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Short Communication

Distribution of Photosynthetic Enzymes between Mesophyll, Specialized Parenchyma and Bundle Sheath Cells of *Arundinella hirta*

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BONNIE J. REGER AND IDA E. YATES
Richard B. Russell Research Center, Science and Education Administration, United States Department of Agriculture, Athens, Georgia 30604

ABSTRACT

Arundinella hirta **L. is a C_4 plant having an unusual C_4 leaf anatomy. Besides mesophyll and bundle sheath cells, *A. hirta* leaves have specialized parenchyma cells which look morphologically like bundle sheath cells but which lack vascular connections and are located between veins, running parallel to them. Activities of phosphoenolpyruvate and ribulose-1,5-bisphosphate carboxylases and phosphoenolpyruvate carboxykinase, NADP- and NAD-malic enzymes were determined for whole leaf extracts and isolated mesophyll protoplasts, specialized parenchyma cells, and bundle sheath cells. The data indicate that *A. hirta* is a NADP-malic enzyme type C_4 species. In addition, specialized parenchyma cells and bundle sheath cells are enzymatically alike. Compartmentation of enzymes followed the C_4 pattern with phosphoenolpyruvate carboxylase being restricted to mesophyll cells while ribulose-1,5-bisphosphate carboxylase and decarboxylating enzymes were restricted to bundle sheath and specialized parenchyma cells.**

Plants displaying C_4 photosynthesis[1] generally have a specific anatomical leaf structure known as Kranz anatomy (9). This anatomical pattern consists of a radial arrangement of chlorenchyma around vascular bundles. The chlorenchyma is differentiated into an inner layer of thick walled cells comprising the bundle sheath and one or more outer layers of thin walled mesophyll cells.

Variations of this typical C_4 leaf anatomy have been found. Shomer-Ilan *et al.* (10) characterized *Suaeda monoica* as a C_4 plant species whose succulent leaves lack typical bundle sheaths. They found that *Suaeda* leaves have outer and inner layers of chlorenchymatous cells and that the inner layer surrounds water tissue rather than vascular tissue. They suggested that a chlorenchymatous cell layer need not be adjacent to vascular tissue for the operation of the C_4 pathway.

Tateoka (11) found the internal leaf anatomy of several *Garnotia* and *Arundinella* species to be similar in that many species of each genus had "distinctive cells," chloroplast-containing bundle sheath type cells scattered throughout the mesophyll. Apparently, *Garnotia* and *Arundinella* are the only two genera of the Gramineae to share this feature (11). Crookston and Moss (1) characterized these cells anatomically in the leaves of *Arundinella hirta*, a C_4 plant species (1, 12). They determined that the specialized parenchyma cells appeared morphologically identical to bundle sheath cells, that they ran parallel to vascular bundles but were not associated with vascular tissue, that they lacked plasmodesmata between like cells but did have abundant plasmodesmata with mesophyll cells, and that they stored starch. They also found (1) that the chloroplasts of both the bundle sheath and specialized parenchyma cells had underdeveloped grana and contained many starch grains as opposed to mesophyll chloroplasts which had well developed grana and very little starch. Chloroplasts in the bundle sheath and specialized parenchyma cells were arranged centrifugally, *i.e.* directed outward from the cell center toward the surrounding mesophyll cells (1).

A. hirta's specialized parenchyma cells were of obvious interest since operation of the C_4 system requires rigid compartmentation and transport between mesophyll and bundle sheath cells (4) and since translocation would be favored by the typical C_4 anatomy. We used the cell separation technique of Edwards *et al.* (7, 8) to isolate mesophyll protoplasts, specialized parenchyma cells, and bundle sheath cells of *A. hirta* leaves, and determined the enzyme content of each.

MATERIALS AND METHODS

Seeds of *Arundinella hirta* L., accessions 246756 Japan and 263693 Korea, were obtained from Regional Plant Introduction Station at Experiment, Georgia. Seedlings were grown from seeds sown in potting soil (mixture of equal parts of pinebark, sand, and sandy loam) contained in pans (31 × 21 cm). Following germination in a greenhouse mist chamber these were placed in growth chambers with 30 and 20 C day-night temperatures, a 16-hr day length, and a quantum flux density from fluorescent and incandescent lamps of about 127 μE m^{-2} sec^{-1} between 400 and 700 nm. Plants were fertilized weekly with Miller's[2] 20-20-20 and Sequestrene iron chelate.

