Journal of Experimental Botany, Vol. 62, No. 15, pp. 5347–5355, 2011
doi:10.1093/jxb/err187 Advance Access publication 8 August, 2011
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Structural and biochemical characterization of the C_3–C_4 intermediate *Brassica gravinae* and relatives, with particular reference to cellular distribution of Rubisco

Osamu Ueno*

Laboratory of Plant Production Physiology, Faculty of Agriculture, Kyushu University, Hakozaki, Fukuoka 812-8581, Japan

* To whom correspondence should be addressed. E-mail: uenoos@agr.kyushu-u.ac.jp

Received 3 February 2011; Revised 13 May 2011; Accepted 16 May 2011

Abstract

On the basis of its CO_2 compensation concentration, *Brassica gravinae* Ten. has been reported to be a C_3–C_4 intermediate. This study investigated the structural and biochemical features of photosynthetic metabolism in *B. gravinae*. The cellular distribution of ribulose 1,5-*bis*phosphate carboxylase/oxygenase (Rubisco) was also examined in *B. gravinae*, *B. napus* L. (C_3), *Raphanus sativus* L. (C_3), and *Diplotaxis tenuifolia* (L.) DC. (C_3–C_4) by immunogold electron microscopy to elucidate Rubisco expression during the evolution from C_3 to C_3–C_4 intermediate plants. The bundle sheath (BS) cells of *B. gravinae* contained centrifugally located chloroplasts as well as centripetally located chloroplasts and mitochondria. Glycine decarboxylase P-protein was localized in the BS mitochondria. *Brassica gravinae* had low C_4 enzyme activities and high activities of Rubisco and photorespiratory enzymes, suggesting that it reduces photorespiratory CO_2 loss by the glycine shuttle. In *B. gravinae*, the labelling density of Rubisco was higher in the mesophyll chloroplasts than in the BS chloroplasts. A similar cellular pattern was found in other Brassicaceae species. These data demonstrate that, during the evolution from C_3 to C_3–C_4 intermediate plants, the intercellular pattern of Rubisco expression did not change greatly, although the amount of chloroplasts in the BS cells increased. It also appears that intracellular variation in Rubisco distribution may occur within the BS cells of *B. gravinae.*

Key words: *Brassica gravinae*, Brassicaceae, bundle sheath cell, C_3–C_4 intermediate plant, glycine decarboxylase, leaf anatomy, photorespiration, ribulose 1,5-*bis*phosphate carboxylase/oxygenase.

Introduction

C_3–C_4 intermediate plants have photosynthetic characteristics intermediate between those of C_3 and C_4 plants. Their leaf anatomy and CO_2 exchange characteristics, including CO_2 compensation concentrations and O_2 inhibition of photosynthesis, are in between those of C_3 and C_4 plants. Low rates of photorespiration in C_3–C_4 intermediates result mainly from the intercellular localization of glycine decarboxylase (GDC), a key photorespiratory enzyme that is involved in the decarboxylation of glycine (Rawsthorne, 1992; Monson and Rawsthorne, 2000). GDC is a multienzyme complex consisting of four components, P-, T-, L-, and H-protein, all of which are required for GDC activity (Douce *et al.*, 2001; Bauwe, 2011). In the leaves of C_3 plants, all four subunits of GDC occur in the mitochondria of photosynthetic cells, primarily the mesophyll (M) cells. In the leaves of C_3–C_4 intermediate plants, however, at least the P-protein of GDC is lacking in the mitochondria of M cells, although all of the subunits are present in the mitochondria of the bundle sheath (BS) cells (Morgan *et al.*, 1993). Thus, the intermediate metabolite glycine, which is generated in the M cells, must be transferred into the BS cells to be decarboxylated by GDC. This biochemical mechanism is accompanied by cellular specialization of the BS cells, which includes the presence of high numbers of chloroplasts and mitochondria, and enables the recapture of CO_2 released by the decarboxylation of glycine.

Abbreviations: BS, bundle sheath; GDC, glycine decarboxylase; LS, large subunit; M, mesophyll; Rubisco, ribulose 1,5-*bis*phosphate carboxylase/oxygenase.

This pathway, called the glycine shuttle, functions as a recycling system for photorespiratory CO_2 in the BS cells (Rawsthorne, 1992; Monson and Rawsthorne, 2000). This photosynthetic mechanism to reduce photorespiration occurs in at least nine families of higher plants (Bauwe, 2011). In some C_3–C_4 intermediate species of *Flaveria* (Asteraceae), a C_4 cycle operates together with the glycine shuttle (Monson and Rawsthorne, 2000).

Most species in the Brassicaceae are C_3 type, but some C_3–C_4 intermediate species occur in the genera *Moricandia*, *Diplotaxis*, and *Brassica* (Apel *et al.*, 1997). The C_3–C_4 intermediates in *Moricandia* and *Diplotaxis* reduce photorespiration only by the glycine shuttle, without a C_4 cycle (Hunt *et al.*, 1987; Rawsthorne, 1992; Ueno *et al.*, 2003). In *Brassica*, *B. gravinae* has been reported to be a C_3–C_4 intermediate species on the basis of its low value of CO_2 compensation concentration (22.7 μmol mol^{-1}) and the presence of many chloroplasts in the BS cells (Apel *et al.*, 1997). However, it is still unknown whether this species reduces photorespiration only by the glycine shuttle, as in the C_3–C_4 intermediate species of *Moricandia* and *Diplotaxis*, or by both the shuttle and a C_4 cycle, as in some species of *Flaveria*. The genus *Brassica* includes many vegetable and oil C_3 crops with high agronomic value, and within the Brassicaceae inter- and intrageneric hybridization is relatively easy (Apel *et al.*, 1984; Bang *et al.*, 1996, 2009; O'Neill *et al.*, 1996; Yan *et al.*, 1999). In the future, *B. gravinae* may become a valuable genetic resource as a parent plant with C_3–C_4 intermediate photosynthesis for use in the improvement of commercially important *Brassica* species.

