Synaptotagmin 5 Controls SYP132-VAMP721/722 Interaction for Arabidopsis Immunity to Pseudomonas syringae pv tomato DC3000

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INTRODUCTION

Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) are now regarded as the minimal core factors to drive the vesicle fusion in eukaryotes including plants (Jahn and Scheller, 2006; Lipka et al., 2007; Yun and Kwon, 2017). To overcome energetically unfavorable lipid fusion, they form a SNARE complex in which four α-helices are bundled. Based on the conserved central amino acid, SNAREs are classified into the glutamine-conserved Q-SNARE group that is localized to a target membrane, and the arginine-conserved R-SNARE group that is located on a vesicle membrane (Jahn and Scheller, 2006; Lipka et al., 2007; Yun and Kwon, 2017). Based on their positions in a SNARE complex, Q-SNAREs are further subgrouped into Qa-SNARE, Qb-SNARE, Qc-SNARE, and Qbc-SNARE (which contains two SNARE domains) (Jahn and Scheller, 2006; Lipka et al., 2007; Yun and Kwon, 2017). In general, three distinct SNAREs (Qa + Qbc + R) form a ternary SNARE complex for exocytosis, whereas four SNAREs do a quaternary SNARE complex for intracellular vesicle fusion (Jahn and Scheller, 2006; Kwon et al., 2020a; Lipka et al., 2007; Yun and Kwon, 2017).

The first identified SNARE components to form a biologically relevant SNARE complex in plants are the plasma...
membrane (PM)-localized SYP121 (syntaxin of plant 121), also called PEN1) Qa-SNARE, the PM-attached SNAP33 Qbc-SNARE and the vesicle-residing VAMP721/722 (vesicle-associated membrane proteins 721 and 722) R-SNAREs (Collins et al., 2003; Kwon et al., 2008). The SYP121-SNAP33-VAMP721/722 ternary SNARE complex drives an immune exocytosis. Interestingly, while SYP121 function is limited to defense against fungal pathogens, VAMP721/722 are additionally required for resistance to oomycete pathogens (Kwon et al., 2008). VAMP721/722 are also required for growth, cell division and abiotic stress responses (El Kasmi et al., 2013; Ichikawa et al., 2014; Kim et al., 2019; Kwon et al., 2008; Yi et al., 2013; Yun et al., 2013). Promiscuous SNARE complex formation of VAMP721/722 with distinct Qa-SNAREs such as SYT1, SYP111 (KNOLLE), SYP132 and SYP133 (El Kasmi et al., 2013; Ichikawa et al., 2014; Kwon et al., 2008; Yun et al., 2013) strongly suggests that at least one regulatory protein should control the SNARE complex formation of VAMP721/722 with a specific Qa-SNARE in a particular biological process in plants. This can be additionally supported by promiscuous SNARE complex formation of SYP121 with members in the VAMP72 R-SNARE group (Kwon et al., 2008).

In plants, three groups of regulatory proteins such as Sec1/Munc18 (SM), small GTPase and synaptotagmin (SYT) have been studied for modulating the trafficking functionality of SNAREs. The KEULE (also called SEC11) SM protein controls the SNARE complex formation of VAMP721/722 with KNOLLE for cytokinesis (Karnahl et al., 2018; Park et al., 2012), but with SYP121 for potassium uptake and growth (Karnik et al., 2013; 2015; Waghmare et al., 2019; Zhang et al., 2019) by binding to the N-terminus of respective Qa-SNARE. Recently, a KEULE paralog, SEC1B, was found to be predominantly engaged in regulating the SYP132-dependent secretion during pollen growth (Karnahl et al., 2018). The plant-specific ARAL GTPase is regarded to switch the interaction of SYP121 between VAMP721/722 and VAMP727 (Ebine et al., 2011). SYP121 is known to continuously cycle between the PM and endosomes (Reichardt et al., 2011). Focal accumulation of endocytosed SYT121 or its barley ortholog, ROR2, to fungal entry sites was found to require the GNOM ADP-ribosylation factor-guanine nucleotide exchange factor (ARF-GEF) in Arabidopsis or ARFAIb/c GTPases in barley, respectively (Bohlenius et al., 2010; Nielsen et al., 2012). SYT1 preferentially residing in the endoplasmic reticulum (ER) (Levy et al., 2015; Perez-Sancho et al., 2015) was originally found to repair damaged PM by abiotic stresses and control endocytosis often resulting in promoting cell-to-cell movement of viral movement proteins (Lewis and Lazarowitz, 2010; Schapire et al., 2008; Yamazaki et al., 2008). SYT1 together with SYT5 was recently found to regulate the intactness and rearrangement of ER-PM contact sites for stress responses to rare earth elements and viral pathogens in plants (Shikawa et al., 2020; Lee et al., 2020).

