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Rice XB15, a Protein Phosphatase 2C, Negatively Regulates Cell Death and XA21-Mediated Innate Immunity

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Perception of extracellular signals by cell surface receptors is of central importance to eukaryotic development and immunity. Kinases that are associated with the receptors or are part of the receptors themselves modulate signaling through phosphorylation events. The rice (Oryza sativa L.) XA21 receptor kinase is a key recognition and signaling determinant in the innate immune response. A yeast two-hybrid screen using the intracellular portion of XA21, including the juxtamembrane (JM) and kinase domain as bait, identified a protein phosphatase 2C (PP2C), called XA21 binding protein 15 (XB15). The interaction of XA21 and XB15 was confirmed in vitro and in vivo by glutathione-S-transferase (GST) pull-down and co-immunoprecipitation assays, respectively. XB15 fusion proteins purified from Escherichia coli and from transgenic rice carry PP2C activity. Autophosphorylated XA21 can be dephosphorylated by XB15 in a temporal- and dosage-dependent manner. A serine residue in the XA21 JM domain is required for XB15 binding. Xb15 mutants display a severe cell death phenotype, induction of pathogenesis-related genes, and enhanced XA21-mediated resistance. Overexpression of Xb15 in an XA21 rice line compromises resistance to the bacterial pathogen Xanthomonas oryzae pv. oryzae. These results demonstrate that Xb15 encodes a PP2C that negatively regulates the XA21-mediated innate immune response.

Introduction

Protein kinases regulate most cellular signal transduction pathways including cell growth and proliferation, cellular differentiation, morphogenesis, gene transcription, and immunity [1–3]. Adaptive immunity, restricted to vertebrates, is characterized by the creation of antigen-specific receptors through somatic recombination in maturing lymphocytes [4]. In contrast, innate immunity, common to both animals and plants, is mediated by a set of defined receptors referred to as pathogen recognition receptors (PRRs). Recognition of pathogen-associated molecular patterns (also called microbe-associated molecular patterns) or pathogen-derived avirulence (Avr) molecules by PRRs triggers signal transduction pathways mediated by activation of mitogen-associated protein kinase (MAPK) cascades and transcription factors [4,5]. These pathways lead to a core set of defense responses including accumulation of defense related molecules, increases in reactive oxygen species, calcium fluxes, and programmed cell death (PCD) [4–6].

In animals, recognition of pathogen-associated molecular patterns in extracellular compartments or at the cell surface is largely carried out by the Toll-like receptor (TLR) family containing leucine rich repeats (LRRs) in the extracellular domain [6]. TLRs associate with the interleukin-1 receptor-associated kinase (IRAK) family [7] and with receptor interacting-protein (RIP) kinases [8] via adaptor proteins. In plants, cell surface recognition of pathogen-associated molecular patterns or pathogen-derived Avr molecules is largely carried out by the non-RD class of receptor kinases (RKs) [6,9].

These “non-RD” kinases typically carry a cysteine (C) or glycine (G) before the conserved aspartate (D) residue. In contrast, the larger group of “RD” kinases have an arginine (R) immediately preceding the conserved catalytic aspartate (D) [10,11]. The RD class of kinases includes nearly all receptor tyrosine kinases and most characterized plant receptor serine/threonine kinases [10]. The non-RD class includes members of human IRAKs and RIs, Drosophila Pelle, and members of plant RKs belonging to the IRAK family [10,12,13].

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Abbreviations: 18S rRNA, 18S ribosomal RNA; Avr, avirulence; Betv1, Betula verrucosa 1; cfu/leaf, colony-forming units per leaf; DAI, days after inoculation; GFP, green fluorescent protein; IRAK, interleukin-1 receptor-associated kinase; KAPP, kinase-associated protein phosphatase; JM, juxtamembrane domain; LRR, leucine rich repeats; MAPK, mitogen-associated protein kinase; NTAP, N-terminal tandem affinity purification; PAP, peroxidase-anti-peroxidase; PBEZT, probenazole-inducible protein 1; PCD, programmed cell death; POL, POL-like protein; PP, protein phosphatase; PP2C, protein phosphatase 2C; PRR, pathogen recognition receptor; RK, receptor kinase; RNAi, RNA interference; RT-PCR, reverse transcription-PCR; TAK1, transforming-growth-factor-β-activated kinase 1; TLR, Toll-like receptor; Xb15, XA21 binding protein 15; Xoo, X. oryzae pv. oryzae Philippine race 6

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Author Summary

Resistance to pathogens is critical to plant and animal survival. Plants, unlike animals, lack an adaptive immune system and instead rely on the innate immune response to protect against infection. To elucidate the molecular mechanism of plant innate immunity, we are studying the signaling cascade mediated by the rice pathogen recognition receptor kinase XA21, which confers resistance to the bacterial pathogen Xanthomomas oryzae pv. oryzae. We demonstrate that XA21 binding protein 15 (a protein phosphatase 2C) negatively regulates XA21-mediated signaling resistance. This finding provides significant insight into regulation of receptor kinase-mediated immunity.

Plant genome analyses have revealed the presence of a large family of these non-RD IRAK kinases, with more than 45 encoded in the Arabidopsis genome and more than 370 found in the rice genome [10,14]. Members include the Arabidopsis flagellin RK (FLS2), the Arabidopsis elongation factor Tu RK (EFR) [15,16], the rice XA26 and Pi-d2 RKs [17–19], and the rice XA21 RK that mediates recognition of the Gram negative bacteria Xanthomomas oryzae pv. oryzae (Xoo) [18,20]. Because the presence of the non-RD motif in IRAK kinases is correlated with a role of the protein in pathogen recognition, there is great interest in understanding how they are regulated [10,21]. Unlike the majority of RD kinases, where phosphorylation of the activation loop is critical for activation [22], the mechanism of non-RD kinase regulation, in which many non-RD kinases do not autophosphorylate the activation loop, remains to be elucidated [10]. The juxtamembrane (JM) domain, a region of the RK that is N terminal to the kinase domain, has been suggested to be important for regulation of non-RD RKs and to serve as a high affinity binding site for downstream signaling proteins [10,23].

Although non-RD IRAK kinases are clearly essential for innate immunity in both plants and animals, sustained or highly induced immune response can be harmful [24]. It is therefore necessary that PRR signaling through non-RD kinases be under tight negative regulation. For example, misregulation of the non-RD IRAK1 in mice induces activation of nuclear factor kB (NF-kB) and increases inflammatory responses to bacterial infection [25,26].

Dephosphorylation of kinases by protein phosphatases (PPs) is a common mechanism for downregulating kinase-mediated signaling [27]. PPs are classified into two major classes: tyrosine phosphatases and serine/threonine phosphatases, depending on their substrates [27,28]. In humans, a group of MAPK phosphatases (MKPs) including MKP1, MKP5, and dual specificity PPs, negatively regulate the innate immune response [24,27,29].

In contrast to animal systems, negative regulation of kinases involved in plant innate immunity is not well understood. One important class of negative regulators are PP 2Cs (PP2Cs), a group of serine/threonine phosphatases that function as monomers and require Mn2+ and/or Mg2+ for activity [28,30]. One of the best characterized plant PP2Cs is the Arabidopsis kinase-associated PP (KAPP), which interacts with many RKs including FLS2, CLAVATA1 (CLV1), somatic embryogenesis RK 1, brassinosteroid-insensitive 1 (BR11), and BR11-associated RK 1 [31–36]. Overexpression of KAPP in Arabidopsis results in loss of sensitivity to flagellin treatment, suggesting that KAPP negatively regulates the FLS2-mediated defense response [33]. Arabidopsis CLV1 controls stem cell identity in shoot and flower meristems. In vitro, a CLV1 fusion protein can phosphorylate KAPP. Conversely, KAPP dephosphorylates the kinase domain of CLV1 in vitro [3,31,37,38]. So far, KAPP is the only PP known to be involved in regulation of RK-mediated signaling and to be associated with RKs in plants [31,33].

