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NUCLEAR DNA CONTENT AND CHROMOSOME NUMBERS IN SWITCHGRASS

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ABSTRACT—Switchgrass, *Panicum virgatum* L., one of the three dominant grasses of the North American tall grass prairie, is a genetically and morphologically diverse species with an array of ploidy levels, or set of chromosomes, and ecotypes. The relationship between DNA content and ploidy level has been controversial. The objectives of this study were to provide clear photodocumentation of switchgrass chromosome numbers and to clarify the relationship between nuclear DNA content and

chromosome number. Defining the relationship between ploidy level and nuclear DNA content will facilitate the use of molecular biology techniques, such as flow cytometry, in plant breeding and evolutionary biology. The switchgrass tetraploids examined, which contain 4 sets of chromosomes, had 36 chromosomes with a nuclear DNA content of 3.1 pg/nuclei, while octaploids (8 sets of chromosomes) had 72 chromosomes with 6.1 pg/nuclei. Tetraploid plants from lowland ecotypes had the same nuclear DNA content as tetraploid plants from upland ecotypes. Normal diploid chromosome pairing occurred at meiosis for all tetraploid and octaploid plants examined. Our results indicate that the lowland and upland ecotypes have the same basic genome, and that the octaploids most likely evolved from the tetraploids by a natural doubling of chromosomes, and did so long enough ago for meiosis to be stabilized. Further research is needed to explore the evolutionary origins of switchgrass.

Introduction

Switchgrass, one of the three dominant grasses of the North American tall grass prairie, was found in all areas of North America east of the Rocky Mountains and south of 44° N Lat. (Hitchcock 1951). To facilitate its agronomic use in the Great Plains, switchgrass cultivars have been developed in plant breeding programs (Moser and Vogel 1995). These switchgrass cultivars are currently being used in the temperate regions of the USA in warm-season pastures and conservation programs, such as the current Conservation Reserve Program administered by the U.S. Department of Agriculture. Also because of its high herbage yields and its broad adaptability, it has been identified by the U.S. Department of Energy as a plant species that could be used as a biomass fuel crop (Moser and Vogel 1995).

Switchgrass has two main ecotypes, upland and lowland, that differ in morphology and habitat (Moser and Vogel 1995; Barnett and Carver 1967; Brunken and Estes 1975). Cultivars such as Blackwell and Trailblazer, which are based on upland ecotypes, are used primarily for pastures. Lowland cultivars such as Kanlow and Alamo have the most potential for use as biomass fuel plants. The genetic relationship between these ecotypes is unclear. Recently, Hultquist et al. (1995) used DNA marker technologies (chloroplast DNA restriction fragment length polymorphisms or cpDNA RFLP's) to show that these upland and lowland ecotypes are genetically different in chloroplast DNA. Since switchgrass is cross-pollinated and largely self-incompatible, or it does not produce seed when self-pollinated

(Moser and Vogel 1995). Because of this sexual, outcrossing mode of reproduction, all switchgrass cultivars are populations of genetically different but related individuals, and there can be considerable genetic variation (heterogeneity) among the plants within a cultivar.

In addition to being highly heterogenous and having two different types of chloroplast DNA, switchgrass cultivars also can have different numbers of chromosomes. As defined cytogenetically, the single or haploid set of chromosomes of a diploid species is its genome, and it is designated as x . A normal diploid organism, such as corn or maize (*Zea mays* L.), has two complete sets of chromosomes ($2x$). When in its sexual cycle, diploid plants produce sexual cells, pollen grains and ovules or egg cells in the ovaries, that contain only one set of chromosomes (x). Polyploid plant, however, can have multiple sets of chromosomes, including tetraploids ($4x$ or XXXX where X is the base genome), hexaploids ($6x$), octaploids ($8x$) and higher ploidy levels. The reported polyploid series in switchgrass ranges from diploid ($2x=18$ chromosomes) to duodecaploid ($12x=108$ chromosomes) (Church 1940; Nielson 1944; McMillan and Weiler 1959; Barnett and Carver 1967; Riley and Vogel 1982). The base chromosome number of switchgrass is $n=9$ (Gould 1975). The only report of a diploid switchgrass is from a single site in Wisconsin (Nielsen 1944). To date, all lowland ecotypes examined are tetraploids ($4x=36$), while upland ecotypes are tetraploids, hexaploids, or octaploids (Barnett and Carver 1967; Brunken and Estes 1975; Porter 1966).

