Transcriptomic reprogramming in soybean seedlings under salt stress

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ABSTRACT
To obtain a comprehensive understanding of transcriptomic reprogramming under salt stress, we performed whole-transcriptome sequencing on the leaf and root of soybean seedlings subjected to salt treatment in a time-course experiment (0h, 1h, 2h, 4h, 24h and 48h). This time series dataset enabled us to identify important hubs and connections of gene expressions. We highlighted the analysis on phytohormone signaling pathways and their possible cross-talks. Differential expressions were also found among those genes involved in carbon and nitrogen metabolism. In general, the salt-treated seedlings slowed down their photosynthetic functions and ramped up sugar catabolism to provide extra energy for survival. Primary nitrogen assimilation was shut down while nitrogen resources were redistributed. Overall, the results from the transcriptomic analyses indicate that the plant uses a multi-pronged approach to overcome salt stress, with both fast-acting, immediate physiological responses and longer-term reactions that may involve metabolic adjustment.

Brief Summary Statement:
We performed time-course experiments to analyse the changes in gene expression profiles in conjunction with physiological adaptations and metabolic adjustments under salt stress. Using multiple time points, we are able to identify important hubs and connections of gene expressions. Our data indicate that soybean seedlings uses a multi-pronged approach to overcome salt stress, with both fast-acting, immediate physiological responses and longer-term reactions that may involve metabolic adjustments.

Running title: Soybean transcriptome under salt stress

Keywords: soybean; salinity; transcriptome; gene expression reprogramming; phytohormone signaling; metabolic adjustment
INTRODUCTION

Worldwide, salinity has affected 20% of irrigated lands that produce 40% of the world’s food (Pimentel et al. 2004), and caused a marked reduction in soybean production and seed quality (Miransari 2015; Specht et al. 1999). Soybean is classified as a moderately salt-sensitive crop (Munns and Tester 2008), with estimated salt tolerance thresholds ranging from 5.2 to 8.0g/L NaCl (Pantalone et al. 1997).

Salt treatment leads to an early-onset osmotic response in roots, that subsequently affects the whole plant, and a slower ionic response in leaves due to the accumulation of Na\(^+\) ions (Munns and Tester 2008). Under salt shock when the plant is suddenly exposed to a high level of salt (Shavrukov 2012), the transpiration rates of clover plants reached their lowest values after 2h of treatment with 200mM NaCl, and then raised slowly over the next 24h (Abogadallah 2010b). When subjected to 150mM NaCl, the leaves of cotton seedlings were partially dehydrated initially, followed by a gradual recovery (Zhang et al. 2011). Similarly, we have previously observed that the leaves of soybean seedlings drooped in the first hour under 0.9% NaCl treatment and started to partially recover after several hours (Phang et al. 2008). All these physiological observations indicate that there are two distinct stresses (osmotic and ionic) for plants to adapt to when subjected to salt shock (Dragičević 2015).

Multiple signal transduction pathways are activated to cope with salt stress, such as the phytohormone-mediated, Ca\(^{2+}\)-dependent, and phosphatidylinositol (PI) signals (Ji et al. 2013; Xue et al. 2009; Fernando and Schroeder 2016). These signaling pathways activate other regulators, such as transcription factors (TFs) to amplify the signals for gene regulation, and finally initiate protective mechanisms through the induction or repression of functional genes (Lata et al. 2011).

For the initial osmotic stress, plants attempt to adjust the water potential and maintain cell turgor by regulating stomatal conductance and increasing the intracellular concentrations of osmolytes (Munns and Tester 2008). After NaCl application for 24 to 72h, cytosolic Na\(^+\) will rise to toxic levels. Other strategies will therefore have to be employed to alleviate Na\(^+\) toxicity, e.g. by sequestering or compartmentalizing excess Na\(^+\) in the vacuole to maintain a high cytosolic K\(^+\)/Na\(^+\) ratio (Assaha et al. 2017). On the other hand, metabolic adjustments also play important roles in attaining a new balance of energy and metabolites (Obata and Fernie 2012). For instance, the accumulation of carbohydrates, sugar alcohols, organic acids, and N-containing compounds could be a way to counteract a lower water potential under a NaCl-rich environment (Yang et al. 2017). Comparative proteomic analyses also showed that when soybean was subjected to salt stress, the differentially expressed proteins are enriched...
among those involved in the processes of carbohydrate and energy metabolism, protein synthesis, and redox homeostasis (Ji et al. 2016).

We hypothesize that the changes in signal transductions, metabolic adjustments, and other protection mechanisms are tightly associated with the genome-wide transcriptomic reprogramming. Transcriptome analysis has become a very powerful approach to explore genome-wide gene expression reprogramming in response to different stresses (Khraiwesh et al. 2012; Severin et al. 2010). A number of transcriptomic studies have been done on salt stress with different species (Song et al. 2016; Tripathi et al. 2015; Marcolino-Gomes et al. 2015). However, there are relatively little transcriptomic researches in soybean that involve multiple time points, which should give a more comprehensive picture on the changes in the expression profiles of early- and late-responsive genes under salt stress, and allow the identification of important hubs and connections of gene expressions.

In this work, we investigated and compared gene expression reprogramming under short-term (osmotic phase; 1 to 4h) and prolonged (ionic phase; 24 to 48h) salt stress. We identified gene co-expression networks and important hubs. Since there are numerous pathways involved in salt stress responses, here we focus our analysis on phytohormone signaling and metabolic adjustments.

MATERIALS AND METHODS

Plant materials and library preparation

Seeds of soybean (Glycine max [L.] Merr., accession C08); (Lam et al. 2010) were sown in sterile vermiculite for germination. Seven-day-old seedlings were transferred to half-strength Hoagland's nutrient solution (Hoagland and Arnon 1950) in a greenhouse, under natural sunlight with supplementary lighting to maintain a 12h/12h light/dark cycle in an ambient temperature of 25-28°C. When the primary leaves were fully opened, seedlings were transferred to half-strength Hoagland solution supplemented with 0.9% (w/w) (~150mM) NaCl. Primary leaf and root samples were harvested at 0h, 1h, 2h, 4h, 24h, and 48h after treatment, immediately frozen in liquid nitrogen, and stored at -80°C. Three independent sets of samples were collected for each time point.

Total RNA was extracted from the samples ground in liquid nitrogen using a modified phenol/chloroform/isoamylalcohol (P:C:I=25:24:1, v/v) mediated extraction protocol (Jackson and Larkins, 1976). The library construction and sequencing were performed by
BGI-Shenzhen Company (Shenzhen, China) on the Illumina HiSeq 2000 platform by 2x90bp pair-end mode. The data can be accessed from SRA database: SRP132150.

**Quality control and differential expression analyses**

The Illumina RNA-Seq adapters were searched and removed by Cutadapt using default parameters (Martin 2011). The mapping results were assembled into transcripts using a reference guided method based on Williams 82 gene pool by StringTie (Pertea et al. 2016), followed by differential expression test using time course mode in the Ballgown package (Frazee et al. 2015). Fragments per kilobase per million mapped reads (FPKM) values were calculated by Ballgown, and only those genes with a FPKM value larger than one in any sample were retained for subsequent analyses. Differential expression analyses were performed by comparing the expression of a gene at each time point to 0h and selected by a false discover rate (FDR) value of 0.05.

