APPLICATION ARTICLE

Applications
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Whole-genomic DNA amplifications from individually isolated sweet sorghum microspores

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Abstract

Premise: Sorghum is a multi-use crop, the efficient breeding of which requires the development of new genetic tools. One such tool could be the genetic assessment of free microspores, which are released just after the tetrad stage of pollen development. Microspores are ideal for DNA isolation as they have underdeveloped cell walls and can be readily lysed as natural protoplasts.

Methods: Four cultivars of *Sorghum bicolor* ('Achi Turi', 'Dale', 'Local', and 'Topper 76-6') were grown in a greenhouse until flowering (7.7–11.5 cm flag leaf internode length), after which 30 immature microspores were isolated from each line. Plant height, time to flowering, boot radius, and spikelet maturation were recorded for each cultivar. The exine development of the microspores was observed under an inverted Nikon microscope, and those with underdeveloped exine were subjected to whole-genome amplification and sequencing.

Results: Microspores in the early uninucleate to early binucleate stages had underdeveloped exine, and were therefore ideal for DNA extraction. High-quality DNA was obtained from these single-cell gametophytes. The average DNA concentration was 2902 ng/μL, with fragment sizes comparable to those obtained from leaf tissue extractions.

Discussion: Harvesting panicles with immature microspores means the entire gametic population is accessible for DNA analyses. This is the first amplification of whole-genome DNA fragments from sorghum single-cell microspores isolated during gametogenesis.

KEYWORDS

DNA extraction, immature pollen, microspore isolation, single gametophyte, *Sorghum bicolor*, whole-genome amplification

Sorghum bicolor (L.) Moench is the fifth most important cereal and is cultivated globally as a source of food, syrup, feed, and biomass. It is a C_4 plant capable of producing high yields through its photosynthetic efficiency and drought resistance, even on nonarable land with few agricultural inputs (Garner et al., 2016). In addition, due to the easy extractability of fermentable sugars that accumulate in its stems, sweet sorghum offers an efficient processing pathway for biofuel (ethanol) production (Dahlberg et al., 2011; Mathur et al., 2017). Some cultivars can provide juice yields up to 78% of total plant biomass, with 15–23% °Brix

contents for sugars composed mostly of sucrose (~75%), fructose, and glucose (Kawahigashi et al., 2013; Vinutha et al., 2014).

Sweet sorghum has been the subject of genetic and genomic studies for almost a century (Smith and Frederiksen, 2000). Several genetic maps have been constructed using biparental sorghum populations (Berhan et al., 1993; Klein et al., 2000; Menz et al., 2002), and the reference genome is publicly available. The commonly used BTx623 genome database has been updated utilizing next-generation sequencing (McCormick et al., 2018), and sorghum chloroplast and

mitochondrial genomes are available (Chase and Pring, 1985; Dang and Pring, 1986). Immature pollen grains represent natural haploids and can be used for genetic studies (Aziz et al., 2017); however, only 60–70% of successfully germinated pollens captured are available for subsequent DNA analyses, as 30–40% of individually isolated pollen either did not germinate or did not provide genomic DNA (Aziz and Sauve, 2008). Haplotype maps have been developed for the hexaploid genome of wheat (Jordan et al., 2015). By targeting microspores, which are early-stage pollen, haplotype genetic information could also be revealed for sorghum.

Direct DNA analyses require microspores to be harvested as natural protoplasts. By extracting and amplifying the genomes of individual immature microspores treated with a REPLI-g single-cell kit (Qiagen, Hilden, Germany), a 100% genome coverage can be obtained (Li et al., 2015). A method was established for the identification of different pollen developmental stages without dissection in maize (*Zea mays* L.) using the leaf collar method (Begcy and Dresselhaus, 2017). Other parameters, such as flag leaf internode length, flag leaf deployment time, thickness of the panicle and spikelet, and anther lengths, were observed to be associated with microspore developmental stages in various monocots and have been used as the basis for microspore isolation protocols (Chang and Neuffer, 1989; Jahne and Lorz, 1995). Early unicellular to mid-binucleate microspores are essentially free protoplast cells (Christensen et al., 1972; Yamamoto et al., 2003), and these gametophytes can be captured for DNA isolation and sequencing after observing sorghum panicle characteristics after flag leaf deployment. Although technically difficult (Efroni and Birnbaum, 2016), mechanical micromanipulation is a simple and relatively affordable method for isolating single cells through capillary action (Wang and Song, 2017). The process of single-cell DNA sequencing (scDNA-seq) can be more challenging due to the limited amount of DNA; however, multiple displacement amplification (MDA) allows for whole-genome amplification (WGA) while generating high genome coverage (>90%) with a low (~10^{-7}) false positive rate (Wang and Navin, 2015). In this report, immature sweet sorghum gametophytes were harvested after adapting a flag leaf–based protocol and tracking the reproductive stages. We also standardized individual cell isolation and MDA-based WGA protocols for sweet sorghum, facilitating downstream studies and applications such as genetic analyses and androgenesis.

