Early-Generation Evaluation of Marker-assistedtransferof *bmr6* and *bmr12* alleles into sweet and high-biomass sorghum

Abstract

The effects of transferring brown midrib (*bmr*) alleles in sweet and high biomass sorghums (SS/HBM) are not fully understood, and available works produced mixed results depending upon the genetic background of the recurrent parent used. Early-generation testing of the effects of the two mutations in the background of the recurrent parents can help predict the final products and make appropriate early go/no-go breeding decisions, yet, this information is missing. The present study was therefore carried out to reduce these gaps. Marker-assisted transfer of the *bmr6* and *bmr12* was performed using four (SSV84, ICSB474, ICSV18003, ICSV100324) SS and one (ICSV15024) HBM wild type (WT) lines as recurrent parents,and one bmr6 (N609) and two bmr12 (ICSV101039 and N600) lines as donor parents. Crossing and backcrossing techniques were used to transfer the *bmr* alleles to develop $BC_2F_2$and F_3populations. Kompetitive allele-specific PCR (KASP) marker genotyping assays were performed and implemented for the indirect selection of the *bmr* transfer in F_1 and BC_F_1 populations. While the *bmr6* markers were polymorphic, the *bmr12* markers were monomorphic. The successful *bmr6* transfer was therefore confirmed by markers in F_1 and BC_F_1, while for *bmr12* the transfer was confirmed after segregation assessment in F_2 and BC_F_2 populations. The transfer of *bmr12* improved the cellulose content relative to WT genotypes, and *bmr12* populations were shorter-statured relative to *bmr6* individuals, meaning that *bmr12* gene can be considered in sorghum breeding to boost 2G bioethanol bioconversion and to control lodging, and hence favouring mechanical harvesting and limiting yield losses. The observed earliness in the *bmr* lines is an attractive trait conferring adaptation in the drylands, particularly in the world's semi-arid tropics characterized by terminal drought stress. Both *bmr*alleles did not show any negative trade-offs relative to the must-have sorghum plant characteristics, implying that they can safely be deployed in sorghum breeding for bioenergy production purposes. The observed superior bmr lines with farmer-preferred phenotypic acceptance are candidates not only for advancement in breeding pipelines but also for subsequent pyramiding *bmr6* and *bmr12* in the same genetic background, further improving the *bmr*-conferred benefits. The successfully used molecular

markers will be uploaded in the public domain and can help breed sorghum products other than SS/HBM such as high-quality forage sorghums.

Key words: KASPar markers,*bm6*,*bmr12*,Sweet sorghum, High biomass sorghum, Fiber components, Cellulose, Marker-assisted backcrossing.

1. Introduction

Sorghum bicolor **(**L.)Moenchis one of the important cereal crops; it can be used for food, fodder, and biofuel production.It belongs to the Poaceaefamily with five races (caudatum, bicolor, durra, guinea, and kafir) and several hybrids therefrom. Itis an annual crop with a minimal life cycleof 3-4 months(Reddy *et al.,* 2005),and canbe grown in almost all seasons i.e., rainy, post-rainy andsummer, because of its wide adaptability (Oliveira *et al*. 2020; da Silva *et al*. 2020).The increase in biomass productivity depends upon many characteristics like the plant height, maturity, and fresh and dry stalk yields(Nagaiah*et al.,* 2012).Unlike other crop species used for renewable energy bioconversion, sweet sorghum producesbiofuels in a sustainable way and without compromising food production(Mathur *et al.,* 2017)because it is possible to produce both grain and 1G and 2G bioethanol (Mask and Morris 1991; Bennett and Anex 2009).Sweet and high biomass sorghum yields 23% more fermentable carbohydrates, uses 37% less nitrogen fertilizer and 17% less irrigation water, and yields more ethanolthan maize (*Zea mays*)(Putnam *et al.,* 1991; Hills *et al.,* 1990).

Sweet and high biomass sorghum is the prominent feedstock for second-generation (2G) lignocellulosic production of biofuel whichcan produce about288 L of ethanol per each ton of dry biomass by fermenting both C5 and C6 sugars (Umakanth *et al.,* 2022); this is substantial as SS/HBM dry biomass yieldsof 30-35t/ha were reported in previous works (Habyarimana *et al*., 2004). According to Kim and Day (2011), the primary components of lignocellulosic material are lignin (20–30%), hemicellulose (30–40%), cellulose (15–40%), and pectin. The composition of these structural polymers differs across primary and secondary cell walls, various plant tissues, and various plant species (Kim and Day, 2011; Welker *et al.,* 2015). All plant cell types contain primary cell walls (PCW); however only a few cell types, including tracheary elements (TE) and sclerenchymal cells, have the secondary cell walls (SCW)which are present in few plants like Sorghum (Scavuzzo-Duggan *et al.,* 2021). The SCWs are the major components of plant biomass, and they are therefore

central to second-generation biofuels production (Kumar *et al.,* 2016). Cellulose is the common polymer on Earth, which is a homopolymer of -(1,4)-linked glucose monomers, whereas, hemicelluloses are branched heteropolymers made of monomers of pentose and hexose sugar (Cosgrove, 2005; Somerville, 2006). Pectin is a complex polymer with a -(1-4)-linked D-galacturonic acid backbone which is another polysaccharide that is predominant in primordial cell walls. Based on the type of sugars present on the branches, three different types of pectins called rhamnogalacturonans-I (RG-I), rhamnogalacturonans-II (RG-II) and homogalacturonans (HG), have been identified in plants (Burton *et al.,* 2010). The microfibrils of cellulose are cross-linked with different matrices of polysaccharides such as pectins and hemicelluloses (Cosgrove, 2005; Muthamilarasan *et al.,* 2015). Lignin has a significant function in plants, and is concentrated in plant secondary cell walls together with cellulose, hemicellulose, and pectin. Lignin is a complex phenolic polymer that increases the stiffness and hydrophobicity of the cell wallas well as the transport of minerals through the plant's vascular bundles (Schuetz *et al.,* 2014). In addition, lignin also has a protective effect against pathogens and pests (Ithal *et al.,* 2007).However, increased lignin concentration limits enzymatic digestibility and biofuel recovery, suggesting that reducing the lignin quantity in biomass can be a game changer in biofuel productionindustry (Oliveira *et al.,* 2020).