**Co-expression network construction**

Differentially expressed genes (DEGs) (FDR ≤ 0.05, max FPKM ≥ 1) were used to construct a network by weighted correlation network analysis (WGCNA) (Langfelder and Horvath 2008). Log-transformed FPKM values of DEGs were used to calculate the adjacency matrix. Soft threshold power was selected to make the whole network fit the scale-free topology. Signed networks were built for leaf and root respectively. Intramodular connectivity (sum of the weight of intramodular edges) of each gene was calculated and used for ranking members in each module, with the top 10 members selected as hub genes. Module eigengene (the first principal component) was calculated and used to study the correlational relationships among modules. For the ease of visualization, subnetworks of selected modules were reduced by filtering at a correlation of 0.85 and visualized in Cytoscape 3.5. Soybean transcription factor (TF) family information was extracted from plantTFDB (Jin et al. 2017), expression patterns of TFs were generated by dynamic regulatory events miner (DREM) (Schulz et al. 2012). KEGG (Kyoto Encyclopedia Genes and Genomes) pathway enrichment analysis was performed on each module by KOBAS2.0 (Xie et al. 2011). Gene lists corresponding to each component of the pathways of interest were extracted according to the phytozome annotations of the Williams 82 genome (version a2v1), based on PANTHER annotation followed by the manual checking of PFAM motifs. Enzymes involved in metabolism were selected by Enzyme Code. The protein localization was predicted by ProtComp9.0 (http://www.softberry.com/berry.phtml?topic=index&group=programs&subgroup=proloc).
**Physiological indicator measurements**

Photosynthesis rates and stomatal conductance were measured for the primary leaves of eight treated and four control plants by the LI-6400XT portable photosynthesis system (LI-COR; Lincoln, Nebraska, USA), with 5 technical repeats, at 0h, 1h, 2h, 4h, 6h, 8h, 24h, 26h, 28h, 30h, 32h, and 48h after the initiation of treatment. The control and treated seedlings were randomly arranged to eliminate possible position effects. Leaf surface temperatures were recorded using the InfRec camera system (InfReC R300SR) under the same conditions for 24h. Two of each control and salt-treated seedlings were subjected to imaging and detection at the same time, and three focusing areas were selected randomly on each primary leaf. To determine the Na\(^+\) and K\(^+\) ion contents of primary leaves and roots, the fresh weights (FW) of tissues were measured immediately after sample collection. Water-soluble ions were extracted using 2 ml 1% acetic acid after grinding in liquid nitrogen. The extract was centrifuged at 14,000 x g for 30min at room temperature, and the supernatant was diluted for Na\(^+\) and K\(^+\) ion measurements using atomic absorption spectrometry (Z2300 flame; Hitachi, Tokyo, Japan).

**RESULTS**

**Physiological changes under salinity treatment**

Soybean seedlings were subjected to salinity shock (0.9% NaCl) for 48h. Leaves started drooping after 30min, together with an elevation in leaf surface temperature. Recovery from drooping started after 4h, and stopped at 24h. The difference between leaf temperature of treated plant and control plant increased significantly from 1h to 6h for ~2\(^\circ\)C, and this temperature elevation was maintained up to 24h (Fig. 1a, and 1b). Compared to untreated seedlings, the photosynthetic rates and stomata conductance of salt-treated seedlings exhibited a sharp drop in the early phase (starting at 1h), and the photosynthetic rates partially recovered after 24h but to a lower value than at 0h. From 24h to 48h, treated seedlings exhibited a consistently lower photosynthetic rate and stomata conductance (Fig. 1c and d).

The Na\(^+\) contents in root started to accumulate at 1h whereas the levels of Na\(^+\) in leaf only started to rise at 24h and 48h (Fig. 1e and f). In subsequent transcriptomic analyses, we focused on two typical phases: early (osmotic) phase and late (ionic) phase, and selected 6 time points: 0h, 1h, 2h, and 4h for early phase, and 24h and 48h for late phase, respectively.
Genome-wide changes in the mRNA profile under salt stress
We generated RNA-Seq data from two tissue types (leaf and root) at 6 time points with 3 biological replicates. Thirty-six pair-end libraries with over 1.41 billion raw reads were obtained in total, attaining an average of 20x2 million high-quality (Q20, 99% accuracy of base call) reads per library. After the quality control steps, there were an average of 79.5% reads remaining in each library. The average mapping rate per library reached up to 97.8%. The assembly step generated 39,085 expressed genes (max FPKM≥1) together with 3,641 in the intergenic regions, representing 71.6% of total genes. We further identified DEGs under salt treatment by comparing each time point with time zero. A total of 15,997 and 15,494 DEGs were obtained in the leaf and root, respectively.

Co-expression networks reveal modules with diverse expression patterns under salt treatment
To investigate the interrelationships between salt-responsive genes, we constructed weighted co-expression networks with time-series expressions of all DEGs. A total of 21 modules were obtained in the leaf and 24 modules in the root (Table S1). The patterns of some modules may be correlated to the adaptation processes under salt stress.

In leaf, the biggest module L-M1, containing 5,750 DEGs, exhibited an increasing trend from early to late phase (Table 1). DEGs of L-M1 are mainly enriched in ribosomal proteins, ribosomal biogenesis, protein processing and amino acid metabolism (degradation of lysine, valine, leucine and isoleucine, and biosynthesis of proline), suggesting that protein turnover and changes in amino acid profiles occurred in the leaves of salt-treated seedlings.

The second biggest module L-M2 (3,278 DEGs) exhibited an opposite pattern which showed a consistent decreasing trend to 48h. KEGG enrichment of DEGs in this module revealed that they are mainly involved in the photosynthetic process, carbon fixation and primary nitrogen metabolism.

L-M4 (1,076 DEGs) induced only at 1h and 2h after salt treatment is enriched in genes for plant-pathogen interactions, plant hormone signal transduction pathways, and protein processing in the endoplasmic reticulum (ER) (Table 1). On the other hand, L-M7 (576 DEGs) showed an early-phase repression pattern and the members mainly function in plant hormone signal transduction pathways. These data suggested that in early phase, some phytohormone signals were turned on while some others were turned off.

L-M13 (182 DEGs) and L-M16 (132 DEGs) consist of up-regulated and down-regulated genes, respectively, in the late phase. Zeatin biosynthesis (cytokinin [CK]
degradation) and plant hormone signal transduction-related genes were enriched in the late phase in leaf, while aminoacyl-tRNA biosynthesis-related and ribosomal proteins were down-regulated. The module L-M10 (302 DEGs), showing a biphasic induction (first at 1h, and then again at 24h and 48h), is enriched in genes related to plant-pathogen interactions.

