Longin R-SNARE is retrieved from the plasma membrane by ANTH domain-containing proteins in Arabidopsis

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The plasma membrane (PM) acts as the interface between intra- and extracellular environments and exhibits a tightly regulated molecular composition. The composition and amount of PM proteins are regulated by balancing endocytic and exocytic trafficking in a cargo-specific manner, according to the demands of specific cellular states and developmental processes. In plant cells, retrieval of membrane proteins from the PM depends largely on clathrin-mediated endocytosis (CME). However, the mechanisms for sorting PM proteins during CME remain ambiguous. In this study, we identified a homologous pair of ANTH domain-containing proteins, PICALM1a and PICALM1b, as adaptors for proteins of CME of the secretory vesicle-associated longin-type R-SNARE VAMP72 group. PICALM1 interacted with the SNARE domain of VAMP72 and clathrin at the PM. The loss of function of PICALM1 resulted in faulty retrieval of VAMP72, whereas general endocytosis was not considerably affected by this mutation. The double mutant of PICALM1 exhibited impaired vegetative development, indicating the requirement of VAMP72 recycling for normal plant growth. In the mammalian system, VAMP7, which is homologous to plant VAMP72, is retrieved from the PM via the interaction with a clathrin adaptor HIV Rev-binding protein in the longin domain during CME, which is not functional in the plant system, whereas retrieval of brevin-type R-SNARE members is dependent on a PICALM1 homolog. These results indicate that ANTH domain-containing proteins have evolved to be recruited distinctly for recycling R-SNARE proteins and are critical to eukaryote physiology.

Significance

The plasma membrane (PM) acts as the interface between intra- and extracellular environments and is thus important for intercellular communication and extracellular signal perception. The composition and amounts of PM proteins are tightly regulated, by molecular mechanisms that remain largely unknown in plant cells. We identified a pair of ANTH domain-containing proteins functioning as adaptors for the retrieval of VAMP72 members, which are components of the membrane fusion machinery, during clathrin-mediated endocytosis. Our results further indicate that the recycling mechanisms of homologous VAMP7 proteins are different in plants and animals, suggesting a divergence of the endocytosis mechanism between these two kingdoms.
membrane fusion between the synaptic vesicle and the presynaptic membrane), and similar brevin-type R-SNAREs have been identified in other opisthokonts, such as yeasts, other eukaryotic lineages do not possess R-SNAREs with a similar N-terminal structure (5), suggesting that brevin was acquired in an ancestor of opisthokonts. Conversely, longins are also broadly conserved in eukaryotic lineages and play important roles in membrane trafficking in several organisms. Longins are also distributed throughout the plant lineages, with notable expansion of a subgroup, i.e., the VAMP7 group, during land plant evolution (6, 7). While animals possess only one or few VAMP7 members, Arabidopsis harbors 11 VAMP7 members, which are further classified into two subgroups, namely, VAMP71 and VAMP72. These VAMPs participate in distinct membrane fusion events. VAMP71 mediates homotypic fusion of the vacuolar membrane in Arabidopsis (8), and remarkable functional differentiation has been reported in the VAMP72 group. Of the seven VAMP72 members in Arabidopsis, VAMP727 is localized mainly to the multivesicular bodies (MVBs), mediating the fusion of MVBs with the vacuolar and plasma membranes (9, 10), whereas the other VAMP72 members (VAMP721 to 726) localize to the trans-Golgi network (TGN), secretory vesicles, forming cell plates, and PM, reflecting their major role in the secretory pathway (11–14).

In animal cells, VAMP72 is also mediating membrane fusion at several locations, including the PM and autophagosomes (15, 16). Thus, VAMP72 underwent functional diversification/specialization in a lineage-specific manner during evolution, and this underpins various cellular functions in diverse organisms.