The rice XA21 RK is one of a few plant PRRs that has been studied in depth [18]. Despite the clear biological role for XA21 in the rice innate immune response, very little is known about the mechanism by which XA21-mediated resistance is regulated. Although the rice KAPP protein emerged as a good candidate for being a negative regulator of the XA21-mediated innate immune response, it does not interact with XA21 [39]. This suggests the presence of another protein that negatively regulates XA21-mediated signaling pathway.

In this study, we report the identification and characterization of rice XA21 binding protein 15 (XB15), which encodes a novel PP2C. Transgenic rice lines overexpressing Xb15 display compromised Xa21-mediated resistance to Xoo strains carrying AvrXa21 activity. Conversely, Xb15 Toss1 insertion mutants display cell death, constitutive induction of PR genes, and enhanced Xa21-mediated resistance upon Xoo infection. Subsequent biochemical experiments show that XB15 can dephosphorylate autophosphorylated XA21 in a temporal- and dosage-dependent manner and that a serine residue in the XA21 JM domain is required for XB15 binding. Our findings are consistent with a model in which XB15 associates with the XA21 JM domain to negatively regulate the XA21-mediated innate immune response and cell death.

Results

XB15 Is an XA21 Binding Protein

The rice XA21 protein is representative of the large class of non-RD RKs predicted to be involved in plant innate immunity [10,18,20]. To elucidate the mechanism of XA21-mediated resistance and to identify its interaction partners, we performed a GAL4-based yeast two-hybrid screen using a rice cDNA library constructed from Oryza sativa spp. Indica line, IRkb21. IRBb21 is an isogenic line of the Indica variety IR24 carrying an intragenome at the Xa21 locus [40]. It has been previously reported that both the JM region (the portion of the cytoplasmic domain between the transmembrane sequence and the kinase domain) and the C-terminal region of RKs, can serve as high affinity binding sites for downstream signaling proteins [23]. Therefore, the entire XA21 predicted intracellular region, including the JM, kinase, and C-terminal domains, called XA21K668 (668–1,025 amino acids), was used as bait for this screen. Of the 7 × 10^7 transformants screened, a total of eight unique clones both grew on selective media lacking histidine and tested positive for β-galactosidase reporter gene activity. These clones were named XA21 binding proteins (XBs). We have previously reported characterization of another Xb, called Xb10, which encodes a WRKY transcription factor [41], and Xb13, which encodes an ubiquitin ligase [42]. No RKs were isolated in this screen.

Here, we report the functional characterization of one of the isolated interacting proteins, XB15, the only PP among the isolated XB5s. To assess whether the XA21 JM domain was important for the physical interaction of XB15 with XA21, we
generated a new construct, XA21K(TDG) (residues 705–803), which lacks the JM domain. XA21K(TDG) was then used as bait in a yeast two-hybrid assay with XB15 (Figure 1A). Activation of the lacZ reporter gene was dependent on the simultaneous presence of pAD-XB15 and pLexA-XA21K(TDG). XA21K(TDG) lacking the JM domain did not interact with XB15. This result suggests that the JM domain contains specific amino acids needed for XB15 binding in yeast and that these residues may serve as a docking site for XB15. The JM region (XA21JM) lacking the kinase domain was not sufficient for the interaction with XB15 (Figure 1A). XB15 also failed to interact with the catalytically inactive mutant XA21K(736E), in which lysine 736 is substituted with glutamic acid [43], suggesting that XA21 kinase activity is also important for the interaction.

On the basis of these results, we hypothesized that a residue(s) in the JM domain phosphorylated by XA21 may serve as a docking site for XB15. It has previously been reported that XA21 autophosphorylates residues Ser686, Thr688, and Ser689 in vitro [44]. To test if these residues are important for XB15 binding, we assessed whether mutations in these sites affected binding activity. We found that all three XA21 single mutants, XA21S686A, XA21T688A, and XA21S689A, as well as the triple mutant XA21S686A,T688A,S689A, maintained interaction with XB15. We next assessed the role of three previously uncharacterized Ser and Thr residues (Ser697, Ser699, and Thr705) in the JM region of XA21. When Ser697 is mutated to Alanine, interaction with XB15 is abolished (Figure 1A). This result indicates that Ser697 in the XA21 JM domain is critical for interaction with XB15.

To confirm that the absence of interaction was not due to lack of expression of the fusion constructs in yeast cells, we performed protein gel blot analysis with anti-XB15 or anti-LexA antibodies. All yeast cells transformed with AD-XB15 carried an 80-kDa band cross-reacting with the anti-XB15 antibody (unpublished data). In Western blot analysis using an Anti-LexA antibody, yeast cells expressing LexA-XA21K668 or -XA21 variants, displayed bands with molecular weights corresponding to the correct size of each fusion protein (Figure 1B). Yeast cells expressing the vector control produced a protein of 24 kDa that reacted with the anti-LexA antibody.

**XB15 Encodes a PP2C with Similarity to Arabidopsis POLTERGEIST**

XB15 carries a 3,219-bp open reading frame that consists of three introns with lengths of 94 bp, 1,070 bp, and 138 bp and four exons. The ORF is predicted to encode a 639 amino acid protein (Figure 2A) with a molecular mass of 69.2 kDa and an isoelectric point of 5.4. XB15 is similar in overall structure to known plant PP2Cs, with a conserved C terminus (240–630 amino acids), which is predicted to have phosphatase catalytic activity (Figure 2A, underlines). It has a unique N terminus (1–239 amino acids) with no similarity to proteins with known function. The catalytic domain is interrupted by approximately 100 amino acids with no similarity to any sequences in the database (Figure 2A, with italics). XB15 contains six amino acid residues (four aspartic acids, one glutamic acid, and one glycine) known to interact with two Mg²⁺ or Mn²⁺ ions to form a binuclear metal center in Arabidopsis POL and human PP2Ca [45–47].

We next compared the PP2C domain of XB15 with other evolutionary related proteins from the reference plant species rice and Arabidopsis, such as Arabidopsis POL and POL-like proteins (PLLs) (Figure 2B). XB15 shows significant similarity to PP2Cs from other organisms including Arabidopsis POL (47% identity) and shares several features with the Arabidopsis POL and PLLs such as unique intron/exon boundaries and an insertion in the same region of the PP2C catalytic domain. Figure 2B shows the phylogenetic relationship among POL and PLL genes from rice and Arabidopsis based on the amino acid sequence of the last three exons. Arabidopsis POL and PLL1 cluster in one branch of the tree, consistent with the similar primary phenotype of pol and pll mutants—phenotypic suppression of clv mutants [48,49]. These two proteins have also been shown to regulate the balance between stem-cell maintenance and differentiation and are closely related to Wuschel, which encodes a homeodomain transcription factor expressed in shoot meristems [50]. XB15 groups with Arabidopsis PLL2, PLL3, PLL4, and PLL5, showing the greatest similarity to PLL4 and PLL5.
with 57.5% and 56.3% identity, respectively. \(\text{pll4}^\text{and} \text{pll5}\) mutants develop abnormal leaves that are altered in shape [48]. To date, no function has been reported for \(\text{Arabidopsis} \) \(\text{PLL2} \) or \(\text{PLL3} \), or any of the rice \(\text{PLLs} \) including \(\text{XB15} \), leaving the functional role of these proteins unknown.

