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Promoter Trapping of a Novel Medium-chain Acyl-CoA Oxidase, Which Is Induced Transcriptionally during *Arabidopsis* Seed Germination*

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The first step of peroxisomal fatty acid β-oxidation is catalyzed by a family of acyl-CoA oxidase isozymes with distinct fatty acyl-CoA chain-length specificities. Here we identify a new acyl-CoA oxidase gene from *Arabidopsis* (*AtACX3*) following the isolation of a promoter-trapped mutant in which β-glucuronidase expression was initially detected in the root meristem. In *acx3* mutant seedlings medium-chain acyl-CoA oxidase activity was reduced by 95%, whereas long- and short-chain activities were unchanged. Despite this reduction in activity lipid catabolism and seedling development were not perturbed. *AtACX3* was cloned and expressed in *Escherichia coli*. The recombinant enzyme displayed medium-chain acyl-CoA substrate specificity. Analysis of β-glucuronidase activity in *acx3* revealed that, in addition to constitutive expression in the root axis, *AtACX3* is also up-regulated strongly in the hypocotyl and cotyledons of germinating seedlings. This suggests that β-oxidation is regulated predominantly at the level of transcription in germinating oilseeds. After the discovery of *AtACX3*, the *Arabidopsis* acyl-CoA oxidase gene family now comprises four isozymes with substrate specificities that encompass the full range of acyl-CoA chain lengths that exist *in vivo*.

Peroxisomal β-oxidation is the primary pathway of fatty acid catabolism in plants. The pathway plays a fundamental role in breaking down stored lipid reserves to provide metabolic energy and carbon skeletons during processes such as oilseed germination, leaf senescence, and starvation (1, 2). β-Oxidation may also play a constitutive role in membrane lipid turnover and be involved in the synthesis of important fatty acid-derived signals such as jasmonic acid (3) and traumatin (4). Recently an *Arabidopsis* mutant (*aim1*) has been isolated that is deficient in a multifunctional protein isoform (5). The fact that *aim1* displays an altered inflorescence meristem phenotype has lead to the suggestion that β-oxidation may be involved in flower development (5).

Peroxisomal β-oxidation consists of three components: (i) acyl-CoA oxidase, (ii) the multifunctional protein (which exhibits 2-*trans*-enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, D-3-hydroxyacyl-CoA epimerase, and Δ^3,Δ^2-enoyl-CoA isomerase activities), and (iii) L-3-ketoacyl-CoA thiolase. Together these enzymes are capable of the complete degradation of both saturated and unsaturated long-chain fatty acyl-CoAs to acetyl-CoA (6). The process involves the repeated cleavage of acetate units from the thiol end of the fatty acid.

Acyl-CoA oxidase (ACX,[1] EC 1.3.3.6) catalyzes the conversion of fatty acyl-CoAs to trans-2-enoyl-CoAs. The reaction requires FAD as a cofactor, which is subsequently re-oxidized by O_2 to form H_2O_2. This first step is believed to be predominant in exerting control over the rate of carbon flux through the pathway (7, 8). Biochemical evidence suggests that plants contain a family of acyl-CoA oxidase isozymes with distinct but partially overlapping substrate specificities (9–11). cDNA clones of several acyl-CoA oxidase homologues have been identified in plants (12–14).

However, direct evidence of their identity has only recently been obtained by overexpression and characterization of the recombinant proteins. Hooks *et al.* (15) have identified and characterized two long-chain acyl-CoA oxidases from the oilseed *Arabidopsis thaliana* (*AtACX1* and *AtACX2*). The preferred substrate of *AtACX1* is myristoyl-CoA (C14:0), whereas that of *AtACX2* is oleoyl-CoA (C18:1). In addition Hayashi *et al.* (16) have recently described a gene (referred to here as *AtACX4*) that encodes an enzyme with specificity for the short-chain substrate hexanoyl-CoA (C6:0). A comparison of the substrate specificities of known acyl-CoA oxidases from *Arabidopsis* suggests that a gene encoding a medium-chain acyl-CoA oxidase with a preference for decanoyl-CoA (C10:0) and lauroyl-CoA (C12:0) remains to be discovered (15).

Three acyl-CoA oxidase genes, a multifunctional protein, and a 3-keto acyl-CoA thiolase are up-regulated co-ordinately dur-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF253474.

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[1] The abbreviations used are: ACX, acyl-CoA oxidase; T-DNA, transfer DNA; GUS, β-glucuronidase; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; UTR, untranslated region; PS, protein signature; 2,4-DB, 2,4-dichlorophenoxybutyric acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; FAD, flavin adenosine dinucleotide; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; bp, base pair(s).

ing *Arabidopsis* seed germination and post-germinative growth, correlating with the period of most rapid fatty acid degradation (Refs. 15–18, respectively). In addition genes encoding glyoxylate cycle and gluconeogenesis enzymes are also induced during oilseed germination (19, 20). Although transcription is known to play a major role in the regulation of the glyoxylate cycle (19, 21, 22), the level at which peroxisomal β-oxidation is regulated has not yet been determined.

The regulation of β-oxidation in plants may be important for a variety of biotechnological applications. In the majority of cases where crops have been genetically engineered to produce novel fatty acids, the accumulation of these products is much lower than that required for commercial exploitation (23, 24). It is believed that in these plants the novel fatty acids are synthesized at significant rates but are subsequently degraded by peroxisomal β-oxidation (25, 26). Moreover, Eccleston and Ohlrogge (25) provide evidence that in the case of plants producing medium-chain fatty acids, medium-chain acyl-CoA oxidase activity is up-regulated to facilitate this degradation. The down-regulation of acyl-CoA oxidases may therefore promote the accumulation of unusual fatty acids in genetically modified crops.

