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Ectopic expression of rice Xa21 overcomes developmentally controlled resistance to Xanthomonas oryzae pv. oryzae

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1. Introduction

At least 10% of global food production is lost to plant disease [1]. Resistance to many of these diseases is often developmentally controlled such that only adult plants are resistant [2]. For example, development-controlled disease resistance is mentally controlled such that only adult plants are resistant [1]. Resistance to many of these diseases is often developmentally controlled. For example, development-controlled disease resistance is observed in wheat/Puccinia recondita [3], Maize/Puccinia sorghi [4], tomato/Cladosporium fulvum [5], and Arabidopsis/Pseudomonas syringae interactions [6]. Despite the economic importance of seedling resistance, little is known about the biochemical and molecular mechanisms involved in this regulation.

Plant innate immune systems rely on monitoring the presence of pathogen through PRRs (which recognize PAMPs) and nucleotide-binding site leucine-rich repeat (NB-LRR) type proteins (which recognize pathogen effectors) [7–9]. The rice PRR, XA21, recognizes the PAMP Ax21 (Activator of XA21-mediated immunity), present in all Xanthomonas and Xylella species [10–12]. XA21-mediated resistance has been shown to be under developmental control with incomplete resistance in early stages that gradually increases with development [13]. XA3/XA26-mediated resistance is also developmentally regulated [14]. The developmentally controlled resistance is hypothesized to be due to transcript levels of Xa21 and Xa3/Xa26 [2,14]. Their expressions are low at the two-leaf stage, and then gradually increase with development. The highest level of expression is reached at the maximum-tillering stage, consistent with an increased resistance at this stage. Based on the expression result of Xa21, we hypothesized that ectopic expression of Xa21 may overcome the developmental control of XA21-mediated resistance. Ectopic expression experiments are specifically designed to increase the abundance of the desired transcript through introduction of a transgene into the host. This strategy was applied to the study of several NB-LRR and PRR proteins. In the case of Arabidopsis RPS2, tomato Pto, and rice Xa2, overexpression leads to constitutive activation of downstream defense responses even in the absence of the corresponding effector or PAMPs [14–17].

Here we show that transgenic plants overexpressing Xa21 display significantly increased resistance at the seedling stage. These results support the hypothesis that Xa21 transcript levels are rate limiting in early stages of development.

2. Materials and methods

2.1. Plant material and growth conditions

Rice (Oryza sativa L.) plants (cultivar Kitaake (Kit)) were maintained in the greenhouse. The growth chamber was set on a 16 h light and 8 h dark photoperiod, 28/26 °C temperature cycle, and 85/90% humidity. Healthy and well-expanded leaves from three-
to construct the Ubi Myc-Xa21 plasmid, a 5' fragment of Myc-Xa21 was PCR-amplified using primers, 5'-AAAGATCC-
AAATCTCTGGCTCTT-3'/5'-GGTGAGCCTCCGGTGAT-3' and template pCH22-cMyc-Xa21 [18,19]. This 420 bp 5'-fragment was cut with BamHI/Sacl at the ends and cloned, together with a 4.2 kb Sacl/Spel Xa21 3'-fragment, into the pBluescript II SK-vector to create a promoterless full-length Myc-Xa21 gene. The 5' end of this gene was confirmed by sequencing. This Myc-Xa21 gene was excised with BamHI/Spel and subcloned into the Ubi-CAMBIA-1300 vector using the same enzyme sites to generate plasmid Ubi Myc-Xa21/C1300.