We previously found that SYT1 additionally down-regulates SYP121 abundance possibly via endocytosis to control plant disease resistance to fungal pathogens (Kim et al., 2016). We therefore examined whether SYT5 also functions in plant immunity. Based on specific impairment of resistance to Pseudomonas syringae pv tomato (Pst) DC3000 bacterium but not to Erysiphe pisi pea powdery mildew fungus in syt5 plants, we found that SYT5 interacts with the PM-localized SYP132 which is required for plant defense against bacteria but not fungi (Kalde et al., 2007). Elevated growth of surface-inoculated Pst DC3000 in VAMP721/722-depleted plants indicates that VAMP721/722 are required for previously unrevealed plant immunity to epiphytic bacteria. In vitro stimulation of SYP132-VAMP722 interaction by SYT5, and reduced SYP132-VAMP721/722 interaction in syt5 plants suggest that SYT5 positively regulates the SYP132-VAMP721/722 immune exocytosis to bacteria.

**MATERIALS AND METHODS**

**Plant materials**

Plants used for experiments were grown at 22℃ with 10-h light/14-h dark photoperiod. To isolate T-DNA-inserted syt5-1 and syt5-2, SALK_036961 and GABL_679H10 were obtained from ABRC and GABI-Kat. Homozygous mutant plants were selected by genomic DNA polymerase chain reaction (PCR) with T-DNA-specific (5’-GGCTGGACCCTGCTGCAACAC and 5’-ATATGGACCATCATACCTCAGG) and SYT5-specific primers (5’-GCTATGATTTCCAGCAATTTCGAGC and 5’-TAGTTTTCGACGAAAAGGCC), and further analyzed by immunoblot with anti-SYT5 antibody.

**Purification of recombinant proteins**

To express recombinant proteins, CDAs corresponding to SYP132, VAMP722, SYT5ΔTM, SYP111, SYT121, and SYP123 were amplified by PCR and introduced into the pGEX-6p-1 vector (GE Healthcare Life Sciences, USA). To express HA-SYP132, HA-corresponding DNA sequence was added to a primer to amplify recombinant HA-SYP132. Purification of recombinant proteins

**In vitro protein interaction assay**

To test the interaction between SYP132 and SYT5, equimolar recombinant HA-SYP132 and GST-SYT5ΔTM were incubated in the absence or presence of 1 mM CaCl2. To assess the effect of Ca2+ on SYT5-SYP132 interaction, 1 mM EDTA was added during incubation. To test the interaction between SYP132 and VAMP722, equimolar recombinant HA-SYP132 and GST-VAMP722 were mixed together with GST-free SYT5ΔTM in the absence or presence of 1 mM CaCl2. To analyze whether the Ca2+-promoted SYT5ΔTM-SYP132 interaction affects SYP132-VAMP722 interaction, 1 mM EDTA was added during incubation. Interacted proteins were then precipitated with glutathione-Sepharose 4B and the precipitates were analyzed by immunoblot with anti-HA antibody to de-
In vivo protein interaction assay
Proteins were extracted from the indicated genotype plants by suspending ground plant materials in 1× phosphate-buffered saline (PBS) containing 1% Triton X-100. Protein amounts were measured by the Bio-Rad protein assay (Bio-Rad, USA). Protein extracts were first pre-cleared with Protein A/G-agarose beads (Santa Cruz Biotechnology, USA) and incubated with anti-SYT5 or anti-VAMP721/722 antibody. Anti-SYT5 antibody-bound or anti-VAMP721/722 antibody-bound proteins were then retrieved by precipitation with Protein A/G-agarose beads. The immunoprecipitates were finally analyzed by immunoblot with anti-SYP132 antibody. A part (3%) of protein extracts used for immunoprecipitation were subject to immunoblot with anti-SYT5, anti-SYP121, anti-SYP132, anti-VAMP721/722 antibody, or anti-SNAP33 antiserum for showing steady state levels of SYTs, SYP121, SYP132, VAMP721/722, or SNAP33, respectively.

