Theor Appl Genet (2001) 103:953–961

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Molecular mapping of gene *Gm-6*(*t*) which confers resistance against four biotypes of Asian rice gall midge in China

Received: 18 May 2000 / Accepted: 9 March 2001

Abstract The Chinese rice cultivar Duokang #1 carries a single dominant gene *Gm-6*(*t*) that confers resistance to the four biotypes of Asian rice gall midge (*Orseolia oryzae* Wood-Mason) known in China. Bulked segregant analysis was performed on progeny of a cross between Duokang #1 and the gall midge-susceptible cultivar Feng Yin Zhan using the RAPD method. The RAPD marker $OPM06_{(1400)}$ amplified a locus linked to *Gm-6*(*t*). The locus was subsequently mapped to rice chromosome 4 in a region flanked by cloned RFLP markers RG214 and RG163. Fine mapping of *Gm-6*(*t*) revealed that markers RG214 and RG476 flanked the gene at distances of 1.0 and 2.3 cM, respectively. Another gall midge resistance gene, *Gm-2*, mapped previously to chromosome 4, is located about 16 cM from *Gm-6*(*t*), to judge by data from a segregating population derived from a cross between Duokang #1 and the Indian cultivar Phalguna that carries *Gm-2*. We developed a PCR-based marker-assisted selection kit for transfer of the *Gm-6*(*t*) gene into Ming Hui 63 and IR50404, two parental lines commonly used in hybrid rice production in China. The kit contains PCR primer pairs based on the terminal sequences of the RG214 and RG476 clones. Polymorphism between Duokang #1 and the hybrid parental lines was found at these markers after digestion of the PCR products with specific restriction endonucleases. The kit will accelerate introduction of gall midge resistance into hybrid rice in China.

Keywords Rice (*Oryza sativa*) · Gall midge · *Orseolia oryzae* · RAPD · Mapping · Sequence tagged sites

Communicated by P. Langridge

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Introduction

Asian rice gall midge (*Orseolia oryzae* Wood-Mason) is a significant pest in China, India, Sri Lanka and several other countries (Lai et al. 1984; Kudagamage and Gunawardena 1989; Katiyar et al. 1995). Neonate larvae emerge from eggs laid on leaf blades and move to the shoot meristem where they feed from, and kill, the shoot meristem. The tiller fails to produce a panicle and forms instead a cylindrical gall that feeds and protects the insect until eclosion of the adult. Rice plants have little capacity to compensate for this loss of tillers.

Host plant resistance provides an efficient, economical and safe means of crop protection against gall midge (Heinrichs 1994). Seven characterized resistance genes and numerous other uncharacterized genes are used in rice-improvement programs in Asia (Katiyar et al. 2000). Resistant cultivars, usually containing a single gene for resistance, have been widely deployed in China, India and Sri Lanka. Scientists in other gall midge-affected countries such as Cambodia, Laos, Thailand and Vietnam are now attempting to identify suitable resistance genes for deployment against local biotypes. Hybrid rice in China and India and IRRI's New Plant Types for the irrigated and rainfed lowland ecosystems (Khush 1995) are also susceptible to gall midge and must be protected from this insect. Resistance-breaking biotypes of rice gall midge have emerged in India, China and Sri Lanka by mutation or migration, and have come to dominate local gall midge populations (Katiyar et al. 2001). Breeding programs must discover additional resistance genes and deploy them in pyramids or rotations that will be

less-readily overcome than individual genes released sequentially (Cohen et al. 2001).

Breeding for gall midge resistance has, in the past, relied on field screening in endemic areas where climatic and agricultural conditions provide high insect pressure. Particularly conducive to the development of an epidemic are such factors as continuous high humidity, the overlap of rice growing seasons in a locality, and the presence of alternate hosts in the form of grasses such as *Leersia hexandra*. When these conditions are not met, breeding for gall midge resistance may be either delayed or rendered prohibitively expensive because of the need to conduct phenotyping in greenhouses or at distant locations. It is in this context that DNA marker-assisted selection (MAS) becomes an attractive option for improving gall midge resistance. MAS increases the efficiency of resistance breeding by allowing selection to be conducted when the insect pressure is low (e.g., in relatively dry seasons). It also permits selection in locations where the gall midge is absent or where the prevalent biotype differs from that in the target environments. Finally, MAS is ideal for pyramiding genes to achieve more-durable resistance against a specific biotype or broad resistance against several biotypes. MAS has been used at IRRI for pyramiding genes for bacterial blight resistance (Huang et al. 1997).

To be adopted, MAS must be reliable, convenient, cost-effective and capable of dealing with the number of plants that breeders wish to screen in the time available. Although MAS is reliable when based on restriction fragment length polymorphism (RFLP) analysis, it fails to meet the other three criteria. MAS based on the polymerase chain reaction (PCR) is more likely to satisfy all of the above criteria. However, as it relies on finding polymorphisms in a smaller stretch of DNA than RFLP-based MAS (<2 kbp compared with <50 kbp), PCR-based MAS may require a greater effort to find flanking markers that are polymorphic between the resistant donor and the recipient lines. Thus, identification of suitable polymorphic flanking markers is an integral component for the development of a PCR-based MAS kit.