**XB15 Functions as a Negative Regulator in the XA21-Mediated Innate Immune Response**

To explore the role of XB15 in resistance, we examined whether constitutive overexpression of XB15 would affect resistance to \(\text{Xoo} \). We first generated an \(\text{XB15} \) tagged N-terminal tandem affinity purification (NTAP) construct under the control of the ubiquitin promoter \((\text{Ubi}) \) [51,52] and then introduced this construct into the rice cultivar \(\text{Kitaake} \) using our established transformation procedures [53,54]. Six-week-old progeny from self-pollinated \(\text{XA21/XB15} 17\text{A-18}, 17\text{A-21}, 19\text{A-64}, \) and 19A-72 were then inoculated with \(\text{Xoo} \) PR6 strain PXO99 expressing AvrXA21 and examined for cosegregation of genotype with phenotype by PCR analysis and measurement of the length of \(\text{Xoo} \)-induced lesions. All
Myc-Xa21 plants overexpressing Ntap-Xb15 (+/+) by PCR analysis showed accumulation of NTAP-XB15 in protein gel blot analysis and displayed enhanced susceptibility to Xoo PR6 compared to transgenic plants carrying Myc-Xa21 alone (++/−) (Figure 3B). Western blot analysis revealed a higher accumulation of NTAP-XB15 protein in XA21/XB15 19A line compared to XA21/XB15 17A, which correlated with longer leaf lesions developed by Xoo PR6, indicating that XB15 negatively regulates the XA21-mediated defense pathway in a dosage-dependent manner.

We could barely distinguish a difference in XA21 accumulation with homozygous or heterozygous Myc-XA21 transgenic plants (Figure S1). Therefore, to rule out the possibility that this phenotypic difference is caused by a Xa21 dosage effect, we selected segregants (F2) carrying Myc-Xa21 and analyzed the next generation (F3) to determine which segregants were heterozygous or homozygous for Myc-Xa21. After PCR genotyping 20 progeny from each line, we identified 17A-18-1, 17A-18-2, and 19A-72-5 as homozygous for Myc-Xa21 (unpublished data). The remaining segregants are heterozygous for Myc-Xa21.

Inoculation analysis revealed that there is no correlation between the number of copies of Myc-Xa21 and...
the enhanced susceptibility phenotype in the NTAP-XB15 overexpression lines (Figure 3B). These results indicate that the enhanced susceptibility observed in the crossing population (F2) is caused by NTAP-XB15 overexpression and is not due to the presence of fewer copies of Myc-Xa21.

Figure 3C shows a picture of two typical leaves from each of the following inoculated rice plants: F2 segregants of XA21/XB15 17A and 19A, and transgenic plants overexpressing NTAP only (NTAP) 16 d after Xoo PR6 inoculation. The Myc-XA21 plants lacking Ntap-Xb15 (++) displayed a cell death phenotype similar to that observed in the homozygous individuals (++). In contrast, all the defense-related genes tested, PR1a, PR1b, PR10, Betula

PP2C Regulates Rice Innate Immunity

To further investigate the in vivo function of Xb15, a knockout mutant line was identified from a collection of rice mutants generated by random insertion of the endogenous retrotransposon Tos17 (http://tos.nias.affrc.go.jp/~miyao/pub/tos17/) [55]. One mutant line (NF9014) carried a Tos17 insertion in the third exon of the Xb15 gene (Figure 5A). This insertion resulted in a lack of expression of the full-length Xb15 mRNA compared to the wild type Nipponbare plants when tested with appropriate primers by reverse transcription-PCR (RT-PCR) (Figure 5B). 18S ribosomal RNA (18S rRNA) was used as an internal control. The homozygous insertion mutant line (--) showed a severe cell death phenotype marked by the appearance of small necrotic lesions that were apparent at the vegetative stage (Figure 5C). Of the 24 segregants of 6-16-30, six out of eight of the heterozygotes (++) displayed the cell death phenotype. Two of the heterozygotes displayed a severe cell death phenotype similar to that observed in the homozygous individuals (--). In contrast, all nine homozygous (--) plants displayed a cell death phenotype and seven of these were very severe (Figure S2).

We next tested if the cell death phenotype correlated with alterations in PR gene expression. Total RNA was extracted from the mutant line and the Nipponbare control. RT-PCR was performed with primers targeting PR genes as molecular markers. Figure 5D shows that the expression of full-length Xb15 was not detected in the Tos17 mutant line. In contrast, all the defense-related genes tested, PR1a, PR1b, PR10, Betula

A Retrotransposon Insertional Line of Xb15 Exhibits Cell Death

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verrucosa 1 (Betv1, major birch allergen), and probenazole-inducible protein 1 (PBZ1) were highly expressed in the insertion mutant lines (+/C0) but barely expressed in Nipponbare wild type (WT) or the no-insertion null plants (+/+). Both the internal controls (18S rRNA and elongation factor 1α [EF1α]) showed constitutive expressions in all tested plants.

To confirm that the cell death phenotype of the Tos17 insertion mutant is due to the loss of function of XB15, we constructed a plasmid for the gene-specific knockdown of XB15 by RNA interference (RNAi) with an inverted repeat of a specific fragment of XB15 cDNA under control of the Ubi promoter. The protein level of XB15 in XB15 RNAi transgenic rice plants was severely reduced compared with the Kitaake control (Figure S3A). We observed a similar cell death to that detected in the Tos17 insertion mutant line in several independent RNAi lines (Figure S3B). Taken together, these results show that loss-of-function of XB15 induces expression of defense-related genes and elicits a cell death phenotype, suggesting a negative regulatory role for XB15 in the defense signaling pathway.

A Retrotransposon Insertional Xb15 Mutant Carrying Xa21 Shows Enhanced Resistance

We have shown that the Xb15 Tos17 insertion mutant line (NF9014) accumulates PR gene transcripts (Figure 5). On the basis of this result, we hypothesized that these lines would be more resistant to Xoo PR6 and because Nipponbare plants are already moderately resistant to Xoo PR6 and because we are pushing the limits of sensitivity of our bacterial assay, we were unable to detect enhanced resistance in these lines (unpublished data).

To further explore the role of Xb15 in resistance, we carried out additional experiments in the Kitaake genetic background, which is highly susceptible to Xoo PR6 (Figures 3 and 4). In this experiment, we crossed the Xb15 Tos17 insertion mutant line (NF9014–1) (pollen recipient) with a Kitaake line containing Myc-XA21 under control of its native promoter (pollen donor). We then tested if the loss of XB15 function alters the XA21-mediated defense response. We were able to simultaneously test for both reduced and enhanced resistance by taking advantage of the fact that the resistance conferred by XA21 is developmentally regulated. At the juvenile two-leaf stage (~2 wk old), the plants are fully susceptible. Full resistance develops only at the adult stage [56]. We therefore inoculated progeny (F4) from the cross (NF9014–1/Myc-XA21) at 3 wk old when XA21 resistance is not yet fully active. At this stage of development, XA21 rice plants are only partially resistant (approximately 40% of resistance to that of 6-wk-old plants) [56]. Because lesion development continues to 16 d (Figure 4), we measured the lesion lengths at 18 d to capture as large a difference as possible (Figure 6A).

We found that progeny carrying Xa21 and the Xb15 Tos17
resistance caused by the putative PP2C activity of XB15. Full length XB15 (GST-XB15FL) tagged with an N-terminal tagged glutathione-S-transferase (GST) recombinant fusion protein and GST protein alone were expressed in E. coli. A smaller clone expressing the PP2C catalytic region alone, without the N-terminal extension (GST-XB15ΔN) was also expressed to check its possible regulatory function. We purified and assayed the recombinant proteins for phosphatase activity by measuring the release of phosphate from a phosphorylated synthetic peptide [57]. Figure 7A shows that in the presence of the substrate, both GST-XB15FL and GST-XB15ΔN, but not GST alone, catalyze the release of 240–350 pmoles of phosphate. This reaction was inhibited by the serine/threonine phosphatase inhibitor, sodium fluoride (NaF), but not by vanadate, a tyrosine phosphatase inhibitor. These results indicate that XB15 encodes a protein serine/threonine phosphatase not a tyrosine phosphatase, as predicted on the basis of a sequence analysis that places XB15 in the PP2C clade.

