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Developmental and Environmental Effects on the Expression of the C_3-C_4 Intermediate Phenotype in *Moricandia arvensis*[1]

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Cellular anatomy and expression of glycine decarboxylase (GDC) protein were studied during leaf development of the C_3-C_4 intermediate species *Moricandia arvensis*. Leaf anatomy was initially C_3-like and the number and profile area of mitochondria in the bundle-sheath cells were the same as those in adjacent mesophyll cells. Between a leaf length of 6 and 12 mm there was a bundle-sheath-specific, 4-fold increase in the number of mitochondrial profiles, followed by a doubling of their individual profile areas as the leaves expanded further. Subunits of GDC were present in whole-leaf extracts before the anatomical development of bundle-sheath cells. Whereas the GDC H-protein content of leaves increased steadily throughout development, the increase in GDC P-protein was synchronous with the development of mitochondria in the bundle sheath. The P-protein was confined to bundle-sheath mitochondria throughout leaf development, and its content in individual mitochondria increased before the anatomical development of the bundle sheath. Anatomical and biochemical attributes of the C_3-C_4 character were present in the cotyledons and sepals but not in other photosynthetic organs/tissues. In leaves and cotyledons that developed in the dark, the expression of the P-protein and the organellar development were reduced but the bundle-sheath cell specificity was retained.

Moricandia arvensis is a species that has C_3-C_4 intermediate photosynthesis and is characterized by having a CO_2 compensation concentration value between that of the C_3 and C_4 species. This is the result of both anatomical and biochemical adaptations (Hunt et al., 1987; Rawsthorne et al., 1988a, 1988b). The mature leaves of C_3-C_4 intermediate species exhibit a marked Kranz-like arrangement of organelles within the cells that surround the vascular bundle. In these bundle-sheath cells, mitochondria and peroxisomes are distributed centripetally against the cell wall adjacent to the vascular bundle, and are overlain by chloroplasts (Brown and Hattersley, 1989). This spatial arrangement of organelles is emphasized by a 4-fold greater number of mitochondrial profiles in the bundle-sheath cells compared with that in mesophyll cells (Brown and Hattersley, 1989). Furthermore, in those C_3-C_4 intermediate species studied to date the bundle-sheath cell mitochondria have twice the profile area of that in the mesophyll cells (Hylton et al., 1988).

In C_3-C_4 intermediate species in the genera *Alternanthera*, *Flaveria*, *Mollugo*, *Moricandia*, and *Panicum*, GDC protein and/or activity has been shown to be confined to the bundle-sheath cells in the leaf (Hylton et al., 1988; Moore et al., 1988; Rawsthorne et al., 1988a; Devi et al., 1995). In contrast, GDC is found in all photosynthetic cells in the leaves of C_3 species (Hylton et al., 1988; Rawsthorne et al., 1988a; Tobin et al., 1989). GDC is a key mitochondrial enzyme in the photorespiratory pathway. It comprises four heterologous protein subunits, P, H, T, and L (Bourguignon et al., 1988; Oliver et al., 1990) and, together with Ser hydroxymethyltransferase, it catalyzes the oxidative conversion of Gly to Ser, NH_3, and CO_2 (Neuburger et al., 1986). In C_3-C_4 intermediate species the confinement of GDC to the bundle-sheath cells, combined with the Kranz-like leaf anatomy, is proposed to enhance recycling of photorespiratory CO_2, and so reduce CO_2 compensation concentration compared with that in C_3 species (Rawsthorne et al., 1988a). *M. arvensis* is unique among C_3-C_4 intermediate species that have been studied to date in that it lacks only the P-protein of GDC in the mesophyll cells (Morgan et al., 1993). For all of the other C_3-C_4 intermediates studied so far, all four GDC proteins are absent from the mesophyll cells (Morgan et al., 1993). In all of these C_3-C_4 intermediate species Gly decarboxylation in the mesophyll would be lost, because GDC activity requires all four GDC proteins (Rawsthorne et al., 1995).

Studies of leaf development in wheat show that the activities of GDC (Rogers et al., 1991) and Rubisco (Dean and Leech, 1982) increase with leaf development. However, although photosynthesis and photorespiration in a mature C_3 leaf are intrinsically linked metabolically, the expression of the two pathways is not necessarily synchronous during leaf development. Recent studies have shown a lag between the initial appearance of Rubisco and GDC protein during the early stages of expansion of pea leaves (Vauclare et al., 1996). The specialized Kranz-like anatomy and

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Abbreviations: GDC, Gly decarboxylase; GO, glycolate oxidase; RbcL, large subunit protein of Rubisco.

biochemistry found in *M. arvensis* are central to the reduction of photorespiratory CO_2 loss, but very little is known about the coordination of these two components during leaf development in a C_3-C_4 intermediate species. The only previous study of leaf development in a C_3-C_4 intermediate was of the monocotyledonous species *Panicum milioides*, which has no visible changes in bundle-sheath cell anatomy from the uncurled leaves within the sheath to maturity (Fladung and Hesselbach, 1987).

