The Plant Cell, Vol. 1, 3–13, January 1989, © American Society of Plant Physiologists

Patterns of Leaf Development in C4 Plants

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REVIEW

INTRODUCTION

The differentiation of cell types in plants depends on the continuous interpretation of positional information. Plant organs are established by patterns of cell division that are often highly variable, yet the final arrangement of cell types within each organ remains the same. A current challenge is to understand the means by which cells of varying clonal history arrive at the same differentiated fate in the development of a plant organ. The ideal system for the study of this developmental control would be an organ with few cell types, with defined patterns of cell origin, and with molecular markers for the differentiation of the individual cell types. The developing C4 leaf is one system that satisfies these requirements. In the leaves of plants capable of C4 carbon fixation, two major photosynthetic cell types—bundle sheath and mesophyll—differentiate as the leaf vascular system is established. The data suggest that bundle sheath (BS) and mesophyll (M) cells must interpret positional information distributed around each vein to express correctly cell-specific genes. Light plays a crucial role in the generation or interpretation of this positional information. This article reviews the available data on the differentiation of these cell types and on the development of the C4 system.

C4 Carbon Fixation

C4 carbon fixation depends on cell-specific gene expression. Neighboring photosynthetic BS and M cells interact in C4 plants to eliminate two inefficiencies of the ribulose bisphosphate carboxylase (RuBPCase)-catalyzed fixation of CO_2. In most (C3) plants, O_2 competes with CO_2 for RuBPCase and the resulting fixed O_2 wastes energy via the process of photorespiration. (For reviews on C4 physiology and biochemistry, see Hatch, 1978; Edwards and Huber, 1981; Edwards and Walker, 1983; Furbank and Foyer, 1988.) C4 plants eliminate this O_2 inhibition by compartmentalizing the O_2-sensitive RuBPCase step away from O_2 in internal BS cells and by saturating RuBPCase with high levels of CO_2. In a two-cell shuttle, CO_2 is first fixed into the C4 acid oxaloacetate by an O_2-insensitive carboxylase (phosphoenolpyruvate carboxylase, PEPCase) in M cells. After conversion to malate or aspartate, the C4 compound is then passed rapidly to neighboring BS cells for release of CO_2 and refixation by RuBPCase. A C3 compound is returned to M cells to balance the cycle. C4 carbon metabolism is generally more efficient than the conventional C3 scheme under conditions of high temperature and light intensity. The C4 system is nearly always associated with Kranz leaf anatomy, in which photosynthetic BS and M cells form successive layers around the veins (Hatch and Osmond, 1976). This anatomy is also associated with increased efficiency in water use and is a distinct advantage in arid conditions. The C4 system costs energy, however, and some studies suggest that certain C4 plants can also function as C3 plants when use of the C3 pathway is energetically favorable (Khanna and Sinha, 1973; Ueno et al., 1988).

The C4 system has evolved independently in many plant families, both monocotyledonous and dicotyledonous, and appears to be a spatial re-regulation of genes for metabolic enzymes also present in plants not using the C4 shuttle (C3-type) (Hatch, 1978). Three interspecific variations of the C4 scheme predominate, which differ primarily in the C4 compound shuttled (malate or aspartate) and in the C4 decarboxylating enzyme in BS cells (NADP-malic enzyme [NADP-ME], NAD-malic enzyme [NAD-ME], or phosphoenolpyruvate carboxykinase [PEP-CK]) (Edwards and Walker, 1983). The NADP-ME subtype of the C4 cycle requires the compartmentalization of at least two enzyme activities in M cells (PEPCase, NADP-malate dehydrogenase [NADP-MDH]) and two enzymes in BS cells (RuBPCase and NADP-ME) (Hatch and Osmond, 1976; Edwards and Huber, 1979; Edwards and Walker, 1983). Another C4 pathway enzyme, pyruvate,Pi dikinase (PPdK), is found at high levels in M cells and at lower levels in BS cells (Aoyagi and Nakamoto, 1985). The other two decarboxylation subtypes exhibit similar cell-specific localization of activities (Hatch, 1978). The corresponding proteins are accumulated only in the correct cell type, as shown in both cell-separation (Huber et al., 1976; Kirchanski and Park, 1976; Broglie et al., 1984; Aoyagi and Nakamoto, 1985; Sheen and Bogorad, 1987b) and immunolocalization experiments (Hattersley et al., 1977; Matsumoto et al., 1977; Perrot-Rechenmann et al., 1982, 1983; Langdale et al., 1987).

