Molecular Plant • Volume 3 • Number 5 • Pages 917–926 • September 2010 RESEARCH ARTICLE

Plasma Membrane Localization and Potential Endocytosis of Constitutively Expressed XA21 Proteins in Transgenic Rice

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ABSTRACT The rice pattern recognition receptor (PRR) XA21 confers race-specific resistance in leaf infection by bacterial blight *Xathomonas oryzae* pv. *oryzae* (*Xoo*), and was shown to be primarily localized to the endoplasmic reticulum (ER) when expressed with its native promoter or overexpressed in the protoplast. However, whether the protein is still ER-localization in the intact cell when overexpressed remains to be identified. Here, we showed that XA21, its kinase-dead mutant XA21P^{K736EP}, and the triple autophosphorylation mutant XA21P$^{S686A/T688A/S699A}$ GFP fusions were primarily localized to the plasma membrane (PM) when overexpressed in the intact transgenic rice cell, and also localized to the ER in the transgenic protoplast. The transgenic plants constitutively expressing the wild-type XA21 or its GFP fusion displayed race-specific resistance to *Xoo* at the adult and seedling stages. XA21 and XA21P^{K736EP} could be internalized probably via the SCAMP-positive early endosomal compartment in the protoplast, suggesting that XA21 might be endocytosed to initiate resistance responses during pathogen infection. We also established a root infection system and demonstrated that XA21 also mediated race-specific resistance responses to *Xoo* in the root. Our current study provides an insight into the nature of the XA21-mediated resistance and a practical approach using the root cell system to further dissect the cellular signaling of the PRR during the rice–*Xoo* interaction.

Key words: Rice; XA21; pattern recognition receptor; bacterial bight; cellular localization; internalization; root infection; race-specific resistance.

INTRODUCTION

Plant innate immunity to pathogens relies on the specific sensing of pathogen-associated molecules by defined pattern recognition receptors (PRRs) or resistance (R) proteins that recognize pathogen effectors (i.e. avirulence protein, Avr) at the cell surface or in the cytoplasm (Dangl and Jones, 2001; Ausubel, 2005; Chisholm et al., 2006). Over the past 15 years, more than 60 R genes have been isolated from diverse plants, of which most encode intracellular R proteins with nucleotide-binding site-leucine-rich repeats (NBS-LRR). Several R proteins belong to the cytoplasmic serine/threonine protein kinase class or PM-bound LRR (eLRR) proteins that possess a single transmembrane domain and a short intracellular tail or a protein kinase domain (Hammond-Kosack and Parker, 2003; DeYoung and Innes, 2006). Growing evidence indicates that these R proteins directly or indirectly detect pathogen effectors and rapidly activate defense signaling pathways following infection.

It is particularly interesting that some PRR or R proteins are receptor kinases (RLK or eLRR-kinase) such as the rice XA21 and XA26 that confer race-specific resistance to bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Song et al., 1995; Sun et al., 2004). The best characterized PRR, FLS2, is also

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doi: 10.1093/mp/ssq038, Advance Access publication 8 July 2010
Received 1 May 2010; accepted 2 June 2010

a RLK that recognizes bacterial flagellin, a pathogen-associated molecular pattern (PAMP) to activate innate immunity in *Arabidopsis* (Gomez-Gomez and Boller, 2000, 2002). These RLK all belong to the non-RD kinase class, whose activation does not require autophosphorylation in the activation loop (Dardick and Ronald, 2006). It has been shown that FLS2 is necessary for flagellin binding and that both the LRR domain and the kinase activity are required for flagellin binding and defense signaling. Furthermore, the FLS2 kinase activity is required for protein stability, which, in turn, impacts binding of flg22 (Gomez-Gomez et al., 2001).

Recently, XA21 was identified as a PRR that recognizes the type I-secreted sulfated peptide Ax21 and also a R protein because it is the single polymorphic determinant in rice that confers resistance to strains of bacteria expressing sulfated Ax21 (Lee et al., 2009). It was shown that XA21 can autophosphorylate the residues Ser686, Thr688, and Ser689 *in vitro* and substitution of these residues with alanines destabilized the protein *in vitro* and *in vivo* (Xu et al., 2006). Transgenic plants expressing this mutant protein were compromised in their resistance to avirulent *Xoo* Philippine race 6 (P6, strain PXO99A). Interestingly, both the wild-type XA21 and the XA21P^{K736EP} mutant carrying the Lys736–Glu mutation were also proteolytically degraded in protein extracts. Using the yeast two-hybrid system, one XA21-binding protein XB15, a protein phosphatase 2C, was identified to negatively regulate cell death and XA21-mediated innate immune response, further confirming that XA21 signals through phosphorylation events (Park et al., 2009). Interestingly, another XA21-binding protein 3 (XB3), an E3 ubiquitin ligase, physically associates with XA21 and acts as a substrate for the XA21 kinase; reduction in *Xb3* levels led to decreased levels of the XA21 protein, indicating that *Xb3* is important for XA21 stability and for the XA21-mediated disease resistance (Wang et al., 2006). However, the molecular mechanism by which the XA21–XB3 complex activates the downstream events of defense signaling remains unknown. Through immuno-precipitating and sequencing the XA21 complex, another XA21-interacting protein was identified that is an ER chaperone, BiP3, which compromises XA21-mediated immunity when overexpressed (Park et al., 2010). It is puzzling that XA21 was shown to be glycosylated and primarily localized to the ER when expressed under its native promoter and also partially to the PM when overexpressed in the rice protoplast (Park et al., 2010). This ER-localization of XA21 is contrary to the widely recognized fact that RLKs are localized to the PM.