In this study we investigated the expression of GDC P-protein and the Kranz-like anatomy throughout leaf development in *M. arvensis*. The organ specificity of the character was also studied, as were environmental signals that might potentially contribute to its expression. Light has been shown to play a major role in the expression of GDC genes (Rawsthorne et al., 1995), and the effect of light was studied in comparisons of light- and dark-grown plants. Recently, it has also been shown that Gly induces the expression of GDC P-protein in the yeast *Saccharomyces cerevisiae* (Sinclair et al., 1996). Therefore, it is possible that leaf Gly content, which is determined by photorespiratory metabolism (Rawsthorne and Hylton, 1991), could act as a signal to induce the expression of GDC in higher plants. To investigate this possibility we have compared GDC P-protein expression and leaf anatomy in plants grown in atmospheric CO_2 concentrations or under elevated CO_2 to suppress photorespiration.

MATERIALS AND METHODS

Plant Material

Seeds of *Moricandia arvensis* (L.) DC and of *Moricandia moricandioides* (Boiss.) Heywood were from stocks held at the John Innes Centre, obtained originally from Professor C. Gomez-Campo (University of Madrid). Plants were grown in a 3:1 (v/v) mixture of John Innes no. 3 potting compost:perlite in a controlled-environment cabinet with day and night temperatures of 25°C and 18°C, respectively, and a 14-h photoperiod. The PPFD was 750 μmol m^{-2} s^{-1}. Seeds for plants used to study the effect of light on GDC distribution and anatomy in seedlings were surface-sterilized in a 1.2% (w/v chlorine) sodium hypochlorite solution for 10 min and sown onto Murashige and Skoog (1962) medium with 1% (w/v) bactoagar and 3% (w/v) Suc in Magenta pots (Sigma). For dark-grown seedlings the jars were double-wrapped in aluminum foil. The Magenta pots were placed in a controlled-environment cabinet at 20°C, with a 16-h photoperiod and a PPFD of 350 μmol m^{-2} s^{-1}. In studies using elevated CO_2, 4-week-old plants at the three- to four-leaf stage were placed in a Perspex chamber with a PPFD of 300 μmol m^{-2} s^{-1}. Air enriched with CO_2 to 1000 μL L^{-1} was pumped through the chamber. This concentration of CO_2 was 10% to 20% in excess of that required to give CO_2-saturated rates of CO_2 assimilation by attached *M. arvensis* leaves under a saturating PPFD (Rylott, 1997). The CO_2 concentration of the gas leaving the chamber was monitored with an IR gas analyzer. Control plants were grown alongside the chamber. The temperatures and photoperiod were as described above. The plants were grown for an additional 4 weeks before sampling of the youngest fully expanded leaves.

Immunological Techniques

The immunoglobulin fraction of a polyclonal antiserum raised against GDC P-protein from pea (kindly provided by Dr. D.J. Oliver, Iowa State University, Ames) was used for the immunolocalization studies of leaf development. For the studies of organ specificity and environmental effects, and for probing western blots of total proteins during leaf development, the IgG fraction of a separate polyclonal antiserum raised against GDC P-protein from pea was used (Morgan et al., 1993). Antibodies raised against the L- and H-proteins of GDC were as described by Morgan et al. (1993). The anti-Rubisco (large subunit) and anti-GO antibodies were as described by Rawsthorne et al. (1988a). All of these antibody preparations have been shown to be monospecific, giving rise to single bands on western blots of total leaf proteins of *M. arvensis*. Preparation of leaf sections and immunolocalization techniques were as described by Morgan et al. (1993). Extraction of leaf proteins and western-blotting techniques were as described by Morgan et al. (1993). Dilutions of IgG or antiserum are given below as appropriate.

RESULTS

To study the expression of the C_3-C_4 character during leaf development, experiments were carried out on expanding leaves with lengths between 2 and 12 mm and at full expansion. Leaves were taken from approximately the same node on the developing plants. Sections were made through vascular bundles from the midpoint of each leaf blade and were subjected to ultrastructural and immunocytochemical analysis.

