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ORIGINAL RESEARCH

Genomic Characterization of Interspecific Hybrids and an Admixture Population Derived from *Panicum amarum* × *P. virgatum*

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Abstract

Switchgrass (*Panicum virgatum* L.) and its relatives are regarded as top bioenergy crop candidates; however, one critical barrier is the introduction of useful genetic diversity and the development of new cultivars and hybrids. Combining genomes from related cultivars and species provides an opportunity to introduce new traits. In switchgrass, a breeding advantage would be achieved by combining the genomes of intervarietal ecotypes or interspecific hybrids. The recovery of wide crosses, however, is often tedious and may involve complicated embryo rescue and numerous backcrosses. Here, we demonstrate a straightforward approach to wide crosses involving the use of a selectable transgene for recovery of interspecific [*P. virgatum* cv. Alamo × *Panicum amarum* Ell. var *amarulum* or Atlantic Coastal Panicgrass (ACP)] F_1 hybrids followed by backcrossing to generate a nontransgenic admixture population. A nontransgenic herbicide-sensitive (HbS) admixture population of 83 F_1BC_1 progeny was analyzed by genotyping-by-sequencing (GBS) to characterize local ancestry, parental contribution, and patterns of recombination. These results demonstrate a widely applicable breeding strategy that makes use of transgenic selectable resistance to identify and recover true hybrids.

SWITCHGRASS ecotypes and related species have become dominant candidates as feedstock for cellulosic biomass that will be used for the production of various types of biofuels. Switchgrass is native and widespread throughout most of North America (Casler, 2012, 2011; Moser and Vogel, 1995) and, importantly, grows on marginal lands that are not in competition with food production resources. Switchgrass is widely recognized as a top candidate for biofuel feedstock and crop improvement; however, hybrid systems, trait selection, and domestication must be put in place to achieve a level of performance suitable for commercial production (Casler, 2012; Vogel and Burson, 2004). One problem is the limited ability to introgress and combine useful traits into regionally selected germplasm. Switchgrass cultivars and their related species are highly heterozygous, anemophilous, obligate outcrossers with both prefertilization and postfertilization incompatibility systems present (Martinez-Reyna and Vogel, 2002, 2008).

The key to identifying potential targets for introgression has been the recent efforts toward understanding switchgrass diversity. In the last few years, large amounts of phylogenetic and taxonomic information have accumulated about switchgrass cultivars and populations (Casler et al., 2007, 2011; Huang et al., 2011; Lu et al.,

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Abbreviations: ACP, Atlantic Coastal Panicgrass; EST, expressed sequence tag; GBS, genotyping-by-sequencing; GMO, genetically modified organism; HbR, herbicide-resistant; HbS, herbicide-sensitive; PCR, polymerase chain reaction; QTL, quantitative trait loci; SNP, single nucleotide polymorphism; T-DNA, transfer DNA.

2013). Two main ecotypes have been characterized. Upland types are indigenous to upland areas in North America with a low flood risk and lowland types are common to the flood plain regions of North America. These two ecotypes show distinct morphological and physiological characteristics (Casler, 2005; Eberhardt and Newell, 1959; McMillan and Weiler, 1959) and the distinctiveness of their nuclear genomes has been established on the basis of cluster analysis of random amplified polymorphic DNA markers (Gunter et al., 1996), identification of restriction fragment length polymorphism markers (Missaoui et al., 2006) and of expressed sequence tag (EST)–simple sequence repeat markers (Narasimhamoorthy et al., 2008).

In addition to switchgrass ecotypes, there are several closely related species that are also indigenous to North America, including *Panicum coloratum* L.*, Panicum dichotomiflorum* Michx.*, Panicum hallii* Vasey*, Panicum obtusum* Kunth, and *P. amarum*. One species, *P. amarum* var. *amarulum* or ACP, is of particular interest. Atlantic Coastal Panicgrass is a tetraploid with many shared characteristics consistent with both lowland and upland switchgrass ecotypes (Triplett et al., 2012). Using molecular markers and dendrogram results, Huang et al. (2011) found *P. amarum* to be a sister taxa to *P. virgatum,* the most common commercial variety of switchgrass. Despite their close relationship, each species possesses independent traits that are invaluable for the biofuel industry (Huang et al., 2011). For example, *P. amarum* is known to be tolerant to heat, drought, and humidity and has a different range with respect to lowland ecotypes such as *P. virgatum* cv. Alamo, which is a high biomass variety. The abiotic stress resistance of ACP combined with the commercial yields of Alamo switchgrass would clearly be desirable to integrate into biofuel germplasm resources.

An obstacle to hybrid development in switchgrass varieties is the time-consuming and laborious process of recovering hybrid plants and fertile alloploids (Hultquist et al., 1996; Martinez-Reyna and Vogel, 2002). Controlled hybridization techniques, based on floral emasculation and mutual pollination by bagging inflorescences, have been used for generating hybrids within and between the upland and lowland switchgrass ecotypes (Hultquist et al., 1996; Martinez-Reyna and Vogel, 1998, 2002, 2008). Through these techniques, intraspecific crosses between spatially separated populations have yielded viable hybrid plants that display heterosis (Hultquist et al., 1996; Martinez-Reyna and Vogel, 1998, 2002, 2008). Although these methods are accurate and promising, they are tedious and time-consuming and produce low numbers of candidate progeny. Additionally, analysis and verification of hybrid plants requires extensive phenotypic observation and measurements based on morphological characteristics as well as molecular analysis to verify the hybrid genotype.

Although the phenotypic diversity characterized in switchgrass and its relatives justifies extensive hybrid development, taking advantage of these genetic resources requires molecular and computational tools to identify quantitative trait loci (QTL) for accelerated selections. Mapping populations of switchgrass have been genotyped using standard molecular marker analysis (Missaoui et al., 2005; Okada et al., 2010); however, single nucleotide polymorphisms (SNPs) are likely to provide the most useful approach for long-term breeding and genetic studies. A reduced representation gDNA sequencing method for SNP discovery, GBS, has been used in several crop species including switchgrass (Baird et al., 2008; Cao et al., 2011; Eaton and Ree, 2013; Elshire et al., 2011; Lu et al., 2013; Poland et al., 2011; Wang et al., 2013; Ward et al., 2013). Genotyping-by-sequencing has been shown to be suitable for generating robust marker datasets from large, repetitive polyploidy genomes such as switchgrass at low cost (Lu et al., 2013). Its high multiplexing capacity and low cost per sample make it especially suitable for population studies.

In this paper, we demonstrate the use of a selectable transgene to identify and recover interspecific hybrids of ACP and Alamo switchgrass without extensive phenotypic observation or measurement. The hybrid F_1 was backcrossed to wild-type Alamo switchgrass and segregation was used to remove the transgenic sequence and facilitate recovery of nontransgenic F_1BC_1 progeny, resulting in a novel admixture population. A recently modified GBS protocol (Heffelfinger et al., 2014) was employed to analyze the genetic admixture of the F_1BC_1 lines and to demonstrate the transmission and heritability of genomic segments from both the Alamo and ACP parents and recombination in the offspring. This study confirms the use of transgenic selectable markers as a method to identify robust, wide-cross switchgrass populations suitable for both mapping studies and introgression of genetic diversity.

