Theor Appl Genet (1998) 96: 922–927

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Cellular expression pattern of the glycine decarboxylase P protein in leaves of an intergeneric hybrid between the C_3-C_4 intermediate species *Moricandia nitens* and the C_3 species *Brassica napus*

Received: 23 October 1997 / Accepted: 28 November 1997

Abstract An intergeneric hybrid plant was produced between the C_3-C_4 intermediate species *Moricandia nitens* and the C_3 species *Brassica napus* by sexual hybridization and in vitro embryo rescue. The hybrid nature of the plant was apparent in its morphology and flower pigmentation and was confirmed by leaf isozyme patterns. The overall plant morphology and the shape and thickness of leaves of the hybrid plant were intermediate between those of the parent species. However, the bundle-sheath cells of the hybrid resembled those of the C_3 parent and lacked the organelle development of the C_3-C_4 intermediate parent. Immunogold labelling for the presence of the P subunit of the mitochondrial glycine decarboxylase complex revealed a very similar labelling density on mitochondria in bundle-sheath and mesophyll cells in *B. napus*, while in *M. nitens* the P subunit was only detectable in bundle sheath cells. In the hybrid the labelling density on mesophyll cell mitochondria was almost half of that on the bundle-sheath mitochondria. The CO_2 compensation point of the hybrid was significantly less than that of the C_3 parent but was not as low, nor as responsive to changes in light intensity, as for the C_3-C_4 parent.

Key words *Brassica napus* · C_3-C_4 intermediate photosynthesis · Glycine decarboxylase · Intergeneric hybrid · *Moricandia arvensis*

Communicated by K. Glimelius

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Introduction

The C_3-C_4 intermediate photosynthetic mechanism has been documented in a range of species from several higher plant genera (Rawsthorne 1992). Plants which use C_3-C_4 photosynthesis have a lower CO_2 compensation point (Γ) than related C_3 species, and their Γs decrease in response to light intensity whereas those of C_3 plants do not (Rawsthorne 1992). Most C_3-C_4 intermediate species do not have any C_4 metabolism but they are differentiated from those with C_3 photosynthesis by two principal characters which centre on an increased role of the bundle-sheath cells in the leaf (Rawsthorne 1992). The first is an increase in the number of organelles, primarily of mitochondria, in the bundle-sheath cells and the alignment of these mitochondria at the centripetal wall of the cells. Chloroplasts are distributed around the cell periphery but overlie the mitochondria at the centripetal face. The second is the loss of the glycine decarboxylase complex (GDC) from the mitochondria of the mesophyll cells (Rawsthorne 1992). In C_3 species the enzyme is found in all photosynthetic cells, and its activity releases CO_2 during photorespiratory metabolism (Hylton et al. 1988; Rawsthorne et al. 1988a; Tobin et al. 1989). The loss of glycine decarboxylase protein and activity from the mesophyll cells of a C_3-C_4 intermediate species was first demonstrated in *Moricandia arvensis* (Rawsthorne et al. 1988a, b). Later studies revealed that this loss was due specifically to a lack of the P subunit protein (Morgan et al. 1993). Loss of mesophyll GDC was proposed to lead to confinement of photorespiratory CO_2 release to the bundle-sheath cells where the close association between mitochondria and chloroplasts would enhance the recapture of the CO_2 before it left the leaf (Rawsthorne et al. 1988a).

The transpirational efficiency (TE; CO_2 uptake per unit water lost through the stomata during photosynthesis) of C_3 Brassicaceous plants was previously

