Sphingolipids Containing Very-Long-Chain Fatty Acids Define a Secretory Pathway for Specific Polar Plasma Membrane Protein Targeting in *Arabidopsis*

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Sphingolipids Containing Very-Long-Chain Fatty Acids Define a Secretory Pathway for Specific Polar Plasma Membrane Protein Targeting in Arabidopsis

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Abstract
Sphingolipids are a class of structural membrane lipids involved in membrane trafficking and cell polarity. Functional analysis of the ceramide synthase family in Arabidopsis thaliana demonstrates the existence of two activities selective for the length of the acyl chains. Very-long-acyl-chain (C > 18 carbons) but not long-chain sphingolipids are essential for plant development. Reduction of very-long-chain fatty acid sphingolipid levels leads in particular to auxin-dependent inhibition of lateral root emergence that is associated with selective aggregation of the plasma membrane auxin carriers AUX1 and PIN1 in the cytosol. Defective targeting of polar auxin carriers is characterized by specific aggregation of Rab-A20– and Rab-A15–labeled early endosomes along the secretory pathway. This aggregates correlate with the accumulation of membrane structures and vesicle fragmentation in the cytosol. In conclusion, sphingolipids with very long acyl chains define a trafficking pathway with specific endomembrane compartments and polar auxin transport protein cargoes.

Introduction
Sphingolipids are essential for eukaryotic life (Holthuis et al., 2001). Current theory suggests this is due, at least in part, to their role in protein sorting and secretion. Evidence indicates that, within the diverse membrane composition of the Golgi body, sphingolipids coalesce into microdomains or lipid rafts where, together with cholesterol and saturated sphingolipids, they attract a unique subset of proteins and together are transported to the plasma membrane (PM; Klemm et al., 2009). In animal epithelial cells, this property of sphingolipids is exploited to maintain cell polarity through the regulation of vesicle trafficking and endocytosis at the apical membrane (Maier and Hoekstra, 2003; Nyasae et al., 2003). Sphingolipid sterol–rich microdomains are similarly recruited in the budding yeast Saccharomyces cerevisiae to establish cell polarity during mating and budding (Bagnat and Simons, 2002).

The ability of sphingolipids to form microdomains may be attributed to their unique physical properties compared with the glycerolipids. Sphingolipids consist of three primary components: an acyl amino alcohol or long-chain base (LCB), a fatty acid attached via the amino group, and a head group attached to carbon-1 (C1) of the LCB. Additional hydroxyl groups at C2 on the fatty acid and C4 on the LCB promote hydrogen bonding between sphingolipids that is not available to glycerolipids (Pascher, 1976). Furthermore, the fatty acid component of sphingolipids often consists of a saturated or monounsaturated very-long-chain fatty acid (VLFA) of >18 carbons and up to 26 carbons in length (C26). The presence of VLCFA in sphingolipids increases their hydrophobicity, membrane leaflet interdigitation, and the transition from a fluid to a gel phase, which is a requirement for microdomain formation. This important property of VLCFA in membrane organization is supported by the observation that S. cerevisiae mutants unable to synthesize sphingolipids can be rescued by the SLC1-1 mutation, which allows for the transfer of C26 fatty acids to the sn-2 position of glycerolipids. C22 fatty acids are unable to support the functions performed by C26 fatty acids, indicating the sensitivity of the system to fatty acid chain length (Gaigg et al., 2005, 2006). As a result, yeast mutants in which fatty acid elongation beyond C16 is abolished are not viable (Oh et al., 1997).

The incorporation of fatty acids into sphingolipids is catalyzed by the enzyme ceramide synthase (sphinganine N-acetyltransferase). Ceramide synthases are encoded by the LAG1 gene family (named after longevity assurance gene 1); members of which have been found in all eukaryotes so far examined from fungi to animals and plants (Winter and Ponting, 2002). In animals, several ceramide synthases have been characterized (CERS1-6) and shown to have different substrate specificities with respect to the length of the acyl chain of the fatty acid (Riebeling et al., 2003; Mizutani et al., 2005, 2006). S. cerevisiae contains two LAG1 family mem-
bers, LAG1 and LAC1, and deletion of both of these genes causes strongly reduced cell growth, cell wall defects, and delayed endoplasmic reticulum (ER)-to-Golgi transport of glycosylphosphatidylinositol-anchored proteins (Barz and Walter, 1999; Guillais et al., 2001; Schorling et al., 2001). These phenotypes can be complemented by homologous genes from Homo sapiens, Caenorhabditis elegans, and Solanum lycopersicum (Jiang et al., 1998; Spassieva et al., 2002), indicating that LAG1 homologs serve as ceramide synthases. Recent studies on the role of the mammalian ceramide synthase CERS2 indicate that it is responsible for the incorporation of the majority of VLCFAs into the sphingolipids of the liver and brain (Imgrund et al., 2009; Pewzner-Jung et al., 2010). Interestingly, the alteration in sphingolipid profile resulting from CERS2 disruption bears some resemblance to that obtained when challenged by the ceramide synthase inhibitor fumonisin B1 (FB1), suggesting that FB1 may specifically inhibit the incorporation of VLCFA into sphingolipids, thereby mimicking the disruption of CERS2 activity (Pewzner-Jung et al., 2010).

Recent studies have identified several mutants of acyl-CoA elongation in plants that show phenotypes that have been attributed to depletion of VLCFA in sphingolipids. These mutants include cer10 (Zheng et al., 2005), gurke/pasticcino3 (pas3; Baud et al., 2004), pas2 (Bach et al., 2008), and pas1 (Roudier et al., 2010). In all these mutants, the level of VLCFA in sphingolipids is reduced, and this reduction is coupled with important morphological changes in the plant. In the cer10 mutant, which is deficient in elongation-specific enoyl reductase, endosomal compartments were shown to accumulate, indicating abnormal vesicle trafficking (Zheng et al., 2005). In the case of the pas1 mutant, abnormal trafficking of the auxin polar efflux carrier PIN1 was associated with decreased VLCFA in sphingolipids (Roudier et al., 2010). PIN1 is one of several proteins with a polar localization within the root tissues of Arabidopsis thaliana responsible for forming auxin gradients that in turn control root elongation and lateral root formation. While VLCFAs are involved in other metabolic processes in plants, such as wax biosynthesis or seed storage lipid biosynthesis, defects in neither of these lead to the extreme defects in plant and seed morphology in these elongase mutants leaving sphingolipids as the prime candidate.

Several lines of evidence suggest that sphingolipid cerol-rich microdomains exist in plant membranes and that they may be involved in the polar targeting of proteins to the PM (Fischer et al., 2004). However, the precise role of sphingolipids in such processes has only been tangentially addressed in plants. As in animals, plant sphingolipids are a complex family of lipids with various acyl chain lengths, LCBs, and head groups moieties, although the specific role of each moiety appears to be evolutionarily distinct from LOH1 and LOH3. In Arabidopsis, the LOH1 homologs have different acyl chain substrate specificities and that VLCFA sphingolipids are essential for plant development. By combining genetic and pharmacological approaches, we show that VLCFA-containing sphingolipids are required for organogenesis and, more specifically, for auxin-dependent lateral root outgrowth. Finally, we demonstrate that the depletion of ceramides led to defective secretory targeting of AUX1 and PIN1 to the PM by altering the Rab-A2a early endosomal pathway, suggesting that VLCFA sphingolipids define specific endomembrane compartments for targeting specific polar cargo proteins.

Results

Identification of Genes for Ceramide Synthase in Arabidopsis

The first ceramide synthase identified from plants is the tomato gene Asc-1 (for Alternaria stem cancer resistance-1) that gives resistance to the ceramide synthase inhibitor FB1 (Brandwaagt et al., 2000; Spassieva et al., 2002). A tBLASTn search of the completed Arabidopsis genome identified three Asc-1/LAG1 homologs in Arabidopsis, At3g25540, At3g19260, and At1g13580, subsequently referred to as LOH1, LOH2, and LOH3, respectively (LAG One Homologue). A potential fourth homolog (At1g26200) was also identified; however, it contains some highly variable sequences not found in any other LAG1 homologs that may suggest a different function yet to be discerned (see Supplemental Figure 1A). The protein sequences of the identified LAG1 homologs were aligned with the known full-length proteins from tomato and the LAG1 and LAC1 proteins from yeast to identify conserved structural features (Figure 1A). The alignments show strong conservation in the LAG1p motif and predicted transmembrane helices, suggesting that the LOH genes encode ceramide synthases. A phylogenetic tree derived from an alignment of all known full-length protein sequences of the eudicot plant LAG1 homologs along with the LAG1/LAC1 proteins from yeast and the CerS proteins from human was constructed (see Supplemental Figures 1A and 1B and Supplemental Data Set 1). The tree showed that LOH1 and LOH3 are closely related and clustered into a group along with the majority of other plant LAG1 homologs (see Supplemental Figure 1B). On the other hand, LOH2 appears to be evolutionarily distinct from LOH1 and LOH3. In order to investigate the function of the LOH genes, genetic disruptions were characterized for each gene.

Arabidopsis LAG1 Homologs LOH1 and LOH3 Are Essential for Plant Growth

T-DNA insertion mutants were identified from the Wisconsin (Krysan et al., 1999) (loh1-1, loh2-2, and loh3-1) and Salk (Alonso et al., 2003) (loh1-2, loh2-2, and loh3-2) collections for each of the three Arabidopsis LOH genes (Figure 1B). Both alleles loh1-1 and loh3-1 are insertions in the 5′-untranslated region of their respective genes (at −3 and −10 bp from the ATG Codon).
start, respectively) leading to knockdown of wild-type mRNA levels (see Supplemental Figure 2A). All other mutants are caused by insertions in an exon of their respective genes and result in undetectable wild-type mRNAs and are therefore true knockouts (see Supplemental Figures 1A and 1B).

Despite this, each single mutant showed a normal phenotype under standard growth conditions (see Supplemental Figure 2C). Similarly, combinations of double mutants between loh2 and loh1 or loh3 null alleles did not show any significant differences of growth or development (see Supplemental Figure 2C). By contrast, the cross between the knockout alleles loh1-2 and loh3-2 resulted in ~1:16 seeds that were darker and smaller with a poor germination rate in the F2 progeny (n = 300). This phenotype was confirmed in the F3 progeny of the sesqui-mutant combinations loh1-2+/− loh3-2+/− (n = 96) or loh1-2+/− loh3-2−/− (n = 79), which pro-
duced 18 and 12% abnormal seeds, respectively (Figure 1C). The embryo morphology in these seeds was often strongly altered with thicker hypocotyls and a single cotyledon (Figure 1C). No double loh1-2 loh3-2 mutant was recovered from seeds sown on soil, suggesting that the combination of mutations is lethal. However, when sown on solid media containing Suc, seedlings of the loh1-2+/− loh3-2+/− genotype could sometimes be recovered, albeit after a prolong incubation of several weeks. The resulting seedlings showed severely deformed plants with no roots and a deformed leaf shape that did not further develop, indicating that LOH1 and LOH3 are essential for root growth (Figure 1D). Despite transfer to fresh media, none of the seedlings were able to survive longer than 4 weeks.

