

Analyzing serial cDNA libraries revealed reactive oxygen species and gibberellins signaling pathways in the salt response of Upland cotton (*Gossypium hirsutum* L.)

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Abstract

Key message By comparing series full-length cDNA libraries stressed and control, the dynamic process of salt stress response in Upland cotton was studied, and reactive oxygen species and gibberellins signaling pathways were proposed.

Abstract The Upland cotton is the most important fiber plant with highly salt tolerance. However, the molecular mechanism underlying salt tolerance in domesticated cotton was unclear. Here, seven full-length cDNA libraries were constructed for seedling roots of Upland cotton ‘Zhong G 5’ at 0, 3, 12 and 48 h after the treatment of control or 150 mM NaCl stress. About 3300 colonies in

each library were selected robotically for 5′-end pyrosequencing, resulting in 20,358 expressed sequence tags (ESTs) totally. And 8516 uniESTs were then assembled, including 2914 contigs and 5602 singletons, and explored for Gene Ontology (GO) function. GO comparison between serial stress libraries and control reflected the growth regulation, stimulus response, signal transduction and biology regulation processes were conducted dynamically in response to salt stress. MYB, MYB-related, WRKY, bHLH, GRAS and ERF families of transcription factors were significantly enriched in the early response. 65 differentially expressed genes (DEGs), mainly associated with reactive oxygen species (ROS) scavenging, gibberellins (GAs) metabolism, signal transduction, transcription regulation, stress response and transmembrane transport, were identified and confirmed by quantitative real-time PCR. Overexpression of selected DEGs increased tolerance against salt stress in transgenic yeast. Results in this study supported that a ROS–GAs interacting signaling pathway of salt stress response was activated in Upland cotton. Our results provided valuable gene resources for further investigation of the molecular mechanism of salinity tolerance.

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Abbreviations

ABA	Abscisic acid
BP	Biological process
BR	Brassinosteroids
CC	Cellular components
CDPK	Calcium-dependent protein kinase
CK	Control library
DEGs	Differentially expressed genes

Dof	DNA binding with one finger
ESTs	Expressed sequence tags
GAs	Gibberellins
GO	Gene Ontology
GRAS	GAI [(Gibberellic acid)-insensitive], RGA (Repressor of GAI) and SCR (SCARECROW)
MAPK	Mitogen-activated protein kinase
MF	Molecular functions
NR	Stress library
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
ROS	Reactive oxygen species
TFs	Transcription factors

Introduction

Soil salinization is one of the major constraints to crop production. Soils with 4 dS/m (i.e. 40 mM NaCl) or higher electrical conductivity of the saturated paste extract are classified as saline (Chinnusamy et al. 2006). Most crops are glycophytes and susceptible to soil salinity. It is essential to screen plant germplasm with tolerance to soil salinization and to reveal the mechanism of salinity tolerance in crop plants. Upland cotton (*Gossypium hirsutum* L.), the extremely important textile fiber and edible oil crop worldwide, served as a potential pioneer species for marginal saline land, as well as a model plant for salinity tolerance study for tolerating a salinity threshold of 7.7 dS/m (77 mM NaCl) (Chinnusamy, et al. 2006).

Salt stress induced osmotic, ionic toxicity and oxidative stress disrupted plant homeostasis (Chinnusamy et al. 2006). Under stress, the sensor systems in plant triggered downstream signaling and transcriptional control cascades, which resulted in extensive changes in cellular gene expression (Cabello et al. 2014; Deinlein et al. 2014). In response to salt stress, numerous transcription factors (TFs) were up-regulated, such as members of basic leucine zipper (bZIP), WRKY, ETHYLENE RESPONSE FACTOR (ERF), MYB, basic helix–loop–helix (bHLH) and NAC families (Golldack et al. 2011), while other TFs were down-regulated, such as members of Dof and ARR-B that played critical roles as transcriptional regulators in plant growth and development in response to phytohormones GAs, IAA or CK (Peng et al. 2014). In addition, dependent or independent on TFs, microRNAs play important roles in plant salt tolerance by regulating gene expression at post-transcriptional level through degrading the target mRNAs (Kumar 2014). And there were differences in miRNA expression profiles between two cotton varieties with distinct salt resistance (Yin et al. 2012).

Multiple stress signals were induced and multiple signaling pathways were activated in response to salt stress, including ROS signaling pathway (Suzuki et al. 2012) and mitogen-activated protein kinase (MAPK) cascades signaling pathway (Zhang et al. 2012b), phytohormone signaling pathways such as abscisic acid (Gallego-Bartolome et al. 2012; Yang et al. 2014), ethylene (Li et al. 2014a), brassinosteroids (BR) (Divi et al. 2010), and gibberellins (GAs) (Colebrook et al. 2014), and the Ca²⁺ signal coupled calcium-dependent protein kinase (CDPK) signaling pathway (Boudsocq and Sheen 2013). Different pathways interacted with each other as a complex signaling network in response to abiotic stresses (Bahin et al. 2011; Baxter et al. 2014; Mittler et al. 2011; Petrov and Van Breusegem 2012). For instance, CDPK-dependent NADPH oxidase produced ROS under salt stress (Boudsocq and Sheen 2013), meanwhile ROS activated MAPK cascade signaling that translated the stress signal into an appropriate expressional response resulting in salt tolerance (Schmidt et al. 2013). Furthermore, the cellular ROS level was highly integrated with phytohormones metabolism, such as GAs, ABA, SA (Ishibashi et al. 2012; Khokon et al. 2011). Being the output or input of different signaling pathways, or influencing with each other, ROS signaling serving as central player integrated different stress response signaling pathways into stress response network (Baxter et al. 2014; Mittler et al. 2011). For example, *Arabidopsis* increased ROS levels in root vasculature under salt stress, by monitoring the ROS signals thereby limited Na concentrations in xylem sap, and in turn protected shoot cells from transpiration-dependent delivery of excess Na (Jiang et al. 2012).

The DELLAs-dependent GAs signaling was also acknowledged as another hub integrating different stress signals such as ROS and phytohormones under adverse conditions (An et al. 2012; Achard et al. 2006; Colebrook et al. 2014; Gallego-Bartolome et al. 2012; Golldack et al. 2013; Hou et al. 2010; Navarro et al. 2008; Willige et al. 2011). DELLAs belonged to a plant-special GRAS TFs family, which functioned as master growth repressors inhibiting all aspects of GAs responses and were targeted for degradation in the presence of GAs (Middleton et al. 2012; Sun et al. 2012; Zhang et al. 2011c). DELLAs mediated GAs signaling pathway responding to salt stress was reported through regulating the GAs and ROS metabolism (Achard et al. 2008; Colebrook et al. 2014). Under salt stress, *Arabidopsis* reduced the bioactive GAs levels and accumulated DELLAs that restrained growth and promoted plant survival by activating the downstream ROS-detoxification genes and reducing the levels of ROS (Achard et al. 2006, 2008). The reduced GAs allowed DELLAs accumulation to promote GA biosynthesis in feedback (Middleton et al. 2012; Zhang et al. 2011c). The GA-DELLAs mediated stress response mechanism was thought to be land-plant

specific by evolution that permitted plant to adapt to environmental fluctuation (Yasumura et al. 2007).

Modern cultivated cotton originated from wild perennial plant adapting to various environments and experienced periodic salinity, drought and temperature extremes (Wendel, et al. 2010). More than 5000 years domestication process had re-wired the cotton transcriptome profiling for the remarkably elongated fibers and other advantageous characters fitting for cultivation (Yoo and Wendel 2014). Comparative transcriptome analysis revealed that ROS scavenging and signaling process as well as GAs signaling process were significantly enhanced in domesticated cotton species by human selection to avoid earlier onset of programmed cell death and prolong the duration of fiber elongation (Chaudhary et al. 2008, 2009; Shi et al. 2006). Other researches supported that ROS responding process was responsible for the enhanced abiotic stress tolerance of cotton (Park et al. 2012; Ranjan et al. 2012; Shi et al. 2011; Zhang et al. 2011b, 2012a). For example, the ROS-related genes were the major parts of functional pathways induced by water deficit stress in Upland cotton (Park et al. 2012), and higher expression of antioxidant genes contributed to stronger tolerance to drought of Levant cottons (*Gossypium herbaceum* L.) (Ranjan et al. 2012). However, no report showed that ROS responds and GAs signaling participated in salt stress response in Upland cotton.

Plant root was commonly the first organ perceiving the salt stress (Munns and Tester 2008). Hence, a time-series root transcriptome analysis would be helpful for understanding the stress response in plant. Full-length cDNA library is a useful tool for transcriptome analysis and functional genes cloning (Liu et al. 2013). There had been some libraries of cotton, such as leaf library for leaf senescence (Lin et al. 2013), anther library for anther development (Ma et al. 2012) and tissues mixed library for salt tolerance (Xu et al. 2013). However, no full-length cDNA library of Upland cotton root at time series was reported for salt stress.

In this work, three full-length cDNA libraries for seedling roots of Upland cotton ‘Zhong G 5’ after salt stressed 3, 12 and 48 h under 150 mM NaCl, and other four control (3 corresponding ones, and additional one at 0 h) were constructed. The time-serial transcriptomes responding to salt stress were analyzed to elucidate the dynamic stress response in Upland cotton.

