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C_4 Photosynthetic Gene Expression in Light- and Dark-Grown Amaranth Cotyledons[1]

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The patterns of expression for genes encoding several C_4 photosynthetic enzymes were examined in light-grown and dark-grown (etiolated) cotyledons of amaranth (*Amaranthus hypochondriacus*), a dicotyledonous C_4 plant. The large subunit and small subunit of ribulose-1,5-bisphosphate carboxylase (RuBPCase), phospho*enol*pyruvate carboxylase (PEPCase), and pyruvate orthophosphate dikinase (PPdK) were all present in the cotyledons by d 2 after planting when the seedlings first emerged from the seed coat. Kranz anatomy was apparent in light-grown cotyledons throughout development, and the overall patterns of C_4 gene expression were similar to those recently described for developing amaranth leaves (J.L. Wang, D.F. Klessig, J.O. Berry [1992] Plant Cell 4: 173–184). RuBPCase mRNA and proteins were present in both bundle sheath and mesophyll cells in a C_3-like pattern during early development and became progressively more localized to bundle sheath cells in the C_4-type pattern as the cotyledons expanded over 2 to 7 d. PEPCase and PPdK polypeptides were localized to mesophyll cells throughout development, even though PEPCase transcripts were detected in both bundle sheath and mesophyll cells. Kranz anatomy also developed in cotyledons grown in complete darkness. In 7-d-old dark-grown cotyledons, RuBPCase, PPdK, and PEPCase were all localized to the appropriate cell types, although at somewhat lower levels than in light-grown cotyledons. These findings demonstrate that the leaves and postembryonic cotyledons of amaranth undergo common developmental programs of C_4 gene expression during maturation. Furthermore, light is not required for the cell-type-specific expression of genes encoding RuBPCase and other photosynthetic enzymes in this dicotyledonous C_4 plant.

C_4 plant species possess a specialized Kranz-type leaf anatomy consisting of two photosynthetically active cell types, mesophyll and bundle sheath, which differ in their photosynthetic roles (Hatch and Slack, 1970; Edwards and Huber, 1981). In C_4 leaves, bundle sheath cells occur as a layer of cells around each vein, with one or more layers of mesophyll cells surrounding each ring of bundle sheath cells. In C_4 plant species, the initial fixation of atmospheric CO_2 is accomplished by the enzyme PEPCase, which is found only in mesophyll cells. PEPCase and other mesophyll-specific enzymes such as PPdK, which produces the substrate for PEPCase, work together to produce C_4 acids during the carboxylation phase of the C_4 pathway. C_4 acids that are produced in the mesophyll cells are transported to the bundle sheath cells for decarboxylation and subsequent refixation by RuBPCase, which is localized specifically to bundle sheath cell chloroplasts. The reactions of the C_4 pathway concentrate CO_2 in the vicinity of RuBPCase, thereby reducing metabolically wasteful photorespiration caused by the oxygenase activity of this enzyme (Miziorko and Lorimer, 1983). C_4 plants are especially efficient at high temperatures and light intensities (Laetsch, 1968; Hatch, 1978; Edwards and Huber, 1981).

We recently described the patterns of expression for genes encoding several bundle sheath- and mesophyll-specific C_4 pathway enzymes during leaf development in amaranth (*Amaranthus hypochondriacus*), a dicotyledonous C_4 plant (Wang et al., 1992, 1993). Of particular interest was the developmental transition that occurred for RuBPCase gene expression. In very young (less than 5 mm long) leaves of light-grown amaranth seedlings, the chloroplast-encoded LSU and nuclear-encoded SSU genes of RuBPCase are expressed in both bundle sheath and mesophyll cells in a C_3-type pattern. The RuBPCase proteins and mRNAs become specifically localized to bundle sheath cells in the characteristic C_4-type pattern as the leaves expand to 10 mm in length over 24 to 36 h. This C_3-to-C_4 transition in the pattern of RuBPCase gene expression occurs in the basipetal (apex to base) direction and is coordinated with the carbon sink-to-source transition of these leaves. These findings link together two significant developmental transitions and suggest that developmental processes associated with changes in photoassimilate transport status may influence patterns of photosynthetic gene expression in C_4 dicot leaves.

In the C_4 monocot maize, light has been shown to be a major signal that influences bundle sheath cell-specific RuBPCase gene expression (Sheen and Bogorad, 1985, 1986, 1987a, 1987b; Langdale et al., 1988b; Langdale and Nelson, 1991). In dark-grown (etiolated) maize seedlings, RuBPCase mRNAs and proteins are found in both bundle sheath and mesophyll cells in a C_3-like pattern. When the etiolated seedlings are illuminated, RuBPCase decreases in mesophyll and increases in bundle sheath cells in a manner similar to that observed in the C_3-to-C_4 transition zone in amaranth. Although developmental signals, independent of light, appear to signal the C_3-to-C_4 transition in RuBPCase gene

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Abbreviations: LSU, large subunit of RuBPCase; PEPCase, phospho*enol*pyruvate carboxylase; PPdK, pyruvate orthophosphate dikinase; RuBPCase, ribulose-1,5-bisphosphate carboxylase; SSU, small subunit of RuBPCase.

expression in amaranth leaves (Wang et al., 1992, 1993), we have not previously determined what effect the complete absence of light would have on the cell-type-specific expression of genes encoding RuBPCase or other C_4 pathway enzymes.