As the loh1-2 loh3-2 double mutant is lethal, the cross between the knockdown alleles loh1-1 and loh3-1 was also examined. Like the loh1-2 loh3-2 seeds, loh1-1 loh3-1 seeds showed reduced viability, and those that did germinate gave rise to seedlings with highly variable phenotype ranging from triple cotyledon, single deformed cotyledon, or undeveloped cotyledon (Figure 1E). Plants with relatively normal development showed smaller, wrinkled leaves, early senescence, delayed flowering, and reduced primary bolt length (Figure 1F; see Supplemental Figures 2G and 3D). Embryos dissected from seeds that did not germinate showed very strong alteration of cotyledon morphology with often single cotyledon embryos (Figure 1E) similar to the phenotypes observed in the progeny of sesqui-mutant combinations. To confirm that these phenotypes were indeed caused by the loss of LOH1 and LOH3 function, native LOH1 and LOH3 genes were transformed into the loh1-1 loh3-1 or sesqui loh1-2 3-2+/− mutants under the control of the native promoter (see Supplemental Figures 3A and 3E). Plants with a wild-type phenotype were recovered upon transformation with either LOH1 or LOH3 (see Supplemental Figures 3B to 3D), indicating that the mutations in the LOH1 and LOH3 genes are responsible for the phenotypes observed in each of the double mutants. Overall, these data demonstrate that, out of the three different LOH genes, LOH1 and LOH3 are redundant and essential for plant growth, while LOH2 has no obvious effect on plant development, indicative of a different role for LOH2 versus LOH1 and LOH3 in sphingolipid metabolism.

**LOH Genes Encode Ceramide Synthases with Different Specific Activities**

To investigate the role of the different LOH genes in sphingolipid metabolism and the biochemical basis for the phenotypes of the loh mutants, a complete sphingolipid analysis was performed for each line (Markham and Jaworski, 2007). For the lethal loh1-2 loh3-2 double mutant, sphingolipid analysis performed on 2-week-old germinated seeds grown on solid media showed that all the classes of sphingolipids from these mutants were almost completely devoid of VLCFA (Figure 2A; see Supplemental Figure 4) and instead contained mostly 16:0 fatty acids. To confirm these results, sphingolipid analysis was performed on seedlings from the knockdown loh1-1 loh3-1 double mutant. As expected, this mutant combination showed a less drastic reduction in VLCFA containing sphingolipids, ~30 mol % in total (Figure 2B; see Supplemental Figure 4A). Single loh1-2 or loh3-2 mutants had sphingolipid profiles almost identical to the wild type, indicating that LOH1 and LOH3 have redundant activities specifically targeted toward VLCFA sphingolipids (see Supplemental Figure 4A). The combined loss of LOH1 and LOH3 was also associated with a very strong accumulation of saturated free LCBs consistent with reduced ceramide synthase activity in these mutants (Figure 2C). Free LCBs also accumulated in the loh1-1 loh3-1 mutants, albeit less pronounced than for loh1-2 loh3-2 (see Supplemental Figure 4B).

Because disruption of LOH1 and LOH3 leads to an almost complete absence of VLCFA in sphingolipids, this suggests that the remaining ceramide synthase in these plants, LOH2, must be specific for 16:0. In support of this, sphingolipid profiling of loh2-1 and loh2-2 seedlings showed a strong reduction of 16:0 fatty acid in ceramide and other sphingolipids and a significant increase in VLCFA containing sphingolipids (Figure 2A; see Supplemental Figure 4A). Altogether, these results demonstrate the existence of at least two different ceramide synthase activities in Arabidopsis: LOH1 and LOH3 are specific for VLCFA, while LOH2 is specific for 16:0 fatty acids. Interestingly, disruption of LOH1 and LOH3 leads to high accumulation of free LCBs, similar to what was seen when CERS2 is disrupted in mice (Pewzner-Jung et al., 2010) and reminiscent of the effect of ceramide synthase inhibitors such as FB1 and AAL toxin (Abbas et al., 1994).

**FB1 Mimics the Disruption of VLCFA-Specific Ceramide Synthases**

FB1 and AAL toxins are sphingoid-base analog mycotoxins that have a strong and specific inhibitory effect on ceramide synthases (Merrill et al., 1993; Spassieva et al., 2002). Previous studies in animal systems have suggested that FB1 may be selective in its inhibition of ceramide synthase activity because treatment with FB1 changes the profile of fatty acids incorporated into sphingolipids (Venkataraman et al., 2002). The effect of 0.5 μM FB1 on the sphingolipid profile of Arabidopsis seedlings was examined (Figure 2D). Counterintuitively, after 9 d of growth on FB1, Arabidopsis seedlings contained higher amount of total sphingolipids than control plants, but the majority of the extra sphingolipids consisted of 16:0 species with a decrease in VLCFA containing species (Figure 2D). Exposure to FB1 for 24 h or less did not cause excessive 16:0:ceramide to be synthesized; however, a small, but significant, reduction in VLCFA-containing ceramide was noticeable by 16 to 24 h (see Supplemental Figure 5A). Inhibition of ceramide synthase by FB1 was detectable even after short exposure to FB1, judging by the rapid accumulation of the free trihydroxy (t) LCBs t18:0 after 4 h of treatment (see Supplemental Figures 5B and 5C). Accumulation of t18:0 and the dihydroxy (d) d18:0 was directly correlated with the time of exposure to FB1 with a maximum at 8 and 16 h for t18:0 and d18:0, respectively. Application of FB1 for 16 h and longer was saturating as shown by free LCB levels (see Supplemental Figure 5B). The effect of FB1 was also dose dependent, as shown by the analysis of total LCB hydrolyzed from sphingolipids after 9 d exposure to 0.4 and 0.8 μM FB1 (see Supplemental Figure 5D). Together, these data indicate that FB1 exposure rapidly inhibits its ceramide synthase, elevating free LCB levels and depleting VLCFA-containing ceramides. Eventually, free LCBs are rerouted into 16:0-ceramides similar to the disruption of sphingo-
lipid metabolism seen in the loh1 loh3 double mutants. As expected, knockdown loh1-1 loh3-1 double mutants treated with FB1 showed stronger depletion of VLCFA sphingolipids and enhanced accumulation of 16:0 sphingolipids, confirming the specific targeting of LOH1 and LOH3 ceramide synthases by FB1 (see Supplemental Figure 5E).

Wild-type seedlings germinated for 9 d on FB1 concentrations of 1 μM or higher showed global inhibition of growth (Figure 3A). At 0.5 μM FB1, primary root length was only moderately reduced (30% reduction compared with untreated control), but a strong reduction of lateral root emergence could be observed on 1 μM FB1 with 80% reduction compared with untreated control (Figure 3B). Likewise, knockdown loh1-1 loh3-1 mutants, displaying abnormal cotyledon symmetry, also showed a 60% reduction in lateral root numbers compared with wild-type controls, as did the two different combinations of sesqui-mutants involving the loh1-2 loh3-2 alleles (loh1-2−/− loh3-2−/− or loh1-2−/− loh3-2−/−) (Figure 3C; see Supplemental Figure 2D). The weakest mutant phenotype could be mimicked by decreasing FB1 concentration to 0.25 μM, leading to 50% inhibition of lateral root growth (Figure 3B). FB1 application reduced lateral root primordia outgrowth but not the initiation stages as detected by the auxin reporter DR5:β-glucuronidase (GUS) (see Supplemental Figure 2E). Lateral root formation and growth is known to be very dependent on auxin, suggesting that the strong morphological changes observed in the loh1/loh3 mutants could be related to an altered auxin physiology. Interestingly, the pas1 mutant has reduced VLCFAs in sphingolipids and showed auxin-related inhibition of lateral root formation (Roudier et al., 2010). Primary root growth of pas1 was found to be more sensitive to FB1 compared with the wild type, supporting the involvement of VLCFA sphingolipids in auxin-dependent lateral root formation (see Supplemental Figure 6A).

**Very-Long-Chain Ceramides Are Required for Polar Auxin Distribution**

In order to analyze the effect of sphingolipid composition on auxin distribution, we analyzed DR5:GUS expression in roots grown in presence of FB1 (Swarup et al., 2008). DR5:GUS ex-
Expression was reduced in root tips of seedlings grown for 9 d in the presence of 1 μM FB1 (see Supplemental Figure 2F) or simply treated with 2.5 μM FB1 for 16 h (Figure 3D). To check if auxin was directly involved in the root response to inhibition of ceramide synthase, we tested the effect of the permeable auxin analog naphthalene acetic acid (NAA) on lateral root development of FB1-treated seedlings and loh1-1 loh3-1 double mutants. In both cases, NAA was able to restore lateral root outgrowth, suggesting that auxin transport and not auxin response was most likely impaired in FB1-treated roots (Figures 3E and 3F).

To investigate the role of VLCFA-containing sphingolipids in auxin transport, the transport of radiolabeled auxin along stem segments of wild-type and loh1-1 loh3-1 mutants was measured. Stem segments of 2.5 cm in length were incubated for 16 h in the dark with the top/flower end (basipetal) or bottom/rosette end (acropetal) in a 20-μL solution of 14C-labeled indole-3-acetic acid, the native auxin. The basipetal or acropetal auxin movements were subsequently quantified by measuring the amount of labeled auxin in the distal 5 mm along the stem. Controls for ruling out possible diffusion or metabolism of indole-3-acetic acid were performed by including 10 μM naphthalenic acid (NPA), an inhibitor of polar auxin transport. As expected, labeled auxin showed significant, NPA-sensitive, basipetal transport along the different sections of the wild-type stem (Figure 3G). In the loh1-1 loh3-1 mutant, the auxin...
flux was strongly affected compared with the wild type with the amount of auxin transported reduced by 50 to 75% along the stem (Figure 3G). Together, these data show that polar auxin transport is impaired by the reduction in VLCFA in the loh1-1 loh3-1 mutant or by the inhibition of ceramide synthase in the FB1-treated plants.

**Sphingolipid Synthesis Is Required for the Targeting of Specific Auxin Carriers to the PM**

Polar auxin transport is mediated through a family of PM polar auxin transporters involved in cellular influx (AUX1 family members) or efflux of auxin (PIN family members). Polar localization of auxin transporters was thus investigated in the context of sphingolipid depletion either in the loh1 loh3 mutant or in FB1-treated seedlings. To minimize possible artifactual response at the cellular level induced by long FB1 treatment, we designed a shorter FB1 treatment leading to ceramide synthase inhibition. We found that the application of 2.5 μM FB1 for 16 h (subsequently referred as FB1-16h) led to an accumulation of 118:0 and d18:0 that was identical to that induced by 9 d of treatment with 0.5 μM (see Supplemental Figure 5B). Since FB1 was described to induce cell death at high concentration, we verified that our short FB1 treatment was not impairing cell viability. We monitored cell viability in both primary and lateral roots using the fluorodiniacetate (FDA) test, which labels viable cells by monitoring fluorescence release from ester bond hydrolysis of apolar FDA. In our conditions, FB1-treated roots were still showing fluorescence labeling, demonstrating their viability (see Supplemental Figure 6B). This experimental setting allowed the study of cellular responses in lateral root tip, which was found to be more sensitive to ceramide synthase inhibition than the primary root (Figures 3A and 3B).

We first analyzed AUX1-yellow fluorescent protein (YFP) distribution since aux1 mutants showed reduced lateral root development similar to FB1-treated plants (Marchant et al., 2002). FB1 treatment for 16 h led to an accumulation of pAUX1:AUX1-YFP fluorescence inside meristematic, protophloem, and epidermal cells of lateral roots (Figures 4A and 4B). Similar aggregation of AUX1-YFP was observed in the primary root of sesqui loh1-2/-loh3-2/- mutants but not in the weak loh1-1 loh3-2 (Figure 4C). PM targeting of AUX1-YFP was also impaired in lateral root primordia of loh1-2/-loh3-2/- (see Supplemental Figure 7A). Interestingly, FB1 treatment led to partial relocation of AUX1-YFP into lateral membrane of epidermal and protophloem cells (Figures 4A and 4C, inset, arrows). We also investigated whether inhibition of ceramide synthase would have a similar effect on the subcellular distribution of LAX3-green fluorescent protein (GFP), since this AUX1-like influx carrier was described to be also essential for lateral root outgrowth (Swarup et al., 2008). But contrary to AUX1-YFP, FB1 did not modify LAX3-GFP targeting to the PM (see Supplemental Figures 7B and 7C).

