Production of *Raphanus sativus* (C₃)-*Moricandia arvensis* (C₃-C₄ intermediate) Monosomic and Disomic Addition Lines with Each Parental Cytoplasmic Background and their Photorespiratory Characteristics

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Abstract : We are maintaining five *Moricandia arvensis* monosomic addition lines of *Raphanus sativus* carrying R. sativus cytoplasm (autoplasmic MALs) and twelve *M. arvensis* MALs of R. sativus carrying M. arvensis cytoplasm (alloplasmic MALs) from BC₀ to BC₉ generation, and newly produced five *M. arvensis* disomic addition lines of R. sativus (autoplasmic DALs) and seven *M. arvensis* DALs of R. sativus carrying M. arvensis cytoplasm (alloplasmic DALs) from selfing and sib-crossing of the MALs and DALs in S₂BC₆ and S₃BC₆ generations. The structural, biochemical and physiological characteristics related to photorespiration of these MALs and DALs were compared to study the genetic mechanisms of the C₃-C₄ intermediate photosynthesis in the individual chromosomes of *M. arvensis*. The CO₂ compensation point of the autoplasmic and alloplasmic disomic DALs (RM-a-b and MaR-b DALs) with one pair of *M. arvensis* 'b' chromosome were 29.4 and 30.1 μmol mol⁻¹, respectively, which were significantly lower than that of other DALs and MALs as well as R. sativus (34.5 μmol mol⁻¹). An immunogold electron microscopic study of the P-protein of glycine decarboxylase (GDC) in photosynthetic cells of the RMa-b DAL revealed that the bundle sheath cell (BSC) mitochondria were more intensively labeled for the protein than the mesophyll cell (MC) mitochondria. The ratio of the labeling density of the BSC mitochondria to that of the MC mitochondria was 1.13, which lies between values in *M. arvensis* (2.66) and *R. sativus* (0.76). These data suggest that the 'b' chromosome of *M. arvensis* genome controls the expression of C₃-C₄ intermediate characteristics.

Key words : C₃-C₄ intermediate plant, CO₂ compensation point, Disomic addition line (DAL), Monosomic addition line (MAL), *Moricandia arvensis*, Photorespiration.

*Moricandia arvensis* (L.) DC. (2n=28, MaMa) is one of the C₃-C₄ intermediate species of Brassicaceae. Among the species of Brassicaceae in which C₃ species have not yet been found, other C₃-C₄ intermediate species have also been found within the genera *Moricandia*, *Diplotaxis* and *Brassica* (Apel et al., 1997). *M. arvensis*, which was the first-reported C₃-C₄ intermediate species in the Brassicaceae (Apel et al., 1978; Holaday et al., 1981), has attracted the attention of breeders because its traits can be introgressed into cultivated crops to improve their photosynthetic efficiency (Apel et al., 1984; Toriyama et al., 1987; Takahata, 1990; Takahata and Takeda, 1990; Kirii et al., 1992; Takahata et al., 1993; Remzioo et al., 1996; Bang et al., 1996, 2002, 2007; Ishikawa et al., 2003).

CO₂ release by photorespiration in C₃ plants is estimated to be about 25% of photosynthetically fixed CO₂ in ordinary air (Sharkey, 1988). *M. arvensis* shows low a CO₂ compensation point (F₃) because a large part of the CO₂ released from the mitochondria in bundle sheath cells (BSCs) is recaptured by the chloroplasts before it escapes from the BSCs (Rawsthorne et al., 1988; Monson and Rawsthorne, 2000). The photorespiratory metabolism unique to *M. arvensis* results from the Kranz-like leaf anatomy and the specific expression of glycine decarboxylase (GDC), a key enzyme of the photorespiratory (glycolate) cycle, within photosynthetic cells (Rawsthorne et al., 1988; Hylton et al., 1988; Morgan et al., 1993). The Kranz-like leaf anatomy and biochemical components of *M. arvensis* are controlled by different genetic mechanisms (Rylott et al., 1998). The four subunits of GDC, including the P-protein, are encoded in the nuclear genome (Douce and Heldt, 2000), and the development of chloroplast and mitochondria is also controlled by the nuclear genome (Leon et al., 1998). With respect to the expression of C₃-C₄ intermediate characteristics, Apel et al. (1984) first reported that the intergeneric F₃ and
The C₄-C₃ intermediate genome to the C₃ genome in confirmed. It is suggested that the constitution ratio in their hybrids, but the cytoplasmic effect was not genome according to the ratio of genome constitution compensation point were controlled by the nuclear characteristics. Among the Brassicaceae species, *M. arvensis* (2n=28) and *D. tenuifolia* (2n=22) belong to the same “Rapa/Oleracea” lineage at the level of molecular phylogeny based on chloroplast DNA restriction site variation (Warwick et al., 1992; Warwick and Black, 1994). This research suggested the existence of a common phylogenetic ancestor and monophyletic evolution of the C₄-C₃ intermediate characteristics in Brassicaceae (Apel et al., 1997). The C₄-C₃ intermediate species monosomic and disomic addition lines of C₃ species (MALs and DALs) could provide more valuable information on the evolution and the genetic system of the C₄-C₃ intermediate characteristics under the control of individual chromosome. In this study, we produced five *M. arvensis* DALs of autoplasmic *R. sativus* and seven *M. arvensis* DALs of allotomplasmic *R. sativus* by selfing and sib-crossing of the MALs and DALs, and also maintained five autoplasmic MALs and 12 allotomplasmic MALs of *R. sativus*. The structural, biochemical and physiological characteristics related to photorespiration of these MALs and DALs were examined to study the genetic mechanisms of C₄-C₃ intermediate photosynthesis at each chromosome level.

