Inheritance of C₃–C₄ Intermediate Photosynthesis in Reciprocal Hybrids between Moricandia arvensis (C₃–C₄) and Brassica oleracea (C₃) that Differ in their Genome Constitution

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Abstract: To elucidate the genetic mechanisms underlying C₃–C₄ intermediate photosynthesis, we investigated the structural and photosynthetic characteristics of leaves of reciprocal hybrids between the C₃–C₄ intermediate species Moricandia arvensis (L.) DC. (MaMa) and the C₃ species Brassica oleracea L. (cabbage; CC), which differ in genome constitution. Moricandia arvensis bundle sheath (BS) cells included many centripetally located chloroplasts and mitochondria, whereas those of cabbage had few organelles. Hybrid leaves were structurally intermediate between those of the parents and showed stronger intermediate C₃–C₄ features as the proportion of the Ma genome increased. The P-protein of glycine decarboxylase (GDC) was confined mainly to BS mitochondria in M. arvensis, but accumulated more in the mesophyll (M) of cabbage. In the hybrids, the accumulation of GDC in BS cells increased with an increasing Ma:C ratio. Hybrids exhibited gradients in structural and biochemical features, even in reciprocal crosses. The CO₂ compensation point of reciprocal hybrids with high Ma:C ratios was lower than that of cabbage but higher than that of M. arvensis. Thus, the structural and biochemical features in hybrid leaves reduced photorespiration. Moricandia arvensis had a higher photosynthetic rate than cabbage, but the photosynthetic rates of hybrids were intermediate between those of the parents or comparable to that of M. arvensis. Our results demonstrate that the C₃–C₄ intermediate characteristics are inherited based on the ratio of the parent genomes, and that there is no evidence of cytoplasmic inheritance in these characteristics.

Key words: Brassicaceae, C₃ species, C₃–C₄ intermediate species, CO₂ exchange, Glycine decarboxylase, Leaf anatomy, Photorespiration, Reciprocal hybrids.

In C₃ plants, atmospheric CO₂ is fixed by the carboxylase reaction of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in the C₃ cycle. The oxygenase reaction of Rubisco occurs simultaneously, generating glycolate-2-phosphate, an initial compound of the photorespiratory (glycolate) pathway. The CO₂ released during photorespiration comes from glycolate oxidation in the mitochondria by glycine decarboxylase (GDC) (Douce and Heldt, 2000; Wingler et al., 2000; Keys and Leegood, 2002). In C₃ plants, the CO₂ release by photorespiration in ordinary air is estimated to be about 25% of photosynthetically fixed CO₂, and this loss decreases the efficiency of photosynthesis (Sharkey, 1988). Thus, the regulation of photorespiration has been the subject of intense investigation for plant physiologists and agricultural researchers for many decades (reviewed in Zelitch, 1992; Wingler et al., 2000; Keys and Leegood, 2002).

On the other hand, C₄ plants have a mechanism for concentrating CO₂ that results in suppression of the oxygenase reaction of Rubisco in the bundle sheath (BS) cells.

C₃–C₄ intermediate plants are intermediate between C₃ and C₄ plants in their CO₂ exchange characteristics, including their CO₂ compensation point (Γ) and the inhibition of photosynthesis by O₂. Their leaves have Kranz-like anatomy, and the BS cells include numerous chloroplasts and mitochondria. Rubisco occurs in both mesophyll (M) and BS cells. However, the M mitochondria lack at least the P-protein of the four protein subunits (P, H, L, and T) that comprise the GDC complex, whereas the BS mitochondria contain all four subunits (Monson and Rawsthorne, 2000). Thus, glycin generated in the M cells must be transported into the BS mitochondria to be decarboxylated by GDC.
because GDC requires the concerted effects of all four subunits to complete its reactions. As a result, the release of CO₂ by GDC is restricted to the BS cells. This permits C₃–C₄ intermediates to effectively recapture CO₂ released from mitochondria by the chloroplasts of the BS cells before it escapes, thereby reducing the impact of photospiration (CO₂ loss) on photosynthesis (Monson and Rawsthorne, 2000). Some C₃–C₄ intermediates of 
*Flaveria* exhibit a partially functioning C₁ cycle together with the C₃ glycinede-carboxylation system (Monson and Rawsthorne, 2000). The increased efficiency of CO₂ recapture in C₃–C₄ intermediates may improve photosynthetic performance at warm temperatures and may provide higher water-use efficiency than in C₃ plants (McVetty et al., 1989; Monson and Rawsthorne, 2000).

