGGTACGCACCTTACT	CCAAGGTCAACTTCAGCCAT	STS	60	500	
409	93.8	AC217820.3	STS378	TGCAGCAGGTTCATGTTTAT	TTCCAACTTATCAGCGACGA	STS	60	567	
409	94.3	AC207764.1	STS373	CATCAAGTTACCCCTGGTTT	GCCAACGAGAGTAGCAGTCT	STS	60	177	
409	94.5	AC199598.4	STS400	TTGACATTACACCACTTTCT	CGTATAATTTAGAGCGTGTT	STS	60	292	
409	94.5	AC199598.4	STS414	CCATAGACCAGTCGCACATT	GCCTGACATCACGTACCAGT	STS	64	396	
409	94.5	AC199598.4	STS416	TCCGTTCGACCTGTGCCATT	CCGAATGTCCAGCCTTACAA	STS	62	180	
409	94.7	AC199190.2	STS444	ACTGGATGGAATGGATGGAT	GACGAATAATGATGGCTGCT	STS	60	669	
409	94.7	AC199190.2	STS446	TAGTGCAACACGTCCGATCT	GCCTAGCGTAGCCATTCAAT	STS	64	584	
409	94.8	AC208625.2	STS434	GTGATATTCCGGTGCCTAGT	CATGACATGGTGCTTGATCT	STS	60	429	
409	96.2	AC200323.2	STS02	ATCTACAACGGCACGCTGAT	CCTCCAGTTCTAGCCAGCTT	STS	60	1500	
412	114	AC199372.2	STS03	CTTGTATCATCAGCTAGGGCATGT	GTGATCTGAACGCCAACCTC	STS	58	300	
412	114	AC199372.2	STS04	GTGCCAAGGACAGTGTCAAT	CTTCAGGACCATCGAACAGA	STS	60	1200	
412	115.8	AC193656.3	STS06	CGACCAACACATTTCCTACG	CAAGAAGGACCCAGAGAACG	STS	53	250	

