Promoter mutations of an essential gene for pollen development result in disease resistance in rice

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Disease resistance and sexual reproductive development are generally considered as separate biological processes, regulated by different sets of genes. Here we show that xa13, a recessive allele conferring disease resistance against bacterial blight, one of the most devastating rice diseases worldwide, plays a key role in both disease resistance and pollen development. The dominant allele, Xa13, is required for both bacterial growth and pollen development. Promoter mutations in Xa13 cause down-regulation of expression during host-pathogen interaction, resulting in the fully recessive xa13 that confers race-specific resistance. The recessive xa13 allele represents a new type of plant disease resistance.

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Both disease resistance and sexual reproductive development are essential for plants to complete their life cycles. They are generally considered as separate and independent biological processes, regulated by different sets of genes (Martin et al. 2003; Ma 2005). Although >40 disease resistance (R) genes have been characterized (Martin et al. 2003), none of these genes was identified as being involved in reproductive development. It is generally accepted that the R proteins encoded by dominant R genes recognize specific pathogen effectors and trigger signal transduction cascades that lead to rapid disease resistance in the host plant (Dangl and Jones 2001; Martin et al. 2003; Belkhadir et al. 2004). However, the function of R proteins inherited recessively is not generally understood. R genes often show a constitutive expression pattern in either uninfected or infected plants, in agreement with their common role in the pathogen recognition step. In a few cases, specific additional induction of R gene expression is observed upon infection (Yoshimura et al. 1998; Piffanelli et al. 2002, Levy et al. 2004, Gu et al. 2005). These results suggest that, in most cases, the basal level of R proteins pre-existing in cells is sufficient for effective initiation of the resistance pathway while, in a few cases, more R protein is produced upon infection to help amplify the resistance response. Even with the R gene functioning as a mutation of a negative effector in disease resistance, induced expression upon infection has been observed (Buschges et al. 1997, Piffanelli et al. 2002).

Thirty R genes conferring resistance against various strains of Xanthomonas oryzae pv. oryzae [Xoo], the causal agent of bacterial blight, have been reported in rice, of which nine were identified as recessively inherited. So far, five of the 30 R genes have been characterized as encoding four types of proteins, suggesting multiple mechanisms of R-gene-mediated Xoo resistance (Song et al. 1995; Yoshimura et al. 1998; Iyer and Mc-Couch 2004; Sun et al. 2004; Gu et al. 2005). R gene xa13 is fully recessive, conferring resistance only in the homozygous status to Philippine Xoo race 6 [strain PXO99], which is virulent to most R genes (Khush and Angeles 1999). Here, by cloning and functional analysis of the xa13, we report that the product of the same gene plays key roles in both disease resistance and pollen development. The resistant [recessive] and susceptible [dominant] alleles of xa13 can encode identical proteins, but have crucial sequence differences in their promoter regions. Suppressing expression of either the dominant or recessive allele of xa13 enhanced the resistance, but caused male sterility of the plants, indicating that the product of this gene acted both as a bacterial growth-dependent modulator and as an essential constituent of pollen development.

Results and Discussion

We have previously mapped xa13 to a 14.8-kb fragment using three F2 populations from a cross between near-isogenic lines IRBB13 [resistant, carrying xa13] and IR24 [susceptible, carrying the dominant allele Xa13] (Chu et al. 2006). Using the same mapping populations and a newly developed molecular marker, ST12, we further mapped the xa13 locus to a DNA fragment of 9.2 kb, flanked by markers RP7 and ST12 [Fig. 1A]. Analysis of the 9.2-kb sequence of BAC clone 14L03 from rice line IR64, carrying the dominant allele Xa13, revealed only one predicted gene, therefore regarded as the candidate of dominant Xa13 [GenBank accession no. DQ421395]. The homologous fragment of the Xa13 candidate from IR24 [GenBank accession no. DQ421396] had an identical sequence with that of IR64. Transformation of IRBB13 with the DNA fragment encompassing the Xa13 candidate, including the promoter region isolated from IR64, produced 29 independent transformatants. Sixteen of the 29 T0 transgenic plants were susceptible upon infection by PXO99, with average lesion lengths of 16.7 ± 2.95 cm as compared with 1.4 ± 0.23 cm measured for the untransformed control IRBB13 and 20.7 ± 2.74 cm measured for IR24. PCR analysis demonstrated that all of the

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susceptible transgenic plants had the band derived from the Xa13 candidate fragment, while none of the resistant plants amplified this band (Fig. 1B). To confirm that the susceptibility of the transgenic plants was caused by the transgene, T₀ plants derived from four of the susceptible T₀ plants were investigated individually for resistance and the marker genotype. The susceptibility cosegregated perfectly with the transgene in all four families, indicating that the gene in this fragment was indeed Xa13 [Supplementary Table S1].