**Materials and Methods**

1. **Plant materials and production of MALs and DALs**

*Photorespiratory Characteristics of R. sativus (C₃) – M. arvensis (C₄-C₃) MALs and DALs*  

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BC₃ hybrids between *M. arvensis* and *Brassica alboglabra* (C₃) showed intermediate gas exchange traits. In recent studies with the intergeneric hybrids between *Diplotaxis tenuifolia* (C₅-C₃) and *Raphanus sativus* (C₃) and the reciprocal intergeneric hybrids between *M. arvensis* (C₃-C₅) and *B. oleracea* (C₅), Ueno et al. (2003, 2007) demonstrated that the C₅-C₃ intermediate characteristics such as the BSC-dominant expression of the GDC P-protein, the inclusion of numerous chloroplasts and mitochondria in the BSCs, and reduced CO₂ compensation point were controlled by the nuclear genome according to the ratio of genome constitution in their hybrids, but the cytoplasmic effect was not confirmed. It is suggested that the constitution ratio of the C₅-C₃ intermediate genome to the C₃ genome in their hybrids is the most important factor determining the degree of expression of the C₅-C₃ intermediate characteristics.

Among the Brassicaceae species, *M. arvensis* (2n=28) and *D. tenuifolia* (2n=22) belong to the same “Rapa/Oleracea” lineage at the level of molecular phylogeny based on chloroplast DNA restriction site variation (Warwick et al., 1992; Warwick and Black, 1994). This research suggested the existence of a common phylogenetic ancestor and monophyletic evolution of the C₅-C₃ intermediate characteristics in Brassicaceae (Apel et al., 1997). The C₅-C₃ intermediate species monosomic and disomic addition lines of C₃ species (MALs and DALs) could provide more valuable information on

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**Fig. 1.** Schematic diagram of producing the autoplasmic and alloplasmic MALs and DALs which were used as plant materials in this study.
Production of autoplasmic and alloplasmic DALs from selfing and sib-crossing of each MAL and DAL.

| Cross combination                  | No. of flowers pollinated | No. of seeds obtained | No. of seeds sown | Somatic chromosome number (2n) | No. of plants observed |
|------------------------------------|---------------------------|-----------------------|-------------------|-------------------------------|------------------------|
| R. sativus cytoplasm               |                           |                       |                   |                               |                        |
| RMa-b DAL (20) selfing             | 30                        | 5                     | 5                 | 4                             | 1                      |
| RMa-d MAL (19) × RMa-d DAL (20)    | 154                       | 104                   | 20                | 12                            | 9                      |
| RMa-e DAL (20) selfing             | 90                        | 14                    | 9                 | 6                             | 2                      |
| RMa-f MAL (19) sib cross           | 140                       | 125                   | 20                | 19                            | 9                      |
| RMa-I MAL (19) sib cross           | 121                       | 214                   | 20                | 18                            | 5                      |
| M. arvensis cytoplasm              |                           |                       |                   |                               |                        |
| MaR-a MAL (19) × MaR-a DAL (20)    | 96                        | 65                    | 11                | 10                            | 1                      |
| MaR-b MAL (19) × RMa-b DAL (20)    | 124                       | 137                   | 10                | 9                             | 7                      |
| MaR-c MAL (19) sib cross           | 84                        | 24                    | 20                | 15                            | 4                      |
| MaR-d MAL (19) × MaR-d DAL (20)    | 471                       | 20                    | 20                | 16                            | 12                     |
| MaR-e MAL (19) × RMa-e DAL (20)    | 62                        | 22                    | 10                | 10                            | 9                      |
| MaR-f MAL (19) sib cross           | 26                        | 89                    | 10                | 8                             | 5                      |
| MaR-I MAL (19) × RMa-I DAL (20)    | 405                       | 32                    | 10                | 6                             | 1                      |

3. Anatomical and ultrastructural studies of leaves

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 hours. They were then washed with phosphate buffer and post-fixed in 2% OsO$_4$ in phosphate buffer for 2 hours. The samples were dehydrated by an acetone series and then embedded in Spurr’s resin. Transverse ultrathin sections were stained with lead citrate and viewed under a transmission electron microscope (Hitachi H-7000, Hitachi Co., Ltd., Tokyo, Japan). Semi-thin sections (about 1 μm) were stained with toluidine blue O. The chloroplasts per cell profile of BSCs were counted using semithin sections. The centripetal chloroplasts of the BSCs were also counted.

4. Protein A-immunogold electron microscopy of glycine decarboxylase (GDC)

Small segments of leaves were fixed with 3% (v/v) glutaraldehyde in 50 mM sodium phosphate buffer (pH 6.8), dehydrated by an ethanol series and embedded in Lowicryl K4M resin (Chemische Werke Lowi GmbH, Waldkirburg, Germany), as previously described (Ueno et al., 2003). Ultrathin sections were immunolabeled with an antisera to the P protein of GDC and with protein A-colloidal gold particles (EY Lab. Inc., San Mateo, CA, USA), as previously described (Ueno et
was used at a dilution of 1:500. The density of labeling
provided by Dr. D. J. Oliver, University of Idaho, was
calculated as the means of 19 to 55 measurements. For controls, the antiserum was replaced with non-immune serum. The antiserum was kindly provided by Dr. D. J. Oliver, University of Idaho, was raised against the P protein of GDC isolated from pea leaf mitochondria. For immunolabeling, 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 25000x magnification and calculating the number per unit area (μm²). Between 8 and 23 individual cells were examined in each of several immunolabeled sections. The density of labeling was calculated as the means of 19 to 55 measurements.

