Genes for degradation of storage oil and their application to oil biotechnology

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Abstract

cDNAs for long- and short-chain acyl-CoA oxidases in fatty acid β-oxidation were isolated and were characterized their enzymatical and molecular properties. Both oxidases were exclusively localized in glyoxysomes, indicating that glyoxysomes can completely metabolize fatty acids to acyl-CoA by their cooperative action. In order to clarify the regulatory mechanisms underlying degradation of storage oil, , we tried to obtain glyoxysome-deficient mutants of Arabidopsis. We screened 2,4-dichlorophenoxybutyric acid (2,4-DB) mutants of Arabidopsis which have defects in glyoxysomal fatty acid β-oxidation. Four mutants can be classified as carrying alleles at three independent loci, which we designated *ped1*, *ped2*, and *ped3*, respectively (where ped stands for peroxisome defective). The characteristics of these *ped* mutants are described.

Oilseed plants convert reserve oil to sucrose after germination. This unique type of gluconeogenesis occurs in the storage tissues of oil seeds, such as endosperms or cotyledons [1]. The metabolic pathway involves many enzymes in several subcellular compartments, including lipid bodies, glyoxysomes (a specialized peroxisome), mitochondria, and the cytosol. Within the entire gluconeogenic pathway, the conversion of a fatty acid to succinate takes place within the glyoxysomes, which contain enzymes for fatty acid β-oxidation and the glyoxylate cycle.

Glyoxysomes and leaf peroxisomes are members of a group of organelles called peroxisomes [2]. In the glyoxysomes, fatty acid are first activated to fatty acyl-CoA by fatty acyl-CoA synthetase [14]. Fatty acyl-CoA is the substrate for fatty acid β-oxidation, which consists of four enzymatic reactions [20]. The first reaction is catalyzed by acyl-CoA oxidase. The second and third enzymatic reactions are catalyzed by a single enzyme that possesses enoyl-CoA hydratase and β-hydroxy-acyl-CoA dehydrogenase activities [26]. The fourth reaction is catalyzed by 3-ketoacyl-CoA thiolase (thiolase) [27]. Acetyl-CoA, an end product of fatty acid β-oxidation, is metabolized further to produce succinate by the glyoxylate cycle.

In mammalian cells, both peroxisomes and mitochondria contain a functional fatty acid β-oxidation system. In peroxisomes, the first enzyme of fatty acid β-oxidation, acyl-CoA oxidase, donates electrons to molecular oxygen producing hydrogen peroxide [7]. Mammalian peroxisomes oxidize long-chain fatty acids, but are inactive with fatty acids shorter than octanoic acid (C8). This is mainly the consequence of the exclusive presence of long-chain acyl-CoA oxidases and the absence of acyl-CoA oxidases that are active on short-chain acyl-CoAs. In contrast, mammalian mitochondria are capable of complete oxidization of fatty acids to acetyl-CoA [4], the first step of fatty acid β-oxidation is accomplished by long-, medium- and short-chain acyl-CoA dehydrogenases, and electrons generated by the dehydrogenases are transferred to the mitochondrial respiratory chain. By analogy, Thomas and coworkers have postulated the existence of plant mitochondrial β-oxidation [23, 24, 29], but the presence of acyl-CoA dehydrogenase was not investigated or not detected [13]. In contrast, data reported by the group of Gerhardt have suggested that glyoxysomes in plants can completely metabolize fatty acids to acetyl-CoA [5, 6, 21]. We characterized acyl-CoA oxidases that are active on long- and short-chain acyl-CoA in glyoxysomes, respectively [8, 9]. We also discuss the unique features of fatty acid β-oxidation accomplished by these acyl-CoA oxidases in plant cells.
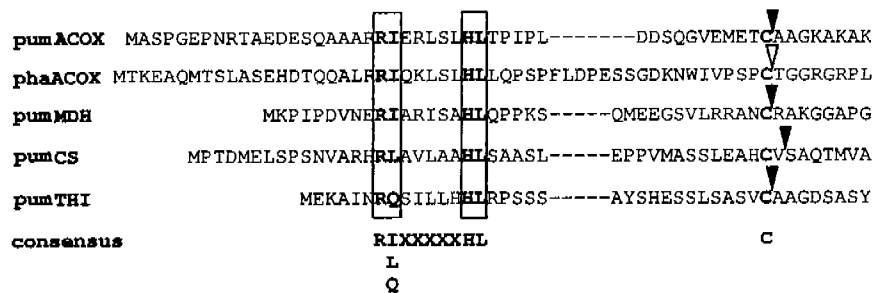
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pumACOX    MASPGEPNRTAEDESQAAARIERLSLHITPIPL-------DDSQGVEMETCAAGKAKAK

