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REVIEW

Characterization and molecular mapping of *RsrR*, a resistant gene to maize head smut

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Abstract Maize head smut (MHS) caused by the fungi *Sporisorium reilianum* (Kühn) Landon and Fullerton (*S. reilianum*) is the most serious disease occurred in China recently. There are only a few reports concerning the genetics of this disease and the resistant gene of maize. In this paper a new dominant resistant gene to MHS caused by the pathogen Shenyang-1 of *S. reilianum* was discovered from a newly developed resistant inbred line 'R24' and was named *RsrR*. *RsrR* gene was molecular tagged and mapped on the long arm of maize chromosome 1 via simple sequence repeat-bulked segregant analysis (SSR-BSA) and sequence related amplified polymorphism (SRAP)-BSA analysis, the nearest linkage marker is 2.5 cM apart from the *RsrR* gene. The gene *RsrR* has been transferred into two elite maize inbred lines, 'Huangzao4' and 'Qi319', through traditional hybridization and marker assisted selection. The converted lines can be used in maize MHS-resistance breeding.

Keywords Gene mapping · Maize head smut resistant gene · Marker assisted selection · SRAP markers · SSR markers

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Introduction

Maize head smut (MHS) caused by the fungi *Sporisorium reilianum*, mainly jeopardizes ears and causes significant damage to maize production. It has been reported that MHS occurred in Australia, Mexico, France, Germany, Brazil, Russia, China and other maize growing areas of the world (Al-Sohaily et al. 1963; Frederiksen 1977; Pataky 1999; Lu and Brewbaker 1999). Since the 1990s of the twentieth century, MHS has developed quickly and increased year by year. Recently, MHS became one of the most important maize diseases in China. Therefore, more and more maize breeders and scientists are involved in the development of new resistant varieties, studying the

genetics of MHS trait, molecular tagging, mapping and cloning of the MHS-resistant genes.

In previous research, some results indicated that the MHS-resistance was controlled by dominant nuclear gene, recessive gene or major quantitative trait locus (QTL), respectively. Oh et al. (1994) reported that a restriction fragment length polymorphism (RFLP) marker tam1294 and a randomly amplified polymorphic DNA (RAPD) marker OPGS-2 were linked to a single dominant MHS-resistant gene, with genetic distance of 13.6 and 11.2 cM respectively. Shi et al. (2005) detected an over-dominant resistance locus on chromosome 2 (bin 2.09). Shi et al. (2009) developed a sequence characterized amplified region (SCAR) marker S130, which was highly associated with the MHS-resistant gene and located at chromosome bin 2.09. Chen et al. (2008) mapped the MHS-resistant gene at the position bin 2.09 and delimited into an interval of ~2 Mb, the MHS-resistant gene was flanked by markers SSR148152 and STS661.

However, some results suggested that MHS-resistance was controlled by multiple loci or QTLs. Shi et al. (2005) detected QTLs on the maize chromosome 1, 2, 3, 4 and 7, which could explain 15.1, 13.21, 13.1, 11.4 and 7.1 % of the phenotypic variation respectively. Lu and Brewbaker (1999) mapped the MHS-resistant genes on chromosome 1, 2, 9 and 10 using a recombinant inbred line (RIL) mapping population. Lübberstedt et al. (1999) mapped eight MHS-resistant QTLs, which can explain 44 % of the phenotypic variation.

Although the previous genetic and mapping results of MHS-resistant genes reported by different researchers were not consistent, but in one point they have a common opinion, that is to develop or to screen out good MHS-resistant germplasm is important and necessary for MHS-resistance breeding and theoretical research.

A new MHS-resistant line 'R24', which was developed by the Institute of Plant Protection, Liaoning Academy of Agricultural Sciences, conferred high MHS-resistance and showed good agronomic performance. Now 'R24' has become the most important parent in MHS-resistance breeding in China. The purpose of this study is to characterize the MHS-resistant gene in the inbred line 'R24' through genetic analysis by artificial inoculation in the field, and to screen molecular markers linked to the MHS-resistant gene through SSR0-BSA and SRAP-BSA analyses, and to improve the MHS-resistance of the MHS-susceptible maize inbred lines by using the MHS-resistant gene of the inbred line 'R24'.

