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Requirement of Vesicle-Associated Membrane Protein 721 and 722 for Sustained Growth during Immune Responses in Arabidopsis

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Extracellular immune responses to ascomycete and oomycete pathogens in Arabidopsis are dependent on vesicle-associated secretion mediated by the SNARE proteins PEN1 syntaxin, SNAP33 and endomembrane-resident VAMP721/722. Continuous movement of functional GFP-VAMP722 to and from the plasma membrane in non-stimulated cells reflects the second proposed function of VAMP721/722 in constitutive secretion during plant growth and development. Application of the bacterium-derived elicitor flg22 stabilizes VAMP721/722 that are otherwise constitutively degraded *via* the 26S proteasome pathway. Depletion of VAMP721/722 levels by reducing *VAMP721/722* gene dosage enhances flg22-induced seedling growth inhibition in spite of elevated VAMP721/722 abundance. We therefore propose that plants prioritize the deployment of the corresponding secretory pathway for defense over plant growth. Interstingly, VAMP721/722 specifically interact *in vitro* and *in vivo* with the plasma membrane syntaxin SYP132 that is required for plant growth and resistance to bacteria. This suggests that the plant growth/immunity-involved VAMP721/722 form SNARE complexes with multiple plasma membrane syntaxins to discharge cue-dependent cargo molecules.

INTRODUCTION

Intra- and intercellular communication between different subcellular compartments and between adjacent cells *via* small endomembrane compartments is an essential feature of many processes in eukaryotes. It is generally accepted that members of the soluble *N*-ethylmaleimide sensitive factor attachment protein receptor (SNARE) superfamily are key factors to drive vesicle fusion events in eukaryotes including yeasts, animals and plants (Jahn and Scheller, 2006). Plant genomes contain a larger number of *SNARE* genes than yeast and animals, implying a higher degree of sub-functionalization as illustrated by the engagement of distinct *SNARE* genes in different biological processes such as cytokinesis, gravitropism and disease resistance (Lipka et al., 2007; Sanderfoot, 2007).

It has long been known that plant cells secrete a variety of peptides and secondary metabolites in response to pathogen attack (Dixon, 2001; van Loon et al., 2006), but how these molecules are extruded and how this process is regulated is still poorly understood. Mutations in secretion-related endoplasmic reticulum (ER)-resident genes such as *Bip2*, *Sec61α* or *Dad1* correlate with significantly reduced secretion of pathogenesis-related protein 1 (PR-1) and impair the ability of Arabidopsis to limit the growth of a virulent bacterium, *Pseudomonas syringae* pv. *maculicula* in salicylic acid (SA)-dependent resistance (Wang et al., 2005). Similarly, dysfunction in ER *N*-linked glycosylation by mutations in genes of UDP-glucose:glyco-protein glycosyltransferase (UGGT) and STT3A, a component of oligosaccharyltransferase complex, also reduces SA-induced defence against *P. syringae* pv. *maculicula* (Saijo et al., 2009). These reports indicate that a secretory pathway that traverses the ER and Golgi is critical for successful defense responses in plants. The bacterial effector HopM1, which is required for proliferation of *P. syringae* in the apoplast of the leaf interior, has been shown to degrade AtMIN7 in Arabidopsis (Nomura et al., 2006). Since AtMIN7 is an ADP ribosylation factor guanine nucleotide exchange factor (ARF-GEF) involved in controlling vesicle trafficking (Nomura et al., 2006), this finding supports the idea that the host secretion machinery plays an important role in repulsing pathogens. In *Nicotiana benthamiana*, gene silencing of the plasma membrane (PM)-resident syntaxin *NbSYP132* compromises multiple forms of immune responses to *P. syringae* pv *tabaci* (*Pstab*), including resistance (*R*) gene-triggered immunity and chemically-induced defense associated with salicylic acid (SA) (Kalde et al., 2007). In addition, *NbSYP132*-

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silenced plants allow enhanced growth of nonpathogenic *Agrobacterium* as well as a mutant strain of *P. syringae* pv tomato DC3000 that is defective in type-III effector-dependent delivery (Kalde et al., 2007). These impaired immune responses correlated with reduced secretion of the SA-dependent defense marker PR-1 (Kalde et al., 2007). Since syntaxins belong to the SNARE superfamily that are core factors to drive vesicle fusions in eukaryotic cells (Jahn and Scheller, 2006), this suggests that a NbSYP132-dependent process is engaged by multiple defense pathways to bacterial pathogens that includes the secretion of PR-1.

At least two secretory pathways operate in Arabidopsis disease resistance to non-adapted powdery mildew fungi (Kwon et al., 2008a; 2008b). One pathway consists of the P450 monooxygenase CYP81F2, the myrosinase PEN2, and the ABC transporter PEN3. CYP81F2 is necessary for the generation of substrates of PEN2, which in turn initiates the metabolism of a group of tryptophan-derived compounds, known as indole glucosinolates, to release potential antimicrobial products by the PEN3 transporter into the extracellular space (Bednarek et al., 2009; Lipka et al., 2005; Stein et al., 2006). The other consists of the PM-anchored syntaxin PEN1, the PM-bound adaptor SNAP33, and the vesicle-associated membrane proteins (VAMPs) 721 and 722 that share 96% identical amino acid sequences (Collins et al., 2003; Kwon et al., 2008c; Pajonk et al., 2008). These proteins are proposed to operate in an exocytic plant defense pathway by forming ternary SNARE complexes (Kwon et al., 2008c). We additionally showed that VAMP721/722 have a broader role in plant defense responses because their depletion in haplo-insufficient $VAMP721^{-/-}$ $VAMP722^{+/-}$ and $VAMP721^{+/-}$ $VAMP722^{-/-}$ genotypes leads to impaired defense to a virulent oomycete, *Hyaloperonospora arabidopsidis*, as well as to the non-adapted *Erysiphe pisi* and the host-adapted *Golovinomyces orontii* powdery mildew fungi (Kwon et al., 2008c). Since loss of PEN1 specifically allows enhanced plant cell entry of non-adapted powdery mildews (Collins et al., 2003), this points to the possible involvement of other syntaxins than PEN1 in defense responses against *H. arabidopsidis* and *G. orontii*.

