Plant Physiol. (1996) 111: 999–1010

Photosynthetic Gene Expression in Meristems and during Initial Leaf Development in a C_4 Dicotyledonous Plant[1]

Vincent C. Ramsperger, Robert G. Summers, and James O. Berry*

Department of Biological Sciences (V.C.R., J.O.B), and Department of Anatomy and Cell Biology (R.G.S.), State University of New York, Buffalo, New York 14260

Immunolocalization and fluorescent in situ hybridization were used with confocal microscopy to examine patterns of photosynthetic gene expression during initial stages of leaf development in the C_4 dicot *Amaranthus hypochondriacus*. mRNAs encoding the large and small subunit of ribulose-1,5-bisphosphate carboxylase were present in the apical dome and in all cells of the leaf primordia. In contrast, these polypeptides were detected only in cells of the ground meristem, with no accumulation detected in the apical dome or in other leaf primordia cells. The ribulose-1,5-bisphosphate carboxylase transcripts showed very little cell-type specificity as leaf structures began to differentiate, whereas their polypeptides accumulated primarily in bundle-sheath precursor cells. Phospho*enol*pyruvate carboxylase and pyruvate orthophosphate dikinase mRNAs were abundant in meristems and leaf primordia, although their corresponding polypeptides did not accumulate in leaves until the leaf vascular system began to differentiate. These polypeptides were mostly restricted to premesophyll cells from their earliest detection, whereas their transcripts remained present in nearly all leaf cells. These findings indicate that individual C_4 genes are independently regulated as they become initially localized to their appropriate cell types. Furthermore, posttranscriptional regulation plays a major role in determining early patterns of C_4 gene expression.

The highly efficient C_4 photosynthetic pathway functions as a CO_2 pump to localize and concentrate CO_2 in one type of specialized photosynthetic leaf cell. This eliminates two major disadvantages of direct carbon fixation by RuBPCase, low CO_2 affinity, and metabolically wasteful photorespiration resulting from the oxygenase activity of this enzyme (Hatch and Slack, 1970; Edwards and Huber, 1981; Hatch, 1987; Furbank and Taylor, 1995). C_4 photosynthesis is often found in plant species that inhabit desert or arid tropical environments, because the C_4 pathway is superior to the more common and less specialized C_3 pathway under hot, dry, and light-intense conditions. C_4 plants have a Kranz-type leaf anatomy consisting of two morphologically distinct cell types, mesophyll and bundle sheath, which serve to compartmentalize the two distinct biochemical phases (carboxylation and decarboxylation/fixation) required for this pathway to function (Hatch and Slack, 1970; Edwards and Huber, 1981; Hatch, 1987). In mature C_4 leaves, specialized photosynthetic bundle-sheath cells occur as a single or double layer of cells forming a ring around each vein, with layers of mesophyll cells surrounding each ring of bundle-sheath cells. The initial fixation of atmospheric CO_2 in C_4 leaves occurs in mesophyll cells, where three-carbon PEP is carboxylated by the mesophyll-specific enzyme PEPCase. C_4 acids that are produced during the carboxylation reactions are then transported from mesophyll cells to the neighboring bundle-sheath cells, where they are decarboxylated and CO_2 is released for refixation by the bundle-sheath-specific RuBPCase.

C_4 photosynthesis requires the coordination of adaptations at several levels (Hatch, 1987; Furbank and Taylor, 1995). These adaptations include morphological development (specialized leaf anatomy), biochemical metabolism (an additional photosynthetic pathway), and gene expression (cell-type-specific expression of genes encoding C_4 enzymes). The developmental signals and molecular mechanisms that initiate and maintain these C_4 growth patterns are not as yet well understood. We have previously shown that in leaves and cotyledons of *Amaranthus hyochondriacus*, a C_4 dicot, developmental processes can influence the establishment of cell-type-specific C_4 gene expression (Wang et al., 1992, 1993a, 1993b). These processes appear to work independently of light, since cell-specific accumulation of C_4 enzymes and their corresponding mRNAs occurs in dark-grown as well as in light-grown plants (Wang et al., 1993a).

Our previous studies have demonstrated that bundle sheath and mesophyll cell-specific enzymes, and their corresponding mRNAs, show independent patterns of accumulation in young amaranth leaves that are between 5 and 10 mm in length (5–10% of their final expansion) (Wang et al., 1992, 1993b). Leaves at the 5-mm stage appeared to be fully differentiated in terms of overall morphology, vascular development, and differentiation of leaf cell types (including bundle-sheath and mesophyll cells). However, some patterns of C_4 gene expression characteristic of mature amaranth leaves had not yet become established. For the bundle-sheath-specific enzyme RuBPCase, the LSU and SSU were found to be present in both bundle-sheath and

[1] This work was supported by U.S. Department of Agriculture grant no. 93-37306-9041 and by National Science Foundation grant no. MCB 9316806 to J.O.B.

* Corresponding author; e-mail camjob@ubvms.cc.buffalo.edu; fax 1-716-645-2975.

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

mesophyll cells in a "C_3-like" pattern in the 5-mm leaves. Bundle-sheath cell-specific localization of both subunits and their corresponding mRNAs occurred during a particular developmental stage as the leaves expanded from 5 to 10 mm in length. Cell-specific localization was initiated at the leaf tip and then progressed rapidly down the leaf in the basipetal (apex to base) direction, occurring in coordination with the carbon sink-to-source transition. These findings establish a link between photosynthetic carbon metabolism and the establishment of cell-type-specific RuBPCase gene expression in C_4 plants.

Two other C_4 enzymes, PPdK (the enzyme that produces PEP) and PEPCase, both of which function in carboxylation reactions, were localized exclusively to mesophyll cells of the morphologically differentiated 5-mm-long leaves (Wang et al., 1992). Since the cell-specific accumulation of these enzymes occurred before the C_3-to-C_4 transition in RuBPCase gene expression had been initiated, it is likely that developmental processes responsible for establishing mesophyll-specific gene expression act at an earlier stage of leaf development than those responsible for bundle-sheath-specific RuBPCase expression.

The relationship between the very early events of leaf morphogenesis and the initiation of C_4 gene expression has been previously examined in the C_4 monocot maize (*Zea mays*) (Langdale et al., 1987, 1988a, 1988b; Langdale and Nelson, 1991; Nelson and Dengler, 1992). These studies have indicated that the position of a cell within an expanding leaf, relative to a developing vascular center and within the monocot leaf developmental gradient, determines the initial accumulation of C_4 mRNAs and polypeptides. However, many differences exist between C_4 monocots such as maize and C_4 dicots in terms of leaf development, morphogenesis of Kranz anatomy, cellular organization, physiology, and the regulation of genes encoding C_4 photosynthetic enzymes (Gutierrez et al., 1974; Wang et al., 1992, 1993a, 1993b; Long et al., 1994). Therefore, factors involved with initiating C_4 gene expression in dicots may not necessarily be the same as in monocots. Developmental processes and signals associated with the establishment of bundle-sheath cell-specific RuBPCase gene expression have already been shown to differ between these two groups, one significant difference being that the initiation of bundle-sheath cell-specific RuBPCase localization is light-dependent in maize and light-independent in amaranth (Sheen and Bogorad, 1985, 1986, 1987a, 1987b; Langdale et al., 1988b; Wang et al., 1993a, 1993b). Other unique events in early dicot leaf development that influence the initial expression of all of the photosynthetic genes and the establishment of mesophyll cell-specific gene expression might be elucidated by examining the earliest identifiable phase of development, when the leaves are differentiating from the shoot meristem.