Materials and methods

MHS resistant line 'R24'

'R24' is a maize MHS-resistant line used in this study. 'R24' not only confers high resistance against MHS, but also shows good agronomic performance, it has been widely used in MHS-resistance breeding in China (Xu 2009).

Populations

For genetic statistic study, 'R24' was crossed with five MHS-susceptible lines ('Huangzao4', 'Qi319', 'Lu2548', 'H9' and 'Zheng22') respectively. Then, F_1, F_2, BC_1F_1 and $F_{2:3}$ populations were generated from the progenies of each cross.

Two crosses ("R24 × Huangzao4" and "Qi319 × R24") were used for studying the MHS-resistant gene transfer and the genetic stability of the MHS-resistance gene in the converted plants. Some MHS-resistant individuals were selected from their BC_1F_1 populations, and then backcrossed with MHS-susceptible parents for two generations to generate the BC_3F_1 individuals, which were then self-pollinated for two generations to generate the BC_3F_3 individuals. The MHS-resistant individuals were identified by double selections with MAS and field inoculation test in each generation.

Pathogen inoculation and the disease incidence investigation

MHS pathogen, the race Shenyang-1 of *S. reilianum* was used in MHS-resistance studies, which was isolated and identified from infected maize by the Institute of Plant Protection, Liaoning Academy of Agricultural Sciences. Now, it is the most widely used pathogen in MHS resistance breeding in China. Field inoculation trials with Shenyang-1 of *S. reilianum* were carried out from 2006 to 2009 in Shenyang city, Liaoning province, China. The pathogen teliospores were collected from the diseased maize in autumn every year. In late April next year, the pathogen

teliospores were mixed with fine soil (prepared according to the ratio of 0.6 % in fine soil, teliospores powder/fine soil). The testing maize seeds were sowed by bunch planting and the seeds per hole were covered with 100 g of mixture of fine soil and teliospores, and then covered with field soil on the surface. Field management was carried on as usual. The disease incidence was surveyed and scored for individual plant during milky maturity stage of maize.

DNA preparation

Genomic DNA was extracted from young leaves of individual plant and dissolved in TE solution as our previous report (Chen et al. 2004). DNA concentrations were estimated and adjusted to 200 ng/μl. All DNA samples were stored in −20 °C before use.

BSA analysis

The BSA method was used to screen and evaluate molecular markers associated with MHS resistance (Michelmore et al. 1991). The MHS-resistant bulk (B_R) contained equal amount of DNA from each of 15 resistant F_2 individuals, and the MHS-susceptible bulk (B_S) contained equal amount of DNA from each of 15 susceptible F_2 individuals. In BSA analysis, if a PCR product exhibiting the same polymorphism between the two parents (P_R and P_S) and the two bulks (B_R and B_S), this product was considered as potential linkage marker, which was further analyzed with a large F_2 population to confirm its linkage relation to the MHS-resistant gene.

Molecular marker analysis

SSR analysis was performed as our previous report (Chen et al. 2004). SSR products were separated on 3.0 % agarose gel stained with ethidium bromide (EB) or on 6 % poly-acrylamide gel electrophoresis (PAGE) sequencing gel stained by the rapid silver staining method (Xu et al. 2002).

SRAP analysis was carried out as described (Li and Quiros 2001). Amplification was conducted with the primer-combinations paired from the five forward primers and the six reverse primers shown in Table 1. The amplification products were separated on a 6 % PAGE sequencing gel run at 80 W for 2.5 h after pre-electrophoresis for 15 min. The gels were stained by rapid silver-staining method (Xu et al. 2002).

Linkage analysis and construction of a local linkage map

Based on the SSR and SRAP data combined with MHS phenotypic data, linkage analysis was performed with MAPMAKER version 3.0 at a LOD threshold of 3.0 (Lander et al. 1987). The Kosambi function was applied to convert recombination fractions into map distance (centimorgan, cM) (Kosambi 1994).

Alternatively, anchor marker method was used in primary mapping (Chantret et al. 2000; Zhao et al. 2010). In brief, to analyze the linkage relation between the target gene and a relative large amount of mapped molecular markers, if any of the analyzed markers is found to be linked with the target gene, the target gene should be located on the same chromosome with this linkage marker. Thus the target gene is primary mapped.

