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Microreview

Elucidation of XA21-mediated innate immunity

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Summary

In the early 1970s, the *Xa21* gene from the wild rice species *Oryza longistaminata* drew attention of rice breeders because of its broad-spectrum resistance to diverse strains of a serious bacterial disease of rice in Asia and Africa, called 'bacterial blight disease', caused by the Gram-negative bacterium, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). In 1995, we isolated the gene controlling this resistance and in 2009 demonstrated that XA21 recognizes a highly conserved peptide, called 'Ax21' (activator of XA21-mediated immunity). Tyrosine sulfation of Ax21 is required for recognition by rice XA21. A decade of genetic, molecular and biochemical studies have uncovered key components of the XA21-mediated signalling cascade. Ax21 recognition by XA21 at the cell surface induces phosphorylation-mediated events, which are predicted to alter subcellular localization and/or DNA-binding activity of a WRKY family of transcription factors. Because XA21 is representative of the large number of predicted pattern recognition receptors (PRRs) in rice ($n = 328$), *Arabidopsis* ($n = 35$) and other plant species, further characterization of XA21-mediated signalling pathways will contribute to elucidation of these important defence responses.

Introduction

Recognition of conserved microbial signatures [also called 'pathogen-associated molecular patterns' (PAMPs)] by host sensors [(also called 'pattern recognition receptors' (PRRs)] activates innate immune response. Plant and animal PRRs share conserved domains, such as leucine-rich repeats (LRRs) for PAMP recognition (Anderson *et al*., 1985; Song *et al*., 1995; Poltorak *et al*., 1998; Wang *et al*., 1998; Gomez-Gomez and Boller, 2000; Boller and Felix, 2009) and non-RD kinase domains that are either integral to the receptor (plants) or associated with it (animals) (Dardick and Ronald, 2006). In plants, three PRR/PAMP interactions have been well characterized. These are rice XA21 (Song *et al*., 1995), *Arabidopsis* flagellin sensitive 2 (FLS2) (Gomez-Gomez and Boller, 2000) and the *Arabidopsis* elongation factor (EF)-Tu receptor (EFR) (Zipfel *et al*., 2006). XA21, FLS2 and EFR recognize a sulfated peptide (axY^{S}22) derived from the N-terminal region of Ax21 (Lee *et al*., 2009), the flg22 peptide derived from bacterial flagellin (Gomez-Gomez and Boller, 2000) and the elf18 peptide, derived from the EF-Tu protein (Zipfel *et al*., 2006) respectively.

In animals, positional cloning of a spontaneous mutation that caused lipopolysaccharide resistance and susceptibility to Gram-negative infection led to the isolation of Toll-like receptor 4 (TLR4), which shared striking structural similarities to XA21 (Song *et al*., 1995; Poltorak *et al*., 1998) and, like XA21, is an essential host sensor of microbial infection. To date, 13 human TLRs have now been described (Mishra *et al*., 2008) and all recognize PAMPs presented in invading microbes and activate corresponding PRR-mediated signalling pathways (Hornef *et al*., 2008).

In this review, we discuss the isolation and characterization of Ax21 and present a model for XA21-mediated immunity based on recent results (Fig. 1).

Ax21 (activator of XA21-mediated immunity)

A screen for *Xoo* mutants defective in genes required for activation of XA21-mediated immunity (the *rax* genes) led to the identification of the *raxA*, *raxB* and *raxC* encoding components of a bacterial type I secretion system. *Xoo* mutants carrying knockouts in any of these genes lose the ability to trigger XA21-mediated immunity and are no longer able to secrete Ax21 (Lee *et al*., 2006). Another class of *rax* mutants involved in sulfation were also isolated. These include *raxST*, which encodes a protein with similarity to mammalian tyrosine sulfotransferases (da Silva *et al*., 2004) and the *raxR* and *raxP* genes, which encode genes critical for synthesis of 3′-phosphoadenosine 5′-phosphosulfate (PAPS). Based