Table 2. Photosynthetic and photorespiratory characteristics of autoplasmic and alloplasmic DALs and MALs.

| Line       | Photosynthesis rate (μmol m⁻²s⁻¹) | CO₂ compensation point (μmol mol⁻¹) |
|------------|-----------------------------------|-------------------------------------|
|            | High light | Low light | High/low light |
| Autoplasmic DAL |          |           |               |
| RMa-b      | 11.8 ± 2.5* | 29.4 ± 3.7* | 38.4 ± 0.5 | 0.763 ± 0.087* |
| RMa-d      | 12.5 ± 2.3* | 43.7 ± 1.8* | 45.6 ± 4.5 | 0.962 ± 0.055 |
| RMa-e      | 14.3 ± 2.4  | 44.1 ± 6.3  | 44.7 ± 2.7 | 0.983 ± 0.089 |
| RMa-f      | 15.9 ± 1.5  | 38.3 ± 2.5  | 44.0 ± 1.0* | 0.869 ± 0.040 |
| RMa-l      | 13.8 ± 1.8* | 42.1 ± 3.7  | 47.5 ± 1.0* | 0.887 ± 0.096 |
| Alloplasmic DAL |         |           |               |
| MaRa       | 22.4 ± 3.9  | 33.6 ± 1.4  | 38.5 ± 1.9 | 0.874 ± 0.064 |
| MaRb       | 22.7 ± 4.4  | 30.1 ± 1.8* | 37.9 ± 2.5 | 0.797 ± 0.065* |
| MaRc       | 16.6 ± 0.8  | 37.6 ± 4.7  | 46.4 ± 4.4* | 0.823 ± 0.174 |
| MaRd       | 17.0 ± 1.9  | 37.7 ± 1.8  | 41.1 ± 1.0  | 0.916 ± 0.034 |
| MaRe       | 18.1 ± 3.2  | 38.3 ± 0.9* | 41.4 ± 2.0  | 0.927 ± 0.031 |
| MaRf       | 11.5 ± 4.2* | 37.5 ± 2.2  | 43.6 ± 1.6* | 0.862 ± 0.067 |
| MaRl       | 16.8 ± 0.8  | 37.8 ± 2.8  | 44.6 ± 2.1  | 0.846 ± 0.022 |
| Autoplasmic MAL |       |           |               |
| RMa-b      | 14.8 ± 3.1  | 33.0 ± 2.1  | 40.2 ± 2.2  | 0.822 ± 0.044 |
| RMa-d      | 12.0 ± 2.1* | 40.0 ± 6.3  | 43.2 ± 5.1  | 0.929 ± 0.062 |
| RMa-e      | 11.4 ± 0.4* | 47.4 ± 2.0  | 47.3 ± 5.9  | 1.008 ± 0.083 |
| RMa-f      | 16.9 ± 5.5  | 37.5 ± 5.0  | 42.1 ± 3.1  | 0.888 ± 0.054 |
| RMa-l      | 17.6 ± 2.8  | 39.4 ± 3.3  | 42.1 ± 0.2* | 0.936 ± 0.084 |
| Alloplasmic MAL |      |           |               |
| MaRa       | 16.8 ± 3.5  | 37.6 ± 1.8  | 38.9 ± 3.5  | 0.971 ± 0.054 |
| MaRb       | 17.2 ± 1.9  | 40.9 ± 3.9* | 45.7 ± 6.0  | 0.912 ± 0.178 |
| MaRc       | 19.0 ± 2.1  | 40.2 ± 5.5  | 43.6 ± 6.4  | 0.937 ± 0.193 |
| MaRd       | 20.5 ± 4.1  | 35.0 ± 0.9  | 40.9 ± 5.0  | 0.863 ± 0.089 |
| MaRe       | 14.4 ± 3.3  | 35.0 ± 3.0  | 41.3 ± 3.3  | 0.847 ± 0.018 |
| MaRf       | 18.3 ± 2.5  | 34.1 ± 1.5  | 43.2 ± 3.7  | 0.794 ± 0.065 |
| MaRg       | 20.8 ± 0.8  | 37.3 ± 3.7  | 45.5 ± 3.9  | 0.820 ± 0.074 |
| MaRh       | 19.7 ± 2.2  | 35.3 ± 1.0  | 36.9 ± 2.2  | 0.958 ± 0.042 |
| MaRi       | 18.4 ± 3.0  | 34.9 ± 2.4  | 37.2 ± 7.9  | 0.975 ± 0.242 |
| MaRj       | 17.3 ± 2.2  | 38.9 ± 2.8  | 43.2 ± 4.5  | 0.907 ± 0.107 |
| MaRk       | 21.8 ± 1.2  | 39.6 ± 2.6  | 43.7 ± 3.2  | 0.907 ± 0.047 |
| MaRl       | 16.4 ± 1.5  | 37.6 ± 0.8  | 41.3 ± 2.3  | 0.904 ± 0.060 |
| M. arvensis | 17.3 ± 4.0  | 18.7 ± 1.8* | 36.5 ± 3.6  | 0.517 ± 0.072* |
| R. sativus  | 22.1 ± 4.8  | 34.5 ± 0.9  | 39.0 ± 0.8  | 0.884 ± 0.023 |

High and low light are 1000 and 300 μmol m⁻²s⁻¹, respectively. Values are given as the means ± SD of four measurements. Asterisks represent significant differences at P < 0.05 between R. sativus and other plants.
The diameters of mitochondria were determined using the same electron micrographs as those used for the measurement of labeling density of GDC. The mitochondrial diameters represent the means of 19 to 58 measurements.

