cDNA cloning and expression of a gene for 3-ketoacyl-CoA thiolase in pumpkin cotyledons

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Abstract

A cDNA clone for 3-ketoacyl-CoA thiolase (EC 2.3.1.16) was isolated from a λgt11 cDNA library constructed from the poly(A)⁺ RNA of etiolated pumpkin cotyledons. The cDNA insert contained 1682 nucleotides and encoded 461 amino acid residues. A study of the expression in vitro of the cDNA and analysis of the amino-terminal sequence of the protein indicated that pumpkin thiolase is synthesized as a precursor which has a cleavable amino-terminal presequence of 33 amino acids. The amino-terminal presequence was highly homologous to typical amino-terminal signals that target proteins to microbodies. Immunoblot analysis showed that the amount of thiolase increased markedly during germination but decreased dramatically during the light-inducible transition of microbodies from glyoxysomes to leaf peroxisomes. By contrast, the amount of mRNA increased temporarily during the early stage of germination. In senescing cotyledons, the levels of the thiolase mRNA and protein increased again with the reverse transition of microbodies from leaf peroxisomes to glyoxysomes, but the pattern of accumulation of the protein was slightly different from that of malate synthase. These results indicate that expression of the thiolase is regulated in a similar manner to that of other glyoxysomal enzymes, such as malate synthase and citrate synthase, during seed germination and post-germination growth. By contrast, during senescence, expression of the thiolase is regulated in a different manner from that of other glyoxysomal enzymes.

Introduction

Microbodies in higher plants are of three types, namely, glyoxysomes, leaf peroxisomes and unspecialized microbodies [15]. Glyoxysomes contain enzymes for β-oxidation and the glyoxylate cycle that participate in the conversion of lipid to sucrose, whereas leaf peroxisomes function together with chloroplasts and mitochondria in photorespiratory glycolate metabolism. In cotyledons of some oil seeds, such as those of pumpkin and watermelon, glyoxysomes are transformed to leaf peroxisomes during the greening of cotyledons. Immunocytochemical studies have demonstrated that glyoxysomes are directly transformed to leaf peroxisomes during the microbody transition [24, 30]. We have shown previously that the expression of glyoxysomal enzymes and leaf peroxisomal enzymes is regulated at the transcriptional level and the post-transcriptional level, respectively, during the microbody transition. The levels of transcripts for glyoxysomal enzymes, such as malate synthase [22] and citrate synthase [16], decrease during the transition, whereas those of transcripts for leaf peroxisomal enzymes, such as glyceraldehyde oxidase [32], increase markedly. Furthermore, malate synthase is specifically degraded in microbodies at the transition stage [21], and citrate synthase seems also to be degraded during the transition [16]. Recently, we showed that

The nucleotide sequence data reported will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases under the accession number D70895.
membrane proteins in microbodies change dramatically during the transition, as do the enzymes in the matrix [35]. The reverse transition of microbodies from leaf peroxisomes to glyoxysomes is observed during senescence of leaves. In senescing cotyledons of pumpkin, glyoxysomal enzymes accumulate once again in the microbodies and leaf peroxisomal enzymes disappear concomitantly [5]. We have also demonstrated by immunoelectron microscopic analysis that leaf peroxisomes are transformed directly into glyoxysomes in senescing cotyledons [25].

The glyoxylate cycle metabolizes acetyl-CoA which is produced by the \( \beta \)-oxidation of fatty acids. Therefore, enzymes for \( \beta \)-oxidation seem likely to be regulated in a similar manner to enzymes of the glyoxylate cycle. We have cloned a cDNA for 3-ketoseryl-CoA thiolase (referred to herein simply as thiolase), which catalyzes a terminal step in \( \beta \)-oxidation, as part of study of the regulation of \( \beta \)-oxidation during the microbody transition. We report here the nucleotide and deduced amino acid sequences of the cDNA for the thiolase, as well as the relative levels of the mRNA and protein during germination, post-germination growth and leaf senescence in pumpkin cotyledons. Since the peroxisomal thiolase is known as an enzyme with a presequence that functions as a targeting signal to microbodies [29], we also discuss this signal.

**Materials and methods**

**Plant materials**

Pumpkin (Cucurbita sp. Kurokawa Amakuri) seeds were purchased from Aisan Seed Company (Aichi, Japan). Seeds were soaked in running tap water overnight and germinated in Rock-Fiber soil (66R; Nitto Boseki, Chiba, Japan) at 25 °C in darkness. Some of the seedlings were transferred to the light after 5 days. Ten halves of cotyledons were harvested at various stages of germination. Senescing cotyledons were prepared by the method of Nishimura et al. [25]. Pumpkin seeds were soaked overnight, planted in vermiculite and seedlings were grown in a greenhouse for 20 days. On day 0 of senescence, green cotyledons were harvested and placed on moistened filter paper disks in 10 cm plastic Petri dishes, which were incubated in darkness at 25 °C.

**Screening and sequencing of cDNA**

The cDNA library was constructed with mRNA that had been extracted from etiolated cotyledons using the \( \lambda \)gt11 system [16]. Immunological screening and plaque hybridization were performed by the standard techniques [28]. The insert of the isolated phage clone was subcloned into the plasmid vector Bluescript II SK(−) (Stratagene, La Jolla, CA). A series of unidirectional deletion clones was constructed with a deletion kit (Takara Shuzo, Kyoto, Japan) and DNA sequencing was performed by the method of Sanger et al. [27]. DNA sequences were analyzed with GeneWorks Release 2.2 computer software (IntelliGenetics, Mountain View, CA) and we accessed amino acid sequence databases using the BLAST e-mail server [1] for the analysis of homologies among proteins. Alignment of several thiolases was performed using Clustal V software [12].

**Construction of a gene for a fusion protein and preparation of specific antiserum**

A DNA fragment of pumpkin thiolase, from Asn-201 to Thr-228, was produced by the polymerase chain reaction (PCR), performed as described previously [16]. The primers used were Oligo-10 (sense, 5'-CGCGGATCCTGTTGCCCAGCGTTTTGGG-3') and Oligo-11 (antisense, 5'-CATGAATTCCCTAAGTGGCAGCTGCAGCCTTCC-3'). These primers were conjugated with BamHI and EcoRI restriction sites, respectively. The DNA fragment obtained after PCR was digested by appropriate restriction enzymes and ligated into the multiple cloning site of pGEX-2T vector (Pharmacia, Uppsala, Sweden). The fusion protein, consisting of glutathione \( \beta \)-transferase (GST) and the sequence of the thiolase from Asn-201 to Thr-228 was expressed in *Escherichia coli* cells and purified by column chromatography on glutathione-Sepharose 4B (Pharmacia). The purified fusion protein was used to immunize a rabbit as described previously [16].

**Cell-free protein synthesis**

Poly(A)+ RNA (5 μg), prepared from pumpkin etiolated cotyledons, was translated in a rabbit reticulocyte lysate (40 μl; Amersham Japan, Tokyo) that contained \([^{35}S]\)methionine (50 μCi; Amersham Japan) in a total volume of 50 μl. Immunoprecipitation was performed by the method of Anderson and Blobel [2] using protein A-cellululose (Seikagaku, Tokyo, Japan) and an