To fuse the XA21 protein to the cyan fluorescent protein (CFP), a 380 bp 3' fragment of the Xa21 gene was PCR-amplified using primers, 5'-TGGATCCAGCATAGAGAATCTTCCGGTGGTAT-3'/5'-AAATCCATGGAATCCCAGCTCCACCATC-3', this fragment removed the stop codon and the EcoRl site located immediately in front of the stop codon. EcoRl was used to digest the 5' region and NcoI to digest the 3' region of Xa21 3' PCR product. Meanwhile, the CFP gene was excised from the pECFP plasmid (Clontech) using NcoI/Spel. These two fragments were joined at the Ncol site and cloned into pBluescript II SK, digested with EcoRl/Spel, to create plasmid Xa21 3'-CFP/SK. The Xa21 portion and the junction were confirmed by sequencing. The Xa21 3'-CFP fragment was excised with EcoRl (the second EcoRl site, coming from the pECFP plasmid, is located downstream next to the end of the CFP gene) and used to replace the 380 bp EcoRl fragment in the original promoterless (as described above) Xa21 gene, creating Xa21-CFP. To create the Ubi Xa21-CFP/C1300 construct, the Xa21-CFP fragment was excised with BamHI/Spel and cloned into the Ubi-CAMBIA-1300 vector predigested with BamHI/Spel as described above.

For reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, total RNAs were extracted from leaves using TRIZol® reagent (Invitrogen) after each treatment. Then the RT reaction was performed following the manual for QuantumRNA 18S Internal Standards (Ambion). PCR analyses were performed with primers pairs, 5'-TCATCATCTACTACTGTTATA-3'/5'-GAATTCAGAGGCTCCCCACCC-3' (for Xa21utr), 5'-GTITATTGCGACATCATTGAG-3'/5'-GAAAGCCTCCCACCACCTCTACTCTATAC-3' (for Xa21utr). After 28 cycles, the amplified products were resolved by gel electrophoresis.

For qRT-PCR, the total RNA was treated with RNase free DNase (Promega), purified using Macherey-Nagel Nucleospin RNA II kit and quantified using ND-1000 spectrophotometer (Nanodrop). cDNA was synthesized from 10 μg of total RNA in 40 μl volume using M-MLV RT (Invitrogen) followed by RNase treatment (NEB). The cDNA was cleaned using Zymo DNA clean and concentrator kit and eluted in 100 μl of 1 mM Tris–HCl. The CDNA was diluted to 20 fols and 4 μl of it was used for each reaction. Gene-specific primers were designed using PRIMER EXPRESS version 2.0 (PE Applied Biosystems, USA) and checked for their specificity using Blast tool of National Center for Biotechnology Information (NCBI). Primer sequences have been given in Supplementary Table 1.

3. Results

3.1. Xa21 expression is induced by Xoo infection

To determine if Xa21 expression is modulated by Xoo strain PX099Az expressing Ax21, its expression pattern was monitored in six-week-old transgenic rice plants carrying Xa21 gene under the control of its native promoter (Nat XA21 23A-1-14, homozygous T2) (Fig. 1A). For reverse transcriptase (RT)-PCR analysis, specific primer sets in 3'-untranslated region (utr) and coding sequence (cds) of Xa21 were used. As expected, transcripts corresponding to Xa21 in wild type Kitaake (Kit) were not detectable at any time point after Xoo PX099Az inoculation, indicating that primers for Xa21 are specific (data not shown). In contrast, Nat XA21 plant wounded by cutting the leaf tip with scissors induced Xa21 gene to moderate level. In Xoo-inoculated Nat XA21, Xa21 was induced in RT-PCR analysis, performed with both primer pairs (labeled as Xa21utr and Xa21cds) with maximum accumulation at 72 h after inoculation (HAI). This result suggests that Ax21 and XA21 interaction regulates XA21-mediated immune response as well as the transcription level of Xa21. 18S ribosomal RNA (18S rRNA) was used as an internal control.
To investigate if accumulation of Xa21 transcripts correlates with the XA21 protein after Xoo PX099Az inoculation, we generated transgenic Kit lines possessing an N-terminal Myc-epitope-tagged XA21 (Nat Myc-XA21) or a C-terminal CFP-tagged XA21 (Nat XA21-CFP), under the control of its native promoter. At the six-week-old adult stage (9–10 leaves), non-transgenic Kit plants are susceptible to Xoo PX099Az. In contrast, the transgenic Nat Myc-XA21 (T3, 20–1) and Nat XA21-CFP (T1, 15A-1) plants were fully resistant to Xoo, indicating that the proteins are biologically equivalent to the native XA21 protein (Fig. S1A and B). We then investigated if XA21 is accumulated using anti-Myc or anti-GFP antibodies after Xoo PX099Az inoculation (Fig. 1B and C). In the absence of Xoo (0 time point), Xyc-XA21 protein of 140 kDa could be detected slightly, but not in Kit control plants (Fig. 1B). At 6 HAI, the protein started to accumulate and continuously increased till 18 HAI. We have previously reported that the XA21 protein accumulation is independent of mRNA levels after Xoo inoculation, because the stability of the XA21 protein is regulated through ER quality control mechanisms [20]. The anti-actin antibody has been used as an internal control to show equal loading in each lane. Anti-GFP antibody detected a 180-kDa polypeptide in transgenic plants carrying Nat XA21-CFP but not in the control line Kit (Fig. 1C). Similar accumulation pattern of XA21-CFP protein was observed after PX099Az inoculation, indicating that XA21 protein synthesis and/or stability is increased during the defense response.

