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Rapid Paper

Ped3p is a Peroxisomal ATP-Binding Cassette Transporter that might Supply Substrates for Fatty Acid β-Oxidation

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Glyoxysomes, a group of specialized peroxisomes, are organelles that degrade fatty acids by the combination of fatty acid β-oxidation and glyoxylate cycle. However, the mechanism underlying the transport of the fatty acids across the peroxisomal membrane is still obscure in higher plant cells. We identified and analyzed the *PED3* gene and its gene product, Ped3p. The phenotype of the Arabidopsis *ped3* mutant indicated that the mutation in the *PED3* gene inhibits the activity of fatty acid β-oxidation. Ped3p is a 149-kDa protein that exists in peroxisomal membranes. The amino acid sequence of Ped3p had a typical characteristic for "full-size" ATP-binding cassette (ABC) transporter consisting of two transmembrane regions and two ATP-binding regions. This protein was divided into two parts, that had 32% identical amino acid sequences. Each part showed a significant sequence similarity with peroxisomal "half" ABC transporters so far identified in mammals and yeast. Ped3p may contribute to the transport of fatty acids and their derivatives across the peroxisomal membrane.

Key words: ABC transporter — *Arabidopsis thaliana* — Fatty acid β-oxidation — Glyoxysome — Leaf peroxisome — Peroxisome.

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4-DB, 2,4-dichlorophenoxybutyric acid.

The nucleotide sequence reported in this paper has been submitted to the DDBJ, EMBL and GenBank under accession numbers AB070615 (*PED3*) and AB070616 (*PED3* cDNA).

Introduction

Peroxisomes in higher plant cells are known to differentiate into at least three different classes, namely glyoxysomes, leaf peroxisomes and unspecialized peroxisomes (Beevers 1982). Each organelle contains a unique set of enzymes that provides special functions in various organs in higher plants. Glyoxysomes are present in cells of storage organs, such as endosperms and cotyledons during post-germinative growth of oil-seed plants, as well as in senescent organs (Nishimura et al. 1986, Nishimura et al. 1993, Nishimura et al. 1996). They contain enzymes for fatty acid β-oxidation and the glyoxylate cycle, and play a pivotal role in the conversion of seed-reserved lipids into sucrose. The seed-reserved lipids are deposited in lipid bodies as triacylglycerols. In general, these triacylglycerols mainly contain long-chain fatty acids such as palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid (Trelease and Doman 1984). The fatty acids produced from the seed-reserved lipids are thought to be exclusively degraded in glyoxysomes (i.e. not in mitochondria) during germination and post-germinative growth (Beevers 1982). By contrast, leaf peroxisomes are widely found in cells of photosynthetic organs. Some of the enzymes responsible for photorespiration are localized in leaf peroxisomes even though the entire photorespiratory process involves a combination of enzymatic reactions that occur in chloroplasts, leaf peroxisomes and mitochondria (Tolbert 1982).

To identify the genes regulating the peroxisomal function in plant cells, we isolated mutants with defective peroxisomes. To screen such mutants, we used 2,4-dichlorophenoxybutyric acid (2,4-DB) as a compound for detecting Arabidopsis mutants with defects in glyoxysomal fatty acid β-oxidation (Hayashi et al. 1998b). We expected that two methylene groups of the butyric side chain in 2,4-DB would be removed by the action of glyoxysomal fatty acid β-oxidation to produce a herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), in wild-type plants, whereas the mutants no longer produce a toxic level of 2,4-D from 2,4-DB, because of the defect in fatty acid β-oxidation. We succeeded in identifying four mutants that were classified as carrying alleles at three independent loci. These loci were designated as *ped1*, *ped2*, and *ped3*, respectively, where *ped* stands for peroxisome defective.

Extensive studies of *ped2* mutant revealed that the *PED2* gene encodes Arabidopsis ortholog of *PEX14* (Hayashi et al.

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2000). *At*Pex14p, a *PED2* gene product, is involved in the peroxisomal protein import machinery, and maintains peroxisomal functions by determining protein targeting to all kinds of plant peroxisomes. Therefore, the *ped2* mutant has deduced activity of not only glyoxysomal function but also leaf peroxisomal function, i.e. photorespiration. By contrast, it has been known that *ped1* mutant has a defect in a gene encoding one of the enzymes for fatty acid β-oxidation, 3-ketoacyl CoA thiolase (Hayashi et al. 1998b). The loss of 3-ketoacyl-CoA thiolase in the *ped1* mutant has recently been reported to affect the morphology of the glyoxysomes, while leaf peroxisomes and unspecialized peroxisomes are normal (Hayashi et al. 2001). Ultrastructural analyses revealed that glyoxysomes in the *ped1* mutant become enlarged organelles, probably because of accumulation of a metabolic intermediate induced by the lack of 3-ketoacyl-CoA thiolase. Detailed morphological observations suggested a direct interaction between glyoxysomes and lipid bodies during the post-germinative growth of the seedlings. The direct interaction is implicated in incorporating the fatty acids produced from the seed-reserved lipids into glyoxysomes. However, the mechanism underlying the transport of the fatty acids across the glyoxysomal membrane is still obscure in cells of higher plants.

In mammalian and yeast cells, it has been speculated that the peroxisomal half ATP-binding cassette (ABC) transporter plays an important role for the transport of fatty acids across peroxisomal membranes (Shani and Valle 1998). The existence of an ABC transporter in peroxisomal membranes was first elucidated in mammalian cells. Kamijo et al. (1990) cloned the rat PMP70 gene, and showed that it encoded a 70-kDa polypeptide containing one transmembrane region and one cytosolic ATP binding region, so-called peroxisomal "half" ABC transporter. Since then, attention has been paid to the peroxisomal ABC transporter, especially in relation to the human peroxisomal disorder. Mosser et al. (1993) reported a gene responsible for a human peroxisomal disorder, X-linked adrenoleukodystrophy. ALDP, a product of the gene, is a second "half" peroxisomal ABC transporter that has significant similarity to PMP70, more so than any other ABC transporter known at the time. At present, four peroxisomal ABC transporters have been identified in mammals. They are PMP70, ALDP and their homologues P70R and ALDR (Lombardplatet et al. 1996, Shani et al. 1997). In addition to these mammalian proteins, two orthologs of yeast, *Saccharomyces cerevisiae*, peroxisomal ABC transporters have been identified and characterized. They are called *PXA1* and *PXA2* (also called *PAT2* and *PAT1*, respectively) (Shani et al. 1995, Hettema et al. 1996, Shani and Valle 1998).

Here we report the identification the *PED3* gene. The *PED3* gene encodes a novel type of peroxisomal ABC transporter with a molecular mass of 149 kDa. Ped3p, a *PED3* gene product, exists in the glyoxysomal membrane, and is involved in the mechanism for transporting fatty acids produced from the seed-reserved lipids. We also discuss the possibility of a broad substrate specificity of Ped3p.

