THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 40, pp. 26974–26984, October 3, 2008

Activity Determinants and Functional Specialization of *Arabidopsis* PEN1 Syntaxin in Innate Immunity*[S]

Received for publication, July 10, 2008, and in revised form, July 25, 2008 Published, JBC Papers in Press, August 4, 2008, DOI 10.1074/jbc.M805236200

Simone Pajonk[1], **Chian Kwon, Natascha Clemens, Ralph Panstruga, and Paul Schulze-Lefert**[2]

From the Max Planck Institute for Plant Breeding Research, Department of Plant Microbe Interactions, D-50829 Köln, Germany

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)[3] 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 oligoheteromeric 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 rely on plasma membrane-resident syntaxins of the same subfamily, PEN1 (*At*SYP121) in *Arabidopsis* and *Nb*SYP132 in *Nicotiana benthamiana* (6, 7). PEN1, its *Nicotiana tabacum* ortholog *Nt*SYP121, and *At*SYP132 (the presumed *Arabidopsis* ortholog of *Nb*SYP132) 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

* This work was supported by funds from the Max Planck Society and Deutsche Forschungsgemeinschaft Grants SPP1212 (to C. K. and P. S.-L.) and SFB670 (to R. P. and P. S.-L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1 and Table 1.

[1] Supported by an International Max Planck Research School Ph.D. fellowship from the Max Planck Society.

[2] To whom correspondence should be addressed. Tel.: 49-2215062-350; Fax: 49-2215062–353; E-mail: schlef@mpiz-koeln.mpg.de.

[3] The abbreviations used are: SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor; VAMP, vesicle-associated membrane protein; mYFP, monomeric yellow fluorescent protein; cCFP, cerulean fluorescent protein; hpi, h post-inoculation; WT, wild type; SM, Sec1/Munc; GST, glutathione *S*-transferase; HA, hemagglutinin; GUS, β-glucuronidase.

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 F_1 plants were self-fertilized to produce segregating F_2 siblings. Homo- or heterozygosity at the *PEN1* and *SYP122* loci was determined by PCR on genomic DNA isolated from F_2 individuals. The *pen1*-1 mutation was identified using primer CAPS-F (5′-CAACGAAACACTCTCTTCATGTCACGC-3′) and CAPS-R (5′-CATCAATTTCTTCCTGAGAC-3′) for amplification and subsequent amplicon digestion with MluI. The T-DNA insertion present in the *syp122*-1 allele was determined by PCR using genomic DNA from F_2 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 ApaI. 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-GW[4] 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. T_1-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 antiserum (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 His_6 (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-

[4] B. Ülker, unpublished information.
[5] S. Pajonk, unpublished information.

rose 4B (Amersham Biosciences). To remove the N-terminal GST moiety (in case of GST/His_6-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_6-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_5 (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 72 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 inspected by 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 *HvSNAP34Δ25*) 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-glucoronidase activity by vacuum infiltration of 5-bromo-4-chloro-3-indolyl-β-D-glucoronid (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. 1*A*). 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. $PEN1^{-/-}$ $SYP122^{+/+}$ and $PEN1^{-/-}$ $SYP122^{+/-}$ plants were severely defense-compromised (74–82% entry rates), whereas $PEN1^{+/+}$ $SYP122^{+/+}$, $PEN1^{+/+}$ $SYP122^{-/-}$, $PEN1^{+/-}$ $SYP122^{+/-}$, and even $PEN1^{+/-}$ $SYP122^{-/-}$ genotypes showed similar levels of resistance (about 16–24%; Fig. 1*A*). 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 $SYP122^{-/-}$ $PEN1^{+/-}$ and $SYP122^{+/-}$ $PEN1^{+/-}$ plants (Fig. 1*A*, *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 $PEN1^{+/-}$ $SYP122^{-/-}$ plants may account for the slight increase in *B. g. hordei* entry rates in this genotype, although the increase was statistically not significant (Fig. 1*A*).

