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The Expression of 2-Oxoglutarate/Malate Translocator in the Bundle-Sheath Mitochondria of *Panicum miliaceum*, a NAD-Malic Enzyme-Type C_4 Plant, Is Regulated by Light and Development[1]

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The bundle-sheath mitochondria in NAD-malic enzyme-type C_4 plants participate in the C_4 dicarboxylate cycle and require high capacities of translocators to accommodate the high rates of exchange of metabolites involved in photosynthesis. In *Panicum miliaceum*, a NAD-malic enzyme-type C_4 plant, the steady-state level of mRNA for the mitochondrial 2-oxoglutarate (2-OG)/malate translocator was higher in leaves than in nonphotosynthetic tissues. Furthermore, the expression of the gene for the mitochondrial 2-OG/malate translocator was restricted to bundle-sheath cells (BSC) but not mesophyll cells. The transcript level of the BSC-located mitochondrial 2-OG/malate translocator increased during greening in accordance with levels of photosynthetic genes, although the relative transcript levels of other mitochondrial membrane proteins decreased. The specific activities of C_4 photosynthetic enzymes and the relative abundance of the 2-OG/malate translocator protein in bundle-sheath mitochondria increased in successive sections from the basal meristem to the distal tip, whereas the specific activities of mitochondrial respiratory enzymes remained constant or decreased. These findings indicate that the specific 2-OG/malate translocator in BSC mitochondria of *P. miliaceum* is expressed in concert with C_4 enzymes during the differentiation of BSC and parallels the capacity of C_4 photosynthesis. Most unusual, northern analysis showed that significant amounts of unspliced mRNAs, the levels of which are variable during greening, were present in leaf tissues. It is possible that this incomplete splicing is involved in posttranscriptional regulation of expression of this gene.

C_4 plants possess two photosynthetic cells, MC and BSC. Through the coordination of the two photosynthetic cells, the C_4 dicarboxylate cycle acts as a CO_2 pump to concentrate CO_2 in BSC. In NAD-ME-type C_4 plants aspartate is transported from MC to bundle-sheath mitochondria, where it is transaminated to oxaloacetate by mitochondrial aspartate aminotransferase (Fig. 1). Oxaloacetate is further reduced to malate, which is then decarboxylated to pyruvate by NAD-ME in the bundle-sheath mitochondria. The CO_2 released is refixed by RuBPCase localized in the bundle-sheath chloroplasts. The decarboxylation product, pyruvate, is transported to cytosol, transaminated to Ala, and transported back to MC (for review, see Edwards and Walker, 1983; Hatch, 1988). Therefore, the bundle-sheath mitochondria in this C_4 subgroup are functionally differentiated into photosynthetic organelles containing enzymes that operate in the C_4 dicarboxylate cycle.

The bundle-sheath mitochondria in NAD-ME-type species have much higher activities of enzymes involved in the C_4 pathway (e.g. aspartate aminotransferase and NAD-ME) compared with their counterpart activities in the other types of C_4 plants (Hatch et al., 1975). Moreover, the bundle-sheath mitochondria contain photorespiratory enzymes such as Ser hydroxymethyltransferase and Gly decarboxylase complex, which are absent in mesophyll mitochondria (Ohnishi and Kanai, 1983). By contrast, the activities of respiratory enzymes (Cyt *c* oxidase, fumarate hydratase, and citrate synthase) in the bundle-sheath mitochondria are comparable in the different subgroups of C_4 plants and in C_3 plants. These observations indicate that only enzymes involved in photosynthesis are differentially accumulated in the bundle-sheath mitochondria of NAD-ME-type species during cell differentiation. Consistent with its role in C_4 photosynthesis, mitochondria in BSC of NAD-ME-type species are much more abundant and develop more cristae membrane structures than those in MC and in other types of C_4 plants (Hatch et al., 1975).

In addition, it has been reported that the number of bundle-sheath mitochondria per cell increased significantly during the developmental divergence of MC and BSC, whereas the number of mitochondria in MC changed less dramatically (Dengler et al., 1986). The enlargement of the surface area of the bundle-sheath mitochondria in NAD-ME-type C_4 plants is thought to facilitate the large metabolite fluxes across mitochondrial membranes (Hatch et al., 1975). Several mitochondrial translocators are thought to be involved in the C_4 photosynthetic cycle to accommodate the high rates of exchange of metabolites across mitochon-

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Abbreviations: BSC, bundle-sheath cell; MC, mesophyll cell; ME, malic enzyme; 2-OG, 2-oxoglutarate; PEPC, PEP carboxylase; PPDK, pyruvate,orthophosphate dikinase; RuBPCase, ribulose-1,5-bisphosphate carboxylase.