Results

Identification and characterization of four ped3 alleles

We previously identified two allelic mutations in *PED3* locus that are *ped3-1* and *ped3-2* on an *Arabidopsis thaliana*, ecotype Landsberg *erecta* background (Hayashi et al. 1998b). These mutant lines were identified by their resistance to the presence of the toxic level of 2,4-DB. By employing the same screening technique, we identified two additional alleles, which we designated as *ped3-3* and *ped3-4*. These *ped3* mutants showed resistance specifically to 2,4-DB (Fig. 1A) but not to 2,4-D (data not shown). By contrast, 2,4-DB inhibits the growth of wild-type Arabidopsis, because 2,4-DB is metabolized to produce a herbicide, 2,4-D, by the action of peroxisomal fatty acid β-oxidation (Fig. 1A). This suggests that the mutations in *PED3* locus reduce or inhibit the activity of peroxisomal fatty acid β-oxidation.

In oil-seed plants, the most important physiological function of fatty acid β-oxidation is a gluconeogenesis from seed-reserved lipids during post-germinative growth. To determine the activity of fatty acid β-oxidation, we examined the effect of sucrose on growth of these mutant lines (Fig. 1B), because defects in fatty acid β-oxidation seem to inhibit the conversion of seed-reserved lipids into sucrose that is required for heterotrophic growth. The wild-type Arabidopsis seedlings germinated and grew normally regardless of the presence or absence of sucrose in the growth medium. None of the *ped3* mutants could expand their green cotyledons and leaves when sucrose was removed from the growth medium (Fig. 1B). The inhibitory effects on germination and post-germinative growth varied depending on the mutant. Germination of *ped3-1* and *ped3-3* embryos was most severely inhibited, and seedlings never emerged from their seed coats on growth medium without sucrose. By contrast, *ped3-2* and *ped3-4* seedlings emerged from their seed coats but could no longer grow further. Roots of these mutants did not elongate and their leaves did not develop.

The growth inhibition that occurred in the absence of sucrose may be due to the suppression of gluconeogenesis from seed-reserved lipids during post-germinative growth. A mature seed of Arabidopsis contains approximately 5 μg of triacylglycerol as seed-reserved lipids (Fig. 1C). Seed-reserved lipids in the wild-type seed were rapidly degraded within 5 d after germination and used for gluconeogenesis (Fig. 1C). By contrast, seedlings of all the *ped3* mutants grown for 5 d still contained a significant amount of seed-reserved lipids (Fig. 1C). This suggests that all the *ped3* mutants have a defect in degrading the seed-reserved lipids. These findings indicated that all *ped3* mutant lines have a defect in gluconeogenesis from the seed-reserved lipids that is necessary for the post-germinative growth.

Despite the requirement of sucrose for germination and post-germinative growth, none of the *ped3* mutants required

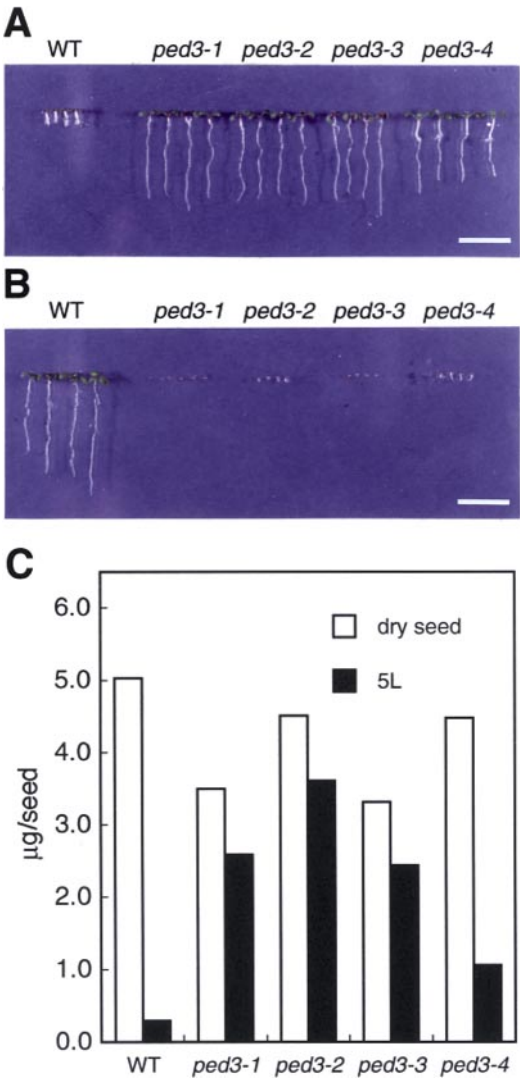


Fig. 1 Phenotypes of four *ped3* mutant lines. (A) Effects of 2,4-dichlorophenoxybutyric acid (2,4-DB) on the growth of four *ped3* mutant lines. Wild-type Arabidopsis (WT), *ped3-1*, *ped3-2*, *ped3-3* and *ped3-4* were grown for 7 d on growth medium containing 0.25 µg ml^{-1} of 2,4-DB under constant illumination. Photographs were taken after the seedlings were removed from the medium and rearranged on agar plates. (B) Effects of sucrose on the growth of four *ped3* mutant lines. Wild-type Arabidopsis (WT), *ped3-1*, *ped3-2*, *ped3-3* and *ped3-4* were grown for 7 d on growth medium without sucrose under constant illumination. Photographs were taken after the seedlings were removed from the medium and rearranged on agar plates. (C) Four *ped3* mutant lines have defect in degrading seed-reserved lipids during post-germinative growth. The amount of seed-reserved lipids remaining in a 5-day-old seedling grown under constant illumination (black bar) is compared with that in a dry seed (white bar). Wild-type Arabidopsis rapidly degrades seed-reserved lipids, while all *ped3* mutants (*ped3-1*, *ped3-2*, *ped3-3* and *ped3-4*) still contained larger amount of seed-reserved lipids 5 d after germination.

sucrose after they expanded green leaves on the growth medium containing sucrose. Therefore, they could grow and

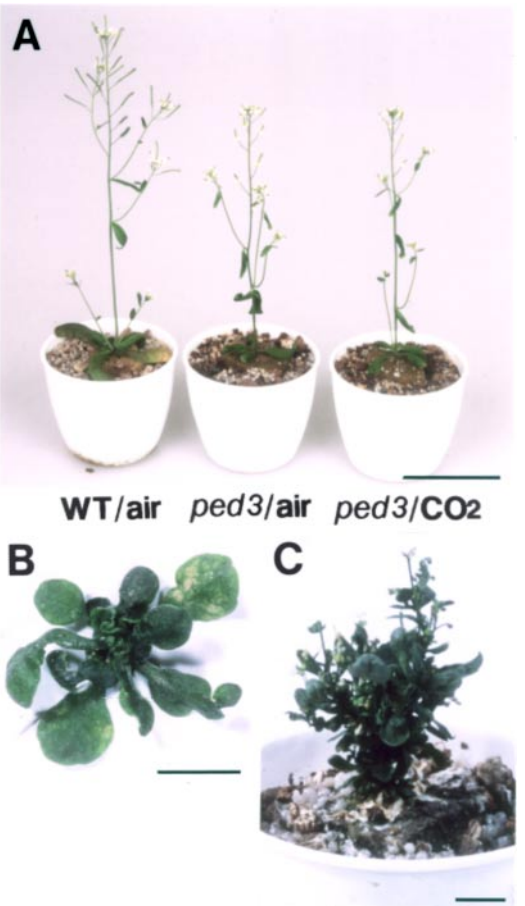
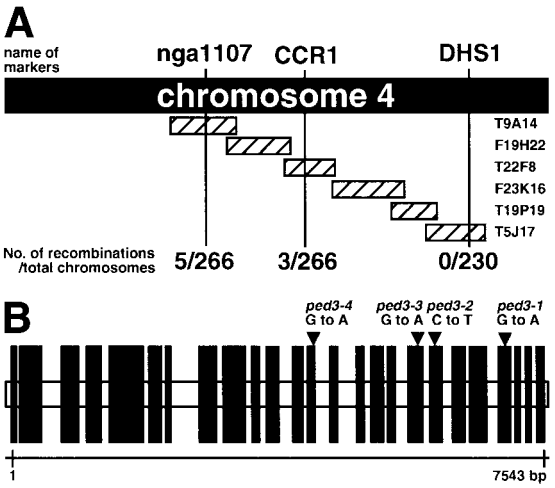


