Deletion of Glycine Decarboxylase in Arabidopsis Is Lethal under Nonphotorespiratory Conditions[1][W][OA]

Nadja Engel, Kirsten van den Daele, Üner Kolukisaoglu[2], Katja Morgenthal, Wolfram Weckwerth, Tiit Pärnik, Olav Keerberg, and Hermann Bauwe*

Department of Plant Physiology, University of Rostock, D–18059 Rostock, Germany (N.E., K.v.d.D., Ü.K., H.B.); Max Planck Institute of Molecular Plant Physiology, D–14476 Golm, Germany (K.M., W.W.); and Estonian University of Life Sciences, Institute of Agricultural and Environmental Sciences, 51014 Tartu, Estonia (T.P., O.K.)

The mitochondrial multienzyme glycine decarboxylase (GDC) catalyzes the tetrahydrofolate-dependent catabolism of glycine to 5,10-methylene-tetrahydrofolate and the side products NADH, CO_2, and NH_3. This reaction forms part of the photorespiratory cycle and contributes to one-carbon metabolism. While the important role of GDC for these two metabolic pathways is well established, the existence of bypassing reactions has also been suggested. Therefore, it is not clear to what extent GDC is obligatory for these processes. Here, we report on features of individual and combined T-DNA insertion mutants for one of the GDC subunits, P protein, which is encoded by two genes in Arabidopsis (*Arabidopsis thaliana*). The individual knockout of either of these two genes does not significantly alter metabolism and photosynthetic performance indicating functional redundancy. In contrast, the double mutant does not develop beyond the cotyledon stage in air enriched with 0.9% CO_2. Rosette leaves do not appear and the seedlings do not survive for longer than about 3 to 4 weeks under these nonphotorespiratory conditions. This feature distinguishes the GDC-lacking double mutant from all other known photorespiratory mutants and provides evidence for the nonreplaceable function of GDC in vital metabolic processes other than photorespiration.

The mitochondrial multienzyme complex Gly decarboxylase (GDC) contributes to the two strategically important metabolic pathways of (1) photorespiration in all photosynthesizing organs and (2) one-carbon metabolism in all biosynthetically active tissues. In each of these two metabolic contexts, GDC closely cooperates with a second mitochondrial enzyme, Ser hydroxymethyltransferase (SHM), in the conversion of Gly to Ser. In the course of the tetrahydrofolate (THF)-dependent GDC reaction cycle comprising three individual reactions, CO_2 and NH_3 are released, and NAD^+ becomes reduced to NADH. The remaining methylene moiety becomes attached to THF to produce the one-carbon donor compound 5,10-methylene-THF (CH_2-THF). SHM subsequently synthesizes Ser from CH_2-THF and a second molecule of Gly in a fully reversible reaction (Douce et al., 2001; Hanson and Roje, 2001).

The combined GDC/SHM reaction represents the mitochondrial part of the photorespiratory C_2 cycle, which occurs in all photosynthesizing tissues of C_3 plants, extends over three cellular compartments, and converts Rubisco-generated 2-phosphoglycolate into the Calvin cycle metabolite 3-phosphoglycerate (Tolbert, 1997; Douce and Neuburger, 1999). The importance of GDC and SHM for photorespiration becomes apparent from the fact that all as yet-reported mutants and antisense plants show strong metabolic disturbations in normal air, but grow well in the nonphotorespiratory conditions of approximately 1% CO_2 (Somerville and Ogren, 1981, 1982; Blackwell et al., 1990; Artus et al., 1994; Dever et al., 1995; Wingler et al., 1999; Heineke et al., 2001; Voll et al., 2006). Notably, the exact nature of the genetic defect is not known for any of the reported GDC-deficient mutants and it is unclear whether GDC is completely deleted in these mutants.

In contrast to the homotetrameric SHM (Schirch and Szebenyi, 2005), GDC comprises four proteins, which are usually named P, T, L, and H protein (Walker and Oliver, 1986). The presence of all four proteins is necessary for catalytic activity of the complex. H protein carries no enzymatic activity but interacts as a mobile substrate via its lipoamide arm one after the other with P, T, and L protein (Douce et al., 2001). In Arabidopsis (*Arabidopsis thaliana*), these four proteins are encoded by a total of seven genes, two genes each for P and L protein, three genes for H protein, and one

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[2] Present address: Adeborsweg 33, D–18184 Neuendorf, Germany.

* Corresponding author; e-mail hermann.bauwe@uni-rostock.de; fax 49–381–498–6112.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Hermann Bauwe (hermann.bauwe@uni-rostock.de).

