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Communication

DNA and RNA Levels in Bundle Sheath and Mesophyll Cells of Pearl Millet (*Pennisetum americanum*)[1]

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CAROLE L. BASSETT*, JAMES R. Y. RAWSON, AND JUDITH A. JERNSTEDT
United States Department of Agriculture, Russell Research Center, Athens, Georgia 30613 (C.L.B.); *BP America Research and Development, Cleveland, OH 44128* (J.R.Y.R.); *and Department of Agronomy and Range Science, University of California, Davis, California 95616* (J.A.J.)

ABSTRACT

The DNA content of bundle sheath cells and mesophyll protoplasts from the C_4 plant pearl millet (*Pennisetum americanum*, Tift 23DB) was determined by microspectrophotometry to be 1.8 to 2.3 and 3.2 to 4.0 picograms/nucleus, respectively. Measurement of RNA by ultraviolet spectroscopy indicated that bundle sheath cells contain twice as much RNA as mesophyll cells.

A common assumption among researchers is that nuclear DNA contents are the same in cells of mature, differentiated plant tissues comprising a given organ. Reports in the literature, however, suggest caution in applying this assumption to diverse experimental systems. For example, Evans and Van't Hof (15) compared ploidy levels in various tissues of three angiosperm genera. No polyploid cells were found in any tissue of *Helianthus annuus* examined, but polyploidy in *Pisum sativum* tissues was the rule, rather than the exception. Likewise, cells in the cotyledons of developing soybean seedlings differed significantly in DNA content depending on the age of the tissue sampled (13). These results indicate that individual cells within a single tissue may differ in nuclear DNA content, as may tissues within a single organ. The primary objective of our work, therefore, was to compare the relative DNA and RNA contents of the two photosynthetically active cell types, bundle sheath and mesophyll, in the leaves of the C_4 grass pearl millet. The limitations and possible significance of these results are presented here, and the difference in nuclear DNA content between the cell types is discussed in light of current information about cell differentiation, cell cycle progression, and the role of polyploidy in plant growth and development.

MATERIALS AND METHODS

Growth of Plant Material. Seeds of pearl millet (*Pennisetum americanum*, Tift 23DB) were planted in flats of Fafard No. 3 and germinated in the dark for four days at 25°C. Etiolated seedlings were transferred into constant fluorescent light (92 μmol m^{-2} s^{-1}) and grown at 25°C for 17 to 46 d. The plants were treated once a week with soluble fertilizer (Peters Fertilizer Products, Fogelsville, PA) according to manufacturer's directions.

Enzymes and Reagents. Cellulysin (10 units/mg) was purchased from Calbiochem (San Diego, CA);[2] pectinase (6 units/mg) was obtained from Sigma Chemical Co. (St Louis, MO). Unless otherwise stated, all other chemicals and reagents were purchased from Sigma Chemical Co.

Preparation of Mesophyll Protoplasts. Mature, fully expanded leaves of 40- to 50-d-old plants were harvested and cut crosswise into 2 to 3 mm segments. Protoplasts were isolated following the procedure of Kanai and Edwards (20). The isolation (I) medium was 0.4 M sorbitol, 1 mM $CaCl_2$ (pH 5.5) containing 2% cellulysin, 0.1% BSA, and 2.5% pectinase. Purified protoplasts were collected and examined in the light microscope for purity and intactness. No contaminating cell types or ruptured protoplasts were observed, and chloroplasts in these preparations had well developed grana typical of mesophyll cells. Yields averaged around 8×10^3 protoplasts/g fresh leaf weight.