Fig. 2 Phenotypes of the *ped3* mutant and *ped1*/*ped3* double mutant. (A) Wild-type Arabidopsis (WT/air), and *ped3-2* mutant (ped3/air) were grown for 6 weeks in a normal atmosphere (36 Pa CO_2). The *ped3-2* mutant was also grown for 6 weeks in an atmosphere containing 1,000 Pa CO_2 (ped3/CO_2). Bar = 5 cm. (B) Rosette leaves of the *ped1*/*ped3* double mutant grown for 3 weeks. Bar = 1 cm. (C) *ped1*/*ped3* double mutant was grown for 6 weeks. Bar = 1 cm.

produce seeds on soil without supplying sucrose (Fig. 2A). *ped3* mutants grew equally under a normal atmosphere and an atmosphere enriched to 1% CO_2, suggesting that the *ped3* mutants do not have defects in leaf peroxisomal function (Fig. 2A). By contrast, the *ped2* mutant, that has a defect in the gene encoding the Arabidopsis ortholog of *PEX14*, has reduced activity of not only glyoxysomal function but also leaf peroxisomal function. Because of this defect, the *ped2* mutant showed a growth defect and has yellowish leaves in a normal atmosphere, but grew normally in a high CO_2 concentration (Hayashi et al. 2000).

To investigate the functional relationship between the *PED3* gene and fatty acid β-oxidation, we generated a *ped1*/*ped3* double mutant by outcrossing the *ped3-2* mutant to the *ped1* mutant that has a defect in 3-ketoacyl CoA thiolase. In the F3 generation, we identified 11 *ped1*/*ped3* double mutants. All these mutants showed similar vegetative and reproductive phe-



C

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MPSLQLLQLTERGRGLVASRRKSILLAAGIVAAGGTAVYL      40
KSRVASRRPDSSRLCNGQSDDDEALEKLTATDQNAKITTK      80
KKKGGGLKSLQVLTAILLSQMGKMGARDLLALVATVVFRT     120
ALSNRLAKVQGFLFRAAFLRRAPLFLRLISENIMLCFMLS     160
TLHSTSKYITGALSLRFRKILTKIIHSHYFENMVYYKISH     200
VDGRITHPEQRIASDVPRFSSELSDLILDDLTAVTDGILY     240
AWRLCSYASPKYIFWILAYVLGAGTAIRNFSPSFGKLMSK     280
EQQLEGEYRQLHSRLRTHSESIAFYGGETREESHIQQKFK     320
NLVSHMSHVLHDHWWFGMIQDFLLKYLGATVAVILIIEPF     360
FSGHLRPDDSTLGRAEMLSNIRYHTSVIISLFQALGTLSI     400
SSRRLNRLSGYADRIHELMAVSRELSGDDKSSFQRNRSRN     440
YLSEANYVEFSDVKVVTPTGNVLVEDLTLRVEQGSNLLIT     480
GPNGSGKSSLFRVLGGLWPLVSGHIVKPGVGSDLNKEIFY     520
VPQRPYMAVGTLRDQLIYPLTSGQESELLTEIGMVELLKN     560
VDLEYLLDRYQPEKEVNWGDELSLGEQQRLGMARLFYHKP     600
KFAILDECTSAVTTDMEERFAAKVRAMGTSCITISHRPAL     640
VAFHDVVLSLDGEGGWSVHYKRDDSALLTDAEIDSVKSSD     680
TDRQNDAMVVQRAFAAARKESATNSKAQSYQTQLIARSPV     720
VDKSVVLPRFPQPQTSQRALPSRVAAMLNVLIPTIFDKQG     760
AQLLAVACLVVSRTLISDRIASLNGTTVKYVLEQDKAAFV     800
RLIGLSVLQSGASSIIAPSLRHLTQRLALGWRIRLTQHLL     840
        (N; ped3-4)
RNYLRNNAFYKVFHMSGNSIDADQRLTRDLEKLTADLSGL     880
LTGMVKPSVDILWFTWRMKLLTGQRGVAILYTYMLLGLGF     920
LRRVAPDFGDLAGEEQQLEGKFRFMHERLNTHAESIAFFG     960
GGAREKAMVDKKFRALLDHSLMLLRKKWLYGILDDFVTKQ    1000
LPNNVIWGLSLLYALEHKGDRALVSTQGELAHALRYLASV    1040
    (X; ped3-3)                (W; ped3-2)
VSQSFMAFGDILELHKKFLELSGGINRIFELDEFLDASQS    1080
GVTSENQTSRLDSQDLLSFSEVDIITPAQKLMASKLSCEI    1120
VSGKSLLVTGPNGSGKTSVFRVLRDIWPTVCGRLTKPSLD    1160
IKELGSGNGMFFVPQRPYTCLGTLRDQIIYPLSKEEAEKR    1200
AAKLYTSGESSTEAGSILDSHLKTILENVRLVYLLERDVG    1240
GWDATTNWEDILSLGEQQRLGMARLFFHRPKFGVLDECTN    1280
(X; ped3-1)
ATSVDVEEQLYRVARDMGVTFITSSQRPALIPFHSLELRL    1320
IDGEGNWELRSIEQTTE                           1337
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D

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Ped3p  (298-312)   HSESIAFYGGETREE
Ped3p  (952-966)   HAESIAFFGGGARER
Pxa1p  (292-306)   NSEEIAFYQGTAVER
Pxa2p  (320-334)   SNEEIALLRGQKREL
PMP70 (275-289)    NSEEIAFYNGNKREK
ALDP   (289-303)   NSEEIAFYGGHEVEL
Consensus            E IA   G   E
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notypes. They had wavy leaves with irregular shapes (Fig. 2B). Influorescence of the double mutants was difficult to develop, but they occasionally had dwarf inflorescences with abnormal structure (Fig. 2C). Although the inflorescences had some flowers, they were sterile. These phenotypes were not found in their parents.