Leaf Anatomy

At the 2- and 3-mm stages the leaves were visibly achlorophyllous, and greening commenced at the 4-mm stage. During these stages the bundle-sheath cells were cytoplasmically dense and contained small vacuoles. The chloroplasts were not fully developed, as shown by the fact that they had few appressed thylakoids and showed no distinct granal stacking (Fig. 1a). By the 5-mm stage the bundle-sheath cells became vacuolate and thylakoid stacking was visible in the chloroplasts. The development of chloroplasts was well established by the 6-mm stage (Figs. 1, b and c, and 2a). Up to the 6-mm stage, the number of mitochondria per bundle-sheath cell and their mean profile areas did not differ significantly from these same parameters in the mesophyll cells (Fig. 3, a and b). Between the 6-mm and mature-leaf stage the number of mitochondrial profiles per bundle-sheath cell increased by a total of 4-fold (Fig. 3b). Furthermore, the centripetal arrangement of mitochondria along the bundle-sheath cell wall adjacent to the vascular cells also became clearly visible (Fig. 2), leading to the appearance of the characteristic C_3-C_4 intermediate Kranz-like anatomy. In contrast, the number of mitochondrial

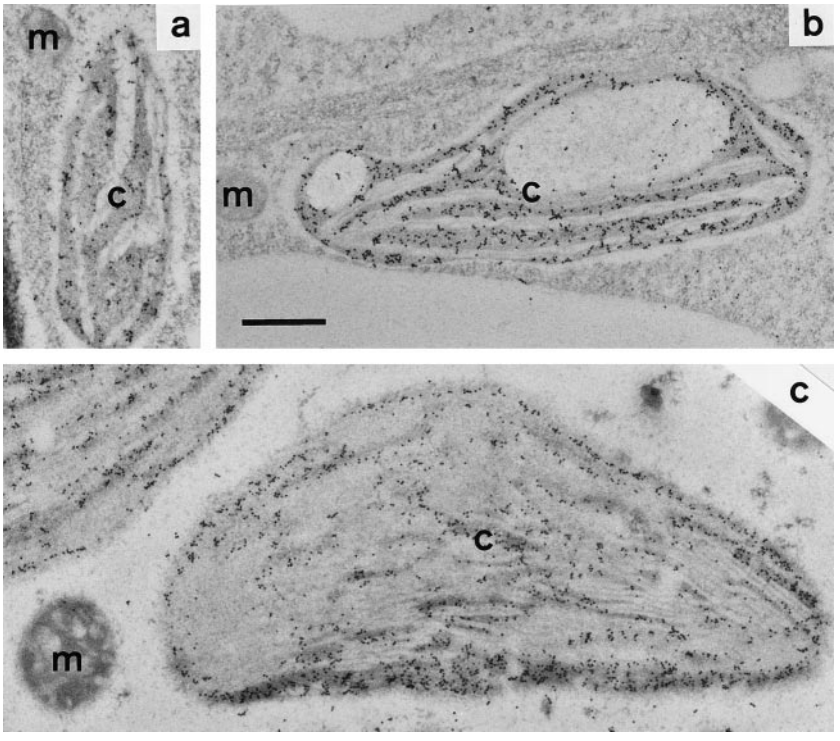

Figure 1. Mitochondrial and chloroplast development in bundle-sheath cells of *M. arvensis* leaves. Sections from the 4-mm (a) and 6-mm (b) stages and from mature leaves (c) were labeled with antiserum to RbcL at a dilution of 1:500. m, Mitochondrion; c, chloroplast. Scale bar = 0.5 μm.

profiles remained unchanged in the mesophyll cells (see legend to Fig. 3).

There was relatively little change in the profile areas of individual mitochondria and chloroplasts, or of the bundle-sheath cells up to the 12-mm stage (Figs. 2 and 3, a and d). Between the 12-mm stage and full leaf expansion there was a 7.5-fold increase in the bundle-sheath cell profile area (Fig. 3d). Coincident with this cell expansion the individual chloroplast and mitochondrial profile areas more than doubled, with the increases in mitochondrial size being confined to the bundle-sheath cells (Figs. 2 and 3a).