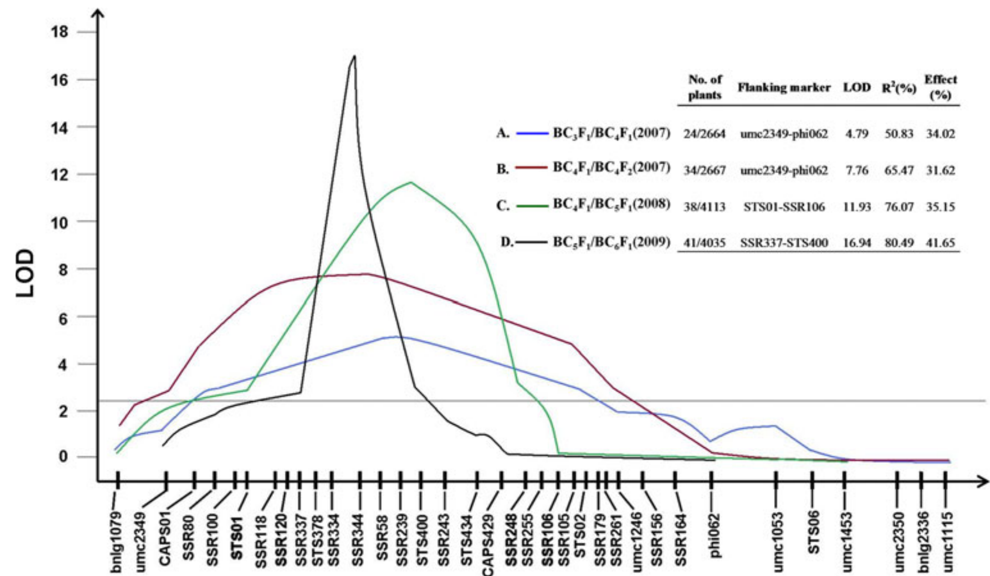


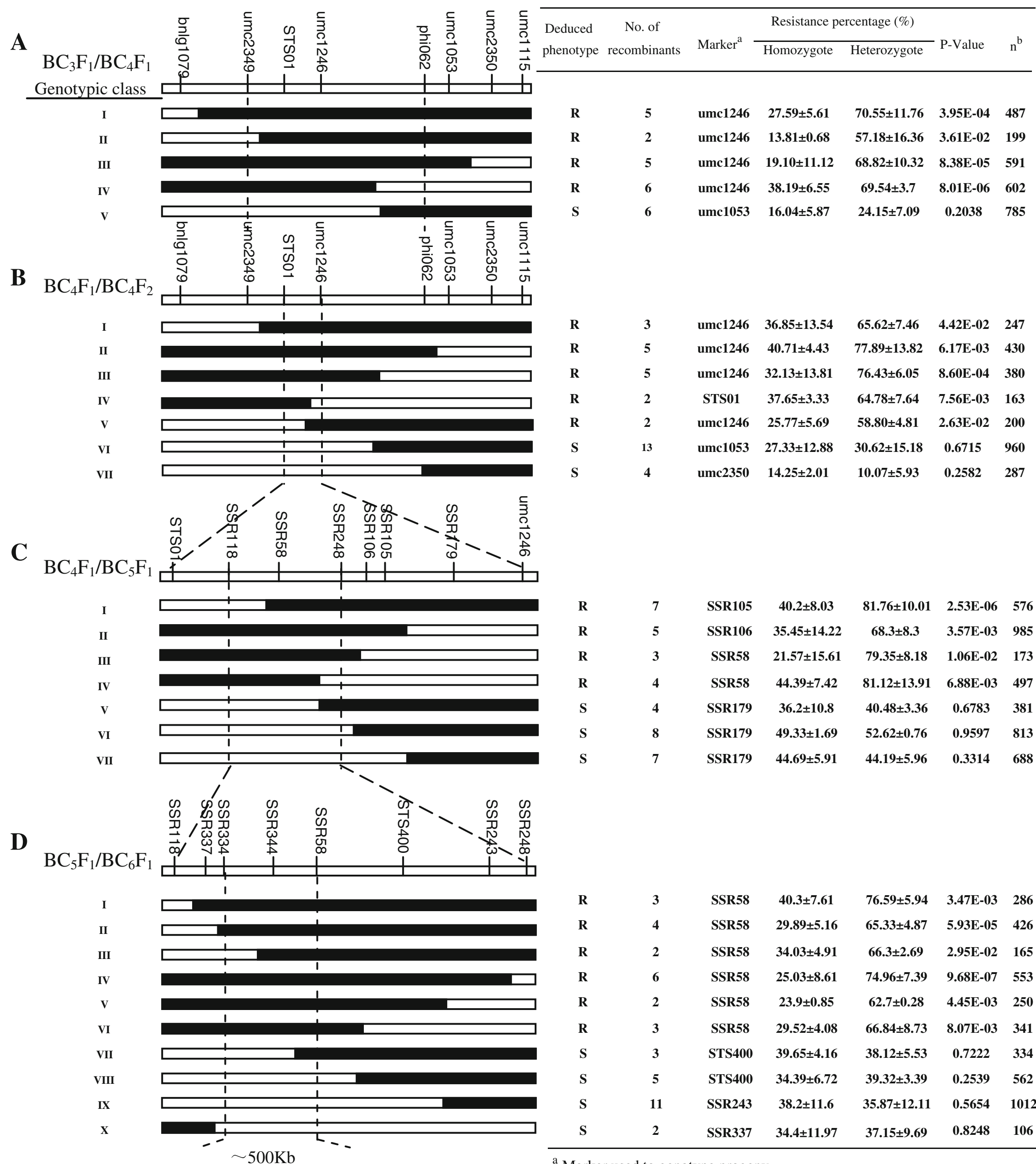
Fig. 2 Diagram of the *qRfg1* plot in four backcross generations. By using QTL cartographer version 2.5, logarithm of odds (LOD) profile, relative position of *qRfg1,* and relevant markers are displayed. **a** QTL plot of 24 $BC_{3:4}$ families consisting of 2664 BC_4F_1 plants. **b** QTL plot of 34 $BC_4F_{1:2}$ families consisting of 2667 BC_4F_2 plants. **c** QTL plot of 38 $BC_{4:5}$ families consisting of 4113 BC_5F_1 plants. **d** QTL plot of 41 $BC_{5:6}$ families consisting of 4035 BC_6F_1 plants

