Biochemical and Molecular Characterization of ACH2, an Acyl-CoA Thioesterase from Arabidopsis thaliana*

By using computer-based homology searches of the Arabidopsis genome, we identified the gene for ACH2, a putative acyl-CoA thioesterase. With the exception of a unique 129-amino acid N-terminal extension, the ACH2 protein is 17–38% identical to members of a family of acyl-CoA thioesterases that are found in both prokaryotes and eukaryotes. The eukaryotic homologs of ACH2 are peroxisomal acyl-CoA thioesterases that are up-regulated during times of increased fatty acid oxidation, suggesting potential roles in peroxisomal \( \beta \)-oxidation. We investigated ACH2 to determine whether it has a similar role in the plant cell. Like its eukaryotic homologs, ACH2 carries a putative type 1 peroxisomal targeting sequence (-SKLCOOH), and maintains all the catalytic residues typical of this family of acyl-CoA thioesterases. Analytical ultracentrifugation of recombinant ACH2–6His shows that it associates as a 196-kDa homotetramer in vitro, a result that is significant in light of the cooperative kinetics demonstrated by ACH2–6His in vitro. The cooperative effects are most pronounced with medium chain acyl-CoAs, where the Hill coefficient is 3.8 for lauroyl-CoA, but decrease for long chain acyl-CoAs, where the Hill coefficient is only 1.9 for oleoyl-CoA. ACH2–6His hydrolyzes both medium and long chain fatty acyl-CoAs but has highest activity toward the long chain unsaturated fatty acyl-CoAs. Maximum rates were found with palmitoleoyl-CoA, which is hydrolyzed at 21 \( \mu \)mol/min/mg protein. Additionally, ACH2–6His is insensitive to feedback inhibition by free CoASH levels as high as 100 \( \mu \)M. ACH2 is most highly expressed in mature tissues such as young leaves and flowers rather than in germinating seedlings where \( \beta \)-oxidation is rapidly proceeding. Taken together, these results suggest that ACH2 activity is not linked to fatty acid oxidation as has been suggested for its eukaryotic homologs, but rather has a unique role in the plant cell.

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Acyl-CoA thioesterases (EC 3.1.2.2) catalyze the hydrolysis of fatty acyl-CoAs to free fatty acids and CoASH. They are an extremely diverse set of enzymes, and five distinct families of acyl-CoA thioesterase genes have been cloned from prokaryotic and eukaryotic organisms. The activities of at least four other types of acyl-CoA thioesterases have also been observed, although these enzymes have yet to be identified. In eukaryotes, acyl-CoA thioesterases are distributed to a variety of subcellular locations, including the cytosol (1, 2), endoplasmic reticulum (3, 4), mitochondrion (5, 6), and peroxisome (7–9). Prokaryotic acyl-CoA thioesterases, such as Escherichia coli TesA and TesB, can be found in the periplasm and cytosol, respectively (10). The great diversity and ubiquitous presence of acyl-CoA thioesterases throughout the cell are indicative of important functions for these enzymes. Surprisingly, despite 2 decades of research, the physiological roles of acyl-CoA thioesterases remain unclear.

Recent work has been interpreted to suggest that acyl-CoA thioesterases have a possible role in fatty acid oxidation. Three of the five cloned families of acyl-CoA thioesterases are up-regulated under conditions where fatty acids are being rapidly degraded. These three families, the type I, type II, and acyl-CoA thioesterases related to E. coli TesB, can be induced in mice and rats by administering peroxisome proliferator drugs (8, 11, 12), which are chemical compounds that cause peroxisome proliferation and up-regulation of genes involved in fatty acid degradation (13). Gene up-regulation of these three families can also be seen in fasting animals that are oxidizing fat stores for energy (2, 14). Furthermore, yeast cells that are grown in media enriched in fatty acids have elevated levels of acyl-CoA thioesterase gene expression, suggesting that acyl-CoA thioesterases are needed for efficient fatty acid metabolism (15).

The suggestion that acyl-CoA thioesterases might be involved in fatty acid oxidation is biologically perplexing. Fatty acid substrates require covalent attachment to CoASH during \( \beta \)-oxidation, making the increased expression of acyl-CoA thioesterases appear counterproductive. Nevertheless, members of each of the three up-regulated acyl-CoA thioesterase families are localized to the very organelles that house the reactions of fatty acid oxidation, the mitochondrion and peroxisome.

