ActivityDeterminantsandFunctionalSpecializationof
ArabidopsisPEN1SyntaxinInnateImmunity

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In eukaryotes, proteins of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) family are believed to have a general role for the fusion of intracellular transport vesicles with acceptor membranes. Arabidopsis thaliana PEN1 syntaxin resides in the plasma membrane and was previously shown to act together with its partner SNAREs, the adaptor protein SNAP33, and endomembrane-anchored VAMP721/722 in the execution of secretory immune responses against powdery mildew fungi. We conducted a structure-function analysis of PEN1 and show that N-terminal phospho-mimicking and non-phosphorylatable variants neither affected binary nor ternary SNARE complex formation with cognate partners in vitro. However, expression of these syntaxin variants at native protein levels in a pen1 mutant background suggests that phosphorylation is required for full resistance activity in planta. All tested site-directed substitutions of SNARE domain or “linker region” residues reduced PEN1 defense activity. Two of the variants failed to form ternary complexes with the partner SNAREs in vitro, possibly explaining their diminished in planta activity. However, impaired pathogen defense in plants expressing a linker region variant is likely because of PEN1 destabilization. Although Arabidopsis PEN1 and SYP122 syntaxins share overlapping functions in plant growth and development, PEN1 activity in disease resistance is apparently the result of a complete functional specialization. Our findings are consistent with the hypothesis that PEN1 acts in plant defense through the formation of ternary SNARE complexes and point to the existence of unknown regulatory factors. Our data indirectly support structural inferences that the four-helical coiled coil bundle in ternary SNARE complexes is formed in a sequential order from the N- to C-terminal direction.

Genome-wide phylogenetic analysis of eukaryotic soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins in animals, plants, protists, and fungi identified in each kingdom a splitting of four main branches (1, 2). These four phylogenetic groups coincide with four distinctive SNARE motif profiles (designated Qa-, Qb-, Qc-, and R-SNAREs (1)), which likely reflect the principal nature of the structural arrangement of oligomeric SNARE proteins into canonical four-helix bundle structures across eukaryotic kingdoms. Compared with yeast and animals, higher plant genomes encode a disproportionately large number of SNARE proteins, which might be indicative of a greater level of functional diversification and possible engagements in plant-specific vesicle transport processes (3, 4). Indeed, different plant SNAREs have been assigned functions in cytokinesis, shoot gravitropism, pathogen defense, symbiosis, abiotic stress responses, and trafficking to protein storage or lytic vacuoles (3).

Plants have evolved an elaborate immune system that detects non-self structures through a diversified repertoire of membrane-resident cell surface and intracellular immune receptors (5). Recent studies revealed the existence of secretory machineries that become engaged in the execution of extracellular immune responses. At least two SNARE protein-mediated exocytosis pathways appear to drive focal and/or non-directional secretion of antimicrobials, comprising defense-related proteins, and/or cell wall building blocks into the apoplastic space to terminate the pathogenesis of fungal or bacterial intruders (6–8). Both of these secretory pathways have additional functions in plant development and might have been co-opted for immune responses (for review, see Ref. 9).

Genetic and biochemical experiments revealed that the two pathogen-induced secretory machineries depend on plasma membrane-resident syntaxins of the same subfamily, PEN1 (AtSYP121) in Arabidopsis and NbSYP132 in Nicotiana benthamiana (6, 7). PEN1, its Nicotiana tabacum ortholog NtSYP121, and AtSYP132 (the presumed Arabidopsis ortholog of NbSYP132) become differentially phosphorylated within minutes upon treatment of plant cells with microbial elicitors such as the bacterial flagellin-derived peptide flg22 (7, 10–12). Whether these post-translational modifications affect syntaxin activity during plant defense responses remains unclear. Genetically supported evidence for additional SNARE proteins in Arabidopsis pathogen defense is at present limited to vesicle-associated membrane protein (VAMP) 721 and 722. PEN1 has been shown to interact and...
form ternary SNARE complexes in vitro and in vivo with synaptosomal-associated protein of 33 kDa (SNAP33) and VAMP721/722 (8). In monocotyledonous barley, the disease resistance function of their presumed orthologs (HvROR2 syntaxin, HvSNAP34, and HvVAMP721) is conserved, including SNARE complex formation of the gene products in vivo during transient co-expression of the respective partner SNAREs in single leaf epidermal cells (6, 8, 13).

Among the 18 Arabidopsis thaliana Qa-type family members (syntaxins) (4), PEN1 shares the highest level of sequence relatedness with SYP122 (63% amino acid identity). Both syntaxins reside in the plasma membrane and exert overlapping functions in growth and development because pen1 syp122 double mutant plants, but not the respective single mutants, are severely dwarfed and necrotic (14). PEN1 exerts an additional function in plant innate immunity because pen1 mutants allow enhanced entry of non-adapted powdery mildew fungi such as Blumeria graminis and Erysiphe pisi into leaf epidermal cells (6). Despite focal co-accumulation of PEN1 and SYP122 beneath incipient B. graminis f. sp. hordei (B. g. hordei) entry sites (14, 15), syp122 plants retain effective disease resistance to the fungal parasite, suggesting functional specialization of PEN1 in plant immune responses. However, SYP122 could share another redundant defense function with PEN1 that would be detectable only in pen1 syp122 plants. Unfortunately, the double mutants cannot be used for meaningful infection experiments because of severe pleiotropic effects (14, 16).

