**chy1**, an Arabidopsis Mutant with Impaired β-Oxidation, Is Defective in a Peroxisomal β-Hydroxyisobutyryl-CoA Hydrolase*  

Received for publication, May 22, 2001, and in revised form, June 11, 2001 Published, JBC Papers in Press, June 12, 2001 DOI 10.1074/jbc.M104679200

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The Arabidopsis chy1 mutant is resistant to indole-3-butyric acid, a naturally occurring form of the plant hormone auxin. Because the mutant also has defects in peroxisomal β-oxidation, this resistance presumably results from a reduced conversion of indole-3-butyric acid to indole-3-acetic acid. We have cloned CHY1, which appears to encode a peroxisomal protein 43% identical to a mammalian valine catabolic enzyme that hydrolyzes β-hydroxyisobutyryl-CoA. We demonstrated that a human β-hydroxyisobutyryl-CoA hydrolase functionally complements chy1 when redirected from the mitochondria to the peroxisomes. We expressed CHY1 as a glutathione S-transferase (GST) fusion protein and demonstrated that purified GST-CHY1 hydrolyzes β-hydroxyisobutyryl-CoA. Mutagenesis studies showed that a glutamate that is catalytically essential in homologous enoyl-CoA hydratases was also essential in CHY1. Mutating a residue that is differentially conserved between hydratases and hydratases established that this position is relevant to the catalytic distinction between the enzyme classes. It is likely that CHY1 acts in peroxisomal valine catabolism and that accumulation of a toxic intermediate, methacrylyl-CoA, causes the altered accumulation of a toxic intermediate, methacrylyl-CoA, that a glutamate that is catalytically essential in homologous enoyl-CoA hydratases was also essential in CHY1. Mutating a residue that is differentially conserved between hydratases and hydratases established that this position is relevant to the catalytic distinction between the enzyme classes. It is likely that CHY1 acts in peroxisomal valine catabolism and that accumulation of a toxic intermediate, methacrylyl-CoA, causes the altered β-oxidation phenotypes of the chy1 mutant. Our results support the hypothesis that the energy-intensive sequence unique to valine catabolism, where an intermediate CoA ester is hydrolyzed and a new CoA ester is formed two steps later, avoids methacrylyl-CoA accumulation.

Germinating seedlings of oilseed plants β-oxidize long-chain fatty acids as an energy source until photosynthesis begins. Enzymes that catalyze each step of fatty acid β-oxidation have been identified, and different isozymes may participate during distinct developmental stages or in different tissues. Although mammals β-oxidize fatty acids in both the mitochondria and peroxisomes (reviewed in Refs. 1, 2), plant fatty acid β-oxidation is exclusively peroxisomal (reviewed in Refs. 2–5). Peroxisomes are small organelles bounded by a single lipid bilayer that contains β-oxidation enzymes and catalases to inactivate H₂O₂ (reviewed in Refs. 3, 5). Leaf peroxisomes also house the enzymes acting in photosynthesis, and specialized peroxisomes in seedlings and senescing tissues called glyoxysomes contain the enzymes required for the glyoxylate cycle, which converts acetyl-CoA to succinate (reviewed in Refs. 3, 5).

The branched chain amino acids (BCAAs)¹ valine, leucine, and isoleucine can be broken down to generate energy during germination, senescence, or carbon starvation; the end products of these reactions enter the citric acid cycle and fuel respiration (6–8). The subcellular localization of BCAA catabolism in plants remains controversial. Although BCAA catabolism is mitochondrial in mammals, peroxisomes isolated from mung bean and sunflower cotyledons can convert labeled BCAAs to propionyl-CoA and acetyl-CoA (9, 10). The initial catabolic enzymes are shared by the three BCAAs. The amino acids are first oxidatively transaminated to form the α-keto acids (see Fig. 1; step 1), which undergo oxidative decarboxylation and esterification to form the acyl-CoA esters (step 2). Then, the catabolic mechanisms of the three BCAAs diverge. The isobutyryl-CoA made from Val is desaturated to form the α,β-unsaturated thioester methacryl-CoA (step 3), which can react with nucleophiles such as free thiols and cause damage within the organelle (11). Hydration to β-hydroxyisobutyryl-CoA (HIBYL-CoA; step 4) and thioester hydrolysis (step 5) forms diffusible, transportable β-hydroxyisobutyrate (12–14). This β-hydroxy acid is oxidized to methylmalonyl semialdehyde (step 6) and thioesterified to form propionyl-CoA (step 7). Leu and Ile catabolic pathways exclude the CoA hydrolysis step, perhaps because the corresponding intermediates are less reactive than methacrylyl-CoA.

