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A secondary suppression subtractive hybridization method for isolation and identification of some salt-induced genes in soybean (*Glycine max* L. Merr)

Weiqi Wang[1], Liang Li[2], Shan Huang[1], Shi Sun[1], Cunxiang Wu[1], Tianfu Han[1], Wensheng Hou[1]*

[1]The National Key Facility for Crop Gene Resources and Genetic Improvement (NFCRI), Institute of Crop Science, Chinese Academy of Agricultural Sciences, 12 Zhongguancun South Street, Beijing 100081, China

[2]Daqing Branches of Heilongjiang Academy of Agricultural Sciences, Daqing 163319, China

***Corresponding author: houwsh@caas.net.cn**

Abstract

The yield of soybean (*Glycine max* L. Merr.) has been seriously challenged by increased salinization of the land. Continuous efforts are needed to understand the salt tolerance mechanism in soybean using suitable biotechnological tools. Although the suppression subtractive hybridization (SSH) method was a powerful technique and has been successful in some cases, it may retain some less effective genes which make screening difficult. In order to enrich the target sequences and improve screening efficiency, a secondary subtraction was implemented. In this study, three SSH cDNA libraries were constructed and 1500 clean EST sequences were obtained from forward SSH libraries for further analysis. Using real-time PCR, the expression of 16 representative candidate genes was examined and their differential expressions in NaCl treated *Wenfeng7* (salt-tolerant) and *Union* (salt-sensitive) was confirmed. Dot blot hybridization analysis showed that the retention of common EST sequences were significantly reduced or eliminated in the SSH3 library. GO (gene ontology) annotation and COG (cluster of orthologous group) functional classification revealed that development-related genes and defense mechanism genes were removed from the SSH3 library and salt-tolerance related EST sequences were further enriched, suggesting that the secondary subtraction is a simple and effective way to enrich response-specific genes and improve screening efficiency.

Keywords: Soybean, Suppression subtractive hybridization, Salinity stress.
Abbreviations: SSH- suppression subtractive hybridization; GO- gene ontology; COG- cluster of orthologous group; DDRT-PCR- differential display reverse transcription PCR; cDNA-AFLP- cDNA amplified fragment length polymorphism; MIPs- major intrinsic proteins; HSPs- heat shock proteins; *GmUBC2*- soybean ubiquitin-conjugating enzyme gene; ABC- ATP-binding cassette.

Introduction

Salinity is one of the major abiotic stresses that severely affect plant growth. Globally, salt-affected land, mostly resulting from natural salinity, accounts for more than 6% of the total land area and close to 20% of irrigated land (Li et al., 2010). Salinization becomes even worse with the land clearing and irrigation, both of which lead to a rise of water tables and enrichment of salts in the root zone. Soybean (*Glycine max* L. Merr.) is one of the most important crops in the world. As an important source for protein and vegetable oil for human and animal nutrition, the agronomical importance of soybean has been steadily increased. In addition, soybean serves as a valuable renewable agricultural source for industrial products, e.g. lubricating oil, printing ink and biodiesel (Umezawa et al., 2008). Soybean has been recognized as a salt-sensitive plant according to the relative growth ratio under high-salt conditions (Ayako et al., 2005). The increased soil salinization has necessitated the identification of soybean traits/genes that confer resistance to salinity. The traditional breeding strategies are limited by the complexity of stress tolerance traits, low genetic variance of yield components under stress conditions and the lack of efficient selection techniques. With the great progress of molecular biology, introducing some functional genes of interest to soybean by genetic engineering seems to be a shortcut to improve stress tolerance (Ji et al., 2006). Therefore, it's crucial to identify and clone salt-related genes which can potentially increase the crop salt tolerance. Several techniques have been employed in identifying the genes expressed in response to salt stress. These techniques include differential display reverse transcription PCR (DDRT-PCR) (Liu et al., 2007), cDNA-amplified fragment length polymorphism (cDNA-AFLP) (Jayaraman et al., 2008) and suppression subtractive hybridization (SSH) (Varshney et al., 2009). Compare to DDRT-PCR and cDNA-AFLP techniques, SSH is a powerful tool that enables researchers to compare two populations of mRNAs and obtain clones of genes that are expressed in one population but not in the other. By using two specific hybridizations and two PCR specific amplifications, SSH has the advantage of much lower false positive rate, higher degree of target sequence enrichment and higher consistency (Diatchenko et al., 1996). In particular, SSH technique has gained favorite in gene identification of eukaryotic organisms in recent years (Ji et al., 2006; Ouyang et al., 2007; Varshney et al., 2009; Jin et al., 2010). This method can develop libraries that include both scarce and abundant genes, potentially yielding a more diverse gene pool than other

techniques that isolate response-specific genes (Susan et al., 2008). To this day, SSH has been largely used to identify stress responsive genes. Attempts have been made to identify the salt stress regulated genes by SSH in Alfalfa (Jin et al., 2010), chickpea (Varshney et al., 2009), *Suaeda maritima* (Sahu and Shaw 2009), *Caragana intermedia* (Shi et al., 2010), rice (Sahi et al., 2003), tomato (Ouyang et al., 2007) and *Glycine soja* (Ji et al., 2006). However, data suggested that the SSH method also has some drawbacks. Research by Sahu and Shaw (2009) showed that 167 uingenes were grouped into 19 functional sub-categories, among which genes encoding proteins for cell rescue and systemic developmental processes accounted for 3.6% and 1.8%, respectively in the total unigene population, indicating that irrelevant target genes were obtained using the SSH approach. Research by Varshney et al. (2009) also showed that a high retention of genes related to developmental processes in the total unigene population of a chickpea SSH cDNA library. To obtain the response specific target genes, a large number of EST sequences need to be analyzed.

