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Ultrastructural Aspects of Kranz Anatomy in *Digitaria sanguinalis* and *Setaria viridis* (Poaceae)

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Structural differentiation of Kranz anatomy has been investigated in leaf cross sections of two C-4 Poaceae: *Digitaria sanguinalis* and *Setaria viridis*. The study mainly focused on cellular and interfacial features of bundle sheath (BS) and mesophyll (MS) cells of the C-4 structure. Prominent BS, spaced by only two MS cells apart, were surrounded concentrically by a layer of MS cells. BS cells of *S. viridis* had centrifugally arranged relatively large chloroplasts containing much starch, but the chloroplasts had agrana to rudimentary grana. Structural and size dimorphisms, when starch was present, were detected between BS and MS chloroplasts. Loosely arranged MS cells had peripherally displaced smaller chloroplasts containing little to none starch. BS chloroplasts of *D. sanguinalis* were similar to those of *S. viridis*, but had very little starch and well-developed long agranal stroma lamella. Features of MS cells were similar in both species, but well-defined peripheral reticulum (PR) was easily recognized in MS chloroplasts of *S. viridis*. Virtually no PR was developed in BS chloroplasts examined. BS cells contained more mitochondria and microbodies, but no structural dimorphism was noticed. The electron-dense suberized lamella were often observed between BS and MS cells, especially in the primary wall of BS cells. It was most frequently found at the BS and MS cell interfaces and terminated in radial walls of the adjacent BS cells. Prominent pits with plasmodesmata (pd) were seen in the walls of both cells. There also were numerous pd in outer tangential walls of the BS cells. The number of pd ranged from 20 to 60. The pd trasversed a segment of cell wall much thinner than the adjacent wall. The current cellular data have been compared to the ultrastructural features known in leaves of other C-4 plants, especially NADP-ME species.

Key words: C-4 Poaceae, *Digitaria sanguinalis*, *Setaria viridis*, Kranz anatomy, ultrastructure

Plants can be divided into two groups, C-3 and C-4, by their photosynthetic pathway. Characteristically C-4 plants have high levels of C-4 acids as their early photosynthetic products and Kranz anatomy. The C-4 system is nearly always associated with a specialized leaf Kranz anatomy (Hatch, 1987), a structure of concentrically arranged chlorenchymatous bundle sheath (BS) and mesophyll (MS) cells around vascular bundles. Anatomically the C-4 pathway is expected to require spatial coordination between these two cell types with appropriate relative abundances of cells and organelles in MS and BS tissues (Hattersley, 1984). The C-4 pathway occurs relatively widely in flowering plants and it has been reported in over 1,000 species from 17 different families (Voznesenskaya and Gamalei, 1986). The Poaceae is one of the large family which contains a large number of C-4 species.

Since the discovery of C-4 photosynthesis in mid 1960's, correlation of various morphological as well as physiological characteristics with this mechanism have been established. C-4 species are further divided into three groups of NADP-malic enzyme (NADP-ME), NAD-ME, and PCK type by the differing mechanisms operating to decarboxylate C-4 acids in BS cells (Hatch *et al.*, 1975). Each biochemical C-4 type is characterized by a suite of anatomical and ultrastructural features in C-4 Poaceae. These three C-4 groups are mostly distinguished by characteristics of BS chloroplasts. NADP-ME species lack well-developed grana in BS chloroplasts and the BS chloroplasts are in the centrifugal position. NAD-ME species have BS chloroplasts in the centripetal position and contain grana. PCK species have BS chloroplasts

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in the centrifugal position and they contain grana. The comparative ultrastructure of the BS and MS cells in C-4 species shows general taxonomic correlations within Poaceae whose character syndromes of leaf anatomy has been described by the BS types (Carolin *et al.*, 1973). In Poaceae, the different types have been closely correlated with distinct leaf anatomical and physiological characteristics. NADP-ME species of the Panicoideae have been characterized by chloroplasts in BS cell located in the centrifugal position. This group is known to show considerable variation in their ultrastructure (Laetsch, 1974; Brown, 1975; Voznesenskaya and Gamalei, 1986). Structurally BS cells are distinguished from MS cells by various charateristics of chloroplasts, mitochondria, cell wall thickness, and features of plasmodesmata and suberized lamella (Dengler *et al.*, 1986; Robinson-Beers and Evert 1991; Botha, 1992).