MATERIALS AND METHODS

Transgenics

Primary Alamo transgenics were generated according to Somleva et al. (2002) using *Agrobacterium tumefaciens* strain LBA4404 containing a ~13-kb transformation vector derived from the intermediate binary vector pSB11 (Komari et al., 1996) (Genbank accession: AB027256). The transfer DNA (T-DNA) region contains a selectable marker cassette comprised of the *bar* gene (Thompson et al., 1987) under control of a rice (*Oryza sativa* L.) ubiquitin promoter and the nopaline synthase terminator sequence (Depicker et al., 1982). The complete sequence of the T-DNA region is provided in GenBank (Genbank accession: KP724690).

Interspecific Hybridization and Backcrossing of Alamo Switchgrass and ACP

The F_1 hybrid population was made using a transgenic Alamo parent (T85–2) pollen donor carrying a single T-DNA insertion and a wild-type ACP parent as the pollen recipient. Pollen cage frames were constructed using 2.54 cm polyvinyl chloride pipe in the dimensions

114.3 by 63.5 by 63.5 cm. Fitted three-way 1" polyvinyl chloride connectors were used to fit all polyvinyl chloride lengths together in a rectangular box. Each cage was wrapped with a double layer of summer-weight Agribon (Agribon, San Luis Potosi, Mexico) row-cover. In six independent pollen cage experiments, parental types were set up in interspecific combinations using three vegetative clones of T85–2 as a pollen donor with a wild-type ACP plant. The T85–2 parent was removed after pollen shed had ceased. Seed was harvested from the ACP parent. Control crosses of wild-type Alamo and ACP were similarly conducted. Interspecific F_1 hybrids [1684 total, 644 herbicide-resistant (HbR), 1040 HbS] were identified using a nondestructive herbicide leaf paint assay [3% Finale (Bayer Environmental Science, Research Triangle Park, NC), a.i. glufosinate (2-amino-4-[hydroxymethylphosphinyl]butanoic acid)] and one HbR was backcrossed to a wild-type Alamo pollen donor. F_1BC_1 seed was collected, germinated, and rescreened for herbicide resistance. From an original population of 86, 83 *bar*-negative, HbS F_1BC_1 plants survived transplantation and were further characterized.

Phenotype Measurement

Iodine potassium iodide staining of select F_1 and parental plants was performed according to published protocols (Johansen, 1940). Stomata count was made on 10.5 mm^2 of leaf tissue. Chlorophyll concentration was measured using published protocols (Edelman et al., 1982) on 20 g of fresh leaf tissue. Three technical replicates were performed for each measurement.

Molecular Analysis

Southern blot analyses were performed using 20 μg of genomic DNA digested with the restriction endonuclease EcoRV (New England Biolabs, Ipswich, MA) or NcoI (New England Biolabs) for F_1 single-copy validation. Wild-type genomic DNA samples from Alamo switchgrass and ACP were included as negative hybridization controls. The digested DNA was size-fractionated by agarose gel electrophoresis in a 0.8% (w/v) agarose gel and transferred to either a positively charged Roche Nylon Membrane (Roche Applied Science, Indianapolis, IN) or (NcoI only) a Hybond N+ positively charged nylon membrane (GE Healthcare, Piscataway, NJ) by high-salt capillary transfer. The resulting membrane was hybridized to either a (EcoRV) 513-bp or (NcoI) a 213-bp digoxigenin-labeled *bar* probe or (NcoI) a 213 -bp digoxigenin-labeled bar probe generated using the PCR DIG Probe Synthesis Kit (Roche Applied Science, Indianapolis, IN). Probe-hybridized fragments were detected by enzyme immunoassay and an enzyme-catalyzed chemiluminescent reaction according the manufacturer's instructions. Digital images of the membranes were captured using a Kodak Image Station 4000MM (Kodak, Rochester, NY) and viewed with molecular imaging software (GE Healthcare).

A 49-bp deletion in the *tDNA-leucine* gene of the chloroplast was used as a maternal marker (Missaoui et al., 2006). The wild-type state was present in ACP and the deletion state was present in Alamo. The product was amplified in Alamo (T85–2) and ACP parents, the F_1 offspring, and four F_1BC_1 offspring using the primers 5'-GGTAATGGAACTCCCTCGAAATTA-3' (forward) and 5'-GGACTCTCTCTTTATCCTCGTTCG-3' (reverse) at a final concentration of 0.5 μM and Phusion HF master mix (New England Biolabs). Polymerase chain reaction (PCR) conditions were 98°C for 2 min and 30 cycles of 98°C for 30 s, 64°C for 15 s, and 72°C for 20 s, followed by 72°C for 2 min. Products were visualized using 2% Agarose Resolute GPG (American Bio Inc., Natick, MA) gel and Sybr Green dye (Life Technologies, Carlsbad, CA).

A 202-bp region of the *bar* transgene was amplified in the Alamo (T85–2) and ACP parents, F_1 offspring, and four F_1BC_1 offspring using the primers 5'-ACTGGGCTCCACGCTCTA-3' (forward) and 5'-GAAGTCCAGCTGCCAGAAAC-3' (reverse) at a final concentration of 0.5 μM and Phusion HF master mix (New England Biolabs). Polymerase chain reaction conditions were 98°C for 2 min and 30 cycles of 98°C for 30 s, 62°C for 15 s, and 72°C for 20 s, followed by 72°C for 2 min. Productions were visualized using 2% Agarose Resolute GPG (American Bio) gel and Sybr Green dye (Life Technologies).

To confirm segregation of the *bar* transgene in an F_1BC_1 hybrid switchgrass population, PCR assays were performed on F_1BC_1 individuals. A 513-bp fragment was amplified in transgenic Alamo (T85–2), ACP wild-type, F_1, four HbS and four HbR F_1BC_1 offspring. For the positive control, 100 ρg of the transformation construct was used. The primers were 5'-GGATCTACCATGAGCCCAGA-3' (forward) and 5'-GAAGTCCAGCTGCCAGAAAC-3' (reverse). Samples were prepared via the Harris Unicore System (ThermoScientific, Waltham, MA) in conjunction with the KAPA 3G Plant PCR kit (KAPA Biosystems, Wilmington, MA) following the manufacturer's instructions for a crude sample (50 μL sample preparation). Polymerase chain reaction conditions were 95°C for 10 min and 30 cycles of 95°C for 30 s, 58 for 15 s, and 72°C for 30 s, followed by 72°C for 1 min. Products were visualized on 1.2% agarose gel.

GBS Library Preparation and Sequencing

Genomic DNA was isolated from leaf tissue using published methods (Chen and Dellaporta, 1994). Approximately 1 μg of gDNA from each sample was digested with RsaI (New England Biolabs) according to conditions recommended by manufacturer and GBS samples were processed for sequencing according to the method described (Heffelfinger et al., 2014). Atlantic Coastal Panicgrass and Alamo parents, F_1 offspring, and 83 F_1BC_1 samples were paired-end (2 × 75 bp) sequenced on one lane of an Illumina HiSeq 2000 system (Illumina, San Diego, CA) by the Yale Center for Genome Analysis.

