

Characterization of Two cDNAs Encoding Mitochondrial Lipoamide Dehydrogenase from Arabidopsis¹

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In contrast to peas (*Pisum sativum*), where mitochondrial lipoamide dehydrogenase is encoded by a single gene and shared between the α -ketoacid dehydrogenase complexes and the Gly decarboxylase complex, Arabidopsis has two genes encoding for two mitochondrial lipoamide dehydrogenases. Northern-blot analysis revealed different levels of RNA expression for the two genes in different organs; *mtLPD1* had higher RNA levels in green leaves compared with the much lower level in roots. The mRNA for *mtLPD2* shows the inverse pattern. The other organs examined showed nearly equal RNA expressions for both genes. Analysis of etiolated seedlings transferred to light showed a strong induction of RNA expression for *mtLPD1* but only a moderate induction of *mtLPD2*. Based on the organ and light-dependent expression patterns, we hypothesize that *mtLPD1* encodes the protein most often associated with the Gly decarboxylase complex, and *mtLPD2* encodes the protein incorporated into α -ketoacid dehydrogenase complexes. Due to the high level of sequence conservation between the two mtLPDs, we assume that the proteins, once in the mitochondrial matrix, are interchangeable among the different multienzyme complexes. If present at high levels, one mtLPD might substitute for the other. Supporting this hypothesis are results obtained with a T-DNA knockout mutant, *mtlpd2*, which shows no apparent phenotypic change under laboratory growth conditions. This indicates that mtLPD1 can substitute for mtLPD2 and associate with all these multienzyme complexes.

Lipoamide dehydrogenase is part of the α -ketoacid dehydrogenase complexes, the pyruvate dehydrogenase complex (PDC; Luethy et al., 1996), the α -ketoglutarate dehydrogenase complex, and the branched-chain α -ketoacid dehydrogenase complex, as well as the Gly decarboxylase complex (GDC; Oliver, 1994). These multienzyme complexes consist of three to four subunits (component enzymes) that are in different stoichiometric arrangements non-covalently associated with each other. The α -ketoacid dehydrogenase complexes consist of three protein components, E1 (the actual α -ketoacid dehydrogenase), E2 (the dihydrolipoyl transacylase), and E3 (the lipoamide dehydrogenase also called dihydrolipoyl dehydrogenase). The structural organization of PDC has been studied in mammals and *Escherichia coli*. In mammals, PDC exists in a dodecahedral form with 60 monomers of E2 as a core unit, surrounded by 30 E1 heterodimers (E1 α and E1 β) and six E3 homodimers (Reed, 1998). Yeast (*Saccharomyces cerevisiae*) and mammalian PDC also contain an E3 binding protein that is implicated in stabilizing the complex (Lawson et al., 1991; Stoops et al., 1997).

The GDC consist of four subunits, namely the P protein (pyridoxal 5-phosphate-dependent Gly decarboxylase), the H protein (the hydrogen carrier with the covalent bound lipoamide cofactor), the T

protein (a tetrahydrofolate transferase), and the L protein (the lipoamide dehydrogenase). This complex exists in a stoichiometric arrangement of 2 P protein homodimers, 27 H protein monomers, nine T protein monomers, and one L protein homodimer with H proteins building the center core (Oliver et al., 1990b).

In these multienzyme complexes, lipoamide dehydrogenase catalyzes the reoxidation of the covalently bound lipoamide cofactor of E2 or the H protein. As a flavoprotein disulfide oxidoreductase, the homodimeric lipoamide dehydrogenase (LPD) uses FAD as cofactor and NAD⁺ as final electron acceptor. The electrons flow from the dihydrolipoamide to the catalytic Cys residues of one LPD subunit, supported by the active base His and its hydrogen partner, glutamate, of the other subunit, to the cofactor FAD ending up reducing NAD⁺ in a ping-pong bi-bi mechanism (Williams, 1992; Vettakkorumakankav and Patel, 1996).

All these multienzyme complexes are found in the mitochondria with pyruvate dehydrogenase also occurring in plastids. Because the role of lipoamide dehydrogenase is the same in all four complexes, it is not too surprising that in pea (*Pisum sativum*) mitochondria only one single lipoamide dehydrogenase, encoded by a single copy gene, has been found (Bourguignon et al., 1992; Turner et al., 1992b). This finding confirmed earlier work dealing with the purification of the subunits from the GDC from pea, where a monoclonal antibody against the L protein not only inhibits GDC but also PDC activity (Walker and Oliver, 1986b). It was demonstrated more recently by mass spectrometry that in pea mitochon-

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dria, the same lipoamide dehydrogenase protein is shared between the GDC and the PDC (Bourguignon et al., 1996). Finally, there is no structural interaction between the H protein and the L protein; rather, the L protein only recognizes the lipoyl moiety bound to the H protein (Faure et al., 2000; Neuburger et al., 2000). Thus, it seems reasonable that moderate differences in protein structure will not change the effectiveness of this LPD as a subunit in the complex.

