Euphytica (2020) 216:95
https://doi.org/10.1007/s10681-020-02635-5

Introgression of the *bmr6* allele in biomass sorghum lines for bioenergy production

Michele Jorge da Silva · Cynthia Maria Borges Damasceno · Cláudia Teixeira Guimarães · Marcos de Oliveira Pinto · Beatriz de Almeida Barros · José Eustáquio de Souza Carneiro · Robert Eugene Schaffert · Rafael Augusto da Costa Parrella

Received: 25 March 2020 / Accepted: 22 May 2020 / Published online: 30 May 2020

Abstract *Sorghum bicolor* (L.) *Moench* is a crop that has high potential to be used for bioenergy generation. The objective of this study was to introgression of the *bmr6* allele in elite lines of biomass sorghum and to obtain experimental "brown midrib" hybrids. Three genetic materials belonging to the Embrapa Maize and Sorghum Breeding Program were used. Two backcross programs were conducted separately, in which the CMSXS170 line was the donor of the *bmr6* allele and the CMSXS652 and IS23777 lines were the recurrent ones. Through molecular markers specific for the *bmr6* allele, the assisted selection was utilized for the brown midrib characteristic in the BC_1F_1 and BC_2F_1 generations. Polymorphic SNP markers were distributed throughout the genome of sorghum to accelerate the recovery of the recurrent genome. After the confirmation of the bmr genotypes, the lines were crossed with line A (female), to obtain the hybrid seeds and evaluated under field conditions. As result, it was possible to perform the introgression of the *bmr6* allele in lines of biomass sorghum. The SNPs markers were efficient in identifying individuals with a higher rate of recurrence of the recurrent genome. Experimental "brown midrib" hybrids were obtained and demonstrated satisfactory potential for bioenergy production.

Keywords Ethanol · Lignin · Recurrent · SNPs · *Sorghum bicolor* (L.) *Moench*

M. J. da Silva (✉)
Departament of Biology, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil
e-mail: michelejorgesilva@gmail.com

C. M. B. Damasceno · C. T. Guimarães · M. de Oliveira Pinto · B. de Almeida Barros · R. E. Schaffert · R. A. da Costa Parrella (✉)
Embrapa Maize and Sorghum, Sete Lagoas, Minas Gerais, Brazil
e-mail: rafael.parrella@embrapa.br

J. E. de Souza Carneiro
Departament of Plant Sciences, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil

Introduction

Sorghum bicolor (L.) *Moench* is a crop that has high potential to be used for bioenergy generation. The crop has a short cycle (150–180 days), wide adaptability, seed propagation, fully mechanized production, and high productivity, which reaches 150 t/ha of green mass (da Silva et al. 2020; Oliveira et al. 2019). Other advantages include its tolerance to drought, an established agricultural production system, good aptitude for tropical and temperate regions and its biomass can be used in direct combustion (second-generation bioethanol) to generate energy (Parrella et al. 2010).

Sorghum is primarily self-pollinated but can also accept pollen from other sorghum plants (House 1985). The discovery of the cytoplasmic genetic

male-sterility system based on the milo-kafir system was a milestone in sorghum breeding and research (Stephens and Holland 1954) to enabled the development of commercial hybrid seeds. Also, it is widely used in the commercial exploitation of heterosis. In the generation of sorghum hybrids, three types of lines, called A, B and R (House 1985) are required. The lines A and B are isogenic and differentiate only by the cytoplasm: line A has a cytoplasm that confers the male-sterility phenotype when associated with recessive nuclear genes for fertility restoration, and line B has normal cytoplasm and therefore, the plant exhibits the fertile male part, even with nuclear recessive alleles for fertility restoration (Smith and Frederiksen 2000). Thus, the hybrid is obtained from the cross between a male-sterile A (female) line, with an R (restorer) line that has dominant alleles for fertility restoration genes.

The brown midrib (bmr) mutation of sorghum leads to decreased lignin content and altered lignin composition (Barrière et al. 2007; Saballos et al. 2009). Phenotypically, the presence of the bmr gene is characterized by brown coloration in mid-leaf veins in the sorghum plant. The reduction of the lignin content represents a positive impact on the conversion of bmr biomass sorghum into simple sugars, which makes the second-generation ethanol production process more efficient. Brown midrib sorghums, both forage and biomass types, were shown to exhibit improved sugar conversion into ethanol of their biomass compared to conventional sorghums (Cotton et al. 2013; Dien et al. 2009). Therefore, the development of sorghum cultivars with lower lignin accumulation is an important strategy for bioenergy production (Corredor et al. 2009; Anderson and Akin 2008).

Sensitivity to photoperiod is also a trait of interest for biomass sorghum. Biomass sorghum plants susceptible to photoperiod when sown in September or October in regions tropical with photoperiod greater than 12 h and 20 min, will initiate the development of the floral only from March 21 of the following year, increasing the vegetative cycle and, simultaneously, allowing biomass production per hectare/cycle in comparison to cultivars insensitive to photoperiod (Parrella et al. 2010; Rooney and Aydin 1999).

The backcrossing method is used to improve elite genotypes, in the traits in which they are deficient, through the crossing with genotypes carrying the traits that one wishes to introduce. And to accelerate the backcrossing process, selection assisted by molecular markers is a strategy that has been widely used, consisting of the use of markers to follow the introgression of the locus of interest (Frisch and Melchinger 2005).

The molecular genotyping of the individuals allows the selection of those more similar to the recurrent genotype and with better conversion in the region close to the introduced gene. Among the most current methodologies, we have the competitive allele specific PCR (KASP®, Kompetitive Allele-Specific PCR), which allows the genotyping with SNP (Single Nucleotide Polymorphism) markers, whose analysis shows high specificity and sensitivity (Openshaw et al. 1994). The KASP, technology uses two specific forward allele primers containing a specific tail primer for each of them, a common reverse primer, and specific probes to ring in the complementary sequence to the tail of the primers, containing different fluorophils (FAM and HEX). After the synthesis of the tapes, regions complementary to the tails of the specific allele primers are generated. The probes, which are normally bound to a quencher, bind to these complementary regions, enabling them to emit fluorescence (LGC Group®).

In view of the above, the objective of this study was to introgression of the *bmr6* allele in elite lines of biomass sorghum, to assist the development of hybrids of biomass sorghum with lower lignin content and therefore with great potential for the bioenergy production.