The goal of this study was to determine the initial onset of photosynthetic gene expression during the earliest stages of leaf differentiation in the C_4 dicot amaranth. We have examined the expression of genes encoding the bundle-sheath-specific RuBPCase LSU and SSU polypeptides and the mesophyll-specific enzymes PEPCase and PPdK in apical meristems and in very young leaves (2 mm or less in length) that have started to differentiate from these meristems. Our findings indicate that the establishment of mesophyll-specific expression and bundle-sheath-specific gene expression are separate events that occur during different stages of leaf development. Furthermore, distinct regulatory processes determine the cell-type-specific expression of individual photosynthetic genes during very early leaf differentiation in this C_4 dicotyledonous plant.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of *Amaranthus hyochondriacus* var 1023 were germinated, and plants were grown in a Conviron (Asheville, NC) growth chamber at 24°C with 14 h/d illumination at an approximate intensity of 170 to 200 μE m^{-2} s^{-1}. Shoot tips, along with the first, second, and/or third leaves to emerge after the cotyledons, were harvested from the plants 5 to 7 d after planting and immediately placed in fixative (3:1 ethanol:acetic acid).

Immunolocalization Analysis of C_4 Photosynthetic Proteins

Antibodies raised against the LSU and SSU of RuBPCase (Berry et al., 1985) and PEPCase (Wang et al., 1992) have been described previously. Antiserum raised against maize PPdK was generously provided by W.C. Taylor (Commonwealth Scientific and Industrial Research Organization, Division of Plant Industry, Canberra, Australia). Fixed shoot tip and leaf samples were embedded in paraffin, sectioned, and reacted with antiserum 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 a rhodamine-conjugated secondary antibody (Organon Teknika, West Chester, PA).

Sections were analyzed using a confocal imaging system (MRC 1000, Bio-Rad) in conjunction with an epifluorescence microscope (Optiphot, Nikon). A krypton-argon laser was used to produce red excitation at 560 nm (emission filters were 590 nm). Images were digitized and printed on a Codonics NP1600 dye sublimation color printer.

In Situ Localization of mRNAs Encoding C_4 Photosynthetic Proteins

Plasmids used for generating sense and antisense probes to detect mRNAs encoding 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 an anti-digoxigenin antiserum conjugated to rhodamine (Or-

ganon Teknika) according to the manufacturer's recommendations. Hybridized transcripts were analyzed using the confocal imaging system as described above.

RESULTS

Analysis of Early C_4 Gene Expression by Confocal Imaging

In young amaranth seedlings, the first emerging leaf (after the cotyledons) can be detected approximately 5 d after planting. All of the cross-sections used in this study were taken from 5- to 7-d-old amaranth seedlings. Figures 1 and 2 show median sections taken from 6-d-old shoot apical meristems. Sections shown in Figures 3 and 4 are from the base or midsection (midway between the apex and base) of very young, 2-mm-long first, second, or third emerging leaves. Although dicot leaves do not show strong developmental polarity in their overall patterns of cell growth and expansion, a general overall polarity in which lineages of cells are produced that tend to be younger near the leaf base and older near the leaf edge does occur (Sunderland, 1960; Scott and Possingham, 1982; Poethig, 1987; Steeves and Sussex, 1988). The tissues used in this study thus represent three distinct stages of initial C_4 dicot leaf development. The developmental stages shown here occur approximately 2 to 3 d earlier than the more advanced stages described in our previous studies (Wang et al., 1992).

To determine the initial onset of C_4 gene expression, meristems and 2-mm-long leaves were fixed and sectioned for use in immunolocalization and in situ hybridization analysis. During our initial preparations, these young amaranth tissue samples were found to be much more fragile than the older leaf tissues used in our previous studies (Wang et al., 1992, 1993b), and it was often difficult to maintain ideal morphological integrity during sectioning or under conditions used for antibody reaction and RNA probe hybridization. To increase the durability, integrity, and resolution of sections prepared from these tissues, we found it necessary to significantly increase the sectioning thickness from 4 or 5 to 10 μm. These thicker sections maintained the structural integrity of the meristems and early leaves, while still allowing for complete antibody penetration into the tissues.

The precise optical sectioning capabilities offered by confocal laser scanning microscopy make it possible to accurately localize fluorescent probes within thicker sections such as the ones used in this current study, without the blurred haze often associated with standard fluorescent microscopy techniques. For immunolocalization analysis, the sections were first reacted with primary antisera raised against the individual C_4 enzymes and then with rhodamine-conjugated secondary antibodies. For fluorescent in situ hybridization analysis, sections were first hybridized to antisense C_4 gene transcripts labeled with digoxigenin-11-UTP and then reacted with anti-digoxigenin antibodies conjugated with rhodamine. Specific fluorescence signals produced by the secondary antibodies used in both procedures were clearly detected and quantified by the confocal imaging system and are displayed in the figures using a color intensity scale (indicated by a color bar displayed in each of the figures). The strongest reactions are clearly indicated by green or red, and the absence of a specific signal is shown as dark purple.

Production of the two RuBPCase subunits is coordinately regulated during light-mediated development in amaranth at the levels of mRNA accumulation, protein synthesis, and protein accumulation (Berry et al., 1985, 1986, 1988, 1990). Similarly, for each phase of leaf development shown, changes in the accumulation of one RuBPCase subunit polypeptide corresponded to changes in the accumulation of the other subunit; changes in levels of the transcripts encoding the two subunits correlated with each other as well. Data for only one of the two subunits or transcripts are shown for each developmental stage represented in the figures, but these data are representative of both RuBPCase subunits at that stage. As negative controls, representative sections from each developmental phase failed to show any reaction with preimmune serum or sense-strand RNA probes (data not shown).

Localization of C_4 mRNAs and Polypeptides in the Apical Meristems

The structure of the amaranth apical meristem is shown in Figures 1A and 2A. The apical dome (a) can be clearly observed, surrounded by two expanding leaf primordia (l), which will become the second and third emerging leaves of the seedling. Within the apical dome, the small cells of the peripheral tunica layers can be visualized, as well as the larger, more vacuolated cells within the central corpus region.

The two primordia differentiating from the meristem represent the earliest observable stage of leaf development (slightly larger primordia are shown in Fig. 2 than in Fig. 1 to more clearly demonstrate the distinct cellular distribution patterns of the RuBPCase polypeptides). Although some morphological cell differentiation could be discerned within the primordia, the major precursor cell types were identified primarily by their relative position within the developing leaves. In these early tissues, differentiation of the major veins from the procambium had not yet been initiated, and differentiation of the specialized photosynthetic cells had not yet occurred.

