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High resolution genetic mapping and candidate gene identification at the *xa5* locus for bacterial blight resistance in rice (*Oryza sativa* L.)

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Abstract The *xa5* resistance gene from rice provides recessive, race-specific resistance to bacterial blight of rice caused by the pathogen *Xanthomonas oryzae* pv *oryzae*. A high-resolution genetic map of the chromosomal region surrounding *xa5* was developed by placing 44 DNA markers on the distal end of rice chromosome 5. The basis for mapping was a PCR-based screening of 1,016 F_2 individuals derived from a cross between a near-isogenic line (NIL) and its corresponding recurrent parent to identify recombinants in the region. Recombinant F_2 individuals were progeny tested using F_3 families inoculated with the Philippine strain PXO 61 of bacterial blight pathogen. The *xa5* gene was mapped to a 0.5-cM interval between the markers RS7 and RM611, which spanned an interval of approximately 70 kb and contained a total of 11 open reading frames. Sequence data for the locus was generated from an *Indica* (the IR24 isoline, IRBB21) BAC covering part of the region and compared to other overlapping *Indica* (cv 93-11) and *Japonica* (cv Nipponbare) sequences. Candidate-gene analysis revealed that a basal transcription factor (TFIIa), an ABC transporter, a tRNA synthase, a MAP kinase and a cysteine protease, as well as four unknown, hypothetical or putative proteins, are encoded at the locus and could be potential candidates for the resistance gene product. The mechanism by which these genes could provide recessive, race-specific resistance will be elucidated by map-based cloning of the *xa5* gene.

Keywords *Xanthomonas oryzae* pv *oryzae* · Recessive resistance gene · High-resolution mapping · Single nucleotide polymorphism · Simple sequence repeat markers

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Introduction

Bacterial blight of rice, caused by *Xanthomonas oryzae* pv *oryzae*, is a serious disease of rice in tropical lowland rice environments (Mew et al. 1993; Gnanamanickam et al. 1999) and has become a model system for the study of diseases caused by bacterial pathogens on monocotyledonous hosts (Ronald 1997). Research has focused on the roles of systemic-acquired resistance (Chern et al. 2001) as well as gene-for-gene resistance, including the characterization of the bacterial blight resistance genes in rice, the avirulence genes in the bacteria and the molecular processes occurring during compatible/susceptible or incompatible/resistant host-strain interactions. Over 20 genes for bacterial blight resistance have been phenotypically identified in rice, most of them providing vertical, race-specific resistance (Ogawa et al. 1988). Although the majority of these genes are dominant, six are recessive, including *xa5*, *xa8*, *xa9*, *xa13*, *xa19* and *xa20* (Kinoshita 1995). Map-based cloning approaches have been successfully applied to isolate two of the dominant genes, *Xa21* (Song et al. 1995) and *Xa1* (Yoshimura et al. 1996), a leucine-rich repeat (LRR)/kinase receptor protein and a nucleotide binding site (NBS)/LLR protein, respectively. Efforts are underway to isolate several other dominant bacterial blight resistance genes (Wang et al. 2001).

The *xa5* gene is one of the recessively inherited resistance genes that provide race-specific resistance to

bacterial blight. This gene is found at the end of the short arm of chromosome 5 and several researchers have located or developed molecular markers that are closely linked to it (McCouch 1990; Yoshimura et al. 1995; Blair and McCouch 1997; Huang et al. 1997; Sanchez et al. 2000; Singh et al. 2001) or constructed physical contigs around the gene (Saji et al. 1996; Yang et al. 1998). *xa5* is unlinked to any other known genes for resistance to diseases or insects, but to-date the gene has not been isolated. Isolation of the *xa5* gene is of interest in order to understand how this recessive gene functions in pathogen recognition and plant defense responses. Given that no other recessive, race-specific resistance genes have been cloned in plants, it is likely that isolating the *xa5* gene will provide new insights into the molecular mechanisms governing host-pathogen recognition processes that will be useful in plant improvement. Recessive bacterial blight resistance genes are of particular interest because they are likely to be different in nature from their dominant counterparts. Recessive resistance genes generally do not cluster with other resistance genes in rice (Ronald 1997, 1998; Richter and Ronald 2000) nor do they map to the same location as resistance gene analogs and other candidate genes as many of the dominant genes do (Ilag et al. 2000; Wang et al. 2001).

High-resolution mapping has been crucial to the success of map-based cloning projects in rice (e.g., Song et al. 1995; Yoshimura et al. 1998; Ashikari et al. 1999; Yano et al. 2000; Monna et al. 2002). High-resolution maps are useful for the precise placement of a gene of interest and the analysis of regional and sub-regional rates of recombination (Miyamoto et al. 1996; Rybka et al. 1997; Fridman et al. 2000). They can also be used to select appropriate combinations of markers for marker-assisted selection in plant-breeding programs. In our previous work we mapped two restriction fragment length polymorphisms (RFLPs) and three simple sequence repeats (SSRs) that were closely linked to the *xa5* gene (Blair and McCouch 1997).

The underlying objective of this research was to lay the groundwork for positional cloning of *xa5* through the development and fine mapping of new SSR and single nucleotide polymorphism (SNP) markers that are closely linked to the gene. The markers developed in this study allowed us to narrow-down the area of interest to 70 kb around the *xa5* gene, define the frequency of recombination occurring in regions flanking the gene and identify candidate genes.

Materials and methods

Plant materials, DNA extraction

An F_2 population consisting of 1,016 plants derived from the cross IRBB5 × IR24 was used for segregation analysis. IRBB5 is the near-isogenic line (NIL) for the *xa5* gene. The original donor of *xa5* was the genotype DZ192, an *Aus* variety originating in Bangladesh, which is an ecotype of the *Indica* subspecies and belongs to isozyme group II (Glaszmann 1987). A derived line, IR1545-339, containing the DZ192 introgression carrying *xa5*, was used to construct the NIL, IRBB5 (Ogawa et al. 1988). IR24, the susceptible recurrent parent for the IRBB5 isoline, is an *Indica* variety (non-*Aus*) of isozyme group I, developed at the International Rice Research Institute (IRRI) in the Philippines.