On the other hand, these multienzyme complexes all play key roles in different biochemical pathways with the PDC and α -ketoglutarate dehydrogenase complex controlling carbon flow in the citric acid cycle and the GDC an essential enzyme of photorespiration. This suggests different mechanisms of regulation. The genes encoding for the subunits of GDC are strongly light induced. Being part of photorespiration, GDC occurs mainly in photosynthetically active organs such as leaves and stems. mtPDC is essential in all organs and is expressed fairly evenly throughout the plant. The branched-chain α -ketoacid dehydrogenase complex in Arabidopsis has been suggested as an alternative carbon energy source important during the degradation of branched-chain amino acids during leaf senescence and during stress-induced sugar starvation (Fujiki et al., 2000).

The central role of LPD in so many pathways raises the question of how the regulation of a single copy gene encoding this protein could fulfill all these different requirements. The aim of this paper was to address this question in Arabidopsis by cloning the cDNA, performing molecular and biochemical analysis, and investigating transgenic plants.

RESULTS

Isolation and Characterization of Two cDNAs Encoding Mitochondrial Lipoamide Dehydrogenases

Two similar, but not identical, expressed sequence tag (EST) clones for mitochondrial lipoamide dehydrogenase were identified for Arabidopsis. A full-length, 1,918-bp cDNA clone was obtained for *mtLPD2* that is identical to the partial EST clone T43970 (GenBank accession no. AF228640). No full-length clone identical to the second EST 120K5T7 was obtained. Reverse transcriptase (RT)-PCR with a theoretical forward primer allowed us to obtain the missing 5' coding information of this cDNA. This cDNA was named *mtLPD1* and can be found in GenBank (accession no. AF228639). Because the chromosomal information is now available, the cDNA sequence has been confirmed and updated, containing 1,734 bp, and lacking only the 5'-untranslated region (UTR).

Comparing this 1,734-bp *mtLPD1* with the 1,918-bp *mtLPD2*, both *mtLPD* cDNAs have a coding sequence of 1,524 bp with a nucleotide identity of 83%. The 3'-UTR of *mtLPD1* consists of 189 bp, whereas the one from *mtLPD2* is 272 bp long. The identity be-

tween the two 3'-UTR is only 12%, but has stretches with perfect matches up to 14 bp long. The cloned 5'-UTR from *mtLPD2* is 80 bp long.

mtLPD1 is on chromosome 1 (BAC F21D18, GenBank accession no. AC023673) and *mtLPD2* on chromosome 3 (P1 clone MGD8, GenBank accession no. AB022216). Alignments of the cDNAs with their genomic sequences revealed two introns in each gene. In both cases, the first intron is 270 bp after the start codon and consists of 186 bp (*mtLPD1*) and 365 bp (*mtLPD2*). There are short stretches of identities between the two introns of up to 9-bp perfect matches with an overall 31% identity. The second intron is 11 bp (*mtLPD1*) or 10 bp (*mtLPD2*) after the stop codon consisting of 100 and 97 bp, respectively. In this case, the overall identity is 51% with up to 11 bp of perfect matches. The identities between the two genes (including within the UTRs) clearly points to recent gene duplication.

Comparison of the Two Deduced Amino Acid Sequences from the *mtLPDs* with LPD from Peas and Other Species Confirms Their Identity and Strongly Suggests Mitochondrial Targeting

MtLPD1 consists of 507 amino acids with a calculated molecular mass of 53,984 D. MtLPD2 is also 507 amino acids long with a calculated molecular mass of 53,982 D. The identity between the two proteins is 92%. The two *mtLPDs* were 85% identical to the mitochondrial LPD from peas, 53% identical to the human protein, 55% identical to the yeast protein, and 40% identical to E3 from *E. coli*. All conserved domains characterizing this protein can be found in both *mtLPDs* from Arabidopsis. The FAD-binding domain (amino acids 37–184) with its functional motif (GxGxxG/AxxxG/A) for dinucleotide binding, in the Rossmann fold, and its disulfide active site (CL/VNxGC) are present. The NAD⁺-binding domain (amino acids 185–315) with the motif GxGxIGxExxx-VxxxxG, followed by the central domain from amino acids 316 through 384, are also present. The interface domain (amino acids 385–507) contains the active base His and the stabilizing hydrogen bond partner Glu in the signature motif (HAHPTxxE; Williams, 1992; Vettakkorumakankav and Patel, 1996).

