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Tissue-Specific and Light-Mediated Expression of the C_4 Photosynthetic NAD-Dependent Malic Enzyme of Amaranth Mitochondria[1]

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In the C_4 dicotyledonous grain plant amaranth (*Amaranthus hypochondriacus*), a mitochondrial NAD-dependent malic enzyme (NAD-ME; EC 1.1.1.39) serves a specialized and essential role in photosynthetic carbon fixation. In this study we have examined specialized photosynthetic gene expression patterns for the NAD-ME α subunit. We show here that the α subunit gene is preferentially expressed in leaves and cotyledons (the most photosynthetically active tissues), and this expression is specific to the bundle-sheath cells of these tissues from the earliest stages of development. Synthesis of the α subunit polypeptide and accumulation of its corresponding mRNA are strongly light-dependent, but this regulation is also influenced by seedling development. In addition, light-dependent accumulation of the α subunit mRNA is regulated at transcriptional as well as posttranscriptional levels. Our findings demonstrate that the NAD-ME of amaranth has acquired numerous complex tissue-specific and light-mediated regulation patterns that define its specialized function as a key enzyme in the C_4 photosynthetic pathway.

Malic enzymes occur in all organisms and catalyze the conversion of malate into pyruvate, which results in the release of CO_2. All of the known malic enzymes have been categorized into one of three classes (Artus and Edwards, 1985; Wedding, 1989). The first class, NADP-ME (EC 1.1.1.40), is defined by its preferential use of NADP as a cofactor and its ability to decarboxylate OAA. This form has been shown to occur in all eukaryotes and can be found in the cytosol, chloroplasts, and mitochondria. The second class, NAD-ME (EC 1.1.1.38), utilizes NAD as a cofactor, has the ability to decarboxylate OAA, and has been found in the cytosol of bacteria and yeast. The third class, NAD-ME (EC1.1.1.39), utilizes NAD as a cofactor, has the characteristic inability to decarboxylate OAA, and is localized to the mitochondria of plants and animals.

The importance of malic enzymes in metabolism is reinforced by their universal occurrence. All malic enzyme forms are believed to function in supplemental respiration pathways by providing pyruvate for the tricarboxylic acid cycle if glycolysis becomes inhibited or by oxidizing pools of excess tricarboxylic acid cycle intermediates. Malic enzymes also can provide reducing equivalents for biosynthetic pathways such as fatty acid biosynthesis (Artus and Edwards, 1985). In addition to their roles as metabolic enzymes, malic enzymes have been recruited in some plants to function in certain specialized processes such as thermogenesis in species of the Araceae (ap Rees et al., 1983). The best-known specialization of malic enzyme function occurs in plants that utilize the highly efficient C_4 pathway of photosynthesis, in which the NADP-ME (EC1.1.1.40) or NAD-ME (EC1.1.1.39) form of malic enzyme has acquired key roles in the assimilation of atmospheric CO_2 into biological matter (Gutierrez et al., 1974; Hatch et al., 1974; Hatch, 1987).

The leaves (and cotyledons in dicots) of C_4 plants possess a specialized Kranz-type anatomy, which consists of a vascular bundle surrounded by a ring of bundle-sheath cells, which, in turn, are surrounded by layers of mesophyll cells (Edwards and Huber, 1981; Hatch, 1987; Wang et al., 1992, 1993a; Furbank and Taylor, 1995). In the initial carbon fixation step, 3-carbon PEP is combined with atmospheric CO_2 to form 4-carbon acids, which are then transported into the inner ring of bundle-sheath cells. Here they are decarboxylated by the photosynthetic form of NADP-ME (in most C_4 monocots) or NAD-ME (in many C_4 dicots). This reaction releases CO_2, which is then incorporated into the Calvin cycle of photosynthesis by RuBPCase. The specialized anatomical and biochemical specializations in C_4 plants work together to eliminate the oxygenation reaction of RuBPCase and the resulting metabolically wasteful photorespiration pathway, thereby greatly increasing the efficiency of carboxylation by RuBPCase (Hatch and Slack, 1970; Edwards and Huber, 1981; Hatch, 1987; Furbank and Taylor, 1995). C_4 plants are especially efficient in marginal growing conditions such as very hot climates or arid environments.

Amaranth is classified as an NAD-ME-type C_4 plant, and its photosynthetic pathway is defined by the unique photosynthetic NAD-ME used for decarboxylating malate in leaf bundle-sheath cells (Long et al., 1994). This mitochondrial NAD-ME, like the nonphotosynthetic forms found in

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Abbreviations: NAD-ME, NAD-dependent malic enzyme; NADP-ME, NADP-dependent malic enzyme; OAA, oxaloacetate; PEPCase, PEP carboxylase; PPdK, pyruvate Pi dikinase; RuPBCase, ribulose-1,5-bisphosphate carboxylase.

other plant species, consists of two nonidentical subunits, α and β. The two subunits can function in dimeric, tetrameric, or octameric form, with equivalent numbers of each subunit occurring in each complex (Artus and Edwards, 1985; Wedding, 1989). The two-subunit composition of plant mitochondrial NAD-MEs contrasts with that of all other forms of plant, animal, and bacterial malic enzymes, which occur as tetramers of a single subunit. We have previously shown that the amaranth NAD-ME α subunit polypeptide contains all of the motifs required for a complete and functional malic enzyme (Long et al., 1994). Furthermore, the β subunit of the mitochondrial NAD-ME from potato mitochondria (Winning et al., 1994) and from amaranth (J.J. Long and J.O. Berry, unpublished data) has been shown to be similar to the α subunit, representing a distinct and slightly smaller malic enzyme polypeptide. It is not known why this form of NAD-ME requires a structure composed of the two related subunits, although it has been shown that both subunits are required for enzymatic activity (Willeford and Wedding, 1987).

Like the RuBPCase small subunit of RuBPCase, the NAD-ME subunits are nuclear-encoded polypeptides that accumulate and function only in bundle-sheath cells. Although they are targeted to different compartments within the cells, these two enzymes catalyze consecutive steps (decarboxylation and CO_2 fixation) in the NAD-ME-type C_4 pathway. Thus, it might be expected that genes encoding the two photosynthetic enzymes would be coordinately regulated during seedling development and leaf differentiation or in response to environmental signals such as light. In this study we have examined developmental, cell-type-specific and light-mediated control of NAD-ME α subunit gene expression. Our results presented here show that, like RuBPCase, α subunit gene expression is established only in bundle-sheath cells and requires illumination, with both processes being strongly influenced by development. However, the NAD-ME shows distinct and independent patterns of expression in response to these developmental and environmental cues, with regulation occurring at transcriptional as well as posttranscriptional levels.

MATERIALS AND METHODS

Seeds of *Amaranthus hypochondriacus* var 1023 were germinated, and plants were grown in a growth chamber (Conviron, Asheville, NC) at 24°C with 14 h d^{-1} illumination at an approximate intensity of 170 to 200 $\mu E\ m^{-2}\ s^{-1}$. Leaves, cotyledons, and other tissues from plants grown under these standard light-grown illumination conditions were harvested at the appropriate times or developmental stage, as indicated in the figures. Dark-grown (etiolated) seedlings were germinated and grown in a darkroom within light-proof boxes, and cotyledons were harvested at the appropriate times in complete darkness. For light-shift (greening) experiments, 7- or 8-d-old etiolated seedlings were transferred to an illuminated growth chamber, and cotyledons were harvested after 5 h of illumination. For dark-shift experiments, 7- or 8-d-old light-grown seedlings were placed inside of a darkroom within light-proof boxes for the indicated periods, and cotyledons were harvested in complete darkness.

