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Research article

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

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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 *Arabidopsis thaliana* 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

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Abbreviations: P_{clamp}, clamp pressure exerted by magnets; P_c, turgor pressure; P_p, recorded patch pressure; P_m, the maximum value of recorded patch pressure; FDR, false discovery rate; SE, standard error; DTG, differentially transcribed gene; ABA, abscisic acid

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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, Sümer 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 ZIM 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 (P_m) occurs around noon and is a potential parameter to determine whether plants are suffering a water deficit. Under water stress, the P_m 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 plants' 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 (Lü 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 ZIM 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

Arabidopsis 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 $\mu mol\ m^{-2}\ s^{-1}$) in the light and 18 °C (0 $\mu mol\ m^{-2}\ s^{-1}$) in the dark. Maize was cultivated at 26 °C (200 $\mu mol\ m^{-2}\ s^{-1}$) for 16 h and 18 °C (0 $\mu mol\ m^{-2}\ s^{-1}$) for 8 h in a growth chamber. Arabidopsis was cultivated in hydroponic culture including 1 mM KH_2PO_4, 0.25 mM K_2SO_4, 1 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 50 μM KCl, 5 μM $MnSO_4$, 1 μM $ZnSO_4$, 1 μM $CuSO_4$, 0.1 μM $NiSO_4$, 0.7 μM $(NH_4)_6Mo_7O_{24}$, 30 μM H_3BO_3, and 100 μM FeNaEDTA. Maize was grown in hydroponic culture with 2 mM $Ca(NO_3)_2$, 1 mM K_2SO_4, 0.75 mM $MgSO_4$, 2 mM $CaCl_2$, 0.2 mM KH_2PO_4, 0.2 mM FeNaEDTA, 2 μM $MnSO_4$, 0.5 μM $ZnSO_4$, 0.3 μM $CuSO_4$, 0.1 μM $NiSO_4$, 0.01 μM $(NH_4)_6Mo_7O_{24}$, and 1 μM H_3BO_3.

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 ZIM YARA water sensors which were clamped to leaves via two magnets. The clamp pressure (P_{clamp}) exerted by magnets is constant during the measurements and can be adjusted by screwing the movable magnet. The leaf turgor pressure (P_c) is opposed to clamp pressure in the leaf patch. The output of sensors (patch pressure, P_p) is a power function of the turgor pressure P_c (Zimmermann et al., 2008):

$$Pp = \left(\frac{b}{aPc + b}\right)^{\frac{1}{a}} \times Fa \times Pclamp \qquad (1)$$

Among these, a and b are constants. F_a is a leaf-specific attenuation factor. Equation (1) shows that the recorded P_p pressure is inversely correlated with leaf turgor pressure P_c, which means that P_p is large when P_c 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 P_p 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^+, Ca^{2+}, K^+, and Mg^{2+} concentrations, samples were prepared by dry-ashing (550 °C) overnight and cations were measured with an atomic absorption spectrophotometer (SpectrAA220 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 DNase Set (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

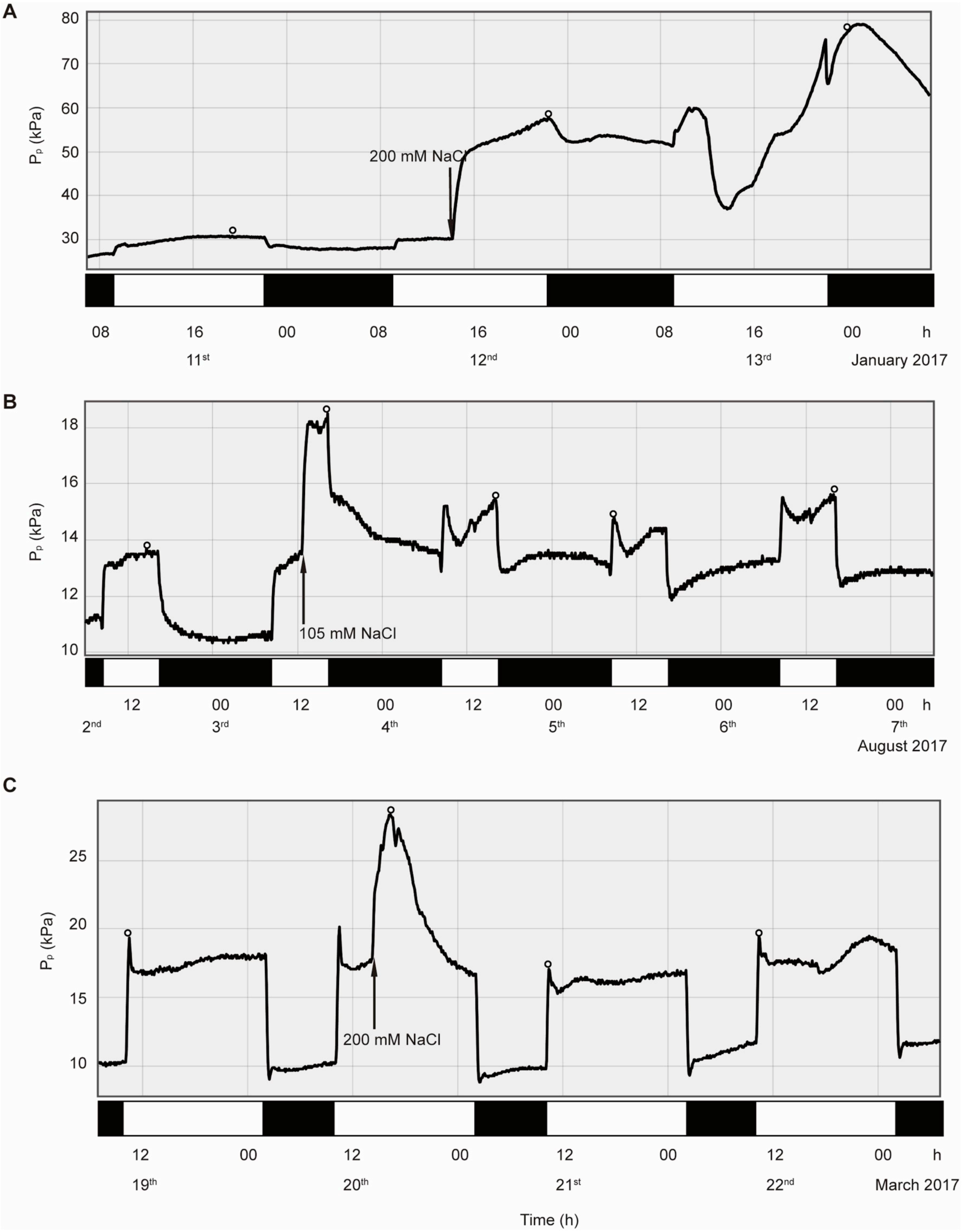

Fig. 1. Concomitant recordings of P_p in *Arabidopsis thaliana* (**A, B**) and *Zea mays* (**C**) during Phase 0. The leaf turgor pressure was measured with ZIM YARA water sensors. Note that leaf turgor pressure and the output patch pressure (P_p) are inversely proportional. Thus, higher P_p values mean lower leaf turgor pressure. 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/B.** 200 mM NaCl (**A**) and 105 mM NaCl (**B**) were added to Arabidopsis at one time. **C.** 200 mM NaCl were added to maize at one time. Representative readings of three biological replicates.

