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Physiological response to drought and Dehydration responsive transcripts (DRTs) from the leaves of water-deficit Indian soybean [Glycine max (L.) Merrill cv NRC7]

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Abstract

Drought is a major reason for reduced productivity in principal food crops including soybean. Development of drought tolerant varieties is imperative to tide over erratic rainfall conditions in Indian context. Here we studied molecular mechanism governing drought tolerance in Indian soybean cultivar NRC7 to identify drought responsive genic biomarkers. Drought associated physiological parameters like relative water content (RWC) and electrolytic leakage studies under water-deficit condition revealed very low water status in the stressed plants. In addition, membrane damage studies on electrolytic leakages revealed that the stressed plants exhibited greater membrane damage than control plants. Differential display RT-PCR (DD-RT-PCR) identified two drought responsive transcripts (gmDRT1 and gmDRT2) from the soybean cultivar NRC7. The identified transcripts were found to have sequence homology with dehydrin protein and ion ATPase transporters. These biomarkers identified for drought tolerance would also help in engineering drought tolerant soybean cultivars.

Keywords: Biomarkers; Drought tolerance; Differential display; Gene regulation

Introduction

Drought is a major factor that limits productivity of the crops worldwide [1]. Climate change studies further indicates that water is going to be an increasingly limiting factor thus drought is a looming threat that would significantly affect the productivity levels of major crops [2]. Despite its significance, molecular mechanisms underlying the plant's response to drought conditions are not well understood. The genetics and molecular biology of the plant growth and developmental processes under the influence of drought would help in devising appropriate mitigation strategies. Drought mitigation strategy essentially involves development of drought tolerant crop genotypes. Genetic markers (SSRs and SNPs) aided QTL analysis and mapping of drought tolerance traits in plants is an approach to develop drought tolerant cultivars [3]. Alternatively, gene expression profiling studies in plants under drought status help dissect the dehydration responsive genes, *cis*-regulatory elements, non-coding RNAs etc in order to decipher the complete molecular cascades functioning under water-deficit conditions [4,5]. Soybean is an important oilseed crop, and a grain legume that is grown extensively in central Indian regions of the country. At present India occupies fourth position, globally, in terms of soybean production (Annual report, ICAR-DSR 2013-14). Soybean cultivation under Indian conditions is mainly associated with low productivity and the two main reasons attributed to this low productivity are short growing period and narrow genetic base [6]. Narrow genetic base of the present day genotypes is a major cause for their susceptibility to various biotic and abiotic stresses. Among the abiotic factors, drought is considered to be of prime importance for its low productivity in India. In soybean, drought has been shown to reduce the yield by about 40% [7]. Observed shift in the rainfall pattern, intensity, and erratic nature of precipitation in the central Indian region where soybean is grown extensively as a rain fed crop warrants introduction of drought tolerant cultivars without compromising yield potential. However the bottleneck in attaining drought tolerant or resistant genotypes of soybean is greatly hindered by availability of genes or transcriptional factors (TFs) conferring drought resistance. This prompted us to device a study to isolate and characterize drought responsive transcripts in soybean that may be utilized as biomarkers for drought tolerance, and its ultimate introgression into cultivated genotypes. As a first step in this direction, the molecular basis of drought tolerance in Indian soybean cultivar NRC7 was studied employing transcript expression profiling tool differential display reverse transcription PCR (DD-RT-PCR) [8]. Differential Display Polymerase chain reaction (DDRT-PCR) is a sensitive, simple and powerful technique for screening cDNA and is also useful in characterizing tissue or developmental stage specific gene expression patterns. Thus the ultimate goal of this study is to utilize the candidate genes, promoter elements for engineering drought tolerant soybean for Indian conditions.

Materials and Methods

Plant material and growth conditions

Seeds of soybean cultivar NRC7 characterised as drought tolerant genotype were sown in the pots containing potting soil and irrigated regularly. The plants were grown under green house conditions at 26°C with 16 h day and 8 h night.