We report here the tagging and mapping of the *Gm-6*(*t*) gene for gall midge resistance in China and the development of a PCR-based MAS kit suitable for transferring the gene to Ming Hui 63 and IR50404, two parental lines commonly used in hybrid rice production. Ming Hui 63 and IR50404 are restorer lines used in three-line and two-line hybrid production, respectively. Four biotypes of gall midge are recognized in southern China (Huang et al. 2001). Several sources of resistance are known against bioypes 1–3, but biotype 4, the most recently identified variant, was found to be virulent against all commonly used gall midge differentials (W1263, OB677, Ptb21, Siam 29 and Leuang 152) in Mexian, Wuhua, Fenkai and Xinyi regions of China (Lai et al. 1984; Tan et al. 1993). Recently, a new gall midge resistance gene, *Gm-6*(*t*), that confers resistance against all four Chinese biotypes, was identified in a Chinese germplasm line, Daqiuqi, and the derived line Duokang #1 (Tan et al. 1993). This dominant gene is being used in breeding programs in Guangdong province (Huang et al. 2000) as the only source of resistances against biotype 4. At present no hybrid rice in China carries gall midge resistance. To facilitate the transfer of *Gm-6*(*t*) to hybrid rice, we developed a PCR-based MAS kit with two closely flanking markers that are polymorphic between Duokang #1 and both Ming Hui 63 and IR50404.

Materials and methods

Gall midge biotypes, rice cultivars and screening protocol

This study employed two gall midge biotypes. Chinese biotype 4 was recovered from field-grown hybrid rice plants in the Yangshan district and was maintained on susceptible cultivar Feng Yin Zhang in an outdoor chamber walled with fine plastic mesh at the Plant Protection Institute of the Guangdong Academy of Agricultural Sciences (GDAAS) in Guangzhou. Indian biotype 1 was recovered from field-grown plants in the Raipur district and was maintained on susceptible cultivar TN1 in the field and in a glass house at the Indira Gandhi Agricultural University (IGAU) in Raipur. Two other susceptible cultivars, Ming Hui 63 and IR50404, were also used in the study, along with two resistant cultivars: Duokang #1 carries the *Gm-6*(*t*) gene for resistance against Chinese biotypes 1, 2, 3 and 4, whereas Phalguna carries the *Gm2* gene for resistance to Indian biotypes 1 and 2 (Mohan et al. 1994; Rajyashri et al. 1998).

Two populations segregating for gall midge resistance were studied. One population consisted of 160 F_3 lines derived from a cross between Duokang #1 and Feng Yin Zhan. This population was used for RAPD tagging and fine-mapping of the *Gm-6*(*t*) gene. Lines were assessed for resistance to Chinese biotype 4 at GDAAS. Rows of at least 20 plants from each line were sown in seedbeds in a mesh-covered chamber. Rows of resistant and susceptible parents were located after every 20 lines. Seedlings were infested 7 days after sowing, and reactions were assessed 25 days later, using the Standard Evaluation System (Anonymous 1996). Seeds from 160 F_3 lines of this cross were also grown at IRRI and the leaves of 6-week-old plants were harvested for DNA extraction.

The second segregating population consisted of 417 F_3 families derived from a cross between Duokang #1 [donor of *Gm-6*(*t*)) and Phalguna (donor of *Gm-2*)]. This population was used to study the allelic relationship between *Gm-6*(*t*) and *Gm-2*. The F_3 seeds harvested from 417 individual F_2 plants were divided into two sets (approximately 60 seeds per set per F_3 family) and screened at GDAAS and IGAU, respectively. At GDAAS, infestation with Chinese biotype 4 was conducted as described above. At IGAU, seedlings sown in trays were infested with Indian biotype 1 at 7 days after sowing. Reactions were evaluated 21 days later, when 100% of plants of Duokang #1 showed silver shoots. Again, the Standard Evaluation System of IRRI was employed (Anonymous 1996).

In addition, we used a doubled-haploid population of 113 lines derived from the cross IR64×Azucena (Guiderdoni et al. 1992). This population is commonly employed at the International Rice Research Institute and elsewhere to map isolated genes or DNA markers (Huang et al. 1994). We used the population to map a RAPD marker $OPM06_{(1400)}$ that showed linkage to *Gm-6*(*t*) gene (see below). This population was not evaluated for gall midge resistance.

DNA isolation

Total rice genomic DNA was extracted from 6-week-old leaves of the Duokang #1/Feng Yin Zhan mapping population by the method described by Tai and Tanksley (1990). The purity and concen-

tration of DNA extracted from individual lines was assessed by spectrophotometry. The acceptable absorbance ratio (A_{260}/A_{280}) was 1.8, and an absorbance at 260 nm of 1 was taken to correspond to 50 µg of double-stranded DNA/ml.

RAPD bulk segregant analysis

After the Duokang #1/Feng Yin Zhan F_3 population had been scored for gall midge resistance (see above), lines homozygous for resistance or homozygous for susceptibility were identified and used for DNA extraction. DNA aliquots (2 µg) from each of 12 resistant lines and each of 12 susceptible lines were pooled to make resistant and susceptible bulks, respectively (Michaelmore et al. 1991). These two bulk DNA preparations were used as target DNAs for RAPD analysis along with DNAs from the parental cultivars, Duokang #1 and Feng Yin Zhan. The bulked DNA samples and the parental DNA samples were used to identify co-segregating molecular markers amplified using arbitrary decanucleotide primers obtained from Operon Technologies Inc. (Almada, Calif.). The amplification reaction conditions were as described by Williams et al. (1990) with minor modifications as follows. DNA samples (5 µl) containing approximately 100 ng of genomic DNA template were used in a 25-µl reaction that contained 0.5 units of *Taq* polymerase, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatine, 100 µM each of dATP, dCTP, dGTP and dTTP, and a single dodecamer primer (5 pmol). The mixtures were overlaid with 25 µl of mineral oil before amplification. Amplification was carried out for 45 cycles, each of 1 min at 94°C, 1 min at 34°C and 2 min at 72°C, using the fastest available ramp in a Perkin-Elmer Cetus thermal cycler model 480. Amplification products were resolved by electrophoresis on a 1.5% agarose gel in 1× TAE buffer, stained with ethidium bromide and visualized with UV illumination. Where bands of interest were observed, replicate PCR was performed to confirm amplification patterns. A 1400-bp band amplified with primer OPM06 was produced from DNA of the resistant parent and the resistant bulk but was absent from DNA of the susceptible parent and the susceptible bulk. This RAPD marker was studied in detail.