Transverse leaf segments, about 0.5 mm wide, were cut by hand from 1.0-g young leaves of accession 246756 and 263693. Enzyme and wash media of Huber and Edwards (7) were used to separate cells. Leaf segments were incubated in enzyme solution at 30 C with illumination. After 2-hr incubation leaf segments were appropriately washed to release protoplasts and other cells. Cells in wash medium were filtered successively through a 35-mesh steel sieve and 80-, 35-, 30-, and 20-μm nylon nets. The filtrate consisted mainly of protoplasts but also some chloroplasts which were

[1] Abbreviations: C_4 photosynthesis: C_4-dicarboxylic acid pathway; RuBP: ribulose 1,5-bisphosphate; PEP: phosphoenolpyruvate.

[2] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

eliminated by discarding supernatant fractions following pelleting and washing of protoplasts at 300*g* for 1.5 min. The 20-μm net prevented contamination of protoplasts by a few single rectangular cells freed from either bundle sheath strands or specialized parenchyma strands. These cells were discarded. Specialized parenchyma strands collected on the 30- and 35-μm nets; some bundle sheath strands also collected on the 35-μm net. Bundle sheath strands tended to be heavier than specialized parenchyma strands and settled out upon standing for a couple min or upon centrifuging at 100*g* for 10 to 20 sec. Specialized parenchyma strands from the 35-μm net were purified by combining supernatant fractions from short centrifugations at 100*g* and were combined with the specialized parenchyma strands from the 30-μm net. A mixture of bundle sheath strands and specialized parenchyma strands collected on the 80-μm net. While some specialized parenchyma strands were eliminated from this fraction by discarding supernatant fractions upon settling of bundle sheath strands naturally or by short centrifugations at 100*g*, others remained linked to bundle sheath strands by adhering to cross-veins. Cell fractions were washed off nets and collected by centrifugation at about 300*g* for 1.5 min in a clinical centrifuge with a swinging bucket rotor. Cell fractions were broken using a glass homogenizer and the breaking medium of Kanai and Edwards (8). RuBP carboxylase and PEP carboxylase were assayed at 30 C according to Willmer *et al.* (13). PEP carboxykinase was assayed at 30 C by the exchange reaction according to Dittrich *et al.* (2). NADP-malic and NAD-malic enzymes were assayed spectrophotometrically at room temperature (13). Chl was determined by the method of Wintermans and DeMots (14).

Whole leaf extracts were obtained by grinding 1 g of tissue, transverse segments about 1 mm wide, in a mortar and pestle with acid-washed sand and medium of Kanai and Edwards (8). The ground material was squeezed through two layers of Miracloth and centrifuged at about 300*g* for 45 sec. The supernatant fraction was used in enzyme assays.

RESULTS AND DISCUSSION

Figures 1, 2, and 3 are representative of the isolated cell fractions—mesophyll protoplasts, specialized parenchyma strands, and bundle sheath strands, respectively. As shown in Figure 3, the bundle sheath enriched fraction was contaminated, about 20% generally, with specialized parenchyma strands (three are evident though out of focus). This contamination was due to specialized parenchyma cells adhering tightly to cross-veins. Rapid vibration using a Vortex mixer loosened some specialized parenchyma strands but failed to eliminate them effectively. Specialized parenchyma strands were easily isolated and purified (Fig. 2). Bundle sheath and specialized parenchyma strands remained distinguishable due to their resistance to cellulase and pectinase even upon incubation much longer than used here. Mesophyll protoplasts were very fragile and were easily damaged by extensive purification; therefore, protoplasts were handled very carefully and simply washed two to three times. Membrane integrity of the various cell fractions was determined by exclusion of Evan's blue dye (8).

Enzyme activities of whole leaf extracts (Table I) indicate that *A. hirta* is a NADP-malic enzyme type C_4 plant species. This is in accord with the ultrastructural and biochemical relationship discovered by Gutierrez *et al.* (3) for Gramineae species. According to Gutierrez *et al.* (3) NADP-malic enzyme species of the Gramineae lack well developed grana in bundle sheath chloroplasts (grana reduced) and the bundle sheath chloroplasts are located in the centrifugal position. These ultrastructural features were shown in *A. hirta* by Crookston and Moss (1). *Arundinella* species lack a mesotome sheath which is also indicative of a NADP-malic enzyme C_4 species (5). Bundle sheath and specialized parenchyma cells have similar Chl *a/b* ratios (Table I) and their *a/b* ratios are higher than the *a/b* ratio of mesophyll protoplasts, which is characteristic of C_4 plants with agranal bundle sheath chloroplasts.

