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ORIGINAL PAPER

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Tagging and mapping of a rice gall midge resistance gene, *Gm8*, and development of SCARs for use in marker-aided selection and gene pyramiding

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Abstract Using amplified fragment length polymorphisms (AFLPs) and random amplified polymorphic DNAs (RAPDs), we have tagged and mapped *Gm8*, a gene conferring resistance to the rice gall midge (*Orseolia oryzae*), a major insect pest of rice, onto rice chromosome 8. Using AFLPs, two fragments, AR257 and AS168, were identified that were linked to the resistant and susceptible phenotypes, respectively. Another resistant phenotype-specific marker, $AP19_{587}$, was also identified using RAPDs. SCAR primers based on the sequence of the fragments AR257 and AS168 failed to reveal polymorphism between the resistant and the susceptible parents. However, PCR using primers based on the regions flanking AR257 revealed polymorphism that was phenotype-specific. In contrast, PCR carried out using primers flanking the susceptible phenotype-associated fragment AS168 produced a monomorphic fragment. Restriction digestion of these monomorphic fragments revealed polymorphism between the susceptible and resistant parents. Nucleotide BLAST searches revealed that the three fragments show strong homology to rice PAC and BAC clones that formed a contig representing the short arm of chromosome 8. PCR amplification using the above-mentioned primers on a larger population, derived from a cross between two *indica* rice varieties, Jhitpiti (resistant parent) and TN1 (susceptible parent), showed that there is a tight linkage between the markers and the *Gm8* locus. These markers, therefore, have potential for use in marker-aided selection and pyramiding of *Gm8* along with other previously tagged gall midge resistance genes [*Gm2*, *Gm4* (*t*), and *Gm7*].

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The nucleotide sequence data reported here will appear in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession numbers AY545920–AY545923

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Introduction

Rice gall infestation is a serious rice disease caused by a dipteran insect pest known as gall midge (*Orseolia oryzae*). The disease is prevalent in India, China, Southeast Asia and Africa. In Asia alone, the damage caused by gall midge is more than US $550 million per year (Bentur et al. 2003). In India, gall midge infestation is most prevalent in the states of Madhya Pradesh, Chhattisgarh, Bihar, Jharkhand, Orissa, Andhra Pradesh and Maharashtra. Recent reports show that it is becoming a serious threat in Kerala and some northeastern states (Bentur et al. 2003).

The gall midge problem in rice is further compounded by the fact that there are many biotypes of this insect and new biotypes are continuously evolving. In India, until recently, five biotypes of gall midge were known to exist but now a new biotype has been reported from the northeast (Bentur et al. 2003). Different biotypes of gall midge are distributed in different regions of the country. Resistance in a rice variety for a particular biotype is usually governed by a single dominant gene, and a total of nine non-allelic resistance genes have been identified from different varieties of rice that confer resistance against different biotypes of the pest (Kumar et al. 1998; Sardesai et al. 2001). Genetic studies have revealed that there is a gene-for-gene interaction between the different resistance genes and their respective biotypes of gall midge (Harris et al. 2003). The deployment of these resistant genes will not only be environment-friendly but is also likely to provide durable resistance.

The identification and development of DNA-based molecular markers that are tightly linked to a resistance gene enables one to follow the gene in a cross intended to breed new resistant varieties any time of the year without depending on the annual occurrence of insects (Mohan et al. 1997a). Of the total nine gall midge resistance genes that are known (*Gm1* to *Gm9*) so far, four [*Gm2*, *Gm4*(*t*), *Gm6*(*t*) and *Gm7*] have been tagged and mapped (Mohan et al. 1994, 1997b; Nair et al. 1995, 1996; Katiyar et al. 2001; Sardesai et al. 2002). The development of these markers has been made possible using various molecular marker techniques (Mohan et al. 1994, 1997b; Nair et al. 1995, 1996). Amplified fragment length polymorphisms (AFLP) have been widely used as a DNA fingerprinting technique (Vos et al. 1995) in plant genetic studies (Hill et al. 1996; Mackill et al. 1996; Maughen et al. 1996; Hongtrakul et al. 1997; Zhu et al. 1998; Bonnema et al. 2002). Owing to its higher marker index and the potential to scan a wider area of the genome for polymorphisms, the AFLP technique, which is also known to produce highly specific and reproducible results, has been employed (Ellis et al. 1997; Singh et al. 1999). In the present study, using AFLPs and random amplified polymorphic DNA (RAPDs), we have developed two sequence-characterized amplified region (SCAR) markers that show very tight linkage to a gall midge resistance gene locus, *Gm8*, in rice.

