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Isolation and characterization of drought-responsive genes from peanut roots by suppression subtractive hybridization

Hong Ding, Zhi Meng Zhang *, Fei Fei Qin, Liang Xiang Dai, Chun Juan Li, Dun Wei Ci, Wen Wu Song

Shandong Peanut Research Institute, 126 Wannianquan Rd., Qingdao 266100, China

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ABSTRACT

Background: Peanut (*Arachis hypogaea* L.) is an important economic and oilseed crop. Long-term rainless conditions and seasonal droughts can limit peanut yields and were conducive to preharvest aflatoxin contamination. To elucidate the molecular mechanisms by which peanut responds and adapts to water limited conditions, we isolated and characterized several drought-induced genes from peanut roots using a suppression subtractive hybridization (SSH) technique.
Results: RNA was extracted from peanut roots subjected to a water stress treatment (45% field capacity) and from control plants (75% field capacity), and used to generate an SSH cDNA library. A total of 111 non-redundant sequences were obtained, with 80 unique transcripts showing homology to known genes and 31 clones with no similarity to either hypothetical or known proteins. GO and KEGG analyses of these differentially expressed ESTs indicated that drought-related responses in peanut could mainly be attributed to genes involved in cellular structure and metabolism. In addition, we examined the expression patterns of seven differentially expressed candidate genes using real-time reverse transcription-PCR (qRT-PCR) and confirmed that all were up-regulated in roots in response to drought stress, but to differing extents.
Conclusions: We successfully constructed an SSH cDNA library in peanut roots and identified several drought-related genes. Our results serve as a foundation for future studies into the elucidation of the drought stress response mechanisms of peanut.

1. Introduction

Peanut (*Arachis hypogaea* L.) is an important economic and oilseed crop, which is mainly grown under rain-fed conditions in arid and semi-arid regions. Consequently, drought is a major production constraint since rainfall is generally both erratic and inadequate [1,2]. Hence, improving the drought tolerance of peanut is a key objective. Genetic engineering is one approach that could be used, but requires prior information about drought stress-related genes in peanut. However, the molecular mechanisms by which peanut adapts to water stress are not well described. The peanut genome is very large in comparison to other plant species, making it difficult to study. Thus, a detailed understanding of peanut water stress tolerance would be highly informative and, moreover, the altered expression of key genes may enhance peanut drought tolerance.

Studies into the mechanisms of peanut drought resistance have previously focused on aboveground plant tissues. For instance, nearly 700 genes were identified as being enriched in a subtractive cDNA library generated from peanut leaves exposed to a gradual drought stress treatment [3]; and a proteomic analysis of the water-deficit stress response in three contrasting peanut genotypes implicated a variety of stress response mechanisms as being active in peanut [4]. Dang et al. [5] analyzed the gene expression of twelve transcription factors from two drought tolerant peanut genotypes under drought conditions and identified the expression patterns of drought-inducible transcripts.

As the major interface between the plant and the various biotic and abiotic factors in the soil environment, root tissues may produce root-to-shoot chemical signals that regulate stomatal closure and thus reduce transpiration [6,7]. However, there is currently limited information on the root responses of peanut under water deficit conditions, particularly at the molecular level. Suppression subtractive hybridization (SSH) is a powerful technique for the identification of differentially expressed genes and for the enrichment of genes with low expression levels [8]. There are several examples in the literature where the SSH approach has been successfully employed to screen for candidate genes, including the identification of chilling-responsive transcripts in peanut [9], and the isolation of a submergence-induced gene, *OsGGT* (glycogenin glucosyltransferase) in rice [10]. Hence, we

* Corresponding author.
E-mail address: qinhdao@126.com (Z.M. Zhang).
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Table 1
Sequences of qRT-PCR primers used in this study.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
STPK	TCCAAATGGGCAAATGAAACC	ATTCCATCGTTCGTCTGTTTCG
ANN	TTTGTGGCAGCGGTTATTATGTC	ATCCCAACCCAAACCACCTACAT
P5CS	GTCCTGTAGGAGTTGAGGGTTTG	TTTAGTGGCAGTTCTTTATGAGTGT
GolS	GGTTCACTATTGTGCTGCTGGGT	CCTCATATATCTCCCACCATTTCTTA
ADH	CGAATGATGCACCTGATGG	CCCGAACCGATCTTCCTAAT
MnSOD	TATGCCAGCGAAGTGTATGAAAAAG	GTCTTATATGCCACATTACATCCTTTT
Gsi-83	GACGGTGCCGAGGGTGAGA	AGCAAGCAGTAATGGCGGAGA
ACT11	TTGGAATGGGTCAGAAGGATGC	AGTGGTGCCTCAGTAAGAAGC

utilized an SSH strategy to isolate and characterize drought-induced transcripts from peanut roots. A better understanding of the key genes involved in peanut stress response is vital for the development of plants that can maintain high yields under drought conditions, and the cultivation of drought-resistant peanut varieties.

