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Identification of chilling-responsive transcripts in peanut (*Arachis hypogaea* L.)

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Abstract To isolate differentially expressed peanut genes responsive to chilling, a suppression subtractive hybridization (SSH) cDNA library was constructed for a chilling tolerant peanut cultivar A4 with mRNAs extracted from the seeds imbibed at 2ºC and 15ºC, respectively, for 24 hrs. A total of 466 cDNA clones were sequenced, from which 193 unique transcripts (73 contigs and 120 singlets) were assembled. Of these unique transcripts, 132 (68.4%) were significantly similar to the sequences in GenBank non-redundant (nr) protein database, which belonged to diverse functional categories including metabolism, signal transduction, stress response, cell defense and transcriptional regulation. The remaining 61 (31.6%) showed no similarity to either hypothetical or known proteins. Six differentially expressed transcripts were further confirmed with real-time quantitative PCR (RT-qPCR).

Keywords: chilling response, peanut, real-time quantitative PCR, suppression subtractive hybridization

INTRODUCTION

Low temperature (LT) is a major environmental factor limiting plant growth, distribution, and productivity (Boyer, 1982). Different plant species, even individual genotypes of a single species, may respond differently to LT stress. In the long course of evolution, some plant genotypes have developed resistance mechanisms that protect themselves from being severely affected by LT.

Thus far, a large number of genes related to LT stress have been identified. These mainly include, late embryogenesis abundant (*LEA*) genes, *oleosin* genes, C-repeat/dehydration-responsive element binding factor (CBFs/DREB1s), NAC, WRKY-type, basic leucine zipper (b-ZIP) and MYB transcription factors (Thomashow, 1999; Shimada et al. 2008; Survila et al. 2010). Dehydrins are probably the best characterized group of LEA protein and have been demonstrated to be responsive to LT in several plant species (Survila et al. 2010). Simultaneous over-expression of two different *Arabidopsis* dehydrins resulted in enhanced tolerance of transgenic *Arabidopsis* plants to freezing (Puhakainen et al. 2004). Similarly, a citrus (*Citrus unshiu Marcov.*) dehydrin, when expressed in tobacco, led to improved chilling tolerance (Hara et al. 2003). *Oleosin* genes were associated with chilling tolerance in *Arabidopsis* (Shimada et al. 2008). Over-expression of *OsDREB1A* in transgenic *Arabidopsis* induced over-expression of target stress-inducible genes of *Arabidopsis DREB1A* resulting in plants with higher tolerance to drought, high-salt and freezing stresses (Dubouzet et al. 2003). Over-expression of NAC-coding genes in rice enhanced cold tolerance (Hu et al. 2008). Of 64 WRKY-type transcription factors

identified in soybean, 8 were low temperature responsive, and over-expression of *GmWRKY21 in Arabidopsis* increased tolerance to freezing (Zhou et al. 2008). Over-expression of *GmbZIP44*, *GmbZIP62*, *GmbZIP78* and *GmMYB76* from soybean in *Arabidopsis* improved freezing tolerance of the plants (Liao et al. 2008a; Liao et al. 2008b).

In contrast to the abundant information on LT stress in other plant species, in peanut (*Arachis* spp), there are scanty reports in this regard, even though in many portions of the world, LT is a major constraint to peanut production, especially during seeding period. Studies aimed at screening for peanut genotypes with chilling tolerance either at the period of seeding or emergence were conducted and differential responses were noted (Wang et al. 1985; Feng, 1991; Upadhyaya et al. 2001; Upadhyaya et al. 2009). Dave and Mitra (1998) isolated a peanut cold shock protein (AHCSP33) which was secreted into leaf apoplast during low temperature exposure. They also identified a putative *Ahlti* (*A. hypogaea* low temperature induced) gene in a leaf cDNA library (Dave and Mitra, 2000). However, to the best of our knowledge, there is no report on genes related to chilling tolerance at the seeding period in peanut.

The objective of the present study is to isolate and characterize chilling responsive transcripts from peanut with chilling tolerance by exploiting a suppression substractive hybridization (SSH) strategy, which is of relevance to the development of peanut cultivars with stabilized high yields.