We have re-examined the potential functional specialization of PEN1 and SYP122 in defense responses to the non-adapted powdery mildew fungus B. g. hordei by gene dosage experiments and a chimeric syntaxin. Because the contribution of conserved SNARE motif residues to secretion has been extensively studied in syntaxins of vertebrates, Caenorhabditis elegans, and Drosophila melanogaster, but not in plants, we have conducted a structure-function analysis of PEN1 syntaxin in planta and in vitro. This revealed a critical role of the corresponding residues in the PEN1 linker region and the SNARE motif for immune responses. We show that equivalent amino acid substitutions in syntaxins from different kingdoms can have different functional consequences in exocytosis, which might reflect different modes of regulating secretory SNARE complexes. Using phospho-mimicking and non-phosphorylatable PEN1 variants, we also provide evidence that phosphorylation of N-terminal PEN1 syntaxins is required for full defense activity. Our study underlines the importance of conducting structure-function analysis of SNAREs in a whole organism context at native expression levels.

**EXPERIMENTAL PROCEDURES**

**Plant Materials**—Arabidopsis and barley plants were soil-grown in growth chambers at 20–23 °C with a 10-h photoperiod and a light intensity of ~150 μE m–2 s–1. Total proteins were extracted by grinding leaves from 3- or 4-week-old plants in liquid nitrogen and resuspending in 1× phosphate-buffered saline containing 1% Triton X-100. The extracts were centrifuged at 13,000 rpm for 30 min at 4 °C to remove cellular debris. Protein concentration in the supernatant was measured by the Bradford assay (Bio-Rad).

To generate Arabidopsis plants containing different PEN1 and SYP122 gene dosages, homozygous pen1-1 (6) and syp122-1 (SALK_008617 (14)) single mutants were crossed, and the resulting F1 plants were self-fertilized to produce segregating F2 siblings. Homo- or heterozygosity at the PEN1 and SYP122 loci was determined by PCR on genomic DNA isolated from F2 individuals. The pen1-1 mutation was identified using primer CAPS-F (5′-CAACGAAAACACTCTTCTTATGTCGGCC-3′) and CAPS-R (5′-CATCAATTCTCTTCTTCTTGAGA-C-3′) for amplification and subsequent ampiclon digestion with MluI. The T-DNA insertion present in the syp122-1 allele was determined by PCR using genomic DNA from F2 individuals as previously described (14).

To generate PEN1 variants, splicing by overlap extension PCR was performed using oligonucleotides encoding the desired site-directed substitutions (supplemental Table 1). The resulting PCR fragments were introduced into pDONR (Invitrogen). To generate a chimeric syntaxin protein (2211) composed of the N-terminal 175 amino acids of SYP122 followed by the C-terminal 171 amino acids of PEN1, PEN1 and SYP122 cDNAs in pDONR vector were digested with SgrAI and Apal. The digested DNA fragment encoding SYP122 N terminus was re-ligated with the reciprocal fragment of pDONR-PEN1 encoding the PEN1 C terminus.

To produce N-terminal fluorochrome-tagged versions of the PEN1 and SYP122 variants, the respective coding sequences in pDONR vector were introduced into a binary plant transformation vector based on pAM-PAT-GW4 by gateway cloning, containing either monomeric yellow (mYFP) or cerulean fluorescent protein (cCFP) coding sequence in frame with the gateway cassette (GW). 1.2 kilobases of PEN1 5′ regulatory DNA sequence (pPEN1) or 35 S promoter was engineered to drive the expression of the transgene, resulting in pPEN1::mYFP-GW or 35S::cCFP-GW. Stable transgenic Arabidopsis lines were generated by Agrobacterium tumefaciens-mediated transformation of flowering pen1-1 plants. T1-transformants were selected by Basta (Bayer) application, and the expression level of the engineered proteins was examined by immunoblot analysis of total plant leaf extracts using anti-PEN1 or anti-SYP122 antisera (12, 16).

**Purification of Recombinant Proteins**—To express and purify recombinant proteins, coding sequences of PEN1 variants (supplemental Table 1), SYP122, the chimeric syntaxin (2211), VAMP722, and SNAP33/SNAP33Δ25 were introduced into pDONR to generate GW entry clones. Subsequently, coding sequences were shuttled by LR recombination reactions either into the GW-compatible pGEX-6p-1 (Amersham Biosciences) GST fusion vector (SNAP33/SNAP33Δ25) or into derivatives of this vector,5 harboring in addition to the N-terminal GST label either an additional His6 (syntaxin) or HA (VAMP722) tag. After verification by DNA sequencing, plasmid DNA constructs were introduced into Escherichia coli Rosetta (Invitrogen), and protein expression was induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside. The recombinant proteins were purified by affinity chromatography using glutathione-Sepha-

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4 B. Ulker, unpublished information.

5 S. Pajonk, unpublished information.
rose 4B (Amersham Biosciences). To remove the N-terminal GST moiety (in case of GST/His<sub>e</sub>-double tagged syntaxins and the GST/HA-double tagged VAMP722), Precision protease (Amersham Biosciences) was used, and the eluted GST-free proteins were collected. The resulting purified recombinant GST-SNAP33/SNAP33Δ25 (immobilized to glutathione-Sepharose 4B), His<sub>e</sub>-syntaxin, and HA-VAMP722 polypeptides were used for GST pulldown assays as described below.

