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An ATPase promotes autophosphorylation of the pattern recognition receptor XA21 and inhibits XA21-mediated immunity

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Cell-surface pattern recognition receptors (PRRs) are key components of the innate immune response in animals and plants. These receptors typically carry or associate with non-RD kinases to control early events of innate immunity signaling. Despite their importance, the mode of regulation of PRRs is largely unknown. Here we show that the rice PRR, XA21, interacts with XA21 binding protein 24 (XB24), a previously undescribed ATPase. XB24 promotes autophosphorylation of XA21 through its ATPase activity. Rice lines silenced for *Xb24* display enhanced XA21-mediated immunity, whereas rice lines overexpressing XB24 are compromised for immunity. XB24 ATPase enzyme activity is required for XB24 function. XA21 is degraded in the presence of the pathogen-associated molecular pattern Ax21 when XB24 is overexpressed. These results demonstrate a function for this large class of broadly conserved ATPases in PRR-mediated immunity.

non-RD kinase | pathogen-associated molecular pattern | pattern recognition receptor | ATPase | rice

Innate immunity is the first line of defense against pathogen attack and is activated rapidly following infection. In contrast to the adaptive immune system that depends on somatic gene rearrangements for the generation of antigen receptors with random specificities, the innate immune system uses a set of defined receptors for recognition of pathogen-associated molecular patterns (PAMPs) (1).

Cell-surface pattern recognition receptors (PRRs) are key components of the innate immune response in animals and plants (2–8). In animals, recognition of PAMPs at the cell surface is largely carried out by the Toll-like receptor (TLR) family that contains leucine-rich repeats (LRRs) in the extracellular domain and a Toll-interleukin receptor (TIR) intracellular domain (9). Although TLRs recognize diverse molecules, they activate a common signaling pathway via association with non-RD (arginine-aspartic acid) kinases to induce a core set of defense responses (10, 11). For example, TLRs 1, 3, 5, 6, 7, 8, and 9 function through interleukin-1 receptor-associated kinase (IRAK1), whereas TLR3 and TLR4 function through receptor interacting-protein 1 (RIP1). Both IRAK1 and RIP1 are non-RD kinases (10). In plants, cell-surface recognition of PAMPs is carried out by PRRs that carry non-RD IRAK kinases integral to the receptor (*ca.* 35 in *Arabidopsis* and 328 in rice) (10). For example, rice XA21, XA26, *Arabidopsis* flagellin sensitive 2 (FLS2), elongation factor-Tu receptor (EFR), and barley Rpg1 (conferring stem rust-resistance) all contain an intracellular non-RD Ser/Thr kinase (8, 12–15).

Non-RD kinases typically carry a cysteine or glycine (in place of the arginine) before the catalytic aspartate residue and control early events of innate immunity signaling in animals and plants (16). Whereas RD kinases are regulated by autophosphorylation of the activation segment—a centrally located loop that sits close to the catalytic center—non-RD kinases are not autophosphorylated in the activation segment (17). These results suggest that non-RD kinases are activated in a manner different from RD kinases.

The rice PRR, XA21, recognizes the PAMP, Ax21 (Activator of XA21-mediated immunity), which is highly conserved in all sequenced genomes of *Xanthomonas* and in *Xylella* (12, 18–20). Previous studies have shown that the intracellular non-RD cytoplasmic kinase domain of XA21 contains intrinsic kinase activity (17). Phosphorylation of amino acids Ser-686, Thr-688, and Ser-689 of XA21 is required to stabilize the XA21 protein (21). To date, three XA21 binding (XB) proteins—XB3 (an E3 ubiquitin ligase), XB10 (OsWRKY62), and XB15 (a PP2C phosphatase)—have been shown to regulate XA21-mediated immunity (22–24).

Here, we report the isolation of an ATPase, called XB24, that associates with XA21 in vivo and modulates XA21 function. XB24 belongs to a large class of broadly conserved ATPases of unknown function. The association between XB24 and XA21 is compromised upon inoculation of the *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strain PXO99, which secretes the Ax21 PAMP (19). XB24 promotes autophosphorylation of XA21 through its ATPase activity. Rice plants silenced for *Xb24* display enhanced XA21-mediated immunity, whereas rice plants overexpressing XB24 are compromised for immunity. XA21 is degraded in the presence of Ax21 when XB24 is overexpressed. These findings reveal that XB24 negatively regulates XA21 PRR function.

