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Molecular markers linked to stem rot resistance in rice

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Abstract Stem rot (*Sclerotium oryzae*) is an important disease constraint in Californian rice production. Measurement of resistance is laborious, and the low heritability of the trait limits the effectiveness of selection in breeding programs. Molecular markers linked to the trait would therefore provide a superior selection screen to assist in transferring resistance into improved cultivars. The genetics of resistance to stem rot was studied in the germplasm line 87-Y-550 (PI566666), which inherited its resistance from the wild species *Oryza rufipogon*. Four crosses of 87-Y-550 with susceptible lines were made and recombinant inbred lines of only the most-resistant and most-susceptible progeny within each cross were advanced for late-generation testing. Approximately 900 AFLP (amplified fragment length polymorphism) primer combinations were applied to resistant and susceptible bulks within each cross. One AFLP marker showed significant association with stem rot resistance and accounted for approximately 45.0% of the phenotypic variation in 59 progenies. This marker was mapped on rice chromosome 2 between the RFLP markers RZ166 and RG139 by using F_2-reference population information. The accuracy of AFLP marker mapping was validated by size and sequence comparison of AFLP bands from 87-Y-550 and the reference population. With the strategy of selective genotyping combined with a parental survey, two microsatellite markers, RM232 and RM251, on chromosome 3 were also found associated with stem rot resistance and accounted for 41.1% and 37.9% of the phenotypic variation, respectively. The multiple linear regression model included TAA/GTA167 on chromosome 2 and RM232 on chromosome 3 and cumulatively explained 49.3% of total variation. The molecular markers linked to stem rot resistance should facilitate selection for this recalcitrant trait in rice breeding programs by eliminating the need for early generation screening.

Keywords Rice · *Oryza sativa* · AFLP · Microsatellite · Disease resistance

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Introduction

Stem rot, caused by the fungal pathogen *Sclerotium oryzae* Catt. [perfect state=*Magnaporthe salvinii* (Cattaneo) Krause & R.K. Webser, conidial state=*Nakataea sigmoidea* (Cavara) K. Hara] is found in many rice-growing areas (Ou 1985). The disease causes yield losses through reduced tillering, unfilled panicles, chalky grain, decreased milling yields, and increased lodging. In most countries, this is not regarded as a serious constraint to rice production. In California, stem rot has been the most important rice disease. A yield loss of up to 75% has been reported (Ou 1985), but annual losses from the disease are estimated at 5–10% (Krause and Webster 1973). Yield losses up to 22% (Krause and Webster 1973) and 30% (Cother and Nicol 1999) were reported under field conditions. With the legislated phase-out of rice straw burning there is concern that stem rot incidence will increase.

Resistant cultivars have been identified in India (Singh and Dodan 1995) and the Philippines (IRRI 1966). In California, differences in resistance among cultivars have been observed, with the resistance being quantitatively inherited (Ferriera and Webster 1975). The differences in resistance among cultivars, however, are small.

An accession of the ancestor of Asian cultivated rice, *Oryza rufipogon*, was identified with a useful level of resistance (Figoni et al. 1983). Genetic studies indicated that the resistance was controlled by at least two genes

(Figoni 1981; Oster 1990). Resistance was transferred from this accession into a Californian japonica background, and a high yielding breeding line, 87-Y-550 (PI566666), was released as germplasm (Tseng and Oster 1994). While not possessing complete resistance, this accession reduces sclerotia production and is sufficient to reduce inoculum and disease severity (Oster 1992).

The assessment of disease levels in breeding lines is complicated by the quantitative nature of resistance and the large effect of the environment. Accurate disease measurement requires replication over space and time. Disease measurements on individual plants are not accurate enough for the selection of resistance in segregating populations. Transferring resistance into elite cultivars would be facilitated by an easily scored marker linked to the gene or genes controlling resistance. In the present paper we report the molecular markers linked to stem rot resistance that can be used in selection.