In roots, the largest module R-M1 (2,206 DEGs) consists of genes induced in the early phase. It is enriched in genes for α-linolenic acid metabolism (jasmonic acid [JA] biosynthesis), flavonoid biosynthesis, circadian rhythm, and plant-pathogen interactions (Table 1). R-M3 (2,037 DEGs) was also induced in the early phase with a peak at 1h, comprising genes enriched in starch and sucrose metabolism, cyanoamino acid metabolism, and phenylpropanoid biosynthesis (lignin biosynthesis). It is consistent with previous studies that soluble sugars such as glucose and trehalose, as well as lignin and flavonoid biosynthesis pathways were enhanced as a reaction to salt stress (Das et al. 2017).

The second largest module in root, R-M2 (2,110 DEGs), was induced in the late phase. It contains genes for fatty acid metabolism, protein processing in ER, and phenylpropanoid biosynthesis. Lignin is a major component of secondary cell wall and fatty acids are essential for membrane lipids (Van Acker et al. 2013; Mikami and Murata 2003). The enrichment results suggested that cell wall and cell membrane composition of root might undergo modifications in the late phase of salt stress responses.

R-M6 (1,294 DEGs) showed early repression and is enriched in genes for photosynthesis and plant hormone signal transduction. R-M9 (366 DEGs) showed repression of genes involved in alanine, aspartate and glutamate metabolism and primary nitrogen metabolism, suggesting that nitrogen and amino acid metabolism were affected in salt-treated roots.

R-M11 (186 DEGs) showed an induction pattern only at 48h, and most members function in structural component biosynthesis, e.g. cutin, suberine, and wax. Cuticular wax is a major component of cuticle which plays an important protective role under salt stress (Lee and Suh 2015).

Another interesting module, R-M7 (1,191 DEGs), which showed induction in early phase, is enriched in glutathione metabolism pathway (glutathione redox cycle), indicating that ROS scavenging was activated in salt-treated roots.
Phytohormone signals are involved in expression hubs

To reveal possible key regulators under salt stress, we identified major expression hubs which showed strong association to DEGs in each module. Several of these hubs are related to phytohormone signaling (Fig. 2a, and 2b; Table S2).

In leaf, SKP2 (Glyma.13G036600) (in L-M1) encodes a homologue of an Arabidopsis auxin-binding F-box protein involved in cell division and its degradation is mediated by auxin (Jurado et al. 2010). Glyma.08G142500 (in L-M2) encodes a GATA-type TF which is found to be involved in the cross-talk between GA and auxin in A. thaliana and in regulating nitrogen metabolism in soybean (Richter et al. 2013; Zhang et al. 2015). Lipoxygenase 1 (LOX1) (in L-M13) plays an important role in JA biosynthesis and its expression can be stimulated by abscisic acid (ABA) and methyl-JA (Melan et al. 1993).

In root, Glyma.08G362700 (in R-M1) is the homologue of AtDGL that encodes galactolipase which is involved in the initial step of JA biosynthesis (Hyun et al. 2008). It is consistent with the accumulation of JA in salt-treated roots (Du et al. 2013). GA insensitive DWARF1 (GID1) in R-M6, repressed in the early phase, encodes the receptor of gibberellin (GA), suggesting that GA signaling may be inhibited in early responses toward salt stress in root. The hubs in the module R-M7 include genes encoding ATP-binding cassette (ABC) transporters, Gretchen Hagen 3 (GH3), ABA-insensitive 5 (ABI5) and phospholipase C (PLC), indicating persistent ABA, auxin and PI signals were involved in early salt stress responses in roots (Fig. 2b). ABI5 is a core component of ABA signaling (Skubacz et al. 2016). Its neighbour genes include the auxin response protein GH3, a CK-activating enzyme, CK riboside 5’-monophosphate phosphoribohydrolase (LOG7), and a GA catabolic enzyme, gibberellin 2 beta-dioxygenase 2 (GA2OX2) (Fig. 2b). It supports the notion of cross-talks between ABA signaling and the metabolism of CK and GA under salt stress (Weiss and Ori 2007; Nishiyama et al. 2011).

For the module R-M11, 7 out of 10 hubs were genes encoding FASCICLIN-like arabinogalactan-proteins (FLA). AtFLA4 positively regulates cell wall biosynthesis and root growth by modulating ABA signaling in A. thaliana (Seifert et al. 2014). Moreover, Glyma.10G080000 (in R-M18) encodes a hydroxyproline-rich glycoprotein family protein which is a major structural protein in the cell wall (Table S2). This gene has been shown to be induced by ABA and salt stress in rice (Tseng et al. 2013).

Besides phytohormone signals, there are other hubs that indicate highly coordinated gene expressions. In leaf, the module L-M16 contains multiple hubs encoding ribosomal proteins, including L17, L6, S5, and S10 family proteins, that were repressed in the late phase.
Moreover, there are also hubs involving key metabolic genes, possibly related to a global change in metabolic profiles under salt stress. One hub of the module L-M1 is the gene Glyma.14G211000 encoding isocitrate dehydrogenase (IDH), a key enzyme producing 2-oxoglutarate which is a central metabolite for both C and N metabolism and can be used as an indicator for the status of C-N balance (Lam et al. 1996).

**Complex phytohormone signals triggered by salt stress**

To further dissect signaling reprogramming and to reveal alternate regulations in the early and late phases, we investigated the regulations of phytohormones, ABA, auxin, CK, GA, ethylene, and JA. The changes in the gene expressions of the biosynthesis enzymes for each phytohormone, hormone-responsive genes, and their regulating TFs are shown in Table 2. In general, upon salt treatment, most phytohormone signaling pathways, such as ABA, auxin, CK, GA, JA and ethylene, showed dynamic changes in both the early and late phases in both root and leaf.

Genes for the key enzymes in ABA biosynthesis, 9-cis-epoxycarotenoid dioxygenase (NCED) and ABA-aldehyde oxidase (AAO3), as well as the ABA-responsive genes, RD29B and RD22 (Tuteja 2007) showed induction in both the early and late phases in leaf and root. Moreover, the ABF gene encoding a major TF participating in the ABA signaling pathway (Choi et al. 2000) was induced mainly during the osmotic phase in root but in both the osmotic and ionic phases in leaf (Table 2).

ABA binds to ABA receptors that interact with protein phosphatase 2C (PP2C) to lift the inhibition of SNF1-related kinases (SnRKs) by PP2C. SnRKs then trigger downstream components that are mainly inhibitory in nature (Hubbard et al. 2010). In the guard cell, Open Stomata 1 protein kinase (OST1/SnRK2.6) released from ABI1 (ABI1/PP2C) then activates the S-type anion channel, SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1) to effect stomata closure (Geiger et al. 2009).

