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Identification of differentially expressed transcripts in groundnut genotypes subjected to different levels of mid-season moisture stress

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Abstract
Groundnut is mostly cultivated under rainfed situation in India and drought tolerance is one of the major breeding objectives. Irrespective of the drought tolerance levels in groundnut genotypes, a significant increase in proline concentration under drought stress situation and progressively higher level of proline was accumulated in drought sensitive genotypes like Kadiri 6 and Narayani than the drought tolerant genotypes like TCGS 1157 and Kadiri 1725. Transcriptome analysis by cDNA-RAPD revealed a total of 712 drought responsive Transcript Derived Fragments (TDFs) were identified in four groundnut genotypes with 20 RAPD primers among the four genotypes at different levels of moisture stress and in comparison with their respective control. Out of 712 TDFs, 180 TDFs were quantitatively expressed, 514 TDFs were qualitatively expressed and 18 TDFs were showed without any difference in the level of expression. Two moisture stress responsive transcripts were identified with OPA2 (700 bp TDF) and OPA4 (450 bp TDF) markers only under drought stress in resistant genotypes (TCGS1157 & Kadiri1725) and absent susceptible genotypes (Kadiri 6 & Narayani). Further, characterization of these transcripts by sequencing will greatly helps in understanding nature of the genes and the mechanism by which groundnut plants respond to drought stress. These genes will be highly useful for the development of drought tolerant groundnut genotypes through molecular breeding.

Keywords: Drought, proline, cDNA-RAPD, TDFs, transcriptome

Introduction
Groundnut (*Arachis hypogaea* L.) popularly known as 'king of oil seeds' cultivated all over the world. It is touted as functional food owing to rich in minerals, vitamins, antioxidants and health improving bioactive compounds such as resveratrol, tocopherol, and arginine (Mukhtar, 2009) [14]. In India, it is mostly grown under rainfed situation in kharif season and drought is one of the important production constraints limiting yield and quality of the produce. Global warming and climate change is anticipated to further decrease rainfall in many semi-arid regions in next decades (Bates *et al*., 2008) [3]. Therefore, understanding the mechanisms of drought tolerance and development of drought tolerant varieties are key strategies for sustainable yield under rainfed situation.

Drought is a complex phenomenon and plants have developed a wide array of strategies either to avoid or cope with the stress conditions (Bartels *et al*., 2005) [2]. Response to drought stress also depends on the type of species and genotypes. The main physiological drought stress responses in groundnut include folding of leaves, stomatal closure, repression of cell growth and photosynthesis, and activation of respiration. At the biochemical level, many plants accumulate osmoprotectants such as sugars (sucrose, raffinose, and trehalose), sugar alcohols (sorbitol and mannitol), amino acids (proline) amines (glycine betaine and polyamines) and enzymes (Peroxidase, polyphenol oxidase, catalase and superoxide dismutase) (Seki *et al*., 2007; Chakraborty *et al* 2015, Aparna *et al*., 2018) [19, 4, 1].

At molecular level, the drought stress tolerance is conditioned by a large number of up- and down- regulated genes inducing multiple signaling pathways which intern strictly control the physiological and biochemical responses to the drought stress (Sreenivasulu *et al*. 2007) [20]. The identification and characterization of genes induced under drought stress is a common approach to understanding the molecular mechanisms of stress tolerance in plants. Several methods are being employed to identify genes involved in stress responses such as differential display reverse transcription-polymerase chain reaction (DDRT-PCR), serial analysis of gene expression (SAGE), suppression subtractive hybridization (SSH), cDNA –RAPD, cDNA - AFLP and cDNA microarray are available for transcriptomic analysis. High throughput sequencing (Myles *et al*., 2010) [15] and cDNA Microarray (Duressa *et al*., 2011) [6] technologies

have proved to be the efficient way to study genome-wide analysis of differentially expressed genes.

Comparing with these methods, cDNA based randomly amplified polymorphic DNA (cDNA-RAPD) is much more economical and time-saving. The cDNA-RAPD is employed to study differential gene expression in Triticum aestivum L. (Mizumoto *et al*., 2009) [13], Gossypium hirsutum L. (Jagadeesh *et al*., 2009) [9] Cucurbita pepo L. (Guo *et al*., 2011) [7] Soy bean (Huang *et al*., 2015) [8] and Mungbean (Lovejot *et al*., 2015) [11]. Comparative analysis by combining groundnut genotypes with contrasting drought tolerance capacities will be much more effective for mining drought responsive genes. In addition, information on drought stress responses at a particular stage during which water deficit condition leads to economic yield loss will be useful to guide breeders to modify varieties for improvement in water use efficiency (WUE). Therefore, the present work used cDNA-RAPD method to identify the potential genes that really conferred to drought response by using two drought tolerant and two drought sensitive groundnut cultivars. Here we report a number of transcript-deririved fragments (TDFs) in cultivated groundnut that were found to be activated (up-regulated) or suppressed (down-regulated) during the drought stress. The differentially expressed TDFs could be employted as molecular markersfor screening groundnut germplasm for drought tolerance and further in crop improvement programs.