Brown midrib (*bmr*) mutants arecharacterized phenotypically by the presence of brown vascular tissuesobserved on the leaf midribs and stems. These *bmr* mutations appeared in sorghum and maize throughchemical or spontaneous mutagenesis.From the intense brown coloration (compared with wild-type green or white leaf midrib colour) of leaf midrib in sorghumderives the name brown midrib (*bmr*) trait, whichis correlated with the reduction in lignin content in the stover when compared with wildtype sorghums (Porter *et al*., 1978; Oliver *et al*., 2005). However, the intensity of the brown colouris not relatedtofurther ligninreduction,butit is anindicatorof the presence of *bmr* alleles(Sattler *et al.,* 2014). This phenotype (reddish brown coloration) was found to be positively correlated with two homologous loci *Bm*1and*Bm*3, that are commonly found in maize (*Zea mays*) and *bmr6*and*bmr12* in sorghum (Sattler *et al.,* 2014). The introgressionof *bmr6* and*bmr12*showed the ability to reducelignin concentration and improve cellulose and hemicellulose contents in the aboveground biomass(Diakité*et al.,* 2021).Various *bmr*lociwere identified and were extensively investigated in sorghum (*Sorghum bicolar*) andmaize (*Zea mays)*by many researchers (Sattler *et al.,* 2010; Rao *et al.,* 2012; Adeyanju *et al.,* 2021).The*bmr* plants exhibit less polymerized lignin with fewer phenolic monomers, which positively affects

digestion e.g., increased digestibility in the livestock, and can enhance 2G biofuel bioconversion process yields **(**Fahey, 1983; Habyarimana *et al.,* 2016; Anderson and Akin 2008; Corredor *et al*., 2009).

The *bmr*mutationsaregenerally associated with reducedlignin in crops like sorghum, maize and pearl millet (Kuc and Nelson, 1964),but most available literature was reported in crops used as ruminant forage (Oliver *et al.,* 2004; Wahyono *et al.,* 2021), while fewer information is available in sweet sorghum.In addition, the *bmr* mutations can reduce the concentration of lignin in the sorghum biomass, but this is not in a systematic way meaning that there are instances where lignin was not reduced and other instances where traits other than lignin were affected (Oliver et al., 2005). In most available literature, backcrossing of bmr mutations were evaluated later (>F3) in the breeding pipelines (Kawahigashi et al. 2022; Rivera-Burgos *et al*. 2019; Deshavath et al. 2018; Cotton et al. 2013; Theerarattananoon et al. 2010; Pedersen et al. 2008; Pedersen et al. 2006; Oliver et al. 2005; Oliver *et al.* 2004; Bout and Vermerris 2003; Grant et al. 1995; Pillonel et al. 1991), which can lead to wasting resources on inferior families that will not be selected. The present study was therefore carried out to reduce this gap. Inferences in this work were based on the results from early-generation (EGT) testing F_3families derived from several unique crosses between bmr sorghum donor parents and sweet and high biomass recurrent sorghum parental lines. EGT is a robust breeding technique (Jones and Smith, 2006) used to identify superior segregating populations containing the greatest frequency of favorable genotypes and to discard inferior populations with limited promise.In sweet and high-biomasssorghum the sweet stem trait and the *bmr* mutation were found to act in an additive manner to reduce lignin content in the stover; the outcome of the introgression of bmr alleles into this type of cropcannot therefore be readily predicted (Velmurugan *et al.,* 2020; Espinal, 2009). More research is needed to shed light on the issues related with *bmr* deployment in sorghum breeding and on the best approaches to optimize sweet/high-biomass sorghum must-have and long-term traits that are relevant to bioethanol production. Thus, the aim of this study was to understand the impact of *bmr6*and *bmr12*alleles on agronomic and quality traits in SS/HBM, and produce plant materials and molecular data information that are meaningful for downstream research works in *bmr* sorghum cultivar development.

2. **Materials and Methods**

The present research was carried out in five seasons (from postrainy 2020 to postrainy 2022) with as an outcome the development of $BC_2F_2$and F_3populationsof sweet sorghum and high-biomass sorghum lines. The experimental sites were located at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) Patancheru, Hyderabad, India (17^o27'N and 78^o28'E).

2.1 Plant materials

The experiments were set up to transfer*bmr6* and *bmr12* in diverse recurrent parent genetic backgrounds with the aim of developinghigh-biomass sweet sorghum lines with improved bioethanol production traits. Theparental linesincluded four and one Bmr WT sweet sorghum varieties (SSV84, ICSV18003, ICSB474, and ICSV100324) and high-biomasssorghumvariety (ICSV15024), respectively, one and two *bmr6*(N609) and *bmr12*(ICSV101039 and N600) mutants, respectively, as *bmr* trait donor parents(Table 1).

Table 1: Parental lines used in the *bmr*transfer experiments.

S. N.	Parents	*bmr*/Bmr	Sorghum type
1	SSV84	Bmr(RP,WT)	SS/HBM
2	ICSV15024	Bmr(RP,WT)	HBM
3	ICSV18003	Bmr(RP,WT)	SS
4	ICSB474	Bmr(RP,WT)	SS/HBM
5	ICSV100324	Bmr(RP,WT)	SS
6	N609	*bmr*(DP)	*bmr6*
7	ICSV101039	*bmr(DP)*	*bmr12*
8	N600	*bmr(DP)*	*bmr12*

SS: Sweet Sorghum, HBM: High-BiomassSorghum, *bmr* - brown midrib mutation,
Bmr: Wild Type (WT),DP : Donor Parent, RP: Recurrent Parent.