Materials and methods

Plant materials

Four populations were generated from crosses of the stem rot-resistant line '87-Y-550' (PI 566666) (Tseng and Oster 1994) with adapted long grain lines (R17277=90-Y-479/87-Y-550, R17278=90-Y-375/87-Y-550, R17281=87-Y-550/8940113, and R17636=87-Y-550/9031535). In 1992, 400 F_2 plants per population were transplanted, 15 per row. Plants were spaced 0.3-m within rows and 0.5-m between rows. Appropriate parental checks were inserted as rows between populations. This was continued until the F_6 (the F_3 and F_5 generations were advanced in the greenhouse), and population size was reduced to 200 plants per cross for the F_7 to the F_9. In 1994, two replicate F_5 rows were planted from seed obtained from each 1993 F_4 transplants. Rows 1.2-m long and 0.5-m apart were each planted with 2 g of seed. Based on transplant and row data, 50 plants with the highest and 50 plants with the lowest stem rot ratings were selected for line testing in the F_7 to F_8 (three replications) and the F_9 (four replications).

All field research was conducted at the Rice Experiment Station, Biggs, California, on Stockton clay adobe soil. Standard cultural practices for rice production in California were followed (Rutger and Brandon 1981), with fertilization ranging from 98 to 121 kg N/ha (basal plus topdressing). The insecticide Furadan (Carbofuran) was pre-plant incorporated at the rate of 0.6 kg/ha. The grass herbicide Ordram (Molinate) and broadleaf herbicide Londax (Bensulfuronmethyl) were broadcast by airplane into the water, at the rates of 4.5 kg ai/ha and 0.07 kg ai/ha, 8–13 days after seeding.

Inoculation and disease evaluation

Inoculum production techniques involved growth of nine single-spore isolates on autoclaved rough rice medium, and harvested with a grain sheller and screens of various pore sizes (Krause and Webster 1973; Oster 1990). The inoculum consisted almost entirely of sclerotia. Cultures were prepared for long-term storage by growing on a soil-wheat bran medium, drying, and placing in a freezer at 0°C (Butler 1980). Transplants and rows were inoculated by broadcasting sclerotia with a fertilizer spreader at the rate of 2.6 g (2.0×10^5 sclerotia) per m^2 between 60 and 66 days after seeding when plants were close to the sensitive early internode elongation stage (Krause and Webster 1973). Sclerotia were dispersed on the water surface and contacted plants at the waterline.

For transplants, the ten most-diseased tillers were scored for each plant 42 days after flowering. For rows, 15 tillers per replicate were scored 42 days after flowering. Plants were rated for stem rot severity on a scale of 0–10, where 0 represented no infection, and 10 represented culm penetration and tiller death (Krause and Webster 1973; Oster 1990). All data were transformed in an attempt to minimize between year differences in disease severity. The normalization formula was:

$$\frac{\text{(susceptible parent score)} - \text{(line score)}}{\text{(susceptible parent score)} - \text{(87-Y-550 score)}}$$

A score of zero indicated that a line was as diseased as its susceptible parent, and a score of '1' indicated it was as resistant as 87-Y-550.

AFLP marker analysis

AFLP marker analysis was performed based on the procedures described by Vos et al. (1995) and Mackill et al. (1996). Briefly, genomic DNA was digested with two restriction enzymes (*Eco*RI and *Mse*I), ligated to adapters, and amplified with two primers with 3-bp selective nucleotides at the 3′ end. Fragments were separated on 4.5% denaturing polyacrylamide gels. A total of 900 primer combinations were employed. Based on the average number of bands observed for +3/+3 primer combinations (Mackill et al. 1996), this would represent about 27000 bands. AFLP markers were initially assessed on bulks of the resistant and susceptible lines within a cross. Primer combinations that showed a differential reaction between the bulks were tested on individual lines.

The AFLP band originating from 87-Y-550, TAA/GTA_{167}, was associated with stem rot resistance. As the populations used to identify markers associated with stem rot resistance were from relatively narrow crosses and could not be used to map the marker of interest, another indica×japonica F_2 population was used as the reference mapping population PI543851×IR40931–26. This reference population was previously used to map submergence tolerance (Xu and Mackill 1996). In this reference population, a fragment identical to TAA/GTA_{167} was also segregating and was mapped using the Mapmaker 2.0 for Macintosh.

