

A Conserved Threonine Residue in the Juxtamembrane Domain of the XA21 Pattern Recognition Receptor Is Critical for Kinase Autophosphorylation and XA21-mediated Immunity^{*[S]}

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Xuwei Chen, Mawsheng Chern, Patrick E. Canlas, Caiying Jiang¹, Deling Ruan, Peijian Cao, and Pamela C. Ronald²

From the Department of Plant Pathology, University of California, Davis, California 95616

Despite the key role that pattern recognition receptors (PRRs) play in regulating immunity in plants and animals, the mechanism of activation of the associated non-arginine-aspartate (non-RD) kinases is unknown. The rice PRR XA21 recognizes the pathogen-associated molecular pattern, Ax21 (activator of XA21-mediated immunity). Here we show that the XA21 juxtamembrane (JM) domain is required for kinase autophosphorylation. Threonine 705 in the XA21 JM domain is essential for XA21 autophosphorylation *in vitro* and XA21-mediated innate immunity *in vivo*. The replacement of Thr⁷⁰⁵ by an alanine or glutamic acid abolishes XA21 autophosphorylation and eliminates interactions between XA21 and four XA21-binding proteins in yeast and rice. Although threonine residues analogous to Thr⁷⁰⁵ of XA21 are present in the JM domains of most RD and non-RD plant receptor-like kinases, this residue is not required for autophosphorylation of the *Arabidopsis* RD RLK BRI1 (brassinosteroid insensitive 1). The threonine 705 of XA21 is conserved only in the JM domains of plant RLKs but not in those of fly, human, or mouse suggesting distinct regulatory mechanisms. These results contribute to growing knowledge regarding the mechanism by which non-RD RLKs function in plant.

Receptor-like kinases (RLKs)³ play important roles in many biological processes, including development and immunity responses, in both animals and plants. Most RLKs possess intrinsic protein kinase activity and regulate downstream signaling through phosphorylation and dephosphorylation events (1–5). Kinases are classified as arginine-aspartate (RD) or non-RD kinases. RD kinases carry a conserved arginine (Arg) immediately preceding the catalytic aspartate (Asp) (6). RD

kinases are regulated by autophosphorylation of the activation segment, a centrally located loop positioned close to the catalytic center.

In contrast to RD kinases, non-RD kinases typically carry a cysteine or glycine in place of the arginine. We have previously reported that non-RD kinases are associated with the control of early signaling events in both plant and animal innate immunity (7). For example, in humans, non-RD kinases associate with proteins belonging to the Toll-like receptor (TLR) family, which contain leucine-rich repeats in the extracellular domain and Toll-interleukin receptor intracellular domains (8). TLRs recognize pathogen-associated molecular patterns at the cell surface and then activate a common signaling pathway through an association with non-RD kinases to induce a core set of defense responses (9). TLR1, TLR3, TLR5, TLR6, TLR7, TLR8, and TLR9 function through the non-RD interleukin-1 receptor-associated kinase (IRAK1), whereas TLR3 and TLR4 function through the non-RD receptor interacting-protein 1 (10). In plants, RLKs demonstrated to function in mediating innate immunity fall into the non-RD class (10) or are associated with non-RD RLKs (11–13). These include the *Arabidopsis* pattern recognition receptors (PRRs), flagellin sensitive 2 (FLS2) (14) and elongation factor-Tu receptor (15), the rice PRRs, such as XA21 (16), XA26 (17), and Pid2 (or called Pi-d2) (18), the barley PRG1 (resistance to *Puccinia graminis* f. sp. *tritici*) (19), and the *Arabidopsis* RD RLK BAK1 that associates with the non-RD RLK FLS2 (11). Unlike RD kinases, non-RD kinases do not autophosphorylate their activation segments (6, 10, 20). Given the demonstrated importance of the non-RD class of kinases in innate immunity, there is great interest in understanding their mode of action.

RLKs contain a juxtamembrane (JM) domain located between the transmembrane and kinase domains. It is now clear that the JM domain can play an important role in regulating the function of kinase. For example, deletion of the JM domain of the epidermal growth factor (ErbB-1) kinase (an RD RLK) results in a severe loss of tyrosine phosphorylation (1). Two conserved tyrosine phosphorylation sites Tyr⁶⁰⁵ and Tyr⁶¹¹ of EphB2 are essential for EphB2 kinase autophosphorylation and biological responses (21, 22). Phosphorylation of the JM domain of the type I transforming growth factor- β (T β R-I, an RD RLK) eliminates the binding site for the FKBP12 (12-kDa FK506-binding protein) inhibitor protein, leading to activation of the T β R-I kinase (23, 24). To date, the role of the

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¹ Present address: College of Life Science, Zhejiang Sci-Tech University, Hangzhou 310018, China.

² To whom correspondence should be addressed. Tel.: 530-752-1654; Fax: 530-752-6088; E-mail: pcronald@ucdavis.edu.

³ The abbreviations used are: RLK, receptor-like kinase; TLR, Toll-like receptor; PRR, pattern recognition receptor; EFR, elongation factor-Tu receptor; FLS2, flagellin sensing 2; JM, juxtamembrane; GST, glutathione S-transferase; PBS, phosphate-buffered saline; XB, XA21-binding protein; BRI, brassinosteroid insensitive 1; IRS1, insulin receptor substrate 1.

JM domain in non-RD RLK phosphorylation has not yet been elucidated. The rice PRR XA21 is a non-RD RLK that binds the sulfated peptide, called Ax21 (activator of XA21-mediated immunity) (25, 26). XA21 is not autophosphorylated in the activation segment (20). Here we investigate the role of the XA21 JM domain in regulating XA21 autophosphorylation and XA21-mediated immunity. We found that the JM domain is essential for XA21 autophosphorylation *in vitro*. We identified Thr⁷⁰⁵ in the JM domain as an essential site for XA21 autophosphorylation and XA21-mediated innate immunity. We further identified that Thr⁷⁰⁵ is an autophosphorylation site and proposed a model for Thr⁷⁰⁵ to activate XA21 function. Although the RLKs of most plant species surveyed contain a residue analogous to Thr⁷⁰⁵, this threonine is not critical for autophosphorylation of the *Arabidopsis* RD RLK BRI1.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Site-directed Mutagenesis—For the GST fusion constructs, XA21K668, XA21K690, XA21K705, and XA21JM were amplified by the primer pairs 5'-TCTAGA-ATGTCATCACTCTACTTGCTTATA-3'/5'-GTCGACTC-AGAATTCAAGGCTCCACCTTC-3' (for XA21K668), 5'-TCTAGAATGAAAGGCCACCCATTGGTCTCT-3'/5'-GTCGACTCAGAAATTCAGGCTCCACCTTC-3' (for XA21K690), 5'-TCTAGAATGACAGATGGTTTCGCGCCGACC-3'/5'-GTCGACTCAGAAATTCAGGCTCCACCTTC-3' (for XA21K705), and 5'-TCTAGAATGTCATCACTCTACTTGCTTATA-3'/5'-GTCGACTCAGGCGCGAAACCATCTGTTGC-3' (for XA21JM), respectively (XbaI and SalI recognition sites are, respectively, underlined), and cloned into pGEMTM-T Easy vector (Promega). After the sequences were verified, they were digested by XbaI/SalI and cloned into the pGEX-KG-1 vector pre-digested with XbaI/SalI. The pGEX-KG-1 plasmid was modified from pGEX-KG (kindly provided by Chang-Jin Park) by inserting an A nucleotide base to create a frameshift mutation in the multiple cloning site. The resulting constructs were designated as pEGX-KG-1:XA21K668, pEGX-KG-1:XA21K690, pEGX-KG-1:XA21K705, and pEGX-KG-1:XA21JM. The single or triple amino acids mutants, GST-XA21K668^{K736E}, GST-XA21K668^{S697A}, GST-XA21K668^{S697D}, GST-XA21K668^{T705A}, GST-XA21K668^{T705E}, GST-XA21K668^{T680A}, GST-XA21K668^{S686A/T688A/S689A}, and GST-XA21K668^{S699A}, were created by QuikChange[®] site-directed mutagenesis (Stratagene) using pEGX-KG-1:XA21K668 as template. The primer pairs used are 5'-GTTGCAGTGGGAAGTACTA-AAGCTTGAAATCC-3'/5'-GGATTTTCAAGCTTTAGTACTTCCACTGCAAC-3' (for K736E), 5'-CACCCATTG-GTCGCTTATTCGCAGTTG-3'/5'-CAACTGCGAATAA-GCGACCAATGGGTG-3' (for S697A), 5'-CACCCATTG-GTCGATTATTCGCAGTTG-3'/5'-CAACTGCGAATAA-TCGACCAATGGGTG-3' (for S697D), 5'-TTGGTAAAA-GCAGCAGATGGTTTCGC-3'/5'-GCGAAACCATCTGCTGCTTTTACCAA-3' (for T705A), 5'-TTGGTAAAAAGCAG-AAGATGGTTTCGC-3'/5'-GCGAAACCATCTTCTGCTTTTACCAA-3' (for T705E), 5'-TGGCACAAGAGAGCTAAAAA-GGGAG-3'/5'-CTCCCTTTTCTAGCTCTCTTGTGCCA-3' (for T680A), 5'-AAGGGAGCCCCTGCAAGAGCTGCCATGAA-AGGCCAC-3'/5'-GTGGCCTTTTCATGGCAGCTCTTGCA-

