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Dihaploid Stocks of Switchgrass Isolated by a Screening Approach

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Abstract Manipulation of ploidy in switchgrass has potential to accelerate inbred production and to provide insight about genome structure through either sequencing or cytogenetic approaches. We have identified two dihaploid individuals isolated from among the progeny of a controlled cross between two individuals of the cultivars Alamo and Kanlow. The dihaploid lines were initially distinguished from the parental lines by their reduced heterozygosity and were subsequently confirmed through estimation of *C* values by flow cytometry and chromosome counts of metaphase root tip squash preparations. These plants are functionally sterile, with floral bracts that remain closed and inviable pollen. They can be easily distinguished from tetraploid individuals by their reduced stature, smaller epidermal cell size, and lower number of chloroplasts per guard cell. Aberrant meiosis in these individuals is evidenced by a lack of regular pairing at diakinesis and metaphase I and suggests that the non-homologous genomes are distinct from one another. The reduced genome size of these dihaploids will facilitate basic genome studies and genetic analyses that are impossible or problematic in polyploid accessions.

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Introduction

Due to its highly productive perennial habit, water use efficiency, and low nitrogen requirements, switchgrass (*Panicum virgatum* L.) is viewed as a promising source of biomass for renewable energy production. The base chromosome number for switchgrass and other *Panicum* sp. is believed to be 9 [16], but the species is a complex of different cytotypes. Tetraploid ($2n=4x=36$) and octoploid ($2n=8x=72$) cytotypes predominate, although diploids, hexaploids, duodecaploids, and aneuploids have been reported [9, 12, 17, 30, 33, 35, 38]. It is still unclear if gene flow between cytotypes is common in natural populations. Artificial crosses between octoploids and tetraploids are not usually successful [28, 41]. Lowland ecotypes are mainly tetraploid and are found in the southern range of the species distribution, while upland ecotypes are associated with several different cytotypes and have a wide geographical distribution. Some populations appear to exist as mixtures of ploidy levels. Mixed ploidy has been observed in seed lots obtained from germplasm repositories and commercial seed sources [32] as well as collections from remnant prairie sites [18].

Genetic and population studies in switchgrass are complicated by polyploidy because infrequent pairing of non-homologous chromosomes may lead to polysomic inheritance patterns and the formation of multivalents can cause double reduction. Usefulness of marker data or sequence data alone to infer population structure can be compromised by these complexities, particularly if gene

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flow occurs between cytotypes. Therefore, knowledge of ploidy relationships and chromosomal architecture in switchgrass is important for evaluating germplasm and mobilizing it effectively in breeding programs. These relationships between and among different cytotypes can be evaluated through the observation of chromosome pairing during meiosis and cytogenetic techniques. Meiotic pairing during metaphase I in switchgrass has been reported to be primarily bivalent in tetraploids with a low occurrence of univalents, while in octoploids and aneuploids, multivalents were observed with a low frequency [4]. There is a need to perform cytogenetic studies on hybrids of existing cytotypes to understand distinctions between genomes and to determine if genetic exchange occurs between these gene pools. However, elaborate measures such as embryo rescue may be needed to generate these F1 hybrids and to characterize their meiotic behavior relative to naturally occurring hexaploids. In contrast, fertile hybrids between morphologically distinct upland and lowland ecotypes that are both tetraploid undergo regular meiotic pairing, which indicates overall genome similarity between these populations. Heterotic effects have also been observed [27, 29, 47].

Cytogenetic analysis of closely related diploid stocks and *Panicum* collections within the sections *Virgata* and *Urvilleana* might help resolve genome interrelationships in switchgrass and could also define a secondary gene pool, providing additional traits for breeding. However, these species are not well represented in germplasm collections. A single diploid individual from switchgrass collections in Wisconsin was identified, but this individual no longer exists and therefore cannot be studied further [33]. It may have represented a rare genotype from a polyembryonic seedling or product of gynogenesis. Artificial diploids (or dihaploids if originally from a polyploid) have been generated experimentally in many species and have allowed rapid creation of inbreds and interploidal hybridization. Experimental dihaploid production from switchgrass anther culture has been reported, but was inefficient [13]. In this report, we describe the identification through screening and initial characterization of two dihaploid switchgrass lines. We present genotypic and phenotypic data distinguishing these lines from similar tetraploid individuals.