Bioinformatics

Eighty-six samples consisting of male (Alamo) and female (ACP) parents, a *bar*-positive F_1 individual, and 83

F_1BC_1 offspring were sequenced on one Illumina HiSeq 2000 lane (Illumina) to a mean of 1965,020 ± 958,961 2 × 75-bp reads per sample. Reads were aligned against the draft *P. virgatum* version 1.1 reference genome (US Department of Energy Joint Genome Institute, http://www.phytozome.net/panicumvirgatum, accessed 6 Mar. 2015) (Goodstein et al., 2012) using Bowtie2 (Langmead and Salzberg, 2012) and variants were called using the mpileup function of Samtools (Li et al., 2009).

Genotyping markers were filtered on the basis of several criteria. Retained markers were (i) homozygous within and polymorphic between Alamo and ACP parents, (ii) heterozygous in the F_1 offspring, (iii) typed by at least two reads within parental plants and the F_1 offspring as well as in at least 20 F_1BC_1 offspring, (iv) aligned with a mapping quality of ≥20, and (v) a base call quality score of ≥30. These filtering steps were performed via custom Perl scripts.

Genotypes were imputed using the least-squares algorithm described in Heffelfinger et al. (2014). Briefly, variants were placed in 5-Mbp bins across the genome and a "mean genotype" was calculated. Proximal bins with matching genotypes were merged. Bins with differing calls were reanalyzed using a sliding, 1-Mbp window in the forward and reverse direction to identify breakpoints. Although only homozygous Alamo and heterozygous calls were possible, given the cross, homozygous ACP calls were maintained in the dataset to indicate the error rate and error-prone regions of the genome. Circos (Krzywinski et al., 2009) was used to display raw and imputed genome-wide marker datasets for parents, F_1, and offspring.

To estimate the recombination rate within the population, the total number of recombination events minus recombination events leading to a homozygous ACP state was determined. This value was then divided by size of the tested F_1BC_1 population (83) and multiplied by 100 to obtain the genetic distance in cM.

RESULTS

Analysis of Transgenics and Generation of F_1 and F_1BC_1 Populations

In a series of four transformation experiments using the Alamo embryogenic callus, 117 T_0 independent events were recovered and over 10 plants were regenerated per event. These primary T_0 Alamo regenerated plantlets were grown to maturity in the greenhouse. All T_0 plants that tested positive by PCR for the *bar* transgene exhibited no damage in leaf paint assays using 3% (v/v) Finale herbicide and were fertile as judged by seed set. T-DNA insertion number as estimated by Southern blot analyses showed (58.1%) likely single T-DNA insertion events, (12.9%) with at least two T-DNA insertions, and (29.0%) multiple copy events (data not shown). Transgenic line T85–2 was characterized as carrying a single T-DNA insertion (Supplementary Fig. 1).

Generation of the F_1 hybrid population used vegetative clones of the transgenic (HbR or *bar*-positive) T85–2 Alamo as paternal pollen donors to a wild-type (HbS or *bar*-negative) ACP in an isolated pollen cage. Recovered seedlings (F_1) were treated with 3% Finale and scored for the resulting resistant and sensitive plants after 7 d to reveal HbR (*bar*-positive) and HbS (*bar*-negative) populations. There were a total of 1684 F_1 seeds collected from the maternal ACP plant. Of the F_1 plants, 644 were HbR and 1040 were HbS. Plants were screened by Southern blot and only those with single gene insertions were selected. Of the F_1 selected for analysis, all plants showed good seed set with high rates of germination, indicating female fertility. Male fertility in the F_1 was tested indirectly by iodine potassium iodide staining. On the basis of this assay, only a low rate of defective pollen was detected (data not shown), suggesting that the F_1 was also male fertile. Control crosses yielded zero resistant plants. A subset of the hybrid F_1 plants was then backcrossed to paternal pollen donor wild-type Alamo to generate the F_1BC_1 progeny. Of the original 86 F_1BC_1 hybrid plants, 83 HbS survived transplantation and were used for genomic characterization (Fig. 1).

Characterization of Parental and F_1 Hybrid Plants

P. virgatum cv Alamo and *Pa. amarum* var. *amarulum* (ACP) are taxonomically described as different species yet the results here indicate that they cross to produce fertile offspring. Therefore, a characterization of parental and F_1 hybrid molecular and phenotypic characteristics was conducted to describe and confirm their differences and validate the F_1 as a hybrid. All Finale-resistant F_1 plants retained the HbR phenotype to floral maturity.

Plant morphological characteristics for the parental Alamo and ACP plants were compared with the F_1 hybrid. Multiple characteristics show individuation of Alamo, ACP, and the F_1 hybrid. Alamo is a taller plant with expanded panicles, ACP is shorter with compressed panicles, and the F_1 hybrid shows intermediate characteristics (Fig. 2A, B), except that the F_1 hybrid has increased tillering and expanded and smaller panicles than either parent. The inflorescence of Alamo has dispersed spikelets, ACP is compact, and the hybrid is intermediate (Fig. 2C). Individual spikelets of Alamo have deep red stigmas, ACP is white, and that of the hybrids is light red to pink (Fig. 2D).

Epicuticular wax patterns as observed via scanning electron microscopy on the adaxial surface of Alamo leaves are relatively smooth in comparison to ACP and the hybrid is intermediate (Fig. 2E). There are two types of trichomes on the surface on Alamo leaves: those comprised of a single cell and those with two cells, whereas ACP has only the two-celled trichomes. The hybrid leaves have only two-celled trichomes. All leaf observations and measurements were made on samples from the second flag leaf 2.5 cm from the axil during anthesis. Furthermore, the leaves of Alamo are a deep greenish-blue, whereas those of ACP have a distinctive bluish hue and the F_1 hybrid is intermediate in color under greenhouse conditions. The chlorophyll content of Alamo is similar to that of the hybrid and less than that of the ACP (Supplementary Fig. 2A), yet the leaf anatomy and bundle sheath appearance of these plants are similar (Fig. 2F).