To experimentally test which class of serine/threonine phosphatases XB15 falls into, we incubated the recombinant GST-XB15FL, GST-XB15ΔN, and GST proteins with the substrate in various reaction buffers optimized for activity for different protein PP classes, PP2A, PP2B, and PP2C (Figure 7B) [57]. As predicted, XB15 enzyme activity was detected in PP2C buffer but not in PP2A and PP2B buffer. The presence of Mg2+ in the PP2C buffer was required for full activity. Significant inhibition was observed in PP2B buffer with saturating amounts of calmodulin and additional Ca2+. While GST-XB15FL showed a slightly lower PP activity than GST-XB15ΔN, unlike POL, inhibition of phosphatase activity by the N-terminal domain appears to be minimal (Figure 7A and 7B).

To test whether plant-expressed XB15 has PP2C activity, we used the NTAP-XB15 transgenic plants described above. The NTAP tag includes two IgG binding domains from Staphylococcus aureus protein A and a calmodulin binding peptide linked by a TEV protease cleavage site [51,52]. This tag has been successfully used for purification of native protein complexes from yeast, plants, and animals [52,58,59]. The NTAP-tagged XB15 (NTAP-XB15) and NTAP alone were purified with IgG-agarose and assayed for their phosphatase activity in PP2C buffer. Figure 7C shows that NTAP-XB15 purified from transgenic rice plants carries PP2C activity, but not purified NTAP alone or boiled NTAP-XB15. These experiments demonstrate that XB15 encodes a serine/threonine protein PP2C.

## XB15 Interacts withXA21 In Vitro and In Vivo

XB15 was originally isolated as one of XA21 binding proteins using the yeast two-hybrid screen. To rule out the possibility of false positive caused by nonspecific binding to XA21, the specificity of the interaction between XA21 and XB15 was confirmed using a GST pull-down assay with immobilized GST-XA21K668. GST-XA21K668 and HIS-

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**Figure 6.** AXA21 Rice Plants Carrying XB15 Tos17 Gene Exhibit Increased Resistance of Xoo

(A) Three-week-old segregants (F4) of NF9014–1 (Tos17 XB15) and Myc-XA21 were inoculated with Xoo PR6 and lesion lengths were measured 18 DAL. Each data point represents the average and standard deviation of at least three samples.

(B) Bacterial populations were determined at 0 and 18 DAL. For each time point, the mean of the bacterial cfu/leaf from three plants per genotype is shown. Error bars indicate the standard deviation. The experiment was repeated three times with similar results.

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mutation (xb15xb15, Xa21l–), displayed enhanced resistance to Xoo PR6 with lesions ranging length from 1–2 cm compared to the segregants carrying Xa21 alone (Xb15Xb15, Xa21l–), which showed approximately 4-cm lesion lengths. The difference was statistically significant. In contrast, although enhanced resistance was observed in the Xb15 Tos17 mutant lines lacking Xa21 (xb15xb15, Xa21l–) as compared to the wild-type Xb15 line (Xb15Xb15, Xa21l–), this difference was not statistically significant (Figure 6A). This result indicates that, using our currently available methods, the enhanced resistance caused by the Xb15 mutation can be detected only in the presence of XA21.

To further validate the enhanced resistance phenotype conferred by the Xb15 Tos17 mutation in the XA21 genetic background, we measured bacterial populations in these lines. In Figure 6B, we show that Xoo populations multiplied to 9.2 × 10^7 cfu/leaf in the XA21 plants carrying the Xb15 Tos17 mutation (xb15xb15, Xa21l–). In contrast, in the absence of the Xb15 Tos17 mutation (Xb15Xb15, Xa21l–), bacterial populations reached to 3.5 × 10^6 cfu/leaf a 3.8-fold increase compared to the Xb15 Tos17 mutant lines. These results clearly show that loss of XB15 function enhances XA21-mediated resistance.

**XB15 Has PP2C Activity**

To elucidate the mechanism of XB15 negative regulation of XA21-mediated resistance, we initiated in vitro biochemical studies. We first examined whether XB15 encodes a functional PP2C and if alterations in XA21-mediated resistance are caused by the putative PP2C activity of XB15. The NTAP tag includes two IgG binding domains from Staphylococcus aureus. A smaller clone of the NTAP-XB15 (NTAP-XB15ΔN) was also expressed to check its possible regulatory function. The purified and assayed the recombinant proteins for phosphatase activity by measuring the release of phosphate from a phosphorylated synthetic peptide [57]. Figure 7A shows that in the presence of the substrate, both GST-XB15FL and GST-XB15ΔN, but not GST alone, catalyze the release of 240–350 pmoles of phosphate. This reaction was inhibited by the serine/threonine phosphatase inhibitor, sodium fluoride (NaF), but not by vanadate, a tyrosine phosphatase inhibitor. These results indicate that XB15 encodes a protein serine/threonine phosphatase not a tyrosine phosphatase, as predicted on the basis of a sequence analysis that places XB15 in the PP2C clade.

To experimentally test which class of serine/threonine phosphatases XB15 falls into, we incubated the recombinant GST-XB15FL, GST-XB15ΔN, and GST proteins with the substrate in various reaction buffers optimized for activity for different protein PP classes, PP2A, PP2B, and PP2C (Figure 7B) [57]. As predicted, XB15 enzyme activity was detected in PP2C buffer but not in PP2A and PP2B buffer. The presence of Mg2+ in the PP2C buffer was required for full activity. Significant inhibition was observed in PP2B buffer with saturating amounts of calmodulin and additional Ca2+. While GST-XB15FL showed a slightly lower PP activity than GST-XB15ΔN, unlike POL, inhibition of phosphatase activity by the N-terminal domain appears to be minimal (Figure 7A and 7B).

To test whether plant-expressed XB15 has PP2C activity, we used the NTAP-XB15 transgenic plants described above. The NTAP tag includes two IgG binding domains from Staphylococcus aureus protein A and a calmodulin binding peptide linked by a TEV protease cleavage site [51,52]. This tag has been successfully used for purification of native protein complexes from yeast, plants, and animals [52,58,59]. The NTAP-tagged XB15 (NTAP-XB15) and NTAP alone were purified with IgG-agarose and assayed for their phosphatase activity in PP2C buffer. Figure 7C shows that NTAP-XB15 purified from transgenic rice plants carries PP2C activity, but not purified NTAP alone or boiled NTAP-XB15. These experiments demonstrate that XB15 encodes a serine/threonine protein PP2C.
XB15ΔN were expressed in *E. coli* and purified using glutathione and nickel nitritolriacetic (Ni-NTA) agarose beads, respectively. The purified recombinant HIS-XB15ΔN protein was incubated with glutathione beads bound to either GST-XA21K668 or GST alone in a binding buffer containing 2 mM MgCl₂. Figure 8A shows that the recombinant protein HIS-XB15ΔN specifically interacts with GST-XA21K668 (lane 2) but not GST (lane 3) or glutathione beads (lane 1).