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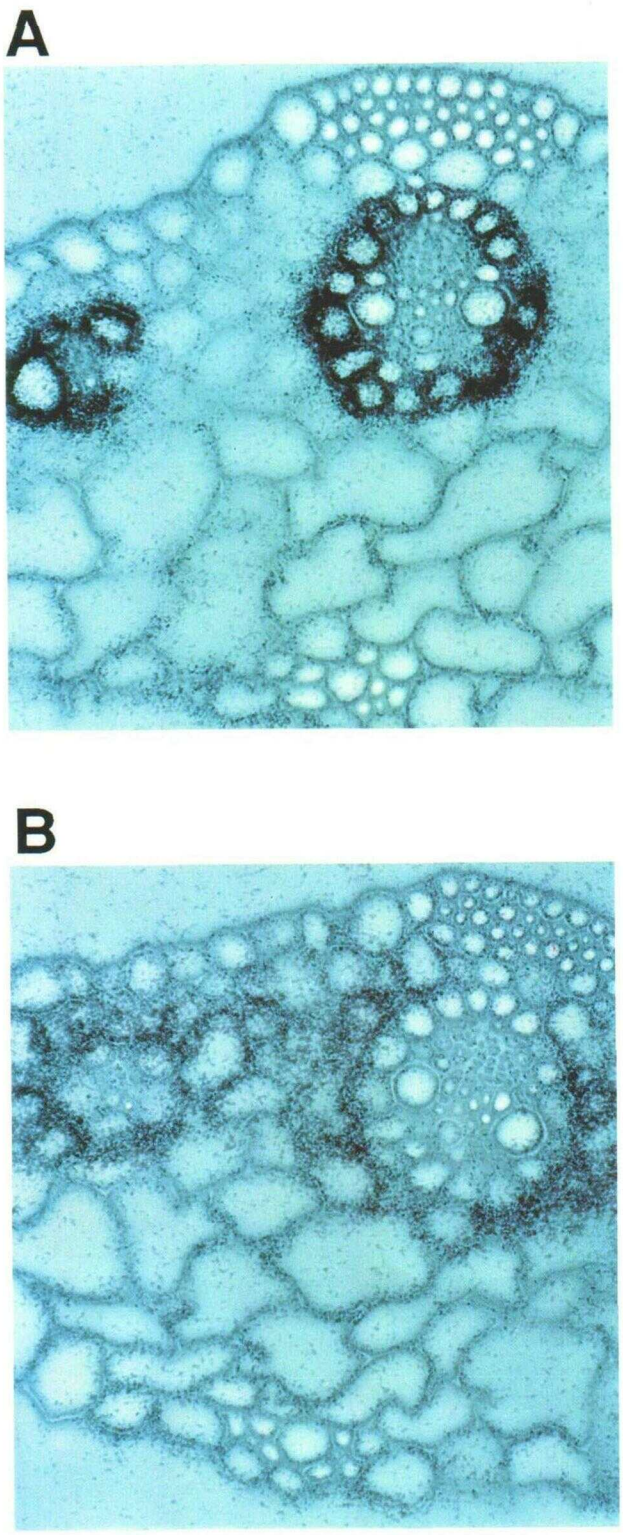

Most of the C4 genes have been cloned (Hague et al., 1983; Nelson et al., 1984; Sheen and Bogorad, 1986, 1987b; Harpster and Taylor, 1986; Hudspeth et al., 1986; Izui et al., 1986; Cretin et al., 1988; Langdale et al., 1988a). Several of these cloning studies have suggested that C4 genes are members of small gene families whose individual members have distinct metabolic roles and localizations. Several groups have now shown by cell separation (Link et al., 1978; Broglie et al., 1984; Martineau and Taylor, 1985, 1986; Sheen and Bogorad, 1987a, 1987b) and in situ hybridization (Martineau and Taylor, 1986; Langdale et al., 1988a) that the basis for the compartmentalization of activities is the cell-specific expression of C4 genes in BS or M cells. Figure 1 shows an example of the cell-specific localization of two of the C4 mRNAs. How is C4 gene expression spatially regulated? How is the development of C4 gene function linked to the development of Kranz anatomy? The following pages summarize our current understanding of these topics and highlight areas deserving further attention.

Ontogeny of Photosynthetic Cells

Leaves are derived from the outer two layers (monocots) or three layers (dicots) of the shoot apical meristem (for review, see Steeves and Sussex, 1988). This derivation is most obvious in periclinal chimeras, in which cells of different apical layers are genetically distinct (Tilney-Bassett, 1986; Poethig, 1987). Cell divisions in the leaf primordium are primarily anticlinal, such that derivatives of the outermost (L1) layer of the shoot apex form the leaf epidermal layers, whereas derivatives of the apical subsurface (L2 and L3) layers form the mesophyll. Certain cells of all three layers undergo photosynthetic differentiation, although the L1 photosynthetic derivatives usually include only stomatal guard cells in the epidermis and some mesophyll at the leaf margins. The conservation of cell layers through anticlinal division patterns is seen both in the orientation of cell divisions in histological sections and in sectorial chimeras, in which clones of leaf cells are genetically marked (Tilney-Bassett, 1986). The meristematic activities that generate most cells of monocot leaves are located at the base of each leaf primordium, and more or less regular files of cell clones extend from the base. However, in dicots, such as tobacco (Poethig, 1984) and cockleburr (Maksymowych, 1973), intercalary cell divisions occur

Figure 1. In Situ Localization of C4 mRNAs Around the Midrib of a Maize Leaf.

(A) BS cell-specific compartmentalization of RuBPCase Large Subunit (LSu) mRNA.

(B) M cell-specific compartmentalization of PEPCase mRNA. The large cells below the veins are non-photosynthetic. Tissue was taken from the middle of a third leaf blade and embedded in paraffin. Sections were hybridized with ^{35}S-labeled antisense RNA probes and autoradiographed for 16 and 72 hr, respectively.

throughout the developing leaf. Further leaf growth, through expansion and elongation of existing cells, preserves the layered structure of the organ.

Photosynthetic Differentiation

Photosynthetic differentiation occurs according to cell position. Experiments with both spontaneous and radiation-induced sectors have shown that occasional periclinal divisions contribute cells from one layer to an adjacent layer (Stewart and Derman, 1975, 1979). In every case, these cells differentiate according to their position, rather than their cell layer history. Cells derived from the L1 layer, which would ordinarily form epidermal cells, can undergo periclinal divisions to generate cells that differentiate as photosynthetic mesophyll cells. The importance of positional information in photosynthetic differentiation is also illustrated in the epidermal cell layer. Non-photosynthetic epidermal cells generate photosynthetic guard cells at regularly spaced positions (Sachs, 1984). The next sections consider the establishment of positional landmarks that influence the differentiation of BS and M cells in C4 leaves.