We are examining the metabolism and function of the phytohormone auxin, which affects virtually all plant developmental processes, including root elongation and lateral root initiation (15). Indole-3-butyrates (IBA) is a naturally occurring auxin that is converted to the more abundant auxin indole-3-acetic acid (IAA) in several plant species (reviewed in Ref. 16). Because even but not odd-chain length IAA derivatives have auxin activity (17), the mechanism of this conversion probably parallels fatty acid β-oxidation. Previously, we described a group of Arabidopsis thaliana mutants that are resistant to the

* This work was supported by National Science Foundation Grant IBN-9982611, the Robert A. Welch Foundation (C-1309), a Houston Livestock Show and Rodeo scholarship (to M. M. A.), and an American Society of Pharmacognosy scholarship (to K. A. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: BCAA, branched-chain amino acid; HIBYL, β-hydroxyisobutyryl; IBA, indole-3-butyric acid; IAA, indole-3-acetic acid; PN, plant nutrient medium; PNS, PNS medium plus sucrose; Kan, kanamycin; HIBCH, HIBYL-CoA hydrolase; PCR, polymerase chain reaction; kb, kilobase(s); bp, base pair(s); GST, glutathione S-transferase.
inhibitory effects of IBA on root elongation but respond normally to IAA (15). Mutants in several β-oxidation enzymes, including an acyl-CoA oxidase (ACX3 (19)), a multifunctional protein (AIM1 (20)), and a thiolase (PED1 (21)) are IBA-resistant and IAA-sensitive (18). In addition, a mutant defective in PEX5, a receptor required to import peroxisomal matrix proteins, is IBA-resistant (18). The isolation and characterization of these mutants provide compelling evidence that Arabidopsis peroxisomes convert IBA to IAA in a mechanism similar to fatty acid chain shortening and demonstrate that screening for IBA resistance is a powerful method to identify mutants defective in β-oxidation and peroxisomal function.

Here we describe the cloning and characterization of the gene defective in the Arabidopsis chy1 mutant, which is IBA-resistant both in root elongation and lateral root initiation and has defects in the peroxisomal β-oxidation of fatty acids. CHY1 encodes a β-hydroxyisobutyryl-CoA hydrolase, which may act in peroxisomal Val catabolism. We hypothesize that the loss of CHY1 indirectly disrupts both fatty acid β-oxidation and the conversion of IBA to IAA because the toxic intermediate, methacrylyl-CoA, accumulates in the peroxisome.

**EXPERIMENTAL PROCEDURES**

**Plant Materials and Growth Conditions—**A. thaliana accessions Columbia (Col-0), Landsberg erecta tt4 (Ler), and Wassilewskija (Ws) were used. Plants were grown in soil (Metromix 200, Scotts, Marysville OH) under continuous illumination by Sylvania Cool White fluorescent bulbs at 22–25°C. Plants were grown aseptically on PNS (plant nutrient medium with 0.5% sucrose (22)) solidified with 0.6% agar, either alone or supplemented with hormones (from 1 or 100 mM stocks in ethanol), kanamycin (Kan; from a 25 mg/ml aqueous stock), or glu- phosinate ammonium (Basta; from a 50 mg/ml stock in 25% ethanol; Crescent Chemical Co., Hauppauge, NY). Plates were wrapped with gas-permeable surgical tape (LeeTec Corp., Minnetonka, MN) and grown at 22°C under continuous yellow-filtered light to prevent breakdown of indolc compounds (22). Isolation of the IBA-resistant mutants was described previously (18). chyl-1 (B17) and chyl-3 (B7) were generated by ethyl methanesulfonate in the Col-0 accession, and chyl-1-2 (B52) is an untagged transferred DNA-induced allele in the Ws accession (24). Mutants were backcrossed to the parental accession at least once prior to phenotypic analysis.

**Phenotypic Assays—**Seeds were surface-sterilized and plated on PNS with the indicated hormone concentration. Seedlings were grown for the indicated time and removed from the agar, and the primary root was measured. Data are expressed as percent elongation on supplemented versus unsupplemented media. To assay lateral root initiation, seeds were germinated and grown on PNS for 4 days, then transferred to media containing IBA, IAA, or no hormone and grown for 4 additional days after which roots were counted using a dissecting microscope. For hypocotyl elongation assays, seeds were plated on PN (without sucrose) or PNS and incubated for 24 h under white light before being transferred to the dark. Hypocotyl elongation was measured after 5 days in the dark and is expressed as percent elongation on medium without sucrose versus medium with sucrose.

**Genetic Analysis—**The mutants were mapped after outcrossing. The resulting F2 seeds were plated on 15 μm IBA, and DNA was isolated (25) from resistant individuals. Mutants were mapped using published Simple Sequence Length Polymorphism (SSLP (26)) and Cleaved Amplified Polymorphic Sequence (CAPS (27)) markers. New markers were identified by PCR-amplifying and sequencing ~1.3-kb genomic DNA fragments from different accessions and by identifying polymorphisms that altered fragment sizes or restriction enzyme recognition sites (Table I).

