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Generation of Octaploid Switchgrass by Seedling Treatment with Mitotic Inhibitors

Sangwoong Yoon[1] · **Sheyla Aucar**[1] · **Bradley J. Hernlem**[2] · **Serge Edme**[3] · **Nathan Palmer**[3] · **Gautam Sarath**[3] · **Robert Mitchell**[3] · **Eduardo Blumwald**[1] · **Christian M . Tobias**[2]

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Abstract Switchgrass (*Panicum virgatum* L.) exists as multiple cytotypes with octaploid (8*x*) and tetraploid (4*x*) populations occupying distinct, overlapping ranges. These cytotypes tend to show differences in adaptation, yield potential, and other characters, but the specific result of whole-genome duplication is not clear and 8*x* and 4*x* switchgrass populations are reproductively isolated with limited genetic exchange. To create new opportunities for population improvement and to study the effects of whole genome duplication on switchgrass, seedling treatment of the tetraploid cultivar *Liberty* with microtubule inhibitors was used to generate an octaploid population. Resulting octaploids, tetraploids, and cytochimeras were resolved by intercrossing octaploid sectors to produce a population of 19 octaploid families. Fertility of octaploid sectors was significantly reduced relative to tetraploid sectors and caryopsis size significantly increased. Cell size was significantly increased which resulted in quantitative changes to leaf anatomy. During seedling and early vegetative growth stages, no differences in vigor or tillering ability were seen. This technique resulted in efficient genome doubling and was simple to perform. However, aneuploids were also identified with both larger and smaller than expected genome sizes.

Keywords Colchicine · Bioenergy · Polyploidy · Cytochimera · Tetraploid · Autopolyploid

✉ Christian M . Tobias
christian.tobias@ars.usda.gov

[1] Department of Plant Sciences, University of California, 1 Shields Ave, Davis, CA 95616, USA

[2] USDA-ARS Crop Improvement and Genetics Research Unit, Western Regional Research Center, 800 Buchanan Street, Albany, CA 94710, USA

[3] USDA-ARS Grain Forage and Bioenergy Research Unit, 251 Filley Hall University of Nebraska, East Campus, Lincoln, NE 68583, USA

Introduction

Switchgrass is a perennial, C4 grass native to North America that is used as forage, for restoration plantings, as an ornament, and for biomass production. Its desirability as a biomass crop arises from its ability to produce high yields in marginal conditions, its low input requirements, and its compatibility with existing harvest and seeding equipment [1–4]. Past whole-genome duplications (WGD) have played a significant role in switchgrass evolution, and extensive genome size variation exists in natural subpopulations of switchgrass [5–7]. Tetraploid, octaploid, and hexaploid cytotypes are most prevalent with aneuploidy common among octaploids [8]. Tetraploid cytotypes ($2n = 4x = 36$) are alloploids with two distinctly different subgenomes resulting from an initial WGD event that arose in common with its relatives in closely related *Panicum* sections *virgata* and *urvilleana* prior to speciation [9]. Octaploid cytotypes ($2n = 8x = 72$) appeared more recently, and likely arose repeatedly. Studies of morphological and physiological variation between switchgrass populations of differing ploidy have demonstrated differences in many character traits [10–12]. However, in these cases, the contribution of genetic variation prior to, or evolution after WGD event(s) were unknown and high levels of morphological variation within cytotypes make analysis difficult.

It is recognized that mechanisms such as polyembryony or production of fertile unreduced gametes can lead to limited gene flow between different cytotypes and can often restore fertility in sterile hybrids that are meiotically unstable. Recently, increased collection efforts across its natural range and application of marker data have begun to provide

evidence for this in switchgrass. Relatively rare 8*x* individuals in 4*x* lowland populations and individuals with hybrid upland/lowland origin have been identified based on morphological classification and molecular markers [13]. Marker analysis has demonstrated that genetically distinct, northern upland 4*x* and 8*x* populations are related and have undergone substantial admixture [14, 15], and further analysis of the relative likelihoods of alternate-origin hypothesis indicate that the northern upland 4*x* population was likely derived via genome reduction from the northern upland 8*x* population [16].