recombinants in the STS01/SSR106 interval were screened and genotyped at the 12 high-density markers (SSR120, SSR337, STS378, SSR334, SSR344, SSR58, STS400, SSR243, STS434, CAPS429, SSR248, and SSR255) (Fig. 2). A total of 4340 BC_6F_1 progeny developed from the 41 BC_5F_1 recombinants were investigated for their phenotypes and genotypes. QTL analysis of these BC_5F_1 recombinants and BC_6F_1 progeny pinpointed presence of a sharp QTL peak within the confidence interval of SSR337/STS400 with an even higher LOD score (16.94) and a higher *qRfg1* contribution (80.49%) to the total phenotypic variation. Similarly, *qRfg1* could enhance frequency of resistant plants, by 41.65%, in these advanced backcross populations (Fig. 2).

In addition to QTL analysis, a progeny test was performed via the two-way ANOVA *t*-test ($P < 0.05$) to deduce the phenotype of a given parental recombinant. Following genotyping for the *qRfg1* region using 13 markers, 24 BC_3F_1 recombinants could be classified into five genotypic types. Five type I and two type II recombinants carried the donor regions downstream of bnlg1079 and umc2349, respectively, and exhibited resistance to *Gibberella* stalk rot with $P < 0.01$. On the other hand, five type III and six type IV recombinants carried the donor regions upstream of markers umc2350 and phi062, and also exhibited *Gibberella* stalk rot resistance ($P < 0.01$) (Fig. 3a). Six type V recombinants carried donor regions downstream of umc1246 and showed susceptible reactions ($P > 0.05$). Altogether, these results indicated that *qRfg1* was located in the chromosomal segment between umc2349 and phi062. A similar analysis was conducted using 34 BC_4F_1 recombinants whose phenotypes were deduced from their self-pollinated 2667 BC_4F_2 plants. Two type IV recombinants (resistant) and two type V recombinants (resistant) had their nearest crossovers around the *qRfg1* locus, and this allowed fine mapping of *qRfg1* and narrowing it to the STS01/umc1246 interval (Fig. 3b). Further narrowing down of the *qRfg1* locus based on the 38 BC_4F_1 recombinants and their BC_5F_1 progeny resulted in a relative short *qRfg1* region of ~1.3 Mb, and flanked by SSR118 and SSR248 (Fig. 3c). Within the defined *qRfg1* region, additional markers were developed (Table 4), and 41 BC_5F_1 recombinants were also screened for fine-mapping. Among 41 BC_5F_1 recombinants, two type III recombinants had the nearest crossovers left to the *qRfg1* locus and showed resistance; while, three type VII recombinants had the nearest crossovers right to the *qRfg1* locus and showed susceptibility (Fig. 3d). It could be concluded that *qRfg1* must reside within the SSR334/SSR58 interval. Based on the maize genome sequence (http://www.maizesequence.org), the physical distance between SSR334 and SSR58 was estimated to be ~500 kb.

Estimation of the QTL effect

The genetic effect of *qRfg1* was investigated using BC_3F_1, BC_4F_1, BC_4F_2, BC_5F_1, and BC_6F_1 populations during the years 2006–2009. As expected, plants carrying the *qRfg1* region showed higher resistance than those without *qRfg1*. In 2006, a total of 2182 BC_3F_1 plants were investigated, and the frequency of resistant plants was estimated to be 66.2% for BC_3F_1 plants carrying the *qRfg1* region compared to 33.9% for those plants lacking the *qRfg1* region. Of the 2664 BC_4F_1 individuals grown in 2007, the frequencies of resistant plants with and without the *qRfg1* region were calculated to be 66.5 and 23.2%, respectively. In 2008, frequencies of 77.6% and 40.9% resistant plants were observed in the BC_5F_1 population (consisting of 4113



