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Sugarcane-specific drought responsive candidate genes belonging to ABA-independent pathway identified from tolerant and susceptible clones of *Saccharum* and *Erianthus* species

P. J. Priji and G. Hemaprabha*

Sugarcane Breeding Institute, Coimbatore 641 007

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Abstract

Drought is a major limitation to sustainable sugarcane production worldwide and the study of candidate genes provide a productive approach for establishing the basic responses of plants to drought. In present study, 14 putative candidate genes belonging to abscisic acid (ABA) independent pathway were screened on a set of tolerant species clones belonging to *Saccharum spontaneum*, *S. barberi*, *S. sinense, S. robustum* and *Erianthus* and the susceptible species clones of *S. officinarum*. Out of these, 11 genes *viz*., *DREB 1A, DRF 1, Hep2, HRD, NAC 2, NIT 1, PIN 3, SHN 1, S1Z 1, Snac 1* and *Wrky 38* were present in *Saccharum* and *Erianthus,* highlighting the role of these genes in drought tolerance. The number of candidate genes detected in *S. officinarum* ranged from zero to three. Three typical clones *viz*., Awela 68, Laukona 15 and 57 NG 215 did not show the presence of any of the drought specific genes studied, showing their pure *S. officinarum* nature, while the rest are suspected to be introgressed forms evolved in their place of origin/diversity. All the drought tolerant species clones exhibited eight to eleven genes indicating the role of these genes in imparting drought tolerance in sugarcane. All genes were uniformly present in drought tolerant species clones *viz*., Iritty 2, *S. spontaneum* (Coimbatore) and SES 600 (*S. spontaneum)*, NG 77-59 (*S. robustum*), Kewali and Khatuia (*S. barberi*), Ikhri (*S. sinense*) and IK 76-62 and IK 76-48 (*Erianthus* sp.). These clones could serve as potential donors in pre-breeding activities for genetic improvement of drought tolerance in sugarcane and the eleven sugarcane specific drought responsive genes can serve as novel and functional candidate gene markers in marker-assisted introgression breeding programmes.

Key words: Drought, candidate genes, ABA independent pathway, *Saccharum*, *Erianthus*

Introduction

Sugarcane (*Saccharum* spp.) is an outstanding crop throughout the tropical regions of the world providing sugar and bioenergy source apart from other uses [1]. The genus traditionally comprises of six distinct, yet intercrossable species *viz S. officinarum, S. barberi, S. sinense, S. spontaneum*, *S. robustum* and *S. edule*. *S. officinarum* is a sucrose rich cultivated species but is prone to biotic and abiotic stresses, while the remaining species clones are widely adapted with disease and stress resistance especially drought [2]. Modern sugarcane cultivars are complex hybrids of interspecific origin involving *S. officinarum*, *S. spontaneum*, *S. barberi* and *S. sinense*. Among these, *S. spontaneum* is an important wild species that has contributed to disease resistance, tolerance to abiotic stress and high ratooning potential in the sugarcane cultivars, while *S. officinarum* is associated with sugar accumulation [3]. *Erianthus* is a wild genus, readily crossable with *Saccharum* and several intergeneric hybrids with improved performance at problem situations and as energy canes are being developed by cane breeders [4].

Drought stress is one of the most important abiotic stress factors that influences sugarcane growth and development at all stages, more so during the critical water demand period (60-150 days of age) adversely affecting yield and sugar content [5]. Identification of genetic factors involved in plant responses to drought stress will provide a solid foundation to breed plants with improved drought

*Corresponding author's e-mail: hemaprabha@sify.com
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tolerance [6]. In this regard, candidate genes are useful, and their identification starting with selection of some target genes based on the biochemical pathway could pave the way for developing an effective breeding approach for drought tolerance. A number of genes have been reported to be induced by drought, high salinity, and low-temperature stresses and their products are thought to function in stress tolerance response [7, 8]. Most of these genes are induced by the plant hormone ABA, in contrast to several others being irresponsive to ABA [9], which points to the existence of complex regulatory mechanisms between perception of abiotic stress signals and gene expression [8]. ABA-dependent and ABA-independent pathways lead to rapid responses to drought or cold and function through members of the AP2/ERF family of transcription factors [10]. In this study, ABA-independent genes identified in other plant species were tested for their role in drought tolerance in sugarcane, based on the analysis on a set of drought tolerant and susceptible basic species clones.