MATERIALS AND METHODS

Plant materials and chilling treatment

The seeds of A4, a chilling tolerant peanut cultivar of Valencia type, were surface sterilized with 75% (v/v) ethanol, rinsed with distilled water and imbibed in a Petri dish. For chilling treatment, the seeds were kept in a growth chamber set at 2ºC for 1, 6 and 24 hrs, respectively. The control seeds were kept in a growth chamber set at 15ºC. The seeds were frozen in liquid nitrogen at appropriate times and stored at -70ºC for further analysis.

Isolation of total RNA and mRNA

Total RNA was isolated from the frozen seeds using RNAprep pure Plant Kit (Tiangen, Beijing, China) with its quantity and quality determined with spectrophotometery and agarose gel electrophoresis and Gelred (Biotium, USA) staining. The poly $(A)^+$ RNA was purified from total RNA using Oligotex mRNA Mini Kit (Qiagen, Germany).

SSH library construction

A subtraction cDNA library was constructed using the PCR Select^{TM} cDNA subtraction kit (Clontech, Mountain View, CA, USA) basically following the manufacturer's instructions. Two micrograms of poly$(A)^+$ RNA were used to synthesize cDNA. The cDNAs of A4 (2ºC, 24 hrs) and A4 (15ºC, 24 hrs) were used as tester and driver, respectively. PCR amplification was conducted using the Advantage 2 Polymerase Mix (Clontech, Mountain View, CA, USA). The subtracted and enriched DNA fragments were purified by QIAquick PCR Purification Kit (Qiagen, Germany). The differential cDNA fragments derived from SSH forward subtractive library were cloned into pGEM-T easy vector (Promega, USA). The positive colonies were identified with colony-PCR method. PCR products were resolved on a 2% agarose gel to confirm the positive colonies and analyze the size of inserts.

Sequencing and sequence analysis

The clones were sequenced in an ABI3700 DNA Sequencer from two ends with SP6 and T7 primers, respectively. Quality read were trimmed with DNAStar (DNASTAR Inc., London, UK) and assembled using CAP3 program (http://deepc2.psi.iastate.edu/aat/cap/cap.html) with default parameters. Each contig or singlet was assumed to represent a unique transcript.

Transcript annotation and functional assignment were done with BLAST2GO (http://blast2go.org) and GenBank nr database. Of the hits with an e-value of < 1.0E-6, the most similar one was considered as the homologue.

RT-qPCR analysis

Total RNA for RT-qPCR analysis was extracted from chilling-treated (1, 6, 24 hrs) and untreated (control) seeds (1, 6, 24 hrs). First-strand cDNA was synthesized using an oligo (dT)18 primer (TaKaRa, Japan) and reverse transcriptase M-MLV (RNase H^-) (TaKaRa, Japan).

Primer pairs were designed with the Beacon Designer 7.91 (Table 1) and checked for specificity by aligning with peanut DNA sequences. RT-qPCR was carried out in a Lightcycler 2.0 (Roche, USA) PCR machine. Thermal cycling profile was 95ºC for 30 sec, followed by 45 cycles of 95ºC for 5 sec, 60ºC for 20 sec, and 72ºC for 15 sec. Melting curves were obtained by slow heating from 65 to 95ºC at 0.1ºC/s and continuously monitoring the fluorescence signal. A negative control without a cDNA template was run with each analysis to evaluate the overall specificity. The reaction mixture (20 µl) contained 2 µl of cDNA solution, 10 µl SYBR *Premix Ex Taq* ^{TM}(TaKaRa, Japan), and 5 pmol of each primer. The reactions were performed in triplicate with the resultant data averaged. Fold changes of RNA transcripts were calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using β-actin gene as an internal control.

Table 1. Sequences of RT-qPCR primers used in this study.