High-resolution mapping of PED3 locus

We outcrossed the *ped3-2* mutant that has a Landsberg *erecta* ecotype background to wild-type Arabidopsis, ecotype Columbia, and identified 133 F2 progenies that have homozygous *ped3-2* alleles for high-resolution mapping. These progenies were subsequently scored according to their genetic background with a series of molecular markers (Fig. 3A). The number of chromosomes that showed a Colombia background represents the number of recombinations that occurred between the *PED3* locus and the position of each molecular marker, since the genomic DNA of the *ped3* mutant has a Landsberg *erecta* background. As summarized in Fig. 3A, high-resolution mapping revealed that the *PED3* locus is located on the right side of CCR1. The nearest marker to the *PED3* locus is DHS1 that is known to contain a BAC clone, T5J17 (accession no. AL035708). T5J17 represents the terminal end sequence of the lower arm of chromosome 4 in the Arabidopsis genome sequencing project (http://www.arabidopsis.org/). We searched for nucleotide sequences of T5J17, as well as T19P19, F23K16 and T22F8. These BAC clones are known to represent the

Fig. 3 Positional cloning of the *PED3* gene. (A) High-resolution mapping of *PED3* on chromosome 4. Names and positions of the molecular markers used in this study are indicated at the top of the illustration. Hatched bars represent the regions covered by BAC clones. We analyzed 133 F_2 progenies (266 chromosomes) having homozygous *ped3-2* alleles. The numbers of recombinations that occurred between the *PED3* locus and the molecular markers are indicated at the bottom of the illustration. Mapping results with molecular markers, nga1107, CCR1 and DHS1 are summarized schematically and indicate that the *PED3* locus is most likely to be located within a single BAC clone, T5J17. (B) Schematic diagram of a 7,543-bp genomic DNA fragment that is involved in the BAC clone, T5J17. The twenty five black bars represent protein coding regions determined from the cDNA sequence. Four arrowheads on the black bars indicate single nucleotide substitutions that occur in *ped3-1*, *ped3-2*, *ped3-3* and *ped3-4* alleles. Nucleotide residue 1 corresponds to an adenine of the first methionine codon. (C) Amino acid sequence of Ped3p. Amino acid sequence of Ped3p deduced from the nucleotide sequence of the *PED3* cDNA is indicated as single letter codes. Underlines indicate ATP/GTP-binding site motif A (P-loop) (prosite accession no. PS00017), while two double underlines indicate ABC transporters family signature (prosite accession no. PS00211). ^{810}Ser, ^{1007}Trp, ^{1035}Arg and ^{1242}Trp are substituted to Asn in *ped3-4*, stop codon (X) in *ped3-3*, Trp in *ped3-2,* and stop codon (X) in *ped3-1*, respectively. (D) Alignment of EAA-like motifs found in peroxisomal ABC transporters. Amino acid sequences of Ped3p, *S. cerevisiae* Pxa1p and Pex2p, human PMP70 and ALDP are shown with the number of amino acid residues. Similar amino acids are boxed in black, and identical amino acids are indicated as consensus.

genomic sequence for the right side of CCR1 (Fig. 3A). Based on these nucleotide sequences, we designed a set of oligonucleotide primers that could amplify one of the predicted genes by using the polymerase chain reaction (PCR). This gene is located within the 7543-bp DNA fragment contained in T5J17 (Fig. 3B). DNA fragments corresponding to this region were amplified from genomic DNAs of wild-type Arabidopsis, *ped3-1*, *ped3-2*, *ped3-3* and *ped3-4* mutants using the same primer set, and were fully sequenced. The nucleotide sequences of these DNA fragments are identical except for single nucleotide substitutions from ^{6999}G in the wild-type plant to A in the *ped3-1* mutant, ^{5986}C to T in *ped3-2* mutant, ^{5805}G to A in *ped3-3* mutant, ^{4352}G to A in *ped3-4* mutant (Fig. 3B, arrowheads). These results strongly indicated that the 7,543-bp DNA fragment contained the *PED3* gene (accession No. AB070615).

PED3 gene encodes an ABC transporter

To deduce the amino acid sequence of the *PED3* gene product, Ped3p, we searched the Arabidopsis expressed sequence tag (EST) database (http://www.kazusa.or.jp/) for the nucleotide sequence of the *PED3* gene, and found an EST clone called APZL37d10 (GenBank accession no. AV523713). This clone contained a partial cDNA encoding a peptide starting from Phe^{336} to the carboxy-terminal end. Therefore, we generated the full-length cDNA using reverse transcriptase-PCR with total RNA isolated from wild-type plants. We determined the nucleotide sequence of the cDNA (accession No. AB070616). Comparison of the nucleotide sequences for the cDNA and genomic DNA revealed that the *PED3* gene contains at least 25 exons (Fig. 3B). The deduced amino acid sequence of the Ped3p is composed of 1,337 amino acid residues with calculated a molecular mass of 149.4 kDa (Fig. 3C). Two alleles, *ped3-1* and *ped3-3*, have a nonsense mutation at the codon encoding ^{1242}Trp and ^{1007}Trp, respectively, while other alleles, *ped3-2* and *ped3-4*, have an amino acid substitution of ^{1035}Arg to Trp, and ^{810}Ser to Asn, respectively (Fig. 3C). It is worth emphasizing that germination of *ped3-1* and *ped3-3* embryos, that have a nonsense mutation in *PED3* locus, was the most severely inhibited, while *ped3-2* and *ped3-4* seedlings, that have a missense mutation at the *PED3* locus, showed milder phenotypes on the growth medium without sucrose (Fig. 3C).

Analyses of the amino acid sequence revealed that Ped3p could be divided into two parts at around ^{668}Cys. The amino acid sequences of these repeat sequences were 32% identical. In addition, each repeat has significant similarity with peroxisomal membrane proteins identified in mammals and yeast, such as PMP70, ALDP, and Pxa1p. The highest similarity was found in Rat PMP70 (Fig. 4A). Each repeat was further divided into two regions. One corresponds to the amino-terminal two thirds of the repeat (Fig. 4B, C; thick bars), while the other corresponds to the rest (Fig. 4B, C; open boxes). The former region is highly hydrophobic, and contains approximately six putative membrane-spanning segments. It also contains an

