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The *Arabidopsis acbp1acbp2* double mutant lacking acyl-CoA-binding proteins ACBP1 and ACBP2 is embryo lethal

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Summary

- In *Arabidopsis thaliana*, the amino acid sequences of membrane-associated acyl-CoA-binding proteins ACBP1 and ACBP2 are highly-conserved. We have previously shown that in developing seeds, ACBP1 accumulates in the cotyledonary cells of embryos and ACBP1 was proposed to be involved in lipid transfer. We show here by immunolocalization using ACBP2-specific antibodies, that ACBP2 is also expressed in the embryos at various stages of seed development in *Arabidopsis*.
- Phenotypic analyses of *acbp1* and *acbp2* single mutants revealed that knockout of either *ACBP1* or *ACBP2* alone did not affect their life cycle since both single mutants exhibited normal growth and development similar to wild type. However, the *acbp1acbp2* double mutant was embryo lethal and was also defective in callus induction.
- On lipid and acyl-CoA profiling analyses, siliques but not leaves of the *acbp1* mutant were shown to accumulate galactolipid monogalactosyldiacylglycerol (MGDG) and 18:0-CoA but the levels of most polyunsaturated species of phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) had declined.
- Since recombinant ACBP1 and ACBP2 bind unsaturated PC and acyl-CoA esters *in vitro*, we propose that ACBP1 and ACBP2 are essential in lipid transfer during early embryogenesis in *Arabidopsis*.

Keywords

acyl-CoA measurement; acyl-CoA-binding protein; embryo development; lipid metabolism; phosphatidylcholine; phospholipids

Introduction

During embryogenesis, which is an early essential step in the life cycle of higher plants, cell differentiation and division occur and a multicellular organism is produced from one single

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cell (Yeung & Meinke, 1993; Devic, 2008). In *Arabidopsis thaliana*, seed development can be divided into two major phases: embryo morphogenesis and maturation. Embryo morphogenesis is initiated by the fusion of the male and female gametes forming a zygote, after which rapid cell division occurs and the embryo pattern is soon established. Subsequently, during embryo maturation, cell expansion and accumulation of reserves follow. Finally, the seed undergoes desiccation and dormancy sets in (Harada, 1997; Fan *et al*., 2008; Baud & Lepiniec, 2009).

To date, a large number of *Arabidopsis* genes essential in seed development (*EMBs*) have been identified using forward- and reverse-genetic approaches (Tzafrir *et al*., 2004; Meinke *et al*., 2008). Analyses of embryo development in these mutants have indicated that the majority display aborted phenotypes at or before the globular stage, reinforcing this to be most critical in embryo development (Devic, 2008). Some *EMB* genes affect lipid transport and metabolism (9 genes), or CoA transport and metabolism (4 genes), implicating the essential role of lipids in plant embryo development (Tzafrir *et al*., 2003; http://www.seedgenes.org). Studies have revealed that depletion of *LPAAT1* causes embryo lethality in *Arabidopsis*, with embryo development arrested at the heart-torpedo stage (Kim & Huang, 2004). LPAAT1 encodes a plastid-localized lysophosphatidic acid acyltransferase for the acylation of the *sn*-2 position of lysophosphatidic acid in phosphatidic acid biosynthesis and was deemed essential for heart-torpedo stage establishment in embryo development in *Arabidopsis* (Kim & Huang, 2004). In contrast, the knockout mutant of *Arabidopsis LPAT2* lacking an endoplasmic reticulum (ER)-located lysophosphatidic acid acyltransferase is defective in female, but not male, gametophyte development (Kim *et al*., 2005). Downregulation of *ACCase* in *Brassica napus* by an antisense approach produced wrinkled seeds accompanied by a decline in lipid content (Sellwood *et al*., 2000). In *Arabidopsis*, two genes *ACC1* and *ACC2* encode multifunctional isoforms of ACCase (Yanai *et al*., 1995) and knockout mutants of *ACC1* were embryo lethal (Baud *et al*., 2003). These results demonstrate that processes affecting plant lipid metabolism, especially those related to very long-chain fatty acid elongation are important during the early stages of seed development.

ACBPs are a family of proteins that facilitate the binding of long-chain acyl-CoA esters at a conserved acyl-CoA-binding domain (Xiao & Chye, 2009). In *Arabidopsis*, six genes encode ACBPs which are subcellularly localized in different compartments (Chye *et al*., 1999; Li & Chye, 2003; Chen *et al*., 2008; Xiao *et al*., 2008a). Since they bind different acyl-CoA esters with varying affinities, they do not seem to have redundant roles *in vivo* (Chye, 1998; Chye *et al*., 2000; Leung *et al*., 2004; 2006; Gao *et al*., 2009; Xiao *et al*., 2009). Besides the presence of the conserved ACBP domain, some ACBPs contain other functional domains, such as ankyrin repeats in ACBP1 and ACBP2, and kelch motifs in ACBP4 and ACBP5, which mediate interaction with protein partners (Li & Chye; 2004; Li *et al*., 2008; Gao *et al*., 2009). Three cytosolic ACBPs (ACBP4, ACBP5 and ACBP6) are known to bind phosphatidylcholine *in vitro* (Chen *et al*., 2008; Xiao *et al*., 2009). Immunogold labeling analysis has previously indicated that ACBP1 is localized to the plasma membrane of epidermal cells and in the cotyledonary cells at various stages (heart, torpedo and cotyledon) of embryo development in developing seeds (Chye *et al*., 1999). It

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has been proposed that ACBP1 may be involved in lipid transfer originating from the endoplasmic reticulum (ER) to the plasma membrane during lipid metabolism in these seeds (Chye *et al*., 1999). Consistently, ACBP1-GFP and ACBP2-GFP fusion proteins were localized at the plasma membrane and ER in onion epidermal cells by particle bombardment and results were confirmed using subcellular fractionation followed by western blot analysis (Li & Chye, 2003). The *N*-terminal transmembrane domains, in both ACBP1 and ACBP2, are essential for their membrane association (Li & Chye, 2003). Given the importance of proteins/enzymes related to lipid metabolism in *Arabidopsis* seed development (Baud *et al*., 2003; Rylott *et al*., 2003), the accumulation of ACBP1 in seeds and embryos suggests possible roles in embryo development or seedling establishment. Thus, it was pertinent to investigate whether ACBP1 and/or ACBP2 affect early development by analysis of single mutants as well as the *acbp1acbp2* double mutant.

Materials and methods

Plant materials and growth conditions

The *acbp2* T-DNA insertion mutant was identified from a T-DNA insertional library from the Torrey Mesa Research Institute of Syngenta (www.tmri.org). After surface-sterilization and chilling at 4°C for 2 days, seeds of *Arabidopsis thaliana* wild-type (ecotype Columbia), *acbp1* and *acbp2* mutants were germinated and grown on MS medium (Murashige & Skoog, 1962) supplemented with 2% sucrose grown under cycles of 8 h dark (21°C) and 16 h light (23°C). Soil-grown plants were also grown under 8 h dark (21°C) and 16 h light (23°C) cycles.

Immunohistochemical localization of ACBP2 using light microscopy

Immunohistochemical localization of ACBP2 using the anti-ACBP2 specific antibodies (Chye *et al*., 2000; Li & Chye, 2003) was performed as previous described (Chye *et al*., 1999). Briefly, the *Arabidopsis* siliques containing developing seeds at various stages of embryos were fixed and embedded in paraffin following the procedure described by Chye *et al*. (1999). Blocking and antibody reactions were carried out in 1% BSA in phosphate buffered saline (PBS). The sections were incubated for 5 min with PBS and 0.1% saponin (Sigma) before incubation with PBS containing 0.1% saponin, 1% BSA and 2% goat serum for 1 h at room temperature. Sections were incubated with rabbit anti-ACBP2 specific antibodies (1:1000 [v/v]) at 4°C overnight and with the secondary antibody biotinylated alkaline-phosphatase-conjugated goat anti-rabbit antibodies (1:1000 [v/v]; BioRad) at room temperature for 2 h. Levamisole (1 mM; Sigma) was included in the alkaline phosphatase reaction to inhibit endogenous phosphatase activity and this reaction was carried out following the instructions of manufacturer (BioRad).