Given the current model of fatty acid oxidation in mitochondria and peroxisomes, there is no direct role for an acyl-CoA thioesterase. One of the principal theories concerning their physiological function is that mitochondrial and peroxisomal acyl-CoA thioesterases are important for maintaining CoASH at optimal levels during periods of increased fatty acid oxidation (15–17). As \( \beta \)-oxidation demands increase, the activity of acyl-CoA thioesterases may prevent CoASH from being sequestered in intermediates that cannot be metabolized or are oxidized slowly, allowing the liberated CoASH to be used for fatty acid degradation. Conversely, the mitochondrial and peroxisomal acyl-CoA thioesterases may prevent over-accumulation of
CoASH into fatty acyl-CoA molecules, providing CoASH for other CoASH-dependent pathways such as the citric acid cycle. In order to better understand how acyl-CoA thioesterases are related to fatty acid oxidation and to investigate their role in a general sense, we have begun to study their activity in the oilseed plant *Arabidopsis thaliana*. We have focused on peroxisomal isoforms of acyl-CoA thioesterases because plant fatty acid oxidation is exclusively peroxisomal. This paper presents evidence for other higher eukaryotes that fatty acid degradation takes place in both mitochondria and peroxisomes. Oilseed plants produce seeds that contain up to 30% triacylglycerol by weight, and the fatty acids stored in the triacylglycerols are used during germination to provide the carbon skeletons and energy needed to achieve photosynthetic competence. Over 90% of the fatty acids in this triacylglycerol pool are oxidized during the first 4 days of germination (18). This intense period of fatty acid turnover is useful for characterizing enzymes related to peroxisomal fatty acid oxidation and is conducive to studying the involvement of acyl-CoA thioesterases in this process.

Here we report the cloning and biochemical characterization of ACH2, a putative peroxisomal acyl-CoA thioesterase from *A. thaliana*. This is the first acyl-CoA thioesterase to be cloned from plants. ACH2 was identified through its homology with a unique family of acyl-CoA thioesterases that is conserved in both prokaryotes and eukaryotes. Enzymes that have been cloned and characterized in this family of acyl-CoA thioesterases, include *E. coli* TesB, yeast PTE1, mouse PTE-2, and human ACH2. All the members of the TesB family, it is unlikely to be involved in fatty acid oxidation. These results suggest that plant acyl-CoA thioesterases have unique physiological roles distinct from other eukaryotic acyl-CoA thioesterases.

**EXPERIMENTAL PROCEDURES**

**Plant Material and Growth Conditions**—*A. thaliana* seedlings (Wasilewskija ecotype) were grown on plates containing 0.4% Phytagel, 1 M Murashige and Skoog salts (Invitrogen), and 1% sucrose. Seeds were sown on these plates in 0.1% agarose and then stored in the dark at 4 °C for 3 days before being placed in a growth chamber with a 16/8 h light:hours dark) photoperiod. Plants were also grown to maturity on commercial potting soil in a growth chamber with a 16:8 (hours light:hours dark) photoperiod or under constant light.

**Gene Identification and Plasmid Construction**—The *E. coli* acyl-CoA thioesterase TesB protein sequence was used to search the Arabidopsis Genome Initiative data base (currently found at the Arabidopsis Biological Resource Center, leading to the identification of the full-length ACH2 gene. A cDNA clone from Genome Systems Inc. (GenBank accession number A100858) was also identified, confirming the full-length sequence of the ACH2 cDNA. A protein overexpression plasmid was constructed using sticky-end PCR (21) to introduce the full-length ACH2 cDNA between the NcoI and Xhol sites of the pET-24d (Invitrogen), in-frame with a coding region for a 6-histidine (6His) tag at the C terminus of the protein.