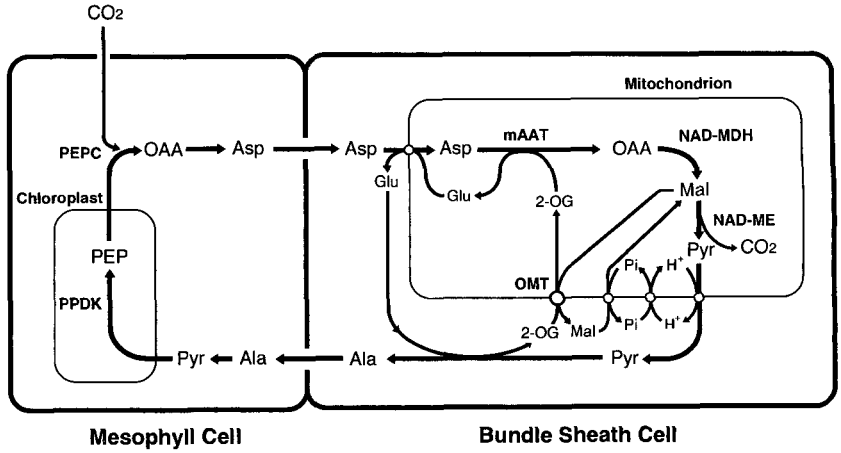

Figure 1. A scheme showing the C_4 dicarboxylate cycle and the proposed metabolite transport processes across bundle-sheath mitochondrial membrane in NAD-ME-type C_4 plants. Aspartate moved from MC is imported into bundle-sheath mitochondria via an Asp/Glu translocator. Glu exported to the cytosol is transaminated to 2-OG and imported back to mitochondria by 2-OG/malate translocator. 2-OG is further transaminated to Glu. (From Hatch and Carnal, 1992.) mAAT, Mitochondrial aspartate aminotransferase; Mal, malate; MDH, malate dehydrogenase; OAA, oxaloacetate; OMT, 2-OG/malate translocator; Pyr, pyruvate.

drial membranes. It is thus assumed that an Asp/Glu translocator, a pyruvate translocator, and a 2-OG/malate translocator function in concert with the C_4 dicarboxylate cycle in the bundle-sheath mitochondria of NAD-ME-type C_4 plants (Fig. 1) (Furbank et al., 1990). In this proposed scheme, Glu/2-OG shuttle across the mitochondrial membrane via the Asp/Glu translocator and the 2-OG/malate translocator is linked to the C_4 dicarboxylate cycle. With regard to these translocators, several questions have been raised: Are these translocators functionally differentiated from those in MC and other types of C_4 plants? Is the C_4 photosynthetic capacity regulated by these translocators? Are the expression of these genes co-regulated with other C_4 photosynthetic genes?

Recently, we cloned cDNAs that encode 32-kD proteins localized on mitochondrial membranes from leaves of *Panicum miliaceum* L. (proso millet), a NAD-ME-type C_4 plant (Taniguchi and Sugiyama, 1996). The predicted amino acid sequence showed a high similarity to that of 2-OG/malate translocator from mammalian mitochondria (Runswick et al., 1990), but no homology to that of the chloroplastic 2-OG/malate translocator from spinach (Weber et al., 1995). The hydrophobic profile of the amino acid sequence predicts the existence of six transmembrane α helices that are a common property of members of the mitochondrial transporter family. However, the profile is quite different from the 12-helix motif of the chloroplastic 2-OG/malate translocator. Moreover, the recombinant protein derived from the isolated cDNA efficiently transported malate and 2-OG in a reconstituted liposome system (Taniguchi and Sugiyama, 1996). These findings led us to conclude that the putative cDNAs encode specific mitochondrial 2-OG/malate translocators.

The goal of our study on this group of C_4 plants is to understand the molecular mechanism of photosynthetic differentiation of the bundle-sheath mitochondria relative to their metabolite translocators. This study was undertaken with the intent of examining the correlation between mitochondrial development and expression of mitochondrial 2-OG/malate translocator using the cDNA clone and specific antibodies. cDNA clones encoding mitochondrial 2-OG/malate translocator have also been isolated from human (Iacobazzi et al., 1992), bovine (Runswick et al., 1990), rat (Dolce et al., 1994), and nematode cells (Runswick et al., 1994). Chloroplastic 2-OG/malate translocator has been cloned only from spinach (Weber et al., 1995). However, the pattern and regulatory mechanism of gene expression for these translocators have not been examined so far. The results in the present study show that the gene for the mitochondrial 2-OG/malate translocator expresses preferentially in BSC in a light-dependent and developmentally regulated manner. Moreover, it was found that significant amounts of unspliced mRNAs, the levels of which are variable during greening, were present in leaf tissues.

MATERIALS AND METHODS

Panicum miliaceum was grown in vermiculite in a growth chamber for 10 d with 14 h of illumination (35,000 lux) at 28°C and 10 h of darkness at 20°C. In the greening experiment, the seeds were grown in the dark at 23°C for 7 d and then exposed to continuous fluorescent light at an intensity of about 9,000 lux at 26°C for 19.5 h.

RNA Isolation

Total RNA was prepared from roots, mesocotyls, and leaves of 10-d-old plants by the guanidine thiocyanate procedure (McGookin, 1984). Total RNA from MC and BSC were isolated by the procedure of Nelson (1994). MC protoplasts were isolated by digestion of 10-d-old second green leaves (Ohnishi and Kanai, 1983). BSC strands were mechanically isolated with a Polytron homogenizer. During both isolation procedures, 1 mM aurintricarboxylic acid was added as an inhibitor of nucleases (Hallick et al., 1977). Poly(A)$^+$ RNA was purified with Oligotex-dT30 (Takara, Otsu, Shiga, Japan).