Materials and methods

Plant materials, treatments and cDNA library construction

Upland cotton cultivar ‘Zhong G 5’ was the one we used before and plant cultivation, salt stress treatment followed

the previous procedure (Zhang et al. 2011b). In short, in our pre-experiment, different salt concentrations were checked to judge the optimum stress level. Cotton seedlings at three-leaf-stage suffered NaCl gradient at 50, 100, 150 and 200 for 0 h to 3 days. Salt stress was initiated at 11:00 am in Beijing. No significant phenotype variation was observed when treated with 50 and 100 mM NaCl. Seedling leaves were partially dehydrated under 150 mM NaCl treatment and recovered gradually. But seedlings under 200 mM NaCl stress were severely dehydrated, and only some could recover tardily. Hence, 150 mM NaCl was chosen. And to select appropriate sampling time, we performed another pre-experiment to analysis the differentially expressed genes (DEGs) in ‘Zhong G 5’ seedlings root exposing to 150 mM NaCl at 0, 1, 3, 6, 12, 24, 48 and 72 h using GeneChip Cotton Genome Array (Affymetrix). Using two-fold change as cutoff to screen DEGs, the numbers of DEGs from 1 to 12 h were continuously increased. DEGs were detected much more at 3 h than that detected initially at 1 h, and the number of new DEGs increased continuously and reached the peak value at 12 h. After 12 h the amounts of DEGs decreased. The amounts of DEGs at 24, 48 and 72 h were approximately close to each other; however, less down-regulated and more up-regulated DEGs were detected at 48 h (Data not shown). Hence, roots from each three control and stress plants were sampled at 0, 3, 12 and 48 h after stress application of 150 mM NaCl and immediately frozen in liquid nitrogen. Frozen tissues were stored at -80°C until use.

Total RNA was extracted using Trizol agent (Invitrogen, USA) according to the manufacturer’s instructions. And mRNA was subsequently purified using the mRNA Poly-Atract@mRNA Isolation System (Promega, USA). The full-length cDNA libraries were constructed using the Creator Smart cDNA Library Construction Kit (Clontech, USA, Cat.No. 634903) followed the manufacturer’s protocol. In short, the first strand cDNA was synthesized with dC tailing by RT using CDS III primer (*Sfi* IB) in the Kit, and then the second strand cDNA was synthesized by Long-Distance PCR with SMART IV Oligonucleotide primers (*Sfi* IA). Then the double-stranded cDNA was subjected to *Sfi*I digestion, and fragments larger than 500 bp were collected and ligated with the pDNR-LIB vector. After transformed into *Escherichia coli* competent cells, single colonies from the cDNA libraries were cultured on LB-chloramphenicol plates.

ESTs sequencing, assembly and A_t/D_t subgenome categorization

About 3300 colonies were selected robotically from each library for 5′-end single-end sequencing on a 3730 × 1

DNA analyzer (Applied Biosystems, BGI Sequencing, Shenzhen, China) using the T7 universal primer. Totally 22,769 raw ESTs reads were generated. Vector trimming, and elimination of microbial sequence contamination, primer sequences, poly A/T tails, low complexity sequences and sequences with lengths under 100 nt were performed using Seqclean in TIGR Gene Indices clustering tools. The 20,358 valid ESTs was parsed into A or D homoeolog-specific bins (A_t or D_t) using PolyCat based on the *Gossypium* SNP index version 2.0 (Page, et al. 2013; Udall, et al. 2006). And 20,358 valid ESTs were then assembled into uniESTs using CAP3 with the command '-p 92 -y 10 -o 100' (Huang and Madan 1999). The whole genome CDs sequences of *G. raimondii* (D5) and *G. arboreum* (A2) were downloaded from the Cotton Genome Project (CGP: <http://cgp.genomics.org.cn/page/species/index.jsp>). BLASTn the uniESTs in this study with the CDs sequences of A2 and D5 genome and the newly integrated uniESTs in CottonGen (Yu et al. 2014) with an E value cutoff $1e-6$.

GO annotations and KEGG mapping

GO terms were assigned after blastx search of uniESTs sequences using Blast2GO (Conesa et al. 2005) based on sequence similarity in GenBank non-redundant protein (nr) database, the UniProt database and domains in the pfam database. Threshold cutoff was at E value $1e-6$ and the alignment length of 33 amino acids. The gene proportions of GO terms between control and stress libraries were compared in biological process (BP), molecular function (MF) and cellular compartment (CC) at level 3. The KEGG pathways mapping was performed based on information from the KEGG database (Kanehisa and Goto 2000). BLASTx uniESTs with the transcription factors in the PlantTFDB (<http://planttfdb.cbi.pku.edu.cn>) with cutoff $1e-5$ to identify the transcription factors.

Identification of differentially expressed genes (DEGs)

DEGs after stress at each time point were identified by the two-tailed Fisher's exact test (Audic and Claverie 1997) with p value <0.05 as significant. Because some uniESTs contained no EST in some library, its change fold cannot be calculated by division. The fisher test p value at each time point was $-\log_{10}$ -transformed and multiplied by ± 1 (+1 and -1 for up- and down-regulation, respectively) as the regulation parameter of DEGs for clustering. The DEGs regulation was clustered using TIGR MeV (<http://www.tm4.org/mev>) in an uncentered Pearson correlation matrix with average linkage.

Quantitative real-time PCR and salinity tolerance ability validation of DEGs

The expressions of DEGs were verified by quantitative real-time PCR (qRT-PCR) using SYBR Green Master Mix on an ABI 7500 sequencer detection system (Applied Biosystems, Beijing, China) following the manufacturer's protocol. The qRT-PCR reaction contained 0.5 μ g of 1st cDNA, 1 U ExTaq, 10 pM dNTPs, 5 pM $MgCl_2$ and 10 pM gene-specific primers (Supplementary 8). The gene-specific primers were designed using PRIMER 3. *GhUBI1* (GenBank: EU604080) were used as the internal control. Transcriptional expression levels were calculated by the comparative ΔC_T method. Each sample was repeated three times at least. The gene relative expression value was \log_2 -transformed and compared with Fisher's exact test results.

The salinity tolerance of ten selected DEGs (Supplementary 6) was tested by heterologous overexpression in yeast (Y23346C, uracil deficient) undergoing 150 mM NaCl stress. The selected DEGs were cloned into yeast overexpression vector *p426-HXT7-6His* and then transformed into yeast. The transformed yeasts were first selected on SD medium (uracil-deficient yeast nitrogen-base; Difco, Detroit, USA) containing 5 mM $(NH_4)_2SO_4$ as N source and then tested on yeast extract peptone dextrose agar medium (YPD) containing 150 mM NaCl plate for salinity tolerance validation. The Y23346C and blank vector *p426*-transformed Y23346C yeast were used as controls.

Analysis based on public data

We mapped the uniESTs to the locus ID of *Arabidopsis* Tair10 data (www.arabidopsis.org) by BLASTx. Microarray databases for studying salt stress response genes in Upland cotton TM-1 root (Yao et al. 2011) and DELLAs regulated salt stress response genes in *Arabidopsis* root (Achard et al. 2008) were employed to evaluate the homology of salt stress response genes in this study. For investigating the regulation of GAs metabolism in response to the cellular ROS fluctuation in plant, transcriptome data of *Arabidopsis*, including 29 groups with enhanced level of ROS and two groups (OXUPB1-2, SOS1 OX) with reduced cellular ROS, were collected from the Genevestigator microarray database (<https://www.genevestigator.com/>).

Results

The features of cDNA library and uniESTs assembly

Seven full-length cDNA libraries of Upland cotton seedling roots were constructed. There were three stress libraries

sampled at 3, 12 and 48 h after being treated with 150 mM NaCl (termed NR 3, NR 12, and NR 48, respectively), and three corresponding control libraries at 3, 12 and 48 h (termed CK 3, CK 12, CK 48, respectively) and one initial blank library at stress 0 h (CK 0). The titer of every library was larger than 1×10^6 pfu/ml (Table 1).

About 3300 colonies were randomly selected from each library for 5'-end single-pass 454 pyrosequencing, resulting in 22,769 raw ESTs. After vector trimming and low-quality sequence (<100 bp) elimination, 20,358 high-quality ESTs remained. Finally, totally 8516 uniESTs (putative unigenes) were assembled, consisting of 2914 contigs (ESTs number ≥ 2 , Supplementary 1) and 5602 singletons. A_t/D_t subgenome read categorization results showed that more A_t ESTs than D_t were expressed (A_t versus D_t was about 1.25 \times , Table 1). The mean GC content of the sequenced uniESTs was 43.6 %, which was close to that in *G. barbadense* L. (43.1 %) (Yuan et al. 2011). These high-quality ESTs were deposited and could be accessed in GenBank dbEST under Accession Numbers JZ368371–JZ388728.

BLASTn 8516 uniESTs against the genome CDs of D5 and A2 showed that there were 7947 uniESTs with a homologous gene in D5 genome, while 7958 uniESTs with a homologous gene in A2 genome. There were 443 uniESTs

without homologous gene in either genome that were mainly functional unknown genes and might be special for Upland cotton (Supplementary 2). BLASTn against the newly integrated cotton uniESTs in CottonGen (Yu et al. 2014) showed that 6721 uniESTs (78.9 %) were highly homologous to entries in the newly integrated cotton uniESTs (E value $<1E-20$ and more than 50 % identity); the remaining 1795 uniESTs (21.1 %) with no hits were considered as newly reported sequences in Upland cotton (Supplementary 2).

The functional annotation of the uniESTs was performed with BLAST2GO after BLASTx against NCBI non-redundant (nr) protein database. 7528 uniESTs (88.4 %) matched entries in the database, in which 5933 (69.7 %) uniESTs were assigned one or more GO terms of biological process (BP), molecular functions (MF), and cellular components (CC) (Table 1).

The CK 0 library always shared fewer common genes with the stressed library than those with the control at three time points (Fig. 1), which reflected the changes in transcriptome of the cotton root under salt stress. And the common genes shared between the initial control (CK 0) library and stressed libraries decreased (449, 445 and 432) as stress prolonged, which might reflect a serial adaptation process in the cotton root under salt stress.