VAMP7 members mediate membrane fusion at the PM in both animals and plants. After membrane fusion, VAMP7 must be retrieved from the PM, through the endocytic pathway, for subsequent fusion events. In animal cells, VAMP7 is sorted into clathrin-coated vesicles, through the interaction of its longin domain with a clathrin adaptor, known as HIV Rev-binding protein (Hrb or AGFG1) (17). However, the mechanism for the retrieval of VAMP7 members from the plant PM remains unknown. An Hrb homolog with a similar domain composition is not encoded in the Arabidopsis genome; therefore, VAMP72 must be loaded into endocytic vesicles in a distinct manner from the animal system. Brevin-type secretory R-SNAREs should also be retrieved from the PM via endocytic transport. In animals, the retrieval of brevin-type R-SNAREs, including VAMPs, VAMP2, and VAMP2, from the PM is mediated by another class of clathrin adaptor protein containing the AP180 N-terminal Homology (ANTH) domain, that is, CALM/PICALM (18, 19). In plants, the PICALM family has also expanded during evolution (20), and some of its members are required for normal pollen tube growth (21–23). However, it is still not known if any of the PICALM members are involved in the recycling of SNARE proteins in plants.

In the present study, we attempted to identify a clathrin adaptor responsible for recycling VAMP72 from the PM in Arabidopsis. We further revealed divergent retrieval mechanisms of secretory longin-type SNAREs in plants and animals.

Results

ANTH-Domain Containing Proteins Interact with the SNARE Domain of VAMP72. To determine the molecules interacting with VAMP72 proteins in Arabidopsis, we performed yeast two-hybrid screening using the cytoplasmic domain of VAMP72 as bait, which allowed us to identify several candidate interacting partners. We hypothesized that the adaptor protein mediating the retrieval of VAMP72 members from the PM would also interact with other VAMP72 members; therefore, we performed a second screening using the cytoplasmic domain of VAMP72 as bait, and an ANTH domain-containing protein, PICALM1a/ECA1, was identified to interact with the VAMP72 members (Fig. 1A and SI Appendix, Fig. S1). This interaction should be specific to the VAMP72 group, because we did not detect an interaction between PICALM1a and a

vacular membrane-residing VAMP7 member, VAMP713, in the yeast two-hybrid assay (Fig. 1A). The PICALM family expanded during land plant evolution. Eighteen PICALM proteins are encoded in the Arabidopsis genome (20), in addition to putative clathrin adaptor proteins with the Epsin N-terminal Homology (ENTH) domain, which has a similar structure to the ANTH domain. We further tested whether VAMP72 could interact with other E/ANTH-containing proteins or whether its interaction was specific to PICALM1. As shown in Fig. 1B, VAMP72 was found to interact with PICALM1b, a PICALM1a paralog (20), but not with other E/ANTH members in the yeast two-hybrid assay. We then investigated whether PICALM1 interacted with the longin or the SNARE domain of the VAMP72 members by splitting the cytoplasmic part of VAMP721 and VAMP727 into each domain. In the yeast two-hybrid assay, PICALM1a interacted with the SNARE domain of VAMP721 and VAMP727, but not with their longin domains (Fig. 1B and SI Appendix, Fig. S1).

We further investigated the in planta interaction between VAMP721 and PICALM1a. We constructed transgenic plants expressing His6-tagged PICALM1a and GFP-tagged VAMP721 or VAMP713, which were subjected to immunoprecipitation using the anti-GFP antibody. Although comparable amounts of GFP-VAMP713 and GFP-VAMP721 were precipitated, PICALM1a-His6 was coprecipitated with only VAMP721 (Fig. 1C), indicating the specificity of interaction between PICALM1 and VAMP72 in plants. Because ANTH domain-containing proteins are predicted to act as clathrin adaptors, we examined the interaction between PICALM1a and clathrin in plants. We generated transgenic plants expressing GFP-tagged PICALM1a, which was found to retain its function (as described below) and performed an immunoprecipitation analysis using the anti-GFP antibody. As shown in Fig. 1D, PICALM1a-GFP was coprecipitated with the clathrin heavy chain (CHC), whereas GFP alone was not. This result indicates that PICALM1a interacts with clathrin, probably during clathrin-mediated vesicle formation in Arabidopsis.