Materials and methods

Genetic materials

To assist assisted introgression in aiming the development of biomass sorghum hybrids with lower lignin content, three genetic materials from the Embrapa Maize and Sorghum Genetic Breeding Program were used. Two backcrossing programs were conducted separately, in which the CMSXS170 line was the donor of the *bmr6* allele, which is a small, graniferous photoperiod insensitive line, and the CMSXS652 and IS23777 lines were the recurrents, as they are elite materials, with high biomass production and photoperiod sensitive.

The backcrossing program

The crossings between the donor parental (DP) CMSXS170 and the two recurrent parental (RP), CMSXS652 and IS23777 were performed to obtain F_1 generation (Fig. 1), which were backcrossed with the respective recurrent parent to obtain the first generation of backcross (BC_1F_1). A second backcrossing cycle (BC_2F_1) was performed, followed by a self-fertilization cycle, generating the BC_2F_2 generation. Molecular markers were used to identify heterozygous and most similar individuals with recurrent parenting in the BC_1F_1 and BC_2F_1 generations (Fig. 1).

DNA extraction

After obtaining the BC_1F_1 and BC_2F_1 generations, plant samples were collected in the greenhouse. Sorghum seeds were sterilized with 0.525% sodium hypochlorite for 10 min under constant agitation. Then the seeds germinated in Petri dishes containing germination paper moistened with distilled water and stored in a growth chamber with an mean daytime temperature of 27 ± 3 °C, a nighttime temperature of 20 ± 3 °C, and a 12-h photoperiod. After 2 days, the seedlings obtained were transplanted to Styrofoam trays containing substrate in a greenhouse. After 2 weeks, four 0.5 cm leaf tissue discs were collected and subjected to lyophilization for 48 h and then used to isolate genomic DNA, according to the method described by Saghai-Maroof et al. (1984). Then DNA was quantified by NanoDrop 1000 (Thermo Fisher Scientific®, Waltham, MA) and diluted to 30 ng/μL use concentration.

Use of CAPS markers in the backcross generations

Through molecular markers specific for the bmr6 allele, assisted selection for the brown midrib characteristic in the BC_1F_1 and BC_2F_1 generations was employed. After DNA extraction, a PCR reaction was performed, according to Sattler et al. (2009) with primers specific for the bmr6 allele. The amplification products were then cleaved with a specific restriction enzyme (BsaAI) to the bmr6 mutation site. The amplified and cleaved DNA fragments were further separated by agarose gel electrophoresis and visualized for identification of the analyzed genotype *Bmr6/Bmr6*; *Bmr6/bmr6; bmr6/bmr6*.

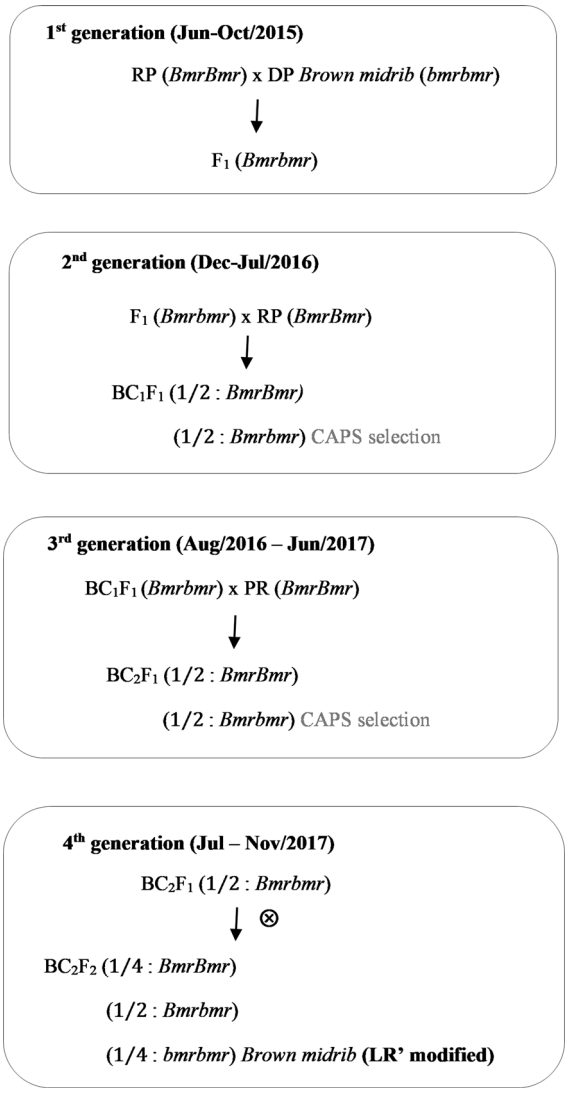

Fig. 1 Biomass sorghum backcrossing program with the recurrent (RP) and donor (DP) parentals

Use of SNPs markers to accelerate recovery of recurrent lines

DNA extracted from each plant was diluted to the use concentration of 10 ng/μL. Polymorphic SNPs markers distributed throughout the sorghum genome were used to accelerate the recovery of the recurrent genome. The total of 99 KASP (Kompetitive Allele-Specific—PCR, LGC Genomics®) SNP markers were screened between the parental lines to identify polymorphic markers and 42 polymorphic primers and lines were identified for CMSXS652 and IS23777 40

polymorphic primers were identified. For the analysis of BC_1F_1 generation individuals, 16 polymorphic markers were selected from the CMSXS652 parent line and 14 polymorphic markers from the IS23777 parent line. For generation BC_2F_1, 11 polymorphic markers were selected from the CMSXS652 parent line and 9 polymorphic markers from the IS23777 line. Thus, total of 27 markers for CMSXS652 and 23 for IS23777, homogeneously distributed in the sorghum genome. Heterozygous markers in BC_1F_1 generation plants were reevaluated in BC_2F_1 individuals.

After selecting the polymorphic markers in each generation of backcrossing, genotyping was performed between the parents and the line plants, based on the KASP. The amplification reaction was performed with 3 µL of Kasp Master Mix, 30 ng of DNA and 0.084 µL of Kasp Assay Mix containing the primers. Amplification cycles were: initial denaturation at 94 °C for 15 min, followed by 10 cycles at 94 °C for 20 s, 61 °C for 1 min reducing 0.6 °C per cycle, followed by another 26 cycles of 94 °C for 20 s and 55 °C for 1 min. The fluorescence intensity of the samples was quantified using the FLUOstar Omega® Filter-based multi-mode microplate reader (BMG Labtech, Ortenberg, Germany) microplate reader using ROX in signal normalization. Genotyping was performed using KlusterCaller® 1.1 software (LGC Genomics, Teddington, England).