Pathogenicity test
Plants were grown in soil for 4-5 weeks to be inoculated with E. pisi or dip-inoculated with Pst DC3000 (1 × 10^7 cfu). To be liquid-inoculated with Pst DC3000 (1 × 10^7 cfu), plants were grown for 10 days in liquid Murashige and Skoog (MS) medium containing 1% sucrose. For measuring E. pisi entry rate, inoculated leaves were stained with 0.6% Coomassie blue in 100% ethanol and subject to light microscopy. Conidiospores with secondary epiphytic hyphae were counted as successful fungal entry. Bacterial numbers (in liquid-inoculated seedlings at 2 days post-inoculation [dpi] or in dip-inoculated leaves at 3 hours post-inoculation [hpi] and 3 dpi) were counted by serial dilution plating.

Confocal microscopy
For immunostaining, 7-day-old transgenic Arabidopsis seedlings expressing either GFP-SYP132 or mRFP-VAMP722 were fixed in MTSB buffer (50 mM PIPES, 5 mM EGTA and 5 mM MgSO4, pH 7.0 adjusted with KOH) containing 4% paraformaldehyde for 1 h at room temperature under vacuum infiltration (Sauer et al., 2006). Fixed seedlings were placed onto poly-L-Lys-coated glass slides, and washed with MTSB buffer containing 0.1% Triton X-100 and with deionized water. Fixed seedlings were then incubated with 2% driselase in MTSB buffer for 40 min to digest their cell wall, and incubated with PBS containing 20% DMSO and 3% NP40 for 1 h. Following washing with MTSB buffer containing 0.1% Triton and with deionized water, fixed seedlings were pre-incubated with a blocking buffer (PBS containing 5% BSA) at 37°C for 1 h and incubated with anti-SYT5 antibody in the blocking buffer at 4°C overnight. After washing with PBS containing 0.1% Triton X-100, fixed seedlings were incubated with either Alexa 488- or Alexa 546-conjugated rabbit IgG antibody (Invitrogen, USA) in the blocking buffer at room temperature for 3 h. Following washing with PBS containing 0.1% Triton X-100, these seedlings were finally transferred into a mounting medium (100 mM Tris [pH 8.5] containing 25% glycerol) containing Mowiol 4-88 (Calbiochem, USA). All fluorescent images were taken by LSM780 confocal microscope (Zeiss, Germany) equipped with a 40× objective (C-Apochromat 40 ×/1.1 W) and processed by Zen 2011 software (Zeiss) and Adobe Photoshop CS5 (Adobe, USA). GFP and Alexa 488 were excited with an argon laser at 488 nm, and mRFP and Alexa 546 were at 561 nm. For two-color imaging, multitracking was configured to avoid cross-talk between fluorescence channels.

RESULTS
It was reported that transiently expressed SYT5-GFP is localized to endomembrane compartments in Arabidopsis protoplasts (Yamazaki et al., 2010). SYT5-GFP was also reported to be localized to the ER/PM contact sites by transient expression in Nicotiana benthamiana (Ishikawa et al., 2020). SYT5 together with SYT1 is suggested to regulate the ER-PM interactions and plasmodesmata (PD) conductance in Arabidopsis (Ishikawa et al., 2020; Lee et al., 2020). We previously reported that SYT1, which is localized to the PM and ER/PM contact sites, negatively controls plant disease resistance to powdery mildew fungi by modulating SYP121 levels (Kim et al., 2016). Therefore, we tested whether SYT5 is also required for immune responses to phytopathogens. For this, we isolated two independent T-DNA insertion syt5 mutant plants, syt5-1 and syt5-2. Although T-DNAs are inserted in introns in both syt5-1 and syt5-2 (Supplementary Fig. S1), we found that SYT5 proteins were barely detectable in those mutant plants by immunoblot with anti-SYT5 antibody (Fig. 1A). We then inoculated syt5-1 and syt5-2 plants with the Arabidopsis-nonadapted E. pisi pea powdery mildew fungus. However, we found no significant difference in fungal entry rates between wild-type (WT) and syt5 plants (Fig. 1B), indicating no requirement of SYT5 for plant immunity to E. pisi. We next dip-inoculated the above-mentioned mutant plants with the Arabidopsis-pathogenic Pst DC3000 bacterium. At 3 dpi, we interestingly found more bacterial growth in both syt5-1 and syt5-2 plants than in WT (Fig. 1C). This suggests that SYT5 is involved in plant immunity to Pst DC3000 bacterium but not to E. pisi fungus.