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

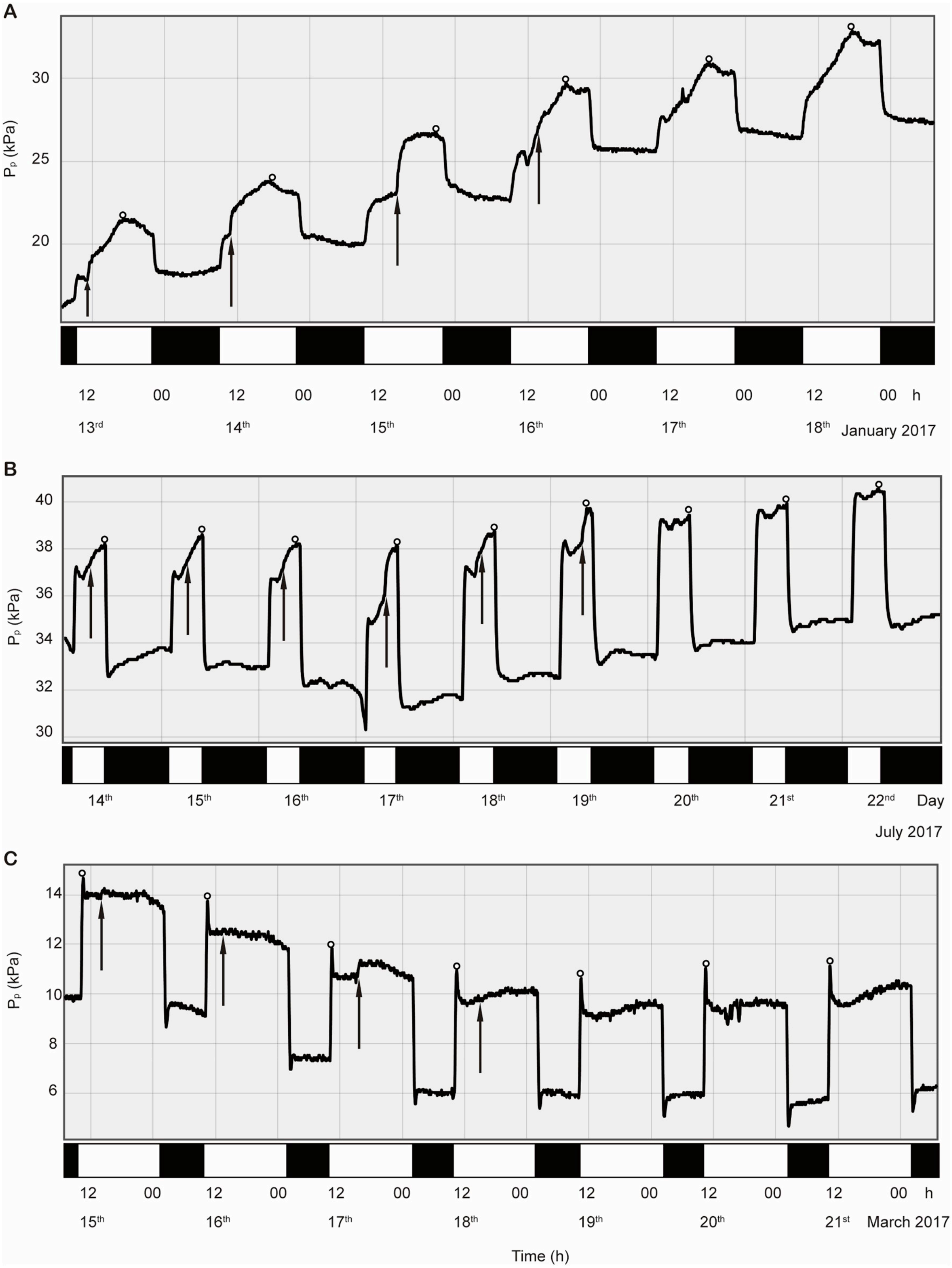

Fig. 2. Concomitant recordings of P_p in *Arabidopsis thaliana* (**A, B**) and *Zea mays* (**C**) during Phase 1. Arrows indicate NaCl-adding points; open circles indicate the maximum P_p values (P_m values) during the day. White blanks represent the day; black blanks represent the night.
A/C. 100 mM 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.

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) ≤ 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