**Overexpression and Purification of ACH2–6His**—Recombinant ACH2–6His was overexpressed in 100-ml cultures of BL-21 (DE3) *E. coli* cells (Novagen) carrying the pET-24d/ACH2–6His plasmid. Cultures were grown at 37 °C to an optical density of 0.5–0.6, then cooled to room temperature, and induced with 0.8 mM isopropyl-1-thio-β-D-galactopyranoside. The cells were shaken at room temperature for 2 days at 300 rpm, collected by centrifugation, and frozen. Frozen cells were thawed and lysed by three 30-s bursts with a Virsonic 475 sonicator in binding buffer (50 mM KPi, pH 5.8, 500 mM NaCl, 5 mM imidazole). Insoluble material was removed by centrifuging the lysate at 39,000 × g for 30 min. The soluble portion was then loaded through a 0.45-μm filter onto a His-Bind resin (Novagen) gravity flow column that was charged with NiSO4. The column was washed with 10 volumes of binding buffer (50 mM KPi, pH 5.8, 500 mM NaCl, 60 mM imidazole), and eluted in 500-μl fractions with elution buffer (50 mM KPi, pH 5.8, 500 mM NaCl, 500 mM imidazole). The fractions containing the most ACH2–6His were pooled and dialyzed overnight at 4 °C against 50 mM KPi, pH 5.8, 100 mM NaCl in order to remove the imidazole and excess salt. The dialyzed protein was further dialyzed with 20 mM KPi, pH 5.8, 20 mM NaCl before being lyophilized and stored at −20 °C.

**Analytical Sedimentation Equilibrium Centrifugation**—Sedimentation equilibrium experiments were done with three different concentrations of ACH2–6His. Dialyzed ACH2–6His was serially diluted to 15, 5, and 1.65 μM with dialysis buffer. From each dilution, 110 μl was loaded onto a charcoal-filled Epon six-channel sedimentation equilbrium centrifuge using an An-60 Ti four-plate rotor at 3000, 6000, and 9000 rpm. Absorbance at 280 nm was taken as a function of radial distance every hour until the scans became superimposable, at which point equilibriums were considered attained for that speed. Equilibrium experiments were analyzed using the program NONLIN (22) which simultaneously performs a nonlinear least squares fit with data from the different concentrations and centrifugation speeds.

**Acyl-CoA Thioesterase Enzyme Assays**—The acyl-CoA thioesterase activity of recombinant ACH2–6His was measured spectrophotometrically using 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). Briefly, 2 μg of recombinant ACH2–6His was added to a 750-μL assay volume composed of 200 μM DTNB (Sigma) in 50 mM potassium phosphate buffer, pH 8.0, acyl-CoA substrate (Sigma and ICN), and BSA (Sigma). Acyl-CoA stock concentrations were verified by their absorbance at 260 nM in either potassium phosphate buffer (pH 6.0–8.0) or Tris-Cl buffer (pH 8.5–9.5). Coenzyme A inhibition was tested in a 50-μL reaction containing 20 μM radiolabeled 18:1-CoA (PerkinElmer Life Sciences), 166 μg/mL BSA, coenzyme A (Sigma), and 80 ng of recombinant ACH2–6His in 50 mM KPi, pH 8.0. The thioesterase activity was stopped after 1 min by addition of 100 μL of isopropyl alcohol:acetic acid (9:1) and then overlaid with 1 mL of hexane. The reactions were mixed and centrifuged, and radiolabeled free 18:1 was measured in the hexane layer by liquid scintillation counting.

**Real Time Quantitative PCR**—For germinating seedling tissues, seeds were surface-sterilized and plated on 1× MS salts, 1% sucrose, 0.4% Phytagel. The plates were left in the dark at 4 °C for 3 days and then transferred to a 16:8 (hours light:hours dark) growth cabinet until ready to harvest. All tissue was frozen immediately in liquid nitrogen after harvesting. Green silique tissue was harvested at all stages of development. Flower tissues were taken from all stages of flower development, including unopened buds and mature flowers. Young leaf tissue consisted of leaves less than 1 cm long. Total RNA was extracted using a lithium chloride extraction protocol (23) or TRIzol® reagent (Invitrogen). In our experience, TRIzol® reagent does not effectively extract RNA from seeds, young seedlings, or silique tissue, although it was used successfully for RNA extraction of leaves and flowers. Total RNA was DNase-treated using the DNA-free™ kit from Ambion, and 2–5 μg of DNase-treated RNA was used for reverse transcription with random hexamers using the Invitrogen SuperScript™ II QPCR Core Reagent kit. Double-strand cDNA synthesis kit. SuperScript™ III was used for LAC56 experiments.

