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SCREENING OF SORGHUM (*SORGHUM BICOLOR* (L). MOENCH) FOR DROUGHT TOLERANCE USING PEG AND DROUGHT ASSOCIATED EST MARKERS

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ABSTRACT

Drought is one of the main environmental factors affecting growth and yield of sorghum in arid and semi-arid areas of the world. In vitro selection of thirty *Sorghum bicolor* accessions for drought tolerance was undertaken by the use of shoot and root length variations under polyethylene glycol (PEG) stress. Data were recorded at two PEG-6000 levels (0 and -0.7Mpa) on shoot length (SL) and root length (RL). Sorghum accessions WadAkar, Gishish, E315, F.508, F.509, F.511 and KU439 showed low reductions on shoot and considerable increase on root length under PEG stress. A set of 10 EST-SSRs primers related to drought stress was used to assess this trait among sorghum accessions. Six out of the 10 EST were polymorphic (60%). The marker Dsenhsbm99, which code for drought-induced hydrophobic protein, has the highest abundance among the PEG-tolerant accessions. The genetic similarity (GS) for pairs of sorghum accessions was calculated using Jaccard's coefficients. The genetic similarity among these accessions ranged from 0.26 to 0.88. Similarity coefficient matrices based on the data of EST-SSRs, were used to construct a dendrogram. The results obtained from the analysis of the EST markers were in accordance with the results of osmotic stress experiment using PEG. These results may help in breeding more drought-tolerant sorghum accessions in the near future.

INTRODUCTION

Abiotic stress factors remain a major constraint to the growth and productivity of crops. The single greatest abiotic stress factor that limits crop growth worldwide is water availability (Araus *et al*., 2002). Plants have evolved a number of mechanisms to adapt to and survive water stress, including drought avoidance, dehydration avoidance, or dehydration tolerance. Such adaptive mechanisms are the results of a multitude of morphoanatomical, physiological, biochemical, and molecular changes (Ashraf *et al*., 2012).

Sorghum (*Sorghum bicolor* (L.) Moench is the fifth most economically important cereal crop grown in many parts of the world (Doggett, 1988). It is the second most important feed grain (Dahlberg *et al*., 1995), and is a staple food used in porridges and breads in parts of Africa and Asia (Mann *et al*., 1983). For instance, the harvested area of sorghum in Africa and Asia accounted for 81% of the world according to 2013 data (FAO, 2015). Sorghum is usually grown under rain-fed conditions in drought-prone hot regions of Africa and Asia. Although it is one of the most drought tolerant cereals, water stress is one of the major constraints for its stability and reliable production in these environments. Therefore, identification and understanding the mechanisms of drought tolerance in sorghum have been major goals of plant physiologists and breeders (Bibi *et al*., 2012).

Selection for physiological traits related to drought tolerance is essential as it can increase selection efficiency (Yohannes *et al*., 2014). Field experiments related to water stress has been difficult to handle due to significant environmental or drought interactions with other abiotic stresses (Rauf, 2008). An alternative approach is to use polyethylene glycol (PEG) to induce plant water deficit for germplasm screening (Nepomuceno *et al*., 1998; Kulkarni and Deshpande, 2007; Khodarahmpour, 2011). Polyethylene glycol with molecular mass of 6000 and above are non-ionic, water soluble polymers which are not expected to penetrate intact plant tissues rapidly. PEG solution interferes with the ability of plant roots to absorb water due to reduction of osmotic potential (Dodd and Donovan, 1999; Sidari *et al*., 2008). PEG was used to evaluate sorghum for genetic potential to drought tolerance at seedling stage by many authors (Bibi *et al*., 2012; Gill *et al*., 2002; Bibi *et al*., 2010).

Genomic screening for drought tolerance in sorghum is becoming one of the most interesting research activities for sorghum breeders. Sorghum is an important target of genome