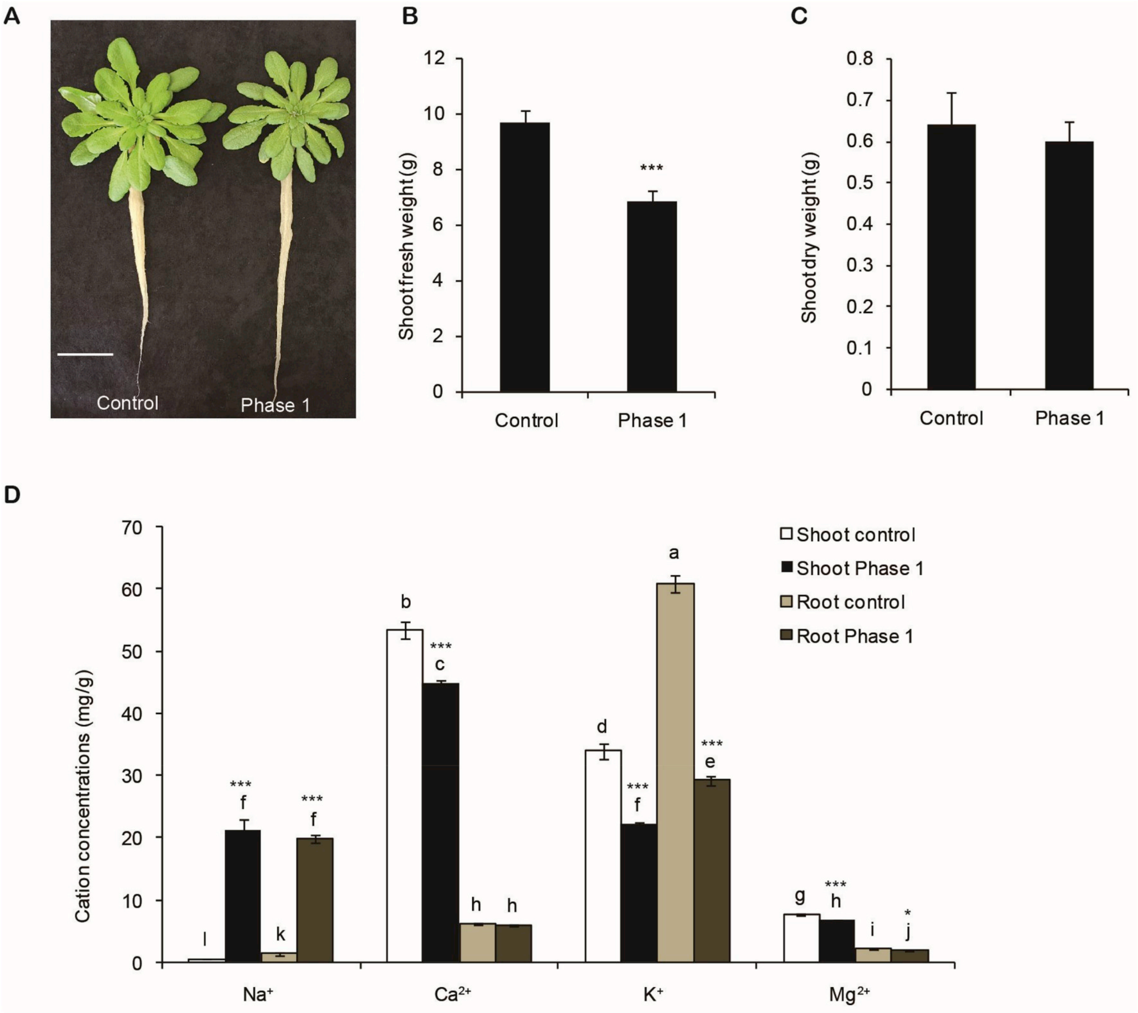

Fig. 3. Growth responses of *Arabidopsis thaliana* to 90 mM NaCl after a stepwise salt acclimation (15 mM NaCl per day).
A. Phenotypes of Arabidopsis plants exposed to 90 mM NaCl for 2 days. White bar = 2 cm.
B/C. Quantification of shoot fresh weight (**B**), and shoot dry weight (**C**) of Arabidopsis plants treated with 90 mM NaCl for 2 days. Significant differences are indicated by asterisks (*, **, *** significant differences of shoot fresh weight during Phase 1 in comparison to the control with $P < 5\%$, 1%, 0.1%, respectively; *t*-test). According to *t*-test, no significant difference of shoot dry weight was observed during Phase 1 in comparison to the control.
D. Cation concentrations (Na^{+}, Ca^{2+}, K^{+}, and Mg^{2+}) in Arabidopsis plants shoots and roots were quantified after 2 days treatment with 90 mM NaCl. Significant differences among Na^{+}, Ca^{2+}, K^{+}, and Mg^{2+} concentrations in shoots and roots are indicated by different letters ($P < 5\%$; one-way ANOVA and Post-hoc analysis using Tukey's Honestly Significant Difference Method). Significant differences of Na^{+}, Ca^{2+}, K^{+}, or Mg^{2+} concentrations in shoots or roots after salt treatment in comparison to Na^{+}, Ca^{2+}, K^{+}, or Mg^{2+} concentrations in shoots or roots under control conditions are indicated by asterisks (*$P < 5\%$, **$P < 1\%$, ***$P < 0.1\%$, *t*-test). Data show means of four replicates. Error bars represent the standard errors (SE).

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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 $2^{-\triangle Ct}$. $\triangle$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*

(Supplemental Fig. S2). Thus, YARA water sensors are suitable for the measurements of turgor pressure in Arabidopsis and maize.

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_p 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 (P_m) 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 (Ca^{2+}, K^+, and Mg^{2+}) 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).

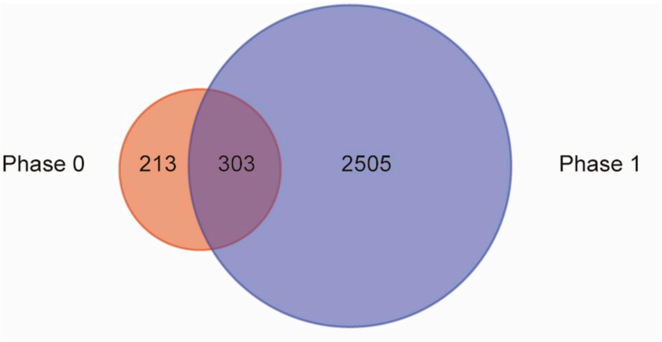

Fig. 4. RNA-sequence analysis of differentially transcribed genes (DTGs) of *Arabidopsis thaliana* in Phase 0 and Phase 1. Venn diagram showing the specially affected DTGs in Phase 0, Phase 1, and the overlap of DTGs between groups. False discovery rate (FDR) ≤ 0.05 and the value $|\log_2 \text{FoldChange (Phase 0/Control)}| \geq 1$ or $|\log_2 \text{FoldChange (Phase 1/Control)}| \geq 1$ are used as cut-off to evaluate DTGs.

