Acyl-CoA-Binding Protein ACBP1 Modulates Sterol Synthesis during Embryogenesis

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Fatty acids (FAs) and sterols are primary metabolites that exert interrelated functions as structural and signaling lipids. Despite their common syntheses from acetyl-coenzyme A, homeostatic cross talk remains enigmatic. Six Arabidopsis (Arabidopsis thaliana) acyl-coenzyme A-binding proteins (ACBP’s) are involved in FA metabolism. ACBP1 interacts with PHOSPHOLIPASE Dα1 and regulates phospholipid composition. Here, its specific role in the negative modulation of sterol synthesis during embryogenesis is reported. ACBP1, likely in a liganded state, interacts with STEROL C4-METHYL OXIDASE1-1 (SMO1-1), a rate-limiting enzyme in the sterol pathway. Proembryo abortion in the double mutant indicated that the ACBP1-SMO1-1 interaction is synthetic lethal, corroborating with their strong promoter activities in developing ovules. Gas chromatography-mass spectrometry revealed quantitative and compositional changes in FAs and sterols upon overexpression or mutation of ACBP1 and/or SMO1-1. aberrant levels of these metabolites may account for the downstream defect in lipid signaling: GLABRA2 (GL2), encoding a phospholipid/sterol-binding homeodomain transcription factor, was up-regulated in developing seeds of acbp1, smo1-1, and ACBP1+/−/− smo1-1 in comparison with the wild type. Consistent with the corresponding transcriptional alteration of GL2 targets, high-oil, low-mucilage phenotypes of gl2 were phenocopied in ACBP1+/−/− smo1-1. Thus, ACBP1 appears to modulate the metabolism of two important lipid classes (FAs and sterols) influencing cellular signaling.

Sterols are structural components that control eukaryotic membrane fluidity and permeability (Schaller, 2004). Unlike cholesterol, which is a principal animal sterol, there exist more than 100 plant sterols (i.e. phytosterols), prevalently as sitosterol, campesterol, and stigmasterol (Hartmann, 1998). Sterols are primary metabolites synthesized from cycloartenol, the first stable tetracyclic derivative from acetyl-CoA via the isoprenoid pathway (Bach, 1995). Functional sterols are produced after the removal of two C4 methyl groups from cycloartenol, which is catalyzed successively by a multienzyme complex containing STEROL C4-METHYL OXIDASE (SMO) in animals and fungi (Li and Kaplan, 1996). In plants, demethylation occurs nonconsecutively under the catalysis of nonhomologous SMO1 and SMO2, which are encoded by three and two loci in Arabidopsis (Arabidopsis thaliana), respectively (Darnet and Rahier, 2004). Five of the six steps in the early sterol pathway have been investigated using Arabidopsis mutants that exhibited embryonic and vascular patterning defects (Diener et al., 2000; Jang et al., 2000; Schrick et al., 2000, 2002; Souter et al., 2002; Willemsen et al., 2003; Kim et al., 2005; Men et al., 2008). SMO1 catalyzes the remaining one that has not been studied by reverse genetics but shown to be rate limiting, representing a potential target for enhancing flux toward higher phytosterol accumulation (Lange et al., 2015). Its catalytic product also is known to regulate polar auxin transport (Mialoundama et al., 2013). Studies on sterol biosynthetic mutants have indicated the existence of other sterol-derived signals that shape plant development, although many remain unknown (Carland et al., 2010; Qian et al., 2013; Zhang et al., 2015). Its catalytic product also is known to regulate polar auxin transport (Mialoundama et al., 2013). Studies on sterol biosynthetic mutants have indicated the existence of other sterol-derived signals that shape plant development, although many remain unknown (Carland et al., 2010; Qian et al., 2013; Zhang et al., 2015).

Downstream protein participants in sterol-mediated signaling are better understood. Sterols are bound by the steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) domain (Ponting and Aravind, 1999), which facilitates StAR-mediated cholesterol transport in animal mitochondria (Stocco, 2001). In plants, this domain is distributed mostly within the homeodomain (HD)-Leu zipper transcription factor (TF) family (Schrick et al., 2004), of which the...
unidentified ligands are deemed important developmental and stress regulators (Yu et al., 2008). The Arabidopsis genome encodes 21 HD-START TFs, most of which regulate cell fate determination and patterning (Schrick et al., 2014). Among them, GLABRA2 (GL2) is the sole known member that negatively regulates seed oil biosynthesis and promotes seed coat mucilage formation (Shen et al., 2006; Shi et al., 2012). Whether the START domain is essential for GL2 function had remained unclear until recent findings that its truncation or site-directed mutagenesis affected the complementation of Arabidopsis gl2 phenotypes (Schrick et al., 2014). The START domain of GL2 was shown recently to bind phospholipids (PLs) in addition to sterols (Schrick et al., 2014), corroborating earlier evidence that GL2 modulates PL signaling in root hair patterning (Ohashi et al., 2003). While PLs and sterols functionally converge on the same downstream target (START domain) in signal transduction, their close metabolic relationship also is inferred from a reaction that esterifies them into fatty acyl sterol esters (Schaller, 2004; Bouvier-Navé et al., 2010).