In this study we identify and characterize a promoter-trapped *Arabidopsis* mutant disrupted in a gene encoding a new member of the acyl-CoA oxidase family with medium-chain substrate specificity. This gene is up-regulated at the level of transcription during seed germination. Combined with the three genes previously identified and characterized, our data reveal that *Arabidopsis* comprises a family of isoforms that together are capable of utilizing the full range of fatty acyl-CoA chain lengths present *in vivo*.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions—A T-DNA-mutagenized *A. thaliana* (ecotype Wassilewskija) population consisting of 10,000 lines (27) transformed with the pGKB5 vector designed for promoter trapping (28) was screened for transformants exhibiting GUS expression in the roots. Seeds were surface-sterilized and germinated on modified Hoagland solution (1 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 1.7 mM KNO_3, 0.5 mM $NH_4H_2PO_4$, 1.6 mM $FeSO_47H_2O$, 46.2 μM H_3BO_3, 9.1 μM $MnCl_2$, 0.87 μM $ZnSO_4$, 0.32 μM $CuSO_4$, and 1.03 μM Na_2MoO_4) plus 0.8% (w/v) agar. After 7 days of growth in a 16-h photoperiod (23 °C light/18 °C dark) seedlings were stained for GUS as described below. For subsequent analysis of line Rm 328, seeds were germinated in continuous light on 0.8% (w/v) agar plates containing half-strength Murashige and Skoog media (29) (plus 1% (w/v) sucrose where indicated) at 20 °C after 4 days of imbibition at 4 °C in the dark. For experiments with etiolated seedlings, plates were transferred back to the dark at 20 °C after 30 min exposure to white light.

Isolation of T-DNA Flanking Sequences—The sequence flanking the right border of the T-DNA inserts in lines displaying GUS expression in the roots was obtained by inverse PCR. Genomic DNA was digested with *Eco*RI and ligated using T4 DNA ligase. PCR was then carried out using the primers GUS1 (5′-CCAGACTGAATGCCCACACGCCGTC) and inverse PCR 2200 (5′-GTATCACCGCGTCTTTGATCGCGTC). The conditions for amplification were 15 s at 94 °C, 30 s at 65 °C, and 30 s at 68 °C, repeated 38 times followed by a 2-min extension. The products were sequenced directly using the nested primer GUS2 (5′-TCACGGGTTGGGGTTTCTACAGGAC).

Isolation of cDNA Clones—A partial clone of *AtACX3* was isolated from a λZAPII cDNA library constructed from 3-day-old etiolated hypocotyls (30) by 3′ RACE using the gene specific primer ACX3S (5′-AGGAGTATTTATCACAAACTCTTCAG) in combination with the T7 primer. The library was then screened by colony hybridization according to Sambrook *et al.* (31) using the partial clone as a probe (15). Four full-length clones were identified and sequenced. RNA was prepared from 2-day-old seedlings (see below), and the 5′-UTR was mapped by 5′ RACE using the 5′ RACE System Version 2 (Life Technologies, Inc.) following the manufacturer's protocols. GSP1 and GSP2 were (5′-ACTTTACAGCATTACCCCACAGGA) and (5′-GTGATCATAAATCCCGCAAACCT), respectively. Three 5′ RACE products from separate PCR reactions were cloned and sequenced.

Expression of AtACX3—One *AtACX3* cDNA clone was determined to be in-frame with the N terminus of the pBluescript β-galactosidase gene and could be expressed as a fusion protein following induction of *Escherichia coli* XL1-blue MRF™ cells with isopropyl-β-D-thiogalactoside. Cells were grown at 37 °C to an optical density of 0.5 at 600 nm in Luria broth media. Isopropyl-β-D-thiogalactoside (0.4 mM) was then added to the culture, and the cells grown overnight at 28 °C. The culture was centrifuged at 700 × *g* for 10 min, and the pellet was resuspended in extraction buffer (150 mM Tris/HCl, pH 7.5, 10 mM KCl, 1 mm EDTA, 1 mm dithiothreitol, 10 mm FAD, 10% (v/v) glycerol). The cells were lysed by sonication using a Soniprep 150 ultrasonic disintegrator (Sanyo Gallenkamp PLC, Leicester, UK), and cell debris was removed by centrifugation at 21,000 × *g* for 10 min. Assays were performed on the supernatant.

Tissue Extraction and Subcellular Fractionation—Crude tissue extracts were prepared from approximately 2000 2-day-old seedlings. The tissue was ground in 1 ml of extraction buffer using a glass homogenizer. The extract was then centrifuged at 13,000 × *g* for 10 min, and the supernatant was desalted using a Sephadex G-50 spin column as described by Hooks *et al.* (15).

For subcellular fractionation experiments, approximately 10,000 2-day-old seedlings were homogenized in 3 ml of a medium containing: 150 Tricine/KOH, pH 7.5, 1 mM EDTA, 0.5 M sucrose using an Ultra-Turrax (Janke and Kunkel KG). The homogenate was filtered through four layers of mira cloth, and 2 ml of homogenate was layered on top of a gradient consisting of a 1-ml cushion of 60% (w/v) sucrose plus 8 ml of a 60 to 30% linear sucrose gradient. The gradient was centrifuged at 30,000 × *g* for 3 h in a Sorvall OTD55B centrifuge using a TST 41.14 swing out rotor. 0.5-ml fractions were removed and assayed for various enzyme activities. The sucrose concentration of the fractions was determined using a refractometer.

Enzyme Assays, Histochemical Staining, and Fatty Acid Measurement—ACX assays were performed on plant tissue and bacterial cell extracts according to the method of Hryb and Hogg (32) using 50 μM acyl-CoAs as substrate. GUS assays and histochemical staining were carried out as described by Jefferson (33). Catalase and cytochrome *c* oxidase were assayed according to Takahashi *et al.* (34) and Denyer and Smith (35), respectively. Protein content was determined as described by Bradford (36) using bovine serum albumin as a standard. Fatty acids were measured using the method of Browse *et al.* (37).