**Immunoblot Analysis**—In vitro pairwise or ternary SNARE complex formation assays were performed by incubating equimolar amounts (2 μM each) of purified recombinant proteins in a binding buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 50 mM NaCl, 5% glycerol, and 0.1% Triton X-100. The bound proteins were pulled down by sedimenting immobilized GST-SNAP33 or GST-SNAP33Δ25 and resuspended in sample buffer. After boiling, the supernatant was separated on polyacrylamide gels and probed with anti-His<sub>e</sub> (Qiagen) or anti-HA (Santa Cruz Biotechnology, Inc.) antibody. To detect the SDS-resistant SNARE complex, boiling of the supernatant was omitted before polyacrylamide gel separation.

To assess transgene expression, equal amounts (5–30 μg) of total protein extracts were loaded on a polyacrylamide gel. After transfer to nitrocellulose membranes (Amersham Biosciences), immunoblot analysis was carried out using anti-PEN1 (16) or anti-SYP122 (12) antiserum.

**Confocal Imaging Microscopy**—The intracellular localization of fluorescently tagged PEN1 variant proteins in transgenic Arabidopsis plants after *B. g. hordei* inoculation was inspected using a Leica TCS SP2 AOBS confocal microscope equipped with an argon/helium-neon laser for mYFP excitation at 514 nm and a diode laser of 405 nm (Leica) for cCFP excitation. Overlay and analysis of the images was carried out using the Leica Confocal software (LCS Lite, Leica Microsystems) and Adobe Photoshop 8.0.

**Analysis of Entry Resistance to B. g. hordei in Arabidopsis Leaf Epidermal Cells**—Three- to four-week-old plants were inoculated with *B. g. hordei* conidiospores with a conventional settling tower to maximize equal conidiospore distribution on leaves. Three leaves per plant were harvested at 24 h post-inoculation (hpi) and destained in ethanol for 24 h before overnight staining with 0.01% aniline blue solution (pH 9.5). Leaves were examined using UV-light epifluorescence microscopy. On each leaf ~100 interaction sites between a germinated conidiospore and an attacked epidermal cell were scored. At least three independent lines were tested for each construct with at least three independent repetitions. Student’s t test was performed to test for statistical significance.

**Transient Gene Expression in Barley Leaf Epidermal Cells**—Transient gene expression in single barley leaf epidermal cells was performed by ballistic transformation using coated gold particles as DNA carriers as described previously (17). Briefly, segments of primary barley leaves (resistant *mlo-3* genotype (18)) were placed on agar medium (1% phytoagar, 85 μM benzimidazole) and bombarded with DNA-coated gold particles (1 μM diameter; 5 μg plasmid DNA/bombardment) using a Bio-Rad PDS1000 Hepta Adapter system. Transformation of a plasmid (pUbi-GUS) encoding the β-glucuronidase (GUS) reporter protein allowed the subsequent identification of transformed cells. Plasmids harboring the test genes (*HvMLO*, *HvSNAP34*, or *HvSNAP33Δ25*) were co-transformed with the pUbi-GUS reporter plasmid. Expression of all genes was driven by the constitutive maize (*Zea mays*) ubiquitin promoter. Four hours after transformation leaves were inoculated with a high density of *B. g. hordei* (isolate K1) conidiospores. Forty-eight hours post-inoculation leaves were stained for β-glucuronidase activity by vacuum infiltration of 5-bromo-4-chloro-3-indolyl-β-D-glucuronidase (X-Gluc) solution and overnight incubation at 37 °C. Epiphytic fungal structures were visualized by Coomassie Brilliant Blue staining, and interaction sites were evaluated by light microscopy. Fungal entry rates were calculated as the number of successfully penetrated transformed (GUS-stained) cells (indicated by presence of a fungal haustorium) in relation to all attacked transformed cells.

**RESULTS**

**Sequence Variation between PEN1 and SYP122 Determines Functional Specialization**—To re-examine a potential contribution of *SYP122* to *B. g. hordei* defense responses we performed a gene dosage experiment by selecting five plant genotypes each with different PEN1 and SYP122 doses (Fig. 1A). None of these plants was dwarfed or necrotic. Using a PEN1-specific antiserum, we determined in the respective genotypes PEN1 abundance in leaf protein extracts and scored in 3–4-week-old plants entry rates to *B. g. hordei* conidiospore attack. PEN1<sup>+/+</sup> *SYP122<sup>+/+</sup>* and PEN1<sup>−/−</sup> *SYP122<sup>+/+</sup>* plants were severely defense-compromised (74–82% entry rates), whereas PEN1<sup>−/−</sup> *SYP122<sup>++/+</sup>*, PEN1<sup>−/−</sup> *SYP122<sup>−/−</sup>*, PEN1<sup>−/−</sup> *SYP122<sup>+/−</sup>*, and even PEN1<sup>−/−</sup> *SYP122<sup>−/+</sup>* genotypes showed similar levels of resistance (about 16–24%; Fig. 1A). Retained entry resistance in the latter two genotypes excludes a contribution of *SYP122* to immune responses in the presence of only one PEN1 dose. Reduced gene dosage resulted in reduced PEN1 abundance in *SYP122<sup>−/−</sup> PEN1<sup>−/−</sup>* and *SYP122<sup>−/−</sup> PEN1<sup>−/+</sup>* plants (Fig. 1A, lower panel), indicating that PEN1 activity in defense responses and plant growth is buffered against these variations in steady-state levels. The more pronounced reduction in PEN1 levels in *PEN1<sup>−/−</sup> SYP122<sup>−/−</sup>* plants may account for the slight increase in *B. g. hordei* entry rates in this genotype, although the increase was statistically not significant (Fig. 1A).

