RESEARCH ARTICLE

SACCHARUM AND *ERIANTHUS* SPECIFIC MARKERS BASED ON DROUGHT AND SUCROSE SPECIFIC CANDIDATE GENES FOR HYBRID IDENTIFICATION

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Abstract

Three drought tolerant and one sucrose accumulation genes were studied as candidates in 35 basic species clones of *Saccharum* and *Erianthus* and 15 intergeneric hybrids involving the two genera. Gene specific primers designed were used for PCR amplification of genomic DNA that showed variable multiallelic profiles among the clones studied. It was possible to identify species specific markers in *Saccharum* and genus specific markers in *Erianthus*. ABA-independent DREB exhibited three alleles, of which a 250 bp amplificate was absent in *Erianthus* but present in *Saccharum,* while a band of 142 bp was specifically present in *Erianthus* species. HRD gene showed distinct *Erianthus* specific markers of 240 and 591 bp. WRKY transcription factor 35 could yield *Saccharum* and *Erianthus* specific markers of 306 and 835 bp, respectively. The specific primers of sucrose phosphate synthase (SuPS) amplified three alleles, of which a 660 bp band was specific to *Erianthus*, while a 820 bp band showed *Saccharum* specificity. The efficiency of the five *Erianthus* specific markers namely HRD_{240}, HRD_{591}, WRKY 38_{306}, DREB $1A_{140}$ and $SuPS(b)_{660}$ in hybrid identification was tested on a set of 15 progenies derived from intergeneric hybridization. Six out of 15 progenies could be confirmed as hybrids. The result indicated the inefficiency of candidate gene based markers for the purpose due to fewer alleles of the gene of interest compared to non-gene specific markers. However, these gene based markers would be advantageous in trait specific pre-breeding activities to ensure the presence of the specific desirable alleles, apart from confirming the hybrid nature of wide cross derived progenies as a marker assisted backcross.

Key words: *Saccharum, Erianthus*, true hybrids, candidate genes, drought, sucrose, markers

Introduction

Sugarcane breeding is advancing in a broad direction with product diversification including sugar, energy and fuel from the crop as comprehensive goals. Climate change is bringing in increasing intensities of stress factors that warrant the use of new germplasm sources in breeding programmes. *Saccharum* species are one of the most genetically complex plants due to polyploidy and relatively large genome size (Arumuganathan and Earle 1991). These features, together with the high heterozygous nature, make planned genetic improvement programmes guided by the principles of genetics a difficult exercise. Being intercrossable, all the species within the "*Saccharum* Complex" are considered the primary gene pool for sugarcane breeding but recent molecular data do not support this assumption (D'Hont et al. 2008). However, intergeneric hybridization was not a serious option till recently in view of the success of the interspecific hybrids and their derivatives. Of late, there has been

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renewed interest in the utilization of related genera in sugarcane varietal improvement as some of them are considered potential sources for important characters like earliness (*Sorghum*), high biomass production, ratoonability, disease and pest resistance (*Erianthus*), water logging resistance (*Sclerostachya*), cold tolerance and disease resistance (*Miscanthus*) (Nair et al. 2006). Utilization of *Erianthus* had been seriously pursued in India, Australia, USA, Fiji and West Indies over the past two decades. Genetic base broadening programmes need a systematic approach starting with crossing complementary parents. Identification of genuine intergeneric hybrids among the progeny is difficult due to barriers in wide hybridization and a high level of selfing of the female *Saccharum* parent. Apart from this, the hybrids resemble the *Saccharum* parent which contributes the major part of the hybrid genome. Genus specific molecular markers have been reported to be effective in identifying genuine hybrids of *Saccharum* with related genera (Alix et al. 1999).

A large number of genomic regions of this crop's germplasm can be examined for their breeding value through marker-assisted breeding, which enables the breeder to pool genes of diverse origins (Vinh and Paterson 2005). Identification of specific genes for major economic traits in sugarcane has been facilitated by the advances made in related genera. Identification of genetic factors involved in key traits and in plant's response to stress factors including drought will provide a solid foundation to breed better genotypes with improved performance under a given condition. In this regard, identification of candidate genes, beginning with selection of some target genes based on the biochemical pathway, could pave the way for the development of an effective breeding approach for different traits as well as the development of transgenics. Another major application of candidate genes is their deployment as genus and species specific molecular markers in base broadening programmes. The present study is carried out to explore the feasibility of three sugarcane specific drought responsive candidate genes, viz. Hardy transcription factor (HRD), WRKY transcription factor 38 and Dehydration response element binding factor1A (DREB1A), and Sucrose Phosphate Synthase (SuPS), a gene involved in sucrose accumulation, as species/genus specific markers in introgression breeding in sugarcane. An attempt was also made to identify true intergeneric hybrids based on the gene specific markers of the four candidate genes.