METHODS

Plant materials

Seeds of two cultivars of *S. bicolor*, 'Dale' (Plant Introduction [PI] number: 651495) and 'Topper76-6' (PI number: 583832), both of which have been promoted for biofuel use in Tennessee by Delta Biorenewables (Memphis, Tennessee, USA), were obtained from the United States Department of Agriculture–Agricultural Research Service's Plant Genetic Resources Conservation Unit (Griffin, Georgia, USA). Two additional sorghum cultivars, 'Dasht Local' and 'Achi Turi', were recommended for this study by the Agriculture Research Institute (Quetta, Balochistan, Pakistan) due to their value as biofuels, and their certified seeds were provided by Dr. M. Y. Khan Barozai (University of Balochistan, Quetta, Pakistan) as per the Pakistan–U.S. Science and Technology Cooperation Program (international joint research) funded through the National Academies of Sciences, Engineering, and Medicine.

The seeds were germinated at room temperature in Petri dishes and then embedded in 4.5-L nursery pots containing Miracle-Gro potting mix (Scotts Company, Marysville, Ohio, USA). For aphid control, a half tablespoon of the systemic pesticide Marathon (OHP, Morrisville, North Carolina, USA) was added per container following the manufacturer's applications and formulations protocol. Thirteen to 26 plants of each cultivar were maintained at $30 \pm 3°C$ during the day in a greenhouse, with night temperatures of $24 \pm 3°C$. The plants did not require fertilization, and the pots were watered by hand as needed. The plants were grown at the Tennessee State University Agricultural Research and Education Center (36.12′N, 86.89′W) under natural light conditions: vernal equinox (12 h), estival solstice (14.5 h), and hibernal solstice (9.5 h), with corresponding average (from 09:00 hours to 15:00 hours) lux readings of 12,000, 18,000 and 10,000, respectively. The emerging panicles were scrutinized daily at the beginning of the booting stage after the emergence of the flag leaf. Sheaths covering the immature inflorescence containing the developing pollen grains were gently peeled open to observe the physical appearance of the panicles. All inflorescence-related measurements were replicated at least 10 times. The results were comparatively analyzed, and the figures were generated using the R statistical computing environment (R Project version 3.6.1; R Core Team, 2021).

Identification of microspore developmental stages

Panicles were harvested by snipping the flag leaf stem two internodes beneath the crown of the plant, while recording the planting date, plant height, flag leaf length, and days since flag leaf deployment. After collection, the panicles were stored in resealable polypropylene bags at 4°C in a refrigerator until use. Before use, the panicles were sterilized for 2 min in bleach solution containing 1% NaClO and then washed with sterilized H_2O. Three spikelets were then harvested along every 1 cm length of the panicle from its proximal to its distal end, sequentially representing all microspore developmental stages. Spikelet and anther lengths were recorded to be correlated with the growing microspore diameters as per their maturation stages. After establishing the harvesting parameters for each microspore maturation stage, each spikelet was dissected using a

sterilized scalpel and the three retrieved anthers were placed on a glass slide. The anthers were aseptically cut in half with care taken to minimize excessive debris, and then gently pressed a few times to release the free microspores in 2 μL of 6% sorbitol (MilliporeSigma, St. Louis, Missouri, USA). The density and viability of the microspores obtained from vigorous healthy plants were observed using a hemocytometer (Hausser Scientific, Horsham, Pennsylvania, USA) and staining with Trypan Blue (MilliporeSigma). Aliquots of the 6% sorbitol solution containing microspores at different developmental stages were then used for subsequent stainings. To observe the developing exine under a microscope (Inverted Diaphot-TMD; Nikon, Tokyo, Japan), 0.0005% Auramine O (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to stain the cell wall. The nuclei of the microspores were stained using DAPI (Keyence, Osaka, Japan). All spikelet, anther, and microspore measurements were replicated 10 times and subjected to a comparative analysis using the R statistical computing environment (R Project version 3.6.1; R Core Team, 2021).

