A Variation of C$_4$ Leaf Anatomy in *Arundinella hirta* (Gramineae)[1]

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ABSTRACT

The species *Arundinella hirta* L. posseses a striking variation of the leaf anatomy that is characteristic of C$_4$ grasses. In addition to a sheath of large, bright green cells around the vascular bundles, there are strands of large parenchyma cells which appear identical to the bundle sheath cells and which run parallel to the vascular bundles, but which are not associated with any vascular tissue. This species may be useful for studying the cellular compartmentalization associated with the C$_4$ pathway and should provide interesting material for determining the role of translocation in the functioning of the C$_4$ system.

The radial arrangement of leaf mesophyll cells and the bright green appearance of the bundle sheath chloroplasts which are typical of C$_4$ species were described and illustrated by such early workers as Heinricher, 1884 (10); Volkens, 1896 (18); and Haberlandt, 1914 (7). In 1944, Rhoades and Carvalho (14) attributed physiological significance to the presence of the prominent sheath, suggesting that the chloroplasts in mesophyll and bundle sheath cells had different roles in the photosynthetic process. It was not until workers such as Downton and Tregunna (5) pointed out the relationship between leaf anatomy and the C$_4$ pathway of photosynthesis, however, that widespread interest in the specialized leaf structure of C$_4$ plants developed among plant physiologists.

A close correlation between the specialized leaf anatomy and the C$_4$ pathway has since become widely accepted. Several workers have described the C$_4$ system as a rigidly compartmentalized biochemical cycle which involves reaction sequences requiring major fluxes of reactants between the mesophyll and bundle sheath cells (1, 8, 9, 15). Not only have the two cell types appeared to have a close functional association, but their spatial arrangement within the leaf has always seemed prescribed to a definite order also. Only the bundle sheath cells of C$_4$ species contained the larger plastids which were frequently agranal and specialized for starch formation (5, 11, 12). The positions of the two cell types were apparently not interchangeable; *i.e.*, the cells containing specialized chloroplasts were always located next to vascular tissue.

A report exists, however, of a species which we believed had the C$_4$ pathway for photosynthesis but which appeared to have "bundle sheath-type" cells not associated directly with vascular bundles. Brown (2) reported that "in the genus *Arundinella* cells which are similar to parenchyma sheath cells and form starch are scattered in the chlorenchyma." Tregunna *et al.* (17) reported that *Arundinella hirta* was a C$_4$ species.

We confirmed that the C$_4$ system functions in *A. hirta* and attempted to characterize its specialized starch-storing leaf cells which were not associated with vascular tissue.

MATERIALS AND METHODS

Plants were grown from seed that was sown in a 2:1 mixture of loam and peat moss in 13-cm clay pots. These were placed in growth chambers with 27 to 21 C day-night temperatures, a 16-hr day length, and a light intensity from incandescent and fluorescent lamps of 0.1 cal cm^{-2} min^{-1} (400–700 nm).

CO$_2$ compensation measurements were made using leaves from plants that were approximately 6 weeks old. The CO$_2$ compensation system has been described in a previous report (13).

Fully expanded leaves were used for all anatomical observations. Freshly cut cross sections were stained with I$_2$-KI to observe starch storage patterns. Leaf tissue was also prepared for examination in the electron microscope. Leaves were cut into segments approximately 1 mm square and fixed for 2 hr in a 2.5% glutaraldehyde solution freshly treated with BaCO$_3$ and buffered with 0.05 M sodium phosphate buffer (pH 6.8). The segments were then washed in buffer, postfixed in OsO$_4$ (1% aqueous solution) for 1 hr, dehydrated with a water-acetone series ending in dry acetone, and embedded in Spurr's (16) embedding mixture. Silver sections were cut with a diamond knife and a Sorvall MT-2 ultramicrotome. The sections were mounted on copper grids, stained in uranyl acetate (10 min) and lead citrate (3 min), and viewed with a Phillips 300 electron microscope operated at 80 kv.