We previously reported that SYT1 interacts with the PM residing SYP121 syntaxin to modulate plant immune responses by controlling SYP121 level in plants (Kim et al., 2016). We therefore tested whether SYT5 is also able to interact with a PM syntaxin. It is known that SYP121 is required for defense against powdery mildew fungi, whereas SYP132 is for resistance to bacteria (Kalde et al., 2007; Kwon et al., 2008). We found that SYP121 levels in both syt5-1 and syt5-2 are indistinguishable from that in WT by immunoblot with anti-SYP121 antibody (Fig. 1A). We therefore investigated an interaction between SYT5 and SYP132, because SYT5 is required for immunity to Pst DC3000 bacterium (Fig. 1C). We first tested their direct interaction in vitro, using hemagglutinin-tagged SYP132 (HA-SYP132) and transmembrane motif-lacking SYT5 fused with glutathione-S-transferase (GST-SYT5TM) that were purified from E. coli. After mixing HA-SYP132 and GST-SYT5TM, we precipitated GST-SYT5TM with glutathione-agarose and analyzed the precipitates by immunoblot with anti-HA antibody. Detection of HA-SYP132
The indicated genotype plants were marked 100 bp plants were
synt5 and synt5-2 plants in immunoblot (Fig. 1A), indicative of its specificity to SYT5. Unlike synt5 plants, syt132 plants are lethal (Kalde et al., 2007), leading to difficulty in testing an in vivo specificity of anti-SYP132 antibody. Therefore, to test the specificity of anti-SYP132 antibody, four different PM syntaxins belonging to the SYP1 group (SYP111, SYP121, SYP123, and SYP132) were purified from E. coli (Supplementary Fig. S1) and subject to immunoblot. Immunoblot results with anti-SYP132 antibody also indicate that the generated anti-SYP132 antibody can discriminate SYP132 from the other tested PM syntaxins (Supplementary Fig. S1). In spite of this specificity of anti-SYP132 antibody, the chicken IgY nonspecifically reacts with SYT5 and VAMP721/722 (data not shown). Therefore, we performed a co-immunoprecipitation (Co-IP) assay with WT protein extracts using anti-SYT5 antibody for immunoprecipitation and anti-SYP132 antibody for immunoblot. Detection of SYP132 in the SYT5 precipitates but not in the rabbit IgG ones (Supplementary Fig. S2A) indicates that endogenous SYT5 and SYP132 interact in plants. SYP132 specifically interacts with VAMP721/722 that reside in the trans-Golgi network (TGN) and secretory vesicles (Ichikawa et al., 2014; Yun et al., 2013). Since SYT5 interacts with SYP132 (Fig. 2A, Supplementary Fig. S2A), we then tested whether SYT5 is able to regulate SYP132-VAMP721/722 interaction. We first examined the effect of SYT5 on SYP132-VAMP722 interaction in vitro using E. coli-purified recombinant proteins, SYT5TM, HA-SYP132 and GST-fused VAMP722 (GST-VAMP722). We failed to detect HA-SYP132 in the GST-VAMP722 precipitates even in the presence of SYT5TM (Fig. 2B). Similarly, we previously reported that SYT121 alone scarcely interacts with VAMP722 in vitro (Kwon et al., 2008). When Ca²⁺ was added during their incubation, we unexpectedly detected significant amount of SYP132 in the GST-VAMP722 precipitates (Fig. 2B). Interestingly, this Ca²⁺-aided SYP132-VAMP722 interaction by SYT5 was completely abolished by EDTA addition (Fig. 2B). Since Ca²⁺ greatly increased SYT5-SYP132 interaction (Fig. 2A), these results suggest that Ca²⁺-bound SYT5 induces SYP132-VAMP722 complex. This additionally suggests that SYT5 may stimulate a secretory pathway involving SYP132 and VAMP721/722 in plants. Similar levels of HA-SYP132 in the GST-VAMP722 precipitates with more amounts of SYT5TM (Fig. 2B) suggest that SYT5 affects SYP132-VAMP722 interaction with equimolar ratio.

