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DNA markers tightly linked to a gall midge resistance gene (*Gm2*) are potentially useful for marker-aided selection in rice breeding

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Abstract We have developed a polymerase chain reaction (PCR)-based assay that could effectively reduce the time period required to screen and select for Gall Midge-resistant rice lines under field conditions. The primers for the assay were designed on the basis of sequence information of two phenotype specific random amplified polymorphic DNA fragments which were found to be tightly linked to Gall Midge biotype-1 resistance gene (*Gm2*). The two RAPD fragments, $F8_{1700}$ in the susceptible parent 'ARC6650' and $F10_{600}$ in the resistant parent 'Phalguna', were identified after screening 5450 loci using 520 random primers on genomic DNAs of 'ARC6650' and 'Phalguna'. These primers, when used in a multiplexed PCR, amplified specifically a 1.7-kb and 0.6-kb fragment in the susceptible and resistant parents, respectively. When this assay was performed on genomic DNAs of 44 recombinant inbred lines derived from 'ARC6650' × 'Phalguna' and 5 lines derived from other crosses where one of the parents was 'Phalguna', 'ARC6650' or their derivatives, the primers amplified a 1.7-kb fragment in all of the susceptible lines or a 0.6-kb fragment in all of the resistant ones. These markers can be of potential use in the marker-aided selection of Gall Midge biotype-1 resistant phenotypes. As screening for resistance can now be conducted independent of the availability of insects, the breeding of resistant varieties can be hastened.

Key words PCR · RAPDs · *Oryza sativa* · Insect resistance · Marker-aided selection

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Introduction

The breeding of new varieties of rice resistant to diseases and pests by employing traditional methods of selection have been quite effective even though the release of a new variety can take anywhere between 7 and 10 years. One of the primary reasons for this is that screening based on the natural occurrence of the pest is limited to one particular time of the year, which is during the annual build-up of the insect population. This is particularly true of Gall Midge, whose occurrence is predominant during the months just following monsoon and when the plants are in the heading stage.

Gall Midge (*Orseolia oryzae* Wood-Mason) is a major dipteran pest of rice. It occurs in several countries of Asia, and an estimated US$550 million worth of yield is lost as a result of this pest alone (Herdt 1991). An infestation by Gall Midge begins with the larvae lacerating the growing tips of the susceptible rice plants. The feeding larvae cause the conversion of these apices (which in a mature plant would have been a grain-bearing panicle) to galls often referred to as 'onion shoots' or 'silver shoots' (Hill 1987). The infestation causes the rice plants to produce secondary tillers, which may themselves get infected. This leads to a substantial loss in yields.

Previously we have mapped a resistance gene (*Gm2*) in rice that confers resistance to Gall Midge Biotype 1 using restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers (Mohan et al. 1994). Though the mapping information is useful in map-based cloning and to a limited extent in trailing an introgressed gene in a plant breeding programme, the routine use of RFLPs is still quite cumbersome and tedious considering the number of processes involved in this technique (Williams et al. 1991). RAPD analysis is a quick and efficient screening for DNA sequence-based polymorphisms at a large number of loci. These markers are dominant, as they can only be scored for presence or absence of an allele. Besides being simple and fast, it does not involve radio-

active isotopes and can be scaled up to analyse a large number of samples. RAPD markers are increasingly being used for tagging genes of agronomic importance in many crop plants (Martin et al. 1991; Michelmore et al. 1991; Haley et al. 1993; Paran and Michelmore 1993; Penner et al. 1993; Williamson et al. 1994). Although phenotype-specific RAPDs could be used to screen plants in the field, RAPDs are very sensitive to template DNA concentration and $MgCl_2$ concentration, as well as to other reaction parameters, which can lead to erroneous results. To overcome these drawbacks and yet retain the polymerase chain reaction (PCR) nature of the assay we have designed specific 24-bp oligonucleotide primers based on sequence information of two phenotype-specific RAPDs. Using these primers we were able to achieve reliable phenotype-specific PCR amplifications.

