The very-long-chain hydroxy fatty acyl-CoA dehydratase PASTICCINO2 is essential and limiting for plant development

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Very-long-chain fatty acids (VLCFAs) are synthesized as acyl-CoAs by the endoplasmic reticulum-localized elongase multienzyme complex. Two Arabidopsis genes are putative homologues of the recently identified yeast 3-hydroxy-acyl-CoA dehydratase (PHS1), the third enzyme of the elongase complex. We showed that Arabidopsis PASTICCINO2 (PAS2) was able to restore phs1 cytokinesis defects and sphingolipid long chain base overaccumulation. Conversely, the expression of PHS1 was able to complement the developmental defects and the accumulation of long chain bases of the pas2–1 mutant. The pas2–1 mutant was characterized by a general reduction of VLCFA pools in seed storage triacylglycerols, cuticular waxes, and complex sphingolipids. Most strikingly, the defective elongation cycle resulted in the accumulation of 3-cuticular waxes, and complex sphingolipids. Most strikingly, the general reduction of VLCFA pools in seed storage triacylglycerols, cuticular wax, leaf development

Very-long-chain fatty acids (VLCFAs) are components of euukaryotic cells and are composed of 20 or more carbons (i.e., >C18). VLCFAs are involved in many different physiological functions in different organisms. They are abundant constituents of some tissues like the brain (myelin) or plant seeds (storage triacylglycerols). VLCFAs are components of the lipid barrier of the skin and the plant cuticular waxes (1). VLCFAs are also involved in the secretory pathway for protein trafficking and for the synthesis of GPI lipid anchor (2). Finally, VLCFAs are components of sphingolipids that are both membrane constituents and signaling molecules (3).

In yeast, VLCFA synthesis is catalyzed in the endoplasmic reticulum (ER) by a membrane-bound multienzyme protein complex referred as the elongase (4). The elongase complex catalyzes the cyclic addition of a C2-moiety obtained from malonyl-CoA to an acyl-CoA. VLCFAs (C20, C22, C24, or higher) are produced from shorter fatty acids (usually C16 or C18) made by the cytosolic fatty acid synthase complex. The two-carbon addition during the elongation cycle requires four independent but sequential enzymatic steps. The first step involves the condensation of the malonyl-CoA with an acyl-CoA precursor, resulting in a 3-ketoacyl-CoA intermediate, which is reduced to form a 3-hydroxy-acyl-CoA. The third enzymatic step is the dehydration of the 3-hydroxy-acyl-CoA to an enoyl-CoA, which is finally reduced to yield an acyl

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can rescue biochemical phenotypes of the ybr159Δ mutant, the in vivo role of the plant gene remains to be determined (14). The yeast dehydratase PHS1/Y3L097w gene also shares significant similarity with the Arabidopsis PASTICCINO2 (PAS2) gene, which was shown to rescue psh1Δ lethality (15). Mutations in the PAS2 gene lead to strong developmental defects associated with ectopic cell division, cell differentiation, and hormonal responses (15–18). Finally, PAS2 was demonstrated to be able to interact with phosphorylated cyclin-dependent kinase (CDK) and subsequently to prevent its dephosphorylation by CDC25-like phosphatase(s), preventing premature entry in mitosis (19). However, the fact that PAS2 shows significant sequence similarity with Phs1p and that it was able to rescue phs1 mutant argues in favor of PAS2 being the dehydratase component of the microsomal elongase complex. Here, we show by reciprocal complementation experiments that PAS2 and PHS1 are functionally exchangeable. The pas2 mutant was also characterized by global reduction of VLCFAs and by the specific accumulation of 3-hydroxacyl-CoA substrate of the dehydratase. Moreover, PAS2 was found to be associated with ER and to physically interact with the reductase CER10. Collectively our results demonstrate that PAS2 is required for the synthesis of VLCFAs and that these fatty acids are indispensable components of several specific lipid classes.