GGGGCTCCCTT-3' (for S686A/T688A/S689A), and 5'-TTGGTCTCTTATGCGCAGTTGGTA-3'/5'-TACCAACT-GCGCATAAGAGACCAA-3' (for S699A). All constructs were verified by sequencing.

For construction of pNlexA-XA21K668, the XA21K668 sequence was amplified by PCR using the specific primer pairs 5'-CACCATGTCATCACTCTACTTGCTTATA-3'/5'-TCAGAATTCAAGGCTCCACCTTC-3 and cloned into pENTRTM/D-TOPO[®] (Invitrogen). After the sequence was verified, it was subcloned into pNlexAgtw (a gateway compatible vector modified from pNlexA (Clontech)) using Gateway[®] LRTM Clonase Enzyme Mix (Invitrogen). The resulting plasmid was designated BD vector pNlexAXA21-K668. The single amino acid mutations in pNlexA-XA21K668^{T705A} and pNlexA-XA21K668^{T705E} were introduced by mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene), following the manufacturer's instructions. The primer pairs used for site-directed mutagenesis were 5'-TTGGTA-AAAGCAGCAGATGGTTTCGC-3'/5'-GCGAAACCATCTGCTGCTTTTACCAA-3' (for T705A), and 5'-TTGGTA-AAAGCAGAAGATGGTTTCGC-3'/5'-GCGAAACCATCTTCTGCTTTTACCAA-3' (for T705E). pAD-Gal4-XB3, pAD-Gal4-XB10, pAD-Gal4-XB15, and pAD-Gal4-XB24 clones were isolated from the cDNA library.

We created the stable plant transformation vector pCambia1300:pXA21-Myc-Xa21 according to the method described previously (27). Briefly, the DNA fragment encoding the 13-amino acid Myc peptide was synthesized and cloned into the unique DraIII site in pC822 to create plasmid pC822-Myc. The KpnI fragment containing Myc-Xa21 was cloned into pCAMBIA1300 to create pCambia1300:pXA21:Myc-XA21 (designated pC1300:Myc-XA21). The 1641-base pair DNA fragment covering the region encoding the JM domain was recovered from plasmid pC1300:Myc-XA21 by NcoI digestion and cloned, after sequence verification, into pGEMTM-T Easy vector pre-digested with NcoI, yielding construct pEGM-T:XA21-1641bp. After the 1641-bp sequence was removed from pCAMBIA1300:pXA21:Myc-XA21 by NcoI, the product was designated pC1300:Myc-XA21-8.3kb. The single amino acid mutants, pEGM-T:XA21-1641bp^{T705A} and pEGM-T:XA21-1641bp^{T705E}, were obtained by site-directed mutagenesis using pEGM-T:XA21-1641bp as the template DNA and primer pairs for T705A and T705E, respectively, as described above. After the sequences were verified, the DNA fragments of XA21-1641bp containing the single amino acid mutations were recovered by NcoI digestion and cloned into vector pC1300:Myc-XA21-8.3kb separately. After the sequence and orientations were verified by digestion and sequencing, the resulting constructs were designated pC1300:Myc-XA21^{T705A} and pC1300:Myc-XA21^{T705E}, respectively.

Yeast Two-hybrid Assay—The Matchmaker LexA two-hybrid system (Clontech) was used for our yeast two-hybrid assay. Briefly, pEGY48/p8op-lacZ (Clontech) yeast cells were co-transformed with the BD and AD vectors using the yeast transformation kit, Frozen-EZ yeast transformation II (Zymol Research), and spread onto plates containing SD/-His/-Leu/-Ura medium. After the plates were incubated for 2–3 days, the clones were picked and streaked onto new SD/-His/-Leu/-Ura plates. The yeast clones

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were then streaked onto test plates containing SD/Gal/Raf/-His/-Leu/-Ura/+X-Gal+BU medium, which allowed LexA reporter selection.

Expression and Purification of GST-fused Proteins—BL21 CodonPlus *Escherichia coli* cells (Invitrogen) were transformed with the recombinant GST plasmids and grown to an A_{600} of 0.6. Transformed bacterial cells in 200 ml of medium were induced with 200 μ M isopropyl β -D-thiogalactopyranoside for 7 h at 30 °C. Cells were harvested by centrifugation and resuspended into 1 \times PBS buffer (125 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4 , and 2 mM KH_2PO_4 , pH 7.4) supplemented with 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. The resuspended cells were lysed by sonication, followed by addition of Triton X-100 to a final concentration of 0.1% and gentle agitation at 4 °C for 15 min. The lysate was centrifuged at 13,000 \times g for 15 min at 4 °C. The supernatant was mixed with glutathione-agarose beads (Sigma) followed by incubation for 1 h at 4 °C. After washing twice with 1 \times PBS buffer containing 0.05% Triton X-100 and once with 0.5 \times PBS buffer, some of the glutathione-agarose beads bound with proteins were subjected to an immunodetection assay using anti-GST, whereas others were stored on ice or at 4 °C for later use.

Stable Transformation of Rice—Rice transformation was performed as described previously (28). The *Xa21* gene was isolated from the wild rice *Oryza longistaminata* (16), most rice cultivars including Kitaake, do not contain *Xa21*. Thus, we used Kitaake as the transformation recipient rice in our study. *Agrobacterium* EHA105 was used to infect calli from the rice Kitaake for transformation. Transformants of the rice carrying *Myc-XA21*, *Myc-XA21*^{T705A}, and *Myc-XA21*^{T705E} were selected using hygromycin.

PCR-based Genotyping and Development of Homozygous Transgenic Lines—The presence of the transgene in genomic DNA was examined by PCR using primer pairs 5'-ATAGCA-ACTGATTGCTTGG-3'/5'-CGATCGGTATAACAGCAAA-AC-3'. There were two major PCR products after 0.8% agarose gel separation. One product was 1.2 kb and was specific to transgenic *Myc-XA21* or its site mutants, *Myc-Xa21*^{T705A} and *Myc-Xa21*^{T705E}. The other product was about 1.1 kb, which was a nonspecific band amplified from rice genomic DNA. The transgenic lines with a segregation ratio of \sim 3:1 (transgene: non-transgene) in the T1 generation were selected to develop the homozygous lines. The homozygous lines were obtained from the T3 generation, of which all the progeny plants contained the target transgene.

RNA Extraction and Reverse Transcription-PCR—Total RNA was extracted from leaf tissue using TRIzol reagent (Invitrogen) and reverse transcribed to cDNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen). PCR analyses were performed using *Xa21*-specific primer pairs 5'-TTGTAACTGTGATATTAATC-3'/5'-TCAGAATTCA-AGGCTCCACCTTC-3', which could amplify the 666-bp cDNA transcript from a 1408-bp genomic DNA sequence with a 742-bp intron of *Xa21*.