The GDC with its LPD has been characterized at the biochemical level in peas (Walker and Oliver, 1986a, 1986b; Oliver et al., 1990a, 1990b; Bourguignon et al., 1996; Neuburger et al., 2000). The 31-amino acids-long mitochondrial targeting sequence from peas is 70% identical to the one from Arabidopsis, suggesting a mitochondrial location for the two Arabidopsis *mtLPDs*. Further analysis using TargetP (<http://www.cbs.dtu.dk/services/TargetP>) confirmed mitochondrial location with a score of 0.919 for *mtLPD1* and 0.902 for *mtLPD2* and also predicted its cleavage site to be between amino acids 36 and 37 (Phe and Ala) exactly as established in peas. This means

that both mature proteins are 471 amino acids long with a calculated molecular mass of 49,918 D for mtLPD1 and 49,868 D for mtLPD2.

We recently have identified the cDNAs encoding the two plastidic lipoamide dehydrogenases from Arabidopsis and have verified their location by a chloroplast uptake assay (Lutziger and Oliver, 2000). The Arabidopsis mtLPDs showed only 33% identity to the Arabidopsis ptLPDs.

Northern Analysis of *mtLPD1* and *mtLPD2* Showed Differences in Organ-Specific RNA Expressions with *mtLPD1* RNA Expression Being Strongly Light Induced

To obtain some insight into why there are two genes encoding mitochondrial lipoamide dehydrogenase, northern analyses were performed. Different organs from mature Arabidopsis plants were isolated and analyzed. Specific normalized 3'-UTR probes of each gene were used allowing direct comparison of the signals. RNA expression of *mtLPD1* was much stronger in leaves compared with *mtLPD2*. On the other hand, much stronger RNA expression of *mtLPD2* was found in roots. All other organs showed about equal RNA expressions of the two *mtLPD* genes (Fig. 1).

To examine the light dependence of the mRNA levels for *mtLPD1* and *mtLPD2*, Arabidopsis plants were grown on plates either in complete darkness or under continuous light for 1 week. The plates were then transferred from the dark into light or vice versa. This transfer was set as time zero. At the time indicated after this transfer, RNA was isolated and analyzed. As can be seen in Figure 2, *mtLPD1* RNA expression was strongly light induced and within 8 h reached near-maximum expression consisting of a severalfold increase. The *mtLPD1* RNA expression declined rapidly in plants transferred into the dark.

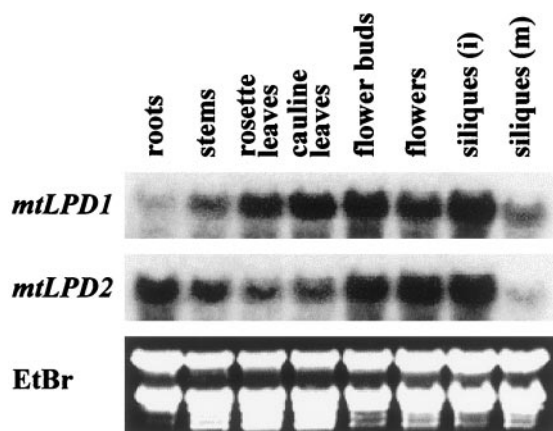


Figure 1. Differential expression of *mtLPD1* and *mtLPD2* mRNA in organs. Northern blot representing 5 μ g of RNA from different organs (i, immature; m, mature) in each lane was hybridized with the normalized specific 3'-UTR probe of each gene. Ethidium bromide staining of the gel is shown for equal loading.

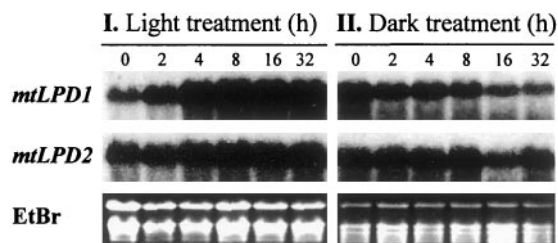


Figure 2. Light-dependent mRNA expression of *mtLPD1* and *mtLPD2* in Arabidopsis. Arabidopsis plants were grown in the dark for 1 week as described in "Materials and Methods" and then transferred to light (I) or grown in the light for 1 week and then transferred to the dark (II). RNA samples were taken at different times as indicated on top of each lane. Ethidium bromide staining of the gel is shown for equal loading.

For comparison, there were only very slight light-dependent changes in RNA expression for *mtLPD2*.

