SEIPIN Isoforms Interact with the Membrane-Tethering Protein VAP27-1 for Lipid Droplet Formation

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SEIPIN proteins are localized to endoplasmic reticulum (ER)–lipid droplet (LD) junctions where they mediate the directional formation of LDs into the cytoplasm in eukaryotic cells. Unlike in animal and yeast cells, which have single SEIPIN genes, plants have three distinct SEIPIN isoforms encoded by separate genes. The mechanism of SEIPIN action remains poorly understood, and here we demonstrate that part of the function of two SEIPIN isoforms in Arabidopsis (Arabidopsis thaliana), AtSEIPIN2 and AtSEIPIN3, may depend on their interaction with the vesicle-associated membrane protein (VAMP)–associated protein (VAP) family member AtVAP27-1. VAPs have well-established roles in the formation of membrane contact sites and lipid transfer between the ER and other organelles, and here, we used a combination of biochemical, cell biology, and genetics approaches to show that AtVAP27-1 interacts with the N termini of AtSEIPIN2 and AtSEIPIN3 and likely supports the normal formation of LDs. This insight indicates that the ER membrane tethering machinery in plant cells could play a role with select SEIPIN isoforms in LD biogenesis at the ER, and additional experimental evidence in Saccharomyces cerevisiae supports the possibility that this interaction may be important in other eukaryotic systems.

INTRODUCTION

Little is known about the mechanisms by which SEIPIN proteins mediate the formation of cytoplasmic lipid droplets (LDs) at the endoplasmic reticulum (ER); yet, it is clear that, in all eukaryotic organisms investigated to date (including yeast, animals, and plants), disruption of SEIPIN function results in aberrant LD deposition in the cytoplasm (Cartwright and Goodman, 2012; Chapman et al., 2019; Bohnert, 2020). Recently, partial three-dimensional structures for insect and mammalian SEIPINs revealed by cryo-electron microscopy (Sui et al., 2018) confirmed previous studies from yeast (Birnb et al., 2010) showing that SEIPIN forms large, oligomeric complexes in the ER membrane. These ring-like structures, composed of 11 or 12 subunits, are stabilized by interactions between conserved domains located within each SEIPIN monomer on the lumenal side of the ER (Sui et al., 2018; Yan et al., 2018). These conserved regions include both amphipathic helices that are capable of binding to LDs as well as a lipid binding domain. The resolved three-dimensional structures of the SEIPIN proteins examined to date, however, did not reveal any specific lipidic border to these locations. Notably, there was no definitive structural information provided for the N- or C-terminal portions of the SEIPIN proteins. The N- and C-terminal ends of the SEIPIN proteins are hypothesized to extend through the ER bilayer and into the cytoplasm, where at least the N terminus of the Drosophila (Drosophila melanogaster) protein has been proposed to interact with the LD monolayer and thereby tether the emerging LD to the ER surface (Sui et al., 2018).

Surprisingly, unlike yeast, insects, or mammals, plants express multiple SEIPIN genes, suggesting an elaboration of the LD biogenetic machinery in plants compared with other eukaryotes (Cai et al., 2015; Chapman et al., 2019). In Arabidopsis (Arabidopsis thaliana), loss of function of SEIPINs results in aberrant LD formation in seeds and pollen (Taurino et al., 2018), both of which are composed of cell types wherein neutral lipid (e.g., triacylglycerol [TAG]) synthesis and storage are intensely active. The three different Arabidopsis SEIPIN isoforms—AtSEIPIN1, AtSEIPIN2, and AtSEIPIN3—promote LD formation when expressed alone or in combination in a leaf cell background (a plant cell type with relatively few LDs; Chapman et al., 2012). That is, when expressed individually, each AtSEIPIN isoform directed the formation of LDs of different sizes in leaves: AtSEIPIN1 produced

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larger, super-sized LDs; AtSEIPIN3 produced smaller sized LDs; and AtSEIPIN2 produced generally intermediate-sized LDs (Cai et al., 2015). While the C-terminal two-thirds of the three AtSEIPINs isoforms are mostly conserved, the N-terminal sequences are substantially diverged. Furthermore, domain-swaps between the N termini of AtSEIPIN1 and AtSEIPIN3 revealed that the N terminus of AtSEIPIN3 was responsible for the production of smaller sized LDs, while the shorter N terminus of AtSEIPIN1 was associated with production of larger LDs (Cai et al., 2015). Based on these observations, we reasoned that perhaps the N terminus of AtSEIPIN3 interacts with some additional protein factor(s) to facilitate formation of small LDs and that this interaction is missing or different in AtSEIPIN1.

To begin to test the hypothesis that the N-terminal sequence of AtSEIPIN3 facilitates LD biogenesis by interacting with other proteins, we performed affinity-capture experiments with mCherry-tagged AtSEIPIN3 expressed in Nicotiana benthamiana leaf cells (where small LDs are proliferated by expression of AtSEIPIN3; Cai et al., 2015). At the same time, we searched publicly available protein interactome databases for putative AtSEIPIN3 binding partners. Both strategies converged on the vesicle-associated membrane protein (VAMP)–associated proteins (VAPs), particularly the Arabidopsis VAP isoform AtVAP27-1. The presence of VAPs in plant LD proteomes (Brocard et al., 2017; Zhi et al., 2017; Kretzschmar et al., 2018) further attracted our attention. Here, we show that the N terminus of both AtSEIPIN2 and AtSEIPIN3 interact specifically and directly with AtVAP27-1 in yeast two-hybrid (Y2H) assays and in plant cells using colocalization and bimolecular fluorescence complementation (BiFC) analysis. Furthermore, seeds from two independent Arabidopsis vtap27-1 T-DNA knockout lines showed abnormal, large LDs, similar to the aberrant LD phenotype in sepin2 seipin3 double mutant seeds. AtVAP27-1 has been shown by others to be involved in the formation of membrane contact sites (MCSs) between ER and plasma membrane in plants (Siao et al., 2016) and also to interact with clathrin subunits in support of endocytic vesicle trafficking (Stefano et al., 2018). Our results suggest a mechanistic cooperation between SEIPIN proteins, the membrane tethering and vesicular trafficking machinery of plant cells, and ER-LD connections that are likely to be required for proper filling and expansion of nascent LDs at the ER surface.

RESULTS

Interactions of Plant SEIPINs and VAPs In Planta and in Yeast Cells

Prior studies have shown that overexpression of AtSEIPIN3 in N. benthamiana leaves resulted in the protein’s localization to ER-LD junctions and induced a proliferation of small-sized LDs (Cai et al., 2015). In an attempt to identify proteins that might work together with AtSEIPIN3 to facilitate this process, mCherry-tagged, full-length AtSEIPIN3 was transiently produced in N. benthamiana leaves. Three days later, leaves were homogenized in nonionic detergent, and mCherry-AtSEIPIN3 and associated proteins were affinity captured using mCherry antibodies conjugated to agarose beads. Transiently produced mCherry alone (lacking AtSEIPIN3) was used as a control to help rule out nonspecific protein interactions. Proteins in the precipitated fractions were identified by tryptic digestions and mass spectrometry analysis, as described previously (Cai et al., 2019). As shown in Figure 1A, peptides corresponding to N. benthamiana VAP proteins NbVAP1-2 and NbVAP1-3 were identified in mCherry-AtSEIPIN3 pull-downs, but
they were absent from the mCherry control. Although some proteins from chloroplasts likely contaminated these pull-down fractions (Supplemental Data Set 1), only a few other ER candidate proteins were identified in mCherry-AtSEIPIN3 fractions, the subcellular site where AtSEIPIN3 protein is located (Cai et al., 2015). Affinity-capture experiments also were conducted by co-expressing mCherry-AtSEIPIN3 with either Arabidopsis LEAFY COTYLEDON2 (AtLEC2) or diacylglycerol acyltransferase1 (AtDGAT1), which are both known to increase TAG content and LD abundance when transiently produced in plant leaves (Vanhercke et al., 2014; Cai et al., 2019). The N. benthamiana NbVAP1-2 and NbVAP1-3 proteins, as well as NbVAP2-1, were identified as AtSEIPIN3-interacting proteins under these lipid-loading conditions (Figure 1A).