Primer ID	Forward primer(5'-3')	Reverse primer(5'-3')
TS36	CCACAGGATGCTCAAGTT	TTAGTCAACCTCTTCCATCTT
TS110	ATCTTGTTCTCTTGCTCTT	CTTCCATCTTGCTTCCTT
TC50	GCAGACATGGCGGATTAC	TGACCTCTTAACATCCTGGG
TC36	GTCAACGAGATGGAACAG	AACTATACACAGCCTAACCTA
TS54	GGTGCTGATTGGGACATAGATT	TTGCTGAACATGCTAGTGAGG
TS98	GTTCCTGGCTCCTCTTCTA	ACATAGTTCCTTGCGATTCC
β-actin	TTGGAATGGGTCAGAAGGATGC	AGTGGTGCCTCAGTAAGAAGC

RESULTS AND DISCUSSION

Construction of SSH cDNA library

After subtraction and transformation, 500 well isolated clones were randomly selected and checked by colony PCR prior to sequencing. Agarose gel electrophoresis showed that the length of cDNA inserts ranged from 100 to 1500 bp (Figure 1). Plasmid DNAs were then extracted from these clones and inserts sequenced, resulting in 466 high-quality reads, which were assembled into 193 unique cDNAs (73 contigs and 120 singlets)

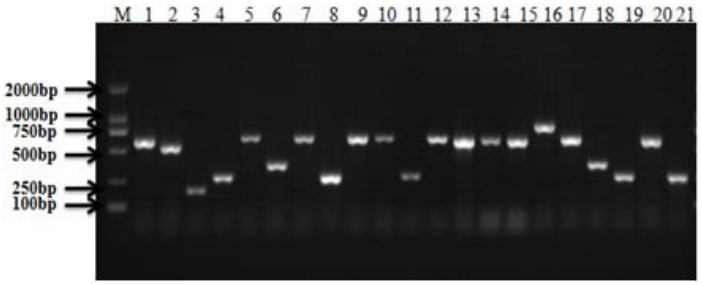

Fig. 1 Individual colonies harbouring cDNA inserts of varied sizes. M: Tiangen D2000 DNA marker 1-21: PCR product of isolated colonies.

Annotation and functional assignment

Each unique transcript was aligned with the sequences in GenBank nr protein database. As showed in Table 2, 132 transcripts (68.4% of the total) were significantly similar to the sequences in GenBank nr database, while the remaining 61 (31.6%) were unclassified.

Table 2. Homology analysis of the 193 unique transcripts.

Seq. Name	Seq. Description	Genbank Accession No.	Seq. Length(bp)	Min. EValue	Mean Similarity
TC1	late embryogenesis abundant	AAA91965	1033	1.25E-39	54.30%
TC2	short chain	ABD32398	1017	1.96E-138	88.60%
TC3	vicilin 47k	AAT00596	883	1.14E-48	86.70%
TC4	af117723_1seed maturation protein pm27	ACU20077	380	5.04E-12	78.50%
TC5	seed maturation protein lea 4	ADQ91841	550	2.69E-29	73.20%
TC6	protein	ACU14938	756	4.41E-53	76.20%
TC7	unnamed protein product (*Vitis vinifera*)	CBI25770	412	1.23E-10	60.00%
TC8	protein	XP_002304373	351	7.25E-27	63.15%
TC9	phosphatidylethanolamine binding protein	XP_002530493	461	6.18E-55	78.60%
TC10	late embryogenesis abundant protein	AAW33981	413	1.22E-34	57.63%
TC11	protein	XP_002531881	968	1.63E-38	58.35%
TC12	---NA---		402		
TC13	nad transhydrogenase beta subunit	CAQ43070	968	1.17E-36	86.45%
TC14	af446875_1 at5g05220 k18i23_2	ACU13567	240	8.51E-20	81.54%
TC15	ara h i	P43238	1554	6.11E-97	81.20%
TC16	galactose-binding lectin precursor	P02872	413	1.38E-54	78.55%
TC17	---NA---		275		
TC18	---NA---		450		
TC19	defensin	AAV85438	452	2.57E-24	74.75%
TC20	cyclophilin	ACT63839	459	3.17E-59	95.75%
TC21	2 (arabidopsis thaliana seed imbibition 2) hydrolyzing o-glycosyl compounds	XP_002308957	443	2.54E-25	87.40%
TC22	1-cys peroxiredoxin	ACU19072	303	1.31E-28	84.25%
TC23	conglutin	ACN62248	567	1.58E-46	93.65%
TC24	---NA---		270		
TC25	nucleolar protein	CAC27161	267	2.85E-15	70.50%
TC26	heat shock protein 70	AAB42159	273	5.66E-40	95.45%
TC27	metallothionein-like protein	ABG57066	242	1.50E-32	73.15%
TC28	f-box family protein	XP_002514931	316	3.38E-48	83.80%
TC29	at5g18400 f20l16_120	B7FNA9	353	4.85E-31	69.14%
TC30	---NA---		157		
TC31	acireductone dioxygenase	CAL49076	309	9.57E-48	92.15%
TC32	myo-inositol oxygenase	ACU18263	322	1.78E-49	89.75%