phaACOX    MTKEAQMTSLASEHDTQQALRIQKLSIHILQPSPFLDPESSGDKNWIVPSPCTGGRGRPL

pumMDH        MKPIPDVNERIARISAHIQPPKS-----QMEEGSVLRRANCRAKGGAPG

pumCS      MPTDMELSPSNVARHRIAVLAAHISAASL-----EPPVMASSLEAHCVSAQTMVA

pumTHI        MEKAINRQSILLHIRPSSS------AYSHESSLSASVCAAGDSASY

consensus           RIXXXXXHL                    C
                    L
                    Q
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Figure 1. Alignment of the amino-terminal presequence of pumpkin ACOX with other presequences of microbody proteins that are synthesized as larger precursors: pumACOX, pumpkin ACOX; phaACOX, *Phalaenopsis* ACOX [3]; pumMDH, pumpkin glyoxysomal malate dehydrogenase [19]; pumCS, pumpkin glyoxysomal citrate synthase [17]; pumTHI, pumpkin glyoxysomal 3-keto-acyl-CoA thiolase [18]. Conserved amino acids are shown in bold. Processing sites of presequences, determined by sequencing of the amino-terminal amino acids of mature proteins, are shown by arrowheads. These sequences function as targeting signal to peroxisomes and are designated as PTS (peroxisomal targeting signal) 2 [15, 16]. The other targeting signal to peroxisomes is a carboxy-terminal tripeptide, [C/A/S/R]-{K/R]-[I/L/M], and is designated as PTS 1 [10, 11, 22].

Characterization of long- and short-chain acyl-CoA oxidases

A cDNA clone for pumpkin acyl-CoA oxidase (EC 1.3.3.6; ACOX) was isolated from a λgt11 cDNA library constructed from poly $(A)^+$ RNA extracted from etiolated cotyledons. The inserted cDNA clone contains 2313 nucleotides and encodes a polypeptide of 690 amino acids. Analysis of the amino-terminal sequence of the protein indicates that pumpkin the acyl-CoA oxidase protein is synthesized as a larger precursor containing a cleavable amino-terminal presequence of 45 amino acids. As shown in Figure 1, the presequence shows high similarity to the typical peroxisomal targeting signal (PTS2). Western blot analysis following cell fractionation in a sucrose gradient revealed that ACOX is localized in glyoxysomes. A partial purification of ACOX from etiolated pumpkin cotyledons indicated that the ACOX cDNA codes for a long-chain acyl-CoA oxidase. The amount of ACOX increased and reached to the maximum activity by day 5 of germination but decreased about 4-fold on the following days during the subsequent microbody transition from glyoxysomes to leaf peroxisomes. By contrast, the amount of mRNA was already high at day 1 of germination, increased by about 30% at day 3 and faded completely by day 7. These data indicated that the expression pattern of ACOX was very similar to that of the glyoxysomal enzyme 3-ketoacyl-CoA thiolase, another marker enzyme of the β-oxidation spiral, during germination and suggested that the expression of each enzyme of β-oxidation is coordinately regulated.

Short-chain acyl-CoA oxidase is an β-oxidation enzyme that is active on short-chain acyl-CoAs and appear to be present in higher plant peroxisomes and absent in mammalian peroxisomes. Therefore, plant peroxisomes are capable of performing complete β-oxidation of acyl-CoA chains, whereas mammalian peroxisomes can perform β-oxidation of only those acyl-CoA chains that are larger than octanoyl-CoA (C8). We have shown that a novel acyl-CoA oxidase can oxidize short-chain acyl-CoA in plant peroxisomes [8]. A peroxisomal short-chain acyl-CoA oxidase from *Arabidopsis* was purified following the expression of the *Arabidopsis* cDNA in a baculovirus expression system. The purified enzyme was active on butyryl-CoA (C4), hexanoyl-CoA (C6), and octanoyl-CoA (C8) (Figure 2). Cell fractionation and immunocytochemical analysis revealed that the short-chain acyl-CoA oxidase is exclusively localized in peroxisomes. The expression pattern of the short-chain acyl-CoA oxidase was similar to that of peroxisomal 3-ketoacyl-CoA thiolase, a marker enzyme of fatty acid β-oxidation, during post-germinative growth. Although the molecular structure and amino acid sequence of the enzyme are similar to those of mammalian mitochondrial acyl-CoA dehydrogenase, the purified enzyme has no activity as acyl-CoA dehydrogenase. These results indicate that the short-chain acyl-CoA oxidases function in fatty acid β-oxidation in plant peroxisomes and by the cooperative action of long-chain and short-chain acyl-CoA oxidases, plant peroxisomes are capable of performing the complete β-oxidation of acyl-CoA.