Materials and methods

Species clones and hybrid derivatives

A set of 35 basic species clones of *Saccharum* (*S. officinarum*: 13; *S. robustum*: 5; *S. spontaneum*: 5; *S. sinense/ S. barberi*: 7), four accessions of *Erianthus arundinaceus* and one accession of *E. bengalensis* and 15 intergeneric hybrids (Table 1) were taken for the study.

Candidate genes

Four candidate genes - three responsible for drought tolerance, viz. Hardy transcription factor (HRD), WRKY transcription factor 38 (WRKY 38) and Dehydration response element binding factor1A (DREB 1A), and Sucrose Phosphate Synthase (SuPS), a gene involved in sucrose accumulation - were screened on the species clones and hybrids. The sequences of these genes were obtained from Genbank databases and specific primers were designed using the Primer3 software (Rozen and Skaletsky 2000). The genes and the specific forward and reverse primers are given in Table 2.

PCR amplification and electrophoresis

DNA from all the clones was isolated using CTAB method (Doyle and Doyle 1987) and quantified in

Table 1. *Saccharum* and *Erianthus* species clones and intergeneric hybrids used for candidate gene analysis

a. *Saccharum* and *Erianthus* species clones

S. officinarum	*S. spontaneum*	*S. robustum*	*S. barberi*	*S. sinense*	*Erianthus sp.*
57 NG 122	SES 515/7	NG 77-73	Saretha	Uba White	*E. arundinaceus*:
57 NG 136	SES 563	NG 77-59	Pathri	Chuckche	IK 76-48
57 NG 215	IND 90-813	NG 77-28	Kewali	Ikhri	IK 76-91
57 NG 110	Iritty-1	IJ 76-336	Khatuia		IK 76-99
28 NG 224	Iritty-2	IJ 76-337			IK 76-62
28 NG 2					
28 NG 210					*E. bengalensis*:
Awela-68					IND 84-394
Penang					
Laukona 15					
Keong					
Fiji-B					
Fiji-30					

b. Intergeneric hybrids

Hybrid	Progeny	Parentage
GUK 01-934	BC_1	(CP 52-68 x IK76-91) x Co 96011
96 GUK 578	F_1	28NG210 x IK 76-99
GUK 00-1054	F_1	IK 76-91 x Co 98007
GUK 01-925	BC_1	(CP 52-68 x IK 76-91) x Co 96011
GUK 01-919	BC_1	(CP 52-68 x IK 76-91) x Co 96011
GUK 00-1058	F_1	IK 76-91 x Co 98007
GUK 01-882	F_1	Co 740 x IS 76-142
CYM 04-420	F_1	IK 76-62 x Iritty-2
CYM 04-387	F_1	Iritty-2 x IK 76-99
CYM 08-903	BC_2	{(IK 76-62 x Iritty-2)x Co 775)} x CoC 671
CYM 06-1226	BC_2	{(Iritty-2 x IK 76-99) x (IK 76-48}x CoC 671
CYM 05-106	BC_1	(Iritty-2 x IK 76-99) x Co 775
CYM 06-1308	BC_1	{[(Iritty-2 x IK 76-99)x Co 775)]x Co 775)}
CYM 08-997	BC_2	{[(Iritty-2 x IK 76-99)x Co 775)]x Co 775}x CoC 671]
CYM 05-461	BC_2	(28NG 210 x IK 76-99)x IK 76-91

Nanodrop 100 DNA quantifier. PCR reactions were performed in Eppendorf master cycler with a total reaction volume of 10 µl reaction mixture containing 0.33 mM dNTPs, 2.5 mM Mg, 0.15 µM of each primer, 0.5 U of Taq polymerase and 50ng of template DNA. PCR cycling conditions included a

