Transcription profile analysis identifies marker genes to distinguish salt shock and salt stress after stepwise acclimation in *Arabidopsis thaliana* and *Zea mays*

Wenting Zhao* , Stephan Jung, Sven Schubert

Institute of Plant Nutrition (iFZ), Justus Liebig University, Heinrich-Buff-Ring 26-32, 35392, Giessen, Germany

ARTICLE INFO

Keywords: *Arabidopsis thaliana*  
Marker genes  
Salt shock  
Stepwise acclimation  
Turgor pressure  
*Zea mays*

ABSTRACT

Many physiological and molecular responses to salt stress have been investigated after a salt shock. However, salt shock rarely happens in agricultural practice. In the field, salts accumulate gradually due to poor agricultural management. Thus in salinity research, it is more reasonable to investigate plant reaction after stepwise acclimation to salt stress. Previous studies demonstrate that salt shock induces Phase 0, a short-term effect that shows transient water loss and rapid turgor decrease; salt stress after stepwise acclimation avoids Phase 0 effects and induces Phase 1. During Phase 1, plants show maintenance of turgor. In this study, salt shock and stepwise acclimation to salt stress were separated at physiological and transcriptional levels. Four major experiments were conducted: 1) leaf turgor changes were monitored in real-time after salt application to separate Phase 0 and Phase 1 effects at the physiological level, 2) RNA-sequence analysis was conducted in *Arabidopsisthaliana* L. to identify potential marker genes that are involved in plant water relations to distinguish Phase 0 and Phase 1 at transcript level, 3) these selected marker gene candidates were identified in Arabidopsis at different Phase 0 and Phase 1 time points via qRT-PCR, 4) these candidates were further evaluated in *Zea mays* L. (a model plant for applied research in plant physiology and an important crop plant) via qRT-PCR. In future salinity research, marker genes that are both applicable in Arabidopsis and maize have the potential to differentiate salt shock and stepwise acclimation to salt stress.

1. Introduction

Salinity is considered a major threat to crop yield worldwide because most crops are sensitive to salt stress (Munns and Tester, 2008). Several practical strategies have been developed to alleviate damages caused by salt stress on crops, including conventional plant breeding (Schubert et al., 2009) and transgene technology (Roy et al., 2014). However, many approaches have not successfully increased crop yield or salt resistance. One reason for this failure might be that most physiological and molecular responses to salt stress are investigated after salt shock where plants are suddenly exposed to high levels of salt. However, salt shock does not usually occur in agricultural practice (Shavrukov, 2013). In the field, salts accumulate gradually because of high-salt irrigation water and poor agricultural management that allows ground water to rise to soil surface (Rengasamy, 2006). Therefore, it is more conducive to investigate salt stress in a way that allows plants to stepwise acclimate to salt treatment like in the field.

According to Munns (1993) and Munns and Tester (2008), plants suffer salt stress in two phases: one is an early, also called osmotic phase (Phase 1) that physiologically inhibits growth of young leaves; the other is a late ion-toxicity phase (Phase 2) that accelerates senescence in mature leaves. In addition, Schubert (2011) suggested to add a transient phase (Phase 0) before Phase 1 under salt shock situations. During Phase 0, plants show transient decreases in turgor and growth rate in the first few minutes and hours after the imposition of salt. These changes are transient and recover after some time (Thiel et al., 1988; Munns, 1993). The extent of recovery depends on the levels of applied salts, the sensitivity of plants, and the salt acclimation capacity of plants (Munns and Tester, 2008). It is important to avoid Phase 0 effects in salinity research because its transient character is not useful in plant breeding (Schubert, 2011). During Phase 1, ion accumulation surrounding the root medium reduces the water availability to plants and...
plants suffer osmotic stress (Munns and Tester, 2008; Vetterlein et al., 2004). Plant growth is inhibited during this phase and the growth inhibition showed in this phase is similar to that induced by other osmotica such as PEG, mannitol, and KCl (Munns, 1993). Thus, this effect is not salt-specific. During this phase, carbohydrate supply is sufficient (Schubert, 2011) and turgor is maintained because of osmotic adjustment (De Costa et al., 2007; Thiel et al., 1988). However, Sümér et al. (2004) found that Na+ also contributes to the decreased growth in maize during Phase 1. During Phase 2, plants develop chlorosis or even necrosis resulting from ion toxicity (Munns and Tester, 2008).

Turgor is the driving force of plant cell expansion and elongation (Cosgrove, 2005). In earlier research, turgor was calculated by measuring the total water potential and osmotic potential separately via psychrometer and osmometer, respectively. In those methods, tissues under test are destroyed, which inevitably impacts the following research. Recently, a novel method conducted by ZYM YARA water sensors has been developed which can measure turgor pressure in real time without damaging the plants (Zimmermann et al., 2008). The maximum value of recorded patch pressure (Pp) occurs around noon and is a potential parameter to determine whether plants are suffering a water deficit. Under water stress, the Pp value increases. This parameter has been successfully used in grapevines (Westhoff et al., 2009), wheat (Bramley et al., 2013), and Arabidopsis (Ache et al., 2010) under experimental and field conditions.

In the early stages of salt stress (Phase 0 and Phase 1), the plant’s first response is to alleviate salt stress-induced water deficiency by reducing leaf water loss (Deinlein et al., 2014). Leaf transpiration and leaf hydraulic conductance are two key processes that mediate leaf water loss (Defraeye et al., 2014; Kosma et al., 2009; Pou et al., 2013). When plants experience water stress, they reduce the leaf water loss mainly by decreasing stomatal transpiration through closing stomata (Defraeye et al., 2014). The rest of transpired leaf water loss is determined by cuticular transpiration (Kosma et al., 2009). The cuticle is a hydrophobic layer on epidermal cells, comprised of two major components (cutin and cuticular waxes). It has been demonstrated that water deficit increases the amount of cuticular wax per unit area and leaf cuticle thickness in Arabidopsis plants to enhance their resistance (Li et al., 2012). In addition, the efficiency of water transport within leaves is reduced under water stress conditions (Pou et al., 2013), and it is closely related to the transcript abundance of aquaporins in sunflowers (Nardini et al., 2005), walnuts (Cochard et al., 2007), and grapevines (Pou et al., 2013).