The evolutionary processes giving rise to C_3–C_4 intermediate and C_4 plants remain to be elucidated. Previous studies have suggested that C_4 plants gradually evolved from C_3 plants through various stages of C_3–C_4 intermediates. This progression included the structural and biochemical modification of leaves: the development of organelles in the BS cells, the localization of GDC in the BS mitochondria, enhanced expression and appropriate distribution of the C_4 enzymes in the M and BS cells, and exclusive distribution of Rubisco in the BS chloroplasts (reviewed in Sage, 2004). However, less is known about whether a change in the intercellular expression of Rubisco occurred between M and BS chloroplasts during the evolution from C_3 to C_3–C_4 intermediate plants. There are several plants that have C_4-like characteristics but still accumulate small amounts of Rubisco in the M chloroplasts (Bauwe, 1984*a*; Reed and Chollet, 1985; Ueno and Wakayama, 2004; Ueno and Sentoku, 2006). The existence of C_4-like plants suggests that, during the evolutionary course from C_3–C_4 intermediate through C_4-like to true C_4 plants, the levels of Rubisco were reduced in the M chloroplasts relative to those in the BS chloroplasts, and Rubisco was finally restricted to the BS chloroplasts.

The BS cells of C_3–C_4 intermediate plants in the Brassicaceae include centrifugally located chloroplasts as well as centripetally located chloroplasts (Ueno *et al.*, 2003, 2007). It is still unknown whether these two types of chloroplasts play distinct functional roles within the BS cell. However, it is likely that the centripetal chloroplasts play a role in the recapture of photorespiratory CO_2 released from the mitochondria, whereas the centrifugal chloroplasts are responsible mainly for the fixation of CO_2 from the intercellular spaces (as in the M chloroplasts) rather than for the refixation of photorespiratory CO_2 (Ueno *et al.*, 2003). Thus, it is of interest whether the level of Rubisco is the same or different in centrifugal and centripetal chloroplasts.

This study reports the structural features and activities and intercellular localization of photosynthetic and photorespiratory enzymes in leaves of *B. gravinae* and characterizes the photosynthetic metabolism in this species. The study also investigated whether the intercellular patterns of Rubisco accumulation in M and BS cells differ between C_3 and C_3–C_4 intermediate species in the Brassicaceae. In addition, the intracellular accumulation of Rubisco within a BS cell was examined for some species. These findings would contribute to a better understanding of the cellular regulation of Rubisco expression during the course of evolution from C_3 to C_3–C_4 intermediate plants.

Materials and methods

Plant materials

Five C_3 and C_3–C_4 intermediate species in the Brassicaceae were examined: *Brassica napus* L. (C_3), *B. gravinae* Ten. (C_3–C_4), *B. rapa* L. (C_3), *Raphanus sativus* L. (C_3), and *Diplotaxis tenuifolia* (L.) DC. (C_3–C_4). Seeds of *B. gravinae* were provided by the National Germplasm Resources Laboratory of the United States Department of Agriculture (USDA), Agricultural Research Service, Beltsville, Maryland, USA. Seeds were sown in 8.0 l pots filled with a commercial soil mix for vegetables (ISEKI, Tokyo, Japan). Plants were grown in a growth chamber with temperatures maintained at 27 °C in the light (14 h) and 20 °C in the dark (10 h). Photon irradiance was provided by metal halide lamps at a photon flux density of 350 μmol m^{-2} s^{-1} (wavelength 400–700 nm). For the enzyme assay, *Panicum maximum* Jacq. was also grown in the chamber as a control C_4 plant. Plants were watered daily. Fully expanded uppermost leaves were examined 1–1.5 months after planting.

Anatomical and ultrastructural studies

A single leaf was examined from each of three plants of *B. gravinae* and each of two plants of *B. napus*. Samples taken from the midsections of leaves were fixed in 3% (v/v) glutaraldehyde in 50 mM sodium phosphate buffer (pH 6.8) and post-fixed in 2% OsO_4 in phosphate buffer. Samples were then dehydrated through an acetone series and embedded in Spurr's resin, as described by Ueno *et al.* (2003). 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 (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 sizes of the mitochondria and chloroplasts in the M and BS cells were measured for each leaf, because the sizes of the organelles differed somewhat between leaves of different plants, even within a species, but the ratios of organelle sizes between BS and M cells were similar in different leaves of the same species. Mitochondrial diameter was determined by using electron micrographs at ×25 000 magnification and represented the means of 28–66 measurements. Chloroplast length (long axis) was determined from electron micrographs at ×2000 or ×3000 magnification and represented the means

of 21–40 measurements. For BS cells, centrifugally and centripetally located chloroplasts were separately measured. Centrifugally located chloroplasts were defined as those located at the outer wall (wall adjacent to intercellular space) and the outer half of the radial walls (walls adjacent to neighbouring BS cells). The centripetally located chloroplasts were those located at the inner tangential walls (walls adjacent to vascular tissue) and the inner half of the radial walls.

Protein A–immunogold electron microscopy

Small segments of leaves were fixed with 3% (v/v) glutaraldehyde in 50 mM sodium phosphate (pH 6.8), dehydrated through an ethanol series, and embedded in Lowicryl K4M resin (Chemische Werke Lowi GmbH, Waldkraiburg, Germany), as described by Ueno *et al.* (2003). Ultrathin sections were immunolabelled with an antiserum to the P-protein of GDC or to the large subunit (LS) of Rubisco 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 against the P-protein of GDC isolated from pea leaf mitochondria was kindly provided by Dr DJ Oliver (University of Idaho, Moscow, ID, USA); the antiserum against the LS of Rubisco isolated from pea leaves was kindly provided by Dr S Muto (Nagoya University, Nagoya, Japan; now deceased). The antiserum against GDC P-protein was used at a dilution of 1:500, whereas that against Rubisco LS was at a dilution of 1:1000. In a preliminary examination, it was confirmed that such dilution ratios of the antisera produced the most reliable results, as was found in our previous studies (Ueno *et al.*, 2003; Ueno and Wakayama, 2004; Yoshimura *et al.*, 2004; Ueno and Sentoku, 2006). The density of labelling for these enzyme proteins was determined by counting the gold particles on electron micrographs at ×25 000 magnification and calculating the number per unit area (μm^{-2}).

Intercellular distribution of GDC P-protein and Rubisco LS

In the immunolabelling study, one leaf from each of two plants was examined for *B. gravinae*, *B. napus*, and *D. tenuifolia*, and one leaf from a single plant was examined in the case of *R. sativus*. The labelling density was measured on several immunolabelled sections of each leaf, because the labelling densities showed similar ratios between BS and M cells within a leaf but varied among leaves, probably because of differences in the amount of protein in each leaf. To assess the intercellular distribution of P-protein, between 12 and 26 individual cells were examined. The labelling density was calculated as the mean of 27–65 measurements of mitochondria. To assess the intercellular distribution of Rubisco LS, between 5 and 11 individual cells were examined. The labelling density was calculated as the mean of 9–17 measurements of chloroplasts. Areas occupied by starch grains were excluded from the estimations of the sectional areas of chloroplasts.