Plant Cultivation, Xoo Inoculation, and Disease Resistance Determination—Rice plants were grown in the greenhouse until 6 weeks of age and transferred to the growth chamber before *Xoo* inoculation. Bacterial suspensions (A_{600} of 0.5) of

Xoo strains, PXO99AZ carrying Ax21 (25, 26), were used to inoculate rice by the scissors-dip method (16) separately. The disease lesion length and bacterial population accumulated in rice leaves were evaluated according to previously reported methods (16).

Total Protein Extraction and Immunodetection—Total rice leaf protein extracts were isolated by grinding leaf tissue in liquid nitrogen and thawing in an equal volume of extraction buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, 10 mM β -mercaptoethanol, 20 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1% protease mixture (Sigma), 2 μ M leupeptin (Sigma), 2 μ M antipain (Sigma), and 2 μ M aprotinin (Sigma)). Cell debris was removed by centrifuging twice at 12,000 \times g for 10 min at 4 °C. The protein concentration was measured with Bio-Rad protein assays. Immunodetection of Myc-XA21 or its mutant proteins was carried out with anti-Myc antibodies (Santa Cruz).

For immunodetection of AD- or BD-protein expression in yeast cells, the yeast cells co-transformed with AD and BD vectors were grown in liquid medium (20 g/liter of galactose, 10 g/liter of raffinose, 6.7 g/liter of Difco™ yeast nitrogen base without amino acids (BD Bioscience), and 0.7 g/liter of drop out supplement -Ura/-His/-Leu (BD Bioscience)) to an A_{600} of 1.0, and then harvested. The yeast cells were washed twice with cold 1 \times PBS, and resuspended in 1 \times Laemmli loading buffer and boiled for 5 min. SDS-PAGE (10% gel) was used to separate the proteins. Western blotting was performed as described previously (29) using anti-LexA (Clontech), anti-XB24 (Pacific Immunology), or anti-XB15 (Pacific Immunology) antibodies.

Immunoprecipitation of Rice Proteins—To immunoprecipitate MycXA21 and its mutant proteins, 10 g of rice leaf tissue was harvested from 5-week-old transgenic plants carrying pC1300:Myc-XA21 or its site mutation proteins pC1300:Myc-XA21^{T705A} or pC1300:Myc-XA21^{T705E}. Protein extracts were isolated by grinding leaf tissue in liquid nitrogen and thawing in 20 ml of extraction buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10 mM β -mercaptoethanol, 20 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1% protease mixture (Sigma), 10 μ M leupeptin, and 5% glycerol). After filtering through double layers of Miracloth (Calbiochem) followed by centrifugation twice at 13,000 \times g for 15 min at 4 °C, the supernatant was mixed with 50 μ l of agarose-conjugated anti-Myc antibodies (Santa Cruz) and incubated at 4 °C for 1 h. The agarose beads were washed four times each in 1 ml of extraction buffer lacking protease inhibitors. Some of the washed beads were boiled, and the proteins were separated by SDS-PAGE followed by immunodetection with anti-Myc (Santa Cruz), anti-XB15, and anti-XB24 antibodies, respectively. The other beads were stored on ice or at 4 °C for later use.

In Vitro Kinase Autophosphorylation Assay—The purified GST- or Myc-fused proteins bound to agarose beads were washed twice with kinase buffer (50 mM HEPES, pH 7.4, 10 mM MgCl_2 , 10 mM MnCl_2 , 1 mM dithiothreitol). The autophosphorylation assay was carried out in a 30- μ l reaction mixture containing 20 μ l of agarose bead-bound proteins and 15 μ Ci of [γ -³²P]ATP (PerkinElmer Life Sciences). The reaction was stopped after 1 h by addition of cold (4 °C) washing buffer (0.1 \times PBS containing 2 mM EDTA) and washed five times. The pro-

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teins bound to agarose beads were separated by SDS-PAGE (10 or 7% gels) after addition of 10 μ l of Laemmli loading buffer (4 times) and boiling for 5 min. After staining with Coomassie Brilliant Blue G-250 (for the GST-fused proteins), the gel was dried and exposed to x-ray film.

Mass Spectrometry (MS) and Identification of Phosphorylation Sites—LC-MS/MS-based protein identification was carried out at the Genome Center Proteomics Core at the University of California, Davis. Proteins were prepared for MS analysis using standard reduction, alkylation, and tryptic digest procedures (30). Digested peptides were analyzed by LC-MS/MS on a LTQ-FT with a Waters UPLC. Peptides were identified by searching the MS/MS spectra against a data base containing XA21 protein sequence using TurboSEQUEST as provided in BioWorks 3.3 (Thermo Finnigan). All of the spectra were analyzed using the software Scaffold Viewer 2.0 following the way as previously reported (31).

Determination of the Threonine Sites Analogous to Thr⁷⁰⁵ of XA21 in Human, Arabidopsis, and Rice RLKs—The amino acid sequences of fly, human, and mouse were from kinases.com (32, 33). The transmembrane helices were predicted using TMHMM Serve version 2. Only those containing transmembrane helices were considered RLKs for JM domain analyses. The sequences of rice and *Arabidopsis* RLKs were obtained from published reports (10). The amino acid residues that were located one (similar as that of Pid2) or three amino acids before the kinase domains of RLKs were sites equivalent to Thr⁷⁰⁵ of XA21. The kinase domains of RLKs were determined through PFAM domain research using the simple modular architecture research tool (SMART). The list of RLKs is provided in supplemental Fig. S1.

RESULTS

Both the JM and Kinase Domains of XA21 Are Required for XA21 Autophosphorylation in Vitro—We have previously shown that XA21K690, a truncated XA21 protein containing a truncated JM and the entire kinase domain, possesses kinase activity (20). To determine whether the JM domain is essential for XA21 autophosphorylation, we expressed and purified several variants of the XA21 proteins: XA21K668 (containing the entire JM and kinase domains), XA21K690, XA21K705 (containing the last three amino acids of the JM domain and the entire kinase domain), XA21K668^{K736E} containing a catalytically inactive mutant (20, 34), and XA21JM (containing only the JM domain). These proteins were expressed as GST fusion proteins and used for autophosphorylation assays (Fig. 1A). We found that GST-XA21K668 and GST-XA21K690 undergo *in vitro* autophosphorylation, as previously demonstrated (20) (Fig. 1B). In contrast, GST-XA21K705 (labeled K705), XA21JM (labeled JM), and GST-K668^{K736E} (labeled K668^{K736E}) all lost the ability to autophosphorylate *in vitro*. These results indicate that the presence of both the JM and kinase domains of XA21 as well as residue Lys⁷³⁶ in the kinase domain are required for XA21 autophosphorylation *in vitro*.

Thr⁷⁰⁵ Is Essential for Kinase Autophosphorylation—It was previously reported that the triple sites mutation of the phosphorylation residues, Ser⁶⁸⁶, Thr⁶⁸⁸, and Ser⁶⁸⁹ in the JM domain does not affect XA21 autophosphorylation (35). To

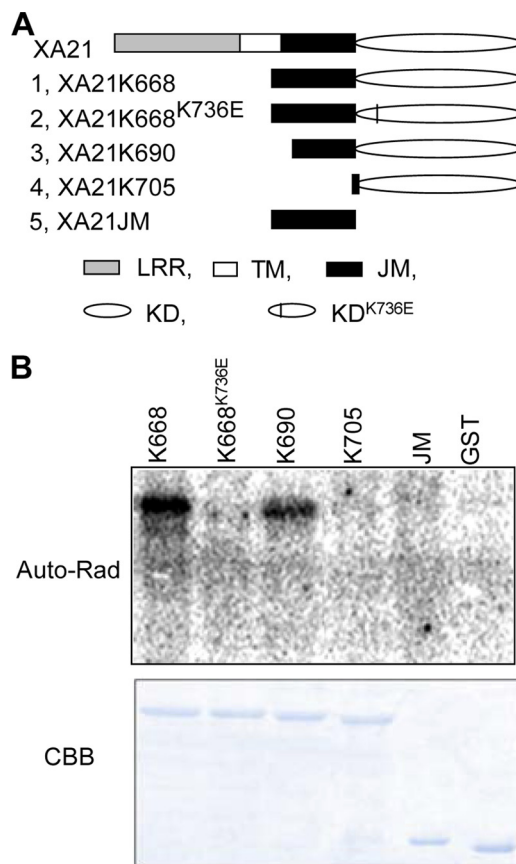


FIGURE 1. Kinase autophosphorylation assay for the XA21 variants. A, the structures of XA21 variants. B, the autophosphorylation assay for the *E. coli*-expressed, GST-fused XA21 variant proteins. The upper panel shows the autoradiograph (auto-rad) and the lower panel is a photograph of the gel stained with Coomassie Brilliant Blue (CBB). K668, K668^{K736E}, K690, K705, and JM denote GST-XA21K668, GST-XA21K668^{K736E}, GST-XA21K690, GST-XA21K705, and GST-XA21K668JM, respectively, and GST denotes GST alone.