It has been long recognized that eukaryotic cells respond to external signals by regulating the abundance and distribution of PM proteins that comprise the functional interface with the external environment. The removal and compartmentalization of PM proteins are regulated by endocytosis (internalization) and/or endocytotic cycling, an essential eukaryotic process whereby cells take up extracellular substances and/or internalize PM proteins for transport to endosomes, providing necessary spatial and temporal dimensions to signaling (Murphy et al., 2005; Polo and Di Fiore, 2006). However, the occurrence of endocytic processes in plants has been investigated only recently. Studies on PM cycling of the PIN auxin efflux carrier family and the KAT1 potassium channel demonstrated occurrence of endocytosis in plants (Geldner et al., 2001; Hurst et al., 2004; Peer et al., 2004). It was suggested that there are at least two forms of endocytosis operating in plants: clathrin-dependent (clathrin-mediated endocytosis, CME) and independent (e.g. raft/caveloae endocytosis, RCE) (Murphy et al., 2005; Polo and Di Fiore, 2006). Endocytosis is also required for signaling mediated by plant RLKs. In *Arabidopsis*, two such RLKs, BRASSINOSTEROID INSENSITIVE1 (BRI1) and BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1), both required for brassinosteroid (BR) signaling, preferentially heterodimerize in the endosomes (Russinova et al., 2004). Exogenous ligand BRs do not cause difference in BRI1 intracellular transport, suggesting constitutive endocytic trafficking of BRI1 (Geldner et al., 2007). Interestingly, one of the plant PRRs, FLS2, can also undergo endocytosis. In contrast to BRI1–BAK1, FLS2 undergoes endocytosis and degradation only upon stimulation with the flagellin epitope flg22 (Robatzek et al., 2006).

Interestingly, *Xa21* confers disease resistance to leaf infection by *Xoo*, with developmental regulation of resistance in seedling and adult plants (Century et al., 1999; Xu et al., 2006). However, it is also known that *Xoo* can survive on roots of rice stubble and its infection is often associated with root wounds and occasionally causes seedling disease called Kresek in paddy fields (Nyvall, 1999). Whether the *Xoo* root infection also induces race-specific resistance response remains unknown. In this study, we developed transgenic rice with XA21–GFP fusion proteins to demonstrate the primary PM-localization feature of XA21 that confers race-specific disease resistance in adult plants and seedlings when constitutively expressed. We showed that XA21 was internalized likely via early endosome compartments in root cells. We also established a root infection system that showed that *Xoo* also induced race-specific resistance in the root, together providing a reliable system for further investigation of XA21 endocytosis during interaction with *Xoo*.

RESULTS

Overexpression of XA21–GFP Fusion and Wild-Type XA21 Exhibits Race-Specific Disease Resistance in Adult Plants and Seedlings

We generated transgenic rice constitutively expressing the *Xa21–GFP* fusion and the kinase-dead *Xa21*^{K736E}*–GFP* mutant driven by the *35S* promoter (Supplemental Figure 1). We inoculated 8-week-old plants with bacterial blight *Xoo* races P6 (strain PXO99A) and K1 (strain DY89031) that are incompatible and compatible with *Xa21*, respectively (da Silva et al., 2004; Yuan et al., 2007). These stable XA21–GFP transgenic lines still exhibited race-specific resistance to *Xoo*, which were resistant

to P6 and susceptible to K1, respectively, in comparison with wild-type TP309 and the control line 106 containing the *Xa21* gene (Figure 1A and 1B, two representative lines are shown). Consistent with a previous report that the XA21^{K736E} mutant conferred partial resistance to *Xoo* P6 (Andaya and Ronald, 2003), a few transgenic lines containing the XA21^{K736E}–GFP fusion displayed partial resistance to P6 (Figure 1B). To confirm that GFP fusion did not affect *Xa21*-mediated resistance, we also developed stable transgenic lines overexpressing the wild-type *Xa21* and the *Xa21^{K736E}* mutant (Supplemental Figure 1). Similar race-specific resistance was conferred by overexpression of the wild-type *Xa21* gene and partial resistance was observed in some lines overexpressing *Xa21^{K736E}* (Figure 1C and 1D, two representative lines are shown). Thus, the XA21–GFP fusion is biologically equivalent to the wild-type XA21.

The *Xa21*-mediated resistance is developmentally regulated and 4-week-old seedlings (four to five full expanded leaves) of line 106 exhibited 50–75% resistance in comparison with adult plants (Century et al., 1999). We further investigated bacterial resistance in seedlings of these transgenic lines expressing the XA21–GFP fusions and found that overexpression of *Xa21–GFP* but not *Xa21^{K736E}–GFP*, like the *Xa21*-carrying line 106, was able to confer race-specific resistance to P6 in young seedlings at 10–12 d post inoculation (dpi) (Figure 2A–2C). Therefore, seedling inoculation provides an alternative system for studying the rice–*Xoo* interaction.