Interestingly, PEN1 exerts together with the closely related SYP122 syntaxin an overlapping function in growth and development because *pen1 syp122* double mutant plants, but not the respective single mutants, are severely dwarfed and necrotic (Assaad et al., 2004). Similarly, the *VAMP721/722*-co-silenced plants are dwarfed, and the *snap33* and the *vamp721 vamp722* double mutant genotypes are lethal at early seedling stage (Assaad et al., 2004; Heese et al., 2001; Kwon et al., 2008c; Zhang et al., 2007), suggesting that the PEN1-SNAP33-VAMP721/722 pathway also serves a role in constitutive secretion during plant growth, transporting materials required for cellular viability to the PM. We here describe a function of VAMP721/722 in sustained growth during immune responses in plants. We found in VAMP721/722-depleted lines upon application of the bacterial epitope flg22, derived from flagellin, an enhanced seedling growth retardation concomitant with reduced accumulation of VAMP721/722 proteins. Confocal imaging of GFP-VAMP722 in transgenic plants and co-labeling with the endocytic tracer FM-4-64 detected the fusion protein in vesicle-like compartments of the secretory and endocytic pathways, suggesting that VAMP722 continuously moves to and is retrieved from the PM. By *in vitro* ternary SNARE complex formation assays and *in vivo* split luciferase assays, we also show that the PM-resident syntaxin SYP132 constitutively and preferentially interacts with VAMP721/722. Our study suggests that VAMP721/722 participate in secretory pathways for plant growth through the formation of SNARE complexes with multiple PM-localized syntaxins to release the contents of their endomembrane compartments.

MATERIALS AND METHODS

Plant materials

Cultured Arabidopsis cells were grown in modified B5 medium as previously described (Kombrink and Hahlbrock, 1986). All plants used for experiments were Arabidopsis Col-0 background and grown in growth chambers at 20-23°C with a 10h photoperiod. Plants were germinated on solid Murashige and Skoog (MS) medium containing 0.25% sucrose and grown for two weeks. The indicated genotypes were selected by PCR analysis of genomic DNA and selected plants were transferred to liquid MS medium. After growth for further two days to allow adaptation, the indicated amount of flg22 or MG132 was applied for the indicated time. For subcellular localization assays of VAMP722, transgenic plants expressing functional GFP-VAMP722 driven by its 5′ regulatory sequences (Kwon et al., 2008c) were used. To analyze the extent of growth inhibition by flg22, plants of the indicated genotypes were grown for one week, treated with 100 nM flg22 and allowed to grow five more days. Seedlings of the indicated genotypes were then weighed. To assess the effect of flg22 on cell growth, the size of leaf meshophyll cells was measured. Cell size was calculated by dividing the area of microscopic window by the numbers of mesophyll cells. All experiments were performed at least three times independently and the collected results were tested by the student's *t*-test.

Confocal microscopy

Two-week-old Arabidopsis seedlings grown as described above were used for confocal microscopy. To label the plasma membrane and components of the endocytic pathway, seedlings were exposed to 4.4 μM FM 4-64 (Invitrogen). Fluorescence signals were detected using a LSM 510 META microscope system (ZEISS) with either a × 40 C-Apochromat water immersive or a × 100 Plan-Neofluar iris oil immersive lens with numerical apertures of respectively 1.2 and 1.3 (ZEISS). For spectral imaging of GFP, the samples were excited with the 488 nm laser-line of the Argon-ion laser and the emission light was detected with the META detector array from 494 to 580 nm (ZEISS). FM 4-64 and GFP were simultaneously excited with the 488 nm laser-line of the Argon-ion laser. Emission light was separated by a 545 nm dichroic filter, GFP and FM 4-64 fluorescence were filtered through a 500-530 nm and a 650-710 nm band pass filter respectively before detection. No significant bleedthrough of FM 4-64 signal in the GFP detection channel was observed. Pinhole diameters were set to an identical optical slice thickness of <1.5 μm for both FM 4-64 and GFP detection. For the GFP-VAMP722/FM 4-64 co-localization studies, differentiated epidermal root cells were used. Images were analyzed and processed with the microscope manufacturers' software, Image J 1.42 (National Institute of Health) and the Adobe CS software package (Adobe Systems Incorporated), respectively.

Immunoblot analysis

Total proteins were extracted from the indicated tissues of Arabidopsis seedlings grown as described above or from cultured cells. Equal amounts of proteins were separated on polyacrylamide gels and analyzed with anti-PEN1, anti-VAMP721/ 722 or anti-HSP70 antibody. To monitor the formation of ternary

SNARE complexes *in vitro*, HA-tagged SYP132 (HA-SYP132), GST-fused SNAP33 (GST-SNAP33), VAMP721 and VAMP722 were expressed in *Escherichia coli* and purified as previously described (Yoo et al., 2010). Equimolar amounts of the indicated recombinant proteins were incubated and protein complexes were retrieved by adsorbing GST-SNAP33 to glutathione Sepharose 4B (Amersham). To detect SDS-resistant ternary SNARE complexes, protein samples were not subjected to boiling before loading.