Leaf Organization

Monocot and dicot leaves differ in their spatial organization. The monocot leaf is polarized in both cell division and expansion. In monocots such as maize (C4) and wheat (C3), a basal meristem generates files of cells that remain aligned from the base to the tip of the leaf (Poethig, 1984). Clonal and histological studies in maize have demonstrated that only the outer two layers (L1 and L2) of the shoot apex contribute to leaf primordium (Poethig, 1984). A central third layer is generated subsequently and maintained within the primordium (Sharman, 1942; Esau, 1943; Langdale et al., 1989). Files near the midvein tend to run from base to tip, whereas more marginal files fan out toward the leaf edges. Cellular differentiation proceeds in a basipetal direction. Cells at the tip (the oldest) are the most photosynthetically developed in a young leaf, whereas leaf segments at more basal locations contain cells at progressively younger stages of differentiation (reviewed in Leech, 1985). Many workers have taken advantage of this basipetal gradient of cell maturation in monocot leaves for studies of plastid development, photosynthetic development, and morphological development of cell types (reviewed in Leech, 1985).

The dicot leaf is less polarized in its cell division patterns. Three layers of the dicot shoot apex (L1, L2, and L3) contribute to each leaf primordium (Steeves and Sussex, 1988). Clonal analyses in tobacco have shown that divisions generate files of cells that are generally polarized from the base to leaf edges (fan-like), but with further intercalary anticlinal divisions occurring in all directions (reviewed in Poethig, 1984, 1987). This pattern makes the dicot leaf a spatial mosaic of cell ages rather than the simpler gradient observed in monocots. This mosaicism has been particularly well illustrated in the work of Maksymowych (1973) on *Xanthium* (cockleburr). Despite this age mosaicism, cells of the dicot leaf also tend to mature in a basipetal direction.

Kranz Anatomy

Concurrent with leaf expansion, the vascular system and its derivative cells are generated by divisions throughout the leaf mesophyll (itself a derivative of the L2 and L3) that define procambial or vascular meristems (reviewed in Aloni, 1987). The timing and placement of these procambial divisions are of particular importance to C4 plants, yet in few of these plants have vein spacing and initiation been studied. There are abundant data implicating plant growth regulators, and auxins in particular, in vascular differentiation, but little is known of the initiation of localized cell divisions that give the vein a discrete position. It is assumed that polar, channeled transport of auxin leads in some way to the formation of veins, since, for example, vascular wounds regenerate most rapidly on the high side of an auxin gradient. However, the means by which such "channels of stimulus" might assume a regular spacing and by which they might act on individual cells are at present unknown.

The vascular system is established in a hierarchical fashion as the leaf develops. The initiation and organization of veins have been described in detail in the case of the monocot maize (Sharman, 1942; Esau, 1943; Russell and Evert, 1985). In maize, the midvein is established first by divisions progressing through the primordium in an acropetal direction. Other major bundles ("laterals") are established on each side, differentiating both acropetally toward the tip of the leaf blade and basipetally into the leaf sheath. As the leaf increases in width, first intermediate and then minor veins are initiated in the increasing spaces between each previous set of veins. These, and the small transverse veins which subsequently interconnect the three parallel types into a network, differentiate basipetally. The procambial divisions that generate major (early) veins are induced in alignment with files of cells generated from the leaf base, whereas intermediate and minor veins are discontinuously initiated and are polyclonal (Langdale et al., 1989). The mature monocot leaf is therefore divided lengthwise by parallel elements—laterals, intermediates, and minors—of at least three ages. This hierarchy is reflected in the progressive differentiation of photosynthetic cells around each vein type (Langdale et al., 1987). In dicot leaves, the pattern of the veins is an irregular network, with minor veins joined to majors as tributaries rather than running parallel to them (Fahn, 1982).

In C4 monocots and dicots, the vascular system is the framework around which two photosynthetic cell types are arranged in a pattern referred to as Kranz anatomy

(Laetsch, 1974; Brown, 1975; Edwards and Walker, 1983). Each vein, whether major or minor, is surrounded by a photosynthetic BS. Photosynthetic M cells and air spaces occupy the remaining mesophyll space. The leaves of C4 plants are highly vascularized, often with veins separated by as few as four photosynthetic cells (vein-BS-M-M-BS-vein). Although a BS layer of photosynthetic cells exists in some C3 plants, the BS in C4 plants is distinguished by the presence of thick cell walls, numerous chloroplasts (which are nearly agranal in some species, such as maize), the accumulation of starch, and the accumulation of certain photosynthetic enzymes (Laetsch, 1974; Brown, 1975). The remaining mesophyll cells of C4 leaves are similar in appearance to the chlorenchymal cells of C3 plants, with numerous granal plastids. The plastids in BS cells may be arranged centripetally, centrifugally, or distributed throughout the cytoplasm relative to the veins the BS cells surround. C4 dicots generally exhibit centripetally arranged BS chloroplasts. In C4 grasses, the three arrangements of plastids can be correlated with the three decarboxylation types. Plastids of the NADP-ME type are arranged centrifugally, those of the NAD-ME type centripetally, and those of the PEP-CK type distributed throughout (Edwards and Walker, 1983). The physiological importance of this correspondence in the *Gramineae* is unknown.

