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Screening and analysis of soda saline-alkali stress induced up-regulated genes in sugar sorghum

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Abstract

Soil salinization severely constrains the growth of crops, which ultimately leads to reduced yields. Because *Sorghum dochna* (common name sugar sorghum) has the advantageous properties of excellent salt stress resistance, high biomass, and tremendous flexibility for utilization as food, livestock feed, and industrial products, this species holds great potential to be further developed as a primary alternative crop. To elucidate the molecular mechanism that governs sugar sorghum's adaptation to high salinity environments, we constructed a suppression subtractive hybridization (SSH) cDNA library from sugar sorghum transcripts that contains the soda saline-alkali induced up-regulated genes from the resistant variety M-81E. The SSH cDNA library was screened by using the colony hybridization method, and the ESTs obtained were sequenced and analyzed. A total of 200 EST clones were identified, representing 127 unigenes (6 contigs and 121 singlets). A Blast analysis showed that 48 ESTs (46.6%) have annotated functions in GenBank, 55 ESTs (53.4%) have unknown functions (or encode hypothetical proteins), and 24 ESTs (18.9%) have no blast hits. The majority of the hypothetical ESTs from the cDNA library displayed very high sequence similarity with their homologs found through GenBank. A clustering analysis of the ESTs with known functions indicated that a wide variety of genes were induced during the salt stress treatment. These genes were found to function in photosynthesis, material and energy metabolism (carbohydrates, lipids, amino acids, co-enzymes, ions, etc.), synthesis or maintenance of constituents of the cell wall and cell membrane, signal transduction, transcriptional regulation, and as water channels. This indicates that sugar sorghum tolerance to soda saline-alkali stress results from the coordinated functions of many genes.

Keywords: sugar sorghum, SSH, soda saline-alkali stress, up-regulated genes, EST

Introduction

Soil salinization has been a challenging ecological problem for agriculture globally, and this process has become one of the main factors limiting crop yield (Wei et al, 2003). China is one of the major countries affected by salinization, with an estimated land area of approximately 3.46×107 Ha affected by high soil salinity, including nearly 7.6×10^6 Ha that constitute 1/5 of China's arable land area. The salinized area in the west Songnen plain is estimated to be 3.0×10^6 Ha, which is one of the three largest continuous areas of soda saline-alkali soils in the world. This type of inland soda saline-alkali soil contains Na_2CO_3 and $NaHCO_3$ as the major salt constituents, which carries the properties of high pH value and poor physical and chemical conditions (Wang et al, 2008). Plants grown on such soils are stressed not only by excess Na^+ but also by their low water potential and high pH value. Therefore, only limited varieties of crops can adapt and survive in such soils. Thus, it would be of practical benefit both ecologically and economically to search for crops that are capable of growing in soda saline-alkali soils. Sugar sorghum is a prime candidate for this purpose because the crop possesses many excellent characteristics. Such characteristics include being a C4 plant that produces a relatively high amount of biomass; its potential as a source of food, feedstock, and alternative fuel; and its tolerance to stresses induced by drought, flooding, salinity, nutrient deficiency, and elevated temperature.

The phenotype of salt tolerance has been shown to be controlled by multiple genes, including not only genes acting in stress signaling and transcriptional regulation but also genes functioning directly in the processes of protection, defense, and stress tolerance. Therefore, to deeply understand the mechanism underlying the tolerance to salt stress, systematic and integrative investigations at the genomic level are needed. Differential expression of genes in a spatio-temporal manner is the molecular basis of plant development, differentiation, senescence, and resistance to adverse stimuli. The determination of differentially expressed genes and subsequent identification of their functions has long been proven to be

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an effective way to study functional genomics.