We found striking differences in PEN1 and *SYP122* abundance changes in time-course experiments after pathogen challenge. Constitutively high PEN1 levels were detected before *B. g. hordei* inoculation (0 hpi) followed by a slight and sustained increase between 24 and 72 hpi (Fig. 1B). In contrast, low *SYP122* levels in healthy plants were markedly and transiently elevated with a peak at 24 h after pathogen challenge (Fig. 1C). This extends previous observations on a marked *SYP122* transcriptional up-regulation by fungal, bacterial, and viral pathogens (14). Because termination of *B. g. hordei* entry attempts occurs at ~12 hpi, this raised the possibility that the low amount of *SYP122* at early time points is insufficient to confer effective disease resistance. To test this, we determined *B. g. hordei* entry rates in transgenic lines overexpressing func-
tional cCFP-tagged SYP122 (35S::cCFP-SYP122 (14)) in the pen1 null mutant background. Although the encoded fusion protein accumulated to a high level in these plants, cCFP-SYP122 failed to complement the pen1 immune defect (Fig. 2, A and B). Because cCFP-SYP122 rescues the growth defect in a syp122 pen1 double mutant background (14) and, thus, retains functionality, we conclude that the functional specialization between SYP122 and PEN1 in entry resistance to B. g. hordei is complete and is likely determined by amino acid sequence variations between the two syntaxins. Although unlikely, it remains possible that the fusion protein complements the growth/development defect but not the pathogen defense function.

To further examine the proposed functional specialization, we generated transgenic lines overexpressing a chimeric syntaxin in which 175 N-terminal PEN1 residues were substituted by corresponding SYP122 sequences (35S::cCFP-2211) in pen1-1 and pen1-1 syp122-1 backgrounds (Fig. 2A). Although the chimeric protein accumulated at fungal entry sites (supplemental Fig. 1), cCFP-2211-expressing plants neither rescued dwarfism in the pen1 syp122 background (tested in F2 progeny derived from crosses between pen1-1 plants expressing...
in response to elicitation with the bacterial flagellin-derived peptide flg22 at S6 and S8 positions of SYP122 and at S7 of PEN1 (10, 12). This provides direct evidence for rapid changes of post-translational modifications of the syntaxins in response to non-self recognition. To test the functional significance of serine phosphorylation sites, we generated phspho-mimic (serine to aspartate) and non-phosphorylatable (serine to alanine) variants (PEN1S7G, PEN1S6A,S7A,S8A, and PEN1S6D,S7D,S8D, Fig. 3A; supplemental Table 1). We also created a PEN1S7G variant to examine a potential functional contribution of glycine 7 located at the N terminus of SYP122 (Fig. 3A). The corresponding PEN1 derivatives were constructed by site-directed mutagenesis (“Experimental Procedures”) and expressed as N-terminal fusions to mYFP driven by 1.2 kilobases of native 5’ regulatory PEN1 sequences in transgenic Arabidopsis lines carrying a pen1-1 null mutation. Immunoblot analysis of leaf protein extracts with a PEN1-specific antiserum revealed an accumulation of the expected 67-kDa mYFP-PEN1 fusion proteins (Fig. 3B). Three independent lines of each derivative typically showing wild-type-like or lower than PEN1 wild-type levels were selected for further biological activity assays. Three- to four-week-old seedlings of T2 and T3 progeny were inoculated with B. g. hordei conidiospores, and fungal entry rates were microscopically scored at 72 hpi, thereby providing a direct measure of PEN1 defense activity (Fig. 3B).

Of three control transgenic lines expressing wild-type PEN1 as mYFP-PEN1 fusion, two (lines #2 and #7) almost fully complemented the pen1-1 mutant phenotype despite substantially lower abundance of the fusion protein in comparison to PEN1 levels in wild type (WT). One line (#4) permitted fungal entry rates that were significantly higher (26%) than in WT but still lower than those in partially defense-compromised pen1-3 plants (39%, Fig. 3B) (6, 8). This confirms previous data that a similar GFP-PEN1 fusion protein is functional (6) and is consistent with the above described gene dosage experiment showing that PEN1 function is retained at reduced gene dosage and reduced steady-state levels (Fig. 1A).
Two of three transgenic lines expressing the mYFP-PEN1\(^{S7G}\) derivative at low levels almost completely rescued the \(pen1^{-1}\) phenotype, whereas a third line (\#4) showed fungal entry rates between those found in \(pen1^{-1}\) and WT, suggesting that the S7 phosphorylation site alone is dispensable for PEN1 disease-resistance activity (Fig. 3B). However, each of three mYFP-PEN1\(^{S6A,S7A,S8A}\) containing lines was defense-compromised to \(B. g.\ hordei\) (Fig. 3B). Fungal entry rates were in one line (\#1) slightly lower and in two lines (\#2 and \#4) higher than in \(pen1^{-1}\) mutant plants. Because the respective mYFP-PEN1\(^{S6A,S7A,S8A}\) levels were only in line \#1, similar to PEN1 abundance in WT plants (Fig. 3B), this is indicative of a dose-dependent activity of the mutant protein. Thus, the Ser-6, Ser-7, and Ser-8 phosphorylation sites together appear to be critical for full PEN1 activity. Consistent with this, all three tested lines expressing the phospho-mimic mYFP-PEN1\(^{S6D,S7D,S8D}\) derivative fully complement the \(pen1^{-1}\) phenotype even at low protein levels (lines \#3 and \#4; Fig. 3B). The latter finding makes it unlikely that impaired mYFP-PEN1\(^{S6A,S7A,S8A}\) activity is the result of an indirect effect such as a perturbation of PEN1 structure.

