Biochemical Characterization of the Kinase Domain of the Rice Disease Resistance Receptor-like Kinase XA21*

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The rice disease resistance gene, Xa21, encodes a receptor kinase-like protein consisting of leucine-rich repeats in the putative extracellular domain and a serine/threonine kinase in the putative intracellular domain. The putative XA21 kinase domain was expressed as maltose-binding and glutathione S-transferase fusion proteins in Escherichia coli. The fusion proteins are capable of autophosphorylation. Phosphoamino acid analysis of the glutathione S-transferase fusion protein indicates that only serine and threonine residues are phosphorylated. The relative phosphorylation rate of the XA21 kinase against increasing enzyme concentrations follows a first-order rather than second-order kinetics, indicating an intramolecular phosphorylation mechanism. Moreover, the active XA21 kinase cannot phosphorylate a kinase-deficient mutant of XA21 kinase. The enzymatic activity of the XA21 kinase in a buffer containing Mn²⁺ is at least 15 times higher than that with Mg²⁺. The $K_m$ and $V_{max}$ of XA21 kinase for ATP are 0.3 $\mu$M and 8.4 nmol/min, respectively. Tryptic phosphopeptide mapping reveals that multiple sites on the XA21 kinase are phosphorylated. Finally, our data suggest that the region of XA21 kinase corresponding to the RD kinase activation domain is not phosphorylated, revealing a distinct mode of action compared with the tomato Pto serine/threonine kinase conferring disease resistance.

Plants are continually under attack by a variety of pathogens and have developed a wide array of defense mechanisms to protect themselves. Genetic analyses of plant-pathogen interactions have revealed that the resistance reactions, in many cases, are controlled by two dominant loci: an avirulence (avr) gene in the pathogen and a corresponding resistance (R) gene in the plants. Lack of either gene results in the development of disease symptoms. These genetic interactions are defined by the gene-for-gene theory (1).

A number of plant R genes, which confer gene-for-gene type resistance, have been cloned and characterized from diverse plants. The encoded proteins can be grouped into six classes based on structure: a serine/threonine kinase, proteins with a nucleotide-binding site and leucine-rich repeats (LRR),¹ presumed extracellular LRR-containing proteins with or without a transmembrane domain, a serine/threonine receptor-like kinase (RLK), and a protein without significant homology to known proteins (2–5).

Among all of the cloned resistance genes, only two encode protein kinases. The tomato Pto gene conferring resistance to Pseudomonas syringae pv. tomato containing the avr gene avrPto, encodes a serine/threonine kinase (Pto) that interacts with avrPto and several other proteins known as Pts (6–10). The Pto kinase can autophosphorylate eight serine and threonine residues and the autophosphorylation proceeds via an intramolecular mechanism (11, 12). The predicted amino acid sequence of Pto indicates that it belongs to a large subfamily of protein kinases known as RD kinases, which have an arginine immediately preceding the conserved catalytic aspartate (13). Consistent with other RD kinases whose activation requires phosphorylation of the activation domain, a region spanning the conserved sequences DFG and PE (13), Pto autophosphorylates its activation domain in vitro (12). Moreover, specific amino acid substitutions in this region result in constitutive induction of the hypersensitive response in the absence of avrPto (14).

The rice bacterial blight disease resistance gene, Xa21, encodes a RLK protein (15). The putative extracellular domain is composed of 23 LRRs, whereas the putative intracellular domain (XA21K) contains all the invariant amino acid residues characteristic of serine/threonine protein kinases. Based on the mode of action of animal receptor-tyrosine kinases (RTKs), we have hypothesized that the XA21 LRR domain acts as receptor for a plant or pathogen-produced ligand and that upon ligand binding, XA21K undergoes autophosphorylation by an intra- or intermolecular mechanism (16). In this report, we demonstrate that XA21K is an active serine/threonine kinase, that autophosphorylation of multiple residues occurs exclusively via an intramolecular mechanism, and that the XA21K region corresponding to the RD kinase activation domain is not phosphorylated.