Xa13 consists of five exons and encodes a protein of 307 amino acids [Supplementary Fig. S1] that is located to the plasma membrane (Fig. 1C). Analysis of the Xa13 sequence from IRBB13 [GenBank accession no. DQ421394] detected three nucleotide substitutions in the coding region that caused one amino acid change, as compared with the predicted product of Xa13. To determine whether this amino acid difference is the cause of the resistance, the DNA fragments corresponding to the Xa13 genomic region from 10 additional Xa13-carrying varieties, Chinsurah Boro 2 (11484), Tepa 1, Chinsurah Boro 2 (11760), Aus 274, AC 19-1-1, Long Grain (35023), Kalimekri 77-5, Chinsurah Boro 2 (50930), Long Grain (64950), and BJ 1 [Khush and Angeles 1999], and five additional Xa13 carrying varieties, Minghui 63, Zhonghua 11, Mudanjiang 8, 93-11, and Nipponbare, were isolated and sequenced. Sequence analysis revealed that although there were various substitutions, deletions, and insertions in the Xa13 region of the latter five varieties compared with IR24 and IR64, the Xa13 alleles of all the seven varieties encode an identical protein [Supplementary Fig. S1]. The additional 10 Xa13 carrying varieties showed approximately the same level of resistance as IRBB13 upon PXO99 infection [Supplementary Table S2]. The Xa13 alleles from only eight of the varieties encode proteins with one to three amino acid differences from that of the susceptible allele Xa13, while the predicted protein sequences of the Xa13 alleles from Aus 274 and Kalimekri 77-5 were identical to that of Xa13 [Supplementary Fig. S1]. These results suggest that amino acid change is not the determinant of Xa13-mediated resistance.

To characterize the function of Xa13, we suppressed the expression of Xa13 or xα13 in different rice lines using an RNA interference [RNAi] strategy. Suppression of Xa13 expression in cultivar Zhonghua 11 significantly increased the resistance to PXO99 in nine of the 12 transgenic plants examined. The level of increased resistance was associated with the reduced accumulation of Xa13 transcripts [Fig. 2A]. The correlation between lesion length and Xa13 expression level was 0.78, significant at $P < 0.01$ ($n = 12$). Similar results were also observed in Xa13-suppressed plants in the Minghui 63 genetic background [Supplementary Table S3]. More interestingly, suppressing the expression of xα13 in IRBB13 further enhanced xα13-mediated resistance [Fig. 2B]. The lesion length for eight of the 14 transgenic plants was significantly reduced upon PXO99 infection, and the correlation coefficient between the lesion length and the xα13 transcript level was 0.64 ($P < 0.01$, $n = 14$). These results suggest that resistance to PXO99 depends on the expression level of the gene, such that the lower the expression level of either xα13 or Xa13, the more resistant the plants become.

To examine the expression profile of this gene, we analyzed the expression patterns of xα13 and Xa13 in the near-isogenic lines IRBB13 and IR24 by quantitative RT–PCR. The transcript levels of xα13 and Xa13 were very low in leaves but quite high in panicles and anthers [Fig. 2C]. The expression of Xa13 was greatly elevated after PXO99 inoculation, but not mock inoculation [Fig. 2D]. Xa13 expression in PXO99-challenged IR24 plants increased by sixfold at 8 h post-inoculation and 47-fold at 72 h post-inoculation compared with the nontreatment control. However, PXO99 inoculation did not induce the expression of xα13. We further compared the xα13- and Xa13-transcript levels in other rice lines. The Xa13-transcripts also accumulated upon PXO99 infection in rice lines Minghui 63 and IRBB4 in a similar pattern as in IR24, while the xα13-transcript level was not obviously increased by PXO99 in rice lines Tepa 1, Aus 274, AC19-1-1, Long Grain (35023), and Kalimekri 77-5 as in IRBB13 [Supplementary Fig. S2]. These results further confirmed that a low expression level of this gene is the basis of Xa13-mediated resistance.