A phylogenetic analysis was conducted to delineate the relationships of these three full-length NbVAP polypeptide sequences with Arabidopsis VAP protein family members (Wang et al., 2016b), and with yeast VAPs Saccharomyces cerevisiae suppressor of choline sensitivity2 (SCS2) and SCS22 (Figure 1B; Supplemental Figures 1 and 2; Manford et al., 2012). Notably,

| Accession No. | Name                                             | mCherry | mCherry + AILDC2 | mCherry + AIDGAT1 | mCherry-AtSEIPIN3 + AILDC2 | mCherry-AtSEIPIN3 + AIDGAT1 |
|---------------|--------------------------------------------------|---------|------------------|---------------------|---------------------------|---------------------------|
| -             | mCherry                                         | 439.5   | 483.9            | 507.5               | 154.8                     | 82.6                      | 87.4                      |
| -             | AtSEIPIN3                                       | 0       | 0                | 0                   | 36.1                      | 11.8                      | 17.2                      |
| NbS0011956g004.1 | Vesicle-associated protein 1-2                  | 0       | 0                | 0                   | 1.3                       | 2.4                       | 1.2                       |
| NbS0001985g0007.1 | Vesicle-associated protein 1-3                  | 0       | 0                | 0                   | 3.9                       | 2.4                       | 2.5                       |
| NbS00006602g0001.1 | Vesicle-associated protein 2-1                  | 0       | 0                | 0                   | 0                         | 2.4                       | 1.2                       |

**Figure 1.** Identification of Candidate AtSEIPIN3-Interacting Proteins and Phylogenetic Analysis of the VAP Protein Family. (A) Proteins listed across the top of the table were transiently coexpressed in *N. benthamiana* leaves and then mCherry or mCherry-tagged fusion proteins were immunoprecipitated using RFP-Trap_A beads. Copurifying proteins were identified by mass spectrometry, as described in Methods. Shown is a select group of affinity-captured proteins, including the three *N. benthamiana* VAPs; a complete list of the coprecipitating proteins is provided in Supplemental Data Set 1. The accession numbers and descriptions of *N. benthamiana* proteins were acquired from the *N. benthamiana* genome database at Sol Genomics Network (v0.4.4; www.solgenomics.net). Normalized spectral counts represent quantities of coimmunoprecipitated proteins. (B) Phylogenetic analysis of VAP protein families (unrooted tree) from Arabidopsis, *N. benthamiana*, and *S. cerevisiae*. The three *N. benthamiana* VAPs identified by affinity capture with mCherry-AtSEIPIN3 (refer to [A]) are indicated with asterisks; AtVAP27-1 is highlighted. The percent bootstrap support for 1000 replicates is shown below each branch with >50% support. For alignment used to make the tree and the tree in Newick format, see Supplemental Figure 1 and Supplemental Figure 2, respectively.
among the Arabidopsis VAPs most similar to NbVAP1-2 is AtVAP27-1. AtVAP27-1, based on information from a publicly available protein interaction database (https://thebiogrid.org; Arabidopsis Interactome Mapping Consortium; Arabidopsis Interactome Mapping, 2011), is a putative interactor with AtSEIPIN3; consequently, we focused our subsequent studies on AtVAP27-1.

The predicted topologies of the three AtSEIPIN isoforms and AtVAP 27-1 in the ER membrane are shown in Figure 2. The ER lumenal region in all three AtSEIPIN isoforms is flanked by two putative transmembrane domains (TMDs). Both AtSEIPIN2 and AtSEIPIN3 are also predicted to have long N and C termini that extend into the cytoplasm, while AtSEIPIN1 has a relatively short N terminus that is predicted to orient into the ER lumen (Figure 2). For AtSEIPIN3, the N-terminal 170 amino acids are both necessary and sufficient to direct the formation of small-sized LDs (Cai et al., 2015). Both of the N-terminal ~30 amino acids of AtSEIPIN2 and AtSEIPIN3 possess an FFAT motif (Figure 2B, shaded), which is well known to be important for mediating protein interactions with VAPs and was originally defined as consisting of two phenylalanesines (FF) in an acidic tract, but is now recognized as possessing a large number of possible amino acid variants (Loewen and Levine, 2005; Murphy and Levine, 2016). Also highlighted in the topology prediction for AtVAP27-1 is the major sperm domain (MSD; Figure 2A), a conserved region of VAP proteins known to interact with corresponding FFAT motifs (Loewen and Levine, 2005). Based on these observations, we hypothesized that the N-terminal region of AtSEIPIN3 (and AtSEIPIN2), perhaps via its FFAT motif, is responsible for interacting with the AtVAP27-1 protein and, furthermore, that AtSEIPIN1, which lacks an obvious FFAT motif, is unlikely to interact, at least not directly, with AtVAP27-1.

Affinity-capture experiments can help to identify proteins that physically interact as well as proteins that are associated together in larger complexes, where the individual protein components might not interact directly with all other components. Consequently, to determine whether AtSEIPINs and AtVAP27-1 interact directly, Y2H assays were conducted with all three AtSEIPINs and AtVAP27-1. We also tested the interaction of AtVAP27-1 with a chimeric construct where the N terminus of AtSEIPIN1 was swapped with the N terminus of AtSEIPIN3 (AtSEIPINChim; Figure 3D) as well as various truncation mutants of AtSEIPIN2 and AtSEIPIN3 (positions of truncations are marked by arrowheads in Figures 2 and 3B). As shown in Figure 3A, both AtSEIPIN2 and AtSEIPIN3, but not AtSEIPIN1, showed positive interactions with AtVAP27-1, based on yeast growth on stringent-selection media in Y2H assays. By contrast, removal of the N terminus, but not the C terminus, from AtSEIPIN3 or AtSEIPIN2, eliminated the interactions with AtVAP27-1 (Figures 3C and 3E). Specifically, removal of the N-terminal 25 or 29 amino acids, containing the putative FFAT motifs of AtSEIPIN3 or AtSEIPIN2, respectively, abolished interaction with AtVAP27-1. In addition, the chimeric construct AtSEIPINChim, wherein the AtSEIPIN1 N terminus was replaced with the AtSEIPIN3 N terminus, conferred a positive interaction with AtVAP27-1 (Figure 3D).

Figure 2. Predicted Topological Orientations of Arabidopsis SEIPINs in the ER Membrane.

(A) Topologies of AtSEIPIN1-3 and AtVAP27-1 were predicted using PROTTER (http://wlab.ethz.ch/protter/start/; Omasits et al., 2014) and are generally consistent with the topologies reported for other SEIPIN proteins (Binns et al., 2010; Sui et al., 2018; Yan et al., 2018; Chapman et al., 2019). Both AtSEIPIN2 and AtSEIPIN3 are predicted to have an N terminus that extends into the cytoplasm and includes an FFAT motif located within the first 30 amino acids (refer to [B]). The MSD of AtVAP27-1 is highlighted in gray.

(B) Polypeptide sequences of the N-terminal 30 amino acids of AtSEIPIN2 and AtSEIPIN3, along with their predicted FFAT motifs (highlighted gray), which are based on Mikita and Levine (2012). Both AtSEIPIN2 and AtSEIPIN3 possess an FFAT-like motif score of >2 (with a score of <3, the FFAT-like motif is considered strong and specific according to Murphy and Levine [2016]). Black and white arrowheads in (A) and (B) indicate the locations of N- and C-terminal truncations, respectively, that were tested in this study in Y2H or in planta assays, and numbers indicate the corresponding amino acid positions in each (truncated) protein.
Figure 3. Y2H Protein Interaction Analysis of AtSEIPINs and AtVAP27-1.

(A) to (F) Sequences for full-length AtSEIPINs or various N- or C-terminal truncations or chimeric and mutated AtSEIPIN proteins were coexpressed with coding sequences for AtVAP27-1 in yeast cells, and then cells were plated on either plasmid-selection conditions (DDO) or higher stringency conditions (QDO) where yeast cell growth was dependent on Y2H protein interactions. Empty plasmid (negative) controls included pGBK or pGAD. Plasmid combinations are shown to the left in (A and C–F), and images of the corresponding cell culture dilution series on DDO or QDO plates are shown on the right. Illustrations depicting full-length or various truncation mutants of AtSEIPIN2/3 are shown in (B), with black and white arrowheads, as well as the corresponding amino acid numbers, denoting the positions of N- or C-terminal truncation mutants, respectively. The putative FFAT motif near the N terminus of each protein is highlighted black (see also Figure 2). Shown in (D) is an illustration and the Y2H results for the chimeric protein AtSEIPINChim, whereby the
However, because the interaction was not eliminated by these mutations, we cannot rule out that other amino acids in the N terminus or elsewhere in AtSEIPIN2 could participate in the interaction with AtVAP27-1. In a complementary experiment, removal of the MSD (i.e., FFAT-interacting domain) from AtVAP27-1 eliminated the interaction with AtSEIPIN2 (Figure 3F). Taken together, the results from 2H assays support a physical interaction between AtSEIPIN3 or AtSEIPIN2 and AtVAP27-1, specifically through their N-terminal regions that contain FFAT motifs. Furthermore, the AtSEIPIN3 N-terminal sequence is both necessary and sufficient (when fused to AtSEIPIN1) to facilitate an interaction with AtVAP27-1.