PICALM1 Acts in CME. Clathrin acts at multiple locations in the post-Golgi trafficking network, including the PM and TGN in plant cells (24), and VAMP72 also localizes to the PM and TGN (12, 14). Thus, we examined the location of these proteins in transgenic Arabidopsis plants expressing Hr-TagRFP by confocal microscopy, we found that GFP-VAMP721 and PICALM1a-GFP were coprecipitated with the clathrin heavy chain (CHC), whereas GFP alone was not. This result indicates that PICALM1a interacts with clathrin, probably during clathrin-mediated vesicle formation in Arabidopsis.
due to the multiple localization of VAMP721 near the PM (i.e., secretory vesicles, sites of secretion, and clathrin-coated pits), although we do not rule out the possibility that PICALM1 could also be involved in CME of other cargo molecules, besides the VAMP72 proteins. Noticeable localization of fluorescently tagged PICALM1a was not detected at the vacuolar membrane visualized with GFP-VAMP713, on punctate compartments bearing CLC-mKO in the cytoplasm, on the TGN labeled with VHAa1-mRFP, or on MVBs visualized with mRFP-ARA7/RABF2b (SI Appendix, Fig. S2). The subcellular localization of PICALM1a at the PM, together with its interaction with clathrin and VAMP721, strongly suggested that PICALM1 acts as an adaptor for sorting VAMP721 to clathrin-coated vesicles at the PM.

**PICALM1 Is Required for Normal Vegetative Development.** To determine the function of PICALM1 in plants, we analyzed the effects of loss-of-function mutations in the *PICALM1a/b* genes. Single mutants of *PICALM1a* (SALK_043625) and *PICALM1b* (GABI_026G05) did not exhibit macroscopic phenotypic abnormalities, although full-length transcripts were not detected by RT-PCR in these mutants (Fig. 3 A and B and SI Appendix, Figs. S3A and S4A). We therefore obtained the double mutant by crossing the single mutants. The *picalm1a/b* double mutant exhibited semidwarfism throughout its vegetative growth (Fig. 3B and SI Appendix, Figs. S3 A and B and S4 B-D), indicating important and redundant functions of PICALM1a and PICALM1b in the vegetative growth of *Arabidopsis*. The growth defect was rescued by expressing PICALM1a-GFP under the regulation of PICALM1a regulatory elements (promoter and introns) (Fig. 3B and SI Appendix, Fig. S3 A and B), indicating that the semidwarfism of the *picalm1a/b* mutant is due to mutations in the *PICALM1* genes, further demonstrating that GFP-tagged PICALM1a retains its original function. The double mutant was fertile and produced seeds, although the siliques were shorter than that of the wild type, indicating that PICALM1 does not play a critical role in reproductive processes, distinguishing it from PICALMS members that are required for sustainable pollen tube growth (23). The size and cortical cell number in the root meristematic region were reduced in the *picalm1a/b* double mutant, whereas the final cell size was comparable with that in wild-type plants (Fig. 3C and SI Appendix, Fig. S4 E–G), suggesting that the semidwarfism caused by the *picalm1a/b* mutation is mainly due to a compromised meristematic activity, resulting in a reduced cell number, rather than a reduced cell size.

A volcano-shaped secondary cell wall structure of mucilage secretory cells in the seed coat was rarely observed in the *picalm1a/b* mutant (SI Appendix, Fig. S3C), whereas the seed coat of the mutant complemented with PICALM1a-GFP harbored the volcano-shaped structure, similar to wild-type plants. We then examined the extrusion of mucilage after the imbition of seeds by ruthenium red staining, which demonstrated that the amount of mucilage was substantially reduced in the *picalm1a/b* mutant (Fig. 3D and SI Appendix, Fig. S4H). Given that defective mucilage deposition and/or extrusion may also occur due to the impairment of secretion-related proteins such as ECHIDNA and subunits of the exocyst complex (30, 31), the data indicate that the secretory activity is compromised by the *picalm1a/b* mutation. This is also consistent with the notion that PICALM1 acts in the recycling of VAMP72 members acting in the secretory pathway.

