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ORIGINAL PAPER

Identification and fine-mapping of a major QTL conferring resistance against head smut in maize

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Abstract Head smut is one of the most devastating diseases in maize, causing severe yield loss worldwide. Here we report identification and fine-mapping of a major quantitative trait locus (QTL) conferring resistance to head smut. Two inbred lines 'Ji1037' (donor parent, highly resistant) and 'Huangzao4' (recurrent parent, highly susceptible) were crossed and then backcrossed to 'Huangzao4' to generate BC populations. Four putative resistance QTLs were detected in the BC_1 population, in which the major one, designated as qHSR1, was mapped on bin 2.09. The anchored ESTs, IDPs, RGAs, BAC and BAC-end sequences in bin 2.09 were exploited to develop markers to saturate the qHSR1 region. The recombinants in the qHSR1 region were obtained by screening the BC_2 population and then backcrossed again to 'Huangzao4' to produce 59 $BC_{2:3}$ families or selfed to generate nine BC_2F_2 families. Individuals from each $BC_{2:3}$ or BC_2F_2 family were evaluated for their resistances to head smut and genotypes at qHSR1. Analysis of genotypes between the resistant and susceptible groups within the same family allows deduction of phenotype of its parental BC_2 recombinant. Based on the 68 BC_2 recombinants, the major resistance QTL, qHSR1, was delimited into an interval of ~2 Mb, flanked by the newly developed markers SSR148152 and STS661. A large-scale survey of $BC_{2:3}$ and BC_2F_2 progeny indicated that qHSR1 could exert its genetic effect by reducing the disease incidence by ~25%.

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Introduction

Head smut, caused by the host-specific fungus *Sphacelotheca reiliana* (Kühn) Clint, is a soil-borne and systemic disease in maize (Frederiksen 1977). The teliospores from sori buried in soil are the primary source of infection, and can survive 3 years in soil without loss of any infection capacity (Wu et al. 1981). The fungus infects seedlings through root or coleoptile during and after seed emergence (Krüger 1962). If an infection of susceptible variety is ensured, the plants can continue normal vegetative growth, but some may be stunted (Matyac and Kommedahl 1985a). At maturity stage, sori would replace ears or tassels of the infected plants, which results in nearly no yield for the plant. In an individual environment, the portion of infected plants could amount to 80% (Frederiksen 1977). Jin et al. (2000) reported the incidence of this disease varied from 7.0 to 35.0%, some even reached to 62.0%, resulting from the cultivation of susceptible cultivars. In Northern China, head smut causes yield loss of up to 0.3 million tons annually (Bai et al. 1994). It was reported that maize in Southern Europe, North America, and Asia also seriously suffered from this disease (Xu et al. 1999). Considering both

economic and ecological elements, cultivation of resistant varieties is an effective way to control epidemics of head smut, and pyramiding of resistant genes/quantitative trait loci (QTLs) against head smut into elite varieties would be a promising way to improve the resistance of maize against this disease.

Up to date, many researches have been conducted to study the genetic models with regard to resistance against head smut. Mei et al. (1982) reported that resistance against head smut was controlled by partial dominant nuclear genes with no difference being found in reciprocal crosses. Ma et al. (1983) reported maize resistance to head smut was a quantitative trait, affected by partial resistance genes and their non-allelic interactions. Stromberg et al. (1984) discovered that F_1 population showed an intermediate disease incidence between resistant and susceptible parents. Ali and Baggett (1990) reported additive and dominant genetic actions were preponderant under different treatments. Bernardo et al. (1992) studied genetic effect of resistance gene(s) by using generation mean analysis, suggesting that additive effect is decisive, while the dominant and epistatic effects are weak. Shi et al. (2005) reported that apart from additive effect, over-dominance also plays a key role in resistance against head smut. It is obvious that resistance against head smut in maize may involve in a number of genetic elements and act in a complex way.

Genotyping a mapping population consisting of 100 recombinant inbred lines (derived from Hi34 and TZil7) with 120 markers, Lu and Brewbaker (1999) mapped four QTLs on chromosomes 1, 3, 9, and 10, among them, the QTL near the RFLP marker asg30 was the major one. Based on a population of 220 F_3 families produced from the cross of two European elite inbreds (D32 and D145), Lübberstedt et al. (1999) reported three QTLs in France, while eight QTLs in China, among them, an individual QTL could explain up to 14% phenotypic variation. With the 191 $F_{2:3}$ families derived from the 'Mo17' × 'Huangzao4' cross, Shi et al. (2005) detected five QTLs on chrs 1, 2, 3, 8, and 9 at one location and five QTLs on chrs 1, 2, 3, 4, and 7 at another location. A better understanding of the genetic basis underlying resistance against head smut and mapping of the resistance gene would facilitate cloning of the resistance gene and application of the resistance gene in maize breeding program.

Up to date, more than 50 resistance genes from diverse plant species have been cloned, mostly via map-based cloning and transposon tagging approaches. In maize, four resistance genes have been cloned so far, among them, *Hm1* and *Rp1-D*, conferring resistance to respective fungus *Cochliobolus carbonum* race 1 and maize common rust (*Puccinia sorghi*), were cloned via transposon tagging method (Johal and Briggs 1992; Collins et al. 1999), a resistance QTL against *Colletotrichum graminicola* was isolated via map-based cloning (Wolters et al. 2006), and the *Rxo1* gene that conditions a resistance reaction to rice bacterial streak disease was cloned via candidate gene approach (Zhao et al. 2005). With the rapid progress in maize genome sequencing program, map-based cloning would become a predominant approach in future to clone functional genes in maize, especially those resistance genes.

To delimit a QTL/gene into an interval as short as possible is the crucial step to identify the candidate gene(s) (Salvi and Tuberosa 2005). This fine-mapping step relies on development of high-density markers in the target region, which in turn depends on sequence diversity between parental lines. Fortunately, sequence analysis reveals that inserts/deletions (InDels) and single nucleotide polymorphisms (SNPs) are in abundance in maize genomes. Sequence polymorphisms between any two maize inbreds may exceed those between a human being and a chimpanzee (Buckler et al. 2006). The level of diversity between two randomly selected maize genotypes from 25 lines, averaged one SNP per 104 bp, is higher than that of human and *Drosophila melanogaster* (Tenaillon et al. 2001). For the transcribed regions in maize genome, an average of one InDel per 85 bp and one SNP per 31 bp exists in the noncoding regions, respectively; while, one SNP per 124 bp was present in the coding region (Ching et al. 2002). All these sequence divergences provide a resource for developing high-density markers, which would in turn greatly facilitate fine-mapping of the target gene(s)/QTL and eventually lead to isolation of the genes of interest.

