Arabidopsis Mutants in Short- and Medium-chain Acyl-CoA Oxidase Activities Accumulate Acyl-CoAs and Reveal That Fatty Acid β-Oxidation Is Essential for Embryo Development*

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The short-chain acyl-CoA oxidase (ACX4) is one of a family of ACX genes that together catalyze the first step of peroxisomal fatty acid β-oxidation during early, post-germinative growth in oilseed species. Here we have isolated and characterized an Arabidopsis thaliana mutant containing a T-DNA insert in ACX4. In acx4 seedlings, short-chain acyl-CoA oxidase activity was reduced by greater than 98%, whereas medium-chain activity was unchanged from wild type levels. Despite the almost complete loss of short-chain activity, lipid catabolism and seedling growth and establishment were unaltered in the acx4 mutant. However, the acx4 seedlings accumulated high levels (31 mol %) of short-chain acyl-CoAs and showed resistance to 2,4-dichlorophenoxybutyric acid, which is converted to the herbicide and auxin analogue 2,4-dichlorophenoxyacetic acid by β-oxidation. A mutant in medium-chain length acyl-CoA activity (acx3) (1) shows a similar phenotype to acx4, and we show here that acx3 seedlings accumulate medium-chain length acyl-CoAs (16.4 mol %). The acx3 and acx4 mutants were crossed together, and remarkably, the acx3acx4 double mutants aborted during the first phase of embryo development. We propose that acx3acx4 double mutants are nonviable because they have a complete block in short-chain acyl-CoA oxidase activity. This is the first demonstration of the effects of eliminating (short-chain) β-oxidation capacity in plants and shows that a functional β-oxidation cycle is essential in the early stages of embryo development.

In oilseed plants, seed lipid reserves are catabolized to provide metabolic energy and carbon skeletons to fuel germination and early post-germinative growth (2). Peroxisomal β-oxidation plays an essential role in the catabolism of fatty acids from storage lipid during this phase. This pathway plays additional roles including general housekeeping functions in membrane lipid turnover in all cells and as a salvage pathway of primary galactolipids during foliar senescence, reviewed by Graham and Eastmond (3). Additionally, β-oxidation is induced to supply respiratory substrates in carbohydrate-deprived maize root tips (4, 5) and is also involved in the synthesis of fatty acid-derived signaling molecules such as jasmonic acid (6) and traumatin (7). A mutation in the β-oxidation gene aim1 displays abnormal inflorescence meristems, suggesting that β-oxidation may be involved in floral development (8), and more recently, the increased expression of an acyl-CoA oxidase (ACX2) gene of β-oxidation in response to UV light suggests that the pathway may be induced to provide acetyl-CoA substrate for secondary metabolism (9, 10).

The β-oxidation pathway breaks down acyl-CoAs to acetyl-CoA via the repeated cleavage of acetate units from the thiol end of the acyl-CoA molecule. With each round of β-oxidation, the substrate chain length shortens by 2 carbon units. The acyl-CoA units produced are converted to succinate by the glyoxylate cycle, and this is then metabolized into sugars by gluconeogenesis. In Arabidopsis, the degradation of common, straight chain fatty acids involves the sequential activity of three gene families: the acyl-CoA oxidasases (ACX), multifunctional proteins (MFP), and 3-ketoacyl-CoA thiolases. The ACX gene family products catalyze the conversion of fatty acyl-CoAs to trans-2-enoyl CoAs (EC 1.3.3.6). This first step is believed to be predominant in exerting control over the rate of carbon flux through the pathway (11, 12).

There are six ACX genes identified in the Arabidopsis genome (3). The ACX1 gene (Munich Information center for Protein Sequences (MIPS)1 code At4g16760) is a medium- to long-chain ACX with a substrate optimum of C14:0,1 whereas the ACX2 gene (MIPS code At5g56110) has optimum activity with long-chain saturated and unsaturated acyl-CoAs (C14:0 to C20:0) (13). The ACX3 gene (MIPS code At1g06290/At1g06300) exhibits medium chain length substrate specificity (C8:0- C14:0) (1, 14), and AtSCX (ACX4) (MIPS code At3g51840) exhibits short-chain (C4:0-C8:0) substrate specificity (15). Two additional ACX genes, ACX5 (MIPS code At2g35690) and ACX6 (MIPS code At1g06310) are present in the Arabidopsis genome. ACX5 exhibits 85% identity with ACX1, and one expressed sequence tag currently exists in the MIPS Arabidopsis data base (AV786007). Expression analysis using RT-PCR indicates that the level of ACX5 expression in 2-day-old seedlings is less than 10% that of ACX1.3 The ACX6 gene occurs adjacent with ACX3 on chromosome 1 and exhibits 87% identity with it. However, there are no expressed sequence tags reported in the MIPS data base for ACX6, and its expression was undetectable in 2-day-old seedlings using RT-PCR analysis.3 All six Arabi-