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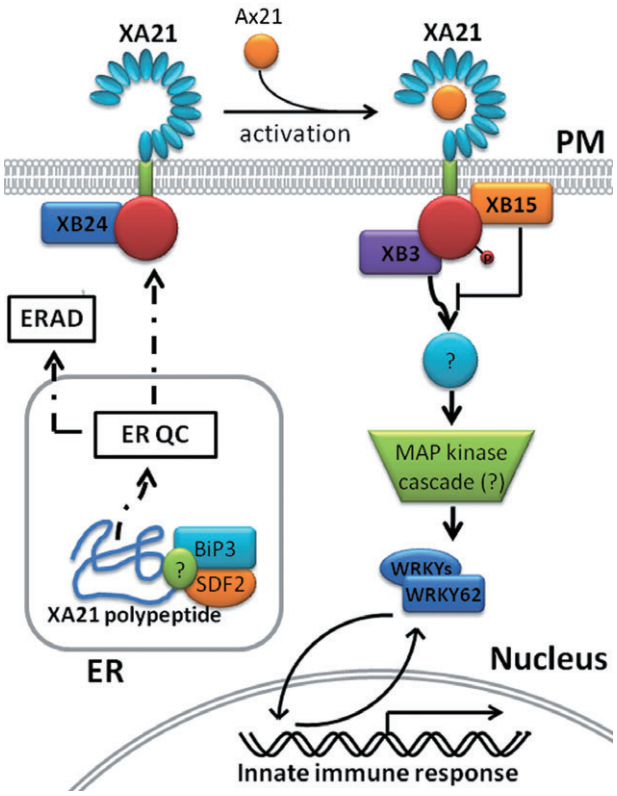

Fig. 1. A model XA21-mediated immunity. ER chaperones and co-chaperones such as BiP3 and SDF2 are involved in XA21 biogenesis (Park *et al*., 2010). XA21 is processed through the ER and translocated to the PM. XB24 physically associates with the XA21 JM domain, promotes autophosphorylation, and keeps XA21 in an inactive state (Chen *et al*., 2010b). Ax21 binding to the XA21 LRR domain induces dissociation of XA21 from XB24 and activates the XA21 non-RD kinase domain. (Wang *et al*., 1998; Lee *et al*., 2009; Chen *et al*., 2010b). Autophosphorylated Thr705 transfers its phosphoryl group to another XA21 residues, activating XA21 (Chen *et al*., 2010a). XA21 transphosphorylates downstream target proteins that have not yet been identified. XB3 may serve to activate a downstream MAPK cascade (Wang *et al*., 2006). In the nucleus, WRKY transcription factors regulate defence-related genes, such as *PR1* and *PR10*, either positively or negatively (Peng *et al*., 2008; Peng *et al*., 2010). Recruitment of XB15 to Ser697 in the XA21 JM domain and subsequent dephosphorylation of phosphorylated residue(s) attenuates the XA21-mediated immune response.

on these results, we hypothesized that RaxST utilizes PAPS to transfer a sulfuryl group to Ax21 (Lee *et al*., 2006).

These genetic screens were non-saturating because of the labour involved in the screen. It was necessary to grow rice plants for 6 weeks before inoculation because XA21-mediated resistance is only expressed at the adult stage and because scoring required another 10 days to assay symptom development. To quantify the response, we measured the length of bacterial induced lesions because a hypersensitive response, which is typical of many plant defence responses, cannot easily be observed in the XA21/Ax21 interaction. Thus, although the screens led to the identification of key genes controlling Ax21 activity and allowed us to establish increasingly focused models on the function of the putative Ax21, we failed to identify Ax21 itself.

Based on our model that Ax21 was likely a type I secreted, sulfated peptide, we switched to a biochemical approach. This was made possible both by the establishment of a new bioassay system (Lee *et al*., 2006) and by advances in proteomic analyses. Ax21 was isolated by analysis of bioactive fractions from *Xoo* strain PXO99Az using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The identified peptides were derived from a 194-amino-acid protein encoded by a gene designated *ax21* (Lee *et al*., 2009). An *Xoo* mutant strain lacking *ax21* is unable to trigger XA21-mediated immunity.

