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Synaptotagmin 1 Negatively Controls the Two Distinct Immune Secretory Pathways to Powdery Mildew Fungi in Arabidopsis

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PEN1, one of the plasma membrane (PM) syntaxins, comprises an immune exocytic pathway by forming the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex with SNAP33 and VAMP721/722 in plants. Although this secretory pathway is also involved in plant growth and development, how plants control their exocytic activity is as yet poorly understood. Since constitutive PEN1 cycling between the PM and endocytosed vesicles is critical for its immune activity, we studied here the relationship of PEN1 to synaptotagmin 1 (SYT1) that is known to regulate endocytosis at the PM. Interestingly, *syt1* plants showed enhanced disease resistance to the Arabidopsis-adapted *Golovinomyces orontii* fungus, and elevated protein but not transcript levels of *PEN1*. Calcium-dependent promotion of PEN1–SYT1 interaction suggests that SYT1 controls defense activities of the PEN1-associated secretory pathway by post-translationally modulating PEN1. Increased PEN1–SYT1 interaction and inhibited PEN1 SNARE complex induction by *G. orontii* additionally suggest that the adaption of phytopathogens to host plants might partly result from effective suppression of the PEN1-related secretory pathway. Further genetic analyses revealed that SYT1 also regulates the atypical peroxisomal myrosinase PEN2-associated secretory pathway.

Keywords: PEN1 • PEN2 • Plant immunity • Secretory pathway • SYT1.

Abbreviations: ARF, ADP-ribosylation factor; BFA, brefeldin A; ER, endoplasmic reticulum; GEF, GDP/GDP exchange factor; GFP, green fluorescent protein; GST, glutathione *S*-transferase; PM, plasma membrane; SA, salicylic acid; SM, Sec1/Munc18; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; SYT, synaptotagmin; TM, transmembrane motif; VAMP, vesicle-associated membrane protein; WT, wild type.

Introduction

In plants, as in other eukaryotes, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins are core factors to drive membrane mergence by SNARE complex formation between a vesicle and a target subcellular compartment (Lipka et al. 2007, Sanderfoot 2007). Based on the conserved central amino acid, SNAREs are classified into four target site-localized Q (glutamine)-SNAREs (Q_a, Q_b, Q_c and Q_{b+c}) and one vesicle-residing R (arginine)-SNARE. To drive vesicle fusion, equimolecular distinct SNAREs ($Q_a + Q_b + Q_c + R$ or $Q_a + Q_{b+c} + R$) form the SNARE complex containing four α-helical SNARE domains (Fasshauer et al. 1998, Bock et al. 2001).

In yeast, it is thought that the interaction or fusion specificity resides in a SNARE itself (Jahn and Scheller 2006). However, the in vitro promiscuous interactions between animal SNAREs suggest that the participation of a SNARE in a specific vesicle fusion in animals should be determined by regulatory proteins at various levels such as tissue/development-specific expression, intracellular localization and complex formation, rather than SNAREs themselves (Jahn and Scheller 2006). Likewise, fusion specificity of plant SNAREs is thought to be controlled by accessory proteins, because they also promiscuously form SNARE complexes with biologically non-relevant partners. Although PEN1 in plants forms the SNARE complex during defense against powdery mildew fungi with its cognate partners, vesicle-associated membrane proteins (VAMPs) 721 and 722, it also forms SNARE complexes in vitro with VAMP724 and VAMP727 which are not related to plant immunity (Kwon et al. 2008b). Engagement in distinct biological processes of the same SNAREs additionally supports the importance of regulatory factors in intracellular vesicle trafficking in plants. For instance, VAMP721 and 722, which are involved in resistance to powdery mildew fungi together with PEN1 and through carrying the powdery mildew resistance protein RPW8.2 on their vesicles to the extrahaustorial membrane (Kim et al. 2014), also play

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a role in plant growth and abiotic stress responses (Kwon et al. 2008b, Yi et al. 2013).

Major animal regulators of vesicle fusion are small GTPases, Sec1/Munc18 (SM) proteins and synaptotagmins (SYTs) (Jahn and Scheller 2006, Sudhof and Rothman 2009, Jahn and Fasshauer 2012). Plant counterparts were also reported to regulate vesicle trafficking. In plants, the Sec1 homolog KEULE (also called SEC11) binds KNOLLE and renders it better at interacting with other SNAREs or fusion competent at the cell plate-forming area in dividing cells (Assaad et al. 2001, Park et al. 2012). KEULE was also reported to compete with SNAP33 and VAMP721 for PEN1 interaction to control the PEN1-dependent secretion (Karnik et al. 2013, Karnik et al. 2015). The plant-specific Rab GTPase Ara6 regulates the SNARE complex formation of VAMP727 with SYP22, SYP51 and VTI11 at the vacuole but with PEN1 at the PM (Ebine et al. 2011). The barley ADP-ribosylation factor (ARF) GTPase ARFA1b/1c modulates disease resistance to powdery mildew fungi by controlling focal accumulation of Ror2 that is the barley PEN1 ortholog at fungal entry sites (Bohlenius et al. 2010). Similarly, the ARF-GDP/GDP exchange factor (GEF) GNOM was reported to control focal accumulation of PEN1 at fungal entry sites in Arabidopsis in a timely fashion (Nielsen et al. 2012). Recent studies have shown that SYT1 is preferentially localized to endoplasmic reticulum (ER)–PM contact sites (Levy et al. 2015, Perez-Sancho et al. 2015), which is aided by reticulon proteins (Kriechbaumer et al. 2015). It plays an important role in the repair and/or stabilization of the PM under salt, cold and mechanical stresses (Schapire et al. 2008, Yamazaki et al. 2008, Perez-Sancho et al. 2015), and defense against viruses (Levy et al. 2015). Studies with dominant-negative SYT1 forms also suggest that SYT1 controls endocytosis and the recycling of endosomes to the PM (Lewis and Lazarowitz 2010).