Suppression subtractive hybridization (SSH) is an approach that has been widely adopted among researchers due to its low rate of false-positive results, high sensitivity, high efficiency, and simplicity (Ji et al, 2002; Lévesque et al, 2003). Developed by Diatchenko (Diatchenko et al, 1996), SSH is based on the use of a cDNA library to screen for unknown genes that show differential expression levels between multiple conditions. By using SSH, cDNAs of differentially expressed genes can be quickly obtained, and even very low abundance transcripts can be enriched by over 1000-fold, thus allowing marginally expressed gene sequences to be detected. To date, many differentially expressed genes that are associated with abiotic stress resistance have been obtained by screening SSH libraries (e.g., genes related to resistance to salt stress (Ouyang et al, 2007; Basyuni et al, 2011), cold stress (Nguyen et al, 2009), ozone (Sävenstrand et al, 2000), high-temperature (Tian et al, 2009), heavy metal contamination (Srivastava et al, 2007), osmotic pressure (Bae et al, 2010), and flooding (Zheng et al, 2004). The cloning of genes involved in these traits greatly expands the resource pool of stress resistance genes.

Currently, understanding of the mechanism underlying sugar sorghum resistance to saline-alkali stress is still restricted to physiological or biochemical reactions, and deep understanding of the molecular mechanism has not yet been reported. In this study, we constructed an SSH library consisting of sugar sorghum cDNAs that were up-regulated in response to soda saline-alkali stress. The EST sequences obtained by SSH screening were also analyzed by using bioinformatics tools. We believe our work will facilitate further mechanistic studies of sugar sorghum adaptation to salt stress. Furthermore, the cloning of the saline-alkali tolerance genes identified in this study may guide the isolation of other corresponding genes in the Gramineae family (especially cereals) and may also provide genetic resources for molecular breeding to improve plant saline-alkali resistance.

Materials and Methods

Plant culture and stress treatment

This study utilized the experimental sugar sorghum variety M-81E provided by the Institute of Hebei Agriculture & Forest Science, which has excellent tolerance to saline-alkali stress proven by our previous studies (Dai et al, 2012a; 2012b). Large, plump seeds were surface sterilized by soaking in 0.1% $HgCl_2$ for 5 min, then flushed thoroughly with deionized water, and finally soaked in water for 12 h. The swollen seeds were germinated on Whatman filter papers. The germinated seeds were then transplanted to plastic flower containers (22 cm in height and 20.5 cm in diameter) that were each filled with 7.5 kg of sterilized silica sand. Each container was planted with 30 seedlings, and six containers were assigned to each treatment. The seedlings were grown under natural sunlight, protected from falling rain-water, and watered every other day with Hoagland nutrient broth.

The saline-alkali stress solution was prepared by using Hoagland broth as solvent, and $NaHCO_3$ and Na_2CO_3 in a molar ratio of 5:1 were dissolved together to a concentration of 100 mmol l^{-1} (pH 9.33, salinity 5.92%). When the seedlings reached the stage of four nascent leaves, the control group continued to be watered with Hoagland broth, while the stress-treated group was thereafter watered with the saline stress solution every other day. Beginning from the time of the initial stress treatment, the whole seedlings from both the control and stress-treated groups were sampled at 0 h, 4 h, 12 h, 24 h, 36 h, 48 h, and 72 h, and then flash frozen in liquid nitrogen for later use.

Isolation of mRNA

Total RNA was first extracted with the TRIzol kit (Invitrogen Life Technologies, Carlsbad, CA, USA), and the preparations from different time points were pooled in equal amounts. Purification of mRNA was then performed from the pool by using PolyA Tract mRNA Isolation Systems (Promega Corp, Madison, WI, USA) according to the manufacturer's protocol.

Construction and screening of the SSH cDNA library

The library was constructed by using the PCR SelectTM cDNA Subtraction Kit (Clontech, Takara Bio Inc., Shiga, Japan) according to the manual. mRNA from the stress-treated group was designated as the tester, while mRNA from the control group served as the driver. Double-stranded cDNA was synthesized and digested with Rsa I. The tester cDNA was then ligated with adapters at both ends, and subjected to two rounds of hybridization and two rounds of selective PCR amplification in order to enrich the differentially expressed genes. The PCR product was purified, cloned into a pMD18-T vector (Takara Bio Inc), and transformed into E. coli DH5α cells. The white colonies were selected and further confirmed to be positive by nested PCR using primers NPP1 and NPP2R.

