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Molecular mapping of a resistance-specific PCR-based marker linked to a gall midge resistance gene (*Gm4t*) in rice

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Abstract A PCR-based marker ($E20_{570}$) linked to the gene *Gm4t*, which confers resistance to a dipteran pest gall midge (*Orseolia oryzae*), has been mapped using the restriction fragment length polymorphism (RFLP) technique in rice. *Gm4t* is a dominant resistance gene. We initially failed to detect useful polymorphism for this marker in a F_3 mapping population derived from a cross between two *indica* parents, 'Abhaya' × 'Shyamala', with as many as 35 restriction enzymes. 'Abhaya' carries the resistance gene *Gm4t* and 'Shyamala' is susceptible to gall midge. Subsequently, $E20_{570}$ was mapped using another mapping population represented by a F_2 progeny from a cross between 'Nipponbare', a *japonica* variety, and 'Kasalath', an *indica* variety, in which the gene *Gm4t* was not known to be present. *Gm4t* mapped onto chromosome 8 between markers R1813 and S1633B. Our method, thus, presents an alternative way of mapping genes which otherwise would be difficult to map because of a lack of polymorphism between closely related parents differing in desired agronomic traits.

Key words RFLP · Insect resistance · *Oryza sativa* · *Orseolia oryzae* · Linkage analysis

Introduction

Gall midge (*Orseolia oryzae*) is a major dipteran insect pest of rice. This insect is known to have five biotypes in India (Kalode and Bentur 1989; Nair and Ambika Devi 1994). Currently, many resistance genes have been identified in rice which confer resistance to the different biotypes. Genetic studies of gall midge resistance indicate that in most cases the resistance is governed by a single dominant gene (Chaudhary et al. 1986; Srivastava et al. 1994; Mohan et al. 1994). However, it is still not clear whether a single gene can confer resistance to more than one biotype. In all of the studies from which data is available, resistance was found to be monogenic.

The *Gm2* gene, which confers resistance against biotype 1, has been mapped onto chromosome 4 of rice (Mohan et al.1994). Marker-assisted selection of *Gm2* has also been successfully demonstrated (Nair et al. 1995). Recently, polymerase chain reaction (PCR)-based markers were developed which were found to be linked to *Gm4t*, a resistance gene in *indica* rice variety 'Abhaya' (Nair et al. 1996). It has also been demonstrated that genes conferring resistance to different biotypes of gall midge are in most cases non-allelic (Srivastava et al. 1994).

Restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNAs (RAPDs) are powerful techniques used to map or tag agronomically important genes including resistance genes against viruses, bacteria, fungi, nematodes and insects (Mohan et al. 1997). RFLP markers have served as tools in the identification of genes in many plants such as those for resistance to *Pseudomonas syringae*, tobacco mosaic virus and tomato spotted wilt virus (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), black root rot of tobacco (Bai et al. 1995), blackleg resistance in *Brassica napus* (Dion et al. 1995), wheat leaf rust resistance gene (Schachermayr et al. 1995), nematode resistance genes in sugar beet, soybean and wheat (Jung et al. 1990; Salentijn et al. 1995; Webb et al. 1995; Vierling et al. 1996; Williams et al. 1994) and root knot nematode in tomato (Klein-Lankhorst et al. 1991;

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Yaghoobi et al. 1995), downy mildew resistance in lettuce (Michelmore et al. 1991) and Hessian fly resistance genes in wheat (Ma et al. 1993). In rice, genes have been mapped for resistance to rice blast (Yu et al. 1991), bacterial leaf blight (Ronald and Tanksley 1991; Yoshimura et al. 1992), and brown planthopper resistance gene (Ishii et al. 1994) using this procedure. We have also successfully exploited this technique in mapping the *Gm2* gene conferring resistance to gall midge biotype 1 onto chromosome 4 of rice (Mohan et al. 1994) .

