LW7 marker application for sweet sorghum breeding in BC_3F_2

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ABSTRACT: In conventional breeding, test cross of segregating populations is required to identify the B-line plants during line development processes. The objectives of this study were to apply the LW7 molecular marker associated with the restoration of male fertility gene in sorghum to facilitate the selection for B-line plants in the BC_3F_2 generation of the cross between grain sorghum "KU03B" (maintainer line or B-line) and sweet sorghum "SSV84" (restorer line or R-line), and to use phenotypic selection of individual plants from F_2 to BC_3F_2 generations of backcrossing for total soluble solid (TSS) and other agronomic traits. The LW7 marker successfully identified B-line plants in the BC_3F_2 generation with the ratio of 1 sterile plant to 3 fertile plants. On the successive backcrossing for four generations, segregation patterns of the backcross generations shifted towards high TSS, indicating that backcrossing increased the chance to obtain the B-lines with higher TSS. Based on B-line, TSS and other agronomic traits, 23 plants in the BC_3F_2 generation were selected for further conversion to A3 cytoplasm specific to *rf4* gene and hybrid development.
Keywords: backcrossing, male sterility, phenotypic selection, segregation pattern, brix value

Introduction

High price of fossil fuels increases the importance of energy from renewable sources. The use of bio-ethanol from fermentation of agricultural crop such as sugarcane (Pfeiffer et al., 2010; Ratnavathi et al., 2011; Zhong et al., 2010; Reddy et al., 2008; Ali et al., 2008) corn (Ali et al., 2008; Reddy et al., 2008; Hettinga et al., 2009; Wang et al., 2009a; Person et al., 2010) and cassava (Zhong et al., 2010) for transportation fuel is successful in many countries. These agricultural crops can serve human as food and fuel (Ratnavathi et al., 2011). Therefore, shortage of raw materials and food security are major concerns. The use of alternative or supplement crops is thus important for production efficiency of bio-ethanol industry and increase in food security. Sweet sorghum [*Sorghum bicolor* (L.) Moench] is a candidate crop for use as an alternative feedstock for ethanol production because of its sweet juice in the stems (Ratnavathi et al., 2011), high biomass production (Wang et al., 2009b) and early maturity (Prasad et al., 2007; Ratnavathi et al., 2011). The crop is therefore suitable to fill the gap of raw material supply chain especially during the shortage of raw materials.

More recently, research on sweet sorghum as energy crop has been carried out (Reddy et al., 2005; Zhong et al., 2010; Reddy et al., 2011). At initial stage of the research, pure line varieties were used for commercial production but sugar yield was rather low (Pfeiffer et al., 2010). Research on sweet sorghum hybrids has been carried out in China (Hana et al., 2011), United State (Pfeiffer et al., 2010; Peterson et al., 2012) and India (Reddy et al., 2005; Umakanth et al.,

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2012). Sorghum hybrid varieties showed considerable increase in biomass and sugar yield (Makanda et al., 2009; Pfeiffer et al., 2010; Umakanth et al., 2012; Peterson et al., 2012), and the use of hybrid varieties is a means to increase the potential of sweet sorghum as an energy crop.

Since the discovery of cytoplasmic genetic male sterility in sorghum (Stephen and Holland, 1954), male sterility facilitates development of commercial F_1 hybrids in grain sorghum. However, research on sweet sorghum hybrids has been initiated recently. Heterosis in sweet sorghum for brix and juice yield has been reported, and the hybrids could produce more syrup or ethanol than current pure lines (Makanda et al., 2009; Pfeiffer et al., 2010). In our earlier work, the hybrids L103/KKU 40 and L103/Urja were promising for stalk yield, juice yield and ethanol yield (Pothisoong and Jaisil, 2011). However, the development of these hybrids especially for development of B lines through conventional backcrossing is tedious, laborious and time-consuming. Molecular breeding to assist the development of B lines can reduce time and labor cost.

