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ORIGINAL ARTICLE

Transcript profiling and gene expression analysis under drought stress in *Ziziphus nummularia* (Burm.f.) Wright & Arn.

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Abstract

Drought is one of the prime abiotic stresses responsible for limiting agricultural productivity. A number of drought responsive genes have been isolated and functionally characterized but these studies have been restricted to a few model plant systems. Very few drought responsive genes have been reported till date from non model drought tolerant plants. The present study aimed at identifying differentially expressed genes from a drought tolerant, non-model plant, *Ziziphus nummularia* (Burm.f.) Wight & Arn. One month old seedlings of *Z. nummularia* were subjected to drought stress by 30% Polyethylene glycol (PEG 6000) treatment for 6, 12, 24, 48 and 72 h. A significant reduction in RWC and increase in proline was observed at 24 h and 48 h of treatment. Suppression subtractive hybridization (SSH) library was constructed with drought stressed seedlings after 24 h and 48 h of PEG 6000 treatment. A total of 142 and 530 unigenes from 24 h and 48 h library were identified respectively. Gene ontology studies revealed that about 9.78% and 15.07% unigenes from 24 h and 48 h SSH libraries were expressed in "response to stress". Fifteen putative drought responsive genes identified in SSH library were validated for drought responsive differential expression by RT-qPCR. Significant changes in fold expressions were observed with time in the treated samples compared to the control. A heat map revealing the expression profile of genes was constructed by hierarchical clustering. Various genes identified in SSH libraries can serve as a resource for marker discovery and selection of candidate genes to improve drought tolerance in other susceptible crops.

Keywords *Z. nummularia* · SSH library · BLASTx · Unigenes · Gene ontology · RT-qPCR

Introduction

Drought is a meteorological event which implies the absence of rainfall for a period of time, long enough to cause moisture depletion in soil and plant tissues. It acts as a serious limiting factor in agricultural production by restricting the crop from reaching the genetically determined theoretical maximum yield [1]. Most of the plants are sensitive to water deficits, particularly during flowering to seed development stage. Certain plants are endowed with inherent metabolic mechanism which makes them capable of growing under drought conditions. Drought tolerance is a very complex molecular mechanism involving a number of metabolic pathways which are controlled by interplay of various drought responsive genes. Such drought tolerant plants are good genetic resources to understand the genetic and metabolic control of their adaptation to drought. Identification and characterization of drought responsive genes from naturally drought tolerant plant species could be an effective approach to gain insights into the molecular mechanisms of drought tolerance.

Ziziphus species belonging to Rhamnaceae family comprises of more than two hundred species of spiny shrubs and small trees, among them 17 species are native to India [2]. To effectively defer dehydration and thereby survive in adverse conditions, *Ziziphus* species exhibit a combination of mechanisms for drought avoidance and drought tolerance like effective photoprotective and stimulation of antioxidative metabolic pathways, osmotic adjustment and sensitive stomatal closure

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under stress [3, 4]. *Ziziphus nummularia* (Burm.f.) Wight & Arn. an important species of genus *Ziziphus* is native to temperate and tropical area of Asia and is highly tolerant to low moisture stress and can survive well in areas with an annual rainfall as low as 100 mm [5]. *Z. nummularia* is horticultural plant of economic significance as it provides fodder, fuel wood and fruits even during harsh environmental conditions like drought, salinity and high temperatures [6, 7] through a number of changes at physiological and developmental levels. Owing to its capability to thrive in extreme habitat, *Z. nummularia* is a good genetic resource for understanding molecular basis of stress tolerance mechanism in plants.

Studies exploring physiological basis of drought responsiveness in *Z. nummularia* have been conducted [8] but studies involving molecular basis of drought tolerance are scarce [9, 10]. Identification of differentially regulated genes in plant can be achieved by a range of techniques like cDNA-amplification fragment length polymorphism (cDNA-AFLP) [11], differential display PCR [12], PCR-based cDNA-representational difference analysis (cDNA-RDA) [13] and suppression subtractive hybridization [14, 15]. Among these techniques construction of SSH library and analysis of ESTs is one of the primary tools for discovery of drought responsive and novel genes, especially in non-model plants like *Z. nummularia*. SSH is a technology which allows PCR-based amplification of cDNA fragments from mRNA differentially expressed only in experimental transcriptome as compared to control. The EST sequence information provides a core resource for various genome scale experiments and represents a platform for understanding the complex cellular processes and mechanisms of plant adaptation to a variety of biotic and abiotic factors [16].

The present study aimed at transcript profiling in *Z. nummularia* for identification of drought responsive genes. Two SSH libraries were constructed from one month old *Z. nummularia* seedlings which were subjected to drought stress through 30% Polyethylene glycol (PEG 6000) treatment for 24 h and 48 h. A total of 672 ESTs were identified and annotated for gene ontology (GO) categorization. Fourteen drought responsive genes were validated by reverse transcription-quantitative PCR (RT-qPCR) at different duration of 30% PEG 6000 treatment. Transcript pattern of genes showed significant upregulations with time in treated samples compared to control. The genes differentially upregulated in response to drought stress can serve as candidate genes for development of drought tolerant transgenics in food crops.

Materials and methods

Seed materials and treatment

Seeds of *Z. nummularia* (National identity number IC0598427), provided by the Central Institute of Arid Horticulture (CIAH), Bikaner, Rajasthan, India were germinated in pots (15 cm diameter) containing autoclaved soilrite at 30 °C with 16 h light/8 h dark photoperiod at the National Phytotron Facility, Indian Agricultural Research Institute, New Delhi, India. One month old seedlings (twelve seedlings per treatment) were subjected to 30% PEG 6000 (Himedia, India) treatment in 0.5× Murashige and Skoog media (w/v) (Himedia, India) supplemented with 1 mM 2-(N-morpholino) ethanesulfonic acid (MES) (w/v) (Himedia, India) for different duration of PEG induced stress (0, 6, 12, 24, 48 and 72 h) to stimulate drought stress. The whole seedlings after treatment were harvested, flash frozen in liquid nitrogen and stored at − 80 °C for further down-stream processing. The seedling samples (three seedlings per treatment) harvested at 0 h as control (driver) and 24, 48 h as experimental (tester) were used for SSH library construction.