In etiolated amaranth seedlings, leaves will not develop. However, we have previously shown that amaranth cotyledons from both light-grown and dark-grown seedlings can be used to study the regulation of genes encoding RuBPCase (Berry et al., 1985, 1986, 1988, 1990). RuBPCase LSU and SSU and their corresponding mRNAs are present in 2- through 8-d-old light-grown cotyledons and, at somewhat lower levels, in 2- through 8-d-old dark-grown cotyledons as well (Berry et al., 1985). Cotyledons from light-grown amaranth seedlings are very similar in appearance to true leaves, although they have a separate origin (embryonic as opposed to vegetative) and differ from leaves in several aspects of development and gene expression (Scott and Possingham, 1982; Meinke, 1992). Amaranth cotyledons thus provide a system in which to examine patterns of expression for genes encoding RuBPCase and other C_4 pathway enzymes in a photosynthetic tissue other than leaves, with a different origin and distinct developmental processes. In addition, cotyledons can be used to determine the role of light in establishing patterns of photosynthetic gene expression in C_4 plants.

There were two goals of this current investigation: (a) to characterize the expression patterns of genes encoding the C_4 pathway enzymes RuBPCase, PEPCase, and PPdK in postembryonic cotyledons of light-grown amaranth seedlings and to determine whether their patterns of expression in cotyledons are similar to those recently reported for the developing leaves (Wang et al., 1992); and (b) to determine what effect, if any, growing cotyledons in the absence of light would have on the cell-type-specific expression of genes encoding RuBPCase and the other C_4 pathway enzymes. Our findings indicate that, with minor differences, the same pattern of C_4 gene expression that occurs during early stages of leaf development also occurs in the cotyledons. Furthermore, light is not required for the establishment of cell-type-specific C_4 gene expression in amaranth cotyledons.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of *Amaranthus hypochondriacus* var 1023 were germinated, and plants were grown in a Conviron growth chamber at 24°C with 14 h d^{-1} illumination at an approximate intensity of 170 to 200 $\mu E\ m^{-2}\ s^{-1}$. Cotyledons were harvested from the plants at the appropriate times. Dark-grown plants were germinated and grown in light-proof boxes, which were placed in a dark room. Extreme care was taken during the growth of the dark-grown seedlings to avoid any exposure to light. Dark-grown cotyledons were harvested under a dim green safelight and immediately placed in fixative (3:1 ethanol:acetic acid).

Immunolocalization Analysis

Antibodies raised against the LSU and SSU of RuBPCase (Berry et al., 1985) and PEPCase (Wang et al., 1992) have been previously described. Antisera raised against maize PPdK was generously provided by W.C. Taylor (Commonwealth Scientific and Industrial Research Organization, Division of Plant Industry, Canberra, Australia).

Cotyledon samples were fixed, embedded in paraffin, sectioned, and reacted with antisera as previously described (Wang et al., 1992). Briefly, primary antiserum against RuBPCase LSU, SSU, PEPCase, or PPdK was applied to the sections, followed by the application of R-phycoerythrin-conjugated secondary antibody (Sigma). Sections were visualized and photographed using a ×20 objective with a Zeiss Axiovert 10 microscope using a 450 to 490, FT510, LP520 filter system.

In Situ Localization of mRNAs Encoding C_4 Photosynthetic Proteins

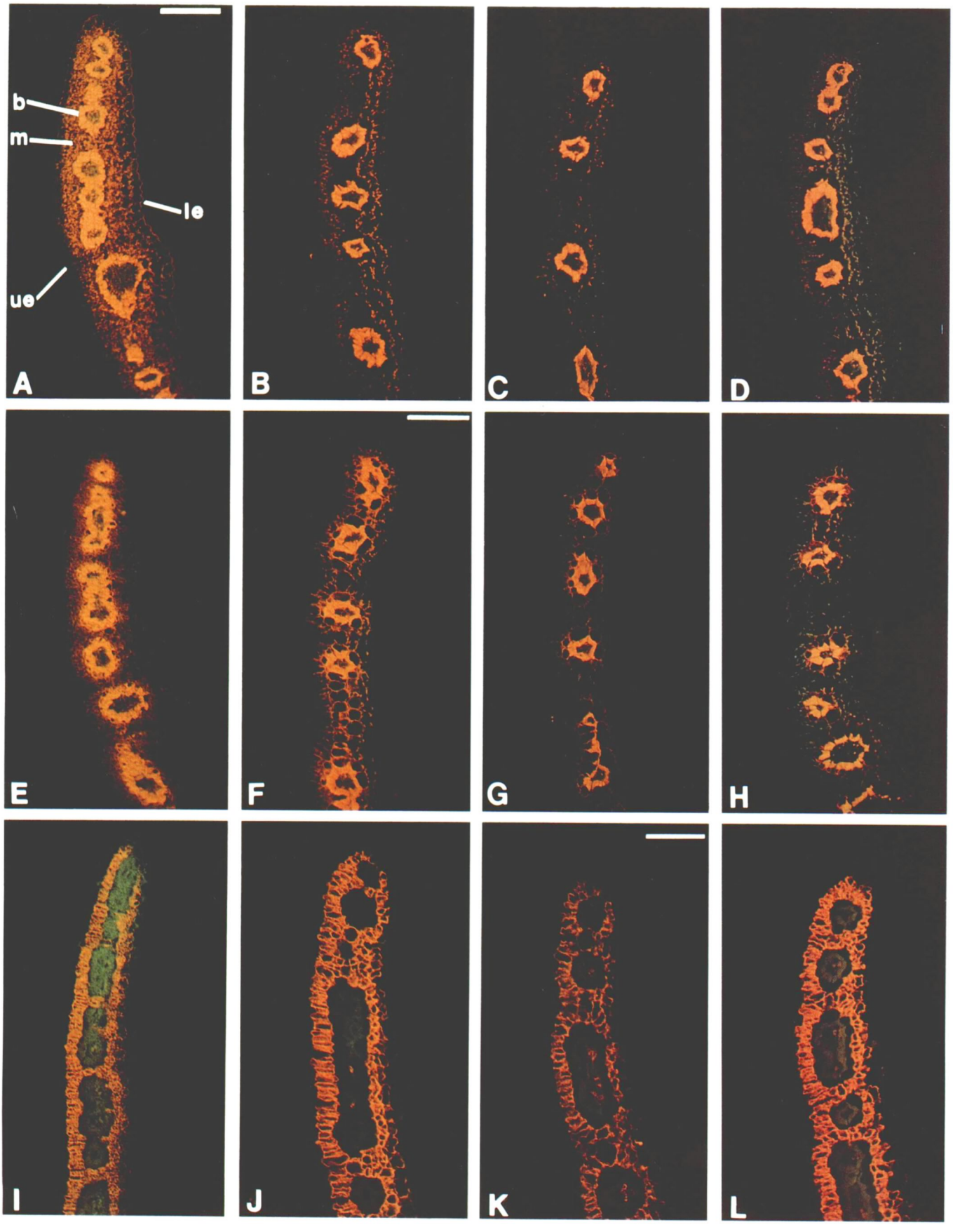
Plasmids used for generating sense and antisense RNA probes for RuBPCase SSU and LSU, PEPCase, and PPdK have been described (Wang et al., 1992). Sense and antisense transcripts for the various C_4 clones were synthesized and labeled in vitro with digoxigenin-11-UTP (Boehringer Mannheim) using T7 or T3 polymerase. Sections were prepared for in situ hybridization analysis according to the methods of Langdale et al. (1987, 1988a) and hybridized as previously described (Wang et al., 1992). Hybridized transcripts were detected using anti-digoxigenin antisera conjugated to alkaline phosphatase in combination with a phosphatase detection system (Boehringer Mannheim) according to the manufacturer's recommendations. Hybridizations were photographed with an Olympus BH-2 microscope using a ×10 objective.