**Identification of the Defects in the chyl Mutants—**Genomic DNA extracted from chyl mutant plants was amplified using two pairs of oligonucleotides: K14B20-1 (5'-CCACAGACGTAAACAATAGTGCTC-3') plus K14B20-2 (5'-GCGTGCGTCTATTTCGTAAGAC-3') and K14B20-3 (5'-CAACTTCTTAACATTGACGGGTGAAG-3') plus K14B20-4 (5'-GCTCTTACGAAATAGACGGCGACCC-3'). Alternatively, DNA was amplified with K14B20-1 plus K14B20-2, K14B20-5 (5'-CGAGGCTGAAATGGTATCGCATXGCATGC-3'), and K14B20-6 (5'-GGGTTGTACCTATTACAGGATGG-3') plus K14B20-7 (5'-CGAAATCGACCATGGACGGCGACCC-3'). Primers were designed to amplify overlapping fragments, which covered the gene from 110 bp upstream of the putative translation start site to 40 bp downstream of the stop codon. Amplification products were purified by sequential ethanol, polyethylene glycol, and ethanol precipitations (28) and sequenced directly using an automated DNA sequencer (Lone Star Laboratories, Houston, TX or Rice Sequencing Facility, Rice University, Houston, TX) with the primers used for amplification.

A full-length CHY1 cDNA was isolated from a plasmid-based cDNA library (29) by colony hybridization with a 600-bp probe amplified from genomic DNA with K14B20-5 plus K14B20-7. The resulting cDNA was subcloned into the Nol I site of pBluescript II KS (+) (Stratagene) to form pKS-CHY1 and was sequenced with both internal and vector-derived primers (GenBank accession number AF276301).

A CHY1 genomic clone was constructed by isolating a 5.9-kb BamHI fragment (2.4-kb 5'-untranslated region, 2.5-kb coding sequence, and 1-kb 3'-untranslated region) from the K14B20 TAC clone (30) and subcloning it into the BamHI site of the plant transformation vector pBIN19 (31) to give pBIN-CHY1. This plasmid was electroporated (28) into Agrobacterium tumefaciens strain GV3101 (32), which was used to infiltrate chyl mutant plants (33). Transformants were selected for the ability to develop on PNS containing 12 μg/ml Kan (chy1-1; chy1-3) or on PN in the dark (chy1-2), which contains an unlinked T-DNA conferring Kan resistance.

**Construction of Human HIBYL-CoA Hydrolase Constructs—**A human HIBYL-CoA hydrolase (HIBHCH) cDNA (14) in the Lamin B vector was obtained from Image Consortium, Lawrence Livermore National Laboratory (IMAGE Consortium clone ID 32783 (34)). The mito-
chondrial targeting signal was removed by mutating the HIBCH cdNA with the oligonucleotide 5′-GTGTGGCTTGGACATCTCGCG-3′ (altered residues underlined), which created a BamHI site 5′ of a natural ATG at the end of the mitochondrial targeting signal. A peroxisomal targeting signal was added at the C terminus of the human protein by first mutating with the oligonucleotide 5′-AAAAAGCTTAAACCGGTATTCAATCTTC-3′ (altered residues are underlined) to create an Smal site 13 codons upstream of the stop codon. The 3′-end of the CHY1 cdNA was then excised with ScaI and NotI and inserted into the mutagenized HIBCH cdNA cut with Smal and NotI, replacing the last 13 amino acids of the human protein with the C-terminal 28 amino acids from CHY1. The modified cdNA was excised with BanHI and NotI and ligated into pbLueScript II KS(+) cut with the same enzymes, then digested with XhoI and NotI and ligated into the plant transformation vector 35SpBARN (35) cut with the same enzymes to give the construct 35S-HIBCH-ALK. Three control plasmids also were constructed in 35SpBARN: the full-length human cdNA with the mitochondrial targeting signal (35S-mHIBCH), the human cdNA lacking the N-terminal mitochondrial targeting sequence (35S-HIBCH), and the full-length human cdNA with the CHY1 C-terminal region (35S-mHIBCH-ALK). We also subcloned the full-length Arabidopsis CHY1 cdNA as an NotI fragment into 35SpBARN to make 35S-CHY1. Inserts of all constructs were sequenced to verify that no unintended changes were introduced during the mutagenesis or subcloning. Of the constructs was electroporated into Agrobacterium and transformed into chy1-1 mutant plants (see above). Transformants were selected after growth on PN in the dark for 5 days or on PNS plates supplemented with 7.5 μg/ml glucoseamine under white light for 10 days. Lines homozygous for the transgenes were selected by examining the pattern of glucoseamine ammonium resistance in the T2 generation.

**CHY1 Protein Expression and Enzyme Assays—**Mutagenesis was performed on pKS-CHY1 to add an Ndel site immediately 5′ of the initiator ATG using the oligonucleotide 5′-GGCCATCTCGACTGC-GATATGCTTACTGGTCAGATTCG-3′ (altered residues are underlined). In addition, two CHY1 amino acids were separately altered using oligonucleotide-directed mutagenesis: Glu-141 was changed to Gly using the oligonucleotide 5′-CCCCAGACTGTCGCCAGATG-GCAAAAACC-3′, and Asp-149 was changed to Gly using the oligonucleotide-directed mutagenesis: Glu-141 was changed to Gly using the oligonucleotide 5′-GAGGCGCCTACCGCGGAGGCTCGGAGGCG-3′ (altered residues are underlined). The Ndel-NotI fragments of the wild-type and mutagenized cdDNAs were ligated into the protein expression vector pGEX-KTO (36) cut with the same enzymes.

