

RESEARCH PAPER

Structural and biochemical characterization of the C₃–C₄ intermediate *Brassica gravinae* and relatives, with particular reference to cellular distribution of Rubisco

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Abstract

On the basis of its CO₂ compensation concentration, *Brassica gravinae* Ten. has been reported to be a C₃–C₄ intermediate. This study investigated the structural and biochemical features of photosynthetic metabolism in *B. gravinae*. The cellular distribution of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) was also examined in *B. gravinae*, *B. napus* L. (C₃), *Raphanus sativus* L. (C₃), and *Diplotaxis tenuifolia* (L.) DC. (C₃–C₄) by immunogold electron microscopy to elucidate Rubisco expression during the evolution from C₃ to C₃–C₄ 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 C₄ enzyme activities and high activities of Rubisco and photorespiratory enzymes, suggesting that it reduces photorespiratory CO₂ 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 C₃ to C₃–C₄ 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, C₃–C₄ intermediate plant, glycine decarboxylase, leaf anatomy, photorespiration, ribulose 1,5-bisphosphate carboxylase/oxygenase.

Introduction

C₃–C₄ intermediate plants have photosynthetic characteristics intermediate between those of C₃ and C₄ plants. Their leaf anatomy and CO₂ exchange characteristics, including CO₂ compensation concentrations and O₂ inhibition of photosynthesis, are in between those of C₃ and C₄ plants. Low rates of photorespiration in C₃–C₄ 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 C₃

plants, all four subunits of GDC occur in the mitochondria of photosynthetic cells, primarily the mesophyll (M) cells. In the leaves of C₃–C₄ 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 CO₂ released by the decarboxylation of glycine.

This pathway, called the glycine shuttle, functions as a recycling system for photorespiratory CO₂ 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 C₃–C₄ intermediate species of *Flaveria* (Asteraceae), a C₄ cycle operates together with the glycine shuttle (Monson and Rawsthorne, 2000).

Most species in the Brassicaceae are C₃ type, but some C₃–C₄ intermediate species occur in the genera *Moricandia*, *Diplotaxis*, and *Brassica* (Apel *et al.*, 1997). The C₃–C₄ intermediates in *Moricandia* and *Diplotaxis* reduce photorespiration only by the glycine shuttle, without a C₄ cycle (Hunt *et al.*, 1987; Rawsthorne, 1992; Ueno *et al.*, 2003). In *Brassica*, *B. gravinae* has been reported to be a C₃–C₄ intermediate species on the basis of its low value of CO₂ 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 C₃–C₄ intermediate species of *Moricandia* and *Diplotaxis*, or by both the shuttle and a C₄ cycle, as in some species of *Flaveria*. The genus *Brassica* includes many vegetable and oil C₃ 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 C₃–C₄ intermediate photosynthesis for use in the improvement of commercially important *Brassica* species.

The evolutionary processes giving rise to C₃–C₄ intermediate and C₄ plants remain to be elucidated. Previous studies have suggested that C₄ plants gradually evolved from C₃ plants through various stages of C₃–C₄ 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 C₄ 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 C₃ to C₃–C₄ intermediate plants. There are several plants that have C₄-like characteristics but still accumulate small amounts of Rubisco in the M chloroplasts (Bauwe, 1984a; Reed and Chollet, 1985; Ueno and Wakayama, 2004; Ueno and Sentoku, 2006). The existence of C₄-like plants suggests that, during the evolutionary course from C₃–C₄ intermediate through C₄-like to true C₄ 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 C₃–C₄ 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 CO₂ released from the mitochondria, whereas the centrifugal chloroplasts are responsible mainly for the fixation of CO₂ from the intercellular spaces (as in the M chloroplasts) rather than for the refixation of photorespiratory CO₂ (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 C₃ and C₃–C₄ 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 C₃ to C₃–C₄ intermediate plants.

