Genomics 98 (2011) 47–55

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

# Transcriptome analysis reveals salt-stress-regulated biological processes and key pathways in roots of cotton (*Gossypium hirsutum* L.)

Dongxia Yao [a,1], Xueyan Zhang [b,1], Xinhua Zhao [b,1], Chuanliang Liu [b], Chunchao Wang [a], Zhenghai Zhang [a], Chaojun Zhang [b], Qiang Wei [a], Qianhua Wang [b], Hong Yan [a], Fuguang Li [b,*], Zhen Su [a,**]

[a] State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China Agricultural University, Beijing 100193, PR China
[b] Key Laboratory of Cotton Genetic Improvement, Ministry of Agriculture, Cotton Research Institute, Chinese Academy of Agricultural Sciences, Anyang, Henan 455000, PR China

ARTICLE INFO

*Article history:*
Received 25 February 2011
Accepted 15 April 2011
Available online 30 April 2011

*Keywords:*
TM-1 upland cotton
Salt stress
Root
Transcriptome map
Signal transduction pathways
Hormone

ABSTRACT

High salinity is one of the main factors limiting cotton growth and productivity. The genes that regulate salt stress in TM-1 upland cotton were monitored using microarray and real-time PCR (RT-PCR) with samples taken from roots. Microarray analysis showed that 1503 probe sets were up-regulated and 1490 probe sets were down-regulated in plants exposed for 3 h to 100 mM NaCl, and RT-PCR analysis validated 42 relevant/related genes. The distribution of enriched gene ontology terms showed such important processes as the response to water stress and pathways of hormone metabolism and signal transduction were induced by the NaCl treatment. Some key regulatory gene families involved in abiotic and biotic sources of stress such as WRKY, ERF, and JAZ were differentially expressed. Our transcriptome analysis might provide some useful insights into salt-mediated signal transduction pathways in cotton and offer a number of candidate genes as potential markers of tolerance to salt stress.



## 1. Introduction

Cotton (*Gossypium hirsutum* L.) produces an essential commodity, namely fibers for use in textiles, and cotton seed is a source of oil. Although cotton is moderately tolerant to salt, with a salinity threshold of 7.7 dS $m^{-1}$[1,2], growth especially of seedlings and productivity are severely reduced under high salinity [3,4]. The high salinity is one of the major factors that limit photosynthesis and respiration, flowering, boll and fiber quality, and ion uptake in cotton, resulting in significant losses in yield [4,5]. Breeders have sought to make cotton more tolerant to salt through various methods including traditional plant breeding and biotechnological approaches such as creating transgenic cotton in which vacuolar H1-PPase from *Thellungiella halophila* and *AtNHX1*, a vacuolar $Na^+/H^+$ antiporter, is over-expressed [6]. Arbuscular mycorrhizae (AM) that colonize cotton roots have also been deployed for the purpose, a measure that has proved particularly useful in growing cotton in saline–alkali soils [7,8].

Generally, plant response to high salinity is governed mainly by pathways involved in ionic and osmotic homeostasis signaling, detoxification, and growth regulation [9–11]. Although sensors that detect salt stress are yet to be identified, the mechanisms of salt tolerance have been studied extensively using several different approaches including genetic analysis, especially in Arabidopsis. A pathway, namely *SOS* (salt overly sensitive), that controls ionic homeostasis was identified, in which ion transporters such as *SOS1* are regulated by the calcium-responsive protein kinase *SOS3–SOS2* complex [12]. *SOS3* is a myristoylated calcium-binding protein and senses changes in cytosolic calcium during salt stress [13–15]. In addition, the $K^+/Na^+$ homeostasis under salt stress is also critical to stress tolerance, and *SKC1* was identified as a sodium transporter that regulates $K^+/Na^+$ homeostasis under salt stress [16].