The F_2 individuals were genotyped based on micro-preparation DNA that was extracted from either a 4-cm section of the inner leaf whorl of a single tiller or from the first leaf of week-old seedlings by a method modified from Cho et al. (1995). Recombinant F_2 individuals were selected with molecular markers as described below and grown in the Guterman greenhouse at Cornell University until 55 days after planting, whereupon they were transferred to a controlled environment chamber for inoculation and evaluation for resistance or susceptibility, bacterial inoculation and disease evaluation.

Bacterial blight resistance to Philippine race 1 *X. oryzae* pv *oryzae* (strain PXO 61) was evaluated by scissors-clipping six of the youngest leaves of four tillers at 5 cm below the leaf tips with a bacterial suspension having an OD_{600} = 0.6. The inoculum was prepared from bacteria revived from glycerol stocks and grown for 48 h in nutrient yeast sucrose broth at 30 °C. After inoculation the plants were maintained in a growth chamber with 11 h of light, relative humidity above 70%, night temperatures of 28 °C and day temperatures of 32 °C. At 14 days after inoculation the plants were scored as resistant or susceptible and lesion length was measured for the six inoculated leaves.

Plants with lesion length $\leq$3 cm were scored as resistant while those with lesion length $\geq$3 cm were considered susceptible. Leaf tissue of these plants was collected for large-scale DNA extraction according to the method of Causse et al. (1994) from a portion of the uninoculated tissue, which was harvested at the time of bacterial blight inoculation. The F_3 seed was harvested individually from each recombinant plant. Twenty plants per recombinant F_3 family were submitted to phenotypic analysis using the method described above. At 14 days after inoculation each F_3 family was scored to determine if the family was segregating or fixed for resistance or susceptibility. The controls used during inoculation were both parents of the population.

RFLP and STS marker analysis

RFLP survey filters were made from DNAs of the recurrent parent (IR24), isoline (IRBB5) and donor parent (IR1545-339) digested with nine restriction enzymes (*Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, *Sca*I and *Xba*I). Southern analysis was conducted according to Causse et al. (1994) using 15 RFLP clones from the short arm of chromosome 5 on rice maps published by Causse et al. (1994), Blair and McCouch (1997) and Harushima et al. (1998). Marker G396 was monomorphic based on Southern hybridization, so we used the sequence tagged site (STS) primers for this clone described by Inoue et al. (1994) and detected polymorphism after *Alu*I restriction digestion of the PCR product followed by polyacrylamide-gel electrophoresis (PAGE) in 4% gels.

Genomic sequencing of IR24 BAC clone

A single *Indica* BAC clone, UCD-4D7, approximately 90 kb in length, was sequenced (GenBank Accession: AF532975). The BAC clone was identified from a 3 × large-insert bacterial artificial chromosome (BAC) library made for the genotype IRBB21 by Wang et al. (1995). IRBB21 is an isoline of the *Indica* variety IR24 carrying an introgression at the *Xa21* locus, but with IR24 DNA on the short arm of chromosome 5 (Ronald et al. 1992). This clone was selected because it hybridized with four RFLPs that cosegregated or were closely-linked with *xa5* (C568, C919, RG207 and RG556). Random shotgun subclones were created for this BAC by sonicating 5 μg of *Not*I-digested insert DNA that had been separated from the pBELO vector by pulsed-field gel electrophoresis and cloning the blunt-ended fragments into *Sma*I-digested pBluescript (SK+) plasmid vector. A total of 384 sub-clones with

Table 1 Rice microsatellites (RM) and single nucleotide polymorphism (RS) markers used in the genetic mapping of the *xa*5 gene with their locus name, product size in IR24, simple sequence repeat motif or DHPLC-run temperature and forward and reverse primers

Rice microsatellites (RM)				
Locus name	Product size in IR24	Motif and length	Forward primer (5′–3′)	Reverse primer (5′–3′)
RM601	182	(GA)14	CCGGGGGTGTTGGGCTTAT	TCACTGGCTCTACTTCCGCTTCAC
RM602	287	(AT)31	GTTCTGTTCTGATAGATAAGATAG	TTAGTGTGATTCCATTACCAAT
RA603	139	(CCT)5-(CT)4	AGATTTAAGGGAGGGAGATG	CTGCAGATTTGAGAAAAAGG
RM604	116	(CTTT)5	AGAGAAGCTAGGGTTAACGAA	AGGTGACCATCCTCTTTCTT
RM605	167	(AT)3-(ATT)5	TGCAATATCCCTCAGAACTC	CGTTGTCATTCGATTTTCTT
RM606	224	(CA)6-(CA)7	CTGGGCACACGCACATCACA	TTGCCATTGCCATCGCCATTC
RM607	211	(GA)3-(GA)5	CCGTGAAGTGTGTGACAGTA	ATCTGTCTGCTTTTTGAGGA
RM608	187	(CA)6(TA)5	CGGACAAAATTTCAATTTCCA	TCAAATTCGACGGCCCTAT
RM609	289	(ATT)18	GCGATGAAATGGTTTCTGAG	TCTCGGTGCTATCCCAACAT
RM610	207	CT(10)-(CT)9	TTCCCATCAACCTCTGCAAT	ACAAAAGGAGCAACGACGAC
RM611	213	(GA)19	CAACAAGATGGCCTCTTACC	TACAAACAAACAGCTTGTGC
SNP marker (RS)				
Locus name	Product size in IR24	Temperature for resolution of heteroduplexes on dHPLC	Forward primer (5′–3′)	Reverse primer (5′–3′)
RS1	292	50	AAGGCGTATTAGCTTTTTATACCA	GCACACAAATTTTGAATAAGACGA
RS2	400	50	AACGCATATGCTGGTTTGTG	TGATTCGTGTAACATGGGTTAAAA
RS3	436	50	TTATTGCAGGCTGAAGCTGA	ACAAACATGGATGCACAAGC
RS4	419	52	AGCTGCACCCTATAGCCATC	CCTCGCAATTTTCTTTCTCG
RS5	238	55	AGCTGCAAAAGAGGTTATCA	CAGCCATATCCTTCAGCTAC
RS6	343	62	CACCCACCCATGTATCACAA	CTGAATGAGGTGTCCGAGGT
RS7	440	57	ATCACTTTTGGGAGGCATGA	AGATTTGCAGCCTCGTTCTC
RS8	269	54	CGGTGGACAGGTTCCATTAT	CGGACCAGAGCCTAAATCTG
RS9	252	54	AGCAAGATGACCAAAGTCTG	AAAACCACTTGTCGACATTC
RS10	399	Monomorphic	TGCAATTGTCCATACAGCAAA	ACCAGTTTCTGCCTGAGGTT
RS11	301	51	TTGGGAATGAAAATCCACTTG	CGATAGTCTCGCGATATGCT
RS12	431	57	ATACCACATTGCGGCAAAC	TCCTTGGATATCAAGCCTGTC
RS13	355	57	TTTCCTCCTGCTGATGCTTT	AGCCTGATCAAGGCACTTTC
RS14	131	57	AATGTACAAATGGCCAACCA	GCCGGTTGCACTATCTCAAG
RS15	300	54	GGGATGGCAGCATTTTAGTT	CCATGTGTCTCTTCTTGTTCATC
RS16	277	57	GCATGCATGTGCTCAATTTC	GCGAAATGCCTGGAGTTTTA
RS17	300	54	CGACCGAAAGTGAGAAGCAT	CGTCCAGCTTTAATCCAACA
RS18	289	56	GGTGCCATGGAGCTGTTTAG	AGGGAGAAAAACAAGGAGGTG
RS19	399	58	TGTGAGTGTTTGTTTGGCTCA	AACATGTGCGTTGGATCAAG
RS20	310	Monomorphic	ATGGGATCGAGCAAAATCAA	ACAAAACACAGACGCATGGA
RS21	399	58	TGTGAGTGTTTGTTTGGCTCA	AACATGTGCGTTGGATCAAG