Stress induction and assessment of physiological parameters

Seedlings at the age of 30 days after sowing (DAS) (at vegetative stage) were subjected to moisture stress until plants exhibit symptoms of wilting by withholding irrigation. Control plants were watered regularly until 45 days. Physiological parameters associated with drought tolerant phenomenon such as relative water content (RWC) and membrane integrity studies were documented using these controls and stressed leaves. RWC was measured as follows: fresh weight of five

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leaf discs, in triplicates, was recorded from control and stressed leaf samples. Then the leaf discs were floated in 10 ml of water for six hours and allowed to gain turgidity. Turgid weights are recorded and dried in hot oven at 80°C to a constant weight to record dry weight. RWC was estimated using the formula

RWC=(Fresh weight - Dry weight)/(Turgid weight - Dry weight) × 100

In order to assess membrane integrity, control and stressed leaf discs of 1 cm diameter were collected, washed in distilled water, blotted on filter paper and incubated in 25 ml of water with continuous shaking for 2 hours. Initial electrical conductivity (EC) was taken using EC-tds analyzer (Eutech). The leaf discs were then boiled for 30 minutes and final EC were recorded. Percentage of cell leakage is computed using the formula

Percentage leakage=(Initial EC/ Final EC) × 100

RNA extraction and differential display RT-PCR

Total RNA was extracted from the leaves of control and stressed plants by using RNA easy plant minikit (Qiagen). Concentration and integrity of the extracted RNA were assessed using spectrophotometer (Shimazdu) and in denaturing agarose gel electrophoresis (Figure 1).

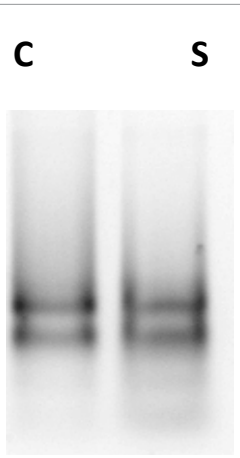

Figure 1: Total RNA isolated from NRC7 control (C) and stressed (S) leaf samples resolved in 1% denaturing agarose gel electrophoresis using formaldehyde.

S. No	Anchored primers	Sequence
1	H-T11 G	AAGCTTTTTTTTTTG
2	H-T11 A	AAGCTTTTTTTTTTA
3	H-T11 C	AAGCTTTTTTTTTTC

Table 1a: List of anchored primers.

S.No	Arbitrary primers	Sequence
1	H-AP9	AAGCTTGATTGCC
2	H-AP10	AAGCTTCGACTGT
3	H-AP11	AAGCTTTGGTCAG
4	H-AP12	AAGCTTCTCAACG
5	H-AP13	AAGCTTAGTAGGC
6	H-AP14	AAGCTTGCACCAT
7	H-AP15	AAGCTTAACGAGG
8	H-AP16	AAGCTTTTACCGC

Table 1b: List of arbitrary primers. PCR primers and its sequence used in DDRT-PCR analysis. (Source: GenHunter Corporation™).

Further to avoid any genomic DNA contamination, extracted RNA was subjected to DNAase I treatment (Thermo Scientific, MA USA). Total RNA extracted and about 2 µg of RNA was reverse transcribed in 3 separate reactions using each of the anchored oligodT primers supplied with RNAimage kit, following prescribed protocol (GenHunter Corp., Nashville, TN, USA) (Table 1). Generated cDNA was quantified and equal quantity of cDNA was used as a template for DD-PCR using the eight different kinds of arbitrary primers (H-AP9 to H-AP16) supplied with the kit. Hence a total of 24 primer combinations (using 3 anchored primers and 8 arbitrary primers) of DD-RT-PCR were carried out. Total reaction volume of DD-RT-PCR is 20 µl comprising 2 µl cDNA, 0.5 µl of *Taq* DNA Polymerase (Fermentas), 1 µl of anchored primer, 1 µl of arbitrary primer, 1.0 µl of dNTPs, 1 µl $MgCl_2$, 2 µl of *Taq* buffer (10X) and volume was made up to 20 µl with sterile water. Temperature profile in the thermocycler for performing PCR was as follows: 1 cycle for denaturation 94°C for 4 minutes, followed by 30 cycles comprising 3 segments each of denaturation 94°C for 30 seconds, annealing 40°C for 2 minutes followed by extension at 72°C for 30 seconds. Final extension was kept at 72°C for 5 minutes. PCR amplified products were then resolved in 16% polyacrylamide gels with 5X TBE buffer and silver stained following the procedure as described earlier [9]. Differentially expressed bands were cut and re-amplified for sequence characterisation.