Identification of the *acbp2* mutant

The homozygous *acbp2* mutant was isolated by PCR amplification using 2 primer pairs (i) *ACBP2* gene-specific forward primer ML251 (5′-ATCGGCGTTGGTTTTTCGTTTTTGAGAAT-3′) with reverse primer ML252 (5′-TTGCCGCCAAAGTCGGTTATTTATTCGTT-3′) and (ii) ML205 (5′-CGTCACCCAGAGGAGTC-3′) with the T-DNA left border primer Oligo113 (O113; 5′-

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TAGCATCTGAATTTCATAACCAATCTCGATACAC-3′). The PCR products were separated by electrophoresis on 0.8% agarose and DNA was transferred to a nylon membrane (Hybond-N, Amersham). The blot was hybridized overnight at 42 °C to a random-primed ^{32}P-labeled full-length *ACBP2* gene probe. The blot was washed in 0.1 × SSC, 0.1% SDS at 65 °C for 10 min. The position of the T-DNA insertion was confirmed by DNA sequence analysis of the resultant PCR products.

Western blot analysis

Total plant protein was extracted (Chye *et al*., 1999) from mature silique-bearing plants of wild-type *Arabidopsis* or the *acbp2* mutant. Protein concentration was determined using the Bio-Rad Protein Assay Kit following the method of Bradford (1976). Ten μg of total protein was loaded per well in SDS-polyacrylamide gel electrophoresis. The proteins were electrophoretically transferred to Hybond-C membrane (Amersham) from the SDS-PAGE gel using the Trans-Blot cell (Bio-Rad). Affinity-column purified ACBP2-specific antibodies (Chye *et al*., 2000; Li & Chye, 2003) were used in Western blot analysis. The Amplified Alkaline Phosphatase Goat Anti-rabbit Immuno-blot Assay Kit (BioRad) was used following the instructions of the manufacturer in detection of cross-reacting bands.

Semi-quantitative reverse transcription (RT)-PCR analysis

Total RNA was isolated using TRIzol reagent (Invitrogen, Cat No. 15596-018) from rosettes and siliques of 7-week-old wild-type, *acbp1* and *acbp2* plants. First-strand synthesis was carried out using the Superscript™ First-strand synthesis system (Invitrogen, Cat No. 12371-019). Gene-specific primers for RT-PCR were used as described previously (Xiao *et al*., 2008b) and the cycle numbers of amplification with each primer pair was adjusted to be within the linear range.

Screening of the *acbp1acbp2* double mutant

The *acbp1* (Xiao *et al*., 2008b) and *acbp2* homozygous mutants were crossed and their resultant F_2 population was screened for *acbp1acbp2* double mutants. F_2 seeds were sterilized and grown on kanamycin-containing MS medium. From kanamycin resistant (for *acbp2* allele) plants, DNA was extracted and primer combinations ML179/ML209 and ML179/SLB1 (Xiao *et al*., 2008b), O113/ML206 (5′-TCGGGGTGGGGATGA TGC-3′) and ML206/ML252 (Fig. 3a) were used in PCR to screen for the *acbp1* and *acbp2* alleles, respectively. Since *acbp1acbp2* double mutants were not obtained from >200 F_2 plants screened, *acbp1ACBP2+/−* (i.e., homozygous for *acbp1* and heterozygous for *acbp2*) and *ACBP1+/−acbp2* (i.e., heterozygous for *acbp1* and homozygous for *acbp2*) plants were subsequently generated. The self-fertilized F_3 seeds of *acbp1ACBP2+/−* or *ACBP1+/−acbp2* plants were compared to WT by light microscopy, the percentages of aborted ovules in open siliques from WT and *acbp1ACBP2+/−* or *ACBP1+/− acbp2* plants were calculated and their whole-mount embryo development observed. For complementation testing, transgenic line *acbp1::35S-ACBP1* (*cACBP1-2*; Xiao *et al*., 2008b) was crossed to *acbp1ACBP2+/−* plants and the F_1 progenies were used for further analysis.

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Microscopy

Developing seeds or excised embryos were placed in Herr's solution (Herr, 1971) composed of 85% lactic acid, chloral hydrate, phenol, clove oil and xylene (2:2:2:2:1), for 2 h to overnight. Slides were viewed subsequently using a Leitz photomicroscope using differential interference contrast optics.

Callus induction

Callus induction was carried out according to Liu *et al*. (2004). Aborted embryos of 4-DAF *acbp1ACBP2+/–* plants were excised under the microscope, placed onto callus-inducing medium containing 2,4-D (0.5 mg L^{-1}), indoleacetic acid (2 mg L^{-1}), and 2-iP(*N*6-[2-isopentenyl]adenine; 0.5 mg L^{-1}), and were cultured for 3 weeks at 22°C in the dark. Embryos from wild-type, *acbp1* and *acbp2* plants at similar developmental stage were excised and grown on callus-inducing medium under the same conditions.

Lipid and acyl-CoA profiling

Total lipid extraction was carried out according to Welti *et al*. (2002) and lipids were dissolved in chloroform for analysis. The profiles of membrane lipids were measured by an automated electrospray ionization tandem mass spectrometry method (Devaiah *et al*., 2006).

Acyl-CoA profiling was carried out as described previously (Larson & Graham, 2001; Larson *et al*., 2002). Briefly, acyl-CoAs from the frozen samples of 7-week-old rosettes and siliques were extracted, dried under vacuum at 40°C and subsequently reacted with 50 μl buffered chloroacetaldehyde reagent to form fluorescent acyl *etheno* CoA derivatives. The acyl-CoA standards purchased from Avanti (Avanti Polar Lipids, Inc. USA) were similarly treated. The derived standards and acyl-*etheno*-CoA samples were separated and quantified by a reversed-phased HPLC on a LUNA phenyl-hexyl column (Phenomenex, 150×2.0 mm, 5 μm) together with a 4×2 mm phenyl-propyl guard column. The solvent system was identical with the longer gradient conditions reported by Larson & Graham in 2001..

Lipid binding assay

$(His)_6$-ACBP1 and $(His)_6$-ACBP2 were expressed in the soluble fraction and inclusion bodies, respectively, of *E. coli* extracts and were each purified through an affinity column of Ni-NTA Agarose (Qiagen, Valencia, CA, USA) as described (Chye, 1998; Chye *et al*., 2000). Binding of $(His)_6$-ACBP1 and $(His)_6$-ACBP2 to various lipids on filters was carried out according to Chen *et al*. (2008).

To further confirm the interactions of $(His)_6$-ACBP1 and $(His)_6$-ACBP2 with PC, the Lipidex 1000 competition assay was carried out to determine if PC was capable of competing with $[^{14}C]$linoleoyl-CoA (American Radiolabelled Chemicals, https://www.arcincusa.com) in binding $(His)_6$-ACBP1 and $(His)_6$-ACBP2 (Rosendal *et al*., 1993; Gao *et al*., 2009). In the Lipidex 1000 binding assay, the incubation medium contains both ACBP1/ACBP2 and radiolabelled acyl-CoA. Subsequently, unbound radiolabelled acyl-CoA was removed from the incubation medium by Lipidex 1000. The bound radiolabelled acyl-CoA remain in the supernatant and was detected by measurement of radioactivity counts. In Lipidex 1000 competition assays, PC liposome was further added to the

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incubation medium containing both ACBP1/ACBP2 and radiolabelled acyl-CoA. If PC liposome successfully competes with acyl-CoA in binding to ACBP1/ACBP2, bound radiolabelled acyl-CoA will decrease and a decline in radioactivity count would result.