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gene for T protein (Arabidopsis Genome Initiative, 2000; Bauwe and Kolukisaoglu, 2003).

While GDC is restricted to mitochondria, nonmitochondrial isoforms of SHM exist that reside in the cytosol, in plastids, and possibly in the nucleus (Turner et al., 1992; Besson et al., 1995; Neuburger et al., 1996; McClung et al., 2000). According to current hypotheses on plant one-carbon metabolism, Ser acts as a vehicle transporting CH_2 units from the mitochondria to other compartments of the cell, mainly the cytosol, where CH_2-THF is resynthesized by specific SHM isoforms to subsequently feed a multitude of biosynthetic reactions. The remaining Gly becomes reconverted into Ser by GDC and mitochondrial SHM. It has been suggested that this Gly-Ser cycle may be obligatory for one-carbon metabolism (Mouillon et al., 1999).

For several reasons, this hypothesis is not undisputed (Hanson and Roje, 2001). First, the aforementioned curing effect of elevated CO_2 on the growth of GDC-deficient mutants appears reasonable for a blockade in the photorespiratory C_2 cycle, but is in apparent contradiction with the suggested irreplaceable function of GDC in one-carbon metabolism. It shall be noted that residual GDC activity has been reported for such mutants (for example, Li et al., 2003). Moreover, it was reported that CH_2-THF and Ser can be synthesized through multiple routes including the so-called C1-THF synthase/SHM pathway (Prabhu et al., 1996; Li et al., 2003). In this GDC-bypassing pathway, formate would be converted into CH_2-THF via two enzymes, 10-formyl-THF synthetase and the bifunctional enzyme CH_2-THF dehydrogenase/CH_2-THF cyclohydrolase. In the context of the photorespiratory cycle, it was suggested that formate could originate from the hydrogen peroxide-dependent nonenzymatic decomposition of glyoxylate (Grodzinski, 1978; Igamberdiev et al., 1999). While the in vivo significance of this reaction for higher plants is questioned by some authors (Yokota et al., 1985), others have suggested that a C1-THF synthase-based alternative photorespiratory pathway could bypass the GDC reaction (Wingler et al., 1999).

In this report, we intend to bring more clarity into the question whether GDC is obligatory for processes other than photorespiration. To this end, we have isolated and characterized T-DNA insertion mutants for each of the two Arabidopsis P-protein genes. We find that the individual knockout of any of the two genes does not significantly affect growth or other properties of the respective mutants. In contrast, the combined knockout of both P-protein genes leads to an arrest of seedling development at the cotyledon stage even under the nonphotorespiratory conditions of 0.9% CO_2. This indicates the possibility that all hitherto reported GDC mutants may be leaky. We suggest that the GDC reaction very likely cannot be bypassed in higher plants and that some GDC activity is indispensable for crucial metabolic processes other than photorespiration, most likely Gly-Ser cycling during one-carbon metabolism.

RESULTS AND DISCUSSION

GDC is important for at least two areas of plant primary metabolism, photorespiration, and one-carbon metabolism. However, possible bypass reactions have been suggested (Wingler et al., 1999; Wingler et al., 2000), and it is not known whether GDC is absolutely obligatory in these two areas. This uncertainty results from the fact that all as yet-reported GDC-deficient mutants grow healthy in air supplemented with approximately 1% CO_2, i.e. under conditions that strongly suppress the synthesis of 2-phosphoglycolate by Rubisco. As already mentioned, evidence for the functional total knockout of GDC has not been provided for any of these mutants.

Two Redundant P-Protein Genes Are Present in Arabidopsis

To produce a genetically defined GDC-knockout mutant in Arabidopsis, we choose to isolate T-DNA insertion lines for the two P-protein genes, *AtGLDP1* (*At4g33010*) and *AtGLDP2* (*At2g26080*), for subsequent crossing. Focusing on the P-protein genes appeared feasible for several reasons. First, T-DNA knockout lines for the single gene encoding T protein in Arabidopsis are not available from stock centers. Second, the combined knockout of the three genes encoding H protein is possible but less practical. Third, L protein is a component of other multienzyme complexes, too (Bourguignon et al., 1996; Mooney et al., 2002).