The objectives of this research were to identify the major QTL conferring resistance against head smut in maize, to develop new markers to saturate the major QTL region, and to fine map the resistance gene. This research contributes to improve maize resistance to head smut by providing newly developed markers for marker assisted selection (MAS) breeding program.

Materials and methods

Plant materials

Two inbred lines, 'Ji1037' (donor parent) and 'Huangzao4' (recurrent parent), which differ wildly in resistance to the host-specific fungus *S. reiliana* Clint were used as parental lines to develop all mapping populations in this study. All plant materials tested in the present study were artificially inoculated with *S. reiliana* Clint. 'Ji1037' shows fully resistant to head smut and no any susceptible individual has ever been observed in the field; while, 'Huangzao4', an elite Chinese inbred line, is highly susceptible to head smut with ~75% susceptible individuals in the field. In 2004, a BC_1

population consisting of 314 individuals along with two parents was grown in the experimental farm of the Jilin Academy of Agricultural Sciences, Gongzhulin. Each BC_1 individual was evaluated for its resistance against head smut. Resistant BC_1 individuals were backcrossed to 'Hungzao4' to generate $BC_{1:2}$ families (BC_2 population). In 2005, ~20 plants from each $BC_{1:2}$ family were grown in a single plot to evaluate their resistances to head smut. Recombinant individuals from BC_2 population were identified and backcrossed to 'Hungzao4' to generate $BC_{2:3}$ families or self-pollinated to produce BC_2F_2 families. In 2006, approximately 80 individuals from each of the 59 $BC_{2:3}$ and nine BC_2F2 families were grown in the experimental farm of the Jilin Academy of Agricultural Sciences for investigating their resistances to head smut.

Artificial inoculation and resistant scoring in the field

The sori containing teliospores of *S. reliana* were collected from the field in the previous growing season and stored in cloth bag in a dry and well ventilated environment. Before planting, spores were removed from the sori, filtered, and then mixed with soil at a ratio of 1:1,000. The mixture of soil and teliospores were used to cover maize kernels when sowing seeds to conduct artificial inoculation. Plants at maturity stage were scored for the presence/absence of sorus in either ear or tassels as an indicator for susceptibility/resistance.

DNA extraction

Leaf tissues from 1-month-old plants were harvested and ground to a powder in liquid nitrogen. Genomic DNA was extracted followed the method described by Murray and Thompson (1980).

Genotyping at SSR markers and linkage map construction

SSR markers were firstly employed to check their polymorphisms between two parents 'Ji1037' and 'Huangzao4'. Only those SSR markers that showed unambiguously polymorphic bands and evenly distributed across ten chromosomes were used to genotype segregating populations. PCR reactions were performed as follows: denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 30 s, and with a final extension step at 72°C for 10 min. The PCR products were subjected to electrophoresis on 6% polyacrylamide gel, followed by sliver-staining for visualization.

A total of 94 BC_1 individuals were randomly selected from the BC_1 generation and assayed for their genotypes at the 113 polymorphic SSR markers. A PCR band was marked as '2' if it is the same as that of the donor parent, and scored as '1' if it is identical to that of the recurrent parent. The ratio of homozygotes (1/1) to heterozygotes (1/2) in the BC_1 backcross population was analyzed for its consistency of 1:1 at each SSR marker by χ^2 test. The genetic distances between SSR markers were estimated by MAPMAKER/Exp version 3.0b (Lincoln et al. 1992). By the way, some markers on chromosome 2 were genotyped in different scales of populations, and their genetic positions were adjusted with the integration data in the JoinMap software.

Data analysis and QTL/gene mapping

Putative QTLs conferring resistance to head smut were identified according to design III of Trait-Based Analysis (Lebowitz et al. 1987). Briefly, BC_1 individuals with the resistance QTL are expected to be more resistant to head smut than those without the resistance QTL. Consequently, a marker allele adjacent to the resistance QTL in coupling would show higher frequency in the resistant group than that in the susceptible group. A tetrad grids χ^2 test (SAS 8.2 version) was used to test allele frequencies at all markers between the resistant and susceptible groups to scan putative QTL across whole genome. Thereafter, a number of methods were employed to confirm the major QTL region and its effectiveness in resistance to head smut. First, the SSR markers in the putative major QTL region were used to genotype all BC_1 individuals to confirm the presence of the major QTL. Second, infection percentages of BC_1 individuals were estimated based on their $BC_{1:2}$ progenies to confirm the putative major QTL by single-factor analysis of variance. Third, putative QTL was identified across the ten chromosomes by the composite interval mapping method (Windows QTL Cartographer Version 2.0 software). Finally, the major QTL was further confirmed by estimating its genetic effect in reducing disease incidence.

Development of the region-specific markers

Sequences available in the major resistance QTL region, including the anchored EST, IDP, RGA, BAC, and BAC-end sequences, were used to develop high-density markers. These sequences were compared to NCBI and MAGI databases via tBLASTn to obtain possible longer sequences. Primer was designed using the PRIMER5.0 software (http://www-genome.wi.mit.Edu/ftp/pub/software/primer5.0) in accordance with the following parameters: 20 nucleotides in length, GC content of 40–60%, no secondary structure, and no consecutive tracts of a single nucleotide.