The Brassicaceae family is agronomically important, and includes many oilseeds and vegetable crops such as rapeseed, cabbage, and radish. Although most members of this family are C₃ plants, the family also includes several C₃–C₄ intermediate species in the genera *Moricandia*, *Diplotaxis*, and *Brassica* (Apel et al., 1997). These C₃–C₄ intermediate species reduce photospiration by the glycinede-carboxylation system without adding a C₁ cycle (Monson and Rawsthorne, 2000; Ueno et al., 2003). In this family, interspecific and intergeneric crosses are relatively easier to achieve than in other families (e.g., Bang et al., 1996, 2003; reviewed in Matsuzawa et al., 1996). As a result, studies of artificial hybridization between C₃ and C₄ intermediate species in this family have been attempted to genetically improve carbon and water economies by the introduction of C₃–C₄ intermediate characteristics into C₄ plants (Apel et al., 1984; O’Neill et al., 1996; Razmjoo et al., 1996; Rawsthorne et al., 1998; Yan et al., 1999; Zhang et al., 2004). In an early study, Apel et al. (1984) reported that hybrids showed Γ values intermediate between those of the parent C₃ plants and C₃–C₄ intermediate species. However, the underlying genetic mechanism has not yet been fully understood.

Recently, we studied the inheritance of C₃–C₄ intermediacy using hybrids between the C₃–C₄ intermediate *Diplotaxis tenuifolia* (L.) DC. (DtDt) and the C₄ species *Raphanus sativus* L. (radish; RR) as the maternal and pollen parents, respectively, which differ in genomic constitution (Ueno et al., 2003). With respect to the structures of their M and BS cells and the distribution of the GDC P-protein, the hybrids showed various features intermediate between those of the parents, depending on the Dt:R genome ratio; hybrids with a higher Dt:R genome ratio had features most similar to those of *D. tenuifolia*. Gradients were also found in the Γ values and in photosynthetic rates. Based on these data, we concluded that the anatomical, biochemical, and physiological characteristics of C₃–C₄ intermediate photosynthesis are inherited in hybrids as a function of the ratio of the parent genomes in the hybrid. Leaf chloroplasts and mitochondria of C₃–C₄ intermediate plants have peculiar structural and biochemical characteristics. These organelles have individual genomes (Leon et al., 1998). However, it has not been sufficiently elucidated whether cytoplasmic inheritance as well as nuclear inheritance is involved in the genetics of C₃–C₄ intermediacy (Brown and Bouton, 1993). Unfortunately, it has not yet been possible to produce hybrids with radish and *D. tenuifolia* as the maternal and pollen parents, respectively.

In the present paper, we report the results of a study of the structural, biochemical, and physiological characteristics of the leaves of hybrids differing in their genome constitution that were produced in crosses between the C₃–C₄ intermediate *Moricandia arvensis* (L.) DC. and the C₄ species *Brassica oleracea* L. (cabbage). *Moricandia arvensis* is a wild crucifer that grows in semi-arid areas of the Mediterranean region, Africa, and Asia (Apel et al., 1984). It is possible to produce reciprocal hybrids between *M. arvensis* and *B. oleracea* (Matsuzawa et al., 1999). The aims of our study were to assess (1) whether the inheritance pattern in C₃–C₄ intermediate photosynthesis found in our previous study (Ueno et al., 2003) was also present in hybrids of other species in the Brassicaceae and (2) whether...
the inheritance of $C_3$–$C_4$ intermediacy exhibited cytoplasmic inheritance.

