

# Structural and Biochemical Dissection of Photorespiration in Hybrids Differing in Genome Constitution between *Diplotaxis tenuifolia* (C<sub>3</sub>-C<sub>4</sub>) and Radish (C<sub>3</sub>)<sup>1</sup>

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We compared the structural, biochemical, and physiological characteristics involved in photorespiration of intergeneric hybrids differing in genome constitution (DtDtR, DtDtRR, and DtRR) between the C<sub>3</sub>-C<sub>4</sub> intermediate species *Diplotaxis tenuifolia* (DtDt) and the C<sub>3</sub> species radish (*Raphanus sativus*; RR). The bundle sheath (BS) cells in *D. tenuifolia* included many centripetally located chloroplasts and mitochondria, but those of radish had only a few chloroplasts and mitochondria. In the hybrids, the numbers of chloroplasts and mitochondria, the ratio of centripetally located organelles to total organelles, and the mitochondrial size in the BS cells increased with an increase in the constitution ratio of the Dt:R genome. The P-protein of glycine decarboxylase (GDC) was confined to the BS mitochondria in *D. tenuifolia*, whereas in radish, it accumulated more densely in the mesophyll than in the BS mitochondria. In the hybrids, more intense accumulation of GDC in the BS relative to the mesophyll mitochondria occurred with an increase in the Dt:R ratio. These structural and biochemical features in the hybrids were reflected in the gas exchange characteristics of leaves, such as the CO<sub>2</sub> compensation point. Our data indicate that the leaf structure, the intercellular pattern of GDC expression, and the gas exchange characteristics of C<sub>3</sub>-C<sub>4</sub> intermediate photosynthesis are inherited in the hybrids depending on the constitution ratio of the parent genomes. Our findings also demonstrate that the apparent reduced photorespiration in C<sub>3</sub>-C<sub>4</sub> intermediate plants is mainly due to the structural differentiation of mitochondria and chloroplasts in the BS cells combined with the BS-dominant expression of GDC.

In ordinary air, Rubisco catalyzes bifunctional reactions, namely, the carboxylation and oxygenation of ribulose-1,5-bisphosphate. Glycolate-2-phosphate produced by the oxygenase activity of Rubisco is recycled into glycerate-3-phosphate via the photorespiratory pathway, and glycerate-3-phosphate is used for the regeneration of ribulose-1,5-bisphosphate via the C<sub>3</sub> cycle (Leegood et al., 1995; Tolbert, 1997; Douce and Neuburger, 1999). This oxidative photosynthetic carbon cycle involves three subcellular compartments: chloroplasts, peroxisomes, and mitochondria. The CO<sub>2</sub> released during photorespiration comes from Gly oxidation (Oliver, 1994; Douce et al., 2001). In C<sub>3</sub> plants, the release of CO<sub>2</sub> by photorespiration in ordinary air is estimated to be approximately 25% of photosynthetically fixed

CO<sub>2</sub> (Sharkey, 1988) and decreases the photosynthetic efficiency (Zelitch, 1992). However, some plants have evolved biochemical mechanisms, such as the C<sub>4</sub> photosynthetic pathway, to suppress photorespiration.

The leaves of C<sub>3</sub>-C<sub>4</sub> intermediate plants have anatomical and physiological characteristics between those of C<sub>3</sub> and C<sub>4</sub> plants (Edwards and Ku, 1987; Brown and Hattersley, 1989; Monson and Rawsthorne, 2000). The leaves of C<sub>3</sub>-C<sub>4</sub> intermediate plants possess a Kranz-like anatomy, in which the bundle sheath (BS) cells include numerous chloroplasts and mitochondria. The gas exchange characteristics, such as the CO<sub>2</sub> compensation point ( $\Gamma$ ) and O<sub>2</sub> inhibition of photosynthesis, of C<sub>3</sub>-C<sub>4</sub> intermediate plants are intermediate between those of C<sub>3</sub> and C<sub>4</sub> plants (Edwards and Ku, 1987; Monson and Rawsthorne, 2000). It is thought that the reduction in photorespiratory CO<sub>2</sub> loss results from the cellular compartmentalization of Gly decarboxylation (Hylton et al., 1988; Monson and Rawsthorne, 2000).

Together with Ser hydroxymethyltransferase, Gly decarboxylase (GDC) catalyzes the oxidative conversion of Gly to Ser, NH<sub>3</sub>, and CO<sub>2</sub>. GDC is a complex of four heterologous protein subunits—P, H, L, and T—and requires the concerted effects of these four proteins to complete its reactions (Oliver, 1994;

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Douce et al., 2001). The mitochondria of the BS cells of C<sub>3</sub>-C<sub>4</sub> intermediate plants contain all of the proteins of GDC, but those of the mesophyll cells substantially lack at least the P-protein (Hylton et al., 1988; Morgan et al., 1993). This situation means that Gly generated in the mesophyll cells must be transported into the BS cells to be decarboxylated by GDC. In the BS cells, the mitochondria are surrounded by chloroplasts. Thus, a large part of the CO<sub>2</sub> released from the mitochondria is recaptured by the chloroplasts before it escapes from the BS cells. The increased efficiency of CO<sub>2</sub> recapture in C<sub>3</sub>-C<sub>4</sub> intermediate plants may give rise to better photosynthetic performance at warm temperatures and improved water-use efficiency, as compared with C<sub>3</sub> plants (McVetty et al., 1989; Monson and Rawsthorne, 2000). C<sub>3</sub>-C<sub>4</sub> intermediate species have been found in several genera including *Mollugo*, *Panicum*, *Moricandia*, and *Flaveria* (Edwards and Ku, 1987; Monson and Rawsthorne, 2000). Furthermore, some C<sub>3</sub>-C<sub>4</sub> intermediate species of *Flaveria* use a partially functioning C<sub>4</sub> cycle as well as the Gly decarboxylation system to reduce photorespiration, suggesting an evolutionary link to C<sub>4</sub> plants (Edwards and Ku, 1987).