Since the SSH method may produce less specific genes in the subtracted library, which makes the screening difficult, a secondary suppression-subtracted cDNA library was constructed using SSH libraries from salt-tolerant (SSH1) and salt-sensitive (SSH2) soybean cultivars (Fig.1). The secondary PCR products of forward SSH1 were treated as tester and the secondary PCR products of forward SSH2 were treated as driver. After the tester and driver cDNAs were hybridized, and the hybrid sequences were removed, a third suppression-subtracted cDNA library (SSH3) was obtained. Dot blot hybridization, GO annotation and COG functional classification analysis of three libraries demonstrated that common genes in the SSH1 and SSH2 libraries were removed in the SSH3 library and the screening efficiency was improved. Therefore, this approach is a simple and effective way for improving the efficiency of screening.

Results

SSH library construction

The secondary PCR products from subtracted and unsubtracted cDNAs were approximately 600 bp in length (Fig. 2). Since subtraction efficiency is vital for successful construction of subtracted cDNA libraries, the subtraction efficiency was evaluated by amplifying a housekeeping gene in both unsubtracted and subtracted samples via PCR. Results (Fig. 3a, b) showed that 33 cycles were required to detect the housekeeping gene in the SSH1 and SSH2 subtracted cDNA libraries, whereas only 23 cycles were required for the SSH1 and SSH2 unsubtracted samples. The difference in the number of cycles for detection suggests that 5 cycle difference corresponds roughly to 20-fold cDNA enrichment. Therefore, the difference in the number of cycles required for equal amplification of the corresponding PCR product indicated high efficiency of subtraction in both SSH libraries. The PCR products from SSH were then cloned into the pMD18-T vector and transformed into DH5α cells. Sequencing of the inserts from randomly selected cDNA library clones revealed that approximately 95% of the transformants contained inserts and the insert size ranged between 200 bp and 1500 bp, with an average size of 600 bp. The average size of the inserts was similar to that of the secondary PCR products from the subtracted cDNAs (Fig. 2). Therefore, data indicated the successful construction of SSH cDNA libraries.

Generation of salt-responsive ESTs

In this study, cloned ESTs from only the forward SSH cDNA libraries were sequenced as they represent the up-regulated genes in response to the NaCl treatment and hence could be more relevant for the identification of salt tolerance genes. 600 clones in SSH1, 550 clones in SSH2 and 600 clones in SSH3 were randomly selected respectively. A total of 1750 clones were sequenced, and 1500 clean EST sequences (513 ESTs in forward SSH1, 453 ESTs in forward SSH2 and 534 ESTs in forward SSH3) were generated for further analysis after trimming off the vector sequence and removing of sequences shorter than 100 bp. All of the EST sequences are available at NCBI (GenBank ID: HO759947-HO761446).

Real-time PCR analysis of selected unigenes

Based on the GO annotation and the COG categorization, 16 unigenes (Table 2) related to salt-tolerance were selected for quantitative PCR analysis. Total RNAs were extracted from roots of both *Wenfeng7* and *Union* soybean cultivars after the first pair of leaves fully expanded. Results of real-time PCR (Fig. 4a) showed that the expression levels of HO761284, HO760009, HO760535, HO760167 and HO760162 were much higher in *Wenfeng7* after salt stress treatment. However, the expression of these genes decreased in *Union* after salt stress treatment. The expressions of HO760128 and HO761330 were up-regulated in both *Wenfeng7* and *Union* cultivars after treatment, although the expression of HO761330 in *Union* was much higher than that in *Wenfeng7*. The expression levels of HO761128, HO761311 and HO760071 were up-regulated in *Union* after salt stress treatment and down-regulated in *Wenfeng7*. The expression levels of HO761314, HO760884, HO76759948, HO761433, HO760044 and HO761228 were decreased in both *Wenfeng7* and *Union* after salt stress treatment (Fig. 4b).

Differential screening of the common ESTs

The ClustalW2 program was used to analyze the EST sequences in the SSH1 and SSH2 libraries. Analysis showed that 42 ESTs in SSH1 library and 47 ESTs in SSH2 library had similarities of more than 90%. The BLASTX search of the SSH1 and SSH2 cDNA libraries against the Uniprot database suggested that 21 EST sequences in the SSH1 library and 19 EST sequences in the SSH2 library may come from the same protein. All the identified common ESTs were analyzed by DNA gel blotting. After hybridization, results showed that most of the signals were from the SSH1 and SSH2 cDNA probes, while only few signals were seen when the SSH3 cDNA probe was used. This result suggested that the retention of common EST sequences in the SSH3 library were significantly reduced or eliminated (Fig. 5). Therefore, the SSH3 library probably enriched of salt-tolerance related genes and this secondary suppression subtraction method likely improved the efficiency of screening.

Sequence annotation of the cDNA libraries

The GO annotation classifies transcripts into three principal ontologies: molecular function, biological process and cellular component. These genes cover a broad range of the GO functional categories. However, due to the lack of gene product information, many transcripts could not be functionally

Table 1. List of real-time PCR primers for representative differentially expressed gene transcripts in the SSH libraries.