In order to validate the mapping of TAA/GTA_{167} by using the reference population, the bands from 87-Y-550 and the reference mapping population were subjected to a size and sequence comparison. Briefly, the bands from both sources were excised directly from the dried silver-stained denaturing polyacrylamide gels on the glass plate using a razor blade. These bands were used directly as templates in a total PCR reaction volume of 50 µl. The PCR profile and reaction components were the same as those described above. These fresh PCR products were analyzed on 1% agarose and 5% LongRanger gels to compare the band size. Then, they were ligated into the TA cloning vector (pCR 2.1 vector, *Invitrogen* Corporation, Cat. No. K2000–01) according to the manufacturer's instructions. DNA sequencing was carried out by a 377 automated DNA sequencer using the ABI Primer Big-Dye Terminator Cycle Sequencing Ready kit (PE applied Biosystems).

Microsatellite marker analysis

As the population from the cross R17277 had the widest divergence for stem rot scores, the parental DNA from this cross was firstly used to detect the polymorphism of 109 rice microsatellite primer pairs (Research Genetics, Huntsville, Ala., USA) as described in Panaud et al. (1996) and Chen et al. (1997). Forty two polymorphic microsatellite markers were further assessed on selective genotypes of seven highly tolerant and eight susceptible lines from the same population. The primer pairs that showed a differential reaction between selective genotypes were tested on individual lines. These selected primer pairs were also used for the parental survey and individual evaluation of the other three populations.

The polymerase chain reaction (PCR) for microsatellite analysis followed ABI Prism 377 GeneScan protocols with minor modifications. It was performed in 15 µl of a mixture containing 50 ng of DNA, 330 nM of each primer, 250 µmol of each dNTP, 0.6 U of

Taq DNA polymerase in reaction buffer (20 mM TRIS pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 50% glycerol). Three hundred and thirty nanometers of fluorescent d UTPs labeled with a rhodamine dye (R110, R6G) were incorporated into the PCR products to enable detection of the fragments in the ABI377 automated sequencing system (Perkin-Elmer). The PCR was run on the GeneAmp PCR System 9700 thermocycler as follows: an initial denaturation step of 3 min at 94°C followed by 35 cycles of 94°C for 1 min, 55°C for 2 min, 72°C for 1.5 min and concluded with a final extension step for 5 min at 72°C. PCR products were analyzed on a sequencing gel (5% LongRanger, 1×TBE buffer, 6 M urea) in an automated ABI377 sequencing apparatus (Perkin-Elmer). Fragment lengths were estimated using internal size standards [GeneScan-500 (ROX), Cat. No. 401734] by GeneScan Analysis Software (Perkin-Elmer).

Statistical and QTL analysis

The individual lines from four populations consisted of resistant and susceptible groups based on selection for response to stem rot over several generations. Chi-square analysis was performed to determine independence of stem rot resistance and the marker class. For QTL analysis, the scores for stem rot severity were used after being transformed by the normalization procedure described above. Single-marker locus analysis (SAS GLM, SAS Institute Inc 1989) at a probability of less than 0.005 for error I was employed for detecting marker loci associated with the variations in the transformed row score data. The proportion of the phenotypic variance explained by the marker loci linked to the QTLs detected was investigated by regression analysis. Experiment-wise error was estimated by using 1000 permutations of the marker data (Churchill and Doerge 1994) as calculated by the program QGENE for Macintosh (Nelson 1997).

