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PCR-based DNA markers linked to a gall midge resistance gene, *Gm4t*, has potential for marker-aided selection in rice

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Abstract Rice DNAs from a gall midge resistant variety, 'Abhaya', a susceptible variety, 'Tulsi' and their F_3 progeny were screened using 500 random primers in conjunction with bulked-segregant analysis in a polymerase chain reaction (PCR) with a view to detecting random amplified polymorphic DNAs (RAPDs) linked to the gene, *Gm4t*, which confers resistance to gall midge, a dipteran insect pest of rice. A total of 454 primers were able to produce a distinct amplification pattern, and 3695 bands/loci were amplified between the phenotypically different parents. Of these, 304 bands were polymorphic between the parents, with 19 being phenotype-specific. One of these primers, E20, amplified 2 bands, $E20_{570}$ and $E20_{583}$, which are tightly linked to resistance and susceptibility, respectively. These specific bands were cloned and sequenced, and a 94% sequence homology was found between the two fragments. Two specific 20-mer oligonucleotides were synthesized, based on the sequence information of $E20_{583}$, for use in PCR amplification directly from genomic DNAs. These PCR primers were able to amplify phenotype-specific bands, a 583-bp fragment in susceptible F_3 lines and a 570-bp fragment in resistant F_3 lines that had been derived from a cross between the parents, indicating their potential and utility for marker-aided selection of the *Gm4t* gene in rice. Its use would facilitate the early and efficient selection of resistant genes in plant breeding programmes and even in those areas where the insect is not known to occur. These phenotype-specific bands are single-copy sequences and are being mapped to ascertain their chromosomal location in rice.

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Introduction

Rice plants are prone to attack from many pests and pathogens. Over 200 insects attack rice, and a dipteran insect, gall midge (*Orseolia oryzae*), poses a major problem (Hill 1987). Like all major pathogens and pests that have strains/pathovars or biotypes, gall midge too is known to have biotypes, four (Kalode and Bentur 1989), and recently a fifth biotype has been identified on the western coast of India (Nair and Ambika Devi 1994). Though genes conferring resistance to gall midge have been identified in different varieties of rice, the product of the resistance gene is still unknown. Studies on the genetics of resistance have revealed that resistance against gall midge is due to a single major gene. The various genes conferring resistance to the different biotypes of the insect are in most cases non-allelic (Srivastava et al. 1994). This insect causes the conversion of the potential grain-bearing tillers into 'onion shoot'-like galls that do not produce seeds and, therefore, yield is severely reduced (Hidaka 1974).

The biotyping of gall midge is done by observing the infectivity pattern on a set of rice differentials/varieties. Dependent on the differential reaction, the biotypes are classified from biotype one through five. In India, the different biotypes are distributed throughout the various rice-growing regions of India. It is not currently possible to differentiate the biotypes on the basis of morphology of the insects and, thus, biotyping has been solely based on differential infestation patterns on specific rice hosts. This has slowed down the process of biotype identification, and consequently the selection for rice plants resistant to more than one biotype of gall midge. For example, if plants resistant to biotype 1 have to be screened for resistance to another biotype, they will have to be screened at the second site where the second biotype is prevalent. As the time of the annual appear-

ance of all biotypes in India is more or less same, (i.e. rainy season), screening for the second biotype will have to be postponed to the following year. As an artificial diet for culturing gall midge has yet to be identified, screening still depends on field-based screening methods and to a limited extent on glasshouse-based systems. This situation considerably slows the process of breeding new resistant rice varieties.

An alternative to the labour-intensive and time-consuming screening procedure would be to screen the population of interest using genetic markers. These markers could be either molecular or morphological. Unfortunately, there are only a limited number of phenotypic markers available, and the linkage of the morphological markers to the gene of interest is usually not very tight. Thus, molecular markers, whether based on restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) or microsatellites would prove to be ideal under the above circumstances. Consequently, upon identification of a suitable molecular marker closely linked to an insect resistance gene, one could easily follow the gene in a cross intended to breed new resistant varieties any time of the year without depending on the annual occurrence of the insects. Moreover, the screening for a biotype could be conducted in a breeding station where the biotype being screened for is not known to be a problem. This would speed up breeding for new gall midge resistant varieties.