Screening of the SSH cDNA library was carried out by using colony hybridization. The probe was labeled with a DIG kit (Shenzhen Yinuojin Biotech, Shenzhen, China). Both forward and reverse probes were synthesized. The PCR product from the first round of SSH was diluted 50-fold and used as a template, and NPP1 and NPP2R were used as primers. For the forward probe, mRNA from the stress-treated group was used as the tester, and the control group was used as the driver. For the reverse probe, the control group mRNA was used as the tester, and the stress-treated group was used as the driver. Following membrane hybridization, washing, and visualization of the signal, candidates for differentially expressed genes were screened. After comparison

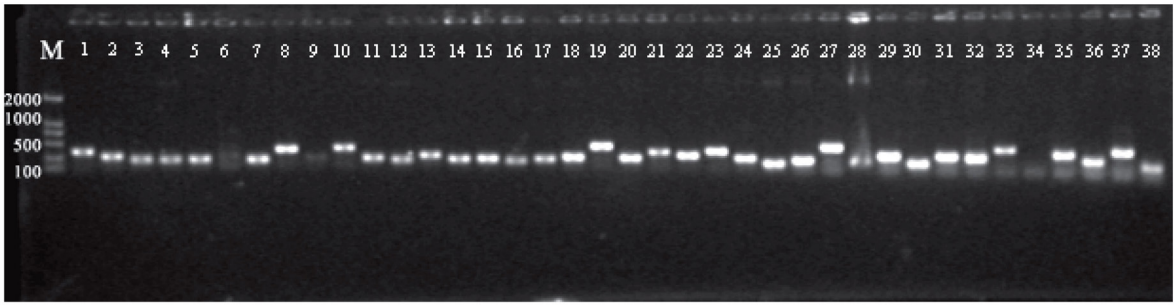

Figure 1 - Confirmative PCR amplification of the inserts from the library. M, DNA ladder; Lane 1~38, randomly selected individual clones from the SSH library.

of the signals on the forward versus reverse membranes, the forward membrane was selected for further analysis, followed by sequencing of the original corresponding clone.

Analysis of differentially expressed ESTs

The following software packages were used to extract the unique non-redundant EST sequences: Vecscreen, DNAstar, and DNAMAN. The representative sequences were subjected to BLASTn and BLASTx searches in GenBank in order to obtain the functional information from the annotations of the hits. Further bioinformatics analysis was performed using the software GO (Gene Ontology; http://geneontology.org/; Consortium GO, 2015) and COGs (Cluster of Orthologous Groups of proteins; http://www.ncbi.nlm.nih.gov/COG; Tatusov et al, 2001).

Quality assessment of the SSH cDNA library

Eight randomly chosen EST sequences from the subtracted cDNA library were used to assess the quality of the library. The whole seedling RNA samples isolated from the 12 h time-point after treatment for both stressed and control plants were used for the RT-PCR analysis. A sorghum actin gene was used as an internal control for the RT-PCR analysis. All primers for the candidate genes and actin were designed using the PRIMER5 software (Lalitha, 2000). The relative levels of gene transcripts in sorghum from the control and stressed groups were monitored by real-time PCR using a CFX ConnectTM apparatus (BIO-RAD, Hercules, CA, USA). PCR conditions consisted of DNA denaturation (3 min, 95°C) followed by 40 amplification cycles (10 s at 95°C and 20 s at 55°C). A melting curve of PCR products (65 to 95°C) was performed to ensure the detection of a single specific product.

Results

The size distribution of EST inserts from the SSH cDNA library

Thirty-eight clones were randomly selected from the cDNA library (Figure 1). PCR was performed using nested primers and the bacteria cells as the template. The cDNA inserts had a size distribution of 0.2-0.5 kb. There were four clones that failed to show the insert PCR band, so the insertion rate of the library was calculated to be 89.5% (34/38), which satisfied the general requirement for the construction of an SSH library.

Screening of differentially expressed clones by colony hybridization

Two nitrocellulose membranes were blotted by stamping on LB plates that were spread with equal amounts of bacterial culture and co-incubated overnight. The transferred bacteria on the membrane were alkaline-lysed, and the released DNA was heat-immobilized on the membrane.

