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Identification and mapping of an AFLP marker linked to *Gm7*, a gall midge resistance gene and its conversion to a SCAR marker for its utility in marker aided selection in rice

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Abstract We have identified an AFLP marker SA598 that is linked to *Gm7*, a gene conferring resistance to biotypes 1, 2 and 4 of the gall midge (*Orseolia oryzae*), a major dipteran pest of rice. A set of PCR primers specific to an RFLP marker, previously identified to be linked to another gall midge resistance gene *Gm2*, also amplified a 1.5-kb (F8LB) fragment that is linked to *Gm7*. *Gm7* is a dominant gene and non-allelic to *Gm2*. Hybridization experiments with clones from a YAC library of Nipponbare, a japonica variety, a BAC library of IR-BB21, an indica variety, and cosmid clones encompassing *Gm2* from Phalguna, an indica variety, with F8LB and SA598 as probes, revealed that *Gm7* is tightly linked to *Gm2* and is located on chromosome 4 of rice. SA598 was sequenced and the sequence information was used to design sequence-characterized amplified region (SCAR) primers. The potential use of these SCAR primers in marker-aided selection of *Gm7* in a rice breeding program has been demonstrated.

Keywords Random amplified polymorphic DNA (RAPD) · Insect resistance gene · Gene cluster · *Oryza sativa* · *Orseolia oryzae*

Introduction

Rice is the most important crop in the world with over 1.5 billion hectares under paddy cultivation and a worldwide production of over 596 million tons (FAO 1999). The rice crop in the field is subjected to attack by a number of insect pests, pathogens, weeds and other harmful organisms. Several studies have reported that major yield losses of rice are often caused by insects alone. Of these insects, the dipteran pest, gall midge (*Orseolia oryzae* Wood-Mason), alone is responsible for a loss of more than US$ 550 millions annually (Herdt 1991).

Developing new rice varieties resistant to gall midge using conventional breeding methods is the most effective way of controlling this pest. At least seven non-allelic gall midge resistance genes have been identified (Chaudhary et al. 1986; Shrivastava et al. 1993; Kumar et al. 1998, 1999) that confer resistance to five biotypes of rice gall midge known to occur in India (Bentur and Amudhan 1996; Behura et al. 1999; see review by Sardesai et al. 2001). As the different biotypes of the pest are distributed in different regions of the country with two or more biotypes not occurring at the same geographical location, the selection of rice plants resistant to more than one biotype becomes time consuming in a single season. However, the development of molecular markers that are tightly linked to the gene of interest would enable one to follow the gene in a cross intended to develop new resistant varieties any time of the year without depending on the annual occurrence of insects (Mohan et al. 1997a). Thus, screening and pyramiding of gall midge resistance is possible in a fewer number of breeding cycles as compared to conventional breeding methods.

Previously, other gall midge resistance genes have been tagged and mapped (Mohan et al. 1994; Nair et al. 1995, 1996; Mohan et al. 1997b). The potential use of DNA markers linked to the gall midge resistance genes in marker-aided selection (MAS) has been demonstrated (Nair et al. 1995, 1996). We report here the identification of an AFLP marker linked to the gall midge resistance gene, *Gm7* (a dominant gene that confers resistance to biotypes 1, 2 and 4) in rice, that has potential application in marker-aided selection. *Gm7* has been mapped using a strategy involving hybridization to different rice genomic libraries in the Bacterial Artificial Chromosome (BAC), the Yeast Artificial Chromosome (YAC) and

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subsequently to cosmids that encompass the *Gm2* gene, in the indica rice variety Phalguna. Mapping of *Gm7* has revealed a linkage between *Gm7* and *Gm2*.

Materials and methods

Plant materials

The F_5 population used in this study was derived from a cross between the two indica rice varieties, RP2333 (containing *Gm7*; resistant to gall midge biotypes 1, 2 and 4; Kumar et al. 1999) and Shyamala (susceptible to all gall midge biotypes). The scoring for resistance and susceptibility was done under field conditions at the Indira Gandhi Agricultural University, Raipur, India. The plants were screened for the presence and absence of galls. Plants without any galls were scored as resistant and those having even one gall per plant were scored as susceptible. The scored plants were subsequently harvested for DNA extraction. RP2333, carrying the *Gm7* gene, was also crossed with varieties carrying other gall midge resistance genes to study the allelic relationship between *Gm7* and other previously identified gall midge resistance genes (Kumar et al. 1999).