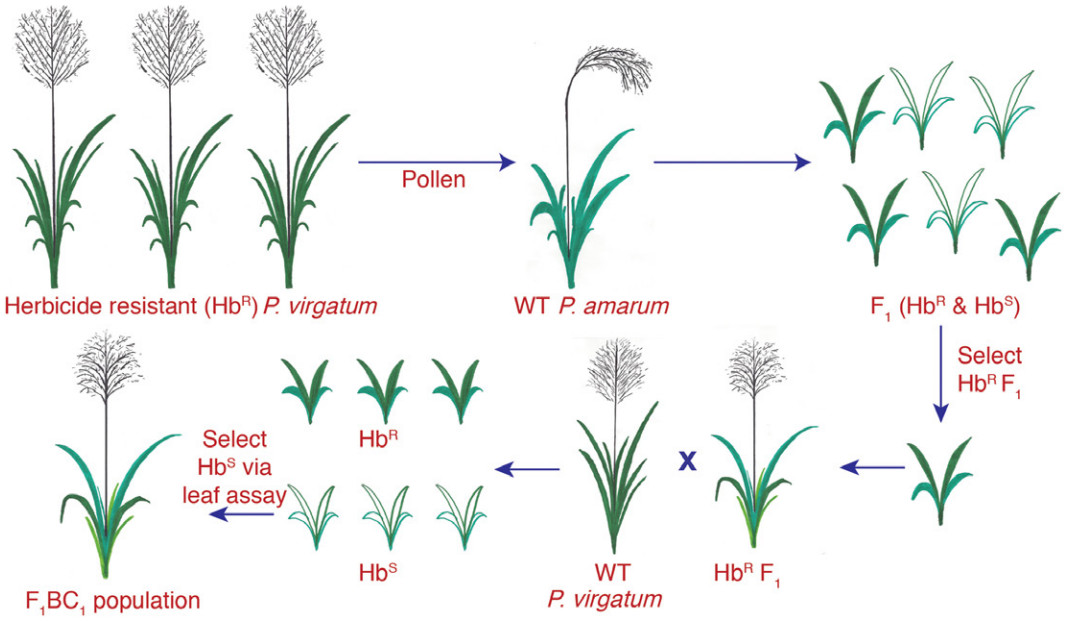

Figure 1. Recovery of wide interspecific crosses using transgenic herbicide selection. Three parental clones of a single transgenic Alamo switchgrass event (upper left) are herbicide-resistant (HbR, bar+) and used as pollen donors in pollen cages crossed with maternal wild-type Atlantic Coastal Panicgrass (ACP) (upper center). Seed is harvested only from the maternal ACP parent and germinated, providing a F_1 population consisting of herbicide-resistant (HbR, green) and herbicide-sensitive (HbS, white) seedlings (upper right). These are scored after 21 d and a single F_1 resistant individual is selected and grown to floral maturity. Wild-type Alamo plants are used as paternal pollen donors to this HbR F_1 (lower center). The resultant seed is recovered and germinated. The F_1BC_1 seedling population is treated with 3% (v/v) Finale using a "paint assay" and scored for herbicide resistant (bar+, green) and herbicide sensitive (bar–, white) populations (lower left).

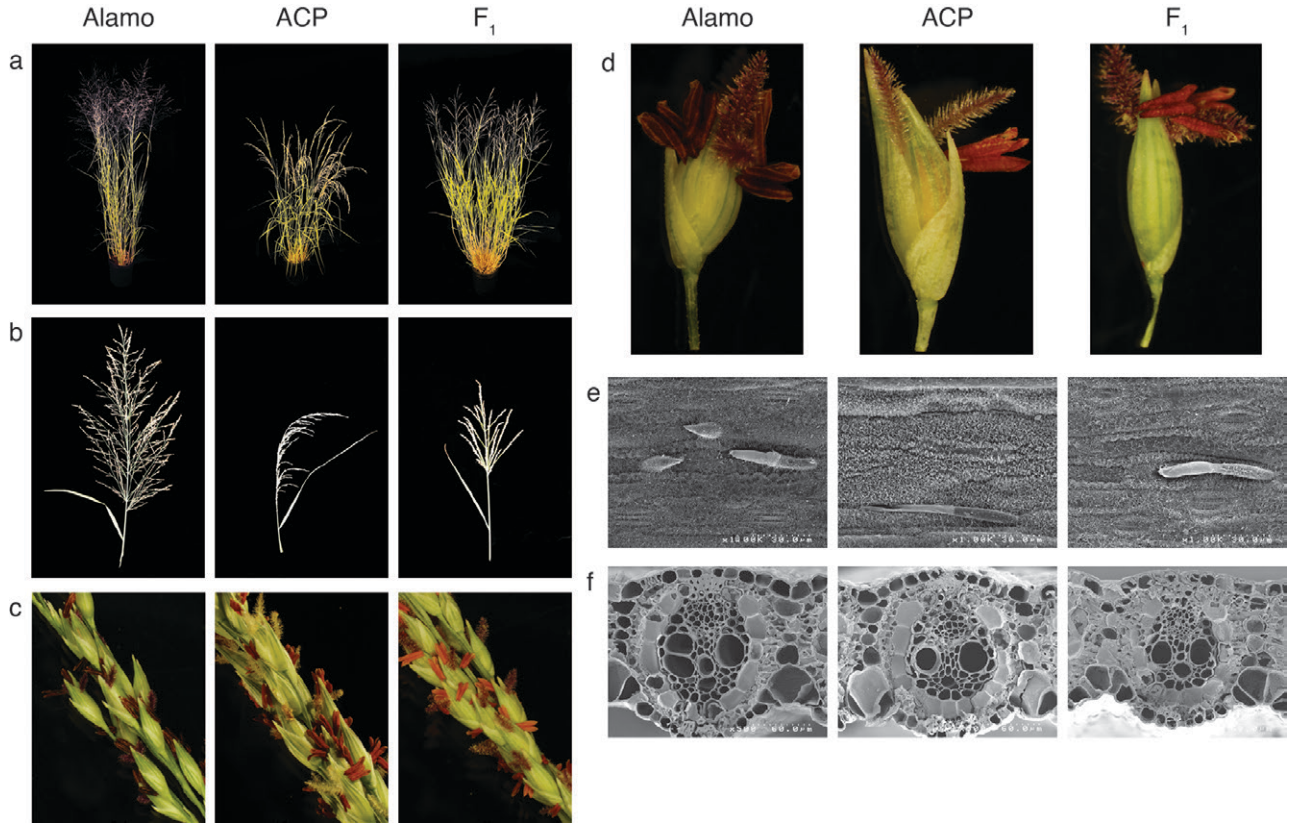

Figure 2. Parental and F_1 hybrid plant phenotypes. (A–F) *Pancicum virgatum* L. cv. Alamo (left), *Panicum amarum* Ell. var. *amarulum* [Atlantic Coastal Panicgrass (ACP), center], and the female ACP × male Alamo F_1 hybrid (right). (A) Plant morphology, (B) panicle architecture variation, (C) spikelet arrangement on the rachis, (D) spikelets with variations in stigma colors, (E) scanning electron microscopy of epicuticular wax patterns, and (F) scanning electron microscopy of bundle sheath appearance.

Additional phenotyped plant characteristics showing similarities and differences among both the Alamo and ACP parents and the F_1 hybrid include the rachis (Supplementary Fig. 2B), spikelet (Supplementary Fig. 2C), and seed set (Supplementary Fig. 2D) number per inflorescence. Adaxial (Supplementary Fig. 2E) and abaxial (Supplementary Fig. 2F) leaf stomata counts were also measured and were similar among the three plant groups.