Materials and methods

Plant materials

The F_4 population used in the present study consisted of rice lines derived from a cross between the two *indica* rice varieties; 'Jhitpiti' (carrying *Gm8*; resistant to gall midge biotype 1) and 'TN1' (susceptible to gall midge). Of the 608 F_2 plants derived from the above cross, 265 random plants were sown as single-plant progeny in F_3. The reaction of each of the individual F_3 lines was recorded as homozygous resistant, segregating, or homozygous susceptible. From each F_3 progeny scored, one resistant or susceptible plant [i.e. (1) resistant plants from progenies showing homozygous resistance reaction; or (2) susceptible plants from progenies showing homozygous susceptible reaction; or (3) resistant/susceptible plants from progenies showing segregation for resistance/susceptibility)] was selected for advancing to F_4. Thus, we had 265 F_3 progenies from which we selected 265 F_4 individual plants. These F_4 plants were grown again as individuals (lines). Individual plants were scored in each line (resistant plants were tagged from progenies showing homozygous resistance reaction, susceptible plants were tagged from progenies showing homozygous susceptible reaction) and DNA was isolated from leaves of scored individual resistant/susceptible plants.

Plant reaction for resistance and susceptibility towards the gall midge was observed under field conditions based on the natural occurrence of the insect at the Indira Gandhi Agricultural University, Raipur, Chhattisgarh, India. The plants were screened for the presence or absence of galls. The plants without any gall formation were scored as resistant and those with even one gall were recorded as susceptible.

DNA extraction and preparation of resistant and susceptible bulks

Total genomic DNA was isolated from the leaves of 40 field-grown F_4 plants (10-week old) along with leaves from parent plants using the modified CTAB method of Murray and Thompson (1980). For bulked segregant analysis, an equal quantity of DNA from 12 resistant and 12 susceptible F_4 individuals was pooled to form the resistant and susceptible bulks, respectively (Michelmore et al. 1991; Mohan et al. 1994). The concentration of DNA of the two bulks and the two parental DNAs was adjusted to 10 ng/μl.

Random amplified polymorphic DNA analysis

The amplification conditions have been described previously (Williams et al. 1990), with certain modifications (Mohan et al. 1994). A total of 1,200 RAPD primers (Operon Technologies, Alameda, Calif., USA) belonging to the A to Z, AA to AZ and BA to BH series were used in this study. The RAPD products (7.5 μl out of a 25 μl reaction volume) were separated on 1.1% agarose gels in 1× TBE buffer and stained with ethidium bromide at a concentration of 0.5 μg/ml. The gels were visualized and photographed on a UV transilluminator using Polaroid film (Type 667).

Amplified fragment length polymorphism analysis

Amplified fragment length polymorphism reactions were performed as described by Vos et al. (1995) with some minor modifications (Sardesai et al. 2002). A total of 105 selective enzyme-primer combinations were tried in this study. After PCR, 20 μl of formamide dye (98% formamide, 10 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol) was added to the reaction. The samples were heat-denatured for 5 min, snap-cooled on ice and loaded onto a 6% sequencing gel containing 8 M urea. The gel was dried and exposed overnight to Bio Max MR film (Kodak) at −80°C.

Isolation, cloning and sequencing of the phenotype-specific AFLP and RAPD fragments

The phenotype-specific AFLP fragments were cut out from the gels by first aligning the respective autoradiograms on the dried gels. DNA from the gel fragments was isolated and reamplified as described by Behura et al. (2000). The PCR products were gel-purified using a

Qiagen gel extraction kit (Qiagen, Hilder, Germany) and cloned into the PCR-4-TOPO vector (Invitrogen, Calif., USA). Two clones each of the resistant phenotype-specific fragment and the susceptible phenotype-specific AFLP fragments were sequenced and named AR257 (254 bp) and AS168 (168 bp), respectively. The resistant phenotype-specific RAPD fragment was directly gel purified and cloned as mentioned above. The clone was named $AP19_{587}$ (587 bp) and was partially sequenced from the two termini. Sequencing of these clones was done by dideoxy chain termination method (Sanger et al. 1977) using a Sequenase Ver. 2.0 sequencing Kit (USB, Cleveland, Ohio, USA). The sequences of the AFLP fragments were used to develop SCAR primers.

Southern hybridization of the AFLP fragments

Genomic DNA (5 µg) of the rice varieties Jhitpiti and TN1 were digested with 10 U of each of *Bam*H I, *Bgl* II, *Cla* I, *Eco*R I, *Eco*R V, *Hin*d III, *Pst* I, *Sal* I and *Xba* I at 37°C overnight. The digested DNA was run on a 0.8% agarose gel and blotted onto a nylon membrane (GeneScreen Plus, NEN Life Sciences, Boston, USA) as described by Williams et al. (1991). The membrane was probed with the AR257 and AS168 probes separately. The probes were labeled with [^{32}P]-dCTP using a nick translation Kit (Bethesda Research Laboratories, Life Technologies, USA). After hybridization for 20 h at 65°C, the membrane was washed under stringent conditions (Mohan et al. 1994) and kept for autoradiography.

