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Receptor-like Cytoplasmic Kinases Integrate Signaling from Multiple Plant Immune Receptors and Are Targeted by a *Pseudomonas syringae* Effector

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SUMMARY

Cell-surface-localized plant immune receptors, such as FLS2, detect pathogen-associated molecular patterns (PAMPs) and initiate PAMP-triggered immunity (PTI) through poorly understood signal-transduction pathways. The pathogenic *Pseudomonas syringae* effector AvrPphB, a cysteine protease, cleaves the *Arabidopsis* receptor-like cytoplasmic kinase PBS1 to trigger cytoplasmic immune receptor RPS5-specified effector-triggered immunity (ETI). Analyzing the function of AvrPphB in plants lacking *RPS5*, we find that AvrPphB can inhibit PTI by cleaving additional PBS1-like (PBL) kinases, including BIK1, PBL1, and PBL2. In unstimulated plants, BIK1 and PBL1 interact with FLS2 and are rapidly phosphorylated upon FLS2 activation by its ligand flg22. Genetic and molecular analyses indicate that BIK1, and possibly PBL1, PBL2, and PBS1, integrate immune signaling from multiple immune receptors. Whereas AvrPphB-mediated degradation of one of these kinases, PBS1, is monitored by RPS5 to initiate ETI, this pathogenic effector targets other PBL kinases for PTI inhibition.

INTRODUCTION

Plants use a suite of cell-surface-localized pattern-recognition receptors to detect various pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) and trigger immune responses (Schwessinger and Zipfel, 2008). PAMP-triggered immunity (PTI) is critical for the survival of land plants under constant threat from numerous potential pathogenic microbes. The signal-transduction mechanism underlying PTI, however, is not well understood. The best-studied PTI pathway is initiated by the receptor kinase FLS2. Upon binding to the bacterial flagellar peptide flg22 (Chinchilla et al., 2006), FLS2 rapidly associates with another receptor-like kinase, BAK1, to activate defenses (Chinchilla et al., 2007; Heese et al., 2007). Similarly, the receptor kinase EFR binds the bacterial elongation factor-Tu (EF-Tu) peptide elf18 to trigger immune responses (Zipfel et al., 2006). Another receptor-like kinase, CERK1, is required for defenses in response to chitin, a fungal cell-wall component. CERK1 possesses three LysM domains that are thought to bind chitin (Miya et al., 2007; Wan et al., 2008). CERK1 is also required for plant resistance to the bacterial pathogen *Pseudomonas syringae* (Gimenez-Ibanez et al., 2009), although the corresponding PAMP remains to be identified. Downstream, two MAP kinase cascades are activated. MEKK1, MKK1, MKK2, and MPK4 constitute a cascade negatively regulating PTI defenses (Ichimura et al., 2006; Qiu et al., 2008; Suarez-Rodriguez et al., 2007), whereas MPK3 and MPK6 are thought to positively regulate PTI defenses (Schwessinger and Zipfel, 2008). We have no knowledge of additional components that act in early phases of the signal transduction. Furthermore, it is not known how signals from distinct immune receptors are integrated to activate an overlapping set of downstream defense responses.

P. syringae secretes a large number of effector proteins into host cells to assist its proliferation in plants (Cunnac et al., 2009). Many of these effector proteins are capable of targeting components of the PTI signaling pathway to suppress plant immunity (Block et al., 2008; Fu et al., 2007; Gimenez-Ibanez et al., 2009; Göhre et al., 2008; Kim et al., 2005; Li et al., 2005; Nomura et al., 2006; Xiang et al., 2008; Zhang et al., 2007; Zhou and Chai, 2008). For example, the *P. syringae* effector AvrPto acts as a kinase inhibitor to directly block immune signaling from FLS2 and EFR (Xiang et al., 2008). Another *P. syringae* effector, AvrPtoB, structurally and functionally mimics E3 ubiquitin ligase (Janjusevic et al., 2006; Abramovitch et al., 2006) and inhibits PTI by targeting FLS2 (Göhre et al., 2008) and CERK1 (Gimenez-Ibanez et al., 2009) for degradation. Shan et al. (2008) suggested that both AvrPto and AvrPtoB target BAK1. In addition, the *P. syringae* effector HopAI1 uses phosphothreonine lyase activity to "dephosphorylate" *Arabidopsis* MPK3 and MPK6, thereby permanently inactivating the MAP kinases (Zhang et al., 2007). The fact that many of the *P. syringae* effectors target important signaling components to inhibit PTI suggests that they can be used as powerful molecular probes

to identify PTI signaling components. Indeed, analyses of host targets for the *P. syringae* effectors HopU1 and HopM1 have led to the identification of GRP7 and MIN7 as mediators of plant immunity (Fu et al., 2007; Nomura et al., 2006).

Some pathogen effectors trigger immunity mediated by cytoplasmic immune receptors that are primarily nucleotide-binding, leucine-rich repeat (NB-LRR) proteins. The recognition of effectors by NB-LRR proteins is often indirectly mediated by other host proteins of diverse biochemical functions (Jones and Dangl, 2006). For example, the recognition of AvrPto by the NB-LRR protein Prf in tomato plants is mediated by the protein kinase Pto (Mucyn et al., 2006; Tang et al., 1996). Likewise, the *P. syringae* effector AvrPphB, a cysteine protease, triggers RPS5-specified disease resistance by proteolytically cleaving the cytoplasmic receptor-like kinase PBS1 (Ade et al., 2007; Shao et al., 2003). Thus, the Prf-Pto and RPS5-PBS1 protein complexes act as a conformational switch that is activated only when the corresponding effector proteins are present. However, it is not understood why different host proteins are deployed as sensors for effector-triggered immunity (ETI).