3.2. Generation of transgenic rice plants overexpressing Xa21

Based on the results of Xoo PX099Az inoculation in Fig. 1, we hypothesized that accumulated Xa21 transcripts and XA21 proteins may affect the resistance level to Xoo. To evaluate the hypothesis, we transferred Xa21 driven by a strong constitutive promoter, maize ubiquitin gene promoter (Ubi) into Kitaake wild type and generated 21 independent transgenic lines carrying Myc-Xa21 under the control of the Ubi promoter (Ubi Myc-XA21). We then analyzed the transgenic lines (T0 generation) at six weeks of age for alterations in resistance to Xoo PX099Az. Fourteen Ubi Myc-XA21 lines displayed similar level of enhanced resistance to Xoo. Seven lines showed no difference compared to Kitaake (data not shown). We generated T1 progeny from each of the T0 lines displaying enhanced resistance and analyzed the T1 progeny for Mendelian segregation of the transgene with the enhanced resistance phenotype. 2A, 7A, 8A, and 9A of lines were chosen for further analysis. The protein levels of Myc-XA21 in six-week-old transgenic lines (T1) displaying the enhanced resistance (2A-1, 7A-1, 8A-2, and 9A-1) were examined by Western blot analysis using anti-Myc antibody (Fig. 2A). The Ubi Myc-XA21 transgenic lines (T1) overexpress Myc-XA21 protein compared to the Nat Myc-XA21 lines. Anti-actin antibody was used as an internal loading control. Total RNAs from Kit, homozygous Nat Myc-XA21 (20-1), and homozygous Ubi Myc-XA21 (T3, 7A-8-101-1 and -2) were extracted. Then we performed RT-PCR with primers targeting specifically Xa21 (Fig. 2B). Internal control, 18S rRNA, showed constitutive expressions in all tested plant lines. Although the Xa21 was detected in Nat Myc-XA21 to moderate level, the levels of transcripts and protein were significantly increased in Ubi Myc-XA21, demonstrating that Xa21 under control of Ubi promoter was overexpressed constitutively.