Real time PCR experiments were done on a Stratagene Mx4000™ quantitative PCR instrument using the Brilliant™ SYBR® Green QPCR Core Reagent kit from Stratagene and analyzed using Stratagene software. ACH2 and 18S primers were designed using Primer Express, a primer design program by Applied Biosystems, which de-
signed primers for amplifying 80-bp amplicons of the ACH2 and 18 S cDNAs. ACH2 and 18 S PCR products were amplified and cloned into pCRII-Script Amp (Stratagene) and used as standards for quantitative PCRs. LACS6 primers and full-length cDNA were provided by Dr. Jay Shockey. Reverse transcriptase reactions (2 μl) were used as template for the PCRs. ACH2 and LACS6 amplification from each tissue was normalized by comparing amplification of the 18 S amplicon.

RESULTS
Cloning and Sequence Analysis—Homology sequence comparisons between the E. coli TesB protein sequence and the Arabidopsis Genome Initiative data base identified an expressed sequence tag (EST) (GenBank™ accession number T04836) that provided 250 bp of the 3' end of the putative ACH2 cDNA. The reported sequence was sufficient to create a nucleic acid probe that hybridized to a genomic clone in the CD4-8 library from the Arabidopsis Biological Research Center. After completely sequencing the ACH2 gene, we identified potential exon/intron junctions using the NetPlantGene web-site (www.cbs.dtu.dk/services/NetPGene) that predicts splice junctions in plant DNA. By using the TesB protein as a guide to identify the most likely open reading frames, we predicted that the full-length ACH2 open reading frame was 993 bp. An ACH2 cDNA clone from Genome Systems Inc. (GenBank™ accession number A1999643) was identified and sequenced, revealing that the actual open reading frame is 291 bp longer than our predicted sequence, adding 97 amino acids to the N-terminus. The 5'-untranslated region (UTR) of this cDNA clone did not have an in-frame stop codon, leaving the location of the correct start codon in question. Subsequent analysis of the Institute of Genomic Research Arabidopsis EST contig assemblies provided additional 5'-UTR from EST AU238185 that contained an in-frame stop codon, allowing us to identify the correct start codon. The full-length cDNA has an open reading frame of 1284 bp flanked by 122 bp of 5'-UTR and at least 180 bp of 3'-UTR (Fig. 1). The encoded ACH2 protein has a predicted mass of 48.1 kDa. In pairwise comparisons, ACH2 showed 36% sequence identity to E. coli TesB (GenBank™ accession number P23911), 38% sequence identity to the human (GenBank™ accession number AAB71665) and mouse (GenBank™ accession number NP_573503) PTE-2, but only 17% sequence identity to yeast PTE1 (GenBank™ accession number P41903) (Fig. 2).

ACH2 possesses a catalytic triad that is similar to those in other enzymes of the TesB family. The E. coli crystal structure determined that Asp-204, Gln-278, and Thr-228 are the crucial amino acids for TesB acyl-CoA thioesterase activity (24). The corresponding ACH2 residues are Asp-337, Gln-409, and Ser-359 (Fig. 3). Replacement of threonine with serine in the catalytic triad is common among the eukaryotic isoforms of the TesB-like acyl-CoA thioesterases. In addition, the eukaryotic proteins are localized to the peroxisome via a type 1 peroxisomal targeting sequence.
The C-terminal tripeptide of ACH2, -SKLCOOH, is the consensus sequence for PTS1 signals in eukaryotes and is sufficient for protein localization to the peroxisome in plants, thus indicating that ACH2 is a peroxisomal enzyme.

The 129 amino acids at the ACH2 N terminus are completely unique and are not part of any other known acyl-CoA thioesterase. PSORT (psort.ims.u-tokyo.ac.jp) and TargetP (www.cbs.dtu.dk/services/TargetP), which are programs that analyze protein sequences for subcellular targeting information, indicate that the 129-amino acid extension does not conform to a mitochondrial, chloroplast, endoplasmic reticulum, or type II peroxisomal organelle targeting sequence. The N-terminal extension does share homology with cyclic nucleotide (cAMP and cGMP) binding pockets of cyclic nucleotide-dependent protein kinases and channels (Pfam PF0027) with an E value score of 0.026. However, only two of the six invariant residues that are conserved in cyclic nucleotide binding domains are found in ACH2. Because plant proteins that bind cyclic nucleotides do not always conform to the consensus binding domain in animals (29), we sent recombinant ACH2–6His to Dr. Jackie Corbin (Vanderbilt University, Nashville, TN) for cyclic nucleotide binding analysis.