Although there have been many structural studies of C-4 Poaceae species (See Voznesenskaya and Gamalei, 1986), relatively few of them have been entirely focused on the cosmopolitan Panicoideae species, *Digitaria sanguinalis* and *Setaria viridis*, in their ultrastructure. Hence, the present study of structural differentiation in leaves of *D. sanguinalis* and *S. viridis* was undertaken to examine their cellular features associated with C-4 Kranz anatomy. The work presents cellular and interfacial features of BS and MS cells and these have been compared to the ultrastructural characteristics known in leaves of other C-4 plants.

MATERIALS AND METHODS

Five to ten plants of *Digitaria sanguinalis* and *Setaria viridis* were collected from low lands of Taegu were used in this study. Healthy mature leaves were sampled and processed immediately for the experiment. Small pieces of tissue, ca. 1 mm, were dissected from the middle portion of leaves, avoiding midrib. The tissues were fixed in a mixture of 3% paraformaldehyde and 3% glutaraldehyde in 0.1 M phosphate buffer (pH 6.8-7.2) at room temperature. After fixation for 3 hours followed by a short rinse in buffer, the material was postfixed with 2% OsO_4 in the same buffer for 2 hours to overnight at 4°C. After postfixation followed by 4 times of 15 min rinses, specimens were dehydrated in a graded alcohol series, substituted with acetone, and embedded in Spurr's low viscosity resin (Spurr, 1969). Ca. 1 μm thick sections were made and stained in 5% toluidine blue. Thin sections (60-90 nm) were cut on a RMC 7000 ultramicrotome with diamond knives. The sections were stained with 2% aqueous uranyl acetate for 45 min followed by a 45 min staining with lead citrate. Ultrathin sections were viewed in a Phillips EM201 transmission electron microscope at 60 kV.

RESULTS

The leaf anatomy of *Digitaria sanguinalis* and *Se-*

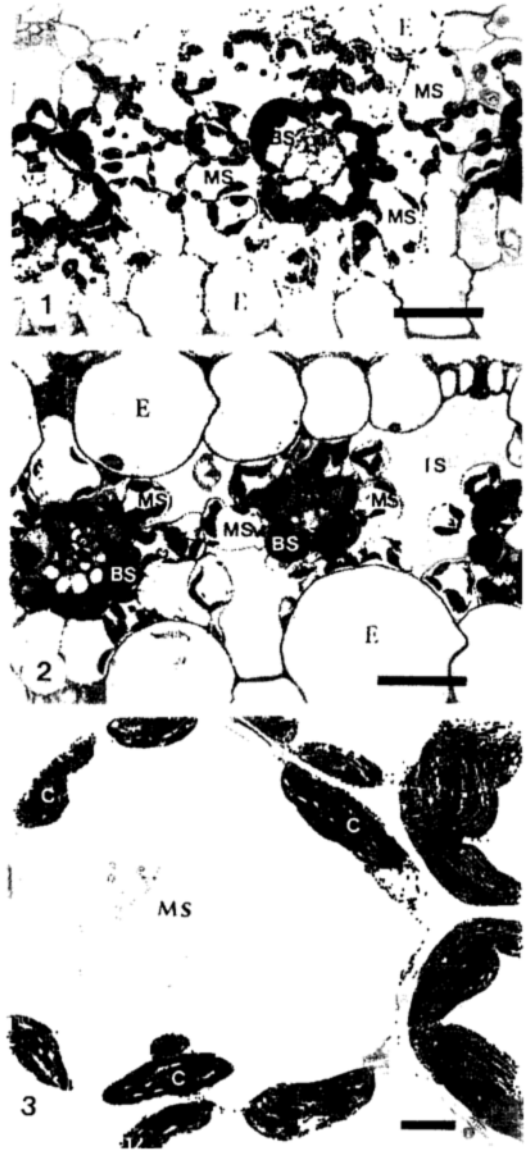

Fig. 1-3. 1. Leaf cross-section of *D. sanguinalis* showing typical Kranz anatomy. E=Epidermis, BS=Bundle sheath cell, MS=Mesophyll cell. Scale=0.1 mm. 2. Leaf cross-section of *S. viridis* with the Kranz anatomy. Notice the loose arrangement of mesophyll cells between vascular bundles. IS=Intercellular space. Scale=0.1 mm. 3. Peripheral arrangement of organelles in the mesophyll cell of *D. sanguinalis*. C=Chloroplast. Scale=3 μm.