TC33	---NA---		125		
TC34	---NA---		367		
TC35	protein phosphatase 2c	ACU19436	393	1.99E-32	92.65%
TC36	nac domain ipr003441	ACD39411	1221	1.19E-58	70.70%
TC37	---NA---		304		
TC38	DNA binding	ABR28329	733	9.98E-31	58.15%
TC39	group 3 late embryogenesis abundant protein	ADQ91835	509	2.35E-44	62.90%
TC40	---NA---		239		
TC41	af479304_1secretory protein	AAO33586	300	1.90E-19	100.00%
TC42	arachin ahy-4	ABI17154	926	1.98E-131	81.90%
TC43	---NA---		380		
TC44	---NA---		369		
TC45	---NA---		245		
TC46	---NA---		229		
TC47	putative defensin 3.1 (*Medicago sativa*)	AAT66095	276	7.44E-08	68.00%
TC48	amino acid transporter family protein	XP_002316182	352	1.92E-11	80.50%
TC49	---NA---		380		
TC50	oleosin high molecular weight isoform	AAK13450	230	9.24E-14	83.35%
TC51	late embryogenesis abundant protein group 1 protein	ADQ91831	716	1.89E-47	91.60%
TC52	af117723_1seed maturation protein pm27	ACU20077	310	1.22E-26	69.10%
TC53	transcription regulator not2 not3 not5 family protein	ABN09819	641	4.56E-97	77.90%
TC54	lactoylglutathione lyase	ACF74334	294	9.70E-21	75.68%
TC55	---NA---		205		
TC56	---NA---		168		
TC57	plasma membrane associated protein	ADQ91848	438	7.29E-56	87.90%
TC58	reticulon family protein	ACU18528	417	2.42E-26	70.79%
TC59	unknown (*Glycine max*)	ACU20570	402	2.86E-07	86.00%
TC60	malate dehydrogenase	BAG09381	257	8.97E-25	95.25%
TC61	autophagy protein 9	XP_002532369	595	1.00E-73	74.20%
TC62	amino acid binding	XP_002525301	610	5.67E-43	69.35%
TC63	lipoxygenase 2	AAY87056	498	1.56E-90	93.85%
TC64	heat shock	XP_002279101	438	2.14E-31	82.00%
TC65	heat shock protein 83	XP_002305263	485	3.58E-66	97.00%
TC66	---NA---		332		
TC67	kda class ii heat shock protein	CBI35818	309	2.46E-19	79.50%

