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Markers for selection of the rice *Xa21* disease resistance gene

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Abstract Six molecular markers were mapped to a 7.4-cM region of rice chromosome 11 containing the *Xa21* gene, which confers resistance to the pathogen *Xanthomonas oryzae* pv *oryzae*. Three markers, RG103, 248 and 818, co-segregated with *Xa21* in a population of 1141 plants. Multiple copies of all marker loci were present within the region that was introgressed from *Oryza longistaminata* into *O. sativa*. The marker loci were cloned and primers were designed that defined sequence-tagged sites. Physical mapping of the three tightly linked central markers revealed that RG103, the marker that hybridizes to the *Xa21* gene, resides on a separate DNA fragment from the other two markers.

Key words Rice · *Xa21* · Disease resistance · Linkage map · Physical map

Introduction

The genomic region of rice plants containing *Xa21*, which confers resistance to most isolates of *Xanthomonas oryzae* pv *oryzae* (*Xoo*), includes eight tightly linked fragments that cross-hybridize with the *Xa21* genomic clone (Ronald et al. 1992; Song et al. 1995). Seven of these fragments were introgressed from the resistant donor line, whereas the eighth fragment was already in residence in the susceptible backcross line. At least one of the eight copies confers a high level of resistance to race 6 in transformed plants (Song et al. 1995). The other copies may include genes for resistance to other *Xoo* races, newly evolving or defunct genes, related pseudo-gene sequences, or genes for yet undetermined resistances. For example, in rice lines that lack *Xa21*, genes for blast (fungal) resistance (Wang et al. 1994) and stripe virus resistance (Hayano-Saito et al. 1993) map to the corresponding *Xa21* chromosomal region. In addition, *Xanthomonas* resistance genes *Xa3*, *Xa4* and *Xa10* (Yoshimura et al. 1992; Causse et al. 1994) map to the same arm of chromosome 11. All of these genes may have common origins with *Xa21*.

The present paper builds on previous work by Ronald et al. (1992) in describing the chromosomal region containing the rice *Xa21* locus. *Xa21* was mapped with respect to three RFLP markers and four sequence-tagged sites (STSs). Like the *Xa21* gene family, all of the linked markers are present in multiple copies within the genome, suggesting that duplication and divergence have occurred in this region. Used together in breeding programs, these markers could be applied to the introgression of *Xa21* into a variety of cultivars while de-selecting for linked regions from the donor line.

Materials and methods

Plant material

In order to generate over 1000 plants for mapping, three related populations were constructed from nearly isogenic lines IRBB21 (resistant) and IR24 (susceptible). A set of 47 BC_5F_2 plants (population 1) was obtained by crossing a *Xa21*-containing accession of *Oryza longistaminata* to the susceptible recurrent parent *O. sativa* (Khush et al. 1991). To generate a related population of 540 BC_5F_3 plants (population 2), plants from population 1 that were heterozygous at

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Table 1 Markers linked to *Xa21*

Marker	Origin	RFLP bands enz./sus./het./res./dom. or co-dom.	STS bands sus./het./res./dom. or co-dom.
AB9	RAPD	*Eco*RI/2/2/1/dom.	11/12/12/dom.
248	RAPD	*Xba*I/1/3/2/co-dom.[a]	1/1/3/co-dom.[a]
818	RAPD	*Xba*I/1/2/2/dom.	1/2/2/dom.[a]
RG103	Genomic clone	*Hin*dIII/2/9/8/co-dom[a]	1/3/3/dom.
560	RAPD	*Hin*dIII/2/2/1/dom.	7/9/7/dom.
AE3	RAPD	*Hin*dIII/8/9/9/dom.	5/9/4/co-dom.

[a] RFLPs for 248, 818 and RG103 reported in Ronald et al. (1992). STS 248 reported as generated by primers PB7 and PB8 (Chunwongse et al. 1993)

all mapping loci were selfed. In addition, heterozygous plants from a related BC_6F_5 generation were selfed to produce 554 segregating BC_6F_6 plants (Ronald et al. 1992, population 3). Because these three closely related populations were generated from selfing non-recombinant plants that were known to be heterozygous at all mapping loci, data from the three populations were combined and dealt with for mapping purposes as if arising from a single F_2 population.