Molecular data analysis

To evaluate the genotypic segregation of populations in BC_1F_1 and BC_2F_1, the proportions obtained were compared with expected proportions (1:2:1) by the Chi square test (X^2). The GENES software (Cruz 2016) was used to perform the analyses. Then, to verify the recovery proportion of the recurrent parent, molecular data were organized by coding the homozygous loci for the recurrent line as "A" and those in heterozygous as "H" (Benchimol et al. 2005). Thus, % recurrent parent recovery (RR) was calculated using the following expression 1:

$$\%RR = [A + (0.5H)/(A + H)] \times 100 \quad (1)$$

Confirmation of genotype predicted by CAPS marker

The heterozygous (*Bmr6*/*bmr6*) genotypes identified in the generation of BC_2F_1 were self-fertilized to obtain BC_2F_2 individuals. Thus, it was possible to confirm the genotype observed by the classification as segregating (from a heterozygous plant *Bmr6*/*bmr6*) and non-segregating (from a homozygous plant *Bmr6*/*Bmr6*).

Brown midrib line multiplication and obtaining experimental hybrids

After confirming the obtention of brown midrib genotypes (*bmr6*/*bmr6*), they were cross-bred with line A (BR008A *bmr*) to obtain hybrids seeds, i.e. materials with the introgression of the *bmr6* allele. Tests were performed to compare normal line and brown midrib, with an evaluation of several agronomic traits of interest, such as flowering, plant height, stand, stem diameter, lodging, total green mass production, and total dry mass production.

Field evaluations of experimental hybrids

The bmr lines obtained were planted under field conditions at the experimental unit of Embrapa Maize and Sorghum, located in Sete Lagoas, Minas Gerais, Brazil. The planting occurred on November 2018 consisting of 30 plants, distributed in 3 m rows, spaced at 0.7 m. The following traits were evaluated: days to flowering (FLOW, in number of days), which consists of the days between sowing and the pollen liberation of 50% of the plants in the plot; plant height (PH, in meters), which is the mean height of the plants within the plot, measured from the soil surface to the top of the panicle; and fresh biomass yield (FBY, in t/ha), which was determined by weighing all plants of the useful area.

Results

Physical and genetic distribution of markers

The physical and genetic positions of the available SNP markers in the sorghum genome was the first step

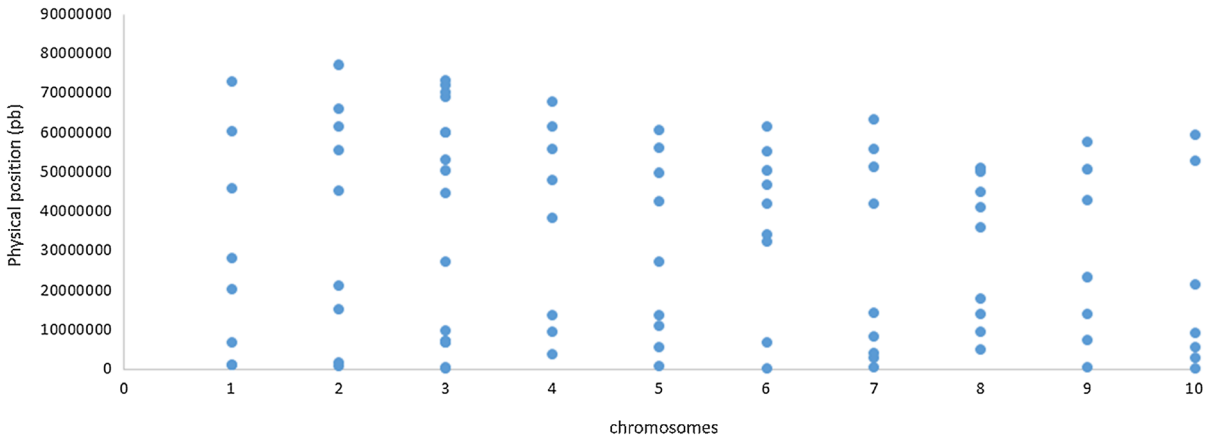

Fig. 2 Physical distribution of the KASP-type SNPs markers used for background selection in the sorghum genome. Positions were obtained by sequence similarity analysis (BLAST) of SNP primers with the sorghum genome (http://www.phytozome.net/sorghum)

in selecting to maximize recurrent genome recovery (Fig. 2).

The Fig. 2 indicates a good distribution of the SNPs markers along the sorghum genome. Recurrent genome monitoring requires a greater number of random markers in the genome, reducing the number of cycles for adequate recovery of recurrent parental. All available genetic position information for these markers were then compiled using the consensus sorghum map published by Mace et al. (2009). Genetic distances for markers with unavailable information were inferred based on the physical and genetic distances of neighboring loci. After primer screening, the polymorphic markers for each inbred line were selected so that the sorghum genome was uniformly covered.

Genotyping of first-generation backcrossing

Regarding the first generation of backcrossing, part of the genotyping result for the mutant allele *bmr6* (CMSXS170 backcrossed with the recurrent line CMSXS652) is shown in Fig. 3, in which 14 of the 60 BC_1F_1 backcross plants can be visualized after gel

