SEED LIPID DROPLET PROTEIN1, SEED LIPID DROPLET PROTEIN2, and LIPID DROPLET PLASMA MEMBRANE ADAPTOR mediate lipid droplet–plasma membrane tethering

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Abstract

Membrane contact sites (MCSs) are interorganellar connections that allow for the direct exchange of molecules, such as lipids or Ca2+ between organelles, but can also serve to tether organelles at specific locations within cells. Here, we identified and characterized three proteins of Arabidopsis thaliana that form a lipid droplet (LD)–plasma membrane (PM) tethering complex in plant cells, namely LD-localized SEED LD PROTEIN (SLDP) 1 and SLDP2 and PM-localized LD-PLASMA MEMBRANE ADAPTOR (LIPA). Using proteomics and different protein–protein interaction assays, we show that both SLDPs associate with LIPA. Disruption of either SLDP1 and SLDP2 expression, or that of LIPA, leads to an aberrant clustering of LDs in Arabidopsis seedlings. Ectopic co-expression of one of the SLDPs with LIPA is sufficient to reconstitute LD–PM tethering in Nicotiana tabacum pollen tubes, a cell type characterized by dynamically moving LDs in the cytosolic...
Introduction

As knowledge on organelle-specific functions and their proteomes has expanded in recent years, there has been ever mounting interest in interorganelle interactions that are in part facilitated by membrane contact sites (MCSs; Prinz et al., 2020). MCSs foster physical interactions and the exchange of molecules between organelles without the need of membrane fusion events. The transient connections are established through tethering proteins, connecting the membranes of interacting organelles and allowing for direct exchange of lipids, cellular signals (e.g. Ca^{2+}, reactive oxygen species [ROS], etc.) and/or other molecules (Baillie et al., 2020; Prinz et al., 2020; Rossini et al., 2020).

MCSs can form between nearly all organelles (Eisenberg-Bord et al., 2016; Valm et al., 2017; Shai et al., 2018; Baillie et al., 2020). The endoplasmic reticulum (ER) and peroxisomes, for example, are organelles with well-described inter-actomes (Shai et al., 2016; Zang et al., 2020). Also, several multiorganelle MCSs have been described, including the three-way connection between mitochondria, ER, and lipid droplets (LDs) that promotes de novo lipogenesis in human adipocytes (Freyre et al., 2019).

Although ER-derived LDs can engage in various MCSs, the LD interactome is less well described than that of other organelles (Bohnert, 2020). LDs consist of a lipophilic core of neutral lipids, such as triacylglycerols (TAGs) and sterol esters, surrounded by a phospholipid monolayer and a variety of surface-associated “coat” proteins. Long believed to be an inert storage organelle, it is now widely accepted that LDs actively participate in a wide range of cellular processes involving lipids and their derivatives (Thiam and Beller, 2017; Welte and Gould, 2017; Ischebeck et al., 2020). As such, rather than just housing storage lipids, LDs are now considered to be dynamic hubs for lipid homeostasis and specialized metabolism (Schaffer, 2003). Furthermore, LDs can serve as a sink for reducing cytosolic free fatty acids (Fan et al., 2017; Olzmann and Carvalho, 2019; de Vries and Ischebeck, 2020) and ROS (Muliyil et al., 2020), and also sequester potentially harmful proteins (Geltinger et al., 2020) or histone complexes at the LD surface (Johnson et al., 2018).

Given the established role(s) of MCSs in the nonvesicular transport of lipids (Cockcroft and Raghu, 2018), it is not surprising that LDs form contacts with many other organelles (Gao and Goodman, 2015; Schuldiner and Bohnert, 2017; Valm et al., 2017; Bohnert, 2020; Rakotonirina-Ricquebourg et al., 2022). The majority of these described LD MCSs, however, were found in mammalian or yeast cells. Knowledge in plants is so far still limited to LD–ER and LD–peroxisome MCSs, which are involved in storage lipid accumulation (Cai et al., 2015; Greer et al., 2020; Pyc et al., 2021) and
breakdown (Eastmond, 2006; Cui et al., 2016), respectively. For instance, while an LD–plasma membrane (PM) MCS was described in fly (Drosophila melanogaster; Ugrankar et al., 2019), no such connection has been described for plants.

SEED LD PROTEIN 1 (SLDP1) was recently reported as a plant-specific LD protein (Kretzschmar et al., 2020). Arabidopsis thaliana contains SLDP1 and a close homolog, SLDP2, although their function(s) are unknown. Here, we report that LDs in sldp1 sldp2 double mutants display aberrant subcellular positioning (i.e. clustering) during early seedling growth. In proteomic analyses of sldp mutants, we identified a PM-localized protein that serves as an interaction partner of SLDPs and which we termed LIPID DROPLET PLASMA MEMBRANE ADAPTOR (LIPA). Consistent with this role, subcellular localization studies in Nicotiana tabacum pollen tubes showed that ectopically expressed LIPA localizes to the PM, but is relocated to apparent PM–LD污染 tubes. As shown in Figure 1B, both SLDP1.3 and SLDP2.1, as well as their related (spliced) protein variants, share a predicted amphipathic α-helix sequence near their N-termini (amino acids 19–26 and 13–30, respectively) and an adjacent hydrophobic region of ~40 uncharged amino acids (amino acids residues 31–69 and 25–62, respectively; Figure 1, A, C, D; Supplemental Figure S2), both of which are key features of the LD targeting signals found in other LD proteins (Kory et al., 2016; Olarte et al., 2022). To test if these regions are indeed involved in LD targeting of SLDPs, we generated various truncated versions of SLDP1.3 and SLDP 2.1 and expressed them individually in N. tabacum pollen tubes. As shown in Figure 1B, both SLDP1.319–81-mVenus and SLDP2.113–75-mVenus, each including a putative amphipathic α-helix and a hydrophobic sequence, targeted to Nile red-stained LDs, similar to their full-length protein counterparts. In contrast, SLDP1.31–81-mVenus and SLDP2.11–75-mVenus, lacking the corresponding N-terminal portion of each protein, did not target LDs, but instead localized to the cytosol (Figure 1B). Based on these findings, both SLPD1 and SLPD2 proteins appear to share similar LD targeting information.

**Knockout of SLDP expression interferes with the subcellular distribution of LDs during post-germinative seedling growth**

According to transcriptome data available at Arabidopsis AtGenExpress (Nakabayashi et al., 2005; Schmid et al., 2005; Winter et al., 2007; Waese et al., 2017) and the Klepikova eFP browser (Klepikova et al., 2016; Waese et al., 2017), SLPD1 and SLPD2 are highly expressed in seeds. Consistent with these data, we confirmed the expression of all splice variants of SLDP1 and SLDP2 in seeds via two-step reverse transcriptase–quantitative polymerase chain reaction (RT–qPCR; Supplemental Figure S3). Furthermore, based on proteomic data, both proteins are also found in seedlings (Kretzschmar et al., 2020). We therefore reasoned that SLDPs might play a role in LD biology during these stages of development, and we investigated this possibility using a gene loss-of-function approach. Two sets of one Arabidopsis T-DNA and one clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 knockout mutant line each for SLDP1 and SLDP2 were generated (Figure 2A) and confirmed by genotyping (Supplemental Data Set S1). In addition, corresponding double knockout mutant lines of SLDP1 and SLDP2 were generated. RT–qPCR analyses confirmed that no full-length SLDP1 or SLDP2 transcripts were detectable in the sldp1-1 and sldp2-1 T-DNA mutant lines, respectively, or in the sldp1-1 sldp2-1 double mutant (Supplemental Figure S4). SLDP1 and SLDP2 transcript levels were not significantly altered in the CRISPR/Cas9 sldp1-2 and sldp2-2 single or double mutant lines (relative to wild-type [WT] plants; Supplemental Figure S4). However, cloning and sequencing of the respective cDNAs from these mutant lines confirmed CRISPR/Cas9-induced mutations (i.e. deletions) that resulted in premature stop codons in the

**Results**

SLDP1 and SLDP2 are members of a plant-specific LD protein family and contain a similar LD targeting signal

We recently investigated the proteomes of LD-enriched fractions of Arabidopsis siliques, seeds, and seedlings and, in doing so, identified several LD protein families (Kretzschmar et al., 2020). Notably, some of these proteins were unique to plants, such that they had no obvious homologs in mammals and/or yeast, and they were also annotated to be of unknown function, suggesting that they serve novel roles related to LDs in plants.