Primer pairs were used to amplify the corresponding segments from both parents. The cycling parameters were set up the same as those described above except for the

annealing temperature that was adjusted according to different primer pairs. Only those amplicons with the same or bigger than predicted were cut down from gel and purified with Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). The purified PCR products were then cloned into the vector pGEM-T (Promega, Madison, USA). Normally, three to five positive clones for each amplicon were selected for sequencing to avoid any contamination or mismatch. The amplicon sequence was firstly compared with the original one from which it was derived to make sure the right one was obtained, and then comparison was conducted to search for sequence divergence between two parents by using DNAMAN software. The InDels were amenable for developing sequence-tagged site (STS) markers; while single nuclear polymorphism (SNP) can be used to develop either SNP marker or CAPS marker (cleaved-amplified polymorphic sequence). A CAPS marker is developed if the SNP is related to a given restriction site. In developing SNP marker, a SNPpicker program of SeqVISTA software was used to see if it was possible to create a specific restriction site by introducing a mismatch base pair into primer to alter a 'half-site' to a 'full-site' for a specific restriction site, following the method described by Niu and Hu (2004).

The primer pairs were used to amplify the two parents to develop high-density markers. For STS markers, polymorphic PCR bands should appear after electrophoresis on agarose or polyacrylamide gel. For those CAPS and SNP markers, polymorphic bands could be observed on agarose or polyacrylamide gel after digestion with certain restriction endonucleases.

Fine mapping

Recombinant individuals from the BC_2 population were screened out with the SSR markers in the major QTL region. Due to partial penetrance for head smut resistance, it would be at high risk to judge whether or not a BC_2 recombinant carries the resistance gene based on performance of a single individual. Hence, we adopted a more robust method to judge the presence/absence of the resistance gene for a single BC_2 recombinant based on both genotypes and phenotypes of its progeny. If there is no resistance gene in the donor region for a certain BC_2 recombinant, its progeny with donor regions would show no difference with those without donor regions in resistance to head smut. On the contrary, if the donor region harbors the resistance gene, the progeny with the donor regions would show significantly higher resistant than those without the donor regions. By comparing the insert sizes of the 'resistant' and 'non-resistant' donor regions, we could fix on an interval where the resistance gene resides on. With an application of the newly developed high-density markers, we could definitely define the donor regions harboring the resistance gene and therefore narrow down the resistance region into a very short interval. In all comparisons, significant differences were estimated on SAS software using χ^2 test.

Results

Construction of the SSR linkage map

A total of 700 SSR markers (http://www.maizeGDB.org) were checked for their polymorphisms between 'Ji1037' and 'Huangzao4'. Among the 347 polymorphic SSR markers, 113 markers evenly distributed across ten chromosomes were selected to genotype the BC_1 mapping population. Of these 113 markers, 33 (29.2%) showed distortion segregation at $P < 0.05$ or at $P < 0.01$. Generally, markers showing genetic distortion had no negative impact on QTL detection. Therefore, a linkage map was constructed using all 113 SSR markers. The map was ~1753.4 cM in length with one marker in every 14.6 cM averagely.

Mapping putative QTLs

According to the Design III of TB analysis (Lebowitz et al. 1987), each of the 113 SSR markers was tested for its frequency at 1/2 (heterozygote) and 1/1 (homozygote) in both the resistant and susceptible groups. The significant biases at frequencies between the resistant and susceptible groups were observed for those markers located on the four chromosomal regions (bins 1.02/3, 2.08/9, 6.07, and 10.03/4), suggesting the presence of four putative QTLs (Table 1). For instance, the markers on bin 2.09 showed no distortion from 1:1 ratios of heterozygote to homozygote in the whole BC_1 population. However, percentages of heterozygote at these markers significantly differ between the resistant and susceptible groups with the P values < 0.0001 (Table 1). The result strongly indicated the presence of a major QTL (named as qHSR1) in this region. Markers on both bin 10.03/4 and bin 1.02/3 had the P values < 0.01 (Table 1), implying the presence of putative QTLs with less effects in these two regions. Markers on bin 6.07 also showed skewness with the P values < 0.05 (Table 1), suggesting the presence of a possible minor QTL. In addition, only one marker on bin 4.01 or bin 5.03 was found to show frequency skewness between the resistant and susceptible groups (Table 1), it was, therefore, difficult to judge whether or not a QTL was actually present in these two bins.

Percentages of heterozygote (1/2) in bin 2.09 and bin 10.03/4 were significant higher in the resistant group than those in the susceptible group, suggesting the resistance alleles were derived from the donor parent 'Ji1037'. On the contrary, heterozygotes (1/2) in bin 1.02/3 and bin 6.07 had

Table 1 Scanning putative QTL across the whole genome via a tetrad grids χ^2 test at the 113 SSR markers

bins	Markers	Percentage of heterozygote (%)		$\chi 2$	*P* values	Putative QTL
		In R group	In S group			
1.02	bnlg1614	48.65	71.43	4.93	0.0265	Yes
1.02	bnlg1083	50.00	72.73	5.00	0.0253	
1.03	umc1403	44.74	76.36	9.69	0.0019	
2.08	bnlg1141	65.63	36.36	6.95	0.0084	Yes
2.08/09	umc1230	68.57	40.38	6.66	0.0099	
2.09	bnlg1520	72.22	36.36	11.19	0.0008	
2.09	umc1525	81.08	33.93	19.87	<0.0001	
2.09	umc1736	86.11	30.00	26.49	<0.0001	
2.09	bnlg1893	91.67	26.00	36.28	<0.0001	
2.09	umc1207	91.67	26.53	35.46	<0.0001	
2.09	phi427434	91.43	29.63	32.64	<0.0001	
2.09	umc2184	94.74	30.19	37.65	<0.0001	
2.09	umc2077	94.59	28.85	37.96	<0.0001	
2.09	umc2214	92.11	34.55	30.58	<0.0001	
4.01	umc1164	60.00	37.21	4.02	0.045	?
5.03	umc1447	56.76	34.00	4.48	0.0344	?
6.07	umc1063	34.21	57.14	4.78	0.0289	Yes
6.07	phi299852	33.33	56.36	4.638	0.0314	
10.03	umc1938	76.47	34.69	14.038	0.0002	Yes
10.04	phi062	72.97	41.07	9.128	0.0025	

SSR markers on each bin are ordered according to their positions on the genetic linkage map of the present study

R group, resistant group; S group, susceptible group; *P* value, probability of H0 hypothesis that is independent between genotype and trait

lower percentages in the resistant group compared with those in the susceptible group, indicating that the resistance alleles were derived from the susceptible parent 'Huangzao 4'.