Identification of a T-DNA Knockout Mutant, *mtlpd2*

To determine the roles of these two mitochondrial lipoamide dehydrogenases in Arabidopsis, the Feldmann and Jack T-DNA-tagged mutant lines were screened for a line containing a T-DNA insertion into either *mtLPD* gene. A T-DNA-tagged *mtlpd2* mutant was obtained and all further investigations were performed with a homozygous line for T-DNA-tagged *mtlpd2*. PCR amplification and sequencing revealed a T-DNA insertion into the first intron of *mtlpd2* (Fig. 3A). A Southern blot (Fig. 3B) confirmed T-DNA insertion into *mtlpd2* with a shift to increased fragment sizes, compared with wild type, with several restriction endonucleases. Southern analyses with the *nptII* marker gene of the T-DNA insert revealed that there were two copies of the T-DNA in *mtlpd2* (Fig. 3B). It is not clear whether there are two T-DNA copies at the very same insertion site or at two different linked loci, but the two inserts never segregated through several generations.

RT-PCR analysis was used to ensure the disruption of the *mtlpd2* gene and complete absence of mRNA expression. As can be seen in Figure 4A, lane 4, no cDNA amplification product was visible, using *mtLPD2*-specific primers with the *mtlpd2* mutant, but strong amplification was seen in the wild type (lane 2). As a positive control, RT-PCR was also performed with gene-specific primers for the *mtLPD1* gene (lane 1 and 3). Both the wild-type plant and the *mtlpd2* mutant showed the expected amplification product. Furthermore, this gel and a control gel containing a 500 \times more concentrated load from the RT-PCR reactions of the *mtlpd2* mutant lane, were blotted on a membrane and hybridized with the gene-specific probe for *mtLPD2*. In both cases, strong signals could be observed in the wild-type lane (Fig. 4B), whereas no signal occurred in the mutant lanes even after exposing the blot for several days. Chromosomal contamination was visible in the control (lane 5) of

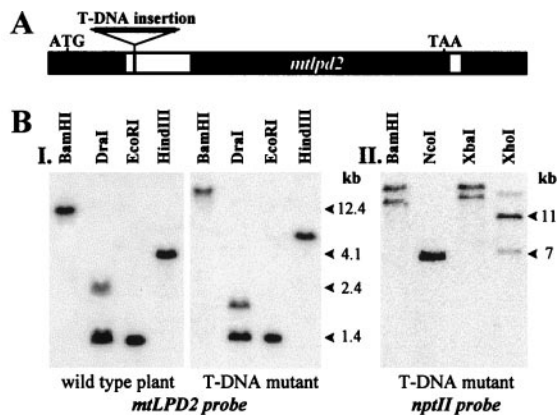


Figure 3. A, Schematic representation of the T-DNA insertion into *mtlpd2*. Black boxes represent exons and white boxes represent introns. B, Southern blot confirms T-DNA insertion into *mtlpd2*. Five micrograms DNA from wild-type cv Wassilewskija as well as mutant containing the T-DNA insertion in *mtlpd2* were digested with the enzymes as indicated. Hybridization in I was performed with the 3'-UTR of *mtLPD2*. In II, 5 μ g DNA was digested with different enzymes and hybridized with a *nptII* probe to reveal the number of T-DNA insertions.

the wild type only. These results strongly suggest that there is no *mtLPD2* expression in this mutant.

T-DNA-Tagged Knockout *mtlpd2* Mutant Had No Apparent Morphological Phenotype

The T-DNA-tagged knockout *mtlpd2* mutant was investigated for potential phenotypes. Mutant and wild-type plants were grown to maturity in a mosaic order (alternating order of wild-type and mutant plants in a tray) in a growth chamber at 21°C under continuous light. No apparent phenotype was observed at any developmental stage. The total weight of the different organs and the developmental time course were measured for a number of single plants and no significant differences were found. Because *mtLPD1* was strongly light induced, the growth of the mutant plants in the dark was measured. There were no significant differences in germination or growth of the etiolated plants. Because *mtLPD2* is more strongly expressed in roots than *mtLPD1*, root growth was analyzed in liquid shaker culture. Mutant and wild-type plants were grown in the dark in liquid culture containing one-half-strength Murashige and Skoog salts. There were no differences in growth rate or root biomass over a 4-week period.

It is possible that stronger expression of *mtLPD1* in the *mtlpd2* mutant than in wild-type plants could compensate for the lack of *mtLPD2*. mRNA levels were examined in different organs in mutant and wild-type plants (Fig. 5). The *mtlpd2* mutant plants did not show elevated mRNA except for a slight increase in mRNA levels in flower buds or flowers. As a control, it can be seen that *mtLPD2* mRNA is only present in wild-type plants.

Total LPD activity was measured from roots and etiolated plants of the *mtlpd2* mutant and wild-type plants to see if there were any biochemical phenotypes associated with the mutation. Two-week-old roots grown in liquid culture from *mtlpd2* mutants had only 38% of the LPD activity found in wild-type plants. Four- to 6-week-old liquid culture *mtlpd2* mutant roots had 85% of the wild-type LPD activity. Etiolated *mtlpd2* mutant plants showed 71% of the LPD activity compared with wild-type plants (Table I).