Intracellular distribution of Rubisco LS within a BS cell

Labelling density was measured on several immunolabelled sections of the same leaf from a representative plant of each species. For the C_3–C_4 intermediate species *B. gravinae* and *D. tenuifolia*, three representative cells were selected from the BS cells surrounding the same small vascular bundle. The centrifugally and centripetally located chloroplasts within a BS cell were examined for labelling density of Rubisco LS. The number of chloroplasts examined per BS cell was between five and 10 in the centrifugal location and between six and 11 in the centripetal location. *Brassica napus* (C_3) had fewer centripetally located chloroplasts in the BS cells than did the C_3–C_4 intermediate species; the number was insufficient for comparing the labelling densities of Rubisco LS among chloroplasts within individual BS cells. Thus, mean values were calculated from six centrifugally located chloroplasts and four centripetally located chloroplasts within three BS cells selected from the BS cells surrounding the same small vascular bundle.

Enzyme assays

Three plants each of *B. gravinae* and *B. napus* and one plant each of *B. rapa* and *P. maximum* were used for the enzyme assays. Leaf samples were frozen in liquid nitrogen and stored in a deep freeze (approximately –80 °C) until enzyme extraction. Leaves (0.25 g) were ground on ice, using a pestle in a mortar containing 0.5 g of sea sand, 25 mg of polyvinylpyrrolidone, and 1 ml of grinding medium. The grinding medium contained 50 mM HEPES-KOH (pH 7.5), 0.2 mM dithiothreitol, and 0.7% (w/v) bovine serum albumin. The homogenates were filtered through gauze, the filtrates were centrifuged at 10 000 *g* for 5 min at 4 °C, and the supernatants were used for the enzyme assays. All enzymes were assayed spectrophotometrically in 1 ml reaction mixtures at 25 °C, as described by Ueno *et al.* (2003, 2005).

Statistical analysis

Student's *t* test was used to test the significance (P <0.01) of any differences in the sizes of mitochondria and chloroplasts between the M and BS cells, differences in labelling densities of GDC P-protein and Rubisco LS between the M and BS cells, and differences in labelling densities between the centrifugally and centripetally located chloroplasts within a BS cell.

Results

Leaf anatomy of B. gravinae *and* B. napus

The mesophyll in leaves of *B. gravinae* was differentiated into palisade and spongy tissues (Fig. 1A), as seen in the leaves of the C_3 species *B. napus* (Fig. 1B). The BS cells of *B. gravinae* exhibited an elongated shape, which is somewhat similar to the adjacent M cells (Fig. 1A, C), and they contained a large group of chloroplasts in the centripetal position (Fig. 1C, G). In the area of the BS cell exposed to intercellular space (the centrifugal location), chloroplasts were arranged in a single row along the wall, as in the M cells (Fig. 1C, E). The BS cells of *B. napus* contained only a few chloroplasts in the centripetal position (Fig. 1D, H) but many chloroplasts in the centrifugal location (Fig. 1D, F). The BS cells of *B. gravinae* contained many mitochondria which were located between the inner tangential walls adjacent to the vascular tissue and the centripetally located chloroplasts (Fig. 1G). By contrast, the BS cells of *B. napus* contained fewer mitochondria which were distributed throughout the cell (Fig. 1H).

In *B. gravinae* the BS mitochondria were significantly larger than the M mitochondria (Table 1) whereas, in *B. napus*, the BS mitochondria were significantly smaller than the M mitochondria. In both species, the BS chloroplasts were smaller than the M chloroplasts (Table 1). Within the BS cells of *B. gravinae*, there was no significant difference in size between the centrifugally and centripetally located chloroplasts. Within the BS cells of *B. napus*, however, the centrifugally located chloroplasts were significantly larger than the centripetally located chloroplasts (P <0.01).

Intercellular distribution of GDC P-protein in M and BS cells

In *B. gravinae* the BS mitochondria were densely labelled for GDC P-protein, whereas the M mitochondria contained

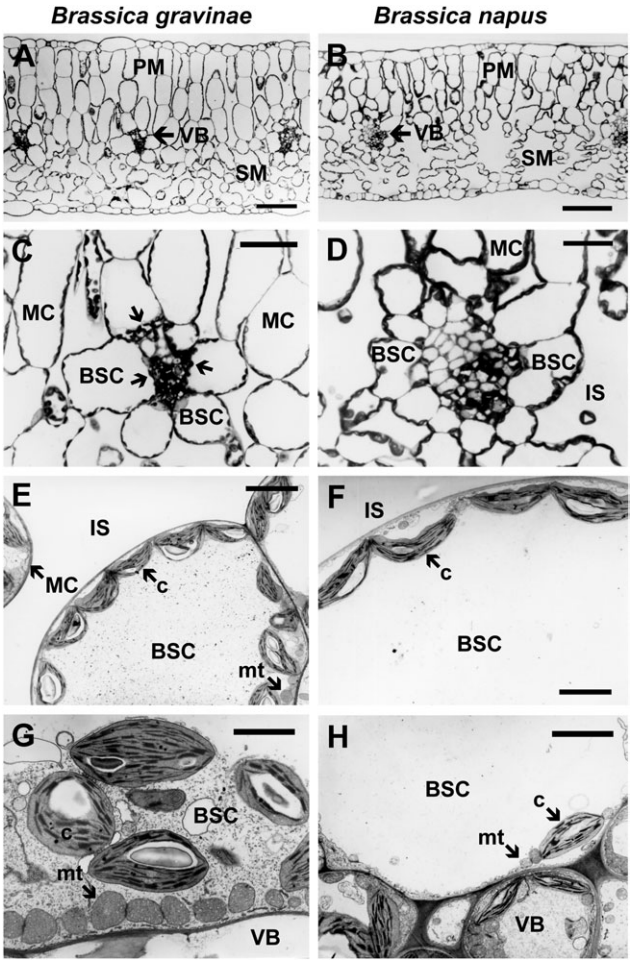

Fig. 1. Leaf anatomy of *B. gravinae* (A, C, E, G) and *B. napus* (B, D, F, H). (A, B) Leaf anatomical structures. (C, D) BS cells surrounding a vascular bundle. Arrows in (C) indicate centripetally located chloroplasts in the BS cells of *B. gravinae*. (E) Centrifugally and centripetally located chloroplasts in a BS cell. Centrifugally located chloroplasts are at the upper left, adjacent to the intracellular space; centripetally located chloroplasts are at the bottom right. Mitochondria are at the bottom right. (F) Centrifugally located chloroplasts in a BS cell. (G, H) Centripetally located chloroplasts and mitochondria in a BS cell. BSC, BS cell; c, chloroplast; IS, intercellular space; MC, M cell; mit, mitochondrion; PM, palisade M; SM, spongy M; VB, vascular bundle. Bars=100 μm (A, B), 25 μm (C, D), 5 μm (E), 3 μm (F, H), and 2 μm (G).