TC68	acch2_arath ame: full=1-aminocyclopropane-1-carboxylate oxidase homolog 2	XP_002315711	276	3.32E-32	74.75%
TC69	cbs domain-containing protein	XP_002298798	217	2.53E-16	88.30%
TC70	---NA---		186		
TC71	---NA---		103		
TC72	---NA---		82		
TC73	short chain	ABD32398	632	1.06E-66	90.70%
TS1	---NA---		207		
TS2	---NA---		311		
TS3	udp-galactose 4-epimerase-like protein	ACU24580	1124	3.93E-106	90.45%
TS4	ATP-dependent helicase	XP_002332671	992	1.10E-53	74.45%
TS5	---NA---		191		
TS6	---NA---		186		
TS7	gamma-glutamylcysteine synthetase	XP_002298035	561	1.38E-39	59.30%
TS8	---NA---		368		
TS9	dj-1 family protein	XP_002316868	581	3.09E-77	86.80%
TS10	---NA---		159		
TS11	---NA---		270		
TS12	---NA---		219		
TS13	protein	XP_002521588	440	3.73E-68	89.75%
TS14	peptidylprolyl isomerase	ACU23964	180	7.63E-13	89.00%
TS15	---NA---		157		
TS16	---NA---		150		
TS17	elegans protein confirmed by transcript evidence	CBI17507	684	3.63E-37	51.25%
TS18	sensory transduction histidine	XP_002281776	601	5.56E-27	61.70%
TS19	protein phosphatase	BAJ33949	288	2.27E-36	96.00%
TS20	---NA---		130		
TS21	protein	XP_002283631	540	2.97E-33	70.10%
TS22	---NA---		139		
TS23	protein	XP_002276985	300	2.47E-43	87.10%
TS24	protein	XP_002515824	372	4.86E-47	86.65%
TS25	cytosolic phosphoglycerate kinase 1	ACJ11718	838	7.86E-113	94.55%
TS26	dihydrolipoyllysine-residue acetyltransferase component of pyruvatedehydrogenase complex	XP_002315510	117	6.42E-07	81.46%
TS27	11-beta-hydroxysteroid dehydrogenase-like	ACU17782	369	2.68E-45	77.05%
TS28	---NA---		341		

TS29	---NA---		153		
TS30	xylose isomerase	ACJ84879	516	1.20E-22	87.75%
TS31	af117723_1seed maturation protein pm27	ACU20077	138	8.76E-09	78.81%
TS32	---NA---		237		
TS33	---NA---		160		
TS34	---NA---		356		
TS35	mitochondrial carrier protein	XP_002519295	516	7.27E-20	85.45%
TS36	heat shock protein 82	BAH97107	390	4.05E-14	88.00%
TS37	---NA---		292		
TS38	---NA---		414		
TS39	cupin family protein	ABU45197	442	1.99E-24	66.05%
TS40	embryonic abundant protein precursor-like protein	ADQ91849	525	6.73E-77	72.20%
TS41	protein	XP_002279785	345	3.86E-44	88.20%
TS42	unknown (*Glycine max*)	ACU13190	247	1.15E-08	73.00%
TS43	protein	XP_002513433	504	7.93E-82	88.50%
TS44	f-box family protein	ACJ85491	460	1.64E-31	80.00%
TS45	---NA---		249		
TS46	cbs domain-containing protein	AAO61675	575	1.64E-38	69.30%
TS47	eukaryotic translation initiation factor 2 beta subunit	XP_002319977	377	3.97E-41	96.70%
TS48	---NA---		456		
TS49	40s ribosomal protein	ACU15827	252	2.14E-34	97.65%
TS50	protein	ACU23431	662	2.72E-39	69.65%
TS51	---NA---		138		
TS52	beta-alanine n-methyltransferase related	ACJ84599	246	2.02E-05	72.83%
TS53	protein	ACU24270	221	6.04E-21	75.90%
TS54	late embryogenesis abundant protein	ADQ91846	319	4.01E-25	91.40%
TS55	60s ribosomal protein	ACU15099	236	6.98E-38	98.55%
TS56	26s protease regulatory subunit	XP_002969838	190	1.74E-28	99.80%
TS57	---NA---		123		
TS58	glutathione transferase 10	AAG34813	462	2.12E-34	82.80%
TS59	asparaginyl endopeptidase rep-2	CAB42651	347	9.16E-54	88.70%
TS60	malate dehydrogenase	CAH60894	436	2.21E-44	91.95%
TS61	sgt1-like protein	ACJ84554	206	7.46E-24	85.60%
TS62	amino acid transporter family protein	ACU19726	279	1.75E-33	78.45%
TS63	---NA---		290		

