Rice XA21 Binding Protein 3 Is a Ubiquitin Ligase Required for Full Xa21-Mediated Disease Resistance

Yong-Sheng Wang, a,1 Li-Ya Pi, a,1 Xiuhua Chen, a,1 Pranjib K. Chakrabarty, a,2 Junda Jiang, b,3 Alfred Lopez De Leon, c,4 Guo-Zhen Liu, a,5 Liangcai Li, d Ulla Benny, a James Oard, b Pamela C. Ronald, c and Wen-Yuan Song a,6

a Department of Plant Pathology, University of Florida, Gainesville, Florida 32611
b Department of Agronomy, Louisiana State University, Baton Rouge, Louisiana 70803
c Department of Plant Pathology, University of California, Davis, California 95616
d Department of Crop Science, North Carolina State University, Raleigh, North Carolina 27695

XA21 is a receptor-like kinase protein in rice (Oryza sativa) that confers gene-for-gene resistance to specific races of the causal agent of bacterial blight disease, Xanthomonas oryzae pv oryzae. We identified XA21 binding protein 3 (XB3), an E3 ubiquitin ligase, as a substrate for the XA21 Ser and Thr kinase. The interaction between XB3 and the kinase domain of XA21 has been shown in yeast and in vitro, and the physical association between XB3 and XA21 in vivo has also been confirmed by coimmunoprecipitation assays. XB3 contains an ankyrin repeat domain and a RING finger motif that is sufficient for its interaction with the kinase domain of XA21 and for its E3 ubiquitin ligase activity, respectively. Transgenic plants with reduced expression of the Xb3 gene are compromised in resistance to the avirulent race of X. oryzae pv oryzae. Furthermore, reduced levels of Xb3 lead to decreased levels of the XA21 protein. These results indicate that Xb3 is necessary for full accumulation of the XA21 protein and for Xa21-mediated resistance.

INTRODUCTION

Plant immunity is often governed by dominant resistance (R) genes. Over the past decade, a large number of R genes have been characterized from diverse plant species (Dangl and Jones, 2001; Staskawicz et al., 2001). The majority of these encode proteins with a nucleotide binding site and leucine-rich repeats (NB-LRRs). The NB-LRR class of R proteins can be divided into two subgroups based on the N-terminal domains: coiled-coil NB-LRRs carrying a coil-coil structure and TIR-NB-LRRs containing the TIR domain that was originally identified in the intracellular regions of the Drosophila melanogaster transmembrane receptor Toll and the mammalian interleukin 1 receptor (IL-1R). In the evolutionarily conserved animal innate immunity pathways, the TIR domains of Toll and IL-1R form similar protein complexes, including the Ser and Thr kinase Pelle or IRAK (Hoffmann and Reichhart, 2002). While it is still unclear whether the TIR domain of the R proteins also recruits a Pelle-like kinase in the defense response, a number of receptor-like kinases (RLKs) containing a Pelle-related kinase domain have been implicated in plant disease resistance (Song et al., 1995; Brueggeman et al., 2002; Scheer and Ryan, 2002; Godiard et al., 2003; Sun et al., 2004; Zipfel et al., 2004; Diener and Ausubel, 2005; Llorente et al., 2005; Chen et al., 2006). These RLKs appear to be equivalent to the receptor/kinase complexes in animal innate immunity.

Ubiquitin-mediated protein modification regulates many cellular processes, including homeostasis, development, cell division, growth, and hormone and stress responses (Smalle and Vierstra, 2004). Ubiquitinated proteins can then be degraded by the 26S proteasome or can assume a substrate by the ubiquitin-activating enzyme E1, transferred to the ubiquitin-conjugating enzyme E2, and finally linked to a target substrate by the ubiquitin ligase E3 (Smalle and Vierstra, 2004). Characterization of a number of E3 proteins in both animal and plant systems indicates that the zinc binding domain RING (for Really Interesting New Gene) finger (RF) is essential for many ubiquitin-mediated protein modification events (Joazeiro and Weissman, 2000; Osterlund et al., 2000). Ubiquitinated proteins can then be degraded by the 26S proteasome or can assume a role in other proteolysis-independent processes (Ben-Neriah, 2002; Smalle and Vierstra, 2004). It has been suggested that polyubiquitination of the RF-containing protein TRAF6 activates the downstream protein kinase TAK1 in the IL-1–mediated innate immunity pathway (Wang et al., 2001).