Confirmation of *bar* Segregation in F_1BC_1 Offspring

Segregation of the *bar* transgene was confirmed in the F_1BC_1 offspring via PCR assay and Southern blot. In the PCR assay, the *bar* transgene was amplified in the HbR Alamo parent (T85–2), the HbS ACP parent, the HbR F_1 offspring, four HbS F_1BC_1 offspring, and four HbR F_1BC_1 offspring (Supplementary Fig. 3). The Southern blot was performed on the transgenic HbR Alamo parent, a wild-type HbS Alamo individual, a wild-type HbS ACP individual, the HbR F_1 offspring, four HbS F_1BC_1 offspring, and four HbR F_1BC_1 offspring (Supplementary Fig. 1). In both the PCR and the Southern blot assays, the *bar* transgene was only detected in HbR individuals.

Chloroplast DNA and *bar* Transgene Markers

A chloroplast DNA *tRNA-leucine* 49-bp deletion in Alamo, which is present in the wild-type state in ACP, served as a marker to confirm maternal contribution from ACP (Supplementary Fig. 4A) (Missaoui et al., 2006), whereas the presence of the *bar* transgene confirmed parental contribution from transgenic Alamo (Supplementary Fig. 4B). Polymerase chain reaction amplification of the 49-bp Alamo *tDNA-leucine* deletion identified the wild-type state in the ACP maternal parent, the F_1 offspring, and four F_1BC_1 offspring. The deletion state was only observed in the Alamo parent. The *bar* transgene was observed, however, in both the Alamo parent and the F_1 offspring. This confirms maternal inheritance of the chloroplast marker from the ACP parent and the inheritance of the HbR transgene from Alamo pollen donor in the F_1 hybrid. These data supported the identity of the F_1 as an interspecific ACP–Alamo hybrid.

Identification of Useful Markers

Key to confirming and quantifying the genomic contribution of the Alamo and ACP parents to the F_1BC_1 population was the development of a robust set of genetic markers from the GBS sequencing data. In total, 92.45% of the 170,553,076 paired-end 75-bp reads were successfully mapped to the version 1.1 assembly of the *P. virgatum* genome across all samples. Variants were identified using Samtools mpileup (Li et al., 2009) and filtered on the basis of homozygous, differing inter-parental calls and heterozygosity in the F_1. Furthermore, markers were required to be called by a minimum of two reads in at least 20 F_1BC_1 samples and have a mapping quality of at least 20 and a base call quality score of at least 30. By these criteria, a total of 3646,353 variant calls resulted in 35,170 filtered markers (Supplementary Table 1). All samples contained at least 3779 called markers, with a per-sample mean of 17,732 (SD = ±6484.2) markers.

Postimputation Genotypes

Genotypes were imputed using a least-squares methodology from the postfilter ordered variants in each F_1BC_1 offspring (Fig. 3A) as described (Heffelfinger et al., 2014). Regions of the genome were called either homozygous Alamo, homozygous ACP, or heterozygous. Because of the nature of the F_1 backcross to Alamo, only homozygous Alamo and heterozygous genotypes were possible; nevertheless, homozygous ACP calls were useful for identifying the error rate and error-prone regions of the genome. Observation of the postimputed genotypes identified recombination in all F_1BC_1 samples (Fig. 3B). The number of detected recombination events was 1341 for a total genetic distance of 1615.7 cM.

Atlantic Coastal Panicgrass's and Alamo's Genetic Contribution to the F_1BC_1 Offspring

The contribution of the ACP and Alamo parents was measured in the F_1BC_1 offspring after imputation. The contribution from the ACP parent across all F_1BC_1 individuals was found to be 31.62% (SD = ±6.35%) (Supplementary Fig. 5). This was slightly above the expected value of 25%. The fraction of samples with a heterozygous call tended to be slightly higher than the fraction of samples with an Alamo homozygous call in many regions of the genome. Furthermore, some regions of the genome, such as most of chromosome 6a and parts of 7b and 9a were greatly enriched for heterozygous markers (Fig. 4). This may be evidence of segregation distortion and it was expected that at least one region would be entirely heterozygous because of selection against the *bar* insertion site coming from the Alamo parent.

The fraction of samples with a postimputation ACP homozygous call for a region was also considered. Regions with a homozygous ACP call tended to be small and proximal to the telomeres. No region contained an ACP homozygous fraction >20%; in most, it was <10% or absent entirely. The total ACP homozygous fraction was 1.92% across samples. Although a true homozygous ACP genotype was not possible in the F_1BC_1 offspring, such calls may be indicative of regions prone to errors in genotyping.

Identification of the *bar* Transgene from Sequencing Data

Sequencing data were used to test for the presence of the *bar* transgene and plasmid backbone in parents, F_1, and F_1BC_1 samples. To do this, sequences ranging from 34 to 61 bp that flanked RsaI (New England Biolabs) restriction sites in the transgene and plasmid backbone were queried against the sequencing datasets from all samples (Supplementary Table 2). Sequences originating from the transgene were positively identified only in the transgenic Alamo parent (10 reads) and F_1 offspring (11 reads). No sequence from the plasmid backbone was identified

