Photosynthesis, Leaf Anatomy, and Cellular Constituents in the Polyplloid C₄ Grass Panicum virgatum

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ABSTRACT

Photosynthetic gas exchange, activities of six key C₄ cycle enzymes, amounts of soluble protein, chlorophyll, and DNA, and various leaf anatomical and structural features were measured in naturally occurring tetraploid and octoploid plants of the NAD-malic enzyme type C₄ grass Panicum virgatum L. On a leaf area basis, the photosynthetic rate and concentrations of DNA, soluble protein, and chlorophyll were 40 to 50% higher, and enzyme activities 20 to 70% higher in the octoploid than in the tetraploid. Photosynthetic cells in the octoploid were only 17 to 19% larger in volume, yet contained 33 to 38% more chloroplasts than cells in the tetraploid. On a per cell basis the contents of DNA, soluble protein, and chlorophyll, activities of carboxylating photosynthetic enzymes, and carbon assimilation rate were all doubled in octoploid compared with tetraploid cells. Since cellular volume did not double with genome doubling, cellular constituents were more concentrated in the cells of the octoploid. The influence of polyploidy were balanced between mesophyll and bundle sheath cells since the changes in physical and biochemical parameters with ploidy level were similar in both cell types. We conclude that photosynthetic activity in these two polyploid genotypes of P. virgatum is determined by enzyme activities and concentrations of biochemical constituents, and that selection for smaller cell volume has led to higher photosynthetic rates per unit leaf area in the octoploid. The ratio of DNA content to cellular volume is a major factor determining the concentrations of gene products in cells. The number of chloroplasts, however, is controlled more by cellular volume than by the number of nuclear chromosomes.

Polyploidy is widespread in higher plants and plays a major role in the evolution of plant species (15). Polyploid plants often occur in more diverse habitats than their diploid ancestors (16). The success of polyploid plants has been attributed to increased enzyme concentrations due to expression of additional genomes (15), and to increased biochemical diversity due to increased heterozygosity and the production of novel heteromeric enzymes (15, 27). Polyploidy also affects anatomical and structural characteristics such as leaf size, cell size, and density of stomata, and number of chloroplasts per cell (10, 17). These effects of polyploidy can influence physiological activities such as photosynthesis.

The effects of polyploidy on photosynthetic capacity have been demonstrated in several C₄ plants, but have not been studied in C₄ plants. Photosynthetic rates increase with ploidy level in naturally occurring allopolyploid Festuca arundinacea (18), naturally occurring autopolyploid Agropyron cristatum (14), and in synthetic autopolyploid Lolium perenne (26) and Medicago sativa (21). However, photosynthetic rates decrease or do not change in higher ploidy levels of autotetraploid Triticum (2), naturally occurring autopolyploid Tolmiea menziesii (D Solis, MSB Ku, unpublished data), and the synthetic autopolyploids Datura stramonium (12), Lycopersicon esculentum (1), Ricinus communis (28), and Phlox drummondii (5). Except for the studies of M. sativa (21) and Triticum (2) where photosynthetic rates were expressed per cell, photosynthesis in these studies was expressed on the basis of leaf area (2, 5, 12, 14, 18), Chl (26, 28), or leaf protein (1). In many studies the photosynthetic performance of polyploids was not analyzed in relation to changes in leaf anatomy and biochemical activities in the photosynthetic cells. It is also difficult to equate changes in photosynthetic rates with either the type of polyploidy involved (auto- or allopolyploidy), or the origin of the polyploidy (natural or induced). Isogenic autopolyploids to which heterozygosity has been restored are preferable for studying the direct effects of polyploidy on photosynthesis (21), but such material has been developed for only a few C₃ species, and for no C₄ species.

In leaves of C₄ plants, the activities of mesophyll and bundle sheath cells and the compartmentalized pathways of CO₂ assimilation are coordinated to provide high rates of photosynthesis. Leaf biochemical and anatomical changes in a C₄ plant due to polyploidy may affect the way that mesophyll and bundle sheath cells interact to perform photosynthesis. The efficiency of carbon assimilation might be affected if there are changes in the relative numbers and sizes of the two cell types and their organsoles, or the relative activities of key photosynthetic enzymes which are compartmented differentially in the two cell types. Alternatively, polyploidy may affect the structure and biochemistry of the two photosynthetic cell types to the same extent.

The present study was undertaken to investigate differences in photosynthetic rates between naturally occurring tetraploid and octoploid races of the NAD-malic enzyme type C₄ grass Panicum virgatum, and to assess the physical and biochemical bases for the observed differences.