The ubiquitin-mediated protein modification system also plays a role in plant defense mechanisms. For example, SGT1 and RAR1 are required for the function of multiple R genes (Shirasu et al., 1999; Austin et al., 2002; Azevedo et al., 2002; Liu et al., 2002; Tor et al., 2002; Tornerio et al., 2002). SGT1 associates
RESULTS

**Xb3 Encodes a Putative E3 Protein with Multiple Domains**

A truncated kinase domain of XA21 (XA21K_{690}) was used to screen a yeast two-hybrid rice cDNA library to identify XA21-interacting proteins. XA21K_{690} spans the entire intracellular region of XA21 (XA21K) except the first 13 amino acids of the JM domain that contains the autophosphorylated Ser-686, Thr-688, and Ser-689 (Figure 1A). Seven classes of XA21K_{690} interacting proteins were Identified (data not shown). Among them, Xb3 is encoded by the cDNA 98-2 that contains a complete open reading frame and 65 bp of 5’ untranslated region.

In addition to XA21K_{690}, Xb3 interacted with XA21K and the autophosphorylation mutant XA21K^{S666A/T666A/S669A} (Figure 1B; Xu et al., 2006), indicating that these three autophosphorylated residues are not required for the binding of Xb3 in yeast. By contrast, Xb3 failed to interact with the catalytically inactive mutant XA21K^{K736E} (Figure 1B; Liu et al., 2002b). Additionally, XA21K did not interact with the closest rice homolog of Xb3, XBOS31 (69% identity and 76% similarity) (see Supplemental Figure 1 and Supplemental Table 1 online). Thus, the interaction between XA21K and Xb3 is specific.

The open reading frame of Xb3 and its 5’ sequence were fused in frame to the activation domain of the GAL4 transcription factor in the cDNA 98-2. Xb3 has 450 amino acids (Figure 1C). The N terminus carries a putative N-myristoylation site (Resh, 1999). Amino acids 11 to 305 carry eight imperfect copies of ankyrin repeats that have been implicated in protein-protein interactions (Sedgwick and Smerdon, 1999). Following the ankyrin domain is a region (amino acids 323 to 371) sharing the conserved Cys and His residues characteristic of RF motifs. Because a number of RF-containing proteins carry E3 activities (Lorick et al., 1999), we hypothesized that Xb3 is an E3 enzyme. The C terminus of Xb3 has the potential to form a coiled-coil structure.

**The Ankyrin Repeat Domain of Xb3 Is Sufficient for in Vitro Interactions with XA21K**

Because the C-terminal half of Xb3, spanning the RF motif and the tail, did not interact with XA21K in the yeast two-hybrid system (data not shown), we tested whether the ankyrin domain physically binds to XA21K. Xb3 and the ankyrin repeats of Xb3 (not including the 65 bp 5’ untranslated region in the cDNA 98-2) were expressed as maltose binding protein (MBP) fusions, and the bacterially expressed proteins (MBP-Xb3 and MBP-XB3ankyrin) were bound to amylose resin. MBP alone was used as a control. XA21K, produced and labeled with [35S]Met by an in vitro transcription and translation reaction, was mixed with the above resin-bound MBP fusion proteins. After extensive washing, proteins in the resin-bound fractions were eluted and resolved by SDS-PAGE. The 35S-labeled XA21K was only detected in the MBP-Xb3 and MBP-Xb3 ankyrin fractions but not in the MBP control (Figure 2), indicating that the ankyrin repeat domain of Xb3 is sufficient for binding to XA21K.

**Xb3 Interacts with Xa21 in Vivo**

We generated polyclonal antibodies against Xb3 (anti-Xb3M) and demonstrated their specificity against Xb3. As shown in Figure 3A, anti-Xb3M detected a major band at the position of 54 kD, which is slightly larger than the predicted Xb3 (48 kD). The quantity of this product is reduced in the Xb3 RNA interference (RNAiXb3) lines when compared with the recipient line Taipei309 (TP309) but significantly enhanced in an Xb3-FLAG overexpression line (Figures 3A and 3B). We then immunoprecipitated the Xb3-FLAG from the overexpression line using anti-FLAG M2 agarose followed by detection with anti-Xb3M. A single product of 54 kD was only found from the Xb3-FLAG overexpression line (Figure 3B). To rule out the possibility that the 54-kD product could be XBOS31, we expressed XB3 and XBOS31 as FLAG-tagged fusion proteins in *Escherichia coli*. Protein gel blot analyses of bacterial extracts revealed that anti-Xb3M only recognizes...
FLAG-XB3, not FLAG-XBOS31 (Figure 3C). As a control, the anti-FLAG M2 antibody detected both XB3 and XBOS31 in the same extracts. These results indicate that anti-XB3M is specific for XB3 and the 54-kD product in rice extracts is XB3.