**Materials and methods**

1. **Plant material**

Seeds of *M. arvensis* and *B. oleracea* were sown in 8-L pots filled with sufficiently fertilized field soil. We produced various types of reciprocal hybrids differing in genome constitution by using *M. arvensis* (MaMa, $2n = 28$) and *B. oleracea* (CC, $2n = 18$) as the maternal and pollen parents and vice versa (Fig. 1), following the method of Matsuzawa et al. (1999). In summary, the $F_1$ plant MaC ($2n = 23$) was obtained by means of embryo rescue culture. To induce the amphidiploid MaMaCC ($2n = 46$), colchicine was applied to the apical meristems of the $F_1$ seedlings. Backcrossings of the $F_1$ plants with the parent plants were performed to obtain two other progeny: MaMaC ($2n = 37$) and MaCC ($2n = 32$). The amphidiploid $F_1$ plant CCMaMa ($2n = 46$) was obtained through embryo rescue culture.
and colchicine treatment. Backcrossings of this second amphidiploid F1 plant with the parent plants were performed to obtain the two final progeny: CMaMa (2n = 37) and CCMa (2n = 32).

Plants were grown at the National Institute of Agrobiological Sciences (Tsukuba, Ibaraki, Japan) and the Faculty of Agriculture of Utsunomiya University (Tochigi, Japan). In the Tsukuba study, plants were grown in a naturally illuminated greenhouse with temperatures maintained at 25 to 28°C during the day and 15 to 18°C during the night in the spring. We examined leaf anatomy and performed immunocytochemical studies of GDC levels from February to April of 2002 and 2004. In the Utsunomiya study, plants were grown in a naturally illuminated greenhouse in the spring, but without temperature maintenance, and we performed a gas exchange study in May of 2005. Both groups of plants were watered daily. Fully expanded mature leaves of plants were examined 2 to 3 months after planting.

2. Anatomical and ultrastructural studies

Samples taken 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. They were then washed with phosphate buffer and post-fixed in 2% OsO4 in phosphate buffer for 2 h. Samples were dehydrated through an acetone series and then embedded in Spurr’s resin. Transverse ultrathin sections of the leaves were stained with lead citrate or with phosphotungstic acid followed by lead citrate and viewed under a transmission electron microscope.
electron microscope (Hitachi H-7000, Hitachi Co. Ltd., Tokyo, Japan) at 75 kV. Semithin sections (about 1 µm) of leaves on glass slides were stained with toluidine blue O. The chloroplasts per cell profile were counted for 20 BS cells using semithin sections. The centripetal chloroplasts (i.e., those located in the inner tangential walls and the inner half of the radial walls) of the BS cells were also counted. The diameters of mitochondria were determined using electron micrographs at 25000 × magnification. The mitochondrial diameters represent the means of 28 to 66 measurements.

3. Protein A–immunogold electron microscopy of GDC

Small segments of the 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 described by Ueno et al. (2003). Ultrathin sections were immunolabeled with an antiserum to the P-protein of GDC and with protein A–colloidal gold particles (EY Laboratories Inc., San Mateo, CA, USA) as described by Ueno et al. (2003). For controls, the antiserum was replaced by non-immune serum. The antiserum was against the P-protein of GDC isolated from pea leaf mitochondria and was kindly provided by Dr. D. J. Oliver (University of Idaho, Moscow, ID, USA). This antiserum was the same as that used in our previous immunocytochemical studies (Ueno et al., 2003, 2006b). The density of labeling for GDC P-protein was determined by counting the gold particles on electron micrographs at 25000 × magnification and calculating the number per unit area (µm²). Between 12 and 26 individual cells were examined in each of several immunolabeled sections. The density of labeling was calculated as the means of 27 to 65 measurements of mitochondria. In the M mitochondria, there was a gradient in the labeling density of GDC P-protein: the mitochondria of the upper layer of the M cells showed relatively higher labeling densities than those of the lower layer of M cells. Thus, we measured the labeling densities of the mitochondria mainly in the middle layer of M cells.

4. Gas exchange measurements

The net photosynthetic rate was measured using an
LI-6400 portable photosynthesis system (Li-Cor Inc., Lincoln, Nebraska, USA) as described by Ueno et al. (2003). Measurements were made at a photosynthetic photon flux density (PPFD) of 1000 µmol m$^{-2}$ s$^{-1}$, a leaf temperature of 20°C, and a CO$_2$ concentration of 350 µL L$^{-1}$. Light within the chamber was provided by a 6400-02 LED Light Source (Li-Cor Inc.). The $Γ$ value was determined by changing the CO$_2$ concentration in the chamber. Measurements of $Γ$ were obtained at PPFDs of 300 and 1000 µmol m$^{-2}$ s$^{-1}$; leaf temperature was maintained at 20°C during these measurements. The photosynthetic values reported in this paper are the means of four measurements.