PCR and RFLP markers

In a previous *Xa21* mapping study, Ronald et al (1992) describes the cloning of RAPD 248 and RAPD 818 (random amplified polymorphic DNA markers, Williams et al. 1990) and their use as RFLP markers along with the already mapped RG103 clone. Additional RAPD markers were identified for the current study by screening DNA samples from the two nearly isogenic lines, IRBB21 (resistant) and IR24 (susceptible), with 10-nucleotide primers from Operon Technologies. The screen included 720 primers resulting in about 3200 amplified bands, three of which were polymorphic and linked to *Xa21* (Table 1). The three RFLP markers, plus the three RAPD markers, were converted into sequence-tagged site markers (STS, Olson et al. 1989; STS 248, Chunwongse et al. 1993) in order to specifically target the polymorphic locus for amplification (Table 2). Ultimately, all markers existed as cloned inserts that could be used in the hybridization of Southern blots, as well as STSs that could be used in PCR.

Molecular techniques

Small DNA preparations, amplification by PCR, and band visualization were as described elsewhere (Williams and Ronald 1994). For RFLP analysis, DNA was isolated (Osborn et al. 1987) from leaves of single plants or from 30 pooled progeny of plants in mapping population 3. Samples were digested with *Xba*I (for RFLP 248 and 818) and with *Hin*dIII (for RG103) and were separated on gels composed of 1×TBE and 0.7% agarose, at 1.7 V/cm for 16 h, before being transferred to MagnaCharge membrane (Micron Separations, Inc.) and hybridized according to the manufacturer's directions.

Disease screen

Xa21 confers resistance to most Philippine races of *Xanthomonas oryzae*, pv *oryzae* (*Xoo*) (Khush et al. 1989; Ikeda et al. 1990). Inoculations and scoring of disease symptoms with *Xoo* race 6 (strain PX099) were done by the standard leaf clipping method (Kauffman et al. 1973) in growth chambers, with daytime conditions of 10 h of light at 28°C and 85% humidity and a night temp of 24°C and 95% humidity.

Data collection

Populations 1 and 3 were scored with RFLP markers RG103, 248 and 818 (Ronald et al. 1992), STS markers AB9, 248, 560 and AE3, plus were screened for the *Xa21*-resistant phenotype. These data, plus the study by Ronald et al (1992), indicated that the RFLP markers RG103, 248 and 818 co-segregated, so we used only the STS markers AB9, 248, 560 and AE3 to score population 2. All population-2 individuals found to be recombinant with the STS markers (52 individuals) were allowed to self and 30 individuals from each family of BC_5F_4 plants were pooled and screened with RFLP markers RG103, 248 and 818, and then re-screened with the four STS markers. In addition, nine individuals from each BC_5F_4 family were screened for the *Xa21*-resistant phenotype. In this way, we determined the genotype at each locus for all recombinants in population 2 by scoring their progeny.

Map construction

The data were analyzed with Mapmaker for the Macintosh programme (Lander et al. 1987; obtained from S. V. Tingey, E. I. duPont Demour Corp., Wilmington, Del.). We first determined the orders for marker loci 248, AB9 and AE3. This process resulted in a framework map composed of the two most widely spaced markers (AB9 and AE3) plus one central marker (248), giving a high degree of certainty to the marker order. Then, marker locus 560 was added to this framework map by using the "try" command. The order was confirmed by determining the difference in likelihoods of maps that resulted from all possible permutations of adjacent triplets (the "ripple" command). *Xa21*, RG103 and 818 co-segregated with 248, and so were assigned the same map position.