According to Munns and Tester (2008) and Schubert (2011), turgor pressure decreases dramatically after salt shock (Phase 0) and is maintained after stepwise acclimation to salt stress (Phase 1). Our first goal of this study was to distinguish these two types of salt stress at physiological level. For this purpose, turgor pressure changes were measured in a non-invasive online-monitoring way via ZYM YARA water sensor in Arabidopsis thaliana (a model plant for genetics and molecular biology) and Zea mays (an important crop plant). In a second step, to separate Phase 0 and Phase 1 at transcriptional level, RNA-sequence analysis was conducted in Arabidopsis to select marker gene candidates that are highly and specifically affected by each type of salt stress. Third, only genes involved in the regulation of plant water relations were selected as marker genes in Arabidopsis to avoid genes that are related to Phase 2 (ion toxicity) getting involved. To test whether the marker candidates allow for differentiation between Phase 0 and Phase 1, they were first evaluated in Arabidopsis by means of qRT-PCR at different time points of Phase 0 and Phase 1, and then validated in maize plants via qRT-PCR. We hypothesized that there are marker genes which can be used to differentiate Phase 0 (salt shock) from Phase 1 (stepwise acclimation to salt stress).

2. Materials and methods

a) Plant materials and growth conditions

Abacidopsis thaliana L. (Columbia-0, salt-sensitive ecotype) and Zea mays L. (cv. Pioneer 3906, salt-resistant hybrid) were used throughout the study. Arabidopsis was cultivated with two patterns in a growth chamber; one was 8 h light/16 h dark short-day cycle; another one was 13 h light/11 h dark long-day cycle; both were 21°C (200 μmol m−2 s−1) in the light and 18°C (0 μmol m−2 s−1) in the dark. Maize was cultivated at 26°C (200 μmol m−2 s−1) for 16 h and 18°C (0 μmol m−2 s−1) for 8 h in a growth chamber. Arabidopsis was cultivated in hydroponic culture including 1 mM KH2PO4, 0.25 mM K2SO4, 1 mM MgSO4, 2 mM Ca(NO3)2, 50 μM KCl, 5 μM MnSO4, 1 μM ZnSO4, 1 μM CuSO4, 0.1 μM NiSO4, 0.7 μM (NH4)2MoO4, 30 μM H3BO3, and 100 μM FeNaEDTA. Maize was grown in hydroponic culture with 2 mM Ca(NO3)2, 1 mM K2SO4, 0.75 mM MgSO4, 2 mM CaCl2, 0.2 mM KH2PO4, 0.2 mM FeNaEDTA, 2 μM MnSO4, 0.5 μM ZnSO4, 0.3 μM CuSO4, 0.1 μM NiSO4, 0.01 μM (NH4)2MoO4, and 1 μM H3BO3.

b) Long-term leaf turgor pressure measurements

To induce Phase 0 (salt shock), 5-week-old Arabidopsis plants were treated with 105, 125, and 200 mM NaCl shocks, respectively; 2-week-old maize plants were exposed to 200 mM NaCl. To induce Phase 1 (stepwise acclimation), 90 mM NaCl (15 mM increments per day) and 100 mM NaCl (25 mM increments per day) were applied stepwise to Arabidopsis plants; 100 mM NaCl (25 mM increments per day) were added gradually to maize plants.

The relative turgor pressure was monitored by ZYM YARA water sensors which were clipped to leaves via two magnets. The clamp pressure (Pclamp) exerted by magnets is constant during the measurements and can be adjusted by screwing the movable magnet. The leaf turgor pressure (Pp) is opposed to clamp pressure in the leaf patch. The output of sensors (patch pressure, Pp) is a power function of the turgor pressure Pp (Zimmermann et al., 2008):

\[ Pp = \left( \frac{b}{aPc + b} \right)^2 \times Fa \times Pclamp \]  

(1)

Among these, a and b are constants. Fp is a leaf-specific attenuation factor. Equation (1) shows that the recorded Pp pressure is inversely correlated with leaf turgor pressure Pp, which means that Pp is large when Pp is small and vice versa.

Sensors were attached to fully expanded rosette leaves of Arabidopsis and the 4th leaves of maize to measure leaf turgor pressure in real time. Moreover, sensors were applied to the leaves 2 days prior to the treatments for accommodation. Due to individual compressibility of leaves and pressure applied through sensors, the initial Pp value varied among leaves.

c) Quantification of cation and anion concentrations

Five-week-old Arabidopsis thaliana plants were harvested after 2 days of 90 mM NaCl treatment (stepwise acclimated, 15 mM per day). Plant shoots and roots were harvested and dried at 80°C separately. In order to determine Na+, Ca2+, K+, and Mg2+ concentrations, samples were prepared by dry-ashing (550°C) overnight and cations were measured with an atomic absorption spectrophotometer (SpectraAA220 FS, Varian, Mulgrave, Victoria, Australia).

d) RNA sequence

RNA sequence was performed by Max Planck Institute for Heart and Lung (Bad Nauheim, Germany). The total RNA was isolated from rosette leaves according to manufacturer protocols of RNeasy Plant Mini Kit (Qiagen). Genomic DNA was removed using RNase-Free DNsSet (Qiagen) to avoid contamination. Four milligrams of total RNA were used as input for Truseq Stranded mRNA Library preparation following the low sample protocol (Illumina). Sequencing was performed on the NextSeq500 instrument (Illumina) using v2 chemistry, resulting on
average in 30 Mb reads per library with 1 × 75 bp single end setup. The resulting raw reads (Table S1) were assessed for quality, adapter content and duplication rates with FastQC (FastQC: a quality control tool for high throughput sequence data. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Reaper version 13–100 was employed to trim reads after a quality drop below a mean of Q20 in a window of 10 nucleotides. Only reads between 30 and 150 nucleotides were cleared for further analyses. Trimmed and filtered reads were aligned versus the Ensembl Arabidopsis genome version TAIR10 (version 34) using STAR 2.4.0a with the parameter “--outFilterMismatchNoverLmax 0.1” to increase the maximum ratio of mismatches to mapped length to 10%. The number of reads aligning to genes was counted with featureCounts 1.4.5-p1 tool from the Subread package. Only reads mapping at least partially inside exons were...
admitted and aggregated per gene. Reads overlapping multiple genes or aligning to multiple regions were excluded. Differentially expressed genes were identified using DESeq2 version 1.62. Only genes with $|\log_2 \text{FoldChange}| \geq 1$, false discovery rate (FDR) $\leq 0.05$, and a minimum combined mean of five reads were deemed to be significantly differentially transcribed. The Ensemble annotation was enriched with UniProt data (release 06.06.2014) based on Ensembl gene identifiers (Activities at the Universal Protein Resource (UniProt)). According to the regularized log transformation (R-Log transformation) in DESeq2, the replicates in each group work well (Supplemental Fig. S1). The RNA-sequence data can be found in the NCBI Gene Expression Omnibus (GEO) database under accession number GSE113138. It is currently