5. Statistical analysis

We tested the significance at $P < 0.05$ of any differences in the sizes and the labeling densities of GDC between the M and BS mitochondria using Student’s t-test, and of differences in gas exchange characteristics between plant types by means of one-way ANOVA with the Tukey and Kramer HSD test.

Results

1. Gross morphology

The leaf shape differed between the parents: *M. arvensis* has entire leaves, whereas cabbage has crenate leaves, and the cabbage leaves were larger. The leaves of the hybrids showed various shapes and sizes intermediate between those of the parents, but became increasingly similar to those of *M. arvensis* with an increase in the MaC genome ratio. The flowers of *M. arvensis* and cabbage had violet and yellow petals, respectively, but the flowers of the hybrids generally had yellowish-white petals.

2. Inner leaf structure

The leaves of *M. arvensis* contained large BS cells (Figs. 2A, 3A) that included numerous centripetally located chloroplasts surrounding the vascular bundle (Fig. 4A, Table 1). Both the adaxial and abaxial M cells were elongated (Fig. 2A). The mitochondria in the BS cells were also centripetally located, and most were adjacent to the inner walls of BS cells and overlain by numerous chloroplasts (Fig. 4A). The BS mitochondria were significantly larger than the M mitochondria (Table 2). In contrast, the leaves of cabbage showed a typical C$_4$ anatomy (Fig. 2B). Although the BS cells of cabbage also surrounded the vascular bundle, they contained few chloroplasts and mitochondria (Figs. 3B and 4B, Table 1). The M cells were clearly differentiated into palisade and spongy parenchyma between the adaxial and abaxial sides (Fig. 2B). In contrast to the mitochondria of *M. arvensis*, the BS mitochondria of cabbage were significantly smaller than the M mitochondria (Table 2).

The inner structures of the leaves of the hybrid plants showed a range of anatomies intermediate between those of the parents (Figs. 2C–H, 3C–H). In general, the M cells were elongated (Fig. 2G–H). However, in the hybrids with a relatively high proportion of the C genome, the abaxial M cells showed some characteristics of spongy parenchyma, as illustrated by the MaCC hybrid (Fig. 2E). There was a gradient in the number of chloroplasts in the BS cells among the hybrids (Fig. 3C–H, Table 1). The number of chloroplasts in the BS cells of the MaMaC and CMaMa hybrids (Fig. 3C, F, Table 1) was similar to that in the BS cells of *M. arvensis* (Fig. 3A). The BS cells of the MaCC and CCMa hybrids (Fig. 3E, H, Table 1) included more chloroplasts than the BS cells of cabbage (Fig. 3B), but the numbers were less than in the MaMaC and CMaMa hybrids. The numbers of chloroplasts in the BS cells of the MaMaCC and CCMaMa hybrids (Fig. 3D, G, Table 1) were intermediate between those found in the BS cells of the hybrids with a higher proportion of Ma (MaMaC and CMaMa) and of the hybrids with a higher proportion of C (MaCC and CCMa). The MaC hybrid

| Plant and genotype | Number of chloroplasts | Cp (%) |
|--------------------|------------------------|--------|
| *M. arvensis* (MaMa) | $12.5 \pm 5.0$ | $61.8 \pm 18.9$ |
| *B. oleracea* (CC) | $2.4 \pm 1.7$ | $3.7 \pm 9.2$ |
| MaMaC hybrid | $11.5 \pm 2.1$ | $47.1 \pm 12.5$ |
| MaMaCC hybrid | $8.1 \pm 2.8$ | $37.8 \pm 18.8$ |
| MaC hybrid | $8.8 \pm 2.2$ | $36.4 \pm 13.9$ |
| MaCC hybrid | $6.3 \pm 2.5$ | $30.2 \pm 17.6$ |
| C MaMa hybrid | $10.5 \pm 2.6$ | $43.5 \pm 19.3$ |
| CCMaMa hybrid | $8.5 \pm 2.8$ | $37.0 \pm 15.5$ |
| CCMa hybrid | $6.6 \pm 2.9$ | $30.1 \pm 9.8$ |

Values are given as the means ± SD of 20 cells. Cp, percentage of centripetally located chloroplasts in the BS cells.
had a similar M structure (data not shown) and similar BS chloroplasts (Table 1) to those of the MaMaCC and CCMaMa hybrids. There was also a gradient in the ratios of centripetally located chloroplasts in the BS cells among the hybrids: the values increased with an increase in the proportion of the Ma genome in the hybrids (Fig. 3C–H, Table 1).