One of the families of Arabidopsis LD proteins we identified with no apparent homology to other proteins and no known function(s) included SLDP1 (AT1G65090) and SLDP2 (AT5G36100). There are three and two splice variants of Arabidopsis SLDP1 and SLDP2, respectively, sharing 35.4%–46.7% sequence identity (Figure 1A; Supplemental Figure S1). We previously showed that SLDP1.3 targets LDs (Kretzschmar et al., 2020) and we confirmed the same LD localization for SLDP2.1 by transiently expressing the mVenus-tagged protein (i.e. SLDP2.1-mVenus) in N. tabacum pollen tubes (Figure 1B), which provide a well-established model plant cell system for studying LD protein localization (Müller et al., 2017; Müller and Ischebeck, 2018; Kretzschmar et al., 2018, 2020). As indicated by bioinformatic analyses, both SLDP1.3 and SLDP2.1, as well as their related (spliced) protein variants, share a predicted amphipathic α-helix sequence near their N-termini (amino acids 19–26 and 13–30, respectively) and an adjacent hydrophobic region of ~40 uncharged amino acids (amino acids residues 31–69 and 25–62, respectively; Figure 1, A, C, D; Supplemental Figure S2), both of which are key features of the LD targeting signals found in other LD proteins (Kory et al., 2016; Olarte et al., 2022). To test if these regions are indeed involved in LD targeting of SLDPs, we generated various truncated versions of SLDP1.3 and SLDP 2.1 and expressed them individually in N. tabacum pollen tubes. As shown in Figure 1B, both SLDP1.319–81-mVenus and SLDP2.113–75-mVenus, each including a putative amphipathic α-helix and a hydrophobic sequence, targeted to Nile red-stained LDs, similar to their full-length protein counterparts. In contrast, SLDP1.31–81-mVenus and SLDP2.11–75-mVenus, lacking the corresponding N-terminal portion of each protein, did not target LDs, but instead localized to the cytosol (Figure 1B). Based on these findings, both SLPD1 and SLPD2 proteins appear to share similar LD targeting information.

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SLDP1 and SLDP2 open reading frames (Supplemental Data Set S1).

In comparison to WT plants, neither single nor double T-DNA mutant lines of SLDP displayed any obvious growth or developmental phenotypes, including hypocotyl elongation in light- and dark-grown seedlings (Supplemental Figure S5, A and B), and were also not strongly affected in the levels of the total fatty acids in seeds (Supplemental Figure S5C). With respect to the 1,000 seed weight, the double T-DNA insertion line was also only slightly affected (19.4 ± 0.2 mg in WT and 20.8 ± 0.2 in sldp1-1 and sldp2-1).

Furthermore, the degradation of total fatty acids during...
Figure 2  Time-course analysis of LDs in sldp mutant Arabidopsis seeds and seedlings. A, Illustration depicting the Arabidopsis SLDP1.3 and SLDP2.1 genes based on information provided at TAIR. Indicated are the relative positions of the 5'- and 3'-untranslated regions (gray boxes), exons (black boxes), introns (black line), T-DNA insertion sites (triangle, arrow indicating direction of T-DNA), and the regions deleted with CRISPR/Cas9-based genome editing (red lines). B, CLSM images of rehydrated seeds and seedling cotyledon cells from WT and various sldp1 and sldp2 mutant Arabidopsis lines. Seeds were rehydrated for 1 h or stratified for 4 days at 4°C in the dark. LDs were stained with Nile red after rehydration, or 12, 24, 36, and 48 h (+2 h) after stratification. Arrowheads indicate obvious examples of LD clusters in sldp2 single and sldp1 sldp2 double mutant seedling. Images are single plane images from the middle of the cell (similar planes were chosen for all images). Images are representative micrographs of at least five seeds and seedlings for each plant line and time point. Bar, 10 μm, applies to all images in the panels.
seed germination and early seedling growth in the sldp mutants was not significantly altered compared to WT (Supplemental Figure S5C), including the degradation of eicosenoic acid, which is specifically incorporated into TAGs in Arabidopsis seeds (Rylott et al., 2003; Supplemental Figure S5D). However, an aberrant LD phenotype was readily observed in seedlings from both sldp2 single and both sldp1 sldp2 double mutant lines during germination and post-germinative growth.

As shown in Figure 2B, and consistent with results presented in other studies of LDs in WT Arabidopsis seeds and seedlings (Cai et al., 2015; Gidda et al., 2016; Kretzschmar et al., 2018), Nile red-stained LDs displayed a “typical” subcellular distribution in rehydrated (mature) seeds and in seedlings from 12 h to 48 h after stratification. That is, in WT seeds and seedlings at 12 h, LDs in cotyledon cells (Figure 2B), as well as those in hypocotyl cells (Supplemental Figure S6) were often closely appressed. At the same stages, no obvious difference in LD morphology or distribution was observed between WT and any of the sldp mutant lines (Figure 2B), nor were there any obvious differences in storage vacuole morphology or distribution (Supplemental Figure S7). By 24 h to 48 h, after the completion of germination in Arabidopsis (Bewley, 1997), LDs in cotyledon cells in the WT and both sldp1 single mutant lines were mostly positioned in close proximity to the PM (Figure 2B). However, at 36 h and 48 h in cotyledon cells of sldp2 single and particularly sldp1 sldp2 double mutants, LDs were not evenly distributed along the PM, but instead were noticeably clustered near the center of the cell (Figure 2B).

A similar clustered LD phenotype was observed in hypocotyl cells in the sldp1 sldp2 double mutants at 24 h to 48 h, but hypocotyls of the sldp2 single mutants are less severely affected than its cotyledons (Supplemental Figure S6). Notably, Z-stack images of cotyledon and hypocotyl cells at 36 h, when the LD distribution phenotype was well pronounced (Figure 2B), and subsequent quantification of the LD distribution in these cells, confirmed that a significantly smaller proportion of LDs were located at cell periphery in the sldp2 single and sldp1 sldp2 double mutants compared to the WT (Supplemental Figure S8). The LD clustering phenotype in cotyledon cells of the sldp1-1 sldp2-1 double mutant was also observed by electron microscopy, whereas close contacts between LDs and the PM were almost solely observed in WT cotyledon cells (Supplemental Figure S9). Taken together, these data indicate that SLDP1 and SLDP2 are involved in subcellular distribution of LDs during post-germinative seedling growth in Arabidopsis.

Comparative proteomic analyses reveal LIPA as a potential interaction partner of SLDP2

To further explore the functions of SLDPs during post-germinative seedling growth in Arabidopsis, we tested if the loss of either SLDP1 or SLDP2, or both proteins, has an influence on the composition of the LD proteome. To this end, proteomic analyses of the WT, sldp1-1, sldp2-1, and sldp1-1 sldp2-1 mutant seedlings (36 h after seed stratification) were performed on LD-enriched fractions, as well as total cellular extracts (abbreviated as TE; Supplemental Figure S10, A–C). Initially, protein levels of SLDP1 and SLDP2 in the LD proteomes of the sldp mutant lines were assessed. As shown in Figure 3A, SLDP1 protein was nearly absent from LD-enriched fractions derived from sldp1-1 and sldp1-1 sldp2-1 mutant seedlings. Similarly, SLDP2 was not detected in the sldp2-1 and sldp1-1 sldp2-1 LD proteomes, but also in the WT and sldp1-1 derived LD proteomes it was only detectable in one of three replicates each. This is consistent with SLDP2 (and SLDP1) being a low abundance LD protein (Kretzschmar et al., 2020).

We then compared the TE and LD-enriched fractions to assess any differences in protein composition between WT and sldp mutant seedlings. For this, stringent filters (i.e. proteins only detected at least three times in at least one group and identified by at least two peptides) were applied to the whole data set (see Supplemental Data Set S2 for raw LFQs and Supplemental Data Set S3 for normalized and filtered LFQs). Imputations were performed so that fold changes could also be calculated for proteins absent in one of the fractions (Supplemental Data Set S4). Comparisons of the TE fractions did not reveal any statistically significant differences between the WT and any of the sldp mutants (Supplemental Figure S10D). Likewise, comparisons of known LD proteins from LD-enriched fractions of WT and sldp mutants revealed no significant changes (Supplemental Data Set S4). However, two other proteins were found to be significantly differentially abundant in LD fractions of WT and sldp mutant seedlings: RING DOMAIN LIGASE2 (RGL2) and LIPA (Figure 3B), with LIPA being absent in LD fractions from both sldp2-1 and sldp1-1 sldp2-1 seedlings and RGL22 being more abundant in sldp1-1 sldp2-1 seedling LD fractions (Figure 3B; Supplemental Figure S10E). Moreover, in WT and sldp1-1, LIPA was significantly enriched in LD fractions (i.e. absent from TE fractions, Figure 3, C and D; Supplemental Figure S10F). As expected, a similar enrichment of LIPA was not found in LD fractions from sldp2-1 and sldp1-1 sldp2-1, as LIPA was not detected in these samples (Figure 3D). Taken together, these data suggest that LIPA is associated with isolated LDs. They further indicate that SLDPs might act as a link between isolated LDs and LIPA and that LIPA is therefore important to mediate some of the functions of SLDPs. The link of LIPA to a higher abundance of RGL22 is, however, harder to interpret. Furthermore, LIPA, in comparison to RGL2 (Cheng et al., 2012; Yu et al., 2021), has previously not been explored in any detail. In conclusion, we aimed to further elucidate the function of LIPA, while the role of RGL22 could be studied in the future.

Co-expressed LIPA and SLDP2 mutually influence their subcellular localization

Based on information provided by The Arabidopsis Information Resource (TAIR; Berardini et al., 2015), LIPA has no annotated functions. While LIPA homologs exist in...
other plant species, they are absent in nonplant species, indicating that LIPA, like SLDP, is a plant-specific protein. LIPA is a 144 amino acid-long protein with no described protein domains/motifs or putative transmembrane domains, nor any other obvious physicochemical features consistent with an LD targeting signal (e.g. putative amphipathic helix and/or hydrophobic sequence, Supplemental Figure S11, A–C).