Fig. 2. Concomitant recordings of $P_p$ in Arabidopsis thaliana (A, B) and Zea mays (C) during Phase 1. Arrows indicate NaCl-adding points; open circles indicate the maximum $P_p$ values ($P_m$ values) during the day. White blanks represent the day; black blanks represent the night.
A/C. 100 mM NaCl were added to Arabidopsis (A) and maize (C) with a stepwise acclimation (25 mM per day).
B. 90 mM NaCl were added to Arabidopsis by a stepwise acclimation (15 mM NaCl per day). Representative readings of three biological replicates.
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e) qRT-PCR

Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen), and was reversely transcribed with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer protocols. Real-time PCR was performed in a StepOnePlus system (Applied Biosystems). Relative quantification of gene expression in mRNA level was calculated using equation by $2^{-\Delta\Delta Ct}$. $\Delta Ct$ is the target gene Ct subtracted by the geometric mean of the endogenous reference genes Ct. At-actin2 and Zm-GaPDh were considered as endogenous reference genes in Arabidopsis thaliana and Zea mays, respectively. Primers used for qRT-PCR are listed in Table S2.

f) Statistical analysis

Turgor measurements and RNA-sequence analysis were conducted in three replicates. The shoot fresh weight, shoot dry weight, cation concentrations, and qRT-PCR were conducted in four biological replicates. In the diagrams, means ± standard errors (SE) are given. The t-test was carried out and significant differences are indicated by asterisks ($^* P < 5\%$, **$P < 1\%$, ***$P < 0.1\%$, ****$P < 0.01\%$). One-way ANOVA with Post-hoc analysis using Tukey’s Honestly Significant Difference (HSD) method (SciStatCalc: http://scistatcalc.blogspot.com/2013/11/home.html) was conducted and significant differences are indicated by different letters ($P < 5\%$).

3. Results

3.1. Diurnal changes of leaf turgor pressure in phase 0

The relative leaf turgor changes were measured in real-time by ZIM YARA water sensors in a non-invasive way. After 2 weeks, the leaf patches beneath the sensors were brighter than the surroundings but had no signs of necrosis both in Arabidopsis thaliana and Zea mays.
To induce Phase 0 in Arabidopsis plants, salt shocks induced by 105 mM, 125 mM, and 200 mM NaCl were conducted. When 200 mM NaCl were added to Arabidopsis plants, \( P_p \) increased rapidly within minutes but could not recover from this shock (Fig. 1A). Similarly, \( P_o \) could not recover from 125 mM NaCl salt shock in Arabidopsis either (Supplemental Fig. S3). When the strength of treatment was reduced to 105 mM NaCl, \( P_p \) value showed a rapid rise, just like after the 200 mM and 105 mM NaCl shocks, and reached its maximum value after 1 h (Fig. 1B). After the peak, \( P_p \) recovered within one day and the maximum value of \( P_p \) increased a bit in the following days (Fig. 1B). When maize plants were treated with 200 mM NaCl salt shock, their \( P_p \) increased rapidly within minutes and peaked after 2 h (Fig. 1C). The \( P_p \) and \( P_m \) values of maize plants both returned to the original levels 1 day after (Fig. 1C).

### 3.2. Diurnal changes of leaf turgor pressure in phase 1

When NaCl was added gradually, \( P_p \) showed a different response to salt stress. NaCl was added to nutrient solution of Arabidopsis in daily increments of 25 mM, and reached a final concentration of 100 mM 4 days later. The \( P_m \) value of Arabidopsis continued to rise with increasing NaCl, even after the addition was stopped (Fig. 2A). Actually, the turgor should be maintained in this period. If the daily application was decreased from 25 mM to 15 mM NaCl and the addition period was increased from 4 days to 6 days, \( P_m \) showed a slight increase after each 15 mM NaCl application, and fully recovered within 1 day (Fig. 2B). The \( P_m \) value remained mostly stable in the first 6 days and only had a slight increase when NaCl addition was stopped in Arabidopsis (Fig. 2B). When maize plants were stepwise acclimated to 100 mM NaCl (25 mM NaCl per day), the \( P_m \) value decreased slightly when 25 mM NaCl were added daily, and was maintained when NaCl application was stopped (Fig. 2C).

The growth of Arabidopsis was inhibited after 90 mM NaCl treatment (stepwise adapted, as described above) (Fig. 3A). Under salt stress, shoot fresh weight decreased which was 70.8% relative to the control (Fig. 3B). However, shoot dry weight showed no significant change (Fig. 3C). The Na⁺ concentrations in shoots and roots increased after salt treatment, whereas other cation (Ca²⁺, K⁺, and Mg²⁺) concentrations decreased (Fig. 3D). However, no toxicity and deficiency symptoms were observed in these plants.

### 3.3. Differentially transcribed genes in phase 0 and phase 1 for Arabidopsis thaliana

Nine samples from Arabidopsis thaliana were harvested and used for RNA-sequence analysis with three biological replicates for each treatment. One group was harvested under control conditions; another group was harvested 1 h after 105 mM NaCl treatment when \( P_p \) reached its peak value; and the last group was harvested 2 days after reaching 90 mM NaCl when the \( P_m \) value remained stable using the stepwise acclimation as described above. Based on the selection conditions (see "Materials and methods"), 213 differentially transcribed genes (DTGs) were identified in Phase 0, 2505 DTGs were observed in Phase 1, and 303 DTGs were affected both in Phase 0 and Phase 1 (Fig. 4).

### 3.4. Responses of DTGs involved in the inhibition of leaf water loss

To alleviate the salt stress-induced water deficiency, plants have developed several strategies to reduce leaf water loss such as reducing leaf transpiration and leaf hydraulic conductance (Defraeye et al., 2014; Kosma et al., 2009; Pou et al., 2013).