DNA extraction and preparation of susceptible and resistant bulks

DNA was isolated from field-grown plants (10-week-old) using the modified CTAB method of Murray and Thompson (1980). An equal quantity of DNA from 12 resistant and 12 susceptible F_5 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 amplification of polymorphic DNA (RAPD) analysis

The amplification conditions were as described previously (Williams et al. 1990) with certain modifications (Mohan et al. 1994). The RAPD primers were from the Operon 10-mer Kits (Operon Technologies, Alameda, Calif.). Five hundred and twenty random primers of Kits A to Z were utilized in this study. The RAPD products were separated on a 1.1% agarose gel in 1 × TBE buffer and stained with ethidium bromide at a concentration of 0.5 µg/ml to observe and photograph the products.

Amplified fragment length polymorphism (AFLP)

AFLP reactions were performed as described by Vos et al. (1995). Briefly, genomic DNAs (500 ng) from RP2333, Shyamala, and the resistant and susceptible bulks and progenies, were digested in a reaction volume of 25 µl. The digested and adapter ligated DNA was amplified with *Eco*RI or *Pst*I and *Mse*I non-selective primer pairs in a 50 µl reaction. The amplification profile was 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min for 30 cycles followed by an extension at 72 °C for 5 min. The amplified products were diluted 10-fold in TE (10 mM Tris, pH 8.0; 1 mM EDTA) and used for selective amplification. *Eco*RI or *Pst*I primers used in the selective amplification were radiolabeled separately by kinasing 100 ng of each primer with [γ-^{32}P]-ATP. Selective amplification was carried out with a 5 µl diluted preamplification product, 1 µl of labeled *Eco*RI/*Pst*I primer and 50 ng of *Mse*I primer. The reaction volume was 20 µl. A total of 157 primer (*Mse*I and *Eco*RI/*Pst*I) combinations were used in this study. After PCR, 20 µl of formamide dye (containing 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 X-OMAT-AR film (Kodak) at room temperature.

Isolation, cloning and sequencing of the AFLP fragment

The AFLP fragment considered to be putatively linked to *Gm7* was first marked out on the dried gel by aligning it with the autoradiogram and then cut out from the gel. The DNA from this gel fragment was isolated as described earlier (Behura et al. 2000). The isolated DNA was pelleted, washed with 85% ethanol, dried and dissolved in 10 µl of sterile dH_2O. The DNA (5 µl) was then PCR amplified using the same primer pairs that generated the AFLP fragment. The PCR products were run on a 0.8% agarose gel, gel-purified using a Qiagen gel extraction kit (Qiagen, Hilder, Germany) and cloned into pGEM(T) (Promega, Madison, Wis.). The clone containing the AFLP fragment was named SA598 (susceptibility specific AFLP fragment of 598 bp). Sequencing of this clone was done by the di-deoxy chain-termination method (Sanger et al. 1977) using a Sequenase Version 2.0 kit (USB, Cleveland, Ohio). The DNA sequence of the fragment was used to develop sequence-characterized amplified region (SCAR) primers.

Southern hybridisation of the AFLP fragment with parental DNA

Genomic DNA (5 µg) of RP2333 and Shyamala was digested with 40 U each of *Hin*dIII and *Dra*I at 37 °C overnight. The digested DNA was run on a 0.8% agarose gel and blotted onto a Hybond nylon membrane as described by Williams et al. (1991). The membrane was probed with SA598 labeled with [α^{32}P]dCTP using the Random Primers DNA Labeling System (Bethesda Research Laboratories, Life Technologies, USA). After hybridisation for 20 h at 65 °C, the membrane was washed under stringent conditions (twice in 2 × SSC at room temperature for 15 min each; once in 2 × SSC and 0.1% SDS at 65 °C for 20 min; once in 0.25 × SSC and 0.1% SDS at 65 °C for 15 min; and once briefly in 2 × SSC at room temperature and autoradiographed.