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. 1*B*). In contrast, low SYP122 levels in healthy plants were markedly and transiently elevated with a peak at 24 h after pathogen challenge (Fig. 1*C*). 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-

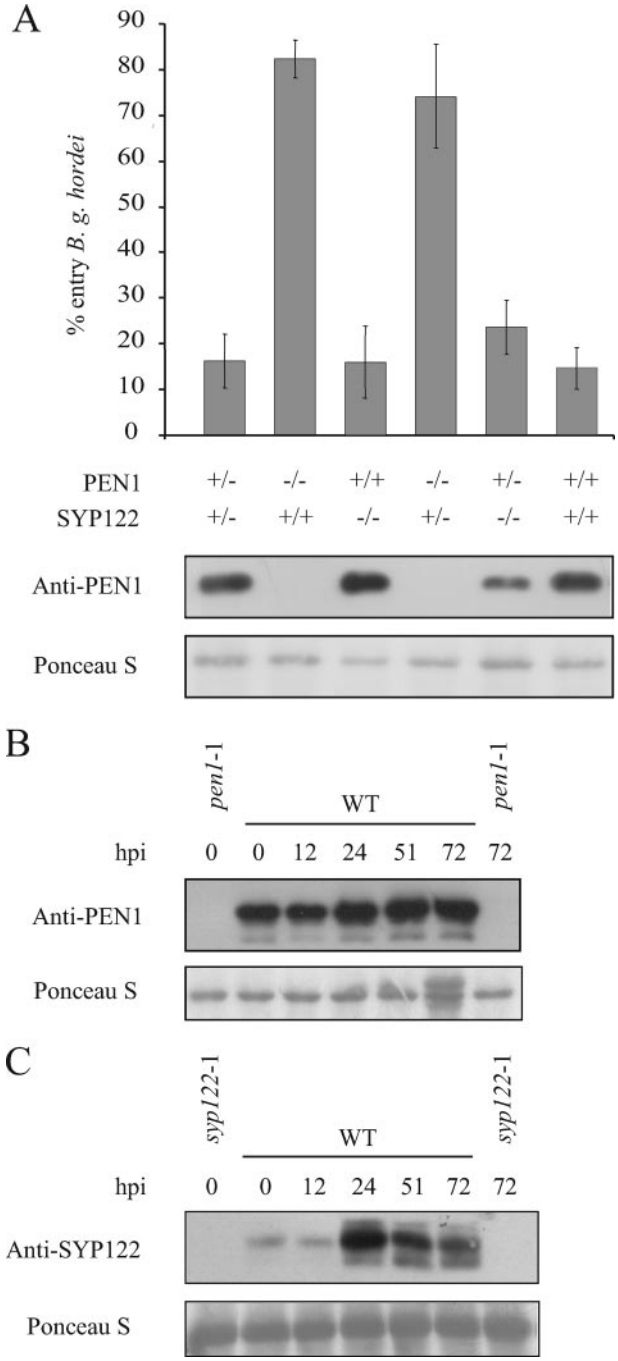

FIGURE 1. **Defense activity and changes of PEN1 and SYP122 accumulation patterns in response to *B. g. hordei* inoculation.** *A*, a single dose of *PEN1* is sufficient for effective defense responses in the *syp122* null mutant background. Fungal entry rates (*upper panel*) were determined by UV-light epifluorescence microscopy of *Arabidopsis* leaves at 72 hpi with *B. g. hordei* conidiospores. PEN1 steady-state levels (*lower panel*) of the indicated plant genotypes were examined by immunoblot analysis with anti-PEN1 antiserum in leaf extracts of non-inoculated plants. Data shown are the average ± S.E. of at least three independent experiments. *B* and *C*, time-course analysis of PEN1 and SYP122 accumulation in response to *B. g. hordei* inoculation. Leaf protein extracts were isolated from pathogen-treated and untreated samples at the indicated time points and separated by polyacrylamide gel electrophoresis. Immunoblots were probed with anti-PEN1 or anti-SYP122 antiserum. Extracts from *pen1*-1 and *syp122*-1 plants served as controls for antiserum specificity. Ponceau S staining was used to confirm uniform protein loading.