The Ax21 protein carries two predicted tyrosine sulfation sites. An Ax21-derived synthetic peptide (17-amino-acid) containing a sulfated tyrosine-22 (axY^{S}22) is sufficient for Ax21 activity, whereas peptides lacking tyrosine sulfation and peptide variants carrying alanine in place of the tyrosine are inactive (Lee *et al*., 2009). *In vivo* co-immunoprecipitation experiments demonstrated that axY^{S}22 binds to XA21 in transgenic plants expressing an N-terminal Myc-epitope-tagged XA21 (Lee *et al*., 2009). Although all *Xanthomonas species* tested carry *ax21* (Lee *et al*., 2009), *Xoo* strains lacking the sulfation and/or secretion systems can no longer elicit the XA21-mediated defence response (da Silva *et al*., 2004). These results indicate that sulfation on the axY22 peptide is critical for XA21/Ax21 recognition in rice.

Ax21 is present in all sequenced *Xanthomonas* species, in *Xylella fastidiosa*, the causal agent of Pierce's disease on grapes, and in the human pathogen, *Stenotrophomonas maltophilia* (Lee *et al*., 2009). The amino acid sequence of axY^{S}22 peptide is 100% conserved in all sequenced *Xanthomonas* species. *X. fastidiosa* and *S. maltophilia* peptides show 77% and 65% identity respectively (Lee *et al*., 2009).

Thus, Ax21 satisfies a key aspect of the definition of PAMPs: it is conserved within a class of microbes (Medzhitov, 2001; Beutler, 2004). The specificity conferred by a post-translational modification, Tyr22 sulfation of axY^{S}22, supports an emerging theme for PAMPs – that sequence variation and post-translational modifications, such as glycosylation, acetylation and sulfation, can modulate PRR-dependent recognition (Taguchi *et al*., 2003; Kunze *et al*., 2004; Sun *et al*., 2006).

The non-RD kinase domain

XA21 is a receptor kinase, which consists of LRR, transmembrane, juxtamembrane (JM) and intracellular kinase domains (Song *et al*., 1995). Kinases are classified as arginine-aspartate (RD) or non-RD kinases. RD kinases carry a conserved arginine (R) immediately preceding

the catalytic aspartate (D) (Dardick and Ronald, 2006). In contrast to RD kinases, non-RD kinases typically carry a cysteine or glycine in place of the arginine. We previously reported that non-RD kinases are associated with the control of early signalling events in both plant and animal innate immunity (Dardick and Ronald, 2006). For example, in humans, recognition of PAMPs at the cell surface is largely carried out by TLRs (Nurnberger and Brunner, 2002). TLR1, TLR3, TLR5, TLR6, TLR7, TLR8 and TLR9 associate with the non-RD interleukin-1 receptor associated kinase (IRAK) family (Akira and Takeda, 2004), and TLR3 and TLR4 associate with the non-RD receptor interacting-protein (RIP) kinases (Meylan *et al*., 2004) via adaptor proteins.

In plants, receptor kinases demonstrated to function in mediating innate immunity also fall into the non-RD class (Dardick and Ronald, 2006) or are associated with non-RD receptor kinases (Chinchilla *et al*., 2007; Miya *et al*., 2007; Wan *et al*., 2008). Plant genome analyses have revealed the presence of a large family of the non-RD receptor kinases at the cell surface, with 35 encoded in the *Arabidopsis* genome and 328 found in the rice genome (Dardick and Ronald, 2006). These include the *Arabidopsis* PRRs FLS2 and EFR (Gomez-Gomez and Boller, 2000; Zipfel *et al*., 2006), the rice XA26, Pid2 (Sun *et al*., 2004; Chen *et al*., 2006) and XA21 (Song *et al*., 1995). The *Arabidopsis* BRI1-associated receptor kinase 1 (BAK1) that associates with FLS2 is an RD kinase (Li *et al*., 2002; Chinchilla *et al*., 2007), suggesting that RD receptor kinases may need to associate with non-RD PRRs to transduce the immune response.

The majority of RD receptor kinases are regulated by autophosphorylation of the activation loop, a centrally located domain that is positioned close to the catalytic centre (Adams, 2003). In contrast, non-RD receptor kinases, the activation loop is not autophosphorylated. These results suggest that this important class of non-RD kinases use alternative mechanisms for activation (Dardick and Ronald, 2006).