2.2 Population development

The selection of parents and successive crossing wasstartedin the post-rainy 2020, by hand-emasculation of the recurrent parents and pollinating it with pollen collected from the *bmr6*and*bmr12*donorparents separately. The resulting physiologically mature crossed seeds were collected from the recurrent parents and F_{1s}were backcrossedto the recurrent parentsduring second season of rainy 2021 to generate the BC_1F_1populationsfollowed by selfing to obtain BC_1F_2 populations. In thethird season post-rainy 2021, theBC_1F_{1s}were further backcrossed for generating BC_2F_1populations that were selfed to produce BC_2F_{2s}duringfourthseason i.e., rainy 2022. In parallel with the backcrossing pipeline,

F_3populations were developed to evaluate fiber production potential and other agronomic traits (Table 6). TheBC_1F_2swere used to assess the expected*bmr* and Bmr segregation in 3:1ratio during the fifth season, post-rainy 2022.

These$F_1$and BC_1F_1populationswere confirmed for heterozygosity at the *bmr6*locus using validated KASPar SNP markers; *bmr12*markers were monomorphic in our populations. To identify successful *bmr12*transfer, aliquots F_1 seeds from all crosses were planted and selfed to observe the segregation of the recessive *bmr12* allele in the $F_{2s}$and BC_1F_{2s}. True F_1 or BC_1F_1heterozygotes at the *bmr12* locus were subsequently used for advancement or for further backcrossing with the recurrent parents. For *bmr6*, only $F_1$and BC_1F_1 plantsthat showed SNP polymorphism (heterozygosity) at the Bmr6locus were used in backcrossing.

2.3 DNA extraction

After obtaining BC_1F_1 generations, the phenol-chloroform method was used for genomic DNA extraction(Xia *et al.,* 2019) where, one ml of DNA extraction buffer (150 mM NaCl, 20 g SDS/l, 25 mM EDTA, 100 mM Tris/HCl, pH 8.0) was added to 100 mg of grounded sorghum seedling leaf samples, followed bythe addition of 10 µl of Proteinase K-10 mg/ml and incubated at 65 °C for 1 hour, while being stirred every 10 minutes. Then, the reaction mixture was centrifuged at 12000 xgfor 10 min and the supernatant was extracted twice using Phenol: Chloroform: Isoamyl alcohol (25: 24: 1, v/v/v) and Chloroform: Isoamyl alcohol (24: 1, v/v) respectively. Then, 0.1 volume of potassium acetate solution (3 M, pH 5.5) and ethanol (double volume with 95%, v/v, 20°C) were added to the upper aqueous phase (first precipitation), which was then gently inverted and vortexed at 15000 g for 10 min to pellet DNA. The pellet was washed twice with 70%, v/v, ethanol solution at 20°C and allowed to air dry for five minutes. The pellet was then dissolved in 400 µl of Tris/EDTA buffer (10 mM Tris, 1 mM EDTA). The mixture was treated with 10 mg of RNase, and any residual RNA was removed by incubating it at 37 °C for 30 min. Further, another extraction was carried out using C:I (third extraction) for removing protein from the DNA solution.

2.4 Marker-assisted selection

The above genomic DNA was analyzed for CAPS for *bmr6*and *bmr12*, by PCR amplificationusing specific primers in a 20-µl reaction (Sattler *et al*., 2009). Further, the foreground marker-assisted selection across the donor-recurrent parent combinationswas

performed by identifying the presence of KASPar SNP (snpSB00519_CALL)*BMR6*-132and KASPar SNP (snpSB00520_CALL) *BMR12*-129markers in the target *bmr6*and *bmr12*locion chromosomes 4 and 7, respectively (Gorthy *et al.,* 2013), adapted from Burow *et al.* (2019). The primers used in this study are listed in Table 2. The final volume of the reaction mixture used for genotyping was 10 µl which consists of 10–20 ng of DNA along with 1x KASP Reaction Mix, 1 µl of the assay mix containing the two allele-specific SNP primers as well as the common reverse primer. Fifteen minutes at 94 °C, 10 touchdown cycles of 20 s at 94 °C, 60 s at 63-55 °C (dropping 0.8 °C every cycle), and 30 cycles of 20 s at 94 °C, 60 s at 55 °C were the PCR cycling conditions.

2.4.1 KASPar assays for SNP validation and foreground marker-assisted backcrossing

KASPar assays were performed for all the identified SNPs in the *bmr*transferred plants. KASPar genotyping assays were performed in parents and off-springs (F_1 and BC_1F_1) at the four-leaf stage, using specific KASP markers i.e., *BMR6*-132 (snpSB00519_CALL) for *bmr6* and *BMR12*-129 (snpSB00520_CALL) for *bmr12*. PCR products were size fractionated using capillary electrophoresis on an ABI3700 automatic DNA sequencer (Applied Biosystems USA). The KASPar SNP genotyping of sorghum samples was done at Intertek Hyderabad. KASPar-SNP markersfor *bmr12* allele resulted monomorphic. The designed *bmr6* allele specific KASPar-SNP markers, on the other hand, were polymorphic and used as proxy to screen and classify the F_1, BC_1F_1, and BC_2F_2 individuals into *bmr6* heterozygotes, homozygotes, and Bmr; the BC_2F_{2s} derived from self-fertilizing true BC_2F_{1s}.

Table2:List of primers used in the study

S.N.	SNP markers	Primers	Primer sequences	Reference
1	*BMR6*-132	Wild-type allele Mutant allele Common primer	5'- GGCGAAGCCGCCCTG-3' 5'-GGCGAAGCCGCCCTA-'3 3'-GCAACAAGAAGATCTGGTCCT-5'	Burrow *et al.,* 2019
2	*BMR12*-129	Wild-type allele Mutant allele Common primer	5'-TCTCCATGAGGACCTTGTCATG-3' 5'-TCTCCATGAGGACCTTGTCATA-3' 3'-GCTCACCCCTAACGAGGAC-5'	Burrow *et al.,* 2019