Measurement of RNA

For analysis of mRNA accumulation in various tissues with northern blotting, total RNA or poly(A)$^+$ RNA samples were denatured in formaldehyde, subjected to electrophoresis on a 1.2% agarose gel containing formaldehyde

(Sambrook et al., 1989), and blotted on a nylon membrane (Hybond-N^+, Amersham). In the case of dot-blot analysis, 10 μg of total RNA was dot-blotted on the nylon membrane. After alkaline fixation the membranes were hybridized with ^{32}P-labeled probes. The probe was labeled with [α-^{32}P]dCTP using the Multiprime DNA Labeling System (Amersham). The full-size insert or the 3′ untranslated region (nucleotides 1132–1458) of pOMT134 (Taniguchi and Sugiyama, 1996) was used to detect transcripts for the 2-OG/malate translocator. For detection of unspliced mRNA of the translocator, the *Bam*HI-*Sal*I fragment (nucleotides 495–870) and the *Ssp*I-*Bgl*II fragment (nucleotides 1325–1771) from pOMT103 were used as specific probes for the second and third insertions, respectively (see Fig. 7A). It was confirmed that the probes for detecting the insertions hybridized specifically only with the corresponding regions (data not shown). Other cDNAs used as probes were a full-size insert of maize pZmSSu1025 for the small subunit of RuBPCase (Matsuoka et al., 1987), a 1.7-kb *Pst*I fragment of maize pZmPPDK1062 for PPDK (Matsuoka et al., 1988), a full-size insert of *P. miliaceum* pmAAT3 for mitochondrial aspartate aminotransferase (Taniguchi et al., 1992), a 3.7-kb *Eco*RI fragment of *Vicia faba* VER17 for 25S rRNA (Yukura and Tanifuji, 1983), and a full-size insert of *P. miliaceum* ppAAT1 for plastidic aspartate aminotransferase (Taniguchi et al., 1995). A probe for mitochondrial adenylate translocator is a cDNA fragment from a rice clone (accession no. D10430) isolated by the Rice Genome Research Program of the National Institute of Agrobiological Resources in Tsukuba, Japan. A probe for the ATP synthase β-subunit is a cDNA fragment from a maize clone (accession no. T18684) isolated by the Maize Restriction Fragment Length Polymorphism Laboratory at the University of Missouri, Columbia. Hybridization and washing were performed as previously described (Taniguchi et al., 1992). The hybridization signal was scanned by a bioimaging analyzer (BAS 2000, Fujix, Tokyo, Japan).

Isolation of Crude Total Membranes from MC and BSC

MC protoplasts and BSC strands were isolated from 11-d-old second green leaves by enzymatic digestion (Ohnishi and Kanai, 1983). The cross-contamination in the preparations was less than 1%, as estimated by the activities of PEPC and NAD-ME, the respective marker enzymes for MC and BSC. The isolated cells were macerated in 50 mM Hepes-KOH (pH 7.2) and 1 mM PMSF, and the homogenates were sonicated (Branson [Danbury, CT] sonifier model 250: microtip, 30%; line voltage, 30%; duty cycle, 50 s) and centrifuged at 600*g* for 10 min. The supernatant containing mitochondrial membranes was collected and the activity of Cyt *c* oxidase was assayed.

Preparation of BSC Proteins from Successive Leaf Sections

The following isolation procedure for BSC strands was carried out at 4°C. The 13-d-old second green leaves (7–8 cm long) were cut into six sections: below 1 cm; 1 to 2 cm; 2 to 3 cm; 3 to 4 cm; 4 to 5.5 cm; and above 5.5 cm. Each section was sliced with a razor blade and blended twice in a precooled blending medium (0.35 M sorbitol, 25 mM Hepes-KOH [pH 7.5], 2 mM $MgCl_2$, and 2 mM potassium phosphate) with a polytron homogenizer for 30 s at speed 10. The homogenate was then filtrated through two layers of Miracloth (Calbiochem) and washed with the blending medium. The same blending procedure was repeated, and the residual BSC strands on Miracloth were recovered. For preparation of soluble proteins, the BSC strands were ground in a grinding medium (50 mM Hepes-KOH [pH 7.5], 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF) with a mortar and pestle. The homogenate was centrifuged at 12,000*g* for 10 min, and the supernatant was used as soluble protein. For preparation of membrane proteins, the BSC strands were ground in a grinding medium containing 50 mM Hepes-KOH (pH 7.2) and 1 mM PMSF. The homogenate was filtered through two layers of Miracloth and the filtrate was centrifuged at 117,000*g* for 1 h. The pellets were suspended in 50 mM Hepes-KOH (pH 7.2) and used as membrane protein.