Isolation and whole-genome amplification of single-cell DNA

The anthers were crushed in 2 μL of 6% sorbitol on a glass slide, as described above, and 30 microspores per cultivar were individually isolated in 0.2-mL centrifuge tubes through a pulled glass capillary (Harvard Apparatus, Holliston, Massachusetts, USA) using a Cell-Tram microinjector (Eppendorf, Hamburg, Germany) mounted on a Märzhäuser (HS-6) micromanipulator (Narishige International, Tokyo, Japan). Each tube was then centrifuged at 5000 rpm for 5 min after phosphate-buffer saline (8 g/L NaCl, 200 mg/L KCl, 1.44 g/L Na_2HPO_4, and 240 mg/L KH_2PO_4) was added to a final volume of 4 μL. Next, 6 μL of reconstituted extraction buffer from the REPLI-g single cell kit (Qiagen) was added into the microcentrifuge tubes before a 10-min incubation at 65°C. Afterward, 3 μL stop solution (Qiagen) was added, and then the WGA reaction was performed. The final 50-μL reaction volume comprised 9 μL H_2O, 29 μL REPLI-g Reaction Buffer (Qiagen), and 2 μL DNA polymerase (Qiagen). The tubes were then centrifuged and incubated at 30°C for 8 h in a thermocycler, followed by 3-min inactivation of the polymerase at 65°C. The resulting MDA products were then quantified using a NanoDrop One spectrophotometer (Thermo Fisher Scientific).

Leaf DNA extraction

Young leaves from healthy 60- to 75-day-old plants were harvested and immediately subjected to DNA extraction using a DNeasy Plant Mini Kit (Qiagen) with slight modifications to the standard manufacturer's protocol. All DNA products were visualized and photographed by placing the gel on a UV transilluminator (Alpha Innotech, San Leandro, California, USA) after staining with 5× GelRed (Biotium, Fremont, California, USA). The resulting leaf DNA extraction products were then quantified using a NanoDrop One spectrophotometer (Thermo Fisher Scientific).

RESULTS

Sorghum booting stage characteristics

Thirteen to 26 plants each of four sweet sorghum cultivars were grown from germinated seeds through early spring to fall in the years 2018 and 2019. By staggering the planting dates, we were able to observe the effect of planting time on flowering, while expanding the availability of gametophytes for the staggered collection of fresh microspores. Plant growth characteristics, such as the flag leaf emergence time, plant height, flag leaf internode length, and flag leaf boot radius, were recorded at the time of panicle harvest (Table 1). At the booting stage initiation, a bulge formed within the sheath containing the panicle, which was observed just before the emergence of the flag leaf. The flag leaf emergence proved to be the main indicator for

TABLE 1 The five important growth indicators used to predict the accurate timeframe for harvesting *Sorghum bicolor* panicles containing microspores at naturally protoplast-like developmental stages. The number of days needed for the corresponding growth and flag leaf–related trait sizes (in centimeters or millimeters) were recorded for each sweet sorghum cultivar, allowing for the consistent harvesting of panicles containing free immature microspores. Different letters denote a significant difference at $P < 0.05$ within the row.

Growth indicators	*Sorghum bicolor* cultivar[*]			
	'Achi Turi' (N = 13)	'Dale' (N = 18)	'Dasht Local' (N = 23)	'Topper 76-6' (N = 26)
Days from planting to flag leaf emergence	143.2[a]	147.2[a]	152.5[a]	144.3[a]
Days from flag leaf emergence to harvest date	4.3[b]	5.3[a]	5[ab]	5.4[a]
Plant height (cm) at harvest	261.8[a]	86.5[b]	56.4[c]	61.2[c]
Flag leaf internode length (cm) at harvest	7.7[b]	11.5[a]	11.5[a]	7.9[b]
Flag leaf boot radius (mm) at harvest	11.1[a]	7.4[b]	8.7[b]	8.5[b]

[*]Data from at least 10 biological replicates of each cultivar, which were greenhouse grown from early spring to fall of 2018 and 2019. The mean values are across all planting dates.

predicting and setting the timeline for harvesting the panicles. On the third day after the flag leaf emerged, a small vertical cut was made on the sheath to observe the maturing panicles harboring spikelets and anthers. It was observed that, once the flag leaf deployed, the panicles containing immature microspores were ready to be harvested within 3–7 days, depending on the planting date and environmental factors.