The XA21 LRR recognizes Ax21

Based on models for animal receptor kinase function, we proposed that the LRR domain of XA21 recognizes Ax21 and that this recognition activates downstream phosphorylation events (Ronald, 1997). In support of this hypothesis, we showed that a natural variant of XA21, called 'XA21 family member D' (designated XA21D), which lacks the transmembrane and kinase domains, is able to confer partial resistance to *Xoo* expressing Ax21 (Wang *et al*., 1998). *Xa21D* is 99% identical to the *Xa21 LRR* in nucleotide sequence and confers Ax21-specific resistance. Based on these results, we hypothesized that the XA21D and XA21 LRR domains bind directly to Ax21 (Wang *et al*., 1998). Due to the lack of both the transmembrane and kinase domains, the secreted XA21D was predicted form a heterodimer with an unidentified, endogenous receptor kinase (Wang *et al*., 1998). We hypothesized that, upon Ax21 binding to XA21D, the unidentified intracellular domain of the unidentified receptor kinase would be activated, partially transducing the defence response and leading to partial resistance phenotype (Wang *et al*., 1998).

In other words, the kinase activity of XA21 is at least partially dispensable for the innate immune response. Supporting this hypothesis, we subsequently demonstrated that a mutation in the conserved Lys736 residue (XA21K736E) in the XA21 kinase domain that is required for catalytic activity can still partially function in resistance, with levels of resistance similar to that observed for that of XA21D (Liu *et al*., 2002).

Despite this hypothesis, we have not yet identified an XA21 co-regulator. An important discovery in 2002 by two independent research groups identified such a co-regulator in *Arabidopsis*, called 'BAK1' (Li *et al*., 2002, Nam and Li, 2002). *Arabidopsis* FLS2 form heterodimers with BAK1, demonstrating that BAK1 serves as a co-regulator of *Arabidopsis* PRR-mediated immunity (Chinchilla *et al*., 2007; Heese *et al*., 2007). Further investigations demonstrated that BAK1 also functions with multiple PRRs including EFR (Chinchilla *et al*., 2007; Heese *et al*., 2007; Kemmerling *et al*., 2007; Shan *et al*., 2008). Taken together, the results of XA21D, XA21K736E and the studies of *Arabidopsis* BAK1 support the existence of a co-regulator functioning with XA21. Whether or not this hypothetical co-regulator can associate with the other predicted 328 non-RD receptor kinases in rice is an important question.

Activation of XA21 is regulated by the JM domain

It is now clear that the JM domain of receptor kinases can play an important role in regulating the function of kinase. For example, in animals, deletion of the JM domain of the ErbB-1 (epidermal growth factor receptor, an RD receptor kinase) results in a severe loss of tyrosine phosphorylation (Thiel and Carpenter, 2007). Two conserved tyrosine phosphorylation sites Tyr605 and Tyr611 of EphB2 (Eph receptor B2) are essential for EphB2 kinase autophosphorylation and biological responses (Binns *et al*., 2000; Zisch *et al*., 2000). Phosphorylation of the JM domain of the TβR-I (transforming growth factor β receptor, an RD receptor kinase) eliminates the binding site for the FKBP12 (12 kDa FK506-binding protein) inhibitor protein, leading to activation of the TβR-I kinase (Hubbard, 2001; Huse *et al*., 2001).

XA21/Ax21 binding is hypothesized to activate the non-RD kinase domain via JM domain regulation,