Western Analysis of 2-OG/Malate Translocator Protein

Protein was precipitated by adding TCA to a final concentration of 10% (w/v) to the samples, kept on ice for 25 min, and then centrifuged at 13,000*g* for 10 min. The pellets were resuspended in 50 μL of urea solution (9 M urea, 2% [w/v] Triton X-100, and 5% [v/v] 2-mercaptoethanol), and sonicated until the pellets were totally redissolved. Next, 12.5 μL of 10% (w/v) lithium dodecylsulfate and 0.1% (w/v) bromphenol blue were added, and the resulting solution was neutralized using 2 M Tris. The protein samples were subjected to SDS-PAGE on a 12.5% gel (Laemmli, 1970), and western analysis was carried out (Towbin et al., 1979) using affinity-purified antibodies raised against the 2-OG/malate translocator (Taniguchi and Sugiyama, 1996). Antibody binding was detected with anti-mouse IgG conjugated with alkaline phosphatase (Bio-Rad). In semiquantitative analysis for 2-OG/malate translocator protein, the serially diluted protein samples were also applied to the same gel and intensities of developed bands were measured by an ImageMaster 2-D System (Pharmacia). Judging from the linearity of the intensities of the diluted sample's bands, the intensities of the developed bands in the original samples were confirmed to be within the limited linear response range with the blots.

Other Procedures

Enzymes were assayed spectrophotometrically according to published methods: citrate synthase (Stitt, 1984), Cyt *c* oxidase (Yonetani, 1967), NAD-ME (Hatch et al., 1982), PEPC (Hatch and Oliver, 1978), and Ser hydroxymethyltransferase (Taylor and Weissbach, 1965). The assay for NAD-malate dehydrogenase contained 50 mM Hepes-KOH (pH 8.0), 2.5 mM EDTA, 5 mM DTT, 0.1 mM NADH, 0.05% (w/v) Triton X-100, and 2 mM oxaloacetate. The activity of mitochondrial aspartate aminotransferase was measured by densitometric scanning of activity-stained bands on native PAGE (Taniguchi et al., 1995). Protein concentration

was determined by the method of Bradford (1976) with BSA as a standard.

RESULTS

Cell-Specific Expression of the 2-OG/Malate Translocator Gene

We have investigated tissue and cell distribution of mRNA encoding our cloned mitochondrial 2-OG/malate translocator to evaluate its physiological function. Total RNA was isolated from various tissues (roots, mesocotyls, and leaves of etiolated, greening, and green seedlings) of *P. miliaceum* and analyzed by northern hybridization. As shown in Figure 2A, a high level of approximately 1.6-kb transcripts of 2-OG/malate translocator was detected in greening and green leaves, and a somewhat lower expression level was found in etiolated leaves. The level of the 2-OG/malate translocator mRNA observed in the mesocotyl was significantly lower than that in the leaves, and in root tissues, a very weak signal was detected.

The accumulation of mRNA for the 2-OG/malate translocator between the two types of C_4 photosynthetic cells was examined (Fig. 3). Total RNA was prepared from MC protoplasts and mechanically isolated BSC strands of green leaves. The transcript of PPDK was detectable only in MC, and that of the small subunit of RuBPCase was preferentially associated with BSC. These findings were consistent with the results from northern analysis with maize (Sheen and Bogorad, 1987) and, therefore, the prepared RNA samples had little cross-contamination. The mRNA for the 2-OG/malate translocator was detected in both cell types when a full-size insert of the cDNA encoding the 2-OG/malate translocator was used as a probe. However, a cDNA fragment from the 3′ untranslated region hybridized only with the BSC RNA. Moreover, intercellular localization of the 2-OG/malate translocator protein was ascertained by western analysis (Fig. 4). Antibody raised against *P. miliaceum* 2-OG/malate translocator cross-reacted with a 32-kD protein from the crude mitochondrial membrane fraction extracted from BSC but not from MC. These findings indicate that the gene we isolated for the mitochondrial 2-OG/malate translocator is specifically expressed in BSC.

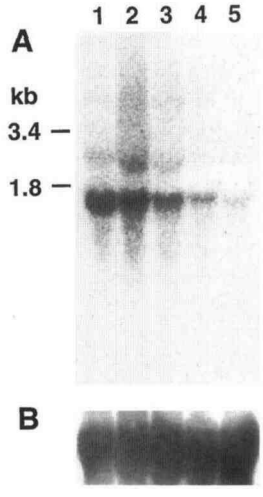

Figure 2. Distribution of the mitochondrial 2-OG/malate translocator mRNAs in different tissues of *P. miliaceum*. A, Thirty micrograms of total RNAs from green leaves (lane 1), greening leaves (lane 2), etiolated leaves (lane 3), mesocotyls (lane 4), and roots (lane 5) was electrophoresed, transferred to a nylon membrane, and hybridized with a full-sized cDNA insert of pOMT134 encoding *P. miliaceum* 2-OG/malate translocator. B, The blot was rehybridized with *V. faba* 25S rDNA probe.

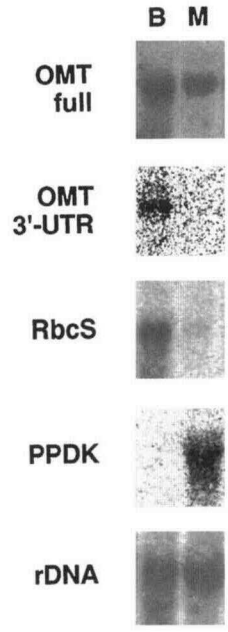

Figure 3. Intercellular distribution of the mitochondrial 2-OG/malate translocator mRNAs between BSC and MC. Twenty micrograms of total RNAs, extracted from separated bundle-sheath strands (lane B) and mesophyll protoplasts (lane M) of green leaves, was analyzed by northern analysis and hybridization with ^{32}P-labeled cDNA probes. A full-size insert (OMT full) or 3′ untranslated region (OMT 3′-UTR) of pOMT134 (see Fig. 6A), small subunit of RuBPCase (RbcS), PPDK, and rDNA were analyzed.