**Experimental Procedures**

Construction of XA21K Expression Plasmids—XA21K (amino acids 677–1025) (15) was PCR-amplified with primer 1 (5'-GGATCCGGAC- AAGAGAATCTAAAAAAGGGAGGC-3') and primer 2 (5'-CAGAAGTCTGAT- CTGAAGTGTGGCA-3'), cloned, sequenced to confirm that no PCR error was introduced, and subcloned into the pGTP vector, which was

¹ The abbreviations used are: LRR, leucine-rich repeats; GST, glutathione S-transferase; MBP, maltose-binding protein; RLK, receptor-like kinase; RTK, receptor-tyrosine kinase; XA21K, XA21 kinase; PP1, protein phosphatase 1.

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modified from pGEX-2T (Amersham Biosciences) (17). The resulting plasmid was designated as pGST-XA21K and used for protein expression.

A single amino acid mutation in XA21K was introduced using the method of Dawid et al. Two primer pairs (primer 3, 5'-GGATCCGTCGACCCGGGACGACTACAAAAGGAGG-3') and primer 6 (5'-GGATCCGACCGCGGGAGCCGACTACAAAAGGAGG-3') were used to introduce mutations in the vector for mutant selection and also to substitute the lysine 736 (Lys-736) codon of XA21K. The lysine mutation creates a single substitution of glutamic acid for Lys-736. Candidates were confirmed by DNA sequencing and cloned into the pGTX vector. The resulting construct, designated pGST-XA21K-K736E, was used to express the kinase-deficient form of XA21K.

To express XA21K as a maltose-binding protein (MBP) fusion protein, plasmids pMBP-XA21K and pMBP-XA21K-K736E were created. Primer 5 (5'-GGATCCGTCGACCCGGGACGACTACAAAAGGAGG-3') and primer 6 (5'-GGATCCGACCGCGGGAGCCGACTACAAAAGGAGG-3') were used to amplify XA21K and its kinase-deficient mutant using pGST-XA21K and pGST-XA21K-K736E as templates. The PCR products were cloned into the pMAL-c2x expression vector (New England Biolabs, Beverly, MA) and confirmed by sequencing.

All other XA21K mutants used in this study were created according to the methods from Stratagene (La Jolla, CA). pGST-XA21K was used as a template for primer pairs 5' (5'-GGATCCGACCGCGGGAGCCGACTACAAAAGGAGG-3') and 6 (5'-GGATCCGACCGCGGGAGCCGACTACAAAAGGAGG-3'). 5'-AGATCTGGTTCCGCCG-3' and 5'-GATCTGGTTCCGCCG-3' were used to amplify XA21K and its kinase-deficient mutant using pGST-XA21K and pGST-XA21K-K736E as templates. The PCR products were cloned into the pMal-c2x expression vector (New England Biolabs, Beverly, MA) and confirmed by sequencing.

Expression and Purification of Fusion Proteins—pMBP-XA21K and pMBP-XA21K-K736E were transformed into Escherichia coli strain ER2566 (New England Biolabs). Bacterial cells were grown in 50 ml of LB supplemented with glucose (2 g/liter) and ampicillin (50 mg/ml) to an = 0.5–0.6. To induce expression of the fusion proteins, isopropyl-1-thio-D-galactopyranoside was added to a final concentration of 0.4 mM and incubated for 8 h at room temperature. Cells were harvested by centrifugation, resuspended into column buffer (20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA), supplemented with phenylmethylsulfonyl fluoride (2 mM) and dithiothreitol (1 mM), and lysed by sonication at 4°C. The lysate was centrifuged (14,000 g for 10 min) at 4°C. The supernatant was mixed with 60 μl of amylase resin (New England Biolabs) by incubation for 1 h at 4°C. After washing with column buffer extensively, the fusion protein was eluted with column buffer containing 3.6 mg/ml maltose.