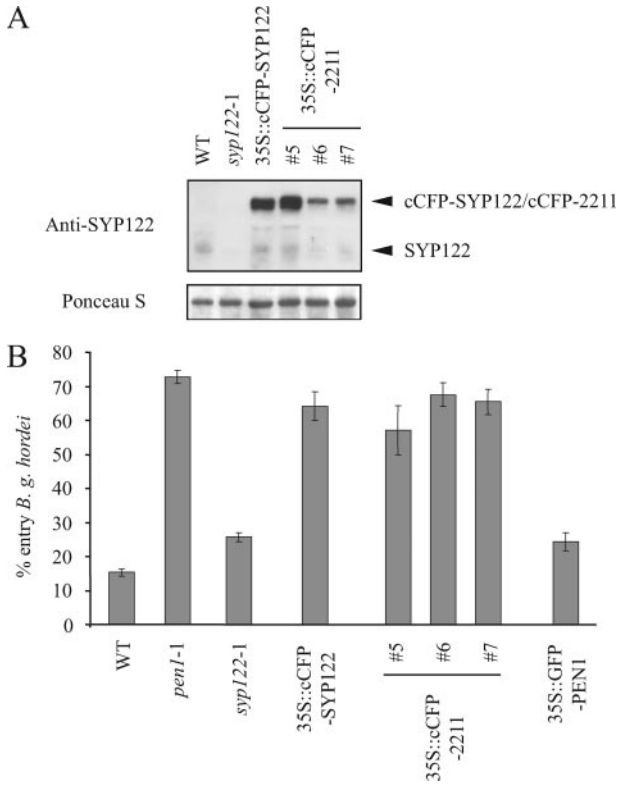

FIGURE 2. **Functional specialization of PEN1 in defense responses to *B. g. hordei*.** *A*, accumulation of the indicated fusion proteins in *pen1*-1 plants. Equal amounts of total protein extracted from leaves of the indicated transgenic plants were probed in immunoblot analysis with anti-SYP122 antiserum raised against the N terminus of SYP122. This antiserum also detects the chimeric protein cCFP-2211 consisting of the N-terminal half of SYP122 and the C-terminal half of PEN1. *syp122*-1 plants served as control for antiserum specificity. Note that the signal of endogenous SYP122 is weak in comparison to that of the transgene products. Ponceau S staining was used to confirm uniform protein loading. *B*, overexpression of SYP122 or the hybrid protein 2211 fails to rescue the *pen1*-1 phenotype. Fungal entry rates were determined in the indicated plant lines by UV-light epifluorescence microscopy of *Arabidopsis* leaves at 72 hpi with *B. g. hordei* conidiospores. Data shown are the average ± S.E. of at least three independent experiments.

tional cCFP-tagged SYP122 (35S::cCFP-SYP122 (14)) in the *pen1*-1 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. 2*A*). 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 F_2 progeny derived from crosses between *pen1*-1 plants expressing