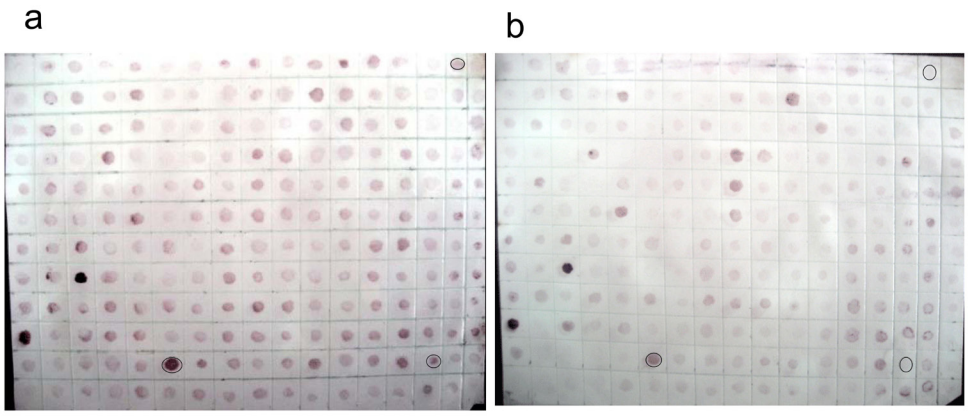

Figure 2 - Colony hybridization. A) Hybridization with forward probe. B) hybridization with reverse probe.

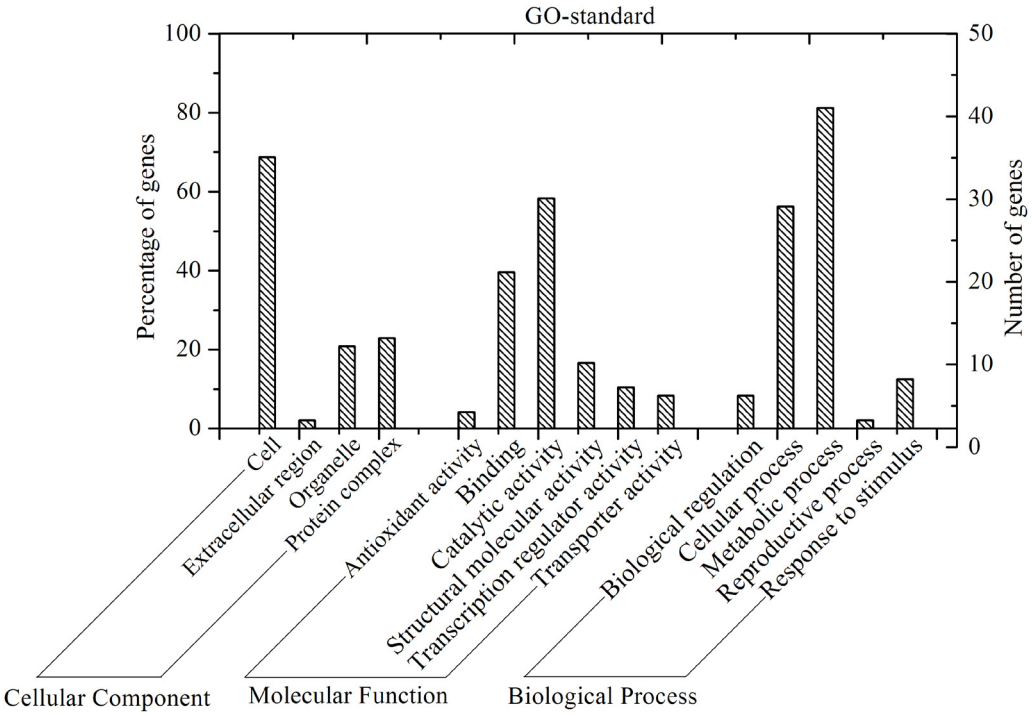

Figure 3 - Classification of EST functions according to Gene Ontology method.

The colony hybridization probes were synthesized by using the DIG DNA labeling kit. After digestion with the enzyme Rsa I to remove the adapters on both ends, the probes were further purified by agarose electrophoresis. The final concentration of the purified forward probe was 50 ng μl^{-1}, and the reverse probe concentration was 35 ng μl^{-1}.