We then checked the effect of FB1-16h treatment on the subcellular distribution of auxin efflux carriers in protophloem and epidermal cells. Like AUX1-YFP, PIN1-GFP was found to aggregate both in vascular and root meristem cells of lateral roots in presence of FB1 or in the most affected primary root of the weak loh1-1 loh3-1 mutant (Figure 4D; see Supplemental Figures 8A and 8B). In the most severe cases, PIN1-GFP or PIN1 immunolocalization labeling showed PIN1 almost exclusively in cytosolic aggregates and no or only a weak signal could be detected at the PM (Figure 4D; see Supplemental Figure 8C). However, subcellular distributions of other efflux carriers, such as the ABC1-GFP and ABC19-GFP auxin pumps, were not affected by FB1-16h treatment (Figures 4E and 4F). The auxin efflux carrier PIN2-GFP was also insensitive to FB1 (see Supplemental Figure 7D); however, this was unrelated to its epidermal localization because PIN1-GFP expression in the epidermis led to aggregates as in the stele (see Supplemental Figure 7E). We also checked PM proteins not involved in auxin transport, such as the nonpolar LT16b-GFP and PIP2-GFP fusion proteins. As for ABCs, PIN2, or LAX3 proteins, the localization of these markers was insensitive to FB1 treatment (see Supplemental Figures 7F and 7G). Altogether, these results indicate that ceramide synthase inhibition modified membrane targeting of specific protein cargoes. Interestingly, even if AUX1 and PIN1 are targeted to different sites within the PM, both proteins gathered partially into the same or closely associate aggregates upon FB1 application (Figure 4G; see Supplemental Figure 8C).

Inactivation of LOH activity led not only to decreased VLCFA-containing ceramide and sphingolipid contents but also to the accumulation of free LCBs. To exclude a possible role of free LCBs in the aggregation of AUX1 and PIN1 proteins, we treated Arabidopsis AUX1-YFP and PIN1-GFP seedlings with myriocin, an inhibitor of serine palmitoyltransferase, the first step of sphingolipid synthesis, leading to the depletion of both free LCBs and ceramides (Miyake et al., 1995). Like FB1, myriocin treatment led to similar accumulation of AUX1-YFP in cytosolic aggregates, demonstrating that inhibition of de novo sphingolipid synthesis and not LCB accumulation was responsible for the specific mistargeting of protein cargoes (see Supplemental Figure 8D). In conclusion, our results showed that inhibition of sphingolipid synthesis impaired normal trafficking of AUX1-YFP and PIN1-GFP proteins in the protophloem, meristem, and epidermal root cells, explaining the reduction of polar auxin transport and auxin-dependent lateral root outgrowth.

**Inhibition of Sphingolipid Synthesis Disrupts Early Endosomes**

Inhibition of ceramide synthase induced the aggregation of membrane and proteins in a structure that could be referred to as FB1 compartment by analogy with compartments induced by the anterograde transport inhibitor brefeldin A (BFA). We therefore investigated the different endomembrane markers recruited in the formation of these compartments. GFP marker lines for different endomembrane compartments were tested for their sensitivity to FB1. First, we investigated the effect of FB1 on the ER, since it is the site of de novo LCB, ceramide, and probably glucosylceramide biosynthesis (Marion et al., 2008). We examined the distribution of the ER markers YFP-NIP1 and AXR4-GFP. AXR4-GFP was of special interest since axr4 mutant aggregated AUX1 into cytosolic compartments, blocking its targeting to the PM (Dharmasiri et al., 2006). No obvious ER containing aggregates could be observed upon FB1-16h treatment for both markers, although enhanced AXR4-GFP signal was often observed in the perinuclear region (Figure 5A). We then evaluated the localization
of several markers labeling the ER/cis-Golgi and Golgi apparatus, but none of them showed any significant aggregation in presence of FB1 (Figure 5). By contrast, the early endosomal markers YFP-Rab-A2α and YFP-RabA1α were clearly aggregated by FB1-16h treatment (Figure 5). The RabA2α compartment extensively overlapped with the trans-Golgi network (TGN)-resident VHAa1 (Chow et al., 2008). As expected, partial colocalization of YFP-RabA2α and VHAa1-RFP could also be observed in FB1-induced aggregates (Figure 5B). Endosomal subpopulations labeled by YFP-ARA7/RabF2β or SNX1-GFP, defined as late endosomes, were insensitive to FB1 application. Similarly, late endosome vacuolar YFP-RabG3Cβ or YFP-RabG3Fβ or specific vacuolar marker YFP-VAMP711 were also insensitive to FB1 (Figure 5A; see Supplemental Figure 8E). Altogether, these results indicated that inhibition of ceramide synthase modified subcellular distribution only of the early endosomal/TGN resident markers, suggesting the involvement of sphingolipid synthesis in the maintenance of specific endomembrane compartments.

The modifications of endomembrane structures observed with GFP markers were confirmed by transmission electron microscopy. The organization of the Golgi apparatus as well as the mitochondria and plastids were not modified by FB1-16h treatment and by loh1-1 loh3-1 mutations (Figure 6; see Supplemental Figures 9A and 9B). The ER network was clearly visible, and it seemed to be larger and more branched, an observation that could correlate with higher fluorescence of ER markers after FB1 treatment. The most dramatic changes were the presence of small vesicular structures looking like dismantled vacuole or fused vesicles (Figure 6D). Since fluorescent vacuolar markers did not show major macroscopic changes in vacuolar structure while early endosomal markers formed large aggregates, these accumulations of membrane materials could result from fusion of endosomal compartments.

**Sphingolipid Synthesis Is Not Required for Endocytosis**

AUX1-YFP and PIN1-GFP aggregates in the cytosol most probably result from altered TGN and/or early endosomal trafficking. However, PIN1 and AUX1, apart from being localized in the opposite side of the cell, are characterized by different dynamics with PIN1 undergoing active recycling, while AUX1 showed a slower turnover at the PM (Kleine-Vehn et al., 2006). We thus reasoned that the aggregation of both proteins upon ceramide synthesis inhibition would preferentially result from secretory pathway impairment rather than

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**Figure 4.** Inhibition of Ceramide Synthesis Alters the AUX1-YFP and PIN1-GFP Targeting in Lateral Roots.

(A) and (B) AUX1-YFP aggregates (arrowheads) in the cytosol in presence of 2.5 μM FB1 for 16 h (A). Detail of untreated (left, −) and treated lateral root cells (right, +). Details of epidermal cells are given in insets. Details of protophloem region of untreated and treated lateral roots are shown in (B).

(C) AUX1-YFP aggregates (arrowheads) in the cytosol of meristematic and protophloem cells in the primary root of sesqui-mutants loh1-2−/− loh3-2−/− (right) compared with the wild type (wt; left).

(D) PIN1-GFP aggregates (arrowheads) in the cytosol of FB1-treated cells (bottom, +) compared with control (top, −). PIN1 aggregates were also confirmed by immunolocalization (anti-PIN1). Note that FB1 treatment depleted PIN1 from PM.

(E) ABC1-GFP expression in untreated (left, −) and FB1-treated cells (right, +).

(F) ABC19-GFP expression in untreated (left, −) and FB1-treated cells (right, +).

(G) AUX1-YFP (red) and PIN1-GFP (green) distribution in absence (left, −FB1) and in presence of FB1 (right, +FB1). Merged images are also shown. AUX1-YFP and PIN1-GFP aggregates colocalize partially (arrowheads).

Bars = 20 μm in (A), (F), and (G) and 10 μm in inset of (A) to (E) and (H).
PM recycling. To first evaluate the involvement of recycling membrane material in ceramide-depleted endomembrane pools, we checked whether endocytosed membrane material was associated with FB1 compartments. Early endosomes like Rab-A2a rapidly colocalized with endocytosed FM4-64 (Chow et al., 2008). However, when treated with FB1, the aggregated YFP-Rab-A2a-labeled endosomes showed close proximity with FM4-64 but did not seem to colocalize, indicating that endocytosis provides only a minor contribution to the formation of FB1 compartments (see Supplemental Figure 10A). This was confirmed by comparing BOR1-GFP internalization upon boron application in presence or not of FB1-16h treatment (Takano et al., 2005). BOR1-GFP–labeled endosomes appeared with similar kinetics in the presence or absence of FB1, indicating that endocytosis was not severely impaired by FB1-16h treatment (see Supplemental Figure 10B). The origin of the FB1 compartment was directly probed using point mutations in Rab-A2a that modify its subcellular distribution (Chow et al., 2008). To rule out possible interaction of GTPase activity with FB1 treatment, we monitored the subcellular distribution in a YFP-Rab-A2a[N125I] mutant that is a nucleotide-free dominant-negative mutant that has the same subcellular distribution as wild-type YFP-Rab-A2a but lower expression levels. YFP-Rab-A2a[N125I] was as sensitive to FB1 as wild-type YFP-Rab-A2a, demonstrating that FB1 sensitivity was independent of GTPase ac-

Figure 5. Inhibition of Ceramide Synthase Affects Specific Endomembrane Compartments.
Figure 5. Inhibition of Ceramide Synthase Affects Specific Endomembrane Compartments.
(A) Fluorescence micrographs showing the subcellular localization of different endomembrane marker-XFP fusions in the lateral roots of untreated transgenic lines (−FB1) or after treatment with FB1 (+FB1). The subcellular compartment to which the marker has previously been localized is annotated far right or left, and the marker-XFP fusion being examined is shown in the top left of each set of micrographs. Note the aggregations under FB1 treatment for the early endosome markers (arrowheads). Bars = 5 μm.
(B) TGN marker VHAa1-RFP partially colocalizes with YFP-RabA2a in the presence of FB1. Bars = 5 μm.
Then, we analyzed the S26N or Q71L mutations that stabilize YFP-Rab-A2a into the inactive GDP-bound state or the constitutively active GTP-bound state. These mutations relocate YFP-Rab-A2a mainly to the Golgi for YFP-Rab-A2a[S26N] and the PM for YFP-Rab-A2a[Q71L]. The Golgi YFP-RabA2a[S26N] was still sensitive to FB1 by forming aggregates, while the PM-localized YFP-Rab-A2a[Q71L] was insensitive, demonstrating that FB1 aggregates recruited YFP-Rab-A2a from the Golgi but not the PM (see Supplemental Figure 10C). Altogether, these results indicate that FB1-16h compartments are not formed from recycled PM materials.

**Sphingolipid Synthesis Is Required for the AUX1 and PIN1 Secretory Pathway**

The alternative pathway for FB1 compartments would be the secretory route. BFA inhibits anterograde transport, resulting also in the aggregation of early endosomes with the TGN to form BFA compartments (Ritzenhaller et al., 2002; Geldner et al., 2003). BFA was thus applied to FB1-treated PIN-GFP and AUX1-YFP roots stained with FM4-64. In vascular cells, AUX1-YFP and PIN1-GFP FB1-induced aggregates appeared to be distinct from BFA compartments, suggesting that FB1 impaired a distinct post-Golgi compartment.
than did BFA (Figures 7A to 7D; see Supplemental Figures 11A to 11D). To address the involvement of de novo synthesis in FB1 aggregates, we directly monitored aggregate formation through whole-cell fluorescence recovery after photobleaching (FRAP) analysis. We performed FRAP analysis of FB1-16h–treated PIN1-GFP and AUX1-YFP secondary root cells. FB1-16h–treated cells with PIN1-GFP localized in aggregates and at the PM were completely bleached (Figure 7E). Recovery of PIN1-GFP in aggregates was clearly visible after 30 min, and PM labeling was observed after aggregate recovery usually 30 to 45 min after bleaching. A similar finding was observed with AUX1-YFP, albeit with a reduced recovery (see Supplemental Figure 11E). FRAP analysis showed that FB1 delayed but also reduced PIN1-GFP recovery at the PM (Figure 7E, left). However, PIN1-GFP recovery in aggregates showed similar kinetics than the recovery of PIN1-GFP at PM in untreated samples (Figure 7E, right), indicating that the primary effect of FB1 is to block secretion by aggregating PIN1-GFP prior to its PM localization. Altogether, these results show that PIN1-GFP, like AUX1-YFP, aggregation occurs along the secretory pathway.

