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2,4-Dichlorophenoxybutyric Acid–Resistant Mutants of Arabidopsis Have Defects in Glyoxysomal Fatty Acid β-Oxidation

Makoto Hayashi, Kanako Toriyama, Maki Kondo, and Mikio Nishimura[1]

Department of Cell Biology, National Institute for Basic Biology, Okazaki 444, Japan

It has been demonstrated previously that 2,4-dichlorophenoxybutyric acid (2,4-DB) is metabolized to produce a herbicide, 2,4-D, by the action of peroxisomal fatty acid β-oxidation in higher plants. To isolate mutants that have defects in peroxisomal fatty acid β-oxidation, we screened mutant lines of Arabidopsis seedlings for growth in the presence of toxic levels of 2,4-DB. Twelve of the mutants survived; of these, four required sucrose for postgerminative growth. This result suggests that these mutants have defects in peroxisomal fatty acid β-oxidation, because peroxisomal fatty acid β-oxidation plays an important role in producing sucrose from storage lipids during germination. Genetic analysis revealed that these 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 *ped1* mutant lacks the thiolase protein, an enzyme involved in fatty acid β-oxidation during germination and subsequent seedling growth, whereas the *ped2* mutant has a defect in the intracellular transport of thiolase from the cytosol to glyoxysomes. Etiolated cotyledons of both *ped1* and *ped2* mutants have glyoxysomes with abnormal morphology.

INTRODUCTION

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 (Beevers, 1982). The metabolic pathway involves many enzymes in several subcellular compartments, including lipid bodies, glyoxysomes, mitochondria, and the cytosol. Within the entire gluconeogenic pathway, the conversion of fatty acid to succinate takes place within the glyoxysomes, which contain enzymes for fatty acid β-oxidation and the glyoxylate cycle.

In the glyoxysomes, fatty acids are first activated to fatty acyl CoA by fatty acyl CoA synthetase (Huang et al., 1983). Fatty acyl CoA is the substrate for fatty acid β-oxidation, which consists of four enzymatic reactions (Kindl, 1993). 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-hydroxyacyl CoA dehydrogenase activities (Preisig-Muller et al., 1994). The fourth reaction is catalyzed by 3-ketoacyl CoA thiolase (referred to herein simply as thiolase) (Preisig-Muller and Kindl, 1993; Kato et al., 1996b). Acetyl CoA, an end product of fatty acid β-oxidation, is metabolized further to produce succinate by the action of five enzymes of the glyoxylate cycle involving isocitrate lyase.

[1]To whom correspondence should be addressed. E-mail mikosome @nibb.ac.jp; fax 81-564-55-7505.

The existence of fatty acid β-oxidation in plant cells was first elucidated by demonstrating the plant growth-regulating activities of a homologous series of 2,4-dichlorophenoxyalkylcarboxylic acids (Wain and Wightman, 1954). It has been demonstrated clearly that an odd number of aliphatic side chain methylene groups ($n = 3$, 5, and 7) in 2,4-dichlorophenoxyalkylcarboxylic acids are degraded to produce 2,4-D and show growth-regulating activity. From these results, the authors concluded that the aliphatic side chain of these acids is degraded by fatty acid β-oxidation (Wain and Wightman, 1954).

Glyoxysomes and leaf peroxisomes are members of a group of organelles called peroxisomes (Beevers, 1979). During seedling growth, glyoxysomes and leaf peroxisomes in cotyledonary cells are interconverted. Glyoxysomes in etiolated cotyledons are transformed directly to leaf peroxisomes, which play a crucial role in photorespiration in combination with chloroplasts and mitochondria during the greening of cotyledons (Titus and Becker, 1985; Nishimura et al., 1986). During this process, glyoxysomal enzymes, such as malate synthase and isocitrate lyase, are specifically degraded (Mori and Nishimura, 1989), and leaf peroxisomal enzymes, such as glycolate oxidase and hydroxypyruvate reductase, are newly synthesized and transported into the organelle. Leaf peroxisomes in green cotyledons are subsequently converted to glyoxysomes when the cotyledons undergo senescence (De Bellis and Nishimura, 1991; Nishimura

et al., 1993). It has been suggested that the functional conversion between glyoxysomes and leaf peroxisomes is controlled by gene expression, protein translocation, and protein degradation, although the detailed mechanisms underlying these processes still need to be clarified (Nishimura et al., 1996).

A genetic approach may be an effective strategy toward understanding the regulatory mechanism(s) of peroxisomal function at the level of gene expression, protein translocation, and protein degradation. In this study, we describe the isolation and characterization of 2,4-dichlorophenoxybutyric acid (2,4-DB)–resistant mutants. Based on these data, we have identified the *ped1*, *ped2*, and *ped3* loci (where *ped* stands for peroxisome defective), which are necessary to maintain glyoxysomal function in plant cells.

RESULTS

Identification of 2,4-DB–Resistant Mutants

It has been demonstrated that 2,4-DB is metabolized to produce 2,4-D by the action of peroxisomal fatty acid β-oxidation (Wain and Wightman, 1954). Because 2,4-D is known to inhibit root elongation of Arabidopsis at an early stage of seedling growth (Estelle and Somerville, 1987), we assumed that 2,4-DB also affects the root elongation of wild-type Arabidopsis by its conversion to 2,4-D and that mutants that have defects in peroxisomal fatty acid β-oxidation become resistant to 2,4-DB.

On the basis of this hypothesis, we isolated Arabidopsis mutants showing resistance specifically to 2,4-DB but not to 2,4-D. When wild-type Arabidopsis seeds were placed on growth media containing 0.2 μg/mL 2,4-DB, the seedlings emerged from the seed coat and accumulated chlorophyll to the same extent as occurred in the absence of 2,4-DB, but the roots of the seedlings did not elongate properly (Figure 1, WT). A similar inhibition of root elongation was observed when wild-type Arabidopsis seedlings were germinated on growth media containing 0.05 μg/mL 2,4-D. In contrast, roots of *aux1-7*, a mutant resistant to 2,4-D (Pickett et al., 1990), elongated both in the presence of 0.2 μg/mL 2,4-DB and 0.05 μg/mL 2,4-D (Figure 1, AUX1-7). To identify mutants resistant to 2,4-DB but sensitive to 2,4-D, we distributed 70,000 M_2 seeds onto plates containing growth media supplemented with 0.2 μg/mL 2,4-DB. Plants that showed significant root elongation on the selective media were recovered and grown on soil until they produced M_3 seeds. The resistance of the M_3 seedlings to 0.2 μg/mL 2,4-DB and 0.05 μg/mL 2,4-D during germination and growth was analyzed. Many of the progenies showed 2,4-D resistance because they had significant root elongation in the presence of 2,4-D as well as 2,4-DB. For some of them, however, such as LR40 (LR; long root in the presence of 2,4-DB), root elongation was inhibited by 2,4-D, whereas these mutants had elongated roots in the presence of 2,4-DB (Figure 1, LR40).

Based on these criteria, we isolated 12 Arabidopsis mutants (LR11, LR24, LR27, LR40, LR43, LR47, LR53, LR77, LR81, LR91, LR92, and LR98) that showed resistance specifically to 2,4-DB. As shown in Figure 2, root elongation of these mutants was inhibited to various extents by 2,4-DB. LR40, LR43, and LR81 showed the greatest resistance to 2,4-DB. Roots of these mutants elongated to similar lengths in the presence and absence of 2,4-DB. Root elongation of other mutants, however, was inhibited by 57 to 82% in the presence of 2,4-DB.