PEN1 is known to cycle continuously between the PM and endosomes (Reichardt et al. 2011). In addition, interference with endosome recycling to the PM by brefeldin A (BFA) severely inhibits the PEN1 focal accumulation at fungal entry sites which accompanies delayed callose deposition and compromised defense against *Blumeria graminis* powdery mildew fungus (Nielsen et al. 2012). These results strongly suggest that the endocytosis-associated recycling of PEN1 at the PM is critical for its immune function. Interestingly, the endocytosis-controlling SYT1 was reported to be concentrated around oomycete haustoria like PEN1 (Meyer et al. 2009, Lu et al. 2012, Bozkurt et al. 2014). Therefore, we investigated a possible relationship between PEN1 and SYT1 using biochemical and genetic approaches. Here we report that the lack of SYT1 causes an elevation of pre-invasive resistance to powdery mildew fungi, which is partially dependent on increased PEN1 protein levels. In Arabidopsis, the peroxisomal PEN2 myrosinase and the PEN3 ABC transporter are in parallel with the PEN1 exocytic pathway engaged in pre-invasive resistance to powdery mildew fungi by generating and secreting toxic indole glucosinolate(s) (Lipka et al. 2005, Stein et al. 2006, Bednarek et al. 2009). Additional requirement of PEN2 for enhanced defense in *syt1* plants indicates that SYT1 controls both the PEN1- and PEN2-associated secretory pathways. Increased interactions of PEN1 with SYT1, and suppressed induction of the PEN1 SNARE complex formation by the adapted *Golovinomyces orontii* fungus additionally suggest that this adapted pathogen overcomes the host plant immune responses by inhibiting PEN1 and PEN2 immune secretory pathways.

Results and Discussion

PEN1 is known to cycle continuously between the PM and endosomes (Reichardt et al. 2011). Indeed, inhibition of PEN1 recycling from endosomes to the PM by BFA compromises its focal accumulation at fungal entry sites, resulting in impaired pre-invasive resistance to powdery mildew fungi (Nielsen et al. 2012). Since SYT1 was reported to localize in ER–PM contact sites (Levy et al. 2015, Perez-Sancho et al. 2015), to regulate the endocytosis between the PM and endosomes (Lewis and Lazarowitz 2010) and to be accumulated around oomycete haustoria (Lu et al. 2012, Bozkurt et al. 2014), we tested a relationship between PEN1 and SYT1 in pre-invasive resistance in Arabidopsis. We first examined resistance to the Arabidopsis-adapted *G. orontii* powdery mildew fungus in *syt1* plants. Interestingly, we found that *G. orontii* growth was markedly reduced in *syt1* plants compared with the wild type (WT) (**Fig. 1A**). Plants possess two distinct layers of defense against powdery mildew fungi (Kwon et al. 2008a). Pre-invasive resistance contributed by the PEN1 PM syntaxin and the PEN2 atypical myrosinase mainly stops fungal initial entry via blocking the formation of fungal haustoria (Collins et al. 2003, Lipka et al. 2005, Kwon et al. 2008b, Bednarek et al. 2009). However, post-invasive resistance governed by salicylic acid (SA) typically limits fungal growth after haustorial formation (Lipka et al. 2005). Therefore, we then investigated the entry rate of *G. orontii* and found that the fungal entry rate was significantly reduced in *syt1* plants compared with the WT (**Fig. 1B**). These results suggest that SYT1 plays a negative role in pre-invasive resistance to powdery mildew fungi in plants.

While PEN1 is localized to the PM, PEN2 is attached to peroxisomes (Collins et al. 2003, Lipka et al. 2005, Meyer et al. 2009). Since SYT1 is mainly located at ER–PM contact sites (Levy et al. 2015, Perez-Sancho et al. 2015) and the fungal entry rate was altered in *syt1* plants (**Fig. 1B**), we then tested whether the amount of PEN1 protein was changed in *syt1* plants. By analyzing PEN1 protein levels with immunobloting with anti-PEN1 antibody, we found that PEN1 protein is more accumulated in *syt1* plants than in WT plants (**Fig. 2A**). However, *PEN1* transcript levels were similar in WT and *syt1* plants (**Supplementary Fig. S1**), indicating that SYT1 negatively regulates the steady-state levels of PEN1 protein without changing the expression of *PEN1*. Interestingly, inhibition of protein degradation using the 26S proteasome inhibitor MG132 remarkably increased PEN1 levels in WT plants (**Supplementary Fig. S2**), supporting an important post-translational control of PEN1.

PEN1 is a component of the ternary PEN1–SNAP33–VAMP721/722 SNARE complex (Kwon et al. 2008b). Therefore, we next examined the abundance of

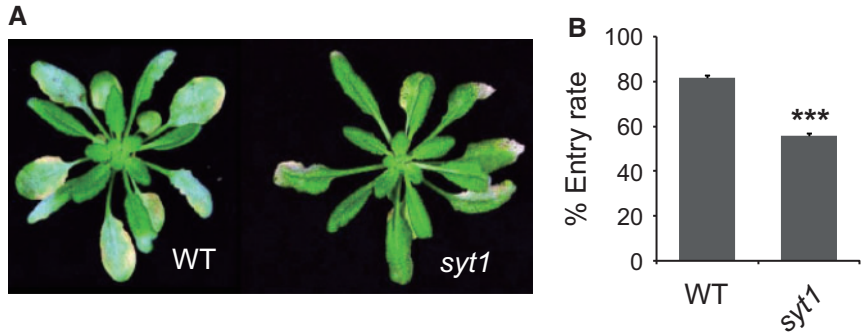

Fig. 1 Elevated disease resistance to *G. orontii* in *syt1* plants is associated with enhanced pre-invasive resistance. (A) Reduced growth of *G. orontii* on *syt1* plants. The plants of the indicated genotypes were inoculated with *G. orontii* conidiospores and the fungal growth was macroscopically observed at 9 d post-inoculation. (B) Increased pre-invasive resistance in *syt1* plants. The entry rate of *G. orontii* was measured at 48 h post-inoculation by counting the fungal secondary epiphytic hyphae formed in interacted plant cells. Successfully invaded conidiospores with epiphytic hyphae of the indicated genotype plants were stained with Coomassie blue and analyzed by light microscopy. ***$P < 0.001$ in comparison with the WT. Bar, mean ± SD from four independent biological replicates (five technical replicates for each biological replicate and $n > 100$ interaction cells for each technical replicate).