Split luciferase complementation assays

Protoplasts were prepared from three to four week-old Arabidopsis Col-0 plants and transformed as previously described (Fujikawa and Kato, 2007). Freshly prepared protoplasts were transformed with equal amounts (0.5 μg) of the indicated cDNAs fused to the C-terminal end of the sequences coding for either the N-terminal or C-terminal fragment of *Renilla* luciferase, which were cloned into pDuExAn6 or pDuExD7 vectors (Fujikawa and Kato, 2007). To measure the complemented luciferase activity, 12 M of ViviRen (Promega), a *Renilla* luciferase substrate, was added. To normalize the interaction-dependent luciferase activity, the protoplasts were co-transformed with the pMONT vector (Kato et al., 2010) expressing beetle red luciferase (Promega). The activity of beetle red luciferase was measured through a red filter (Kodak Wratten filter No. 29, Kodak, USA) by adding 150 M of luciferin after assaying the complementary *Renilla* luciferase activity. Luminescence from ViviRen did not bleed-through to the red filter (Kato et al., 2010). The interaction activity was expressed in normalized relative luminescence unit (RLU) calculated by dividing the split luciferase activity by the beetle red luciferase activity.

RESULTS

VAMP721/722 are required for sustained seedling growth during immune responses

Plants mount microbe-associated molecular pattern (MAMP)-triggered immunity (MTI) at the cost of other physiological processes such as growth (Gomez-Gomez et al., 1999; Zipfel et al., 2006). The FLS2 receptor, which detects the presence of bacterial flagellin, is known to restrict bacterial growth in part by limiting entry of the bacteria into the leaf interior through stomata (Melotto et al., 2006; Zipfel et al., 2004). To test a potential involvement of VAMP721/722 in growth during MTI, we first examined FLS2 receptor-dependent growth inhibition in 7 day-old seedlings upon treatment with flg22, the elicitor-active peptide derived from flagellin (Gomez-Gomez et al., 1999). Consistent with the original finding (Gomez-Gomez et al., 1999), *fls2* plants show markedly reduced seedling growth retardation in comparison to WT in the presence of flg22 (Fig. 1A). This growth retardation was associated with a flg22-induced reduction in mesophyll cell size (Fig. 1B and Supplementary Fig. 1). Growth retardation of *vamp721* and *vamp722* single mutants in response to flg22 was indistinguishable from WT (Fig. 1A). Since *vamp721 vamp722* double mutants are early seedling lethal (Zhang et al., 2011), we inspected flg22-triggered growth retardation in progeny of haplo-insufficient *VAMP721*$^{-/-}$ *VAMP722*$^{+/-}$ and *VAMP721*$^{+/-}$ *VAMP722*$^{-/-}$ plants. It has been shown that 2/3 of the selfed progeny has the desired *VAMP721*$^{-/-}$ *VAMP722*$^{+/-}$ or *VAMP721*$^{+/-}$ *VAMP722*$^{-/-}$ genotype (Kwon et al., 2008c). We included at least 16 siblings per genotype in each biological replicate. Progeny seedlings of selfed *VAMP721*$^{-/-}$ *VAMP722*$^{+/-}$ and *VAMP721*$^{+/-}$ *VAMP722*$^{-/-}$ plants showed an enhanced flg22-induced seedling growth retardation and mesophyll cell size reduction compared to WT (Figs. 1A and 1B, and Supplementary Fig. 1), suggesting the engagement of VAMP721/722 in sustained growth during FLS2-triggered immune responses. The WT-like growth retardation seen in *vamp721* and *vamp722* single mutants is likely due to the functional redundancy of the corresponding WT genes in plant growth.

To examine a possible role of VAMP721/722 in flg22-triggered secretory processes, we used Arabidopsis suspension-cultured cells, previously shown to respond to flg22 treatment (Benschop et al., 2007; Nuhse et al., 2003). VAMP721/722 were barely detectable in untreated cultured cells (Fig. 2A). Unexpectedly, flg22 treatment dramatically increased the steady-state levels of