Thicker sections (1 μ) of the same material were also cut with glass knives for light microscopy. These sections were dried onto glass slides, stained with 0.05% toluidine blue for 30 sec (80 C), rinsed and dried again, and mounted under cover slips with a permanent mounting medium. They were photographed with a Zeiss research microscope.

Leaves were also cleared and stained for paradermal viewing. Fresh leaf material was placed in boiling 80% (v/v) ethanol until the chlorophyll had been extracted. It was then placed in 10% aqueous NaOH solution and left until it had cleared. The cleared material was rinsed in distilled water and then stained with I$_2$-KI solution. Stained tissue was placed on a glass slide in water, covered with a cover glass, and examined with the light microscope.

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RESULTS AND DISCUSSION

As measured in our system, the CO_2 compensation point of *Arundinella hirta* was less than 5 μl CO_2/liter. The leaves had vascular bundles surrounded with prominent thick-walled starch-storing sheaths. We therefore concluded (3) that *A. hirta* was a C_4 species.

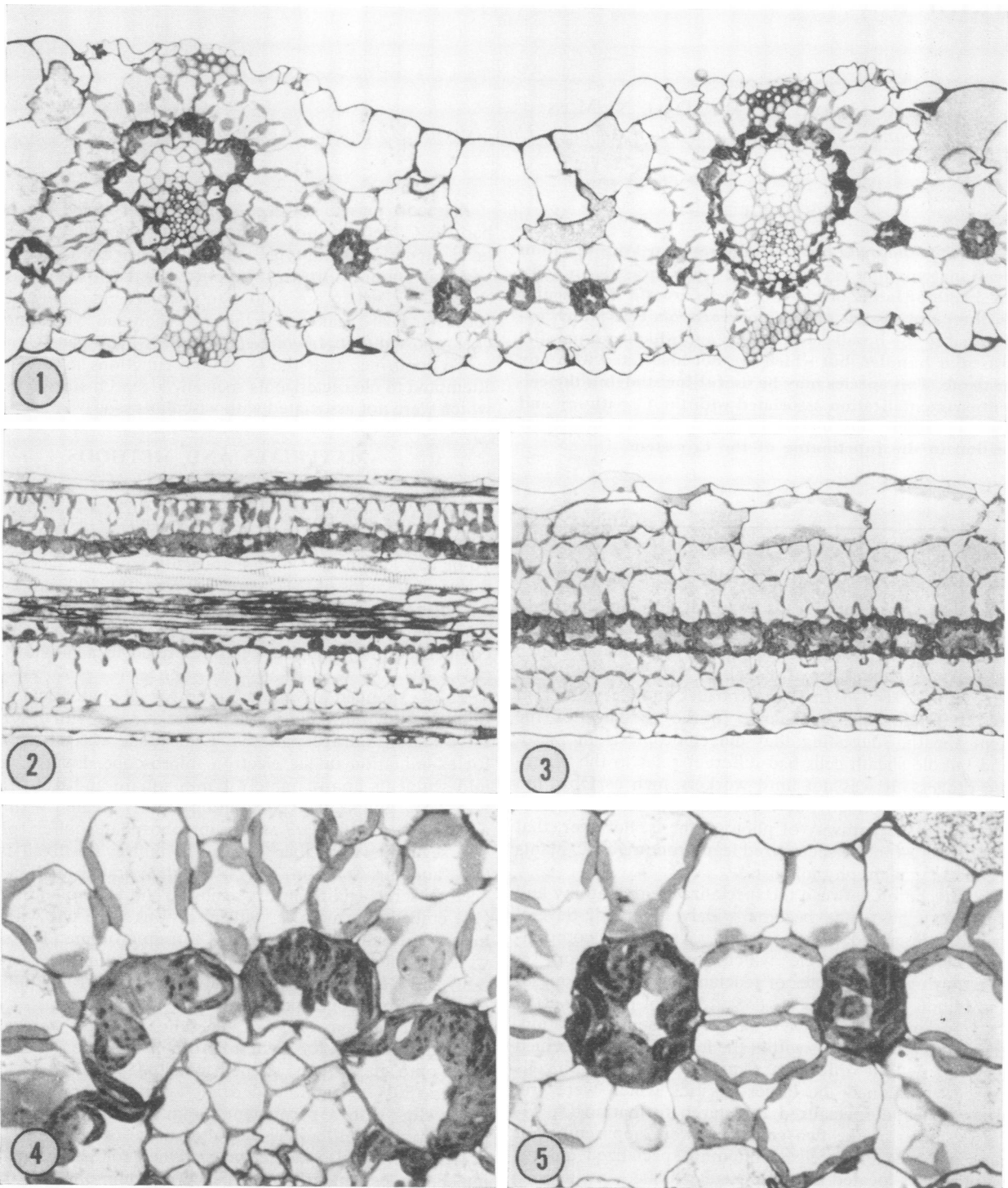Figure 1 is a cross-sectional view of an *A. hirta* leaf, illus-