Immunolocalization Analysis

Antiserum raised against the α subunit of NAD-ME (Long et al., 1994) was used for immunolocalization analysis according to the methods of Wang et al. (1992). Briefly, 5- or 10-mm-long leaves were embedded in paraffin and sectioned to a thickness of 4 to 5 μm. The sections were reacted with the NAD-ME α subunit antiserum, followed by reaction to an R-phycoerythrin-conjugated secondary antibody (Sigma). The reacted sections were visualized and photographed with a fluorescence microscope (Olympus BH-2) using the fluorescein isothiocyanate excitation/emission range and an ×10 objective.

In Situ Localization of mRNAs Encoding the NAD-ME α Subunit Gene

pMel1 contains a full-length cDNA of the NAD-ME α subunit cloned into Bluescript SK− (Stratagene) (Long et al., 1994). This plasmid was linearized and used to generate sense and antisense RNA probes labeled with digoxygenin-11-UTP (Boehringer Mannheim) in vitro using T3 or T7 polymerase. For in situ localizing of α subunit mRNAs, the labeled transcripts were hybridized to paraffin-embedded fixed leaf sections according to the procedures of Wang et al. (1992). Hybridized transcripts containing digoxygenin-11-UTP were detected using alkaline phosphatase-conjugated antisera in conjunction with a color detection system (Boehringer Mannheim). Hybridized and reacted sections were visualized with a microscope (Olympus BH-2) using an ×10 objective.

RNA Extraction and Northern Analysis

Total RNA was extracted from the various plant tissues or from the cotyledons of seedlings grown under the appropriate light conditions using methods previously described (Berry et al., 1985). Equivalent amounts of total RNA were loaded into each lane of an agarose-formaldehyde denaturing gel, separated by electrophoresis, and transferred to a nitrocellulose membrane as previously described (Berry et al., 1985; Sambrook et al., 1989). The blots were hybridized to a gel-purified *Xho*I-*Eco*RI fragment of pMel1 that was ^{32}P-labeled using a DNA-labeling kit (Rapidprime, Amersham).

Analysis of Protein Synthesis

The rates of in vivo protein synthesis were determined by radioactive labeling. Seedlings (approximately 10 per labeling) were cut at ground level, and their stems were placed into 200 μL of a solution containing 100 μCi of [^{35}S]Met in water. The seedlings were incubated for 2 h, either under illumination in a growth chamber or in complete darkness. Labeled proteins were extracted from the cotyledons, immunoprecipitated from equal amounts of incorporated radioactivity, and analyzed by SDS-PAGE as previously described (Berry et al., 1985).

Nuclear Run-On Transcription Analysis

The preparation of nuclei, the quantification of DNA in the nuclei, nuclear run-on transcription assays, and hybridization of the run-on transcripts were performed using the procedures of Wanner and Gruissem (1991), with a few modifications. Nuclei were isolated from the cotyledons of 7-d-old seedlings grown under the appropriate light conditions. They were initially suspended in 200 mL of buffer (10 mM Tris, 1 mM EDTA), pH 8.0, and an equivalent number of nuclei (based on equivalent DNA content) were used for each run-on reaction. Transcription was reinitiated by adding 2 mL of RNasin, 250 μCi of [α-^{32}P]UTP, and 56.8 mL of 5× transcription mixture to the 200-mL nuclear suspension and incubating at 25°C for 20 min. The 5× transcription mixture contained 250 mM Tris-HCl (pH 8.0), 375 mM KCl, 25 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 5 mM ATP, 2.5 mM GTP, 2.5 mM CTP, and 0.05 mM UTP. Transcription was terminated by adding $CaCl_2$, proteinase K, and RNase-free DNase I to a final concentration of 2.5 mM, 0.02 mg/mL, and 40 ng/mL, respectively, and incubating for 30 min at 37°C. An equal volume of a solution (containing 0.1 M Tris-HCl, pH 8.0, 0.05 M EDTA, 1 M NaCl, 2% sodium sarcosine, and 0.25 mg/mL proteinase K) was added, and incubation was continued for 30 min. This solution was extracted with phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and again with chloroform:isoamyl alcohol (24:1, v/v). The labeled RNA was precipitated overnight at −20°C in an equal volume of isopropanol, and 2 μg of tRNA was added as the carrier. RNA was pelleted and resuspended in 100 μL of resuspension buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 10 mM NaCl).

A restriction digest of the NAD-ME α subunit cDNA clone (pMEL1) and a PCR-amplified fragment of the 18S rRNA gene were separated by agarose gel electrophoresis and transferred to a nitrocellulose membrane as described by Sambrook et al. (1989). Blots were prehybridized for 24 h in hybridization solution (5× Denhardt's solution, 1% sodium sarcosine, 10 mM sodium phosphate, pH 7.0, and 0.1 mg/mL sheared calf thymus DNA) and hybridized for 2 d in fresh hybridization solution containing 100 μL of the ^{32}P-labeled run-on RNAs. Blots were rinsed briefly in 2× SSC, 0.8% sodium sarcosine, followed by washes in 2× SSC, 0.8% sodium sarcosine at 65°C for 30 min and in 0.5× SSC, 0.4% sodium sarcosine at 65°C for 30 min. The blots were visualized, and the relative intensity of hybridization to each band was quantified using a phosphorimager (Molecular Dynamics, Sunnyvale, CA) equipped with ImageQuant, version 4.2, software (Molecular Dynamics). Digitized images were printed using a dye-sublimation printer (Coloreaze, Kodak).

RESULTS

Cellular Localization of NAD-ME α Polypeptide and mRNA in Developing Leaves

Cell-specific patterns of the NAD-ME α subunit polypeptide and mRNA accumulation were examined in amaranth leaf sections by immunolocalization with NAD-ME antisera and by in situ hybridization analysis using an NAD-ME antisense RNA probe (as controls, preimmune serum or an NAD-ME sense strand RNA failed to react with or hybridize to the leaf sections; data not shown). Our previous findings demonstrated that the NAD-ME α subunit occurs exclusively in the mitochondrial matrix in the bundle-sheath cells of mature amaranth leaves (Long et al., 1994). Figure 1 shows that this bundle-sheath cell specificity was established during the earliest stages of leaf development. Figure 1, A and C, shows a very early stage of development when the leaves were 5 mm in length, or approximately one-twentieth of their final expansion. As previously described (Wang et al., 1992; Long et al., 1994), the morphologically distinguishable Kranz anatomy characteristic of NAD-ME-type C_4 dicots was already apparent in these very young leaves. There was a single layer of bundle-sheath cells surrounding each vascular center, with one or more layers of mesophyll cells surrounding each of the rings of the bundle-sheath cells. The bundle-sheath mitochondria were observed to be tightly clustered together with the chloroplasts in the centripetal portion of the cells (toward the vascular tissues). Antisera raised against the NAD-ME α subunit reacted specifically to the mitochondria of bundle-sheath cells in the 5-mm leaves (Fig. 1A), with a minimal amount of reaction detected in mesophyll cells or in any other leaf tissues. In situ hybridization of the 5-mm leaf sections to an NAD-ME antisense probe indicated that mRNAs encoding this enzyme accumulated primarily in the leaf bundle-sheath cells (Fig. 1C). However, a detectable amount of hybridization to the NAD-ME antisense probe was observed in the mesophyll cells as well. Thus, bundle-sheath-specific accumulation of the NAD-ME α subunit polypeptide appears to be determined separately from the NAD-ME transcript, suggesting the possibility of posttranscriptional control of bundle-sheath cell specificity in these very young leaves. Alternatively, transcripts encoding a related form of malic enzyme, not recognized by our α subunit antisera, could have been present and cross-hybridized to the NAD-ME antisense RNA probe.