GenBank_Accn		Real-time primers
HO760009	F	5'-GTTCCGGTTCTGTGGGATAAG -3'
	R	5'-TCAGTGGGATACAAGTCCAAGGT -3'
HO760128	F	5'-ATCAGTTTGTGTGGTGTTGTGTTCT -3'
	R	5'-TTTGTAATCAGGGCAGGTGTTG -3'
HO760167	F	5'-TGCCATTGACCCCAAGAAG -3'
	R	5'-GCCCCACCCAACAAAATG -3'
HO760162	F	5'-GCCAGGATCAGTGCTTGAGA -3'
	R	5'-ATGAGGGCGGAGGCAAA -3'
HO760535	F	5'-TTGTAGGAGTTGTGGTGAATGTTGT -3'
	R	5'-ATTCTGGCTGGAGAAAACTGATG -3'
HO759948	F	5'-ATGGCGGAAGAGGGACAAG -3'
	R	5'-GCACCAAGAAGCAGTAAAATCCA -3'
HO760044	F	5'-ACTTTCAGAAGGCATTGCACAC -3'
	R	5'-AGCCCTTGAGACCACCACA -3'
HO760071	F	5'-CCGAGTTTAGGACTTGTTCGTGT -3'
	R	5'-AATGGATGGCTGGTAGGTTTGT -3'
HO760884	F	5'-AGGTGCCTACAGCAGCATCA -3'
	R	5'-CATAAACCACAACTCGCAGACC -3'
HO761433	F	5'-CCGCAGTAAAGTGGATAAGGAAA -3'
	R	5'-GCTCGCATTATGGTGAGTGTTG -3'
HO761128	F	5'-GGTATCCTTCAGGTCCGTGCT -3'
	R	5'-TTGACTTCACATCTAGCCCATGTT -3'
HO761311	F	5'-TCCATTCACTGCTGGTGCTAA -3'
	R	5'-TCAGATAGGGCATTGAGAGAGTTG -3'
HO761314	F	5'-TATTGGGGTTGCCTTGGACTT -3'
	R	5'-GGCTTGATGTGGACGATGTAGAG -3'
HO761330	F	5'-TGATTAGCATTCTCACCCAGGAA -3'
	R	5'-GGGAATCGGAAATAGAAGCGTAG -3'
HO761228	F	5'-TGCTGAAGATGGGGCTAATG -3'
	R	5'- AGCATTGTTCTGTTTGGTGAGTG-3'
HO761284	F	5'-CATAACTTTCTTCATTCCCCTAGCC -3'
	R	5'-AGAAGCGAAGGTGCGTGAG -3'

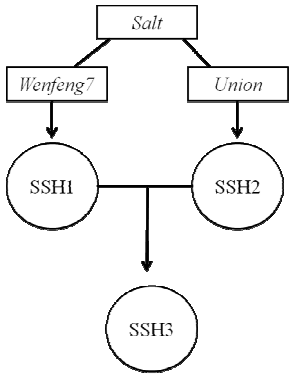


Fig 1. Scheme of secondary subtraction from two SSH cDNA libraries. The SSH1 and SSH2 cDNA libraries were constructed using the suppression subtractive hybridization (SSH) method from NaCl treated (0.9% W/V for 24 h) seedlings of the salt-tolerant soybean cultivar *Wenfeng7* and the salt-sensitive soybean cultivar *Union*, respectively. The secondary PCR products of forward SSH1 and forward SSH2 were then used as tester and driver respectively for cDNA hybridization. After the hybrid sequences were removed, the third suppression-subtracted cDNA library (SSH3) was obtained. The remaining unhybridized cDNAs represent genes that were expressed in the forward SSH1 library yet absent from the forward SSH2 library. Constructing a third cDNA library from salt-tolerant and salt-sensitive soybean varieties by SSH method can remove the common genes from SSH1 and SSH2 libraries and improve the screening efficiency.

categorized. Earlier studies suggested that these 'unknown' genes are likely the source of candidate salt-tolerance genes and further functional analysis would help to elucidate their specific roles in salt tolerance (Mehta et al., 2005). Similar to an earlier study in this regard (Ji et al., 2006), one gene product could be assigned to multiple parental categories. Thus, the total number of GO mappings in each of the three ontologies exceeded the number of unigenes analyzed. In SSH1, 92 unigenes were successfully classified to cellular components, 306 unigenes to have molecular functions, and 273 unigenes to be involved in biological processes. As shown in Fig. 6a, in the category of cellular components, majority of the genes are cell related (56, 12.96%); in the category of molecular function, majority of the unigenes participate in catalytic activities (127, 29.40%) or binding (122, 28.24%); and in the category of biological processes, the groups of physiological process (138, 31.94%) and cellular process (104, 24.07%) account for most of the annotated unigenes. In SSH3, 126, 303 and 297 unigenes were classified to the categories of cellular components, molecular function and biological processes, respectively. As shown in Fig. 6b,c, the proportion of unigenes in all the groups of the cellular components ontology was higher in the SSH3 library than in the SSH1 library, especially in the group of organelle and protein complex. In the molecular function ontology, the percentage of unigenes coding for the antioxidant activity, enzyme regulator activity and structural molecular activity proteins was higher in the SSH3 library than in the SSH1 library. In the biological processes ontology, the proportion of the unigenes in most groups except for development was higher in the SSH3 library than in the SSH1 library. In SSH3, no unigene was found in the group of development.

