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Production of *Raphanus sativus* (C_3)-*Moricandia arvensis* (C_3-C_4 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 BC_6 to BC_8 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 S_3BC_5 and S_2BC_6 generations. The structural, biochemical and physiological characteristics related to photorespiration of these MALs and DALs were compared to study the genetic mechanisms of the C_3-C_4 intermediate photosynthesis in the individual chromosomes of *M. arvensis*. The CO_2 compensation point of the autoplasmic and alloplasmic DALs (RMa-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 C_3-C_4 intermediate characteristics.

Key words : C_3-C_4 intermediate plant, CO_2 compensation point, Disomic addition line (DAL), Monosomic addition line (MAL), *Moricandia arvensis*, Photorespiration.

Moricandia arvensis (L.) DC. ($2n$=28, MaMa) is one of the C_3-C_4 intermediate species of Brassicaceae. Among the species of Brassicaceae in which C_4 species have not yet been found, other C_3-C_4 intermediate species have also been found within the genera *Moricandia*, *Diplotaxis* and *Brassica* (Apel et al., 1997). *M. arvensis*, which was the first-reported C_3-C_4 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; Kirti et al., 1992; Takahata et al., 1993; Razmjoo et al., 1996; Bang et al., 1996, 2002, 2007; Ishikawa et al., 2003).

CO_2 release by photorespiration in C_3 plants is estimated to be about 25% of photosynthetically fixed CO_2 in ordinary air (Sharkey, 1988). *M. arvensis* shows low a CO_2 compensation point (Γ) because a large part of the CO_2 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 C_3-C_4 intermediate characteristics, Apel et al. (1984) first reported that the intergeneric F_1 and

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Abbreviations : DAL, disomic addition line; MAL, monosomic addition line; MC, mesophyll cell; BSC, bundle sheath cell.