5. Statistical analysis

We tested the significance at P<0.05 of any difference in gas exchange characteristics between *R. sativus* and other plants, and in the size and the labeling densities of GDC between the MC and BSC mitochondria using Student’s t-test.

Results

1. Production of MALs and DALs and their cytogenetic characteristics

Five autoplasmic MALs (RMa-b, -d, -e, -f and -l) were maintained by backcrossing each MAL to *R. sativus* cv. ‘Pink ball’ (data not shown). Five autoplasmic DALs (RMa-b, -d, -e, -f and -l) were produced by sibfying of DAL (2n=20), sib-crossing of MAL (2n=19) and crossing between MAL and DAL (Table 1). Each RMa-b and RMa-e DAL was produced by sibfying of each DAL (2n=20) that was obtained in SBC₁ and SBC₂ generations, respectively. Three RMa-d DALs were obtained by crossing between RMa-d (MAL, 2n=19) and alloplasmic MaR-d (DAL, 2n=20). One RMa-f DAL and two RMa-l DALs were produced by sib-crossing of MAL (2n=19).

Twelve alloplasmic MALs (MaR-a~l) were maintained by backcrossing MAL to *R. sativus* cv. ‘Pink ball’ (data not shown). Seven alloplasmic DALs (MaR-a, -b, -c, -d, -e, -f and -l) were obtained by sibfying of MAL and crossing between MAL and DAL, and crossing between MAL and alloplasmic DAL in which two added chromosomes are homologous chromosomes. Two DALs of each of MaR-a, -b and -l, and one MaR-e DAL were produced by crossing between MAL and DAL. Two MaR-c DALs and three MaR-f DALs were obtained by sibfying of MAL. Another four MaR-d DALs were produced by crossing between MAL and DAL.

2. Gas exchange characteristics

The photosynthetic rates in the *R. sativus* (22.1 μmol m⁻² s⁻¹) and *M. arvensis* (17.3 μmol m⁻² s⁻¹) were not significantly different (Table 2). The photosynthetic rates in the alloplasmic DALs and MALs were mostly not significantly different from those in the parents, but those in some autoplasmic DALs and MALs were significantly lower than that in *R. sativus*. Under high-intensity light (1000 μmol m⁻² s⁻¹), the CO₂ compensation point (Γ) of *M. arvensis* was a typical C₃-C₄ intermediate value (18.7 μmol mol⁻¹), and the value in *R. sativus* (34.5 μmol mol⁻¹) was higher than in *M. arvensis* and was within the range for C₃ plants (Edwards and Ku, 1987). Under low light (300 μmol m⁻² s⁻¹), however, Γ values in *M. arvensis* and *R. sativus* were 36.5 and 39.0 μmol mol⁻¹, respectively. As a result, the high/low-intensity light ratio of Γ was significantly lower in *M. arvensis* (0.517) than in *R. sativus* (0.884). These patterns of responses of Γ to light intensity are typical of C₃-C₄ intermediate and C₃ plants (Holaday et al., 1982).

Under high-intensity light, the Γ value in almost all the auto- and alloplasmic MALs and DALs was not
significantly different from that in \textit{R. sativus} (34.5 μmol mol$^{-1}$). However, the $\Gamma$ in RMa-b and MaR-b DALs was 29.4 and 30.1 μmol mol$^{-1}$, respectively, which was significantly different from that in \textit{R. sativus}. The RMa-b and MaR-b DALs possess the same individual chromosomes but have different cytoplasm. Under low-intensity light, $\Gamma$ in almost all DALs and MALs were not significantly different from that in \textit{R. sativus}. The high/low-intensity light ratios of $\Gamma$ in all DALs and MALs were not significantly different from that in \textit{R. sativus}, but the ratios in both RMa-b and MaR-b DAL were significantly lower than that in \textit{R. sativus}. These results suggest that the individual ‘b’ chromosome added to RMa-b and MaR-b DALs might control the expression of the C$_3$-C$_4$ intermediate characteristics in \textit{M. arvensis}.

### Table 3. The number of chloroplasts per cell profile and the ratio of centripetally located chloroplasts in the BSCs of autoplasmic and alloplasmic DALs, \textit{M. arvensis} and \textit{R. sativus}.

| Line     | Number of chloroplasts | Cp (%) |
|----------|-------------------------|--------|
| RMa-b    | 8.3 ± 2.9 (14)          | 36.2   |
| \textit{M. arvensis} | 9.5 ± 4.0 (22) | 57.4   |
| \textit{R. sativus} | 6.0 ± 2.2 (20) | 15.0   |

Values are given as the means ± SD. The number of BSCs examined is given in parentheses. Cp, percentage of centripetally located chloroplasts in the BSCs.