taria viridis, as shown in Figs 1-2, exhibited a typical C-4 Kranz anatomy that characterized by two concentric layers of chlorenchyma surround the vascular bundles. Their Kranz anatomy was similar and resembled that of the panicoid species. There were about 5-8 BS cells and 10-15 MS cells around the vascular bundle. Small vascular bundles were often surrounded by only four BS cells. Bundle sheath variation was not observed in both species. Most cells were in contact with BS cells, but sometimes there were number of cells between the radial files that were not connected to the BS cells. In most of the leaf blade, only two layers of MS cells intervened the adjacent vascular bundles (vein-BS-MS-MS-BS-vein). The MS cells were highly vacuolate (Fig. 3) and huge intercellular spaces occupied a large portion of the leaf volume, especially in the MS of *S. viridis*.

The cell ultrastructure of these species was characteristic of C-4 monocotyledonous leaf, since the BS cells having a very small vacuole contained a substantial number of chloroplasts and other organelles (Figs. 4-5). In some BS cells, the vacuole could not be identified since organells appeared to fill the entire cell. Most of organelles in the BS cells were located close to the MS cells, whereas those in MS cells were in a peripheral orientation. The BS cells contain numerous chloroplasts in a centrifugal posi-

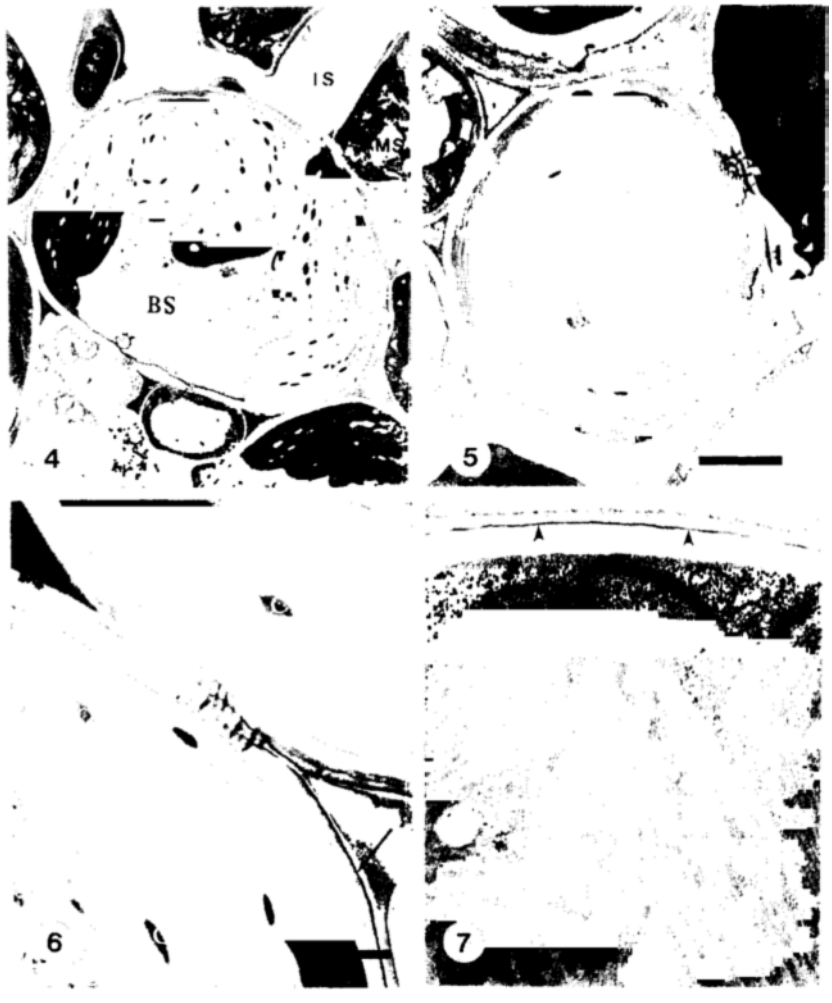

Fig. 4-7. 4. A bundle sheath cell exhibiting centrifugally arranged chloroplasts in *D. sanguinalis*. Scale=4 μm. 5. A bundle sheath cell entirely filled with chloroplasts in *D. sanguinalis*. Arrow indicates the suberized lamella. Scale=2 μm. 6. Plasmodesmata and suberized lamella (arrow) in the walls between two bundle sheath cells of *D. sanguinalis*. Notice the agranal thylakoids in the chloroplasts. Scale=1 μm. 7. Part of a bundle sheath chloroplast showing circular agranal thylakoids (arrow) in the peripheral stroma of *D. sanguinalis*. Arrow heads point to the suberized lamella. M=Mitochondria. Scale=0.5 μm.