*Targeted Exchange of Conserved Amino Acids in the PEN1 Linker Region and the SNARE Domain—* A subset of conserved residues in the SNARE domain or in the adjacent N-terminal linker region has previously been shown to contribute to the activities of rat (19), \(D. melanogaster\) (20), and \(C. elegans\) syntaxins in exocytosis (21, 22). *Arabidopsis* transgenic lines expressing mYFP-PEN1\(^{I227A,E228A}\) exhibited either severely or partially impaired entry resistance (Fig. 3B). Because the corresponding substitutions in the linker region between the Hc and SNARE domains (Fig. 3A) specifically disrupted the closed conformation of rat syntaxin 1A and *in vitro* binding to mammalian Munc18-1 (19), this could be evidence for the existence of PEN1 complexes containing *Arabidopsis* Munc homologs and/or PEN1 activity-dependent conformational switches. However, the corresponding substitutions in \(C. elegans\) syntaxin resulted in a functional but constitutively active version of the protein, which overcomes the requirement of the positive fusion regulator UNC-13 (21, 22). The contribution of these linker amino acids to syntaxin functions in three species representing different kingdoms points to the existence of a conserved role of the linker region in secretory processes, which in turn might be differentially modulated by species- and/or family member-specific regulatory factors. Notably, of all derivatives tested, recovered transgenic mYFP-PEN1\(^{L185A,D186A}\) plants showed the lowest syntaxin steady-state levels (Fig. 3B), suggesting that *in planta* these substitutions render the variant unstable. The destabilizing effect of substitutions in the linker region might have escaped previous detection because both rat syntaxin 1A\(^{L166A,E167A}\) and \(C. elegans\) syntaxin\(^{L166A,E167A}\) activities were tested only during overexpression in mammalian COS7 cells and in worms, respectively (19, 22). Although transgenic plants expressing other PEN1 derivatives at low levels retain functionality (*e.g.* mYFP-PEN1\(^{S6D,S7D,S8D}\) lines \#3 and \#4), it is likely that the non-functionality of mYFP-PEN1\(^{L185A,D186A}\) is at least in part caused by insufficient cellular syntaxin amounts.

Transgenic plants expressing the mYFP-PEN1\(^{D227A,E228A}\) derivative, which affect residues in the N-terminal region of the SNARE domain, showed noticeable syntaxin level-dependent infection phenotypes (Fig. 3B). Only line \#5, which accumulates wild-type-like mYFP-PEN1\(^{D227A,E228A}\) amounts, rescued the \(pen1^{-1}\) phenotype to entry rates between \(pen1^{-1}\) and WT, whereas lower levels detected in lines \#1 and \#3 were insufficient to restore disease resistance (Fig. 3B). The corresponding residues in the SNARE domain are in direct contact with SNAP-25 in the crystal structure of the rat core ternary SNARE complex (supplemental Table 1) (23). We tested *in vitro* binding of PEN1\(^{D227A,E228A}\) to SNAP33 and found reduced binary SNARE complex formation (Fig. 4A), suggesting reduced affinity of the two SNARE partners or
lowered stability of the respective binary SNARE complex. Surprisingly, PEN1<sup>1227A,E228A</sup> retained unaltered in vitro ternary SNARE complex formation in the presence of SNAP33 and VAMP722 (Fig. 4B), suggesting that intramolecular protein-protein interactions conferred by VAMP722 promote and/or stabilize the ternary complex. Altered SNARE complex formation and/or stability, thus, likely account for the dosage-dependent complementation of the pen1 phenotype by the PEN1<sup>1227A,E228A</sup> variant (Fig. 3B).

Unlike mYFP-PEN1<sup>1227A,E228A</sup> transgenic lines, the disease resistance activity of plants expressing the mYFP-PEN1<sup>1255A</sup> variant appears largely insensitive to varying syntaxin levels (Fig. 3B). All three tested lines partially complemented the pen1-1 null mutation and conferred resistance activity that is indistinguishable from pen1-3 plants with known residual wild-type syntaxin activity (8). The corresponding mutation in the Drosophila syntaxin 1A gene (syx<sup>3</sup>) specifically impairs binding to Ras opposite (ROP) (20), the Drosophila homolog of Sec1/Munc18, which regulates SYX-dependent neurotransmitter release (24). Flies expressing the SYX3 variant exhibit a dramatic increase in evoked neurotransmitter release in comparison to wild type ("hyperactivity"), suggesting an inhibitory role of ROP in this process (20). This demonstrates opposite functional consequences of corresponding substitutions in the Arabidopsis and Drosophila syntaxins in secretory processes (reduced and hyperactivity, respectively). One possibility is that neurosecretion in animals/flies and pathogen-triggered exocytosis in plants is subject to different modes of regulation conferred by different regulatory proteins.