In vitro experiments with recombinant ACH2–6His showed that the protein does not bind either cAMP or cGMP. Furthermore, in our experiments the acyl-CoA thioesterase activity of recombinant ACH2–6His is not affected by the presence of cAMP (10 nM to 100 μM) or cGMP (0–500 μM) (data not shown). Truncated ACH2 that is missing 97 amino acids of the N-terminal region still maintains 20% of its native acyl-CoA thioesterase activity (data not shown), indicating that the ACH2 N terminus is not essential for catalysis.

Structure and Acyl-CoA Thioesterase Activity of Recombinant ACH2–6His—In order to characterize the activity of ACH2, we overexpressed the protein in-frame with a C-terminal 6-histidine (6His) tag and purified the soluble ACH2–6His by Ni2+-affinity chromatography. Overexpression of ACH2–6His at 37°C yielded only insoluble protein, but growing the induced culture at room temperature allowed us to obtain nearly pure soluble ACH2–6His from a 100-ml culture of E. coli cells (Fig. 3A). ACH2–6His was stable in the elution buffer, which contains 500 mM imidazole, 375 mM NaCl, and 50 mM KPi, pH 8.9, but began to precipitate when the salt and imidazole were removed by dialysis unless the pH was lowered to 6.0. This is similar to the recombinant yeast peroxisomal acyl-CoA thioesterase PTE1 that is stable in vitro only at low pH (15).

The availability of purified protein allowed us to determine the molecular weight of native ACH2–6His by sedimentation equilibrium analytical centrifugation. Fig. 3B shows the concentration of 15, 5, and 1.65 nM ACH2–6His as a function of the radius of the centrifuge cell at 9000 rpm. Data were also collected at 6000 and 3000 rpm (not shown). Simultaneous curve-fitting at each concentration and speed using NONLIN software (22) indicated ACH2–6His associates as a homotetramer with a molecular mass of 196 kDa. The residual plot (Fig. 3C) shows the deviation of the data points from the calculated points predicted by NONLIN. The residual points are randomly distributed, indicating a good fit, although at very high concentra-

FIG. 2. Sequence alignment of homologous acyl-CoA thioesterases. Arabidopsis ACH2, human PTE-2, mouse PTE-2, yeast PTE1, and E. coli TesB were aligned using the Blossum62 method with AlignX (Informax, Inc., Frederick, MD). Identical residues are shaded in black and conserved residues are shaded in gray. The three catalytic residues are marked with an asterisk, and type 1 peroxisome targeting sequences are underlined.
trations there may be some non-ideal association that is not tetrameric.

During the course of optimizing the in vitro assays to characterize the activity of ACH2–6His, we found that the enzyme activity remained constant during storage at 4 °C at pH 6.0 for 3 days and retained 90% of the original activity when stored at pH 8.0 at 4 °C for the same amount of time. The pH optimum for ACH2–6His activity is between pH 8.0 and 9.0 (Fig. 4), which is consistent with the basic pH of the peroxisomal lumen (30). Because acyl-CoAs are susceptible to base-catalyzed hydrolysis, all of the rates in Fig. 5 are corrected for non-enzymatic hydrolysis. Non-enzymatic hydrolysis rates are very low at all of the pH values examined, and even at pH 9.5 represent less than 1% of the enzyme-catalyzed hydrolysis. In addition, ACH2–6His has seven cysteine residues that are potentially reactive with the DTNB that is added to the assay to detect increases in free CoASH. We found that DTNB inhibits the activity of ACH2–6His but only at concentrations greater than 300 μM, well above the 200 μM DTNB we used in our in vitro assays.

ACH2–6His demonstrated high levels of fatty acyl-CoA thioesterase activity against a wide range of substrates but has highest activity toward long chain, unsaturated acyl-CoAs. Acyl-CoAs longer than 12:0-CoA inhibit ACH2–6His at concentrations as low as 5 μM, but the inhibitory effects can be reversed by lipid-binding molecules such as BSA or α-casein.