leading to XA21 autophosphorylation and/or transphosphorylation of downstream target proteins (Xu *et al*., 2006a; Wang *et al*., 2006). In support of this hypothesis several key residues have recently been shown to be critical for autophosphorylation or transphosphorylation. For example, autophosphorylation of the XA21 JM residues Ser686, Thr688 and Ser689 are important stabilizers of the XA21 protein (Xu *et al*., 2006a). Transgenic rice expressing XA21 mutants with either single or triple alanine-replacement mutant of these three sites display slightly compromised resistance compared with the wild-type XA21 (Xu *et al*., 2006a). In addition, yeast two-hybrid studies have been shown that Thr705 in the XA21 JM region is required for binding to XA21 binding protein (XBs) including XB3, XB10, XB15 and XB24 (Park *et al*., 2008; Chen *et al*., 2010a). More recently, we have shown that the XA21 JM residue Thr705 is essential for XA21 autophosphorylation and XA21-mediated immunity (Chen *et al*., 2010a). The replacement of Thr705 by an alanine or a glutamic acid abolishes XA21 autophosphorylation and eliminates the interactions between XA21 and XB3, XB10, XB15 and XB24 in yeast or rice. These results suggest that after being autophosphorylated, Thr705 may transfer its phosphoryl group to another XA21 residue, which would activate XA21. Although Thr residues analogous to Thr705 of XA21 are present in the JM domains of most RD and non-RD receptor kinases in plants, this residue is not required for autophosphorylation of the *Arabidopsis* RD receptor kinase BRI1 (Chen *et al*., 2010a, Wang and Chory, 2006). Additional research is needed to assess whether Thr705 autophosporylation is critical for function of other non-RD receptor kinases.

XA21-mediated signalling components: XB3, XA21 binding protein 3, a RING finger ubiquitin ligase

In animals, TLR1, TLR2, TLR4 and TLR6 signalling proceeds through adaptor molecule myeloid differentiation factor 88 (MyD88) (Brikos and O'Neill, 2008). MyD88 associates with TLRs to recruit the non-RD serine/threonine kinase, IRAK1. IRAK1 associates with tumour necrosis factor receptor associated factor 6 (TRAF6), a RING (really interesting new gene) finger ubiquitin ligase (Muzio *et al*., 2000). TRAF6 autoubiquitinates and activates downstream mitogen-activated protein kinase (MAPK) cascades, which mediate downstream events, such as degradation of inhibitor of nuclear factor κB (IκB) and release of nuclear factor κB (Suzuki *et al*., 2002; Bochud *et al*., 2007).

Similarly, *in vitro* assays have shown that the XA21 kinase transphosphorylates the RING finger ubiquitin ligase XB3 and that XB3 is autoubiquitinated *in vitro*. XB3 is required for effective XA21-mediated resistance (Wang *et al*., 2006). Given the functional and structural parallels between XB3 and TRAF6, it is tempting to speculate that XB3 also activates a MAPK cascade. In support of MAPK-mediated signaling in plant innate immunity, two *Arabidopsis* MAPK cascades have been demonstrated to function downstream of the flagellin receptor FLS2. MEKK1 is not required for flagellin-triggered activation of MPK3 and MPK6 but is essential for activation of MPK4 (Asai *et al*., 2002; Ichimura *et al*., 2006). EFR-mediated immunity also induces a rapid activation of MAPKs (Zipfel *et al*., 2006). A direct role for a MAPK cascade in Xa21-mediated immunity has not yet been demonstrated.

XB10, a WRKY transcriptional factor

In animals, one of key mechanisms of PRR-triggered innate immunity is the activation of defence-related genes, as mediated by transcription factors (Arancibia *et al*., 2007). For example, PAMP-triggered TLRs leads to the activation of transcription factor NF-κB and the expression of immune response genes (Wan and Lenardo, 2010, Arancibia *et al*., 2007). In plants, which lack NR-κB orthologues, studies have shown that instead WRKY transcription factors are the key regulators (Eulgem, 2005). For example, in *Arabidopsis*, WRKY22 and WRKY29 function downstream of the FLS2-mediated immune response. Overexpression of the *AtWRKY29* constitutively activates the plant defence response against bacterial invasion (Asai *et al*., 2002). Also in *Arabidopsis*, loss of WRKY70 function compromises both basal defence responses to bacterial and fungal pathogens and RPP4 (recognition of *Peronospora parasitica* 4)-mediated race-specific resistance to *Hyaloperonospora parasitica* (Li *et al*., 2004; 2006; Knoth *et al*., 2007). In barley, overexpression of either *HvWRKY1* or *HvWRKY2* compromises both the basal defence response and MLA10-mediated race-specific resistance to *Blumeria graminis* (Shen *et al*., 2007).