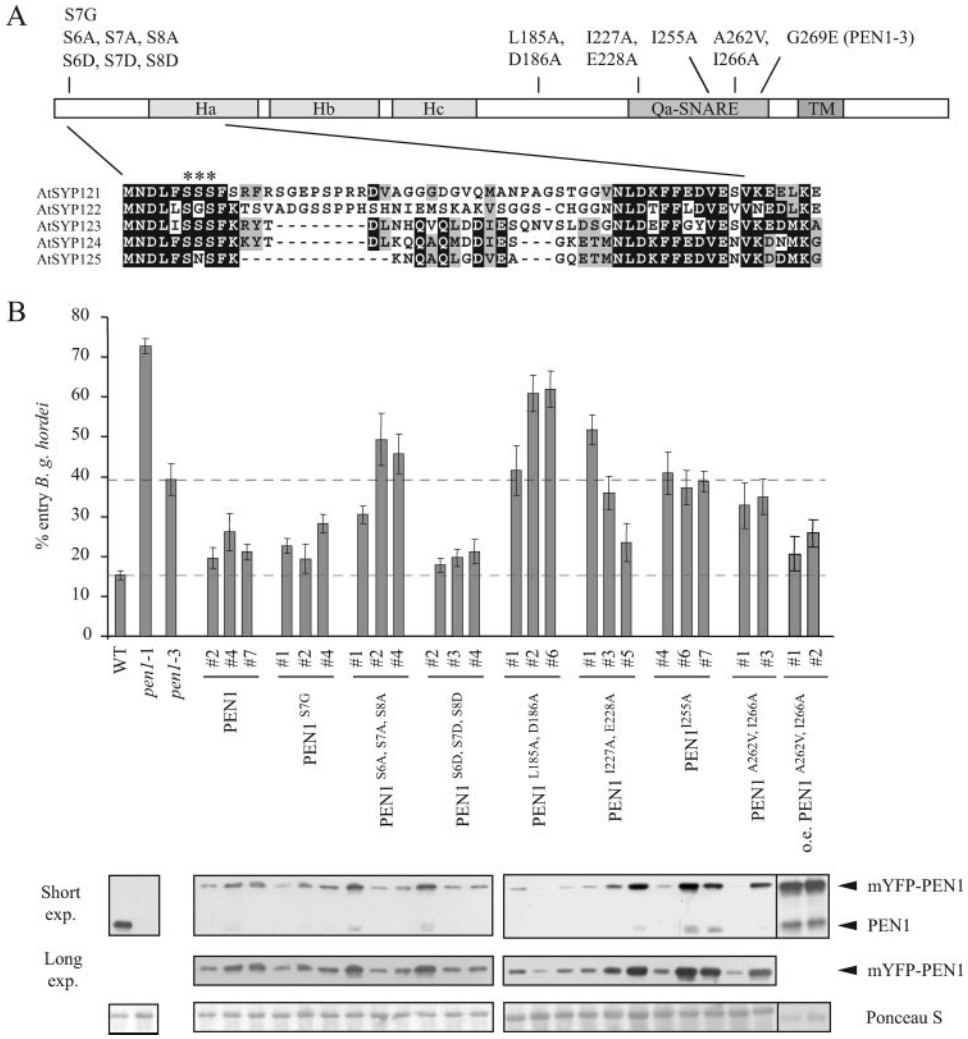

FIGURE 3. **Structure-function analysis of PEN1 variants in defense responses to *B. g. hordei*.** *A*, schematic representation of deduced PEN1 domains. The close-up shows an alignment of N-terminal amino acid sequences of the *At*SYP12 group members (4). The alignment was constructed on the basis of the ClustalW algorithm. Three conserved potential serine phosphorylation sites (*S*) are marked by *asterisks*. *B*, defense activities of the indicated PEN1 variants upon inoculation with *B. g. hordei* conidiospores. Fungal entry rates were determined in the indicated plant lines by UV-light epifluorescence microscopy of *Arabidopsis* leaves at 72 hpi with *B. g. hordei* conidiospores. The tested transgenic lines (denoted by #) represent plants of T_2 and T_3 generations. Entry rates of WT (*lower dashed line*) or *pen1*-3 (*upper dashed line*) plants served as controls. Accumulation of the indicated fusion proteins in *pen1*-1 plants was analyzed by immunoblot with anti-PEN1 antiserum using total proteins extracted from healthy leaves of the indicated transgenic plants. Protein extracts of *syp122*-1 plants served as control for antiserum specificity. *Short exp.*, short exposure; *long exp.*, longer exposure; *o. e.*, overexpression, Ponceau S staining was used to confirm uniform protein loading. Data shown are the average ± S.E. of at least three independent experiments.

35S::cCFP-2211 and the *syp122*-1 mutant, data not shown) nor resistance to *B. g. hordei* entry in the *pen1*-1 null mutant background in each of three independent transgenic lines tested (*#5*, *#6*, *#7*; Fig. 2*B*). This reveals potential intramolecular domain interactions between the N- and C-terminal parts that are critical for functionality of the respective wild-type proteins and limits the utility of domain swap experiments to delineate sequence motifs contributing to the functional specialization between the two syntaxin family members.