To assess the subcellular localization of LIPA in plant cells, we transiently expressed the protein as an mVenus-tagged fusion protein in *N. tabacum* pollen tubes. As shown in Figure 4A, C-terminal mVenus-tagged LIPA (LIPA-mVenus) localized to the cytosol, while its N-terminal mVenus-tagged counterpart, mVenus-LIPA, localized predominantly to the PM. Notably, in *Agrobacterium*-transformed *N. benthamiana* leaves, another well-established plant cell model system for studying protein localization (Sparkes et al., 2006), no apparent differences in localization were observed between N- or C-terminal-GFP-tagged LIPA. Both LIPA fusion proteins localized to the PM and also the cytosol, which are closely appressed in these cells due to the presence of the large central vacuole (Supplemental Figure S12A).

While LIPA is localized to the PM (and cytosol) in transiently transformed plant cells (Figure 4A; Supplemental Figure S12A), proteomic analysis indicated that LIPA co-purified with SLDP2-containing LDs, but not with LDs...
lacking SLDP2 (Figure 3B; Supplemental Figure S10, E and F). Hence, LIPA appears to be a PM protein that also associates with LDs in an SLDP2-dependent manner. To further test this hypothesis, we co-expressed Arabidopsis SLDP2.1 and LIPA. Nicotiana tabacum pollen tubes, lacking homologs of both SLDPs and LIPA based on previous proteomic data (Kretzschmar et al., 2018), were chosen as the expression system to avoid endogenous SLDP and/or LIPA from potentially interfering with localization analyses. SLDP2.1 (and SLDP1.3) tagged with mCherry showed the same LD-localization in pollen tubes as its mVenus-tagged counterparts (compare images in Supplemental Figure S13A and Figure 1C). As shown in Figure 4B, the co-expression of SLDP2.1-mCherry and LIPA-mVenus (C-terminally tagged) in pollen tubes resulted in LIPA being re-localized from the cytosol to LDs (compare with images of LIPA-mVenus expressed on its own; Figure 4A). A similar relocation was observed when LIPA-mVenus was co-expressed with either SLDP1.3-mCherry or the nontagged (native) versions of SLDP1.3 or SLDP2.1 (Supplemental Figure S13B).

Next, we assessed the localization of co-expressed SLDP2.1-mCherry and mVenus-LIPA (N-terminally tagged). As shown in Figure 4B, this co-expression resulted in a change of localization of both proteins: co-expressed SLDP2.1 and LIPA were both dually localized to LDs and the PM (Figure 4B), unlike their localization exclusively to LDs or the PM, respectively, when they were expressed on their own (Figures 1, B and 4, A). Furthermore and as discussed below, a significant proportion of LDs decorated with SLDP2.1-mCherry and mVenus-LIPA appeared to be positioned close to the PM instead of distributed in the cytosol (Figure 4B). One-way analysis of variance (ANOVA) was performed, followed by Tukey post hoc analysis ($F_{(4,81)} = 23.37, P = 7.24e−13, n = as indicated$). Note that only the SLDP2.1 + mVenus-LIPA co-bombardment increased the number of pLDs compared to the single bombardment controls. Statistical results are presented as compact letter display of all pairwise comparisons.

Taking together these data indicate that SLDPs and LIPA can influence each other’s localization in plant cells, such that SLDP2.1 and SLDP1.3 can recruit LIPA to LDs, while LIPA can recruit at least SLDP2.1 and LDs to the PM.

LIPA can immobilize SLDP2-containing LDs at the PM in pollen tubes

A consistent observation from experiments involving co-expressed SLDP2.1 and LIPA in pollen tubes (Figure 4B) was the distinct positioning of LDs at the PM in these cells. That is, in pollen tubes co-expressing SLDP2.1-mCherry and mVenus-LIPA, LDs appeared to be located in close proximity...
to the PM more often as compared to cells expressing either protein on its own (Figure 4B; compare also with images in Figures 1, B and 4, A). This suggests that SLDP2.1 and LIPA together are involved in the positioning of LDs, or at least a subset thereof, at the PM. To quantify this positioning, LDs in vicinity to the PM were manually counted, and put in relation to the pollen tube length in the taken micrographs for all transient expression combinations with SLDP2.1 and/or LIPA (Figure 4C). Consistent with the observations described above, the co-expression of SLDP2.1-mCherry and mVenus-LIPA significantly increased the number of LDs in proximity to the PM, compared to either protein expressed alone. However, pollen tubes co-expressing SLDP2.1-mCherry and C-terminal-tagged LIPA-mVenus (or LIPA-mVenus alone) did not appear to differ in the number of LDs in proximity to PM, reinforcing our earlier conclusion that the C-terminus of LIPA is important for its association with the PM (Figure 4C).

To further test the premise that SLDPs and LIPA are important for the positioning of LDs at the PM, time-lapse imaging of Nile red-stained LDs in LIPA and SLDP2.1 co-transformed pollen tubes was performed. Pollen tube

![mVenus-LIPA](image1)

![SLDP2.1-mVenus](image2)

![SLDP2.1-mVenus + mVenus-LIPA](image3)

**Figure 5** LD mobility analysis of LIPA- and SLDP2.1-transformed tobacco pollen tubes. CLSM images of mVenus-LIPA and SLDP2.1-mVenus transiently expressed either alone or together in *N. tabacum* pollen tubes. Pollen tubes were stained with Nile red and LD dynamics were recorded over the indicated time course. Images are representative of time-course series of five transformed pollen tubes with each of the indicated fusion constructs. Note in mVenus-LIPA and SLDP2.1-mVenus-transformed pollen tubes, LDs display dynamic cytoplasmic streaming, while in mVenus-LIPA and SLDP2.1-mVenus co-transformed pollen tubes LDs were predominantly immobilized at the PM. Bars, 10 μm.
growth was extended by 3 h in comparison to previous experiments in order to give the tubes more time for protein expression and potential protein interactions. In control pollen tubes expressing mVenus alone (Supplemental Figure S13C and Supplemental Movie S1), as well as in pollen tubes expressing mVenus-LIPA or SLDP2.1-mVenus alone (Figure 5; Supplemental Movies S2 and S3), LDs moved dynamically via cytoplasmic streaming. In contrast, in pollen tubes co-expressing mVenus-LIPA and SLDP2.1-mVenus, LDs were mostly localized and immobilized at the PM (Figure 5; Supplemental Figure S4), as were, to a lesser extent, LDs in pollen tubes co-expressing SLDP1.3-mCherry and mVenus-LIPA (Supplemental Figure S13C and Supplemental Movies S5 and S6). Notably, the observed immobilization of LDs at the PM was not due to the fluorophore appended to LIPA and SLDP. That is, the co-expression of native (notagged) variants of LIPA and SLDP1.3 or SLDP2.1, along with mVenus alone serving as a cell transformation marker, yielded similar results and, in fact, even a more pronounced association of LDs with the PM in SLDP1.3 and LIPA co-expressing pollen tubes (Supplemental Figure S13C and Supplemental Movies S7 and S8).

A coiled-coil domain in LIPA mediates its interaction with SLDP2 at LDs

While in silico analysis of Arabidopsis LIPA did not yield any known protein functional domains/motifs, the prediction program COILS (Lupas et al., 1991) revealed a putative coiled-coil domain in LIPA at residues 60–113 (Supplemental Figure S11D). Further structural predictions using the AlphaFold2 algorithm (Jumper et al., 2021; Varadi (Supplemental Figure S11D). The predicted structure of LIPA was also assessed using the AlphaFold2 algorithm (Supplemental Figure S11D). Although the algorithm is generally not well suited for predicting effects of individual point mutations (Akdel et al., 2021), helix H3 was still predicted to be significantly shorter in the mutant protein than in native LIPA (Figure 6A). Notably, LIPA $^{L80P/V98P}$ mutant protein was re-localized to LDs upon SLDP2.1-mCherry co-expression.

Prediction of LIPA $^{L80P/V98P}$ mutant protein shown in Supplemental Figure S11D)

The predicted structure of LIPA $^{L80P/V98P}$ mutant protein was also assessed using the AlphaFold2 algorithm (Mirdita et al., 2021). Although the algorithm is generally not well suited for predicting effects of individual point mutations (Akdel et al., 2021), helix H3 was still predicted to be significantly shorter in the mutant protein than in native LIPA (Figure 6A). Notably, LIPA $^{L80P/V98P}$ mutant protein with either N- or C-terminally appended mVenus, irrespective of co-expression with SLDP, localized in a similar manner as its native LIPA counterparts, i.e. to the PM and/or cytosol, but not to LDs.
Thus, the predicted coiled-coil region of LIPA appears to be both sufficient and necessary to induce LIPA-mediated relocation of SLDP2-containing LDs.

**Figure 6C; Supplemental Figure S14.** Thus, the predicted coiled-coil region of LIPA appears to be both sufficient and necessary to induce LIPA-mediated relocation of SLDP2-containing LDs.