Results

Genotype identification and genetic analysis

In field, the disease symptoms were observed mainly appeared on ears (Fig. 1a) and tassels (Fig. 1b) of the maize plants infected by the MHS pathogen. The symptom on the infected tassel performed as branched when the infection limited to an individual spikelet or as leaf-shaped when the infection spread to whole tassel, the infected tassel could form diseased galls filled with smut sori. The infected ear could not spit silks, and became a diseased gall filling of black powder except the husk leaves, then the husk breached and the black spore powder scattered (Xu 2009). The maize plants with above symptoms were marked as susceptible individuals, while the resistant plants showed no sign of infection. The phenotypic identification is easy and reliable.

The numbers of resistant and susceptible plants were counted in F_1, F_2 and BC_1F_1 populations generated from the cross “R24 × Huangzao4”. Statistics analysis was carried out in multiple years and locations. Results indicated that all F_1 plants were resistant to MHS and the segregation ratio in F_3 generation of the F_2 resistant individuals was 2:1 (data were not

Table 1 Sequences of the 11 primers used for SRAP analysis

Forward primer	Sequence (5′ to 3′)	Reverse primer	Sequence (5′ to 3′)
ME1	TGAGTCCAAACCGGATA	EM1	GACTGCGTACGAATTAAT
ME2	TGAGTCCAAACCGGAGC	EM2	GACTGCGTACGAATTTGC
ME3	TGAGTCCAAACCGGAAT	EM3	GACTGCGTACGAATTGAC
ME4	TGAGTCCAAACCGGACC	EM4	GACTGCGTACGAATTTGA
ME5	TGAGTCCAAACCGGAAG	EM5	GACTGCGTACGAATTAAC
		EM6	GACTGCGTACGAATTGCA

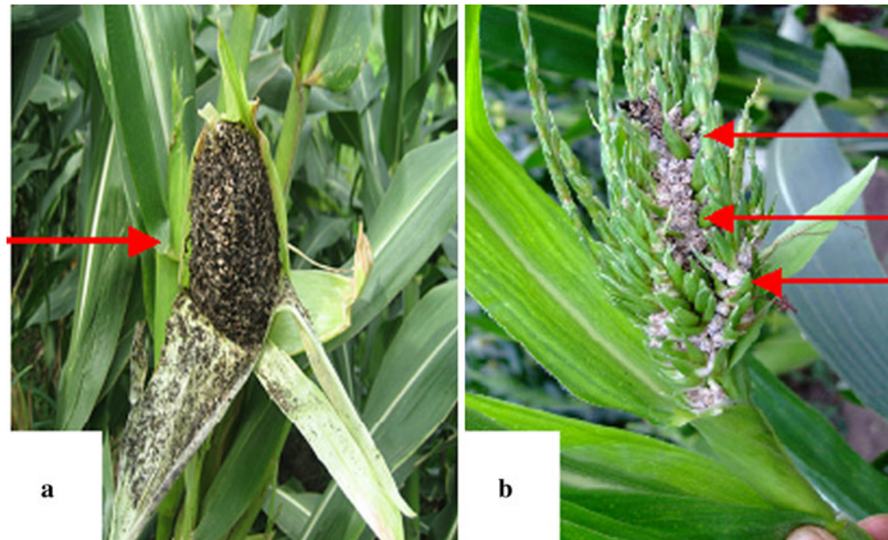

Fig. 1 Symptoms of ear (**a**) and tassel (**b**) of maize plants infected by the pathogen race Shenyang-1 of *S. reilianum. Arrows* indicate the diseased ear or tassel