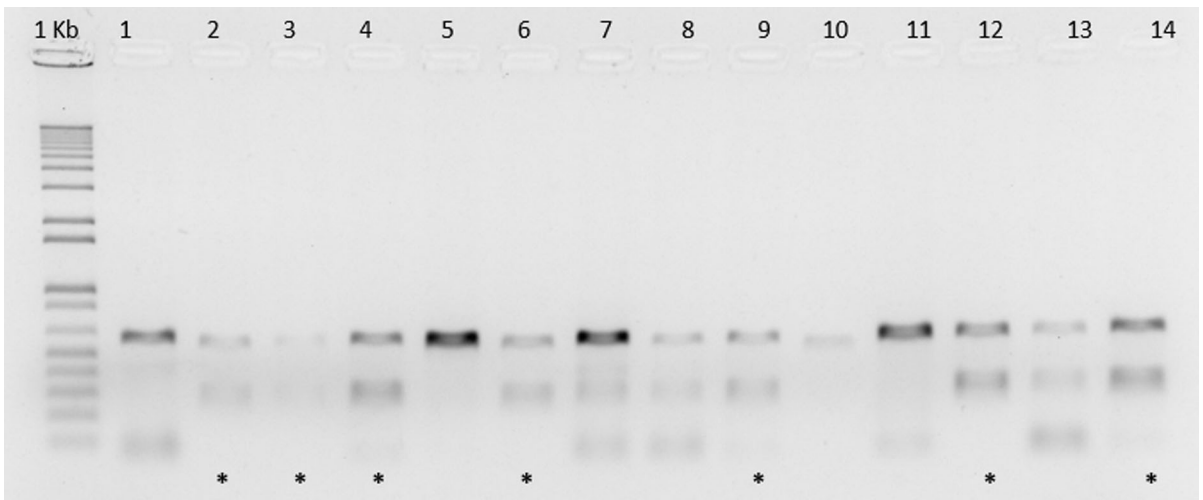

Fig. 3 Genotyping of BC_1F_1 plants, with the CAPS marker for the *bmr6* allele. The PCR amplified fragments were cleaved with the *BsaAI* enzyme and analyzed by agarose gel electrophoresis (1.2%, 1X TAE). CAPS *bmr6* primers amplified a 613 bp fragment of the *bmr6* allele. After cleavage with *BsaAI*, only the *bmr6* mutant allele fragment resulted in two 333 and 280 bp fragments, with the non-mutant allele remaining intact. Heterozygous plants are identified by an asterisk (*)

electrophoretic analysis of agarose. In the BC_1F_1 population from line CMSXS652, 30 heterozygous individuals were identified in a total of 60 individuals. Already for the recurrent line IS23777, 31 heterozygous individuals in BC_1F_1 were identified, also in a total of 60 individuals. Each of the two backcrosses showed segregation very close to the expected, 1:1 of dominant homozygous plants (*Bmr6*/*Bmr6*) to heterozygous plants (*Bmr6*/*bmr6*).

Recurrent genotype recovery in BC_1F_1 generation

Genotyping between parent lines and heterozygous individuals, based on the Kompetitive Allele-Specific (PCR) assay was efficient to accelerate the recovery of the recurrent genome. One example is illustrated in Fig. 4, in which one of the polymorphic markers (SB_07057) for both recurrent lines was used. It was noted that it was possible to identify the classes of homozygous and heterozygous individuals, allowing to verify the rate of recovery of the recurrent parent (Fig. 4).

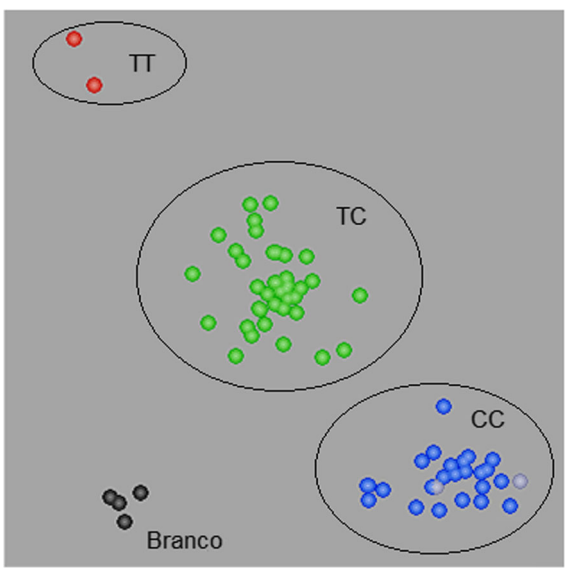

Fig. 4 Genotyping of BC_1F_1 individuals with the KASP marker (SB_07057). Homozygous individuals with C alleles, derived from parentheses IS23777 and CMSXS652, are shown in blue. Heterozygous individuals with T:C (H) alleles are represented in green; in red is represented the CMSXS170 parental used as homozygous control with T (B) alleles and in black the negative control (water added reaction mix) is represented

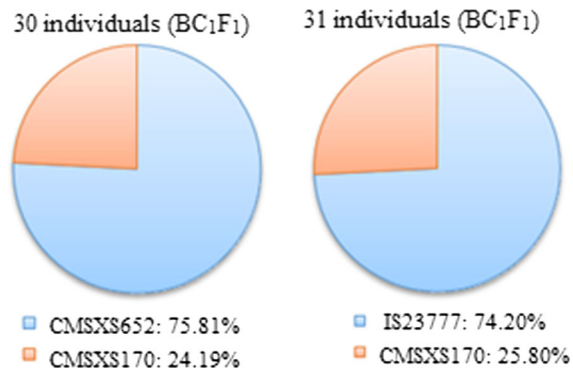

Fig. 5 Mean recurrent genome recovery of the 30 BC_1F_1 individuals (CMSXS652) and 31 BC_1F_1 individuals (IS23777). Blue is the proportion of the homozygous genome to the recurrent parent and red the proportion of the heterozygous genome

The mean recurrent genome recovery (BC_1F_1) for the CMSXS652 line was 75.81% and for the IS23777 line, the recovery mean was 74.20% (Fig. 5).

The result obtained is in agreement with the expected mean in the BC_1F_1 generation, which is 75%. In terms of individual recovery of the recurrent genome, progeny 201632B057 presented 86.67% of the genome of CMSXS652 line, and progeny 201632B032 showed 84.62% of genome recovery of IS23777 line. These individuals presented an advance of 9.62% and 11.7% of the conventional crossing and were therefore selected for the next backcross cycle. The individual recovery of the recurrent genome as well as the mean recovery in BC_1F_1, is shown in Table 1. From the selected individuals, whose recovery was superior to the others, the second-generation of backcrossing with the respective recurrent parent was performed to obtain the BC_2F_1 generation.

From the selected individuals, whose recovery was superior to the others, the second-generation of backcrossing was performed with the respective recurrent parent to obtain the BC_2F_1 generation.