A sequence comparison revealed 91% sequence identity for the two predicted mitochondrial precursor P proteins. Similar to the two described P-protein genes of *Flaveria pringlei* (Bauwe et al., 1995), an additional intron in *AtGLDP2* represents the most obvious structural difference between the two Arabidopsis *GLDP* genes (Fig. 2A). These and other structural data gave no hint on possible functional differences. To find out whether any of the two Arabidopsis P-protein genes might be of particular importance for the photorespiratory cycle, we next examined transcript abundances in different Arabidopsis organs by reverse transcriptase (RT)-mediated PCR (Fig. 1). This semiquantitative analysis revealed that both transcripts are present in all examined organs with a slight dominance of *AtGLDP1* transcripts in photosynthesising organs. Rosette and cauline leaves show higher transcript levels for both genes in comparison with nonphotorespiring organs. These results correspond well with electronic northern data (https://www.genevestigator.ethz.ch/at/) that indicate a high *AtGLDP1*/*AtGLDP2* transcript ratio (two for rosette leaves, three for cauline leaves) for photosynthesising organs. In contrast, *AtGLDP2* transcripts dominate about 3-fold in roots (Supplemental Fig. S1; Zimmermann et al., 2004).

In light of the consistency of these RT-PCR and in silico expression data, which did not reveal strong dominance of one of the two *AtGLDP* genes in photosynthetic tissue, we next examined the effects

of individual knockouts for each of the two genes. To this end, allelic T-DNA insertion lines were isolated for both genes (Fig. 2A). The presence and location of the T-DNA insertions were verified by PCR with genomic DNA (for an example of *Atgldp1-1* and *Atgldp2-2* see Fig. 2B) and sequencing of PCR products. RT-PCR with total leaf RNA and gene-specific primers revealed complete absence of the respective *GLDP* transcript for all homozygous mutants (for *Atgldp1-1* and *Atgldp2-2* as examples, Fig. 2C). However, this was not reflected on the P-protein level, because western-blotting analyses did not show a clear reduction of the P-protein content in leaves of any of the individual knockout lines (Fig. 2D). In agreement with this wild-type-like P-protein content, the general appearance and growth of the mutants was unaltered in comparison with wild-type plants grown under identical conditions (Fig. 4A). The slightly different appearance of the *Atgldp1-2* mutant plants is related to the different genetic background (ecotype Wassilewskija instead of Columbia). All these findings suggested that the complete loss of either P-protein gene can be well compensated by the remaining intact gene.

To further support this result, we next determined leaf Gly/Ser ratios that provide a very sensitive measure of any disorder in the photorespiratory Gly-to-Ser conversion (Heineke et al., 2001) and are correlated with photorespiratory rates (Novitskaya et al., 2002). In accordance with the unaltered phenotype and growth of the mutants, the analysis of plants grown under standard light intensity and temperature revealed only minor differences in the Gly/Ser ratio between wild-type and mutant plants (Fig. 3, top). To boost up possible effects of the mutations on photorespiratory metabolism, a second set of plants was exposed for 4 h to high light intensities of 800 to 1,000 μmol s^{-1} m^{-2} in combination with a 4°C to 5°C higher temperature. Notably, this treatment led to a distinctly

Figure 1. *GLDP1* and *GLDP2* transcripts are present in all examined tissues of Arabidopsis but dominate in photosynthesizing organs. The figure shows a typical RT-PCR experiment using transcript-specific primers with RNA from roots (RT), rosette leaves (RL), stems (ST), cauline leaves (CL), flowers (FL), and siliques (SI). The same sense primer (P12-S2) and different gene-specific antisense primers (P1-A2RT for *AtGLDP1*, and P2-A2RT for *AtGLDP2*) were used for PCR. Signals of the constitutively expressed *At2g09990* mRNA were used for internal calibration (primers S16-S and S16-A).

