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Development of the Kranz Structure during Leaf Growth in C_4 *Euphorbia maculata*

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The development of the Kranz structure was investigated in leaves of C_4 *Euphorbia maculata* using electron microscopy. Four leaf stages, i.e., primordial, immature, young, and mature, were examined, based on the photosynthetic tissue that surrounded the veins. The examination revealed how cells differentiated into distinct bundle sheath cells (BSCs) and mesophyll cells (MCs). Specialization of the BSCs was invariably associated with the development of the veins as well as the MCs. Precursors for BSC and MC were recognizable fairly early, at the immature stage, according to their position and differential enlargement. Once these precursors were delimited from the procambial area, differentiation into each cell type occurred synchronously, in a coordinated manner. All cells enlarged as they were displaced from the Kranz precursor area, but the BSC precursors were initially larger and remained relatively larger than the other cell types throughout leaf development. The developmental changes sharply distinguished BSCs from the adjacent MCs at the onset of Kranz formation and continued until maturity. Chloroplast enlargement also occurred during cell displacement, but the rate of enlargement was greater in BSCs, resulting in larger chloroplasts at later stages. However, no significant structural differences were detected among the chloroplasts of BSC and MC in the early stages. Most of the specialized features appeared at the young-leaf stage; structural dimorphism became prominent at the later stages. This enhanced development of the BSC chloroplasts was correlated with asymmetric distribution of cellular components. In addition, the BSC formed thin primary pit fields with numerous plasmodesmata. Peripheral reticulum was present, but generally was not conspicuous. We also discuss the characteristics of leaf anatomy and ultrastructure in *E. maculata* as they relate to the C_4 photosynthetic pathway.

Keywords: C_4 species, cell differentiation, *Euphorbia maculata*, Kranz development, ultrastructure

One of the distinguishing structural features of C_4 plants is the differentiation of two different cell types in the photosynthetic tissues, i.e., the bundle sheath cell (BSC) and the mesophyll cell (MC). These form the Kranz structure around the vascular tissues. Within the Kranz structure, the chlorenchymatous BS and radiating MCs are well defined, making this characteristic the most common criterion when identifying C_4 plants (Laetsch, 1971, 1974; Kim and Fisher, 1990; Kim, 1997; Kim et al., 1997, 1999; Dengler and Nelson, 1999). The spatial configuration and specialization of the two photosynthetic cell types with a short photosynthetic diffusion path are certain anatomical features undoubtedly associated with the C_4 syndrome (Dengler and Nelson, 1999).

The primary determinant of photosynthetic cell fate is cell position within the ground meristem; cell-to-cell communication is considered essential for interpreting that position (Dengler and Nelson, 1999). In C_4 plants the two carboxylation systems of photosynthesis operate simultaneously, but are spatially separated in the two cell types. The MCs radiating from BSCs are specialized as initial, primary carbon assimilation tissue, whereas the BSCs at the periphery of the vascular tissue are secondary photosynthetic carbon-reduction tissues (Hatch, 1987; Dengler and Nelson, 1999). Accordingly, this division of labor makes C_4 photosynthetic activities more efficient than other photosynthetic pathways (Hatch, 1987; Nelson and Langdale, 1992; Dengler and Nelson, 1999).

Ontogeny of the discrete Kranz pattern in C_4 plants has become an intriguing model for studying the origin of tissue type and the differentiation of specialized cell lineage during leaf development (Dengler et al., 1985, 1995, 1996; Nelson and Dengler, 1992). Differentiation of the Kranz structural components has been studied in a few C_4 dicotyledons (see Dengler and Nelson, 1999), but no such studies have been carried out for C_4 *Euphorbia* plants. In the present work we used electron microscopy to examine *Euphorbia maculata*, a species having the characteristic Kranz

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anatomy. Ontogeny of the Kranz pattern revealed cell differentiation into BSCs and MCs within the structure. We also followed some of the ultrastructural changes that occurred during Kranz development.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of *E. maculata* L. (Lee, 1982, 1996) were germinated in a growth chamber at 25°C, under continuous illumination (160 μmol m^{-2} s^{-1}). They had been incubated beforehand for three to five days at 4°C on moist filter paper. The seedlings and mature plants were then maintained in a greenhouse at 25/20°C (day/night), with a 14-h photoperiod. Plants were watered daily and fertilized every two weeks with nutrient solutions. Five seedlings each were used for dissecting either the first leaf and/or leaf primordia that had just emerged from the shoot apical meristem. Based on the progress of the developing photosynthetic tissues and vascular elements, four stages of Kranz differentiation in leaves were identified: primordial, immature, young, and mature. Leaf samples for light and electron miscroscopy were taken from plants at each of these stages during one to three months of growth in the greenhouse.