2. Materials and methods

2.1. Plant growth and drought stress treatment

A. hypogaea cv Huayu 25 were used in this study. Plants were grown in a growth chamber at 28°C/18°C (day/night), and 300 μmoL m^{-2} s^{-1} light intensity provided by reflector sunlight dysprosium lamps (DDF 400, Nanjing, China). The water stress treatment was as described by Govind et al. [3]. The amount of water held by the soil is expressed as a mass percentage, and it is considered as 100% field capacity (FC) of soil. Three different water treatments were considered in this study: 75%, 45% and 20% FC with 75% FC serving as the control treatment. Plants were held at one of the three different water treatments (75%, 45% and 20% FC) for the plants planted at 75% FC for 25 d after sowing. The water stress treatment was maintained for a total of 5 d and was monitored gravimetrically by weighing the pots twice daily. The fresh roots, first nodal leaves and the first main stem were harvested at the end of the stress period from three treated plants for RNA isolation. The second fully expanded leaves were harvested for the measurement of leaf relative water content (RWC). The RWC was calculated as described by Barrs and Weatherly [11]:

$$\mathrm{RWC}(\%) = [(\text{Fresh wt} - \text{dry wt})/(\text{Turgid wt} - \text{dry wt})] \times 100.$$

2.2. Isolation of total RNA and cDNA synthesis

Total RNA was isolated from the frozen roots using RNAprep pure Plant Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. RNA was treated with recombinant RNase-free DNaseI (Takara, Toyoto, Japan) to avoid genomic DNA contamination before cDNA synthesis. RNA integrity was verified by 1% agarose gel electrophoresis, with only RNA preparations having an A260/A280 ratio of 1.8–2.0 and an A260/A230 ratio >2.0 used for subsequent

B

	Leaf relative water content (%)	SE
CK	85. 91	0. 66
45%FC	40. 36	0. 84
20%FC	25. 62	2. 71

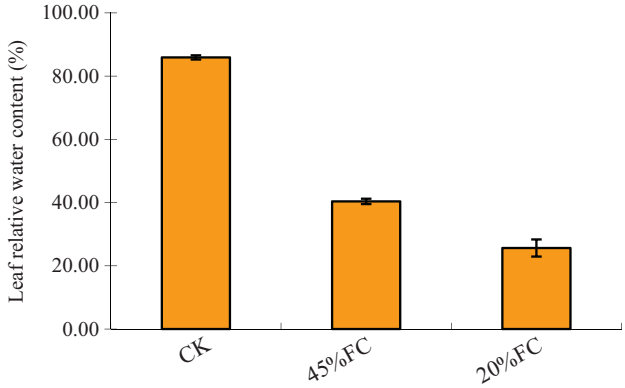

Fig. 1. (a) Phenotype of peanut plants exposed to different levels of water deficit. Leaf rolling and leaf thinning were observed in drought stressed plants but not in control plants; (b) changes in RWC of peanut leaves subjected to different water deficit treatments for 5 d. RWC was measured in the upper fully expanded leaves. Bars represent mean ± SD of three samples.

Table 2
Homology analysis of the 111 unique transcripts.