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*, *EXPA1*, and *RZPF34* showed decreased transcription abundance. Seventeen DTGs were specially influenced in Phase 1. The transcription of *BGLU18*, *ArathEULS3*, *RBOHF*, *GLR2*, *ABCC5*, *MAPKKK18*, *ABCC4*, *ZIFL1*, and *TOD1* was up-influenced; *PHOT1*, *CRY2*, *BHLH128*, *SYP22*, *GRP7*, *APK1B*, *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 eceriferum family genes (*CER1*, *CER3*, *CER4*, *CER8*, 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 ($|\log_2 \text{FoldChange (Phase 0/Control)}| \geq 2$) 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	$\log_2$ FoldChange (Phase 0 /Control)	$\log_2$ FoldChange (Phase 1 /Control)	Mutant phenotypes
Genes only affected in Phase 0					
AT2G20880	ERF053	Integrase-type DNA-binding superfamily protein 53	2.79	-	larger stomata aperture in the presence of ABA, drought sensitive
AT5G10720	AHK5	Histidine kinase 5	2.63	-	larger stomatal aperture in the presence of H_2O_2
AT4G26080	ABI1	Protein phosphatase 2C family protein	1.29	-	larger stomata aperture in the presence of ABA
Genes affected both in Phase 0 and Phase 1					
AT5G59220	HAI-1 (SAG113)	Clade A protein phosphatases type 2C	4.00	5.90	smaller aperture in the presence of ABA
AT2G33380	RD20	Caleosin-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
Genes only affected in Phase 1					
AT2G47800	ABCC4 (MRP4)	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 kinase 18	-	1.96	larger stomata aperture under normal conditions, slower ABA-induced stomatal closure

(*continued on next page*)

Table 1 (*continued*)

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	iPGAM1	Phosphoglycerate mutase	-	-1.16	hyposensitive to blue light or low CO_2 induced of stomatal opening
AT1G05805	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
AT5G46860	SYP22	Syntaxin/t-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

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 ($|\log_2$ FoldChange (Phase 1/Control)$| \geq 5$) were observed (Table 4). Among these, *UF3GT*, *LDOX*, *TT8*, *GSTF12*, *SWEET15*, and AT5G62360 were selected as marker genes based on the transcriptional changes. The transcriptional levels of *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

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* increased after 1 h, 2 h and 3 h of 105 mM NaCl shock, but the fold changes were getting smaller with the prolonged treatment time (Fig. 5A).

To evaluate the Phase 1 marker gene candidates, Arabidopsis samples harvested after 1 day, 2 days, and 3 days of 90 mM NaCl treatment (stepwise acclimated, 15 mM NaCl per day) were analyzed using qRT-PCR. The absolute value of fold change at transcriptional level of all these Phase 1 candidates (except *GSTF12*) increased with the elongated treating time (Fig. 5B). The absolute value of $\log_2$ FoldChange ≥ 1 was used as cutoff to evaluate significant differences in gene transcription. All these Phase 0 and Phase 1 marker candidates were beyond the cutoff at transcriptional level after 1 h, 2 h, and 3 h of 105 mM NaCl shock or 1 day, 2 days, and 3 days of 90 mM NaCl treatment (Fig. 5), indicating high reliability of the suggested marker gene candidates.

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 RefGen_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, GRMZM2G125023, and GRMZM2G027098 exceeded the cutoff (Fig. 6A). 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, GRMZM2G345717, GRMZM2G042733, 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_p value could not recover after the peak which indicates that Arabidopsis had lost the ability to adjust turgor. Second, the diurnal change of P_p was abolished in the following days. Generally, turgor decreased during the day because of transpiration and was restored at night. As P_p is inversely correlated with turgor, P_p increased during the day and recovered at night under normal conditions. The irregular diurnal changes of P_p 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_p 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_m 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^+ 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^+ 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^+ exclusion and Na^+ compartmentation; Phase 2) may also have responded in Phase 1. Therefore, the following analyses based on RNA-

Table 2

Genes related to wax biosynthesis and water transportation were differentially transcribed in Phase 0 and Phase 1.

ID	Gene	Gene description	$\log_2$ FoldChange (Phase 0/Control)	$\log_2$ FoldChange (Phase 1/Control)
Wax biosynthesis				
AT2G16280	KCS9	3-Ketoacyl-CoA synthase 9	-1.10	-
AT5G37300	WSD1	O-Acyltransferase (WSD1-like) family protein	2.86	5.62
AT1G51140	CFLAP1	Basic helix-loop-helix (bHLH) DNA-binding superfamily protein	1.71	2.90
AT5G61590	DEWAX	Integrase-type DNA-binding superfamily protein	-1.51	-4.24
AT1G02205	CER1	Aldehyde decarbonylase	-	3.47
AT5G57800	CER3 (WAX2)	Fatty acid hydroxylase superfamily	-	1.31
AT4G33790	CER4	Alcohol-forming fatty acyl-CoA reductase	-	1.28
AT2G47240	CER8 (LACS1)	Acyl-CoA synthetase	-	-1.17
AT1G25450	CER60 (KCS5)	3-Ketoacyl-CoA synthase 5	-	-1.14
Water channels (aquaporin)				
AT3G16240	TIP2-1	Delta tonoplast integral protein	-2.01	-
AT4G35100	PIP2-7	Plasma membrane intrinsic protein 3	-1.15	-
AT4G23400	PIP1-5	Plasma membrane intrinsic protein 1-5	-1.10	-1.03
AT3G53420	PIP2-1	Plasma membrane intrinsic protein 2A	-	1.02
AT4G01470	TIP1-3	Tonoplast intrinsic protein 1-3	-	2.60

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 regulate stomatal movement through the ABA pathway, including *ABI1*, *HAI-1*, *RD20*, *PP2CA*, *AKS1*, *ABI2*, *RZPF34*, *BGLU18*, *RBOHF*, and *BHLH128*. Apart from the ABA pathway, plants can mediate stomatal movement via ion channels especially through Ca^{2+} channels (*e.g. PCaP1*, *ArathEULS3*, *GLR2*, *PHOT1*, and *CRY2*). Moreover, *EXPA1* and *SYP22* take part in stomatal opening through influencing the volumetric elastic modulus and vacuole fusion, respectively. *ABCC4*, *APK1B*, and *ELF3* showed in Table 1 are involved in the regulation of H^+-ATPase activity which is a key regulator of stomatal opening. *TOD1* and *ABCC5* participate in stomatal movement by influencing the inositol hexakisphosphate and sphingosine-1-phosphate metabolic processes, respectively.