Mapping of the phenotype-specific AFLP and RAPD fragments

The sequences of both phenotype-specific AFLP fragments, AR257 and AS168, and the RAPD fragment, $AP19_{587}$, were subjected to homology searches using the rice database at National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov), The Institute for Genomic Research (TIGR; http://www.tigr.org/tdb/e2 k1/osa1) and the Rice Genome Research Program (RGP; http://rgp.dna.affrc.go.jp) to map them to the chromosomal location in the rice genome.

Design of the SCAR primers and PCR

For conversion of the AFLP and RAPD fragments into PCR-based SCAR markers, forward and reverse SCAR primers were designed based on the sequence of the AFLP fragments AR257 and AS168 and the RAPD fragment $AP19_{587}$ using Oligo 4.0 software (National Biosciences) and were synthesized by Microsynth (Balagad, Switzerland). In addition, another set of SCAR primers (forward and reverse) were designed from the region flanking each AFLP fragment. These regions were identified using the rice database at RGP. The details of the AFLP-based SCAR primers used in this study are given in Table 1.

Table 1 Sequences of the SCAR primers designed for the different phenotype specific markers used in this study. *F* Forward, *R* reverse

Marker	Primer sequence	
AR257	5′-ATCGAAGGAGGAGCCTTTGC-3′	*F*
	5′-AACGTATCATACCTTACCCATAAACCA-3′	*R*
AS168	5′-ATATTTACTTGAATTTACAGATG-3′	*F*
	5′-AATAGGGCTTAGCTTGATGATG-3′	*R*
Flanking AR257	5′-ACAAAATCAAATGTGAAACTAGG-3′	*F*
	5′-AGTCCGCTTCGTCCGTCGTT-3′	*R*
Flanking AS168	5′-TGATGTTTCCCTTGCTTTTCTT-3′	*F*
	5′-TACGGACGGAGATGAACTGT-3′	*R*

Polymerase chain reaction was carried out using genomic DNA of the resistant and susceptible parents as well as resistant and susceptible individuals of the F_4 progeny in a 50 µl reaction volume containing 10 mM of Tris-Cl (pH 8.0), 50 mM of KCl, 1.5 mM of $MgCl_2$, 0.01% gelatin, 200 µM of each dNTP, 450 nM of each primer, 200 ng of template DNA and 2.5 U of *Taq* DNA polymerase. The amplification conditions were 94°C for 1 min, 56°C for 1 min and 72°C for 1 min for 30 cycles, except that the annealing temperature was kept at 59°C to amplify $AP19_{587}$. The PCR products were electrophoresed on 1.2–1.3% agarose gels in 1× TBE.

Restriction of AFLP-derived SCAR-amplified products

Single and double-digestions of a SCAR product were carried out using the *Pst* I and/or *Mse* I enzyme(s). Ten microlitres of the amplified products were digested using 10 U of a restriction enzyme in a 20 µl restriction volume. In case of double digestions, 10 U each of both the enzymes were used in a restriction reaction.

Results

Random amplified polymorphic DNAs

We screened 1,200 RAPD primers to identify markers tightly linked to the gall midge resistance gene, *Gm8*. We observed 1,112 polymorphic bands between the parents. Of these, 115 were resistant/susceptible bulk-specific. Only one RAPD fragment, $AP19_{587}$, showed tight linkage with the resistance phenotype. It amplified a 587 bp fragment in the resistant parent and in the resistant bulk. When tested on F_4 individuals, 18 out of 19 resistant lines and two out of 20 susceptible lines amplified the fragment linked to the resistant phenotype (data not shown).

Amplified fragment length polymorphisms

The 105 enzyme-primer combinations used in this study revealed a total of 24 fragments that amplified in phenotype-specific manner. Of these, three fragments were found to be tightly linked to the resistant/susceptible phenotype. While one enzyme-primer combination (*Pst* I-AT+*Mse* I-CGT) generated a 254 bp resistant phenotype-specific fragment (AR257) (Fig. 1a), the other enzyme-primer combination (*Pst* I-AA+*Mse* I-CAC) yielded two fragments, 168 bp (AS168) and 135 bp, associated with the susceptible phenotype (Fig. 1b).

The resistant phenotype-specific fragment, AR257, amplified only from the resistant parent and the resistant bulk. Amplification of DNA from individual F_4 lines (a total of 40, including the 24 lines that constituted the bulks) using the same enzyme-primer combination revealed the presence of AR257 in all resistant individuals and its absence in all susceptible individuals (except 201S and 215S) (Fig. 1a). For the susceptible phenotype-specific enzyme-primer combination, the fragment AS168 amplified in all susceptible individuals (i.e. the TN1 parent and all susceptible individuals that constituted the susceptible bulk) whereas it was absent from all the resistant individuals (Fig. 1b). The AFLP screening results showed that two lines (i.e. 201S and 215S) did not show phenotype-specific amplification (Fig. 1a).