Protein expression was performed as described previously (36), except that cells were allowed to grow 4 h after induction with isopropyl-β-D-thiogalactopyranoside. Following purification, the free glutathione was removed by dialyzing proteins against phosphate buffer (25 mM KPO4, pH 6.0, 0.1 mM EDTA, 10% glycerol) overnight. Protein expression, purification, and quantification were confirmed by SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining (28). CoA-ester hydrolysis was assayed essentially as described previously (12–14). GST-CHY1 or GST was incubated at 25 °C with each substrate in 1.5 ml of 25 mM phosphate buffer, 50 mM NaCl, 1 mM EDTA, 0.1% Triton X-100. To assay HIBYL-CoA hydrolysis, 10 units of crotonase (Sigma Chemical Co.) was incubated with methacrylyl-CoA prior to addition of 60 ng of GST-CHY1 or 600 ng of GST. Other substrates were tested with 200 ng of GST-CHY1 or GST. At each time point, 450 μl was removed for spectrophotometric determination of liberated CoA. Determination of the pH optimum was done using 25 mM potassium phosphate buffers from pH 5 to 9. Substrate specificity assays were done either at pH 7.5 or 5.0. Methacrylyl-CoA was synthesized as described (13). IAA-N-acetylsteammine, IBA-N-acetylsteammine, and IAA-CoA were synthesized by benzotriazolyloxytris(pyrrrolidino)phosphonium hexafluorophosphate-mediated coupling (37) of IAA or IBA to N-acetylstea'mmine or CoA using established protocols (38). Acetyl-, benzoyl-, β-hydroxybutyryl-, butyryl-, crotonyl-, isobutyryl-, isovaleryl-, malonyl-, methylmalonyl-, oleoyl-, phenylacetyl-, and propionyl-CoA esters were from Sigma. Kinetic values were determined using the Igor-Pro program (WaveMetrics).

**RESULTS**

**Auxin-response Phenotypes of chy1 Mutants—**Fourteen Arabidopsis IBA-response mutants were previously identified (18). We show here that three of these mutants (B7, B17, and B52) are alleles of the same gene, which we named CHY1 (for HIBYL-CoA hydrolyase; see below). Whereas exogenous IBA and IAA both inhibit wild-type root elongation, the chy1 alleles are resistant to IBA (Fig. 2A) but remain sensitive to IAA (Fig. 2B) over a range of concentrations. Similarly, chy1 mutants form typical numbers of lateral roots when induced on IAA but form few or no lateral roots when grown on IBA (Fig. 2C). In contrast, chy1 mutants respond like wild-type to other phytohormones tested, including the synthetic auxins 2,4-dichlorophenoxyacetic acid (18) and 1-naphthaleneacetic acid, the ethylene precursor 1-aminoacyclopropane-1-carboxylic acid, and the cytokinin benzyladene (data not shown). The chy1 mutation is recessive; F1 progeny from a backcross of chy1-1 to Col-0 or chy1-2 toWs respond normally to IBA (data not shown).

**Positional Cloning of CHY1—**We previously showed that chy1 belongs to the subset of IBA-response mutants that develop poorly in the dark in the absence of supplemental sucrose and that this defect correlates with slower fatty acid β-oxidation during germination (18). This phenotype suggested that the IBA-response defects in chy1 resulted from a reduced ability to convert IBA to IAA in a peroxisomal chain-shortening process. To determine the molecular nature of this defect, we used positional information to clone the gene defective in the chy1 mutants. We mapped the three chy1 alleles to an ~800-kb interval on the bottom of chromosome 5, between MNA5 (40) and K919 (41), south of the previously described peroxisome-defective pex5 (18) and ped2 (21, 42) mutants (Fig. 3A). Because the chy1 mutant phenotype suggested peroxisomal defects (18), we examined the sequence in this interval for genes that might be involved in peroxisomal biogenesis or function. The clone K14B20 (30) contains a gene encoding a protein 43% identical to a mammalian enzyme that hydrolyzes HIBYL-CoA to β-hydroxyisobutyrate and CoA during Val catabolism (Fig. 1, step 5) (12–14). Unlike the mammalian enzyme, which is mitochondrial (12–14), the Arabidopsis protein was predicted to be per-
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Fig. 2. Auxin-response phenotypes of chy1 mutants. A, root elongation on IBA. 8-day-old seedlings grown on the indicated concentration of IBA under yellow-filtered light were removed from the agar, and the length of the primary root was measured. The percent elongation of plants grown on hormone versus hormone-free media is shown. chy1-1 and chy1-3 are in the Col-0 background; chy1-2 is in theWs background. Error bars indicate the standard errors (n > 14). B, root elongation on IAA. Root elongation was measured as described above. Error bars indicate the standard errors (n > 11). For A and B, average root lengths on unsupplemented medium were 19, 17, 12, 17, and 19 mm for Col-0, Ws, chy1-1, chy1-2, and chy1-3, respectively. C, lateral root initiation. Seedlings were grown for 4 days on hormone-free medium and transferred to media containing the indicated concentrations of IBA or IAA or no hormone. After 4 additional days, plants were removed from the agar and the number of lateral roots counted. Error bars indicate the standard deviations (n > 14).