Recent development of molecular markers (LW7, LW8 and LW9) linked to male fertility gene (*Rf*4) in sorghum facilitates the use of molecular breeding for B lines development (Wen et al., 2002). The markers LW7, LW8 and LW9 were tested in our earlier study and LW7 was proven to be usable (Lunmat et al., 2008). LW7 was then used in screening of sweet sorghum breeding lines (B-line) from F_2 to BC_2F_2 generations (Bunphan et al., 2013), and at the same time, the B-lines were also selected based on agronomic traits performances.

In this study, screening of B-lines was finalized in the BC_3F_2 generation. The objective of this research was to use LW7 marker to identify B-line plants in the BC_3F_2 population and to use TSS as a selection criterion for sweetness in segregating generation from F_2 to BC_3F_2. The results of this study are important for sweet sorghum hybrid breeding aiming to improve yield and incorporate male sterility traits using conventional and molecular breeding methods.

Materials and Methods

Plant materials

In order to transfer male sterility from grain sorghum to sweet sorghum, a 'KU03B' maintainer line or B-line of grain sorghum was used as female parent, and it was crossed with a sweet sorghum cv. 'SSV84' (*Rf*4*Rf*4) or R-line. The line 'KU03B' was developed from Kasetsart University, Thailand. This line has recessive gene for male sterility in nucleus, but it has genes conferring male sterility and dwarfing in cytoplasm. The line 'SSV84' was kindly donated from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT). It is tall and has high sugar concentration and high stalk yield. The resulting F_1 hybrid was self-pollinated to generate a segregating F_2 population.

The experiment was initially conducted at the Field Crops Research Station, Khon Kaen University. In the rainy season 2009, 315 plants in the F_2 generation were planted with a spacing 50 cm between rows and 30 cm between plants within row and screened for B-lines using LW7 marker. The B-lines are referred to the plants with recessive gene for male sterility in the nucleus and male

fertile gene in cytoplasm. Individual plants were simultaneously evaluated for sweetness at harvesting stage using a portable hand-held refractometer. The data of TSS at flowering were used for selecting the plants for backcrossing. LW7 marker was used for screening the plants for male sterility gene using as described below.

DNA extraction

The plants with one-month old were used for DNA extraction. The entries consisted of the single plants in the F_2 population derived from the cross of 'KU03B' with 'SSV84'. The BC_3F_2 population was used for screening of progenies that carry male sterile genes in nucleus.

The extraction method was described previously (Dellaporta et al., 1983), leaf samples of about 3 gram for each sample were collected from the plants with 3-4 week old grown in the field, and the samples were snap-frozen in liquid nitrogen. The DNA extraction method was used with minor alteration to extract DNA. 1% agarose gel electrophoresis was used to cross checked quantity and quality of DNA compared to λ-DNA, standard check.

PCR conditions and gel electrophoresis

PCR was carried out in 20 μl reaction volume containing 50 ng of DNA template, 1X PCR buffer, 0.2 mM dNTPs, 1.8 mM $MgCl_2$, 1 Unit *Taq* polymerase, 0.25μl E-CTA/5'-GATCTACATATGT-GGAGCTC-3' and M CAC/5' AGATCTCGGCGCT-GCGGAGCAGAA-3' of LW7 marker (Wen et al. 2002) and 6.96 μl of dH_2O.

The standardized amplification/PCR condition was set with minor modification at pre-denaturation at 95 °C for 3 minutes, 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 60 seconds (changed from 60 °C for 90 seconds), extension at 72 °C for 60 seconds (changed from 120 seconds) followed by final extension at 72 °C for 5 minutes.

The amplified PCR products were then separated on 2% agarose gel electrophoresis. The amplified fragments of LW7 marker were scored as "1" and "0", where "1" indicated the presence of specific allele (band) and "0" indicated its absence at 779 bp (Wen et al., 2002). The data of PCR reactions were recorded as "1" or "0" for the presence of 779 bp or absence of the bands, respectively.

Once genotypes of the plants were known, only B-line plants were backcrossed with 'SSV84' to generate BC_1F_1 hybrid. At harvest, the individual plants were selected base on high TSS. The seeds of the BC_1F_1 plants were grown, and the same procedure of backcrossing was repeated to generate the segregating BC_1F_2 population.