RuBPCase *rbc*S (Fig. 1B) and *rbc*L (data not shown) mRNAs were present throughout the meristematic region in the leaf primordia as well as within the apical dome itself. Similarly, mRNAs encoding PEPCase (Fig. 1C) and PPdK (Fig. 1D), two enzymes specific to mesophyll cells of mature amaranth leaves, were present within the meristem itself and in regions surrounding it. RuBPCase and PEPCase transcripts showed a similar distribution pattern throughout the meristem region, being less abundant in the apical dome than in the leaf primordia and more abundant near the leaf apex than near the leaf base. In Figure 1C, significant levels of PEPCase mRNA can be observed in a newly emerging primordium (p), indicating that this transcript is present in leaves from the earliest possible stage of differentia-

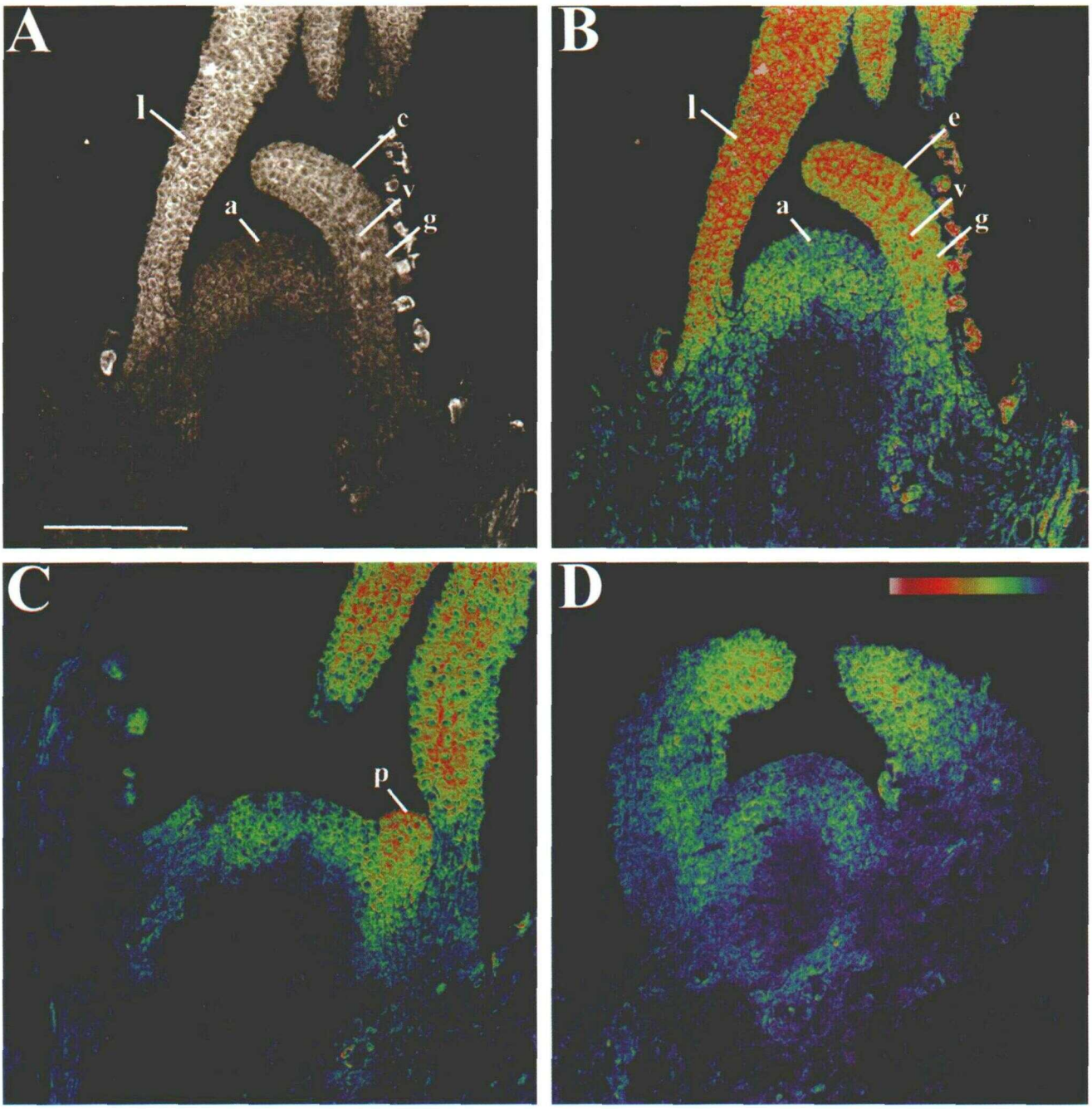

Figure 1. Confocal sections showing C_4 mRNA accumulation in meristems and in leaf primordia. A, Black and white image of the section shown in B showing cellular structures within the meristem. B, Meristem section hybridized to *rbc*S antisense RNA with fluorescence signal converted to a color intensity scale. C, Meristem section hybridized to PEPCase antisense RNA. D, Meristem section hybridized to PPdK antisense RNA. A series of apical meristems median sections from 6-d-old amaranth seedlings were prepared and hybridized with digoxigenin-labeled antisense transcripts and rhodamine-conjugated anti-digoxigenin antibody. The specific reaction of the antibodies was quantified in these micrographs using a color intensity scale, as indicated by the color bar in D. The strongest reactions are indicated by green or red, and the absence of a specific signal is indicated by dark purple. The scale is the same for all micrographs. Bar = 100 μm. a, Apical meristem; l, primordia of the second and third emerging leaves; e, protoderm; v, procambium; g, ground meristem; p, a newly emerging leaf primordium.

tion. PPdK mRNAs were preferentially located in the peripheral zone of the meristem and near the tips of the leaf primordia (Fig. 1D).

Unlike the RuBPCase transcripts, LSU (Fig. 2B) and SSU (data not shown) polypeptides were not detected within the apical dome itself, although significant levels of both subunits were present in tissues surrounding the meristem. Within the leaf primordia, the LSU and SSU were present and displayed an early tissue-specific pattern of distribution. RuBPCase polypeptides did not accumulate in pre-epidermal protoderm cells (e) or in cells of the prevascular procambium (v), but this enzyme was very abundant in