Relative water content

Relative water content (RWC) of seedlings subjected to drought stress was determined by the method described by Barrs and Weatherley [17]. A set of treated seedling (three seedlings per treatment) samples were weighed to obtain fresh weight (FW) and then placed in double distilled water to full turgidity for 4 h under normal room light and temperature. After hydration the fully turgid weight (TW) was obtained by weighing the samples immediately. Samples were then dried in oven at 80 °C for 24 h and weighed to obtain their dry weight (DW). RWC was calculated as:

$$\mathrm{RWC}(\%) = \left[(\mathrm{FW} - \mathrm{DW})/(\mathrm{TW} - \mathrm{DW})\right] \times 100.$$

Proline assay

Proline accumulation was estimated as per the protocol given by Bates et al. [18]. Briefly, samples from each treatment were homogenized in 5 ml of sulphosalicylic acid (3%) and proline was assayed by the acid ninhydrin reagent. Absorbance was recorded against toluene blank at 520 nm in spectrophotometer and concentration of proline was estimated by referring to the standard curve drawn from known concentrations of proline.

Statistical analysis

RWC and proline estimation were carried out with three independent biological replicates from each treatment. One way analysis of variance (ANOVA) was performed and comparison of mean values were analyzed by Tukey test at P value 0.05 by Origin pro software (version 8.0).

Extraction of total RNA and SMART cDNA synthesis

Total plant RNA was isolated from 100 mg seedling treated to different duration of PEG induced stress (0, 6, 12, 24, 48 and 72 h) using Spectrum™ plant total RNA kit (Sigma–Aldrich, USA) following manufacturer's instructions. The quality and quantity of extracted RNA was determined by agarose gel electrophoresis and UV spectrophotometer (NanoDrop 2000, Thermo Scientific, USA). RNA isolated from 0, 24 and 48 h of treatment were used for construction of two independent SSH libraries in forward direction and termed as 24 h and 48 h libraries respectively. The first strand cDNA was synthesized by adding 1 µg of RNA from each control (0 h) and stressed (24 h and 48 h) samples using SMARTer™ PCR cDNA synthesis Kit (Clontech laboratories, Inc, USA) as described in user manual. Double stranded cDNA (ds-cDNA) was generated by long distance (LD) PCR with advantage 2 polymerase mix (Clontech laboratories, Inc, USA), using first strand cDNA as template.

Construction of SSH library

SSH libraries were constructed by using PCR-Select™ cDNA Subtraction kit (Clonetech, USA) according to manufacturer's instructions with certain modifications. Double stranded cDNA from 24 h and 48 h of treatment designed as tester and 0 h as driver were used for SSH library construction. Tester and driver cDNAs were digested with *Rsa*I and divided in two equal volumes followed by ligation with two different cDNA adaptors. After two rounds of hybridizations, primary PCR was performed to amplify differentially expressed genes and secondary PCR was performed with nested primers to enrich differentially expressed sequences. Purified secondary PCR products were ligated into pGEM®-T Easy vector (Promega, USA) and transformed into E. coli strain DH10B (New England Biolabs, UK) by electroporation. Positive clones from Luria Broth-agar plates supplemented with isopropyl-β-D-thiogalactopyranoside (IPTG) (0.5 mM), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (80 µg/ml) and ampicillin (100 µg/ml) were selected for colony PCR amplification.

Screening and sequencing of recombinant clone

The putative recombinant colonies were confirmed by colony PCR [19] with T7: 5′ TAATACGACTCACTATAGG 3′ and SP6: 5′ TATTTAGGTGACACTATAG 3′ promoter specific primers (Promega, USA). Colony PCR products with insert size of more than 250 bp were purified by ExoSAP-IT® (Affymetrix, US) and sequenced by BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) by following user guidelines, in single direction with sequencing primer T7 promoter on ABI 3730 XL DNA analyzer (Applied Biosystems, USA).

Sequence analysis and gene ontology categorization

Raw reads of sequences obtained were screened and vector/adaptor sequences were trimmed manually by using VecScreen tool (http://www.ncbi.nlm.nih.gov/Vecscreen). The high quality edited expressed sequence tags (ESTs) which had more than 100 bp in length were assembled and clustered into contigs and singletons using CodonCode Aligner. The contigs and singletons were annotated against non redundant protein database of Arabidopsis, The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org/) through BLASTx to identify the similarity with known ESTs in Arabidopsis. GO and functional categorization analysis of annotated transcripts was performed by gene ontology tool at TAIR (https://www.arabidopsis.org/tools/bulk/go/index.jsp) by using locus IDs obtained through BLASTx of TAIR10.

Expression analysis by RT-qPCR

RT-qPCR was carried out for randomly selected fourteen drought responsive genes which were expressed in 24 and 48 h forward SSH library. Specific primers for each gene were designed using the Primer Quest software of Integrated DNA Technology (IDT) at default parameter; primer sequences of genes and their respective library are listed in Table 1. The first strand cDNA was synthesized with the SuperScript®III first strand synthesis system (Invitrogen, USA) using oligo (dT) primer. RT-qPCR amplification mixtures (10 µl) containing 100 ng template cDNA, 0.5 µM of each primer and 2X KAPA SYBR FAST qPCR master mix buffer (5 µl) (KAPA Biosystems, USA) were run on the LightCycler® 480 II (Roche, Switzerland). RT-qPCR experiments were performed in three biological and three technical replicates for each treatment. Ct (threshold cycles) values were calculated by the $2^{-\Delta\Delta C_t}$ method [20]. The *Ziziphus jujuba* elongation factor1 (*Zjef*1) gene (accession no. EU916202) was used as internal control [21]. Ct values from replicates were averaged and normalized with