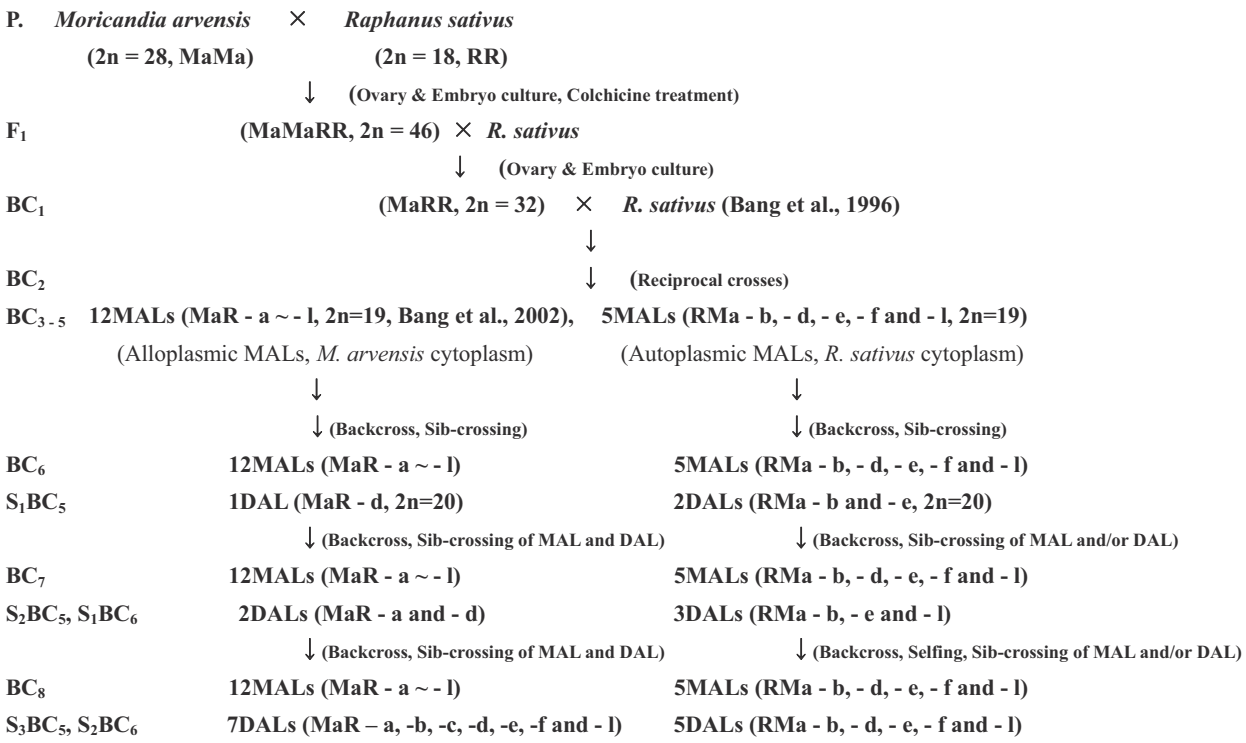

Fig. 1. Schematic diagram of producing the autoplasmic and alloplasmic MALs and DALs which were used as plant materials in this study.

BC_1 hybrids between *M. arvensis* and *Brassica alboglabra* (C_3) showed intermediate gas exchange traits. In recent studies with the intergeneric hybrids between *Diplotaxis tenuifolia* (C_3-C_4) and *Raphanus sativus* (C_3) and the reciprocal intergeneric hybrids between *M. arvensis* (C_3-C_4) and *B. oleracea* (C_3), Ueno et al. (2003, 2007) demonstrated that the C_3-C_4 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 CO_2 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 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_3-C_4 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 C_3-C_4 intermediate characteristics in Brassicaceae (Apel et al., 1997). The C_3-C_4 intermediate species monosomic and disomic addition lines of C_3 species (MALs and DALs) could provide more valuable information on the evolution and the genetic system of the C_3-C_4 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 alloplasmic *R. sativus* by selfing and sib-crossing of the MALs and DALs, and also maintained five autoplasmic MALs and 12 alloplasmic 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 C_3-C_4 intermediate photosynthesis at each chromosome level.

Materials and Methods

1. Plant materials and production of MALs and DALs

Moricandia arvensis ($2n$=28, MaMa, strain 4) is an accession of Cruciferae genetic stocks from the Laboratory of Plant Breeding, Tohoku University, Japan. *Raphanus sativus* cv. 'Pink ball' ($2n$=18, RR) for the recurrent parent is an accession from the Laboratory of Plant Breeding, Utsunomiya University, Japan. As shown in Fig. 1, *M. arvensis* MALs of alloplasmic *R. sativus* having *M. arvensis* cytoplasm were produced and they were classified into twelve types (MaR-a～-l, $2n$=19) by their morphological, physiological and cytological characteristics (Bang et al., 2002). The twelve types have been maintained by backcrossing to *R. sativus* cv. 'Pink ball' from BC_6 to BC_8 generation. The five *M. arvensis* MALs of *R. sativus* carrying *R.*

Table 1. Production of autoplasmic and alloplasmic DALs from selfing and sib-crossing of each MAL and DAL.

Cross combination (2*n*)	No. of flowers pollinated	No. of seeds obtained	No. of seeds sown	Somatic chromosome number (2*n*)			
				No. of plants observed	18	19	20
R. sativus cytoplasm							
RMa-b DAL (20) selfing	30	5	5	4		3	1
RMa-d MAL (19) × MaR-d DAL (20)	154	104	20	12		9	3
RMa-e DAL (20) selfing	90	14	9	9	6	2	1
RMa-f MAL (19) sib cross	140	125	20	19	9	9	1
RMa-l MAL (19) sib cross	121	214	20	18	5	11	2
M. arvensis cytoplasm							
MaR-a MAL (19) × MaR-a DAL (20)	96	65	11	10	1	7	2
MaR-b MAL (19) × RMa-b DAL (20)	124	137	10	9		7	2
MaR-c MAL (19) sib cross	84	24	20	15	4	10	1
MaR-d MAL (19) × MaR-d DAL (20)	471	20	20	16		12	4
MaR-e MAL (19) × RMa-e DAL (20)	62	22	10	10		9	1
MaR-f MAL (19) sib cross	26	89	10	8		5	3
MaR-l MAL (19) × RMa–l DAL (20)	405	32	10	6	1	3	2