Since PXO99 infection can induce the expression of Xa13 but not xα13 in two rice lines with the same genetic background [Fig. 2D], it is highly likely that the
differential expression of \(Xa_{13}\) and \(xa_{13}\) results from differences in the promoter regions of the \(Xa_{13}\) and \(xa_{13}\) alleles of the gene. To examine this conjecture, we analyzed the promoter regions (\(\sim 1.4\) kb) of \(Xa_{13}\) from seven rice lines and \(xa_{13}\) from 11 rice lines. The sequences of the \(Xa_{13}\) promoter region are identical between IR24 and IR64, but various nucleotide substitutions, deletions, and insertions were observed in the promoter regions of other rice lines, compared with IR24 and IR64 (Fig. 3). While the majority of the mutation sites are not allele specific, all of the 11 \(xa_{13}\)-carrying lines had insertion, deletion, or substitution of 1–252 base pairs (bp) within a region of 18 bp corresponding to the –69 to –86 region of the \(Xa_{13}\) promoter in the seven rice lines (Fig. 3). Thus, mutations in this region abolished the induction activity of \(xa_{13}\) expression by pathogen infection, suggesting that this region is required for the \(Xa_{13}\) induction by the pathogen. This induction could be an indirect outcome of pathogen infection, or might be from direct interaction with a pathogen factor that up-regulates \(Xa_{13}\), as in the case of avirulence \(avr\) protein \(AvrXa27\) from \(Xoo\), which acts as a nuclear-localized regulator of rice \(R\) gene \(Xa27\) expression [Gu et al. 2005]. Related pathogen factors with features similar to transcription activators have also been identified both as elicitors and as virulence factors in host–pathogen interaction [White et al. 2000].

The \(xa_{13}\) protein has no sequence similarity with any known \(R\) proteins, but it shows 50% sequence identity and 68% sequence similarity (E value \(10^{-52}\)) to the product of a nodulin \(MtN3\) gene (NCBI protein database accession no. CAA69976) that is induced by \(Rhizobium\) in legume during nodule development [Gamas et al. 1996]. The differential expression of \(xa_{13}\) and \(Xa_{13}\) upon bac-
Histological analysis showed that while no abnormality was observed with the anther wall in Xa13-suppressed plants, the development of the microspores stopped at the unicellular pollen grain stage and gradually degenerated afterward (Fig. 5A). To assess whether the reduced fertility of these plants was associated with the reduced accumulation of Xa13 transcripts, we examined the expression of Xa13 in anthers by in situ hybridization. No Xa13 expression was detectable at early stages of anther development [e.g., microspore mother cell and dyad stage] (Fig. 5B). In wild-type plants, Xa13 transcripts accumulated to high levels in pollen grains, tapetal cells, and middle-layer cells of the anther wall. Moreover, it was observed with the anther wall in Xa13-suppressed plants, the development of the microspores stopped at the unicellular pollen grain stage and gradually degenerated afterward (Fig. 5A). To assess whether the reduced fertility of these plants was associated with the reduced accumulation of Xa13 transcripts, we examined the expression of Xa13 in anthers by in situ hybridization. No Xa13 expression was detectable at early stages of anther development [e.g., microspore mother cell and dyad stage] (Fig. 5B). In wild-type plants, Xa13 transcripts accumulated to high levels in pollen grains, tapetal cells, and middle-layer cells of the anther wall. Moreover, it

Figure 4. Bacterial growth analysis and the expression pattern of Xa13 in leaves. (A) Growth of Xoo strain PXO99 in leaves of wild-type IRBB13 and T0 transgenic line D0903-7 carrying Xa13. Bacterial population was determined from three leaves at each time point by counting colony-forming units (cfu) [Sun et al. 2004]. (B) Xa13 promoter-ß-glucuronidase (GUS) expression in transgenic rice plant. The blue color indicates that GUS was preferentially expressed in the parenchyma cells surrounding the vascular element (V). Bar, 30 µm.

Figure 5. Association of Xa13 expression and pollen development. Bars, 30 µm. (Bp) Bicellular pollen grain; (D) dyad; (Dp) degenerated pollen grain; (E) epidermis; (En) endothecium; (Ml) middle layer; (Up) unicellular pollen grain; (T) tapetum; (Tp) tricellular pollen grain; (V) vascular element; (W) anther wall. (A) Pollen development at different stages in wild-type Minghui 63 and Xa13-suppressed T0 plant D75RMH2, showing that there is no apparent difference between the wild-type and transgenic plants at the unicellular pollen grain stage, whereas various abnormalities are observed at bi- and tricellular pollen grain stages. (B) Expression of Xa13 in anthers of wild-type Minghui 63 and T0 plants D75RMH2 and D75RMH5 examined by in situ hybridization. The dark blue color in the pollen grains, the cells of anther wall, and the connective vascular element indicates hybridization of Xa13 transcripts.
was also found at high levels in connective tissue cells and the connective vascular element at the unicellular-to-bicellular pollen grain stages and occurred only in the cells of connective vascular elements at the tricellular pollen grain stage [Fig. 5B]. The hybridization signal in these tissues was very low or undetectable in the Xa13-suppressed transgenic plants [Fig. 5B]. The coincidence in time between the accumulation of Xa13 transcripts in wild-type plants and the appearance of pollen abortion in Xa13-suppressed plants during pollen development suggests an indispensable role of Xa13 in pollen development.