To study the physiological, biochemical, and molecular effects of WGD directly, natural or induced neopolyploids have been used extensively. The first artificially generated polyploids were produced in 1916 by Winkler [17], and induced polyploidy has been an established breeding method for over 50 years. Both allopolyploids and autopolyploids can be produced in many species, and the process has been conducted using methods including, grafting, NO_2, colchicine, and other microtubule inhibitors [18]. In switchgrass, octaploids have been generated via tissue culture from a limited number of individuals [19]. Though polyploids comprise approximately 50 % of angiosperm species, the promise of induced polyploidy in the field of plant breeding has not been fully realized because polyploids tend to exhibit seed sterility due to aberrant meiosis and/or somatic instability. Frequently, induced polyploids are found to be agronomically inferior. However, seed sterility is less of an issue when vegetative plant parts are utilized such as for biomass or forage. A large number of physiological and biochemical processes are impacted in neopolyploids including rates of photosynthesis, enzyme activity, and accumulation of secondary metabolites. These changes can be desirable in some cases and have led to improvements in forage quality. For example, hybrids between induced polyploids of tall fescue and giant fescue had greater dry matter disappearance, less acid detergent lignin, and less neutral detergent fiber than their diploid hybrids [20]. Induction of polyploidy has played an important role in stabilizing interspecific genetic introgression lines that are otherwise infertile.

The objective of this study was to generate a population of octaploid switchgrass from the tetraploid cultivar *Liberty*, and to determine if WGD by seedling treatment is a suitable breeding technique. Octaploids obtained through chromosome doubling would be useful for intercrossing tetraploid and octaploid germplasm pools and for future studies of the effects of WGD on gene expression and genome structure.

Materials and Methods

Plant Materials and Seedling Treatment Seed of the cultivar *Liberty* (reg. no. CV-271, PI 669371) [21] was harvested in 2012 from a seed increase nursery at the Agricultural Research and Development Center located near Mead and Ithaca, NE (41° 09′ N, 96° 25′ W) and was used for all treatments. Natural octaploid accessions used were obtained from the National Plant Germplasm System. A procedure adapted from that of Subrahmanyam and Kasha [18] for barley doubled haploid production was applied to switchgrass. Approximately 1000 *Liberty* caryopses were stratified for 3 weeks at 4 °C in large petri plates lined with blotting paper. Caryopses were then transferred to pots containing moistened vermiculite in a greenhouse with supplemental LED illumination and were irrigated with dilute fertilizer solution until reaching the 1- or 2-leaf stage (9 days after transfer to greenhouse). Prior to treatment, seedlings were washed free of vermiculite, placed in 125-ml Erlenmeyer flasks, and immersed in enough treatment solution to submerge the crown for 5 h at room temperature in daylight. Colchicine (CC) was obtained from Acros Organics. Amiprophos methyl (APM) was obtained from Sigma-Aldrich. Dimethyl sulfoxide (DMSO) was obtained from EK industries (Joliet, IL). The seedlings were treated as follows: (1) 0.1 % (*w*/*v*) CC, 2 % (*v*/*v*) DMSO; (2) 0.2 % (*w*/*v*) CC, 2 % (*v*/*v*) DMSO; (3) 10 μM APM, 2 % (*v*/*v*) DMSO; (4) 20 μM APM, 2 % (*v*/*v*) DMSO; and (5) 2 % (*v*/*v*) DMSO. They were subsequently washed continuously with gently flowing tap water for 3 h prior to potting in soil and returning to the greenhouse.

Plants were grown together on the same bench in a greenhouse maintained between 21 and 35 °C under 16:8 h light/dark conditions with supplemental LED lighting (Lumigrow, Novato, CA) and continuous air circulation. Plant nutrients and irrigation were supplied through an injector-equipped drip system at 100 ppm N using Jack's Peat-Lite 15-16-17 (JR Peters Inc., Allentown, PA). Plants were allowed to freely intercross with each other.

Flow Cytometry Procedures described by Arumuganathan and Earle (1991) [22] were used to determine DNA content per cell. Prepared and propidium iodide stained nuclei samples were analyzed using a BD FACSAria Fusion flow cytometer (BD Biosciences, San Jose, CA). The fluorescent stain was excited using the 488-nm laser, and fluorescence was measured using the 585/42 and 616/23 bandpass filter detector channels. Nuclei were gated from debris based on fluorescence peak area and laser side scatter. Logarithmic histogram analysis of the fluorescence peak area of the nuclei was used to quantify DNA content and to identify octaploid samples. The mean DNA content per sample was based on at least 1000 events. The internal standard used for comparison was rice cv. *Nipponbare* with a 2C genome content of 0.9 pg as estimated by flow cytometry (FCM) and using the average value of 980 Mbp = 1 pg [23–25]. Each line was sampled by excising a 3–4-cm segment from the midpoint of the uppermost collared leaf blade of the primary tiller 10 weeks after treatment. Further screening was conducted in a similar

manner on the flag leaf of up to 12 samples per plant or alternatively from 10 to 12 pooled caryopses arising from the same panicle.