Effect of Sucrose on Germination of 2,4-DB–Resistant Mutants

Because 2,4-DB is known to be metabolized to produce 2,4-D by fatty acid β-oxidation, we assumed that these 12

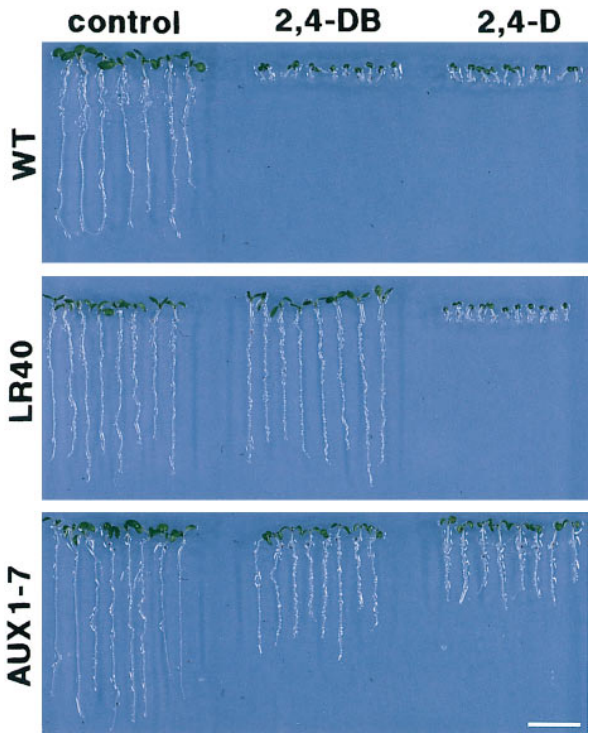

Figure 1. Effects of 2,4-DB and 2,4-D on the Growth of Mutants and Wild-Type Seedlings.

Wild-type Arabidopsis (WT), LR40, and the 2,4-D–resistant mutant (AUX1-7) were grown for 7 days on growth medium (control), growth medium containing 0.2 μg/mL 2,4-DB (2,4-DB), or growth medium containing 0.05 μg/mL 2,4-D (2,4-D) under constant illumination. Photographs were taken after the seedlings were removed from the media and rearranged on agar plates. Bar = 1 cm.

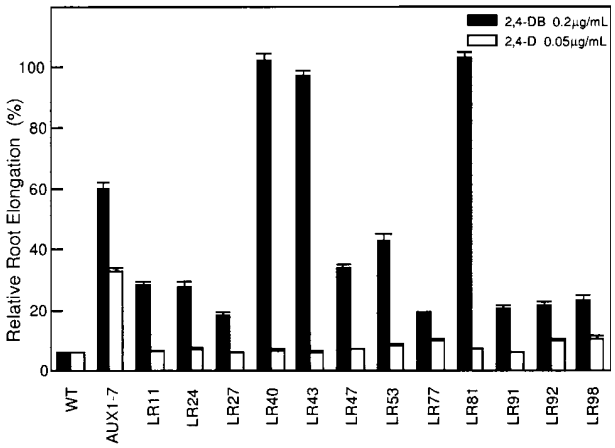

Figure 2. Root Elongation of Mutants and Wild-Type Seedlings in the Presence of 2,4-DB or 2,4-D.

Seedlings were grown for 7 days on growth medium containing 0.2 μg/mL 2,4-DB (solid bars) or 0.05 μg/mL 2,4-D (open bars) under constant illumination. Seedlings were also grown for 7 days on growth medium as a control. Roots of seedlings on each plate were measured. The effect of 2,4-DB and 2,4-D on root elongation was expressed relative to the mean root elongation of the same genotype on growth medium. Each value represents the mean of measurements of at least 10 seedlings ±SE. WT, wild type.

mutants would be good candidates for mutants that are defective in glyoxysomal fatty acid β-oxidation. To determine the function of glyoxysomes in these candidates, we examined the effect of sucrose on their growth because defects in fatty acid β-oxidation seem to inhibit the conversion of seed storage lipids into sucrose that is required for heterotrophic growth.

As shown in Figure 3 (WT), wild-type Arabidopsis seedlings germinated and grew normally, regardless of the presence or absence of sucrose in the growth medium. Most of the 2,4-DB–resistant mutants grew on the growth medium without sucrose as well as the wild-type plants did. However, four of the mutants—LR40, LR43, LR47, and LR81—could expand their green cotyledons and leaves only when sucrose was supplied to the growth medium (Figure 3, LR40, LR43, LR47, and LR81). The inhibitory effects on germination and postgerminative growth varied depending on the mutants. Germination of LR43 embryos was the most severely inhibited, and they never emerged from their seed coats on growth medium without sucrose. In contrast, LR40, LR47, and LR81 seedlings emerged from their seed coats but could grow no further. Roots of these mutants did not elongate, and their leaves did not develop. The cotyledons of LR40 and LR81 did not accumulate chlorophyll, whereas the cotyledons of LR47 accumulated low levels of chlorophyll. Despite the requirement of sucrose for germination and postgerminative growth, these four mutants (LR40, LR43, LR47, and LR81) did not require sucrose after they expanded green leaves on the growth medium containing sucrose. Therefore, they could be grown and have seeds on soil without supplying sucrose. Although LR47 showed a weak dwarf phenotype, no other obvious vegetative or reproductive phenotype was observed in these mutants.

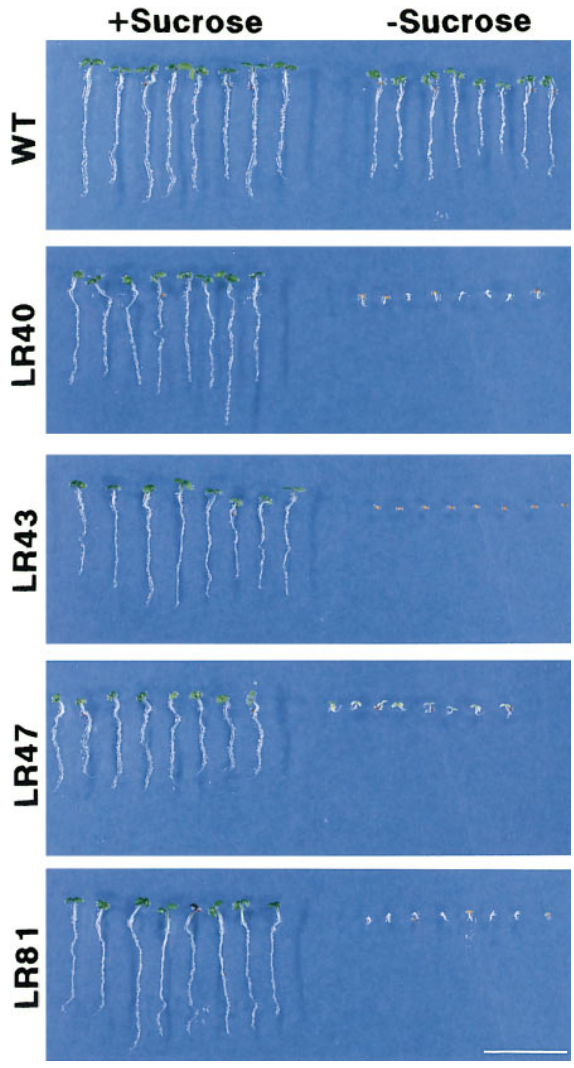

Figure 3. Effect of Sucrose on Mutants and Growth of Wild-Type Seedlings.