It is interesting that, in contrast to the NAD-ME, during this same 5-mm stage of leaf development the RuBPCase polypeptides and RNAs have not as yet become specific to bundle-sheath cells but occur throughout the leaf in a pattern more similar to that occurring in C_3 plants (Wang et al., 1992, 1993b). Therefore, cell-type-specific NAD-ME gene expression is established at an earlier stage than is RuBPCase, indicating that genes encoding these two bundle-sheath-specific enzymes are regulated independently during C_4 dicot leaf development.

The 10-mm leaf stage shown in Figure 1, B and D, occurs approximately 24 h later than the 5-mm stage. At this phase of development many characteristics associated with fully expanded amaranth leaves have become established, including the completion of the photosynthetic carbon sink-source transition and the establishment of bundle-sheath-specific RuBPCase gene expression (Wang et al., 1992, 1993b). By this stage the NAD-ME α subunit mRNAs (Fig. 1D), as well as the polypeptide (Fig. 1B), had become entirely specific to bundle-sheath cells in a pattern that

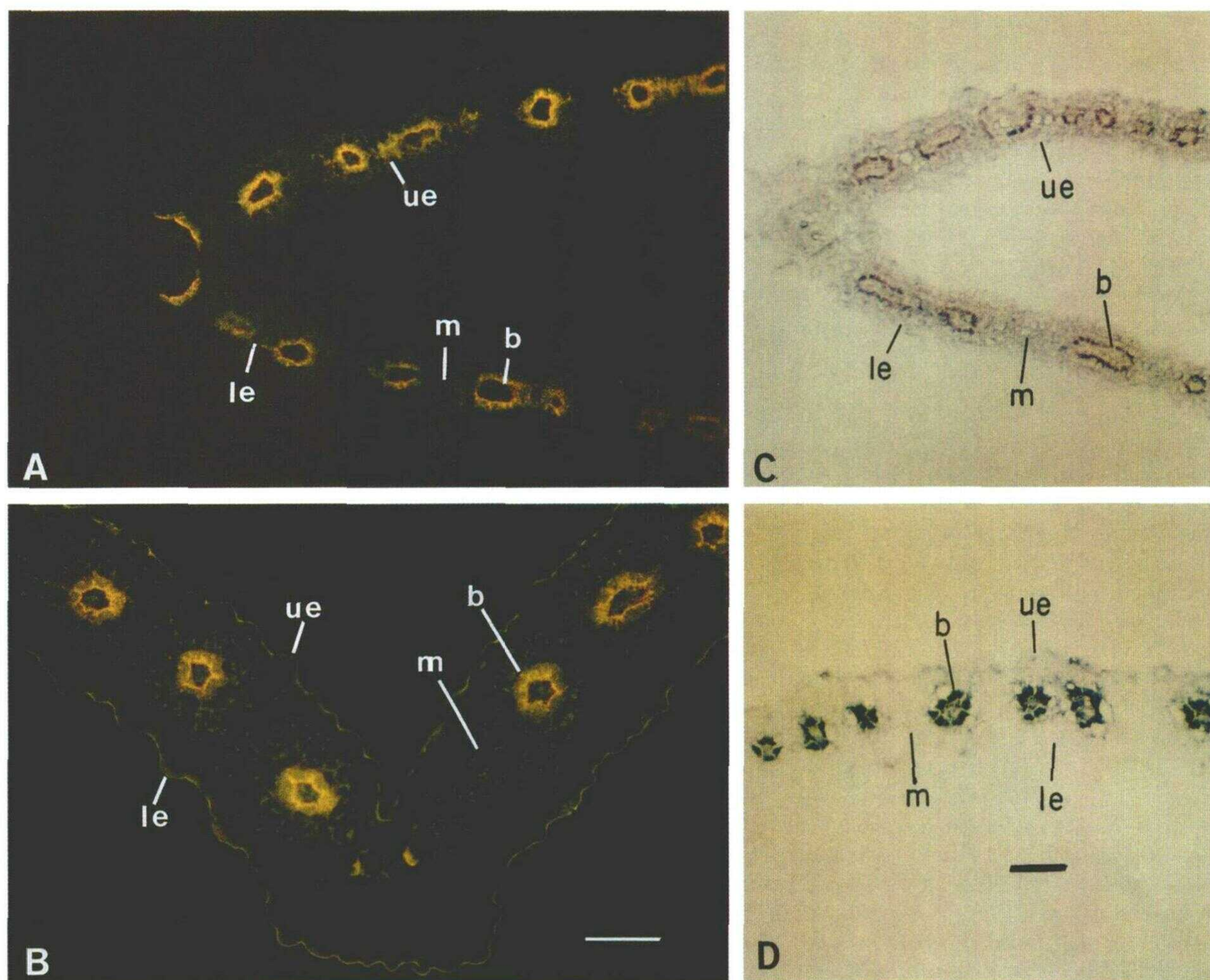

Figure 1. Immunolocalization of NAD-ME α subunit polypeptide and in situ localization of NAD-ME α subunit mRNA in developing leaves. For immunofluorescence, specific reaction of the antisera was observed as a yellow-orange fluorescence, whereas unreacted regions exhibited a characteristic green autofluorescence. For in situ hybridizations, specific hybridization was observed as a blue-purple color. ue, Upper epidermal cells; le, lower epidermal cells; m, mesophyll cells; b, bundle-sheath cells. A, Leaf section (5 mm) reacted with NAD-ME α subunit antiserum. B, Leaf section (10 mm) reacted with antiserum. C, Leaf section (5 mm) hybridized to malic enzyme α subunit antisense RNA probe. D, Leaf section (10 mm) hybridized to antisense probe. Bars = 100 μm.

remains throughout the later stages of leaf development (Long et al., 1994). Unlike RuBPCase, the final reduction of NAD-ME transcripts in mesophyll cells did not occur in the basipetal direction and did not correlate with any observable leaf developmental process (data not shown).

NAD-ME Gene Expression in Photosynthetic and Nonphotosynthetic Tissues

The localization of the NAD-ME α subunit protein and its corresponding mRNA to leaf bundle-sheath cells is essential for the function of this enzyme in the C_4 pathway. To determine whether genes encoding this photosynthetic form of malic enzyme are expressed in any tissues other than leaves, total RNA was isolated from various plant tissues and levels of α subunit mRNA were determined by northern analysis (Fig. 2). NAD-ME α subunit mRNA was most abundant in leaves and cotyledons, both of which serve as the primary photosynthetic organs in amaranth (Wang et al., 1993a, 1993b). In contrast, transcripts encoding this enzyme were much less abundant in tissues that do not have a primary role as photosynthetic organs. NAD-ME α subunit mRNA levels were barely detectable or below the level of detection in roots, stems, petioles, and flowers (Fig. 2), although much longer exposures of this and duplicate blots revealed low levels of hybridization to mRNA from all of the tissue types examined.