A similar protocol was used to express and purify GST-XA21K and GST-XA21K-K736E except that a shorter time period (40 min) was used for induction at 4°C. The GST-XA21K recombinant fusion protein was oxidized in 50 mM HEPES (pH 7.4), 10 mM MgCl2, 1 mM dithiothreitol, and glutathione-agarose beads (Sigma) were used for isopropyl-1-thio-D-galactopyranoside induction and that the GST buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1 mM dithiothreitol) and glutathione-agarose beads (Sigma) were used for purification.

Auto phosphorylation and Dephosphorylation Assays—The resin-bound fusion proteins purified above were washed with kinase buffer (50 mM HEPES (pH 7.4), 10 mM MgCl2, 1 mM dithiothreitol). Auto phosphorylation experiments were carried out in a 50-μl reaction mixture containing 20 μl of resin-bound protein (5 μg) and 20 μCi of [γ-32P]ATP (600 Ci/mmole) (PerkinElmer Life Science, Boston, MA). The reaction was stopped after 30 min by adding 10 μl of Laemmli loading buffer (×4) and boiling for 5 min. The proteins were separated by SDS-PAGE (7.5 or 10%). After staining with Coomassie Brilliant Blue G-250, the gel was dried and exposed to x-ray film.

To dephosphorylate the phosphorylated fusion proteins, the [32P]-labeled XA21K proteins were washed with protein phosphatase 1 (PP1) buffer and incubated with PP1 (New England Biolabs) according to the manufacturer’s protocol. The resulting proteins were resolved by SDS-PAGE as described above.

For the dinitrophenyl-1-thio-D-galactopyranoside induction and that the GST buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1 mM dithiothreitol) and glutathione-agarose beads (Sigma) were used for purification.

Results

The dinitrophenyl-1-thio-D-galactopyranoside induction and that the GST buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1 mM dithiothreitol) and glutathione-agarose beads (Sigma) were used for purification.

Results

XA21K Is a Functional Kinase—To test the hypothesis that Xa21 encodes a protein kinase, we expressed and purified MBP-XA21K as a MBP recombinant fusion protein and assayed for auto phosphorylation. Fig. 1 shows that the fusion protein (MBP-XA21K) is capable of autophosphorylation. To confirm that the phosphorylated protein was MBP-XA21K rather than a contaminating bacterial protein, a mutated MBP-XA21K construct was generated using the site-directed mutagenesis approach. A single base substitution replaced Lys-736 with a glutamic acid residue. Because Lys-

FIG. 1. Autophosphorylation of MBP-XA21K and MBP-XA21K-K736E. Equal amounts of affinity-purified proteins were incubated with [γ-32P]ATP in the kinase buffer described under "Experimental Procedures" for 30 min at room temperature. Samples were then resolved by 10% SDS-PAGE gel. Both the Coomassie Blue-stained gel (left) and autoradiogram (right) of the same gel are shown.

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736 is essential for phosphotransfer and highly conserved in all protein kinases, the K736E mutation is expected to inactivate XA21K. Indeed, autophosphorylation analysis of the MBP-XA21K-K736E mutant revealed no autophosphorylation activity (Fig. 1). Similar results were observed when XA21K was expressed as GST fusion protein (data not shown). Because MBP and GST alone do not have any detectable kinase activity, these results indicate that Xa21 encodes an active kinase capable of autophosphorylation in vitro.