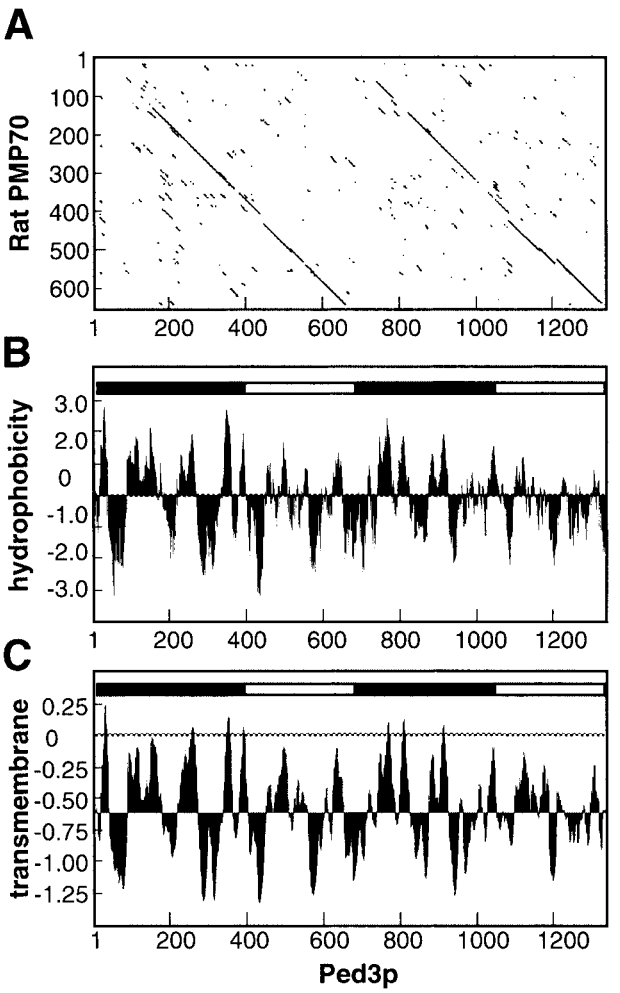

Fig. 4 Characterization of amino acid sequence of Ped3p. (A) Graphical comparison between amino acid sequences of Ped3p (vertical line) and rat PMP70 (horizontal line). A dot represents a stretch of 30 amino acids that aligned at least 11 similar amino acids. Numbers on each axis represent numbers of amino acid residues. (B) Hydropathy profile of Ped3p. The spots are generated using the algorithm of Kyte and Doolittle with a window size of 16 amino acid residues. Thick bars and open boxes in the panel represent two hydrophobic regions containing putative transmembrane segments, and two hydrophilic regions containing ATP-binding site, respectively. (C) Prediction of transmembrane helices in Ped3p. The spots are generated using the algorithm of von Heijne. Thick bars and open boxes in the panel represent two hydrophobic regions containing putative transmembrane segments, and two hydrophilic regions containing ATP-binding site, respectively.

EAA-like motif highly conserved among peroxisomal ABC transporters (Fig. 4D). EAA-like motif is similar in sequence and location to a motif previously described only in prokaryotic ABC transporters known as the EAA motif (Shani et al. 1996). Two missense and two single codon deletions were found within the EAA-like motif of ALDP (^{291}Glu) in four unrelated X-linked adrenoleukodystrophy patients. It has also been demonstrated that the yeast possessing missense mutation

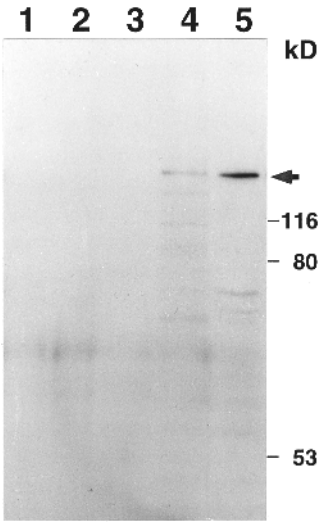

Fig. 5 Immunodetection of Ped3p in etiolated cotyledons of wild-type Arabidopsis and the *ped3* mutants. Extracts were prepared from etiolated cotyledons of *ped3-1* (line 1), *ped3-2* (lane 2), *ped3-3* (lane 3), *ped3-4* (lane 4), and wild-type Arabidopsis (lane 5). For each sample, 10 μg of total protein was subjected to SDS-PAGE. Immunoblot analysis was performed using an antibody raised against Ped3p. Arrow indicates the position of Ped3p. Positions of molecular markers are shown on the right with molecular masses in kDa.

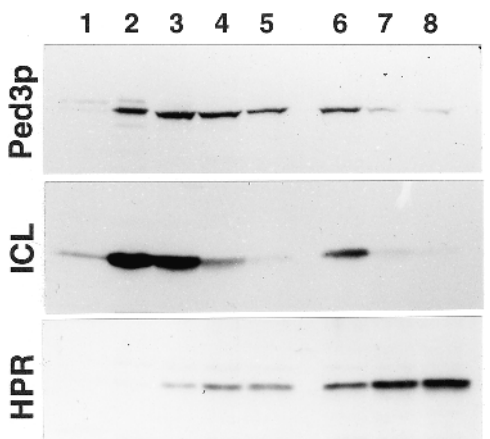

Fig. 6 Developmental changes of the Ped3p. The samples contained in each lane were prepared from etiolated cotyledons grown for 1 d in the dark (lane 1), 3 d in the dark (lane 2), 5 d in the dark (lane 3), 7 d in the dark (lane 4), 9 d in the dark. Green cotyledons obtained from the seedlings grown 4 d in the dark followed by 1 d in the light (lane 6), 4 d in the dark followed by 3 d in the light (lane 7), 4 d in the dark followed by 5 d in the light (lane 8) were also analyzed. For each sample, 10 μg of total protein prepared from cotyledons was subjected to SDS-PAGE. Immunoblot analyses were performed using the antibodies raised against Ped3p, isocitrate lyase (ICL) and hydroxypyruvate reductase (HPR).

in the EAA-like motif of PXA1 (^{294}Glu and ^{301}Gly) could not grow on medium containing 0.1% oleic acid as a sole carbon source, and had reduced fatty acid β-oxidation activity. These results indicated that the EAA-like motif is important for the

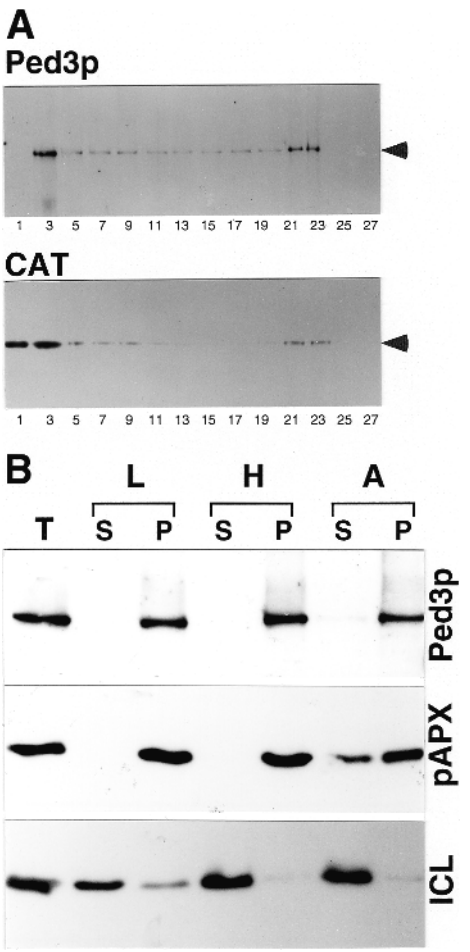

Fig. 7 Subcellular localization of Ped3p. (A) Subcellular fractionation of Arabidopsis etiolated cotyledons was performed by 30–60% sucrose density gradient centrifugation. Fraction number 1 represents the top fraction of the gradient. Ped3p and catalase in each fraction were detected by immunoblot analyses using antibodies raised against Ped3p and catalase (CAT). (B) Intact glyoxysomes isolated from pumpkin etiolated cotyledons were resuspended in either low salt buffer (L), high salt buffer (H) or alkaline solution (A). Each buffer consists of 10 mM HEPES-KOH (pH 7.2), 500 mM KCl with 10 mM HEPES-KOH (pH 7.2), and 0.1 M Na_2CO_3, respectively. These samples were then centrifuged, and separated into soluble (S) and insoluble (P) fractions. T represents total proteins of the intact glyoxysomes. Ped3p, peroxisomal ascorbate peroxidase (pAPX) and isocitrate lyase (ICL) were detected by immunoblot analysis.

function of peroxisomal ABC transporters, although the precise function of the EAA-like motif in peroxisomal ABC transporters remains to be determined. From these observation, conserved amino acid residues in the EAA-like motif of Ped3p, such as ^{300}Glu, ^{954}Glu, ^{307}Gly and ^{961}Gly, seemed to be important for its function as a peroxisomal ABC transporter. The latter region contains an ATP-binding site (Fig. 3C). It is hydrophilic, and may exist in cytosolic surface of the peroxisomal membrane (Fig. 4B).