**Figure 7 SLDP and LIPA interaction assays by FRET–FLIM and Y2H.** A, Full-length versions of SLDPs tagged with mVenus (mV) were expressed in tobacco pollen tubes either alone or in combination with the cytosolic LIPA-mCherry (LIPA-mC). Co-expression led to a recruitment of LIPA-mCherry to the LDs and a significant reduction of the donor lifetime. One-way ANOVA was performed, followed by Tukey post hoc analysis (left: $F(1,27) = 43.85, P = 4.18 \times 10^{-7}, n_1 = 15, n_2 = 14$; right: $F(1,28) = 27.33, P = 1.49 \times 10^{-5}, n = 15$). Statistical results are presented as compact letter display of all pairwise comparisons. B, The expression of truncated cytosolic versions of the SLDPs with cytosolic LIPA-mCherry also led to a reduction of the donor lifetime in comparison to the expression of the SLDPs alone, or of the SLDPs in combination with mCherry. One-way ANOVA was performed, followed by Tukey post hoc analysis ($F(5,114) = 94.57, P = 6.68 \times 10^{-39}, n = 20$). Statistical results are presented as compact letter display of all pairwise comparisons. C, Y2H interaction analysis of SLDP1, SLDP2, and LIPA. Yeast (*S. cerevisiae*) were co-transformed with bait (pGBKT7) plasmids containing full-length SLDP1 or SLDP2 and prey (pGADT7) plasmids containing LIPA or modified versions thereof, or with the corresponding empty plasmids serving as negative controls. Serial dilutions of transformed yeast cell cultures were then plated onto either plasmid-selection conditions (DDO medium), or higher stringency selection conditions (QDO medium) where yeast cell growth requires an Y2H protein–protein interaction. Note that only yeast cells co-expressing SLDP1 or SLDP2 and LIPA or LIPA64–113, but not LIPA L80P V98P, grew on QDO plates. Results shown are representative of three separate co-transformations of yeast with each plasmid combination.

Both FRET/FLIM and Y2H assays confirm SLDP–LIPA interaction
To further test the hypothesis that SLDPs and LIPA interact, both FRET-FLIM and Y2H analyses were performed.
FRET-FLIM experiments were carried out in tobacco pollen tubes. As shown in Figure 7A, the co-expression of SLDP1.3-mVen or SLDP2.1-mVen with mCherry-LIPA led to a significant decrease in the fluorescence lifetime of mVen in comparison to the expression of the SLDP1.3-mVen and SLDP2.1-mVen on their own. These results indicate that SLDP and LIPA come in close proximity at the surface of LDs. The putative interaction of SLDP and LIPA was also assessed testing truncated versions of SLDP1 and 2 that mislocalize to the cytosol in pollen tubes, i.e. SLDP1.3Δ1–81 and SLDP2.1Δ1–75 (refer to Figure 1B). Similar to full-length SLDP1.3-mVen and SLDP2.1-mVen, the co-expression of SLDP1.3Δ1–81 or SLDP2.1Δ1–75 with LIPA-mCherry led to a significant reduction in the fluorescence lifetime of mVen, while the co-expression with mCherry alone did not (Figure 7B). These results indicate that the N-termini of the SLDPs are not required for the interaction with LIPA and that an interaction does not depend on the localization to LDs.

Additionally, the interaction of SLDP and LIPA or mutant versions thereof was also addressed in Y2H assays. As shown in Figure 7C, results showed that both SLDP1.3 and SLDP2.1 interact with full-length LIPA or the putative coiled-coil domain of LIPA (LIPAΔ61–113), but not with LIPA L107–144 (Figure 7C). As expected, yeast expressing either SLDP1, SLDP2, or LIPA with only the corresponding “empty” vector did not grow on selection media. Furthermore, Y2H assays revealed that SLDP1.3, SLDP2.1, and LIPA do not self-associate, nor do SLDP1.3 and SLDP2.1 associate with each other (Supplemental Figure S15).

LIPA targets the PM via its C-terminal polybasic region

Our experiments suggest that the putative coiled-coil region of LIPA (residues 60–113) is involved in binding SLDPs but is not sufficient for its localization to the PM (Figure 6B). To determine the region(s) in LIPA required for PM targeting, several truncated versions of the protein were generated and expressed as N-terminal mVenus fusions in tobacco pollen tubes. As shown in Figure 8A, the C-terminus of LIPA (residues 107–144), which includes a polybasic region (residues 107–134; Figure 8B), localized to the PM similar to full-length LIPA. In contrast, a shorter C-terminal region of LIPA (residues 120–144) was mislocalized to the cytosol, indicating that the polybasic region in LIPA is necessary for its PM targeting (Figure 8B). We also removed additional amino acids from the C-terminal end of the LIPA in the context of the LIPAΔ107–144 mutant, including the C-terminal cysteine residue (LIPAΔ107–147), which could potentially serve as a lipid-anchor site (Xie et al., 2016), and the C-terminal 10 or 21 amino acids (LIPAΔ107–134 and LIPAΔ107–123, respectively). Overall, the localization results for these LIPA mutants indicated that the C-terminal 10 amino acids are not essential for PM targeting, while deletions of residues within the polybasic region abolished the PM targeting of LIPA (Figure 8B). Taken together, the amino acids 107–134 are able to bind the PM.

To further investigate the interaction of LIPA with the PM at the molecular level, we utilized coarse-grained molecular dynamics (MD) simulations. This computational approach has been successfully used to study the interaction of a wide variety of membrane proteins with a lipid bilayer (Corradi et al., 2019; Marrink et al., 2019). Here, we used the recently released version (3.0) of the Martini force field (Souza et al., 2021), which was shown to accurately describe the membrane-binding behavior of several membrane proteins and to correctly identify pivotal amino acid residues involved in the interaction (Srinivasan et al., 2021). To study LIPA, the simulated system contained one molecule of LIPA, ions, water molecules, and a complex phospholipid bilayer with a composition mimicking the negatively charged plant cell PM (Wassenaar et al., 2015). We performed five independent MD simulations with different starting velocities, resulting in a total of 5-μs simulation time. During the simulations, LIPA displayed an on/off membrane binding similarly to the behavior reported for other membrane proteins (Srinivasan et al., 2021; Supplemental Figure S16). Figure 8C shows selected snapshots from the MD simulations depicting unbound and membrane-bound states of LIPA (see also Supplemental Movie S9). We then quantified the binding events by generating a probability density distribution using the kernel density estimation method and, as shown in Figure 8D, the calculated probability density distribution revealed a significant population of LIPA in the membrane-bound state. Next, we investigated amino acid residues of LIPA potentially involved in the interaction with negatively charged phospholipids. In agreement with the results from our truncation analyses of LIPA (Figure 8A), the highest number of contacts is located at the C-terminus of LIPA (Figure 8E). In addition, we also observed a contribution of H1 and, to a lesser extent, of the adjacent flexible region, to the interaction of LIPA with negatively charged phospholipids (Figure 8E). Interestingly, the region corresponding to the helical/coiled-coil domain (i.e. H3) involved in the LIPA–SLDP interaction (Figure 6) showed no interaction with the phospholipids (Figure 8E), further corroborating the role of this region in protein–protein interactions (Figure 8E).

**lipa mutants phenocopy sldp1 sldp2 mutants in their aberrant subcellular distribution of LDs during post-germinative seedling growth**

Given that SLDP and LIPA appear to act together in the positioning of LDs at the PM, we next tested whether disruption of LIPA would have a similar effect on the subcellular distribution of LDs, as observed upon disruption of SLDP (Figure 2). To this end, two independent Arabidopsis mutant lines of LIPA were generated, a T-DNA insertional line, lipa-1, and a CRISPR/Cas9 deletion line, lipa-2, which is devoid of most of the LIPA open reading frame (Figure 6A; Supplemental Data Set S1). RT–qPCR analyses confirmed a lack of full-length LIPA transcripts in both mutant lines.
Supplemental Figure S17. Time-course imaging of Nile red-stained LDs in cotyledon and hypocotyl cells in WT and both lipa-1 and lipa-2 rehydrated (mature) seeds and seedlings after stratification, was performed as described above for the sldp mutants (Figure 9B; Supplemental Figure S6; compare with images presented in Figure 2B). While again no obvious differences in LD distribution or storage vacuole appearance were observed in WT and lipa mutant rehydrated seeds (Supplemental Figure S7), an LD-clustering phenotype similar to sldp2 and sldp1 sldp2 seedlings at 24 h and 36 h was readily observed (Figure 9B; Supplemental Figure S6B). Additionally, as described for sldp mutants,
Z-stack images of cotyledon and hypocotyl cells in lipa mutants at the 36-h time point and quantification of the LD distribution in these cells were performed and revealed significant differences in the LD distribution as compared to WT cells (Supplemental Figure S8). These results provide further evidence that SLDP2 and LIPA act together in the proper positioning of LDs at the PM during post-germinative seedling growth.

Both mGFP-LIPA and SLDP-mCherry localize to contact sites between LDs and the PM

Previous results hint at a putative MCS between LDs and the PM, formed through interaction of LIPA and SLDP. On that account, we next investigated whether these proteins are specifically enriched at LD–PM contact sites in seedlings. Therefore, Arabidopsis transgenic lines stably expressing SLDP1.3 and SLDP2.1 (under control of the 35S promoter) appended to a C-terminal mCherry were assayed for their subcellular localization in hypocotyl cells of 40-h old seedlings. As shown in Figure 10, A and B, both SLDP1.3-mCherry and SLDP2.1-mCherry localized to the surface of LDs, as evidenced by the torus-shaped fluorescence patterns surrounding the fluorescence attributable to the BODIPY-stained neutral lipids inside the LDs. However, SLDP fluorescence, particularly SLDP2.1-mCherry, was often enriched at distinct sites on the LD surface that were presumably adjacent to the PM (Figure 10B), suggesting that especially SLDP2.1 preferentially localizes at LD–PM contact sites. Similarly, eGFP-LIPA stably expressed in the Arabidopsis lipa-1 background localized to the surface of LDs in hypocotyl cells and was often enriched at apparent LD–PM contact sites (as shown by staining of LDs with Nile Red and the PM with FM4–64).