Results

XB24 Physically Associates with XA21 in Vivo. We isolated XB24 as an XA21 interacting protein through yeast two-hybrid screening (23). The XB24 cDNA is expressed from a unique rice gene, *Os01g56470* (Fig. S1*A*), and encodes a 198-aa protein. The predicted secondary structure has no significant motifs except for a C-terminal ATP synthase α- and β-subunits signature (ATPase) motif with the sequence PSINERESSS (Fig. S1*B*). Although 38 human proteins, 43 *Arabidopsis* proteins, and 67 additional rice proteins are annotated to contain a conserved ATPase motif (Fig. S2), none share similarity beyond the ATPase motif with XB24 and most are not functionally characterized. Thus, XB24 belongs to a previously uncharacterized class of ATPases.

To confirm the specificity of the XB24-XA21 interaction, we performed yeast two-hybrid analysis and found that XB24 associates with XA21K668 (containing the entire juxtamembrane and the kinase domains of XA21) but not with XA21K668^{K736E} (17), a catalytically inactive mutant of XA21K668 (Fig. 1*A Left*). These results indicate that the association between XB24 and XA21 requires XA21 kinase activity. The ATPase motif of XB24 is not required for the XB24-XA21 interaction in yeast because XB24(1-146), lacking the ATPase motif, retains the ability to interact with

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The authors declare no conflict of interest.

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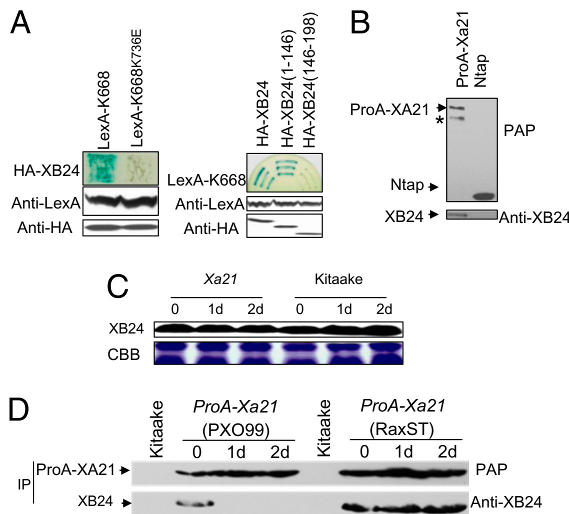

Fig. 1. Association of XB24 with XA21 in yeast and in rice plants. (*A*) Interaction of XB24 with XA21K668 in yeast. K668, truncated XA21 (XA21K668) containing the entire JM and kinase domains (23); $K668^{K736E}$, kinase catalytically inactive mutant $XA21K668^{K736E}$; XB24(1-146), truncated XB24 containing amino acids 1–146; XB24(146-198), truncated XB24 containing amino acids 146–198 including the ATPase motif. Blue, positive interaction. Expression proteins were detected using antibodies as indicated in Western blotting. (*B*) Detection of XA21 and XB24 in immunoprecipitates of ProA-XA21 from rice tissues using the Peroxidase Anti-Peroxidase (PAP) probe and anti-XB24, respectively. *, Cleaved form of ProA-XA21 (23, 24). (*C*) Analysis of XB24 protein levels in plants before and after PXO99 inoculation using anti-XB24 in Western blot analysis. A duplicate protein gel was stained with Coomassie brilliant blue (CBB) as loading control. (*D*) Dissociation of XB24 from XA21 in response to PXO99 inoculation. Detection of ProA-XA21 and XB24 in the immunoprecipitates of ProA-XA21 from rice leaf tissues not treated or treated (1 or 2 days) with *Xoo* strains as indicated. IP, immunoprecipitate.

XA21, whereas XB24(146-198), containing the ATPase motif, is incapable of interacting with XA21 (Fig. 1*A Right*).

To determine whether XB24 physically associates with XA21 in vivo, we created transgenic plants that express a protein A domain-tagged XA21 (ProA-XA21) under control of the native *Xa21* promoter in the rice cultivar Kitaake. We established a homozygous line, A114, with a single transgene insertion and demonstrated that it confers full resistance to *Xoo* strain PXO99 (Fig. S3). A complex associated with ProA-XA21 was immunoprecipitated from total extracts from A114 leaves. Ntap (N-terminal tandem affinity purification, which contains the same protein A domain) transgenic plants, under control of the maize Ubi-1 promoter, were used as the control. The immunoprecipitates were separated on an SDS/PAGE gel and analyzed by Western blotting using the PAP antibody to probe ProA-XA21 and Ntap, and anti-XB24 antibody for XB24, separately. The PAP probe detected full-length ProA-XA21 and a cleaved XA21 product (marked by an asterisk in Fig. 1*B*) in the ProA-XA21 immunoprecipitate. A clear band of endogenous XB24 was detected from the immunoprecipitate of ProA-XA21 but not from the precipitates of Ntap (Fig. 1*B*).