Sequence no	Length	Homology	Species	Accession no	E-value
DR2	436	Alcohol dehydrogenase 1	*Phaseolus vulgaris*	AGV54356.1	9e-27
DR3	869	Cellulose synthase-like protein G1-like	*Cicer arietinum*	XP_004499569.1	1e-140
DR 5	288	Chitinase (class II)	*Arachis hypogaea*	CAA57774.1	3e-04
DR 6	445	NA			
DR 7	501	Hypothetical protein PHAVU_007G280500g	*Phaseolus vulgaris*	ESW17931.1	2e-37
DR 8	1087	Uncharacterized protein LOC100806287	*Glycine max*	XP_003554538.1	8e-09
DR 10	625	Annexin 1	*Theobroma cacao*	EOY16019.1	3e-129
DR 11	840	Tobamovirus multiplication protein 2A isoform X1	*Glycine max*	XP_003524459.1	2e-80
DR 13	572	Annexin D1-like isoform X1	*Cicer arietinum*	XP_004516176.1	5e-33
DR 14	323	Hypothetical protein M569_00407	*Genlisea aurea*	EPS74345.1	1e-50
DR 15	343	Vacuolar amino acid transporter 1-like	*Glycine max*	XP_006591247.1	9e-27
DR 16	440	NA			
DR 17	1123	3-Hydroxyisobutyryl-CoA hydrolase-like protein 3, mitochondrial-like isoform X2	*Cicer arietinum*	XP_004503424.1	1E-146
DR 18	592	Uncharacterized protein LOC100306273 isoform X1	*Glycine max*	XP_006576151.1	5e-56
DR 19	416	Predicted: protein ROS1-like	*Cicer arietinum*	XP_004497617.1	3e-07
DR 22	145	Protein phosphatase 2C 16-like	*Fragaria vesca subsp. vesca*	XP_004303490.1	6e-10
DR 25	494	NAD-dependent protein deacetylase SRT2-like	*Glycine max*	XP_003528059.2	2e-97
DR 26	612	Histidine kinase 3-like isoform X1	*Glycine max*	XP_003531201.1	2e-09
DR 34	364	NA			
DR 36	1047	Probable ubiquitin-conjugating enzyme E2 26-like isoform X1	*Glycine max*	XP_006580093.1	2e-61
DR 37	217	Secretory protein	*Arachis hypogaea*	AAO33586.1	3e-21
DR 45	809	Uncharacterized protein LOC101500555	*Cicer arietinum*	XP_004503811.1	1e-66
DR 47	265	Hypothetical protein EUTSA_v10002144mg	*Eutrema salsugineum*	XP_006408892.1	4e-05
DR 49	227	Type 4 metallothionein	*Arachis hypogaea*	ABG57066.1	6e-27
DR 51	514	Hypothetical protein PHAVU_008G286500g	*Phaseolus vulgaris*	ESW14501.1	2e-61
DR 68	916	Hypothetical protein, partial	*Bacteroides dorei*	WP_007851439.1	6e-04
DR 69	899	Mitochondrial-processing peptidase subunit alpha	*Medicago truncatula*	XP_003630686.1	1e-48
DR 73	540	Uncharacterized protein LOC100778245	*Glycine max*	NP_001239643.1	3e-37
DR 76	585	NA			
DR 77	285	WAT1-related protein At5g40240-like isoform X2	*Glycine max*	XP_006586197.1	2e-26
DR 82	497	Unknown	*Lotus japonicus*	AFK49522.1	1e-53
DR 83	1048	NA			
DR 86	1035	Serine/threonine-protein kinase HT1-like	*Cicer arietinum*	XP_004485788.1	2e-17
DR 87	126	NA			
DR 90	1036	Protein GIGANTEA	*Medicago truncatula*	XP_003592047.1	2e-124
DR 92	412	Uncharacterized protein LOC101506019 isoform X1	*Cicer arietinum*	XP_004485727.1	2e-08
DR 93	909	Epidermal growth factor receptor substrate 15-like	*Glycine max*	XP_003527306.1	6e-43
DR 98	556	Starch branching enzyme I	*Pisum sativum*	CAA56319.1	4e-34
DR 102	971	Epidermal growth factor receptor substrate 15-like	*Glycine max*	XM_004500802.1	1e-43
DR 105	247	DNA/RNA-binding protein KIN17-like	*Cicer arietinum*	XP_004491366.1	1e-44
DR 111	109	NA			
DR 117	645	Plasma membrane H+-ATPase	*Sesbania rostrata*	BAC77533.1	2e-130
DR 118	937	DEMETER	*Citrus sinensis*	AGU16984.1	1e-13
DR 121	475	Lipoxygenase	*Phaseolus vulgaris*	AAB18970.2	1e-70
DR 122	932	Carotenoid cleavage dioxygenase	*Eustoma exaltatum*	BAK22396.1	1e-44
DR 123	429	Universal stress protein A-like protein	*Medicago truncatula*	XP_003603940.1	7e-74
DR 125	1064	Protein ROS1-like isoform X1	*Glycine max*	XP_006588820.1	6e-29
DR 126	454	Putative cold stress responsive protein	*Arachis hypogaea*	AAO33592.1	5e-07
DR 128	849	Methyl-CpG-binding domain-containing protein 10-like	*Glycine max*	XP_003543681.1	8e-60
DR 136	592	Glutamic acid-rich protein-like	*Glycine max*	XP_003548693.1	2e-05
DR 137	541	Galactinol synthase 2	*Glycine max*	XP_003555792.1	1e-41
DR 138	338	NA			
DR 139	1121	Protein ROS1-like isoform X1	*Glycine max*	XP_006594195.1	9e-20
DR 141	558	Lea4	*Glycine tomentella*	AAU94909.1	7e-42
DR 145	190	NA			
DR 154	1119	Serine/threonine–protein kinase HT1	*Glycine max*	XP_003543042.1	1e-50
DR 157	1121	3-Hydroxyisobutyryl-CoA hydrolase-like protein 3, mitochondrial-like isoform 1	*Glycine max*	XP_003525261.1	5e-144
DR 159	247	Nitrate transporter 1.1 isoform 1	*Theobroma cacao*	EOY24389.1	1e-36
DR 167	313	NA			
DR 170	579	Hypothetical protein PHAVU_001G146200g	*Phaseolus vulgaris*	ESW34362.1	8e-27
DR 172	457	NA			
DR 176	553	Late embryogenesis abundant protein group 4 protein	*Arachis hypogaea*	ADQ91841.1	6e-35
DR 181	362	Expansin-like B1-like	*Glycine max*	XP_003517398.1	2e-67
DR 182	342	NA			
DR 188	559	Gigantean	*Arachis hypogaea*	ACF74296.1	2e-23
DR 194	1072	Protein ROS1-like isoform X4	*Glycine max*	XP_006588823.1	1e-30
DR 195	510	Thylakoidal ascorbate peroxidase	*Jatropha curcas*	AGW52121.1	6e-16
DR 197	435	Carotenoid cleavage dioxygenase 1	*Medicago truncatula*	CAR57918.1	1e-70
DR 203	367	NA			
DR 208	143	NA			
DR 215	423	Hypothetical protein EUTSA_v10004562mg	*Eutrema salsugineum*	XP_006395044	9e-04
DR 220	509	Manganese superoxide dismutase, partial	*Trifolium repens*	AFV96160.1	5e-45
DR 227	363	NA			
DR 230	181	NA			
DR 233	152	NA			