Protein Expression

To examine how the expression of GDC protein was related to changes in leaf anatomy, western blots of total protein extracts from leaves of increasing length were probed with antibodies raised against the P-, L-, and H-protein subunits of GDC. Expression patterns were compared with those of other photosynthetic and photorespiratory enzymes, RbcL, and GO. All of the proteins were detectable at the 2-mm stage (data not shown). The relative abundance of GDC P-protein changed very little up to the 5-mm stage and then increased markedly thereafter (Fig. 4). In contrast, the abundance of GDC H-protein, and of RbcL and GO, increased progressively from the 3-mm stage until the mature-leaf stage, with no abrupt transition (Fig. 4). The GDC L-protein was readily detected at the 3-mm stage, and during leaf development its abundance increased relatively little compared with that of the P- and H-proteins (Fig. 4). This differential response for the P- and H-proteins versus the L-protein has been observed in greening pea leaves (Turner et al., 1993) and developing wheat leaves (Rogers et al., 1991). It is believed to be caused by the role of the L-protein in other oxo-acid dehydrogenase complexes that are present in the mitochondria of all cells, and the expression of this subunit is therefore not linked specifically to photorespiratory activity of the tissue (Bourguignon et al., 1996).

Immunogold labeling was used to study the cellular localization of expression of GDC P-protein and Rubisco. The GDC P-protein was detectable only in the mitochondria of the bundle-sheath cells at each of the developmental stages studied (data not shown). There was a noticeable increase in the density of immunogold particles between the 4- and 5-mm stages (Fig. 3c), with relatively little change thereafter. Chloroplasts of both bundle-sheath and mesophyll cells contained Rubisco throughout development, and an increase in immunolabeling density was also seen early in leaf development (Fig. 1; data not shown for mesophyll cells).

Organ Specificity of C_3-C_4 Intermediate Anatomy and Biochemistry

To examine whether the C_3-C_4 intermediate character was expressed in organs other than true leaves, we used ultrastructural and immunocytochemical techniques to study stems, silique walls, sepals, petals, and cotyledons during embryo development and after germination. Comparisons were also made with leaves of the related C_3 species *M. moricandioides*. In leaves, cotyledons of seedlings, and sepals of *M. arvensis*, the profile areas of individual mitochondria in the bundle-sheath cells were significantly greater (by between 2.0- and 5.5-fold) than for mitochondria in the mesophyll cells of these organs. This

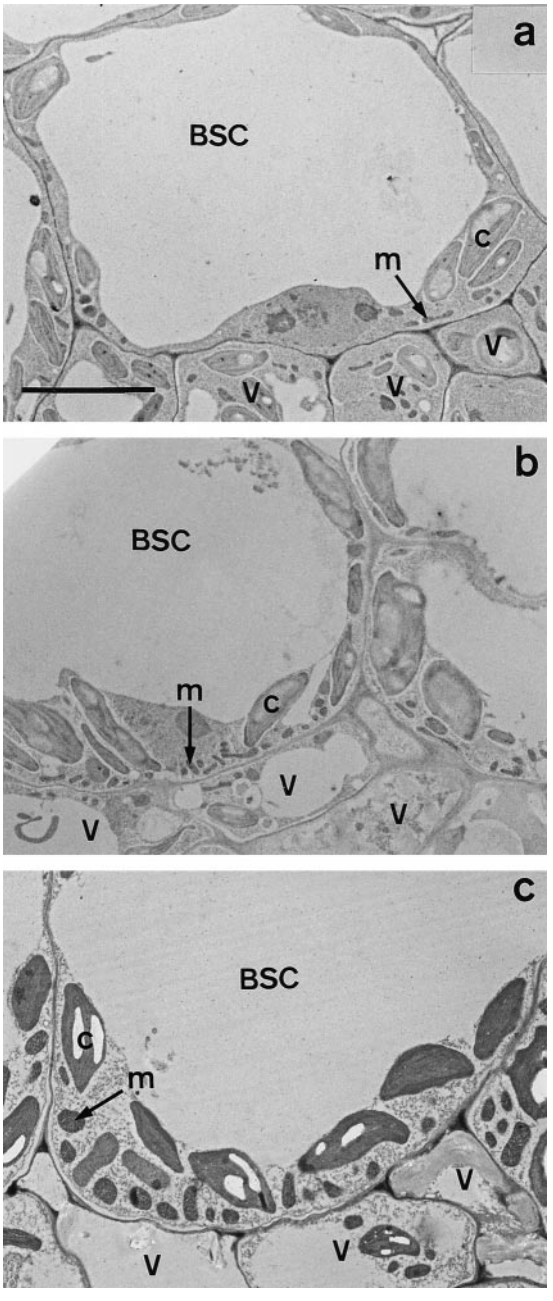

Figure 2. Bundle-sheath-cell development in leaves of *M. arvensis*. Sections were taken from the 6-mm (a) and 12-mm (b) stages and from mature leaves (c). BSC, Bundle-sheath cell; V, vascular cell; m, mitochondrion; c, chloroplast. Scale bar = 5 μm.

mitochondrial development in the bundle-sheath cells of these organs resulted in 17-, 10-, and 5-fold increases, respectively, in the total mitochondrial profile area per cell profile compared with that in the respective mesophyll cells (Fig. 5a). For leaves of *M. moricandioides* the total mitochondrial profile areas of the bundle-sheath and mesophyll cells did not differ (Fig. 5a). Stems, silique walls, petals, and cotyledons of developing embryos of *M. arvensis* did not have the characteristic C_3-C_4 intermediate anatomy. In these organs there were no clearly visible differences in mitochondrial profile area, numbers of mitochondria per bundle-sheath cell, or organelle localization compared with adjacent mesophyll cells (data not shown).