**PICALM1 Is Required for Loading VAMP721 to Clathrin-Coated Endocytic Vesicles.** If PICALM1 is an adaptor mediating VAMP72 loading to clathrin-coated vesicles, VAMP72 would accumulate at the PM in the *picalm1a/b* double mutant because of defective retrieval from the PM. To verify this, we expressed GFP-VAMP721 in *picalm1a/b* and observed its localization in root epidermal cells. We observed that GFP-VAMP721 was localized almost exclusively to the PM in the double mutant, contrary to the punctate cytoplasmic localization with minor localization at the PM in wild-type plants (Fig. 4A). Thus, PICALM1 is a major factor for retrieving the longin-type SNARE VAMP721 in *Arabidopsis*, which strongly supports the notion that PICALM1 is the adaptor protein bridging the clathrin cage to the VAMP72 proteins during CME.

The ANTH domain is also known to bind phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2), which recruits ANTH domain proteins to the membrane domain containing PtdIns(4,5)P2 in animal cells (32). This would also be the case for *Arabidopsis* PICALM1, because treatment with wortmannin (WM) or p-nitrophenylsine oxide (PAO), which act as inhibitors of phosphatidylinositol 3- and 4-kinases or phosphatidylinositol 4-kinase in plant cells, respectively, caused PICALM1a-GFP to dissociate from the PM and disperse in the cytosol (Fig. 4B and SI Appendix, Fig. S5). We further investigated whether the WM treatment hampered the retrieval of VAMP721 from the PM. If PICALM1 indeed acts as an adaptor in CME of VAMP721, WM
Fig. 2. Subcellular localization of PICALM1. (A) Subcellular localization of GFP-VAMP721 and PICALM1a-TagRFP in root epidermal cells observed by confocal microscopy. (B) Subcellular localization of PICALM1a-GFP and PICALM1b-TagRFP in root epidermal cells observed by confocal microscopy. (C) Localization of PICALM1a-GFP and CLATHRIN LIGHT CHAIN 2 tagged with mKO (CLC-mKO) near the PM observed by VIAFM. (D) Localization of GFP-VAMP721 and PICALM1b-TagRFP near the PM observed by VIAFM. The arrowheads indicate a dot bearing both GFP and TagRFP signals. (E) Quantification of colocalization between PICALM1a-GFP and CLC-mKO (Left) and GFP-VAMP721 and PICALM1b-TagRFP (Right) observed by VIAFM. The 90°-rotated CLC-mKO or PICALM1b-TagRFP images were used to test whether the detected colocalization was random or not. Wilcoxon signed rank test was used for statistical analyses. ***P < 0.001. n = 38 images for PICALM1a-GFP and CLC-mKO and 40 images for GFP-VAMP721 and PICALM1b-TagRFP. (Scale bars: 10 μm in A and B; 5 μm in C and D.)
treatment would hamper this process by inhibiting the association of PICALM1 with the PM. As expected, there was a substantial reduction in the amount of GFP-VAMP721 on punctate compartments in the cytoplasm of root epidermal cells treated with WM, and GFP-VAMP721 accumulated almost exclusively at the PM (Fig. 4C). Conversely, WM did not affect the localization of GFP-VAMP721 to the PM in the picalm1a/b double mutant. The vacuolar membrane localization of VAMP713 was not affected by WM in either wild-type or the double mutant plants. These results further support that PICALM1 is an adaptor protein required for the retrieval of VAMP72 from the PM in Arabidopsis.

**PICALM1 Is Not Required for General Endocytosis.** We further investigated whether the picalm1a/b mutation hampered general endocytosis, by monitoring the internalization of the lipophilic endocytic tracer dye FM4-64 in root epidermal cells. In nondividing cells, internalization of FM4-64 was not markedly affected by the double mutation (Fig. 5A and SI Appendix, Fig. S6A), indicating that endocytic flow from the PM to endosomes was not severely affected by this mutation. Consistent with this notion, FM4-64 accumulated rapidly at the forming cell plate in wild-type and double mutant plants, as previously reported (33), suggesting that endocytic transport to the forming cell plate was also not severely affected by the picalm1a/b mutation (Fig. 5B). However, the picalm1a/b mutation affected the localization of VAMP721 at the forming cell plate, which presented lower density of GFP-VAMP721 in the picalm1a/b mutant than in the wild-type plant expressing GFP-VAMP721 (Fig. 5B). This effect of the double mutation reflects the contribution of VAMP721 recycled from the PM via PICALM1-dependent retrieval in membrane fusion at the forming cell plate in wild-type plants. Whereas, VAMP721 synthesized de novo could somehow accomplish cytokinesis in the picalm1a/b mutant, considering that the double mutant did not exhibit a severe cytokinesis defect like the vmp712 vamp722 double mutant, which is defective in cell plate formation (14).