Table 2 shows that genes related to wax biosynthesis were mostly influenced in Phase 1 at transcriptional level especially the eceriferum family genes (*e.g. CER1*, *CER3*, *CER4*, *CER8*, and *CER60*). *CER1* and *CER3* both encode fatty acid hydroxylase superfamily proteins. Overexpressing *CER1* in Arabidopsis increased the odd-carbon-numbered alkanes and reduced soil water deficit susceptibility (Bourdenx et al., 2011). *CER4* encodes an alcohol-forming fatty acyl-CoA reductase. Arabidopsis *cer4* mutants displayed a decreased primary alcohols and wax esters in stem, indicating that *CER4* was involved in cuticular wax biosynthesis (Rowland et al., 2006). *CER8* encodes an acyl-CoA synthetase; the Arabidopsis *cer8* mutants reduced the accumulation of wax in the stem and leaf, indicating that *CER8* was involved in cuticular wax and cutin biosynthesis (Lü et al., 2009). *CER60* encodes a member of the 3-ketoacyl-CoA synthase family which was involved in the biosynthesis of very long chain fatty acids (Trenkamp et al., 2004).

The aquaporin activity showed contrasting transcription patterns between Phase 0 and Phase 1 (Table 2). The salinity-induced short-term down-influenced and long-term up-influenced aquaporins are consistent with the findings in Arabidopsis (Jang et al., 2004) and rice (Kawasaki et al., 2001). Moreover, Hill et al. (2004) suggested that the initially down-regulated aquaporins may contribute to decreased water loss in the early stages of salt stress, and the later up-influenced aquaporins may play a role in the subsequent water uptake to maintain water homeostasis.

Table 3

Differentially transcribed genes with statistically significant changes in Phase 0. False discovery rate (FDR) ≤ 0.05 and the maximum value of $|\log_2$ FoldChange (Phase 0/Control)$| \ge 2$ 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 water transportation.

ID	Gene	$\log_2$ FoldChange (Phase 0/Control)	Gene description
AT4G28140	AT4G28140	3.87	△ Integrase-type DNA-binding superfamily protein
AT1G70640	AT1G70640	3.34	Octicosapeptide/Phox/Bem1p (PB1) domain-containing protein
AT2G20880	ERF053	2.79	△ Ethylene-responsive transcription factor ERF053
AT5G10720	AHK5	2.63	△ Histidine kinase 5
AT3G55500	EXPA16	2.58	Expansin-A16
AT1G61120	GES	2.57	(E,E)-geranyllinalool synthase
AT5G57010	IQM5	2.35	IQ domain-containing protein IQM5
AT1G52890	NAC019	2.28	NAC domain-containing protein 19
AT2G08575	AT2G08575	2.25	Natural antisense transcript overlaps with AT2G33390
AT2G25297	AT2G25297	2.19	Transmembrane protein
AT1G47890	AT1G47890	2.18	Disease resistance protein, putative
AT4G37540	LBD39	−2.01	LOB domain-containing protein 39
AT3G16240	TIP2-1	−2.01	▲ Aquaporin TIP2-1
AT2G08220	AT2G08220	−2.22	NA
AT3G52450	PUB22	−2.55	Plant U-box 22
AT5G65730	XTH6	−2.56	Xyloglucan endotransglucosylase/hydrolase
AT4G08950	EXO	−2.59	Phosphate-responsive 1 family protein
AT4G08040	ACS11	−2.91	1-Aminocyclopropane-1-carboxylate synthase 11

Table 4

Differentially transcribed genes with statistically significant changes in Phase 1. False discovery rate (FDR) ≤ 0.05 and the maximum value of $|\log_2$ FoldChange (Phase 1/Control)$| \geq 5$ 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	$\log_2$ FoldChange (Phase 1/Control)	Gene description
AT5G40790	AT5G40790	7.54	Hypothetical protein
AT4G08570	HIPP24	7.49	Heavy metal-associated isoprenylated plant protein 24
AT5G24880	AT5G24880	7.12	Chromo domain cec-like protein
AT3G24750	AT3G24750	6.55	Hypothetical protein
AT5G38130	AT5G38130	6.39	HXXXD-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	6.01	△ Glutathione S-transferase F12
AT1G01060	LHY	5.99	Homeodomain-like superfamily protein
AT4G14690	ELIP2	5.76	Early light-induced protein 2, chloroplastic
AT5G41315	GL3	5.72	Basic helix-loop-helix DNA-binding superfamily protein
AT5G59320	LTP3	5.61	Non-specific lipid-transfer protein 3
AT2G37770	AKR4C9	5.59	Aldo-keto reductase family 4 member C9
AT1G69480	AT1G69480	5.58	EXS (ERD1/XPR1/SYG1) family protein
AT5G59310	LTP4	5.56	Non-specific lipid-transfer protein 4
AT5G09930	ABCF2	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
AT3G22275	JAZ13	5.13	Protein JAZ13
AT4G27360	AT4G27360	5.11	Dynein 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	ILL5	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 endotransglucosylase/hydrolase protein 33
AT5G06690	WCRKC1	−5.43	WCRKC thioredoxin 1
AT3G51400	AT3G51400	−5.97	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	APRR5	−6.35	Two-component response regulator-like APRR5
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 methylesterase inhibitor superfamily protein

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. Overexpressing *AtERF053* in Arabidopsis leads to more sensitive stomatal behavior and greater drought tolerance (Cheng et al., 2012). The role of AT4G28140 in stomatal movement requires further investigation. *AHK5* encodes a member of the histidine kinase family and mediates stomatal closure in an ABA-independent way (Desikan et al., 2008). *TIP2-1* encodes a tonoplast water channel. 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 methylesterase 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) via 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