Results

PASTICCINO2 is the Arabidopsis Orthologue of Yeast PHS1. PAS2 shares 35% identity with a similar protein in Arabidopsis coded by the gene At5g59770, and both proteins showed a similar degree of similarities with Phs1p (33% and 32% identity, respectively, for PAS2 and At5g59770 coding protein). However, At5g59770 was not able to suppress the lethality of phs1 [supporting information (SI) Fig. S1]. Therefore, PAS2 represents a bona fide functional orthologue of PHS1 in Arabidopsis. However, it was still not clear whether this complementation by PAS2 was associated with the rescue of both cell division and lipid defects present in phs1. To address these questions, we used a phs1 haploid strain expressing the inducible pGAL1::PAS2 construct (designated D1B), which was able to grow only on galactose- and not on glucose-supplemented medium (Fig. S1) (15). When grown on galactose, D1B cells showed wild-type phenotype. Conversely, cells grown on glucose presented the characteristic phs1 multibudded or large-budded phenotypes (Fig. L4). Likewise, galactose-grown D1B cells displayed a restoration of the normal low levels of free (i.e., nonacylated) long chain bases (LCBs) that accumulated in phs1 mutant (Fig. 1B and Fig. S2). The expression of the Arabidopsis PAS2 gene is thus able to rescue both cell division and lipid defects of the yeast phs1 mutant.

One scenario we considered was that the plant PAS2 function might still have evolved, compared with its yeast orthologue, by the acquisition of new biochemical functions such as the regulation of cell cycle proteins (15, 19). We thus investigated whether the yeast PHS1 gene could complement the developmental phenotype of the plant pas2 mutant. PHS1 was therefore expressed in the heterozygous pas2+/+ plant under the control of the constitutive 35S promoter (Fig. S3). Segregation of pas2/+ plants carrying 35S:PHS1 construct showed that the phenotype of pas2 mutants expressing PHS1 reverted from small dwarf seedlings with fused leaves to plants almost indistinguishable from wild type (Fig. 1C). PHS1 complementation of pas2 phenotype was also observed at the levels of free LCBs that accumulated in pas2 and was almost completely restored to wild-type levels by expression of PHS1 (Fig. 1D and Fig. S2). In conclusion, yeast PHS1 is able to complement pas2 developmental defects and LCB accumulation, demonstrating that both proteins are exchangeable and that PAS2 represents the true PHS1 orthologue. More importantly, PHS1 complementation indicates that pas2 developmental defects are most likely caused by a defective dehydratase activity in the VLCFA microsomal elongase complex.

pas2-1 Mutant Is Characterized by Low VLCFAs. In plants, VLCFAs are involved in several classes of complex lipids, such as seed storage triacylglycerols, cuticular waxes, and sphingolipids, but could also be found in the phospholipid fraction (20). Analysis of VLCFAs in seeds showed that pas2 seeds accumulated lower levels of 22:1 and 20:1 and higher levels of short chains 16:0, 18:1, 18:2, and 18:3 compared with wild type (Fig. 2A). Wax deposition was modified in pas2 mutant stems with a lower density of wax crystals, which were mainly of globule shapes instead of flakes (Fig. S4). This result was consistent with the observation in pas2 of postgenital organ fusion characteristic of cuticle mutants (15). Accordingly, all of the aliphatic components of cuticular waxes were dramatically reduced in the pas2 mutant compared with wild type in both stems and leaves (Fig. 2B and Fig. S5). Finally, VLCFA content from complex sphingolipids was lower in pas2 mutant compared with wild type (Fig. 2C and Fig. S6). The glucosylceramide pool showed an almost complete absence of VLCFAs, whereas in contrast, the glycosyl-inositol-phosphoceramide (GIPC) pool showed only small VLCFA reduction. Almost no difference could be observed in the simple sphingolipid fractions except for 16:0 ceramide and hydroxy-ceramide levels, which were increased in pas2 mutant (Fig. S6). Thus, in agreement with the PAS2 role in fatty acid elongation, three lipid fractions of Arabidopsis that contain VLCFAs were perturbed in the pas2 mutant.