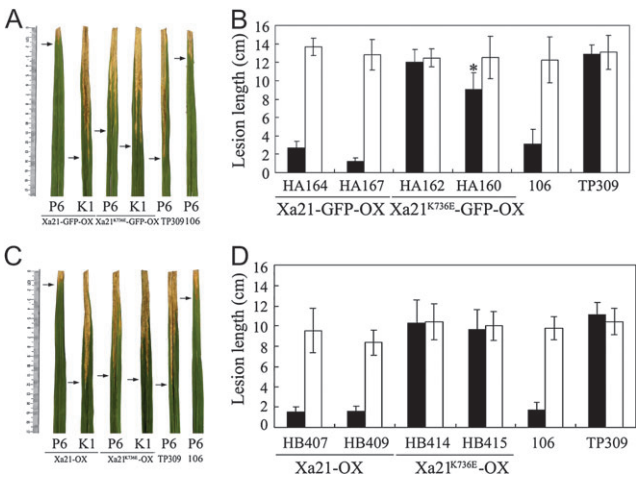

Figure 1. Overexpression of *Xa21–GFP* and *Xa21* Confers Race-Specific Resistance to *Xoo*.

(A) Representative lesions of 8-week-old XA21–GFP–OX plants inoculated with P6 (1) and K1 (2), XA21^{K736E}–GFP–OX plants with P6 (3) and K1 (4), wild-type TP309 with P6 (5), and line 106 with P6 (6). Arrows indicate bottoms of lesions.

(B) Statistic analysis of lesion length of transgenic lines, TP309 and line 106. Two representative lines of each transgenic rice are shown. Filled bars, lesion length with P6 inoculation; open bars, lesion length with K1 inoculation; *, significant difference in comparison with TP309 ($P < 0.05$). Experiments were repeated three times, with similar results.

(C) Representative lesions of 8-week-old XA21–OX plants inoculated with P6 (1) and K1 (2), XA21^{K736E}–OX plants with P6 (3) and K1 (4), TP309 with P6 (5), and line 106 with P6 (6). Arrows indicate the bottoms of lesions.

(D) Statistic analysis of lesion length of transgenic lines, TP309 and line 106. Two representative lines of each transgenic rice are shown. Filled bars, lesion length with P6 inoculation; open bars, lesion length with K1 inoculation.

PM- and ER-Localization of XA21, XA21^{K736E}, and XA21$^{S686A/T688A/S689A}$ in Root Cells and Protoplasts

To determine the sub-cellular localization of XA21 in rice cells, we observed GFP signal in rice root cells of wild-type and *Xa21–GFP* and *Xa21^{K736E}–GFP* transgenic plants with a confocal microscopy. As shown in Figure 3, both XA21–GFP and the kinase-dead XA21^{K736E}–GFP were primarily localized to the PM of root cells, in comparison with the GFP alone control that was localized at the cytoplasm and nuclear. Similar PM-localization was also observed for XA21–GFP and XA21^{K736E}–GFP in rice protoplasts (see below). Therefore, our current study indicated that the majority of XA21 is translocated to the PM when overexpressed, in contrast to its primary ER-location when expressed under the native promoter (Park et al., 2010). A previous study showed that the auto-phosphorylated Ser686, Thr688, and Ser689 residues in the XA21 intracellular juxtamembrane domain required for phosphorylation were important for

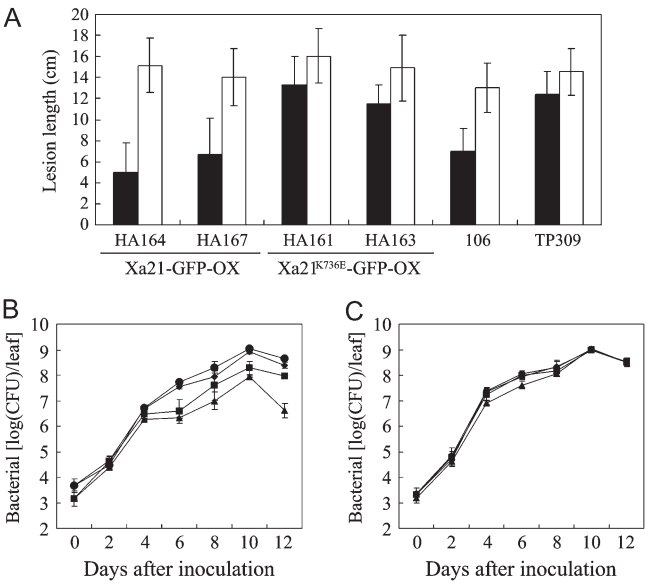

Figure 2. Disease Resistance of Transgenic Rice Seedlings to *Xoo*.

(A) Statistic analysis of lesion length in 4-week-old rice seedlings of XA21–GFP–OX and XA21^{K736E}–GFP–OX with wild-type TP309 and line 106 as controls, with P6 (filled bars) and K1 (open bars) inoculation.

(B) Bacterial growth of P6 in representative XA21–GFP–OX and XA21^{K736E}–GFP–OX lines.