Discussion

While plant LD research has yielded a number of significant advancements in recent years (Lundquist et al., 2020; Ischebeck et al., 2020; Kang et al., 2021), many important questions related to plant LD biology remain unanswered, including if and how they interact with other organelles and subcellular structures. Here, we took advantage of the recent proteomics-based identification of several LD-associated proteins (Kretzschmar et al., 2020) and characterized two members of the plant-specific SLDP family, SLDP1 and SLDP2. We
showed that the LD-association of SLDPs is mediated by an N-terminal predicted amphipathic and hydrophobic region (Figure 1), similar to LD targeting sequences reported previously for other LD-localized proteins (Wilfling et al., 2013; Pyc et al., 2017; Kretzschmar et al., 2018). Moreover, in Arabidopsis sldp mutant seedlings, LDs display an aberrant subcellular distribution, with LDs clustering in the center of the cell and not, as observed in WT seedlings, aligning along the PM (Figure 2).

We further showed that LIPA is associated with isolated LDs, depending on the presence of SLDP2 (Figure 3) and LDs in Arabidopsis lipa mutant seedlings displayed an aberrant clustering phenotype, similar to that observed in sldp mutant seedlings. Microscopic analyses revealed that transiently expressed LIPA localizes to the PM in pollen tubes, but, upon co-expression with SLDP, is also found at LDs. Conversely, SLDP is partially re-located to the PM upon co-expression with LIPA (Figure 4). Moreover, we observed that at least a subset of LDs in LIPA and SLDP co-expressing pollen tubes are conspicuously immobilized at the PM and not streaming throughout the cytoplasm (Figure 4). How exactly LIPA associates with the PM remains unclear, as no putative transmembrane domains were detected within the LIPA protein sequence. Based on structural modeling and truncation analyses, though, we suggest that LIPA might bind the plasma membrane through electrostatic interactions of a positively charged polybasic sequence near LIPA’s C-terminus (in the region of amino acids 107–134) which

Figure 10 Localization of stably expressed SLDP and LIPA in Arabidopsis seedling hypocotyls. SLDP1.3-mCherry and SLDP2.1-mCherry (A and B) or eGFP-LIPA (C and D) were stably expressed under the 35 S promoter in Arabidopsis Col-0 (A and B) or lipa-1 mutant (C and D) plants. Fusion protein localization was monitored in 38-h-old seedlings by CLSM after staining with either BODIPY 493/503 (A and B), Nile Red (C), or FM4-64 (D). The panels on the right display portions of the cells at higher magnification in the panels to the right. Note that the fluorescence attributable to SLDP1.3-mCherry predominantly encircled LDs (A), while for SLDP2.1-mCherry and eGFP-LIPA, fluorescence was enriched at putative LD–PM MCSs (B–D). Images are representative of at least five seedlings from each of three (A and B) or two independent plant lines (C and D). Bars, 10 μm and 2 μm in low and high magnified images, respectively, and applies to all the corresponding images in the other panels.
reserves in LDs via peroxisomal β-oxidation (Esnay et al., 2020). Our results extend this work by showcasing a possible additional LD-involving MCS during post-germinative seedling growth: LDs in seedlings might be needed at the PM, either to provide lipids or to buffer (i.e. store) excess and potentially cytotoxic lipids produced during membrane repair and/or growth. More generally, LD–PM MCSs might be required for maintaining PM lipid homeostasis, as has been shown, for example, LD–ER MCSs (Velázquez et al., 2016). The importance of this LD–PM MCS might therefore only come into effect upon stress conditions (such as salt, freezing, mechanical, etc.), when membrane composition has to be remodeled. This would explain the lack of any obvious growth and/or developmental phenotypes in the sldp and lipa mutants, which were examined under laboratory conditions in this study, despite their striking cellular (LD) phenotype.

Recently, several tri-organelar contact sites involving LDs and the ER have been described in mammals, yeast and insects (Freyre et al., 2019; Hariri et al., 2019; Ugrankar et al., 2019). As LDs are often associated with the ER (Hugenroth and Bohnert, 2020), another interesting question arising is whether the ER might be involved in the observed LD–PM MCSs, as well. While the work presented here does not provide direct evidence for this hypothesis, it was recently found that SEIPIN, an ER-membrane protein that participates in LD biogenesis (Sui et al., 2018), interacts with the ER-associated protein VAP27-1 (vesicle-associated membrane protein-associated protein 27-1) at ER–LD MCSs (Greer et al., 2020). VAP27-1 is also involved in tethering the ER to the PM via interaction with SYT1 (Siao et al., 2016), a homolog of mammalian extended synaptotagmins, which is known to be important during abiotic stress responses (Yamazaki et al., 2008; Schapire et al., 2008). Given that mammalian extended synaptotagmins are involved in lipid transfer (Schauder et al., 2014; Yu et al., 2016), a three-way MCS involving LD, ER, and PM could serve as a key hub for lipid homeostasis at the plant PM.

Future work will now be aimed at investigating these possibilities by uncovering the mechanistic details underlying the putative SLDP-LIPA tethering complex reported here, as well as elucidating the physiological importance of LD–PM MCSs in seedlings and other tissues.

**Experimental procedures**

**Plant material and growth conditions**

All *A. thaliana* (L.) plants employed the ecotype Col-0 or were derived from it in the case of T-DNA and CRISPR/Cas9 mutant lines. Plants on soil (Einheitserde SPECIAL Vermehrung, Patzer Erden, Sinntal-Altengronau, Germany; medium clay content, contains peat, perlite, 1% nutrient salts, trace elements and iron, pH 5.8) were grown in a climate chamber (York) in 60% relative humidity, with a constant temperature of 23°C and under a 16-h/8-h day/night cycle with a daytime light intensity of 120–150 μmol
photon m$^{-2}$ s$^{-1}$ (the climate chamber was equipped with LuxLine Plus F36W 830 Warm White de Luxe fluorescent tubes or Ledvance STBA VUN Warm white LED tubes; both Osram, Berlin, Germany). Seeds for growth under sterile conditions were surface sterilized in 6% sodium hypochlorite solution for 15–20 min. Plants from these seeds were grown on half-strength MS medium (Murashige and Skoog, 1962) supplemented with 0.8% (w/v) agar with or without 1% (w/v) sucrose (as indicated) in a growth chamber at 22°C and under 100 μmol photons m$^{-2}$ s$^{-1}$ (Ledvance STBA EM, LED tubes, Osram). For hygromycin selection, half-strength MS plates were supplemented with 25 μg mL$^{-1}$ hygromycin and 1% (w/v) sucrose, stratified for 2 days at 4°C, subjected to light for 4 h and then kept vertically in the dark for 3 days. Hygromycin-resistant seedlings were transferred to half-strength MS + 1% (w/v) sucrose without hygromycin for 1 week prior to transplanting them into soil.

Tobacco (N. tabacum L. cv. Samsun-NN) plants were grown in the greenhouse on the same soil as Arabidopsis and as previously described in order to collect pollen (Rotsch et al., 2017). Plants were kept under 14 h of light from mercury-vapor lamps in addition to sunlight. Light intensities reached 150–300 μmol m$^{-2}$ s$^{-1}$ at the flowers and 50–100 μmol m$^{-2}$ s$^{-1}$ at leaves at mid-height. Temperature was set to 16°C at night and 21°C during the day with a humidity of 57%–68%.

Nicotiana benthamiana plants were grown Pro-mix BX soil (Premier Horticulture Ltd; Rivière-du-Loup, QC, Canada) at 22°C with a 16-h/8-h day/night cycle and 50–100 μmol photons m$^{-2}$ s$^{-1}$ intensity from fluorescent T8 bulbs (Sylvania, London, UK).

**T-DNA lines**

Knockout lines of SLDP1, SLDP2, and LIPA were generated. The commercially available T-DNA insertional lines SALK_204434C (sldp1-1, T-DNA inserted in intronic region behind base 1028) and SALK_068917 (sldp2-1, T-DNA inserted in first exon behind base 42) and Gabi-KAT 723C08 (lipa-1, T-DNA inserted behind base 20) were used, and CRISPR/Cas9 was used to generate sldp1-2, sldp2-2, and lipa-2 (see below). Sequence alignments and predicted protein products of all analyzed mutant lines are shown in Supplemental Data Set S1.

**CRISPR/Cas9**

To generate CRISPR/Cas9 mutants, single guide RNAs (sgRNAs) were designed using the Cas-Designer and CasOFFinder at http://www.rgenome.net/ for a SpCas9 protospacer adjacent motif (PAM) sequence and with a length of 19 bp (without PAM) against the A. thaliana (TAIR10) genome (Bae et al., 2014; Park et al., 2015). Cloning was performed as described previously (Xing et al., 2014). As template for the sgRNA cassette (including one sgRNA backbone, one U6–26 terminator and one U6–29 promoter), pCBC DT1T2 was used and the generated PCR product was cloned into pHEE401E via BsaI restriction sites, between a U6–26 promoter on one side and a second

sgRNA backbone and a U6–29 terminator on the other side (as described previously (Xing et al., 2014; Wang et al., 2015). This way, a CRISPR/Cas9 construct containing two sgRNAs under two U6 promoters and a Cas9 under the egg-cell specific EC1.2 promoter was obtained. To knockout one gene, two different sgRNAs were targeted at it, aiming at deleting the whole gene stretch between the target sequences. This made it possible to screen for mutant plants via PCR. For this, gDNA was extracted from rosette leaves, the area of interest was amplified via REDTaq-PCR and screened for the desired smaller PCR products that indicated a deletion. Homozygous mutants were obtained in the T2 and T1 generation for SLDP1 and SLDP2, respectively. To remove the Cas9-transgene, homozygous mutants were backcrossed to WT plants (and Cas9-loss was confirmed by PCR with U6- and hygromycin resistance gene-specific primers).