TS64	protein phosphatase	XP_002327730	435	6.87E-46	92.95%
TS65	soul heme-binding family protein	ACU19835	436	9.27E-51	73.95%
TS66	t-complex protein 11	XP_002530684	150	5.28E-09	76.72%
TS67	heat shock protein 101	XP_002328643	342	1.85E-54	97.45%
TS68	---NA---		201		
TS69	allantoate amidohydrolase	ACN87318	147	4.80E-10	88.00%
TS70	---NA---		129		
TS71	glucose-6-phosphate phosphate translocator-related	XP_002300993	171	1.01E-12	81.88%
TS72	---NA---		176		
TS73	---NA---		204		
TS74	---NA---		181		
TS75	nac domain ipr003441	ACI42833	293	2.49E-51	96.20%
TS76	-like protein	BAF62127	499	3.79E-28	68.80%
TS77	---NA---		183		
TS78	---NA---		148		
TS79	---NA---		226		
TS80	polyubiquitin-like protein	XP_002322526	216	2.44E-27	76.80%
TS81	---NA---		279		
TS82	protein	XP_002523309	481	2.90E-20	69.25%
TS83	---NA---		196		
TS84	kelch repeat-containing serine threonine phosphoesterase family protein	CBI24435	281	9.29E-35	95.50%
TS85	ara h 3 allergen	ADQ53859	121	9.24E-06	100.00%
TS86	protein	XP_002522599	327	8.51E-36	71.10%
TS87	---NA---		144		
TS88	unknown (*Populus trichocarpa*)	ABK93665	331	1.32E-04	58.00%
TS89	splicing factor	ACJ85781	418	2.95E-52	80.25%
TS90	vacuolar-processing enzyme precursor	XP_002509577	286	1.25E-34	79.80%
TS91	galactosyltransferase family protein	XP_002511491	253	6.64E-36	89.70%
TS92	short chain	ACU23410	432	4.76E-63	82.30%
TS93	protein	XP_002270163	326	1.77E-49	92.15%
TS94	emb1135 (embryo defective 1135) dna binding protein binding zinc ion binding	XP_002518826	128	1.41E-14	94.00%
TS95	late embryogenesis abundant protein	AAA33985	486	7.51E-32	52.80%
TS96	protein kinase adk1	XP_002329062	421	1.23E-74	99.00%

TS97	bah domain containing protein	XP_002299533	403	8.86E-25	54.00%
TS98	myb family transcription factor	ABH02845	526	5.32E-82	87.65%
TS99	protein	XP_002515280	382	2.40E-30	52.35%
TS100	binding protein	XP_002527926	342	3.30E-19	82.89%
TS101	---NA---		201		
TS102	---NA---		255		
TS103	---NA---		377		
TS104	manganese superoxide dismutase	ACU16940	582	7.69E-92	88.45%
TS105	g10-like protein	ACU13359	349	5.94E-37	93.15%
TS106	senescence-associated protein	ACU20029	558	2.23E-66	76.20%
TS107	amino acid binding	CBI35746	119	1.38E-09	84.74%
TS108	proton-dependent oligopeptide transport family protein	XP_002529119	235	4.68E-34	86.15%
TS109	unknown (*Glycine max*)	ACU17782	252	7.65E-08	78.00%
TS110	heat shock protein 90	ACI31551	453	3.92E-41	95.20%
TS111	---NA---		211		
TS112	kiaa0052 protein	XP_002527838	427	1.26E-63	80.50%
TS113	aldehyde dehydrogenase family 7 member a1	XP_002278093	265	1.32E-28	94.85%
TS114	aldehyde dehydrogenase	XP_002283132	137	5.15E-09	96.55%
TS115	---NA---		177		
TS116	high mobility group family	O04235	495	3.93E-33	76.75%
TS117	pre-mrna cleavage factor 25kd	XP_002279095	226	2.52E-19	83.80%
TS118	protein	CAA07228	415	8.67E-41	80.40%
TS119	xyloglucan endotransglucosylase hydrolase protein 2	ACU19067	600	1.55E-53	83.30%
TS120	---NA---		87		

NA = not available.

The transcripts with known functions involved in metabolism, signal transduction, stress response, cell defence and transcriptional regulation.