Acetyl-CoA also fuels the synthesis of fatty acids (FAs), which are used for acyl-lipid (e.g. PLs and triacylglycerols) assembly after their activation into CoA esters (Block et al., 1983). In the cytosol, these esters are maintained homeostatically by nonenzymic proteins such as acyl-CoA-binding proteins (ACBPs; Xiao and Chye, 2011). Apart from cytosolic members, there exist other plant ACBPs that also are conserved to bind acyl-CoA esters and PLs (Lung and Chye, 2016a). The six Arabidopsis ACBPs are grouped into four classes by size and domain architecture (Meng et al., 2011), which are linked differently to plant development and stress responses (Xiao and Chye, 2011; Du et al., 2016; Lung and Chye, 2016b). ACBP1 and its homolog, ACBP2, are targeted to the endoplasmic reticulum (ER) and the plasma membrane (Chye, 1998; Chye et al., 1999; Li and Chye, 2003). Both proteins are highly expressed during seed development, and the acbp1acbpb2 double mutant is embryo lethal (Chen et al., 2010). While ACBP1 binds very-long-chain acyl-CoA esters and contributes to stem cuticle synthesis (Xue et al., 2014), its affinity to phosphatidylcholine (PC) and phosphatic acid (PA) is pertinent to PL signal transduction (Du et al., 2010, 2013). ACBP1 interacts with PHOSPHOLIPASE Dα1 (PLD1α) at the plasma membrane to regulate the generation of PA, a messenger for abscisic acid signaling (Du et al., 2013). acbp1 is more freezing tolerant, as its rosettes contain a lower level of PLD1α-derived PA, a damaging agent of biomembranes during cold stress (Du et al., 2010). In siliques, acbp1 exhibits an aberrant PL composition (Chen et al., 2010).

Other than its roles in PL metabolism and signaling, ACBP1 is reported here to negatively modulate sterol synthesis during embryogenesis via protein-protein interaction (PPI) with another partner, SMO1-1. Reverse genetics studies revealed a relationship between FA homeostasis and sterol production as linked by ACBP1. The aberrant FA and sterol compositions of mutant siliques may account for the downstream defect in lipid-mediated signaling that involves GL2.

**RESULTS**

ACBP1 and SMO1-1 Are ER-Localized Protein Partners

Yeast two-hybrid (Y2H) screens of an Arabidopsis cDNA library using ACBP1 as bait had identified a partial in-frame sequence of SMO1-1 (Tse, 2005). This PPI was confirmed by Y2H analysis using the soluble region (i.e. residues 41–338) of ACBP1 and the open reading frame (ORF) encoding SMO1-1 (Fig. 1A). SMO1-1 did not bind ACBP1<sub>32–212</sub>, lacking the C-terminal ANK domain (Fig. 1A), a PPI structure (Vo et al., 2015). The absence of blue colonies using ANK alone (i.e. ACBP1<sub>217–338</sub>) further suggested that both ACB and ANK domains are essential for PPI (Fig. 1A). To study if a functional ACB domain is required, the conserved Tyr at 171 was substituted with Ala, because an equivalent substitution had interrupted 16:0-CoA binding to recombiant ACBP2 (Chye et al., 2000). In Y2H screens, this mutation weakened PPI, implying that a ligand form of ACBP1 may facilitate SMO1-1 interaction (Fig. 1A).

To investigate the PPI site, SMO1-1 was expressed in fusion with an enhanced GFP (EGFP) in transgenic Arabidopsis. SMO1-1:EGFP was detected at the perinuclear ER in guard cells and ER bodies in root cells, apart from the tubular ER network, by confocal laser scanning microscopy (Fig. 1B, top). In root hair cells, signals were visualized at the plasma membrane and colocalized at the membrane of ER-derived vesicles with the ER marker (Fig. 1B, bottom). In Arabidopsis seedlings, SMO1-1:EGFP was colocalized with DsRed:ACBP1 at the membrane of ER-derived vesicles in root cells and the ER cisterna in hypocotyl cells, in addition to the plasma membrane of both cell types (Fig. 1C). In agroinfiltrated tobacco (*Nicotiana tabacum*) leaf epidermal cells, the signals were colocalized at the plasma membrane, reticular ER network, and perinuclear ER (Fig. 1C, bottom). These observations coincided well with the predicted location of SMO1-1 (Supplemental Fig. S1A) and the reports of ACBP1 (Chye, 1998; Li and Chye, 2003) at the ER, the site of sterol biosynthesis (Benveniste, 2004).

To further detect the ACBP1-SMO1-1 complex, Arabidopsis was transformed with 35S::SMO1-1:HA:StrepII for Strep-Tactin pull-down and coimmunoprecipitation assays. These plants also served as overexpressors (OEs) in subsequent studies. First, subcellular fractionation showed that SMO1-1:HA:StrepII was enriched in the membrane fraction and less abundant in the fraction of large particles containing mitochondria, plastsids, and peroxisomes (Fig. 1D). The trace amount of nucleus-associated signals (Fig. 1D) could be attributed to the perinuclear localization arising from the connection of nuclear envelopes with ER membranes (Fig. 1, B and C). A similar subcellular distribution of ACBP1 was observed (Fig. 1D, right), consistent with a previous observation.
Subsequently, SMO1-1:HA:StrepII enriched in the membrane fraction was solubilized and captured on Strep-Tactin beads for pull-down assays. Western-blot analysis of coeluted proteins using ACBP1 antibodies produced a cross-reacting band from SMO1-1:HA:StrepII but not the vector control (Fig. 1E). Reciprocally, ACBP1 antibodies coimmunoprecipitated SMO1-1:HA:StrepII from the solubilized membrane fraction (Fig. 1F).