Similar to mYFP-PEN1<sup>1227A,E228A</sup>, plants expressing cCFP-PEN1<sup>1262V, I266A</sup> partially complemented the pen1-1 phenotype in a protein level-dependent manner (Fig. 3B). At levels of cCFP-PEN1<sup>1262V, I266A</sup> that were similar to or lower than PEN1 in WT plants, the mutant protein partially restored fungal resistance to entry rates, which are slightly lower than in pen1-3 plants (lines #1 and #3). However, two tested 35S:cCFP-PEN1<sup>1262V, I266A</sup> overexpressor lines showed entry rates similar to mYFP-PEN1-expressing plants. Thus, the functional impairment caused by these amino acid exchanges can be compensated by non-physiologically high cCFP-PEN1<sup>1262V, I266A</sup> levels. This could be explained by a reduced binding affinity of PEN1<sup>1262V, I266A</sup> to its SNARE partners, which becomes compensated in the presence of excess amounts of the syntaxin variant. The corresponding substitutions in Drosophila syx<sup>4</sup> mutants specifically and severely impair excitation-secretion coupling of SYX-dependent neurotransmitter release (reduction by ~90%) if expressed at endogenous levels, whereas constitutive SYX-dependent secretion of cuticle proteins remained unaffected (25). SYX<sup>4</sup> ternary complexes were detectable in vitro but are heat-labile, which is indicative of impaired complex stability (25). Similarly, we failed to detect in vitro ternary SNARE complexes containing PEN1<sup>1262V, I266A</sup> in the presence of SNAP33 and VAMP722 (Fig. 4B), which likely reflects impaired complex stability. The only other tested PEN1 variant that was unable to form in vitro ternary SNARE complexes was pen1-3 (Fig. 4B). However, residual in planta resistance activities of PEN1<sup>1262V, I266A</sup> (Fig. 3B) as well as pen1-3 (8) indicate that functional ternary SNARE complexes can be formed with low efficiency in vivo.

A C-terminal-truncated SNAP Variant Exerts a Dominant Negative Effect on Innate Immunity—To further unravel structure-function relationships of SNARE complex formation in plant defense responses, we analyzed the effect of a C-terminal-truncated SNAP mutant variant on innate immunity. Previous studies in yeast and human cells indicated that amino acid residues at the C terminus of SNAP-25 and its yeast homolog, Sec9, are critical for the formation of a stable ternary SNARE complex and efficient exocytosis (26, 27). Moreover, overexpression of a C-terminal-truncated Sec9 variant in yeast was found to exert a dominant-negative effect on secretion (27). We generated the AtSNAP33Δ25 derivative, in which the relative position of the deletion within the second SNARE domain is equivalent to the yeast Sec9-Δ38 variant, and tested the ability of the encoded recombinant protein to form binary and ternary SNARE complexes in vitro. We found that AtSNAP33Δ25 exhibits a moderate reduction in the ability to form stable binary SNARE complexes with the PEN1 syntaxin (Fig. 5A) but, in contrast to AtSNAP33, fully fails to form a stable ternary SNARE complex with PEN1 and the ArvAMP722 R-SNARE (Fig. 5B). This is reminiscent of the results in Saccharomyces cerevisiae expressing Sec9-Δ38 (27).

Homozygous null mutations in Arabidopsis SNAP33 result in severe dwarfism and seedling lethality, preventing the analysis of the SNAP33 mutant in plant defense (28). The HvROR2 syntaxin, HvSNAP34, and the R-SNARE HvVAMP721 have been previously identified as the presumptive barley orthologs of Arabidopsis PEN1/SYP122, SNAP33, and VAMP721/722 (8). Pairwise protein-protein interactions between the three barley SNARE polypeptides in single cell Förster resonance energy transfer-fluorescence lifetime imaging (FRET-FLIM) measurements indicate that the three SNAP33, as their Arabidopsis orthologs, form an authentic ternary SNARE complex in vivo (8). A mutation in HvROR2 or transient gene silencing of HvSNAP34 in single leaf epidermal cells partially compromised the near-complete powdery mildew immunity of barley mlo mutants (6, 13, 29). Resistance conferred by Arabidopsis and barley loss-of-function mlo alleles has been previously suggested to share essential characteristics and likely a common mechanistic basis with plant innate immune responses (30). We, thus, exploited the tight powdery mildew resistance of barley mlo mutants to gain insight on the role of the SNAP C terminus in plant defense responses by overexpressing a HvSNAP34Δ25 variant. We observed a partial restoration of B. g. hordei host cell entry (23%) in the otherwise highly resistant mlo mutant that was not observed upon overexpression of a construct encoding full-length HvSNAP34 (2.3%, Fig. 5C). These results suggest that the C-terminally-deleted version of HvSNAP34 exerts a dominant negative effect on the formation of functional SNARE complexes that are essential for the execution of cell-autonomous antifungal defense at the cell periphery.

DISCUSSION

Here we have shown that the functional specialization between SYP122 and PEN1 in entry resistance to B. g. hordei is
Available except *Arabidopsis* (4) implicate that the gene duplication event and subsequent functional specialization of PEN1 and SYP122 in *Arabidopsis* antifungal defense occurred recently.