The numbers of mitochondria in the BS cells of the hybrids depended on the Ma:C genome ratio (only the BS cells of the CMaMa, CCMaMa, and CCMa hybrids are shown in Fig. 4C–E). In all hybrids except MaCC and CCMa, the BS mitochondria were larger than the M mitochondria (Table 2). In the MaCC hybrid, the BS mitochondria were significantly smaller than the
M mitochondria, whereas in the CCMa hybrid there was no significant difference in size between the M and BS mitochondria. We also observed a gradient in the ratios of the size of the BS mitochondria to that of the M mitochondria: the values roughly increased with an increase in the proportion of the Ma genome in the hybrid (Table 2). In all plants, there was a high and significant positive correlation between the ratio of centripetally located chloroplasts in the BS cells and the ratio of mitochondrial size (BS:M) (r = 0.871, P < 0.01).

### 3. Immunogold localization of the P-protein of GDC

Table 3 shows the labeling density of the P-protein of GDC in the M and BS mitochondria of M. arvensis, cabbage, and their hybrids. In M. arvensis, the BS mitochondria showed intense labeling for the GDC P-protein (Fig. 5B), whereas the M mitochondria showed only weak labeling (Fig. 5A). The ratio of the labeling density of the BS mitochondria to that of the M mitochondria was 7.6 (Table 3). In cabbage, both the M and BS mitochondria showed intense labeling of the GDC P-protein (Fig. 5E, F), but the labeling density was significantly higher in the M mitochondria than in the BS mitochondria (Table 3). As a result, the ratio of labeling density (BS:M) was very low (0.5; Table 3). In all hybrids, labeling of the GDC P-protein occurred in both the M and BS mitochondria (Fig. 5C, D), and the ratios of labeling density (BS:M) in the mitochondria were intermediate between those of the parents (Table 3). In addition, there was a gradient in the pattern of labeling in the hybrids. The ratios of labeling density (BS:M) increased with increasing proportion of the Ma genome, irrespective of whether M. arvensis was the maternal or pollen parent.

In all plants, there were high and significant positive correlations between the ratio of centripetally located chloroplasts in the BS cells and the ratio of labeling density of the GDC P-protein (BS:M) (r = 0.811, P < 0.01) and between the ratio of mitochondrial size (BS:M) and the ratio of labeling density of the GDC P-protein (BS:M) (r = 0.838, P < 0.01).

### 4. Gas-exchange characteristics

The net photosynthetic rate was significantly higher in M. arvensis than in cabbage (Table 4). The net photosynthetic rates of most hybrids were intermediate between those of the parents or were close to that of M. arvensis, but that of the CCMaMa hybrid was somewhat higher (but not significantly so) than that of M. arvensis (Table 4). Under high PPFD, M. arvensis showed a Γ value typical of C₃-C₄ intermediate plants, whereas that of cabbage was typical for C₃ plants (Table 4; Holaday et al., 1982). The MaC, CMaMa, and CCMaMa hybrids had significantly lower Γ values than cabbage at high PPFD. This was especially clear in the Γ value for the CCMa hybrid. However, the Γ values of the MaCC and CCMa hybrids (i.e., hybrids with a higher proportion of the C genome) were not significantly different from that of cabbage at high PPFD. The Γ of C₃-C₄ intermediate plants (M. arvensis and Panicum milioides Nees ex. Trin.) increases as PPFD decreases, whereas that of C₃ plants is essentially unaffected (Holaday et al., 1982). This may be due to a light-dependent mechanism for photorespiratory CO₂ re-assimilation in the C₃-C₄ intermediate plants (Hunt et al., 1987). When measured at low PPFD, the Γ values of all plants increased to a greater or lesser extent (Table 4). However, the magnitude of the increase was clearly largest in M. arvensis and was slightly larger in the CMaMa and CCMaMa hybrids (i.e., hybrids with a higher proportion of the Ma genome) than in the