Progress has been made in mapping and tagging many agriculturally important genes with molecular markers, which forms the foundation for marker-aided selection (MAS). Recently, sequence-tagged sites (STS) have been developed for a few resistance genes. Using near-isogenic lines of tomato, Williamson et al. (1994) developed STS for resistance genes on introgressed segments from wild relatives using RAPD markers. The use of the RAPDs in marker-aided selection of rust resistance in common beans has also been proposed (Johnson et al. 1995).

Recently, RFLP and RAPD techniques in conjunction with bulked-segregant analysis have been used to map and tag many disease and pest resistance genes against viruses, bacteria, fungi, nematodes and insects in many crop plants. DNA markers have been used to identify genes for resistance to *Pseudomonas syringae*, tobacco mosaic virus and tomato spotted wilt virus in tomato (Martin et al. 1991; Ohmori et al. 1995; Stevens et al. 1995), *Pseudomonas syringae* in *Arabidopsis* (Debener et al. 1992), powdery mildew in barley (Hinze et al. 1991), a nematode resistance gene in sugar beet (Jung et al. 1990) and root knot nematode in tomato (Klein-Lankhorst et al. 1991). Genes have been mapped for resistance to rice blast (Yu et al. 1991), bacterial leaf blight (Ronald and Tanksley 1991; Yoshimura et al. 1992), downy mildew resistance in lettuce (Michelmore et al. 1991), Hessian fly in wheat (Ma et al. 1993), brown planthopper in rice (Ishii et al. 1994) and gall midge in rice (Mohan et al. 1994).

In a continuing effort to tag and map other gall midge resistance genes we report here the tagging of another gene, *Gm4t*, which is non-allelic to *Gm2* and is responsible for conferring resistance against biotype 1, 2, 3 and 4 of the insect. Tagging was achieved after screening parental DNAs with 500 RAPD primers in combination with bulked-segregant analysis on a F_3 population derived from a cross between two indica rice varieties, 'Abhaya' (resistant parent) and 'Tulsi' (susceptible parent). The tag was identified as a 570-bp RAPD fragment found in the resistant plants and the resistant progenies and a 583-bp fragment found in the susceptible parent and all the susceptible progenies. Further, both fragments were sequenced and specific primers designed to directly PCR amplify the fragment from genomic DNAs. This was done with the idea that marker-aided selection of the *Gm4t* gene would be useful in a breeding programme. The screening of F_3 individuals from this cross using these specific primers demonstrates the suitability of these primers in achieving an allele-specific PCR viz-a-viz the resistance/susceptible phenotype.

Materials and methods

Plant material and entomology

The F_3 population used in this study was derived from a cross between two *indica* rice varieties, 'Abhaya' (R296 selections; resistant to biotypes 1, 2, 3 and 4 of gall midge; Kalode et al. 1993; Rao and Kandalkar 1992) and 'Tulsi' (susceptible to all biotypes of gall midge). The scoring for resistance and susceptibility was done under field conditions at the Indira Gandhi Agricultural University, Raipur, India. Plants were screened for the presence or absence of galls. Plants with even one gall per plant were scored as susceptible and those without galls were scored as resistant. Scored plants were subsequently harvested for DNA extraction.

DNA extraction and preparation of susceptible and resistant bulks

DNA was isolated from field-grown material (10 weeks old) using the method of Walbot (1988). An equal quantity of DNA from 12 resistant and 12 susceptible F_3 individuals was pooled to form the resistant and susceptible bulks, respectively (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 DNAs (RAPDs)

The amplification conditions were as described by Williams et al. (1990) with the only exception being that the denaturation time was kept at 5 s. Twenty nanograms of template was taken for a 25-μl reaction. PCR was carried out on a Perkin-Elmer Cetus DNA Thermal Cycler. *Taq* polymerase was from Strategene (La Jolla, Calif.). The 500 random primers (10-mers; Kits A–J and L–Z) used in this study were from Operon Technologies, Alameda, Calif. The RAPD products were separated electrophoretically on a 1.1% agarose gel in 1 × TBE buffer with 7.5 μl of the 25-μl reaction being loaded onto the gel. Gel and buffer contained ethidium bromide at a concentration of 0.5 μg/ml. Gels were photographed on a UV transilluminator using Polaroid film (Type 667).