Figure 2. Isolation of T-DNA insertion mutants for the Arabidopsis genes encoding P protein of GDC. A, Schematic presentation of genes *AtGLDP1* and *AtGLDP2* and approximate positions of T-DNA insertions including that of the knockdown allele *Atgldp2-1* (triangles). B, PCR with genomic DNA shows absence of the intact *AtGLDP1* gene in two homozygous *Atgldp1-1* mutant plants (1 and 2) and its presence in wild type (W). This is due to the insertion of T-DNA (3 and 4 for the same two plants) that does not occur in the wild-type control (W). Analogous results were obtained for *Atgldp2-2* (5 and 6, absence of *AtGLDP2* in two homozygous knockout plants; W, Wild type; 7 and 8, presence of the T-DNA insertion in the same two plants). Absence of *AtGLDP1* and *AtGLDP2*, respectively, was also verified for homozygous *Atgldp1-2* and *Atgldp2-3* plants (data not shown). Primer pairs for PCR were P12-S1 and P1-A1n (*Atgldp1-1*) and P2-2Check-S and P2-A2n (*Atgldp2-2*). DNA size markers are shown. C, RT-PCR shows absence of the respective transcripts in homozygous plants of *Atgldp1-1* (1-1) and *Atgldp2-2* (2-2) and their presence in wild type (W). The same sense primer (P12-S2) and different gene-specific antisense primers (P1-A2RT for *AtGLDP1*, and P2-A2RT for *AtGLDP2*) were used for PCR. Analogous results were obtained for homozygous *Atgldp1-2* and *Atgldp2-3* plants (data not shown). D, Western-blotting analysis with a specific antiserum shows no difference in leaf P-protein content in comparison to wild type (WT) and the individual homozygous *Atgldp1-1* (*ho1-1*) and *Atgldp2-2* mutants (*ho2-2*), but a reduction in the homozygous *Atgldp1-1*/heterozygous *Atgldp2-2* double mutant (*ho1/he2*, two individual plants). Lanes of a 15% SDS-polyacrylamide gel were loaded with 20 μg total leaf protein each (Bradford, 1976).

Figure 3. Leaf contents of Gly and Ser, except slightly more Gly in the *Atgldp1* mutants, remain essentially unchanged in either of individual P-protein knockout mutants in comparison with wild-type plants grown under 150 to 180 μE m^{-2} s^{-1} in normal air (top). Exposure to a 5-fold higher light intensity of 800 to 1,000 μE m^{-2} s^{-1} and a 4°C to 5°C higher temperature for 4 h results in distinctly elevated leaf Gly levels in the *Atgldp1* mutants but not in *Atgldp2* (bottom). Grey and white columns represent Gly and Ser, respectively. Note different scales. Error bars represent ±SD.

stronger increase in leaf Gly content of the *Atgldp1-1* mutant in comparison with both *Atgldp2-2* and wild-type plants (Fig. 3, bottom). In accordance with the expression data discussed above, these data suggest a somewhat higher contribution of AtGLDP1 to the photorespiratory catabolism of Gly. Clear differences between wild-type and mutant plants in the contents of amino acids other than Gly and Ser were not observed under both conditions (data not shown).

The Redundancy of Arabidopsis P-Protein Genes Is Reflected by Metabolite Profiling Analyses and Photosynthetic Performance

To further investigate the role of the two P-protein genes in Arabidopsis, we individually performed gas chromatography-coupled time-of-flight mass spectrometric-based metabolite profiling analyses of the isolated T-DNA insertion lines *Atgldp1-1*, *Atgldp1-2*, *Atgldp2-2*, and *Atgldp2-3*. These experiments resulted in the relative quantification of 80 polar metabolites, mainly amino acids, intermediates of the tricarboxylic acid cycle, and soluble sugars. No significant differences in the metabolite profiles and the Gly/Ser ratio between wild-type and mutant plants grown under low photorespiratory conditions (approximately 0.14% CO_2) could be detected. Growth in normal air led only to slight differences in the content of a very few selected metabolites. In accordance with the amino acid data shown in Figure 3, clear differences could be identified for the leaf contents of Gly for *Atgldp1-1* and *Atgldp1-2*, which were significantly elevated under photorespiratory conditions. In contrast to our recent investigations on glycerate kinase photorespiratory mutants, which allowed unambiguous classification by metabolite profiling (Boldt et al., 2005), these analyses of P-protein mutant plants grown under photorespiratory and low photorespiratory conditions revealed gene redundancy and similarity to the wild-type plants. This finding is in agreement with the results discussed above and also suggests that the complete loss of either of the two P proteins in Arabidopsis can be compensated by the remaining other P protein.

Because of these clear though still moderate knockout effects on the Gly/Ser ratio of *Atgldp1* allelic mutants under high light intensity, we wanted to find out whether the mutation affects photosynthetic gas exchange to any measurable extent. However, even by using a very sensitive radiogasometric method (Pärnik and Keerberg, 2007), we were unable to detect a significant difference in photosynthetic performance relative to wild-type plants (6.84 ± 0.26 versus 6.72 ± 0.25 μmol CO_2 m^{-2} s^{-1} true photosynthesis and 1.66 ± 0.06 versus 1.73 ± 0.07 μmol CO_2 m^{-2} s^{-1} decarboxylation in the light in wild-type and *Atgldp1-1* knockout plants, respectively).