The protective mechanisms induced during cold acclimation include alterations in membrane structure, solute biosynthesis and production of protective proteins (Survila et al. 2010). Gene expression regulation under chilling stress is also crucial for plant survival; as it is involved in signal transduction in cold acclimation, transcriptional/post-transcriptional regulation and abscisic acid-dependent cold signal pathway (Survila et al. 2010). To date, a large number of chilling responsive genes have been identified from plants (Survila et al. 2010), which can be divided into two categories. Those genes in category I encode proteins directly relating to the improvement in chilling resistance, for example, the LEA proteins that enhance the ability of resisting freezing injury and cellular dehydration, the membrane proteins that protect enzymes, heat shock proteins (HSPs), proline synthase, chaperones that prevent degeneration of proteins and membranes, FAD8 that increases the content of unsaturated

fatty acids and decreases the temperature of phase transition, and the kinase-regulated proteins that stabilize RNAs. Those in category II are involved in the regulation of signal transduction, expression of chilling-resistant genes, and proteins with chilling-resistant activities. Those in category II also include transcription factors such as MYB, bZIP, WRKY, AP2/EREBP, Zinc finger protein and protein kinases. The differential cDNAs obtained in this study almost covered all the chilling tolerance/resistance relating components described above (Table 2).

Verification of differential transcripts

Six transcripts encoding heat shock related proteins, oleosin, LEA, NAC family transcription factor and MYB family transcription factor, respectively, were verified with RT-qPCR. The results showed that these transcripts existed differentially in stressed and control peanut seeds (with overall relative expression ranging from 0.65 to 5.40) (Figure 2). Specifically, relative expression of the 6 genes ranged from 0.65 to 2.23 at 1 hr, 1.06 to 1.62 at 6 hrs, and 2.26 to 5.40 at 24 hrs, respectively, indicating that there was no remarkable difference in the relative expression level of the 6 genes at the earlier stages (1 hr and 6 hrs).

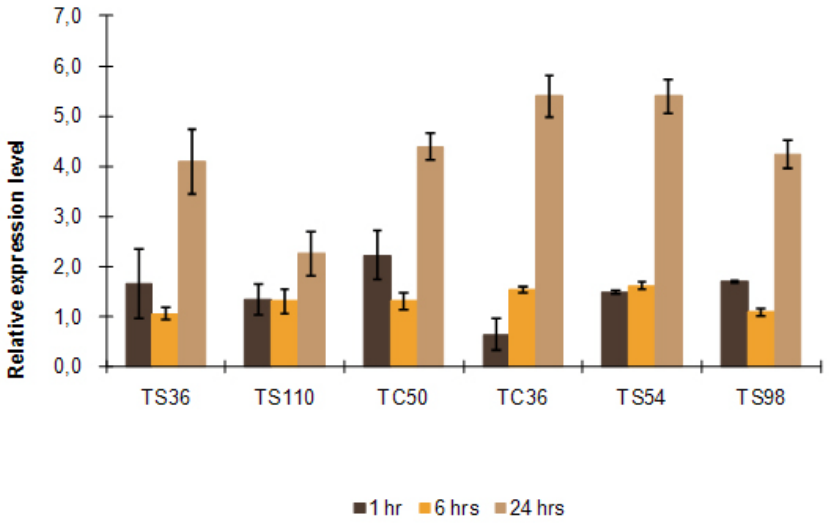

Fig. 2 Relative expression of 6 genes in peanut seeds imbibed at 2ºC and 15°C for 1 hr, 6 hrs and 24 hrs, respectively. Relative expression (1 hr, 6 hrsand 24 hrs) at 2°C is computed based on the corresponding gene expression (1 hr, 6 hrs and 24 hrs) at 15°C. The error bars indicate standard deviation of mean.

To investigate changes in gene expression in stressed seeds over time, the expression of the 6 genes at 2ºC (1 hr, 6 hrs) relative to that at 24 hrs was analyzed. Gene expression across 1 hr, 6 hrs and 24 hrs as illustrated in Figure 3 exhibited 3 different tendencies: decrease (TS36 and TS54); increase and then decrease (TS110 and TC36); and decrease and then increase (TC50 and TS98). For TS110, TC36, TC50 and TS98, the relative expression level was lower than 2.0, indicating a narrow range of variation in expression; for TS36 and TS54, however, the relative expression level was higher than 2.4, showing that the expression at 1 hrs and 6 hrs was significantly higher than that at 24 hrs. Though the expression of the 6 genes in stressed seeds exhibited a narrow range of fluctuation or remarkably decreased (Figure 3), its relative expression at 24 hrs relative to that in the control (15ºC) was much higher (Figure 2), due to drastic decline in expression of the genes under unstressed condition, possibly suggesting their protective roles against LT injury.