Preparation of Bundle Sheath Cells. Leaves from 40- to 50-d-old plants were harvested and cut into segments as before. Chopped segments (30–35 g) were added to 300 ml of I medium, blended in a Waring blender for 10 s, and filtered through cheesecloth (9). The debris was blended a second time for 5 min, filtered, and rinsed extensively with water to remove mesophyll debris. The strands were incubated in I medium plus enzymes with gentle shaking for 6 to 8 h, washed three times in I medium alone, and vigorously stirred for 15 min. The material was filtered through 35 μm mesh nylon and bundle sheath cells were collected by centrifugation at 300*g*. In some experiments, the bundle sheath cells were further purified by layering the resuspended pellet on a step-gradient consisting of 10, 15, and 20% Ficoll (w/v) in I medium. The band at the 15/20% Ficoll interface was collected and centrifuged as before. The yield of purified bundle sheath cells was between 10 to 50 cells/g fresh leaf weight.

Preparation of Bundle Sheath Strands and Mesophyll Lysates. Bundle sheath strands and mesophyll cell lysates were prepared by differential homogenization following a modification of the procedure described by Chollet and Ogren (9). Mature, fully expanded leaves (50–100 g) of 21-d-old plants were harvested as before and blended for 10 s at 4°C in 200 ml extraction buffer (50 mM Tris-HCl [pH 9], 100 mM NaCl, 100 mM 2-mercaptoethanol, and 5 mM diethyldithiocarbamic acid). The material was filtered through cheesecloth, and the filtrate (mesophyll lysate)

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[2] Mention of trademark or a proprietary product does not constitute a guarantee or warrant 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.

was stored at −80°C. Debris retained by the cheesecloth was returned to the blender and blended an additional 5 min to remove any remaining mesophyll cells. Bundle sheath strands prepared by this method were essentially devoid of any adhering mesophyll cells, and no mesophyll cells or protoplasts survived the 5 min blending.

Purity of the separated tissues was determined by measuring the amounts of PEPC[3] monomeric subunit and the small subunit of Rubisco in protein extracts from both tissues using a competition ELISA procedure (21) with the following modifications. For protein extraction, 3 ml of mesophyll cell lysate were adjusted to 2% (w/v) SDS. The lysate was ground with 0.2 g sand for 2 min in a mortar and pestle on ice, and the sample was centrifuged at 37,000*g* for 15 min. The supernatant was removed, and proteins were precipitated with 25 volumes of cold acetone at −20°C for 12 to 16 h. Proteins were extracted from bundle sheath strands (0.1 g wet weight in 3 ml T buffer [50 mM Tris-HCl (pH 8.0), 0.143 M 2-mercaptoethanol, 2% (w/v) SDS]) identically. The protein precipitates from each cell type were suspended in 0.5 ml of 0.1 N NaOH, sonicated briefly, and neutralized with 1.2 N HCl. Tris-HCl was added to a final concentration of 50 mM, and the samples were stored at −80°C. Protein concentrations were determined using the Bradford procedure (6). Rabbit antiserum specific to either the monomeric subunit of PEPC (1000-fold dilution) or the small subunit of Rubisco (2000-fold dilution) from pearl millet (2) was used in the competitive ELISA. Purified PEPC and small subunit of Rubisco from pearl millet were used to generate standard curves. Purities of six different preparations of bundle sheath strands and mesophyll cell lysates were calculated by assuming that PEPC and Rubisco were expressed solely in mesophyll or bundle sheath cells, respectively (Table I). The purities ranged from 93 to 98% (bundle sheath strands) and from 88 to 96% (mesophyll lysates). The purity of bundle sheath strand preparations may be slightly overestimated, since contributions of other vascular bundle cell types to the extracted proteins could not be determined.