Materials and methods

Plant material and entomology

The population used in this study was a set of 44 recombinant inbred (RI) lines (F_5–F_6 generation) derived from a cross between two *indica* rice varieties, 'ARC6650' (susceptible to Gall Midge biotype 1) and 'Phalguna' (resistant to Gall Midge biotype 1), and 5 RI lines from other crosses where one of the parents was 'Phalguna', 'ARC6650' or their derivatives. The scoring for resistance against Gall Midge biotype 1 on the two parents and the 49 RI lines was done under glasshouse conditions. All those lines which showed a damage score of less than 10% were scored as resistant and the rest as susceptible. The damage scores were consistent in three trials conducted during the period 1991–1993.

DNA extraction and preparation of the susceptible and resistant bulks

DNA was isolated from 14-day-old seedlings grown in the dark as described previously (Walbot 1988). An equal concentration of DNAs from 12 susceptible RI lines that showed a consistently high damage score against Gall Midge infestation and 12 resistant RI lines that showed a consistently low damage score was pooled to form the 'susceptible bulk' and the 'resistant bulk', respectively. The concentration of DNA in the two bulks was adjusted to 10 ng/μl.

Random amplification of polymorphic DNAs (RAPDs)

The amplification conditions were as described previously (Williams et al. 1990) except that the denaturation time was kept to 5 s. The reaction volume was 25 μl, and 20 ng template DNA was used per reaction. All reactions were carried out on a Perkin-Elmer Cetus DNA Thermal Cycler. *Taq* DNA polymerase was from Stratagene (La Jolla, Calif.). The RAPD primers used were from the Operon 10-mer Kits (Operon Technologies, Alameda, Calif.). Kits A to Z were utilised in this study. The RAPD products were size-fractionated on a 1.1% agarose gel (Agarose NA; Pharmacia, Milwaukee, Wis.) by electrophoresis in 1 × TBE buffer, with 7.5 μl out of a 25 μl reaction loaded onto the gel. The gel and the buffer contained ethidium bromide at a concentration of 0.5 μg/ml. The DNA fragments were visualised on a UV transilluminator and photographed using Polaroid film (Type 667).

Southern transfer and hybridisation

DNA was transferred from agarose gels onto nylon membranes (GeneScreen Plus; Du Pont, USA) as described by Williams et al. (1991). All probes were radiolabelled with α–[^{32}P]-dCTP using a nick-translation kit (BRL, Life Technologies, USA) to a specific activity of greater than 1×10^8 cpm/μg as per the manufacturer's instructions. Hybridisation conditions were the same as those described earlier (Mohan et al. 1994).

Isolation and cloning of RAPD fragments from agarose gels

The polymorphic RAPD band was cut of the gel with a surgical blade. The agarose piece containing the DNA fragment was passed through a 1-ml syringe (without needle), and the crushed agarose was collected in a microfuge tube. An equal volume of phenol was added and mixed thoroughly by inverting the tube many times. The tube was kept at −80 °C for 15 min and then centrifuged at 14 000 rpm for 15 min at room temperature. The aqueous phase was extracted with an equal volume of phenol:choloroform:isoamyl alcohol (25:24:1) followed by a single extraction with chloroform. The DNA was precipitated using 0.8 *M* lithium chloride and 2.5 volumes of ethanol at −80 °C. The DNA pellet was washed with 75% ethanol, dried and quantitated before being used in the cloning step. The RAPD fragments of interest were cloned into a plasmid vector pCITE-T (Novagen, Madison, Wis.) using the pCITE-T vector kit as per the manufactuer's instructions.

Plasmid DNA preparation and sequencing

Plasmid DNAs containing RAPD inserts were prepared by the alkaline lysis method (Sambrook et al. 1989). The inserts were partially sequenced by the dideoxy chain termination method using the Sequenase Version 1.0 kit (USB, Cleveland, Ohio) and double-stranded plasmid DNA. The forward CITE primer (5′-CACGGGGACGTGGTTTTCCT-3′) was synthesised at ICGEB, and the reverse T3 promotor primer (5′-ATTAACCCTCACTAAAGGGA-3′) was from New England BioLabs, Beverly, Mass.

Synthesis of PCR primers

Based on the sequence information of the cloned RAPD fragments, we synthesised primers on an Applied Biosysystems 380B DNA synthesizer, deprotected with ammonia and precipitated with ethanol. The primers were subsequently purified on a polyacrylamide gel (Sambrook et al. 1989). The primers synthesised were all 24-mers with the first 10 bases being the sequence of the original random primer that produced the respective RAPD fragments.