determine whether the other four candidate phosphorylation sites, Thr⁶⁸⁰, Ser⁶⁹⁷, Ser⁶⁹⁹, and Thr⁷⁰⁵ in the JM domain affect(s) XA21 kinase autophosphorylation, we changed these sites into alanine, or the negatively charged aspartic acid or glutamic acid, one by one in GST-XA21K668 and obtained a set of single amino acid mutations of GST-XA21K668. We performed kinase autophosphorylation assays on the *E. coli* produced proteins of these mutants and found that only one residue, Thr⁷⁰⁵, abolished XA21 autophosphorylation *in vitro* (*E. coli*-produced GST-K668 and GST-K668^{S686A/T688A/S689A} were included as positive controls and the catalytic dead mutant GST-K668^{K736E} as negative control). As expected, GST-K668 and GST-K668^{S686A/T688A/S689A} show autophosphorylation, whereas GST-K668^{K736E} does not (Fig. 2). The mutation of Ser⁶⁹⁷ into alanine or aspartic acid slightly reduces XA21 autophosphorylation, whereas the mutation of Thr⁶⁸⁰ or Ser⁶⁹⁹ enhances XA21 autophosphorylation. More interestingly, both mutations of Thr⁷⁰⁵ to alanine and glutamic acid abolished XA21 autophosphorylation (Fig. 2). The same results were obtained from three repeats of kinase autophosphorylation analyses for the purified protein GST-XA21K668^{T705A} and GST-XA21K668^{T705E} (Fig. 3A).

To determine whether the XA21 protein purified from rice was affected by T705A and T705E mutations as observed in

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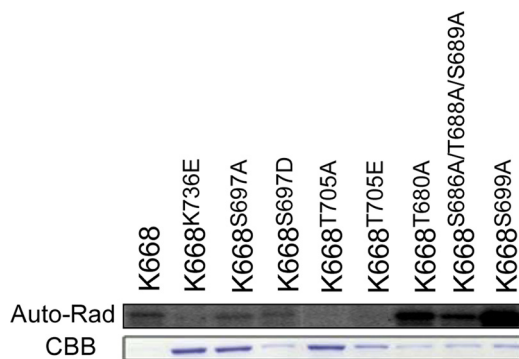


FIGURE 2. Kinase autophosphorylation assay for the GST-XA21K668 variants. The autophosphorylation assay was performed for the *E. coli*-expressed, GST-fused XA21K668 variant proteins. The upper panel shows the autoradiograph (autorad) and the lower panel is a photograph of the gel stained with Coomassie Brilliant Blue (CBB). K668, K668^{K736E}, K668^{S697A}, K668^{S697D}, K668^{T705A}, K668^{T705E}, K668^{T680A}, K668^{S686A/T688A/S689A}, and K668^{S699A}, denote their XA21 GST fusion proteins, respectively.

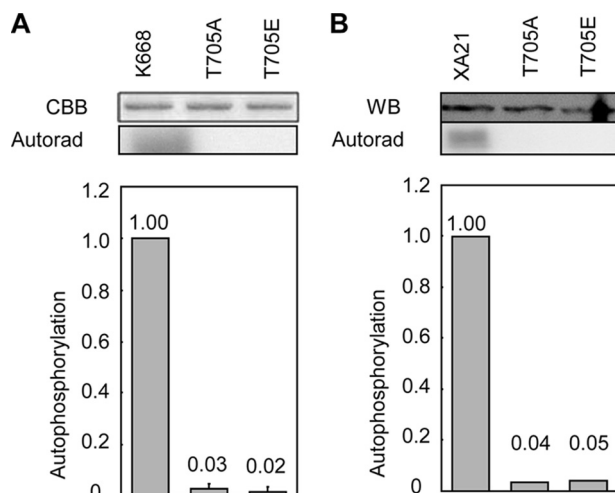


FIGURE 3. Kinase autophosphorylation analyses of XA21K668 and its single amino acid mutants T705A and T705E. A, kinase autophosphorylation was determined for *E. coli*-expressed wild-type GST-XA21K668 (K668) and the GST-XA21K668^{T705A} (T705A), and GST-XA21K668^{T705E} (T705E) mutants. B, kinase autophosphorylation was determined for rice-expressed Myc-XA21 (XA21) and mutants Myc-XA21^{T705A} (T705A) and Myc-XA21^{T705E} (T705E). The upper panel in A shows a Coomassie Brilliant Blue-stained gel and the upper panel in B shows a Western blot (WB) probed with an anti-Myc antibody to normalize protein loading. The middle panels show typical autoradiographs of the indicated proteins, and the bottom panels show quantification of kinase autophosphorylation. Error bars indicate the standard deviations from three independent experiments.

the *E. coli*-produced, truncated XA21, we generated transgenic rice lines expressing Myc-XA21^{T705A}, Myc-XA21^{T705E}, and the control protein Myc-XA21 in Kitaake background. Because the Kitaake rice cultivar does not contain *Xa21* and is susceptible to *Xoo* pathogens, it was used as a recipient in transformation experiments to study the function of *Xa21* (29, 36, 37). In these lines, the transgenes are under control of the *Xa21* native promoter. The (T0) lines were genotyped by PCR, and transgene expression was confirmed by reverse transcription-PCR. We immunoprecipitated Myc-tagged proteins (Myc-XA21, Myc-XA21^{T705A}, and Myc-XA21^{T705E}) from 10 g of rice leaf tissue of the Myc-XA21, Myc-XA21^{T705A}, and Myc-XA21^{T705E} transgenic lines, respectively, by using agarose-conjugated anti-Myc antibody. The Myc-tagged proteins were then subjected to kinase autophosphorylation assays. We found that although the

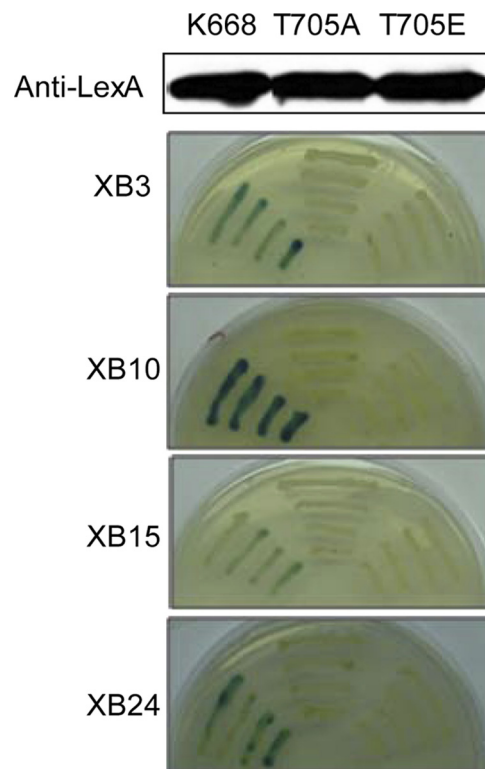


FIGURE 4. Test on the protein interactions of XA21K668 variants (XA21K668^{T705A} and XA21K668^{T705E}) with XBs in yeast. The cytoplasmic domain of XA21 (XA21K668), containing both the JM and kinase domains, and its variants XA21K668^{T705A} and XA21K668^{T705E} were used as baits in the yeast two-hybrid analysis. Upper panel, Western blot of yeast-expressed proteins. LexA-XA21K668 (K668), LexA-XA21K668^{T705A} (T705A), and LexA-XA21K668^{T705E} (T705E) proteins expressed in yeast were extracted and probed with an anti-LexA antibody. Lower panel, summary of protein interaction results. The XB proteins were fused to the activation domain. Blue color indicates the positive interaction.