The findings presented here demonstrate that the C_4 NAD-ME gene expression occurs primarily in the tissues (leaves and cotyledons) in which the specialized photosynthetic function of this enzyme is required. In addition, Figure 1 shows that within the photosynthetic organs showing enhanced levels of NAD-ME gene expression the accumulation of the NAD-ME polypeptide and mRNA occurs only in bundle-sheath cells. Therefore, the attainment of photosynthetic function by the amaranth mitochondrial NAD-ME was accompanied by enhanced levels

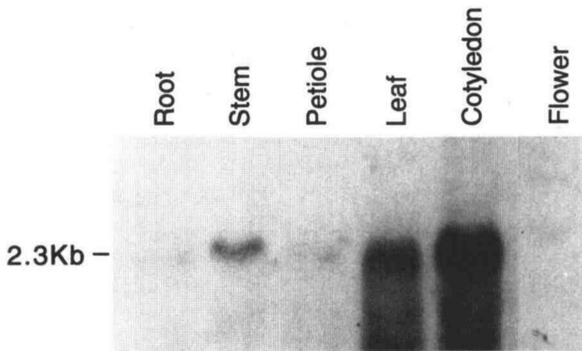

Figure 2. NAD-ME mRNA accumulation in photosynthetic and nonphotosynthetic tissues. Total RNA was extracted from the root, stem, petiole, leaf, cotyledon, and flower. Equal amounts of total RNA were loaded in each lane, electrophoresed on an agarose-formaldehyde gel, and subjected to northern analysis using pMel1 as a probe.

of expression in the primary photosynthetic tissues (relative to nonphotosynthetic tissues), as well as the restriction of expression to one specialized cell type within these tissues.

Developmental Control of Light-Dependent NAD-ME Gene Expression

In addition to having NAD-ME production enhanced and restricted to one photosynthetic cell type, it might also be expected that the expression levels of genes encoding this enzyme would be significantly reduced during extended periods of darkness when energy produced by photosynthetic electron transport is not available. To determine the effects of light on NAD-ME α subunit gene expression, we used cotyledons of young amaranth seedlings that were grown in the presence or absence of light for up to 8 d after planting. Cotyledons were used for the light-regulation experiments because etiolated amaranth seedlings do not produce leaves. Previous studies from our laboratory have demonstrated that amaranth cotyledons provide a good model system to study the light-mediated regulation of C_4 gene expression (Berry et al., 1985, 1986, 1988, 1990; Wang et al., 1993a).

It was necessary to determine whether NAD-ME gene expression was influenced by light and whether seedling development played a role in this regulation. In vivo protein synthesis was analyzed by using cotyledons from seedlings, which were labeled with [^{35}S]Met. Proteins were extracted from the labeled cotyledons, and the NAD-ME α subunit was immunoprecipitated from equal amounts of incorporated radioactivity. NAD-ME synthesis was initiated in both light- and dark-grown plants 2 d after sowing (data not shown) and remained relatively high in the cotyledons of seedlings grown under both illumination conditions up to 5 and 6 d after planting (Fig. 3, top). In light-grown seedlings, synthesis of the polypeptide remained at this same level through d 8. In contrast, in dark-grown seedlings synthesis of the NAD-ME α subunit was reduced to below the level of detection after 7 d of growth in the absence of light and remained at this same very low level through d 8.

Northern analysis of total amaranth RNA using the pMel1 probe revealed a single hybridizing transcript of 2.3 kb. Like the α subunit polypeptide, these mRNAs were first detected 2 d after planting, with corresponding levels occurring in both light- and dark-grown seedlings (data not shown). In light-grown seedlings, the NAD-ME transcripts increased and reached a plateau at d 5 and 6, remaining at the same abundant levels through d 8 (Fig. 3, bottom). In the absence of light, NAD-ME transcripts also reached a maximum at d 5 and 6 but then decreased dramatically so that only a minimal amount of this mRNA could be detected on or after d 7.

The developmental changes in NAD-ME protein synthesis that were observed in the dark-grown seedlings corresponded closely with changes in the levels of the transcripts encoding this enzyme. It is therefore likely that the developmental pattern of the NAD-ME gene expression that occurs in etiolated seedlings is determined at the level of mRNA accumulation.

Light-Induced Changes in NAD-ME Gene Expression

The light-mediated induction of the NAD-ME α subunit gene expression was examined by illuminating amaranth seedlings grown in the absence of light for 7 or 8 d (when protein synthesis and mRNA accumulation were reduced to undetectable levels in the etiolated cotyledons; Fig. 3). When 8-d-old etiolated seedlings were transferred to light, NAD-ME α subunit protein synthesis increased rapidly, from undetectable amounts in the dark to nearly the same abundant levels observed in light-grown seedlings, within 5 h postillumination (Fig. 4A).

NAD-ME mRNA levels were enhanced significantly within the first 5 h of illumination (Fig. 4B). These transcripts were easily observed in 8-d-old light-grown cotyledons and were not detectable in dark-grown cotyledons. NAD-ME mRNA levels increased rapidly when the dark-

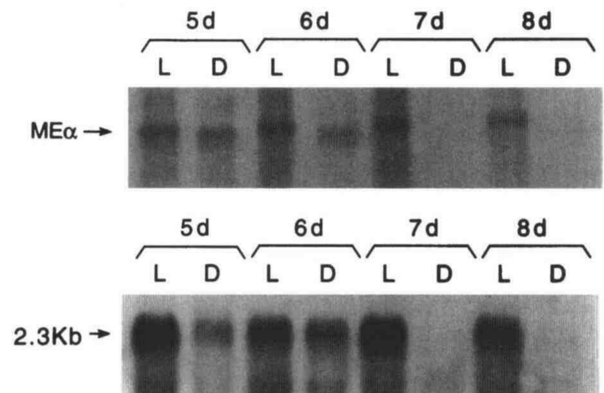

Figure 3. NAD-ME protein synthesis and mRNA accumulation in 5- to 8-d-old light-grown (L) and dark-grown (D) amaranth cotyledons. For in vivo protein synthesis, seedlings were labeled with [^{35}S]Met as described in the text. NAD-ME α subunit was immunoprecipitated from equal amounts of incorporated radioactivity, separated by SDS-PAGE, and fluorographed. For northern analysis, equal amounts of total RNA extracted from cotyledons were electrophoresed and probed with pMel1 as described for Figure 2.