Finally, we investigated the involvement of VLCFA sphingolipids in protein secretion in the apoplast by analyzing the localization of tomato arabinoxylan fusion protein LeAGP1-GFP (see Supplemental Figure 11F). It was previously demonstrated that this fusion protein provided a robust marker for secretion in Arabidopsis (Estévez et al., 2006). FB1-16h treatment did not modify LeAGP1 accumulation in the apoplast, confirming that VLCFA sphingolipids are involved in the traffic of specific cargoes along the secretory pathway.

Discussion

Functional analysis of Arabidopsis LAG1 homologs 1 through 3 demonstrated that the three LOH genes encode ceramide synthases. The three LOH proteins showed high sequence identity with the yeast LAG1p and LAC1p but also with tomato Le Asc1, which was demonstrated to complement yeast lag mutants (Spassieva et al., 2002). Sphingolipid analysis of the different loh mutants demonstrated how the different LOH proteins contribute to ceramide biosynthesis and more specifically, the sphingolipid fatty acid chain length. Complete loss of LOH2 function led to specific reduction in C16-ceramide, whereas the absence of both LOH1 and LOH3 activity caused complete depletion of ceramides with an acyl chain longer than C18. Altogether, these results show that Arabidopsis possesses two different ceramide synthase activities. LOH2 encodes long-acyl-chain ceramide synthase, whereas LOH1 and LOH3 encode ceramide synthases with very-long-acyl-chain specificity. The role of the distinct LAG1 homolog At2g26200 in plant biology remains somewhat of an enigma as LOH1, 2, and 3 would appear to account for VLCFA and c16-specific ceramide synthase activity in leaf tissues. This functional distinction is supported by the phylogenetic analysis of plant LOH proteins, with LOH2 representative of a distinct clade. It will be interesting to see if other members of the LOH2 clade are also specific for C16-fatty acids and to dissect the amino acid differences between LOH2 and VLCFA-specific ceramide synthases. Similar
differences of acyl chain specificity were also described for mammalian ceramide synthase CERS (Teufel et al., 2009). CERS5 and 6, like LOH2, display a preference for long-chain acyl-CoA, while CERS2 or 4 is specifically involved in the synthesis of C22- and C22-C24-ceramides, respectively.

In yeast, both ceramide synthases are specific to very-long-chain ceramides with mainly C26 acyl chains (Guillas et al., 2001). However, this specificity can be modified by disrupting the enzymes responsible for VLCFA synthesis, namely, ELO2 and ELO3. Similarly, in the Arabidopsis pas3 mutant, elongation is affected by the reduced cytoplasmic acetyl-CoA carboxylase activity, and increased levels of C20 and C18 fatty acids are found in sphingolipids (Roudier et al., 2010). These observations indicate that there is a close relationship between the specificity of the ceramide synthase reaction and the acyl-CoA pool feeding that reaction; however, the nature of that relationship remains to be understood.

Disruption of LOH1 and LOH3 also has a marked affect on the total amount of sphingolipids. Free LCBs accumulate to high levels and drive the synthesis of excessive amounts of 16:0-containing sphingolipids. Similar findings were observed in the CERS2 knockout mouse, which is depleted in VLCFA sphingolipids but has overall normal sphingolipid content due to the accumulation of C16-sphingolipids (Pewzner-Jung et al., 2010). Likewise, the reduction of the VLCFA-CoA pool in the elongation mutants pas1 and pas3 led to an increase of C16-ceramides (Roudier et al., 2010). Interestingly, sphingoid base hydroxylases mutants that lacked t18:0 showed also enhanced C16-sphingolipids (Chen et al., 2008). In that article, a model was proposed whereby disruption of t18:0 biosynthesis caused d18:0 to accumulate, which drove the synthesis of C16:0-containing sphingolipids through a C16:0-dependent ceramide synthase. It was also suggested that t18:0-VLCFA–containing sphingolipids negatively regulate serine palmitoyltransferase activity, regulating the amount of sphingolipid that the cell synthesizes. This work further refines that idea by identifying the C16:0-dependent ceramide synthase as LOH2 and indicating that VLCFA-containing sphingolipids are capable of affecting total sphingolipid content.

Our work demonstrated that the length of the ceramide acyl chain has an important effect on the biological function of the sphingolipid molecules. While loss of LOH2 function did not lead to any obvious developmental phenotype, the loh1-2 loh3-2 double mutant was impaired in embryo development and germination, indicating that very-long-chain but not long-chain ceramides are critical for plant growth. Fine-tuning of ceramide synthase activity could be efficiently achieved with the sphingoid base analog FB1. FB1 is a mycotoxin from Fusarium moniliforme that inhibits ceramide synthase and is responsible for several naturally and experimentally induced animal and human diseases (Desai et al., 2002; Stockmann-Juvalala and Savolainen, 2008). FB1 was found to inhibit ceramide synthesis and to lead to free LCB accumulation (Abbas et al., 1994; Spassieva et al., 2002). Sensitivity to FB1 was also described to directly correlate with the activity of tomato LAG1 homolog Asc-1 (Brandwagt et al., 2000, 2002; Spassieva et al., 2002).

Here, we demonstrated that FB1 selectively inhibits very-long-acyl-chain ceramide synthesis in Arabidopsis. Moreover, our results demonstrate that, at the concentrations used, the most of the effects of FB1 can be attributed to the inhibition of 

de novo ceramide synthesis since many FB1 phenotypes were observed in the loh1 loh3 mutant. Interestingly, lateral roots were more sensitive to FB1 than primary roots. Contrary to primary roots that are of embryogenic origin, lateral roots are developed from nondividing pericycle cells and exhibit extensive plasticity associated with differential response to environmental cues, like phosphate or nitrate (Linkoehr et al., 2002; Jain et al., 2007). Lateral roots could thus be more sensitive to protein secretion at the PM to respond to these different environmental stimuli.

The use of FB1 to inhibit ceramide synthesis in a short window of time demonstrates the role of these lipids in the secretory pathway in particular for two specific cargoes, PIN1-GFP and AUX1-YFP. The normal expression and subcellular distribution of AXR4-GFP in the presence of FB1 indicates that the effect of sphingolipid depletion on AUX1-YFP targeting is independent of the AXR4 pathway. Interestingly, PIN1, but not AUX1, targeting was also impaired in VLCFA-defective mutants pas1 and 3 (Roudier et al., 2010). The fact that PIN1-GFP was more sensitive to BFA in pas1 mutant suggests that PIN1 recycling was selectively altered. The differences observed between pas1 and FB1 or loh1 loh3 plants could be explained by the nature of the VLCFA lipids involved since pas1 mutation impacts potentially all the VLCFA-containing lipids and even among sphingolipids pas1 was found to reduce more the levels of glucosyleramides than glucosylinositolphosphorylceramides (Roudier et al., 2010). PIN1 membrane localization was found to be dependent on sterol levels, as illustrated by polarity defects in sterol methyltransferase mutant smt1 (Willemsen et al., 2003). The role of sterols in AUX1 polarity is debated since conflicting results were obtained with smt1 (Willemsen et al., 2003; Kleine-Vehn et al., 2006). Nonetheless, the application of filipin, a specific sterol binding drug, was able to specifically reduce sterol levels at the PM and resulted in changes in AUX1 polar distribution and caused its cytosolic aggregation similarly to what was found with FB1 (Kleine-Vehn et al., 2006).

Interestingly, PIN2 polarity was also dependent on sterol levels since PIN2 localized to apical, basal, and lateral membranes in the sterol biosynthetic mutant cpi-1 (Men et al., 2008). In contrast with defects in sterol biosynthesis, inhibition of ceramide synthase did not seem to alter PIN2-GFP targeting, indicating a potentially differential sensitivity of the membrane proteins to the nature of their lipid environment. Similarly, we showed that FB1 treatment specifically altered the Rab-A2µ– and Rab-A1µ–labeled early endosome populations. The fact that both markers are targeted to the cell plate (Chow et al., 2008) indicates that VLCFA sphingolipids might directly impact cytokinesis, as recently suggested in BY2 cells (Aubert et al., 2011). The existence of lipid sorting at the TGN was recently demonstrated in yeast with specific enrichment of sphingolipids and sterols in post-Golgi vesicles transporting raft proteins (Klemm et al., 2009). Not surprisingly, PIN1 was found to be associated with detergent-resistant membrane fractions that are usually rich in sterols and sphingolipids (Mongrand et al., 2004; Borner et al., 2005; Titaipawanakun et al., 2009). Detergent-resistant membrane, enriched in sterol and sphingolipids, cofractionate with PM and Golgi but not with ER proteins (Lai et al., 2007). Inhibition of ceramide synthase did not seem to alter the ER and Golgi structure even though Arabidopsis ceramide synthases were found to localize in the ER (Marion et al., 2008). However, the reduction of ceramide syn-
thesis could impair the assembly of lipid domains in the ER or Golgi, leading to subsequent disorganization of specific endosomal populations.

Recently, FB1 was reported to induce ER-derived aggregates and to block ER to Golgi cargo delivery in BY2 cells (Aubert et al., 2011). The nature of the sphingolipids involved remain to be found, but the acyl chain length was found to be critical for polar targeting and trafficking and could not be compensated for by shorter chain ceramides. Importantly, very-long-chain ceramides were a determinant of protein cargoes and endosomal specificity. Very-long-chain fatty acyl chains were also described to be involved in polar auxin transport in Arabidopsis (Roudier et al., 2010). Reduction of the acyl chain length in particular in sphingolipids resulted in PIN1-GFP but not AUX1-YFP cytosolic aggregation. The relative insensitivity of AUX1-YFP to VCLCA depletion in that report might be explained by a lesser reduction of very-long-acyl-chain sphingolipids compared with that achieved in this work by FB1 treatment or the effect of the loh1-1 loh3-1 double mutant. However, enhanced sensitivity of PIN1 compared with AUX1 was also observed in this work, as PIN1 was aggregated in both weak and strong loh1 loh3 double mutants, while AUX1 distribution was only impaired in the strong loh1-2 loh3-2 mutant. These data suggest that AUX1 sensitivity to lipid environment might be more complex than for PIN1.

The VCLCA content of lipids has been shown to affect membrane bending during yeast nuclear pore formation and membrane plasticity during furrow ingestion in Drosophila melanogaster (Schneider et al., 1996; Szafer-Blusman et al., 2008). Simulation of lipid blayers comprising common sphingomyelins showed that increasing acyl chain length enhances blayer thickness and lipid packing but reduces lateral diffusion because of acyl chain interdigitations (Niemelä et al., 2006). Impairment of yeast fatty acid elongation leading to reduced C26-sphingolipid levels decreased lipid packing and membrane order in yeast cell but also prevented liquid ordered phase separation in model membranes from total lipid extracts (Klose et al., 2010). Such structural properties of the acyl chains of sphingolipids could infer specific membrane features like vesicular mobility or fusions that could define some endosomal populations and PM subdomains. However, the molecular mechanisms underlying these specificities remain to be explored to fully understand the structural role of sphingolipids in membrane dynamics.