RESULTS

Localization of C_4 Enzymes in Light-Grown Cotyledons

Amaranth cotyledons emerge from the seed coat by the 2nd d after planting and appear above the soil at the tip of

Figure 1 (on facing page). Immunolocalization of RuBPCase and PEPCase in developing cotyledons. Cotyledon sections were prepared and incubated first with the indicated primary antiserum and then with phycoerythrin-conjugated second antibody. The cross-sections were taken from regions midway between the apex and base of cotyledons. b, Bundle sheath cell; m, mesophyll cell; ue, upper epidermal cell; le, lower epidermal cell. Specific reaction of the antibodies is detected in these photographs as yellow-orange fluorescence, distinct from the normal green autofluorescence of the cotyledon tissue under a fluorescein isothiocyanate excitation field. A to D (top row), LSU antiserum reacted to sections of cotyledons from plants at 2, 3, 5, 7 d, respectively, after sowing. E to H (middle row), SSU antiserum reacted to the cotyledon sections. I to L (bottom row), PEPCase antiserum reacted to the cotyledon sections. Scale is the same for all micrographs, bar = 100 μm.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an extended hypocotyl by d 3. To determine the cellular localization of the C_4 pathway enzymes during cotyledon development, polyclonal antisera against RuBPCase LSU or SSU, PEPCase, or PPdK polypeptides were reacted with sections prepared from cotyledons of light-grown seedlings 2, 3, 5, and 7 d after planting. The cross-sections shown in Figure 1 were taken from regions midway between the apex and base of cotyledons.

The Kranz anatomy observed in the amaranth cotyledons at all of these stages was identical with that observed in leaves and is characteristic of NAD-dependent malic enzyme type C_4 dicots, as described previously (Laetsch, 1968; Gutierrez et al., 1974; Wang et al., 1992). Around each vascular center was a single layer of bundle sheath cells, with one or more layers of mesophyll cells surrounding each ring of bundle sheath cells. In mesophyll cells, the chloroplasts were evenly distributed around the edges of the cells, whereas in bundle sheath cells, the chloroplasts were larger and clustered together in the centripetal position (in toward the vascular tissue). This specialized C_4 cotyledon anatomy could be distinguished as early as d 2 after planting, when the cotyledons had first emerged from the seed coat.

In Figure 1, specific reaction of the C_4 antibodies is indicated by the yellow-orange fluorescence of the phycoerythrin-conjugated second antibody, clearly distinct from the normal green autofluorescence of the cotyledon tissue under a fluorescein isothiocyanate excitation field. In cotyledons from 2-d-old amaranth seedlings, LSU (Fig. 1A) and SSU (Fig. 1E) polypeptides were found in both bundle sheath cells and mesophyll cells. Although the highest levels were observed in bundle sheath cells, significant levels of both polypeptides were present in mesophyll cells as well. At d 3, as the cotyledons expanded and developed larger vacuoles, levels of LSU and SSU became slightly reduced in cotyledon mesophyll cells and remained constant in bundle sheath cells (Fig. 1, B and F). At d 5, the cells of the cotyledons had expanded further, with LSU and SSU levels in mesophyll cells becoming further reduced (Fig. 1, C and G). In d 7 and older cotyledons, there were slightly higher amounts of mesophyll cell autofluorescence observed (green fluorescence) than in earlier stages. In the 7-d-old cotyledons, reaction with LSU and SSU antisera was observed primarily in the bundle sheath cells (Fig. 1, D and H), although slight amounts of LSU polypeptides appeared to be present in the mesophyll cells as well. These findings demonstrate that both RuBPCase polypeptides became increasingly more specific to bundle sheath cells as the cotyledons expanded and developed over 2 to 7 d.

