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Transcriptome analysis reveals that distinct metabolic pathways operate in salt-tolerant and salt-sensitive upland cotton varieties subjected to salinity stress

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ABSTRACT

Salinity stress is one of the most devastating abiotic stresses in crop plants. As a moderately salt-tolerant crop, upland cotton (*Gossypium hirsutum* L.) is a major cash crop in saline areas and a suitable model for salt stress tolerance research. In this study, we compared the transcriptome changes between the salt-tolerant upland cotton cultivar Zhong 07 and salt-sensitive cultivar Zhong G5 in response to NaCl treatments. Transcriptional regulation, signal transduction and secondary metabolism in two varieties showed significant differences, all of which might be related to mechanisms underlying salt stress tolerance. The transcriptional profiles presented here provide a foundation for deciphering the mechanism underlying salt tolerance. Based on our findings, we proposed several candidate genes that might be used to improve salt tolerance in upland cotton.

1. Introduction

Salinity is a major abiotic stress limiting crop productivity and quality worldwide [1–3]. Understanding the molecular mechanisms underlying the plant's response to salinity stress will facilitate efforts to develop crop plants with enhanced resistance to high salinity [2–6]. Upland cotton (*Gossypium hirsutum* L.) is an important natural fiber and oil crop and provides edible protein for livestock feed. Although upland cotton is a moderately salt-tolerant crop, with a salinity threshold of 7.7 dS m^{-1} [7], the yield is drastically reduced with increased salinity. The tolerance to salinity varies considerably among cotton varieties, and identifying the molecular mechanisms associated with increased salt tolerance may pave the way for developing lines with improved salt tolerance [6,8].

Plants have evolved various biochemical and physiological mechanisms to withstand salt stress. Generally, mechanisms underlying salinity tolerance in plants involve maintaining homeostasis (including ionic and osmotic homeostasis), detoxification, and growth regulation [9], and these mechanisms have been reported at the molecular level, cellular level, and whole plant level [10]. Previous studies identified a possible mechanism involved in the salt-stress response in cotton [6,11–14] and *Arabidopsis* [15–17]. When exposed to salt stress, plants maintain ionic homeostasis (i.e., a low concentration of Na^+ and a high concentration of K^+) in the cytosol [18]. In *Arabidopsis*, the SOS (salt overly sensitive) pathway regulates the response to salinity stress, and genes involved in this pathway include *SOS1*, *SOS2*, *SOS3*, *NHX1*, and *HKT1* [16,17,19–21]. Salt stress caused increases in intracellular Ca^{2+}, which is sensed by the calcium sensor SOS3. A protein kinase complex, Ca^{2+}-activated SOS2–SOS3, phosphorylates SOS1 (a Na^+/H^+ antiporter), which pumps out excess Na^+ to reestablish cellular ionic homeostasis [16,22]. In addition, the SOS2–SOS3 complex may also influence other transporters, such as NHX1 [9,15] and AtHKT1 [20], to restore ionic homeostasis.

Salinity stress not only causes ionic and osmotic imbalances in plants, but also results in the accumulation of ROS (reactive

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oxygen species), which leads to oxidative stress. Many plants respond to osmotic stress by accumulating osmoprotectants, and some plants contain antioxidant mechanisms that eliminate ROS [23]. The ROS-scavenging systems in plants consist of diverse antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR), and some non-enzyme antioxidants, such as reduced glutathione (GSH) and ascorbic acid (AsA) [5,24]. Furthermore, some growth regulation-related genes are induced or repressed to adapt to changing environments. All of these mechanisms, together with some that remain to be identified, promote plant growth under stress conditions. Numerous genes are activated or modulated when plants are exposed to salt stress such as hormone. Identifying salt-tolerant genes is an important part of salt-tolerant crops breeding through genetic engineering. However, only a few salt stress-inducible genes have been documented in cotton, such as *GhNHX1* [25], *GhDREB* [26], *GhERF2–GhERF6* [27–29], *GhNAC1–GhNAC13* [30,31], *GhMT3a* [32], *GhMPK2* [33], *GhMKK5* [34], *GhZFP1* [35], *GhCIPK6* [36], *GhWRKY17* [37], *GhWRKY39-1* [38], and *GbRLK* [39]. With the success of the cotton whole-genome sequencing [40,41], and the widespread use of microarrays and next-generation sequencing, some salt-related regulatory factors and genes have been identified in cotton at genome-wide level [6,42–44]. For example, some members of transcription factor families such as WRKY, NAC, MYB, and C2H2 have been reported to play an important role in salt tolerance of cotton [6,42–44]. Yan et al. [37] found that *GhWRKY17* was induced after exposure to drought, salt, H_2O_2, and ABA. The phytohormone, such as auxin, cytokinin (CK), gibberellin (GA), abscisic acid (ABA), ethylene, brassinosteroid (BR), jasmonate (JA), and salicylic acid (SA), have been documented to be involved in the adaption abiotic stress, including salinity stress. Some members in the hormone signaling pathway have been identified to participate in the response to salt stress. It has been reported that overexpressing of *G. hirsutum* sucrose non-fermenting 1-related protein kinase 2 (*GhSnRK2*), which acts as a positive regulator in ABA signaling pathway, exhibited increased tolerance to ABA and salt stresses [45]. However, the molecular basis of cotton tolerance to salt stress remains to be discovered.