Materials and Methods

Experimental material and drought stress imposition

The experiment was conducted under field conditions at Regional Agricultural Research Station, Tirupati, Acharya N. G. Ranga Agricultural University, India. Groundnut genotypes with different levels of drought tolerance were procured from Regional Agricultural Research Station, ANGRAU, Tirupati. Two groundnut genotypes *viz*., TCGS 1157 and Kadiri 1725 as drought tolerant and Narayani and kadiri 6 as comparatively less drought tolerant were selected based on the water use efficiency, SCMR and SLA for this study. The experiment was conducted under field conditions. The seeds were sown in the field in two treatments of three replications each. Drought stress was imposed on 50-day-old plants by withholding water for 30 days which coincides with critical growth stages of the crop like pegging and pod filling, while control treatment were irrigated 10 days interval.

RNA isolation and cDNA synthesis

Leaf samples from both stressed and their respective controls were collected at three sampling times *viz*., 10 days, 20 days and 30 days after the initiation of the moisture stress treatment (50 days after sowing). The samples were collected separately in aluminum foil and plunged into liquid nitrogen to avoid RNA degradation.

The total RNA was extracted from leaf samples by using Triazol method according to the procedure of Mac Rae (2007) with minor modifications. Purity, integrity of RNA and potential DNA contamination was determined by running 3 µL of total RNA in agarose gel (0.8 % agarose gel prepared 1X TBE buffer treated with Guanidine Thiocyanate @ 2.3 gm GTC in 1 lit of 1X TBE buffer along with ethidium bromide @ 3.5µl/100ml) and quantified with Nanodrop spectrophotometer (Nanodrop, ND-1000, USA). After the quality and quantity check procedures, cDNA was synthesized from 20 µg of total RNA was used initially for first strand synthesis followed by second strand synthesis using RevertAid First strand cDNA synthesis kit (Thermo Scientific) in accordance with the manufacturer's instructions.

RAPD Reaction with cDNA templates and profile scoring

PCR amplification of cDNA from both stressed and their respective control plant samples (60, 70 and 80 DAS) were carried out using 10-mer RAPD primers (OPA, C, D, F, G, S and T, Operon Technologies, Inc., Alameda, CA). The PCR reaction mixture (25 µl) contained 10 ng of cDNA, 2.5 µl of 10x PCR buffer, 2.5 mM MgCl2, 2 mM dNTPs, 1 picomoles of primer and 1 Unit of Taq Polymerase (Fermentas). Amplifications were performed in Eppendorf thermal cycler programmed for 45 cycles: 1st cycle of 4.5 min at 92ºC, 1 min at 35ºC, 2 min at 72ºC; followed by 44 cycles each of 1 min at 92ºC, 0.5 min at 35ºC, 2 min at 72ºC followed by final extension for 15 min at 72ºC. For analysis of differentially expressed trnscripts, the PCR products were resolved on 1.5% agarose in 1x TBE buffer. Clearly resolved bands of both stressed (60, 70 and 80 DAS) and respective control samples were scored manually on the basis of their presence/absence or intensity of the bands and were assigned up- and down-regulated in comparison with their respective controls.