To examine whether the loss of function of PICALM1 affected the formation and/or distribution of clathrin-coated pits at the PM, we observed CLC-GFP in wild-type and picalm1a/b mutant plants by VIAFM and found no significant abnormality in the mutant (Fig. 5C and SI Appendix, Fig. S6B). This result suggested that PICALM1 is not required for the assembly of and vesicle formation by clathrin at the PM, consistent with unaffected general endocytosis visualized with FM4-64. To test whether PICALM1 is generally involved in the retrieval of membrane proteins from the PM, we also examined whether a component of the cellulose synthase complex involved in primary cell wall synthesis, CESA2, which is cycled between the PM and intracellular compartments through CME (34–36), was affected in the picalm1a/b mutant. We found that mRFP-tagged CESA2 exhibited similar localization in root epidermal cells of both wild-type and double mutant plants (Fig. 5D). These results indicated that PICALM1 is required for the CME of the VAMP72 proteins, acting as the adaptor protein during the sorting of VAMP72 into clathrin-coated pits/vesicles at the PM, although they are not required for general endocytosis.

**Discussion**

In this study, we demonstrated that a pair of paralogous ANTH domain-containing proteins, PICALM1a and PICALM1b, act as adaptors for loading the longin-type R-SNARE VAMP72 to clathrin-coated vesicles at the PM in Arabidopsis. In mammalian cells, ANTH domain-containing CALM/PICALM mediates the sorting of R-SNARE proteins into clathrin-coated vesicles at the PM by recognizing the SNARE domain (18, 19). However, only brevin-type R-SNAREs, such as VAMP8, VAMP3, and VAMP2, which are the short versions of R-SNAREs, are recycled by...
Mammalian VAMP7, which is a longin-type R-SNARE orthologous to Arabidopsis VAMP72, is recycled distinctly from brevins; the clathrin adaptor Hrb binds to the longin domain, but not to the SNARE domain, of VAMP7, to load it to clathrin-coated vesicles formed at the PM. Thus, the mechanisms of retrieving VAMP7 are different between animals and plants. This is surprising, considering that the distribution of brevins is restricted in eukaryotic lineages, and longin is widely conserved and is believed to have an ancient origin (5). In Dictyostelium discoideum, an ANTH-domain protein has been reported to interact with VAMP7B, further regulating its localization at contractile vacuoles (37). This might imply that the ANTH-domain protein was originally involved in recycling VAMP7 proteins, and the Hrb-dependent system in mammals could have been acquired in restricted lineages during evolution. However, it remains unknown whether the PM-resident VAMP7 is retrieved by ANTH-domain proteins in D. discoideum. Investigation of VAMP7 recycling mechanisms in other organisms is required to reconstitute the diversification of longin-SNARE retrieval systems during evolution.

Our findings provide interesting insights into the functional differentiation of ANTH domain-containing proteins during plant evolution. Four ANTH-domain proteins are encoded in the human genome (38), and the genome of the basal land plant, the liverwort Marchantia polymorpha, harbors three genes encoding ANTH proteins (39). Surprisingly, the Arabidopsis genome encodes 18 ANTH proteins, suggesting divergent functions of this protein family in Arabidopsis. A comparative genomic analysis further suggested that this group expanded during land plant evolution (20). However, although distinct expression patterns and subcellular localizations have been reported for these proteins (21, 22, 25, 40), their molecular functions remain largely ambiguous in plants. Recently, we found that one subgroup of the Arabidopsis PICALM family, PICALM5, is responsible for CME of ANXUR receptor kinases, which is required for sustained pollen tube growth and therefore, normal fertility (23). The present study revealed the molecular function of another subgroup of ANTH proteins, PICALM1, which was found to mediate CME of VAMP72 and was required for normal vegetative development. Together with distinctive expression patterns and subcellular localizations of related members (21, 22, 25, 40), our results indicated that PICALM family members have diverged for the retrieval of distinct cargo proteins via CME during land plant evolution and are involved in various physiological events during both vegetative and reproductive developmental processes in angiosperms.