Moreover, there is an increasing emphasis on studying the temporal changes in gene expression in response to environmental cues. For example, microarrays were used to study transcriptome differences in the roots of soybean plants exposed to $NaHCO_3$ stress for 3, 6, 12, and 24 h [46]. Some molecular processes, such as signal transduction, secondary metabolism, and regulation of transcription, were found to be induced at earlier time points, and the genes involved in these processes decreased after 12 h of stress treatment [46]. Therefore, temporal dynamic changes should be considered when evaluating a plant's response to a stress factor. Since salt stress is initially perceived by the root, the root is the ideal target for studying the mechanisms underlying plant salinity stress tolerance and adaptation [3,47,48]. However, few studies have compared the transcriptional profiles of two cotton varieties with different levels of salt resistance subjected to salt stress [6].

In this study, we sought to generate a transcription map of two cotton varieties with distinct performances in response to salt stress. We examined the expression profiles of the salt-tolerant variety, Zhong 07, and salt-sensitive variety, Zhong G5, using an Affymetrix® Cotton Genome Array representing 21,854 cotton transcripts. We identified genes that were differentially expressed in the two varieties after 3, 12, and 48 h of exposure to salt stress and compared our findings with published data, including GO (gene ontology) enrichment, and regulatory network data. Furthermore, we identified candidate genes involved in salt tolerance that may be used in molecular breeding programs to improve salt tolerance in upland cotton.

2. Materials and methods

2.1. Plant material, cultivation, and salt stress treatment

Two upland cotton (*G. hirsutum*) genotypes: salt-tolerant Zhong 07 and salt-sensitive Zhong G5 (provided by Cotton Research Institute, Chinese Academy of Agricultural Sciences) were employed in this study. It has been reported that the salinity-resistance index (SRI) of Zhong 07 was 58.37% and it was seen as a salt-tolerant variety [49]. Seeds of the two varieties were immersed in water for 8 h at 37 °C, and then germinated in sand at 29 °C in darkness. After 3–4 days, similar seedlings were transferred to black plastic tanks filled with aerated 1/2 Hoagland nutrient solution (Table S1) and allowed to grow until they had 3–5 leaves. Some of the seedlings were then placed in nutrient solution containing 150 mM NaCl and others were transferred to tanks filled with nutrient solution (without added salt) to serve as the control. After exposing the seedlings to salt stress for 3, 12, and 48 h, the roots of the seedlings were harvested, immediately frozen in liquid nitrogen, and then stored at −80 °C for use. Root samples of the control plants were also harvested.

2.2. Measurement of electric conductivity

To measure the relative electric conductivity under salt stress conditions, we punched 30 disks from the first three true leaves of each plant and then placed the disks into a tube containing 10 ml of distilled water. The tube was then shaken for 12 h at 180 rpm and the initial electric conductivity of the solution (S1) was measured by conductivity meter DDSJ-308A (Shanghai REX Instrument Factory, China). The tube was placed in a water bath, and the solution was heated to 100 °C for 10 min and then cooled to room temperature. The final electric conductivity (S2) was then measured. The relative electric conductivity (REC) was calculated as follows: REC (%) =S1/S2 × 100.

2.3. Total RNA extraction and real-time RCR validation

Total RNA was extracted using the modified CTAB method and then purified. Purified RNA was digested with DNase I (TaKaRa, Japan) at 37 °C for 0.5 h. Two micrograms of each total RNA sample was reverse transcribed using MMLV Transcriptase (Promega, USA). The cDNA samples were diluted to 8 ng/μl for qRT-PCR. The assays were performed in triplicate on 1 μl cDNA dilution (8 ng/μl) using the SYBR Green Master Mix (Applied Biosystems; PN 4309155) with an ABI 7500 Sequence Detection System (Applied Biosystems) as described in the manufacturer's protocol. 18S rRNA was used as an internal control to normalize all data, and the relative quantification method (ΔΔCT) was used to evaluate the variation between replicates. The qRT-PCR primers were designed using PRIMER3 (http://frodo.wi.mit.edu/primer3/input.htm; Table S2).

2.4. Microarray experiment and data analysis

The salt-tolerant cultivar Zhong 07 and salt-sensitive cultivar Zhong G5 were used to analyze the salt stress transcriptome response. Sets of Affymetrix cotton genome arrays were used to generate transcription profiles of the plant's response to NaCl treatment. Samples of root tissues were collected from both treated and untreated plants (the control) after 3, 12, and 48 h of exposure to 150 mM NaCl. Three biological replicates of every treatment were collected separately and a total of 36 cotton chips were used in the analysis. For GeneChip (Affymetrix, USA) analysis, total RNA from each sample was used to construct biotin-labeled cRNA targets according to the GeneChip standard protocol. Affymetrix GCOS software was used to read the signal intensity of each probe set. The