MATERIALS AND METHODS

Plant Material. Rhizomes of Panicum virgatum L. were collected from naturally growing plants in populations that were previously studied by Brunken and Estes (8) and Porter (25). Tetraploids were growing on the bank of the Canadian River in Norman, OK; octoploids were growing in the University of Oklahoma Grassland Investigation Plot west of Norman. Rhizomes from a single clone for each ploidy level were used to propagate the plants used in this study. Plants of both ploidy levels were grown under identical conditions of light and temperature in the Washington State University Botany Greenhouse. Temperatures were from 20 to 26°C in the daytime and 15°C at...
night. Daytime irradiance was between 500 and 1200 \(\mu\)mol photons/m\(^2\)-s. Plants were grown in 3.8 L plastic pots filled with potting soil, watered regularly, and fertilized twice a week with Peter’s fertilizer (20-20-20) or with a Hoagland solution. Ploidy levels were confirmed by chromosome counts of pollen mother cells in stages of meiosis. Anthers were fixed in ethanol/glacial acetic acid (3:1, v/v) and stained with acetic-carmine.

**Leaf Anatomical Studies.** Cross-sections were taken from mature leaf blades half-way between the ligule and the tip. Tissue was fixed in glutaraldehyde, embedded in L. R. White resin, and stained with Stevenel’s blue. Leaf clearings were prepared by treatment with 70% ethanol followed by 10% NaOH. Clearings were stained with permanganate and infiltrated with xylene prior to mounting.

To determine the number of mesophyll and bundle sheath cells per unit leaf area, leaf tissues were macerated by digesting small (0.6 mm diameter) glutaraldehyde-fixed leaf disks in 5% chromic acid prepared in 1 N HCl for 1 h at 60°C. The entire digested leaf disk was teased apart on a glass slide and covered with a small cover slip. Mesophyll and bundle sheath cells were distinguished in a light microscope by their distinctive shapes and thickness of cell walls. For each ploidy level, the number of both cell types was counted in three or four replicate leaf disks. Number of chloroplasts in mesophyll and bundle sheath cells was counted in 10 different cells from each of four replicates. To determine cell volumes, length and width were measured on 10 different cells for each cell type in four replicate tissue macerations. Cell width and thickness were measured in three different cross-sections.

For stomatal counts and measurements, upper and lower epidermis of living leaves were photographed through a light microscope under epillumination and the resulting negative images projected on a screen where guard cells were counted and measured. Stomata were counted on three replicate images, and measurements taken on 26 guard cell pairs in each of three images.

**Gas Exchange Measurements.** Photosynthetic gas exchange was measured on the youngest fully expanded leaves (fourth or fifth leaf on shoot) using an Anarad AR-6000 infrared gas analyzer in an open system equipped with an air-sealed cuvette as described by Wolf et al. (31). Temperature was controlled by a water jacket around the cuvette that was attached to a circulating water bath. The Anarad analyzer simultaneously measures changes in both \(\text{CO}_2\) and \(\text{H}_2\text{O}\) vapor concentrations in the air stream passing over the leaf. Photon flux density during measurements was 850 \(\mu\)mol/m\(^2\)-s at the surface of the cuvette as measured by a LiCor quantum sensor. Air flow rate was 0.78 L/min in the cuvette, and the leaf areas enclosed were approximately 10 to 12 cm\(^2\). The concentration of \(\text{CO}_2\) was maintained at 300 to 315 \(\mu\)L/L in the cuvette using outside air. Leaves were allowed to reach steady state photosynthesis at temperatures ranging from 19 to 41°C. Temperature response of photosynthesis was determined in three separate experiments for each ploidy level.

**Enzyme Assays.** Enzyme assays were performed on extracts made from leaves equivalent to those used for gas exchange measurements. Plants were preilluminated with 850 \(\mu\)mol photons/m\(^2\)-s at 24°C for 1 h, then approximately 10 cm\(^2\) of the leaf blade was quickly frozen in liquid \(\text{N}_2\) and stored at -80°C. Frozen leaf tissue was ground in 20 volumes per unit leaf fresh weight of extraction buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM MgCl\(_2\), 1 mM MnCl\(_2\), 10 mM \(\beta\)-mercaptoethanol, and 2% (w/v) insoluble PVP using a mortar and pestle. The homogenate was filtered through one layer of Miracloth, and the volume (approximately 5 ml) measured. An aliquot of this crude extract was taken for Chl determination using the method of Wintermans and De Mots (30). The crude extract was centrifuged at 14,000g for 5 min, and the resulting supernatant used for enzyme assays and measurement of soluble protein. All enzymes were assayed spectrophotometrically (29), except for RuBP carboxylase which was assayed by incorporation of \(^{14}\text{C}\)-radioactivity into acid-stable products (24). All assays were at 24°C.