Screening of the Bacterial Artificial Chromosome (BAC), the Yeast Artificial Chromosome (YAC) and Cosmid libraries with SA598

A BAC library constructed from the high-molecular-weight nuclear DNA of the rice variety IR-BB21 (Wang et al. 1995) was probed with [α^{32}P]dCTP-labeled SA598 as above. Blots of a contiguous stretch of *Dra*I-digested YAC DNAs from japonica rice variety, Nipponbare (Rajyashri et al. 1998), and of cosmid DNAs digested with *Dra*I, from indica variety, Phalguna, encompassing the *Gm2* gene were also probed with radiolabeled SA598. The filters were hybridized at 65 °C for 20 h and washed under stringent conditions as above.

Design of SCAR primers and PCR

Forward and reverse primers internal to the 5′ and 3′ ends of the cloned AFLP fragment (SA598) were designed from its sequence using the Oligo 4.0 software (National Biosciences) and were synthesized by Integrated DNA Technology Inc. (USA). The following primers SCAR Forward 5′-GATCATTGGAGCAACATTCTG-3′ (position 44–64) and SCAR Reverse 5′-CATTTCTAATTCTTTC-TTCAA-3′ (position 559–579) were used to amplify genomic DNA from the resistant and susceptible parents as well as resistant and susceptible individuals of the F_5 progeny. PCR was carried 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, 380 nM of each primer, 125 ng of template DNA and 2.5 U of *Taq* DNA polymerase. PCR conditions were 94 °C for 1 min, 45 °C for 1 min and 72 °C for 1 min; for 30 cycles.

PCR amplification of parental and bulked DNA with primers specific to the *Xa1* gene and RFLP markers (RG329, RG476, RG214, F8, F10) linked to the *Gm2* gene

The genomic DNA of RP2333, Shyamala, and the resistant and susceptible bulks was amplified with primers specific to RG329, RG476, RG214 (Williams et al. 1991), F8, F10 (Nair et al. 1995) and primers from the 3′ end of *Xa1*, a bacterial blight resistance gene from rice (Yoshimura et al. 1996, 1998). Two hundred nano grams of template DNA were taken for the PCR reaction. PCR conditions were 94 °C for 30 s, 51 °C for 45 s and 72 °C for 1 min; for 30 cycles.

Since only the F8-specific primers (Nair et al. 1995) showed polymorphism between RP2333 and Shyamala, genomic DNA of the resistant and susceptible individuals of the F_5 population (raised from a cross between RP2333 and Shyamala) was amplified using the same primers. The composition of the reaction mixture and PCR conditions were as described above. The PCR products were electrophoresed on a 1% agarose gel in 1 × TBE. The polymorphic band amplified from the resistant parent using the F8 primers was excised from the agarose gel as mentioned above, and isolated using a Qiagen gel extraction kit (Qiagen, Hilder, Germany), and called F8LB (F8 resistance linked band).

Southern hybridization of a YAC forming contig encompassing an allele of *Gm2* gene with F8LB

A blot containing YAC DNAs, from clones that encompass an allele of the *Gm2* gene, digested with *Dra*I (Rajyashri et al. 1998), was hybridized to the gel-eluted F8 polymorphic band (F8LB) after labeling with [α^{32}P]dCTP using the Random Primers DNA Labeling System (Bethesda Research Laboratories, Life Technologies, USA). Hybridisation and washing conditions were the same as above.

Cross hybridisation of SA598 with F8LB

PCR was carried out using 100 ng of genomic DNA of RP2333, Shyamala, and seven each of the resistant and susceptible individuals of the F_5 population with the SCAR primers and the F8 primers in two separate reactions. PCR conditions were as mentioned for the respective sets of primers. The PCR products were run on a 1% agarose gel in 1 × TBE and blotted as described above. Southern hybridization of the blot of F8-primer-amplified PCR products was carried out with SA598 labeled with [α^{32}P]dCTP using the Random Primers DNA Labeling System (Bethesda Research Laboratories, Life Technologies, USA), whereas, the blot of the SCAR-primer-amplified PCR product was hybridized with radiolabeled F8LB. Hybridization was carried out at 65 °C for 20 h and the filters washed under stringent conditions (twice in 2 × SSC at room temperature for 20 min each; once in 2 × SSC and 0.1% SDS at 65 °C for 20 min; once in 0.1 × SSC and 0.1% SDS at 65 °C for 20 min; and once in 2 × SSC at room temperature briefly) and autoradiographed.