PICALM1 is required for the retrieval of VAMP72 from the PM, and the loss of this function resulted in the accumulation of VAMP72 at the PM. Because PICALM1 also interacted with VAMP72 in yeast two-hybrid analysis, it may mediate recycling of other VAMP72 members, in addition to VAMP721, in Arabidopsis. VAMP72 members are involved in fundamental cellular activities, in Arabidopsis, including cytokinesis, and the double mutation in VAMP721 and VAMP722 results in seedling lethality (11, 14). However, the picalm1a/b mutant completed its life cycle, although it did exhibit semidwarfism. This mitigative effect could be attributed to the VAMP72 members synthesized de novo, which could partially perform their functions, whereas reuse of this R-SNARE is required for complete vegetative growth in Arabidopsis. Because secretory trafficking is also critical to reproductive processes, such as the pollen tube growth, and is strictly regulated (41), VAMP72 members may also play pivotal roles in reproductive processes. Expression data deposited in the public database also indicate that VAMP725 and VAMP726 are highly expressed in mature pollen (bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). An absence of any detectable defect in the reproduction of picalm1a/b mutants might reflect the involvement of other PICALM members in the recycling of these CALM.
pICALM members have also been identified as components of liverworts and mosses, can effectively reconstitute the functional analyses of ANTH-domain proteins in basal land plants, such as chloroplast-coated pits/vesicles during CME. Functional coordination of plant ANTH proteins with other clathrin adaptors is important to understand the unique regulatory mechanisms of CME in plant cells.

Materials and Methods

Plant Materials, Growth Condition, and Plasmids. Arabidopsis thaliana plants from the Col-0 accession were used in this study. Arabidopsis seedlings were grown on half-strength Murashige and Skoog (1/2 MS) agar medium containing 1% sucrose, at 23 °C under continuous light. Fourteen-day-old plants were planted to soil and grown at 23 °C under a 16-h-long day/8-h-dark cycle (16 h light and 8 h dark). Arabidopsis picalm1a (SALK_043625) and picalm1b (GABI_026G05) mutants were obtained from the Arabidopsis Biological Resource Center (ABRC) and German plant genetics research program-Kölner Arabidopsis T-DNA lines (GABI-Kat) (48, 49), respectively. The mutants were backcrossed to wild-type Arabidopsis (Col-0) at least three times. To construct transgenic plants expressing fluorescently or His-tagged PICALM1a and PICALM1b under the regulation of their own regulatory elements, GFP CDS, mRFP, or 6xHis tag was inserted in front of the stop codon of the 4.5-kb genomic fragment of PICALM1a or the 5.7-kb genomic fragment of PICALM1b. These genomic fragments contained the coding regions, introns, and 1.1 kb and 2.1 kb of 5′-flanking sequences of PICALM1a and PICALM1b, respectively. The chimeric genomic fragments of PICALM1a and PICALM1b were subcloned into binary vectors, i.e., pB7WG, pGW84, pGW859, pGW8659, or pGW1352 (28, 50–52). To construct transgenic plants expressing fluorescently tagged pGW-GVAMP721, VAMP721, ARA7, and CLC-GFP near the PM observed by fluorescence microscopy using the T7 primer.