Table 2 *(continued)*

Sequence no	Length	Homology	Species	Accession no	E-value
DR 241	322	NA			
DR 242	965	Aldose reductase-like	*Glycine max*	XP_003551585.1	6e-168
DR 262	523	Metallothionein-like protein	*Arachis hypogaea*	AAZ20291.1	8e-20
DR 278	486	Uncharacterized protein LOC101508994	*Cicer arietinum*	XP_004500002.1	2e-60
DR 284	166	NA			
DR 285	1011	Transcriptional activator DEMETER-like	*Cucumis sativus*	XP_004150492.1	5e-13
DR 289	219	NA			
DR 291	886	Delta-1-pyrroline-5-carboxylate synthase	*Medicago sativa*	CAA67070.1	1e-84
DR 316	337	NA			
DR 318	1075	Ferrochelatase-2, chloroplastic-like isoform X2	*Glycine max*	XP_006580371.1	3e-76
DR 324	489	NA			
DR 338	569	Mannose glucose binding lectin precursor	*Arachis hypogaea*	AAV33364.1	3e-29
DR 339	342	NA			
DR 341	529	Annexin AnxGb3	*Gossypium barbadense*	AGG75999.1	3e-101
DR 379	441	NA			
DR 382	259	NA			
DR 383	378	Small acidic protein 1-like	*Glycine max*	XP_003555729.1	1e-06
DR 395	261	Alcohol dehydrogenase 1-like	*Cicer arietinum*	XP_004502579.1	1e-33
DR 400	444	NA			
DR 403	258	Chaperone protein dnaJ 49-like	*Cicer arietinum*	XP_004488532.1	5e-13
DR 404	734	Cyclin-dependent kinase G-2-like	*Glycine max*	XP_006601445.1	4e-40
DR 405	298	MOB kinase activator-like 1-like isoform X1	*Cicer arietinum*	XP_004512415.1	8e-45
DR 408	379	Annexin D1-like isoform X2	*Cicer arietinum*	XP_004516177.1	7e-35
DR 412	326	Manganese superoxide dismutase 2	*Prunus persica*	CAC19487.1	3e-23
DR 423	187	Enolase	*Medicago truncatula*	NP_003617922.1	1E-03
DR 425	594	Lea protein 3	*Arachis hypogaea*	AAZ20280.1	6e-60
DR 430	248	NA			
DR 432	459	NA			
DR 435	295	Lipoxygenase LoxN2	*Pisum sativum*	AAD08700.1	4e-30
DR 449	337	Hypothetical protein ZEAMMB73_103592	*Zea mays*	AFW74002.1	6e-20
DR 451	1076	Protein ROS1-like isoform X2	*Glycine max*	XP_006588821.1	2e-20
DR 464	363	NA			
DR 465	257	NA			
DR 470	344	NA			
DR 471	522	Alternative oxidase 2b	*Glycine max*	AAP68983.1	9e-58
DR 472	268	Class II chitinase	*Arachis hypogaea*	AEO14153.1	4E-05