Figure 1. PPI of ACBP1 with SMO1-1. A, Y2H assays. The soluble domain of ACBP1, its derivative without the ankyrin repeat (ANK) or acyl-CoA-binding (ACB) domains, and its mutated (Y171A) versions were cloned into the bait vector. The full-length SMO1-1 sequence was cloned into the prey vector. Cotransformants of bait and prey constructs were verified on double dropout (DDO) plates. The appearance of blue colonies on triple dropout selection plates (TDO/X/A) indicates PPI. The pGADT7-T construct was cotransformed with pGBK7-53 and pGBK7-Lam as positive and negative controls, respectively. B, Localization of SMO1-1:EGFP in transgenic Arabidopsis. Leaf epidermal cells of 3-week-old plants and root cells of 1-week-old seedlings were imaged by confocal laser scanning microscopy. Signals were detected at the perinuclear ER (white arrowheads), ER bodies (open arrowheads), and tubular ER network throughout the cells. Signals were colocalized at the membrane of ER-derived vesicles (red arrowheads) using the ER-Tracker in root hair cells (bottom). Bars = 20 μm. C, Colocalization of SMO1-1:EGFP with DsRed:ACBP1. Root and hypocotyl cells of 1-week-old transgenic Arabidopsis seedlings and agroinfiltrated tobacco leaf epidermal cells were imaged by confocal laser scanning microscopy. Signals were colocalized at the plasma membrane, membrane of ER-derived vesicles (red arrowheads), ER cisternae (arrows), and perinuclear ER (white arrowheads). Bars = 20 μm. D, Subcellular fractionation of SMO1-1:HA:StrepII and ACBP1. Proteins (20 μg per lane) from total crude extracts (T), nuclei (N), membranes (M), large particles including mitochondria, plastids, and peroxisomes (LP), and soluble fractions (S) were prepared from aboveground tissues of 6-week-old Arabidopsis and analyzed by western-blot analysis and Coomassie Blue-stained gels. Total proteins from an empty vector line (VC) served as a negative control. Arrowheads indicate the positions of the expected bands (37 kD for SMO1-1:HA:StrepII and 38 kD for ACBP1). E, Strep-Tactin pull-down assays. Membrane proteins were isolated from transgenic Arabidopsis of SMO1-1:HA:StrepII and the vector control, solubilized, and incubated with Strep-Tactin beads. Eluents were analyzed by western-blot analysis using anti-ACBP1 and anti-HA antibodies. F, Coimmunoprecipitation of SMO1-1:ACBP1 complexes. Membrane proteins were prepared as described in E and incubated with anti-ACBP1 antibodies that had been covalently coupled to Affi-Gel 10 beads. Eluents were analyzed by western-blot analysis using anti-ACBP1 and anti-HA antibodies.
together, it is concluded that both ACB and ANK domains of ACBP1 are essential for its association with SMO1-1 at the ER, which is likely facilitated if ACBP1 is in a liganded state.

SMO1-1 Is Expressed in Floral Organs and Developing Seeds

Quantitative real-time (qRT)-PCR detected SMO1-1 transcripts in rosettes, stems, roots, floral buds, flowers, and siliques (Fig. 2A). Its expression was up- or down-regulated in all aboveground tissue samples of acbp1, indicating the functional relevance of SMO1-1 with ACBP1 (Fig. 2A). Microarray data showed that SMO1-1 and ACBP1 were expressed in seeds and dissected embryos from zygotic to maturation stages throughout embryogenesis (Supplemental Fig. S2, B and C). Promoter-driven GUS assays had indicated ACBP1 expression in embryos and floral parts, including stigmas, styles, ovaries, and sepalas (Du et al., 2013). SMO1-1pro:GUS lines were generated for comparative studies. Signals were detected in pollen at all floral stages and in pollen tubes at anthesis (Fig. 2B). Microscopically, SMO1-1pro:GUS was observed in guard cells of sepalas and anthers (Fig. 2, C and D) and the micropylar end of fertilized ovules at stage 13 (Fig. 2E). At stage 14, it was found in the egg apparatus and, to a lesser extent, in elongated embryo sacs (Fig. 2F). At the quadrant embryo stage, SMO1-1pro:GUS was visualized in the basal suspensor cells and chalazal endosperm (Supplemental Fig. S2A). At maturation, it was expressed in cotyledons of embryos (Supplemental Fig. S2, D–F), consistent with its immunolocalization pattern (Chye et al., 1999). Hence, microarray data mining, qRT-PCR, and GUS assays suggested the coexpression of SMO1-1 and ACBP1 in reproductive tissues, where functional significance was addressed subsequently by reverse genetics.

ACBP1 and SMO1-1 Mutation Led to Reproductive Phenotypes

A T-DNA insertion line was first characterized to be a knockdown mutant of smo1-1 by reverse transcription (RT)-PCR (Fig. 3, A–C). qRT-PCR revealed that its SMO1-1 levels in various tissues were 70% to 80% lower than in the wild type (Fig. 3D). This mutant was crossed subsequently with the acbp1 knockout mutant. After genotyping more than 100 F2 and F3 progeny, ACBP1+/- smo1-1 (i.e. hemizygous for acbp1 and homozygous for smo1-1) and acbp1SMO1-1+/- were obtained without encountering double homozygotes. The locations of ACBP1 and SMO1-1 on different chromosomes (chromosomes 5 and 4, respectively) ruled out a linkage relationship, implying a synthetic-lethal interaction of the two loci. The double mutant was propagated.
as ACBP1+/−smo1-1 (since acbp1 was Basta resistant and smo1-1 lacked antibiotic resistance), of which selfed progeny gave a Basta-resistant to Basta-sensitive ratio of 2.19:1, deviating from the 3:1 Mendelian ratio (Supplemental Table S1). As controls, selfed progeny of double hemizygotes and those of ACBP1+/−smo1-1 complemented with 35S:SMO1-1 (cSMO1-1) exhibited a 3:1 ratio (Supplemental Table S1). In mature green siliques of selfed ACBP1+/−smo1-1, high frequencies of aborted and senesced ovules were observed (Fig. 4A). The 25% abortion rate was higher than in the wild type and complemented lines (2.4%–2.8%) and close to the 1:3 Mendelian ratio (Fig. 4B). These results implied fatal arrest postfertilization rather than gametophytic sterility, which would have yielded a 1:1 ratio. In selfed ACBP1+/−smo1-1 at 3 to 4 d after flowering (DAF), some fertilized ovules had shrunken while others were normal (Fig. 4C). aborted acbp1smo1-1 proembryos displayed distorted-shaped suspensors (Fig. 4D). To further discriminate embryo lethality from gametophytic defects, ACBP1+/−smo1-1 was crossed reciprocally with the wild type. Irrespective of whether ACBP1+/−smo1-1 was a pollen donor or a maternal line, ACBP1smo1-1 and acbp1smo1-1 gametes were carried forward at efficiencies not different (P > 0.05) from the hypothesized 1:1 ratio (Fig. 4E). Taken together, the data indicated that acbp1smo1-1 was synthetic lethal at the proembryo stage.