Determination of DNA and RNA Content by Ultraviolet Spectroscopy. Nucleic acids were extracted according to Fleck and Munro (16) with the following modifications: 10 ml of mesophyll lysate were brought to 10% TCA by the addition of 5.0 ml 30% TCA. Bundle sheath strands (0.5 g wet weight) were ground to a powder in liquid nitrogen and suspended in 5.0 ml extraction buffer and 2.5 ml 30% TCA. The suspensions were incubated on ice for 30 min and centrifuged at 12,000*g*, and the pellets were washed two times each with cold 10% TCA, acetone, ethanol/chloroform (3:1), and 95% ethanol to remove Chl. The pellets were resuspended in 1.0 ml (mesophyll) or 2.0 ml (bundle sheath) 0.3 N KOH, heated to 37°C for 1 h, and centrifuged as before. This treatment effectively avoids interference from protein degradation products that have been shown to occur in samples treated for longer times in more concentrated base (16). The supernatants were removed, neutralized with 9 N HCl, and precipitated on ice 45 min by the addition of 1 volume 10% TCA. Each precipitate was washed twice with 5% TCA, and the washes (containing RNA hydrolysis products) were combined with the supernatant. The pellets (DNA) were resuspended in 1.0 ml 1 N NaOH and used immediately. DNA and RNA were measured by ultraviolet absorption using λ-DNA and *Escherichia coli* ribosomal RNA as standards. The standards were separately solubilized in 0.3 N KOH and thereafter treated in the same manner as the unknown samples. DNA obtained by this method was fully recovered, since longer extraction times in cold acid or boiling briefly in dilute acid failed to increased the DNA yield.

Microspectrophotometry. Mesophyll protoplasts and bundle sheath cells prepared as described above were resuspended in water, spotted onto glass slides coated with chrome alum gelatin adhesive (5), dried for 1 h at 60°C, and cooled to room temperature. A drop of blood from a young white leghorn rooster previously had been smeared onto each slide to serve as an internal standard. Cells and protoplasts on slides were fixed in 3:1 absolute ethanol/glacial acetic acid for 2 h in the dark, rinsed for 8 h in three changes of deionized water, and stored in the dark to dry. Schiff's reagent was prepared according to Berlyn and Miksche (5). Material was hydrolyzed in 5 N HCl for 20 min at 22°C, stained for 90 min in Schiff's reagent in the dark, rinsed briefly in 4°C deionized water, rinsed in three changes of potassium metabisulfate-water, dehydrated through an ethanol series, and mounted in Euparal. Absorbance measurements of Feulgen stained nuclei were made at 550 nm with a Zeiss Zonax MPM 03 scanning microspectrophotometer using BIOSCAN software (version 84-0701), following standard procedures to minimize experimental error (3, 4, 17). Relative DNA content was determined for 160 bundle sheath nuclei, 152 mesophyll nuclei, and 90 red blood cell nuclei. Mean relative DNA contents of bundle sheath and mesophyll nuclei were compared using the *z* test statistic.

Table I. *Estimation of Purity of Bundle Sheath Strands and Mesophyll Cell Extracts*

Tissue	PEPC[a]	Small Subunit[a] of Rubisco	Average Cell Purity[b]
	μg/mg total protein		%
Bundle sheath strands	2.6 ± 0.53	55 ± 4.30	95
Mesophyll cell extracts	48 ± 15.4	3.9 ± 0.85	93

[a] The concentrations of monomeric subunit of PEPC and small subunit of Rubisco were determined by the competition ELISA procedure. The data for six independent preparations of bundle sheath strands and mesophyll cell extracts were averaged and the standard error of the mean was calculated for each. [b] % Purity bundle sheath strands was calculated as

$$1 - \left(\frac{\mu g \text{ PEPC bundle sheath}}{\mu g \text{ PEPC mesophyll}}\right) \times 100$$

% purity mesophyll cell extracts was calculated as

$$1 - \left(\frac{\mu g \text{ SS Rubisco mesophyll}}{\mu g \text{ SS Rubisco bundle sheath}}\right) \times 100$$

[3] Abbreviations used: PEPC, phosphoenolpyruvate carboxylase; Rubisco, ribulose-1,5-bisphosphate carboxylase; ELISA, enzyme-linked immunosorbent assay; Kbp, kilo base pairs; C, the quantity of DNA in the unreplicated haploid genome.