Results and Discussion

The present study interested in the drought responsive genes that showed differential expression in both drought tolerant and drought sensitive genotypes under irrigated and stress treatments. Contrasting genotypes with respect to drought *viz*., TCGS 1157, Kadiri 1725 (drought tolerant), Narayani and Kadiri 6 (Drought sensitive) were identified previously by Aparna *et al*. (2018) [1] based on physiological traits were subjected to mid-season drought stress at 50 to 80 DAS. The mid- season drought stress led to severe yield losses and reduction in yield attributes and quality, as against crop receiving full irrigation during the whole crop duration, (Suvarna *et al*., 2006) [21]. Within 10 days of drought stress, drought stress symptoms like drooping and folding of leaflets were observed in drought sensitive genotypes *viz*., Kadiri 6 and Narayani, whereas drought tolerant genotypes *viz*., Kadiri 1725 and TCGS1157 exhibited water saving mechanisms like leaflet folding. With progressive increase in drought stress up to 30 days, all the genotypes exhibited drought stress symptoms, but drooping and withering was more severe in Kadiri 6 and Narayani. In addition, there was a remarkable difference was noticed between control and moisture stress imposed treatments like reduced vegetative growth decreased chlorophyll content and increased levels of proline as a defence mechanism (Table 1). The SLA decreased more significantly in drought tolerant genotypes (TCGS 1157 and Kadiri 1725) than in drought sensitive genotypes (Kadiri 6 and Narayani) as compared to respective controls. These results suggests that the genotypes with low SLA and leaflet folding under mid-season drought stress are more useful traits for breeders to effect selection in segregating lines for development of drought tolerant varieties(Maiti *et al.,* 2000; Tardieu, 2012; Chakrabarthy *et al.,* 2015) [12, 22].

Proline accumulation in drought stressed plants is a primary defense response to maintain the osmotic pressure in a cell. There was a significant increase in proline concentration in drought stressed as compared to its respective control, however proline level was noticeably increased more in 30 days drought stressed genotypes in comparison with 10 and 20 days drought stress (Table 1). Higher levels of proline in response to drought stress play an adaptive role in mediating osmotic adjustment and protecting the sub-cellular structures (Ranganayakulu *et al.,* 2015) [17]. A progressively higher level

of proline was accumulated in drought sensitive genotypes like Kadiri 6 and Narayani as they did not show any water saving mechanisms and thereby more proline was required to maintain osmotic potential. Therefore, the increased levels of proline under drought stress can be better considered as a stress indicator in plants (Sairam & Tyagi, 2004; Kaneria and Bishi, 2015) [18, 10]. A positive correlation between the accumulation of proline and drought stress tolerance in different crops has been noted (Maiti *et al.*, 2000; Ranganayakulu *et al.*, 2015) [12, 17].

Table 1: Effect of mid-season drought stress on SCMR, SLA total chlorophyll content and proline content in groundnut. The data are mean values ± SE for ten plants in three replicates (* significant at 1% level)

Physico-biochemical parameters	Genotypes	60DAS		70DAS		80DAS	
		Control	10days Drought stress	Control	20 days Drought stress	Control	30 days Drought stress
SLA	Kadiri1725	128.3±0.3	113.8±11.8	129.4±0.4	114.7±3.3*	151.1±1.7	123.9±2.0*
	TCGS1157	118.3±3.1	117.9±1.4	138.7±3.1	128.6±2.9*	161.7±1.4	133.1±2.2*
	Kadiri 6	130.3±2.0	125.0±0.2	140.7±1.9	137.6±5.4*	146.6±0.3	133.2±1.5*
	Narayani	120.0±4.0	120.0±1.1	135.7±4.2	128.2±0.4*	144.8±3.3	136.5±0.4
Proline content	Kadiri1725	8.5±0.6	136.6±1.6*	25.5±2.5*	179.0±2.5*	37.3±1.5	262.7±1.8*
	TCGS1157	16.1±1.9	168.3±1.6*	26.1±2.5	241.1±12.0*	39.9±1.8	247.2±5.3*
	Kadiri 6	5.5±0.9	134.9±3.4*	15.7±0.4	288.3±5.3*	32.0±0.2	409.6±3.2*
	Narayani	3.8±1.0	211.0±2.0*	18.0±3.3	322.8±2.0*	69.0±1.7	432.7±10.8*

Transcriptome profiling under mid-season drought in groundnut

To unravel the molecular mechanisms conditioning drought tolerance in groundnut, transcript me of leaves was analysed by cDNA-RAPD under imposed moisture stress of 10 (60DAS), 20 (70DAS) and 30 (80DAS) days by using 35 random decamer primers (Table 2). The cDNA-RAPD profile developed with RAPD marker OPA4 in four genotypes at different moisture regimes and their respective controls was depicted in figure 1. The transcript sizes ranged between 180 to 2500bp from RAPD primers. Twenty out of 35 RAPD primers produced 712 reproducible and scorable TDFs with both qualitative, quantitative differences in expression levels in response to various levels of mid-season drought (Table 2). Out of 35 RAPD primers, 15 primers resulted in non reproducible banding pattern and were not counted for the differential gene expression analysis. Similar kind of differential expression in transcripts was reported in many crops including groundnut. Ding et.al (2014) identified a total of 111 differentially expressed sequence tags in groundnut of which 80 transcripts encoded proteins with drought-related functions. Maximum number of 78 TDFs was amplified by OPA2 and least number of only 10 TDFs were identified by OPD16.