Southern transfer and hybridisation

DNA was transferred from agarose gels onto nylon membranes (Gene Screen Plus; Du Pont, USA) as described by Williams et al. (1991). Probes were radio-labelled with α-[^{32}P]-dCTP using nick-translation kit (BRL, Life Technologies, USA) to a specific activity of more than 1×10^8 cpm/μg as per manufacturer's instructions. Hybridisation conditions were according to Mohan et al. (1994).

Isolation and cloning of RAPD fragments from agarose gels

RAPD bands of interest were isolated from the agarose gels as described by Nair et al. (1995) and cloned into plasmid vector pGEM-T (Promega, Madison, Wis.) as per the manufacturer's instructions.

Plasmid DNA preparation and sequencing

Plasmids containing RAPD inserts were prepared by the alkaline lysis method (Sambrook et al. 1989). Inserts were completely sequenced by the dideoxy chain termination method using the Sequenase Versin 2.0 kit (USB, Cleveland, Ohio) and double-stranded plasmid DNA. The forward sequencing primer was T7 (5'-GTAATACGACTCACTATAGGGC-3') and the reverse was PGEMT(R) (5'-GCATGCAACGCGTTGGGAGC-3'). Based on the sequence data obtained using the above primers we synthesised two internal primers, IP43F(5'-CTTACGGCTTTCAGGCACAA-3') and IP43R(5'-TTTGTCGAACCGTTATTGTA-3'), in order to completely sequence the inserts.

PCR primers

In order to specifically amplify the RAPD fragment of interest directly from rice genomic DNAs we synthesised two specific primers based on the sequence information that we had obtained with respect to the termini of the cloned RAPD fragments. Both of the synthesised primers were 20 mers: 43F(5'-TTATTGATGAGGACTTAGGG-3') and 43R(5'-TGGATAGGTTAGCAGAGCTG-3').

Polymerase chain reaction (PCR)

PCR was carried out directly on genomic DNAs in 50-μl reaction volumes 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). PCR parameters were 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min for 30 cycles. Ten microlitres of each reaction was loaded onto a 2.0% agarose gel containing ethidium bromide at 0.5 μg/ml for analysis of the PCR products.

Results

Genetic analysis of gall midge resistance in 'Abhaya'

It has been established in many national coordinated trials that the F_1 of the cross between 'Abhaya' (resistant parent) and 'Tulsi' (susceptible parent) are resistant to biotypes 1, 2, 3 and 4 of gall midge (Kalode et al. 1993; Rao and Kandalkar, 1992). The F_2 population of this cross segregated 3:1 for resistance:susceptibility, indicating that resistance is determined by a single dominant gene that has been designated as *Gm4t* (Srivastava et al. 1994). This gene is non-allelic to another resistance gene, *Gm2*, which confers resistance to biotype 1 of gall midge and which has been mapped and tagged in another resistant variety, 'Phalguna' (Mohan et al. 1994).

RAPD and bulked-segregant analysis

The two parental DNAs along with the resistant and susceptible bulks were screened using 500 RAPD primers to identify RAPD fragments linked to the gall midge resistance gene. Of the 500 primers 454 produced amplification products, while the remainder failed to amplify. During this survey we identified 304 RAPDs that differentiated 'Abhaya' from 'Tulsi', of which 19 were specific for the resistant parent and the resistant bulk or for the susceptible parent and the susceptible bulk. The primers that produced phenotype-specific