(C) Bacterial growth of K1 in representative XA21–GFP–OX and XA21^{K736E}–GFP–OX lines. Experiments were repeated twice, with similar results (a–c). Squares, XA21–GFP–OX; diamonds, XA21^{K736E}–GFP–OX; circles, TP309; triangles, line106 (b, c).

the XA21 function and stability (Xu et al., 2006). To determine whether these sites affect the sub-cellular localization of XA21, we also generated transgenic rice overexpressing XA21$^{S686A/T688A/S689A}$–GFP that conferred partial resistance to P6 (Supplemental Figure 2A and 2C). We observed that the triple mutant isoform was also primarily localized to the PM (Supplemental Figure 2B). Taken together, our results suggest that kinase or phosphorylation activity does not impact its sub-cellular localization, although the disruption of kinase or phosphorylation activity destabilizes the XA21 protein (Xu et al., 2006).

Endocytotic Compartments of XA21 and XA21^{K736E} are BFA-Sensitive in Root Cells

We did not observe similar constitutive endocytic trafficking of XA21 as observed for BRI1 (Geldner et al., 2007). We then studied internalization of XA21 in the cells treated with Brefeldin A (BFA) that inhibits endosomal recycling to the PM and post-Golgi vesicle trafficking and leads to rapid accumulation of plasma membrane proteins in the so-called 'BFA compartment' (Peyroche et al., 1999; Geldner et al., 2001). To eliminate the exocytotic compartments of newly synthesized GFP fusion proteins, we also pretreated these transgenic roots for 30 min with cycloheximide (CHX) that inhibits *de novo* protein synthesis. As shown in Figure 4, pretreatment with CHX (50 μM) for 30 min followed by BFA incubation resulted in retention of both XA21–GFP and XA21^{K736E}–GFP in the BFA compartments. Therefore, we concluded that XA21, like other RLKs, is capably internalized via a BFA-sensitive vesicle.

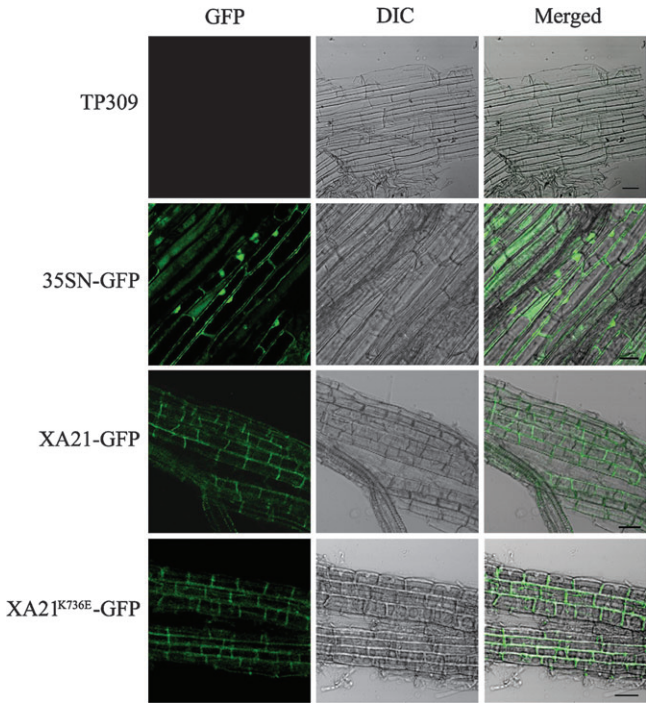

Figure 3. Plasma Membrane-Localization of XA21–GFP and XA21^{K736E}–GFP in Root Cells.

Confocol images of the PM-located XA21–GFP, the kinase-dead mutant XA21^{K736E}–GFP isoform in transgenic rice root cells, with GFP alone as a control, which was localized in the cytoplasm and nuclear. Note that wild-type (TP309) cells did not display autofluorescence under the confocol image. Bar = 20 μm.

Endocytosis of XA21 and XA21^{K736E} Likely Through an Early Endosome

To further determine the identity of these cytosolic XA21 and XA21K^{736E} compartments, we next compared the nature and dynamics of the XA21–GFP or XA21K^{736E}–GFP-positive organelles to those of the early endosome containing the rice secretory carrier mRFP–SCAMP1 (Lam et al., 2007a), using protoplasts derived from rice suspension cells (Miao and Jiang, 2007). When transiently expressed in rice protoplasts, both XA21–GFP and XA21K^{736E}–GFP were localized to both the PM and the ER-like structure (Figure 5), similar to the previous report (Park et al., 2010), whereas mRFP–SCAMP1 was localized to both the PM and the cytosolic early endosomes (Figure 5, upper panel). In addition, upon BFA treatment, similar cytosolic aggregates were formed in rice cells expressing mRFP–SCAMP1 and XA21–GFP or XA21K^{736E}–GFP (Figure 5, middle panel), as observed in the root cells (Figure 4).