In maize and sugarcane, the plastids of both BS and M cells appear morphologically similar early in development and then gradually become distinct (Laetsch and Price, 1969; Kirchanski, 1975). This has been summarized as a "de-differentiation" of chloroplasts in BS cells because a reduction in the stacking of grana is one element of this differentiation. However, as will be discussed below, the plastids of both BS and M cells undergo specialized development. Plasmodesmata become abundant on the cell wall separating neighboring BS and M cells (Evert et al., 1977), presumably to expedite intracellular transfer of metabolites. C4 metabolism is virtually always associated with the development of Kranz anatomy (Brown, 1975; Edwards and Walker, 1983), although C3 plants have been described with Kranz anatomy (Edwards and Ku, 1987), and some C4 plants have considerable variations on the basic Kranz scheme (Olesen, 1974; Shomer-Ilan et al., 1975). At present, all plants characterized with true C4 metabolism distribute steps of the pathway between specialized BS and mesophyll cells (Hatch and Osmond, 1976), although BS and M plastids are not always dimorphic (Laetsch, 1974).

Bundle Sheath Ontogeny

The bundle sheath can arise from ground or vascular leaf meristems. Since the specialized bundle sheath is a distinguishing feature of C4 plants and varies in morphology with different C4 biochemical subtypes, it is of interest to understand its ontogeny in various C4 groups. Histological comparisons of C4 grass species have revealed that photosynthetic BS cells can arise from divisions in the procambium or in the "ground" cells of the mesophyll. This difference is correlated with the presence of a single or double sheath around veins (Brown, 1975; Dengler et al., 1985). C4 grasses of the NADP-ME type always have a single BS that appears to be derived from the procambium. NAD-ME and PEP-CK subtypes, however, exhibit a double sheath. In these species the inner mestome sheath, which is non-photosynthetic, is derived from the procambium, but the outer sheath, which is photosynthetic, is derived from the ground mesophyll cells. These differences suggest that the developmental pathways of BS and M cells are distinct much earlier in single sheath species than in double sheath species. However, when this hypothesis was tested using *Panicum effusum* (NAD-ME, M, and BS derived from ground meristem) and *P. bulbosum* (NADP-ME, BS from procambium, M from ground meristem), very few differences were observed in the timing of photosynthetic development (Dengler et al., 1986).

Two more general approaches have been taken to study the interdependence of Kranz anatomy with gene expression in C4 plants. In one approach, species that display physiology and anatomy intermediate to C3 and C4 species have been analyzed. In a second approach, the ontogeny of the C4 system has been compared with the appearance of Kranz anatomy. Each of these approaches has been useful in describing the spatial control of the C4 system.

C3-C4 Intermediates

C3-C4 intermediate plants show correlated differences in anatomy and gene expression. Species with anatomical and physiological characteristics intermediate between C3 and C4 exist in many plant families (reviewed in Rathnam and Chollet, 1980; Edwards and Ku, 1987). Models for the stepwise evolution of the C4 system from C3 ancestors have been proposed that postulate that the characteristics of these intermediate species represent evolutionary steps between C3 and C4 plants (Powell, 1978; Moore, 1982; Edwards and Ku, 1987). Certain genera (e.g. *Flaveria*) contain C3, C4, and C3-C4 intermediate species and therefore provide the opportunity to study the concordance of changes in anatomy with physiology among related species. Comparisons thus far have been primarily at the physiological level. Among *Flaveria* species, there is not a direct correlation between degree of Kranz anatomy and C4 physiological characteristics, such as reduction of photorespiration. However, these species might be of considerable use in understanding the spatial control of the C4 genes and their C3 counterparts. For example, the C3-C4 intermediate *F. brownii* exhibits Kranz anatomy with typical thick-walled BS cells surrounded by two layers of M cells. PEPCase and RuBPCase are abundant in *F. brownii* leaves

but incompletely compartmentalized. Rather, there is a gradient of PEPCase activity in the three cell types decreasing toward the nearest vein and a gradient of RuBPCase activity increasing toward the nearest vein (Edwards and Ku, 1987). Clearly, these species provide potentially useful variations in the spatial control of C4 genes that should be further exploited.

Developmental Regulation of C4 Genes

The C4 system and Kranz anatomy develop coordinately during leaf ontogeny. Another approach used to study the spatial regulation of the C4 genes and BS/M differentiation is to follow the appearance of anatomical, physiological, and gene expression features during leaf ontogeny. For such studies, the RNAs and proteins responsible for C4 activities satisfy the requirements for BS and M cell-differentiation markers; they are easily detected at the time of morphological differentiation, are stable, and are cell-specific. The appearance of C4 activities and the corresponding proteins and mRNAs has been established relative to the morphological development of Kranz anatomy in studies of whole leaves, leaf age gradients, differentiating callus, and leaf sections.

Whole-leaf developmental studies have included analysis of leaves of increasing age and of the same leaf at various developmental stages. The appearance of C4 activities has been measured in leaves 1 to 5 of maize seedlings (Crespo et al., 1979). In this study, the first leaf initiated (leaf 1) was more C3 in character, while the last measured (leaf 5) was fully C4. Other workers (Kennedy and Laetsch, 1973; Khanna and Sinha, 1973; Williams and Kennedy, 1977; Imai and Murata, 1979; Thiagarajah et al., 1981; Moore et al., 1986) have shown in a variety of C4 species that relative use of the C4 and C3 scheme varies from young to mature to senescent leaves of the same plant, showing that the C4 system is a continuously regulated one. Combined with studies on the appearance of Kranz anatomy in developing leaves (Miranda et al., 1981a; Dengler et al., 1985), these whole-leaf physiological and biochemical studies indicate that the system appears at a time consistent with the maturity of Kranz anatomy and suggest that exclusive use of the C3 photosynthetic pathway may occur prior to the full differentiation of Kranz anatomy.