PC liposome was prepared according to Sano *et al*. (1998). Different concentrations of PC liposome (0–5 μM) were mixed with 0.8 μM [^{14}C]linoleoyl-CoA and 0.2 μM $(His)_6$-ACBP1 or $(His)_6$-ACBP2. Each mixture was incubated for 30 min at 37°C, and 400 μl of ice-cold 50% slurry of Lipidex 1000 (PerkinElmer, http://www.perkinelmer.com) and binding buffer were added. Samples were centrifuged at 12,000 × *g* for 5 min at 4°C and a 200-μl aliquot of the supernatant was taken for analysis of radioactivity counts using a LS 6500 liquid scintillation counter (Beckman). Assays were performed in triplicates, with blanks, at each concentration of PC liposome.

Results

Expression of *ACBP1* and *ACBP2* mRNAs during *Arabidopsis* development

Our previous studies have indicated that *ACBP1* (Chye, 1998) and *ACBP2* (Chye *et al*., 2000; Gao *et al*., 2009) mRNAs are expressed in all plant organs while ACBP1 protein accumulates in developing seeds (Chye, 1998), and ACBP2, in flowers and siliques (Kojima *et al*., 2007). To further understand the dynamic expression patterns of *ACBP1* and *ACBP2* during *Arabidopsis* development, data of their expression at various stages in development were retrieved from the microarray database Gene Chronologer (Zimmermann *et al*., 2004; https://3.met.genevestigator.com/at/index.php?page=home). Expression of both *ACBP1* and *ACBP2* was relatively high in early developmental stage 1 (DS1; refers to seedlings aged 1 to 5.9 days post-germination). Expression decreased from stages DS2 to DS8, spanning day 6 to day 44.9 (Fig. 1a). However, at DS9 (representing the seed set developmental stage), both genes again show high expression (Fig. 1a). Further investigation on *ACBP1* and *ACBP2* expression during seed development was performed by analyses of their expression at different seed stages (SS) using the e-FP Browser (Winter *et al*., 2007; http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) database. Results suggest that the expression of *ACBP1* and *ACBP2* increased from stages SS5 (walking-stick stage of development) to SS7 (late cotyledonary stage) or SS8 (green cotyledonary stage) and were relatively high in dry seeds (SS9) (Fig. 1b).

Immunolocalization of ACBP2 in *Arabidopsis* embryos using light microscopy

Since immunolocalization using anti-ACBP1 polyclonal antibodies has revealed that ACBP1 protein accumulates in the cotyledonary cells of embryos in developing seeds (Chye *et al*., 1999), we were interested to investigate if ACBP2 accumulates in seeds. Longitudinal sections of siliques at various stages of development were immunolocalized using anti-ACBP2 specific antibodies (Chye *et al*., 2000; Li & Chye, 2003) followed by light microscopy. Results revealed that embryos at globular (Fig. 2a), heart (Fig. 2b), heart-to-torpedo transition (Fig. 2c) and mature cotyledonary (Fig. 2d) stages were immunostained and express ACBP2. In contrast, similar stage embryos using preimmune serum from the same rabbit as a negative control did not show any staining (Figs. 2e to 2h).

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Characterization of an *acbp2* knockout mutant

In this study, an *acbp2* T-DNA insertional mutant was isolated from CS19943 (a T-DNA mutagenized *Arabidopsis* seed pool generated by Thomas Jack; ecotype Col-6) using a combination of gene-specific and T-DNA border-specific primers (Winkler *et al*., 1998). The location of the T-DNA insertion in *ACBP2* was confirmed by PCR using *ACBP2* gene-specific primers ML251 and ML252, as well as gene-specific forward primer ML205 and reverse primer Oligo113 (O113) that maps to the T-DNA left border (Figs. 3a and 3b). By further DNA sequencing analysis, the insertion site was mapped to intron 5 on *ACBP2* (Fig. 3a). Western blot analysis using ACBP2-specific antibodies (Fig. 3c) and RT-PCR analysis using gene-specific primers ML206/ML192 (Fig. 3d) confirmed the mutant to be a functional knockout line. Since we have previously characterized a T-DNA insertional knockout mutant of *ACBP1* (Xiao *et al*., 2008b), we were able to compare the expression of *ACBP1* in the *acbp2* mutant and that of *ACBP2* in the *acbp1* mutant. Semi-quantitative RT-PCR results show that in rosettes, both of the expression of *ACBP1* in the *acbp2* mutant and the expression of *ACBP2* in the *acbp1* mutant were up-regulated in comparison to wild type (Fig. 3d). Consistent with microarray data (Fig. 1), the expression levels of *ACBP1* and *ACBP2* in wild-type siliques were significantly higher than rosettes, while up-regulation of *ACBP1* in the *acbp2* mutant and vice verse was not evident (Fig. 3d).

Growth and development of the *acbp2* and the *acbp1* mutants were compared. At early development (2-week-old seedling) and late development (5-week-old plants), both *acbp1* and *acbp2* mutants were no different from wild type under normal growth conditions (Fig. S1a and b). To gain a better insight into the functions of ACBP1 and ACBP2 in seed development, embryos from the *acbp1* and *acbp2* mutants were carefully analyzed. Siliques of the *acbp1* and *acbp2* mutants produced normal seeds, similar to wild type (Fig. S1c). Whole-mount examination of embryo development in wild-type, *acbp1* and *acbp2* siliques (Fig. S1d) indicated that *acbp1* and *acbp2* embryos reached heart stage at 4 days after fertilization (DAF) and bent-cotyledonary stage at 6 DAF. These results suggest that the *acbp1* and *acbp2* single mutants displayed no obvious morphological changes in embryonic and seed development in *Arabidopsis* and are indistinguishable from wild type.

The *acbp2acbp2* double mutant is embryo lethal

Since ACBP1 and ACBP2 are highly conserved (82% identity) and are both expressed in embryos, we next investigated if they function redundantly in lipid metabolism during seed development. To test this possibility, a combination of *acbp1* and *acbp2* mutations would be necessary. Hence, we crossed the two single *acbp1* and *acbp2* mutants, generated the F_1 progeny, and subsequently screened their F_2 populations for *acbp1acbp2* double mutants. However, following genotyping of more than 200 F_2 plants, *acbp1acbp2* double mutants were not encountered. We were only able to identify genotypes of either *acbp1ACBP2+/−* (i.e., homozygous for *acbp1* and heterozygous for *acbp2*; lane 1 in Fig. 4a) or *ACBP1+/−acbp2* (i.e., heterozygous for *acbp1* and homozygous for *acbp2*; lanes 6, 9 and 11 in Fig. 4a).

The morphologies of seeds produced from the self-pollinated *acbp1ACBP2+/−* and *ACBP1+/−acbp2* plants were further examined. As shown in Fig. 4c, in contrast to wild-

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type siliques which contain almost all normal seeds that were large and green, the siliques of *acbp1ACBP2+/−* and *ACBP1+/−acbp2* plants contain both normal seeds which were large and green, as well as a portion of aborted seeds (Fig. 4c). The percentage of aborted seeds in *acbp1ACBP2+/−* and *ACBP1+/−acbp2* plants of around 35% (Table 1), correlated well with a 3:1 segregation ratio. However, when the *ACBP1* full-length cDNA was introduced into an *acbp1ACBP2+/−* background by crossing *acbp1ACBP2+/−* with *cACBP1-2* (previously verified in Xiao *et al.*, (2008b) to be a complemented line for the *acbp1* mutant), only 5.7% of seeds were aborted (Table 1). This indicates that *acbp1acbp2* embryos could be rescued by introduction of an *ACBP1* cDNA. Also, by taking advantage of the kanamycin resistance phenotype conferred by the T-DNA in the *acbp2* mutant, the ratios of resistant: sensitive progenies from both *acbp1ACBP2+/−* and *ACBP1+/−acbp2* were about 2: 1 when grown on MS medium containing kanamycin (Table 2). These results confirm that a combination of *acbp1* and *acbp2* mutations affect embryo development.