Various attempts have been made to genetically improve the photosynthetic efficiency of C<sub>3</sub> plants (Zelitch, 1992; Brown and Bouton, 1993; Leegood, 2002; Surridge, 2002). The transfer of C<sub>3</sub>-C<sub>4</sub> intermediate characteristics into C<sub>3</sub> plants may be the method of choice (Leegood, 2002), because the mechanism underlying C<sub>3</sub>-C<sub>4</sub> intermediate photosynthesis is less complicated than that of C<sub>4</sub> photosynthesis. Until now, artificial interspecific hybrids have been produced between C<sub>3</sub> and C<sub>3</sub>-C<sub>4</sub> intermediate species (Brown and Bouton, 1993). In early studies, hybrids were found to exhibit photosynthetic characteristics intermediate between the parents (Apel et al., 1984; Brown et al., 1985). The Brassicaceae include C<sub>3</sub>-C<sub>4</sub> intermediate species in the genera *Moricandia*, *Diplotaxis*, and *Brassica* as well as C<sub>3</sub> species such as oilseed (*Brassica napus*), cabbage (*Brassica capitata*), and radish (*Raphanus sativus*; Apel, 1996; Apel et al., 1997). Hybridization has been successfully carried out between C<sub>3</sub>-C<sub>4</sub> intermediate species of *Moricandia* and C<sub>3</sub> species of other cruciferous genera (Apel et al., 1984; Bang et al., 1996; O'Neill et al., 1996; Razmjoo et al., 1996; Rawsthorne et al., 1998; Yan et al., 1999). These hybrids provide not only valuable materials for the breeding of agronomically important crops but also unique opportunities to study the genetic mechanisms of C<sub>3</sub>-C<sub>4</sub> intermediate photosynthesis.

Here, we report the anatomical and biochemical features of the leaves of hybrids produced between the C<sub>3</sub>-C<sub>4</sub> intermediate *Diplotaxis tenuifolia* and the C<sub>3</sub> species radish and that differ in their genome constitution. The data obtained clearly show that these anatomical and biochemical characteristics are correctly inherited from the parents by the hybrids according to the genome constitution. Our results also

demonstrate that the apparent reduction of photorespiration in C<sub>3</sub>-C<sub>4</sub> intermediate plants is caused by the anatomical differentiation of leaf cells combined with the intercellular location of GDC accumulation.

## RESULTS

### Gross Morphology

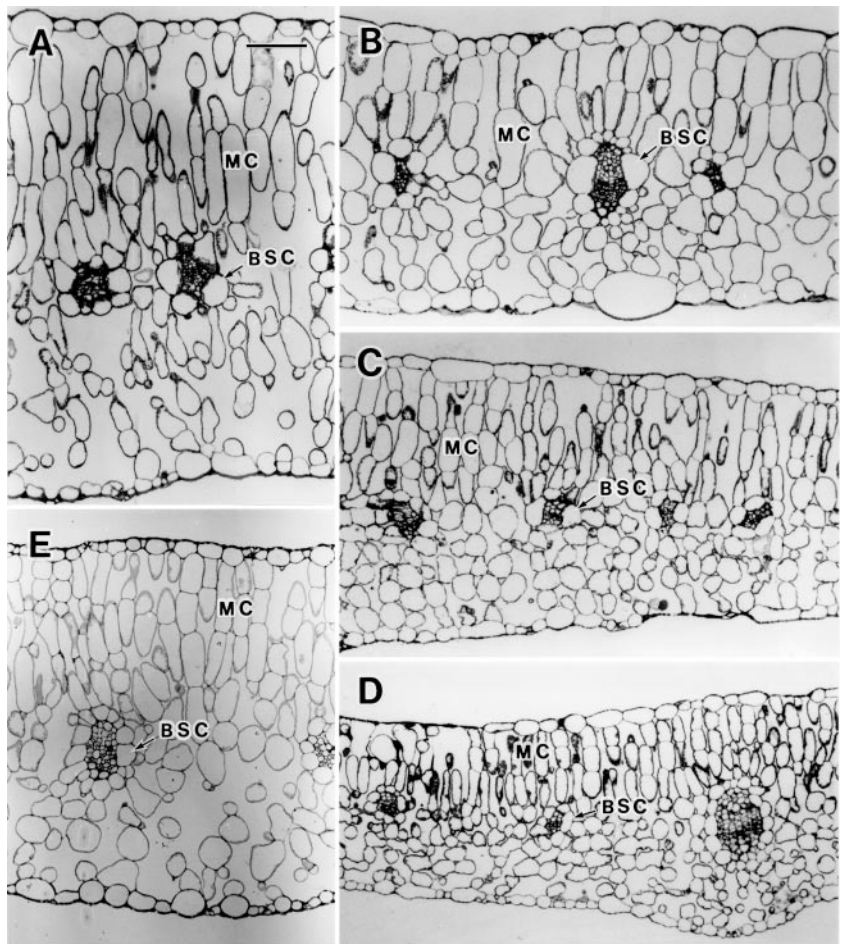
The gross morphology of the hybrids and the method for producing them have been reported in detail by Bang et al. (2000). In brief, *D. tenuifolia* (DtDt) and radish (RR) were used as the female parent and pollen parent, respectively. Three types of hybrids differing in genome constitution (DtDtR, DtDtRR, and DtRR) were produced through ovary culture followed by embryo culture (Bang et al., 2000). The leaf shape differs between the parents: *D. tenuifolia* has crenate leaves, and radish has lacerate leaves. The leaf shape of the DtDtRR hybrid was intermediate between those of the parents, whereas those of the DtDtR hybrid were more like those of *D. tenuifolia*, and those of the DtRR hybrid more resembled those of radish. The flowers of *D. tenuifolia* and radish had yellow and white petals, respectively. The petals of the three hybrids showed different levels of intermediate color depending on the Dt:R genome constitution.

### Internal Leaf Structure

The leaves of the C<sub>3</sub>-C<sub>4</sub> intermediate species *D. tenuifolia* contained large BS cells that included numerous organelles surrounding the vascular bundle (Fig. 1A). Their mitochondria were centripetally located, and most were adjacent to the inner walls of BS cells and were closely overlaid by numerous chloroplasts (Fig. 2A). The adaxial mesophyll cells exhibited an elongated shape, whereas the abaxial mesophyll cells were somewhat similar to spongy parenchyma. The abaxial mesophyll cells adjacent to BS cells were somewhat radially arranged. The leaves of radish showed a typical C<sub>3</sub> anatomy (Fig. 1E). Although the BS cells of radish also surrounded the vascular bundle, they contained only a few chloroplasts and mitochondria (Fig. 2E). The mesophyll was clearly differentiated into palisade and spongy parenchyma between the adaxial and abaxial sides, respectively.