XB24 Dissociates from XA21 in Response to PXO99 Inoculation. To determine whether XB24 is degraded in response to *Xoo* strain PXO99 inoculation, we performed a Western blot analysis to detect the XB24 protein before and after inoculation. We found that a similar amount of XB24 protein was detected in Xa21 and Kitaake plants before inoculation and 1 day or 2 days after inoculation (Fig. 1*C*). This result shows that XB24 is not degraded in response to Ax21. We next investigated whether Ax21 recognition affects the interaction of XA21 and XB24. We performed coimmunoprecipitation experiments with PAP (targeting ProA-XA21) using rice leaf tissues from the Xa21 line inoculated with *Xoo* strain PXO99 or *Xoo* strain PXO99Δ*raxST*, which lacks Ax21 activity due to a knockout of the *raxST* gene (18, 19, 25). We then carried out immunoblotting to detect XB24. A similar coimmunoprecipitation was performed using Kitaake rice leaves as a control. As shown in Fig. 1*D*, we observed a sharp decrease in the amount of XB24 associated with ProA-XA21 post-PXO99 inoculation, whereas, no decrease in the amount of XB24 associated with ProA-XA21 was observed after PXO99Δ*raxST* inoculation. These results clearly indicate that the physical interaction between XB24 and XA21 disassociates specifically in response to *Xoo* strains expressing Ax21 activity.

XB24 Possesses ATPase Activity. Because XB24 contains a C-terminal ATPase motif and the residue serine (Ser) 154 is a predicted key site for this motif, we tested whether it indeed possesses intrinsic ATPase activity. We purified Ntap-XB24 and $Ntap\text{-}XB24^{S154A}$ (containing a single amino acid change of Serine 154 by Alanine) from Ntap-Xb24 and $Ntap\text{-}XB24^{S154A}$ transgenic plants, respectively, and performed the ATP hydrolysis assay. As shown in Fig. 2*A*, Ntap-XB24 displayed significant ATP hydrolysis activity, whereas $Ntap\text{-}XB24^{S154A}$ had only negligible ATPase activity. We also found that *E. coli*-produced recombinant protein His-XB24 possesses ATPase activity and that the S154A mutant completely abolished the ATPase activity of XB24 (Fig. S4). Taken together, these results show that the XB24 protein possesses an ATPase activity and that amino acid S154 is essential for its ATPase activity.

XB24 ATPase Enhances Autophosphorylation of XA21K668. We next tested whether XB24 is a substrate of XA21 or affects XA21 kinase

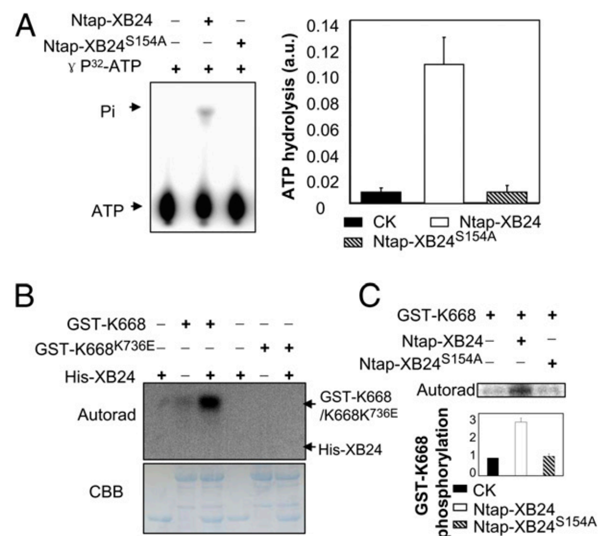

Fig. 2. An ATPase activity is associated with XB24 and effects XA21 autophosphorylation. (*A*) ATPase activity assay on purified Ntap-XB24 and $Ntap\text{-}XB24^{S154A}$ protein from transgenic plants. The same amount of proteins was used. (*Left*) Representative autoradiogram. (*Right*) Quantitative results of three independent experiments. ATP hydrolysis was quantified based on radioactivity of the reaction product Pi. Error bars indicate SDs. (*B*) Effects of XB24 on XA21 autophosphorylation. In vitro autophosphorylation assays were performed on GST-XA21K668 and $GST\text{-}XA21K668^{K736E}$, respectively, in the presence of the purified His-XB24 protein. (*C*) Effects of XB24 ATPase on XA21 autophosphorylation. An in vitro autophosphorylation assay was performed on GST-XA21K668 in the presence of the same amount of rice-expressed Ntap-XB24 or $Ntap\text{-}XB24^{S154A}$. (*Upper*) Representative autoradiogram. (*Lower*) Quantitative results (mean + SD) from three independent experiments. CK, control provided using autophosphorylation assay on GST-XA21K668 in the absence of XB24. The autophosphorylation level from CK was arbitrarily set as "1."