In rice, OsWRKY62 (XB10) regulates XA21-mediated immune response (Peng *et al*., 2008), indicating another level of conservation between the *Arabidopsis* and rice PRR signalling pathways. Transgenic rice plants overexpressing *OsWRKY62* are compromised in XA21-mediated immunity to *Xoo*, suppressing the activation of defence-related genes including *OsPR1* and *OsPR10* (Peng *et al*., 2008). These results indicate that OsWRKY62 can function as a negative regulator of innate immunity.

OsWRKY28, OsWRKY71 and OsWRKY76, together with OsWRKY62, comprise the rice WRKY IIa subfamily (Peng *et al*., 2010). Transgenic lines overexpressing all four genes showed resistance against *Xoo*, displaying activation of *OsPR10* expression. These results indicate a

functional interaction between WRKY IIa members in regulating plant innate immunity (Peng *et al*., 2010). WRKY IIa proteins contain putative leucine zipper motifs at the N-terminus, suggesting potential dimerizations between proteins. It has been shown that leucine zipper motifs are critical for the physical interaction of WRKY IIa protein in *Arabidopsis* (Xu *et al*., 2006b). Therefore, it may be that different combinatorial dimers formed by WRKY IIa proteins may exhibit different functions in regulating target gene expression (Peng *et al*., 2010). Although this study suggests a functional link between OsWRKYs and XA21 in XA21-mediated immunity to *Xoo*, the physical location of the *in vivo* interaction remains to be elucidated.

XB15, a protein phosphatase 2C

Although PRR-mediated immune responses are clearly essential for innate immunity in both plants and animals, sustained or highly induced immune response can be harmful (Lang and Mansell, 2007). It is therefore necessary that PRR signalling through non-RD kinases be under tight negative regulation.

In contrast to animals, where negative regulators have been shown to act at multiple levels within TLR signalling cascades, negative regulation of plant innate immunity is not well understood. One important class of negative regulators are protein phosphatase 2Cs (PP2Cs), a group of serine/threonine phosphatases (Schweighofer *et al*., 2004). *Arabidopsis* PP2C, kinase-associated PP (KAPP), interacts with many receptor kinases, including CLAVATA1 (CLV1), somatic embryogenesis receptor kinase 1, BRI1, BAK1 and FLS2 (Braun *et al*., 1997; Stone *et al*., 1998; Gomez-Gomez *et al*., 2001; Shah *et al*., 2002; Ding *et al*., 2007). Overexpression of KAPP in *Arabidopsis* results in loss of sensitivity to flagellin treatment, suggesting that KAPP negatively regulates the FLS2-mediated immune response (Gomez-Gomez *et al*., 2001). Although the rice KAPP protein emerged as a good candidate for being a negative regulator of the XA21-mediated innate immune response, it does not interact with XA21 (van der Knaap *et al*., 1999). Instead, another PP2C (XB15) was isolated from yeast two-hybrid screen using the intracellular portion of XA21 as bait (Park *et al*., 2008). Additional *in vitro* biochemical experiments showed that XB15 can effectively dephosphorylate XA21 in a temporal- and dosage-dependent manner. *Xb15* mutant and *Xb15* RNAi lines display spontaneous cell death in the absence of obvious stress and disease with constitutive expression of defence-related *OsPR* genes (Park *et al*., 2008). Overexpression of the *Xb15* in an XA21 rice line compromised resistance to the *Xoo*, demonstrating that XB15 negatively regulates the XA21-mediated innate immune response (Park *et al*., 2008).

XB24, a novel ATPase

Recently, we showed that XB24, a previously uncharacterized ATPase, interacts with XA21 and regulates XA21-mediated immunity (Chen *et al*., 2010b). XA24 has no significant annotated motifs except for a C-terminal ATP synthase alpha- and beta-subunits signature (ATPase) motif with sequence PSINERESSS. None of plants and human proteins containing a conserved ATPase motif shares similarity beyond the ATPase motif with XB24. XB24 displays significant ATP hydrolysis activity, while XB24 mutant containing a single amino acid change Ser154 with Ala had only negligible ATPase activity, indicating that the XB24 protein possesses an ATPase activity and that amino acid Ser154 is essential for its ATPase activity (Chen *et al*., 2010b). XB24 promotes autophosphorylation of the XA21 protein *in vitro*. XB24 is not transphosphorylated by the XA21 protein in the absence or presence of *Xoo* expressing Ax21 (Chen *et al*., 2010b). Autophosphorylation of XA21 is enhanced in the presence of rice-expressed XB24 but not in the XB24 mutant, demonstrating that XB24 enhances XA21 autophosphorylation and that its ATPase activity is required for this function. *In planta* silencing of *Xb24* expression enhances XA21-mediated disease resistance (Chen *et al*., 2010b).