tion. BS chloroplasts usually had agrana or rudimentary grana, but regions where two thylakoids appressed were occasionally observed. Single stroma lamellae were extremely numerous in BS chloroplasts of *D. sanguinalis* (Figs. 6-9). BS chloroplasts contained a little starch grains in *D. sanguinalis* whereas those in *S. viridis* had prominent starch grains. The BS cell chloroplasts appeared to be larger than MS cell chloroplasts. MS chloroplastids genarally contained quite reduced starch grains in their size and quantity. Structural and size dimorphisms, when starch was present, were detected between BS and MS chloroplasts.

The BS cell walls thicker than those of the MS with deepened numerous pit fields were observed (Fig. 8). They were most frequently found in the walls of BS cells neighboring the MS cells (Figs. 8, 9, 11, 12). The number of pd ranged from 20 to 60 (Figs. 10-11) and the pd trasversed a segment of cell wall much thinner than the adjacent wall. They were, in all instances, constricted where they trasversed suberized lamellae at the MS-BS, MS-MS, BS-BS, and BS-vascular parenchyma (Figs. 12-14). Pd are most numerous at BS-MS interfaces. All BS cells of *D. sanguinalis* and *S. viridis* possessed an electron-dense suberized lamella (Figs. 8, 9, 11, 12). Between BS and MS cells, it was detected mostly in the primary wall of the BS cells. It was frequently found in the wall adjacent to MS cells and terminated in radial walls of adjacent BS cells. It completed in the outer tangential wall and extended along 1/2-1/3 of the radial walls. In small veins, the inner tangential walls of BS cells were usually devoid of suberized lamella, even around pd. In large veins, a suberized lamella was present even in inner tangential walls.

Mitochondria and microbodies of BS cells in *D. sanguinalis* and *S. viridis* were very closely associated with chloroplasts (Figs. 15-16). No significant structural dimorphism of mitochondria was detected between BS and MS cells (Figs. 15-17). BS mitochondria were slightly larger than those of MS, but more translucent cristae were found (Figs. 15-16). MS mitochondria were small and sparse (Fig. 17). Almost no peripheral reticulum (PR) was observed in BS chloroplasts in general, but well-defined PR was detected in chloroplasts of MS cells, the most being in MS cells of *S. viridis* (Fig. 18).

DISCUSSION

Leaf anatomy has been one of the most useful characteristics to recognize the C-4 system in Poaceae. It is now realized that all grasses with a single BS in their major leaf veins are C-4 species (Brown, 1975; Dengler *et al.*, 1985). The single sheath C-4 species also appear to be consistently of the NADP-ME type (Dengler *et al.*, 1985). The feature noticed in *Digitaria sanguinalis* and *Setaria viridis* was the concentration of organelles in a centrifugal position. The physiological importance of the position of BS chloroplasts in various C-4 species is unknown (Nelson and Langdale, 1989). However, the centrifugal arrangement of the chloroplasts appears to be ideal for the exchange of the photosynthetic intermediates between these chloroplasts and those of the MS cells (Brown *et al.*, 1983). An important feature of C-4 photosynthesis is the compartmentation of metabolism between MS and BS cells. In C-4 plants the initial phase of the C-4 pathway is localized in MS cells and the secondary carboxylative phase in the BS cells. Accumulation of C-4 protein associated with a vein and BS anatomy was revealed in C-4 monocot species (Langdale *et al.*, 1987; Sinha and Kellogg, 1996). The number and concentration of chloroplasts, mitochondria, and microbodies in the BS cells is the most reliable anatomical criterion for determining C-4 features structurally in plant species (Laetsch, 1974; Brown *et al.*, 1983). This greater concentration of organelles in BS cell profiles in these C-4 species suggests an important metabolic role (Brown *et al.*, 1983; Hatch, 1987). As noted previously, grana are deficient or absent in BS chloroplasts of NADP-ME species. Carolin *et al.* (1973) suggested that the panicoid BS are more metabolically active than the MS cells, since in the BS cells the number of cellular organelles is increased, while in the MS cells it is decreased. In general, NADP-ME species had slightly different BS mitochondria than those in MS cells. The internal membrane systems of the MS mitochondria were generally slightly different compared to their counterparts in BS cells. MS and BS cells are believed to attain their specific fates early in leaf development before the onset of C-4 photosynthetic maturity (Dengler *et al.*, 1995, 1996; Taylor, 1996). If we accept the loss of grana in the BS chloroplasts is the most specialized situation in C-4 grasses, then NADP-ME species with centrifugal chloroplasts and varying degrees of grana reduction in BS chloroplasts is the advanced group within the C-4 Poaceae (Gutierrez *et al.*, 1974; Sinha and Kellogg, 1996).