Electron Microscopy

Leaf primordia as well as leaf segments from the latter three developmental stages were sampled for transmission electron microscopy as follows: The middle region, excluding mid-veins, of fully expanded leaves at each stage was used. To compare starch contents in the chloroplasts, mature leaves were collected at both 07:00-08:00 h and 13:00-15:00 h. Approximately 1 mm^2 samples were prepared and processed, following the modified procedures of Kim and Fisher (1990) and Kim (1997). After being fixed in 3% glutaraldehyde in 0.01 M phosphate buffer (pH 6.8) for 3 h at room temperature, they were post-fixed in 2% osmium tetroxide for several hours at 4°C. The fixed tissues were rinsed four times, at 15-min intervals, in the same buffer after the primary- and post-fixation. The specimens were then dehydrated in a graded acetone series and embedded in Spurr resin. Semi-thick sections, 0.5-1.0 μm, were made with a histo-diamond knife, then stained with toluidine blue O, and examined under a Zeiss photomicroscope. Ca. 60-90 nm ultra-thin sections were cut with a diamond knife on a Reichert Ultracut-S ultramicrotome and mounted on 0.35% formvar in dichloroethane-coated grids. The sections were stained with 2-4% uranyl acetate and lead citrate, then viewed at the Korea Basic Science Institute (KBSI) using an Hitachi H-7100 transmission electron microscope operated at 75 kV.