To further study the cause for embryo lethality in the *acbp1acbp2* double mutant, embryo development at various stages in *acbp1ACBP2+/−* and wild-type plants were investigated. In the siliques of wild type, most embryos reached globular (Fig. 5g) and heart (Figs. 5h and 5i) stages by 3 DAF (days after fertilization). However, significant variation in embryo development was observed in different seeds derived from a single silique of the *acbp1ACBP2+/−* line. As shown in Fig. 5, in addition to "normal" seeds in *acbp1ACBP2+/ −* siliques which develop to globular (Fig. 5d) and heart (Figs. 5e and 5f) stages, there were also aborted ovules arrested at either zygote (Fig. 5a), 2-cell (Fig. 5d) or 8-cell (Fig. 5c) stage in the *acbp1ACBP2+/−* siliques. Further analysis revealed that 161 of 225 (72%) embryos in *acbp1ACBP2+/−* siliques reached globular and heart stages at 3 DAF, while the remaining 64 embryos (28%) were aborted early at either zygote, 2-cell or 8-cell stage (Fig. 5j). The percentage of aborted embryos in siliques of *acbp1ACBP2+/−* plants would be in agreement with an expected lethality in the *acbp1acbp2* double mutation. In summary, these results demonstrate that ACBP1 and ACBP2 are essential for embryo and seed development and embryos of the *acbp1acbp2* double mutant were aborted at early embryo development.

Aborted embryos from *acbp1ACBP2+/−* are defective in callus induction

To further investigate the nature of embryo aberration in the *acbp1ACBP2+/−* plants, callus induction was performed. To this end, the aborted embryos from 4-DAF *acbp1ACBP2+/−* plants were excised and subsequently cultured on a callus induction medium. As controls, embryos from wild type, *acbp1* and *acbp2* at a similar developmental stage were also excised and cultured. As shown in Fig. S2, wild-type, *acbp1*, and *acbp2* embryos formed callus tissue after a 3-week induction period, while the aborted embryos, which should be *acbp1acbp2* double mutants, failed to form calli.

Lipid and acyl-CoA profile changes in the *acbp1* and *acbp2* mutants

To investigate whether the altered expression of *ACBP1* or *ACBP2* in the mutants affect plant lipid metabolism, lipid composition in both rosettes and siliques of 7-week-old wild type (Col), *acbp1* and *acbp2* mutants were analyzed using electrospray ionization tandem mass spectrometry (ESI MS/MS; Welti *et al.*, 2002; Devaiah *et al.*, 2006). From lipid profiling data, we observed that wild-type plants showed different patterns of lipid profiles

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in rosettes and siliques (Table 3). Specifically, the total contents of galactolipids digalactosyldiacylglycerol (DGDG) and monogalactosyldiacylglycerol (MGDG) were significantly lower in siliques while those of phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI), as well as lysoPC and lysoPE were significantly higher in comparison to rosettes (Table 3). There were little differences between rosettes of wild type and the single *acbp1* or *acbp2* knockout mutant in the total amounts of all lipid species analyzed including DGDG, MGDG, phosphatidylglycerol (PG), PC, PE, PI, phosphatidylserine (PS), phosphatidic acid (PA), lysoPC, lysoPE and lysoPG (Table 3). However, in comparison to wild type siliques, significantly higher levels of MGDG ($P < 0.01$) and significantly lower amounts of the phospholipids PC, PI and PS ($P < 0.05$ or $P < 0.01$) were observed in the *acbp1* mutant, but not the *acbp2* mutant (Table 3). Further, analyses of the lipid profiles of wild type and *acbp1* mutant siliques revealed that galactolipid species such as 34:6- and 34:3-MGDG, DGDG, and 36:6-MGDG significantly increased in the *acbp1* mutant, while those of 36:4-, 38:5- and 38:4- MGDG, DGDG and 38:6-MGDG, 36:5-DGDG significantly decreased in comparison to wild type (Fig. 6). In contrast, most of the polyunsaturated species of phospholipids such as 34:2-, 36:4-, 36:3-, 38:5-, 38:4-, 38:3-, 40:5-, 40:4-, 40:3-PC; 36:4-, 38:5-, 38:4-, 38:3-, 40:3-PE, and 34:2-, 36:5-, 36:4-, 36:3- and 36:2-PI were significantly lower than wild type, except for the species of 34:3- and 36:6-PC which were higher in the *acbp1* mutant siliques (Fig. 6).

Analyses of acyl-CoA content of rosettes from both *acbp1* and *acbp2* single knockout mutants did not reveal any differences in acyl-CoA profile in comparison to wild type (Fig. 7, upper column). In siliques, only 18:0-CoA in the *acbp1* mutant was higher than wild type (Fig. 7, bottom column). The acyl-CoA profile of rosettes and siliques from the *acbp1ACBP2+/−* plants resembled the *acbp1* mutant (Fig. 7).

His-tagged ACBP1 and ACBP2 recombinant proteins bind to unsaturated PC and acyl-CoA esters *in vitro*

We have previously used *in vitro* filter-binding assays to show that recombinant ACBP4, ACBP5 and ACBP6 binds PC, and not PE, PI, PA and lysoPC (Chen *et al*., 2008; Xiao *et al*., 2009). To explore whether recombinant ACBP1 and ACBP2 bind to phospholipids, purified ACBP1 and ACBP2 recombinant proteins (Chye, 1998; Chye *et al*., 2000; Gao *et al*., 2009) were similarly tested with various concentrations of PC, PE, PI and PS as well as MGDG. These phospholipids were chosen because their contents had been altered in the siliques of the *acbp1* mutant in comparison to wild type (Table 3 and Fig. 6). Results indicated that $(His)_6$-ACBP1 and $(His)_6$-ACBP2 bind PC, but not MGDG in a dose-dependent manner (Fig. 8a). They also do not bind PE, PI and PS (data not shown). As the PC used in Fig. 8a was 1,2-diacyl-sn-glocero-3-phosphatidylcholine, consisting of 33% 16:0, 13% 18:0, 31% 18:1 and 15% 18:2 fatty acids, the binding of several fatty acid species of PC to $(His)_6$-ACBP1 and $(His)_6$-ACBP2 were subsequently tested. Results showed that both $(His)_6$-ACBP1 and $(His)_6$-ACBP2 bind unsaturated species of PC (18:1-PC, 18:2-PC) tested, but unlike $(His)_6$-ACBP4 (Xiao *et al*., 2009), $(His)_6$-ACBP5 (Xiao *et al*., 2009), and $(His)_6$-ACBP6 (Chen *et al*., 2008), they did not bind saturated species of PC (16:0-PC and 18:0-PC) (Fig. 8b).

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Lipidex assays have previously shown that $(His)_6$-ACBP1 and $(His)_6$-ACBP2 binds $[^{14}C]$18:2-CoA and $[^{14}C]$18:3-CoA (Gao *et al*., 2009). Since they now also bind to 18:2-PC, Lipidex competition assays were used to determine if 18:2-PC liposome can compete with $[^{14}C]$18:2-CoA in binding $(His)_6$-ACBP1 and $(His)_6$-ACBP2. The binding of $[^{14}C]$18:2-CoA to both $(His)_6$-ACBP1 and $(His)_6$-ACBP2 (Fig. 8c) were observed to decrease in the presence of 18:2-PC liposome, implying that binding was displaced by the addition of 18:2-PC liposome.

Discussion

Given that both recombinant ACBP1 and ACBP2 proteins have been shown recently to bind linoleoyl-CoA and linolenoyl-CoA esters *in vitro*, the precursors in phospholipid membrane repair, we have suggested that they may share a role in repair of the phospholipid bilayer membrane following heavy metal stress (Xiao *et al*., 2008b; Gao *et al*., 2009). We further demonstrate that *Arabidopsis* ACBP1 (Chye *et al*., 1999) and ACBP2 (this study in Figs. 1 and 2) accumulate in the embryos of developing seeds, and they likely play essential roles in embryogenesis, possibly in membrane biogenesis. Northern blot and western blot analyses showed that ACBP1 and ACBP2 mRNAs and proteins are not seed-specific (Chye, 1998; Chye *et al*., 2000; Gao *et al*., 2009) and this would support the dual functions of these two ACBPs in both plant development and plant stress response.