Wild-type Arabidopsis (WT), LR40, LR43, LR47, and LR81 were grown for 7 days on a growth medium with (+Sucrose) or without (−Sucrose) sucrose under constant illumination. Photographs were taken after the seedlings were removed from the media and rearranged on agar plates. Bar = 1 cm.

Genetic Analyses

To determine the genetic basis for these mutations in LR40, LR43, LR47, and LR81, mutants were crossed with wild-type plants and their progenies were analyzed (Table 1). All F_1 plants obtained from the crosses were 2,4-DB resistant. F_2 seedlings, the progenies obtained by self-fertilization of F_1 plants, segregated at a ratio of 3:1 (2,4-DB resistant to 2,4-DB sensitive). Thus, the mutant alleles in these mutants are dominant for 0.2 μg/mL 2,4-DB resistance and segregated in a manner most consistent with a single Mendelian gene. These results indicate that the mutations in these mutants are inherited as single dominant loci.

In contrast, the F_1 heterozygous plants could germinate and expand green cotyledons and leaves on growth medium without sucrose, despite the inhibitory effect on the growth of these parents. F_2 seedlings segregated at a ratio of 3:1, and approximately three-fourths of the F_2 seedlings could germinate and grow as well as the wild-type plants did in the absence of sucrose. One-fourth of the F_2 seedlings could not grow in the absence of sucrose. However, their growth, which was inhibited in the absence of sucrose, was recovered when the seedlings were transferred to growth medium containing sucrose, and their progenies showed 2,4-DB resistance. These results indicate that these mutations are recessive for growth in the absence of sucrose.

To determine allelism of these mutations, F_1 progenies were obtained from the crosses between two of the four mutants (Table 1). All F_1 hybrids, except LR43 × LR81, could germinate and expand green cotyledons and leaves on growth medium without sucrose. The result indicated that LR40, LR43, and LR47 are nonallelic mutations, whereas mutations in LR43 and LR81 are allelic. Based on these data, we designated *ped1* as LR40, *ped2* as LR47, *ped3-1* as LR43, and *ped3-2* as LR81, respectively.

Mapping of the *PED1*, *PED2*, and *PED3* Loci

The locations of the *PED1*, *PED2*, and *PED3* loci were mapped using F_2 plants that had *ped1*/*ped1*, *ped2*/*ped2*, and *ped3*/*ped3* genotypes obtained from the LR40 × Columbia (Col-0) ecotype, LR47 × Col-0, and LR81 × Col-0 crosses, respectively. Linkage data for *PED1*, *PED2*, and *PED3* loci were determined by an analysis using cleaved amplified polymorphic sequence markers (Konieczny and Ausubel, 1993) and simple sequence length polymorphism markers (Bell and Ecker, 1994). The initial analyses using the

Table 1. Genetic Segregation of 2,4-DB Resistance and Sucrose Response

	Number of Plants			Number of Plants		
Cross	2,4-DB[a] (Elongated)	2,4-DB[a] (Shortened)	$\chi^{2\,b}$	Sucrose[c] (Normal)	Sucrose[c] (Inhibited)	$\chi^{2\,b}$
LR40 × LR40	57	0		0	143	
LR40 (♂) × WT (♀) F_1	65	0		28	1	
F_1 × F_1	161	66	2.01[d]	69	18	0.86[d]
LR43 × LR43	49	0		0	129	
LR43 (♂) × WT (♀) F_1	55	1		30	1	
F_1 × F_1	190	58	0.34[d]	102	25	0.86[d]
LR47 × LR47	68	0		0	129	
LR47 (♂) × WT (♀) F_1	68	0		30	1	
F_1 × F_1	169	47	3.25[d]	102	25	1.91[d]
LR81 × LR81	96	2		0	26	
LR81 (♂) × WT (♀) F_1	54	2		48	1	
F_1 × F_1	73	25	0.01[d]	78	27	0.03[d]
LR40 × LR43 F_1				67	0	
LR40 × LR47 F_1				52	1	
LR40 × LR81 F_1				66	0	
LR43 × LR47 F_1				63	1	
LR43 × LR81 F_1				0	122	
LR47 × LR81 F_1				25	0	

[a] Plants were grown for 5 days on medium containing 0.2 μg/mL 2,4-DB and scored for elongated or shortened roots.
[b] χ^2 values are calculated based on an expected ratio of 3 resistant to 1 sensitive.
[c] Plants were grown for 5 days on medium without sucrose and scored for normal or inhibited growth.
[d] $P > 0.05$.

markers for all five chromosomes revealed that the *PED1*, *PED2*, and *PED3* loci map to chromosome 2, chromosome 5, and chromosome 4, respectively. Further analysis indicated that the *PED1* locus is located between the constitutive photomorphogenic COP1 and m429 markers on chromosome 2 and that the distance between COP1 and *PED1* is ~1.1 centimorgans (cM). Among all of the markers that we tested, the marker nearest to the *PED2* locus was g2368 on chromosome 5. The position of *PED2* is ~2.4 cM from g2368 and is ~3.3 cM from the leafy LFY3. Because the genetic distance between g2368 and LFY3 has been calculated as 6.1 cM (Arabidopsis database; http://genome-www.stanford.edu/Arabidopsis), it is most likely that the *MID2* locus is located between LFY3 and g2369. In contrast, the marker nearest to the *PED3* locus is DHS1 on chromosome 4. The position of *PED3* is calculated as 0 cM from DHS1 and 3.4 cM from nga1107. Because the genetic distance between DHS1 and nga1107 has been calculated as 3.7 cM (Arabidopsis database; http://genome-www.stanford.edu/Arabidopsis), it is most likely that the *PED3* locus is located close to DHS1.

Defect in Thiolase in *ped1* and *ped2* Mutants

To characterize the phenotypes of the mutants, we analyzed glyoxysomal enzymes in 5-day-old etiolated cotyledons by using an immunoblot technique. The glyoxysomal enzymes analyzed in this study were thiolase and isocitrate lyase. The former is one of the enzymes for fatty acid β-oxidation, and the latter is one of the enzymes for glyoxylate cycle. As shown in Figure 4, two mutations, *ped1* in LR40 and *ped2* in LR47, showed thiolase patterns that were different from that of the wild-type plant, and the amount of isocitrate lyase in LR77 was less than that in the wild-type plant. Because thiolase is involved in glyoxysomal fatty acid β-oxidation, we decided to analyze *ped1* and *ped2* mutants further.

We have demonstrated that the amount of thiolase in etiolated pumpkin cotyledons increased until 2 days after germination and then declined during seedling growth (Kato et al., 1996b). As shown in Figure 5 (WT), etiolated cotyledons of wild-type Arabidopsis grown 3 days in darkness already accumulated a high amount of thiolase. The amount of thiolase in dark-grown cotyledons then declined during seedling growth. When the seedlings were exposed to light, the amount of thiolase decreased rapidly. In the cotyledons of the *ped1* mutant, however, no accumulation of thiolase was observed at any stage of postgerminative growth, regardless of the light conditions (Figure 5, *ped1*). In contrast, cotyledons of the *ped2* mutant contained two types of thiolase. 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) (Figure 5, *ped2*).