an average insert size of 2.0 kb were mini-prepped and sequenced from both insert ends with dRhodamine, dye-terminator cycle-sequencing kits (Applied Biosystems Inc., Foster City, Calif.). The sequences were assembled using a combination of Phred, Phrap (Ewing and Green 1998; Ewing et al. 1998) and Consed 2.0 (Gordon et al. 1998) to provide 4.3 × sequence coverage of the UCD-4D7 BAC.

Sequence alignment

After sequencing, the UCD-4D7 *Indica* BAC sequence (Genbank Accession: AF532975) was aligned with sequences from P0574H01, a *Japonica* (cv Nipponbare) PAC clone that was obtained by the International Rice Genome Sequencing Project (Genbank Accession: AC079022), and with contigs 36, 1677 and 11301 from the whole-genome shotgun sequencing of the *Indica* cultivar 93-11, downloaded from the Beijing Genomics Initiative (210.83.138.53/rice/) on May 22, 2002. Contig 1677 was reverse-complemented prior to alignment with the Nipponbare PAC. Sequence alignments (of ACO90722, AF532975 and 93-11 contigs) were done with ClustalW (http://www.ebi.ac.uk/clustalw/) and the Megalign program of DNAstar (GeneCodes, Ann Arbor, Mich).

SSR marker analysis

A total of 16 SSRs were analyzed: five previously reported markers for the initial recombinant screen and 11 newly designed markers (RM601–RM611, Table 1) using sequence information derived from the UCD-4D7 and Nipponbare large-insert clones. Six of the new markers were useful for higher resolution mapping while five proved to be monomorphic. Previously reported SSRs included RM13, RM122 and RM153 (Chen et al. 1997; Temnykh et al. 2000; http://www.gramene.org/microsat/microsats.txt), RM390, which was designed directly from the RFLP marker, RZ390, as described in Blair and McCouch (1997), and OSR59, a marker originally reported by Akagi et al. (1996), for which we re-designed primers (OSR59 and RM611) to provide better annealing temperature and improved amplification. Both primer pairs, OSR59 and RM611, are based on the same perfect $(GA)_{19}$ simple-sequence repeat identified in the Genbank accession D48905. SSR markers used in the initial recombinant screen were amplified according to Temnykh et al. (2001) and were analyzed with silver-stained polyacrylamide gels as described in Panaud et al. (1996). The ten additional markers were fluorescently labeled and combined into panels that were run on 5% polyacrylamide gels as described in Blair et al. (2002).

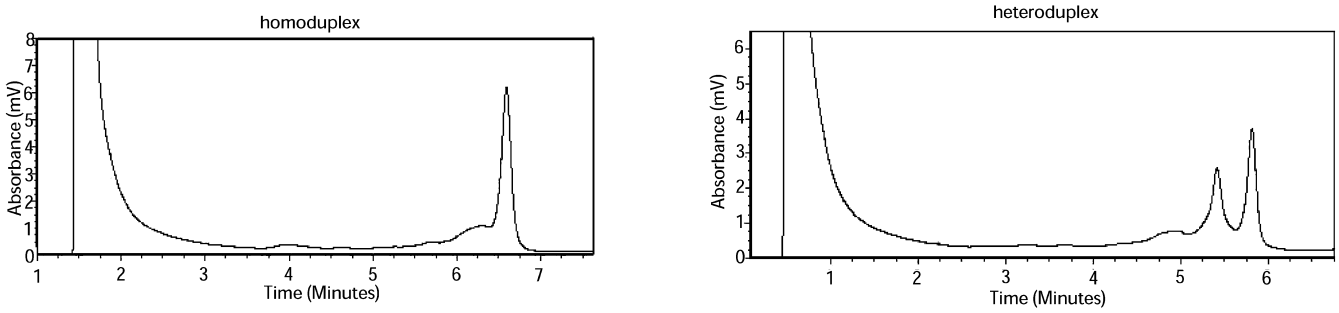

Fig. 1 Chromatograms from dHPLC analysis of SNP marker, RS5, showing a single peak, corresponding to homoduplex molecules (amplified from homozygotes) in the graph on the left and additional peaks, corresponding to the heteroduplex molecules (amplified from heterozygotes) in the graph at the right (RS5: 238 bp in length; G → T at position 33; T → C at position 150; analyzed at 55 °C).