Besides embryogenesis, developmental phenotypes of ACBP1+/−smo1-1 were examined. Macroscopic examination of inflorescences showed fewer floral buds than in the wild type, while the size and morphology of their floral buds and open flowers were normal (Fig. 5A). ACBP1+/−smo1-1 showed a decline in silique number (−66%) and length (−8%) and a lighter seed weight (−10%), accompanied by the reduced germination rate (89.5%), when compared with the wild type (94.5%; Fig. 5, B–E). The lower seed quality also compromised postgerminative growth, as reflected by the shorter (−41%) primary root length of seedlings (Fig. 5F).

**Sterols and FAs Were Altered in Mutant and OE Lines of ACBP1 and SMO1-1**

To justify the phenotypic aberrance of ACBP1+/−smo1-1 by a shift in FA-sterol homeostasis, sterols were analyzed in siliques by gas chromatography-mass spectrometry (GC-MS). The total content and composition of major sterols in the mutant, complemented, and OE lines of SMO1-1 and ACBP1 were compared with those of the wild type. In mature green siliques, the level of β-sitosterol, a predominant species in Arabidopsis, was reduced in smo1-1 (−36%) but elevated in acbp1 (+54%), while their opposing effects were negated in ACBP1+/−smo1-1 (Fig. 6A). Campesterol was less abundant in smo1-1 (−36%) but enriched in SMO1-1 OEs (+21% to +25%), acbp1 (+40%), and ACBP1+/−smo1-1 (+21%; Fig. 6B), Stigmastanol was elevated in mature green siliques of acbp1 (+110%) and ACBP1+/−smo1-1 (+110%) as well as in 1- to 2-DAF siliques of acbp1 (+30%) and ACBP1+/−smo1-1 (+48%; Fig. 6C), whereas their cholesterol levels remained unchanged (Fig. 6D). In fact, SMO1-1 does not act on...
cycloartenol (a cholesterol precursor), but its C24 methylated toward sitosterol, campesterol, and stigmasterol production (Diener et al., 2000). Overall, the total sterol content in mature green siliques was higher in acbp1 (+51%) and ACBP1+/−smo1-1 (+20%) and lower in smo1-1 (−35%; Fig. 6 E). FA methyl esters from mutants and OEs also were compared with the wild type by GC-MS. The total FA level was higher (+24%) in mature green siliques of acbp1, while the 1- to 2-DAF and mature green siliques of smo1-1 (+27% and +11%) and ACBP1+/−smo1-1 (+20% and +9%) also accumulated more FAs (Table I). With respect to acyl composition, no change was observed with FA species derived from the ER-localized eukaryotic pathway, including 18:2-, 18:3-, 20:0-, 20:1-, 20:2-, 20:3-, 22:0-, and 22:1-FAs (Table I). In 1- to 2-DAF siliques, mutation in SMO1-1 and/or ACBP1 increased 16:3-FA at the expense of 18:0-FA, whereas 18:1Δ9-FA was elevated in smo1-1 and ACBP1 OEs (Table I). In mature green siliques, the 35S:ACBP1 transgene enhanced 18:0-FA, while plastidal 16:1- and 16:3-FAs decreased in smo1-1 but increased in SMO1-1 OEs at the expense of 18:0-FA (Table I). Similar changes were not detected in ACBP1+/−smo1-1, possibly due to the negating effect of ACBP1 mutation that boosted 16:1- and 16:3-FAs (Table I). Taken together, the homeostasis of sterols and FAs was affected quantitatively and compositionally upon manipulating SMO1-1 and/or ACBP1 expression.

ACBP1 and SMO1-1 Mutation Affected GL2 Function

The higher FA accumulation in mature green siliques of smo1-1, acbp1, and ACBP1+/−smo1-1 (Table I) coincided with the high-oil phenotype of gl2 seeds (Shen
et al., 2006; Shi et al., 2012). Hence, it was investigated if the aberrant sterol and FA compositions affected the activity of PL/sterol-binding GL2, a downstream participant in lipid signaling. qRT-PCR revealed a significant \( (P, \leq 0.01) \) up-regulation of \( GL2 \) in developing seeds and siliques of \( smo1-1 \), \( acbp1 \), and \( ACBP1+/- smo1-1 \) over the wild type (Fig. 7A). The expression of a GL2 target, \( MUCILAGE MODIFIED4 (MUM4; Shi et al., 2012) \), was reduced in \( smo1-1 \) and \( acbp1 \) and more drastically in \( ACBP1+/- smo1-1 \) over the wild type (Fig. 7A). The expression of a GL2 target, \( MUCILAGE MODIFIED4 (MUM4; Shi et al., 2012) \), was reduced in \( smo1-1 \) and \( acbp1 \) and more drastically in \( ACBP1+/- smo1-1 \) over the wild type (Fig. 7A). Consistently, \( ACBP1+/- smo1-1 \) seeds failed to accumulate mucilage, as indicated by Ruthenium Red staining (Fig. 7B). In agreement with the direct suppression of the \( PLD\alpha1 \) promoter by \( GL2 \) (Liu et al., 2014), \( PLD\alpha1 \) levels were higher in \( smo1-1 \), \( acbp1 \), and \( ACBP1+/- smo1-1 \) (Fig. 7A). In these genotypes, up-regulation of \( PHOSPHOLIPID:STEROL ACYLTRANSFERASE1 (PSAT1) \) further indicated a change in PL and sterol metabolism (Fig. 7A).