Results

Genetic analyses of gall midge resistance in RP2333

F_2 and F_3 segregation data for resistance to biotype 1 of the gall midge in crosses involving the resistant variety RP2333 and susceptible parents, Shyamala and R2270, revealed that the resistance in RP2333 is determined by a single dominant gene (Kumar et al. 1999). Allelic crosses between RP2333 and varieties Samridhi, Phalguna, Abhaya and ARC5984 having the gall midge resistance genes *Gm1*, *Gm2*, *Gm4* and *Gm5*, respectively, showed a segregation ratio of 15:1 for resistance:susceptibility in the F_2 and of 7:8:1 for resistance:segregating:susceptible progenies in the F_3, indicating the independent segregation of two dominant resistance genes. Crossing between RP2333 with RP2068-18-3-5, the variety harbouring the recessive *gm3* gene, showed a segregation ratio of 13:3 for resistance:susceptibility in the F_2 and 7:8:1 in the F_3 progenies (Kumar et al. 1999), indicating the independent segregation of *Gm7*, the dominant resistance gene, and *gm3*, the recessive resistance gene. This shows that the *Gm7* gene is non-allelic to the other gall midge resistance genes reported from India.

RAPDs

The two parental DNAs along with the resistant and susceptible bulks were screened using 520 RAPD primers in order to identify markers linked to *Gm7*. Of these, 488 primers produced amplification products while the remainder failed to amplify. Twenty four primers produced resistance-specific products and eight produced susceptible-specific products in the parents and the bulked DNAs. These primers were further used to screen each of the 12 different individual DNAs that constituted each of the two bulked DNAs. However, none of these primers amplified in a phenotype-specific manner in the individual lines that constituted the bulk, thereby indicating that these markers are not closely linked to *Gm7*.

AFLP

In order to identify additional phenotype-specific polymorphisms we employed AFLP. Of the 157 primer combinations used we identified one resistance-specific amplification and four showed susceptibility linked amplification using the *Eco*RI/*Mse*I combination. Further screening of the resistant and the susceptible individuals of the F_5 progeny (constituting the respective bulked DNAs) using these primer combinations revealed the absence of phenotype-specific amplifications. Using the *Pst*I/*Mse*I primer combinations, 20 combinations identified resistance-specific fragments and five combinations showed susceptible-specific amplification in the parents and the bulked DNAs. Analysis of the amplification pattern of the individuals forming the bulks using these primer combinations also showed the absence of phenotype-specific amplification except for the primers combination P-CG × M-CTG which amplified a susceptible-specific fragment in 22 of the 24 susceptible individuals (Fig. 1).

Cloning and Southern Hybridization

The susceptible-specific AFLP fragment (SA598) was eluted from the gel, cloned into pGEMT vector and se-

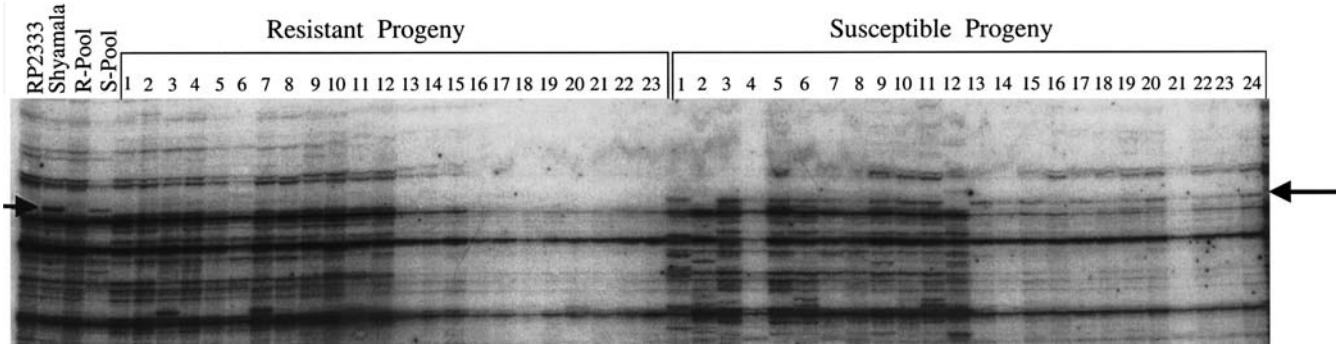


Fig. 1 AFLP fragment (subsequently called SA598) segregating with the susceptible phenotype (*arrows*), using primer combination P-CG (5′ GACTGCGTACATGCACG 3′) and M-CTG (5′ GATGAGTCCTGAGTAACTG 3′). The first two lanes are the parents, RP2333 and Shyamala, respectively, followed by the resistant (R-pool) and susceptible (S-pool) bulks. The remaining lanes are the resistant and susceptible progeny of the F_5 population