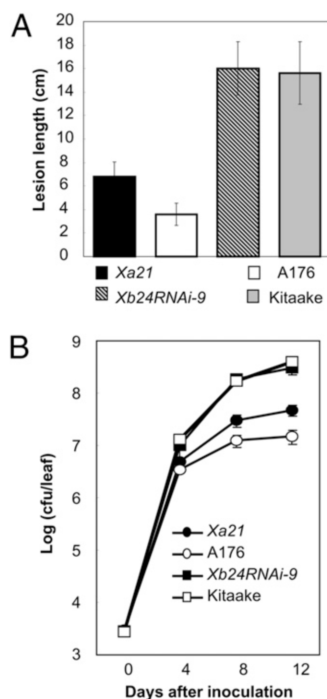

Fig. 3. Effects of reduced expression of Xb24 on Xa21-mediated resistance. (*A*) Quantitative lesion length measurements of rice leaves at 14 days after PXO99 inoculation. The means ± SD of each sample was calculated from 24 infected leaves of 8 plants. (*B*) Bacterial growth curves after PXO99 inoculation. Error bars indicate SDs.

autophosphorylation. Purified His-XB24 and GST-XA21K668 were co-incubated in the presence of [^{32}P]ATP for kinase analysis. For a control, the purified His-XB24 was co-incubated with GST-XA21K668^{K736E}, a catalytically inactive mutant (17). As Fig. 2*B* shows, the GST-XA21K668 autophosphorylates as expected, whereas His-XB24 does not autophosphorylate or become transphosphorylated by GST-XA21K668. The phosphorylation of GST-XA21K668 is highly enhanced in the presence of His-XB24 protein. No phosphorylation of GST-XA21K668^{K736E} can be detected in reactions carried out in the presence of absence of His-XB24. These results demonstrate that XB24 promotes XA21K668 autophosphorylation. To test whether XB24 promotes autophosphorylation of intact, native XA21 protein, the immunoprecipitated ProA-XA21 protein from rice tissue described above (0, 1, or 2 days post-PXO99 inoculation) was co-incubated with the purified His-XB24 for kinase autophosphorylation analyses. These results demonstrate that XB24 promotes autophosphorylation of the native XA21 protein. Furthermore, XB24 is not transphosphorylated by the XA21 protein with or without PXO99 inoculation (Fig. S5). To test whether the ATPase activity of XB24 is required for promoting XA21K668 autophosphorylation, the purified Ntap-XB24 and NtapXB24^{S154A} were incubated with GST-tagged XA21K668 in the presence of [^{32}P]ATP for kinase analyses. Autophosphorylation of GST-XA21K668 is enhanced in the presence of rice-expressed Ntap-XB24 but not Ntap-XB24^{S154A} (Fig. 2*C*). Autophosphorylation of the GST-XA21K668 fusion protein is also enhanced in the presence of the His-XB24 protein but not His-XB24^{S154A} (Fig. S6). These results demonstrate that XB24 enhances XA21 autophosphorylation and that its ATPase activity is required for this function.

Silencing of *Xb24* Enhances *Xa21*-Mediated Resistance. To investigate the biological function of XB24, we used the RNA interference (RNAi) approach (26, 27) to silence the *Xb24* gene and monitored its effects on disease resistance. We developed two independent lines, *Xb24RNAi-3* and *Xb24RNAi-9*, each containing a single-locus insertion, using the rice cultivar Kitaake as the transgene recipient. RT-PCR analysis revealed that *Xb24* transcript levels were significantly reduced in these two lines (Fig. S7*A*). Both lines show similar disease lesion lengths compared to the control line Kitaake after challenge with PXO99 (Fig. S7*B*), indicating that silencing of *Xb24* does not affect the susceptibility of Kitaake to *Xoo*.