Functional classification of the cDNA libraries

This study also analyzed the homology of EST coded proteins to those found in the COG database. In SSH1 (SSH3) library, only 135 (130) ESTs were found to have significant protein homologs (E value < 1e-05) and were sorted into 20 (19) groups (excluding general function prediction only and function unknown) according to the functional categories of the database (Fig. 7a, b). Among these ESTs, those related to transport, metabolism, enzyme, cell wall and membrane had higher representations in SSH1 and SSH3 libraries. Compared with the SSH1 library, the SSH3 library has fewer genes involved in amino acid transport and metabolism, carbohydrate transport and metabolism, replication, recombination and repair (Fig. 7c), but more genes involved in posttranslational modification, protein turnover, chaperones, translation, ribosomal structure and biogenesis, energy production and conversion, secondary metabolites biosynthesis, transport and catabolism, lipid transport and metabolism, signal transduction mechanisms, inorganic ion transport and metabolism, cell wall/membrane/envelope biogenesis, chromatin structure and dynamics, coenzyme transport and metabolism, and cytoskeleton. Genes related to cell cycle control, cell division, chromosome partitioning and defense mechanisms were only found in the SSH1 library. Genes related to transcription, intracellular trafficking, secretion, and vesicular transport were only found in the SSH3 library.

Discussion

Plants are known to use more than one mechanism to adapt to unfavorable environmental conditions. For example, under salt conditions, soybean plants may enhance their salt stress tolerance through maintenance of ion homeostasis, osmotic adjustment, restoration of oxidative balance and other metabolic and structural adaptations (Phang et al., 2008). Plant roots are the primary sites for injury and other signal sensing during various water stresses, such as high salinity and drought. In many circumstances, it is the stress sensitivity of the root that limits the productivity of the entire plant (Varshney et al., 2009). Therefore, in this study root tissues were selected for generation of salt-responsive ESTs. Most ESTs identified in the SSH3 library have homologies to genes related to abiotic or biotic stress, or specifically to salt stress. Real-time PCR analysis (Fig. 4a, b) showed that the expression of these unigenes in *Wenfeng7* was significantly difference from that in *Union* after salt treatment. In our study, the uingene of HO760167 was found to have high homology to aquaporins. Real-time PCR showed that the expression of HO760167 in *Wenfeng7* was significantly higher than that in *Union* after salt treatment. Major intrinsic proteins (MIPs) consist of a large family of highly conserved membrane-spanning proteins that are mainly represented in plants by the aquaporins (Brien et al., 2002). In plants, aquaporins are abundant in the plasma membrane and in the vacuolar membrane (Johansson et al., 2000). Previous studies (Maurel, 2007) demonstrated that aquaporins are involved in multiple functions in plants, including nutrient acquisition, carbon fixation, cell signaling and stress responses. Aquaporins-like proteins transport water across cellular membranes and play vital roles in all organisms under salt stress (Kamal et al., 2010). Such genes could be used as references in molecular markers and transgenic technologies to generate salt-tolerant soybean varieties with stable yield. DNA gel blotting hybridization results showed that most of the hybridized signals were from the SSH1 or the SSH2 cDNA library probe. Only a few hybridization signals were observed when the SSH3 cDNA library probe was used, suggesting that the expression of common EST sequences in the SSH3 library has been significantly reduced (Fig. 5). Therefore, the SSH3 library is more representative of the true salt-tolerance related genes. To confirm that the third cDNA library is indeed an enrichment of salt-related genes and that the secondary SSH method is a more efficient screening approach, 600 clones were randomly selected from each of the SSH1 and SSH3 libraries for sequencing. 1,047 clean EST sequences (513 in forward SSH1 and 534 in forward SSH3) were obtained for further bioinformatics analysis. Based on the GO annotation, all the unigenes were classified to three ontologies: cellular components, molecular functions and biological processes. Several unigenes were classified to all three ontologies. Therefore, a gene product may be associated with or located in one or more cellular components; it may be active in one or more biological processes, during which it may perform one or more molecular functions. As shown in Fig. 6c, the SSH3 library has high expression of unigenes in all subcategories in the cellular components ontology than the SSH1 library. For majority of the groups in the molecular function ontology, the percentage of the unigenes in the SSH3 library was similar to the SSH1 library. Genes involved in antioxidant activity, enzyme regulator activity and structural molecular activity have higher representations in SSH3 library than the SSH1 library. The SSH3 library also had a higher abundance of all groups (except for development) in the biological processes ontology, but the SSH3 library had no unigenes involved in development. The gene encoding eyes absent protein (HO760098) was found in the development subcategory of the biological processes ontology in SSH1. The eyes absent (eya) gene in animals

Table2. Catalogue of selected unigenes function.

Acc no.	Length (bp)	Similar to	Identity (%)
HO760044	363	Annexin	80
HO760071	339	KUP10; potassium ion transmembrane transporter	86
HO759948	312	General substrate transporter	87
HO760535	420	Intracellular chloride channel	81
HO760162	235	SLT1 (sodium- and lithium-tolerant 1)	84
HO760128	210	Probable aquaporin TIP1-1	86
HO761284	871	Disease resistance-responsive family protein	49
HO761314	920	Universal stress protein	72
HO760884	555	Vacuolar protein sorting	94
HO761128	436	Calcium ion binding protein, putative	55
HO761433	358	26S proteasome AAA-ATPase subunit RPT4a	94
HO761311	737	Ca^{2+}-ATPase	88
HO761228	595	Pectinesterase inhibitor, putative	49
HO761330	565	Zinc finger, C_3HC_4 type family protein	45
HO760167	188	Serine/threonine kinase	87
HO760009	358	Transmembrane protein	78