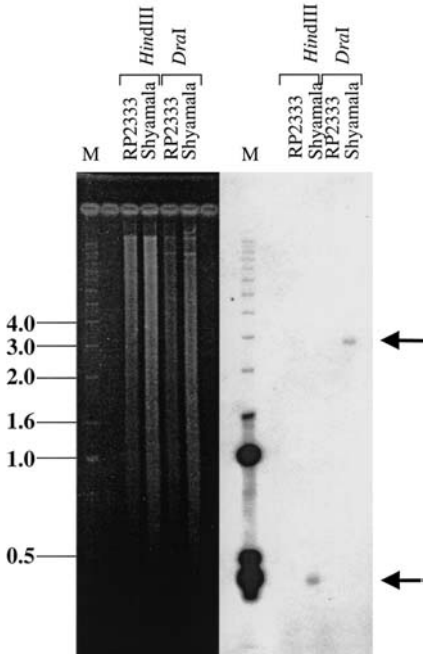


Fig. 2 Gel and Southern hybridization of *Hin*dIII- and *Dra*I-digested DNA of RP2333 and Shyamala probed with SA598. *M* 1-kb DNA marker ladder. *Arrows* indicate regions of hybridization. Note that the probe shows a hybridization signal only in the Shyamala lanes. Figures on the left represent the molecular weight in kb

quenced (GenBank Accession # AF455275). Southern hybridization of *Hin*dIII- and *Dra*I-digested genomic DNAs of the parents, RP2333 and Shyamala, with SA598 revealed that it is present as a single copy in the susceptible parent. However, the probe did not show any hybridization signal with the genomic DNA of the resistant parent (Fig. 2).

PCR using SCAR primers

SCAR primers were designed from the sequence information of SA598 and used in a PCR assay with the DNA of the parents and resistant and susceptible individuals of the F_5 progeny of the population. The primers amplified a 0.55-kb fragment in the susceptible individuals. However, it also amplified this fragment in some of the resistant individuals screened (Fig. 3).

Southern hybridization with the BAC library, *Dra*I-digested YAC and Cosmid DNA with SA598

In order to ascertain the chromosomal location of *Gm7*, we attempted to map SA598. The IR-BB21 BAC library was screened with this clone. Screening identified BACs that were a subset of the clones that hybridized to YAC probes, Y5212L and F8 [data not included; these mark-

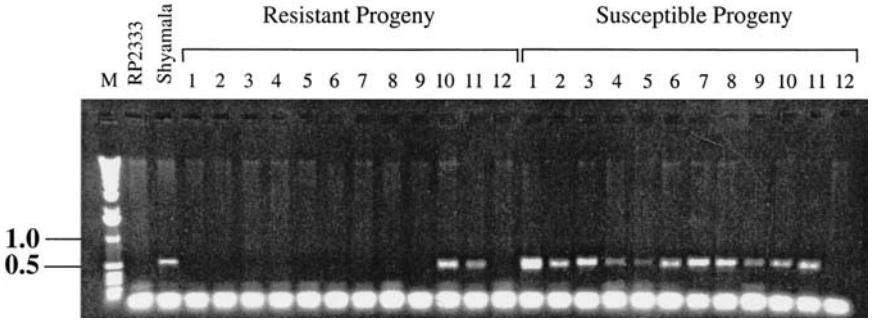


Fig. 3 PCR-based screening for gall midge-resistant and susceptible progeny in the F_5 population derived from a cross between RP2333 and Shyamala using the susceptible phenotype-specific SCAR primers. *Numbers on the top* represent different individuals of the F_5 population. *Lane M* represents a 1-kb DNA marker ladder. *Figures on the left* represent the molecular weight in kb