Polymerase chain reaction (PCR)

PCR was carried out in reaction volumes of 50 μl containing 10 m*M* Tris-Cl (pH 8.8), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% gelatin, 200 μ*M* each of the four dNTPs, 380 n*M* of each primer, 100–200 ng of template DNA and 2.5 units of *Taq* polymerase (Stratagene, La Jolla, Calif.). Amplification consisted of 30 cycles of 1 min denaturation at 94 °C, 1 min annealing at 55 °C and 4 min extension at 72 °C. Ten microliters of each reaction was loaded onto a 0.8% agarose gel to visualise the products.

Results

RAPDs and bulked segregant analysis

We have screened 520 RAPD primers in order to identify markers by which to effectively tag the Gall Midge resistance gene (*Gm2*) using a set of 40 recombinant inbred lines derived from a cross between two *indica* rice varieties, 'ARC6650' (susceptible to Gall Midge biotype

1) and 'Phalguna' (resistant to Gall Midge biotype 1). The RAPD tagging was done in conjunction with bulked segregant analysis (Mohan et al. 1994). Out of 520, 10-base oligonucleotides used, 506 primers were successful in amplifying loci from parental DNAs. A total of 5450 bands were amplified, of which 579 were polymorphic between the parents. Of these, 65 primers amplified bulk-specific RAPDs. The amplification patterns of few primers are shown in Fig. 1. When 25 of these primers were used to amplify the DNAs from the 12 individual susceptible RI lines and 12 individual resistant RI lines that constituted the susceptible and resistant bulks, respectively, we identified two unique bands amplified by F08 and F10 RAPD primers that were phenotype-specific. Primer F08 amplified a 1.7-kbp fragment from the susceptible parent 'ARC6650' and the 12 susceptible RI lines. In contrast, primer F10 amplified a 0.6-kbp fragment from the resistant parent 'Phalguna' and the 12 resistant RI lines. The other 23 primers, which produced bulk-specific amplifications, failed to amplify in a phenotype-specific manner and were not used further in this study (Table 1).

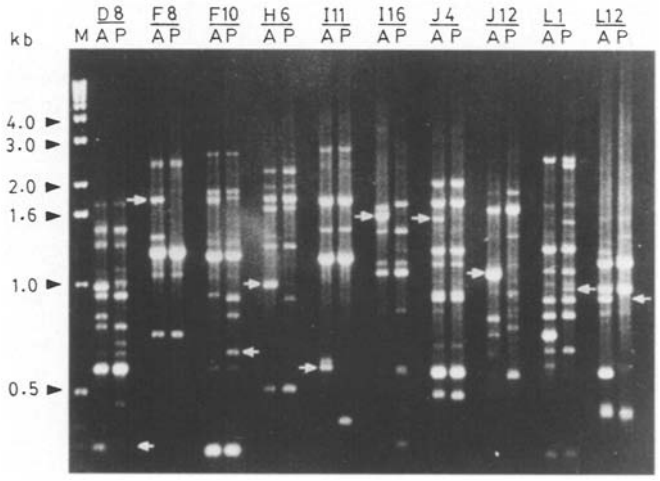

Fig. 1 Amplification products of 'ARC6650' (*A*) and 'Phalguna' (*P*) produced by a few of the RAPD primers screened. *White arrows* indicate polymorphisms that are bulk-specific and likely to be linked to the *Gm2* gene. Figures on the *left* indicate molecular weights in kilobase pairs (kb). Primer numbers are indicated on *top* of the lanes [*M* markers; 1-kb ladder (BRL)]

Cloning and Southern hybridisation

The $F8_{1700}$ and $F10_{600}$ fragments were gel-purified and cloned into pCITE-T vector. The authenticity of the cloned inserts was verified by Southern hybridisation (Fig 2B, D). The $F8_{1700}$ fragment hybridised only to the 'ARC6650' and susceptible bulk-specific fragment and

Table 1 Segregation data of polymorphic and bulk specific RAPD fragments for 24 individuals (12 susceptible RI lines and 12 resistant RI lines) as amplified by 25 RAPD primers. The DNAs used were from parents 'ARC6650'(A) and 'Phalguna' (P), the resistant bulk (R), susceptible bulk (S), the 12 individual RI lines that constituted the susceptible bulk (1-12) and the 12 individual RI lines that constituted the resistant pool (1-12)