Materials and methods

The plant materials were obtained from the germplasm collection maintained at Sugarcane Breeding Institute, Coimbatore. Table 1 illustrates the list of drought tolerant and susceptible species belonging to *Saccharum* and *Erianthus* species and clones taken for the study.

Candidate genes

In order to find the putative drought responsive genes, the public database was exhaustively searched for gene sequences and/or regulatory sequences important for enhanced drought resistance along with an elaborate literature survey. The putative gene sequences were identified by reviewing published literature in several academic database search engines such as PubMed®, Web of Knowledge® and Google search. Fourteen candidate genes belonging to ABA independent pathway were selected as candidates to study drought response in sugarcane. These genes included Dehydration response element binding factor 1A (*DREB 1A*), Dehydration-responsive factor 1 (*DRF 1*), Heparanase (*Hep2*), Hardy transcription factor (*HRD*), Beta-carotene hydroxylase (*HYD3*), NAC-like transcription factor 2 (*NAC 2*), NIT 1 (*Nitrilase 1*), Polar auxin transport genes (*PIN 3*), Chitinase (*POM1*), SHINE 1 Transcriptional factor (*SHN 1*), Sumo ligase 1 (*S1Z 1*), Stress responsive nac1 (*Snac1*), Wrky transcription factor 38 (*Wrky 38*) and Zinc finger protein 1 (*ZF1*). The genes studied fell into groups such as drought responsive regulatory factors, dehydration responsive element transcription factors for drought and salt stress, auxin mediated regulatory factors and genes encoding protective factors. Table 2 shows the list of fourteen genes associated with drought tolerance genes, forward and reverse primers and the pathway to which they belong.

Genomic DNA isolation and purification

Tender leaf samples were collected from the species clones given in Table 1 and DNA isolation and purification were carried out by CTAB method [11]. The quality, quantity and purity of DNA were checked in Nano Drop ND-1000- DNA / RNA quantifier.

Designing the candidate genes

The nucleotide sequences of the genes of interest were identified by using the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). The forward and reverse primers of the respective genes were designed using the Primer 3 software (http://frodo.wi.mit.edu/cgi bin/primer3/primer3_www.cgi) [12].

Table 1. List of drought tolerant and susceptible clones belonging to *Saccharum* and *Erianthus* species

Drought susceptible	Drought tolerant				
S. officinarum	*S. spontaneum*	*S. robustum*	*S. barberi*	*S. sinense*	*Erianthus*
Awela 68, Penang, Laukona15, 57 NG 136, 57 NG 215, 28 NG 224, Keong, Mia Moi, 21 NG 2, 28 NG 210, Fiji B, Fiji 30	SES 168, Iritty 2, SES600, SES 106 B, *S. spontaneum* Coimbatore, SES51517, SES 561, IND90 - 813	NG 77-59, NG77-122, IJ 76 336, IJ 76 337	Saretha, Pathri, Kewali, Khatuia	Chuckche Uba white Ikhri	IK 76 -99, IK 76- 91, IND 84 - 363, IK 76-62, IK 76-48

PCR amplification and electrophoresis for candidate gene evaluation

PCR reactions were performed in all the clones using the primers of 14 drought tolerant genes in a MJ thermal cycler PT 100. Each PCR reaction was carried out with a total reaction volume of 10 µl containing 25 ng of genomic DNA, 1X *Taq* buffer with $MgCl_2$, 100 µM dNTPs, 0.3 unit of *Taq* DNA polymerase enzyme and 20-30 pmol of each forward and reverse primer. The amplification conditions included an initial denaturation at 94°C for five minutes, followed by 35 cycles of 94° C for 30 seconds for denaturation, primer annealing at 58-62°C (based on the T^m value of gene specific primers used for the study) for 30 seconds, 72°C for 30 seconds for extension and a final extension at 72°C for seven minutes. Upon completion of the PCR cycles, the amplified products were mixed with 2µl of loading dye (6X) and were resolved on 1% agarose gel (low EEO) containing Ethidium bromide (0.2mg/ml) for 40 minutes at 100 W. The gels were observed using Alpha Imager Imaging system (Alpha Innotech) and drought specific bands were identified.