Many enzymes/proteins involved in plant lipid metabolism such as acyl-CoA oxidases (ACXs), CoA biosynthetic enzymes (HAL3s), **lysophosphatidic acid acyltransferase (LPAAT) and** acetyl-CoA carboxylase (ACCase) are essential for either early embryogenesis or seedling development in *Arabidopsis* (Baud *et al*., 2003; Rylott *et al*., 2003; Kim & Huang, 2004; Kim *et al*., 2005; Rubio *et al*., 2006). More interestingly, impairment of CoA biosynthesis in the *Arabidopsis hal3a-1halb* double mutant caused embryo lethality at early/midglobular stage which precedes triacylglycerol deposition, suggesting that CoA is crucial in early embryo development (Rubio *et al*., 2006). Also, complete breakdown of short-chain acyl-CoA oxidase activity in the *Arabidopsis acx3-1acx4-1* double mutant arrested embryo development at a very early stage (Rylott *et al*., 2003). Possible explanations for the embryo defects observed in the *acx3-1acx4-1* double mutant include the accumulation of acyl-CoA or short chain fatty acid to toxic levels, and the failure in CoA transfer to the acyl-CoA pool or to the production of fatty acid- or lipid-based signaling molecules (Rylott *et al*., 2003). The redundant functions of ACBP1 and ACBP2 in seed development share the characteristics of these previously reported proteins because embryo lethality in the *acbp1acbp2* double mutant resembles those of *hal3a-1halb* and *acx3-1acx4-1*. Observations of early embryo arrestment and lack of callus induction indicate that the development of the *acbp1acbp2* embryo is stalled during embryo morphogenesis which precedes fatty acid accumulation and lipid storage.

During early seed development, one of the major plant membrane lipids, PC serves as a main substrate for desaturation in the ER and plays a central role in assembly of unsaturated TAG (Browse, 1997). An acyl-CoA:lysophosphatidylcholine acyltransferase catalyses the exchange of polyunsaturated fatty acyl chains on *sn*-2 of PC with linoleoyl-CoA (18:2-CoA) and linolenoyl-CoA (18:3-CoA) in the acyl-CoA pool. Subsequently, glycerol-3-phosphate

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acyltransferase (GPAT) and lysophophatidic acid acyltransferase (LPAAT) are involved in the formation of polyunsaturated diacylglycerol (DAG) and acyl-CoA:*sn*-1,2-diacylglycerol acyltransferase in the acylation of the *sn*-3 position of DAG to produce triacylglycerol (TAG; Browse, 1997). Previous evidence has suggested that acyl-CoA preference of mitochondrial GPAT in young rat liver is regulated by the presence of an ACBP bound to acyl-CoA (Kannan *et al*., 2003). The 10-kDa *Brassica napus* ACBP which was the first ACBP identified from plants, is highly expressed in embryos, and has also been demonstrated to activate GPAT activity *in vitro* (Hills *et al*., 1994; Brown *et al*., 1998). A recent study has revealed that the overexpression of *BnACBP* in *Arabidopsis* seeds increased polyunsaturated (18:2 and 18:3) and decreased saturated and monounsaturated (16:0, 18:0 and 20:1) fatty acids (Yurchenko *et al*., 2009). Further, recombinant BnACBP is important for the activity of lysophosphatidylcholine acyltransferase (LPCAT) in the exchange of the acyl group between the acyl-CoA pool and PC (Yurchenko *et al*., 2009). Given that only one 10-kDa ACBP has been isolated from *Brassica* so far (Hills *et al*., 1994; Yurchenko *et al*., 2009) while a family of six ACBPs exists in *Arabidopsis* (Xiao & Chye, 2009), it is possible the larger ACBP1 and ACBP2, which are membrane-associated and accumulate in embryos, play a more significant role in this event in *Arabidopsis*, rather than the 10-kDa ACBP6.

Furthermore, comparison of the lipid profiles in rosettes and siliques of wild-type *Arabidopsis* has shown that extra-plastidial phospholipids (PC, PE, PI and PS) significantly accumulate in siliques (Table 3), consistent with a previous report (Devaiah *et al*., 2006), and confirm the crucial role for these phospholipids in embryo development. Our biochemical data have also revealed that siliques (but not rosettes) of the *acbp1* mutant show higher levels of galactolipid MGDG and lower levels of polyunsaturated PC, PE, PI and PS species in comparison to wild-type, demonstrating that knockout of one of these two genes affect phospholipid composition in siliques although these changes per se may not effectively stall embryo development. Lack of significant changes in phospholipid in siliques of the *acbp2* mutant, indicates that ACBP1 may play a more prominent role in this event than its redundant homologue, ACBP2. Consistently, acyl-CoA profiling revealed that the level of 18:0-CoA was higher in siliques of the *acbp1* mutant (but not in the *acbp2* mutant), suggesting that similar to BnACBP (Yurchenko *et al*., 2009), ACBP1 may play a role in the exchange of acyl-CoAs, especially 18:0-CoA, affecting phospholipid content in siliques. This exchange was blocked to a certain extent in the *acbp1* mutant and an accumulation of acyl-CoA in *acbp1* siliques corresponded to a decline in phospholipids. Also, upregulation of *ACBP1* in the *acbp2* mutant, and vice verse, occurred only in rosettes (but not siliques), indicating that these two ACBPs may share both redundant and distinct cellular functions during plant development. Moreover, *in vitro* evidence showed that recombinant ACBP1 and ACBP2 bind unsaturated PC (18:1-PC and 18:2-PC) and acyl-CoAs (18:2-CoA and 18:3-CoA), further supporting their roles in phospholipid metabolism. These results also reinforce ACBP1 and ACBP2 function in phospholipid membrane biogenesis (Chye *et al*., 1998; Xiao *et al*., 2008b; Gao *et al*., 2009) which would be crucial in early embryo development. Taken together, we propose that the combination of *acbp1* and *acbp2* mutations likely abolish formation of an acyl-CoA pool in the ER or disrupt acyl-CoAs/lipid trafficking between the ER and the plasma membrane during early embryo embryogenesis.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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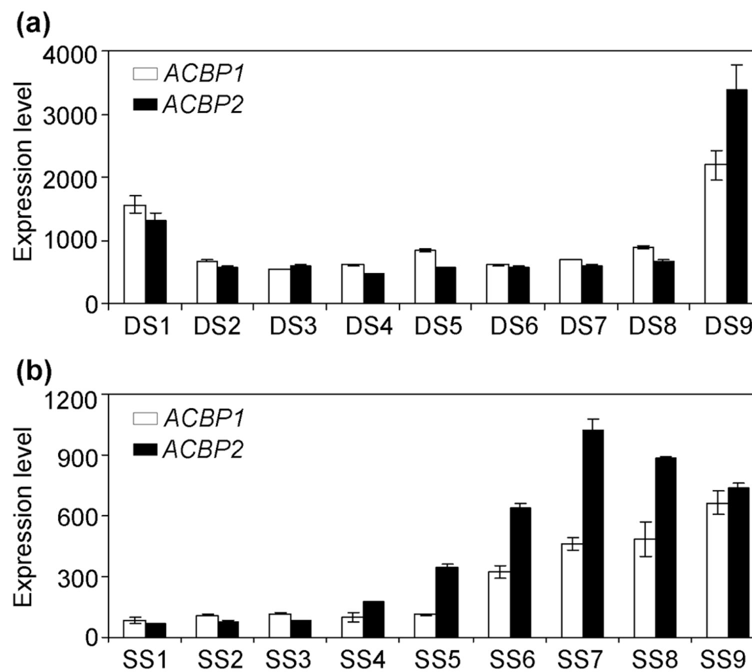

Fig. 1. Microarray data showing the expression patterns of *ACBP1* and *ACBP2* mRNAs

(a) Expression of *ACBP1* and *ACBP2* at various developmental stages (DS). DS1, seedlings of 1–5.9 days; DS2, seedlings of 6–13.9 days; DS3, seedlings of 14–17.9 days; DS4, plants of 18–20.9 days; DS5, plants of 21–24.9 days; DS6, plants of 25–28.9 days; DS7, plants of 29–35.9 days; DS8, plants of 36–44.9 days; DS9, 45–50 days (seeds).