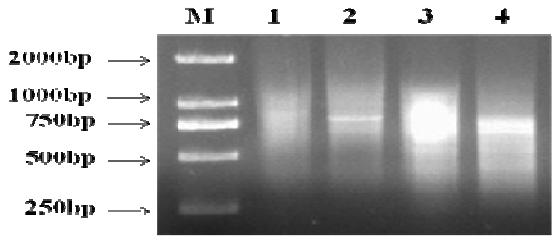


Fig 2. PCR-select cDNA subtraction analysis. The subtracted and unsubtracted cDNA samples were amplified using nested PCR primers. The secondary PCR products from subtracted and unsubtracted cDNAs were approximately 600 bp in length, but the length of the PCR products from subtracted cDNA was smaller than unsubtracted cDNA. Lane 1: SSH1 secondary PCR products from unsubtracted cDNA. Lane 2: SSH1 secondary PCR products from subtracted cDNA. Lane 3: SSH2 secondary PCR products from unsubtracted cDNA. Lane 4: SSH2 secondary PCR products from subtracted cDNA. Lane M: DNA size marker.

functions at multiple stages in the development of organs and its role in the genetic network that regulates eye development in *Drosophila* has been extensively analyzed recently (Tootle et al., 2003). The homolog of eyes absent gene was also identified in rice (Takeda et al., 1999), corn (Alexandrov et al., 2009) and *Arabidopsis* (Lin et al., 1999). Studies showed that the rice *OSEya1* gene was expressed in the embryo, shoot apex, and caryopsis of rice and it might regulate the morphogenesis of lateral organs as a subunit of a transcription factor (Takeda et al., 1999). Genes that encode peroxidase, auxin response factor, late embryogenesis abundant protein, stress and pathogenesis-related protein, serine/threonine kinase and cationic peroxidase were found in the subcategory of "response to stimulus" in the biological processes ontology. Our results showed that 3.9% of the unigenes in the SSH1 library were "response to stimulus" genes, while it was 9.4% in the SSH3 library and most of these unigenes were stress response genes. By constructing the SSH3 library, the non-specific development-related genes were removed, and salt-tolerance related EST sequences were further enriched. Therefore, a secondary subtractive hybridization can probably enrich salt-tolerance genes and improve the screening efficiency.

This study also analyzed the homology of EST coded proteins to those found in COG database. Molecular chaperones are key components of cellular homeostasis. These proteins are important for the stabilization of other proteins and membranes, and can assist in protein refolding under stress conditions (Wang et al., 2004). Unigenes encoding heat shock proteins (HSPs) were found in both the SSH1 and the SSH3 libraries. The induction of heat shock protein is an important mechanism for plant acclimation to stressful conditions via protein folding, assembly, translocation, and degradation (Mayer and Bukau, 2005). Previous studies showed that the over-expression of HSP in *Arabidopsis thaliana* enhanced plant sensitivity to salt and drought stresses (Song et al., 2009). Genes encoding the ubiquitin-conjugation enzyme and the 26S proteasome were also found in SSH1 and SSH3 libraries. Previous studies have shown that ubiquitination plays important roles in plant abiotic stress responses and is involved in the regulation of ion homeostasis, osmolyte synthesis, and oxidative stress responses (Devoto et al., 2003). Zhou et al. (2010) demonstrated that *GmUBC2* (soybean ubiquitin-conjugating enzyme gene) was expressed in all tissues of soybean and was up-regulated by drought or salt stress. Additionally, *Arabidopsis* plants over-expressing *GmUBC2* were more tolerant to salinity and drought compared with the control plants (Zhou et al., 2010). In our study, such unigenes in the category of post-translational modification, protein turnover and chaperone functions were much more abundant in the SSH3 library than in the SSH1 library. In the defense mechanisms category, found that most of the unigenes belong to the white-brown-complex ABC transporter family. ATP-binding cassette (ABC) proteins constitute a large, diverse and ubiquitous superfamily (Verrier et al., 2008). In recent years, various biochemical and physiological functions of ABC proteins in plants have been reported. ABC proteins are firmly established as key players in the physiology and development of plants. ABC genes are associated with the defense mechanisms against biotic and abiotic stresses, and other basic functions necessary for the maintenance of plant life (Yazaki et al., 2009). In the SSH1 library, three unigenes were found in this category, while there was none in the SSH3 library, suggesting that the suppression

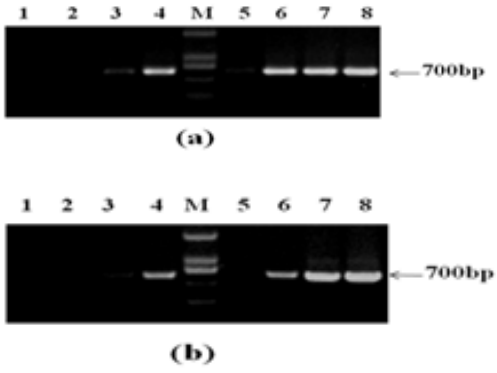


Fig 3. Evaluation of subtraction efficiency by PCR. The subtracted and unsubtracted cDNA pools were amplified using primers for the constitutively expressed housekeeping gene. PCR was performed with the subtracted (Lanes 1–4) or unsubtracted (Lanes 5–8) secondary PCR products using the housekeeping gene primers. 33 cycles were required to detect the housekeeping gene in the SSH1 and SSH2 subtracted cDNA libraries, whereas only 23 cycles were required for the SSH1 and SSH2 unsubtracted samples. Lanes 1 and 5: 18 cycles; Lanes 2 and 6: 23 cycles; Lanes 3 and 7: 28 cycles; Lanes 4 and 8: 33 cycles. (a): the SSH1 cDNA library; (b): the SSH2 cDNA library.

subtractive hybridization using secondary PCR products of forward SSH1 (tester) and forward SSH2 (driver) has indeed removed some genes involved in defense mechanisms. Therefore, our data suggested that secondary SSH likely enriched the salt-tolerance related genes and improved the screening efficiency.