analysis. cDNA was synthesized using SMARTer™ PCR cDNA Synthesis Kit (Clontech, Mountain View, CA, USA) as described by the manufacturer. The cDNA was purified by column chromatography and digested with RsaI for SSH library construction.

2.3. Construction of an SSH cDNA library

A subtractive cDNA library was constructed using the PCR Select™ cDNA subtraction kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. The 45% FC root cDNA was used as the tester and the 75% FC root cDNA as the driver for SSH. The digested cDNA were ligated to adapters 1 and 2R supplied with the PCR-Select cDNA Subtraction Kit. After two rounds of hybridization and PCR amplification, the differentially expressed cDNAs were normalized and enriched. The subtracted and enriched DNA fragments were purified by QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The PCR products were ligated to pGEM-T Easy vector (Promega Co., USA) and transformed into DH5α cells using heat shock. Transformants were isolated from white colonies on X-gal/isopropyl-beta-D-thio-galatopyranoside agar plates. Positive colonies were identified by colony PCR. PCR products were separated on a 2% agarose gel to detect the amplification quality and quantity.

2.4. Sequencing and sequence analysis

The clones were sequenced by Sangon (Shanghai, China). The vector and adaptor sequences were removed using the DNAman software, and masked repeats, rRNA and low complicity sequences were eliminated using RepeatMasker. The sequences were searched against the NCBI database using BLASTN and BLASTX. Transcript annotation and functional assignment were performed using Blast2GO (http://blast2go.org).

2.5. Quantitative real time PCR analysis (qRT-PCR)

Total RNA for qRT-PCR analysis was treated with recombinant RNase-free DNaseI (Takara, Toyoto, Japan) to remove any contaminating genomic DNA. First-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen, USA). Primer pairs were designed using the Primer 5.0 software (Table 1). *ACT11* was used as a reference gene for the normalization of all data [12]. qRT-PCR was carried out in a Lightcycler 2.0 PCR machine (Roche, USA) based on SYBR Premix Ex Taq polymerase (Takara, Toyoto, Japan). The thermal protocol consisted of 95°C for 30 s, then 40 cycles of amplification at 95°C for 5 s, 60°C for 20 s, and 72°C for 15 s. Melting curves were obtained by slow heating from 65°C to 95°C at 0.1°C/s and continuous monitoring of the fluorescence signal. The reactions were performed in 20 μL volumes containing 2 μL of cDNA solution, 10 μL 2 × SYBR Premix and 0.4 μL (10 μM) of each primer. Each experiment was replicated three times. The comparative Ct method was applied.

3. Results

3.1. Performance of peanut under drought stress

Huayu 25 has been identified as a peanut variety with strong drought tolerance. An obvious difference in phenotype was observed between plants subjected to drought stress and well-watered plants (Fig. 1a). Visible symptoms such as leaf rolling and leaf thinning were seen in the plants subjected to drought stress, and the leaves of the control plants were greener than those of the stressed plants. The