Mapping and fine-mapping of the *Gm-6*(*t*) gene

The location of RAPD marker $OPM06_{(1400)}$ on the rice genetic map was determined by RFLP analysis with doubled-haploid (DH) lines derived from the cross IR64×Azucena, as described by Huang et al. (1994). $OPM06_{(1400)}$ was excised from the agarose gel, purified using the Gene-Clean kit (Qiagen Inc.), re-amplified with primer OPM06, and radioactively labeled by the random primer method (Feinberg and Vogelstein 1983). The probe was hybridized to membranes containing *Hin*dIII digests of DNA extracted from 113 DH lines and the two parental lines, and detected a clear RFLP between the parental DNAs. The pattern of segregation of the RFLP among the DH lines was analyzed using the MAPMAKER program (Lander et al. 1987) in conjunction with a database of 175 RFLP markers, most of which had previously been mapped by Huang et al. (1994). $OPM06_{(1400)}$ was found to reside between RG214 and RG163 on chromosome 4.

To fine-map *Gm-6*(*t*), the surrounding RFLP markers RG214, RG476 and RG329 on chromosome 4 were converted into sequence tagged sites (STSs) as described by Robeniol et al. (1996). The sequences of the STS primers are given in Table 1. PCR-amplification of the genomic DNA of Duokang #1 and Feng Yin Zhan with the STS primers was carried out in 25 µl of the same reaction mix as for the RAPD amplification except that STS primers were used. The PCR conditions, which optimized STS amplification, include a hot start at 94°C for 2 min, 40 cycles of 94°C denaturing for 1 min; 60°C annealing for 1 min and 72°C extension for 2 min; and a 7-min extension at 72°C. To detect polymorphisms between the two parents at the three marker loci, the PCR products were digested at the appropriate temperature with a panel of restriction endonucleases. The enzymes recognized sites containing either four base pairs (A*lu*I, D*pn*I, H*ae*III, H*ha*I, H*pa*II, M*bo*I, M*sp*I, M*vn*I, R*sa*I, S*au*3A, S*pe*I and T*aq*I), five base pairs (B*st*NI, E*co*RII and H*in*fI) or six base pairs (B*am*HI, B*gl*II, D*ra*I, E*co*RI, E*co*RV, H*inc*II, H*ind*III, P*st*I, S*ca*I, X*ba*I and X*ho*I). The digestion products were analyzed on 1.5% agarose gels in 1× TAE buffer. Polymorphisms were detected with *Alu*I for RG476 and RG329 but no polymorphism was detected for RG214. Allelic segregation at RG476 and RG329 in the Duokang #1/Feng Yin Zhan mapping population was analyzed by *Alu*I digestion of PCR products amplified from DNA of F_3 individuals, followed by electrophoresis on 1.5% agarose gels in 1× TAE buffer.

Table 1 Primer sequences for STS loci. F: forward primer, R: reverse primer

Locus	Primer sequence
RG214	F: 5′ GGT AGA CAC GCG GGC GAG GTT G 3′ R: 5′ CAC GCT CAA TCC AGG TGG ACA C 3′
RG476	F: 5′ GAT GGC AAG CCA ATC AGA TCG 3′ R: 5′ GAA GTG AGG AAG CCT ACA GTA AGCC 3′
RG329	F: 5′ GCT GCA TTA GTC CAC AAC 3′ R: 5′ AAT CTC TCA CAC CCA AGG C 3′

Linkage analysis

For the Duokang#1× Feng Yin Zhan population, full multipoint linkage analysis for the segregating polymorphic markers and the gall midge resistance phenotypic data was conducted using MAPMAKER V. 3.0 (Lander et al. 1987). All map distances (centiMorgans, cM) are reported in Kosambi units (Kosambi 1944). The RFLP and AFLP loci were mapped using MAPMAKER, with a threshold logarithm of odds (LOD) score of 3.0. Linkage groups were based on the reference molecular map of rice (Huang et al. 1994), but marker order and distances were obtained using the F_2 segregating algorithm in the MAPMAKER program based on the DNA data for the F2 generation and the phenotypic data for the F_3 families. For the Duokang#1× Phalguna population, the segregation of alleles into genotypic classes at the *Gm-6*(*t*) and *Gm-2* loci was checked against the expected 1:2:1 ratio for an F_3 segregating population using a χ^2-test with a significance level of 5%.

Results

Inheritance of gall midge resistance

Duokang #1 and its parent Daiqiuqi carry *Gm-6*(*t*), the only gene known to give resistance to Chinese biotype 4 of the Asian rice gall midge (Tan et al. 1993). To map *Gm-6*(*t*), Duokang #1 was crossed with the susceptible line Feng Yin Zhan and generations F_1–F_3 were prepared. The susceptible parent Feng Yin Zhan showed galls on 100% of the plants, whereas galls failed to develop on plants of Duokang #1. F_1 plants were also free of galls. Phenotyping of F_3 progeny lines identified the genotypes of the F_2 plants at the *Gm-6*(*t*) locus. The ratio of homozygous resistant: heterozygous segregating: homozygous susceptible plants in the F_2 generation was the 1:2:1 expected for segregation of a single dominant gene (in χ^2–test, $P>0.75$).