1 The abbreviations used are: MIPS, Munich Information center for Protein Sequences; T-DNA, transfer DNA; 2,4-D, 2,4-dichlorophenoxyacetic acid; PTS1 and 2, peroxisomal targeting signal type 1 and 2, respectively; TAG, triacylglycerol; GFP, green fluorescent protein.
2 Fatty acid nomenclature is as follows. xy indicates that the fatty acid contains x number of carbon atoms and y number of double bonds; z indicates that a double bond is positioned at the rth carbon atom from the carboxyl terminus.
3 E. L. Rylott and I. A. Graham, unpublished results.
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do2. ACX isoforms contain two common motifs (PROSITE numbers PS00072 and PS00073, available on the World Wide Web at ca.expasy.org) that are homologous to the conserved signatures from mammalian acyl-CoA dehydrogenases (16), and these regions may be important in substrate interaction (15). There are two MFP genes identified in the Arabidopsis genome; MFP2 (MIPS code At3g06860) shows a significant induction during germination and seedling growth (17), whereas AIM1 (MIPS code At4g29010) is expressed predominantly in the silique and flower (8). The predominantly expressed thiolase during early postgerminative growth in Arabidopsis is ketocarboxy-CoA thiolase 2 (KAT2/PED1) (MIPS code At2g33150).

The ACX1, ACX2, ACX3, ACX4, MFP2, and KAT2 genes are all up-regulated coordinately during Arabidopsis seed germination and early postgerminative growth (18), correlating with the period of most rapid fatty acid degradation in Arabidopsis (19). The β-oxidation proteins are targeted to the peroxisome by a variety of signaling motifs. The KAT2 protein contains a peroxisomal targeting signal type 2 (PTS2) N-terminal sequence that is cleaved upon import into the peroxisome (20), and ACX2 also contains a putative PTS2 sequence (13). The AIM1 protein contains a PTS1, ending in the amino acids Ser, Lys, and Leu, and the ACX1 contains a putative PTS1, Ala, Arg, and Leu (13). MFP2 and ACX4 (15) both have putative PTS1s ending with the tripeptide Ser, Arg, Leu, which has been demonstrated to target proteins to the peroxisome (21). The ACX3 protein lacks motifs diagnostic of either PTS1 or PTS2, demonstrated to target proteins to the peroxisome (21). The Lys, and Leu, and the ACX1 contains a putative PTS1, Ala, Arg, and Leu (13). MFP2 and ACX4 (15) both have putative PTS1s ending with the tripeptide Ser, Arg, Leu, which has been demonstrated to target proteins to the peroxisome (21). The ACX3 protein lacks motifs diagnostic of either PTS1 or PTS2, and its mode of import is unknown (1).

Two allelic mutants disrupted in the KAT2 gene, ped1 (22) and kat2 (19), along with mutants in acx3 (1) and aim1 (8) have been described. In acx3 seedlings, there is a large reduction (>95% of wild type) in medium-chain, acyl-CoA oxidase activity. However, the phenotype of acx3 is unaltered throughout the life cycle of the plant, and seedling lipid breakdown is not affected, most probably due to the overlapping substrate specificities of the ACX1 and ACX4 gene products (1). The aim1 mutant shows abnormal inflorescence development (8) indicating that β-oxidation may play a role in floral development. However, seedling growth is not compromised in the aim1 mutant, presumably because the AtMFP2 gene encodes the predominant multifunctional protein responsible for fatty acid β-oxidation at this stage. In both the ped1 and kat2 mutants, postgerminative seedling growth arrests in the absence of an exogenous supply of sucrose. In addition, seedlings of kat2 are unable to catabolize storage triacylglycerol (TAG) and accumulate long-chain (C16:0-C20:0) acyl-CoA units (19). Five-day-old ped1 and kat2 seedlings grown in the light also have enlarged peroxisomes with lipid bodies still present in the coryledons (19, 22). In addition, a mutant in the yeast Yarrowia lipolytica disrupted in multifunctional enzyme type 2 (MFE2), a homologue of MFP2, also has enlarged peroxisomes (23).