Thus, xa13, in addition to being a fully recessive R gene functioning by expression loss-of-function from rice, is also an essential gene for pollen development. The dual function of this gene provides a unique opportunity for exploring the cross-talk between disease resistance and male gamete development pathways. Although there have been a few reports on the functional overlap between pathogen-induced defense signaling and plant development (Holt et al. 2002; Montoya et al. 2002; Godiard et al. 2003; Nakashita et al. 2003), none of these genes have major effects on race-specific disease resistance. The complete recessivity of the resistance conferred by xa13 indicates that Xoo resistance may only be a fortuitous by-product of this gene. Thus, mutation of the regulatory element[s] of an essential gene for pollen development results in a novel recessive gene for disease resistance in rice. Although it is not clear how these processes might be linked, the similarities in the mechanisms of pollen/pathogen recognition and development should be investigated in future studies.

Materials and methods

Characterization of resistant and susceptible alleles of xa13

A cleaved amplification polymorphism sequence marker, ST12, detected by PCR amplification using the primers ST12F (5'-AGTAGCCAGTACC CGCCACACA-3') and ST12R (5'-TGATCCGAGAAGGGAGAATTG-3') and digestion by Sau3AI, was used to screen the five recombinant F2 individuals that defined the location of xa13 locus (Chu et al. 2006) for fine mapping of this gene. A DNA fragment containing the dominant allele Xa13 and its upstream (5021 bp) and downstream (6314 bp) regions from rice line IR64 was inserted into pCAMBIA1301 vector and introduced to rice line IRBB13 with Agrobacterium-mediated transformation. GUS histochemical staining of the leaves from T0 transgenic plants was assayed as described previously (Wu et al. 2003). The stained leaves were then sectioned using a razor blade.

Pathogen inoculation

Three to five uppermost fully expanded leaves of each plant were inoculated with Philippine Xoo race 6 (PX099) using the leaf-clipping method (Kauffman et al. 1973) at tillering to booting stages. Lesion length (centimeters) was measured at 14–21 days after inoculation, based on which the mean and standard deviation were calculated.

Analyses of xa13 function

To construct a RNAi vector for Xa13, a 650-bp cDNA fragment of Xa13 was amplified using primers del1.2F and dsl2.R [Supplementary Table S4] from cDNA clone E174B06 of rice line Minghui 63 and was inserted into the pDS1301 vector, which was constructed by inserting the cassette-producing double-strand RNA of the pMCG161 vector [McGinnis et al. 2005] into the pCAMBIA1301 vector. The expression levels of Xa13 and xa13 were determined by quantitative RT-PCR. RT–PCR was carried out in a two-step reaction (Zhou et al. 2002) using gene-specific primers [Supplementary Table S4]. Quantitative PCR was performed using ABI 7500 Real-Time PCR System [Applied Biosystems] according to the manufacturer’s protocol. The expression level of each gene in each sample was calculated relative to the expression level of the actin gene and was the average of three repetitions.

Xa13 promoter–GUS analysis

The promoter region of Xa13 from IR24 [-1.4 kb] [Fig. 3] was fused with the GUS gene and cloned into pCAMBIA1381 vector. The vector was introduced to rice line IRBB13 with Agrobacterium-mediated transformation. GUS histochemical staining of the leaves from T0 transgenic plants was assayed as described previously [Wu et al. 2003]. The stained leaves were then sectioned using a razor blade.

In situ hybridization and histochemical staining

Anthers were fixed in a solution containing 5% glacial acetic acid, 1.85% formaldehyde, 63% ethanol, and 30.15% water at room temperature for 24 h. After dehydration and infiltration, the samples were embedded in paraffin and cut into 10-µm-thick sections. The expression of Xa13 in anthers was assayed using in situ hybridization analysis (Drews 1998). For synthesizing the hybridization probe, a 552-bp DNA fragment was amplified from Minghui 63 cDNA clone 74B06 using Xa13-specific primers X13-QF and X13-R1 (Supplementary Table S4) and ligated into vector pGEM-T. A digoxigenin-labeled antisense RNA probe was synthesized using SP6 RNA polymerase and digoxigenin-labeled sense RNA [control] probe was synthesized using T7 RNA polymerase. Hybridization was as described previously [Drews 1998]. The tissue samples were stained with Ehrlich’s haematoxylin solution before dehydration for structural analysis and with 1% eosin in 95% ethanol during dehydration for in situ hybridization.

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