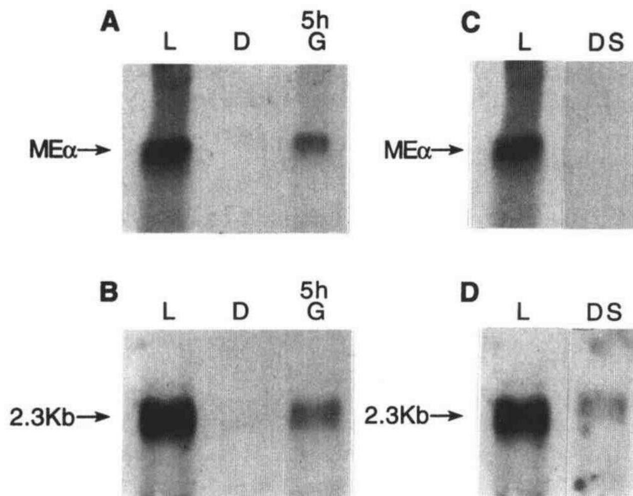

Figure 4. NAD-ME protein synthesis and mRNA accumulation in response to light. A and B, Light shift. Eight-day-old seedlings were grown under normal illumination (L), in complete darkness (D), or in complete darkness and transferred to light for 5 h (5hG). A, In vivo NAD-ME protein synthesis. B, NAD-ME mRNA accumulation. C and D, Dark shift. Eight-day-old seedlings were grown under illumination (L) or grown in light and transferred to complete darkness for 4 h (DS). C, In vivo protein synthesis. D, mRNA accumulation. Methods were as described in Figures 2 and 3.

grown seedlings were shifted into the light and showed the same timing and pattern of induction as NAD-ME protein synthesis. These results demonstrate that C_4 NAD-ME gene expression is rapidly induced by light and that the induction of synthesis of the α subunit polypeptide appears to be determined at the level of mRNA accumulation.

The effects of light on NAD-ME production were investigated further by transferring seedlings grown under standard illumination conditions into complete darkness (dark-shift). When 8-d-old light-grown seedlings were dark-shifted for 4 h, NAD-ME α subunit protein synthesis was rapidly decreased to below the level of detection (Fig. 4C). In addition, the levels of NAD-ME α subunit mRNA were decreased rapidly, indicating that dark-induced reductions in mRNA abundance were responsible for the reductions in synthesis of the polypeptide (Fig. 4D). However, some NAD-ME α subunit mRNA was still observed in the cotyledons 5 h following the dark shift. Because NAD-ME protein synthesis was not detectable in the 5-h dark-shifted cotyledons, it was difficult to quantitatively compare the changes in synthesis with the changes in transcript abundance. However, it appears that the reductions in synthesis of the protein were greater than the reductions in transcript abundance, suggesting that regulation at the level of protein synthesis also might contribute to the decreased production of NAD-ME in the cotyledons of dark-shifted seedlings.

Transcriptional Control of Light-Regulated NAD-ME Expression

Figures 3 and 4 clearly demonstrate that the light-mediated NAD-ME α subunit gene expression patterns were determined primarily at the level of mRNA accumulation. This implies that the transcription of the NAD-ME α subunit mRNAs was regulated by light or, alternatively, that light affected the stability or processing of these transcripts. Examples of both forms of regulation have been shown to be involved in determining the expression of photosynthetic genes (Fluhr et al., 1986; Mullet and Klein, 1987; Shirley et al., 1990; Silverthorne and Tobin, 1990; Wanner and Gruissem, 1991; Schaffner and Sheen, 1991; Boinski et al., 1993).

Nuclear run-on transcription analysis was used to investigate the effects of light on the transcription of the NAD-ME α subunit mRNA. Nuclear extracts were prepared from the cotyledons of 7-d-old light-grown, dark-grown, 5-h greening, and 4-h dark-shifted seedlings. Run-on transcription assays were performed so that only those transcripts that were already in the process of being transcribed in vivo were reinitiated and ^{32}P-labeled in vitro. The run-on transcripts were hybridized to equalized excess amounts of a pMel1 2.3-kb DNA fragment and, as a control, a 0.35-kb DNA fragment was PCR-amplified from amaranth genomic DNA corresponding to an 18S rRNA gene. Relative levels of hybridization to each DNA fragment were quantified using a phosphorimager equipped with image analysis software. The results of these experiments are shown in Figure 5, and a quantification of these results is presented in Table I.

Hybridization to the 18S rRNA gene was used as an internal standard, since the transcription level of this housekeeping gene should not be specifically affected by light. Individual values for levels of transcription of the NAD-ME α subunit gene under each illumination condition were first normalized to the 18S hybridization values. Since the light-grown seedlings were grown under standard illumination conditions, the levels of NAD-ME transcription occurring under all other illumination conditions, as presented in Table I, are given relative to that which occurred in the light.

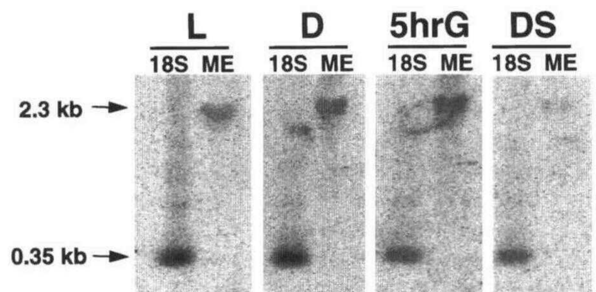

Figure 5. NAD-ME transcription in response to light. ^{32}P-UTP-labeled transcripts were generated in vitro using nuclear extracts from the cotyledons of amaranth seedlings grown for 7 d in light (L), darkness (D), darkness plus 5 h of illumination (5hrG), or light followed by 4 h of darkness (DS). The in vitro labeled transcripts were used to probe DNA fragments that had been blotted onto nitrocellulose membranes. Each lane designated ME contained a restriction digest of pMel1, which resulted in 3 μg of a 2.3-kb NAD-ME α fragment. Each lane labeled 18S contained 3 μg of a 0.35-kb PCR fragment of the amaranth 18S rRNA gene. Hybridization signals were recorded on a phosphorimager and printed using a dye-sublimation printer.

Table I. *Relative levels of NAD-ME α subunit transcription in response to various light conditions*

Levels of transcription for each condition were determined by quantifying the intensity of the bands from three nuclear run-on transcription experiments. Individual values for malic enzyme were normalized to 18S. The values presented (with SE) are normalized levels of transcription relative to the normalized levels occurring in the light.

Light Condition	Transcription of NAD-ME α Subunit Relative to Light-Grown Seedlings
Light-grown	1
Dark-grown	0.89 ± 0.20
5-h Greening	2.00 ± 0.50
Dark-shift	0.53 ± 0.08

The dark-grown cotyledons showed only a slight reduction in NAD-ME transcription activity, approximately 0.89 times the level of transcription of light-grown cotyledons (Fig. 5; Table I). Although it is difficult to determine whether this slight decrease had an effect on the final levels of NAD-ME transcript accumulation in the 7-d-old dark-grown seedlings, it was clearly not enough of a decrease to account for the undetectably low levels of NAD-ME α subunit mRNA present in dark-grown cotyledons on and after d 7 (as seen in Fig. 3). In the cotyledons of dark-grown seedlings that were shifted into the light for 5 h, transcription of the NAD-ME α subunit gene increased to a level approximately 2-fold higher than that observed in light-grown cotyledons and approximately 2.2-fold higher than that observed in dark-grown cotyledons (Fig. 5; Table I). This moderate increase suggests that at least some light-mediated regulation did occur at the transcriptional level. In the cotyledons of light-grown seedlings that were shifted into darkness for 4 h, the level of NAD-ME transcription decreased approximately 2-fold relative to light (Fig. 5; Table I), again suggesting that light did have some effect on the rates of NAD-ME transcription.

The relative changes in the rates of transcription for the NAD-ME in seedlings exposed to changes in illumination did not correspond to the dramatic changes in levels of NAD-ME mRNA accumulation observed under these same conditions. This evidence suggests that the differential accumulation of NAD-ME transcripts in the cotyledons of 7-d and older light- and dark-grown amaranth seedlings and the rapid light-induced changes in NAD-ME gene expression that occurred in these seedlings were not due to changes in transcription. It is therefore likely that these light-dependent differences and changes in gene expression were caused by alterations in the processing and/or stability of the NAD-ME transcripts.