Yeast Two-Hybrid Assays. Yeast two-hybrid screening was performed using the Matchmaker Two-Hybrid Library Construction and Screening Kit (Clontech) and the normalized Arabidopsis cDNA library (Mate & Plate Library, Universal Arabidopsis, Clontech), containing cDNA sequences derived from seedlings, flowers, buds, pollen, leaves, siliques, and stems, introduced into the yeast strain Y187. The cDNA fragment for the cytoplasmic domain of VAMP727 was subcloned into pGBK7, which was then transformed into the yeast strain Y2H Gold. After mating between the Y2H Gold strain expressing VAMP72 and the Y187 strain with the expression library, resultant zygotes were selected by culturing on SD-Leu−/−Trp−/−His−/−Aureobasidin A plate for 5 d at 25 °C. Plasmids released from positive clones were then sequenced using the T7 primer.

To construct GAL4 AD fusion-expressing vectors, open reading frames (ORFs) for ANTH/ENTH-domain proteins (PICALM1a/ECA11/AT1G01600, PICALM1b/AT1G4910, PICALM2a/AT5G57200, PICALM2b/AT4G25490, PICALM3/AT5G35320, PICALM4a/AT1G25490, PICALM5a/ECA2/AT1G03050, PICALM5b/AT4G02650, PICALM6/AT1G52050, EPSIN1/AT4G17170, EPSIN2/AT2G63160, and EPSIN3/AT3G59290) were subcloned into pAD-GAL4-CWF. These members were selected according to the reaction of Holstein and Olivius (57); in addition, an ANTH protein close to the PICALM1 group, PICALM2a, was included. To construct GAL4 BD fusion-expressing vectors, ORFs for the cytoplasmic domains of VAMP7 proteins (VAMP713, VAMP727, and VAMP721) and the long or SNARE domains of VAMP72 proteins (VAMP727 and VAMP721) were subcloned into pBD-GAL4-CWF and pBD-GAL4-CWF and pBD-GAL4-CWF were provided by T. Demura, Nara Institute of Science and Technology (NAIST), Ikoma, Japan. Empty vectors, i.e., pAD-GAL4-CWF and pBD-GAL4-CAM, were used as negative controls. Each pair of the pAD-GAL4-CWF- and the TPLATE/TSET complex, which is a clathrin adaptor complex conserved only in plants and some limited clades of eu-
pBD-GAL4-GWRFc-derived vectors was introduced into Saccharomyces cerevisiae strain AH109 (Clontech). The AH109 transformants were selected on SD−Leu−Trp plates, cultured in SD−Leu−Trp liquid medium for 24 h at 28 °C, and then diluted with water to achieve an OD600 of 5.0. Then, 5 μL of each culture was spotted on SD−Leu−Trp plates cultured in SD−Leu−Trp−His and incubated for 72 h at 28 °C. Transformation was performed independently at least twice, and at least two colonies per transformation were checked for interaction.

Primers. Primers used for genotyping, cloning, plasmid construction, and RT−PCR are listed in SI Appendix, Table S1.

RT−PCR. Total RNA was extracted from 10-d-old seedlings with genotypes presented in SI Appendix, Fig. S3 A, using NEasy Plant Mini Kit (Qiagen) and TURBO DNA-free Kit (Ambion, Thermo Fisher Scientific), according to the manufacturer’s instructions. The extracted RNA (400 ng) was then reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen), and ORFs of the indicated genes were amplified using PrimeSTAR Max DNA Polymerase (Takara Bio) with gene-specific primers listed in SI Appendix, Table S1.

Confocal Microscopy. Root epidermal or lateral root cap cells of seedlings expressing fluorescently tagged PICALM1a, PICALM1b, VAMP713, VAMP721, ARAY, VHAa1, CESa2, and/or ΔClc were observed using the LSM780 confocal microscope (Carl Zeiss) with a 63× oil immersion lens (N.A. = 1.40), operated using ZEN software (Carl Zeiss). GFP and mRFP/TagRFP/FM4-64 were excited with 488 nm Ar/Kr and 561 nm diode lasers, respectively. For WM treatment, the seedlings were stained with 1% ruthenium red (Wako) for 1 h. The stained seeds were observed using Miniscope TM-1000 (Hitachi). To visualize the mucilage released from seeds, the seed coats of dry seeds were observed using a BX60 (Olympus) equipped with a UPlanApo 10× lens (N.A. = 0.40). Images were acquired using a DP73 digital camera (Olympus) operated using cellSens standard software (Olympus).