#### 3.4.1. DTGs involved in leaf transpiration

The leaf transpiration rate depends on stomatal transpiration and cuticular transpiration which are governed by stomatal movement and cuticular layers, respectively (Defraeye et al., 2014; Kosma et al., 2009). Twenty-eight DTGs involved in the regulation of stomatal movement were identified in Phase 0 and Phase 1 (Table 1). Among these, transcription of ERF053, AHK5, and ABI1 was especially influenced in Phase 0. Nine DTGs were observed both in Phase 0 and Phase 1; HAI-1, PP2CA, ABI2, NHX2, AKS1, and RD20 showed increased transcription abundance; Pcap1, EXP1, and RZPF34 showed decreased transcription abundance. Seventeen DTGs were specially influenced in Phase 1. The transcription of BGLU18, ArathEULS3, RBOHF, GLR2, ABC55, MAPK118, ABC4, ZIF1, and TOD1 was up-influenced; PHOT1, CRY2, BHLH128, SYP22, GRP7, APK18, iPGAM1, and ELF3 were down-influenced in Phase 1. The function of those genes has been experimentally studied using mutant-based research approaches in previous researches (Table 1).

Several wax biosynthesis-related genes were affected in Phase 0 and Phase 1 (Table 2). KCS9 was especially down-influenced in Phase 0. WSD1, CFLAP1, and DEWAX were affected both in Phase 0 and Phase 1. WSD1 and CFLAP1 showed increased transcription abundance. DEWAX showed decreased transcription abundance. Five eceriferae family genes (CER1, CER3, CER4, COR8, and CER60) were affected only in Phase 1. Among those, CER1, CER3, and CER4 were up-regulated; CER8 and CER60 were down-regulated in transcript level.

#### 3.4.2. DTGs involved in the regulation of leaf hydraulic conductance

Leaf hydraulic resistance contributes 30% or more to the whole plant hydraulic resistance (Pou et al., 2013). Water transport in leaves includes an apoplastic and a cell-to-cell pathway (Pou et al., 2013). The cell-to-cell pathway is mainly triggered by water channels (aquaporins). Transcriptional changes in several water channels were observed in Phase 0 and Phase 1 (Table 2). Two aquaporin genes were down-influenced in Phase 0; aquaporin PIP2-7, located in the plasma membrane had 2.2-fold lower transcript abundance compared to controls; aquaporin TIP2-1, located in tonoplast membrane had 4.0-fold lower transcript levels than under control conditions. One aquaporin gene, PIP1D, was down-influenced both in Phase 0 and Phase 1, and the transcript levels were 2.1 and 2.0-fold lower than controls, respectively. Nevertheless, two aquaporin genes (PIP2A and TIP1-3) were up-influenced in Phase 1. The transcription level of PIP2A was 2.0-fold higher than that in controls, and TIP1-3 was 6.0-fold higher.

#### 3.5. Candidate marker genes for phase 0 and phase 1 in Arabidopsis thaliana

In Phase 0, 16 highly induced or inhibited DTGs (|log₂ FoldChange (Phase 0/Control)| ≥ 2) were identified and these are listed in Table 3.
Table 1
Genes related to stomatal movement differentially transcribed in Phase 0 and Phase 1. Pink indicates genes involved in ABA pathway; yellow indicates genes affecting ion channels (Ca\(^{2+}\) and K\(^{+}\)) localized in plasma membrane and tonoplast; green indicates genes influencing the dynamic changes of apoplast and tonoplast in guard cells (e.g. volumetric elastic modulus and vacuole fusion); purple indicates genes affecting low molecular weight compounds (e.g. inositol hexakisphosphate and sphingosine-1-phosphate); blue indicates genes correlated with H\(^{+}\)-ATPase.

| ID       | Gene       | Gene description                          | log2 FoldChange (Phase 0/Control) | log2 FoldChange (Phase 1/Control) | Mutant phenotypes                                                                 |
|----------|------------|-------------------------------------------|-----------------------------------|-----------------------------------|-----------------------------------------------------------------------------------|
| AT2G20880| ERF053     | Integrase-type DNA-binding superfamly 53   | 2.79                              | -                                 | larger stomata aperture in the presence of ABA, drought sensitive                 |
| AT5G10720| AHK5       | Histidine kinase 5                        | 2.63                              | -                                 | larger stomata aperture in the presence of H\(_2\)O\(_2\)                        |
| AT4G26080| ABI1       | Protein phosphatase 2C family protein      | 1.29                              | -                                 | larger stomata aperture in the presence of ABA                                    |
| AT5G92220| HAI1       | Clade A protein phosphatases type 2C      | 4.00                              | 5.90                              | smaller aperture in the presence of ABA                                          |
| AT2G33380| RD20       | Calexin-related family protein            | 2.10                              | 4.50                              | higher transpiration rate, drought hypersensitive                                 |
| AT3G11410| PP2CA      | Protein phosphatase 2CA                   | 1.92                              | 1.85                              | decreased water loss, enhanced drought resistance                                 |
| AT1G51140| AKS1       | ABA-responsive kinase substrate 1         | 1.71                              | 2.90                              | decreased light-induced stomatal opening                                          |
| AT5G57050| ABI2       | Protein phosphatase 2C family protein      | 1.43                              | 2.00                              | decreased light and low CO\(_2\) induced stomatal opening                       |
| AT3G05030| NHX2       | Sodium hydrogen exchanger 2               | 1.06                              | 1.76                              | delayed stomatal closure, enhanced stomatal conductance                          |
| AT5G22920| RZPF34     | RING-type Zinc finger protein             | -1.05                             | -3.80                             | smaller stomata aperture under non-stress conditions                              |
| AT4G20260| PCaP1      | Plasma-membrane associated cation-binding protein 1 | -1.63                             | -2.96                             | opened stomata under osmotic stress                                              |
| AT1G69530| ExPA1      | Expansin A1                               | -1.66                             | -1.79                             | decreased light-induced stomatal opening                                          |
| AT2G47800| ABCC4      | Multidrug resistance-associated protein 4 | -                                  | 1.97                              | enhanced water loss, larger stomata aperture in response to light and dark        |
| AT1G05100| MAPKKK18   | Mitogen-activated protein kinase kinase 18 | -                                  | 1.96                              | larger stomata aperture under normal conditions, slower ABA-induced stomatal closure |

(continued on next page)
The transcript levels of AT4G28140, ERF053, and AHK5 were 14.6, 6.9, and 6.2-fold higher than controls, and they are all involved in the regulation of stomatal movement. TIP2-1, a tonoplast water channel, was 4.0-fold lower compared to controls in transcriptional level. In addition, these four genes showed no significant transcript changes in Phase 1.