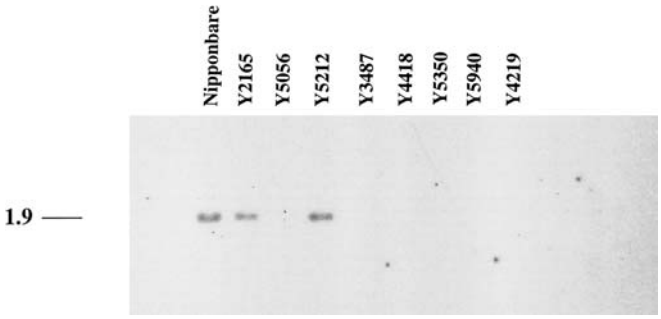


Fig. 4 Southern hybridization of *Dra*I-digested Nipponbare and YAC DNA, forming a contig encompassing an allele of the *Gm2* gene, with SA598. The figure on the left represents the molecular weight in kb

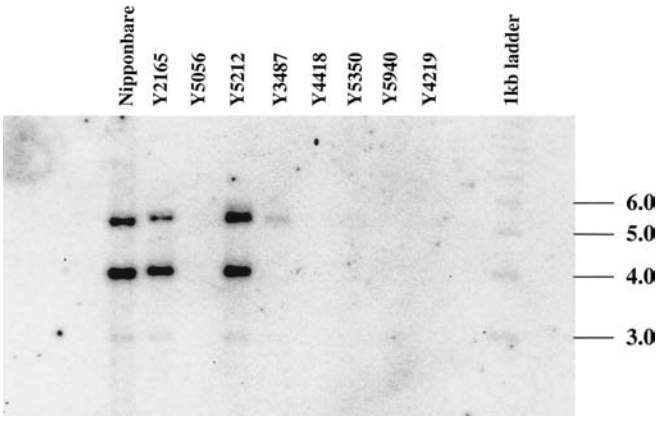


Fig. 6 Southern hybridization of *Dra*I-digested Nipponbare and YAC DNAs, forming a contig encompassing an allele of the *Gm2* gene, with F8LB. *Figures* on the right represent the molecular weight in kb

ers were earlier shown to flank *Gm2* (see Rajyashri et al. 1998)]. Southern hybridization of the Nipponbare YAC DNAs with SA598 showed the presence of a single copy of this marker in the japonica variety Nipponbare and the YAC clones, Y2165 and Y5212 (Fig. 4). The insert hybridized to two overlapping cosmids that was previously shown to encompass the *Gm2* gene from the indica variety Phalguna (data not shown).

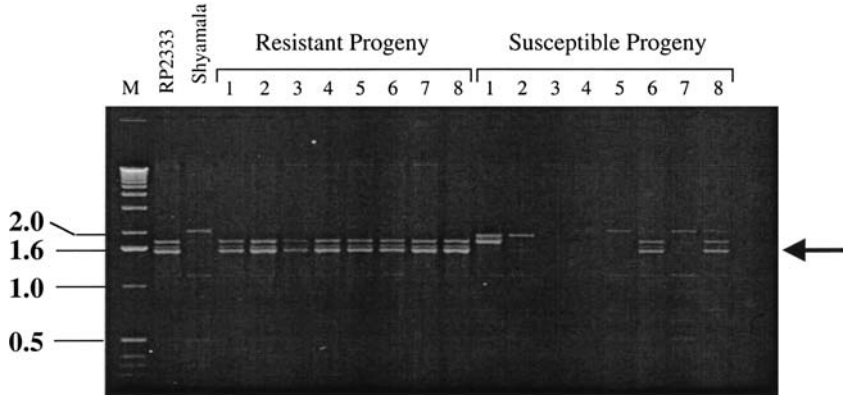
PCR amplification of DNAs from parents and bulks with primers specific to the 3′ end of *Xa1* and RFLP markers linked to *Gm2*

As *Gm7* was shown to be linked to F8, a marker previously identified to be linked to *Gm2* (Mohan et al. 1994; Rajyashri et al. 1998), we wanted to determine if any of the other markers linked to *Gm2* are linked to *Gm7* as well. With the parental and the bulk DNA as templates, primers specific to RG329, RG476, RG214 and *Xa1* amplified fragments of the expected size, but the products did not reveal any amplification-length polymorphism between the resistant and the susceptible phenotype. The primer set F10 failed to amplify at all. However, the primer set F8 amplified a 1.5-kb fragment that was specific to the resistant phenotype (Fig. 5). PCR amplification of resistant and susceptible individuals of the F_5 progeny with the F8 set of primers revealed that the 1.5-kb fragment amplified in all the 23 resistant individuals tested except two, and also amplified in four of the susceptible lines (Fig. 5).