**Interactions of AtSEIPIN2/3 and AtVAP27-1 in Plant Cells**

The interactions between AtSEIPIN2/3 and AtVAP27-1 suggest that, in addition to roles in ER–plasma membrane MCS formation (Wang et al., 2017) and clathrin-mediated vesicular trafficking (Stefano et al., 2018), AtVAP27-1 also may play a role in LD dynamics. To begin to investigate this potential relationship between AtVAP27-1 and LDs in plant cells, the coding sequence for green fluorescent protein (GFP)-tagged AtVAP27-1 (AtVAP27-1-GFP) was transiently expressed in *N. benthamiana* leaves and the subcellular localization pattern for AtVAP27-1-GFP was compared with the fluorescence pattern of LDs stained with the neutral lipid–selective dye monodansylpentane (MDH; Figure 4A; Yang et al., 2012). As expected, based on similar results reported recently by Stefano et al. (2018), the AtVAP27-1-GFP fusion protein was located in leaf epidermal cells throughout the reticular ER network and also showed enrichment in discrete foci considered to be subdomains of the ER (see Stefano et al., 2018, Figure 2). Notably, many of the MDH-stained LDs were closely associated with the AtVAP27-1–enriched ER regions (Figure 4A). A similar relationship was observed when the coding sequence for AtVAP27-1-GFP was coexpressed with the coding sequence for mCherry-tagged Arabidopsis LD-associated protein1 (AtLDAP1-mCherry), which is known to be located on the LD surface in plant cells (Figure 4B; Gidda et al., 2016). Surface renderings of a zoomed-in region of leaf cells showed the close apposition of LDs with AtVAP27-1 foci in greater detail (Figures 4A and 4B, far right panels). Furthermore, time-lapse imaging revealed that the ER foci enriched in AtVAP27-1-GFP were often closely associated with LDAP1-mCherry–labeled LDs (Supplemental Movie). As an additional reference for the observed localization of AtVAP27-1–GFP to ER subdomains, the coding sequence for AtVAP27-1-GFP was coexpressed with the coding sequence for mCherry fused to the N terminus of the Arabidopsis clathrin heavy chain 2 subunit (mCherry-CH2) and served as a reference control to mark the position of the ER–plasma membrane MCSs known to be enriched in AtVAP27-1 (Figure 4C; Stefano et al., 2018). In general, the subcellular relationship between AtVAP27-1 and LDs was similar in juxtaposition to AtVAP27-1 foci like those found in ER–plasma membrane MCSs. Taken together, these data support the concept that ER-localized AtVAP27-1 might serve to tether the ER to LDs in plant cells. The similarity in the subcellular distributions between the LDAP1/AtVAP27-1 proximity and the CHC2/AtVAP27-1 proximity may point also to intriguing possibilities that clathrins could be associated with LD formation, but this remains to be examined in more detail.

To further explore the relationships between AtSEIPIN3 or AtSEIPIN2 and AtVAP27-1, coding sequences for both sets of proteins were transiently coexpressed in *N. benthamiana* leaves and compared with the distribution of MDH-stained LDs. As shown in Figure 5, considerable overlap was observed in the fluorescent staining patterns attributable to mCherry-AtSEIPIN3 or mCherry-SEIPIN2 and AtVAP27-1-GFP in the ER in leaf cells, and LDs were often intimately associated with the ER network, particularly in ER regions where mCherry-AtSEIPIN2 or mCherry-AtSEIPIN3 and AtVAP27-1-GFP appeared to coalesce (Figure 5, arrowheads). mCherry-SEIPIN1 also appeared to codistribute in the ER with AtVAP27-1-GFP (Figure 5, bottom row); so, although we did not obtain evidence for a direct interaction between these two proteins in Y2H (Figure 3A), we cannot rule out an indirect association between AtVAP27-1 and AtSEIPIN1 in planta.

Similar results were obtained when coding sequences for mCherry-AtSEIPIN2 or AtSEIPIN3 and AtVAP27-1-GFP were transiently coexpressed in another plant cell type (i.e., *Nicotiana tabacum* pollen tubes; Supplemental Figure 3). However, production of mCherry-AtSEIPIN3 (Supplemental Figure 3D) and, albeit to a lesser extent, mCherry-AtSEIPIN2 (Supplemental Figure 3E) resulted in unusual ER membrane structures, which are reminiscent of ER membrane whorls (Gong et al., 1996; Koning et al., 1996) and probably due to ectopic overproduction of these membrane proteins in the ER. Nevertheless, in both of the two different plant cell types examined (i.e., leaf epidermal cells and pollen tubes), AtVAP27-1 colocalized with both AtSEIPIN3 and AtSEIPIN2, along with an association with LDs. It is also important to point out that the distribution of AtVAP27-1 when coexpressed with AtSEIPIN2/3 (Figure 5) was different from that when AtVAP27-1 was expressed alone (or with CHC2 or LDAP1; Figure 4), suggesting that AtSEIPIN2/3 can redirect the distribution of AtVAP27-1 in the ER to a subcellular localization pattern closely associated with AtSEIPIN2/3.
fluorophores consisting of the C-terminal half of the cyan fluorescent protein (cCFP) and the N-terminal half of the Venus fluorescent protein (nVenus) were used to evaluate the direct interaction of AtSEIPIN and AtVAP27-1 in planta. For these experiments, we focused our attention on AtSEIPIN2. We were cognizant that BiFC assays need to be conducted with caution due to protein overexpression artifacts and false positives (Kudla and Bock, 2016), and here, the quantified reconstituted fluorescence signals were compared with negative controls to provide supportive evidence of protein interactions in vivo.

N. benthamiana leaves were coinfiltrated with Agrobacterium tumefaciens (Agrobacterium)–containing plasmids encoding cCFP-AtVAP27-1 and either nVenus-tagged full-length AtSEIPIN2 (nVenus-AtSEIPIN2) or a truncated version of AtSEIPIN2 that lacked the N-terminal 29 amino acids (nVenus-AtSEIPIN2D1-29) and that was necessary for the interaction of AtSEIPIN2 and AtVAP27-1 in Y2H assays (Figure 3E). An mCherry-peroxisomal fusion protein (mCherry-Perox) was also coinfiltrated and served as a marker for cell transformation, as in our previously reported BiFC studies (Pyc et al., 2017). As shown in Figure 6, coexpression of nVenus-AtSEIPIN2 and cCFP-AtVAP27-1 yielded an obvious BiFC fluorescence signal in leaves, indicating that AtSEIPIN2 and AtVAP27 interact directly in planta. By contrast, coexpression of coding sequences for nVenus-SEIPIN2D1-29 and cCFP-AtVAP27-1 resulted in a substantial reduction in the BiFC fluorescence signal (Figure 6), suggesting that the AtSEIPIN2-AtVAP27-1 interaction is dependent on the N terminus of AtSEIPIN2, which includes the protein’s FFAT motif (Figure 2B). These BiFC results were consistent with the results obtained from the Y2H analysis (Figure 3). RT-PCR analysis confirmed similar expression levels of the BiFC fusion constructs (i.e., nVenus-AtSEIPIN2, nVenus-AtSEIPIN2D1-29, and cCFP-AtVAP27-1) in plant leaves (Supplemental Figure 4).

The N-Terminal Domain of AtSEIPIN2 Localizes to LDs and Promotes the Relocalization of Coexpressed AtVAP27-1 to LDs in Plant Cells

To further dissect the region of AtSEIPIN involved in interaction with AtVAP27-1, we focused on a minimal portion of AtSEIPIN2 (amino acids 1 to 238, AtSEIPIN2D1-238) that includes the N-terminal
region with the first putative TMD, but lacks the conserved ER lumenal domain and C-terminal portion of the protein (refer to illustrations of AtSEIPIN2 in Figures 2A and 3B). Strikingly, this truncated protein (mCherry-AtSEIPIN21-238) localized predominantly to LDs in N. benthamiana leaf epidermal cells (Figure 7A), which is different from the localization of full-length AtSEIPIN2 (mCherry-AtSEIPIN2) to the ER (compared with Figures 7A and 5). Additional deletion of the N-terminal 29 amino acids from mCherry-AtSEIPIN21-238 (i.e., mCherry-AtSEIPIN230-238), which removes the FFAT motif, resulted in a similar localization to LDs (Figure 7A). These results reveal that the N-terminal region of AtSEIPIN2, which is predicted to face the cytoplasm (Figure 2A; Chapman et al., 2019), contains an LD targeting sequence(s). Furthermore, this targeting information does not include the protein’s first 29 amino acids. Notably, a similar N-terminal LD targeting sequence, which includes an amphipathic \( \alpha \) helix, was recently identified in the Drosophila SEIPIN protein (Wang et al., 2016a; Sui et al., 2018).