Table 1 Description of primers used for validation of genes by RT-qPCR

Primer code	Representing library	Primer sequence (5′–3′)	Product size
Zjef	Housekeeping gene	F: GCTGACTGTGCTGTTCTCATC	111
		R: GACACCAAGAGTGAAAGCGAG	
CCD1	24 h	F: CGGTGCTGATGCTTTCTTCTG	154
		R: AGTAAATGCGATCACTTGCCG	
Dehydrin	24 h	F: GAAGGTGCTGTTGAAACCCAGG	162
		R: TTCCTCGTCCTTGCGTTCCTC	
Dnaj	24 h	F: TCGACCATTCCTCTGACTCCC	163
		R: TCAGCCTCATGAGCTTGTTGG	
DREB	24 h	F: TTACAAGGGCGTGAGGATGAGGAA	86
		R: AACCCAACCATATTCTGTGTCGGC	
Expansin	24 h	F: AAACACAGCAGCTTTGAGCACG	177
		R: TTACACCAGCCTCCAGCGTTG	
GolS	24 h	F: GCACTTGGATATGGTCGTCAGG	160
		R: AGGATTGGCTAAGGGTCTGCG	
C2H2 zinc finger	24 h	F: CACAACACACACGAACAGCCG	135
		R: GGCTTCTTCGTCGGCAGAGG	
Uncharacterized	48 h	F: CGGTTAAGTCGGGCAAGAAA	99
		R: CCTTATCCCTAAATCGGCTGAAG	
CP	48 h	F: GTGGCGTCCATTGATGATTAC	115
		R: GGAAATCTCTACCACCTCCTTC	
Inositol-1,4,5-trisphosphate 5-phosphatase	48 h	F: TCGGAAAGACCTTCACTCTCTA	90
		R: ACAGTCGCAGCCATCATT	
PIP	48 h	F: GAGGGTAAGGAAGAAGATGTTAAA	101
		R: TGTAGTCCTTGCTGTCATCTTG	
NCED	48 h	F: GGGCTGACGGTGATCTTT	123
		R: CGTCGTAGCTTAGAGTGTGTAG	
Osmotin	48 h	F: TGGCAGAATACGCTCTCAAC	115
		R: ATTCCTCTACACCCTCCTGAT	
P5CS	48 h	F: TAAGTGCAGAGGGTGGGA	110
		R: CACTACATGACCAGTTCCTCTAAG	

the Ct values of the internal control *Zjef*1, standard deviations and errors were calculated. Furthermore, a heat map was generated to investigate expression profile of unigenes, found in SSH libraries under drought stress by using the Genevestigator™ database [22]. Genes are hierarchically clustered according to expression level by using orthologous probe of *Arabidopsis thaliana*.

Results

Analysis of RWC and proline assay

The RWC values decreased significantly with increase in the duration of PEG 6000 treatment, showing the maximum reduction 56.85% and 40.91%, respectively at 24 h and 48 h of treatment in comparison to the untreated (control) samples (Fig. 1a). There was significant increase in proline levels with the duration of drought stress (Fig. 1b) in comparison to control. Concentration of proline (per gram fresh weight) was observed as 34.40 µmol, 51.82 and 57.66 µmol at 24, 48 and 72 h of treatment respectively (Fig. 1b).

Construction and screening of SSH libraries

Good quality of RNA was successfully isolated from treated seedlings of *Z. nummularia* and first strand of cDNA was synthesized for SSH library construction. During first strand cDNA synthesis 3′ SMART CDS Primer II A primes the first strand reaction and SMARTer II A Oligonucleotide flanks as a short, extended site at 5′ end of RNA template. Therefore, first strand of cDNA has complete 5′ end of mRNA and the complementary sequence of SMARTer II A Oligonucleotide works as long distance polymerase chain reaction (LD-PCR) priming site for amplification of full length cDNA. Desirable ds-cDNA PCR product was obtained at 24 cycle of LD-PCR.

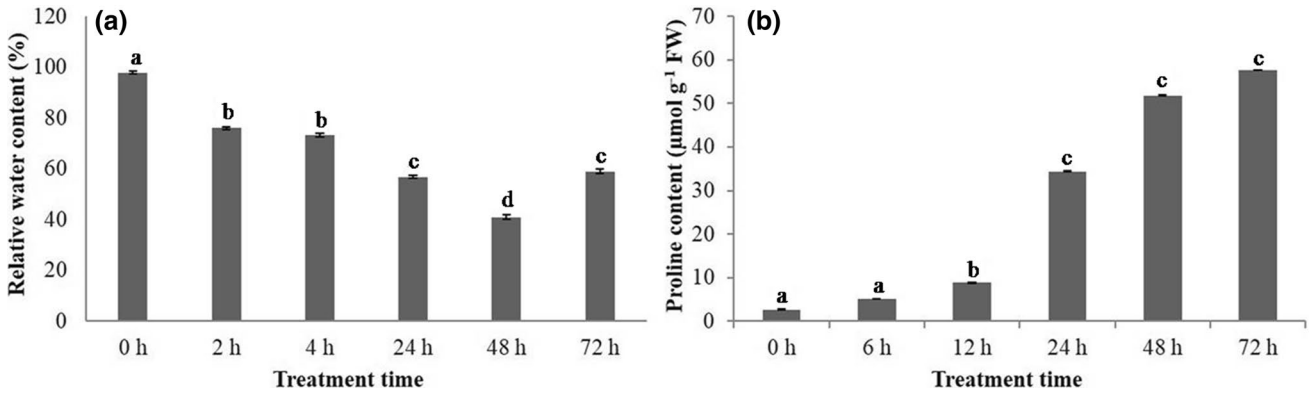

Fig. 1 Estimation of relative water content (**a**) and proline (**b**) under water stress induced by 30% PEG 6000 treatment for different time periods in *Z. nummularia*. Vertical lines indicate the calculated standard error. Bar represents mean values ± SE (n = 3) at P = 0.05 calculated by Tukey test. Means in different lower case letters are significantly different

Table 2 Summarized description of cDNA libraries constructed by suppression subtractive hybridization