We also generated transgenic plants expressing either a c-Myc–tagged or a double ProA-tagged XA21 for coimmunoprecipitation assays. Each of the tagged Xa21 variants was flanked by 2204-bp native 5' and 3787-bp 3' regulatory sequences that are sufficient for supporting Xa21-mediated resistance (Song et al., 1995). Transgenic plants carrying each of the constructs were resistant to Xoo Philippine race 6 (Xoo PR6), indicating that the tagged Xa21 variants are functional (data not shown). Anti-c-Myc antibody detected a 140-kD polypeptide in the resistant transgenic plants but not in the susceptible recipient line TP309 (Figure 3D). We concluded that the 140-kD polypeptide is Myc-XA21. A homozygous Myc-XA21 line (4021-3) was identified and used in this study. No segregation for the resistance was observed in the progeny of 4021-3 with >50 individuals (Xu et al., 2006). A similar strategy was used to specify ProA-XA21 in the rice protein extracts using the peroxidase-antiperoxidase (PAP) antibody (Figure 3E).

To demonstrate that XB3 interacts with XA21 in vivo, we used IgG sepharose beads to immunoprecipitate XA21 from the ProA-XA21 line (carrying only the native XB3). The ProA domains have proven to be a high-affinity tag for recovering protein complexes from a complex mixture (Rigaut et al., 1999; Rohila et al., 2004). Indeed, ProA-XA21 was strongly recognized by the PAP antibody in the precipitates, indicating that the ProA tag can efficiently recover the XA21 protein from plant extracts (Figure 3F). Anti-XB3M was then used to detect XB3 in the IgG precipitates. Figure 3F shows that a major band of 54 kD, identical to XB3, was detected in the ProA-XA21 line but not in the TP309 line. To exclude the possibility that XB3 could interact with the 128–amino acid ProA tag, we used the transgenic line A6 that expresses a tandem affinity purification (TAP)-tagged kinase (Os06g48590) unrelated to XA21. Similar to ProA-XA21, a ProA tag was placed at the N terminus of this kinase. No product of 54 kD was detected in the IgG precipitates prepared from the A6 plants. As a control, similar amounts of XB3 were present in the supernatant of these three reactions. These results are consistent with our in vitro observations and indicate that XB3 interacts with XA21 in vivo.

**XB3 Is a Substrate of XA21K**

The physical interaction between XA21 and XB3 suggests that XB3 may be a substrate of XA21K. To test this hypothesis, the...
Xb3 Is Required for Stability of the XA21 Protein and for Xa21-Mediated Resistance

We used RNAi to downregulate the expression of Xb3 in vivo. The RNAiXB3 construct, driven by the maize (Zea mays) ubiquitin promoter, contains both sense and antisense Xb3 sequences separated by a 979-bp uidA sequence. The Xb3 probe is generated using a 302-bp region derived from the 3’ end of the gene, including part of the last exon (see Supplemental Figure 2 online). This region shares <50% identity with the corresponding region of Xbos31. No sequence stretches of >11 bp are identical within the 302-bp region between Xb3 and Xbos31. Moreover, DNA gel blot analyses indicated that the 302-bp sequence only hybridizes to Xb3, not to Xbos31 (see Supplemental Figure 3 online). Therefore, RNAiXB3 should specifically downregulate the Xb3 gene.

The RNAiXB3 construct was transformed into the susceptible cultivar TP309 using Agrobacterium tumefaciens–mediated transformation. More than 60 independent lines were generated and inoculated with Xoo PR6. All the plants were fully susceptible to Xoo PR6 (data not shown). Two RNAiXB3 lines (37 and A13) with drastically reduced levels of Xb3 RNA transcripts were chosen for further characterization (Figure 6A). Protein blot analyses confirmed that in comparison to TP309, Xb3 was decreased in these two lines (Figure 3A).

To test the effects of reduced Xb3 on Xa21-mediated resistance, the RNAiXB3 lines 37 and A13 were used as the pollen recipient parents in crosses with the homozygous Myc-XA21 line 4021-3 (pollen donor). All the progeny tested contained the Xa21 gene, as shown by PCR analyses (Figure 6B, results from six representative F1 plants are shown). We have previously shown that the protein levels of XA21 are developmentally regulated with higher levels at the seedling stage (Xu et al., 2006), although the expression of the Xa21 gene at the RNA level is independent of developmental processes (Century et al., 1999). We then monitored the levels of XA21 at two developmental stages by protein blot analyses. XA21 accumulated at comparable levels at the seedling stage (Figure 6C), but in 4-month-old plants, XA21 protein blot analyses. XA21 accumulated at a significantly lower level in seven of the 12 F1 plants tested (Figure 6D, results from six representative F1 plants are shown). The observed reduction in the XA21 protein is not due to decreases in the Xa21 transcripts but strictly correlates with the decreases in the Xb3 transcripts (Figures 6A and 6E). Furthermore, this reduction also correlates with compromised resistance to Xoo PR6 (Figures 6F and 6G). Bacterial growth curve analyses confirmed that Xoo PR6 achieved a higher level in the F1 progeny with reduced XA21 levels than that generated from a cross of TP309 and 4021-3 (Figure 6H). Taken together, we conclude that Xb3 is required for an abundance of the XA21 protein and Xa21-mediated resistance.