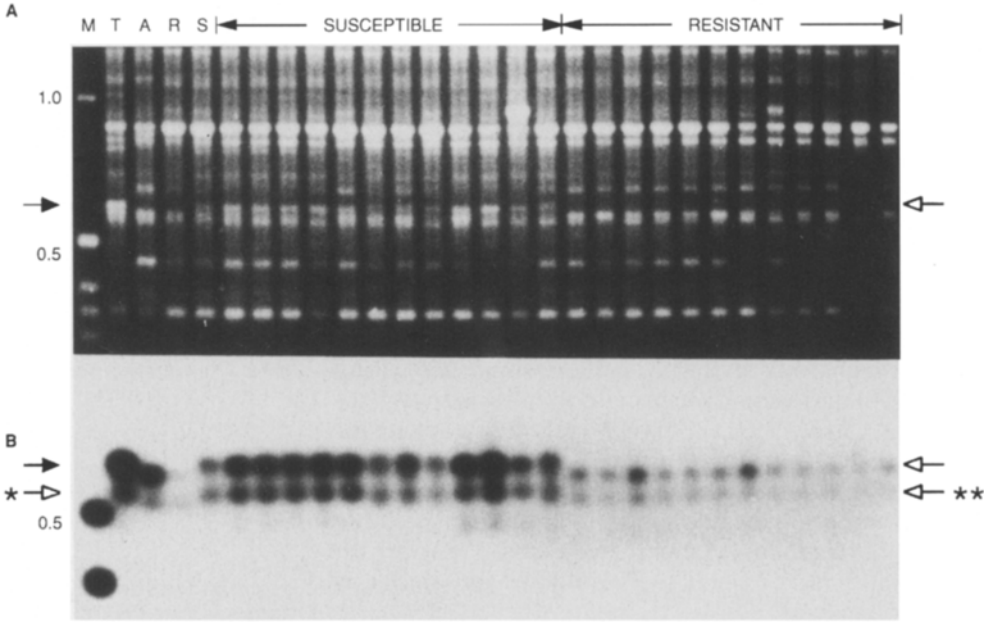


Fig. 1A RAPD amplification of genomic DNAs using primer E20. **B** Hybridization of RAPD products in panel A with cloned fragment $E20_{583}$. *T* 'Tulsi' (susceptible parent), *A* 'Abhaya' (resistant parent), *S* susceptible bulk, *R* resistant bulk, *M* molecular weight marker, 123-bp ladder. *Arrows* indicate the phenotype-specific RAPD fragment: *closed arrows* indicate the 583-bp fragment and the *open arrows* indicate the 570-bp fragment. *Open arrows* with *single* and *double asterix* are additional bands showing homology to the cloned fragment $E20_{583}$. *Numbers* on the *left* represent molecular weights in kb

RAPDs were further used to screen each of the 12 different individual DNAs that constituted each of the two bulked DNAs. RAPDs that failed to amplify in a phenotype-specific manner were not pursued further. Of the 19 primers that amplified RAPDs in a bulk-specific manner, only 1 primer, E20, produced both a 583-bp fragment in the susceptible parent, susceptible bulk and in all 12 individuals that constituted this bulk and a 570-bp fragment in the resistant parent, resistant bulk and in all 12 individuals that constituted the resistant bulk (Fig. 1A). The remaining 18 specific primers that failed to amplify in a phenotype-specific manner in individual lines that constituted the bulks were not pursued further in this study. In addition, we could also see the amplification of 13 non-parental bands in the resistant and susceptible pools.

Cloning and Southern hybridisation

The $E20_{583}$ fragment was gel-purified and cloned into the pGEM-T vector. The cloning was confirmed by Southern hybridisation. The $E20_{583}$ fragment hybridised to both the 'Tulsi' and the susceptible bulk-specific fragment. Interestingly, it also hybridised to a slightly smaller (570-bp) fragment in 'Abhaya', the resistant bulk and the 12 individual resistant lines that constituted the resistant bulk. In addition, $E20_{583}$ hybridised to a 550-bp fragment in 'Tulsi', the susceptible bulk and susceptible individuals and to a 535-bp fragment in 'Abhaya', the resistant bulk and the resistant individuals (Fig. 1B).

The cloned $E20_{583}$ fragment was sequenced completely and on the basis of the sequence information obtained, specific primers (20-mers) were synthesised (see Materials and methods).

PCR amplification of DNAs from parents and individual F_3 lines

When the above primer set was used in a PCR with the parental DNAs as template it amplified a 583-bp and a 570-bp fragment in 'Tulsi' and 'Abhaya', respectively. Similarly, when DNAs of individual F_3 lines chosen at random were used as the template, the primers amplified the 583-bp fragment in all of the susceptible lines and the 570-bp fragment in all of the resistant lines (Fig. 2); in some cases we observed co-amplification of both bands in the resistant lines. The only exceptions were lanes numbers 20 and 31, which show heterozygous situation with reference to the amplified products but were scored susceptible based on entomological screening. These lines should have been resistant since *Gm4t* is a dominant gene.