sativus cytoplasm (2*n*=19, RMa-b, -d, -e, -f and -l; autoplasmic MALs) were produced by the reciprocal cross between *R. sativus* and each *M. arvensis* MALs of alloplasmic *R. sativus* (MaR-b, -d, -e, -f and -l) in BC_3 to BC_5 generation, and then have been maintained by backcrossing to *R. sativus* cv. 'Pink ball' from BC_6 to BC_8 generation. Seven *M. arvensis* DALs of *R. sativus* carrying *M. arvensis* cytoplasm (MaR-a, -b, -c, -d, -e, -f and -l; alloplasmic MALs) and five *M. arvensis* DALs of *R. sativus* (RMa-b, -d, -e, -f and -l; autoplasmic DALs) were produced by selfing of DAL, and sib-crossing of MAL and crossing between MAL and DAL in S_3BC_5 and S_2BC_6 generations. Each MAL and DAL has enough pollen fertility for using as pollen parent. These materials were grown from smaller seeds selected according to the method of Kaneko et al. (1991). These smaller seeds were sown in small pots and grown in a greenhouse after being transplanted to ceramic pots 18 cm in diameter. Flower buds were emasculated one day before anthesis, pollinated immediately with fresh pollen, and then bagged. Each MAL and DAL was identified based on the specific morphological and physiological characteristics of their leaves, roots and flowers, and their somatic chromosome number. Somatic chromosomes were observed using the Feulgen stain squash method followed by 1% acetocarmine staining of root tip cells. Pollen fertility was ascertained by observing 1000 pollen grains after staining with 1% acetocarmine.

2. Gas exchange measurements

The net photosynthetic rate was measured with a LI-6400 portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA). Measurements were made at a photon flux density of 1000 μmol m^{-2} s^{-1}, a leaf temperature of 20.0±0.2°C, and a CO_2 concentration of 350 μL L^{-1}. Light within the chamber was provided from a 6400-02 LED light source (LI-COR). The CO_2 compensation point was measured at photon flux densities of 300 and 1000 μmol m^{-2} s^{-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 (pH 6.8), dehydrated by an ethanol series and embedded in Lowicryl K4M resin (Chemische Werke Lowi GmbH, Waldkriburg, Germany), as previously described (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 Lab. Inc., San Mateo, CA, USA), as previously described (Ueno et

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

Line	Photosynthesis rate (μmol $m^{-2}s^{-1}$)	CO_2 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 ± 4.3	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				
MaR-a	22.4 ± 3.9	33.6 ± 1.4	38.5 ± 1.9	0.874 ± 0.064
MaR-b	22.7 ± 4.4	30.1 ± 1.8*	37.9 ± 2.5	0.797 ± 0.065*
MaR-c	16.6 ± 0.8	37.6 ± 4.7	46.4 ± 4.4*	0.823 ± 0.174
MaR-d	17.0 ± 1.9	37.7 ± 1.8	41.1 ± 1.0	0.916 ± 0.034
MaR-e	18.1 ± 3.2	38.3 ± 0.9*	41.4 ± 2.0	0.927 ± 0.031
MaR-f	11.5 ± 4.2*	37.5 ± 2.2	43.6 ± 1.6*	0.862 ± 0.067
MaR-l	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	0.923 ± 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				
MaR-a	16.8 ± 3.5	37.6 ± 1.8	38.9 ± 3.5	0.971 ± 0.054
MaR-b	17.2 ± 1.9	40.9 ± 3.9*	45.7 ± 6.0	0.912 ± 0.178
MaR-c	19.0 ± 2.1	40.2 ± 5.5	43.6 ± 6.4	0.937 ± 0.193
MaR-d	20.5 ± 4.1	35.0 ± 0.9	40.9 ± 5.0	0.863 ± 0.089
MaR-e	14.4 ± 3.3	35.0 ± 3.0	41.3 ± 3.3	0.847 ± 0.018
MaR-f	18.3 ± 2.5	34.1 ± 1.5	43.2 ± 3.7	0.794 ± 0.065
MaR-g	20.8 ± 0.8	37.3 ± 3.7	45.5 ± 3.9	0.820 ± 0.074
MaR-h	19.7 ± 2.2	35.3 ± 1.0	36.9 ± 2.2	0.958 ± 0.042
MaR-i	18.4 ± 3.0	34.9 ± 2.4	37.2 ± 7.9	0.975 ± 0.242
MaR-j	17.3 ± 2.2	38.9 ± 2.8	43.2 ± 4.5	0.907 ± 0.107
MaR-k	21.8 ± 1.2	39.6 ± 2.6	43.7 ± 3.2	0.907 ± 0.047
MaR-l	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 $m^{-2}s^{-1}$, 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.