### 3. Anatomical and ultrastructural features of leaves

We observed anatomical and ultrastructural features of leaves in six autoplasmic and alloplasmic DALs including the RMa-b DAL, and also in the parent species. The leaves of \textit{M. arvensis} had a typical C$_3$-C$_4$ intermediate anatomy (Fig. 2A). The BSCs included numerous centripetally located chloroplasts surrounding the vascular bundle (Fig. 3A), whereas both the adaxial and abaxial mesopyll cells were elongated (Fig. 2A). The leaves of \textit{R. sativus} had a typical C$_3$ anatomy (Fig. 3B). The BSCs included only a few chloroplasts (Fig. 3B, Table 3), whereas the MCs were differentiated into palisade and spongy parenchyma (Fig. 2B). All the DALs except for the RMa-b DAL also showed anatomical features of the BSCs and MCs similar to those of \textit{R. sativus} (Figs. 2D, 3D). However, the BSCs of the RMa-b DAL included more chloroplasts in the centripetal position than those in other DALs (Figs. 3C, 4D, Table 3). The leaves of RMa-b DAL were thicker than those of \textit{R. sativus} and other DALs, and had elongated palisade MCs (Fig. 2C).
Electron microscopic observation revealed that the BSCs of the RMa-b DAL generally included a considerable number of mitochondria in the centripetal position (Fig. 4C, D), although the number varied among the BSCs. The number of mitochondria was somewhat larger than that in *R. sativus* (Fig. 4B) but much smaller than that in *M. arvensis* (Fig. 4A). In the BSCs of other DALs, no increase in the number of mitochondria was observed (data not shown).

In *M. arvensis*, the mitochondria were significantly larger in the BSCs than in the MCs, whereas in *R. sativus*, the difference in the size between the two cell types was not significant (Table 4). In the RMa-b DAL, the mitochondria were significantly larger in the BSCs than in the MCs. The ratio of the size of mitochondria in the BSCs to that in the MCs in the RMa-b DAL was 1.37, which was also intermediate of those in *M. arvensis* (1.71) and *R. sativus* (0.94) (Table 4). In RMa-e, MaR-a and MaR-l DALs, the mitochondria were significantly larger in the MCs than in the BSCs. In RMa-l and MaR-d DALs, mitochondrial size was not significantly different between the two cell types. In our previous study, the BSC/MC ratio of size of mitochondria in *R. sativus* was also 0.83 to 0.84. Thus, the BSC/MC ratios of mitochondrial size in these DALs were similar to those in *R. sativus*.

4. Localization of the immunogold-labeled P-protein of glycine decarboxylase (GDC)

Table 5 shows the labeling densities of the P-protein of GDC in photosynthetic cells of six autoplasmic and alloplasmic DALs.

| Line          | Number of gold particles (μm⁻²) | Ratio (BSC : MC) | MC   | BSC                     |
|---------------|--------------------------------|------------------|------|-------------------------|
|               | Mitochondria                   |                  |      |                         |
| RMa-b         | 63.9 ± 25.6 (33)               | ND (14)          | 71.9 ± 15.5 (32)* | ND (11) | 1.13 |
| RMa-e         | 146.7 ± 33.0 (46)              | 0.3 ± 0.2 (11)   | 71.2 ± 24.6 (38)* | 0.1 ± 0.2 (16) | 0.49 |
| RMa-l         | 93.1 ± 25.9 (37)               | ND (10)          | 45.8 ± 17.7 (28)* | ND (10) | 0.49 |
| MaR-a         | 98.8 ± 18.9 (39)               | 0.1 ± 0.2 (16)   | 62.6 ± 14.5 (26)* | 0.1 ± 0.3 (11) | 0.63 |
| MaR-d         | 143.8 ± 30.7 (42)              | 0.7 ± 0.4 (11)   | 85.8 ± 22.9 (30)* | 0.9 ± 0.7 (20) | 0.60 |
| MaR-l         | 133.1 ± 30.2 (37)              | ND (11)          | 80.3 ± 22.4 (19)* | ND (8)  | 0.60 |
| *M. arvensis* | 29.5 ± 13.5 (45)               | 0.1 ± 0.1 (23)   | 78.4 ± 12.5 (55)* | 0.8 ± 0.6 (16) | 2.66 |
| *R. sativus*  | 130.0 ± 33.3 (39)              | 2.1 ± 1.3 (8)    | 98.7 ± 29.3 (24)* | 2.5 ± 1.7 (18) | 0.76 |

Values are given as the means ± SD. The number of mitochondrial or cell profiles examined is given in parentheses. ND, not detectable. ns, not significant. Asterisks represent significant differences at $P < 0.05$ between the MC and BSC mitochondria.
was similar to that in *R. sativus*. In the RMa-b DAL, however, there was no significant difference in the labeling density of the P-protein of GDC between BSC and MC mitochondria, and the labeling density ratio was 1.13 which lied between those of *R. sativus* and *M. arvensis*.

**Discussion**

Chromosome addition lines such as MALs were used for the analysis of agronomic traits and gene(s) that were assumed to be located on the chromosome added. The advantages of using MALs include the possibilities of assigning species-specific gene(s) and/or characteristics to particular chromosomes, and transferring desirable agronomic traits between species (Namai, 1987; McGrath and Quiros, 1990; Matuszawa et al., 1996; Kynast et al., 2004). The intergeneric hybrids between *C*₃-*C*₄ intermediate species and *C*₃ species with various genome constitutions and the MALs could provide valuable information to help our understanding of the genetic system of the *C*₃-*C*₄ intermediate characteristics. In previous investigations, the inner leaf structure, the intercellular pattern of GDC expression and the gas exchange characteristics of *C*₃-*C*₄ intermediate photosynthesis were inherited in the hybrids according to the constitution ratio of the *C*₃-*C*₄ intermediate genome to the *C*₃ genome (Apel et al., 1984; Razmjoor et al., 1996; Ueno et al., 2003). The amphidiploid (DtDtRR) had characteristics intermediate between the *C*₃-*C*₄ intermediate (*D. tenuifolia*, DtDt) and *C*₃ (*R. sativus*, RR) parents, the sesquidiploid (DtDtR) had the characteristics close to the *C*₃-*C*₄ intermediate parent and other sesquidiploid (DtRR) had strongly *C*₃ parent characteristics (Ueno et al., 2003). Such a mode of inheritance in the *C*₃-*C*₄ intermediate and *C*₃ characteristics has also been demonstrated in the hybrids between *Diplotaxis muralis* (DtDtDvDv) and its ancestral species *D. tenuifolia* (DtDt) or *D. viminalis* (DvDv), and in the reciprocal hybrids between *M. arvensis* and *B. oleracea* (*C*₃) with various genome constitutions (Ueno et al., 2006; 2007). It is suggested that the constitution ratio of the *C*₃-*C*₄ intermediate genome to the *C*₃ genome in their hybrids is the most important factor determining the degree of expression of the *C*₂-*C*₄ intermediate characteristics.