Genotyping of the second-generation of backcrossing

In the second-generation of backcrossing, a total of 60 individuals were obtained for each backcross population. For the population from the CMSXS652 recurrent line, 27 heterozygous individuals were identified and to the IS23777 recurrent line, 26 heterozygous individuals were identified. Segregation was also close

Table 1 Percentage of recurrent genome recovery in BC_1F_1 generation in the 30 individuals derived from the CMSXS652 line and the 31 individuals derived from the IS23777 line

BC_1F_1 CMSXS652	Recurrent genome (%)	BC_1F_1 IS23777	Recurrent genome (%)
201632B057[a]	86.67	201632B032[a]	84.62
201632B051	84.62	201632B001	82.14
201632B049	84.38	201632B004	82.14
201632B059	84.38	201632B008	82.14
201632B068	84.38	201632B023	82.14
201632B058	82.14	201632B027	82.14
201632B066	82.14	201632B005	78.57
201632B067	82.14	201632B013	78.57
201632B035	78.13	201632B018	78.57
201632B041	76.92	201632B031	78.57
201632B042	76.67	201632B034	78.57
201632B044	76.67	201632B006	75.00
201632B050	76.67	201632B007	75.00
201632B065	76.67	201632B017	75.00
201632B038	75.00	201632B026	75.00
201632B043	75.00	201632B011	73.08
201632B055	75.00	201632B012	73.08
201632B056	75.00	201632B002	71.43
201632B039	73.33	201632B009	71.43
201632B047	71.88	201632B014	71.43
201632B061	71.88	201632B015	71.43
201632B040	71.43	201632B022	71.43
201632B054	70.00	201632B025	71.43
201632B037	68.75	201632B010	70.83
201632B045	68.75	201632B021	70.83
201632B052	68.75	201632B029	69.23
201632B062	68.75	201632B030	69.23
201632B046	66.67	201632B024	67.86
201632B053	65.63	201632B020	64.29
201632B060	64.29	201632B028	64.29
–	–	201632B033	60.71
General mean	75.81	General mean	74.20

[a]Selected individuals in BC_1F_1

to expected (1:1), as occurred in the previous backcross generation.

Recurrence genotype recovery in BC_2F_1 generation

Regarding the recovery of the recurrent parent, the mean recovery in BC_2F_1 was 93.86% (CMSXS652) and 89.93% (IS23777), as shown in Fig. 6. The result was quite satisfactory, as the mean expected in this case is 87.5%. The individual recovery in this cycle was 99.98% for the CMSXS652 line and 95.45% for the IS23777 line. Individual recovery of the recurrent genome, as well as mean recovery in $BC2F_1$, is shown in Table 2.

After the identification of heterozygous genotypes (Bmr6/bmr6) in BC_2F_1 generation, these individuals self-fertilized, resulting in the BC_2F_2 individuals. Then, three individuals in BC_2F_2 were selected from each population of the recurrent CMSXS652 and IS23777 lines, which presented a recurrent genome recovery mean greater than 93.86% and 89.93%, indicating superiority to the general mean (Table 2).

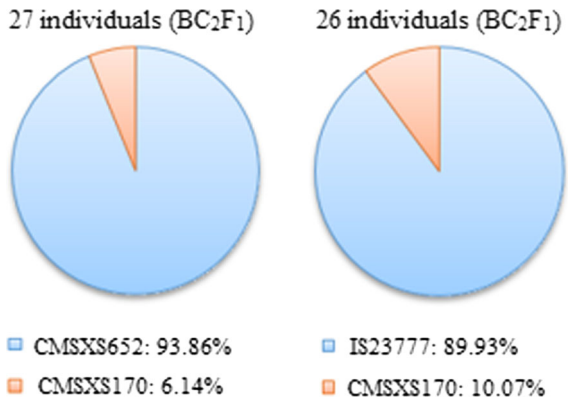

Fig. 6 Mean recurrent genome recovery of 27 BC_2F_1 individuals (CMSXS652) and 26 BC_2F_1 individuals (IS23777). Blue is the proportion of the homozygous genome to the recurrent parent and red the proportion of the heterozygous genome

Experimental "brown midrib" hybrids production

After obtaining the restorative lines of male sterility with introgression of the *bmr6* allele (modified recurrent line), the manual crossing with the female line (LA) BR008A *bmr* was performed, obtaining the experimental hybrid "brown midrib", i.e. the hybrids CMSXS652 *bmr* and IS23777 *bmr*.

Potential agronomic of experimental "brown midrib" hybrids

The biomass sorghum experimental hybrids demonstrated satisfactory potential for bioenergy production, according to the desirable traits in the sorghum

Table 2 Percentage recurrent genome recovery in BC_2F_1 generation in the 27 individuals derived from the CMSXS652 line and in the 26 individuals derived from the IS23777 line

BC_1F_1 CMSXS652	Recurrent genome (%)	BC_1F_1 IS23777	Recurrent genome (%)
201725B024[a]	99.98	201725B005_P5B[a]	95.45
201725B027[a]	99.97	201725B006_P6A[a]	95.45
201725B023[a]	98.15	201725B001	93.18
201725B027	98.15	201725B004	93.18
201725B040	98.08	201725B007	93.18
201725B028	97.92	201725B008	93.18
201725B021	96.30	201725B008	93.18
201725B022	96.30	201725B014	93.18
201725B027	96.30	201725B003	90.91
201725B037	96.15	201725B004	90.91
201725B028	93.75	201725B019	90.91
201725B035	93.48	201725B011	90.48
201725B021	92.59	201725B006	88.64
201725B023	92.59	201725B007	88.64
201725B025	92.59	201725B009	88.64
201725B025	92.59	201725B010	88.64
201725B026	92.59	201725B016	88.64
201725B040	92.31	201725B018	88.64
201725B039	92.00	201725B018	88.64
201725B038	91.30	201725B020	88.64
201725B024	90.74	201725B020	88.64
201725B025	90.74	201725B008	86.36
201725B029	90.74	201725B016	86.36
201725B029	90.74	201725B016	86.36
201725B039	90.38	201725B011	84.09
201725B030	88.89	201725B012	84.09
201725B033	88.89	–	–
General mean	93.86	General mean	89.93