It has been demonstrated that the mature form of thiolase (45 kD) is synthesized in the cytosol as a precursor protein (48 kD) and that the N-terminal presequence is removed during the translocation of the protein into glyoxysomes (Preisig-Muller and Kindl, 1993; Kato et al., 1996b). The molecular mass of the additional protein coincided with that of the precursor form of thiolase. The precursor form of thiolase accumulated at early stages of postgerminative growth and rapidly disappeared during subsequent seedling growth (Figure 5, *ped2*). In contrast, the amount of the mature form of thiolase in etiolated cotyledons increased until 6 to 7 days after germination and then rapidly declined. Exposure of the seedlings to light accelerated the reduction of both mature and precursor forms of thiolase.

Figure 6 shows the result of subcellular fractionation by using sucrose density gradient centrifugation. The glyoxysomes isolated from cells of 5-day-old etiolated cotyledons were analyzed by using an immunoblot technique with antibodies raised against thiolase, isocitrate lyase, and catalase. In the wild-type plant, thiolase, isocitrate lyase, and catalase were detected in fractions 21 to 23, whose densities were 1.25 g/cm^3. Although these enzymes were also detected in

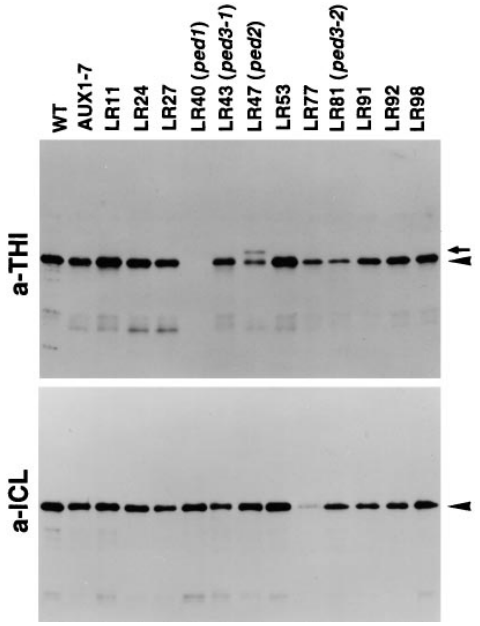

Figure 4. Immunodetection of Thiolase and Isocitrate Lyase in Etiolated Cotyledons of Wild-Type Arabidopsis and 2,4-DB–Resistant Mutants.

Extracts were prepared from 5-day-old etiolated cotyledons of wild-type Arabidopsis (WT), the 2,4-D–resistant mutant (AUX1-7), and 2,4-DB–resistant mutants. For each sample, 5 μg of total protein was subjected to SDS-PAGE, and immunoblot analysis was performed using antibodies raised against thiolase (a-THI) and isocitrate lyase (a-ICL). Arrowheads indicate the positions of thiolase and isocitrate lyase; the arrow indicates the position of the precursor form of thiolase. Mutant alleles in LR40, LR43, LR47, and LR81 are designated as *ped1*, *ped3-1*, *ped2*, and *ped3-2*, respectively.

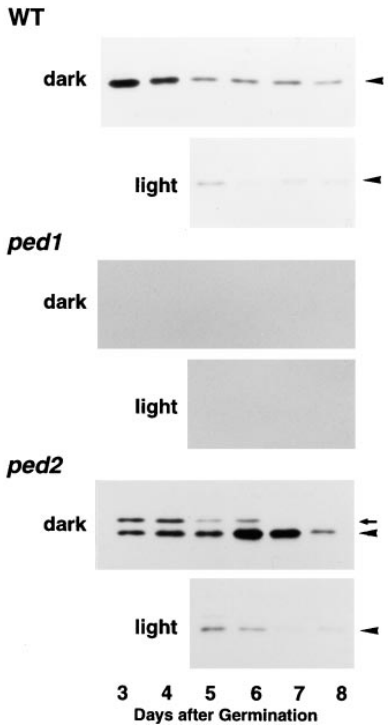

Figure 5. Developmental Changes in Levels of Thiolase in Cotyledons of Wild-Type Arabidopsis and *ped1* and *ped2* Mutants after Germination.

Seedlings of wild-type Arabidopsis (WT) and *ped1* and *ped2* mutants were grown in continuous darkness for 8 days or in darkness for 4 days and then under continuous illumination for 4 days. Cotyledons were harvested every day, and crude extracts containing 5 μg of total protein were subjected to immunoblotting using an antibody raised against thiolase. Dark and light represent the levels of thiolase in the cotyledons grown in continuous darkness and grown under constant illumination after being grown for 4 days in darkness, respectively. The number of days after germination are shown at the bottom. Arrowheads indicate the positions of the mature form of thiolase; the arrow indicates the position of the precursor form of thiolase.

the first few fractions (top of the gradient), this may be due to disruption of the glyoxysomes during homogenization and cell fractionation (Figure 6, WT). The distribution of isocitrate lyase and catalase revealed that glyoxysomes of the *ped1* mutant were also distributed in fractions 21 to 23 (Figure 6, *ped1*). However, these fractions did not contain a detectable amount of thiolase. In contrast, the distribution patterns of thiolase, isocitrate lyase, and catalase in the *ped2* mutant were different from those of the wild-type plant and the *ped1* mutant. All of these enzymes were concentrated in fractions 9 to 13, whose densities were ~1.19 g/cm^3 (Figure 6, *ped2*). The results clearly indicate that the glyoxysomes of the *ped2* mutant have a lower density than those of the wild-type Arabidopsis plant.

It should be emphasized that glyoxysomal fractions of the *ped2* mutant contained only the mature form of thiolase. Therefore, no band corresponding to the precursor form of thiolase was detected after immunoblot analysis of fraction 11 (Figure 6, *ped2*) concentrated 10 times using Centricon 10 concentrators (Amicon, Beverly, MA), despite the fact that soluble fractions, such as fraction 3, contained two types of thiolase that corresponded to the mature and precursor proteins (data not shown). These results suggest that the precursor form of thiolase accumulated in the cytosol of the *ped2* mutant, whereas the mature protein was localized in the glyoxysomes.

Comparison of Thiolase Genes in the Wild-Type Plant and *ped1* Mutant

To compare the nucleotide sequence of thiolase genes in the wild-type plant and *ped1* mutant, the expressed sequence tag bank at the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH) was searched to identify a thiolase cDNA. We determined the full-length nucleotide sequence of clone 91A18T7 (accession number AB008854) and found that the cDNA encodes thiolase composed of 462 amino acids. The amino acid sequence of the Arabidopsis thiolase was 87.4% identical with pumpkin peroxisomal thiolase (Kato et al., 1996b). Based on this nucleotide sequence, we designed a set of oligonucleotide primers that could amplify the thiolase gene by using polymerase chain reactions. Thiolase genes were amplified from genomic DNAs of a wild-type plant and *ped1* mutant by using the primer set, and nucleotide sequences of these genes were determined. The wild-type thiolase gene (accession number AB008855) has 14 exons and encodes a thiolase completely identical to that encoded by cDNA clone 91A18T7.