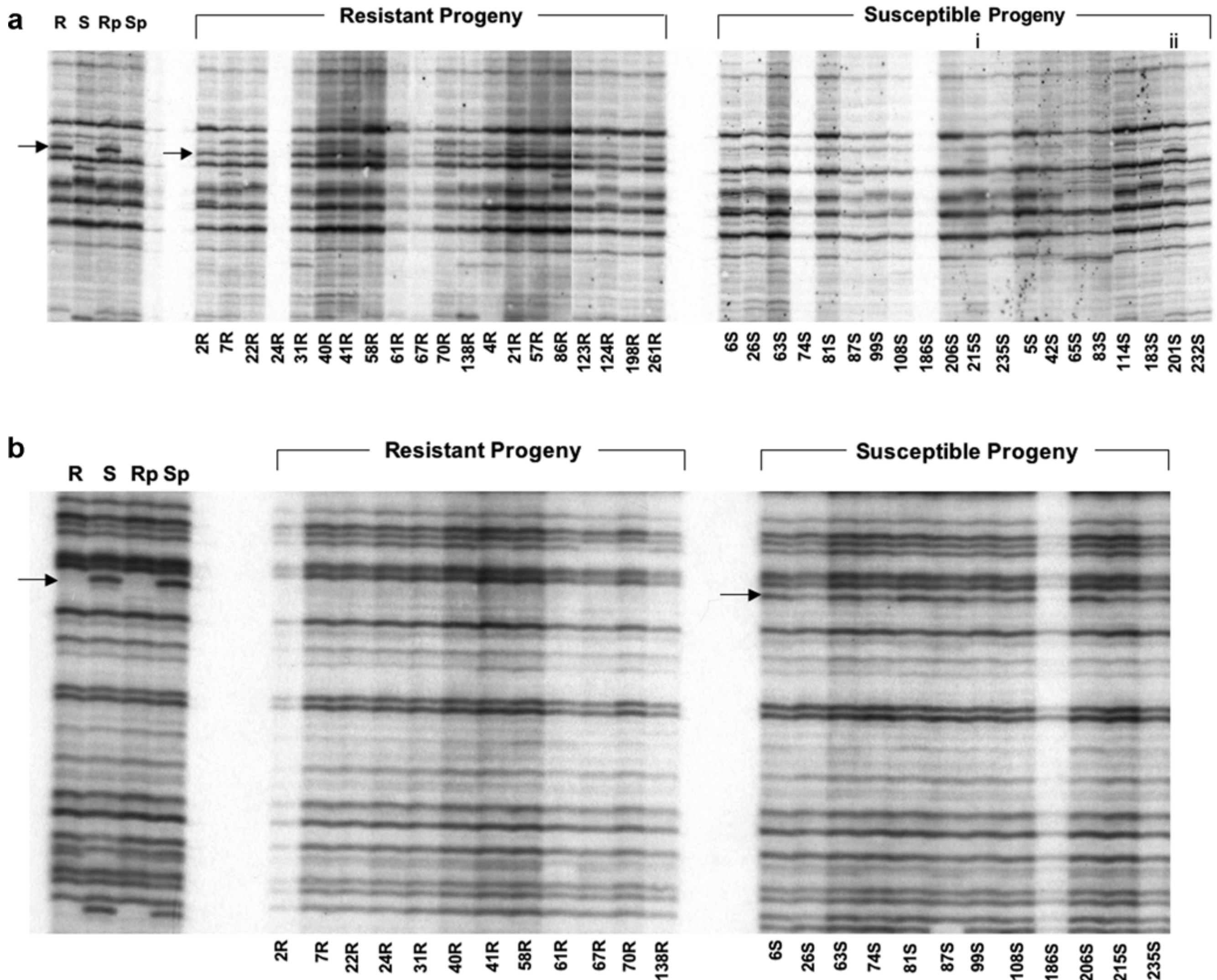

Fig. 1 a Amplified fragment length polymorphism fragment (AR257) segregating with the resistant phenotype (*arrows*), using primer combination *Pst* I-AT (5′-GACTGCGTACATGCAAT-3′) and *Mse* I-CGT (5′-GATGAGTCCTGAGTAACGT-3′). The first two lanes are the resistant (*R*) and susceptible (*S*) parents, Jhitpiti and TN1, respectively, followed by the resistant (*Rp*) and susceptible (*Sp*) bulks. *Lanes i* and *ii* represent lines 215S and 201S, respectively. *Labels* at the bottom of the figure indicate individual F_4 line numbers. **b** AFLP fragment (AS168) segregating with the susceptible phenotype (*arrows*), using primer combination *Pst* I-AA (5′-GACTGCGTACATGCAAA-3′) and *Mse* I-CAC (5′-GATGAGTCCTGAGTAACAC-3′). The first two lanes are the resistant parents, Jhitpiti and TN1, respectively, followed by the resistant (*Rp*) and susceptible (*Sp*) bulks. *Labels* at the bottom of the figure indicate individual F_4 line numbers

Cloning and Southern hybridization

The two AFLP fragments AR257 and AS168 were eluted and cloned into the PCR-4-TOPO vector, then sequenced. Southern analysis of genomic DNA isolated from the parents Jhitpiti and TN1, then digested with nine different restriction enzymes and hybridized with AR257 and AS168 as probes, revealed polymorphisms between the parents. The hybridization signals using the AR257 and AS168 probes revealed that these regions were present as single or low-copy sequences in both the parents (data not shown).