Plant response to osmotic stress also affects ABA metabolic and signal transduction pathways [12,17,18], and salt tolerance and drought resistance are significantly and positively correlated, indicating that similar mechanisms of osmotic regulation are involved in plant response to both salinity and drought [19]. Proline and trehalose are two important osmolytes. The P5CS gene, coding for pyrroline 5-carboxylate synthetase, and the TPS gene, coding for trehalose 6-phosphate-synthase, were recently isolated and characterized from *Gossypium arboreum* "Shixiya I," a cultivar known for its drought tolerance, and were reported to be over-expressed in *G.hirsutum*[20].

Osmotic homeostasis was reported to be regulated also by some MAP kinases (MAPK) cascades [2,21,22], which transduce signals into adaptive and programmed responses. Plant MAPK cascades were identified in the regulation of stress and hormonal responses, innate immunity, and developmental programs. Several MAP kinases were reported to be activated by hyperosmotic stress.

When exposed to salt stress, many genes are induced that either protect the plant from salt stress directly or regulate the expression of

* Corresponding author. Fax: +86 372 2562256.
** Corresponding author. Fax: +86 10 62731380.
*E-mail addresses:* aylifug@hotmail.com (F. Li), zhensu@cau.edu.cn (Z. Su).
[1] These authors contributed equally to this work.

0888-7543/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygeno.2011.04.007

other target genes. Plant transcriptome mapping has become a popular method of studying the possible mechanisms of response to salt stress and of elucidating the pathways involved in transducing the signals that convey salt stress. Recently, microarray-based analyses of the response to NaCl of many plant species have been published in more than 50 reports from the NCBI GEO data sets available at <www.ncbi.nlm.nih.gov>. These studies have analyzed cell cultures, whole plants, or specific plant organs such as roots and leaves. Several microarray platforms are available for development- and stress-related transcriptome studies of cotton [23–30]; for example, the Affymetrix cotton genome array was used for studying transcription proofing in cotton roots and leaves responding to stress in the form of water-logging [29].

When plants are exposed to stress in the form of excess salt, roots are the first site at which salinity is detected. In cotton seedlings exposed to salt stress (administered as NaCl), the length and fresh weight of roots were reduced to a greater extent than those of hypocotyls [31]. However, little is known about possible signal transduction pathways related to salt stress in cotton roots. Global transcriptome analysis is also limited. Therefore, we used GeneChip® Cotton Genome Array representing 21,854 cotton transcripts to monitor the expression profiles of genes related to the plant's response, taking root samples, and validated our results with real-time RT-PCR. The biological processes involved in the response of cotton roots to salt stress are being studied using several approaches including gene ontology (GO) enrichment analysis, MapMan, and network analysis for the up- or down-regulated genes. This paper attempts an overview of the transcription map of cotton roots under salt stress, which may yield some useful insights into salt-mediated signal transduction pathways in plant roots and offer a number of candidate genes as potential markers of tolerance to salt stress.

## 2. Materials and methods

### 2.1. Plant material and growth conditions

Cotton (*G. hirsutum* L. "TM-1") seeds were kept immersed in water for 1 day at 30 °C and then placed for germination on sterilized soil in plates maintained under the following conditions: 28 °C/25 °C as day and night temperatures, 12 h of light alternating with 12 h of darkness, and relative humidity of 80%. After 3–4 days, properly germinated seeds were transferred to black plastic tanks filled with a nutrient solution (the composition is given in Supplemental Table 1) and allowed to grow until they had produced 3–6 leaves. Seedlings showing normal growth were then placed into tanks filled with a 100 mM solution of NaCl in water; some of the seedlings were transferred to tanks filled with plain water to serve as controls. After exposing the seedlings to salt stress for varying durations (1 h, 3 h, 6 h, and 24 h), roots of the upland cotton seedlings were harvested. Roots of the control plants were also harvested at the same times.

### 2.2. Measurement of electric conductivity

To measure the relative electric conductivity after exposure to stress, 30 disks punched from the first 3 true leaves from each plant were put into a tube containing 10 ml distilled water and shaken for 12 h at 180 rpm and the initial electric conductivity of the solution (S1) was measured. The solution was then heated to 100 °C for 10 min, cooled to room temperature, and the final electric conductivity (S2) was measured. The relative electric conductivity (REC) was calculated as follows: $\mathrm{REC}\ (\%) = S1/S2 \times 100$.