PAS2 Is Involved in 3-hydroxy acyl-CoA Dehydration During VLCFA Elongation. In yeast, the microsomal elongase complex is localized in the ER, as demonstrated by biochemical and subcellular data (4). In plants, the elongase synthases and the two reductases were also found to be ER-associated proteins (21). Subcellular
S1), three additional peaks present in the acyl-CoA pool were detected between 14:0 to 22:1 could originate from the elongation cycle. Although no significant quantitative differences between nonhydroxylated acyl-CoAs from 14:0 to 22:1 could be observed in the complemented pas2–1, 35S:PHS1 line, confirming that the presence of 3-hydroxyacyl-CoAs was caused by a defective dehydratase activity (Fig. S9). These data are not only in agreement with the proposed role of PAS2 as the microsomal elongase dehydratase but also report the presence of intermediates of the elongation cycle in the acyl-CoA pool. Interestingly, the presence of 3-hydroxy-C18:0-CoA in dehydratase-deficient mutants strongly suggests that acyl-CoA with an acyl chain as short as C16 could be a substrate of the Arabidopsis elongase complex.

Acyl-CoA Dehydratase Is Essential for Plant Development and Is Limiting for VLFA Synthesis. The pas2–1 and pas2–2 mutations were characterized as leaky alleles because both mutants were still able to accumulate low levels of wild-type transcript (15, 17). We therefore checked the effect of the Salk T-DNA insertion line N617051, which contains an insertion in the 5′ UTR, 174 bases before the initiating methionine of PAS2. Heterozygous plants for this T-DNA insertion mutant were genotypically (Fig. S10), and no homozygous plants could be identified in the progeny. Siliques of heterozygous plants contained about 25% of aborted seeds, suggesting that homozygous pas2–3 mutants were embryo lethal and could not be recovered (Fig. 4A).

Because the Arabidopsis dehydratase corresponds to a single gene and its inactivation is lethal, we investigated whether PAS2 might represent a limiting step for VLFA synthesis. To limit possible transgene-derived silencing associated with the overexpression of PAS2, we used the orthologous yeast PHS1 to monitor the effect of increasing dehydratase activity on VLFA levels and on plant development. Several independent lines expressing PHS1 under the 35S promoter showed clear growth retardation associated with abnormal leaf development. Leaves from transgenic lines were smaller and crinkled, with pronounced serration and often an asymmetric development compared with that of control plants (Fig. 4B). Epidermal cells from PHS1-expressing transgenic leaves showed a large heterogeneity in cell sizes and shapes (Fig. 4C). Moreover, the surface of PHS1-expressing leaf epidermal cells was decorated with wax crystals, suggesting an increase in cuticular waxes in contrast to wild type (Fig. 4C). Flower development was also modified by PHS1 expression with, for instance, misshapen and unfused carpels (Fig. S11). Detailed analysis of cell surface of unfused carpel showed high accumulation of cuticular waxes (Fig. S11).

To confirm that ectopic expression of PHS1 could modify VLFA content, we analyzed fatty acid content of roots of young seedlings. The 35S:PHS1 seedlings showed significant changes in the relative distribution of VLFAs, with higher levels of 22:0 compared with wild type (Fig. 4D). Because VLFAs are also normally found in mature seeds, we investigated the effect of...
PHS1 expression on seed size and total fatty acid levels. Expression of PHS1 led to slightly larger seeds, whereas pas2 mutant showed smaller seeds compared with wild type (Fig. S11). Similar to the observation with seedlings, PHS1-expressing seeds showed an increase in VLCFAs, mostly 22:1 (Fig. 4E).

In conclusion, VLCFA dehydratase is not only an essential enzyme for plant growth and development, but it is also a limiting step for VLCFA synthesis because an increased dehydratase expression resulted in enhanced levels of VLCFAs in both vegetative and seed tissues.