Results and discussion

In order to understand the molecular basis of drought tolerance, it is important to identify plant genes that respond to drought tolerance. Candidate gene analysis was used to identify drought specific genes in tolerant genotypes. In the present study, out of fourteen drought responsive genes independent of ABA pathway analyzed, eleven genes were amplified in the species clones, showing their specificity to drought tolerance in sugarcane, while three candidate genes *viz*., *POM1* essential for tolerance to heat, salt and drought stresses [13] and Zinc finger protein 1 (*ZF1*) inducing abiotic stress proteins [14] in rice and *HYD3* (Beta carotene hydrolyase) conferring drought and oxidative stress resistance [15] in barely did not exhibit any amplification in the species clones studied. The genes whose presence could be detected in *S. officinarum*, *S. spontaneum*, *S. barberi*, *S. sinense, S. robustum* and *Erianthus* are listed in Table 3 and are shown in Fig. 1(A-K).

S. officinarum

The drought susceptible *S. officinarum* species clones exhibited variation for the presence of the genes. Out of the eleven candidate genes amplified in sugarcane, six genes *viz*., *DREB1A, NAC2, Snac1, SHN 1, SIZ 1* and *PIN 3* gene could be detected in a few *S. officinarum* clones. *DREB 1A* that exhibited 408 bp amplicon in Fiji B and Fiji 30 clones (Fig. 1A) was reported to induce a set of abiotic stress responsive genes and maintenance of water balance in plant systems [16], while *NAC 2* gene that showed 305 bp band in 28 NG 224, Keong, 21 NG 2, Fiji B and Fiji 30 clones (Fig. 1B) conferred cold and salt tolerance in rice [17]. The genes *Snac1, SHN 1* and *SIZ 1* clearly showed presence of bands of 270 bp in 28 NG 210, 502 bp in Penang and 300 bp in Keong, respectively (Figs. 1C, 1D and 1E). Earlier studies showed that these three genes are likely to be involved in promoting drought tolerance in other crops, for instance, *Snac1* gene specifically reduced water loss in rice by increasing stomatal sensitivity to ABA [18], *SHN 1* gene was involved in wax biosynthesis [19] and *SIZ 1* conferred innate immunity, regulation of plant growth, drought responses and freezing tolerance [20]. *PIN 3* gene that produced a 400 bp band in seven clones *viz*., 57 NG 136, 28 NG 224, Keong, Mia Moi, 28 NG 210, Fiji B and Fiji 30 clones (Fig. 1F) is a regulator of auxin efflux that was found to decrease in the presence of alloxan [21].

In contrast, five specific genes *viz*., *DRF 1* - an ABA inducible late-embryogenesis abundant protein gene [22], *HRD* - a drought and salt tolerance gene [23], *Hep2* - a drought tolerant gene identified in rice (S. Subramanian, personal communication), *NIT 1* - a crucial enzyme in auxin biosynthesis [24] and *Wrky 38* involved in cold- and drought response in barley [25] failed to amplify in this species. The markers specific to these genes were noticed in the tolerant species as 300 bp with the primers specific for *DRF 1* (Fig. 1G), 420 bp for *HRD* (Fig. 1H), 550 bp for *Hep2* (Fig. 1I), 300 bp for *NIT 1* (Fig. 1J) and 500 bp for *Wrky 38* (Fig. 1K). The absence of these genes with proven role in drought tolerance in related crops in *S. officinarum* and their presence in the drought tolerant *Saccharum* and *Erianthus* species highlighted the role of these genes in imparting drought tolerance in sugarcane and susceptibility of the *S. officinarum* species to drought.

It may be noted that *S. officinarum* includes typical forms with 2n = 80 and atypical forms with chromosome number deviating from 2n = 80. The latter are considered to be natural hybrids with the introgression of related grasses of the closely bred *Saccharum* complex in the natural habitat. Among the typical *S. officinarum clones viz*., Awela 68, Laukona 15, 57 NG 136, 57 NG 215, 28 NG 224, Mia Moi, 21 NG 2, 28 NG 210, Fiji B, Fiji 30, three clones *viz*., Awela 68, Laukona 15 and 57 NG 215 did not