As shown in Figure 7B, the subcellular location of AtVAP27-1 in N. benthamiana leaf cells was dramatically altered by the coexpression of coding sequences for AtSEIPIN21-238 and was dependent on the protein’s N-terminal FFAT motif-containing region. That is, AtVAP27-1-GFP localized predominantly to LDs when coexpressed with the coding sequence for mCherry-AtSEIPIN21-238, which contains the FFAT motif. However, when AtVAP27-1-GFP was coexpressed with the coding sequence for the mCherry-AtSEIPIN230-238, which lacks the FFAT motif, there was no apparent change in the subcellular distribution of AtVAP27-1 (i.e., AtVAP27-1 localized to general ER and ER subdomains, similar to its location when expressed either on its own or with other proteins; Figures 4 and 5). Notably, AtVAP27-1 did not colocalize with AtSEIPIN230-238 at LDs. These data provide evidence in support of a role for the FFAT motif in AtSEIPIN2 in recruiting AtVAP27-1 to regions of ER that also include LDs. In support of this premise, similar results were obtained when coding sequences for the same mCherry-AtSEIPIN2 truncation mutants and AtVAP27-1-GFP were transiently coexpressed in N. tabacum pollen tubes (Supplemental Figure 5). In addition, in transient expression assays in leaves (Figure 7C), appending the N-terminal 30 amino acids of AtSEIPIN2 to mCherry (mCherry-AtSEIPIN21-30), redirected mCherry from its cytoplasmic location (when produced on its own) to colocalize with AtVAP27-1-GFP when coexpressed with the coding sequence for AtVAP27-1-GFP. These data altogether demonstrate that the N-terminal sequence of AtSEIPIN2 is both necessary and sufficient for interaction with AtVAP27-1. Collectively, these findings reveal dissectible structure–function relationships within the N terminus of AtSEIPIN2 that include a targeting signal for localization of AtSEIPIN2 to LDs as well as a second region (i.e., containing the FFAT motif) that is involved in the interaction with AtVAP27-1.

**AtVAP27-1 Is Required for Proper LD Formation in Seeds**

The interactions of AtSEIPIN2/3 with AtVAP27-1 predict a functional role for AtVAP27-1 in SEIPIN-mediated LD dynamics. Prior studies revealed that loss of AtSEIPIN2 and AtSEIPIN3 (seipin2 seipin3 double mutant) resulted in the formation of aberrant, larger
might phenocopy the functioning of AtSEIPIN2/3 in plant cells, the disruption of AtVAP27-1 (Taurino et al., 2018). If AtVAP27-1 is indeed important for normal function, abnormal morphology of cytoplasmic LDs in mutant Arabidopsis seeds (Taurino et al., 2018). Overall, these results provide genetic evidence that supports the concept that AtSEIPIN2/3 and AtVAP27-1 proteins work together in the normal formation of LDs from the ER. Although the mutations in these two different classes of proteins similarly disrupted normal LD formation, the total amount of neutral lipids in dry seeds was not dramatically reduced, nor was oil breakdown following seed germination or seedling establishment, based on hypocotyl elongation, adversely affected in a dramatic manner (Figure 9). These observations suggest also that the functional interaction of AtSEIPIN2/3 and AtVAP27-1 is restricted primarily to the process of LD formation and not to the synthesis or turnover of TAGs.

**VAPs Are Involved in LD Formation in Yeast**

To determine whether VAPs might also be involved in production of cytoplasmic LDs in other eukaryotes outside of the plant kingdom, experiments were performed using vap-disrupted strains of *S. cerevisiae*. There are two VAP genes in *S. cerevisiae*, SCS2 and SCS22 (Manford et al., 2012), and similar to plants, these VAPs are involved in various ER-membrane-tethering and vesicle-trafficking processes, including formation of ER-plasma membrane MCSs (Manford et al., 2012). Here, we showed that when the wild-type and scs2/22Δ double mutant yeast cells were cultivated in dextrose-rich medium, there were no obvious differences in the average number or sizes of BODIPY 493/503–stained LDs in yeast cells (Figure 10). However, when cells were cultivated in oleic acid-rich medium, which stimulates LD formation (Veenhuis et al., 1987), the number of LDs in scs2/22Δ mutant yeast cells was significantly lower, as well as significantly larger, than in the wild-type cells (Figure 10). These results support a broader role for VAP proteins in LD biogenesis in eukaryotes, particularly under conditions of enhanced lipid storage.

**DISCUSSION**

The formation of cytoplasmic LDs from the surface of the ER is a process that is broadly conserved in all eukaryotes; however, many of the underlying protein components vary among organisms, or even among tissues in the same organism (Chapman et al., 2012, 2019; Pyc et al., 2017; Ischebeck et al., 2020). One conserved component that is present across all kingdoms investigated to date is the SEIPIN protein complex. SEIPIN is an ER-

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**Figure 6.** Interaction of AtSEIPIN2 and AtVAP27-1 in the BiFC Assay in Plant Cells.

(A) Representative confocal micrographs of *N. benthamiana* leaves coexpressing (as indicated by labels) sequences for cCFP-AtVAP27-1 and either nVenus-tagged, full-length AtSEIPIN2, or AtSEIPIN2 lacking its N-terminal 30 amino acids (nVenus-AtSEIPIN2Δ1-29), as well as with mCherry-Perox, which served as a transformation control. Also shown for each set of images is the corresponding differential interference contrast (DIC) image. Note the relative abundance of BiFC puncta (see [B]) in cells coexpressing nVenus-AtSEIPIN2 and cCFP-AtVAP27-1 (top row), compared with cells coexpressing nVenus-AtSEIPIN2Δ1-29 and cCFP-AtVAP27. Note that the decrease in BiFC fluorescence in the latter cells was not due to a difference in transgene expression; refer to RT-PCR analysis of expression of BiFC fusion constructs in Supplemental Figure 4. Bar (top row) = 20 μm.

(B) Quantification of BiFC assays with AtSEIPIN2 and AtVAP27-1 in *N. benthamiana* leaf cells. Results from 30 areas of transformed epidermal leaf cells, similar to those shown in (A), were analyzed from three independent experiments, and the mean number of BiFC puncta per area (± so) are shown. Asterisks (****) indicate significant differences at *P* ≤ 0.0001, as determined by a Student’s *t* test. A summary of the statistical analysis is shown in Supplemental Data Set 2. Confirmation of transient expression is shown in Supplemental Figure 4.
Figure 7. AtSEIPIN2\textsubscript{1-238} Localizes to LDs and Promotes the Relocalization of Coexpressed AtVAP27-1 to LDs in Plant Cells.

(A) to (C) Representative confocal micrographs of \textit{N. benthamiana} leaf cells transiently expressing (as indicated by labels) truncated versions of mCherry-AtSEIPIN2 (mCherry-AtSEIPIN2\textsubscript{1-238} and mCherry-AtSEIPIN2\textsubscript{230-238} and mCherry-AtSEIPIN2\textsubscript{1-30}) along with or without coexpressed AtVAP27-1-GFP. The numbers in the name of each AtSEIPIN2 truncated protein denote the amino acid residues in AtSEIPIN2 that were fused to mCherry. All cells were also stained with the neutral lipid dye MDH. Shown also in (A) and (B) are the corresponding merged images, including, in the far-right panel in (B), the merged images for mCherry-AtSEIPIN2\textsubscript{1-238} or mCherry-AtSEIPIN2\textsubscript{230-238}, AtVAP27-1-GFP, and MDH. Note in (A) the obvious colocalization of both mCherry-AtSEIPIN2 truncation proteins with the MDH-stained LDs, compared with the ER localization of full-length AtSEIPIN2 (mCherry-AtSEIPIN2; see Figure 5). Note also the relocalization of AtVAP27-1-GFP to LDs in cells coexpressing mCherry-AtSEIPIN2\textsubscript{1-238} but not mCherry-AtSEIPIN2\textsubscript{230-238}, which lacks the protein’s FFAT motif (see Figure 2B). Arrowheads indicate obvious examples of colocalization. Coding sequences for mCherry and mCherry-AtSEIPIN2\textsubscript{1-30} expressed individually show the same cytoplasmic localization in (C); however, when coexpressed with the coding sequence for AtVAP27-1-GFP, mCherry-AtSEIPIN2\textsubscript{1-30} is relocalized to ER and adjacent to LDs. Boxes represent the portion of the cells shown at higher magnification in the insets. Bar in (A) to (C) = 20 \textmu m; bar in insets = 5 \textmu m. Supplemental Figure 5 shows replicated results for (A) and (B) with these same constructs in tobacco pollen tubes.
localized integral membrane protein that associates into large ring-like structures through interaction of conserved ER lumenal domains (Sui et al., 2018; Yan et al., 2018; Chapman et al., 2019; Chung et al., 2019). The SEIPIN complex is essential for LD biogenesis and plays a critical role in formation of the ER-LD junction (Bohnert, 2020). This intimate relationship between ER and LDs, where the outer leaflet of the ER is contiguous with the LD monolayer, is thought to be important not only for the proper filling of LDs with neutral lipids but also for the acquisition of the phospholipids and proteins that associate with the LD surface and comprise the LD coat. But how SEIPIN coordinates these various processes is not well understood.