shown here). The segregation ratios in F_2 and BC_1F_1 populations were calculated and analyzed using a χ^2 test. In 2006, from the 729 individuals of F_2 population, 550 were scored as resistant and 179 were scored as susceptible, this result was accorded with the 3:1 ratio by χ^2 test. In the BC_1F_1 population including 58 individuals, 30 were scored as resistant and 28 were scored as susceptible, which tend to the 1:1 ratio by χ^2 test (Table 2). To check the preliminary results obtained in 2006, the inoculation test was repeated with larger scale of F_2 and BC_1F_1 populations in 2007. Among the 1,671 individuals in the F_2 population, 1,275 were scored as resistant and 396 were scored as susceptible, while among the 159 individuals in the BC_1F_1 population, 86 were scored as resistant and 73 were scored as susceptible. The result of χ^2 test indicated that the segregation ratio of resistant to susceptible individuals in F_2 and BC_1F_1 populations was in accord with the 3:1 and 1:1 ratio, respectively (Table 2). In 2008, the statistics were repeated with fewer individuals, the obtained result was similar as previous (Table 2). The genetic statistic results of field inoculation tests in the three continuous years indicated that the highly resistance of maize inbred line ‘R24’ against the pathogen race Shenyang-1 was controlled by a single dominant gene, which was named *RsrR* later.

In 2007, genetic statistic study was performed with four other F_2 populations derived from the crosses of ‘R24’ with four maize inbred lines (‘Lu2548’, ‘Qi319’, ‘H9’ and ‘Zheng22’). Results indicated that the segregation ratio of resistant individuals to susceptible individuals tend to the 3:1 ratio in three of the four F_2 populations, with one exception of the F_2 populations derived from the cross “R24 × Zheng22” (Table 3).

Table 2 Segregation of resistant to susceptible individuals in F_2 and BC_1F_1 populations generated from the cross “R24 × Huangzao4”

Population	Years	Total plants	Resistant plants	Susceptible plants	R/S ratio	χ^2	χ^2 test
F_2	2006	729	550	179	3.07	0.075	ns
	2007	1,671	1,275	396	3.22	1.509	ns
	2008	291	216	75	2.88	0.084	ns
BC_1F_1	2006	58	30	28	1.07	0.086	ns
	2007	159	86	73	1.18	1.062	ns
	2008	224	120	104	1.15	1.063	ns

ns Not significantly different

Table 3 Segregation of resistant to susceptible individuals in F_2 populations generated by the cross combinations of ‘R24’ with the other four susceptible maize inbred lines

Cross	Total plants	Resistant plants	Susceptible plants	R/S ratio	χ^2	χ^2 test
Lu2548/R24	31	23	8	2.88	0.011	ns
Qi319/R24	45	34	11	3.09	0.017	ns
H9/R24	36	27	9	3.00	0	ns
R24/Zheng22	19	17	2	8.5		

ns Not significantly different

SSR analysis

At first, screening of molecular markers linked to the MHS-resistant gene was carried out by SSR strategy. In total, 100 mapped SSR markers distributed evenly on the 10 maize chromosomes were selected and screened regarding their linkage relation with the *RsrR* gene by SSR-BSA analysis among the two parents and the two bulks. Thirty-three primer-pairs amplified polymorphisms between the two parents, but only one of them, umc1331 located on chromosome 1, amplified identical polymorphism between the two parents and the two bulks. The SSR marker umc1331 was considered as a potential linkage marker of the *RsrR* gene. Then the linkage relationship was checked and confirmed by analyzing with 150 susceptible F_2 individuals (Zhang et al. 1994). Results indicated that umc1331 was really linked to the resistant gene *RsrR* (Fig. 2a), and the *RsrR* gene was primary mapped on chromosome 1.

In order to fine map the *RsrR* gene, forty-two SSR primer-pairs flanked the marker umc1331 on the long arm of chromosome 1 were analyzed regarding their relationship to the *RsrR* gene. Results indicated that six SSR markers showed potential linkage with *RsrR* gene, their linkage relationship was further checked and confirmed by analyzing with 150 susceptible individuals of the F_2 population (Zhang et al. 1994). It was observed that only four SSR markers (bnlg667, umc2241, umc1538 and bnlg1055) were really linked with the *RsrR* gene, and that the nearest linkage marker bnlg667 linked to *RsrR* gene with a genetic distance of 8.5 cM (Fig. 2).