Polyploids can evolve by a doubling of the chromosomes of a single species, resulting in an autopolyploid, or by the intermating of two different species and a subsequent doubling of the chromosomes, resulting in the development of an allopolyploid. As an example, an autotetraploid would have the genomic configuration NNNN where N is the base genome, while an allotetraploid would have the genomic configuration of NNPP where N and P are the base genomes. Previous cytogenetic work at several locations indicated that the principal cultivars of switchgrass currently used in the central Great Plains were tetraploids or hexaploids (Alderson and Sharp 1994; Riley and Vogel 1982; Vogel et al. 1991). However, Hopkins et al. (1996) recently reported that several cultivars previously characterized as hexaploid contained primarily octaploid plants. One way to determine which is correct is to match DNA content with direct counts of chromosomes.

All chromosomes are located in the cell nucleus of plants enabling nuclear DNA content to be used as an estimate of ploidy level. The recent development of flow cytometry enables plant scientists to determine DNA content of a large number of individual cells of plants. In DNA content

assays, the results are reported as pg/2C. A picogram or pg is 10^{-12} gram. The letter C stands for "constant" or the amount of DNA in a haploid nucleus or haploid genome; 2C values represent the DNA content of a diploid somatic nucleus (Bennett and Leitch 1995). In the Hopkins et al. (1996) report, the nuclear DNA content of tetraploids, hexaploids, and octaploids averaged 3.1pg/2C, 4.1 pg/2C, and 5.2 pg/2C, respectively. Since the DNA content of octaploids is substantially less than two times the amount in tetraploids in the results of Hopkins et al. (1996), their data would suggest that octaploids are possibly allo-octaploids, i.e., they are derived from two different tetraploid species. In contrast, DNA content measurements by Hultquist et al. (1996; 1997) and Wullschleger et al. (1996) are consistent with octaploids containing twice the DNA per 2C nucleus of tetraploids, suggesting that the octaploids could have evolved by the simple doubling of the chromosomes of the tetraploid switchgrasses. Unfortunately, chromosome counts were not included in the Hultquist et al. (1996; 1997) and Wullschleger et al. (1996) reports. Photographic documentation of chromosome number of mitotic or meiotic cells of switchgrass cultivars were not provided in any previous reports, including the recent report by Hopkins et al. (1996), so that the reported chromosome numbers can not be verified visually.

Attempts to hybridize or intermate plants that differ in ploidy level can result in no viable progeny or genetically unstable progeny (Vogel and Pedersen 1993), so knowledge of ploidy level of individual plants or populations of plants is important for both plant breeders and evolutionary biologists. To effectively utilize the potential of flow cytometry analyses in plant genetics and evolutionary biology research, the relationship between ploidy level and DNA content in a species must be known. The objectives of this study were to provide clear photodocumentation of switchgrass chromosome numbers, clarify the relationship between DNA content and chromosome number in tetraploid and octaploid switchgrass plants, and to characterize the DNA content of a large number of plants from populations previously classified as either hexaploid or octaploid (e.g., Trailblazer and Pathfinder) to determine if these populations contain both hexaploid and octaploid individuals.

Materials and Methods

Sixteen switchgrass plants were used to clarify the relationship between DNA content and ploidy level. They included 7 tetraploid plants (2 Summer and 5 Kanlow), 6 hexaploid plants (3 Blackwell and 3 Pathfinder),

and 3 plants with unknown ploidy level (1 Cave-in-Rock and 2 Shawnee). In addition, we determined DNA content of 100 plants per population from Shawnee, Summer, Pathfinder, Trailblazer, and Kanlow, using flow cytometry. Kanlow is a lowland ecotype/cytotype (Moser and Vogel 1995) with lowland type chloroplast DNA (Hultquist et al. 1996) while the other cultivars are the upland ecotype/cytotype. Plants used in the study were grown in a greenhouse on East Campus of the University of Nebraska. The area of origin of the germplasm from which the cultivars were derived is: Nebraska for Summer; Nebraska and Kansas for Pathfinder and Trailblazer; Oklahoma for Blackwell and Kanlow; and, southern Illinois for Cave-in-Rock and Shawnee (Moser and Vogel 1995).

