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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/mg/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), 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 Ptis (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-AAGAGAATCTAAAAAGGGAGGC-3') and primer 2 (5'-CAGAAGTCGAT-CTGAAGTGTTGGCA-3'), cloned, sequenced to confirm that no PCR error was introduced, and subcloned into the pGTR vector, which was used for expression in Escherichia coli (17).

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 DeMartino and coworkers (primer 3, 5′-AGTCTGTTGGTAATCTCCACAG-3′; primer 4, 5′-AAGCTTTGATACCCGATTCGATCTGAAAGTGGCA-3′) were designed 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 pGEX 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 S(5′-GATCCGCGACACAAAGAAGACGACGTTGGAATTCCCG-3′) and primer 6 (5′-GGATCCGTCGACCCCGGGCAGAAGTCGATCTGGTTGAATACTCACCAG-3′) were designed to introduce mutations in the vector for mutant 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′-GGATCCGCGACACAAAGAAGACGACGTTGGAATTCCCG-3′) and primer 6 (5′-GGATCCGTCGACCCCGGGCAGAAGTCGATCTGGTTGAATACTCACCAG-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 ER2556 (New England Biolabs). Bacterial cells were grown in 50 ml of LB supplemented with glucose (2 g/liter) and ampicillin (50 μg/ml) to A600 of 0.2–0.6. To induce expression of the fusion proteins, isopropyl-thiogalactoside (1 mM) was added to each 50 ml culture. The cultures were incubated at 37 °C for 12 h in phosphopeptide buffer (75:50:15:60, pH 1.9, 1.5 kV, 40 min) followed by ascending chromatography about 12 h in phosphopeptide buffer (75:50:15:60, n-butanol:pyridine:acetic acid:water). The plate was subjected to autoradiography to visualize the phosphoproteidcs.

**RESULTS**

**XA21K Is a Functional Kinase**—To test the hypothesis that Xa21 encodes a protein kinase, we expressed and purified MBP-XA21K and MBP-za21K-K736E and used the purified proteins for autophosphorylation and phosphopeptide mapping. We found that MBP-XA21K and MBP-za21K-K736E are both 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-
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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 μM and 8.4 nmol/mg/min, respectively (Fig. 2E).

The $K_m$ and $V_{max}$ values for ATP illustrated a slope of 1.03 (logarithm of 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 μM (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 μM 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 32P-labeled GST-XA21K was hydrolyzed with HCl and subjected to two-dimensional phosphoamino acid assays. Dashed circles indicate the positions of standard phosphorylated serine, threonine, and tyrosine. B, dephosphorylation of MBP-XA21K by the serine/threonine phosphatase PP1. The 32P-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.

**Fig. 5.** XA21Kt cannot phosphorylate the GST protein. **A**, schematic diagram showing structural domains of GST-XA21K. The 22 amino acids on XA21Kt are underlined. S (8) and S (21) are shown in **B**, XA21Kt released by thrombin digestion. 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 11001/8. A, GST-XA21K; B, XA21Kt; C, GST-XA21K-S (21)A; D, GST-XA21K-S (8)A.

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.
XA21Kt. Bands of predicted sizes of XA21Kt and GST were observed after thrombin digestion (Fig. 5B). Autoradiography analysis showed that a protein with the predicted size of XA21Kt was strongly phosphorylated, indicating that this protein is XA21Kt (Fig. 5B). No radiolabeled products at the position corresponding to GST were found. The weak bands observed on both the Coomassie Blue-stained gel and the autoradiogram in the GST-XA21Kt lanes may be the results of partial degradation of XA21Kt.

Both GST-XA21K and XA21Kt were subjected to phosphopeptide mapping assays to determine the phosphopeptide pattern. The 32P-labeled proteins were digested with trypsin, and the resulting peptides were loaded onto TLC plates and resolved horizontally by electrophoresis and vertically by ascending chromatography. Multiple labeled spots were observed on the autoradiograms generated from both GST-XA21K and XA21Kt (Fig. 6, A and B). Interestingly, three spots were absent in the tryptic pattern of XA21Kt when compared with that of GST-XA21K. Because the thrombin-released GST is not labeled as shown in Fig. 5B, we hypothesized that the absence of three GST-XA21K-specific spots was because of the cleavage of a phosphorylated residue by thrombin. Indeed, the S(−21) residue on the 22-amino acid peptide of XA21Kt is part of the thrombin recognition site (Fig. 5A). To test this hypothesis, we mutated S(−21) to alanine on GST-XA21K, and the mutant was subjected to phosphopeptide mapping. As shown in Fig. 6C, the phosphopeptide pattern of GST-XA21K-S(−21)A is identical to that of XA21Kt. This result indicates that S(−21) is phosphorylated by the XA21 kinase and also confirms that the GST portion does not contain any phosphorylation sites.

In addition to S(−21), there is another serine residue (S(−8)) located on the 22-amino acid peptide between GST and the XA21 kinase domain (Fig. 5A). We mutated S(−8) to alanine to test whether this residue is also phosphorylated. Phosphopeptide mapping of the GST-XA21K-S(−8)A showed that S(−8) accounts for four phosphospots as shown in Fig. 6D. Interestingly, S(−8) is located at the protein kinase A recognition sequence. Taken together, these results indicate that at least 20 of 27 phosphospots on the GST-XA21K peptide map are because of autophosphorylation of XA21K, thereby strongly suggesting that multiple residues on XA21K are autophosphorylated.

The Activation Domain of XA21K Does Not Carry Any Phosphorylation Sites—There are seven serine and threonine residues located in the presumed activation domain of XA21K, and all of them are included in a single tryptic peptide (Fig. 7). To determine whether any of the residues can be autophosphorylated by XA21K, we mutated arginine 865 (Arg-865) to histidine, which abolishes a trypsin recognition site upstream of the seven serine and threonine residues. Because this mutation will significantly increase the size of the tryptic peptide carrying the seven serine and threonine residues, a shift of a labeled spot(s) on the two-dimensional autoradiogram should be observed if the peptide contained a phosphorylated residue(s). However, tryptic mapping of the GST-XA21K-R865H mutant showed no detectable changes on the autoradiogram when compared with that of GST-XA21K (data not shown). To confirm this result, we further mutated histidine 903 (His-903), downstream of the activation domain, to arginine to create a new trypsin recognition site. Consistent with the result from the GST-XA21K-R865H mutant, the GST-XA21K-H903R mutant showed an identical tryptic pattern to that of the wild type GST-XA21K (data not shown). These results suggest that the activation domain of XA21K is not autophosphorylated in vitro.

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 Kcat 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 Mn2+ and Mg2+ but not Ca2+ (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 intramolecular phosphorylation of the Tyr667 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.2 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-

2 L.-Y. Pi and W.-Y. Song, unpublished data.
rlation 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 XA21 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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