Light-Dependent Expression of the 2-OG/Malate Translocator

When dark-grown plants are transferred to light, the expression of many photosynthetic genes is known to be coordinately induced. If the mitochondrial 2-OG/malate translocator participates in the C_4 dicarboxylate cycle, it may be induced during greening to accommodate the high rates of transport of metabolites. Figure 5 shows changes of mRNA levels for various photosynthetic enzymes and mitochondrial membrane proteins during greening of the etiolated *P. miliaceum* seedlings. Dot-blot analysis using the 3′ untranslated region of the 2-OG/malate translocator cDNA as a probe showed that the transcript level for the translocator in bundle-sheath mitochondria rapidly increased with periods of illumination shorter than 24 h. The increase paralleled those of the small subunit of RuBPCase and mitochondrial aspartate aminotransferase. The small subunit of RuBPCase is well known for its responsiveness to light. The mitochondrial aspartate aminotransferase functions in the C_4 photosynthetic pathway in this group of

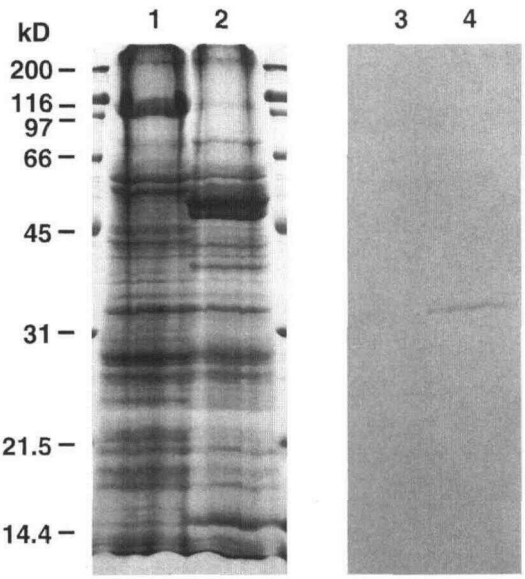

Figure 4. Intercellular distribution of the mitochondrial 2-OG/malate translocator protein between MC and BSC. Crude total membrane proteins from MC (lanes 1 and 3) and BSC (lanes 2 and 4) were isolated and subjected to SDS-PAGE and analyzed by Coomassie brilliant blue R-250 staining (lanes 1 and 2) and western analysis with the affinity-purified antibodies against *P. miliaceum* 2-OG/malate translocator (lanes 3 and 4). Each lane contains equal Cyt *c* oxidase activity (20 milliunits).

plants is also known to be induced by light (Taniguchi et al., 1995). In contrast, the levels of other mitochondrial membrane proteins, such as adenylate translocator and the β subunit of ATP synthase, decreased during greening.

Expression of the 2-OG/Malate Translocator during Cell Maturation

The monocotyledonous leaf contains a linear gradient of cellular development and differentiation between leaf base and tip. To investigate the change in expression of the 2-OG/malate translocator during leaf development, specific activities of mitochondrial enzymes and the amount of the 2-OG/malate translocator protein in BSC were measured from successive sections of the second green leaves (Fig. 6). The specific activities of mitochondrial aspartate aminotransferase, NAD-ME, and NAD-malate dehydrogenase, which function in the C_4 dicarboxylate cycle in bundle-sheath mitochondria, were relatively high at the leaf base and showed gradual increases in activity toward a maximum at the tip (Fig. 6A). These findings indicate that photosynthetic capacity develops in accordance with the maturation of BSC. In contrast, the activity level of the photorespiratory enzyme Ser hydroxymethyltransferase remained constant along the leaf blade. In the case of citrate synthase, a matrix enzyme functioning in tricarboxylate cycle, its specific activity decreased progressively toward the tip. Figure 6B shows the changes in membrane proteins of BSC mitochondria. Although the specific activity of Cyt *c* oxidase remained relatively constant, the relative abundance of the 2-OG/malate translocator protein in BSC mitochondria rose progressively, to reach a maximum in the distal section.

Accumulation of Unspliced mRNA in Seedlings

In a previous study we reported that some of the isolated cDNAs encoding the 2-OG/malate translocator contain insertional sequences (Taniguchi and Sugiyama, 1996). These insertions seem to be introns, since the sequences surrounding the 5′ and 3′ boundaries of the insertional sequences are similar to the consensus sequences of the 5′ and 3′ intron boundaries in monocotyledonous plants and the insertional sequences contain in-frame stop codons (Taniguchi and Sugiyama, 1996). A cDNA clone, pOMT103, contains the second and third insertions in the coding region and a pOMT134 clone contains the first insertions in the 5′ noncoding region (Fig. 7A). It is likely that these cDNA clones were derived from incompletely spliced transcripts in *P. miliaceum* leaves and, therefore, a considerable amount of the unspliced transcripts may accumulate in vivo. Poly(A)$^+$ RNA was prepared from green, greening, and etiolated leaves, and northern analysis was conducted. To indicate that constant amounts of RNA had been applied, the blot was hybridized with a cDNA insert encoding plastidic aspartate aminotransferase as a probe. Taniguchi et al. (1995) previously reported that the mRNA level for plastidic aspartate aminotransferase in *P. mili-*