PEPCase was specifically localized to cotyledon mesophyll cells throughout development (Fig. 1, I–L). Mesophyll cell-specific localization of PEPCase was initially detected at d 2. As the cotyledons expanded, PEPCase localization remained unchanged, while RuBPCase localization changed from occurrence in both cell types to specific accumulation in bundle sheath cells. Similar results were observed when the cotyledons were reacted with antisera against PPdK. PPdK was localized specifically to the chloroplasts of cotyledon mesophyll cells at all of the developmental stages examined, with no reaction observed in leaf bundle sheath cells (data not shown).

Localization of C_4 Transcripts in Light-Grown Cotyledons

The localization of RuBPCase LSU and SSU polypeptides changed as the cotyledons expanded, becoming more specifically localized to bundle sheath cells during the period of 2 through 5 d after planting. To determine at what level RuBPCase gene expression is regulated during this time, the cellular localization of LSU and SSU mRNAs was analyzed by in situ hybridization of digoxigenin-11-UTP-labeled antisense LSU (pBls1) and SSU (pBas1) RNA probes to sections from 2- and 5-d-old light-grown cotyledons. As a negative control, sense strands generated from these plasmids failed to hybridize with the amaranth cotyledon sections as shown in Figure 2, A and B. In Figure 2, C to F, specific hybridization of the antisense probes is observed as a dark purple color. The labeling patterns for the LSU and SSU antisense probes were very similar, such that both probes appeared to hybridize to the same location in the cell (Fig. 2, C–F). This labeling pattern is due to the presence of the large cell vacuole, which occupies most of the volume of the cell. The cytoplasm and organelles are compressed around the edges of the cell (in mesophyll cells) or in the centripetal portion of the cell (in bundle sheath cells), which causes a strong localized signal in these regions for both RuBPCase probes.

During early cotyledon development, LSU and SSU transcripts were initially detected in both mesophyll and bundle sheath cells. On the 2nd d after planting, SSU and LSU transcripts were detected in bundle sheath cells and, at somewhat lower levels, in mesophyll cells (Fig. 2, C and D, respectively). As the cotyledons continued to expand and develop, the RuBPCase transcripts became progressively more localized to cotyledon bundle sheath cells so that by d 5 very little, if any, of either RuBPCase transcript was detected in cotyledon mesophyll cells (Fig. 2, E and F). These results demonstrate that cotyledons, like leaves (Wang et al., 1992, 1993), undergo a C_3-to-C_4 transition in RuBPCase gene expression. This transition occurs as the cotyledons develop in the light between 2 and 5 d after planting.

Whereas the cellular localization of the RuBPCase polypeptides changed as the cotyledons developed, the PEPCase and PPdK polypeptides remained localized to one cell type, the mesophyll cells, throughout this time. As expected, antisense RNA generated from the PPdK gene probe (pApk1) hybridized specifically to cotyledon mesophyll cells (Fig. 2G). However, specific hybridization of the amaranth PEPCase antisense RNA probe occurred in bundle sheath cells as well as in mesophyll cells (Fig. 2H). This same result was obtained when amaranth leaf sections were hybridized with PEPCase probes (Wang et al., 1992), and it indicates that in both leaves and photosynthetic cotyledons transcripts with homology to the amaranth PEPCase gene are present in bundle sheath and mesophyll cells, even though the C_4 PEPCase enzyme is present only in mesophyll cells.

Localization of C_4 Enzymes in Dark-Grown Cotyledons

As in light-grown seedlings, the cotyledons of dark-grown seedlings first emerge from the seed coat on the 1st d after planting and appear above the soil by the end of d 3. The dark-grown cotyledons show very little morphological de-