Other acyl-CoA thioesterases are similarly inhibited by their substrates (7, 31), which is generally attributed to the detergent-like effects of acyl-CoAs. In order to analyze the kinetics of ACH2–6His enzyme activity, we empirically determined the optimum substrate:BSA ratio for a range of concentrations of 18:1-CoA. We found that the optimum 18:1-CoA to BSA ratio we used in our in vitro assays.

Arabidopsis ACH2 Characterization
ACYL–COA thioesterases that is conserved in both prokaryotes and eu-

eryotes. Like the eukaryotic members of the TesB family, 

ACH2–6His activity toward 14C-labeled 18:1-CoA was measured in 

the presence of increasing amounts of free CoASH. The average of two 

assays at each CoASH concentration is presented relative to ACH2–

6His activity in the absence of free CoASH.

| [CoASH] | Activity |
|---------|----------|
| μM     | %        |
| 0       | 100      |
| 5       | 115      |
| 10      | 105      |
| 25      | 106      |
| 50      | 98       |
| 100     | 95       |

PCR assays show that the highest ACH2 transcript levels are in 

young leaves and inflorescences (Fig. 7, left side), which have 

two and a half times the amount of ACH2 transcript levels than the 

highest expressing germinating seedlings. The right side of Fig. 7 

shows the relative expression of the peroxisomal long chain acyl-CoA synthetase 6 (LACS6), which has an expression 

pattern typical of genes that code for important proteins in-

volved in the fatty acid β-oxidation process (33).

**DISCUSSION**

The discovery of *Arabidopsis ACH2* represents the first plant 

acyl-CoA thioesterase to be cloned and characterized. ACH2 

was identified through its homology with the TesB family of 

acyl-CoA thioesterases (Fig. 2), a unique family of acyl-CoA 

thioesterases that is conserved in both prokaryotes and eu-

karyotes. Like the eukaryotic members of the TesB family, 

ACH2 carries a consensus C-terminal type 1 peroxisomal tar-

geting sequence, strongly suggesting that it is localized to the 

peroxisome. The Asp, Gln, and Ser catalytic triad, which is a 

defining feature of the TesB family of acyl-CoA thioesterases, is 

likewise conserved in ACH2. With the exception of a unique 

129-amino acid N terminus, ACH2 is between 17–38% identical 

to TesB-type acyl-CoA thioesterases from *E. coli*, yeast, and 

mammals. Although the actual function of the unique ACH2 N 

terminus has not been elucidated, it is interesting to note that
deletion of the first 97 amino acids causes ACH2–6His activity to drop to levels that are equivalent to those reported for mammalian TesB acyl-CoA thioesterases (data not shown).

The conservation of sequence between plant, bacterial, yeast, and mammalian acyl-CoA thioesterases suggests a similar function for the TesB family members. The eukaryotic homologs of the TesB family are up-regulated under conditions that favor fatty acid β-oxidation. For example, yeast PTE1 is up-regulated ~5-fold when yeast cells are grown in media containing oleate as a carbon source. Because all fatty acid oxidation in yeast is peroxisomal, PTE1 up-regulation suggests that the acyl-CoA thioesterase has a part in metabolizing fatty acids. This is further supported by the fact that pte1 mutants have impaired growth rates in the same oleate media (15). Mouse PTE-2 is up-regulated >10-fold when mice are fed peroxisome proliferators (8), which are drugs that cause peroxisome proliferation and up-regulation of fatty acid degradation genes (13). Similar up-regulation can be seen in fasting mice that are converting fat stores into energy (8).