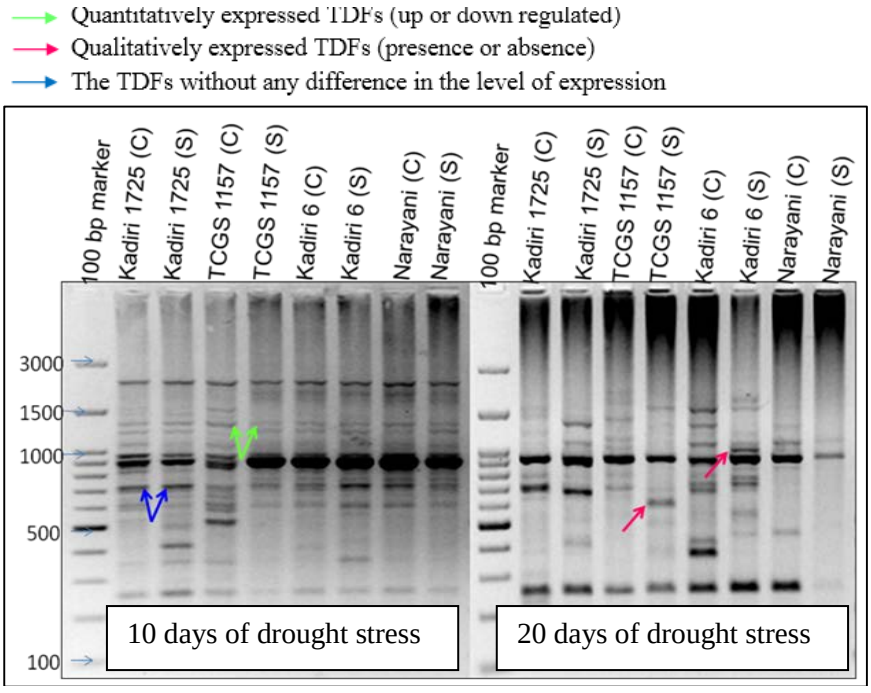

Fig 1: Representative amplification profiles generated by RAPD primers OPA4 using cDNA templates of groundnut cultivars TCGS 1157, Kadiri1725, Kadiri 6 and Narayani showing the differentially expressed TDFs under different stages (10 and 20 DAS) of drought stress

Table 2: RAPD primers for the development of cDNA profiles in selected groundnut genotypes.

S. No.	Primer ID	Primer Sequence	Total no. of transcripts
1	OPA-02	TGCCGAGCTG	78
2	OPA-04	AATCGGGCTG	73
3	OPA-07	GAAACGGGTG	54
4	OPA-09	GGGTAACGCC	0
5	OPA-10	GTGATCGCAG	0
6	OPA-18	AGGTGACCGT	35

7	OPA-19	CAAACGTCGG	0
8	OPA-20	GTTGCGATCC	16
9	OPC-03	GGGGGTCTTT	32
10	OPC-04	CCGCATCTAC	46
11	OPC-05	GATGACCGCC	51
12	OPC-10	TGTCTGGGTG	67
13	OPC-14	TGCGTGCTTG	42
14	OPC-07	GTCCCGACGA	37
15	OPC-09	CTCACCGTCC	18
16	OPD-01	ACCGCGAAGG	0
17	OPD -02	GGACCCAACC	0
18	OPD-07	TTGGCACGGG	13
19	OPD-08	GTGTGCCCCA	0
20	OPD-09	CTCTGGAGAC	0
21	OPD-10	GGTCTACACC	16
22	OPD-11	AGCGCCATTG	11
23	OPD-16	AGGGCGTAAG	10
24	OPD- 17	TTTCCCACGG	35
25	OPD-18	GAGAGCCAAC	0
26	OPF-09	CCAAGCTTCC	17
27	OPF-16	GGAGTACTGG	24
28	OPG-08	TCACGTCCAC	0
29	OPG-10	AGGGCCGTCT	0
30	OPS-01	CTACTGCGCT	37
31	OPS-02	CCTCTGACTG	0
32	OPT-09	CACCCCTGAG	0
33	OPT-10	CCTTCGGAAG	0
34	OPT-11	TTCCCCGCGA	0
35	OPT-12	GGGTGTGTAG	0
Total			712

