Identification of Molecular Markers Linked to Head Smut Resistance Gene (*Shs*) in Sorghum by RFLP and RAPD Analyses

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ABSTRACT

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Restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) methods were used to find markers linked to a head smut resistance gene (*Shs*) in sorghum. To select parents for a mapping population, RFLPs were identified between four resistant accessions (Lahoma Sudan, White Kafir, SC325, and CS3541) and four susceptible accessions (RTx7078, SC170-6-17, BTx399, and BTx623) for three restriction enzymes (*Eco*RI, *Eco*RV, and *Hin*dIII) with 43 maize genomic clones. Since SC325 (resistant) and RTx7078 (susceptible) showed the maximum RFLP frequency, these accessions were selected for mapping. Fifty-two F_2 progenies from a selfed cross between accessions SC325 and RTx7078 were used to map the *Shs* locus. One hundred twenty-four sorghum genomic clones with five restriction enzymes (*Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, and *Xba*I) and 326 RAPD markers were used for linkage analysis with *Shs*. Linkage of RFLP and RAPD loci with *Shs* was verified by using F_3 lines to determine the *Shs* genotypes of the F_2 plants after inoculation with a race 5 isolate of *Sporisorium reilianum*. RFLP and RAPD analyses revealed that RFLP loci detected by probes pSbTXS560 and pSbTXS1294 and one RAPD locus from primer OPG5 were linked to *Shs*.

Additional keywords: bulked segregant analysis, sorghum hybrid breeding.

Sorghum head smut, caused by *Sporisorium reilianum* (Kühn) Langdon & Fullerton, is an important disease of sorghum (*Sorghum bicolor* (L.) Moench) in Africa, Asia, Australia, Europe, and North America. Chemical control and effective agronomic practices are not sufficient to efficiently reduce disease incidence. Resistant cultivars are the most effective method to control this disease, but head smut still remains a potentially important disease because of the pathogen's variability (6). In the United States, four physiological races have been defined among sorghum isolates of *S. reilianum* on the basis of their reactions on a series of host differentials (7). An isolate that originated in Taylor, Texas, was determined to be race 5 of *S. reilianum*. Resistance to race 5 of *S. reilianum* in several sorghum accessions was expressed as a dominant trait (20). However, there is no information on linkage of any genes for sorghum head smut resistance.

Since the recognition of restriction fragment length polymorphisms (RFLPs) as a useful tool for genome mapping by Botstein et al (2), RFLP markers have been introduced into general plant breeding programs (9) and used to tag disease resistance genes (29). Disease resistance genes linked to RFLP markers have been identified in several important crops, including maize (1), potato (13,25), rice (26), and tomato (10,14).

Recently, Williams et al (30) developed a procedure that employs random DNA primers in a polymerase chain reaction (PCR) to rapidly generate polymorphic markers that can be used to create genetic linkage maps. Since then, random amplified polymorphic DNAs (RAPDs) have been used to produce maps in several plant species (23,24,30) and to tag major genes for disease resistance in common bean (8,17), lettuce (21), and tomato (14,15). Michelmore et al (16) proposed using bulked segregant analysis for the identification of RFLP or RAPD markers linked to important genes in plants where near-isogenic lines are not available. The combination of RAPDs and bulked segregant analysis serves to enhance the identification of markers that are tightly linked to the gene of interest.

One of the goals in our sorghum hybrid breeding is to improve the ability to select and combine genes for sorghum head smut resistance via their linkage to easily detectable RFLP or RAPD markers. This study reports two RFLP markers and one RAPD marker that are linked to a sorghum head smut resistance gene in accession SC325.

MATERIALS AND METHODS

Parental lines. Four resistant accessions (Lahoma Sudan, White Kafir [PI48770], SC325 [IS2462der.], and CS3541 [IS3541der.]) and four susceptible accessions (RTx7078 [IS415], SC170-6-17 [IS12661der.], BTx399 [IS169], and BTx623) were used to select parents for a mapping population of a gene for sorghum head smut resistance (*Shs*). These accessions confer resistance whether they have been naturally inoculated or injected with sporidia, but it has not been determined whether they have the same *Shs* resistance genes.