The ABI1 protein is a feedback inhibitor of ABA signaling and the ABII gene is induced by an increased ABA level. Our data showed that two G. max ABII homologues (Glyma.05G227100, Glyma08G033800) were induced in the early phase and reached their highest expression levels in the late phase. On the other hand, two of the three soybean homologues of OST1 were repressed in the early phase and induced in the late phase. We also identified two differentially expressed SLAC homolog 2 (SLAH2), Glyma.16G173500 and Glyma.16G173600. The expression of Glyma.16G173500 was greatly reduced in the early phase, and dropped from FPKM 15.7 at 0h to near zero (FPKM 0.43) at 48h (Fig. S1).
Genes encoding essential enzymes of auxin biosynthesis, L-tryptophan-pyruvate aminotransferase (TAA) and flavin-containing monooxygenases (YUCCAs), were repressed in the late phase in root and in both early and late phases in leaf (Table 2). The gene for the auxin-responsive TF, auxin response factor (ARF), was repressed in the late phase in root and in the early phase in leaf respectively (Table 2). Moreover, the auxin responsive genes, auxin/indole acetic acid (AUX/IAA), GH3, and small auxin-up RNA (SAUR) showed dynamic changes in both the early and late phases, indicating the important and complex roles of auxin under salt stress.

The gene encoding the rate-limiting, ADP/ATP-dependent enzyme, isopentenyltransferase (IPT) involved in CK biosynthesis was repressed in both the early and late phases in the leaf. The type-A Arabidopsis Response Regulator genes (type-A ARRs) are marker genes of the CK pathway (Brenner et al. 2012). The down-regulation of type B-ARRs and up-regulation of its inhibitors, type A ARRs, imply the activation of CK signaling in the early phase in leaf and in both phases in root (Table 2).

JA signaling associated with plant growth inhibition is crucial for plants to coordinate growth and adaption to environmental stresses (Wasternack and Hause 2013). GA exhibits antagonistic cross-talks with JA through the modulating interactions between DELLAs and JA ZIM-domain (JAZ) proteins (Hou et al. 2013). Based on our data, the induction of JAZ1 and the repression of DELLA genes suggest that the activation of JA signaling may inhibit GA-mediated growth through downstream TFs (Table 2).

Ethylene also regulates plant responses to salt stress (Tao et al. 2015), by down-regulating Ethylene Insensitive 3-Binding F-Box Protein (EBF1/2), leading to the accumulation of Ethylene Insensitive 3 (EIN3) proteins which subsequently initiate a transcriptional cascade mediated by several ethylene response factors (ERFs) (An et al. 2010). Our data showed a downregulation of EBF1/2 and upregulation of EIN3, supporting the notion that ethylene signaling was activated in the early phase in the root but not in the leaf of salt-treated seedlings (Table 2).

**Shutting down of photosynthesis and carbon assimilation under prolonged salt stress**

In order for the plant to survive and adapt to salt stress, the expression and functioning of genes controlling physiology and metabolism will eventually need to be adjusted. Therefore, we next looked into genes that play central roles in physiology and metabolism.

Photosynthesis is one of the primary processes affected by salt stress (Ghassemi-Golezani and Taifeh-Noori 2011). Compared to untreated controls, the photosynthetic rate of
the leaf of salt-treated seedlings decreased significantly at 1h and then stabilized at a significantly lower level (Fig. 1c).

To understand how the photosynthetic genes responded to salt stress in leaf, we extracted the expression information for genes encoding the proteins involved in the photosynthetic process: photosystem I (PSI), light harvesting complex I (LHCI), photosystem II (PSII), light harvesting complex II (LHCII), and cytochrome b_{6f} (Cyt b_{6f}) (Fig. 3a and 3b, Table S3). The core of the PSII reaction center consists of a heterodimer, the D1 and D2 proteins encoded by PsbA and PsbD, respectively (Caffarri et al. 2014). With the exception of the three homologues of PsbA, the expressions of which showed an increase of 4 folds at 24h, the expressions of most of the other Psb’s decreased by 2-4 folds 24h after salt treatment. The expressions of all six subunits of the PSII-associated LHCII were also down-regulated in the late phase of salt stress (Fig. 3b). LHCII subunit proteins are also called antenna proteins, and they together with PSII, are responsible for photon capture.

Consistent with the PSII and LHCII, the overall gene expressions of both PSI and LHCI were also inhibited in the late phase, probably due to ionic stress (Munns and Tester 2008). The expressions of all the genes encoding the subunits of PSI (Psas) were repressed in the late phase, and PsA and PsB, encoding the large subunits of the core complex in PSI which carries the cofactors for the electron transport chain (Caffarri et al. 2014), were also repressed from 1-2h at the early phase (Fig. 3a). Expressions of most of the genes encoding the Cyt b_{6f} subunits were also repressed in the late phase (Fig. 3a).

Besides having negative effects on the light reaction of photosynthesis, salt shock also blocked carbon assimilation in the dark reaction by impeding the Calvin-Benson cycle (Flexas et al. 2006). When the soybean seedlings were subjected to salt shock, stomatal conductance dropped in the early phase (Fig. 1d), leading to reduced CO_{2} availability to the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Genes encoding the Rubisco large subunit (RbcL, Glyma.12G061600) and one of five Rubisco small subunits (RbcSs, Glyma.19G047000) were repressed at 1h and all five RbcSs were repressed at 24h after stress (Fig. 3c). This is consistent with the lower levels of Rubisco proteins observed under salt stress in soybean (He et al. 2014).

The expression of genes encoding the other four Calvin-Benson cycle enzymes: photosynthetic glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoribulokinase (PRK), fructose-1, 6-bisphosphatase (FBPase), and sedoheptulose-1, 7-bisphosphatase (SBPase), were all down-regulated in the late phase (Fig. 3c).
The repression of genes encoding major components of the photosystems, especially in the late phase, indicated that prolonged salt treatment will eventually shut down this vital energy generating process.

**Enhanced carbon catabolism under salt stress**

We next investigated carbon catabolic pathways which are important for generating metabolic energy by breaking down sugars. Glycolytic GAPDH (GAPC) is a rate-limiting enzyme of the glycolysis pathway (Shestov et al. 2014). The expressions of two GAPDH homologues were induced 2-4 folds at 24-48h under salt treatment (Fig. S2a). In addition, we also calculated the total FPKM values for all isoforms of a few other possible rate-limiting enzymes. We found increase in hexokinase (HK) genes, phosphofructokinase (PFK) genes, while no significant changes in pyruvate kinase (PK) genes in late phase.

We analyzed all genes encoding TCA enzymes which were predicted to localize in mitochondria (through the presence of mitochondrion transit peptide sequences) (Table S3), including citrate synthase (CS; 2 genes), aconitase (1 gene), isocitrate dehydrogenase (IDH; 6 genes), succinyl-CoA synthetase (4 genes), and malate dehydrogenase (MDH; 2 genes) in leaf. Overall, with the exception of MDH (no effect), fumarase (1 homologue being repressed in the late phase and another one repressed in the early phase) and IDH (either no effect on some homologues and repressed for others), all the other genes involved were induced in the late phase. CS is the rate-limiting enzyme of the TCA cycle, and its gene expression increased at both the early and late phases under salt treatment (Fig. S2b). Our results suggest intensified carbohydrate catabolism via glycolysis/TCA cycle, especially under prolonged salt stress.