Methods

Plant Material

An F1 mapping population was produced from a controlled greenhouse cross in July 2005. The male parent was an individual Alamo (ALBA4) plant that showed good response to tissue culture and was from seed obtained from colleagues in Lincoln, NE. The female parent was a randomly selected Kanlow (ALBK5) individual from a commercial seed source (Osenbaugh Grass Seed, Lucas, IA). F1 seed were stratified for 3 months at 4°C and 275 seed were germinated on filter paper before transplanting to the greenhouse. An additional 300 seed were subsequently germinated and genotyped with select microsatellite markers. A true hybrid F1 tetraploid (ALB881) was phenotyped in lieu of ALBK5 for which genomic DNA was available but no plant material. ALB881 was representative of other tetraploids in the F1 population in both phenotype and cytology.

Flow Cytometry

Procedures described by Arumuganathan and Earle [3] were used to determine DNA content per cell. The prepared material was analyzed using a BD FACSVantage flow cytometer (BD Biosciences, San Jose, CA). The propidium iodide-stained samples were excited with 150-mW laser light at 488 nm from a Coherent Enterprise II Argon ion laser (Coherent, Palo Alto, CA). Red fluorescence was collected through a 610/30 bandpass filter (Omega Optical, Brattleboro, VT). Flow cytometric analysis of integral peak and log fluorescence and forward scatter was performed. The mean DNA content per plant was based on at least 1,000 nuclei. The internal standard used for comparison was rice cv. “Nipponbare” with a 2C genome content of 0.9 pg as estimated by flow cytometry and using the average value of 980 Mbp=1 pg [5, 10, 46].

Chromosome Squashes

For chromosome counts, root tips approximately 0.5 cm long were collected and pretreated at room temperature with 0.05% (*w*/*v*) colchicine in deionized water for 4 h. Root tips were then fixed either for 4 h or overnight in 3:1 ethanol/glacial acetic acid. Slide preparations were made by the Feulgen staining method according to Marks [26], with the addition of a 30- to 60-min digestion step with 0.05 gL^{-1} Onuzuka R-10 cellulase and 0.01 gL^{-1} pectolyase Y-23 (Phytotechnology Labs, Shawnee Mission, KS). Digestion time varied according to the thickness and degree of lignification of the roots. Squashes were made permanent by coverslip removal, drying the slides, and mounting with Permount (Fisher Scientific, Pittsburgh, PA). Meiotic spreads were made in a similar fashion from immature florets using anthers approximately 0.4–0.7 mm in length.

Genetic Analysis

DNA was extracted using a small-scale modified cetyltrimethylammonium bromide procedure [11] on young leaf

tissue dried in silica gel desiccant and ground to a powder in a Retch mill. Expressed sequence tag–simple sequence repeat (EST-SSR) markers and conserved intron spanning primers were produced as described [43]. Forward primers were appended at the 5′ end with M13 to allow indirect labeling reactions. Reverse primers were appended with a variable number of bases at the 5′ end to match the consensus GTTTV. This sequence is found to promote non-templated (A) addition and facilitated subsequent genotyping [8]. Products were amplified in 5–10 μl PCR reactions, PEG precipitated, and sized on an ABI3730xl using homemade size standards labeled with PET. Markers were scored using Genemapper v. 3.7 (Applied Biosystems, Foster City, CA).

Phenotypic Analysis

All phenotypic data were collected on plants grown to maturity in the greenhouse under a 16:8-h day/night (~26–30°C/~22–26°C) growth regimen using supplemental lighting from halide lamps (200 mol photons per square meter per second). Interstomatal distances were measured by excising abaxial epidermis from fully expanded flag leaves, mounting in water, and documenting by digital photomicrography with an Olympus BX51 research microscope. Pixels were subsequently converted to micrometers for data analysis and distances recorded for 98–108 stomata per line. The number of chloroplasts in individual guard cells was determined on separate abaxial epidermal peels using fluorescence microscopy to visualize chloroplast autofluorescence (exc. 470 nm em. 500 long pass). A total of 42–66 individual guard cells were counted per line. Plant height was measured at a stage when all florets were fully visible as the distance from soil level to the collar of the fully expanded flag leaf. Stem diameter was measured at the thickest point of the peduncle using four to ten individual tillers per line. Significant differences were determined using a chi-square test for independence (chloroplast counts) or a Welch two-sample *t* test for all other data.