Table 1 Summary of seven full-length cDNA libraries

	CK0*	CK3	NR3	CK12	NR12	CK48	NR48	Total
Library titer	5.6×10^6	5.5×10^6	2.4×10^6	3.8×10^6	8.0×10^6	7.8×10^6	4.0×10^6	
Sequenced colonies	3215	3194	3191	3370	3235	3310	3254	22,769
High-quality sequences ESTs	2761	2848	2813	3196	3081	2858	2801	20,358
Average ESTs length (bp)	524	515	513	519	512	526	521	518
A_t	1276	1334	1259	1431	1468	1304	1233	9305
D_t	990	1028	1046	1186	1089	1063	1064	7466
N	495	488	509	580	524	493	504	3593
A_t/D_t	1.29	1.30	1.20	1.21	1.35	1.23	1.16	1.25
Contigs	348	324	368	429	449	325	344	2914
Singletons	1518	1560	1431	1888	1638	1491	1563	5602
Total uniESTs (unigenes)	1866	1884	1799	2317	2087	1816	1907	8516
Average ESTs per contig	4	4	4	3	3	4	4	5
Redundancy rate ^a	32.4 %	33.8 %	36.0 %	27.5 %	32.3 %	36.5 %	31.9 %	58.2 %
Homologous to identified cotton genes ^b	1593	1571	1500	1886	1741	1532	1566	6721
Homologous to identified genes ^c	1735	1732	1619	2097	1904	1669	1725	7528
With a GO term ^d	1408	1413	1290	1726	1499	1365	1393	5933

*CK0, CK3, CK12, CK48 control library at 0, 3, 12, 48 h, NR3, NR12, NR48 stress library at 3, 12, 48 h, A_t A-homoeolog, D_t D-homoeolog, N no homoeolog-specific

^a Redundancy rate was calculated by formula: $(1 - \text{number of uniESTs}/\text{number of total ESTs}) \times 100 \%$

^b BLASTn against the newly integrated upland cotton unigenes in CottonGen V1 (Yu et al. 2014)

^c Blastx against non-redundant protein database with an E value $1e-6$

^d GO annotation with the default parameters of Blast2GO program

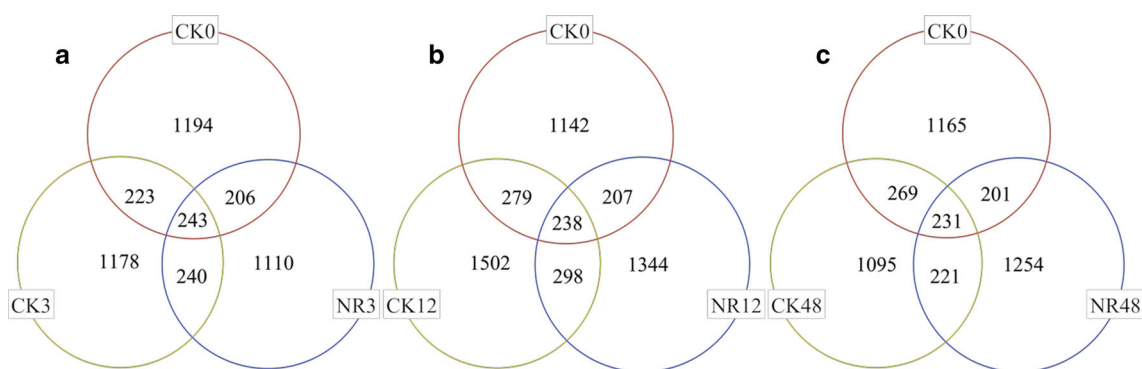


Fig. 1 Venn diagrams of uniESTs distribution among the libraries. Intersections denote uniESTs shared by two or more libraries. CK 0–48, control library at 0, 3, 12, 48 h; NR 3–48, stress library at 3, 12,

48 h; **a** among CK 0, CK 3 and NR 3 libraries; **b** among CK 0, CK 12 and NR 12 libraries; **c** among CK 0, CK 48 and NR 48

Gene ontology comparative analysis between stress and control library

The GO terms changes after stress would provide clues to assess the stress response process. In our study, ESTs in each library were randomly sequenced in the similar depth (Table 1), when comparing the corresponding stress with the control, changes in gene proportion of a GO term might reflect how this GO term genes functioned essentially in the salinity stress response. So the gene proportions of GO terms for BP, MF and CC were compared at level 3 between the corresponding stress and control libraries (Table 2). Compared with the control library, 10 % changes in the gene proportions of a GO term in stress library were considered as obvious change.

When plant encountered an emergent stress, growth restraint enabled the redirection of resources and energy to support mechanisms that promoted survival of adversity (Achar et al. 2006). In this study, multiple GO terms involved in cellular development and foundational metabolism process, such as ‘Reproductive process’, ‘Catabolic process’, ‘Oxidation–reduction process’, ‘Macromolecule metabolic process’ and ‘Multicellular organismal development’ etc., diminished their gene proportions initially after stress, in contrast, the gene proportion of ‘Hydrolase activity’ functioned in energy product and resources redirection increased (Table 2, NR 3). What’s more, KEGG analysis showed that more unigenes in NR 12 were enriched in ‘Glycolysis’ pathway for energy production and in NR 48 were enriched ‘Fatty acid degradation’ pathway that was known as energy production (Supplementary 3).

Rapid stress signals generation and transduction were important for stress response and tolerance in plant. ROS and the secondary metabolites such as phenylpropanoids were identified increasingly as key stress signals (Baxter et al. 2014; Vogt 2010) in plant stress response. In our study, the ‘Oxidation–reduction process’ in BP and the

‘Oxidoreductase activity’ in MF diminished gene proportions in NR 3, which would induce ROS production. On the contrary, the GO ‘Secondary metabolic process’ increased gene proportion in NR 3, and KEGG analysis revealed that genes of ‘Secondary metabolic process’ in NR 3 were enriched in ‘Phenylpropanoid biosynthesis’ and ‘Flavonoid biosynthesis pathways’ (Supplementary 3). In addition, the GO terms involving in stress signaling, such as ‘Vitamin binding’ and ‘Cofactor binding’, increased gene proportions in NR 3. And when the plant adaptation went on to stress for 12 h and/or 48 h, GO terms ‘Cell communication’ in BP, ‘Signal transducer activity’, ‘Ligase activity’ and ‘Transferase activity’ in MF involving in signal transduction increased gene proportions, which reflected promoted signals transduction was conducted in cotton root (Table 2).

Salt stress caused osmotic and ionic stress in plant (Munns and Tester 2008). Modulating the water and ionic transmembrane transport to avoid the dehydration and high level of sodium into cellular should be necessary for plant survival. After 3 h stress, GO terms ‘Transmembrane transporter activity’ and ‘Substrate-specific transporter activity’ in MF increased gene proportions. And in CC, more genes were sequenced in GO term ‘Apoplast’ in all three stress libraries, which would facilitate the water and solutes transport and intake in the root under stress (Table 2). The selective transmembrane transport ability seemed to be aroused rapidly in response to the osmotic and ionic stress.

For survival under salinity conditions, plant would alter transcriptional expression of some genes to activate the stress response and regulate the non-adaptive biological process to adapt to the adversity. Compared with control libraries, more genes belonged to ‘Sequence-specific DNA-binding TFs activity’ were sequenced in three stress libraries, which reflected transcriptional regulation was conducted in response to stress. The GO term ‘Regulation of biological processes’ also increased gene proportion in

Table 2 Compare the gene proportions of GO terms in biological process, molecular function and cellular component between control and stress library

	CK3	NR3	CK12	NR12	CK48	NR48
GO terms of biological process at level 3						
Secondary metabolic process	0.42	1.17	1.07	0.60	1.00	1.04
Response to stress	5.43	6.04	6.06	5.88	6.03	6.70
Response to endogenous stimulus	2.55	2.97	2.53	2.16	2.47	2.95
Cellular localization	1.87	2.25	2.26	1.93	1.92	2.23
Macromolecule localization	1.95	2.52	2.40	2.23	2.19	2.47
Response to biotic stimulus	1.19	1.71	1.60	1.79	1.10	1.59
Response to other organism	1.10	1.71	1.53	1.71	1.00	1.59
Response to external stimulus	0.85	1.08	0.80	1.19	0.73	1.44
Cellular response to stimulus	5.35	4.77	5.13	5.80	5.30	5.18
Cell communication	4.41	3.87	3.99	4.76	4.02	4.07
Regulation of biological process	10.87	10.72	10.25	11.28	10.27	11.56
Response to abiotic stimulus	3.23	3.33	3.66	3.42	3.11	3.43
Regulation of molecular function	1.27	0.81	1.33	0.97	0.73	0.96
Reproductive process	3.48	1.89	2.60	2.16	1.83	3.19
Catabolic process	7.05	5.77	5.79	5.88	5.21	5.26
Macromolecule metabolic process	21.73	18.83	20.91	21.28	22.56	20.81
Cellular developmental process	1.10	0.99	0.93	0.67	1.00	1.04
Oxidation–reduction process	11.71	9.10	8.92	7.22	7.85	8.29
Multicellular organismal development	5.77	4.32	4.06	3.50	4.38	4.63
Anatomical structure development	4.67	3.42	3.26	2.68	3.65	3.91
Cellular component assembly	1.61	1.17	1.40	0.45	1.37	1.04
Establishment of localization	7.47	8.11	7.92	8.11	8.95	7.26
Cellular metabolic process	31.07	28.74	30.03	28.42	31.32	30.94
Primary metabolic process	30.56	27.66	30.29	28.65	32.05	29.27
Small molecule metabolic process	9.59	9.28	9.52	8.48	9.41	8.77
Biosynthetic process	15.53	14.14	15.45	13.99	16.26	14.99
Response to chemical stimulus	6.37	6.04	6.52	6.03	5.84	6.14
GO terms of molecular function at level 3						
Transmembrane transporter activity	2.72	3.60	4.26	4.02	4.11	3.59
Substrate-specific transporter activity	2.46	3.42	4.19	3.65	4.11	3.67
Hydrolase activity	10.53	12.16	12.25	11.98	12.24	9.57
Vitamin binding	0.93	1.53	0.73	0.52	1.02	0.64
Cofactor binding	3.65	4.59	3.46	2.68	2.92	3.11
Isomerase activity	0.68	0.99	1.53	1.04	1.28	1.59
Sequence-specific DNA-binding TF activity	2.60	2.90	2.26	2.53	2.56	3.35
Ligase activity	3.23	2.70	2.40	2.68	2.28	2.55
Signal transducer activity	1.27	0.92	1.07	1.34	0.64	1.04
Transferase activity	14.26	12.34	13.18	13.47	13.33	14.67
Lyase activity	2.46	1.62	2.01	2.08	2.01	1.91
Nucleotide binding	13.50	11.89	13.58	12.43	13.70	12.68
Ion binding	14.26	13.06	13.38	11.24	11.32	11.96
Oxidoreductase activity	12.99	10.09	10.52	8.26	9.13	9.57
Protein binding	7.72	6.58	7.39	6.25	6.39	6.06
Tetrapyrrole binding	2.21	1.17	1.26	1.34	1.55	0.96
GO Term of cellular component at level 3						
Apoplast	1.17	1.33	1.04	1.21	0.91	1.15

