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

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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 (Bevers, 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.

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-dichlorophenoxyalkyloxcarboxylic 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-dichlorophenoxyalkyloxcarboxylic 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 (Bevers, 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 hydroxyppyruvate 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

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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 M2 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 M3 seeds. The resistance of the M3 seedlings to 2,4-DB was not observed at the seedling stage.

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 mutants that had been isolated were also resistant to 2,4-D. We tested this hypothesis by germinating the mutants on growth media containing 0.2 μg/mL 2,4-DB in the presence of sucrose. The results showed that the presence of sucrose did not affect root elongation of the mutants in the presence of 2,4-DB. The presence of sucrose also did not affect root elongation of the mutants in the absence of 2,4-DB. These results suggest that the resistance of the mutants to 2,4-DB is not due to the presence of sucrose.

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.
mutants would be good candidates for mutants that are defective in glyoxysomal fatty acid \( \beta \)-oxidation. To determine the function of glyoxysomes in these candidates, we examined the effect of sucrose on their growth because defects in fatty acid \( \beta \)-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 the 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 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 \( \mu \)g/mL 2,4-DB (solid bars) or 0.05 \( \mu \)g/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 \( \pm SE \). WT, wild type.

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 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

<table>
<thead>
<tr>
<th>Cross</th>
<th>2,4-DB^a (Elongated)</th>
<th>2,4-DB^a (Shortened)</th>
<th>x^2^b</th>
<th>2,4-DB^c (Normal)</th>
<th>2,4-DB^c (Inhibited)</th>
<th>x^2^b</th>
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<td>F_1 × F_1</td>
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<td>66</td>
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<td>F_1 × F_1</td>
<td>73</td>
<td>25</td>
<td>0.01^a</td>
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^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 \(\sim 1.1\) 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 \(\sim 2.4\) cM from g2368 and is \(\sim 3.3\) 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 \(\beta\)-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 \(\beta\)-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. The reaction products 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/cm³. Although these enzymes were also detected in
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 lyase,
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 \( \beta \)-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 \( \beta \)-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 \( \beta \)-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 \( \beta \)-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 \( \beta \)-oxidation, because

![Figure 6. Subcellular Localization of Thiolase in Etiolated Cotyledons of Wild-Type Arabidopsis and ped1 and ped2 Mutants.](image-url)

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.
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 $\beta$-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 $\beta$-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 $\beta$-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 $\beta$-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 $\beta$-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 $\beta$-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 $\beta$-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 $\beta$-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 $\beta$-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)-X5-(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 thiolute 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 thiolute 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 thaliana* 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, 0.3 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 (Nacara, 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 3 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; Ikawa 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-
According 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 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 semidy 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 blotting detection kit (ECL; Amersham, 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 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 were 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 microfilters 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 Sneed 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, J apan), a 5’ primer (ATGGGAAAGCGATCGAGAACA), a 3’ primer (CTCAGTACGAGACCATGCACAA), 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 J apan, Chiba, J apan) 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 J apan), 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, Rechert 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) 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 J apan), according to the manufacturer’s instructions.

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