(b) Expression of *ACBP1* and *ACBP2* at various seed stages (SS). SS1, globular; SS2, early heart; SS3, late heart; SS4, torpedo; SS5, walking-stick; SS6, early curled cotyledons; SS7, late cotyledons; SS8, green cotyledons; SS9, dry seeds.

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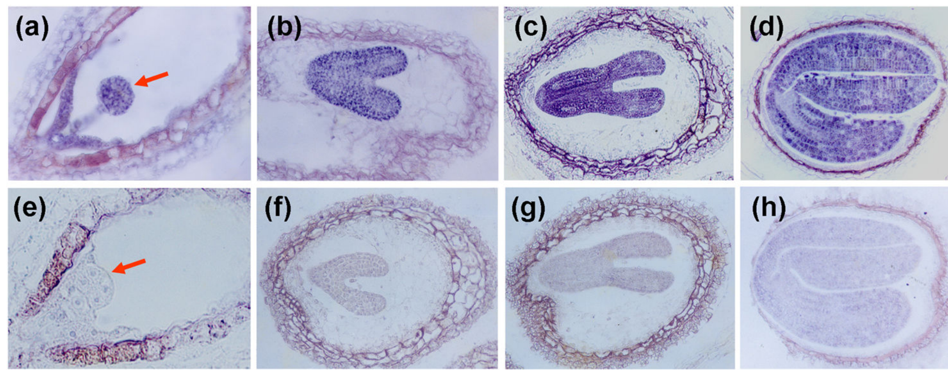

Fig. 2. Immunolocalization of ACBP2 using anti-ACBP2 antibodies in developing seeds of various embryo stages

(a) to (d) Longitudinal sections of seed at globular (a, indicated by arrow), heart (b), heart to torpedo transition (c) and cotyledon (d) stages showing the embryos stained with anti-ACBP2 primary antibody followed by biotinylated goat anti-rabbit antibody and reacted with streptavidin-biotinylated alkaline phosphatase.

(e) to (h) Longitudinal section of seed at globular (e, indicated by arrow), heart (f), heart to torpedo transition (g) and cotyledon (h) stages showing embryos stained with preimmune serum followed by biotinylated goat anti-rabbit antibody and reacted with streptavidin-biotinylated alkaline phosphatase.

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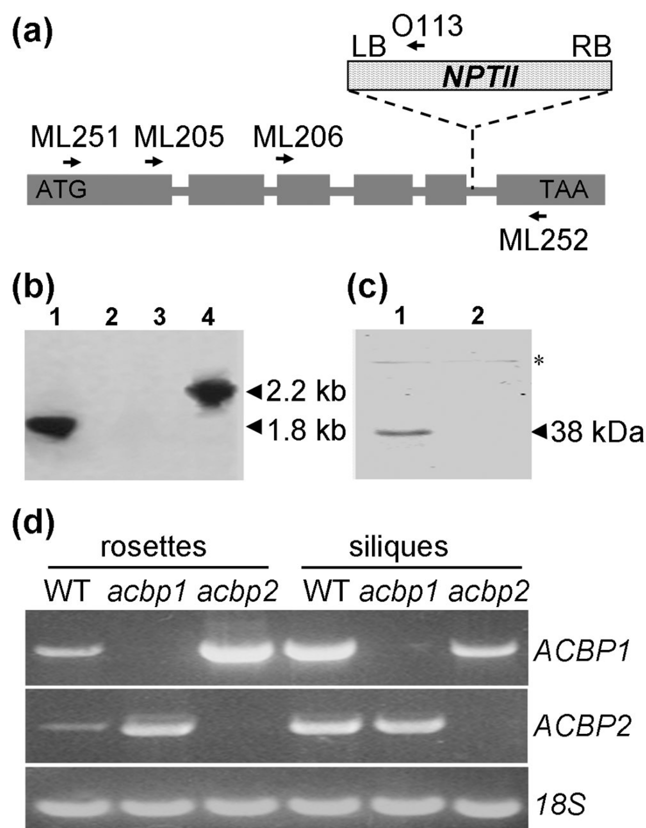

Fig. 3. Characterization of *acbp1* and *acbp2* knockout mutants

(a) Diagram of T-DNA insertion into *ACBP2*, and the primer locations used for PCR genotyping of the *acbp2* allele.

(b) Southern blot analysis of PCR products amplified from *acbp2* mutant DNA (lanes 1 and 2) or wild-type *Arabidopsis* DNA (lanes 3 and 4) using primer pair, ML205 and O113 (lanes 1 and 3) or ML251 and ML252 (lanes 2 and 4). The gel was blotted on Hybond-N and hybridized to ^{32}P-labeled *ACBP2* cDNA.

(c) Western blot analysis of total protein from the silique-bearing *acbp2* mutant (lane 1) or wild-type *Arabidopsis* (lane 2) using the ACBP2-specific antibodies. Ten μg of total proteins were loaded each lane on a 10% SDS- polyacrylamide gel. Asterisk indicates the position of a nonspecific immunoreacting band.

(d) Semi-quantitative reverse transcription (RT)-PCR analysis on the expression of *ACBP1* and *ACBP2* in wild type, *acbp1* and *acbp2* mutants. Total RNA was extracted from rosettes and siliques of 7-week-old wild-type, *acbp1* and *acbp2* plants. The *18S* transcript was used as a control.

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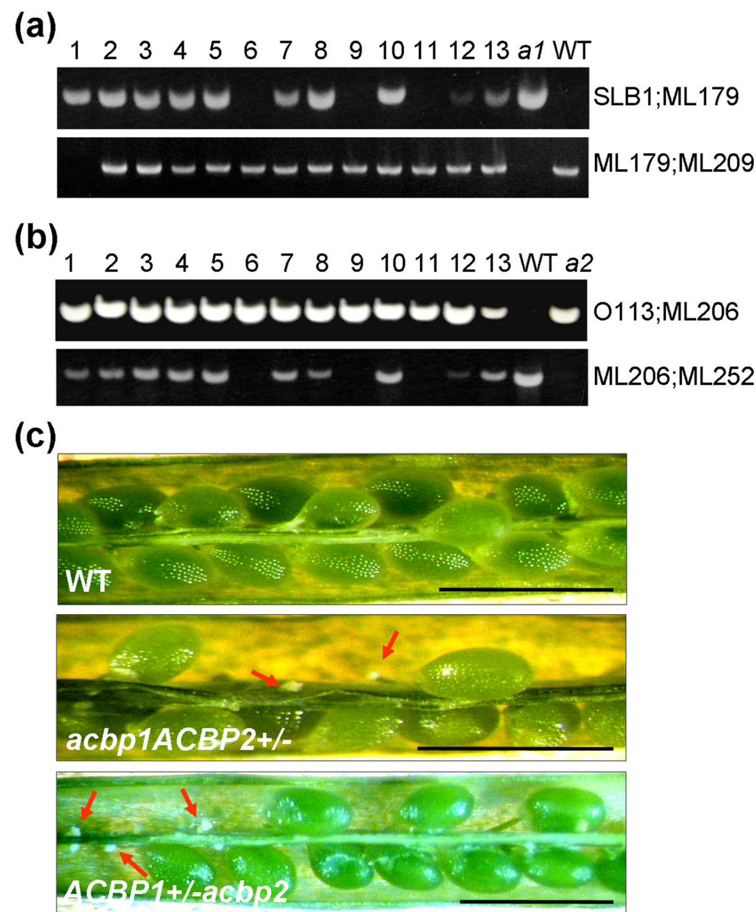