Table 2 continued

	CK3	NR3	CK12	NR12	CK48	NR48
Protein complex	6.05	5.56	5.78	7.16	5.94	5.93
Organelle part	10.08	9.34	10.23	9.47	9.20	10.12
Non-membrane-bounded organelle	3.82	2.22	3.63	3.36	2.59	3.15
Cell part	44.69	42.41	45.10	43.78	43.99	43.94
Membrane-bounded organelle	22.77	21.57	23.91	22.63	23.43	23.86

The bold and italic number indicate 10 % increase or decrease in the genes numbers of the GO term in stress library compared with the control. GO terms that contain more than 1 % of total genes were included

NR 12 and NR 48 (Table 2). In addition, GO terms concerning stimulus response, such as ‘Response to stress’, ‘Response to biotic/endogenous/external stimulus’, ‘Response to abiotic stimulus’ and ‘Cellular response to stimulus’ etc. increased gene proportions after stress at one or more time points (Table 2), which meant that the stress response ability was improved in cotton root.

Through upper regulation, majority of GO terms that involving in development and foundational metabolism process diminished gene proportions initially after stress recovered and accompanied with enhanced stress tolerance in NR 48 (Table 2). It seemed that after a period of response and adaption, a new cellular homeostasis under stress was re-established and a reduced growth might be recovered in plant.

Transcription factors regulation under salt stress

The preceding GO analysis showed that the transcription regulation process was always enhanced under stress (Table 2), hence the transcription factors were analyzed further. We mapped the uniESTs to the transcription factors in the PlantTFDB. And 758 transcription factors were identified that belonged to 47 TF families (Supplementary 4). Comparing the numbers of the ESTs belong to different TF families between control and stress library showed that the MYB, MYB-related, WRKY, bHLH, GRAS and ERF families were significantly enriched in early stress response (Fig. 2, Supplementary 4), while NAC transcription factor family was enriched in both NR12 and NR48 (Fig. 2, Supplementary 4).

GRAS protein is a plant-specific TFs family that plays essential roles in transcriptional regulation and various signal transduction pathways in a GA-dependent manner (Sun et al. 2012). The well-studied plant DELLA proteins belonged to the GRAS family, participating in major negative regulators of GA signaling in plant development and stress tolerance (Achard et al. 2008). In our study, highly expressed uniESTs reflected that the phytohormone GA biosynthesis was prevailing in the cotton root (Supplementary 2). Hence, it was not hard to imagine the GAs

signal response TFs would be regulated significantly after stress.

Differentially expressed genes (DEGs) responded to salt stress

DEGs under salt stress at three time points were identified using Fisher’s exact test (Table 3, Supplementary 5). Hierarchical clustering the regulation parameter of all DEGs reflected that the salt stress response process in cotton was of time-course characteristics (Fig. 3).

Agreed with our previous speculation that the oxidation–reduction balance and osmotic homeostasis would be broken initially under stress, the prevailing GAs biosynthesis should be restrained and the downstream transcriptional regulation would be effected, 14 genes were significantly down-regulated after salt stress 3 h, including six redox regulation genes (contig 492, contig 1877, contig 1296, contig 2077, contig 1813, contig 1907), two aquaporin genes *TIP1-1* and *TIP 2-6* (contig 1087, contig 201), two gibberellins (GAs) synthesizing gene (contig 180, contig 1121), and four other functional genes. And meanwhile, there were 12 up-regulated genes, including two GAs-inhibited transcription factors *GRAS* and *bHLH30* (-contig 1590 and contig 1393), a transcription regulation gene BSD domain-containing transcription regulation gene (contig 1203), two osmoregulation genes stachyose synthase precursor (contig 857) and *GhPIP 2-1* (contig 1746), a mevalonate pathway rate-controlling enzyme the HMG CoA reductase encoding gene, and six amino acids/protein metabolism-related genes.

Specially, after salt stress 12 h, seven antioxidant genes (contig 1564, contig 384, contig 1648, contig 1813, contig 2457, contig 926 and contig 351) that mainly functioned in ROS scavenging were up-regulated. In addition, two bioactive GAs synthesis genes (contig 351, contig 2816) were up-regulated in NR 12, which was contrary to the down-regulation of GAs synthesis genes observed in NR 3. All these results resembled that in *Arabidopsis* subjected to salt stress, plant reduced the cellular GAs level and

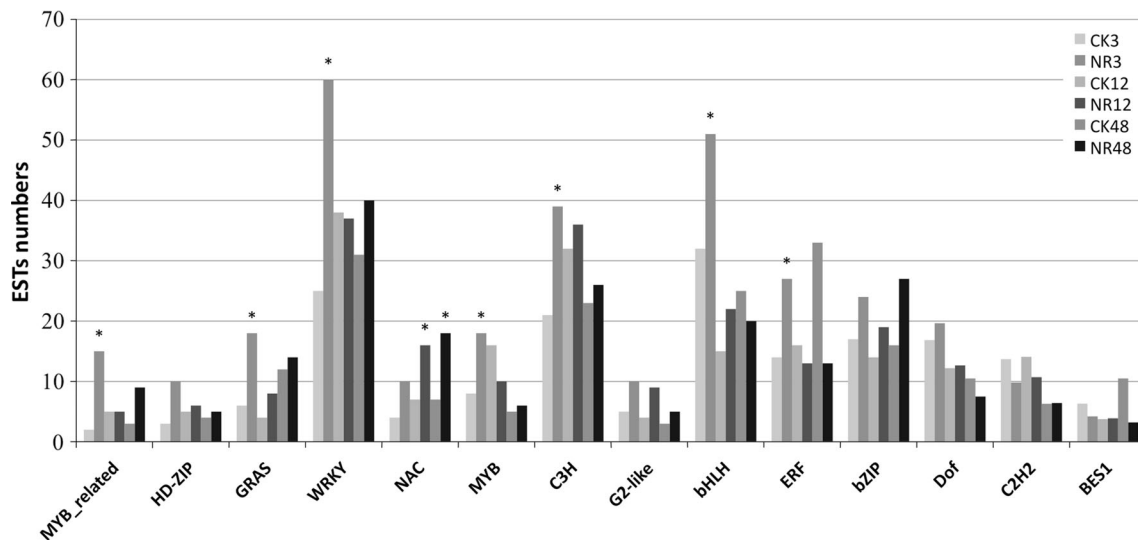


Fig. 2 ESTs number in different TFs families between control and stressed libraries. Asterisk represents enriched TFs families in stressed library relative to its counterpart (proportion test; $P < 0.05$)

accumulated the DELLAs, which activated the downstream ROS-detoxification enzymes encoding genes and promoted plant survival of adversity by reducing the levels of ROS (Achard, et al. 2006, 2008) and accumulated DELLAs-activated GAs biosynthesis in feedback (Middleton et al. 2012; Zhang et al. 2011c). Other significantly regulated genes at 12 h functioned in amino acids/protein metabolism (contig 34, contig 2289, contig 2508, contig 458), repairing (contig 403, contig 1673), growth control (contig 34, contig 328) and osmoregulation (contig 37, contig 299, contig 346, contig 2159, contig 626).

Up-regulated DEGs after stress 48 h were mainly related to amino acids/protein metabolism (contig 639, contig 769, contig 728, contig 450, contig 665, contig 1666, contig 1801) and redox homeostasis (contig 1648, contig 2398, contig 2733, contig 239). The down-regulated genes in NR 48 were mainly involved in transcriptional regulation (contig 77, contig 328, contig 212, contig 236, contig 1237, contig 92, contig 1393), energy storage (contig 81, contig 226, contig 183, contig 790, contig 2296) and hormone metabolism (contig 4 and contig 176). In contrast with the down-regulation of GA synthesizing genes and up-regulation of GA-suppressed transcription factors in NR 3, GA-inactivating gene (contig 176) and GA signal inhibited transcription factor *bHLH30* (contig 1393) were down-regulated in NR 48. In addition, along with the down-regulation of the ethylene synthesizing ACC oxidase gene (contig 4), two ethylene response transcription factors (contig 77, contig 328) were down-regulated. The DEGs observed here were highly consistent with the salt stress response genes regulated by GAs-DELLAs in *Arabidopsis* (Achard, et al. 2008) and most shared the same response mode (Table 3).