To further investigate the association between XA21 and XB15 in vivo, we used the Myc-XA21 transgenic plants described above. Anti-Myc antibody detected a 140-kDa polypeptide in transgenic plants carrying Myc-Xa21 but not in the control line Kitaake (Kit) (Figure 8B). When the Myc-XA21 protein is enriched after immunoprecipitation (IP) with agarose conjugated anti-Myc antibody, an additional 100-kDa product was detected. We have previously reported that the 140-kDa polypeptide is Myc-XA21 and the 100-kDa polypeptide is a proteolytic cleavage product of Myc-XA21 (Myc-XA21cp) [42]. We then developed a polyclonal antibody against a synthetic peptide of XB15 (anti-XB15) to detect the XB15 protein in planta and confirmed its specificity against XB15 from rice extracts. Anti-XB15 detected a major band of 70 kDa, which is close to the predicted size of XB15 (69.2 kDa) (Figure 8C). In the NTAP-XB15 overexpression line 17A and 19A, there was a significant increase in NTAP-XB15 (95 kDa) compared to endogenous XB15 (70 kDa).

When rice protein extracts were incubated with agarose conjugated anti-Myc antibody, an immune complex containing a 140-kDa polypeptide was precipitated and detected after western blot analysis with anti-Myc antibody in Myc-XA21 transgenic plants inoculated with *Xoo* PR6 but not in the Kitaake control (Figure 8D). Although the same amount of total protein extract was used for each immunoprecipitation, the amount of Myc-XA21 protein precipitated by anti-Myc-antibody accumulated to greater amounts at 12 and 24 h after *Xoo* PR6 inoculation as compared to the 0-h time point. Next, we examined whether XB15 was co-immunoprecipitated with XA21 in the immune complex. A 70-kDa polypeptide was detected with the anti-XB15 antibody in *Xoo* PR6-inoculated protein extracts. This band was not detected in Kitaake lacking Myc-Xa21 and was barely detectable in mock-treated Myc-XA21 plants. The association between XB15 and XA21 was detectable by 12 h after inoculation with *Xoo* PR6 and had significantly increased after 24 h. As a control, similar experiments were performed with agarose-conjugated anti-Myc antibody presaturated with Myc peptide to check for a nonspecific interaction of XB15 with the Myc peptide (unpublished data). These results demonstrate a direct interaction between XB15 and XA21 in leaves, consistent with our in vitro observations.

**XA21 Is a Substrate of XB15**

We have previously shown that XA21 encodes a protein kinase and that autophosphorylation of the XA21 kinase domain occurs via an intramolecular mechanism [43]. Because XB15 is associated with XA21 both in vitro and in vivo, because the overexpression of XB15 compromises the

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**Figure 7. XB15 Possesses PP2C Catalytic Activity**

(A) XB15 protein is a serine/threonine phosphatase. Five micrograms of GST-XB15FL (full length XB15), GST-XB15ΔN (catalytic domain only), or GST was incubated with: no substrate, 100 μM substrate, 100 μM substrate plus 1 mM sodium vanadate, 100 μM substrate plus 50 mM NaF.

(B) XB15 protein is a PP2C. Five micrograms of GST-XB15FL, GST-XB15ΔN, or GST was incubated with 100 μM substrate in each of the three buffers specific for PP2A, PP2B, and PP2C activity.

(C) The XB15 protein purified from rice plants carries a PP2C activity. NTAP-XB15 and NTAP were purified from transgenic plants and 1 μg of each protein was incubated with 100 μM substrate in PP2C buffer. For the boiled NTAP-XB15, 1 μg of NTAP-XB15 was boiled in water for 20 min. The data are average value of three experiments. Error bars represent standard deviations.

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PP2C Regulates Rice Innate Immunity
XA21-mediated resistance, and because XB15 possesses PP2C activity, we hypothesized that XB15 could directly dephosphorylate XA21. We tested this hypothesis by checking if XB15 could dephosphorylate the in vitro autophosphorylated XA21. After expression in *E. coli* and purification, GST-XA21K668 bound to glutathione beads was autophosphorylated with $[^{32}P]_\gamma$ in vitro. Autophosphorylation was monitored by radioactive incorporation of $[^{32}P]$-ATP into the bead-binding fraction (unpublished data) and by SDS-PAGE. The exposure to X-ray film shows that the fusion protein, GST-XA21K668 is capable of autophosphorylation (Figure 9A, 0 min).

The $^{32}P$-autophosphorylated GST-XA21K668 was incubated with or without 1 µg of purified recombinant HIS-XB15AN in the presence of PP2C buffer for the indicated time (Figure 9A). While XA21K668 remained labeled for over 60 min in the absence of XB15 (unpublished data), dephosphorylation of XA21K668 was detectable after 1 min of incubation with XB15. Additionally, dephosphorylation of XA21K668 by HIS-XB15AN was not detected in the presence of serine/threonine PP inhibitor, NaF (20 mM) or when HIS-XB15AN (1 µg) was boiled (unpublished data) suggesting that the dephosphorylation of XA21 is caused by the PP2C activity of XB15. In further support of this conclusion, increasing the amount of HIS-XB15AN (1–10 µg) resulted in a dosagedependent dephosphorylation of GST-XA21K668 (Figure 9B).

Although XA21-dependent phosphorylation of XB15 was examined with recombinant proteins purified from *E. coli* at various time points and different conditions, we did not detect transphosphorylation of XB15 by XA21 (unpublished data). From these results, we conclude that XA21 is a substrate of XB15 and that the XB15 phosphatase can effectively dephosphorylate XA21.

**An XB15-smGFP2 Fusion Protein Localizes to the Plasma Membrane**

To further elucidate XB15 function, we assessed its subcellular localization. According to MultiLoc (http://www-bs.informatik.uni-tuebingen.de/Services/MultiLoc) [60] and PSORT (http://www.psort.org/) [61], XB15 has a putative nuclear-localization sequence (Figure 2) and is predicted to be a nuclear protein. To investigate the in vivo cellular distribution of XB15, a targeting experiment was performed using smGFP2, as a fluorescent marker [62]. We fused the entire *Xb15* coding region without the termination codon to the *smGFP2* gene (Figure S4A), and the resulting construct was introduced into rice *Kitaake* protoplast cells by PEG-mediated transformation [63,64]. Localization of the fusion protein was determined by visualization with an Olympus FV1000 confocal microscope. We introduced the *smGFP2* gene alone into protoplast cells as a control. As shown in Figure S4B, the fusion protein was mainly localized to the plasma membrane of the rice protoplast cell, whereas the control *smGFP2* was uniformly distributed throughout the cell except the large central vacuole. As a positive control for plasma membrane targeting, we cotransfected protoplasts with vector expressing a H–ATPase-red fluorescent protein (dsRed) fusion protein, which also localized to the plasma membrane [65]. A close overlap was observed between the green and red fluorescent signals of XB15-smGFP2 and H–ATPase-dsRed, respectively. This experiment was repeated with protoplasts extracted from transgenic rice carrying *xa21* gene and same localization of XB15 to the plasma membrane was observed (unpublished data).

We next tested if the large green fluorescent protein (GFP) tag would inhibit XB15 phosphatase activity. XB15-smGFP2 and smGFP2 alone were purified from transiently trans-
Coomassie brilliant blue R250 and destained (Coomassie). For the localization of the intact XA21 protein [44].

**Recruitment of XB15 to the JM Region of XA21**

In animals, some receptor tyrosine kinases are controlled through autophosphorylation of their JM domains. For example, the mouse ephrin and KIT receptors, and human Fms (Feline McDonough Sarcoma)-like tyrosine kinase 3, interact with protein tyrosine phosphatases via phosphorylated tyrosine residues in their JM domains. These interactions negatively regulate receptor tyrosine kinase-mediated signaling [68–71]. In plants, only a few RKs have been shown to autophosphorylate residues in their JM regions. These include barley HvLysMR1, legume symbiosis RK, *Arabidopsis* BRI1, and rice XA21 [43,72–76]. In spite of the presumed importance of the JM domain in plant RK-mediated signaling, no downstream target proteins that bind the JM regions and regulate RK signaling in a phosphorylation-dependent manner have yet been identified.