Fig. 4. Genotyping and initial characterization of *acbp1acbp2* double mutant

(a) and (b) Specificity of the primer combinations (at right) used in PCR to screen the *acbp1acbp2* double mutant from the kanamycin resistant (for *acbp2* allele) F_2 population. ML179 and ML209 are *ACBP1* gene-specific primers and SLB1 is T-DNA-sepcific primer used for genotyping the *acbp1* allele (Xiao *et al*., 2008b). No *acbp1acbp2* double mutants were obtained from over 200 F_2 plants screened, so *acbp1ACBP2+/−* (i.e., homozygous for *acbp1* and heterozygous for *acbp2*; lane 1 in a) and *ACBP1+/− acbp2* (i.e., heterozygous for *acbp1* and homozygous for *acbp2*; lanes 6, 9 and 11 in b) were subsequently generated.

(c) The self-fertilized F_3 seeds of *acbp1ACBP2+/−* and *ACBP1+/−acbp2* plants were compared with WT by light microscopy (top). Ovules in open siliques from *acbp1ACBP2+/ −* plants showing aborted ovules (indicated by arrows).

WT, wild type. Bars: 1mm.

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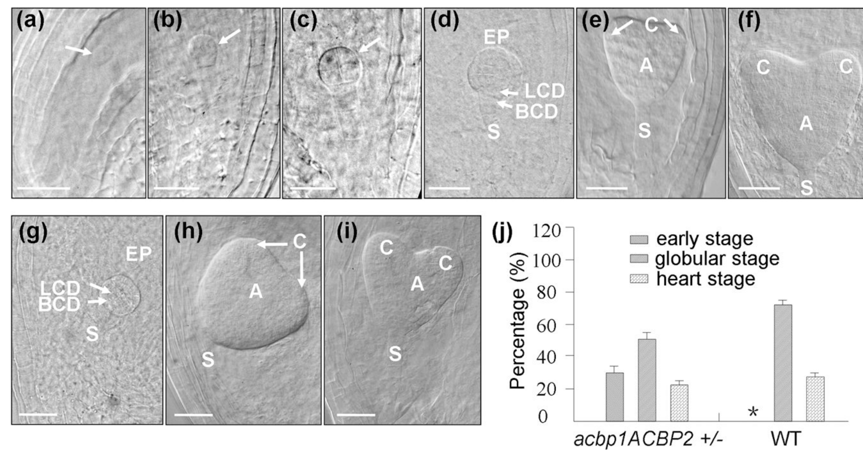

Fig. 5. A combination of *acbp1* and *acbp2* mutations affects embryo development

(a) to (i) Embryo development of 3-DAF (days after fertilization) ovules of *acbp1ACBP2+/−* (a–f) and WT (g–i) plants. At this stage, most of WT embryos have reached globular (g) or heart stages (h and i), while in the *acbp1ACBP2+/−* siliques, aborted ovules were arrested at zygote (a), 2-cell (b) or 8-cell (c) stage. Normal seeds in the same *acbp1ACBP2+/−* siliques develop to globular (d) and heart (e and f) stages. Arrows in (a)–(c) indicate arrested embryos.

(j) Percentage of different embryo stages (early stage including zygote, 4-cell or 8-cell stage, globular stage and heart stage) in *acbp1ACBP2+/−* (28%, 50% and 22%, respectively) and WT (3%, 73% and 24%, respectively) plants.

WT, wild type; A, axis; BCD, basal cell descendant; C, cotyledon; LCD, lens cell descendant; S, suspensor; Bars: 25 μm.

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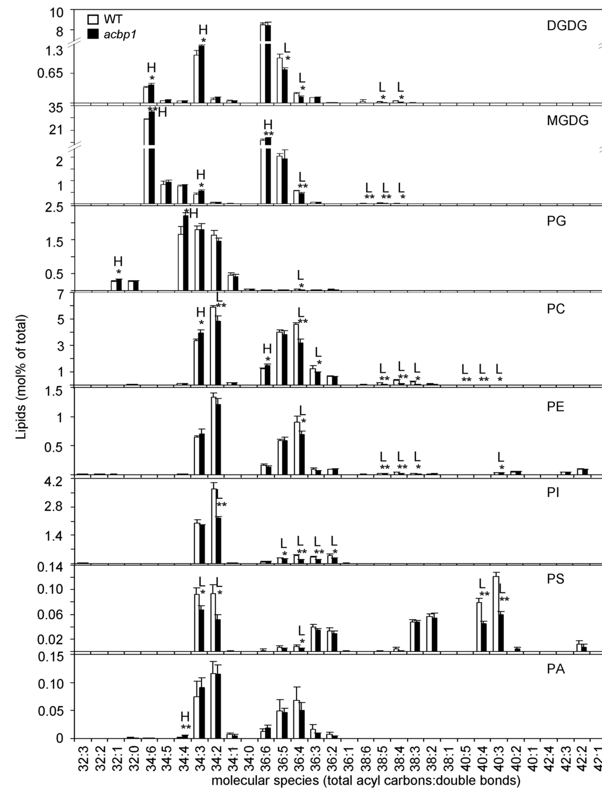

Fig. 6. Membrane lipid content (% of total glycerolipids analysed) in siliques of 7-week-old wild type (WT) and *acbp1* mutant

H indicates value of *acbp1* mutant higher than WT; L indicates value of mutant lower than WT (* $P < 0.05$ or ** $P < 0.01$). Values are the means ± SD (n = 3).

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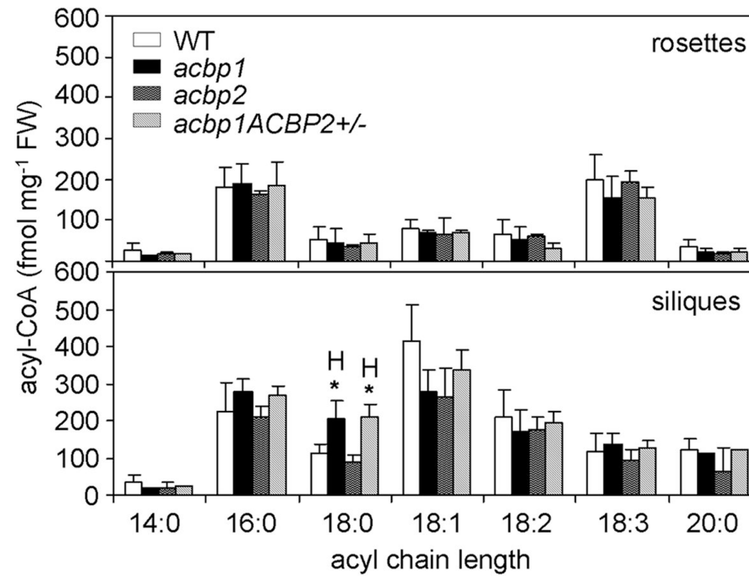

Fig. 7. Acyl-CoA content (fmole mg^{-1} FW) in rosettes and siliques of 7-week-old wild type (WT), *acbp1*, *acbp2* and *acbp1ACBP2+/−* mutants

H indicates value of *acbp1* mutant and *acbp1ACBP2+/−* plants higher than WT (* $P < 0.05$). Values are the means ± SD (n = 3).