A second approach has been to exploit the developmental age gradients in monocot leaves. Such studies have characterized the time of appearance of C4 activities (Williams and Kennedy, 1978; Perchorowicz and Gibbs, 1980; Miranda et al., 1981b), proteins (Mayfield and Taylor, 1984; Martineau and Taylor, 1985; Langdale et al., 1987), and mRNAs (Martineau and Taylor, 1985; Langdale et al., 1988a). These studies suggest that C4 function occurs early in the region of leaf blade expansion and is present to lesser extents in the sheath region. Levels of C4 proteins increase toward the tip of the leaf, whereas levels of the corresponding mRNAs peak near the base of the blade and decrease toward the tip, as shown in Figure 2. The regions displaying detectable C4 function and C4 enzymes in the developing leaf are already well vascularized.

Callus cultures derived from C4 plants do not exhibit a functioning C4 pathway until cells organize into shoots and vascular development is underway. A correlation between vascularization and C4 protein accumulation was made by Aoyagi and Bassham (1986), who showed that C4 enzymes do not appear in green regenerating maize callus until some vascularization has occurred. In contrast, callus cultures derived from the C4 dicot *Portulaca oleracea* exhibited reduction of photorespiration without obvious vascularization or Kranz anatomy (Kennedy, 1976). However, the reduction in photorespiration may have been due to intracellular mechanisms analogous to those functioning in certain C3-C4 intermediates, without C4 carboxylation and cellular compartmentalization (Edwards and Ku, 1987). Other green callus or suspension cultures from C4 plants were found to contain C4 enzymes (Laetsch and Kortschak, 1972) and to fix carbon dioxide into C4 primary products (Seeni and Gnanam, 1983; Usada et al., 1971), but without full function of the C4 pathway. These studies suggest that Kranz anatomy is an essential element for the efficient accumulation of C4 enzymes.

Immunolocalization methods have been used to visualize the cellular distribution of C4 enzymes in leaf sections of varying developmental age (Langdale et al., 1987). These studies have revealed that C4 protein accumulation is always associated with a mature vein and BS anatomy, even in relatively young (basal) regions of a developing maize leaf. When photosynthetic development is retarded, as in the mutant *argentia*, BS and M cells accumulate C4 proteins first around major veins, then around intermediate veins, and finally around minor veins. This suggests that BS and M development is controlled locally, within a distance of a few cells, or that cells must attain a particular age before differentiating. The process is stochastic, with individual BS and M cells or cell clusters able to accumulate all cell-specific C4 proteins before neighboring cells accumulate any. In similar immunochemical studies, mutants with clonal sectors of defective cells adjacent to normal cells show slight neighbor effects across sector boundaries, but mutant cells do not prevent normal neighbors from differentiating (Langdale et al., 1989). For example, an M cell can accumulate C4 enzymes adjacent to a non-photosynthetic BS cell. This suggests that the signal to accumulate C4 proteins is either positional or involves a non-photosynthetic metabolic interaction with the BS cell neighbor.

Recently, analogous in situ hybridization experiments have permitted the visualization of the cellular pattern of C4 mRNA accumulation during development (Langdale et al., 1988a). Certain C4 genes are expressed concurrent with provascular cell divisions, but before extensive