RESULTS

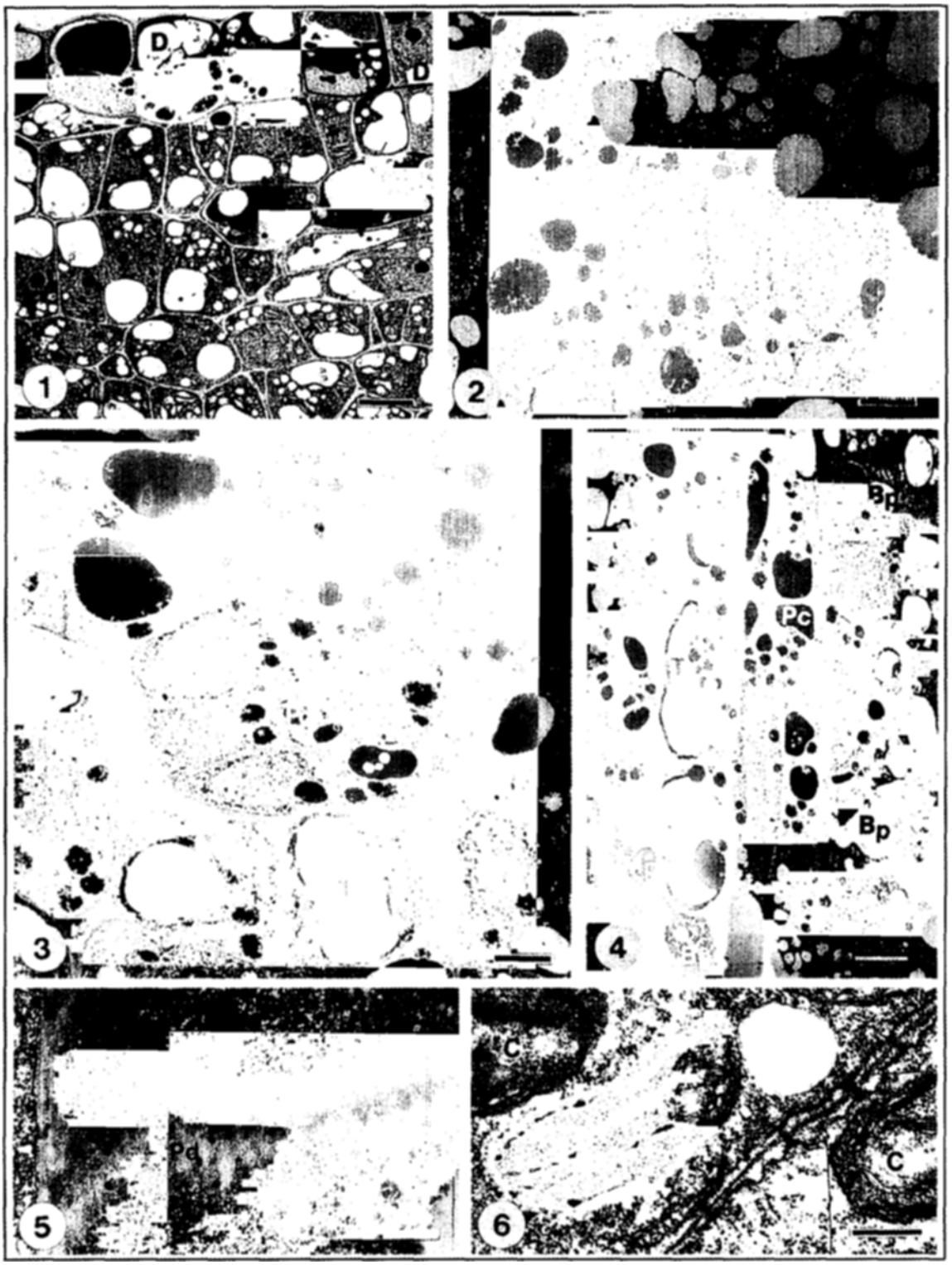
Leaf Anatomy

Tissue differentiation began at the leaf apex upon emergence of the leaves from the apical meristem. Maturation progressed downward and laterally during leaf growth. The mid-vein formed first, followed by almost simultaneous major vein development. Other minor, smaller veins developed later. An early sign of Kranz formation, defined by vein procambium in a polygonal fashion with surrounding precursor cells, was visible in the ground meristem five to six days after germination, in ca. 0.8-1.5 mm-long leaves. Cell differentiation of those precursor cells into BS (i.e., Kranz BS) and MCs appeared 10 to 12 days after germination. This occurred during the late immature to early young leaf stage, when leaves were ca. five to seven mm long. Kranz development extended into the young leaves, with the typical structure being well demonstrated from the later young to the fully mature stages. *E. maculata* are erect, 25-55 cm, annual herbaceous plants with thin leaves, ca. 8-11 mm wide and 12-18 mm long, at maturity.

Ultrastructure

In leaf primordia, the rudimentary lamina consisted of layers of ground meristem and protoderm. At this stage, no precursors of any cell type, except the protoderm, could be identified on a structural basis. The ground meristematic cells were rather homogeneous with numerous vacuoles in the cytoplasm, but the adaxial and abaxial protoderms were often filled with osmiophilic, probably tanniferous, electron-dense deposits (Fig. 1).

Initial periclinal divisions, occurring in the second or third layer of the ground meristem (Fig. 2), as well as succeeding anticlinal divisions, eventually led to the establishment of procambial precursors during the immature stage (Fig. 3). BSC precursors were inferred only by cell position and by the accumulation of osmiophilic deposits. BSC precursors, slightly larger



Figures 1-6. Ultrastructural features of the leaf primordia (1-2) and immature leaf stage (3-6). **1.** A lamina at a primordial stage with rather homogeneous meristematic cells. D = protoderm. Scale = 4.4 μm. **2.** Periclinally dividing cells in the ground meristem where a vein will develop. N = nucleus. Scale = 1.9 μm. **3.** Initiation of the procambial tissue (Pc). T = osmiophilic tanniferous deposit. Scale = 1.7 μm. **4.** Longitudinal section around the procambial tissue shown in Figure 3. Bp = BSC precursor, Mp = MC precursor. Scale = 3.3 μm. **5.** The BSC-MC wall interface demonstrating cortical microtubules (arrow heads) and numerous plasmodesmata (Pd). Scale = 0.7 μm. **6.** BSC chloroplasts (C). Scale = 1.0 μm.

than their surrounding cells, were directly in contact with the procambial precursors, and conspicuous tanniferous compounds had accumulated in the vacuoles (Figs. 3 and 4). Cells neighboring both adaxially and abaxially to these BSC precursors were the MC precursors. These two cell type precursors were well interconnected by numerous simple plasmodesmata (Fig. 5). However, no differences were detected in plastids at this early stage. Both BSC and MC precursors exhibited plastids in similar structures. The plastids were small and contained only a few rudimentary grana having no more than three or four thylakoids (Fig. 6).

Subsequent cell differentiation of BSC and MC precursors into the characteristic Kranz pattern proceeded very rapidly following delimitation of the vein procambium in a polygonal fashion (Fig. 7). In fact, it was only after the formation of vascular elements that BSC precursors appeared delimited in relation to the veins. Approximately 6-10 large cells, next to the procambial tissue, became BSCs; surrounding cells resulted in MCs. A larger procambial strand was produced when a great number of divisions took place in the precursors of vascular elements. An early form of the Kranz pattern became clearly visible at this stage. Large veins occasionally produced more than one cell within the Kranz BS, but development of xylem and phloem elements always preceded cell differentiation into each type.