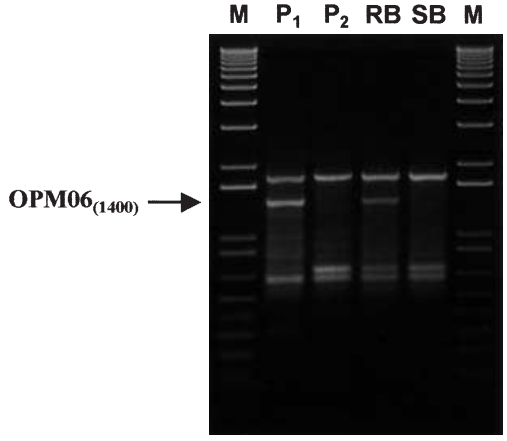

Fig. 1 Identification of a RAPD marker for the rice *Gm-6*(*t*) gene using bulked segregant analysis. The RAPD primer was OPM06. *M* molecular-weight markers, *P1* resistant parent (Duokang #1), *P2* susceptible parent (Feng Yin Zhan), *RB* resistant bulk, and *SB* susceptible bulk. Bulks were constructed from DNA extracted from 12 homozygous resistant and 12 homozygous susceptible F_3 lines from the cross Duokang #1× Feng Yin Zhan. *Arrow* marker $OPM06_{(1400)}$, linked with gall midge resistance

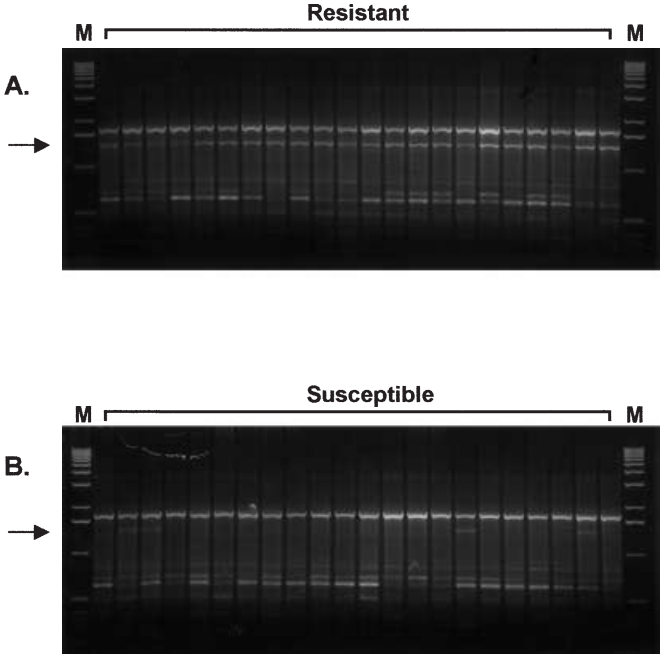

Fig. 2A, B Co-segregation analysis confirming linkage of the $OPM06_{(1400)}$ marker with the *Gm-6*(*t*) gene. Mapping population: F_3 lines from the cross Duokang #1× Feng Yin Zhan. **A** 22 homozygous resistant lines. **B** 22 homozygous susceptible lines. *Arrow* RAPD marker $OPM06_{(1400)}$ linked to resistance. *M* molecular weight markers

Tagging of the *Gm-6*(*t*) gene

DNA was extracted from leaves of the homozygous resistant and homozygous susceptible F_2 plants of the Duokang #1/Feng Yin Zhan population and from the two parents. DNA samples from 12 homozygous resistant plants and from 12 homozygous susceptible plants were bulked and amplified with 150 random 10-mer primers to find a polymorphic marker linked to the *Gm-6*(*t*) gene. On average, each primer amplified about five bands that ranged in size from 300 to 2000 bp. One primer (OPM06, 5′-CTGGGCAACT-3′) generated a 1.4-kb DNA band (designated as $OPM06_{(1400)}$) that was amplified from DNA of Duokang #1 and the resistant bulk, but was not amplified from DNA of Feng Yin Zhan and the susceptible bulk (Fig. 1). When OPM06 was used to amplify DNA from the individual plants constituting the bulks, $OPM06_{(1400)}$ appeared for all 12 resistant plants and for none of the susceptible lines (data not shown), suggesting close linkage between the RAPD marker and the gene *Gm-6*(*t*). When we extended this analysis to 22 homozygous resistant plants (no galls visible) and 22 homozygous susceptible lines (91–100% of plants infested), $OPM06_{(1400)}$ appeared for all resistant plants and four of the 22 susceptible plants (Fig. 2). The four susceptible plants positive for the RAPD marker presumably experienced recombination between *Gm-6*(*t*) and the marker locus. As single-recombination events are more likely than double-recombination events, these four plants are probably heterozygous rather than homozygous for the presence of $OPM06_{(1400)}$. Thus, four recombination events out of the 44 informative meiotic events contributing to the 22 susceptible lines indicate that *Gm-6*(*t*) and the locus defined by $OPM06_{(1400)}$ are about 9 cM apart on the rice genome. The presence of $OPM06_{(1400)}$ for all 22 homozygous resistant plants also argues against the occurrence of double-recombination events.