Panel	Genotypic class	Deduced phenotype	No. of recombinants	Marker[a]	Resistance percentage (%) Homozygote	Resistance percentage (%) Heterozygote	P-Value	n[b]
A	I	R	5	umc1246	27.59±5.61	70.55±11.76	3.95E-04	487
A	II	R	2	umc1246	13.81±0.68	57.18±16.36	3.61E-02	199
A	III	R	5	umc1246	19.10±11.12	68.82±10.32	8.38E-05	591
A	IV	R	6	umc1246	38.19±6.55	69.54±3.7	8.01E-06	602
A	V	S	6	umc1053	16.04±5.87	24.15±7.09	0.2038	785
B	I	R	3	umc1246	36.85±13.54	65.62±7.46	4.42E-02	247
B	II	R	5	umc1246	40.71±4.43	77.89±13.82	6.17E-03	430
B	III	R	5	umc1246	32.13±13.81	76.43±6.05	8.60E-04	380
B	IV	R	2	STS01	37.65±3.33	64.78±7.64	7.56E-03	163
B	V	R	2	umc1246	25.77±5.69	58.80±4.81	2.63E-02	200
B	VI	S	13	umc1053	27.33±12.88	30.62±15.18	0.6715	960
B	VII	S	4	umc2350	14.25±2.01	10.07±5.93	0.2582	287
C	I	R	7	SSR105	40.2±8.03	81.76±10.01	2.53E-06	576
C	II	R	5	SSR106	35.45±14.22	68.3±8.3	3.57E-03	985
C	III	R	3	SSR58	21.57±15.61	79.35±8.18	1.06E-02	173
C	IV	R	4	SSR58	44.39±7.42	81.12±13.91	6.88E-03	497
C	V	S	4	SSR179	36.2±10.8	40.48±3.36	0.6783	381
C	VI	S	8	SSR179	49.33±1.69	52.62±0.76	0.9597	813
C	VII	S	7	SSR179	44.69±5.91	44.19±5.96	0.3314	688
D	I	R	3	SSR58	40.3±7.61	76.59±5.94	3.47E-03	286
D	II	R	4	SSR58	29.89±5.16	65.33±4.87	5.93E-05	426
D	III	R	2	SSR58	34.03±4.91	66.3±2.69	2.95E-02	165
D	IV	R	6	SSR58	25.03±8.61	74.96±7.39	9.68E-07	553
D	V	R	2	SSR58	23.9±0.85	62.7±0.28	4.45E-03	250
D	VI	R	3	SSR58	29.52±4.08	66.84±8.73	8.07E-03	341
D	VII	S	3	STS400	39.65±4.16	38.12±5.53	0.7222	334
D	VIII	S	5	STS400	34.39±6.72	39.32±3.39	0.2539	562
D	IX	S	11	SSR243	38.2±11.6	35.87±12.11	0.5654	1012
D	X	S	2	SSR337	34.4±11.97	37.15±9.69	0.8248	106

[a] Marker used to genotype progeny.

[b] Total number of progeny derived from the same genotypic class.

Fig. 3 Sequential fine-mapping of *qRfg1*. The genetic structure for each recombinant type is depicted as blank and filled rectangles, corresponding to homozygous 'Y331'/'Y331' alleles and heterozygous '1145'/'Y331' alleles, respectively. The frequency of resistant plants in progeny with or without donor regions are listed in the table (on the right). Significant differences ($P < 0.05$) between the two subgroups correspond to presence of *qRfg1* in the donor region, and thus their corresponding recombinant types are deemed to be resistant (R). No significant differences ($P > 0.05$) between the two subgroups suggest a lack of *qRfg1* in the donor region, and thereby, their corresponding recombinant type is deemed to be susceptible (S). Analysis of both the donor region and the phenotype of each recombinant type has permitted fine-mapping of the *qRfg1*. **a** Fine-mapping using 24 BC_3F_1 recombinants narrowed down the *qRfg1* to the umc2349/phi062 interval. **b** Fine-mapping using 34 BC_4F_1 recombinants delimited *qRfg1* to the STS01/umc1246 interval. C: Fine-mapping using 38 BC_4F_1 recombinants delimited *qRfg1* to the SSR118/SSR248 interval. D: Fine-mapping using 41 BC_5F_1 recombinants delimited *qRfg1* to the SSR334/SSR58 interval. [a]Markers used in analysis of genotypes of each progeny. [b]The total number of progeny from each genotypic types