almost no label (Fig. 2A, C; Table 2). In *B. napus* both M and BS mitochondria were labelled for GDC P-protein (Fig. 2B, D). The labelling density was somewhat higher in the M mitochondria than in the BS mitochondria (Table 2).

Intercellular distribution of Rubisco LS in M and BS cells

In *B. gravinae* both M and BS chloroplasts were densely labelled for Rubisco LS (Fig. 3A, C, E), but the labelling density was somewhat lower in the BS chloroplasts than in the M chloroplasts (Table 2). The results for *B. napus* were similar: the chloroplasts of both M and BS cells were densely labelled for Rubisco LS (Fig. 3B, D, F), and the

Table 1. Sizes of organelles in the M and BS cells of *Brassica* species

Species and organelle	M cells (μm)		BS cells (μm)	Ratio (BS:M cell size)
B. gravinae (C_3–C_4)				
Mitochondria	0.31±0.07 (35)		0.62±0.13 (35)**	2.00
Chloroplasts	5.33±0.95 (40)	Cf	4.13±0.89 (40)**	0.77
		Cp	3.98±0.63 (40)**	0.75
B. napus (C_3)				
Mitochondria	0.79±0.25 (33)		0.49±0.11 (31)**	0.62
Chloroplasts	7.73±1.43 (40)	Cf	5.74±1.21 (40)**	0.74
		Cp	4.30±0.81 (21)**	0.56

Values are given as means ±SD. Numbers in parentheses show the numbers of organelles examined. Representative data obtained from several sections of the same leaf are shown. M, mesophyll; BS, bundle sheath; Cf, centrifugally located chloroplasts; Cp, centripetally located chloroplasts. Asterisks indicate a significant difference between the M and BS cells at P <0.01.

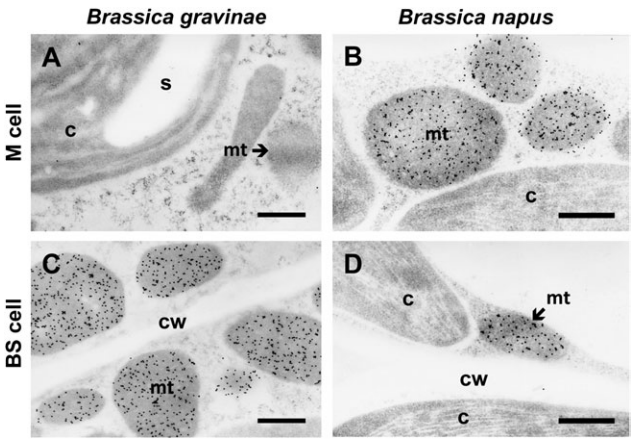

Fig. 2. Immunogold labelling of the P-protein of GDC in M and BS cells of *B. gravinae* (A, C) and *B. napus* (B, D). (A, B) M cells; (C, D) BS cells. c, Chloroplast; cw, cell wall; mit, mitochondrion; s, starch grain. Bars=0.5 μm.

labelling density was somewhat lower in the BS chloroplasts than in the M chloroplasts (Table 2). Similar patterns of intercellular enzyme distribution were also obtained for the C_3–C_4 intermediate species *D. tenuifolia* and the C_3 species *R. sativus* (Table 2). The ratio of labelling density of Rubisco LS in the BS chloroplasts to that in the M chloroplasts did not differ widely among the four species, although it was somewhat lower in the C_3–C_4 intermediate species than in the C_3 species.

Intracellular distribution of Rubisco LS within individual BS cells

In *B. gravinae*, three BS cells surrounding the same vascular bundle were examined for the intracellular distribution of Rubisco LS (Fig. 3C, E; Table 3). In these cells, the labelling density of Rubisco LS was significantly higher in centrifugally located chloroplasts than in centripetally located chloroplasts (Table 3). The ratios of labelling

Table 2. Immunogold labelling of the GDC P-protein and Rubisco LS in the M and BS cells of Brassicaceae species

Species and enzyme	Cell fraction	Number of gold particles (μm^{-2})		Ratio (BS:M cell label density)
		M cells	BS cells	
B. gravinae (C_3–C_4)				
GDC P-protein	Mitochondria	10.4±11.7 (34)	350.7±64.2 (36)**	33.7
	Cyt+other	0.8±0.4 (13)	0.3±0.4 (7)	
Rubisco LS	Chloroplasts	381.9±48.5 (9)	275.2±41.9 (9)**	0.72
	Cyt+other	ND (6)	ND (5)	
B. napus (C_3)				
GDC P-protein	Mitochondria	265.5±60.0 (29)	205.8±80.9 (30)**	0.78
	Cyt+other	1.8±1.1 (14)	0.6±0.7 (14)	
Rubisco LS	Chloroplasts	338.8±28.6 (9)	288.0±31.3 (9)**	0.85
	Cyt+other	4.3±4.2 (7)	ND (9)	
D. tenuifolia (C_3–C_4)				
Rubisco LS	Chloroplasts	207.9±25.5 (15)	139.5±19.0 (17)**	0.67
	Cyt+other	1.1±1.7 (10)	1.3±1.5 (10)	
R. sativus (C_3)				
Rubisco LS	Chloroplasts	70.9±11.0 (17)	58.9±6.1 (10)**	0.83
	Cyt+other	0.3±0.6 (11)	0.1±0.3 (9)	

Numbers of gold particles per unit area (μm^{-2}) are given as means ±SD. Data obtained from several immunolabelled sections of the same leaf are shown. Numbers in parentheses show the numbers of organelles or cell profiles examined. Cyt+other, cytosol+other organelles; ND, not detectable. Asterisks indicate a significant difference between the M and BS cells at P <0.01.