Arabidopsis mutants in β-oxidation genes show resistance to the compound 2,4-dichlorophenoxybutyric acid (2,4-DB), which is metabolized to the herbicide and auxin analogue 2,4-dichlorophenoxyacetic acid by β-oxidation (22, 24). The ped1kat2 seedlings exhibit a high level of resistance to 2,4-DB (up to 10 μM) whereas acx3 and aim1 seedlings are only resistant at lower concentrations (up to 2 μM) (1, 8).

There are several characteristics of the ACX4 gene that distinguish it from the ACX1, -2, and -3 genes. Intriguingly, the ACX4 gene sequence is more closely related to mammalian mitochondrial acyl-CoA dehydrogenases than to other ACX genes. Mammalian peroxisomes are unable to β-oxidize short-chain acyl-CoAs, and fatty acids are shuttled to the mitochondria instead for completion of the process involving acyl-CoA dehydrogenase (23). Furthermore, the ACX1, -2, and -3 isozymes share common characteristics; they are all dimers, with similar subunit molecular masses, pH optima, and respective substrate affinities. However, ACX4 exists as a homotetramer with a K_m double that of ACX1, -2, and -3 (25). In addition, the activity of short-chain (C6:0) ACX in 2-day-old Arabidopsis seedlings is significantly higher than medium-chain (C12:0) ACX (Fig. 2b) and Ref. 1) and 17-fold higher than long-chain activity (C18:0) ACX activity (1).

In mammals and yeast, coordinate regulation of peroxisomal β-oxidation genes is via free fatty acids or acyl-CoAs (26–28). In plants, although there is some correlative evidence suggesting that acyl-CoAs are involved in controlling peroxisomal size and morphology in Arabidopsis seedlings (29), peroxisomal β-oxidation regulation is not yet understood. Together, these factors made ACX4 an interesting target for reverse genetics analysis.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions—Seeds were surface-sterilized and germinated in continuous light on 1% (w/v) agar plates containing half-strength Murashige and Skoog medium (30) (plus 20 mM sucrose, were indicated) at 20 °C following 4 days of incubation at 4 °C in the dark. For experiments with etiolated seedlings, plates were transferred back to the dark at 20 °C after a 1-h exposure to white light.

Mutant Isolation—We screened the T-DNA-mutagenized Arabidopsis thaliana (ecotype Wassilewskija) population at the University of Wisconsin-Madison Biotechnology Center (available on the World Wide Web at www.biotech.wisc.edu/Arabidopsis/) (31), using primers to the ACX4 gene, 5'-GTC CGC AAT TTC TCC GTC TCT GAC ATC ATC ATC-3' and 5'-GTT AAC AAA TGG TAT TTC CCC CAG TAC AA-3', the MFP2 gene, 5'-GGG CCA AGG ATT AAG AGA CAC TTT TAC TAT GGG TGC TC-3' and 5'-GAA AGC AGC CAA CGG TGC TC-3', the KAT2 gene, 5'-GAA AGC AGC CAA CGG TGC TC-3' and 5'-GCC GGT TTG AAA AGC AGC CAA GC-3', and the act2 gene, 5'-TTG GGG CCG CTA ACT GCA TCT GCT C-3' and 5'-GTA CA-3', P4 (5'-GCC GGT TTG AAA AGC AGC CAA GC-3'), P1 (5'-GTT GGG CCG CTA ACT GCA TCT GCT C-3'), and P2 (5'-GCT TGG GGC TAT GGG TGT TG). Soluble protein was measured on the same extracts as those used for enzyme assays, according to the method of Bradford (35). Western blot analysis was carried out using an antibody raised against the Arabidopsis ACX4 protein as previously described by Hayashi et al. (15). This antibody was shown to recognize specifically recombinant Arabidopsis ACX4 protein (15).

Fatty Acid and Acyl-CoA Measurement—Fatty acids were measured using the method of Browse et al. (37), and acyl-CoAs were measured using the method of Larson and Graham (38) with the modifications of Larson et al. (39).

RT-PCR—Total RNA from various tissues was isolated using the RNaseasy isolation kit (Qiagen), and cDNA was made using Invitrogen reverse transcriptase and reagents. For RT-PCR, the primers used were acx2a (5'-GTC CGC AAT TTC TCC GTC TCT GAC ATC ATC ATC-3'), act2s (5'-TTG GGG CCG CTA ACT GCA TCT GCT C-3'), P4 (5'-GCC GGT TTG AAA AGC AGC CAA GC-3'), and act2s (5'-GTA CA-3').