The process of identifying B-line plants, phenotyping for TSS, backcrossing the B-line plants and high soluble solid with 'SSV84' line and generating the segregating population were repeated in 2010, 2011 and 2012. In summary, the plants used for screening were 315 in F_2 population (in 2009), 622 in the BC_1F_2 population (in 2010) and 537 in the BC_2F_2 population (in 2011) and 141 in the BC_3F_2 population (2012). However, for each generation, some plants did not survive at harvest. Finally, 43 B-line plants in the BC_3F_2 generation were selected, and only 23 plants had high soluble solid.

Phenotypic measurements

Phenotypic characters of individual plants in segregating populations from F_2 generation to BC_3F_2 generation were measured at physiological maturity of individual plants which was 30 days after 50% flowering. In sweet sorghum, this growth stage is associated with the highest accumulation of sugar in the stem. In the F_2 and BC_1F_2 generations, only TSS was measured. However, in the later generations for BC_2F_2 and BC_3F_2, the data were also recorded for days to flowering, plant height, stalk diameter, stripped stalk weight and TSS.

At harvest, the stems were cut at the soil surface and transported immediately to the shelter. Then the traits were measured in the shelter. Plant height in cm was measured from the ground surface to the panicle tip. Stem diameter in millimeters was measured using digital vernier caliper at middle inter-node in single plant. Stripped stalk weight in g/plant of whole plant removed panicle, fresh leaves and leave sheath was determined using a digital balance. Total soluble was measured with a hand-held refractometer from the composite juice sample from three positions along the stems at the third inter-node from base, middle of the stems and tip of the stems, and the single value of each plant was averaged from three positions.

Data analysis

The data of segregating population was used for selecting B-line plants. The binary data of PCR reactions for 141 plants the BC_3F_2 population were analyzed using Chi-square (χ^2) test (Gomez and Gomez, 1984) as follow;

$\chi^2 = \sum_{i=1}^{k} \frac{(Oi-Ei)^2}{Oi}$, where χ^2 = Chi-square, O_i = the frequencies of observed value, E_i = the frequencies of expected value. The Chi-square test would verify the segregating models of the progenies.

Table 1 Segregation ratio of non B-lines to B-line plants of sweet sorghum in the BC_3F_2 population.

Generation	Total	Expected ratio	Observed ratio	Test ratio	c^2	Probability (0.01)
BC_3F_2	141	106:35	98:43	3:1	1.82*ns*	0.25-0.10

ns=non-significant

Table 2 Total soluble solid (TSS) of selected B- lines in F_2, BC_1F_2, BC_2F_2 and BC_3F_2 generations

F_2		BC_1F_2		BC_2F_2		BC_3F_2	
Selected lines no.	TSS (°brix)	Selected lines no.	TSS (°brix)	Selected lines no.	TSS (° brix)	Selected lines no.	TSS (° brix)
2	16.7	26	18.3	13	15.6	1	14.0
14	17.4	250	16.3	24	16.8	11	14.0
50	14.5	272	15.7	35	14.0	12	19.0
56	18.7	284	18.0	38	15.0	14	16.0
58	19.3	310	16.7	39	13.0	24	17.0
60	20.0	317	16.3	42	16.0	26	15.0
62	16.0	344	16.0	44	17.0	43	17.0
68	16.0	345	17.0	50	13.0	45	15.4
75	15.0	359	14.3	74	14.0	48	16.0
89	17.0	360	14.3	85	13.6	53	15.0
90	20.0	362	15.3	115	17.4	54	17.0
96	15.0	371	18.0	122	18.0	57	17.0
97	16.0	375	17.3	123	19.0	59	18.0
106	15.0	377	15.7	125	13.0	64	15.0
123	14.5	392	18.3	130	15.4	65	15.0
141	16.7	396	15.5	153	16.6	87	13.4
158	16.0	410	16.1	154	16.4	90	13.0
160	19.0	417	18.0	202	15.0	99	14.0
163	18.0	420	18.0	212	15.6	103	14.0
173	16.0	421	17.7	217	15.0	105	15.0
195	17.7	423	18.3	222	15.0	111	16.0
237	17.0	463	17.4	320	14.0	120	16.2
264	16.0	541	17.8			139	14.0
Total plants	275		401		204		104
Mean	16.8		16.8		15.4		15.5
S.D.	4.30		3.20		2.85		2.82