Northern Analysis, Southern Analysis, and Reverse Transcriptase-PCR—Total RNA from various tissues was isolated using the Purescript RNA isolation kit (Flowgen) or the hot phenol method (38). Ten μg of total RNA was separated by electrophoresis using a 1.1% formaldehyde gel and alkaline-blotted onto Zeta-Probe membrane (Bio-Rad) using 50 mM NaOH. Genomic DNA was purified using the Puregene DNA isolation kit (Flowgen). Three μg of DNA were digested using *Eco*RI, separated on a 0.8% agarose gel, and blotted using 0.4 M NaOH. Probes were prepared from *AtACX3* and *gus*A, and membranes were hybridized using the digoxygenin system (Roche Molecular Biochemicals) following the manufacturer protocols. Bands were detected using a ChemiImager (Alpha Innotech Corp.). Reverse transcriptase-PCR was performed using the Reverse-iT kit from Advanced Biotechnologies Ltd. The primers used were GUS1, ACX3S, and ACX3A (5′-GAAACATCAGCAACTGCATTCAAC).

RESULTS

Mutant Isolation—As part of a research program focused on root-rhizosphere interactions, *Arabidopsis* promoter trapped lines that displayed GUS expression in the roots of 7-day-old seedlings were isolated from the INRA-Versailles T-DNA-mutagenized population (27). Genomic DNA sequences that flank the right borders of the T-DNAs were obtained by inverse PCR. One of two inverse PCR products from a line with GUS expression in the root meristem (Rm 328) was found to be homologous to acyl-CoA oxidases. These enzymes catalyze the first step in the pathway of peroxisomal β-oxidation and are known to be highly active in the root tips of maize (2). This putative acyl-CoA oxidase was different from those previously identified in *Arabidopsis*, and the new gene and mutant were designated *AtACX3* and *acx3*, respectively.

Cloning of AtACX3—A partial *AtACX3* cDNA clone was isolated by performing 3′ RACE on a cDNA library constructed from 3-day-old etiolated seedling hypocotyls (30). This clone was used as a homologous probe to screen the same library by colony hybridization. Four full-length cDNAs were isolated and