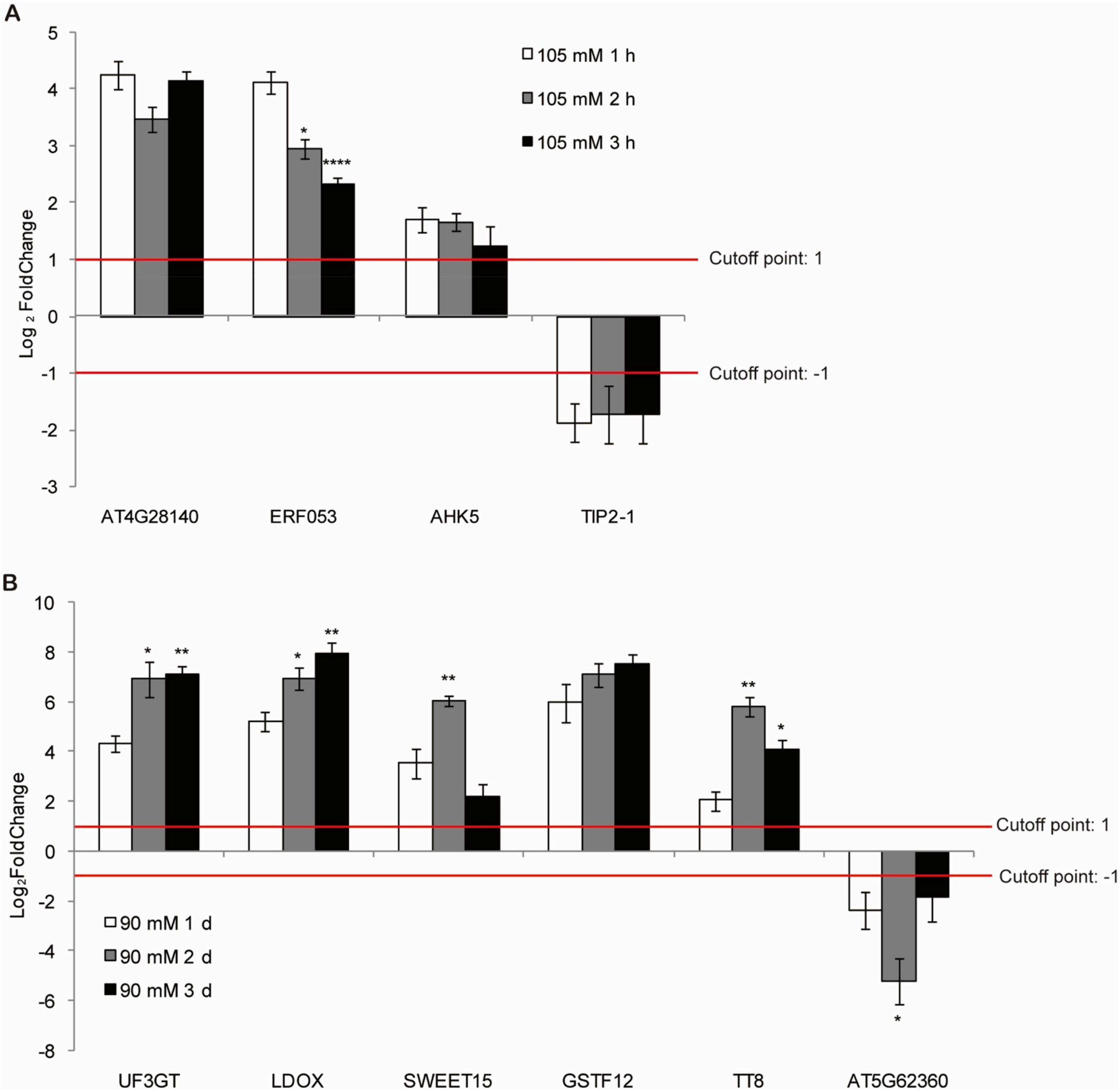

Fig. 5. Based on the RNA-sequence analysis, the selected Phase 0 and Phase 1 marker gene candidates were further tested via qRT-PCR.
Five-week-old Arabidopsis plants were treated with 105 mM NaCl shock and harvested after 1 h, 2 h, and 3 h treatments to test the Phase 0 marker gene candidates (**A**). Five-week-old Arabidopsis plants were stepwise acclimated to 90 mM NaCl (15 mM per day) and harvested after 1 day, 2 days, and 3 days treatments under 90 mM NaCl to test Phase 1 marker gene candidates (**B**). The y-axis values are the $\log_2$ FoldChange of transcript level after salt treatments in comparison to the control. Error bars represent standard errors (SE). Significant differences of each gene's relative transcription after 2 h and 3 h of 105 mM NaCl shock or 2 days and 3 days of 90 mM NaCl treatment in comparison to the transcript level after 1 h of 105 mM NaCl or 1 day of 90 mM NaCl treatment are indicated by asterisks ($^*P < 5\%$, $^{**}P < 1\%$, $^{***}P < 0.1\%$, $^{****}P < 0.01\%$, *t*-test). $|\log_2 \text{FoldChange}| \geq 1$ was used as cutoff to evaluate differentially transcribed genes. Red lines indicate cutoff points.

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. 1 C, 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. 1 A). Though Arabidopsis can endure 105 mM NaCl shock, the P_m value in Arabidopsis plants cannot completely recover on the next day (Fig. 1 B). 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