Chromosome Squashing/Staining Metaphase spreads were visualized by fixation of colchicine-treated root tips fixed in 3:1 ethanol/acetic acid followed by digestion and spreading using the procedure of Kirov et al. [26]. Spreads were mounted in Vectashield (Burlingame, CA) containing 1 μg/ml DAPI and visualized with a Zeiss Axiovert microscope. Accurate chromosome counts were obtained from at least one non-overlapping mitotic figure for each plant evaluated.

Leaf Anatomy and Pollen Measurements Leaf cross sections, epidermal peels, and epidermal impressions were made from the lamina of the fourth true leaf midway from the leaf tip to the beginning of the sheath. Leaf cross sections were prepared by hand sectioning; epidermal peels were made as described previously [27]. Tetraploid and octaploid samples were taken with a paper punch and fixed in 1 % formaldehyde in PBS, washed several times with water, and then incubated with 0.1 % pectinolyase Y-23 overnight at room temperature. The next day, the epidermis was carefully removed, stained with 0.05 % toluidine blue, pH 4.5, washed, and photographed using brightfield optics. Leaf anatomical measurements were made for 4–8 individuals per accession. For pollen measurements, freshly shed pollen was collected onto microscope slides and stained with I/KI and photographed. Measurements of pollen size were then collected from multiple images using ImageJ software tools [28].

Results

Ten weeks after treatment with 0.1 and 0.2 % CC, 48 and 46 % of the *Liberty* seedlings, respectively, survived while *Liberty* seedlings treated with either 10 or 20 μM APM survived at rates of 86 %. A summary of the experimental treatments is presented in Table 1. Nuclei isolated from the blade of the youngest collared leaf along the primary axis at 10 weeks after treatment was spiked with rice cv. *Nipponbare* nuclei as an internal standard and subjected to FCM to estimate genome size. Preliminary analysis of aligned forward vs. side scatter in Fig. 1a (upper panels) and corresponding flow histograms (Fig. 1a, lower panels) of untreated 4*x Liberty* and untreated 8*x Trailblazer* indicated that the percentage of leaf nuclei in G2 was typically 3–15 %. The resulting flow histograms of treated *Liberty* seedlings in Fig. 1b showed that the plants did not respond uniformly to treatment and tended to produce cytochimeras. The tetraploid and octaploid genome sizes were defined by positions P2 and P3, respectively, while the peak corresponding to the rice standard was defined by peak position P1. Using the published genome size of rice, the 2C DNA content of tetraploid switchgrass nuclei was estimated to be 2.66 pg of DNA while that of octaploid switchgrass nuclei was 5.34 pg. Because both tetraploid G2 phase nuclei and octaploid G1 phase nuclei in cytochimeric tissues fall into peak position P3, we could not discriminate these separate events.

The histograms were classified to assist further selection into "tetraploid," "chimeric," "octaploid," and "other" groups. The tetraploid group is typified by sample 1-02 where the sub-population of events in P2 (4*x*) was at least double the number in P3 (8*x*). These individuals were discarded. Similarly, the octaploid group, typified by sample 1-08, had a sub-population of events in P3 that was at least double the number in P2 and these plants were retained for further analysis. The chimeric group included samples in between these ranges where the number of events within P2 and P3 peaks were similar. This chimeric group is typified by sample 1-18. If the sub-population of nuclei in P3 was greater than in P2, the plant was retained for further analysis. Three samples were classified as "other" due to the presence of significant numbers of events outside of P2 or P3, such as sample 2-42 that contained a large sub-population in P6. This corresponded to a 2C DNA content of 9.94 pg and was predicted to be a hexadecaploid. Two samples treated with APM produced peaks corresponding to a 2C DNA content of 3.98 and 4.05 pg, respectively. These two lines were not further analyzed, but corresponded to *C* values predicted of hexaploids. The number of individuals in each group is shown in Table 1.

Figure 1c shows the overall effect of the different treatments after 10 weeks. Samples are plotted by treatment and as the fraction of events in P3 relative to total events in P2 + P3. Colchicine applied at concentrations of 0.1 and 0.2 % in

Table 1 Number of seedlings treated and survival after 10-week recovery in greenhouse

Treatment	Seedlings *n*	% survival (*n*)	tetrap.	cytochimeric	octap.	Other
0.1 % CC, 2 % DMSO	174	48 % (84)	56	20	8	0
0.2 % CC, 2 % DMSO	108	46 % (50)	16	19	14	1
10 μM APM, 2 % DMSO	105	86 % (91)	89	1	0	1
20 μM APM, 2 % DMSO	97	86 % (97)	81	1	0	1
2 % DMSO	117	ND	29	0	0	0