SRAP analysis

Subsequently SRAP-BSA analysis was performed to enrich molecular markers in the *RsrR* gene encompassing region. In total 30 SRAP primer-combinations (paired from five forward primers and six reverse primers shown in Table 1) were analyzed, seven primer-combinations (ME1/EM4, ME1/EM5, ME2/EM1, ME3/EM4, ME3/EM5, ME3/EM6 and ME4/EM1) amplified polymorphism showing possible linkage with the *RsrR* gene (Table 4 and Supplementary Fig. 1). The seven possible linkage markers were checked and confirmed with susceptible individuals of the F_2 population (Zhang et al. 1994), result showed that only two products amplified by primer-combinations $ME3/EM6_{390}$ and $ME3/EM5_{420}$ were truly linked to the *RsrR* gene, at a genetic distance of 2.5 and 6.25 cM, respectively (Fig. 3, Supplementary Figs. 2 and 3).

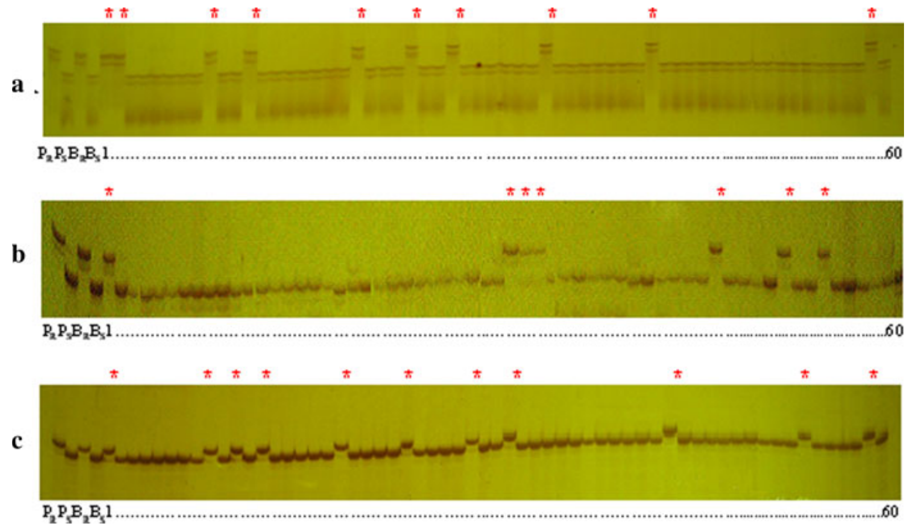

Fig. 2 The electrophoretic patterns amplified by SSR marker umc1331 (**a**), bnlg667 (**b**), and umc2241 (**c**) on the susceptible individuals of the F_2 population developed from the cross "R24 × Huangzao4". P_R, resistant parent 'R24'; P_S, susceptible parent 'Huangzao4'; B_R, resistant bulk; B_S, susceptible bulk; samples 1–60, F_2 susceptible individuals; Stars indicate the recombinants

Table 4 BSA analysis with 30 SRAP primer-combinations

	ME1	ME2	ME3	ME4	ME5
EM1		√		√	
EM2					
EM3					
EM4	√		√		
EM5	√		√		
EM6			√		

√ indicates the primer-combinations that generated the same polymorphic bands between the two parents ('R24' and 'Huangzao4') and the two bulks (resistant bulk and susceptible bulk)

Construction of the local linkage map of *RsrR* gene-encompassing region

Based on the obtained SSR and SRAP data combined with MHS phenotypic data, linkage analysis was performed as described in "method" section. As a result, a local genetic linkage map of the *RsrR* gene-encompassing region on the long arm of chromosome 1 was constructed (Fig. 3). In total, seven linkage markers were identified; the *RsrR* gene was located on the locus between SRAP markers ME3/$EM6_{390}$ and ME3/$EM5_{420}$ at genetic distances of 2.5 and 6.25 cM, respectively. On one side of the target gene, located linkage markers ME3/$EM6_{390}$, bnlg667, bnlg1055, umc1538 and umc2241 at genetic distances of 2.5, 8.5, 15, 17.25 and 19.25 cM respectively; while on the other side located linkage SRAP marker ME3/$EM5_{420}$ and SSR marker umc1331 with a genetic distance of