Table 2 Sequences of forward (f) and reverse (r) primers for six STSs linked to *Xa21*

Primer	Length	Sequence
AB9f	20 bases	5′-GGG CGA CTA CTA CAA AAC AT-3′
AB9r	18 bases	5′-GGG CGA CTA CAG AGT TCA-3′
248f	24 bases	5′-AGA CGC GGA AGG GTG GTT CCC GGA-3′
248r	24 bases	5′-AGA CGC GGT AAT CGA AAG ATG AAA-3′
818f	24 bases	5′-CAT CAC AAA GAC GTG CTA ATG ACA-3′
818r	24 bases	5′-CGA GGA TAT GTA CTT GCA ATC TGA-3′
RG103f	24 bases	5′-AAC CAA GCT AGG ACC AAT GAC CAT-3′
RG103r	24 bases	5′-GAG AGG AGA TGT TCC AAA TAG GAT-3′
560f	24 bases	5′-GAT TTT CGC TGA TTT AGG TGC TTA-3′
560r	24 bases	5′-ACA GGA CCA GGT AAC GCT TGT AAT-3′
AE3f	24 bases	5′-GCT GGT ACG GTT CTG GGA GGA TTG-3′
AE3r	24 bases	5′-GGG GGA GGA GGA AGA AAA TAA AAG-3′

Physical mapping

High-molecular-weight DNA was isolated, imbedded in agarose plugs, digested to completion and loaded in gels (Wang et al. 1995). Fragments were separated on a 1% pulsed field gel at 150 V with a 20 s switching time at 16°C for 54 h. The DNA was fragmented by exposing the gel to UV light (254 nm) at a distance of 10 cm for 1 min. DNA was transferred to Hybond N^+ filters (Amersham) in a 0.4 N NaOH, 1.5 M NaCl solution for 2 days. Hybridization conditions were the same as described above for RFLPs.

Results and discussion

STS markers

The STS versions of the markers mapped to the same genomic locations and were more easily scored than their progenitor RFLP or RAPD markers. However, due to the complex nature of repeats in the region containing *Xa21*, all markers except 248 resulted in the amplification of monomorphic bands (present in both resistant and susceptible plants) in addition to polymorphic bands (Fig. 1, Table 1). When multiple polymorphic bands were generated, they co-segregated. The 248 and AE3 STSs generated additional heteroduplex bands, in samples from heterozygous individuals, that were not present in samples from either type of homozygote.

Linkage map of the introgressed region containing *Xa21*

The order of the framework map, composed of STS marker data for AB9, 248 and AE3 from all three populations, was unambiguous (Fig. 2). The likelihood of the map with this order exceeded all others by LOD 8.0. However, the position of marker locus 560 could not be determined with a LOD 3.0 confidence.

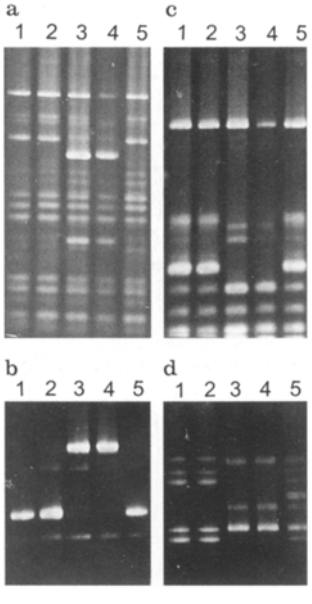

Fig. 1a–d STS amplification patterns. *Lanes* on all gels are as follows: *1 and 2* contain amplification products from homozygous-susceptible individuals; *3 and 4* contain amplification products from homozygous-resistant individuals; *5* contains amplification products from a recombinant individual that is homozygous or heterozygous for alleles at AB9 (**a**- indistinguishable due to dominance of marker), homozygous for 248 alleles from the resistant line (**b**), homozygous or heterozygous for the alleles at 560 (**c**), and heterozygous for the AE3 alleles due to a recombination somewhere between 248 and AE3 (**d**). Heteroduplex bands found only in heterozygotes are present in *lane 5* (**d**)

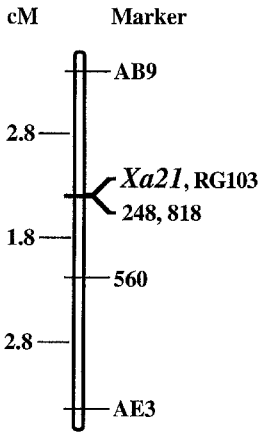

Fig. 2 Linkage map of the introgressed region containing *Xa21* and six marker loci on chromosome 11. Three populations totaling 1141 individuals were used to generate data for the map. The distance between the markers is shown at the left and totals 7.4 cM. Markers AB9, 248 and AE3 were ordered at LOD 8. Marker 560 was positioned between AB9 and AE3 with respect to 248 at lod 1.8. No recombination was found between *Xa21*, RG103, 818 and 248