PEN1 Phosphorylation Is Needed for Full Disease Resistance Activity—N-terminal serine residues that are shared among the *Arabidopsis* SYP12 group (comprising SYP121 (PEN1), SYP122, SYP123, SYP124, and SYP125; Fig. 3*A*) were previously shown to be differentially phosphorylated within minutes 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 phospho-mimic (serine to aspartate) and non-phosphorylatable (serine to alanine) variants ($PEN1^{S7G}$, $PEN1^{S6A,S7A,S8A}$, and $PEN1^{S6D,S7D,S8D}$, Fig. 3*A*; supplemental Table 1). We also created a $PEN1^{S7G}$ variant to examine a potential functional contribution of glycine 7 located at the N terminus of SYP122 (Fig. 3*A*). 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. 3*B*). 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 T_2 and T_3 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. 3*B*).

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. 3*B*) (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. 1*A*).

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*-3 and WT, suggesting that the S7 phosphorylation site alone is dispensable for PEN1 disease-resistance activity (Fig. 3*B*). However, each of three mYFP-PEN1^{S6A,S7A,S8A} containing lines was defense-compromised to *B. g. hordei* (Fig. 3*B*). Fungal entry rates were in one line (#1) slightly lower and in two lines (#2 and *#4*) higher than in *pen1*-3 mutant plants. Because the respective mYFP-PEN1^{S6A,S7A,S8A} levels were only in line #1, similar to PEN1 abundance in WT plants (Fig. 3*B*), 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. 3*B*). 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^{L185A,D186A} exhibited either severely or partially impaired entry resistance (Fig. 3*B*). Because the corresponding substitutions in the linker region between the Hc and SNARE domains (Fig. 3*A*) specifically disrupt 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. 3*B*), 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^{L165A,E166A} 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^{I227A,E228A} derivative, which affect residues in the N-terminal region of the SNARE domain, showed noticeable syntaxin level-dependent infection phenotypes (Fig. 3*B*). Only *line #5*, which accumulates wild-type-like mYFP-PEN1^{I227A,E228A} amounts, rescued the *pen1*-1 phenotype to entry rates between *pen1*-3 and WT, whereas lower levels detected in lines #1 and #3 were insufficient to restore disease resistance (Fig. 3*B*). 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^{I227A,E228A} to SNAP33 and found reduced binary SNARE complex formation (Fig. 4*A*), suggesting reduced affinity of the two SNARE partners or