The Combined Knockout of Both P-Protein Genes Is Lethal

To analyze the effects of a total P-protein knockout on metabolism, we combined the individual *Atgldp* mutations in a double-knockout line. To this end, homozygous *Atgldp1-1* (kanamycine-resistant enhancer trap line, Campisi et al., 1999) was crossed with homozygous *Atgldp2-2* plants (BASTA-resistant SAIL line, Sessions et al., 2002). F2 seeds were then germinated and grown in air enriched with approximately 0.14% CO_2, i.e. under conditions that allow satisfactory growth of all other known photorespiratory mutants. Next, 51 F2 plants were examined for the presence of both the T-DNA and the respective wild-type *AtGLDP* genes by PCR with genomic DNA and specific primers. No double knockouts were found at this stage; however, five plants were homozygous with respect to *Atgldp1-1* and heterozygous for *Atgldp2-2*. The leaf P-protein content in these plants was distinctly reduced (Fig. 2D), but clear phenotypic alterations did not exist in comparison with wild-type plants (Fig. 4B, left). Next, these F2 plants were selfed and the F3 progeny germinated on Murashige and Skoog medium in a CO_2 concentration close to 1% in combination with moderate light intensity. Under these conditions, which very strongly suppress 2-phosphoglycolate synthesis by Rubisco, several very small plants with whitish leaves and a growth arrest at the cotyledon stage segregated (Fig. 4B, middle). Shadowing by leaves of neighboring plants resulted in minor chlorophyll biosynthesis, but growth was not significantly improved (Fig. 4B, right). PCR analysis of genomic DNA confirmed the absence of *AtGLDP* wild-type alleles in these plants (Fig. 4C), and RT-PCR showed absence of the respective transcripts (data not shown). Typically,

Figure 4. Complete deletion of P protein is lethal, while knockout of the two individual P-protein genes shows no visible effects. A, Individual homozygous mutants grow similarly as wild-type plants in normal air. B, The *Atgldp1-1xAtgldp2-2* double-homozygous GDC-knockout plants do not germinate in normal air (data not shown) and do not develop beyond the cotyledon stage in air enriched with 0.9% CO_2 (nonphotorespiratory conditions). Left, Wild-type-like plant that is homozygous in *Atgldp1-1* and heterozygous in *Atgldp2-2*; middle, development of the double-homozygous mutant is arrested at the cotyledon stage; right, double-homozygous seedlings (arrow) develop somewhat better and seem to accumulate minor amounts of chlorophyll when the mutant is shadowed by other plants. Seeds were germinated and kept on Murashige and Skoog medium for two weeks, but seedlings do not survive for longer than three to four weeks. C, PCR with genomic DNA isolated from a double-homozygous mutant with identical phenotype as shown in B (middle) using gene-specific and T-DNA border-specific primers for *AtGLDP1* and *AtGLDP2*, respectively. Lanes show the absence of gene-specific signals (1 and 5 for *AtGLDP1* and *AtGLDP2*, respectively) in comparison with wild-type DNA (2 and 6) and the presence of the T-DNA insertion (3 and 7 for *AtGLDP1* and *AtGLDP2*, respectively) in comparison with wild-type DNA (4 and 8). Primer pairs for PCR were P12-S1 and P1-A1n (*Atgldp1-1*) and P2-2Check-S and P2-A2n (*Atgldp2-2*). DNA size markers are shown (M). Transcripts were undetectable for either of the two P-protein genes (data not shown).

these double-mutant plants do not survive for longer than 3 to 4 weeks even under the nonphotorespiratory growth conditions of 0.9% CO_2 in combination with low light intensity.

From studies on one-carbon metabolism in Arabidopsis, it has been suggested that Ser synthesis can occur using CH_2-THF synthesized from formate via the C1-THF synthase system thus providing an alternative one-carbon source and an important means of compensating for the lack of GDC activity (Li et al., 2003). Similarly, the analysis of GDC-deficient barley (*Hordeum vulgare*) and Arabidopsis mutants led to the conclusion that the hydrogen peroxide-dependent nonenzymatic decomposition of glyoxylate to formate in combination with the C1-THF synthase system may bypass GDC and act as an alternative route to the normal photorespiratory pathway (Wingler et al., 1999). We therefore added different concentrations of 0.1 to 2 mM formate to the medium, but could not vitalize the double-knockout plants by this treatment (data not shown). Due to the lethality of the GDC knockout and the failure to chemically complement the double mutants, we have not yet been able to grow and analyze these plants any further.