A comparison of thiolase genes from the wild-type plant and the *ped1* mutant revealed that the ATT codon for Ile-100 at the fourth exon of the wild-type thiolase gene is changed to ATGG in the thiolase gene of the *ped1* mutant (accession number AB008856). A nucleotide substitution of T to GG causes a frameshift and produces a stop codon within the fourth exon. Therefore, the thiolase gene in the *ped1* mutant encodes a smaller protein composed of 114 amino acids, and the first 99 amino acids of the protein are identical to that of wild-type thiolase.

Morphology of Glyoxysomes in *ped1* and *ped2* Mutants

Figure 7 shows electron microscopic analysis of glyoxysomes in 5-day-old etiolated cotyledons of the wild-type plant and *ped1* and *ped2* mutants. As shown in Figure 7A, glyoxysomes in the wild-type plant are ~0.5 μm in diameter and have a round or oval shape containing a uniform matrix. Because the glyoxysomes contain thiolase and isocitrate

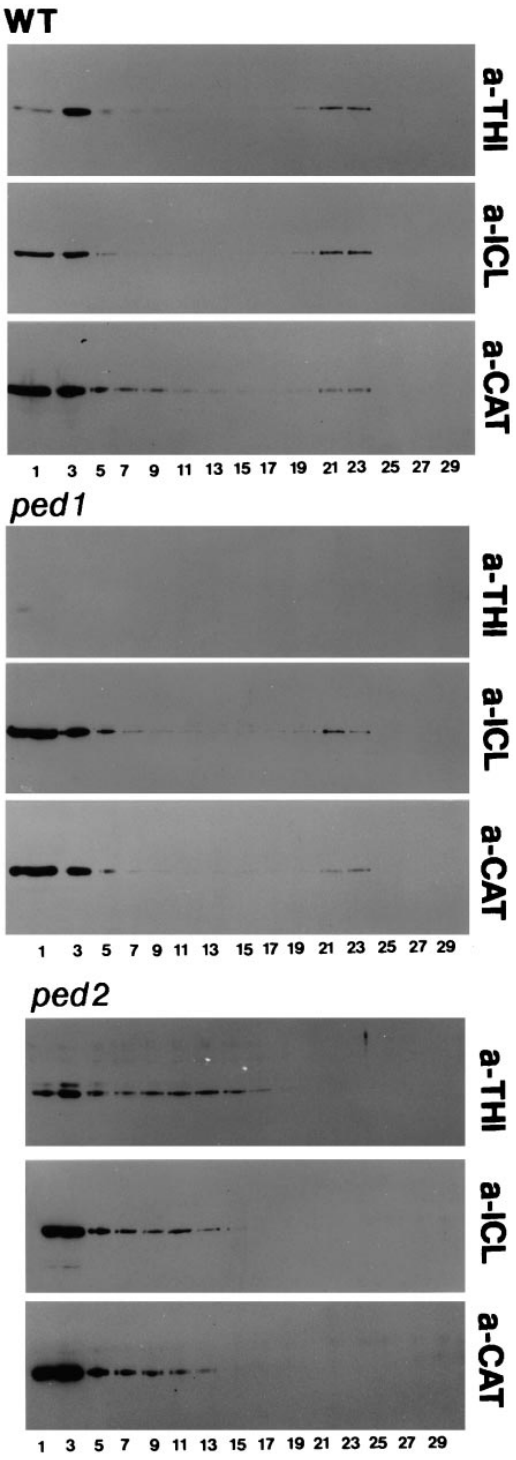

Figure 6. Subcellular Localization of Thiolase in Etiolated Cotyledons of Wild-Type Arabidopsis and *ped1* and *ped2* Mutants.

Extracts prepared from 5-day-old etiolated cotyledons were fractionated by sucrose density gradient centrifugation, and 20 μL of each fraction was analyzed by immunoblotting using antibodies raised against thiolase (a-THI), isocitrate lyase (a-ICL), and catalase (a-CAT). Fraction 1 is at the top of the gradient. WT, wild type.

lyase, gold particles were localized exclusively on the glyoxysomes after staining with the immunogold labeling technique using antibodies raised against thiolase (Figure 7B) and isocitrate lyase (Figure 7C).

In contrast, glyoxysomes in the *ped1* mutant were two or three times greater in diameter than those in the wild-type plant and contained vesicle-like structures (Figure 7D). Because the *ped1* mutant lacks thiolase, immunogold labeling experiments revealed that the organelles contain isocitrate lyase but not thiolase (cf. Figures 7E and 7F).

Most of the glyoxysomes found in the cells of the *ped2* mutant were shrunken and not round. Therefore, they looked very different from glyoxysomes of the wild-type plant (Figure 7G). However, the glyoxysomes were stained with antibodies raised against thiolase (Figure 7H) and isocitrate lyase (Figure 7I). Although immunoblot analysis revealed that the *ped2* mutant accumulated the precursor form of thiolase in the cytosol, no significant signal corresponding to the precursor protein was observed in the cytosol. This might be due to the dilution of the proteins in the cytosol, as we have reported previously (Hayashi et al., 1996).

DISCUSSION

2,4-DB as a Chemical for Screening Mutants

This study was designed to identify the genes responsible for regulation of peroxisomal function in plant cells by using a genetic approach. For this purpose, we attempted to isolate mutants with defective peroxisomes. To screen such mutants, we used 2,4-DB as a compound for detecting Arabidopsis mutants that have reduced activity of glyoxysomal fatty acid β-oxidation during postgerminative growth, which is one of the important functions of plant peroxisomes. 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 the acetic side chain in 2,4-D in wild-type plants, whereas the mutants would no longer produce toxic levels of 2,4-D from 2,4-DB because of the reduced activity for fatty acid β-oxidation.

The screening was successful, because two (*ped1* and *ped2* mutants) of 12 2,4-DB–resistant mutants have a defective thiolase, which is an enzyme involved in fatty acid β-oxidation. In addition to these mutants, two allelic mutants, namely, *ped3-1* and *ped3-2*, showed inhibited growth in the absence of sucrose. The requirement of sucrose for postgerminative growth of these mutations seems to indicate a reduction in the activity of glyoxysomal fatty acid β-oxidation, because