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(5'-CAT TTT ATA ATA ACG CTG CGG ACA TCT AC-3') to amplify across the T-DNA region. The presence of the acx3 T-DNA in the progeny, determined using PCR, was confirmed using GUS staining of leaves, as described by Eastmond et al. (1).

Peroxisome Morphology—The acx3, acx4 and kat2 mutants were crossed into a line (A5) containing GFP fused to a truncated MFP2 cDNA that targets GFP to the peroxisomes (40) (see supplementary information on the World Wide Web at deepgreen.stanford.edu). Lines homozygous for acx3, acx4, and kat2 were identified by PCR and by resistance to kanamycin. The acx3:GFP-MFP2, acx4:GFP-MFP2 and kat2:GFP-MFP2 lines were germinated on half-strength Murashige and Skoog medium plus 20 mM sucrose plates. Peroxisomes were visualized in 4-day-old seedlings using a Nikon Optiphot-2 confocal microscope with mercury lamp and epifluorescence filter set. GFP fluorescence was detected at 510–532 nm with excitation at 488 nm.

RESULTS

Mutant Isolation and Genotypic Characterization—The acx4 mutant was isolated from the T-DNA-tagged population described by Sussman et al. (31) following procedures detailed under “Experimental Procedures.” The acx4 mutant was back-crossed, and the F1 population shown to segregate 3:1 for kanamycin resistance. PCR-based analysis of the F2 progeny showed that kanamycin resistance segregated with T-DNA insertion in the ACX4 gene and that only those plants that were homozygous for T-DNA insertion in the ACX4 gene were affected in short-chain acyl-CoA oxidase activity. Sequencing of the left and right borders and flanking regions indicated that the T-DNA was situated in the last exon of the acx4 gene, 3315 bp 3′ from the ATG (Fig. 1a). PCR and sequencing analysis indicated that the T-DNA insert consisted of a truncated, 400-bp, left border fragment followed by an inverted, complete T-DNA. The 13 amino acids at the carboxyl terminus of the ACX4 protein, including the putative PTS1 Ser, Arg, and Leu, are predicted to be replaced with a 23-amino acid sequence, encoded by the T-DNA insert and ending in the amino acids Lys, Lys, Lys, and Gln (Fig. 1a). RT-PCR analysis using ACX4-specific primers, 5′ of the T-DNA insert, amplified the expected sized fragment from both wild type and acx4 seedling RNA. Primers that spanned the T-DNA insert failed to amplify a product for the acx4 cDNA (Fig. 1b). These results suggest that a truncated or modified transcript of ACX4 is produced in acx4.

Activity of ACX4—To investigate whether the production and integrity of the ACX4 protein was perturbed in acx4, a Western blot of protein extracts from 0–5-day-old wild type and acx4 seedlings was challenged with an antibody raised against Arabidopsis ACX4 (15). A band corresponding to the predicted molecular mass of 47.7 kDa for ACX4 was detected in the wild type, but no bands were detected in acx4 seedling extracts (Fig. 2a). To investigate whether the acx4 mutant displayed altered acyl-CoA oxidase activity, enzyme activity was measured in 2-day-old germinating seedlings (Fig. 2b) and rosette leaves (Fig. 2c). In seedlings and leaves, the acx4 mutant exhibited less than 2% of wild type short-chain acyl-CoA oxidase activity (measured using hexanoyl-CoA, C6:0 substrate), whereas medium chain activity was unaltered (measured using lauroyl-CoA, C12:0 substrate).

Phenotypic Analysis—Arabidopsis mutants in the β-oxidation genes, ped1/kat2, acx3, and aim1 exhibit varying degrees of resistance to 2,4-DB, indicating that they have impaired β-oxidation activity (1, 8, 19, 22). To quantify the level of resistance in acx4 seedlings, root length of seedlings germinated on half-strength Murashige and Skoog medium (30) containing 1.5 μM 2,4-DB was measured. As shown in Fig. 3, a and b, homozygous acx4 seedlings were significantly more resistant to 2,4-DB than wild type seedlings, although acx4 seedlings were not as resistant as kat2 seedlings. Both acx4 and wild type seedlings were susceptible to the auxin 2,4-dichlorophenoxyacetic acid (results not shown), demonstrating that the resistance to 2,4-DB in the mutant is specific to the impaired β-oxidation activity in acx4. Heterozygous acx4 seedlings were sensitive to 2,4-DB, indicating that 2,4-DB resistance is a recessive phenotype.