CONCLUSION

The Arabidopsis genome harbors two essentially redundant P-protein genes that are both expressed in all organs, although in different ratios. *AtGLDP1* transcripts dominate in photosynthesizing organs while *AtGLDP2* transcripts are more abundant in roots. The corresponding P proteins, AtGLDP1 and AtGLDP2, can more or less fully substitute each other under standard growth conditions. This can be inferred from the very similar phenotype and growth behavior of knockout mutants for both genes in comparison with wild-type plants and is further supported by biochemical and gas-exchange data. Differences become apparent only under conditions that promote photorespiration, such as high light intensity and elevated temperature. While AtGLDP1 clearly carries a somewhat higher photorespiratory workload than AtGLDP2, the two types of mutants neither show any clear photorespiratory phenotype nor do they require elevated CO_2 for adequate growth. Therefore, the reported GDC-deficient Arabidopsis mutants *gld1* and *gld2* (Somerville and Ogren, 1982; Artus et al., 1994) cannot be defective in P-protein biosynthesis. Recent data from our laboratory point to incomplete lipoylation of H protein caused by defective mitochondrial lipoate biosynthesis in the *gld1* mutant (R. Ewald, U. Kolukisaoglu, U. Bauwe, S. Mikkat, and H. Bauwe, unpublished data). The genetic defect in *gld2* is not exactly known either, but, due to its location on Arabidopsis chromosome 5 (Artus et al., 1994), could be related to posttranslational processes as well. It must be noted that, except the attachment of the pyridoxal phosphate, posttranslational modification of P protein has not been reported.

In clear contrast to the individual T-DNA insertion mutants, the double-homozygous *Atgldp1xAtgldp2* mutant does not survive beyond the cotyledon stage and cannot be recovered by growth under nonphotorespiratory conditions. This unique feature distinguishes the GDC-lacking mutant described in this report from all other known photorespiratory mutants, for example, Arabidopsis mutants lacking 2-phosphoglycolate phosphatase (Somerville and Ogren, 1979), glycerate kinase (Boldt et al., 2005), or mitochondrial SHM (Voll et al., 2006). It appears that a higher plant mutant

Table I. *Oligonucleotides used for the isolation of Atgldp T-DNA insertion mutants and for RT-PCR experiments*

The lower part of the table lists the oligonucleotides specific for the T-DNA mutagenized populations or individual lines used in this study (INRA, Bechtold et al., 1993; Tom-Jack, Campisi et al., 1999; SAIL, Sessions et al., 2002). LB, Left border.

Name	Gene or Population	Border	Sequence (5′ → 3′)
P12-A1	*AtGLDP1* and *2*		GTTTATCGACATCATCCAAGGTGGTTG
P12-S1	*AtGLDP1* and *2*		GCTCATGCTAATGGTGTTAAGGTTG
P12-S2	*AtGLDP1* and *2*		TACTTACATTGCCATGATGGGATCTG
P1-A1n	*AtGLDP1*		CAAATCTGTATGATGCTATGAGAG
P2-A1n	*AtGLDP2*		GCAACAGTAATCTTAAAGAACAAG
P1-A2n	*AtGLDP1*		CCCTGAGAAAACAAACAACCACC
P2-A2n	*AtGLDP2*		AGTATCAGACTAGAGGGCTAGTC
P1-A2RT	*AtGLDP1*		GCAGCTGCGACTTGTTCTTCCTCT
P2-A2RT	*AtGLDP2*		CAGCTGCAGCCTGTTCTTCGTTC
P2-2Check-S	*AtGLDP2*		CAATGTCACTTTTGGGTCAATCCAC
S16-S	*At2g09990*		GGCGACACAACCAGCTACTGA
S16-A	*At2g09990*		CGGTAACTCTTCTGGTAACGA
VsLB1	INRA	LB	CTACAAATTGCCTTTTCTTATCGACCATG
SAIL-LB1	SAIL	LB	GCCTTTTCAGAAATGGATAAATAGCCTGCTTCC
SynLB1	SAIL	LB	GCAGACTTTGCTCATGTTACCGATG
TJLB1	Tom-Jack	LB	GAACATCGGTCTCAATGCAAAAGGGGAAC

lacking GDC cannot be easily isolated by conventional low CO_2/high CO_2 screens as they were used in earlier pioneering studies not only for Arabidopsis but also for other plants (Blackwell et al., 1988). We therefore believe that all as yet-reported GDC-deficient mutants are leaky with some residual GDC activity.