Subsequently, the 570-bp resistant-specific fragment (produced by PCR amplification of the genomic DNA of 'Abhaya' using the above-mentioned specific primers) was cloned and also sequenced completely. The plasmids containing the 583-bp (from 'Tulsi') and the 570-bp (from 'Abhaya') inserts were designated as pTu and pAb, respectively.

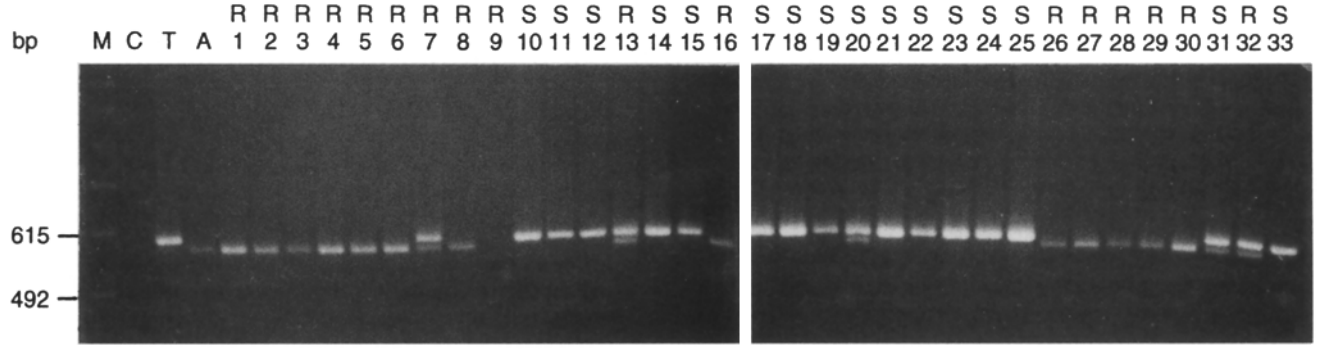
Sequence analysis of clones pTu and pAb

Complete sequencing revealed that pAb and pTu were 570 bp and 583 bp, respectively. In general, both sequences were AT rich (>65%). Sequence homology searches revealed that pAb and pTu have a sequence homology of 94%, with the difference in size being primarily due to deletions in pAb (total of 23 bp) and pTu (total 10 bp). The largest single contiguous deletion was of 16 bp in pAb when compared to pTu. Some deletions are in the regions of simple sequence repeats (data not included).

Discussion

We have tagged a gall midge resistance gene, *Gm4t*, on the basis of RAPD analyses of bulked-segregants derived from a cross between the resistant parent, 'Ab-

Fig. 2 PCR-based screening for *Gm4t*. PCR amplification was done using the primers mentioned in the Materials and methods. Template DNAs were 'Tulsi' (*T*), 'Abhaya' (*A*) and 33 lines representing the F_3 generation of the cross between 'Abhaya' and 'Tulsi' (*lanes 1–33*). *M* Molecular weight marker, 123-bp ladder, *C* minus DNA control, *T* 'Tulsi' (susceptible parent), *A* 'Abhaya' (resistant parent), *R* and *S* on *top* of lane numbers represent resistant and susceptible phenotypes (as determined by entomological tests), respectively. *Numbers* on the *left* represent molecular weights in bp. Note that *lane 9* did not show any amplified products



haya', and the susceptible parent, 'Tulsi'. Though numerous RAPDs revealed polymorphism between the parental DNAs, only 2 RAPD fragments, $E20_{570}$ and $E20_{583}$, were amplified in an phenotype-specific manner.

This latter RAPD fragment was subsequently converted to sequence-characterised amplified regions (SCARs) or sequence-tagged sites (STSs) for greater robustness during PCR. The primers designed after sequencing the cloned RAPD fragment amplified a 583-bp fragment in the susceptible parent and all of the susceptible F_3 individuals tested in contrast to a 570-bp fragment produced in the resistant parent and all of the resistant individuals tested. This clearly indicates that this pair of primers is ideal for screening individuals in a breeding programme by means of marker-aided selection. However, we did observe the co-amplification of both bands in susceptible lines (Fig. 2; lanes 20 and 31), and this may be due to a recombination event between the gene block and the markers. A similar approach has already been demonstrated for another gall midge resistance gene, *Gm2*, in rice (Nair et al. 1995). RFLP and RAPD markers with sequence information can be helpful in directly amplifying the mapped regions by PCR. Williams et al. (1991) were able to convert many rice genomic clones into STS or SCARs that were then used in a PCR to detect polymorphism between many rice varieties without resorting to Southern hybridisation. This approach is relevant with respect to an acceleration of plant breeding through the molecular approach. Recently, Niewöhner et al. (1995) developed PCR assays diagnostic for RFLP marker alleles which are closely linked to alleles *Gro1* and *H1* conferring resistance to the root cyst nematode *Globodera rostochiensis* in potato. A large number of mapped STSs also makes it possible to construct a saturated genetic physical map (Monna et al. 1994).