The internal structures of the leaves of the hybrid plants showed various intermediate anatomies between those of the parents (Fig. 1, B–D). This diversity was particularly evident in the structure of the BS cells. The BS cells of the DtDtR hybrid included numerous chloroplasts and mitochondria in the centripetal position; thus this configuration approached that of the BS cells of *D. tenuifolia* (Fig. 2B). The BS cells of the DtRR hybrid were similar to those of radish but included several chloroplasts and mitochondria in the centripetal position (Fig. 2D). The

**Figure 1.** Leaf anatomy of *D. tenuifolia* (A), the hybrids (B, DtDtR; C, DtDtRR; D, DtRR), and radish (E). BSC, BS cell; MC, mesophyll cell. Bar, 100  $\mu\text{m}$ .



pattern of the BS cells of the DtDtRR hybrid was intermediate between the other two hybrids (Fig. 2C). In addition, the shape and arrangement of mesophyll cells showed a gradation from the  $C_3$ - $C_4$  intermediate to the  $C_3$  parent.

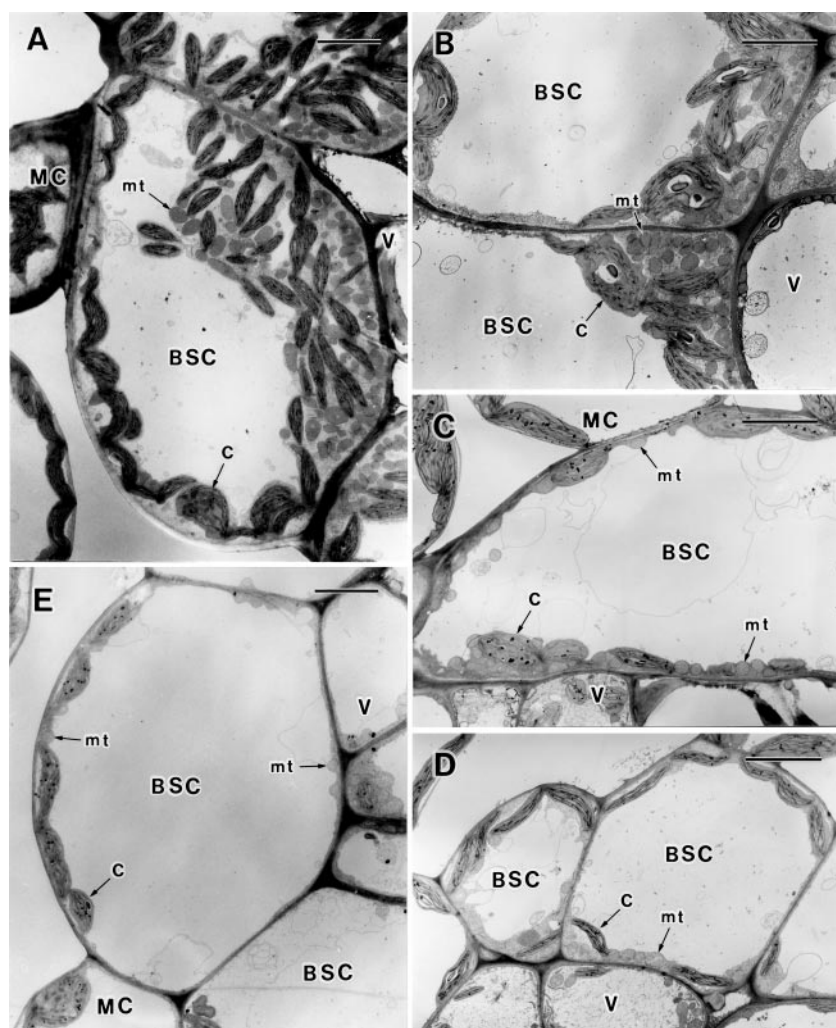
The interveinal distance was shorter in *D. tenuifolia* than in radish, and the values for the three hybrids were intermediate between those of the parents (Table I). The photosynthetic cells were larger in *D. tenuifolia* than in radish (Table I). In the DtDtR hybrid, the size of the mesophyll cells was intermediate to those of the parents, but the size of the BS cells was almost the same as that of the  $C_4$  parent. In the DtDtRR and DtRR hybrids, all types of photosynthetic cells, except for the BS cells of DtDtRR, were smaller than those in the  $C_3$  parent (Table I).

#### Quantification of Organelles Involved in Photosynthesis and Photorespiration

The number of chloroplasts per BS cell in *D. tenuifolia* was about five times higher than that in radish (Table II), and the values for the hybrids were intermediate between the parents and showed a gradation. In *D. tenuifolia*, 56% of chloroplasts in the BS cells were centripetally located, but the remaining

chloroplasts were centrifugal. In radish, only 15% of chloroplasts were centripetal (Table II). In addition, the number of chloroplasts per mesophyll cell was higher in *D. tenuifolia* than in radish, whereas the values for the hybrids were similar to that for radish. The number of mitochondria per BS cell differed greatly (six times) between the two parents (Table II), and the values for the hybrids again showed a gradation. In *D. tenuifolia*, all mitochondria in the BS cells were centripetally located. In the DtDtR and DtDtRR hybrids, almost all mitochondria were centripetal, as were 80% of those in the BS cells of radish (Table II). The number of mitochondria per mesophyll cell did not differ markedly among the plants, except that the DtRR hybrid had few. As a result, the concentration of mitochondria in the BS cells increased with an increase in the Dt:R genome constitution ratio. However, we did not observe such a trend in the number of peroxisomes (Table II). In all plants, the number of peroxisomes per cell was lower in BS cells than in mesophyll cells. The intracellular location of peroxisomes in the BS cells varied among the plants (Table II).

In all plants, the chloroplasts in the BS cells generally were smaller than those in the mesophyll cells (Table III). The size of chloroplasts in the various



**Figure 2.** Ultrastructure of bundle sheath cells of *D. tenuifolia* (A), the hybrids (B, DtDtR; C, DtDtRR; D, DtRR), and radish (E). BSC, BS cell; V, vascular bundle; C, chloroplast; mt, mitochondrion. Bar = 5  $\mu\text{m}$ .

types of photosynthetic cells varied greatly among plants. Except for those in radish, the mitochondria in the BS cells were larger than those in the mesophyll cells (Table III). The size of mitochondria in the BS cells tended to increase as the constitution ratio of the Dt to R genome increased. In *D. tenuifolia*, the peroxisomes in the BS cells were larger than those in

the mesophyll cells, whereas in radish, the opposite trend occurred (Table III). The peroxisomal sizes of the BS cells and mesophyll cells did not vary notably among the three hybrids.

**Table I.** Interveneal distance and size of photosynthetic cells in leaves of *D. tenuifolia*, radish, and their hybrids

MC, Mesophyll cells; BSC, BS cells. Values are given as the mean  $\pm$  SD of 30 to 40 measurements for interveneal distance and 40 measurements for cell size.