shown to be dependent on the thickness of the leaf, but the TE of *M. arvensis* is greater than that predicted from the linear relationship between leaf thickness and TE (McVetty et al. 1989). The genus *Moricandia* is closely related to *Brassica* crop species (Warwick et al. 1992), and it may be possible to introduce the C_3-C_4 intermediate character into these crops to improve their carbon and water economies through alterations to photorespiratory CO_2 release and TE. Hybrids have been produced between *Moricandia* and *Brassica* species through both somatic hybridization and ovary culture to rescue embryos of sexual crosses (Apel et al. 1984; Toriyama et al. 1987, 1988; Takahata 1990; Takahata and Takeda 1990; Takahata et al. 1993; O'Neill et al. 1996). In only a few cases has any degree of transfer of the C_3-C_4 character been demonstrated. A single hybrid between *B. alboglabra* and *M. arvensis* had a Γ value that was intermediate between those of the two parents (Apel et al. 1984), while three hybrids between *B. napus* and *M. arvensis* also had Γ values between those of the parents (O'Neill et al. 1996). In this paper we describe for the first time in a C_3-C_4 intermediate $\times$ C_3 intergeneric hybrid (*M. nitens* $\times$ *B. napus*) the expression of biochemical characteristics of photorespiratory metabolism which are found in C_3-C_4 intermediate species.

Materials and methods

Plant culture

Seeds of *Moricandia nitens* (Viviana) Durd. et Barr. were obtained from Prof. C. Gomez-Campo (University of Madrid, Spain) while *Brassica napus* L. cv 'Duplo' was from a commercial source. Plants were grown as described in Morgan et al. (1993). Between October and April supplementary illumination (16 h) was provided during the natural photoperiod by high pressure sodium lamps.

Production of hybrid plants

Approximately 300 pollinations of flowers of *M. nitens* were made using pollen collected from *B. napus* cv 'Duplo' using bee sticks (Williams 1980). The *M. nitens* flowers were emasculated 1 day prior to the opening of the buds and pollinated immediately. After pollination the flowers were covered with glassine paper bags to prevent any other accidental pollinations. When pod-set occurred on the *M. nitens* plants development was allowed to continue for 2–3 weeks. After this time the developing ovules were dissected aseptically from the ovaries and placed into tissue culture on nutrient-supplemented agar medium (Monnier 1978). Plantlets which developed from the embryos were transferred to half-strength M&S medium (Murashige and Skoog 1962) after 3–4 weeks. Plantlets which reached a sufficient size and were rooted were washed free of agar medium and transferred to small pots containing a mixture of John Innes No. 1 compost and medium grade vermiculite (50 : 50, v : v). The pots were placed in a controlled temperature and light tissue culture room, under shading, with a cover to increase the atmospheric humidity. The shading and humidity were reduced progressively, and plants were ultimately transferred to the glasshouse. Replicate plants of the hybrid were produced by vegetative propagation of young shoots (length 7–10 cm).

Measurement of CO_2 compensation point

All measurements were made on attached, fully expanded leaves using the apparatus described by Hunt et al. (1987). To ensure standardization we made the measurements at the same time in the photoperiod and on leaves at similar positions on young plants. Measurements were made at atmospheric oxygen concentrations at 25°C, and at a range of light intensities generated by interposing neutral density filters between the light source and the leaf chamber.

Leaf anatomical characters and immunocytochemical techniques

Leaf tissue pieces were excised from mature, fully expanded leaves under fixative and embedded into L.R. White resin as described previously (Rawsthorne et al. 1988a). Immunogold labelling was performed using an antibody raised against the P subunit of the glycine decarboxylase complex from pea (*Pisum sativum* L.: kindly provided by Dr. D. Oliver, University of Moscow, Idaho, USA) using methods described by Rawsthorne et al. (1988a). The antibody had been shown previously to cross-react with the P subunit of several species from a diverse range of genera, including those from *Moricandia*, and we were therefore confident that it could be used as a specific probe in this investigation (Rawsthorne et al. 1988a). Sections were viewed using a light microscope for anatomical comparisons and using an electron microscope for the immunogold analysis. The density of immunogold particles on cell organelles was determined directly from photomicrographs using a computer graphics tablet.

Leaf isozyme analysis

Preparation of leaf extracts and isoelectric focussing in gels was carried out according to O'Neill and Mathias (1995). The gels contained a mixture of pH 3–5 and pH 4–8 ampholytes (Pharmacia) in a ratio of 2 : 1 (v : v) and were stained for esterase and acid phosphatase activity (Tanksley and Orton 1983).