For marker-based screening to work effectively one has to have all the genes conferring resistance to the various biotypes tagged. This would hasten the pyramiding of resistance genes against various biotypes into an elite cultivar. The pyramiding of genes is an important strategy in plant breeding with bearing on the development of new varieties with a durable resistance to several biotypes of an insect pest. With this view in mind, we previously mapped and tagged *Gm2*, which confers resistance to biotype 1 of gall midge, using RAPDs and RFLPs (Mohan et al. 1994). We also showed that screening for resistance in a large breeding programme can be hastened by converting the molecular tags to sequence-tagged sites (STS). This makes the whole process amenable to automation using the polymerase chain reaction (PCR). We were able to demonstrate that marker-aided selection is a possibility as far as *Gm2* is concerned (Nair et al. 1995), and now we have demonstrated this for another gene, *Gm4t*.

In a plant breeding programme after the initial cross(es) the breeder follows up with many rounds of selfing the hybrid or backcrossing it to one of the parents. The resulting segregating population is screened for resistance, usually by utilising the natural occurrence of the insect pest. The breeder then uses, the resistant plants for the next round of crossing. If for some reason insect pressure is not enough, the breeder might carry forward false positives.

The availability of molecular markers would enable breeders to ascertain which of the individuals from a cross are resistant or susceptible to the insect pest in question. Thus, with MAS it is now possible for the breeder to conduct many rounds of selection in a year without depending on the natural occurrence of the pest and, theoretically, without the pest at all. As envisaged above, in the case of the selection of plants resistant to gall midge, the breeder requires only small amounts of DNA (Langridge et al. 1991) from the individuals he wants tested, he uses the above-mentioned sets of primers in a PCR, he runs the products of the reaction on an agarose gel and he directly ascertains the genotype of the individuals viz-a-viz resistance/susceptibility to gall midge.

It was interesting to note that both of the cloned RAPD fragments, pAb and pTu, hybridised to additional fragments (Fig. 1B). However, when the specific primers were synthesised and used in a PCR using parental and F_3 DNAs, only the allele-specific band amplified or, in the case of a heterozygote, both alleles could be seen. This shows that the hybridisations of the RAPD fragments to additional fragments is due to internal homologies rather than homologies at the termini as the specific primers failed to amplify these additional bands. An identical situation was noticed in a previous study involving the tagging of *Gm2* gene (Nair et al. 1995).

There is a 94% homology between the two clones, pTu and pAb, and the differences between them are mainly due to deletions, some of them in reiterated sequences in the simple sequence repeat regions. This would lend credence to various reports that the mapping effort would benefit from mapping various microsatellites that are able to reveal polymorphisms even between closely related individuals. Recently, Wu and Tanksley (1993) found an abundance of microsatellites in rice, many of which were mapped on to the rice chromosomes. Microsatellites or simple sequence repeats have been found to be linked to the soybean mosaic virus resistance gene in soybean (Yu et al. 1994). The present study also demonstrates that a microsatellite region is linked to a gene of interest and that a judicious selection of primers can make the assay for the gene more rapid and less cumbersome (data not included).

We also noticed the amplification of non-parental bands with many RAPD primers in F_3 lines that were not present in either of the parents. It has been suggested that these non-parental bands arise as a result of a mixing of allelic sequences followed by denaturation and annealing in the absence of polymerase activity, or in some RAPD reactions, heteroduplex formation could also be between two allelic sequences of different sizes as

has been shown to be the case in a study conducted on *Melampsora lini*, which causes flax rust (Ayliffe et al. 1994).

Work is currently in progress towards the mapping of this gene and the RAPD tags to one of the 12 linkage groups of rice.

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