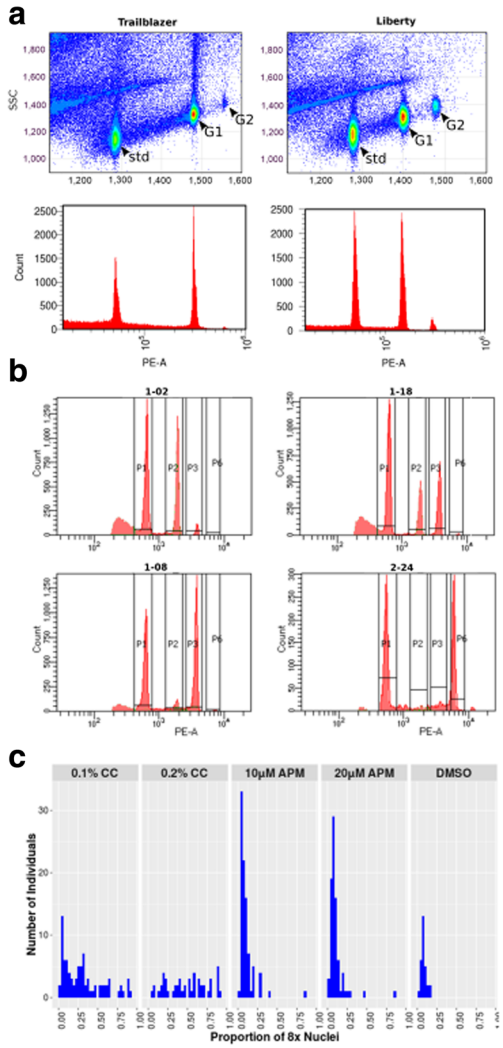

Fig. 1 Flow cytometry of switchgrass nuclei stained with propidium iodide using rice cv. *Nipponbare* used as an internal standard (std) for fluorescence calibration in all samples. Data has been log transformed and appropriately gated. (**a**) Dot density plot (*upper panels*) and corresponding FCM histograms (*lower panels*) of forward (PE-A) vs. side scatter (SSC) collected using a 585/40 nm bandpass filter. *Left panels, Trailblazer*; *right panels, Liberty*. Populations of G1 and G2 nuclei are indicated as well as the rice nuclei used for internal calibration. **b** *Clockwise from upper left* FCM histograms of leaf sample nuclei isolated from colchicine-treated *Liberty* specimens 1-02, 1-18, 1-08, and 2-42. Regions P1, P2, P3, and P6 in each histogram bracket the *O. sativa* 2*x*, switchgrass 4*x*, 8*x*, and 16*x* peaks, respectively. **b** Summary of seedling treatments displaying the fraction of total switchgrass nuclei present in tetraploid and octaploid regions of FCM histograms that were octaploid

2 % DMSO was more effective at inducing WGD than amiprophos methyl at 10 or 20 μM in 2 % DMSO or 2 % DMSO alone.

The presence of large numbers of nuclei falling within P2 even among lines tentatively classified as octaploid and follow-up ploidy analysis indicated that cytochimeras were prevalent within the population. Therefore, extensive phenotypic analysis was not attempted. Instead, after further maturation in the greenhouse, 8–12 individual shoots from each line were tagged and separately reassayed by FCM using nuclei isolated from flag leaf sections. Panicles from all shoots were allowed to open pollinate in the greenhouse, and mature caryopses (hereafter referred to as seed) were harvested individually from each tagged shoot and later pooled according to the results of FCM. Alternatively, approximately 10 mature seeds harvested from the same panicle were assayed by FCM to assign *C*- values. Table 2 summarizes these results.

Of the 32 individuals that had multiple shoot or seed samples evaluated by FCM, 12 individual samples were 100 % tetraploid. Two individual samples were 100 % octaploid. The remainder of the individuals produced some tillers with *C* values consistent with octaploid genome size and some consistent with tetraploid genome size.

A significant number of panicles from the treated individuals did not produce viable seed. Variation in seed production across lines and individual panicles was high with a mean of 95 and a standard deviation of 115 seeds per panicle. Mean seed production for octaploid samples and tetraploid samples across all lines was 44.7 and 123.91 seeds, respectively. This difference was highly significant (t = 8.977, p value = 2.2×10^{-16}). Mean 100 seed weight for seeds pooled by estimated ploidy and individual was 0.158 g for octaploid and 0.137 g for tetraploid samples. This difference was significant using either paired (t = 2.29, p value = 0.017) or unpaired (t = 2.59, p value = 0.014) t tests. This data is summarized in Table 2. Differences in seed size were visually apparent and Fig. 2a, b show two seed samples at the same enlargement factor taken from different ploidy sectors of cytochimeric individual 1-82.

