Direct interaction between glyoxysomes and lipid bodies in cotyledons of the *Arabidopsis thaliana pedl* mutant

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Summary. During germination and subsequent growth of fatty seeds, higher plants obtain energy from the glyconeogenic pathway in which fatty acids are converted to succinate in glyoxysomes, which contain enzymes for fatty acid β-oxidation and the glyoxylate cycle. The *Arabidopsis thaliana pedl* gene encodes a 3-ketoacyl-CoA thiolase (EC 2.3.1.16) involved in fatty acid β-oxidation. The *pedl* mutant shows normal germination and seedling growth under white light. However, etiolated cotyledons of the *pedl* mutant grow poorly in the dark and have small cotyledons. To elucidate the mechanisms of lipid degradation during germination in the *pedl* mutant, we examined the morphology of the *pedl* mutant. The glyoxysomes in etiolated cotyledons of the *pedl* mutant appeared abnormal, having tubular structures that contained many vesicles. Electron microscopic analysis revealed that the tubular structures in glyoxysomes are derived from invagination of the glyoxysomal membrane. By immunoelectron microscopic analysis, acyl-CoA synthetase (EC 6.2.1.3), which was located on the membranes of glyoxysomes in wild-type plants, was located on the membranes of the tubular structures in the glyoxysomes in the *pedl* mutant. These invagination sites were always in contact with lipid bodies. The tubular structure had many vesicles containing substances with the same electron density as those in the lipid bodies. From these results, we propose a model in which there is a direct mechanism of transporting lipids from the lipid bodies to glyoxysomes during fatty acid β-oxidation.

Keywords: *Arabidopsis thaliana*; Electron microscopy; Glyoxysome; Fatty acid β-oxidation; Lipid body.

Introduction

Glyoxysomes and leaf peroxisomes are members of organelles called peroxisomes (Beevers 1979) and are interconverted during seedling growth, greening, and senescence in oil-seed plants. In greening pumpkin and cucumber cotyledons, glyoxysomes are transformed directly to leaf peroxisomes, which play a crucial role in photorespiration in combination with chloroplasts and mitochondria (Titus and Becker 1985, Nishimura et al. 1986). The shape of the peroxisome does not change during this transformation. Leaf peroxisomal enzymes are imported into preexisting glyoxysomes, whereas glyoxysomal enzymes, such as malate synthase and isocitrate lyase, are degraded (Comai et al. 1989; Graham et al. 1989; Mori et al. 1991; Rodriguez et al. 1990; Turley et al. 1990a,b; Beeching and Northcote 1987; Mano et al. 1996; Zhang et al. 1993), leaf peroxisomal enzymes, such as glycolate oxidase and hydroxypyruvate reductase, are accumulated during conversion of glyoxysomes to leaf peroxisomes (Tugeki et al. 1993, Volokita and Somerville 1987, Greenler et al. 1989, M. Hayashi et al. 1996, Nishimura et al. 1993).

Glyoxysomes contain enzymes for fatty acid β-oxidation and the glyoxylate cycle to convert fatty acids to succinate. Fatty acids are stored in lipid bodies in seeds and germinating cotyledons. In the glyoxysomes, fatty acyl-CoA synthetase (ACS) (EC 6.2.1.3) activates fatty acids to fatty acyl-CoAs (Huang et al. 1983). Fatty acyl-CoAs are the substrate for fatty acid β-oxidation, which consists of four enzymatic reactions (Kindl 1993). In the first step of the β-oxidation spiral, acyl-CoA oxidase converts acyl-CoA to trans-2-enoyl-CoA. acyl-CoA oxidases have been shown to have different substrate specificities against long, medium, and short acyl-CoAs (H. Hayashi et al. 1998, 1999). In the second step, a bifunctional protein works for two enzymatic reactions as an enoyl-CoA hydratase and a 3-hydroxy acyl-CoA dehydrogenase (Preisig-Muller...
In the last step, a 3-ketoacyl-CoA thiolase (referred to herein simply as thiolase; EC 2.3.1.16) produces acetyl-CoA (Presig-Müller and Kindl 1993, Kato et al. 1996). By the action of five enzymes of the glyoxylate cycle, an acetyl-CoA, which is the final product of fatty acid β-oxidation, is metabolized further to produce succinate. The regulatory mechanism of lipid degradation remains obscure: how fatty acids are transported from lipid bodies to glyoxysomes and whether fatty acids are degraded only in glyoxysomes still remains to be resolved.

Recently, we have been investigating the regulatory mechanisms of peroxisomal function at the level of gene expression, protein translocation, and protein degradation (Nishimura et al. 1996, 1998). In a previous study, we screened 2,4-dichlorophenoxybutyric acid (2,4-DB)-resistant mutants that have defects in fatty acid β-oxidation in glyoxysomes (M. Hayashi et al. 1998). In higher plants, 2,4-DB is metabolized to produce a herbicide, 2,4-dichlorophenoxybutyric acid, by fatty acid β-oxidation in the peroxisomes (Wain and Wightman 1954). Therefore, Arabidopsis thaliana mutants that can grow in the presence of toxic levels of 2,4-DB may be deficient in fatty acid β-oxidation.