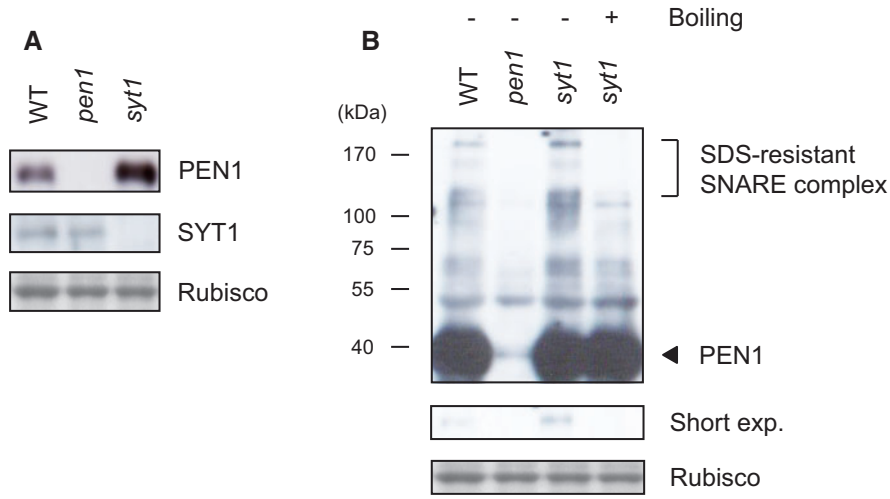

Fig. 2 Deletion of SYT1 increases levels of both PEN1 and the PEN1 SNARE complex. (A) Elevated abundance of PEN1 in *syt1* plants. PEN1 or SYT1 protein levels were analyzed by immunoblot with anti-PEN1 or anti-SYT1 antibody, respectively, using protein extracts from plants of the indicated genotype. (B) Increased SNARE complex containing PEN1 in *syt1* plants. The PEN1 SNARE complexes were analyzed by immuniblot with anti-PEN1 antibody. To detect the SNARE complex that is SDS-resistant but heat-labile, protein samples were not boiled before loading. Total proteins were extracted from plants of the indicated genotype grown for 10 d in liquid MS medium. Equal loading was visualized by staining Rubisco with Coomassie blue. Short exp., short exposure corresponding to the SNARE complex.

PEN1-containing SNARE complex in *syt1* plants. As the ternary SNARE complex is SDS-resistant but heat-sensitive (Hayashi et al. 1994, Kwon et al. 2008b), we detected the PEN1-containing SNARE complex in WT plants by comparing the band disappearance by heat (boiling) in immunoblot with anti-PEN1 antibody (**Fig. 2B**). The PEN1-containing SNARE complex was also observed in *syt1* plants (**Fig. 2B**). However, we detected higher levels of the SNARE complex in *syt1* plants than in WT plants (**Fig. 2B**), which is consistent with the higher levels of PEN1 monomer in *syt1* plants. Since the formation of PEN1 ternary SNARE complex is critical for plant pre-invasive resistance to powdery mildew fungi (Kwon et al. 2008b), these data suggest that elevated defense in *syt1* plants against *G. orontii* fungus (**Fig. 1**) can be attributed to the increased levels of the PEN1 SNARE complex.

Since the presence or absence of SYT1 influenced plant immunity to fungal pathogens concomitant with altered PEN1 levels (**Figs. 1, 2**), we then examined a relationship between PEN1 and SYT1 during defense responses. We first tested their interaction using purified recombinant proteins that were expressed in *Escherichia coli*. We incubated PEN1 and the transmembrane motif (TM)-lacking SYT1 fused with glutathione-*S*-transferase (GST–SYT1ΔTM), precipitated GST–SYT1ΔTM with glutathione–Sepharose 4B, and analyzed the precipitates by immunoblot with anti-PEN1 antibody. The presence of PEN1 in the GST–SYT1ΔTM precipitates (**Fig. 3A**) indicates