Characterization of XA21K—The kinase activity of XA21K requires the presence of divalent cations. A linear increase in the enzymatic activity of XA21K was observed in the range of 0.1–5 mM MnCl₂, followed by a plateau of the autophosphorylation when the concentration of MnCl₂ was further increased (Fig. 2A). Magnesium chloride maintains the linear increase of the autophosphorylation up to the highest concentration (20 mM) used in this assay (Fig. 2B). The kinase activity of XA21K was, however, at least 15 times higher in the presence of MnCl₂ than in MgCl₂ (Fig. 2C). No detectable kinase activity was observed when CaCl₂ was used as a source of divalent cation. Time course experiments with 10 mM MnCl₂ and MgCl₂ indicated that the autophosphorylation of XA21K reached a plateau after 30–40 min (Fig. 2D).

MBP-XA21K exhibits standard Michaelis-Menten kinetics with respect to ATP. The \( K_m \) and \( V_{max} \) values for ATP, determined by a double-reciprocal plot, are 0.3 \( \mu \)M and 8.4 nmol/mg/min, respectively (Fig. 2E).

**XA21K is Serine/Threonine-specific—**Based on the sequence of amino acids in kinase subdomains VI and VIII, Xa21 was presumed to encode a serine/threonine protein kinase (15). To confirm the serine/threonine specificity of XA21K, phosphoamino acid assays were performed. In these assays, serine and threonine residues were phosphorylated, whereas no detectable tyrosine residues were labeled (Fig. 3A). Moreover, the autophosphorylated XA21K can be dephosphorylated by the serine/threonine phosphatase PP1 (Fig. 3B). These results indicated that XA21K carries serine/threonine specificity.

**The XA21K Autophosphorylation Occurs through an Intramolecular Mechanism—**To test whether the autophosphorylation of XA21K proceeds via an intramolecular (first-order with respect to enzyme concentration) or intermolecular (second-order with respect to enzyme concentration) mechanism, the autophosphorylation reaction was carried out in the presence of increasing concentrations of XA21K. As shown in Fig. 4A, the relative phosphorylation rate increases linearly with the increasing enzyme concentration. This result indicated that autophosphorylation of MBP-XA21K follows first-order rather than second-order reaction kinetics. Moreover, the phosphate incorporation per molecule of MBP-XA21K was at the same level when the MBP-XA21K concentration in the reaction varied from 0.7 to 44.8 \( \mu \)M (64 times) (Fig. 4B). The van’t Hoff analysis of autophosphorylation (logarithm of phosphorylation rate versus logarithm of enzyme concentration) illustrated a slope of 1.03 ± 0.02 and a correlation coefficient of 0.997 for linear regression (Fig. 4C). Our data sug-

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**Fig. 2.** Enzymatic properties of XA21K. Autophosphorylation of MBP-XA21K was carried out for 30 min at room temperature in kinase buffer containing different concentrations of MnCl₂ (A), MgCl₂ (B), and 10 mM of distinct divalent cations (C). Time course experiments of MBP-XA21K were performed with 10 mM MnCl₂ and 10 mM MgCl₂ (D). Samples were then resolved by 10% SDS-PAGE. Both the Coomassie Blue-stained gel (upper) and autoradiogram (lower) of the same gel are shown. E, the kinetics of XA21K autophosphorylation with respect to ATP. The double-reciprocal plot showed that the \( K_m \) and \( V_{max} \) are 0.3 \( \mu \)M and 8.4 nmol/mg/min, respectively.

**Fig. 3.** XA21K is a serine/threonine kinase. A, phosphoamino acid assays of the autophosphorylated GST-XA21K. The \( ^{32} \text{P} \)-labeled GST-XA21K was hydrolyzed with HCl and subjected to two-dimensional analysis as described under “Experimental Procedures.” The autoradiogram spots indicate the positions of standard phosphorylated serine and threonine residues. Dashed circles indicate the positions of standard phosphorylated serine, threonine, and tyrosine. B, dephosphorylation of MBP-XA21K by the serine/threonine phosphatase PP1. The \( ^{32} \text{P} \)-labeled MBP-XA21K was incubated with and without PP1 phosphatase for 30 min at room temperature. The samples were then resolved by 10% SDS-PAGE. Both the Coomassie Blue-stained gel (left) and autoradiogram (right) of the same gel are shown.
suggested that the MBP-XA21K autophosphorylation occurs via an intramolecular mechanism.