From the total of 1141 progeny, the 52 individuals from population 2 that were recombinant between the outside markers AB9 and AE3 were subjected to further analysis to confirm marker order with respect to *Xa21*. Pooled progeny of these individuals were used in RFLP analysis to confirm the genotypes of their BC_5F_2 parents for the tightly linked loci RG103, 248 and 818. The progeny were also used in a disease screen with *Xoo* to determine the parental *Xa21* genotype. The resulting map clearly specified the marker order to be AB9-248–560-AE3, but no recombination occurred between 248, 818, *Xa21* (disease phenotype) or RG103. We found that all loci except 560 (chi-square= 16.82, $P<0.001$) showed no bias in segregation ratios. The recessive allele of 560 was more difficult to score and thus contributed to missing data more often than the dominant 560 allele. As a result, the homozygous recessive 560 class was under-represented, causing a deviation from the expected 3:1 ratio and less certainty in the map position of 560.

In comparing map distances between intra- and interspecific crosses, Causse et al. (1994) report that intervals averaged 25% shorter in a cross between *O. longistaminata* and *O. sativa* than in an intraspecific *O. sativa* cross. However, a comparison of the region containing *Xa21* among four different mapping populations gives no clear trend. The interval between RG303 and RG103 in an intraspecific *O. sativa* cross (McCouch et al. 1988) is reported as 18 cM, whereas the same interval in an interspecific *O. sativa* × *O. longistaminata* cross (Ronald et al. 1992) is 21.4 cM. In three crosses between *O. sativa* and an *O. sativa* line containing the introgressed *Xa21* region from *O. longistaminata* (Abenes et al. 1994) that same region is 17.9, 12.3 and 13.1 cM, respectively. We identified no recombination between *Xa21* and the three central markers RG103, 248 and 818. Abenes et al. (1994) report recombination in this region flanking *Xa21* (818 to *Xa21* was 2.2 cM and *Xa21* to RG103 was 3.8 cM in one cross, and 818 to *Xa21* was 2.7 cM and *Xa21* to RG103 was 2.7 cM in another cross). However, these authors did not confirm recombinants by scoring their progeny for resistance and RFLP markers. In addition, they mapped in small popula-

tions (35 and 63 individuals). With small populations, a single individual mis-scored for the *Xa21* resistance phenotype would introduce erroneous double crossovers into a data set.

Physical analysis

Physical linkage between copies of 248 and 818 in the resistant line was clearly shown by pulsed-field gel electrophoresis (PFGE) studies (Ronald et al. 1992). These studies suggested that one copy of 818 and RG103 reside on the same 270-kb DNA fragment generated by *Sfi*I. However, through higher resolution PFGE, we demonstrated here that 818 hybridized to a 270-kb fragment, whereas RG103 hybridized to a distinct 230-kb *Sfi*I genomic fragment in DNA from the resistant line (data not shown). Thus, these experiments indicated that all eight genomic *Hin*dIII fragments hybridizing to RG103, the marker that hybridizes to the *Xa21* gene, are physically separated from fragments containing 248 and 818. The intensity of the 230-kb fragment indicates that a majority of the eight *Hin*dIII fragments that hybridize to RG103 reside together on that one fragment. The physical distance between RG103 and 248/818 actually may be quite small since no recombination was identified between the markers during genetic mapping.

Xa21 confers broad-spectrum resistance to most isolates of *Xoo*, making it a valuable gene for plant breeding (Khush et al. 1989; Ikeda et al. 1990). The six molecular markers described here will be useful in marker-assisted breeding because they will allow more efficient selection of *Xa21* with concurrent counter selection of linked markers from the *O. longistaminata* donor parent. In addition, the availability of six markers broadens the range of crosses in which polymorphisms can be identified.

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