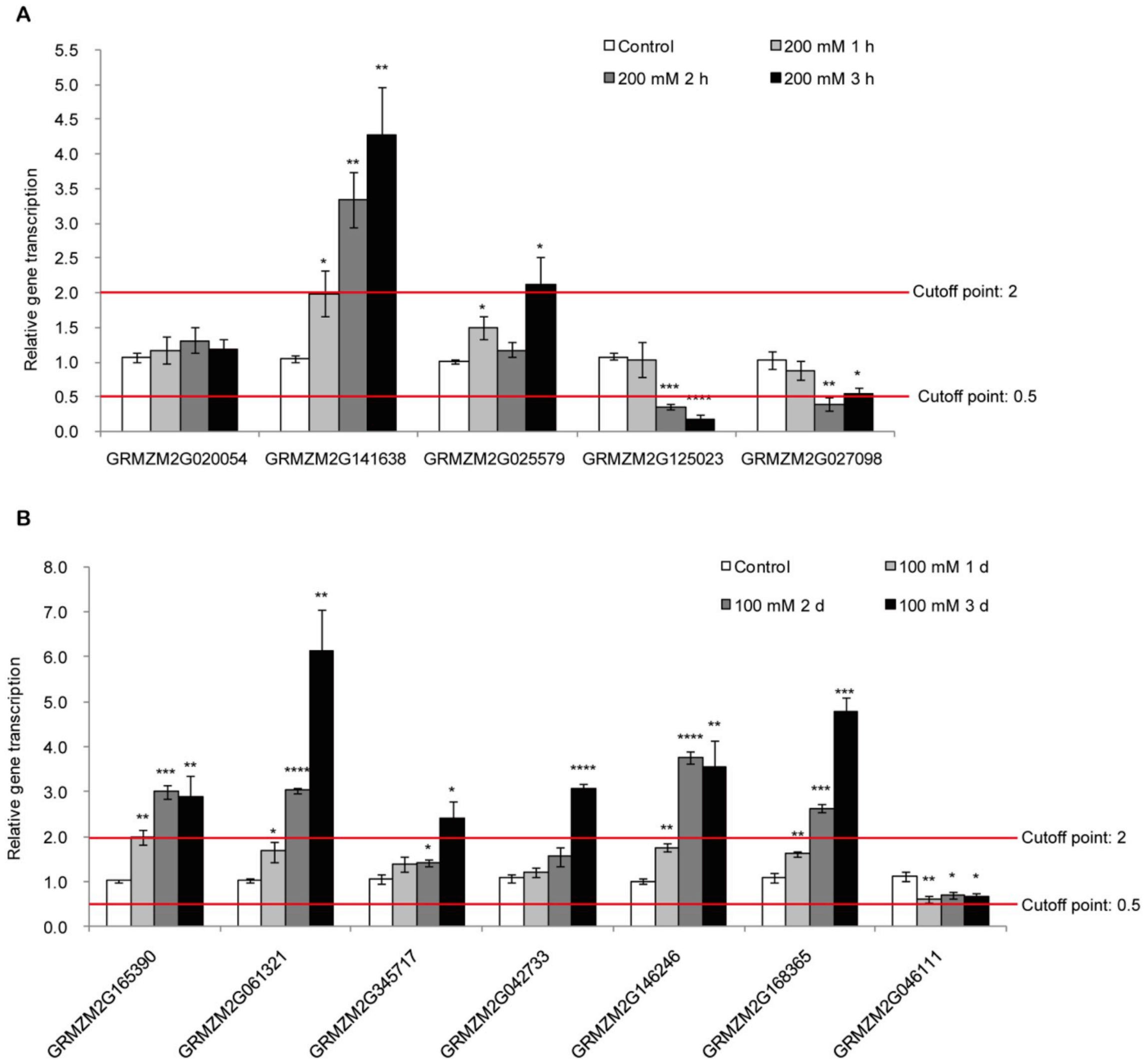

Fig. 6. Test marker gene candidates in *Zea mays*. Two-week-old Pioneer 3906 plants were treated with 200 mM NaCl shock and 100 mM NaCl by stepwise application (25 mM NaCl per day), respectively. The Phase 0 samples were harvested after 1 h, 2 h, and 3 h of 200 mM NaCl shock. The Phase 1 samples were harvested after 1 day, 2 days, and 3 days of 100 mM NaCl treatment. The graphs (y-axis) indicate the x-fold transcription of the genes during Phase 0 (**A**) and Phase 1 (**B**) as compared to the control (0 mM NaCl). Data show means of four biological replicates. Error bars represent the standard errors (SE). Significant differences are indicated by asterisks (*, **, ***, **** significant differences of each gene's relative transcription during Phase 0 and Phase 1 in comparison to the control with $P < 5\%$, 1%, 0.1%, 0.01%, respectively, *t*-test). The relative transcript level ≥2 or ≤0.5 were used as cutoff to evaluate significant up-regulated or down-regulated genes, respectively. Red lines indicate cutoff points. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

conditions, otherwise plants cannot recover from these stresses. At the transcriptional level, genes or analogs of *ERF053*, *TIP2-1*, *UF3GT*, *GSTF12*, and *SWEET 15* 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.

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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.

References

Ache, P., Bauer, H., Kollist, H., Al-Rasheid, K.A., Lautner, S., Hartung, W., Hedrich, R., 2010. Stomatal action directly feeds back on leaf turgor: new insights into the regulation of the plant water status from non-invasive pressure probe measurements. Plant J. 62, 1072–1082. https://doi.org/10.1111/j.1365-313x.2010.04213.x.

Bourdenx, B., Bernard, A., Domergue, F., Pascal, S., Léger, A., Roby, D., Pervent, M., Vile, D., Haslam, R.P., Napier, J.A., Lessire, R., Joubès, J., 2011. Overexpression of Arabidopsis ECERIFERUM1 promotes wax very-long-chain alkane biosynthesis and influences plant response to biotic and abiotic stresses. Plant Physiol. 156, 29–45. https://doi.org/10.1104/pp.111.172320.

Bramley, H., Ehrenberger, W., Zimmermann, U., Palta, J.A., Rüger, S., Siddique, K.H.M., 2013. Non-invasive pressure probes magnetically clamped to leaves to monitor the water status of wheat. Plant Soil 369, 257–268. https://doi.org/10.1007/s11104-012-1568-x.

Chalker-Scott, L., 2002. Do anthocyanins function as osmoregulators in leaf tissues? Adv. Bot. Res. 37, 104–129. https://doi.org/10.1016/S0065-2296(02)37046-0.

Cheng, M.C., Hsieh, E.J., Chen, J.H., Chen, H.Y., Lin, T.P., 2012. Arabidopsis RGLG2, functioning as a RING E3 ligase, interacts with AtERF53 and negatively regulates the plant drought stress response. Plant Physiol. 158, 367–375. https://doi.org/10.1104/pp.111.189738.

Cochard, H., Venisse, J.S., Barigah, T.S., Brunel, N., Herbette, S., Guilliot, A., Tyree, M.T., Sakr, S., 2007. Putative role of aquaporins in variable hydraulic conductance of leaves in response to light. Plant Physiol. 143, 122–133. https://doi.org/10.1104/pp.106.090092.

Cosgrove, D.J., 2005. Growth of the plant cell wall. Nat. Rev. Mol. Cell Biol. 6, 850–861. https://doi.org/10.1038/nrm1746.

De Costa, W., Zörb, C., Hartung, W., Schubert, S., 2007. Salt resistance is determined by osmotic adjustment and abscisic acid in newly developed maize hybrids in the first phase of salt stress. Physiol. Plant. 131, 311–321. https://doi.org/10.1111/j.1399-3054.2007.00962.x.

Defraeye, T., Derome, D., Verboven, P., Carmeliet, J., Nicolai, B., 2014. Cross-scale modeling of transpiration from stomata via the leaf boundary layer. Ann. Bot. 114, 711–723. https://doi.org/10.1093/aob/mct313.

Deinlein, U., Stephan, A.B., Horie, T., Luo, W., Xu, G., Schroeder, J.I., 2014. Plant salt-tolerance mechanisms. Trends Plant Sci. 19, 371–379. https://doi.org/10.1016/j.tplants.2014.02.001.

Desikan, R., Horák, J., Chaban, C., Mira-Rodado, V., Witthöft, J., Elgass, K., Grefen, C., Cheung, M.K., Meixner, A.J., Hooley, R., Neill, S.J., Hancock, J.T., Harter, K., 2008. The histidine kinase AHK5 integrates endogenous and environmental signals in Arabidopsis guard cells. PLoS One 3https://doi.org/10.1371/journal.pone.0002491. e2491.

Hanway, J.J., 1963. Growth stages of corn (*Zea mays*, L.). Agron. J. 55, 487–492. https://doi.org/10.2134/agronj1963.00021962005500050024x.