To explore the role of XB24 in XA21-mediated signaling, we crossed *Xb24RNAi-3* and *Xb24RNAi-9* with *Xa21* lines and obtained one progeny form the *Xa21*/*Xb24RNAi-3* cross and three from the *Xa21*/*Xb24RNAi-9* cross. Our initial results indicated that silencing of *Xb24* enhanced resistance (Fig. S7*B*). To confirm these results, we developed an F_4 line (A176) from one of the F_1 plants. The A176 line carries homozygous *Xa21* and homozygous *Xb24RNAi-9*. We then inoculated 3-week-old A176 plants. As shown in Fig. 3*A*, these plants developed much shorter lesion lengths (3 ± 0.9 cm) than the wild-type *Xa21* plants (6.8 ± 1.2 cm), which show only partial resistance at the 3-weeks-old (tilling) stage (28). A *t* test gave a *P* value of 8.62×10^{-13}, showing a highly significant difference. Rice line *Xb24RNAi-9* showed similar disease lesion lengths (16 ± 2.5 cm) as Kitaake ($P = 0.56$). Bacterial growth curve analysis revealed that *Xa21*/*Xb24RNAi-9* lines harbor 3.2-fold less *Xoo* bacteria ($1.48 \times 10^7 \pm 1.2 \times 10^6$) in their leaves than the *Xa21* lines ($4.8 \times 10^7 \pm 4.4 \times 10^6$) at 12 days postinoculation (Fig. 3*B*), consistent with the leaf lesion length measurements described above. This experiment was repeated three times, and similar results were obtained each time. These results demonstrate that silencing of *Xb24* expression enhances XA21-mediated disease resistance.

Overexpression of XB24 Compromises XA21-Mediated Resistance. To further investigate the involvement of XB24 in the XA21-mediated signaling, we created construct *Ubi-Xb24* to overexpress XB24 using the maize Ubi-1 promoter. We introduced the *Ubi-Xb24*

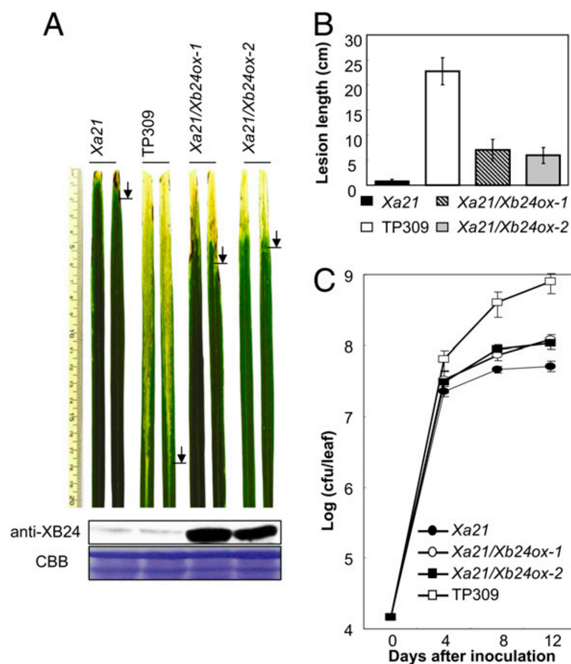

Fig. 4. Effects of overexpression of *Xb24* on *Xa21*-mediated resistance. (*A*) Photograph of rice leaves 14 days after inoculation with PXO99 (*Top*). The disease lesions are indicated from the top of the leaf cuts to the arrows. The XB24 protein was detected by anti-XB24 (*Middle*). A duplicate protein gel was stained with CBB as control (*Bottom*). (*B*) Quantitative lesion length measurements of rice leaves at 14 days after *Xoo* inoculation. The average of each sample was calculated from 40 infected leaves of 10 plants. (*C*) Growth curves of *Xoo* postinoculation. Error bars in *B* and *C* indicate SDs.

construct directly into an *Xa21* (in the TP309 genetic background) line by *Agrobacterium*-mediated transformation using mannose selection (29) and generated five independent T_0 plants. After PCR-based genotyping and RT-PCR-based transcripts expression analyses to confirm that *Xb24* is overexpressed, we challenged 6-week-old Xa21 lines with PXO99. We found that all of the five lines have longer disease lesion lengths compared with the wild-type Xa21 plants (Fig. S8).