A sharp decrease in vacuole area corresponding to the dramatic increase in chloroplast area was fre-

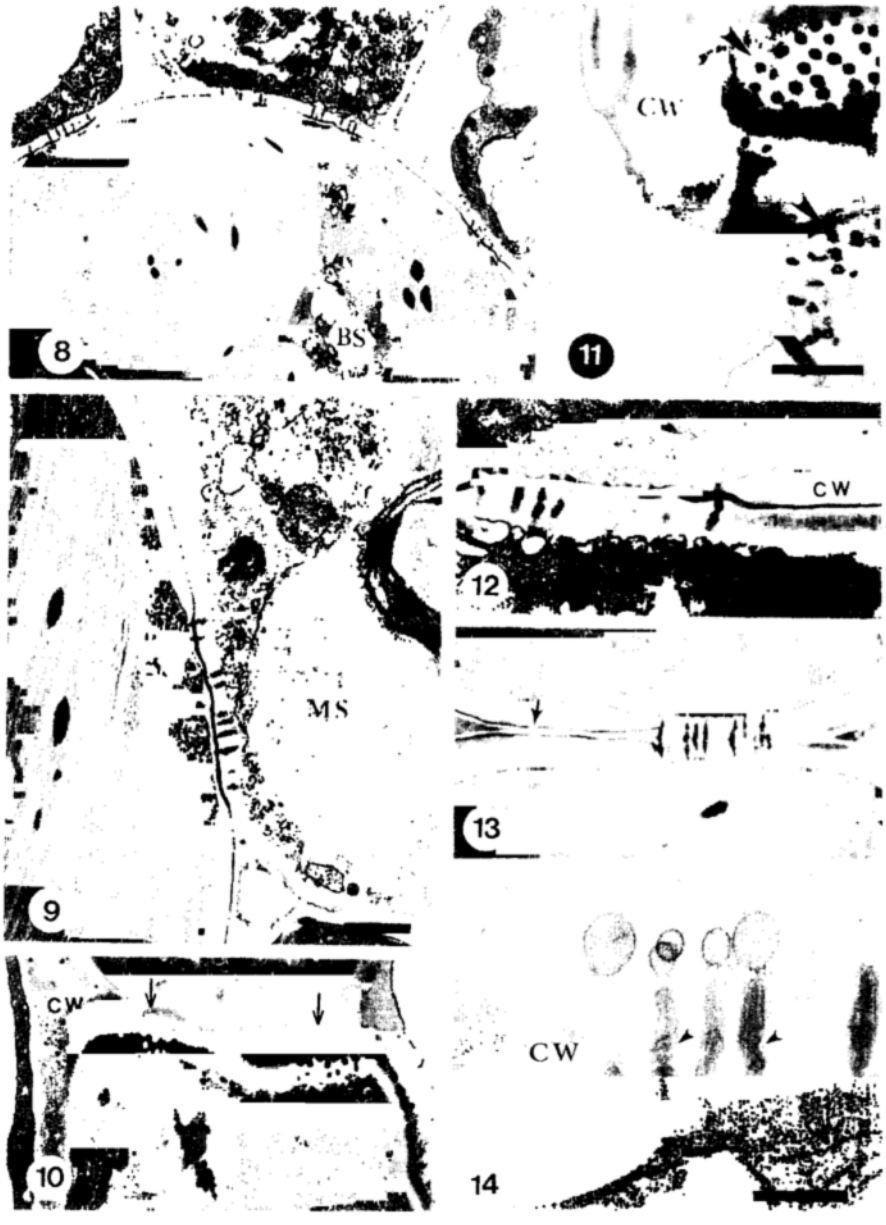

Fig. 8-14. 8. Numerous plasmodesmata in the pit fields between bundle sheath (below) and mesophyll cells (above) of *D. sanguinalis*. Scale=2 μm. 9. A pit field with several plasmodesmata and suberized lamella between bundle sheath and mesophyll cell in *D. sanguinalis*. Scale=1 μm. 10. Plasmodesmata (arrows) observed on the interface between mesophyll cells in *S. viridis*. Scale=1 μm. 11. Numerous plasmodesmata (arrow heads) in the bundle sheath cell of *D. sanguinalis*. CW=Cell wall. Scale=0.5 μm. 12. Plasmodesmata and suberized lamella between bundle sheath and mesophyll cell of *D. sanguinalis*. Note the difference in an electron-dense layer of well-defined subeirzed lamella in the cell walls. Also notice the suberized lamella in the wall of bundle sheath cell with plasmodesmata traversing this layer. Scale=0.5 μm. 13. Plasmodesmata and suberized lamella between bundle sheath cells of *D. sanguinalis*. Arrow indicates the suberized lamella. Scale=1 μm. 14. Plasmodesmata involved in secretion from the bundle sheath cell (bottom) to the sieve element (top) in *D. sanguinalis*. Note the open and convoluted desmotubule (arrow heads). Scale=0.2 μm.