Inoculation and resistance assessment. In order to obtain sporidia, teliospores of *S. reilianum* were surface sterilized in 95% ethanol, air dried, and streaked onto acidified potato-dextrose agar for germination. Colonies of sporidia from individually germinated teliospores were maintained separately and incubated at room temperature for 4 days. Sporidia of a single teliospore colony were transferred to potato-dextrose broth and increased on a rotary shaker at room temperature and 100 rpm for 4 days. The sporidia were then filtered through four layers of cheesecloth. The sporidia from three individual teliospores were mixed before inoculation. A hypodermic syringe was used to introduce the mixed sporidia (5×10^3/ml) into 4-wk-old sorghum seedlings immediately below the apical meristem (4).

F_3 lines were used for progeny tests to identify the genotype of F_2 plants on the basis of resistance or susceptibility to race 5 of *S. reilianum*. At least 10 F_3 plants were scored per F_2 plant.

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Susceptible plants developed smut sori and/or sterile panicles. Plants that did not develop smut sori and/or sterile panicles more than 2 mo after inoculation were scored as resistant.

DNA extraction. Total DNA was extracted from freeze-dried leaves of four resistant accessions, four susceptible accessions, and F_2 and F_3 plants from SC325 × RTx7078 by the method of Murray and Thompson (18) as modified by Saghai-Maroof et al (27) with minor modifications. Freeze-dried and ground leaf tissue (300–500 mg) was mixed with CTAB (cetyltrimethylammonium bromide) extraction buffer (7.4 ml of distilled water, 1 ml of 1 M Tris [pH 8.0], 1.4 ml of 5 M NaCl, 0.2 ml of 0.5 M EDTA, 0.1 ml of β-mercaptoethanol, and 0.1 g of CTAB) and incubated for 3 h at 65 C with occasional mixing. This mixture was extracted with chloroform and octanol (24:1) and centrifuged, and the aqueous phase was transferred to a new tube. The DNA was precipitated with cold isopropanol, spooled, rinsed (in 76% ethanol plus 0.2 M NaOAc and then in 76% ethanol plus 10 mM NH_4OAc), dried, and dissolved in Tris-EDTA buffer. The DNA concentration was measured with a TKO 100 DNA fluorometer (Hoefer Instruments, San Francisco, CA).

RFLP analysis. As a preliminary step, 43 maize genomic clones obtained from the University of Missouri were used with three restriction enzymes (*Eco*RI, *Eco*RV, and *Hin*dIII) to identify polymorphic parents suitable for mapping population.

The 124 sorghum genomic clones used to search for the *Shs* gene in SC325 are located at intervals of approximately 15–20 centimorgans (cM) throughout the sorghum genome (31). To increase the probability of detecting polymorphisms in the parents, five restriction enzymes (*Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, and *Xba*I) were used.

The cloned inserts were digested with *Pst*I and electrophoresed on 0.8% low-melting agarose gels. The appropriate band was excised and labeled with ^{32}P-dATP by the random primer method (5). Five micrograms of sorghum DNA was digested with *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, and *Xba*I. Digested DNA was separated by electrophoresis on 0.8% agarose gels, denatured (0.25 N HCl for 15 min), and blotted onto Zetaprobe membranes with 0.4 N NaOH as the transfer solution (28).

Prehybridization and hybridization were carried out in sealed plastic pouches at 60 C for maize RFLP probes and at 65 C for sorghum RFLP probes. Hybridization was carried out overnight at 60 or 65 C with gentle agitation. Filters were washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.5% sodium dodecyl sulfate (SDS) at room temperature for 5 min and then in 0.1× SSC plus 0.1% SDS at 60 or 65 C for 30 min. The washed filters were exposed to X-ray films for 3–4 days at −80 C. Probes were removed from the membrane by washing in 0.2 N NaOH at room temperature for 30 min and then in 0.25 M Tris (pH 7.5), 0.1× SSC, and 0.1% SDS at room temperature.