Materials and methods

Plant materials

Five C₃ and C₃–C₄ intermediate species in the Brassicaceae were examined: *Brassica napus* L. (C₃), *B. gravinae* Ten. (C₃–C₄), *B. rapa* L. (C₃), *Raphanus sativus* L. (C₃), and *Diplotaxis tenuifolia* (L.) DC. (C₃–C₄). 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 C₄ 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% OsO₄ 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 $\times 25\,000$ 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 C₃-C₄ 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* (C₃) had fewer centripetally located chloroplasts in the BS cells than did the C₃-C₄ 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 $-80\text{ }^{\circ}\text{C}$) 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 $^{\circ}\text{C}$, and the supernatants were used for the enzyme assays. All enzymes were assayed spectrophotometrically in 1 ml reaction mixtures at 25 $^{\circ}\text{C}$, 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 C₃ 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

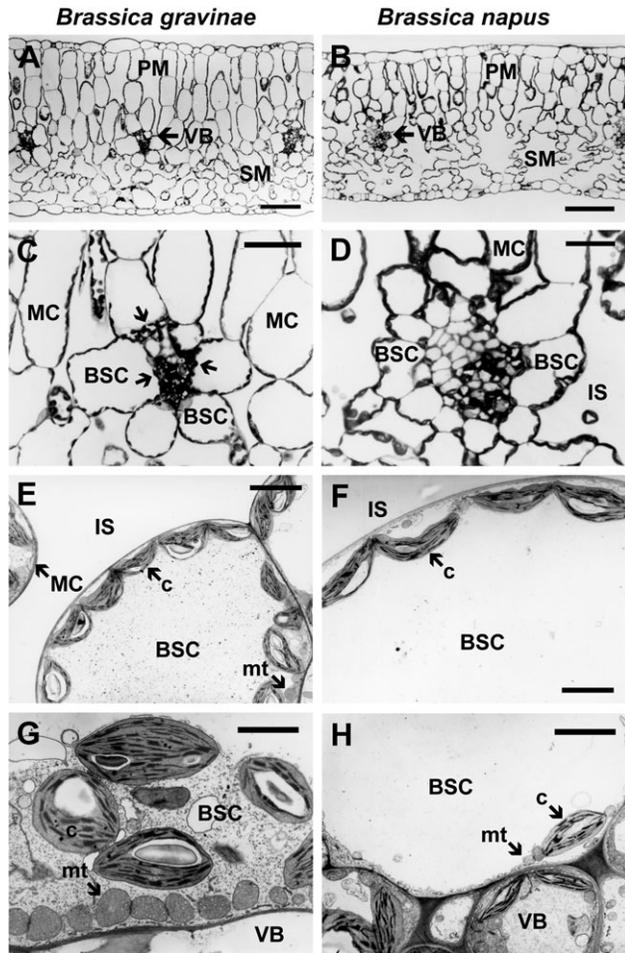


Fig. 1. Leaf anatomy of *B. gravinae* (A, C, E, G) and *B. napus* (B, D, F, H). (A, B) Leaf anatomical structures. (C, D) BS cells surrounding a vascular bundle. Arrows in (C) indicate centripetally located chloroplasts in the BS cells of *B. gravinae*. (E) Centrifugally and centripetally located chloroplasts 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)
<i>B. gravinae</i> (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
<i>B. napus</i> (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 \pm 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 $P < 0.01$.

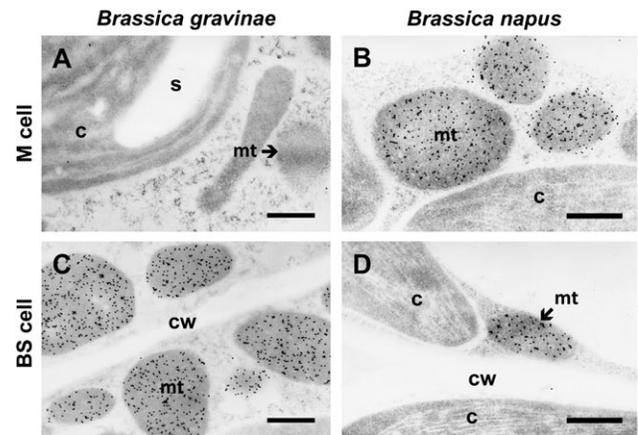


Fig. 2. Immunogold labelling of the P-protein of GDC in M and BS cells of *B. gravinae* (A, C) and *B. napus* (B, D). (A, B) M cells; (C, D) BS cells. c, Chloroplast; cw, cell wall; mit, mitochondrion; s, starch grain. Bars=0.5 μm .

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	
<i>B. gravinae</i> (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)	
<i>B. napus</i> (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)	
<i>D. tenuifolia</i> (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)	
<i>R. sativus</i> (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 \pm 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 $P < 0.01$.