The aim of the investigation presented here was to map *Gm4t* in 'Abhaya', using RFLP analysis. However, this gene could not be mapped in the population derived from an *indica* × *indica* cross ('Abhaya' × 'Shyamala') in which the gene segregated due to the lack of sufficient polymorphism. To overcome this hurdle, we mapped the marker linked to *Gm4t* in another population obtained from a *japonica* × *indica* cross ('Nipponbare' × 'Kasalath') where sufficient polymorphism did exist. This paper discusses the possibility of being able to map a marker linked to a gene of importance but for which sufficient RFLPs do not exist in the population of interest (in which the resistance gene was segregating) by mapping it in another population where adequate polymorphism does exist. Our RFLP analysis of the *japonica* × *indica* cross also yielded new markers linked to the PCR-based marker. The mapping population in which *Gm4t* was segregating was a set of 184 F_3 lines derived from a cross between 'Abhaya', a resistant variety, and 'Shyamala', a susceptible variety. The population used for mapping the resistance-specific marker was a set of 186 F_2 lines derived from a cross between 'Nipponbare', a *japonica* variety, and 'Kasalath' an *indica* variety. This represents an alternate strategy for mapping resistance genes using a population not harboring the gene. We had to resort to this strategy as the 'Abhaya' × 'Shyamala' population, in which the gene segregated, failed to reveal enough RFLPs to be useful in mapping. Here, we report the mapping of a PCR-based DNA marker, $E20_{570}$, linked to *Gm4t*.

Materials and methods

Plant materials

The rice lines were grown either in field or in pot cultures. For the mapping experiment, 184 F_3 lines were derived from a cross between 'Abhaya' and 'Shyamala'. This population was raised for the mapping of gene/s that confer(s) resistance against biotype 1, 2, 3 and 4 of gall midge. 'Abhaya' is resistant to many biotypes of gall midge (R296 selections; resistant to biotypes 1, 2, 3 and 4; Kalode et al. 1993; Rao and Kandalkar 1992), and 'Shyamala' is susceptible to all the known biotypes in India. The gene for resistance in 'Abhaya' was introgressed from a resistant landrace, Ptb10 (Kalode et al. 1993; Rao and Kandalkar 1992). The other mapping population used in this study was a set of 186 F_2 lines derived from a cross between 'Nipponbare', a *japonica* variety, and 'Kasalath', an *indica* variety.

Evaluation of gall midge resistance

The scoring for the presence of galls or 'silver-shoots' (symptoms of pest infection) against biotype 1 of gall midge was carried out on the F_3 lines of 'Abhaya' × 'Shyamala' when the plants were reaching the heading stage in the experimental field at IGAU, Raipur, India. The landrace Ptb10, donor of the *Gm4t* gene, was also screened against biotypes 1, 2, 3 and 4 of gall midge (Rao and Kandalkar 1992). The level of infestation was very high – the susceptible varieties 'TN1', 'Tulsi', 'ARC6650' and 'Shyamala' showed 100 % infestation.

DNA extraction and Southern hybridization

DNA from the parental lines, 'Abhaya', 'Shyamala' and 184 F_3 progeny lines was isolated by a modified CTAB method of Murray and Thompson (1980). Genomic DNA restriction, Southern transfer and hybridization were either as described in Mohan et al. (1994) or Kurata et al. (1994). The following set of 35 restriction endonucleases were used to detect polymorphisms: *Aat*II, *Alu*I, *Apa*I, *Ava*I, *Ban*II, *Bam*HI, *Bgl*II, *Cla*I, *Dra*I, *Eco*RI, *Eco*RV, *Eco*T14I, *Eco*T22I, *Eco*0109I, *Fba*I, *Hae*III, *Hin*cII, *Hin*dIII, *Hin*fI, *Hpa*I, *Kas*I, *Kpn*I, *Mbo*I, *Msc*I, *Mva*I, *Nde*I, *Nco*I, *Sac*I, *Sal*I, *Sma*I, *Sau*3AI, *Sca*I, *Taq*I, *Xba*I and *Xho*I.

RFLP probes

A total of over 100 single-copy DNA probes distributed over chromosomes 4, 8, 9 and 11 of rice were selected for the RFLP analysis. These clones were random genomic clones originally selected from a *Pst*I genomic library of rice (McCouch et al. 1988) and were kindly provided by Dr. S. D. Tanksley of Cornell University, N.Y., USA. The genomic DNA clones, cDNA clones from root, callus and *Not*I-linking clones were developed by the Rice Genome Research Programme of Japan (Kurata et al. 1994). In addition, we also used a PCR-based single-copy DNA marker linked to *Gm4t* as described in Nair et al. 1996.