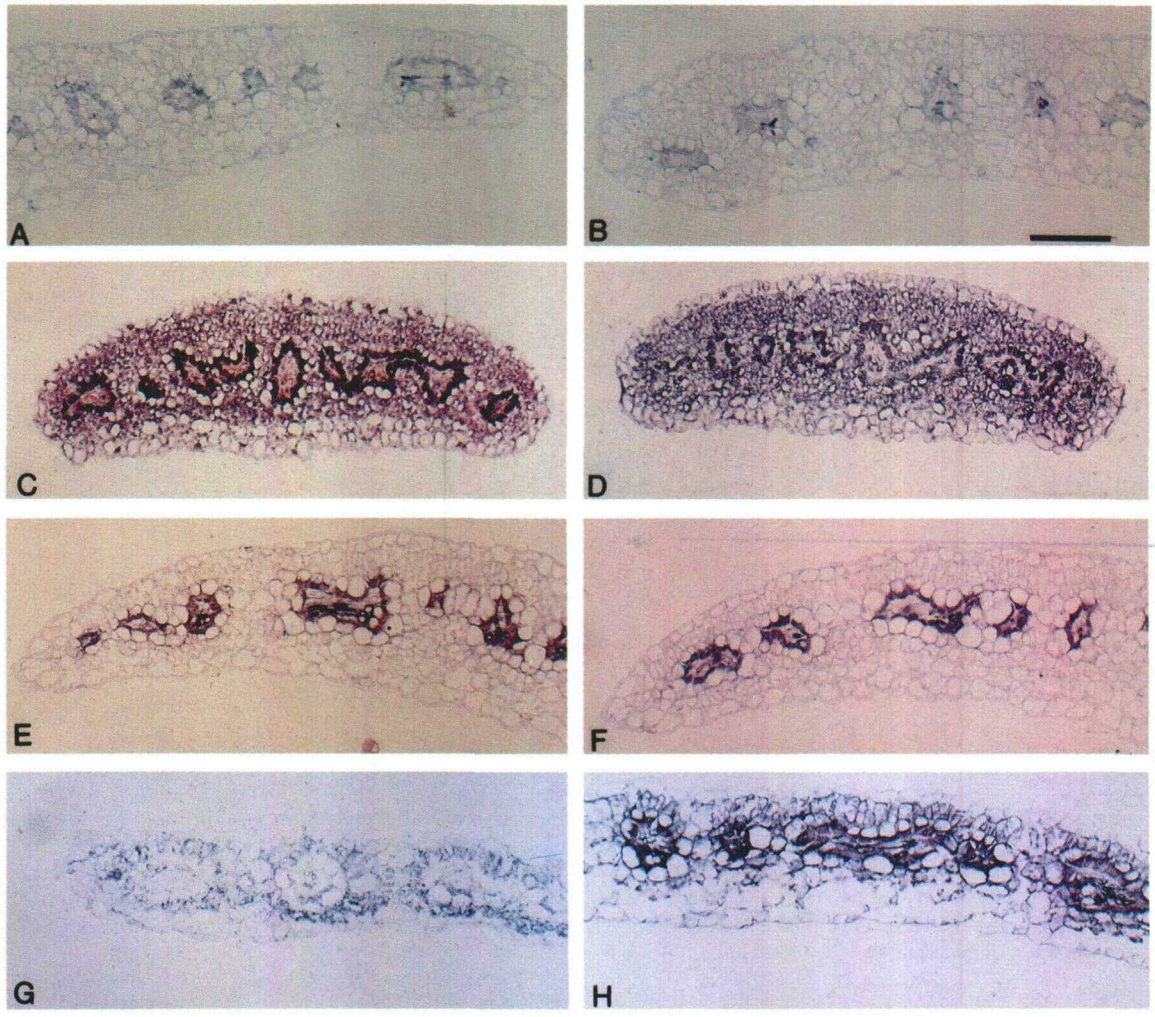

Figure 2. Accumulation of RuBPCase mRNAs in developing cotyledons from light-grown seedlings. Sections are from 2- and 5-d-old cotyledons. Sections were prepared and hybridized with labeled LSU or SSU transcripts. Transcripts were synthesized and labeled in vitro with digoxigenin-11-UTP using T7 or T3 polymerase. Hybridized transcripts were detected using anti-digoxigenin antisera conjugated to alkaline phosphatase in combination with a color detection system. In these micrographs, specific hybridization is observed as a dark purple color. A, Cross-section of 5-d-old cotyledon hybridized to LSU sense RNA. B, Five-day-old cotyledon hybridized to SSU sense RNA. C, Two-day-old cotyledon hybridized to LSU antisense RNA. D, Two-day-old cotyledon hybridized to SSU antisense RNA. E, Five-day-old cotyledon hybridized to LSU antisense RNA. F, Five-day-old cotyledon hybridized to SSU antisense RNA. G, Seven-day-old cotyledon hybridized to PPdK antisense RNA. H, Seven-day-old cotyledon hybridized to PEPCase antisense RNA. The control hybridizations with sense strand RNA probes (A and B) were developed five to 10 times longer than in the sections hybridized with specific antisense probe RNAs, and the micrographs were printed darker, to show clearly the cotyledon Kranz anatomy. Scale is the same for all micrographs, bar = 100 μm.

velopment or expansion beyond the 3rd d after planting, and the first true leaf does not develop unless the seedlings are illuminated. Our previous work has shown that both RuBPCase subunits and their corresponding mRNAs are present at low levels in 2- through 7-d-old dark-grown cotyledons, even though synthesis of the two subunits stops after d 5 (Berry et al., 1986).

As shown in Figure 3, morphologically distinguishable Kranz anatomy develops in cotyledons grown in complete darkness. In 7-d-old dark-grown cotyledons, the RuBPCase LSU is localized primarily to bundle sheath cells, with only very slight amounts of this protein detected in the mesophyll cells (Fig. 3A). The RuBPCase SSU is also primarily localized to the bundle sheath cells of 7-d-old dark-grown cotyledons, although very low levels above background appeared to be present in mesophyll cells as well (Fig. 3B). Both of the RuBPCase subunits appeared to be present at lower levels in dark-grown cotyledons when directly compared with similar sections from the light-grown cotyledons (based on estimated intensity of fluorescence), although it is difficult to quantitatively compare primary and secondary antibody reactions in the separately prepared tissue sections. In line with these