Table 2. Four candidate genes and sequences of forward and reverse primers

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Hardy transcription factor (HRD)	TGGCGGCAATA GCCTATGAC	CTATTCATGCAAGCCACACCAC
WRKY transcription factor 38(WRKY 38)	CGTGGTGTTTGAGGGACCAA	GTACGTCGCCACGAGTATGG
Dehydration response elementbinding factor1A (DREB 1A)	AGATGTGCGGGATCAAGCAG	TCGCGTAGTACAGGTCCCAG
Sucrose Phosphate Synthase(SuPS)	ATTCTGGTGAAACGCCAAAC	AAGTCATCCGTGGTGTAGCC

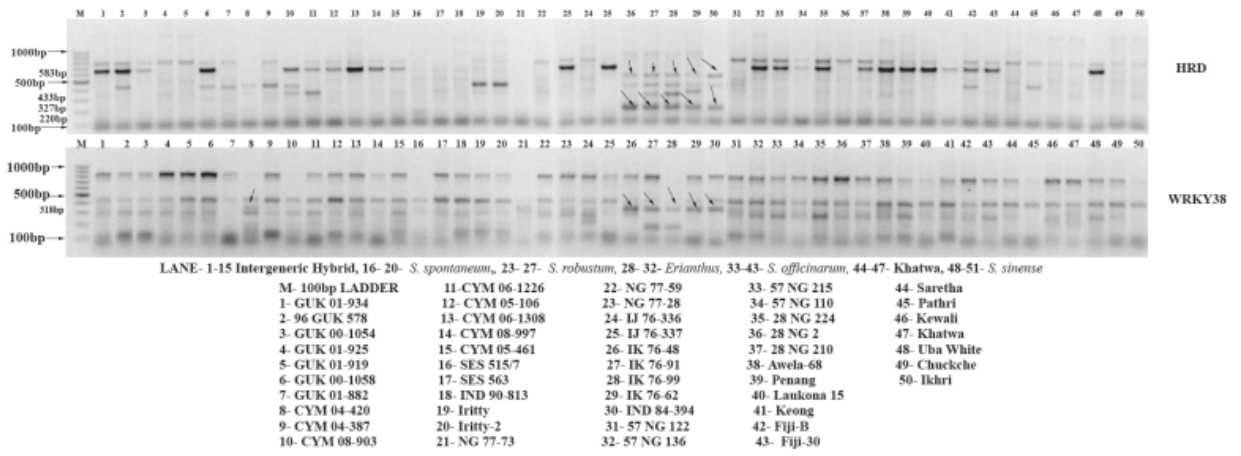
single cycle of 4 minutes at 94ºC, followed by 35 cycles of 94ºC for 45 seconds, annealing temperature of 48°C for HRD, WRKY38 and SuPS and 54°C for DREB1A for 45 seconds and 72°C for one minute, with a final extension of 72°C for seven minutes. PCR products were resolved on 1% agarose gel using 1X TBE buffer and stained with ethidium bromide (0.2mg/ml) adopting standard protocols. The gels were visualized in UV in a gel documentation system (Alpha Innotech).

Results and discussion

The present study was focused towards identifying species and genus specific markers associated with drought and sucrose specific candidate genes. Analysis of 30 drought specific genes from other crops has yielded 12 sugarcane responsive drought specific genes (NCBI/Nucleotide/ Hemaprabha and Priji 2013). For this study, three candidate genes responsible for drought tolerance, viz. Hardy transcription factor (HRD), WRKY transcription factor 38 (Wrky 38) and Dehydration response element binding factor 1A (DREB 1A) were selected along with Sucrose phosphate Synthase (SuPS), a gene involved in sucrose accumulation.