Ped3p is abundant during post-germinative growth

To analyze Ped3p, a *PED3* gene product, we prepared an antiserum raised against a fusion protein containing a partial amino acid sequence of Ped3p (^{384}His-^{780}Ile). This antiserum recognized an ~149-kD protein in the wild-type plant (Fig. 5; lane 5), while no cross-reactive band that exhibited the comparable intensity was detected in the *ped3* mutants (Fig. 5; lanes 1–4). These data indicate that Ped3p is the 149-kDa protein.

Immunoblot analyses revealed that the amount of Ped3p in Arabidopsis etiolated cotyledons increased until 5 d after germination, and then declined during seedling growth under constant darkness (Fig. 6; Ped3p, lanes 1–5). The amount of Ped3p in the seedlings germinated in the dark decreased rapidly by illumination from the 4th day after germination (Fig. 6; Ped3p, lanes 6–8). The appearance and disappearance of Ped3p during post-germinative growth of the wild-type plant is similar to that of other glyoxysomal matrix proteins such as isocitrate lyase (Fig. 6; ICL), and different from leaf peroxisomal enzymes, such as hydroxypyruvate reductase (Fig. 6; HPR).

Ped3p is a glyoxysomal integral membrane protein

To investigate the subcellular localization of the Ped3p in wild-type plant cells, we subjected homogenates prepared from etiolated Arabidopsis cotyledons to sucrose density gradient centrifugation. Fractions thus obtained were analyzed using an immunoblot technique with an antibody raised against Ped3p. The 149-kDa protein was detected in fractions 21–23, whose densities were 1.25 g cm^{-3} (Fig. 7A). These fractions also contained other glyoxysomal marker enzymes, such as catalase (Fig. 7A) and isocitrate lyase (data not shown).

Fig. 7B represents the result of the extensive subfractionation studies performed by the treatment of intact glyoxysomes isolated from etiolated pumpkin cotyledons with various solutions. Isocitrate lyase, a marker enzyme for glyoxysomal matrix, was completely dissolved both in high-salt buffer and alkaline solution. By contrast, Ped3p were found only in the insoluble fraction even after the treatment with alkaline solution. This suggests the tight integration of Ped3p into the glyoxysomal membrane, since ascorbate peroxidase, a marker enzyme for peroxisomal membranes (Yamaguchi et al. 1995, Bunkelmann and Trelease 1996, Nito et al. 2001), was detected both in soluble and insoluble fractions after the treatment with an alkaline solution.

Morphology of glyoxysomes in the ped3 mutant

Fig. 8 shows an electron microscopic analysis of glyoxysomes in wild-type plants and the *ped3* mutants. As shown in Fig. 8A, glyoxysomes found in the 5-day-old etiolated cotyledons of wild-type plants are approximately 0.5 μm in diame-

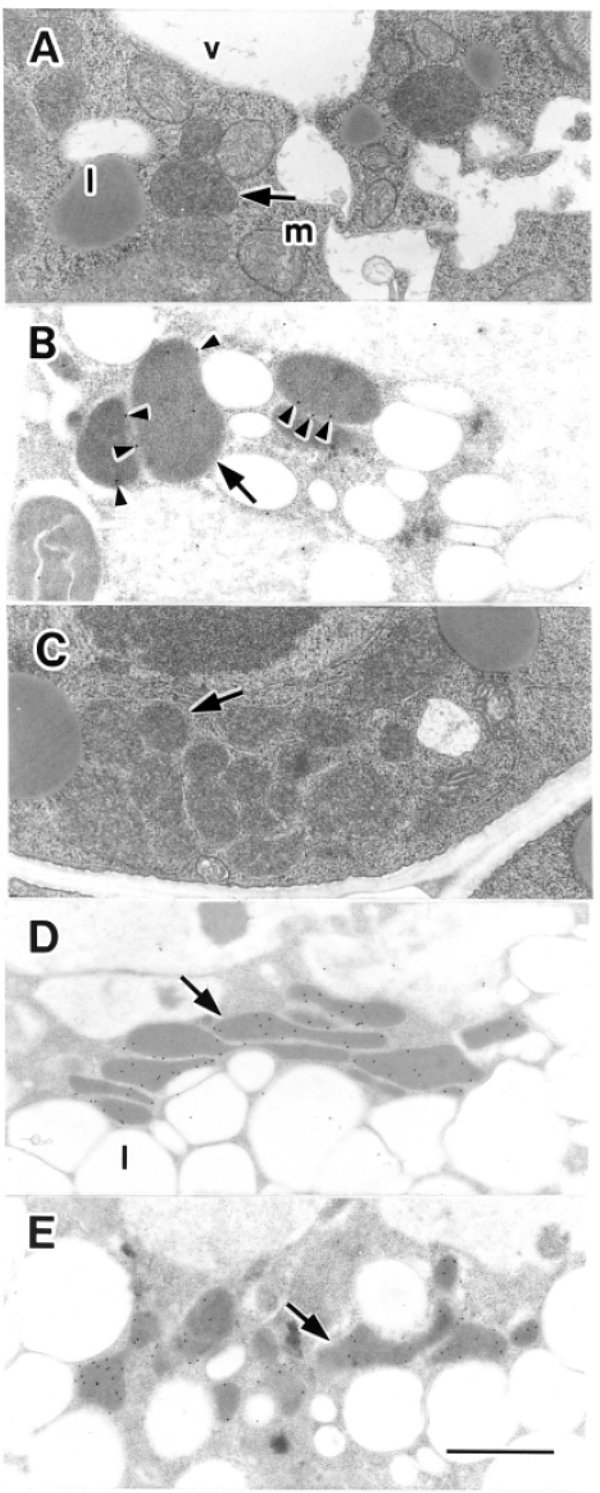

Fig. 8 Electron microscopic analyses of glyoxysomes in the cells of 5-day-old etiolated cotyledons. (A) A cotyledonary cell of wild-type plant. (B) Immunogold labeling of wild-type plant using an antibody raised against Ped3p. (C) A cotyledonary cell of *ped3-2*. (D) Immunogold labeling of *ped3-2* using an antibody raised against 3-ketoacyl CoA thiolase. (E) Immunogold labeling of *ped3-2* using an antibody raised against isocitrate lyase. Arrowhead, peroxisome; m, mitochondrion; l, lipid body. Bar in (D) = 1 μm. Magnification of (A) to (E) is the same.

ter, and have a round or oval shape containing a uniform matrix. When the cells were immunogold-labeled using antibodies raised against Ped3p, the gold particles were mostly localized on the glyoxysomal membrane (Fig. 8B, arrowheads). Glyoxysomes in the *ped3* mutant are morphologically normal (Fig. 8C), while other 2,4DB-resistant mutants, such as *ped1* and *ped2* have glyoxysomes with abnormal structures (Hayashi et al. 1998b, Hayashi et al. 2000, Hayashi et al. 2001). The glyoxysomes in the *ped3* mutants contain detectable amounts of enzymes for fatty acid β-oxidation and glyoxylate cycle, such as 3-ketoacyl CoA thiolase (Fig. 8D) and isocitrate lyase (Fig. 8E), respectively.