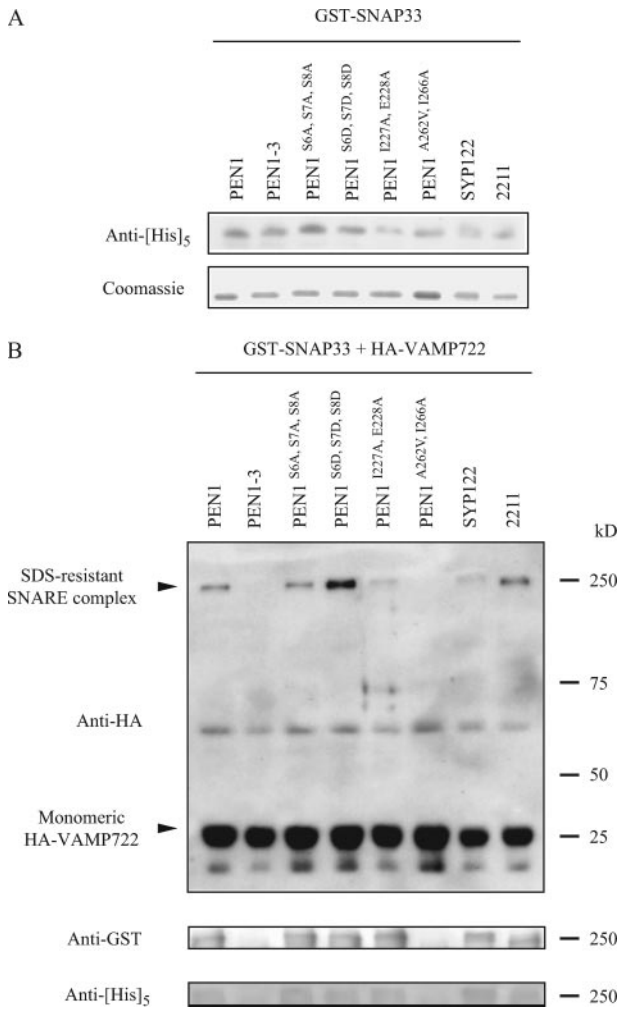

FIGURE 4. **Binary and ternary SNARE complexes formed by PEN1 variants *in vitro*.** *A*, pairwise interactions of recombinant PEN1 variants with SNAP33. The indicated His$_6$-tagged syntaxins were incubated with GST-SNAP33 immobilized to glutathione-Sepharose 4B, and the pulled-down precipitates were subjected to immunoblot with anti-His$_5$ antibody. The purity of recombinant syntaxins used in this experiment was shown by separating purified proteins on a polyacrylamide gel followed by Coomassie Blue staining (Coomassie). *B*, SDS-resistant ternary SNARE complex formation of PEN1 variants. The indicated His$_6$-tagged syntaxins were incubated with HA-VAMP722 and immobilized GST-SNAP33, and the pulled-down precipitates were subjected to immunoblot with anti-HA, anti-GST, or anti-His$_5$ antibody. The precipitates, resuspended in sample buffer, were not boiled before separation on a polyacrylamide gel. Note that the different band intensities of ternary SNARE complexes detected by the anti-HA immunoblot are likely because of differential accessibility of the antibody because the signal intensities of these complexes are indistinguishable in the anti-GST or anti-His$_5$ immunoblots, which detect GST-SNAP33 or His$_6$-PEN1 variants, respectively.

lowered stability of the respective binary SNARE complex. Surprisingly, PEN1^{I227A,E228A} retained unaltered *in vitro* ternary SNARE complex formation in the presence of SNAP33 and VAMP722 (Fig. 4*B*), suggesting that intermolecular 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^{I227A,E228A} variant (Fig. 3*B*).

Unlike mYFP-PEN1^{I227A,E228A} transgenic lines, the disease resistance activity of plants expressing the mYFP-PEN1^{I255A} variant appears largely insensitive to varying syntaxin levels (Fig. 3*B*). 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*^{3}) 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^{I227A,E228A} plants, transgenic lines expressing cCFP-PEN1$^{A262V,\ I266A}$ partially complemented the *pen1*-1 phenotype in a protein level-dependent manner (Fig. 3*B*). At levels of cCFP-PEN1$^{A262V,\ I266A}$ 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$^{A262V,\ I266A}$ 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$^{A262V,\ I266A}$ levels. This could be explained by a reduced binding affinity of PEN1$^{A262V,\ I266A}$ to its SNARE partners, which becomes compensated in the presence of excess amounts of the syntaxin variant. The corresponding substitutions in *Drosophila syx4* 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). SYX4 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$^{A262V,\ I266A}$ in the presence of SNAP33 and VAMP722 (Fig. 4*B*), 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. 4*B*). However, residual *in planta* resistance activities of PEN1$^{A262V,\ I266A}$ (Fig. 3*B*) 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 *At*SNAP33Δ25 exhibits a moderate reduction in the ability to form stable binary SNARE complexes with the PEN1 syntaxin (Fig. 5*A*) but, in contrast to *At*SNAP33, fully fails to form a stable ternary SNARE complex with PEN1 and the *At*VAMP722 R-SNARE (Fig. 5*B*). 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 *Hv*ROR2 syntaxin, *Hv*SNAP34, and the R-SNARE *Hv*VAMP721 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 SNARE proteins, 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 *Hv*SNAP34 (2.3%, Fig. 5*C*). These results suggest that the C-terminally-deleted version of *Hv*SNAP34 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