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acaacgtcactttcttcccggataattgaaatcggtgatcggaatccctagaaatgtcgg   60
                                                    M  S  D
ataatcgtgcactccgacgagctcatgttctcgccaatcacatactccaatcaaatcctc  120
  N  R  A  L  R  R  A  H  V  L  A  N  H  I  L  Q  S  N  P  P
catcttcgaacccgtccctgtcgcgcgagttatgtttgcagtactctccaccggagctca  180
  S  S  N  P  S  L  S  R  E  L  C  L  Q  Y  S  P  P  E  L  N
acgagagctatggattcgatgtcaaggagatgagaaaattacttgacggacacaacgtgg  240
  E  S  Y  G  F  D  V  K  E  M  R  K  L  L  D  G  H  N  V  V
tggatcgggactggatttatggactcatgatgcagagcaatctgtttaatcggaaggaga  300
  D  R  D  W  I  Y  G  L  M  M  Q  S  N  L  F  N  R  K  E  R
gaggaggtaagattttcgtgtcgccggattacaatcagacgatggagcagcagcgtgaga  360
  G  G  K  I  F  V  S  P  D  Y  N  Q  T  M  E  Q  Q  R  E  I
tcacaatgaaacggatctggtacttgcttgagaatggggttttcaaaggatggttgacgg  420
  T  M  K  R  I  W  Y  L  L  E  N  G  V  F  K  G  W  L  T  E
agacaggtcctgaggccgagctcaggaaattagctctgcttgaggtttgcgggatttatg  480
  T  G  P  E  A  E  L  R  K  L  A  L  L  E  V  C  G  I  Y  D
atcactccgtctccatcaaagttggtgtgcatttcttcctgtggggtaatgctgtaaagt  540
  H  S  V  S  I  K  V  G  V  H  F  F  L  W  G  N  A  V  K  F
tttttggaacaaagcgtcaccatgaaaagtggctgaagaacaccgaagattatgttgtca  600
  F  G  T  K  R  H  H  E  K  W  L  K  N  T  E  D  Y  V  V  K
agggctgttttgctatgactgagctaggccatggaagtaatgtacggggaattgaaacag  660
  G  C  F  A  M  T  E  L  G  H  G  S  N  V  R  G  I  E  T  V
tgacaacttatgacccaaaaactgaagagtttgtgataaatactccttgtgaatctgctc  720
  T  T  Y  D  P  K  T  E  E  F  V  I  N  T  P  C  E  S  A  Q
agaagtattggattggtggggcagctaatcatgcaacccacacaattgtgttttcacagc  780
  K  Y  W  I  G  G  A  A  N  H  A  T  H  T  I  V  F  S  Q  L
atcatatcaacggaaccaaccaggggtccatgcctttatcgcccaaatcagggatcaag  840
  H  I  N  G  T  N  Q  G  V  H  A  F  I  A  Q  I  R  D  Q  D
atggcagcatatgtccaaatatccgcattgctgactgtggacacaaaattggtctaaatg  900
  G  S  I  C  P  N  I  R  I  A  D  C  G  H  K  I  G  L  N  G
gtgttgacaatggccggatctggtttgataatcttcgaattccaagagagaatttgttga  960
  V  D  N  G  R  I  W  F  D  N  L  R  I  P  R  E  N  L  L  N
atgcagttgctgatgtttcgtctgatgggaagtatgttagctcaattaaagatcctgatc 1020
  A  V  A  D  V  S  S  D  G  K  Y  V  S  S  I  K  D  P  D  Q
agagatttggagcattcatggccccttgacttctggccgagtcacaattgcatcaagtg 1080
  R  F  G  A  F  M  A  P  L  T  S  G  R  V  T  I  A  S  S  A
caatttattctgcaaaggtcggattatctattgctataaggtactcattatcgagaagag 1140
  I  Y  S  A  K  V  G  L  S  I  A  I  R  Y  S  L  S  R  R  A
ccttctctgttacagctaatggtcctgaagtcctcctccttgattacccaagccatcaaa 1200
  F  S  V  T  A  N  G  P  E  V  L  L  L  D  Y  P  S  H  Q  R
ggcgactgctaccactcctagcaaagacatatgctatgagttttgctgcaaatgaattga 1260
  R  L  P  L  L  A  K  T  Y  A  M  S  F  A  A  N  E  L  K
agatgatttacgtgaagagaacaccggagaccaacaaagccatccacgttgtttcaagtg 1320
  M  I  Y  V  K  R  T  P  E  T  N  K  A  I  H  V  V  S  S  G
ggttcaaagctgttctcacctggcacaatatgcacacacttcaggaatgtcgagaagctg 1380
  F  K  A  V  L  T  W  H  N  M  H  T  L  Q  E  C  R  E  A  V
tcggagggcaaggtgtgaaaacagaaaatctagttggtcagttgaaaggtgaatttgatg 1440
  G  G  Q  G  V  K  T  E  N  L  V  G  Q  L  K  G  E  F  D  V
tgcagactacatttgagggtgacaatgtattgatgcagcaggtgagcaaggcgctttt 1500
  Q  T  T  F  E  G  D  N  N  V  L  M  Q  Q  V  S  K  A  L  F
tcgctgaatatgtatcgtgtaagaagagaaacaaaccttttcaagggactgggattggagc 1560
  A  E  Y  V  S  C  K  K  R  N  K  P  F  K  G  L  G  L  E  H
acatgaacagtccacgtcctgtattaccgactcaactcacatcatcaaccctcagatgca 1620
  M  N  S  P  R  P  V  L  P  T  Q  L  T  S  S  T  L  R  C  S
gccaattccagacaaatgtgttctgcttaagagagcgagatcttctggagcaatttactt 1680
  Q  F  Q  T  N  V  F  C  L  R  E  R  D  L  L  E  Q  F  T  S
ctgaagttgcacagcttcaaggagaggagaaagtcgagaattctctttcctcctgagtc 1740
  E  V  A  Q  L  Q  G  R  G  E  S  R  E  F  S  F  L  L  S  H
atcaacttgctgaagacttaggtaaagctttcacagagaaagcaatacttcaaaccattt 1800
  Q  L  A  E  D  L  G  K  A  F  T  E  K  A  I  L  Q  T  I  L
tggatgctgaggcaaaactacctactggctcagtaaaggatgtgttgggtcttgtaagat 1860
  D  A  E  A  K  L  P  T  G  S  V  K  D  V  L  G  L  V  R  S
caatgtacgcattgatcagcttggaagaagatccatcgttgctgcgatatggttacctat 1920
  M  Y  A  L  I  S  L  E  E  D  P  S  L  L  R  Y  G  Y  L  S
ctcaggataatgttggagatgtgaggagagaagtttcaaagctctgtggagagcttagac 1980
  Q  D  N  V  G  D  V  R  R  E  V  S  K  L  C  G  E  L  R  P
cacacgcgcttgcactcgtcacttcattcggcattccagactccttcttgagtccaattg 2040
  H  A  L  A  L  V  T  S  F  G  I  P  D  S  F  L  S  P  I  A
cattcaactgggtcgaagccaatgcttggtcttcagtttagttacctgctagtaacaatc 2100
  F  N  W  V  E  A  N  A  W  S  S  V
tcttccataattcccatatatctcttataaaagttatgtttccacaatatctacagaaac 2160
tgttgtaagctcgtcataataaagggtatatatgatatgtatccagggtttattcttta 2220
cttaaactggtccaaacaaaaatgtt                                   2246
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FIG. 1. **Nucleotide and predicted amino acid sequence of AtACX3.** Features of the cDNA sequence are *underlined*. Amino acids that are common to protein signatures of acyl-CoA dehydrogenase (PS1 and PS2) are *double underlined*.

sequenced. Finally the 5′-UTR was mapped using 5′ RACE. The assembled sequence was submitted to the GenBank data base. The cDNA was 2246 bp long and contained a 2028-bp putative open reading frame (Fig. 1). The 5′-UTR extended 53 bp 5′ of the start of translation. The 3′-UTR contained a putative polyadenylation signal (AATAAA) 96 bp 3′ of the stop codon.

The deduced protein was 675 amino acids long (Fig. 1) with a calculated molecular mass of 75676.33 Da and an isoelectric point (pI) of 8.17. Both the molecular weight and pI values are similar to those of long-chain acyl-CoA oxidases from *Arabidopsis* (15). A similarity search of available data bases revealed that expressed sequence tags homologous to AtACX3 (>70% amino acid identity) are present in a variety of plant species