Wortmannin, a specific inhibitor of PI-3K kinase, has been widely used to study organelle dynamics and endocytosis in plant cells (Samaj et al., 2005; Lam et al., 2007b). We thus treated the transgenic protoplasts with wortmannin (33 μM) for 30 min. The numbers of cytosolic vesicles containing

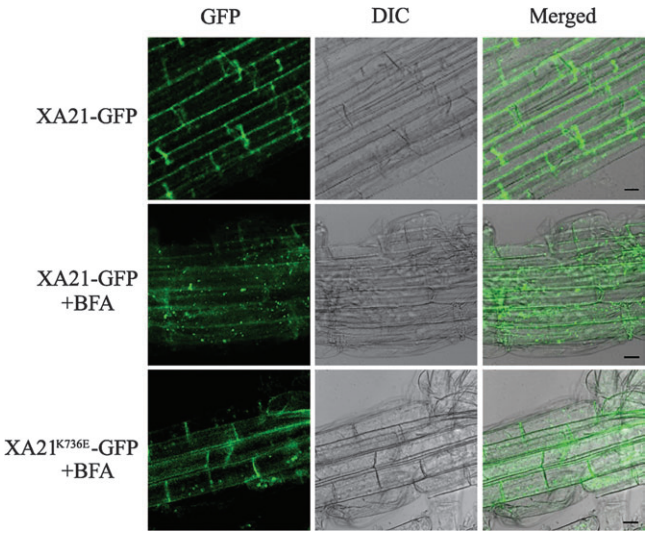

Figure 4. Internalization of the XA21–GFP and XA21^{K736E}–GFP Proteins by BFA Treatment.

BFA-induced relocation of the XA21–GFP and XA21^{K736E}–GFP proteins from the PM to the BFA compartments. Seedling roots were pretreated with CHX (50 μM) for 30 min to inhibit *de novo* protein synthesis, followed by treatment with BFA (50 μg ml^{-1}) for 30 min as described in Methods. Note that relative GFP signals in the PM decreased in the XA21–GFP and XA21^{K736E}–GFP cells after the BFA compartments formed. Bar = 10 μm.

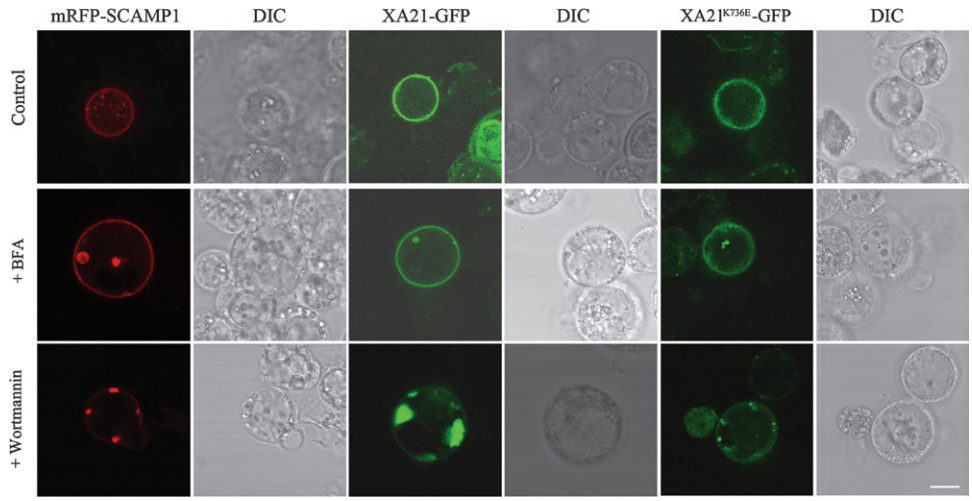

Figure 5. Identical Vesicles of XA21–GFP, XA21^{K736E}–GFP, and mRFP–SCAMP1 in Rice Protoplasts.
The XA21–GFP, XA21^{K736E}–GFP, and mRFP–SCAMP1 fusions were transiently expressed in rice protoplasts (upper panel). The protoplasts were treated with BFA (10 μg ml^{-1}) (middle panel) or wortmannin (33 μM) (bottom panel) as described in Methods. Note that the vesicles of XA21–GFP, XA21^{K736E}–GFP were identical to those of mRFP–SCAMP1.

XA21–GFP or XA21K^{736E}–GFP increased upon wortmannin treatment (Figure 5, bottom panel); similar cytosolic aggregates were also formed in the rice cell expressing mRFP–SCAMP1, suggesting identical features of these compartments. Although a direct comparison between mRFP–SCAMP1 and XA21–GFP or XA21K^{736E}–GFP in the same cell would have a more conclusive result, we failed to co-express pairs of mRFP–SCAMP1 and XA21–GFP together after many attempts. We also failed to detect reliable endosomal uptake of FM4-64, an endocytotic tracker, since the rice cell has strong uptake of FM4-64 in the PM and different sub-cellular compartments (data not shown). Since BFA and wortmannin treatments caused similar aggregate formation in the rice cells expressing XA21–GFP or XA21K^{736E}–GFP or mRFP–SCAMP1, we proposed that the XA21–GFP/XA21K^{736E}–GFP-containing organelles are likely the same as the early endosomes containing mRFP–SCAMP1 in the rice cell (Lam et al., 2007a).

Establishment of *Xoo* Root Inoculation System

In order to further study the predicted XA21 endocytosic signaling during the rice–*Xoo* interaction, and also to investigate root resistance response to *Xoo*, we established a root infection system, given the fact that *Xoo* can survive on roots of rice stubble and occasionally causes seedling disease called Kresek in paddy field (Nyvall, 1999). First, we transformed *Xoo* P6 with a GUS expression cassette (P6–GUS) to visibly detect *Xoo* infection, which produced strong GUS activity and did not change its virulence on line 106 and TP309, compared with wild-type P6 (Supplemental Figure 3A and 3B). Second, we performed root inoculation at the seedling stage (2 weeks old) and observed that P6–GUS rapidly massed in wound roots with maximum GUS staining at 12 hpi and could grow in roots for over 10 dpi, spread upwards to stems, and occasionally to leaves, as detected by GUS staining (Supplemental Figure 3C and 3D). Histological assays of GUS activity in thin-sectioned root samples indicated that P6–GUS could effectively infect and grow inside the rice root (Figure 6A). Therefore, this root inoculation system likely mimics natural root infection by *Xoo*.