Discussion
Collectively, our data demonstrated that the fatty acyl-CoA dehydratase is encoded by the PASTICCINO2 gene, and that it is an essential activity for plants. Similarly, the ketoacyl-CoA reductase activity in the double gl8ag8b maize mutant was also found to be essential (24). However, the loss of function of the enoyl-CoA reductase CER10, which is involved in the ultimate step in the acyl-CoA elongation cycle, is not lethal (unlike the yeast orthologue TSC13), suggesting that there is at least another partially redundant CER10 homologue in Arabidopsis (13). The weak pas2–1 allele is still able to produce some very-long-chain acyl-CoAs, resulting in strong reduction but not complete absence of VLCFAs. However, the channeling of these rare VLCFAs into different lipid classes is not unselective in pas2–1, indicating a previously unobserved level of regulation for the homoeostasis of different lipid types. The most straightforward explanation is that a leaky mutation such as pas2–1 would result in significant perturbations to the lipid pools with the highest turnovers. Another hypothesis is that small changes in some lipid pools (like GPCs) might lead to severe physiologic effects and that its homeostasis is maintained at the expense of less sensitive pools (like glucosyleramides). Alternatively, VLCFAs synthesis is compartmentalized differently and channeled independently for the different lipid pools. It was suggested, for instance, that VLCFA could be channeled into sphingolipids via an association of ceramide synthases with the elongase complex. This hypothesis was raised to explain the accumulation of medium-chain ceramide observed in yeast elongase mutants as in pas2 (25). It was previously thought that the limiting step in the elongase complex involved only the condensing enzymes (10). We demonstrated here that the dehydratase activity is also limiting for VLCFA synthesis. Interestingly, the overexpression of the condensing enzyme FAE1, as with PHS1, led to similar developmental alteration, such as asymmetric leaf shape. However, PHS1 enhanced wax deposition whereas FAE1 suppressed it, suggesting that both enzymes, despite being part of the same complex, could modify VLCFA homeostasis in different ways (20).

Contrary to the cer10 mutant, pas2–1 showed very severe developmental defects. In particular, pas2 was characterized by abnormal cell division that was enhanced in the presence of cytokinins, leading to callus-like structure (15, 16). It has to be noted that a similar phenotype was observed with weak mutations in the PAS3/GURKE gene, which codes the cytosolic acetylCoA carboxylase required for providing the malonyl-CoA to the elongase complex (26). The link between VLCFA and cell division was also reported in yeast (7, 27). In plants, the overexpression of PAS2 delayed cell cycle progression, in particular during mitosis (19). PAS2 was described as interacting directly with phosphorylated cell cycle regulator CDK1 (19). The presence of a protein kinase (PK) domain is conserved in eukaryotes led originally to the definition of the PAS2/Phs1 family as PKT-like proteins. However, recent structure/function analysis of Phs1p identified the catalytic residues involved in the dehydratase activity, and they do not belong to the PKT motif (9). We cannot exclude the possibility that the dehydratase function evolved recently from a PKT ancestor and that the PKT motif remained conserved across the PAS2/Phs1 proteins might still be involved in phosphorylation-related processes. First, protein alignment of 31 members of PAS2/Phs1p showed amino acid conservation of the PKT motif (9).

Then, the mutation of PAS2/Phs1p mammalian homolog PTPLA led to centronuclear myopathy in dogs, a disease related to mutations in the phosphoinositide phosphatase myotubularin MTM1 (28). PHS1 had the strongest epistatic interaction in yeast with the LCB phosphatase LCB3 (6). Moreover, the stability of the LCB kinase LCB4p is tightly regulated by the CDK PHOS5p (29). The involvement of CDK-dependent PTPC in the regulation of LCB or VLCFA metabolic enzymes remains to be investigated in plants, but it would provide an attractive model reunifying the apparent divergent PAS2 functions.

The nature of the PAS2-mediated VLCFA pathway that regulates cell division and cell differentiation is still unclear. Mutations downstream in the LCB and sphingolipid pathway will help in understanding the functional role of these different lipids in plant development. The fact that PAS2 fulfills a nonredundant essential activity also opens up the possibility of using tissue-specific RNAi inactivation to probe and better define the multiple roles of VLCFAs in plant form and function.