Mapping and fine mapping

Band $OPM06_{(1400)}$ was used to probe a gel blot of *Hin*dIII-digested DNA from 135 doubled-haploid lines from a cross between IR64 and Azucena (Huang et al. 1994). These lines are a standard segregating population used for mapping isolated genes, RAPD markers and other genomic fragments. Analysis of the hybridization data using the MAPMAKER program indicated that the locus hybridizing to $OPM06_{(1400)}$ maps to the region between markers RG214 and RG163 on chromosome 4 (Fig. 3A). The probe hybridized closer to RG214 (7 cM) than to RG163 (15 cM).

Three STS markers (RG214, RG476 and RG329) were available within the RG214-RG163 interval (Robeniol et al. 1996). The sequences of the corresponding primers are given in Table 1. The primer pair for RG476 yielded a 700-bp PCR product from the DNA of both Duokang #1 and Feng Yin Zhan. *Alu*I digestion of these products revealed a clear polymorphism: cleavage of the Duokang #1 amplicon to fragments of 600 kb and 100 kb, but no cleavage for the Feng Yin Zhan amplicon (Fig. 4). Analysis of this polymorphism in ten resistant lines and ten susceptible lines of the Duokang #1/Feng Yin Zhan segregating population confirmed close linkage between

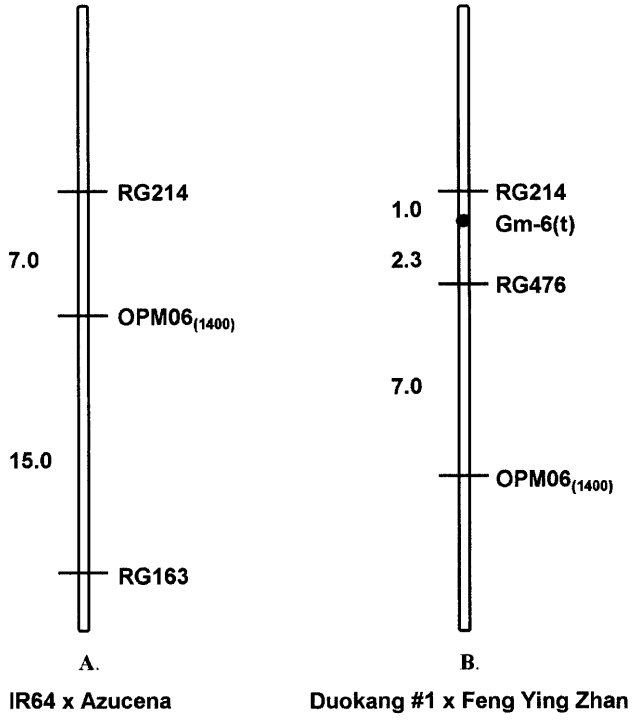

Fig. 3A, B Two-step mapping of *Gm-6*(*t*) to chromosome 4 of rice. **A** The IR64×Azucena population was used to establish that $OPM06_{(1400)}$, a RAPD marker for *Gm-6*(*t*), maps to a site between markers RG214 and RG163. **B** The Duokang #1× Feng Yin Zhan population allowed fine-mapping of *Gm-6*(*t*) to a location between RG214 and RG476. *Figures* to the left of the maps are genetic recombination distances (in cM). LOD scores were above the threshold of 3.0

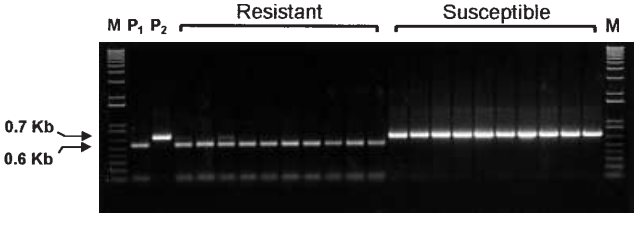

Fig. 4 Co-segregation analysis of marker RG476 with *Gm-6*(*t*) using PCR. Products amplified by RG476-specific primers were restricted with A*lu*I to reveal polymorphism between the resistant parent (P_1= Duokang #1) and the susceptible parent (P_2=Feng Yin Zhan). The ten homozygous resistant and ten homozygous susceptible lines were F_3 lines from the cross Duokang #1× Feng Yin Zhan. *M* molecular-weight markers

Gm-6(*t*) and this region of chromosome 4 (Fig. 4). When this protocol was extended to 152 of 160 F_3 lines, MAPMAKER analysis indicated that RG476 and *Gm-6*(*t*) are about 2.3 cM apart (Fig. 3B). We have not yet been able to find a restriction endonuclease giving PCR-based polymorphism between STS amplicons of Duokang #1 and Feng Yin Zhan at locus RG214. We therefore resorted to RFLP analysis of this locus using gel blots of DNA digested with *Hind*III (Fig. 5). The results showed co-segregation of RG214 and *Gm-6*(*t*) for 12 resistant and 12 susceptible lines. When the analysis

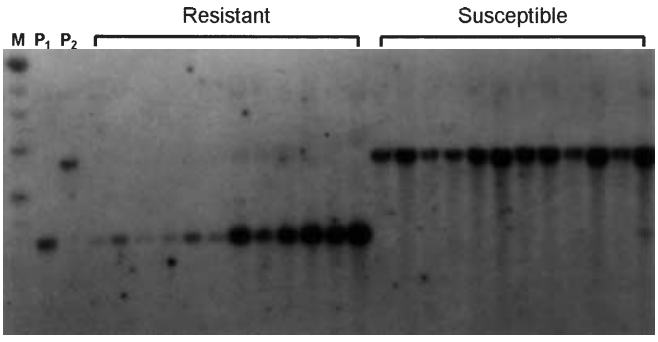

Fig. 5 Co-segregation analysis of marker RG214 with *Gm-6*(*t*) using RFLP analysis. DNA was extracted from the resistant parent (P_1=Duokang #1) and the susceptible parent (P_2=Feng Yin Zhan), together with 12 homozygous resistant and 12 homozygous susceptible F_3 lines from the cross Duokang #1× Feng Yin Zhan. The DNA was digested with *Hind*III and probed with a ^{32}P-labeled clone of marker RG214. *M* molecular-weight markers

was extended to the 106 F_3 lines, the map distance between RG214 and *Gm-6*(*t*) was calculated to be about 1.0 cM (Fig. 3B). We conclude that the *Gm-6*(*t*) gene is located on the long arm of chromosome 4 flanked closely by RG214 and RG476.