Equal amounts of forward and reverse probes were used to separately hybridize two nitrocellulose membranes (Figure 2). After aligning the spots on each hybridized membrane and referring to the original size of the colony, we harvested and sequenced those colonies that produced stronger forward hybridization versus reverse hybridization signals. Presumably, these colonies represent the genes that are up-regulated after soda saline-alkali stress.

Functional analysis of the differentially expressed ESTs

Out of the 200 colonies that were sequenced, 127 unigenes were ultimately identified, which excluded the ESTs that were shorter than 100 bp (14) or redundant (22). These unigenes belonged to 6 contigs and 121 singlets.

The unigenes were then used as an input to search for their homologs in GenBank based on the BLASTn and BLASTx algorithms, and the functions of the unigenes were deduced from the annotations of their hits in GenBank. Among the 127 unigenes, 103 had homologous hits, and 24 (18.9%) did not have any known homologs. Among the homologs, only 48 (46.6%) had functional annotations; the remainder were either unknown sequences or encoded hypothetical proteins. Furthermore, most of the hypothetical homologs had very high sequence similarities with the ESTs from sorghum cDNA expressed after adverse stimuli, including water stress, drought, nitrogen shortage, salt stress, heat shock, acid/alkaline treatment, ion stress, etc. Interestingly, some homologs had sequence similarity with the sorghum ESTs expressed following GA3 or brassinolide treatment.

The homologs that were obtained are listed in Table 1. They are either saline-alkali induced and have known functions, or are associated purely with saline-alkali stress resistance but with unknown functions. Among the homologs with known functional annotations, 23 (47.9%) participate in basic metabolism and energy dynamics, 14 (29.2%) are involved in signaling pathways or transcriptional regulation, 4 (8.3%) function in protein synthesis and cellular defense, and 3 (6.25%) have roles in transmembrane molecular transport.

Categorization of the functions of the differentially expressed ESTs

To understand the functions of the genes involved in sugar sorghum resistance to soda saline-alkali stress, all the EST with known functions were categorized by the GO system and grouped by cellular component, molecular function, and biological process (Figure 3). In the cellular component group, most of the ESTs are cell-related (33 ESTs), followed by protein complex (11 ESTs) and organelle (10 ESTs). In the molecular function group, the majority are involved in catalytic activity (28 ESTs) and binding (19 ESTs), followed by structural molecular activ-

ity (8 ESTs), transcription regulator activity (5 ESTs), transporter activity (4 ESTs), and antioxidant activity (2 ESTs). In the biological process group, the majority are associated with metabolic process (39 ESTs) and cellular process (27 ESTs), followed by response to stimulus (6 ESTs) and biological regulation (4 ESTs).

When analyzed by COGs, the functions of the ESTs can be assigned as information storage and processing (I), cellular process and signaling (C), and metabolism (M; Figure 4). Most of the sugar sorghum genes that were induced by saline-alkali stress are components of metabolism (43.75%), including metabolism of carbohydrates (8.3%), lipids (2.1%), amino acids (8.3%), coenzymes (4.2%), and inorganic ions (4.2%), as well as energy dynamics (16.7%). The second largest cluster of ESTs occurred in the cellular process and signaling category (22.9%), including signal transduction (10.4%) and post-translational modifications (6.25%). The ESTs that fell into the information storage and processing group account for 18.75%, with 6.25% being involved in transcription and 12.5% involved in translation. The last group of ESTs represented those genes for which very little information is known about their functions (14.6%).

Evaluation of the SSH library quality

To assess the quality of the subtractive hybridized library, we randomly picked eight non-redundant ESTs from the library and compared their relative transcript abundance between the saline-alkali stress-treated group and the control group (Figure 5). Among the eight genes randomly selected after 12 h of stress, six of them had increased expression in the stress group compared to the control. Two of these six genes displayed a more than hundred-fold increase, suggesting that they are induced and up-regulated by saline-alkali stress. The other two genes did not display any variation in expression between the two conditions (data not shown). These results demonstrate that SSH is an efficient way to create a high-quality library of sugar sorghum genes that are differentially expressed under saline-alkali stress.