In Phase 1, 33 DTGs ($|\log_2 \text{FoldChange (Phase 1/Control)}| \geq 5$) were observed (Table 4). Among these, UF3GT, LDOX, TT8, and GSTF12 were 77, 70, 64, 37-fold higher in Phase 1 compared to the control. These four genes are involved in anthocyanin metabolism. The transcript level of SWEET15 was 64-fold higher than the control and AT5G62360 showed 141-fold decrease on transcript abundance compared to controls. SWEET15 and AT5G62360 are involved in sucrose metabolism.

To evaluate the selected Phase 0 marker gene candidates, qRT-PCR was conducted in samples harvested after 1 h, 2 h, and 3 h of 105 mM

| AT1G52400 | BGLU18 | Beta glucosidase 18 | - | 1.83 | ABA deficiency, defective stomatal closure in response to dark |
| AT2G39050 | ArathEULS3 | Hydroxyproline-rich glycoprotein family protein | - | 1.34 | larger stomatal aperture in the presence of ABA |
| AT5G13750 | ZIFL1 | Zinc induced facilitator-like 1 | - | 1.22 | larger stomata aperture in response to ABA and light |
| AT5G46220 | TOD1 | Turgor regulation defect 1 | - | 1.12 | insensitive to abscisic acid (ABA)-induced stomatal closure |
| AT2G17260 | GLR2 | Glutamate receptor 2 | - | 1.11 | lower levels of cytosolic Ca$^{2+}$, defective Ca$^{2+}$ programmed stomatal closure |
| AT1G04120 | ABCC5 (MRP5) | Multidrug resistance associated protein 5 | - | 1.09 | decreased light and glibenclamide induced stomatal opening |
| AT1G64060 | RBOHF | Respiratory burst oxidase protein F | - | 1.07 | decreased ABA-induced stomatal closure |
| AT3G45780 | PHOT1 | Phototropin 1 | - | -1.00 | reduced stomatal opening in response to blue light |
| AT1G04400 | CRY2 | Cryptochrome 2 | - | -1.11 | reduced stomatal opening in response to blue and red light |
| AT1G09780 | PIGAM1 | Phosphoglycerate mutase | - | -1.16 | hyposensitive to blue light or low CO$_2$ induced of stomatal opening |
| AT1G05800 | BHLH128 (AKS2) | ABA-responsive kinase substrate 2 | - | -1.63 | reduced stomatal opening in response to light |
| AT2G28930 | APK1B | Protein kinase 1B | - | -1.83 | decreased light-induced stomatal opening |
| AT5G48860 | SYP22 | Syntaxin/SNARE family protein | - | -1.84 | slower stomatal opening in response to light |
| AT2G25930 | ELF3 | Hydroxyproline-rich glycoprotein family protein | - | -2.62 | larger stomata aperture under dark and light conditions |
| AT2G21660 | GRP7 | Glycine rich protein 7 | - | -6.17 | larger stomata aperture in the presence of ABA |
NaCl shock. The qRT-PCR results show that the transcriptional level of AT4G28140, AHK5, and TIP2-1 had no significant differences with the elongated treated time (Fig. 5A). The transcription of ERF053, NaCl shock. The qRT-PCR results show that the transcriptional level of W.Zhao, et al. Plant Physiology and Biochemistry 143 (2019) 232–245

3.6. Marker genes tested in Zea mays

To evaluate the applicability of the selected marker genes, qRT-PCR was conducted in an important crop plant, Zea mays. The Phase 0 samples were harvested after 1 h, 2 h, and 3 h of 200 mM NaCl shock. Likewise, the Phase 1 samples were harvested after 1 day, 2 days, and 3 days of 100 mM NaCl treatment (4 days acclimation: 25 mM NaCl added daily). Using the amino acid sequences of the suggested marker genes (Arabidopsis thaliana) as templates, a BLAST was conducted in Zea mays database (B73 RelGen_v4). Analogs with the highest similarity were selected as the marker gene candidates in Zea mays, and details of these analogs are presented in Table S3. The relative transcript levels ≥2 or ≤0.5 were used as cutoff to evaluate the significant up-regulated or down-regulated genes, respectively.

In Phase 0, the transcript levels of all the Phase 0 candidates in maize were not in the range ≥2 or ≤0.5 after 1 h of 200 mM NaCl shock (Fig. 6A). After 2 h of 200 mM NaCl shock, the transcript levels of GRMZM2G141638, GRMZM2G025579, and GRMZM2G125023 were in the range ≥2 or ≤0.5 after 3 h of 200 mM NaCl shock (Fig. 6A). These results indicate that GRMZM2G141638 and GRMZM2G125023 whose transcript levels exceeded the cutoff after 2 h and 3 h of 200 mM NaCl shock are suitable Phase 0 marker genes for maize.

In Phase 1, only GRMZM2G165390 exceeded the cutoff at transcriptional level after 1 day of 100 mM NaCl treatment (stepwise acclimated, 25 mM NaCl per day) (Fig. 6B). The transcript levels of GRMZM2G165390, GRMZM2G061321, GRMZM2G146246, and GRMZM2G168365 were in the range ≥2 or ≤0.5 after 2 days of 100 mM NaCl treatment (Fig. 6B). After 3 days of 100 mM NaCl treatment, GRMZM2G165390, GRMZM2G061321, GRMZM2G146246, and GRMZM2G168365 exceeded the cutoff (Fig. 6B). The qRT-PCR experiments indicate that GRMZM2G165390, GRMZM2G061321, GRMZM2G146246, and GRMZM2G168365 are suitable Phase 1 marker genes for maize.