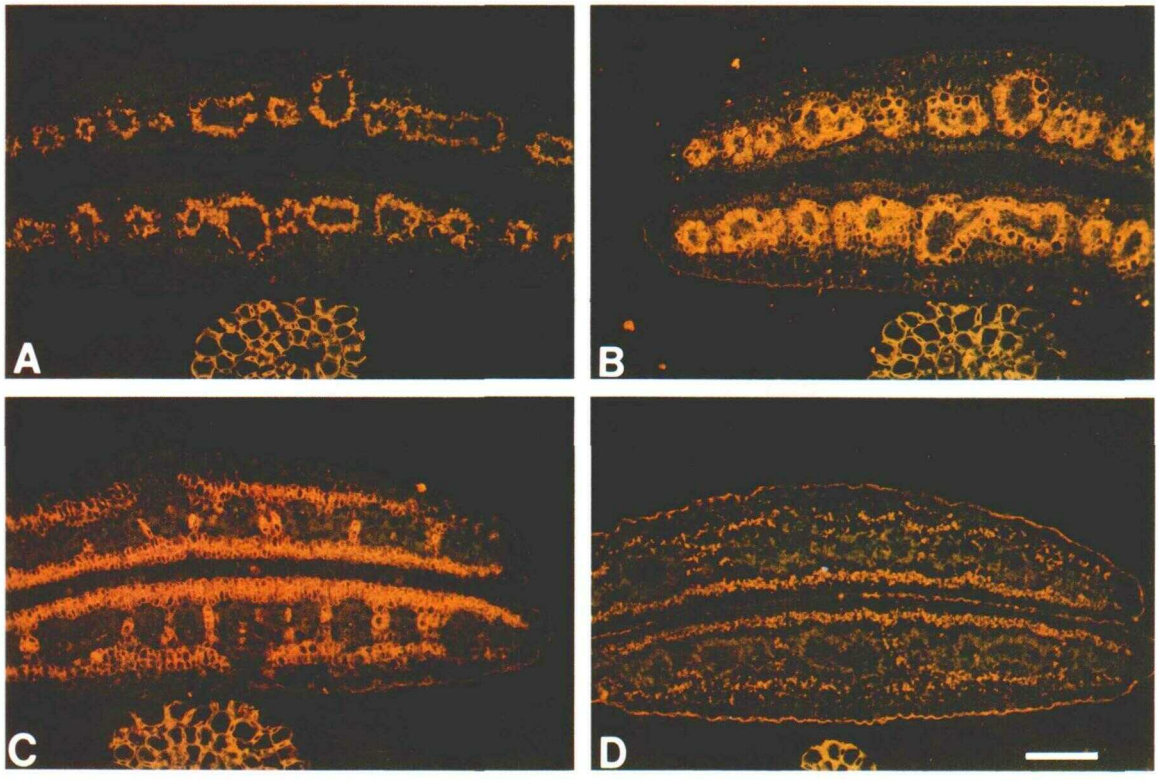

Figure 3. Immunolocalization of C_4 enzymes in 7-d-old dark-grown (etiolated) cotyledons. Sections from the etiolated cotyledons were prepared and reacted with primary and secondary antiserum as described in the legend to Figure 1. The cross-sections were taken from regions approximately midway between the apex and base of cotyledons. A, Cotyledon cross-section reacted with LSU antiserum. B, Cotyledon cross-section reacted with SSU antiserum. C, Cotyledon cross-section reacted with PEPCase antiserum. D, Cotyledon cross-section reacted with PPdK antiserum. Scale is the same for all micrographs, bar = 100 μm.

observations, previous western analysis has indicated that LSU levels are approximately 3-fold lower in 7-d-old dark-grown cotyledons than in 7-d-old light-grown cotyledons, and SSU levels are approximately 10-fold lower in the dark (Berry et al., 1985).

The mesophyll cell-specific enzymes PPdK and PEPCase were also localized to the appropriate cell type in 7-d-old dark-grown cotyledons (Fig. 3, C and D, respectively) and appeared to be present at similar to slightly lower levels when compared with the light-grown cotyledons (PPdK also showed nonspecific hybridization to the outer surface of the cotyledon epidermal cells). These results demonstrate that light is not required for the correct compartmentalization of the C_4 photosynthetic enzymes, although light may affect their final levels of accumulation.

RuBPCase Transcripts in Dark-Grown Cotyledons

Cotyledons from 2-d-old etiolated seedlings are identical with those from 2-d-old seedlings grown in an illuminated growth chamber (as shown in Fig. 2, C and D). Similarly, the 2-d-old etiolated cotyledons show identical patterns of LSU and SSU transcript accumulation, with LSU and SSU transcripts detected in both bundle sheath and mesophyll cells (data not shown). It was expected that 2-d-old cotyledons would not be greatly affected by illumination conditions, because at this early stage the seedlings were still buried beneath the soil and were capable of receiving only extremely low amounts of light.

Like the LSU polypeptide, LSU mRNAs were localized primarily to the bundle sheath cells of 7-d-old etiolated cotyledons (Fig. 4B), even though very small amounts above background, as determined by hybridization to sense-strand probes (Fig. 4A), also appeared to be present in mesophyll cells as well. Although the LSU transcripts were compartmentalized primarily to the correct cell type, they appeared to be present in the bundle sheath cells of dark-grown cotyledons at lower levels than those observed in the bundle sheath cells of light-grown cotyledons (based on intensities of staining). Previous northern analysis supports this observation and indicates that LSU mRNA levels are approximately 3- to 5-fold lower in 7-d-old dark-grown cotyledons than in 7-d-old light-grown cotyledons (Berry et al., 1985).

RuBPCase SSU mRNAs were also correctly compartmentalized to bundle sheath cells in 7-d-old dark-grown cotyledons but at greatly reduced levels when compared with light-grown cotyledons (Fig. 4C). This is consistent with previous northern analysis, indicating that there is approximately 10- to 20-fold less SSU mRNA present in 7-d-old dark-grown