**DISCUSSION**

**ACBP1 Cooperates with SMO1-1 during Early Embryogenesis**

Previously, knockouts of \( ACBP1 \) and its homolog \( ACBP2 \) arrested embryo development, possibly due to an aberrant PL composition (Chen et al., 2010). In this study, a discrete role of \( ACBP1 \) in modulating sterol synthesis via PPI with SMO1-1 is reported. The importance of their interaction is reflected by the severe proembryo abortion phenotype of \( acbp1 smo1-1 \) (Fig. 4). \( acbp1 smo1-1 \) differed from all other early sterol
Figure 6. Major sterol content of mutant, complemented, and OE lines of SMO1-1 and ACBP1. Phytosterols were extracted from young (1–2 DAF) and mature green siliques of 6- to 7-week-old Arabidopsis and analyzed by GC-MS. Each bar represents the mean of four replicates ± SE. H and L indicate statistically significant (P < 0.01) elevation and reduction, respectively, in comparison with the wild type by Student’s t test. A, β-Sitosterol. B, Campesterol. C, Stigmasterol. D, Cholesterol. E, Total phytosterols. DW, Dry weight.
### Table 1. Fatty acid content and composition of T- to 2-DAF and mature green siliques from mutant complemented, and OF lines of SMO1-1 and ACBP1

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>16:0</th>
<th>16:1</th>
<th>16:3</th>
<th>18:0</th>
<th>18:1</th>
<th>20:2</th>
<th>20:3</th>
<th>22:0</th>
<th>22:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature green</td>
<td>0.13</td>
<td>0.03</td>
<td>0.01</td>
<td>0.26</td>
<td>1.02</td>
<td>0.06</td>
<td>0.02</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
<td>Mature</td>
<td>0.17</td>
<td>0.04</td>
<td>0.01</td>
<td>0.26</td>
<td>1.02</td>
<td>0.06</td>
<td>0.02</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
<td>cACBP1</td>
<td>0.13</td>
<td>0.03</td>
<td>0.01</td>
<td>0.26</td>
<td>1.02</td>
<td>0.06</td>
<td>0.02</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
<td>ACBP1+/-</td>
<td>0.17</td>
<td>0.04</td>
<td>0.01</td>
<td>0.26</td>
<td>1.02</td>
<td>0.06</td>
<td>0.02</td>
<td>0.07</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Note: ND indicates not detected.
biosynthetic mutants with embryonic abnormalities detected at or beyond the globular stage, and their lethality (if any) occurred postembionically (Diener et al., 2000; Jang et al., 2000; Schrick et al., 2000, 2002; Souter et al., 2002; Willemsen et al., 2003; Kim et al., 2005; Men et al., 2008). Coincidentally, smo2-1smo2-2 is the only embryo-lethal genotype among all mutants in the entire phytosterol pathway characterized thus far (Zhang et al., 2016), although SMO1 and SMO2 are catalytically independent (Darnet and Rahier, 2004). Unlike the arrest of acbp1smo1-1 at the preglobular stage (Fig. 4D), smo2-1smo2-2 embryos were aborted at globular to heart-like stages (Zhang et al., 2016). Consistent with specific SMO1-1pro:GUS signals at the micropylar end in the egg apparatus (Fig. 2, E and F) and basal cells (Supplemental Fig. S2A), acbp1smo1-1-proembryos were aborted with collapsed suspensors (Fig. 4D), likely from loss in cell turgidity (Almagro et al., 2013).
As sterols regulate membrane fluidity and osmotic permeability (Schaller, 2004), the distortion of suspensor cells could arise from malfunctioning membranes with nonphysiological sterol content, reminiscent of the increased pathogen susceptibility upon knockout of STEROL METHYLTRANSFERASE2 (SMT2; Wang et al., 2012). Suspensor cell vacuolation could be affected by abnormal sterol-lipid distribution, analogous to the vacuolar fragmentation in yeast lacking oxysterol-binding proteins (Beh and Rine, 2004). Besides, the sterol-mediated formation of microdomains to which sterol-dependent proteins bind may be affected by an imbalance in sterol homeostasis (Hartmann, 1998). Recently, the smt1 proteome revealed changes in the microdomain association of many transporters and enzymes, including those in FA and acyl-lipid synthesis, sterol glycosylation, and vesicle trafficking (Zauber et al., 2014). Similar changes may account for the severe acbp1smo1-1 phenotype (Fig. 4).

Furthermore, in several sterol biosynthetic mutants, cell signaling is compromised by altered membrane sterol composition, which affects endocytosis and polar auxin transport (Men et al., 2008; Zhang et al., 2016). Phospholipases as lipid signal generators rely on microdomain association (Gardiner and Marc, 2013). For instance, phospholipase C was less active in smt1 when it became less abundant in the sterol-associated membrane fraction (Zauber et al., 2014). ACBP1 cooperates with PLDα1 and PLDδ in the freezing stress response (Du et al., 2010) and partners with PLDα1 in abscisic acid signaling (Du et al., 2013). Considering its interactions with PLDα1 (Du et al., 2013) and SMO1-1 (Fig. 1), which generate PL (Wang, 2001) and sterol (Mialoundama et al., 2013) signals, respectively, ACBP1 is positioned to modulate cross talk between the two lipogenic signaling pathways. The magnitude of PL or sterol change may not be drastic enough to affect embryogenesis in single mutants. In acbp1smo1-1, the cumulative variation of both lipidic signals may result in proembryo lethality when the dual signaling pathways converge on shared downstream targets, as represented by HD-START TFs (Schrick et al., 2004).