Results

The 275 individuals of the F1 population and two parents were scored with 137 markers consisting of 91 EST-SSRs [42, 43], 29 genomic SSRs (Lee et al., unpublished), and 17 conserved intron-spanning primers. In each genotype, alleles were identified as present in either the female (ALBK5) parent only, the male (ALBA4) parent only, or in both parents. Several lines did not conform to expectations for a true cross as they had no or very few alleles unique to ALBA4 and fewer amplifiable alleles per marker. In total, 13 lines showed this anomalous behavior and were initially classified as being the result of uncontrolled self-pollination or cross-fertilization by contaminating pollen. The initial screening is summarized in Table 1. ALB280

Table 1 Summary of screening to identify potential diploids

Genotype	Probes assayed, *n*	Number of alleles detected per probe, *x*	Number of shared alleles[a]			
			ALBA4	ALBK5	Both	Total
1.1 Initial screen						
ALBA4	80	2.01	77	0	84	161
ALBK5	81	1.98	0	72	84	156
ALB213	45	1.67	3	36	36	75
ALB242	56	1.43	1	41	38	80
ALB280	72	1.44	2	40	59	101
ALB282	79	1.73	5	57	72	134
ALB297	65	1.82	6	44	64	114
ALB308	64	1.70	3	44	59	106
ALB313	68	1.76	3	48	65	116
ALB315	63	1.67	6	36	59	101
ALB390	66	1.77	11	41	60	112
1.2 Second screen						
ALBA4	22	2.68	37	0	22	59
ALBK5	22	2.77	0	39	22	61
ALB280	22	1.45	0	20	12	32
ALB5B	22	1.55	0	23	11	34

[a] Alleles present in the indicated genotypes that are unique to one parent or common to both parents

was the second least diverse of these individuals, while the least diverse, genotype ALB242, died and was not analyzed further. Additional progeny from the initial cross were sown and screened with one highly informative marker (SWW348), which amplified a total of eight different products (four in each ALBA4 and ALBK5). This marker amplified two rather than the expected four products from several individuals, indicating the possibility of genome reduction.

A more detailed second marker analysis of ALBK5, ALBA4, ALB5B, and ALB280 used a combination of 22 markers fully described in Table 2. These results, summarized in Table 1, confirmed that alleles present in ALB5B and ALB280 were all derived from ALBK5, eliminating the possibility of cross-pollination or mis-scoring.

All viable individuals identified from the initial genotyping screens and secondary genotyping with SWW348 were subjected to flow cytometry. The analysis showed that two individuals, ALB280 and ALB5B, had 2C values of 1.48 and 1.35 pg, respectively, approximately half that of a tetraploid F1 reference individual (ALB881) and the male parent (ALBA4) which had 2C values of 2.61 and 2.75 pg, respectively. These results are presented in Table 3. Values taken from existing literature for two related C4 grasses (*Sorghum bicolor* and *Setaria italica*) and *Populus trichocarpa* [7, 24, 37, 45] are included for comparative purposes.

Somatic chromosome counts of ALB881, ALB280, and ALB5B were determined from metaphase cells in root tip squash preparations. These results, shown in Fig. 1a–c, confirm the presence of $2n=36$ metaphase chromosomes in ALB881, which agrees with published data for tetraploid cultivars [17, 25]. Metaphase chromosome numbers for ALB280 and ALB5B were determined to be $2n=18$ based on counts obtained from more than 15 root tip squash preparations each, and greater than 30 cells in metaphase.

