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A catalytically impaired mutant of the rice Xa21 receptor kinase confers partial resistance to *Xanthomonas oryzae* pv *oryzae*

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Abstract

The rice *Xa21* gene that confers resistance against *Xanthomonas oryzae* pv *oryzae* encodes a receptor-like kinase protein consisting of a presumed extracellular domain, a transmembrane domain and a cytoplasmic protein kinase. To determine if the XA21 kinase domain is essential for transducing the defense response, we mutated the Lysine 736 of *Xa21* to Glutamic acid (K736E) to construct XA21K736E. Transgenic lines expressing XA21K736E display partial resistance to *Xoo* PR6. Bacterial growth in the XA21K736E mutants is intermediate to that of transgenic lines expressing the wild type *Xa21* gene and the susceptible control, Taipei 309. The partial resistance phenotype was transmitted to the progeny of two independent lines. Thus, although catalytic activity of XA21 is essential for wild type resistance levels, the catalytically impaired mutant maintains partial resistance activity.

Keywords: Heterodimerization; Leucine-rich repeat domains; Non-RD kinases; Phosphorylation; RD kinases

1. Introduction

Plants are equipped with innate immune defense mechanisms as protection against pathogen attack. In gene-for-gene mediated resistance, both the dominant resistance (R) gene from the host and a complementary avirulence (Avr) gene from the pathogen must be present for disease resistance to occur. Absence of either partner leads to disease [4].

To date, about 30 resistance genes have been cloned from both monocotyledonous and dicotyledonous plants [13,24]. The rice gene *Xa21* belongs to a unique class of disease resistance genes. It encodes a receptor-like kinase (RLK) with leucine-rich repeats (LRR) in the presumed extracellular domain, a transmembrane domain and a serine–threonine protein kinase domain [10,23]. Xa21 confers resistance to multiple races of *Xanthomonas oryzae* pv *oryzae* (*Xoo*) [23,27]. Aside from Xa21, only one other resistance gene, Pto, carries a protein kinase domain [13,24]. Pto confers resistance to *Pseudomonas syringae* pv *tomato* (*Pst*) [12].

A lysine residue is conserved in all protein kinases and is critical for phospho-transfer. The importance of this lysine for kinase function has been demonstrated in vitro for both Pto and Xa21. Substitution of Lysine 736 of Xa21 with glutamic acid (K736E) or substitution of Lysine 69 of Pto with asparagine (K69N), abolished autophosphorylation of the respective kinase domains in vitro [10,17]. The Pto K69N mutation also eliminated the ability of Pto to bind the corresponding Avr protein, AvrPto, in the yeast two-hybrid system [5,15,17,25]. Furthermore, substitution of Lysine 69 with glutamine (K69E) impairs interaction with Pti1, a serine threonine kinase that physically interacts with Pto in the yeast two-hybrid system [30].

Several studies suggest that Pto catalytic activity is required for *Pst* resistance. Tomato and tobacco plants carrying the PtoK69N mutation failed to display a hypersensitive response (HR) and disease resistance in transient assays [15]. Pto phosphorylation is required for its interaction with the transcription factors Pti4/5/6, which are thought to drive expression of pathogenesis related-genes [21]. Phosphorylation of Pti4 by Pto in vitro enhanced the ability of this transcription factor to bind a GCC box element, present in the promoter regions of many pathogenesis related (PR) genes [6].

Pto belongs to a class of RLKs called RD kinases where an arginine (R) immediately precedes the conserved catalytic aspartate (D). Activation of RD kinases requires

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phosphorylation of the activation domain, a region spanning the conserved sequences DFG and PE. In vitro evidence shows that Pto autophosphorylates on serine and threonine residues located in the Pto activation domain and that these phosphorylation sites are important for *Pst* resistance [5,19,20]. Rathjen and associates [15] observed that specific mutations in the Pto activation domain lead to constitutive induction of HR in the absence of AvrPto. In contrast to Pto, Xa21 belongs to a small subclass of non-RD kinases where the arginine is replaced by a cysteine or glutamic acid. In Xa21, the region corresponding to the activation domain is not autophosphorylated in vitro suggesting a distinct mode of action for the XA21 kinase [10].