Discussion

We successfully constructed a high-quality sugar sorghum SSH library and screened for the genes induced by soda saline-alkali stress using colony hybridization. The lengths of the ESTs in the library are relatively short and do not cover the entire length of most cDNA sequences. Nevertheless, SSH is still an effective system for isolating the full-length sequences of differentially expressed genes if this strategy is used in combination with other methods (e.g., rapid-amplification of cDNA ends [RACE], genomic walking, or in silico cloning). Without a doubt, SSH technology will have a significant role in the cloning of new genes, the investigation of gene expression regulation, and understanding the molecular mechanisms of plant growth and development.

Many metabolic pathways of plants are known to be affected by plant resistance to adverse stimuli. Consistent with this assumption, most of the genes discovered in this study are related to a metabolic pathway. Some examples of such genes products include Nudix hydrolytic enzymes, E3 ubiquitin ligase, dihydroneopterin aldolase, ATP phosphoribosyltransferase, cytochrome C oxidase, hydroxyproline-rich glycoproteins, galactosyltransferase, carbohydrate esterase, cell division protease, acetyl-CoA synthase, choline/ethanolamine kinase, NADH dehydrogenase, arabinogalactan endo-1,4-β-galactosidase, indole-3-glycerol phosphate synthase, 1,4,5-phosphoinositide phosphatase, carboxypeptidase, photosynthetic PSII oxygen-evolving complex, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), etc.

Currently, many studies have shown that the enzymes and proteins highlighted in this study are involved in plant adaptation to abiotic stresses. Hy-

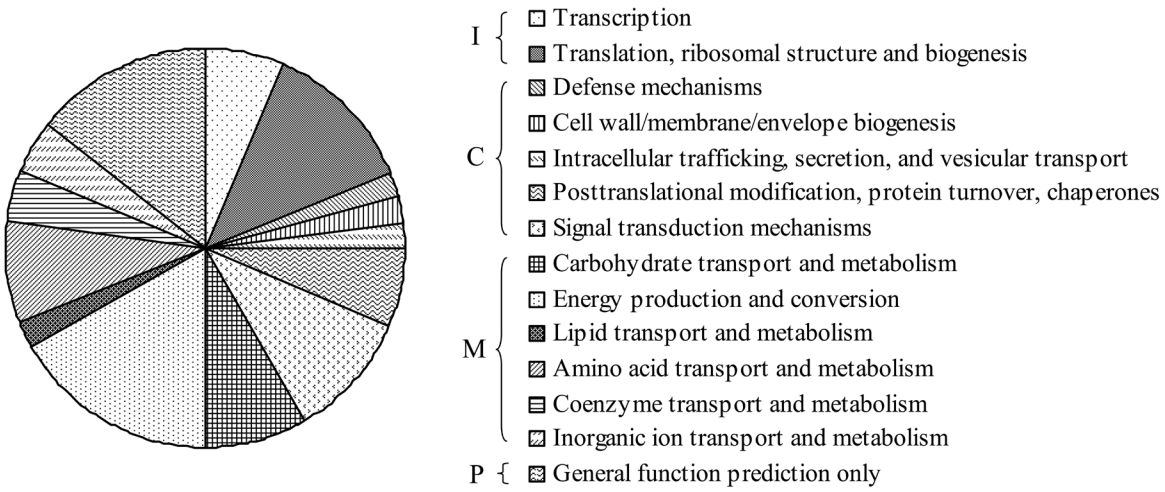

Figure 4 - Classification of EST functions according to COGs method. I, Information storage and processing; C, Cellular process and signaling; M, Metabolism; P, Unknown function.