Chromosomal location and relative map position of the AFLP and RAPD fragments in the rice genetic map

BLAST searches of the NCBI, TIGR and RGP databases with the sequences of the two cloned AFLP fragments showed tight homology with individual PAC clones belonging to the short arm of rice chromosome 8. While AR257 showed 96% homology (a score of 432 and an E value of e^{-120}) to a PAC clone (AP004705) at the 48.8 cM position of chromosome 8 (short arm), AS168 showed 95% homology (a score of 224 and an E value of $2e^{-57}$) to a PAC clone (AP004690) at the 50.8 cM position of chromosome 8 (short arm) in the rice database. The RAPD fragment, $AP19_{587}$, showed 99% homology to adjacent overlapping rice clones AP005440 and AP004646 (a score of 456 and an E value of e^{-127}) in the rice databank. The map positions of these markers, along with other associated markers on chromosome 8 are shown in Fig. 2.

In order to ascertain if these fragments were part of a gene, we also carried out a BLAST search with the phenotype-specific sequences against the sequences in the EST data bank. Of the three fragments identified in this study, only the resistant phenotype-specific AFLP marker, AR257, showed significant homology to a sequence in the EST library. This fragment had strong homology (96%; E value e^{-120}) to an EST clone (no. CB674118) from *an Oryza sativa* var. *japonica* leaf cDNA library. This EST has been identified as being involved in the interaction between rice and its fungal pathogen, *Magnaporthe grisea*.

SCAR amplification and generation of phenotype-specific polymorphisms

No polymorphism could be observed between the parents when the SCAR primer pairs derived from the end-sequences of AR257 or AS168 were used. Even cutting the monomorphic amplified products with five different restriction enzymes located within their sequences (data not shown) did not yield any phenotype-specific restriction length polymorphisms. However, a distinct phenotype-specific SCAR amplification profile was obtained when primers based on the sequences flanking AR257

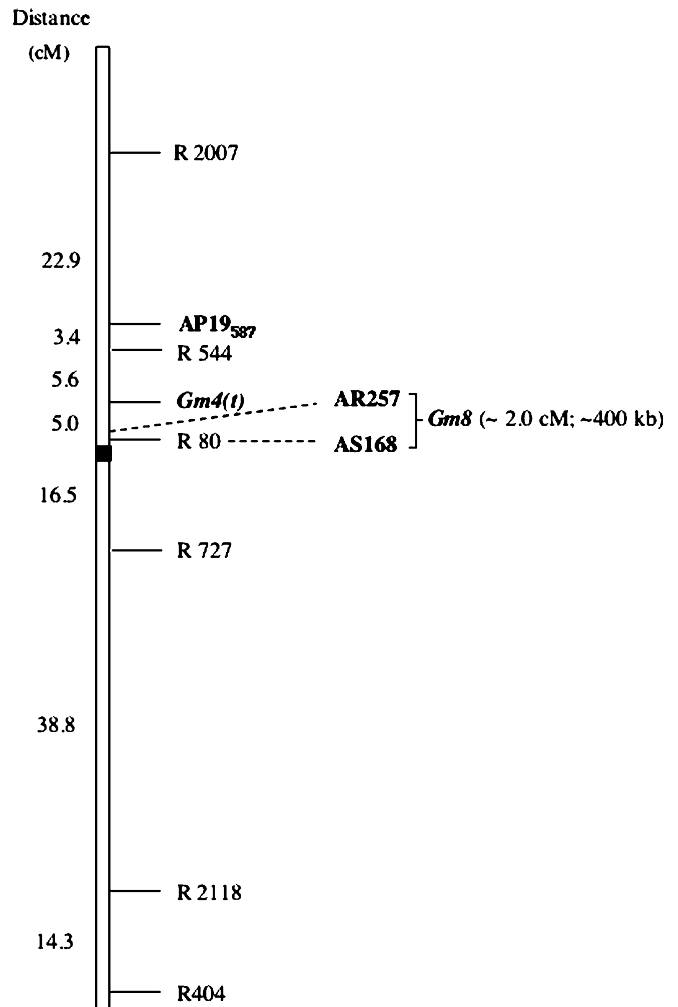

Fig. 2 Mapping of the gall midge resistance gene, *Gm8*, on the molecular linkage map of rice chromosome 8. *Numbers* on the left show genetic distances (cM). *AR257* and *AS168* are the AFLP markers, and $AP19_{587}$ is the RAPD marker identified in this study. Map position of a previously mapped (see Mohan et al. 1997b) gall midge resistance gene *Gm4*(*t*) is also shown. The genetic distances are based on the Rice Genome Program map (http://rgp.dna.affrc.go.jp/publicdata/geneticmap2000/chr08.html). Map not to scale