4. Discussion

4.1. Turgor decrease in phase 0 and maintenance in phase 1

According to Fig. 1A and Supplemental Fig. S3, Arabidopsis cannot endure 200 mM and 125 mM NaCl shocks. First, the P<sub>t</sub> value could not recover after the peak which indicates that Arabidopsis had lost the ability to adjust turgor. Second, the diurnal change of P<sub>t</sub> was abolished in the following days. Generally, turgor decreased during the day because of transpiration and was restored at night. As P<sub>t</sub> is inversely correlated with turgor, P<sub>t</sub> increased during the day and recovered at night under normal conditions. The irregular diurnal changes of P<sub>t</sub> in Arabidopsis indicate that plants cannot recover from salt shocks induced by 200 mM and 125 mM NaCl. In contrast, maize plants can endure 200 mM NaCl shock and Arabidopsis plants can recover from the 105 mM NaCl shock. Under proper salt shock treatments, the P<sub>t</sub> value showed transient increase in the first few minutes and recovered within 1 day both in Arabidopsis and maize plants (Fig. 1B and C). This indicates that turgor pressure decreased rapidly at first, and then recovered both in Arabidopsis and maize during Phase 0. This is consistent with the results obtained in barley and maize root tissue (Pritchard et al., 1996; Thiel et al., 1988).

During Phase 1 (salt stress after stepwise acclimation), Arabidopsis could not tolerate 100 mM NaCl treatment (25 mM increments per day) because the P<sub>t</sub> value kept increasing before and after NaCl addition was stopped (Fig. 2A). This indicates that turgor decreased after 100 mM NaCl treatment. In contrast, turgor in Arabidopsis almost remained constant during and after the addition of 90 mM NaCl (15 mM increments per day) (Fig. 2B). Moreover, turgor in maize increased during NaCl adaptation (100 mM; 25 mM increments per day) and was maintained after stopping addition (Fig. 2C). The maintained turgor observed in Arabidopsis and maize under proper salt stress is in agreement with studies conducted in barley leaves with different levels of NaCl treatments (Thiel et al., 1988), maize root tissues under osmotic stress (Pritchard et al., 1996), and maize leaves with stepwise-acclimated salt stress (De Costa et al., 2007). These studies demonstrate that the decreased osmotic potential contributes to the turgor recovery. Moreover, the different responses of turgor to NaCl application between Arabidopsis and maize may be attributed to their different growth rate. According to Hanway (1963), maize plants with four or six fully emerged leaves are in the stage 1 or 1.5, respectively. In this study, maize plants developed five leaves, when the ZIM YARA water sensors were first applied. So the tested 4th leaves were still growing, undergoing cell expansion. This result agrees with the increased turgor pressure observed in expanding tissues by Thiel et al. (1988) and Pritchard et al. (1996).

In conclusion, the turgor measurements conducted in Arabidopsis and maize indicate that these two plant species have different capacity of salt acclimation. Moreover, salts need to be carefully applied under experimental conditions to induce salt shock or stepwise acclimation to salt stress. As shown in (Figs. 1), 125 mM and 200 mM NaCl shocks were too strong for Arabidopsis plants to survive. However, the NaCl concentration used in many Arabidopsis salinity researches exceeded 125 mM. To induce stepwise acclimation to salt stress, the daily increment of NaCl differs between Arabidopsis and maize. For Arabidopsis, the daily increment is 15 mM NaCl; and for maize, it is 25 mM NaCl. However, this is not always the case, and it may differ for different Arabidopsis ecotypes and maize cultivars.

4.2. Growth response to salt stress

After 2 days of 90 mM NaCl treatment (stepwise acclimated as described in Materials and Methods), the shoot fresh weight of Arabidopsis plants decreased (Fig. 3B), which indicates that the water uptake and transport were inhibited during this period. However, shoot dry weight had no significant difference after salt treatment (Fig. 3C). This indicates that the photosynthesis was not inhibited and the carbohydrate supply was sufficient in this period. The Na<sup>+</sup> concentration in shoots was around 20 mg/g on dry matter basis after salt treatment (Fig. 3D). As far as we know, there is no literature demonstrating the Na<sup>+</sup> toxic level for Arabidopsis thaliana. Furthermore, Arabidopsis plants did not show any toxicity and nutrient deficiency symptoms during this period. The turgor pressure of Arabidopsis plants was maintained after 2 days of 90 mM NaCl treatment (Fig. 2B). Thus, we demonstrate that Arabidopsis plants were still in Phase 1 rather than Phase 2 after 2 days of 90 mM NaCl treatment. However, we cannot exclude that genes related to ion toxicity (e.g. genes involved in Na<sup>+</sup> exclusion and Na<sup>+</sup> compartmentation; Phase 2) may also have responded in Phase 1. Therefore, the following analyses based on RNA-
sequence were mainly focusing on the genes related to plant water relations (e.g. water transport and transpiration) to avoid the genes related to Phase 2 getting involved.

### 4.3. The inhibition of leaf water loss

The genes involved in the regulation of stomatal closure were mostly affected in Phase 1 (Table 1). Plants regulate stomatal movement in different ways. Among those, ABA (abscisic acid)-induced stomatal closure is the most functional strategy and has been well studied (Kim et al., 2010). One-third of the genes listed in Table 1 were mainly focusing on the genes related to plant water homeostasis.

| ID          | Gene          | Gene description                  | log₂ FoldChange (Phase 0/Control) | log₂ FoldChange (Phase 1/Control) |
|-------------|---------------|-----------------------------------|-----------------------------------|-----------------------------------|
| AT4G28140   | AT4G28140     | 3-Ketoacyl-CoA synthase 9         | -1.10                             |                                   |
| AT5G07640   | AT5G07640     | 1-Aminocyclopropane-1-carboxylate synthase 11 | 3.47                             |                                   |
| AT1G34200   | EXPA1         | Plasma membrane intrinsic protein 3 | -1.15                             | -1.03                             |
| AT1G52890   | NES1          |Integrase-type DNA-binding superfamily protein | -1.10                             | -1.03                             |
| AT2G25297   | AT2G25297     | Natural antisense transcript overlaps with AT2G33390 | 1.25                             |                                   |
| AT3G53420   | PIP2-1        | Delta tonoplast integral protein  | -2.01                             |                                   |
| AT4G35100   | PIP2-7        | Plasma membrane intrinsic protein 3 | -1.15                             | -1.03                             |
| AT4G28140   | AT4G28140     | 3-Ketoacyl-CoA synthase 9         | -1.10                             | -1.03                             |
| AT3G53420   | PIP2-1        | Plasma membrane intrinsic protein 2A | -1.46                             | 1.02                              |
| AT4G01470   | TIP1-3        | Tonoplastic intrinsic protein 1-3 | -2.60                             |                                   |

**Table 1**

Genes related to water transport were differentially transcribed in Phase 0 and Phase 1.