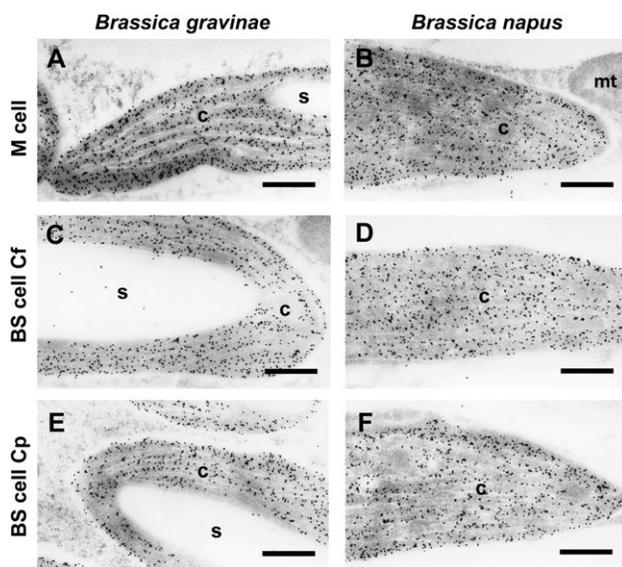


Fig. 3. Immunogold labelling of Rubisco LS in M and BS cells of *B. gravinae* (A, C, E) and *B. napus* (B, D, F). (A, B) Chloroplasts in M cells; (C, D) centrifugally located chloroplasts within BS cells; (E, F) centripetally located chloroplasts within BS cells. c, chloroplast; cw, cell wall; mit, mitochondrion; 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 C₄ plant, *P. maximum*, although the activity was somewhat lower than those of the control C₃ plants, *B. napus* and *B. rapa* (Table 4). The activity of the C₄ photosynthetic enzyme phosphoenolpyruvate carboxylase of *B. gravinae* was similar to that measured for C₃ plants. This was also the case for the activities of three C₄-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 C₃ 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 C₃ and C₄ 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 C₃-C₄ 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 C₃–C₄ intermediate and C₃ species

Species and BS cells		Cell fraction	Number of gold particles (μm^{-2})		Ratio (Cp:Cf label density)
			Cf	Cp	
<i>B. gravinae</i> (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)	
<i>B. napus</i> (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)	
<i>D. tenuifolia</i> (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 \pm 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 C₃ and C₄ plants

Enzyme	Activity ($\mu\text{mol mg}^{-1} \text{Chl h}^{-1}$)			
	<i>B. gravinae</i> (C ₃ –C ₄)	<i>B. napus</i> (C ₃)	<i>B. rapa</i> (C ₃)	<i>P. maximum</i> (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 \pm 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 C₄ photosynthetic enzymes. These data suggest that the C₄ 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 C₄ grasses than in C₃ grasses (Ueno *et al.*, 2005). *Brassica gravinae* also had higher activities of these photorespiratory enzymes than the control C₄ species. The activity levels are comparable with those found in the C₃ species *B. napus* and *B. rapa*. Previous studies of C₃–C₄ 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 C₃–C₄ intermediate species that reduces photorespiratory CO₂ loss by using the glycine shuttle. It seems that the photosynthetic carbon metabolism is similar to that of the C₃–C₄ intermediate species of *Moricandia* and *Diploaxis*.

In leaves of *B. gravinae*, the M layer was clearly differentiated into palisade and spongy tissues. This structure differed from that found in the C₃-C₄ 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 C₃-C₄ intermediacy relative to that in the C₃-C₄ intermediates of *Moricandia* and *Diplotaxis*, because the M layers of C₃ 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 CO₂ 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 C₃-C₄ intermediacy in *B. gravinae*.

Cellular distribution of Rubisco during the evolution from C₃ to C₃-C₄ 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 C₃-C₄ intermediate and C₃ 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* (C₃) and *B. gravinae* (C₃-C₄), 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 C₃ to C₃-C₄ intermediate plants, without large modification. However, an increase in chloroplast number occurred in the BS cells of C₃-C₄ 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 C₃-C₄ intermediate plants as compared with C₃ 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 C₃ to C₃-C₄ intermediate plants. This structural event could also have contributed to the increased partitioning of Rubisco into the BS cells of C₃-C₄ 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 C₃-C₄ intermediate with C₄-cycle activity. Bauwe (1984b) showed that there was no large difference in the kinetic properties of Rubisco between C₃ and C₃-C₄ intermediate species of *Moricandia*. In the leaves of other C₃-C₄ intermediates, the kinetic constants of Rubisco are C₃-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 C₃-C₄ intermediate plants.

As discussed earlier, the BS cells of C₃-C₄ 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 C₄ 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. C₃ 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 C₃-C₄ 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* (C₃-C₄). In *B. napus* (C₃), 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 C₄ 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 C₃ 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.

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