### ACBP1 Links FA Homeostasis to Sterol Production

To provide insight into the biochemical significance of the ACBP1-SMO1-1 interaction, sterols (Fig. 6) and FAs (Table I) were analyzed in mutant, complemented, and OE lines. Resembling other sterol biosynthetic mutants (Diener et al., 2000; Jang et al., 2000; Schrick et al., 2000, 2002; Kim et al., 2005; Men et al., 2008; Carland et al., 2010; Qian et al., 2013; Zhang et al., 2016) and SMO1-silenced tomato (Solanum lycopersicum) and Nicotiana benthamiana (Sonawane et al., 2016), smo1-1 accumulated abnormal levels of end-product sterols (Fig. 6). The altered sterol profile of acbp1 suggested a role for ACBP1 in sterol metabolism, and changes in ACBP1+/-smo1-1 further revealed semidominant effects of the ACBP1 mutation (Fig. 6). Differences in the sterol composition of ACBP1+/-smo1-1 in comparison with acbp1 and smo1-1 (Fig. 6) explained its phenotypic aberrance in plant development not seen in single mutants (Fig. 5). For instance, the poor silique development of ACBP1+/-smo1-1 (Fig. 5) was consistent with its shifted ratio of campesterol to sitosterol (Fig. 6), a crucial factor for Arabidopsis growth (Schaeffer et al., 2001). Among all genotypes tested, ACBP1+/-smo1-1 siliques also contained the highest level of stigmasterol (Fig. 6), a cell proliferation modulator (Hartmann, 1998). In mature green siliques, down-regulation of SMO1-1 reduced the total sterol content, whereas ACBP1 mutation produced an opposing effect and complemented the change in smo1-1, demonstrating that ACBP1 inhibits SMO1-1 function (Fig. 6). The inhibitory effect of ACBP1 likely occurs in its liganded form, because site-directed mutagenesis at its ACB domain weakened the interaction with SMO1-1 (Fig. 1A). Accordingly, the ligand-binding status of ACBP1 may be a cue that reflects the cellular levels of FA esters and determines the sterol synthetic rate, indicating a link between FA homeostasis and sterol production in plants. This PPI-mediated regulation contrasts with mammalian systems, in which ABCPs regulate cholesterol homeostasis at the transcriptional level in conjunction with sterol regulatory element-binding protein1 (Nees et al., 2006; Oikari et al., 2008), which modulates STAR gene expression (Yokoyama et al., 1993).

ABCs are analogous to some ATP-binding cassette (ABC) transporters that mobilize acyl-CoA esters and PLs (Du et al., 2016). Recently, ABCG9, ABCG11, and ABCG14 were postulated to mediate the long-distance transport of sterols or sterol conjugates in the phloem sap (Le Hir et al., 2013), besides their roles in cuticle formation (Hwang et al., 2016). Given the functional resemblance of ACBP1 in stem cuticle synthesis and its high expression in the vasculature (Du et al., 2013; Xue et al., 2014), it is pertinent to study whether these plasma membrane-localized proteins cooperate in sterol transport. Similar to other sterol biosynthetic mutants (Zauber et al., 2014), abcg9, abcg11, and abcg14 exhibited FA compositional changes (Le Hir et al., 2013), supporting an interrelationship of sterol and FA metabolism. In this study, FA profiles (particularly 16:0- and 18:0-FAs) were significantly altered in mutants and OE of SMO1-1 and ACBP1 (Table I). As 16:0- and 18:0-FAs were the two major species elevated in Arabidopsis OEs of acyl-CoA:sterol acyltransferase with substrate specificity for 16:0- and 18:0-CoA esters (Chen et al., 2007), our results suggest a regulatory role for ACBP1 in sterol acylation via its association with these acyl-CoA species. Deviations of 16:1- and 16:3-FA levels in mutants and OEs of SMO1-1 and ACBP1 also revealed a compositional change in plastidial lipids (Table I). This observation is consistent with a previous analysis of acbp1 silicates indicating acyl compositional variations in galactolipids and phosphatidylglycerol, which are crucial thylakoid membrane components for embryo and cotyledon development (Chen et al., 2010). Although ACBP1 does not function in plastids, the
The Potential Role of the ACBP1-SMO1-1 Interaction in Lipid Signaling

Among several transcriptional regulators of seed oil synthesis, GL2 is the only known TF that binds lipids and sterols via its START domain (Schrick et al., 2014). Similar to the boosted FA levels (9%-24%) in mature green siliques of acbp1, smo1-1, and ACBP1+/-smo1-1 (Table I), Arabidopsis gl2 seeds accumulated 8% more oil than the wild type (Shen et al., 2006; Shi et al., 2012). Resembling gl2 (Shi et al., 2012), a loss in seed coat mucilage was observed in ACBP1+/-smo1-1 but not in acbp1 and smo1-1 single mutants (Fig. 7B), indicating a semidominant mutation of ACBP1 when SMO1-1 was knocked down simultaneously. Possibly, a cumulative change in ACBP1 and SMO1-1 protein levels was sufficient to affect lipid signaling, in agreement with the ectopic root hair formation in hemizygous gl2 mutants in a semidominant fashion (Masucci et al., 1996). Considering the concomitant transcriptional regulation of GL2 and its downstream targets (Fig. 7A), the high-oil, low-mucilage phenotype of ACBP1+/-smo1-1 may be attributed to a reduction in GL2 activity when concentrations of its ligands (PL and sterols) are suboptimal. The up-regulation of GL2 at the mRNA level may be a compensatory response to its loss of transactivating activity at the protein level, given that GL2 expression is regulated transcriptionally via a positive feedback loop involving other TFs (Khosla et al., 2014). In our proposed model (Fig. 7C), ACBP1 may sense the metabolic status of a cell by its ability to bind acyl-CoA esters and PLs (Chye, 1998; Chen et al., 2010; Du et al., 2010, 2013). It may then modulate the synthesis of PC and sterol signals via PPI with PLDα1 (Du et al., 2013) and SMO1-1 (Fig. 1), respectively.

The fate of PC and sterol signals also may be influenced by PSAT1 activity, given its transcriptional regulation in acbp1 and smo1-1 mutants (Fig. 7A). By altering the cellular levels of these lipid signals, ACBP1 may modulate the transcriptional regulatory activity of downstream targets, including GL2. Schrick et al. (2014) reported that deletion of the START domain or site-directed mutagenesis of its lipid-binding residues abolished GL2 activity. By analogy, varying its ligand concentrations may result in similar effects, as shown here. In fact, the involvement of GL2 in PL- and steroid-mediated signaling has been proposed previously (Ohashi et al., 2003; Kuppusamy et al., 2009). Besides GL2, other HD-START TFs are promising subjects for future studies. The proembryo abortion of acbp1smo1-1 (Fig. 4) may be linked to some HD-START TFs. For instance, ARABIDOPSIS THALIANA MERISTEM LAYER1 and PROTODERMAL FACTOR2 have been ascribed a role in embryogenesis, as double mutants were arrested at the globular stage (Ogawa et al., 2015). Triple mutants lacking CORONA, PHABULOSA, and PHAVOLUTA also displayed ovule abnormalities (Kelley et al., 2009). HOMEODOMAIN GLABROUS9 is another candidate, given its specific expression in the embryo sacs (Nakamura et al., 2006), coinciding with the SMO1-proGUS expression pattern (Fig. 2, E and F). Sterol intermediates and derivatives per se could act as signals for embryonic and postembryonic development (Carland et al., 2010; Qian et al., 2013; Zhang et al., 2016), including the catalytic product of SMO1 (Mialoundama et al., 2013). It remains to be explored whether the ACBP1-SMO1-1 interaction regulates the generation of an as yet unidentified signal that binds to any HD-START protein(s) during embryogenesis.