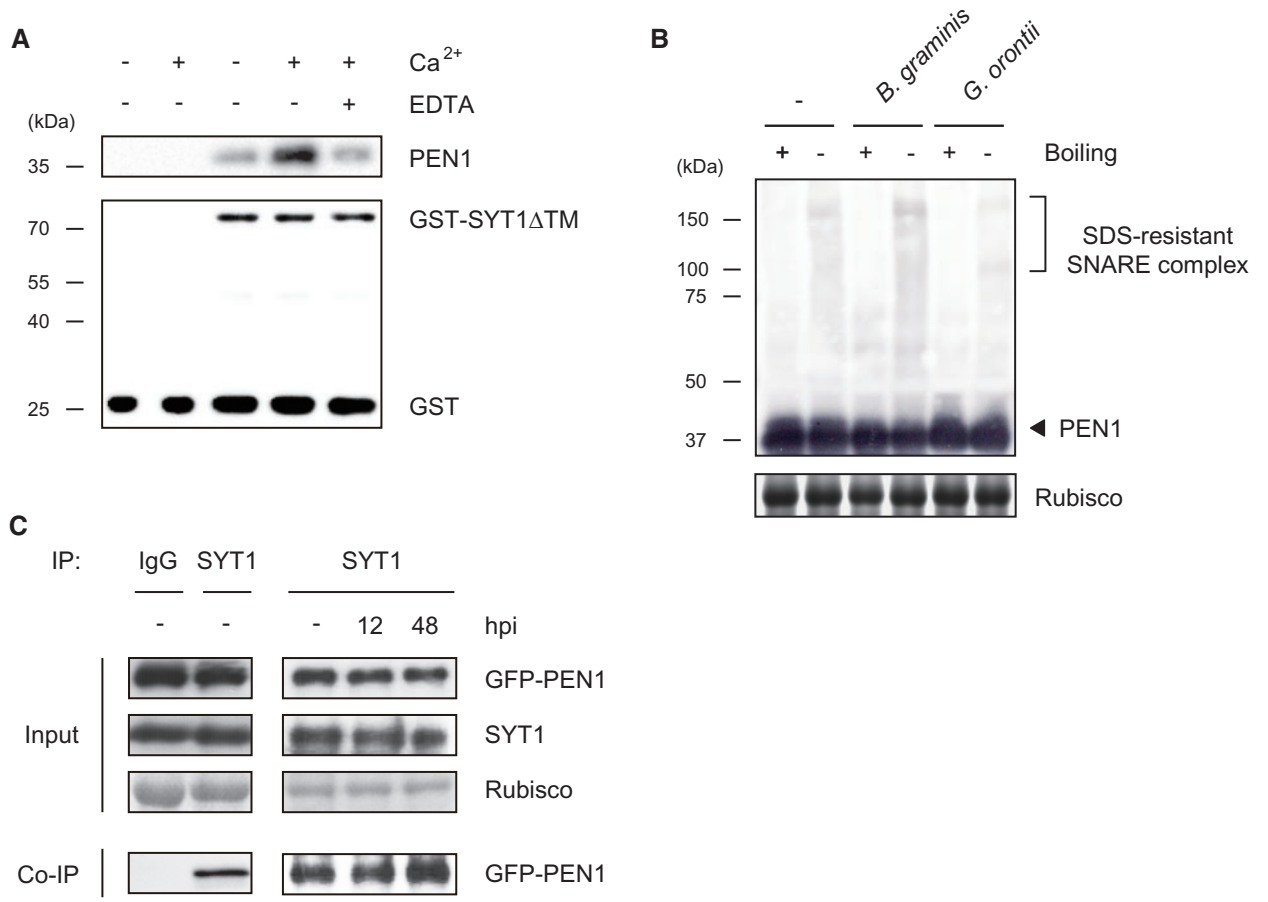

Fig. 3 *G. orontii* modulates the PEN1–SYT1 interaction and suppresses the induction of PEN1 SNARE complex formation. (A) Ca2+-stimulated direct interaction between PEN1 and SYT1. Equimolar recombinant PEN1 and the TM-lacking GST-fused SYT1 (GST–SYT1ΔTM) purified from *E. coli* were incubated in the presence or absence of 10 mM CaCl2. Their interaction was analyzed by immunoblot with anti-PEN1 antibody using the precipitates with GST–SYT1ΔTM. To test a Ca2+ effect on their interaction, 1 mM EDTA was added during incubation. Equal loading was visualized by immunoblot with anti-GST antibody to detect GST–SYT1ΔTM. GST was used as a negative control. (B) Non-induced abundance of the PEN1 SNARE complex by *G. orontii*. WT plants were inoculated with conidiospores of *B. graminis* or *G. orontii*. Proteins extracted from 24 h inoculated plant leaves were subject to immunoblot with anti-PEN1 antibody. To detect the SNARE complex, boiled and non-boiled protein samples were compared. Note that the PEN1 SNARE complex formation was induced by the non-adapted *B. graminis* but not by the adapted *G. orontii*. Equal loading was visualized by staining Rubisco with Coomassie blue. (C) Stimulated in planta interaction between PEN1 and SYT1 by *G. orontii*. Transgenic plants ectopically expressing functional GFP–PEN1 were inoculated with *G. orontii* conidiospores for the indicated time. Extracted proteins from inoculated leaves were precipitated with anti-SYT1 antibody, and the precipitates (Co-IP) were analyzed by immunoblot with anti-GFP antibody to detect GFP–PEN1. To show expression levels of GFP–PEN1 and SYT1, 1% of protein extracts used for co-immunoprecipitation were subject to immunoblot with anti-GFP or anti-SYT1 antibody. Equal loading was visualized by staining Rubisco with Coomassie blue. hpi, hours post-inoculation. Rabbit IgG (IgG) was used as a negative control.

that they directly interact. Since the SYT1-involved PM repair is Ca^{2+}-dependent (Schapire et al. 2008, Yamazaki et al. 2008), we also investigated the PEN1–SYT1 interactions in the presence of Ca^{2+}. Interestingly, we detected greatly increased amounts of PEN1 in the precipitates when Ca^{2+} was added during incubation (**Fig. 3A**). However, this Ca^{2+}-induced elevated interaction between PEN1 and SYT1 was abolished by adding the cation chelator EDTA (**Fig. 3A**. Thus, our in vitro results indicate that Ca^{2+} stimulates the PEN1–SYT1 interaction.

We previously reported that the non-adapted *B. graminis* fungus stimulated the accumulation of PEN1-containing SNARE complexes in plants (Kwon et al. 2008b). Therefore, we tested whether or not the adapted *G. orontii* fungus can modulate the abundance of PEN1 and its SNARE complex to subvert pre-invasive resistance in plants. When plants were inoculated with *B. graminis*, we detected elevated levels of PEN1 SNARE complex which is SDS-resistant but heat-labile in immunoblot using anti-PEN1 antibody as previously reported (**Fig. 3B**). In *G. orontii*-inoculated plants, we found, however, no increased levels of the PEN1 SNARE complex (**Fig. 3B**). This suggests that adapted fungal pathogens may overcome plant pre-invasive resistance at least in part by suppressing the induction of SNARE complex formation probably to inhibit the immune exocytic pathway.