Plant Growth Condition and Drug Treatments

Seedlings were grown on Arabidopsis thaliana agar medium (Es- tellle and Somerville, 1987) for phenotyping, genotyping, and drug treatment. FB1 (Sigma-Aldrich) short treatment was performed by incubating 9-d-old seedlings in liquid Arabidopsis media for 16 to 20 h in the presence of 2.5 μM FB1. Due to batch variability, each FB1 stock solution was calibrated with a root growth response curve and variation of drug concentration never exceeded 2.5 ± 1 μM for a short incubation. For long FB1 treatment, seedling were directly germinated on Arabidopsis solid media supplemented with 0.25 to 3.5 μM FB1. BFA (Sigma-Aldrich), which inhibits anterograde vesicular transport, was used in liquid Arabidopsis media at 50 μM for 1 h on 9-d-old seedlings. After each drug treatment, seedlings where washed before mounting for imaging.

Complementation of the lateral root defect of the loh1-1 loh3-1 mutant or FB1-treated seedlings was obtained with 0.1 mM NAA (Sigma-Aldrich) supplemented in solid Arabidopsis media with or without 0.5 μM FB1.

The different transgenic lines used in this study were as follows: pPIN1:PIN1-GFP (Benková et al., 2003), pPIN2:PIN2-GFP (Abas et al., 2006), pPIN2:PIN1-GFP (Wünsniewska et al., 2006), pAUX1:AUX1-YFP (Swarup et al., 2004), pLAX2:LAX2-GFP (Swarup et al., 2008), 35S:PIN2:GFP and 35S:SL6:GFP (Cutler et al., 2000), pABC19:ABC19-GFP and pABC1:ABC1-GFP (Titilap- watanakun et al., 2009), pSNX1:SNX1-GFP (Jaililais et al., 2006), and pAXR4:AXR4-GFP (Dharmasiri et al., 2006). The lines from the Wave Marker collection lines containing the UBQ10 promoter were more sensitive as follows: wave6 (YFP-NP1), wave29Y (YFP-ARA5/RabD2), wave33Y (YFP-RABG3)/a wave18Y (YFP-GOT1), wave127Y (YFP-MEpb), wave22Y (YFP-SYP32), wave34Y (YFP-RAB1b), wave2Y (YFP-ARA7), wave5Y (YFP-RABG3b), wave9Y (YFP-VAMP711), and wave11Y (YFP-RabG3c) (Geldner et al., 2009). The Rab-A2a mutant constructs were the following: pRab-A2a:YFP-pRab-A2a, pRab-A2a:YFP-Rab-A2a*N125I, pRab-A2a:YFP-Rab-A2a*S26N, pRab-A2a:YFP-Rab-A2a*Q71L (Chow et al., 2008), pBOR1-BOR1-GFP (Takano et al., 2005), and p35S:LeAQP1-GFP (Estévez et al., 2006).

Methods

Bioinformatics and Phylogenetic Analysis

GenBank and the tomato genome database (http://mips.helmholtz- muenchen.de/plant/tomato/index.jsp) were searched using the tBLASTn algorithm and the Asc-1 protein sequence AJ312131 using default parameters, except the filter for low complexity was turned off. The GenBank sequence for Arabidopsis lyrata XM_002890624 was manually extended using the A. lyrata sequence available at http://genome.jgi-psf.org/Araly1/Araly1.home.html. Full-length eudicot sequences were identified and the protein sequences aligned using ClustalW. Gaps were condensed and edited using the BioEdit sequence software. Phylogenetic relationships between sequences were established using the MEGA4 software program with parameters set to the default values (Tamura et al., 2007). Transmembrane domains were predicted with TMPred (Hofmann and Stoffel, 1993).

Insertion Mutants

Two Arabidopsis ecotypes Columbia-0 (Col-0) and Wassilewskija (Ws) were used. loh1-1, loh2-1, and loh3-1 are in the Ws background and were isolated by screening the original collection of 60,480 T-DNA insertions described by Krysan et al. (1999) using the following primers: loh1-1 (5′-CTTTTTACATTCTCTCTGCT- GCT-3′; 5′-GAACCAAAACGCCCAAGAATCCAT-3′), loh2-1 (5′-AGTCAGTAAGGTAATGAAGCTGAAAACAA-3′; 5′-AAAAAGAAAGGAAGCTGTGAGATCCCAA-3′), and loh3-1 (5′-ATTCGATTCTTCTCCGATAGTCTCTCTT-3′; 5′-GACCTGACCTGCTTACTACCT-3′; 5′-ACAAGCTGCTTATTA- CATTCTATACCTAC-3′) in combination with the left border primer JLR202 (5′-CTTTTATACGTGACCGATCTAC-3′), loh1-2 (N580371), loh2-2 (N524192), and loh3-2 (N650849) were obtained from the Salk collection. The genotyping of SALK loh was performed with the following primers: loh1 (5′-TTCTCTCTTGTTAGGTAAAGAGAA-3′; 5′-AAAAACCGAAAGAATCTTATTAG-3′), loh2 (5′-TTTCTGATCCTCTCTGTTA-3′; 5′-AAAGACATCGCATCG-3′), and loh3 (5′-TGTCTTTTAAAGAGATTAGATTACCCG-3′; 5′-CAAATTTGTGACACTGACTAACT-3′).

Lipid Analysis

Free and total LCB analysis was performed by HPLC after fluorescent derivatization (Bach et al., 2008). Complete sphingo- lipid analysis was performed by mass spectrometry as described (Markham and Jaworski, 2007).
Auxin Transport Measurements

Auxin transport was measured through sections cut from 12- to 15-cm-long inflorescence bolts as previously described (Brown et al., 2001).Ws and loh1-1 loh3-1 plants were grown for 5 to 6 weeks until the bolts were 12 to 15 cm high. Sections 25 mm in length were cut using a sharp razor blade from the bottom, the middle, and 10 mm below the top of the bolt. The stem section was immediately placed in a tube containing 20 μL of 5 mM MES, pH 5.5, 1% Suc, 0.05% Tween 20, 19 mCi 1-14C-indole acetic acid (9 mCi/mmol) with or without the addition of 10 μM NPA such that one end of the stem segment was immersed in the solution. To measure basipetal transport, the stem segment was placed in the inverted orientation, that is, with the top of the stem segment in the solution. Stem segments were incubated for 16 h in the dark after which the upper 5 mm of the stem was excised, immersed in scintillation fluid, and the dpm measured in a scintillation counter.

Live-Cell Imaging

Excitation/emission of YFP (514 nm, 520 to 560 nm) GFP (488 nm, 490 to 550 nm), and FM4-64 (488 or 514 nm, 600 to 700 nm) was performed on LSM 710 (Zeiss) and SP2 (Leica) confocal microscopes. Root tip imaging was performed with seedlings mounted in water, and high magnification images were taken with a ×63 objective lens (water immersion) with a line averaging of 8. Roots were stained with 2 μM FM4-64 and directly mounted on the microscope slide. Spectral separation of PIN1:GFP and AUX1:YFP was conducted according the GFP and YFP emission profile and the LSM software spectral mode. GUS and propidium iodide staining was performed as described previously (Harrar et al., 2003; Truemit et al., 2008). FRAP analysis of PIN1-GFP and AUX1-YFP was performed on 10- to 12-d-old secondary root tips of pPIN1:PIN1-GFP and pAUX1:AUX1-YFP seedlings treated with 2.5 μM FB1. The FRAP experiment was performed using a Zeiss LSM710 with a ×40 oil objective. A prebleach scan was done with either a 488-nm laser excitation (5%) for PIN1-GFP or a 514-nm laser excitation (3.5%) for AUX1-YFP with a pinhole of 82.2 and a 512 × 512 (8-bit) acquisition mode with a pixel dwell of 1.27 μs, 8 line average, and a 2× zoom. Fluorescence bleaching was performed after three prebleach scans with a double scan at 100% laser 488 and 514 nm and a pixel dwell of 177.32 μs. Confocal pictures were processed using ImageJ V1.44m. The pictures corresponding to prebleach, postbleach, and each recovery time point (15, 30, 45, and 60 min) were assembled into a time-lapse picture stack. Occasional drifting of the sample was corrected using the StackReg plugin. About two to four cells were quantified per root. Fluorescence intensity was measured on cell apical/basal membranes either as rectangular region of interest (ROI) in the case of PMs or as circular ROI in the case of pAUX1:AUX1-YFP seedlings grown for 5 d on agar medium were treated or not with 2.5 mM FB1 (16 h) in water and mounted (t = 0) in 100 μM boric acid (±2.5 μM FB1) (see Supplemental Figure 10B). To test the effect of FB1 treatment on secretion, 12-d-old seedlings (35S:LeAGP1-GFP) treated or not with 2.5 mM FB1 (16 h) were plasmolysed using 0.4 mM mannitol with 3 μM propidium Iodide during 5 min and then mounted (see Supplemental Figure 11F).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL database or the Arabidopsis Genome Initiative database under the following accession numbers: Arabidopsis (At) LOH1 (At3g25540), LOH1 (At3g19260), LOH3 (At1g13580), and LOH4 (At1g26200). Accession numbers of tomato (Le) Asc1, Asc2, and Asc3 and yeast Lag1p and Lac1p used in Figure 1A are AJ312131, SL1.005002793_15.1.1, SL1.005002749_302.1.1, SCU08133, and NM_001179574, respectively. The accession numbers of the Arabidopsis mutants used in this study are as follows: loh1-2 (N580371), loh1-2 (N524192), loh3-2 (N650849), loh1-1 (CS66113), loh2-1 (CS66114), and loh3-1 (CS66115).

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Supplemental Data

The following materials are presented following the References to this article:

Supplemental Figure 1. Protein Sequence Alignment of 29 LAG1 Homologs and Phylogenetic Relationship among LAG1 Homologs.

Supplemental Figure 2. Characterization of loh Mutants.

Supplemental Figure 3. Complementation of the loh1 loh3 Double Mutant.

Supplemental Figure 4. Fatty Acid Content of Total Sphingolipids and Free LCBDs from loh Mutants.

Supplemental Figure 5. Sphingolipid Analysis of FB1-Treated Seedlings.

Supplemental Figure 6. Effect of FB1 on Root Growth and Viability.

Supplemental Figure 7. Subcellular Distribution of PIN1-GFP and AUX1-YFP and YFP-VAMP711 upon Sphingolipid Depletion.

Supplemental Figure 8. Endomembrane Ultrastructure Is Altered in loh1-1/loh3-1 Root Cells.

Supplemental Figure 9. Involvement of Endocytosis in YFP-RabA2a Aggregation.

Supplemental Figure 10. Involvement of Endocytic and Secretory Pathways in YFP-RabA2a Aggregation.

Supplemental Data Set 1. Text Files and Alignment Used for the Phylogenetic Analysis Shown in Supplemental Figure 1B.

References


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Figure S1A. Protein sequence alignment of 29 LAG1 homologs. Full length protein eudicot sequences are aligned with the CerS homologs from human (HsCerS1-6; #NM_021267, #NM_181746, #NM_178842, #NM_024552, #NM_147190, #NM_203463) and the LAG1 (SCU08133) / LAC1 (#NM_001179574) sequences from S.cerevisiae. The two tomato sequences SlAsc-2 (#SL1.00sc02793_15.1.1) and SlAsc-3 (#SL1.00sc02749_302.1.1) are available from the tomato genome database http://mips.helmholtz-muenchen.de/plant/tomato/index.jsp. Other proteins are from Arabidopsis lyrata, (AlLOH3 # XM_002892714, AlLOH4 # XM_002890624); Arabidopsis thaliana, (AtLOH1-4, #NM_113450, #NM_112813, #NM_001035960 and #NM_001160896); Brassica rapa (BrLOH1 #EU186328); Glycine max (GmLOH1 #BT097014 and GmLOH3 #BT093554); Orobanche cerunae (OcLOH1 #GQ181109 and OcLOH3 #GQ181110); Populus trichocarpa (PtLOH1a #XM_002311388, PtLOH1b #XM_002315949, PtLOH2 #XM_002330290, PtLOH3 #XM_002328643); Ricinus communis (RcLOH3 #XM_002511975); Solanum lycopersicon (SlAsc-1 #AJ312131); Sorghum bicolor (SbLOH2 #XM_002465463); Vitis vinifera (VvLOH1 #XM_002270764) Sequences are listed in Supplemental Dataset 1 online.