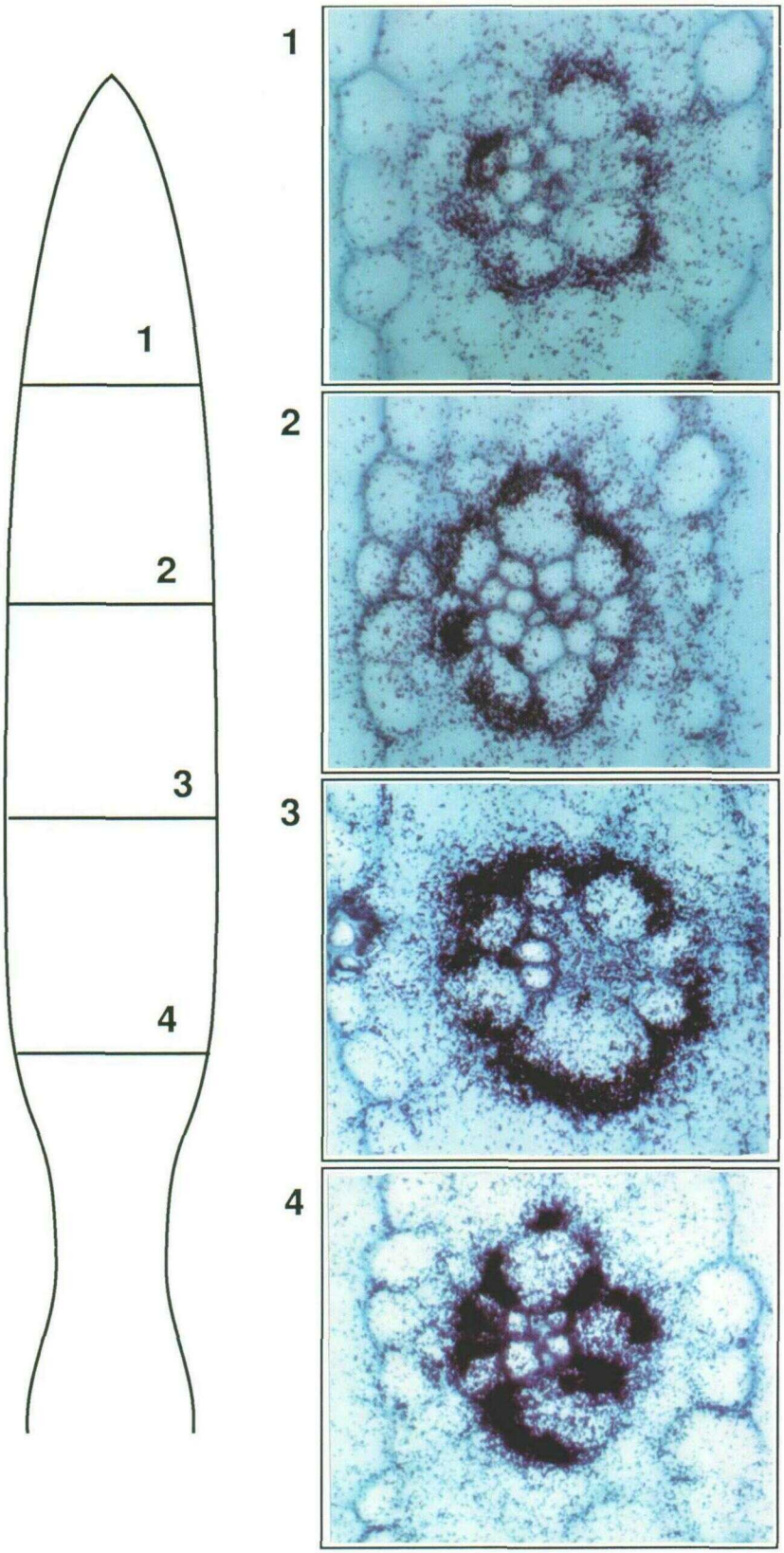

Figure 2. RuBPCase LSu mRNA Accumulation in the Developing Maize Leaf.

Sections were taken from the length of leaf 3, as indicated, and hybridized with a ^{35}S-labeled antisense RNA probe. Steady-state levels of LSu mRNA decrease toward the tip of the leaf.

vascular differentiation. RuBPCase large and small subunit genes are expressed in a ring of cells tightly surrounding the region of provascular cell divisions, as exemplified in Figure 3. It is not yet known which of the several RuBPCase genes (Sheen and Bogorad, 1986) are expressed at this time. The NADP-MDH gene is expressed locally in the same region, but at a greater radial distance from the vein. This position-specific expression occurs at a time in the leaf primordium when neither BS nor M is morphologically distinct and before chloroplasts are distinguishable. Expression of the genes for PEPCase and NADP-ME reach equivalent local levels of expression when the veins are further differentiated. These studies show that positional control of BS and M photosynthetic development must begin very early in the leaf primordium, concurrent with or just after the initiation of veins. At this time Kranz anatomy is not yet evident.

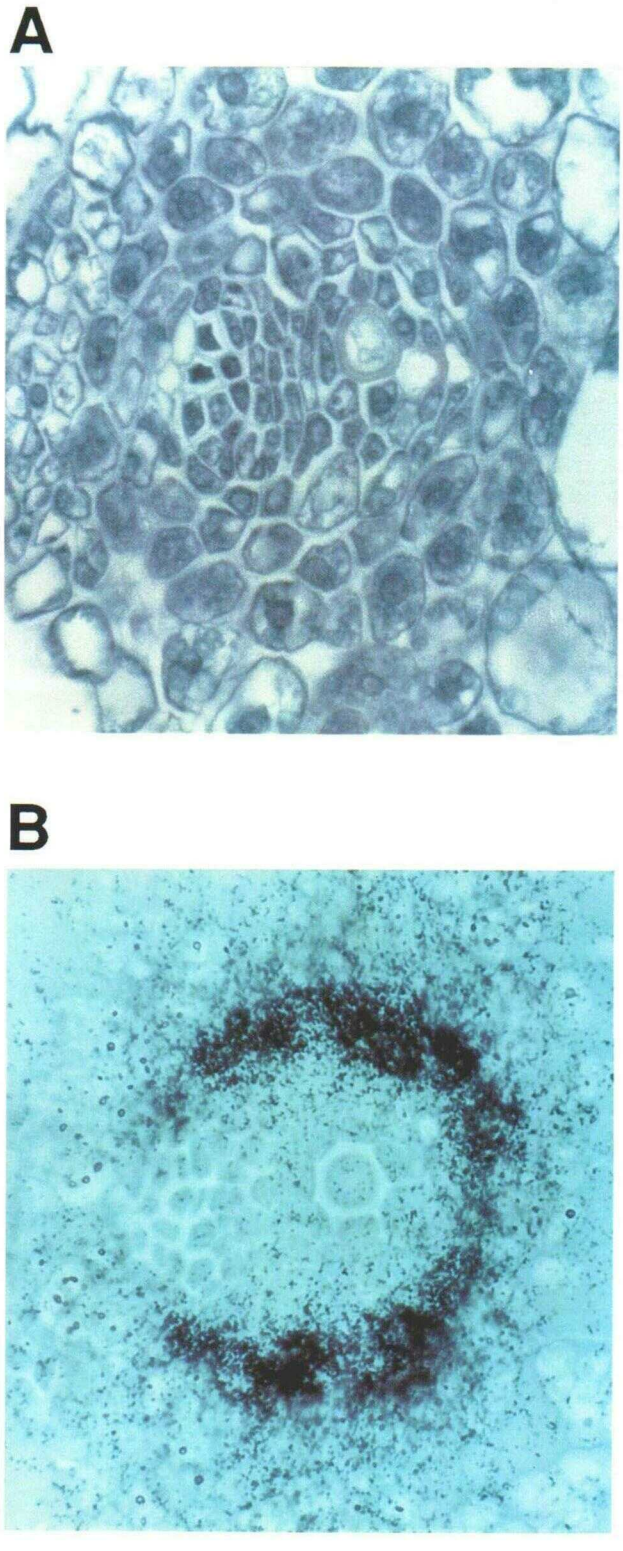

Figure 3. Accumulation of RuBPCase mRNA around Provascular Tissue in the Maize Leaf Primordium.