Xa21-Mediated Resistance Shows a Gene Dosage Effect

We crossed TP309 (pollen recipient) with 4021-3 (pollen donor) to determine whether Xa21-mediated resistance is gene dosage dependent. Eight F1 plants were characterized further. All the eight plants had similar levels of XA21, which were slightly lower than that of the homozygous parent 4021-3 (Figure 7A). These
Figure 3. XB3 Interacts with XA21 in Vivo.

(A) Immunodetection of XB3 in the Xb3 RNAi lines. Equal amounts of total protein extracts isolated from the recipient line TP309 and four individuals of the T1 progeny of the RNAiXB3 lines 37 and A13 were immunoblotted with anti-XB3M (top). The XB3 band is indicated. The Ponceau S–stained blot is shown as the loading control (bottom).

(B) Immunodetection of XB3 in the Xb3 overexpression line. Left panels: Equal amounts of total protein extracts isolated from TP309 and the transgenic line 6-1 overexpressing a FLAG-tagged Xb3 were immunoblotted with anti-XB3M (top). Coomassie blue–stained gel of identical protein samples as a loading control (bottom). Right panel: Equal amounts of total protein extracts from TP309 and 6-1 were immunoprecipitated with anti-FLAG M2 agarose followed by detection with anti-XB3M.

(C) Anti-XB3M does not recognize bacterially expressed XBOS31. Protein extracts from the bacterial cultures expressing the indicated constructs were immunoblotted with anti-XB3M (top) or anti-FLAG M2 (bottom) antibodies.

(D) and (E) Immunodetection of Myc-XA21 and ProA-XA21, respectively. Equal amounts of total protein extracts from TP309 and the transgenic lines 4021-3 (carrying Myc-Xa21) or 716-1 (carrying ProA-Xa21) were immunoblotted with anti-c-Myc (D) or PAP (E) antibodies. The tagged XA21 proteins are indicated. n.s., nonspecific products.

(F) XB3 is coimmunoprecipitated with XA21. Twenty-five milliliters of total protein extracts from 5 g of leaf tissue of TP309, 716-1, and A6 (carrying TAP-Os06g48590) were immunoprecipitated with IgG sepharose beads. One-fifth of the precipitates were subjected to protein blot analyses using PAP (top) or anti-XB3M (bottom) antibodies. One microliter of total protein extracts from TP309, 716-1, and A6 was used as a control. XB3 is indicated. The XB3 band was not detectable in the A6 pellet even when 10 times more immunoprecipitates were used for the protein blot analyses (data not shown). The asterisks denote products degraded from XA21. Dots indicate nonspecific products. In the A6 line, the TAP-tagged kinase can be detected by anti-XB3M antibodies. The experiments were repeated three times with similar results.
results exclude the possibility that the reduced XA21 protein in the above RNAiXB3/4021-3 plants was the result of a segregation of the functional copies of \textit{Xa21} and confirm that 4021-3 is homozygous for \textit{Xa21}. To evaluate resistance, the eight F1 plants were inoculated with \textit{Xoo} PR6. Although these plants and 4021-3 showed a typical resistance response, most of the F1 plants exhibited slightly longer lesions than 4021-3 (Figure 7B). Given that the F1 progeny only contain half the amount of the functional \textit{Xa21} gene, these results indicate that, over a certain range, \textit{Xa21} shows dosage-dependent resistance.

**DISCUSSION**

We characterized a ubiquitin ligase XB3 containing eight ankyrin repeats and an RF motif for its role in \textit{Xa21}-mediated disease resistance. The ankyrin domain is sufficient for binding to XA21K in vitro. Ankyrin repeats exist in a large number of proteins and interact with diverse partners, including Ser and Thr kinases (Mosavi et al., 2004). Coimmunoprecipitation experiments confirmed that XB3 interacts with XA21 in vivo. Because the leaf tissues used for the coimmunoprecipitation experiments were from uninoculated, healthy plants, the data strongly suggest that the ubiquitin ligase XB3 forms a protein complex with XA21 in planta.