The procedures described by Arumuganathan and Earle (1991) were used to determine DNA content per nucleus. Briefly, the procedure consists of preparing suspensions of intact nuclei by chopping plant tissues and lysing protoplasts in a MgSO_4 buffer mixed with DNA standards and stained with propidium iodide (PI) in a solution containing DNAase-free RNAase. Fluorescence intensities of the stained nuclei are measured by a flow cytometer. Values for nuclear DNA content are estimated by comparing fluorescence intensities of the nuclei of the test population with those of an appropriate internal DNA standard that is included with the tissue being tested. We used Stark spring barley (*Hordeum vulgare* L.) as the internal standard. This cultivar is a diploid ($2x=14$) that has a $2C$ complement of DNA of 10.68 pg per nucleus. Specifically for the flow cytometry analysis, 50 mg fresh leaf tissue was excised from each plant and placed on ice in a sterile 35 x 10 mm plastic petri dish. Barley seedling leaf tissue (20 mg) was added as the standard. The tissue was chopped into 0.25 to 1.0 mm segments in 1 ml solution A [24 ml MgSO_4 buffer (ice-cold); 25 mg dithiothreitol; 500 μl propidium iodide stock (5.0 mg propidium iodide in 1.0 ml double distilled H_2O); 625 μl Triton X-100 stock (1.0g Triton x-100 in 10 ml distilled, dionized H_2O)]. The solution and tissue were filtered through a 30 μm nylon mesh into a micro-centrifuge tube and centrifuged at high speed (13,000 RPM) for 20 seconds. The supernatant was discarded; the pellet was resuspended in 400 μl solution B [7.5 ml solution A; 17.5 μl DNase-free RNAse (Calbiochem, La Jolla, CA)] and incubated for 15 minutes at 37EC before measurement with a FACScan flow cytometer (Beckton-Dickinson, San Jose, CA). Samples stained with propidium iodide (PI) were excited with a 15 mW Argon ion laser at 488 nm. Red PI fluorescence area signals (FL2A) from nuclei were collected through 582/42 nm band pass filter in FL2 channel. Data were collected, stored, and analyzed with Cellquest software

(Becton-Dickinson, San Jose, CA) on a Macintosh computer. Mean DNA content per sample was based on analysis of 1000 or more nuclei per sample. Three samples were collected and analyzed per plant for DNA content for the 16 plants on which chromosome counts were made. A single sample was taken from each plant in the population study.

The method suggested by Jewell and Islam-Faridi (1994) was used for preparation of the root-tip slides for chromosome counts in the mitotic phase. The terminal 1 cm of new growing roots was excised and then washed in distilled deionized water [ddH₂O]. Clean root-tips were pre-treated in 0.0004 g L⁻¹ 8- hydroxyquinoline for 3.5 hours at 24°C to accumulate mitotic cells. Root-tips were fixed in 3:1 of 0.95L L⁻¹ ethanol:glacial acetic acid and stored at 4°C. Root-tips then were washed twice in ddH₂O at room temperature for 5 minutes, and were soaked in 0.01 M citrate buffer (4°C) for 20 minutes. The root-tips were transferred to a glass slide and the meristematic portion (solid white tip) was separated from the rest of the root tissue. Excess buffer near the meristematic portion was removed and root-tips were transferred to a small microfuge tube (0.5 ml) containing about 100 µl enzyme solution {[0.05 g L⁻¹ cellulase Onozuka R-10 (Yakult Honsha Co., Ltd., Japan) and 0.01g L⁻¹ pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd., Japan)] in 0.01 M citrate buffer [1.47g trisodium citrate-dihydrate (Na₃C₆H₅O₇·2H₂O) and 1.05g citric acid monohydrate (C₆H₈O₇·7H₂O) in ddH₂O up to 500 ml]}.

The tube containing the treated root tips was incubated at 37°C for 60 to 120 minutes, depending on the thickness and lignification of roots. Root-tips were agitated very gently with a Pasteur pipet once or twice during the incubation. After digestion, the cell slurry was washed once with a 0.01 M citrate buffer and then re-suspended in the same buffer. The root-tips were transferred to a small watch glass containing distilled water and soaked for 10 to 20 minutes before slide preparation. Root-tips were stained in acetocarmine (Smith 1947) . A single drop of slurry containing mitotic cells was transferred onto an ethanol-washed glass slide. Excess water was removed and a drop of acetocarmine stain was placed on the root-tip. The tissue was macerated and immediately spread over the slide and covered with a cover slip. Filter paper was used to soak up the excess solution. Chromosomes were counted using a Zeiss microscope and photographed for documentation.

Chromosome analysis of meiotic cells was conducted on immature anthers. A hand lens was used to select florets that had anthers that were light colored and not fully developed. A portion of the inflorescence containing

the selected and adjacent florets was excised and fixed in a screw-top plastic tube with Farmer's solution (Sass 1958). The solution was changed to 0.70 L L⁻¹ ethanol after two days and the sample was stored at room temperature until observations were made. The acetocarmine squash procedure described above (Smith 1947) also was used to stain and prepare anthers for observation.