Potential Applications for Plant Oil Modification

In the interest of promoting phytosterol intake for lowering blood cholesterol (Moreau et al., 2002) and cancer therapies (Woyengo et al., 2009), higher phytosterol content in seeds is a nutraceutically valuable trait in plant seed oil production. In this study, the phytosterol increase in mature green siliques of acbp1 and ACBP1+/-smo1-1 (Fig. 6) opens up a new strategy for boosting phytosterol production in seed crops. It is plausible to identify and deplete a SMO1-interacting equivalent of ACBP1 in crop species to eliminate its inhibitory effect on sterol biosynthesis, as is the case in Arabidopsis (Fig. 6). It may present a promising approach to push the flux toward end-product sterol production, because SMO1 catalyzes one of the rate-limiting steps in sterol biosynthesis (Lange et al., 2015). If the carbon partitioning of photoassimilates in developing seeds shifts toward a higher accumulation of lipids (sterols and FAs), as observed in acbp1 and ACBP1+/-smo1-1 (Table I; Fig. 6), the genetically modified crops will exhibit a high-oil phenotype of great agronomic significance. Nonetheless, considering the versatile roles of ACBP1 in other aspects of plant development and stress responses, the drawbacks of ACBP1 depletion in Arabidopsis, such as defective stem cuticle formation (Xue et al., 2014) and higher sensitivity to heavy metal stress (Xiao et al., 2008), should be taken into consideration. The negative impacts of ACBP1/SMO1-1 cosilencing on reproductive development also present some concerns (Fig. 5). Whether these issues can be solved by seed-specific silencing awaits further investigations.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (Arabidopsis thaliana) ecotype Columbia-0 was used in this study. Seeds were stratified at 4°C for 2 d and germinated on MS plates with 2% (w/v) Suc (unless specified otherwise) at 22°C under a 16-h-light/8-h-dark cycle. Potted plants were grown with a day/night regime of 16/8 h and...
23°C/21°C. The acbp1 (Basta-resistant) mutant, cACBP1, and ACBP1 OE s have been described previously (Xiao et al., 2008). The smo1-1 mutant (SALK, 021394) was selected from the SALK collection of TAIR, and its T-DNA was identified by PCR using the left border primer LBA1 and the gene-specific primer ML2265 (Fig. 3A). By sequencing the PCR product, the T-DNA insert was mapped to the 5′ untranslated region at 102 bp upstream of the translation start site (Fig. 3A). The homozygous mutant was confirmed by PCR using the primer pair ML2251/ML2265 (Fig. 3A). All primers used in this study are listed in Supplementary Table S2. Transgenic lines were generated by Agrobacterium tumefaciens-mediated transformation of wild-type Arabidopsis by the floral dip method (Clough and Bent, 1998).

**Generation of OE and Complemented Lines**

The full-length SMO1-1 ORF was PCR amplified using the primer pair ML2804/ML2805. The 900-bp product was blunt-end ligated into the Snu1 sites on pXCS-HAStrep (Witte et al., 2004) to generate plasmid pATX30 for Arabidopsis transformation. The homozygous mutant was confirmed by PCR using the primer pair ML2251/ML2265 (Fig. 3A). All primers used in this study are listed in Supplementary Table S2. Transgenic lines were generated by Agrobacterium tumefaciens-mediated transformation of wild-type Arabidopsis by the floral dip method (Clough and Bent, 1998).

**Genetic Crosses**

Homozygous smo1-1 and acbp1 were genetically crossed. With no double homozygote identified after genotyping more than 100 F2 progeny, the double mutant was maintained as ACBP1+/+–smo1-1. From the F3 progeny of these plants, the absence of the acbp1 homozygote was further confirmed by PCR and Basta selection. To complement the embryo lethality of acbp1 smo1-1 by over-expression of ACBP1 or SMO1-1 ORFs, ACBP1+/+–smo1-1 was crossed with cACBP1 or cSMO1-1, respectively. Homozygous lines were identified from F2 progeny by genotyping. The seed abortion rate of each line was scored in the F3 progeny by PCR using the left border primer LBa1 and the gene-speciﬁc primer ML2265 (Fig. 3A). All primers used in this study are listed in Supplementary Table S2. Transgenic lines were generated by Agrobacterium tumefaciens-mediated transformation of wild-type Arabidopsis by the floral dip method (Clough and Bent, 1998).

**Generation of SMO1-1EGFP and DsRed:ACBP1 Lines**

The full-length SMO1-1 ORF was PCR amplified using the primer pair ML2237/ML2238. The 900-bp product was cloned in frame into the BanHI site on pb1211-EGFP (Shi et al., 2005) to generate plasmid pAT735 for Arabidopsis transformation. The homozygous T3 plants were identiﬁed by kanamycin/calcium selection and PCR genotyping. The DsRed:ACBP1 fragment was PCR amplified from plasmid pAT226 (Li and Chye, 2004) using the primer pair ML2433/ML2434. The 1.9-kb product was digested with Sall and HindIII and cloned into Xhol and HindIII sites on pXCS-HAStrep (Witte et al., 2004) to generate plasmid pAT763. The full-length ACBP1 ORF was PCR amplified using the primer pair ML2435/ML2436 and blunt-end ligated into Snu1 sites on plasmid pAT763 (replacing ACBP1) to generate plasmid pAT764, which was introduced into SMO1-1:EGFP lines. After conﬁrmation by Basta selection and PCR genotyping, homozygous T3 plants were used for confocal laser scanning microscopy.