To confirm that intermolecular autophosphorylation does not occur, a kinase-deficient form of XA21K (MBP-XA21K-K736E) was used as a potential substrate for phosphorylation by GST-XA21K. Free MBP-XA21K-K736E was incubated with free GST-XA21K. Consistent with the results described above, GST-XA21K was unable to transphosphorylate MBP-XA21K-K736E (Fig. 4D). Similar results were obtained by using GST-XA21K-K736E as a potential substrate for phosphorylation by MBP-XA21K (data not shown). These results, together with the observations from the intramolecular phosphorylation assays described above, support the notion that autophosphorylation of XA21 exclusively occurs through an intramolecular mechanism in vitro.

Multiple Serine/Threonine Residues on XA21K Are Autophosphorylated in Vitro—To investigate the number of autophosphorylated serine and threonine residues, phosphopeptide mapping was carried out using both GST-XA21K and the XA21K protein without GST. XA21K was released from GST-XA21K by digestion with thrombin (Fig. 5). Because the thrombin-released XA21K contains a 22-amino acid peptide derived from the GTK vector, we designated the released XA21K as XA21Kt. The 32P-labeled GST-XA21K and its mutants were digested with trypsin and subjected to two-dimensional electrophoresis and chromatography analysis as described under “Experimental Procedures.” The autoradiogram spots are the phosphorylated peptides generated from trypsin digestion. The origin is indicated by "\( S\)". A, GST-XA21K; B, XA21Kt; C, GST-XA21K-S(21)A; D, GST-XA21K-S(8)A.

FIG. 4. Intramolecular phosphorylation assays of the XA21K. Increased concentrations of MBP-XA21K (from 0.7 to 44.8 \( \mu M \)) were used to perform autophosphorylation as described in the legend to Fig. 1. A, plot of phosphate incorporation rate versus MBP-XA21K concentration. B, specific activity of MBP-XA21K expressed as phosphate incorporation rate per pmol of MBP-XA21K. C, van’t Hoff plot of log velocity versus the log of MBP-XA21K concentration has a slope of 1.03 ± 0.02 and a correlation coefficient of 0.997. Data in A–C are the mean ± S.E. (n = 3). D, GST-XA21K cannot transphosphorylate the kinase-deficient mutant MBP-XA21K-K736E. Free GST-XA21K was incubated with free MBP-XA21K-K736E and \( {\gamma}^{32P}\)ATP in kinase buffer for 30 min at room temperature. Autophosphorylations of GST-XA21K and MBP-XA21K-K736E were performed as controls. Samples were resolved by 7.5% SDS-PAGE. Coomassie Blue staining (left) and autoradiogram (right) of the same gel are shown.
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**Fig. 7. Sequence comparison of the activation domain of XA21K and Pto.** Identified phosphorylation sites on Pto (12) are in bold. Mutated residues in the XA21K mutants (R865H and H903R) are underlined. Arginine and lysine recognized by trypsin on XA21K and the mutants are indicated in italic. Tryptic peptides are boxed. X represents the amino acids that are not shown. — denotes gaps for maximal alignment.

DISCUSSION

It has long been hypothesized that protein phosphorylation plays a key role in R gene-mediated disease resistance (20). In this paper, we demonstrate that the presumed intracellular domain encoded by the rice disease resistance gene Xa21 is an active serine/threonine kinase capable of autophosphorylation. Like other protein kinases, XA21K activity can be abolished by a single substitution of the invariant lysine residue that is responsible for phosphotransfer. The enzymatic properties of XA21K are also similar to those of other characterized kinases. For instance, the MBP-XA21K $K_m$ for ATP (0.3 μM) is comparable with the values obtained for the epidermal growth factor receptor (0.2–3 μM), the Catharanthus roseus CrRLK1 (2–2.5 μM), and the Arabidopsis RLK5 kinases (15.2–17.8 μM) (19, 21). Similar to CrRLK1 and the Arabidopsis BRI1 kinases, the activity of XA21K requires Mn$^{2+}$ and Mg$^{2+}$ but not Ca$^{2+}$ (21, 22). These results suggest that autophosphorylation of XA21K may be an important step in the XA21-mediated signaling.