A total of 712 transcripts were amplified by 20 RAPD primers (Table 2). Out of 712 TDFs, 347 TDFs were up regulated and 347 TDFs were down regulated among the four genotypes at different levels of imposed drought stress and in comparison with their respective control. A maximum 251 transcripts were identified by the OPA series followed by OPC (298), OPD (85), OPF (41) and OPS (39) series respectively. Out of 712 TDFs, 18 TDFs were without any difference in the level of expression between control and 10, 30 and 30 days drought stressed imposed treatments. A total of 347 TDFs were found to be up-regulated and 347 TDFs were found to be down regulated out of which 72 in 10 Days while 173 in 20 Days and 102 in 30 days stressed plants (Table 3).

Table 3: Summary of cDNA-RAPD scoring and analysis

RAPD primer series	Monomorphic TDFs	TDFs Up/ switched on in all	TDFs Up/ switched on at 10 days stress	TDFs Up/ switched on at 20 days stress	TDFs Up/ switched on at 30 days stress	TDFs Down/ switched off in all	TDFs Down/ switched off 10 days stress	TDFs Down/ switched off at 20 days stress	TDFs Down/ switched off at 30 days stress	Total
OPA	5	133	23	30	80	118	36	36	46	251
OPC	6	142	8	78	56	150	16	78	56	292
OPD	7	45	0	45	0	30	0	30	0	75
OPF	0	12	0	12	0	29	0	29	0	41
OPS	0	15	3	12	0	20	20	0	0	35
Total	18	347	34	177	136	347	72	173	102	712

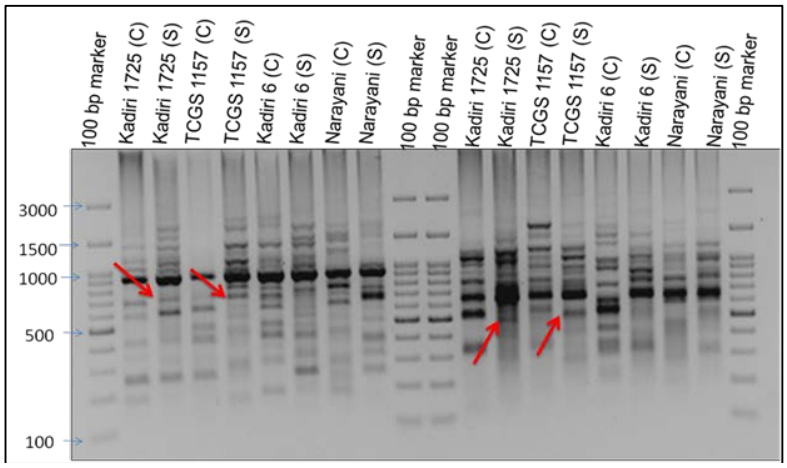

Fig 2: cDNA- RAPD profiling of OPA2 and OPA4 primer at 30 days after stress imposition.

Two moisture stress responsive transcripts were identified with OPA2 (700 bp TDF) and OPA4 (450bp TDF) markers only under drought stress in resistant genotypes (TCGS1157 & kadiri 1725) and absent susceptible genotypes (Kadiri 6 & Narayani) and also in well-watered control of all the four genotypes three moisture stress regimes (figure 2). Further, sequencing and characterization of these transcripts and identification of functional domains will greatly help in understanding nature of the genes and the mechanism by which groundnut plants respond to drought stress (Pagariya, *et al*., 2010) [16].

After 10 days of drought stress, in both drought tolerant and sensitive genotypes twice the number of TDFs were switched off (61) when compared to novel TDFs expressed (29). Whereas, a total of 28 TDFs had modulated their expression levels of either up (16) or down (12) regulated. After 20 days of drought stress, in both drought tolerant and sensitive genotypes, more numbers of TDFs were switched off (150) when compared to novel TDFs expressed (119). Whereas, a total of 80 TDFs had modulated their expression levels of either up (53) or down (27) regulated. At extreme drought stress of 30 days of drought stress, in both drought tolerant and sensitive genotypes, a number of 80 TDFs where switched off when compared to novel TDFs expressed (87). Whereas, a total of 80 TDFs had modulated their expression levels of either up (59) or down (21) regulated (figure 2). Similar kind of modulation in gene expression levels at two intervals of 15 and 30 days of salinity stress in sugarcane were reported by Pagariya, *et al*., 2010 [16]. They identified a total of 335 differentially expressed transcript-derived fragments out of that 156 up- and 85 down-regulated were reamplified and sequence to characterized their functions.