Crosstalking between ROS and GAs signals in plant

In this study, along with the down-regulation of six redox regulation genes under early stress (NR3), two GAs biosynthesis genes were repressed and two GA-inhibited transcription factors were up-regulated (Table 3), which agreed with the general knowledge that ROS production was notably exacerbated and plant reduced bioactive GAs level under salt stress (Achard, et al. 2006, 2008). Interestingly, when stress response lasted (NR 12, NR 48), accompanying with the up-regulation of ROS scavenging and redox homeostasis genes, the GA biosynthesis genes were activated and the GA-repressed transcription factor was down-regulated. It seemed that there might be crosstalking between plant ROS and GAs signals under salt stress.

For lack of the cotton transcriptome data, the public datasets of *Arabidopsis thaliana* in response to different level of ROS were employed to investigate the relationship between the ROS fluctuation and GAs metabolism in plant (Fig. 4). While ROS level rose, whatever resulted from abiotic stresses, genetic modification or exogenous application, the GAs deactivation GA2ox-type genes were up-regulated, but the GAs biosynthesis GA3/20ox-type genes were down-regulated. In contrast, the reduced level of ROS induced the opposite regulation pattern (Fig. 4). The enhanced level of ROS accompanying the suppression of the GAs biosynthesis and reduced ROS along with the activation of GAs production were similar to the salt stress response process in cotton observed here. And together with the existing experimental conclusions that GAs treatment induced the production of ROS in plant (Ishibashi et al. 2012) and retarded GAs biosynthesis suppressed the accumulation of cellular ROS (Achard et al.

Table 3 Differentially expressed genes identified at three time intervals using Fisher's exact test

Time	Contig	Description	CK	NR	<i>p</i> value	TAIR10 hits	E value	
3H	Contig 1203	BSD domain-containing protein (<i>BSD</i>)	0 ^a	5	0.030	AT3G49800	8E–40	
	Contig 1590	GRAS family transcription factor (<i>GRAS</i>)	0	8	0.004	AT1G21450 ^b	2E–107	
	Contig 1393	bHLH30 transcription factor (<i>bHLH30</i>)	1	9	0.011	AT3G25710 ^b	4E–05	
	Contig 857	Stachyose synthase precursor	0	5	0.030	AT5G40390 ^b	0	
	Contig 1746	GhPIP 2-1	63	124	0.000	AT4G35100 ^b	4E–129	
	Contig 239	HMG CoA reductase	1	9	0.011	AT1G76490	0	
	Contig 2	Asparagine synthetase	11	35	0.001	AT3G47340 ^b	0	
	Contig 769	Lysine/histidine transporter (<i>LHT</i>)	3	11	0.034	AT1G77380 ^b	1E–23	
	Contig 715	Cysteine proteinase	0	6	0.015	AT5G43060 ^b	0	
	Contig 665	Protein transport protein sec61 subunit α -like	0	5	0.030	AT2G34250	3E–88	
	Contig 23	26S proteasome regulatory subunit S3	0	5	0.030	AT5G19990 ^b	1E–129	
	Contig 1801	Ubiquitin-like protein (<i>UBQ10</i>)	1	13	0.001	AT3G09790 ^b	4E–156	
	Contig 1087	GhTIP 1-1	13	3	0.021	AT2G36830 ^b	9E–129	
	Contig 201	GhTIP 2-6	52	30	0.019	AT4G17340 ^b	3E–121	
	Contig 1121	Gibberellin 3- β -dioxygenase	13	4	0.049	AT4G25420 ^b	2E–23	
	Contig 180	Gibberellin 3-hydroxylase 1	6	0	0.031	AT1G15550 ^b	2E–10	
	Contig 492	Short chain alcohol dehydrogenase	13	4	0.049	AT2G29340 ^b	3E–12	
	Contig 1877	Short chain alcohol dehydrogenase	12	3	0.035	AT2G29340 ^b	3E–22	
	Contig 1296	Peroxidase 21	8	1	0.039	AT2G37130 ^b	2E–136	
	Contig 2077	Class III peroxidase	6	0	0.031	AT3G49110 ^b	4E–34	
	Contig 1813	Apoplastic anionic guaiacol peroxidase	9	1	0.021	AT3G49110 ^b	5E–54	
	Contig 1907	Multicopper oxidase	6	0	0.031	AT5G48100	1E–33	
	Contig 811	Glycoamidase	11	1	0.006	AT3G54580 ^b	5E–09	
	Contig 607	Benzoyl coenzyme A	18	2	0.000	AT3G48720 ^b	8E–62	
	Contig 81	Sucrose synthase	9	1	0.021	AT5G11110 ^b	1E–34	
	Contig 1816	Actin 7 isoform 2	6	0	0.031	AT2G42100	4E–69	
	12H	Contig 34	Thiol protease aleurain	2	12	0.007	AT3G45310 ^b	3E–164
		Contig 2289	Arginase	0	7	0.007	AT4G08900	7E–109
		Contig 2508	Asparagine synthetase	0	7	0.007	AT3G47340 ^b	0
		Contig 403	DNA repair protein RAD23-3-like	0	5	0.029	AT2G17200 ^b	1E–07
		Contig 1036	Auxin-repressed protein/Dormancy	0	7	0.007	AT1G56220 ^b	5E–12
		Contig 346	Tonoplast dicarboxylate transporter	0	9	0.002	AT5G47560 ^b	5E–64
Contig 2159		Epidermis-specific secreted glycoprotein	1	11	0.003	AT5G18470 ^b	1E–08	
Contig 626		Raffinose synthase family protein	1	8	0.019	AT5G20250 ^b	0	
Contig 1564		Epoxide hydrolase (<i>EPHX</i>)	6	19	0.008	AT3G03990 ^b	7E–07	
Contig 384		Short chain alcohol dehydrogenase	0	5	0.029	AT1G52340	6E–31	
Contig 1648		3-ketoacyl-CoA thiolase 2, peroxisomal	0	5	0.029	AT2G33150 ^b	4E–116	
Contig 1813		Apoplastic anionic guaiacol peroxidase	6	25	0.000	AT3G49110 ^b	3E–102	
Contig 2457		3-hydroxybutyryl-dehydrogenase	0	7	0.007	AT3G15290 ^b	2E–61	
Contig 926		Catalase	0	5	0.029	AT1G60940 ^b	4E–61	
Contig 351		Cytochrome p450	0	5	0.029	AT2G26710 ^b	6E–64	
Contig 2816		Gibberellin 3- β -dioxygenase	5	14	0.038	AT4G25420 ^b	2E–22	
Contig 299		High affinity nitrate transporter	13	0	0.000	AT3G45060	1E–116	
Contig 37		GDP-mannose pyrophosphorylase	6	0	0.031	AT2G39770	4E–66	
Contig 328		Ethylene response factor 2	6	0	0.031	AT1G53910 ^b	6E–31	
Contig 1673		Heat shock protein 90	9	1	0.022	AT5G56030 ^b	0	
Contig 458		Subtilisin-type protease precursor	9	1	0.022	AT2G04160 ^b	3E–155	

Table 3 continued

Time	Contig	Description	CK	NR	<i>p</i> value	TAIR10 hits	E value
48H	Contig 639	Alanine-glyoxylate aminotransferase	0	5	0.030	AT3G08860	4E–163
	Contig 769	Lysine/histidine transporter	1	8	0.020	AT1G77380 ^b	1E–23
	Contig 728	Granulin repeat cysteine protease	0	6	0.015	AT5G43060 ^b	2E–59
	Contig 450	26S proteasome regulatory subunit S3	0	6	0.015	AT5G14250	8E–91
	Contig 665	Protein transport protein sec61 subunit α -like	1	9	0.011	AT2G34250	3E–88
	Contig 1666	Octicosapeptide/Phox/Bem1p contain protein	1	7	0.037	AT5G16220 ^b	1E–14
	Contig 1801	Ubiquitin-like protein	1	11	0.003	AT3G09790 ^b	4E–156
	Contig 799	ADP/ATP carrier 3 protein	0	7	0.007	AT3G54110 ^b	5E–17
	Contig 626	Raffinose synthase	1	8	0.020	AT5G20250 ^b	0
	Contig 239	HMG CoA reductase	2	9	0.037	AT1G76490	0
	Contig 1648	3-ketoacyl-CoA thiolase 2, peroxisomal	0	5	0.030	AT2G33150 ^b	4E–116
	Contig 2398	NADH dehydrogenase subunit	0	7	0.007	AT3G54360	9E–69
	Contig 2733	Aldehyde dehydrogenase	2	13	0.004	AT3G24503 ^b	0
	Contig 77	Ethylene-responsive transcription factor 11	6	0	0.031	AT5G47230 ^b	3E–07
	Contig 328	Ethylene response transcription factor 2	9	1	0.021	AT1G53910 ^b	6E–31
	Contig 212	Global transcription factor group E7	6	0	0.031	AT5G65630	1E–43
	Contig 236	Zinc finger family protein	8	1	0.039	AT2G40140 ^b	4E–41
	Contig 1237	General regulatory factor 8	6	0	0.031	AT5G65430	9E–71
	Contig 92	BSD domain-containing protein	6	0	0.031	AT1G69030	1E–40
	Contig 1393	bHLH30 transcription factor (<i>bHLH30</i>)	13	4	0.049	AT3G25710 ^b	4E–05
	Contig 4	1-aminocyclopropane-1-carboxylate oxidase	10	1	0.012	AT1G05010 ^b	0
	Contig 176	Gibberellin 20-oxidase 1	6	0	0.031	AT1G30040	1E–16
	Contig 2	Asparagine synthetase	47	12	0.000	AT3G47340 ^b	0
	Contig 81	Sucrose synthase	10	2	0.038	AT5G11110 ^b	1E–34
	Contig 226	ATP synthase beta chain	7	0	0.016	AT5G08690 ^b	0
	Contig 183	Xyloglucan endotransglycosylase	6	0	0.031	AT4G30270 ^b	7E–39
	Contig 790	Malate dehydrogenase	7	0	0.016	AT5G58330 ^b	4E–64
	Contig 2296	Glyceraldehyde 3-phosphate dehydrogenase	21	7	0.012	AT1G42970 ^b	2E–78
	Contig 1296	Peroxidase 21	12	2	0.013	AT2G37130 ^b	2E–136
	Contig 201	GhTIP2-6	64	30	0.001	AT4G17340 ^b	3E–121
	Contig 1910	GhTIP1-3	26	5	0.000	AT2G36830 ^b	1E–114

^a The ESTs distribution of contigs in the control and stress libraries, respectively. The ESTs distribution and Fisher test *p* value of DEGs at the three time points are shown in Table S4

^b Homologous genes of *Arabidopsis thaliana* regulated by DELLAs under salt stress identified by Achard et al. (2008) with identical response pattern

2008), all these results partly supported our speculation that there might be a crosstalking between plant ROS production and GAs signals under salt stress.