Allelic relationship of *Gm-6*(*t*) with *Gm-2*

Another gall midge resistance gene, *Gm-2*, identified in cultivars Siam 29 and Phalguna, has also been mapped to the RG214-RG476 interval (Mohan et al. 1994; Rajyashri et al. 1998). To examine the allelic relationship between *Gm2* and *Gm-6(t)* we conducted segregation analysis of a Duokang #1/Phalguna mapping population of 417 F_3 lines (Table 3). Phenotyping was done on F_3 plants in Guangdong using Chinese biotype 4 and in Raipur using Indian biotype 1. As both resistance genes are dominant, Duokang #1 may be represented as *gm2gm2*/*Gm6Gm6* and Phalguna as *Gm2Gm2*/*gm6gm6*. None of the F_2 plants was homozygous for resistance to both biotypes, so there was no double recombinant of the type *Gm2Gm2*/*Gm6Gm6*. There was only one double-recombinant of the type *gm2gm2*/*gm6gm6*, homozygous for susceptibility to both biotypes. However, many single recombinants of the types *Gm2Gm2*/*Gm6gm6* and *Gm2gm2*/*Gm6Gm6* were detected on the basis of phenotyping data collected on the F_3 generation. From the segregation data, we calculated the distance between *Gm-2* and *Gm-6*(*t*) to be about 16.2±3 cM using the method described by Allard (1956). Thus, these two genes are not allelic.

Development of PCR-based markers and of this MAS kits

Although no PCR-based polymorphism was found between Duokang #1 and Feng Yin Zhan at locus RG214, there was no such difficulty with Duokang #1 and Ming Hui 63 and IR50404, two gall midge-susceptible parental

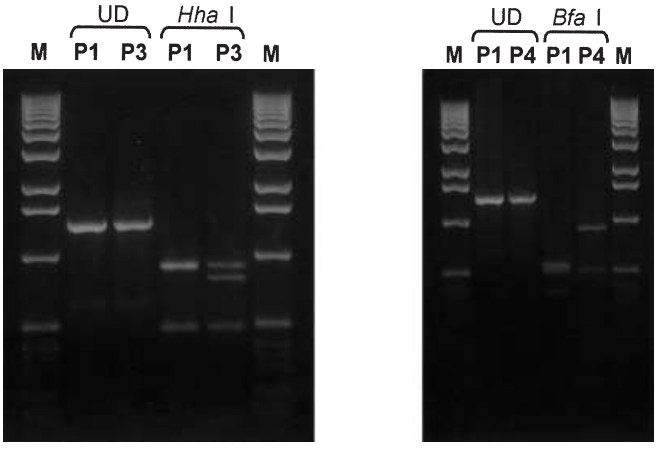

Fig. 6 PCR-based marker-assisted selection kits for the transfer of *Gm-6*(*t*) to hybrid rice parental lines IR50404 and Ming Hui 63 using marker RG214. *P1* Duokang #1. *P3* IR 50404. *P4* Ming Hui 63. *UD* undigested STS product. *Hha*I, *Bfa*I PCR products digested with respective restriction endonucleases

lines used in hybrid rice production in China (Fig. 6). A polymorphism between the amplicons of Duokang #1 (P1) and IR50404 (P3) was revealed by digestion with restriction enzyme *Hha*I, while polymorphism between the amplicons of Duokang #1 and Ming Hui 63 (P4) was revealed by digestion with *Bfa*I. At locus RG476, polymorphism between Duokang #1 and the two A lines was revealed using A*lu*I and *Sca*I, respectively (data not shown). Two MAS kits for PCR-based transfer of the *Gm-6*(*t*) gene into hybrid rice were prepared using these data.

Discussion

DNA marker-assisted selection

Our data establish that *Gm-6*(*t*) is located in the short interval between markers RG214 and RG476 on chromosome 4. These markers may now be used in MAS for gall midge resistance. The fact that they are located 1.0 and 2.3 cM, respectively, from *Gm-6*(*t*) suggests that used separately they will allow the correct genotype to be predicted in all but 1% and 2.3% of assays, respectively. However, if the two markers are used in combination, the error should fall to 2.3 in 10^4, allowing correct prediction of the genotype at the *Gm-6*(*t*) locus in >99.97% of assays.

PCR provides the most convenient protocol for MAS. Compared with RFLP analysis, it is a cheaper and faster method for analyzing each line and thus more readily applied to populations of the size used by breeders. It is clearer and more robust then the RAPD analysis, which amplifies more bands and uses less-stringent annealing temperatures and less-specific primers. PCR-based MAS kits have been developed for transferring *Gm-6*(*t*) from the donor Duokang #1 to the recipient lines, Ming Hui 63 and IR 50404. The kits contain primers based on the terminal sequences of RG214 and RG476 (Robeniol et al. 1996). To detect introgression of the *Gm-6*(*t*) gene into the Ming Hui 63 background, the amplified products at RG214 and RG476 should be digested with *Bfa*I and *Alu*I, respectively, prior to agarose-gel analysis. Similarly, to detect introgression of the gene into the IR50404 background, the amplified products should be digested with *Hha*I and *Sca*I, respectively.