Table 2 Primers used in this study

Marker[a]	Type	Forward primer sequence (5′-3′)[b]	Reverse primer sequence (5′-3′)[c]	Genbank	Similarity
SWW1394	SSR	*M13*-TATGATAACCCAAAGGGCAAC	*PIG*-ACACCCTCGTCATCATCCTC	FL943365	Unknown
SWW1414	SSR	*M13*-AACCAAACCTATGCACACACC	*PIG*-GATCAAGGACAAGTGCCAGAA	DN148383	Unknown
SWW2670	ISP	*M13*-GCGGGAGCTCATCTCAAA	*PIG*-CCGTCAGGCTCTCGAAC	FL871819	heat shock protein HSP82
SWW2682	ISP	*M13*-ATCGCCAGCAAGAACTGC	*PIG*-CGTAAGTGCCAGCGTCA	FE632066	Peroxisomal Ascorbate Peroxidase
SWW2777	ISP	*M13*-GGAGATCTGCCCATGCTG	*PIG*-CGGAGTATCCTTCAGCCC	GD048117	SIR2-like histone deacetylase
SWW2786	ISP	*M13*-CAGCCACTGCAAAGGGAT	*PIG*-GCTCGAAGTGGGTCTTGC	FL817205	LIM domain containing protein
SWW2833	ISP	*M13*-TCCTCCCTGTACTAGTTGTCA	*PIG*-AAATTCCTCGCTTCATACTTT	FL876264	Tonoplast intrinsic protein
SWW2859	SSR	*M13*-CTCAACCTCTACCTCCAAAAC	*PIG*-CGTCTATGATGATTGTCCAT	FL794786	Unknown
SWW2864	SSR	*M13*-GTGGTTAGGGTTAGGGTTTG	*PIG*-CCTCTCCTGCCTTTTAAGC	FL924161	Unknown
SWW2893	SSR	*M13*-CTCCTGAGCTCCTCTTTACAT	*PIG*-AAGCTTCTAGAGGACAGGAAA	FE599118	DNA-binding protein S1FA2
SWW2931	SSR	*M13*-GAAGGGGAAGATGGTGGT	*PIG*-GGAGGATGTTGCTGAGGT	FL777999	Unknown
SWW3005	SSR	*M13*-TTACTGCAACGACAACTACCT	*PIG*-ATTGCAAGCTATCAAACTGTG	FL970172	Cupin superfamily enzyme
SWW3027	SSR	*M13*-AACTCTCCTTTGATTTCTTGG	*PIG*-GTTCTTAATCGATCTCTTGC	FE635157	DNA binding protein
SWW348	SSR	*M13*-TCCATCCTCTCCCTTTGCTA	*PIG*-CTTAGAACAATCGGATGTGGGAG	FL924155	Protein phosphatase regulatory subunit
SWW1918	SSR	*M13*-GAAGCTCCTGCTGTCAGTTTG	*PIG*-GACACCACAGAATTCACCTG	FE639712	Myo-inositol oxidase
SWW2059	SSR	*M13*-CGCCAACTGCTGAGAGATTTA	*PIG*-CGAGAGCACATATCACAACGA	FE65927	Unknown
SWW2132	SSR	*M13*-CATAAGCCATCCAATTTGACC	*PIG*-AAGTGGGGGAACAGTAAAGGA	FE650441	Basic helix-loop-helix family protein
SWW2221	SSR	*M13*-AAGACCAAGCAGGAGAAGAGG	*PIG*-CTTGAGGAAGAAGCACAGCAC	FE630051	Cellulose-synthase 3
SWW2351	SSR	*M13*-GATCTCCAGCTCCTGCTTGAT	*PIG*-CTGAGGGTGTCCAACTTTGTC	FE623083	Unknown
SWW2362	SSR	*M13*-AGTTTTTCCCTGTCACCGATT	*PIG*-CCCTGGTTTTCTCTTTGGTTC	FE624009	Unknown
SWW2402	SSR	*M13*-GCCGCAGAGGTTTTCAAAT	*PIG*-GCACAGGAACCGGTATATGCT	FE651971	Ca^{2+}-dependent DNase
SWW2405	SSR	*M13*-CGGTATACACAGCCCTTCCTT	*PIG*-ATAACAACCATCCCTCCCAAT	FE652200	Unknown
SWW2480	SSR	*M13*-CCCCTCTGGACTACGACGAC	*PIG*-CTCCGGAAGACTCTCCTGATT	FE619779	Unknown

[a] Markers indicated by italics and underscored initially described in Tobias et al. [43]

[b] All forward primers used in this study were appended with the sequence 5′-CACGACGTTGTAAAACGAC-3′ (*M13*)

[c] All reverse primers used in this study were tailed to match the consensus 5′-GTTTV-3′ (PIG)