Since SYT1 negatively controls the abundance of PEN1 monomer and its SNARE complex (**Fig. 2**), we next investigated

the in vivo interaction between PEN1 and SYT1 in response to *G. orontii* challenge using co-immunoprecipitation. To exclude any differences in *PEN1* expression which could be caused by *G. orontii* inoculation, we used transgenic plants where functional green fluorescent protein (GFP)-fused PEN1 (GFP–PEN1) is expressed under the 35S promoter (Collins et al. 2003). In these plants, GFP–PEN1 and SYT1 levels were not altered by *G. orontii* inoculation (**Fig. 3C**). Detection of GFP–PEN1 in the immunoprecipitates with anti-SYT1 antibody indicates the in planta PEN1–SYT1 interaction (**Fig. 3C**). Based on the alteration patterns of the PEN1 SNARE complex in response to *B. graminis* inoculation (**Supplementary Fig. S3A**), we investigated PEN1–SYT1 interactions in *G. orontii*-inoculated plants at two different time points, at 12 hours post-inoculation (hpi) at which the PEN1 SNARE complex level was not increased or was comparable with the level in non-inoculated plants, and at 48 hpi at which the PEN1 SNARE complex level was clearly and markedly elevated by *B. graminis* but not by *G. orontii* inoculation (**Supplementary Fig. S3A, B**). While PEN1–SYT1 interaction at 12 hpi after *G. orontii* inoculation was not different from that in non-inoculated plants, their interaction at 48 hpi was enhanced (**Fig. 3C**). Since the abundance of PEN1 and its SNARE complex is elevated in *syt1* plants (**Fig. 2**), this suggests that SYT1 modulates PEN1 levels through direct interaction. Increased interaction between PEN1 and SYT1 (**Fig. 3C**), but non-induced formation of PEN1 SNARE complex by *G. orontii* (**Fig. 3B**), additionally implies that adapted fungal pathogens may manipulate the PEN1-associated immune exocytosis via PEN1–SYT1 interactions to overcome pre-invasive resistance in plants.

Because *G. orontii* probably reduces the Arabidopsis pre-invasive resistance through promoted PEN1–SYT1 interactions (**Fig. 3B, C**), we next investigated whether PEN1 is required for the elevated defense in *syt1* plants. We therefore generated the *pen1 syt1* double mutant plants and examined the *G. orontii* entry rate which was diminished in *syt1* plants compared with the WT (**Fig. 1**). As shown in **Fig. 4A**, the fungal entry rate was significantly higher in the *pen1 syt1* double mutant than in the *syt1* single mutant, but still lower than in WT plants. This suggests that PEN1 contributes partially to the enhanced resistance to *G. orontii* in *syt1* plants.

The peroxisomal PEN2 myrosinase and the PEN3 ABC transporter comprise a distinct secretory pathway from the PEN1 exocytosis (Lipka et al. 2005, Stein et al. 2006, Bednarek et al. 2009). Since the PEN1 immune activity is partially responsible for the elevated defense in *syt1* plants, we then investigated a possible interaction between PEN2 and SYT1 by generating the *pen2 syt1* double mutant. Surprisingly, introduction of the *PEN2* mutation into *syt1* plants completely restored the fungal entry rate to the WT level (**Fig. 4A**), suggesting that PEN2 rather than PEN1 is mostly responsible for SYT1-related defense regulation against *G. orontii*. Macroscopic growth of *G. orontii* in *pen1 syt1* plants was indistinguishable from that in *syt1* plants, although the fungal entry rate was significantly elevated in *pen1 syt1* plants (**Fig. 4A, B**). Because *G. orontii* grew similarly well in *pen2 syt1* plants to WT and *pen2* plants (**Fig. 4B**), it seems that the increased fungal entry rate in *pen1 syt1* plants compared with *syt1* plants does not reach a threshold to affect post-invasive fungal growth. A non-adapted powdery mildew fungus *Erysiphe pisi* can form conidiophores in *pen2 pad1 sag101* and *pen3 eds1* plants where both the PEN2/PEN3 secretory pathway and SA response are abrogated but not in *pen2* and *pen3* single mutant plants (Lipka et al. 2005, Stein et al. 2006). This indicates that post-invasive resistance related to cell death is primarily contributed by SA. Therefore, WT-like growth of *G. orontii* in the *pen2 syt1* plants additionally suggests that the elevated defense in *syt1* plants (**Fig. 1**) is not due to increased cell death but rather to elevated pre-invasive resistance.

Next we investigated relationships between SYT1 and PEN1/PEN2 in pre-invasive resistance to non-adapted powdery mildew fungi, *E. pisi* and *B. graminis*. The entry rate of these fungi was not reduced in *syt1* plants compared with the WT (**Fig. 4C, D**), probably due to maximal or saturated immune activities of PEN1 and PEN2. Greatly elevated entry rates of *E. pisi* and *B. graminis* in *pen1* and *pen2* plants (**Fig. 4C, D**) support the important function of PEN1 and PEN2 in pre-invasive resistance to these fungi. We again observed that introducing a *PEN1* or *PEN2* mutation into the *syt1* background re-elevated the fungal entry rate (**Fig. 4C, D**), indicating that both PEN1 and PEN2 are required for heightened pre-invasive immunity in *syt1* plants. The entry rate of *E. pisi* in *pen2 syt1* plants was higher than that in *pen1 syt1* plants (**Fig. 4C**), while the entry rate of *B. graminis* in *pen2 syt1* plants was lower than that in *pen1 syt1* plants (**Fig. 4D**). In Arabidopsis WT plants, the entry rate of *E. pisi* is higher than that of *B. graminis* (**Fig. 4C, D**) (Lipka et al. 2005), indicating that the former is slightly more adapted to Arabidopsis. Taken together, it is therefore likely that the PEN1-associated secretory pathway and the PEN2/PEN3-related pathway distinctly contribute to pre-invasive resistance depending on the degree of fungal adaptation to a host plant species. The higher entry rate of *B. graminis* in *pen1* plants than in *pen2* plants (**Fig. 4D**) (Lipka et al. 2005) suggests that as fungal pathogens become more adapted to host plants, the PEN2/PEN3 secretory pathway has a more important function than the PEN1-associated exocytic pathway in plant defense against those pathogens. Due to lethality of the *pen1 pen2 syt1* triple mutant before seed production in our growth conditions (data not shown), we were unable to include this genotype plant in our genetic analyses.