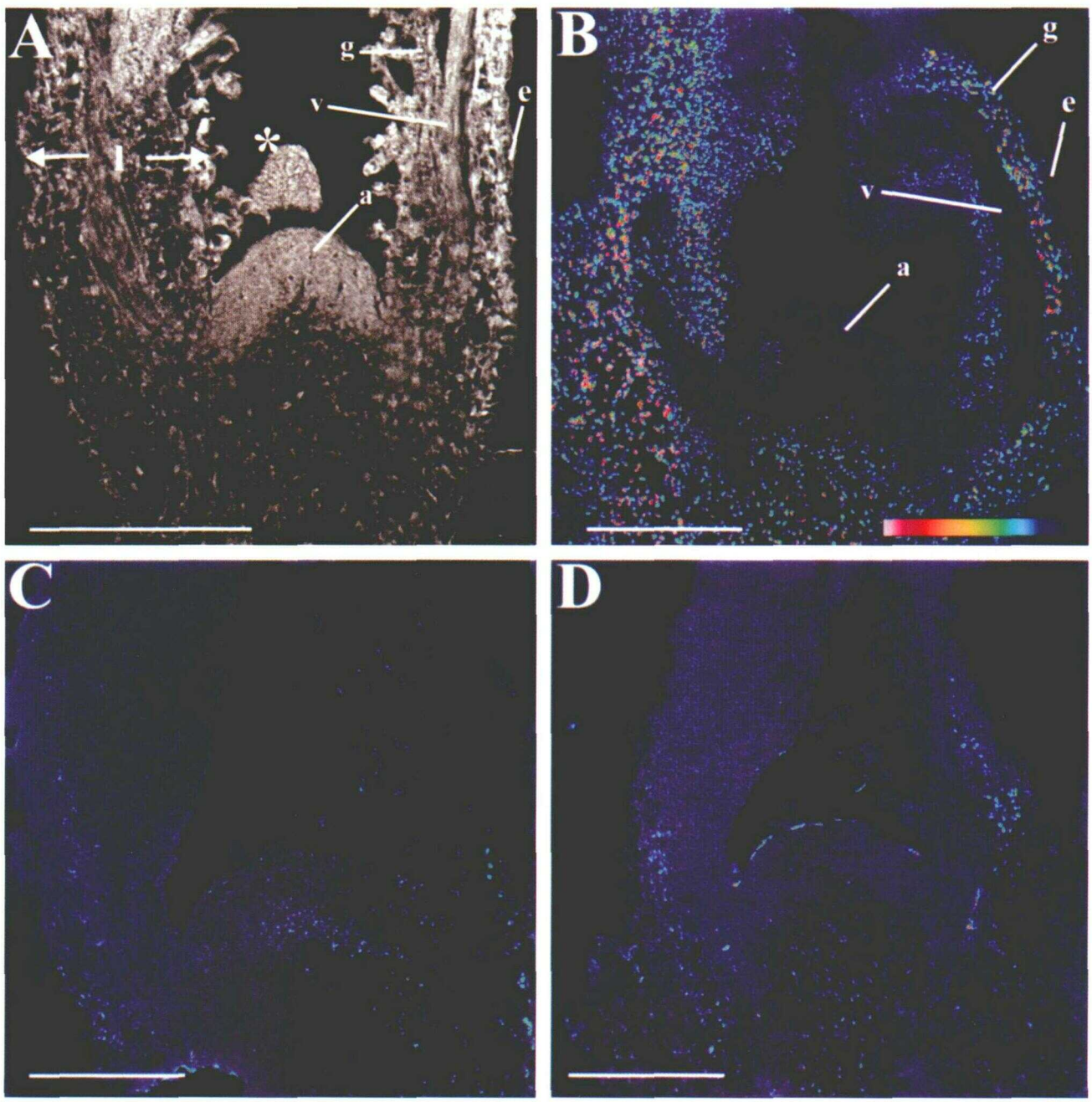

Figure 2. Confocal sections showing C_4 polypeptide accumulation in meristems and in leaf primordia. A, Structure of the amaranth apical meristem, as viewed by autofluorescence. The asterisk (*) indicates a folded over portion of the first emerging leaf and is not part of the meristem structure. B, Meristem section reacted with LSU antiserum. C, Meristem section reacted with PEPCase antiserum. D, Meristem section reacted with PPdK antiserum. A series of apical meristem median sections from 6-d-old amaranth seedlings were prepared and reacted with primary antiserum and rhodamine-conjugated secondary antibody. Specific reactions of the antibodies were quantified and converted to a color scale as described for Figure 1 and are indicated by the color bar in B. The scale is the same for all micrographs. Bar = 100 μm. Meristem tissues are indicated as described for Figure 1.

layers of prephotosynthetic ground meristem cells (g) located between these two tissues.

Figure 2B shows that RuBPCase polypeptides were also present in some very young stem tissues immediately below the meristem, even though their corresponding transcripts were not detected in the equivalent area of Figure 1B. Although we have no definitive answer for the occurrence of RuBPCase polypeptides in these cells, it is possible that they are the products of transcripts present in very low amounts and not detected in our hybridization assays. Alternatively, they might be left over from an earlier stage of development, when these stem cells were located closer to the expanding regions of the meristem and contained RuBPCase transcripts.

In contrast to their corresponding transcripts, PEPCase and PPdK did not accumulate in the apical dome or in any regions surrounding the meristem (Fig. 2, C and D, respectively). For the most part, these enzymes were also undetectable in the early leaf primordia, although very slight amounts of PPdK had begun to accumulate in a few internal cells of one developing leaf (Fig. 2D, right leaf primordium). Therefore, whereas RuBPCase was present in regions immediately surrounding the meristem and in leaf primordia from the earliest stages of development, production of the other C_4 enzymes from their already-present transcripts appears to be delayed until a more advanced stage of leaf development.

All four of the photosynthetic transcripts showed different patterns of distribution than their corresponding polypeptides. For RuBPCase, *rbc*L and *rbc*S transcripts were present in the apical dome and within internal cells of the leaf primordia that correspond to the prevascular procambium, whereas the two subunit proteins were not detected in these tissues. For PEPCase and PPdK, neither polypeptide accumulated in the meristem or in the early leaf primordia, even though their mRNAs were present at very abundant levels. From these findings, it is apparent that at least some photosynthetic gene transcription occurs in the amaranth apical meristem and that there is an initial increase in mRNA production as the leaf primordia begin to differentiate. For PEPCase and PPdK, utilization of the transcripts for production of the enzymes appears to be delayed until later stages of leaf development, providing evidence for early posttranscriptional regulation. In addition, some early patterns of tissue-specific RuBPCase accumulation also appear to be regulated at posttranscriptional levels.

Cellular Localization of C_4 mRNAs and Polypeptides in 2-mm Leaves

Sections taken from the base of 2-mm-long leaves (shown in Figs. 3A and 4, A and C–E) represent a slightly later stage of development than that shown in Figures 1 and 2 and a much earlier stage than those represented by the morphologically differentiated older leaves used in our previous studies (Wang et al., 1992, 1993b). At this stage the overall shape of the leaf blade was just starting to become apparent. However, most of the cells within the new leaf were of a similar size and shape, so that very little morphological differentiation of individual cell types (including bundle-sheath and mesophyll precursor cells) could be detected. The most noticeable region of differentiation was the large central vein at the base of the leaf; some initial stages of vascular differentiation could also be observed further away from the basal region and out toward the leaf edge. Differentiation of individual cell types and of major vascular centers had progressed only slightly further in sections taken midway between the apex and base of the 2-mm-long leaves (shown in Figs. 3, B–D, and 4, B and F–H). As can be observed in Figure 3D, at this slightly later developmental stage there was still very little difference in size or appearance between individual cell types, including bundle-sheath or mesophyll precursor cells.

Transcripts encoding the C_4 enzymes showed very little cell-type specificity during early stages of development in the 2-mm-long leaves. In the leaf base (Fig. 3A) and in the more developmentally advanced leaf midregion (Fig. 3, B and C), the accumulation patterns for all four C_4 transcripts were nearly identical. Transcripts encoding the LSU (Fig. 3A), the SSU (data not shown), PEPCase (Fig. 3B), and PPdK (Fig. 3C) were found at very abundant levels in most, but not all, internal cells of the developing leaf. All four C_4 mRNAs showed reduced levels of accumulation within new regions of vascular differentiation, and very little if any of these transcripts were detected within the central veins or within the more developmentally advanced vascular centers (v). The C_4 transcripts were also not present (or occurred in greatly reduced amounts) in developing sclerenchyma strands(s) immediately below some of the developing vascular centers (adjacent to the lower epidermis) or in most epidermal cells (ue and le). Apart from these specified tissues, the C_4 transcripts showed a very uniform distribution throughout most of the internal cells of the 2-mm leaves.

Unlike their corresponding transcripts, all of the C_4 polypeptides showed cell-type-specific patterns of accumulation in the base and midregions of 2-mm leaves. In the basal regions, the RuBPCase SSU (data not shown) and LSU (Fig. 4C) polypeptides had begun to show a strong cell-specific pattern of accumulation. Antiserum against both subunits reacted most strongly to a clearly observable ring of cells, although very slight amounts of the proteins could also be detected in cells located between the rings. Although there was little morphological cell differentiation observable at this stage, the rings containing high levels of RuBPCase were identified as the bundle-sheath cell precursors based on their position within the leaf and on the similarity of this pattern to that observed in more mature leaves.