PCR-based DNA marker

The DNA marker, $E20_{570}$, linked to the *Gm4t* gene, was obtained from genomic DNA of 'Abhaya' by screening RAPD primers in conjunction with bulked-segregant analysis as described in Nair et al. (1996).

Computing

The MAPMAKER Macintosh Version 1.0 program (E.I. duPont de Nemours and Company, Copyright 1990), based on the MAPMAKER program of Lander et al. (1987), was used for the analysis of segregation data.

Chromosome nomenclature

The numbering system used in this paper is that of Causse et al. (1994) and Kurata et al. (1994).

Results

Genetic analysis of gall midge resistance

It has been shown previously that 'Abhaya' is resistant to many biotypes of gall midge and that the resistance in 'Abhaya' is controlled by a single dominant gene (Srivastava et al. 1994). The resistance in 'Abhaya' has been introgressed from Ptb10 through CR157-392. 'Abhaya' is a cross between CR157-392 and OR57-21. Ptb10, CR157-392 and 'Abhaya' are resistant to all four biotypes of gall midge (Kalode et al. 1993; Rao and Kandalkar 1992). 'Shyamala' is purple-pigmented and susceptible to all four biotypes. It is a cross between two susceptible lines, namely, R60-2713 and R2385.

Table 1 shows the reaction of the rice varieties studied to the different biotypes of gall midge in India. Table 2 summarizes F_2 segregation data for resistance against gall midge in many crosses featuring resistant variety 'Phalguna' containing the *Gm2* gene, which confers resistance against biotypes 1 and 2 (Mohan et al. 1994), and 'Abhaya' containing *Gm4t* gene, which confers resistance against biotypes 1, 2, 3, and 4 (Srivastava et al. 1994). *Gm2* gene in 'Phalguna' has been introgressed from 'Siam 29' (Sastry et al. 1984). The F_2 populations from the crosses of 'Abhaya' or 'Phalguna' with any susceptible variety of rice segregated in the ratio of 3 resistant to 1 susceptible, indicating the presence of a single dominant gene conferring resistance in these varieties. In contrast, the analysis of the F_2 populations from a cross 'Abhaya' × 'Phalguna' segregated in a ratio of 15 resistant to 1 susceptible. This shows that the resistance gene in 'Abhaya' is nonallelic to the other resistance gene, *Gm2*, in 'Phalguna'. It also indicates that *Gm2* or *Gm4t* alone can confer resistance to the biotype used in the current screening. The resistance gene in 'Abhaya' has been designated as *Gm4t* by Srivastava et al. (1994).

Table 1 Reactions of some rice varieties to gall mide[a] (*R* resistant; *S* susceptible)

Variety	Biotypes			
	1	2	3	4
Tulsi	S	S	S	S
ARC6650	S	S	S	S
Shyamala	S	S	S	S
TN1	S	S	S	S
Phalguna	R	R	S	S
Abhaya	R	R	R	R

[a] After Srivastava et al. (1994); Rao and Kandalkar (1992); Mohan et al. (1994)

Mapping population

RFLP mapping of the *Gm4t* gene was performed on 184 F_3 plants derived from an 'Abhaya' × 'Shyamala' cross. As mentioned earlier, 'Abhaya' is resistant to the four biotypes of gall midge and 'Shyamala' is susceptible to all the known biotypes in India. The fact that both parents are *indica* varieties severely reduced the frequency of useful DNA polymorphisms. Out of over 100 cloned probes tested, only 30 (30%) showed RFLPs with a set of 35 restriction enzymes. We could map only 22 out of 30 polymorphic markers. The remaining 8 markers could not be mapped as the polymorphic bands were too close to be scored unambiguously.