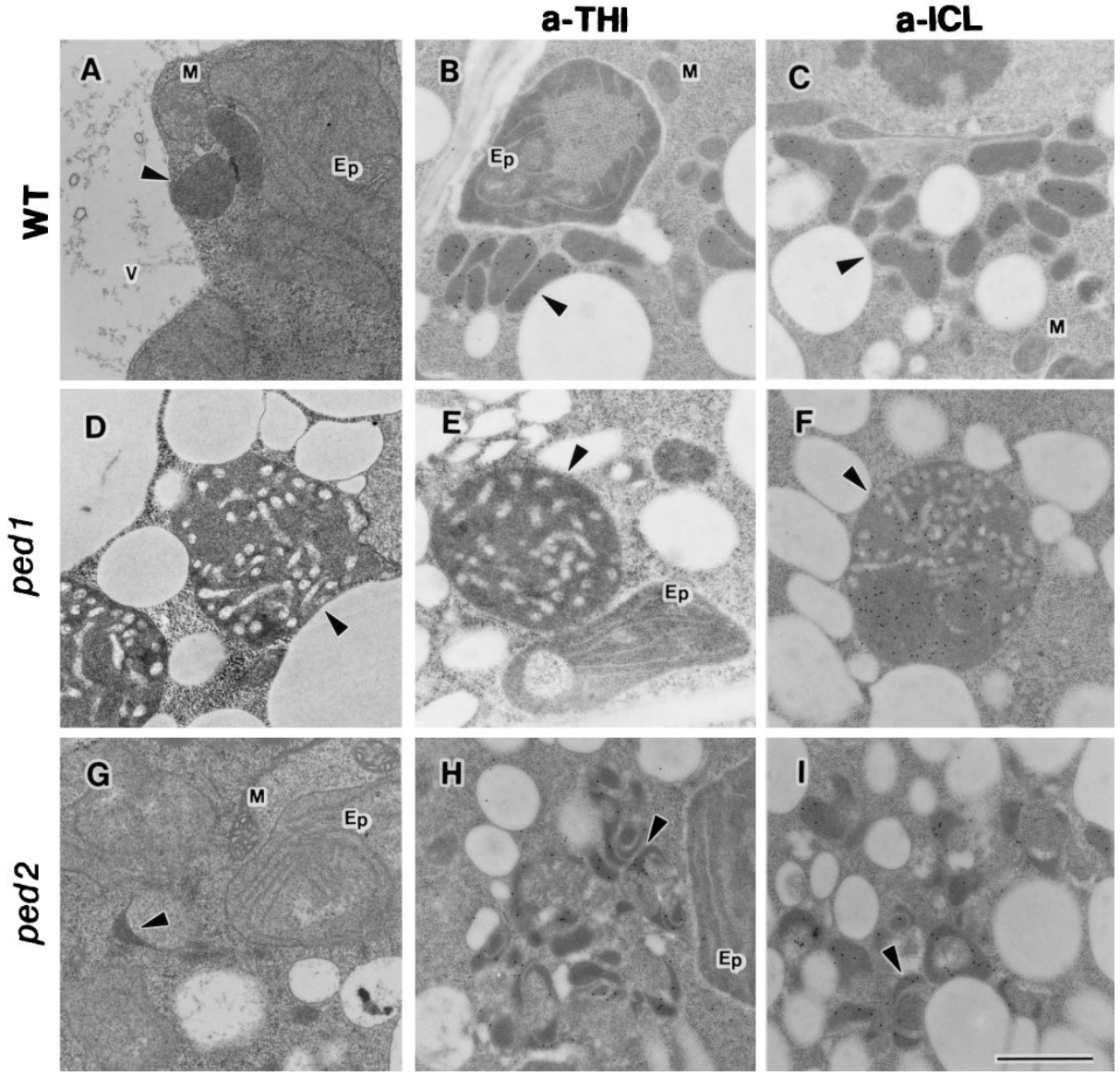

Figure 7. Electron Microscopic Analysis of Glyoxysomes in the Cells of 5-Day-Old Etiolated Cotyledons.

(A) A cotyledonary cell of a wild-type (WT) plant.
(B) Immunogold labeling of a wild-type plant, using an antibody raised against thiolase (a-THI).
(C) Immunogold labeling of a wild-type plant, using an antibody raised against isocitrate lyase (a-ICL).
(D) A cotyledonary cell of *ped1* mutant.
(E) Immunogold labeling of *ped1* mutant, using an antibody raised against thiolase.
(F) Immunogold labeling of *ped1* mutant, using an antibody raised against isocitrate lyase.
(G) A cotyledonary cell of *ped2* mutant.
(H) Immunogold labeling of *ped2* mutant, using an antibody raised against thiolase.
(I) Immunogold labeling of *ped2* mutant, using an antibody raised against isocitrate lyase.
Arrowheads indicate glyoxysomes; Ep, etioplast; M, mitochondrion; V, vacuole. Bar in **(I)** = 1 μm for **(A)** to **(I)**.

glyoxysomal fatty acid β-oxidation is known to play a role in producing sucrose from storage lipids. Therefore, we believe that not only *ped1* and *ped2* but also *ped3* mutants have defective fatty acid β-oxidation activity, although we have not yet identified the mechanism for the defect occurring in *ped3* mutants.

Genetic analysis revealed that all three mutations (*ped1*, *ped2*, and *ped3*) are dominant for 2,4-DB sensitivity, despite the fact that they are recessive for a sucrose requirement for postgerminative growth. We assumed that reduced activity of fatty acid β-oxidation in the heterozygous plants is enough to produce sucrose from storage lipid that is essential for postgerminative growth. However, it is not enough to produce toxic levels of 2,4-D from 2,4-DB, because we used the lowest concentration of 2,4-DB that could detect weak mutations.

In contrast to these four mutants, the rest of the 2,4-DB–resistant mutants have expanded green cotyledons and leaves in the absence of sucrose. However, it is possible that they also have defects in glyoxysomal function(s). For example, LR77 contains a reduced amount of isocitrate lyase, which is a glyoxysomal enzyme of the glyoxylate cycle, although the mechanism for 2,4-DB resistance in this mutant has not been determined. Currently, we have analyzed only two glyoxysomal enzymes, thiolase and isocitrate lyase. However, glyoxysomes are known to contain various enzymes, including other enzymes for fatty acid β-oxidation and the glyoxylate cycle (Huang et al., 1983). Thus, the possibility that other glyoxysomal enzymes might be defective needs to be examined.

Relationship between the *PED1* Gene and Phenotype of *ped1*/*ped1* Plants

Recently, the full-length nucleotide sequence of the bacterial artificial chromosome clone F25I18 was submitted to the database (accession number AC002334). It has been known that this clone covers a part of the genomic DNA contained in the yeast artificial chromosome clone CIC10F7, which includes the COP1 marker on chromosome 2 (Arabidopsis database; http://genome-www.stanford.edu/Arabidopsis). We found that clone F25I18 contains a nucleotide sequence that is identical with that of the thiolase gene (accession number AB008855) reported in this study. Although two single nucleotide substitutions and a 32-bp insertion were found in clone F25I18, this may be due to a difference in the ecotypes used (Col-0 for F25I18 and Landsberg *erecta* for the thiolase gene). The amino acid sequences of the thiolases encoded by both DNAs are completely identical. This result suggests that the thiolase gene is located close to the COP1 marker.

Because we determined that the *PED1* gene is also located close to the COP1 marker, *PED1* seems to encode thiolase itself or to be located close to the thiolase gene. Indeed, we could identify the nucleotide substitution (T to GG) within the thiolase gene of the *ped1* mutant (accession number AB008856). Because of the substitution, the thiolase gene in the *ped1* mutant encodes smaller protein that might be unstable in the cell. Overall, this result suggests that the *PED1* locus is most likely to be the thiolase gene.

ped1 homozygous plants lack a detectable amount of thiolase in glyoxysomes at all stages of seedling growth. This result is reasonable if *PED1* encodes thiolase. The abnormal small protein translated from the *ped1* gene is unstable and is degraded rapidly in the cells of *ped1*/*ped1* plants, and the loss of thiolase in the glyoxysomes may inhibit fatty acid β-oxidation. Therefore, *ped1*/*ped1* plants do not produce 2,4-D from 2,4-DB and show resistance to 2,4-DB during postgerminative growth. At the same time, they do not expand green cotyledons and leaves in the absence of sucrose, because the lack of fatty acid β-oxidation activity prevents the production of sucrose from storage lipids. The loss of thiolase also affected the morphology of the glyoxysomes. Glyoxysomes without thiolase may accumulate a metabolic intermediate(s) for fatty acid β-oxidation and become enlarged organelles containing vesicle-like structures.