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analysis among the C4 grasses because the sorghum genome is relatively small (~730 Mbp) (Price *et al*., 2005), and consequently numerous sorghum genetic and comparative maps have been constructed (Tao *et al*., 1998; Boivin *et al*., 1999; Peng *et al*., 1999; Klein *et al*., 2000; Haussmann *et al*., 2002a; Menz *et al*., 2002; Bowers *et al*., 2003). Also, a sorghum EST project (Reddy *et al*., 2008) and associated microarray analyses of sorghum gene expression have been carried out (Buchanan *et al*., 2005). As for drought tolerance, quantitative trait loci (QTLs) associated with stay green have been identified and mapped in sorghum (Tuinstra *et al*., 1997; Crasta *et al*., 1999; Xu *et al*., 2000). The determination of the consistency of stay green QTLs across different genetic backgrounds would be important in improving sorghum drought tolerance. Drought response in sorghum has been classified into two distinct stages; pre-flowering drought response that occurs prior to anthesis and post-flowering drought response that is observed when water limitation occurs during the grain-filling stage (Rosenow and Clark, 1995). The already available large collection of expressed sequence tags (ESTs) from genes which are expressed during these two drought stress phases in sorghum (Pratt *et al*., 2005), provides invaluable opportunity for identification of candidate genes for drought tolerance. This study is to screen sorghum accessions for drought tolerance using different physiological and genetical characters.

MATERIALS AND METHODS

Germplasm

Thirty sorghum accessions were obtained from the Faculty of Agriculture, University of Khartoum, Sudan (8 accessions) and from Sorghum and Corn Research Center Kasetsart University, Thailand (22 accessions).

Osmotic stress induction by polyethylene glycol

Seeds of each accession were sown at a depth of 1cm in pots containing 0.5 kg sand soil saturated with Hoagland solution. Each accession was raised in these pots under drought stress conditions induced by adding PEG-6000 (80g/kg) to the Hoagland solution in order to create an osmotic potential of -0.07MPa. A control set raised under non-stress conditions was irrigated daily with Hoagland solution which creates 0.0MPa osmotic potential as suggested by Michel and Kaufman (1973).

The experiment was replicated three times.

Ten days after sowing, measurements on shoot and root lengths were made and data were analyzed using T-test.

Molecular analysis of sorghum accessions

A set of 10 EST-SSR primers (Srinivas *et al*., 2009) related to drought stress were used to assess the genetic diversity between sorghum accessions these are:

DNA extraction

Sorghum genomic DNA was extracted from the 30 accessions using a modified CTAB procedure as described by Hoisington *et al*. (1994). The DNA samples were purified and checked for quality and quantity in a 1.5% agarose gel.

EST-SSR PCR amplification

PCR amplifications were performed in 25 µl reaction mixtures containing 15 ng genomic DNA, 1 unit *Taq* polymerase, 10mM Tris-HCl (pH 9.0), 50mM KCl, 0.1% TritonX-100, 1.5mM $MgCl_2$, 1mM dNTPs, 0.2µM of each primer. The PCR was carried out on a DNA thermo cycler programmed as initial denaturation (95°C for 5 min), followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 50-55°C for 1 min, extension at 72°C for 2 min and a final extension (72°C for 7 min). The PCR products were separated on 2% agarose gel stained with ethidium bromide (10ng/100 ml) and the gels were photographed under UV light. Results were scored for the presence (1) and absence (0) of bands from top to the bottom of each lane. The sizes of bands were estimated by using 1 Kb ladder. Major allele frequency was calculated according to following equation:

$$\text{Major allele frequency} = \frac{\text{Number of genotypes having major allele}}{\text{Total number of genotypes}} \times 100$$

The data was subjected to statistical analysis using SPSS software to calculate Jaccards'. similarity coefficient which converted into distances matrix and dendrogram using Unweighted Paired Group of Arithmetic Means (UPGMA).

RESULTS AND DISCUSSION

Osmotic stress induced by polyethylene glycol (PEG)

The ability of sorghum accessions to tolerate drought was assessed under negative osmotic potential using PEG stress. Table 1 showed significant differences (p<0.05) among the sorghum accessions in shoot and root lengths among different sorghum accessions (Figure 1).