**Alterations in nitrogen assimilation and amino acid metabolism under salt stress**

In the absence of nitrogen fixation under our experimental conditions, nitrate is the main source of nitrogen for the seedlings, which will be first converted to ammonia by the concerted actions of nitrate reductase (NR) and nitrite reductase (NiR) (Lam et al. 1996). In salt-treated root, the expressions of most of the NR and both of the NiR homologues were repressed at both early and late phases (Fig. S3a, Table S3). Whereas in the leaf, two of the four NR homologues showed a slight induction at 1 and 2h while all NR and NiR homologues were repressed in the late phase. The NiR homologues and two of the four NR homologues were also repressed in the early phase (Fig. S3b). This implies a reduction in the assimilation of nitrogen through nitrate reduction especially in the late phase under salt stress.
Ammonia is primarily assimilated into glutamate through the glutamate synthase (GS)-glutamate synthetase (Ferredoxin [Fd]-GOGAT, NADH-GOGAT) cycle, or alternatively by the glutamate dehydrogenase (GDH) pathway (Coruzzi 2003). The cytosolic GS (GS1)/NADH-GOGAT isoenzymes contribute to the assimilation of non-photorespiratory ammonium, and chloroplastic GS2/Fd-GOGAT are the predominant enzymes in leaf plastids (Suzuki and Knaff 2005; Lam et al. 1996). Based on our data on the root, there is no clear pattern in gene induction/repression with respect to GS1, with an equal split in either direction among its homologues, while three out of four NADH-GOGAT homologues were induced in the early phase. In the leaf, all three GS2 genes were down-regulated in the late phase, along with one of two Fd-GOGAT homologues (Fig. S3b).

Under salt treatment, stress-related ammonia could be accumulated, which may induce the expression of genes encoding GDH or asparagine synthetase 2/3 (ASN2/3) (Melo-Oliveira et al. 1996; Wong et al. 2004). In the root, expression of two out of three GDH homologues were induced around 16 folds at 2h and over 4 folds at 24h (Fig. S3a), while one other was repressed in the late phase. In the leaf, the change in gene expressions of the GDH homologues was much less pronounced, with only one (Glyma.19G111000) being slightly repressed in both early and late phases and no effect on the other two. On the contrary, the effects on ASN2/3 expression were much less significant in both the leaf and the root. There was almost no effect except for a slight induction in the leaf after 48h. This may imply that the effects of salt stress-induced ammonia production were probably neutralized by the action of GDH as an early response in the root, which is the first organ to encounter the excess salt.

Asparagine is one of the key amino acids for the long-range translocation of organic nitrogen from source to sink, in which asparagine synthetase 1 (ASN1) plays an important role in biosynthesis (Wan et al. 2006; Lam et al. 2003) and asparaginase is responsible for catabolism of asparagine (Yabuki et al. 2017).

In the leaf, three of five ASN1 genes were induced in early phase. In late phase, while two of five ASN1 genes were induced, the remaining three were repressed. One ASN1 gene (Glyma.18G061100) was induced over 80 folds at 2h and 48h (based on FPKM value), suggesting that it is a major stress-responsive ASN1 gene. This adjustment may be important to maintain the biosynthesis of asparagine under stress.

In contrast, two out of five genes encoding asparaginase were down-regulated in both the early and late phases in the leaf while the other three were unaffected (Fig. 4). These results suggested that under salt stress, the seedlings will maintain the translocation of organic nitrogen in the form of asparagine to cope with the changes in nitrogen metabolism.
Besides long-range translocation, plant cells could also redistribute their limited nitrogen resources to establish a new cellular metabolic profile (Miller et al. 2010). Glutamate is a key substrate for transaminases that transfer nitrogen to and from amino acids. It is particularly important for redirecting nitrogen resources into different pathways (Brosnan 2000). The expressions of genes encoding aminotransferases were thus analyzed (Fig. 4, Table S3). In the leaf, the genes encoding alanine aminotransferase and branched-chain amino acid aminotransferase for the conversion of alanine and leucine/valine/isoleucine to glutamate were induced in both the early and late phases. In contrast, the genes encoding tryptophan aminotransferase and tyrosine aminotransferase which catalyse tryptophan and tyrosine to glutamate, were found to be repressed in both the early and late phases.

Glutamate is the substrate of two stress-related metabolites: proline and γ-aminobutyric acid (GABA) (Bender 2012). There are two routes for proline synthesis, one involves pyrroline-5-carboxylate synthetase (P5CS) and P5C reductase (P5CR) (Szabados and Savouré 2010; Verbruggen et al. 1993) and the other requires the δ-ornithine aminotransferase (δ-OAT) (Trovato et al. 2008). Our result showed that in the leaf, the P5CS, P5CR and δ-OAT genes were all induced, most of all in the late phase (Fig. 5, Table S3). This adjustment is important for producing the osmolyte, proline, to protect the plant cells from osmotic stress.

GABA is an important intermediate involved in ROS scavenging under abiotic stress (Liu et al. 2011). GABA accumulation is mainly processed by the GABA shunt pathway and alternatively by the polyamine degradation pathway (Yang et al. 2013). Most of the expressions of the genes for the key enzymes, glutamate decarboxylase (GAD) and GABA transaminase (GABA-T), in GABA shunt as well as those for polyamine oxidase (PAO) in the polyamine degradation pathway were induced under salt stress, especially in the late phase in the leaf (Fig. 5).

Due to the impairment of photosynthesis and the increased demand for organic carbon for respiratory energy (see above), the catabolism of some amino acids may be enhanced to provide organic acids. The catabolic genes involved in the initial steps of converting lysine, methionine, and branched amino acids into organic acids were found to be induced mainly in the late phase, and the ones for lysine catabolism were all induced much earlier, starting at 4h and lasting into the late phase (Fig. S4). The genes encoding deaminases and lyases that generate carbon backbones by removing the nitrogen from amino acids, such as threonine deaminase, serine deaminase, and methionine gamma-lyase, were strongly induced at the late
phase (Fig. 4). More comprehensive results on the genes involved in other amino acid metabolic pathways are presented in detail in Fig. S4.

DISCUSSION

It is important to identify major stress tolerance mechanisms in plants to help formulate strategies for crop improvement. To this end, we tracked the transcriptomic reprogramming in soybean seedlings under salt stress over a period of 48h. Based on the physiological changes, the responses can be divided into two main phases: the early osmotic phase (1-4h) that was signified by the sharp drop in stomatal conductance and photosynthesis; and the late ionic phase (24h and beyond) in which the seedlings entered a new physiological state with lower photosynthetic rates and stomatal conductance, followed by the accumulation of Na\(^+\) in the leaf that could be detrimental to the seedlings. The seedlings made physiological and metabolic adjustments to the environmental shock in the early phase and strived for survival in the late phase, through extensive transcriptomic reprogramming.

Co-expression network analyses revealed diverse expression patterns. In general, there are modules that were differentially expressed (induced or repressed) in early, late, or both phases, probably in relation to different adaptive mechanisms. There are expression hubs that are associated with a large number of genes, suggesting their key positions in salt stress responses.