droxyproline-rich glycoprotein (HRGP), for example, is a unique structural component of the plant cell wall. HRGP has roles in plant defense and resistance to pathogens and abiotic stress (Roby et al, 1985). The HRGP content increases when the plant is subjected to pathogens, inducers, or ethylene treatment as well as mechanical damage. The enzyme 1,4,5-phosphoinositide phosphatase converts its substrate to inositol, a class of metabolites that play important roles in nearly every biological process. Inositol and its derivatives can regulate plant growth, control membrane formation, enhance resistance to osmotic stress, and participate in signaling pathways (Loewus and Murthy, 2000). We discovered some ESTs coding for enzymes that catalyze inositol metabolism. Cytochrome C oxidase, NADH dehydrogenase, and ubiquinone are electron carriers in the process of oxidative phosphorylation and the concurrent electron transport chain. Coupled with electron transport is the formation of a trans-mitochondrial-membrane electrochemical potential gradient, which drives the synthesis of ATP in the mitochondria. Three of the twenty-two ESTs identified as being related to metabolism in this study are directly related to energy metabolism, suggesting that it takes a substantial amount of energy for sugar sorghum to resist soda saline-alkali stress and the intermediates of distinct biochemical reactions may be co-opted for participation in other metabolic processes. The Rubisco enzyme catalyzes both carbon fixation and carbon oxidation during photorespiration. Cucumber leaves increase their expression of Rubisco following low temperature induction (Xu et al, 2009). Rubisco was also found to be highly enriched in leaves after salt stress in a proteomics study (Huo et al, 2004). Considering these two observations, Rubisco is implicated in plant resistance to adverse stimuli. In this study, many material- and energy metabolism-related genes of sugar sorghum were induced by soda saline-alkali stress. The products of these inducible genes extensively participate in many physiological and biochemical processes, including photosynthetic carbon fixation and energy metabolism, biosynthesis of the cell wall and cell membrane, metabolism of lipids, amino acids, carbohydrates, and hormones, and the synthesis/degradation of proteins.

A certain amount of plant genes function in signal transduction and transcriptional regulation in response to environmental changes, especially adverse conditions. The signal transduction-related ESTs discovered in this study include F-box proteins, zinc-finger proteins, WRKY transcription factors, DAG protein, phytochrome A, gibberellin regulators, protein kinases, RAB GTPase, translation initiation factor eIF-5A, polyA binding protein, and leucine-rich repeat serine/threonine-like protein kinases.

The expression of F-box proteins can be affected by adverse surrounding signals, thereby enabling them to intervene in the plant response to stress. The expression of F-box genes can be induced by cold in *Citrus reticulata* Blanco (Zhang et al, 2005) and by $NaHCO_3$ alkaline stress in Tamatrix androssowii (Yang et al, 2004). In this study, we found that an E3 ubiquitin ligase and an F-box protein were both overexpressed after soda saline-alkali stress, indicating that ubiquitin-mediated protein degradation may alleviate the harm caused by saline stress in sugar sorghum.

The first characterized eukaryotic salt stress-tolerant zinc-finger protein was STZ, which was cloned from Arabidopsis by screening a cDNA library. STZ has the typical plant C2H2 zinc-finger structure. Lippuner et al (2000) demonstrated that NaCl treatment increased the expression of STZ and STZ-like proteins in the Arabidopsis root. STZ may enhance the salt tolerance of plants by regulating the expression of downstream salt tolerance genes. Yinghui Guo et al (2010) isolated another zinc-finger protein, CCCH-

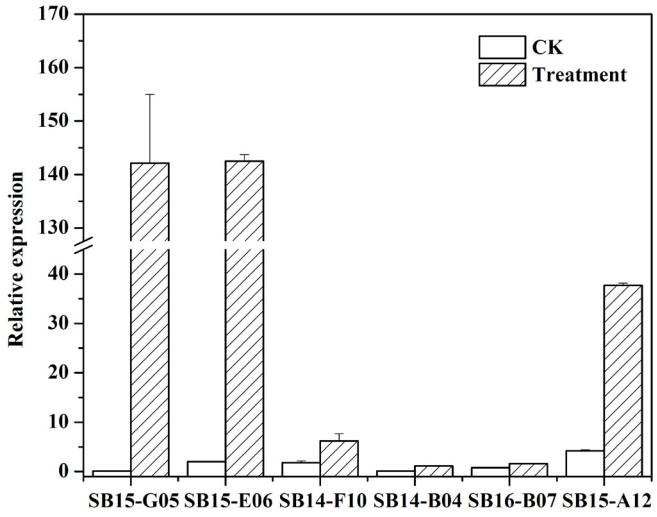

Figure 5 - Quality assessment of SSH library using RT-PCR.