Although the initially treated lines included cytochimeras, chromosome estimates were obtained from root tips of several individuals. Root tip squashes display 36 visible chromosomes in the case of line 1-17 (Fig. 2c) while approximately 70 chromosomes are visible with some overlapping figures for line 1-8 (Fig. 2d).

Seed from octaploid tillers of 19 individuals listed on Table 2 was pooled by line and germinated, and ploidy determinations were made as before on leaf nuclei. A significant number of tetraploid individuals were present in some families, family 1-69 had similar numbers of tetraploid and octaploid genotypes. Further chromosome counts were made on from one to five octaploid progeny of 11 out of the 19 individuals indicated on Table 2; however, the chromosome counts of individuals were variable even apparently within a single root tip. In total, 126 mitotic figures analyzed produced an average of 64.38 chromosomes with a range between 39 and 76 chromosomes.

Differences in leaf anatomy among tetraploid *Liberty*, natural octaploid switchgrass accessions, and induced octaploid *Liberty* were examined. Figure 3a–d shows the leaf blade epidermis from a tetraploid *Liberty* individual and three

Table 2 Evaluation of individual tiller fertility and seed weight of treated *Liberty* seedlings

Line	Tillers evaluated (*n*)		Caryopsis/panicle (mean)		100 seed weight (g)		Chromosome range[a]
	4*x*	8*x*	4*x*	8*x*	4*x*	8*x*	
1-06	8	0	117.1	–	0.136	–	ND
1-08	7	2	15.6	5.0	0.164	0.140	67–71
1-09	12	0	224.1	–	0.128	–	ND
1-10	6	3	233.1	11.3	0.129	0.216	42–71
1-11	12	0	101.3	ND	0.086	ND	ND
1-14	3	5	40.0	28.0	0.158	0.190	57–69
1-18	9	7	108.1	72.4	0.136	0.147	63–75
1-20	12	0	102.0	–	0.141	–	ND
1-21	1	1	34.0	145.0	0.094	0.130	ND
1-25	11	1	46.6	75.0	0.136	0.185	ND
1-30	1	0	209.0	–	0.112	–	ND
1-41	12	0	108.7	–	0.144	–	ND
1-42	12	0	113.2	–	0.131	–	ND
1-43	1	0	20.0	–	ND	–	ND
1-45	8	0	192.8	–	0.181	–	ND
1-51	7	0	244.3	–	0.124	–	ND
1-59	4	0	103.3	–	ND	–	ND
1-62	6	6	31.5	103.3	0.143	0.192	67–71
1-67	12	0	194.2	–	0.128	–	ND
1-69	0	6	ND	44.5	–	0.185	55–71
1-72	7	3	79.9	29.7	0.127	0.172	64–71
1-78	10	3	39.6	74.3	0.124	0.136	ND
1-82	7	4	91.3	25.8	0.134	0.168	ND
2-10	16	2	86.6	76.5	0.126	0.149	ND
2-15	1	5	69.0	51.4	0.139	0.134	ND
2-17	1	3	427.0	2.0	0.153	0.187	ND
2-22	5	4	142.4	36.8	0.101	0.099	51–76
2-24	0	3	ND	21.6	ND	0.193	54–67
2-26	3	0	157.0	–	0.181	–	ND
2-36	2	2	348.0	93.5	0.147	0.133	62–74
2-37	8	2	179.5	59.0	0.151	0.132	60–72
2-43	3	7	63.7	39.9	0.137	0.164	ND
Average			130.8	52.4	0.135	0.161	

ND not determined

[a] Range of prometaphase or metaphase chromosomes observed in root tips of 1–5 progeny plants

different octaploid individuals (1-54-4, 2-22-7, and 1-82-8) at the same magnification with differences is guard cell density evident. The greenhouse grown plants showed minor differences with respect to flowering time and height (Fig. 3e). After confirming ploidy by FCM, comparison of leaf anatomy with natural octaploid individuals was done to determine if differences seen between 4*x Liberty* and induced 8*x Liberty* were likely to be a general phenomenon of all octaploid switchgrass. Four progeny each from 4*x* and 8*x* sectors of a single treated individual (1-69) were used to represent Liberty of different ploidy. These results are presented in Table 3. Compared with 4*x Liberty*, induced 8*x Liberty* individual's adaxial and abaxial guard cells were an average of 21 and 36 % longer, respectively, while adaxial and abaxial stomatal densities were 52 and 45 % lower, respectively. These differences were highly significant. ($p < .0001$). Guard cell length was longer and stomatal density were also lower in natural 8*x* accessions than in 4*x Liberty*. Differences in interveinal distance and leaf thickness were significant between accessions, but were not correlated with ploidy.