oxisomal by the PSORT program (43) because of the presence of a type 1 (C-terminal) peroxisomal targeting signal (44). We hypothesized that disruption of Val catabolism might indirectly disrupt peroxisomal β-oxidation (see below) and cause an IAA-resistant, IAA-sensitive mutant phenotype. To determine if chy1 is defective in this HIBYL-CoA hydrolase homolog, we PCR-amplified and sequenced this gene from genomic DNA prepared from the mutant alleles (see “Experimental Procedures”). chy1-1 has a G to A mutation at position 518 (where the A of the initiator ATG is position 1; Fig. 3A), chy1-2 has a 34-bp deletion from positions 1084 to 1118 (Fig. 3A), and chy1-3 has a G to A mutation at position 407, changing a conserved Gly to Ser (see Fig. 4).

Fig. 3. Positional cloning of CHY1. A, positions of PCR-based markers (LFY (27), MBK5, MNA5 (Table I), and K919 (Table I)) and peroxisome-defective mutants (pez3 (18), ped2 (21, 42), and chy1) are shown above the chromosome. The recombination frequency (number of recombinant/number of chromosomes scored) for each allele is shown below the chromosome. Examination of the sequenced clones in this region (thick lines) revealed the CHY1 gene on K14B20. CHY1 exons are shown by rectangles and introns by thin lines. chy1-1 has a G to A mutation at position 518 that alters the 3′-splice site of the fourth intron. chy1-2 has a 34-bp deletion from positions 1084 to 1118, which alters the 5′-splice site of the eighth intron. B and C, pBIN-CHY1 rescues chy1 mutant phenotypes. B, wild-type (Col-0), chy1-1, and homozygous T1 progeny of chy1-1 plants transformed with the pBIN-CHY1 construct were analyzed for sucrose dependence by growing seedlings on media with or without sucrose for 1 day in the light and 5 days in the dark, removing them from the agar, and measuring hypocotyl length. The percent elongation on sucrose-free medium compared with medium containing sucrose is shown. Average hypocotyl lengths on sucrose-containing medium were 16, 12, and 14 mm for Col-0, chy1-1, and chy1-1 (pBIN-CHY1), respectively. Error bars indicate the standard deviations (n > 9). C, wild-type, mutant, and transformant seeds were tested for root elongation on 10 μM IBA as described in the legend to Fig. 2A. Average root lengths on sucrose-containing medium were 19, 12, and 14 mm for Col-0, chy1-1, and chy1-1 (pBIN-CHY1), respectively. Error bars indicate the standard deviations (n > 14).

To determine the splicing pattern of CHY1, we isolated the corresponding full-length cDNA (see “Experimental Procedures”). Sequencing this cDNA revealed that CHY1 contains 12 introns and that the chy1-1 defect was in the 3′-splice site of the fourth intron (Fig. 3A). This mutation changes an essential G (45), which should alter splicing in the mutant and thus disrupt the encoded protein. The chy1-2 deletion disrupts the 5′-splice site of the eighth intron, which should also alter splicing (45) and prematurely terminate the protein (Fig. 3A). To ascertain how the chy1-1 and chy1-2 mutations affect splicing, we attempted to use reverse transcription-PCR to amplify RNA from wild-type and mutant plants with primers that spanned the lesions (see “Experimental Procedures”). Whereas Col-0 andWs RNA produced a single band of the predicted size, we failed to amplify discrete bands in either the chy1-1 or chy1-2 mutant (data not shown), suggesting that the mutant tran-
scripts are unstable or aberrantly processed into multiple forms. To verify that the nucleotide changes in the chy1 mutant alleles caused the IBA-resistant phenotype, we tested a 5.9-kb CHY1 genomic fragment for complementation of the chy1-1, chy1-2, and chy1-3 phenotypes (see “Experimental Procedures”). Transformation of this clone (pBIN-CHY1) into mutant plants restored wild-type hypocotyl elongation in the dark (Fig. 3B and data not shown), as well as IBA sensitivity in root elongation (Fig. 3C and data not shown) and lateral root initiation (data not shown), confirming that we have identified the gene responsible for the mutant phenotype.