type GhZFP1, from a cDNA library of cotton seedlings that were under salt stress. Over-expression of GhZFP1 in transgenic tobacco significantly improved the resistance to stress caused by salt and pathogens. Jiang and Deyholos (2006) analyzed the transcription profile of Arabidopsis roots following NaCl stress induction and found that 18 out of 35 WRKY transcription factors were up-regulated by at least 1.5-fold. Qiu and Yu (2009) demonstrated that the rice WRKY45 expression was markedly induced in response to the stress-related hormone abscisic acid (ABA) and abiotic stresses (salt, drought, cold, and osmosis), and the transgenic Arabidopsis plants that over-expressed OsWRKY45 gained increased tolerance to salt and drought. All of these studies suggest that WRKY transcription factors play important roles in stress resistance and they can enhance plant adaptability to a complex and frequently changing environment.

By far, the signaling pathways that are found to be most associated with plant resistance to salt stress include SOS (Zhu, 2000), the Ca^{2+}/calmodulin pathway (Shi et al, 2003), and the protein kinase/protein phosphatase pathway (Zhang and Klessig, 2001), etc. This study found that diacylglycerol (DAG), together with inositol triphosphate (IP3), may participate in the Ca^{2+}/calmodulin pathway. Serine/threonine protein kinases (S/TPK) are a major class of receptor-like kinases that phosphorylate other proteins, and phosphorylation is a major mechanism of cellular signal transduction. This study detected a protein kinase and leucine-rich repeat serine/threonine-like protein kinase that may participate in the protein kinase/phosphatase signaling pathway.

Several defense-related genes were also identified in this study, including catalase (CAT), defensin-like proteins, and glutathione reductase (GR). Reactive oxygen species (ROS) can cause lipid peroxidation, thereby damaging cellular components, such as enzymes, DNA, and membranes. The plant anti-oxidation enzymes, including superoxide dismutase (SOD), peroxidase (POD), CAT, and GR, are capable of clearing the ROS, and the overall activity of plant anti-oxidation enzymes is closely related to stress resistance. Our research also found that the ESTs of CAT and GR had increased expression after saline stress, indicating that the elevated expression level of the anti-oxidation enzymes was helpful in improving sorghum performance in terms of salt stress resistance.

Synthesis of ATP in mitochondria requires two important proteins, ATP synthase and a phosphate carrier. The synthesis process is influenced by the availability of Pi and ADP that are imported into the mitochondria. The mitochondrial concentration of Pi is determined by the activity of the phosphate carrier. Hamel et al (2004) showed that the activity of mitochondrial phosphate carrier PIC2 was high during heat stress conditions, and their postulate was that PIC2 plays important roles under some stress conditions. Here we found that genes encoding the ATP synthase F1 subunit and phosphate carrier had increased expression, suggesting that sugar sorghum needs more energy during the process of adaption to saline-alkali stress.

Most aquaporins (AQPs) also function in the plant response to environmental stress stimuli, as many studies have found. By adjusting the activity of AQPs, the plant is able to resist the stress from drought, cold, high salt, osmotic pressure, and other abiotic stresses (Li et al, 2015; Šurbanovski et al, 2013; Vera-Estrella et al, 2004; Aroca et al, 2007). Under these stress conditions, the expression of PIP aquaporins is decreased at both the transcriptional and protein levels, and the activity of the channel goes down or even disappears (Secchi et al, 2007; Jang et al, 2004; Boursiac et al, 2005). Closure of AQPs generally believed to restrict the loss of water from the plant, thereby maintaining equilibrium and enhancing the plant tolerance to stress stimuli (Alexandersson et al, 2005; Aroca et al, 2011).

In this study, the screened ESTs were first classified based on their functions according to the GO method. The results indicated that following soda saline-alkali stress the expression pattern in sugar sorghum changed dramatically with respect to cellular components, molecular functions, and biological processes. Most ESTs in the molecular function group are involved in binding and catalyzing. Most ESTs in the biological process group are related to metabolic and cellular processes, and most ESTs in the cellular components group belong to cell- or organelle-related genes. However, when the COGs classification method was used to analyze the data, most of the saline-alkali stress induced ESTs were classified as belonging to metabolism, followed by cellular processes and signaling, and then by information storage and processing.

Acknowledgements

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