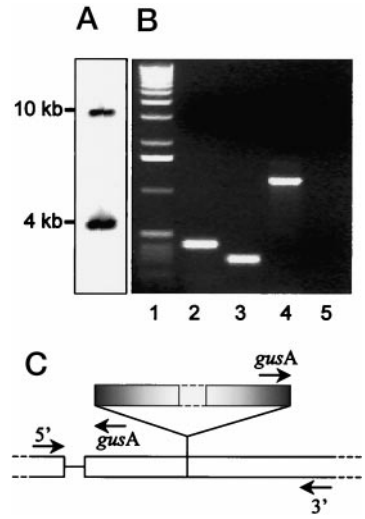

FIG. 2. **Characterization of the T-DNA copies in *acx3*.** *A*, a Southern blot of an *Eco*RI digest of *acx3* genomic DNA probed with *gus*A. *B*, PCR carried out on genomic DNA (2, 4) and cDNA (3, 5) from *acx3* seedlings using primers either 5′ (2, 3) or 3′ (4, 5) to the insertion in *AtACX3* in combination with a *gus*A-specific primer. 1 = 1-kilobase (*kb*) ladder. *C*, a diagram of the inverted tandem T-DNA repeat inserted in *AtACX3*. *Lines* and *bars* are introns and exons, respectively. *Shaded bars* are copies of the T-DNA. The relative positions of the *AtACX3* 5′ and 3′ primers and the *gus*A primer are shown.

including *Glycine max* and *Gossypium hirsutum*. Comparison of the *Arabidopsis* acyl-CoA oxidase proteins shows that AtACX3 shares 28, 23, and 14% identity at the amino acid level with AtACX2, AtACX1 (15), and AtACX4 (16), respectively. All four *Arabidopsis* acyl-CoA oxidases have regions homologous to the mammalian acyl-CoA dehydrogenase protein signatures PS1 ((G/A/C)(L/I/V/M)(S/T)EX_2(G/S/A/N)GSDX_2(G/S/A)) and PS2 (Q/E)X_2G(G/S)XG(L/I/V/M/F/Y)X_2(D/E/N)X_4(K/R)X_3(D/E)) (39). In the AtACX3 sequence, 7 of the 9 positions in PS1 and 7 of 8 positions in PS2 are conserved (Fig. 1). Consensus motifs characteristic of type 1 or 2 peroxisomal targeting signals (14, 40) are not obvious in the AtACX3 amino acid sequence.

Characterization of the acx3 Locus and Expression of gusA—Southern analysis using a probe to *gusA* revealed that *acx3* contained three copies of the T-DNA (data not shown). As shown in Fig. 2*A*, one copy was segregated out by back-crossing *acx3*. The remaining two copies formed a tandem inverted repeat inserted in *AtACX3*. In Fig. 2*B* this was demonstrated by a PCR experiment on *acx3* genomic DNA. Primers 5′ or 3′ of the site of insertion in *AtACX3* were used in combination with a *gus*A primer to demonstrate that *gus*A is present at both borders. The PCR products were sequenced, and comparison with the *AtACX3* cDNA sequence revealed that the insertion is situated in an exon, 806 bp 3′ of the putative start of transcription.

In Fig. 2*B* a reverse transcriptase-PCR experiment using the same primer combinations on RNA from 2-day-old *acx3* seedlings demonstrated also that the 5′ end of *AtACX3* is expressed *in vivo* as a *gus*A transcriptional fusion. In contrast, the *gusA* copy bordering the 3′ end of *AtACX3* was not expressed. No product was detected when primers specific to a region of *AtACX3* that is 3′ of the insertion site were used in combination (data not shown). These data showed that wild type transcripts are absent from *acx3* mutant seedlings.

Phenotypic Analysis—Arabidopsis mutants defective in peroxisomal β-oxidation have previously been selected by their resistance to 2,4-dichlorophenoxybutyric acid (2,4-DB) (18, 41). This compound is bio-activated to the herbicide and auxin analogue 2,4-dichlorophenoxyacetic acid (2,4-D) by β-oxidation

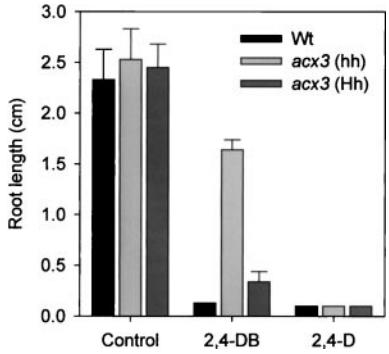

FIG. 3. **Effect of 2,4-DB and 2,4-D on *acx3* seedling growth.** Root length of 7-day-old *acx3* homozygous (*hh*), heterozygous (*Hh*), and wild type (*Wt*) seedlings is shown. Values are the mean ± S.E. of measurements made on three separate batches of 25 seedlings. 2,4-DB and 2,4-D concentrations were 1.5 μM and 0.15 μM, respectively. Sucrose (1% w/v) was included in the medium.

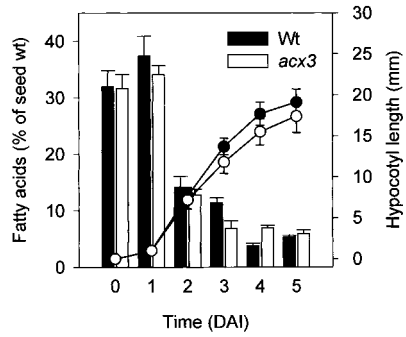

FIG. 4. **Post-germinative growth of *acx3* mutant seedlings.** Hypocotyl length (●) and total lipid content (*bars*) of *acx3* mutant and wild type etiolated seedlings grown in the dark on medium without sucrose. *DAI* is days after imbibition. Values are the mean ± S.E. of measurements made on three separate batches of 25 seedlings.

(5, 18, 41). To investigate whether *acx3* is impaired in β-oxidation, seeds were germinated on media containing 1.5 μM 2,4-DB (41), and root growth was used as an indicator of resistance. As shown in Fig. 3, homozygous *acx3* seedlings were significantly more resistant to 2,4-DB than the wild type. In contrast, heterozygous *acx3* seedling were sensitive to 2,4-DB, showing that the phenotype is recessive (Fig. 3). Both *acx3* and wild type seedlings were susceptible to 2,4-D (Fig. 3).