(A) Fast Green-stained section around the midvein of a developing leaf primordium.

(B) Serial section of **(A)** hybridized with a ^{35}S-labeled LSu antisense RNA probe. LSu mRNA is localized in the ring of cells immediately adjacent to the provascular tissue. Kranz anatomy is not apparent.

Spatial Regulation

C4 gene expression depends on positional signals. Another surprising observation from in situ studies is that the expression of the C4 system is concentrated near veins. In a recent study (Langdale et al., 1988b), a variety of leaf types on the maize plant were compared with respect to the development of BS and M cells and the functioning of the C4 pathway. These studies included the foliar leaves used in earlier experiments, as well as husk leaves, coleoptiles, glumes, and other structures exhibiting Kranz anatomy with altered vein spacing patterns. Husk leaves, for example, may have as many as 20 photosynthetic cells between adjacent veins, whereas foliar leaves have only four. The general observation, exemplified in Figure 4, was that C4 development occurs adjacent to veins in all leaf types in equivalent illumination, but that, beyond a several-cell radius, M cells develop as conventional C3 chlorenchyma. The more distant M cells express the genes for RuBPCase and light-harvesting chlorophyll-protein complex (as do normal C3 cells), whereas vein-proximal M cells express PEPCase, PPdK, and NADP-MDH (as do C4 M cells). These experiments suggest that positional control of M cell development acts locally within a small radius of each vein and that C3-type photosynthetic development is the default scheme. Physiological measurements of oxygen inhibition of photosynthesis suggested that the observed patterns of gene expression reflected the function of the C4 carbon fixation pathway near veins and the C3 pathway at more distant locations (Langdale et al., 1988b).

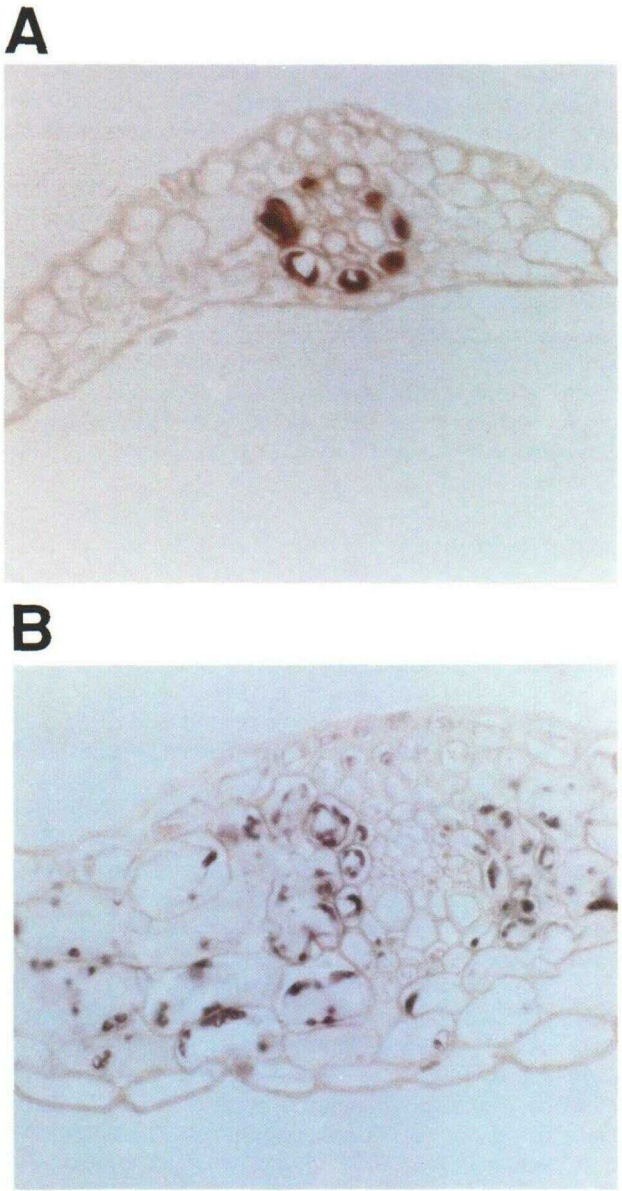

Figure 4. Immunolocalization of RuBPCase in the Glumes of Normal and *Tunicate* Maize.

Paraffin-embedded sections were reacted with primary antiserum and a biotin-streptavidin-peroxidase detection system. In normal glumes **(A),** RuBPCase accumulates specifically in the BS. In these structures, adjacent vascular bundles are separated by strings of epidermal cells such that all M cells are close to a vein. In glumes of the maize mutant *Tunicate* **(B),** however, adjacent veins are separated by up to 20 M cells. This altered morphology is correlated with the accumulation of RuBPCase in both BS and M cells.

Light Regulation

C4 positional regulation depends on light. Steady-state levels of C4 enzymes and their mRNAs increase several-fold if dark-grown plants are illuminated (Nelson et al., 1984; Sheen and Bogorad, 1987a, 1987b). This is an increase from low levels that are developmentally induced even in darkness. Sheen and Bogorad (1985) observed that the low levels of RuBPCase found in dark-grown maize seedlings represent accumulation in both BS and M cells. If dark-grown plants are illuminated, RuBPCase accumulation is restricted to BS cells and M-localized RuBPCase is turned over. This suggests that illumination is an essential component of the positional system that represses RuBPCase in M cells of C4 plants.