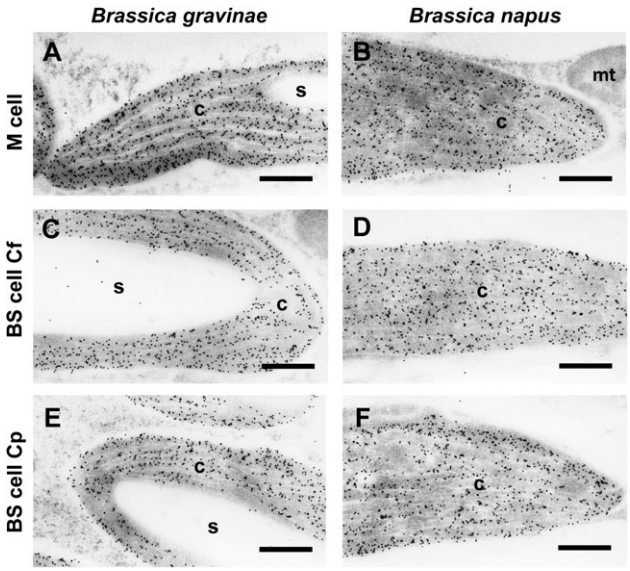

Fig. 3. Immunogold labelling of Rubisco LS in M and BS cells of *B. gravinae* (A, C, E) and *B. napus* (B, D, F). (A, B) Chloroplasts in M cells; (C, D) centrifugally located chloroplasts within BS cells; (E, F) centripetally located chloroplasts within BS cells. c, chloroplast; cw, cell wall; mit, mitochondrion; s, starch grain. Bars=0.5 μm.

density of the centripetal to centrifugal chloroplasts ranged from 0.78 to 0.86. BS cells of *B. napus* showed no significant difference in the labelling densities of Rubisco LS between the centrifugally and centripetally located chloroplasts (Fig. 3D, F; Table 3). As in *B. napus*, the BS cells of *D. tenuifolia* showed no significant difference in the labelling densities of Rubisco LS in chloroplasts differing in intracellular location (Table 3). In the analysis of *B. gravinae* and *D. tenuifolia*, the BS cells examined were located in different positions around the same vascular bundle, but there were no large differences in the ratios of labelling density of the centripetal to centrifugal chloroplasts among the BS cells within each species (Table 3).

Activities of photosynthetic and photorespiratory enzymes

Brassica gravinae had higher activity of Rubisco than the control C_4 plant, *P. maximum*, although the activity was somewhat lower than those of the control C_3 plants, *B. napus* and *B. rapa* (Table 4). The activity of the C_4 photosynthetic enzyme phosphoenolpyruvate carboxylase of *B. gravinae* was similar to that measured for C_3 plants. This was also the case for the activities of three C_4-acid decarboxylating enzymes: NADP-malic enzyme, NAD-malic enzyme, and phosphoenolpyruvate carboxykinase (Table 4). The activities of glycolate oxidase and catalase, which are involved in the glycolate pathway, were also comparable with those of the control C_3 plants but higher than those of *P. maximum*. There was no large difference in activity of another glycolate pathway enzyme, hydroxypyruvate reductase, among *B. gravinae* and the control C_3 and C_4 plants (Table 4).

Discussion

Structural and biochemical characteristics of photosynthesis in B. gravinae

This study confirmed that *B. gravinae* has a leaf anatomical structure typical of C_3–C_4 intermediate plants, as observed by Apel *et al.* (1997). Electron microscopic observation was performed to characterize the more detailed structural

Table 3. Comparison of labelling densities of Rubisco LS in centrifugally and centripetally located chloroplasts within BS cells of C_3–C_4 intermediate and C_3 species

Species and BS cells		Cell fraction	Number of gold particles (νm^{-2})		Ratio
			Cf	Cp	(Cp:Cf label density)
B. gravinae (C_3–C_4)					
BS cell 1	Adaxial cell	Chloroplasts	368.3±48.2 (5)	318.3±34.1 (9)**	0.86
		Cyt+other	ND (5)	0.3±0.8 (6)	
BS cell 2	Abaxial cell	Chloroplasts	347.9±28.6 (6)	297.8±22.4 (8)**	0.86
		Cyt+other	ND (4)	ND (6)	
BS cell 3	Abaxial cell	Chloroplasts	329.5±30.2 (10)	258.1±46.0 (11)**	0.78
		Cyt+other	ND (8)	ND (5)	
B. napus (C_3)					
BS cells	3 abaxial cells	Chloroplasts	314.5±29.3 (6)	318.7±25.4 (4) NS	1.01
		Cyt+other	1.1±2.7 (6)	ND (4)	
D. tenuifolia (C_3–C_4)					
BS cell 1	Adaxial cell	Chloroplasts	135.4±4.6 (6)	145.4±13.5 (8) NS	1.07
		Cyt+other	0.2±0.4 (6)	0.2±0.2 (5)	
BS cell 2	Lateral cell	Chloroplasts	144.8±14.7 (7)	138.8±3.4 (7) NS	0.96
		Cyt+other	ND (7)	0.2±0.1 (6)	
BS cell 3	Abaxial cell	Chloroplasts	130.9±8.2 (5)	128.7±15.5 (9) NS	0.98
		Cyt+other	ND (5)	0.4±0.3 (6)	

Numbers of gold particles per unit area (μm^{-2}) are given as means ±SD. Numbers in parentheses show the numbers of chloroplasts or cell profiles examined. Measurements were made for BS cells surrounding the same vascular bundle on several immunolabelled sections of the same leaf. Cf, centrifugally located chloroplasts; Cp, centripetally located chloroplasts; Cyt+other, cytosol+other organelles; ND, not detectable. Asterisks indicate a significant difference between the centrifugally and centripetally located chloroplasts at P <0.01. NS, not significant.