FIG. 1. A cross-sectional view of an *Arundinella hirta* leaf. Two vascular bundles are shown enclosed by sheaths of prominent cells containing numerous dark chloroplasts. Between the vascular bundles are individual prominent cells which resemble the bundle sheath cells (*i.e.*, contain numerous dark chloroplasts), but which are not associated with vascular tissue. $\times$ 200.

FIG. 2. A longitudinal section through a vascular bundle of *A. hirta*. $\times$ 200.

FIG. 3. A longitudinal section through a row of specialized parenchyma cells of *A. hirta*. $\times$ 200.

FIG. 4. A cross-sectional view of some vascular bundle sheath and mesophyll cells of *A. hirta*. $\times$ 650.

FIG. 5. A cross-sectional view of two specialized parenchyma cells and some mesophyll cells of *A. hirta*. $\times$ 650.

trating the irregular arrangement of bundle sheath-type leaf cells as reported by Brown (2). In addition to the regular sheath of large, bright green cells around the vascular bundle, there are single large parenchyma cells which appear identical to the bundle sheath cells, but which are not associated with vascular tissue. Hereafter, we will refer to these cells as "specialized parenchyma cells." Figure 2 shows a longitudinal section through a vascular bundle of *A. hirta*. Figure 3 shows a longitudinal section through the specialized parenchyma cell area and illustrates the continuous, end-to-end nature of the arrangement of these cells. Figures 4 and 5 are close-ups of portions of Figure 1 and show some bundle sheath cells and two of the specialized parenchyma cells at a higher magnification. The similarity between the two cells (bundle sheath and specialized parenchyma) is clear. The chloroplasts of both are large, dark, and closely packed and contain numerous starch grains (black spots). They contrast markedly with the smaller chloroplasts of the mesophyll cells which are lighter, less crowded, and relatively free of starch. The chloroplasts of both the bundle sheath and specialized parenchyma cells have a centrifugal arrangement (toward the surrounding mesophyll). The specialized parenchyma cells are not associated with any vascular tissue, however.

Figure 6 shows a paradermal view of an *A. hirta* leaf that

has been cleared with NaOH and stained with I₂-KI. The large dark rows are vascular bundles with sheaths of surrounding parenchyma cells specialized for starch storage. Several rows of specialized parenchyma cells can be seen running in single strands parallel to the vascular bundles. These strands were generally continuous (end to end) throughout all leaf segments. Occasionally, however, one row ended and another began, or became double (extreme lower right of Fig. 6). The lack of association of the specialized parenchyma cells with vascular tissue is again apparent.