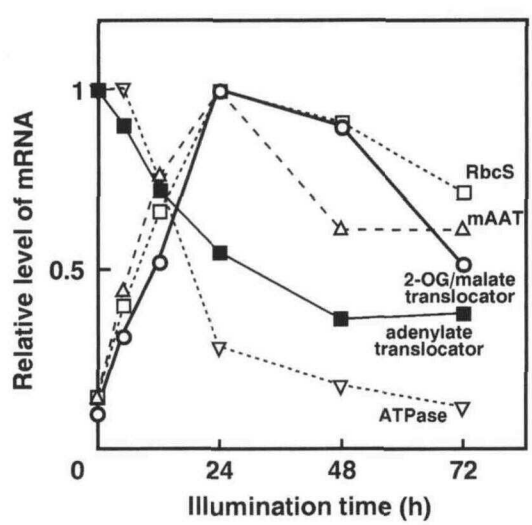

Figure 5. Changes of mRNA levels for photosynthetic enzymes and mitochondrial membrane proteins during the greening of etiolated *P. miliaceum* seedlings. *P. miliaceum* seedlings, grown in the dark for 7 d, were illuminated with continuous white light (9000 lux), and leaf tissues were harvested at the times indicated. Total RNA was extracted and 10 μg was dot-blotted on a nylon membrane and probed with the gene probes for 2-OG/malate translocator (3′ untranslated region of pOMT134, ○), small subunit of RuBPCase (RbcS, □), mitochondrial aspartate aminotransferase (mAAT, △), adenylate translocator (■), and ATP synthase β-subunit (ATPase, ▽). The mRNA level of each protein was estimated with a bioimaging analyzer. The blot was rehybridized with a *V. faba* 25S rDNA probe, and hybridized radioactivities were also quantified. To standardize the amount of RNA blotted to the membrane, the hybridized radioactivity of each protein was corrected with that of rRNA.

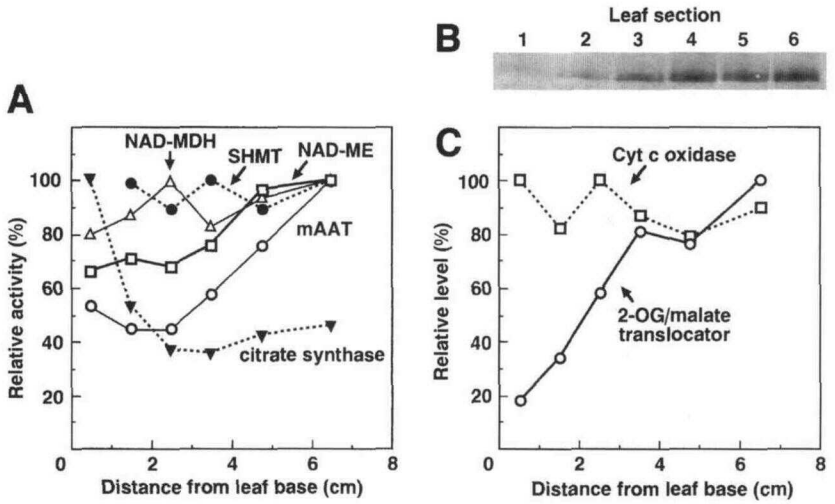

Figure 6. Changes in activities of mitochondrial enzymes and protein amount of mitochondrial 2-OG/malate translocator during development of *P. miliaceum* BSC. BSC strands were prepared from six sections (section 1, below 1 cm; section 2, 1–2 cm; section 3, 2–3 cm; section 4, 3–4 cm; section 5, 4–5.5 cm; section 6, above 5.5 cm) of the second green leaves, and soluble and membrane proteins were extracted. A, The enzyme activities of soluble proteins (mitochondrial aspartate aminotransferase [mAAT], ○; NAD-ME, □; NAD-malate dehydrogenase [NAD-MDH], △; citrate synthase, ▼; and Ser hydroxymethyltransferase [SHMT], ●) were measured in each fraction. The 100% activities (μmol min^{-1} mg^{-1} of soluble protein) were 0.88 (NAD-ME), 8.1 (NAD-malate dehydrogenase), 0.040 (citrate synthase), and 0.0052 (Ser hydroxymethyltransferase). B, Twenty micrograms of BSC membrane proteins was subjected to SDS-PAGE and western-blotted with antibodies against mitochondrial 2-OG/malate translocator. C, Activity of Cyt *c* oxidase (□) was measured in each membrane fraction. The 100% activity of Cyt *c* oxidase is 0.15 μmol min^{-1} mg^{-1} membrane protein. Relative protein amounts of 2-OG/malate translocator (○) were quantified from the western blot shown in B within the limited linear response range. The quantification procedure is detailed in "Materials and Methods."