XB15 failed to interact with the XA21K(TDG) mutant lacking the XA21 JM region and the catalytically inactive mutant XA21K668K736E (Figure 1), suggesting that XA21 autophosphorylation in the JM region is critical for the XB15/XA21 interaction [43]. Although autophosphorylation of XA21 has not been demonstrated in yeast, there have been many reports showing that plant proteins maintain their function when they are expressed in yeast as heterologous proteins [77–79]. For example, an *Arabidopsis* kinase, GSK3/3 shaggy-like protein rescues the phenotype of its yeast homolog *mck1*, suggesting that plant kinases in yeast cells undergo the necessary post-translational modification for their in vivo functions [78]. Autophosphorylation of the JM residues Ser686, Thr688, and Ser689 was previously shown to be important stabilizers of XA21 protein levels [44]. XA21 proteins with mutations in these residues maintained interaction with Xb15, suggesting that these phosphorylation sites mainly function in the stability of XA21 protein but are not directly involved with mediating the downstream signal transduction cascade. In contrast, when Ser697 is mutated to knockout mutants for *pll4* and *pll5* and transgenic plants overexpressing *Pl1* [48]. Despite the sequence similarity with POL and PLLs, we observed no disorders of leaf development in the Xb15 knockout mutant and RNAi plants. Additionally, transgenic plants overexpressing Xb15 displayed normal leaf development. These results indicate that XB15 has distinct functions to that of the *Arabidopsis* POL and PLLs.

The apparent differences in the function of XB15 and *Arabidopsis* POLs may be caused by their different localizations. *Arabidopsis* POL contains a putative nuclear-localization motif [47] and is predicted to be a nuclear protein according to the prediction methods for subcellular location, MultiLoc, and PSORT. In contrast, the fusion protein, XB15-smGFP2 is mainly localized to the plasma membrane of rice protoplast cells, suggesting that the in vivo function of XB15 is primarily related to the plasma membrane itself or to other membrane-localized proteins (Figure S4). Based on sequence analysis, XA21 is predicted to localize to the plasma membrane like other RKs, but its subcellular localization has not yet been demonstrated in vivo. Our in vivo data of XB15 combined with the putative localization of XA21 suggest that the interaction between XB15 and XA21 occurs at the plasma membrane, and may be similar to KAPP, which interacts with its target RKs at the plasma membrane to attenuate their signaling [34,36,67].

**Discussion**

Recognition of pathogen-associated molecules by PRRs is a critical component of innate immunity in animals and plants [66]. The rice XA21 protein is representative of a very large class of plant PRRs that carry the non-RD motif in the kinase domain [10]. The regulation of this important subclass of kinases has not yet been elucidated. Here, we have shown that rice employs a novel PP2C, XB15, to attenuate the XA21-mediated innate immune response.

**Functions and Subcellular Localization of XB15 Versus Arabidopsis POLs**

XB15 possesses an active PP2C domain in its C-terminal region. On the basis of sequence comparison and phylogenetic analysis among PP2Cs from the rice and *Arabidopsis* databases, XB15 shows relatively high similarity with *Arabidopsis* POL, PLL4, and PLL5 (Figure 2B). All cluster into a small subgroup of the rice/*Arabidopsis* phylogenetic tree.

Abnormal leaf development was observed in *Arabidopsis*
Alanine, interaction with XB15 is abolished, indicating that Ser697 is essential for XA21 binding with XB15 in yeast. The Ser697 mutation does not affect kinase activity of XA21S697A, suggesting that Ser697 serves as a high affinity binding site rather than as a regulator of kinase activity (unpublished data, X. Chen, P.E. Canlas, M. Chern, D. Ruan, R. Bart, et al.). Taken together, these results suggest that XA21 requires its own kinase activity and residues in the JM region for interaction with XB15.

In support of this hypothesis, our preliminary results suggest that transgenic rice carrying the XA21 variant, XA21S697A, display enhanced resistance to Xoo PR6 at the 3-wk-old stage (unpublished data, X. Chen, P.E. Canlas, M. Chern, D. Ruan, R. Bart, et al.). These results indicate that Ser697 is essential for XA21 binding with XB15 and that in the absence of XB15 binding to XA21, negative regulation is compromised leading to enhanced resistance.

**Figure 10.** Comparison of Mammal, Arabidopsis, and Rice PRR-Mediated Signaling

The LRR domain of TLR4 recognizes lipopolysaccharide signal (LPS) and transduces the signal across the cell membrane [91]. An adaptor molecule myeloid differentiation factor 88 (MyD88) associated with TLR4 recruits the non-RD serine/threonine kinase, IRAK1, which associates with tumor necrosis factor receptor associated factor 6 (TRAF6) in cytoplasm [102]. TRAF6 forms a complex with TAK1 [93] in which the kinase activity of, TAK1 mediates downstream events, such as inhibitor of kinase complex and NF-κB [5,95].

In Arabidopsis, flag22 binds to FLS2, which transduces the pathogen signal through its cytoplasmic non-RD kinase domain, and activates MAPK cascade [15]. Several WRKY transcription factors acting downstream of MAPK cascade to induce defense-related genes including PR genes [97]. KAPP, a PP2C, blocks the activated FLS signaling and attenuates the downstream innate immune response [33]. In rice, AvrXa21 binding to XA21 activates its cytoplasmic non-RD kinase [18,20]. XA21 trans-phosphorylates proteins such as X3B, which are required for the defense response [42]. In the nucleus, WRKY transcription factors activate defense-related genes such as PR1 and PR10 of XA21 signaling pathway. Recruitment of XB15 to Ser697 in the XA21 JM domain and subsequent dephosphorylation of phosphorylated residue(s) attenuates XA21 signaling.

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**Xb15 Negatively Regulates Cell Death and the XA21-Mediated Innate Immune Response**

In both animals and plants, PCD is a highly regulated process involved in immunity and other functions [66]. Many mutants exhibiting spontaneous PCD, initially isolated in maize [80], have now been identified in other plants, including Arabidopsis, barley, and rice [80–86]. Analysis of these mutants has led to the identification of genes that regulate cell death [87].

The Xb15 Tos17 insertion mutant line, NF9014, and Xb15 RNAi transgenic rice lines, RNAiXB15, display spontaneous cell death on the leaves during development under green house condition (26–28 ºC) in the absence of obvious stress or disease. All of the tested defense-related PR genes commonly associated with the PCD and defense response [87–89] are constitutively expressed in the NF9014 mutant. In addition, progeny (F4) carrying both Xa21 and the Xb15 Tos17 mutation derived from the cross (NF9014–1/Myc-XA21) shows enhanced resistance to Xoo PR6, indicating that XA21-mediated resistance is enhanced in the absence of XB15 (Figure 6). In the absence of XA21, we do see enhanced resistance, although this difference is not statistically significant. We believe this unexpected result is due to the limitations in sensitivity of our assay.

The PCD-like phenotype observed in plants lacking Xa21 suggests the presence of an additional signaling cascade that is negatively regulated by a functional XB15 (Figure 5). There are many examples of PP2Cs that function as negative regulators of multiple kinase cascades. In addition to Arabidopsis KAPP, which associates with at least five RKs [31–36], mouse PP2Cα dephosphorylates and negatively regulates MKKks, apoptosis signal-regulating kinase 1 and transforming-growth-factor-β-activated kinase 1 (TAK1) [90]. In this report, we provide evidence that XB15 regulates at least one, but likely more, RK-mediated pathways, controlling PCD-like lesions.