The kat2 mutant is impaired in the ability to catabolize storage lipid through β-oxidation, and both ped1 and kat2 mutants require an exogenous supply of sucrose for seedling establishment (19, 22). To investigate whether the acx4 mutation affects germination and seedling establishment, wild type and acx4 seedlings were grown for 4 weeks on half-strength Murashige and Skoog medium plates with and without 20 mM sucrose under a range of photoperiods (8, 12, 16, and 24 h) and decreasing light intensities (160, 80, 40, 20, and 10 μmolm−2s−1 and total darkness). No significant difference in seedling growth or germination was seen between wild type and acx4 under any of the growth conditions tested (results not shown). In addition, despite the fact that the short-chain ACOX activity had dropped to less than 2% of wild type levels in rosette leaf tissue extracts (Fig. 2c), no alterations in vegetative or reproductive phenotype were seen throughout the life cycle of acx4 plants. To examine whether lipid breakdown was compromised in the acx4 mutant, lipid levels were measured in
and acx4 mutant. To investigate specifically the rate of storage TAG breakdown in acx4 seedlings, we measured the levels of eicosenoic acid (C20:1). Eicosenoic acid is specific to storage TAG in Arabidopsis (41). Using the levels of eicosenoic acid as a marker for the rate of TAG breakdown, the data in Fig. 4c show that the overall rate of TAG breakdown in acx4 seedlings was not significantly different from wild type, although 3-day-old acx4 seedlings did contain significantly more eicosenoic acid than wild type seedlings.

Although the overall rate of lipid catabolism was unaffected, acx4 seedlings exhibited resistance to 1.5 μM 2,4-DB, demonstrating that the flux at some point through the β-oxidation pathway had been restricted. Acyl-CoAs are both β-oxidation intermediates and ACOX substrates, and we have developed a new method for their measurement (38, 39). Using this methodology, we observed a 9-fold increase in the quantity of short-chain acyl-CoAs (C4:0 and C6:0) in 3-day-old seedlings of acx4 (31 mol % for short-chain (C4:0 and C6:0) acyl-CoAs) relative to wild type (2.1 mol %) (Fig. 4b). In addition, peaks of unidenti-
than 200 F2 progeny plants, germinated on medium containing 20 mM sucrose, was determined. The acx3 line contains a promoter-trapped T-DNA and expresses the β-glucuronidase reporter gene under the control of the ACX3 promoter (1). β-Glucuronidase staining was initially used to identify F2 progeny with a T-DNA in the ACX3 gene, and these plants were further genotyped at the ACX3 and ACX4 loci by PCR. The T-DNA present in the acx4 mutant also contains a β-glucuronidase gene; however, it is under the control of a small apetala promoter fragment (32), and expression is mainly restricted to the floral parts of the acx4 plant and was not detectable in leaves. No acx3acx4 double mutant plants were identified using this approach.

Seedlings of ped1kat2 are blocked in fatty acid breakdown and are resistant to higher concentrations of 2,4-DB (up to 10 μM) than either acx3 or acx4 seedlings individually (up to 2 μM). Based on the assumption that double homozygous seedlings might be more resistant to 2,4-DB than either acx3 or acx4 individually, larger (>10,000) numbers of seedlings were screened for enhanced resistance on medium containing 1.5 and 5 μM 2,4-DB. No seedlings with increased resistance to 2,4-DB were found. Seedlings of ped1kat2 also require an exogenous supply of sucrose for seedling establishment; hence, we also screened large (>10,000) numbers of F2 seeds for sucrose dependence. Seedlings that failed to establish (<5%) were transferred to medium containing 20 mM sucrose, and the genotype was determined using PCR. Again, no double homozygous acx3acx4 plants were identified. Siliques of plants homozygous for acx4 and heterozygous for acx3 contained ~25% less seed than wild type, acx3, or acx4 plants. Examination of embryos from plants homozygous for acx3 or acx4 but heterozygous for acx4 or acx3, respectively, at the middle to late cotyledonary stage revealed gaps in the rows of developing embryos. Further examination revealed these to be embryos that had aborted during the phase of embryo differentiation and pattern formation (34). The number of aborted embryos per silique in these plants accounted for ~26–33% of the embryos per silique (Fig. 7). The number of aborted embryos per silique in wild type, acx3, and acx4 plants was less than 4%. The ratio of heterozygous (ACX3acx3) to wild type (ACX3ACX3) plants in progeny from self-fertilized acx3acx4 ACX3acx3 plants recorded in Table I is close to the 2:1 ratio expected if homozygous acx3 embryos were nonviable in the acx4 homozygous background (the χ² test value = 8.50, p = 0.1%). These results indicate that the combined removal of both short- and medium-chain ACX activity is lethal to the embryo at an early stage of development. To investigate the level of ACX expression and activity in developing embryos, semiquantitative RT-PCR and ACX assays were performed on developing wild type Arabidopsis embryos. Specific stages representative of the three main phases of embryo development (34) were selected. The first phase is differentiation and pattern formation represented by heart and Torpedo stage. The second phase is maturation (cell expansion and storage reserve accumulation) represented by cotyledon stage. The third phase is desiccation represented by desiccating and dry seeds. Fig. 8a demonstrates that transcripts for both ACX3 and ACX4 are produced throughout these stages of embryo development with ACX3 transcript levels being consistently lower than ACX4. Fig. 8b shows that both short and medium chain ACX activity was also detectable from the heart stage of embryo development onwards. The ACX4
activity, on a protein basis, in heart stage embryos is ~33% of that found in 2-day-old wild type seedlings.