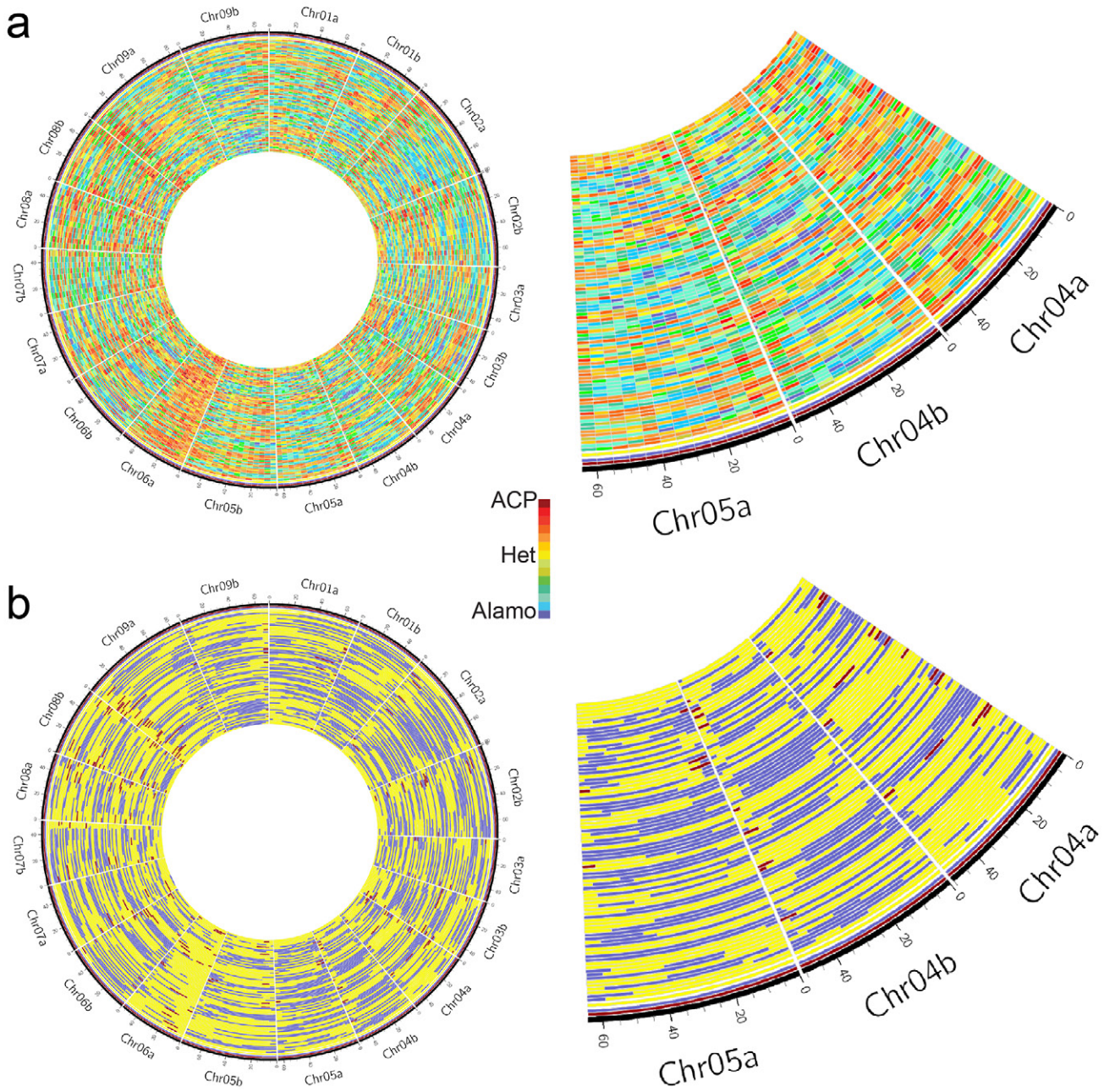

Figure 3. Parental genetic contribution in a switchgrass F_1BC_1 population. The genome-wide parental contribution to 83 F_1BC_1 offspring was measured using 26,131 markers. Outermost to innermost, the rings represent the ACP parent, the Alamo parent, F_1 offspring, and finally the F_1BC_1 offspring. Graphing was done via Circos. (A) Markers were grouped into 5-Mb bins and mean genotype was determined. Warm heat map colors indicate high ACP contribution and cool colors indicate high Alamo contribution. (B) Genotypes were imputed via a least-squares methodology, with recombination breakpoints resolved via a forward and reverse five-marker sliding window across the recombination interval.

in any individual. No sequence from the *bar* transgene was found in any HbS F_1BC_1 individual.

DISCUSSION

Switchgrass and its relatives have the potential to be important bioenergy crops as feedstock, yet germplasm improvement remains a critical barrier to this goal. The development of improved cultivars and hybrids would benefit from the ability to combine the genetic variation of intervarietal ecotypes as well as related species. The ultimate goal would be to expand the pool of available genetic resources further through the identification and introgression of valuable biofuel traits. Here we demonstrate the recovery of an interspecific crosses between transgenic herbicide-resistant *P. virgatum* cv. Alamo switchgrass and wild-type *P. aramrum* var. *amarulum* (ACP) and the subsequent generation of a fertile admixture population.

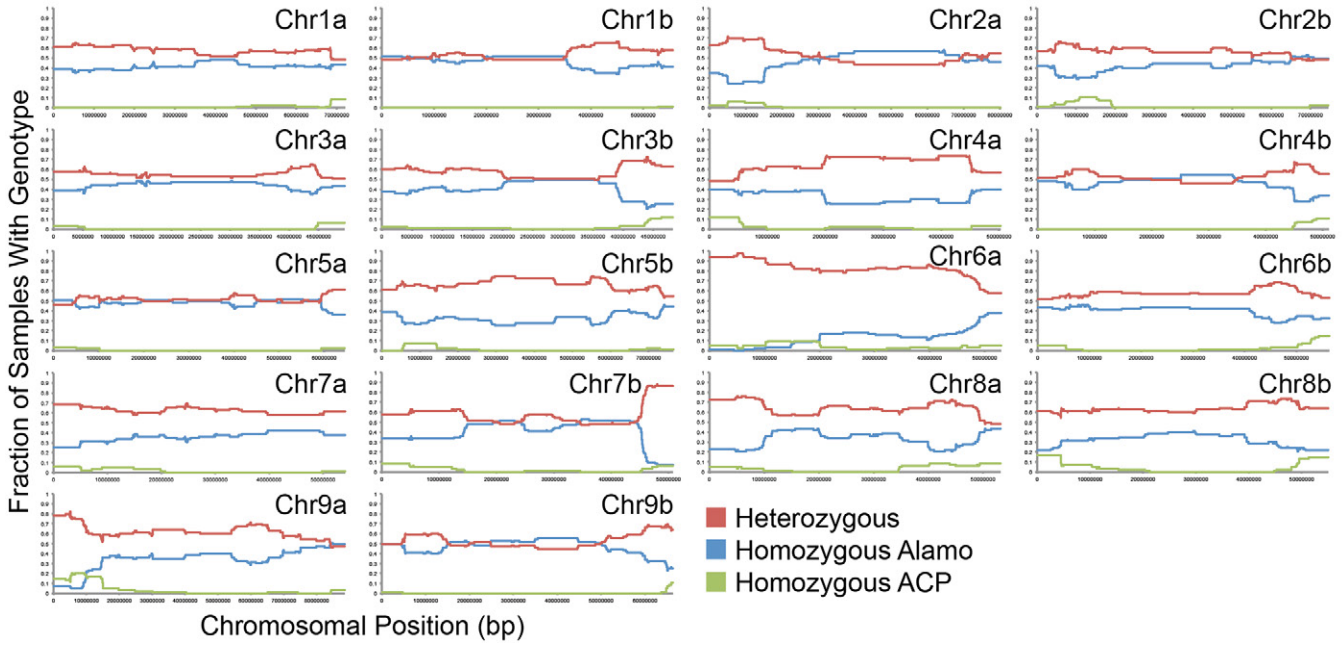

Figure 4. Genotype fractions in F_1BC_1 offspring across the switchgrass genome. The F_1BC_1 fraction of each postimputation genotype (heterozygous, homozygous Alamo, and homozygous ACP) was determined across the entire switchgrass genome. Homozygous ACP calls, though not possible due to the nature of the cross, are retained to identify error-prone regions.

Phylogenetics in Switchgrass and Related Species

There are approximately 500 species in the genus *Panicum* (Huang et al., 2011). Genomics on switchgrass and its relatives is relatively recent (Lu et al., 2013), yet there has been considerable clarification in the phylogenetic and taxanomic relationships among species from genomic studies (Casler et al., 2007, 2011; Huang et al., 2011; Lu et al., 2013). More recently, switchgrass genomic diversity, ploidy, and evolution were explored using a network-based SNP discovery protocol (Lu et al., 2013). These studies yielded a linkage map, an EST database, and a set of SNP markers across 18 linkage groups and bacterial artificial chromosome libraries. These results found that the switchgrass genome is highly syntenous with the genomes of rice, sorghum (*Sorghum bicolor* (L.) Moench), and *Brachypodium distachyon* (L.) P.Beauv. (Casler et al., 2011) and illustrate isolation by distance and by ploidy between switchgrass populations (Lu et al., 2013).