Supp. Fig. 1A. Protein sequence alignment of 29 LAG1 homologs.

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Supp. Fig. 1B. Phylogenetic relationship among LAG1 homologs.

The alignment shown in Fig. S1A was used to examine the phylogenetic relationship between LAG1 protein sequence homologs. Significant groupings are highlighted in colour. The region from 179 to 417 on the alignment ruler was used to establish the tree using Maximum Parsimony methods, gaps were excluded. The bootstrap consensus tree (1000 replicates) is shown, branches where divisions produced less than 50% of the time are collapsed. The percentage of trees where a particular branch occurred is shown next to each branch point. CERS1 is an unique link between the mammalian and fungal sequences and was selected as the root branch. Similar tree arrangements were produced using Maximum Likelihood and Neighbour-Joining algorithms. Alignment is shown in Supplemental Dataset 1 online.
Supp. Fig. 2. Characterization of loh mutants

(A) Northern blot with RNA from loh1-1, loh2-1 and loh3-1 and hybridized with LOH1, LOH2 and LOH3 probes. Transcript size (kb) are indicated by solid arrow and the abnormal LOH3 transcript by an open arrow.

(B) Molecular analysis of loh alleles. RT-PCR of total RNAs from loh1-2, loh2-2, loh3-2 with T-DNA flanking primers.

(C) Phenotype of 3 week-old rosettes of loh mutant. Scale bar, 1cm.

(D) Lateral root development is inhibited in sesqui mutants loh1-2-/+;loh3-2-/- and loh1-2-/-;loh3-2-/+.

Plants were grown in vitro for two weeks. Scale bar, 1mm.

(E) Lateral root outgrowth but not initiation is inhibited by FB1 treatment. Number of lateral roots expressing DR5::GUS were classified according to different developmental stages (defined arbitrarily as I, first pericycle divisions; II, first periclinal divisions; III, primordia formation; IV, primordia outgrowth) in seedlings treated for 9 days with different concentrations of FB1. Data represent the mean of total lateral roots for 30 seedlings as shown in Fig.S2D. Significant differences between means (Student’s t-test) are indicated by one (*P<0.05) or two (**P<0.01) asterisks.

(F) GUS staining in wild-type primary root meristems containing the DR5::GUS auxin reporter that were grown for 9 days in the presence of various concentrations (0, 0.5 and 1μM) of FB1. Scale bar, 100μm.

(G) Rosette leaves from wild-type (WS - top) and loh1-1/loh3-1 mutant plants (bottom). Mutant leaves are smaller, wrinkled and show premature senescence. Scale bar, 10mm.
**Supp. Fig. 3. Complementation of loh1/loh3 double mutant.**

A. Location of primers used to create LOH complementation constructs and analyze the complemented plants. Primers are shown as green arrows, the position of exons is indicated by blue boxes, the position of the T-DNA insertions in the loh1-2 and loh3-2 allele are indicated.

B. Complementation of loh1-2/loh3-2 by LOH3. Shown are a Col-0 wild-type plant, loh1-2/loh3-2 seedlings and complemented loh1-2/loh3-2 plants containing a transgenic copy of the LOH3 allele.

C. PCR analysis of genomic DNA extracted from the plants shown in B. PCR reactions used the primers indicated on the right. Primers 175 and 014 detect the wild-type LOH3 allele and the complementing LOH3 containing T-DNA. Primers 175 and 174 detect only the wild-type LOH3 allele.

D. Complementation of loh1-1/loh3-1 by LOH1. A loh1-1/loh3-1 plant is shown in the left with typical delayed flowering and reduced rosette size. Plants containing the complementing LOH1 T-DNA do not show these phenotypes.

E. Primers used in making and analyzing the complemented plants.
Figure S4. Fatty acid content of total sphingolipids and free LCBs from loh mutants. (A) Total sphingolipids content of loh mutants compared to their related control (WS for loh1-1, loh2-1 and loh3-1; Col0 for loh1-2 and loh3-2). Sphingolipid content is shown according to the length and saturation of the fatty acid chain. (B) Free LCB content of loh1-1/loh3-1 compared to its related control (WS). Analyses were performed on 3-5 biological replicates. Data are the mean and standard error (n = 5).

Supp. Fig. 4. Fatty acid content of total sphingolipids and free LCBs from loh mutants. (A) Total sphingolipids Total sphingolipid content of loh mutants compared to their related control (WS for loh1-1, loh2-1 and loh3-1; Col0 for loh1-2 and loh3-2). Sphingolipid content is shown according to the length and saturation of the fatty acid chain. (B) Free LCB content of loh 1-1/loh3-1 compared to its related control (WS). Analyses were performed on 3-5 biological replicates. Data are the mean and standard error (n = 5).
Supp. Fig. 5. Sphingolipid analysis of FB1 treated seedlings.

(A) Ceramide content in seedlings grown for several hours (16h, 24h) or 9 days (9d) on medium supplemented with 0.5 μM FB1 and compared to untreated seedlings.

(B) Free LCB content in seedlings grown for several hours (16h, 24h) or 9 days (9d) on medium supplemented with 0.5 μM FB1 and compared to untreated seedlings.

(C) Free t18:0 and d18:0 levels during time course treatment with 1 μM FB1 (h, hours).

(D) Total LCB from seedlings treated with two FB1 concentrations for 9 days compared to untreated seedlings.

(E) Total sphingolipid content of WS and loh1-1/loh3-1 double mutant treated (+FB1) or not with 0.5 μM FB1. Data show mean plus one standard error and significant differences between means (P ≤ 0.05, Student’s t-test) are indicated by an asterisk.
Supp. Fig. 6. Effect of FB1 on root growth and viability.

(A)  *Pasticcino1* mutant root growth is more sensitive to FB1 than wild type. Seedlings were germinated and grown vertically on different concentrations of FB1 for 10 days. Data show root length relative to untreated control seedlings. Data are the mean of 25 to 33 seedling roots.

(B) Primary and secondary root viability upon FB1 treatment. Root tips treated or not with FB1 were stained with FM4-64 and FDA. Scale bar, 20μm.
Supp. Fig. 7. FB1 has no effect on several membrane markers.

(A) Expression of pAUX1:AUX1-YFP in lateral root primordia of Col0 and loh1-2/loh3-2 double mutant. Note the absence of membrane localization of AUX1-YFP in loh1-2/loh3-2. Scale bar, 10μm.

(B-C) Expression of pLAX3:LAX3-GFP in FB1-16h treated lateral roots (+) compared to untreated roots (-). LAX3-GFP expression was observed at two different developmental stages. Scale bar, 10μm. 

(B) the first anticlinal division of pericycle cells, and (C) the first periclinal cell divisions leading to the lateral root emergence.

(D-G) Plasma membrane markers in untreated (-) and FB1-16h treated (+) epidermal cells from lateral roots. (D) pPIN2:PIN2-GFP, (E) pPIN2:PIN1-GFP, (F) 35S:LTi6b-GFP and (G) 35S:PIP2-GFP. Scale bar, 10μm.
Supp. Fig. 8. Subcellular Distribution of PIN1-GFP and AUX1-YFP and YFP-VAMP711 upon sphingolipid depletion

(A) Expression of pPIN1:PIN1-GFP in control (ctrl) and loh1-1/loh3-1 double mutant. Arrows indicate the columella and arrows head show the presence of PIN1-GFP aggregates.

(B) Detail of pPIN1:PIN1-GFP in loh1-1/loh3-1 double mutant.

(C) Immunolocalization of PIN1 in pAUX1:AUX1-YFP secondary root cells treated (+FB1) or not (-FB1) with 2.5μM FB1 for 16h. Cells were counterstained with DAPI. Note that PIN1 aggregates in presence of FB1 and partially colocalize with AUX1-YFP.

(D) Expression of the vacuolar marker YFP-VAMP711 (Wave 9Y) in the root stele in presence (+FB1) or not of FB1-16h treatment.

Scale bar. 20μm in A, 10 μm in B and D, 2μm in C.
Supp. Fig. 9. Endomembrane ultrastructure is altered in loh1-1/loh3-1 root cells.

(A) Control cells exhibiting characteristic tubular endoplasmic reticulum (ER), a Golgi stack (GA) and a large vacuole (V).

(B-C) In loh 1 mutant (B) and loh1-1/3-1 mutant (C), Golgi ultrastructure is not affected, cytoplasm is often filled with small convoluted vacuole-like structures, with modified ER network.

Scale bar, 200nm
Supp. Fig. 10. Involvement of endocytosis in YFP-RabA2a aggregation

(A) Proximity but no colocalisation between YFP-Rab-A2a (green) and FM4-64 (red) in presence of FB1.

(B) Kinetics of BOR1-GFP endocytosis in the presence of boron. Five day-old BOR1-GFP seedlings were treated with 2.5μM FB1 for 16h and incubated in the presence of 100μM boric acid (t=0). Internalization of BOR1-GFP was then monitored in FB1 treated seedlings (+FB1) compared to untreated (- FB1). BOR1-GFP are clearly visible in both conditions 10 min after boron application.

(C) Subcellular distribution of Rab-A2a mutants. YFP-Rab-A2a[N125I] is a nucleotide free mutant showing the same subcellular distribution as wild type YFP-Rab-A2a but with lower protein levels. YFP-Rab-A2a[S26N] is the GDP-bound inactive isoform localized exclusively in the cytosol and the Golgi. YFP-Rab-A2a[Q71L] is the GTP-bound constitutively active isoform present in the cytosol and at the plasma membrane.

Scale bar : A and C, 5μm; B ,10μm.
Supp. Fig. 11. Involvement of endocytic and secretory pathways in YFP-RabA2a aggregation

(A) Partial localization of pPIN1:PIN1-GFP (left) and FM4-64 (right) staining in presence of BFA.

(B) Detail of BFA compartments with presence (open arrow head) or absence (closed arrow head) of colocalization between FM4-64 and PIN1-GFP.

(C) FM4-64 staining of aggregates in FB1-16h treated pPIN1:PIN1-GFP cells in presence of BFA. Partial colocalization is observed (arrow heads).

(D) Detail of FM4-64 staining of aggregates in FB1-treated pPIN1:PIN1-GFP cells showing partial colocalisation of PIN1-GFP with FM4-64 in FB1/BFA compartments.

(E) FRAP analysis of FB1-16h cells expressing pAUX1:AUX1-YFP. AUX1-YFP expressing lateral root (pre-bleach) was bleached and recovery of AUX1-YFP was monitored for 60min.

(F) LeAGP1-GFP expression in absence (-FB1) or presence of 2.5μM FB1 (+ FB1) in plasmolysed epidermal root cells. Cell wall was stained with propidium Iodide (PI).

Scale bar is 10 μm in A, C, E and F and 5μm in B and D.