SNP marker analysis

A total of 21 SNP markers were developed for mapping in this population based on the sequence information for the *Indica* BAC and *Japonica* PAC clones described above. Primers were designed to generate PCR fragments ranging in length from 186 to 440 bp (Table 1). SNPs were analyzed on an automated denaturing high-performance liquid chromatography (dHPLC) instrument (Wave; Transgenomic, Omaha, Neb.). The stationary phase consisted of non-porous alkylated polystyrene-divinylbenzene copolymers (DNASep column; Transgenomic, Omaha, Neb.). Samples were eluted with 0.1 M triethylammonium acetate (TEAA) buffer at pH 7.0. In order to induce the formation of heteroduplex molecules, PCR products were denatured by heating to 95 °C for 4 min and re-annealed slowly by cooling from 95 to 35 °C over 30 min in a thermal cycler. The injected sample was eluted with the acetonitrile gradient predicted by Wavemaker 3.1 software (Transgenomic, Omaha, Neb.) at a flow rate of 0.9 ml/min. The optimum temperature for resolution of heteroduplex peaks was established empirically. Amplified fragments from the recombinant individuals were screened alone to identify heterozygotes (9 μl injected) and in a mixture with the amplified product from IR24 (18 μl injected) to distinguish between the two homozygous classes (Fig. 1).

Genetic analysis

Multipoint linkage analysis for the segregating polymorphic markers and *xa5* resistance was conducted with Mapmaker v. 2.0 (Lander and Green 1987). The maximum-likelihood map order for markers was determined with a LOD score threshold of 3.0, and all map distances (cM) are reported in Kosambi units. Chi-square tests were used to evaluate segregation distortion for the *xa5* gene in both the F_2 and F_3 generations and for the markers linked to this gene.

Gene annotation

Open reading frames (ORFs) and potential exon/intron boundaries were predicted for the sequences described above using FgenesH (http://genomic.sanger.ac.uk), GeneMark (http://opal.biology.gatech.edu/GeneMark/), and GeneScan (http://genes.mit.edu/GENSCAN) programs. The potential identities of predicted coding sequences were found by searching against the non-redundant protein and DNA databases, and against the species-specific (*Oryza sativa*) EST databases at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), Gramene (http://www.gramene.org) and The Institute for Genomics Research (TIGR) (http://www.tigr.org/) using the BLASTN and BLASTP programs (Altschul et al. 1997). The rice repeat database at TIGR (http://www.tigr.org/tdb/e2k1/osa1/blastsearch.shtml) was searched to locate repeats within the sequences and positive hits were classified as either retrotransposons, transposons or miniature inverted repeat transposable elements (MITES).

Results

High-resolution genetic map of the *xa5* region and detection of genetic recombination events near the *xa5* locus

A precise linkage map was constructed for the region containing *xa*5 based on the scoring of 2,032 gametes from a cross between the resistant isoline, IRBB5, and the susceptible recurrent parent, IR24, and 21 polymorphic RFLP and SSR markers (Fig. 2). Recombinant F_2 individuals were selected by a sequential screening process based on this genetic map. In the first screening, two SSR loci (RM153 and RM13) that define a genetic interval of 17.8 cM were used to screen for recombination events in the target region and a total of 286 out of the 1,016 F_2 plants were identified as recombinants. In the second screening, RM122 and G396 were used to reduce the number of recombinant individuals that required phenotyping for the disease reaction. A total of 146 individuals were found to be recombinant in the 7.8-cM interval from RM122 to G396 that contained the *xa5* gene, four of which were double recombinants. In the adjacent two intervals, 37 plants showed crossovers in the distal 2-cM interval between RM153 and RM122, and 103 plants showed cross-overs in the proximal 8 cM region between RM13 and G396. No double recombinants were detected by intervening markers in either of these flanking regions. The mapping of the flanking regions was of interest because they oriented the search for large-insert clones and sequence information available from the international rice genome sequencing project (http://rgp.dna.affrc.go.jp/) and Genbank (http://www.ncbi.nlm.nih.gov).

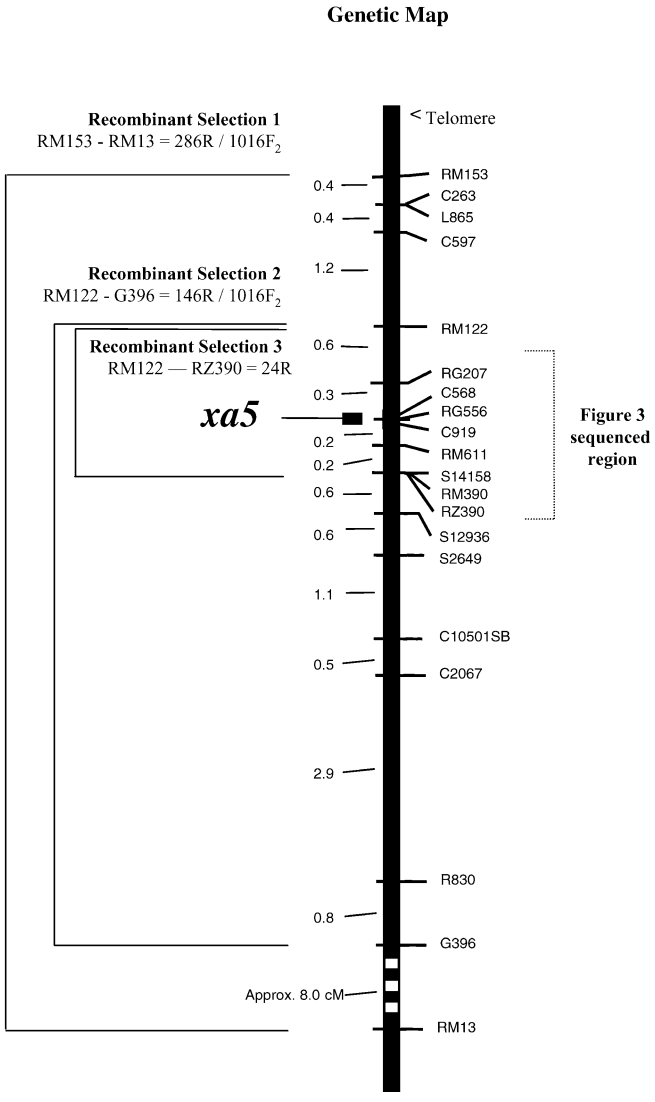

Fig. 2 High resolution genetic map of the *xa5* region on the short arm of chromosome 5. The chromosome is represented as a filled bar with the telomere on top. SSR and RFLP markers used in this phase of the study are indicated to the right of the bar; the distance between markers is indicated to the left. The location of the sequenced region described in greater detail in Figure 3 is shown with a bracket to the far right. Brackets on the left designate the size of the windows used in sequential screens for recombinants. Recombinant selection steps 1–3 are as described in the text