Quantitative real-time PCR and salt tolerance verification

All DEGs were validated by quantitative real-time PCR with gene-specific primers (Supplementary 8). The real-time PCR results were highly in agreement with the DGE results (Fig. 3). Furthermore, ten genes, including *BSD*, *GRAS*, *bHLH30*, *LHT*, *UBQ10*, *EP1* and four ROS-scavenging genes *EPHX*, *KAT2*, *pod10*, *p450* (Supplementary

6), were selected to test the salinity tolerance function by heterologous overexpression in yeast. Undergoing 150 mM NaCl stress, eight genes (*bHLH30*, *GRAS*, *p450*, *KAT2*, *UBQ10*, *pod10*, *EPHX* and *LHT*) could enhance the salinity tolerance in yeast in various degrees (Fig. 5).

Discussion

Dynamic salt stress response in Upland cotton

The sessile lifestyle of plant necessitates the potential to induce different transcriptomes depending on the change

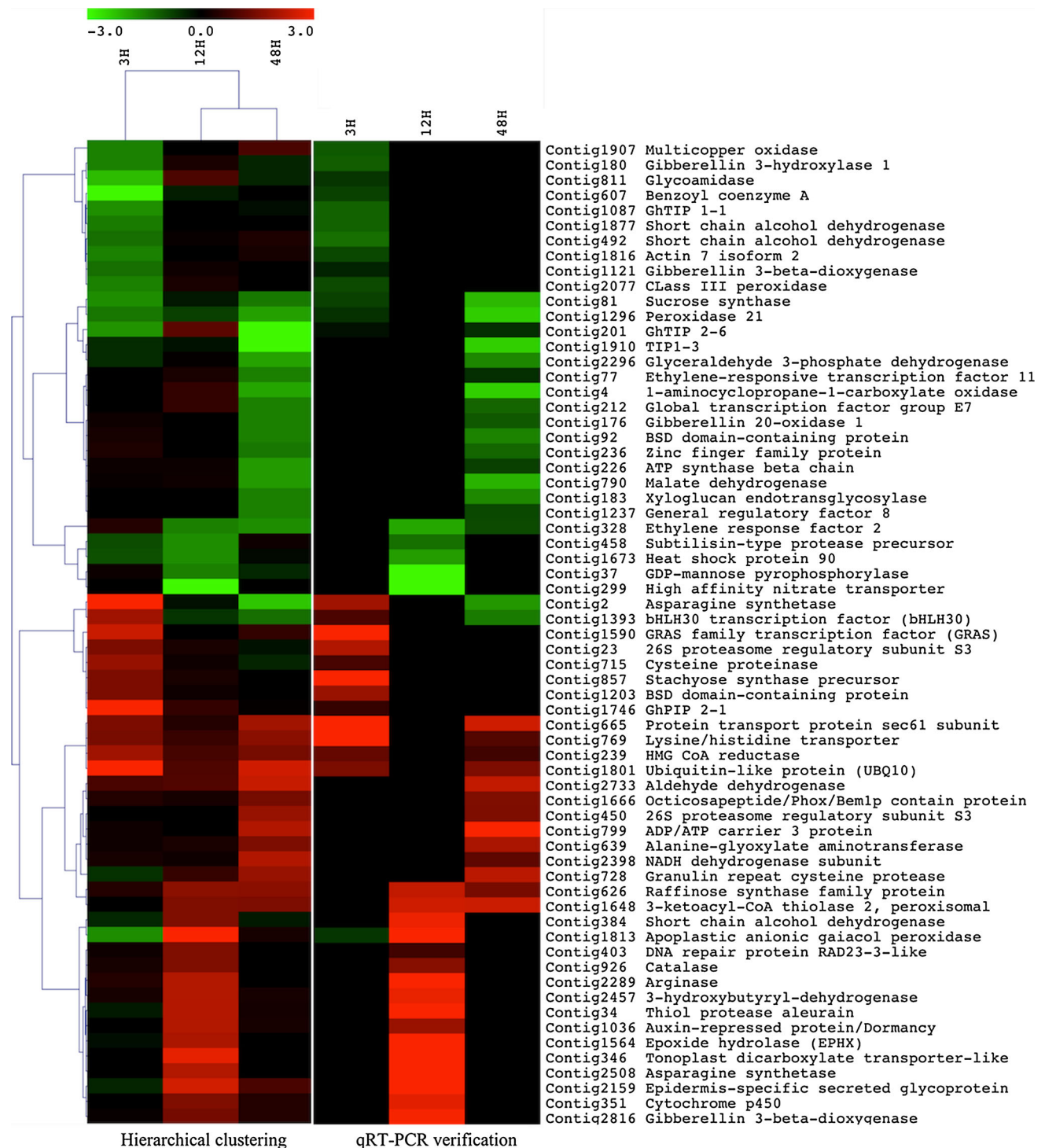


Fig. 3 Hierarchical clustering and quantitative real-time PCR validation of DEGs. The 65 DEGs were clustered using hierarchical clustering with Pearson correlation matrix with average linkage. The

relative expression levels of DEGs verified by quantitative real-time PCR were \log_2 -transformed and listed without clustering

of the surrounding environment (Rymen and Sugimoto 2012). Under salt stress, plant should initiate a series of responses to adapt to the adversity (Munns and Tester 2008). In this study, we designed a time-course

transcriptome analysis for better understanding the salt stress response in cotton. The serial GO comparing and DEGs analysis revealed a dynamic salt stress response process in cotton root.

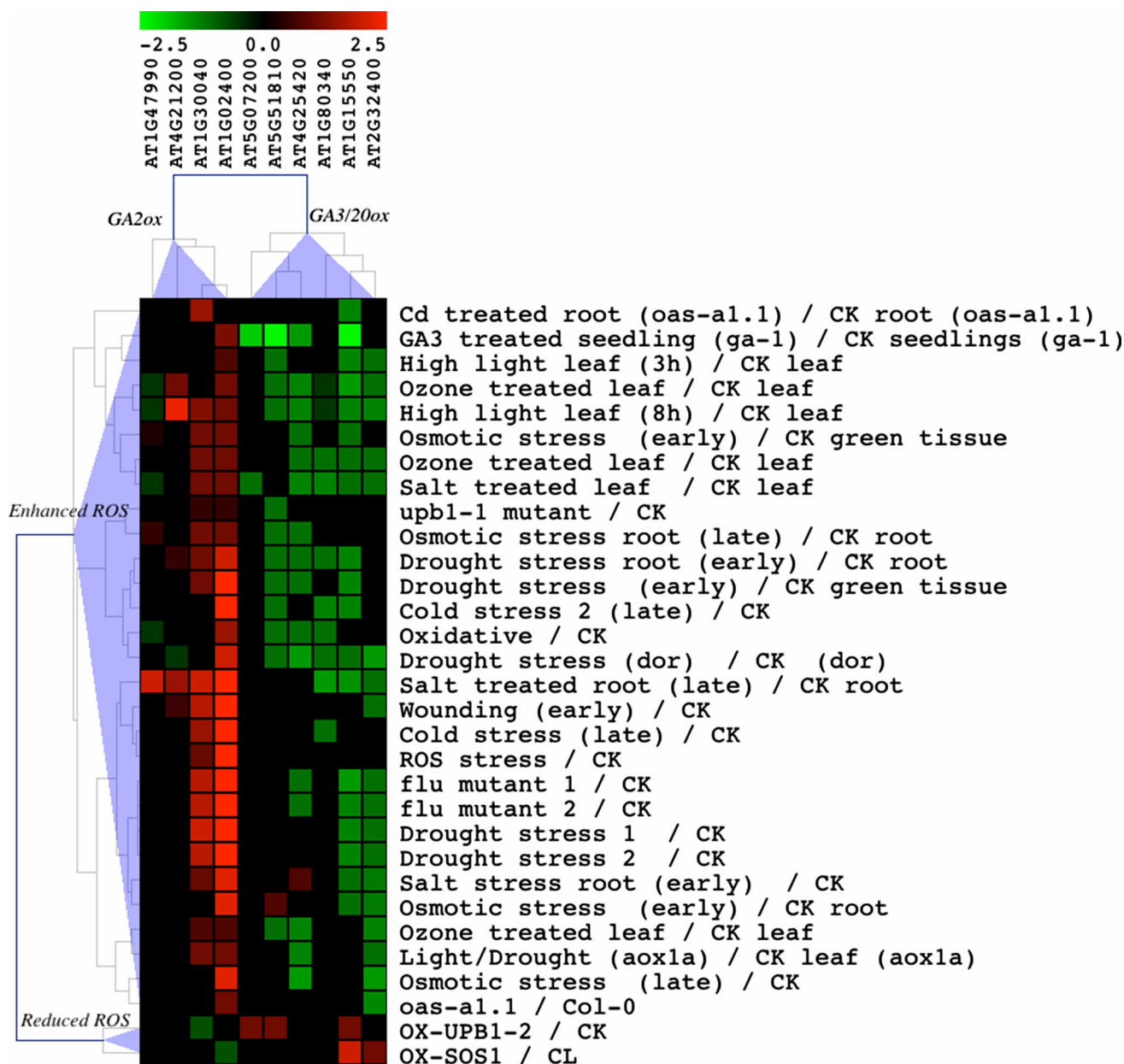


Fig. 4 Regulation of gibberellins metabolic genes in response to the cellular ROS fluctuation. Data were collected from the Genevestigator microarray database. Compared with control, the upper 29 groups

showed enhanced level of ROS, and the other two groups (OXUPB1-2, SOS1 OX) with reduced level of cellular ROS