We have hypothesized that extracellular ligand recognition activates the Xa21 signaling pathway [26]. XA21 may function as part of a heterodimeric complex that transduces the resistance response upon recognition of an *Xoo*-produced molecule. Activation of the XA21 kinase may create phosphorylated amino acids that serve as binding sites for interacting proteins. These proteins would then directly or indirectly transduce various defense responses such as transcription of defense related genes. In support of this hypothesis, XA21 can autophosphorylate itself and can transphosphorylate an Xa21 binding protein; however, XA21 cannot transphosphorylate XA21K736E [10] (Pi et al., submitted).

Xa21D, an *Xa21* gene family member, encodes a predicted protein consisting of a signal peptide and a LRR domain, which is highly similar to the LRR domain of XA21 [22]. Although XA21D lacks a kinase domain, it confers an identical resistance spectrum as *Xa21* against various strains of *Xoo* [26]. Interestingly Xa21D confers partial race specific resistance suggesting that the XA21 catalytic domain is needed for full resistance. These data also suggest the presence of another molecule that can partially complement for the lack of the XA21D kinase domain.

Here we report that transgenic lines expressing the XA21K736E display partial resistance to *Xoo* Philippine race 6 (PR6). The resistance level of the catalytically impaired mutant ranges between the transgenic lines containing the wild type *Xa21* gene and the susceptible control, Taipei 309. The partial resistance is comparable to that of transgenic lines expressing Xa21D, indicating that kinase activity of XA21 is essential for full resistance.

2. Materials and methods

2.1. Generation of catalytically impaired Xa21 mutant transgenic line

A mutation in the invariant lysine (K736E) of *Xa21* was created by site-directed mutagenesis (data not shown). The Kpn1 fragment of *Xa21* genomic clone was subcloned into pCAMBIA 1300 binary vector (courtesy of Dr Richard Jefferson) and transformed into *Agrobacterium* EHA 105 (data not shown).

Agrobacterium transformation was carried out using the protocol of Hiei et al. [7] with the following modifications. One month-old seed calli of Taipei 309 were infected by *Agrobacterium* and co-cultivated for 3 days in MS medium with 2 mg l^{-1} 2,4-D and 19·62 mg l^{-1} acetosyringone. Calli were washed in water and then plated on MS medium with 2,4-D, carbenicillin (250 mg l^{-1}) and hygromycin (50 mg l^{-1}) for selection for at least one month. Resistant calli were transferred to MS medium + 3 mg l^{-1} BAP + 0·5 mg l^{-1} NAA. Plantlets were then transferred to MS medium without hormones to induce root formation.

2.2. Plant maintenance

The plantlets (T_0 plants) were planted in the greenhouse after 2 days of acclimatization. The plants were grown using the standard rice growing protocol in our lab (Ronald, unpublished). Dry seeds from selected T_0 plants were germinated on filter paper lined-petri plates at 30°C. Germinated seeds (now referred to as T_1 plants) were transferred to the soil. Three plants were planted per pot. The plants were maintained as described above until ready for inoculation.

2.3. Pathogen infection

Plants were transferred to the UCD Controlled Environment Facility (CEF) after 4–5 weeks in the greenhouse. The chamber settings were as follows: 28°C and 85% RH for 10 h with light [metal halide (MH), high pressure sodium (HPS) and incandescent light bulbs (OSRAM Sylvania Inc., Manchester, NH)] and 24°C and 90% RH for 14 h without light.

The plants were inoculated with *Xoo* PR6 using the leaf clip method [8]. Sterile scissors were dipped in bacterial solution (OD_{600} of 0·5) and leaves were cut 1–2 cm from the tip of the leaf blade. After 14 days, lesion lengths were measured. Lesions were measured from the cut surface at the tip to the distal-most position exhibiting gray, chlorotic or water soaked lesion. Lesion lengths in Table 1 represent the mean and standard deviation of at least three leaves from each of three plants. Analysis of variance and *t*-test were performed.