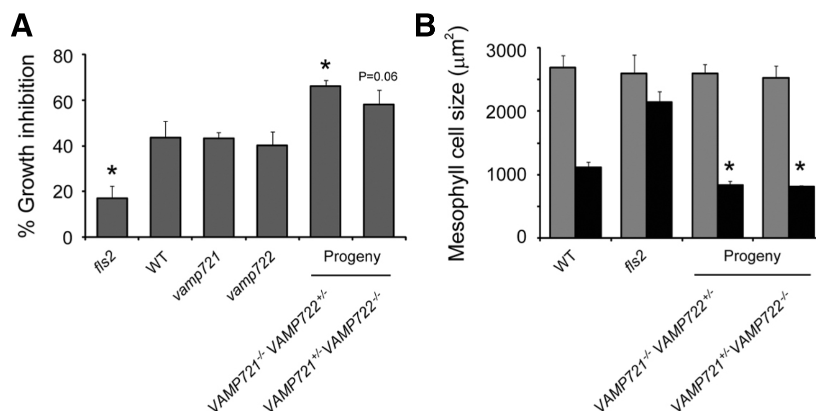

Fig. 1. Depletion of VAMP721/722 enhances flg22-induced seedling growth inhibition. (A) Arabidopsis growth inhibition, stimulated by flg22 application, is enhanced in *VAMP721*$^{-/-}$ *VAMP722*$^{+/-}$ and *VAMP721*$^{+/-}$ *VAMP722*$^{-/-}$ seedlings. Seeds of the indicated genotypes were germinated and grown in liquid MS medium for 7 days. flg22 (100 nM) was then added to the medium and plants were grown for five further days. The degree of growth inhibition in each genotype was calculated by dividing the difference in the weight between non-treated and flg22-treated seedlings by that of non-treated plants (at least 16 plantlets per genotype). Note that because detachment of a leaf from the one-week old plantlets for PCR-based genotyping prior to the growth inhibition assay affected plant growth, we treated intact progeny plantlets derived from selfed *VAMP721*$^{-/-}$ *VAMP722*$^{+/-}$ and *VAMP721*$^{+/-}$ *VAMP722*$^{-/-}$ plants with flg22. We have previously shown a 1:2 segregation ratio of *VAMP721*$^{-/-}$ *VAMP722*$^{+/+}$ and *VAMP721*$^{-/-}$ *VAMP722*$^{+/-}$ genotypes among progeny of selfed *VAMP721*$^{-/-}$ *VAMP722*$^{+/-}$ plants or *VAMP721*$^{+/+}$ *VAMP722*$^{-/-}$ and *VAMP721*$^{+/-}$ *VAMP722*$^{-/-}$ genotypes among progeny of selfed *VAMP721*$^{+/-}$ *VAMP722*$^{-/-}$ plants (Kwon et al., 2008c). (B) Exogenously added flg22 (100 nM) reduces meshophyll cell expansion. The average size of mesophyll cells of the indicated plant genotypes was calculated by dividing the area of microscopic window by cell numbers (19-69 cells per window). Note that cell growth was inhibited more strongly by flg22 in the seedling progeny of selfed *VAMP721*$^{-/-}$ *VAMP722*$^{+/-}$ and *VAMP721*$^{+/-}$ *VAMP722*$^{-/-}$ plants. Gray bars, untreated; black bars, flg22-treated plants. Bar, mean ± standard deviation; asterisk, $P < 0.05$ in comparison to WT.

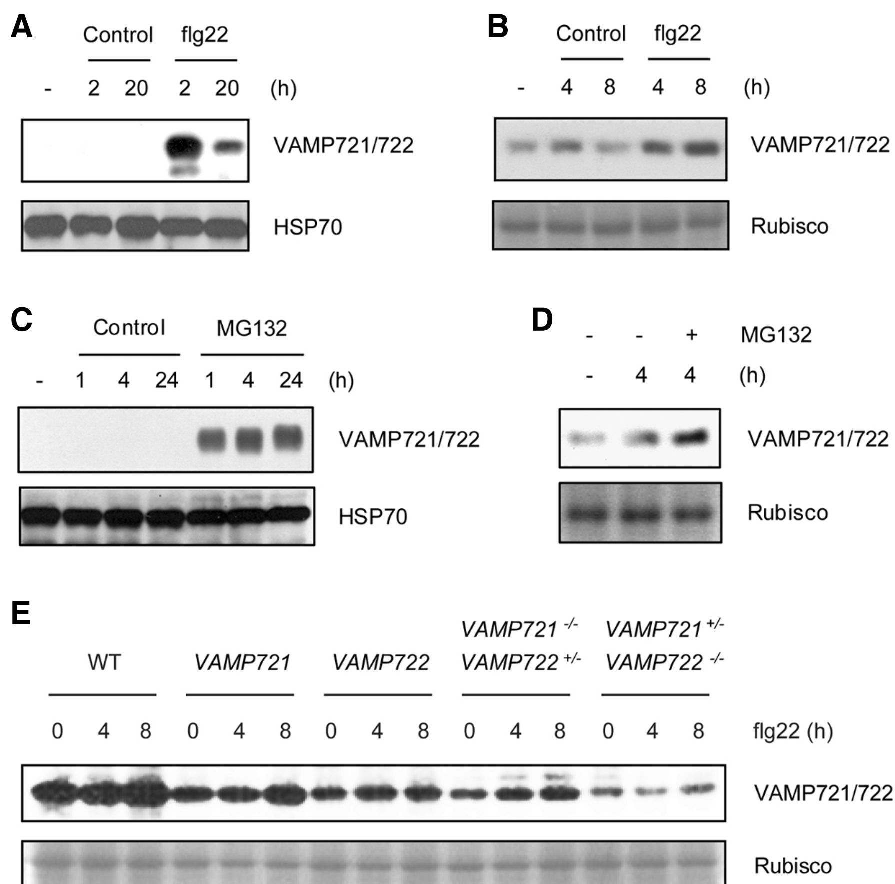

Fig. 2. Bacterial flagellin stabilizes VAMP721/722. Arabidopsis suspension-cultured cells (A, C) or intact seedlings grown in liquid MS medium (B, D) were treated with an active flagellin fragment, flg22, at a concentration of 1 μM (A) or 10 μM (B) or with 10 nM (B) or 100 nM (D) MG132, a 26S proteasome inhibitor for the indicated time. (E) Reduced and delayed accumulation of VAMP721/722 proteins by flg22 in *VAMP721*$^{-/-}$ *VAMP722*$^{+/+}$ and *VAMP721*$^{-/-}$ *VAMP722*$^{+/-}$ genotype plants. The indicated genotype plants were grown in a sterile condition and treated with 10 μM flg22 for the indicated time. Proteins were extracted with 1× PBS containing 1% Triton X-100. Equal amounts of total proteins were separated on polyacrylamide gels and probed with the indicated antibodies. HSP70 (A and C) or Rubisco (B, D, and E) was used as a control for equal loading.