DISCUSSION

The C_4 pathway of photosynthesis involves the morphological development of a specialized Kranz leaf anatomy, as well the coordinated action of several key C_4 enzymes. The C_4 pathway is hypothesized to have evolved as a more efficient alternative to the normal C_3 pathway in response to arid or high-temperature conditions (Hatch and Slack, 1970; Hatch et al., 1974; Edwards and Huber, 1981; Hatch, 1987). During the evolution of this pathway many nonphotosynthetic enzymes were recruited to serve a new photosynthetic role, and with the exception of RuBPCase, all of the C_4 enzymes, including NAD-ME, were originally metabolic enzymes and still exist in the C_4 species in their original nonphotosynthetic form (Sheen, 1991; Furbank and Taylor, 1995). Our previous work demonstrated that the α subunit of the amaranth C_4 NAD-ME was very similar along its entire length to other forms of malic enzymes present in both plants and animals (Long et al., 1994), with the polypeptide showing no significant new characteristics that could be associated with its acquisition of photosynthetic function. It is thus apparent that alterations in the regulation patterns of the genes encoding this malic enzyme, rather than structural changes to the enzyme itself, are primarily responsible for its specialized involvement in CO_2 fixation. The purpose of this current study was to characterize the complex gene expression patterns that have enabled the NAD-ME of amaranth mitochondria to function as a key enzyme of the C_4 photosynthetic pathway.

As with all C_4 enzymes, compartmentalization of NAD-ME is essential for its efficient function in the C_4 pathway. Previous studies have shown that C_4 photosynthetic NAD-ME activity occurs only in leaf bundle-sheath cells (Artus and Edwards, 1985), and we have recently demonstrated that the NAD-ME α subunit protein of mature amaranth leaves is indeed found only in the mitochondrial matrix of bundle-sheath cells (Long et al., 1994). The results presented here show that bundle-sheath cell-specific localization of the NAD-ME α subunit is established very early in leaf development. Even as early as the 5-mm leaf stage, NAD-ME α subunit polypeptide was detected only in the bundle-sheath cells, although low levels of an mRNA with homology to the NAD-ME α subunit antisense probe were also detected in the mesophyll cells of these young leaves. It is possible that bundle-sheath cell specificity at this early stage of leaf development is determined by transcriptional as well as posttranscriptional processes. Similar developmental patterns of C_4 gene expression, with C_4 mRNAs accumulating in tissues and cells that do not accumulate their corresponding polypeptides, have been observed (Wang et al., 1992; Ramsperger et al., 1996). However, from these data we cannot exclude the possibility that the pMel1 probe had hybridized to mRNAs produced from a gene encoding a related form of malic enzyme that was not recognized by our NAD-ME antisera.

Approximately 1 d later, when the leaves had expanded to 10 mm in length, NAD-ME α subunit mRNA was reduced to undetectable levels in mesophyll cells but remained at the same abundant levels in bundle-sheath cells, a pattern that remains throughout leaf maturity. Thus, in leaves 10 mm or greater in length, bundle-sheath cell-specific expression of the α subunit gene appears to be determined exclusively by the cell-specific accumulation of its corresponding transcript. As an alternative interpretation of the data, it is also possible that the expression of a second, non-cell-type-specific malic enzyme gene had be-

come reduced or eliminated by the 10-mm stage of leaf development, so that only the bundle-sheath-specific C_4 NAD-ME transcript could be detected in the older leaves.

The temporal patterns of the NAD-ME α subunit gene expression that occur during amaranth leaf development are more similar to that observed for the genes encoding the mesophyll cell-specific C_4 enzymes PPdK and PEPCase (two other metabolic enzymes recruited to a photosynthetic role in amaranth) than to the bundle-sheath cell-specific RuBPCase (Wang et al., 1992; Ramsperger et al., 1996). PPdK and PEPCase polypeptides are both detected only in leaf mesophyll cells from the earliest stages of development, even though their corresponding mRNAs initially accumulate in both photosynthetic cell types. Like the NAD-ME transcripts, PPdK and PEPCase mRNAs become localized exclusively to the one photosynthetic cell type during the later stages of leaf development, after the cell-type-specific localization of their encoded polypeptides have been established.

The establishment of cell-type-specific gene expression for NAD-ME, PPdK, and PEPCase differs from the pattern observed for the large and small subunits of RuBPCase genes of RuBPCase. The two RuBPCase subunits, as well as their corresponding mRNAs, initially accumulate in both photosynthetic cell types in a "C_3-like" default pattern until the 5-mm leaf stage (when NAD-ME and the other C_4 enzymes have already become cell-type-specific; Wang et al., 1992). The establishment of bundle-sheath cell-specific RuBPCase gene expression in amaranth leaves occurs in the basipetal direction when the leaves are between 5 and 10 mm in length, and this process is tightly coordinated with the photosynthetic carbon sink-to-source transition (Wang et al., 1993b). Since RuBPCase serves a photosynthetic function in all plants, it is likely that the regulation of the genes encoding this enzyme would be intimately associated with other types of photosynthetic processes, such as those that occur during the sink-to-source transition (Turgeon, 1989). In fact, there are many examples of regulation of the RuBPCase genes by photosynthetic metabolism (Sheen, 1990; Sonnewald and Willimitzer, 1992; Krapp et al., 1993). The C_4 forms of NAD-ME, PPdK, and PEPCase all have separate origins as nonphotosynthetic enzymes that have been recruited to serve a photosynthetic function in amaranth (Hatch, 1987; Furbank and Taylor, 1995). It is therefore not surprising that the patterns of gene expression for these three C_4 enzymes are more similar to each other than to RuBPCase and that their expression is more independent of photosynthetic metabolism.

The developmental signals that might signal and regulate the bundle-sheath cell-specific gene expression of NAD-ME are not yet known. Such a signal might be associated with a basic leaf developmental process such as vascular differentiation. There is evidence that the development of leaf vascular centers does influence the establishment of cell-type-specific C_4 gene expression in developing maize leaves (Langdale et al., 1987; Nelson and Langdale, 1989, 1992). Alternatively, a more complex series of metabolic or developmental signals, similar to those involved in regulating RuBPCase gene expression (Sheen, 1990; Sonnewald and Willimitzer, 1992; Krapp et al., 1993), could act together to determine bundle-sheath cell-specific NAD-ME α subunit gene expression.

In addition to correct compartmentalization within the leaves, efficient energy utilization in a C_4 plant might also be expected to result in additional levels of spatial regulation for an enzyme such as the photosynthetic NAD-ME, so that it would accumulate at high levels only in those plant tissues in which its specialized activity was required. In fact, the NAD-ME α subunit mRNA gene expression is clearly most abundant in photosynthetically active tissues (leaves and cotyledons) and less abundant (although still detectable) in roots, stems, petioles, and flowers, tissues that have little or no photosynthetic activity. The small amounts of NAD-ME mRNA occurring in non-leaf or cotyledon tissues could be required to support low levels of photosynthetic activity for the stem, petiole, and flower, although this could not account for the small amount of transcript detected in roots. An alternative explanation is that the low amount of NAD-ME gene expression represents basal levels of the same enzyme used in photosynthesis, or normal levels of a related form of malic enzyme, required for metabolic activity in these tissues. The various forms of malic enzyme show relatively high levels of similarity in their DNA sequence, even among different organisms. For example, the amaranth NAD-ME α subunit gene shares between 50 and 53% similarity with genes encoding NADP-MEs from organisms as diverse as maize and humans (along their entire length, with some very highly conserved regions; Long et al., 1994). It is therefore possible that, if mRNAs encoding another form of amaranth malic enzyme were present in these other tissues, our probe might have been able to hybridize to them.