Discussion

The chemically induced *ped3* mutant lines belong to a series of *A. thaliana* mutants that has defects in peroxisomal fatty acid β-oxidation. Positional cloning and subsequent analyses revealed that the *PED3* gene encodes a 149-kDa glyoxysomal membrane protein that possesses a typical characteristic of ABC transporter. Present data indicated that two *ped3* alleles, *ped3-1* and *ped3-3*, with nonsense mutations in *PED3* locus showed a stronger defect than the rest of two alleles, *ped3-2* and *ped3-4*, that have missense mutations during postgerminative growth without supplemental sucrose (Fig. 1B, 3C). In addition, analyses of the Arabidopsis genome sequence near the end of the lower arm of chromosome 4 revealed that there are no putative genes encoding a polypeptide that has similarity to the known enzymes for fatty acid β-oxidation, such as acyl CoA synthetase, acyl CoA oxidase, the bifunctional enzyme, 3-ketoacyl CoA thiolase (http://pedant.mips.biochem.mpg.de/, data not shown). These data strongly support the conclusion that the loss of the 149-kDa peroxisomal ABC transporter reduces the activity for fatty acid β-oxidation in the *ped3* mutants.

Many ABC transporters have been identified to date, in taxa ranging from bacteria to higher organisms. These proteins have been known to transport a variety of substrates across various membranes by utilizing the energy of ATP hydrolysis, and are grouped into the ABC superfamily (Higgins 1992). The designation of ABC transporters recognizes a highly conserved ATP-binding cassette, which is the most characteristic feature of this superfamily. The typical transporter consists of two copies each of two structural units. One of these domains is highly hydrophobic and each consists of six membrane-spanning segments. These domains form a pathway through which the substrate crosses the membrane and are believed to determine the substrate specificity of the transporter. The other domains are peripherally located at the cytoplasmic face of the membrane, bind ATP, and couple ATP hydrolysis to the transport process. The domain organization of ABC transporters is various. The most frequent arrangement is four domains fused in a single polypeptide, referred to herein as "full-size" ABC transporter. However, there are many ABC transporters that are expressed as separate subunits, which contains a single repeat sequence or a single domain.

Several peroxisomal ABC transporters in mammalian and yeast cells have been identified: PMP70, ALDP, P70R, ALDR, *PXA1* and *PXA2* (Shani and Valle 1998). All these proteins have a molecular mass near 70 kDa, and contain one transmembrane domain and one ATP-binding region. Because of this unique structure, these proteins are often called peroxisomal "half" ABC transporters. By contrast, analysis of the amino acid sequence revealed that Ped3p is grouped into the "full-size" ABC transporter in spite of the fact that it exists in peroxisomal membranes. Ped3p is the first "full-size" ABC transporter so far identified in peroxisomal membranes. A BLAST search failed to reveal an Arabidopsis "half" ABC transporter that is more similar to rat PMP70 than Ped3p. We also failed to find a peroxisomal candidate for "full-size" ABC transporter in other organisms, including the yeast genome, which has sequence similarity to Ped3p. Therefore, it is possible that the domain structure of the peroxisomal ABC transporters varies depending on the organisms. Since the ABC transporters are thought to have a common evolutionary origin (Ames and Higgins 1983, Ames 1986), the difference of the domain structure in peroxisomal ABC transporters may reflect the evolution of peroxisomes. Since peroxisomal ABC transporters have been identified from only a limited number of organisms at present, further identification and detailed structural comparison of the peroxisomal ABC transporters from various organisms may help to clarify the evolution of the peroxisomes.

A human peroxisomal disorder, X-linked adrenoleukodystrophy is known to be induced by a defect in the peroxisomal half ABC transporter, ALDP. This genetic disease is characterized by the accumulation of very long-chain fatty acids (VLCFA) in serum due to a decreased peroxisomal VLCFA β-oxidation capacity. Because of its characteristic of ABC transporter and symptoms of the disease as well as the existence of VLCFA CoA synthetase activity on the cytoplasmic side of the peroxisomal membrane, ALDP is anticipated to be involved in the transport of CoA-esterified VLCFA across the peroxisomal membrane (Shani and Valle 1998). In yeast, two peroxisomal ABC transporters, Pxa1p and Pxa2p, form a heterodimer (Shani and Valle 1996), and are involved in the transport of CoA-esterified VLCFA (Hettema et al. 1996, Verleur et al. 1997). By inference, the Ped3p may function as a transporter of either fatty acids or CoA-esterified fatty acids across the peroxisomal membrane in higher plants. In the life cycle of the higher plants, fatty acids are most actively produced from seed-reserved lipids deposited in lipid bodies during post-germinative growth of the seedlings, when peroxisomes are functionally differentiated into glyoxysomes. These fatty acids or CoA-esterified fatty acids may be imported into the glyoxysomes by the action of Ped3p. The loss of Ped3p prevents the import of fatty acids into glyoxysomes, and subsequent gluconeogenesis that is necessary for the growth of the seedlings. The observed

phenotypes in the *ped3* mutants support this conclusion.

This assumption is also supported by the morphological difference of glyoxysomes in the *ped3* and *ped1* mutants. It has been known that glyoxysomes in the *ped1* mutant become enlarged organelles with abnormal structure (Hayashi et al. 1998b, Hayashi et al. 2001). Although fatty acids are imported into the glyoxysomes, the loss of 3-ketoacyl-CoA thiolase may induce the enlargement of the glyoxysomes by the accumulation of a metabolic intermediate for fatty acid β-oxidation. A similar phenomenon is induced by a deletion of another enzyme for fatty acid β-oxidation, the multifunctional enzyme, in yeast *Yarrowia lipolytica* (Smith et al. 2000). By contrast, glyoxysomes in *ped3* mutants did not show any morphological defect. One possible interpretation is that import the incompetency of fatty acids prevents subsequent metabolism, but does not affect the environment inside the glyoxysomes of the *ped3* mutant.