individuals) for those with and without the *qRfg1* region. Finally, 4035 BC_6F_1 plants were evaluated in 2009, and a frequency of 68.8% resistant plants was observed for plants carrying the *qRfg1* region compared to 34.3% for plants lacking *qRfg1* (Fig. 4). These data suggested that a single *qRfg1* allele derived from '1145' could steadily enhance the frequency of resistant plants by 32.3 to 43.3% in the 'Y331' background. Moreover, these results implied that the

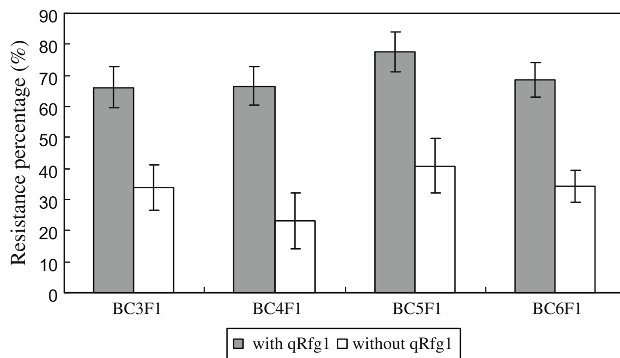


Fig. 4 The effect of *qRfg1* in maize resistance to *Gibberella* stalk rot. The *filled column* corresponds to a heterozygous '1145'/'Y331' genotype for *qRfg1*; while, the *blank column* corresponds to the homologous 'Y331'/'Y331' genotype for *qRfg1*. A single '1145' allele at *qRfg1* could steadily enhance frequency of resistant plants by ~34% in the 'Y331' genetic background

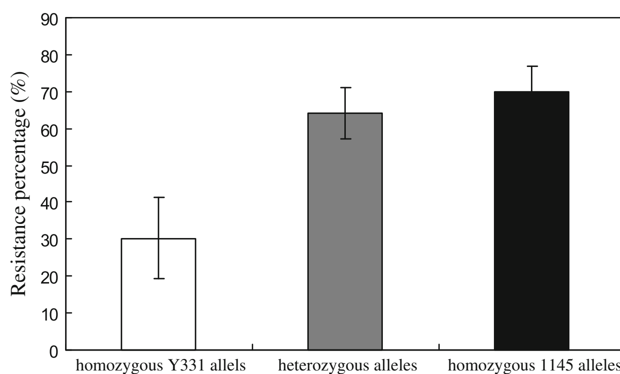


Fig. 5 Frequency of resistant plants in three genotypes of BC_4F_2 family for *qRfg1*

genetic effect of *qRfg1* could steadily be passed down to subsequent generations.

A total of 2667 BC_4F_2 plants were evaluated for their resistance to *Gibberella* stalk rot. The frequency of resistant plants with homologous *qRfg1* alleles was estimated to be 69.8%, slightly higher than those carrying a single *qRfg1* allele (64.0%). However, plants lacking *qRfg1* exhibited a low frequency of resistant plants (30.3%) (Fig. 5). These data suggested that *qRfg1* acted in a partially dominant manner.