Progeny testing and placement of the *xa5* resistance gene

The exact location of the *xa5* resistance gene on the molecular map was determined by progeny testing the 146 recombinant F_2 individuals via phenotypic evaluation of their F_3 families. Progeny tests served to distinguish heterozygous from homozygous susceptible F_2 individuals and to confirm the phenotype of resistant F_2 plants. The ratio of susceptible to resistant F_2 individuals was evaluated using chi-square tests, and the observed segregation ratio fit the expected 3:1 ratio of susceptible to resistant plants (P = 0.8494). The segregation ratio of susceptible, segregating and resistant F_3 families also fit the expected ratio of 1:2:1 (P = 0.5899). Normal Mendelian segregation ratios were also observed within all of the F_3 families of susceptible heterozygous F_2 individuals (data not shown) and there were no cases in which progeny tests were unable to clearly distinguish between homozygous and heterozygous F_2 individuals. All of the F_3 families of homozygous-resistant F_2 individuals were also found to be 100% resistant, as expected. There was a clear agreement between the disease reaction scores in the F_2 and F_3 generations, showing that the heritability of the disease reaction under these conditions was high.

Lesion length was a good measure of resistance or susceptibility, and the F_2 plants could be reliably scored based on the evaluation of six leaves from the four inoculated tillers. The lesion lengths of the susceptible F_2 plants that were homozygous dominant (16.3 cm ± SD = 5.62) were not significantly different from that of those that were heterozygous (15.3 cm ± SD = 3.94) for the *xa5* gene (unpaired *t*-test, P = 0.269). Meanwhile the lesion length of the resistant F_2 plants that were homozygous recessive (1.36 cm ± SD = 2.82) was significantly below that of the susceptible F_2 plants. Therefore, against the PXO61 (race 1) isolate of the pathogen, the *xa5* gene was confirmed to act as a recessive resistance gene. Since the heterozygous individuals were as susceptible as the homozygous dominant ones and only homozygous recessive individuals expressed resistance, the gene action for the *Xa5* susceptibility allele would be one of complete dominance.

The *xa5* resistance gene co-segregated with the markers C568, RG556 and C919, and could be precisely located to a 0.5-cM genetic interval between the markers RG207 and RM611 (Fig. 2). Furthermore, the *xa5* gene was found to be located at a cumulative distance of less than 5 cM from the telomere of the short arm of chromosome 5, based on mapping of the RFLP markers C263, L865 and C597 in comparison to a telomere repeat-containing RFLP probe named TEL (Kurata et al. 1994). Therefore the region as a whole could be considered subtelomeric.

Normal segregation was observed for the marker loci around the *xa5* gene from RM153 to S2649 as indicated by chi-square tests for goodness of fit to a 1:2:1 ratio. The F_2 individuals that were observed to be heterozygous for *xa5* resistance also were heterozygous at the genetic marker loci C568, RG556 and C919. Mild segregation distortion favoring the IRBB5 alleles did occur for markers in the more proximal region, beginning at the marker C2067 and continuing on to RM13.

Generation of additional codominant markers to saturate the high-density genetic map

An additional 32 new primer pairs were generated using sequence information from GenBank accessions, AF532975 and AC079022, the *Indica* BAC and *Japonica* PAC clones from the *xa5* region, of which 11 bracketed