S.D. = Standard deviation

Table 3 Days to flowering, plant height, stalk diameter and stripped stalk weight of selected B-lines in BC_2F_2 and BC_3F_2 generations

BC_2F_2					BC_3F_2				
Selected lines no.	50 % days to flowering	Plant height (cm)	Stalk diameter (mm)	Stripped stalk weight (g/pl)	Selected lines no.	50 % days to flowering (days)	Plant height (cm)	Stalk diameter (mm)	Stripped stalk weight (g/pl)
13	93	245.0	14.89	503.36	1	75	215.0	14.12	322.62
24	72	220.0	14.60	377.31	11	84	228.0	12.61	211.55
35	83	224.0	16.05	374.75	12	71	288.0	18.03	663.52
38	70	205.0	11.40	188.49	14	71	311.0	20.40	843.46
39	87	203.0	10.94	186.55	24	79	241.0	12.70	274.93
42	80	305.0	16.61	632.69	26	74	215.0	26.06	923.82
44	83	238.0	14.27	398.89	43	77	165.0	13.37	293.4
50	71	200.0	12.60	279.28	45	77	224.0	9.97	348.59
74	78	272.0	13.66	318.56	48	73	281.0	16.18	484.76
85	74	223.0	16.32	371.19	53	87	280.0	15.24	375.59
115	71	255.0	13.42	313.28	54	88	280.0	18.27	624.55
122	84	217.8	13.55	282.05	57	86	260.0	16.90	563.92
123	74	292.0	26.94	573.83	59	74	265.0	15.39	300.73
125	90	215.0	18.12	419.49	64	76	281.0	14.47	332.43
130	80	213.0	12.83	185.10	65	82	288.0	14.51	342.72
153	68	215.0	13.53	299.41	87	79	274.0	17.12	531.45
154	71	242.0	14.23	458.57	90	81	265.0	12.57	195.57
202	90	229.4	13.32	263.11	99	84	220.0	16.32	414.35
212	71	258.2	16.49	304.61	103	76	287.0	15.29	328.65
217	79	275.8	16.88	488.65	105	81	304.0	19.76	780.18
222	72	275.0	14.35	423.91	111	82	228.0	14.87	365.15
320	64	211.0	15.61	435.02	120	83	277.0	19.56	691.15
					139	76	305.0	18.06	669.66
Total	204	204	204	204	104	104	104	104	104
Mean	78	237.9	15.02	366.28	Mean	79	260.1	16.16	473.16
S.D.	12	46.05	3.88	230.75	S.D.	8	49.68	3.48	251.84