Methods

Plant Material and Growth Conditions. The pas2–1 mutants are ethyl methane sulfonate alleles in Col-0 background that were maintained as heterozygous stocks. Plants were grown in vitro and in a greenhouse in soil as described previously (30). The pPAS2::PAS2-GFP construct corresponds to the PAS2 genomic sequence with 1014 bp of promoter cloned into pMDC107. The pas2–3 T-DNA insertion line N617051 from the SALK collection was genotyped by PCR with the PAS2-specific primers F20 (5′-AAAAAGGAGCGTC-GAGCTGTTAGCTAC-3′) and R549 (5′-ACCGAAATCTCCAAATATC-3′) or T-DNA-specific primers Lb1 (5′-TTGTTACGAGATGGCCATCG-3′). Yeast strains and growth conditions were carried out as described previously (15).

Cytologic Analyses. Observations were carried out using an inverted TCS SP2-AOB5 spectral confocal laser microscope (Leica Microsystems) using either
Lipid Analyses. For mass quantification of lipid species, GPCs, glucosylceramides, and ceramides from A. thaliana wild type and mutant seedlings were extracted, isolated, and quantified as detailed in (31). Analysis of free long-chain bases of sphingolipids was adapted from Lester and Dickson (32). Cuticular lipids were extracted and analyzed as described previously (33). Total seed and leaf fatty acid were analyzed as reported by Li et al. (34). AcylCoA profiling and MS/MS analysis were carried out as described previously (8, 35). Detailed description of lipid analysis can be found in the SI Text.

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Supporting Information

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SI Methods

Neutral Lipids. To isolate neutral lipids, total lipids were loaded onto high-performance thin-layer chromatography plates developed in hexane/ethyl ether/acetic acid (90:15:2, vol/vol) and separated into diacylglycerols (RF 0.08), sterols (RF 0.17), fatty alcohols (RF 0.22), and free fatty acids (RF 0.29). Lipids were identified by comigration with known standards and quantified by densitometry analysis using a TLC scanner 3 (CAMAG).

Cuticular Waxes. Cuticular waxes were extracted by immersing stems and leaves of 2-month-old plants grown in glass-house tissues for 30 s in 20 ml of chloroform containing 20 µg of docosane as internal standard and subsequently dried to determine dry weights. Extracts were dried under a gentle stream of nitrogen, dissolved into 100 µl of BSTFA-TMCS [N,O-bis(trimethylsilyl)trifluoroacetamide]/trimethylchlorosilane (99:1)] and derivatized at 80°C for 1 h. Surplus BSTFA-TMCS was evaporated under nitrogen, and samples were dissolved in 200 µl hexane for analysis using an Agilent 6850 gas chromatograph and helium as carrier gas (1.5 ml/min). The gas chromatograph was programmed with an initial temperature of 80°C for 1 min and increased at 15°C/min to 260°C, held for 10 min at 200°C; increased again at 5°C/min to 320°C, and held for 15 min at 320°C. Qualitative analyses were performed using an HP-5MS column (30 m × 0.25 mm × 0.25 µm) and an Agilent 5975 MS detector (70eV, mass to charge ratio 50 to 750). Quantitative analyses based on peak areas and internal standard docosane was performed using an HP-1 column (30 m × 0.32 mm × 0.25 µm) and a flame ionization detector.

Acyl-CoA Profiling. Fifteen-milligram portions of plant material were frozen in liquid nitrogen and extracted for subsequent analysis of fluorescent acyl-etheno-CoA derivatives by HPLC (Agilent 1100 LC system; Phenomenex LUNA 150 × 2-mm C18(2) column). The identities of different acyl-CoA esters and their acyl-etheno-CoA derivatives were confirmed by electrospray ionization MS. Peak identities were confirmed by LC/MS carried out on a Thermoquest LCQ system with an electrospray ionization source according to a method adapted from Larson TR, Graham IA (2001) Plant J. 25(1):115–125. Standards, where available, were obtained from Sigma (>70% recovery from extraction).