VAMP721/722 (Fig. 2A). Next we examined potential MAMP-triggered changes of VAMP721/722 abundance *in planta*. We treated two week-old seedlings grown in liquid medium with flg22. In contrast to the root-derived Arabidopsis cell culture, we found in untreated seedlings significant amounts of VAMP721/722 (Fig. 2B). Upon flg22 treatment, VAMP721/722 levels were elevated in the seedlings (4 and 8 h time points, Fig. 2B). Interestingly, in suspension cultured cells flg22 treatment dramatically increased the steady-state levels of VAMP721/722 within 10 min (Supplementary Fig. 2). Transcriptional activation of *VAMP721/722* is unlikely to explain the rapid increase of VAMP721/722 abundance within 10 min after flg22 application. We thus suspected the involvement of a post-translational control mechanism. We investigated the effect of MG132, an inhibitor of the 26S proteasome, in the suspension cultured cells. Indeed, a single application of MG132 increased the abundance of VAMP721/722 within 1 h (Fig. 2C), indicating the constitutive degradation of VAMP721/722 by the ubiquitin-proteasome pathway in non-stimulated cells. These results also suggest that the recognition of bacterial flagellin rapidly stabilizes VAMP721/722 by inhibiting 26S proteasome activity. The protein levels of VAMP721/722 were diminished at 20 h after flg22 treatment (Fig. 2A). Since the early up-regulation of VAMP721/722 by flg22 was maintained until 6 h (Supplementary Fig. 2), this suggests that VAMP721/722 stability is decreased between 6 and 20 h after flg22 recognition in plants. MG132 treatment of the seedlings also increased the abundance of VAMP721/722 (Fig. 2D), which is reminiscent of the 26S proteasome inhibitor-induced stabilization in the suspension-cultured cells.

Since flg22 treatment stabilized VAMP721/722 proteins although it triggered hyper-sensitive growth inhibition in both VAMP721/722-depleted lines (*VAMP721*$^{-/-}$ *VAMP722*$^{+/-}$ and *VAMP721*$^{+/-}$ *VAMP722*$^{-/-}$) (Fig. 1A), we then investigated the protein levels of VAMP721/722 in plants containing different dosages of *VAMP721/722* genes. As we previously reported (Kwon et al., 2008c), the abundance of VAMP721/722 is greatly reduced in *VAMP721*$^{-/-}$ *VAMP722*$^{+/-}$ and *VAMP721*$^{+/-}$ *VAMP722*$^{-/-}$ plants (Fig. 2E). Although a slight increase of VAMP721/722 levels were observed, the absolute levels of VAMP721/722 in *VAMP721*$^{-/-}$ *VAMP722*$^{+/-}$ and *VAMP721*$^{+/-}$ *VAMP722*$^{-/-}$ plants were significantly low compared to WT plants (Fig. 2E). Since VAMP721/722 are involved in plant growth and immunity (Kwon et al., 2008c), this suggests that less allocation of VAMP721/722 to plant growth in *VAMP721*$^{-/-}$ *VAMP722*$^{+/-}$ and *VAMP721*$^{+/-}$ *VAMP722*$^{-/-}$ plants in the presence of flg22 may result in hyper-sensitive growth inhibition. This also suggests that VAMP721/722 play an important role in sustained growth during immune responses triggered by flg22.

In vitro and *in vivo* interactions between SYP132 and VAMP721/722

VAMP721/722 form the ternary SNARE complex with the PM-localized PEN1 syntaxin for extracellular immune responses to filamentous fungal pathogens (Collins et al., 2003; Kwon et al., 2008c). Since the depletion of VAMP721/722 in *VAMP721*$^{-/-}$