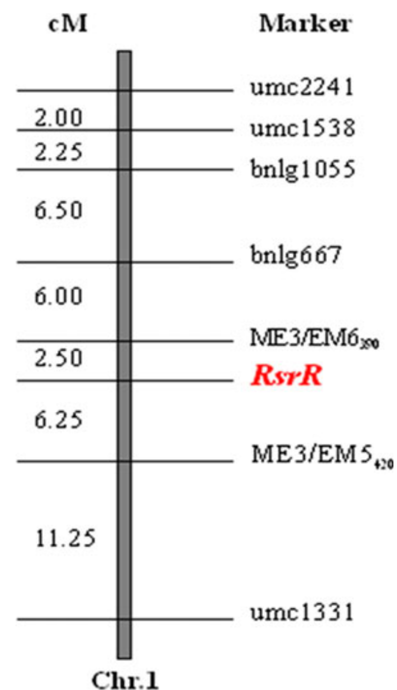

Fig. 3 Map showing the *RsrR* gene and its linkage markers on maize chromosome 1

6.25 and 17.5 cM to the *RsrR* gene, respectively (Fig. 3).

Transfer the *RsrR* gene into MHS-susceptible inbred lines

'Huangzao4' and 'Qi319' are the most popular backbone inbred lines used in maize breeding in

China (Zeng et al. 1996; Ye 2000), but they are susceptible to MHS. In order to improve their MHS-resistance, we performed transferring the MHS-resistant gene from inbred line ‘R24’ into inbred lines ‘Huangzao4’ and ‘Qi319’ respectively through traditional hybridization and MAS by using the obtained linked markers ME3/EM5$_{420}$ and ME3/EM6$_{390}$. Supplementary Fig. 3 shows the MAS testing results of the ‘Huangzao4’ converted lines in different generations. Now, the ‘Huangzao4’ converted line is up to the stage BC_3F_3, it is stable and can be used in maize breeding instead of the original ‘Huangzao4’. Similar results were obtained in MAS testing of the ‘Qi319’ converted lines in different generations. Transferring the *RsrR* gene into other elite maize inbred lines is undergoing.

Discussions

Several research groups detected single MHS-resistant gene or major QTL in the locus bin 2.09 on chromosome 2 (Shi et al. 2005; Chen et al. 2008; Shi et al. 2009). Some scientists detected MHS-resistant gene on chromosome 1 (Shi et al. 2005; Lu and Brewbaker 1999). In this study a new MHS-resistant gene *RsrR* was discovered and mapped on chromosome 1. These results point out that there are important regions associated with MHS-resistance on chromosome 2 and chromosome 1.

Overview the previous studies the MHS-resistant trait was reported to be related to eight of the ten maize chromosomes, comparatively concentrating on chromosome 2 and 1. The mapping results were scattered and inconsistent. This may be related to the follow facts: (1) The experiments were carried out with different research materials, methods and in different environmental conditions; (2) The research materials used in their studies may carry different resistant genes; (3) The pathogen caused MHS may has high diversity, the races of the pathogen used in artificial inoculation by different researchers might be different from each other. Some papers had indicated that there were different pathogen races parasitizing in different maize lines. He et al. (2006) reported that the MHS pathogen had seven types of pathogenicity using six maize inbred lines as hosts. Hua et al. (1995) reported that there were five races with five maize inbred lines as identifying host.

In genetic statistic analysis of the MHS-resistant gene *RsrR,* a F_2 and a BC_1F_1 populations derived from the cross “R24 × Huangzao4” were used, the statistic analysis of MHS resistance trait was performed in multiple years and locations through artificial inoculation with the pathogen race Shenyang-1 of *S. reilianum*. The obtained results indicated that the MHS-resistant gene in ‘R24’ was controlled by a single dominant resistant gene. This conclusion was further proved by the genetic analysis with other three F_2 populations.

A number of molecular markers linked to the newly discovered *RsrR* gene were identified through SSR-BSA and SRAP-BSA analyses and used to map the *RsrR* gene. The *RsrR* gene was finally mapped at the position bin 1.10 on the long arm of maize chromosome 1 and flanked by markers ME3/EM6$_{390}$ and ME3/EM5$_{420}$. The successful development of these linked molecular markers may provide available option for marker assisted selection(MAS) in maize MHS-resistance breeding. The newly released maize genome sequencing data will further provide great help for developing molecular markers more closely linked to MHS-resistant gene.