Soluble protein was determined by the Bradford method (7) using the Bio-Rad protein assay reagent. DNA was measured in separate assays using fluorescent spectroscopy according to Baer et al. (3).

**RESULTS**

Earlier studies (8, 22, 23, 25) showed some morphological, physiological, and ecological variations associated with different ploidy levels in *P. virgatum*. For example, tetraploid plants have larger culms and longer and wider leaf blades than octaploid plants. We found no difference in leaf thickness or interveinal distance between the two ploidy levels, but both the size and density of stomata were greater in the octaploid (Table I).

Photosynthetic rate on a leaf area basis was about 40% higher in the octaploid than in the tetraploid over a range of temperatures (Fig. 1; Table II). Rates of transpiration were proportional to photosynthesis for both ploidy levels at all temperatures, and water use efficiency (g CO\(_2\) fixed/h \(\text{H}_2\text{O}\) transpired) was similar for both ploidy levels (data not shown). Under atmospheric conditions at 20°C, intercellular concentrations of \(\text{CO}_2\) were about 260 \(\mu\)L/L for the tetraploid, and about 270 \(\mu\)L/L for the octaploid. These concentrations are sufficient to saturate photosynthesis in \(\text{C}_3\) plants (6).

In octaploid plants Chl and soluble protein contents were approximately 40% higher per unit leaf area, and DNA content 50% higher than in tetraploids (Table II). Activities of major photosynthetic enzymes on a Chl basis were slightly lower in the octaploid, and were similar on a protein basis (data not shown), but were 20 to 70% higher on a leaf area basis (Table III). On a leaf area basis the activities of RuBP carboxylase, PEP carboxylase, and NAD-malic enzyme were 31, 47, and 70% higher, respectively, in the octaploid than in the tetraploid. The photosynthetic rate was 37% higher in the octaploid on a leaf area basis at the temperature used for enzyme assays (Table II). Therefore, on a leaf area basis, higher enzyme activities and contents of soluble protein and Chl correlate with a higher

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**Table I. Anatomical Comparison of Tetraploid and Octaploid *P. virgatum* Leaves**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ploidy Level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4x</td>
</tr>
<tr>
<td>Number of guard cells/mm(^2)</td>
<td>99 ± 2</td>
</tr>
<tr>
<td>Adaxial</td>
<td>85 ± 5</td>
</tr>
<tr>
<td>Abaxial</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>Guard cell length ((\mu)m)</td>
<td>40 ± 1</td>
</tr>
<tr>
<td>Adaxial</td>
<td>205 ± 5</td>
</tr>
<tr>
<td>Abaxial</td>
<td>161 ± 4</td>
</tr>
</tbody>
</table>

* and **, significant difference between ploidy levels at 5% and 0.5% levels, respectively (Student's t-test).

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2 Abbreviations: RuBP, ribulose 1,5-bisphosphate; PEP, phosphoenolpyruvate.
The photosynthetic rate of the octaploid

Octoploid photosynthetic cells were 17 to 19% larger in volume and contained 33 to 38% more chloroplasts than tetraploid cells (Fig. 2; Table IV). The octaploid had 32% fewer photosynthetic cells per unit leaf area. The tetraploid may have more nonphotosynthetic tissue than the octaploid (Fig. 2).

When expressed on a per cell basis, the contents of DNA, soluble protein and Chl, the activities of photosynthetic CO₂-fixing enzymes, and the rate of carbon uptake were all doubled in octaploid compared with tetraploid cells (Table V). The amount of DNA per unit cell volume was also doubled in octaploid cells, apparently producing a higher concentration of cellular constituents resulting in a doubling of the carbon uptake rate per cell.

**DISCUSSION**

Because of the leaf anatomical and biochemical changes associated with polyploidy, it is important to express photosynthetic activities on a per cell or DNA basis rather than on the basis of leaf area, weight, or Chl (20). Photosynthetic rates can be determined per cell for isolated protoplasts, but these rates are usually lower than for whole leaves, and these methods are not suitable for use with C₄ plants which have two different photosynthetic cell types. Activities that are measured for whole leaves or whole leaf extracts can be expressed per cell when cell numbers per leaf area or weight are known. Cellular volumes can also be precisely determined from measurements of macerated cells combined with measurements of cells in cross-sections.