We were able to verify the utility of RG476 as a marker for *Gm-6*(*t*) in an alternative genetic background (B.C. Huang, H. Li, S Constantino and J. Bennett, unpublished data). In this study, we examined F_4 progeny derived from a cross between Kangwenqinzhan, a gall midge-resistant derivative of Duokang #1 (Huang et al. 2000), and Gui99, a restorer line for hybrid rice breeding. DNA was extracted from the two parents and more than 50 F_4 lines, and was amplified with STS primers for RG476. The PCR products were identical in size but when they were digested with *Alu*I, a polymorphism was seen between Kangwenqinzhan and Qui99. This polymorphism co-segregated in the F_4 population precisely with resistance to biotype 4.

Enhancing the durability of the *Gm-6*(*t*) gene

Gm-6(*t*) is a very valuable gene because it provides resistance against all four biotypes of gall midge known in southern China (Tan et al. 1993; Katiyar et al. 1995). It would be unfortunate if it were rendered ineffective by

Table 2 Asian rice gall midge (*O. oryzae*) resistance genes and their reaction against different biotypes in China and India. R=Resistant, S=Susceptible, MR=Moderately resistant, –=Reaction not known, * gene not yet designated

Gene	Cultivar	Chinese GM biotypes				Indian GM biotypes					
		1	2	3	4	1	2	3	4	5	6
Gm-1	Samridhi/W1263	MR	S	R	S	R	S	R	S	R	R
Gm-2	Phalguna/Siam 29	R	R	S	S	R	R	S	S	R	S
gm-3	RP-2068–18–5-3	–	–	–	–	R	S	S	R	–	–
Gm-4	Abhaya	–	–	–	–	R	R	S	R	S	–
Gm-5	ARC-5984	R	S	–	–	R	R	S	S	R	–
Gm-6(*t*)	Duokang #1	R	R	R	R	S	S	S	S	–	–
Gm-?*	IR-36/Ptb 21	S	S	S	S	R	R	R	R	S	S

Table 3 Allelism test for *Gm-6*(*t*) and *Gm-2* genes for gall midge resistance in rice. Genotypes of the F_2 generation from a Duokang #1× Phalguna cross were determined from the reaction of F_3 lines to two biotypes of gall midge: Chinese biotype 4 and Indian biotype 1, respectively . A total of 417 F_3 lines were phenotyped at Guangdong, China, and at Raipur, India. Each column shows the number of lines attributed to each genotype

Gene	*Gm2Gm2*	*Gm2gm2*	*gm2gm2*
Gm6Gm6	0	24	72
Gm6gm6	38	158	32
gm6gm6	56	36	1

the emergence of a new resistance breaking biotype in this region. We are therefore eager to pyramid *Gm-6*(*t*) with other *Gm* genes by MAS to achieve more durable resistance. Although several resistance genes are known to be effective against Chinese biotypes 1–3 (Table 2), until recently only *Gm-6*(*t*) was known to provide resistance against biotype 4. Now, however, an Assam land race ARC5984 has been shown to contain one or more genes effective against biotype 4 (Huang et al. 2001). We are currently determining the genetic basis of this resistance to assess the feasibility of pyramiding it with *Gm-6*(*t*).

Physical mapping of *Gm6*

On average, a genetic distance of 1 cM corresponds to a physical distance of 363 kb in the rice genome (Goff 1999). The 1-cM distance between *Gm-6*(*t*) and RG214 should therefore be bridged by small contigs of YAC or BAC clones initiated at RG214. An IR64 BAC library constructed at IRRI (Yang et al. 1997) has been used to define many contigs on chromosome 4. BAC clone 24E21 has been found to hybridize with the marker RG214. This BAC clone is now being used as a landmark for physical mapping and positional cloning of *Gm-6*(*t*).

Relationship between *Gm-6*(*t*) and *Gm-2*

The map position of *Gm-6*(*t*) places it close to *Gm-2*, a gene for resistance to gall midge biotypes 1, 2 and 5 in India (Table 2) and carried by the popular cultivar Phalguna (Mohan et al. 1994; Rajyashri et al. 1998). Using a large population derived from the cross Duokang #1× Phalguna, we showed that recombination takes place rarely between the *Gm-2* and *Gm-6*(*t*) loci, equivalent to a separation of 16.3 cM. We conclude that, although the two loci are close to one another, they are not allelic. The resistance alleles of these two genes could therefore be pyramided to provide resistance to gall midge biotypes in southern China and central and eastern India. It would be appropriate to advance single recombinant lines of our Duokang #1× Phalguna population to select double recombinants of the type *Gm6Gm6Gm2Gm2*.

We mapped *Gm-6(t)* to a 3.3-cM RG214-RG476 interval, and Mohan et al. (1994) mapped *Gm-2* to a 7.3-cM RG214-RG476 interval, and yet our genetic study indicates that the genetic distance between *Gm-6*(*t*) and *Gm-2* is about 16 cM. These discrepancies could be due to the fact that the data were obtained with three different populations, which could differ in recombination frequency. Rajyashri et al. (1998) made a similar point. The same authors also noted that physical mapping of the same chromosomal region using YAC clones suggested that the markers RG214 and RG476 are much closer than would be predicted from recombination analysis. Thus, the recombination frequency in this region of chromosome 4 may be less than the average frequency for the genome as a whole and may also show genotype-dependent differences in recombinational repression.