al., 2003). 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 (μm^{-2}). 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.

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 selfing 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 selfing of each DAL ($2n=20$) that was obtained in S_2BC_5 and S_1BC_6 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 sib-crossing 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 sib-crossing 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 CO_2 compensation point (Γ) of *M. arvensis* was a typical C_3-C_4 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 C_3 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 C_3-C_4 intermediate and C_3 plants (Holaday et al., 1982).

Under high-intensity light, the Γ value in almost all the auto- and alloplasmic MALs and DALs was not

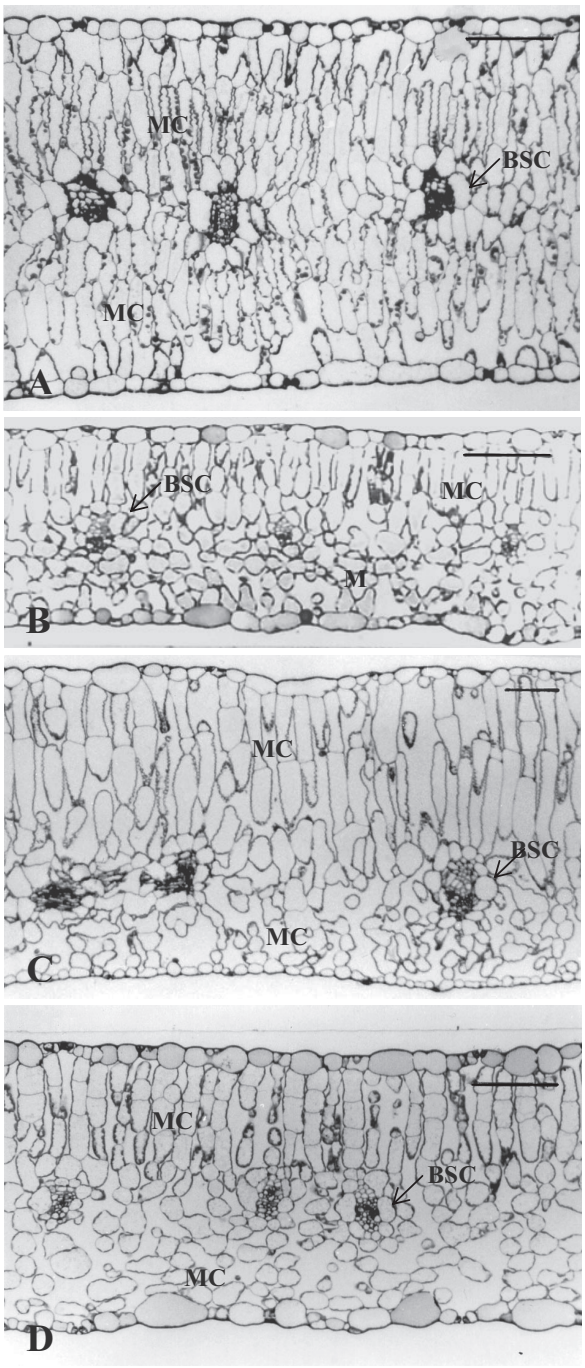

Fig. 2. Comparison of the leaf inner structures of *M. arvensis*, *R. sativus* and autoplasmic and alloplasmic DALs. A, *M. arvensis*; B. *R. sativus*; C, RMa-b; D, MaR-d. BSC, bundle sheath cell; MC, mesophyll cell. Bars = 100 μm.