We previously showed that PEN2 is localized to peroxisomes whereas PEN3 is localized to the PM (Lipka et al. 2005, Stein et al. 2006). Since SYT1 is preferentially located at the PM–ER contact sites (Levy et al. 2015, Perez-Sancho et al. 2015), one possibility is that SYT1 might regulate the abundance of the PM-localized PEN3 rather than peroxisomal PEN2 as in the case of the PM-residing PEN1 (**Fig. 2**). We first investigated the expression of *PEN2* and *PEN3* in *syt1* plants. By real-time reverse transcription–PCR (RT–PCR), we found that the transcript levels of *PEN2* and *PEN3* are similar in WT and *syt1* plants, like *PEN1* (**Supplementary Fig. S4A, B**), indicating that SYT1 depletion has no effect on *PEN2* and *PEN3* transcription. We then examined PEN3 protein levels by immunoblot with the anti-PEN3 antibody (Lu et al. 2015) and found that unlike PEN1,

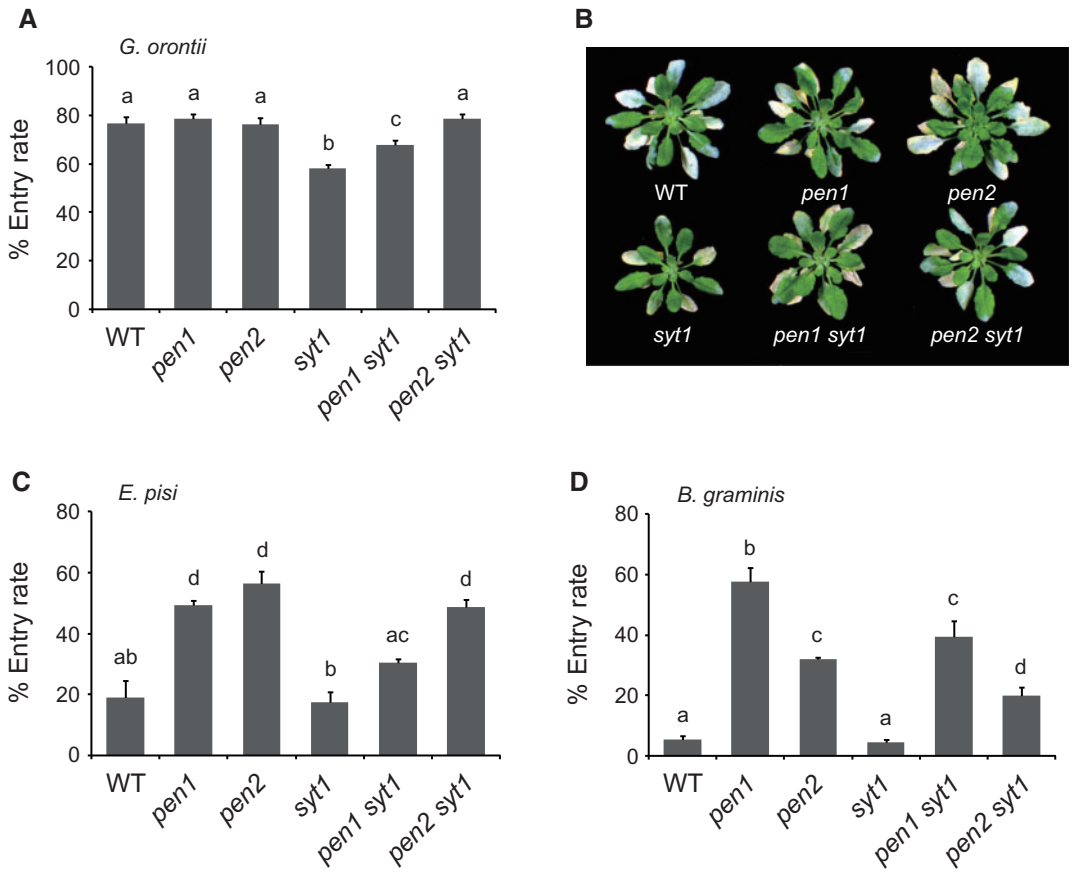

Fig. 4 SYT1 controls pre-invasive resistance contributed by both the PEN1- and the PEN2-related secretory pathways. Introduction of the *PEN1* or *PEN2* mutation into *syt1* plants elevated pre-invasive resistance to *G. orontii* (A), *E. pisi* (C) and *B. graminis* (D). Plants of the indicated genotype were inoculated with conidiospores of *G. orontii* (A), *E. pisi* (C) and *B. graminis* (D). Inoculated leaf materials were stained with Commassie blue for *G. orontii* at 2 days post-inoculation (dpi) and *E. pisi* at 3 dpi and with aniline blue for *B. graminis* at 3 dpi. The entry rate of the indicated powdery mildew fungus in the indicated genotype plant cells was analyzed with fungal haustoria for *B. graminis* and with epiphytic hyphae for *E. pisi* and *G. orontii*. (B) Re-increased and WT-like growth of *G. orontii* in *pen2 syt1* plants compared with *syt1* plants. The plants of the indicated genotype were inoculated with *G. orontii* conidiospores and the fungal growth was macroscopically observed at 9 dpi. Different letters represent significant differences analyzed by one-way ANOVA with Turkey's post-hoc test ($P < 0.05$). Bar, mean ± SD from four independent biological replicates (five technical replicates for each biological replicate and $n > 100$ interaction cells for each technical replicate).

the PEN3 abundance between WT and *syt1* plants is indistinguishable (**Supplementary Fig. S4C**). This indicates that elevated PEN1 levels in *syt1* plants do not result from non-specific interference with 26S proteasome activity. It was reported that focal accumulation of PEN3 at pathogen-attacking sites is BFA insensitive (Underwood and Somerville 2013), suggesting that PEN3 might be recruited to its action sites by a mechanism distinct from PEN1. Taken together, our results suggest that SYT1 post-translationally regulates PEN1 abundance but not that of PEN3. Hence, it is likely that SYT1 controls the PEN2-related immune activity in the PEN2/PEN3 secretory pathway. It is also possible that SYT1 regulates the cellular localization of PEN2 and PEN3.