Plant and Genotype	Interveneal Distance	Cell Size		
		Adaxial MC	Abaxial MC	BSC
		$\mu\text{m}$		
<i>D. tenuifolia</i> (DtDt)	238 $\pm$ 52	116 $\pm$ 24	78 $\pm$ 19	40 $\pm$ 9
DtDtR hybrid	245 $\pm$ 66	83 $\pm$ 17	61 $\pm$ 10	39 $\pm$ 10
DtDtRR hybrid	311 $\pm$ 91	60 $\pm$ 12	44 $\pm$ 6	31 $\pm$ 7
DtRR hybrid	243 $\pm$ 93	49 $\pm$ 13	36 $\pm$ 11	19 $\pm$ 5
Radish (RR)	340 $\pm$ 138	67 $\pm$ 21	56 $\pm$ 11	27 $\pm$ 5

#### Immunogold Localization of the P-Protein of GDC

In *D. tenuifolia*, the BS mitochondria (Fig. 3B) but not the mesophyll mitochondria (Fig. 3A) showed intense labeling for the P-protein of GDC. The ratio of the labeling density for the BS mitochondria to that of the mesophyll mitochondria was 28.0 (Table IV). In radish, the mesophyll mitochondria (Fig. 3E) showed more intense labeling than did the BS mitochondria (Fig. 3F), and the ratio of labeling density for the BS:mesophyll mitochondria was very low (0.4; Table IV). Although marked labeling of the P-protein of GDC occurred in mesophyll as well as BS mitochondria of all three hybrids, there was a gradation in the cellular pattern of the labeling (Fig. 3, C and D). The labeling density of the mesophyll mitochondria decreased and that of the BS mitochondria increased as the constitution ratio of the Dt to R genome in-

**Table II.** Numbers of organelles per cell profile in photosynthetic cells and ratios of centripetally located organelles in the BS cells in leaves of *D. tenuifolia*, radish, and their hybrids. MC, Mesophyll cells; BSC, BS cells; Cp, percentage of centripetally located organelles in the BS cells. Numbers of organelles per cell profile are given as means  $\pm$  SD of 20 measurements.

Plant and Genotype	Chloroplasts				Mitochondria				Peroxisomes			
	Adaxial MC	Abaxial MC	BSC	Cp	Adaxial MC	Abaxial MC	BSC	Cp	Adaxial MC	Abaxial MC	BSC	Cp
	%				%				%			
<i>D. tenuifolia</i> (DtDt)	38.9 $\pm$ 7.9	31.6 $\pm$ 5.2	32.4 $\pm$ 8.1	56.2	22.3 $\pm$ 5.9	13.6 $\pm$ 5.0	60.4 $\pm$ 21.2	100.0	3.9 $\pm$ 2.8	1.8 $\pm$ 1.0	0.5 $\pm$ 0.9	20.0
Hybrid (DtDtR)	21.6 $\pm$ 4.6	20.1 $\pm$ 4.3	16.9 $\pm$ 8.0	47.3	22.7 $\pm$ 7.5	14.9 $\pm$ 4.4	27.0 $\pm$ 10.9	99.6	3.4 $\pm$ 1.5	2.7 $\pm$ 1.3	2.0 $\pm$ 2.6	60.0
Hybrid (DtDtRR)	19.8 $\pm$ 4.3	16.6 $\pm$ 4.3	9.5 $\pm$ 1.9	35.5	22.0 $\pm$ 7.3	16.2 $\pm$ 5.0	21.9 $\pm$ 9.0	98.2	3.0 $\pm$ 1.8	3.1 $\pm$ 3.2	0.8 $\pm$ 0.7	50.0
Hybrid (DtRR)	15.5 $\pm$ 3.7	11.9 $\pm$ 2.9	8.8 $\pm$ 2.2	34.1	15.9 $\pm$ 6.7	7.9 $\pm$ 3.7	12.5 $\pm$ 4.6	80.0	1.6 $\pm$ 1.0	0.7 $\pm$ 0.8	0.8 $\pm$ 1.3	62.5
Radish (RR)	20.0 $\pm$ 5.9	16.5 $\pm$ 4.5	6.0 $\pm$ 2.2	15.0	20.4 $\pm$ 7.7	15.9 $\pm$ 5.1	9.5 $\pm$ 4.0	80.0	2.7 $\pm$ 2.1	1.9 $\pm$ 1.6	1.5 $\pm$ 1.2	25.0

creased; for example, the ratio of labeling density for the BS:mesophyll mitochondria ranged from 1.5 in the DtRR hybrid to 2.7 in DtDtRR to 4.0 in DtDtR (Table IV).

### Activities of Photosynthetic Enzymes

The photosynthetic carbon metabolism of *D. tenuifolia* has not yet been characterized fully, although the leaf anatomy and CO<sub>2</sub> gas exchange characteristics have been reported (Apel, 1996; Apel et al., 1997). Therefore, we compared the activities of the C<sub>3</sub> and C<sub>4</sub> photosynthetic enzymes in leaves of this C<sub>3</sub>-C<sub>4</sub> intermediate species with those of the C<sub>3</sub> parent radish and the DtDtRR hybrid (Table V). The activity of the key C<sub>4</sub> enzyme phosphoenolpyruvate (PEP) carboxylase was higher in *D. tenuifolia* than in radish. That of pyruvate, Pi dikinase, was very low in both species. Regarding the three types of decarboxylating enzymes, the activities of NADP- and NAD-malic enzymes were higher in *D. tenuifolia* than in radish, whereas PEP carboxykinase activity was undetectable in both species. Rubisco showed high activity in both species. In the DtDtRR plants, the activities of PEP carboxylase and NADP-malic enzyme were intermediate between those of both parents, whereas the activities of the other enzymes generally were lower than in the C<sub>3</sub> species.

### Gas Exchange Characteristics

The photosynthetic rate was higher in *D. tenuifolia* than in radish (Table VI). The rates of the DtDtRR and DtRR hybrids were intermediate between those of the parents, and that of DtDtR was somewhat higher than that of *D. tenuifolia*. Under high PPFD, *D. tenuifolia* showed a  $\Gamma$  value typical of C<sub>3</sub>-C<sub>4</sub> intermediate plants, whereas that of radish was within the range for C<sub>3</sub> plants (Table VI). The  $\Gamma$  values of the DtDtR and DtDtRR hybrids were intermediate between those of the parents, whereas that of DtRR was the same as that of the C<sub>3</sub> parent. It is known that the  $\Gamma$  of C<sub>3</sub>-C<sub>4</sub> intermediate plants increases as the PPFD is reduced, but that of C<sub>3</sub> plants is unaffected (Holaday et al., 1982). When measured at low PPFD, the  $\Gamma$  values of all plants examined were somewhat increased (Table VI). However, the magnitude of the increase was larger in *D. tenuifolia* and the DtDtR hybrid than in the other plants.