RESULTS AND DISCUSSION

Mesophyll protoplasts and bundle sheath cells were separated from fully expanded leaves of pearl millet by enzymic digestion or by a combination of enzymic digestion and mechanical disruption (Fig. 1, A and B). Relative DNA values were determined microspectrophotometrically for bundle sheath (Fig. 1C) and mesophyll cell nuclei, and the distributions of relative DNA contents of the two cell types are shown in Figure 2. Absolute DNA content was estimated by comparison with literature values (24) for chicken erythrocytes (2.6–3.3 pg/nucleus). Bundle sheath cells contained 1.8 to 2.3 pg DNA per nucleus; mesophyll cells contained 3.2 to 4.0 pg DNA per nucleus. Mean relative DNA content of the two cell types was significantly different ($P < 0.01$), with mesophyll cells averaging approximately 1.7 times more DNA than bundle sheath cells. No large differences in heterochromatin between bundle sheath and mesophyll nuclei were detectable by microscopic examination, nor were mitotic figures observed in either cell type.

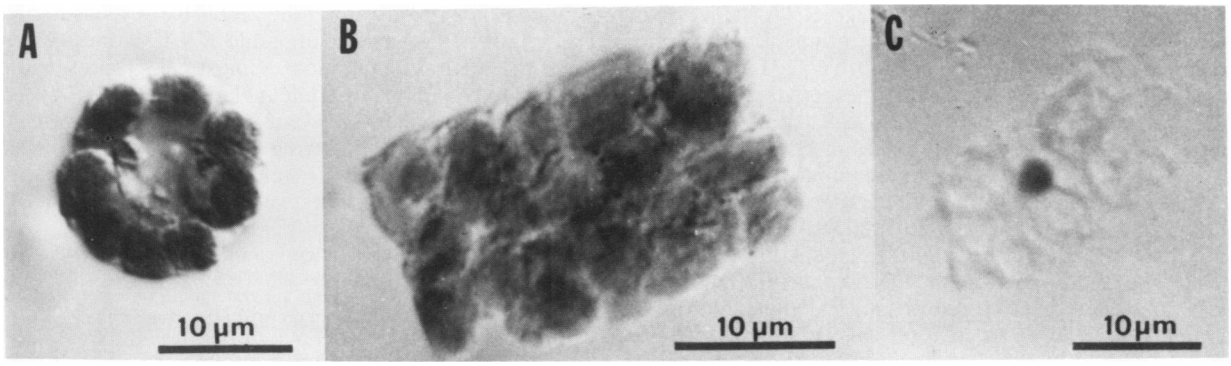

FIG. 1. Isolated mesophyll and bundle sheath cells used for microspectrophotometric DNA determinations. A, Leaf mesophyll protoplast from *P. americanum* (Tift 23DB); differential interference contrast (DIC) optics. B, Bundle sheath cell from millet leaf digestion; DIC optics. C, Bundle sheath cell after fixation and staining with Feulgen reagent, showing conspicuous nucleus; DIC optics.