In the present study, the Γ value under high-intensity light of almost all the auto- and alloplasmic MALs and DALs were not significantly different from that of *R. sativus* (*C*₃). However, the Γ of the auto- and alloplasmic DALs carrying one pair of ‘b’ *M. arvensis* chromosomes (RMa-b and MaR-b DALs) was significantly different from that of *R. sativus* (*C*₃), although that of the auto- and alloplasmic MALs with one ‘b’ *M. arvensis* chromosome was not significantly different from that of *R. sativus* (*C*₃). As the amphidiploid showed a Γ intermediate between that of the *C*₂-*C*₄ intermediate and *C*₃ parents (Ueno et al., 2003; 2006; 2007), the present two DALs (RMa-b and MaR-b DALs) also showed intermediate values. When the constitution ratio of the DALs was considered at the individual chromosome level, the constitution ratio of the *C*₂-*C*₄ intermediate chromosome to the *C*₃ chromosome was 1:1 because they have one pair of homologous chromosomes of the *C*₂-*C*₄ intermediate (*M. arvensis*) and the *C*₃ plant (*R. sativus*). Based on the results of the present and the previous studies (Ueno et al., 2003; 2006; 2007), the *C*₂-*C*₄ intermediate characteristics such as the gas-exchange characteristics might be expressed in the hybrid plants on the basis of the constitution ratio of each individual chromosome as well as the parent genomes. Therefore, the DAL would be useful material for investigating the genetic mechanisms of the *C*₂-*C*₄ intermediate characteristics.

The structural differentiation of mitochondria and chloroplasts in the BSCs and also the BSC-dominant expression of GDC are essential prerequisites for the low Γ in *C*₂-*C*₄ intermediate plants. However, the complete suppression of the expression of the P-protein of GDC in the MGs is not required to reduce Γ (Ueno et al., 2003). In the RMa-b DAL showing significantly lower Γ than *R. sativus* (*C*₃), the BSC/MC ratio of mitochondrial size and that of the GDC P-protein labeling density were intermediate between those in *M. arvensis* and *R. sativus*. Such structural and biochemical characteristics were also observed in the reciprocal amphidiploids between the *C*₂-*C*₄ intermediate and *C*₃ parents having equivalent genome constitution (Ueno et al., 2003, 2007). In the present study, we determined the Γ in the seven DALs with one pair of the individual chromosomes of the *M. arvensis* genome (n=14). Although the Γ in the remaining seven DALs existing theoretically has not been measured, it is suggested that the ‘b’ chromosome of the *M. arvensis* genome controls the Kranz-like leaf anatomy and biochemical components of *C*₂-*C*₄ intermediate photosynthesis, resulting in a lower Γ than in *C*₃ plants.

As mentioned above, the DAL is potential material for investigating the genetic mechanisms of the *C*₂-*C*₄ intermediate characteristics. However, we were able to produce only seven alloplasmic DALs (MaRa-a, -b, -c, -d, -e, -f and -l, 2n=20) by sib-cossing of the MALs and/or DALs in *S. bicolor* to *S. bicolor* generations, because the twelve alloplasmic MALs showed pollen fertility ranging from 85.6% to complete male sterility and alloplasmic *R. sativus* carrying *M. arvensis* cytoplasm exhibited complete male sterility (Bang et al., 2002). Prakash et al. (1998) observed that the cytoplasmic substitution line of *B. juncea* having the cytoplasm of *M. arvensis* showed male sterility. In other MALs in Brassicaceae, the complete series of MALs corresponding to the species chromosome number has not been developed (Kaneko et al., 1987; 2001; Quiros et al., 1987; 1988; Jahier et al., 1989; Chen et al., 1992; Srinivasan et al., 1998). Therefore, it seems to be extremely difficult to
produce the complete series of DALs corresponding to the *M. arvensis* genome (*n* = 14) on the background of the *R. sativus* genome due to the pre- and post-fertilization barriers in this intergeneric hybridization. In our recent study, the intergeneric F$_1$ hybrids between *B. oleracea* and *M. arvensis* were able to produce using the *B. oleracea* as a pistillate parent, and the BC$_3$ plants carrying one to a few *M. arvensis* chromosomes were obtained by successive backcrossing to *B. oleracea* (Bang et al., 2007). If the DALs carrying the remaining seven chromosomes of *M. arvensis* could be obtained by selfing the BC$_3$ plants in the next generation, more valuable information could be obtained to help understand the evolution and the genetic system of the C$_3$-C$_4$ intermediate characteristics in the individual chromosomes.