It has also been demonstrated that the post-germinative growth of *Arabidopsis* mutants disrupted in storage lipid breakdown can be prevented if exogenous sugars are not supplied to the seedling (18, 42). To determine if the germination or post-germinative growth of *acx3* is impaired, the rate of hypocotyl elongation and fatty acid breakdown were measured in etiolated seedlings in the absence of exogenous sucrose. The data in Fig. 4 show that the rate of hypocotyl growth and fatty acid breakdown were not significantly different from wild type over the course of 5 days following germination. Furthermore, no visible vegetative or reproductive phenotype was observed throughout the life cycle of *acx3* plants.

Activity of AtACX3—To investigate whether the *acx3* mutant displayed altered acyl-CoA oxidase activity, the enzyme was measured in 2-day-old germinating seedlings (Table I). Saturated acyl-CoAs ranging from 4 to 20 carbons in length were used at a saturating concentration (50 μM). The *acx*3 mutant was almost deficient in medium-chain acyl-CoA oxidase activity (<5% wild type), whereas short- and long-chain activities were unchanged (Table I). The maximal difference between mutant and wild type activity was observed using lauroyl-CoA (C12:0) as substrate (Table I). In wild type seedlings, acyl-CoA oxidase activity declined with increasing substrate chain length. Long-chain acyl-CoA oxidase activity was approximately 8- and 10-fold lower than that of medium- and short-chain activities, respectively (Table I).

To confirm the function of *AtACX3*, the cDNA was expressed in *E. coli* as a β-galactosidase fusion protein transcribed from the pBluescript cloning vector upon induction with isopropyl-β-D-thiogalactoside. As previously reported (15), no inducible acyl-CoA oxidase activity was observed in extracts of *E. coli* harboring pBluescript without insert and endogenous levels of acyl-CoA oxidase activity were below the limits of detection. There was also no induction when the *AtACX3* cDNA is in the antisense orientation (data not shown). As shown in Fig. 5*A*, *AtACX3* expressed in the correct orientation encoded a protein with medium-chain acyl-CoA oxidase activity. The optimum substrate was lauroyl-CoA (C12:0). No activity was detected with substrates of chain length greater than C14:0. In Fig. 5*B*,

TABLE I

Acyl-CoA oxidase activity in 2-day-old acx3 and wild type seedlings

Activity was measured using saturated acyl-CoAs of varying chain length at a saturating concentration of 50 μM. Values are the mean ± S.E. of measurements made on three separate batches of seedlings. NS, not significant ($p > 0.01$).

Substrate carbon chain length	Activity		Wild type activity minus *acx3* activity
	acx3	Wild type	
	nmol mg^{-1} protein min^{-1}		
4	6.6 ± 0.1	6.6 ± 1.1	NS
6	33.8 ± 3.7	42.9 ± 7.4	NS
8	2.5 ± 0.3	14.8 ± 1.4	12
10	0.6 ± 0.1	18.8 ± 2.2	18
12	3.6 ± 1.3	30.3 ± 4.3	27
14	5.5 ± 1.3	9.6 ± 1.0	4
16	2.8 ± 0.1	2.8 ± 0.3	NS
18	2.4 ± 0.1	2.5 ± 0.2	NS
20	2.4 ± 0.2	2.3 ± 0.2	NS

kinetic analysis of recombinant AtACX3 showed that the apparent K_m value of the enzyme for lauroyl-CoA was 3.7 μM. The optimum pH was between 8.5 and 9.0 and activity was dependent on the provision of the cofactor FAD (data not shown).

Subcellular Localization—To investigate whether AtACX3 is a peroxisomal protein, the subcellular location of medium-chain acyl-CoA oxidase activity was determined. The *acx3* mutant specifically lacks this activity (Table I), making it a reliable marker for the subcellular localization of the protein in wild type. A homogenate of two-day-old wild type seedlings was fractionated on a sucrose density gradient by centrifugation to separate the subcellular compartments. Catalase and cytochrome *c* oxidase activities were used as peroxisomal and mitochondrial markers, respectively. As shown in Fig. 6, medium-chain acyl-CoA oxidase activity co-localized with that of catalase (fraction 16), suggesting that the majority of AtACX3 is located in the peroxisome. Both activities were present in the supernatant as well as in the peroxisomal fraction. This is likely to be due to a proportion of the organelles rupturing during the tissue homogenization step.

Expression of AtACX3—In Fig. 7, Northern blot analysis of total RNA from imbibed seeds, germinating seedlings, and various tissues from wild type plants showed that *AtACX3* is expressed at low levels in all tissues but is up-regulated strongly during germination and leaf senescence. *AtACX3* transcripts were detectable in imbibed seeds before radicle emergence. Steady-state *AtACX3* mRNA levels increased during germination to a maximum between 2 and 3 days after imbibition (*DAI*) and subsequently decreased (Fig. 7*A*).

The analysis of GUS expression in *acx3*, displayed in Fig. 8*A*, revealed that the *AtACX::gus*A transcriptional fusion is ex-

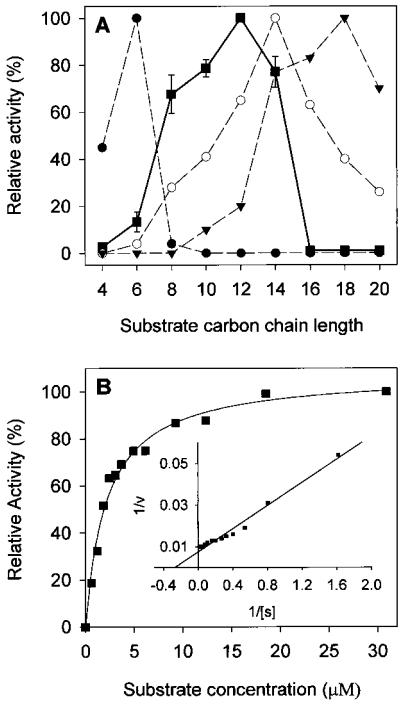

FIG. 5. **Acyl-CoA oxidase activity of recombinant AtACX3 fusion protein.** *A*, substrate specificity of AtACX3 (■) *versus* AtACX1 (○), AtACX2 (▼) (from Hooks *et al.* (15)), and AtACX4 (●) (from Hayashi *et al.* (16)). Values are the mean ± S.E. of measurements made on three separate protein extracts. *B*, saturation kinetics of AtACX3 using lauroyl-CoA as substrate.