were used to amplify DNA from the parents and their F_4 progeny. All resistant individuals (Jhitpiti and the resistant F_4 lines) showed the amplification of two bands of 550 and 640 bp in their profiles, whereas all susceptible individuals (TN1 and the susceptible F_4 lines) except for 201S, showed the presence of only one band of 500 bp (Fig. 3a). Two lines, one resistant (198R) and one susceptible (215S), showed amplification of three bands in a co-dominant manner.

In contrast, when PCR was carried out using primers based on the sequences flanking AS168 (these sequences were obtained from the rice genome sequence data bank), it did not show a phenotype-specific polymorphism initially. However, when restricted with *Pst* I and *Mse* I, the monomorphic amplification product (276 bp) gave a phenotype-specific restriction length polymorphism with all resistant individuals showing the presence of a distinct

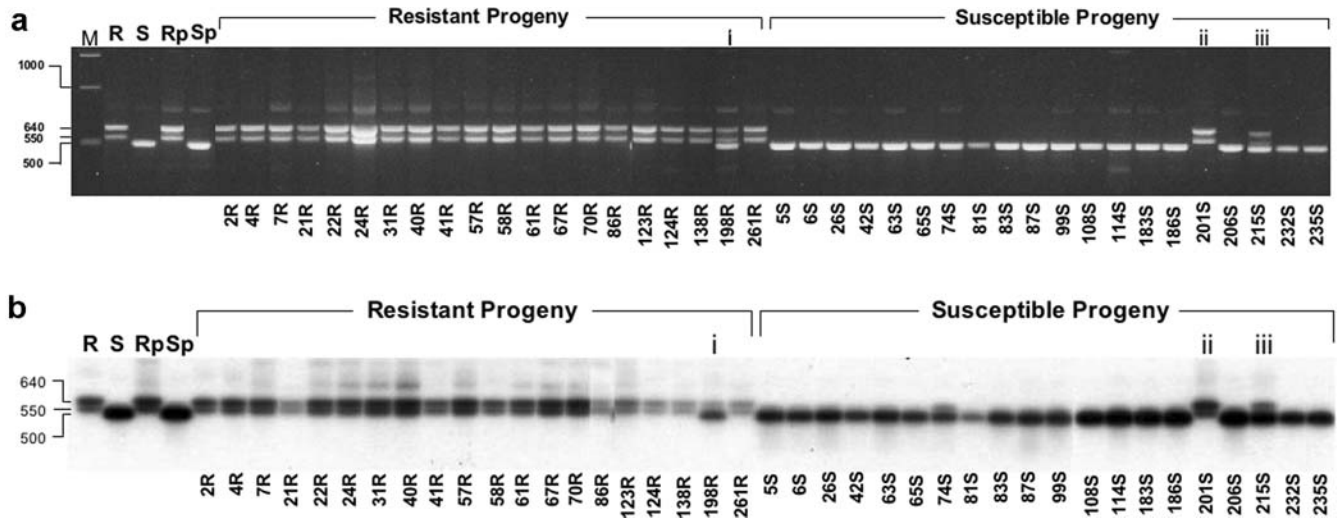

Fig. 3 **a** Polymerase chain reaction-based screening for gall midge resistant and susceptible progeny in the F_4 population, derived from a cross between Jhitpiti and TN1, using SCAR primers flanking the resistance phenotype-specific marker, AR257. *Lanes i*, *ii* and *iii* represent lines 198R, 201S and 215S, respectively. Lane *M* represents the 1 kb DNA marker ladder. *Figures* on the left represent the molecular weights in bp. *R*, Resistant parent; *S*, susceptible parent; *Rp* and *Sp*, resistant and susceptible bulks, respectively. *Labels* at the bottom of the figure indicate the individual F_4 line numbers. **b** Southern hybridization of the gel shown in (**a**) using the susceptible-specific fragment, amplified by the SCAR primers flanking AR257, as probe. *Lanes i*, *ii* and *iii* represent 198R, 201S and 215S, respectively. *Figures* on the left represent the molecular weight in bp. *R*, Resistant parent; *S*, susceptible parent; *Rp* and *Sp*, resistant and susceptible bulks, respectively. *Labels* at the bottom of the figure indicate the individual F_4 line numbers

225 bp fragment and all susceptible individuals showing the occurrence of a distinct 168 bp fragment (Fig. 4). Four F_4 lines, i.e. one resistant (198R) and three susceptible (99S, 186S, 215S), showed the amplification of both resistance- and susceptible-associated fragments (Fig. 4); and one susceptible line (201S) showed the presence of only the resistance-associated fragment. It is interesting to note that these five F_4 lines included the three lines (198R, 201S and 215S) that had earlier shown similar results (co-dominant amplification in case of 198R and 215S and resistance-associated amplification in case of 201S) with the resistance-associated SCAR primers also. However, the SCAR primers designed for the RAPD marker $AP19_{587}$ failed to amplify in a phenotype-specific manner.