2.5 Fiber components analysis

The proximate fiber components analysis was carried out in the F_3generation. The different fiber quality traits (Table 6) i.e., cellulose, hemicellulose, acid detergent fiber (ADF),lignin (ADL), neutral detergent fiber (NDF), metabolizable energy, nitrogen, *in vitro* organic matter digestibility (IVOMD), and ash weremeasured using Near-Infrared Reflectance Spectroscopy (NIRS) as adapted from Rivera-Burgos *et al.,*(2019). The Near infrared spectroscopy (NIRS) is by now a well-developed and mature technology used cost-effectively by the scientific community. NIRS was widely applied as a rapid and non-destructive analysis tool for several products e.g., meat, fruit, or biomass feedstocks. Our samples were analyzed by the International Livestock Research Institute (ILRI), in their lab at the International Crops Research Institute for the Semi-Arid Tropics, headquarters, India, using FOSS DS 2500 Forage analyzer and WinISI calibration software. The ILRI reported Determination Coefficients (R^2) were satisfactory (i.e., 0.83, 0.91, 0.82, 0.91, and 0.9, respectively for NDF, ADF, ADL, ME, and IVOMOD) and greater than previously reported works (e.g., Otero *et al*., 2023)

2.6 Data analysis

The genotypic segregation of individualsin $BC_1F_{2s}$was assessed usingChi-square test (χ^2) and theobtained proportionswere compared with expected codominant (marker) segregation (1:2:1) for *bmr6*. In addition, the goodness of fit for phenotypic segregation(*bmr6* and *bmr12*) was assessed against the 3:1 monohybrid phenotypic ratio. The statistical analyses were carried out using the GENES program (Cruz,2016), ASREML-R (Butler *et al.,* 2009) as implemented under the 'R' software environment *version 4.3.1* (R Core Team., 2023)

3.Results

3.1 Development of *bmr6* hybrids through marker-assisted backcrossing

Marker-assisted transfer of the *bmr6*and *bmr12*was performed using four (SSV84, ICSB474, ICSV18003, ICSV100324) sweet sorghum and one (ICSV15024) high-biomass sorghum wild type (WT) lines as recurrent parents, and one and two *bmr6* (N609) and *bmr12*(ICSV101039 and N600) lines, respectively, as donor parents.

3.2 Phenotypic characteristics

The *bmr6*and*bmr12*transferredplants showed brown midrib colour in the matured plant (Fig. 1). However, the brown midribphenotype was observed only in F_2 and BC_1F_2 generations but not in F_{1s}, confirming the recessive inheritance of the trait. Among the WT x *bmr6*derived F_2 populations, 20% of individuals were transferred with *bmr*allele, whereasin BC_1F_2 population 14.6% weretransferred.For the *bmr12*trait, 22.8% of F_2 individuals were *bmr*transferred, whereas in BC_1F_2 population 20.35% of the individuals were found to be *bmr*transferred. The phenotypic data collected from $F_2$and BC_1F_2populations were presented in Table 3.

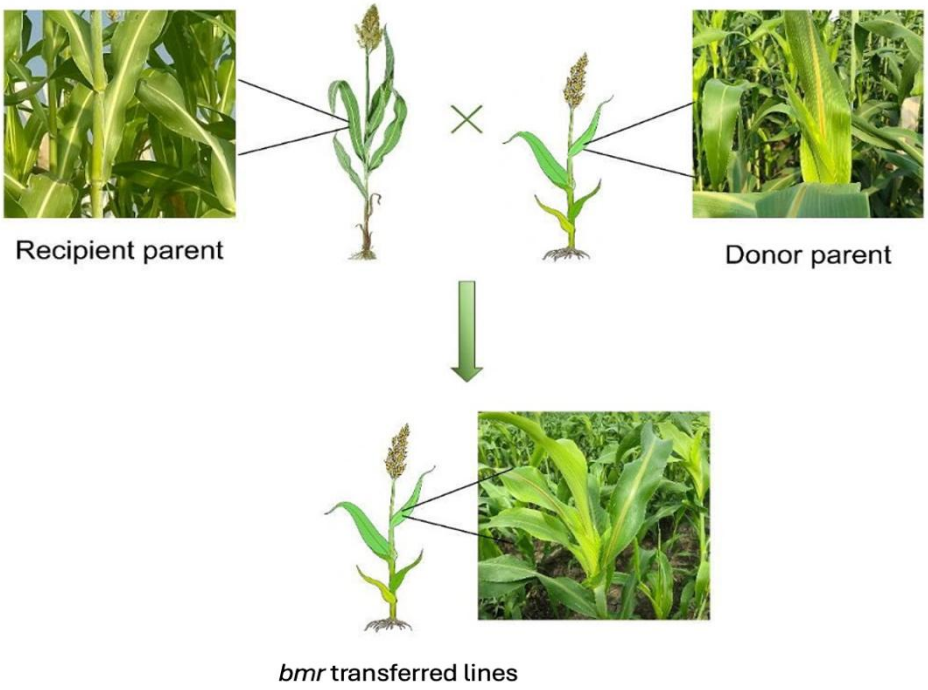


Figure1-Display of the recurrent and the donor parents along with *bmr* transferredlines

Table 3:Phenotypic dataof *bmr6and bmr12* in F_2 and BC_1F_2 Populations

S. N.	Cross Combination	Stage	*bmr*	*bmr* plants	Bmr Plants	Total Plants
1	SSV84 × N609	F_2	*bmr6*	7	15	22
2	ICSV18003 × N609	F_2	*bmr6*	3	19	22
3	ICSV15024 × N609	F_2	*bmr6*	3	18	21
4	{(SSV84 × N609)× SSV84}	BC_1F_2	*bmr6*	28	180	208
7	{(ICSV18003 × N609)× ICSV18003}	BC_1F_2	*bmr6*	10	46	56
8	{(ICSV15024 × N609)× ICSV15024}	BC_1F_2	*bmr6*	35	200	235

1	SSV84 × ICSV101039	F_2	*bmr12*	6	15	21
2	ICSB474 × ICSV101039	F_2	*bmr12*	5	16	21
3	ICSB474 × N600	F_2	*bmr12*	3	17	20
4	ICSV100324× ICSV101039	F_2	*bmr12*	5	16	21
5	{(SSV84 × ICSV101039)× SSV84}	BC_1F_2	*bmr12*	13	103	116
6	{(ICSB474 × ICSV101039)× ICSB474}	BC_1F_2	*bmr12*	8	49	57
7	{(ICSB474 × N600)× ICSB474}	BC_1F_2	*bmr12*	12	69	81
8	{(ICSV100324× ICSV101039) ×ICSV100324}	BC_1F_2	*bmr12*	103	311	414

3.3 KASPar assays for *bmr6* specific SNP

The parent N609 harbors homozygous SNP mutation for *bmr6* as expected and the SSV84 parent is WT. The resulting F_1- SSV84 ×N609 was heterozygous for *bmr6*, and similarresults were obtained with the other recipient recurrent parents ICSV18003 and ICSV15024 (Table 4). The F_1 individuals with *bmr*locus in heterozygous state were further backcrossedto increase the dose of the recurrent parents.