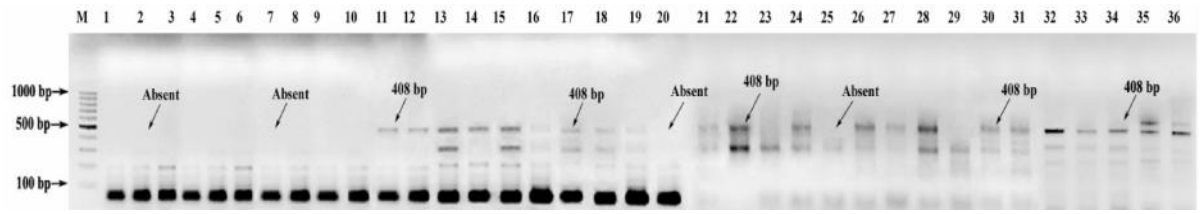

Fig. 1A. DREBIA

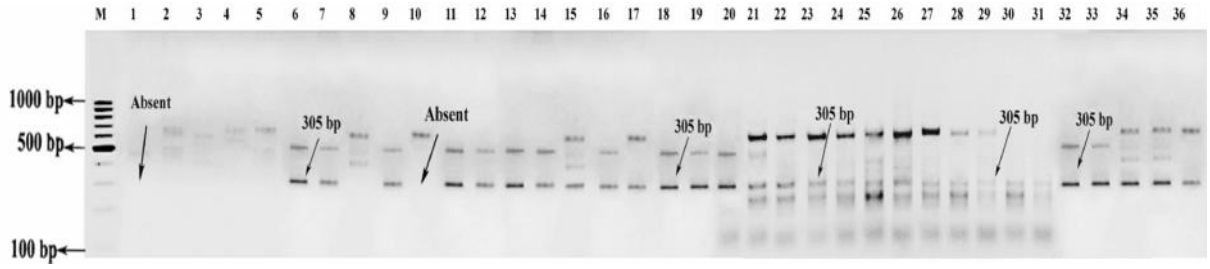

Fig. 1B. NAC 2

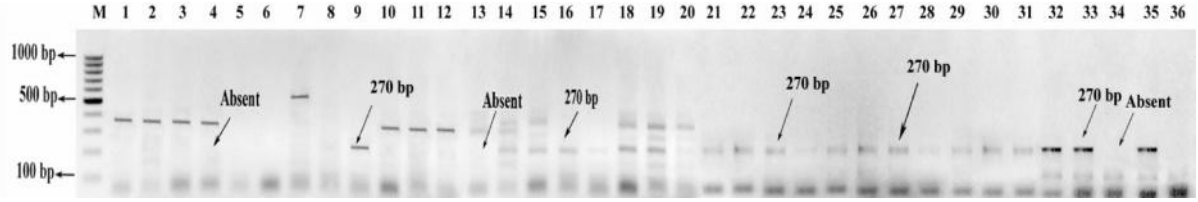

Fig. 1C. Snac1

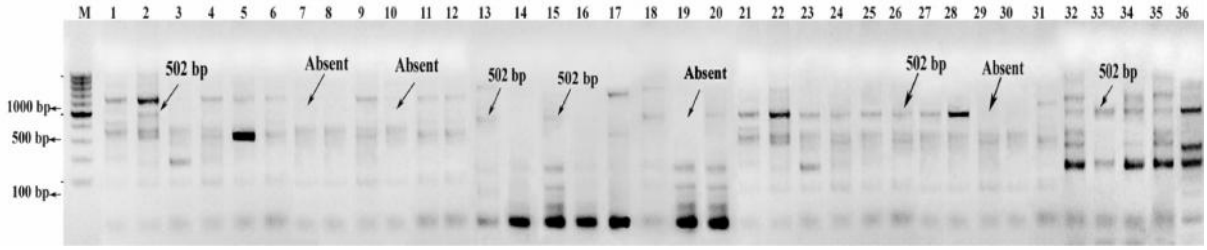

Fig. 1D. SHN1

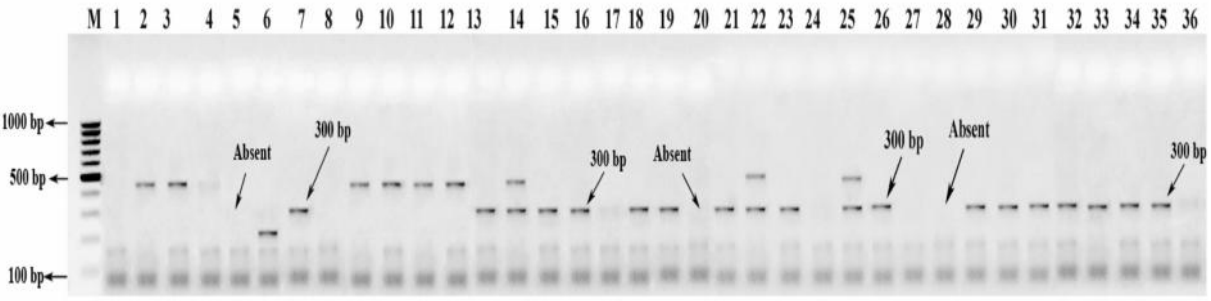

Fig. 1E. SIZ 1

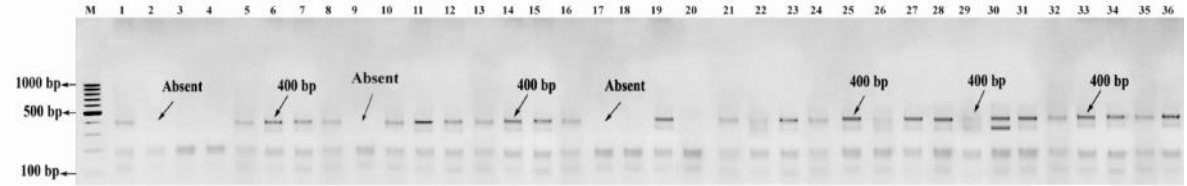

Fig. 1F. PIN 3

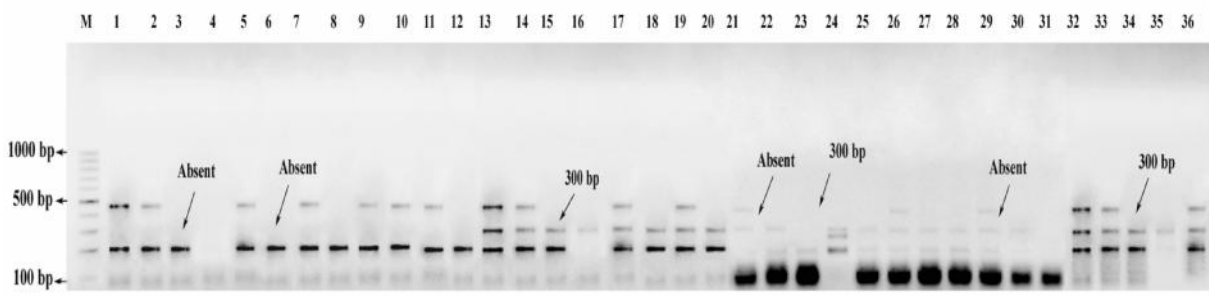

Fig. 1G. DRF1

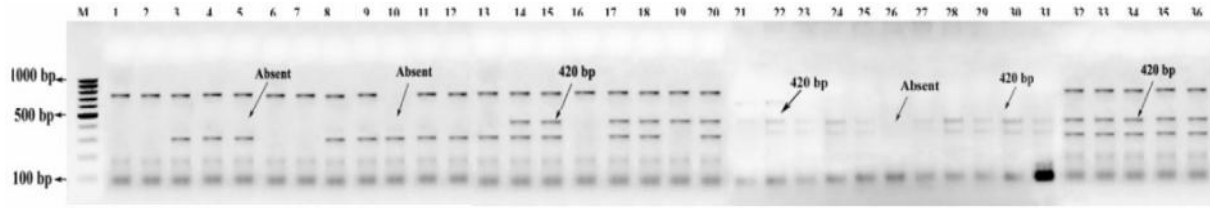

Fig. 1H. HRD

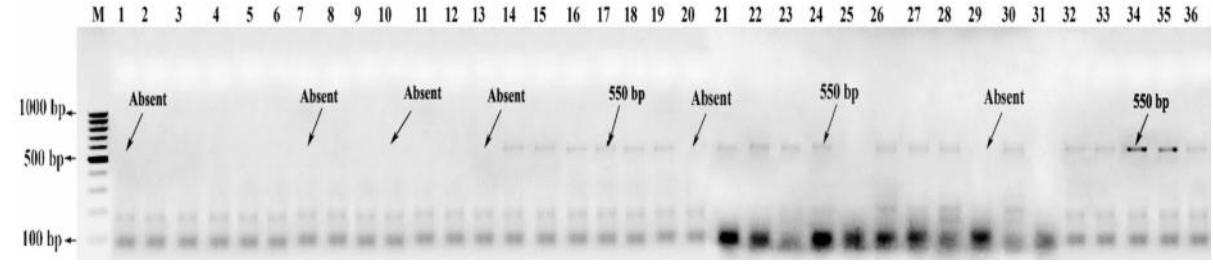

Fig. 1I. Hep 2

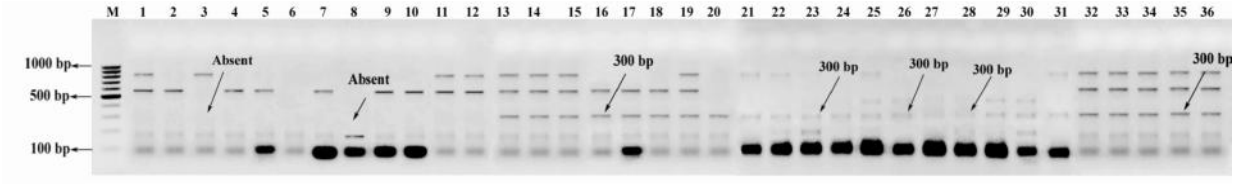

Fig. 1J. NIT 1

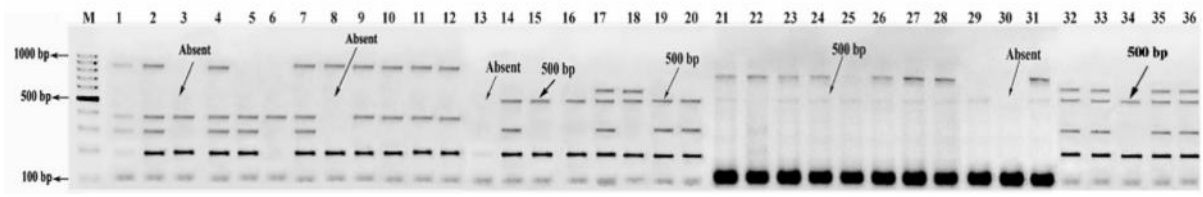

Fig. 1K. Wrky 38

Fig. 1A-K. Presence/absence of eleven drought responsive candidate genes in *Saccharum* and *Erianthus* species clones. M – 100 bp Ladder, Lane 1 - 12 – *S. officinarum clones*, Lane 