The specialized parenchyma cells did attach to vascular tissue under certain conditions, however. Figure 7 is a segment of a cleared *A. hirta* leaf showing a cross-vein which runs from one vascular bundle to another (lower left to upper right). As the cross-vein passes the rows of specialized parenchyma cells, some of the cells become appressed to the cross-vein, much like a normal bundle sheath. This happened with all the traversing veins which we observed. These cross-veins were few in number, however, and provided a vascular-bundle association for only an insignificant proportion of the specialized parenchyma cells. Figure 8 illustrates the same type of association of specialized parenchyma cells with vascular tissue as is seen in Figure 7, except the view is cross-sectional.

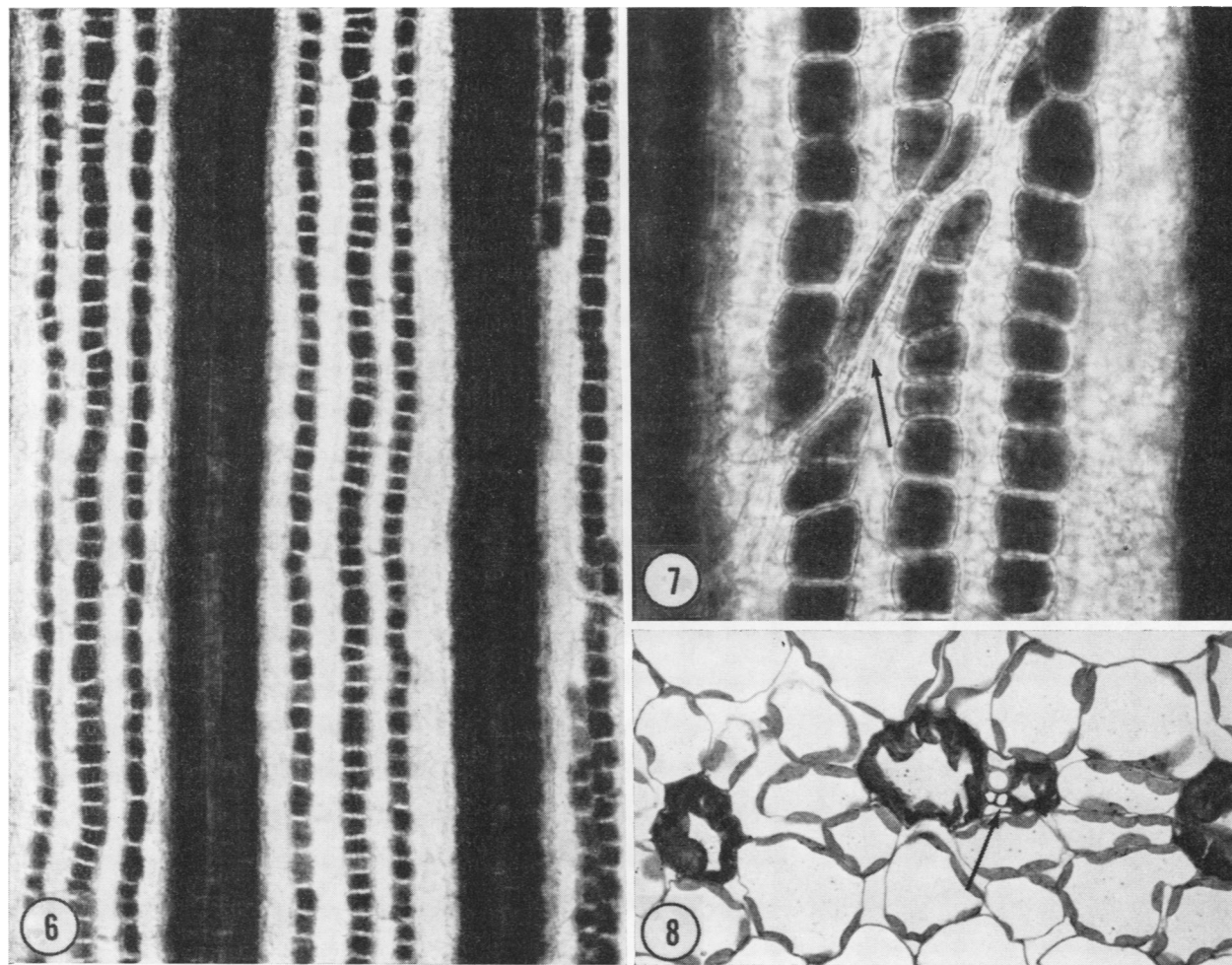Figure 9 is an electron micrograph of bundle sheath and

FIG. 6. A paradermal view of a portion of an *A. hirta* leaf that has been cleared and stained with I₂-KI. The starch-containing vascular bundle sheath and rows of specialized cells show clearly. × 125.

