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Phosphorylation is required for the pathogen defense function of the Arabidopsis PEN3 ABC transporter

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ABSTRACT

The Arabidopsis PEN3 ABC transporter accumulates at sites of pathogen detection, where it is involved in defense against a number of pathogens. Perception of PAMPs by pattern recognition receptors initiates recruitment of PEN3 and also leads to PEN3 phosphorylation at multiple amino acid residues. Whether PAMP-induced phosphorylation of PEN3 is important for its defense function or focal recruitment has not been addressed. In this study, we evaluated the role of PEN3 phosphorylation in modulating the localization and defense function of the transporter. We report that PEN3 phosphorylation is critical for its function in defense, but dispensable for recruitment to powdery mildew penetration sites. These results indicate that PAMP-induced phosphorylation is likely to regulate the transport activity of PEN3.

The plant antimicrobial immune system is activated upon detection of potentially pathogenic microorganisms mediated primarily by two major classes of immune receptors, pattern recognition receptors (PRRs) that perceive conserved elicitor molecules characteristic of microorganisms, termed pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs), or nucleotide-binding leucine-rich repeat (NB-LRR) type resistance (R) proteins that detect pathogen virulence factors or their activities.[7,11,13,14] Stimulation of PRRs by PAMP perception results in the activation of a number of characteristic defense outputs, including local reinforcement of the cell wall through construction of papillae, a process that involves deposition of callose, accumulation of antimicrobial metabolites, and recruitment of defense-associated proteins.[21-23] The Arabidopsis PENE-TRATION3 (PEN3; synonyms ABCG36 and PDR8) ATP-binding cassette (ABC) transporter is required for full resistance to cell wall penetration and subsequent haustorium formation by the non-host barley powdery mildew pathogen *Blumeria graminis* f. sp. *hordei* (*Bgh*) and contributes to resistance to numerous other fungal and oomycete pathogens.[8,9,11,12,18] PEN3 is recruited to sites of papilla deposition upon perception of PAMPs and is required for full pattern-triggered immunity against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000, indicating that the pathway involving the PEN3 transporter participates in the pattern-triggered immune response.[22,24] In accordance with these findings, phosphoproteomics studies identified amino acid residues of the PEN3 transporter that were differentially phosphorylated in response to perception of the PAMPs flg22, an elicitor active peptide derived from

bacterial flagellin, oligo-galacturonide (OG) fragments derived from the cell wall polymer pectin, or fungal xylanase.[4,10,15] However, whether PAMP-induced phosphorylation of PEN3 is required for the defense function of the transporter has not been addressed. In this study, we generated mutant PEN3 variants at proteomically supported phosphorylation sites and evaluated their ability to restore penetration defense in the *pen3* mutant and to be recruited to powdery mildew penetration sites.

Several phosphoproteomic studies of Arabidopsis PM proteins have identified phosphorylation sites in the PEN3 transporter.[4,10,15,16] An evaluation of PM phosphoproteins enriched from a mixture of control- and flg22-treated suspension culture cells unambiguously identified phosphorylation events at serine residues S^{37}, S^{40}, S^{45}, S^{841}, and S^{844} as well as threonine residue T^{43}.[14] Additionally, serine residue S^{38} was identified as a putative phosphorylated residue that could not be unambiguously identified. Subsequent quantitative studies revealed that serine residues S^{40} and S^{45} are differentially phosphorylated in response to perception of flg22 or fungal xylanase and identified an additional serine residue, S^{825}, that is marginally differentially phosphorylated (significant at $P = 0.1$) in response to flg22 treatment.[4,15] Additionally, serines S^{37} and S^{38} could not be unambiguously ruled out as potential sites of differential phosphorylation in response to flg22.[15] Similarly, quantitative analysis of phosphorylation events in response to perception of OGs by Arabidopsis seedlings identified differential PEN3 phosphorylation at a single ambiguous residue, either serine S^{37} or S^{40}.[10]