Phylogenetic analyses (Lu et al., 2013) of switchgrass indicate a tendency of south-to-north migration in North America. Ploidy levels vary within switchgrass ecotypes (Hopkins et al., 1996; Lu et al., 1998; Narasimhamoorthy et al., 2008), ranging from diploid ($2n = 2\times = 18$) to duodecaploid ($2n = 12\times = 108$) (Nielsen, 1944). All lowland ecotypes have been identified as tetraploids ($2n = 4\times = 36$); upland types can be tetraploids or octaploids ($2n = 8\times = 72$) (Hultquist et al., 1996). Mixed ploidy levels among accessions and within cultivars have also been observed (Narasimhamoorthy et al., 2008). Both geographic isolation and sexual incompatibility related to ploidy have resulted in both varietal and species-specific diversification. The switchgrass accession used in this study, Alamo, is a tetraploid member of the lowland ecotype (Serba et al., 2013). Using sequence-related amplified polymorphism and EST–simple sequence repeat markers Huang et al. (2011) shows that *P. amarum* is a sister taxon to *P. virgatum*. Close genetic proximity and multiple abiotic stress resistance and yield traits result in them being excellent candidates for hybridization.

Interspecific F_1 hybrids between HbR transgenic Alamo switchgrass and wild-type ACP were generated in this study. Phenotypic and molecular comparisons identified clear differences between the parents themselves and between the parents and F_1 offspring, indicating the likely origin of F_1 and $F1BC_1$ individuals as interspecific hybrids. Molecular and genomic analysis of the F_1 offspring indicated the presence of an ACP-specific maternal chloroplast allele and the paternal *bar* transgene, providing further confirmation of the F_1 hybrid genome. A hybrid F_1 plant was backcrossed to the wild-type Alamo and an F_1BC_1 population of 83 herbicide-sensitive progeny was recovered. All F_1BC_1 progeny were identified as backcross hybrids by genotyping, demonstrating the robustness to this approach for screening. Phenotypically each of these individuals exhibited unique characteristics and were stable to floral maturity. As predicted, the majority of the HbS plants no longer contained the *bar* transgene as evaluated by PCR assay and sequencing. With the application of further testing for transgenic sequence in select F_1BC_1 individuals, this method may allow the recovery of nontransgenic and, arguably, offspring that are not genetically modified organisms (GMOs) from wide crosses. This method provides a proof of concept for

efficient selection of interspecific hybrids using a selectable transgenic as an intermediate. It is also likely that this approach will also extend to intervarietal crosses.

Accuracy of Variant Identification and Imputation

Although well-established methods for molecular marker analysis have been described previously for the development of mapping populations (Missaoui et al., 2005; Okada et al., 2010) and phylogenetic comparisons (Gunter et al., 1996; Hultquist et al., 1996; Tobias et al., 2005, 2006, 2008), here, we have applied the GBS approach for marker identification and data analysis as previously described (Heffelfinger et al., 2014). The major strength of this method is that it allows a considerably higher resolution of recombination via a denser panel of markers than traditional nonsequencing-based methodologies. Although the results quantitatively assess the hybrid contribution of both parents and identify patterns of recombination in the F_1BC_1 individuals, questions over the rate of error remain.

Beyond the specific concerns of variant identification with GBS and low coverage sequencing in general (Elshire et al., 2011; Heffelfinger et al., 2014), the switchgrass reference genome presents additional problems. The current state of the *P. virgatum* reference assembly is scaffolded contigs with synteny established against the related species *P. hallii* genome (*P. virgatum* version 1.1, http://www.phytozome.net/panicumvirgatum, accessed 6 Mar. 2015). Compounding this difficulty is that switchgrass is an allotetraploid, resulting in a highly repetitive genome. The result of this is that few variants achieve a high mapping quality (>30); of those that do, the possibility of misalignment remains because of the draft nature of the assembly.

To resolve confounding issues as best as possible, a stringent set of variant filtering metrics was applied to retained markers. These included that variants were required to be homozygous within the parents, polymorphic between the parents, and heterozygous in the F_1 offspring. The requirement that all three possible marker states had to be observed reduced the likelihood of PCR, sequencing, and mapping artifacts. No expectation for allele frequency or segregation was applied to the F_1BC_1 however, as segregation distortion would be masked.

Another concern, not related to the reference genome but caused instead by the high degree of multiplexing, was false homozygosity. False homozygosity results when only one allele of a heterozygous site is observed in the sequencing data. False homozygosity was primarily solved through the imputation and error correction method, which is relatively insensitive to a single genotype call and instead determines a regional genotype based on a "mean" value from a set of calls (Heffelfinger et al., 2014). As long as homozygous miscalls did not randomly skew toward ACP or Alamo for a given region, the rate of erroneously imputed homozygous sites should be low.

A partial estimate of this error rate can be obtained from the amount of the genome across F_1BC_1 individuals called as ACP homozygous. Due to the nature of the cross, this genotype state is not physically possible, but may nonetheless result from false homozygous calls. Across all samples, approximately 1.92% of the genome is called as ACP homozygous. Assuming this error rate results in the same percentage of the genome being miscalled as Alamo homozygous, the total postimputation error rate caused by false homozygosity is under 4%. In reality, the percentage of the genome miscalled as Alamo homozygous or heterozygous is probably higher than 4%, however, as erroneously mapped reads may result in regions of the genome being "placed" incorrectly, even if the genotype is technically correct.

Evidence of Segregation Distortion in the F_1BC_1 Offspring

A primary concern in interspecific crosses is the segregation distortion caused by genetic incompatibility. If such forces were active, one would expect ACP alleles to be selectively lost in the F_1BC_1 because of backcrossing to the Alamo parent. Such regions with a fixed homozygous Alamo state would present severe obstacles to introgressive hybridization and trait mapping because of a local lack of ACP genetic variation and recombination. However, when the data were analyzed for such regions, there was no evidence of any segregation distortion favoring a homozygous Alamo state in the postimputation F_1BC_1 offspring.

Instead, the results suggested segregation distortion toward the heterozygous state in some regions of the genome. One region of heterozygosity was expected because of the selection against herbicide resistance. Selection for herbicide sensitivity would cause the T-DNA insertion site from the Alamo genome to be absent in all F_1BC_1 individuals. Nevertheless, other regions of fixed heterozygosity may represent segregation distortion in favor of alleles from the non-backcross ACP parent. Switchgrass exhibits both prefertilization and postfertilization self-incompatibility systems (Martinez-Reyna and Vogel, 2002, Martinez-Reyna and Vogel, 2008). Gametophytic self-incompatibility in grasses is controlled by two loci, S and Z (Lundqvist, 1962). In switchgrass Martinez-Reyna and Vogel (2002) showed that in controlled octoploid × octoploid, octoploid × tetraploid, and tetraploid × octoploid crosses, postfertilization abortion occurs in many cases, 20–40 d after pollination. On the basis of this study, self-compatibility is estimated to be between 0.35 and 1.39%. None of the self-compatibility genes have been cloned (Aguirre et al., 2012).