quently observed in this study. Some of the BS cells did not have the large vacuole considered typical of fully differentiated cells. In some BS cells of these species, the vacuole could not be identified since organells appeared to fill the entire cell. Recently, the functional significance of vacuole membrane involved in metabolic transport by regulating ions and enzyme activity between BS and MS cells has been

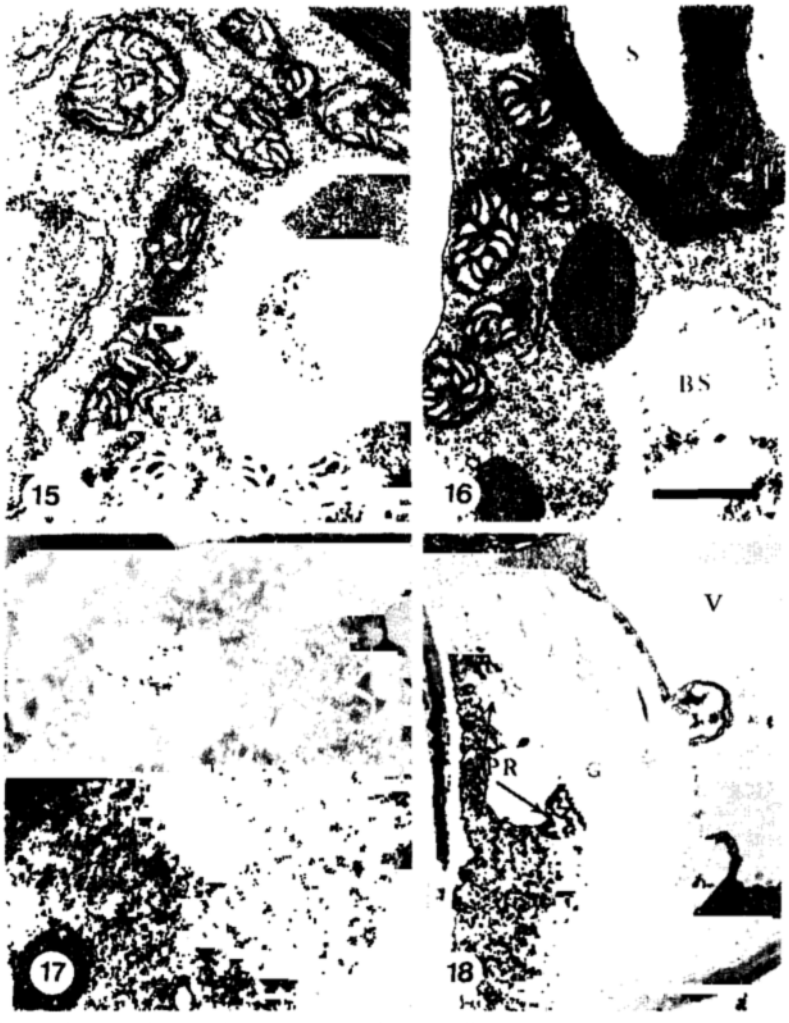

Fig. 15-18. 15. Abundant mitochodria (M) in the bundle sheath cell of *D. sanguinalis*. The cell contains slightly more mitochondria and these show only moderate development of internal membranes. Scale=0.5 μm. 16. Microbodies (Mb) in the bundle sheath cell of *D. sanguinalis*. S=Starch. Scale=0.5 μm. 17. Mitochodria in the mesophyll cell of *D. sanguinalis*. Scale=0.5 μm. 18. A mesophyll chloroplast with well-developed peripheral reticulum (PR) in *S. viridis*. V=Vacuole, G=Grana. Scale=1 μm.

stressed (Giglioli-Guivarc'h *et al.*, 1996).