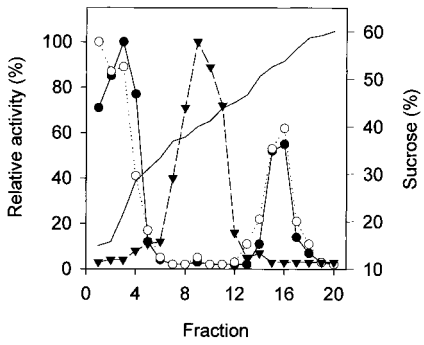

FIG. 6. **Subcellular localization of medium-chain acyl-CoA oxidase activity in 2-day-old seedlings.** A crude homogenate of 2-day-old wild type seedlings was fractionated on a sucrose gradient (*line*), and the distribution of acyl-CoA oxidase activity using decanoyl-CoA as substrate (○) was compared with that of catalase (●) and cytochrome *c* oxidase (▼).

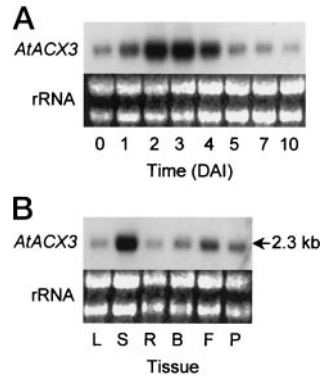

FIG. 7. **A Northern blot of *AtACX3* mRNA in various tissues.** *A*, germinating seedlings from 0 to 10 days after imbibition. *B*, young leaves (*L*), senescing leaves (*S*), roots (*R*), bolts (*B*), inflorescences (*F*), and siliques (*P*). rRNA is shown as a loading control. *kb*, kilobases.

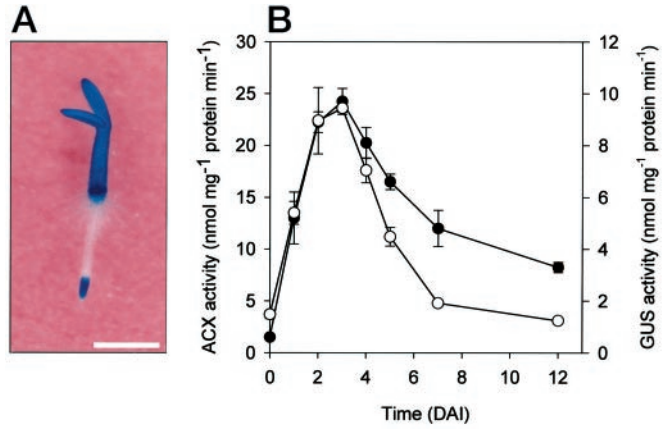

FIG. 8. **GUS expression in the *acx3* mutant.** *A*, GUS staining (*blue*) of 2-day-old seedlings. *Scale bar* is 1 mm. *B*, activity of GUS (●) and medium-chain acyl-CoA oxidase (wild type) (○) measured over the course of germination. *DAI* is days after imbibition. Values are the mean ± S.E. of measurements made on three separate batches of seedlings.

DISCUSSION

In this study we identify a new member of the ACX gene family from *Arabidopsis* using a promoter-trapping strategy. Hooks *et al.* (2) previously showed that in maize, acyl-CoA oxidases are highly active in the root tips. A promoter-less GUS T-DNA line was identified in which GUS activity was localized to the root meristem of 7-day-old seedlings. This line contained an *in vivo* transcriptional fusion between the *gus*A gene and the 5′ end of a new acyl-CoA oxidase homologue designated *AtACX3*.

The *AtACX3* gene was cloned and characterized. A comparison of the predicted amino acid sequence with those of AtACX1, AtACX2 (15), and AtACX4 (16) revealed that AtACX3 shares a significant level of homology (28–14% identity). Furthermore, all four *Arabidopsis* acyl-CoA oxidase isoforms contain common motifs that are homologous to the conserved signatures from mammalian acyl-CoA dehydrogenases (39). It has been suggested that these regions may be important for interaction with the substrates (16).

Two independent lines of evidence demonstrated that *AtACX3* encodes an acyl-CoA oxidase with medium-chain acyl-CoA substrate specificity. First, the *acx3* mutant was specifically deficient in medium-chain activity, whereas long- and short-chain activities were normal. Second, when expressed in *E. coli*, the recombinant protein displayed maximum activity using medium-chain acyl-CoAs as a substrate. The K_m value, pH optima, and cofactor requirements of AtACX3 are very

pressed in the cotyledons, hypocotyl, and root tip of young seedlings. GUS expression in the root axis was constitutive, whereas the cotyledons and hypocotyl showed transient expression during early post-germinative growth. This is reflected by the change in the level of total GUS activity, as shown in Fig. 8*B*. The activity was detectable before radicle emergence, increased rapidly during germination, peaked at 2 to 3 days after imbibition (*DAI*), and then declined (Fig. 8*B*). This pattern of expression correlated positively with medium-chain acyl-CoA oxidase activity in wild type seedlings, although GUS levels in *acx3* declined more slowly after the activity peaked at day 3 (Fig. 8*B*). The retention of GUS is likely to be a result of the relative stability of the protein *in vivo*. An increase in GUS expression was also observed in senescing leaves (data not shown).

similar to those previously reported for both long- and short-chain acyl-CoA oxidases from *Arabidopsis* (15, 16).