Immunogold localization studies of the GDC P-protein in sepals revealed that the pattern of labeling in the two cell types was very similar to that found in mature leaves of *M. arvensis* (Fig. 5b). The labeling density on the mitochondria in the bundle-sheath cells of sepals was 12-fold higher than on those in the mesophyll cells. The same cell-specific pattern of immunolabeling for GDC P-protein was seen in cotyledons after germination in the light (data not shown).

Effects of Light and CO_2 Concentration

To investigate the effect of light on the development of the C_3-C_4 intermediate character, cotyledons of seedlings and true leaves on young seedling plants were allowed to develop in the presence or absence of light. In both leaf types, the bundle-sheath-cell specificity of GDC P-protein expression was retained in the absence of light (data for mesophyll cells not shown), although the level of expression was reduced, as indicated by the lower labeling densities for the protein (Table I). The profile areas of individ-

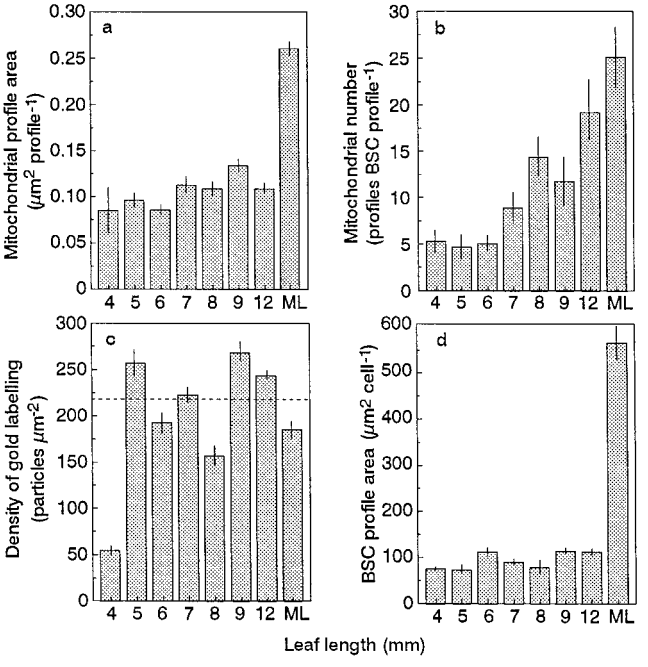

Figure 3. Developmental changes in mitochondrial and cellular parameters of bundle-sheath cells in *M. arvensis* leaves. Data were determined from sections taken from leaves with a length of 4 mm up to the mature-leaf (ML) stage. a, Mitochondrial profile area; b, number of mitochondrial profiles per bundle-sheath cell (BSC); c, immunogold labeling of bundle-sheath-cell mitochondria with antiserum to the P-protein of GDC at a dilution of 1:2000; the dashed horizontal line represents the average of all values from 5-mm to mature leaves; and (d) profile area of bundle-sheath cells. Measurements were taken from two leaves for each stage from a minimum of 20 mitochondrial and 10 bundle-sheath-cell profiles. Error bars represent ±SE. Throughout development in the mesophyll cells there were 5.4 ± 1.0 mitochondrial profiles per cell (b), each with a profile area of 0.07 ± 0.01 μm^2 (a).

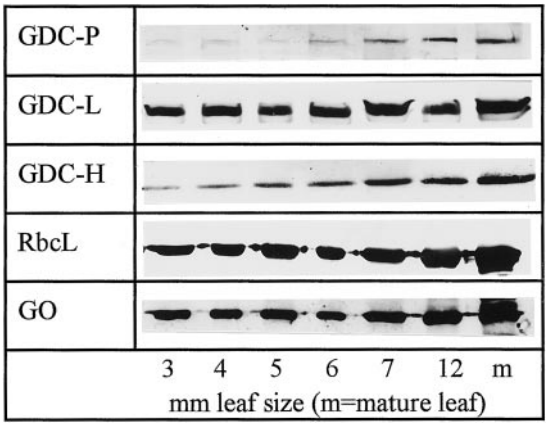

Figure 4. Expression of GDC P-, L-, and H-proteins and RbcL and GO during leaf development in *M. arvensis*. Total leaf proteins were extracted from leaves of between 3 mm and maturity. Proteins (10 μg per lane) were resolved by SDS-PAGE, electroblotted, and probed with monospecific antisera raised against the GDC P-, L-, and H-proteins, RbcL, and GO. Antibody dilutions were 1:200 for P, 1:1000 for L, H, and GO, and 1:2000 for RbcL. The P, L, and H profiles are from the same blot. The GO and RbcL profiles are from a second replicate blot.