Marker	Type(s) of SSR and Number of repeats	Forward primer (5`- 3`)	Reverse primer (5` - 3`)
Dsenhsbm4	(TG)7	CCAAGGCTGAGGTCAAGAAG	AGCCGAGCTCAACATACAGG
Dsenhsbm19	(GCA)8	CATGATGCAGCAACAACAGC	GAAACCAGAACCGAACCTGA
Dsenhsbm22	(CT)8	GAGGTCGACCAGTACGAGGA	GCAATTGCCAAGAGAGGAAC
Dsenhsbm24	(GA)8 + (GA)9	CGTCAATAGCAAACCACCAG	CCCCTCGAGACTAGTTCTCTCT
Dsenhsbm30	(TGA)8	AGTTTGTGTGTGCGCTCGT	CTCCCCATCACGCATCTAGT
Dsenhsbm52	(TA)22	GCTACGGCGATAACTTGGAC	CGTATACGCCACTGTCGTTG
Dsenhsbm58	(TCC)7	GCGTGACCAAGAAATCAAGA	GGAGGACCAAGATGATCCA
Dsenhsbm75	(GCT)7	AGAGGCAGCAAAGCGAGAC	ACTGGTGGGAGTCCGTGTAG
Dsenhsbm89	(GCAACG)6	TAAATCGGAGAGCAGGAGGA	TGAACAAGTTGGAGCTGCTG
Dsenhsbm99	(GCA)6	GCCAAGGCAGAGAAGAAGAAG	CGACGACGACTACTTGGTGA

Osmotic stress reduced shoot length with the highest reductions observed for F.504, followed by F.509, F.511, F.513 and F.514 (Figure 1).

Table 1 Means values of shoot length (SL) and root length (RL) under control and PEG stress conditions.

Treatment	Shoot length	Root length
Control	11.29 ± 0.212	4.04 ± 0.203
PEG	8.4 ±0.327	5.61 ± 0.195

*values are given as a mean of three replicates ± SE

The lowest reduction in Shoot Length was observed in Botana, WadAhmed, WadAkar, E315 and F508. Four accessions dried up and died after one week of treatment, which reflect their sensitivity to osmotic stress. Reduction in shoot length in cereal crops is mostly indicates drought tolerance (Bibi *et al*., 2012). The decrease in shoot Length in the studied accessions may be due to osmotic regulation, which enables them to maintain cell turgor to assist growth under severe stress conditions. The variability in shoot length reductions between the accessions indicates a genotypic variability in response to water deficit. Similar findings were reported in sorghum by Raziuddin *et al*. (2010), Khodarahumpour (2011), Bibi *et al*., (2010); Ali *et al*., (2011). Bibi *et al*. (2010) observed that most of the morphological and physiological characters at seedling stage are affected by osmotic stress in sorghum. Drought stress suppressed shoot growth more than root growth and in certain cases root growth increased (Younis *et al*., 2000; Okçu *et al*., 2005; Bibi *et al*., 2010). Water uptake by the roots is a complex parameter that depends on root structure, root anatomy, and the pattern by which different parts of the root contribute to overall water transport (Cruz *et al*., 1992). Therefore, comparative elongation of the root under water stress is an important indication for drought tolerance.

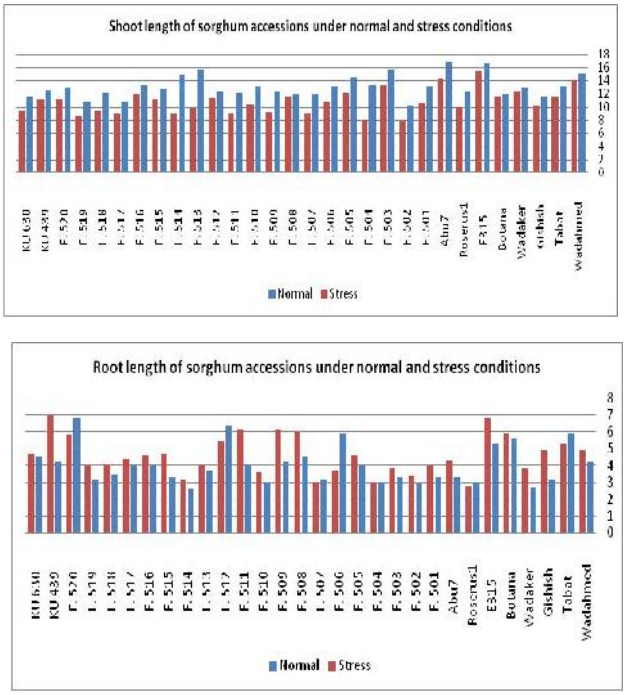

Figure 1 Effects of PEG stress on shoot and root lengths of sorghum accessions

Low reductions in shoot and considerable increase in root lengths under PEG stress were recorded for WadAkar, Gishish, E315, F.508, F.509, F.511 and KU439 sorghum accessions (Figure 1), therefore they were considered as tolerant genotypes. Similar observations and conclusions were also reported by Bibi *et al*. (2012) and Marmar *et al*. (2013) on sorghum and wheat, respectively.