### Table 3. Immunogold labeling of the P-protein of GDC in the M and BS cells of Moricandia arvensis, Brassica oleracea, and their hybrids.

| Plant and genotype | Number of gold particles (µm²) | Ratio (BS : M cells) |
|--------------------|-------------------------------|---------------------|
|                    | Mitochondria | Cytosol + others | Mitochondria | Cytosol + others |                        |
| M. arvensis (MaMa) | 12.1 ± 9.2 (31) | 0.3 ± 0.3 (16) | 91.6 ± 19.3 (43)* | 0.5 ± 0.8 (8) | 7.6 |
| B. oleracea (CC)   | 92.1 ± 23.4 (54) | 0.4 ± 0.4 (16) | 49.2 ± 11.8 (27)* | 0.3 ± 0.4 (16) | 0.5 |
| MaMaC hybrid       | 43.2 ± 14.4 (46) | 0.5 ± 0.4 (18) | 100.5 ± 16.1 (47)* | 0.6 ± 0.5 (14) | 2.3 |
| MaMaCC hybrid      | 57.7 ± 17.4 (49) | 0.4 ± 0.4 (14) | 114.0 ± 20.5 (37)* | 0.2 ± 0.3 (14) | 2.0 |
| MaC hybrid         | 42.5 ± 17.4 (53) | 0.4 ± 0.6 (12) | 76.6 ± 19.9 (47)* | 0.1 ± 0.1 (10) | 1.8 |
| MaCC hybrid        | 70.4 ± 17.9 (58) | 0.3 ± 0.3 (16) | 61.7 ± 20.3 (65)* | 0.2 ± 0.4 (25) | 0.9 |
| CMMaMa hybrid      | 32.5 ± 8.0 (42)  | 0.2 ± 0.2 (26) | 94.0 ± 21.9 (57)* | 0.3 ± 0.3 (13) | 2.9 |
| CCMaMa hybrid      | 39.8 ± 16.8 (47) | 0.1 ± 0.3 (18) | 85.6 ± 18.9 (41)* | 0.2 ± 0.4 (12) | 2.2 |
| CCMa hybrid        | 59.5 ± 17.6 (43) | 0.2 ± 0.3 (18) | 75.3 ± 19.4 (37)* | 0.2 ± 0.4 (15) | 1.3 |

The number of gold particles per µm² is given as the mean ± SD. The number of organelles or cell profiles examined is given in parentheses. Asterisks represent significant differences at P < 0.05 between the M and BS mitochondria.
Table 4. Photosynthetic rates and the effect of photosynthetic photon flux density (PPFD) on the CO₂ compensation point (Γ) in the leaves of *Moricandia arvensis*, *Brassica oleracea*, and their hybrids.

| Species and genotype   | Photosynthetic rate (µmol m⁻² s⁻¹) | Γ (µmol mol⁻¹)       | Ratio of Γ at high : low PPFD |
|------------------------|-------------------------------------|----------------------|-------------------------------|
|                        |                                     | High PPFD            | Low PPFD                      |                               |
| *M. arvensis* (MaMa)   | 21.4 ± 4.6ab                       | 21.7 ± 2.2a          | 35.7 ± 2.7a                   | 0.61 ± 0.06a                  |
| *B. oleracea* (CC)     | 10.5 ± 0.4a                        | 39.6 ± 1.5*          | 46.1 ± 1.9*                   | 0.86 ± 0.02ab                 |
| MaC hybrid             | 16.1 ± 1.4bc                       | 35.9 ± 1.7b          | 40.4 ± 1.9b                   | 0.89 ± 0.01*                  |
| MaCC hybrid            | 16.5 ± 3.1bc                       | 39.6 ± 0.9c          | 45.5 ± 2.4c                   | 0.87 ± 0.05c                  |
| CMaMa hybrid           | 17.1 ± 3.3abc                      | 27.9 ± 1.3c          | 35.3 ± 1.3c                   | 0.80 ± 0.02bc                 |
| CCMaMa hybrid          | 23.9 ± 2.6c                        | 34.3 ± 1.6b          | 43.5 ± 2.1ab                  | 0.79 ± 0.02c                  |
| CCMa hybrid            | 18.3 ± 3.4ab                       | 36.7 ± 0.8ab         | 43.1 ± 1.5b                   | 0.85 ± 0.02abc                |

Values are given as the mean ± SD of four measurements. High and low PPFD are 1000 and 300 µmol m⁻² s⁻¹, respectively. Values in a column followed by the same letter do not differ significantly at P < 0.05.