ual bundle-sheath-cell mitochondria were reduced by 70% in the etiolated cotyledons and by 46% in the etiolated leaves (Table I). Furthermore, compared with light-grown leaves, the number of mitochondrial profiles per bundle-sheath-cell profile decreased by 64% in the etiolated cotyledons and by 86% in the etiolated leaves (Table I). The resulting total mitochondrial profile areas per bundle-sheath-cell profile were 0.53 μm^2 for etiolated true leaves and 0.50 μm^2 for etiolated cotyledons. These values were much smaller than those for the same organs when they developed in the light (7.1 and 4.7 μm^2, respectively). Despite these marked decreases in mitochondrial development of the bundle-sheath cells in the absence of light, the Kranz-like distribution of organelles was still visible (data not shown). Bundle-sheath-cell profile areas from dark-grown leaves and cotyledons were also smaller than those for the same organs when they developed in the light (by 38% and 72%, respectively; data not shown).

To determine whether photorespiratory metabolism influenced leaf development in *M. arvensis*, plants were grown under elevated CO_2 levels to suppress photorespiration. Compared with plants grown in a normal CO_2 atmosphere, elevated CO_2 did not lead to any differences in mitochondrial morphological parameters in the bundle-sheath cells (data not shown).

DISCUSSION

This study on early leaf development in *M. arvensis* reveals that the initiation of anatomical and biochemical development of the bundle sheath is clearly separated. This provides the first evidence, to our knowledge, that in a C_3-C_4 species these two components of the character are under different levels of control. The immunolocalization studies clearly reveal that the bundle-sheath-specific expression of GDC P-protein occurs before the organellar development of the bundle sheath. Moreover, the probing of western blots of whole-leaf proteins also reveals that the content of an active GDC in the leaves of *M. arvensis* (i.e. all four subunits present) is determined initially by an increase in the GDC P-content of mitochondria, and then by an increase in the number and size of the mitochondria. The latter point is clearly illustrated by the coincidence of the increase in GDC P-abundance in leaf extracts with the increase in mitochondrial development in the bundle sheath, both occurring from the 5-mm stage onward. Although developmental changes in cell anatomy must play a major role in determining GDC content in *M. arvensis*, it has been argued that for pea and wheat the increase in GDC is predominantly through the increase in GDC content of existing mitochondria (Rogers et al., 1991; Tobin and Rogers, 1992; Guinel and Ireland, 1996; Vauclare et al.,

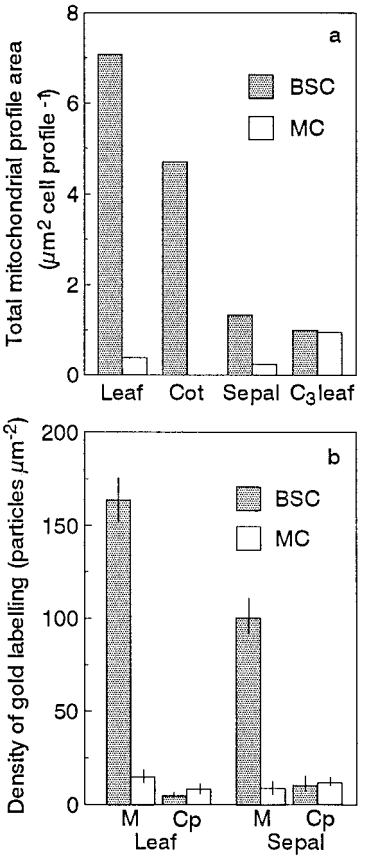

Figure 5. Mitochondrial parameters in bundle-sheath cells (BSC) and mesophyll cells (MC) of leaves, cotyledons (Cot), and sepals of *M. arvensis* and of leaves of the related C_3 species *M. moricandioides* (C_3 leaf). a, Total mitochondrial profile area. The small sample size for mesophyll cells from *M. arvensis* cotyledons precluded presentation for comparison, but based on our limited observations the value would be comparable with that for leaves and sepals. b, Immunogold labeling of mitochondria (M) for the GDC P-protein with the IgG fraction at a dilution of 1:100. We have previously shown that immunolabeling of the chloroplast (Cp) represents the background level (Morgan et al., 1993), and these data are given for each organ and cell type. Error bars represent ±SE.

