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.

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. S1A), 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. S1B). 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 XA21K668ΔB346 (17), a catalytically inactive mutant of XA21K668 (Fig. 1 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 XB24Δ1-146, lacking the ATPase motif, retains the ability to interact with XA21 in vivo.

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.

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using rice leaf tissues from the Xa21 line inoculated with Xoo strain PXO99 or Xoo strain PXO99ΔraxST, which lacks Xa21 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. 1D, 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 PXO99ΔraxST inoculation. These results clearly indicate that the physical interaction between XB24 and XA21 dissociates specifically in response to Xoo strains expressing Xa21 activity.

**XB24 Possesses ATPase Activity.** Because XB24 contains 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 Ntpb-XB24 and Ntpb-XB24S154A (containing a single amino acid change of Serine 154 by Alanine) from Ntpb-Xb24 and Ntpb-XB24S154A transgenic plants, respectively, and performed the ATP hydrolysis assay. As shown in Fig. 2, Ntpb-XB24 displayed significant ATP hydrolysis activity, whereas Ntpb-XB24S154A had only negligible ATPase activity. We also found that *E. coli*-produced recombinant 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 activity of XA21K668 in yeast. K668, truncated XA21 (XA21K668) containing the entire JM and kinase domains (23), K668T328E, kinase catalytically inactive mutant XA21K668T328E, XA21K736E, XA21K668T328E, K736E, 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 strain as indicated. IP, immunoprecipitate.

**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); K668T328E, kinase catalytically inactive mutant XA21K668T328E; XA21K736E, XA21K668T328E, K736E, 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 strain as indicated. IP, immunoprecipitate.

**Fig. 2.** An ATPase activity is associated with XB24 and effects XA21 autophosphorylation. (A) ATPase activity assay on purified Ntpb-XB24 and Ntpb-XB24S154A 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-XA21K668K736E, 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 Ntpb-XB24 or Ntpb-XB24S154A. 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.”
autophosphorylation. Purified His-XB24 and GST-XA21K668 were co-incubated in the presence of [32P]ATP for kinase analysis. For a control, the purified His-XB24 was co-incubated with GST-XA21K668(S154A), a catalytically inactive mutant (17). As Fig. 2B 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(S154A) 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 [32P]ATP for kinase analyses. Autophosphorylation of GST-XA21K668 is enhanced in the presence of rice-expressed Ntap-XB24 but not Ntap-XB24(S154A) (Fig. 2C). 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, Xo24RNAi-3 and Xo24RNAi-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. S7A). Both lines show similar disease lesion lengths compared to the control line Kitaake with challenge with PXO99 (Fig. S7B), 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. S7B). To confirm these results, we developed an F2 line (A176) from one of the F1 plants. The A176 line carries homozygous Xa21 and homozygous Xb24RNAi-9. We then inoculated 3-week-old A176 plants. As shown in Fig. 3A, 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 (filling) stage (28). A t test gave a P value of 8.62 × 10^-10, 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 × 10^9 ± 1.2 × 10^9) in their leaves than the Xa21 lines (4.8 × 10^9 ± 4.4 × 10^9) at 12 days postinoculation (Fig. 3B), 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
construct directly into an Xa21 (in the TP309 genetic background) line by Agrobacterium-mediated transformation using mannose selection (29) and generated five independent T0 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. 4A). 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. 4A and B). The low P values (5.02 × 10−25 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 × 109 ± 1.88 × 108 for Xa21/Xb24ox-1 and 1.08 × 109 ± 1.97 × 108 for Xa21/Xb24ox-2) was clearly higher (>2-fold) than in the Xa21 lines (5.20 × 107 ± 8.9 × 106) (Fig. 4C). 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.

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/Xb24S154Aox plants using Ntap/Xb24ox and Ntap/Xb24S154Aox plants, respectively, to cross with ProAXa21 plants, and inoculated these plants with PXO99. As shown in Fig. 5A, all Xa21/Xb24ox plants display compromised resistance, whereas Xa21/Xb24S154Aox 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 × 10−10), whereas the difference between Xa21 and Xa21/Xb24S154Aox is not (P = 0.12). Bacterial growth curve analysis revealed that the amount of Xoo bacteria accumulation in Xa21/Xb24ox plants (2.65 × 106 ± 5.74 × 105) is higher (∼2.45-fold) than that of Xa21 plants (1.08 × 106 ± 6.55 × 105) at 12 days postinoculation (Fig. 5B). The amount of Xoo bacterial accumulation in Xa21/Xb24S154Aox plants (0.91 × 106 ± 1.65 × 105) is similar to that measured in Xa21 plants (Fig. 5B). The low P values of bacteria accumulation at 12 days postinoculation in Xb24ox plants (0.033 against Xa21 and 0.028 against Xa21/Xb24S154Aox, respectively) indicate that these differences are statistically significant. This experiment was repeated two times and similar results were obtained each time. Because ProAXa21 was expressed to similar levels in Xa21/Xb24ox, Xa21/Xb24S154Aox, 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 Xa21 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. 6A and B, without Xoo inoculation (Mock treatment), over-

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/Xb24S154Aox 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.
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 PXO99arazSt, 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 Xa21-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 (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.

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 superfamilies 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 PSI-NERES<sup>152</sup>SS (Fig. S1B) that is predicted as the ATPase motif, (P-[SAP]-[LIV]-[DNH]-{LKGN}-{F}-{S}-{DCPH}-S) (Fig. S1C). 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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