13 - 20 - *S. spontaneum clones*, Lane 21 – 24 - *S. robustum clones*, Lane 25 - 28 - *S. barberi clones*, Lane 29 – 31 – *S. sinense* clones, Lane 32 – 36 - *Erianthus clones.* 1-Awela 68, 2-Penang, 3-Laukona, 4-57 NG 136, 5-57 NG 215, 6-28 NG 224, 7-Keong, 8-Mia moi, 9-21 NG 2, 10-21 NG 210, 11-Fiji B, 12-Fiji 30, 13-SES 168, 14-Iritty 2, 15-SES 600, 16-SES 106 B, 17-*S. spontaneum* (Coimbatore), 18-SES 51517, 19-SES 561, 20-IND 90-813, 21-NG 77-59, 22-NG 77-122, 23-IJ 76-336, 24-IJ 76-337, 25-Saretha, 26-Pathri, 27-Kewali, 28-Khatuia, 29-Chuckche, 30-Uba white, 31-Ikhri, 32-IK 76-99, 33-IK 76-91, 34-IND 84-363, 35-IK 76-62, 36-IK 76-48

Table 2. List of candidate genes belonging to ABA independent pathway

S.No.	Pathway	Gene/ primer	Forward primer (5'- 3')	Reverse primer (5'- 3')