The demonstration of the XA21–XB3 interaction in vivo may be generally important for understanding RLK complexes in rice and in other plants. In a large-scale yeast two-hybrid analysis, we found that nine of 50 randomly chosen rice RLKs interact with four putative E3 ubiquitin ligases (X. Ding and W.-Y. Song, unpublished data). In \textit{Brassica napus}, the PUB-ARM protein ARC1 interacts with the kinase domain of the \textit{S} receptor kinase in the yeast two-hybrid system and in vitro (Gu et al., 1998). ARC1 is positively involved in the self-incompatibility system (Stone et al., 1999). It has been proposed that ARC1 promotes the ubiquitination and proteasomal degradation of compatibility factors in the pistil (Stone et al., 2003). In tobacco (\textit{Nicotiana tabacum}), the Nt PUB4 protein is also a member of the PUB-ARM family. Yeast two-hybrid analysis has linked Nt PUB4 to the kinase domain of the chitinase-related RLK CHIK1 that may be involved in development and cytokinin homeostasis (Kim et al., 2000; Lee et al., 2003). Thus, many plant RLKs may interact with E3 ubiquitin ligases.

One feature of XA21 in the protein complex is the requirement of XB3 for its accumulation, whereas XB3 does not require XA21 for its stability. In the Xb3 silencing lines, the steady state level of the XA21 protein is reduced at the adult stage; however, the XB3 protein accumulates to a similar level in the plants with or without the Xa21 gene (Figures 3F and 6D). These observations are consistent with our recent hypothesis that XA21 is intrinsically unstable owing to the presence of a putative proteolytic cleavage motif (XA21CS1) in the JM domain of this RLK (Xu et al., 2006). Mutation of three residues (Ser-686, Thr-688, and Ser-689) within XA21CS1 destabilizes XA21 only at the adult stage. It has been hypothesized that autophosphorylation of these residues directly or indirectly protects the resistance protein from cleavage by a developmentally regulated protease (Xu et al., 2006). However, Ser-686, Thr-688, and Ser-689 of XA21 appear

**Figure 4.** XA21K Can Specifically Phosphorylate XB3 in Vitro.

The purified MBP-XA21K was incubated either with purified FLAG-tagged XB3 or XBOX31 in the presence of \[^{32}\text{P}]\text{ATP}. FLAG-XB3 alone and the kinase-deficient mutant MBP-XA21K\[^{K736E}\] were used as negative controls. Samples were resolved with 8% SDS-PAGE. Coomassie blue staining (CBB) and autoradiogram (Autorad) of the same gel are shown. The asterisk denotes FLAG-XBOX31, which has been confirmed by protein blot analysis with anti-FLAG M2 antibodies (Figure 3C). The experiment was repeated three times with similar results. n.s., nonspecific bands.

**Figure 5.** Autoubiquitination of the XB3 and XB3 RF Domain (XB3RF) Fusion Proteins.

GST-ubiquitin was labeled with \[^{32}\text{P}]\text{ATP} by protein kinase A and then digested with thrombin to release the \[^{32}\text{P}]\text{-ubiquitin}. Resin-bound MBP and its fusion proteins were incubated with \[^{32}\text{P}]\text{-ubiquitin}, ATP, and the wheat ubiquitin-activating enzyme E1 in the presence or absence of E2 (Ubch5B). MBP-XB3RF\[^{C323A}\] is a mutant in which conserved Cys-323 is replaced with Ala. Samples were resolved with 8% SDS-PAGE, followed by autoradiography. MBP-XB3 and MBP-XB3RF migrate at \(\sim 89\) and 53 kD (indicated by arrows), respectively. The experiment was repeated three times with similar results.
Figure 6. Reduction of the XA21 Protein Correlates with the Decrease of Xb3 RNA Transcripts in F1 Individuals.

The pollen recipient RNAiXB3 lines 37 and A13 were crossed with the Xa21-containing line 4021-3 (pollen donor). As a positive control, 4021-3 was crossed with the nontransgenic line TP309. Characterization of progeny at the 4-month-old stage is demonstrated.

(A) RNA gel blot analyses showing Xb3 RNA levels in the indicated parental lines and F1 progeny. Total RNA was probed with an Xb3-specific sequence.
to be unnecessary for the binding of XB3. The kinase domain of XA21 used for our original two-hybrid screening does not contain these three residues (Figure 1A). Moreover, mutation of these three residues did not abolish the XA21–XB3 interactions in yeast (Figure 1B). Sequence analysis revealed that XA21 possesses at least two additional stretches inside the kinase domain that fulfill the criteria of the P/GX$_{p,j}$ P/G motif (G. Cory and W.-Y. Song, unpublished data). Similar to XA21CS1, there are Ser and Thr residues within these two sequence stretches. Notably, the dead kinase mutant XA21$^{K736E}$ accumulates at a lower level than the XA21$^{S686A/T688A/S689A}$ mutant, in which autophosphorylation of

Both autoradiogram (top) and agarose gel (bottom) are shown. The Figure 6. (continued).

Figure 6. (continued).

Both autoradiogram (top) and agarose gel (bottom) are shown. The Xb3 band can be visualized in all lines when the film was exposed for a longer time period (data not shown).