Myc-XA21 protein was capable of autophosphorylation, both Myc-XA21^{T705A} and Myc-XA21^{T705E} lost this ability (Fig. 3B). These results confirm that Thr⁷⁰⁵ is required for XA21 autophosphorylation and the phosphorylation mimic state of Thr⁷⁰⁵ cannot retain the autophosphorylation ability of XA21.

Both the XA21^{T705A} and XA21^{T705E} Variants Abolish the Interactions of XA21 with Its Binding Proteins in Yeast—We have previously characterized several XA21-binding proteins (XBs), including XB3, an E3 ubiquitin ligase (27); XB10 (OsWRKY62), a WRKY transcription factor (36); XB15, a protein phosphatase 2C, which negatively regulates cell death and XA21-mediated innate immunity (29); and XB24, an ATPase that modulates XA21-mediated innate immune response.⁴ To determine whether the XA21^{T705A} and XA21^{T705E} variants affect the ability of XA21 to interact with these XBs, we performed yeast two-hybrid analyses. For these experiments, we used XA21K668^{T705A} and XA21K668^{T705E} mutants as baits and XBs as preys in a yeast two-hybrid assay. We found that both XA21K668^{T705A} and XA21K668^{T705E} lost interaction with XB3, XB10, XB15, and XB24 (Fig. 4). These results demonstrate that Thr⁷⁰⁵ is critical for interaction of XA21 with these XBs.

⁴ X. Chen, M. Chern, P. Canlas, D. Ruan, C. Jiang, and P. Ronald, submitted for publication.

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T705A and T705E Abolish the Interaction of XA21 with XB15 and XB24 *in Vivo*—To determine whether Thr⁷⁰⁵ affects the interaction of XA21 with XB15 and XB24 *in vivo* (XB3 and

XB10 were not tested because antibodies are not available), we performed immunoprecipitation experiments using 10 g of rice tissue harvested from each of the Myc-XA21, Myc-XA21^{T705A}, and Myc-XA21^{T705E} transgenic lines. These lines are homozygous with a single transgene insertion. Tissue was harvested 18 h after inoculation with *Xoo* strain PXO99AZ, which carries the sulfated Ax21 pathogen-associated molecular pattern. The anti-Myc antibody was used to immunoprecipitate Myc-tagged XA21 and its variants XA21^{T705A} and XA21^{T705E}. The presence of XB15 and XB24 proteins in the precipitates was monitored with anti-XB15 and anti-XB24 antibodies, respectively. The anti-Myc antibody detected a 140-kDa band corresponding to the Myc-XA21 protein in all three tested lines (labeled XA21, T705A, and T705E in Fig. 5, A and B, upper panels). The anti-XB15 antibody detected the XB15 protein (about 68 kDa) in the precipitates prepared from the wild-type XA21 line, but not in those from XA21^{T705A} or XA21^{T705E} (Fig. 5A, lower panel). As XB24 can be clearly detected in the immunoprecipitates from XA21 rice tissue without inoculation,⁴ we harvested leaf tissue from the three transgenic lines prior to inoculation for co-immunoprecipitation of XB24. Western blotting was performed using anti-Myc and anti-XB24 antibodies. Proteins XA21, XA21^{T705A}, and XA21^{T705E} were detected in the precipitates using the anti-Myc antibody, as described previously (27) (Fig. 5B, upper panel). Similar to the results obtained for XB15, the

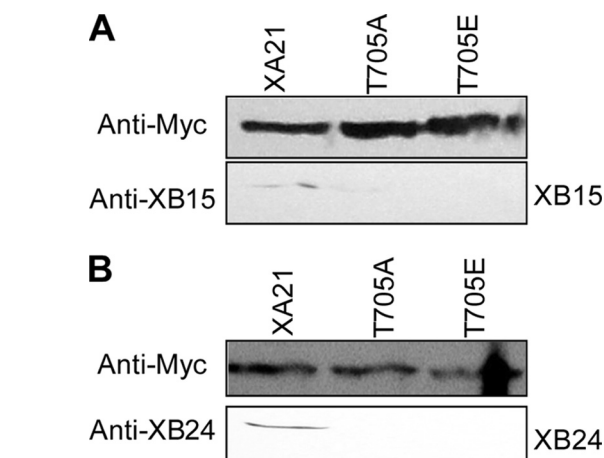


FIGURE 5. Co-immunoprecipitation of XB15 and XB24 with Myc-XA21 and the mutant variants. A, co-immunoprecipitation of XB15. B, co-immunoprecipitation of XB24. Protein extracts from transgenic rice carrying wild-type Myc-XA21, Myc-XA21^{T705A}, or Myc-XA21^{T705E} were mixed with anti-Myc antibody coupled to agarose, and the associated proteins were precipitated. The precipitates were probed with anti-Myc, anti-XB15, or anti-XB24 antibody. Myc-XA21, Myc-XA21^{T705A}, and Myc-XA21^{T705E} proteins represent labeled XA21, T705A, and T705E, respectively.

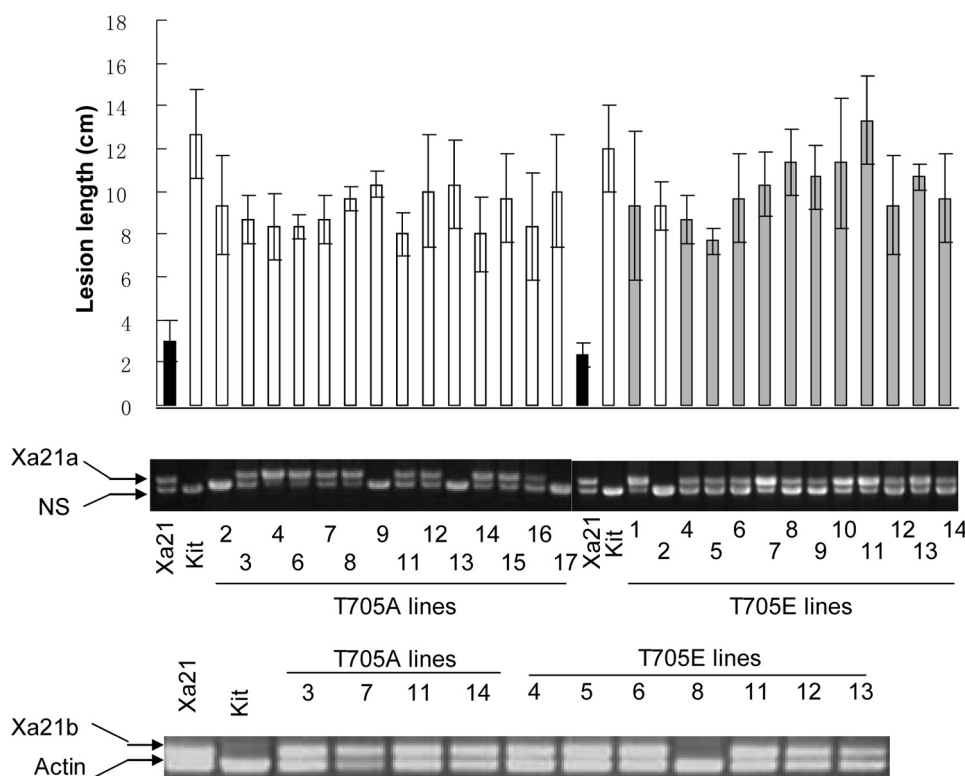


FIGURE 6. Disease resistance evaluation of 6-week-old transgenic plants inoculated with PXO99AZ. The average disease length measured 14 days post-inoculation is shown in the upper panel. The middle panel shows the PCR-based genotyping for the presence of the *Xa21* transgene on the transgenic plants and Myc-*Xa21*, and Kitaake control plants. *Xa21a* designates an *Xa21*-specific amplified fragment and *NS* indicates nonspecific amplification. The bottom panel shows RNA expression of transgenes detected in transgenic lines by reverse transcription-PCR. *Xa21b* indicates the amplification from *Xa21* transcripts. The numbers in the middle and bottom panels represent independent transgenic lines. *Xa21*, *T705A*, and *T705E* represent transgenic plants carrying Myc-*Xa21*, Myc-*Xa21*^{T705A}, and Myc-*Xa21*^{T705E}, respectively. The numbers indicate the progeny lines from each T0 transgenic line used for the analyses. Kitaake is a control. Error bars represent the standard deviation.

anti-XB24 antibody detected the XB24 protein (about 21 kDa) in precipitates of the wild-type XA21 line, but not in those of XA21^{T705A} or XA21^{T705E} (Fig. 5B, lower panel). These results indicate that Thr⁷⁰⁵ is essential for the interaction of XA21 with XB15 and XB24 *in vivo*.