RFLP analysis

All 184 F_3 lines in the mapping population showed distinct scores for resistance or susceptibility to biotype 1 of gall midge in the field trials. The level of infestation was very high as the susceptible parents showed 100% infestation on a single-plant basis. Molecular segregation data for the 'Abhaya' × 'Shyamala', and 'Nipponbare' × 'Kasalath' alleles for all polymorphic RFLP markers were obtained for each line in the F_3 and F_2 progeny, respectively. $E20_{570}$ showed polymorphic bands, 12.4 kb in 'Nipponbare' and 2.6 kb in 'Kasalath', when the respective genomic DNAs were restricted with *Bam*HI. Analysis of the segregation data with the MAPMAKER program (Lander et al. 1987) revealed that the resistance-specific PCR- based DNA marker, $E20_{570}$, mapped onto chromosome 8 and segregated very closely with 4 RFLP markers, C483, S1633B, R1813 and G1010. $E20_{570}$ was calculated to be 0.4 cM from R1813 on one side and 1.4 cM from S1633B on the other side of the chromosome (Fig. 1).

Table 2 Genetic analysis of gall midge resistance in rice (*R* resistant; *S* susceptible)

Cross	F_1 reaction	Plants		F_2 reaction	χ^2
		R	S		
ARC6650 × Phalguna	R	73	25	3R:1S	0.013[a]
Abhaya × Tulsi	R	584	191	3R:1S	0.052
Abhaya × Shyamala	R	340	93	3R:1S	2.856
Abhaya × Phalguna	R	510	36	15R:1S	0.110

[a] Mohan et al. (1994)

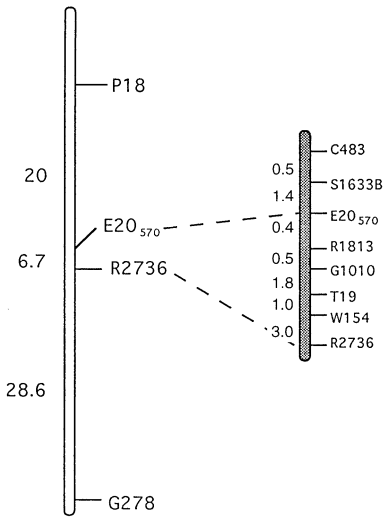

Fig. 1 Linkage map of a portion of chromosome 8. Mapping done on a F_2 population derived from a cross between 'Nipponbare' and 'Kasalath'. RFLP markers linked to the resistance-specific PCR-based marker $E20_{570}$ are shown. Figures to the *left* of the respective maps are map distances in centiMorgans. *Dotted lines* link similar RFLP markers. *Shaded portion* shows relative position of RFLP markers, as mapped by Kurata et al. 1994, around $E20_{570}$. The distances are not to scale

Discussion

We report here the mapping of the *Gm4t* gene in rice, which confers resistance against many biotypes of gall midge including biotype 1. The resistance against biotype 1 is also provided by another gene, *Gm2*. We previously mapped the *Gm2* gene in another rice variety, 'Phalguna', onto chromosome 4 (Mohan et al. 1994). *Gm4t* has now been mapped onto chromosome 8. *Gm2* in 'Phalguna' and *Gm4t* in 'Abhaya' have also been found to be nonallelic (Srivastava et al. 1994). It is interesting to know that genes conferring resistance to different biotypes of an insect have been located on different chromosomes. Similar situations have been reported with resistance genes against bacterial leaf blight resistance genes in rice (Yoshimura et al. 1992), Hessian fly in wheat (Ma et al. 1993), powdery mildew resistance genes in wheat (Hartl et al. 1995) and nematode resistance genes in tomato (Yaghoobi et al. 1995). However, in some instances it has also been observed that different disease resistance genes conferring race specificities are located in clusters (Sudupak et al. 1993; Jahoor and Fischbeck 1993).

Currently, we are unable to demonstrate whether *Gm4t* is also involved in conferring resistance against other biotypes (as 'Abhaya' is resistant to biotypes 1, 2, 3 and 4), or if separate genes are involved in conferring resistance to the other biotypes. To illustrate this one needs to screen the same population of F_3 plants against other biotypes either in the field in different geographical locations of India where the different biotypes are naturally found or in the greenhouse where a high population pressure of these biotypes could be built up. We found it very difficult to conduct the screening because of technical difficulties in transferring the F_3 population to different locations. Besides, the natural occurrence of gall midge is more or less restricted to the same period all over India. It has also been shown that when 'Abhaya' is crossed with many susceptible rice varieties, the progeny, when exposed to other biotypes, showed the presence of a single dominant gene (Kalode et al. 1993; Rao and Kandalkar 1992).