2.4. Nucleic acid manipulation

Standard molecular biology techniques were used [1,16]. For DNA preparation, rice DNA miniprep protocol developed by Chen and Ronald [3] was used. For RNA preparation, RNA was isolated from young leaves of selected T_0 plants using the Gibco Trizol Reagent Protocol (Life Technologies Inc., Rockville, MD).

2.5. PCR analysis

PCR analysis was performed using *Xa21* specific primers, U_1 and I_1 as previously described by Wang et al. [27]. Primer pairs amplified a 1·4-kb band of Xa21 that was polymorphic to fragments amplified from other family members [27]. For Xa21D plants, primer pairs 822-3 and DR-1 were used for PCR [26]. Plants that contain the Xa21D gene amplified 1·9-kb fragment.

PCR was carried out using the following conditions: 1 unit Taq polymerase, 10 mM Tris–HCl, 50 mM KCl, 1·5 mM $MgCl_2$, 10 μM dNTPs, 1 μM forward primer, 1 μM reverse primer. The PCR program consisted of initial denaturation of 2 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 54°C, 2 min and 30 s at 72°C; and a final extension of 10 min. An equal volume of each PCR sample was fractionated on 1·5% agarose gels.

2.6. Reverse transcriptase-PCR

Reverse transcription was done following the protocol provided in the ProSTAR First-Strand RT-PCR kit (Stratagene, La Jolla, CA) using 10 μg of total RNA. PCR was carried out using the same conditions as above. Five microliters (10%) of each reverse transcription reaction were used per PCR sample. *Xa21*-specific primers used were described by Century et al. [2]. The PCR program consisted of initial denaturation of 2 min at 94°C, 40 cycles of 30 s at 94°C, 30 s at 54°C, 2 min at 72°C, then a final extension step of 10 min. An equal volume of each PCR sample was fractionated on 1·5% agarose gels.

PCR products were sequenced by dideoxy chain termination method (ABI Prism Dye Primer Cycle Sequencing Kit and ABI prism Model 1300 Capillary Sequencer; Perkin Elmer, Norwalk, CT, USA). Primers used for sequencing were cyn15 (5′-CCGGGATCTCAATC-CAAGGCAATGCC-3′) and cyn19R (5′-GCTCGAG-CAAATTGTAACTATCTTG-3′), respectively. PCR products were fractionated in 1% agarose gel and 1·3-kb expected band were cut and purified using the Geneclean Spin Kit (BIO 101 Inc., Carlsbad, CA). DNA sequences were analysed by the Sequencer (Gene Codes) software.

2.7. Growth curve analysis

Growth of *Xoo* PR6 on T_1 progeny of transgenic lines Xa21K736E transgenic lines (lines 71 and 79), and control lines (Taipei 309, Xa21 and Xa21D plants) was determined at different time points post-inoculation. For each time point, bacterial populations were determined by grinding three leaves per plant separately, plating on potato sucrose agar (PSA) medium containing azacytidine (200 μM) and cycloheximide (10 mg ml^{-1}). The number of colonies was determined and the colony-forming units per leaf were calculated after 3 days at 30°C. The standard error of the mean is indicated by error bars.

3. Results

We generated transgenic plants expressing XA21K736E, containing a mutation in the invariant Lysine required for phospho-transfer. PCR analysis was carried out on 113 XA21K736E transgenic plants from 34 independent transformed lines (ITLs). Twenty out of 34 lines were confirmed to contain the XA21K736E transgene (data not shown). We selected five ITLs for in-depth analysis (Table 1 and Fig. 1).

3.1. Transgenic plants express the Xa21K736E gene

An RT-PCR strategy was used to detect the expression of XA21 in the Xa21 transgenic control (T106-17-3-37-1) line and XA21K736E in the five ITLs [2]. The Xa21 transgenic control and three lines (71, 72, and 79) carried the expected 1·3-kb band of the Xa21 and XA21K736E transcripts, whereas Taipei 309 and lines 67 and 73 did not (Table 1). No differences were observed in the quantitative expression of XA21K736E transgenic lines compared to the wild-type Xa21 line (data not shown). PCR amplification of the same RT-PCR products using actin primers was performed as an additional control for transcript quality and abundance [29]. Our results showed that all the lines expressed the 143-bp actin RT-PCR product.