Relationship between the *PED2* Gene and Phenotype of *ped2*/*ped2* Plants

In contrast to *ped1* homozygous plants, thiolase was detected in the glyoxysomes of *ped2* homozygous plants. The mature form of thiolase appeared in the glyoxysomes of the mutants at a later stage of postgerminative growth than that of the wild-type plants, whereas the precursor of the protein accumulated in the cytosol specifically at the early stage of postgerminative growth. This result suggests that *ped2*/*ped2* plants have a defect in the intracellular transport of thiolase from the cytosol into the glyoxysomes. Because of the defect in the intracellular transport system, fatty acid β-oxidation activity becomes low at the early stage of postgerminative growth but then increases later. Therefore, *ped2*/*ped2* plants can expand their cotyledons and accumulate low amounts of chlorophyll. However, they cannot expand leaves in the absence of sucrose because of the reduced production of sucrose from storage lipids due to the weak fatty acid β-oxidation activity.

It is known that not only thiolase but also malate dehydrogenase and citrate synthase are synthesized as precursor proteins with an N-terminal presequence in plant cells (Gietl, 1990; Kato et al., 1995). These N-terminal presequences contain the consensus sequence (R)-(L/Q/I)-X_5-(H)-(L) designated PTS2 (where X stands for any of amino acid and PTS stands for peroxisomal targeting signal), which functions as a targeting signal for plant peroxisomes (Kato et al., 1996a). Because these proteins may be imported by the action of common import machinery similar to the yeast system (Waterham and Cregg, 1997), it is possible that glyoxysomes in the *ped2*/*ped2* plants may fail to import a set of proteins containing PTS2. Indeed, we have detected the accumulation of

the precursor form of not only thiolase but also malate dehydrogenase in etiolated cotyledons of *ped2*/*ped2* plants (data not shown). The loss of these proteins may cause a reduction in the glyoxysomal matrix, and glyoxysomes in these mutants would become shrunken structures with a lower density.

There exists another group of peroxisomal proteins that contain a targeting signal at the C terminus, such as isocitrate lyase. Recent studies (Volokita, 1991; Olsen et al., 1993; Hayashi et al., 1996; Trelease et al., 1996) have shown that a unique tripeptide sequence, PTS1, found in the C terminus functions as a targeting signal for plant peroxisomes. The permissible combinations of amino acids for PTS1 in plant cells are (C/A/S/P)-(K/R)-(I/L/M) (Hayashi et al., 1997). The plant PTS1 sequence is similar but not completely identical to mammalian and yeast PTS1 (McNew and Goodman, 1996). Currently, we do not know whether the *ped2* mutation inhibits the intracellular transport of only PTS2-containing proteins or both PTS1- and PTS2-containing proteins, because the fractionation experiments and electron microscopic analysis failed to indicate the transport efficiency of isocitrate lyase in *ped2*/*ped2* plants. Therefore, two possibilities exist for the phenotype of *ped2*/*ped2* plants. One is that *ped2*/*ped2* plants have a defect only in the import of PTS2 proteins. Another possibility is that *ped2*/*ped2* plants have a defect in the import of both PTS1 and PTS2 proteins.

Many yeast mutants that affect protein import and the biogenesis of peroxisomes have been described during the past decade. In these studies, researchers succeeded in identifying more than 17 peroxins (proteins involved in peroxisome biogenesis, including peroxisomal matrix protein import, membrane biogenesis, peroxisome proliferation, and peroxisome inheritance), and the corresponding *PEX* genes have been cloned and characterized (Distel et al., 1996; Subramani, 1997). Among these *PEX* genes, it has been shown that *PEX7* (formerly called *PAS7* and *PEB1*) encodes the PTS2 receptor and that peroxisomes in the *pex7* mutant fail to import thiolase but can import PTS1-containing proteins (Marzioch et al., 1994; Zhang and Lazarow, 1995). If *ped2*/*ped2* plants have a defect only in the import of PTS2-containing proteins, then the PTS2 receptor is a candidate for the protein encoded by the *PED2* gene.

Many other *PEX* mutants that fail to import both PTS1- and PTS2-containing proteins, except for *PEX5,* which encodes the PTS1 receptor, have been described (McNew and Goodman, 1996). It has been suggested that *PEX13* and *PEX14* encode a peroxisomal membrane protein that binds to Pex5p and/or Pex7p (Erdmann and Blobel, 1996; Albertini et al., 1997; Komori et al., 1997). Although other *PEX* gene products may not be directly involved in protein transport machinery, import of proteins in these mutants is presumably inhibited by indirect factors, such as peroxisomal membrane biogenesis, peroxisome proliferation, and peroxisome inheritance. If *ped2*/*ped2* plants have a defect in the import of both PTS1- and PTS2-containing proteins, then *PED2* may be a plant homolog for one of these *PEX* genes.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Landsberg *erecta* was used as the wild-type plant. Ethyl methanesulfonate–mutagenized M_2 seeds of Arabidopsis ecotype Landsberg *erecta* were purchased from Lehle Seeds (Round Rock, TX). Seeds of *aux1-7* plants were kindly sent from the Arabidopsis Biological Resource Center. All seeds were surface sterilized in 2% NaClO and 0.02% Triton X-100 and grown on growth medium (2.3 mg/mL Murashige and Skoog salts [Wako, Osaka, Japan], 1% sucrose, 100 μg/mL myoinositol, 1 μg/mL thiamine–HCl, 0.5 μg/mL pyridoxine, 0.5 μg/mL nicotinic acid, 0.5 mg/mL Mes-KOH, pH 5.7, and 0.2% Gellan gum [Wako]). Seedlings grown for 2 weeks on the growth medium were transferred to a 1:1 mixture of perlite and vermiculite. Plants were grown under constant illumination at 22°C.

Screening of 2,4-Dichlorophenoxybutyric Acid–Resistant Mutants

Ethyl methanesulfonate–mutagenized M_2 seeds were germinated on growth medium containing 0.2 μg/mL 2,4-dichlorophenoxybutyric acid (2,4-DB) (Aldrich Chemical Co., Milwaukee, WI). Five days after germination, seedlings that had roots longer than those of the wild type were separated and grown for an additional 2 weeks on growth medium without 2,4-DB. Plants were then transferred to pots to obtain M_3 seeds. M_3 and M_4 seeds obtained from each mutant were then germinated on growth medium containing 0.2 μg/mL 2,4-DB and on growth medium containing 0.05 μg/mL 2,4-D (Nacarai, Osaka, Japan). Mutants that had expanded roots exclusively on the growth medium containing 2,4-DB were determined to be 2,4-DB–resistant mutants. Homozygous plants of the M_5 generation were used for the analyses. To analyze the requirement of sucrose for growth, we germinated seeds in plant culture jars equipped with gas-permeable membranes (plant culture ware with polytetrafluoroethylene membrane; Iwaki Glass, Chiba, Japan) containing growth medium without sucrose.