1	Drought responsive regulatory factors	DREB 1A	AGATGTGCGGGATCAAGCAG	TCGCGTAGTACAGGTCCCAG
		DRF 1	AGCAAGCTCAAGCAGTCAGT	GGGGTTGGCTGTCAAGCTTA
		HRD	TGGCGGCAATAGCCTATGAC	CTATTCATGCAAGCCACACCAC
		NAC 2	GGTTGCTGGCCACCATTTCT	GCCGTTTGGTACCTTCTGCT
		POM1	CTGATCTGCATTGCGGCTTG	CAGACCCCAAGCTAAAGGCC
		SHN 1	ATCCTCAGCGCCAAACTGAG	GTGGTCGGAGCAAGAATAGCG
		SIZ 1	TGTAGCCAACGGCATGGAAC	TCTCAGACAGGGAACAAACCAG
		Wrky 38	CGTGGTGTTTGAGGGACCAA	GTACGTCGCCACGAGTATGG
		ZF1	ATCAAGTCGACGGTGGAGACT	CCATGGGAAACTCCACTCCG
2	Transcription factor for salt tolerance	Snac1	TACAAGTTCGACCCGTGGGA	GCGACGAGTAGAAGTCGCC
3	Auxin dependent regulatory factors	NIT 1	ATCCCCGTTTACGACACT	ACGAAACATCCACCTTC
		PIN 3	ACGTTTTCGGCGGAGCACCG	TGCCACTGAATTCCCACAAC
4	Enzymes encoding protective factors	Hep2	TGCCTGCCGTTGCATAGATG	TTAGTGCCGTTGAATGTTGCC
		HYD3	TGAGCTGTAACGCTTGGAGGT	GCCATGCCGAACAGCGTAAT