S.D. = Standard deviation

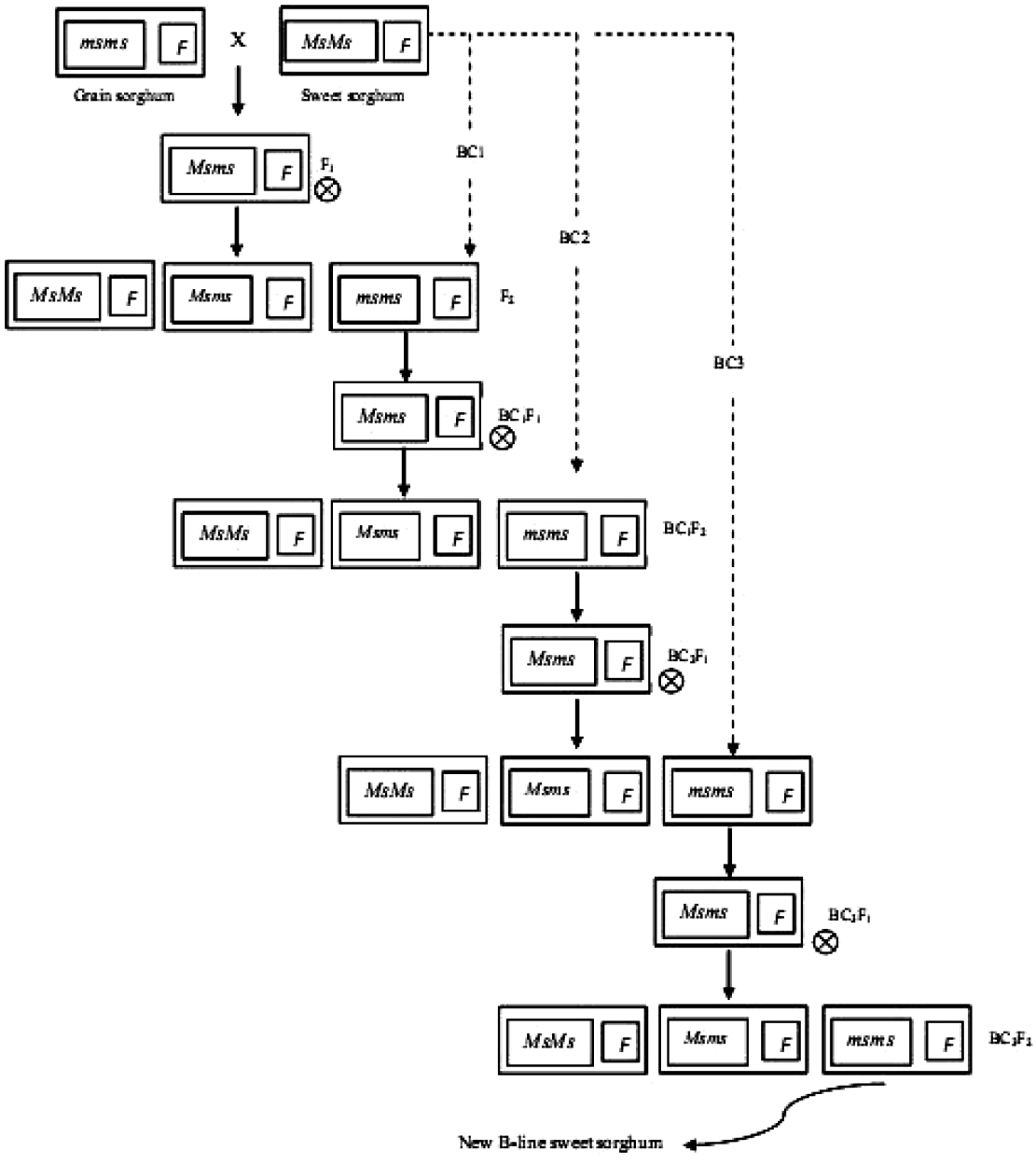


Figure 1 The schematic development of new B-line sweet sorghum using backcrossing method