CO₂ release assays were used to see if the decrease in LPD activity measured in *mtlpd2* mutants was associated with a specific multienzyme complex (Table II). With [1-¹⁴C]pyruvate as the substrate, there were no differences in ¹⁴CO₂ release between wild-type and mutant plants. When [1-¹⁴C]Gly was substrate, mutant plants showed only about 75% the rate of ¹⁴CO₂ release measured with wild-type plants.

DISCUSSION

Molecular and biochemical analyses of lipoamide dehydrogenase in peas indicated a single isozyme encoded by a nuclear single-copy gene (Walker et al., 1986a; Bourguignon et al., 1992, 1996). Because this flavoprotein is a subunit of several multienzyme complexes playing crucial roles in different metabolic pathways, we wanted to investigate the regulation of such a gene. Because transformational techniques are well established in Arabidopsis, in contrast to pea, we investigated this question in this organism.

Arabidopsis has two nuclear-encoded mitochondrial lipoamide dehydrogenase genes. The two *mtLPD* cDNAs are very similar and have the same intron pattern. Both *mtLPDs* show all the domains

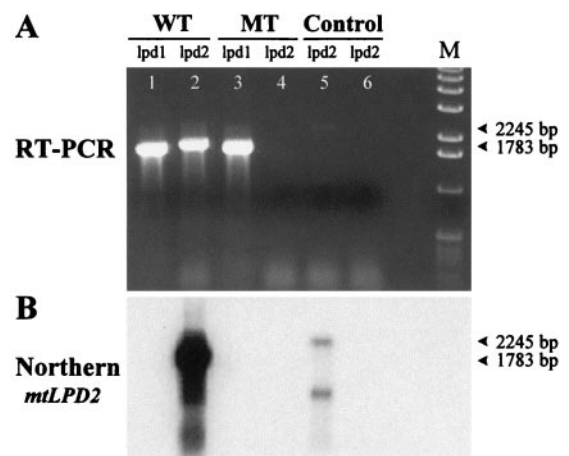


Figure 4. RT-PCR analysis of *mtlpd2* RNA. RT-PCR was performed with a wild-type plant (WT) and a T-DNA mutant (MT) plant, using *mtlpd2*-specific primers (*lpd2*). As a control, *mtLPD1*-specific primers (*lpd1*) were used in lanes 1 and 3. The two controls in lane 5 (wild-type plant) and lane 6 (T-DNA mutant plant) were performed with *mtLPD2*-specific primers by subsiding the RT/Taq Mix with Taq DNA polymerase. The northern blot (B) derived from A was hybridized with 3'-UTR of *mtLPD2*.

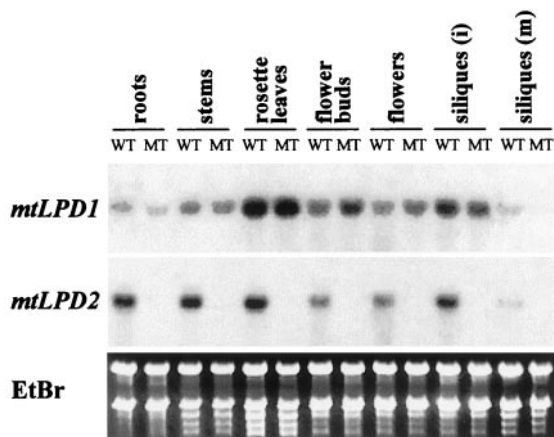


Figure 5. mtLPD RNA expression in different organs comparing wild type and T-DNA mutant, *mtlpd2*. Five micrograms of RNA from different organs was loaded in each lane with WT representing wild type and MT the *mtlpd2* mutant. Hybridization was performed with the 3'-UTR of each gene as indicated. Ethidium bromide staining shows equal RNA loading.

necessary for LPD to perform its enzymatic function (Carothers et al., 1989; Williams, 1992; Vettakkorumakankav and Patel, 1996).

The Arabidopsis mtLPDs have 85% identity to the pea mitochondrial LPD but only 33% identity to their own plastidic counterparts (Lutziger and Oliver, 2000). In plastids, the only known multienzyme complex containing LPD is the plastidic PDC. There are two nuclear-encoded single-copy genes encoding for the plastidic LPDs, *ptLPD1* and *ptLPD2*. The plastidic E2 (Mooney et al., 1999) as well as the plastidic E1 α and E1 β (Johnston et al., 1997) all show approximately 30% identity with the equivalent subunit from the mitochondria but 60% identity with the commensurate cyanobacterial gene. The greater similarity of all the ptPDC subunits to those from cyanobacteria than to the mitochondrial genes with the same functions reinforces the different evolutionary origin of the mitochondrial (endosymbiosis of an ancestral proteobacteria) versus the plastidic (endosymbiosis of an ancestral cyanobacteria) lipoamide dehydrogenases in Arabidopsis.