Cloning and sequencing

Amplified PCR products corresponding to dehydration responsive transcripts were cloned in pGEM-T easy vector (Promega, Madison, USA) and sequenced at Merck Biosciences (Bengaluru, India).

Results and Discussion

Soybean has long been regarded as one of the principal food crops and serves as a major economical source of dietary proteins in the world. Malwa plateau of Central India is the hub for soybean cultivation and has played a significant contribution to yellow revolution in India [10]. When compared to other countries the productivity of soybean is very low in India. This relative low productivity is mainly ascribed to a short growing period and the narrow genetic base of soybean cultivars resulting in susceptibility to biotic and abiotic stresses [6]. Erratic rainfall is the common attribute of the rain fed ecosystem and among several other factors; drought is the prime factor that limits soybean productivity in India. Therefore, understanding the molecular biology of soybean lines/varieties that can survive limited water situations is essential to develop varieties that can mitigate adversity of drought and help in sustainable and improved productivity of soybean. Soybean cultivar NRC7 was subjected to water-deficit stress 30 DAS by withholding irrigation. Leaf samples of control and stressed plants were analyzed for physiological parameters like relative water content (RWC) and membrane damage assay by electrolytic leakage studies. Comparison of relative water status between control and stressed plants showed that stressed plants generally exhibited low relative water content than control plants (Table 2). Membrane damage assay was ascertained from electrolytic leakage pattern in the leaves of control and stressed plants (Table 2). It was evident from the study that plants under moisture-deficit stress exhibited increased electrolytic leakage consequently it is inferred that more membrane lipid damage under stress when compared to control plants. It has been demonstrated recently that electrolyte leakage measurements may be correlated with several physiological and biochemical parameters such as spectral reflectance, antioxidative enzyme synthesis, stomatal resistance, osmotic potential conditioning that imparts the plants with ability to respond to environmental conditions In order to decipher the molecular response

S.No.	Variety/treatment	Fresh wt. (mg)	Turgid wt. (mg)	Dry wt.	RWC
1.	NRC7 Control	189.96	240.66	55.56	72.609%
2.	NRC7 Stress	193.3	269.63	60.66	63.473%

Table 2a: Relative water content (RWC) status of the leaves of control & stressed plants of soybean cultivar NRC7.

S.No.	Variety/Treatment	Initial EC(ECa)	EC@50°C (ECb)	EC@100°C (ECc)	%Electrolyte leakage
1.	NRC7 Control	105.0	208.0	703.0	471.0
2.	NRC7 Stress	106.0	173.0	830.0	619.8

Table 2b: Electrolytic leakage studies on the leaves of control and stressed of soybean cultivar NRC7.

of soybean following prolonged moisture-deficit stress differential display RT-PCR was employed to isolate water-deficit stress related transcripts. DD-RT-PCR has been effectively deployed in identifying a number of differentially expressed genes from plants. DDRT-PCR identified drought tolerant genes in cowpea called as cowpea responsive to dehydration (CPRD) after 10-h dehydration stress [11]. Differentially expressed genes from four varieties of cotton (*Gossypium hirsutum* L) under water deficit condition were isolated using DDRT-PCR. Among the differentially expressed transcripts, one showed homology to NADP (H) oxidase [12]. Here in this study, DNase treated RNA was subjected to reverse transcription using anchored primers supplied with RNAimage kit (GenHunter Corp., Nashville, TN, USA). The resultant cDNA was diluted appropriately and used as a template for PCR amplifications using 8 different arbitrary primers supplied with the RNA image kit (GenHunter Corp., Nashville, TN, USA). The amplifications resulted in differential display of drought responsive transcripts in control and stressed leaves of NRC-7. Analysing the DD-RT-PCR products obtained by screening with 24 primer combinations reveal that most of these combinations were unable to differentiate control and stressed leaf samples of soybean nonetheless some combinations revealed differential expression pattern (Figure 2) Among the potential differentially expressed bands only two of the products were cloned after re-amplification and were named as gmDRT-1 and gmDRT-2 (Figure 3). Cloning and sequencing of the DRT products revealed that gmDRT-1 exhibited nucleotide and amino acids sequence homology with *lea* a class of dehydrin protein whereas gmDRT-2 showed nucleotide and amino acid sequence homology with Ion transporters ATPases in soybean. The sequence attributes and homology related information of these DRTs are provided as supplementary files. The expressed gene tags obtained in this study (gmDRT1 and gmDRT2) could be employed further to characterise the complete gene sequence