Description	24 h SSH library	48 h SSH library
Number of clones	2208	1792
Good quality ESTs	1243	1326
Number of contigs	67	31
Number of singletons	75	499
Number of unigenes	142	530
Accession number	JZ142477-JZ143719	LN556488-LN557813
Database for submission	dbESTs of NCBI	ENA-EMBL

Primary and secondary PCR products of subtracted experimental cDNA were visualized as smear with some distinct bands (Fig. 2). Enrichment of differentially expressed genes of subtractive hybridization was confirmed by band intensities of primary and secondary PCR products as band intensity of secondary PCR products was more than primary PCR products. Randomly, 2208 and 1792 recombinant clone having insert size in the range of 250–1000 bp from 24 h and 48 h SSH libraries respectively were sequenced. The resulting 1243 good quality ESTs from 24 h SSH library and 1326 ESTs from 48 h SSH library were used for assembly. After EST assembly, 142 unigenes containing 67 contigs and 75

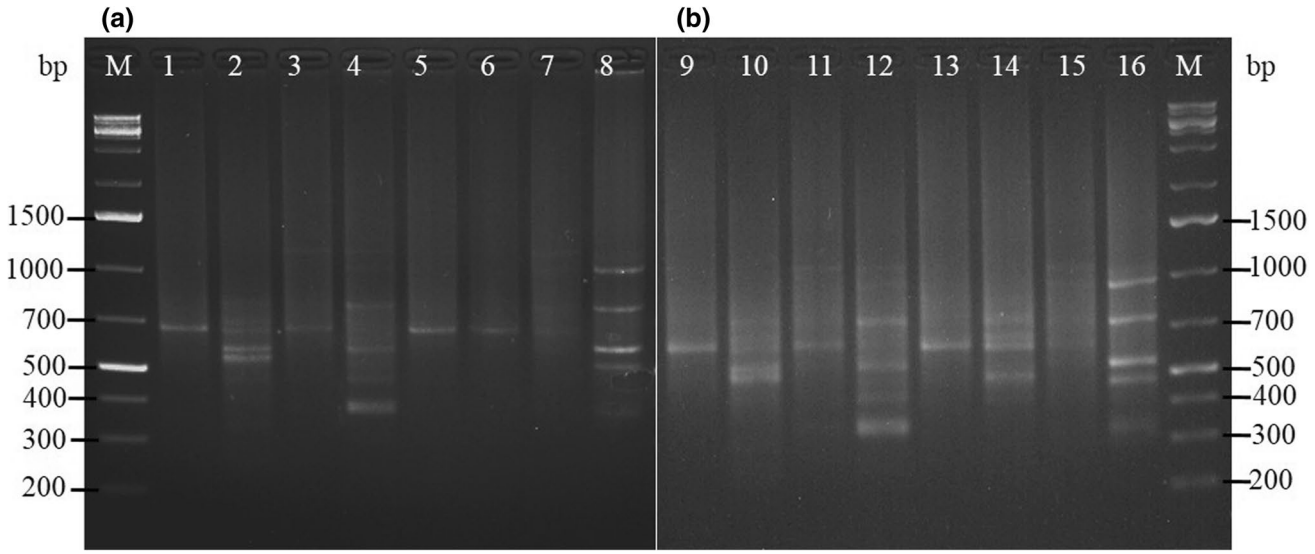

Fig. 2 Agarose gel electrophoresis of primary PCR products (**a**) Lanes (1) unsubtracted 0 h treated samples; (2) subtracted 0 h treated samples; (3) unsubtracted 24 h treated samples; (4) subtracted 24 h treated samples; (5) unsubtracted 0 h treated samples; (6) subtracted 0 h treated samples; (7) unsubtracted 48 h treated samples; (8) subtracted 48 h treated samples; and their corresponding secondary (**b**) PCR products, Lanes 9–16 samples in the same order. Lane M. GeneRuler 1 kb plus DNA Ladder (Thermo Scientific, USA)

singletons from 24 h library and 530 unigenes containing 31 contigs and 499 singletons from 48 h library were identified. All the ESTs from 24 and 48 h libraries were submitted to dbESTs of NCBI (JZ142477- JZ143719) and ENA-EMBL (LN556488- LN557813) databases, respectively. Summarized details of SSH libraries are listed in Table 2.

Annotation and gene ontology analysis

BLASTx analysis of all the unigenes was performed against the non redundant protein database of Arabidopsis (TAIR) to identify the homology of unigenes at threshold E-value $1e^{-5}$ (Supplementary file 1: Table S1 & S2). BLASTx results showed that 130 unigenes (91.54%), 488 unigenes (92.07%) from 24 and 48 h SSH library, respectively are homologous to known genes and 12 unigenes (8.45%), 42 unigenes (7.92%) from 24, 48 h SSH library are homologous to genes with unknown function or without matches in the TAIR database. Among all unigenes, nine were found to be common in both the libraries. Many unigenes were showing similarity to various previously reported abiotic stress responsive genes, some of which are listed in Table 3.

A total of 136 and 497 unigenes, respectively from 24 h and 48 h SSH libraries were subjected to GO analysis and classified into three main GO categories i.e. biological process, molecular function and cellular compartment (Fig. 3). The most abundant class of molecular function category was binding group with 30.70% and 20.54% unigenes from 24 h and 48 h SSH library, respectively followed by enzyme activity, hydrolase activity, unknown molecular function and transferase activity group. In the biological category, the cellular process was found to be prevalent with 24.46% and 21.18% unigenes in 24 h and 48 h SSH library, respectively, followed by metabolic process, biotic and abiotic stimulus and response to stress. In the cellular component category, intracellular component was prevalent for 24 h whereas cytoplasmic component group was observed to be dominant in 48 h library. Among all, under biological category 9.78% and 15.07% unigenes from 24 and 48 h SSH library respectively belongs to group "response to stress".