Species clones

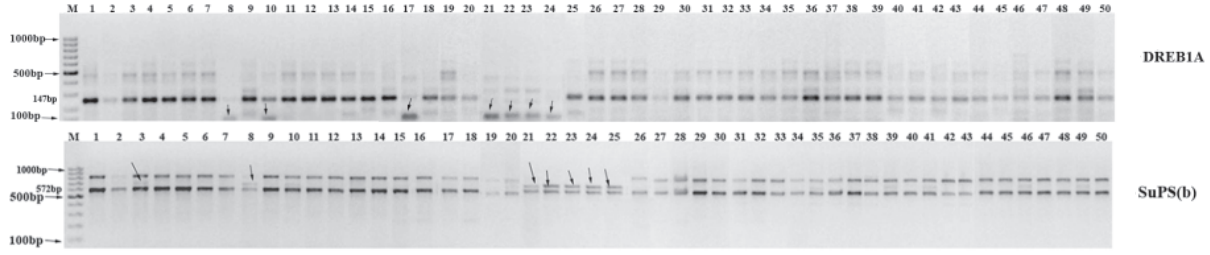
The target genes were tested in 35 germplasm accessions of *Saccharum* and *Erianthus*. Multiple banding pattern with the candidate genes indicated the presence of multiple alleles of the genes studied. HRD gene responsible for drought and salt tolerance in *Arabidopsis thaliana* (Karaba et al. 2007) showed two differentiating markers of 240 and 591 bp specific to *Erianthus*. *Saccharum* species clones studied showed distinct polymorphism with that of *Erianthus* (Fig. 1a). ABA- independent WRKY 38 belonging to the family of proteins that mediate plant stress responses showed two specific markers; a 306 bp marker specific to *Saccharum* and 835 bp specific to *Erianthus* (Fig 1a). ABA-independent DREB IA gene reported to be involved in dehydration tolerance in crop plants (Nakashima et al. 2007) exhibited three alleles of 480 bp, 250 bp and 140 bp, of which 480 bp band was specific to *Saccharum* species except *S. spontaneum* and was absent in *Erianthus*. Polymorphism was observed for 250 bp amplificate, absent in *Erianthus* but present in *Saccharum,* while a band of 140 bp was confined only to *Erianthus* species clones and in a *S. spontaneum* clone (SES 563) (Fig. 1b).



LANE- 1-15 Intergeneric Hybrid, 16- 20- *S. spontaneum*, 23- 27- *S. robustum*, 28- 32- *Erianthus*, 33-43- *S. officinarum*, 44-47- Khatwa, 48-51- *S. sinense*

M- 100bp LADDER	11-CYM 06-1226	22- NG 77-59	33- 57 NG 215	44- Saretha
1- GUK 01-934	12- CYM 05-106	23- NG 77-28	34- 57 NG 110	45- Pathri
2- 96 GUK 578	13- CYM 06-1308	24- IJ 76-336	35- 28 NG 224	46- Kewali
3- GUK 00-1054	14- CYM 08-997	25- IJ 76-337	36- 28 NG 2	47- Khatwa
4- GUK 01-925	15- CYM 05-461	26- IK 76-48	37- 28 NG 210	48- Uba White
5- GUK 01-919	16- SES 515/7	27- IK 76-91	38- Awela-68	49- Chuckche
6- GUK 00-1058	17- SES 563	28- IK 76-99	39- Penang	50- Ikhri
7- GUK 01-882	18- IND 90-813	29- IK 76-62	40- Laukona 15	
8- CYM 04-420	19- Iritty	30- IND 84-394	41- Keong	
9- CYM 04-387	20- Iritty-2	31- 57 NG 122	42- Fiji-B	
10- CYM 08-903	21- NG 77-73	32- 57 NG 136	43- Fiji-30	

Fig 1a. Polymorphism for HRD and WRKY38 candidate genes in *Saccharum* and *Erianthus* species clones and hybrids



Lane 1-15- Intergeneric Hybrids, Lane 16-20- *S.spontaneum*, Lane 21-25- *Erianthus*, Lane 26- 30- *S.robustum*, Lane-31-43- *S.officinarum*, Lane 44-47- *S.barberi*, Lane 48-50- *S.sinense*

M-100BP LADDER	11-CYM 06-1226	22- IK 76-91	33- 28 NG 224	44- Saretha
1- GUK 01-934	12- CYM 05-106	23- IK 76-99	34- 57 NG 110	45- Pathri
2- 96 GUK 578	13- CYM 06-1308	24- IK 76-62	35- 57 NG 122	46- Kewali
3- GUK 00-1054	14- CYM 08-997	25- IND 84-394	36- 57 NG 136	47- Khatwa
4- GUK 01-925	15- CYM 05-461	26- IJ 76-336	37- 57 NG 215	48- Ikhri
5- GUK 01-919	16- IND 90-813	27- IJ 76-337	38- Fiji-B	49- Chuckche
6- GUK 00-1058	17- SES 515/7	28- NG 77-73	39- Fiji-30	50- Ikhri
7- GUK 01-882	18- SES 563	29- NG 77-59	40- Penang	
8- CYM 04-420	19- Iritty-2	30- NG 77-28	41- Laukona 15	
9- CYM 04-387	20- Iritty	31- 28 NG 210	42- Keong	
10- CYM 08-903	21- IK 76-48	32- 28 NG 2	43- Awela-68	