SNARE proteins are now undoubtedly regarded as core factors to drive vesicle fusion events in diverse physiological processes including cell division and responses to biotic and abiotic stresses in plants (Lipka et al. 2007, Sanderfoot 2007). However, unlike yeast SNAREs but rather similar to those of animals, plant SNAREs themselves do not show fusion specificity. For example, PEN1 promiscuously forms the SNARE complex with members in the VAMP72 subgroup in vitro (Kwon et al. 2008b). In addition, the *trans*-Golgi network and secretory vesicle-residing VAMP721/722 specifically interact with the PM syntaxins PEN1 and SYP132 both in vitro and in vivo (Kwon et al. 2008b, Yun et al. 2013a). Furthermore, the cell division-specific syntaxin KNOLLE was reported to form SNARE complexes during cytokinesis either with SNAP33 and VAMP721/722 or with NPSN11, SYP71 and VAMP721/722 (El Kasmi et al. 2013). These indicate that at least an accessory protein should control the SNARE complex formation with cognate partner SNAREs for a specific biological event-associated vesicle trafficking in plants. In addition to the SNARE complex formation, we previously found that a regulatory factor such as an SM or SYT is required for the full immune activity of PEN1 by an amino acid substitution approach in PEN1 protein (Pajonk et al. 2008). Indeed, it was recently reported that the SM protein KEULE controls the SNARE complex formation of both PEN1 and KNOLLE (Park et al. 2012, Karnik et al. 2013, Karnik et al. 2015).

In animal neuronal cells, SYT1 is a representative Ca^{2+}-dependent regulator of exocytosis (Chapman 2002, Jahn and Fasshauer 2012). In addition, SYT1 is also known to play an important but distinct role in endocytosis (Chapman 2002, Yao et al. 2012). In this report, we used biochemical and genetic approaches to show that the Arabidopsis SYT1 which is preferentially localized to ER–PM contact sites negatively controls pre-invasive resistance to powdery mildew fungi by modulating PEN1 abundance. PEN1, which is a component of the ternary SNARE complex driving fusion between secretory vesicles and the target PM, was reported to cycle continually between the PM and endosomal compartments (Collins et al. 2003, Kwon et al. 2008b, Reichardt et al. 2011, Nielsen et al. 2012). Increased PEN1 levels by MG132 (**Supplementary Fig. S2**) suggest that at least a part of endocytosed PEN1 proteins might be degraded via the ubiquitin–proteasome protein degradation pathway. The elevated PEN1 steady-state level in *syt1* plants (**Fig. 2**) and the in planta PEN1–SYT1 interaction (**Fig. 3C**) therefore suggest that SYT1 probably facilitates the PEN1 endocytosis at the PM for degradation.

Interestingly, the enhanced defense against powdery mildew fungi in *syt1* plants additionally requires PEN2 immune activity (**Fig. 4**). Upon *B. graminis* inoculation, BFA inhibited focal accumulation of PEN1 but not PEN3 at fungal entry sites (Nielsen et al. 2012, Underwood and Somerville 2013), indicating that PEN1 and PEN3 are distinctly concentrated at fungal attacking sites. This and the comparable steady-state levels of PEN3 between WT and *syt1* plants (**Supplementary Fig. S4C**) suggests that SYT1 controls PEN2 function rather than PEN3 in the PEN2/PEN3 secretory pathway. Recently, SYT1 was reported to localize to the ER–PM contact sites in which microtubules are depleted due to tight interaction between the ER and PM (Levy et al. 2015, Perez-Sancho et al. 2015). Upon fungal inoculation, PEN2-attached peroxisomes that move along actin filaments (Mano et al. 2002) are redirected to fungal entry sites (Lipka et al. 2005). Therefore, it is possible that the SYT1-driven ER–PM contact sites may slightly hinder targeted movement of PEN2 peroxisomes to fungal entry sites, which can also be applied to directional migration of VAMP721/722 vesicles. It is also possible that SYT1 regulates PEN3 immune activity via modulating its transporting activity or localization.

By testing disease resistance to powdery mildew fungi in *pen1 syt1* and *pen2 syt1* double mutant plants (**Fig. 3**), we were additionally able to narrow down which of the PEN1-associated and PEN2/PEN3-related secretory pathways dominantly work for defense against differentially adapted powdery mildew fungi. Compared with the former exocytic pathway that is evolutionarily conserved between monocots and dicots (Kwon et al. 2008b), the latter is regarded to be recently innovated in Arabidopsis for defense against fungal pathogens (Bednarek et al. 2009). This may explain why the PEN2/PEN3 pathway acts more broadly in defense against tested powdery mildew fungi. The elevated interactions between PEN1 and SYT1 and the inhibited induction of the PEN1 SNARE complex formation by *G. orontii* additionally suggest that adapted pathogens may have an ability to suppress effectively an ancient immune secretory pathway. SYT1 localizes to the ER–PM contact site to repair the damaged PM for tolerant responses to abiotic stresses such as cold, salt and mechanical wounding (Schapire et al. 2008, Yamazaki et al. 2008, Perez-Sancho et al. 2015). PEN1 that is also responsible for delivering a PM aquaporin and a potassium channel (Honsbein et al. 2009, Grefen et al. 2010, Besserer et al. 2012, Hachez et al. 2014), and its partners VAMP721/722 are required for responses to abiotic stresses and the abiotic stress hormone ABA (Yi et al. 2013, Yun et al. 2013b). Therefore, it is likely that SYT1 may have a dual function in abiotic stress responses by directly restoring the damaged PM lipid and by regulating the delivery of mediators of abiotic stress responses.