Discussion

1. The characteristics of C₃–C₄ intermediate photosynthesis are inherited in the hybrids on the basis of the ratio of the parent genomes

Previously, we found that C₃–C₄ intermediate characteristics are inherited by hybrids between *D. tenuifolia* (C₃) and radish (C₄) according to the ratio of the parent genomes (Ueno et al., 2003). Thus, we attempted to assess whether this was also true of hybrids between *M. arvensis* and cabbage, using more varied hybrids in their genome constitution than those examined in our previous study. Our results suggest that the inheritance of C₃–C₄ intermediacy in these hybrids is under genetic control similar to that in hybrids between *D. tenuifolia* and radish.

The leaf inner structure differed greatly between *M. arvensis* and cabbage in the number of organelles in the BS cells and the structure of the M cells, with hybrids exhibiting various levels of intermediacy in these features. The number of chloroplasts and mitochondria in the BS cells and the ratio of centripetally located chloroplasts in the BS cells of hybrids increased with an increasing Ma:C ratio in the genome. In addition, mitochondrial size showed a gradient according to the Ma : C ratio in the genome. In *M. arvensis*, the mitochondria were centripetally located in the BS cells and surrounded by chloroplasts: this morphology allows the chloroplasts to recapture CO₂ released from mitochondria during photorespiration (Monson and Rawsthorne, 2000). In addition, the proportion of the P-protein of GDC in the BS cells relative to that in M cells also increased with increasing Ma : C ratio in the genome. This would increase the relative contribution of the BS mitochondria to photorespiratory CO₂ release within leaves. Finally, the expression of the structural and biochemical features involved in C₃–C₄ intermediacy was reflected in the gas exchange characteristics of the hybrids, in which the Γ value declines with an increase in the Ma:C ratio in the genome. We found that the Γ value was highly and negatively correlated with the ratio of centripetally located chloroplasts in the BS cells and with the ratios (BS : M) of mitochondrial size and of labeling density of the GDC P-protein. It is also important to note that complete suppression of the expression of the GDC P-protein in the M cells is not required to reduce Γ, as revealed in our previous studies (Ueno et al., 2003, 2006b).

Based on the data from the present study and our previous findings (Ueno et al., 2003), the C₃–C₄ intermediate characteristics generally appear to be inherited in hybrid plants on the basis of the ratio of the parent genomes. Chromosome-addition hybrid lines are a powerful tool for dissecting the control of gene expression, inheritance, and syntenic correspondence among different species (Kynast et al., 2004). In cruciferous species, it is possible to produce monosomic and disomic chromosome addition lines (Matsuzawa et al., 1996). Analyses of plants with added chromosomes of C₃-C₄ intermediate species would further improve our understanding of the genetic mechanisms that underlie in C₃-C₄ intermediacy.

2. There is no cytoplasmic inheritance of C₃–C₄ intermediacy

It is not yet fully understood whether cytoplasmic inheritance is involved in the genetics of C₃–C₄ intermediacy and C₄ characteristics (Brown and Bouton, 1993; Ueno et al., 2003). In the present study, we tested this possibility using reciprocal hybrids.
between *M. arvensis* and cabbage. We found that at similar Ma : C ratios in the genome, there was no significant difference in the degree of expression of C₃–C₄ intermediate characteristics between the reciprocal hybrids.

The genes for the large and small subunits of Rubisco are encoded by the chloroplastic and nuclear genomes, respectively. Thus, the gene for the large subunit of Rubisco is maternally inherited (Evans and Austin, 1986; Hudson et al., 1990). However, there is little evidence that the characteristics involved in C₃–C₄ intermediate and C₄ photosynthesis are maternally influenced in hybrids between C₃, C₃–C₄ intermediate, and C₄ species (Araus et al., 1990; Brown and Bouton, 1993). Although the mitochondria contain proteins encoded by both nuclear and mitochondrial genomes (Leon et al., 1998), the four subunits of GDC, including the P-protein, are encoded by the nuclear genome (Douce and Heldt, 2000). It is also known that the development of chloroplasts and mitochondria is controlled by the nuclear genome (Leon et al., 1998). Our data suggest that there is no cytoplasmic effect on the inheritance of C₃–C₄ intermediate characteristics.