Enzyme activities of the various separated fractions (Table I) indicate rigid compartmentation of enzymes which is typical of C_4 plants. PEP carboxylase is restricted to mesophyll cells, while RuBP carboxylase is located in both the bundle sheath cells and the specialized parenchyma cells. Restriction of RuBP carboxylase to these latter cells has been shown previously by Hattersley *et al.* (6) in *A. nepalensis* using *in situ* immunofluorescent labeling. The decarboxylating NADP- and NAD-malic enzymes are also restricted to bundle sheath and specialized parenchyma cells. No PEP carboxykinase activity was detectable in any of the extracts. Inasmuch as the enzyme activities of the bundle sheath cells and the specialized parenchyma cells are similar they seem to be functionally identical.

It is interesting to note the variation of internal leaf anatomy of *Garnotia* and *Arundinella* species. Apparently there are species lacking the so-called "distinctive cells" (specialized parenchyma cells), species showing various transitional states from normal

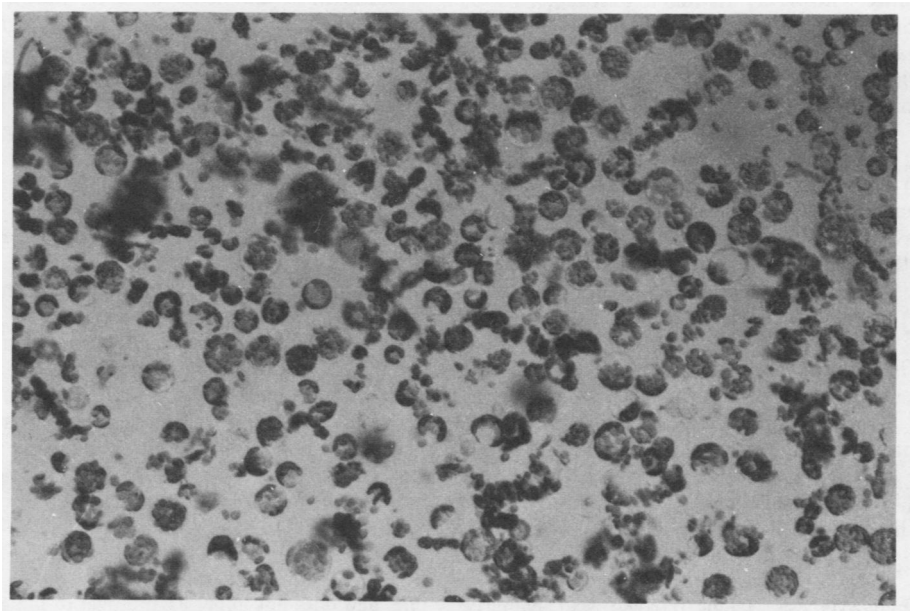

FIG. 1. Light micrograph of a field of isolated mesophyll protoplasts from *A. hirta*, accession 246756 (× 200).