Table 3 DNA content and chromosome number

Species (genotype)	2C[a] DNA content (pg)	SD	Mbp/1C[b]	Counted chromosome no. (2*n*)[c]
P. virgatum (ALB881)	2.61	0.08	1,281	36
P. virgatum (ALBA4)	2.75	0.02	1,346	36
P. virgatum (ALB280)	1.48	0.07	725	18
P. virgatum (ALB5B)	1.35	–	661	18
Sorghum bicolor (Tx623)[d]	1.67	–	818	20
Populus tricocarpa[e]	1.30	–	485	38
Setaria italica[f]	1.00	–	490	18

[a] Somatic cell DNA content regardless of ploidy

[b] 1 pg=980 Mbp [10]

[c] n>15 metaphase cells for ALB881 and ALBA4; n>30 metaphase cells for ALB280 and ALB5B

[d] Reprinted with permission from [37]

[e] Reprinted with permission from[6, 45]

[f] Reprinted with permission from [24]

Morphological data from greenhouse-grown material for ALB280, ALB5B, and ALB881 were compared. Relative to ALB881, there were no observed differences in flowering time for ALB280 and ALB5B. However, the dihaploid lines showed a marked reduction in stature relative to ALB881 or ALBA4 (see Fig. 1j and Table 4). These lines have proven difficult to maintain and to propagate due to reduced vigor. In addition to plant stature, flowering was affected. Inflorescences were more compact with fewer flowers that failed to open and shed no pollen (Fig. 1d–f).

Further measurements of interstomatal distance and chloroplast number per guard cell were taken. These are correlated with ploidy in several species and can be used as alternative screening methods [1]. Data on plant height and stem diameter are also presented in Table 4. There were significant differences in plant height and stem diameter for the dihaploid lines in relation to ALB881. ALB280 and ALB5B also had significantly fewer guard cell chloroplasts and reduced interstomatal distances.

Because a high level of sterility in ALB280 and ALB5B was evident, we were interested if meiosis in these individuals was irregular. Normal pairing in tetraploid switchgrass produces 18 bivalents visible in diakinesis and metaphase I. In both ALB280 and ALB5B, typically 18 but also 19 and 17 univalents were visible during diplotene and diakinesis stages (Fig. 2b, c). Never were fewer than 15 individual chromosomes observed. No chiasmata were visible, whereas in wild-type diplotene stages, cruciform and branched chromosomes were frequently visible. End-to-end, end-to-side (Fig. 2c, arrow), and side-to-side associations between univalents were frequently observed. Pollen inviability was confirmed by isolation from fully expanded anthers and iodine staining to detect starch. Relative to mature pollen collected from ALB881, very little pollen was collected in dihaploids. That which was collected was smaller, collapsed, and non-staining (Fig. 2d–f).

Discussion

Genotype analyses of ALB280 and ALB5B demonstrate a clear reduction in amplifiable alleles per marker. This, in conjunction with flow cytometry data and chromosome counts, indicates that one parental genome complement has been completely eliminated. The DNA content of ALB280 is approximately 1.48 pg/2C, which is close to half that of the tetraploid and similar to the DNA content predicted by Lu et al. [25] for a diploid progenitor grass. In the dihaploid lines described here, loss of vigor, complete sterility, as well as lack of chromosome pairing observed during meiosis all support the conclusion that either there has been functional diploidization of the tetraploid genome or that tetraploid accessions maintain distinct genomes that arose from two ancestral diploids. The assertion that existing lines of switchgrass are autopolyploids derived from a single diploid progenitor is not supported by our analysis of meiosis in ALB280 and ALB5B but may have occurred followed by significant subgenome differentiation. High degrees of preferential pairing seen in linkage analysis of tetraploid switchgrass [31] also indicate functional diploidization or alloploidy. However, this evidence is indirect and the question may be usefully addressed by either applying phylogeny dating methods coupled with sequencing of related taxa [6, 23] or by using fluorescence in situ hybridization techniques [34]. The origins of polyploidy in switchgrass should be resolved in order to fully understand inheritance and intra-genome structural homology. Diploid *Panicum* species include *Panicum*