Table 4. Marker assisted *bmr6*diagnostics and development of*bmr6*transferredpopulations.

S.No	Parent/Cross	Generation	snpSB00519_CALL	Status
1	SSV84	RP	G:G	Polymorphic
2	N609	DP	A:A	Polymorphic
3	SSV84 × N609	F_1	A:G	Polymorphic
4	{(SSV84 × N609)× SSV84}	BC_1F_1	A:G	Polymorphic
5	{(SSV84 × N609) ×(SSV84)× (SSV84)}	BC_2F_1	Advanced for BC_2F_2	
6	ICSV18003	RP	G:G	Polymorphic
7	N609	DP	A:A	Polymorphic
8	ICSV18003 × N609	F_1	A:G	Polymorphic
9	{(ICSV18003 × N609)× ICSV18003}	BC_1F_1	A:G	Polymorphic
10	{(ICSV18003× N609) ×(ICSV18003)× (ICSV18003)}	BC_2F_1	Advanced for BC_2F_2	
11	ICSV15024	RP	G:G	Polymorphic
12	N609	DP	A:A	Polymorphic
13	ICSV15024 × N609	F_1	A:G	Polymorphic
14	{(ICSVI5024 × N609) × ICSV15024}	BC_1F_1	A:G	Polymorphic
15	{(ICSV15024 × N609)×(ICSV15024)×(ICSV15024)}	BC_2F_1	Advanced for BC_2F_2	

RP-Recipient or recurrentparent, DP-Donor parent, F_1-F_1generation without back cross,BC_1F_1-F_1 generation with one back cross, BC_2F_1- F_1generation with two backcrosses, snpSB00519_CALLKASP SNP (BMR6-132) marker for bmr6

3.4Development of *bmr12* hybrids through marker-assisted and phenotypic backcrossing

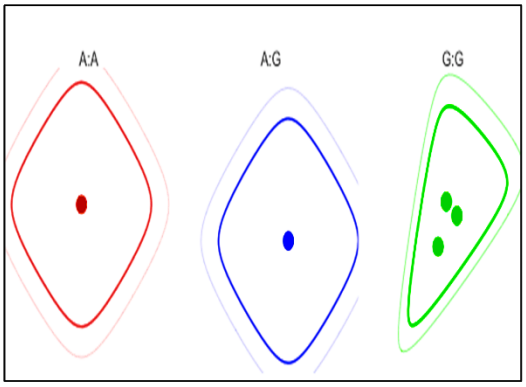
The brown midrib trait, as conferred by *bmr12*expressed in ICSV101039 and N600 (sources), was successfully introgressed into three sweet/high-biomass sorghum lines for developing superior elite bmr transferred lines expressing the *bmr* trait. Foreground marker-assisted selection using KASPar SNP markers was not successful due to lack of polymorphism in our populations. The successful *bmr12* transfer was therefore confirmed by the monohybrid 3:1 Mendelian segregation approach in F_2s and BC-F_2s, accounting for the recessiveness of the *bmr12*allele (Table 5).

Table -5- Development of *bmr12*transferred lines

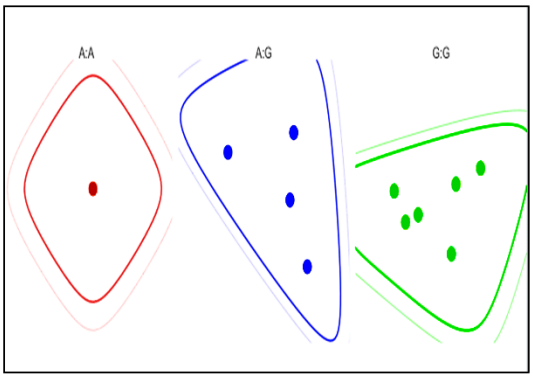
S. No	Cross Combinations	Population	Trait	BC_2F_1 Advanced
1	{(SSV84 × ICSV101039) × (SSV84)×(SSV84)}	BC_2F_1	*bmr12*	BC_2F_2
2	{(ICSB474 × ICSV101039) × (ICSB474)× (ICSB474)}	BC_2F_1	*bmr12*	BC_2F_2
3	(ICSB474 × N600) ×(ICSB474) ×(ICSB474))}	BC_2F_1	*bmr12*	BC_2F_2
4	{(ICSV100324 × ICSV101039) ×(ICSV100324)× (ICSV100324)}	BC_2F_1	*bmr12*	BC_2F_2

3.5Foreground marker-assisted selection for *bmr6*

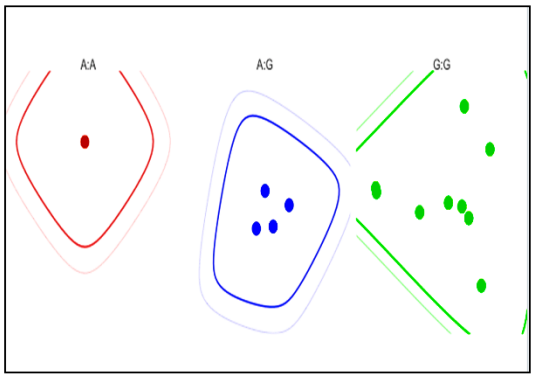
Six recurrent parent (SSV84) plants were crossed withsix (6) donor parent (N609)plants inthe first cross combination (SSV84 × N609), and three true hybrids were obtained in F_1 generation. Similarly for the other recurrent parents, four plants ICSV18003 and seven plants ICSV15024 were crossed with the respective number of donor parents N609 to produce two and four true hybrids in F_1 generation, respectively. Further, the obtained true hybrids were backcrossed with the respective recurrent parent resulting in three true hybrids for {(SSV84×N609)×SSV84}, one in {(ICSV18003 ×N609) ×ICSV18003} and four in {(ICSV15024 × N609)× ICSV15024}in the BC_1F_1 generation (Fig. 2).