Rice Lines Carrying XA21^{T705A} or XA21^{T705E} Are Altered in XA21-mediated Resistance—The discovery that residue Thr⁷⁰⁵ is required for binding of the negative regulators, XB15 and XB24 suggests that Thr⁷⁰⁵ is critical for XA21-mediated innate immunity. We hypothesized that if Thr⁷⁰⁵ was mutated and XA21 could no longer bind the negative regulators *in vivo*, rice lines expressing these mutants would display enhanced resistance. To test this hypothesis, we assayed transgenic plants carrying Myc-XA21^{T705A} and Myc-XA21^{T705E} for resistance to *Xoo*. Six-week-old plants were challenged with *Xoo* strain PXO99AZ. We found that all 10 Myc-XA21^{T705A} and all 12 Myc-XA21^{T705E} primary transgenic (T0) lines were susceptible to *Xoo* (Fig. 6). We then developed homozygous transgenic progeny from these lines carrying single copies of Myc-

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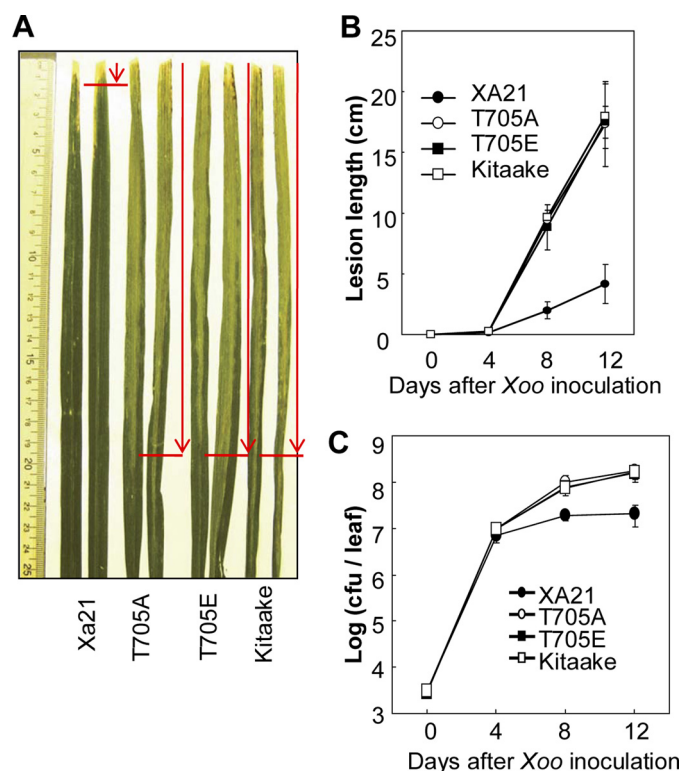


FIGURE 7. Evaluation of transgenic plants carrying XA21, XA21^{T705A}, or XA21^{T705E} on disease resistance to the Xoo strain PXO99AZ. A, photograph of representative leaves taken 14 days after inoculation. B, lesion length development over time. Each data point represents the average and standard deviation of three leaves. C, bacterial growth curves. Each data point represents the average and standard deviation of three leaves. XA21, T705A, and T705E represent transgenic lines expressing wild-type Myc-XA21, Myc-XA21^{T705A}, and Myc-XA21^{T705E}, respectively. Kitaake is the rice recipient used in the transformation experiments.

XA21^{T705A} or Myc-XA21^{T705E} and confirmed that Myc-XA21^{T705A} or Myc-XA21^{T705E} are expressed well. To validate the T0 results, we assayed these homozygous transgenic progeny of Myc-XA21^{T705A} or Myc-XA21^{T705E} by inoculation with PXO99AZ. Myc-XA21 and Kitaake lines were included as controls. We found that both Myc-XA21^{T705A} (labeled T705A in Fig. 7) and Myc-XA21^{T705E} (labeled T705E in Fig. 7) were susceptible to PXO99AZ, similar to the Kitaake control. Fig. 6A shows two typical inoculated leaves (2 weeks post-inoculation) from each of the 6-week-old progeny. As shown in Fig. 7A, both Myc-XA21^{T705A} and Myc-XA21^{T705E} display disease lesion lengths (about 20 cm) similar to that of Kitaake, which is much longer than that (about 2.5 cm) of Myc-XA21 (labeled XA21 in Fig. 7). To more accurately quantify these results, we measured leaf lesion lengths (Fig. 7B) and bacterial populations (Fig. 7C) at different time points (0, 4, 8, and 12 days) post-inoculation. The leaf lesion lengths from both Myc-XA21^{T705A} and Myc-XA21^{T705E} at the 4-, 8-, and 12-day time points are quite similar to that of Kitaake, although much longer than that of Myc-XA21 (Fig. 7B). Consistently, the accumulation of bacterial populations in Myc-XA21^{T705A} and Myc-XA21^{T705E} are similar to that of Kitaake but much higher (≈ 12 times at 12 days) than that of Myc-XA21. These results demonstrate that Thr⁷⁰⁵ in the JM domain is critical for XA21 function, in contrast to our hypothesis.

Thr⁷⁰⁵ Is an Autophosphorylation Site—Because the Thr⁷⁰⁵ residue is essential for XA21 kinase autophosphorylation and required for XA21 function, we were prompted to test whether this residue can be autophosphorylated. To address this question, we performed *in vitro* phosphorylation assays on the GST-K668 protein as described above using excess unlabeled ATP. The *in vitro* autophosphorylated GST-XA21K668 protein was separated by SDS-PAGE after the phosphorylation reaction. In parallel, GST-XA21K668 that was not subjected to the *in vitro* phosphorylation was used as a control and also separated by SDS-PAGE. The GST-XA21K668 bands were excised, digested with trypsin, and subjected to LC/MS/MS analysis. We detected several phosphorylation sites in the XA21 kinase domain (data not shown). But, we could not detect the phosphorylated Thr⁷⁰⁵ after the *in vitro* autophosphorylation treatment. However, when the same *in vitro* phosphorylation experiment was performed in the presence of XB24 (an ATPase), which binds to XA21 and promotes XA21 autophosphorylation,⁴ we detected the phosphorylation of residue Thr⁷⁰⁵ (Fig. 8). Because XB24 is not a kinase but an ATPase that promotes XA21 autophosphorylation, these results indicate that residue Thr⁷⁰⁵ is an autophosphorylation site.

Threonine Sites Analogous to Thr⁷⁰⁵ in XA21 Are Present in Other Plant Receptor-like Kinases—Given the critical importance of Thr⁷⁰⁵ in XA21 autophosphorylation and XA21-mediated immunity, we aligned the XA21 JM sequence with JM sequences of five other known plant RLKs (XA26, Pid2, BRI1, elongation factor-Tu receptor, and FLS2) and found that the other five RLKs contain a threonine residue analogous to Thr⁷⁰⁵ in XA21 at the corresponding location in the JM domain. These include residue Thr⁸⁸⁰ in *Arabidopsis* BRI1 (brassinosteroid insensitive 1), an RD RLK that mediates the brassinosteroid response (38, 39); Thr⁸⁶⁷ in *Arabidopsis* FLS2 (14, 40); Thr⁷⁰⁹ in *Arabidopsis* elongation factor-Tu receptor (15); Thr⁸⁰⁴ in rice XA26 (17); and Thr⁵²⁰ in rice Pid2 (18) (Fig. 9A). We next surveyed the genome sequences of rice, *Arabidopsis*, fly, human, and mouse to determine whether residues analogous to Thr⁷⁰⁵ in XA21 were present and correlated with function of these proteins. We found that 92.7% (507 in 549) of rice RD RLKs and 90.1% (318 in 357) of rice non-RD RLKs contain a threonine residue analogous to Thr⁷⁰⁵ in XA21 at the corresponding location in the JM domain (Fig. 9B). In *Arabidopsis*, 94.2% (425 in 451) of RD RLKs and 84.4% (38 in 45) of non-RD RLKs contained an analogous threonine residue (Fig. 9B). In contrast, only one (the HSER protein) out of 66 mouse RLKs and none of the 22 fly or 71 human RLKs surveyed carried this residue (the JM sequences of these RLKs are described in supplemental Fig. S1). Considering the capability of tyrosine autophosphorylation of the RLKs from fly, human, and mouse, we expected that the JM domains of these RLKs may carry a conserved tyrosine residue, instead of a threonine, which is equivalent to Thr⁷⁰⁵ of XA21. However, we found no RLKs from fly, human, or mouse that contain a tyrosine residue equivalent to Thr⁷⁰⁵ of XA21. These analyses indicate that analogous Thr⁷⁰⁵ residues are highly conserved in plant species but not in fly, human, or mouse RLKs.