The initial appearance of the two mesophyll cell-specific polypeptides, PEPCase and PPdK, occurred at the developmental stage represented in the 2-mm leaf base. PEPCase (Fig. 4D) showed a very strong pattern of cell-specific accumulation from its earliest point of detection. All of the cells showing very high levels of reaction with the PEPCase antiserum were located in layers between the pre-bundle-sheath cells (those containing RuBPCase) and the leaf epi-

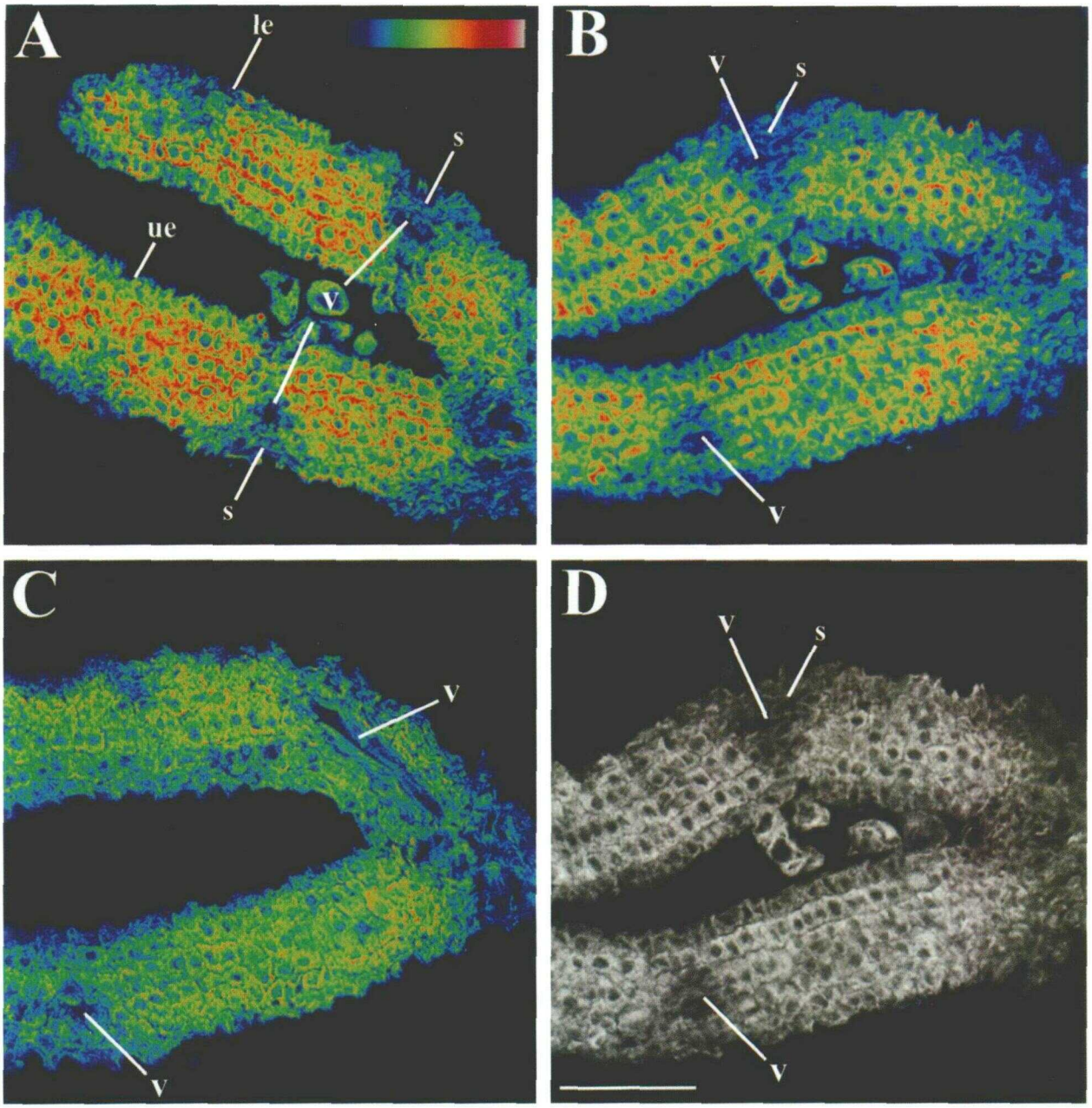

Figure 3. Confocal sections showing C_4 mRNA accumulation in the base and midregions (midway between the leaf apex and base) of 2-mm-long amaranth leaves. A, Two-millimeter leaf base, hybridized to *rbc*L antisense RNA. B, Two-millimeter leaf midregion, hybridized to PEPCase antisense RNA. C, Two-millimeter leaf midregion, hybridized to PPdK antisense RNA. D, Black and white image of the section shown in B showing cellular structure within the developing leaf. A series of 2-mm leaf sections were prepared and analyzed as described for Figure 1. For all micrographs, bar = 50 μm. ue, Upper epidermal cells; le, lower epidermal cells; s, developing sclerenchyma cells; v, developing vascular center.

dermis. These internal cell layers containing very high levels of PEPCase polypeptide were identified as the mesophyll cell precursors based on position and on the similarity of this accumulation pattern to that observed in mature leaves. Unlike PEPCase, the PPdK polypeptide showed little initial cell specificity at this stage of development (Fig. 4E). Although the highest levels of PPdK in the 2-mm leaf base were found in the chloroplasts of premesophyll cells, significant levels of this enzyme were present in the rings of pre-bundle-sheath cells as well.

In the midregions of 2-mm leaves, as in the basal regions, the highest concentrations of RuBPCase LSU (Fig. 4F) and SSU (data not shown) polypeptides occurred in rings of pre-bundle-sheath cells, although significant levels of these polypeptides had begun to accumulate in premesophyll cells as well. These results correlate with previous findings showing significant levels of RuBPCase polypeptides accumulating in mesophyll cells of the older, 5-mm-long amaranth leaves (Wang et al., 1992), although at the earlier stage shown here the polypeptides appeared to show more