Evidence of Race-Specific Resistance Response to *Xoo* in Rice Roots

We next determined whether the rice–*Xoo* interaction induces race-specific responses in roots by dissecting cellular and molecular events during root infection. Most K1-infected roots of line 106 were dead, with deep brown lesion-like symptoms at 4 weeks post inoculation, while P6 infection did not lead to obvious symptoms (Figure 6B). Instead, P6 infection induced a high level of H_2O_2 accumulation at 30 mpi and the hypersensitive response (HR) cell death at 24 hpi (Figure 6C and 6D), which are known to be associated with *Xoo* resistance (He et al., 2000; Chen et al., 2007; Jha et al., 2007). Importantly, the race-specific defense response in roots was also supported at a molecular level by the finding that the rice defense genes *PAL*, *PR1a*, and *PR1b* (He et al., 2000; Yang et al., 2008) were more strongly induced by P6 than by K1 (Figure 6E). Taken together, these experiments have thus established a convenient infection system/pathosystem for dissecting XA21-mediated resistance with seedling roots.

DISCUSSION

Our current study documents that the rice PRR protein, XA21, and its mutant isoforms are primarily localized to the PM of rice root cells and protoplasts when overexpressed as a GFP fusion. Because the less accumulated RLK with its native promoter is likely retained in the ER (Park et al., 2010), we hypothesize

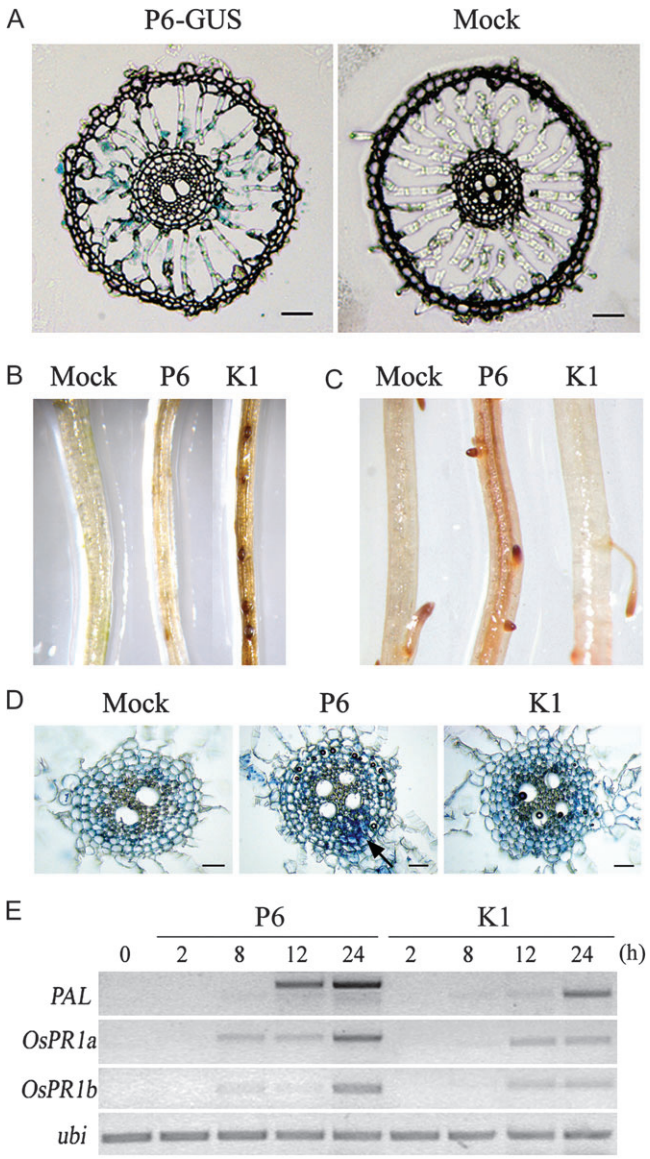

Figure 6. Race-Specific Resistance Responses in Seedling Root Infection.

(A) Microscopic section of TP309 seedling roots. Left, inoculation with P6–GUS for 12 h, showing GUS-staining bacteria (also see Supplemental Figure 3); right, mock inoculation. Bar = 50 μm.

(B) Disease symptoms of line 106 seedling roots infected by P6 and K1 for 1 month; note that K1 infection caused dark-brown lesions.

(C) DAB staining for H_2O_2 detection in line 106 roots at 30 min post inoculation, showing that H_2O_2 greatly accumulated during infection by P6.

(D) Trypan blue staining of line 106 roots infected by P6 and K1 for 24 hpi; arrow indicates cell death occurring with P6 infection. Bar = 25 μm.