Markers[a]	A	P	R	S	Susceptible lines												Resistant lines											
					1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
$D8_{350}$	+[b]	−	−	+	+	+	+	+	+	−	−	+	+	+	+	+	−	−	+	−	+	−	−	−	−	−	−	+
$F8_{1700}$	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
$F10_{600}$	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
$H6_{1000}$	+	−	−	+	−	+	−	+	+	+	+	−	+	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−
$I11_{560}$	+	−	−	+	−	+	−	+	+	+	+	−	+	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−
$I16_{1550}$	+	−	−	+	+	+	+	+	+	−	−	+	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
$J4_{1500}$	+	−	−	+	+	+	+	+	+	−	−	+	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
$J12_{1100}$	+	−	−	+	+	−	+	+	+	+	−	+	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
$L1_{960}$	−	+	+	−	−	−	−	−	−	+	+	−	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
$L12_{800}$	+	−	−	+	+	−	+	+	+	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
$N1_{1200}$	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	−	−	−	−	−	−	−	−	−
$P1_{600}$	+	−	−	+	−	−	−	−	−	+	+	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
$Q9_{1700}$	+	−	−	+	−	+	−	+	+	+	+	−	+	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−
$Q9_{1100}$	+	−	−	+	+	−	+	−	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
$Q18_{500}$	+	−	−	+	−	−	+	+	+	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
$R10_{3200}$	+	−	−	+	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
$R16_{800}$	+	−	−	+	+	+	−	−	−	−	+	+	+	−	−	−	−	+	−	−	−	−	−	−	+	−	+	−
$T3_{400}$	+	−	−	+	+	−	+	+	+	+	−	+	−	+	+	+	−	−	+	−	−	−	−	−	−	−	−	+
$T15_{1000}$	−	+	+	−	−	−	−	−	+	+	−	+	+	−	−	−	−	+	+	−	−	−	−	−	+	+	+	+
$U7_{1100}$	−	+	+	−	−	+	−	+	+	−	−	−	−	+	−	−	−	+	+	−	−	−	−	−	+	+	+	+
$Y5_{1300}$	+	−	−	+	−	+	−	+	+	+	+	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
$Y14_{800}$	−	+	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	+	+	+	+
$Y20_{1400}$	−	+	+	−	−	−	+	−	−	−	−	−	−	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+
$Z16_{1600}$	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	+	+	+	+	+

[a] The markers are designated by the primer number followed by the molecular weight (in bp) of the corresponding RAPD band in subscript. Markers in bold show phenotype-specific amplifications

[b] + indicates amplification of the RAPD, − indicates the absence of RAPD amplification

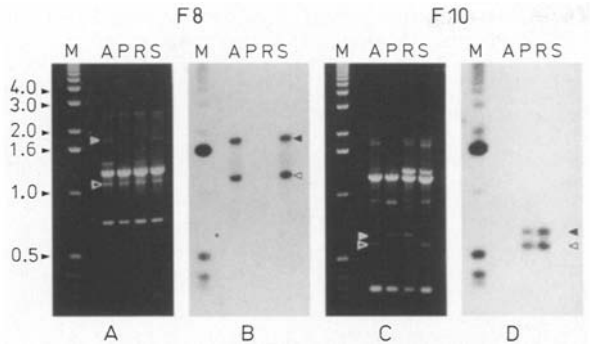

Fig. 2A–D Amplification of genomic DNAs using primer F08 (**panel A**) and F10 (**panel C**). Hybridisation of RAPD products in **panel A** with cloned fragment $F08_{1700}$ (**panel B**) and RAPD products in **panel C** with $F10_{600}$ (**panel D**). *A* 'ARC6650' – susceptible parent, *P* 'Phalguna' – resistant parent, S susceptible bulk. R resistant bulk. *Closed arrow heads* indicate the cloned fragments; *open arrow* indicate additional bands showing homology to the cloned fragments

PF8 (Forward)	5'- GGGATATCGGGGATGAAATGCCAA -3'
PF8 (Reverse)	5'- GGGATATCGGTCATTGCAGTGGAG -3'
PF10 (Forward)	5'- GGAAGCTTGGCTTATAGTAACTAG -3'
PF10 (Reverse)	5'- GGAAGCTTGGAAATGCAAGATCTT -3'