Discussion

To date, there is no standardized method for artificial inoculation and evaluation of maize stalk rot. The 'toothpick' and 'injection' methods have been widely adopted in artificial inoculation due to their simplicity and low-cost (Young 1943; Todd and Kommedahl 1994; Tesso et al. 2004). In this study, the 'toothpick' method was used in the years 2002–2003, and was found to be unreliable in eliciting uniform infection across plants. With both the 'toothpick' and 'injection' methods, inoculum was directly injected into the stem, thus this was different from the natural infection route wherein the pathogen invaded roots and resulted in root damage. In an attempt to reveal essential genetic factors underlying resistance to stalk rot, the 'root-infection' method was adopted in this study as it simulated the natural infection process. Although this method was laborious, in preparing large volumes of inoculum and in conducting field inoculations, it was highly reliable and repeatable (Li et al. 2001). Based on disease severity from the years 2004–2009, typical symptoms were observed on the susceptible parent 'Y331' and no symptoms were observed on the resistant parent '1145'. Moreover, phenotypic disease severity between plants with and without the major QTL was clearly distinguishable. In addition, as this inoculation method relied on root infection, this would aid in localizing functionality of the resistance gene(s), whether this took place in roots or in the stem. To develop a reliable phenotypic analysis in this study, symptoms were assessed three times in the field after 1 month following inoculation. Moreover, the stalk of each plant was split longitudinally to exam mycelial growth and root damage.

As demonstrated in this study, resistance to stalk rot in maize is a complicated trait, as it is influenced by both genetic backgrounds and environmental factors. Furthermore, the major QTL *qRfg1* could only enhance the frequency of resistant plants by 32–43%. The combination of these factors has rendered the *qRfg1*-resistance association unstable. Very often plants with *qRfg1* have exhibited susceptibility; whereas, plants without *qRfg1* did not show symptoms. Thus, it is very necessary to understand the exact phenotype of a recombinant in resistance to stalk rot. Accordingly, continuously backcrossing has been conducted in this study to reduce the 'noise' of the '1145' genetic background to provide a reliable and accurate evaluation of the *qRfg1* role in resistance to *Gibberella* stalk rot. Consequently, recombinants selected from advanced backcross generations displayed more reliable performance in resistance to *Gibberella* stalk rot than those selected in early generation. Moreover, we employed progeny-testing method to deduce phenotype of its parental recombinant. Individuals of the same progeny share almost the same genetic background and are exposed to similar environmental conditions. Based on the whole progeny (with at least 100 or more individuals), the genetic effect of *qRfg1* against stalk rot could be exactly estimated and a correlation between genotype (donor region) and phenotype (resistance) can be exactly determined. Significant (or no significant) differences in resistance reactions between subgroups with and without donor regions indicated presence (or

absence) of *qRfg1* in the donor region. Although it seems impossible to evaluate a single individual plant for resistance to *Gibberella* stalk rot due to incomplete penetrance and phenotypic plasticity, the marker-assisted progeny-testing could unambiguously distinguish resistant (with *qRfg1*) from those susceptible (without *qRfg1*) parents. The accuracy of phenotypic evaluation is of the utmost importance in fine mapping and cloning of the target QTL.

Wide variation in resistance to stalk rot was observed in the BC_1F_1 population. In order to efficiently identify resistance QTLs to stalk rot, a limited number of BC_1F_1 individuals that were either completely resistant or highly susceptible were selected for an initial QTL analysis. This might likely reduce the number of QTLs that could be detected, especially those minor QTLs, and contribute to an over-estimation of major QTL effects (Tanksley 1993; Schön et al. 2004; Visscher and Goddard 2004). However, this was a viable approach for this study to identify QTLs as most marker loci displayed the expected segregation ratio of 1 (homozygote): 1 (heterozygote) in the selected BC_1F_1 mapping population. Two QTLs, the major *qRfg1* and the minor *qRfg2*, were detected in this initial QTL analysis, and this major QTL has been subsequently confirmed using DH, F_2, BC_2F_1, and BC_3F_1 populations (Table 1). This major QTL *qRfg1* here was located in the same region along with a QTL for resistance to *Gibberella* stalk rot (chromosome 10, near molecular marker PIO 15-0013) previously reported by Pè et al. (1993) using a mapping population derived from the cross between the susceptible line 33-16 and the resistant line B89. In other studies, Yang et al. (2004) mapped a QTL for resistance to *Gibberella* stalk rot on chromosome 6. However, this QTL could not be confirmed in our study even though the same parental lines and pathogen were used in both studies. This might be attributed to the use of different sets of genetic markers and to different environments during plant growth and evaluation.