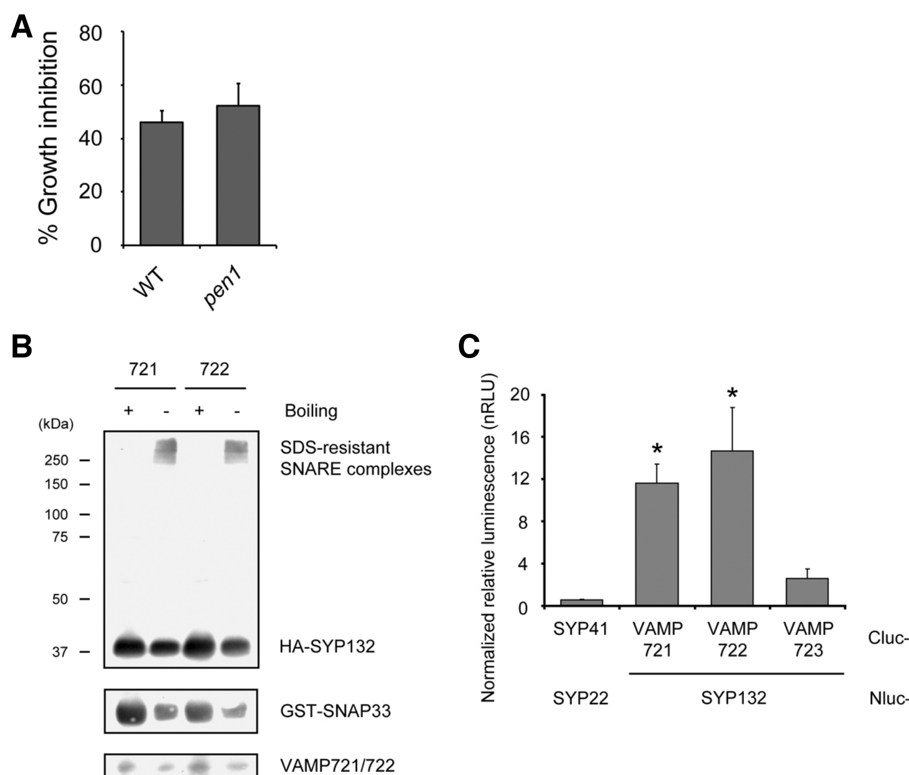

Fig. 3. The plasma membrane syntaxin SYP132 interacts with VAMP721/722 *in vitro* and *in vivo*. (A) No effect of PEN1 removal on growth inhibition by flg22. The indicated genotype plants were grown and the growth inhibition was tested as in Fig. 1A. (B) SYP132 forms SDS-resistant ternary SNARE complexes with SNAP33 and VAMP721/722. Equimolar (1 μM) amounts of the indicated recombinant proteins were mixed and incubated at 4°C overnight. After retrieving the interacted proteins by precipitating GST-SNAP33 with glutathione Sepharose 4B, the matrix-bound complexes were subjected to immunoblot analysis using anti-HA, anti-GST or anti-VAMP721/722 antibody. To detect SDS-resistant but heat-labile ternary SNARE complexes, boiled and non-boiled protein samples were compared. Note that the appearance of high molecular weight complexes correlates with a decrease in levels of the component proteins, HA-SYP132, GST-SNAP33 and VAMP721/722. (C) SYP132 preferentially interacts with VAMP721/722 *in vivo*. SYP132 fused to the N-terminal fragment of luciferase was co-expressed in protoplasts with the indicated VAMP conjugated with the C-terminal fragment of luciferase. Complemented luciferase activities were normalized by the activity of beetle red luciferase that was co-expressed in the protoplasts. As a negative control, the interactions of the TGN-resident Qa SNARE SYP41 and the late endosome-specific Qa SNARE SYP22 were used, and as a cellular compartment control, we used the ER-localizing VAMP723. Bar, mean ± standard deviations from three independent biological replicates. Asterisk, $P < 0.05$ in comparison to the interactions between SYP132 and VAMP723.

VAMP722$^{+/-}$ and *VAMP721*$^{+/-}$ *VAMP722*$^{-/-}$ plants resulted in enhanced growth inhibition by flg22 (Fig. 1A), we then tested the involvement of PEN1 in sustained growth during flg22-driven immune responses. For this, we grew *pen1* plants in a sterile condition, treated with flg22 and measured the degree of growth inhibition. We found that growth inhibition in *pen1* plants did not significantly differ from that in WT plants (Fig. 3A). This suggests that other PM syntaxin(s) than PEN1 is engaged in the sustained growth in the presence of flg22 by forming the SNARE complex with VAMP721/722.

In *N. benthamiana*, gene silencing of *NbSYP132*, but not *NbSYP121*, a *PEN1* ortholog, reduces the secretion of PR-1 as well as the ability to effectively restrict the growth of *Pstab* expressing AvrPto (Kalde et al., 2007). Since PEN1 in Arabidopsis appears to specifically limit the entry of non-adapted powdery mildew fungi into epidermal cells (Collins et al., 2003; Kwon et al., 2008c), NbSYP132 has been proposed to participate in SNARE complex-driven exocytosis required for resistance to bacteria. SYP132 is localized to the PM in Arabidopsis leaves and roots (Enami et al., 2009). Interestingly, the isolation of homozygous *syp132* Arabidopsis plants failed and the *NbSYT132* silencing resulted in severely growth-defective tobacco plants (Enami et al., 2009; Kalde et al., 2007), indicative of an additional SYP132 function in plant growth and development. We thus tested whether Arabidopsis SYP132 can form a ternary SNARE complex with SNAP33 and VAMP721/722 *in vitro*. We expressed, purified, and incubated recombinant HA-tagged SYP132 (HA-SYP132), glutathione-*S*-transferase (GST)-fused SNAP33 (GST-SNAP33), as well as VAMP721 and VAMP722 from *E. coli*. Protein complexes were recovered by adsorption of GST-SNAP33 to glutathione Sepharose 4B, and analyzed for the presence of SDS-resistant ternary SNARE complexes by immunoblot analysis with the anti-HA antibody. The presence of high molecular weight bands that disappeared by boiling prior to electrophoresis indicated that SYP132 formed SDS-resistant ternary SNARE complexes with both VAMP721 and VAMP722 *in vitro* (Fig. 3B). The reduction of retrieved monomeric SYP132, SNAP33 and VAMP721/722 coincident with the appearance of ternary SNARE complex further supports their cooperative involvement in SNARE complex formation (Fig. 3B).

To elucidate their interactions *in vivo*, we transiently and simultaneously expressed SYP132 fused to the N-terminal fragment of luciferase (Nluc-SYP132) and VAMP721 or VAMP722 fused to the C-terminal fragment of luciferase (Cluc-VAMP721 or Cluc-VAMP722) in Arabidopsis protoplasts. Neither fragment of the enzyme possesses luciferase activity, but interactions between two proteins fused to each fragment reconstitute luciferase activities (Fujikawa and Kato, 2007). We therefore assayed for luciferase activity in protoplasts co-expressing Nluc-SYP132 and either Cluc-VAMP721 or Cluc-VAMP722. To investigate potential non-physiological associations due to overexpression, we assayed, as a negative control, the interaction between the trans-Golgi network (TGN)-localized Qa SNARE

SYP41 and the late-endosome Qa SNARE SYP22. Both belong to the same (Qa) class of the SNARE superfamily, but have different subcellular localizations and have been reported not to interact with each other (Sanderfoot et al., 2001; Uemura et al., 2004). In addition, we used the interaction between the PM-localized SYP132 and the ER-resident VAMP723 (Uemura et al., 2004) as a control for compartment specificity. No detectable luciferase activity was observed in protoplasts co-expressing Cluc-SYP41 and Nluc-SYP22 (Fig. 3C), indicating that these Qa SNAREs do not interact with each other in protoplasts. However, high levels of luciferase activity were observed upon co-expression of Nluc-SYP132 and either Cluc-VAMP721 or Cluc-VAMP722 (Fig. 3C), demonstrating that SYP132 interacts with VAMP721/722 *in vivo*. The very low luciferase activity seen in protoplasts co-expressing Nluc-SYP132 and the Cluc-VAMP723 (Fig. 3C) suggests that PM-localized SYP132 predominantly interacts with PM-destined VAMP721/722 (Enami et al., 2009).