For SLDP1, a mutant line with deletion of bases 333–564 in the first exon (resulting in a frameshift and a premature stop codon at position 650–652 for AT1G65090.1 and .2 or at positions 686–688 for AT1G65090.3, producing a potential 139 amino acid protein for AT1G65090.1 and .2 or 152 amino acids for AT1G65090.3) was obtained and called sldp1-2. For SLDP2, a mutant line with deletion of bases 304–379 in the first exon (resulting in frameshift and premature stop codon at position 398–400, producing a potential 107 amino acid protein) was obtained and called sldp2-2. For LIPA a mutant line with deletion of bases Δ94–214 (resulting in a frameshift and premature stop codon at position 230–232 and a potential 36 amino acid protein) was obtained and called lipa-2. Sequence alignments and predicted protein products of all analyzed mutant lines are shown in Supplemental Data Set S1.

**Stable transformation of Arabidopsis plants**

Arabidopsis plants were transformed by the floral dip method (Clough and Bent, 1998) using the Agrobacterium tumefaciens strain EHA105.

**RNA isolation and qPCR**

RNA from was isolated in triplicate from 5 mg of dry seeds (all derived from the same seed batch per line) using an RNA extraction kit (Monarch Total RNA Miniprep Kit, NEB). cDNA synthesis was performed with 900-ng total RNA and 100-pmol oligo(dT) primer using the Maxima Reverse Transcriptase (Thermo Scientific) according to the manufacturer’s instructions. Transcript analysis by qPCR was carried out with AT4G05320 (POLYUBIQUITIN 10) as reference (Czechowski et al., 2005). Amplification and quantification were performed with the Takyon No Rox SYBR MasterMix dTTP Blue Kit (Eurogentec) in the iCycler System (iQ 5 Real-Time PCR Detection System, Bio-Rad). The amplification mix contained 1x Takyon No Rox SYBR MasterMix dTTP Blue, 2-mM primers, and 4-μL cDNA in a final reaction volume of 20 μL. The PCR program consisted of a 3-min denaturation step at 95°C followed by 40 cycles of 10 s at 95°C, 20 s at 58°C, and 40 s at 72°C.
Data analysis was performed using the 2−ΔΔCt method as previously described (Livak and Schmittgen, 2001).

**Plasmid construction**

For localization studies in pollen tubes, coding sequences of the genes of interest were cloned into pLatMVC-GW, pLatMVN-GW, or pLatMCC-GW (Müller et al., 2017) via classical or fast Gateway (Thermo Fisher Scientific) cloning as described before (Müller et al., 2017). All pLat-constructs contain a LAT52 promoter for strong expression in pollen tubes (Twel et al., 1991) and were verified by sequencing. A list of plasmids and primers can be found in Supplemental Data Set S5.

For localization studies in *N. benthamiana* leaves and Arabidopsis seedlings, cloning of pMDC32-ChC/SLDP2, encoding SLDP2 appended at its C-terminus to the red fluorescent protein mCherry (SLDP2-mCherry), pMDC32-GFP/LIPA, encoding LIPA appended at its C-terminus to a monomerized version of GFP (LIPA-mGFP), and pMDC43/LIPA, encoding LIPA appended at its N-terminus to GFP (GFP-LIPA), was performed using Gateway cloning technology (Mueller et al., 2017) and the binary vectors pMDC32-ChC (Kretzschmar et al., 2020), pMDC43 (Curtis and Grossniklaus, 2003), and pMDC32-GFP (described below), respectively. Each binary vector contains the 35S cauliflower mosaic virus promoter and was verified by automated sequencing performed at the University of Guelph Genomics Facility.

The pMDC32-CGFP binary vector contains a Gateway recombination site followed by the full-length mGFP open reading frame, which provides for the expression of a fusion protein with a C-terminal-appended mGFP. To construct pMDC32-CGFP, the mGFP coding sequence was amplified from pRTL2/monoGFP-MCS (Shockey et al., 2006), using primers GFP-FP-PacI (5′- CCAGCCCTTAATTTAAATGAGTA AAGGAGAAGAAGCTTTC-3′) and GFP-RP-SacI (5′-CCGGCC GAGCTCTTATTTGTATGTTTCTACATCCATGCTGCC-3′), which also added 5′ PacI and 3′ SacI restriction sites. The resulting PCR products were digested with PacI and SacI and ligated into similarly digested pMDC32-ChC to yield pMDC32-CGFP.

For Y2H assays, full-length SLDP1.3, SLDP2.1, and LIPA open reading frames, as well as modified versions of the latter, were amplified from the appropriate template plasmids using PCR and primers containing the EcoRl and BamHl restriction digest sites at the 5′- and 3′-ends, respectively. Resulting PCR amplicons were then digested with EcoRl and BamHl and ligated into similarly digested pGBK7T or pGADT7, which contain the GAL4-binding domain and GAL4-activation domain, respectively (Takara Bio Inc.).

**Particle bombardment and pollen tube microscopy**

*Nicotiana tabacum* pollen tubes were transiently transformed using a gene gun. For this, 6 μg of construct DNA was coated onto ~0.9-mg gold particles (1 μm), shot onto freshly harvested *N. tabacum* pollen of five flowers per transformation. Pollen tubes were grown for 5–7 h (8–10 h for LD motility assays) in liquid pollen tube medium on a microscope slide in a humid environment (in detail methods on coating and transformation were described before; Müller et al., 2017). For co-transformation, 6-μg DNA of each construct were pre-mixed and then coated onto the gold particles.

For pollen tube microscopy, pollen tubes were fixed in a final concentration of 1.8% (v/v) formaldehyde in pollen tube medium (Read et al., 1993; 5% w/v sucrose, 12.5% w/v PEG-4000, 15-mM MES-KOH pH 5.9, 1-mM CaCl2, 1-mM KCl, 0.8-mM MgSO4, 0.01% H3BO3 v/v, 30-μM CuSO4) and LDs were stained with a final concentration of 0.25 μg mL−1 Nile red (Sigma-Aldrich, St Louis, MI, USA) or 0.5% Lipi-Blue (Dojindo Molecular Technologies, Rockville, MD, USA), as indicated. Pollen tubes prepared to monitor LD movements were stained in the same manner but no fixative was added. Micrographs were acquired as single z-sections using a Zeiss LSM 510 or a Zeiss LSM780 confocal microscope (Carl Zeiss). For excitation, 405-nm diode was used, Lipi-Blue fluorescence was detected from 443 to 475 nm, Nile red was excited with 488 nm and detected at 583–667 nm. Constructs with mCherry were excited with 561 nm and detected at 571–614 nm, mVenus was excited with 488 nm and detected at 518–550 nm or 497–533 nm when co-imaged with Nile red. HFT 405/514/633-nm major beam splitter (MBS) was used.

**CLSM-based FLIM-FRET Analysis**

Confocal microscopy was performed using a Leica TCS SP8 microscope equipped with the FALCON time-correlated single photon counting system. For fluorescence-lifetime imaging of transiently transformed *N. tabacum* pollen tubes, images were taken with a 20 × 0.75 objective (CS2, HC PL APO). The mVenus-tagged donor proteins and mCherry/LIPA-mCherry acceptors were excited using a pulsed white light laser operating at 514 nm or 561 nm, respectively, with a pulse rate of 40 kHz in a two channel sequential excitation mode. Fluorescence emission was detected at 525–560 nm for mVenus and 580–630 nm for mCherry using Leica HyD SMD detectors. Images were acquired until at least 100 photons per pixel were collected in the brightest channel. The format of the pictures is 512 × 512 pixels. The ROI (region of interest) selection and FLIM data fitting were performed using the LASX Single Molecule Detection software module (v3.5.5). A monoexponential reconvolution model was fitted to all decay curves for calculating the fluorescence lifetime. Donor lifetime data were exported and used for further statistical analysis and plotting in Origin 2020 (OriginLab Corp., Northampton, MA, USA).

**Y2H**

Directed Y2H assays were performed according to Richardson et al. (2011). In brief, both bait (pGBK7T) and prey (pGADT7) vectors were co-transformed into yeast (strain 569-4A) using the lithium acetate transformation method (Gietz and Schiestl, 2007) and then plated on double-drop out (DDO) selection plates, consisting of
synthetic dextrose media containing 2% dextrose, 0.67% yeast nitrogen base, and synthetic complete amino acid and base supplements lacking Leu and Trp (Buffered Inc.). Selected yeast colonies were grown to log phase in liquid DDO at 30°C and 275 rpm, then the optical density at 600 nm (OD600) was adjusted to 0.5 and 1.5 serial dilutions were carried out. Five microliters of each dilution was spotted onto both DDO and quadruple drop-out (synthetic dextrose medium lacking Leu, Trp, Ade, and His) plates and grown at 30°C for 3 days. Results shown are representative of three independent yeast transformations.