Long Chain Bases. Samples (approximately 100 mg fresh weight) were freeze-dried after the addition of 1 nmol of internal standard (d20). Extraction was performed twice by grinding samples in 3 ml isopropanol/hexane/H2O (55:20:25, vol/vol/vol) followed by 15 min incubation at 60°C. After low-speed centrifugation, supernatants were collected, pooled, and dried under N2. Lipids were resuspended in 500 µl extraction buffer, and 100 µl were derivatized with 20 µl 10.5 mM (in acetonitrile) amin-oquinoyl-N-hydroxysuccinimidyl carbamate (AccQ Fluor reagent kit, Waters). Deacylation is achieved by incubating for 30 min at 37°C after addition of 15 µl 1.5N KOH in methanol and neutralized with 15 µl of 1.74N acetic acid in methanol. Finally, aliquots of 5–40 µl were analyzed on a 4.6 × 250-mm XBD-C18 column with a linear gradient (1.5 ml/min) of 75%–90% mobile phase. Similar procedure was used for yeast except that approximately 30 OD600 were fixed three times in cold TCA (%) before extraction.
**Fig. S1.** 
**PAS2** and **PHS1** but not PAS2-like isologue rescued yeast **PHS1** lethality. (A) Expression of **PAS2** restored **PHS1** lethality. (B) Expression of PAS2-like isologue At5g59770 did not rescue **PHS1** lethality.
Fig. S2. Yeast PHS1 and Arabidopsis pas2–1 mutants accumulate free LCBs. Free LCBs were extracted and analyzed by HPLC after AQC derivatization (see Methods). (A) Separation of LCB external standards. (B) Separation of free LCBs from 100 mg of wild-type Col0 plants. (C) Separation of free LCBs from 100 mg of pas2–1 plants. (D and E) Separation of free LCBs from wild-type yeast (D, PHS1) and phs1 mutant (E, phs1; pGAL1:PAS2) transformed with pGAL1:PAS2 grown on galactose-supplemented (Bottom) and glucose-supplemented (Top) rich media.
Fig. S3. Expression of PHS1 in Arabidopsis. Yeast PHS1 was cloned under the control of 35S promoter and transformed into pas2–1/H11001 plants. The expression of PHS1 was analyzed in segregating wild-type and pas2–1/pas2–1 plants by RT-PCR. PHS1 expression was compared with constitutive control EF1α.
Fig. S4. Scanning electron microscopy of stems from wild type (Left) and pas2–1 mutant (Right). (Insets) Details of wax flakes. (Scale bar, 50 μm.)
Fig. S5. Cuticular wax loads on leaves of wild type (Col0, white bars) and pas2−1 (black bars). Wax load was analyzed according to fatty acid chain length and side group, that is, primary alcohols (28OH, 28OH) and alkanes (Alk27, Alk29, Alk31, Alk33).
Fig. S6. Fatty acyl chain length composition of the ceramide (Cer) (A), hydroxyceramide (hCer) (B), and glucosylinositolphosphorylceramide (GIPC) (C) fractions from wild-type (Col0, white bars) and pas2–1 (black bars).
Fig. S7. PAS2 is localized in the ER and interacts with ECR/CER10 in *Nicotiana benthamiana* epidermal cells. (A) pPAS2:PAS2-GFP (Left) and ER marker CD3–959:mCherry (Middle) showed colocalization (merged, Right). (B) pPAS2:PAS2-GFP (Left) and 35S:CER10-mRFP1 (Right) showed colocalization (merged, Right). (C) Coexpression of 35S:YFP\textsuperscript{PH}-PAS2 and 35S:CER10-YFP\textsuperscript{C} (Right) showed BiFC of YFP (green) in presence of chloroplast autofluorescence (red). PAS2-YFP\textsuperscript{PH} and CER10-YFP\textsuperscript{C} did not interact with, respectively, AtDPL1-YFP\textsuperscript{C} (Left) and YFP\textsuperscript{PH}-AtDPL1 (Middle). (Scale bars, 10 μm in A and B, 40 μm in C.)
Fig. S8. MS identification of 3-hydroxy acyl-CoA intermediates in the acyl-etheno-CoAs of the pas 2–1 seedlings. LCMS data in the range m/z 1000–1250 are shown for the acyl-etheno-CoAs of the pas 2–1 seedlings. a, b, and c represent monitoring for an extracted ion over a 4-min window for the [M-H]⁻ ions 1074, 1102, and 1130, respectively, representing 3OHc18:0, 3OHc20:0, and 3OHc22:0, respectively. These ions were only identified in the pas 2–1 sample. Peak values refer to ion current. TIC, total ion current.