### DISCUSSION

*D. tenuifolia* was reported to be a C<sub>3</sub>-C<sub>4</sub> intermediate species (Apel, 1996). Among the Brassicaceae, almost all species are C<sub>3</sub> type; a few C<sub>3</sub>-C<sub>4</sub> intermediate species have been documented, but no C<sub>4</sub> species has been identified (Apel et al., 1997). Our enzyme data indicate that the activities of several C<sub>4</sub> enzymes are somewhat higher in *D. tenuifolia* than in

**Table III.** Sizes of organelles in photosynthetic cells in leaves of *D. tenuifolia*, radish, and their hybrids

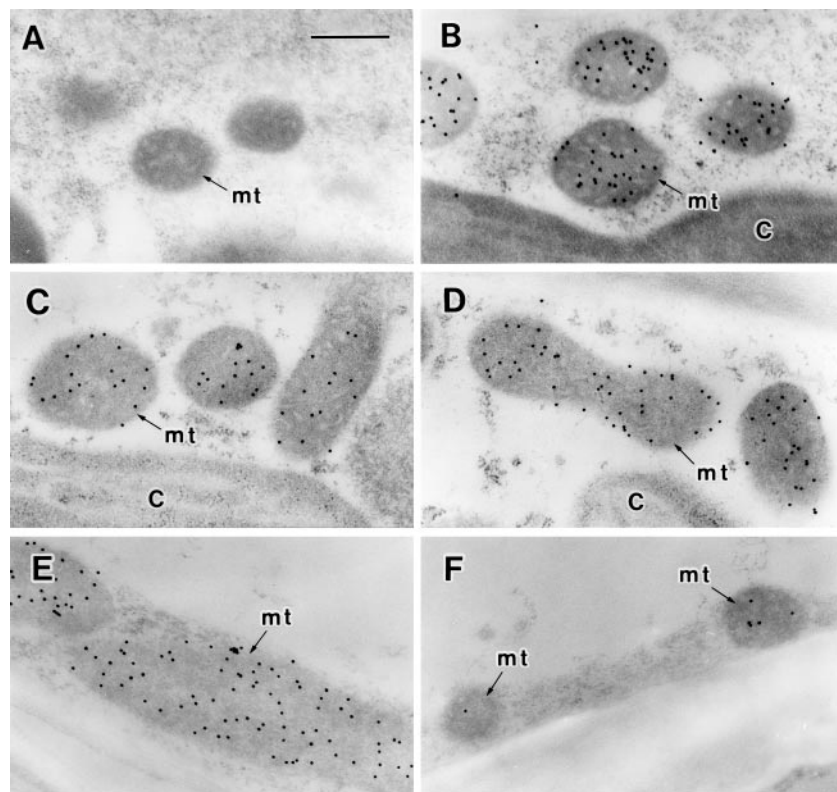
MC, Mesophyll cells; BSC, BS cells. Values are given as the mean  $\pm$  SD of 40 measurements for chloroplasts and mitochondria and 10 measurements for peroxisomes. The long axes and the diameters were measured for chloroplasts and for mitochondria and peroxisomes, respectively.

Plant and Genotype	Chloroplasts			Mitochondria			Peroxisomes		
	Adaxial MC	Abaxial MC	BSC	Adaxial MC	Abaxial MC	BSC	Adaxial MC	Abaxial MC	BSC
	$\mu\text{m}$								
<i>D. tenuifolia</i> (DtDt)	4.8 $\pm$ 0.9	5.5 $\pm$ 1.0	4.8 $\pm$ 1.2	0.57 $\pm$ 0.12	0.58 $\pm$ 0.13	0.73 $\pm$ 0.19	0.79 $\pm$ 0.24	0.71 $\pm$ 0.21	0.91 $\pm$ 0.22
DtDtR hybrid	7.2 $\pm$ 1.5	6.3 $\pm$ 1.2	5.3 $\pm$ 0.9	0.51 $\pm$ 0.08	0.43 $\pm$ 0.06	0.65 $\pm$ 0.17	1.21 $\pm$ 0.24	1.10 $\pm$ 0.19	1.14 $\pm$ 0.22
DtDtRR hybrid	6.2 $\pm$ 1.2	6.3 $\pm$ 1.1	4.8 $\pm$ 0.8	0.46 $\pm$ 0.07	0.46 $\pm$ 0.09	0.68 $\pm$ 0.14	1.12 $\pm$ 0.17	1.20 $\pm$ 0.24	0.97 $\pm$ 0.14
DtRR hybrid	6.5 $\pm$ 1.3	6.1 $\pm$ 0.9	4.6 $\pm$ 1.2	0.50 $\pm$ 0.09	0.52 $\pm$ 0.10	0.61 $\pm$ 0.13	1.12 $\pm$ 0.20	0.87 $\pm$ 0.17	1.03 $\pm$ 0.09
Radish (RR)	6.2 $\pm$ 1.5	5.7 $\pm$ 1.2	4.3 $\pm$ 1.0	0.64 $\pm$ 0.11	0.65 $\pm$ 0.12	0.54 $\pm$ 0.09	0.91 $\pm$ 0.23	0.87 $\pm$ 0.14	0.65 $\pm$ 0.20

the C<sub>3</sub> species radish. The same trend has been found in other C<sub>3</sub>-C<sub>4</sub> intermediate species lacking a C<sub>4</sub> cycle (Holaday et al., 1981; Edwards and Ku, 1987). However, the numbers of organelles in the BS cells of *D. tenuifolia* seem greater than those in *Moricandia arvensis*, a well-known C<sub>3</sub>-C<sub>4</sub> intermediate species (Holaday et al., 1981; Beebe and Evert, 1992). In this regard, *D. tenuifolia* may be more useful than *M. arvensis* as the parent in hybridization studies of C<sub>3</sub>-C<sub>4</sub> intermediacy.