Pollen from freshly shed octaploid and tetraploid *Liberty* individuals was measured and results are presented in Fig. 4.

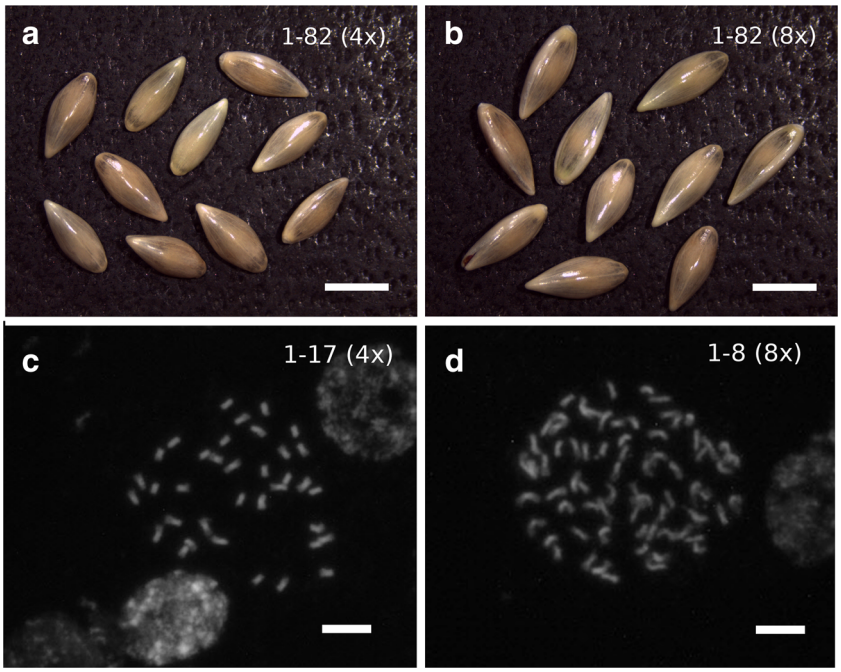

Fig. 2 Seed size and representative chromosome squashes from tetraploid and octaploid samples of *Liberty* switchgrass. Identical scale photographs of seed samples from **a** tetraploid and **b** octaploid sectors of maternal 1-82, a single genotype treated with colchicine at the 2–3 leaf seedling stage (*scale bar* 1 mm). Different genotypes treated with colchicine at the 2–3 leaf seedling stage were used to prepare root tip squashes 6 months after treatment. **c** Line 1-17 with 36 individual chromosomes visible. **d** Line 1-8 with approximately 70 individual chromosomes visible (*scale bar* 5 μm)

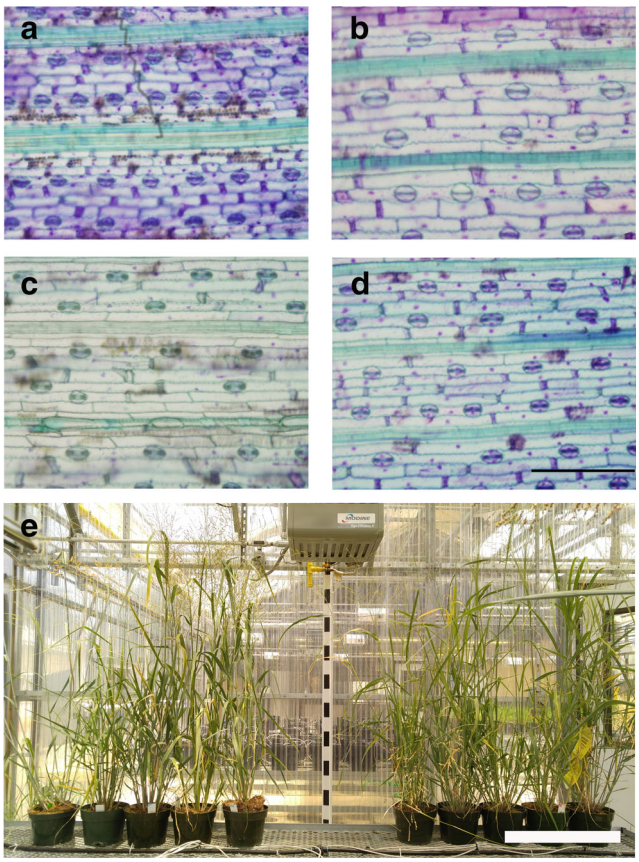

Fig. 3 Epidermal cells of octaploid and tetraploid individuals and whole plant comparisons. Epidermal peels stained with toluidine blue from the lower epidermis of the fourth true leaf are shown. **a** Tetraploid *Liberty* individual; **b**–**d** Octaploid individuals 1-54-4, 2-22-7, and 1-82-8 (*scale bar* 200 μm). Whole plant comparison of greenhouse grown octaploid individuals (five pots on left) and tetraploid individuals (five pots on right) scale bar on lower right is 0.5 m