(a) BC_1F_1 - {(ICSV18003 × N609) × ICSV18003}



(b) BC_1F_1 - {(ICSV15024 × N609) × ICSV15024}



(c) BC_1F_1 - {(SSV84 × N609) × SSV84}

Figure 2-Genotyping of BC_1F_1individuals with the KASP marker (snpSB00519).

From the above diagrammatic representation, *bmr* transferred plants were screened at BC_1F_1 stages using KASPar SNPs. The dots show polymorphism at a locus on the chromosome SBI 4. The red spots represent the homozygous gene (A: A) from the donor parents N609 (*bmr6*) and the blue spots represent the heterozygous allele (A: G) from both the parents, whereas green spots indicate the homozygous gene (G: G) from high-biomass parent and sweet sorghum recurrent parents *i.e.*, ICSV15024, and ICSV18003 and SSV84. Highest number of true hybrids were obtained in the BC_1F_1 cross {(ICSV15024 × N609**)** ×ICSV15024}and {(SSV84× N609) × SSV84} compared with BC_1F_1{(ICSV18003 × N609) × ICSV18003}.

3.6Proximatecomposition of the biomass

The genotypes expressing *bmr6* and *bmr12*matured earlier, showed less metabolizable energy and more ash relative to Bmr populations (Table 6). In addition, *bmr*12 genotypes showed higher cellulose concentration than Bmr populations and were shorter-statured than *bmr*6 genotypes. The two *bmr* alleles had no effect on other must-have and long-term sweet/high-biomass sorghum plant characteristics such as lignin concentration, cellulosic

content (NDF neutral detergent fiber), Brix, and stem girth. The results obtained from the chi-square analysis between the *bmr* and Bmr populations are presented in Table 6.

Table 6. Agronomic and quality traits in F_3 and wild type populations

Trait: Days to flowering								
Group	Means	Group1	Group2	Estimate	StdErr	DF	t Value	Pr > |t|
bmr12	64.50	*bmr12*	*bmr6*	-0.64	1.69	47	-0.38	0.7077
bmr6	65.14	*bmr12*	Bmr	-11.35	3.10	47	-3.66	0.0006
Bmr	75.85	*bmr6*	Bmr	-10.71	3.12	47	-3.43	0.0012
Trait: Days to maturity								
Group	Means	Group1	Group2	Estimate	StdErr	DF	t Value	Pr > |t|
bmr12	86.59	*bmr12*	*bmr6*	-1.28	1.55	47	-0.83	0.4131
bmr6	87.87	*bmr12*	Bmr	-10.68	2.83	47	-3.77	0.0005
Bmr	97.27	*bmr6*	Bmr	-9.40	2.85	47	-3.29	0.0019
Trait: Plant height								
Group	Means	Group1	Group2	Estimate	StdErr	DF	t Value	Pr > |t|
bmr12	270.72	*bmr12*	*bmr6*	-19.30	8.56	47	-2.26	0.0288
bmr6	290.03	*bmr12*	Bmr	-26.14	15.66	47	-1.67	0.1017
Bmr	296.86	*bmr6*	Bmr	-6.84	15.76	47	-0.43	0.6663
Trait: Stem Girth								
Group	Means	Group1	Group2	Estimate	StdErr	DF	t Value	Pr > |t|
bmr12	19.93	*bmr12*	*bmr6*	0.74	0.70	47	1.06	0.2961
bmr6	19.18	*bmr12*	Bmr	2.28	1.29	47	1.78	0.0824
Bmr	17.65	*bmr6*	Bmr	1.54	1.29	47	1.19	0.2401
Trait: Metabolizable energy								
Group	Means	Group1	Group2	Estimate	StdErr	DF	t Value	Pr > |t|
bmr12	6.82	*bmr12*	*bmr6*	-0.16	0.18	47	-0.87	0.3905
bmr6	6.98	*bmr12*	Bmr	-0.82	0.33	47	-2.5	0.0158
Bmr	7.64	*bmr6*	Bmr	-0.67	0.33	47	-2.02	0.0494
Trait: ADF								
Group	Means	Group1	Group2	Estimate	StdErr	DF	t Value	Pr > |t|
bmr12	47.59	*bmr12*	*bmr6*	1.73	1.27	47	1.37	0.1783
bmr6	45.86	*bmr12*	Bmr	6.11	2.32	47	2.63	0.0115
Bmr	41.48	*bmr6*	Bmr	4.38	2.34	47	1.87	0.0673
Trait: ADL								
Group	Means	Group1	Group2	Estimate	StdErr	DF	t Value	Pr > |t|
bmr12	5.45	*bmr12*	*bmr6*	0.44	0.25	47	1.74	0.0883
bmr6	5.01	*bmr12*	Bmr	0.41	0.46	47	0.87	0.3874
Bmr	5.04	*bmr6*	Bmr	-0.04	0.47	47	-0.08	0.9379
Trait: Ash								
Group	Means	Group1	Group2	Estimate	StdErr	DF	t Value	Pr > |t|
bmr12	9.30	*bmr12*	*bmr6*	0.42	0.65	47	0.65	0.5198
bmr6	8.88	*bmr12*	Bmr	3.13	1.19	47	2.63	0.0115
Bmr	6.17	*bmr6*	Bmr	2.71	1.20	47	2.26	0.0284
Trait: Brix								
Group	Means	Group1	Group2	Estimate	StdErr	DF	t Value	Pr > |t|
bmr12	13.98	*bmr12*	*bmr6*	0.11	0.37	47	0.29	0.7746