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**References**

Apel, P., Ticha, I. and Peisker, M. 1978. CO$_2$ compensation concentrations in leaves of *Moricandia arvensis* (L.) DC. at different insertion levels and O$_2$ concentrations. Biochem. Physiol. Pflanz. 172 : 547-552.

Apel, P., Bauwe, H. and Ohle, H. 1984. Hybrids between *Brassica alboglabra* and *Moricandia arvensis* and their photosynthetic properties. Biochem. Physiol. Pflanz. 179 : 793-797.

Apel, P., Horstmann, C. and Pfeffer, M. 1997. The *Moricandia* syndrome in species of the Brassicaceae–evolutionary aspects. Photosynthetica 33 : 205-215.

Bang, S.W., Kaneko, Y. and Matsuzawa, Y. 1996. Production of intergeneric hybrids between *Raphanus* and *Moricandia*. Plant Breed. 115 : 385-390.

Bang, S.W., Kaneko, Y., Matsuzawa, Y. and K.S. Bang, 2002: Breeding of *Moricandia arvensis* monosomic chromosome addition lines (2*n* = 19) of alloplasmic (*M. arvensis*) *Raphanus sativus*. Breed. Sci. 52 : 193-199.

Bang, S.W., Sugihara, K., Jeung, B.H., Kaneko, R., Satake, E., Kaneko, Y. and Matsuzawa, Y. 2007. Production and characterization of intergeneric hybrids between *Brassica oleracea* and a wild relative *Moricandia arvensis*. Plant Breed. 126 : 101-105.

Chen, B.Y., Simonsen, V., Lanner-Herrera, C. and Heneen, W.K. 1992. A *Brassica campestris*–*alboglabra* addition line and its use for gene mapping, intergeneric gene transfer and generation of trisomics. Theor. Appl. Genet. 84 : 592-599.

Douce, R. and Heldt, H.W. 2000. Photospiration. In R.C. Leegood, T.D. Shakey, and S. von Caemmerer eds., Photosynthesis : Physiology and Metabolism. Kluwer Academic Publishers, Dordrecht. 115-116.

Edwards, G.E. and Ku, M.S.B. 1987. Biochemistry of C$_3$-C$_4$ intermediates. In M.D. Hatch and N.K. Boardman eds., The Biochemistry of Plants 10: (Photosynthesis), Academic Press, SanDiego. 275-325.

Holaday, A.S., Shiety, Y.J., Lee, K.W. and Chollet, R. 1981. Anatomical, ultrastructural and enzymic studies of leaves of *Moricandia arvensis*, C$_3$-C$_4$ intermediate species. Biochim Biophys. Acta 637 : 334-341.

Holaday, A.S., Harrison, A.T. and Chollet, R. 1982. Photosynthetic / photospirotor CO$_2$ exchange characteristics of the C$_3$-C$_4$ intermediate species *Moricandia arvensis*. Plant Sci. Lett. 27 : 181-189.

Hilton, C.M., Rawsthorne, S., Smith, A.M., Jones, D.A. and Woolhouse, H.W. 1988. Glycine decarboxylase is confined to the bundle-sheath cells of leaves of C$_3$-C$_4$ intermediate species. Planta 175 : 452-459.

Ishikawa, S., Bang, S.W., Kaneko, Y. and Matsuzawa, Y. 2003. Production and characterization of intergeneric somatic hybrids between *Moricandia arvensis* and *Brassica oleracea*. Plant Breed. 122 : 233-258.

Jahier, J., Chevre, A.M., Tanguy, A.M. and Eber, F. 1989. Extraction of disomic addition lines of *Brassica nigra*. Genome 32 : 408-413.

Kaneko, Y., Matsuzawa, Y. and M. Sarashima, 1987: Breeding of the chromosome addition lines of radish with single kale chromosome. Jpn. J. Breed. 37 : 438-452.

Kaneko, Y., Namai, H., Matsuzawa, Y. and Sarashima, M. 1991. Maintenance and stability of the chromosome addition lines of radish with single kale chromosome. Jpn. J. Breed. 4 : 623-639.

Kaneko, Y., Yano, H., Bang, S.W. and Matsuzawa, Y. 2001. Production and characterization of *Raphanus sativus*-*Brassica rapa* monosomic chromosome addition lines. Plant Breed. 120 : 163-168.

Kirti, P.B., Narasimhulu, S.B., Parkash, S. and Chopra, V.L. 1992. Somatic hybridization between *Brassica juncea* and *Moricandia arvensis* by protoplast fusion. Plant Cell Rep. 11 : 318-321.

Kynast, R.G., Okazaki, R.J., Galatowitsch, M.W., Granath, S.G., Jacobs, M. S., Stec, A.O., Rines, H.W. and Phillips, R.L. 2004. Dissecting the maize genome by using chromosome addition and radiation hybrid lines. Proc. Natl. Acad. Sci. USA 101 : 9921-9926.

Leon, P., Arroyo, A. and Mackenzie, S. 1998. Nuclear control of plastid and mitochondrial development in higher plants. Ann. Rev. Plant Physiol. Plant Mol. Biol. 49 : 453-480.

Matsuzawa, Y., Kaneko, Y. and Bang, S.W. 1996. Prospects of the wide cross for genetics and plant breeding in Brassicaceae. Bull. Coll. Agr. Utsunomiya Univ. 16 : 5-10.

McGrath, J.M. and Quiros, C.F. 1990. Generation of alien chromosome addition lines from synthetic *Brassica napus* morphology, cytology, fertility, and chromosome transmission. Genome 33 : 374-383.