Under early stress, plant growth rate was reduced and the osmotic/ionic homeostasis as well as the oxidation–reduction balance was broken. For adapting adversity, plant enhanced the multiple stress responses, and substrate-specific transport to alleviate drastic damage. Comparing with CK 3, GO terms involved in development, oxidation–reduction, and foundational metabolism process diminished the gene proportions in NR 3, while terms concerning sequence-specific transcription factor activity, stimulus response, transmembrane transport and apoplast transport comprised more genes (Table 2). Agreed with the GO

results, the redox regulation genes, growth hormones GAs synthesizing genes and water transporting genes were down-regulated in NR 3, while transcription factors, osmoregulation genes and amino acids/protein metabolism-related genes were up-regulated (Table 3). As the top 50 genes highly expressed in the control libraries reflected the control of cellular redox homeostasis and GAs biosynthesis was prevailing in cotton root (Supplement 2), it was not difficult to imagine that the oxidative and GAs signals would act as important early stress signals under salt stress. Yun et al. (2010) concluded that the early transcriptional

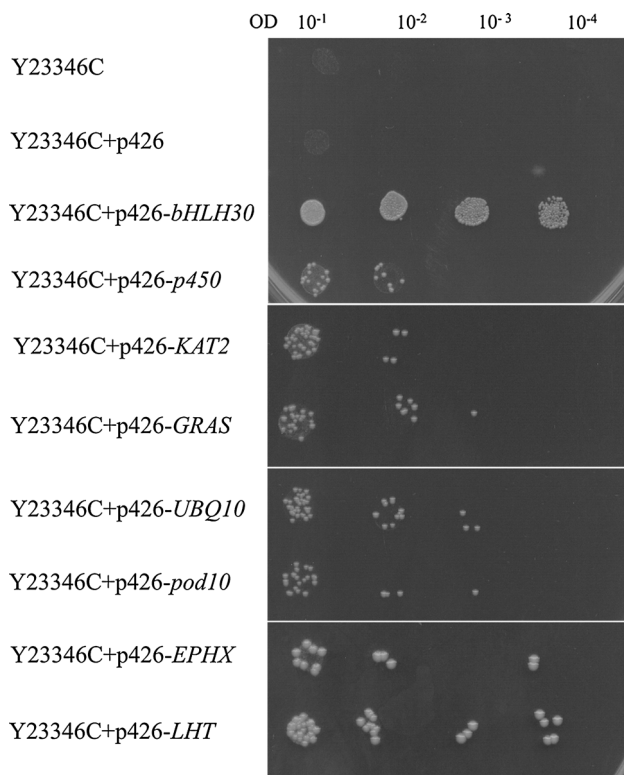


Fig. 5 Salinity tolerance validation in yeast of selected DEGs. Overexpressing eight out of ten selected genes (Supplementary file 6) could enhance the salinity tolerance in transgenic yeast. *bHLH30*: *bHLH30* transcription factor, *p450* cytochrome p450, *KAT2* 3-ketoacyl-CoA thiolase 2, *GRAS* *GRAS* family transcription factor, *UBQ10* Ubiquitin-like protein; *pod10*: apoplastic anionic guaiacol peroxidase, *EPHX* epoxide hydrolase, *LHT* lysine/histidine transporter. Yeast Y23346C is uracil deficient, while yeast overexpression vector p426-HXT7-6His-containing URA3 is growth complemented. SD (uracil-deficient yeast nitrogen-base) containing 5 mM $(\text{NH}_4)_2\text{SO}_4$ was used as selected medium for yeast transformation and complementation test. All transformants were tested on YPD (yeast extract peptone dextrose agar medium) containing 150 mM NaCl plate for genes salinity tolerance validation. The Y23346C and blank vector p426-transformed Y23346C yeast were used as controls

phase in the response of rice to chilling stress appeared to be triggered by oxidative signals.

After salt stress 12 h, cotton strengthened signal transduction, biological regulation and antioxidant ability. Gene percentage of GO term involved in signal transduction and biology regulation increased in NR 12 (Table 2). Mitogen-activated protein kinase (MAPK) genes represented genes in the GO term ‘signal transducer activity’ in NR 12 (Supplement 9). MAPK pathway was known to be involved in the ROS signaling in response to stress in plant (Li et al. 2014b). *GhMPK2*, *GhMPK16*, *GhMCK4* and *GhMCK5* had been proved to participate in cotton salt and others stress response by affecting the ROS signaling (Li et al. 2013, 2014b; Shi et al. 2011; Zhang et al. 2011a; 2012a). The up-regulation of genes for ROS scavenging in NR 12 reflected on-going biological regulation in cotton root for adversity

adaptation (Table 3). As saline stressed process was energy consuming (Munns and Tester 2008), more unigenes enriched in ‘glycolysis’ pathway in NR 12 (Supplementary 3) suggested that the cotton was producing more energy for salt tolerance.

After 2 days acclimatization, a new cellular homeostasis was re-established and strengthened stress adjust ability of plant to recover growth. In NR48 of our study, majority of GO terms that involved in development, oxidation–reduction, foundational metabolism process, and stress response recovered or heightened their gene contents (Table 2). Multiple amino acids/protein metabolism and redox homeostasis genes were up-regulated in NR 48, which reflected the accelerated biomolecule metabolism and redox control in cotton root. The down-regulation of GA-inactivating gene in NR 48 might be another clue for the growth resuming. However, considering of plant allocated more resources and energy to stress tolerance, the resumed growth might be truncated.

Furthermore, it seemed that the salt stress response in cotton was characterized with circadian rhythm. The gene percentages of GO terms concerning the stimuli response increased after 3 h, diminished after 12 h in the night, but increased again after 48 h of salt stress (Table 2). Similarly, DEGs involving in osmoregulation and amino acids/protein metabolism shared similar response pattern between NR 3 and NR 48 (Table 3). As water transport and ion delivery in plant was heavily dependent on the transpiration stream and salt stress disrupted homeostasis in water potential and ion distribution, it was not difficult to understand that plant salt stress response was characterized with circadian rhythm.

Transcription factors response in salt tolerance

Transcription factors are integral in transferring stress signaling into tolerance responses. Multiple TF families such as bZIP, WRKY, ERF, MYB, bHLH, and NAC families are differentially expressed in response to salinity stress (Golldack et al. 2011). In our study, MYB, MYB-related, WRKY, bHLH, GRAS and ERF families were enriched in early stress response, while NAC transcription factor family was enriched in NR 12 and NR 48 (Fig. 2). Identification of stress responsive genes in cotton revealed that WRKY, MYB, NAC, ERF and zinc finger TFs were shown to be involved in the response of different stresses, and a NAC transcription factor was up-regulated under all studied stresses after long time stressed (Zhu et al. 2013). Co-expression of *AtbHLH17* conferred resistance to abiotic stress in *Arabidopsis* (Babitha et al. 2013). Here, a *bHLH* transcription factor was up-regulated under early stress (Table 3) and overexpressing this *bHLH* gene in yeast made it stronger tolerance to salt (Fig. 5).

The GRAS proteins belong to a plant-specific transcription factor protein family role in GA signaling, in which well-studied DELLAs were found to play critical roles in plant stress tolerance (Achard et al. 2006, 2008; Hou et al. 2010; Sun et al. 2012). Under salt stress, plant reduced the GA content and accumulated DELLAs, which promoted plant survival of adversity (Achard et al. 2006, 2008; Hou et al. 2010). Overexpressing a poplar GRAS gene *SCL7* conferred salt and drought tolerance in *Arabidopsis* (Ma et al. 2010). In our study, along with the down-regulation of the GA biosynthesis genes, a *GRAS* gene was up-regulated at early stress. And overexpressing *GRAS* in yeast made it more salt tolerance (Fig. 5).

ROS signals and oxidative stress response in cotton

Metabolic pathways in sessile plant organelles are susceptible to environmental fluctuation, and almost any changes in cellular homeostasis could lead to oxidative stress in cells (Suzuki et al. 2012). Hence, during evolution, plants developed delicate monitoring and scavenging mechanisms to deal with excess ROS and used ROS as good-signaling molecules (Mittler et al. 2011). For example, *Arabidopsis* was able to control the xylem-sap Na concentration by monitoring the ROS signals (Jiang et al. 2012). In NR 3, ‘Oxidation–reduction process’ in BP and ‘Oxidoreductase activity’ in MF diminished gene proportions, and six redox regulation genes were significantly down-regulated, which reflected the ROS production under early stress.