Consistent with a trend of increased cell size in the induced 8*x* *Liberty* (Fig. 3a–d), average pollen diameter in four induced 8*x* individuals was 45.8 ± 4.9 μm. This is 21 % larger than the average pollen diameter of 37.7 ± 3.2 μm measured in 4*x* *Liberty* individuals that consisted of one untreated individual and 4*x* individuals 1-18-10 and 1-69-20 derived from treated *Liberty* seedlings.

Discussion

Induction of polyploidy by seedling treatment with a variety of microtubule inhibitors, and colchicine in particular, has been established as a breeding technique for many years. Colchicine has been used to treat germinating seeds, seedlings, and developing buds. Here, the effectiveness of two different concentrations of colchicine and amiprophos methyl for polyploid induction were compared. It was found that both 10 and 20 μM concentrations of APM were no more effective than control treatments at polyploid induction while both 0.1 and 0.2 % colchicine were highly effective though many of the resulting plants were chimeric. In a previous study, Yang et al. [19], used a 13-day treatment of embryogenic switchgrass calli in liquid media containing 0.04 % colchicine. This method was effective at producing 13 autopolyploid lines. Our method is comparable with the principal differences being that (1) using the seedling treatment technique, chimeric sectors were produced in many lines that required additional flow cytometry to identify and (2) seedling treatment offers the opportunity to produce large number of independent genotypes without a tissue culture requirement. Both methods

Table 3 Leaf traits of natural and induced octaploids

Cultivar	Ploidy	Adaxial		Abaxial		Interveinal Distance (μm)	Leaf Thickness (μm)
		Guard cell length (μm) mean ± SD	Stomatal density (mm^{-2}) mean ± SD	Guard cell length (μm) mean ± SD	Stomatal density (mm^{-2}) mean ± SD	mean ± SD	mean ± SD
Blackwell	8*x*	31.17 ± 3.03 b	101.22 ± 12.44 c	34.44 ± 3.86 b	115.15 ± 10.29 b	153.81 ± 14.06 ab	134.01 ± 14.12 cd
NE NB 994	8*x*	30.91 ± 3.75 b	93.9 ± 7.06 bc	33.37 ± 3.66 a	111.64 ± 16.86 b	150.76 ± 15.15 a	120.93 ± 11.24 b
Shawnee	8*x*	31.36 ± 3.92 bc	117.78 ± 30.18 d	33.45 ± 3.69 a	113.64 ± 28.27 b	150.26 ± 21.23 a	95.41 ± 15.94 a
Trailblazer	8*x*	32.63 ± 3.69 c	86.67 ± 5.47 ab	33.83 ± 3.03 ab	103.51 ± 18.85 b	166.32 ± 20.85 c	140.0 ± 26.32 d
Liberty 8*x*[a]	8*x*	34.0 ± 4.23 d	66.38 ± 7.58 a	45.2 ± 3.82 c	53.9 ± 7.77 a	162.82 ± 17.54 bc	130.69 ± 19.28 bcd
Liberty 4*x*	4*x*	28.44 ± 2.93 a	125.92 ± 24.91 d	33.28 ± 2.63 a	116.09 ± 9.53 b	150.86 ± 15.67 a	125.88 ± 16.35 bc

Same letter in each sample column are not significantly different from each other (Tukey-Kramer test, $P < 0.05$)

[a] *Liberty* 8*x* and *Liberty* 4*x* leaf measurements were taken from progeny of a single colchicine-treated individual (1-69) that were determined to be either 8*x* or 4*x* based on FCM. See text for further details

produced aneuploid individuals with chromosome values differing from the predicted 72 chromosomes for an octaploid. One further difference is that Yang et al. [19] demonstrated the cross compatibility of induced switchgrass autopolyploids derived from a lowland ecotype with an established octaploid upland cultivar whereas in this study induced autopolyploids were allowed to intercross to produce 19 half-sib families.