*Nicotiana benthamiana* infiltration and microscopy

For infiltration, leaves of 4-week-old *N. benthamiana* plants were (co)infiltrated with *A. tumefaciens* (strain LBA4404) harboring a selected binary vector, as described previously (Kretzschmar et al., 2020). All (co)infiltrations also included *A. tumefaciens* transformed with pORE04-35S::P19, which encodes the tomato bushy stunt virus gene to enhance transgene expression (Petrie et al., 2010).

(co)infiltrated *N. benthamiana* leaves were prepared for confocal laser scanning microscopy (CLSM) by first fixing with 4% (w/v) formaldehyde, washing with 50-mM PIPES pH 7.0, and then staining with neutral lipid-specific dye monodansylpentane (MDH) (Abcepta; Yang et al., 2012) at a working concentration of 0.4 mM, as described previously (Gidda et al., 2016). Micrographs of leaf epidermal cells were acquired as single z-sections using a Leica SP5 CLSM (Leica, Germany) at 20× objective and 35× zoom. All images of cells are representative of at least two independent experiments (i.e. infiltrations), including at least three separate (co)transformation of leaf epidermal cells.

Determination of 1,000 seed weight and seed total fatty acid analysis

The 1,000 seed weight was determined by manually counting replicates of 500 seeds and weighing these.

For fatty acid analysis, seeds were sieved to a size of 250–300 μm. Six biological replicates were performed: seeds from five different mother plants were harvested and 25 seeds each were analyzed per time point and genotype, presented results are representative of two other replications of the experiment. Seeds were germinated on wet filter papers soaked in 1.6 mL H2O and put in a petri dish in a humid environment. Apart from seeds for dry seed analysis, seeds were stratified for 4 days at 4°C in the dark prior to imbibition. After 4 days of stratification, 0 day samples were harvested; the other samples were placed into 16-h/8-h day/night cycle in an incubator (CU-36L/D, Percival Scientific Inc., Perry, USA) at 22°C and a light intensity of 120 μmol m−2 s−1. Seeds and seedlings were harvested into 1-mL fatty acid methyl ester (FAME) reagent (2.5% v/v H2SO4, 2% v/v dimethoxypropane in methanol/toluol 2:1, v/v; Miquel and Browse, 1992) with 30 μL of 0.33 mg·mL−1 tri-15:0 TAG (1,2,3-tripentadecanoylglycerol ≥99%, Sigma-Aldrich, St Louis, MI, USA) in toluol (ROTIPURAN ≥99.5%, Carl Roth, Karlsruhe, Deutschland) as internal standard and ground with a glass stick. Samples were then incubated at 80°C in a water bath under constant shaking for 1 h to esterify all FAs to methanol. The reaction was stopped with 1 mL of saturated NaCl solution and vortexing. FAMEs were then extracted twice adding 1 mL of hexane, centrifuging 10 min at 2,000g and transferring the upper phase to a new glass tube. Hexane was evaporated and samples resuspended in 30 μL of acetonitrile (HPLC Gradient grade, Fisher Chemical, Thermo Fisher Scientific, Waltham, MA, USA). Subsequent GC-FID analysis was performed as described in Homung et al. (2002). An Agilent GC 6890 system (Agilent, Waldbronn, Germany) coupled to an FID detector equipped with a capillary HP INNOWAX column (30 m × 0.32 mm, 0.5-μm coating thickness, Agilent, Waldbronn, Germany) was used. Helium served as carrier gas (30 cm·s−1), with an injector temperature of 220°C. The temperature gradient was 150°C for 1 min, 150–200°C at 15°C min−1, 200–250°C at 2°C min−1, and 250°C for 10 min. For quantification, peak integrals were determined using Agilent ChemStation for LC 3D systems (Rev. B.04.03) and used to calculate absolute amounts total fatty acids.

Hypocotyl measurements

All seeds used for hypocotyl analyses were sieved to a size of 250–300 μm prior to analyses and surface-sterilized in 6% sodium hypochlorite solution for 15–20 min and placed on solid half-strength MS medium without sucrose (Murashige and Skoog, 1962). After a stratification for 4 days at 4°C in the dark, seedlings were grown vertically in the light for 7 days under 16-h/8-h day/night regime, or 4 h in the light and then 7 days in the dark in an incubator (CU-36L/D, Percival Scientific Inc., Perry, USA) at 22°C and a light intensity of 120 μmol m−2 s−1, and hypocotyls were recorded with the Ocular scientific image acquisition software (version 1.0, Digital Optics Ltd, Auckland, New Zealand) on a binocular (Olympus SZX12 binocular, Olympus Corporation, Tokyo, Japan) attached to a camera (R6 Retiga camera, QImaging, Surrey, Canada). Hypocotyl length was measured with ImageJ software (1.52p; Rueden et al., 2017) and violin plots with mean points were generated using ggplot2 package (version 3.3.2) in the R environment (version 4.0.1).

Seed and seedling preparation and microscopy

For time-course microscopic analyses, either seeds rehydrated for 30 min were used, or seedlings were stratified and grown on half-strength MS medium without sucrose as described above. They were transferred to light at 07.30 am (light period 7 am to 11 pm, 16-h/8-h day/night cycle) and then analyzed after 12, 24, 36, and 48 h of germination. Rehydrated seeds and seedlings were harvested into 1-mL H2O and directly used for microscopy after removal of seed coats. Fluorescence dyes were used at the following
concentrations 1.5-6 μM Nile Red (Sigma Aldrich), 1.6-μM Bodipy 493/503, 1-μM MDY-64, or 4-μM FM4-64 (Sigma Aldrich). All stock solutions were prepared in DMSO. Seeds were additionally fixed in 1% formaldehyde, when Z-stacks were recorded. Micrographs were taken using a Zeiss LSM780 confocal microscope (Carl Zeiss). For Nile Red excitation, 561 nm laser was used, fluorescence was detected at 571–603 nm with a 488/561 MBS. For microscopy of MDY-64 and Nile Red, laser wavelengths of 458 and 561 nm, respectively, and a 458/561 nm MBS were used, and fluorescence was detected at 463–516 and 552–631 nm, respectively. Bodipy 493/503 and mCherry were excited with a 488- and a 561-nm laser using a 488/561 MBS and detected at range of 493–568 and 586–639, respectively. eGFP and Nile Red or FM4–64 were co-excited with a 488-nm laser and a 488 MBS. Fluorescence was detected at 489–515 and 563–631 nm for eGFP and Nile Red, respectively, and 493–530 and 651–739 nm for eGFP and FM4–64, respectively.

**Proteomic analysis**

*Arabidopsis thaliana* seedlings were surface-sterilized, placed on half-strength MS medium without sucrose, stratified for 72 h at 4°C in the dark and then grown at 22°C under 16-h/8-h of light–dark regime for 38 h. One replicate consisted of seedlings derived from 100 mg of seeds grown in one plate. All seeds from each line derived from the same seed batch harvested from 20 mother plants. Mother plants of all plants were grown side-by-side.

Total protein isolation of TE and LD fractions, LD enrichment, proteomics sample preparation including a tryptic in-gel digest, liquid chromatography-mass spectrometry (LC-MS) analysis, and analysis of MS/MS raw data was performed as previously described (Kretzschmar et al., 2018).

LFQ values were determined using MaxQuant software 1.6.2.10 (Cox and Mann, 2008; Cox et al., 2014). Perseus software (Tyanova et al., 2016) (version 1.6.6.2) was used for data analysis. Principal component analysis (PCA) plots were created from unfiltered raw LFQ values (Supplemental Data Set S2) following the workflow outlined in Horn et al. (2021). The libraries, the meta data file, raw data files, MaxQuant search files, as well as ProteinGroup and Peptide search results created by MaxQuant are available on ProteomeXchange/PRIDE (Vizcaíno et al., 2014) under the identifier PXD022769.

LFQ values were normalized as % of total sum of all LFQs per replicate and log2-transformed (rLFQ) for further analyses. For LD-enrichment analysis within one line, all proteins from TE and LD fractions together were filtered for those detected at least three times in at least one group and identified by at least two peptides (Supplemental Data Set S3).

For differential abundance analysis of LD or TE fractions between the lines, LD fraction and TE fraction were analyzed separately and filtering was performed independently of the respective other fraction (Supplemental Data Set S4).

For enrichment and differential abundance analyses, rLFQ values were imputed: missing values were replaced from normal distribution (for the TE: width 0.3, down shift 1.8; for LD fractions: width 0.5, down shift 1.8; for both fractions together: width 0.8, down shift 1.8; Supplemental Data Set S6). To obtain proteins significantly enriched on LDs, LD fractions were compared to TE fractions and analyzed for proteins enriched in LD fractions. To find differentially abundant proteins between the different lines, LD fractions of mutants were compared to LD fractions of the WT, the same was done for TE fractions. Proteins were considered LD-enriched or differentially abundant, respectively, if the false discovery rate (FDR) < 0.01 and S0 > 2 (as determined by two-sided t test with 250 randomizations). Volcano plots were created to visualize the results. For metadata see Supplemental Data Set S7.