Here we show that AvrPphB inhibits PTI when directly expressed in plants. Interestingly, AvrPphB is capable of proteolytically cleaving a number of PBS1-like (PBL) proteins belonging to the subfamily VII of cytoplasmic receptor-like protein kinases. One of the PBL proteins, BIK1, is required for signaling elicited by flg22, elf18, and chitin and is essential for PAMP-induced resistance to *P. syringae*. Other members including PBL1, PBL2, and PBS1 also contribute to PTI defenses. BIK1 interacts with FLS2, EFR, and CERK1 in unstimulated plants. Treatment of plants with flg22 induces BIK1 phosphorylation in an FLS2- and BAK1-dependent manner. These results indicate that these kinases, particularly BIK1, play a central role in signal integration from multiple surface-localized receptors.

RESULTS

Transgenic AvrPphB Inhibits PTI Signaling

We used a protoplast-based reporter assay (Li et al., 2005; Xiang et al., 2008) to determine whether flg22-induced expression of *FRK1*, a PTI marker gene, can be inhibited by the expression of the *AvrPphB* transgene. Flg22 induces the expression of *FRK1* promoter-firefly luciferase reporter gene (*FRK1::LUC*; Figure 1A). The presence of AvrPphB reduced *FRK1::LUC* expression by 80%, but the protease-compromised AvrPphB^{C98S} mutant (cysteine 98 to serine; Shao et al., 2003) was largely unable to inhibit *FRK1::LUC* expression (Figure 1A; see Figure S1A available online). To further determine whether AvrPphB possesses PTI-inhibitory activity, we introduced a FLAG-tagged *AvrPphB* transgene into the *Arabidopsis rps5-2* mutant. *Arabidopsis* plants exposed to various PAMPs deposit callose at the cell wall and develop a rapid oxidative burst exemplified by the accumulation of H_2O_2 (Felix et al., 1999; Gómez-Gómez et al., 1999; Kunze et al., 2004; Miya et al., 2007). The flg22-induced H_2O_2 accumulation in T1 *AvrPphB* transgenic plants was reduced to 25% compared with the nontransgenic *rps5* control (Figure 1B). Two independent transgenic lines in the T3 generation were further tested for oxidative burst and callose deposition in response to flg22, elf18, and chitin. H_2O_2 accumulation in the two *AvrPphB* transgenic lines was consistently reduced to 20%–40% compared with nontransgenic *rps5* control (Figures 1C–1E). Similarly, PAMP-induced callose deposition in *AvrPphB* transgenic plants was also reduced to 40%–50% compared with control plants (Figure 1F). Together, these results indicated that AvrPphB is capable of inhibiting signaling from all three PAMPs.

PBS1-like Kinases Are AvrPphB Substrates

The cleavage of PBS1 does not appear to account for the PTI-inhibition activity of AvrPphB, because our initial analysis of *pbs1* mutants showed only minimal defects in PTI defenses. As expected, an immunoblot experiment did not detect a cleavage or reduction in abundance of FLS2 in *AvrPphB* transgenic plants (Figure S1B). We therefore hypothesized that AvrPphB may target additional host proteins homologous to PBS1 for PTI inhibition. PBS1 belongs to receptor-like cytoplasmic kinase (RLCK) subfamily VII. The amino acid sequences of the 45 RLCK VII members were therefore aligned and analyzed for the AvrPphB recognition sequence (Figure S2A). In total, we identified 29 putative PBL proteins as potential substrates for AvrPphB. One of the PBL proteins is BIK1, an RLCK required for resistance to *Botrytis cinerea* (Veronese et al., 2006). The remaining PBL proteins are named PBL1–PBL28. The putative AvrPphB substrates were fused to an HA tag and transiently expressed in *Arabidopsis rps5* mutant protoplasts along with AvrPphB. The coexpression of AvrPphB resulted in cleavage of nine of the ten selected proteins (Figure 2A). PBL6, which contains a D-to-S substitution in the GDK motif, was not cleaved. PBL1, which contains a D-to-E substitution in the GDK motif, was cleaved normally, suggesting that an acidic residue in the GDK motif is required for the cleavage. These results are consistent with a previous report that a D-to-A substitution in the GDK motif of PBS1 reduces AvrPphB cleavage by 75% (Shao et al., 2003). BSK1, an RLCK XII family member involved in brassinosteroid signaling (Tang et al., 2008), was not cleaved by AvrPphB, indicating that AvrPphB specifically targets PBS1 and PBL proteins. The protease-compromised AvrPphB mutant did not cleave BIK1 (Figure S2B). To determine whether the AvrPphB protein delivered from the *P. syringae* bacteria is capable of cleaving BIK1, we generated transgenic lines expressing HA-tagged BIK1 under the control of the *BIK1* native promoter in the *rps5* mutant background. Inoculation of *BIK1::BIK1-HA* plants with *P. syringae* carrying *avrPphB* produced a cleaved product, whereas the plants inoculated with the *P. syringae* strain lacking *avrPphB* did not (Figure 2B).

An examination of public microarray data indicated that *BIK1*, *PBL1*, and *PBL2* transcripts are strongly upregulated by flg22. Quantitative RT-PCR confirmed this result with flg22 treatment inducing *BIK1*, *PBL1*, and *PBL2* transcripts by 2.5- to 5.5-fold compared with an H_2O-treated control (Figure S3A). A small but statistically significant induction of *PBS1* by flg22 was also observed. These results suggest that the PBL genes are linked to PTI defenses.

Flg22 Induces BIK1 Phosphorylation

An examination of the protoplast-expressed BIK1 and the PBL1 proteins showed a slower migration following flg22 treatment (Figure 3A). Treatment of the protein samples with a protein