We screened a pedl mutant which has a protein-null mutation in a thiolase gene. The thiolase gene was mapped to the second chromosome of A. thaliana. We identified the nucleotide substitution (T to GG) within the thiolase gene of the pedl mutant (GenBank accession number AB008856, M. Hayashi et al. 1998). This thiolase is involved in fatty acid β-oxidation during germination and subsequent seedling growth (Kato et al. 1996). In etiolated cotyledons of the pedl mutant, glyoxysomes have abnormal structures: They are larger than those in the wild type and contain many electron-lucent regions. In glyoxysomes, a defect in the β-oxidation pathway would be expected to result in the accumulation of intermediates of the degradation of fatty acids. However, the electron-lucent regions in the glyoxysomes of the pedl mutant did not seem to be due to only an accumulation of intermediates (M. Hayashi et al. 1998). Thus, further research is required to identify and determine the origin of the electron-lucent regions in glyoxysomes of the pedl mutant.

In oil seeds, triacylglycerols are stored in lipid bodies. In rape-seed cotyledons, a lipase, which hydrolyzes triglycerides to release fatty acids, was shown to be localized on the spherical appendices of lipid bodies (Theimer and Rosnitschek 1978). The mechanism by which a triacylglycerol (or a fatty acid) is transferred from a lipid body to a microbody is still obscure. The pedl mutant should be useful to elucidate how the lipids in the lipid bodies are metabolized and transported to the glyoxysome for the following reasons: the pedl mutant has a defect in the thiolase gene which encodes an important enzyme for fatty acid β-oxidation; lipid bodies still remain in the cotyledons of the pedl mutant grown in the dark for 5 days; and the abnormal structure does not seem to be caused by a simple accumulation of intermediates inside the glyoxysomes.

In the present study, we analyzed in detail the glyoxysomes by electron microscopy to better understand the formation of defective glyoxysomes in the pedl mutant and showed that direct interaction between glyoxysomes and lipid bodies is involved in the process of fatty acid degradation.

**Material and methods**

**Plant materials and growth conditions**

Arabidopsis thaliana ecotype Landsberg erecta was used as the wild-type plant. The pedl seed was obtained as previously described (M. Hayashi et al. 1998). The surface of the seeds was sterilized with 2% NaClO and 0.02% Triton X-100. Seeds were grown on growth medium (2.3 mg of Murashige and Skoog salts [Wako, Osaka, Japan] per ml, 1% sucrose, 10 μg of myoinositol, 1 μg of thiamine-HCl, 0.5 μg of pyridoxine, 0.5 μg of nicotinic acid, and 0.5 mg of morpholineethanesulfonic acid-KOH per ml, pH 5.7, and 0.2% Gellan gum [Wako]). Etiolated cotyledons were collected from the plants grown on the growth medium for 5 days in the dark at 22 °C. Stems and leaves were collected from plants grown under continuous illumination or in the dark at 22 °C.

**Electron microscopic analysis**

Tissues were cut into blocks with <1 mm thickness. Samples were fixed for 3 h at 4 °C in 0.2 M caducate buffer (pH 7.2) containing 4% paraformaldehyde, 1% glutaraldehyde, and 0.1 M CaCl₂, washed with 0.1 M caducate buffer (pH 7.2) for 1.5 h, postfixed in 2% osmium tetroxide for 1 h on ice, dehydrated serially in ethanol and propylene oxide, embedded in Spurr resin, ultrathin-sectioned, stained with uranium and lead, and observed with an electron microscope (model 1200EX; JEOL Ltd., Tokyo, Japan). For staining of membranes, samples were postfixed with 2% OsO₄ plus 0.8% K₃Fe(CN)₆ in caducate buffer and 1 μM CaCl₂ for 2 h at room temperature (Hepler 1981). For staining lipids in the cell, samples were treated with 1% p-phenylenediamine (Sigma Grade II) in 70% ethanol during dehydration (Boshier et al. 1984).

For immunoelectron microscopy, cotyledons were frozen with a high-pressure freezing machine (model HPM 010; Bal-Tec Inc., Balzers, Liechtenstein) and the ice in the cells was substituted with acetone for 2 days at −85 °C. After being washed with acetone, cotyledons were embedded in London Resin white resin. Sections were treated successively with 1% bovine serum albumin for 30 min and anti-ACS rabbit immunoglobulin G (1:500) (H. Hayashi et al. 1999) for 10 h at 4 °C. After washing with phosphate-buffered saline, sections were treated for 1 h at room temperature with colloidal-gold-conjugated protein A. After washing with 0.1% acetylated bovine serum albumin (BSA-C; Aurion Co., Wageningen, the