VIAM Observation. Root epidermal cells of 7-d-old seedlings expressing fluorescently tagged PICALM1 and VAMP71 or Clc were observed using an IX-71 (Olympus) equipped with a UAPON 100× O TIRF lens (Olympus) for VIAM. The root cells were observed using a BX60 (Olympus) equipped with a UPLANapo 10× lens (N.A. = 0.40). The images were acquired using a DP73 digital camera (Olympus) operated using cellSens standard software (Olympus).

The acquired images were processed using ZEN software (Carl Zeiss) and Photoshop CC (Adobe Systems).

The fluorescence emission spectra were separated using a FF505-DF010−35 LP dichroic mirror (Semrock) and filtered through a FF501-S235 filter (Semrock) for GFP and a FF620/600M filter (Chroma) for mKO and TagRFP, using W-View Confocal Laser Scanning Microscope Imaging Software (Carl Zeiss) and imaging the iXon X3 EMCCD camera (Andor Technology) operated with Metamorph software (Molecular Devices). Each frame was exposed for 200 ms. The acquired images were analyzed using ImageJ and Photoshop CC (Adobe Systems).

Antibodies. Anti-GFP and anti-His-tagged antibodies for the communoprecipitation analysis of PICALM1a and VAMP712 interaction were purchased from MBL (598 and PM032, respectively). The anti-GFP antibody used for the communoprecipitation analysis of PICALM1a and CHC was prepared as described previously (8). The anti-CHC antibody was purchased from Agri-sera (AS10 690). Antibodies were diluted for immunoblotting as follows: anti-GFP, 1:1,000; anti-His-tag, 1:1,000; anti-CHC, 1:2,000.

Immunoprecipitation. For immunoprecipitation followed by Western blotting (Fig. 1 B), 0.8 g of 14-d-old transgenic seedlings expressing PICALM1a-His and GFP-VAMP713 or GFP-VAMP721 was soaked in cross-linking buffer (20 mM Hepes pH 7.5, 1% sucrose, and 1 mM dithiobis [succinimidyl propionate] [DSP] [Thermo Fisher Scientific]) for 1 h at room temperature (RT). Trit-HCl (20 mM, pH 7.5) was then added, and the mixture was incubated for 30 min at RT. Samples were homogenized in grinding buffer (400 mM sucrose, 50 mM Hepes pH 7.5, 50 mM ethylenediamine-N,N',N''-tetraacetic acid (EDTA) pH 8.0, and one tablet of complete EDTA-free [Roche]50 μL). Lysates were centrifuged at 1,000 × g for 10 min and 8,000 × g for 15 min at 4 °C to remove any debris. Supernatants were incubated for 30 min with 1% Triton X-100 at 4 °C for solubilization and then centrifuged at 20,000 × g for 30 min at 4 °C. For analyzing the interaction between PICALM1a and VAMP721 (Fig. 1 C), 0.8 g of 14-d-old transgenic seedlings expressing GFP or PICALM1a-GFP was homogenized in the aforementioned grinding buffer, and the lysates were treated as previously described. Supernatants were incubated for 30 min with 1% Triton X-100 and 1 mM dithiothreitol (DTT) at 4 °C for solubilization and centrifuged at 20,000 × g for 30 min at 4 °C. Immunoprecipitation with detergent extracts was performed using the micro-MACS GFP-tagged protein isolation kit (Miltenyi Biotec), according to the manufacturer’s instructions. Immunoprecipitates were then subjected to immunoblotting.

Statistical Analysis. The Wilcoxon signed rank test (Fig. 2 E) and Wilcoxon rank-sum test (SI Appendix, Fig. S6 B) were used for statistical analyses between the two groups. For statistical analysis among three or more groups, Tukey’s honestly significant difference post hoc test was used for samples with normal distribution (SI Appendix, Fig. S4 B) and the Steel–Dwass test was used for nonparametric sample (other) after one-way ANOVA. Normality of data were tested using the Jarque–Bera test.

Data Availability. All study data are included in the article and SI Appendix.

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