3.3. Constitutive expression of Xa21 shows enhanced resistance to Xoo

We then examined if the increased XA21 protein caused by its constitutive expression results in the enhanced resistance to Xoo. After Xoo PX099Az inoculation to six-week-old plants, the lesion lengths of two independent homozygous lines (Ubi Myc-XA21 T1, 7A-8 and 9A-12) were compared with homozygous Nat Myc-XA21 line (20-1) (Fig. S2A). At 12 days after inoculation (DAI), Kit wild type displayed susceptibility to Xoo PX099Az with long lesions ranging in length over 15 cm, in contrast to the Nat Myc-XA21 line which showed 2–3 cm lesion lengths. Ubi Myc-XA21 displayed shorter lesion lengths (approximately 1 cm) compared to Nat Myc-XA21, indicating that overexpression of Xa21 confers enhanced resistance. To quantify the effect of XA21 overexpression, homozygous Ubi Myc-XA21 (T2, 7A-8-123), Nat Myc-XA21, and Kitaake were inoculated with Xoo PX099Az and bacterial growth was monitored over time (Fig. S2B). At eight DAI, significant decrease in bacterial population was detected in the Ubi Myc-XA21 lines compared with the Nat Myc-XA21. At 12 DAI, Xoo strain PX099Az populations in Ubi Myc-XA21 lines reached to 2.75 × 10^7 colony-forming units per leaf (cfu/leaf), which is approximately five-fold decrease compared to Nat-Myc-XA21 control (1.24 × 10^8 cfu/leaf).

This result was also confirmed with another Ubi Myc-XA21 lines which carry Xa21 tagged with CFP (Ubi Xa21-CFP). Six-week-old progenies (T1) from self-pollinated 7B, 10B, 11B, and 18B were inoculated with Xoo PX099Az. Twelve DAI, we examined for segregation of genotype with phenotype by PCR analysis and performed colony counts. All segregants carrying Ubi Xa21-CFP displayed enhanced resistance to Xoo PX099Az compared to homozygous transgenic plants carrying XA21-CFP under control of native promoter (Nat XA21-CFP 15A-1) (Fig. S3). Segregants lacking Ubi Xa21-CFP showed susceptibility upon Xoo PX099Az inoculation.

3.4. Overexpression of Xa21 overcomes developmentally regulated resistance

The resistance conferred by XA21 progressively increases from the susceptible juvenile two-leaf stage (approximately two-week
old) through later stages, with full resistance only at the adult stage [13]. To test if the enhanced resistance by the overexpression of Xa21 can overcome the juvenile stage susceptibility, we inoculate the three leaf stage plants (three-week old). At this stage of development, XA21 rice plants are only partially resistant (approximately 40% of resistance to that of six-week-old plants) [13]. At 12 DAI, we measured the lesion length of three leaf stage plants inoculated with Xoo PXO99Az (Fig. 3A). The Kit control displayed susceptibility to Xoo PXO99Az with long lesions ranging in length from 14 to 15 cm. Homozygous Nat Myc-XA21 (20-1) developed relatively long lesion lengths (approximately 8–10 cm), confirming that juvenile stage XA21 plant does not possess full-resistance observed in adult-stage XA21 plants. However, significantly enhanced resistance was observed in two independent homozygous lines, Ubi Myc-XA21 (T2, 7A-8-101, 7A-8-102, 9A-12-6, and 9A-12-7) with only 4–5 cm lesion lengths.

We quantified the effect of Xa21 over-expression on bacterial growth by monitoring bacterial populations on homozygous Ubi Myc-XA21 plants (T2, 7A-8-105) inoculated with Xoo PXO99Az (Fig. 3B). For all growth curves until 4 days after inoculation (DAI), there was no significant difference in bacterial populations in any of the lines. However, significant difference in bacterial growth was observed at 12 DAI. Susceptible Kit plants reached approximately 1.97 × 10^8 colony-forming units per leaf (cfu/leaf). The bacterial populations in Nat Myc-XA21 grew to 7.15 × 10^8 cfu/leaf, showing the partial resistance upon Xoo PXO99Az in three leaf stage. In Ubi Myc-XA21 plants (T2, 7A-8-105), the population leveled off at less than 2.05 × 10^8 cfu/leaf, indicating that developmental control of Xa21 can be overcome by its constitutive expression.

3.5. Expression of defense-related genes correlates with Xa21 expression levels

To elucidate the molecular mechanism to confer the enhanced resistance to juvenile and adult stages of Ubi XA21 transgenic lines, we examined if the constitutive expression of Xa21 activates defense signaling pathway. Total RNAs from Kit, homozygous Nat Myc-XA21 (20-1), and homozygous Ubi Myc-XA21 (T1, 7A-8-101-1 and -2) were extracted. We examined the expression of defense related genes in transgenic plants carrying Xa21 under native or Ubi promoter using a real-time qRT-PCR analysis in the absence of Xoo treatment (Fig. 4). The list of genes is given in Table S1.