Results and Discussion

Plants examined from the Summer and Kanlow cultivars were determined to be tetraploid, with $2n=36$ mitotic chromosomes (Table 1; Fig. 1a), which agrees with previous reports (Riley and Vogel 1982). Plants of Cave-in-Rock, Trailblazer, and Blackwell cultivars were determined to be octaploid, with $2n=72$ mitotic chromosomes (Table 1; Fig. 1c), which agrees with Hopkins et al. (1996). Plants of the Shawnee cultivar had 72 chromosomes. Primary bivalent pairing was observed in both tetraploid ($2n=36$) and octaploid ($2n=72$) plants (Figs. 1b and 1d). The bivalent pairing in both the tetraploid and octaploid switchgrass has evolutionary implications. If an autopolyploid is of recent origin, chromosomes will pair in groups of four at meiosis. The bivalent pairing indicates that, if they are autopolyploids, the chromosome doubling occurred long enough ago for bivalent pairing to have evolved.

DNA content (2C) for the tetraploid plants from Summer and Kanlow cultivar on which cytogenetic analysis was also conducted averaged 3.07 ± 0.06 pg per nucleus (Table 1). DNA content of the cytogenetically analyzed octaploid plants from cultivars Cave-in-Rock, Shawnee, Trailblazer, and Blackwell averaged 6.13 ± 0.18 pg per nuclei (Table 1). These results show that octaploid plants had twice the average 2C DNA content of tetraploids, indicating that the component genomes of the tetraploids and octaploid switchgrass were similar in size. These results mean that the octaploids could have evolved by a natural doubling of the chromosomes of tetraploid plants. Since the Summer cultivar had the upland (U) cytotype and Kanlow cultivar had the lowland (L) cytotype (Hultquist 1996), these results also suggest that the nuclear DNA contents for tetraploids of both upland and lowland cytotypes are similar, and contain the same basic component genomes.

Previous reports on Trailblazer and Blackwell cultivar suggested that they were hexaploids (Riley and Vogel 1982; Vogel 1991). The difference in our counts and previous counts could be due to mixtures of hexaploids and

TABLE 1

DNA CONTENT (MEAN \pm STANDARD ERROR) AND CHROMOSOME NUMBERS OF SWITCHGRASS PLANTS FROM SIX CULTIVARS.

Cultivar	Plants n	DNA content (pg/2C)	Chromosome number
Cave-in-rock	1	6.08 \pm 0.21	72
Shawnee	2	6.06 \pm 0.04	72
Trailblazer	3	6.22 \pm 0.13	72
Blackwell	3	6.10 \pm 0.14	72
average		6.13 \pm 0.20	
Summer	2	3.00 \pm 0.03	36
Kanlow	5	3.09 \pm 0.035	36
average		3.07 \pm 0.06	

TABLE 2

ESTIMATES OF MEAN DNA CONTENT OF 100 PLANTS FROM SIX DIFFERENT SWITCHGRASS CULTIVARS.

Cultivar	Plants n	DNA content (pg/2C)		Ploidy level	Estimated chromosome number
		Mean	SD		
Summer	100	2.97	0.10	Tetraploid	36
Kanlow	100	3.00	0.05	Tetraploid	36
Pathfinder	100	5.90	0.18	Octaploid	72
Shawnee	100	6.00	0.18	Octaploid	72
Trailblazer	100	5.92	0.18	Octaploid	72