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Fig. S9. The complemented pas2–1, 35S:PHS1 line does not accumulate 3-hydroxy Acyl-CoA intermediates. The composition of the acyl-CoA pool was determined in wild type (Col0, A) and pas2–1, 35S:PHS1 (B) by extraction, derivatization, and HPLC analysis of acyl-etheno-CoAs. The internal standard (Istd) is 17:0 acyl-CoA.
**Fig. S10.** The mutant pas2–3 is associated with a T-DNA insertion in PAS2 5’ UTR. (A) Structure of PAS2 locus with the position of the T-DNA insertion relative to the two EMS alleles pas2–1 and pas2–2pep. Exons are indicated as gray boxes. Positions of oligonucleotides used for genotyping are indicated with arrows. (B) Genotyping of heterozygous pas2–3/+ plants segregating 1/4 lethal seeds.
**Fig. S11.** Effect of *PHS1* expression on flower and seed development. (A) Ectopic *PHS1* expression modifies carpel development (Top); SEM of wild-type flower with petals (p), anthers (a), and fused carpel (c) (Left) and unfused carpel from *PHS1* plant (Right). Ovules (arrow) are now visible. Bottom: detail of the surface of wild-type carpel with stomata (s) (Left) and *PHS1* carpel with stomata hidden under the wax (Right). (Inset) Detail of wax accumulation. (Scale bar, 500 μm for top, 50 μm for bottom, 2 μm for inset.) (B) Seed dry weight of *pas2−1* and *PHS1*-expressing plants compared with wild type.
Table S1. Relative levels of acyl-etheno-CoA according to the fatty acid chain length

<table>
<thead>
<tr>
<th>Acyl CoA</th>
<th>14:0</th>
<th>16:0</th>
<th>16:1</th>
<th>18:0</th>
<th>18:0−3OH</th>
<th>18:1</th>
<th>18:2</th>
<th>18:3</th>
<th>20:0</th>
<th>20:0−3OH</th>
<th>20:1</th>
<th>22:0−3OH</th>
<th>22:1</th>
</tr>
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<tbody>
<tr>
<td>Col 0</td>
<td>25.6 (7.5)</td>
<td>117.6 (24.7)</td>
<td>9.4 (3.9)</td>
<td>33.4 (11.2)</td>
<td>0</td>
<td>20.2 (5.3)</td>
<td>53.3 (15.4)</td>
<td>77.9 (21.5)</td>
<td>32.3 (6.7)</td>
<td>0</td>
<td>5.4 (1.9)</td>
<td>0</td>
<td>15.1 (5.1)</td>
</tr>
<tr>
<td>pas2-1</td>
<td>22.0 (7.9)</td>
<td>118.1 (33.5)</td>
<td>10.1 (5.1)</td>
<td>42.5 (14.2)</td>
<td>37.6 (15.2)</td>
<td>33.0 (9.2)</td>
<td>45.0 (21.4)</td>
<td>58.1 (30.6)</td>
<td>37.5 (15.3)</td>
<td>55.1 (32.3)</td>
<td>10.1 (3.9)</td>
<td>33.9 (13.7)</td>
<td>15.3 (11.6)</td>
</tr>
</tbody>
</table>

Values are expressed as fmol/mg f.wt⁻¹. Each value is the mean (SD) of several experiments (n = 4).