Insensitivity of the defense activity to changes in PEN1 and SYP122 dose contrasts with the haplo-insufficient disease resistance phenotypes of VAMP721+/− and VAMP722−/− plants (8). Because both PEN1/SYP122 and VAMP721/722 dosage reductions result in decreased steady-state levels of the respective gene products (Fig. 1A), the availability of VAMP721/722, but not PEN1, appears to become rate-limiting for the formation of defense-associated SNARE complexes in planta (8). Because gene dosage-sensitive components often represent regulatory components of a process (31), plants might fine-tune the defense response by influencing VAMP721/722 abundance and/or VAMP721/722 gene expression.

Residues from the four-helical coiled coil SNARE bundle of a ternary complex are grouped into "hydrophobic layers" that are conserved in primary-structure alignments from −7 to +8 (23). Evidence for the contribution of the SNARE domain in PEN1-dependent disease resistance was previously limited to pen1-3 (8). The mutant protein contains a substitution of a conserved glycine residue (G269E) at layer +6 leading to disruption of ternary SNARE complex formation in the presence of SNAP33 and VAMP722 in vitro (Fig. 4, A and B). In *Drosophila* SYX4 syntaxin, substitutions at layer +4 and +5 reduce complex-forming activity and compromise neurotransmitter secretion (25). Similarly, the equivalent PEN1 substitutions in this layer (A262V,1266A) render ternary but not binary SNARE complex formation undetectable in vitro (Fig. 4, A and B) and diminish disease resistance activity (Fig. 3B). Thus, impaired in vivo activities of PEN1A262V,1266A and pen1-3 can be explained by defects in PEN1 ternary SNARE complex formation and/or reduced complex stability.

Not all residues constituting the hydrophobic PEN1 SNAP domain layers are essential for ternary SNARE complex stability as PEN11227A,E228A, containing a substitution of the isoleucine residue at layer −6, formed in vitro ternary assemblies similar to wild-type PEN1 (Fig. 4B). Transgenic plants expressing wild-type-like levels of PEN11227A,E228A fully rescued the pen1-1 mutant phenotype (Fig. 3B). The consequences of mutations in the PEN1 SNARE domain can be rationalized on the basis of recent structure-function studies of secretory SNARE complexes in vertebrate cells, indicating that the four-helical coiled coil is formed between the three SNARE proteins in a sequential order from the N- to the C-terminal direction by means of distinctive assembly intermediates (32, 33).

**Structure-Function Relationships of PEN1 Syntaxin Variants**

![Image](https://example.com/structure-function.png)
vitro pre-complex (33). The latter might explain why the N-terminal layer substitution variant PEN1227A,E228A showed reduced in vivo activity only in transgenic lines expressing lower levels of the derivative compared with wild type plants (Fig. 3B).

Besides single amino acid substitutions in the PEN1 syntaxin, we also investigated the effect of a C-terminal deletion of the SNAP33 protein on SNARE complex formation. In yeast and animal cells, amino acid residues at the C terminus of SNAP polypeptides have been shown to be critical for efficient binary and ternary SNARE complex formation and secretion (26, 27). In human SNAP-25, a leucine residue located at the fourth position from the C terminus was found to be of particular relevance for full exocytotic function (26). This residue is conserved in SNAP proteins across taxa, including plants, and is lacking in the AtSNAP33Δ25 variant. Reminiscent of yeast Sec9Δ38, AtSNAP33Δ25 showed reduced formation of stable binary SNARE complexes and failed to form stable ternary SNARE complexes (Fig. 5, A and B). Overexpression of a plasmid encoding the equivalent HvSNAP34Δ25 variant in single barley leaf epidermal cells of the highly resistant mlo genotype partially restored entry of B. g. hordei (Fig. 5C). Notably, the effect of HvSNAP34Δ25 overexpression significantly exceeds the previously reported effect of HvSNAP34 gene silencing on barley mlo resistance (6, 13). Together, these findings indicate that the HvSNAP34Δ25 variant, like the analogous yeast Sec9Δ38 derivative, likely exerts a dominant negative effect on SNARE complex formation and/or stability. Intramolecular Förster resonance energy transfer of an engineered SNAP-25 expressed in rat PC12 cells was used to monitor the formation of the syntaxin-SNAP-25 dimer and provided evidence that the N-terminal SNARE domain of SNAP-25 joins syntaxin by forming a two-helical bundle, which in turn serves as an acceptor for the C-terminal SNARE domain of SNAP-25 and the R-SNARE syntaxin for exocytosis triggering (37). Because a portion of the deduced C-terminal helix is lacking in HvSNAP34Δ25, it is possible that overexpression HvSNAP34Δ25 sequesters cognate HvROR2 syntaxin away in a non-functional binary SNARE complex.

Different variants have been described for some animal syntaxins, including an open and closed conformation (38). The inhibitory closed conformation is caused by intramolecular interactions between the N-terminal Habc motif and the SNARE domain, which mimics a four-α-helical SNARE complex (19, 38). Mutations in the linker region between the Habc and SNARE domains were found to disrupt the closed conformation of rat syntaxin 1A and in vitro binding to mammalian Munc18–1 (19). Members of the cytosolic Sec1/Munc (SM) family of proteins are essential factors in different intracellular transport steps, during which they functionally interact with the SNARE machinery by different modes of binding to SNARE proteins (39). In contrast to the well documented role for SM family proteins in the regulation of syntaxin-mediated exocytosis in yeast and animal cells, little is known about plant SM proteins (3). The Arabidopsis genome encodes six members of the Sec1 family, of which only one has been functionally characterized to date (3, 40). Because Arabidopsis transgenic lines expressing a PEN1 variant with a mutation in the linker region, PEN1186A,D186A, exhibited either severely or partially impaired entry resistance to B. g. hordei (Fig. 3B), this demonstrates that substitutions outside of the SNARE domain can impair the functionality of a plant syntaxin. This could point to the existence of PEN1-SM complexes and/or PEN1 conformational switches during plant defense responses. Genetic evidence for the engagement of Arabidopsis SM proteins in vesicle trafficking comes from the identification of the Sec1 homolog KEULE, which is required for cytokinesis and interacts with the cytokinesis-specific KNOLLE syntaxin (40).