Fig 1b. Polymorphism for DREB 1A and SuPS(b) candidate genes in *Saccharum* and *Erianthus* species clones and hybrids

Sucrose Phosphate Synthase (SuPS) is responsible for sucrose build-up in crop plants. The specific primers of SuPS(b) amplified three alleles: a 592 bp fragment was present in all the basic species clones of both the genera; a 660 bp fragment was *Erianthus* specific; and 820 bp was specific to *Saccharum*, present in clones of all its species but absent in *Erianthus* (Fig. 1b). In all, nine *Erianthus* and *Saccharum* specific markers were identified (Table 3).

This strategy of developing gene specific markers was different from the earlier reports wherein such markers were either identified from random markers or cytoplasm specific markers. Based on RAPD markers, genuine intergeneric hybrids of *Saccharum* in 12 hybrids of *Saccharum* with *Erianthus* spp., *Sorghum bicolor* and *Zea mays* were identified, though the study could not generate a decisive profile that can identify all the hybrids (Nair et al. 2006). Two PCR diagnostic tests were developed from the inter-Alu-like sequences MsCIR2 and EaCIR6 which proved efficient in identifying intergeneric hybrids of *Saccharum* × *Miscanthus* or *Saccharum* × *Erianthus* respectively (Alix et al. 1999). Pan et al. (2001)

Table 3. *Erianthus* and *Saccharum* specific candidate gene markers in the 35 species accessions

Genus specific markers	Gene	Mol. wt (bp)
Erianthus specific marker	HRD	240
		591
	WRKY 38	306
	DREB 1A	140
	SuPS(b)	660
Saccharum specific marker	DREB 1A	250
		480
	WRKY 38	835
	SuPS(b)	820

developed species-specific PCR markers from the 5S ribosomal DNA spacer for F1 progeny selection involving *S. officinarum, S. spontaneum, Erianthus* spp. (three cytotypes 2n=30, 60 and 90) of *S. giganteum, Miscanthus sinensis,* maize, and sorghum. Selvi et al. (2012) used sugarcane simple sequence repeats developed from enriched genomic libraries to generate *Erianthus* specific markers for hybrid identification. *Erianthus* specific markers accounted for 55.37 % of the markers and were validated in Sugarcane x *Erianthus* hybrids. With the objective of developing new hybrids with the cytoplasm of *Erianthus*, Premachandran et al. (2006) identified chloroplast DNA segments psbc-trnS and trnL intron. Mitochondrial nad4/3-4 that could differentiate the cytoplasm of *Saccharum* and *Erianthus* yielded amplified products of different band sizes of approximately 2 kb for *S. officinarum* and commercial sugarcane varieties, and 1.8 kb for *S. spontaneum* and *Erianthus* clones (Viola et al. 2011).

The presence of *Erianthus* specific marker (DREB $1A_{140}$) was detected in the *S. spontaneum* accession SES 563. This observation supported by gross morphological variations of this accession from the typical *S. spontaneum* characteristics is indicative of its hybrid origin under natural conditions. Such studies would be beneficial in systematic taxonomy within *Saccharum* complex, with the additional benefit of identifying gene sources for various agronomically important traits useful in sugarcane improvement.

Intergeneric hybrids

The occurrence of *Saccharum* and *Erianthus* specific bands in the progenies is presented in Table 4. Out of 16 hybrid derivatives screened with four gene specific primers, six hybrids, namely GUK 00-1054, GUK 01-925, GUK01-919, CYM 04-420, CYM 04-387 and CYM 06-122 showed *Erianthus* specific markers. It was not possible to establish the hybridity of 10 clones. Although more accurate than other marker systems, gene specific markers are fewer in number for the reason that these are the allelic forms of the same gene. Hence, the use of candidate genes as such in hybrid identification has limited applied value. However, gene specific markers have an advantage over the other marker systems in pre-breeding activities, wherein in addition to establishing hybridity, transmission of the desired candidate gene can also be studied. TRAP markers could partially satisfy these two requirements and were successfully employed in identifying polymorphism within the regions of sucrose metabolizing genes on 19 species clones belonging to *Erianthus arundinaceus*, *E. procerus, E. bengalensis, S. spontaneum* and *S. officinarum* (Hemaprabha and Leena 2015). Hence, it could be inferred that for establishing hybridity, TRAP markers or random markers would be advantageous by virtue of generating larger number of polymorphic markers. However, for adopting trait specific improvement involving the wild relatives, for instance