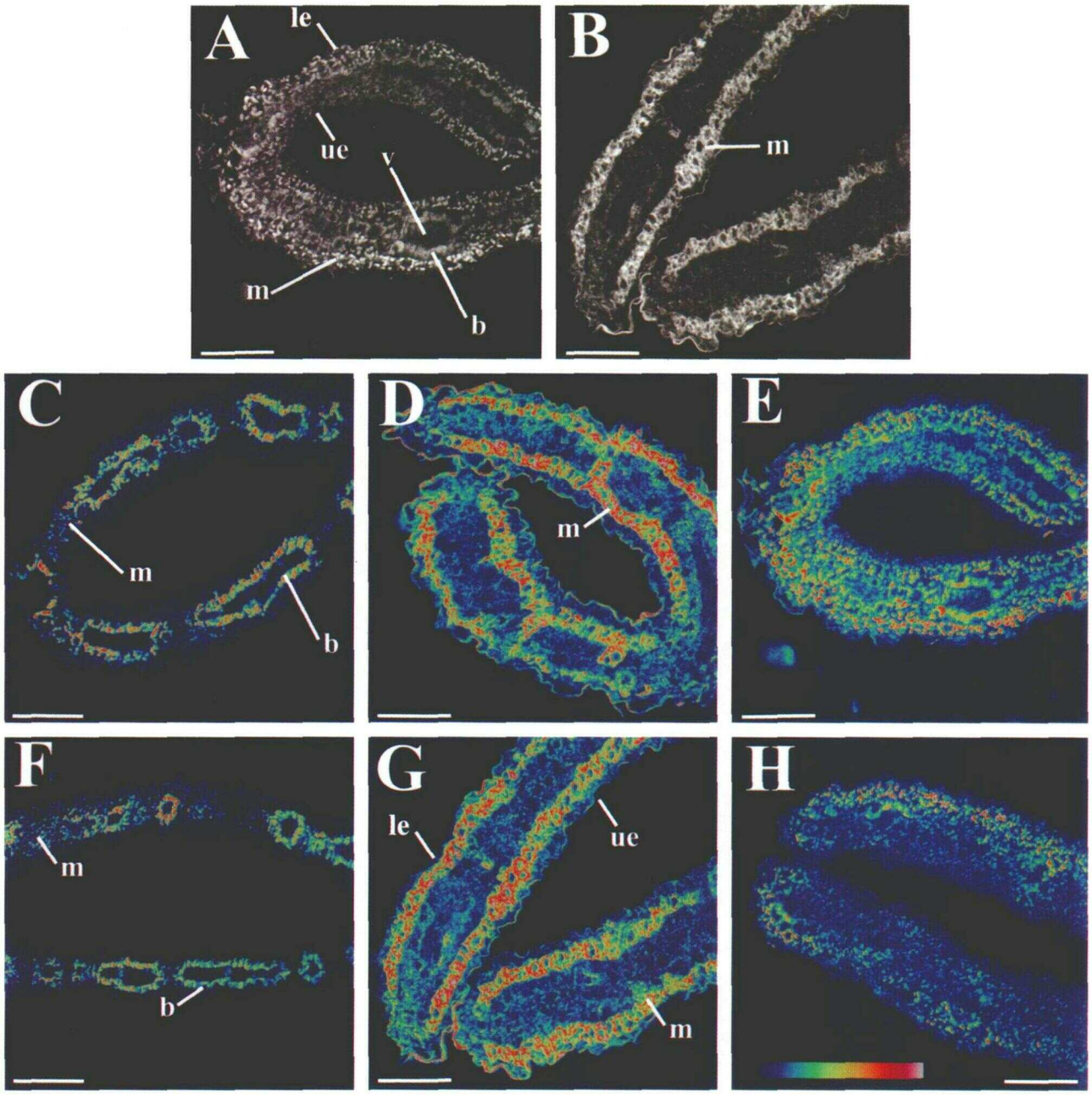

Figure 4. Confocal sections showing C_4 polypeptide accumulation in the base and midregions (midway between the leaf apex and base) of 2-mm-long amaranth leaves. A, Black and white image of the 2-mm leaf base section shown in C to demonstrate the position and structure of pre-bundle-sheath and premesophyll cells (highlighted by the fluorescent PPdK antibody reaction) within the developing leaf. B, Black and white image of a 2-mm leaf midregion section shown in G to demonstrate the position and structure of premesophyll cells (highlighted by the fluorescent PEPCase antibody reaction) within the developing leaf. C, Two-millimeter leaf base, reacted with SSU antiserum. D, Two-millimeter leaf base, reacted with PEPCase antiserum. E, Two-millimeter leaf base, reacted with PPdK antiserum. F, Two-millimeter leaf midregion, reacted with LSU antiserum. G, Two-millimeter leaf midregion, reacted with PEPCase antiserum. H, Two-millimeter leaf midregion, reacted with PPdK antiserum. A series of 2-mm leaf sections were prepared and analyzed as described for Figure 2. For all micrographs, bar = 50 μm. ue, Upper epidermal cells; le, lower epidermal cells; b, pre-bundle-sheath cells; m, premesophyll cells.

cell specificity, being much more abundant in bundle-sheath cells than in mesophyll cells. PEPCase accumulation and specificity remained approximately the same as in the basal regions (Fig. 4G); therefore, reaction to the PEPCase antiserum was observed only in premesophyll cells located midway between the developing vascular centers and the leaf epidermis. Very little, if any, of this enzyme was detected in pre-bundle-sheath cells, developing vascular centers or in epidermal cells. Levels of PPdK polypeptide (Fig. 4H) were significantly reduced in this region when compared with the basal region, possibly as a result of increased chloroplast division and dilution of the accumulated enzyme. In addition, PPdK at this stage showed increased specificity to the premesophyll cells, with the strongest signals detected in premesophyll cells located just beneath the lower leaf epidermis.

The initial cell-specific accumulation patterns of RuBPCase in pre-bundle-sheath cells, or PEPCase and PPdK in premesophyll cells, preceded the morphological differentiation of these cell types. In addition, the cell-specific accumulation of these polypeptides clearly did not correspond with the relatively nonspecific accumulation of the C_4 transcripts during the initial stages of leaf development. Therefore, posttranscriptional control mechanisms appear to operate during the earliest stages of differentiation of both cell types to establish the specific accumulation patterns of C_4 photosynthetic enzymes.

DISCUSSION

The development of C_4 photosynthetic capacity involves changes in the expression patterns of numerous genes. The mechanisms for altering expression are likely to be different for RuBPCase, an enzyme with a photosynthetic function in both C_3 and C_4 plants, and for enzymes such as PEPCase and PPdK, which have a basic metabolic function in C_3 plants but which have been recruited to function as photosynthetic enzymes in C_4 plants. The chloroplast-encoded *rbc*L and nuclear-encoded *rbc*S genes of RuBPCase, which are expressed at very high levels in all plants, are down-regulated in mesophyll cells but not in bundle-sheath cells; therefore, the expression of these genes is restricted to this one specialized photosynthetic cell type.

For genes that encode enzymes with no photosynthetic function in C_3 plants but that have acquired photosynthetic function in C_4 plants, two events must occur. First, expression levels must be greatly enhanced so that these polypeptides can be present at the abundant levels required for their new photosynthetic roles. Second, the expression patterns of these new photosynthetic genes must become selectively restricted so that these enzymes are now present in only one specialized type of cell. If RuBPCase and the other C_4 enzymes are regulated by independent mechanisms or developmental processes, and if the induction of high levels of expression is a separate occurrence from the establishment of cell-specific expression, then it might be expected that these processes could be observed as distinct and separate events during different periods of early leaf differentiation. In this study we examined the expression patterns of genes encoding RuBPCase, PEPCase, and PPdK in meristems, in leaf primordia, and in very young 2-mm-long leaves that have just begun to show morphological, vascular, and photosynthetic cell differentiation. It is during these initial phases of dicot leaf development when mechanisms responsible for the establishment and subsequent maintenance of C_4 gene expression patterns are likely to be initially induced. Taken together with our previous studies (Wang et al., 1992, 1993a, 1993b), these findings present a thorough picture of the establishment of cell-specific C_4 gene expression patterns during specific stages of leaf development, from early primordia through vascular and photosynthetic cell differentiation, in the leaves of a C_4 dicot.

RuBPCase Gene Expression during C_4 Leaf Development

In the C_4 dicot amaranth, the RuBPCase developmental pattern started with LSU and SSU transcripts and polypeptides already present in the meristem region and in the leaf primordia. However, the transcripts and their corresponding polypeptides showed different patterns of accumulation within these tissues. The RuBPCase transcripts occurred in the apical dome and in all of the cells of the early leaf primordia except those of the epidermis, whereas the subunit polypeptides were not present in the apical dome or in cells within regions of the prevascular procambium. Our results showing *rbc*S mRNAs within the apical dome differ somewhat from those reported by Fleming et al. (1993) for *rbc*S gene expression in apical meristem regions of tomato (*Lycopersicon esculentum*) plants. In tomato, transcripts encoding the RuBPCase SSU were found only in the leaf primordia and were not detected in the apical dome. In amaranth, there were lower amounts of *rbc*S mRNA detected in the apical dome than in the developing leaves, and it is possible that the increased sensitivity of the fluorescent probes and the confocal imaging system used in this current study allowed for the detection of very low transcript levels in this region of the meristem. Alternatively, the specialized C_4 *rbc*S genes of amaranth may be controlled differently than those of C_3 plants from the earliest stages of development, even in the undifferentiated cells of the shoot apical meristem.