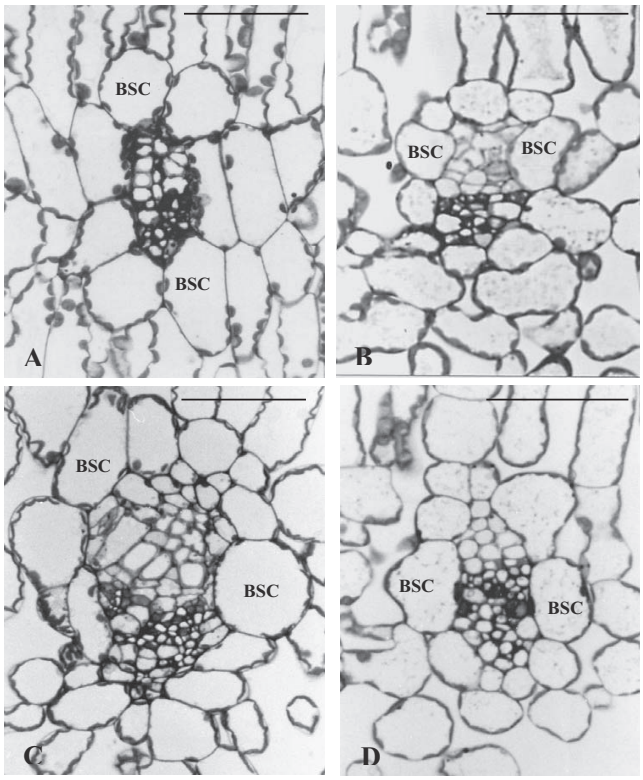

Fig. 3. Comparison of BSCs of *M. arvensis*, *R. sativus* and autoplasmic and alloplasmic DALs. A, *M. arvensis*; B. *R. sativus*; C, RMa-b; D, MaR-d. BSC, bundle sheath cell. Bars = 50 μm.

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

Line	Number of chloroplasts	Cp (%)
RMa-b	8.3±2.9 (14)	36.2
M. arvensis	9.5±4.0 (22)	57.4
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.

significantly different from that in *R. sativus* (34.5 μmol mol^{-1}). However, the Γ in RMa-b and MaR-b DALs was 29.4 and 30.1 μmol mol^{-1}, respectively, which was significantly different from that in *R. sativus*. The RMa-b and MaR-b DALs possess the same individual chromosomes but have different cytoplasm. Under low-intensity light, Γ in almost all DALs and MALs were not significantly different from that in *R. sativus*. The high/low-intensity light ratios of Γ in all DALs and MALs were not significantly different from that in *R. sativus*, but the ratios in both RMa-b and MaR-b DAL were significantly lower than that in *R. sativus*. These results suggest that the individual 'b' chromosome added to RMa-b and MaR-b DALs might control the