Assuming that photosynthetic rate is determined by enzyme activities in different ploidy levels, if both cell size and enzyme activity per cell are doubled when chromosome number is doubled, then rates based on leaf area will show no change, but the rates per cell will be doubled, while the rates per unit DNA will stay the same (21). If, however, when the nuclear genome is doubled, the increase in cell size is less than 2-fold and enzyme activity still doubles, then photosynthetic rates will increase per unit leaf area, but still double per cell, and remain the same per unit DNA.

In this study photosynthesis per unit leaf area was about 40% higher in octaploid than in tetraploid *P. virgatum* over a range of temperatures (Fig. 2; Table II). The octaploid also had correspondingly higher levels of DNA, Chl, soluble protein, and activities of certain major photosynthetic enzymes per unit leaf area (Tables II, III). The volumes of octaploid mesophyll and bundle sheath cells were only 17 to 19% larger than tetraploid cells (Fig. 2; Table IV), so on a per cell basis the photosynthetic rate, carboxylating enzyme activities, and contents of measured cellular constituents showed a 2-fold increase between tetraploid and octaploid plants, corresponding to a doubling of the ratio of DNA content to cell volume (Table V). On a DNA basis the photosynthetic rate, Chl, and protein contents were slightly lower in the octaploid, indicating that somewhat less DNA is expressed in these components in the octaploid.

In some cases photosynthetic rates are inversely proportional to cell size due to lower rates of gas diffusion in larger cells. In this study and in others (4, 17), however, it is apparent that in some species metabolic activities of cells are more important in determining photosynthetic rates than are physical limitations to diffusion based on cell size. Increased gene dosage produces higher concentrations or activities of enzymes in aneuploid *Datura* (11), tetraploid *Phlox* (19), allotetraploid *Tragopogon* (27), and other higher plants (15). Furthermore, increased photosynthesis is correlated with higher concentrations of RuBP carboxylase in polyploid tall fescue (on a leaf area basis) (18), and in alfalfa (on a protoplast basis) (21). Thus, selection for smaller cells after genome doubling can lead to higher photosynthetic rates per unit leaf area (if gene expression is not greatly altered) by enhancing gas diffusion if CO₂ is limiting, and by increasing concentrations of cellular constituents or enzyme activities per cell.

High photosynthesis is correlated with high soluble protein and high activities of key photosynthetic enzymes among 10 C₄ species (29). Also, Baer and Schrader (4) showed that photosynthetic rates in maize genotypes depend more on enzyme activities...
than on the physical limitations to diffusion due to the size of cells. Therefore, it was not surprising that photosynthetic rates increased in *P. virgatum* when enzyme activities per cell doubled in the octaploid.

Our results also agree with previous studies on C₃ plants which show that cellular volume in relation to nuclear DNA content is an important factor governing photosynthetic rates in polyploid plants. Photosynthetic rates on a leaf area basis are inversely proportional to the ratio of mesophyll cell volume to nuclear DNA content (*i.e.* proportional to DNA per cell volume) in different polyploid genotypes of *Triticum* (17). In a polyploid series of tall fescue, the photosynthetic rate is highest in decaploid plants which have the highest ratio of chromosome number to estimated cellular volume (10, 18).

Further insight into the physiological effects of polyploidy can be gained by calculating the relationships among DNA content, cellular volume, and cellular constituents such as the number of chloroplasts. Number of chloroplasts per unit cell volume was 14 to 19% greater in the octaploid, but was 38% less per unit DNA (Table VI). Chloroplast replication or number per cell is apparently more strongly regulated by cell volume as found by Ellis and Leech (13) than by the amount of nuclear DNA as proposed by Butterfass (9). The number of chloroplasts per unit leaf area was actually slightly less in the octaploid, but these chloroplasts contained about 50% more Chl and protein (assuming a similar distribution of total soluble protein between chloroplasts and the rest of the cell) per chloroplast than did those of the tetraploid. Therefore, chloroplasts in the octaploid are either larger, or have Chl and protein much more concentrated than those in the tetraploid, allowing a higher photosynthetic rate per unit leaf area. The photosynthetic rate per chloroplast was 54% higher in the octaploid. Photosynthesis per units of Chl and protein were the same in both ploidy levels, but photosynthesis per unit DNA was slightly lower in the octaploid, as were Chl and protein per unit DNA.