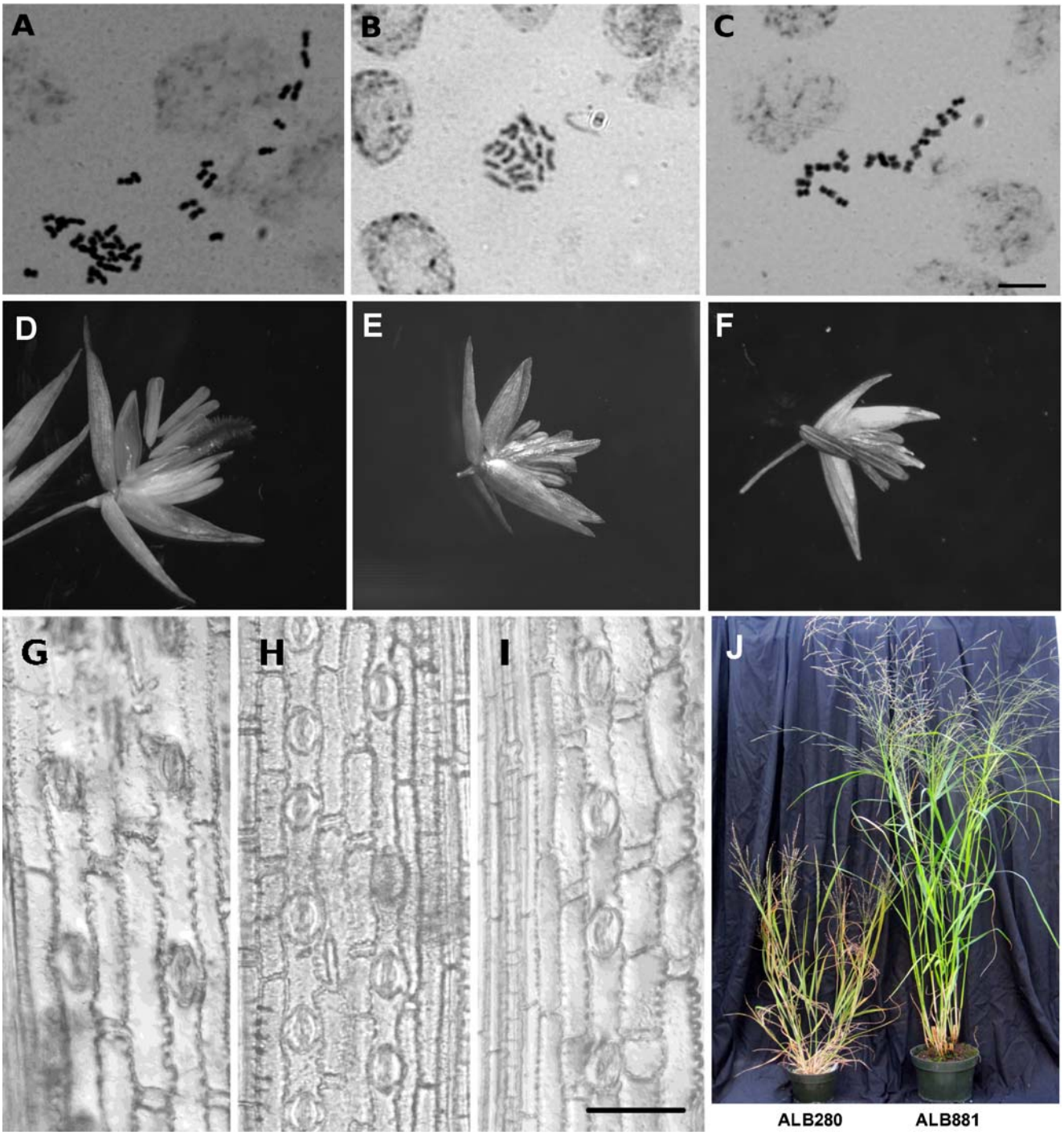

Fig. 1 Abnormal leaf and floral development resulting from genome reduction. ALB881 (**a**, **d**, **g**), ALB5B (**b**, **e**, **h**), and ALB280 (**c**, **f**, **i**) were used for chromosome squashes of root tip cells, and differences between the lines were observed in flowers as well as epidermis. Plant stature and vigor were observed (**j**) for ALB280 and ALB881. Chromosome squashes (**a**–**c**) were prepared from root tips and stained using the Feulgen method (*scale bar* 5 μm). Flowers (**d**–**f**) from different sources were removed and partially dissected to expose anthers and stigma. Epidermal peels (**g**–**i**) from abaxial surface of fully expanded lamina at the approximate midpoint (*scale bar* 50 μm)

capillare, *Panicum laevifolium*, *Panicum antidotale*, and *Panicum coloratum*. However, the 10–12 other species in the *Panicum* section *Virgata* are exclusively polyploid, as are species in the closely related section *Urvilleana* [2]. This would indicate speciation after duplication of the base chromosome number. Sequence comparisons of these dihaploid switchgrass with parental lines and other *Panicum* species would enhance our understanding of