VAMP722 continuously moves to and is retrieved from the plasma membrane

To better understand the role of VAMP721/722 in cellular trafficking, we analyzed their expression and localization patterns. We first compared the abundance of these proteins in roots and leaves by immunoblot analysis with anti-VAMP721/722 antibody and found that their steady-state levels are much higher in roots (Supplementary Fig. 3A). This is consistent with previously observed barely detectable GFP-VAMP722 fluorescence in healthy leaves (Kwon et al., 2008c). By taking advantage of transgenic plants expressing functional *GFP-VAMP722* from native 5′ regulatory sequences (Kwon et al., 2008c), we analyzed the localization of GFP-VAMP722 in the root tissue by confocal microscopy. To examine whether VAMP722-associated endomembrane compartments are destined for exocytosis and/or derived from endocytosis, we treated the GFP-VAMP722-expressing transgenic plants with FM 4-64 a fluorescent lipophylic dye used to trace the endocytic pathway (Dhonukshe et al., 2008) and studied the dynamics of the labeled endomembrane compartments by time-resolved confocal microscopy. After exposure of roots to FM 4-64 for 13 min, GFP-VAMP722 signals were found to mark two distinct populations of mobile vesicle-like structures. One population of GFP-VAMP722 structures co-localized with endomembrane compartments labeled by FM 4-64, but the other population did not (Supplementary Fig. 3B). These two populations were still largely distinguishable after treatment with FM 4-64 for 47 min (Supplementary Fig. 3C). The GFP-VAMP722 compartment diameters show a Gaussian distribution and are, like in leaf epidermal cells, heterogeneous in size, with values ranging from 0.4 to 1.2 μm and an average size of 0.73 μm ± 0.20 μm (mean ± SD) (Supplementary Fig. 3D). The population of FM 4-64 labeled compartments varied in size from 0.2 to 1.0 μm, with an average of 0.50 μm ± 0.18 μm (Supplementary Fig. 3D), which is significantly different from the GFP-VAMP722 labeled population ($P < 0.001$). Quantification of the portion of GFP-VAMP722 structures co-labeling with FM 4-64 over time showed that a large fraction of the GFP-VAMP722 compartments labels with FM 4-64 within 15 min that the labeled fraction varies strongly between cells, but on average only slightly increases in the time interval studied (Supplementary Fig. 3E). A similar trend was observed when GFP-VAMP722 compartments were categorized according to a size above or below the average GFP-VAMP722 compartment size (0.73 μm) (Supplementary Fig. 3E). The rapid labeling of a portion of GFP-VAMP722 vesicles with FM 4-64 suggests that these endomembrane compartments are part of the endocytic pathway. The fraction that does not label with FM 4-64 even after prolonged incubation likely are GFP-VAMP722 containing vesicles of the secretory or intracellular vesicle transport pathways.

DISCUSSION

To maintain secretory activity in eukaryotic cells, efficient retrieval or recycling of components of exocytic vesicles for further re-use is plausible. In human cells, the constitutive secretion of luminal cargo from post-Golgi vesicles is accompanied with a partial or no release of membrane cargo during ‘kiss-and-run’ exocytosis and this is controlled by components of the endocytic machinery (Jaiswal et al., 2009). After neurotransmitter release at nerve synapses (regulated secretion) two distinct but clathrin-dependent endocytic vesicles are formed for a single exocytic vesicle fusion; fast endocytosis to retrieve the components of exocytic vesicles with high fidelity and slow endocytosis to ‘clean up’ the remaining vesicle proteins (Zhu et al., 2009). The fact that VAMP721/722 form *in vivo* SNARE complexes with the PM-resident PEN1 and SYP132 syntaxins (Fig. 3) (Kwon et al., 2008c) suggests that mobile endomembrane compartments marked by GFP-VAMP722 are generated for exocytosis. It was recently reported that VAMP721/722 are co-localized with a trans-Golgi network (TGN)/early endosome marker VHA-a1-GFP (Zhang et al., 2011), indicating the trafficking function of VAMP721/722 at TGN. The intracellular accumulation of a PM marker GFP-Lti6a in the *vamp721 vamp722* plants (Zhang et al., 2011) supports the secretory role of VAMP721/722 from TGN to the PM. We found that a part of GFP-VAMP722 vesicles are also labeled with an endocytic marker FM 4-64 (Supplementary Fig. 3). Since VAMP721/722 are components of a constitutive secretory pathway (Kwon et al., 2008c), the GFP-VAMP722/FM 4-64 co-labeled compartments likely serve a role in retrieving VAMP722 for re-utilization to maintain constitutive cellular trafficking. Interestingly, the GFP-VAMP722 compartments are significantly larger than the GFP-VAMP722/FM 4-64 co-labeled population (Supplementary Fig. 3), suggesting that these GFP-VAMP722-containing compartments might be multivesicular bodies (MVBs) (Otegui and Spitzer, 2008; Richter et al., 2007). The size variation of the GFP-VAMP722 compartments alternatively suggests compound exocytosis in which vesicles fuse with each other before their fusion with the PM (Pickett and Edwardson, 2006).