Simultaneous formation of intercellular spaces was undertaken within the rapidly expanding lamina. This process started in the corner where the abaxial epidermis and MCs joined, then spread to other wall parts (Fig. 8). Prior to further development, the BSCs and MCs were well characterized in their cellular orientation, shape, and size. One or two layers of cells surrounding the Kranz BS had adaxially differentiated into the elongated palisade mesophyll, while those on the abaxial side became irregular spongy parenchyma with huge intercellular spaces.

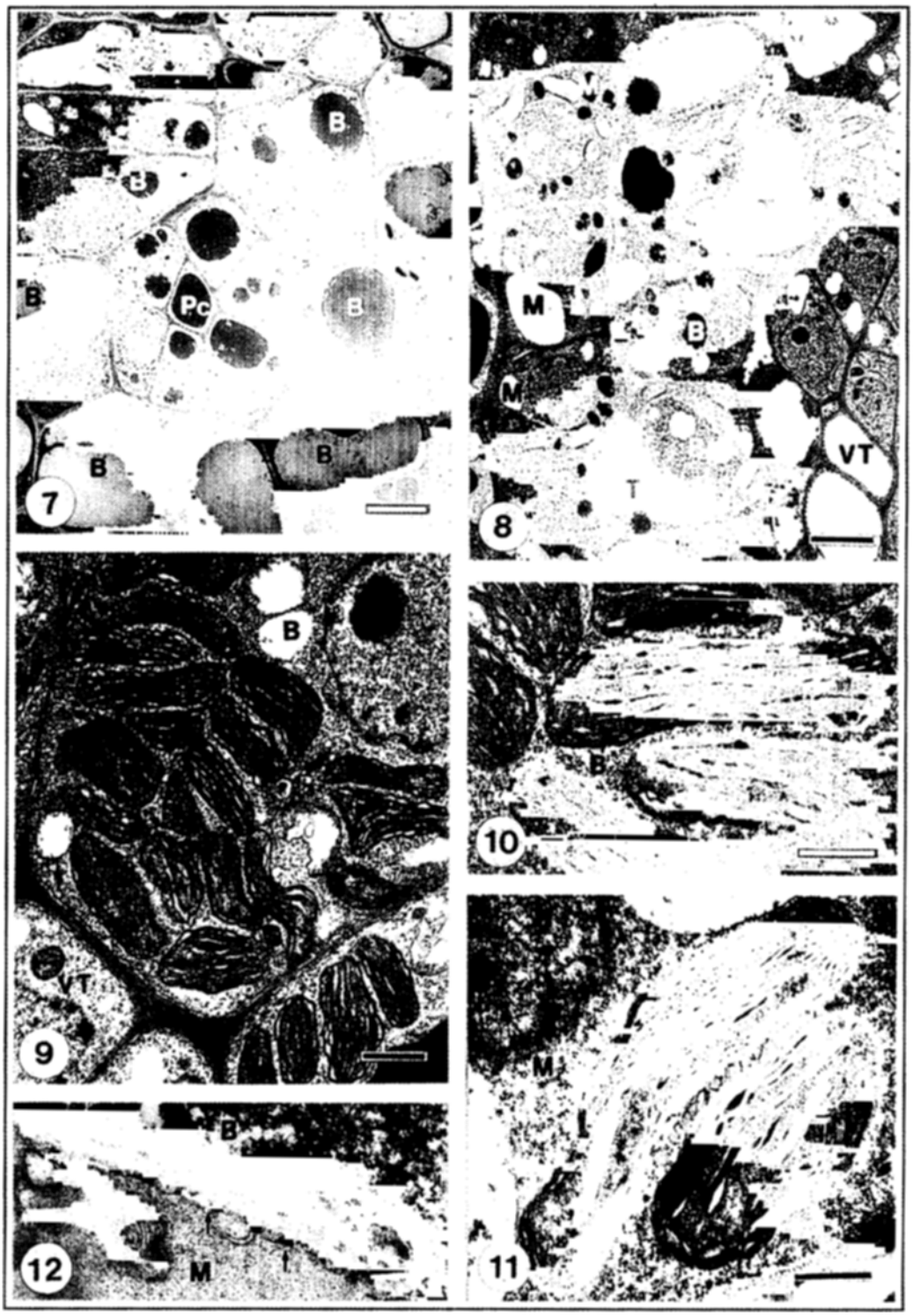
Cellular components in the BSCs were asymmetrical, clustering especially toward the internal vascular tissue (Fig. 9). The chloroplasts had few, but appressed, thylakoids and a weakly developed peripheral reticulum (Fig. 10). However, organelles in both the palisade and spongy MCs were distributed peripherally as cells became more vacuolated. MC chloroplasts showed increased thylakoid density within the grana (Fig. 11). Numerous plasmodesmata developed among the various wall interfaces, but were easily distinguished by their developing primary pit fields at the BSC-MC interfaces (Fig. 12).