The mean fraction of the genome with the ACP allele across all offspring is slightly higher than expected (~25%) at 31.62% (SD = ±6.35%). Furthermore, although across most of the genome, the fraction of offspring with a heterozygous call is similar to that with an Alamo homozygous call, there are several regions with varying degrees of heterozygous enrichment. This enrichment is especially pronounced on chromosome 6a and on parts of 9a and 7b. Chromosomes 4a, 5b, 8a, and 8b show more modest levels of heterozygous enrichment. One of these regions is certainly due to artificial segregation distortion

caused by selection against *bar* in the F_1BC_1 offspring. Chromosome 6a is the most likely candidate, because of the absence of any homozygous Alamo calls in any of the offspring near the telomere. Self-incompatibility loci may be responsible for the other regions showing significant segregation distortion.

Recombination Observed in All F_1BC_1 Offspring

Across all 83 F_1BC_1 individuals, a total of 1341 recombination events were observed for a total genetic distance of 1615.7 cM. This estimate reflects female recombination in the F_1 and is close to previous estimates of the genetic distance of Alamo switchgrass: 1733 cM (female) (Serba et al., 2013) and 1645 cM (male) (Okada et al., 2010). Other estimates from non-Alamo cultivars are 1376 cM (Okada et al., 2010), 1508 cM (Serba et al., 2013), and 2085 cM (Liu et al., 2012). The estimate of 1761.4 cM is likely to be slightly high, however, because of several sources of error within this dataset (Hackett and Broadfoot, 2003).

The first source of error was false homozygosity as a result of low sequencing coverage, resulting in heterozygous regions incorrectly called as ACP or Alamo homozygous. This source of error is common within GBS datasets (Elshire et al., 2011; Heffelfinger et al., 2014). Even though all spurious recombination events involving an ACP homozygous event were removed, spurious Alamo homozygous recombination events were not detectable as errors and were probably retained. On the basis of the number of ACP homozygous calls, there may be as many as ~350 regions incorrectly called as homozygous Alamo.

A second source of error is the early state of the reference genome assembly and the highly repetitive allotetraploid nature of Alamo switchgrass itself. Unlike the markers used in the other switchgrass linkage studies (Liu et al., 2012; Okada et al., 2010; Serba et al., 2013), the markers used in this study were ordered by position on a draft reference genome. This makes our estimate of genetic distance sensitive to errors in the reference genome. The current switchgrass reference genome consists of contigs scaffolded by synteny with the related species *P. hallii* genome (*P. virgatum* version 1.1, http://www.phytozome.net/panicumvirgatum, accessed 6 Mar. 2015). The early state of the reference probably resulted in misplaced contigs and unannotated paralogous artifacts. Both of these events may contribute spurious recombination events.

In spite of these potential sources of error, the observed recombination rate remains well within the expected range. Recombination primarily occurs distal rather than proximal to the centromeres, as has been reported in other species (Akhunov et al., 2003; Tanksley et al., 1992). A detailed map of recombination frequency across the genome of switchgrass would be desirable on the publication and release of the reference genome.

The Question of Nontransgenic F_1BC_1 Offspring

The ability to use transgenics to identify hybrids rapidly would accelerate the breeding process and presents clear advantages over extensive phenotyping and genotyping-based hybrid identification. These advantages may be offset by the increased regulatory difficulties presented by the use of transgenic herbicide resistance. Although the transgenic Alamo parent and the F_1 hybrid are clearly GMOs, the F_1BC_1 population is more difficult to classify. There are two key aspects to the question of the F_1BC_1: whether the plants themselves contain transgenes and whether the absence of a transgenic sequence is sufficient to call them non-GMO when a transgenic sequence was present in the earlier generation.

Selection against *bar*-positive HbR F_1BC_1 offspring should produce a nontransgenic population. Multiple assays suggest that many of the offspring are hybrids with no evidence of transgenic DNA in them. There remains the possibility that gene silencing through methylation or structural polymorphism could disrupt *bar* resistance while maintaining all or part of the transgene. These individuals could be easily identified by a simple PCR assay for the presence of the transgene. The absence of transforming DNA in the genome of the F_1BC_1 individual, however, does not address whether these plants are considered to be non-GMO from a regulatory perspective. Ultimately, it is not likely to be a scientific question but a legal one as to whether this is sufficient to consider these offspring to be non-GMO.

Future Directions

Our results are potentially broadly applicable because they demonstrate the production and recovery of interspecific hybrids using a transgenic (GMO) selectable marker. This approach can be accomplished and applied to most major crop plants and may be useful for expanding breeding opportunities, including making rapid intervarietal crosses for conferring disease and other abiotic resistance traits, interspecific crosses for the combination of broad QTL characteristics, and creating close intergeneric crosses. Such a platform could serve as a basis for combining desirable characteristics by exploiting additive genetic variation and provide a more timely approach to developing novel lines in various crop species.

In addition to the creation of novel hybrid populations, phenotyping and QTL mapping on the extant ACP–Alamo hybrid demonstrated in this study remains a priority. Genotyping-by-sequencing identifies and types markers in a single experiment, thus providing not only a detailed picture of parental contribution and local ancestry but also a framework for downstream genomics applications. A key argument for switchgrass as a bioenergy crop is its ability to thrive in marginal environments (Casler, 2012; Casler et al., 2011; Moser and Vogel, 1995), the introgression of abiotic resistance into commercial varieties would be desirable. Follow-up studies will combine phenotyping of the hybrid population with the marker framework from the GBS dataset to identify and introgress novel trait loci.

CONCLUSIONS

The absence of recombinant inbred lines, well defined breeding strategies, and the inability to combine genomes from related cultivars and species, as exemplified in switchgrass, is a significant barrier to trait mapping and the development of novel germplasm. We show the use of a viable selectable herbicide resistance marker (*bar*) for recovery of interspecific hybrid offspring of transgenic Alamo switchgrass and ACP. Backcrossing to a wild-type parent and selecting against the marker provided a hybrid population. Using improved GBS applications, the results show the hybrid contribution of both parents and the lack of a transgenic sequence in many of the offspring. The entire genomes of both the Alamo and ACP parents are represented across the 83 F_1BC_1 individuals, making this admixture population highly suited to trait mapping and introgression. These results provide the basis for a population breeding strategy in switchgrass and other crops.

The use of transgenic selectable markers provides a useful solution for identification and recovery of embryonic hybrids without time-consuming phenotyping or marker-based validation. The approach we have demonstrated for recovery of F_1 hybrids followed by removal of the transgene by backcrossing to a wild-type parent and selection against the marker may have extended applications for producing heterogeneous breeding and production field populations. This approach may prove especially useful in crosses where a high rate of selfing is expected, where molecular markers for hybrid identification are unavailable, or where phenotyping is insufficient to identify the hybrid.

This platform could serve as a method for combining desirable QTLs by exploiting additive genetic variation and provide a more timely approach to developing novel hybrid populations in various crop species. The method demonstrated here presents a viable approach for the rapid creation of new hybrids using transgenic markers. Selection against the resistance marker, alongside appropriate screening and analysis, may further allow for the creation of non-transgenic hybrid populations. In addition, this approach may be applicable for removal of the transgene from populations where genome editing functions are the goal.

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