We hypothesized that PAMP-induced phosphorylation of PEN3 may contribute to the activation of the PEN3 defense function or to its recruitment to sites of interaction with

invading pathogens. To evaluate the potential roles of specific PEN3 phosphorylated residues, we used site-directed mutagenesis to generate a series of alanine substitution mutants of an existing PEN3 promoter-PEN3-GFP construct,[18] replacing each definitively or putatively phosphorylated residue with alanine to mimic the de-phosphorylated state. Additionally, we created three polymutants. PEN3 phosphorylation sites are not evenly distributed throughout the protein, but fall into two distinct clusters; an N-terminal cluster consisting of serine residues S^{37}, S^{38}, S^{40}, and S^{45} as well as threonine T^{43}, and a central cluster consisting of serine residues S^{825}, S^{841}, and S^{844} (Fig. 1A). We created polymutants containing alanine substitutions at each residue in the two distinct clusters. We refer to these polymutants as "Cluster 1" (5 N-terminal residues) and "Cluster 2" (3 central serines). We also created a double mutant containing alanine substitutions at the PAMP-responsive differentially phosphorylated residues, S^{40} and S^{45}. We transformed each mutant construct into the *pen3-3* mutant (SALK_110926;[1,18]) and assessed the ability of each mutant PEN3 variant to restore penetration resistance in response to *Bgh* at 48 hours post-inoculation (hpi) by creating transgenic plants and evaluating fungal penetration success as previously described.[22] The PEN3 variants S^{37}A, S^{38}A, T^{43}A, S^{825}A, and S^{844}A all were able to restore full penetration resistance against *Bgh* to the *pen3-3* mutant (Fig. 1B), indicating that phosphorylation at these residues is not required for the defense function of PEN3. The PEN3 S^{40}A and S^{45}A variants failed to restore penetration resistance (Fig. 1B), suggesting that

phosphorylation at these residues is important for the defense activity of the transporter. Consistent with a requirement for phosphorylation at serines S^{40} and S^{45}, the S^{40}A/S^{45}A double mutant and Cluster 1 polymutant also failed to restore penetration resistance (Fig. 1B). We were unable to recover any S^{841}A or Cluster 2 polymutant transformants exhibiting detectable levels of GFP fluorescence, suggesting that phosphorylation at serine S^{841} may contribute to protein stability. All other alanine substitution PEN3 variants exhibited GFP fluorescence and localization to the PM that was indistinguishable from wild-type PEN3-GFP (Fig. 2A).

PAMP-induced phosphorylation of PEN3 at serine residues S^{40} and S^{45} could potentially modulate the defense function of the transporter by activating or altering the transport activity of PEN3 or, alternatively, by affecting recruitment of PEN3 to sites of attempted *Bgh* penetration. To determine if disruption of PAMP-induced phosphorylation in our alanine substitution variants altered the ability of PEN3 to accumulate at sites of fungal penetration, we monitored the localization of each GFP-tagged PEN3 variant 24 h after inoculation with *Bgh* using confocal microscopy as previously described.[22] We found that all of the detectable PEN3 alanine substitution variants retained the ability to accumulate at sites of attempted *Bgh* penetration (Fig. 2B). These results suggest that PAMP-induced phosphorylation likely contributes to the activation or alteration of PEN3 transport activity and is not required for recruitment of PEN3 to sites of attempted fungal penetration.

Phosphorylation of ABC transporters by protein kinases is well known to play a role in regulation of both transport activity and protein stability and turnover.[2,19] Here, we identified two PEN3 phosphorylation sites, serines S^{40} and S^{45}, required for the defense function of the transporter that are likely involved in regulating transport activity. In many instances where phosphorylation has been observed to regulate ABC transporter activity, relevant phosphorylation sites have been located within a regulatory (R) or R-like domain present in the cytosolic loop between the first nucleotide binding domain and the second transmembrane domain of the protein.[19] Thus, our finding of relevant regulatory phosphorylation sites N-terminal to the first nucleotide binding domain within PEN3 contrasts with other previous observations that are primarily from humans or yeast. Nonetheless, the PAMP-responsive PEN3 phosphorylation sites are near the first nucleotide binding domain, which is consistent with numerous reports of regulatory phosphorylation events at sites within or adjacent to nucleotide binding domains of other ABC transporters.[19] Additionally, a third PEN3 phosphorylation site, serine S^{841}, appears to affect protein stability. A synthetic peptide encompassing serines S^{37}, S^{38}, S^{40}, S^{45}, and threonine T^{43} was identified as an *in vitro* substrate of the Arabidopsis calcium-dependent protein kinase CPK10.[6] Further work will be required to determine if CPK10 plays a role in the regulation of PEN3 activity upon PAMP perception.