During the latter part of the young to mature stages, both BSCs and MCs increased significantly in size and exhibited prominent structural dimorphism within the Kranz structure. Characteristically large BSCs had centripetally distributed organelles that completely enclosed the vein, whereas the further elongated MCs had radiated from this BS layer (Fig. 13). Encircling the Kranz BS, the palisade MCs radially surrounded the upper half of the vein; spongy MCs were found in the lower half, with more extensive intercellular spaces.

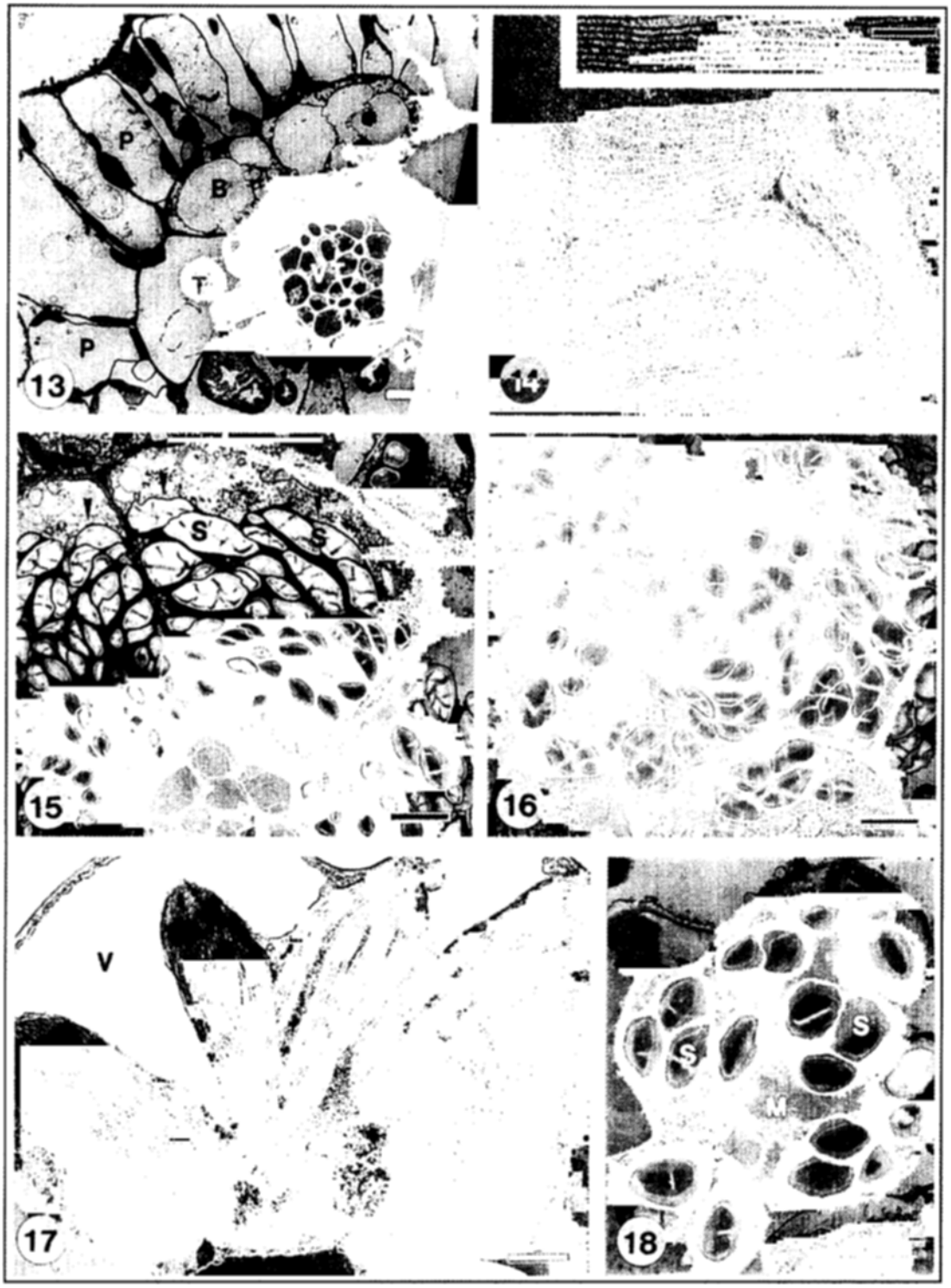
The BS chloroplasts were large, densely stacked, and agranal, with starch grains (not shown) and peripheral reticulum (Fig. 14). In mature leaf samples that had been fixed in the afternoon, the BSCs showed the most striking increase in the size of their starch grains. Large and irregularly shaped starch-laden chloroplasts that occupied almost the entire cytoplasm were fairly common. Chloroplasts toward the outer tangential wall tended to have larger and more starch grains than those close to the vein (Fig. 15). Likewise, the chloroplasts containing such starch grains were strikingly larger than those with few or none. In large veins where Kranz BS developed two or more BSCs in a row, the second cell was usually small and had much reduced cytoplasm relative to the first BSC (Fig. 16). In contrast, MCs became extremely vacuolated and showed lens-shaped small chloroplasts with well-developed grana.