RT-qPCR analysis

Expression patterns of carotenoid cleavage dioxygenase 1 (*CCD1*), dehydrin, *DnaJ*, dehydration responsive element

Table 3 List of some unigenes showing similarity to drought responsive genes

EST code	Locus ID	Gene	E value	Score
Contig 24F_9	AT3G01500.2	Carbonic anhydrase 1	e−107	333
Contig 24F_16	ATCG00150.1	ATPase, F0 complex	e−101	363
Contig 24F_20	AT1G19180.1	Jasmonate-zim-domain	1e−36	150
Contig 24F_38	AT2G41430.5	Dehydration-induced protein	2e−06	49
Contig 24F_45	AT5G25610.1	RD22	4e−12	64
Contig 24F_50	AT1G12460.1	Leucine-rich repeat protein kinase	8e−55	209
Contig 24F_51	AT5G14000.1	NAC domain	1e−29	125
Contig 24F_56	AT5G16400.1	Thioredoxin F2	5e−09	56
Singletons 24F_20	AT2G47370.1	Calcium-dependent phosphotriester	3e−19	90
Singletons 24F_48	AT5G02500.2	HSP70	3e−29	123
Singletons 24F_61	AT2G43150.1	Proline-rich extensin-like family	3e−04	41
Singletons 24F_54	AT4G09350.1	DnaJ-domain superfamily	7e−16	80
Singletons 48F_4	AT4G00430.1	PIP1	3e−26	113
Singletons 48F_16	AT1G78080.1	RAP2.4, related to AP2 4	1e−11	65
Singletons 48F_24	AT1G47128.1	RD21 protein	2e−26	113
Singletons 48F_29	AT4G10960.1	UDP-D-glucose/UDP-D-galactose	1e−42	168
Singletons 48F_31	AT5G10240.2	Asparagine synthetase 3	3e−39	156
Singletons 48F_34	AT5G49360.1	Beta-xylosidase 1 BXL1	7e−56	213
Singletons 48F_41	AT2G39800.3	Delta1-pyrroline-5-carboxylase synthase	2e−17	84
Singletons 48F_54	AT2G21490.1	Dehydrin LEA	3e−06	47
Singletons 48F_67	AT1G71695.1	Peroxidase superfamily protein	8e−06	45
Singletons 48F_71	AT1G56600.1	Galactinol synthase 2	2e−12	68
Singletons 48F_158	AT5G43580.1	Serine protease inhibitor	7e−16	79
Singletons 48F_165	AT1G72610.1	Germin-like protein	1e−45	179

* Score is a numerical value that describes the overall quality of an alignment. Higher numbers correspond to higher similarity

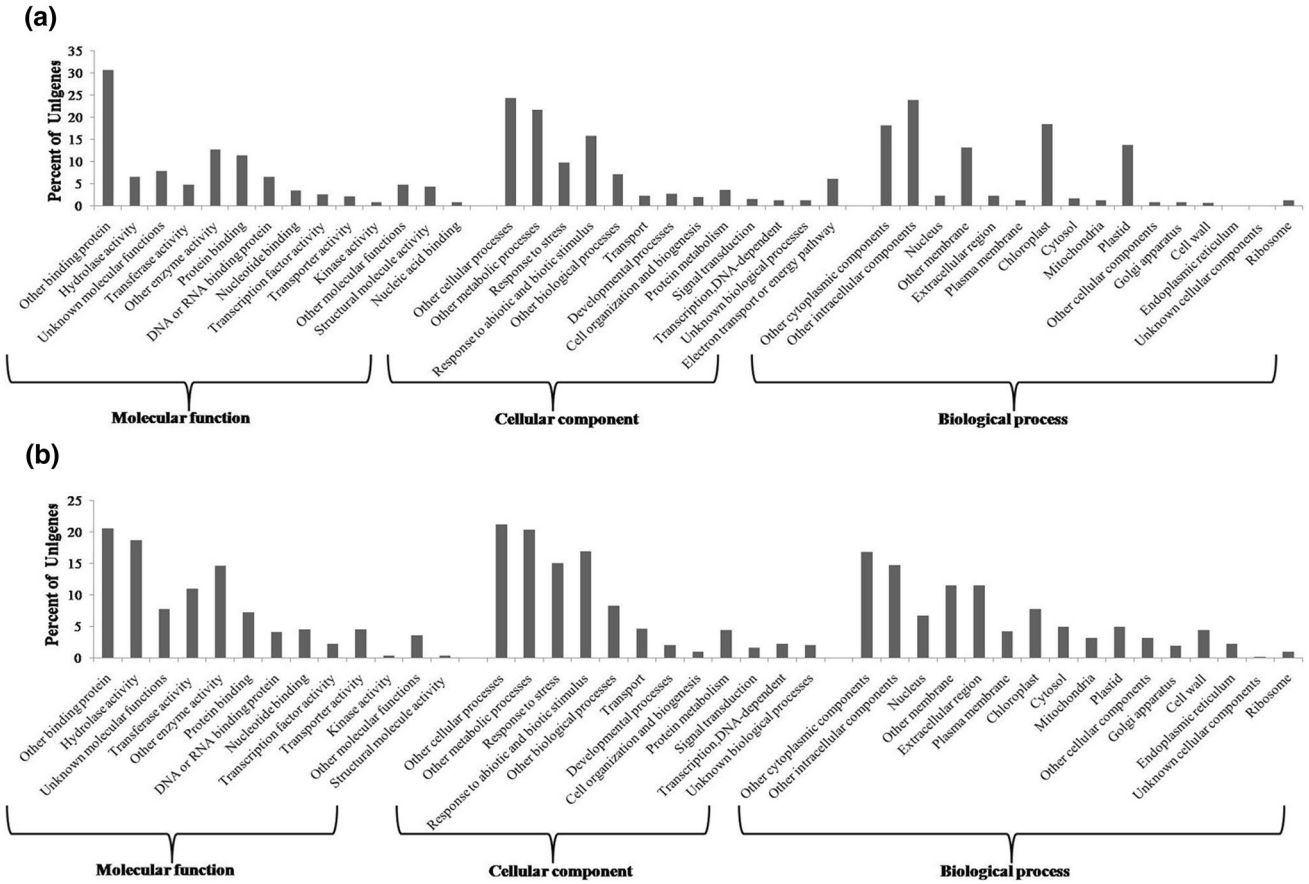

Fig. 3 Gene ontology (GO) classification of unigenes with TAIR database. Bar chart represents the distribution of unigenes (%) in molecular function, biological process and cellular component categories found in 24 h (**a**), 48 h (**b**) SSH library