Two homozygous lines (*Xa21/Xb24ox-1* and *-2*) from two of these five independent lines were then developed. Overexpression of XB24 (XB24ox) in the progeny from these homozygous lines was confirmed by protein gel blotting analysis (Fig. 4*A*). Six-week-old plants were challenged with PXO99. Disease lesion lengths on both the *Xa21/Xb24ox-1* and *-2* lines (7.3 ± 0.5 cm for line 1 and 6.0 ± 0.5 cm for line 2) were longer than those observed on Xa21 lines (1.3 ± 0.4 cm) (Fig. 4 *A* and *B*). The low *P* values (5.02×10^{-21} for *Xa21/Xb24ox-1* and 2.06×10^{-23} for *Xa21/Xb24ox-2*) indicate that these differences are statistically significant. At 12 days postinoculation, the accumulation of bacterial populations, as measured by bacterial growth curve analysis, in the two *Xa21/Xb24ox* lines ($1.23 \times 10^8 \pm 1.88 \times 10^7$ for *Xa21/Xb24ox-1* and $1.08 \times 10^8 \pm 1.97 \times 10^7$ for *Xa21/Xb24ox-2*) was clearly higher (>2-fold) than in the Xa21 lines ($5.20 \times 10^7 \pm 8.9 \times 10^5$) (Fig. 4*C*). Again, the low *P* values (8.27×10^{-4} for *Xa21/Xb24ox-1* and 2.72×10^{-3} for *Xa21/Xb24ox-2*) of bacterial accumulation at 12 days postinoculation indicate that these differences are statistically significant. Rice lines overexpressing *Xb24* display similar levels of susceptibility as control lines lacking overexpressed *Xb24* in three independent biological replicates (Fig. S8). These results demonstrate that overexpression of XB24 compromises XA21-mediated resistance.

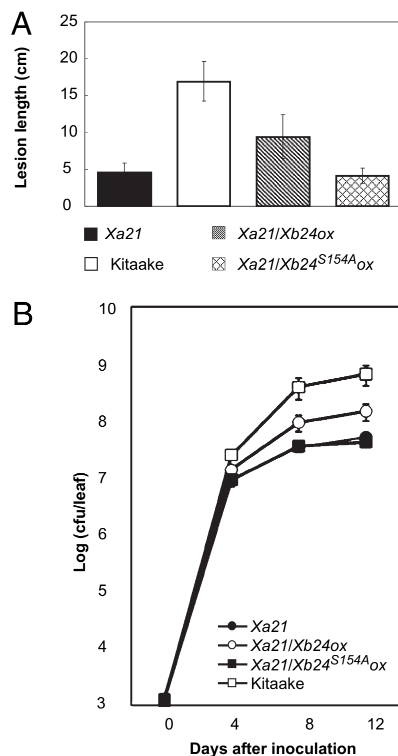

Fig. 5. Requirement of XB24 ATPase activity for regulation of XA21-mediated immunity. (*A*) Lesion lengths were measured for *Xa21*, Kitaake, *Xa21/Xb24ox*, and *Xa21/Xb24*^{S154A}*ox* at 14 days after PXO99 inoculation. The mean and SD of each sample were determined using 32 infected leaves from 8 plants. (*B*) Bacterial growth curve analysis after PXO99 inoculation. Error bars indicate SDs.

ATPase Activity Is Essential for XB24-Mediated Regulation of XA21 Function. We next tested whether XB24 ATPase activity was required for XB24 to regulate XA21 function. We developed *Xa21/Xb24ox* and *Xa21/Xb24*^{S154A}*ox* plants using *NtapXb24ox* and *NtapXb24*^{S154A}*ox* plants, respectively, to cross with *ProAXa21* plants, and inoculated these plants with PXO99. As shown in Fig. 5*A*, all *Xa21/Xb24ox* plants display compromised resistance, whereas *Xa21/Xb24*^{S154A}*ox* plants show similar disease lesion lengths compared to *Xa21* plants. The lesion length difference between *Xa21* and *Xa21/Xb24ox* is highly significant ($P = 1.40 \times 10^{-10}$), whereas the difference between *Xa21* and *Xa21/Xb24*^{S154A}*ox* is not ($P = 0.12$). Bacterial growth curve analysis revealed that the amount of *Xoo* bacteria accumulation in *Xa21/Xb24ox* plants ($2.65 \times 10^8 \pm 5.74 \times 10^7$) is higher (~2.45-fold) than that of *Xa21* plants ($1.08 \times 10^8 \pm 6.55 \times 10^6$) at 12 days postinoculation (Fig. 5*B*). The amount of *Xoo* bacterial accumulation in *Xa21/Xb24*^{S154A}*ox* plants ($0.91 \times 10^8 \pm 1.65 \times 10^7$) is similar to that measured in *Xa21* plants (Fig. 5*B*). The low *P* values of bacteria accumulation at 12 days postinoculation in *Xb24ox* plants (0.033 against *Xa21* and 0.028 against *Xa21/Xb24*^{S154A}*ox*, respectively) indicate that these differences are statistically significant. This experiment was repeated two times and similar results were obtained each time. Because ProA-XA21 was expressed to similar levels in *Xa21/Xb24ox*, *Xa21/Xb24*^{S154A}*ox*, and *Xa21* plants (Fig. S9), these results demonstrate that XB24 requires S154 to repress XA21 function. Thus, we conclude that the ATPase activity of XB24 is essential for XB24 to regulate XA21-mediated defense response.