Since all reported in planta interactions between SYT132 and VAMP721/722 were tested in transgenic plants or protoplasts expressing heterogeneous proteins with tags (El Kasmi et al., 2013; Ichikawa et al., 2014; Yun et al., 2013), we investigated endogenous SYP132-VAMP721/722 interaction with our generated antibodies. In a Co-IP assay with WT protein extracts using anti-SYT5 antibody for immunoprecipitation and anti-SYP132 antibody for immunoblot, we detected SYP132 in the VAMP721/722 precipitates (Supplementary Fig. S2B), indicating their in planta interaction. We then compared SYP132 amounts in the VAMP721/722 precipitates between WT and synt5 plants to examine an in vivo SYT5 function to regulate SYP132-VAMP721/722 interaction. By immunoblot, we found that SYP132, SNAP33 and VAMP721/722 levels are indistinguishable between WT

Fig. 1. SYT5 is required for defense against Pst DC3000 bacterium but not for E. pisi fungus. (A) A schematic view of SYT5 gene. Two independent T-DNA insertion sites for synt5-1 (SALK_036961) and synt5-2 (GABI_679H10) were marked by triangles. LB, left boarder of inserted T-DNA. Box, exon; line, intron. Homozygosity of synt5-1 and synt5-2 plants were tested by immunoblot. Proteins extracted from the indicated genotype plants were subject to immunoblot with anti-SYT5 and anti-SYP121 antibodies. Equal loading was visualized by staining Rubisco with Coomassie blue. (B) Dispensability of SYT5 for resistance to E. pisi. The indicated genotype plants were inoculated with E. pisi conidiospores. At 2 dpi, inoculated leaves were stained with Comassie blue and analyzed for fungal entry rates. Conidiospores with secondary epihyle hyphae were regarded as successfully entered fungi. Values are presented as mean ± SE from three biological replications. (C) Elevated Pst DC3000 growth in synt5 plants. The indicated genotype plants were dip-inoculated with Pst DC3000 (1 × 10⁸ cfu). The leaf bacterial growth was measured at 3 hpi (gray) or at 3 dpi (black). Values are presented as mean ± SE from four biological replications. *P < 0.05; **P < 0.01 in comparison to WT (Student t-test).
and both syt5 lines (Fig. 2C), indicating that SYT5 does not affect the steady-state abundance of SYP132, SNAP33 and VAMP721/722. However, we detected reduced SYP132 levels in the VAMP721/722 precipitates in both syt5-1 and syt5-2 plants compared to WT (Fig. 2C). Together with the in vitro results (Fig. 2B), this suggests that SYT5 can positively control SYP132-VAMP722 interactions in plants. Since SYP132 is required for defense against bacteria (Kalde et al., 2007), elevated bacterial growth in syt5 plants (Fig. 1C) can be explained by reduced SYP132-VAMP721/722 interactions in the absence of SYT5 (Fig. 2C). Similar levels of SYP132, SNAP33 and VAMP721/722 between WT and syt5 plants (Fig. 2C) suggest that the reduced SYP132-VAMP721/722 interactions in syt5 plants does not result from their reduced levels.