Screening for drought tolerance using EST-SSR markers

Ten EST-SSR drought associated markers were used in this study to screen 30 sorghum accessions for drought tolerance. Six markers (Dsenhsbm4, Dsenhsbm22, Dsenhsbm30, Dsenhsbm52, Dsenhsbm89 and Dsenhsbm99) were polymorphic (60%) while the remaining four (Dsenhsbm58, Dsenhsbm75, Dsenhsbm19, Dsenhsbm24) were not (Table 2). Using 70 SSR markers with 33 sorghum accessions from Sudan El Hussein *et al*. (2014) found 71.4% polymorphism. According to Gupta *et al*. (2003) only 55% of the 20 EST-SSR markers used were polymorphic when tested against 52 wheat accessions.

Table 2 shows that two EST-SSR markers (Dsenhsbm19 and Dsenhsbm24) give amplification products with all sorghum accessions. Markers Dsenhsbm22 and Dsenhsbm52 gave positive amplification with most of the tested accessions. Although, Dsenhsbm89 marker gave positive amplicon with some accessions from Sudan, it failed to do so with any of the 22 accessions from Thailand indicating the environmental and geographical effect on the drought response.

The EST used in this study are encoding important functional proteins which are involved in drought tolerance. EST Dsenhsbm19 is known to code for Ethyleneinsensitive3-1 (EIL-1) protein, a key transcription regulator of ethylene biosynthesis (Chao *et al*., 1997; Solano *et al*., 1998), suggesting its role in drought stress adaptation as ethylene is involved in the regulation of leaf senescence (Yang *et al*., 2008). Also, the ESTs Dsenhsbm4 and Dsenhsbm24 were reported to code for heat shock protein and chaperonin, respectively, both are involved in protecting macromolecules such as enzymes and lipids under severe drought stress (Vierling 1991; Zhu *et al*. 1997). In addition, ESTs Dsenhsbm30 and Dsenhsbm99 include genes coding for important regulatory proteins and functional proteins that are involved in stress related metabolism (Yin *et al*., 2014).

Accessions which showed tolerance to osmotic stress are found to encompass different sets of EST marker genes (Table 2). The accession E315 which gave positive amplification with 70 % of the markers used was the most tolerant to PEG stress. This was followed by Wad Ahmed (50%), F.508 (50%) and KU630 (50%). It could be observed from these results that marker Dsenhsbm99, which code for drought-induced hydrophobic protein, has the highest abundance among the PEG-tolerant accessions indicating the role of this gene in stress tolerance. Although Dsenhsbm58 and Dsenhsbm75 markers code for TB2/DP1, HVA22 family protein (ABA responsive) and Zinc finger A20/ AN1 domain-containing stress associated protein 9 (OsSAP9), respectively, it was not detected in any of the PEG-stress tolerant accessions.

ABA inducible transcription factors (AREB/ABF) play a central role in drought-responsive gene expression, and the ABA responsive cis-elements (ABRE; T/CACGTGGC) have been widely found in the upstream regulatory regions of downstream genes (Hirayama and Shinozaki, 2010).

Table 2 Distribution of drought associated EST-markers in 30 sorghum accessions and their putative functions

S. No	Marker	Distribution in Sorghum accessions	allele frequency %	Putative function
1	Dsenhsbm4	Gishish,WadAker, Roserus1, E315, Abu7, F.505, F.508	26.6	Heat shock protein (16.9 KD low molecular weight protein
2	Dsenhsbm19	All	100	Ethyleneinsensitive3- 1 protein
3	Dsenhsbm22	WadAhmed, Tabat, F.501, F.503, F.505, F.506, F.507, F.508, F.510, F.512, F.513, F.514, F.515, F.517, F.520, KU630	53.3	Serine/threonine-protein kinase SAPK5 (Osmotic stress/ abscisic acid-activated protein kinase 5)
4	Dsenhsbm24	All	100	Chaperonin 21 precursor
5	Dsenhsbm30	E315, WadAhmed, Gishish, Botana, F.520, F.515	20	Stress related protein
6	Dsenhsbm52	Roserus1, Tabat, F.501, F.502, F.503, F.504, F.507, F.512, F.513, F.519, KU439, KU630	40	Chitinase
7	Dsenhsbm58	Negative	-	TB2/DP1, HVA22 family protein (ABA responsive)
8	Dsenhsbm75	Negative	-	Zinc finger A20 and AN1 domain-containing stress associated protein 9 (OsSAP9)
9	Dsenhsbm89	WadAker,E315, Roserus1,Abu7	13.3	Glycine-rich RNA-binding protein
10	Dsenhsbm99	E315, WadAhmed, F.508, F.5012, F.516. F.517, F.520, KU439, KU630	30	Drought-induced hydrophobic protein