RAPD analysis. A total of 326 10-base primers (Operon Technologies Inc., Alameda, CA) were surveyed with DNA extracted from SC325 and RTx7078. If a primer showed a polymorphism between SC325 and RTx7078, it was resurveyed with bulks of DNA from the homozygous resistant F_2 plants and from homozygous susceptible F_2 plants. Aliquots (2.0 μg of DNA) of individual F_2 plants were bulked (16). DNA from eight susceptible plants was combined, but the resistant bulk contained DNA from eight to 15 plants. Amplification reactions were in volumes of 10 μl containing 10× PCR buffer, 0.5 mM dNTP, 0.4 mM $MgCl_2$, 0.5 unit of *Taq* DNA polymerase (Promega, Madison, WI), 0.4 μM primer, and 5 ng of genomic DNA. Amplification was performed in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) programmed for 45 cycles of 1 min (5 min for first cycle) at 94 C, 1 min at 36 C, and 2 min at 72 C followed by a final extension of 15 min at 72 C (30).

Linkage analysis. Linkage analysis of RFLP and RAPD markers segregating in the F_2 population was performed with the program MAPMAKER Macintosh V 1.0 (12). RFLP and RAPD markers were first processed by two-point analysis with the logarithm of odds (LOD) = 3.0 and maximum theta = 4.0 to infer a possible linkage group associated with *Shs*. The groups were processed by multiple point analysis (LOD = 3.0) with a maximum of five markers by using the "compare" command to determine an acceptable order for these markers (12). Distances between markers are presented in centimorgans derived with the Kosambi function (11).

RESULTS

Selection of parents to map. RFLPs were identified between four resistant accessions and four susceptible accessions by using three different enzymes (*Eco*RI, *Eco*RV, and *Hin*dIII) and 43 maize genomic clones. Among the 16 pairs of resistant and susceptible accessions, SC325 and RTx7078 showed the maximum level of RFLP that could be easily detected. Therefore, a cross was made between SC325 and RTx7078 to map the *Shs* locus. An F_2 population consisting of 52 progeny was used for linkage mapping.

Segregation analysis of *Shs*. F_3 lines derived from 52 single F_2 plants were used to determine the resistance genotype of the F_2 plants. Of the 52 F_2 plants, 19 were inferred to be homozygous resistant to *S. reilianum,* eight were homozygous susceptible, 23 were heterozygous resistant, and two were scored as missing data for a 1:2:1 ratio ($\chi^2 = 5.038$; $P > 0.05$) (Table 1).

RFLP linkage analysis for *Shs*. To identify RFLP markers for *Shs,* 124 sorghum genomic clones were screened after digestion of sorghum genomic DNA with five different restriction enzymes (*Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, and *Xba*I). Of the genomic clones tested, 102 showed polymorphism between SC325 and RTx7078 with at least one restriction enzyme. To determine which of the 102 RFLP markers were linked to *Shs,* the DNA probes were hybridized to filters that contained DNA from the two parents and 52 F_2 progenies that had been digested with a restriction enzyme that generated easily distinguished polymorphisms.

Evidence of linkage to *Shs* was found with the locus detected by pSbTXS1294 (19), which maps on the end of linkage group A in the sorghum RFLP map (Fig. 1A) (31). In further mapping analysis, the locus detected by pSbTXS560, which is located near *txs1294* (Fig. 1A), was also found to be linked to *Shs*. Linkage analysis showed that the *Shs* locus was linked to two RFLP loci, *txs560* and *txs1294,* at 8.8 and 19.9 cM, respectively (Fig. 1B).

PCR analysis for detecting RAPDs that are linked to *Shs*. Approximately 1,467 discrete products, ranging from 0.3 to 2.6 kilobase pairs, were amplified per parental accession (average 4.5 products per primer). Although the two parents are not near-isogenic lines, the majority of the products were identical in both parents. Nineteen of 326 (5.8%) RAPD primers produced DNA fragments that were polymorphic between the two parents. In the bulked segregant analysis, only primer OPG5 generated one polymorphic product (OPG5-2) that was amplified from the homozygous resistant, but not the susceptible, bulk. OPG5 generated two polymorphic products (OPG5-2 and OPG5-1) that differed between SC325 and RTx7078, but only the former was linked to *Shs* by F_2 segregation (Fig. 2). When each F_2 plant was individually tested, linkage analysis showed that the *Shs* locus was linked to RAPD locus *opg5-2* at 6.0 cM (Fig. 2B).