For using ROS as signals, plants should evolve the capability of transferring ROS as signals throughout the plant and meanwhile tightly control the ROS as toxic agents in particular subcellular localization to avoid oxidative damages (Mittler et al. 2011). Aquaporins played key roles in water transport throughout the plant, and were recognized as important transporters of the ROS signal (Boursiac et al. 2008; Dynowski et al. 2008). TIPs are aquaporins located in the membranes of plant vacuoles that are crucial to the processes of detoxification in responses to abiotic stress (Maurel et al. 2008; Marty 1999). In this study, the three TIPs (*TIP1-1*, *TIP2-6*, and *TIP1-3*) were significantly down-regulated after stress. Studies showed that AtTIP1-1 and AtTIP1-2 were of H₂O₂ permeability (Bienert et al. 2007). As AtTIP1-1 was preferentially localized in the apposing tonoplasts of adjacent vacuoles and functioned in vesicle fusion with the tonoplast (Beebo et al. 2009), suppressing the fusion of H₂O₂-containing vesicles with the tonoplast would improve vacuolar functions and plant salt tolerance (Leshem et al. 2006).

In this study, *GhPIP2-1* was significantly up-regulated under salt stress (Table 3). And the role of *GhPIP2-1* in ROS signaling deserved further research. *AtPIP2-1* was

reported to conduct the H₂O₂ signal in plant stress response (Dynowski et al. 2008). Under salt stress, *AtPIP2-1* was up-regulated and accelerated internalization in intracellular compartments of roots in a ROS-dependent manner (Boursiac et al. 2008). It was speculated that the regulation of aquaporin genes in response to salt stress involved not only in osmotic homeostasis but also in the ROS signaling.

Salt stress induced high levels of ROS were toxic. Enhanced ROS control ability was required necessarily for plant salt tolerance (Deinlein et al. 2014). The cultivated cotton species were domesticated from wild counterparts and human domestication had modified the cellular redox control ability in the cultured cotton (Chaudhary et al. 2008, 2009; Hovav et al. 2008). The modified ROS response ability in cultured cotton had been proved to participate in various stresses tolerance (Park et al. 2012; Ranjan et al. 2012; Shi et al. 2011; Zhang et al. 2011b; 2012a). Ranjan et al. (2012) found that higher expression of antioxidant enzymes genes contributed to drought tolerance in four Levant cotton genotypes. Co-overexpression of *GhSOD1* and *GhCAT1* in cotton chloroplasts caused no damage to the chloroplast under stress and the transgenic plants had higher tolerance to salinity (Luo et al. 2013). In our study, multiple ROS-scavenging genes were up-regulated in NR 12 (Table 3). Four antioxidative genes tested by overexpressing in this study could improve salt tolerance (Fig. 5).

The ROS-GAs interacted signaling in the salt response

Acting as signals as well as a toxic agent, the metabolism and signaling of ROS should be finely controlled by integrating with other signaling pathways in plant (Ishibashi et al. 2012; Rymen and Sugimoto 2012). Crosstalk between ROS with different phytohormones and Ca²⁺ signal in response to abiotic stresses in plant had been reported (Asano et al. 2012; Ishibashi et al. 2012; Petrov and Van Breusegem 2012; Rymen and Sugimoto 2012; Suzuki et al. 2012). The results in the salt response of cotton in this study (Fig. 6) were agreed with that the DELLAs mediated GAs and ROS signaling crosstalk in *Arabidopsis* (Table 3; Fig. 4).

Under normal growth conditions, the balance between ROS-scavenging and ROS-producing systems kept the cellular ROS at an optimal level. As salt stress emerged, cellular homeostasis was disrupted and a rapid ROS burst occurred because of drastic changes to the homeostasis of ions and water (Mittler et al. 2011), just as the down-regulation of redox regulation and osmoregulation genes in NR 3 of this study. The high levels of ROS served as stress signal as well as toxic agents that activated the multiple stimulus responses and suppressed the GAs biosynthesis, which were reflected by the down-regulation of GA

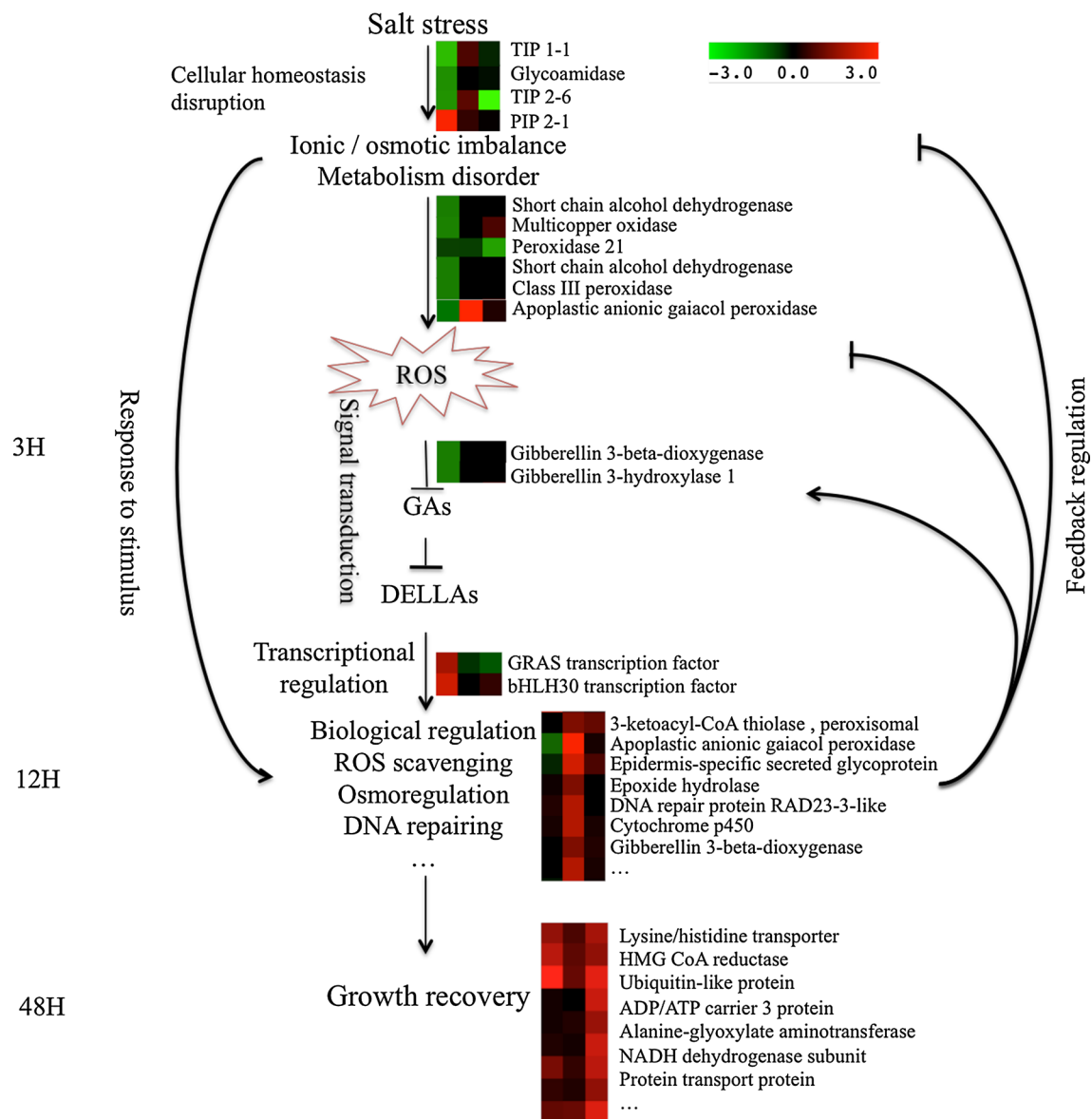


Fig. 6 A model for the ROS-GAs interacting signaling pathway in Upland cotton responded to salt stress

biosynthesis genes and enhancement of stimulus response function in NR 3 (Tables 2, 3). As stress lasted and stress signal transduced, reduced bioactive GAs resulted in the DELLAs accumulation (Achard et al. 2006) and then activated downstream genes involved in ROS-detoxification, osmoregulation and damage repairing in plant (Achard et al. 2008), which could reduce the ROS levels and re-establish the cellular homeostasis, supported by the up-regulation of multiple ROS-scavenging genes in NR 12. Furthermore, the accumulated DELLAs could activate bioactive GAs synthesis in a feedback mechanism (Middleton et al. 2012; Zhang et al. 2011c), agreed with the suppression of GAs synthesis in NR 12 and NR 48. By perceiving and fine-tuning cellular ROS levels and

interacting with other signaling pathways, cotton regulated the transcriptome to re-establish new cellular homeostasis and activated necessary response to adapt to the adversity. This GAs-ROS signaling interacting model deserved our further works to prove.

In this study, 20,358 high-quality ESTs were harvested from seven full-length cDNA libraries of Zhong G5 seedling roots, which were constructed at 0, 3, 12 and 48 h under control and salt stress condition. Total 8516 uniESTs were assembled, in which 1795 uniESTs (21.1 %) with no hits to existing cotton unigenes were reported initially. The full-length cDNA libraries, especially those sequenced clones, supply useful resources for cotton gene cloning and functional study. Genes sequenced from stress libraries

were viewed as candidate genes of salt stress response, the functional data would be highly required besides the DEGs tested in yeast.

Author contribution statement Jinping Hua designed the experiments and revised the manuscript. Xiaoyan Guo prepared the root samples and RNA samples. Gongyao Shi and Xiaoyan Guo maintained the libraries and curated the sequencing data. Gongyao Shi and Jinyan Guo performed the data analysis. Gongyao Shi performed bench experiments. Gongyao Shi prepared the manuscript. Jinping Hua and Laihua Liu provided parts of the experimental platform. All authors approved the final manuscript.

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Conflict of interest The authors declare that they have no conflict of interest.

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