Our study clearly demonstrated that the anatomical and biochemical characteristics of leaves of the parent plants are inherited correctly in the hybrids depending on the constitution ratio of the Dt:R genomes. The hybrids exhibited various levels of intermediacy and provide intriguing materials for dissect-

ing the structural and biochemical features of photosynthesis of C<sub>3</sub>-C<sub>4</sub> intermediate species. The BS cells in the DtDtR and DtDtRR hybrids were larger, and those of DtRR were smaller, than those in radish. However, the chloroplasts and mitochondria in the BS cells of all three hybrids were always larger than those of radish and showed a gradation according to the Dt:R genome constitution. These data suggest that the size of the BS cells may be regulated independently of the sizes of their chloroplasts and mitochondria. The numbers of chloroplasts and mitochondria per BS cell were higher in *D. tenuifolia* than in radish, and those in the hybrids were intermediate between the parents, showing again a gradation depending on the genome constitution. The peroxisomes of leaves are involved in the photorespiratory



**Figure 3.** Immunogold localization of the P-protein of GDC in *D. tenuifolia* (A and B), the DtDtRR hybrid (C and D), and radish (E and F). A, C, and E, Mesophyll cells. B, D, and F, BS cells. C, Chloroplast; mt, mitochondrion. Bar = 0.5  $\mu\text{m}$ .

**Table IV.** Immunogold labeling of the P-protein of GDC in photosynthetic cells of *D. tenuifolia*, radish, and their hybrids

MC, Mesophyll cells, BSC, BS cells. Number of gold particles per square micrometer is given as the mean  $\pm$  SD. The number of mitochondrial or cell profiles examined is given in parentheses.

Plant and Genotype	Number of Gold Particles				Ratio (BSC:MC)
	MC		BSC		
	Mitochondria	Cytosol + Others	Mitochondria	Cytosol + Others	
	$\mu\text{m}^{-2}$				
<i>D. tenuifolia</i> (DtDt)	3.5 $\pm$ 5.3 (29)	0.2 $\pm$ 0.2 (12)	98.1 $\pm$ 17.7 (49)	0.3 $\pm$ 0.1 (10)	28.0
DtDtR hybrid	18.1 $\pm$ 9.4 (54)	0.2 $\pm$ 0.2 (17)	73.1 $\pm$ 18.2 (62)	0.1 $\pm$ 0.1 (14)	4.0
DtDtRR hybrid	23.5 $\pm$ 14.4 (54)	0.2 $\pm$ 0.2 (13)	64.2 $\pm$ 16.1 (58)	0.3 $\pm$ 0.4 (11)	2.7
DtRR hybrid	40.2 $\pm$ 10.9 (51)	0.1 $\pm$ 0.1 (13)	60.0 $\pm$ 15.2 (50)	0.1 $\pm$ 0.2 (14)	1.5
Radish (RR)	75.2 $\pm$ 23.3 (37)	0.1 $\pm$ 0.1 (12)	32.0 $\pm$ 14.4 (43)	0.3 $\pm$ 0.5 (18)	0.4

metabolism (Douce and Neuburger, 1999). In *D. tenuifolia*, the peroxisomes in the BS cells were larger than those in the mesophyll cells, whereas the reverse trend occurred in radish. In the hybrids, the two cell types did not differ notably. In  $C_3$ - $C_4$  intermediate species of *Panicum*, the BS cells contain more peroxisomes than do the mesophyll cells (Brown et al., 1983). In *D. tenuifolia* as well as the hybrid plants that we generated, however, the number of peroxisomes per mesophyll cell was always greater than that per BS cell. Why the cellular pattern of peroxisomes differs between the two groups is unknown. The reason may be related to differences due to evolutionary steps from  $C_3$  type to  $C_3$ - $C_4$  intermediacy.

In the BS cells of  $C_3$ - $C_4$  intermediate plants, the mitochondria are centripetally distributed and are externally surrounded by chloroplasts. This arrangement of organelles may be effective for the recapture of photorespired  $\text{CO}_2$  from mitochondria (Edwards and Ku, 1987; Monson and Rawsthorne, 2000). Similarly, all of the mitochondria in the BS cells of *D. tenuifolia* were located centripetally. The ratios of centripetally located mitochondria in the BS cells of the hybrids were between those of the parents. However, it is interesting to note that even in the  $C_3$  parent radish, 80% of mitochondria in the BS cells were located centripetally. This  $C_3$  species may represent a very early stage in the evolution of  $C_3$ - $C_4$  intermediate photosynthesis, although the structural and functional features of the BS cells in  $C_3$  plants remain to be clarified (Koroleva et al., 2000).

The location of chloroplasts within the BS cells showed a gradation similar to that found for the mitochondria, but the ratios of centripetally located chloroplasts were lower. The structural features of

organelles in the BS cells of most  $C_3$ - $C_4$  intermediate species are similar to those in the BS cells of NAD-malic enzyme-type  $C_4$  plants (Brown and Hattersley, 1989). The BS cells of *D. tenuifolia*, however, had many centrifugal chloroplasts, whereas in NAD-malic enzyme-type  $C_4$  plants, all chloroplasts are located centripetally (Dengler and Nelson, 1999). Therefore, the different intracellular locations of the chloroplasts in the BS cells of  $C_3$ - $C_4$  intermediate plants, such as *D. tenuifolia*, may indicate distinct roles: Centripetal chloroplasts may recapture photorespired  $\text{CO}_2$ , and centrifugal chloroplasts (like their mesophyll counterparts) may fix  $\text{CO}_2$  from the intercellular space. The centrifugal chloroplasts may also recapture  $\text{CO}_2$  leaking from the inner aggregation of organelles. In contrast, we noted no consistent trend regarding the intracellular location of the peroxisomes in the BS cells.

In  $C_3$ - $C_4$  intermediate plants, GDC activity is confined to the BS mitochondria, and this feature is responsible for the reduction of photorespiration, with the structural features of the BS cells (Hylton et al., 1988; Monson and Rawsthorne, 2000). Previous studies on hybridization between  $C_3$  and  $C_3$ - $C_4$  intermediate species have revealed that the  $\Gamma$  values of some hybrids are intermediate between those of the parents (Apel et al., 1984; Brown et al., 1985; O'Neill et al., 1996; Razmjoo et al., 1996; Rawsthorne et al., 1998; Yan et al., 1999). However, except for the study of Rawsthorne et al. (1998), none of these previous studies examined biochemical characteristics such as GDC localization. In the present study, we identified a gradation in the pattern of intercellular accumulation of the P-protein of GDC between the mesophyll and BS mitochondria, although the intercellular pat-

**Table V.** Activities of  $C_3$  and  $C_4$  photosynthetic enzymes in leaves of *D. tenuifolia*, radish, and their DtDtRR hybrid

Values are given as the mean  $\pm$  SD of three or four experiments. ND, Not detectable.