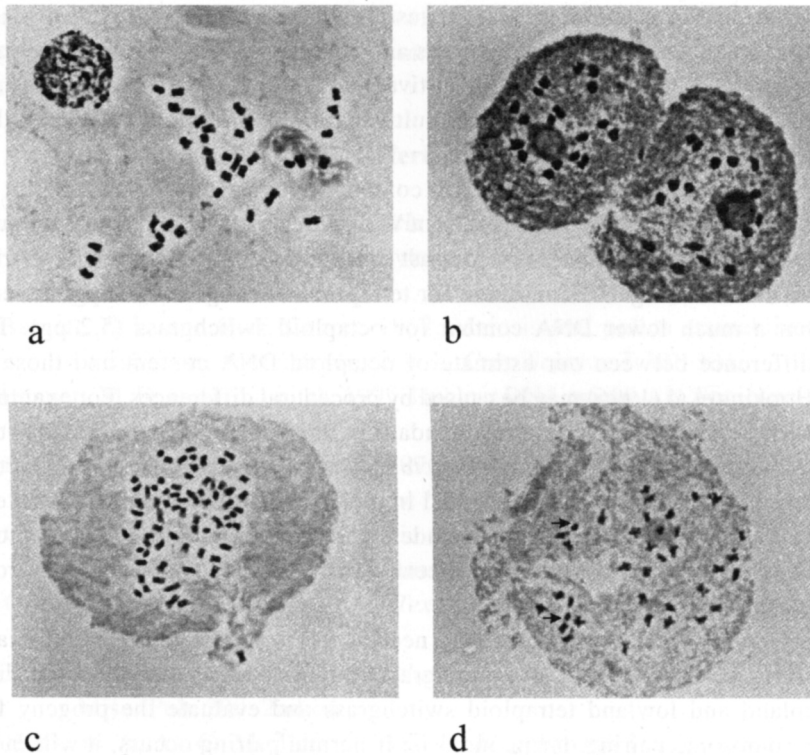


Figure 1. Evidence of chromosome numbers per cell in switchgrass (*Panicum virgatum* L.) (a) A root-tip squash from Kanlow showing 36 chromosomes ($2n=36$) at the metaphase stage of mitosis. DNA content of this plant was 3.07 pg/nucleus. (b) Two meiotic cells at diakinesis from Kanlow showing 18 chromosome pairs in bivalent associations ($2n = 36$) in each cell. (c) A root-tip squash from Shawnee showing 72 chromosomes ($2n=72$) at the metaphase stage of mitosis. DNA content of this plant was 6.03 pg/nucleus. (d) Meiotic cell cell from Trailblazer showing 35 bivalent associations and two univalents (arrows) ($2n=72$). Photos from authors.

octaploids within a cultivar or it may be due to chromosome number determination error caused by difficulties in separating and visualizing the small switchgrass chromosomes. In this study, the DNA content measurement of 300 octaploid plants from Shawnee, Pathfinder, and Trailblazer cultivars (Table 2) revealed no hexaploid plants. This indicates that, at least in the current seed stock of the populations, hexaploids occur at a low frequency, if at all. Plants with a hexaploid DNA content (approximately 4.5 pg/nucleus) were not found in over 250 other plants from 46 populations or accessions

(Hultquist et al. 1996; 1997), suggesting that hexaploid switchgrass plants are likely rare in Midwest cultivars and accessions. All the plants analyzed from the Summer and Kanlow cultivars were tetraploid (Table 2). Thus, in this study, we did not find within cultivar variation for ploidy level in either tetraploid or octaploid cultivars.

Our finding that the DNA content of octaploids is twice that of tetraploids collaborates the results of Wullschleger et al. (1996) and Hultquist et al. (1996; 1997), but is in contrast to Hopkins et al. (1996) who reported a similar average DNA content for tetraploid switchgrass (3.1 pg/nucleus) but a much lower DNA content for octaploid switchgrass (5.2 pg). The difference between our estimate of octaploid DNA content and those of Hopkins et al (1996) may be caused by procedural differences. For example, barley was used as an internal standard in this study whereas Hopkins et al (1996) used catfish (*Ictalurus punctatus* Rafinesque) blood cells. While the precise DNA content is not critical in ploidy determinations, DNA content has important implications in understanding the evolution of octaploid switchgrass. Our results are consistent with the derivation of octaploids from tetraploids.

Additional research will be needed to substantiate the evolutionary origin of the genomes of switchgrass. It will be necessary to hybridize upland and lowland tetraploid switchgrass and evaluate the progeny for chromosome pairing during meiosis. If normal pairing occurs, it will indicate that the upland and lowland tetraploids have the same nuclear genomes. To determine if the octaploids are derived from the tetraploids, it will be necessary to artificially double the chromosome number of tetraploid plants and then hybridize the synthesized octaploid plants with natural octaploids. If normal bivalent pairing occurs, it would indicate that the octaploids evolved by a natural doubling of chromosome of tetraploid progenitors. If some chromosomes do not pair normally, it would indicate instead that the octaploids evolved via hybridization of separate tetraploid species. It will be necessary to hybridize diploid *Panicum* species with tetraploid switchgrasses to attempt to determine the progenitors of this ploidy level. Based on the flow cytometry analysis, potential diploid progenitor species would need to have a nuclear DNA content of approximately 1.5 pg/2C. Relationships among diploid, tetraploid, and octaploid switchgrasses also could be determined by genomic mapping, however, this would require a significant research effort.

Clarification of the relationship of ploidy level and DNA content should enable plant geneticists and population biologists to effectively utilize flow

cytometry analysis in evaluation switchgrass germplasm accessions and populations in genetic, ecological, and evolutionary studies of this important prairie grass.

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