Materials and Methods

Plant materials

All plants used were *Arabidopsis thaliana* Col-0, except *pen2-1* whose background is Col-3 *gl1*. While plants used for fungal inoculation were grown in soil, plants used for detecting PEN1 levels were grown in liquid Murashige and Skoog (MS) medium as previously described (Kwon et al. 2008b, Yi et al. 2013). To generate the *pen1 syt1* and *pen2 syt1* double mutant plants, single mutant plants (*pen1-1*, *pen2-1* or *syt1-2*) were crossed and the offspring siblings were genotyped. To detect the presence of T-DNA in the *SYT1* gene, extracted genomic DNA was subject to PCR using primers 5′-TGGAAGCAAGAAATTCGGTT, 5′-TTCATAACCAATCTCGATACAC and 5′-GTATAGGGGGAAGCTGGAGG as previously described (Schapire et al. 2008). To check the presence of a point mutation in the *PEN1* or *PEN2* gene, genomic DNA was amplified with primers 5′-CAACGAAACACTCTCTTCATGTCACGC and 5′-CATCAATTTCTTCCTGAGAC for *PEN1* or primers 5′-TTTGGAACTGCTTCATCTTCTTATCAGG and 5′-CCTGTACAAGAAATCAATCACAGATCTTCA for *PEN2*, and the amplified DNAs were then digested with *Mlu*I or *Bsp*PI, as previously described (Lipka et al. 2005).

Immunoblot and protein–protein interaction assays

Protein extracts were obtained by suspending ground plant materials in 1× phosphate-buffered saline (PBS) containing 1% Triton X-100 and discarding the pellet after centrifugation. Protein amounts were then measured by the Bio-Rad protein assay (Bio-Rad) Protein extracts were then separated on an acrylamide gel and probed with anti-PEN1, anti-PEN3 or anti-SYT1 antibody. Equal loading was visualized by staining Rubisco with Coomassie blue. To detect the SNARE complex, protein extracts were not boiled before loading because it is SDS-resistant but heat-sensitive (Hayashi et al. 1994).

To test PEN1–SYT1 interactions, coding regions corresponding the PEN1 full-length and the TM-lacking SYT1 (SYT1ΔTM) were separately inserted into the pGEX-6p-1 plasmid vector (GE Healthcare Life Sciences). GST-fused recombinant proteins that were expressed in *E. coli* BL21-CodonPlus (Agilent Technologies) were purified by using glutathione–Sepharose 4B (GE Healthcare Life Sciences). To remove the GST moiety of PEN1, GST-fused recombinant proteins were digested with PreScission Protease (GE Healthcare Life Sciences). Equimolar purified PEN1 and GST–SYT1ΔTM were incubated in the presence or absence of 10 mM CaCl2, and the interacted proteins were obtained by precipitating GST–SYT1ΔTM with glutathione–Sepharose 4B. To detect PEN1 and GST–SYT1ΔTM, the precipitates were subject to immunoblot with anti-PEN1 antibody and anti-GST antibody, respectively. To analyze the effect of Ca^{2+} on the PEN1–SYT1 interaction, 1 mM EDTA was added during incubation. To test in planta PEN1–SYT1 interactions, protein extracts were obtained from non-inoculated or *G. orontii*-inoculated leaves of transgenic plants expressing GFP–PEN1 under the 35S promoter. Protein extracts were first pre-cleared with Protein A/G–agarose beads (Santa Cruz Biotech) and then incubated with anti-SYT1 antibody. The immunoprecipitates from Protein A/G-agarose beads were analyzed by immunoblot using anti-PEN1 antibody.

Pathogenicity test

Plants grown for 4 weeks were inoculated with *B. graminis*, *E. pisi* or *G. orontii* by spreading conidiospores over plants in a open-topped chamber. To measure fungal entry rate of *B. graminis*, 3 d inoculated plant leaves were first cleared with 95% ethanol and stained with 0.01% aniline blue in 15 mM KH_2PO_4 (pH 9.5). By UV microscopy to detect callose that surrounds fungal hasutoria within interacted plant cells, finger-edged forms were counted as successful entry but globular forms as failed ones. For *E. pisi* and *G. orontii*, 3 or 2 d inoculated plant leaves were stained with 0.6% Coomassie blue in 100% ethanol and then subsequently rinsed with distilled water twice. Successfully invaded conidiospores with secondary epiphytic hyphae were counted by light microscopy. To test post-invasive resistance, *G. orontii*-inoculated plants were further grown for 7 d and the degree of white fungal mycelia on plant leaves was macroscopically observed at 9 d post-inoculation.

Gene expression analysis

Total RNA was extracted from WT, *pen1* and *syt1* plants grown in a sterile condition with the Easy-spin™ IIp Plant RNA Extraction Kit (Intron Biotechnology) according to the manufacturer's instruction. Reverse transcribed cDNAs were then subject to PCR with cycles of 10 s at 95°C, 10 s at 55°C and 15 s at 72°C with a LightCycler® Nano System (Roche). Primers used are: 5′-ATGGAAGCTGCTGGAATCCAC and 5′-TTTGCTCATACGGTCAGC GAT for *Actin2*; 5′-ATGTCACGAGCAGACCAAGA and 5′-GAGGAAGAACCA GGTCCACA for *PEN1*; 5′-TAACATGCTTCTAGCGCACGCAG and 5′-CATCTG GATCACTCGGATCATATG for *PEN2*; and 5′-GGTGTTAAGAACAGTCTCGTC AC and 5′-TCTTCTGACCTCCAGATATACC for *PEN3*. Relative transcript amounts of *PEN1*, *PEN2* or *PEN3* normalized against *Actin2* were calculated by LightCycler® Nano Software (Roche).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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