The 6 transcripts were chosen based on their roles in plant chilling tolerance/resistance as described in previous reports (Thomashow, 1999; Zhu et al. 2005; Hu et al. 2008; Shimada et al. 2008; Jan et al. 2009; Duan et al. 2011). In many plants, a large and ubiquitous group of stress influenced genes encoding LEA proteins have been identified, especially under stress conditions such as cold, drought, or high salinity (Thomashow, 1999). HSPs are a class of ubiquitous and highly conserved proteins

which show up-regulated expression in response to various environmental stresses, including heat, cold, heavy metal, water deficit, oxidative stress, and wounding (Duan et al. 2011). NAC (NAM, ATAF, and CUC) family transcription factors have been found to play important roles in plant development and responses to environmental stresses (Ohnishi et al. 2005; Hu et al. 2008). Over expression of cold stress-inducible rice *SNAC2 in transgenic rice resulted in high cell membrane stability under cold stress. Microarray analysis showed up-regulation of several stress regulated genes in SNAC2*-over expressing plants (Hu et al. 2008). These results suggested that several transcriptional networks function during cold acclimation and cold stress in plants. Although *NAC* genes were isolated from peanut (Shao et al. 2008), thus far no research has been conducted on their relationship to cold-resistance. MYB is also an important transcription factor under the cold stress. Over expression of the cold regulated rice transcription factors *MYB4* and *OsMYB3R-2* enhanced freezing tolerance in *Arabidopsis* (Vannini et al. 2004; Zhu et al. 2005). The molecular function of oleosins contributes healthy germination and freezing tolerance to seeds by maintaining nuclear structure (Takashi and Ikuko, 2010).

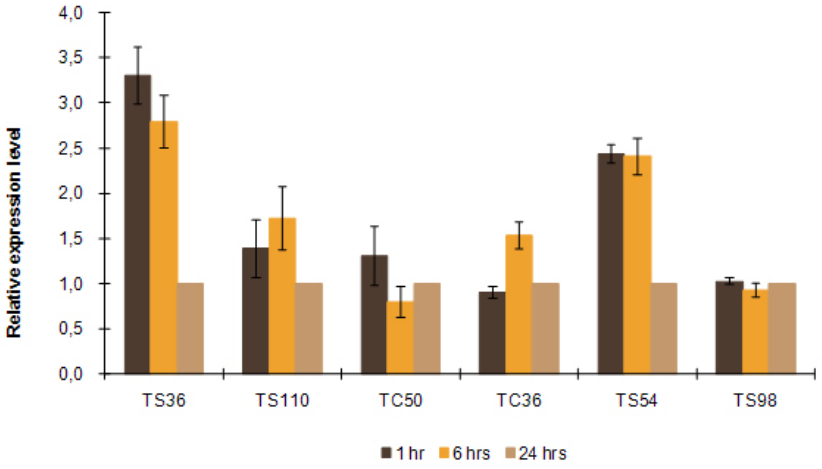

Fig. 3 Expression pattern of 6 genes in peanut seeds imbibed at 2ºC for 1 hr, 6 hrs and 24 hrs, respectively. Relative expression (1 hr, 6 hrs) is computed based on the corresponding gene expression at 24 hrs. The error bars indicate standard deviation of mean.

In this report, a series of chilling responsive genes were identified from peanut, which may provide new insights into the underlying molecular events involved. Nevertheless, the exact function of these genes is still unknown, although the importance of their orthologues to stress responses in other plant species has been well documented. Our future work will focus on the elucidation of the roles played by these genes in peanut using transgenesis. Notably, this study also resulted in some transcripts with unknown functions. It is anticipated that further study on these transcripts may lead to the identification of novel genes involved in chilling tolerance in peanut.

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