We sequenced the RT-PCR products from T_0 lines 71 and 79, and the Xa21 transgenic control. The Xa21 transgenic control and the two T_0 plants (71, 79) were identical in sequence except for the one base pair mutation we introduced (data not shown). Thus, sequence analysis verified that the Xa21K736E plants did indeed carry the K736E mutation.

3.2. Xa21K736E plants display partial resistance to Xoo

The transgenic lines were inoculated with *Xoo* PR6 and assayed for resistance (Table 1). T1 progeny from two lines, 71 and 79, had shorter lesions than the control

Table 1
Lesion length, PCR and RT-PCR results of T_1 plants

Plant	Average lesion length (cm)	SD (cm)	PCR	XA21K736E expressed	Actin expressed
67	21·26	1·92	+	−	+
71	14·69	1·43	+	+	+
72	16·03	0·99	+	+	+
73	21·30	2·03	+	−	+
79	13·76	1·99	+	+	+
Taipei 309	22·98	2·72	−	NT	+
Xa21	4·89	1·86	+	NT	+

Xa21 is the transgenic line T106-17-3-37-1 carrying the Xa21 wild-type gene. Lesion lengths are the mean of at least three leaves from each of three plants. SD = standard deviation. NT = not tested.

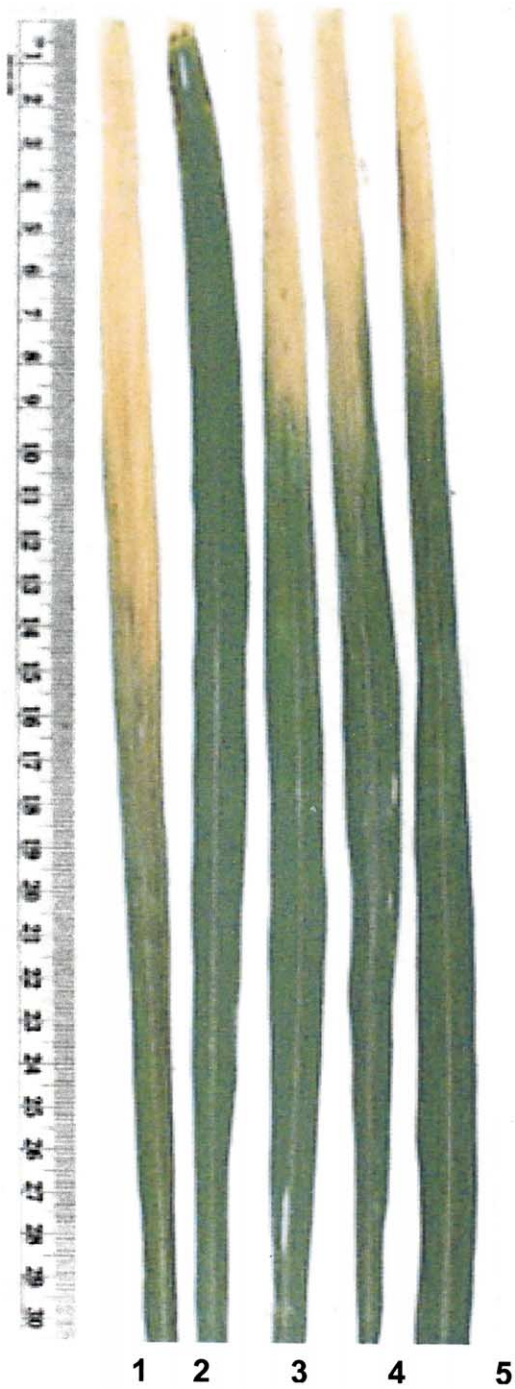

Fig. 1. Inoculation with *Xoo* PR6 of Taipei 309 (1) Xa21 Transgenic line (2), Xa21D line (3), Xa21K376E line 71 (4) and Xa21K736E line 79 (5). Photograph taken 14 days after inoculation.

line Taipei 309. These lines express the Xa21K736E gene as verified from the RT-PCR results. The other two lines (67 and 73) displayed lesion lengths similar to Taipei 309, and did not express Xa21K736E. Thus the presence of the XA21K736E mutation correlates with partial resistance to *Xoo*.