Functional Complementation of chy1 with a Human HIBYL-CoA Hydrolase cDNA—The predicted CHY1 protein is 43% identical to a mammalian Val catabolic enzyme (Fig. 4) that hydrolyzes HIBYL-CoA (12–14). To determine if Arabidopsis CHY1 catalyzes the same reaction (Fig. 1, step 5), we tested whether a human HIBYL-CoA hydrolase (HIBCH) cDNA could functionally complement the chy1 mutant. Because CHY1 is predicted to be peroxisomal (Fig. 4) and the mammalian HIBYL-CoA hydrolase is mitochondrial (12–14), we first removed the N-terminal mitochondrial signal sequence from the human cDNA (see “Experimental Procedures”). We then added a peroxisomal targeting sequence by replacing the C-terminal 13 amino acids of HIBCH with the peroxisomal targeting consensus sequence in CHY1 and two of the homologs, and predicted mitochondrial targeting sequences are boxed. The locations of the splicing defects in chy1-1 and chy1-2 are indicated by arrows. The bar above the sequence denotes the C-terminal “AKL” peroxisomal targeting consensus sequence in CHY1 and two of the homologs, and predicted mitochondrial targeting sequences are boxed. The beginning of T11J7.4 and a few of the intron/exon boundaries in the Arabidopsis proteins were altered from their GenBank annotations using consensus splicing sequences (45, 59, 60) to preserve sequence homology.

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![Fig. 4. Alignment of CHY1 and its homologs. CHY1 was aligned with the human HIBYL-CoA hydrolase (Hs HIBCH; GenBank accession number XP_002278) and related Arabidopsis open reading frames with the MegAlign program (DNASTAR) using the Clustal V method. Amino acid residues identical in at least four of the sequences are shaded, and hyphens indicate gaps introduced to maximize alignment. The seven Arabidopsis homologs were identified in various sequencing projects and are listed according to the GenBank annotation of the bacterial artificial chromosome. The chy1-3 mutant has a Gly to Ser mutation, as shown above the sequence. The locations of the splicing defects in chy1-1 and chy1-2 are indicated by arrows. The bar above the sequence denotes the C-terminal “AKL” peroxisomal targeting consensus sequence in CHY1 and two of the homologs, and predicted mitochondrial targeting sequences are boxed.](http://www.jbc.org/content/383/11/31041/F4)

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with 28 amino acids from CHY1, including the peroxisomal targeting signal "AKL" (44). A construct containing this altered human cDNA driven by the cauliflower mosaic virus 35S promoter (35S-HIBCH-AKL) rescued the chy1 mutant nearly as well as the 35S-CHY1-positive control construct. Sucrose-independent hypocotyl elongation in the dark (Fig. 5A) was fully restored, and IBA sensitivity in both root elongation (Fig. 5B) and lateral root initiation (data not shown) was partially restored. Several control constructs also were tested for chy1 rescue, including the unaltered human cDNA with the mitochondrial targeting signal intact (35S-mHIBCH), without either targeting sequence (35S-HIBCH), and with both mitochondrial and peroxisomal targeting sequences (35S-mHIBCH-AKL). The construct without signal sequences (35S-HIBCH) failed to rescue chy1 defects, and although the mitochondrial derivative (35S-mHIBCH) slightly rescued the mutant defect on medium without sucrose (Fig. 5A), it

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<th>Substrate</th>
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<tr>
<td></td>
<td>GST-CHY1</td>
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<tr>
<td>Methacrylyl-CoA + crotonase (HIBYL-CoA)</td>
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a The limit of detection was 0.1 μmol of CoA released/min/mg.

b ND, not determined.
did not restore sensitivity to IBA (Fig. 5B). Interestingly, the construct with both mitochondrial and peroxisomal tags (35S-mHIBCH-AKL) rescued the mutant phenotype to a similar extent as the 35S-HIBCH-AKL construct. Because the peroxisomally targeted HIBYL-CoA hydrolases can rescue the chy1 mutant defects, it is likely that CHY1 encodes a peroxisomal HIBYL-CoA hydrolase.

**CHY1 Activity in Vitro**—To confirm that CHY1 participates in Val catabolism, we assayed recombinant CHY1 protein in vitro. We expressed and purified CHY1 as a GST fusion in *Escherichia coli* (see “Experimental Procedures”) and determined whether the fusion protein hydrolyzed various CoA esters by spectrophotometrically assaying the free sulfhydryl group of released CoA (12–14). GST-CHY1 hydrolyzed HIBYL-CoA (Table II) with a $K_m$ of 3.7 ± 0.6 μM and a $V_{max}$ of 24.2 ± 0.8 μmol/min/mg (Fig. 6A). GST-CHY1 also hydrolyzed several other substrates but at significantly lower rates (Table II), indicating enzymatic specificity for HIBYL-CoA. No substrates were hydrolyzed when GST-CHY1 was replaced by GST (Table II), even with a 5-fold higher protein concentration (data not shown). To test whether CHY1 participates more directly in auxin metabolism, we also tested IAA-CoA, IAA-N-acetylcysteamine, and IBA-N-acetylcysteamine but detected no hydrolysis (data not shown), indicating CHY1 does not act directly in the conversion of IBA to IAA by hydrolyzing the IAA-CoA formed during the β-oxidation of IBA.