Intermolecular autophosphorylation is particularly important for activation of some RTKs that recognize growth factors in animal systems. Upon ligand binding, these RTKs form homodimers triggering intermolecular phosphorylation of regulatory residues that are essential for activation of the kinases (23). For example, the receptors for platelet-derived growth factor carrying intracellular tyrosine kinase domains are activated by dimerization of two receptors and subsequent intermolecular phosphorylation of the Tyr$^{567}$ residue of the kinases. Here we report that autophosphorylation of XA21K occurs through an intramolecular mechanism. Furthermore, we were unable to detect in vitro intermolecular phosphorylation of the XA21K-K736E mutant protein by XA21K using either resin-bound or free fusion proteins. Similar systems were successfully used to demonstrate that XA21K can transphosphorylate one of the XA21-binding proteins. Thus, our data suggest that intermolecular autophosphorylation is not the mechanism by which XA21K is activated. A possible mechanism for activation of the XA21K is that a second receptor kinase forms a heterodimer with and transphosphorylates XA21K following the infections of Xanthomonas oryzae pv. oryzae, which in turn activates the intramolecular autophosphorylation of XA21K.

Over the last decade, more than 20 plant disease-resistance genes have been cloned from a variety of plant species. Only the rice Xa21 and tomato Pto resistance genes encode protein kinases. Although XA21K shares some common enzymatic properties with Pto, our studies also reveal that these two kinases likely employ distinct mechanisms for signaling. The phospho-
rylation status in the activation domain of XA21K differs from that of the Pto kinase. Four of eight identified phosphorylation sites on Pto are located on the activation domain (12) (Fig. 7). One of them, Ser-198, is critical for avrPto-Pto-mediated elicitation of hypersensitive response. Like the Pto kinase, there are seven serine and threonine residues in the activation domain of XA21K (Fig. 7). Three of them are located in the corresponding positions (including Ser-198) of the Pto phosphorylation sites. However, our data suggest that none of these residues are autophosphorylated, indicating that phosphorylation of the activation domain may not be required for the XA21-mediated signaling. This is consistent with the observations from many other non-RD kinases that lack the conserved arginine preceding the conserved aspartate.

The RLK structure of XA21 suggests that the mode of action of this protein may be more similar to that of RTKs rather than Pto, a receptor-like cytoplasmic kinase (24). For instance, the extracellular domain of RTKs is required for ligand binding. Similarly, the presumed extracellular domain XA21 consists of LRRs that determines race-specific recognition of the pathogen (4). Furthermore, autophosphorylation of multiple tyrosine residues on the kinase domain of RTKs leads to initiation of multiple responses. A classic example is the platelet-derived growth factor receptor-mediated responses (23). Upon platelet-derived growth factor binding to its receptor, nine different tyrosine residues on the receptor are autophosphorylated, resulting in the recruitment of eight distinct intracellular signaling molecules. Because we have shown that XA21K can autophosphorylate multiple serine and threonine residues, it is possible that XA21K can initiate multiple defense responses by the binding of distinct signaling proteins with specific phosphorylated residues on XA21K. In support of this hypothesis, we have found that the active XA21K is capable of binding to at least seven rice proteins in the yeast two-hybrid system; however, these binding proteins showed either no or impaired interactions with the kinase-deficient mutant XA21K-K736E. Thus, we propose that one function of the XA21K autophosphorylation is to create binding sites for recruiting downstream signaling proteins.

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