The number of thylakoid membranes remained high beyond the young leaf stage, with up to 45-50 thylakoids often developing in mature leaves (Fig. 17). The peripheral reticulum was not prominent, but was present in a somewhat discontinuous layer at the chloroplast margin, though not clearly visible when the chloroplasts accumulated starch grains. Moderately large starch grains also formed within the MC chloroplasts of leaf tissues that had been taken at 13:00-15:00 h (Fig. 18). However, these were fewer and smaller than those in the BSCs. The BSCs contained slightly more mitochondria and microbodies than did the MCs, but the differences were not significant.

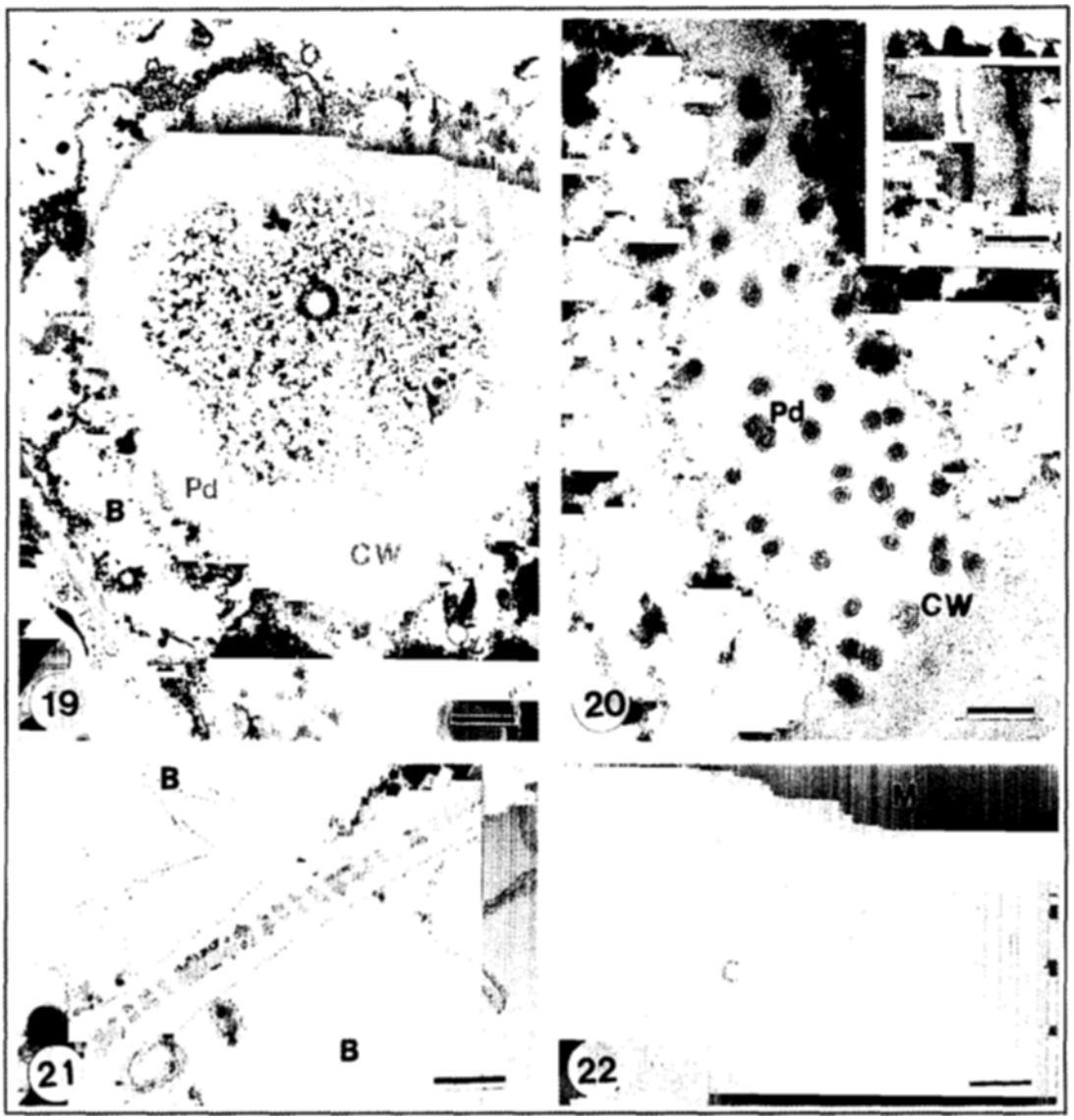
Development of plasmodesmata was the other distinguishing feature among cell types at maturity. These plasmodesmata occurred largely as clusters of simple form (Figs. 19 and 20), but several clusters, with occasional branches, traversed thin areas of the primary pit fields (data not shown), especially often at the BSC-MC interfaces. Adjacent BSCs or MCs were also interconnected by numerous plasmodesmata; pit fields were found quite infrequently in the former (Fig. 21) but with none in the latter (Fig. 22).