[a]Selected individuals in BC_2F_1

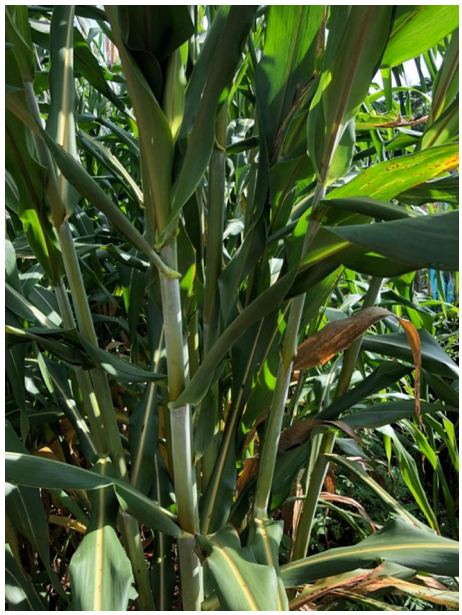

Fig. 7 Experimental "Brown midrib" hybrid (CMSXS652 *bmr*) for bioenergy production—Embrapa Maize and Sorghum, 2019. (Photo by author: Parrella, R.A. da C)

breeding program. The CMSXS652 *bmr* experimental hybrid showed sensitivity to the photoperiod, with a flowering cycle of approximately 150 days. The results due to the sensitivity trait of the genotype to the photoperiod, promoting an increase of the vegetative cycle and a greater accumulation of biomass, reflecting in the productivities of fresh and dry biomass and consecutively in the fiber contents. The mean height of plants was 5 metros and fresh biomass yield of approximately 90 ton/ha. The IS23777 *bmr* experimental hybrid showed a short flowering cycle of approximately 90 days. The height of plants was 3.5–4.0 metros and fresh biomass yield of approximately 60–70 ton/ha. Thus, the CMSXS652 *bmr* experimental hybrid demonstrated agronomic superiority in relation to IS23777 *bmr* experimental hybrid for the bioenergy generation. The Fig. 7 demonstrates the potential of developed experimental bmr lines (CMSXS652 *bmr*).

Discussion

The biomass sorghum, represent a promising source of renewable lignocellulosic materials that are suitable for use as a source of bioenergy feedstock (Sarath et al. 2008; Sattler et al. 2014). To the lignin content, an important trait is that sorghum naturally presents lower lignin contents than sugarcane, in addition to already possessing lignin mutants that can present up to 50% less lignin than the original cultivar depending on the background (Damasceno et al. 2010; Saballos et al. 2009; Barrière et al. 2007). However, low yields represent the main obstacle to the successful use of lignocellulosic materials to obtain second-generation ethanol (Abramson et al. 2010; Oliver et al. 2005; Jung and Allen 1995). Oliver et al. (2005) reported that over a 3-year study, the mean yield of bmr lines was 12% lower on mean when compared to the isogenic lines themselves. Zuber et al. (1977) had already reported a higher incidence of lodging and stem breakage in bmr plants when reaching the maturity stage. But, these negative agricultural factors associated with the *bmr* mutation can be improved through plant breeding (Sattler et al. 2010). On the other hand, some authors demonstrate that they did not observe significant differences in lodging between *bmr* mutants and conventional sorghum and that this trait strongly depends on the interactions between the *bmr* gene and the genetic background of the material (Bean et al. 2013). In this way, the Embrapa Maize and Sorghum Breeding Program is seeking to develop biomass sorghum materials that are more productive and resistant to lodging, through the selection of lines with greater agronomic potential to introduce the bmr mutation that is of great importance for the development cultivars with the best development for the production of second-generation ethanol.

The use of molecular markers in backcrossing programs has been well indicated. In addition to monitoring the introgression of the gene of interest, the molecular genotyping of the individuals allows the selection of those more similar to the recurrent genotype and with better conversion in the region close to the introduced gene. Thus, the number of backcross cycles required for the recovery of the recurrent genotype is reduced, accelerating the development of improved varieties (Openshaw et al. 1994). The application of DNA marker for marker-assisted selection and breeding is still limited in sorghum (Burow et al. 2019). KASP assay in marker-backcrossing in sorghum and other cultivars is aimed at accelerating the development of almost isogenic lines for 3 years compared to 5–6 years using the conventional backcrossing method, being able to improve

overall efficiency, both in cost and accuracy of introgression (Semagn et al. 2014). In this work, the application of the KASP assay was efficient in the identification of individuals with a higher rate of recovery of the recurrent genome in the initial backcross generations, with a gain of up to three cycles compared to that expected in conventional backcrossing. From this result, it was possible to select new genotypes R of biomass sorghum containing the *bmr6* allele, which was used to produce an experimental hybrid.

The Embrapa Maize and Sorghum breeding program develops hybrids with high biomass production and high quality for the production of cellulosic ethanol (da Silva et al. 2017). de Almeida et al. (2019) worked with six biomass sorghum line, all sensitive to photoperiod. Among the materials used, five materials belong to the Embrapa Maize and Sorghum Breeding Program (201556B001, 201556B002, 201556B003, CMSXS7027, and CMSXS7016). In this work, sorghum genotypes were evaluated for agronomic potential and chemical composition favorable to the production of second-generation ethanol. The authors confirmed that mutant bmr line are associated with reduced lignin content, making these genotypes more promising for biomass enzymatic conversion processes. Parrella et al. 2018 developed the work of identifying restorative (R) fertility lines and aluminum tolerant in bmr biomass sorghum by manual crossing between lines IS14351 and CMSS023R. Two biomass sorghum hybrids, 201820B001 and 201820B003, were identified with the bmr6 allele and tolerant to aluminum by the SbMATE gene. Another key point to consider is the number of markers and individuals to be used in assisted backcross programs. Several criteria regarding this issue have been proposed to define the best possible genome sampling (Guimarães et al. 2009; Morris et al. 2003; Frisch et al. 1999; Openshaw et al. 1994). However, the important thing is that the use of these markers is optimized in terms of costs and their operation for the routine conditions of the laboratory.

A few years ago the genetic material change or mutation have been widely used in breeding programs for sorghum around the world. A classic example of this application was carried out in the United States in the 1960s, when the dwarf traits—Dw3dw3 and Dw4dw4 (Quinby 1954) and photoperiod insensitivity—Ma5Ma5ma6ma6 and ma5ma5Ma6Ma6 (Rooney and Aydin 1999) were incorporated into exotic sorghum germplasm through a series of backcrosses using conventional breeding. Thus, currently, the most sorghum cultivated in the USA has a low size and insensitive to photoperiod, except for sorghum used for the production of bioenergy.

Scully et al. (2016) identified and characterized nine new alleles in sorghum lines belonging to the BTX623 population, through the ethyl methanesulfonate (EMS) induced mutation process. The authors concluded that these new bmr alleles are allelic for the previously characterized *bmr6* allele. The lower lignin content was visually identified by the presence of the brown midrib and all lines were tested for the ability to reduce the activity levels of the CAD enzyme and to increase the glucose content produced after saccharification. Besides, some of these lines were associated with higher acid detergent lignin reductions in comparison to the *bmr6* allele. The new *bmr6* lines developed represent new tools to manipulate the composition of biomass and to improve the quality of the raw material for energy production.