In a recent survey of 11 upland cultivars previously reported as octaploid, aneuploids with significantly fewer than 72 chromosomes were prevalent among the lines sampled [8]. Older work has described a widely variable number of different chromosome counts with many upland populations

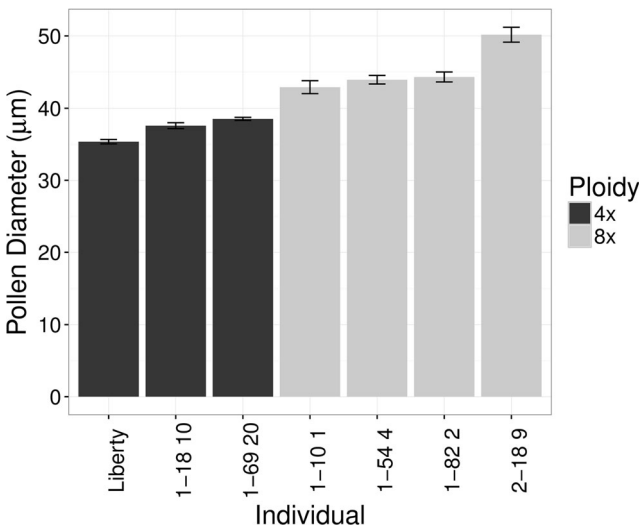

Fig. 4 Pollen diameter of tetraploid and induced octaploid individuals compared to *Liberty* pollen. Error bars ± SE. *Black bars* designate tetraploid plants. *Gray bars* designate octaploid plants

containing mixtures of tetraploid, hexaploid, and octaploid individuals. Dihaploid lines have also been identified and characterized [29]. In the present work, chromosome counts were not confirmed for most of the octaploids that were generated but the count average of 64.38 was closer to the octaploid equivalent of 72 than the tetraploid equivalent of 36. Our counts were likely downwardly biased due to factors such as chromosome loss during slide preparation and miscounting of overlapping figures, but the wide range of observed chromosome numbers suggests aneusomaty [30], perhaps due to genome instability. Nevertheless, it was possible to identify several anomalous individuals based on flow histograms including the presence of two putative hexaploid individuals in the APM treatments and the presence of a putative hexadecaploid in one of the colchicine treatments. These were not further analyzed but support the theory that genome size is not tightly constrained in this species.

In tandem with the whole-genome duplication, 100 seed weight increased by 19.2 % in greenhouse conditions from 0.135 to 0.161 g. Das and Taliaferro [31] reported 100 seed weight for 11 lowland populations including Alamo and Kanlow commercial cultivars grown at 2 locations in Oklahoma. These weights varied between 0.0968 and 0.0868 g. Average 100 seed weight of octaploids in the present study was greater than reported for the upland cv. *Summer* (0.095 g) but less than that reported for another upland cv. *Sunburst* (0.185 g) [32]. A well-established phenotypic correlation exists between seed weight and seedling stand density, seedling vigor, and first-year forage yield in warm and cool season forage grasses. These include sand bluestem, buffalo grass, indian grass, side oats grama, crested wheatgrass, and switchgrass [33–35]. One selection method used in switchgrass breeding efforts has been air column separation to isolate

larger seed classes [36]. Higher germination and emergence rates for larger seed size classes across six different switchgrass cultivars of established tetraploid and octaploid varieties has been demonstrated [37, 38]. Increases in seed weight associated with induced polyploidy may be beneficial in biomass crop development if yield is not negatively impacted. Another trait that was impacted in this study by WTD was epidermal cell size. Epidermal cell width and guard cell subsidiary cell length were increased by 19 and 21 %, respectively. This trait was also similarly affected in autopolyploids induced from embryogenic calli [19]. In environmental selections of differing ploidy from Oklahoma, there were size differences observed in guard cells as well as in mesophyll and bundle sheath cells. Differences were also seen in photosynthesis, leaf anatomy, and activity of multiple enzymes by Warner et al. [12]. Measurements of the same leaf anatomical characters as Warner et al. were taken in this study. While Warner et al. found that the natural octaploid accessions had higher guard cell densities than tetraploid accessions, in the present study, natural octaploid individuals had lower guard cell density than the tetraploid *Liberty* samples while they had higher densities than induced octaploid *Liberty* samples. These studies used different genotypes and were conducted under slightly different conditions so results must be viewed in light of these differences.

A general correlation seen between pollen size and chromosome number in species with polyploid series as well as artificially induced polyploids has been reported in many studies [39, 40], and the present study demonstrates that switchgrass follows this trend with pollen size increasing with genome size.

Seedling treatment could be effective for breeding purposes where genetic exchange between different cytotypes is desired. In this study, the cultivar *Liberty* was chosen because it is of recent hybrid upland/lowland origin, has superior yields, and is adapted to hardiness zones that are considered to be suitable for upland cultivars which are frequently octaploid. Crosses of this material to other distinct octaploid populations are underway.

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