The finding of two genes encoding lipoamide dehydrogenase in Arabidopsis instead of just one as in peas opened the possibility that *mtLPD1* and *mtLPD2* encode subunits for specific multienzyme complexes. Northern analysis of RNA isolated from different organs showed that the expression of *mtLPD1* was favored in leaves, whereas *mtLPD2* expression was higher in roots. In other organs, stems, flower buds, flowers, and siliques, only slight differences were observed. *mtLPD1* expression was also much more light dependent than expression of *mtLPD2*.

mtLPD2 expression seems to be similar to that of the other subunits of the α -ketoacid complexes. The RNA pattern for *mtLPD2* with stronger expression in

roots than in leaves was also observed with the genes encoding mitochondrial E1 α and E1 β of PDC (Luethy et al., 1994, 1995). *mtLPD1* expression is very similar to that of the P protein and H protein of the GDC with higher expression levels in leaves and strong light induction of gene transcription (Kim and Oliver, 1990; Macherel et al., 1990; Turner et al., 1992a, 1993; Vauclare et al., 1996, 1998).

In peas, the level of mRNA (Bourguignon et al., 1992; Turner et al., 1992b) and the enzyme activity (Walker and Oliver, 1986b) for the L protein showed a lower amount of light induction and organ differentiation than for the other component proteins of GDC. In Arabidopsis, *mtLPD1* seems to co-express with the P protein and H protein of GDC, whereas *mtLPD2* has an expression pattern more similar to the α -ketoacid dehydrogenase complexes. The combined amount of *mtLPD1* and *mtLPD2*, however, looks like the expression of the single L protein gene in peas with lower fold changes in the light and less organ-specific expression.

To test this model that *mtLPD1* mainly made L protein for GDC, whereas *mtLPD2* produced E3 for the α -ketoacid dehydrogenases, a T-DNA knockout mutant, *mtlpd2*, was obtained. Although the T-DNA insertion occurred in the first intron of *mtlpd2*, no *mtlpd2* RNA was detectable by RT-PCR in the T-DNA mutants. The mutation had no visible effect on the plants. The mutant plants showed normal developmental growth with no difference in the weight of mature organs. This was even true for roots and etiolated plants where the *mtLPD2* expression was strongest. The only differences observed were at the biochemical level. LPD activity was decreased in the *mtlpd2* mutant. Younger roots, with 38% of the LPD enzyme activity of wild-type roots, were more strongly affected than older roots with 85% of the wild-type activity. This implies that the ratio of the two mtLPDs contributing LPD activity varied at different developmental stages. *mtLPD2* activity was higher in young roots than in older ones.

The only difference observed between the *mtlpd2* mutant and wild-type plants was a 25% decrease in GDC activity measured as $^{14}\text{CO}_2$ release from [1- ^{14}C] Gly and not the change in PDC activity predicted. This could be explained by the fact that the 92% identity between the two mtLPDs does not distinguish them once the proteins are within the mitochondria so that they can be associated with either multienzyme complexes. The subunits of the GDC are not bound together tightly and readily dissociate in vitro. There is no interaction between the H protein and the L protein; the L protein only recognizes the lipoamide moiety bound to the H protein, not the H protein itself (Faure et al., 2000). The situation is different with the PDC. In mammals, and possibly in plants (Luethy et al., 1996), the E3-binding protein is responsible for a tight association of LPD with the E2

Table I. Comparison of LPD activity in wild-type and T-DNA *mtlpd2* mutant plants

The data were obtained in triplicate, from flasks each containing 10 to 20 seedlings, and the whole experiment was repeated twice.

Organs	Wild-Type Arabidopsis cv Wassilewskija	<i>mtlpd2</i> T-DNA Knockout Mutant	Percent ^a
	$\mu\text{mol NADH mg tissue min}^{-1}$		
Roots (young)	0.0220 \pm 0.0048	0.0083 \pm 0.0012	38 ^b
Roots (old)	0.0143 \pm 0.0044	0.0121 \pm 0.0030	85
Etiolated plants	0.0202 \pm 0.0028	0.0143 \pm 0.0038	71 ^b

^a Percentages are calculated based on wild type being 100%. ^b Difference between wild type and mutant significant at $P < 0.05$.

subunit. As a result, the interactions between the different subunits in the α -ketoacid dehydrogenase complexes are more stable than those in GDC and these complexes do not dissociate as readily in vitro. This could explain why the PDC would retain activity in a *mtlpd2* mutant, whereas GDC would not. The tighter interactions of the PDC subunits and the E3-binding protein would keep the mtLPDs associated to these complexes, whereas in the *mtlpd2* mutant only the leftover mtLPDs not in PDC would be available to GDC. GDC activity would decrease, whereas PDC activity would be affected less.