PEN3 is thought to contribute to antimicrobial defense by potentially transporting one or more molecules derived from metabolism of indole glucosinolates,[3,5] however, the specific defense-relevant molecule(s) transported by PEN3 remain unknown. Additionally, PEN3 affects sensitivity of Arabidopsis to the auxin analog 2,4-DB and has been proposed to act
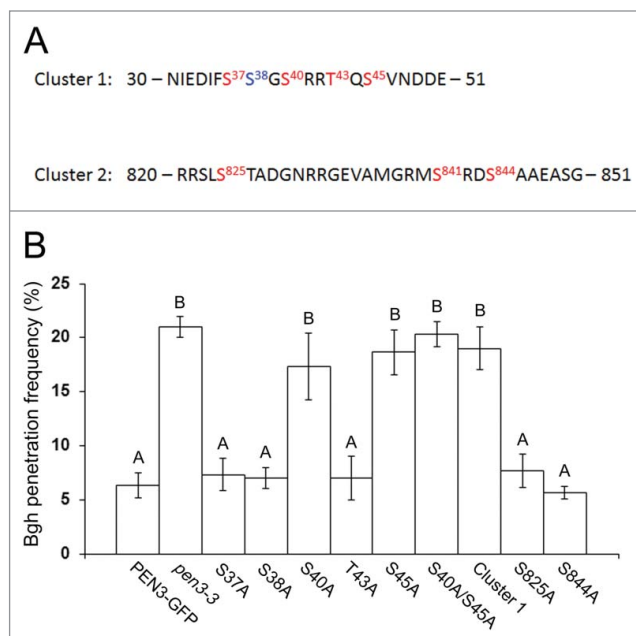