(B) PCR amplification showing the presence of the Xa21 gene in the indicated lines.

(C) and (D) Protein blot analyses showing the steady state levels of XA21 in the indicated lines at the 1-month-old (C) and 4-month-old (D) stages. Equal amounts of total protein extracts were immunoblotted with anti-c-Myc antibody (top). Coomassie blue–stained gel (CBB) of identical protein samples is shown as a loading control (bottom).

(E) Semiquantitative RT-PCR analyses of Xa21 transcripts in the indicated lines. Total RNA was used to amplify a Xa21 region (top) and the actin gene as control (bottom). The genomic contamination will result in a larger Xa21 fragment due to the presence of an intron in the amplified region (data not shown).

(F) Lesion length data of plants inoculated with Xoo PR6. Each data point represents three cDNA individuals with two inoculations per individual. The standard error of the mean is indicated.

(G) Inoculated plants showing lesion development. Leaves 1 and 2, TP309/4021-3 expressing XA21; leaves 3 and 4, TP309; leaves 5 and 6, 37/4021-3-8 expressing a reduced XA21.

(H) Growth of Xoo PR6 in 37/4021-3-8 and control lines. For each time point, the bacterial populations were determined in three leaves separately. Open circles, TP309; triangles, 37/4021-3-8; closed circles, TP309/4021-3. CFU, colony-forming units. Bars indicate standard error.
proteins MLA1 and MLA6 (Bieri et al., 2004). Our results demonstrate that the steady state accumulation of RLK XA21 is also influenced by its binding protein. Therefore, the stability of R proteins may generally rely on the presence of their partners, and the measurement of the steady state levels of R proteins has become a common tool to dissect R gene–mediated signaling pathways.

XB3 can be phosphorylated by XA21K in vitro. Although this finding remains to be confirmed in planta, the direct interaction between XA21 and XB3 in vivo supports such an extrapolation. In previous studies, we have found that XA21K is capable of phosphorylating a 22–amino acid peptide derived from the glutathione S-transferase (GST) vector when the peptide is fused in frame with XA21K (Liu et al., 2002b). XA21K, however, cannot phosphorylate the free GST protein that contains the same peptide (G.-Z. Liu and W.-Y. Song, unpublished data), suggesting that a physical connection between XA21 and the artificial substrate is required for the phosphorylation. The two phosphorylated residues on the 22–amino acid peptide have been mapped (Liu et al., 2002a). Sequence comparisons reveal that the flanking sequences of these two residues share similarities with those of other identified autoprophosphorylation sites inside XA21K (G.-Z. Liu and W.-Y. Song, unpublished data). These observations suggest that XA21-mediated phosphorylation requires the presence of a phosphorylation site and the physical interaction of the substrate with the kinase. XB3 fulfills these criteria. In line with this idea, XA21K was unable to phosphorylate XBOS31, which does not interact with XA21K in yeast. Therefore, protein–protein interactions may contribute to the specificity of XA21 phosphorylation.

When XB3 is reduced, resistance to Xoo PR6 is compromised in the Xa21 lines. The compromised resistance can be attributed to a decrease in the level of the XA21 protein. The dosage effects have been suggested by several previous studies (Song et al., 1995; Zhang et al., 1998; Xu et al., 2006). Additionally, the heterozygous Xa21 plants generated by a cross between 4021-3 and TP309 are slightly less resistant than the homozygous plant 4021-3. Alternatively, but not mutually exclusive, the reduction of Xb3 may directly contribute to the compromised resistance as well. In this case, the Xb3 level is a rate-limiting step for Xa21-mediated resistance.

Based on our data, we propose that the physical interaction of XB3 stabilizes the XA21 protein, thereby maintaining the level of the R protein. Upon pathogen infection, the XA21–XB3 complex is required for activation of XB3, presumably through transphosphorylation. The activated XB3 may ubiquitinate a third protein and target it for degradation. In this scenario, the degraded protein would be a negative regulator of the defense signaling. Alternatively, XB3 may autoubiquitinate, triggering activation of a downstream signaling protein(s). Support for the second model comes from studies in animal innate immunity. Recognition of IL-1 by IL-1R, for example, leads to the formation of a receptor/kinase complex, including IL-1R, MyD88, and the Ser and Thr kinase IRAK (Wu and Arron, 2003). Following this recognition is the activation of TRAF6, an RF-containing ubiquitin ligase. Polyubiquitination of TRAF6 has been suggested to activate the downstream kinase TAK1 (Wang et al., 2001). Our model therefore predicts an interesting parallel between plant and animal defense pathways mediated by a receptor kinase and a receptor/kinase complex. Such a parallel has been recognized based on the fact that a number of plant R proteins share the TIR domain (Baker et al., 1997).