(E) Differential expression of the pathogenesis-related genes *PAL*, *PR1a*, and *PR1b* in line 106 roots inoculated with P6 and K1 up to 24 h. *Ubi*, inner RT–PCR control. Experiments were repeated twice, with similar results.

that XA21 interacts with BIP3 and is retarded within the ER during its process, whereas when over-accumulated/produced, the protein would be guarded/translocated to the PM with a mechanism unrecognized currently. Our current observation is also consistent with the previous result that the majority of XA21 was associated with the membrane fraction (Xu et al., 2006). Nevertheless, our finding is consistent with the feature of a cell surface receptor and with the finding that Ax21 recognition occurs at the PM (Lee et al., 2006, 2009). Equivalent to the wild-type XA21, the XA21–GFP fusion protein also conferred a race-specific resistance in adult plants as well as in young seedlings. Interestingly, root infection induced the *Xa21*-mediated race-specific resistance responses in the rice–*Xoo* interaction, including H_2O_2 accumulation, the HR cell death and defense gene activation. Therefore, our study adds another pathosystem that a rice pathogen that usually infects leaves can also infect roots, in addition to the rice fungal blast that also infects both leaves and roots (Sesma and Osbourn, 2004).

The similarity between the symptoms of the pathogen-infected rice roots and those of the naturally occurring Kresek disease provides some advantages for the study of the mechanism(s) of the *Xa21–Xoo* interaction. First, the root system allows us to observe the sub-cellular location and dynamics of XA21 and other PM proteins with GFP fusions, because the rice cell usually shows obscure GFP fluorescence (Yuan et al., 2007; Wang et al., 2008). Second, we are able to directly observe bacterial growth inside the root with the P6–GUS modified strain, providing an alternative approach to further dissect *Xoo* pathogenicity, of which much remains unknown (Ray et al., 2000; Yang et al., 2000; Rajeshwari et al., 2005; Jha et al., 2007; Aparna et al., 2009).

There is accumulating evidence for endocytosis in plants (Battey et al., 1999; Shah et al., 2002; Samaj et al., 2004; Gifford et al., 2005). The well characterized plant receptor kinase BRI1 constitutively endocytoses (Russinova et al., 2004; Geldner et al., 2007). By contrast, XA21 endocytosis was only observed with BFA or wortmannin treatment (Figures 4 and 5). Therefore, plant RLKs display diverse endocytosis behavior. Recently, endocytosis of PM proteins and hence vesicle trafficking in endocytic process have emerged as the important components of plant innate immune response (Bhat et al., 2005; Koh et al., 2005; Nomura et al., 2006; Lipka et al., 2007; Robatzek, 2007). During infection by the hemibiotrophic fungus *Magnaporthe oryzae*, rice cells also exhibited endocytotic features (Kankanala et al., 2007). Invasive hyphae (IH) were sealed in a plant membrane (the extra-invasive hyphalmembrane, EIHM), which formed distinct membrane caps at the tips of IH. This study revealed new features of cellular mechanisms resulting in disease (Kankanala et al., 2007). In the current study, we observed that the PM-bound XA21 and XA21K^{736E} proteins could be internalized in the presence of BFA or wortmannin, mimicking the rice secretory carrier SCAMP1. The SCAMP1-labeled vesicle represents either an early endosome (EE) or a recycling endosome (RE) (Lam et al.,

2007a). In this aspect, the cellular feature of the XA21 compartment also well resembled an early endosome or a recycling endosome (Figure 5).

One consequence of receptor endocytosis in mammalian cells is that internalized receptors are to be degraded through a late endosome route to vacuole/lysosome or proteasome through the ubiquitination pathway (Haglund et al., 2003). Notably, the mutants XA21^{K736E} and XA21$^{S686A/T688A/S689A}$ did not change their PM location but instead their stability *in vitro* and *in vivo*, a direct linking between disease resistance and PRR degradation (Xu et al., 2006). XA21 might also adopt an endocytosis system to initiate intracellular defense signaling including protein degradation like other RLKs, although we currently could not dissect the endocytosis and recycling behavior of XA21 during pathogen infection because of the technical difficulty and the lack of the established endocytotic system for the rice cell. The further investigation on endocytosis and signaling of XA21 with an established system would provide deep insight into the important PRR-mediated defense responses in the model cereal, in which genome resources are available. It will be of great interest to investigate how endocytosis and/or recycling of XA21 performs in response to Ax21 using the root path system, as observed for the PAMP receptor FLS2 (Robatzek et al., 2006), and how components of endocytosis or endosome assembling would regulate the XA21-mediated resistance, albeit characterization of endocytosis and recycling in plants is still in its very infancy.

METHODS

Construction of Expression Plasmids and Rice Transformation

Xa21/*Xa21*^{K736E} genomic DNA was inserted into the binary vector 35S–C1301 for rice transformation and was also in-frame fused with *mGFP* (accession no. U87974) to make the *Xa21–GFP* and *Xa21*^{K736E}*–GFP* tagged fusions. The triple mutant *XA21*$^{S686A/T688A/S699A}$ was generated by *in vitro* PCR-based mutagenesis using the primer 5′-aaaagggagcccctgcaagagcagccatgaaaggccacc-3′ designed for nucleotides 2042–2080 bp of the *Xa21* genomic DNA as described (Xu et al., 2006). These constructs were transformed into the rice variety Taipei 309 (TP309) calli with the agrobacterium-mediated method to obtain more than 10 independent lines (XA21–OX, XA21^{K736E}–OX, XA21–GFP–OX, XA21^{K736E}–GFP–OX, XA21$^{S686A/T688A/S699A}$–GFP–OX) for each construct. Transgenic plants were detected by RT–PCR or GFP signal. Transgenic progeny generations (T1–T5) were used in all experiments.