DISCUSSION

In theory, combining different genes for head smut resistance may lead to more durable resistance and alleviate the need to continuously search for new sources of disease resistance. However, in order to do so, it will be imperative to easily identify each of the resistance genes that may be present in a particular individual. Current evaluation protocols, including natural infection, hypodermic inoculation (4), and seedling reaction (3), have classified sorghum genotypes as R1, R2, R3, and S1 according to factors for resistance to *S. reilianum* (3), but the genes involved have not been identified. According to the reaction to *S. reilianum* based on natural inoculation and hypodermic inoculation, SC325 is classified as an R3 genotype. Since R3 genotypes are resistant under natural inoculation and by hypodermic inoculation, they have factors for resistance in both nonmeristematic and meri-

stematic tissues that lead to an incompatible host-pathogen interaction. The head smut resistance gene (*Shs*) of SC325 is a valuable source of resistance to *S. reilianum*. RFLP marker loci detected by pSbTXS560 and pSbTXS1294 and a RAPD marker locus from primer OPG5 linked to *Shs* in SC325 may help to identify genotypes of sorghum possessing the resistance allele, to select for other head smut resistance genes, or to breed resistant sorghum hybrids.

Two predictable problems arose in trying to pinpoint the location of the *Shs* allele: 1) probes that revealed polymorphism in the original mapping parents (IS3620C and BTx623) did not detect RFLPs for any of the five enzymes tested in the resistant and susceptible parents (SC325 and RTx7078); and 2) different loci among several fragments detected by the same probe were polymorphic in the two sets of parents.

Four RFLP marker loci, located near *txs560* and *txs1294* on the sorghum linkage group A (31) and detected by pSbTXS2065, pSbTXS1220, pSbTXS1311, and pSbTXS1353, were selected to attempt to better pinpoint the *Shs* locus. Unfortunately, these markers did not reveal linkage to *Shs,* and the end of linkage group A does not have enough markers to find tightly linked markers. On the basis of RFLP analysis, the two RFLP marker loci detected by pSbTXS560 and pSbTXS1294 were not tightly linked to *Shs*. Therefore, to find markers associated with *Shs* and to fill a gap in that region, RAPD and bulked segregant

TABLE 1. The reaction of 52 F_2 progenies from SC325 × RTx7078 to *Sporisorium reilianum* and the segregation data of four loci (*txs560*, *Shs*, *opg-2*, and *txs1294*)

Progeny no.	Reaction[a]	Genotype[b] A	B	C	D
1	seg	2	2	4	2
2	HR	1	1	4	1
3	seg	2	2	4	3
4	HR	2	1	4	2
5	seg	2	2	4	2
6	seg	1	2	4	2
7	seg	2	2	4	2
8	seg	2	2	4	3
9	HR	1	1	3	2
10	HS	3	3	3	3
11	seg	2	2	4	1
12	HR	2	1	4	2
13	HR	1	1	4	1
14	HR	...[c]	1	4	1
15	HR	1	1	4	2
16	HR	2	1	4	1
17	seg	2	2	4	2
18	seg	2	2	4	2
19	HS	3	3	3	2
20	seg	2	2	4	2
21	HR	...	1	4	1
22	seg	2	2	...	...
23	HR	1	1	4	1
24	seg	3	2	4	1
25	HR	1	1	4	1
26	seg	1	2	4	1
27	seg	2	2	4	2
28	seg	2	2	4	2
29	HR	2	1	4	2
30	seg	2	2	4	2
31	HR	1	1	4	1
32	...	2	...	4	2
33	seg	2	2	4	1
34	HR	...	1	4	2
35	...	2	...	4	3
36	seg	2	2	3	3
37	HR	...	1	4	2
38	seg	2	2	4	2
39	HS	3	3	3	3
40	HS	3	3	3	3
41	HR	1	1	4	1
42	seg	2	2	4	3
43	seg	1	2	4	2
44	HS	3	3	3	3
45	seg	1	2	4	2
46	HR	1	1	4	1
47	HR	1	1	4	1
48	HR	1	1	4	1
49	HS	3	3	3	3
50	HS	3	3	3	2
51	HS	3	3	3	...
52	seg	2	2	3	3