Note: *MsMs*-male fertile, *msms*-male sterile, *F*-fertile, *S*-sterile

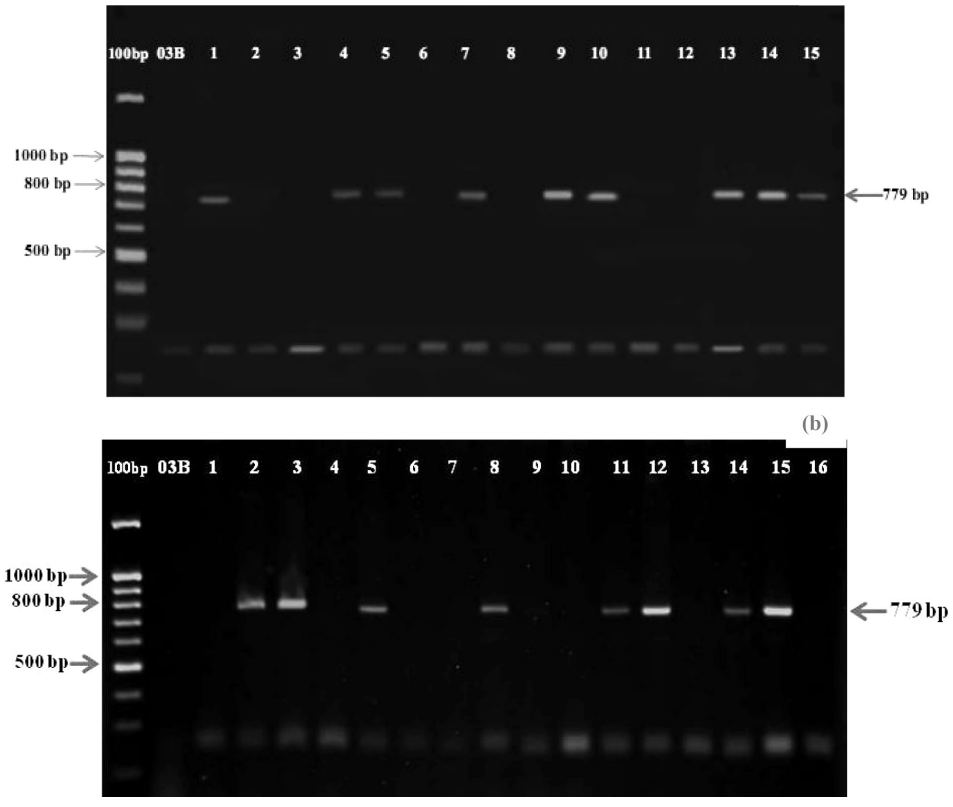


Figure 2 Agarose gel electrophoresis of DNA fragments of individual plants in the BC_3F_2 generation obtained from PCR reactions of LW7 marker

Note: 03B-KU03 B, a B line grain sorghum used as a check line

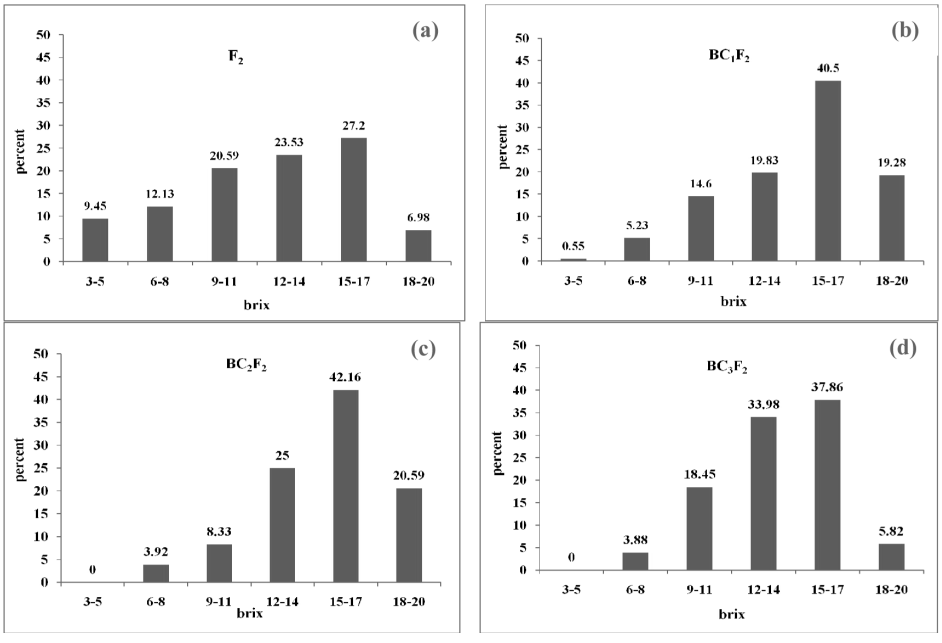


Figure 3 Distribution of TSS (°brix) in the segregating population at harvesting days in F_2 (a), BC_1F_2 (b), BC_2F_2 (c) and BC_3F_2 (d)