Comparisons of the four putative QTLs in the present study with those detected by other groups resulted in two common QTLs. The QTL in bin 1.02/3 in this study was also reported by Shi et al. (2005) and Lu and Brewbaker (1999). The major QTL in bin 2.09 in our study was also detected in Shi's study, in which the mapping population was derived from the cross of 'Huangzao4' × 'Mo17' (Shi et al. 2005). Interestingly, the same susceptible line 'Huangzao4' and a closely related resistant line 'Ji1037' ('Ji1037' was developed from the cross of 'Mo17'/'Suwan') were used to prepare the mapping population in the present study. This may explain why the same major QTL with similar genetic effect was detected in bin 2.09 in both studies. The major QTL in bin 2.09 is, therefore, the best choice for the resistance gene cloning and marker-assisted selection to improve maize resistance to head smut.

Confirmation of the major QTL

To confirm the presence of the major QTL (qHSR1) in bin 2.09 and its genetic effect on resistance to head smut, it is necessary to utilize markers to genotype all BC_1 individuals. The eight SSR markers in bin2.09, including bnlg1520, umc1736, bnlg1893, umc1207, phi427434, umc2184, umc2077, and umc2214, were used to genotype the 118 resistant and 158 susceptible BC_1 plants. Of the 118 resistant individuals, 107 (90.7%) were heterozygotes/recombinants and only 11 (9.3%) were homozygotes at the eight markers. Of the 158 susceptible individuals, however, only 60 (38%) were heterozygotes/recombinants and as many as 98 (62%) were homozygotes. These results showed that the donor region in bin 2.09 could significantly enhance maize resistance to head smut, strongly supporting the presence of the major QTL in bin 2.09. It should be noted that head smut was very serious in 2004 due to drought during the seedling stage. The susceptible 'Huangzao4' had 86% susceptible individuals, compared with ~75% in normal year.

In addition, a total of 97 $BC_{1:2}$ families were produced from the resistant BC_1 individuals. These $BC_{1:2}$ families ranged from 5.9 to 88.3% in disease incidences. Single factor analysis of variance was performed by analyzing both disease incidence and genotype at each of the eight SSR markers on bin 2.09 region. The results showed that these eight SSR markers strongly linked to qHSR1 (Table 2).

Table 2 Single factor analysis of variance of the $BC_{1:2}$ families

SSR markers	b0	b1	LR	F(1,n–2)	pf(F)
umc2214	3.8321	−4.5175	18.6152	20.0983	0.0000**
umc2077	3.8506	−4.5464	18.7612	20.2716	0.0000**
umc2184	3.8534	−4.5509	18.7920	20.3082	0.0000**
phi427434	3.8583	−4.5828	19.0426	20.6065	0.0000**
umc1207	3.8574	−4.5890	19.0812	20.6525	0.0000**
bnlg1893	3.8566	−4.5941	19.1175	20.6959	0.0000**
umc1736	3.8411	−4.7083	20.0836	21.8536	0.0000**
bnlg1520	3.7321	−4.4259	18.1954	19.6013	0.0000**

$y = b0 + b1x + e$; LR = −2log (L0/L1)

** Significant at 0.01% level

Furthermore, the WinQtlCart 2.0 software (Statistical Genetics, North Carolina State University, USA) was used to scan the putative QTLs across the whole genome with the Composite interval mapping (CIM). A major QTL with the LOD value of 11.8 was detected on bin 2.09, bordered by SSR markers umc1736 and umc2184. The QTL could explain ~30% of phenotypic variation.

Developing new markers on bin 2.09 region

In our study, a total of 30 primer pairs were designed based on the sequences available in bin 2.09 to amplify parental lines. Three of the 30 primer pairs have been directly developed into polymorphic STS/SSR markers. Two STS markers, STS1944 and STSrga3195, were developed from the IDP1944 and RGA3195 (Zmtuc03-0811.3195), respectively. The SSR marker SSR148152 was developed from the BAC clone AC148152 (Table 3). Of the remaining 27 primer pairs, 20 gave rise to unambiguous amplicons, which were then cloned and sequenced. Sequence alignments between two parental lines revealed varying degrees of nucleotide variations with regard to different amplicons. No polymorphism was found between two parental lines for those amplicons corresponding to two anchored ESTs. Three SNPs were observed for the amplicons corresponding to three maize sequences (a total length of 2,056 bp) retrieved from the TIGR website. Amplicons corresponding to BAC-end sequences revealed higher divergences with a total of 18 SNPs in the cumulative length of 1,251 bp sequence. Sequence alignment for the four RGA-based amplicons resulted in five InDels and 26 SNPs in a cumulated 3,711 bp sequence. Sequence alignment for five IDP-based amplicons revealed one InDel and 15 SNPs in 2,814 bp. The synteny sequence in rice was also used to develop markers and revealed only one InDel in 2,088 bp. Taken together, seven InDels and 62 SNPs were obtained, resulting in about one InDel per 1,800 bp and one SNP per 200 bp in the qHSR1 region. Based on above polymorphisms, additional six markers have been finally developed, including two SNP markers (SNP140313 and SNP661, developed from the AZM4_140313 and IDP661, respectively) (Fig. 1), one CAPS marker (CAPS25082, developed from IDP25082), and three STS markers (STS171, STSrga840810, and STSsyn1, developed from IDP171, RGA BG840810, and a syntenic rice gene LOC_Os07g07050, respectively) (Table 3).

Of the nine newly developed markers, SNP140313 and STSrga3195 were mapped on chr. 1, and STSsyn1 was mapped on chr. 5. The remaining six markers were authentically mapped on bin 2.09 with five markers (SSR148152, CAPS25082, STS171, SNP661, and STS1944) in and one marker (STSrga840810) out of the resistance qHSR1 region. The newly developed markers would greatly facilitate MAS and fine mapping of the resistance gene (Fig. 2).