Under normal conditions, the *mtLPD1* appears to be regulated to supply L protein when GDC is being made and *mtLPD2* is controlled in such a manner that it is producing E3 protein when the α -ketoacid dehydrogenases are being synthesized. The LPD proteins, however, are so similar that once they are made they can work in either multienzyme complex.

No T-DNA-tagged *mtlpd1* mutants were found. This was not surprising because we assumed that a homozygous knockout *mtlpd1* mutant would probably be lethal. In photosynthetically active leaves, GDC makes up 30% to 50% of the total matrix protein, thus requiring strong expression of the *mtLPD* genes to provide sufficient protein. If this is also the case in Arabidopsis, the low level of *mtLPD2* mRNA expressed in leaves would not be able to satisfy the need for mtLPDs to sustain photorespiration, resulting in a lethal mutation.

Searching the Arabidopsis Database, we found that one gene encoding for the H protein (*GDCH*) as well as one gene encoding for the P protein (*GDCP*) are located on chromosome 2. Another *GDCP* gene was found on chromosome 4 and a second H protein gene on chromosome 1. The gene encoding for the T protein was located on chromosome 1. In peas, it has been shown that the *GDCT* and *GDCL* are linked together on chromosome 7, whereas *GDCP* and *GDCH* can be found on different chromosomes (Turner et al., 1993). Single-copy genes are reported for all of the subunits encoding GDC in peas. This is in contrast to Arabidopsis, where we found two genes encoding for the H, L, and P proteins and one for the T protein.

MATERIALS AND METHODS

Isolation and Characterization of the cDNAs Encoding mtLPDs

The cDNA encoding the L protein from peas (*Pisum sativum*, GenBank accession no. X63464) was used to search for EST clones from Arabidopsis via BLAST at The Arabidopsis Information Resource (<http://www.arabidopsis.org>). Two partial EST clones were obtained from the Arabidopsis Biological Resource Center (ABRC, Columbus, OH; clone 120K5T7, GenBank accession no. T43970; clone 104E16T7, GenBank accession no. T22366). These partial genes were used as probes to screen a λ PRL2 library using

Table II. Comparison of $^{14}\text{CO}_2$ release from $[1-^{14}\text{C}]$ pyruvate or $[1-^{14}\text{C}]$ glycine from wild-type and T-DNA *mtlpd2* mutant plants

Each data set represents independent experiments using at least five different mutant and wild-type root samples. The roots used have the same developmental stage in each data set, but are of increasing age in the following lanes. The nos. in bold represents the average of the data from those three experiments.

	Wild-Type Arabidopsis cv Wassilewskija	<i>mtlpd2</i> T-DNA Knockout Mutant	Percent ^a
	$\mu\text{mole CO}_2 \text{ mg root h}^{-1}$		
$[1-^{14}\text{C}]$ Pyruvate: $^{14}\text{CO}_2$ release	76.1 \pm 13.1	77.3 \pm 5.8	102
	59.5 \pm 8.2	62.3 \pm 3.4	105
	61.4 \pm 7.5	58.8 \pm 4.1	96
	65.6 \pm 12.0	66.1 \pm 9.3	101
$[1-^{14}\text{C}]$ Gly: $^{14}\text{CO}_2$ release	0.263 \pm 0.046	0.207 \pm 0.049	79
	0.212 \pm 0.065	0.165 \pm 0.031	78
	0.142 \pm 0.079	0.103 \pm 0.039	73
	0.179 \pm 0.073	0.134 \pm 0.046	75^b

^a Percentages are calculated based on wild type being 100%. ^b Difference between wild type and mutant significant at $P < 0.05$.

standard techniques (Sambrook et al., 1989) and one full-length clone was isolated. This cDNA was sequenced and named *mtLPD2* (GenBank accession no. AF228640). The missing 5' end of the other gene, *mtLPD1* (GenBank accession no. AF228639), was obtained by RT-PCR as described previously (Lutziger and Oliver, 2000) using a forward primer starting right after the start codon designed according to *mtLPD2* (5'-GCGATGGCGAGCTTAGCTAGG-3').