### 2.3. Isolation of RNA and real-time PCR

All the root samples were homogenized in liquid nitrogen before isolation of RNA. Total RNA was isolated using a modified CTAB method and purified using Qiagen RNeasy columns (Qiagen, Hilden, Germany).

Reverse transcription was performed using an M-MLV kit (Invitrogen). The samples, 10 µl each containing 2 µg of total RNA and 20 pmol of random hexamers (Invitrogen), were maintained at 70 °C for 10 min to denature the RNA and then chilled on ice for 2 min. The reaction buffer and M-MLV enzyme (20 µl of the mixture contained 500 µM dNTPs, 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 5 mM dithiothreitol, 200 U of M-MLV, and 20 pmol random hexamers) were added to the chilled samples and the samples were maintained at 37 °C for 1 h. The cDNA samples were diluted to 8 ng/µl for RT-PCR analysis.

For RT-PCR, assays were performed in triplicate on 1 µl of each cDNA dilution using the SYBR Green Master Mix (Applied Biosystems; PN 4309155) with an ABI 7500 sequence detection system as prescribed in the manufacturer's protocol (Applied Biosystems). The gene-specific primers were designed using PRIMER3 <http://frodo.wi.mit.edu/primer3/input.htm>. The amplification of 18S rRNA was used as an internal control to normalize all data (forward primer, 5′-CGGCTACCACATCCAAGGAA-3′; reverse primer, 5′-TGTCACTACCTCCCCGTGTCA-3′). The gene-specific primers are listed in Supplemental Table 2. The relative quantification method (ΔΔCT) was used for quantitative evaluation of the variation between replicates.

### 2.4. Affymetrix GeneChip experiment and microarray data analysis

Three sets of biological replicates were collected independently and a total of six cotton chips were analyzed. For Affymetrix GeneChip (Affymetrix, Santa Clara, CA, USA) analysis, 8 µg total RNA from each cotton root sample was used for making biotin-labeled cRNA targets. All the processes for cDNA and cRNA synthesis, cRNA fragmentation, hybridization, washing and staining, and scanning were conducted as stipulated in the GeneChip standard protocol (Eukaryotic Target Preparation; Affymetrix). Poly-A RNA Control Kit and the One-Cycle cDNA Synthesis Kit were used in this experiment as described at <www.affymetrix.com/support/technical/manuals.affx>. The signal intensity of each probe set on the GeneChip was read using Affymetrix GCOS software and the TGT (target mean value) was scaled as 500 for each chip. Student's *t*-test analysis and $\log_2$-transformed signal ratio of each probe set were carried out by Partek Genomics Suite (version 6.3). The *q*-value of each probe set was calculated by SAM (significance analysis of microarrays).

To get an updated annotation of the probe set in Affymetrix cotton genome array, we mapped the probe set to the locus ID of Arabidopsis TAIR9 version <www.arabidopsis.org> and the transcription factor ID in the Plant Transcription Factor Database (PlantTFDB, <http://planttfdb.cbi.pku.edu.cn>) by BLAST (basic local alignment and search tool). Within the 23,973 designed probe sets in the Affymetrix cotton genome array, 19,442 were mapped to TAIR9 locus ID and 1270 to the transcription factor ID. Based on the results of the BLAST analysis, we created a MapMan mapping file for the Affymetrix cotton genome array.

Gene ontology (GO) analysis was performed for functional categorization of differentially expressed genes using EasyGO software [32] and the *p*-values corrected by applying the false discovery rate (FDR) correction to control falsely rejected hypotheses during the GO analysis. We selected the Chi-square test as our statistical test, and an FDR corrected *p*-value of $\leq 0.05$, as the cutoff value.

MapMan <http://gabi.rzpd.de/projects/MapMan> was used for key regulation group analysis. The pathway analysis for jasmonic acid, salicylic acid, and ethylene signaling were conducted using Pathway Studio <www.ariadnegenomics.com/products/pathway-studio> and Science Signaling <http://stke.sciencemag.org/index.dtl>, and the corresponding MapMan pathways were created through the mapping files.