**METHODS**

**Yeast Two-Hybrid Screening**

To make the BD-XA21K949 construct for yeast two-hybrid screening, XA21K949 was PCR amplified using the primer pair 5′-AGTCCGACCCGGGAATGAAAGGCCACCCATT-3′/5′-GGGGTACCGGTTCTCTAAAATTCAAGGCTTCACCTTCAA-3′ and cloned into the two-hybrid vector pPC97 carrying the GAL4 BD domain (Chevray and Nathans, 1992; Chern et al., 2001). All the PCR products in this study were confirmed by DNA sequencing. A yeast two-hybrid library constructed from poly(A)+ mRNA harvested from 2-week-old seedlings of the rice (Oryza sativa) line TP309 (Yin et al., 1997) was kindly provided by R. Beachy. Yeast cells carrying BD-XA21 were transformed with the rice library. Transformation efficiency was estimated by plating an aliquot of transformation mixtures onto SD/-Leu-Trp medium. Candidates selected onto SD/-Leu-Trp-His medium were subjected to β-galactosidase assays as described in the manufacturer’s procedure for the Matchmaker GAL4 system (Clontech). Plasmids were recovered from the cells showing the His+ and Lac+ phenotypes and sequenced to determine identity.

To make BD constructs for yeast two-hybrid analyses, XA21K, XB3K736E, and XB3K668A/T688A/S689A were subcloned from their GST versions of constructs into the pPC97 vector (Liu et al., 2002b; Xu et al., 2006).

**In Vitro Binding Assays**

XA21K was PCR amplified with the primer pair 5′-GGATCCGTCGACCA-CAGAGAACATAAAAAGGGAGC-3′/5′-GGATCCGTCGACCCGGGCA-GAATGCGATCGAACTAAAAGGGAGC-3′ and cloned into the pET-28a (Novagen). The resulting plasmid was subjected to the expression of XA21K and labeling using the in vitro transcription and translation kit (Promega). Because the full-length XB3 was poorly expressed as an MBP fusion protein, the cDNA encoding amino acids 1 to 428 of XB3 (missing the last 22 amino acids) was cloned in frame into pMAL-c2X (New England Biolabs) to make MBP-XB3. The ankyrin domain of XB3 was PCR amplified with the primer pair 5′-GGATCCGATATCCTCGAGCGCCACTGCT-3′/5′-GGGATC-GGATCCGATATCCTCGAGCGCCACTGCT-3′ and cloned into pMAL-c2X. The 35S-labeled XA21K was incubated with resin-bound MBP or MBP fusion proteins in binding buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 5 mM MgCl2, 1 mM DTT, 0.1% Triton X-100, 1 mg/mL BSA, and 1× complete protease inhibitors [Roche]) for 120 min at 4°C with gentle shaking. After five washes using buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 5 mM MgCl2, 1 mM DTT, and 0.1% Triton X-100), the bound proteins were eluted with the same buffer supplemented with 10 mM maltose and resolved by 8% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue, dried, and exposed to x-ray film.

**Immunodetection**

For the anti-XB3M antibodies, a region located in the middle of XB3 (amino acids 230 to 365) was PCR amplified with the primer pair 5′-GGATCCGATATCCTCGAGCGCCACTGCT-3′/5′-GGGATCCGATATCCTCGAGCGCCACTGCT-3′ and cloned into the expression vectors pGTK (Liu et al., 2002b) and pMAL-c2X, respectively. The fusion proteins MBP-XB3M and GST–XB3M were expressed in the Escherichia coli strain ER2566 and affinity purified (Liu et al., 2002b). MBP–XB3M was used to immunize rabbits (Cocalico Biologicals). Antisera were subjected to
affinity purification using GST-XB3M according to the method described by Lin et al. (1996).

To create the FLAG-tagged XB3 and XBO31 for bacterial expression, the full-length coding regions of these two genes were PCR amplified with the primer pairs 5′-GGTGAATGATCCGCTCGTTCGTCGTCAGTCTC-3′/5′-GGATTTCTCTACCGGCGACATCTCTA-3′ and 5′-GGTGAATGATCTCCGAATGATCCGCTCGTTCGTCGTCAGTCTC-3′/5′-GGATTTCTCTACCGGCGACATCTCTA-3′ and cloned into pFLAG-M (Sigma-Aldrich). The fusion proteins FLAG-XB3 and FLAG-XBO31 were expressed in the E. coli strain ER2566 (Liu et al., 2002b).