DISCUSSION

We have identified and characterized an Arabidopsis mutant disrupted in the gene encoding the short-chain acyl-CoA oxidase (acx4). Although 2-day-old seedlings of acx4 contained less than 2% of wild type short-chain acyl-CoA activity, lipid breakdown and seedling establishment was not compromised. Seedlings of the acx3 mutant, which contain only 5% of wild type C10:0 activity, are also not compromised in seedling establishment and storage lipid catabolism. Eastmond et al. (1) proposed that lipid breakdown was achieved by residual acyl-CoA oxidase activity from ACXs with substrate specificities overlapping those of acx3. The residual C6:0 activity in acx4 is probably due to the ACX3 protein. That both of these mutants are uninhibited in storage lipid breakdown despite the fact that only residual ACX activities remain is remarkable and suggests that short and medium chain ACXs exert little control on lipid breakdown. The specific activities of these enzymes are significantly higher than the long chain ACXs (1, 13, 15), and whether or not ACX1 and ACX2 exert greater control over β-oxidation flux than ACX3 and ACX4 (in keeping with previous reports (1, 13)) remains to be established. Data on antisense ACX1 lines demonstrated that antisense ACX1 seedlings with 70% less long chain ACX activity germinated and established normally (13).

Both acx3 and acx4 show resistance to 2,4-DB, demonstrating that both mutants are compromised in the flux through β-oxidation. In addition, seedlings of acx4 accumulate short-chain acyl-CoAs, whereas acx3 seedlings accumulate medium-chain acyl-CoAs. This demonstrates that the acx3 and acx4 mutants are restricted in the capacity to β-oxidize short- and medium-chain acyl-CoAs.

**Fig. 5.** Medium-chain length acyl-CoA levels in wild type and acx3 seedlings grown on medium containing 20 mM sucrose. Black bars, wild type; white bars, acx3. a, day 0 after imbibition; b, day 1; c, day 2; d, day 3; e, day 4; f, day 5. In all cases, seedlings were grown on 20 mM sucrose. The values shown are the mean ± S.E. of measurements on five batches of seedlings.

**Fig. 6.** Confocal images of GFP fluorescence in peroxisomes of 4-day-old Arabidopsis seedling expressing a GFP-MFP2 fusion construct from line A5 (40) crossed into kat2, acx3, and acx4. a, line A5; b, kat2:GFP-MFP2; c, acx3:GFP-MFP2; d, acx4:GFP-MFP2. Bar, 25 μm.

**Fig. 7.** Number of aborted embryos in siliques of wild type, acx3, and acx4 plants and in siliques from acx3ACX3 acx4ACX4 plants. The values are the mean from 10 siliques from each of three plants. The developed embryos were at the middle to late cotyledonary stage.
medium-chain acyl-CoAs, respectively, although this restriction is not sufficient to compromise seedling establishment. Two-day-old acx3 seedlings accumulate only 2 times more medium-chain acyl-CoAs than wild type, whereas acx4 seedlings accumulate 9 times more short-chain acyl-CoAs than wild type. Heterologous expression studies in *Escherichia coli* show that ACX1 and ACX2 do not exhibit any detectable activity with C4:0 acyl-CoA substrate (13), whereas ACX3 exhibits a low but detectable activity (1), thus suggesting that this activity is sufficient to allow fatty acid breakdown in the acx4 mutant background. Based on this reasoning, a complete block in the 