### 3. Genetic control of photosynthetic metabolism

Several mechanisms that reduce photorespiration have evolved in plants. In C₃ and C₃–C₄ intermediate photosynthesis in the leaves of terrestrial plants, two cell types are generally required (Leegood, 2002). Even in the single-cell C₄ plants that have recently been discovered, structural differentiation of organelles occurs within a cell (Edwards et al., 2004). Although it appears that there is a genetic distinction between C₃ and other photosynthetic modes, some plants that link the C₃ and C₄ modes, such as *Elaeocharis vivipara* Link (Ueno, 2001) and *Allotropis semialata* (R. Br.) Hitchcock (Ueno and Sentoku, 2006), bridge the genetic gap between C₃ and C₄ species. Currently, attempts have been made to genetically manipulate photosynthesis by introducing the genes for C₄ photosynthetic enzymes into C₃ plants (reviewed in Surridge, 2002). However, there have been many obstacles to these attempts; it seems that spatial compartmentalization of biochemical functions is a prerequisite for efficient operation of the C₄ cycle in terrestrial plants (Leegood, 2002). Our present study and previous studies with hybrids between C₃ and C₃–C₄ intermediate species demonstrated that the impact of photorespiration on photosynthesis can be mitigated if both the structural and biochemical components of C₃–C₄ intermediate photosynthesis are simultaneously and correctly introduced into the C₃ plants.

Although early studies suggested that photorespiration is a wasteful process in plants, more recent research indicated that this process may serve as an energy sink to prevent over-reduction of the photosynthetic electron chain and photoinhibition (Kozaki and Takeba, 1996; Wingler et al., 2000). Photorespiratory metabolism is linked with nitrogen metabolism (Keys and Leegood, 2002), and recent studies have suggested that nitrate assimilation in plant shoots depends on photorespiration (Rachmilevitch et al., 2004). Thus, complete suppression of photorespiration may be detrimental to C₃ plants, as was shown by studies of mutants and antisense plants for GDC P-protein (Wingler et al., 2000; Bauwe and Kolukisaoglu, 2003). There is evidence that potential photorespiration exists even in C₄ plants, although the activity of photorespiration is much lower than in C₃ plants (Yoshimura et al., 2004; Ueno et al., 2005). It should be noted that considerable photorespiration occurs within leaf cells of C₃–C₄ intermediate plants, even though apparent photorespiration is reduced (Monson and Rawsthorne, 2000). Further studies are needed to investigate how the hybrids between *M. arvensis* and cabbage respond to environmental factors such as light, temperature, and water stresses. Recently, Ueno et al. (2006b) found evidence that a wild crucifer, *Diplotaxis muralis* (L.) DC. (2n = 42) was created by natural hybridization between a C₃–C₄ intermediate species *D. tenuifolia* (2n = 22) and a C₄ species *D. viminalis* (L.) DC. (2n = 20). This species has structural, biochemical, and physiological features related to photosynthesis that are intermediate between those of C₃–C₄ intermediate and C₄ species and that resemble those found in the MaMaCC and CCMaMa hybrids. This finding suggests that plants with photosynthetic features intermediate between those of C₃–C₄ intermediate and C₄ species could survive under natural selective pressure (Ueno et al., 2006b).

The mechanisms underlying C₃–C₄ intermediate photosynthesis in *M. arvensis* appear to be less complicated than those that underlie C₄ photosynthesis. The key mechanism in C₃–C₄ intermediate biochemistry is the predominant function of GDC in the BS mitochondria. However, the molecular mechanism that regulates the intercellular expression of the GDC P-protein has not yet been identified (Thole and Rawsthorne, 2003). In the future, the molecular and genetic mechanisms responsible for directing structural differentiation such as the specialized BS cells and dense leaf vasculature found in the leaves of C₃–C₄ intermediate and C₄ plants should also be elucidated (Ueno, 2001; Surridge, 2002; Brown et al., 2005; Ueno et al., 2006a; Wakayama et al., 2006).

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