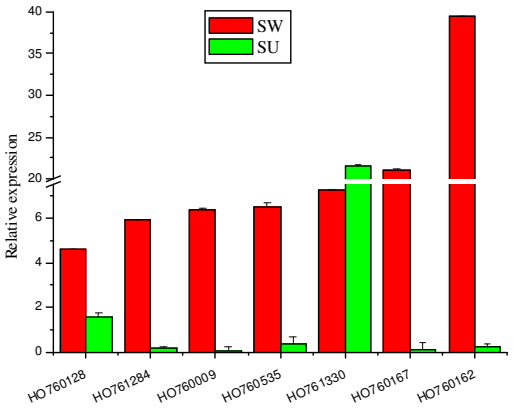
Materials and methods

Plant materials and treatments

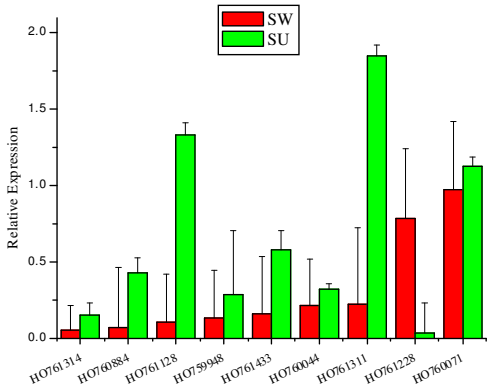
Two soybean cultivars (*Glycine max* L. Merr.), *Wenfeng7* (salt-tolerant) and *Union* (salt-sensitive), were grown with the temperature ranging from 25 to 29 °C, the humidity ranging from 60 to 80% and a short-day photoperiod (12h light/12h dark) in the greenhouse. The seeds were germinated in vermiculite and irrigated with deionized water. After the first pair of leaves fully expanded, the seedlings were transferred to half strength Hoagland's solution and treated with 0.9% (W/V) NaCl. After a stress period of 24 hours, the root tissues from treated and untreated plants of both cultivars were collected for total RNA extraction and cDNA library construction. The total RNA was extracted with Trizol reagent (Invitrogen, USA) and purified with RNeasy plant mini kit (Qiagen, Germany). The absorption ratio of $OD_{260/280}$ was used to verify the quality of RNA. The mRNA was purified with the Oligotex™ mRNA Mini Kit (Qiagen, Germany).

Construction of the Basic SSH cDNA libraries

The forward and reverse SSH cDNA libraries were prepared using the PCR select™ - cDNA Subtraction kit (Clontech, USA). Briefly, double stranded cDNAs prepared from the control and the NaCl treated samples were digested separately by *Rsa*I for 1.5 h and then ligated to adapters 1 and 2R provided in the kit. For the first subtraction, each of the two cDNA tester batches were denatured at 98℃ for 1.5 min and then hybridized at 68℃ for 8 h. Subsequently, the two tester batches were combined and an excess of freshly denatured



(A)



(B)

Fig 4. Quantification of differently expressed genes by Real-time PCR. (a) and (b): Real-time PCR was conducted for 16 uingenes to examine the effect of NaCl treatment on their expressions. SW: salt treated Wenfeng7, SU: salt treated Union. The house keeping gene actin was used as the internal control. Total RNAs were isolated from roots of untreated or NaCl (0.9% W/V, 24h) treated plants. Each bar represents the number of fold increase in the transcript level of a gene in the plant upon NaCl treatment as compare with untreated control. The expression level of each uingene in control soybean was defined as 1.0. The values presented are mean± standard deviation (sd) of three independent Q-PCR analyses.

control cDNA was added into the mixture for a second overnight hybridization at 68℃. Two rounds of hybridization and PCR amplification were processed to normalize and enrich differentially expressed cDNAs. Products of the second hybridization were amplified with two rounds of selective PCR. The SSH1 and SSH2 cDNA libraries were constructed for *Wenfeng7* and *Union* respectively. Secondary PCR products of forward SSH1 and SSH2 were digested separately overnight with *Rsa*I provided in the kit to remove adapters. Subsequently, the digested PCR product of forward SSH1 was used as the tester and the digested PCR product of forward SSH2 was used as the driver. Tester and driver cDNAs were then hybridized and the hybrid sequences were removed to obtain the third suppression-subtracted cDNA library (SSH3). The subtractive