Table I. *Mitochondrial parameters in bundle-sheath cells of light- and dark-grown leaves and cotyledons of M. arvensis*

The presence of GDC P-protein in mitochondria was determined by quantification of immunogold labeling after probing sections with the IgG fraction at a dilution of 1:100. Background labeling (i.e. that on chloroplasts; see Fig. 5 legend) was very similar for all organ/environment combinations and was an average of 58 ± 9 particles μm^{-2}. Mitochondrial profile areas and the number of profiles per cell were determined from at least 10 separate cell profiles. All values are means ± SE.

Parameter	Leaves		Cotyledons	
	Light	Dark	Light	Dark
	particles μm^{-2}			
GDC P-protein	385 ± 10	109 ± 12	397 ± 16	185 ± 12
	μm^2 *profile*$^{-1}$			
Mitochondrial profile area	0.28 ± 0.02	0.15 ± 0.02	0.39 ± 0.25	0.12 ± 0.09
	profiles cell^{-1}			
Mitochondrial no.	25.0 ± 2.1	3.5 ± 3.2	12.1 ± 1.8	4.4 ± 3.6

1996). How mitochondrial and cellular GDC content is regulated is not known precisely, but in pea the accumulation of the GDC complex can reach 40% of the soluble matrix protein (Oliver et al., 1990).

This and previous studies (Rogers et al., 1991; Tobin and Rogers, 1992; Guinel and Ireland, 1996; Vauclare et al., 1996) suggest a common mechanism for GDC expression whereby GDC proteins are imported into existing mitochondria in cells that are developing photosynthetically. However, in *M. arvensis* there are two important differences. First, there is a major increase in mitochondrial development that follows this initial phase, and it is this development that leads to a substantial increase in the GDC content and activity of the bundle-sheath cells. Second, the expression of GDC P-protein in the mesophyll cells is suppressed throughout cell development, despite the otherwise C_3-type photosynthetic development of these cells.

Considerable changes in mitochondrial proliferation occur as leaves of *M. arvensis* expand after cell division. This contrasts with wheat leaves, in which the mitochondrial volume per mesophyll cell remains unchanged after cell division and the number of mitochondrial profiles decreases during leaf cell expansion (i.e. from between 1.5 to 8 cm above the basal meristem of the primary leaf [Tobin and Rogers, 1992]). Therefore, there is a developmental increase in the mitochondrial size of the mesophyll of wheat leaves, which is proposed to arise from the fusion of mitochondria as the volume of the cytoplasmic compartment decreases (Tobin and Rogers, 1992). Cell-specific proliferation and expansion of mitochondria in bundle-sheath cells have been reported for the NAD-malic enzyme C_4 species *Panicum effusum* and *Atriplex rosea* (Dengler et al., 1986; Liu and Dengler, 1994); this allows an interesting parallel to be drawn with *M. arvensis*. In these three species metabolism in the leaf bundle-sheath cell is dependent on a high capacity for decarboxylation reactions that are localized in the mitochondria. In all cases the mitochondria undergo specialized development, and in mature leaves they are confined to the centripetal faces of the bundle-sheath cells. This suggests that these anatomical features are directly related to a common function.

This study has also revealed that in addition to cell-specific and perhaps chloroplast-dependent factors, there are also organ-specific factors involved in determining the expression of the C_3-C_4 intermediate phenotype. The anatomical and biochemical components of the character are coordinately expressed and are found only in true leaves and leaf-like organs of *M. arvensis*. The phenotype is not present in other tissues that are capable of photosynthesis and photorespiration. For example, stems of spinach have been shown to oxidize Gly (Gardeström et al., 1980) and silique walls and developing embryos of the closely related species *Brassica napus* are capable of photosynthesis (Whitfield, 1992; Eastmond et al., 1996). At present we can only speculate on the control of organ-specific expression of the C_3-C_4 intermediate phenotype. Leaves and perhaps cotyledons will make a major contribution to net carbon assimilation at different growth stages, but the sepals are small and are therefore much less likely to do so. The organ specificity of the C_3-C_4 intermediate phenotype in *M. arvensis* is therefore more likely to reside in the "leaf" identity of the organ. It is notable that the developing cotyledons of *M. arvensis* embryos did not have C_3-C_4 intermediate characteristics, whereas the seedling cotyledons did. The same observation was made for the C_4 characteristics in *Amaranthus hypochondriacus* cotyledons (Wang et al., 1993).