**Table 2**

Genes related to wax biosynthesis were differentially transcribed in Phase 0 and Phase 1. False discovery rate (FDR) ≤ 0.05 and the maximum value of |log₂ FoldChange (Phase 0/Control)| ≥ 2 were used as cut-off to evaluate significant differences in transcription. ▲ indicates marker gene candidates related to water transport. ▲ indicates marker gene candidates related to water transport. ▲ indicates marker gene candidates related to water transport. ▲ indicates marker gene candidates related to water transport.

**Table 3**

Differentially transcribed genes with statistically significant changes in Phase 0. False discovery rate (FDR) ≤ 0.05 and the maximum value of |log₂ FoldChange (Phase 0/Control)| ≥ 2 were used as cut-off to evaluate significant differences in transcription. ▲ indicates marker gene candidates related to stomatal movement. ▲ indicates marker gene candidates related to stomatal movement. ▲ indicates marker gene candidates related to stomatal movement.

**Table 4**

Differentially transcribed genes with statistically significant changes in Phase 0. False discovery rate (FDR) ≤ 0.05 and the maximum value of |log₂ FoldChange (Phase 0/Control)| ≥ 2 were used as cut-off to evaluate significant differences in transcription. ▲ indicates marker gene candidates related to stomatal movement.
4.4. Marker genes for phase 0 and phase 1

According to the RNA-sequence analysis in Arabidopsis plants, AT4G28140, ERF053, AHK5, and TIP2-1 were suggested as marker genes for Phase 0. First, they were all differentially and highly influenced at transcriptional level in Phase 0 and had no fundamental changes in Phase 1 (Table 3). Second, they are all involved in the inhibition of leaf water loss according to previous studies. AT4G28140 and ERF053 belong to the ERF/AP2 transcription factor family. Over-expressing AtERF053 in Arabidopsis leads to more sensitive stomatal behavior and greater drought tolerance (Chen et al., 2012). The role of AHK5 encodes a member of the histidine kinase family and mediates stomatal movement requires further investigation. AT5G59520 and AT3G24750 encode a member of the ERF/AP2 transcription factor family. AT5G59520 encodes a member of sucrose uniporter, involved in the transportation of sucrose from source to sink. It was demonstrated that these 10 suggested candidates are suitable marker genes for the differentiation between Phase 0 and Phase 1 in Arabidopsis plants. Leitão et al. (2014) demonstrated that the grapevine TIP2-1 might function as a volume regulator to mediate vacuolar size and shape in response to water deficiency.

The highly and differentially influenced UF3GT, LDOX, TT8, GSTF12, SWEET15, and AT5G62360 were selected as markers for Phase 1 (Table 4). According to the previous studies, UF3GT, LDOX, TT8, and GSTF12 are involved in anthocyanin modification, biosynthesis, and transportation, respectively. Chalker-Scott (2002) demonstrated that anthocyanin is functioning as an osmolyte under salinity and drought stress to fight against osmotic stress. The UDP-glucosyltransferase (UGT) and leucoanthocyanidin dioxygenase (LDOX) proteins are key enzymes mediating biosynthesis of anthocyanin. UF3GT (also called UGT79B1) is a homologous UDP-glucosyltransferase like UGT79B2. Over-expressing UGT79B2 in Arabidopsis leads to anthocyanin accumulation and enhances resistance to drought and salt stresses (Li et al., 2017). Yeast two- and three-hybrid assays indicate that TT8, TT2, and TTG1 work together as a ternary complex to regulate proanthocyanin and anthocyanin biosynthesis (Kitamura et al., 2004). GSTF12 (also called TT19) is a carrier to transport anthocyanins from cytosol to tonoplast (Kitamura et al., 2004). SWEET15 and AT5G62360 are involved in sucrose transport and synthesis, respectively. Sucrose can act as an osmolyte to reduce the intracellular osmotic potential and prevent damage to proteins and cellular structures from salt stress (Deinlein et al., 2014). SWEET15 encodes a member of sucrose uniporter, involved in the transportation of sucrose from source to sink. It was demonstrated that AtSWEET15 is induced under osmotic stress, and alleviates damages by mediating the senescence process (Seo et al., 2011). AT5G62360 encodes a plant invertase/pectin methyltransferase inhibitor, and its transcription was inhibited during Phase 1 (Table 4). This is supported by the findings of Koh et al. (2008), who demonstrated that ABA enhanced invertase activity by down-regulating its inhibitor in water-stressed plants.

These Phase 0 and Phase 1 marker gene candidates were further tested in Arabidopsis samples harvested at different time points of Phase 0 (1 h, 2 h, and 3 h of 105 mM NaCl shock) and Phase 1 (1 day, 2 days, and 3 days of 90 mM NaCl treatment) by qRT-PCR. All these marker candidates showed significant differences at transcriptional level in different Phase 0 and Phase 1 time points (Fig. 5), indicating that these 10 suggested candidates are suitable marker genes for the differentiation between Phase 0 and Phase 1 in Arabidopsis plants.

To evaluate the reliability of the marker genes, they were further tested in Zea mays (a salt-resistant maize cultivar Pioneer 3906 was used). The qRT-PCR results indicate that analogs of ERF053 and TIP2-1 are potential marker genes for Phase 0; likewise, UF3GT, GSTF12, and SWEET 15 are marker genes for Phase 1 in maize (Fig. 6). In conclusion, genes or analogs of ERF053, TIP2-1, UF3GT, GSTF12, and SWEET 15 that are affected in Arabidopsis and maize, can serve as potential

Table 4
Differentially transcribed genes with statistically significant changes in Phase 1. False discovery rate (FDR) ≤ 0.05 and the maximum value of log₂ FoldChange (Phase 1/Control) ≥ 5 were used as cut-off to evaluate significant differences in transcription. ▲ indicates marker gene candidates related to anthocyanin biosynthesis and transport. △ indicates marker gene candidates related to sucrose synthesis and transport.