**Confocal Laser Scanning Microscopy**

For transient expression of EGFP and DsRed fusion proteins, plasmids pAT735 and pAT764 were introduced into Arabidopsis thaliana (L.) Heynh. 1982 (N. tabacum var SRI) according to the manufacturer’s instructions. The bait vector pGBK7 containing the coding region of ACBP1-32-338 (plasmid pAT248) constructed previously (Du et al., 2013) was used. The coding regions of ACBP1Δ32-338 and ACBP1Δ1-338 were PCR ampliﬁed using primer pairs ML791/ML792 and ML2208/ML2209, respectively. The products were cloned into pGEM-T Easy Vector (Promega) to generate plasmids pAT660 and pAT710, respectively, from which EcoRI-BanHI fragments were excised and cloned into similar sites on pGBK7 to generate plasmids pAT661 and pAT713, respectively. A 1.1-kb EcoRI-EcoRI fragment containing the Y171A-mutated coding region of ACBP1Δ32-338 was excised from plasmid pAT280 (Leung et al., 2000) and cloned into the EcoRI site on pGBK7 to generate plasmid pAT760. The full-length SMO1-1 ORF was PCR ampliﬁed using the primer pair ML1794/ML1795. The 900-bp product was cloned in frame into pGEM-T Easy Vector (Promega) to generate plasmid pAT666, from which an EcoRI-Xhol fragment was excised and cloned into similar sites on the prey vector pGAD717 (Promega) to generate plasmid pAT668. The bait and prey constructs were introduced into Y2HGold cells (Clontech) according to the manufacturer’s instructions, using cotransformati-
10 min, then 40 cycles of 95°C for 15 s and 60°C for 30 s. Primer pairs for qRT-PCR were ML1124/ML1125 (ACTIN2), ML2252/ML2253 (SMO1-1), ML2918/ML2919 (PSAT1), ML2922/ML2923 (PLDα1), ML2926/ML2927 (GL2), and ML2934/ML2935 (MUM4).

GUS Staining

The 3-kb 5′-flanking region of SMO1-1 was PCR amplified using the primer pair ML2316/ML2317 and cloned into pGEM-T Easy Vector (Promega) to generate plasmid pAT750, from which a BamHI/HindIII fragment was excised and cloned into the BamHI site on pBI101.3 (Clontech) to generate the SMO1-pro:GUS construct pAT799 for Arabidopsis transformation. After confirmation by kanamyacin selection and PCR genotyping, homozygous T3 plants were used. Arabidopsis ACRBP1pro:GUS plants were generated by Du et al. (2013). GUS staining was performed according to Kim et al. (2006). Seeds were fixed in 90% (v/v) aceton for 20 min and vacuum infiltrated in a substrate solution containing 50 mM sodium phosphate (pH 7), 0.2% (v/v) Triton X-100, 10 mM EDTA, 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide, and 1 mg mL⁻¹ 3-bromo-4-chloro-3-indolyl-b-β-glucuronide for 30 min. The specimens were incubated at 37°C for 6 to 12 h until the development of blue signals and cleared in 70% (v/v) ethanol for photography. Floral stages were numbered according to Smyth et al. (1990). The GUS-stained ovules and developing seeds were further cleared in Herr’s solution as described previously (Chen et al., 2010) prior to differential interference contrast (DIC) microscopy.

Seed Coat Mucilage Staining

Dry seeds were shaken in water for 1 h and stained with an aqueous solution of 0.01% (w/v) Ruthenium Red according to Western et al. (2001). The stained seeds were washed briefly in water and observed with a dissecting microscope.

Light Microscopy

Siliques were vacuum infiltrated in a fixative solution containing 50 mM sodium phosphate (pH 7), 2.5% (v/v) glutaraldehyde, and 1.6% (v/v) paraformaldehyde at room temperature for 1 h and further fixed at 4°C overnight. After dehydration in an ethanol series, the specimens were cleared in Herr’s solution as described previously (Chen et al., 2010). For DIC microscopy, whole-mount tissues were mounted in Hoyer’s medium (Stangeland and Salehian, 2002) and observed with a Nikon Eclipse 80i microscope using DIC optics. For histochemical examination, the specimens were embedded and sectioned in Technovit 7100 (Electron Microscopy Sciences). Sections were stained with the periodic acid-Schiff’s reaction for total insoluble carbohydrates and Amido Black 10B for protein and then imaged with a Leitz photomicroscope using a Nikon DS-F2 digital camera according to Hsiao et al. (2015).

Postgerminative Root Growth Studies

Seeds were germinated for 3 d on MS plates with 1% (w/v) Suc. Germinated seeds (12 per plate; n = 4) were transferred to fresh identical plates and grown for 3 d prior to primary root length measurement. Given that the progeny from ACRBP1+/-;smo1-1 could be segregated into ACRBP1+/-;smo1-1 and smo1-1 (null) genotypes, the presence of the actyl-T DNA in each seedling was determined by PCR using the primer pair ML179/LBL1 following Chen et al. (2010).

GC-MS Analysis of Sterols and FA Methyl Esters

Sterols were extracted according to standard procedures (Liao et al., 2014; Henry et al., 2015) with some modifications. Briefly, 10 mg of freeze-dried siliques at 1 to 2 DAF and 15 mg of freeze-dried mature green siliques were incubated at 37°C for 6 to 12 h until the development of blue signals, and cleared in 70% (v/v) ethanol for photography. Floral stages were numbered according to Smyth et al. (1990). The GUS-stained ovules and developing seeds were further cleared in Herr’s solution as described previously (Chen et al., 2010) prior to differential interference contrast (DIC) microscopy.

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