Plant and Genotype	Rubisco	PEP Carboxylase	Pyruvate, $P_i$ Dikinase	NADP-Malic Enzyme	NAD-Malic Enzyme	PEP Carboxykinase
	$\mu\text{mol mg Chl}^{-1} \text{h}^{-1}$					
<i>D. tenuifolia</i> (DtDt)	1,820 $\pm$ 225	106.9 $\pm$ 20.9	2.3 $\pm$ 0.1	77.7 $\pm$ 10.4	30.3 $\pm$ 2.5	ND
DtDtRR hybrid	1,277 $\pm$ 319	64.2 $\pm$ 6.3	1.4 $\pm$ 0.6	51.8 $\pm$ 11.4	14.5 $\pm$ 8.4	ND
Radish (RR)	1,398 $\pm$ 63	38.8 $\pm$ 3.5	1.4 $\pm$ 0.5	31.6 $\pm$ 3.4	18.2 $\pm$ 3.6	ND

**Table VI.** Photosynthetic rate and effect of photosynthetic photon flux density (PPFD) on the CO<sub>2</sub> compensation point ( $\Gamma$ ) in leaves of *D. tenuifolia*, radish, and their hybrids

Values are given as the mean  $\pm$  SD of four measurements. High and low PPFD are 1,000 and 300  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , respectively.

Plant and Genotype	Photosynthetic Rate $\mu\text{mol m}^{-2} \text{s}^{-1}$	$\Gamma$		Ratio of $\Gamma$ at High/Low PPFD
		High PPFD $\mu\text{mol mol}^{-1}$	Low PPFD $\mu\text{mol mol}^{-1}$	
<i>D. tenuifolia</i> (DtDt)	20.9 $\pm$ 2.8	19.8 $\pm$ 3.2	42.4 $\pm$ 6.5	0.47 $\pm$ 0.04
DtDtR hybrid	22.6 $\pm$ 2.6	28.5 $\pm$ 3.3	38.0 $\pm$ 4.6	0.76 $\pm$ 0.09
DtDtRR hybrid	18.8 $\pm$ 3.3	36.9 $\pm$ 3.9	39.6 $\pm$ 3.8	0.93 $\pm$ 0.07
DtRR hybrid	18.6 $\pm$ 2.6	50.2 $\pm$ 1.6	55.9 $\pm$ 1.4	0.90 $\pm$ 0.01
Radish (RR)	16.7 $\pm$ 1.5	49.7 $\pm$ 2.7	53.2 $\pm$ 2.6	0.93 $\pm$ 0.02

terns of other subunits remain unknown. The gas exchange characteristics of leaves reflected these patterns of localization of the P-protein of GDC and the structural features of the leaf cells. Showing a gradation dependent on the Dt:R genome constitution, the  $\Gamma$  values of our hybrids generally were between those of the parents. We also noted light-dependent changes in  $\Gamma$  for the DtDtR hybrid as well as for *D. tenuifolia*.

Our study suggests that both the structural features of leaves and the intercellular patterns of GDC accumulation are prerequisites for the reduction in  $\Gamma$ . The DtRR hybrid showed slight increases in the number and size of mitochondria and in the ratio of centripetally located chloroplasts in the BS cells but showed a reduction in GDC accumulation in mesophyll cells relative to that in BS cells, compared with those of the C<sub>3</sub> parent radish. However, these structural and biochemical alterations are still insufficient for reducing  $\Gamma$ . Similar results were found in the hybrid between *Moricandia nitens* (C<sub>3</sub>-C<sub>4</sub>) and oilseed (C<sub>3</sub>), in which the ratio of labeling density for the P-protein of GDC in the BS:mesophyll mitochondria was 1.7 and the value of  $\Gamma$  was close to that of the C<sub>3</sub> parent (Rawsthorne et al., 1998). However, we observed a reduction of  $\Gamma$  in the DtDtRR and DtDtR hybrids, in which the P-protein of GDC was expressed more intensely in the BS mitochondria than in the mesophyll mitochondria, as compared with the case of the DtRR hybrid, but the mesophyll mitochondria still accumulated noteworthy amounts of this protein. Our data suggest that complete suppression of expression of the P-protein of GDC in the mesophyll is not required to reduce  $\Gamma$ . To date, all C<sub>3</sub>-C<sub>4</sub> intermediate species examined for GDC localization exhibit BS-specific expression of the P-protein of GDC (Hylton et al., 1988; Morgan et al., 1993; Ueno and Agarie, 1997; Rylott et al., 1998; Voznesenskaya et al., 2001). It would be intriguing to investigate whether C<sub>3</sub>-C<sub>4</sub> intermediate species having the characteristics of the DtDtRR and DtDtR hybrids occur naturally.

If each parent gene contributes equally to the hybrid and if dominance is not involved, then the characteristics of the hybrids should be similar to the mid-parent mean (Brown and Bouton, 1993). It seems that in our hybrids, the expression of structural fea-

tures and cell-specific GDC localization are modulated depending on the constitution ratio of the parent genomes. We cannot evaluate the possible influence of a cytoplasmic effect in the expression of C<sub>3</sub>-C<sub>4</sub> intermediate characteristics, because we did not evaluate the reciprocal hybrids. Although the gene for the large subunit of Rubisco is maternally inherited (Hudson et al., 1990), there is little evidence that other characteristics are maternally influenced in hybrids between C<sub>3</sub>, C<sub>4</sub>, and C<sub>3</sub>-C<sub>4</sub> intermediate species (Araus et al., 1990; Brown and Bouton, 1993). Although mitochondria contain proteins encoded in both the nuclear and mitochondrial genomes (McCabe et al., 2000), all four proteins of GDC are encoded by nuclear genes (Douce et al., 2001). In our hybrid plants, it appears that, irrespective of differences in expression levels, the structural and biochemical components of C<sub>3</sub>-C<sub>4</sub> intermediacy were expressed coordinately. Dense accumulation of GDC in the BS cells relative to that in mesophyll cells occurred with increased partitioning of mitochondria into BS cells relative to mesophyll cells. However, previous studies on *Moricandia* species have suggested that the structural and biochemical components of C<sub>3</sub>-C<sub>4</sub> intermediate characteristics are controlled by different mechanisms (Rawsthorne et al., 1998; Rylott et al., 1998). Furthermore, C<sub>4</sub> photosynthesis may reflect a combination of independently inherited characteristics (Brown and Bouton, 1993; Ueno, 2001a).