aceum leaves is generally constant during greening. As shown in Figure 7B, there was no significant difference among the three RNA preparations and, therefore, we conclude that approximately the same quantity of RNA was applied to the agarose gel. Northern analysis using the full-size inserts of pOMT134 and pOMT103 as probes revealed the presence of two larger transcripts of approximately 2.2 and 2.5 kb above a main 1.6-kb transcript. Hybridization with a subfragment of the pOMT103 specific for the second insertion sequence as a probe showed the presence of the 2.2- and 2.5-kb RNA bands but not the main 1.6-kb band. By contrast, using a subfragment of pOMT103 specific for the third insertion sequence, only the 2.5-kb band was detected. These findings suggest that the 2.5-kb RNA contains both the second and third insertion sequences, whereas the 2.2-kb RNA contains only the second insertion sequence, and that these larger transcripts are unspliced mRNAs. Furthermore, the levels of the larger transcripts were variable during the greening of leaves. The 2.5-kb RNA was more abundant in green and greening leaves than in etiolated leaves, but the 2.2-kb RNA was more abundant in etiolated leaves.

DISCUSSION

In *P. miliaceum* the steady-state level of mRNA for the mitochondrial 2-OG/malate translocator is higher in leaves than in nonphotosynthetic tissues (Fig. 2). When the full-size cDNA fragment of the mitochondrial 2-OG/malate translocator was used as a probe, an approximately 1.6-kb transcript was detected in MC and BSC fractions (Fig. 3). The results suggest that a general mitochondrial 2-OG/malate translocator expresses not only in BSC but also in MC. However, when the 3′ noncoding sequence of the gene was used as a probe, only the transcript from the BSC fraction was detected. This result suggests the existence of a BSC-specific mitochondrial 2-OG/malate translocator that may be involved in the C_4 pathway of photosynthesis in this subgroup. The C_4-type isozymes that accumulate in a cell-specific manner and function in the C_4 pathway have also been found with PEPC (Chollet et al., 1996) and NAD-ME (Long et al., 1994). Apparently, the two types of 2-OG/malate translocator share some homology in the coding region, and the conditions used for hybridization allowed the detection of the general translocator in both cell types. By using the 3′ untranslated cDNA sequence as a probe and specific antibody for the mitochondrial 2-OG/malate translocator localized in BSC (Fig. 4), we examined the expression pattern of the translocator during functional differentiation of bundle-sheath mitochondria to photosynthetic organelles. The accumulation of mRNA for the BSC-located mitochondrial 2-OG/malate translocator is light-inducible in a manner similar to that of genes for photosynthetic carbon-assimilation enzymes (Fig. 5). In contrast, the relative mRNA levels for nucleus-encoded mitochondrial membrane proteins, such as adenylate translocator and the β-subunit of ATP synthase, de-