FIG. 7. A paradermal view of a portion of an *A. hirta* leaf showing a cross-vein (arrow) traversing three rows of specialized cells. Some of the specialized cells have become appressed to the cross-vein. × 300.

FIG. 8. A cross-sectional view of an *A. hirta* leaf showing specialized parenchyma cells appressed to a cross-vein. × 750.
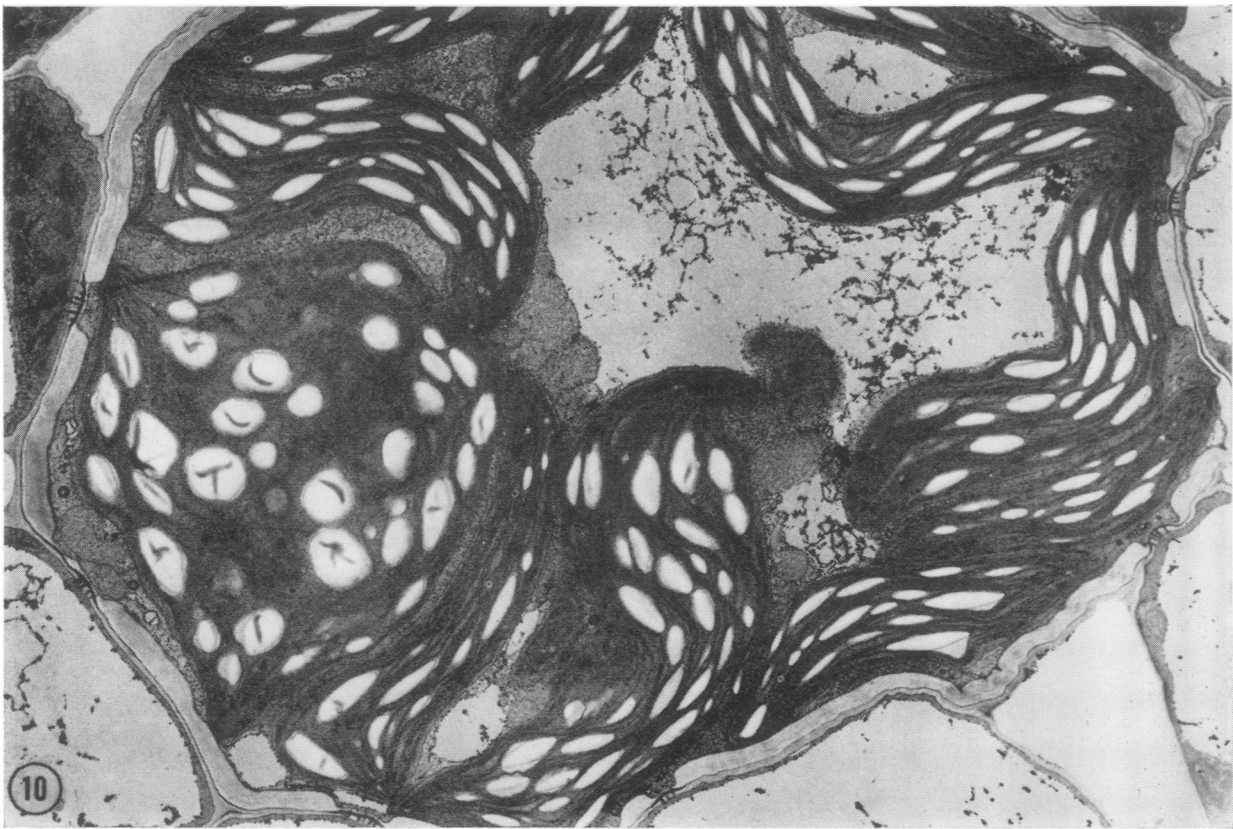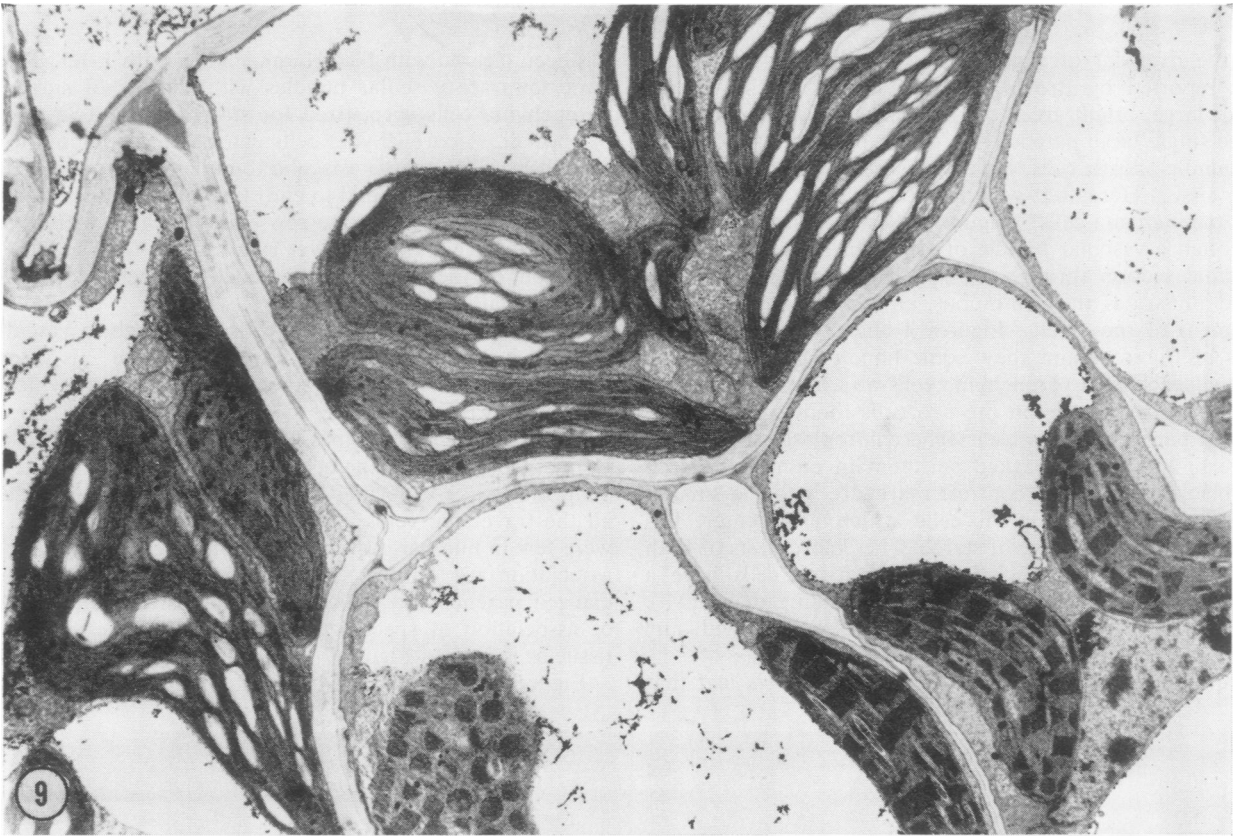
Fig. 9. An electron micrograph of the bundle sheath region of *A. hirta*. Portions of two bundle sheath cells (upper left) are shown in relation to adjoining mesophyll cells. The large chloroplasts of the bundle sheath cells are agranal and contain numerous starch grains. The chloroplasts of the mesophyll cells are free of starch and have well developed grana. × 6400.