We previously reported that VAMP721/722 are indispensable for plant resistance to Pst DC3000 inoculated by vacuum-infiltration (Kwon et al., 2008). However, this type of inoculation allows bacteria to directly enter the leaf tissue to bypass most plant immune responses to epiphytic bacteria. Therefore, to understand an immune function of VAMP721/722 to epiphytic bacteria, we dip-inoculated plants grown in soil with Pst DC3000 and counted the number of multiplied bacteria in leaves. Since the vamp721 vamp722 double null mutant is lethal, we inoculated plants with differentially lowered VAMP721/722 gene dosage (vamp721, 722).
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**DISCUSSION**

Together with SYT1, SYT5 was recently reported to be engaged in stress responses to rare earth elements and viral pathogens by regulating the rearrangement and intactness of ER-PM contact sites (Ishikawa et al., 2020; Lee et al., 2020). We previously found that SYT1 has an additional immune activity to its ER-PM contact-controlling function (Kim et al., 2016). Elevated SYP121 abundance in syt1 plants and reduced resistance to powdery mildew fungi by SYP121 deletion in syt1 plants suggest that SYT1 negatively functions in immune responses to fungal pathogens by regulating SYP121 levels. We in this study show that SYT5 is also involved in plant resistance to Pst DC3000 bacterium. Compromised resistance to Pst DC3000 bacterium but WT-like defense against E. pisi fungus in syt5 plants (Figs. 1B and 1C) suggest that SYT5 plays an important function in immune responses to bacterial pathogens but not to fungal ones. Comparable SYP121 levels between WT and syt5 plants (Fig. 1A) and no detection of SYT5 in the SYT1-detected cell plate (Supplementary Figs. S3D-S3F) support that SYT5 has a dis-

**Fig. 3.** VAMP721/722 are required for plant resistance to surface-inoculated Pst DC3000. (A) Elevated bacterial growth in all four VAMP721/722-related mutant plants (vamp721, vamp722, VAMP721/722, and VAMP721/722). The indicated genotype plants grown in soil were dip-inoculated with Pst DC3000 (1 × 10^5 cfu). The in-leaf bacterial growth was measured at 3 dpi (gray) or at 3 dpi (black). Values are presented as mean ± SE from three biological replications. (B) Increased bacterial growth in all four VAMP721/722-related mutant seedlings. The indicated genotype plants grown in liquid MS medium were liquid-inoculated with Pst DC3000 (1 × 10^5 cfu). The in-seedling bacterial growth was measured at 2 dpi. Values are presented as mean ± SE from three biological replications. *P < 0.05; **P < 0.01 in comparison to WT (Student t-test).

vamp722, VAMP721/722, and VAMP721/722 (Kwon et al., 2008). At 3 dpi, we found more bacterial growth in all four mutant genotypes than in WT plants (Fig. 3A). Since VAMP721 and VAMP722 are regarded to be immunologically redundant, the elevated bacterial growth in vamp721 and vamp722 plants (Fig. 3A) was unexpected. Hence, we additionally examined bacterial growth in VAMP721/722-lowered genotypes by a different inoculation approach. For this, we inoculated liquid-grown plants by adding bacterial suspension to the medium and counted the number of bacteria within plants (Schreiber et al., 2008). At 2 dpi, we again found increased bacterial growth in vamp721 and vamp722 plants as well as in VAMP721/722 and VAMP721/722 plants (Fig. 3B). Recent secretomic analyses revealed that at least a part of cargos transported by VAMP721 and VAMP722 are distinct (Jemura et al., 2019). Taken together, it is therefore likely that at least a part of antibacterial molecules transported by VAMP721 and VAMP722 vesicles might be distinct, which are secreted by SYP132-VAMP721/VAMP722 interactions.