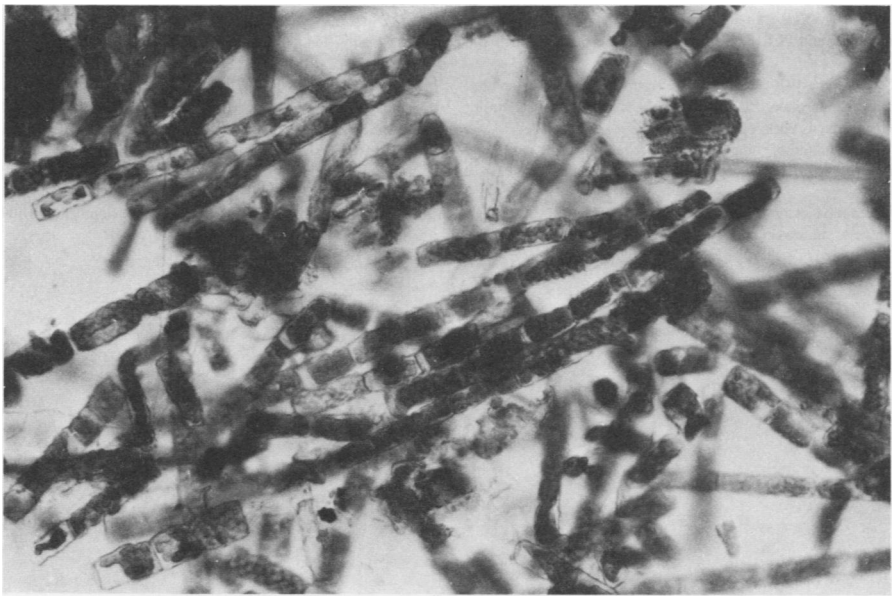

FIG. 2. Light micrograph of a field of isolated specialized parenchyma strands from *A. hirta*, accession 246756 (× 190).

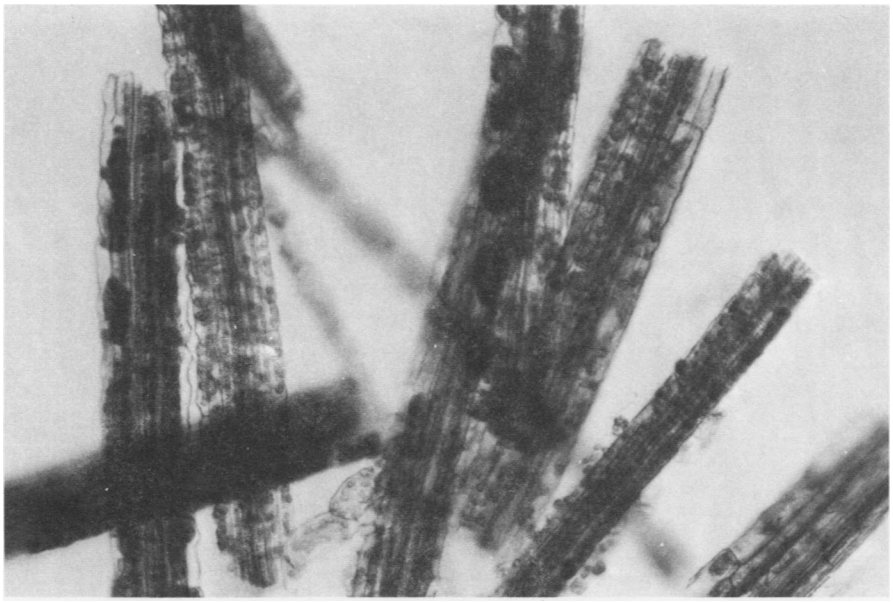

FIG. 3. Light micrograph of a field of isolated bundle sheath strands of *A. hirta*, accession 246756 (× 177).

Table I. Chlorophyll a/b ratios and enzyme distribution of carboxylating and decarboxylating enzymes among mesophyll protoplasts, specialized parenchyma cells, bundle sheath cells, and whole leaf extracts

Preparation	Chl a/b	PEPC[a]	RuBPC	NADP-mal	NAD-mal
		.moles/mg Chl·hr			
Accession 246756 Japan					
Mesophyll protoplasts	2.02	516	0	4	0
Specialized parenchyma cells	2.86	0	120	143	15
Bundle sheath cells	3.03	0	202	306	18
Whole leaf	2.45	367	72	156	8
Accession 263693 Korea					
Mesophyll protoplasts	2.20	699	0	15	1
Specialized parenchyma cells	2.84	0	231	416	25
Bundle sheath cells	2.88	0	294	480	48
Whole leaf	2.34	506	72	234	16

[a] PEPC, PEP carboxylase; RuBPC, RuBP carboxylase; NADP-mal, NADP malic enzyme; NAD-mal, NAD malic enzyme.

vascular bundles to simply a mass of sheath cells, and species with distinctive cells existing either singly or in some species in groups of two or three (11). Species lacking distinctive cells have vascular bundles of various sizes. The transitional aspects displayed by *A. mesophylla* (11) would suggest that distinctive cells are bundle sheath cells which have lost their association with vascular tissue. The existence of C_3 specialized parenchyma cells in *A. hirta* may allow maximal operation of the C_4 pathway in a plant species having extraordinarily large interveinal distances. This remains to be examined and correlated to starch accumulation.

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