The high level of NAD-ME gene expression observed in the photosynthetic cotyledons of amaranth seedlings is dependent on illumination, as well as on development. Consistently high levels of NAD-ME α subunit polypeptide synthesis and mRNA accumulation were observed in the cotyledons of light-grown seedlings up to 8 d after planting. In dark-grown seedlings, NAD-ME mRNA accumulation and protein synthesis were initially observed at levels similar to those in the light but then decreased below the level of detection after 7 d of growth in the absence of light. Thus, NAD-ME gene expression did not require light during the first 6 d of seedling growth. However, light was required for the continuation of NAD-ME gene expression, at the levels of both protein synthesis and mRNA accumulation, on and after d 7.

Light-dependent NAD-ME gene expression is clearly influenced by development in dark-grown seedlings. Previously, we have shown that light-dependent control of RuBPCase gene expression is also dependent on seedling development in the dark (Berry et al., 1985), although the timing and the mechanisms of regulation differ between the two enzymes (translational control for RuBPCase and mRNA accumulation for NAD-ME). The initiation of NAD-ME production in the young seedlings could represent a default pattern induced by seedling germination and cotyledon development. Factor(s) responsible for shutting

down expression in the dark may not be fully active at the very early stages of development represented by 6-d-old and younger seedlings. Alternatively, the expression of the NAD-ME α subunit could become down-regulated in response to the reduced availability of energy (stored as reducing potential) and/or photosynthetic metabolites. Since no light energy is trapped and no new energy-storing molecules are produced in the dark, the resulting low-energy and/or nutrient levels could serve as a signal to reduce NAD-ME α subunit gene expression. The repression of gene expression in 7-d and older etiolated amaranth cotyledons is not universal, however, since the synthesis of some proteins still occurs in the cotyledons of seedlings grown in the absence of light for as long as 8 d (Berry et al., 1985).

The effects of light on the induction of NAD-ME gene expression were examined using seedlings grown in the absence of light for 7 or 8 d, during the period when synthesis of the enzyme and its corresponding mRNAs was greatly reduced relative to light-grown seedlings. When these dark-grown cotyledons were shifted into the light for 5 h, the levels of NAD-ME α subunit mRNA accumulation and protein synthesis increased rapidly in a tightly coordinated fashion, providing a clear example of light-mediated induction of gene expression at the level of mRNA accumulation. Previous studies of light-regulated expression of the NADP-ME (EC 1.1.1.40) gene in maize demonstrated similar patterns of light-mediated regulation. Like the amaranth NAD-ME, there was no synthesis of the maize NADP-ME or any accumulation of its corresponding mRNA detected in the dark (Collins and Hauge, 1983). When 8-d-old dark-grown maize seedlings were shifted into the light, there was a rapid, and presumably coordinated, increase in NADP-ME mRNA accumulation and protein synthesis. Thus, the C_4 photosynthetic forms of malic enzyme in both the dicot amaranth and the monocot maize display similar patterns of induction and regulation during light-dependent seedling development.

A more complex pattern of light regulation was observed when light-grown amaranth cotyledons were shifted into complete darkness. Within the first 5 h after the dark shift there was a dramatic decline in NAD-ME α subunit protein synthesis. This decrease was accompanied by similar but possibly less dramatic reductions in α subunit mRNAs levels. The close correlation in timing between the changes in protein synthesis and mRNA accumulation indicates that significantly reduced mRNA abundance was the primary cause of the shutdown in NAD-ME α subunit protein synthesis. However, the decrease in NAD-ME synthesis from light-grown cotyledons to dark-shifted cotyledons appeared to be greater than the approximately 10- to 20-fold decrease for NAD-ME α subunit mRNA. Thus, regulation at the level of protein synthesis also might be involved in decreasing the synthesis of NAD-ME in the cotyledons of dark-shifted seedlings. Specifically, the fact that some NAD-ME α subunit mRNA was still present in dark-shifted seedlings, but no NAD-ME α subunit protein synthesis was detected, presents the possibility that posttranscriptional (most likely translational) control could work in conjunction with reductions in mRNA accumulation to decrease the production of this photosynthetic malic enzyme in the dark. Similar dual levels of control for RuBPCase and other C_4 enzymes in amaranth have been described (Berry et al., 1988; Wang et al., 1992; Ramsperger et al., 1996).

We have shown here that changes in transcript abundance are primarily responsible for the light-mediated control of NAD-ME α subunit protein synthesis. The changes in mRNA accumulation could result from changes in the rates of transcription or possibly from changes in the stability of the transcripts; our findings indicate that both mechanisms are involved. In the cotyledons of 7-d-old, dark-grown amaranth seedlings, the levels of NAD-ME transcription observed in the run-on assay are only slightly less than the levels observed in light-grown cotyledons, even though NAD-ME mRNAs were not detectable in the dark. Because the reduction in mRNA levels did not correlate with a reduction in transcription, it appears that the change in the stability of the NAD-ME α subunit transcript is the primary mechanism used for down-regulating the production of this enzyme in dark-grown seedlings on and after d 7.

When 7-d-old dark-grown amaranth seedlings were shifted into the light for 5 h, the level of transcription increased only slightly (approximately 2-fold). Whereas the slight increase in transcription rate is indicative of some positive regulation at the transcriptional level, the increase in mRNA accumulation was much greater than the transcriptional increase. Similarly, when light-grown plants were shifted into complete darkness, the level of transcription of the NAD-ME α subunit gene decreased by approximately 2-fold. This decreased transcription was at least temporally correlated to a decrease in levels of NAD-ME α subunit mRNA accumulation, and this result shows that light has a modest effect on NAD-ME transcription. However, the findings presented here clearly demonstrate that the major control mechanism that determines light-dependent NAD-ME gene expression in 7-d and older amaranth cotyledons is posttranscriptional, working through alterations in the processing and/or stability of the NAD-ME transcript.

Posttranscription control of mRNA accumulation has been described for many nuclear- or plastid-encoded plant genes (Mullet and Klein, 1987; Shirley et al., 1990; Silverthorne and Tobin, 1990; Schaffner and Sheen, 1991; Wanner and Gruissem, 1991; Boinski et al., 1993; Furbank and Taylor, 1995). Regulating the accumulation of a photosynthetic transcript at this level might allow for a more rapid adjustment to changes in illumination conditions, particularly in older etiolated seedlings that would need to start synthesizing photosynthetic enzymes as soon as possible after their first exposure to illumination. Rapid adjustment to the expression of photosynthetic genes such as the C_4 NAD-ME or RuBPCase (Berry et al., 1985, 1986, 1988, 1990) at posttranscriptional levels might be one mechanism utilized by seedlings to rapidly adapt to changing environmental conditions during the critical very early stages of the plant development.