Southern hybridization of resistance-derived SCAR (flanking AR257) amplified products

The resistance-derived SCAR primers (flanking AR257) amplified products in a co-dominant manner and it was necessary to ascertain whether the different fragments specifying the individual phenotypes amplified in an allele-specific manner. Since heterozygous lines were not available, sequence homology of the resistant phenotype-specific and susceptible phenotype-specific products was tested as an alternative, by using the gel-eluted susceptible phenotype-specific product (500 bp) as the probe for hybridization to a blot carrying the SCAR primer-amplified PCR products. Results revealed that it hybridized well with both the fragments linked to the resistant phenotype (640 and 550 bp), as well as to the fragment linked to the susceptible phenotype (Fig. 3b).

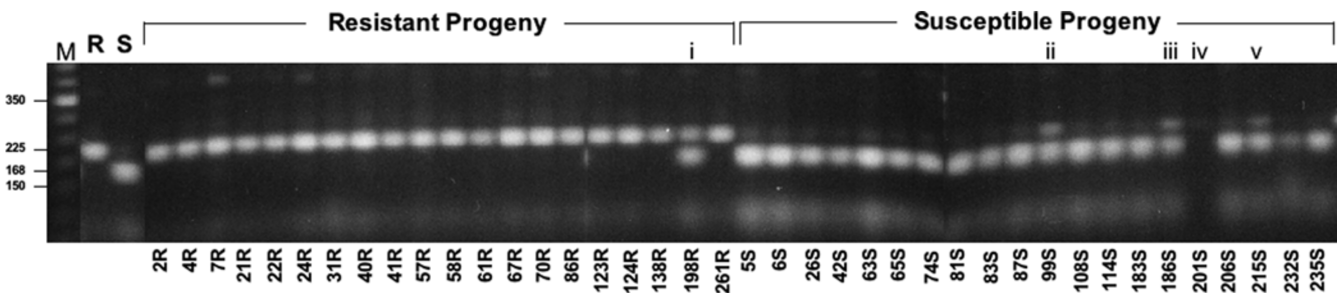

Fig. 4 Restriction profile of the monomorphic PCR products generated using the SCAR primers flanking the susceptible specific AFLP fragment AS168. These fragments, on double digestion with *Mse* I and *Pst* I, resulted in a polymorphism that distinguished the phenotypes. *Lanes i*, *ii*, *iii*, *iv* and *v* represents 198R, 99S, 186S, 201S and 215S, respectively. Lane *M* represents the 50 bp DNA marker ladder. *Figures* on the left represent the molecular weight in bp. *R*, Resistant parent; *S*, susceptible parent. *Labels* at the bottom of the figure indicate individual F_4 line numbers

Discussion

Recent genetic studies have revealed that the gall midge resistance gene *Gm8*, present in the rice variety Jhitpiti, is a dominant gene and is non-allelic to other known gall midge resistance genes [*Gm1*, *Gm2*, *Gm3*, *Gm4*(*t*), *Gm5*, *Gm6*(*t*) and *Gm7*] in rice (Kumar et al. 2000). The occurrence of the resistant phenotype-specific fragment AR257 in two susceptible lines, 201S and 215S, could be due to a recombination event(s) between the AFLP marker locus and the *Gm8* locus. Thus, a high level of phenotype specificity could be obtained using resistance- and susceptible-associated AFLP markers.

BLAST searches using the sequences of AR257 and the susceptible phenotype-specific fragment (AS168), showed near 100% homology to the PAC clones AP004705 and AP004690, respectively, in the RGP database. Both PAC clones are located at a 2.0 cM relative genetic distance on the short arm of rice chromosome 8 (48.8 and 50.8 cM from the short arm end covering a region of approximately 400 kb) (Fig. 2). As both AFLP markers show tight linkage to the resistant and susceptible phenotypes, and high homology to the above mentioned PAC clones, it is therefore inferred that the gall midge resistance gene, *Gm8*, is located on the short arm of rice chromosome 8. Similarly, the RAPD fragment $AP19_{587}$ showed near 100% homology to adjacent overlapping BAC clones AP005440 and AP004646 and both these clones map to the short arm of chromosome 8 (36.8 cM).