Phytohormone signaling is complex and plays important regulatory roles (Pandey 2017). Components of several phytohormone signaling pathways were found to be enriched in different expression modules (Table 1), in which some exhibited opposite expression trends (e.g. L-M4 versus L-M7 in leaf). Furthermore, some components are present as expression hubs. Taking a further look at the signaling pathways of individual hormones resulted in more insights of the relationship between transcriptomic reprogramming and phytohormone signaling (Table 2).

The seedlings faced a dilemma with respect to stomatal closure. While closing the stomata could be an immediate adaptive response for protection against salt shock by reducing water loss, soybean seedlings also need to at least partially resume transpiration and photosynthesis by re-opening them. ABA is a major regulator of stomatal closure and the ABA signaling pathway is well known to be associated with responses to environmental stresses (Fernando and Schroeder 2016). Here we observed the induction of genes for ABA biosynthesis and ABF, a major downstream TF, as well as other ABA response genes such as \(RD29B\) and \(RD22\) (Table 2). It explains how the stomatal conductance was kept low through
the stress period. ABA signals are regulated by feedback inhibitors, such as \textit{ABII} homologues that are induced by high level of ABA (Merlot et al. 2001). Our data suggested that the level of ABA could have continued to build up to induce the expression of \textit{ABII} genes. In the early phase, the \textit{ABII}-mediated negative feedback mechanism, together with the repression of \textit{OSTI} and \textit{SLAH2} genes, would facilitate the re-opening of stomata (Fig. S1). However, such feedback regulations might have broken down in the late phase or superseded by other response mechanisms.

In addition, some ABA signaling pathway components are present in the expression hubs that suggest cross-talks with other phytohormone pathways, e.g. \textit{ABI5} in the module R-M7 is associated with metabolic genes of CK and GA (Fig. 2).

Auxin and CK are important growth regulators. Due to the interruption of growth and development under salt shock, the auxin and CK signaling pathways are expected to make adjustments. There was a general repression of auxin biosynthetic genes, especially in the leaf. This observation is consistent with the reduction in free auxin under salt shock (Du et al. 2013; Junghans et al. 2006). Auxin signaling is mediated by ARF and inhibited by AUX/IAA. Activation of the auxin signaling pathway can be achieved by the degradation of AUX/IAA to release the inhibition on \textit{ARF} (Kazan 2013). Here according to our data from the root, \textit{ARFs} were repressed while \textit{AUX/IAAs} were induced, suggesting a reduction in auxin signals (Table 2).

The induction of type-A \textit{ARR} genes probably further attenuated auxin-dependent cell divisions in the root meristem. Both type-A ARRs and type-B ARRs can perceive the CK signal, and type-B ARRs are TFs that directly promote the expression of nuclear type-A \textit{ARR} and other CK-regulate genes. The type-A ARRs then negatively regulate cell divisions (Hwang et al. 2012). The activation of type-A \textit{ARRs} implied the activation of CK signaling in the root in both the early and late phases, which was consistent with our observation of auxin signaling inhibition in the root at these time points (Table 2).

Besides the cross-talks between auxin and CK signaling, our data also demonstrated the cross-talks between auxin and GA signaling. For instance, a GATA-type TF is present as an expression hub in the module L-M2 (Fig.2 and Table S2). It is consistent with the previous finding in \textit{A. thaliana} that GATA-type TFs are involved in cross-talking between auxin and GA signaling (Richter et al. 2013).

\textit{JA} are lipid-derived signaling molecules involved in salt stress responses. The up-regulated expression of \textit{JAZ1} in the early phase suggested that JA signaling was activated in response to osmotic stress. In Arabidopsis, salt stress causes the JA-mediated inhibition of
root cell elongation in the early response (Valenzuela et al. 2016). JA also displays both synergistic and antagonistic effects with other plant hormones such as auxin, ethylene and ABA (Wasternack 2007).

Here, the gene for a JA biosynthetic enzyme, LOX1, appeared as a hub in the module L-M13, which, together with the first-neighbour gene, IAA15 (auxin responsive protein), indicates the coordination of JA and auxin signaling in the leaf in the late phase of salt stress responses (Fig. 2). JA and auxin share a similar regulatory mechanism via Skp/Cullin/F-box (SCF)-dependent proteasome degradation, with a specific F-box protein for each hormone. It has been reported that JA mediates lateral root formation in an auxin-dependent pathway by triggering the gene expressions of the auxin biosynthesis enzyme, ASA1, and the auxin transporter, PIN2 (Sun et al. 2011; Sun et al. 2009).

Translational control is one possible mechanism in salt stress responses (Park et al. 2012). Ribosomal proteins have been reported to be transcriptionally affected under abiotic stresses and it has been proposed that this leads to changes in ribosomal compositions (Xie 2016; Sormani et al. 2011). Our data revealed that most of the ribosomal protein genes were induced in the leaf in the late phase (module L-M1) while the expressions of ribosomal proteins S10, L29, L24, S13, and L35 (L-M16) showed a reduction in 24h (Table 1 and Table S2). Alterations in the composition of ribosomal proteins could significantly affect translation of other proteins (Xue and Barna 2012).

Unlike the complex nature of phytohormone gene expressions on their respective signaling pathways, our transcriptomic analyses revealed a strong correlation between the expressions of metabolic genes and metabolic adjustments. In general, biosynthetic genes for carbon and nitrogen primary assimilation were repressed and catabolic genes were induced, especially in the late phase.

Gene encoding the components of both the light and dark reactions of photosynthesis were slightly repressed or maintained in the early phase (Fig. 3). The seedlings were still trying to continue with photosynthesis and this was consistent with the partial re-opening of the stomata and partial recovery from leaf drooping.

Expression of genes in the carbon catabolic pathways were found to be induced. The glycolytic GAPDH genes were up-regulated starting at 1h and remained elevated throughout. Using computational modeling and statistical simulations in combination with metabolomics, GAPDH has been proposed to be a rate-limiting enzyme in glycolysis (Shestov et al. 2014). Most genes encoding the TCA cycle components were induced after 2h and reached their highest expression levels in the late phase (Fig. S2b). The TCA cycle is an oxidative
phosphorylation process that generates energy by breaking down sugars, and the intermediates of the TCA cycle also provide carbon skeletons for amino acid synthesis (Fernie et al. 2004). The accumulation of intermediates of the TCA cycle was observed in different metabolomics analyses under drought stress, salt stress, and oxidative stress (Obata and Fernie 2012; Lu et al. 2013; Urano et al. 2009). The enhanced carbon catabolism might be a strategy to produce energy required by the protective mechanisms (Chaves et al. 2009).

In the absence of nitrogen fixation, nitrate and nitrite reduction catalyzed by NR and NiR are important steps to provide ammonia for the GS-GOGAT cycle (Masclaux-Daubresse et al. 2010). We found here that most of the NR genes and NiR genes were turned off in the late phase. In the early phase, there was a slight induction of GS1 and NADH-GOGAT genes in root, suggesting that the seedlings were trying to maintain primary nitrogen assimilation, at least in the early phase of salt stress responses. However, prolonged salt stress would turn off the expressions of most GS2 and Fd-GOGAT genes and hence shut down primary nitrogen assimilation in the late phase in the leaf. As partial compensation, genes for alternative ammonia assimilation, such as ASN2/3 in leaf and GDH in root were induced (Fig. S3).