Our biochemical characterization and gene expression analysis shows that although ACH2 possesses many of the same physical characteristics as other members of the TesB family, its predominant role is unlikely to lie in fatty acid degradation. In Arabidopsis, seed storage oils are rapidly oxidized during the first 4 days of germination, and genes that contribute to breaking down fatty acids are typically up-regulated during the first 2 days (18). LACS6, a peroxisomal long chain fatty acyl-CoA synthetase that activates fatty acids with CoASH in preparation for β-oxidation (33), is expressed at highest levels during the 1st day of seedling germination. LACS6 expression then recedes to half its peak levels in young leaves and flowers (Fig. 7). Like LACS6, ACH2 is up-regulated during the 1st day of germination, but ACH2 transcript levels are nearly 3-fold higher in young leaves and flowers than in germinating seedlings (Fig. 7). Mature tissues conduct only basal levels of fatty acid oxidation demands, presumably by releasing CoA from metabolites that are slowly oxidized. Consistent with this role, mouse PTE-2 is strongly inhibited by free CoASH (IC50 = 10–15 μM), suggesting a method by which the activity of the acyl-CoA thioesterase could be governed to keep CoASH concentrations at an optimum level while preventing unnecessary hydrolysis when sufficient CoASH is available (8). In light of the possibility that ACH2 may have a similar role in the plant peroxisome, we tested the activity of recombinant ACH2–6His in the presence of increasing concentrations of CoASH. Our results show that ACH2–6His is insensitive to any of the CoASH concentrations we tested, ranging from 5 to 100 μM (Table II). Although this result does not totally disqualify ACH2 as a regulator of intraperoxisomal CoASH levels, it does suggest that ACH2 is regulated differently than acyl-CoA thioesterases in mammalian peroxisomes.

The lack of CoASH sensitivity raises the question as to how ACH2 activity is governed. All of the tested members of the TesB family demonstrate broad substrate specificities with activity toward short, medium, and long chain fatty acyl-CoAs (7, 8, 20). Because all of these acyl-CoAs are substrates for β-oxidation, the unregulated activity of an acyl-CoA thioesterase would inhibit the progression of the oxidative pathway by eliminating the substrates. This is especially true for ACH2, which has the highest activity of any of the members of the TesB family toward medium and long chain acyl-CoAs. Because ACH2 is insensitive to free CoASH, other mechanisms may regulate the activity of the enzyme.

Regulation at the protein level may involve the novel N terminus of ACH2. The 129-amino acid N-terminal region of ACH2 strongly differentiates it from other members of the TesB family and is not similar to any other known acyl-CoA thioesterase. Assays of recombinant enzyme missing the first 97 residues show that this portion of the enzyme is not absolutely necessary for acyl-CoA thioesterase activity. The N-terminal region has weak homology to cyclic nucleotide binding domains such as those found in cyclic nucleotide-dependent protein kinases. Both cAMP and cGMP have been detected in plants (35), although neither molecule has an effect on the activity of ACH2–6His. Interestingly, BLAST searches of plant EST data bases reveal that the ACH2 N-terminal region is conserved in all plant TesB-like acyl-CoA thioesterases, indicating that it is a common feature in plants. Discovering the function of the N-terminal domain may be an important step in understanding the role of the peroxisomal acyl-CoA thioesterase.
characte-
ization of the different isozymes of plant acyl-CoA thioesterases shows that these are the primary in vivo substrates, but ACH2 maintains enough short chain acyl-CoA thioesterase activity to prevent possible toxic accumulations of short chain acyl-CoAs.

Characterizing ACH2 mutants will be helpful in identifying the physiological substrate of the enzyme. Recently, mutant screening helped identify a plant peroxisomal thioesterase with specificity toward β-hydroxyisobutyryl-CoA, an intermediate in valine catabolism (36). Seven homologs of the β-hydroxyisobutyryl-CoA thioesterase were also identified in Arabidopsis. None of the seven homologs hydrolyze β-hydroxyisobutyryl-CoA, suggesting that they have specificities for other molecules. Other metabolic pathways in the peroxisome use CoA-ester intermediates, including synthesis of important plant hormones such as jasmonic acid (37, 38), which is presumably matured as a CoA ester but which is present predominantly as the free acid, implicating the activity of an acyl-CoA thioesterase in its synthesis. It has also been suggested that phytic acid, a breakdown product of chlorophyll, is oxidized to some type of regulatory molecule.

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Arabidopsis ACH2 Characterization

Plant hormones such as jasmonic acid (37, 38), which is presumably matured as a CoA ester but which is present predominantly as the free acid, implicating the activity of an acyl-CoA thioesterase in its synthesis. It has also been suggested that phytic acid, a breakdown product of chlorophyll, is oxidized to some type of regulatory molecule.