[a]Reactions were classified as HR for homozygous resistant, seg for segregating, and HS for homozygous susceptible.
[b]Genotypes were classified as 1 for SC325 alleles, 2 for heterozygous, 3 for RTx7078 alleles, and 4 for indistinguishable SC325 or heterozygous. A = *txs560*; B = *Shs*; C = *opg5-2*; and D = *txs1294*.
[c]Missing data.

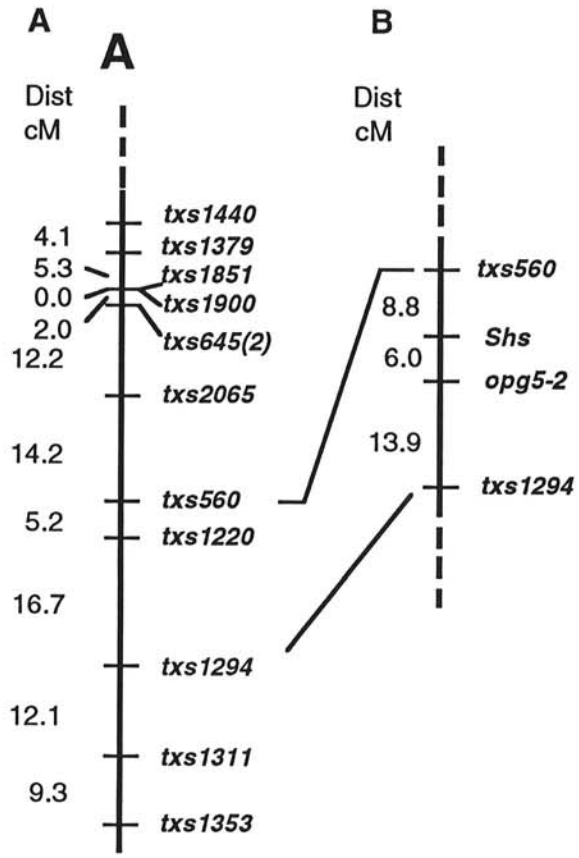

Fig. 1. Position of the *Shs* locus on the restriction fragment length polymorphism (RFLP) linkage map of sorghum. **A,** Linkage group A was derived from the segregating population (IS3620C × BTx623) used for the sorghum RFLP map (31). **B,** A part of the sorghum linkage group showing the linkage maps around the *Shs* locus derived from SC325 (resistant) and RTx7078 (susceptible).

Fig. 2. Segregation analysis by polymerase chain reaction amplification of genomic DNA from 26 F_2 progeny with primer OPG5. A total of 52 F_2 plants derived from SC325 × RTx7078 were scored for their reactions to *Sporisorium reilianum* and for their genotypes with respect to random amplified polymorphic DNA markers selected. Left two lanes are SC325 and RTx7078, respectively. 1 = Homozygous for SC325 alleles (resistant phenotype); 2 = heterozygous (resistant phenotype); 3 = homozygous for RTx7078 alleles (susceptible phenotype); and 4 = not homozygous for RTx7078 alleles (resistant phenotype). M = molecular standard marker (pGEM marker).

analysis were used. One RAPD locus (*opg5-2*) was added between *Shs* and *txs1294*. Although PCR amplification is sensitive to specific reaction conditions, the sensitivity can be offset by converting polymorphic PCR products to reproducible RFLP markers for mapping purposes (15) and to sequence characterized amplified region markers for plant breeding applications such as marker-aided selection and cultivar identification (22). In this study, the combination of RAPDs and bulked segregant analysis proved to be faster and less expensive than the RFLP technique in finding a useful DNA tag for the *Shs* locus.

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