bmr6	13.88	*bmr12*	Bmr	0.01	0.67	47	0.02	0.9828
Bmr	13.97	*bmr6*	Bmr	-0.09	0.68	47	-0.13	0.8933
Trait: NDF								
Group	Means	Group1	Group2	Estimate	StdErr	DF	t Value	Pr > |t|
bmr12	72.53	*bmr12*	*bmr6*	1.70	1.53	47	1.11	0.2724
bmr6	70.83	*bmr12*	Bmr	5.48	2.80	47	1.96	0.0559
Bmr	67.05	*bmr6*	Bmr	3.79	2.82	47	1.34	0.1852
Trait: Nitrogen								
Group	Means	Group1	Group2	Estimate	StdErr	DF	t Value	Pr > |t|
bmr12	0.49	*bmr12*	*bmr6*	0.00	0.05	47	0.03	0.9749
bmr6	0.49	*bmr12*	Bmr	0.13	0.10	47	1.34	0.1871
Bmr	0.36	*bmr6*	Bmr	0.13	0.10	47	1.31	0.1956
Trait: IVOMD								
Group	Means	Group1	Group2	Estimate	StdErr	DF	t Value	Pr > |t|
bmr12	47.06	*bmr12*	*bmr6*	1.07	1.67	47	0.64	0.5259
bmr6	45.99	*bmr12*	Bmr	0.28	3.05	47	0.09	0.9285
Bmr	46.78	*bmr6*	Bmr	-0.79	3.07	47	-0.26	0.798
Trait: Cellulose								
Group	Means	Group1	Group2	Estimate	StdErr	DF	t Value	Pr > |t|
bmr12	41.78	*bmr12*	*bmr6*	0.89	1.23	47	0.73	0.4712
bmr6	40.88	*bmr12*	Bmr	4.84	2.24	47	2.16	0.0363
Bmr	36.94	*bmr6*	Bmr	3.95	2.26	47	1.75	0.0872
Trait: Hemicellulose								
Group	Means	Group1	Group2	Estimate	StdErr	DF	t Value	Pr > |t|
bmr12	25.26	*bmr12*	*bmr6*	0.22	0.65	47	0.34	0.7358
bmr6	25.04	*bmr12*	Bmr	0.11	1.18	47	0.09	0.9261
Bmr	25.15	*bmr6*	Bmr	-0.11	1.19	47	-0.09	0.9273

ADF-Acid detergent fibre, ADL- Acid detergent lignin, NDF- Neutral detergent fibre, IVOMD-Invitro organic matter digestibility

3.7 Discussion

Historically, sorghum was mostly grown for grain and forage (Hoffmann and Rooney., 2013). Grain sorghums are generally shorter (usually having recessive alleles at three of the four Dw genes) relative to biomass sorghums (having recessive alleles at two Dw genes at most), and have been selected to have the grain as the primary sink for photosynthates. Biomass sorghum includes high-biomass sorghum and sweet sorghum (Damasceno *et al*., 2014; Hoffmann and Rooney., 2013; Prakasham *et al*.,2014). Sweet sorghum translocates photosynthates to seeds and stem, their stems are juicy (d recessive to D) instead of dry, and sweet (x recessive to X) instead of nonsweet (Rooney, 2000). Sweet sorghums are high biomass and sugar yielding crops and were traditionally bred for syrup or molasses production. High-biomass and sweet sorghum are mainly bred for sustainable renewable feedstock production targeting different market segments with particular focus on

first and second generation biofuels(Kanbar *et al.,* 2020). The sustainability of sorghum cultivation is imparted by the resilience of this crop to climate adversities, adaptability to harsh environments, resource use efficiency particularly nitrogen use efficiency, and adaptability across latitudes (Mullet *et al.,* 2014, Habyarimana *et al.,* 2020). Sweet/High-biomass sorghumsgive an increasedenergy yield and net gain than sugar beet (*Beta vulgaris* L.) andhave a more net energy balance compared withother plants like grain sorghum and maize (Palumbo *et al.,* 2014). Furthermore, recent studies (Bhattarai et al., 2020) confirmed sorghum's higher silage yield and water use efficiency compared to pearl millet and corn under limited irrigation conditions.

Brown midrib (*bmr*) 6 and 12 in sorghum are loss-of-function mutations that impair the last two enzymatic steps of monolignol synthesis, resulting in reduction of the amount of lignin of diverse structure in the cell wall, particularly in forage sorghums (Sattler et al., 2010). In the present study, we aimed at transferring the *bmr6*and *bmr12*alleles into SS/HBM lines for developing new elite ideotypes with improved agronomic and quality traits for the biofuel market segment. Marker-assisted introgression of the *bmr6*and *bmr12*was performed using four (SSV84, ICSB474, ICSV18003, ICSV100324) sweet sorghum and one (ICSV15024) high-biomass sorghum wild type (WT) lines as recurrent parents, and one and two *bmr6* (N609) and *bmr12*(ICSV101039 and N600) lines, respectively, as donor parents.According to da Silva (2020), sorghum plants can be visually recognised and categorised as either *bmr*or WT phenotypesas early as the 8-leaf growth stage. In this study, we were able to visually identify *bmr* trait in the field at a relatively later growth stage compared to da Silva i.e., at 30-35 days after planting. At this growth and development stage, sorghum plants are 12 to 15 inches tall, have more than eight leaves and, are starting the growing point differentiation that occurs typically at 30 to 40 days after emergence. Clearly, the importance of early generation testing was confirmed in this study and can be recommended in other *bmr* transfer experiments including: (1) crossing and selection in the segregating populations, and (2) conventional and (3) marker-assisted backcrossing.