Sequences (p. 1-5) and alignment (p. 6-10) used for phylogenetic analysis in Supplemental Figure 1B (FASTA format)

>`SlAsc-2
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>`PtLOH2
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>`AtLOH2
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>`SbLOH2
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> PtLOH1b
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>HsCerS3
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EEEEEATKGGEMDCDCLKNRLAERHLPQNGSH4

>HsCerS4
MLSSFNWFWQDRFLWLPNVTWTELEDGRGVRYPHPQDLLAALPLALVLALLAMLAFERFIGGLPLSRWLVG
RQTRQRQVKNATLEKHLTEEGHRPKQPLQSSLAAACQGCLLTQQTQRWRFRRRNQDRQPLTKEASWRF
LFYLSFYGGLSVLYHESSLWLPARVCMWDRYNYQTLSKPSLYWLLPEGYSLSLLRPLFDVKRDKEQG
IHFFAVAVLMFTSAPLNRIGSLVLLHSDADLEAEAKMVNYQOQVCDALFLILFSYFVYTRLVLF
PTQILYTTYESISNRPPFFYYYFNFGLMLQQLLHQFWSCLILRMYSFMMKFGQMEKDIRSDVEESDSS
EEAAAAYEQPQLNKAAGGPRRAPTDGPRSVAGRLTNRHDTAT

>HsCerS5
MATAAQGPLSLLWG
WLWSERFWLENVSWADLEGGYPRHLISIVFLPALAGIFCPGCRG
DCGWGLARRGLAEHAHLAPPELLLLALGALGW
TALRSAATARLFLRKLLASKCYGIRLSWSPFWIDQRWCVHNYPFQPLSLGLYHYILMEAFYSLMFSQFTDK
RKLFLIMFVHHLVTIGLISFYSINNMRVFGTLIMC HDVSDFLLAAEAKLANYA QRLCDTLVFIFS AVF
MVTRLGIYPFWRLLTFESIWEIIFGPYSWLLWNLGLLTLIQHLSIYLARIKALIRGKVDKDDRS
DVESSSEEDVTCTKPSDDNNGANRNVNGHMGGSYWAEE

>HsCerS6
MAGILAWFWNERFLWHNVTWADLKNTEEATFPQAEDLYLALAFPLAFICIFMVLRIFERFVAKPCAIALNQ
ANPQIAAPPNAILEKVFVTAITKHDPERLEGSKLDWVDVRSIQRWFQRRNQEKPLSTRLFCSMSWRF
FYLIFSTYGFRLKTPMLWNTRHCVWNYPQPPLTLDLYYIELESFWSLMSQFQTDIKRRDGIEMFL
HHLVSIIFLFLSYVNNAMRIGSLVLLHSDADLEAEAKMANYAKFQKMDLFLIMFVMAFFVFITRLGIP
LWVLNNTLFESIWEIGPYSWFWFNLWLLVLQQLNCFSWLYLVIKIAKAVSRGKVSDKDDSDIIESSDEE
DSEPPGKNPHATTNTNGSTGNYLLTSGCSDMDD

>HsCerS1
MAAAGPAAGPTGEPMPSYQAQLVQRGWSALAAARGCCTDCGWWGLARRGGAHPLPELLLLALGALGW
TALRSAATARLFLRKLLASKCYGIRLSWSPFWIDQRWCVHNYPFQPLSLGLYHYILMEAFYSLMFSQFTDK
RKLFLIMFVHHLVTIGLISFYSINNMRVFGTLIMC HDVSDFLLAAEAKLANYA QRLCDTLVFIFS AVF
MVTRLGIYPFWRLLTFESIWEIIFGPYSWLLWNLGLLTLIQHLSIYLARIKALIRGKVDKDDRS
DVESSSEEDVTCTKPSDDNNGANRNVNGHMGGSYWAEE

>ScLAC1
MSTIKPSPNNNLKVRSPFRKRSSIGKIDLGDTVPSLGMFETKESKTAAKRRMQRLSEATKNSDLVKK
IWFSFREISYRHAWIAPlMILIAVYSAFTSGNTTKTNVLHRFVAVSYIGDTNAYGKINDLCFVFYPY
IFFTFLREFLMDVIRPFAIRLHVTSKHKRIKREMEQMYAIFYTGVSFGPFGIYCMYHSDLWFNFTKAMYRTYPDFTPNLFKVFYLGCQAFFFFACILVLEKPRKDHSNLFFHHHIVTLHLWSSYVFHFHTKMLPIYITMDVSDLSSFTLNYLDSGLAFFSFAIFVVAVIYLHRYINVILKILWSVLFQFRTEGNVINMFATQYKCIWLPIVFVLIGALQLVNLWLFIRLYRILMRIKDRSDSESDEEEDSSSTPTDSTPTKDI
Alignment used for phylogenetic analysis in Supplemental Figure 1B

SlAsc-2
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PtLOH2
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AtLOH2
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SbLOH2
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SlAsc-1
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SlAsc-3
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PtLOH1a
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PtLOH1b
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VvLOH1
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BrLOH1
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AtLOH3
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AlLOH3
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AlLOH4
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HsCerS5
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HsCerS6
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HsCerS1
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ScLAG1
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AtLOH2
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SbLOH2
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SlAsc-3
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ScLAG1
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SlAsc-3
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PtLOH1b
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AtLOH1
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AtLOH3
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AlLOH3
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OcLOH1
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OcLOH3
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GmLOH1
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GmLOH3
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RcLOH3
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PtLOH3
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AtLOH4
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AlLOH4
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HsCerS2
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VNLTWADLED-RDGRVYAKASDLYITLPLALLFLIVRYFFELVATPLAALLNIKEKTRL

HsCerS3  PTIKWSLED-HDGLVFVKPSLHVTYPIYAFLLLILLIRRFVEKFVASPLASKSIGKETVRK
HsCerS4  PNVTTWTELED-DRGVYHPFDQDIALPLVLLAMRLAERFIFGLPLSRWLGVQDQTRR
HsCerS5  ENVSWADLEGPGYPRGRHLSVFLPAAIGFFVRLLEFRIAKPCALCIGIEDSGPY
HsCerS6  HNTWDDLKN-TEEATFQAEAELYALPFLAFICFVMRLIFERFVAKPCIALNIQANGPQ
HsCerS1  --------------------------MAAAPGPAAGTPEMPYSAQLYQVQRGWSLAARARCTDCGW
ScLAC1  DSQKSDSLWKKWFSYREMYNHRHSWITPPFLILCVCSAYALFSGNRTESNPLHMVFAISYQ
ScLAC1  ATKNDSLVDKKVFSREIWHAIAPLMIANYASYFSGTNTKTVHVRFAVSYQ

S1Asc-2  AVSHLFYAIAFPAGFVQVRLFLFLDFIRRLAVLLRLGTHLTMNDATRGEK----KIVKC
PtLOH2  NSHPFLPPYIAFVFPFLLIFFLIFIRFIRKIAWWLIWYASKAIAISSREDE----IVKC
AtLOH2  EVWHFQIAVYAFPFGFVRFLRLVFLDVFQRIALWLLSTGAIMKLNADATRA----KIVKC
SbLOH2  GQAEVSVALLSAPLLACFALLDLRLVLKPLAVLYFLNTASKMNDRARQG------KIVKC
S1Asc-1  EYQLDIIFLLFALFFPVLRFILDRFIFRRLAVLLLRLGTTHLRNDEATRG-----KIVKC
PtLOH1a  AYEVENFLLPFLFTTFIFRIFMRLLVIFRHLGFLRIFLGLGFLKQDNNEK---KMKNF
VvLOH1  EYGDFIAPVLFAPFFSAFVRFLFQKLGRILIFGKGQQLDDVGLVEZDREK-----KRLKF
BrLOH1  TFTQDFIPLFLAPFFSAPFVLFQKEKAFDRLLVIFGKATPRQDAGNDREK-----KIGKF
AtLOH1  EYQDLGFPFLAVFFPFLRFILDRFIFRRLAVLLLRLGTTHLRNDEATRG-----KIVKC
AtLOH3  VYQDFRVLPLFAVFFPTIRFLLDRFVFEKLAPKRLYLVGRQDMGTQKHERK-----KIRKF
AlLOH3  VYQDFRVLPLFAVFFPTIRFLLDRFVFEKLAPKRLYLVGRQDMGTQKHERK-----KIRKF
OcLOH1  QYEDFLVLPLFAVFFPVRFFLDRFVFQKLEQDLIFGKEHKQTFDRKQKDTPQK---KIRKF
OcLOH3  QYEDFLVLPLFAVFFPVRFFLDRFVFQKLEQDLIFGKEHKQTFDRKQKDTPQK---KIRKF
GmLOH1  AYHDFYFAPFAPFFPSVFLLDRFVFQKLRGLYLVGRQAFDSQKMIKQDFDRK---KISKF
GmLOH3  DFRDSVIFPPPFLFIFPFFIPFPPFFPFLFIFPFFIPFPPFFPFLFIFPFFIPF---KISKF
RcLOH3  EAIDFSAVPFFALFFPSFLRFILDRFLFEKALYLFGRQPSQDVHFMKQDFDRK---KVVKF
PtLOH1a  KESAWKCIYYLFAEILALSVTYDEPWFTDTKYFWVGPQIWPQKMKMLKFLYMGAYR
AtLOH4  DSSDFVLVIFAPFAPFLLRLDDICERAVARLRVLVPGLCA-------DSNERRK-----KVKCF
AtLOH3  VDGTDSYAKGIKDLSFVFFYMIFFTFLREFLMDVVIRPFTVYLNVTSEHRQK------RM
ScLAC1  IGDTNAYGKGJNLCFVFYMIFFTFLREFLMDVVIRPFAAIRLHVTSKHREK------RI