When primary nitrogen assimilation was severely reduced, redistribution of nitrogen is an important strategy to maintain survival. Long-range translocation of nitrogen was enhanced by inducing the ASN1 gene which produced asparagine to carry nitrogen from source to sink (Fig. 4). In soybean, asparagine is a major form of soluble nitrogen which is synthesized in the leaf and transported to the root via the phloem (Ohyama et al. 2017). Redistribution of nitrogen resources could also be achieved by altering the expressions of genes encoding specific transaminases (Fig. 4). To cope with providing enough organic carbon for cellular respiration, the carbon skeletons of some amino acids might have been selectively broken down due to the induction of specific deaminase and lyase genes (Fig. 4, Fig. S4). Our results are consistent with the previously reported massive change in amino acid profiles under stress (Das et al. 2017; Zhang et al. 2016).

Proline and GABA are two derivatives from glutamate that play a role in osmoregulation and ROS scavenging, respectively. They occur widely in higher plants and accumulate in salt-stressed plants (Ábrahám et al. 2003; Xing et al. 2007). While osmotic stress is a result of low intracellular osmotic potential in a high-salt environment, oxidative stress is a secondary stress due to salt treatment (Munns and Tester 2008). Biosynthetic genes for the osmolyte, proline, were mainly induced in the leaf and those for GABA were induced in both the leaf and root, indicating that in the soybean seedlings, proline is mainly involved
in osmoregulation in the leaf and GABA is involved in ROS scavenging in both the leaf and root (Fig. 5 and S5).

In summary, using our time-series transcriptomic dataset, we successfully identified the interplay between important phytohormone signaling networks and illustrated some of the signaling cross-talks in soybean when it is put under salt stress. Adjustments in primary C and N metabolism were also observed and could be largely explained by gene expression reprogramming.

ACKNOWLEDGEMENTS
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CONFLICT OF INTEREST
The authors have no conflict of interest to declare.
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association in rice. Plant Physiology 159, 1111-1124.


<table>
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<tr>
<th>Module name</th>
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<th>KEGG-enriched (FDR ≤ 0.05)</th>
<th>Expression trends *</th>
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<td>Ribosome</td>
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<tr>
<td></td>
<td></td>
<td>Ribosome biogenesis in eukaryotes</td>
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<tr>
<td></td>
<td></td>
<td>Lysine degradation</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Valine, leucine and isoleucine degradation</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Purine metabolism</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Arginine and proline metabolism</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Protein processing in endoplasmic reticulum</td>
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<td></td>
<td></td>
<td>Carbon fixation in photosynthetic organisms</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Metabolic pathways</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Porphyrin and chlorophyll metabolism</td>
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<td></td>
<td></td>
<td>Carbon metabolism</td>
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<tr>
<td></td>
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<td>Pentose phosphate pathway</td>
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<td>Plant hormone signal transduction</td>
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<td></td>
<td></td>
<td>Glyoxylate and dicarboxylate metabolism</td>
<td></td>
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<td></td>
<td></td>
<td>Nitrogen metabolism</td>
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<tr>
<td></td>
<td></td>
<td>Fructose and mannose metabolism</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oxidative phosphorylation</td>
<td></td>
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<td>L-M4</td>
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<tr>
<td></td>
<td></td>
<td>Plant hormone signal transduction</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Protein processing in endoplasmic reticulum</td>
<td></td>
</tr>
<tr>
<td>L-M7</td>
<td>576</td>
<td>Plant hormone signal transduction</td>
<td><img src="image4" alt="Graph" /></td>
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</tbody>
</table>
| R-M1  | 2,206 | alpha-Linolenic acid metabolism  
|              |   | Flavonoid biosynthesis  
|              |   | Circadian rhythm - plant  
|              |   | Plant-pathogen interaction  
|              |   | Linoleic acid metabolism  
| R-M2  | 2,110 | Fatty acid metabolism  
|              |   | Fatty acid biosynthesis  
|              |   | Protein processing in endoplasmic reticulum  
|              |   | Biosynthesis of secondary metabolites  
|              |   | Phenylpropanoid biosynthesis  
|              |   | Monoterpenoid biosynthesis  
|              |   | Phenylalanine metabolism  
| R-M3  | 2,037 | Plant-pathogen interaction  
|              |   | Starch and sucrose metabolism  
|              |   | Cyanoamino acid metabolism  
|              |   | Phenylpropanoid biosynthesis  
| L-M10 | 302  | Plant-pathogen interaction  
| L-M13 | 182  | Zeatin biosynthesis  
|              |   | Plant hormone signal transduction  
| L-M16 | 132  | Aminoacyl-tRNA biosynthesis  
|              |   | Ribosome  
|
**Photosynthesis**
Photosynthesis - antenna proteins
Plant hormone signal transduction

**Glutathione metabolism**

**Alanine, aspartate and glutamate metabolism**
Nitrogen metabolism

**Cutin, suberine and wax biosynthesis**

**Plant-pathogen interaction**

*Expression value was Z-scaled Log₂ (FPKM+1). Blue line indicates the average expression value of the members at each time point.*
Table 2 Changes in the expression of hormone biosynthesis enzymes and regulating genes under salt treatment

<table>
<thead>
<tr>
<th>Phyto-hormones</th>
<th>Key enzymes in hormone biosynthesis*</th>
<th>Transcriptionally responsive genes*</th>
<th>Regulating transcription factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABA</td>
<td>NCED + / (+)</td>
<td>RD29B + / +</td>
<td>ABF in leaf ABF in root</td>
</tr>
<tr>
<td></td>
<td>AAO3 + / +</td>
<td>RD22 + / +</td>
<td></td>
</tr>
<tr>
<td>Auxin</td>
<td>TAA - / -</td>
<td>Aux/IAA ± / ±</td>
<td>ARF in leaf ARF in root</td>
</tr>
<tr>
<td></td>
<td>YUCCA - / -</td>
<td>GH3 ± / ±</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>SAUR ± / ±</td>
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<tr>
<td>CK</td>
<td>IPT - / ±</td>
<td>HK2β - / ±</td>
<td>B-ARR in leaf B-ARR in root</td>
</tr>
<tr>
<td></td>
<td>LOG ± / ±</td>
<td>Type A-ARR ± / ±</td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>KS ± / ±</td>
<td>GA2OX + / +</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>GID1 + / -</td>
<td>DELLA - / ±</td>
<td></td>
</tr>
<tr>
<td>JA</td>
<td>LOX ± / ±</td>
<td>OPR3 + / +</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JAZ1 + / +</td>
<td></td>
</tr>
<tr>
<td>Ethylene</td>
<td>ACO ± / ±</td>
<td>ACS ± / ±</td>
<td>ERF in leaf ERF in root</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EBF1/2 - / +</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>EIN2 + / +</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>EIN3 - / ±</td>
<td></td>
</tr>
</tbody>
</table>