Phenotypic evaluation of the BC_2 recombinants and fine-mapping of the major resistance QTL

Based on genotypes of parental BC_2 recombinants, we used markers STS171 and/or STS1944 to genotype all progeny of the BC_2 recombinants. The percentage of heterozygote was tested for its difference between the resistant and susceptible groups by χ^2 test. The $P \leq 0.05$ (here we set up the threshold at $P = 0.05$) indicates the significant correlation between phenotype (resistance) and genotype (heterozygote), and the parental BC_2 recombinant was then deduced to carry the resistant donor region (Table 4). For example, BC2-64 was inferred to harbor qHSR1 due to the low *P* value (<0.05) at the STS1944 locus. For BC2-50, both STS1944 and STS171 loci showed the very low *P* values, indicating that the parental BC2-50 must harbor qHSR1. On the contrary, no significant difference (as shown by the high *P* value) was observed in percentages of heterozygote between the resistant and susceptible groups for BC2-25, indicating the absence of qHSR1 in the donor region. Taken together, 11 BC_2 recombinants (BC2-64, BC2-50, BC2-65, BC2-27, BC2-19, BC2-46, BC2-66, BC2-60, BC2-43, BC2-37, and BC2-69) were inferred to carry qHSR1 and regarded as the resistant BC_2 recombinants; whereas, five BC_2 recombinants (BC2-67, BC2-68, BC2-49, BC2-25, and BC2-45) were inferred to harbor no qHSR1 and considered to be the susceptible BC_2 recombinants (Table 4).

Based on the deduced phenotypes, the major resistance QTL region could be narrowed down by comparing the donor regions amongst all BC_2 recombinants (Table 4). BC2-50 had a heterogenous genotype in the qHSR1 region and showed high resistance to head smut with the *P* value < 0.01. On the left side, three BC_2 recombinants (BC2-64 and BC2-65, and BC2-27) with their crossover points upstream of bnlg1893 showed resistance to head

Table 3 The names, original sequences, and primer sequences for nine newly developed markers

Markers	Original sequences	Types	Enzymes	Primer pairs (5' → 3')
CAPS25082	IDP25082	CAPS	*Taq*I	L:AAGTCCTTCACGGTCTACCA R:CGGTTAGGACGATGTCAGAA
SNP140313	AZM4_140313 from TIGR	SNP	*Hha*I	L:CAGAGGCATTGAACAGGAAG R:CTGCTATTCCACGAAGTGCT snpL:CTCTTCCACCGAGAATAGCG snpR:CTGCTATTCCACGAAGTGCT
SNP661	IDP661	SNP	*Taq*I	L:CTTCTGTTCTGTGCCAGGTA R:CAAGAACGTAGCAACTCAGC snpL:ATTGTCCCTGAGATGATTCG snpR:CAAGAACGTAGCAACTCAGC
STS1944	IDP1944	STS		L:CATTGGCAACAGGACAAGTG R: GACATCAGCCTCAACATTGG
STS171	IDP171	STS		L:CCAGAGACTTGCGTGAAGAT R: AACAGACTGGTTGTACGTGC
SSR148152	BAC clone AC148152	SSR		L:GTAGGAAGACTGCCGGAGAC R:GACGCTAGAATGACTGAACC
STSrga3195	ZMTUC03-0811.3195 (RGA)	STS		L:CTAGAGGTTCAGGCATATGGCG R:AGCTCCACAGGAATTCGTTGAG
STSrga840810	BG840810(RGA)	STS		L:GCGTCAGGCAGTTCAACTTC R:TGTTCTTGCACTCGCACTTG
STSsyn1	LOC_Os07g07050 from rice	STS		L:GGCACATGGACGTACAAGAT R:GCACAGAGGAAGCTAGGAGA

For SNP markers, a pair of 'L' and 'R' primers was firstly used to amplify genomic DNA and then a pair of 'snpL' (mismatch primer) and 'snpR' primers was used to amplify diluted PCR products from the first step to alter a 'half-site' to 'full-site' for a specific restriction site. Polymorphic bands could be observed after digestion of second-round PCR products with a certain enzyme and subjected to electropherosis on polyacrylamide gel

L, left primer; R, right primer

smut; while, the other five BC_2 recombinants with their crossover points downstream of STS171 (BC2-67, BC2-68, and BC2-49) or SNP 661 (BC2-25 and BC2-45) displayed susceptibility to head smut. On the right side, all seven BC_2 recombinants showed resistance to head smut and they had crossover points downstream of STS1944 (BC2-19, BC2-46, BC2-66, and BC2-60) or SNP661 (BC2-43) or STS171 (BC2-37 and BC2-69). Interestingly, one resistant BC_2 recombinant, BC2-66, had the shortest donor region between SSR148152 and umc2184 and this donor region was assumed to cover qHSR1. It could be concluded from the above analysis that the major resistance QTL (qHSR1) was located in an interval of SSR148152/SNP661, which was estimated to be ~2 Mb based on the physical map available in the website (http://www.genome.arizona.edu/fpc/WebAGCoL/maize/WebFPC).

Estimation of the genetic effect of the major QTL

Theoretically, 93.75% of the genetic background in the $BC_{2:3}$ progeny was reverted to the recurrent parent 'Huangzao4'. Due to the low background noise in $BC_{2:3}$ progeny, the genetic effect of qHSR1 could be definitely estimated by comparison of disease incidences between two groups with/without qHSR1 within the same $BC_{2:3}$ family. A total of 1,524 individuals from 24 $BC_{2:3}$ families were checked for the presence/absence of qHSR1 with markers STS171 and STS1944. The disease incidences were estimated for two groups with/without qHSR1 in each $BC_{2:3}$ family. As a consequence, the group without qHSR1 showed more susceptible than the group with qHSR1 in each $BC_{2:3}$ family with an average difference of $28.6 \pm 10.8\%$. In other word, a single resistance qHSR1 could reduce disease incidence by $28.6 \pm 10.8\%$ (Fig. 2).