Gorthy et al. (2017) carried out a work of introgression of three QTLs associated with the genetic resistance against the fly (*Atherigona soccata* L. *Moench*), through assisted backcrossing in two sorghum elite cultivars. The authors concluded that the development of lines using assisted back-crossing with SSR markers was comparatively faster than conventional breeding. The recovery of the recurrent parent genome was close to 90%. Ouedraogo et al. (2017) also used assisted backcrossing with the use of SSRs to introduce QTLs related to the stay-green trait in sorghum elite cultivars. The authors found two lines with a high level of recovery of the recurrent genome and proposed that these lines are promising for the development of drought-tolerant cultivars, which will be able to guarantee the best yield of the crop in semi-arid regions of West Africa. Besides, sorghum cultivars with higher digestibility in animal feed were developed in Japan by introgression of the bmr genes. Varieties Hazuki (released in 2002), Akidachi (released in 2004), Kazetaka (launched in 2009) and Suzukaze (launched in 2009) were developed by the introgression of the *bmr18* allele (Tsuruta et al. 2015). Thus, backcrossing assisted by molecular markers accelerate the process about gains in time and efficiency in the identification of individuals with a higher percentage of recurrent parental recurrent.

Conclusions

The introgression of the *bmr6* allele in elite lines of biomass sorghum using a marked allele-specific CAPS type was possible, gaining up to three cycles compared to that expected in conventional backcrossing.

Experimental "brown midrib" hybrids will assist the Sorghum Breeding Program for bioenergy production in Brazil.

References

Abramson M, Shoseyov O, Shani Z (2010) Plant cell wall reconstruction toward improved lignocellulosic production and processability. Plant Sci 178:61–72. https://doi.org/10.1016/j.plantsci.2009.11.003

Anderson WF, Akin DE (2008) Structural and chemical properties of grass lignocelluloses related to conversion for biofuels. J Ind Microbiol Biotechnol 35:355–366. https://doi.org/10.1007/s10295-007-0291-8

Barrière Y, Riboulet C, Méchin V, Maltese S, Pichon M, Cardinal AJ, Lapierre C, Lübberstedt T, Martinant JP (2007) Genetics and genomics of lignification in grass cell walls based on maize as a model system. Genes Genomes Genomics 1(2):133–156

Bean BW, Baumhardt RL, McCollum FT, McCuistion KC (2013) Comparison of sorghum classes for grain and forage yield and forage nutritive value. Field Crops Res 142:20–26. https://doi.org/10.1016/j.fcr.2012.11.014

Benchimol LL, Souza CL Jr, Souza AP (2005) Microsatellite-assisted backcross selection in maize. Genet Mol Biol 28:789–797. https://doi.org/10.1590/S1415-47572005000500022

Burow G, Chopra R, Hughes H, Xin Z, Burke J (2019) Marker assisted selection in sorghum using KASP assay for the detection of single nucleotide polymorphism/insertion deletion. Methods Mol Biol 1931:75–84. https://doi.org/10.1007/978-1-4939-9039-9_6

Corredor DY, Salazar JM, Hohn KL, Bean S, Bean B, Wang D (2009) Evaluation and characterization of forage sorghum as feedstock for fermentable sugar production. Appl Biochem Biotechnol 158:164–179. https://doi.org/10.1007/s12010-008-8340-y

Cotton J, Burow GB, Acosta-Martinez V, Moore-Kucera J (2013) Biomass and cellulosic ethanol production of forage sorghum under limited water conditions. Bioenergy Res 6:711–718. https://doi.org/10.1007/s12155-012-9285-0

Cruz CD (2016) Genes software—extended and integrated with the R, Matlab and Selegen. Acta Sci Agron 38(4):547–552. https://doi.org/10.4025/actasciagron.v38i4.32629

da Silva MJ, Pastina MM, de Souza VF, Schaffert RE, Carneiro PCS, Noda RW, Carneiro JES, Damasceno CMB, Parrella RAC (2017) Phenotypic and molecular characterization of sweet sorghum accessions for bioenergy production. PLoS ONE 12(8):e0183504. https://doi.org/10.1371/journal.pone.0183504

da Silva MJ, Damasceno CMB, Carneiro JES, Pereira HD, Carneiro PCS, Schaffert RE, Parrella RAC (2020) Combining ability of biomass sorghum in different crop years and sites for bioenergy generation. Agron J. https://doi.org/10.1002/agj2.20123

Damasceno CMB, Sousa SM de, Noda RW, Parrella RAC, Schaffert RE, Magalhaes JV de. (2010) A importância da lignina para a produção de etanol de segunda geração. Sete Lagoas: Embrapa Milho e Sorgo. Documentos, 108

de Almeida LGF, Parrella RAC, Simeone MLF, Ribeiro PCO, dos Santos AS, da Costa ASV, Guimarães AG, Schaffert RE (2019) Composition and growth of sorghum biomass genotypes for ethanol production. Biomass Bioenergy 122:343–348. https://doi.org/10.1016/j.biombioe.2019.01.030

Dien BS, Sarath G, Pedersen JF, Sattler SE, Chen H, FunnellHarris DL, Nichols NN, Cotta MA (2009) Sugar conversion and sugar yield for forage sorghum (*Sorghum bicolor* L. *Moench*) lines with reduced lignin contents. Bioenergy Res 2:153–164. https://doi.org/10.1016/j.biortech.2007.09.030

Frisch M, Melchinger AE (2005) Selection theory for marker-assisted backcrossing. Genetics 170(2):909–917. https://doi.org/10.1534/genetics.104.035451

Frisch M, Bohn M, Melchinger AE (1999) Minimum sample size and optimum positioning of flanking markers in marker-assisted backcrossing for transfer of a target gene. Crop Sci 39:967–975. https://doi.org/10.2135/cropsci1999.0011183X003900040003x

Gorthy S, Narasu L, Gaddameedi A, Sharma HC, Kotla A, Deshpande P, Are AK (2017) Introgression of shoot fly (*Atherigona soccata* L. Moench) resistance QTLs into elite post-rainy season sorghum varieties using marker assisted backcrossing (MABC). Front Plant Sci 8:1494. https://doi.org/10.3389/fpls.2017.01494