In this study five maize combinations (by crossing ‘R24’ with five MHS-susceptible inbred lines) were investigated in genetic statistic analysis of the MHS-resistance gene. In F_2 populations derived from four crosses (“R24 × Huangzao4”, “Qi319 × R24”, “Lu2548 × R24”, “H9 × R24”), it was observed that the segregation ratios of resistant to susceptible individuals were fit to 3:1 ratio (Table 3), which indicated that a single dominant gene controlled the resistance to MHS in maize ‘R24’. However, in the F_2 population derived from the cross “R24 × Zheng22”, the segregation ratio of resistant to susceptible individuals was not consistent with 3:1 ratio. This result indicated that the genetic traits of the *RsrR* gene were also subjected to the impact of the genetic background of the parent ‘Zheng22’. But, what is the impact and how it affects the *RsrR* gene are not clear yet.

To minimize the error in phenotype determination of a F_2 population, Zhang et al. (1994) developed a method using bulked extreme and recessive F_2 population. The method was used in this study and good results were obtained. The soil inoculation of MHS pathogen by bunch planting method can’t guarantee every maize seed to be inoculated by teliospores. The possibility that a resistant individual is due to failure of

inoculation is existing, which will result in the phenotyping error. However, the susceptible individuals must be infected by inoculated teliospores, thus the phenotyping result of recessive F_2 individuals is absolutely correct and reliable. Therefore, in this study, only the susceptible F_2 individuals were used for linkage marker screening.

In marker screening study, when all of the SSR markers including the existing markers and newly developed markers in accordance with the available maize sequence information were exhausted, but the screened linkage makers were still not close enough to the *RsrR* gene, in this situation we adopted SRAP method to search molecular markers tightly linked with the target gene. Finally, two more closely linked SRAP markers were screened from 30 tested SRAP primer-combinations. This result suggests that SRAP analysis combined with BSA analysis might be a good alternative method for searching molecular markers linked with target gene more closely.

As the rapid development of maize molecular biology, several high density SSR maps have been constructed. The plentiful of SSR markers throughout the 10 chromosomes can be used as anchor loci to map newly discovered genes. By using the SSR-BSA analysis, linkage markers can be screened out without difficulty. As long as some SSR markers linking to the target gene were found, the target gene can be assigned a location immediately (Chantret et al. 2000). In some plants with fine developed SSR maps, SSR-BSA analysis seems to be a fast and effective way for primary mapping a newly discovered gene (Chantret et al. 2000; Zhao et al. 2010). Because all of the screened markers are mapped already, they can be used as anchor markers for mapping a new gene. If any of the screened markers is found to be linked with the target gene, the target gene must be located on the same chromosome with the screened linkage marker. That is to say the target gene is primary mapped.

In the past, by using 'Huangzao4' or 'Qi319' as one parent, numbers of wide-spread maize varieties was developed and used in hybrid maize production in China. Some of them are still planting in quite large area (Zeng et al. 1996; Ye 2000). But 'Huangzao4' and 'Qi319' were found susceptible to MHS recently. Thus, the occurrence and prevalence of MSH has posed a great threat on many varieties derived from these two backbone inbred lines. The discovery of MHS-resistant gene *RsrR* and the successful transferring of the *RsrR* gene into inbred lines 'Huangzao4' and 'Qi319' will make great contribution to decrease the above threat. The successfully transferring *RsrR* gene into inbred lines 'Huangzao4' and 'Qi319' indicates that it is feasible to spread the MHS-resistant gene *RsrR* to other elite inbred lines with various good genetic backgrounds by traditional hybridization combined with MAS technique. The spreading and widely utilization of the *RsrR* gene will greatly facilitate the maize MHS-resistance breeding.

Although the race Shenyang-1 of *S. reilianum* has been applied widely to MHS-resistance breeding in China, the relationship between the race Shenyang-1 of *S. reiliaum* and the other pathogen races used in other reports is not clear yet. It is necessary to perform the allelic analysis among the different MHS-resistance genes.

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