binding (*DREB*), expansin (*EXP*), galactinol synthase (*GolS*), Cys2/His2-type zinc-finger proteins (C2H2 Zinc finger), uncharacterized gene, cysteine protease (*CP*), inositol-1,4,5-trisphosphate 5-phosphatase, aquaporin (*PIP*), 9-cis-epoxycarotenoid dioxygenase (NCED), osmotin and delta-1-pyrroline-5-carboxylate synthase (*P5CS*) genes were analyzed by RT-qPCR (Fig. 4). Dehydrin and *EXP* genes were upregulated up to 5.67 and 5.06 folds, respectively at early stage (6 h) of treatment. Transcript level upregulated up to 4.31, 12.90, and 6.58 folds at 12 h for *CP*, inositol-1,4,5-trisphosphate 5-phosphatase and osmotin genes respectively whereas 8.51 folds change in expression was observed for *DnaJ* gene at 24 h of treatment. *PIP, CCD1, DREB, GolS* and C2H2 zinc finger gene showed up to 5.15, 6.86, 9.95, 25.19 and 2.31 folds change in expression pattern at 48 h of treatment, respectively. Expression level of *P5CS, NCED* genes and uncharacterized protein reached the maximum, up to 31.12, 91.45 and 675.58 folds at late stage of drought stress (72 h of treatment).

The normalized expression intensities of unigenes from SSH libraries and their hierarchical clustering is represented by heat map (Fig. 5). The heat map was generated by clustering unigenes with high expression level of orthologous genes of *A. thaliana* under different drought stress experiments. Hierarchically clustering results showed more genes are differentially expressed in 48 h SSH library in comparison to 24 h library.

Discussion

Drought stress is one of the primary abiotic stresses causing major loss in crop production world over. To understand the mechanism that enables the plants to cope with water deficit conditions, it is quite relevant that plants which are naturally drought stress tolerant are studied to get an insight to the molecular mechanism of drought stress tolerance. Among various physiological parameters relative water content is considered as most meaningful index of dehydration tolerance, which measure water status of plant reflecting the metabolic activity in tissues [23]. RWC values showed significant decrease with increase in duration of PEG 6000 treatment in *Z. nummularia*. The same trend has been reported previously in barley [24], tomato

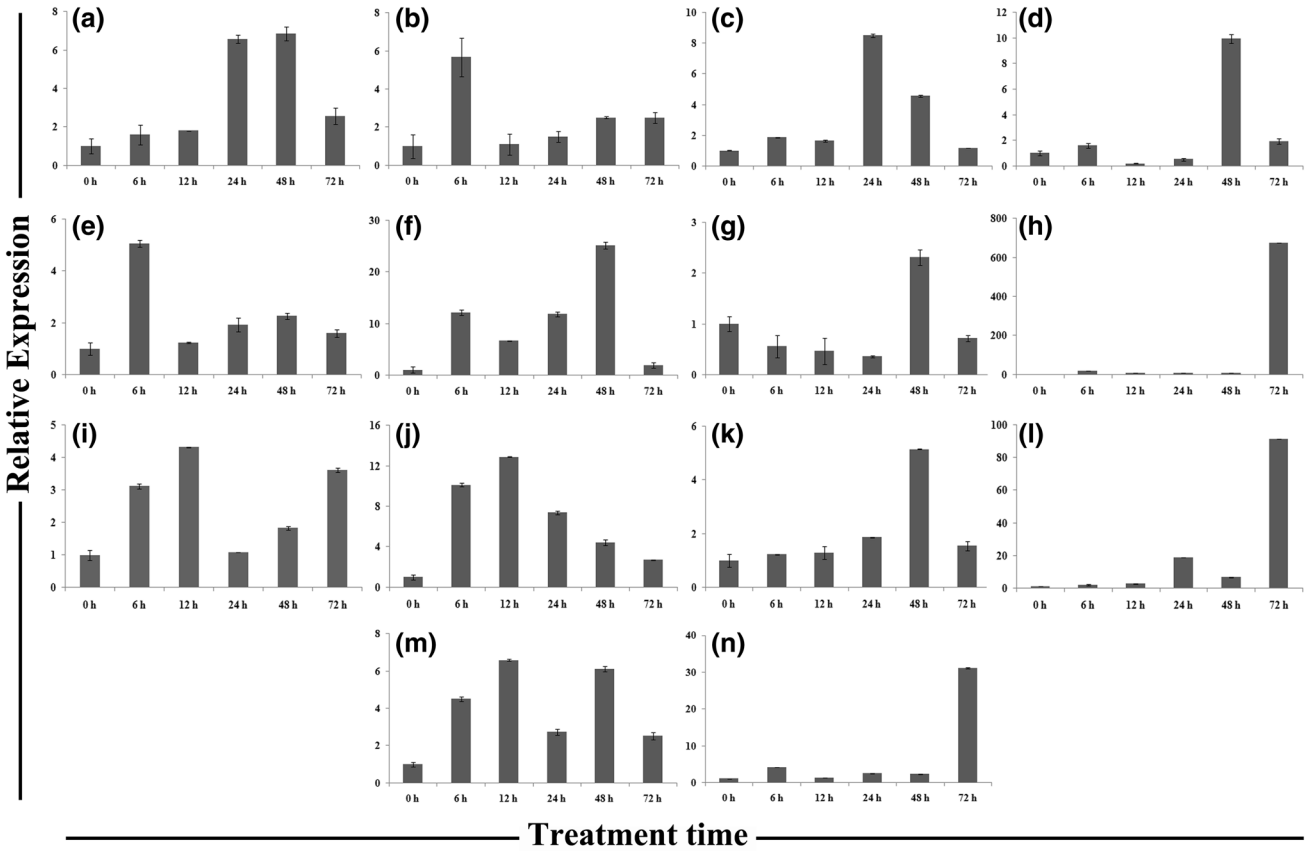

Fig. 4 Expression profile analysis of selected drought responsive genes. RT-qPCR was performed in 30% PEG 6000 treated seedlings for different time durations **a** *CCD1*; **b** dehydrin; **c** *DnaJ*; **d** *DREB*; **e** *EXP*; **f** *GolS*; **g** C2H2 Zinc finger; **h** uncharacterized gene; **i** *CP*; **j** inositol-1,4,5-trisphosphate 5-phosphatase; **k** *PIP*; **l** NCED; **m** osmotin; **n** *P5CS*