The inability of the P-protein double mutant to grow under nonphotorespiratory conditions suggests that deletion of GDC, in addition to the detrimental effect on the photorespiratory C_2 cycle, abolishes other crucial metabolic processes as well. It has been proposed that the mitochondrial Gly-to-Ser conversion, by recycling Gly originating from extramitochondrial SHM reactions, represents an obligatory component of one-carbon metabolism not only in photosynthetic but in all plant tissues (Mouillon et al., 1999). On the other hand, a GDC-independent pathway for the supply of CH_2-THF, driven by the C1-THF synthase system, has been suggested as an important means of compensating for the lack of GDC activity (Prabhu et al., 1996; Wingler et al., 1999; Li et al., 2003). The existence of such pathways will be difficult to prove because of the very early developmental arrest of the GDC-lacking double mutant. While the results described in our report cannot entirely exclude their possible presence in later stages of plant development, they indicate that the GDC reaction cannot be bypassed and strongly support Mouillon et al.'s (1999) notion of an obligatory and indispensable role of GDC in nonphotorespiratory plant metabolism.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotypes Columbia (Columbia-0) and Wassilewskija (Wassilewskija-2) were obtained from the Nottingham Arabidopsis Stock Centre and used for this study as the wild types. T-DNA insertion mutants were obtained from the Nottingham Arabidopsis Stock Centre, the Arabidopsis Biological Resource Center, and from the Syngenta Arabidopsis Insertion Library (Mollier et al., 1995; Bechtold and Pelletier, 1998; Campisi et al., 1999; Sessions et al., 2002). Seeds were incubated at 4°C for at least 2 d to break dormancy prior to germination. Seedlings and adult plants were grown on soil (Type VM, Einheitserdewerk) and vermiculite (4:1 mixture) under a 12-h-light (100–150 $\mu E\ m^{-2}\ s^{-1}$)/12-h-dark cycle (22°C/18°C) in Percival growth chambers and watered with 1× modified Hoagland solution. For some experiments, CO_2 levels were elevated to 0.14% (low photorespiratory condition). Double mutants were germinated and kept on solidified Murashige and Skoog (Murashige and Skoog, 1962) basal medium (Duchefa Biochemie) containing 2% Suc and 1% agar under 0.9% CO_2 (nonphotorespiratory condition) and a 12/12-h light/dark cycle (100–150 $\mu E\ m^{-2}\ s^{-1}$, 22°C/18°C) in SANYO growth cabinets.

Isolation of T-DNA Mutants for *AtGLDP1* and *AtGLDP2*

The T-DNA insertion mutant for *AtGLDP1* (*At4g33010*), *Atgldp1-1*, was identified in a subset of the enhancer trap line collection generated by Campisi et al. (1999) using the gene-specific primers P12-S1 (for primer details see Table I) in combination with the insertion-specific primer TJLB1 in standard PCR reactions (1 min 94°C, 1 min 58°C, 1 min 30 s, 72°C; 35 cycles) with genomic DNA from pooled and individual plants. Zygosity was verified by the same procedure, but using a pair of gene-specific primers flanking the T-DNA insertion (P12-S1 and P1-A1n, sequences in Table I). Similarly, an allelic T-DNA insertion mutant, *Atgldp1-2*, was identified in a subset of the promoter trap Institut National de la Recherche Agronomique (INRA)-Versailles line collection generated by Bechtold et al. (1993; ecotype Wassilewskija) using the gene-specific primer P12-S2 in combination with the insertion-specific primer VSLB1 in standard PCR reactions with genomic DNA. Zygosity was verified by PCR analysis with gene-specific primers flanking the T-DNA insertion (P12-S2 and P1-A2n).

The T-DNA mutants for *AtGLDP2* (*At2g26080*) were identified and isolated from the SAIL collection (Sessions et al., 2002) lines 1261.C02 (*Atgldp2-2*) and 888.D09 (*Atgldp2-3*). To verify the respective T-DNA insertions, leaf DNA was PCR amplified with primers specific for the left or right border of the T-DNA, respectively, and a gene-specific primer (SAIL-LB1 and P2-A2n for *Atgldp2-2*; Syn-LB1 and P12-A1 for *Atgldp2-3*). Homozygous plants were again identified by using a combination of two gene-specific primers encompassing the respective T-DNA insertion for PCR amplification of genomic DNA (P2-2check-S and P2-A2n for *Atgldp2-2*; P12-S1 and P2-A1n for *Atgldp2-3*). Where appropriate, PCR fragments were sequenced to verify the insertion sites.