DISCUSSION

Phosphorylation and dephosphorylation of residues in the JM domain of animal and plant RD RLKs plays an important

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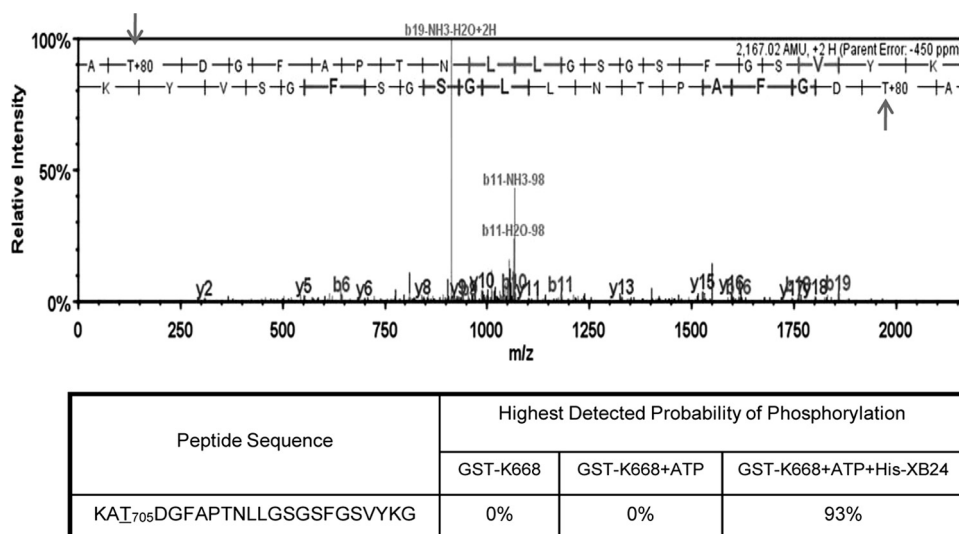


FIGURE 8. Identification of Thr⁷⁰⁵ as an autophosphorylation site by mass spectrometry. GST-XA21K668 protein was incubated with or without the ATPase His-tagged XB24 (*His-XB24*). The purified GST-XA21K668 protein was subject to LC-MS/MS analysis. The top panel shows the spectrum of the peptide containing the Thr⁷⁰⁵ residue from the GST-XA21K668 protein subjected to kinase autophosphorylation in the presence of His-XB24. The spectrum was generated using Scaffold Viewer 2.0. T+80, indicated by the arrows, designates phosphorylated Thr⁷⁰⁵. The bottom panel summarizes the highest probability of phosphorylation of the peptide containing Thr⁷⁰⁵. GST-K668, GST-K668+ATP, and GST-K668+ATP+His-XB24 represent the different treatments of the GST-XA21K668 protein, namely GST-K668 only, GST-K668 subjected to kinase autophosphorylation, and GST-K668 subjected to kinase autophosphorylation in the presence of His-tagged XB24, respectively.

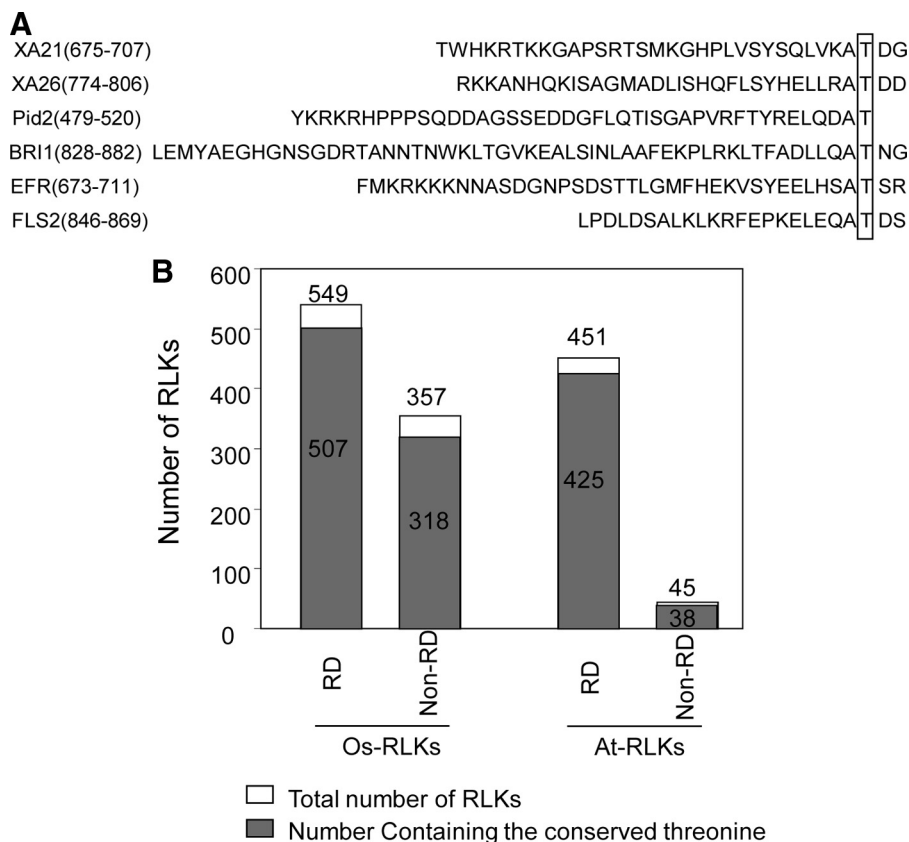


FIGURE 9. Threonine sites in the JM domain of selected plant receptor kinases analogous to Thr⁷⁰⁵ in XA21. A, the JM domains of rice RLKs XA21 (non-RD class), XA26 (non-RD class), and Pid2 (non-RD class), and Arabidopsis RLKs BRI1 (RD-class), FLS2 (non-RD class), and EFR (non-RD class) are listed. The JM sequences shown herein were identified using the SMART program. The boxed conserved threonine amino acid residues are two residues from the beginning of the core S/T kinase domain of all RLKs, except Pid2 (0 residues). B, analyses the equivalent sites to Thr⁷⁰⁵ of XA21 in all RLKs from the kinomes of rice and Arabidopsis. RD and non-RD represent RD and non-RD classes of RLKs, respectively. Os, *Oryza sativa* (rice); At, *Arabidopsis thaliana*.

regulatory role in the signal transduction of these proteins (1–5). It has been previously reported that XA21 JM amino acids Ser⁶⁸⁶, Thr⁶⁸⁸, and Ser⁶⁸⁹ are required to maintain XA21 protein stability (35). Transgenic rice carrying XA21 mutants with either single or triple alanine replacement mutants of these three sites display slightly compromised resistance compared with the wild-type XA21 (35). However, none of these three sites affect XA21 kinase activity to autophosphorylate (35). In this study, we showed that Thr⁷⁰⁵ in the JM domain of XA21 is essential for XA21 autophosphorylation (Figs. 3 and 4) and plays an important biological function. This conclusion is further confirmed by *in vivo* results demonstrating that replacement of Thr⁷⁰⁵ either by alanine or glutamic acid disrupts XA21-mediated immunity.