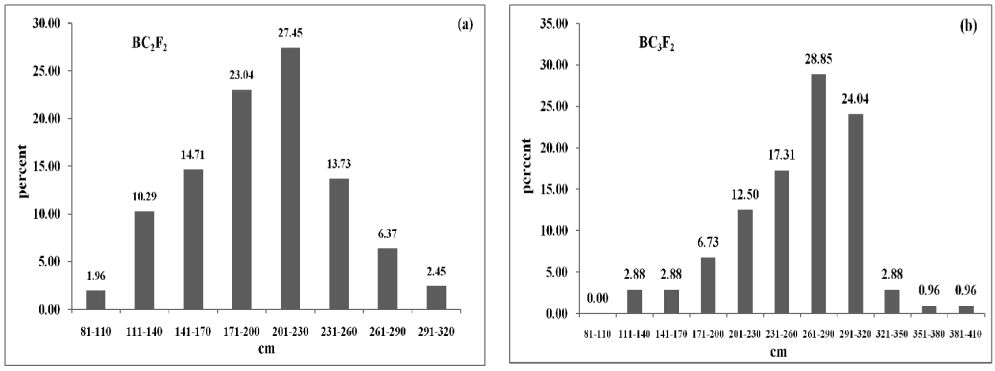


Figure 4 Distribution of plant height in the segregating population at harvesting days in BC_2F_2 and BC_3F_2

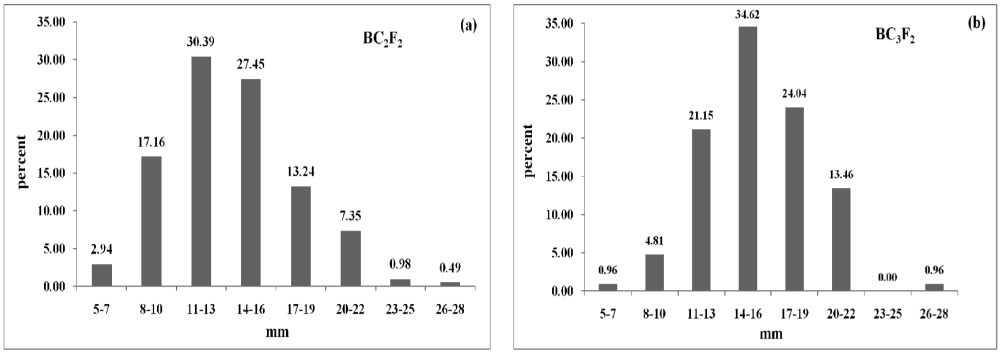


Figure 5 Distribution of stalk diameter in the segregating population at harvesting days in BC_2F_2 and BC_3F_2

Results

LW7 marker was employed in four consecutive generations (F_2, BC_1F_2, BC_2F_2 and BC_3F_2) from the cross of a B-line 'KU03B' of grain sorghum with low sugar concentration and a high yielding and sugar concentration sweet sorghum 'SSV84'. The schematic diagram for backcrossing and selection is presented in **Figure 1**. The genotypic data using molecular selection from F_2, BC_1F_2 and BC_2F_2 generations were reported previously (Bunphan et al. 2013). The data reported herein included genotypic data in the BC_3F_2 generation and phenotypic data in the F_2, BC_1F_2, BC_2F_2 and BC_3F_2 generations.

In the BC_3F_2, 141 plants were screened, and 98 plants show the band of 779 bp but 43 plants did not (**Figure 2**). The segregation ratio of 3:1 also conformed to Mendelian segregation for one gene model (**Table 1**).

In F_2 generation, TSS ranging between 3 to 20.7 °brix was observed, whereas TSS in BC_1F_2, BC_2F_2 and BC_3F_2 generations were between 5.3 to 22.0, 6.0 to 20.6 and 7 to 19 °brix, respectively (**Figure 3**). The segregation patterns indicated that the distribution of progenies in these generations skewed towards high brix values. The segregation also shifted to high TSS because the lowest class (3-5 °brix) was not found in the BC_2F_2 and BC_3F_2 generations.

The TSS of 23 selected male sterile lines in the F_2 generation ranged between 14.5 to 20 °brix, and the mean of the selected plants in this generation was 16.8 °brix (**Table 2**). TSSs of 23 selected plants in the BC_1F_2 generation were from 14.3 to 18.3 °brix and the mean of the TSS was also 16.8 °brix. TSS of 22 selected plants in the BC_2F_2 generation ranged between 13 to 19 °brix and the mean was 15.4 °brix. TSS of 23 selected plants in the BC_3F_2 generation were from 13 to 19 °brix, and the mean was 15.5 °brix. However, means of TSS were higher in the first two generation than in the last two generations.