It is reported that genes with large genetic effects on a quantitative trait could be accurately mapped and isolated by recurrent backcrossing combined with selection for the trait (Darvasi 1998; Salvi et al. 2007). A high-resolution linkage map is required to narrow down the QTL to a small genomic interval (Salvi and Tuberosa 2005). With availability of the maize genome sequence and abundant polymorphisms among different maize inbred lines, development of new markers closely linked to *qRfg1* becomes relatively feasible. In the present study, a large number of PCR-based co-dominant markers, such as SSRs, STS, and CAPS, were developed to saturate the *qRfg1* region. Of the 180 SSR primer pairs, 41 have been successfully developed into SSR markers useful for differentiating the two parental lines ‘1145’ and ‘Y331’. In addition, some SSR markers have been optimized to give rise to distinct polymorphic PCR bands. The perfect PCR-based markers have proved to be effective, timesaving, and cost-effective for screening of recombinants. Moreover, these markers will be very useful in MAS.

In this study, we have generated several BC populations from recombinants screened from a previous BC population. Interestingly, a number of key recombinants have been obtained in advanced BC population. For instance, as many as 41 new recombinants in the STS01/SSR106 interval have been screened from the BC_5F_1 population (Fig. 2). Fine-mapping of the *qRfg1* locus is greatly aided by the high frequency of recombination events that must have occurred in the *qRfg1* region. Following sequential fine mapping across several BC generations, the major QTL region is gradually narrowed down. With the use of 41 BC_5F_1 recombinants and their BC_6F_1 populations, together with high-density markers, the *qRfg1* region is delimited to a region of ~500 kb.

In concomitance with sequential reduction of the *qRfg1* region from BC_4F_1 to BC_6F_1 generations, the LOD score and R^2 increased from 4.7 to 16.94 and from 50.83 to 80.49%, respectively (Fig. 2). This suggested that a reduction of the ‘noise’ ‘1145’ genetic background greatly aided fine-mapping of the major QTL, in which phenotypic variation was largely derived from the major QTL (Li et al. 2004). Commonly, QTL effects are assessed using the same population utilized for QTL mapping, although these effects are often overestimated (Melchinger et al. 1998). When comparing plants heterozygous for *qRfg1* with those lacking *qRfg1*, differences in frequencies of resistant plants remained rather stable, ranging from 31.62 to 41.65% in different years (Fig. 2). It indicated that the presence of the *qRfg1* locus in the ‘Y331’ background significantly enhances the frequency of resistant plants across the generations.

Isolation of genes underlying QTLs is essential for understanding the genetic mechanism of complex traits. The high-resolution mapping of *qRfg1* described in this study provides a solid foundation for its positional cloning. Gene prediction using FGENESH has revealed ~20 putative genes in the B73 sequence in the *qRfg1* region, although no target resistance genes have been found. In addition, there are no counterparts of typical resistance genes found in syntenic regions of both rice and sorghum. Since the ‘1145’ sequence is not available, it is still remains to be seen as to whether or not a typical resistance gene is present in the *qRfg1* region. Based on predicted genes in the B73 sequence, it is likely that some genes related to growth vigor are candidates for the *qRfg1* gene. The *qRfg1* region will be further narrowed down with additional high-density markers and recombinants to pursue the gene(s) involved in resistance to stalk rot.

The utility of QTL for MAS depends primarily on how narrow of a region the QTL is localized (Lauter et al.

2008). Markers tightly linked to disease resistance gene/QTL can potentially be used for MAS in breeding programs (Young 1999; Mackill 2007). In the present study, the major QTL *qRfg1* is restricted to a ~500 kb region and a number of high-density markers have been developed within and around the *qRfg1* region, thus allowing for their utility in MAS to improve maize resistance to *Gibberella* stalk rot. Since the predicted putative genes in the *qRfg1* region may be involved in vigor and were not related to typical receptor-like resistance genes, it can be assumed that the *qRfg1* will exert durable resistance to *Gibberella* stalk rot.

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