Based on these results, we propose that XB24 physically associates with XA21 and promotes autophosphorylation of certain Ser/Thr sites on XA21, keeping the XA21 protein in an inactive state (Chen *et al*., 2010b). Upon recognition of Ax21, the XA21 kinase becomes activated, triggering downstream defence responses. The mechanism(s) for XA21 activation following perception of Ax21 likely requires dissociation of XA21 from XB24 and/or removal of the XB24-promoted phosphorylation. Together with our previously studies that the association between XB24 and XA21 is compromised while the association between XB15 and XA21 is enhanced upon Ax21 triggering (Park *et al*., 2008; Chen *et al*., 2010b), our model suggests that the regulation by XB24 occurs before Ax21 recognition while regulation by XB15 occurs after Ax21 recognition.

Another regulation in the endoplasmic reticulum: quality control of XA21 BiP-heat shock protein 70

In animals, extracellular PRRs are translated on the endoplasmic reticulum (ER) membrane, enter the ER lumen and undergo glycosylation (Ruddock and Molinari, 2006; Akashi-Takamura and Miyake, 2008). For further protein processing, before being translocated to the PM, newly synthesized PRRs interact with different ER chaperones that will assist them to fold properly and to avoid aggregation in a process called ER quality control (ER QC) (Meusser *et al*., 2005; Ruddock and Molinari, 2006). Therefore, most TLRs interact with at least one

ER-resident chaperone for protein folding and trafficking. For example, ER chaperone protein, gp96, is required for functional expression of both intracellular and cell surface TLRs, including TLR2, TLR4, TLR5, TLR7 and TLR9 (Yang *et al*., 2007). In addition to ER chaperones, N-glycosylation, which is essential for the function of TLRs (Leifer *et al*., 2004), is also known to be important for correct protein folding and ER QC (Kleizen and Braakman, 2004, Meusser *et al*., 2005).

In *Arabidopsis*, components in the ER QC, calreticulin3 (CRT3) and UDP-glucose:glycoprotein glycosyltransferase (UGGT), are required for EFR function, as loss of either CRT3 or UGGT leads to complete loss of EFR accumulation (Li *et al*., 2009; Saijo *et al*., 2009). In addition, an ER protein complex compromising stromal-derived factor-2 (SDF2), heat shock protein 70 (HSP70) BiP and co-chaperone HSP40 ERdj3B is indispensible for proper biogenesis of EFR, demonstrating a physiological involvement of ER QC and PRR function in plant (Nekrasov *et al*., 2009).

The involvement of ER QC and ER-associated degradation (ERAD) in XA21-mediated immunity was demonstrated through isolation of an *in vivo* XA21 protein complex (Park *et al*., 2010). An approximately 75 kDa protein co-immunoprecipiated with XA21 was identified as OsBiP3 through LC-MS/MS sequencing. Overexpression of BiP3 compromised XA21-mediated immunity. Transgenic lines overexpressing *OsBiP3* displayed significantly decreased XA21 accumulation and inhibited a protein processing of XA21, suggesting that continuous and/or prolonged binding of overexpressed OsBiP3 results in XA21 degradation possibly via ERAD. This result also suggests that accumulation of BiPs is able to attenuate a receptor-mediated signal transduction pathway causing an ER stress by targeting the receptor to the ERAD. Supporting this hypothesis, BiP has been known to target permanently misfolded proteins for ERAD in mammals and yeast when prolonged ER stress induce excessive loading of unfolded and/or misfolded proteins (Kleizen and Braakman, 2004).