To determine the pH optimum of the enzyme, we monitored GST-CHY1 hydrolysis of HIBYL-CoA over a range of pH values. CHY1 has a broad pH optimum (Fig. 6B), with similarly high hydrolysis rates from pH 7 to 9, consistent with CHY1 functioning in peroxisomes, which have a pH of ~8 (46). Similarly, the mammalian CHY1 homologs are most active above pH 6.5 (12–14).

We also examined the role of two conserved CHY1 residues. We mutagenized the CHY1 cDNA at two sites (Fig. 7A) and expressed the mutant proteins as GST fusions. The completely conserved Glu-141 was converted to Ala and Asp-149 was altered to Gly. These mutant GST-chy1 fusion proteins remained soluble and were purified and tested for HIBYL-CoA hydrolysis. The GST-chy1(D149G) protein had severely reduced activity (~3% of wild-type), whereas the GST-chy1(E141A) protein was completely inactive (Table II).

**DISCUSSION**

The three chy1 mutant alleles were isolated based on resistance to the inhibitory effects of exogenous IBA on root elonga-
tion (Fig. 2A). They also are resistant to lateral root induction by this naturally occurring auxin (Fig. 2C) but respond normally to IAA (Fig. 2B and C). Peroxisomal β-oxidation is also impaired in this class of IBA-response mutants: they exhibit developmental defects in the absence of exogenous sucrose (Fig. 3C) and catabolize seed storage lipids poorly during germination (18).

Here we report that CHY1 is 43% identical to a mammalian mitochondrial CoA-thioester hydrolase that cleaves HIBYL-CoA (12–14). Because it is similar to this Val catabolic enzyme, we propose that CHY1 acts to catabolize Val in Arabidopsis. In support of this hypothesis, GST-CHY1 efficiently hydrolyzes HIBYL-CoA in vitro (Table II, Fig. 6), and the human enzyme functionally complements the chy1 mutants (Fig. 5), suggesting a similar role in plants and animals. The bootstrap method was performed for 100 replicates with a distance optimality criterion, and all characters were weighted equally.

Avoiding this hydrolysis step could shorten the process from four steps to two, similar to the Ile and Leu pathways (12). The Val catabolic sequence, although energy-intensive, avoids accumulation of the potent Michael acceptor methacrylyl-CoA, which reacts rapidly with nucleophiles such as cysteine, cysteamine, glutathione, and CoA (11, 47). This conjugation may consume cofactors, such as CoA, or may attack and covalently inactivate enzymes with active site nucleophiles (11). If HIBYL-CoA hydrolysis is blocked, as in the chy1 mutants, the increasing formation of methacrylyl conjugates would shift the equilibrium from HIBYL-CoA back to methacrylyl-CoA.

chy1 is the first reported plant mutant in a BCAA catabolic enzyme. In humans, disruption of HIBYL-CoA hydrolase is lethal (11). Fibroblasts from an infant born with HIBYL-CoA hydrolase deficiency accumulate S-(2-carboxypropyl)cysteine and S-(2-carboxypropyl)cysteamine, and labeling studies reveal that Val and cysteine are the precursors (48). A reasonable explanation is that HIBYL-CoA hydrolase deficiency causes the
accumulation of its substrate, HIBYL-CoA, which can dehydrate to form methacryloyl-CoA. Carboxypropylcysteine and carboxypropylcysteamine would form by nonenzymatic 1,4-conjugate addition of the nucleophilic sulfur onto the β-position of the methacryloyl-CoA double bond. CoA hydrolysis by the HIBYL-CoA hydrolase normally circumvents this process by consuming HIBYL-CoA in an energetically favorable reaction and drives the equilibrium from methacryloyl-CoA to HIBYL-CoA, which lacks the α,β-unsaturation and consequently does not undergo conjugate addition. Consistent with this model, Ile and Leu proceed through tiglyl-CoA and methylcrotonyl-CoA intermediates. The β-alkyl substituents found in these α,β-unsaturated thioesters should greatly decrease the rate of conjugate addition; these pathways consequently do not clarify thioester hydrolysis and retioesterification.

The chy1 mutants β-oxidize long chain fatty acids inefficiently (18) and have long roots on normally inhibitory IBA concentrations (Fig. 2A) (18). Because IBA is converted to IAA in a process resembling fatty acid β-oxidation, the chy1 mutants are probably blocked in both fatty acid and IBA β-oxidation. Reducing peroxisomal HIBYL-CoA hydrolase activity in chy1 may cause the accumulation of peroxisomal methacryloyl-CoA, which conjugates to free CoA and proteins containing Cys, thereby disturbing β-oxidation either by removing required cofactors or by inactivating required enzymes. One possible target of this inactivation is thiolase, a β-oxidation enzyme that has a catalytic, nucleophilic thiol near C-3 of the substrate (49, 50). As an Arabidopsis thiolase mutant (ped1) has defects in β-oxidation (21) and is IBA-resistant (18), methacryloyl-CoA reaction with the thiolase-active site would likely block the β-oxidation of both fatty acids and IBA.