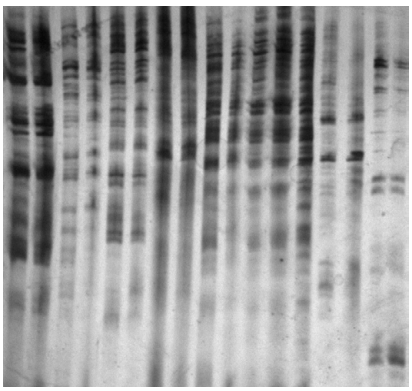

Figure 2: DD-RT-PCR of NRC7 (c) and (s) resolved in 16% denaturing PAGE and silver stained (Odd numbered lanes are NRC7 (C) whereas even numbered lanes are NRC7 (S).

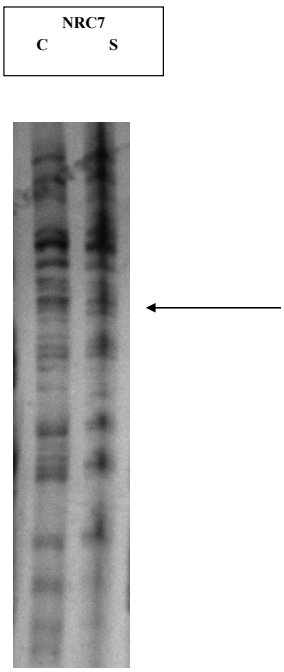

Figure 3: DD-RT-PCR of NRC7 (c) and (s) resolved in 16% denaturing PAGE and silver stained showing the differentially expressed gmDRT1.

including its promoter elements. Hundreds of genes are thought to be involved in abiotic stress responses. The accumulation of heat stable and dehydrin like proteins in developing soybean seeds can be used as an indicator for determining seed maturity in physiological and biochemical. Dehydrin-like Proteins were reported in soybean seeds in response to drought stress during seed filling [13]. Similarly, dissection of molecular basis of drought tolerance in peanut (*Arachis hypogaea L*), identified as many as 1235 differentially modulated transcripts in stressed plants called as Peanut transcripts responsive to drought [14]. Recently salt-stress responsive transcripts from the roots of *Hibiscus subdariffa* were also identified and validated using the technique [15]. In addition the role of dehydrin proteins like *lea* in soybean- arbuscular mycorrhizal fungal symbiosis under the condition of the drought was studied [16]. Transcriptional factors (TFs) are known to play major role in drought response molecular mechanism. In soybean also some of the TFs like (Dehydration-Responsive Element-Binding Factor) *DREB* homologue genes (*GmDREBa*, *GmDREBb* and *GmDREBc*) have also been isolated from soybean (*Glycine max*). More recently, the function of these genes has been validated through their over-expression in transgenic soybean plants where they conferred drought and salinity tolerance [17]. To develop soybean plants with enhanced tolerance to drought stress, an understanding of the physiological and biochemical responses and gene regulatory networks is essential. Hence the results of the study not only enable us to delineate the molecular mechanism behind the drought tolerance but also may be helpful in identifying bio markers associated with drought tolerance and also to engineer the soybean cultivars by expression of these markers to confer drought tolerance.

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