Plant Growth and Inoculation

Eight-week-old (adults) or 4-week-old (seedlings) plants were inoculated with P6 (strain PXO99) and K1 (strain DY89031). Lesion length was measured at 12 dpi. For root inoculation, seedlings were water-incubated for 2 weeks, root tips were cut to make wounding, and incubated with *Xoo* inoculum suspension for different times. For bacterial growth, infected leaves were collected with a time course (0, 2, 4, 6, 8, 10, 12 dpi) and were ground in ddH_2O to release bacteria. The solution was eluted and incubated on PSA medium containing 200 μM azacytidine (Sigma) for 2 d at 28°C, as described (Song et al., 1995). Colony-forming units (cfu) per ml were counted. Three leaves were analyzed for each time point with independent experiments.

Construction of GUS Expressing *Xoo* Strain

The GUS coding region was placed under the promoter of the *Listeria monocytogenes prtA* gene (Mengaud et al., 1991, provided by Dr Yongqiang He). The *prtA–GUS* fusion was inserted into the vector pUFR034 (Defeyter et al., 1990) to generate the plasmid pPRTA–GUS, which was introduced into P6 by electroporation. Transformants were screened with 20 mg l^{-1} kanamycin sulfate and further confirmed by GUS staining. Eight-week-old line 106 and TP309 plants were inoculated with P6–GUS to determine its virulence. TP309 seedlings were inoculated with root incubation to detect P6–GUS infection in rice roots. Leaf, stem, and root tissues of seedlings infected for 10 d by P6–GUS with root inoculation were surface-sterilized and ground in 200 μl ddH_2O for P6–GUS detection.

GUS, DAB, and Trypan Blue Staining

For detection of P6–GUS growth inside the root, seedling roots infected with P6–GUS were extensively washed; histochemical assay for GUS activity was performed with frozen sections under a microscope (Leica CM 1850). For detection of H_2O_2 accumulation in the *Xoo*-infected rice root, the 3,3-diaminobenzidine (DAB) uptake method was adopted, as described (Ning et al., 2004). Two-week-old seedling roots of line 106 were inoculated with P6 and K1 for 24 h and the HR cell death was visualized by the Trypan blue exclusion assay, as described (Ning et al., 2004).

GFP Observation and Drug Treatment

The PM localization and endocytosis of XA21–GFP and XA21^{K736E}–GFP were directly observed immediately after chemical treatment with BFA (Sigma, 50 μg mL^{-1} for root cells and 10 μg mL^{-1} for protoplasts) and Wortmannin (Sigma, 33 μM in DMSO for protoplasts) for 30 min, with a Confocol laser scanning microscope (Zeiss LSM510). To avoid *de novo* protein synthesis and interference from exocytosis, seedling roots were pretreated with CHX (Sigma, 50 μM in water) for 30 min before chemical treatment. GFP fluorescence was detected at λ_{ex} = 488 nm, λ_{em} = 505–530 nm.

Rice Protoplast Preparation and Transient Expression

Rice protoplasts were prepared from rice suspension cells as described (Miao and Jiang, 2007). For transient expression, 10 μg DNA of each construct was mixed with 200 μl rice protoplasts as described (Miao and Jiang, 2007). Transformed protoplasts were collected and observed with Confocol microscopy for image collection.

RNA Analysis

Total RNA was isolated from rice roots by using TRIzol Reagent and the first cDNA strand was synthesized with SuperScript III System (Invitrogen). The primers 5′-attgccagctcttacggt-3′ and 5′-caagtctaagcagccaaaca-3′ were used to detect the *Xa21* and *Xa21*^{K736E} transcripts in different transgenic lines. The rice *ubi-1* was used as a RT–PCR control. The rice defense genes, *PAL*, *PR1a*, and *PR1b* (He et al., 2000; Qiu et al., 2008; Yang et al., 2008), were detected using the primers 5′-TGGATCCTCAAC-TGCATCG-3′ and 5′-GGGTCCATCTCGTTCACCTT-3′ (for *PAL*), 5′-AGTTCGTCGAGCAGGTTATCCT-3′ and 5′-AGATTGGCCGACG-AAGTTG-3′ (for *PR1a*), and 5′-TATCCAAGCTGGCCATTGCTTT-3′ and 5′-TAAGGCCTCTGTCCGACGAA-3′ (for *PR1b*).

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

FUNDING

This work was supported by the grants of the National Natural Science Foundation of China (30730064 and 30721061), the Ministry of Science and Technology of China (2006CB101900), and the Chinese Academy of Sciences (KSCX2-YW-N-005).

ACKNOWLEDGMENTS

We thank Pamela Ronald for providing the *Xa21* constructs, the *Xa21* transgenic rice, useful discussions, and comments on this study, Jianming Li for critical reading of the manuscript, Weuyuan Song for discussion on the experiments, and Jianjun Wang and Linyou Wang for helping plant growth and inoculation. No conflict of interest declared.

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