Our results demonstrate that T705E, a mutation that carries a negatively charged, non-transferable carboxyl group (mimicking a constitutively phosphorylated Thr⁷⁰⁵), has the same effects as T705A, a non-phosphorylatable residue. Both are incapable of transducing XA21-mediated immunity. We also show that phosphorylation of XA21 Thr⁷⁰⁵ can be detected in the XB24 ATPase-promoted autophosphorylated XA21K668 (Fig. 8). These results suggest that the Thr⁷⁰⁵ is an autophosphorylation site and that the phosphophoryl group of Thr⁷⁰⁵ may be transferable as it is not detectable in the XA21K668 protein subjected to kinase autophosphorylation in the absence of XB24 ATPase. All together, these results suggest that Thr⁷⁰⁵ may serve dual roles, to receive and then to donate a phosphoryl group. In other words, after Thr⁷⁰⁵ is autophosphorylated, it may then transfer its phosphoryl group to other XA21 residues, which would activate XA21. In this possible model, a glutamic acid cannot replace Thr⁷⁰⁵ because it cannot serve as a recipient and donor of the phosphoryl group. It is consistent

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with our result that the T705E mutation cannot retain the capability of the XA21 kinase to autophosphorylate. This proposed mechanism is different from most RD kinases (or receptor kinases), whose kinase activation is regulated only by phosphorylation inside the activation segment (6). It is also possible that the T705E mutation may not exactly mimic the stable phosphorylated Thr⁷⁰⁵. If this is the case then the T705E and T705A would be functionally equivalent. In this model Thr⁷⁰⁵ would activate XA21 kinase autophosphorylation through another unknown mechanism.

The mechanism of Thr⁷⁰⁵ function in activation of the XA21 kinase autophosphorylation is different from that of the conserved tyrosine phosphorylation residues of the JM domains of EphB family RD RLKs. First of all, we have shown that the conserved tyrosine phosphorylation residues (for example, Tyr⁶⁰⁵ and Tyr⁶¹¹ in the JM domain of EphB2) (supplemental Fig. S1) (22) are not equivalent to Thr⁷⁰⁵ of XA21. Second, mutation of these tyrosines to phenylalanine impairs kinase activity but mutation to glutamic acid does not disrupt kinase autophosphorylation (21). In contrast, the replacement of Thr⁷⁰⁵ with T705E disrupts XA21 kinase autophosphorylation.

In the case of T β R-I kinase activation, at least three, and perhaps four or five of the serines and threonines in the conserved glycine-serine (GS) region must be phosphorylated by T β R-II upon the binding of transforming growth factor to the T β R-I/T β R-II complex to fully activate T β R-I (23). Phosphorylation of the GS region activates T β R-I not by increasing the activity of the kinase domain but rather by converting the GS region into an efficient recruitment motif for the proper Smad substrate. The mode of XA21 kinase activation via Thr⁷⁰⁵ is most likely distinct from that of the T β R-I RD receptor kinase. Thr⁷⁰⁵ is also required for the binding of the XA21 negative regulators, XB10, XB15, and XB24, representing another difference between XA21 and T β R-I regulation.

Kinase activation of most mammalian RD RLKs (e.g. vascular endothelial growth factor receptor, platelet-derived growth factor receptor; fibroblast growth factor receptor, and epidermal growth factor receptor) is regulated only by phosphorylation inside the activation segment (6). Tyrosine sites in the JM domain of these receptors are not required for kinase activation nor do they appear to be equivalent to Thr⁷⁰⁵. It is unlikely, therefore, that substitution of these tyrosine phosphorylation sites with glutamic acid would constitutively activate these RD receptors. These data suggest that the mechanism by which the JM domain regulates the autophosphorylation and biological function of plant RLKs, especially non-RD RLKs, is different from that of mammalian RLKs. Thus, our findings represent new insights into the mechanism(s) of kinase activation of non-RD receptor kinases. Because, no substrates of XA21 have yet been identified, it is not yet possible to study how phosphorylation of Thr⁷⁰⁵ affects the affinity of XA21 toward its substrates. The failure of XA21 to autophosphorylate results in the inability to interact with XB3, XB10, XB15, and XB24. This result suggests that a conformational change resulting from XA21 autophosphorylation is required for XA21 to interact with its binding proteins. Alternatively, the phosphorylation of XA21 residues may be essential for XBs binding to XA21. For

example, the phosphorylation of residue Ser⁶⁹⁷ may be required for the binding of some XBs like XB15 (29).

It is well known that the conserved lysine site, which is located inside the kinase domain and is required for ATP binding for both RD and non-RD kinases, is essential for kinase autophosphorylation. When this conserved site is mutated, the kinase fails to autophosphorylate and loses its biologic function. For example, the Lys⁹¹¹ residue is essential for the RD RLK BRI to autophosphorylate and mediate the signal of brassinosteroid (39). The Lys⁷³⁶ residue inside the kinase domain is essential for XA21 to autophosphorylate and confer full resistance to *Xoo* (20). However, there are no previous reports showing that a single amino acid in the JM domain of either RD or non-RD RLKs is essential for kinase autophosphorylation. Although residues analogous to Thr⁷⁰⁵ of XA21 are conserved in both RD and non-RD receptor kinases *in planta*, functional studies of these sites are very limited. For example, in RD receptor kinases, the only example in which the equivalent site has been studied is the BRI1 protein. Threonine 880 in BRI1 is analogous to Thr⁷⁰⁵ of XA21 and is an autophosphorylation site. However, the mutation of Thr⁸⁸⁰ to alanine does not affect BRI1 kinase autophosphorylation or biological function (39). In contrast, residue Thr⁸⁶⁷ of FLS2, a non-RD RLK, is analogous to Thr⁷⁰⁵ in XA21 (Fig. 2), and was shown to be essential for the function of FLS2 (40). Transgenic *Ws-0/FLS2^{T867V} Arabidopsis* displays an enhanced disease susceptibility phenotype to *Pseudomonas syringae* (40), suggesting that residue Thr⁸⁶⁷ of FLS2 carries a similar function as that of Thr⁷⁰⁵ in XA21, although further study is needed to demonstrate that the Thr⁸⁶⁷ of FLS2 is essential for FLS2 autophosphorylation. Thus, for the three plant RLKs studied to date, Thr⁷⁰⁵ and Thr⁸⁶⁷ have been demonstrated to be critical for the function of non-RD RLKs XA21 and FLS2, respectively, but not Thr⁸⁸⁰ for the RD RLK BRI1. Whether autophosphorylation of Thr⁷⁰⁵ equivalent sites is critical for other non-RD functions of RLKs remains to be further investigated.

Protein XA21K705, which begins at residue Thr⁷⁰⁵ and contains the whole kinase domain, loses XA21 autophosphorylation activity (Fig. 1). In contrast XA21K690, containing 15 more amino acids at the N terminus, maintains the autophosphorylation activity. These results suggest that the amino acids immediately flanking Thr⁷⁰⁵ are required for the residue to function. This is consistent with previous reports that the flanking sequence is required for kinase autophosphorylation (41, 42).

XA21 autophosphorylation is essential for its interaction with XB3, a positive regulator of XA21 function (27). The fact that XA21 autophosphorylation is also essential for XA21 to bind to negative regulators XB10, XB15, and XB24 suggests that autophosphorylation of XA21 plays an autoregulatory role in XA21 function. Similar examples have been reported. For example, phosphorylation of insulin receptor substrate 1 (IRS1) on serine residues has a dual role, either enhancing or terminating the effects of insulin. The activation of protein kinase B in response to insulin propagates insulin signaling and promotes the phosphorylation of IRS1 on a specific serine residue. Insulin also activates JNK (c-Jun NH₂-terminal kinase), ERK (extracellular signal-regulated kinase), protein kinase C, and mTOR

(mammalian target of rapamycin). These kinases phosphorylate IRS1 on other specific sites, yet, in contrast to protein kinase B, these proteins inhibit IRS1 function. Thus, a delicate balance exists between positive and negative IRS1 serine phosphorylation (43). Similarly, our results suggest that XA21 auto-phosphorylation may lead to differential binding of positive and negative regulators.

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