The *ped3* mutant showed not only a defect in fatty acid β-oxidation but also resistance to a toxic level of 2,4DB, that possesses the butyric side chain within the molecule. This result suggests that Ped3p transports 2,4DB into glyoxysomes, and may function as a transporter with broad substrate specificity from long-chain fatty acids to short-chain fatty acids, and their derivatives. The vegetative and reproductive phenotypes observed only in the *ped1*/*ped3* double mutant may support this idea. Future analyses of the substrate specificity may help to understand the function of Ped3p.

Materials and Methods

Plant materials and growth conditions

Identification of *ped3* mutant lines of *A. thaliana* has been previously described (Hayashi et al. 1998b). Progenies that had been backcrossed three times were used in this study. Arabidopsis ecotype Landsberg *erecta* was used as the wild-type plant. All seeds were surface sterilized in 2% NaClO, 0.02% Triton X-100, and germinated on growth media (2.3 mg ml^{-1} MS salt (Wako, Osaka, Japan), 1% sucrose, 100 μg ml^{-1} myo-inositol, 1 μg ml^{-1} thiamine-HCl, 0.5 μg ml^{-1} pyridoxine, 0.5 μg ml^{-1} nicotinic acid, 0.5 mg ml^{-1} MES-KOH (pH 5.7), 0.8% agar. Seedlings grown for 2 weeks on the growth medium were transferred into a 1 : 1 mixture of perlite and vermiculite. Plants were grown under a 16-h-light (100 μE m^{-2} s^{-1})/8-h-dark cycle at 22°C.

Quantitative analyses of total triacylglycerols

Amount of seed-reserved lipids contained in dry seeds and 5-day-old etiolated seedlings was measured as the amount of total triacylglycerol by using the assay kit, TRIGRYLCERIDE-TEST (Wako, Osaka, Japan). Either 10 dry seeds or 10 seedlings were homogenized in a mortar in 200 μl of water. Homogenates obtained were mixed with 3 ml isopropanol. Free glycerol and other compounds showing a similar color reaction in the sample was removed by an absorbent accompanying with the kit. The amount of triacylglycerols in the sample was measured according to the manufacturer's protocol. The amount of seed-reserved lipids was calculated as the amount of triolein.

Isolation of ped1/ped3 double mutant

The *ped3-2* mutant was outcrossed to *ped1* mutant. F2 progenies, obtained by self-fertilization of the F1 plants, were germinated on growth medium without sucrose. Seedlings that could not grow without sucrose were recovered after transferring these seedlings to medium containing sucrose. The genomic DNA of these F2 plants was individually isolated. The DNA fragment contained in the *PED1* locus was amplified by PCR using the 5′ primer (TGCTCCTGCCTTGAGACACC) and the 3′ primer (CTGCATATCAGAGGACCTCT). The polymorphism between *PED1* and *ped1* alleles was detected by the digestion of the DNA fragment with a restriction enzyme, *Nla*III. We identified one line that had heterozygous *PED1* allele (*PED1*/*ped1*) and homozygous *PED3* allele (*ped3-2*/*ped3-2*), and obtained F3 progenies by self-fertilization. We analyzed the allele at the *PED1* locus of the F3 progenies by the same procedure, and identified the *ped1*/*ped3* double mutant (*ped1*/*ped1*, *ped3-2*/*ped3-2*).

High-resolution mapping of PED3 locus

The *ped3-2* mutant was outcrossed to the wild-type plant (ecotype Columbia [Col-0]). F2 progenies, obtained by self-fertilization of the F1 plants, were germinated on growth medium without sucrose. One hundred thirty three seedlings that could not grow without sucrose recovered growth after they were transferred to medium containing sucrose. The genomic DNA of these F2 plants was individually isolated. Recombinations that occurred between the *PED3* locus and the molecular markers were scored by using the cleaved amplified polymorphic sequence (CAPS) mapping procedure (Konieczny and Ausubel 1993) and simple sequence length polymorphisms (SSLP) mapping procedure described previously (Bell and Ecker 1994).

Sequencing analyses

DNA and RNA extraction, sequence determination, and routine molecular biological techniques were performed by standard techniques (Sambrook et al. 1989). For identification of the *PED3* gene, the DNA fragments were amplified by the polymerase chain reaction (PCR) using 100 ng of genomic DNAs isolated from wild-type plants, *ped3-1*, *ped3-2*, *ped3-3* and *ped3-4* as templates, together with a 5′ primer (TACTCAATTCCAGGCCATGC), and a 3′ primer (TCACTCTGTTGTCTGTTCGATCGAACGG). A *PED3* cDNA clone was generated by reverse transcriptase-PCR with total RNA isolated from 7-day-old cotyledons of wild-type plants using the same primer set. The nucleotide sequences of those DNA fragments were analyzed as reported previously (Hayashi et al. 1998b). DNA and amino acid sequences were analyzed using MacVector and GCG Wisconsin package (Oxford Molecular Group, Inc., Oxford, U.K.).

Immunoblotting

A DNA fragment encoding from ^{384}His to ^{780}Ile of Ped3p was amplified from the *PED3* cDNA by PCR. The amplified DNA was inserted into the pET32 vector (Novagen, Madison, WI, U.S.A.). A fusion protein was synthesized in *Escherichia coli* cells, and used for the production of rabbit antibody raised against Ped3p according to the method previously reported (Hayashi et al. 1999). We also used antibodies raised against caster bean isocitrate lyase (Maeshima et al. 1988), spinach hydroxypyruvate reductase (Mano et al. 1999), pumpkin catalase (Yamaguchi and Nishimura 1984), pumpkin ascorbate peroxidase (Yamaguchi et al. 1995) and pumpkin 3-ketoacyl CoA thiolase (Kato et al. 1996). Immunoblots were analyzed according to protocols described previously (Hayashi et al. 1998a).

Subcellular fractionation and analyses of the intact glyoxysomes

One hundred mg of Arabidopsis seeds were grown on growth medium for 5 d in darkness at 22°C. Subcellular fractionation of 5-day-old etiolated cotyledons was performed using a 30–60% (w/w) sucrose density gradient centrifugation as previously reported (Hayashi et al. 1998b). After the centrifugation, 0.5 ml fractions were col-

lected, and used for immunoblot analyses.

Isolation of intact glyoxysomes from 5-day-old pumpkin etiolated cotyledons (100 g FW) by Percoll density gradient centrifugation has been also reported previously (Hayashi et al. 2000). The intact glyoxysomes (250 μg total protein) were resuspended in 200 μl of either low salt buffer, high salt buffer or alkaline solution. Each solution consisted of 10 mM HEPES-KOH (pH 7.2), 500 mM KCl with 10 mM HEPES-KOH (pH 7.2), and 0.1 M Na_2CO_3 (pH 11), respectively. After centrifugation at 100,000×*g* for 30 min, these samples were separated into the supernatants and the pellets. The pellets were resuspended in 200 μl of 100 mM HEPES-KOH (pH 7.2).

Electron microscopic analysis

Etiolated cotyledons were harvested from plants that were grown for 5 d in the dark. The ultrathin sections, the microscopic analysis and immunogold-labeling technique were prepared according to the protocol described previously (Hayashi et al. 1998b).

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Note Added in Proof

During the reviewing process, the same gene was reported by Zolman et al. (2001) *Plant Physiol*. 127: 1266–1278. The authors designated the gene as *PXA1*.

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