Marker-assisted foreground selection showed the potential to speed up the development of near isogenic lines in sorghum and other crops in 3 years as opposed to 5–6 years using the conventional backcrossing strategy. This was demonstrated in our study and has the promise to increase genetic gain per unit time and cost. The *bmr6* mutation induces change from CAG to T/UAG at amino acid position 132(Glutamine)of the CADgene, resulting in a stop codon (Sattler *et al*. 2009; Li et al., 2015).Similarly, the *bmr12*mutation induces change from CAG to T/UAG at amino acid position 129(Glutamine) of the COMT

gene, resulting in a stop codon (Bout and Vermerris, 2003). Relative to the WT, *bmr6* sorghum mutants display a significant reduction in all three main lignin subunits, p-hydroxyphenyl (H), G, S in the magnitude of 4.8-, 7.3-, and 17.7-fold, respectively; the most significant reduction being in S-lignin, which leads to a reduced S:G ratio (Sattler *et al*., 2009). Thus far, *bmr6*, *Bmr12*, and *bmr18* are among the known *bmr* mutants that can be grown well in sorghum (Kamireddy *et al.*, 2013). According to Oliver *et al*. (2005), Oliveira *et al.* (2020) and Espinal (2009), the allelic genes *bmr12*and *bmr6* inhibit the activity of the enzymes cinnamyl alcohol dehydrogenase (CAD) and caffeic acid o-methyltransferase (COMT) with significant effects on sorghum fiber components. The above was the set of working hypotheses of this work. For each gene, heterozygotes were easily distinguished, and the observed F_2 phenotypes matched the identified genotypic conditions. In the present study, the *bmr6* gene was transferred successfully in sweet sorghum/high-biomass lines and identified in BC_1F_1 generation at chromosome 4 position by utilizing the KASP marker SNP-SB00519.The *bmr12*was transferred using classic approach of backcrossing coupled with selfed progeny to trace back the true heterozygous F_1. The development of *bmr12*marker is underway in our laboratory to assist both the transfer of single alleles as well as the pyramiding of *bmr6* and *bmr12*in the same genetic background.

The genotypes expressing *bmr6* and *bmr12*matured earlier, showed less metabolizable energy and more ash relative to Bmr populations (Table 6). In addition, *bmr12*genotypes showed higher cellulose concentration than Bmrpopulations and were shorter-statured than *bmr6*genotypes. The two*bmr* alleles had no effect on other must-have sweet/high-biomass sorghum plant characteristics such as lignin concentration, cellulosic content (NDF neutral detergent fiber), Brix, and stem girth, implying their good safety for deployment in sorghum breeding pipelines.In this study, the comparative lignin concentration between *bmr* and Bmr populations was not expected (Barrière *et al.*, 2007 and Saballos *et al*. 2009) since the bmr mutations are usually associated with reduced lignin in several cereal crops like sorghum, maize and pearl millet (Kuc and Nelson, 1964).

Our hypothesis was that SS/HBM would behave like forage sorghums (Barrière *et al.*, 2007 and Saballos *et al*. 2009) and it was expected that *bmr* genotypes would express reduced lignin concentration as in forage sorghums (Sattler *et al.*,2010; Oliveira*et al.*, 2020; Wahyono *et al*., 2021). However, our findings are supported by previous research works showing that the bmr mutations can reduce the concentration of lignin in the sorghum biomass, but the pattern was not consistent because there are instances where lignin was not reduced and other instances where traits other than lignin were modified in the recurrent parents (Oliver *et al*.,

2005). More research is needed including using other *bmr*alleles and different sorghum types, in order to get insight into the interaction *bmr*× sweet SS/HBM sweet stem and optimize lignin concentration in this sorghum market segment. In addition, the investigation of the effects of *bmr* mutations on the lignin structure is in order to decipher the extent to which the biomass digestibility can be significantly improved by structural changes in lignin units instead of ponderal reduction of the lignin concentration in the cell wall.

Remarkably, we were able to observe that the transferred*bmr12* improved the cellulose content relative to WT genotypes, and *bmr12* populations were shorter-statured relative to *bmr6*individuals, meaning that *bmr12* gene can be considered in sorghum breeding to boost 2G bioethanol bioconversion and to control lodging, and hence favoring mechanical harvesting and limiting yield losses (Habyarimana*et al.,* 2016, Tse *et al.*, 2021). The observed earliness in the *bmr*lines is another attractive trait as it confers adaptation in the drylands, particularly in the semi-arid tropics characterized by terminal drought stress (Shavrukov *et al.,* 2017). Both *bmr*alleles did not show any deleterious effects on must have traits (e.g., Brix, stem girth, NDF, etc.) and hence they can be safely deployed in sorghum breeding. The observed increased ash in *bmr vs. Bmr* populations can have mixed evaluation. Biomass ash is naturally alkaline which tends to lower the fusion point of ashes leading to fouling, slagging and poor combustion system performance (Carrillo *et al.,* 2014), meaning that *bmr6*and *bmr12*sorghums should not be considered as good candidate for combustion-derived biofuels. However, ash being alkaline and containing phosphorus, potassium, calcium, boron, and other mineral elements,*bmr6*and *bmr12*sorghumsash by-product can be used as to improve soil health and for growing plants healthily. In this work however, the ash percentage was not more than 10%. The requirement for ash content in raw material for bioethanol production is 10% or less, which is accomplished by the*bmr* lines produced in this study. Higher ash concentration beyond 10% is usually undesirable as it might impede the fermentation process and result in crust on the tool during the distillation process (Carrillo *et al.,* 2014). It can therefore be inferred that the *bmr6*and*bmr12*allelescan safelybe deployed in sorghum breeding for bioenergy production purposes.

4. Conclusions

The importance of early generation testing was confirmed in this study and can be suggested in other *bmr* transfer experiments. The *bmr6*and*bmr12*alleles were successfully transferredand true F_{1s} were identified inbackcrosses using SNP markers and phenotypic segregation in F_2s. The transfer of *bmr12* improved the cellulose content relative to WT genotypes, and *bmr12* populations were shorter-statured relative to *bmr6*individuals, meaning

that *bmr12* alleles can be considered in sorghum breeding to boost 2G bioethanol bioconversion and to control lodging, and hence favoring mechanical harvesting and limiting yield losses. The observed earliness in the *bmr*lines is an attractive trait conferring adaptation in the drylands, particularly in the semi-arid tropics characterized by terminal drought stress. Both *bmr*alleles did not show any negative trade-offs relative to must have traits in sorghum, implying that they can safely be deployed in sorghum breeding for bioenergy production purposes.The produced transferred lines can be used to directly pyramid *bmr6*and*bmr12*in the same genetic background, further improving the listed *bmr*-conferred benefits. The *bmr* molecular markers successfully used in this work will be published in the public domain and are expected to help breed sorghum products other than SS/HBM types such as high-quality forage sorghums.

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