[25] and pigeon pea [26]. In most plant species the typical leaf RWC at around initial wilting is about 60–70%, with exceptions and 30–40% in severely desiccated conditions, depending on plant species. In our study maximum value of RWC (97.86%) was observed in control seedling, it decreased significantly (56.85%) after 24 h and continued to decrease (40.91%) at 48 h of treatment. Value of RWC again increased (58.98%) at 72 h, this slight increase (18.07%) in the RWC value may be due to the fact that plant system tried to rebound to normal conditions. Similar phenomenon has been observed in *Pennisetum glaucum* [27]. Proline level significantly accumulated under drought stress imposed by 30% PEG 6000 treatment in *Z. nummularia*. Significant accumulation of proline in mild and severe stresses in root is reported [28]. Proline acts as an osmolite, protects cell membrane against low water potential, protein content and causes osmotic regulation in plant organs. Thus RWC and proline results indicate that *Z. nummularia* requires less water for maintenance under drought conditions.

The response of plants towards water stress is very complex and suggests that a large number of plant genes are involved in stress response pathways, although only a few molecular components have been known so far [29]. Based on RWC and proline assay results, 24 h and 48 h SSH libraries were constructed from *Z. nummularia* to isolate and characterize the drought responsive and novel genes. After BLASTx results, 618 (91.96%) unigenes from both libraries annotated as putative proteins, had significant protein homology to genes from *A. thaliana* database. Among these most of the genes were previously identified as water stress related genes in other plants like ABC transporter [30], 9-*cis*-epoxycarotenoid dioxygenase [31], NAC domain [32], C2H2 zinc finger protein [33–37], plasma membrane intrinsic protein (aquaporin like protein) [38], asparagine synthase [39], Δ1-pyrroline-5-carboxylate synthetase [40], protein kinase super family protein [41], galactinol synthase and germin-like protein [42]. Receptor like protein kinases (*RLKs*) observed in our library is signaling components that mediate plant responses to developmental and environmental stimuli. Some studies reported that *RLKs* like *RPK*1, *GHR*1 and *AtCRK*45 from *A. thaliana* respond to salt, cold and drought stress and play role in ABA and BR signaling pathways [43, 44]. Wu et al. reported that transcription of RLK genes increased under drought stress and suggested that they