| ID       | Gene          | log₂ FoldChange (Phase 1/Control) | Gene description                                      |
|----------|---------------|-----------------------------------|-------------------------------------------------------|
| AT5G40790| AT5G40790     | 7.54                              | Hypothetical protein                                  |
| AT4G08570| H IPP24       | 7.49                              | Heavy metal-associated isoprenylated plant protein 24 |
| AT5G24880| AT5G24880     | 7.12                              | Chromo domain-like protein                            |
| AT3G24750| AT3G24750     | 6.55                              | Hypothetical protein                                  |
| AT5G38130| AT5G38130     | 6.39                              | HXXD1-type acyl-transferase family protein           |
| AT5G54060| UF3GT         | 6.26                              | UDP-glucose:flavonoid 3-o-glucosyltransferase         |
| AT4G22880| LDOX          | 6.13                              | Leucoanthocyanidin dioxygenase                        |
| AT5G13170| SWEET15       | 6.01                              | ▲ Bidirectional sugar transporter SWEET15             |
| AT5G17220| GSTF12        | 6.07                              | △ Glutathione S-transferase F12                       |
| AT1G01060| LIYI          | 5.99                              | Homeodomain-like superfamily protein                  |
| AT4G14690| ELIP2         | 5.76                              | Early light-induced protein 2, chloroplastic          |
| ATSG41315| GL3           | 5.72                              | Basic helix-loop-helix DNA-binding superfamily protein|
| AT5G59520| LTTP          | 5.61                              | Non-specific lip-transfer protein 3                   |
| AT2G37770| AKR4C9        | 5.59                              | Aldo-keto reductase family 4 member C9               |
| AT1G69480| AT1G69480     | 5.58                              | EKS (ERD1/XPR1/SYG1) family protein                  |
| AT5G59310| LTP4          | 5.56                              | Non-specific lipid-transfer protein 4                 |
| AT5G09930| ARCF2         | 5.51                              | ABC transporter F family member 2                     |
| AT3G24463| AT3G24463     | 5.35                              | Nuclear transport factor 2 family protein             |
| AT4G09820| TT8           | 5.21                              | △ Transcription factor TT8                            |
| AT5G22275| JAZ13         | 5.13                              | Protein JAZ13                                         |
| AT4G27360| AT4G27360     | 5.11                              | Dynin light chain type 1 family protein              |
| AT3G09590| AT3G09590     | 5.06                              | CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily protein |
| AT1G51780| BL5           | 5.03                              | IAA-amino acid hydrolase ILR1-like 5                 |
| AT1G43160| RAP2-6        | 5.02                              | Ethylene-responsive transcription factor RAP2-6      |
| AT1G10550| XTH33         | −5.18                             | Probable xyloglucan endotransglycosylase/hydrolase protein 33 |
| AT5G06690| WCRKC1        | −5.43                             | WCRKC thioredoxin 1                                  |
| AT3G51400| AT3G51400     | −5.47                             | Hypothetical protein                                  |
| AT3G16670| AT3G16670     | −6.04                             | Pollen Ole e 1 allergen and extensin family protein  |
| AT2G21660| RBG7          | −6.17                             | Glycine-rich RNA-binding protein 7                    |
| AT5G24470| APR85         | −6.35                             | Two-component response regulator-like APR85         |
| AT2G15890| MEE14         | −6.40                             | Maternal effect embryo arrest 14                     |
| AT2G33830| DRM2          | −6.84                             | Dormancy/auxin associated family protein             |
| AT5G62360| AT5G62360     | −7.14                             | ▲ Plant invertase/pectin methyltransferase inhibitor superfamily protein |
markers to differentiate between Phase 0 and Phase 1 in future salinity research.

The transcriptional changes of marker genes in maize were not as strong as those observed in Arabidopsis under salt stress. This may be attributed to the different salt sensitivity of Arabidopsis and maize plants. The ecotype of *Arabidopsis thaliana* used in this study is Columbia-0, which is a relative salt-sensitive ecotype. The maize cultivar Pioneer 3906 used in this study is salt-resistant, which has strong Na⁺-exclusion ability. It was developed by crossing Pioneer 165 and Pioneer 605 inbred lines, which are efficient in Na⁺ exclusion from the root and shoot, respectively (Schubert et al., 2009). As shown in Fig. 1C, maize plants can endure 200 mM NaCl shock and the $P_m$ value recovered to the original level on the following day. However, Arabidopsis plants cannot live through 200 mM NaCl shock (Fig. 1A). Though Arabidopsis can endure 105 mM NaCl shock, the $P_m$ value in Arabidopsis plants cannot completely recover on the next day (Fig. 1B). These results indicate that Arabidopsis plants are more sensitive to salt stress than maize plants. The different capacity of salt acclimation between Arabidopsis and maize further validates the reliability of marker genes.

5. Conclusions

As salt accumulates gradually in the field, it is more conducive to study salt stress in a stepwise-acclimated way rather than salt shock. Usually plants show wilting symptoms in salt-shock situations, but there are exceptions such as in Arabidopsis and maize under the experimental conditions described in this study. In this case, it is difficult to differentiate these two types of salt stresses in terms of phenotype. In this study, two potential parameters are provided. At the physiological level, our results show that turgor decreased rapidly in a salt-shock situation, but was maintained under stepwise-acclimated salt stress. According to the turgor measurements, we suggest that salts need to be carefully applied both in salt shock and stepwise-acclimated salt stress.
conditions, otherwise plants cannot recover from these stresses. At the transcriptional level, genes or analogs of \textit{ERF053}, \textit{TIP2-1}, \textit{UF3GT}, \textit{GSTF12}, and \textit{SWEET15} are suggested as marker genes to separate these two types of salt stress in Arabidopsis and maize. These marker genes were first selected in Arabidopsis by means of RNA-sequence, then evaluated in Arabidopsis at different time points via qRT-PCR, and finally validated in maize plants via qRT-PCR. These experiments confirmed the reliability of the selected marker genes and we expect that these marker genes are applicable to other plants.

Acknowledgements

WZ thanks the Doctoral fellowship offered by China Scholarship Council (CSC). We thank Prof. Zhimin Yang (College of Life Science, Nanjing Agricultural University) for helpful comments. We thank Lei Wang (Max Planck Institute for Heart and Lung Research) and Guenther Stefan (Max Planck Institute for Heart and Lung Research) for the help of RNA-sequence analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.09.001.

Author contributions

SS and WZ designed the project; SJ and WZ performed the turgor experiments; WZ performed the RNA-sequence analysis; SS and WZ wrote the manuscript; SS provided supervision.

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