Table 4 Phenotypic differences observed with dihaploids compared to a representative tetraploid (ALB881)

Line	Chloroplasts per guard cell	Interstomatal distance (μm)	Plant height (cm)[a]	Stem diameter (mm)
ALB881	3.23±0.73 (n=57)	99.52±19.74 (n=98)	102.43±11.48 (n=7)	2.85±0.25 (n=7)
ALB280	2.17±0.38[b] (n=66)	80.05±15.79[b] (n=108)	47.90±8.02[b] (n=10)	1.53±0.30[b] (n=10)
ALB5B	2.58±0.66[b] (n=42)	65.68±12.64[b] (n=102)	27.15±11.47[b] (n=4)	0.83±0.33[b] (n=4)

Phenotypic differences are average values ± standard deviation

[a] Height to flag leaf

[b] Significant difference relative to ALB881 at $p<0.001$

genome architecture as well as the potential diploid ancestry of this polyploid species.

Haploid embryo production by pollination with distantly related species is well documented in cereals [20, 22]. Diploid (dihaploid) progeny were produced from interspecific crosses between autotetraploid, cultivated barley (*Hordeum vulgare*) and tetraploid *Hordeum bulbosum* [20]. Other possible explanations for how these dihaploids could arise include spontaneous embryo formation from a reduced egg cell via parthenogenesis [21] or through polyembryony in which multiple seedlings emerge from a single seed [14, 39]. We have screened 1,800 germinating seed without finding twin seedlings, indicating that polyembryony is rare. Others have determined that the frequency of polyembryony across a wide range of tetraploid switchgrass germplasm is between 0.00012 and 0.00014 (Price and Casler, 2008, personal communication).

Chromosome spread preparations using meiotic anthers from these dihaploid lines resulted in the almost exclusive occurrence of univalents (Fig. 2). The presence of 18 univalents at diakinesis indicates a lack of homologous chromosome pairing. This meiotic irregularity (which would lead to aberrant disjunction) likely is the cause of the sterility observed. Some counts were below 18 (typically 17), which may have resulted from loose associations between chromosomes. These associations have been documented in the related pearl millet (*Pennisetum typhoides*) [19]. In haploid *P. typhoides*, end-to-end, end-to-side, and side-to-side chromosomal associations form "loose ring" or "loose rod" bivalents [19, 36]. The extent