Initially, SCAR primers derived from the sequences of the AFLP fragments (either AR257 or AS168) failed to generate phenotype-specific amplification and instead produced a single monomorphic band in both parents. However, when SCAR primers flanking AR257 were used, a distinct phenotype-specific PCR amplification was revealed in parents and in their F_4 progeny in a co-dominant manner (Fig. 3a). The occurrence of a heterozygous profile in a susceptible line, 215S, concurs with the results of AFLP screening where this line showed the presence of the resistant phenotype-specific AR257 fragment. This suggests that the susceptible line 215S contains a recombination event that seems to have occurred between the marker and the *Gm8* loci, or is a heterozygous individual mislabeled as susceptible in the field. Also, the presence of all of the three bands (both resistant phenotype-specific and one susceptible phenotype-specific) in the resistant line 198R indicates that the individual could actually be heterozygous. The conversion of the dominant AFLP marker (AR257 specific) to a co-dominant SCAR marker is thus advantageous since the latter can identify a heterozygous individual in the population and is therefore more informative than a dominant marker. It is interesting to observe that in the resistant phenotypes, the SCAR primers flanking AR257 amplified two fragments instead of the one for which they were actually designed. Nonetheless, both fragments amplified in all the resistant individuals. The amplification of the two bands in the resistant phenotypes could be due to a duplication event in a micro-chromosomal segment associated with the *Gm8* locus.

Unlike AR257, PCR using SCAR markers based on the region flanking AS168 produced a monomorphic band of 276 bp across all individuals, which upon double digestion with *Pst* I and *Mse* I distinguished between the susceptible and resistant phenotypes (Fig. 4). Thus, with the use of restriction enzymes, a monomorphic PCR product could be converted to a useful co-dominant marker. Again, the two lines 198R and 215S showed the presence of both resistant phenotype-specific as well as susceptible phenotype-specific bands upon restriction, thereby indicating the heterozygous nature of these lines. In addition, two more susceptible lines, 99S and 186S, revealed the presence of both 225 and 168 bp fragments—a result which does not correspond to the fact that they are susceptible individuals. This could happen if these two individuals are heterozygous for this SCAR marker but not for the *Gm8* locus. Furthermore, restricting the SCAR-amplified monomorphic fragment singly with either *Pst* I or *Mse* I revealed that the polymorphism was actually due to a modification of the *Pst* I site in the resistant individuals (data not shown).This also highlights a strategy for developing SCARs; when SCARs developed from phenotype-specific fragments fail to generate a phenotype-specific amplification, then one could choose to design primers from regions flanking this fragment. This would allow greater success rates for primers for use in marker-aided selection based on phenotype-specific fragments.

In the present study, the SCAR markers developed from the AFLP markers were found to be more robust in terms of both their specificity as well as their greater reliability and are ideally suited as a tool for marker-aided selection in breeding programmes involving the gall midge resistance gene *Gm8*. The conversion of both the tightly linked, phenotype-specific dominant AFLP markers into co-dominant, allele-specific SCAR markers is thus advantageous from the point of view of marker-assisted selection as they can detect the presence of both the alleles in a single PCR reaction using only one set of SCAR primer pairs. This translates to considerable savings for a breeder in terms of time, manpower and test plot area. Markers specific for *Gm8* along with markers specific for *Gm2* (Nair et al. 1995), *Gm4*(*t*) (Nair et al. 1996) and *Gm7* (Sardesai et al. 2002) will be used in pyramiding these genes in different combinations in elite cultivars of rice for developing durable resistance against different biotypes of gall midge prevalent in India. Previous studies have mapped putative resistance genes in the region between *Gm4*(*t*) and *Gm8* (Mohan et al. 1997b; Berruyer et al. 2003) and therefore the possibility exists that these could be potential candidates for the gall midge resistance gene *Gm8*. Interestingly, the resistant phenotype-specific fragment AR257 has strong homology to an EST clone from an *Oryza sativa* var. *japonica* leaf cDNA library. This EST is known to be involved in the interaction between rice and its fungal pathogen, *Magnaporthe grisea*. It is therefore possible that the map position of *Gm8*, as identified on the

short arm of chromosome 8, would help in the isolation of this gene through a map-based cloning strategy.

In this study, SCAR markers were developed with two major aims: (1) for marker-aided selection of *Gm8*, and (2) to localize these markers on the rice genetic map to identify the chromosomal location of *Gm8*. We have also screened the genomic region between markers *Gm4*(*t*) and R 727 (Fig. 2). This covers a region of ~26 cM. We have developed a large number of primer pairs (more than 150, every 50 kb on average) for this region. The primers are based on the sequence of this region available in the TIGR and RGP databases. Primers were selected so as to PCR amplify 1–2 kb regions. After screening 79 pairs of primers we were able to identify only three pairs that amplified fragments in a phenotype-specific manner. On screening 40 F_4 individuals with these primers it was found that none of these SCAR markers were more closely associated than the ones already mentioned earlier in this study (data not included). Identifying markers closer to *Gm8* than the present ones will also help in the map-based gene cloning of *Gm8*.

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