Overexpression of *Xb24* Causes XA21 Instability Following Ax21 Recognition. To gain insight into the mechanism of XB24-mediated regulation of XA21 function, we tested whether XB24 affects the amount of the XA21 protein after *Xoo* inoculation. As shown in Fig. 6 *A* and *B*, without *Xoo* inoculation (Mock treatment), over-

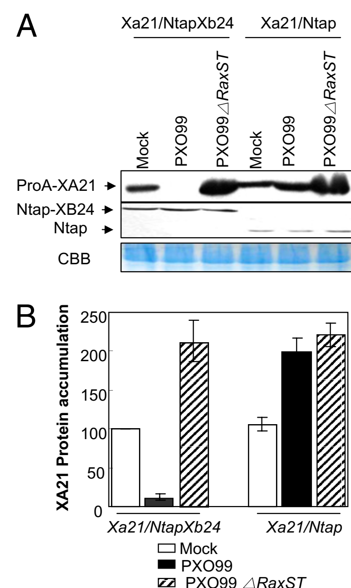

Fig. 6. Effects of excess XB24 on XA21 protein stability. (*A*) Protein immunodetection after SDS/PAGE separation. Protein samples were prepared from *ProA-Xa21* transgenic rice plants overexpressing *Ntap-Xb24* (labeled *Xa21/Ntap-Xb24*) or Ntap (labeled *Xa21/Ntap*) before or after inoculation (1 day) with PXO99 or PXO99Δ*RaxST* (lacking Ax21 activity). Protein accumulation detected by the PAP probe is shown in (*Top*) (for ProA-XA21) and (*Middle*) (for Ntap-XB24 or Ntap), respectively. (*Bottom*) CBB-stained gel as a loading control. (*B*) Quantification of XA21 protein levels. The average and SD are calculated from three biological replicate experiments of (*A*).

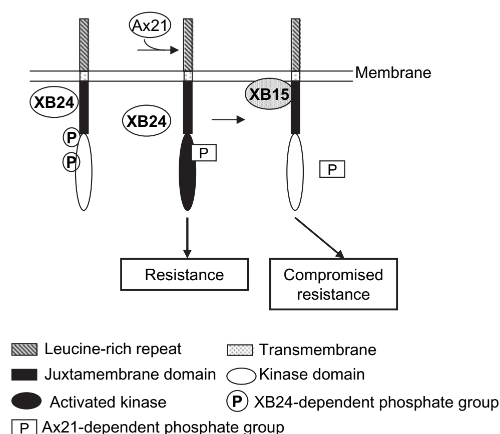

Fig. 7. A model for XB24-mediated regulation of XA21 function. Before Ax21 recognition (left), XB24 physically associates with XA21 and uses ATP to promote phosphorylation of certain Ser/Thr sites on XA21, keeping the XA21 protein in an inactive state. Upon recognition of Ax21 (center), Xb24 dissociates from Xa21 leading to activation of the XA21 kinase, resulting in resistance. Once the signal has been relayed, XA21 binds the XB15 phosphatase (right), which attenuates the immune response, likely by dephosphorylation of amino acids required for XA21 function.

expression of XB24 (*Xa21/Ntap-Xb24*) caused no significant decrease in the ProA-XA21 protein level compared to overexpression of Ntap (*Xa21/Ntap*) alone. In contrast, after inoculation with PXO99, the *Xa21/Xb24ox* line showed a sharp decrease in the ProA-XA21 protein level. The *Xa21/Ntap* control line showed a marked increase. When inoculated with the *Xoo* strain PXO99Δ*raxST*, the *Xa21/Xb24ox* sample showed an increase in the ProA-XA21 level similar to that of the *Xa21/Ntap* control. Similar results were obtained from three biological repeats of this experiment. These results indicate that the sharp decrease in the XA21 protein level is Ax21-specific.

Discussion

A Model for XB24-Mediated Regulation of XA21. Here, we show that XA21 function is enhanced when XB24 expression is reduced and that XA21 function is compromised when XB24 is overexpressed. XB24 regulation on XA21 is tightly associated with its ATPase activity. Thus, we conclude that XB24 regulates XA21 function via its ATPase activity.