Here, we demonstrate that part of the mechanism of SEIPIN function in plants may occur via its interaction with the membrane-tethering protein AtVAP27-1. A combination of biochemical, cell biology, and genetic evidence supports this conclusion. SEIPIN is known to interact with other proteins in mammals and yeast to help facilitate LD biogenesis and related aspects of lipid metabolism (Gao et al., 2019), and this prompted our use of AtSEIPIN3 as bait in affinity-capture experiments to identify potential interacting proteins in plants. AtSEIPIN3 was chosen as bait because it has a relatively long N-terminal region, similar to AtSEIPIN2, but distinct from AtSEIPIN1 (Figure 2; Chapman et al., 2019), and this N-terminal region of AtSEIPIN3 was shown previously to modulate LD size in plants (Cai et al., 2015). Moreover, AtSEIPIN3 has the simplest predicted membrane-spanning organization, with only two predicted TMDs (Figure 2). The affinity-capture experiments with AtSEIPIN3 revealed 22 potential interacting proteins, three of which corresponded to \textit{N. benthamiana} VAP proteins (Figure 1; Supplemental Data Set 1). The presence of VAPs in the pull-down assays was increased when AtSEIPIN3 was coexpressed with AtLEC2 or AtDGAT1 (Figure 1A), which stimulates TAG synthesis and LD formation in plants (Andrianov et al., 2010). We subsequently showed that the closest VAP homolog in Arabidopsis, AtVAP27-1, (1) physically interacted with both AtSEIPIN2 and AtSEIPIN3 in Y2H assays (Figure 3); (2) that the interaction in yeast required the N-terminal \textemdash{}30 amino acids of AtSEIPIN2 or AtSEIPIN3, which both contain an FFAT motif (Figures 2B and 3); (3) that mutations within the predicted FFAT motif weakened this interaction (Figure 3F); (4) that AtVAP27-1 and AtSEIPIN2 also interacted in planta in a BiFC assay (Figure 6); and (5) that the N terminus of SEIPIN2 was both necessary and sufficient to drive the interaction with VAP27-1 in plant cells (Figure 7). Furthermore, when AtVAP27-1 was produced in \textit{N. benthamiana} leaves, the protein was localized to both the general ER and distinct ER puncta that were often adjacent to LDs (Figures 4 and 5; Supplemental Movie). Collectively, these results provide evidence that AtVAP27-1 interacts with AtSEIPIN2 and AtSEIPIN3 presumably at ER-LD junction sites in plant cells. Moreover, a physiological role for AtVAP27-1 in LD formation was obtained using two independent mutant lines of AtVAP27-1, which each showed aberrant, enlarged LDs in cells in mature seeds, similar to loss-of-function double mutants of \textit{seipin2 seipin3} (Figure 8; Taurino et al., 2018).

VAPs have been extensively characterized in yeast and mammals, and more recently in plants, where they have well-established roles in the formation of MCSs between ER and other organelles, as well as facilitating the transfer of lipids between membranes (Lev et al., 2008; Prinz, 2014; Wang et al., 2017; Michaud and Jouhet, 2019). The potential for the presence of VAPs at the ER-LD junction may not be entirely unexpected, and there are interesting similarities and differences when comparing other MCSs of VAPs with the ER-LD junction. VAPs are \textendash{}tail-anchored. ER integral membrane proteins that have three distinct domains: a C-terminal TMD that anchors the protein in the ER membrane; a middle region consisting of a coiled-coil domain often involved in homotypic-heterotypic protein interactions; and an N-terminal region that contains a MSD, which interacts with the FFAT motifs present in many of the VAP binding partners (Murphy and Levine, 2016; Pérez-Sancho et al., 2016; Wang et al., 2016b). Most of these VAP partners have multiple domains that facilitate not only MCS formation but also transfer lipids between the two organelles. For instance, as depicted in Figure 11A for reference purposes (and based on Prinz, 2014), ER-localized VAP is known to interact with a Golgi-localized ceramide transport protein (CERT) to assist with MCS formation. CERT is a multi-domain protein with a C-terminal region that binds to phosphoinositide 4-phosphate located in the Golgi membrane, a middle region with an FFAT motif that interacts with VAP to help stabilize the ER-Golgi MCS, and an N-terminal domain that transfers ceramide from the ER to Golgi membrane (Figure 11A). Similar scenarios are known to exist in various cell types between VAP and other lipid-transfer proteins at MCSs, including ER and plasma membrane, endosomes, and mitochondria (Prinz, 2014). VAPs are also known to interact with proteins associated with vesicular trafficking, including VAMPs, syntaxins, soluble N-ethylmaleimide-sensitive factor attachment proteins, and N-ethylmaleimide-sensitive factors (Lev et al., 2008). In plants, VAPs have been shown recently to mediate ER-PM MCS formation (Wang et al., 2017; Michaud and Jouhet, 2019), and another report demonstrated that VAPs...
recruited clathrin to ER–plasma membrane MCSs to help facilitate the process of vesicle formation and endocytosis (Stefano et al., 2018).

The established roles of VAPs in promoting lipid transfer between organelles, as well as recruitment of proteins associated with vesicle formation and scission from membranes, provide clues for how VAPs might function with SEIPIN in LD formation (see hypothetical model in Figure 11B). One concept for SEIPIN function has recently emerged whereby the SEIPIN complex is initially highly mobile in ER membranes and scans for the presence of a bulge in ER membranes due to a local accumulation of TAG (Wang et al., 2016a). Packing defects in the ER membrane leaflets surrounding the so-called TAG "lens" are recognized by amphipathic helices of SEIPIN present on both the luminal and cytoplasmic sides of the membrane, anchoring the SEIPIN complex at the TAG lens (Sui et al., 2018; Chung et al., 2019). SEIPIN then helps facilitate the directional emergence of a nascent LD into the cytoplasm, where the LD monolayer is contiguous with the outer ER membrane leaflet. In a process that is poorly understood, SEIPIN then promotes the subsequent growth and expansion of the LD through acquisition of additional core neutral lipids, phospholipids, and LD-associated (coat) proteins. Based on this model, as the nascent LD grows in size, the cytoplasmic-facing, N-terminal region of AtSEIPIN2/3, which is associated with the LD, might become more parallel with the ER outer membrane, providing opportunity for the protein’s N-terminal FFAT motif to interact with ER-localized AtVAP27-1 (Figure 11B, inset). The interaction of the AtSEIPIN2/3 N terminus with AtVAP27-1 could then have multiple effects on LD formation. For instance, the additional binding energy might strengthen and stabilize the ER-LD junction, thereby allowing for proper filling and expansion of the nascent LD. The extreme N terminus of AtSEIPIN2/3, upstream of the LD binding region, might also be unstructured in nature (based on the Drosophila and mammalian SEIPIN protein structures; Wang et al., 2016a; Sui et al., 2018); thus, the binding of the N terminus of AtSEIPIN2/3 might reduce the flexible, dynamic motion of this region that would otherwise be destabilizing in the tight confines of the ER-LD junction. Furthermore, the binding of the FFAT motif in the N terminus of AtSEIPIN2/3 to AtVAP27-1 could also pull the LD monolayer toward the ER membrane, thereby promoting the filling of LDs of proper size. VAPs are also known to exist as homo- and heterodimers (Russ and Engelman, 2000), and if one AtVAP27-1 subunit is associated with the FFAT motif of AtSEIPIN2/3, another VAP might recruit additional proteins involved in ER-LD transfer, providing a mechanism for lipid transfer directly from the ER membrane to the monolayer of the LD.

Figure 9. vap27-1 and seipin2 seipin3 Mutants Do Not Display a Marked Deficit in Seed Oil Accumulation or Mobilization, or Seedling Growth.

(A) Oil content measurement by NMR of the wild-type (Col-0), a seipin2 seipin3 double mutant, and two independent vap27-1 mutant (vap27-1 [GK and Wisc]) Arabidopsis dry seeds. Means (± so) of seven different measurements are represented in the graph. DW, dry weight.

(B) Fatty acid (FA) quantity of Col-0 and seipin2 seipin3 and vap27-1 (GK and Wisc) mutant Arabidopsis seedlings 1, 2, and 4 d after germination (DAG). Means (± so) of three different FA extractions are represented in the graph.