Southern hybridizations with the resistance-linked fragment (F8LB)

Probing the *Dra*I-digested YACs, encompassing an allele of *Gm2*, with the resistance-linked F8 fragment (F8LB), revealed the presence of three bands of 5.5, 4.2 and 3.2 kb in Nipponbare, Y2165 and Y5212, and one band of 5.5 kb in Y3487 (Fig. 6). Southern hybridizations between the PCR products generated using the F8 primer with SA598 as a probe, and PCR products generated using the SCAR primers (developed in this study) with

Fig. 5 PCR-based screening for gall midge-resistant and susceptible progeny in the F_5 population derived from a cross between RP2333 and Shyamala using F8-specific primers. *Numbers on the top* represent different individuals of the F_5 population. Only a few of the individuals of the F_5 population tested have been shown here. *Lane M* represents a 1-kb DNA marker ladder. The *arrow* indicates the polymorphic resistance-phenotype-specific (F8LB) fragment amplified by F8. *Figures* on the left represent the molecular weight in kb



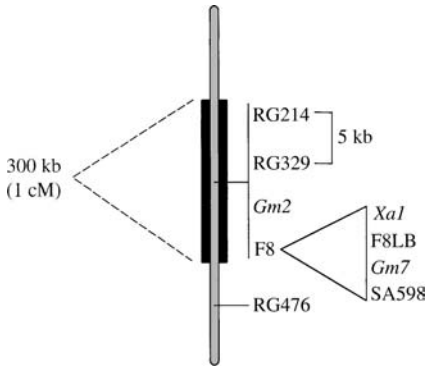


Fig. 7 Graphical representation of a portion of the map of chromosome 4 showing the position of the *Gm7*-linked markers. The *darkly shaded bar* represents the position of the YAC, Y2165. *Numbers* on the left show genetic (cM) and physical distances in this region of chromosome 4. *Gm7*-linked markers are on the right of F8 (they may not be present in the same order as represented here) with the genetic (cM) and physical (kb) distances given on the extreme left, within which they are present. The physical and genetic distances are as given earlier (Rajyashri et al. 1998)

F8LB as a probe, failed to reveal any homology between the two markers (data not shown).

Mapping of the *Gm7* gene

Since SA598, hybridizes to the BACs, YACs and cosmids encompassing the *Gm2* gene, it is concluded that this marker is linked to *Gm2*. Also, as SA598 hybridizes to two of the cosmids to which F8 hybridizes (data not included), it is therefore logical to conclude that SA598 is linked to *Gm7* and is on chromosome 4, and maps along with the F8 and *Xa1* (Yoshimura et al. 1996) and F8LB markers (Fig. 7).

Discussion

Genetic analyses of the gall midge resistance gene, *Gm7*, revealed that it is a dominant gene that is non-allelic to the other known gall midge resistance genes (Kumar et al. 1999). The F_5 population was raised by crossing parents that were different viz-a-viz the reaction to different gall midge biotypes. Screening of the parents with over 520 RAPD primers failed to reveal any polymorphism that co-segregated with either the resistance or the susceptibility trait in the individuals of the mapping population. The lack of consistence between the bulks and the individual line amplifications could be the result of competition in the PCR reaction, as has been demonstrated before (Hallden et al. 1996). The lack of detectable polymorphisms, that were linked to the gall midge resistance between the two parents, could also be due to the fact that both parents, RP2333 and Shyamala, are indica varieties. We, therefore, had to resort to AFLP, which is known to reveal more polymorphisms in closely related varieties.

AFLP has been used as a DNA fingerprinting technique (Vos et al. 1995) with wide usage in plant genetic studies, such as the assessment of genetic diversity in rice (Zhu et al. 1998; Aggarwal et al. 1999), for construction of high-density genetic maps of rice (Mackill et al. 1996; Maheswaran et al. 1997) and for enrichment of DNA markers near a locus of interest in rice (Chen et al. 1999; Dong et al. 2000; Xu et al. 2000). Though, in the present study, screening with over 150 AFLP primer pairs did reveal polymorphisms between the parents, there were very few that were linked to either the resistant or susceptible phenotype in the segregating population. However, AFLP was successful in revealing a 598 base-pair fragment (SA598) that was segregating with the susceptible phenotype in the present study (Fig. 1).