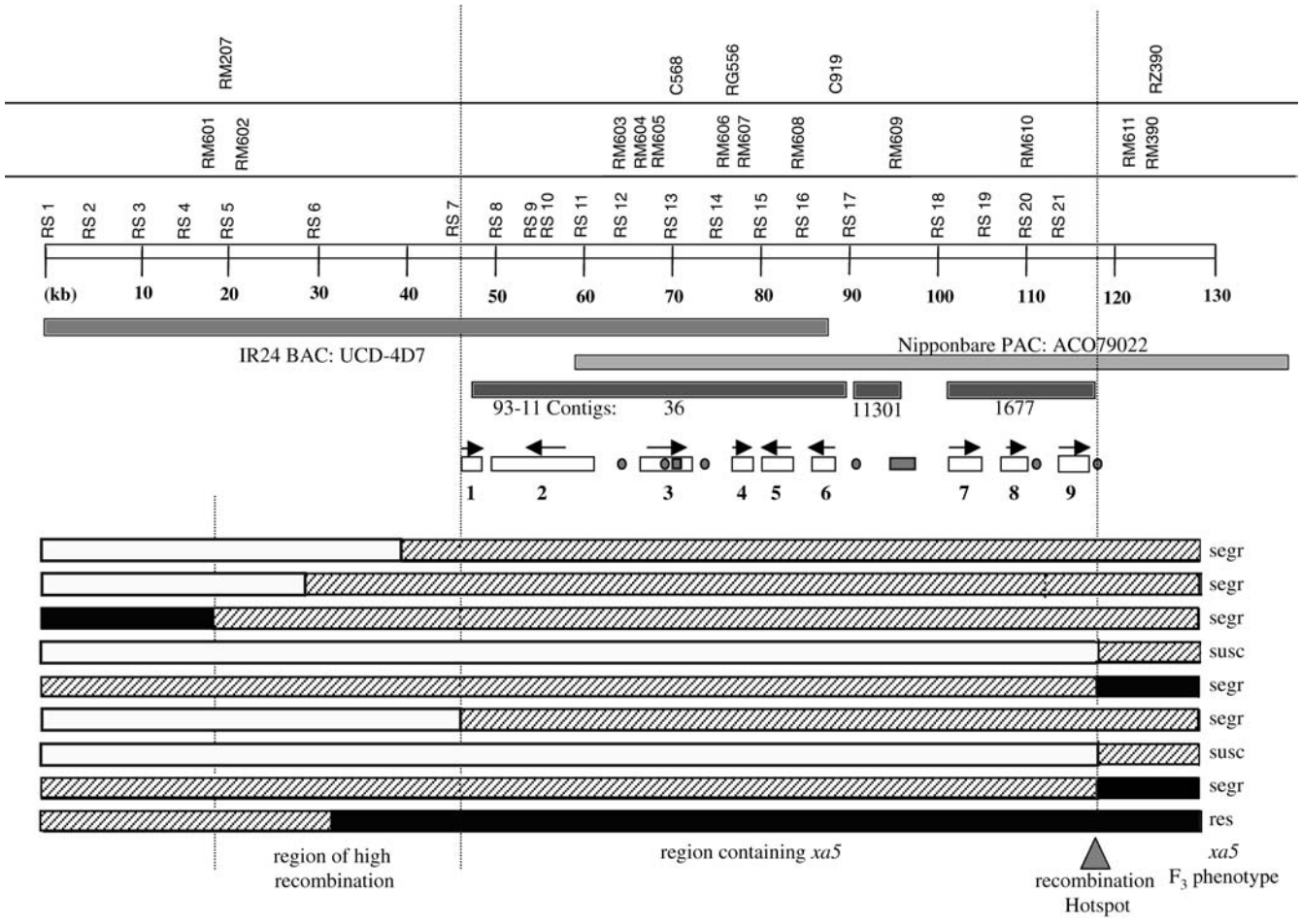

Fig. 3 Locations of RFLP (C, RG or RZ), rice microsatellite (RM) and rice single-nucleotide polymorphism (RS) markers indicated above the physical distance scale (in kb). The sequence-based contig consisting of the IR24 (IRBB21) bacterial artificial chromosome (BAC) clone UCD-4D7 (Accession: AF532975), the Nipponbare P1 artificial chromosome (PAC) clone P0574H01 (Accession: AC079022) and the 93-11 contigs 36, 11301 and 1677, which span 130 kb around the *xa5* resistance gene locus, are shown as *filled bars*. Miniature inverted repeat transposable elements (MITEs) (*circles*), retroelements (*filled rectangles*) and candidate genes (*numbered rectangles*) are described for the 70-kb region containing *xa5*. Candidate genes are as described in Table 2. Graphical genotypes and F_3 family phenotypes are given for nine of the recombinants from the IR24 × IRBB5 F_2 population that had cross-over events between RG207 and RM611; *white* is homozygous for the IRBB5 allele; *black* is homozygous for the IR24 allele; *hatched* is heterozygous

SSRs and 21 were searched for SNPs (Table 1). All of the markers amplified PCR products of the expected sizes in the IR24 and IRBB5 parental genotypes. Of the 21 amplicons tested, two of the putative SNPs (RS10, RS20) and five of the SSRs (RM602, RM604, RM605, RM606 and RM608) were monomorphic or amplified poorly and could not be genetically mapped. In total, 25 new SNP and SSR markers could be mapped on the IR24 × IRBB5 cross.

The physical placement of the new markers relative to six RFLPs and the scaffold of large-insert clones and sequences covering 130 kb of the short arm of chromosome 5 is shown in Fig. 3. Within this 130-kb region, there were an estimated 30 SSR loci (one SSR every 0.23 kb). However, many of these had low numbers of repeat motifs, falling below the threshold that has been empirically determined to demonstrate high levels of polymorphism in rice (Temnykh et al. 2001). The six polymorphic SSRs that were mapped represent an average density of one marker per 21.6 kb (SD = 15 kb). In comparison, SNPs were more abundant, occurring at a density of one every 0.30 kb as calculated from the number of polymorphic fragments divided by the total number of base pairs assayed. Sequence data for some of the amplified fragments showed that more than one nucleotide substitution was detected within the 186–440 bp window assayed by each marker, resulting in an overall SNP frequency for the region of one every 0.12 kb (unpublished data). Compared to this overall frequency, we designed and optimized a SNP marker every 5.7 kb (SD 1.9 kb). The SNP markers could be easily scored as co-dominant markers based on the observation of heteroduplex molecules in each individual recombinant, either alone or together with the IR24 allele on a

Table 2 Candidate genes co-segregating with *xa5* in the region between markers RS7 and RM611

Candidate	Identifier[a]	ESTs[b]	EST source	Number of exons[c]	Gene size (kb)	Coding region % GC content
1. Hypothetical protein	N/A	BI813498 BI811703	Both ESTs: *O. sativa*, mature leaf library, induced by *M. grisea*	5	3.7	45
2. Putative ABC transporter	N/A	BE229765	*O. sativa* cv Milyang23 immature seed	IR24: 29, 93-11: 30	12.3	43.5
3. Putative TFIIa small subunit	Q94HL5	AU057468, D15390 (C568), AU031386	*O. sativa* cv Nipponbare: mature leaf, rice callus, immature leaf	3	0.440	54.3
4. Hypothetical 23.6 kD protein	Q94HL4	AU092639, AU096249	Both ESTs: *O. sativa* cv Nipponbare callus	6	2.04	51.4
5. Putative tRNA synthase	Q94HL3	C72019	*O. sativa* cv Nipponbare panicle at flowering	IR24: 8, 93-11: 7, Nipp: 6	3.22	57.5
6. Hypothetical 46.2 kD protein	Q94HL2	AU181454, C26912, C98168 (C919)	All three ESTs: *O. sativa* cv Nipponbare callus	14	2.85	46.6
7. Putative 61.5 kD kinase	Q94HK9	AV834949, BE600041, BM322652	*Hordeum vulgare* subspecies spontaneum, adult leaves in heading stage; *S. bicolor* Anthracnose-infected leaves from 2-week-old seedlings 48 h after inoculation; *S. bicolor C. graminicola*-infected 4-week-old seedlings	9	4.24	43.5
8. Hypothetical 33.3-kDa protein	Q94HK8	BG558267	*Sorghum propinquum*, rhizomes	1	0.920	72.9
9. Putative cysteine protease	Q94HK7	C25848, AU172507	*O. sativa* cv Nipponbare: rice callus, panicle at flowering stage, respectively	2	1.97	58.5

[a] Identifiers are SwissProtein/TrEMBL accession numbers. Protein designations were based on BLASTP search at the Swiss Institute of Bioinformatics (SIB) on October 7, 2002
[b] ESTs found in GenBank that are at least 85% identical to predicted cDNAs are shown

denaturing HPLC (Fig. 1). The optimum temperature for resolution of heteroduplex peaks was between 50 and 62 °C.