Results and discussion

All crosses were segregating for stem rot resistance, and the four susceptible parents were consistently more susceptible than the resistant parent 87-Y-550 (Fig. 1). The resistance values of the F_6 lines were significantly different at the 0.001 level by the analysis of variance, and the difference between resistant and susceptible lines was also significant at the 0.001 level by the *t*-test. Our original intention was to obtain approximately 20 resistant and 20 susceptible lines within each of the four crosses for molecular-marker analysis. However, many of the lines selected as resistant and susceptible in early generations (F_3–F_4) did not perform consistently in later generations. The number of resistant lines was 7, 7, 2, and 15, and the number of susceptible lines was 16, 4, 6 and 2, for the four crosses R17277, R17278, R17281 and R17636, respectively. In addition to possessing the largest number of selected lines (23), the population from the cross R17277 had the widest divergence for stem rot scores (Table 1).

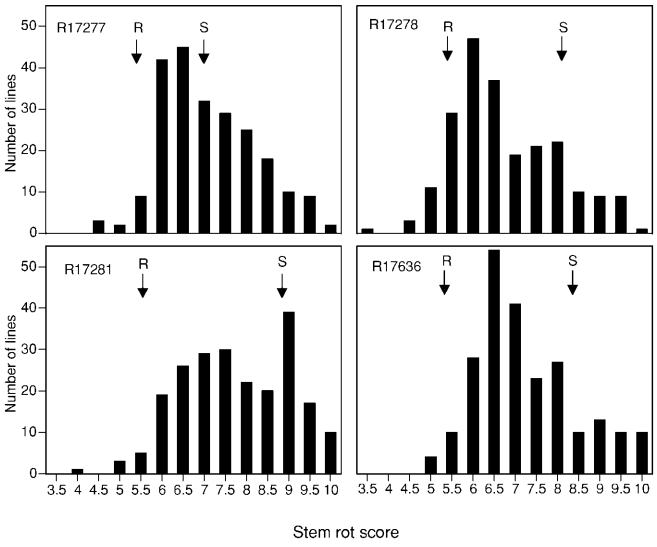

Fig. 1 Distribution of raw stem rot scores in F_6 lines and parents of four crosses scored for resistance (Biggs, Calif., 1994)

Among the 900 primer combinations studied, only nine polymorphic bands showed significant differences between resistant and susceptible RILs by the chi-square test. These markers were analyzed by regression using single-marker analysis. While most of these markers showed probabilities of 0.01 or below, only one marker, TAA/GTA_{167}, was significant using the experiment-wise error value from the permutation test (Churchill and Doerge 1994). When the raw means for stem rot scores were used, this marker accounted for 42.0% of the phenotypic variation observed in the 59 RILs. When normalized scores were used, the R^2 rose to 45.0% (see Table 3). As mentioned previously, the population R17277 had the widest divergence for stem rot reaction among the four crosses. In this population, the AFLP fragment TAA/GTA_{167} was completely associated with resistant individuals (Fig. 2, Table 2).

Using the reference population, we mapped the AFLP marker associated with stem rot resistance, $TAA/GTA_{167,}$

Table 1 Distribution of normalized stem rot scores for recombinant inbred lines of four crosses between an accession resistant to stem rot (PI566666) and susceptible long-grain rice parents

Cross	Suceptible parent	No. of lines in each stem rot class[a]																	
		–1.4	–1.2	–1	–0.8	–0.6	–0.4	–0.2	0	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2
R17277	90Y479	3	1	2	1	1	3	1	1	1	1	0	0	2	0	1	2	0	2
R17278	90Y375	0	0	0	0	0	0	2	1	1	1	0	2	0	3	0	0	0	1
R17281	8940113	0	0	0	0	0	0	1	1	4	0	0	0	2	0	0	0	0	0
R17636	9031535	0	0	0	0	0	0	0	2	0	0	0	5	10	0	0	0	0	0

[a] Normalized row stem rot severity score

Table 2 The segregation of molecular markers (TAA/GTA_{167}, RM232, and RM251) in parents and 23 individuals of the cross 17277. Nd, no data available