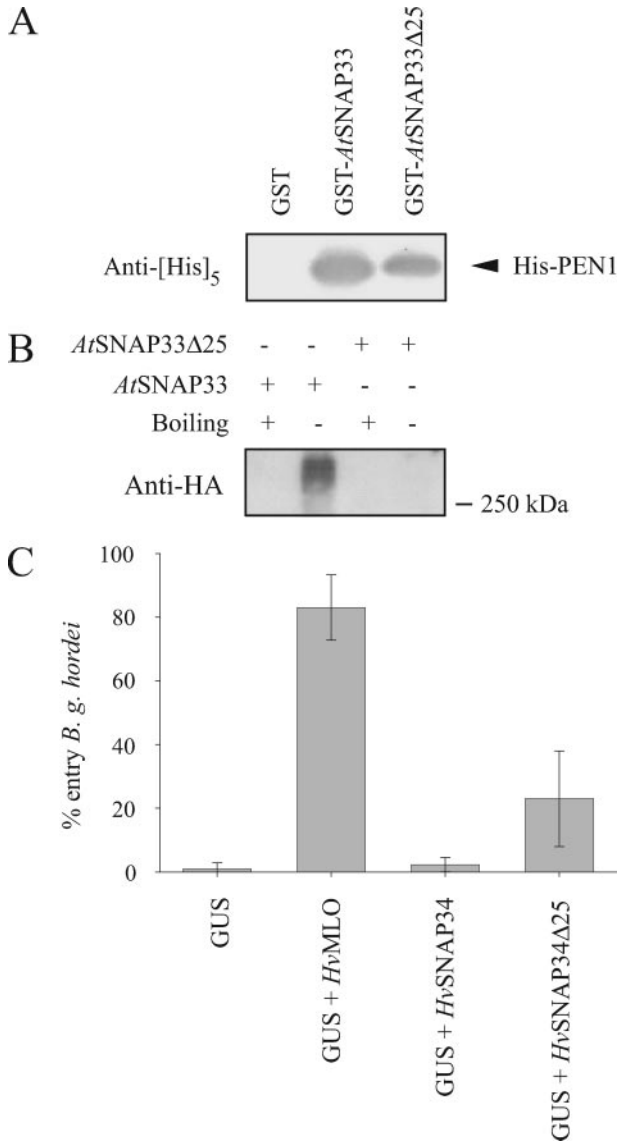

FIGURE 5. **Dominant-negative activity of C-terminal-truncated *At*SNAP33 and *Hv*SNAP34 variants.** *A*, reduced affinity of *At*SNAP33Δ25 to PEN1. Bead-bound GST, GST-*At*SNAP33, or GST-*At*SNAP33Δ25 proteins were incubated with His_6-PEN1 (*His-PEN1*), and the pulled-down precipitates were subjected to immunoblot with anti-His_5 antiserum. *B*, *At*SNAP33Δ25 fails to form a SDS-resistant ternary SNARE complex *in vitro*. The indicated GST-fused *At*SNAP33 or *At*SNAP33Δ25 proteins were incubated with His-PEN1 and HA-VAMP722, and the precipitates were analyzed by immunoblot using anti-HA antiserum. To visualize the SDS-resistant SNARE complex, the precipitates were boiled or non-boiled before loading onto a polyacrylamide gel. *C*, compromised disease resistance to *B. g. hordei* of leaf epidermal cells in *mlo* resistant barley expressing the C-terminal-truncated *Hv*SNAP33 variant that is defective in SNARE complex formation. Expression of the GUS reporter construct served as negative control, whereas overexpression of the defense modulator MLO served as positive control, reinstating successful powdery mildew entry into most transformed cells. Data shown are the average ± S.D. of at least five independent bombardments per construct.

complete, although both syntaxins share an essential role in plant growth and development. Neither did *PEN1* dosage reduction in a *syp122* background diminish the defense activity nor did *SYP122* overexpression in a *pen1* background rescue the susceptible infection phenotype (Fig. 1*A* and 2*B*). The functional redundancy of PEN1 and SYP122 in plant growth and development (14, 16) and the presence of a single *PEN1*-like gene in all plant species for which a full genome sequence 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 $VAMP721^{+/-}$ $VAMP722^{-/-}$ and $VAMP721^{-/-}$ $VAMP722^{+/-}$ plants (8). Because both *PEN1/SYP122* and *VAMP721/722* dosage reductions result in decreased steady-state levels of the respective gene products (Fig. 1*A*), 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,I266A) render ternary but not binary SNARE complex formation undetectable *in vitro* (Fig. 4, *A* and *B*) and diminish disease resistance activity (Fig. 3*B*). Thus, impaired *in vivo* activities of $PEN1^{A262V,I266A}$ 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 SNARE domain layers are essential for ternary SNARE complex stability as $PEN1^{I227A,E228A}$, containing a substitution of the isoleucine residue at layer −6, formed *in vitro* ternary assemblies similar to wild-type PEN1 (Fig. 4*B*). Transgenic plants expressing wild-type-like levels of $PEN1^{I227A,E228A}$ fully rescued the *pen1*-1 mutant phenotype (Fig. 3*B*). 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). In this as well as previous studies, C-terminal layer mutations strongly impaired secretion, whereas N-terminal layer substitutions resulted in milder phenotypes (20, 25, 33–36). It is thought that two functional domains reside within the SNARE helical bundle and that mutations in the C terminus can loosen the complex without affecting the rest of the complex. Accordingly, C-terminal layer mutations appear to specifically impair triggering of vesicle fusion, whereas the initial N-terminal SNARE complex might be driven *in vivo* by specific "priming factors" that overcome the slow kinetics of the syntaxin-SNAP-25 *in*