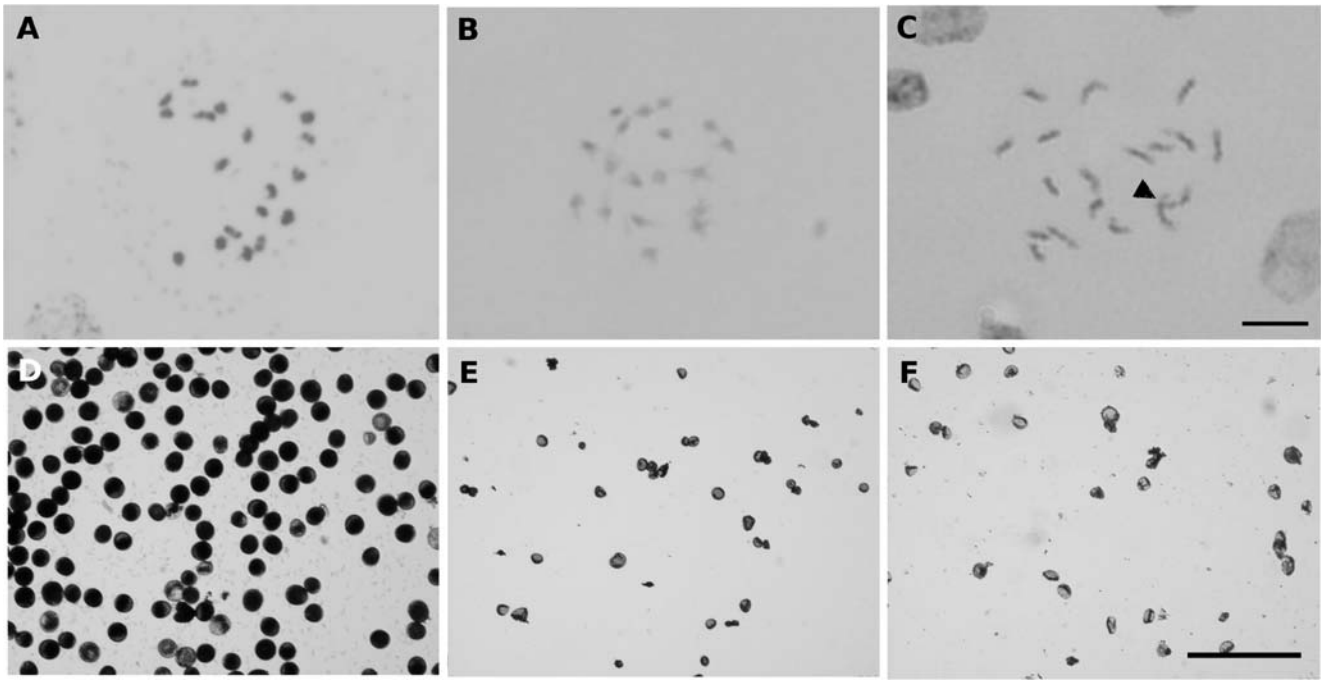

Fig. 2 Meiotic pairing of chromosomes in normal and dihaploid switchgrass and loss of pollen viability. Diakinesis from a tetraploid individual (ALB881) is depicted in **a**, while similar stages from ALB5B and ALB280 are shown in **b** and **c**, respectively (*scale bar* 5 μm). *Arrow* (**c**) indicates chromosome association. Mature pollen from (ALB881) stained with IKI to indicate the presence of starch is shown in **d**. Pollen grains dissected from fully elongated ALB5B (**e**) and ALB280 (**f**) anthers are shown (*scale bar* 200 μm)

of their meiotic associations in haploids and dihaploids is interpreted as internal structural homology and may be correlated with the rates of autosyndetic pairing at meiosis. Our results do not agree with an earlier report that showed nine bivalent pairs and 18 anaphase chromosomes in haploid plants regenerated from anther culture [13]. These androgenic lines were also reported to have extremely poor growth. Loss of a set of homologous as opposed to a set of non-homologous chromosomes could explain these different results. In ALB280 and ALB5B, the loss of two non-pairing homoeologs would lead to univalents, while conversely, the pairing behavior of plants regenerated from anther culture suggests loss of both copies of one set of homologous chromosomes that would lead to normal pairing of the remaining set.

The production of dihaploids provides a novel opportunity to study chromosome architecture and the evolution of polyploidy in switchgrass. The high incidence of bivalents in fertile F1 of lowland × upland crosses of tetraploid cytotypes indicates a high degree of homology within a cytotype between these upland and lowland individuals and suggests that results from studies on dihaploids may be generalizable to both ecotypes [27]. Although previous studies indicated that diploid switchgrass can occur naturally [33], the reduced vigor of both ALB280 and ALB5B and previously reported dihaploids [13] suggests that $2n=18$ lines are not stable.

The development of haploid genotypes has been useful for breeding and genetic research in numerous plant species [15]. Haploids allow for the immediate production of homozygous lines following chromosome doubling, which provides a powerful tool for rapid inbred production and crop improvement [40]. Dihaploid lines have also proven useful for the introgression of genes into hybrids of wheat and barley [44, 48]. The doubling of dihaploids in switchgrass could bypass the plant's self-incompatibility system and enable these approaches. There are several experimental pathways to haploidy, including wide hybridization, delayed pollination, pollination with inactivated pollen, polyembryony, and in vitro techniques of gynogenesis and androgenesis [15]. It is unclear how the dihaploid plants described in this study arose or if the process might be exploited for breeding purposes. This would be more likely if spontaneous or induced chromosome doubling restores fertility or if female fertility is not affected in these plants. Ongoing research to double chromosome numbers via colchicine treatment may lead to fertile homozygous lines. In addition to helping better understand the nature of polyploidy in switchgrass, dihaploid lines provide a resource for simplified karyotyping, sequence assembly, and the integration of genetic, physical, and cytological map data. The dihaploid lines described are being deposited in the National Center for Genetic Resources Preservation and should benefit breeders and researchers alike.

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