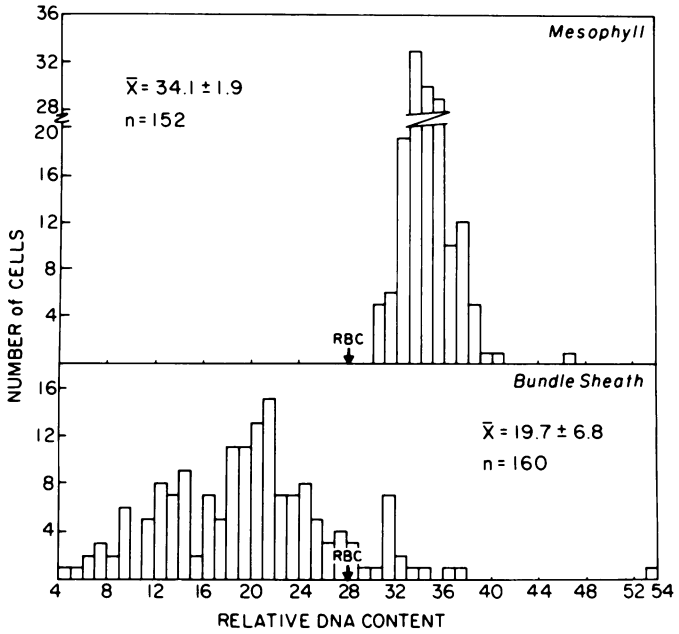

FIG. 2. Histograms of the relative amount of DNA per nucleus in bundle sheath and mesophyll cells of *P. americanum* (Tift 23DB) measured by Feulgen-stain microspectrophotometry. Arrows indicate relative amount of DNA in chicken red blood cells, which served as an internal standard. The mean, standard deviation, and sample size are shown for both cell types.

Although several possibilities can be offered to explain the higher DNA content of mesophyll cells, we are not yet able to determine which accounts for the differences reported here. Among the possibilities are polyploidization, differential cell cycle arrest, selective chromosome duplication or DNA amplification, and/or selective loss of DNA or chromosomes (8, 12, 13, 15). In many plant species, DNA endoreduplication is an important feature of cell development (1), although it is not considered essential for tissue or organ differentiation in all higher plants (15). In the case of developing soybean seedlings, the nuclear DNA content of cotyledons increased approximately 1.5-fold between very early and late maturation stages, with a substantial increase occurring after cessation of cell division (13). This increase was accompanied by a shift in the number of endopolyploid nuclei, resulting in a bimodal distribution of nuclei with 2C and 4C amounts of DNA. However, based on the unimodal distribution of absorbance measurements in each cell type observed here, it seems unlikely that differences in DNA content between bundle sheath and mesophyll cells could be due to endopolyploidy, especially since the DNA contents reported here for both cell types are within the range of 2C values reported for various millet tissues (3, 11), if the probable underestimation of Wimpee and Rawson (27) is excluded, and if differences in tissues used for genome size determinations are taken into account. Likewise, a 1.7-fold difference in DNA content between bundle sheath and mesophyll cells argues against differential arrest in G_1, S, or G_2 cell cycle stages (12), although the absence of metaphase division figures in either cell type makes it difficult to establish unequivocally the 2C or G_1 condition in bundle sheath or mesophyll cells necessary to eliminate this possibility. One likely explanation for the difference in DNA content is the selective amplification or loss of nuclear material in one cell type *versus* the other. Because of the size range of 2C values reported for various millet tissues, we cannot distinguish between amplification or loss of nuclear sequences, and evidence for both has been reported (7, 8). Amplification of selected DNA sequences is generally associated with increased transcription of the amplified region, which might lead to an increase in RNA. This is not observed in mesophyll cells (see below). Loss of specific sequences might not, however, necessarily be tied to a decrease in transcripts, especially if the deleted regions were transcriptionally inactive.

The functional or developmental significance of the larger genome size in mesophyll *versus* bundle sheath is unclear, but it may be related to the observed relationship of variation in genome size with cell volume, cell growth rates, and overall plant growth (18). Although this genome size/growth rate correlation has been observed only at the organismal level, it may also apply at the level of cellular differentiation. In this connection, Dengler *et al.* (10) report that bundle sheath and mesophyll cells in C_4 plants of the NADP-malic enzyme type (like pearl millet) diverge very early from their common developmental pathway. Although the authors did not address this point, it would be interesting to determine which of the two cell types or their precursors first becomes mitotically quiescent and if cell cycle times for bundle sheath and mesophyll precursors differ during leaf histogenesis. Such information could provide insight into the possible developmental significance of the DNA content differences reported here. To date there are no reports of Feulgen microspectrophotometric DNA measurements of bundle sheath and mesophyll nuclei of other species, so the general extent or importance of such histological differences cannot be assessed.