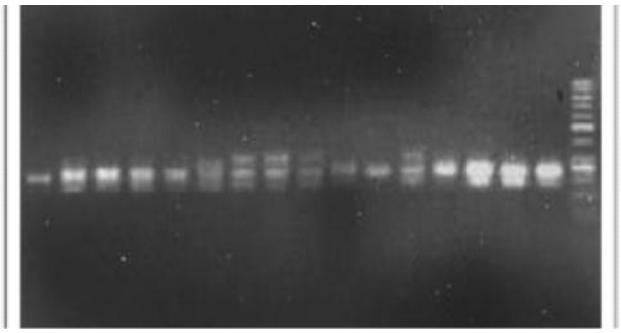

Figure 3 PCR amplification of Dsenhsbm4 EST-primer. M =DNA Ladder.

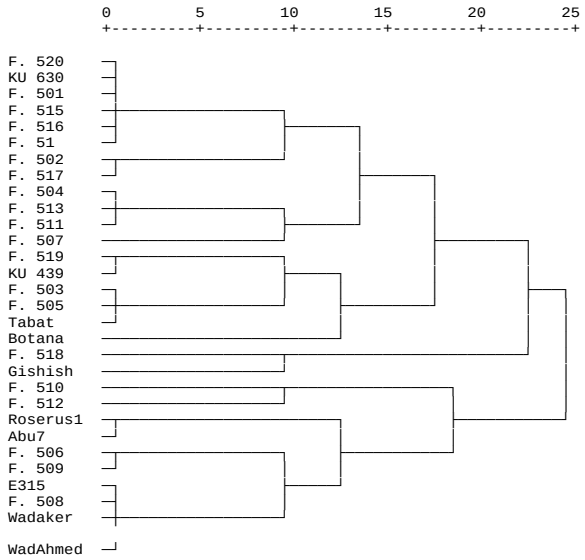

Figure 4 Dendrogram showing the relationship among 30 sorghum accessions based on drought associates EST bands. The scale is based on Dice similarity coefficient.

Transcription factors in the ABA-dependent pathways include 1) ABA-responsive element-binding protein/ factor (AREB/ABF), 2) C-repeat-binding factor 4/dehydration responsive element-binding protein 1D (CBF4/DREB1D), 3) myeloblastosis/myelocytomatosis (MYB/MYC), 4) Cys2His2 zinc-finger proteins (ZFP), and 5) WRKY domain binding transcription factors (WRKY) (Bartels and Sunkar, 2005).

The genetic similarity (GS) for each pair of sorghum accessions was calculated using Jaccard's coefficients. The genetic similarity among these accessions ranged from 0.26 to 0.88, indicating a high degree of genetic homogeneity. The dendrogram was constructed (Fig. 4) using bivariate (1/0) data and Jaccard s' similarity coefficient matrices based on the data of EST-SSRs.

Average Linkage (Between Groups) and SPSS computer program grouped sorghum accessions into seven clusters. The results obtained from the analysis of the EST markers were in accordance with the results of water stress experiment using PEG. The accessions F.504, F. 507, F. 511 and F. 513 which exhibit high response to PEG stress were in the same cluster far from the cluster containing the lines WadAhmed, WadAkar, F. 508 and E315 which tolerate the PEG stress, and accumulate more drought associated ESTs under study.

Based on the results of this study, these markers will provide hypothetically candidate genes that have the potential of being causally linked to the drought tolerance. Therefore, the eight positive ESTs-SSR markers used in the study may assist to study association of the molecular variability of the genes with phenotypic variability of traits related to drought tolerance and other agronomic importance in sorghum.

CONCLUSION

From the result of this study it is concluded that identification of sorghum accessions containing drought tolerance genes using physiological and marker-assisted selection may help in breeding more drought-tolerant sorghum cultivars in the near future and can be useful to speed up sorghum improvement.

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