The new SSR and SNP markers were used to construct a high-resolution map around the *xa5* gene using a total of 24 individuals that were found to have crossover events within a 1.3-cM region between the markers RM122 and RZ390. Figure 3 shows the location of critical recombination events that define the 70-kb window containing the *xa5* gene. The size of the window is determined by the F_3 family phenotypes of the nine recombinants with crossover events flanking the *xa5* gene. The closest distal marker was RS7, which detected a single recombination event, while the closest proximal marker was RM611, which was separated from *xa5* by four recombinants.

Recombination events in the *xa5* region

Recombination was uneven throughout the region, being moderate in the distal region between RG207 and RS8, completely repressed in the region around *xa5* and high in a small proximal interval between RS21and RZ390 (Fig. 3). The physical distance from the last distal cross-over event flanking *xa5* to RG207 was approximately 35 kb, while the proximal cross-over events flanking *xa5* were all clustered within approximately 5 kb of RM611. Five recombinants were observed between RG207 and RS8, while eight recombinants were observed around the RM611 SSR and between the markers RS21and RZ390 (only four of which are illustrated in Fig. 3). No recombinants were observed between RS8 and RS21 in the region that co-segregated with the *xa5* gene. Based on these data, the mean number of recombinations per kb in this approximately 100-kb region between RG207 to RZ390 was 0.13 and the estimate of physical to genetic distance for the interval was 200 kb per cM. These estimates would change depending on the size of the sequence window evaluated.

Candidate gene analysis

Within the 70-kb region there were 11 open reading frames (ORFs) (Fig. 3), nine of which were possible *xa5* candidates, which will be discussed later (Table 2), and

two of which were related to transposon-encoded genes. Among the putative transposons were a retroelement and a pseudogene encoding a region similar to the 3′ end of the transposase 'Mutator', both of which were annotated as repetitive elements. A number of additional repeats, including several MITEs, were found in the introns or inter-genic spaces between the nine other ORFs. Gene size, density and GC content were similar within the region for both the *Indica* and *Japonica* sequences. The average gene size of 3.2 kb was somewhat smaller than the 4.5 kb predicted by Yu et al. (2002) and slightly larger than TIGR predictions of 2.47 kb (http://www.tigr.org). Consistent with these published data, the GC content for coding regions was 40% on average and was higher in exons (52.6%) than introns (35.6%). Variation within genes was such that nearly all candidate cDNAs had a higher GC content at the 5′ protein-coding end compared to the 3′ UTR. Gene density within this region was about one gene every 6.4 kb, lower than published predictions of one gene every 4–5 kb (Yu et al. 2002).

Discussion

In this study we confirmed the recessive nature of the *xa5* resistance gene and the dominance of susceptibility over resistance first reported by Murty and Khush (1972) and Olufowote et al. (1977). Slight differences in average lesion length between the heterozygous F_2 individuals and the homozygous dominant individuals in the cross analyzed here, although not significant in our study, may reflect the partial additivity observed in F_1 individuals of the same cross analyzed by Li et al. (2001). Recessive resistance genes, such as *xa5*, are of interest to us because we hypothesize that they may not conform to the structure and mechanisms that exist for the better-studied, completely dominant resistance genes. Although recessive resistance genes can exhibit gene-for-gene specificity like their dominant counterparts, they tend not to be found in gene clusters or associated with LRR-based resistance gene analogs. This is true of the *xa5* gene, which provides vertical resistance to specific races of the bacterial blight pathogen, but has not been associated with dominant resistance genes or resistance gene-analog clusters in rice (Leister et al. 1999; Ilag et al. 2000; Wang et al. 2001).

The genetic linkage map constructed for the *xa5* region in this study was consistent with the framework of RFLP markers mapped by Causse et al. (1994) and Harushima et al. (1998), and SSR markers mapped by Panaud et al. (1996), Chen et al. (1997) and Temnykh et al. (2000). By way of these co-mapped markers, the map in this study is tied to the physical and sequence map developed by the International Rice Genome Sequencing Project (http://rgp.dna.affrc.go.jp/; http://www.usricegenome.org/; http://genome.arizona.edu/fpc/rice/; http://www.gramene.org/) and the principal mapping populations used by the rice scientific community.

Although SSRs were relatively abundant for the *xa5* region, nearly half (45%) of those selected to subdivide the region of interest were short in length and determined to be monomorphic in this cross. In comparison, SNP markers for the region around *xa5* were both more abundant and more polymorphic than SSRs. SNPs are known to be the most ubiquitous form of polymorphism across the genomes of both plants and animals, making them particularly valuable for high-resolution mapping (Landgren et al. 1998). This is the first report of SNP markers in rice that we know of and their frequency (one SNP per 120 bp) is consistent with that reported in tomato and *Arabidopsis* where they have proven essential for refining the location of cross-over events around genes of interest (Cho et al. 1999; Fridman et al. 2000). The emerging genomic sequence of rice (http://rgp.dna.affrc.go.jp) and the rapid and dependable large-scale extraction technologies for isolating genomic DNA makes the development of SNP (as well as SSR) markers straightforward for the species. The use of dHPLC-based SNP detection was effective in this study, but the analysis was more time consuming than assaying for SSRs because it required the amplification of IR24 DNA as an internal control which needed to be run individually for each recombinant to distinguish between the two homozygous classes. However, several of the technologies available for high through-put SNP and SSR detection, such as oligo nucleotide-based arrays (Cho et al. 1999) and multiplexed arrays in capillary machines (Deforce et al. 1998), respectively, make rapid screening of large populations very routine using either of these technologies.