3.3. Partial resistance conferred by Xa21K736E is stably inherited in progenies of two independently transformed lines

To determine if the partial resistance phenotype was transmitted to the progeny, two lines (71 and 79) were self-pollinated to produce T_1 progeny. Progenies of these lines were analysed for resistance to *Xoo* PR6 (Fig. 1). We included XA21D plants as a partial resistance control [26].

PCR was carried out on the DNA of all the progeny to determine the presence of the XA21K736E gene using Xa21-specific primers [27]. The XA21K736E transgene was detected in all progeny indicating multiple insertions sites in the T_0 parents. The partial resistance phenotype was observed in all progeny of lines 71 and 79 (Fig. 1 and data not shown).

3.4. Bacterial growth is reduced in K736E plants

To more accurately quantitate the partial resistance of XA21K736E plants, *in vivo* bacterial growth of *Xoo* PR6 in the XA21K736E T_1 lines and controls was measured over a ten-day period (Fig. 2). Bacterial growth in Taipei 309 leaves was 10 to 30-fold higher than in the Xa21 transgenic line consistent with earlier studies (Fig. 2). In contrast, bacterial growth in the XA21K736E T_1 lines 71, 79 and Xa21 D plants showed only a two to ten-fold increase in growth as compared to the Xa21 transgenic line. These results indicate that the growth of *Xoo* PR6 in the XA21K736E lines 71 and 79 is comparable to that of Xa21D plants and is intermediate to that of the resistant Xa21 transgenic line and susceptible Taipei 309.

4. Discussion

Protein phosphorylation regulates important physiological processes such as fertilization, cell proliferation, cell migration, cell differentiation and apoptosis [14]. The cloning of two plant resistance genes encoding serine threonine kinases support a central role for protein phosphorylation in gene-for-gene mediated disease resistance [12,18,23] (Pi et al., submitted).

The rice receptor kinase (RK), XA21, is the only known member of a unique class of plant resistance (R) proteins carrying LRRs in the presumed extracellular domain and a serine threonine kinase in the intracellular domain. XA21 displays several properties that differ from PTO. In contrast to the tomato resistance protein PTO and most protein kinases that are regulated by phosphorylation of the activation segment, a centrally located loop that sits close to the catalytic center, Xa21 is not autophosphorylated in this region [10]. Furthermore, unlike PTO, which specifically recognizes and binds the avr products AvrPTO and avrPTO2, the Xa21 kinase domain is not the recognition determinant as demonstrated by previous studies indicating