The sharp (38%) decrease in the number of chloroplasts per DNA in the octaploid (Table VI) shows that intracellular interactions between the chloroplastic and nuclear genomes may have been affected by the increase in the nuclear genome size. However, the effects of polyploidy were more balanced between the two photosynthetic cell types since the ratios of mesophyll to bundle sheath cell volumes, numbers per unit leaf area, and numbers of chloroplasts per cell were within 3%, and activities of PEP carboxylase per cell to RuBP carboxylase per cell were within 10% in each ploidy level.
Table IV. Cellular Volume, Number of Photosynthetic Cells per Unit Leaf Area, and Number of Chloroplasts per Cell in Tetraploid and Octoploid *P. virgatum*

Cellular volume was calculated from measurements of cell length and width in tissue macerations, and from thickness measured on cross-sections. Cell numbers per unit leaf area were determined by counting all photosynthetic cells in macerations of small (0.6 mm diameter) leaf disks. Chloroplasts were counted in cell macerations prepared from chemically fixed leaf disks. Mean ± s.d for n = 4 (except number of cells/leaf disk for 8x, n = 3).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>4x</th>
<th>8x</th>
<th>8x/4x</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell volume (μm² × 10⁻³)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesophyll</td>
<td>5.9 ± 1.3</td>
<td>7.0 ± 0.1</td>
<td>1.17</td>
</tr>
<tr>
<td>Bundle sheath</td>
<td>18 ± 5</td>
<td>21 ± 2</td>
<td>1.17</td>
</tr>
<tr>
<td>Number of cells/leaf disk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesophyll</td>
<td>679 ± 70</td>
<td>458 ± 110</td>
<td>0.67</td>
</tr>
<tr>
<td>Bundle sheath</td>
<td>645 ± 130</td>
<td>446 ± 140</td>
<td>0.69</td>
</tr>
<tr>
<td>Total photosynthetic cells/disk</td>
<td>1324</td>
<td>904</td>
<td>0.68</td>
</tr>
<tr>
<td>Number of chloroplasts/cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesophyll</td>
<td>9.4 ± 0.7</td>
<td>13 ± 0.8</td>
<td>1.38**</td>
</tr>
<tr>
<td>Bundle sheath</td>
<td>15 ± 1.0</td>
<td>20 ± 0.6</td>
<td>1.33**</td>
</tr>
</tbody>
</table>

**. Significant difference between ploidy levels at 0.5% level (Student's t test).

Table V. Concentrations of Cellular Constituents and Carboxylase Activities in Cells of Tetraploid and Octoploid *P. virgatum*

Cellular constituents and enzyme activities determined on a leaf area basis (Tables II, III) were calculated per cell using data from Table IV.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>4x</th>
<th>8x</th>
<th>8x/4x</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble protein/cell (ng)</td>
<td>1.5</td>
<td>3.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Chl/cell (pg)</td>
<td>96</td>
<td>197</td>
<td>2.1</td>
</tr>
<tr>
<td>DNA/cell (pg)</td>
<td>17</td>
<td>37</td>
<td>2.2</td>
</tr>
<tr>
<td>Carbon uptake/细胞 (pmol/min)</td>
<td>0.16</td>
<td>0.33</td>
<td>2.1</td>
</tr>
<tr>
<td>RuBPC (pmol/bundle sheath cell·min)</td>
<td>0.58</td>
<td>1.1</td>
<td>1.9</td>
</tr>
<tr>
<td>PEPC (pmol/mesophyll cell·min)</td>
<td>1.1</td>
<td>2.3</td>
<td>2.1</td>
</tr>
<tr>
<td>Cell DNA/cell volume* (pg/μm² × 10⁻³)</td>
<td>1.4</td>
<td>2.7</td>
<td>1.9</td>
</tr>
</tbody>
</table>

*Overestimated slightly due to the presence of other protein- and DNA-containing cells in whole leaf homogenates that were not counted in chromosome macerations. Degree of overestimation should be comparable in both ploidy levels since leaf structures are similar (Fig. 2).

Abbreviations: RuBPC, RuBP carboxylase; PEPC, PEP carboxylase.

CONCLUSIONS

With a doubling of chromosome number from tetraploid to octoploid, the photosynthetic rate of *Panicum virgatum* increased by 40% on a leaf area basis, doubled per cell, but remained constant per unit DNA. The amount of DNA per cell doubled in the octoploid, but the size of cells increased only 15 to 20%. The result was that Chl, soluble protein, activities of photosynthetic enzymes, and photosynthetic rate per cell all doubled, and since cell volume was not doubled the photosynthetic rate per unit leaf area also increased. In these two genotypes of *P. virgatum* the effects of polyplody on photosynthesis appear to be largely quantitative in nature. The possible qualitative effects of increased heterozygosity or novel enzymes remain to be investigated.

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