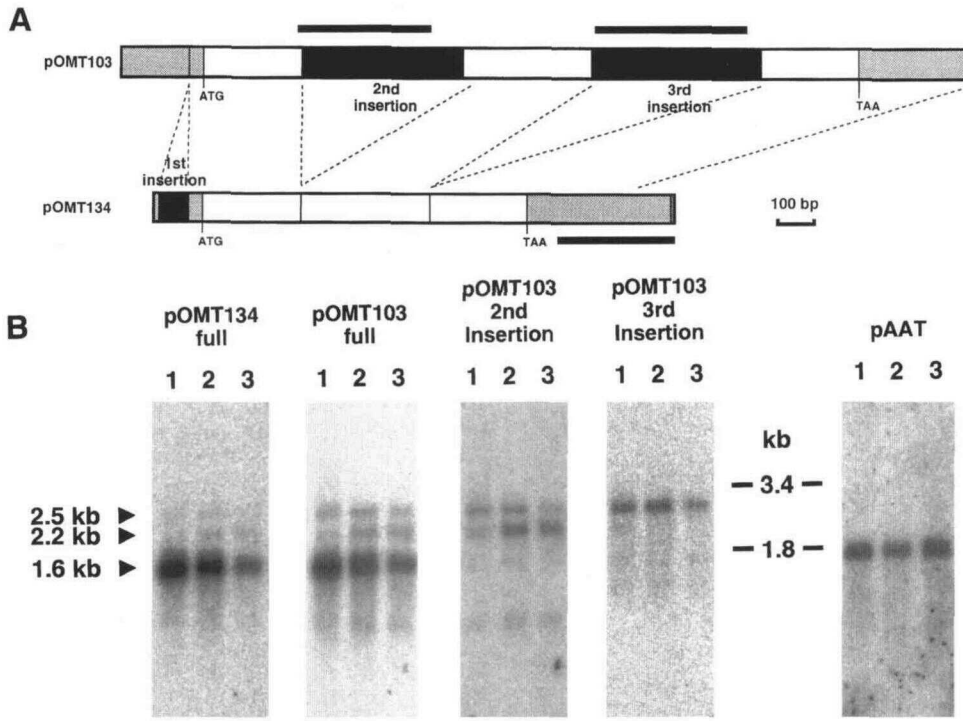

Figure 7. Accumulation of unspliced mRNA for the mitochondrial 2-OG/malate translocator. A, Schematic representation of the structure of cDNAs encoding *P. miliaceum* 2-OG/malate translocator. Black boxes represent the insertional sequences that seem to be introns. Open and shaded regions indicate coding and noncoding regions, respectively. Solid lines above or under the clones denote cDNA fragments of the second and third insertions in pOMT103 and the 3′ noncoding region in pOMT134 used as specific probes for northern analysis. B, Northern analysis of unspliced mRNA. Three micrograms of poly(A)$^+$ RNA prepared from green leaves (lanes 1), greening leaves (lanes 2), and etiolated leaves (lanes 3) was analyzed by northern blotting and hybridization with the full-size cDNA inserts of pOMT134 and pOMT103, the second and third insertion regions of pOMT103 clones, and the full-size cDNA insert of *P. miliaceum* plastidic aspartate aminotransferase (pAAT).

creased during greening. It appears that RNAs for photosynthetic proteins drastically accumulated with illumination, whereas the relative RNA level of the mitochondrial proteins decreased. It is likely that the 2-OG/malate translocator gene contains a light-responsive promoter that functions in a coordinated expression with photosynthetic genes. Among plant translocators, the expression of the chloroplastic triose phosphate translocator gene is reported to be light-dependent and restricted to green tissues (Schulz et al., 1993).

In monocotyledonous C_3 plants such as wheat, the proportion of mesophyll cell occupied by mitochondria remains constant during leaf development (Tobin and Rogers, 1992). Cyt *c* oxidase activity per unit of mitochondrial volume also remains relatively constant. On the other hand, there is an increase in mitochondrial number per BSC of NAD-ME-type C_4 plants during cell maturation. We have examined the expression pattern of mitochondrial 2-OG/malate translocator in successive leaf sections. It has been reported that the basal meristematic regions of maize leaves show obscure Kranz anatomy (Miranda et al., 1981) and lack the CO_2-fixing enzymes of the C_4 pathway (PEPC and RuBPCase) (Mayfield and Taylor, 1984). As shown in Figure 6, the matrix enzymes that function in photosynthetic photosynthesis are present at relatively high activities at the leaf base and show a gradual increase to reach the maximum at the top. This perhaps indicates their function in the basic metabolism other than photosynthesis. The 2-OG/malate translocator protein localized in bundle-sheath mitochondria accumulates at low levels at the leaf base and increases dramatically with cell development. In contrast, the specific activity of citrate synthase decreases and that of Cyt *c* oxidase remains constant during development of BSC. The increase of the 2-OG/malate translocator may be the result of increases in mitochondrial number during cell development. Alternatively, and most likely, the 2-OG/malate translocator preferentially accumulates in bundle-sheath mitochondrial membranes with cell maturation rather than in other membrane proteins such as Cyt *c* oxidase. At later stages of development with increased photosynthetic activity, the transport activity of 2-OG and malate is largely enhanced to accommodate the high rates of exchange of metabolites. These findings suggest that the mitochondrial 2-OG/malate translocator is likely to participate closely in the C_4 photosynthetic pathway. Selective accumulation of mitochondrial proteins involved in C_4 photosynthesis leads to a functional differentiation of bundle-sheath mitochondria during maturation of BSC. To our knowledge, no other substrate translocator has

been documented that is differentially controlled during development.

The morphological differentiation such as amplification of mitochondrial numbers is known to initiate at an early stage of the developmental divergence of MC and BSC (Dengler et al., 1986). In addition, centripetal disposition of bundle-sheath mitochondria and chloroplasts relative to the vascular tissue, which is typical in most NAD-ME-type C_4 plants, is observed even in etiolated leaves and, therefore, seems to be related to the stage of tissue development (Miyake and Yamamoto, 1987). Since the functional differentiation of the bundle-sheath mitochondria is dependent on light, as mentioned above, it is reasonable to assume that it is controlled by regulatory factors distinct from those required in the morphological differentiation (for example, changes in a metabolite's concentration or chloroplast development). Further morphometric analysis is necessary to ascertain the relation between functional and morphological differentiation of the bundle-sheath mitochondria.

Northern analysis of poly(A)$^+$ RNA from leaves showed that there are two larger transcripts that appear to be a result of incomplete splicing (Fig. 7). The 2.5-kb RNA contains both the second and third insertions and the 2.2-kb RNA contains only the second insertion. From the size of the larger transcripts, it is concluded that the third insertion is spliced out first and is followed by the second insertion. The insertional sequences contain in-frame stop codons and, therefore, translated products of the larger transcripts would not have the intended function. Since the accumulation of unspliced mRNA is variable among varieties, under different growth conditions, and at different developmental stages (Nash and Walbot, 1992; Deruére et al., 1994), the "splicing failure" could indicate the existence of a posttranscriptional mechanism regulating the expression of these genes. The accumulation level for the mitochondrial 2-OG/malate translocator is indeed variable during leaf greening. The relative amount of the 2.2-kb RNA is higher in the etiolated leaves than in the greening and green leaves. Therefore, it is concluded that the splicing efficiency of the second insertion is lower in the etiolated leaves. These findings suggest that the expression of *P. miliaceum* mitochondrial translocator may also be posttranscriptionally regulated at the splicing step. Further investigation is needed to address this regulatory mechanism.

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