Plant Number	Response to stem rot	TAA/GTA_{167}[a]	RM232[b]	RM251[c]
87-Y-550	R	1	1	1
90-Y479	S	0	3	3
1	R	1	1	1
2	R	1	1	1
3	R	1	1	1
4	R	1	nd	nd
5	R	1	1	1
6	R	1	1	1
7	R	1	1	1
8	S	0	3	3
9	S	0	3	3
10	S	0	3	3
11	S	0	3	3
12	S	0	3	1
13	S	0	3	3
14	S	1	3	3
15	S	0	3	3
16	S	0	3	3
17	S	0	3	3
18	S	0	3	3
19	S	0	3	3
20	S	0	3	3
21	S	0	3	3
22	S	0	1	1
23	S	0	3	3

[a–c] band size:
[a] 1, 167 bp; 0, no band
[b] 1, 157 bp; 3, 142 bp
[c] 1, 116 bp; 3, 118 bp

Table 3 Molecular markers showing a significant association with stem rot resistance in recombinant inbred lines derived from resistant (PI566666=87-Y-550) by susceptible crosses. The normalized row score for stem rot severity were used for QTL analysis. The experiment-wise error is based on 1000 permutations of the data

Marker	Chromosome	Source	F	R^2	Probability
TAA/GTA_{167}	2	87-Y-550	45.7	0.450	0.000
RM232	3	87-Y-550	39.6	0.414	0.000
RM251	3	87-Y-550	34.3	0.379	0.000
Experiment-wise error (95%)			18.3		

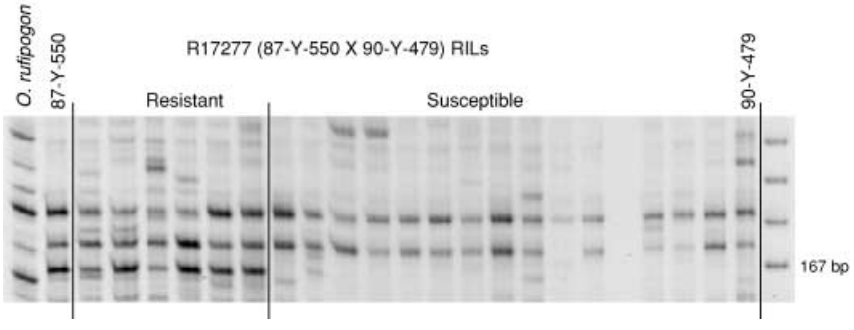

Fig. 2 Section of a gel showing segregation for TAA/GTA_{167}, an AFLP marker linked to stem rot resistance in the cross R17277 (90Y479×87-Y-550)

to rice chromosome 2 (Fig. 3). In general, AFLP markers of similar size, amplified with the same primer-combinations in different populations, can be assumed to be identical (van Eck et al. 1995; Waugh et al. 1997) and it is reliable to use the reference population for mapping the AFLP marker. The fragment-size comparison showed that the PCR products from 87-Y-550 and the reference mapping population were identical in size when analyzed on 1% agarose and 5% LongRanger gels (data not shown). The sequence comparison also showed that the DNA sequences of the cloned inserts representing AFLP products from both sources were exactly the same (Fig. 4). All clones had 167 bp of DNA insert and contained the adapter sequences from the ligation reaction.

Using a selective genotyping strategy combined with the parental survey, two closely linked markers on Chromosome 3, RM232 and RM251, were found related to stem rot resistance. Although these markers are monomorphic in the R17278 and R17636 populations, the close relationship between these markers and stem rot re-

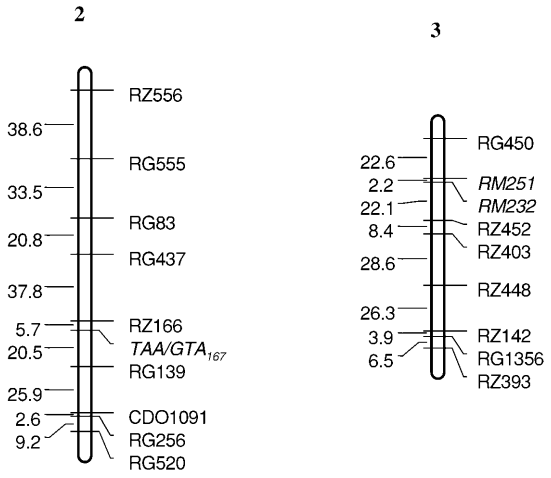

Fig. 3 RFLP linkage map of rice chromosomes 2 and 3 showing the position of TAA/GTA$_{167}$ and RM232 and RM251. The markers were mapped in the japonica/indica cross PI543851×IR40931–26 (Xu and Mackill 1996)