By correlating the dates of planting and flag leaf emergence, a protocol was devised for panicle harvestings. This allowed the development of a schedule for harvesting the panicles at the appropriate time for obtaining the immature microspores. Because no supplemental light source was used, the photoperiod played a critical role on plant growth characteristics for each growing season. The planting date appeared to have no effect on 'Dale' or 'Dasht Local' in terms of the length of the period between planting and flag leaf emergence. As fall approached, the shorter photoperiods hastened 'Topper 76-6' maturity, while delaying it for 'Achi Turi'. For all cultivars, the period between the planting date and harvest and the flag leaf internode length were observed to be curtailed as the fall growing season culminated. Also, it was noted that the height of all cultivars was reduced at harvest when the planting date was delayed. The flag leaf internode length was another morphological indicator used to predict the panicle harvest date; this varied from 6–14 cm, with plants sown after the end of summer or later having reduced heights compared to those initiated in early spring.

## Capturing microspore development stages

It was possible to predict panicle harvesting dates that would yield immature microspores using the four previously mentioned morphological characteristics of developing sweet sorghum plants; however, this had to be individualized for each cultivar based on the environmental conditions during the plant growth seasons. Spikelets, and their corresponding anthers within, were collected and categorized based on physical appearance. These were also correlated with microspore developmental stages as per the harvested panicle portions (distal, middle, and basal). Spikelets containing tetrads, the stage immediately after the meiosis of the pollen mother cells, were small, mostly clear, and had very soft tissue, while the anthers were mostly clear with a slight green tinge at the tip. As the spikelets matured to contain uninucleate microspores, the tip became slightly engorged and developed a light green coloration; similarly, the anthers within turned light green and became slightly enlarged. Toward the end of the uninucleate microspore phase, the external bract of the floret started to harden. During the early binucleate development phase, the spikelet grew larger, the inner tissues developed an orange tint, and the outer tissues hardened. The corresponding anthers yielding binucleate gametophytes also grew longer and their orange tint expanded significantly,

while the tips became dark green and the outer tissue continued to harden and become sticky. When microspores approached the mid-binucleate stage, the spikelet became fully engorged with a completely orange center and a mostly dark green tip from the top to halfway down toward the basal end. At this stage, the spikelets developed a waxy or slippery outer coat and were difficult to pierce, while the anthers within were full and had a darker orange color.

In addition, when the stem was cut two nodes below the penultimate leaf ligule, its regrowth resulted in multiple flag leaves for most of the plants. The panicles obtained from the regrown stems usually developed in a reduced capacity, producing immature microspores after one month (on average). For all cultivars, snipping the stems two internodes below the panicle typically caused them to regrow within a month, although in a reduced state. This allowed for the additional harvesting of panicles containing free microspores from the same plant. During spring and summer, the panicles would regrow after harvesting after one month, and could regrow up to three times, before the plant began to wither. Anther and spikelet lengths and widths were recorded to identify the harvested microspores' developmental stages (Appendix S1). Spikelet length and width, anther length, and microspore diameter across the six micro-gametogenesis developmental stages were compared using one-way ANOVAs, revealing that these characteristics all significantly increased in size across the micro-gametogenesis developmental stages (Figure 1). Furthermore, a strong correlation was found between spikelet length and microspore diameter ($r^2 = 0.74$, Figure 2A), while the spikelet width, which increased from the crown to the base of the panicle, also showed a strong correlation with microspore diameter ($r^2 = 0.75$, Figure 2B). The microspore development stage was found to be directly proportional to the anther length ($r^2 = 0.78$, Figure 2C). The length of the harvested anthers was the best predictive indicator of microspore developmental stage. We also confirmed the previous report that sorghum pollen maturation starts from the crown and proceeds downward along the length of the panicle (Gerik et al., 2003); thus, the distal portion of the panicle contains mature microspores, with progressively immature microspores found toward the basal end.