Northern Analysis

Total RNA from different organs and at different developmental stages were all isolated from Arabidopsis and northern analysis performed as described previously (Lutziger and Oliver, 2000). For the light-dependent expression studies, Arabidopsis seeds were sterilized with 70% (v/v) ethanol for 15 s followed by incubation in 50% to 60% (v/v) bleach containing a few drops of 10% (w/v) SDS for 10 to 15 min and rinsed several times with sterile water. Seeds were then plated out on germination medium (one-half-strength Murashige and Skoog Salt Mixture [Gibco-BRL, Carlsbad, CA], pH 5.6–5.8, and 2 g L⁻¹ phytigel [Sigma, St. Louis]). One-week-old seedlings either grown in the dark at room temperature or in continuous light at 21°C were then transferred, set at time 0 h, either from the dark into the light or vice versa. RNA samples were then taken at different times after the transfer with a control at time zero.

T-DNA-Tagged *mtlpd2* Mutant Isolation

The T-DNA pools containing DNA from about 6,000 T-DNA lines generated by Feldmann, as described in the ABRC Seed and DNA Catalog (1997), with the 3850:1003 Ti plasmid in the Wassilewskija background were obtained from the ABRC at the Ohio State University (stock number: CD5-7). PCR analysis was performed using the provided T-DNA left and right border-specific primers in combination with *mtLPD* gene-specific primers to search for possible T-DNA insertion in either *mtLPD* gene. A strong PCR band of about 500 bp was found in one pool using the left T-DNA border primer and a forward *mtLPD* (5'-GCGATGGCGAGCTTAGCTAGG-3')-specific primer. The PCR fragment was isolated, sequenced, and revealed T-DNA insertion into first intron of *mtlpd2*. This line was isolated.

RT-PCR Analysis of *mtLPD* mRNA

Total RNA was isolated from individual Arabidopsis (ecotype Wassilewskija) and from individual T-DNA *mtlpd2* mutants using the TRIzol LS reagent from Gibco-BRL/Life Technologies (Carlsbad, CA) according to their instructions. The SUPERScript One-Step RT-PCR System (Gibco-BRL/Life Technologies) was then used to examine the presence or absence of *mtLPD* mRNA in the individual plants. The primers used were a common forward primer (5'-GCGATGGCGAGCTTAGCTAGG-3') designed after the start codon of *mtLPD* genes and a 3'-UTR specific

reverse primer with 5'-GATCAGGCTTAACACGTATCTG-3' for *mtLPD1* and 5'-CACCGATCATACTGATTAAATCAC-3' for *mtLPD2*. These primers were chosen to distinguish the cDNA from the genomic DNA with its two introns.

LPD Activity Assay

Sterilized Arabidopsis seeds were germinated in 100-mL Erlenmeyer flasks containing 40 mL liquid culture consisting of one-half-strength Murashige and Skoog salt mixture from GibcoBRL and 2.5 mM MES [2-(*N*-morpholino)-ethanesulfonic acid] adjusted to pH 5.8.

The forward reaction of LPD activity, increase in NADH, was measured spectrophotometrically at 320 nm using a DU 7400 (Beckman, Fullerton, CA). Dihydrolipoamide was prepared as described by Butterworth et al. (1975) and dissolved in ethanol to a final concentration of 10 mM. The enzyme was extracted from organs in 150 mM KPi (pH 8.0) and 0.5% (v/v) Triton X-100. Reaction buffer consisted of 50 mM KPi (pH 8.0), 0.25 mM NAD⁺, and 1.5 mM EDTA. The reaction buffer (970 μL) was mixed with 10 μL of organ extract and the reaction rate was measured for 1 min (control). Then, 20 μL dihydrolipoamide was added and the reaction rate was measured for another minute. Total protein was measured using the bicinchoninic acid protein assay by Pierce (Rockford, IL).

Radioactive CO₂ Release Assay

Sterile Arabidopsis seeds were germinated as described above. After growth on a shaker for 3 to 4 weeks, roots were collected and about 100 mg was distributed into 25-mL Erlenmeyer flasks containing 2 mL of water and either plus 20 μL [1-¹⁴C]pyruvate-Na (2.9 × 10⁵ cpm μmole⁻¹:0.132 mCi mmol⁻¹ measured) or 5 μL [1-¹⁴C]Gly (52.4 mCi mmol⁻¹). After 10 min of vacuum infiltration, the Erlenmeyer flasks were closed with rubber stoppers containing plastic filter holders, in which small pieces of 3-MM paper were inserted. Immediately before capping, 20 μL of 70% (v/v) triethanolamine was added to the filter. The flasks were shaken for 2 h at room temperature, at which time the feeding experiment was stopped by adding 100 μL of 1 M sulfuric acid. After an additional hour of shaking, the filter papers were transferred into 5 mL of liquid-scintillation cocktail (Ready Safe by Beckman Coulter, Fullerton, CA) and radioactive CO₂ release was counted in the Multi-Purpose Scintillation Counter LS 6500 by Beckman. Data were collected and analyzed with InStat Instant Biostatistics by GraphPad Software, Inc. (San Diego).

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