Table 4. Activities of photosynthetic and photorespiratory enzymes in leaves of *B. gravinae* and control C_3 and C_4 plants

Enzyme	Activity ($\mu mol\ mg^{-1}\ Chl\ h^{-1}$)			
	***B. gravinae* (C_3–C_4)**	***B. napus* (C_3)**	***B. rapa* (C_3)**	***P. maximum* (C_4)**
Rubisco	322±28	483±33	628	202
Phosphoenolpyruvate carboxylase	58±10	15±3	52	527
NADP-malic enzyme	34±2	27±4	37	13
NAD-malic enzyme	23±5	16±3	24	83
Phosphoenolpyruvate carboxykinase	16±3	ND	ND	389
Glycolate oxidase	49±12	52±6	102	16
Hydroxypyruvate reductase	526±42	308±11	607	647
Catalase	89 600±5100	54 900±12 100	97 300	16 300

Values of *B. gravinae* and *B. napus* are given as the means ±SD of three plants. Values for *B. rapa* and *P. maximum* were each obtained from a single plant. ND, not detectable.

features of the leaves. The BS cells contained many chloroplasts in the centripetal position, although chloroplasts were also distributed in the centrifugal position. The BS mitochondria were larger than the M mitochondria, and all of the BS mitochondria were located between the centripetal chloroplasts and the inner tangential walls. The immunogold labelling study demonstrated that the GDC P-protein was expressed strongly in the BS mitochondria, whereas the protein was essentially absent from the M mitochondria. *Brassica gravinae* had high Rubisco activity but low activities of C_4 photosynthetic enzymes. These data suggest that the C_4 cycle does not operate to any significant extent in the leaves of *B. gravinae*. Among grass species, the activities of the photorespiratory enzymes glycolate oxidase and catalase are much lower in C_4 grasses than in C_3 grasses (Ueno *et al.*, 2005). *Brassica gravinae* also had higher activities of these photorespiratory enzymes than the control C_4 species. The activity levels are comparable with those found in the C_3 species *B. napus* and *B. rapa*. Previous studies of C_3–C_4 intermediate species, including *Moricandia arvensis*, have reported that they had relatively high activities of photorespiratory enzymes (Rawsthorne *et al.*, 1988; Devi and Raghavendra, 1993). Taken together, the structural and biochemical results suggest that *B. gravinae* is a C_3–C_4 intermediate species that reduces photorespiratory CO_2 loss by using the glycine shuttle. It seems that the photosynthetic carbon metabolism is similar to that of the C_3–C_4 intermediate species of *Moricandia* and *Diplotaxis*.

In leaves of *B. gravinae*, the M layer was clearly differentiated into palisade and spongy tissues. This structure differed from that found in the C_3–C_4 intermediates *M. arvensis* and *D. tenuifolia*, in which the M layer was not clearly differentiated into palisade and spongy tissues (Ueno *et al.*, 2003, 2007). Within the tribe Brassiceae, the genera *Brassica* and *Diplotaxis* belong to the subtribe Brassicinae, and the genus *Moricandia* to the subtribe Moricandiinae (Warwick and Black, 1993, 1994). Thus, it is unlikely that the difference in M structure reflects the phylogenetic relationships among these three genera. The M structure found in *B. gravinae* might represent a primitive feature of C_3–C_4 intermediacy relative to that in the C_3–C_4 intermediates of *Moricandia* and *Diplotaxis*, because the M layers of C_3 species in the Brassicaceae are clearly differentiated into palisade and spongy tissues (Ueno *et al.*, 2003, 2006, 2007). Apel *et al.* (1997) reported that the CO_2 compensation concentration of *B. gravinae* was 22.7 μmol mol^{-1} whereas those of *M. arvensis* and *D. tenuifolia* were 11.1 and 4.9–15.2 μmol mol^{-1}, respectively. These data may also reflect the evolutionary status of C_3–C_4 intermediacy in *B. gravinae*.

Cellular distribution of Rubisco during the evolution from C_3 to C_3–C_4 intermediate plants

The immunogold labelling procedure used here would be the best method with which to evaluate the cellular accumulation of protein accurately. However, it is important to point out that there are limitations in the interpretation of the data. A difference in labelling density for a given protein does not always represent a difference in the absolute quantity of the protein, if compared between different species, because the cross-reactivity of the antiserum may differ between species. Thus, the lower labelling densities of Rubisco LS in *R. sativus* than in other species (Table 2) do not indicate a lower absolute quantity of this protein in *R. sativus*. This is also the case for the analysis of different proteins within the same species. For example, the labelling densities of GDC P-protein and Rubisco LS were similar within *B. napus* (Table 2), but this does not mean that the two proteins are accumulated to similar levels. However, it is possible to assess the relative amounts of a given protein in different cells within the same leaf. Thus, the ratios of labelling density between the BS and M cells could be compared among different species of the Brassicaceae.

This study demonstrated that, in the C_3–C_4 intermediate and C_3 species of the Brassicaceae, the density of Rubisco protein per unit area (unit volume) of chloroplast is lower in BS chloroplasts than in M chloroplasts. In both *B. napus* (C_3) and *B. gravinae* (C_3–C_4), the BS chloroplasts were smaller than the M chloroplasts. In the Brassicaceae, therefore, it is likely that the structural and biochemical characteristics of M and BS chloroplasts were maintained during the evolution from C_3 to C_3–C_4 intermediate plants, without large modification. However, an increase in chloroplast number occurred in the BS cells of C_3–C_4 intermediate plants, accompanied by an increase in the number of mitochondria (Brown and Hattersley, 1989). This increase in chloroplast number would be largely responsible for the increased partitioning of Rubisco into BS cells relative to M cells in C_3–C_4 intermediate plants as compared with C_3 plants. In addition, a comparison of the size ratio of the BS chloroplasts to the M chloroplasts in *B. napus* and *B. gravinae* suggests that enlargement of centripetally located chloroplasts occurred during the evolution of C_3 to C_3–C_4 intermediate plants. This structural event could also have contributed to the increased partitioning of Rubisco into the BS cells of C_3–C_4 intermediates. Moore *et al.* (1988) reported that Rubisco activities on the basis of chlorophyll content were almost the same in the M and BS cells of *Flaveria ramosissima*, a C_3–C_4 intermediate with C_4-cycle activity. Bauwe (1984*b*) showed that there was no large difference in the kinetic properties of Rubisco between C_3 and C_3–C_4 intermediate species of *Moricandia*. In the leaves of other C_3–C_4 intermediates, the kinetic constants of Rubisco are C_3-like (Wessinger *et al.*, 1989; Hudson *et al.*, 1990; Kubien *et al.*, 2008). It is unknown, however, whether the kinetic properties of Rubisco differ between the M and BS cells of C_3–C_4 intermediate plants.