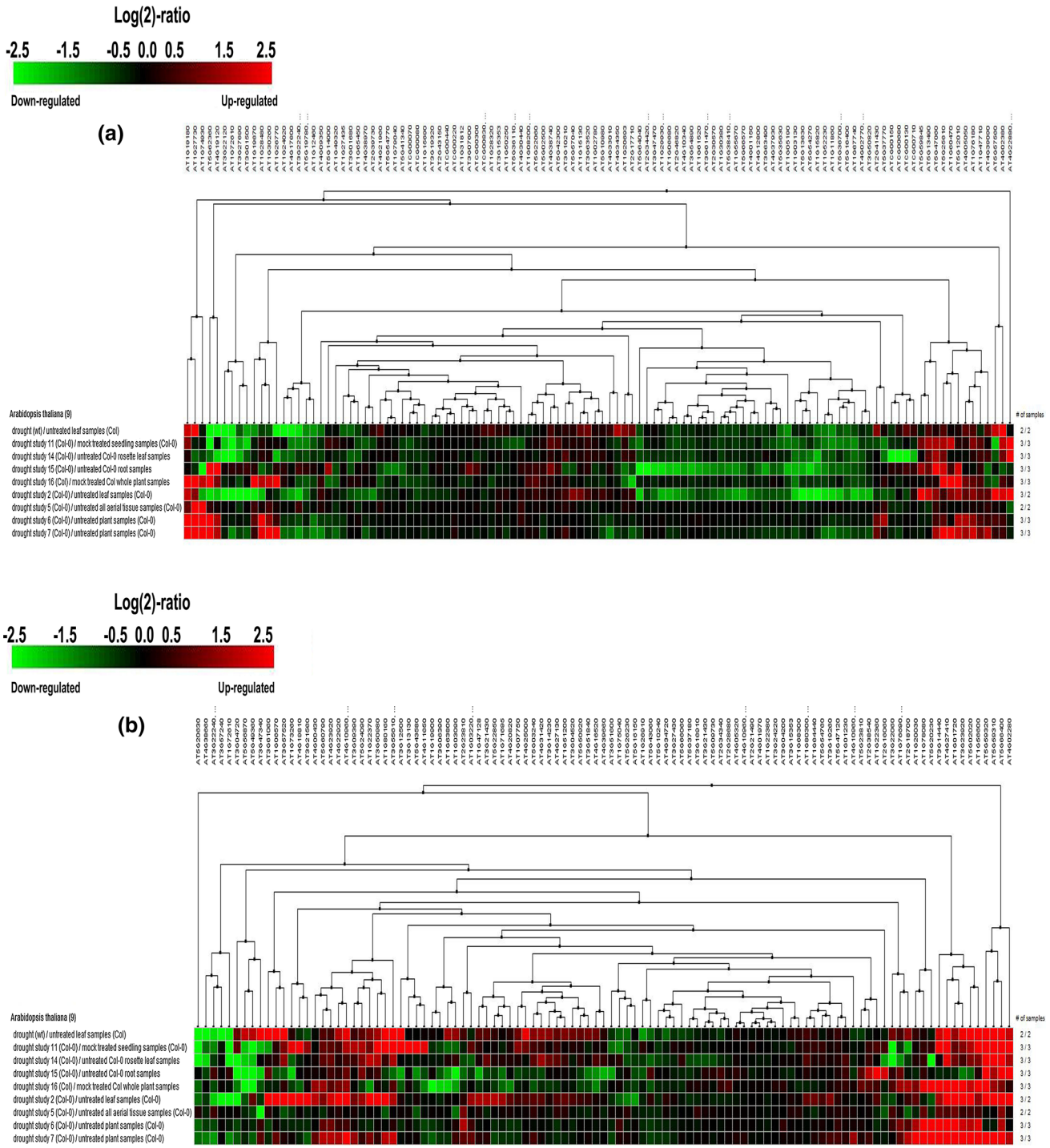

Fig. 5 Heat map of expression values of unigenes from 24 h (**a**) and 48 h (**b**) SSH library. *A. thaliana* probe IDs from different drought stress experiments were used for generation of heat map. Colour scale from green to red indicates the range of expression level. (Color figure online)

may play important role in drought adaptation of *A. mongolicus* [45]. Another group of unigenes (5.80%), not showing similarity to any sequence in TAIR database could be novel, therefore isolation and characterization of those genes would help in understanding the mechanism of drought tolerance in *Z. nummularia*. Some unigenes (2.23%) showing similarity with the genes of unknown function or uncharacterized genes in TAIR database are short length sequences consisting primarily of 3′ UTRs [46] with little to no protein-coding sequence. Assembly and annotation results validate the SSH

libraries constructed at 24 and 48 h of treatment. However, these libraries may not include all drought stress responsive genes expressed in *Z. nummularia* but are good resource to explore the drought responsive genes for development of drought resistant crops.

Unigenes from both libraries were annotated for GO analysis and categorized into biological process, molecular function and cellular compartment category which may help to elucidate the functional relevance of these unigenes under drought stress. In molecular function category unigenes related to other binding proteins, hydrolase activity, transferase activity, unknown molecular functions, enzyme activities, transcription factor activities and transporter activity are dominant, indicating the dominance of gene regulation, signal transduction and enzymatically active processes under drought stress in *Z. nummularia*. Under biological category, cellular process, metabolic processes, response to stress and response to abiotic and biotic stimulus were highly represented, indicating that the plant is undergoing rapid growth and extensive metabolic activity under stress conditions as reported previously [47]. Genes involved in kinase activity, other molecular functions, structural molecule activity and nucleic acid binding were identified in our libraries, suggested the involvement of genes in different molecular and biochemical mechanism to tolerate the drought stress in *Z. nummularia*.

The ESTs of *Z. nummularia* were annotated against well studied genome of model plant Arabidopsis and many ESTs have significant similarity. As ESTs are stretch of coding DNA sequence and there is possibility that complete CDS of ESTs may encode for a protein which participate in drought tolerance mechanism of *Z. nummularia* as *Z. nummularia* is drought tolerant non model plant system. Therefore identification and isolation of genes from *Z. nummularia* has significant role to understand complex mechanism of drought tolerance.

RT-qPCR studies reveled that *CCD1*, dehydrin, *DnaJ, DREB, EXP, GolS*, C2H2 Zinc finger, uncharacterized gene, *CP*, inositol-1,4,5-trisphosphate 5-phosphatase, *PIP*, NCED, osmotin and *P5CS* genes were up regulated at different duration of 30% PEG 6000 treatment. Accumulation of toxic compound in cell including reactive oxygen species (ROS) due to cellular changes during stress conditions are reported, genes belonging to peroxidase family, cystein protease involved in ROS detoxification were found in our library. Expression of *CP* gene was up regulated at 12 h of treatment as previously reported in *Triticum aestivum* (*TaCP*) [48]. Accumulation of cystein protease gene (*CP*) mRNA under drought [49], low temperature [50] and various developmental processes [51, 52] is well studied.

Transcription factors (TFs) play essential roles in response to different abiotic stimuli. Tanscription factors like *RAP*2.4 (member of the *DREB* subfamily A-6 of ERF/AP2 transcription factor family), *RAP*2.3 (ethylene-responsive element binding protein), Basic helix-loop-helix (*bHLH*), C2H2 zinc fingers, *TCP* and *NAC* family were found in our library which have well studied role under drought stress and other abiotic stresses. Among these, expression of *DREB* and C2H2 zinc finger were investigated and higher transcript level was observed at 48 h of treatment. Role of *DREB* [53], C2H2 zinc finger [54, 55] and *NAC* [56, 57] in responses to various abiotic stresses were studied. Over expression of three *Arabidopsis NAC* proteins (*ANAC019, ANAC055* and *ANAC072*/*RD26*) was reported to significantly improve drought tolerance in transgenic plants [58].

Galactinol synthase and *P5CS* catalyze the synthesis of galactinol (from UDP-galactose and myo-inositol) and proline respectively which functions as osmoprotectant, protects cell membrane under abiotic stress conditions. In our study, expression levels of *GolS* and *P5CS* genes were upregulated at 6 h, and the highest fold change was observed at 48 h (in *GolS*) and 72 h (in *P5CS*) post treatment. Iskandar et al. reported that expression of *P5CS1* isolated from *Saccharum officinarum* was significantly upregulated under drought treatment induced by PEG 6000 [59]. Expression level of uncharacterized gene was found up to be 675.58 fold at 72 h of treatment, therefore characterization of gene shall be of interest in future. Thus, the overall expression level of the gene transcripts studied by RT-qPCR validate the differential nature of SSH libraries developed at 24 h and 48 h of treatment. Further, SSH libraries were validated by hierarchically clustered heat map which suggested that 48 h SSH library is rich with differentially expressed genes in comparison to the 24 h SSH library. Quantitative upregulation in genes indicate, complex signaling network activate in *Z. nummularia* on global scale to activate regulated expression of several genes in response to drought stress. Therefore, collection of large number of ESTs from drought responsive SSH library of *Z. nummularia* is a repository to public database and it will be helpful in further studies for better understanding of drought tolerance, development of genetic marker and tagging/map based cloning.

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