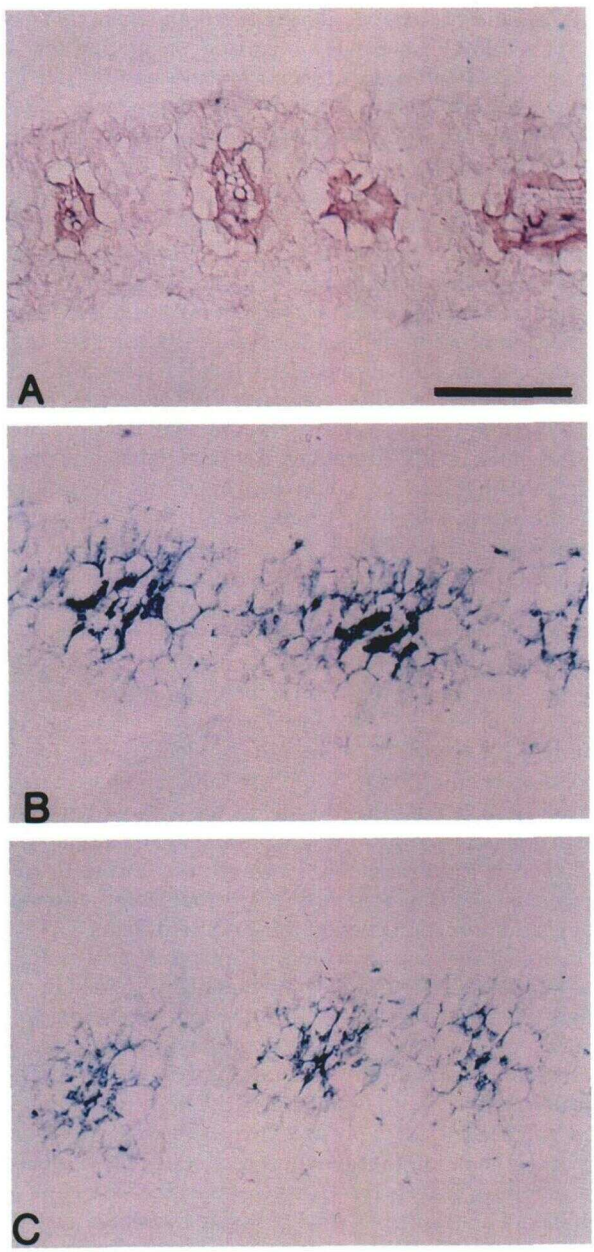

Figure 4. Accumulation of RuBPCase mRNAs in developing cotyledons from 7-d-old dark-grown seedlings. Sections from the etiolated seedlings were prepared and hybridized with labeled LSU or SSU transcripts as described in the legend to Figure 2. A, Cotyledon section hybridized to LSU sense RNA. B, Cotyledon section hybridized to LSU antisense RNA. C, Cotyledon section hybridized to SSU antisense RNA. The control hybridization with LSU sense strand RNA probes (A) was developed three times longer than in the sections hybridized with specific antisense probe RNAs to show clearly the Kranz anatomy of etiolated cotyledons. Scale is the same for all micrographs, bar = 100 μm.

cotyledons than in 7-d-old light-grown cotyledons (Berry et al., 1985).

These findings demonstrate that, even in the absence of light, mRNAs encoding both RuBPCase subunits decreased in mesophyll cells and remained present in bundle sheath cells, thereby becoming cell-type specific as cotyledon development proceeded over 7 d in the dark-grown seedlings. Therefore, although light does affect levels of these transcripts, light is not required for the establishment of cell-type-specific RuBPCase gene expression in developing amaranth cotyledons.

DISCUSSION

Amaranth cotyledons exhibited the characteristic Kranz-type anatomy that is typically found in the leaves of NAD-malic enzyme type C_4 dicotyledonous plants. This specialized C_4 anatomy was detected in cotyledons as early as d 2 after planting when the seedlings first emerged from the seed coat. Enzymes characteristic of the C_4 photosynthetic pathway, RuBPCase, PEPCase, and PPdK, all accumulated in the cotyledons in the appropriate cell types. Genes encoding these proteins followed the same basic developmental patterns of expression that occur during leaf development (Wang et al., 1992). Furthermore, the cotyledons retained these specialized patterns of cell-type-specific C_4 gene expression even when seedlings were grown in the complete absence of light.

Amaranth cotyledons are very similar in appearance to true leaves, although they have a separate origin and developmental program. Unlike leaves, which are formed from vegetative meristems after germination and during seedling growth, cotyledons are formed as a result of cell divisions that take place during embryogenesis within the developing seeds (Scott and Possingham, 1982). Cotyledon development stops during seed desiccation and resumes when seed germination occurs. The growth of cotyledons during and after germination involves primarily cell expansion. Few, if any, cell divisions take place during postembryonic growth and development of cotyledons.

Our findings presented here demonstrate that amaranth cotyledons possess many features that are characteristic of C_4 dicot leaves, including Kranz anatomy, cell-type-specific gene expression, and the C_3-to-C_4 transition in RuBPCase gene expression. Why would cotyledons and leaves share common patterns of C_4 photosynthetic gene expression? Although cotyledons differ from leaves in origin, pattern of development, and many aspects of gene expression (Scott and Possingham, 1982; Meinke, 1992), it is known that cotyledon development can be altered in such a way that the cotyledons acquire many leaf-like characteristics. In unifoliate species of *Streptocarpus*, a single cotyledon enlarges during vegetative growth and is transformed into the single leaf of the mature plant (Hill, 1938; Rosenblum and Basile, 1984). In *Arabidopsis*, a mutation in a single homeotic gene, leafy cotyledon (*LEC*), causes the cotyledons to acquire many leaf-like characteristics (Meinke, 1992). If cotyledons represent a specialization of the basic leaf developmental program, then the leaf-like cotyledons of *Streptocarpus* and the *LEC* mutant of *Arabidopsis* may be due to a reversion back to the basic "default" leaf developmental state. If cotyledons have evolved

as highly specialized leaves, then the regulatory processes responsible for the specialized cotyledon developmental program in amaranth have left intact the complex regulatory processes associated with the development of the C_4 photosynthetic pathway.

Under normal illumination conditions, amaranth cotyledons emerge from the seed coat within 2 d after planting and usually appear above the soil at the tip of an extended hypocotyl by the end of d 3. The first true leaf does not begin to emerge until d 7 and does not begin to act as a photosynthetic source for the seedling until it completes the sink-to-source transition at 8 to 9 d after planting or at 12 to 13 mm in length (Wang et al., 1993). Although the cotyledons serve as a storage organ during early seed germination, in amaranth they must also serve an important photosynthetic function during this period between seed germination and the complete photosynthetic development of the first true leaf. It is likely that the specialized C_4 developmental patterns are retained in amaranth cotyledons because C_4 photosynthetic capacity is required during the time when cotyledons are functioning as the only photosynthetic organs of the young seedlings.