In the BC_2F_2 and BC_3F_2 generations, plant height, stalk diameter, stripped stalk weight and days to flowering were also recorded as additional criteria for selection according to their priority, respectively. The first priority for additional traits was plant height, and the plant shorter than 2.00 m were discarded except for some plants with high TSS and big stalks. Plant heights of two year data ranged between 165 to 311 cm, stalk diameters were from 9.97 to 26.94 mm, stripped stalk weights were from 185.10 to 923.82 g per plant and days to flowerings were from 64 to 93 days. The plants in the BC_3F_2 generation were rather bigger than in the early generation.

Discussion

Marker assisted selection (LW7)

Marker assisted selection for male sterility in sorghum can reduce time for line development in sorghum hybrid breeding program because test cross to identify the plants with male sterility is not necessary. The marker LW7 could effectively identify the plants with male sterility in the F_2, BC_1F_2, BC_2F_2 (Bunphan et al., 2013) and BC_3F_2 (in this study) generations, and the ratio for male fertile to male sterile was consistently 3: 1 across generations, supporting one gene model of Mendelian segregation.

Since the development of molecular markers (LW7, LW8 and LW9) linked to male sterile gene (*rf4*) in sorghum (Wen et al., 2002), the application of these markers in practical breeding has possibly not been reported so far. In our earlier work, only LW7 could identify male sterile plants in sweet sorghum (Lunmat et al., 2008). The results in this study supported previous findings and highlighted the importance of LW7 marker for line development of sweet sorghum hybrid breeding.

TSS and agronomic traits of selected plants

TSS of B-line plants is the major criterion for selection. Selection for this trait was carried out from F_2 generation to BC_3F_2 generation, whereas days to flowering, plant height, stalk diameter and stripped stalk weight were recorded in BC_2F_2 and BC_3F_3 generations only.

TSS was closely related to total sugar content in stalk but was not related to biomass (Ma et al., 1992; Audilakshmi et al., 2010; Guigou et al., 2011). TSSs in the F_2 and BC_1F_2 generations were slightly higher than those in the BC_2F_2 and BC_3F_2 generations because other agronomic traits were also selected in the BC_2F_2 and BC_3F_2 generations.

Days to flowering is related to days to maturity, biomass and yield. In general, the genotypes take longer days to maturity yield better than early mature genotypes. However, genotypes with late maturity may be affected by drought especially when the crop is planted in the late rainy season. However, in this study, medium to late number of days to flowering (64 to 93 days) was selected.

Plant height is an important agronomic trait related to biomass and yield in sweet sorghum (Audilakshmi et al., 2010), and high heterosis for plant height was observed in hybrid sorghum (Makanda et al., 2009). In this study, plant heights of 200 cm or higher were selected. However, shorter plants may be selected if they had larger stalk diameter. Stalk diameter was closely related to biomass and yield in sweet sorghum (Audilakshmi et al., 2010).

Segregation patterns of TSS in backcross generations

In this study, the distributions of TSSs in the segregating generations from F_2 to BC_3F_2 generations were in a similar pattern. All generations had a positive skew towards high TSS, and the later generations shifted towards high TSS. The shift in later generations is not surprising because the population was selected for high TSS for each generation and the plants in each generation were also crossed with high TSS parent (Fehr et al., 1987). The skew towards high TSS in this study would be due to small population size in each generation because some plants did not survive until harvest (Kearsey and Pooni, 1996). However, small population size would not affect selection efficiency in this population because the selected plants were backcrossed to high TSS parent in each generation, and, therefore, the chance to obtain the plants with high TSS in each generation was also high.

Theoretically, there are two strategies of handling segregating populations. The first strategy is to carry the population by successive self-pollination in each generation after sterile plants are identified and the second strategy is to backcross the selected plants in each generation (Fehr et al., 1987). The former strategy needs large population size for effective selection and control pollination is also required. Large population size is not necessary in the latter strategy, but identification of male sterile plants is necessary for each generation. Molecular identification of male sterile plants in this study was effective, and, thus, the time for test-crossing and population size is reduced considerably.

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