To investigate if BiP3 overexpression affects signalling pathways mediated by other receptor kinases, we investigated OsBRI1-mediated responses to brassinolide. Although OsBRI1 shows an overall structural similarity with XA21 (He *et al*., 2000), unlike XA21 it falls into the RD class of kinases. We found that BiP3 overexpressing lines maintain sensitivity to brassinolide, indicating that BiP3 overexpression does not interfere with OsBRI1-mediated signalling. Taken together, these results indicate that altered BiP3 expression does not affect all RK-mediated signalling pathways and does not affect a general ER stress response.

Similar to *Arabidopsis* SDF2, OsSDF2 is involved in XA21-mediated immunity. XA21 transgenic lines silenced for *OsSDF2* displayed severe disease symptoms after *Xoo* inoculation, indicating that OsSDF2 is involved in XA21 biogenesis (C-J. Park, unpubl. data). It has been also shown that both XA21 and EFR are highly glycosylated, which may occur in the ER during maturation (Nekrasov *et al*., 2009; Park *et al*., 2010). Therefore, the conserved requirements for the ER proteins, BiP and SDF2, for both XA21 and EFR biogenesis provide strong evidence that ER QC is involved in plant innate immunity, playing a role in PRR trafficking to the PM.

Perspectives

Recognition of PAMPs by PRRs is critical to both plant and animal survival. We have recently shown that Ax21 is a sulfated peptide that binds the rice PRR, XA21 (Lee *et al*., 2009). The high level of conservation of Ax21 in *Xanthomonas*, in *Xylella* and in the human pathogen *Stenotrophomonas* suggests a critical role for this protein in the biology of these pathogens. Preliminary studies suggest that Ax21 may function in quorum-sensing, a process where bacterial molecules can serve as signals to recognize bacterial population size, leading to changes in expression of specific genes (Bassler and Losick, 2006, Lee *et al*., 2006). Currently, we are further investigating such a function for Ax21.

Although PRR activation processes are believed to cause rapid phosphorylation of many proteins through mostly unknown regulatory networks, no direct targets of PRRs have yet been reported (Gomez-Gomez and Boller, 2002; Boller and Felix, 2009). It is also unknown how PRR phosphorylation can activate ion channels and the NADPH oxidase complex. To answer these fundamental questions and further understand XA21-mediated immune response, identification of additional components in the XA21 complex is essential. One approach is to use phosphoproteomic analysis and quantitative LC-MS/MS phosphopeptide comparisons to identify proteins differentially phosphorylated after Ax21 treatment. Proteins that show unique phosphorylation patterns would be good candidates for XA21 direct target(s).

Because PAMPs are essential for survival or pathogenicity, they cannot be easily mutated without compromising microbial fitness (Gomez-Gomez *et al*., 1999; Kunze *et al*., 2004). Thus, approaches directed at harnessing PRR-mediated immunity will be a useful strategy for enhancing resistance in agricultural crops. For example, rice varieties carrying *Xa21* carry robust resistance to diverse strains of *Xoo* (Wang *et al*., 1996).

The non-RD domain, a newly recognized hallmark of receptor kinases that function as PRRs, is highly expanded in rice compared with *Arabidopsis* (35 in *Arabidopsis* and 328 in rice). For example, the LRR XII subfamily, which includes FLS2, EFR and XA21, contains

over 100 members in rice but only eight in *Arabidopsis* (Dardick and Ronald, 2006). In addition, there are several non-RD receptor kinase subfamilies that are specific to rice and that are lacking in *Arabidopsis*. Thus, although it appears that all *Arabidopsis* subfamilies have a rice counterpart, the converse is not true. Rice carries over 70 members of the WAKa, WAKc and WAKL families; none is present in *Arabidopsis* (Dardick and Ronald, 2006). The large numbers of non-RD receptor kinases in rice suggest that there are probably equally large numbers of extracellular pathogen-derived ligands yet to be discovered. To determine if the observed rice/*Arabidopsis* difference in the number of non-RD receptor kinases is similar between other monocotyledonous and dicotyledonous species, a comprehensive analysis of newly released plant genome sequences, including *Medicago*, Maize, Wheat, *Brassica*, *Sorghum*, *Brachypodium*, is needed (Paterson *et al*., 2009; IBI, 2010).

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