The high-resolution mapping allowed us to narrow down the region containing *xa5* to 70 kb between markers RS7 and RM611. This region was found to contain 11 ORFs, nine of which are considered potential candidate genes: four coded for hypothetical or putative proteins with hits to expressed sequence tags (ESTs) of unknown function, while five coded for proteins with hits to ESTs corresponding to genes of known function (Table 2). All predicted proteins showed hits of at least 85% identity with ESTs from rice, sorghum or barley. Interestingly, the ESTs most similar to the first putative protein listed in Table 2 were found from a rice leaf library induced by the rice blast fungus, *Magnaporthea grisea*.

The five putatively identified candidate genes included an ATP-binding cassette protein (ABC transporter), the small subunit of transcription factor IIa (TFIIA), a tRNA pseudouridine synthase, a protein kinase and a cysteine protease. ABC transporters are a large family of proteins found in a diverse array of organisms such as bacteria, humans and plants (Davies and Coleman 2000). They transport a variety of molecules across biological membranes and are typically composed of two ATP binding domains and two transmembrane domains, which can be found in 1–4 peptides. The putative transporter described here was similar to the *Arabidopsis* PXA1 peroxisomal membrane protein; however, this gene in rice does not appear to contain any transmembrane domains.

TFIIA, a component of the RNA polymerase II holoenzyme complex, facilitates binding of the polymerase to the TATA-box binding protein (Gill 2001). Although none of the basal transcription factors, such as TFIIa, are known to be involved in disease response they have been shown to be targets of signal transduction pathways that regulate genome expression in other eukaryotic organisms. In addition, several other transcription factors from the WRKY (Deslandes et al. 2002) and EREBP families (Sessa and Martin 2000) are important for plant disease resistance.

Protein kinases, especially serine/threonine, receptor-like and mitogen-activated protein (MAP) kinases, are known to be involved in signal transduction and have previously been implicated in plant defense, either as resistance genes or downstream components of defense pathways (Martin et al. 1993; Song et al. 1995; Meskiene and Hirt 2000; Sessa and Martin 2000; Frye et al. 2001). The putative kinase we found had some similarity to MAP kinases from *Arabidopsis* and was highly similar to two ESTs from a pathogen-induced *Sorghum bicolor* library developed from seedlings infected with *Colletotrichum graminicola*; however, no ESTs from rice were found for this gene.

Finally, cysteine proteinases are a large class of peptidases some of which have been shown to be involved in disease resistance pathways (Cervantes et al. 2001; Kruger et al. 2002) or apoptotic events during plant development (Wan et al. 2002). The cysteine protease identified here was similar to several members of this large family in *Arabidopsis* and other plants, and was represented by several significant EST hits.

At present, little is known regarding the molecular mechanisms of recessive resistance, other than the fact that it is thought to differ from that of dominant resistance (Li et al. 2001). However, it is tempting to speculate that one of the *xa5* candidates may function as a negative regulator of plant defense. Several genes implicated in defense are thought to act in such a manner. For example, the *Arabidopsis thaliana edr1* gene, which encodes a putative MAP kinase, is thought to be a negative regulator of salicylic acid- inducible defense responses (Frye et al. 2001). In addition, recessive mutations in the barley gene, *Mlo*, induce spontaneous cell death and provide broad-spectrum resistance to the powdery mildew pathogen *Erysiphe graminis* f.sp *hordei* (Buschges et al. 1997). *Mlo* is thus thought to be a negative regulator of pathogen resistance and/or cell death. The recently discovered recessive gene *RRS1-R*, which provides resistance to several strains of the bacterial blight pathogen *Ralstonia solanacearum* in *Arabidopsis*, and encodes a Toll-IL-1 receptor NBS-LRR protein with a WRKY transcriptional domain may act in the same way, although the mechanism by which the gene functions is not yet fully understood (Deslandes et al. 2002).

An alternative hypothesis for the way recessive resistance genes arise is that they result from mutations in plant genes required for the growth and reproduction of pathogens (Vogel and Somerville 2000). Some such mutations could be envisioned to occur in plant gene products which are targeted by bacterial virulence proteins. Several of the avirulence proteins from the rice bacterial blight pathogen, including Avrxa5, have been shown to have a role in virulence and to be members of the AvrBs3 family of bacterial proteins (Bai et al. 2000). Some members of the AvrBs3 protein family are known to be transported into plant cells and to include functional nuclear localization signals and eukaryotic transcriptional activation domains (White et al. 2000). In this regard it is tempting to hypothesize that one of the candidate gene products for *xa5* could interact with and be the target of the *avrxa*5 encoded protein, that this interaction could take place in either the cytoplasm or the nucleus and that the presence or absence of interaction would lead to susceptibility or resistance in a race-specific manner.

Aside from the candidate gene analysis, the high-resolution map developed in this study allows us to compare the attributes of the *xa5* locus with known dominant and recessive resistance genes. For example, sequence analysis of the *xa5* disease resistance locus revealed no evidence of local gene duplication, showing that this locus is structured differently than many dominant disease resistance loci which commonly contain tandem arrays of homologous genes (Hulbert 1997; Song et al. 1997; Wang et al. 1998; Sicard et al. 1999; Richter and Ronald 2000; Webb et al. 2002). In addition, the lack of recombination around *xa5* observed in this cross contrasts with the increased recombination often associated with dominant resistance gene clusters (McDowell et al. 1998; Collins et al. 1999). In these resistance gene clusters, unequal crossing-over between tandemly arrayed homologues of resistance genes functions in an evolutionary context to create new paralogous genes with new race-specificities.

Without tandem repetition and intensive recombination, the evolutionary trajectory for *xa5* will be likely to differ from the scenario observed for dominant genes (Bergelson et al. 2001). Furthermore, if a recessive resistance gene such as *xa5* does not generate allelic diversity through intergenic recombination, the gene is likely to be more stable than those genes undergoing intensive recombination, unless some other mechanism such as insertion-deletion is responsible for generating new alleles, as was found at a recessive potato virus Y resistance gene locus in tobacco (Noguchi et al. 1999). Conclusions about the *xa5* resistance locus relative to other resistance loci await the map-based cloning and complementation of this gene to confirm which candidate gene(s) encodes resistance, but the cloning of *xa5* is likely to have implications for the study of other recessive resistance in viral, bacterial and fungal pathosystems.

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