Single gametophyte DNA yields

The sorghum microspore developmental stages were determined through the staining of the nuclei and exine layers, as previously reported (Wang et al., 2015). The microscopic observation of the stained nuclei (DAPI) and exine (Auramine O) of the microspores at early developmental stages confirmed that these cells had similar attributes to natural protoplasts (Figure 3). The REPLI-g (Qiagen) kit was used to generate ample and high-quality DNAs from individually isolated sorghum microspores. The REPLI-g kit is designed to work on cells that do not contain
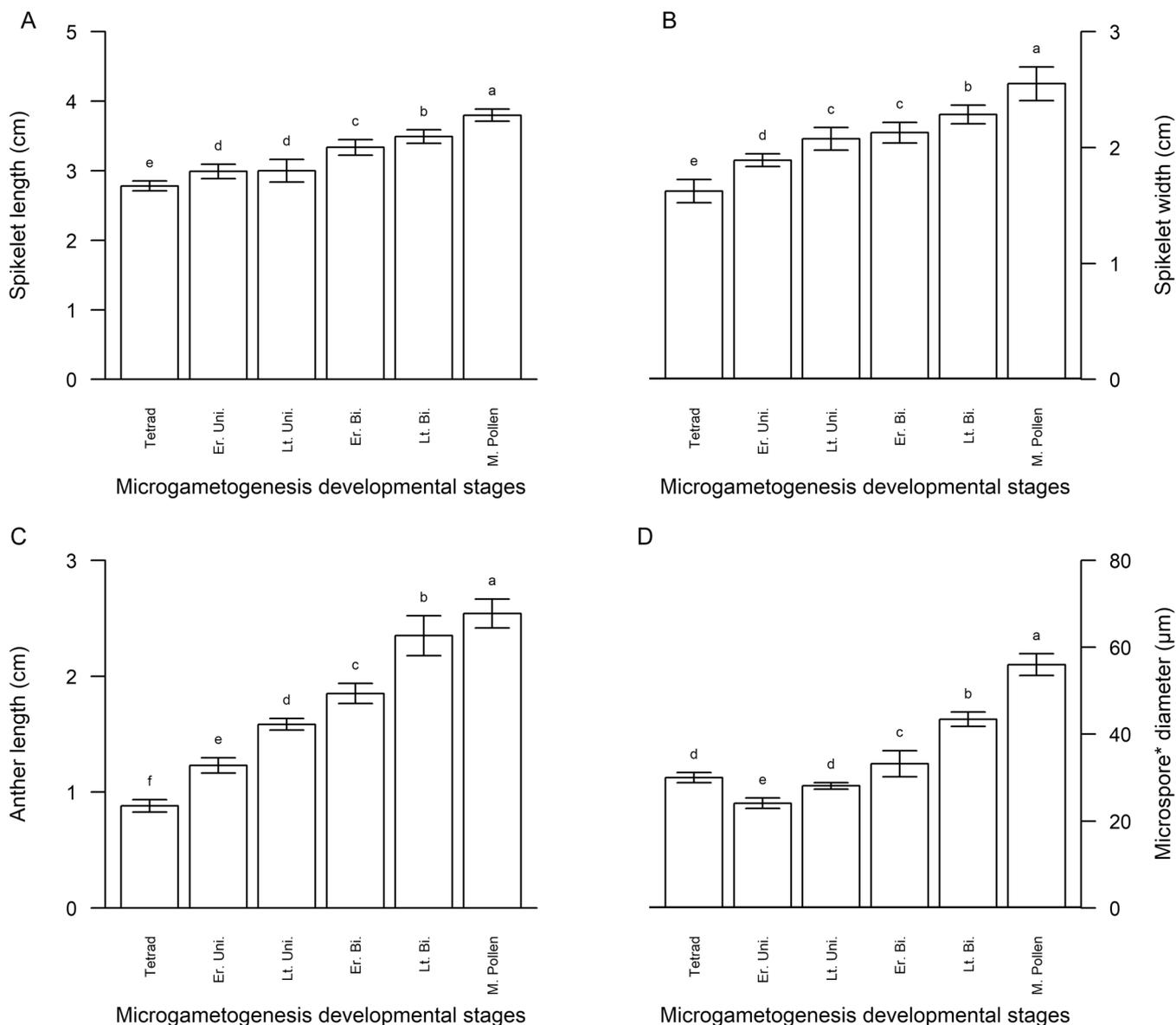
FIGURE 1 Sweet sorghum characteristics across the microgametogenesis developmental stages. Comparisons of typical (A) spikelet length, (B) spikelet width, (C) anther length, and (D) microspore diameter throughout microgametogenesis. Error bars are one standard deviation from the mean. Different letters denote a significant difference at $P < 0.05$. Er. Uni = early uninucleate; Lt. Uni = late uninucleate; Er. Bi. = early binucleate; Lt. Bi. = late binucleate; M. Pollen = mature pollen. *At the tetrad stage, the diameter of the pollen mother cell containing the four microspores was measured.

cell walls; thus, the success of these DNA extractions confirmed that the isolated microspores had an underdeveloped exine. The natural protoplast state of the microspores was therefore verified through the extraction and WGA using MDA protocols.