Results

From the large number of crosses made between *B. napus* and *M. nitens*, very few pods developed and those that did contained only a few embryos. Most embryos failed to develop in tissue culture, and only a single plant was recovered. A number of features reveal that this plant is a hybrid between the two parental species. The morphology of the plant was clearly intermediate between that of its parents. *Brassica napus* cv 'Duplo' has an upright habit with little branching and large, thin, rounded leaves characteristic of this species. *Moricandia nitens* has a compact, branched habit with small, thicker, glaucous leaves which are lanceolate and somewhat serrated. The hybrid has a tendency to branch more than *B. napus*, and the leaves are intermediate in size, shape and thickness (not shown). In addition, the flower pigmentation of the hybrid reflected that of both of the parents. The petals

were pale yellow with purple venation. *Moricandia* species have purple flowers, while the petals in *B. napus* cv 'Duplo' are yellow.

To obtain further evidence as to the hybrid nature of the plant we carried out isozyme analyses on extracts made from leaf tissues of the hybrid and its two parent species. Due to the large numbers of crosses that had been made using many different parent plants it was not possible to use the exact parent plants which had been used to produce the hybrid. Initial tests of the *B. napus* parent showed little plant to plant variation in the isozyme patterns, as expected for an inbred cultivar. Individual *M. nitens* plants were from a wild population, which resulted in small variations in the isozyme patterns (not shown). However, the patterns for *M. nitens* were very distinct from those of *B. napus* (Fig. 1). In both isozyme assays used, stained bands or groups of bands were identified in the hybrid which were derived from each of the parent species (Fig. 1).

The C_3-C_4 intermediate photosynthesis is characterized by (1) a strong development of bundle-sheath cells, (2) localization of glycine decarboxylase (GDC) to the bundle-sheath cells and (3) a CO_2 compensation point (Γ) which is much lower than that of C_3 species and which decreases with increasing photosynthetic photon flux density (PPFD) (Rawsthorne 1992). These three characters were therefore examined in the hybrid. The internal anatomy of the leaves of the hybrid resembled more closely that of the C_3-C_4 parent with less clear differentiation between the shape of palisade and mesophyll parenchyma than in *B. napus* where the palisade and mesophyll are distinct (not shown). However, at higher magnification the bundle-sheath cells of the hybrid resembled those of the C_3 parent and were not as developed as those of the C_3-C_4 parent which contain numerous chloroplasts and other organelles (Fig. 2). In confirmation of this there were 3.5-fold fewer chloroplast profiles in the bundle-sheath cells of the hybrid than in *M. nitens*, although the value for the hybrid was slightly greater ($P < 0.05$) than that for *B. napus* (Table 1). There were no significant differences between the numbers of chloroplast profiles in the mesophyll cells of the different plants examined (Table 1).

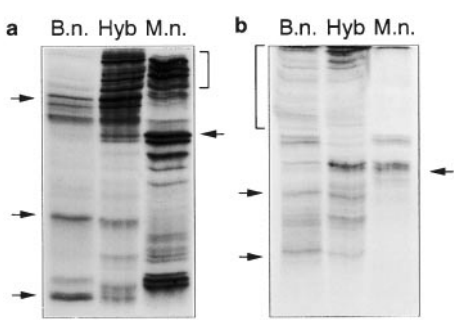

Fig. 1a, b Zymograms for leaf **a** acid phosphatase and **b** esterase of *B. napus* (B.n.), *M. nitens* (M.n.) and the *M. nitens* × *B. napus* hybrid (Hyb). Bands or regions which appear in the hybrid and either of the parents are marked (*arrows*) alongside the lane for the relevant parent. The gels were run with a 2:1 mixture (v:v) of pH 3–5 and 4–8 ampholytes