**Electron microscopy**

High pressure freezing electron microscopic analysis was performed as described before (Hillmer et al., 2012). Plant material was dissected from hypocotyl or cotyledons of 36–48 h germinated seedlings with a biopsy punch (pfm medical, Köln; 2-mm diameter), submerged in freezing medium (200 mM Suc, 10-mM trehalose, and 10-mM Tris buffer, pH 6.6) transferred into planchettes (Wohlwend, Sennwald, Switzerland; types 241 and 242), and frozen in a high pressure freezer (HPM010; Bal-Tec, Liechtenstein). Freeze substitution was performed in a Leica EM AF52 freeze substitution unit in dry acetone supplemented with 0.3% uranyl acetate at −85°C for 16 h before gradually warming up to −50°C over a 5-h period. After washing with 100% ethanol for 60 min, samples were stepwise infiltrated (intermediate steps of 30%, 60% HM20 in ethanol, and twice with 100% HM20 for 1 h each), embedded in Lowicryl HM20 at −50°C and polymerized for 3 days with UV light in the freeze substitution apparatus at −35°C. Ultrathin sections were cut on a Leica UC6 Ultracut and poststained with 3% aqueous uranyl acetate and lead citrate for 3 min each. Micrographs were taken at a Jeol JEM1400 TEM (Jeol Germany, Freising) equipped with a TVIPS TEMCAM F416 digital camera (TVIPS, Gauting) using EMMenu 4 (TVIPS, Gauting).

**Bioinformatics**

For sequence alignments, T-Coffee (Notredame et al., 2000; http://tcoffee.crg.cat/apps/tcoffee/dorregular) was used with default settings. Sequence identity was calculated by Needleman–Wunsch global alignment of two sequences (Needleman and Wunsch, 1970) with EMBoss needle on default settings (https://www.bioinformaticians.nl/cgi-bin/emboss/needle). Helical wheel plots were created by Heliquist (Gautier et al., 2008) (https://helquest.ipmnc.cnrs.fr/cgi-bin/ComputParams.py) with Helix type: alpha and window size: 1_TURN. For hydrophobicity plots, ExPAsy ProtScale (https://web.expasy.org/protscale/) was used with a Kyte&Doolittle scale (Kyte and Doolittle, 1982) and a window size of 9. Charge plots were created by EMBoss
explorer charge (http://www.bioinformatics.nl/cgi-bin/em
boss/charge?_pref_hide_optional=0) with a window length of
5. TMDprediction was performed with ExPASy TMPred
(https://embnet.vital-it.ch/software/TMPRED_form.html).
Coiled-coils were predicted by ExPASy COILS (Lupas
et al., 1991) (https://embnet.vital-it.ch/software/COILS_
form.html) with a window width of 21.

Structure bioinformatics and modeling
Structure of LIPA (Uniprot code Q3EDG6) was downloaded
from the AlphaFold2 structure database (Varadi et al., 2022).
To calculate the structure of LIPA L80P, V98P, ColabFold
with MMseq2 homology search was used (Mirdita et al.,
2021). Electrostatic potential was calculated using the APBS
server (Jurrus et al., 2018).

The structure of LIPA was mapped into the Martini
coarse-grain representation using the martinize2 script with
the ScFix modification (Souza et al., 2021). The phospholipid
bilayer, in total composed of 2042 phospholipid molecules,
containing palmitoyl-oleoyl-phosphatidylcholine:palmityl-
oleoyl-phosphatidylethanolamine:palmityl-oleoyl-phosphati
dyserine:palmityl-oleoyl-phosphatidic acid:palmityl-oleoyl-
phosphatidylinositol 4-phosphate:palmityl-oleoyl-phosphatid-
diyinositol 4,5-bisphosphate (molecular ratio 37:37:10:10:5:1)
was generated using the insane.py script (Wassenaar et al.,
2015). MD simulations were performed with Gromacs2018
(Abraham et al., 2015). Lennard-Jones and electrostatic inter-
actions were cut off at 1.1 nm, with the potentials shifted to
zero at the cutoff. A relative dielectric constant of 15 was
used. The neighbor list was updated every 20 steps using the
Verlet neighbor search algorithm. Simulations were run in the
NPT ensemble. During the production runs, the sys-
tem was subject to pressure scaling to 1 bar using Parrinello–Rahman barostat with temperature scaling to 283
K using the velocity-rescaling method with coupling times of
1.0 and 12.0 ps. Semi-isotropic pressure coupling with a
compressibility of 3 \times 10^{-4} \text{ bar}^{-1} \text{ nm}^{-2}
was employed. Initially, the protein was placed approximately 3.0 nm away from the
membrane. Subsequently, the standard MARTINI water to-
going with Na⁺ and Cl⁻ ions at the concentration of 150
mM were added. Next, additional Na⁺ ions were added to
ensure the electroneutrality of the system. The whole system
was energy-minimized using the steepest descent method
up to the maximum of 5,000 steps and equilibrated for 10
ns with the pressure controlled by the Berendsen barostat.
Production runs were performed for up to 1 \mu s with a time
step of 20 fs. Membrane binding events were analyzed by
monitoring the minimum distance between the protein and
the membrane using the gmx mindist tool in Gromacs.
Membrane binding was subsequently evaluated by comput-
ing the probability density distributions using the kernel
density estimation method (Srinivasan et al., 2021).
Visualization was done using the ChimeraX and VMD pro-
gram (Humphrey et al., 1996).

Accession numbers
Sequence data from this article can be found in the
GenBank/EMBL data libraries under the following accession
numbers: AT1G65090 (SLDP1); AT5G36100 (SLDP2);
AT1G07985 (LIPA).

Supplemental data
The following materials are available in the online version of
this article.

Supplemental Figure S1. Alignment of Arabidopsis
SLDP1 and SLDP2 protein isoforms.
Supplemental Figure S2. Charge plots of SLDP.
Supplemental Figure S3. qPCR analysis of SLDP splice
variants.
Supplemental Figure S4. qPCR analysis of sldp mutant
lines.
Supplemental Figure S5. Images of sldp mutants.
Supplemental Figure S6. Time-course analysis of sldp and
lipa mutant line LDs in hypocotyls.
Supplemental Figure S7. Analysis of storage vacuoles.
Supplemental Figure S8. Time-course analysis of sldp and
lipa mutant line LDs in hypocotyls.
Supplemental Figure S9. Transmission electron micros-
co py of seedlings.
Supplemental Figure S10. Proteomic analyses of sldp
mutants.
Supplementary Figure S11. In silico analysis of LIPA
Supplementary Figure S12. Localization analysis of LIPA in
leaves.
Supplementary Figure S13. Co-expression analysis of SLDP
and LIPA in tobacco pollen tubes.
Supplementary Figure S14. Coiled-coil mutants of LIPA.
Supplementary Figure S15. Y2H assays to test for SLDP and
LIPA self-interaction.
Supplementary Figure S16. Analysis of the MD
simulations.
Supplementary Figure S17. qPCR analysis of LIPA mutant
lines.

Supplemental Data Set S1. Sequence information.
Supplemental Data Set S2. Raw LFQs from proteomic
analyses of LD-enriched and TE fractions of wild-type and
sldp mutant seedlings.
Supplemental Data Set S3: Normalized and filtered LFQs
from proteomic analyses of LD-enriched and TE fractions of
WT and sldp mutant seedlings.
Supplemental Data Set S4. Imputed rLFQs from proteo-
ic analyses of LD-enriched and TE fractions of WT and
sldp mutant seedlings.
Supplemental Data Set S5. List of constructs and
primers.
Supplemental Data Set S6. List of LD-enriched proteins
in WT and sldp mutant seedlings.
Supplemental Data Set S7. Proteomic metadata table.
Supplemental Movie S1. LD movement in pollen tubes
expressing mVenus.
Supplemental Movie S2. LD movement in pollen tubes
expressing mVenus-LIPA.
Supplemental Movie S3. LD movement in pollen tubes expressing SLDP2.1-mVenus.
Supplemental Movie S4. LD movement in pollen tubes co-expressing mVenus-LIPA and SLDP2.1-mVenus.
Supplemental Movie S5. LD movement in pollen tubes expressing SLDP1.3-mVenus.
Supplemental Movie S6. LD movement in pollen tubes co-expressing mVenus-LIPA and SLDP1.3-mVenus.
Supplemental Movie S7. LD movement in pollen tubes co-expressing untagged LIPA and SLDP2.1.
Supplemental Movie S8. LD movement in pollen tubes co-expressing mVenus-LIPA and SLDP1.3.
Supplemental Movie S9. Simulation of LIPA membrane interaction.

Acknowledgments

We would like to thank Julia Matz for help in the lab, the department of Dr Peter Rehling for granting access to their microscope and Karen Linnemann-Stöns for helpful discussions.

Funding

HEK, OV, KS, MW, GB, and TI thank the German research foundation (DFG; Grants IS 273/2-2, IS 273/7-1, IS 273/10-1, IRTG 2172 ProTECT to Till Ischebeck and Marcel Wiermer, BR1502-15-1, and INST 186/1230-1 FUGG to Stefanie Pöggeler, INST 186/1277-1 FUGG to Volker Lipka). PS thanks the Studienstiftung des deutschen Volkes for funding. Research in RTM’s lab was supported by grants from the US Department of Energy, Office of Science, BES-Physical Biosciences program (DE-SC0016536), and the Natural Sciences and Engineering Research Council of Canada (RGPIN-2018-04629). NMD is a recipient of an Ontario Graduate Scholarship.

Conflict of interest statement. None declared.

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