exhibit presence of any genes showing their pure *S. officinarum* nature, while the remaining seven clones exhibited one to three candidate genes, may be as an adaptive mechanism of the species to the harsh environmental conditions, or more likely, the presence of other wild genomes in their genetic constitution. It could be noted that the two Fiji clones *viz*., Fiji B and Fiji 30 showed presence of four genes (*DREB 1A, NAC 2, Snac1* and *PIN 3*) for the specific adaptation under the climatic conditions unique to the island country of Fiji, from where the clones were collected. The atypical forms *viz*., Keong and Penang also showed 1-3 drought responsive genes and based on morphology, these are suspected to be natural hybrids between *S. officinarum and S. robustum*. Thus specific drought responsive candidate genes appear as a useful tool in identifying pure *S. officinarum* clones from the introgressed forms clustered under the group of *S. officinarum*.

S. spontaneum

Four candidate genes *viz., DRF 1, NIT 1, NAC 2* and *Wrky 38* factor were uniformly present in all the eight clones of *S. spontaneum.* Two genes *Snac1* and *Hep2* were absent in *SES 168* alone, while the genes *viz., HRD, SHN 1* and *PIN 3* revealed their existence in all clones except SES 106 B. *PIN 3* and *SHN 1* were also absent in the clones SES 51517 and SES 561, respectively. In contrast, DREB 1A, SIZ 1 and PIN 3 did not exhibit any amplification in the clone IND 90-813. This clone is characterized by robust growth and broader leaves with a morphology deviating from that of typical *S. spontaneum*. The absence of these *S. spontaneum* specific candidate genes could indicate hybrid origin of this clone involving related species with broader leaves. Three clones *viz*., Iritty 2, SES 600 and *S. spontaneum* (Coimbatore) produced amplification with all the eleven genes studied. Thus investigation clearly showed *S. spontaneum* as a species harbouring most of the drought specific genes.

S. robustum

Among the four *S. robustum* clones, IJ 76 33 did not amplify *DREB 1A* and *DRF 1* genes. Furthermore, drought specific genes *viz*., *SIZ 1* and *Wrky 38* were absent in IJ 76 336 and NG 77-122 clones respectively. NG 77-59 is the only *S. robustum* clone that produced amplification with all the eleven candidate genes, while other clones showed the presence of ten genes and therefore the drought tolerance potential of *S. robustum*.

S. barberi and S. sinense

Among the *S. barberi* clones analyzed, Saretha did not exhibit any amplificate with *DREB 1A* and *Hep2* gene, while HRD gene did not amplify in Pathri alone. In *S. sinense* clones, *DREB 1A, DRF 1, HRD* and *SHN 1* were amplified in all the clones except Chuckche. The genes *DRF 1, SHN 1* and *Wrky 38* revealed their absence in Uba White during the drought

Table 3. List of candidate genes and amplified polymorphic bands specific to *Saccharum species* clones