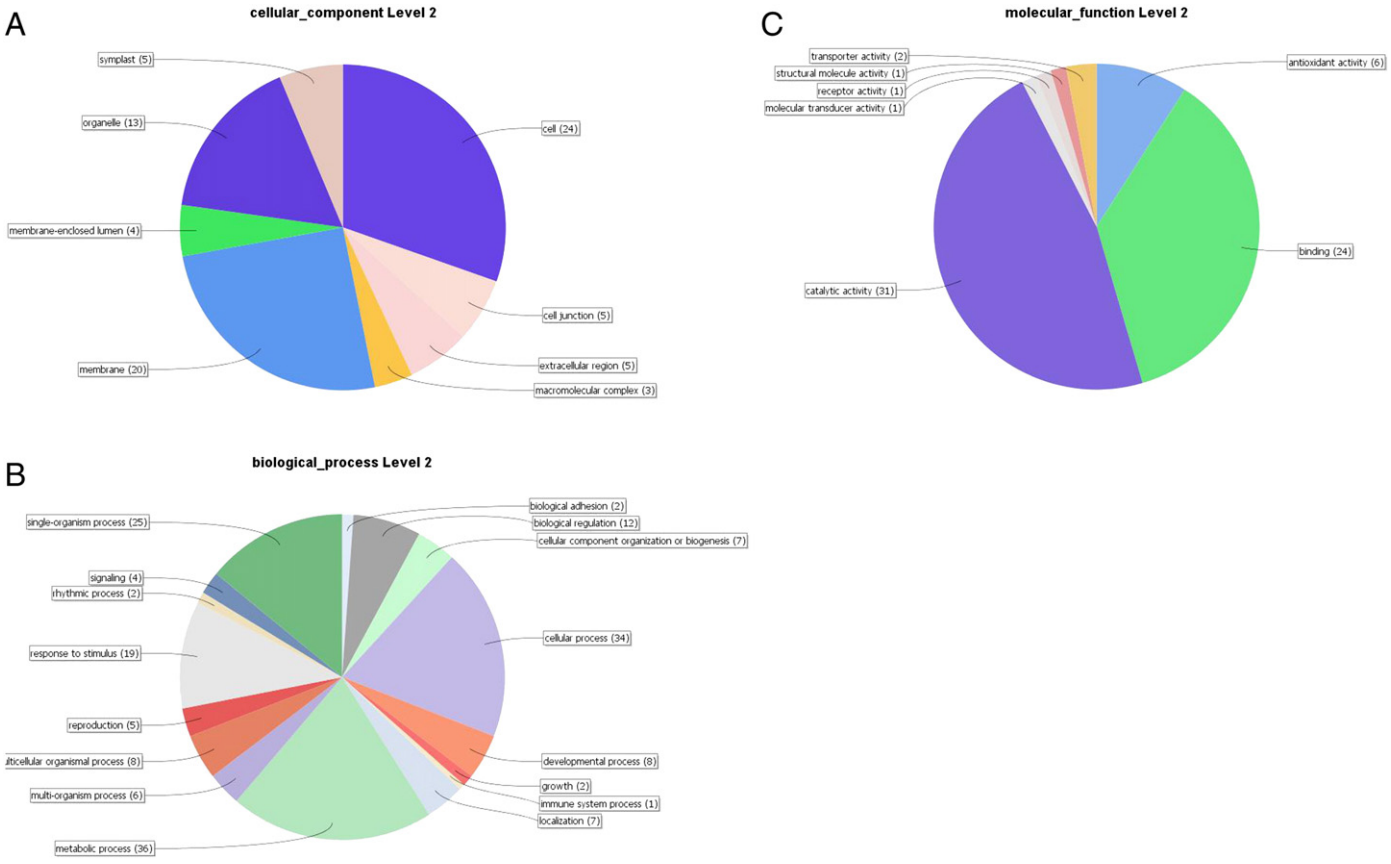

Fig. 2. Functional classification of drought-induced clones in peanut roots identified from subtractive cDNA library. Classification of 80 ESTs based on (a) cellular components, (b) biological process and (c) molecular function using Blast2GO software (http://blast2go.org).

RWC of the leaves decreased in line with the increasing degree of drought stress with the 20% FC treated plants exhibiting a 70.58% decline in comparison to the control plants (Fig. 1b).

3.2. Construction of an SSH cDNA library

A differential expression cDNA library of peanut roots was constructed utilizing Clontech PCR Select Subtraction Kit. After subtraction and transformation, the blue-white spot screening showed that approximately 95% of transformants contained an insert. A total of 576 clones were randomly selected prior to sequencing and were shown to have an insert size of approximately 200–1000 bp. Sequencing of positive clones yielded a total of 360 EST sequences. Thus, we successfully constructed a putative drought-stress specific subtracted cDNA library from peanut roots.

3.3. Analysis of differentially expressed ESTs

After the removal of vector and adaptor sequences and elimination of masked repeats, rRNA and low complicity sequences, 111 non-redundant sequences were obtained. Based on homology searches to the NCBI database, 80 clones (72.07%) were homologous to known genes and 31 clones were homologous to genes with unknown function or had no matches in the NCBI database (Table 2). For functional annotation, Blast2GO was used to classify the ESTs into three principal GO categories: cellular location, molecular function and biological process.