**Model for XB15 Regulation of the XA21-Mediated Defense Response**

Figure 10 compares our working model for XA21-mediated innate immunity with the human TLR4 and Arabidopsis FLS2 signaling cascades. TLR4 contains an extracellular LRR that is critical for transmitting the lipopolysaccharide signal (LPS) across the cell membrane [91]. An adaptor molecule, myeloid differentiation factor 88 (MyD88), associated with the Toll/interculein-1 receptor (TIR) intracellular domain of TLR4, recruits IRAK4 [5]. The activated IRAK4 rapidly phosphorylates the non-RD kinase IRAK1, and leaves the receptor complex to interact with the tumor necrosis factor receptor associated factor 6 (TRAF6) [92]. TRAF6 is autoubiquitinated and forms a complex with TAK1, which functions as a MAPK kinase kinase [93,94]. The activated kinase TAK1 mediates downstream events, such as the activation of inhibitor of kinase complex, p38, and Jun-N-terminal kinases, which lead to the activation of transcription factors including NF-κB and activating protein-1 [5,95].

Similar to TLR4, Arabidopsis FLS2 also carries an extracellular LRR domain that recognizes a pathogen-associated molecule; in this case a small peptide called flag22 [15]. FLS2 contains an intracellular non-RD kinase, which, when activated, transduces the signal to a MAPK cascade [96–98]. WRKY transcription factors in the nucleus are activated by the MAPK cascades and turn on downstream target genes [97]. KAPP has been shown to negatively regulate FLS2-mediated signaling through overexpression studies, although the mechanism has not yet been elucidated [33].

In rice, the XA21 LRR domain is responsible for race-
specific recognition of Xoo strains carrying AvrXa21 [20,99]. Xa21/AvrXa21 binding is hypothesized to activate the non-RD kinase domain leading to Xa21 autophostorylation and/or transphosphorylation of downstream target proteins [20,100]. In support of this hypothesis we have observed a strong interaction between XB15 and Xa21 in Xoo Pr6-inoculated rice plants but not in untreated rice (Figure 8D), suggesting that Xoo inoculation induces Xa21/XB15 complex formation. Our data indicate that Ser697 in the Xa21 JM region is required for XB15 binding.

Xa21 transphosphorylates the RING finger ubiquitin ligase XB3, which is required for effective Xa21-mediated resistance [41,92]. We have also shown a direct, regulatory role of XB10 (OsWRKY62) in the Xa21-mediated response [41], suggesting another layer of conservation between the Arabidopsis FLS2 and rice Xa21 signaling pathways. In the nucleus, XB10 and other WRKY transcription factors (unpublished data, Y. Peng, L.E. Bartley, and P.C. Ronald) [41] either activate or repress defense-related genes such as PR1 and PR10.

The Xa21-mediated defense response is also regulated by rice negative regulator of resistance (NRR) that interacts with NHI [53,54], the rice ortholog of NPR1, a key regulator of the SA-mediated defense pathway (unpublished data). This result demonstrates cross-talk between the Xa21- and NHI-mediated pathways.

In contrast to animal PPs that inactivate phosphorylated MAPKs to negatively regulate the innate immune response [24], XB15 directly interact with the non-RD kinase domain of Xa21. This study suggests that Xa21 is a substrate of XB15 and that the phosphatase activity of XB15 attenuates the Xa21-mediated innate immune response. Future studies will be directed at identifying the Xa21 phosphorylated residue(s) targeted by XB15 for dephosphorylation.

**Materials and Methods**

Plant material and growth conditions. Rice (Oryza sativa L.) plants were maintained in the green house. The growth chamber was set on a 16-h light and 8-h dark photoperiod, a 28/26 °C temperature cycle, and 90% humidity. Healthy and well-expanded leaves from 6-wk-old rice plants were used for Xoo Pr6 inoculation and nucleic acid or protein extraction. Xa21K668 and Xa21K(TD) were amplified using the primer pairs 5’-CACCATGTC AATCTC- TACTTCCAAGAATGCTGCTCCA-3’ and 5’-CACC ACAAAGTAATGCAAGCTGAC-3’; 5’-CACCATGGG CAACAGCTGG-3’. Gene fragments were cloned into pGEM-T Easy and sequenced (pGEM-T Easy kit, Promega). Recombinant Xa21K668 was used to transform the bacterial host strain BL21 (DE3) (Invitrogen) with pLysS (Invitrogen), and expression of protein was induced at midlog phase (1 mL isopropyl β-D-thiogalactoside, 3 h, 28 °C). Recombinant proteins were purified by affinity chromatography using Glutathione Sepharose 4B (Amersham) or Ni-NTA bead (Qiagen). For the purification of the recombinant protein from rice, leaves of 5- to 6-wk-old NTAP-XB15 transgenic plants were harvested essentially as previously described [51,52]. Five grams fresh weight of rice shoots were ground to a fine powder in liquid nitrogen. Crude protein extracts were prepared in four volumes of Extraction Buffer I (20 mM Tris-HCl [pH 8.0], 150 mM ethylene-diamine-tetra-acetic acid (EDTA), 2 mM benzamidine, 0.1% IGEPA, 10 mM β-mercaptoethanol, 20 mM NaF, 1 mM phenylmethanesulfonylfluoride (PMSF), 0.5 M ammonium acetate, pH 5.0) and concentrated by acetone precipitation. The purified NTAP-XB15 protein was used for PP activity assay and protein gel blot analysis.

**In vitro GST pull-down assays.** Purified recombinant proteins, GST-XA21K668, HIS-XB15, and GST were used for pull-down assays as described [101], except that buffers were supplemented with 2 mM MgCl₂. Bound proteins were eluted by boiling in SDS sample buffer, separated by SDSPAGE, and detected by immunoblotting with a anti-histidine antibody (Qiagen). Recombinant GST or GST-XA21K668 were incubated with glutathione-sepharose beads (Amersham) overnight at 4 °C and washed three times for 10 min at 4 °C with phosphate buffer saline (PBS). The beads were equilibrated with Binding Buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 2 mM MgCl₂, 0.5 mM dithiothreitol) to which a protease inhibitor mixture (Roche) was added. Recombinant HIS-XB15 was incubated with GST or GST-XA21K668 (20 μg) bound to glutathione sepharose beads (Amersham) overnight at 4 °C, the beads were washed four times for 20 min in binding buffer. Proteins bound to the bead were eluted with SDS sample buffer, separated by SDSPAGE, and processed for immunoblotting.

**In vivo co-immunoprecipitation.** To co-immunoprecipitate Myc-Xa21 and XB15, total proteins were extracted from 5 g of leaf tissue in 25 mL of ice-cold Extraction Buffer II (0.15 M NaCl, 0.01 M Na-phosphate [pH 7.2], 2 mM EDTA, 1% Triton X-100, 10 mM β-mercaptoethanol, 20 mM NaF, 1 mM PMFS, 1% Protease cocktail Sigma, 5 μg/ml leupeptin, 2 μg/ml antipain, and 2 μg/ml aprotinin). After filtering through Microsol (Calbiochem) followed by centrifugation twice at 13,000 g for 20 min at 4 °C, the supernatant was mixed with 50 μl of agarose conjugated anti-Myc antibody (Santa Cruz) and incubated at 4 °C for 2 h. The beads were then washed four times in 1 mL of Extraction Buffer II without protesase inhibitors. The beads were eluted with 4X Laemmli loading buffer. Protein blot analyses were performed. To co-immunoprecipitate XB15-smGFP2, total proteins were extracted from protoplasts transformed with XB15-smGFP2 in 5 mL of ice-cold Extraction Buffer II and 20 μl of agarose conjugated anti-GFP antibody (Santa Cruz) for 2 h. The beads were washed four times in 1 mL of Extraction Buffer II without protesase inhibitors. The beads were eluted with 4X Laemmli loading buffer. Protein blot analyses were performed. To co-immunoprecipitate XB15-smGFP2, total proteins were extracted from protoplasts transformed with XB15-smGFP2 in 5 mL of ice-cold Extraction Buffer II and 20 μl of agarose conjugated anti-smGFP antibody (Santa Cruz). Rabbit anti-GFP antibody, synthetic peptides and monospecific antibodies were made as a service by Pacific Immunology. Detailed information about their methods can be obtained at Pacific Immunology (http://www. pacificimmunology.com). Epitope selection was directed to hydrophilic regions of the protein. The LAR-TEKFQDSADL was used. Peptides were synthesized and conjugated to keyhole limpet hemocyanin. Rabbits were immunized with peptide in complete Freund’s adjuvant, followed by three boosts in
incomplete Freund's adjuvant. Monospecific antibodies were affinity purified to the synthetic peptides bound to C3-SEP-PAK cartridges. Antibodies were conjugated to horseradish peroxidase for use in Western blots.