The resulting MDA products were quantified, and the quality of the DNA was checked in terms of its A260/A280 (UV absorbance peaks) ratio and A260/A230 readings, in addition to using gel electrophoresis (Figure 4). The DNA yield from each sweet sorghum microspore ranged from 1626 to 3955 ng/μL, averaging 2902 ng/μL for individual cells, while their average A260/A280 ratio was 1.8. The average DNA yield from the leaf extractions was 113.5 ng/μL, while the A260/A280 ratio for these DNAs

was 1.78. The A260/A280 nm ratio obtained from the single sorghum microspore cells indicated that the WGA procedure generating MDA products was an appropriate choice in this study. The WGA yielded DNA fragments from individual sweet sorghum microspores that were of similar size to the sheared DNAs obtained from the leaf tissues (Figure 4). The MDA procedure resulted in ample DNA in large fragments, which should be useful for downstream analyses similar to those performed on DNA obtained from leaf extractions. We successfully extracted DNA from all 30 microspores collected from each of the four sorghum cultivars using the single-cell kit-based DNA extraction and amplification procedures, allowing for subsequent sorghum genomic analyses.
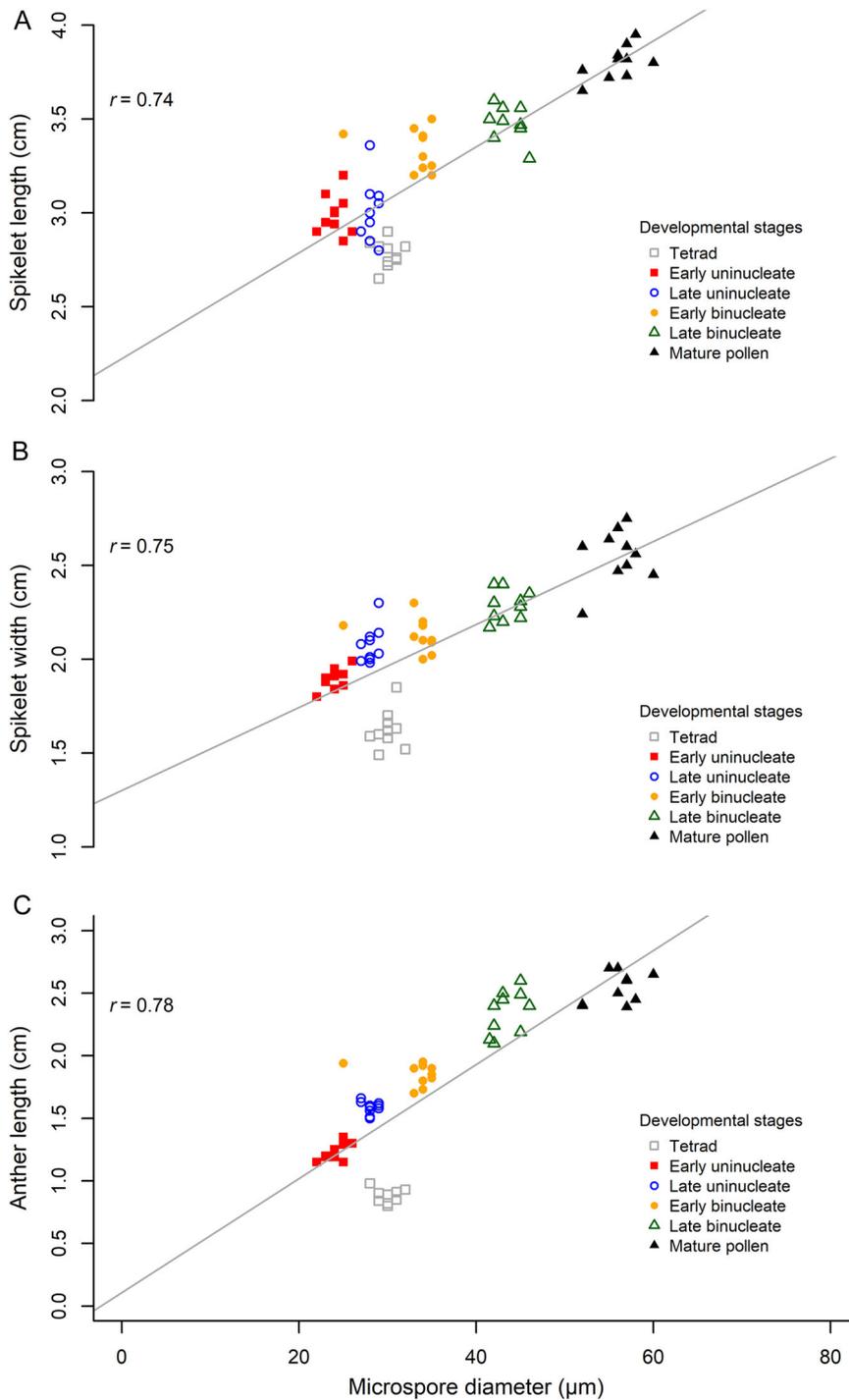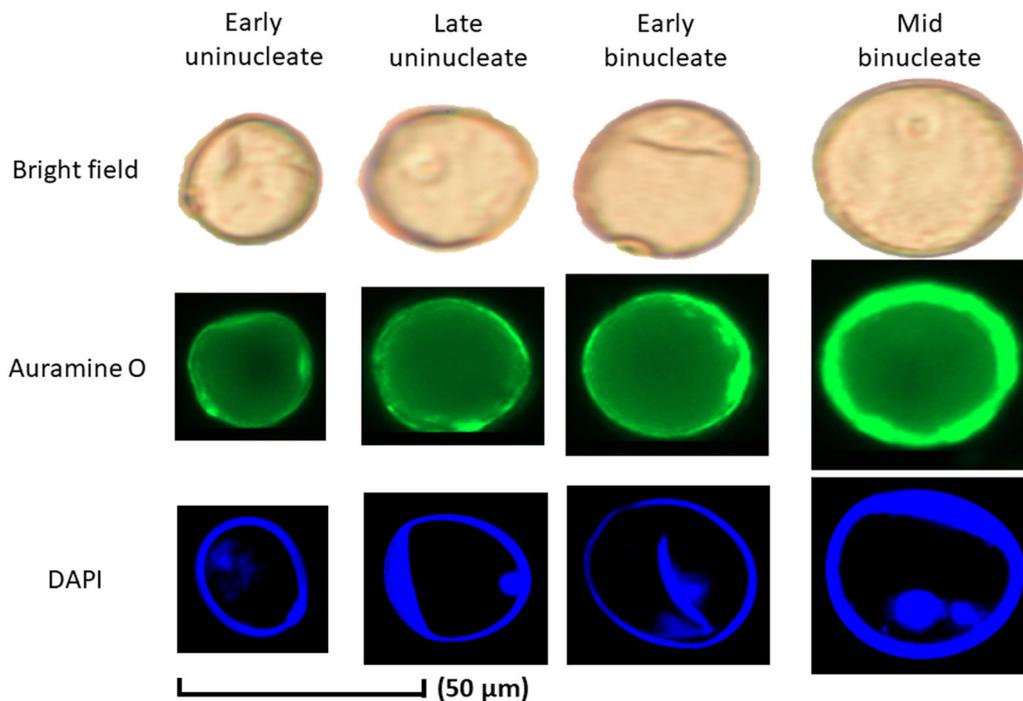
FIGURE 2 Relationship between the sweet sorghum plant traits and the microspore developmental stages. Relationship between typical (A) spikelet length, (B) spikelet width, and (C) anther length and the various (six) microspore developmental stages.