We next examined subcellular localization of endogenous SYT5 in plants. We immuno-fluorescently stained endogenous SYT5 with two distinct secondary antibodies (Alexa 546 with red fluorescence and Alexa 488 with green one) in roots of transgenic plants expressing either the PM-localized GFP-SYP132 or the TGN/vesicle-located mRFP-VAMP722 (Ichikawa et al., 2014). Due to the difficulty in immunostaining root elongating cells leading to smear images (data not shown), we microscopically observed meristematic root cells (Supplementary Fig. S3, Fig. 4). As previously reported (Lee et al., 2020), we detected immuno-fluorescently marked SYT5 in perinuclear area (Figs. 4B and 4E). Distinct localization patterns of SYT5 from GFP-SYP132 (Figs. 4A-4C) and mRFP-VAMP722 (Figs. 4D-4F) indicate that SYT5 is largely localized to the ER. In a dividing root meristematic cell, we interestingly found that SYT5 is not detected in the developing cell plate (Supplementary Fig. S3D-S3F), where SYT1 was reported to be localized (Yamazaki et al., 2010). Since SYP121 level is elevated in syt1 plants (Kim et al., 2016) but not in syt5 plants (Fig. 1A), this implies that SYT5 may play an additional cellular activity to maintaining the ER-PM contact sites together with SYT1.
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**Fig. 4. Endogenous SYT5 is localized to the perinuclear region in Arabidopsis roots.** (A-C) Cellular localization of endogenous SYT5 in roots of transgenic plants expressing the PM-localized GFP-SYP132. Endogenous SYT5 was detected by anti-SYT antibody and Alexa 546 (red fluorescence)-conjugated anti-rabbit IgG antibody. Fluorescent images were analyzed by confocal microscopy. Scale bars = 10 μm. (D-F) Cellular localization of endogenous SYT5 in roots of transgenic plants expressing the TGN/secretory vesicle-localized mRFP-VAMP722. Endogenous SYT5 was detected by anti-SYT antibody and Alexa 488 (green fluorescence)-conjugated anti-rabbit IgG antibody. Fluorescent images were analyzed by confocal microscopy. Scale bars = 10 μm.

VAMP721/722 are the major exocytosis-associated R-SNAREs in Arabidopsis. They participate in a number of physiological processes such as cell division, growth, and biotic/abiotic stress responses even including symbiosis in plants (El Kasmi et al., 2013; Ichikawa et al., 2014; Ivanov et al., 2012; Kim et al., 2008; Sogawa et al., 2019; Yi et al., 2013; Yun et al., 2013). In contrast to VAMP721/722, a plant PM syntaxin is involved rather in a specific biological process. Although how VAMP721/722 can be engaged in such diverse processes is largely unknown, their interactions with distinct PM syntaxins such as SYP111, SYP121, SYP122, SYP123, and SYP132 (El Kasmi et al., 2013; Ichikawa et al., 2014; Kwon et al., 2008; Pajonk et al., 2008; Yun et al., 2013) implicate that VAMP721/722 may work for a cellular activity by interacting with a respective PM syntaxin. It is regarded that SYP121 immune function is restricted to fungal pathogens, whereas SYP132 defense activity is limited to resistance to bacterial pathogens (Kalde et al., 2007; Kwon et al., 2008). Based on elevated bacterial growth in syt5 plants (Fig. 1C), we found that SYT5 interacts with SYP132 both in vitro and in vivo (Fig. 2A, Supplementary Fig. S2A). In addition, we found that SYP132-

VAMP721/722 interactions are diminished in syt5 plants (Fig. 2C). Greatly increased in vitro interaction between SYP132 and VAMP722 by SYT5, which is otherwise rarely detectable in the absence of SYT5 (Fig. 2B), suggests that SYT5 stimulates the interaction between SYP132 and VAMP721/722 in plants. Since VAMP721/722 drive the immune exocytosis, reduced SYP132-VAMP721/722 interactions in syt5 plants (Fig. 2C) may explain why immune responses in syt5 plants to Pst DC3000 are impaired, likely due to disrupted secretion of extracellular immune molecules.

Unlike typical SYTs, all known plant SYTs contain an additional SYT-like mitochondrial lipid-binding (SMP) domain (Saheki and De Camilli, 2017). In animals, these SYTs called extended-SYTs (E-SYTs) are responsible for tethering the ER to the PM to generate the ER-PM contact sites at which lipids are transported through the SMP domain between two distinct membranes (Saheki and De Camilli, 2017). Likewise, plant SYTs, especially SYT1, SYT5, and SYT7 (also called Ca2+- and lipid-binding protein 1 [CLB1]) were found to play a critical role in maintaining the ER-PM contact sites for responses to various biotic/abiotic stresses (Ichikawa et al., 2020; Lee et al., 2020; Levy et al., 2015; Lewis and Lazarowitz, 2010; Perez-Sancho et al., 2015; Schapire et al., 2008; Yamazaki et al., 2008). Interestingly, it was recently reported that a part of endocytosis-associated autophagy pathway is initiated at the ER-PM contact sites (Wang et al., 2019), suggesting that the ER-PM contact sites may control vesicle trafficking in plants. Indeed, SYT2 is suggested to play a role in secession in Arabidopsis (Wang et al., 2015; Zhang et al., 2011). In addition, E-SYTs were recently reported to regulate membrane trafficking even in animals (El Kasmi et al., 2018; Kikuma et al., 2017). In response to pathogen attack, plant cells reorganize all subcellular compartments near to the pathogen-attempted area (Bestwick et al., 1997; Koh et al., 2005; Takemoto et al., 2003), likely for rapid and efficient production and secretion of immune molecules including secondary metabolites (Khare et al., 2020). In this scenario, SYT5 may contribute to plant immunity to bacteria by promoting the VAMP721/722-associted exocytosis at the ER-PM contact sites. The ‘kiss-and-run’ exocytosis may explain the observed distinct localization of SYT5, SYP132, and VAMP722 in plant cells (Fig. 4, Supplementary Fig. S3), in spite of their interactions (Fig. 2, Supplementary Fig. S2).