Apart from $BC_{2:3}$ progeny, BC_2F_2 progeny was also employed to estimate the genetic effect of qHSR1 in the present study. The BC_2 population was firstly genotyped at two markers bnlg1893 and umc2184, resulting in 73 BC_2 plants with qHSR1 and another 31 BC_2 plants without qHSR1. All these BC_2 plants were self-pollinated to produce corresponding BC_2F_2 families. As expected, the BC_2F_2 progeny derived from BC_2 plants with qHSR1

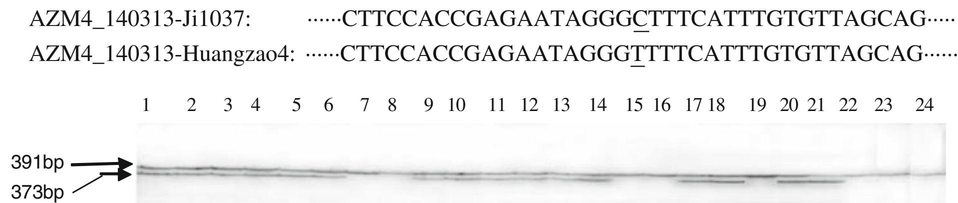

Fig. 1 Development of a SNP marker (SNP140313) for AZM4_140313 (assembled *Zea mays* sequence from TIGR) and its application in genotyping BC populations At the AZM4_140313 locus, a SNP site (*underlined*) was detected whereby nucleotides 'C' (allele 1) and 'T' (allele 2) correspond to parental lines 'Ji1037' and 'Huangzao4', respectively. In the first round of PCR amplification, a pair of normal primers (L:5′-CAGAGGCATTGAACAGGAAG-3′ and R:5′-CTGCTATTCCACGAAGTGCT-3′) was designed to amplify parental lines and BC_1 individuals. In the second round of PCR amplification, a new pair of primers, including the left primer (snpL:5′-CTCTTCCACCGAGAATAG**C**G-3′) with one mismatch nucleotide 'C' (*boldface*) and the right primer (snpR:5′-CTGCTATTCCACGAAGTGCT-3′), was designed to amplify the diluted first-round PCR products. This produced a *Hha*I site 'GCGC' for allele 1, but not allele 2 in the resultant PCR products. PCR products were digested with *Hha*I and then subjected to electrophoresis on 6% polyacrylamide gel. Polymorphic bands were observed in the two parental lines, 'Ji1037' (*line 21*, a digested 373 bp band) and 'Huangzao4' (*line 22*, an intact 391 bp band), and among BC_1 individuals (*lines 1–20*). *Lines 1–20* The randomly selected BC_1 individuals that display either homozygote (1/1, one band having the same size as that of 'Huangzao4') or heterozygote (1/2, two bands corresponding to 'Ji1037' and 'Huangzao4', respectively). *Line 21* The *Hha*I-digested PCR products amplified from 'Ji1037', a digested 373 bp band was observed. *Line 22* The *Hha*I-digested PCR products amplified from 'Huangzao4', an undigested 391 bp band was observed. *Line 23* The undigested PCR products amplified from 'Ji1037'. *Line 24* The undigested PCR products amplified from 'Huangzao4

showed more resistant than those derived from BC_2 plants without qHSR1. Of the 529 BC_2F_2 individuals derived from 31 BC_2 plants without qHSR1, 204 (38.7%) were found to be susceptible. Whereas, 262 (19.3%) of 1,358 BC_2F_2 individuals derived from 73 BC_2 plants with qHSR1 were susceptible. In the BC_2F_2 progeny derived from BC_2 plants with qHSR1, segregation occurred at the qHSR1 locus, resulting in one-fourth BC_2F_2 individuals without qHSR1. These BC_2F_2 individuals without qHSR1 are expected to have the same disease incidence as that estimated from the 31 BC_2F_2 families without qHSR1 (38.7%). For the other three-fourth BC_2F_2 individuals with qHSR1 (one-fourth homozygotes and a half heterozygotes), we needed to estimate its disease incidence. Based on above explanations, we could draw an equation as $3/4X\% + ¼ \times 38.7\% = 19.3\%$; here, '$X$' represents infection percentage for those BC_2F_2 individuals with qHSR1. The 'X' is calculated to be 12.8%. In summary, the qHSR1 locus could reduce disease incidence by 25.9% in the BC_2F_2 progeny, from 38.7% (individuals without qHSR1) to 12.8% (individuals with qHSR1).

Discussion

Segregation distortion at marker loci was a phenomenon frequently observed in constructing genetic linkage map (Lu et al. 2002). It was reported that markers showing segregation distortion could range from 19 to 36% in maize (Lu et al. 2002). In the present study, a fraction of 33 (29.2%) SSR markers showed segregation distortion at $P < 0.05$. These SSR markers cover seven chromosomal regions (at least two linked markers showing distortion) and eight single loci, scattering on all chromosomes except for chrs 4 and 6. It has been shown that distortion markers wouldn't influence construction of genetic map and QTL detection (Lu et al. 2002; Yan et al. 2003). Therefore, all 113 SSR markers in this study were used to construct linkage map and scan the putative QTLs at all single loci. Although genetic distortion at marker loci were observed on bins 1.06, 1.09, 7.05, 7.06, 9.03, and 9.06, no putative QTLs were found in these regions based on TB analysis (Lebowitz et al. 1987). On the contrary, for those regions (bins 2.09 and 6.07) without genetic distortion, marker frequencies were significantly different between the resistant and susceptible groups, suggesting the presence of putative resistance QTLs.

In previous studies, several criteria such as fleck, sorus, and infection percentage at maturity stage were used to evaluate resistance against head smut in maize. Foster and Frederiksen (1977a, b) suggested using fleck as an indicator of infection in greenhouse. However, Mankin (1953) thought that leaf fleck was not a reliable criterion to estimate infection because such plants did not necessarily show definitive infection symptom, that is, ear or tassel was partially or fully replaced by sori at maturity. In this study, the presence/absence of sori on either ear or tassel at maturity stage was used as an indicator.