Fig. 3 Sequence of 24-mer oligonucleotide primers (forward and reverse) PF08 and PF10 linked to resistance gene (*Gm2*). The sequences were obtained after the partial sequencing of RAPD fragments $F08_{1700}$ and $F10_{600}$. The *underlined* portions represent the sequences of primers F08 and F10 that generated the fragments $F8_{1700}$ and $F10_{600}$, respectively

failed to hybridise to any fragments in the 'Phalguna' and resistant bulk lanes, whereas the $F10_{600}$ fragment hybridised only to 'Phalguna' and resistant bulk-specific bands and did not hybridise to any fragments in the 'ARC6650' and susceptible bulk lanes. No signals were observed when the $F8_{1700}$ fragment was used as the probe to hybridise to products amplified by primer F10. A similar result was observed when $F10_{600}$ was used as the probe against products amplified by primer F08 (data not shown). However, the $F8_{1700}$ also hybridised to another fragment (1.2 kb) present in the 'ARC6650' and susceptible bulk lanes, even though only similar-sized fragments are observed in the 'Phalguna' and resistant bulk lanes (Fig. 2A, B). A similar situation was observed with the $F10_{600}$ fragment where it hybridised with another fragment (0.55 kb) amplified in 'Phalguna' and resistant bulk only even though similar-sized fragments are present in 'ARC6650' and susceptible bulk lanes (Fig. 2C, D).

The two inserts were partially sequenced (> 100 bp from their termini) and the sequence information was used to design 24-mer PCR primers (Fig. 3).

PCR amplification of DNAs from parental and individual recombinant inbred lines

When the above two sets of primers were used in a PCR with the parental DNAs as template, primer set PF8 amplified a 1.7-kb fragment from 'ARC6650' DNA and failed to show any amplified product with 'Phalguna' DNA. In contrast, primer set PF10 amplified a 0.6-kb fragment from 'Phalguna' DNA and failed to amplify at all when 'ARC6650' DNA was the template (data not shown). When primer sets PF8 and PF10 were used together in a single PCR (multiplexed) to amplify DNA from the parents, the amplification pattern revealed that 1.7-kb fragment was amplified in the susceptible parent and the 0.6-kb fragment in the resistant parent. The primer sets PF8 and PF10 were used one at a time in separate PCR amplifications on all 44 RI lines available from a cross between 'ARC6650' and 'Phalguna'. The amplification pattern revealed that the 1.7-kb fragment was amplified in all the susceptible lines and the 0.6-kb fragment in all the resistant lines. In a few lines we observed the co-amplification of both bands, which indicated a heterozygous state. Lines that showed both fragments are phenotypically resistant, as the *Gm2* gene is dominant (Mohan et al. 1994), though the 1.7-kb fragment (susceptible-specific fragment) was never the only fragment to be amplified in a resistant line (Fig. 4). We have also used these markers to assay individuals arising from a different cross where one of the parents was either 'Phalguna', 'ARC 6650' or one of their derivatives. However, in 3 susceptible lines (Fig. 4; lanes 20, 48 and 49) the resistant-specific band was amplified.

Discussion

We have identified an allele-specific PCR-based marker linked to the *Gm2* gene in rice. Although we had previously mapped the *Gm2* gene conferring resistance against biotype 1 of Gall Midge (Mohan et al. 1994) using RFLPs, the PCR-based assay described here is faster and easier to conduct in a marker-based selection programme in a rice breeding station.

We converted the RAPD markers to sequence characterised amplified regions (SCARs) for greater reliability as a selection tool. Similar SCARs have been developed for downy mildew resistance genes in lettuce (Paran and Michelmore 1993) and nematode resistance gene in tomato (Williamson et al. 1994). SCARs can also be developed from RFLP markers (Williams et al. 1991). When the SCARs that we have developed in this study are used in a multiplexed PCR situation they have the characteristics of a reliable yes/no assay for the Gall Midge resistance gene. When a multiplexed PCR is performed on a particular sample, at least one of the two expected bands should be amplified. This provides an internal control for the PCR reaction and avoids the problem of false negatives. This is particularly import-

ant during mass screenings where crude DNA preparations involving leaf squashes (Langridge et al. 1991) or other quick methods (Chunwongse et al. 1993) may be employed.