Using the sequence information of the AFLP marker, SA598, primers were synthesized for the sequence-characterized amplified region (SCAR) approach. Earlier, RAPD markers have been successfully converted to SCAR markers to make them more robust and reliable (Paran and Michelmore 1993; Nair et al. 1995, 1996). It may be noted that absence of the susceptible phenotype-specific band in one of the susceptible individuals of the progeny, and the presence of the band in some of the resistant individuals of the progeny, may be either due to a recombination event(s) in these phenotypes or because of some escapes as a result of insufficient insect pressure in the case of the individuals scored as resistant.

Earlier, we had faced difficulties in the mapping of the *Gm4t* gene in the population derived from an indica × indica cross (Abhaya × Shyamala) due to the lack of polymorphism between them (Mohan et al. 1997b). To overcome this difficulty the marker linked to *Gm4t* was mapped to chromosome 8 in another mapping population obtained from a japonica × indica cross (Nipponbare × Kasalath) where sufficient polymorphism did exist (Mohan et al. 1997b). Here, faced with a similar problem of lack of polymorphism between the parents, we have tried mapping of the *Gm7* gene by using YAC, BAC and cosmid clones which have been previously mapped to chromosome 4.

In this study, primers specific to F8, a marker linked to *Gm2* (Mohan et al. 1994), amplified a 1.5-kb fragment (F8LB) in the resistant parent and resistant individuals arising from a cross between RP2333 and Shyamala. This indicates that *Gm7* is in the vicinity of *Gm2*. Further evidence of *Gm7* being present on chromosome 4 comes from the results of hybridization of the resistance specific polymorphic band of F8 (F8LB) obtained in this study to YACs that form part of the contig encompassing an allele of *Gm2* gene (Fig. 6). These results are in concurrence with the Southern hybridization data of F8 with YAC DNAs, where F8 was shown to hybridize to three *Dra*I fragments of 6.0, 4.6 and 3.3 kb in Nipponbare, Y2165 and Y5212, while the 4.6-kb fragment was absent in Y3487 (Rajyashri et al. 1998). Since SA598 hybridized to two cosmid clones, which also hybridize to F8, it was of interest to note that cross-hybridisation studies revealed that there was no homology between F8LB and

SA598, thus indicating that these markers are distinct and separate physical entities linked to *Gm7*. It is interesting to note that markers F8, SA598 and F8LB, along with the *Xa1* gene, all map to the same YAC (Fig. 7). Based on the above hybridization data we conclude that *Gm7* is linked to *Gm2* and maps to chromosome 4.

There are various reports that resistance genes to different pests and pathogens are linked and located in clusters (Salmeron et al. 1996; Meyers et al. 1998; Parniske and Jones 1999; van der Vossen et al. 2000). The markers flanking the gall midge resistance gene *Gm2*, and the bacterial blight resistance gene *Xa1*, hybridize to the same YAC clone, Y2165 (Rajyashri et al. 1998), to which both the resistant (F8LB) and the susceptible (SA598) specific markers linked to *Gm7* also hybridize, indicating a linkage between *Xa1* and the *Gm7* gene as well. Thus, linkage between *Xa1* and *Gm2* and between *Gm2* and *Gm7* suggests the presence of *Gm7*, *Gm2* and *Xa1* as a cluster on chromosome 4, in rice.

For marker-based screening to work effectively it is desirable to have all the genes conferring resistance to the various biotypes of gall midge tagged. This would enable the pyramiding of resistance genes against various biotypes into an elite cultivar. Pyramiding of genes is an important strategy in plant breeding for the development of new varieties with durable resistance to several biotypes of an insect pest (Mohan et al. 1997a). The resistance and susceptibility linked markers can be used effectively in a marker-aided selection programme for the presence of the *Gm7* gene against the gall midge biotypes 1, 2 and 4. With DNA markers also available for two other major gall midge resistance genes (Nair et al. 1995, 1996) there is a potential application for marker-assisted pyramiding of the genes *Gm2*, *Gm4t* and *Gm7* in rice, as has been reported for blast resistance in rice (Hittalmani et al. 2000) and greenbug resistance in wheat (Porter et al. 2000).

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