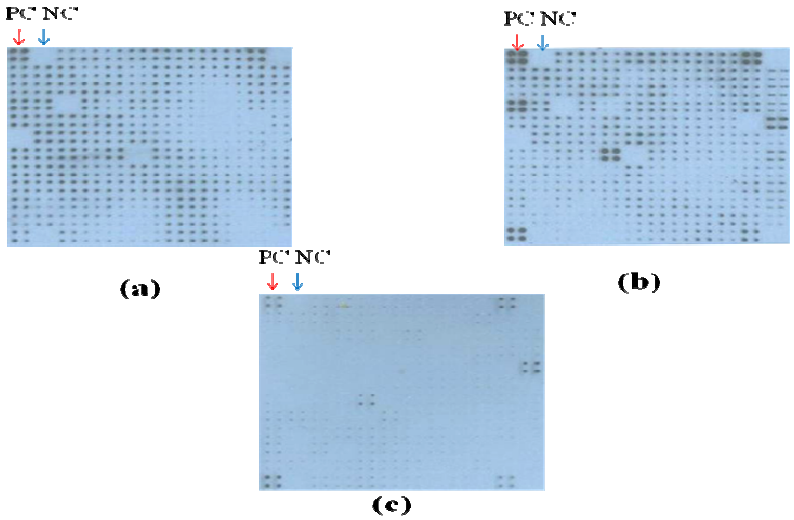


Fig 5. Differential screening of the ESTs. The membranes were blotted with the common EST sequences from SSH1 and SSH2 cDNA libraries. PC: positive control; NC: negative control. (a): the blotted membrane was screened using the probe made from the SSH1 forward subtracted cDNA library. (b): the blotted membrane was screened using the probe made from the SSH2 forward subtracted cDNA library. (c): the blotted membrane was screened using the probe made from the SSH3 forward subtracted cDNA library.

products were purified and inserted into the pMD18-T Vector (Takara, China) and transformed into *Escherichia coli* DH5α cells. The positive clones were then selected and cultured in LB medium containing 100 μg/mL ampicillin in 96-well plates at 37°C for 3-4h. Glycerol stocks were prepared and cells were kept at -80°C until subsequent use.

Amplification of cDNA inserts

The SSH products were amplified by PCR with nested primers provided in the PCR select^{TM} - cDNA Subtraction kit. The reaction mixture contained 7.4 μL sterilized double distilled H_2O (ddH_2O), 10 μL 2× NI-Taq PCR Mix, 0.8 μL of each nested adapter primer 1 (TCGAGCGGCCGCCCGGGCAGGT) and primer 2R (AGCGTGGTCGCGGCCGAGGT) (10 μM each), and 1 μL bacterial culture. PCR was performed as follows: 94°C for 5 min; 30 cycles of 95°C for 30 s, 65°C for 30 s and 72°C for 90 s; and 72°C for 10 min. PCR products were examined on a 1% agarose gel to confirm the amplification quality and quantity.

Bioinformatics sequence analysis

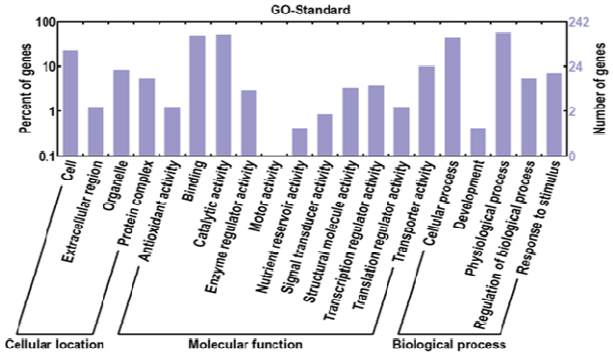
The positive clones identified by PCR were sequenced at Sangon Biotech Company Ltd (Shanghai, China). The vector sequence was then removed from the obtained sequences using the crossmatch software Sequencher (Sequencher, USA). The trimming process, which included the removal of low-quality sequences, poly (A) tails and ribosomal RNA was conducted with minor modifications. Those ESTs of approximately 100 bp or longer were considered for further analysis. The phrap software (Company, USA) was used to perform subsequent steps of clustering, sequence assembly, alignment analysis and consensus partitioning to generate contigs and singletons. The contigs and singletons were termed as unigenes. All of the unigene sequences were searched against the NCBI database with BLASTN and BLASTX (http://www.ncbi.nlm.nih.gov/BLAST/). Functional classification of the ESTs was performed with the GO (http://www.ebi.ac.uk/GO/) and COG (http://www.ncbi.nlm.nih.gov/COG/) tools. The GO annotation and COG classification were analyzed by Beijing Genomics Institute (BGI, China).

Real-time PCR analysis

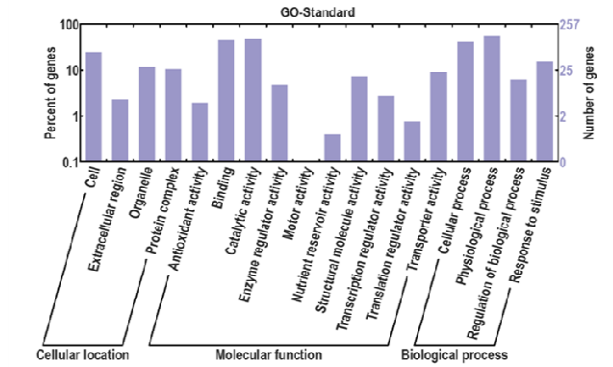
A quantitative determination of the level of differentially expressed transcripts was carried out by Real time RT-PCR. Total RNA was extracted as described above, and cDNA was synthesized using the M-MLV RTase cDNA Synthesis Kit (Takara, China) according to the manufacturer's instructions. Primers of the 16 candidate genes were designed using Primer 3 software and synthesized by Takara. The primer sequences are listed in Table 1. The housekeeping gene was used as the internal control. The SYBR Green real-time PCR assay was carried out in a total volume of 25 μL, containing 12.5 μL of the SYBR Premix Ex Taq^{TM}(2×) (Takara, China), 0.5 μL of each specific primer, 0.5 μL of the ROX Reference Dye (50×), 2.0 μL template cDNA and 9.0 μL of dH_2O. The amplification program consisted of 1 cycle at 95℃ for 10 min, 40 cycles of 95℃ for 5 s and 60℃ for 31s, and a dissociation step. Amplifications were carried out in Fast Reaction Tubes (8Tubes/Strip) in a 7300 Real Time PCR System (Applied Biosystems, USA). The obtained Ct values from biological replicas and two technical replicas of each sample were averaged to obtain the mean, and gene expression of each sample was quantified using the relative quantification $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001) by normalizing to the internal control.