Figure 1. Complementation of *pen3-3* by PEN3 phosphorylation site mutants. (A) PEN3 amino acid sequences in the regions encompassing phosphorylation sites at Cluster 1 (upper panel) and Cluster 2 (lower panel). Proteomically supported phosphorylation sites are colored red. Putative sites that lack unambiguous support are colored blue. (B) *Bgh* penetration frequencies on the wild-type PEN3-GFP line, *pen3-3*, and plants expressing phosphorylation site alanine substitution PEN3-GFP variants are expressed as means of the percentages of haustoria observed relative to total penetration attempts. Penetration frequencies were determined by scoring for presence or absence of haustoria at 100 infection sites per leaf for 3 leaves per plant line 48 hpi with *Bgh*. Error bars represent SD (n = 3). Means indicated by the same letter are not significantly different according to Tukey's post-hoc test (P < 0.01). The experiment was repeated 3 times with similar results.
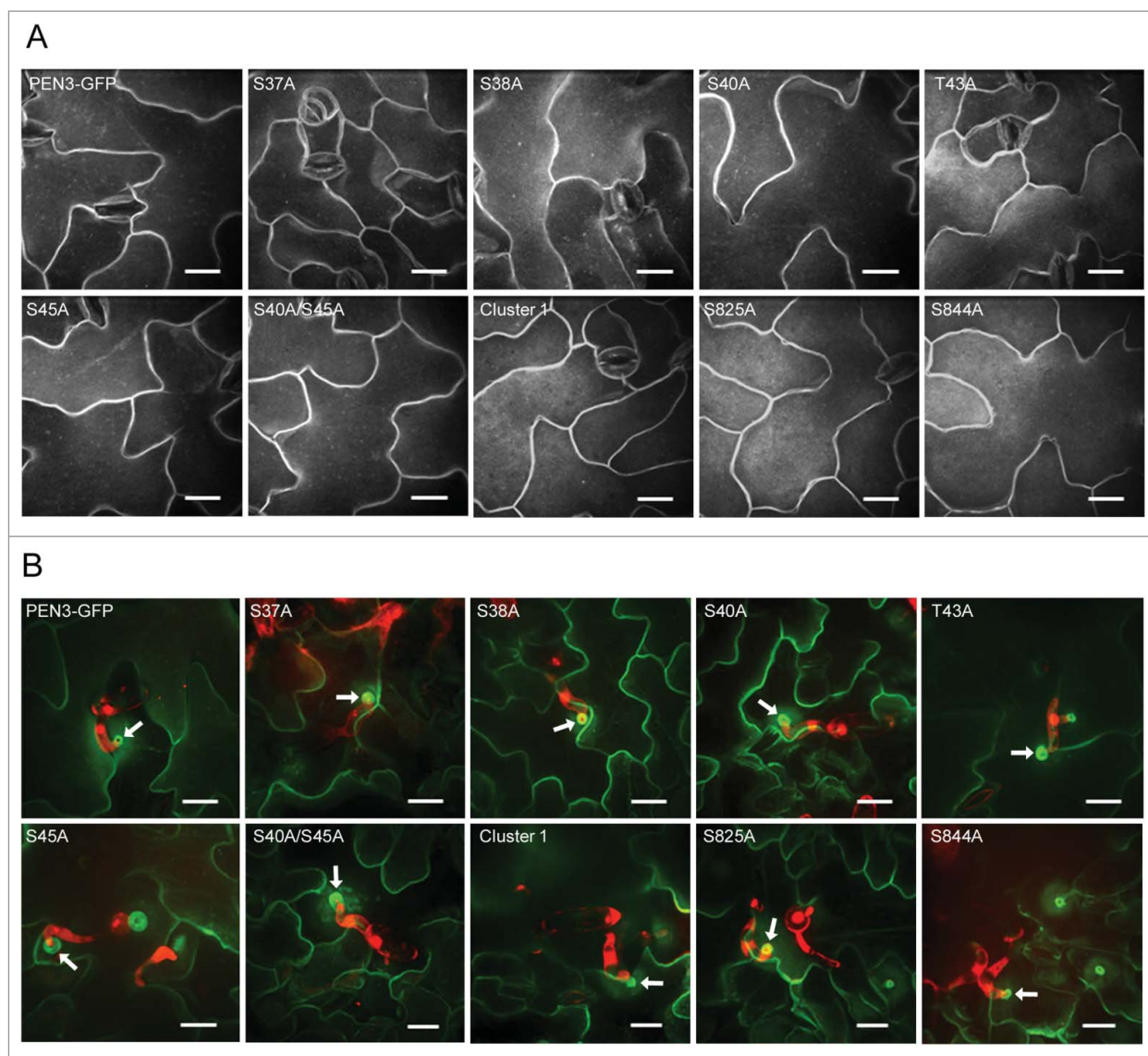
Figure 2. Localization of phosphorylation site alanine substitution variants of PEN3-GFP. (A) Confocal micrographs illustrating the expression and steady-state localization of PEN3-GFP alanine substitution variants in leaf epidermal cells. All variants exhibit PM localization similar to wild-type PEN3-GFP. (B) Confocal micrographs illustrating the localization of PEN3-GFP alanine substation variants in leaf epidermal cells at 24 hpi with *Bgh*. PEN3-GFP and variants appear green, propidium iodide-stained fungal structures appear red. Note that all alanine substitution variants exhibit focal accumulation at powdery mildew penetration sites similar to wild-type PEN3-GFP. At least 3 independent transgenic lines were evaluated for each alanine substitution variant with similar results. All panels are z-projected confocal images. Arrows indicate representative *Bgh* penetration sites. Scale bars = 20 μm.

redundantly with ABCG37 to transport the auxin precursor indole-3-butyric acid.[17,20] It will be interesting to determine whether phosphorylation at S^{40} and S^{45} also regulates transport of auxin-related molecules by PEN3 or if other phosphorylation events modulate PEN3 function in growth and development.

Disclosure of potential conflicts of interest

The authors report no conflict of interest.

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