Two T-DNA insertion mutants, *Atgldp1-1* and *Atgldp2-2*, both of the Columbia genotype, were crossed. Nine out of 51 F2 individuals were homozygous with respect to *Atgldp1-1* and five of these carried a heterozygous *Atgldp2-2* mutation. Double-homozygous plants were not found among the F2 plants. Such plants were selected, after selfing individual F2 plants, from F3 seeds that were germinated as described above (0.9% CO_2, Murashige and Skoog medium with 1% agar and 2% Suc).

Transcript Analysis by RT-PCR

Total RNA was purified (Nucleospin RNA plant kit, Macherey-Nagel) from the specified organs harvested in the middle of the light period, and cDNA was produced from 2.5 μg of RNA with the RevertAid H minus cDNA synthesis kit (MBI Fermentas). Primer specificity was verified by cutting the PCR amplificates with restriction enzymes *Bgl*II (*AtGLDP1*) and *Xcm*I (*AtGLDP2*), respectively. PCR analysis was performed with 28 cycles only and the amounts of cDNA were calibrated according to signal intensities of the 432 bp fragments obtained by PCR amplification of the constitutively expressed *At2g09990* mRNA encoding the 40S ribosomal protein S16 (primers S16-S and S16-A).

Protein Analysis

Protein extracts were prepared in a buffer containing 25 mM HEPES, pH 7.0, 0.5 mM EDTA, 8 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride. Twenty micrograms of total protein per lane (Bradford, 1976) were separated on 15% denaturing polyacrylamide gels (Laemmli, 1970) and subsequently electrotransferred onto a polyvinylidene difluoride membrane. P protein was identified with a specific antiserum raised in rabbits against recombinant potato (*Solanum tuberosum*) P protein (H. Bauwe, unpublished data) in combination with a horseradish peroxidase-conjugated secondary antibody and the enhanced chemiluminescence western-blotting detection kit (GE Healthcare Europe GmbH).

Leaf Soluble Amino Acid Content

For amino acid determination, 100 mg leaf material were ground in liquid nitrogen and extracted in 1.8 mL 80% ethanol for 30 min. After centrifugation, the supernatant was vacuum dried and the dried extract dissolved in 8 mM Na_2PO_4 (pH 6.8) and 0.4% tetrahydrofurane. Individual amino acids were separated by HPLC and quantified as described earlier (Hagemann et al., 2005).

Metabolome Analysis

Mutant and wild-type plants were grown in normal air or in air enriched with 0.14% CO_2. Rosettes of five plants of each line were individually harvested, extracted, and analyzed as described recently (Weckwerth et al., 2004). Gas chromatography-coupled time-of-flight mass spectrometric analysis was performed on an HP 5890 gas chromatograph with deactivated standard split/splitless liners containing glass wool. One microliter of sample was injected in the splitless mode at 230°C injector temperature. GC was operated on a MDN-35 capillary, 30 m × 0.32 mm i.d., 25 μm film (Supelco), at constant flow of 2 mL min^{-1} helium. The temperature program started with 2 min isocratic at 85°C, followed by temperature ramping at 15°C min^{-1} to a final temperature of 360°C that was held for 8 min. Data acquisition was performed on a Pegasus II time-of-flight mass spectrometer (Leco) with an acquisition rate of 20 scans s^{-1} in the mass range of mass-to-charge ratio = 85 to 600. The obtained data were first analyzed by defining a reference chromatogram with the maximum number of detected peaks over a signal/noise threshold of 50. Afterward, all chromatograms were matched against the reference with a minimum match factor of 800. Compounds were annotated by retention index and mass spectra comparison to a user-defined spectra library. Selected unique fragment ions specific for each individual metabolite were used for quantification.

Gas-Exchange Measurements

CO_2 exchange was measured by a $^{14}CO_2$-radiogasometric method in the second half of the photoperiod with fully expanded leaves from 10-week-old plants grown in an 8-h-light/16-h-dark cycle with a photon flux density of 200 μE m^{-2} s^{-1}. Measuring conditions were 400 μL L^{-1} CO_2, 210 mL L^{-1} O_2, 170 μE m^{-2} s^{-1} photon flux density, 25°C chamber temperature, and 23°C to 24°C leaf temperature. Further details of the method have been described elsewhere (Pärnik and Keerberg, 2007).

Supplemental Data

The following material is available in the online version of this article.

Supplemental Figure S1. Expression of *AtGLDP1* and *AtGLDP2* in individual organs (Genevestigator data, Zimmermann et al., 2004).

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