Although we previously reported that VAMP721/722 are dispensable for plant immune responses to Pst DC3000 bacterium (Kwon et al., 2008), we here present that they are required for defense against Pst DC3000 (Fig. 3). Such difference is due to different ways of bacterial inoculation: we infiltrated bacteria in the previous study, whereas surface-inoculated in the present study. Interestingly, it was reported that epiphytic (on-leaf) and apoplastic (in-leaf) bacteria express distinct sets of genes (Yu et al., 2013), likely for dissimilar virulent strategies in considerably different leaf-exterior and -interior environments. Therefore, it is likely that VAMP721/722 are important for plant immune responses to epiphytic bacteria but not to apoplastic ones. Interestingly, we observed elevated bacterial growth even in vamp721 and vamp722 single mutant plants in two distinctly surface-inoculated experiments (Fig. 3). Although VAMP721
and VAMP722 are functionally redundant in general, recent studies revealed their differential activities to deliver cell wall-related proteins and an immune protein, RPM1.2 (Kim et al., 2014; Uemura et al., 2019). Indeed, depending on a pathogen type, plant resistance requires differential dosages of VAMP721 and VAMP722 genes (Kwon et al., 2008). This supports that VAMP721 and VAMP722 vesicles transport at least partly distinct as well as common cargos. No more elevation of bacterial growth in VAMP721−/− VAMP722−/− and VAMP721−/− VAMP722−/− plants than in vmp721 and vmp722 single mutant plants (Fig. 3) suggests that immune molecules differentially secreted by VAMP721 and VAMP722 vesicles might be interchangeable but not additive for defense against epiphytic bacteria.

Although VAMP721/722 are required for immune responses in plants, any other direct immune molecules but RPM1.2 and phospholipase Dα (PLDα) were not identified so far to be transported by VAMP721/722 vesicles (Kim et al., 2014; Xing et al., 2019). Proteomic approaches using VAMP721/722-depleted plants found that mostly cell wall-associated proteins are likely to be delivered by VAMP721/722 vesicles in plants (Kwon et al., 2020b; Uemura et al., 2019). Enhanced resistance to fungal pathogens but not to Pst DC3000 in RPM8.2-expressing plants (Li et al., 2018), and impaired immune responses to E. coli but not to Pst DC3000 in PLDα-deficient plants (Johansson et al., 2014) suggest that plants may modify the cell wall to defend against bacterial pathogens. Indeed, it was reported that the MYB15-governed lignin biosynthetic pathway is important for plant resistance to surface-inoculated Pst DC3000 (Chezem et al., 2017). In addition, extracellular lignin accumulation was recently reported to limit bacterial motility within plant leaves, ultimately resulting in pathogenesis termination (Lee et al., 2019).

Interestingly, bacteria on the leaf surface preferentially express the movement-related genes, compared to leaf-interior bacteria (Yu et al., 2013). This suggests that the growth of epiphytic bacteria depends on their motility. Therefore, it is implied that plants may secrete cell wall-modifying proteins via VAMP721/722 vesicles to restrict bacterial growth on leaf surface likely by disturbing bacterial movement.

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

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AUTHOR CONTRIBUTIONS

All authors (S.K., H.K., K.P., D.J.C., M.K.K., C.K., and H.S.Y.) conceived and performed experiments. H.K., C.K., and H.S.Y. wrote the manuscript.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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