The light dependence of BS- and M-specific gene expression has been further examined in situ (Langdale et al., 1988b). In this study, maize husk leaves with widely spaced veins were allowed to develop under various levels of illumination. In low light, RuBPCase accumulates only in M cells, and other C4 enzymes are absent. In high light, cell-specific C4 enzymes accumulate, principally in cells close to veins. This is consistent with earlier measurements of photosynthetic enzyme levels in maize plants grown under high and low levels of illumination (Bassi and Passera, 1982). These suggested that development in low light levels favored accumulation of the C3 fixation enzyme RuBPCase, whereas higher light levels resulted in greater levels of both RuBPCase and the C4 fixation enzyme PEPCase. In the C4 dicot *Amaranthus*, suppression of RuBPCase in darkness appears to occur at a translational level, rather than at the transcriptional level observed in maize (J. Berry and D. Klessig, personal communication). All of these studies suggest that light has a role both in stimulating overall levels of the C4 gene transcripts and proteins and in suppressing transcription or translation (at least of RuBPCase) in the "incorrect" cell type.

The available evidence suggests that the positional control of BS- and M-specific gene expression is at least partly a negative control. In low light or darkness, RuBPCase accumulates in M and BS cells of C4 plants, yet at higher light levels, expression in M cells is suppressed. The spatial distribution of cells expressing RuBPCase in the leaf primordium suggests that a signal is centered at each developing vascular bundle. This signal may be the same as the one that establishes vein location initially. Other C4 enzymes, including PEPCase, NADP-MDH, and NADP-ME, are expressed abundantly only at high light levels and are cell specific. As reported by others (Aoyagi and Nakamoto, 1985), PPdK is expressed only at high light levels, predominantly in M cells, but also in BS cells.

The light-dependent repression of RuBPCase in M cells but not in BS cells probably represents complex molecular interactions involving a number of RuBPCase genes. In a simplistic form, however, the available data suggest that light induces a diffusible substance, possibly via phytochrome or one of the blue light receptors (reviewed in Nagy et al., 1988; Briggs and Iino, 1983) which is transported to the M cells through the vascular system. This product presumably interacts with a second factor, present only in M cells, either to repress transcription or translation of

RuBPCase or to increase the rate of RuBPCase mRNA or protein destabilization. This M-specific factor may also cause some or all of the M-specific C4 gene products to accumulate. Similarly, the light-induced diffusible product may interact with a BS cell-specific factor to facilitate the accumulation of BS-specific gene products.

Prospects

Several areas are promising for further study. An obvious direction is to determine the molecular nature of the cis regulatory elements flanking C4 genes and of the trans factors that interact with them. Are the elements responsible for spatial localization the same as for light regulation? What is the action spectrum for light-dependent localization? How are trans factors induced in a particular cellular distribution? Are the factors that positionally regulate RuBPCase the same or different from those acting on other C4 genes? Ongoing transient expression studies with sequences flanking the maize PPdK gene suggest that several positive and negative cis elements are present, and that the light regulatory element is a negative one (J.-Y. Sheen, personal communication). This approach should be fruitful for the analysis of cis elements and trans-acting factors regulating all of the C4 genes. Studies in C3 plants (reviewed in Kuhlemeier et al., 1987; Nagy et al., 1988) have shown that the relief of suppression by cis negative control elements is a common mechanism for stimulation of gene expression by light. The modulation by light of trans factors that interact with these cis elements is an area of ongoing research.

Another area worthy of further study is the spatial regulation of C4 gene expression in plants with variations on Kranz anatomy, such as among various C3, C4, and C3-C4 intermediate *Flaveria* species. How do systems of spatial control of RuBPCase, for example, compare at a cell pattern level and at the gene regulation level in plants with different cellular arrangements? Are the trans-acting factors that limit RuBPCase expression in one species effective on another? How have the cis target sequences for regulation changed in plants exhibiting different patterns of C4 gene expression? Do the C3-C4 intermediate species represent intermediates in the evolution of the C3 control scheme to the C4 scheme?

A technically difficult area needing further study is the establishment of the positional landmarks that make specialized BS and M cell differentiation possible. How are veins spaced regularly across the leaf? The initiation of veins divides the developing leaf into longitudinal domains of cells which can be controlled on a fine scale. What are the effectors of this local control? Are they diffusible? Will they act on cell differentiation and gene expression in vitro? The study of vascularization has thus far been primarily physiological, relying on applications of growth regulators such as auxins to effect changes in vascular differentiation (Aloni, 1987). With further advances in research on auxin receptors and organ-specific regulatory elements, it might be possible to construct plants with variations in leaf vascularization. Effectors other than auxins and cytokinins might be crucial in vein spacing and initiation. Alternatively, mutants with altered vascular pattern or differentiation might be identified in genetic screens. In one sense, plants such as maize provide leaf organs with just such varied vascular patterns. However, they provide little opportunity for manipulating the factors causing variations in spacing, and new systems are needed.

The fine-scale positional control of gene expression exhibited in developing BS and M cells should continue to be an experimental system that yields information both about spatial control of gene expression and about patterns of cell differentiation in plant organs.

ACKNOWLEDGMENTS

The authors thank Jen Sheen, Jim Berry, and Dan Klessig for permission to cite their unpublished work. We are also grateful to Leyla Bayraktaroglu for critical reading of this manuscript. Cited work in press or unpublished from the authors' laboratory was supported by grants from the National Institutes of Health (GM33984), The National Science Foundation (DMB8704241), and the McKnight Foundation (to T.N.).

Received October 26, 1988.

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