Differential screening of common clones in the cDNA libraries

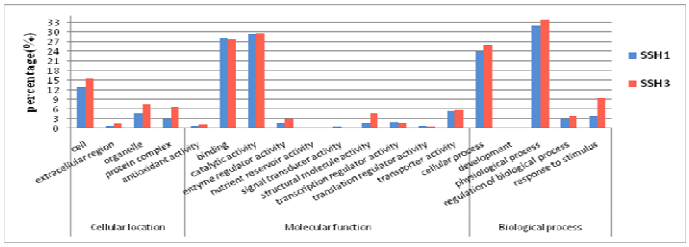
After sequence alignment and analysis, the common ESTs in SSH1 and SSH2 cDNA libraries were obtained. To confirm that these ESTs are not present in SSH3 library, DNA gel dot-blotting was performed. Briefly, cDNA inserts of the common ESTs were amplified by PCR. The amplified products were then spotted on Hybond-N^+ nylon membrane (GE, USA) using a Qpix II robot system (Genetix, UK). Sterilized water



(a)



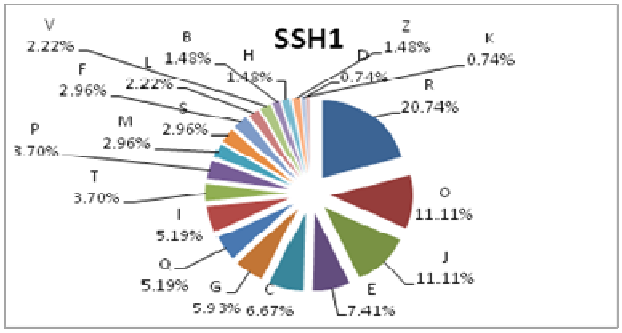
(b)



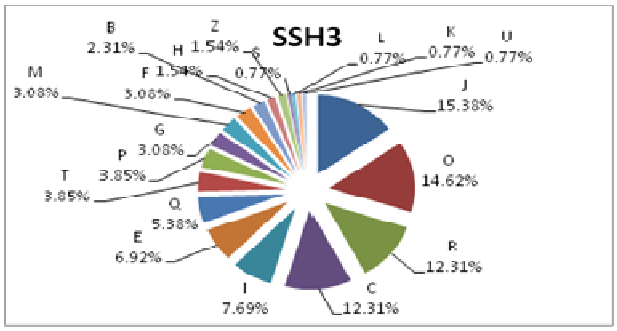
(c)

Fig 6. GO classification of unigenes in the SSH cDNA library
Functional assignment of the uingenes was performed for all sequences with significant hits in the GO database. The bar graph shows the distribution of unigenes in three principal GO categories: cellular location, molecular function and biological process. (a): GO annotation of the SSH1 cDNA library. (b): GO annotation of the SSH3 cDNA library. (c): Comparison of the SSH1 library and the SSH3 library.

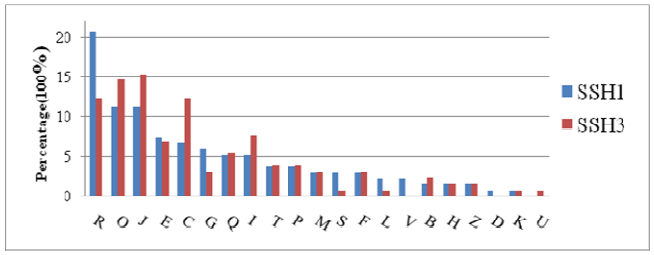
was used as the negative control, and a housekeeping gene was used as the positive control. Four spots were made for each common EST and control, and each spot contained 2 µL of the PCR product. After air drying, the membranes were denatured with 0.3 N NaOH for 5 min, neutralized with 0.5 M Tris-HCl (pH 7.5) for 3 min, rinsed with sterilized water for 3 min, and then baked for 2 h at 80°C. The secondary PCR products of the three forward SSH cDNA libraries were purified by QIA-quick PCR purification kit and labeled separately with the DIG High Primer DNA Labeling and Detection Starter Kit II (Roche, Switzerland). The protocol for dot-blot hybridization was



(a)



(b)



(c)

Fig 7. Functional categorization of unigenes in the SSH cDNA libraries.All of the unigenes were clustered into different functional categories after BLASTing against the COG database. (a): SSH1 cDNA library. (b): SSH3 cDNA library. (c): comparison of the SSH1 library with the SSH3 library. The unigenes were grouped into 19 functional categories: B, chromatin structure and dynamics; C, energy production and conversion; D, cell cycle control and mitosis; E, amino acid metabolism and transport; F, nucleotide metabolism and transport; G, carbohydrate metabolism and transport; H, coenzyme metabolism; I, lipid metabolism; J, translation; K, transcription; L, replication and repair; M, cell wall/membrane/envelope biogenesis; O, post-translational modification, protein turnover, chaperone functions; P, Inorganic ion transport and metabolism; Q, secondary metabolites biosynthesis, transport and catabolism; T, signal transduction; U, intracellular trafficking and secretion; Z, cytoskeleton; V, Defense mechanisms; W, Extra cellular structure; R, general functional prediction only (typically, prediction of biochemical activity); S, function unknown.

followed as Sahu and Shaw (2009). After hybridization, the membranes were exposed to a DIG detection system for 5-20 min at 15-25°C.

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