Besides SM proteins, post-translational modifications of animal syntaxins, such as phosphorylation, can affect associations with other SNARE proteins or their regulators (41–43). PEN1 in Arabidopsis suspension-cultured cells and the tobacco ortholog NtSYP121 in whole plants become differentially phosphorylated within minutes upon treatment with the bacterial PAMP flg22 or the fungal effector Avr9, respectively (10, 11). Whether these rapid phosphorylation/dephosphorylation modifications cause PEN1/NtSYP121 activity changes during plant defense responses remained unclear. Mass spectrometry revealed that serine residues at the N terminus of PEN1 and AtSYP122 are differentially phosphorylated (10, 12). This together with our data presented here (Fig. 3B) strongly suggests that PEN1Ser-6,Ser-7,Ser-8 phosphorylation sites are needed in vivo for full PEN1 activity against B. g. hordei entry. The capability of PEN1S6A,S7A,S8A, and PEN1S6D,S7D,S8D to form in vitro SD5-resistant binary and ternary SNARE complexes with similar efficiency as wild-type PEN1 contrasts with a diminished resistance activity of plants expressing PEN1S6A,S7A,S8A (Figs. 4B and 3B). Reduced in planta activity of PEN1S6A,S7A,S8A cannot be explained by its potentially altered subcellular partitioning because confocal imaging of cells expressing mYFP-PEN1S6A,S7A,S8A, mYFP-PEN1S6D,S7D,S8D, or mYFP-PEN1 produced indistinguishable fluorescence patterns, including a concentration of the variants at incipient fungal entry sites (supplemental Fig. 1). This contrasts with a previous report showing that the phosphorylated form of rat syntaxin 1A localizes to discrete domains of the axonal plasma membrane (42). Similar to the phospho-mimicking and non-phosphorylatable PEN1 variants, in vitro phosphorylation of N-terminal residues in rat syntaxin 1A did not affect binary or ternary SNARE complex formation (43). However, in vitro phosphorylated rat syntaxin 1A enhanced interactions with synaptotagmin, a calcium-sensing vesicular protein that promotes SNARE complex formation (42, 44). It is, thus, possible that the diminished in vivo activity of PEN1S6A,S7A,S8A reflects altered interactions with regulatory factors such as Arabidopsis homologs of synaptotagmin or SM proteins. Alternatively, non-phosphorylatable PEN1S6A,S7A,S8A might alter the kinetics of assembly/disassembly with its partner SNAREs that we have not tested.

Both PEN1 and SYP122 were shown to form SDS-resistant ternary SNARE complexes with SNAP33 and VAMP722 in vitro (Fig. 4). Because each of PEN1/SYP122, SNAP33, and VAMP721/VAMP722 is essential for plant growth and
between PEN1 and SYP122 (cCFP-2211) neither rescued the SNAP33 and VAMP721/722. Despite this, the hybrid protein acts similarly through SNARE complex formation with PEN1 containing ternary SNARE complexes in vitro (Fig. 4B), the hybrid must adopt a fold that is sufficient to engage in intermolecular interactions with other SNARE proteins. Thus, one possibility is that the 2211 hybrid fails to undergo intramolecular transitions between open and closed conformations that are thought to be tightly regulated by SM proteins in vivo (39). Clearly, focal accumulation of cCFP-2211 as well as all other tested non-functional PEN1 derivatives at B. g. hordei entry sites (supplemental Fig. 1), including pen1-3, demonstrates that polar concentration per se does not predict PEN1 functionality. Focal concentration of wild-type and mutant syntaxins is more likely the consequence of a general pathogen-induced host cell polarization process (45) driven by rapid disassembly and polar reassembly of the actin cytoskeleton and subsequent directed movement of cellular compartments, including the nucleus, endoplasmic reticulum, and Golgi (46, 47).

Our study underscores the importance of conducting structure-function analysis in a whole organism context at native protein levels. Remarkably, although PEN1 wild-type activity is insensitive to marked changes in steady-state levels, this appears to be the case only for two of the tested PEN1 variants (PEN1L185A and PEN1L185A,D186A), whereas the activity of several other variants is dose-dependent or has revealed an intrinsic protein instability (PEN1L185A,D186A). Although our data are, in general, consistent with a conserved role of linker and SNARE domain residues in secretory functions across taxa, disparate functional consequences of corresponding substitutions (e.g. opposite functional outcome of PEN1L185A and Drosophila synx1 mutations) might reflect differences in regulatory proteins that modulate neuronal secretion in animals and pathogen-triggered exocytosis in plants. Resolution of this conundrum demands future identification of Arabidopsis proteins regulating the formation of PEN1 containing ternary SNARE complexes during immune responses to pathogens and/or growth/development.

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Structure-Function Relationships of PEN1 Syntaxin Variants
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