Xoo inoculation and determination of bacterial populations. For Xoo inoculation, rice plants were grown in the greenhouse normally until rosette stage, unless stated, to allow the growth of the chamber. The Xooc strain Philippines race 6 (PR6) was used to inoculate rice by the scissors-dip method [18,53]. Only the top two to three expanded leaves of each tiller were inoculated. For Xoo colony counts from inoculated leaves, 20 cm of leaf tissue from the top, including lesions and tissue showing no lesions, was ground up and resuspended in 10 ml water to harvest bacteria. The extract was diluted accordingly and plated out on petri sucrose agar (PSA) plates containing 15 mg/ml cefalexin.

Crossing with NTAP-Xb15 and Myc-XA21. The transgenic lines Mxc-xb15 and Mxc-xb15 were used as the pollen recipient in a cross with pollen donor NTAP-Xb15 17A and 19A. Over 50 seeds were recovered from each of the T330-16-1/17A and T330-16-1/19A crosses. The nature of the F1 hybrid was confirmed by PCR amplification of 828 bp fragment spanning part of the Mxc tag and Xb15 in the Xb15 construction using primers 5'-GAGCAAAAACGCTATTTCTGAGAGGAGGAAT-3'/5'-ACCACCTAGTGTTTTTCTGTGAC-3' and 650-bp fragment spanning part of the NTAP tag and Xa21 in the Xb15 construction using primers 5'-ATGCGCCAGCGGCACACTGCG-3'/5'-CAGTAGCCGAGTTTGACAGTTAACCTGA-3'. The nature of the F1 hybrid was confirmed by PCR amplification of over 50 seeds were crossed with NTAP-XB15 and homozygous Myc-XA21 (F3, 19A-72-5-1 and 17A-18-1-1) and heterozygous Myc-XA21 (F1, 17A-24 and 19A-74) extracted and analyzed by SDS-PAGE, and immunoblotted with anti-Myc. Equal loading of total proteins was confirmed by immunodetection of actin protein with anti-actin antibody. Myc-XA21 gives bands at approximately 140 and a nonspecific band (n.s.) of 95 kDa was detected. Homo, heterozygous for Myc-XA21; Hetero, heterozygous for Myc-XA21.

Rice transformation. Rice transformation was described as constructed previously [53]. Agrobacterium EHA105 was used to infect rice callus for transformation. Transforms of rice cultivars Kitake carrying Np-xb15 or Myc-xb15 were selected using hygromycin.

Immunodetection. For NTAP-Xb15 detection, rabbit PAP was used in the final dilution of 1:5,000 for 2 h. Bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to standard protocol.

Figure S1. Immunodetection of Myc-XA21 in Homozygous and Heterozygous Myc-XA21 Lines

The number below the bar represents individual segregating progeny of cross (Xb15 Xb15). Level 1 indicates no lesion formation. Level 2 indicates a moderate number of lesions (approximately 20–40 lesions per leaf), and level 3 indicates the presence of many lesions (approximately 40–100 lesions per leaf, lesions open spread along the leaf, and lesions without separation). Six of the heterozygotes were used for expression analysis before observation of cell death lesions and are therefore not included in the figure.

PP2C Regulates Rice Innate Immunity

Immunoassay. Phosphatase activity was measured according to the instructions provided by the manufacturer by using a nonradioactive serine/threonine phosphatase assay system (Promega). The color was allowed to be developed for 15 min, and the absorbance was measured at 600 nm with plate reader (Bio-Rad). The colorimetric assay was used in PBS buffer (250 mM imidazole [pH 7.2], 1 mM EDTA, 25 mM MgCl₂, 0.1% 2-mercaptoethanol, 0.5 mg/ml BSA). PP2B 5x buffer (250 mM imidazole [pH 7.2], 1 mM EDTA, 25 mM MgCl₂, 2 mM CaCl₂, 290 μM/ml calmodulin, 0.1% 2-mercaptoethanol), PP2C X buffer (250 mM imidazole [pH 7.2], 1 mM EDTA, 25 mM MgCl₂, 0.1% 2-mercaptoethanol, 0.5 mg/ml BSA).

In vitro phosphatase assays. Purified agarose-bound fusion proteins, GST-XA21K668, were washed with kinase buffer (50 mM HEPES [pH 7.4], 10 mM MgCl₂, 10 mM MnCl₂, 1 mM dithiothreitol). Autophosphorylation experiments were carried out in 30 μl volumes containing 20 μl of agarose-bound protein (5 μg) and 20 μl of [γ-32P]ATP (6,000 Ci/mmol) (PerkinElmer Life Science). The reaction was stopped after 30 min by adding 10 μl of Laemmli loading buffer and boiling for 5 min. The proteins were separated by SDS-PAGE (7.5% or 10%). After staining with Coomassie Brilliant Blue G-250, the gel was dried and exposed to x-ray film.

To dephosphorylate the phosphorylated fusion proteins by HIS-Xb15αN, the 32P-labeled XA21K668 proteins were washed with PP2C buffer and incubated with HIS-Xb15αN. The resulting proteins were resolved by SDS-PAGE as described above.

Plasmid construction for Xb15 overexpression. A 1,950 nt cDNA fragment encoding full-length Xb15 protein was amplified from a rice cDNA using primers 5'-CCACATGGGACACACTCTCAGGTCGTAC-3'/5'-CCATGGGACACACTCTCAGGTCGTAC-3'. The PCR product was cloned into pENTR-DTOPO (Invitrogen) according to the instructions provided by the manufacturer and the insert confirmed by sequencing. For Overexpression in rice, the Xb15 cDNA in pENTR-DTOPO was recombined into the final Ubi-NTAP vector using Gateway LR Clonase (Invitrogen). Ubi-NTAP-Xb15 is a pCAMBIA-1300 (AF324296) derivative with an additional expression cassette containing the maize ubiquitin promoter and a nopaline synthase 3′-polyadenylation region, to which the NTAP Gateway cassette was added [52].

Supporting Information

Figure S2. Homozygous and Heterozygous Progeny (M3) of Xb15 Toss1 Insertion Mutant Lines (NF9014-6-16-30) Display Moderate to Severe Necrotic Lesions

PP2C regulates rice innate immunity.
Our laboratory examined the role of the rice cDNA locus identification numbers (TIGR; http://rice.tigr.org) in innate immunity. We identified two Rice cDNAs, Os12g36880 and Os01g28450, which were expressed in protoplasts incubated with 200 μM substrate in PP2C buffer for 60 min. The boiled XB15-smGFP2, one μg of XB15-smGFP2 was boiled in water for 20 min. The data are average value of three experiments. Error bars represent standard deviations. We identified the rice cDNA locus identification numbers (TIGR; http://rice.tigr.org) as follows: Xb15, Os03g08010, PR1b, Os01g28450, PR1b, Os03g60650, PR1a, Os03g28480, PR1a, Os03g60650, PR1b, Os03g08010. The GenBank (http://www.ncbi.nlm.nih.gov/Genbank) accession number for Xb15 cDNA sequence is NP_001051728.

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