Table 4. *Saccharum* and *Erianthus* specific bands and their occurrence in the progenies studied

Hybrid clone	WRKY 38		HRD		DREB 1A		SuPs(b)		
	Erianthus specific marker	*Saccharum* specific markers	*Erianthus* specific marker		*Erianthus* specific marker	*Saccharum* specific marker	*Erianthus* specific marker		*Saccharum* specific marker
	306 bp	835 bp	240 bp	591 bp	140 bp	250 bp	480 bp	660 bp	820 bp
GUK 01-934	A	P	A	A	A	P	P	A	P
96 GUK 578	A	P	A	A	A	P	A	A	P
GUK 00-1054	A	P	A	A	A	P	P	P	P
GUK 01-925	A	P	A	P	A	P	P	A	P
GUK01-919	A	P	A	P	A	P	P	A	P
GUK 00-1058	A	P	A	A	A	P	P	A	P
GUK01-882	A	P	A	A	A	P	P	A	P
CYM 06-1305	A	P	A	A	A	P	P	A	P
CYM 04-420	P	A	A	A	A	A	A	P	P
CYM 04-387	A	P	A	A	P	P	P	A	P
CYM 08-903	A	P	A	A	A	P	P	A	P
CYM 06-122	A	P	A	A	P	P	P	A	P
CYM 05-166	A	P	A	A	A	P	P	A	P
CYM06-1308	A	P	A	A	A	P	P	A	P
CYM08-997	A	P	A	A	A	P	P	A	P
CYM 05-461	A	P	A	A	A	P	P	A	P

drought, drought specific candidate genes would be more effective in monitoring the transmission of the beneficial alleles to the progenies and ensuring hybridity. This strategy highlights the prospects of marker assisted back cross breeding in sugarcane using candidate genes.

Some of the hybrids which are products of hybridization with *Erianthus* as female parent are expected to have *Erianthus* cytoplasm but could not be confirmed as hybrids in the study. The nuclear genes used in the study could not differentiate *Erianthus* genome from *Saccharum* and this warrants deployment of more efficient ways to detect true hybrids. This assumes greater importance in view of the reports of cytological abnormalities in intergeneric hybrids wherein loss of few chromosomes to *enbloc* elimination of chromosomes of a species are common, while there would also be hybrids retaining the genomes of both genera. The hybrids studied here have chromosome number 2n <100 that indicated chromosomal abnormalities including elimination of chromosomes (Lalitha 2007). While studying three generations of hybrids between *Saccharum* and *Erianthus*, Piperidis et al. (2010) showed that F_1 and BC_2 resulted from n + n chromosome transmission whereas BC_1 resulted from 2n + n transmission. While in the BC_1 clones, the number of *E. arundinaceus* chromosomes ranged from 21 to 30, in the BC_2 clones, the number

ranged from 14 to 15, revealing cases of chromosome loss. This phenomenon can explain the loss of the candidate genes in the hybrids taken for the study. Also, differences in banding intensity were noticed between basic species clones and derived hybrids, as a reflection of the number of loci of each gene. Based on the basic chromosome number of the genera *Saccharum* and *Erianthus* (Heinz 1987), it is expected that 10 loci are present in *S. officinarum* and six in *Erianthus*, to be detected as brighter bands, in contrast to F_1 hybrids with half the number of loci visualized as weak bands; further backcrossing would result in further reduction in loci that makes their detection on PAGE gels difficult. This may be another reason for not establishing hybridity of the hybrids studied here. In both situations, genomic in situ hybridization (GISH) would offer a platform for identifying true hybrids by differentiating the species/genus specific chromosomal locations, in addition to identifying the location as well the extent of chromosomal exchange in interspecific and intergeneric hybrids for a better understanding of introgression processes (D'Hont 2005).

The results of the study on candidate gene markers thus indicate the robustness of the candidate genes in generating genus/species specific bands. These markers could serve as functional markers ensuring the presence of drought responsive/sucrose rich alleles in the hybrids as well as proving their hybrid nature, thus aiding in marker assisted back crossing for trait specific introgression breeding programmes involving *Erianthus* and *S. spontaneum.*

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