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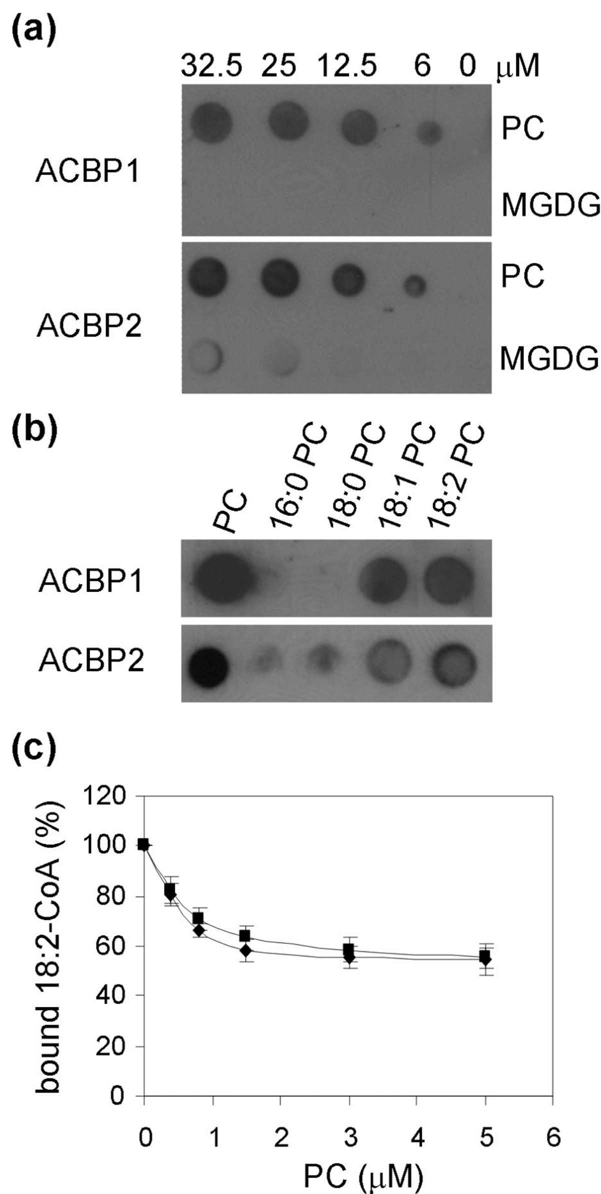

Fig. 8. Interaction of His-tagged ACBP1 and ACBP2 recombinant proteins to PC

(a) $(His)_6$-ACBP1 and $(His)_6$-ACBP2 bind to PC on filters. Various concentrations (0, 6, 12.5, 25.0, 32.5 and 50 μM) of PC and MGDG were spotted onto nitrocellulose and incubated with 1 μg ml^{-1} of purified $(His)_6$-ACBP1 or $(His)_6$-ACBP2 proteins. The $(His)_6$-ACBP/PC binding was detected by immunoblotting with HRP-conjugated anti-penta-His antibodies.

(b) Effect of PC acyl species on $(His)_6$-ACBP/PC binding. Fifty μM lipid (PC, 16:0-PC, 18:0-PC, 18:1-PC or 18:2-PC) spotted onto nitrocellulose was incubated with 1 μg ml^{-1} of purified $(His)_6$-ACBP1 or $(His)_6$-ACBP2 protein. The $(His)_6$-ACBP/lipid binding was detected by immunoblotting with HRP-conjugated anti-penta-His antibodies. PC, 1, 2-diacyl-sn-glocero-3-phosphocholine; 16:0-PC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; 18:0-PC, 1,2-distearoyl-sn-glycero-3-phosphocholine; 18:1-PC, 1,2-

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dioleoyl-sn-glycero-3-phosphocholine; 18:2-PC, 1,2-dilinoleoyl-sn-glycero-3-phosphocholine.

(c) Displacement of [^{14}C]linoleoyl-CoA by PC liposome in Lipidex competition assays. Different concentrations of PC liposome (0, 0.4, 0.8, 1.5, 3.0, 5.0 μM) were incubated with 0.8 μM [^{14}C]linoleoyl-CoA and 0.2 μM $(His)_6$-ACBP1 or $(His)_6$-ACBP2. The mixtures were mixed with Lipidex-1000. Aliquots (200 μl) of the supernatant were taken for analysis of radioactivity. Assays were performed in triplicates, with blanks, at each concentration of PC liposome. The bound acyl-CoAs in the presence of PC liposome (0–5 μM) were expressed relative to the value obtained from reaction containing 0 μM PC liposome (100%). Bars represent SD (n = 3).

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Table 1

Segregation and complementation analyses of *acbp1ACBP2+/−* plants.

	Normal seeds	Aborted seeds	x^2 (hypothesis)
ACBP1+/−acbp2	291	102	0.25 (3:1)
acbp1ACBP2+/−	252	90	0.39 (3:1)
acbp1ACBP2+/− x *cACBP1*	368	21	0.35 (15:1)
wild type	282	8	-

Seeds were analysed from ten siliques of single plants in each genotype. The genotypes of each plant were confirmed by PCR before analysis. Chi-square values were calculated for the hypothesized segregation ratio. The x^2 values showed no significant deviation ($P > 0.05$) from the hypothesized ratio.

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Table 2

Ratio of Kan^r: Kan^s for the progeny from self-pollinated *ACBP1+/−acbp2* and *acbp1ACBP2+/−* plants.

	Kan^r	**Kan^s**	**x^2 (hypothesis)**
ACBP1+/−acbp2	316	166	0.32 (2:1)
acbp1ACBP2+/−	378	180	0.24 (2:1)

Chi-square values are shown for the hypothesized segregation ratio and the x^2 values show no significant deviation ($P > 0.05$) from the hypothesized ratio.

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Table 3

Lipid profiles in rosettes and siliques of 6-week-old wild type (Col), *acbp1* and *acbp2* mutants grown at 16 h light (23°C)/8 h dark (21°C). Values are means ± SD (% of total polar glycerolipids analyzed; n = 3). Significant differences in the siliques of wild type from that of rosettes or in the *acbp1* mutant from that of wild type are bolded (* $P < 0.05$; ** $P < 0.01$).

Lipid class	Rosettes			Siliques		
	WT	*acbp1*	*acbp2*	WT	*acbp1*	*acbp2*
DGDG	13.52±0.65	13.84±0.47	14.43±0.53	**11.46±0.19****[b]	11.33±0.38	12.01±0.14
MGDG	66.84±1.40	68.29±0.14	66.71±0.71	**47.57±0.49****[b]	**52.91±1.39****[c]	48.46±1.02
PG	5.55±0.06	5.59±0.48	5.55±0.66	6.23±0.47	6.55±0.44	5.47±0.25
PC	7.68±0.48	7.73±0.05	7.79±0.37	**22.16±0.71****[a]	**19.35±1.47***[d]	20.83±0.83
PE	1.50±0.24	1.30±0.11	1.42±0.11	**4.18±0.25****[a]	3.77±0.35	4.39±0.09
PI	2.78±0.18	2.77±0.06	3.48±0.28	**7.25±0.58****[a]	**5.15±0.19****[d]	7.70±0.03
PS	1.82±2.83	0.18±0.01	0.18±0.04	0.60±0.04	**0.41±0.04****[d]	0.62±0.04
PA	0.25±0.05	0.24±0.04	0.36±0.32	0.36±0.10	0.35±0.06	0.28±0.06
LysoPC	0.014±0.002	0.013±0.001	0.019±0.004	**0.060±0.007****[a]	0.061±0.013	0.078±0.011
LysoPE	0.046±0.011	0.041±0.008	0.059±0.019	**0.124±0.012****[a]	0.108±0.017	0.142±0.002
LysoPG	0.005±0.001	0.008±0.006	0.005±0.005	0.005±0.004	0.014±0.011	0.017±0.026

[a]Value increase in siliques of wild type when compared rosettes of wild type in the same experiment.

[b]Value decrease in siliques of wild type when compared rosettes of wild type in the same experiment.

[c]Value higher in siliques of *acbp1* mutant when compared to siliques of wild type in the same experiment.

[d]Value lower in siliques of *acbp1* mutant when compared to siliques of wild type in the same experiment.