Reliable phenotyping is the first and most important step towards successful mapping and eventually cloning of the target gene, especially for QTL cloning. As for maize head smut, it seems even more complicated to distinguish resistant from susceptible individuals. The susceptible parent 'Huangzao4' is not 100% infected by *S. reliana* under artificial inoculation. Generally, ~75% infected plants can be observed in the field. On the other hand, individuals carrying

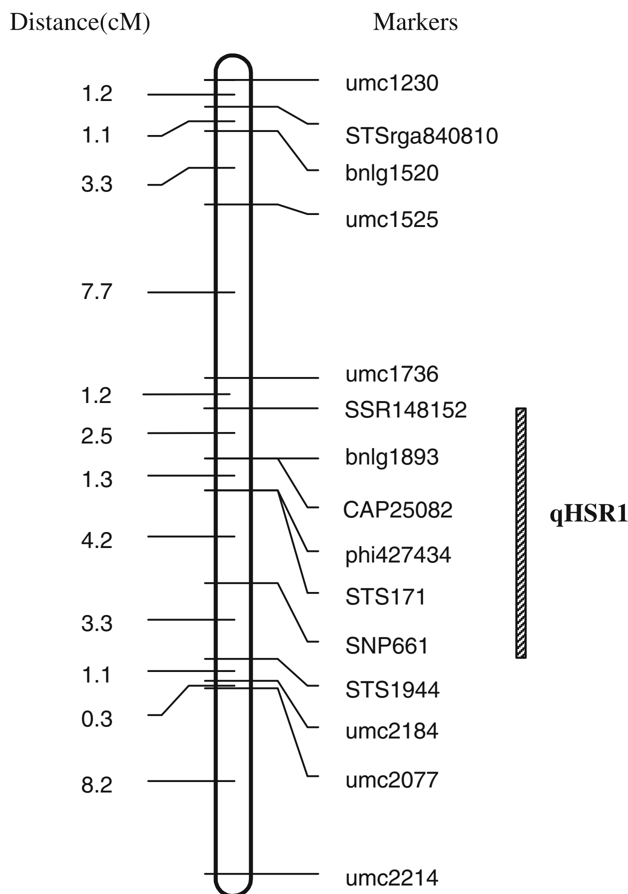

Fig. 2 Genetic-mapping of the newly developed markers on the bin 2.09 region Of the nine newly developed markers, six markers were mapped on bin 2.09 with five markers (SSR148152, CAPS25082, STS171, SNP661, and STS1944) in and one marker (STSrga840810) out of the resistance qHSR1 region. The major resistance QTL, qHSR1, was delimited into an interval of ~2 Mb, flanked by the newly developed markers SSR148152 and STS661

the resistance gene would not show 100% resistance. This partial penetrance makes it very difficult to tell true resistance apart from false resistance for a single individual in a segregation population. For example, out of 121 BC_1 individuals without any symptoms, 13 were found to share the same genotype as that of the susceptible parent in the qHSR1 region. The presence/absence of sori in ear/tassel for a given individual depends on both genetic and environmental factors. First, the major resistance QTL, qHSR1, could only explain ~30% of total variation and it is, therefore, not a decisive factor to confer full resistance to head smut. Second, pathogens may fail to invade some individuals with the susceptible genotypes. Third, many environmental factors, such as temperature, soil moisture, soil type, soil nutrition, water potential, and plant vigor, act in concert on life cycle of the fungi, infection procedure, transmission of disease, and so on, which will finally determine the formation of sori and severity of disease (Baier and Krüger 1962; Foster 1979; Wu et al. 1981; Matyac and Kommedahl 1985b; Martinez et al. 2003).

In the field test, infected plants would significantly increase on condition that plant growth is stunted due to transplanting or drought. We assumed from this phenomenon that the formation of sori in ear/tassel depends on growth competition between plant and pathogen. If hypha reaches to apical or lateral meristems, pathogens will accumulate and finally form sori, if not, no any sori can be observed. Environmental factors would heavily affect plant growth, but not so much on pathogen growth since hypha grows inside the plant. Environmental stress, like transplanting and drought treatment, would slow down plant growth and allow hypha to reach meristem. Ni et al. (2006) reported that resistance of maize against head smut was mainly determined at the stages of invasion and pathogen spread. In their study, specific PCR primer was used to detect *S. reiliana* at different growing stages of both ‘Mo17’ (highly resistant) and ‘Huangzao4’ (highly susceptible). The results indicated that ‘Mo17’ and ‘Huangzao4’ differed in their resistances against pathogen invasion at the seedling stage. For ‘Huangzao4’, once the pathogen was detected in male or ear, the plant would definitely show symptoms, otherwise, no symptom would appear. For ‘Mo17’, due to its resistance at both invasion and spread stages, normally no sori were observed in tassel/ear, though some plants were detected to bear pathogens at the seedling stage.

Due to complicated interactions between pathogen and plant, resistance to head smut for a single individual could only be determined by testing its offspring. Often infection percentage obtained from its offspring was not enough to judge if the parental individual is resistant or susceptible. For example, infection percentages of the BC_2F_2 families ranged from 0 to 41% for those with qHSR1 and from 22 to 75% for those without qHSR1. As a consequence, there are a number of BC_2F_2 families whose infection percentages are close regardless of the presence/absence of the major resistance qHSR1. Just like the susceptible parental line ‘Huangzao4’, infection percentage of the same mapping materials varied from year to year and location to location. Considering all these difficulties, a method was proposed to determine whether or not an individual carry a major resistance QTL by analyzing both genotypes and phenotypes of its progeny, so that the influences from both genetic backgrounds and environmental elements could be diminished to minimum, revealing the actual genetic effect of the major QTL. For instance, apart from the resistance qHSR1, all individuals from the same $BC_{2:3}$ family share the similar genetic backgrounds; moreover, all individuals from the same family were grown in the same place to have the exact same environmental conditions. By comparing infection percentages between the heterozygote (1/2) and

Table 4 Parental BC2 recombinants, their genotypes at the qHSR1 region, χ^2 test in progenies, and deduced BC2 phenotypes