In cultured Arabidopsis cells, VAMP721/722 appear to be constitutively degraded via the 26S proteasome and become rapidly stabilized upon application of the bacterial MAMP flg22 (Figs. 2A and 2C). A qualitatively similar stabilization was observed in intact Arabidopsis seedlings (Fig. 2B and 2D). However, these results were unexpected because we found that VAMP721/722-depleted plants (*VAMP721*$^{-/-}$ *VAMP722*$^{+/-}$ and *VAMP721*$^{+/-}$ *VAMP722*$^{-/-}$ in Fig. 1) show an enhanced cell/seedling growth inhibition phenotype in response to flg22. Transgenic *35S::RNAi*$_{VAMP722}$ gene silencing lines in which both *VAMP721* and *VAMP722* transcript levels are specifically and severely reduced become dwarfed (Kwon et al., 2008c). This dwarfism is also accompanied by a reduction of leaf mesophyll cell size of approximately 60% (Kwon et al., 2008c). This and the early seedling lethality of *vamp721 vamp722* double mutants demonstrate an essential and redundant role of the corresponding WT genes in growth and development (Kwon et al., 2008c; Zhang et al., 2011). The same two proteins are also required for effective growth restriction of powdery mildew fungi

and the oomycete *H. arabidopsidis* (Kwon et al., 2008c). Thus, it is conceivable that in rapidly growing 7 day-old seedlings or in actively dividing suspension-cultured cells stimulation of MTI by flg22 perception causes a conflict on the utilization of VAMP721/722 endomembrane compartments for growth or defense responses. In this scenario, the observed enhanced cell/seedling growth inhibition in *VAMP721*$^{-/-}$ *VAMP722*$^{+/-}$ and *VAMP721*$^{+/-}$ *VAMP722*$^{-/-}$ genotypes could be explained by a prioritization of secretion for immune responses over host cell growth despite the elevated levels of VAMP721/722. Indeed, VAMP721/722 accumulation by flg22 treatment was significantly delayed in the VAMP721/722-depleted plants (Fig. 2E), likely resulting in less utilization of VAMP721/722 for plant growth. Thus, our study has identified VAMP721/722 as shared components contributing to the growth/defense conflict. One possible mechanism underlying this conflict could be a switch from constitutive to cue-induced exocytosis ('kiss-and-run' versus complete fusion with the PM) (Jaiswal et al., 2009; Zhu et al., 2009). Indirect evidence for this comes from the detection of unusual extracellular transport of PEN1 and its interacting partner SNAP33 at pathogen contact sites, possibly involving exosome biogenesis/release (Meyer et al., 2009). Although elevated VAMP721/722 abundance and enhanced growth inhibition of the VAMP721/722-depleted plants (*VAMP721*$^{-/-}$ *VAMP722*$^{+/-}$ and *VAMP721*$^{+/-}$ *VAMP722*$^{-/-}$) in response to flg22 (Figs. 1 and 2) may suggest a possible involvement of VAMP721/722 to defense against bacterial pathogens, we failed to observe a significant difference in bacterial growth after spray-inoculation between WT and the VAMP721/722-depleted plants (data not shown). This might be due to that a residual copy of either *VAMP721* or *VAMP722* gene would be sufficient to secrete antimicrobial cargo for effective resistance to bacterial pathogens. Alternatively, it is possible that in defense against bacterial pathogens more VAMPs than VAMP721/722 function redundantly. It was reported that multiple secretory pathways even within an activated T cell are present to deliver distinct cargos (Huse et al., 2006). Therefore, it is possible that during immune responses although a majority of VAMP721/722 is utilized for releasing antimicrobial cargo a small portion is still involved in secreting growth-related material, leading to sustained growth even during immune responses.

Here we identified the PM-resident syntaxin SYP132 as additional *in vitro* and *in vivo* interacting partner of VAMP721/722 (Fig. 3). Despite the over-expression in protoplasts, essentially undetectable interactions between SYP22 and SYP41 as well as low-level associations between SYP132 and another VAMP72 family member, VAMP723, strongly suggest that the selective pairing between SYP132 and VAMP721/722 is physiologically relevant. Failure to isolate the homozygous *syp132* Arabidopsis plants and severe tobacco growth defect caused by gene silencing of *NbSYP132* (Kalde et al., 2007) suggest that SYP132 is critical for plant growth and development like VAMP721/722. Since no obvious difference in growth inhibition by flg22 between WT and *pen1* plants was observed (Fig. 3A), it is suggested that SYP132 rather than PEN1 is engaged in the secretion of growth-related cargo transported by VAMP721/722 endomembrane containers. However, it was reported that the *pen1 syp122* plants are dwarfed (Zhang et al., 2007), indicating the importance of PEN1 and SYP122 in plant growth. We therefore suggest that the three PM syntaxins PEN1, SYP122 and SYP132 are involved in plant growth and development by forming SNARE complexes with VAMP721/722, although whether VAMP721/722 interact with different PM syntaxins simultaneously or distinctly depending on a cue is unknown. Although SNARE proteins are core factors in the process of vesicle fusions (Weber et al., 1998), completion of vesicle fusions *in vivo* requires additional accessory proteins such as Rab, SM and synaptotagmin (Ungar and Hughson, 2003). It was recently reported that endosomal Arabidopsis VAMP727 forms distinct SNARE complexes with PEN1 at the PM and with SYP22 at the vacuolar membrane (Ebine et al., 2011). Interestingly, the interaction between VAMP727 and PEN1 but not between VAMP727 and SYP22 was reduced in *ara6* mutant plants, suggesting that the plant-unique Rab GTPase Ara6 balances membrane fusions of endomembrane compartments containing VAMP727 with the PM or vacuole by controlling the SYP22-VAMP727 interactions. Therefore, accessory proteins of the secretory machinery could contribute as molecular switches of differential pairings of VAMP721/722 with SYP132 or PEN1.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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