* Key enzymes and regulating transcription factors may vary under salt treatment conditions.
* “+”: up-regulated genes; “−”: down-regulated genes; “±”: both up- and down-regulated genes exist. The four symbols under each gene represent directional changes in expression in: early phase in leaf, late phase in leaf, early phase in root, and late phase in root, respectively. ABA, abscisic acid; CK, cytokinins; GA, gibberellin; JA, jasmonic acid, NCED, 9-cis-epoxycarotenoid dioxygenase; AAO3, abscisic-aldehyde oxidase; RD29B, dehydration responsive protein 29B; RD22, dehydration responsive protein 22; ABF, ABA-responsive element binding factors; TAA, tryptophan aminotransferase of Arabidopsis; YUCCA, flavin-containing monooxygenase; Aux/IAA, auxin/indole acetic acid protein; GH3, Gretchen Hagen 3; SAUR, small auxin-up RNA; ARF, auxin response factor; IPT, isopentenyltransferase; LOG, riboside 5’-monophosphate phosphoribohydrolase; HK2/3, histidine kinase 2/3; TypeA-ARR, typeA-Arabidopsis response regulator; KS, ent-kaurene synthase; GA2OX, gibberellin 2 beta-dioxygenase; GID1, GA insensitive DWARF1; LOX, lipoxygenase; OPR3, 12-oxophytodienoate reductase 3; JAZ1, jasmonate-zim-domain protein 1; ACO, 1-aminocyclopropane-1-carboxylate oxidase; ACS, aminocyclopropane-1-carboxylic acid synthase; EBF1/2, EIN3-binding F-box protein 1/2; EIN2, ethylene-insensitive protein 2; EIN3, ethylene-insensitive protein 3; ERF, ethylene response factor.
Fig. 1 Physiological responses of soybean seedlings under salt stress. Soybean seedlings were either untreated or stressed with 0.9% NaCl. (a) Thermal images; (b) Estimated leaf surface temperatures (t-test, *: p ≤ 0.05; **: p ≤ 0.01); (c) Photosynthesis rates; (d) Stomata conductance; (e) Na⁺ and K⁺ contents in leaves; and (f) Na⁺ and K⁺ contents in roots. (b)-(f) Each bar represents means ± s.d., n=4.
Fig. 2 Selected expression hubs identified in co-expression networks. The schematic representation of selective expression hubs of modules in the leaf (a): L-M1, L-M2, and L-M13, and in the root (b): R-M1, R-M6; and R-M7 are shown. Hub genes are in yellow and edges of hubs are shown in red. Representative neighbour genes are highlighted in blue. For ease of visualization, subnetworks of hub genes and their neighbours in L-M1, L-M2, and R-M1 with edges carrying weights larger than 0.85 were extracted. For L-M13, R-M6, and R-M7, the whole network was zoomed in, and the hubs and surrounding nodes and edges were screen-captured and shown. SKP2, S-phase kinase-associated protein 2; EIF2A, eukaryotic translation initiation factor 2A; CS, citrate synthase; TAF5, transcription initiation factor TFIID subunit 5; SDH1, succinate-semialdehyde dehydrogenase flavoprotein subunit; RPB4, DNA-directed RNA polymerase II subunit RPB4; GATA18, GATA-type zinc finger transcription factor 18; GDC-H3, glycine cleavage system H protein 3; GAPA, glyceraldehyde-3-phosphate dehydrogenase; AMT, aminomethyltransferase; LOX1, lipoxygenase 1; ERF16, ethylene responsive transcription factor 16; IAA15, auxin responsive protein 15; GRX, glutaredoxin; DGL, DONGLE; EXP8, expansin-A8; LEA2, late embryogenesis abundant protein 2; ERF18, ethylene responsive transcription factor 18; PAO3, polyamine oxidase 3; GID1, GA-INSENSITIVE DWARF1; GRXC10, glutaredoxin-C10; FAM213A, family with sequence similarity 213 member A; ATL67, Arabidopsis Tóxicos en Levadura 67; ABI5, ABA-insensitive 5; LOG7, LONELY GUY 7; GA2OX2, gibberellin 2-beta-dioxygenase 2; PLC, phospholipase C; GH3, Gretchen Hagen 3; ABCG21, ABC transporter G family member 21; PP2C27, protein phosphatase 2C 27.
Fig. 3 NaCl-stress induced changes in the expression profiles of key photosynthetic genes. Gradient colours indicate log2 fold-change (FC) in gene expressions at different time points (0h, 1h, 2h, 4h, 24h, and 48h) compared to control (0h) in the leaf. (a) Heatmaps of genes involved in photosystem I; (b) Heatmaps of genes in photosystem II; (c) Heatmaps of genes in the Calvin-Benson cycle. Different rows in each heatmap represent different annotated homologues of the same gene. PSI, photosystem I; PSII, photosystem II; LHCI, light harvesting complex I; LHCII, light harvesting complex II; RbcS, Rubisco small subunit; RbcL, Rubisco large subunit; PGK, phosphoglycerate kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TPI, triose-phosphate isomerase; FBA, fructose 1,6-bisphosphate aldolase; FBPase, fructose-1,6-bisphosphatase; TRK, transketolase; SBPase, sedoheptulose-1,7-bisphosphatase; RPE, ribulose-5-phosphate 3-epimerase; RPI, phosphoribosylisomerase; PRK, phosphoribulokinase.
Fig. 4 NaCl-stress induced changes in the expression profiles of key amino acid metabolic genes. Expression heatmaps of genes involved in the first step of methionine, threonine, serine, glycine, leucine, valine, isoleucine, lysine, alanine, tryptophan, asparagine, and tyrosine catabolism in the leaf are shown. Gradient colours indicate log2 FC in gene expressions at different time points (0h, 1h, 2h, 4h, 24h, and 48h) compared to control (0h). Different rows in each heatmap represent different annotated homologues of the same gene. ASN1, asparagine synthetase 1, GDC, glycine decarboxylase complex; SHMT, serine hydroxyl methyltransferase.
Fig. 5 NaCl-stress induced changes in the expression profiles of the biosynthetic genes of glutamate-derived proline and γ-aminobutyric acid (GABA). Expression heatmaps of genes involved in proline and GABA biosynthesis are shown. Gradient colours indicate log2 FC in gene expressions at different time points (0h, 1h, 2h, 4h, 24h, and 48h) compared to control (0h) in the leaf. Different rows in each heatmap represent different annotated homologues of the same gene. P5CS, pyrroline-5-carboxylate synthetase; δ-OAT, ornithine-δ-aminotransferase; GSA, glutamic-γ-semialdehyde; P5C, 1-pyrroline-5-carboxylic acid; P5C reductase, pyrroline-5-carboxylate reductase; HP, hydroxyl proline; GAD, glutamate decarboxylase; GABA-T, GABA transaminase; SSADH, succinate-semialdehyde dehydrogenase; ODC, ornithine decarboxylase; PAO, polyamine oxidase; SPDS, spermidine synthase; SPMS, spermine synthase.