S1Asc-2  SEWMMKFAAYTIEIFCVKLVYAYEPFLWDVLGYFGS----WPQELTAGIYLMYCCQCG
PtLOH2  SEWMMKLYTATVEICVLKITAICYNPWFNTDKYFEGFR-----WPQELGFPILMFLYMCQCG
AtLOH2  KESLWKLLYAACADFVLQVYHEPARDIKLYFEGFR-----WPQELKLISKLYMYCCQCG
SbLOH2  SESSWKLTFASVQAWLMIKQEPWLDMMQYPFQDG-----WPQPIASSLMLFMYCCQCG
S1Asc-1  SEKSAWKFYPSAALLFELCSVTNEPWFDTSYFVAGPDDVWPNKLMKLKLKLMYMAG
S1Asc-3  KESAWKMFYPSAALLFELCSVTNEPWFDTSYFVAGPDDVWPNKLMKLKLKLMYMAG
PtLOH1a  KESAWKCYFLSAILVLCVTYDPEWNLVTYKFVWGPQSGQAWPDQKMKMLKLKAVMAYAAG
PtLOH1b  KESAWKCYFLSAILVLCVTYDPEWNLVTYKFVWGPQSGQAWPDQKMKMLKLKAVMAYAAG
VvLOH1  KESAWKCYFLSAILVLCVTYDPEWNLVTYKFVWGPQSGQAWPDQKMKMLKLKAVMAYAAG
BrLOH1  KESAWKCYFLSAILVLCVTYDPEWNLVTYKFVWGPQSGQAWPDQKMKMLKLKAVMAYAAG
AtLOH1  KESAWKCYFLSAILVLCVTYDPEWNLVTYKFVWGPQSGQAWPDQKMKMLKLKAVMAYAAG
AtLOH3  KESAWKCYFLSAILVLCVTYDPEWNLVTYKFVWGPQSGQAWPDQKMKMLKLKAVMAYAAG
AlLOH3  KESAWKCYFLSAILVLCVTYDPEWNLVTYKFVWGPQSGQAWPDQKMKMLKLKAVMAYAAG
OcLOH1  KESAWKCYFLSAILVLCVTYDPEWNLVTYKFVWGPQSGQAWPDQKMKMLKLKAVMAYAAG
OcLOH3  KESAWKCYFLSAILVLCVTYDPEWNLVTYKFVWGPQSGQAWPDQKMKMLKLKAVMAYAAG
GmLOH1  KESAWKCYFLSAILVLCVTYDPEWNLVTYKFVWGPQSGQAWPDQKMKMLKLKAVMAYAAG
GmLOH3  KESAWKCYFLSAILVLCVTYDPEWNLVTYKFVWGPQSGQAWPDQKMKMLKLKAVMAYAAG
RcLOH3  KESAWKCYFLSAILVLCVTYDPEWNLVTYKFVWGPQSGQAWPDQKMKMLKLKAVMAYAAG
PtLOH3  KESAWKCYFLSAILVLCVTYDPEWNLVTYKFVWGPQSGQAWPDQKMKMLKLKAVMAYAAG
AtLOH4   KESAWKCLSFSVEAFAVITYKPEFKDTRSFWLPGDQVFDQKIKLKMKGMYMVFGG
AtLOH4   KESAWKCLSFSVEAFAVITYKPEFKDTRSFWLPGDQVFDQKIKLKMKGMYMVFGG
HsCerS2    REASWRFTFYLIAPAGMVAVIKDPFWDYMDKVKVGNV-------YPIQSTPSQWYMMELS
HsCerS3    QEACWRFALMYTAVAGYFELDYPFWYLDWVEWNG------YPKPLPSQWYMYLEMS
HsCerS4    CESAWRFYLFVLSSVGLYSVLEHLSWALPWCMGD------YPNQLTLSWLYMELGG
HsCerS5    CESMRFTYLICFCYGIRFLWSFPWFDIQCWH------YPFQPLSSLHYLYYIMELA
HsCerS6    CESMRFTYLFVLSSVGLYSVLEHLSWALPWCMGD------YPNQLTLSWLYMELGG
HsCerS1    PESAWKFLFYLGSWSYSAYLLFLTYDYPFFHCWPVYD------WTFMAGAVPRDIAAYLQGGS
ScLaG1    LEQMYAIFCGVSGPFLYHMDASNLFLFKTPKQMYRT------YPFITPLFLKIFLYLQGA
ScLaC1    MEQMYAIFCGVSGPFLYHMDASNLFLFKTPKQMYRT------YPFITPLFLKIFLYLQGA
SlAsc-2    FYL-VSIALVWVTRRRKDFAVMSWSHHIVTHTFLSSYILSFRRIGVILALHDSVDVF
PtLOH2    FYI-VSIAALIWETRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
AtLOH2    FYV-YGVAALLAWETRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
SbLOH2    FYV-YGIAVAVIETRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
SlAsc-1    FYF-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
PtLOH1a    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
PtLOH1b    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
VvLOH1    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
BrLOH1    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
AtLOH2    FYV-YGVAALLAWETRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
SbLOH2    FYV-YGIAVAVIETRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
PtLOH3    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
PtLOH1a    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
PtLOH1b    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
VvLOH1    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
BrLOH1    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
AtLOH2    FYV-YGVAALLAWETRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
SbLOH2    FYV-YGIAVAVIETRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
PtLOH3    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
PtLOH1a    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
PtLOH1b    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
VvLOH1    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
BrLOH1    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
AtLOH2    FYV-YGVAALLAWETRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
SbLOH2    FYV-YGIAVAVIETRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
PtLOH3    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
PtLOH1a    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
PtLOH1b    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
VvLOH1    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
BrLOH1    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
AtLOH2    FYV-YGVAALLAWETRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
SbLOH2    FYV-YGIAVAVIETRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
PtLOH3    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
PtLOH1a    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
PtLOH1b    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
VvLOH1    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
BrLOH1    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
AtLOH2    FYV-YGVAALLAWETRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
SbLOH2    FYV-YGIAVAVIETRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
PtLOH3    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
PtLOH1a    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
PtLOH1b    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
VvLOH1    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF
BrLOH1    FYI-VSIALVWVTRRRKDFAVMSWSHHIVTFLSSYILSFRRIGVILALHDSVDVF

GmLOH3    ETGKMSYSGAETTASIAFILFVLCFTVTRIIYYPFWILRSTSYEVVHALKMD---------
RcLOH3    EIAKMSRYEWISSIFFFVLFVSIFWFIRFIYYPFWILRSYEVVFLTDKMK---------
PtLOH3    EIAKMSYGAETMSVFSVFLFVSIFWFIRLIYYPFWILRSYEVVFLTDKMK---------
AtLOH4    EIGKMSYSGAETMVSIFVFLFVSIFWFIRLIYYPFWILRSYEVVFLTDKMK---------
AlLOH4    EIGKMSYSGAETMVSIFVFLFVSIFWFIRLIYYPFWILRSYEVVFLTDKMK---------
HsCerS2   ESAKMFNYAGWKNTCNNIFIVFAIVFIITRLVILPFWILHCTLVYPLELYPAFFG--
HsCerS3   ESAKMFSYAGWTQCNLLIFIVFAIVFIITRLVILPFWILYCTLVILPMYHLEPEFSS--
HsCerS4   EACKMVNYMQYQQVCDALFLIFSFVFFYTRLVILPFWILHCTLVYPLELYPAFFG--
HsCerS5   EAAKLANYAKYQRLCDTLFVIFSVFFYTRLVILPFWILHCTLVQYPLELYPAFFG--
HsCerS6   EAAKMANYAKFQKMCDLLFVMFAMAFVITTRLGIFLPWLNLTLFSEIYMGGFPESS--
HsCerS1   EFTKLNIYFKSFGSFIYVVVFLYFWHYTILFILPFWILHCTLVYPLELYPAFFG--
ScLAG1    SLSKTLNYLNSVFTPFVGLFVYLHVNNIRILNSVTLFEPRTQNFVYVLNFLPATQYK
ScLAC1    SFSKTLNYLNSVFTPFVGLFVYLHVNNIRILNSVTLFEPRTQNFVYVLNFLPATQYK

S1Asc-2    --------TMIYYFFNTMLTLTLVFHYWILYWSMIMKQLRNGQVDIERSDSDED----
PtLOH2    --------LLYFNTMLMLLMLVFHYWILYWSMIMKQLRNGQVDIERSDSDED----
AtLOH2    --------EGTMIFSNTMLMLLVFHYWILYWSMIMKQLRNGQVDIERSDSDED----
SbLOH2    --------PTALYIVNTMLTLTLLVFHYWILYWSMIMKQLRNGQVDIERSDSDED----
S1Asc-1    --------TTGIIYFFNALLICLVLHLFWKILRMVQNLSRGHI1TDVREDSEDSDD-H
S1Asc-3    --------QINGIIYFFNALLICLVLHLFWKILRMVQNLSRGHI1TDVREDSEDSDD-H
PtLOH1a    --------HPVDP gyroryFPFTMYLCVHLLYVQMLAQGRLSDDVREDSEDSDD-H
PtLOH1b    --------HVDP gyroryFPFTMLYGLLVHLHYWMLYRMLVQGRLSDDVREDSEDSDD-H
VvLOH1     --------HVEPFPFNYLFTLCLHLHYWMLYRMLVQGRLSDDVREDSEDSDD-H
BrLOH1     --------HPEIIFYMNFNTMLFLCCLHLHYWMLYRMLVQGRLSDDVREDSEDSDD-H
AtLOH3     --------HPIEIFYMNFNTMLFLCCLHLHYWMLYRMLVQGRLSDDVREDSEDSDD-H
AtLOH4     --------HPIEIFYMNFNTMLFLCCLHLHYWMLYRMLVQGRLSDDVREDSEDSDD-H
AlLOH4     --------HPIEIFYMNFNTMLFLCCLHLHYWMLYRMLVQGRLSDDVREDSEDSDD-H
OcLOH1     --------HRAFPFPFNYVNSLFLVLHYWMLYRMLVQGRLSDDVREDSEDSDD-H
OcLOH3     --------HKVDGPFPFYNTFLLLHLHYWMLYRMLVQGRLSDDVREDSEDSDD-H
GmLOH1     --------HHRFPFPFNYVNSLFLVLLHYWMLYRMLVQGRLSDDVREDSEDSDD-H
GmLOH3     --------LDVPFFYYVNSLFLVLHYWMLYRMLVQGRLSDDVREDSEDSDD-H
RcLOH3     --------HMVDGPLNYYLFLNTLLFCILVHLHYWMLYRMLVQGRLSDDVREDSEDSDD-H
PtLOH3     --------QMVDGISYLYNTFLLLLHLHYWMLYRMLVQGRLSDDVREDSEDSDD-H
AtLOH4     --------ETGPFPFYNTFLLLHLHYWMLYRMLVQGRLSDDVREDSEDSDD-H
AlLOH4     --------ETGPFPFYNTFLLLHLHYWMLYRMLVQGRLSDDVREDSEDSDD-H
HsCerS2    --------YYFFNSMVGLLHYLFWTILLFAYLRRMAKFT-----GKVLEDERSDEESSE
HsCerS3    --------YYFLNLQLMILVQLHLHYWYYILKMLNRICF-----MKSVQDRSDDDEEE
HsCerS4    --------YYFNGMLMAVLLVWLSLMLLMYFMYPMK-----KQMEKDSREDSESES
HsCerS5    --------WWLLGNTTLQVHLHVNYLAIARIALK------RGKVSDDRSDSESES
HsCerS6    --------WWVNNLULLQQLVCLNFWSLILVIAKACAVSRGKVSDDRSDSESES
HsCerS1    --------IPFYFFFNNALLLLLIIILNMLNLYFLIYFAKVLQHHELKLCREYTDARASQ
ScLAG1    C--------WISLPFPVFIALAQVNLNYLFLILRILYRI-QL-IQKDRSDDSESEAE
ScLAC1    C--------WISLPFPVFIALAQVNLNYLFLILRILYRI-QL-IQKDRSDDSESEED

S1Asc-2    -----------------------------------------------
PtLOH2     -----------------------------------------------
AtLOH2     -----------------------------------------------
SbLOH2     -----------------------------------------------
S1Asc-1    -----------------------------------------------
S1Asc-3    -----------------------------------------------
PtLOH1a    -----------------------------------------------
PtLOH1b    -----------------------------------------------
VvLOH1     -----------------------------------------------
BrLOH1     -----------------------------------------------
AtLOH3     -----------------------------------------------
AtLOH4     -----------------------------------------------
AlLOH4     -----------------------------------------------
HsCerS2    -----------------------------------------------
HsCerS3    -----------------------------------------------
HsCerS4    -----------------------------------------------
HsCerS5    -----------------------------------------------
HsCerS6    -----------------------------------------------
HsCerS1    -----------------------------------------------
OcLOH1  ED-----------------------------------------
OcLOH3  DD-----------------------------------------
GmLOH1  EHED-----------------------------------------
GmLOH3  KHEE-----------------------------------------
RcLOH3  DD-----------------------------------------
PtLOH3  DD-----------------------------------------
AtLOH4  QD-----------------------------------------
AlLOH4  HD-----------------------------------------
HsCerS2  GEEAAAGGAKSRPLANGPHILNNHNRKND------------------
HsCerS3  EEEEEATKGEMDCLKNGLRAERHLIPNGQGH-------------
HsCerS4  EAAAAQEPLQLKNGAAGGPAPTDPGPRSRVAGRLTNRHTTAT
HsCerS5  DTTCATKPCDSNNGANRNGM-GSYWAEE---------------
HsCerS6  DSEPPGKNPHTTNGTSGTNGYLLTGSMDDD--------------
HsCerS1  KPSKAEKPLRNLVNDKRF------------------------
ScLAG1  NEESKEKCE-------------------------------------
ScLAC1  ESSTTPTDSTPKDI--------------------------------