The defense related gene, PR10b, PR21, WRKY71, peroxidase, hsp90, and SDF2, were induced in both transgenic plants. In contrast, CO11, a gene induced during involved in jasmonate signaling [21], OsMT2b, a negative regulator of oxidative burst [22], SSI2, implicated in cell death [23], and OsSGT1, a gene encoding a co-chaperone of HSP90, were up-regulated only in the Ubi Myc-XA21 transgenic plants. These results demonstrate that overexpression of XA21 up-regulates a new set of defense-related genes as compared to Nat Myc-XA21, thus enlarging the number of possible target genes that are induced.

3.6. Enhanced resistance mediated by overexpression of Xa21 is Xa21-dependent

To elucidate whether the enhanced resistance mediated by ectopic expression of Xa21 is Xa21-dependent, we inoculated Ubi Myc-XA21 with the Xoo PXO99A/ΔraxST, mutant strain defective in Xa21 biological activity [24]. At 12 DAI, Kit, Nat Myc-XA21, and Ubi Myc-XA21 (T1, 7A-8-117-1 and -2) displayed long lesions ranging in length around 12–14 cm, suggesting that Xa21 overexpression is not able to induce resistance to Xoo strains lacking Ax21 activity (Fig. S4).

We also investigated if overexpression of Xa21 can confer resistance to a normally virulent fungal pathogen (Fig. S5). For this experiment, we used Magnaporthe oryzae isolate R01-1, which is compatible with Kitaake. Ten DAI with M. oryzae, disease levels in Kit, Nat XA21, Nat Myc-XA21, and Ubi Myc-XA21 (T1, 7A-8-1 and 9A-12-1) were evaluated by measuring lesion lengths. No significant difference was observed in the tested rice lines. Taken together, these results indicate that enhanced resistance by overexpression of Xa21 still requires the presence of Ax21.

4. Discussion

4.1. Xa21 expression is regulated upon Xoo infection

It has been previously reported that the expression of PRR and NBS-LRR genes controlling plant immunity are transcriptionally regulated under various conditions, including Arabidopsis RPS2 and
Kitaake wild type (Kit), Nat Myc-XA21, or Ubi Myc-XA21 (T3, 7A-8-101-1 and -2) expression of defense-related genes. Total RNA was extracted from six-week-old plants. qRT-PCR was performed with specific primers for each gene. Gene expression level was normalized using actin as an internal reference.

Several previous reports have demonstrated that ectopic expressions of NB-LRR or PRR genes cause induction of downstream defense responses in the absence of the cognate effector or PAMP [14–17]. For example, overexpression of Pto displayed broad spectrum resistance to bacterial and fungal pathogens including *Pseudomonas syringae* pv. *tomato*, *Xanthomonas campestris* pv. *vesicatoria*, and *Cladosporium fulvum* [15]. Consistent with these previous studies, overexpression of Xa21 confers enhanced resistance and seedling stage and is accompanied by partial induction of defense-related genes. However, Xa21-overexpressing plants are still susceptible to an *Xoo* strain lacking *Ax21* activity and to *M. oryzae*. We hypothesize that, in the absence of *Ax21*, the XA21-mediated resistance pathway in the Ubi Myc-XA21 plants is not fully activated.

In contrasts to Pto-overexpressing tomato plants, which display spontaneous cell death [15], overexpression of Xa21 enhances resistance at the seedling stage with no observable detrimental effects on plant growth or development. Thus constitutive expression of *Xa21* provides a useful strategy to engineer resistance to *Xoo* during multiple developmental stages.

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**Competing interests**

The authors have declared that no competing interests exist.

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2010.07.008.

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