A study of barley (*Hordeum vulgare*) mutants with reduced GDC activities showed that GDC has no control over CO<sub>2</sub> assimilation under normal growth conditions, but appreciable control by GDC becomes apparent under conditions of enhanced photorespiration, such as low CO<sub>2</sub> and high light (Wingler et al., 1997). In contrast, antisense reduction of the P-protein of GDC in potato (*Solanum tuberosum*) plants resulted in a reduction of photosynthetic and growth rates under ordinary air (Heineke et al., 2001). Superficially, photorespiration appears to be a wasteful process, but it may serve as an energy sink to prevent overreduction of the photosynthetic electron chain and photoinhibition (Osmond and Grace, 1995; Kozaki and Takeba, 1996; Wingler et al., 2000). In C<sub>3</sub>-C<sub>4</sub> intermediate plants, considerable photore-



spiration occurs within leaf cells, even though apparent photorespiration is reduced (Edwards and Ku, 1987). The introduction of C<sub>3</sub>-C<sub>4</sub> intermediate characteristic may be one possible option for improving the photosynthesis of C<sub>3</sub> plants, although the molecular and genetic mechanisms responsible for the development of C<sub>3</sub>-C<sub>4</sub> intermediate characteristics remain to be solved.

## MATERIALS AND METHODS

### Plant Material

Seeds of *Diplotaxis tenuifolia* (L.) DC. and radish (*Raphanus sativus*) were sown in 8-L pots filled with sufficiently fertilized field soil. We produced three types of hybrids differing in genome constitution by using *D. tenuifolia* (DtDt, 2n = 22) as the female parent and radish (RR, 2n = 18) as the pollen parent, as described elsewhere (Bang et al., 2000). The F<sub>1</sub> plants were obtained through ovary culture followed by embryo culture. To induce amphidiploidy (DtDtRR, 2n = 40), colchicine was applied to the apical meristems of F<sub>1</sub> seedlings. Backcrossing to parent plants followed by embryo rescue was performed to obtain the other progenies (DtDtR [2n = 31] and DtRR [2n = 29]). Plants were grown in a naturally illuminated greenhouse maintained at 25°C to 28°C/15°C to 18°C (day/night temperature) during the spring. They were watered daily. Fully expanded young leaves of plants were examined 2 to 3 months after planting.

### Anatomical and Ultrastructural Studies

Samples from the midsections of leaves were fixed in 3% (v/v) glutaraldehyde in 50 mM sodium phosphate buffer (pH 6.8) at room temperature for 3 h. After being washed with water, they were hand-sectioned with a razor blade. We determined the mean interveinal distance in these sections from 30 to 40 measurements. Semithin sections were prepared from leaf samples that had been fixed with glutaraldehyde and osmium tetroxide and embedded in Suppur's resin, as described previously (Ueno, 1996). Semithin sections on glass slides were stained with toluidine blue O and were used to determine the sizes of the adaxial and abaxial mesophyll cells and BS cells (n = 40 cells). The sizes of the adaxial and abaxial mesophyll cells were represented by the length of the long axis. That of the BS cells was represented by the diameter of the cells. For BS cells that were not circular in cross-section, the size was estimated as the mean of the longest and shortest diameters.

Ultrathin sections also were prepared from leaf samples embedded in Suppur's resin. These sections were placed on grids and stained with uranyl acetate and lead citrate. The chloroplasts, mitochondria, and peroxisomes per cell profile were counted for 20 cells of each type by using an electron microscope (model HU7000, Hitachi, Tokyo). The centripetal organelles (i.e. those located in the inner tangential walls and the inner half of the radial walls) of the BS cells were counted also. To determine the sizes of organelles, we measured the long axes of chloroplasts and the diameters of mitochondria and peroxisomes from electron micrographs at 4,000× for chloroplasts and 15,000× for mitochondria and peroxisomes. The values given are the means of 40 measurements for chloroplasts and mitochondria and of 10 measurements for peroxisomes.

### Protein A-Immunogold Electron Microscopy of GDC

Small segments of leaves were fixed with 3% (v/v) glutaraldehyde in 50 mM sodium phosphate (pH 6.8), dehydrated through an ethanol series, and embedded in Lowicryl K4M resin (Chemische Werke Lowi GmbH, Waldkraiburg, Germany), as previously described (Ueno, 2001b). Ultrathin sections were immunolabeled with an antiserum to the P-protein of GDC and protein A-colloidal gold particles (EY Lab. Inc., San Mateo, CA) as described (Ueno, 2001b). For controls, the antiserum was replaced by nonimmune serum. The antiserum used was raised against the P-protein of GDC isolated from pea (*Pisum sativum*) leaf mitochondria and was kindly provided by Dr. D. J. Oliver (University of Idaho, Moscow, ID). This antiserum was the same as that used in our previous immunocytochemical studies (Ueno and Aga-

rie, 1997; Ueno, 2001b). The cross-reactivity of the antiserum used was determined by western blotting of crude extracts of leaves after SDS-PAGE, as previously described (Ueno, 2001b). We confirmed that the antiserum used recognized the P-proteins of GDC from *D. tenuifolia*, radish, and their hybrids, generating strong single bands on the blots. For both immunolabeling and western blotting, the antiserum was used at a dilution of 1:500. The density of labeling was determined by counting the gold particles on electron micrographs at 25,000× magnification and calculating the number per unit area (square micrometers). Between 11 and 18 individual cells were examined on several immunolabeled sections.

### Enzymatic Activities

Leaves (0.25 g) were ground by using a mortar and pestle containing 0.5 g of sea sand, 25 mg of polyvinylpyrrolidone, and 1 mL of grinding medium on ice. The grinding medium contained 50 mM HEPES-KOH (pH 7.5), 0.2 mM EDTA, 2.5 mM MgCl<sub>2</sub>, 2.5 mM MnCl<sub>2</sub>, 5 mM dithiothreitol, and 0.7% (w/v) bovine serum albumin. The homogenates were filtered through gauze, the filtrates were centrifuged at 10,000g for 5 min at 4°C, and the supernatants were used for the enzymatic assays. All enzymes were assayed spectrophotometrically as described by Ueno (1998).

### Gas Exchange Measurements

The net photosynthetic rate was measured with an LI-6400 portable photosynthesis system (LI-COR, Lincoln, NE). Measurements were made at a photon flux density of 1,000 μmol m<sup>-2</sup>s<sup>-1</sup>, a leaf temperature of 25°C, and a CO<sub>2</sub> concentration of 350 μL L<sup>-1</sup>. Light within the chamber was provided by a 6400-02 LED light source (LI-COR). The Γ value was determined by changing the CO<sub>2</sub> concentration in the chamber (Apel, 1996). Measurements of Γ were made at photon flux densities of 300 and 1,000 μmol m<sup>-2</sup>s<sup>-1</sup>; leaf temperature was maintained at 25°C.

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