β-oxidation of the shortest chain length acyl-CoAs in an acx3acx4 double mutant would therefore be expected. The results of the χ² test show that the ratio of heterozygous to wild type plants for ACX3 recorded in Table I is not significantly different (p = 0.1%) from the 2:1 ratio expected if homozygous acx3 embryos were lethal in the acx4 homozygous background. Furthermore, the percentage of aborted embryos in siliques of plants with ACX3acx3 acx4acx4 genotypes and plants with acx3acx3 ACX4acx4 genotypes is close to the 25% expected if double acx3acx4 mutant embryos were nonviable. We have also screened large quantities of seed from these plants on both medium containing 2,4-DB and medium without sucrose and have not identified any acx3acx4 double mutants. Thus, we conclude that the nonviable embryos seen in the siliques of self-fertilized plants with ACX3acx3 acx4acx4 and acx3acx3 ACX4acx4 genotypes are acx3acx4 double mutants, and a complete block in short-chain acyl-CoA β-oxidation capacity is lethal during early embryo development. In addition to the accumulation of C4:0 and C6:0 acyl-CoAs shown in Fig. 4b, peaks for other, unidentified short-chain acyl-CoA etheno derivatives were seen (Fig. 4c). Two of these peaks shared retention times with acetyl-CoA (C2:0) and propionyl-CoA (C3:0). Additional peaks may represent short-chain, unsaturated acyl-CoAs (e.g. C6:1) that result from incomplete β-oxidation of long-chain unsaturated fatty acids (e.g. lineolic (Δ⁶,Δ⁹,Δ₁₂−18:3)).

In *kat2* seedlings, storage triacylglycerol breakdown is greatly reduced, and long-chain acyl-CoAs accumulate during early postgerminative growth (19). While there are three additional thiolase genes in the *Arabidopsis* genome, they do not compensate significantly for the thiolase activity in *ped1kat2*, either because they are not expressed sufficiently during early postgerminative seedling growth or they do not share overlapping substrate specificity with *KAT2*. The observation that the *ped1kat2* mutants show no visible phenotype throughout the rest of the life cycle suggests that, after seedling establishment, KAT2 activity is sufficiently compensated for by these additional thiolases to maintain normal growth and development. Unlike genes of the KAT gene family, which appear to encode enzymes with broad substrate specificity, members of the acyl-CoA oxidase gene family encode enzymes with distinct but overlapping activities. Consequently, removal of ACX3 and ACX4 activity through generation of a double mutant would result in the loss of short-chain activity that cannot be compensated for by one of the other ACX proteins, since these do not exhibit activity with short-chain acyl-CoAs.

The gaps seen in siliques of plants homozygous for acx3 or acx4 and heterozygous for acx4 or acx3, respectively, indicate that the acx3acx4 double mutant aborted in the first phase of embryo development. During this stage, the embryo cells are undergoing division and differentiation and establishing the pattern of the embryo. The early stage embryo lethality observed in the double mutant could be due to one of a number of factors including the accumulation to toxic levels of acyl-CoAs or short chain fatty acids, sequestration of CoA to the acyl-CoA pool, or the disruption in the production of fatty acid or lipid-based signaling molecules that are critical for embryogenesis. If the embryo lethality is due to the accumulation of toxic, short-chain acyl-CoAs, the higher specific activities of ACX3 and ACX4 over the longer chain ACXs would ensure that these toxic, shorter chain acyl-CoA intermediates did not accumulate during β-oxidation in wild type plants. However, the accumulation of up to 61 mol % of C12:0 acyl-CoA reported in oilseed rape plants engineered to produce increased levels of medium-chain fatty acids had no effect on late embryo development (39, 42). Furthermore, we have shown here that 3-day-old acx4

### Table I

<table>
<thead>
<tr>
<th>F2 genotype</th>
<th>Frequency of genotype in F3 progeny</th>
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<tr>
<td></td>
<td>acx3 acx3</td>
</tr>
<tr>
<td>ACX3acx3, acx4acx4</td>
<td>0</td>
</tr>
<tr>
<td>Expected frequency for 2:1 ratio</td>
<td>0</td>
</tr>
<tr>
<td>Expected frequency for 1:2:1 ratio</td>
<td>56.5</td>
</tr>
</tbody>
</table>