Genetic Analysis and Mapping

Progenies backcrossed twice with the wild-type plant (ecotype Landsberg *erecta*) were used for the genetic analyses of the mutants. Mutants were crossed with wild-type plants (ecotype Landsberg *erecta*). F_1 and F_2 populations were analyzed by examining 2,4-DB resistance and growth inhibition in the absence of sucrose. For mapping, crosses were made between mutants and the wild-type plant (ecotype Columbia [Col-0]). F_2 seeds, obtained by self-fertilization of F_1 plants, were germinated on the medium without sucrose. Seedlings that could not expand green cotyledons and leaves on the plates were recovered after transferring these seedlings to medium containing sucrose. The genomic DNA of these F_2 plants was individually isolated. The cleaved amplified polymorphic sequence mapping procedure described by Konieczny and Ausubel (1993) and the simple sequence length polymorphism mapping procedure described by Bell and Ecker (1994) were used to determine the map positions of the *PED1*, *PED2*, and *PED3* loci. The percentage of recombination was scored for 44 to 48 plants obtained from the F_2 generation. Map distance in centimorgans (cM) was calculated ac-

cording to the Kosambi function, as described by Koornneef and Stam (1992).

Immunoblotting

Plants were grown on growth medium for 5 days in darkness at 22°C. Seedlings were homogenized in 100 μL of buffer containing 50 mM Tris-HCl, pH 8.3, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged for 15 min at 15,000*g* at 4°C to remove cell debris, and the supernatant was used as the crude extract. The amount of total protein in the crude extract was measured by using a Bio-Rad protein assay kit. Each lane of a 10% SDS–polyacrylamide gel was loaded with a total of 5 μg of protein. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) in a semidry electroblotting system. The membrane was blocked with 3% nonfat dry milk in Tris-buffered saline, pH 7.4, and immunoblotted with a 1:1000 dilution of antibodies raised against thiolase (Kato et al., 1996b) or isocitrate lyase (Maeshima et al., 1988). Bands were visualized with an enhanced chemiluminescence western blotting detection kit (ECL; Amersham Japan, Tokyo, Japan), using a 1:5000 dilution of peroxidase-conjugated goat antibodies against rabbit IgG, following the instructions of the manufacturer.

Subcellular Fractionation

One hundred milligrams of seeds (~5000 seeds) was grown on growth medium for 5 days in darkness at 22°C. Etiolated cotyledons were harvested and chopped with a razor blade in a Petri dish with 2.0 mL of chopping buffer (150 mM Tricine-KOH, pH 7.5, 1 mM EDTA, 0.5 M sucrose, and 1% BSA). The extract was then filtered with a cell strainer (Becton Dickinson, Franklin Lakes, NJ). Two milliliters of the homogenate was layered directly on top of a 16-mL linear sucrose density gradient (30 to 60% [w/w]) that contained 1 mM EDTA. Centrifugation was performed in an SW 28.1 rotor (Beckman Instruments, Palo Alto, CA) at 25,000 rpm for 2.5 hr at 4°C. Half-milliliter fractions were collected with a gradient fractionator (model 185; ISCO, Lincoln, NE). Twenty microliters of each fraction was subjected to the immunoblot analysis by using antibodies raised against thiolase and isocitrate lyase. In some of the experiments, each fraction was concentrated 10 times using Centricon 10 concentrators (Amicon, Beverly, MA), following the instructions of the manufacturer.

Determination of the Nucleotide Sequences of the Thiolase cDNA and Genes

Genomic DNAs of wild-type plants and *ped1* mutants were isolated according to the method reported by Snead et al. (1994). The DNA fragment encoding thiolase was amplified by the polymerase chain reaction, using 100 ng of each genomic DNA as a template. The reaction mixture contained 2.5 units of Takara LA Taq DNA polymerase (Takara Shuzo, Shiga, Japan), a 5′ primer (ATGGAGAAAGCGATCGAGAGACAAC), a 3′ primer (CTCGCTAGAGAGGACCATGCACCAA), and an appropriate buffer in a total volume of 50 μL. Each cycle of the polymerase chain reaction consisted of 95°C for 45 sec, 60°C for 45 sec, and 72°C for 2 min. The polymerase chain reaction product obtained after 25 cycles of amplification was then incubated for 2 hr at 72°C with AmpliTaq DNA polymerase (Perkin-Elmer Japan, Chiba, Japan) to introduce deoxyadenosine at the 3′ ends. The DNA fragment was then subcloned into a T-vector prepared using pBluescript KS+ (Stratagene, La Jolla, CA), as described in a previous report (Marchuk et al., 1990). The expressed sequence tag clone encoding thiolase, whose stock number is 91A18T7, was obtained from the Arabidopsis Biological Resource Center at Ohio State University. The nucleotide sequences of the thiolase cDNA contained in clone 91A18T7 and the thiolase genes amplified from genomic DNAs of the wild-type plant and *ped1* mutant were determined with an automatic DNA sequencer (model 377; Perkin-Elmer Japan), according to the manufacturer's instructions.

Electron Microscopic Analysis

Etiolated cotyledons were obtained from plants grown on growth medium for 5 days in darkness. They were vacuum infiltrated for 1 hr with a fixative that consisted of 4% paraformaldehyde, 1% glutaraldehyde, and 0.06 M sucrose in 0.05 M cacodylate buffer, pH 7.4. The fixed samples were cut into slices of <1 mm thick and treated for another 2 hr with a freshly prepared fixative.

For ultrastructural studies, the fixed samples were then postfixed with 1.5% osmium tetroxide in the same cacodylate buffer for 3 hr. After washing with the same buffer, the specimens were stained in 1% uranyl acetate for 2 hr, with subsequent dehydration in a graded ethanol series at room temperature. The samples were treated with propylene oxide and infiltrated with propylene oxide–Epon (Epon 812 resin; TAAB Laboratories, Aldermaston, UK) solution (propylene oxide–Epon resin, 1:1 [v/v]) overnight. The samples were then embedded in Epon resin that was allowed to polymerize at 60°C for 48 hr. Ultrathin sections were cut on an ultramicrotome (Leica, Reichert Division, Vienna, Austria) and mounted on copper grids. The sections were then stained with 4% uranyl acetate and lead citrate.

Immunoelectron microscopy was performed as described previously (Nishimura et al., 1993). After washing the samples with the same cacodylate buffer, the fixed samples were dehydrated in a graded dimethylformamide series at −20°C and embedded in LR white resin (London Resin Co., Ltd., Basingstoke, UK). Blocks were polymerized under a UV lamp at −20°C for 24 hr. Ultrathin sections were mounted on uncoated nickel grids. The sections were treated with blocking solution (1% BSA in PBS) for 1 hr at room temperature and were then incubated overnight at 4°C in a solution of thiolase-specific and isocitrate lyase–specific antibodies that had been diluted 1:20 and 1:500, respectively, in the blocking solution at 4°C. After washing with PBS, sections were incubated for 30 min at room temperature in a solution of protein A–gold (15 nm; Amersham Japan) that had been diluted 1:20 in the blocking solution. The sections were washed with distilled water and then stained with 4% uranyl acetate and lead citrate. After staining, all sections were examined under a transmission electron microscope (model 1200EX; JEOL, Tokyo, Japan) operated at 80 kV.

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