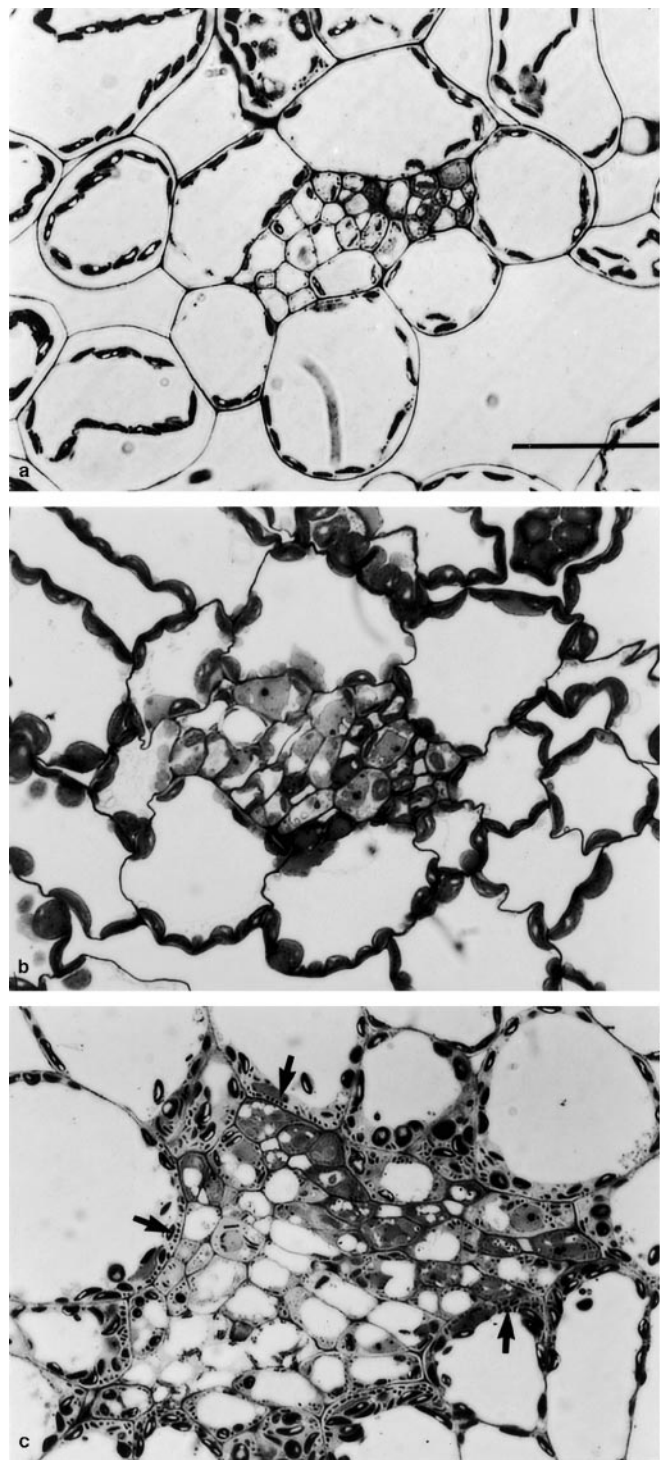

Fig. 2a–c Leaf anatomy of **a** *B. napus*, **c** *M. nitens* and **b** the *M. nitens* × *B. napus* hybrid. The prominent anatomy of the bundle-sheath cells in *M. nitens*, including the numerous mitochondria (*arrowed*) on the centripetal cell faces is clearly visible. Both the hybrid and *B. napus* lack this pronounced development. Magnification is identical for all figures. Bar: 20 μm

Table 1 Chloroplast numbers in mesophyll and bundle-sheath cells of *B. napus*, *M. nitens* and the *M. nitens* × *B. napus* hybrid. The chloroplast profiles were counted directly on photomicrographs and represent the mean ± SE of determinations from at least six separate cells of each type