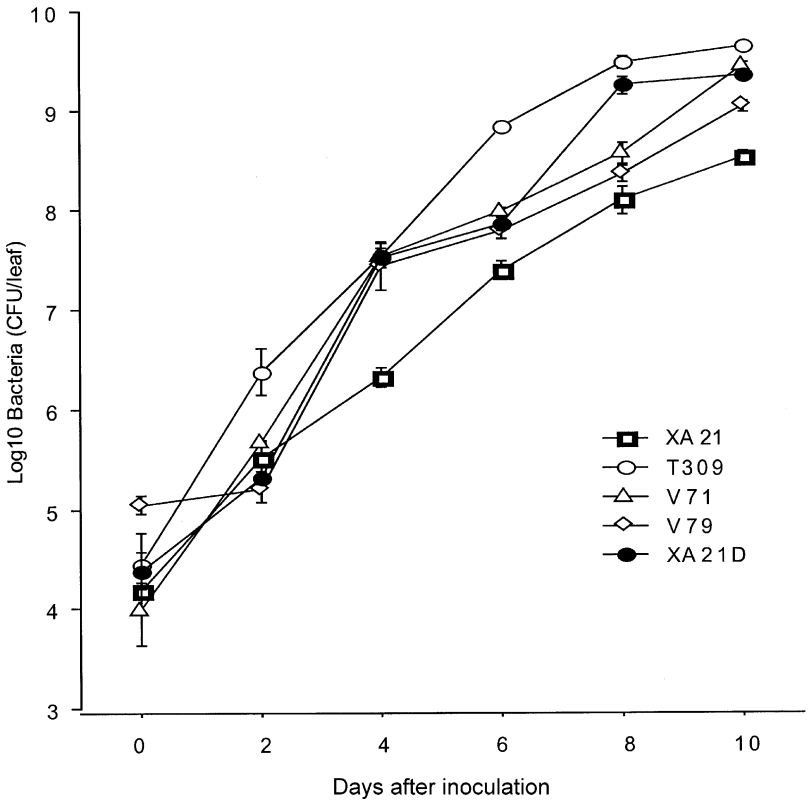

Fig. 2. Growth of *Xoo* PR6 in the XA21 transgenic plants (XA21), Taipei 309 (T309), XA21K736E transgenic line 71 (V71), XA21K736E transgenic line 79 (V79) and the Xa21D transgenic line (XA21D).

that only the LRR domain is required for race specific recognition. The importance of the conserved lysine residue for catalytic activity of Xa21 and PTO has been shown in vitro as lysine mutants cannot autophosphorylate [10]. However, whereas disruption of Pto catalytic activity completely abolished PTO mediated resistance [15], transgenic plants carrying the K736E Xa21 mutation display partial resistance to *Xoo* PR6, indicating that this mutation did not cause a complete loss of function.

Our observation of partial resistance in a catalytically impaired mutant supports earlier data that the LRR domain and not the kinase plays a key role in *Xoo* recognition. In these studies, XA21D, a member of the *Xa21* gene family that encodes an LRR but lacks the transmembrane and kinase domains, was able to confer partial resistance.

Although a functional XA21 kinase domain is not essential for recognition, it is evident that the XA21 catalytic domain plays a critical role in the downstream defense response. Xa21 phosphorylation may be critical for activation of interacting signaling proteins or for creation of phosphorylated residues that can serve as binding sites for docking of downstream binding proteins. In support of this hypothesis, Liu et al. [10] demonstrated that multiple serine and threonine residues of XA21 are autophosphorylated in vitro. Furthermore a Xa21-catalytically impaired mutant is incapable of binding to rice proteins that normally bind to WT XA21 kinase in the yeast two-hybrid system (Pi and Song unpublished; Dardick and Ronald, unpublished). Thus, the lack of full resistance in Xa21K736E plants may be due to the loss of Xa21-specific binding sites for downstream signaling proteins.

How does Xa21K736E transduce a partial resistance response in the absence of catalytic activity? The simplest answer is that another molecule functions with XA21 to transduce the response and that this molecule can partially complement for the loss of Xa21 catalytic activity in the mutant lines. Studies on the platelet-derived growth factor (PDGFR) and transforming growth factor β (TGF-β) receptors signaling support this hypothesis [9]. TGF-β signals through two receptor serine/threonine kinases, TβR-1 and TβR-II. TGF-β binds to TβR-II, allowing this receptor to associate with and phosphorylate TβR-I which then propagates the signal. A catalytically impaired mutant of TβR-I can partially complement the activity of an activation-defective TβR-I mutant that no longer can be phosphorylated by TβR-II. This result suggests that the ligand-induced receptor complex contains two or more TβR-I molecules and that the kinase of one TβR-1 molecule interacts with another enabling its phosphorylation and activation by TβR-II restoring activity. This cooperative interaction is essential for TGF-β signaling [28]. In the case of PDGFR, reciprocal chimeras consisting of the extracellular domain of one isoform of PDGFR (PDGFRα) and the intracellular domain of the other isoform (PDGFRβ)

demonstrated that each isoform activated distinct effectors [11]. Thus, they concluded that distinct information is being transmitted by the two receptors.

In the same manner, ligand-induced heterodimerization of XA21 with another receptor kinase may trigger the activation of two distinct signaling pathways leading to full *Xoo* resistance. In contrast, a heterodimer formation of the X21K736E or Xa21D with such a molecule would transmit through only one signaling pathway leading to partial resistance. In support of this idea we have identified a Xa21 homolog present in normally susceptible lines that could function in a heterodimeric complex [26].

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