At the next stage of leaf development (represented in the basal regions of 2-mm-long leaves), the LSU and SSU polypeptides had begun to show an early C_4-type pattern of cell-specific localization. Abundant levels of both polypeptides could be observed in rings of pre-bundle-sheath cells surrounding the developing vascular centers, in a pattern characteristic of older (10 mm or greater in length) amaranth leaves. This pattern of C_4-type polypeptide accumulation remained evident in the developmentally more advanced midregions of the 2-mm amaranth leaves, although by this stage clearly observable amounts of the two subunits had started to accumulate in the premesophyll cells as well. As in the early leaf primordia, the levels of RuBPCase transcript in the 2-mm-long leaves were similar in pre-bundle-sheath and premesophyll cells

and did not correspond with the accumulation pattern of the RuBPCase polypeptides.

The C_4 RuBPCase developmental sequence is complete when the developing leaves are between 5 and 10 mm in length (Wang et al., 1992, 1993b). At this time the leaves simultaneously undergo two developmental transitions, a C_3-to-C_4 transition in which RuBPCase polypeptides and mRNAs completely disappear from the mesophyll cells, thereby becoming specific to bundle-sheath cells, and a sink-to-source transition in carbon transport. These two transitions (the establishment of bundle-sheath cell-specific expression of LSU and SSU genes and the change in carbon transport status) both initiate at the leaf tip and progress rapidly and coordinately in the basipetal (apex to base) direction. These transitions are complete when the leaves reach 10 mm in length (approximately one-tenth of their final expansion). The leaves remain in the C_4 state with regard to RuBPCase polypeptide and mRNA localization throughout maturity (Wang et al., 1992, 1993b; Boinski et al., 1993).

The occurrence of abundant RuBPCase mRNAs in the premesophyll cells of early amaranth leaves (2–5 mm in length) suggests that posttranscriptional regulation is involved in determining the initial cell-specific accumulation of the LSU and SSU polypeptides. Posttranscriptional mechanisms could act in premesophyll cells by preventing the utilization of accumulated transcripts or by rapidly degrading the RuBPCase polypeptides. We have previously shown that RuBPCase gene expression in amaranth is regulated by light at the translational level (Berry et al., 1986, 1988, 1990), and the evidence presented here suggests that translational regulation might also be involved in determining the initial cell-type-specific patterns of RuBPCase accumulation in differentiating leaves. However, this initial cell-specific regulation appears to be somewhat "leaky," since low levels of LSU and SSU polypeptides were observed in the mesophyll cells of the young, 2-mm-long amaranth leaves. We have shown that this non-cell-specific accumulation of LSU and SSU polypeptides that is first observed in early 2-mm leaves continues and in fact increases until the leaves have expanded to 5 mm in length (Wang et al., 1992). The strict bundle-sheath cell-specific pattern of RuBPCase gene expression that is characteristic of older amaranth leaves occurs only following the C_3-to-C_4 transition, when RuBPCase transcripts, as well as the polypeptides, completely disappear from mesophyll cells.

The patterns of RuBPCase gene expression observed during initial leaf development in the C_4 dicot amaranth have some aspects in common with those observed in the C_4 monocot maize (Martineau and Taylor, 1985; Langdale et al., 1988a, 1988b; Langdale and Nelson, 1991), although there are also many differences between these two species. In each system, the initial accumulation of RuBPCase polypeptides occurs prior to the differentiation of bundle-sheath and mesophyll cells and precedes the accumulation of the other C_4 enzymes. In both amaranth and maize, RuBPCase transcripts are present in the leaf primordia before the differentiation of vascular tissue and the morphological differentiation of the specialized photosynthetic cell types. However, in maize the RuBPCase transcripts accumulate ahead of their corresponding polypeptides, whereas in amaranth leaves RuBPCase polypeptides and mRNAs are present from the earliest stages of development. Although both maize and amaranth leaves show a very early period of cell-specific RuBPCase polypeptide accumulation, in amaranth this specificity is not reflective of transcript localization and becomes more reduced as leaf development proceeds. The C_3-like pattern of RuBPCase mRNA localization ends at a much later developmental stage in amaranth leaves than in maize leaves, occurring during a distinct C_3-to-C_4 transition. Although both types of plants display an early C_3-like pattern of RuBPCase gene expression, in maize the establishment of the specialized C_4-type expression pattern requires both developmental and light signals (Langdale et al., 1988b), whereas in amaranth developmental signals associated with the sink-source transition act independently of light to induce the C_4-type state (Wang et al., 1993a, 1993b).

In a recent study, Dengler et al. (1994) found that in the C_4 dicot *Atriplex rosea* RuBPCase polypeptides were specific to bundle-sheath cell precursors almost from their earliest appearance, although there did appear to be a very early and brief C_3-like phase in which the enzyme was present in mesophyll cell precursors as well. Although transcript levels were not examined, the pattern of RuBPCase polypeptide accumulation in *A. rosea* shows similarities with patterns found in both the dicot amaranth and the monocot maize.

PEPCase and PPdK Gene Expression during C_4 Leaf Development

Like the RuBPCase transcripts, mRNAs encoding the mesophyll-specific enzymes PEPCase and PPdK are detected in apical meristems and leaves from the earliest stages of development. However, the production of these polypeptides lag behind the accumulation of their transcripts and show different initial patterns of accumulation. PEPCase and PPdK polypeptides are not detected in apical meristems or in leaf primordia, although their mRNAs are abundant in these tissues. There is a dramatic cell-specific increase in PEPCase accumulation in the basal regions of 2-mm leaves, accompanied by an equally dramatic but non-cell-specific accumulation of PPdK. In the 2-mm leaf midregions, PEPCase polypeptide levels retain the same abundance and cell-specific pattern as in the basal regions, whereas PPdK polypeptides become less abundant and specific to premesophyll cells. It is interesting that PPdK show higher levels of accumulation in premesophyll cells adjacent to the lower epidermal cells than those adjacent to the upper epidermis. Perhaps the pre-spongy mesophyll cells near the lower surface of the leaf develop slightly ahead of the pre-palisade mesophyll cells near the upper surface.

Except for the reduced PPdK accumulation in upper premesophyll cells, PEPCase and PPdK polypeptide accumulation patterns that are first established in midregions of

2-mm-long leaves remain constant as the leaves continue to expand through 10 mm in length and reach maturity (Wang et al., 1993b). However, patterns of PPdK transcript accumulation do change during early development. As the leaves expand from 2 to 5 mm in length, PPdK transcripts disappear from bundle-sheath cells, thereby becoming mesophyll cell-specific. During this same period, PEPCase transcripts remain abundant in both cell types (Wang et al., 1992). These findings suggest that different levels of regulation may work to maintain the cell-type specificity of these two mesophyll cell-specific enzymes once C_4 patterns of gene expression have been established in older amaranth leaves.

Although it is difficult to directly compare leaf developmental stages in monocots and dicots, it is apparent that the early patterns of mesophyll-specific polypeptide accumulation in amaranth leaves share some similarities and many differences with those observed in the C_4 dicot *A. rosea* and in the C_4 monocot maize. For all three C_4 species, the production of the mesophyll-specific polypeptides is initiated after the first appearance of the bundle-sheath-specific RuBPCase polypeptides (Martineau and Taylor, 1985; Langdale et al., 1988a; Wang et al., 1993b; Dengler et al., 1994). However, in maize leaves PEPCase mRNAs are not present in leaf primordia and do not accumulate until the bundle-sheath and mesophyll cells are fully developed. Furthermore, unlike early amaranth leaves, the maize transcripts are mesophyll cell-specific from their earliest period of detection (Langdale et al., 1988a; Nelson and Dengler, 1992).