Plant	Number of chloroplast profiles per cell		
	Mesophyll		Bundle sheath
	Palisade	Spongy	
B. napus	7.6 ± 2.1	6.6 ± 1.8	3.6 ± 1.5
M. nitens	12.8 ± 4.0	8.6 ± 5.2	25.0 ± 1.5
M.n. × *B.n.*	12.7 ± 3.9	9.9 ± 2.5	7.1 ± 1.8

Table 2 Immunogold labelling for the presence of the P subunit of glycine decarboxylase on mitochondria of bundle-sheath or mesophyll cells of *B. napus*, *M. nitens* and the *M. nitens* × *B. napus* hybrid. Counts of gold particles were made from photomicrographs. Each value represents the mean ± SE (not shown where two or more counts were zero) of counts made from at least 20 mitochondria which were taken from at least three separate cells of each type

Plant	Density of immunogold particles (counts μm^{-2})		
	Mitochondria		Cytoplasm + other organelles[a]
	Bundle sheath	Mesophyll	
B. napus	142.7 ± 6.2	155.1 ± 6.7	6.2 ± 0.5
M. nitens[b]	71.7 ± 5.0	3.6	1.1 ± 0.2
M.n. × *B.n.*	145.1 ± 7.5	86.9 ± 5.0	1.8 ± 0.1

[a] Data from both cell types are combined
[b] Data taken from Hylton et al. (1988)

The distribution of GDC in the leaves of the hybrid was compared to that in the leaves of the parent species using immunolocalization of the P protein of GDC (Morgan et al. 1993). In the leaves of *B. napus* the density of immunogold particles on the mitochondria of bundle-sheath and mesophyll cells was comparable (Table 2). While the mitochondria of *M. nitens* bundle-sheath cells are highly labelled by gold particles, they are almost undetectable on the mitochondria in mesophyll cells (Table 2). The pattern of immunolabelling in the leaf of the hybrid was intermediate between that of the two parent species. On the mitochondria of the mesophyll cells the density of gold particles was significantly ($P < 0.001$) and substantially lower (40% less) than that on the mitochondria of bundle-sheath cells (Table 2).

The Γs of the C_3 species *B. napus* and the hybrid were relatively invariant with the PPFD imposed. In replicated measurements at a PPFD of 350 $\mu mol.m^{-2}.s^{-1}$ the Γ of the hybrid was close to, but significantly less than ($P < 0.05$), that of *B. napus* (Table 3). At a lower PPFD the Γs of *B. napus* and the hybrid were not significantly different (Table 3). In contrast, the Γ of *M. nitens* is highly responsive to the PPFD. At 1200 $\mu mol.m^{-2}.s^{-1}$ the Γ is 10 $\mu l.l^{-1}$, which increases to 19 $\mu l.l^{-1}$ at 350 $\mu mol.m^{-2}.s^{-1}$ and then still further as the light intensity continues to decrease (Hunt 1985).

Table 3 Effect of photosynthetic photon flux density (PPFD) on the CO_2 compensation point (Γ) of attached leaves of *B. napus* and the *M. nitens* × *B. napus* hybrid. Values are the mean ± SE of measurements made on 85–100% fully expanded leaves from at least 4 separate plants. The ratio of Γ at the higher : lower PPFD is given in parenthesis (the ratio for the related C_3-C_4 intermediate species *M. arvensis* = 0.5: O'Neill et al. 1996)

Plant	CO_2 compensation point		
	PPFD ($\mu mol.m^{-2}.s^{-1}$)		
	160	350	
B. napus	44.4 ± 1.4	42.9 ± 1.4	(0.97)
M. nitens × *B. napus*	43.7 ± 1.4	40.5 ± 1.1	(0.93)