Monson, R.K. and Rawsthorne, S. 2000. CO$_2$ assimilation in C$_3$-C$_4$ intermediateplants. In R.C. Leegood, T.D. Sharky and S. von Caemmerer eds., Photosynthesis : Physiology and Metabolism. Kluwer Academic Publishers, Dordrecht. 533-550.

Morgan, C.L., Turner, S.R. and Rawsthorne, S. 1993. Coordination of the cell-specific distribution of the four subunits of glycine decarboxylase and of serine hydroxymethyltransferase in leaves of C$_3$-C$_4$ intermediate species from different genera. Planta 190 : 468-473.

Namai, H. 1987. Inducing cytogenetical alterations by means of interspecific and intergeneric hybridization in brassica crops.
Bang et al. — Photorespiratory Characteristics of \textit{R. sativus} (C\textsubscript{3}) – \textit{M. arvensis} (C\textsubscript{3-C4}) MALs and DALs

Gamma Field Symposia 26 : 41-87.

Prakash, S.H., Kirti, P.B., Bhat, S.R., Gaikwad, K., Kumar, V.D. and Chopra, V.L. 1998. A \textit{Moricandia arvensis} - based cytoplasmic male steril and fertility restoration system in \textit{Brassica juncea}. Theor. Appl. Genet. 97 : 488-492.

Quiros, C.F., Ochoa, O., Kianian, S.F. and Douches, D. 1987. Analysis of the \textit{Brassica oleracea} genome by the generation of \textit{B. campestris-oleracea} chromosome addition lines: characterization by isozymes and rDNA genes. Theor. Appl. Genet. 74 : 758-766.

Quiros, C.F., Ochoa, O. and Douches, D.S. 1988. Exploring the role of x=7 species in \textit{Brassica} evolution: hybridization with \textit{B. nigra} and \textit{B. oleracea}. J. Hered. 79 : 351-358.

Rawsthorne, S., Hylton, C.M., Smith, A.M., Woolhouse, H.W. 1988. Photorespiratory metabolism and immunogold localization of photorespiratory enzymes in leaves of \textit{C}_3 and \textit{C}_3-\textit{C}_4 intermediate species of \textit{Moricandia}. Planta 173 : 298-308.

Razmjoo, K., Toriyama, K., Ishii, R. and Hinata, K. 1996. Photosynthetic properties of hybrids between \textit{Diplotaxis muralis DC}, a \textit{C}_3 species, and \textit{Moricandia arvensis} (L.) DC, a \textit{C}_3-\textit{C}_4 intermediate species in \textit{Brassicaceae}. Genes Genet. Syst. 71 : 189-192.

Rylott, E.L., Metzlaff, K. and Rawsthorne, S. 1998. Developmental and environmental effects on the expression of the \textit{C}_3-\textit{C}_4 intermediate phenotype in \textit{Moricandia arvensis}. Plant Physiol. 118 : 1277-1284.

Sharkey, T.D. 1988. Estimating the rate of photorespiration in leaves. Physiol. Plant. 73 : 147-152.

Srinivasan, K., Malathi, V.G., Kirti, P.B., Prakash, S. and Chopra, V.L. 1998. Generation and characterisation of monosomic chromosome addition lines of \textit{Brassica campestris-B. ozyrithina}. Theor. Appl. Genet. 97 : 976-981.

Takahata, Y. 1990. Production of intergeneric hybrids between a \textit{C}_3-\textit{C}_4 intermediate species \textit{Moricandia arvensis} and a \textit{C}_3 species \textit{Brassica oleracea} through ovary culture. Euphytica 46 : 259-264.

Takahata, Y. and Takeda, T. 1990. Intergeneric (intersubtribe) hybridization between \textit{Moricandia arvensis} and \textit{Brassica A} and B genome species by ovary culture. Theor. Appl. Genet. 80 : 38-42.

Takahata, Y., Takeda, T. and Kaizuma, N. 1993. Wide hybridization between \textit{Moricandia arvensis} and \textit{Brassica} amphidiploid species (\textit{B. napus} and \textit{B. juncea}). Euphytica 69 : 155-160.

Toriyama, K., Hinata, K. and Kameya, T. 1987. Production of somatic hybrid plants, ‘Brassicomoricandi’ through protoplast fusion between \textit{Moricandia arvensis} and \textit{Brassica oleracea}. Plant Sci. 48 : 123-128.

Ueno, O., Bang, S.W., Wada, Y., Kondo, A., Ishihara, K., Kaneko, Y. and Matsuzawa, Y. 2003. Structural and biochemical dissection of photorespiration in hybrids differing in genome constitution between \textit{Diplotaxis tenuifolia} (\textit{C}_3-\textit{C}_4) and radish (\textit{C}_3). Plant Physiol. 132 : 1550-1559.

Warwick, S.I., Black, I.D. and Aguinagalde, I. 1992. Molecular systematics of \textit{Brassica} and allied genera (subtribe Brassicinae, \textit{Brassicaceae})-chloroplast DNA variation in the genus \textit{Diplotaxis}. Theor. Appl. Genet. 83 : 839-850.

Warwick, S.I. and Black, I.D. 1994. Evaluation of the subtribes \textit{Moricidiinae}, \textit{Sauvagnyninae}, \textit{Vellinae}, and \textit{Zillinae} (\textit{Brassicaceae}, tribe \textit{Brassicae}) using chloroplast DNA restriction site variation. Can. J. Bot. 72 : 1692-1701.