**Fig. 8.** ACOX transcript and enzyme levels in wild type embryos. Ht, heart; To, Torpedo; Co, cotyledon; De, desiccating; Ds, dry seed. a, semiquantitative RT-PCR analysis on wild type embryo cDNA. Actin control primers used to amplify the ACT2 gene were Act2a and Act2e; ACX3 primers were ACX3-S and ACX3-A; and ACX4 primers were P2 and P3. Data are representative of two separate experiments. b, ACX activity in wild type embryos using hexanoyl-CoA (C6:0) substrate (gray bar) and lauroyl-CoA (C12:0) substrate (white bars). Soluble protein per embryo is presented as a solid line. Values are the mean ± S.E. of measurements made on three separate batches of 200 embryos.
seedlings accumulate significant C4:0 and C6:0 acyl-CoA (31 mol %) without any detrimental effects on embryo development, and 5-day-old kat2 seedlings, which accumulate 3-fold more long-chain acyl-CoAs than wild type, also exhibit normal embryo development (19).

A role for peroxisomal $\beta$-oxidation in the production of fatty acid or lipid-based signaling molecules may be essential for embryogenesis. For example, $\beta$-oxidation plays an important role in the synthesis of the wound-related signaling molecule jasmonic acid (43). While this compound does not have any known role in embryo development, it is possible that other, as yet uncharacterized fatty acid related signals could operate. In relation to this, it is noteworthy that the $\beta$-oxidation mutant aim1 exhibits abnormal floral development and severely reduced fertility and produces seed of variable size and shape; however, these seeds are still viable (8). In addition, a cross between ped1 and ped3, a mutant in an ATP-binding cassette transporter involved in the transport of fatty acids and their derivatives across the peroxisomal membrane, exhibits a similar phenotype to aim1 with wavy leaves and dwarf, sterile inflorescences with abnormal structure (44). Both aim1 and ped1ped3 phenotypes suggest a role for $\beta$-oxidation in the generation of signals associated with development. However, of the $\beta$-oxidation mutants reported to date, ped1kat2, aim3, and accx4, none have a total block in the peroxisomal $\beta$-oxidation pathway due to redundancy and overlapping substrate specificities within the gene families. The accx3accx4 double mutant is the first demonstration of the effects of eliminating (short-chain) $\beta$-oxidation capacity in plants. Interestingly, there are parallels to the phenotypes reported in Arabidopsis $\beta$-oxidation mutants in mice and humans. Mutations in long-chain acyl-CoA oxidase (ACOX1) and an MFP homologue, $\beta$-bifunctional protein in humans both lead to an accumulation of very long chain fatty acids and severe abnormalities in development (for a review, see Wanders et al. (45)).

Alterations in peroxisomal size and abundance have been reported for both mammals (46) and yeast (23, 47). The genes involved all have roles in either peroxisomal $\beta$-oxidation or peroxisome biogenesis. In yeast, mutants in the $\beta$-oxidation gene MFE2 (a MFP2 homologue) have enlarged peroxisomes (29), and Arabidopsis mutants, ped1 and kat2 also have enlarged peroxisomes (19, 22). Five-day-old Arabidopsis seedlings accumulate long-chain acyl-CoAs, and this increase in acyl-CoAs could be involved in signaling peroxisome development (19). An allele of ped3, comatose (cts1), like kat2, requires an exogenous supply of sucrose for seedling establishment, does not catabolize storage lipid, and also accumulates acyl-CoAs, indicating that the CTS1 ATP-binding cassette protein transports acyl-CoAs (48). However, the cts1 mutant peroxisomes appear normal.

Based on the assumption that kat2 accumulates acyl-CoAs inside the peroxisome and cts1 accumulates acyl-CoAs in the cytosol, the increase in peroxisomal size in kat2 may be due specifically to the peroxisomal concentration of acyl-CoAs (29). Both accx3 and accx4 accumulate acyl-CoAs, and accx4 accumulates more acyl-CoA than kat2, but, unlike kat2, the peroxisomes of accx4 appear normal. The accx4 mutant accumulates short-chain acyl-CoAs, whereas kat2 accumulates long-chain acyl-CoAs. This suggests that it is not the increase in acyl-CoA concentration per se that results in increased peroxisomal size; rather, the long-chain acyl-CoAs are specifically involved in regulating this process.

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Arabidopsis Mutants in Short- and Medium-chain Acyl-CoA Oxidase Activities Accumulate Acyl-CoAs and Reveal That Fatty Acid \( \beta \)-Oxidation Is Essential for Embryo Development

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