RAPDs usually amplify several independent genetic loci (Williams et al. 1993). In the light of this observation it was quite interesting to note that the cloned $F8_{1700}$

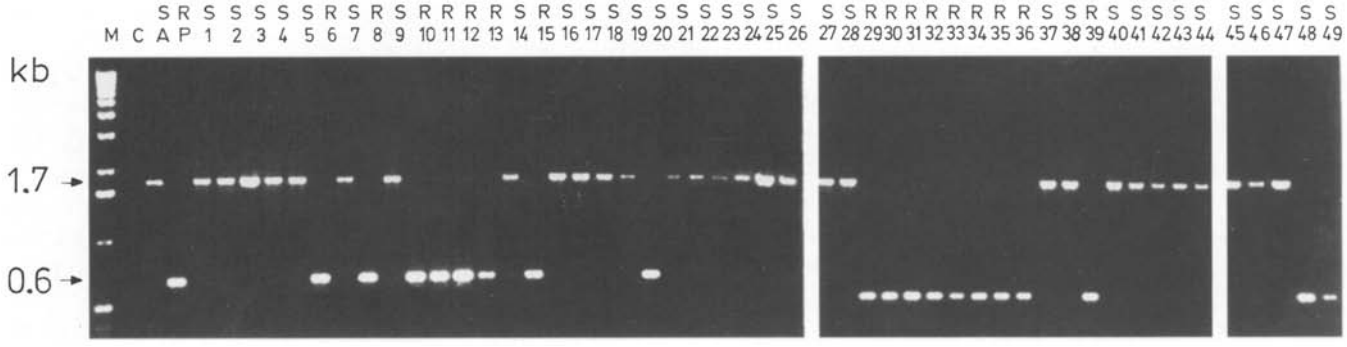

Fig. 4 PCR-based screening for Gall Midge-resistant (*R*) and susceptible (*S*) lines in rice. Multiplexed allele-specific PCR amplification using primer sets PF08 and PF10. Template DNAs were 'ARC6650' (*A*), 'Phalguna' (*P*), 44 recombinant inbred lines representing the F_{5-6} generation of the cross between 'ARC6650' and 'Phalguna' (*lanes 1–44*), and 5 lines from other crosses involving either 'Phalguna' derivatives or 'ARC6650' derivatives as one of the parents (*lanes 45–49*)

fragment hybridised to a 1.2-kb fragment as well as to the 1.7-kb fragment amplified from 'ARC6650'. Though a similar, co-migrating 1.2-kb product was amplified by the RAPD primers in 'Phalguna' and the resistant bulks, this product did not hybridise to the $F8_{1700}$ cloned fragment. A similar situation was observed with $F10_{600}$, which hybridised to a 0.55-kb fragment in addition to the 0.6-kb one in 'Phalguna' and the resistant bulk only. The homology between the 1.7-kb and the 1.2-kb fragments, and the homology between the 0.6-kb and 0.55-kb fragments seems to be internal with sequences that are not conserved in the 14 bp 3′ of the PCR primers as the specific primers designed for the 1.7-kb and the 0.6-kb bands did not amplify the 1.2-kb and the 0.55-kb fragments, respectively.

The amplification of resistant bands in 3 susceptible lines (lanes 20, 48, and 49) is likely due to a recombination event between the gene and the marker. Based on this data, we estimate that the *Gm2* gene is situated 4.1 and 5.4 cM away from markers $F8_{1700}$ and $F10_{600}$, respectively.

Marker-aided selection

One of the major advantages of developing an allele-specific PCR is its potential for marker-aided selection (MAS). In many breeding stations it is currently possible to conduct three rounds of crossing, but the breeder is still constrained by the annual occurrence of the insect. In the case of Gall Midge, its natural occurrence is restricted to a 2- to 4-month period every year. This limitation slows down the breeding programme considerably. Also, low precipitation just preceding the infestation period could result in a reduction in infestation rate leading to poor selection under natural conditions.

Besides, MAS can also be employed to determine the genotype of the individual even at the seed stage itself with the testing of the seed being conducted on a portion while retaining the remaining portion for subsequent seed germination (Chunwongse et al. 1993). MAS would certainly be of immense use to breeding stations involved in developing plants resistant to a particular insect/pathogen of other regions even when that pest/pathogen is not known to occur where the breeding station is located.

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