Interestingly, CHY1 and the mammalian HIBYL-CoA hydrolases are not homologous to other thioester hydrolases but instead resemble enzymes in a superfamily containing enoyl-CoA hydratases and isomerases (Fig. 7) (14, 51). The hydratases and hydrolases both have conserved amino acids near the catalytic site of the structurally characterized rat mitochondrial enoyl-CoA hydratase (Fig. 7A) (52). For example, CHY1 Gly-69 (which is mutated to Ser in chy1-3) is at the position of an Ala involved in ligand interaction (Fig. 7A) in the hydratase crystal structure (52). Glu-141 in CHY1 is absolutely conserved in both families and participates in substrate hydration in the hydratases (51, 52). GST-CHY1 is inactive when Glu-141 is mutated to Ala (Table II), indicating that this amino acid is essential, perhaps because it binds the nucleophilic water molecule.

In addition, several amino acids are differentially conserved between the hydratase and hydrolase families. For example, an active site Ghu residue in the enoyl-CoA hydratases (52) is replaced with Gly (or Ala) in all of the predicted hydrolases (Gly-121 in CHY1, Fig. 7A). Conversely, the predicted hydrolases maintain Asp at positions corresponding to residue 149 in CHY1, but Gly is conserved in the hydratases examined. This Gly is thought to be involved in binding the CoA moiety in the rat enoyl-CoA hydratase (52). Mutating Asp-149 to Gly dramatically reduced GST-CHY1 activity in vitro (Table II). Several sequences with the catalytic Asp have been annotated as hydratases in the data bases. The identity of this residue may be useful in distinguishing these enzyme classes.

Arabidopsis has multiple predicted isozymes for many BCAA catabolic enzymes (data not shown). Seven Arabidopsis genes encoding CHY1-like proteins (31–86% identical at the amino acid level) have been identified (Fig. 4), but only CHY1 has been biochemically characterized to date. PSORT analysis (43) predicts that CHY1 and its two closest homologs (T11L7.4 and T11L7.5; ~86% and 83% identical to CHY1) are peroxisomal, whereas T8B10.170, F11C18.10, and K7M2.13 (~46%, 44%, and 31% identical) are mitochondrial, and F12K11.12 and T9E8.100 (~47% and 39% identical) are cytoplasmic. However, the cytoplasmic localization of F12K11.12 and T9E8.100 is tentative, because cDNAs have not been isolated for these genes and it is possible that targeting sequences are present on upstream exons not identified in the genome annotation. Our ability to repeatedly isolate β-oxidation-defective mutants defective in the CHY1 gene indicates that the CHY1 homologs (including the other peroxisomal enzymes) have substrate specificity or expression differences that preclude their efficient substitution for CHY1 in vivo.

The targeting of multiple apparent CoA-ester hydrolases to different intracellular locations (Fig. 4) may eventually clarify the ongoing debate regarding the compartmentalization of plant BCAA catabolism (9, 10; reviewed in Ref. 5). In vitro assays have demonstrated that BCAA are catabolized in isolated plant peroxisomes (9, 10). However, several potential BCAA catabolic enzymes in Arabidopsis, pea, and soybean have been isolated from mitochondria or contain apparent mitochondrial transit signals (Fig. 4) (6, 53–58). CHY1 is likely to act in Val catabolism, as it hydrolyzes HIBYL-CoA in vitro (Table II; Fig. 6). CHY1 has a peroxisomal targeting sequence, and the human protein rescues the chy1 mutant when it is targeted to peroxisomes (Fig. 5), suggesting that Val catabolism is at least partially peroxisomal in plants. Interestingly, two other eukaryotes with sequenced genomes, Drosophila melanogaster and Caenorhabditis elegans, have only one CHY1 homolog (apparently mitochondrial), suggesting a less complex compartmentalization of Val catabolism in these organisms. Determining the substrate specificity and organelle, tissue, and developmental distribution of the CHY1 family members will illuminate their function and multiplicity and may clarify the role of plant Val catabolism and its intracellular localization.

Amino acid catabolism may provide energy throughout development, especially during periods of high protein turnover, such as seed storage protein utilization during germination, senescence, and starvation (6–8). CHY1 encodes an Arabidopsis HIBYL-CoA hydrolase that may act in Val catabolism. The identification of this mutant provides a unique tool for deciphering the mechanism of Val catabolism in plants. Further examination of the chy1 mutant will allow a better understanding of the timing of Val catabolism, the intracellular localization of this process, and the overall contribution of Val catabolism in Arabidopsis metabolism.

Acknowledgments—We thank Sherry LeClere for the 35S::BARN vector, Dr. Robert Harris for useful discussions, Rachel Leininger for assistance with data analysis, and Jackie Lim and Dr. Rob Last for the MBK5 marker polymorphism. We are grateful to Sherry LeClere, Mónica Magdin, Rebekah Rampey, and Luise Rogg for critical comments on the manuscript.

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doi: 10.1074/jbc.M104679200 originally published online June 12, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M104679200

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