DISCUSSION

Greenhouse data were correlated with pollen developmental stages to facilitate the harvest of anthers containing underdeveloped microspores. Scheduling planting dates over the period from early spring to fall was seen to affect panicle harvesting time, and facilitated the continuous availability of fresh microspores over this nine-month period. We observed several trends based on the relationship of the planting dates to the plant growth data collected at harvesting time. The sweet sorghum panicle harvest periods for each growing season were accurately predicted by correlating the flag leaf emergence time, plant height, flag leaf internode length (7.7–11.5 cm), and flag leaf boot radius (7.4–11.1 mm) to different microspore developmental stages. Similar observations have been made in maize, allowing for the accurate prediction of flowering to enable the harvest of immature microspores at the desired stage (Begcy and Dresselhaus, 2017). Regarding sorghum panicles, incremental seed maturation from the tip toward the base has been reported over a

FIGURE 3 Correlations between the typical sweet sorghum microspore diameters and exine development between the early uninucleate and mid-binucleate stages. Exine development of free microspores is shown by Auramine O staining. Nuclei are stained with DAPI.

FIGURE 4 Whole-genome DNA yields of the sweet sorghum cultivars 'Topper 76-6', 'Dale', 'Achi Turi', and 'Dasht Local'. A 250-ng aliquot was loaded in each sample well after extraction from (A) the leaf tissues using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and (B) the microspores after multiple displacement amplifications using a REPLI-g Advanced DNA Single Cell Kit (Qiagen). 'Mass Ladder' denotes a low-DNA mass ladder (Invitrogen no. 10068013; Thermo Fisher Scientific, Waltham, Massachusetts, USA), and the respective sizes and quantities of the DNA bands are indicated.
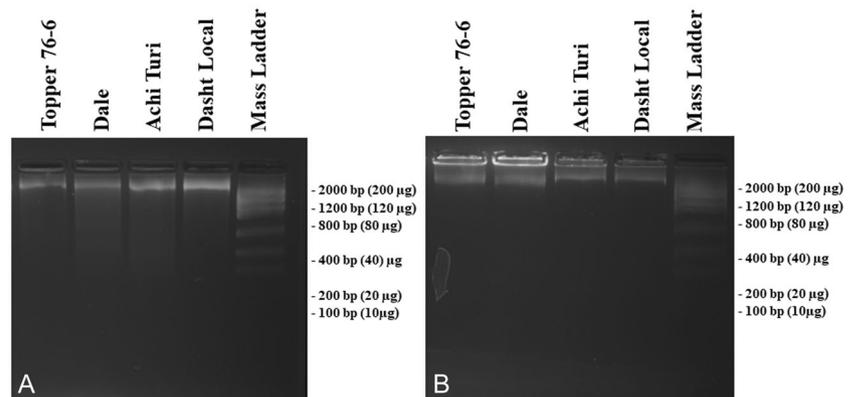
period of 4–9 days (Gerik et al., 2003). Here, the gametogenesis stages of sorghum microspores were categorized by tracking spikelet placement on panicles and the corresponding anther lengths. Microspore developmental stages (tetrad, early uninucleate, mid-uninucleate, early binucleate, mid-binucleate, and mature pollen grain) were correlated to greenhouse growth data with the aim of predicting precise harvesting times. This report confirmed all of the sorghum microspore developmental stages previously recorded by Christensen et al. (1972).

Staining sweet sorghum microspores confirmed that the isolated uninucleate to late binucleate stages lack a fully formed exine (Figure 1), allowing for the use of a single-cell MDA-based REPLI-g kit designed to lyse protoplasts and extract DNA. Li et al. (2015) used the REPLI-g single-cell kit on maize microspores, obtaining sufficient amounts of genomic DNA to perform a genomic analysis. Aziz et al. (2017) also used the same kit for the amplification of genomic DNA from individually isolated microspores of cotton (*Gossypium hirsutum* L.). The sorghum microspores provided ample (~2902 ng/μL) high-quality DNA, as assessed by a 260/280 nm UV absorbance of 1.8, which is generally accepted as "pure" for nucleic acid preparations (Geuther, 2007).

Meiotic products and their genetic analyses are very useful for providing insight into several aspects of inheritance in organisms (Copenhaver et al., 2000; Shi et al., 2019). Natural pollen competition for fertilization leads to

male gametophyte selection, which has implications for female-biased plant population ratios (Lora et al., 2016; Delph, 2019). In addition, only gametophytes responsive to anther culture and pollen germination approaches are available for DNA amplification in subsequent genetic analyses (Tai et al., 2000). The control of the sporophyte over the gametophyte has implications in plant evolution (Lora et al., 2016; Delph, 2019), and this mechanism may be better understood by analyzing unbiased samples from the entire microspore population. This is the first report on whole-genomic DNAs from single-cell *Sorghum* microspores, which could circumvent selection biases and enable subsequent genetic analyses through natural pollination, anther culture, or pollen germination approaches. In addition, it was confirmed that microspore samples contained 15,000 to 20,000 cells on average, with a viability rate of over 95%. These gametophytes harvested as protoplast-like cells can also be used for genetic modification, as was achieved by Lin et al. (2018) on protoplasts from somatic cells, facilitating subsequent androgenesis or mature pollen routes.

AUTHOR CONTRIBUTIONS

A.N.A. conceived the research, acquired the funding, and designed the experiments; A.M.F. performed all the experiments and B.P. conducted all statistical analyses. D.Y. assisted in methodology and performed DNA analyses. A.N.A. and A.M.F. analyzed the data and edited the first draft of the manuscript. All authors approved the final version of the manuscript.

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DATA AVAILABILITY STATEMENT

All supporting data have been provided within the article and supporting information.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Descriptive statistics for spikelet and anther sizes, as well as microspore diameters, across six micro-gametogenesis stages of sweet sorghum (*Sorghum bicolor*).

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