Fig. 10. An electron micrograph of a specialized parenchyma cell. The ultrastructure of the specialized cell appears identical to that of the bundle sheath cells (Fig. 10). An electron-opaque band, which is thickened over the numerous pit fields containing plasmodesmata, is located within the cell wall of both the specialized parenchyma and vascular bundle sheath cells. × 6400.

mesophyll cells. Figure 10 is an electron micrograph of a specialized parenchyma cell. The chloroplasts of both the bundle sheath and specialized parenchyma cells contain numerous starch grains and have underdeveloped grana. The smaller mesophyll chloroplasts contain very little starch but have well developed grana. An electron-opaque band is found in the thick wall of both cells. This band forms a cylinder around the strand of specialized cells much like the cylinder around the vascular bundle in the outer walls of the bundle sheath. Prominent pits with plasmodesmata can be seen in the walls of both cells. It is apparent that the ultrastructural traits of the specialized parenchyma cells are identical to those of the vascular bundle sheath cells. The fact that the chloroplasts of the specialized parenchyma cells of *A. hirta* are rich in starch indicates that these cells have functional as well as structural similarities to bundle sheath cells.

It is interesting to consider the peculiar location of starch-storing parenchyma cells in *A. hirta* in terms of the role of mesophyll and bundle sheath cells in the operation of the C₄ pathway. The proposed pathway (8) requires the close association of bundle sheath and mesophyll cells. Vascular bundles are so frequent in most C₄ grasses that almost all of the

FIG. 11. A longitudinal view of the ultrastructure of the cell wall region between two adjoining specialized parenchyma cells of *A. hirta*. No pit fields or plasmodesmata could be found connecting two specialized cells, although they were plentiful in the walls shared with the surrounding mesophyll (arrow). The electron-opaque band is double for a distance between the 2 cells and then terminates, just as it does between adjacent bundle sheath cells. × 17,000.

mesophyll cells are in direct contact with a bundle sheath cell; *i.e.*, vascular bundle sheaths are usually spaced at intervals only two mesophyll cells apart (4). In contrast, the vascular bundles of *A. hirta* are spaced at an average of seven mesophyll cells apart. The location of specialized parenchyma cells in mid-mesophyll positions in *A. hirta*, therefore, provides a cell which appears to be identical to a bundle sheath cell (except that it lacks a vascular connection) at intervals no greater than two mesophyll cells apart. This restores, in part, the normal C₄ cell pattern and would allow the C₄ pathway of photosynthesis to operate in all the regions of *A. hirta* leaves.

The question arises, however, concerning the role of translocation in the functioning of the C₄ system. That the strands of specialized parenchyma cells do not have vein-like translocational properties is apparent from Figure 11. The cell wall region between two specialized cells is shown (longitudinal view). The view is representative of all such walls observed and shows the impermeable appearance of the connection between these cells. No pit fields or plasmodesmata could be found connecting two specialized cells, although they were plentiful in the walls shared with the surrounding mesophyll. The presence of specialized parenchyma cells in *A. hirta* might not permit the functioning of the C₄ system at a maximal rate for any extended period, therefore, unless the starch-storing capacity of these cells is sufficient to accommodate the entire day's synthesis of carbon from adjacent mesophyll cells. Hydrolysis and translocation via the vascular bundles during the night would then be required to make way for the production of the next day.

The segments of *A. hirta* leaves between vascular bundles are sufficiently large that they could probably be dissected from the leaf, allowing the recovery of tissue containing only normal and specialized parenchyma. Such tissue could be useful in studying both the cellular compartmentalization of the C₄ syndrome and of the role of translocation in the functioning of the C₄ system. It may also be possible to learn more about the differentiation of the leaf anatomy that is characteristic of C₄ plants by studying this interesting species.

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