Guimarães CT, de Magalhães JV, Lanza MA, Schuster I (2009) Marcadores moleculares e suas aplicações no melhoramento genético. Informe Agropecuário, Belo Horizonte 30:253

House LR (1985) A guide to sorghum breeding, 2nd edn. International Crops Research Institute for the Semi-Arid Tropics, Patancheru

Jung HG, Allen MS (1995) Traits of plant cell wall affecting intake and digestibility of forages by ruminants. J Anim Sci 73:2774–2790. https://doi.org/10.2527/1995.7392774x

Mace ES, Rami JF, Bouchet S, Klein PE, Klein RR, Kilian A, Wenzl P, Xia L, Halloran K, Jordan DR (2009) A consensus genetic map of sorghum that integrates multiple component maps and high-throughput Diversity Array Technology (DArT) markers. BMC Plant Biol 9:13. https://doi.org/10.1186/1471-2229-9-13

Morris M, Dreher K, Ribaut JM, Khairallah M (2003) Money matters (II): costs of maize inbred line conversion schemes at CIMMYT using conventional and marker-assisted selection. Mol Breed 11:235–247. https://doi.org/10.1023/A:1022872604743

Oliveira ICM, Marçal TS, Bernardino KC, Ribeiro PCO, Parrella RAC, Carneiro PCS, Schaffert RE, Carneiro JES (2019) Combining ability of biomass sorghum lines for

agroindustrial characters and multitrait selection of photosensitive hybrids for energy cogeneration. Crop Sci 59:1554–1566. https://doi.org/10.2135/cropsci2018.11.0693

Oliver AL, Pedersen JF, Grant RJ, Klopfenstein TJ (2005) Comparative effects of the sorghum *bmr6* and *bmr12* genes: I. Forage sorghum yield and quality. Crop Sci 45:2234–2239. https://doi.org/10.2135/cropsci2004.0644

Openshaw SJ, Jarboe SG, Beavis WD (1994) Marker assisted selection in backcross breeding. In: Symposium analysis of molecular marker data, Corvallis, Oregon. Proceedings. American Society for Horticultural Science/Crop Science Society of America, Corvallis, pp 41–43

Ouedraogo N, Sanou J, Gracen V, Tongoona P (2017) Incorporation of stay-green Quantitative Trait Loci (QTL) in elite sorghum (*Sorghum bicolor* L. *Moench*) variety through marker-assisted selection at early generation. J Appl Biosci 111:10867–10876. https://doi.org/10.4314/jab.v111i1.3

Parrella RAC, Rodrigues JAS, Tardin FD, Damasceno CMB, Schaffert RE (2010) Desenvolvimento de híbridos de sorgo sensíveis ao fotoperíodo visando alta produtividade de biomassa. Boletim de pesquisa e desenvolvimento 28. Embrapa Milho e Sorgo, Sete Lagoas

Parrella RAC, Schaffert RE, Magalhães JV, Menezes CB de, Silva MJA, Virgínia (2018) A importância da lignina para a produção de etanol de segunda geração. Embrapa Milho e Sorgo, Sete Lagoas. Boletim 177

Quinby JR (1974) Sorghum improvement and the genetics of growth. Texas A&M University, College Station

Rooney WL, Aydin S (1999) Genetic control of a photoperiod-sensitive response in *Sorghum bicolor* (L.) *Moench*. Crop Sci 39:397–400. https://doi.org/10.2135/cropsci1999.0011183X0039000200016x

Saballos A, Ejeta G, Sanchez E, Kang C, Vermerris W (2009) A genomewide analysis of the cinnamyl alcohol dehydrogenase family in Sorghum [*Sorghum bicolor* (L.) *Moench*] identifies SbCAD2 as the *Brown midrib 6* gene. Genetics 181:783–795. https://doi.org/10.1534/genetics.108.098996

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNAsepacer-length polymorphism in barley: mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci 81:8014–8019. https://doi.org/10.1073/pnas.81.24.8014

Sarath G, Mitchell RB, Sattler SE, Funnell D, Pederson JF, Graybosch RA, Vogel KP (2008) Opportunities and roadblocks in utilizing forages and small grains for liquid fuels. J Ind Microbiol Biotechnol 35:343–354. https://doi.org/10.1007/s10295-007-0296-3

Sattler SE, Saathoff AJ, Haas EJ, Palmer NA, Funnell-Harris DL, Sarath G, Pedersen JF (2009) A nonsense mutation in a cinnamyl alcohol dehydrogenase gene is responsible for the sorghum brown midrib 6 phenotype. Plant Physiol 150:584–595. https://doi.org/10.1104/pp.109.136408

Sattler SE, Funnell-Harris DL, Pedersen JF (2010) Efficacy of singular and stacked brown midrib 6 and 12 in the modification of lignocellulose and grain chemistry. J Agric Food Chem 58:3611–3616. https://doi.org/10.1021/jf903784j

Sattler SE, Saballos A, Xin Z, Funnell-Harris DL, Vermerris W, Pedersen JF (2014) Characterization of novel sorghum *brown midrib* mutants from an EMS-mutagenized population. G3 Genes Genomes Genet 4:2115–2124. https://doi.org/10.1534/g3.114.014001

Scully ED, Gries T, Funnell-Harris DL, Xin Z, Kovacs FA, Vermerris W (2016) Characterization of novel Brown midrib 6 mutations affecting lignin biosynthesis in sorghum. J Integr Plant Biol 58:136–149. https://doi.org/10.1111/jipb.12375

Semagn K, Babu R, Hearne S, Olsen M (2014) Single nucleotide polymorphism genotyping using Kompetitive Allele Specific PCR (KASP): over view of the technology and its application in crop improvement. Mol Breed 33:1–14. https://doi.org/10.1007/s11032-013-9917-x

Smith CW, Frederiksen RA (2000) Sorghum: origin, history, technology, and production. Wiley series in crop science, series editor. Texas A&M University, College Station

Stephens JC, Holland PF (1954) Cytoplasmic male sterility for line sorghum seed production. Agron J 46:20–23

Tsuruta S, Shimoda S, Kouki K, Ebina M (2015) The present status of C4 tropical grasses breeding and molecular approaches. Jpn Agric Res Quart 49:203–215. https://doi.org/10.6090/jarq.49.203

Zuber MS, Colbert TR, Bauman LF (1977) Effect of brown-midrib-3 mutant in maize (*Zea mays* L.) on stalk strength. Z Pflanzenzucht 79:310–314