As discussed earlier, the BS cells of C_3–C_4 intermediate species in the Brassicaceae possess both centrifugally and centripetally located chloroplasts (Ueno *et al.*, 2003, 2007). This intracellular location of chloroplasts differs from that in the BS cells of NAD-malic enzyme type C_4 plants, in which all chloroplasts are located in the centripetal position (Yoshimura *et al.*, 2004). In *B. gravinae* the size of BS chloroplasts did not differ between the two positions. C_3 plants also include chloroplasts in the BS cells (Yoshimura *et al.*, 2004; Tsutsumi *et al.*, 2008), although there are fewer than in the BS cells of C_3–C_4 intermediate plants (Brown and Hattersley, 1989). In the BS cells of *B. napus*, the centripetally located chloroplasts were smaller than the centrifugally located chloroplasts. Thus, it is evident that a BS cell can develop chloroplasts of different sizes, although the developmental mechanism remains unknown.

This study indicated that the labelling density of Rubisco is the same in the centrifugally and centripetally located chloroplasts within the BS cell of *D. tenuifolia* (C_3–C_4). In *B. napus* (C_3), the same accumulation pattern was found in the two types of BS chloroplasts, although comparisons could not be made within individual BS cells because of the small number of chloroplasts per cell. In *B. gravinae*, however, the labelling density of Rubisco was lower in the centripetally located chloroplasts than in the centrifugally located chloroplasts, indicating that Rubisco may accumulate to different levels among the chloroplasts within a single BS cell. Further studies with a greater number of samples will be required to confirm these results. In single-celled C_4 plants of the Chenopodiaceae, however, it has been reported that Rubisco protein is differentially accumulated in chloroplasts at different locations within a photosynthetic cell (Voznesenskaya *et al.*, 2001, 2002), although the regulatory mechanism is unknown.

The functional role of BS cells in C_3 plants remains elusive (Yoshimura *et al.*, 2004; Leegood, 2008; Tsutsumi *et al.*, 2008; Janacek *et al.*, 2009; Kangasjarvi *et al.*, 2009). This study and others (Ueno *et al.*, 2003; Yoshimura *et al.*, 2004; Tsutsumi *et al.*, 2008) have demonstrated that the BS

cells in C_3 plants accumulate photosynthetic and photorespiratory enzymes, as do the M cells. However, the amounts of enzymes are much lower in the BS cells than in the M cells, suggesting reduced capacities for photosynthetic and photorespiratory functions in the BS cells. In the BS cells of C_3–C_4 intermediate plants, glycine exported from the M cells is decarboxylated by GDC in the mitochondria. Therefore, it is thought that high CO_2 partial pressure is maintained within the BS cells, enabling Rubisco to maintain a reduced level of oxygenase activity (von Caemmerer, 2000; Bauwe, 2011). A gradient of CO_2 partial pressure may occur from the proximal end to the distal end of the BS cell, because glycine decarboxylation occurs in the mitochondria located in the proximal end. The intercellular gradient of density of Rubisco found between the M and BS cells of C_3–C_4 intermediate species, and the possible intracellular gradient of Rubisco observed within the BS cell of *B. gravinae*, may reflect differences in the CO_2 concentration experienced by these photosynthetic cells.

This study examined several aspects of the evolutionary transition from C_3 to C_3–C_4 intermediate plants lacking C_4 cycle activity, with special attention to cellular expression of Rubisco. At present, it is still unknown how the expression of Rubisco became restricted to BS cells during the evolutionary transition from C_3–C_4 intermediate to C_4 plants. Further studies will be required to elucidate the process of cellular compartmentalization of enzymes that accompanied the structural modification of leaf cells during the evolution from C_3 to C_4 plants.

Acknowledgements

The author thanks the Germplasm Resources Laboratory of the USDA for providing the seeds of *B. gravinae*. Part of this study was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No. 20380013).

References

Apel P, Bauwe H, Ohle H. 1984. Hybrids between *Brassica alboglabra* and *Moricandia arvensis* and their photosynthetic properties. *Biochemische und Physiologie der Pflanzen* **179,** 793–797.

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

Bang SW, Kaneko Y, Matsuzawa Y. 1996. Production of intergeneric hybrids between *Raphanus* and *Moricandia*. *Plant Breeding* **115,** 385–390.

Bang SW, Ueno O, Wada Y, Hong SK, Kaneko Y, Matsuzawa Y. 2009. 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. *Plant Production Science* **12,** 70–79.

Bauwe H. 1984*a*. Photosynthetic enzyme activities and immunofluorescence studies on the localization of ribulose 1,5-bisphosphate carboxylase/oxygenase in leaves of C_3, C_4, and C_3–C_4 intermediate species of *Flaveria* (Asteraceae). *Biochemische und Physiologie der Pflanzen* **179,** 253–268.

Bauwe H. 1984*b*. Photosynthetic enzyme activities in C_3 and C_3–C_4 intermediate species of *Moricandia* and in *Panicum milioides*. *Photosynthetica* **18,** 201–209.

Bauwe H. 2011. Photorespiration: the bridge to C_4 photosynthesis. In: Raghavendra AS, Sage RF, eds. *C_4 photosynthesis and related CO_2 concentrating mechanisms*. Dordrecht: Springer, 81–108.

Brown RH, Hattersley PW. 1989. Leaf anatomy of C_3–C_4 species as related to evolution of C_4 photosynthesis. *Plant Physiology* **91,** 1543–1550.

Devi MT, Raghavendra AS. 1993. Partial reduction in activities of photorespiratory enzymes in C_3–C_4 intermediates of *Alternanthera* and *Parthenium*. *Journal of Experimental Botany* **44,** 779–784.

Douce R, Bourguignon J, Neuburger M, Rebeille F. 2001. The glycine decarboxylase system: a fascinating complex. *Current Opinion in Plant Biology* **2,** 214–222.

Hudson GS, Mahon JD, Anderson PA, Gibbs MJ, Badger MR, Andrews TJ, Whitfield PR. 1990. Comparison of rbcL genes for the large subunit of ribulose bisphosphate carboxylase from closely related C_3 and C_4 species. *Journal of Biological Chemistry* **265,** 808–814.

Hunt S, Smith AM, Woolhouse HW. 1987. Evidence for a light-dependent system for reassimilation of photorespiratory CO_2, which does not include a C_4 cycle, in the C_3–C_4 intermediate species *Moricandia arvensis*. *Planta* **171,** 227–234.