BS and MS chloroplast size was found to be similar in *D. sanguinalis* and *S. viridis* as in other NADP-ME species. Ratios of chloroplast lengths in BS cell to MS cell showed a clear pattern, with the highest value of 1.3, and reported a tendency for BS cell chloroplasts in C-4 species to be larger in another C-4 Poaceae species (Brown *et al.*, 1983). There is a differential starch formation in BS chloroplasts by the subtypes known in C-4 Poaceae (Dengler *et al.*, 1986). Distinct starch grains have formed in BS chloroplasts in *Setaria viridis*, and similar observations have been reported in other C-4 monocots (Black and Mollenhauer, 1971). A specialization of labor has been suggested for the structural specialization of chloroplasts of C-4 grasses due to lack of grana and accumulation of starch in the BS chloroplasts, and abundance of grana and virtual absence of starch in the MS chloroplasts (Hatch, 1987). This feature appears to be correlated with functional differences. The size of the BS chloroplasts and their ability to store large amounts of starch suggested that they acts as "sink" for carbohydrate end products of photosynthesis (Hattersley and Browning, 1981). The concentric arrangement of the MS cells around the BSs provide a minimum distance for the transport of photosynthetic materials from one cell layer to the next. Most cells in the MS tis-

sue are no more than one cell layer removed from vascular tissue. The small amount of starch found in these MS chloroplasts suggests a rapid transport of photosynthetic products. Relatively better developed PR is found in the MS chloroplasts of NADP-ME species. They also illustrated the generally greater accumulation of starch in the BS chloroplasts. It has been speculated that the PR is another adaptation for rapid transport (Laetsch, 1974; Taylor, 1996).

Another ultrastructural feature of C-4 grasses is the suberized lamella. It is generally accepted that this exists in the walls of BS cells of all C-4 grasses (Hattersley and Browning, 1981; Hatch, 1987). The suberized lamella of Poaceae leaves appears as a pair of parallel elctron-opaque, osmiophilic bands bounding a translucent, light band. The dark bands are the suberin polymer itself and the light bands the waxes and the latter perhaps constituting the major diffusion barrier to water and other molecules (Kolattukudy, 1980). The architecture of C-4 plant leaves permits gaseous diffusion between MS cells up to the wall of the BS cells. The access of CO_2 to BS chloroplasts is complicated by the presence of a suberized lamellae in the BS cell wall. The suberized layer appears to inhibit the passage outward from the BS in a study of Laetsch (1974), but it is not known if it serves as a barrier to water and small molecuoles. A possibility of an apoplastic transport between adjacent BS cells due to the presence of this layer has been suggested (Hattersley and Browning, 1981). It is unknown why the suberized lamella, when present, is thicker where pd occur than elsewhere in the cell wall. An important function of pd is assumed to be that of symplastic transport of metabolites from cell to cell. An unusually large number of pd linking BS and MS cells was reported in some C-4 Poaceae (Hattersley and Browning 1981; Botha, 1992). A possible causative link between the intercellular transport requirements in C-4 species has been suggested (Hatch, 1987).

Anatomically the dense concentration of cellular organelles in BS cells around the vascular bundles, in general, is well known to be related with a high photosynthetic capacity in C-4 species. The overall quantity and distribution of leaf cellular organelles are believed to be important in C-4 pathway. The C-4 system is nearly always associated with Kranz anatomy and a correlation between such anatomy and environment has been suggested for C-4 Poaceae (Brown, 1975). The grasses possessing features like C-4 pathway and dimorphic chloroplasts are presumed to be of tropical origin (Sinha and Kellogg, 1996) and probable relationship between the efficiency of photosynthesis and the leaf anatomy and chloroplast specialization has been suggested (Laetsch, 1974; Taylor, 1996). A clear elucidation of special functions or activities in bundle sheath cells and mesophyll cells awaits further research. Certainly the investigation on prominent starch formation in BS cells is an intriguing starting point.

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