Table 3
qRT-PCR analysis of representative EST expression in peanut during drought stress treatment.

Gene	Root			Leaf			Stem		
	Control	45% FC	20% FC	Control	45% FC	20% FC	Control	45% FC	20% FC
STPK	1.02 ± 0.13	5.24 ± 0.17	11.62 ± 1.00	1.00 ± 0.04	0.41 ± 0.03	1.30 ± 0.08	1.01 ± 0.08	1.73 ± 0.13	2.08 ± 0.44
P5CS	1.00 ± 0.00	4.08 ± 0.60	5.54 ± 0.31	1.00 ± 0.02	7.52 ± 0.34	40.28 ± 0.52	1.00 ± 0.02	33.67 ± 0.28	2471 ± 0.45
GolS	1.00 ± 0.03	19.69 ± 1.61	45.02 ± 5.17	1.01 ± 0.09	8.90 ± 0.46	179.89 ± 4.57	1.00 ± 0.02	42.20 ± 2.72	1290.17 ± 2.98
Gsi-83	1.00 ± 0.04	9.43 ± 0.04	44.70 ± 2.80	1.00 ± 0.05	5.80 ± 0.47	40.80 ± 0.74	1.00 ± 0.04	16.58 ± 1.00	54.41 ± 2.30
ANN	1.00 ± 0.06	10.14 ± 0.99	25.31 ± 2.33	1.00 ± 0.04	5.25 ± 0.37	14.16 ± 0.32	1.00 ± 0.07	12.22 ± 0.29	10.40 ± 0.32
ADH	1.00 ± 0.06	7.01 ± 0.99	20.60 ± 3.37	1.00 ± 0.05	1.46 ± 0.08	29.87 ± 0.52	1.00 ± 0.04	7.65 ± 0.02	21.02 ± 0.17
MnSOD	1.00 ± 0.00	1.04 ± 0.22	3.99 ± 0.14	1.00 ± 0.04	1.40 ± 0.04	4.91 ± 0.12	1.00 ± 0.05	5.68 ± 0.29	9.69 ± 0.12

Some ESTs were simultaneously annotated into the three categories. Amongst the 80 ESTs with known homologs, 30 (37.5%) were attributed to a cellular component, 45 (56.25%) to a biological process and 36 to a molecular function (45%).

Within the category of cellular component, the highest number of ESTs (24) was obtained for 'cell', followed by 'membrane' (20) (Fig. 2a). Within the category of biological process, 36 ESTs (80%) were assigned to 'metabolic process' and 34 (75.6%) to 'cellular process', which accounted for the majority of the annotated sequences (Fig. 2b). Within the molecular function category, the GO terms with the highest number of ESTs were 'catalytic activity' and 'binding', with 31 and 24 ESTs, respectively (Fig. 2c). Hence, the GO analysis suggested that drought responses in peanut were mainly related to genes involved in cellular structure and metabolism.

3.4. Validation of differential expression using selected SSH clones and qRT-PCR

We selected seven representative ESTs encoding known cold stress-responsive proteins: (*Gsi-83*, colony DR126), annexin (*ANN*, colony DR10), alcohol dehydrogenase (*ADH*, colony DR395), manganese superoxide dismutase (*MnSOD*, colony DR220), serine/threonine-protein kinase HT1 (*STPK*, colony DR154), galactinol synthase 2 (*GolS*, colony DR137) and Δ1-pyrroline-5-carboxylate synthase (*P5CS*, colony DR291), to further evaluate the differential expression of these genes in response to drought stress in peanut.

The expression patterns of the selected SSH clones in peanut roots, leaves and stems under water stress conditions (45% and 20% FC) were analyzed by qRT-PCR. Amongst the seven ESTs, *GolS* showed the greatest degree of up-regulation, with the largest increase in expression levels relative to the control observed in the stems under 20% FC conditions (1290 fold-change). The expression pattern of *STPK* differed in the roots, leaves and stems. In roots subjected to drought stress, the *STPK* transcript level increased approximately five-fold under 45% FC conditions and 11-fold under 20% FC conditions (Table 3). However, in leaves, *STPK* levels decreased significantly in the 45% FC treatment but showed no obvious change in the 20% FC conditions. In stems, *STPK* levels increased approximately two-fold following drought stress. The *MnSOD* gene showed no obvious expression changes in peanut roots and leaves under 45% FC water treatment, but increased between four- and nine-fold in the 20% FC water treatment (Table 3). The expression of *P5CS* in peanut roots and leaves increased with the degree of drought stress, with the highest expression level observed in stems at 45% FC treatment. The remaining three clones (*Gsi-83*, *ANN* and *ADH*) showed a similar pattern of expression in all tissues, with a small increase in the 45% FC treatment and the greatest expression level at 20% FC treatment (Table 3).