Subcellular fractionation experiments indicate that AtACX3 is localized to the peroxisome. However, AtACX3 lacks motifs clearly diagnostic of either PTS1 or PTS2 (14, 40). Both AtACX1 and AtACX4 contain a C-terminal type 1 peroxisomal targeting signal (PTS1), whereas AtACX2 is likely to be targeted via an N-terminal type 2 motif that is cleaved on transit (PTS2) (15, 16). Further work will be required to establish the mechanism of AtACX3 import into the peroxisome.

Interestingly, despite containing less than 5% of wild type medium-chain acyl-CoA oxidase activity, *acx3* mutant seeds were able to germinate, and the seedlings developed normally. No inhibition of storage lipid breakdown was observed following germination. This is in contrast to *ped1*, a mutant disrupted in a putative 3-keto acyl-CoA thiolase, which is incapable of post-germinative growth without the provision of exogenous sugar (16). It is probable that the residual medium-chain acyl-CoA oxidase activity detected in *acx3* is derived from other acyl-CoA oxidase isoforms whose substrate specificities partially overlap AtACX3 (15, 16). This activity is very small but apparently sufficient to allow fatty acid degradation. It can be concluded that medium-chain acyl-CoA oxidase activity exerts little control over the breakdown of storage lipid in germinating seeds. Long-chain acyl-CoA oxidase activity is about 8-fold less than that of medium-chain acyl-CoA oxidase in wild type seedlings (Table I) and leaves (26). If acyl-CoA oxidase plays a significant role in the control of peroxisomal β-oxidation (7, 8), then long-chain acyl-CoA oxidase is likely to predominate.

Disruption of AtACX3 causes resistance to the pro-herbicide 2,4-DB. This chemical is metabolized to the herbicide (and auxin analogue) 2,4-D by β-oxidation (5, 18, 41). Long- and short-chain acyl-CoA oxidase activities are unchanged in *acx3*, suggesting that 2,4-DB is perceived as a medium-chain substrate and selectively metabolized by medium-chain acyl-CoA oxidase in wild type seedlings. a*cx3* is sensitive to 2,4-DB concentrations greater than 2 μM, whereas a second *ped1* mutant allele (*ped1–2*) is resistant to 10 μM 2,4-DB.[2] Furthermore Hayashi *et al.* (18) report that *ped1* is a dominant mutation with respect to 2,4-DB resistance. In contrast, *acx3* is recessive. These differences are consistent with the observation that *acx3* retains a low level of medium-chain acyl-CoA oxidase activity and, therefore, a diminished rather than blocked capacity to metabolize 2,4-DB.

In addition to constitutive expression in the root tip, *AtACX3* was also strongly induced in the cotyledons and hypocotyl of seedlings during germination and in senescing leaves. These tissues are highly active in fatty acid breakdown (1). The temporal and spatial pattern of *AtACX3* expression is similar to other *Arabidopsis* genes encoding enzymes of peroxisomal β-oxidation. These include three additional acyl-CoA oxidase isoforms, a multifunctional enzyme (*AtMFP2*), and a 3-keto acyl-CoA thiolase (Refs. 15–18, respectively). However, not all the genes of β-oxidation are co-ordinately expressed during germination. For example *AtMFP1* transcripts are more abundant in mature plant tissues than in young seedlings (5).

The glyoxylate-cycle enzymes malate synthase and isocitrate lyase are co-ordinately induced during *Arabidopsis* seed germination (42). Reporter gene fusion experiments with both *MS* and *ICL* have revealed that the induction of the glyoxylate cycle occurs at the level of transcription in germinating oilseeds (19, 21, 22). Importantly, in this study the activity of the GUS reporter in *acx3* correlated positively with medium-chain acyl-CoA oxidase activity levels during germination and early post-germinative growth in the wild type, showing that *AtACX3* is also transcriptionally regulated. This is the first demonstration that an enzyme of peroxisomal β-oxidation is primarily controlled at the level of transcription during germination.

In mammals and yeast the enzymes of peroxisomal β-oxidation are co-ordinately regulated by lipid-based signals (either free fatty acids or acyl-CoAs) (43–45). This regulation is mediated via transcription but in each case involves a unique signal transduction pathway (43, 44, 46). Recently Eccleston and Ohlrogge (25) reported that, in transgenic *Brassica napus* embryos engineered to synthesize unusual medium-chain fatty acids, the activity of medium-chain acyl-CoA oxidase is up-regulated. It will be interesting to establish if metabolic signaling from fatty acids (or acyl-CoAs) regulates peroxisomal β-oxidation in higher plants. Alternatively, different mechanisms may operate that involve unique plant signaling molecules.

In conclusion we identified and characterized a promoter trapped *Arabidopsis* mutant disrupted in a gene encoding a new member of the acyl-CoA oxidase family with medium-chain substrate specificity. This enzyme is induced transcriptionally during germination and is also expressed constitutively in the root axis. The discovery of AtACX3 fills a gap in our knowledge of the acyl-CoA oxidase gene family that was apparent from a comparison of the substrate specificities of previously characterized genes (15). In combination, the four acyl-CoA oxidase isoforms identified to date can metabolize a broad range of acyl-CoA chain lengths (C4:0 to C22:0). It will be interesting to determine if this gene family is now complete or whether new acyl-CoA oxidase isoforms have yet to be identified in *Arabidopsis*.

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