Together with our previous findings (Long et al., 1994), we have demonstrated that the photosynthetic NAD-ME is not a structurally unique form of malic enzyme but that the gene encoding the α subunit polypeptide has acquired several novel and complex regulatory patterns that have allowed it to play a key role in the C_4 pathway. These include enhanced production in photosynthetic organs, specific localization to one photosynthetic cell type, and light-dependent expression. The acquisition of these photosynthetic gene expression patterns by the amaranth NAD-ME has involved control mechanisms occurring at transcriptional as well as posttranscriptional levels. We are currently analyzing regulatory sequences on the NAD-ME α subunit gene and factors that interact with these regions to further characterize the processes that determine C_4 photosynthetic capacity in this unique C_4 dicotyledonous plant.

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LITERATURE CITED

ap Rees T, Bryce JH, Wilson M, Green JH (1983) Role and location of NAD malic enzyme in thermogenic tissues of Araceae. Arch Biochem Biophys **227:** 511–521

Artus NN, Edwards GE (1985) NAD-malic enzyme from higher plants. FEBS Lett **182:** 225–233

Berry JO, Brieding DE, Klessig DF (1990) Light-mediated control of translational initiation of ribulose 1,5 bisphosphate carboxylase in amaranth cotyledons. Plant Cell **2:** 795–803

Berry JO, Carr JP, Klessig DF (1988) mRNA's encoding ribulose 1,5-bisphosphate carboxylase remain bound to polysomes but are not translated in amaranth seedlings transferred to darkness. Proc Natl Acad Sci USA **85:** 4190–4194

Berry JO, Nickolau BJ, Carr JP, Klessig DF (1985) Transcriptional and post-transcriptional regulation of ribulose 1,5 bisphosphate carboxylase gene expression in light- and dark-grown amaranth cotyledons. Mol Cell Biol **5:** 2238–2246

Berry JO, Nickolau BJ, Carr JP, Klessig DF (1986) Translational regulation of light-induced ribulose 1,5 bisphosphate carboxylase gene expression in amaranth. Mol Cell Biol **6:** 2347–2353

Boinski JJ, Wang JL, Xu P, Hotchkiss T, Berry JO (1993) Post-transcriptional control of cell type-specific gene expression in bundle sheath and mesophyll chloroplasts of *Amaranthus hypochondriacus*. Plant Mol Biol **22:** 397–410

Collins PD, Hauge DR (1983) Light-stimulated synthesis of NADP malic enzyme in leaves of maize. J Biol Chem **258:** 4012–4018

Edwards GE, Huber SC (1981) The C_4 pathway. *In* MD Hatch, NK Boardman, eds, The Biochemistry of Plants, A Comprehensive Treatise, Vol 8. Academic Press, New York, pp 237–281

Fluhr R, Kuhlemeier C, Nagy F, Chua N-H (1986) Organ-specific and light-induced expression of plant genes. Science **232:** 1106–1112

Furbank RT, Taylor WC (1995) Regulation of photosynthesis in C_3 and C_4 plants: a molecular approach. Plant Cell **7:** 797–807

Gutierrez M, Gracen VE, Edwards GE (1974) Biochemical and cytological relationships in C_4 plants. Planta **119:** 279–300

Hatch MD (1987) C_4 photosynthesis: a unique blend of modified biochemistry, anatomy, and ultrastructure. Biochim Biophys Acta **895:** 81–106

Hatch MD, Mau S-M, Kagawa T (1974) Properties of leaf NAD malic enzyme from plants with C_4 photosynthesis. Arch Biochem Biophys **165:** 188–200

Hatch MD, Slack CR (1970) The C_4-carboxylic acid pathway of photosynthesis. *In* L Reinhold, Y Liwschitz, eds, Progress in Phytochemistry. Wiley-Interscience, London, pp 35–106

Krapp A, Hofmann B, Schafer C, Stitt M (1993) Regulation of the expression of *rbc*S and other photosynthetic gene by carbohydrates: a mechanism for the 'sink regulation' of photosynthesis. Plant J **3:** 817–828

Langdale JA, Metzler MC, Nelson T (1987) The *argentia* mutation delays normal development of photosynthetic cell types in *Zea mays*. Dev Biol **12:** 243–255

Long JJ, Wang J-L, Berry JO (1994) Cloning and analysis of the C_4 photosynthetic NAD-dependent malic enzyme of amaranth mitochondria. J Biol Chem **269:** 2827–2833

Mullet JE, Klein RR (1987) Transcription and RNA stability are important determinants of higher plant chloroplast RNA levels. EMBO J **6:** 1571–1579

Nelson T, Langdale JA (1989) Patterns of development in C_4 plants. Plant Cell **1:** 3–13

Nelson T, Langdale JA (1992) Developmental genetics of C_4 photosynthesis. Annu Rev Plant Physiol Plant Mol Biol **43:** 25–47

Ramsperger VC, Summers RG, Berry JO (1996) Photosynthetic gene expression in meristems and during initial leaf development in a C_4 dicotyledonous plant dicot. Plant Physiol **111:** 999–1010

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Schaffner AR, Sheen J (1991) Maize *rbcs* promoter activity depends on sequence elements not found in dicot *rbcs* promoters. Plant Cell **3:** 997–1012

Sheen J (1990) Metabolic repression of transcription in higher plants. Plant Cell **2:** 1027–1038

Sheen J (1991) Molecular mechanisms underlying the differential expression of maize pyruvate, orthophosphate dikinase genes. Plant Cell **3:** 225–245

Shirley BW, Ham DP, Senecoff JF, Berry-Lowe SL, Zurfluh LL, Shah DM, Meagher RB (1990) Comparison of the expression of two highly homologous members of the soybean ribulose-1,5-bisphosphate carboxylase small subunit gene family. Plant Mol Biol **14:** 909–925

Silverthorne J, Tobin EM (1990) Post-transcriptional regulation of organ-specific expression of individual *rbcS* mRNAs in *Lemna gibba*. Plant Cell **2:** 1181–1190

Sonnewald U, Willmitzer L (1992) Molecular approaches to sink-source interactions. Plant Physiol **99:** 1276–1270

Turgeon R (1989) The sink-source transition in leaves. Annu Rev Plant Physiol Plant Mol Biol **40:** 119–138

Wang J-L, Klessig DF, Berry JO (1992) Regulation of C_4 gene expression in developing amaranth leaves. Plant Cell **4:** 173–184

Wang J-L, Long JJ, Hotchkiss T, Berry JO (1993a) Regulation of C_4 gene expression in light- and dark-grown amaranth cotyledons. Plant Physiol **102:** 1085–1093

Wang J-L, Turgeon R, Carr JP, Berry JO (1993b) Carbon sink-to-source transition is coordinated with establishment of cell-specific gene expression in a C_4 plant. Plant Cell **5:** 289–296

Wanner LA, Gruissem W (1991) Expression dynamics of the tomato rbcS gene family during development. Plant Cell **3:** 1289–1303

Wedding RT (1989) Malic enzymes of higher plants. Characteristics, regulation, and physiological function. Plant Physiol **90:** 367–371

Willeford KO, Wedding RT (1987) Evidence for a multiple subunit composition of plant NAD malic enzyme. J Biol Chem **262:** 8423–8429

Winning BM, Bourguignon J, Leaver CJ (1994) Plant mitochondrial NAD-dependent malic enzyme. J Biol Chem **269:** 4780–4786