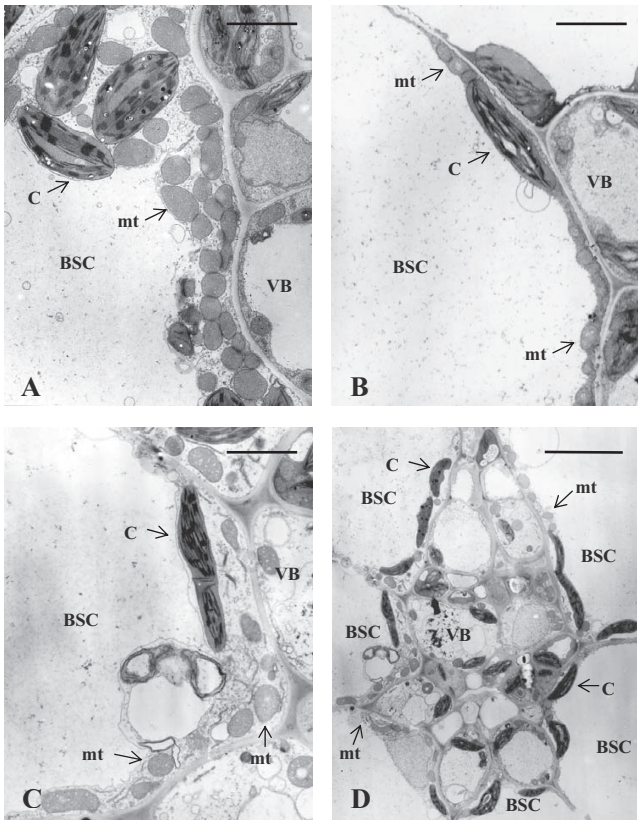

Fig. 4. Ultrastructure of BSCs of *M. arvensis*, *R. sativus* and autoplasmic and alloplasmic DALs. A, *M. arvensis*; B. *R. sativus*; C, RMa-b; D, BSCs surrounding a vascular bundle of the RMa-b. c, chloroplast; BSC, bundle sheath cell; mt, mitochondrion; VB, vascular bundle. Bars for A to C, 3 μm, Bar for D, 10 μm.

expression of the C_3-C_4 intermediate characteristics in *M. arvensis*.

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 *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 *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 *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 *R. sativus* and other DALs, and had elongated palisade MCs (Fig. 2C).

Table 4. Sizes of mitochondria in photosynthetic cells of autoplasmic and alloplasmic DALs.

Line	MC (μm)	BSC (μm)	Ratio (BSC : MC)
RMa-b	0.41±0.11 (33)	0.56±0.11 (34)*	1.37
RMa-e	0.65±0.14 (47)	0.54±0.11 (38)*	0.83
RMa-l	0.45±0.08 (31)	0.37±0.08 (28)^{ns}	0.82
MaR-a	0.71±0.15 (39)	0.61±0.12 (27)*	0.86
MaR-d	0.52±0.10 (38)	0.48±0.08 (30)^{ns}	0.92
MaR-l	0.57±0.16 (36)	0.41±0.09 (19)*	0.72
M. arvensis	0.42±0.09 (48)	0.72±0.19 (58)*	1.71
R. sativus	0.48±0.14 (30)	0.45±0.08 (25) ^{ns}	0.94

Values are given as the means±SD. The number of mitochondrial profiles examined is given in parentheses. Asterisks represent significant differences at $P<0.05$ between the MC and BSC mitochondria.
ns, not significant

Table 5. Immunogold labeling of the P-protein of glycine decarboxylase in photosynthetic cells of autoplasmic and alloplasmic DALs.

Line	Number of gold particles (μm^{-2})				Ratio (BSC : MC)
	MC		BSC		
	Mitochondria	Cytosol+others	Mitochondria	Cytosol+others	
RMa-b	63.9±25.6 (33)	ND (14)	71.9±15.5 (32)^{ns}	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)	85.9±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 BSCmitochondria.

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 and their parent species. In *M. arvensis*, the P-protein of GDC in the MC mitochondria was significantly less labeled than in the BSC mitochondria. The ratio of the labeling density of the BSC mitochondria to that of the MC mitochondria was 2.66. In *R. sativus*, however, the MC mitochondria were significantly more labeled than the BSC mitochondria, and the BSC:MC mitochondria labeling density ratio was very low (0.76). In five DALs (RMa-e, RMa-l, MaR-a, MaR-d and MaR-l), the BSC : MC mitochondria labeling density ratio ranged from 0.49 to 0.63, which

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 laid 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; Matsuzawa 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; Razmjoo 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. viminea* (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_3-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_3-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_3-C_4 intermediate chromosome to the C_3 chromosome was 1:1 because they have one pair of homologous chromosomes of the C_3-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_3-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_3-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_3-C_4 intermediate plants. However, the complete suppression of the expression of the P-protein of GDC in the MCs 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_3-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_3-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_3-C_4 intermediate characteristics. However, we were able to produce only seven alloplasmic DALs (MaR-a, -b, -c, -d, -e, -f and -l, $2n=20$) by sib-crossing of the MALs and/or DALs in S_3BC_5 to S_2BC_6 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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