Further work will be required to determine whether resistance against biotypes 2, 3 and 4 is also conferred by the *Gm4t* gene. We conclude that, based on our results, it is certain that *Gm4t* in 'Abhaya' confers resistance to biotype 1 and that in all probability the same gene confers resistance to other biotypes as well and is different from *Gm2*, which also confers resistance to the same biotype. Map-based cloning of the *Gm2* and *Gm4t* genes would help us to study the biochemical basis of gall midge resistance and biotype specificity.

The mapping of *Gm4t* was difficult as we were not able to score many RFLP markers close to it. This was because of the lack of polymorphism between the *indica* parents. We were also not able to find polymorphism in the amplification of resistance-specific bands using resistance and susceptible specific primers in a PCR reaction from the 'Abhaya' and 'Shyamala' varieties of rice. However, as shown earlier (Nair et al. 1996) we could get amplification fragment length polymorphism (AFLP) from genomic DNAs of 'Abhaya' and 'Tulsi'. 'Tulsi' is a susceptible variety like 'Shyamala', but we did not have a mapping population of desired size from 'Abhaya' and 'Tulsi' and, therefore, it was not possible to map AFLP bands obtained from genomic DNAs of 'Abhaya' and 'Tulsi' in either of the two populations mentioned above. However, it was possible to map the resistance-specific marker $E20_{570}$, amplified from genomic DNA of 'Abhaya', in a different F_2 population consisting of 186 plants between a *japonica* variety 'Nipponbare' and *indica* variety 'Kasalath'.

The resistance-specific fragment, $E20_{570}$, mapped onto chromosome 8 in the 'Nipponbare' and 'Kasalath' population and thereby confirmed the position of the *Gm4t* gene. Nair et al. (1996) had previously shown that $E20_{570}$ is located very close to *Gm4t* gene based on its segregation in F_3 lines of 'Abhaya' × 'Tulsi'. Using the mapping information derived from the 'Nipponbare' × 'Kasalath' population we tried mapping the RFLP markers from chromosome 8 onto the 'Abhaya' × 'Shyamala' population. However, marker R2736, which is only 6.7 cM from the resistance-specific marker, $E20_{570}$, in the 'Nipponbare' × 'Kasalath' population is located 27.2 cM (data not shown) away from the *Gm4t* gene in the 'Abhaya' × 'Shyamala' population (Fig. 1). This is due to the fact that genetic distances often vary

from cross to cross with different recombination frequencies in different mapping populations (Stevens et al. 1995). Alternatively, these distances are not true distances as the region has not been saturated with more markers and it could be that *Gm4t* is located very close to resistance-specific marker, $E20_{570}$, on chromosome 8. This view is, in fact, supported by our tagging data (Nair et al. 1996).

Having mapped and tagged *Gm2* gene in 'Phalguna' (Mohan et al. 1994; Nair et al. 1995) along with developing markers for the marker-assisted selection for *Gm4t* gene (Nair et al. 1996), it will now be easier to pyramid resistance genes in an elite cultivar. The strategy of marker-assisted selection accelerates breeding for pyramiding resistance genes and for optimizing the durability of resistance genes through the combination of many resistance factors. The selection of genotypes carrying two or more gall midge resistance genes using traditional host-pest interactions is very time-consuming and often not possible due to a lack of different biotypes in one geographical location at a given time. The development of molecular markers that are closely linked with the respective resistance genes, therefore, is essential for the selection of such genes and gene combinations in marker-assisted selection programs. This is currently being pursued using PCR-based markers linked to *Gm2* and *Gm4t* (Nair et al. 1995, 1996).

Molecular markers such as RFLPs, RAPDs and microsatellites are the best alternatives to conventional disease screening. Apart from being used in a marker-assisted breeding program, these molecular markers offer a great opportunity in understanding the relationship between resistance genes and the origin and mechanism of resistance (Hartl et al. 1995). Also, closely placed markers will prove useful in the map-based gene cloning of these resistance genes in rice.

This alternative strategy of mapping genes is the first report of its kind, and our method presents an alternative way of mapping genes which are otherwise difficult to map because of the lack of polymorphism between parents differing in desired phenotypes.

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