(C) Hypocotyl length of Col-0, seipin2 seipin3, and vap27-1 (GK and Wisc) seedlings after germination. Means (± so) of n > 127 hypocotyls per mutant from n = 3 experimental replicates. Significant differences in (A) to (C) are indicated as ***P < 0.001, ****P < 0.0001, and *****P < 0.00001, as determined by a Kruskal-Wallis test corrected by a Dunn’s multiple comparison test in (A) and (C) and by a two-way ANOVA in (B) compared with the control at the same time point in (B) (n = 3) and (C) (n > 123). A summary of statistical tests is provided in Supplemental Data Set 2.
Phospholipids can also traffic passively, of course, between the ER and LD due to the continuous nature of their membranes, but perhaps additional proteins are required under conditions of rapid LD expansion, or to help generate specific differences in the phospholipid composition between the ER membrane and LD monolayer. Finally, the interaction of VAPs with proteins involved in vesicle formation and scission, including clathrins and dynamin (Stefano et al., 2018), suggests that VAPs might be important for recruitment of proteins involved in release of LDs from the ER in plants, and, together with AtSEIPIN2/3, help determine the ultimate size of LDs. It may be of interest to note that LDs appear to be larger in cells coexpressing AtSEIPIN3 and AtVAP27-1 (Figure 5, top row) than AtSEIPIN2 and AtVAP27-1 (Figure 5, middle row), despite previous results showing that small LDs are produced in cells expressing coding sequences for AtSEIPIN3 alone (Cai et al., 2015). It is possible therefore that coexpression of AtVAP27-1 with AtSEIPIN3 negates the effects on LD size conferred by SEIPIN3 alone, which may be related to differences in stoichiometric requirements for these two proteins. Alternatively, it could be a result of overproducing both proteins at the same time.

Figure 10. Disruption of VAP Genes in Yeast Influences LD Abundance and Size during Lipid-Storing Conditions.

(A) and (B) Shown are representative micrographs of the wild-type (WT) and VAP gene (SCS2 and SCS22) double mutant (scs2/22Δ) S. cerevisiae cells that were cultivated in either dextrose-rich medium (YPD; see [A]) or oleate-rich medium (YPO; see [B]). LDs were then stained with BODIPY 493/503 and visualized by confocal microscopy. Shown also are the corresponding merged images. Bars = 5 μm.

(C) Violin plot showing the number of LDs per cell (n = 79 cells per treatment from n = 4 experimental replicates) for the wild-type (WT) or scs2/22Δ double mutant cells cultivated in either YPD or YPO medium.

(D) Violin plot showing the size (diameter) of LDs per cell (average and SD of n > 200 LDs per treatment) for the wild-type (WT) or scs2/22Δ double mutant cells cultivated in either YPD or YPO medium. Significant differences in (C) and (D) are indicated as ***P < 0.001, as determined by a Kruskal-Wallis test corrected by a Dunn’s multiple comparison test comparing the wild type (WT) and scs2/22Δ. A summary of the statistical analysis is provided in Supplemental Data Set 2.
time. In any case, the cooperation of AtSEIPIN3 and AtVAP27-1 on LD formation presents new opportunities for further investigation.

Several plant LD proteomics studies have identified VAPs, clathrins, dynamins, and other vesicle-trafficking–related proteins in purified LD fractions (Brocard et al., 2017; Zhi et al., 2017; Kretzschmar et al., 2018). If recruitment of scission-related proteins by AtVAP27-1 to the ER-LD junction is indeed important for formation of LDs in plants, the loss of AtVAP27-1 might result in production of aberrant, large-sized LDs, due to an inability to terminate the process of LD expansion. Notably, enlarged LDs are indeed formed in vap27-1–disrupted seeds (Figure 8). A similar enlarged LD phenotype was observed in seeds from seipin2 seipin3 mutant plants (Figure 8; Taurino et al., 2018). AtSEIPIN1 is also expressed in developing seeds (Cai et al., 2015), but, unlike AtSEIPIN2/3 (Figure 2), we were unable to identify an obvious N-terminal FFAT motif in AtSEIPIN1 that could function in recruitment of AtVAP27-1. Thus, AtSEIPIN1 likely facilitates LD formation in seipin2 seipin3–disrupted seeds but may produce aberrant, enlarged LDs due to an inability to recruit AtVAP27-1 and/or any other associated proteins (e.g., clathrin, dynamin, etc.). These observations further suggest that in the normal, wild-type seeds, AtSEIPIN1 exists in heteromeric complexes with AtSEIPIN2 and/or AtSEIPIN3, which serve to recruit AtVAP27-1 to the AtSEIPIN complex in the ER membrane. This could explain also why AtSEIPIN1 and AtVAP27-1 showed an overlapping distribution in the ER when coexpressed in N. benthamiana cells (Figure 5), despite a lack of direct physical interaction in a Y2H assay (Figure 3A). That is, AtSEIPIN1 might interact with AtSEIPIN2 and/or AtSEIPIN3, which would indirectly bring AtVAP27-1 into a coincident subcellular location. Whether heteromeric (as opposed to homomeric) AtSEIPIN complexes are truly present and whether differences in the stoichiometry of AtSEIPIN subunits...
lead to differences in ability to recruit AtVAP27-1 are the subjects of ongoing investigations in our labs.

It is currently unknown how widely VAPs may be deployed in LD biology in other organisms. The observation that yeast cells disrupted for VAPs have reduced numbers and increased size of LDs in oleate-rich media (Figure 10) suggests an important role for VAPs in LD production in yeast during lipid-loading conditions. But, neither yeast nor human SEIPIN proteins (like AtSEIPIN1) contain any apparent FFAT motifs. However, VAPs might also be recruited to ER-LD junctions by protein(s) other than SEIPIN. Pertinent examples of such proteins include ORP2 and VPS13A, which are localized to LDs in mammalian cells and contain FFAT motifs that might be important for interaction with ER-localized VAPs (Kentala et al., 2015; Yeshaw et al., 2019). Furthermore, there are often multiple protein complexes involved in formation of MCSs, including the well-characterized Rab18/NRZ and FATP/DGAT2 complexes that tether the ER and LDs in mammalian and yeast cells (Wu et al., 2018). These latter two complexes have yet to be identified in plants, and if they indeed do not exist, other membrane tethers (such as SEIPIN and VAP) may have evolved as functional equivalents (Pérez-Sancho et al., 2016). Given that we observed aberrant, enlarged LDs in vap27-1–disrupted seeds (Figure 8) and that VAPs were similarly shown to be important for maintaining normal LD numbers and sizes in yeast cells under lipid-loading conditions (Figure 10), perhaps the VAP-based ER tethers uniquely provide additional support/stability to the ER-LD junction that is required during periods of high neutral lipid synthesis and flux between ER and LDs.

In summary, our work demonstrates that AtSEIPIN2/3 isoforms function, in part, through the interaction with the membrane-tethering protein AtVAP27-1 to promote LD biogenesis. AtVAP27-1 has been shown elsewhere to participate in mediating contact between the ER and the plasma membrane (Wang et al., 2017) and more recently to cooperate in connections with endomembrane trafficking vesicles via clathrin subunits (Stefano et al., 2018). Here, we provide a combination of evidence to indicate that AtVAP27-1 has an additional functional role, one that participates in the formation of LDs in plant cells. Our study identifies AtVAP27-1 as a partner of AtSEIPIN2/3 that is required in part for proper compartmentalization of neutral lipids in seeds. These findings expand not only our understanding of proteins involved in compartmentalization of neutral lipids in plant cells but also provide insight into the role of VAPs in ER-MCS–mediated lipid transfer processes and open new avenues of research for studying integrated aspects of lipid trafficking, sensing, storage, and organellar communication.

METHODS

Plant Materials and Growing Conditions

The Arabidopsis (Arabidopsis thaliana) wild-type (Columbia-0 [Col-0]) background and T-DNA insertion mutants WiscDSloxHs096-064 (vap27-1 knockout), GK_541A03 (vap27-1 knockout), GK_183F09 (seipin2 knockout), and SAIL1286_H09 (seipin3 knockout) were obtained from the Arabidopsis Biological Resource Center (https://abrc.osu.edu). A seipin2 seipin3 double mutant line was generated by crossing single mutants GK_183F09 and SAIL1286_H09 and advancing the F1 progeny to homozygosity. Arabidopsis plants were grown in soil or on plates containing half-strength Murashige and Skoog media (Murashige and Skoog, 1962) in a growth chamber at 21°C with a 16-h-light/8-h-dark cycle and 50 μE m⁻² s⁻¹ light intensity. All mutants were confirmed by genotyping and RT-PCR (Supplemental Figure 6), and the wild type and mutants were grown together at the same time to harvest seed for further studies. The Nicotiana benthamiana plants were grown in soil in a growth room at 22°C under a 16-h-light/8-h-dark cycle. Tobacco (Nicotiana tabacum) plants were grown in soil in the greenhouse, and pollen was harvested from freshly opened anther buds (six flowers of two plants per construct) for pollen transformations as described previously (Rotsch et al., 2017).