Janacek SH, Trenkamp S, Palmer B, *et al*. 2009. Photosynthesis in cells around veins of the C_3 plant *Arabidopsis thaliana* is important for both the shikimate pathway and leaf senescence as well as contributing to plant fitness. *The Plant Journal* **59,** 329–343.

Kangasjarvi S, Murmi M, Tikkanen M, Aro EM. 2009. Cell-specific mechanisms and systemic signaling as emerging themes in light acclimation of C_3 plants. *Plant, Cell and Environment* **32,** 1230–1240.

Kubien DS, Whitney SM, Moore PV, Jasson LK. 2008. The biochemistry of Rubisco in *Flaveria*. *Journal of Experimental Botany* **59,** 1767–1777.

Leegood RC. 2008. Roles of the bundle sheath cells in leaves of C_3 plants. *Journal of Experimental Botany* **59,** 1663–1673.

Monson RK, Rawsthorne S. 2000. CO_2 assimilation in C_3–C_4 intermediate plants. In: Leegood RC, Sharkey TD, von Caemmerer S, eds. *Photosynthesis: physiology and metabolism*. Dordrecht: Kluwer Academic Publishers, 533–550.

Moore BD, Monson RK, Ku MSB, Edwards GE. 1988. Activities of principal photosynthetic and photorespiratory enzymes in leaf mesophyll and bundle sheath protoplasts from the C_3–C_4 intermediate *Flaveria ramosissima*. *Plant and Cell Physiology* **29,** 999–1006.

Morgan CL, Turner SR, 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.

O'Neill CM, Murata T, Morgan CL, Mathias RJ. 1996. Expression of the C_3–C_4 intermediate character in somatic hybrids between *Brassica napus* and the C_3–C_4 species *Moricandia arvensis*. *Theoretical and Applied Genetics* **93,** 1234–1241.

Rawsthorne S. 1992. C_3–C_4 intermediate photosynthesis: linking physiology to gene expression. *The Plant Journal* **2,** 267–274.

Rawsthorne S, Hylton CM, Smith AM, Woolhouse HW. 1988. Distribution of photorespiratory enzymes between bundle-sheath and mesophyll cells in leaves of the C_3–C_4 intermediate species *Moricandia arvensis* (L.) DC. *Planta* **176,** 527–532.

Reed JE, Chollet R. 1985. Immunofluorescent localization of phosphoenolpyruvate carboxylase and ribulose 1,5-bisphosphate carboxylase/oxygenase proteins in leaves of C_3, C_4, and C_3–C_4 intermediate *Flaveria* species. *Planta* **165,** 439–445.

Sage RF. 2004. Evolution of C_4 photosynthesis. *New Phytologist* **161,** 341–370.

Tsutsumi K, Kawasaki M, Taniguchi M, Miyake H. 2008. Gene expression and accumulation of Rubisco in bundle sheath and mesophyll cells during leaf development and senescence in rice, a C_3 plant. *Plant Production Science* **11,** 336–343.

Ueno O, Bang SW, Wada Y, Kobayashi N, Kaneko R, Kaneko Y, Matsuzawa Y. 2007. Inheritance of C_3–C_4 intermediate photosynthesis in reciprocal hybrids between *Moricandia arvensis* (C_3–C_4) and *Brassica oleracea* (C_3) that differ in their genome constitution. *Plant Production Science* **10,** 68–79.

Ueno O, Bang SW, Wada Y, Kondo A, Ishihara K, Kaneko Y, Matsuzawa Y. 2003. Structural and biochemical dissection of photorespiration in hybrids differing in genome constitution between *Diplotaxis tenuifolia* (C_3–C_4) and radish (C_3). *Plant Physiology* **132,** 1550–1559.

Ueno O, Sentoku N. 2006. Comparison of leaf structure and photosynthetic characteristics of C_3 and C_4 *Alloteropsis semialata* subspecies. *Plant, Cell and Environment* **29,** 257–268.

Ueno O, Wada Y, Wakai M, Bang SW. 2006. Evidence from photosynthetic characteristics for the hybrid origin of *Diplotaxis muralis* from a C_3–C_4 intermediate and a C_3 species. *Plant Biology* **8,** 253–259.

Ueno O, Wakayama M. 2004. Cellular expression of C_3 and C_4 photosynthetic enzymes in the amphibious sedge *Eleocharis retroflexa* ssp. *chaetaria*. *Journal of Plant Research* **117,** 433–441.

Ueno O, Yoshimura Y, Sentoku N. 2005. Variation in the activity of some enzymes of photorespiratory metabolism in C_4 grasses. *Annals of Botany* **96,** 863–869.

von Caemmerer S. 2000. *Biochemical models of leaf photosynthesis*. Collingwood: CSIRO Publishing.

Voznesenskaya EV, Franceschi VR, Kiirats O, Artyusheva EG, Freitag H, Edwards GE. 2002. Proof of C_4 photosynthesis without Kranz anatomy in *Bienertia cycloptera* (Chenopodiaceae). *The Plant Journal* **31,** 649–662.

Voznesenskaya EV, Franceschi VR, Kiirats O, Freitag H, Edwards GE. 2001. Kranz anatomy is not essential for terrestrial C_4 plant photosynthesis. *Nature* **414,** 543–546.

Warwick SI, Black LD. 1993. Molecular relationships in subtribe Brassicinae (Cruciferae, tribe Brassiceae). *Canadian Journal of Botany* **71,** 906–918.

Warwick SI, Black LD. 1994. Evaluation of the subtribes Moricandiinae, Savignyinae, Vellinae, and Zillinae (Brassicaceae, tribe Brassiceae) using chloroplast DNA restriction site variation. *Canadian Journal of Botany* **72,** 1692–1701.

Wessinger ME, Edwards GE, Ku MSB. 1989. Quantity and kinetic properties of ribulose 1,5-bisphosphate carboxylase in C_3, C_4 and C_3–C_4 intermediate species of *Flaveria* (Asteraceae). *Plant and Cell Physiology* **30,** 665–671.

Yan Z, Tian Z, Huang B, Huang R, Meng J. 1999. Production of somatic hybrids between *Brassica oleracea* and the C_3–C_4 intermediate species *Moricandia arvensis*. *Theoretical and Applied Genetics* **99,** 1281–1286.

Yoshimura Y, Kubota F, Ueno O. 2004. Structural and biochemical bases of photorespiration in C_4 plants: quantification of organelles and glycine decarboxylase. *Planta* **220,** 307–317.