Figures 7-12. Development of the Kranz structure and cell differentiation at late-immature (7) and young-leaf stages (8-12). **7.** Initiation of the Kranz structure. Six cells in direct contact with the procambial strand (Pc) differentiated into BSCs (B). Scale = 2.5 μm. **8.** Part of the Kranz structure exhibiting already developed vascular tissue (VT) and still differentiating BSCs (B) and MCs (M) at the periphery. Arrows indicate initial intercellular spaces. Scale = 1.0 μm. **9.** Asymmetrically arranged cellular components within BSC (B), especially toward the inner vascular tissue (VT). Scale = 1.0 μm. **10.** BSC chloroplasts with rudimentary thylakoids. Scale = 0.5 μm. **11.** MC chloroplasts having moderately developed grana (G). Scale = 0.5 μm. **12.** Plasmodesmata (arrows) between BSC and MC. Scale = 0.3 μm.



Figures 13-18. Structural dimorphism detected in the Kranz structure of the mature leaves. **13.** Partial Kranz BS (B) with adaxial palisade MCs (P). Organelles were completely appressed centripetally in the BSCs. Scale = 10 µm. **14.** Almost agranal BSC chloroplasts with inconspicuous peripheral reticulum (Pr). C = chloroplast. Scale = 1.0 µm. Inset: Close-up of agranal thylakoids. Scale = 0.5 µm. **15.** Distally located chloroplasts with larger and more starch grains (S, arrow heads) than the proximally situated ones. Scale = 3.3 µm. **16.** Kranz BSCs entirely filled with chloroplasts containing starch grains. The second BSCs were usually smaller in size or had less cytoplasm (2B) than the first cells (1B). Scale = 3.3 µm. **17.** MC chloroplasts with well-developed grana. Scale = 1.0 µm. V = vacuole. **18.** MC chloroplasts with starch grains (S). Scale = 2.1 µm.



Figures 19-22. Plasmodesmata found in various wall interfaces in mature leaves. **19.** Glancing section between BSC and MC showing plasmodesmata (arrows). Cy = cytoplasm. CW = cell wall. Scale = 2 μm. **20.** Numerous plasmodesmata (Pd) in cross section from BSC-MC interface. Scale = 600 nm. Inset: Branched plasmodesmata (arrows). Scale = 250 nm. **21.** Plasmodesmata between adjacent BSCs. Scale = 0.5 μm. **22.** Plasmodesmata (Pd) between two MCs. Note the absence of the primary pit field on the cell wall. I = intercellular space. Scale = 0.3 μm.

DISCUSSION

C_4 photosynthesis is structurally associated with the presence of specialized chlorenchymatous leaf BS within the Kranz structure (Laetsch, 1974; Fahn, 1990). This was well demonstrated in our study of *E. maculata*. During Kranz structure development, differentiation of BSCs was well coordinated with MCs, and a consistent relationship with the vascular tissue was also noted. BSC and MC precursors could be recognized fairly early in leaf development, based on their position and differential enlargement in the rudimentary lamina. Once the precursors of BSC and MC were delimited from the procambial area, cell differentiation into each type occurred synchronously in a coordinated manner within the ground meristem.

All the cells enlarged as they were displaced from the precursor cells of the Kranz structure. However, some unique ultrastructural features appeared in the BSCs well before comparable changes occurred in the MCs early in leaf development. BSC precursors initially were larger, and continued to maintain their

relatively greater size throughout leaf maturation. Similar differences in cellular structure, along with an early accumulation of C_4 cell-specific proteins, have been found in BSC and MC precursors formed early in association with procambial tissues in *Arundinella* (Nelson and Dengler, 1992; Dengler et al., 1995).