Phylogenetic Analysis of VAPs

Phylogenetic relationships of VAPs were determined using the Neighbor-Joining method (Saitou and Nei, 1987). Sequences were aligned using ClustalW and obtained on their following accession numbers: AtVAP27-1 (At3g60600), AtVAP27-2 (At1g08820), AtVAP27-3 (At2g51410), AtVAP27-4 (At5g47180), AtVAP27-5 (At2g23820), AtVAP27-6 (At4g00170), AtVAP27-7 (At1g51270), AtVAP27-8 (At4g12450), AtVAP27-9 (At4g05060), AtVAP27-10 (At5g54110), NbVAP1-2 (NbS00011965g0004.1), NbVAP1-3 (NbS00011965g0007.1), NbVAP2-1 (NbS00000602g0001.1), SCS2 (YER120W), and SCS22 (YBL091C-A). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed, and branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). Evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of amino acid differences per site. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

Plasmid Constructions and Mutagenesis

Molecular biology reagents were purchased from New England Biolabs or Invitrogen. Custom oligonucleotides used for gene cloning and plasmid construction were synthesized by Sigma-Aldrich; sequence information for all primers is available in the Supplemental Table. All DNA constructs were verified using automated sequencing. The full-length open reading frame (ORF) of AtVAP27-1 (At3g60600) was cloned (via PCR) using gene-specific primers and a cDNA library obtained from isolated 3-week-old Arabidopsis leaf mRNA as template. Resulting PCR products were subcloned into pUC18/MCS-mGFP, a plant expression vector containing a multiple cloning site followed by the ORF of the monomerized version of the GFP (Clark et al., 2009), Thereafter, the coding region for VAP27-1-GFP was transferred to the plant expression binary vector pMDC43 using Gateway technology (Curtis and Grossniklaus, 2003). Similarly, pMDC32 binary plasmids encoding the mCherry ORF linked to either full-length AtSEIPIN2 (At1g29760) or AtSEIPIN3 (At2g34380) were constructed by subcloning (via Gateway technology) the AtSEIPIN2/3 ORFs into pMDC23/ChN (Price et al., 2019). Truncated versions of AtSEIPIN2 (i.e., amino acid residues 1 to 30, 1 to 238, or 30 to 238) were generated using PCR with pDONR/AtSEIPIN2 as template, followed by subcloning into pMDC32. pMDC32/mCherry-CHC2, encoding mCherry-CHC2 (At3g08530), was generated by amplifying (via PCR and using pK7m34GW RPS5a:CHC2-GFP [provided by Jenny Russinova; Ortiz-Morea et al., 2016] as template) the CHC2 ORF in two halves, along with Ascl and BamHI or BamHI and Ascl restriction sites. Thereafter, the two DNA fragments were digested with the appropriate restriction enzymes and ligated into similarly-digested pMDC32/ChN. Other plant expression binary vectors used in this study were described elsewhere, including pMDC32/Cherry-Perox, which encodes mCherry linked to a type 1 peroxisomal matrix targeting signal (Ching et al., 2012).
Plasmids used for BiFC assays were generated based on the Gateway-compatible vectors pDEST-VYNE/nVenus and pDEST-SYCE/cCFP, which encode the N-terminal and C-terminal halves of Venus and CFP, respectively (Gehl et al., 2009). Specifically, AStSEIPIN2, a deletion mutant of AtSEIPIN2 missing the N-terminal 29 amino acids (AtSEIPIN2Δ1-29), or AtVAP27-1 were generated by amplifying ORFs using gene-specific primers that included appropriate attB sequences and then fragments were subcloned into pDONR/Zeo using Gateway technology. Sequences were then transferred from entry vectors to BiFC destination vectors (Gehl et al., 2009) to produce pDEST-VYNE/R(AtSEIPIN2, pDEST-VYNE/R/AtSEIPIN2Δ1-29, or pDEST-SYCE/R/AtVAP27-1. Y2H vectors (pGBK and pGAD; Clontech) containing AStSEIPINS (1, 2, or 3), AtVAP27-1, or truncated versions of either AStSEIPIN2 (i.e., amino acids 1 to 432, 30 to 432, 170 to 432, 1 to 238, and 30 to 238) or AStSEIPIN3 (i.e., 25 to 209, 170 to 209, and 1 to 403) were generated by amplifying ORFs (or portions thereof) using the appropriate gene-specific primers along with 5’ and 3’ restriction sites. Resulting PCR products were then subcloned into similarly-digested pGAD and/or pGBK vectors. Molecular cloning of pUC-LAT52/AtVAP27-1-mCherry and pUC-LAT52-based plasmids encoding AStSEIPIN2Δ1-29 or AStSEIPIN2Δ1-29 was performed as described previously (Müller et al., 2017), pGBK:AtSEIPIN2Δ25-290-pGBK:AtSEIPIN2Δ232-432 and pGAD:AtVAP27-1LMD (L23-V142 deletion) were constructed by mutagenesis according to the Q5 Site-Directed Mutagenesis kit protocol (E0554), and the primers were designed using NEBASECHANGER (http://nebasechanger.netb.es). Construction of other plasmids used in tobacco pollen tube localization studies, including those encoding AStSEIPIN2-Venus or AtSEIPIN3-Venus, and ERD2-CFP (consisting of the Arabidopsis ER RETENTION DEFICIENT2 protein fused to CFP), has been described previously (Müller et al., 2017; Kretzschmar et al., 2018).

Y2H and BiFC Assays

Y2H assays were performed using the Y2HGold yeast strain from Clontech. Both prey (pGAD) and bait (pGBK) plasmids were introduced into yeast by cotransformation using the Frozen-EZ Yeast Transformation II kit (Zymo Research) and then grown on double drop-out (DDO) selection plates, which lacked both Leu and Trp. Two colonies per cotransformation with similar size and color were picked and grown in liquid DDO for 4 to 7 d at 30°C and 275 rpm. At saturation, three serial dilutions (1/10 [v/v]) with sterile water were performed, and 10 µL of each dilution (1, 1/10, 1/100, and 1,000) was spotted onto DDO and quadruple drop-out plates, lacking Leu, Trp, His, and Ade (DDO). Yeast were subsequently grown at 30°C for 4 to 7 d and then analyzed.

BiFC assays in N. benthamiana leaves were performed as described previously (Pyc et al., 2017) and based on the guidelines described elsewhere for assessing protein interactions and minimizing the possibility of non-specific interactions using the BiFC assay (Lee et al., 2012; Stefano et al., 2015). Briefly, leaves were infiltrated with Agrobacterium tumefaciens (Agrobacterium)—containing plasmids encoding cCFP-ATVAP27-1 and nVenus appended to either AStSEIPIN2 or AtSEIPIN2Δ1-29. All infiltrations also included mCherry-Perox, serving as a transformation marker. Transformed cells in leaf areas were visualized (via confocal microscopy) based on mCherry fluorescence, and both mCherry and reconstituted BiFC (cCFP/nVenus) fluorescence signals were collected with identical image acquisition settings for all samples analyzed. ImageJ ([https://image.nih.gov/ij; Schneider et al., 2012]) was used to quantify spectral counts from acquired micrographs of at least 20 leaf areas from three separate infiltrations.

Transient Expression and Coimmunoprecipitation Analysis in N. benthamiana Leaves

Genes of interest were transiently expressed in N. benthamiana leaves by infiltrating with A. tumefaciens (GV3101) containing appropriate plasmids as described previously (Cai et al., 2019). A coimmunoprecipitation assay with mCherry-AStSEIPIN3 was performed using RFP-Trap_A (ChromoTek). Approximately 1.5 g of 4-week-old N. benthamiana leaf tissue transiently expressing mCherry or mCherry-AStSEIPIN3, with or without coexpression of AtLEC2 and AIDGAT1, was collected at 3 d of postinfiltration for protein extraction. Leaf tissues were snap frozen in liquid nitrogen and then pulverized and homogenized in 3 mL of extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% [v/v] glycerol, 1% [v/v] Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and two tablets of Roche protease inhibitor cocktail [Sigma-Aldrich] in a total volume of 10 mL), which was used to extract total protein. The homogenate was clarified by centrifugation at 16,000g at 4°C for 20 min, and mCherry-AStSEIPIN3 and associated proteins were captured using 30 µL of RFP-Trap_A beads (ChromoTek), following the manufacturer’s protocol. Copurifying proteins were concentrated by electrophoresis in the top 5 to 10 mm of the SDS-PAGE gel (Bolt 4-12%; Thermo Fisher Scientific). The concentrated protein bands were stained with QC Colloidal Coomassie stain (Bio-Rad Laboratories) and excised for subsequent analysis at the Michigan State University Proteomics Core Facility (https://rtsf.natsci.msu.edu/ proteomics/). The gel-purified proteins were treated with trypsin, and peptides and parental proteins were identified by peptide-mass fingerprinting, as described previously (Cai et al., 2019).

Particle Bombardment of Tobacco Pollen Grains

Transformation of tobacco (N. tabacum) pollen grains by biolistic bombardment and culturation on slides for subsequent microscopy was performed as described previously (Müller et al., 2017).