The data we have presented here show that light is required for the full expression of the C_3-C_4 phenotype in *M. arvensis*. Although in leaves of dark-grown plants the GDC P-protein content and total profile areas of bundle-sheath mitochondria were both reduced compared with leaves of light-grown plants, expression of the GDC P-protein remained cell specific and the bundle-sheath anatomy was still Kranz-like. Light is also required for full expression of C_4 development in *A. hypochondriacus* cotyledons (Wang et al., 1993), whereas in maize leaves it has been shown to directly determine the C_4-type, cell-specific expression patterns (Langdale et al., 1988b). Fladung and Hesselbach (1987) have reported that there is little change in the internal leaf anatomy of the monocotyledonous C_3-C_4 intermediate species *Panicum milioides* before or after emergence from the sheath. From our observations of light and temporal influences on the development of the C_3-C_4

intermediate phenotype in *M. arvensis*, the leaves of *P. milioides* should be reexamined by studying cell anatomy and GDC expression at stages earlier than those assessed by Fladung and Hesselbach (1987). Recent preliminary studies (Rylott, 1997) have revealed clear differences in bundle-sheath-cell development between the basal and mid-leaf sections of primary leaves of *P. milioides*.

The positive role of light in enhancing the expression of the GDC proteins and their mRNAs in C_3 species is well documented (e.g. Arron and Edwards, 1980; Walker and Oliver, 1986; Rogers et al., 1991; Turner et al., 1993). More recently, Vauclare et al. (1996) and Guinel and Ireland (1996) have reported that the GDC content and activity of leaf mitochondria increase on emergence of developing pea leaflets into direct light from between the stipules. Vauclare et al. (1996) argue that this increase in GDC content coincides with direct exposure to light rather than with exposure per se. In *M. arvensis* leaves the GDC P-protein content of the mitochondria increases up to the 5-mm stage, which does not correlate with direct exposure to light. These small leaves remain protected from direct light exposure by other leaves at the shoot apex until about the 6- to 7-mm stage. Furthermore, leaves of 2 to 3 mm contain the GDC P-protein and yet they have not fully initiated light-dependent development because they are achlorophyllous.

There may be factors other than light and organ specificity that control the development of leaf mitochondria. Recent studies have shown that Gly induces the expression of the P-protein and GDC activity in the yeast *Saccharomyces cerevisiae* (Sinclair et al., 1996). The Gly content of leaves is increased markedly by photorespiration (Rawsthorne and Hylton, 1991), and it is postulated that this is the metabolite that moves across from the mesophyll to the bundle-sheath cells in the mature *M. arvensis* leaf during C_3-C_4 intermediate photosynthesis (Rawsthorne et al., 1988a). An increasing Gly content caused by initiation of photorespiratory metabolism in the leaf might therefore be a signal for the development of mitochondria in the bundle sheath. Although we have not determined leaf Gly content directly, the growth of leaves in elevated CO_2 would have suppressed the production of Gly by photorespiration. Growth under these nonphotorespiratory conditions did not lead to any change in the C_3-C_4 phenotype of the mature leaves, and a "signaling" role for Gly, or another metabolite in photorespiration, in C_3-C_4 leaf development therefore seems unlikely.

The temporal separation of biochemical and anatomical events during the specialized leaf development of *M. arvensis* is similar to that seen for the dicotyledonous C_4 plant *A. rosea* (Liu and Dengler, 1994; Dengler et al., 1995). In *A. rosea* the cell-specific expression of Rubisco and PEP carboxylase proteins typical of C_4 species is established before the anatomical and cell-specific specialization. The patterns seen for *A. rosea* and *M. arvensis* contrast with that for another dicotyledonous C_4 plant, *A. hypochondriacus* (Wang et al., 1992). During early leaf development of the latter C_4 species, the anatomical specialization is evident and yet the initial pattern of expression of Rubisco is C_3-like, with mRNA and protein present in both bundle-sheath and mesophyll cells. The C_4-type, cell-specific expression patterns for Rubisco and PEP carboxylase are established later. Notably, the expression of GDC P-protein in *M. arvensis* leaves is always C_3-C_4-like, and we did not see expression in the mesophyll cells at any stage of leaf development. In the monocotyledonous C_4 species maize, the cell-specific expression of Rubisco mRNAs in the bundle-sheath cells precedes their anatomical development (Martineau and Taylor, 1986; Langdale et al., 1988a). However, accumulation of Rubisco protein and anatomical development in maize are broadly coordinated (Langdale et al., 1988a).

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