1	GACTGCGTACCAATTCTAAGCAGAATGTGT
31	ATTAATTAGACATACTGTTATGTGGATCGA
61	TCTTCGGCGTATCGATGAAATGAACAAGTC
91	AGTGCTAGCTGTATATAAGCTAGTAATTAT
121	ATACAGCAAGACAAATCTCTGTGATACTAC
151	TATACTCAGGACTCATC

Fig. 4 The nucleotide sequence of TAA/GTA$_{167}$ originated from 87-Y-550, and the reference population from an indica/japonica cross PI543851×IR40931–26. The adaptor sequences are marked by the *thick underlines*

sistance can be found in the individuals from population R17277 (Table 2) and population R17281 (data not shown), When normalized scores were used, RM232 and RM251 accounted for 41.4% and 37.9% of the phenotypic variation, respectively (Table 3). These two microsatellite markers were also segregating in the reference mapping population and were mapped at the expected positions on rice chromosome 3 (Fig. 3). Using a multiple regression model, the two loci on both chromosome 2 and chromosome 3 cumulatively explained 49.3% of the total phenotypic variation. The finding that two different loci in the rice genome were linked with stem rot resistance agrees with the study of Figoni (1981), who reported that the resistance in *O. rufipogon* was controlled by at least two genes.

The stem rot-resistant breeding line 87-Y-550 was developed by crossing the donor of resistance, *O. rufipogon* Accession 100912, of the International Rice Research Institute, with Californian long-grain breeding lines. It represents the equivalent of three backcrosses to the closely related tropical japonica parents. With such a few number of backcrosses, we expect the introgressed segment to be approximately 35 cM on average (Stam and Zeven 1981). This would be about 2% of the rice genome assuming a genetic map of approximately 1500 cM. It is surprising that there were so few positive AFLP markers. Even though the level of polymorphism between the resistant and susceptible parents, which were both tropical japonica types, was low, the level of polymorphism between the wild species donor and the recurrent parents was high. One explanation could be that many positive markers were missed during the screening process. This could have occurred if the bulks were not really made up of truly resistant and susceptible genotypes. In three of the four crosses, the difference between the susceptible and resistant lines was not very pronounced.

Another explanation could be that the introgressed segment containing the resistance was much smaller than would be expected on average. This has indeed been observed for interspecific crosses in rice. In backcrosses with *O. officinalis* to introduce resistance to the brown planthopper, Jena et al. (1992) observed that the introgressed segments were very small.

In summary, our results suggest that two loci on chromosome 2 and chromosome 3 control stem rot resistance in rice. The identification of the molecular markers linked to stem rot resistance provides the basis for developing a more-efficient screen for early generation breeding populations. Generally, microsatellite markers on chromosome 3 may be used efficiently in marker-assisted selection in rice breeding programs. The fingerprint results in our laboratory also show that these markers are polymorphic in Californian cultivars (data not shown). Unfortunately, the AFLP marker TAA/GTA$_{167}$ may not be ideal for selection purposes. In addition to being somewhat tedious to assay, this marker is not highly polymorphic in japonica cultivars (data not shown). A preliminary screening of Californian japonica cultivars with microsatellite markers mapping in this region (Chen et al. 1997) also indicated that they were all monomorphic (data not shown). This may reflect a low level of polymorphism in general for this chromosome among japonica accessions. Redoña and Mackill (1996) also observed a low level of polymorphism in their RAPD map of a cross between a tropical and temperate japonica. Additional work on saturating this region with markers may allow a more accurate delineation of the introgressed segment that harbors the resistance gene. This will improve the chances of identifying polymorphic markers that can be used in breeding programs.

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