Parental BC2 recombinants	Genotypes at SSR markers for the parental BC2 recombinants						χ^2 test in progenies		Deduced BC2 phenotypes
	SSR148152	bnlg1893	phi427434/STS171	SNP661	STS1944	umc2184	Markers	*P* values	
BC2-50	1/2	1/2	1/2	1/2	1/2	1/2	STS171	0.003	Resistant
							STS1944	0.0002	
BC2-65	1/1	1/2	1/2	1/2	1/2	1/2	STS171	0.042	Resistant
							STS1944	0.051	
BC2-27	1/1	1/2	1/2	1/2	1/2	1/2	STS171	0.006	Resistant
BC2-64	1/1	1/2	1/2	1/2	1/2	1/2	STS1944	0.022	Resistant
BC2-67	1/1	1/1	1/1	1/2	1/2	1/2	STS1944	0.273	Susceptible
BC2-68	1/1	1/1	1/1	1/2	1/2	1/2	STS1944	0.384	Susceptible
BC2-49	1/1	1/1	1/1	1/2	1/2	1/2	STS1944	0.805	Susceptible
BC2-25	1/1	1/1	1/1	1/1	1/2	1/2	STS1944	0.478	Susceptible
BC2-45	1/1	1/1	1/1	1/1	1/2	1/2	STS1944	0.730	Susceptible
BC2-19	1/2	1/2	1/2	1/2	1/2	1/1	STS171	0.033	Resistant
BC2-46	1/2	1/2	1/2	1/2	1/2	1/1	STS171	<0.0001	Resistant
							STS1944	0.0107	
BC2-66	1/1	1/2	1/2	1/2	1/2	1/1	STS1944	0.026	Resistant
BC2-60	1/2	1/2	1/2	1/2	1/2	1/1	STS1944	0.020	Resistant
BC2-43	1/2	1/2	1/2	1/2	1/1	1/1	STS171	0.033	Resistant
BC2-37	1/2	1/2	1/2	1/1	1/1	/	STS171	0.018	Resistant
BC2-69	1/2	1/2	1/2	1/1	1/1	1/1	STS171	0.004	Resistant

homozygote (1/1) groups from the same $BC_{2:3}$ family, we could conclude whether or not the parental BC_2 recombinant carried the resistance qHSR1 in its donor region.

Saturation of the major QTL region with high-density markers is indispensable to transfer a QTL into a QTG (quantitative trait gene) for cloning of the target gene. It is, therefore, very necessary to develop as many markers as possible by taking advantage of all information available. The most advantage to develop marker in maize is the abundance of SNPs and InDels throughout the whole genome as revealed by previous studies (Ching et al. 2002; Tenaillon et al. 2001). In this study, IDP markers, anchored ESTs, BAC and BAC-end sequences, rice syntenic sequence, and RGAs were all used for marker development. The nine markers developed were based on the IDPs (CAPS25082, SNP661, STS1944, and STS171), Assembled *Zea mays* sequence from TIGR (SNP140313), syntenic rice sequence (STSsyn1), BAC sequence (SSR148152), and anchored RGAs (STSrga3195 and STSrga840810). The IDP markers retrieved from the website (http://schnablelab.plantgenomics.iastate.edu/) could not be directly used in our mapping effort due to the lack of polymorphism or even failure in amplification. This requires re-design of IDP markers by amplifying IDP regions from our mapping parents and designing primers based on sequence divergences. The anchored EST is a good source to develop marker, unfortunately, no polymorphism was found in the two anchored ESTs in the present study. Low-copy BAC-end sequence is also a very good source for developing high-density markers. The ongoing maize genome sequencing project provides a huge amount of sequence information to develop high-density markers. As shown in this study, the SSR marker, SSR148152, was developed based on a sequenced BAC clone (AC148152) in the QTL region. In addition, the region covering 230.8–231.4 M of the ctg#109 where the resistance qHSR1 located is syntenic with that from 3.2 to 3.8 M in rice chr. 7. There were about 100 genes in the rice syntenic region, which were used as sequence sources to develop new markers. In this study, one rice gene, LOC_Os07g07050, was used to search for the maize counterpart EST via tBlastn and a new marker was developed. Unfortunately, the marker developed from this approach was mapped on the maize chr. 5 instead of chr. 2. This may result from repetitive sequences frequently present in the maize genome.

With the SSR and newly developed markers on bin 2.09, we could delimit qHSR1 into an interval with the genetic distance of 8 cM by comparing the insert sizes of ‘resistant’ with those ‘susceptible’ donor regions. The physical distance in an interval of SSR148152/SNP661 is estimated to be ~2 Mb. The maize genome is ~2,400 Mb in size and the genetic map is of ~2,400 cM (Arumuganathan and Earle 1991), on average, 1 cM equals to 1,000 kb on whole maize genome. In this study, ~250 kb per cM is found in the

qHSR1 region, which is greatly shorter than an average distance. This phenomenon implies the presence of a possible crossover hotspot in the qHSR1 region, and such hotspot is beneficial to fine-mapping and eventually cloning of the resistance gene. After the BAC contig in the qHSR1 region being constructed and sequenced, the candidate resistance gene(s) could be identified and cloned. During the fine mapping process, a BAC-end sequence located in the qHSR1 region was found to be highly homologous to the *Zea mays* rust resistance protein rp3-1 (rp3-1) gene via Blastn and tBlastx in NCBI. More works are required to confirm this resistance gene analog (RGA) to be the candidate resistance gene for qHSR1.

Resistance to maize head smut is controlled by nuclear genes without any cytoplasmic influence (Mei et al. 1982). Therefore, pyramiding of multiple QTLs/genes is a practical way to strengthen maize resistance to head smut. During the fine-mapping process, we also used the flanking markers to select the individuals harboring qHSR1 to develop near isogenic lines sharing the 'Huangzao4' genetic backgrounds. As a consequence, the isogenic lines showed more resistant than that of the recurrent parent 'Huangzao4' by reducing disease incidence to ~25%. We are now trying to exploit more closely linked markers to improve efficiency for MAS.

In cloning of the resistance qHSR1, near isogenic lines selected via MAS are indispensable for fine-mapping qHSR1. Such isogenic lines have many advantages in fine-mapping of the target QTL by eliminating background noise. It was believed that the power of detecting additive, dominant, and over-dominant QTLs was enhanced in advanced backcross generation (Tanksley and Nelson 1996). Since resistance to head smut is complicated owing to multiple resistance QTLs, genetic interactions, and environmental factors (Ali and Baggett 1990; Mei et al. 1982; Ma et al. 1983; Bernardo et al. 1992), the effect of the major resistance qHSR1 could only be fully detected under the exactly same genetic background. The near isogenic lines from advanced backcross generation could be either self-pollinated or backcrossed to produce a big segregant population for fine-mapping of qHSR1.

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