Genotyping and RT-PCR

Arabidopsis DNA and RNA used for genotyping and RT-PCR were extracted from 4-week-old seedlings. For genotyping Arabidopsis T-DNA insertion mutants pvp27-1, seipin2, and seipin3, DNA was extracted using genotyping kit REDExtract-N-Amp (Sigma-Aldrich) following the manufacturer’s instructions. PCR was performed using the following program: 95°C for 5 min, 35 amplification cycles (95°C for 30 s, 50°C for 30 s, 72°C 1 min/kb), and 72°C for 7 min. RNA from Agrobacterium-infiltrated N. benthamiana leaves, used for RT-PCR, was isolated as described previously (Cai et al., 2015; Gidda et al., 2016). The RT-PCR programs were as follows: for Arabidopsis RNA, 42°C for 20 min, 95°C for 5 min, 35 amplification cycles (94°C for 30 s, 55°C for 30 s, 72°C 1 min/kb), and 72°C for 7 min; for N. benthamiana RNA, the same PCR program was used, except 72°C for 90 s during the amplification cycles. We used 100 ng of Arabidopsis RNA or 1 µg of N. benthamiana RNA for each reaction. Arabidopsis EF1α and N. benthamiana ACTIN were used as the reference genes for RT-PCR. Sequence information for all primers used for RT-PCRs, as well as those used for genotyping of T-DNA insertional transgenic lines (Supplemental Figure 6), are available in the Supplemental Table.

Microscopy

Agrobacterium-infiltrated N. benthamiana leaves, biolistically bombarded tobacco (N. tabacum) pollen tubes, and Arabidopsis (transgenic) seeds were processed for confocal laser-scanning microscopy (CLSM), including staining of LDs either with BODIPY 493/503 (Invitrogen) or MDH (Abgent). As described previously (Cai et al., 2015; Gidda et al., 2016; Müller et al., 2017; Kretzschmar et al., 2020). Micrographs of N. benthamiana leaves were acquired using either a DM RBE microscope equipped with a 63× oil-immersion objective (numerical aperture [NA] = 1.32) and TCS SP2 scanning head, or An SPS microscope equipped with a 63× oil-immersion objective (NA = 1.3) and a Radius 405-nm laser (Leica Microsystems). Micrographs of tobacco pollen tubes were acquired using an LSM510 or LSM780 confocal microscope with a 63× oil-immersion objective.
objective (NA = 1.3 or 1.4, respectively; Carl Zeiss). For Arabidopsis seeds, images were acquired with an LSM710 microscope with a 63× water-immersion objective lens (NA = 1.25; Carl Zeiss). The wild-type and T-DNA mutant Arabidopsis dry seeds were imbibed in water for 20 min, and seed coats were removed from imbibed seeds by pushing embryos out of seed coats under a cover slip. Isolated embryos were stained with 2 μg/mL BODIPY 493/503 in 50 mM PIPES buffer, pH 7.0, for 20 min followed by three washes (10 min each) with 50 mM PIPES buffer. Embryos were then imaged using an LSM710 confocal microscope with a 63× water-immersion objective and a 488-nm laser. Excitation and emission signals for fluorescent proteins and/or chlorophyll autofluorescence were collected sequentially as single optical sections or Z-series in double- or triple-labeling experiments as the same as described previously (Cai et al., 2015; Gidda et al., 2016; Müller et al., 2017, 2020); single-labeling experiments showed no detectable crossover at the settings used for data collection. All fluorescence images of cells shown in individual figures are representative of at least two separate experiments, including at least three separate transformations of N. benthamiana leaf cells and tobacco pollen grains. Images (1024 × 1024 pixels) were used for subsequent quantification of LD size and number (>70 cells analyzed for each cell line and treatment) using the Fiji-plugin-in ImageJ (version 1.52p; Schindelin et al., 2012). Figure compositions were generated using Adobe Photoshop and Adobe Illustrator (versions CS; Adobe Systems).

Total Oil Content and Fatty Acid Analysis

Total oil content in Arabidopsis mature (desiccated) seeds was measured by time-domain, pulsed-field 1H-NMR on a minispec mq20 analyzer (Bruker Optics). At least 50 mg of desiccated seeds was used for each measurement (n = 7), and the oil was quantified as specified by Chapman et al. (2008), except that the instrument was calibrated for Arabidopsis seed oil. For fatty acid analysis, ~5 mg of dry seeds and 50 mg of lyophilized seedlings (1, 2, and 4 d after germination) were used for lipid extractions of three independent samples (n = 3). Total lipids were extracted, and fatty acid methyl esters prepared and analyzed by gas chromatography with flame-ionization detection as described previously (Cai et al., 2015). Heptadecanoic acid (C17; Sigma-Aldrich) was used as an internal standard and added at the first step of the lipid extraction.

Analysis of LDs in VAP-Disrupted Yeast Cells

The wild-type yeast strain SEY6210.1 (MATa leu2-3,112 ura3-52 his3-∆200 trp1-Δ901 lys2-801 suc2-Δ9; Robinson et al., 1988) and a derivative harboring disruptions in both VAP genes (scc2/22Δ; SEY6210.1 scc2/22Δ:TRP1 scc2/22Δ::HIS3); Stefan et al., 2011) were provided by Scott Emr (Manford et al., 2012). Yeast cells were cultured in oleate- or dextrose-rich medium, as described previously (Cartwright et al., 2015), and then LDs were stained with BODIPY 493/503 and visualized by CLSM. Briefly, single yeast colonies were inoculated into 3 mL of minimal Glc medium (2% [v/v] Glc and 0.67% [w/v] yeast nitrogen base; Buffera) and the appropriate amino acid and base supplements (Buffera) and then grown overnight in a shaker/incubator at 300 rpm and 30°C. Cells were then diluted into 3 mL of low Glc minimal medium (as above, but 0.1% [w/v] Glc) at a density of 0.1 OD600 nm/mL and cultured for another 30 h to saturation. Cells were then diluted into either oleate-rich medium (3 g/L yeast extract [Bacto], 16.9 g/L peptone [Bacto], 0.5% potassium phosphate [5% stock buffered to pH 6.0], 0.2% [w/v] Tween 80, and 0.1% oleate) at 1.0 OD600 nm/mL or dextrose-rich medium (same as oleate-rich media except oleate is replaced with 2% [w/v] dextrose) at 0.1 OD600 nm/mL and then grown an additional 18 to 20 h. Cells were then harvested by centrifugation at 500g and 23°C for 5 min, resuspended in 50 mM PIPES buffer, pH 7.0, stained with 0.4 μg/mL BODIPY 493/503 (Invitrogen), and centrifuged again at 500g and 23°C for 5 min, resuspended in water, and visualized by CLSM.

Statistical Analysis

For oil content, fatty acid quantification, hypocotyl length, and LD quantification in yeast, statistical analysis was performed using Prism 8 (GraphPad; www.graphpad.com). Significant differences were determined by a Kruskal-Wallis test corrected by a Dunn’s multiple comparison test. Statistical analysis of BiFC assay quantifications was performed using two-tailed Student’s t test with Prism 8. Summaries of the statistical analysis data are available in Supplemental Data Set 2.

Accession Numbers

The genes can be retrieved using the following accession numbers: AtVAP27-1 (At3g60600); AtSEIPIN1 (At5g16460); AtSEIPIN2 (At1g29760); AtSEIPIN3 (At2g34380); CHC2 (At3g08530); LDAP1 (At1g67360).

Supplemental Data

Supplemental Figure 1. Sequence alignment of Arabidopsis thaliana, Nicotiana benthamiana and Saccharomyces cerevisiae VAP protein families.

Supplemental Figure 2. Phylogenetic tree showed in Figure 1 in Newick format.

Supplemental Figure 3. Co-expressed AtSEIPIN2/3 and AtVAP27-1 co-localize in the ER in tobacco pollen tubes.

Supplemental Figure 4. Confirmation of expression of BiFC fusion constructs in N. benthamiana leaves using RT-PCR.

Supplemental Figure 5. The N terminus of AtSEIPIN2 is required for the relocalization of AtVAP27-1 to LDs in tobacco pollen tubes.

Supplemental Figure 6. Confirmation of Arabidopsis VAP27-1 and SEIPIN2 SEIPIN3 T-DNA insertion mutants.

Supplemental Movie. Dynamics of AtVAP27-1-GFP-containing ER foci and LDAP1-mCherry-labeled LDs in a N. benthamiana leaf cell.

Supplemental Data Set 1. List of N. benthamiana proteins co-immunoprecipitated with mCherry-SEIPIN3 with or without co-expressed Arabidopsis LEC2 and DGAT1.

Supplemental Data Set 2. Statistical analysis data.

Supplemental Table. List of primers.

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AUTHOR CONTRIBUTIONS

M.S.G., Y.C., S.G., N.E., F.K.K., D.S., T.I., and E.M. performed experiments. N.E. coordinated assembly of the figures with assistance of all authors. M.S.G. and K.D.C. prepared a first draft of the article with input from Y.C., T.I., R.T.M., and J.M.D. All authors contributed to editing the article and have read and approved the final version.

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