RNA levels in three independent preparations of bundle sheath strands and mesophyll lysates were determined by the method of Fleck and Munro (16). The concentrations ranged from 276 to 372 μg/ml for bundle sheath strands and 89 to 174 μg/ml for

mesophyll extracts (Table II). The average RNA values were normalized to the DNA content also determined spectrophotometrically for each cell type. As shown in Table II, these ratios were 2.6 and 0.7 for bundle sheath and mesophyll cells, respectively. The difference in ratios between cell types (*i.e.* nearly four-fold) is exaggerated since differences in DNA content/cell (Fig. 2) are not taken into account in this comparison. Therefore, the RNA concentration determined for each tissue was computed on a per cell basis using the amount of DNA per nucleus to determine the total number of cells extracted. This calculation was based on the assumption that the DNA concentrations measured spectrophotometrically represent nuclear plus chloroplast plus mitochondrial DNA. As a result, measurements of total DNA (spectrophotometric measurements) have to be corrected for cytoplasmic DNA content in order to use the amount of DNA/nucleus (microspectrophotometric measurements) to calculate the number of cells. To make this correction, the following observations were considered: (a) the number of DNA molecules/chloroplast is 50 for bundle sheath cells and 10 for mesophyll cells (22); (b) there are approximately 20 chloroplasts/bundle sheath cell and 12 chloroplasts/mesophyll cell (J Jernstedt, unpublished results; 19); (c) the size of the pearl millet chloroplast genome is 135 kbp (25); and (d) the size of the chloroplast DNA is the same in bundle sheath and mesophyll cells (26). Comparable information on the mitochondrial genome is not available for pearl millet, so the contribution of this genome could not be determined. After correcting for chloroplast DNA content, it is estimated that bundle sheath cells have approximately 5.4 pg RNA/cell, whereas mesophyll cells have 2.7 pg RNA/cell (Table II). These values are consistent with total RNA values reported for developing maize leaf cells (23) if differences in leaf age and methodology are considered.

It could be argued that living cells in the vascular bundles (*i.e.* sieve tube members, companion cells, and vascular parenchyma) account for most, if not all, of the RNA differences we observe in these experiments. This seems unlikely. First, dead and empty tracheary elements do not contribute to total RNA. Second, the relative size of the bundle sheath cells compared to the other vascular bundle cell types is 6 to 10 times greater (J Jernstedt, unpublished data). Third, mature sieve elements lack both a nucleus and ribosomes and are assumed to have no cytoplasmic protein synthesis of their own (14). Finally, estimation of cell numbers from sections of pearl millet leaves indicates that companion cells and vascular parenchyma are less than half as numerous as bundle sheath cells (J Jernstedt, unpublished data). As a result, the contribution of cell types other than bundle sheath to the total RNA extracted would be negligible.

The significance of the elevated RNA levels in bundle sheath strands is not readily apparent. Total RNA measurements generally reflect the amount of stable RNA synthesized by a cell, since the mRNA population of most cells represents less than 3% of the total. In this context, increased RNA levels may be indicative of a general increase in metabolic activity associated with photosynthesis and starch formation in bundle sheath cells and may not necessarily be linked directly to qualitative differences in gene expression between the two cell types.

Table II. *DNA and RNA Content of Bundle Sheath Strand and Mesophyll Cell Extracts*

Extracts of bundle sheath strands and mesophyll cell lysates were assayed for DNA and RNA content by the method of Fleck and Munro (16) as described in "Materials and Methods."

Tissue	DNA[a]	RNA[a]	RNA/DNA	RNA/cell[b]
	μg/ml			*pg*
Bundle sheath strands	123 (105–132)	314 (276–372)	2.6	5.4
Mesophyll cell extracts	175 (139–230)	128 (89–174)	0.7	2.7

[a] Values represent the average obtained from three independent preparations of bundle sheath strands and mesophyll cells. The numbers in parentheses are the ranges obtained from the three preparations. [b] The number of bundle sheath or mesophyll cells was computed from total DNA measured spectrophotometrically after correction for differences in chloroplast DNA and using an average value of 2.0 or 3.6 pg DNA/nucleus (*i.e.*, cell), respectively.

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