Prolonged moisture stress has enormous impact on gene expression pattern. In this study, a total of 712 TDFs were analyzed of which 514 transcripts exhibited qualitative difference (switched on: 227 transcripts, switched off: 287 transcripts) and 180 transcripts displayed quantitative differences (up regulated: 124 transcripts, down regulated: 56 transcripts). Similar kind of results were reported by Lovejot *et al*. (2015) [11] in *Vigna radiata* (green gram) and *Vigna umbellate* (rice bean), they identified total of 152 differential transcripts employing the cDNA-RAPD approach to analysis the transcript profile of the genes expression. This implies regulation of expression of different genes during MYMV infection and disease development.

In the initial stages up to 20 days of midseason moisture stress, many novel transcripts were activated along with modulation of gene expression (both up and down regulation) in comparison with well watered control genotypes. Under prolonged drought stress (30 days), the expression of novel transcripts were reduced by 30 % and the up and down regulated transcripts was increased by 15 % (Figure 3). The novel transcripts triggered by moisture stress will play a major role in stress perception, signal transduction, and synthesis of regulators and different compounds associated with drought tolerance mechanism.

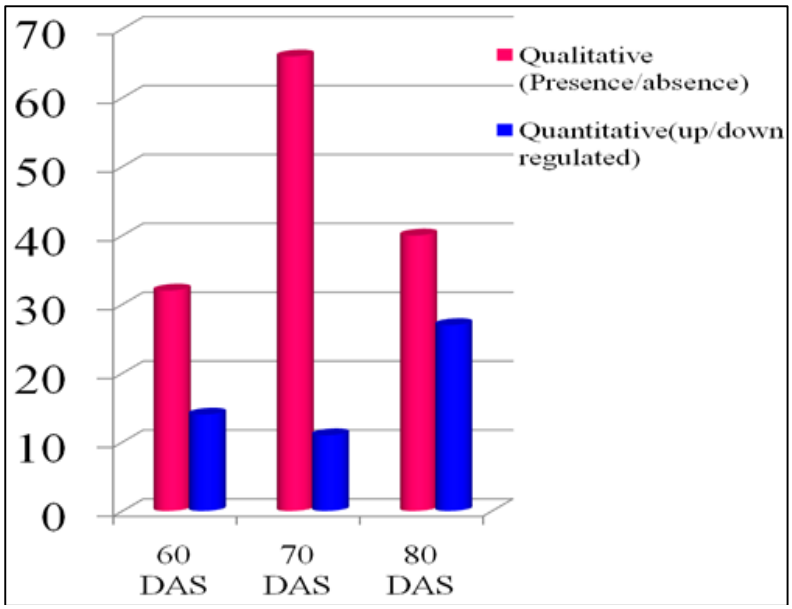

Fig 3: The diversity of qualitative and quantative expression of TDFs at different levels of imposed moisture stress in groundnut genotypes

Conclusion

The present study aimed at identification of differentially expressed transcripts in groundnut genotypes subjected to different levels of mid-season moisture stress. Irrespective of the drought tolerance levels in groundnut genotypes, proline accumulation in cells in drought stressed plants as a defense mechanism was noticed. Further, under prolonged moisture stress, the drought sensitive groundnut genotypes accumulates higher amount of proline than the drought tolerant groundnut genotypes. Hence, under moisture stress situation, proline content can be better used as a drought indicator in groundnut. C DNA-RAPD approach is a simple, fast and inexpensive technique highly suitable for identification of differentially expressed genes. Irrespective of the tolerance to drought tolerance levels, the groundnut genotypes modulated the gene expression levels. A maximum 251 transcripts were identified by the OPA series followed by OPC (298), OPD (85), OPF (41) and OPS (39) series respectively. In the initial stages up to 20 days of midseason moisture stress, many novel transcripts were activated along with the modulation of gene expression. Under prolonged moisture stress imposition, many TDFs were switched off and the TDFs with up and down regulation of gene expression levels were increased. We identified some of TDFs which are specifically expressed in drought tolerant genotypes under drought stress condition and these TDFs can be considered to play an important role in drought tolerance in groundnut. These identified differentially expressed transcripts responsible for the drought tolerance in

groundnut genotypes. Further characterization of these transcripts by sequencing will greatly helps in understanding nature of the genes and the mechanism by which groundnut plants respond to drought stress. These genes will be highly useful for the development of drought tolerant groundnut genotypes through molecular breeding.

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