Glyoxysomal β-oxidation defective mutants of Arabidopsis

A genetic approach may be an effective strategy toward understanding the regulatory mechanism(s) underlying fatty acid β-oxidation at the level of gene
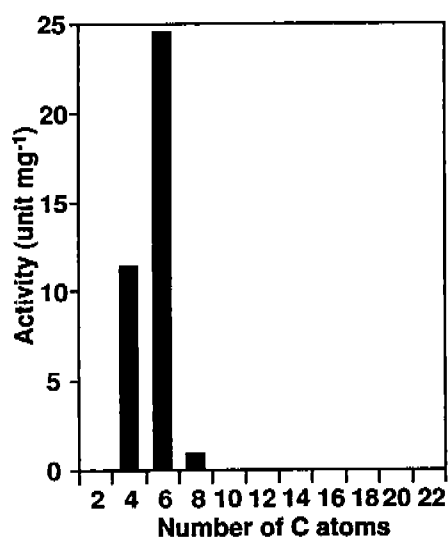
Figure 2. Substrate specificity of *Arabidopsis* short-chain acyl-CoA oxidase produced in a baculovirus expression system. The activity was monitored employing various acyl-CoAs as substrates at a concentration of 25 µM.

expression, protein translocation, and protein degradation. We screened 2,4-dichlorophenoxybutyric acid (2,4-DB)-resistant mutants that have defects in glyoxysomal fatty acid β-oxidation [12]. It has been previously demonstrated that 2,4-DB is metabolized to produce a herbicide, 2,4-D, by the action of fatty acid β-oxidation in higher plants [28]. In order to isolate mutants that have defects in glyoxysomal fatty acid β-oxidation, mutant lines of *Arabidopsis* seedlings were screened for growth in the presence of toxic levels of 2,4-DB. We isolated 12 *Arabidopsis* mutants (LR11, LR24, LR27, LR40, LR43, LR47, LR53, LR77, LR81, LR91, LR92, and LR98: LR; long root in the presence of 2,4-DB) that showed resistance specifically to 2,4-DB, not to 2,4-D.

Most of the 2,4-DB-resistant mutants grew on the growth medium without sucrose as well as the wild-type plants did. But four of the mutants, namely LR40, LR43, LR47, and LR81, could expand their green cotyledons and leaves only when sucrose was supplied to the growth medium, suggesting that these mutants have defects in glyoxysomal fatty acid β-oxidation, since glyoxysomal fatty acid β-oxidation has an important role in converting sucrose from storage lipids during germination. Genetic analysis revealed that LR40, LR43, and LR47 are nonallelic mutations, whereas LR43 and LR 81 are allelic. We designated *ped1* as LR40, *ped2* as LR47, *ped3-1* as LR43, and *ped3-2* as LR81.

To characterize the phenotypes of the mutants, glyoxysomal enzymes in 5-day-old etiolated cotyledons were analyzed by an immunoblot technique using antibody against thiolase which is involved in glyoxysomal fatty acid β-oxidation. Two mutants showed different thiolase patterns from that of the wild-type plant. In the cotyledons of *ped1* mutant, no accumulation of thiolase was observed at any stage of postgerminative growth regardless of the light conditions. In cotyledons of *ped2* mutant, two types of thiolase were detected. One of these had the same molecular mass (45 kD) as that found in the wild-type plant, whereas the other was an additional protein with a higher molecular mass (48 kD), a precursor to the protein.

Etiolated cotyledons of both mutants have glyoxysomes with abnormal morphology. Glyoxysomes in *ped1* mutant were two or three times greater in diameter than those in the wild-type plant and contained vesicle-like structures. By contrast, most of the glyoxysomes found in the cells of *ped2* mutant were shrunken and not round. Further analysis is necessary to clarify the formation of these abnormal glyoxysomes.

Comparison of thiolase genes for wild-type plant and *ped1* mutant revealed that ATT codon for [100]Ile at 4th exon of wild-type thiolase gene (accession No. AB008855) is changed to ATGG in thiolase gene of *ped1* mutant (accession No. AB008856). Nucleotide substitution of T to GG causes frame shift, and produces stop codon within the 4th exon. Therefore thiolase gene in *ped1* mutant encodes a smaller protein composed of 114 amino acids, and first 99 amino acids of the protein are identical to wild-type thiolase. Identification of the genes of other *ped* mutants are now in progress. The functional transformation from glyoxysomes to leaf peroxisomes is also characterized in these mutants. These *ped* mutants provides us useful clues for understanding regulatory mechanisms underlying the degradation of storage oil and reversible transformation between glyoxysomes and leaf peroxisomes [25].

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