4. Discussion

Drought stress cDNA libraries have previously been constructed for peanut, but these correspond to genes expressed in drought stressed leaves [3] or in immature pods [13,14]. Hence, there is limited molecular information on the root responses of peanut subjected to drought stress conditions. In this study, a total of 111 differentially expressed, non-redundant ESTs were identified in the subtractive cDNA library. Of these 111 ESTs, 80 had significant homology to known genes, many of which are associated with drought stress responses previously reported in soybean and chickpea. Some genes, such as those encoding *lea3*, *lea4* and *metallothionein-like protein* had confirmed involvement in drought stress in peanut [15,16]. This suggests that we have successfully constructed an SSH cDNA library and have identified drought-stress responsive genes in peanut roots.

We selected seven ESTs for qRT-PCR analysis in drought-stressed and control peanut roots, leaves and stems. The expressions of *ANN*, *ADH* and *MnSOD* were increased in response to drought stress, especially under the 20% FC condition. These three genes are reported to be involved in water stress responses in other plant species [17,18,19,20]. Our study confirms that these genes are also involved in the drought tolerance mechanism of peanut. Protein kinases are widely detected in living organisms and play important roles in signal perception and transduction in cells. Under environment stress conditions, protein kinases perceive and transmit various signals, and activate transcription factors to regulate the expression of downstream genes [21,22]. The expression patterns of *STPK* differed in the roots, leaves and stems, exhibiting rapid induction in roots under drought stress, but down-regulation in leaves at 45% FC conditions. The expression pattern of this particular protein kinase indicates that its role in the regulation of drought stress response is complex and requires further study.

Some studies have shown that under drought stress conditions, plants can improve their drought tolerance by adjusting the levels of osmoprotectants such as proline [23], galactinol [24] and glycinebetaine [25]. Proline acts as an osmolyte that accumulates when plants are subjected to abiotic stress. P5CS is a key regulatory enzyme that plays a crucial role in proline biosynthesis [26]. Raffinose and galactinol are involved in tolerance to drought, high salinity and cold stress. Stress-inducible GolS plays a key role in the accumulation of galactinol and raffinose under abiotic stress conditions [24]. In this study, the mRNA levels of *P5CS* and *GolS* in the control leaves and stems were significantly reduced in comparison to roots (data not shown). Furthermore, the expression of *P5CS* was significantly increased in all three tissues under drought stress, suggesting that proline accumulation in peanut may form a key defense mechanism against drought stress. The up-regulation of *GolS* under 20% FC conditions was 9-fold, 4.5-fold and 53.8-fold greater than that of *P5CS* in roots, leaves and stems, respectively. This indicates that, in peanut, the osmotic adjustment ability of soluble sucrose is greater than that of proline under drought stress conditions, which is consistent with our previous report [27].

In addition, some of the genes induced under drought stress were found to be associated with other environmental stresses, such as salt, cold and high temperature stress [28,29]. We identified an EST homologous to nitrate transporter 1.1, and a cold stress responsive protein whose expression was marginally increased in peanut under drought stress conditions. This suggests that some genes respond to both drought stress and other abiotic stresses, and thus implies that similar stress tolerance mechanisms and pathways may exist. The gene expression levels analyzed in this study indicate that the response to drought is a very complex physiological and biochemical process involving multiple metabolism pathways.

5. Conclusions

We successfully constructed an SSH cDNA library from peanut roots and identified several transcripts encoding proteins with drought-related functions. These proteins were located in different cellular compartments and were involved in various molecular functions and biological processes during normal and water stress conditions in peanut. Our study contributes to a better understanding of the molecular mechanisms of water-stress tolerance in peanut and would facilitate the genetic manipulation of drought-stress resistance in this species.

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Author contribution

Proposed the theoretical frame: HD, ZMZ; Conceived and designed the experiments: FFQ, LXD; Wrote the paper: HD; Performed the experiments: CJL, DWC; Analyzed the data: WWS.

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