Both the c-Myc and ProA tags were inserted in domain B of the Xa21 gene. The DNA sequences, encoding the 13-amino acid c-Myc and 128-amino acid double ProA epitope tags, were PCR amplified from the plasmids pATGmMyc and pUbi.nc1300.ntapintron.new and cloned into the 128-amino acid double ProA epitope tags, were PCR amplified from the gene. The DNA sequences, encoding the 13–amino acid c-Myc and ProA-Xa21K were expressed in the E. coli strain ER2566 and affinity purified (Liu et al., 2002b).

Ubiquitination assays were performed as described previously (Nodzon et al., 2004).

**RNAiXB3 and Overexpression of the XB3 Gene**

The 3′ end of XB3 was PCR amplified with the following primer pairs: 5′-GAATTTCTCTAGCGGCGACATCTCTA-3′/5′-ACTAGTGATCCCGTTTCTCTACCGGCGACATCTCTA-3′ and 5′-GAATTTCTCTACCGGCGACATCTCTA-3′/5′-ACTAGTGATCTCTACCGGCGACATCTCTA-3′ and 5′-GAATTTCTCTACCGGCGACATCTCTA-3′. The PCR products were ligated to the uidA fragment spanning nucleotides 815 to 1793 in both antisense and sense orientations. The resulting construct was then cloned into the overexpression vector pBHU-1, which contains the hph gene, whose product confers resistance to hygromycin B. At the site between the maize (Zea mays) ubiquitin promoter and the nos 3′ terminator. Rice transformation was performed as described previously (Xu et al., 2006).

A full-length XB3 (lacking the stop codon) was PCR amplified with the primer pair 5′-GGATTTCTCTACCGGCGACATCTCTA-3′/5′-GGATTTCTCTACCGGCGACATCTCTA-3′ and in-frame fused to the FLAG tag (containing a stop codon at the 3′ end). The tagged XB3 was cloned into pCMH1-1, an overexpression vector derived from pBHU-1, for rice transformation.

**Characterization of Transgenic Plants**

DNA and RNA gel blot analyses were performed using standard procedures. Plant inoculation and growth curve analyses were performed as described by Song et al. (1995), except that a bacterial suspension of OD 1.0 was used. Semiquantitative RT-PCR analyses were performed with primer pairs 5′-CAAGAAGTGATCTGAGTGGACTC-3′/5′-GGCAACAGAGACTAA-3′. Semiquantitative RT-PCR analyses were performed with primer pairs 5′-GAATTTCTCTACCGGCGACATCTCTA-3′/5′-ACTAGTGATCCCGTTTCTCTACCGGCGACATCTCTA-3′ and 5′-GAATTTCTCTACCGGCGACATCTCTA-3′. The PCR products were amplified using the SuperScript First-Stand Synthesis system (Invitrogen) followed by PCR amplification for 20, 25, 30, and 35 cycles. The amplified products were then resolved by gel electrophoresis.

The transgenic lines 37 and A13 were used as the pollen recipient parents to cross with pollen donors 4021-3. Eleven seeds were recovered from the 37/4021-3 cross, whereas only one seed was obtained from the A13/4021-3 cross. The nature of the F1 hybrids was confirmed by PCR amplification of a 690-bp fragment spanning the Xa21 and JSS promoters in the pCAMBIA1300-Myc-Xa21 construct using the primer pair 5′-ATTCAGATTGAGACTCGAGTCTCAGTCCAAGACAC-3′/5′-GGTTTCTCTACCGGCGACATCTCTA-3′.

**Sequence Analyses**

The PAUP (version 4.0b10) software package (Swofford, 2002) was used to perform phylogenetic analyses. Neighbor-joining was used to reconstruct the phylogenetic relationships of these amino acids. Bootstrap values were derived from 1000 replicates to quantify the relative support for branches of the inferred phylogenetic tree. The identity and similarity between proteins were calculated using the GAP tool within the Genetics Computer Group program.

**Accession Numbers**

The rice cDNA accession numbers (KOME; http://cdna01.dna.affrc.go.jp/cDNA/) for the Xbos genes are as follows: Xbos31, AK106014; Xbos32, AK112603; Xbos33, AK062233; Xbos34, AK059792; and Xbos35, AK067289. The GenBank accession number for Xbos36 is DQ088999. The accession numbers for the XBAT genes were described previously (Nodzon et al., 2004). The GenBank accession number for XB3 cDNA sequence is AP272660.
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Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Table 1.** Comparisons of Amino Acid Sequences between the XB3-Related Proteins from Rice and Arabidopsis.

**Supplemental Figure 1.** Phylogenetic Tree Based on the Predicted Amino Acid Sequences of XB3 and XB3-Related Proteins from Rice (XBOS) and Arabidopsis (XBAT).

**Supplemental Figure 2.** Schematic Representation of the XB3 and Xbos31 Genomic Gene Structures.

**Supplemental Figure 3.** DNA Gel Blot Analyses Showing Specificity of the RNAiXB3 Probe.

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