S.No.	Species clones	Name of clones and chromosome no	Presence (P) or absence (A) of genes belonging to ABA-independent pathway										
			DREB1A	DRF1	HRD	Hep2	NAC2	NIT1	Snac1	SHN1	SIZ1	PIN3	Wrky38
1	***S. officinarum***	Awela 68(2n=80)	A	A	A	A	A	A	A	A	A	A	A
		Penang (2n>80)	A	A	A	A	A	A	A	P	A	A	A
		Laukona 15(2n=80)	A	A	A	A	A	A	A	A	A	A	A
		57 NG 136(2n=80)	A	A	A	A	A	A	A	A	A	P	A
		57 NG 215(2n=80)	A	A	A	A	A	A	A	A	A	A	A
		28 NG 224(2n=80)	A	A	A	A	P	A	A	A	A	P	A
		Keong (2n>80)	A	A	A	A	P	A	A	A	P	P	A
		Mia Moi (2n=80)	A	A	A	A	A	A	A	A	A	P	A
		21 NG 2 (2n=80)	A	A	A	A	P	A	A	A	A	A	A
		28 NG 210 (2n=80)	A	A	A	A	A	A	P	A	A	P	A
		Fiji B (2n=80)	P	A	A	A	P	A	A	A	A	P	A
		Fiji 30 (2n=80)	P	A	A	A	P	A	A	A	A	P	A
2	***S. spontaneum***	SES 168	P	P	P	A	P	P	A	P	P	P	P
		Iritty 2	P	P	P	P	P	P	P	P	P	P	P
		SES 600	P	P	P	P	P	P	P	P	P	P	P
		SES106 B	P	P	A	P	P	P	P	A	P	A	P
		S. spontaneum(CBE)	P	P	P	P	P	P	P	P	P	P	P
		SES 51517	P	P	P	P	P	P	P	P	P	A	P
		SES 561	P	P	P	P	P	P	P	A	P	P	P
		IND 90 - 813	A	P	P	P	P	P	P	P	A	A	P
3	***S. robustum***	NG 77 - 59	P	P	P	P	P	P	P	P	P	P	P
		NG 77 - 122	P	P	P	P	P	P	P	P	P	P	A
		IJ 76 336	P	P	P	P	P	P	P	P	A	P	P
		IJ 76 337	A	A	P	P	P	P	P	P	P	P	P
4	***S. barberi***	Saretha	A	P	P	A	P	P	P	P	P	P	P
		Pathri	P	P	A	P	P	P	P	P	P	P	P
		Kewali	P	P	P	P	P	P	P	P	P	P	P
		Khatuia	P	P	P	P	P	P	P	P	P	P	P
5	***S. sinense***	Chuckche	A	P	P	A	P	P	P	A	P	P	A
		Uba white	P	A	P	P	P	P	P	A	P	P	P
		Ikhri	P	P	P	P	P	P	P	P	P	P	P
6	***Erianthus***	IK 76 - 99	P	P	P	P	P	P	P	P	P	P	A
		IK 76 - 91	P	P	P	P	P	P	A	P	P	P	P
		IND 84-363	P	P	P	P	P	P	A	P	P	P	P
		IK 76 - 62	P	P	P	P	P	P	P	P	P	P	P
		IK 76 - 48	P	P	P	P	P	P	P	P	P	P	P

stress condition. Kewali and Khatuia of *S. barberi* and Ikhri of *S. sinense* exhibited amplification with all the eleven candidate genes. The presence of majority of the genes studied in these two species, widely grown in the drought affected and high temperature zone of Indo-China, highlighted their capacity to serve as donor for drought tolerance with the added advantage of being a sugar rich *Saccharum* species.

Erianthus species

All the eleven genes detected were present in the five clones of *Erianthus* except Snac1 in IK 76-91, IND 84-363 and Wrky 38 in IK 76-99, respectively. This study revealed the importance of *Erianthus* as a source for drought tolerance. *Erianthus* is now gaining importance as a valuable source for many traits such as tolerance to environmental stresses, multiple ratoonability, disease resistance and potential source for developing energy canes [4].

In the present investigation, eleven out 14 candidate genes belonging to ABA independent pathway of drought tolerance exhibited their presence in *Saccharum* species. The existence of a few drought responsive genes in susceptible accessions of *S. officinarum* and absence of some drought specific genes in tolerant species clones were noticed. This observation leads to the existence of a complex machinery involving several genes in multiple pathways that finally makes a genotype susceptible or tolerant to drought. Also these genes have specific roles in adaptive mechanisms to survive in the long duration spanning round the year in the place of their origin/diversity. The drought tolerant species clones exhibited the presence of a majority of candidate genes ranging from 8-11 in *S. spontaneum*, 9-10 in *S. barberi*, 8-10 in *S. sinense*, 9-11 in both *S. robustum* and *Erianthus* species, respectively. It can furthermore be concluded that *S. spontaneum, S. robustum, S. barberi, S. sinense,* and *Erianthus* species in general, and specifically the clones *viz*., IK 76-62, IK 76-48, Iritty 2, SES 600, *S. spontaneum* (Coimbatore), NG 77-59, Kewali, Khatuia and Ikhri are the best candidate clones for drought tolerance for being good repositories of eleven drought specific genes. Hence, these species would gain importance in prebreeding programs aimed at developing sugarcane varieties for drought tolerance and for isolation of novel drought specific genes. Efforts are on to identify and sequence the candidate genes that would pave way for developing an effective breeding approach for drought tolerance in sugarcane.

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