vitro pre-complex (33). The latter might explain why the N-terminal layer substitution variant PEN1^{I227A,E228A} showed reduced *in vivo* activity only in transgenic lines expressing lower levels of the derivative compared with wild type plants (Fig. 3*B*).

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 *At*SNAP33Δ25 variant. Reminiscent of yeast *Sec9-Δ38*, *At*SNAP33Δ25 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 *Hv*SNAP34Δ25 variant in single barley leaf epidermal cells of the highly resistant *mlo* genotype partially restored entry of *B. g. hordei* (Fig. 5*C*). Notably, the effect of *Hv*SNAP34Δ25 overexpression significantly exceeds the previously reported effect of *HvSNAP34* gene silencing on barley *mlo* resistance (6, 13). Together, these findings indicate that the *Hv*SNAP34Δ25 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 synaptobrevin for exocytosis triggering (37). Because a portion of the deduced C-terminal helix is lacking in *Hv*SNAP34Δ25, it is possible that overexpressed *Hv*SNAP34Δ25 sequesters cognate *Hv*ROR2 syntaxin away in a non-functional binary SNARE complex.

Different conformers 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, PEN1^{L185A,D186A}, exhibited either severely or partially impaired entry resistance to *B. g. hordei* (Fig. 3*B*), 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 *Nt*SYP121 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/*Nt*SYP121 activity changes during plant defense responses remained unclear. Mass spectrometry revealed that serine residues at the N terminus of PEN1 and *At*SYP122 are differentially phosphorylated (10, 12). This together with our data presented here (Fig. 3*B*) strongly suggests that PEN1$^{Ser\text{-}6,Ser\text{-}7,Ser\text{-}8}$ phosphorylation sites are needed *in vivo* for full PEN1 activity against *B. g. hordei* entry. The capability of PEN1^{S6A,S7A,S8A} and PEN1^{S6D,S7D,S8D} to form *in vitro* SDS-resistant binary and ternary SNARE complexes with similar efficiency as wild-type PEN1 contrasts with a diminished resistance activity of plants expressing PEN1^{S6A,S7A,S8A} (Figs. 4*B* and 3*B*). Reduced *in planta* activity of PEN1^{S6A,S7A,S8A} cannot be explained by its potentially altered subcellular partitioning because confocal imaging of cells expressing mYFP-PEN1^{S6A,S7A,S8A}, mYFP-PEN1^{S6D,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 PEN1^{S6A,S7A,S8A} reflects altered interactions with regulatory factors such as *Arabidopsis* homologs of synaptotagmin or SM proteins. Alternatively, non-phosphorylatable PEN1^{S6A,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

development (8, 14, 28), it seems likely that *in vivo* SYP122 acts similarly through SNARE complex formation with SNAP33 and VAMP721/722. Despite this, the hybrid protein between PEN1 and SYP122 (cCFP-2211) neither rescued the *pen1 syp122* developmental nor the *pen1* immune response defect (Fig. 2*B* and data not shown). Because the 2211 chimera is capable of forming ternary SNARE complexes *in vitro* (Fig. 4*B*), 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 ($PEN1^{I255A}$ and $PEN1^{S6D,S7D,S8D}$), whereas the activity of several other variants is dose-dependent or has revealed an intrinsic protein instability ($PEN1^{L185A,D186A}$). 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 $PEN1^{I255A}$ and *Drosophila syx*[3] 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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