It has been demonstrated that pest resistance genes are sometimes linked and located in clusters (Dickinson et al. 1993; Century et al. 1995; Mc Mullen and Simcox 1995; Ohm et al. 1995, Salmeron et al. 1996; Dweikat et al. 1997; Rossi et al. 1998). Such clustering can make allelism testing of dominant genes difficult. To determine whether two genes are alleles of a single locus or distinct genes within a cluster, we must develop a large segregating population that can reveal rare recombination events. The dividend from this analysis will be a better understanding of rice-gall midge interactions and of the relevance of the gene-for-gene hypothesis in this context (Katiyar et al. 1995).

Positional cloning of *Gm-2* and *Gm-6*(*t*) will assist in the development of molecular models of rice-gall midge interactions. Equally relevant are studies on the genetic relationships between gall midge biotypes. We have recently conducted AFLP fingerprinting of gall midge larvae from 15 locations across Asia (Katiyar et al. 2000). The data showed that Asian rice gall midge populations separate into at least two very different groups. Group I comprises the gall midge populations from China, Laos and Manipur in eastern India. Group II comprises populations from the rest of India and from Nepal and Sri Lanka. It is clear that Chinese biotypes 1 and 4 are genetically very similar (>98% similarity score) and that Indian biotypes 1, 2, 3, 4 and 5 are genetically similar (>88% similarity score), but the two groups are only <27% similar as judged by AFLP analysis. It is an interesting situation that *Gm-6*(*t*)) confers resistance to Chinese biotypes 1–4 of gall midge group I, whereas, *Gm-2* confers resistance against Indian biotypes 1,2 and 5 of gall midge group II, and these two genes reside close to each other on chromosome 4.

So far, 30 major genes for insect resistance have been tagged or mapped in six crop species, conferring resistance to species from five orders: Homoptera, Hemiptera, Diptera, Lepidoptera and Coleoptera (Yencho et al. 2000). Isolation of these genes will provide deep insight into the mechanisms of insect resistance in plants. It will be particularly interesting to see how closely related insect resistance genes are to the numerous disease resis-

tance genes that have already been characterized in many species (Michelmore and Meyers (1998).

Cereal synteny and Cecidomyiid resistance genes

Genetic mapping of wheat, maize, rice and other cereals with common DNA markers has revealed a substantial conservation of gene content and gene order (synteny) in genomes differing in basic chromosome number from 5 to 12 and nuclear DNA content from 400 to 6,000 Mb (Gale and Devos 1998). These findings have raised the possibility that gene mapping in one cereal crop may assist gene mapping in other cereals. Examples of conservation of gene location include the waxy (*Wx*) genes in several cereals, the liguleless (*Lg*) locus in barley, maize and rice, genes controlling gibberellin-insensitivity and plant height in wheat (*Rht*) and maize (*d8, d9*) and red grain color in wheat (R) and rice (*Rd*).

Wheat contains at least 27 H genes for resistance to Hessian fly (*Mayetiola destructor*) (Delibes et al. 1997; Dweikat et al. 1997). As Hessian fly and gall midge both belong to the Cecidomyiid family, H genes in wheat may be homologous to Gm genes in rice. Various H genes have been located on chromosome groups 1, 2, 3, 4, 5 and 6 (Raupp et al. 1993; Cox and Hatchett 1994; Knackstedt et al. 1994; Delaney et al. 1995; Ohm et al. 1995, Delibes et al. 1997). These chromosomal assignments are usually made through the use of addition and substitution lines. However, in one case (H27 on chromosome 4) an acid phosphatase isozyme marker was employed to associate H27 with chromosome 4 (Delibes et al. 1997), while in another case representation difference analysis associated an H gene with RFLP markers on chromosome 6 (Delaney et al. 1995). Our finding that *Gm-2* and *Gm-6*(*t*) reside near locus RG214 of chromosome 4 in rice suggests that one or more H genes may be located on the long arm of the homologous chromosome in wheat, chromosome 2 (Kurata et al. 1994). A Hessian fly resistance gene on chromosome 2L of rye has been transferred into wheat (Hatchett et al. 1993). Since this rye chromosome arm is homologous with chromosome arm 2L of wheat, it would be interesting to determine whether RG214 is an effective marker for this H gene in wheat. As Ohm et al. (1995) reported seven H genes on wheat chromosome 5A, some of the known but unmapped gall midge resistance genes may reside at the corresponding loci in rice (possibly chromosomes 2, 3, 11 and 12).

In conclusion, the tagging and fine mapping of the *Gm-6*(*t*) gene provide an opportunity for: (1) MAS-based transfer of the *Gm-6*(*t*) gene to the parents of hybrid rice, (2) MAS-based pyramiding of *Gm-6*(*t*) with other gall midge resistance genes to achieve broader and more durable resistance for gall midge, (3) physical mapping and positional cloning of *Gm-6*(*t*) from a contig of BAC clones, and (4) establishing whether Hessian fly resistance genes in wheat are homologous with Gm genes in rice.

Acknowledgments We gratefully acknowledge financial support from the Asian Development Bank, the German Federal Ministry for Economic Cooperation (BMZ) and the Rockefeller Foundation, USA. S.K.K. was supported by a postdoctoral fellowship from the Rockefeller Foundation. We thank Blesilda Albano for assistance in preparing this manuscript. The experiments comply with the current laws of the countries in which the experiments were performed.

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