Discussion

Hybridization of C_3 and C_3-C_4 intermediate species has been used as a tool to address the genetic control of the C_3-C_4 intermediate character (Bouton et al. 1986) and as a direct means to introduce the potentially beneficial aspects of the character into crop species (e.g. Takahata et al. 1993; O'Neill et al. 1996). In this study we have shown that aspects of the C_3-C_4 intermediate character can be introduced from a *Moricandia* species into a *Moricandia* × *Brassica* hybrid plant. In particular, the expression of the P protein of GDC in the mesophyll of the hybrid plant is reduced compared to that in the bundle sheath. It is also reduced in comparison to that seen in the mesophyll of *B. napus* (Table 2). This is the first report of reduced expression of GDC in the mesophyll of any hybrid between C_3 and C_3-C_4 intermediate species. In this case, although there is clearly greater expression of GDC P protein in the bundle-sheath than in the mesophyll cells, as reported for C_3-C_4 intermediate species generally (Hylton et al. 1988), this has only a limited effect on the Γ of the hybrid. This is an important observation in that hybrids between C_3 and C_3-C_4 intermediate species are commonly screened for expression of the C_3-C_4 intermediate phenotype by measuring Γ. Our data show that measurement of Γ alone is insufficient to detect important underlying changes in other aspects of the overall character.

In leaves of C_3-C_4 intermediate species GDC activity is either absent or present at only very low levels in the mesophyll cells, and it is believed that the localization of GDC activity to the bundle sheath is a key to the decrease in the loss of photorespiratory CO_2 and Γ (Rawsthorne 1992). The results from this study support the hypothesis that complete or greater loss of

GDC from the mesophyll of the hybrid is required in order to prevent or decrease the capacity of the mesophyll to decarboxylate glycine. We would propose that such a decrease is required in order to increase the proportion of whole-leaf photorespiratory CO_2 release in the bundle sheath and so decrease Γ to a level seen in the C_3-C_4 intermediate parent *M. nitens*. However, the anatomical development of the bundle sheath of the hybrid is like that of the C_3 parent and it lacks the organelle development seen in *M. nitens*. Therefore, even with a modest increase in the proportion of the whole-leaf glycine decarboxylation in the bundle-sheath, the CO_2 released in this compartment may not be recaptured as efficiently as it is in C_3-C_4 intermediate species. This could be why the hybrid expresses only a small decrease in Γ compared to the C_3 parent species, *B. napus*. It is notable that the change in GDC expression in the leaves of the hybrid has occurred without coordinate changes in the anatomical development. This suggests that the anatomical and biochemical components of the C_3-C_4 intermediate character are controlled independently.

Interestingly, the study of this hybrid reveals that a significant loss of the GDC P protein can occur from the majority of the leaf photosynthetic cells without any apparent impairment of plant performance. The growth of rooted cuttings of the hybrid plant is vigorous, and photosynthetic gas exchange is comparable to that of the C_3 parent species (unpublished data). Since the P protein catalyses the decarboxylation of glycine (Oliver 1994) it is apparent that losses of total GDC activity can occur without affecting C_3 photosynthesis. This contrasts with C_3 plants which contain null mutations in enzymes of the photorespiratory pathway. Such plants are unable to grow at atmospheric CO_2 concentrations because of the detrimental effect that these lesions have on metabolism in photosynthetic cells (Somerville 1986).

We do not know which genes are expressing GDC P protein in the hybrid plant. Nevertheless, we can speculate that the cell-specific expression of the genes from the C_3-C_4 intermediate species *M. nitens* is at least partially conserved in a genetic background containing chromosomes of the C_3 species *B. napus* and/or that the presence of the *Moricandia* chromosomes is modulating the cellular expression pattern of the *Brassica* GDC P genes. Which of these two possibilities is occurring is certainly intriguing and merits further study in order to elucidate how the cell-specificity of GDC expression is controlled in C_3-C_4 intermediate species.

Acknowledgements The research described in this manuscript was inspired by the late Prof. H. W. Woolhouse. We thank Drs. M. Beschorner and L. Hill for their helpful comments on the manuscript. Dr. D. Oliver is thanked for the kind gift of GDC P antiserum. We are also grateful for the assistance in plant propagation and maintenance provided by staff of the Horticultural Services Department at the John Innes Centre. This research was supported by the Competitive Strategic Grant to the John Innes Centre from the Biotechnology and Biological Sciences Research Council.

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