The expression patterns for PEPCase and PPdK in amaranth leaves indicates that their regulation as C_4 genes does not occur in a single step but that the process has two separate components. The first component of mesophyll cell regulation is an initial, non-cell-type-specific accumulation of transcript encoding these two enzymes in meristems and in leaf primordia. Abundant PEPCase and PPdK transcript accumulation in C_4 plants (relative to C_3 plants) could be accomplished by the addition of more active promoter or enhancer sequences to an already existing metabolic gene and/or by increased stabilization of transcripts. For example, in maize the addition of a new promoter to an already existing nonphotosynthetic form of PPdK gene has led to the differential regulation of overlapping coding regions; therefore, the abundant C_4 photosynthetic form of this enzyme is produced from the same original locus as the less abundant metabolic form of the enzyme (Sheen, 1991). The second component of mesophyll cell regulation is the establishment of cell specificity. This could also result from changes in transcriptional activity, either through the addition of new cell-specific controlling regions or by specifically inactivating existing promoter functions in bundle-sheath cells. The cell-specific expression of the C_4 PEPCase gene of maize appears to be due to regulation at the transcriptional level and is correlated with changes in methylation at a specific site upstream of the gene (Langdale et al., 1991). In contrast, the repression of PEPCase and PPdK polypeptide accumulation in amaranth bundle-sheath cells does not appear to be a result of transcriptional repression, at least initially, since transcripts encoding these enzymes are very abundant in both pre-bundle-sheath and in premesophyll cells, whereas the polypeptides accumulate only in mesophyll cells. It is therefore likely that posttranscriptional processes, either changes in translation or in protein stability, are involved in restricting the localization of PEPCase and PPdK to premesophyll cells during the period when their transcript levels are increasing in both cell types of the very young amaranth leaves.

The Development of C_4 Photosynthetic Capacity

The C_4 pathway is found in many plant species, occurring in both monocots and dicots. Such a wide and diverse distribution implies that the evolution of the C_4 pathway has occurred independently many times (Cockburn, 1983; Hatch, 1987; Furbank and Taylor, 1995). This might further imply that whatever genetic changes are required for the development of the C_4 photosynthetic capacity would be relatively easy to achieve, requiring only a few changes in one or more basic C_3 developmental pathways. However, the findings presented here and elsewhere indicate that C_4 development is much more complex, requiring many independent modifications to the already existing C_3-type expression patterns for numerous metabolic, photosynthetic, and developmental genes. These changes appear to involve multiple levels of regulation, and increasing evidence indicates that posttranscriptional control plays a major role in determining cell-type-specific gene expression in C_4 plants (Boinski et al., 1993; Schaffner and Sheen, 1991). Although specific regulatory genes that control C_4 patterns of development and gene expression have yet to be identified, the elucidation of processes that distinguish development in C_4 plants from development in C_3 plants is a critical step in analyzing the complex mechanisms leading to the functional evolution of the C_4 photosynthetic pathway.

ACKNOWLEDGMENTS

We thank Dr. William Taylor for providing antiserum to PPdK. We are very grateful to Drs. Mary Bisson, Dennis McCormac, Margaret Hollingsworth, and Donald Larson for critical reading of the manuscript, Jim Stamos for assisting with the illustration layouts, and John Long for helpful advice and discussion.

Received December 21, 1995; accepted April 28, 1996.

LITERATURE CITED

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) Translational and post-translational 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

Cockburn W (1983) Stomatal mechanism as the basis of the evolution of CAM and C4 photosynthesis. Plant Cell Environ **6:** 275–279

Dengler NG, Dengler RE, Donnelly PM, Filosa MF (1994) Expression of the C4 pattern of photosynthetic enzyme accumulation during leaf development in *Atriplex rosea* (Chenopodiaceae). Am J Bot **82:** 318–327

Edwards GE, Huber SC (1981) The C4 pathway. *In* MD Hatch, NK Boardman, eds, The Biochemistry of Plants, A Comprehensive Treatise, Vol 8. Academic Press, New York, pp 237–281

Fleming AJ, Mandel T, Roth I, Kuhlemeier C (1993) The patterns of gene expression in the tomato shoot apical meristem. Plant Cell **5:** 297–309

Furbank RT, Taylor WC (1995) Regulation of photosynthesis in C3 and C4 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) C4 photosynthesis: a unique blend of modified biochemistry, anatomy, and ultrastructure. Biochim Biophys Acta **895:** 81–106

Hatch MD, Slack CR (1970) The C4-carboxylic acid pathway of photosynthesis. *In* L Reinhold, Y Liwschitz, eds, Progress in Phytochemistry. Wiley-Interscience, London, pp 35–106

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

Langdale JA, Nelson T (1991) Spatial regulation of photosynthetic development in C4 plants. Trends Genet **7:** 191–196

Langdale JA, Rothermel B, Nelson T (1988a) Cellular pattern of photosynthetic gene expression in developing maize leaves. Genes Dev **2:** 106–115

Langdale JA, Taylor WC, Nelson T (1991) Cell specific accumulation of maize phosphoenolpyruvate carboxylase is correlated with demethylation at a specific site 3kb upstream of the gene. Mol Gen Genet **225:** 49–55

Langdale JA, Zelitch I, Miller E, Nelson T (1988b) Cell position and light influence C4 versus C3 patterns of photosynthetic gene expression in maize. EMBO J **7:** 3643–3651

Long JJ, Wang J-L, Berry JO (1994) Cloning and analysis of the C4 photosynthetic NAD-dependent malic enzyme of amaranth mitochondria. J Biol Chem **269:** 2827–2833

Martineau B, Taylor WC (1985) Photosynthetic gene expression and cellular differentiation in developing maize leaves. Plant Physiol **78:** 399–404

Nelson T, Dengler NG (1992) Photosynthetic tissue differentiation in C4 plants. Int J Plant Sci **153:** S93–S105

Poethig RS (1987) Clonal analysis of cell lineage patterns in plant development. Am J Bot **74:** 581–594

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

Scott NS, Possingham JV (1982) Leaf development. *In* H Smith, D Grierson, eds, The Molecular Biology of Plant Development. University of California Press, Berkeley, CA, pp 223–255

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

Sheen J-Y, Bogorad L (1985) Differential expression of the ribulose bisphosphate carboxylase large subunit gene in bundle sheath and mesophyll cells of developing maize leaves is influenced by light. Plant Physiol **79:** 1072–1076

Sheen J-Y, Bogorad L (1986) Expression of the ribulose 1,5 bisphosphate large subunit gene and three small subunit genes in two cell types of maize leaves. EMBO J **5:** 3417–3422

Sheen J-Y, Bogorad L (1987a) Regulation of levels of nuclear transcripts for C4 photosynthesis in bundle sheath and mesophyll cells of maize leaves. Plant Mol Biol **8:** 227–238

Sheen J-Y, Bogorad L (1987b) Differential of C4 pathway genes in mesophyll and bundle sheath cells of greening maize leaves. J Biol Chem **262:** 11726–11730

Steeves TA, Sussex IM (1988) Patterns in Plant Development. Cambridge University Press, Cambridge, UK

Sunderland N (1960) Cell division and expansion in the growth of the leaf. J Exp Bot **11:** 68–80

Wang J-L, Klessig DF, Berry JO (1992) Regulation of C4 gene expression in developing amaranth leaves. Plant Cell **4:** 173–184

Wang J-L, Long JJ, Hotchkiss T, Berry JO (1993a) Regulation of C4 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 C4 plant. Plant Cell **5:** 289–296