In our *E. maculata*, the differences between cell types appeared around the immature and early young stages, when laminar expansion was noticeable. The developmental changes sharply distinguished BSCs from adjacent MCs at the onset of Kranz formation and continued until leaf maturity. Where differentiation of such highly specialized BSCs has been seen in C_4 *Atriplex*, the requirement for a longer developmental period has been speculated (Liu and Dengler, 1994). Studies of other C_4 monocotyledon species (Dengler et al., 1985, 1986, 1990, 1996) have suggested that cell position, namely positional information, plays a crucial role in regulating structural aspects of cell development. In contrast, cell lineage and delimitation time are considered less influential factors during the later stages of cell differentiation (Dengler and Nelson, 1999).

Chloroplasts enlargement was coordinated in all cell types as cells were displaced from the Kranz precursor area. However, the rate of enlargement was greater in BSCs, resulting in larger chloroplasts at later stages. The current data clearly indicated that maturing BSCs had more and larger chloroplasts, with noticeably increased osmiophilic deposits compared with MCs.

The BSC chloroplasts were further distinguished from those of the MC by continuous starch production late into development and by the association of thylakoids so that developing chloroplasts were nearly agranal. This has been reported in many C_4 species where structural dimorphism is pronounced (Kim and Fisher, 1990; Dengler et al., 1996). Chloroplast dimorphism between BSC and MC, with correlation to one of the C_4 biochemical subtypes, has long been recognized among many C_4 species. It is most conspicuous in NADP-ME species or malate-forming species, where asymmetric, agranal, and starch-laden BS chloroplasts are generally larger, more numerous, and occupy a greater fraction of the cell area (Liu and Dengler, 1994; Dengler et al., 1996; Ueno, 1996). The centripetally located BS chloroplasts with agrana and starch grains are typically found in malate formers -- among them, many are reported to be C_4 *Euphorbia* (Gutierrez et al., 1974, Webster, 1994; Sage et al., 1999). BSCs also tend to have slightly more mitochondria and microbodies than do MCs. However, the two organelles are relatively similar in structure and size for NAD-ME C_4 species (Dengler et al., 1986; Dengler et al., 1990; Kim and Fisher, 1990; Liu and Dengler, 1994), for which the decarboxylation step occurs in distinct BS mitochondria (Hatch, 1987).

One may draw a definite relationship between the vascular tissue and Kranz BS of C_4 plants, based on structural characteristics. Not only were the BSC precursors in direct contact with the procambial strand but also the asymmetric cellular components remained completely enclosed in the vascular tissue, without intercellular spaces, throughout leaf development. The rapid transport of photosynthate to the vein by the typical cellular structure has been suggested, but the exact function associated with this relationship awaits further explanation. The BSCs, frequently with starch grains, presumably serve as a temporary but readily accessible storage center, thus preventing the MCs from becoming logged with photosynthate build-up (Dengler and Nelson, 1999). Being adjacent to the leaf vein, the BSCs have direct access to the transport system during photosynthesis, and they continue to unload the daily accumulation of starch. This was clearly evident in the samples taken at different times of day in our study. The peripheral reticulum of chloroplasts, probably involved in rapid photosynthate movement that enhances photosynthetic efficiency, is usually well-developed in C_4 photosynthetic tissue (Laetsch, 1974; Kim and Fisher, 1990; Kim et al., 1997; Dengler and Nelson, 1999). In our study, the peripheral reticulum was weakly developed in MC chloroplasts and only slightly, though not significantly, better developed in the BSC chloroplasts of *E. maculata*.

Based on our examined ontogeny of the Kranz structure, we speculate that a correlation exists between asymmetric distribution of BS cellular components and the formation of thin primary pit fields with numerous plasmodesmata in the BSC walls. These primary pit fields were clearly shown when the centripetal cellular arrangement was completed toward the later stages of leaf development. The cell wall at the BSC-MC interfaces of C_4 plants is often highly modified, with numerous plasmodesmata connecting BSCs and MCs that probably provide a major pathway for the symplastic diffusion of metabolites (Botha et al., 1993; Dengler and Nelson, 1999). Most of these specialized features appeared at the young-leaf stage, and clearly demonstrated structural dimorphism at maturity.

Another intriguing feature noticed during leaf development of *E. maculata* was the presence of electron-dense deposits in the BSCs. Allelopathy has been demonstrated in *Euphorbia*, and a high concentration of osmiophilic phenolic compounds has been impli-

cated for the allelopathic nature (Elmore and Paul, 1983). The synthesized phenolic compounds are, indeed, an integral part of the Kranz structure in this species. They should be of interest to those working on various structural aspects of *E. maculata* plants.

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