In cotyledons, as in leaves, RuBPCase mRNAs and proteins were initially found in both photosynthetic cell types early in development and became progressively more localized to bundle sheath cells as the cotyledons expanded over 2 to 7 d. This is the same basic pattern as that observed during leaf development (Wang et al., 1992); however, there were some differences. First, we did not observe any developmental polarity in the C_3-to-C_4 transition of cotyledons. In contrast to the basipetal transition that occurs during amaranth leaf development (Wang et al., 1993), it appeared that the C_3-to-C_4 transition in cotyledons occurred throughout the entire length of the tissue at approximately the same time. Second, the developmental switch in RuBPCase expression took much longer in the cotyledons. The change in the cellular localization of the LSU and SSU mRNAs and polypeptides, from a C_3-like pattern in both cell types to bundle sheath cell specificity, required several days in the cotyledons instead of the rapid 24- to 36-h change observed in the leaves (Wang et al., 1992). Cotyledons of light-grown amaranth seedlings do not expand as much, or as rapidly, as the leaves. In general, postembryonic cotyledon development appears to occur at a much slower rate than leaf development, and this is reflected in the longer time required for the establishment of cell-type-specific RuBPCase gene expression.

Another difference between the leaves and cotyledons is that, although in d-7 and older cotyledons LSU and SSU had become localized primarily to cotyledon bundle sheath cells, very low levels of the LSU and SSU polypeptides (above background observed with control preimmune antisera) also appeared to be present in mesophyll cells. In older (10 mm and longer) amaranth leaves, little or no RuBPCase could be detected in mesophyll cells (Wang et al., 1992). If similar regulatory mechanism(s) operate in both leaves and cotyledons to diminish RuBPCase accumulation in mesophyll cells, while allowing continuous accumulation in bundle sheath cells, then these mechanisms may not act as stringently during development of the cotyledons.

The mesophyll cell-specific localization of PPdK and PEPCase did not change during the time when changes in RuBPCase localization were occurring, suggesting that mesophyll and bundle sheath-specific genes are regulated independently of each other during development. PPdK and its corresponding transcripts were both localized primarily to mesophyll cells at all of the developmental stages examined. RNA transcripts with homology to the amaranth PEPCase gene were present in cotyledon bundle sheath cells, even though the PEPCase antibody did not show any reaction with these cells. It is possible that the regulation of genes encoding PEPCase in amaranth might involve the differential expression of different members of a PEPCase gene family. Southern analysis indicated that there are from 10 to 20 copies per genome of the amaranth PEPCase gene (Wang et al., 1992). Alternatively, posttranscriptional regulatory mechanisms, such as RNA turnover, translational control, or specific protein turnover, could be controlling the cell-type-specific expression of PEPCase genes.

The C_4 enzymes RuBPCase, PEPCase, and PPdK all accumulated in the cotyledons of seedlings grown for 7 d in complete darkness. In the dark-grown cotyledons, RuBPCase LSU and SSU were localized primarily to bundle sheath cells, and PEPCase and PPdK were localized specifically to the mesophyll cells. These findings demonstrate that light is not required for the cell-type-specific accumulation of the C_4 pathway enzymes in amaranth cotyledons. In addition, mRNAs encoding both RuBPCase subunits were localized primarily to bundle sheath cells in the 7 d-old dark-grown cotyledons. Therefore, the establishment of bundle sheath cell-specific RuBPCase gene expression in amaranth cotyledons must occur in response to developmental processes or signals that function even in the absence of light.

In both leaves and cotyledons of amaranth, genes encoding the RuBPCase LSU and SSU are initially expressed in mesophyll and bundle sheath cells in the less specialized C_3-like default pattern. A C_3-to-C_4 transition in RuBPCase gene expression occurs in both tissues and results in the bundle sheath cell-specific accumulation of the RuBPCase transcripts and polypeptides. It is possible that there are developmental processes common to leaves and cotyledons that induce this transition. In leaves, the C_3-to-C_4 transition occurs in coordination with the carbon sink-to-source transition, suggesting that developmental processes associated with changes in carbon transport status may influence patterns of photosynthetic gene expression in C_4 plants (Wang et al., 1993). Postembryonic cotyledons most likely serve as carbon source tissue throughout seedling development, initially exporting from storage compounds and later from photoassimilate.

Although very little is known about vascular development and carbon transport in cotyledons, if amaranth cotyledons were to undergo a developmental transition in export capability as they shift from primary storage function to photosynthetic function, then processes associated with changes in transport status (such as minor vein maturation or changes in export compounds) could be associated with patterns in C_4 gene expression in these tissues as well. Light-dependent photosynthetic activity would not necessarily be required for this to occur. Nonphotosynthesizing albino tobacco leaves will undergo a change in transport status and stop importing photoassimilate, even though they never achieve the positive

carbon balance required for export (Turgeon, 1984, 1989). Alternatively, if changes in the accumulation and transport of photosynthetic compounds influence RuBPCase gene expression in leaves (Wang et al., 1992), then patterns of RuBPCase gene expression in cotyledons may be influenced by changes in the levels or transport of storage compounds as these are metabolized or exported during seedling development in the light and in the dark.

Although the C_3-to-C_4 transition in RuBPCase gene expression could be regulated by common developmental processes or signals in amaranth leaves and cotyledons, it is also possible that completely separate processes associated with the distinct developmental patterns of these two tissues regulate this transition. Although the specific regulatory mechanisms have yet to be elucidated, it is clear that developmental processes, independent of light, can determine patterns of C_4 gene expression and the development of C_4 photosynthetic capacity in the leaves and cotyledons of this dicotyledonous C_4 plant.

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