In Fig. 7, we present a model to summarize these results. We hypothesize that the XA21 protein is present on the plasma membrane [after transit from the ER (30)], where it recognizes the Ax21 PAMP. XB24 physically associates with XA21 and uses ATP to promote phosphorylation of certain Ser/Thr sites on XA21, keeping the XA21 protein in an inactive state. Upon recognition of Ax21, the XA21 kinase becomes activated, triggering downstream defense responses. The mechanism(s) for XA21 activation following perception of Ax21 likely requires dissociation of XA21 from XB24 and/or removal of the XB24-promoted autophosphorylation. In this model, XA21 autophosphorylation occurs on multiple residues, some of which stimulate XA21 function and others of which inhibit XA21 function. For example, autophosphorylation of the JM residues, Ser-686, Thr-688, and Ser-689, is required for XA21-mediated resistance (21). Autophosphorylation of Thr-705 is also needed for XA21 function (31). Multisite phosphorylation has been previously demonstrated for the function of insulin receptor substrate 1 (IRS1) in human (32). In this case, the activation of protein kinase B in response to insulin propagates insulin signaling and promotes the phosphorylation of IRS1 on serine residues, generating a positive-feedback loop for insulin action. Insulin also activates other kinases that induce the phosphorylation of IRS1 on specific sites and inhibit its functions. There is thus a delicate balance existing between positive IRS1 tyrosine/serine phosphorylation and negative IRS1 serine phosphorylation, which can regulate the IRS1.

When XB24 is overexpressed (a nonphysiological state), the XA21 protein may not dissociate from XB24 readily or the XB24-promoted phosphorylation may not be easily removed. In this case, binding of Ax21 to XA21 may lead to a conformational change in XA21, exposing the XA21 protein to degradation by endogenous proteases. Alternatively, a protease activity could be induced by Ax21/XA21 binding. In either case, overexpressed XB24 would result in degradation of XA21 when challenged by Ax21.

We have previously reported that XB15, a PP2C phosphatase, dephosphorylates autophosphorylated XA21 and negatively regulates the XA21-mediated innate immune responses (23). Our findings that XB24 promotes XA21 autophosphorylation and inhibits XA21-mediated immune response to the Ax21 PAMP further demonstrate that the phosphorylation state of XA21 is critical for XA21-mediated signaling. Phosphorylation of certain residues on XA21 negatively regulates XA21 function, whereas phosphorylation on other residues may be required for activation of XA21 function. These latter residues are likely dephosphorylated by XB15 to down-regulate XA21 activity. Together with our results that the association between XB24 and XA21 is compromised but the association between XB15 and XA21 is enhanced upon PXO99 inoculation (23), our model suggests that the regulation by XB24 occurs before Ax21 recognition but that regulation by XB15 occurs after Ax21 recognition.

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XB24 Represents a Previously Undescribed Class of ATPases. ATPases are abundant in most species. ATPases have been classified into four superfamilies, F-, V-, A-, and P-ATPases, based on their structures (33–36). There are some other proteins that cannot be classified into these subfamilies but have ATPase activity, such as heat shock proteins (HSPs), including HSP60 (33), HSP70 (34), and HSP72 (35). XB24 does not belong to any of these previously described superfamilies of ATPases or HSPs. The only conserved structure in XB24 is the region composed of 10 amino acids PSINERE**S**154SS (Fig. S1*B*) that is predicted as the ATPase motif, (P-[SAP]-[LIV]-[DNH]-{LKGN}-{F}-{S}-**S**-{DCPH}-S) (Fig. S1*C*). The ATPase motif in the F1, V1, and A1 complexes of F-, V-, and A-ATPases is also essential for ATPase activities, whereas the P-ATPases and the HSPs do not contain this motif. However, whether this motif is enough for the ATPase activity of proteins is unclear. Here, we show that XB24, a protein with an ATPase motif but no other motifs or domains, functions as an ATPase. Proteins with this conserved motif that cannot be classified into the previously identified ATPases exist in many species, including bacteria, fungi, human, *Arabidopsis*, and rice. However, none of these have previously been functionally characterized. Thus, our results demonstrating that XB24 is an ATPase with an important function in XA21-mediated immunity will facilitate functional studies of XB24-type ATPases in other species.

Materials and Methods

The Matchmaker LexA two-hybrid system (Clontech) was used for yeast two-hybrid assays. Rice transformation was performed as described in ref. 36. Purified recombinant proteins from *E. coli* or rice were used for ATPase and kinase analyses. In vitro ATPase and kinase assays were performed as described in refs. 17 and 37. Total protein extraction from yeast and rice as well as Western blotting experiments were also performed as described in ref. 17. Methods for other experiments and antibodies can be found in *SI Materials and Methods*.

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