Hill, A.E., Shachar-Hill, B., Shachar-Hill, Y., 2004. What are aquaporins for? J. Membr. Biol. 197, 1–32. https://doi.org/10.1007/s00232-003-0639-6.

Jang, J.Y., Kim, D.G., Kim, Y.O., Kim, J.S., Kang, H., 2004. An expression analysis of a gene family encoding plasma membrane aquaporins in response to abiotic stresses in *Arabidopsis thaliana*. Plant Mol. Biol. 54, 713–725. https://doi.org/10.1023/b:plan.0000040900.61345.a6.

Kawasaki, S., Borchert, C., Deyholos, M., Wang, H., Brazille, S., Kawai, K., Galbraith, D., Bohnert, H.J., 2001. Gene expression profiles during the initial phase of salt stress in rice. Plant Cell 13, 889–905. https://doi.org/10.1105/tpc.13.4.889.

Kim, T.H., Böhmer, M., Hu, H., Nishimura, N., Schroeder, J.I., 2010. Guard cell signal transduction network: advances in understanding abscisic acid, CO_2, and Ca^{2+} signaling. Annu. Rev. Plant Biol. 61, 561–591. https://doi.org/10.1146/annurev-arplant-042809-112226.

Kitamura, S., Shikazono, N., Tanaka, A., 2004. Transparent Teata 19 is involved in the accumulation of both anthocyanins and proanthocyanidins in Arabidopsis. Plant J. 37, 104–114. https://doi.org/10.1046/j.1365-313x.2003.01943.x.

Koh, E.J., Lee, S.J., Hong, S.W., Lee, H.S., Lee, H., 2008. The ABA effect on the accumulation of an invertase inhibitor transcript that is driven by the CAMV35S promoter in Arabidopsis. Mol. Cells 26, 236–242.

Kosma, D.K., Bourdenx, B., Bernard, A., Parsons, E.P., Lü, S., Joubès, J., Jenks, M.A., 2009. The impact of water deficiency on leaf cuticle lipids of Arabidopsis. Plant Physiol. 151, 1918–1929. https://doi.org/10.1104/pp.109.141911.

Leitão, L., Prista, C., Loureiro-Dias, M.C., Moura, T.F., Soveral, G., 2014. The grapevine tonoplast aquaporin TIP2;1 is a pressure gated water channel. Biochem. Biophys. Res. Commum. 450, 289–294. https://doi.org/10.1016/j.bbrc.2014.05.121.

Li, P., Li, Y.J., Zhang, F.J., Zhang, G.Z., Jiang, X.Y., Yu, H.M., Hou, B.K., 2017. The Arabidopsis UDP-glycosyltransferases UGT79B2 and UGT79B3, contribute to cold, salt and drought stress tolerance via modulating anthocyanin accumulation. Plant J. 89, 85–103. https://doi.org/10.1111/tpj.13324.

Lü, S., Song, T., Kosma, D.K., Parsons, E.P., Rowland, O., Jenks, M.A., 2009. Arabidopsis CER8 encodes Long-Chain Acyl-Coa Synthetase 1 (Lacs1) that has overlapping functions with LACS2 in plant wax and cutin synthesis. Plant J. 59, 553–564. https://doi.org/10.1111/j.1365-313X.2009.03892.x.

Lü, S., Zhao, H., Des Marais, D.L., Parsons, E.P., Wen, X., Xu, X., Bangarusamy, D.K., Wang, G., Rowland, O., Juenger, T., Bressan, R.A., Jenks, M.A., 2012. Arabidopsis ECERIFERUM9 involvement in cuticle formation and maintenance of plant water status. Plant Physiol. 159, 930–944. https://doi.org/10.1104/pp.112.198697.

Munns, R., 1993. Physiological processes limiting plant growth in saline soil: some dogmas and hypotheses. Plant Cell Environ. 16, 15–24. https://doi.org/10.1111/j.1365-3040.1993.tb00840.x.

Munns, R., Tester, M., 2008. Mechanisms of salinity tolerance. Annu. Rev. Plant Biol. 59, 651–681. https://doi.org/10.1146/annurev.arplant.59.032607.092911.

Nardini, A., Salleo, S., Andri, S., 2005. Circadian regulation of leaf hydraulic conductance in sunflower (*Helianthus annuus* L. cv Margot). Plant Cell Environ. 28, 750–759. https://doi.org/10.1111/j.1365-3040.2005.01320.x.

Pou, A., Medrano, H., Flexas, J., Tyerman, S.D., 2013. A putative role for TIP and PIP aquaporins in dynamics of leaf hydraulic and stomatalconductances in grapevine under water stress and re-watering. Plant Cell Environ. 36, 828–843. https://doi.org/10.1111/pce.12019.

Pritchard, J., Fricke, W., Tomos, D., 1996. Turgor-regulation during extension growth and osmotic stress of maize roots. An example of single-cell mapping. Plant Soil 187, 11–21. https://doi.org/10.1007/bf00011653.

Rengasamy, P., 2006. World salinization with emphasis on Australia. J. Exp. Bot. 57, 1017–1023. https://doi.org/10.1093/jxb/erj108.

Rowland, O., Zheng, H., Hepworth, S.R., Lam, P., Jetter, R., Kunst, L., 2006. CER4 encodes an alcohol-forming fatty acyl-coenzyme A reductase involved in cuticular wax production in Arabidopsis. Plant Physiol. 142, 866–877. https://doi.org/10.1104/pp.106.086785.

Roy, S.J., Negrão, S., Tester, M., 2014. Salt resistant crop plants. Curr. Opin. Biotechnol. 26, 115–124. https://doi.org/10.1016/j.copbio.2013.12.004.

Schubert, S., 2011. Salt resistance of crop plants: physiological characterization of a multigenic trait. In: Hawkesford, M.J., Barraclough, P. (Eds.), The Molecular and Physiological Basis of Nutrient Use Efficiency in Crops. Wiley Blackwell, Ames, USA, pp. 443–455.

Schubert, S., Neubert, A., Schierholt, A., Sümer, A., Zörb, C., 2009. Development of salt-resistant maize hybrids: the combination of physiological strategies using conventional breeding methods. Plant Sci. 177, 196–202. https://doi.org/10.1016/j.plantsci.2009.05.011.

Seo, P.J., Park, J.M., Kang, S.K., Kim, S.G., Park, C.M., 2011. An Arabidopsis senescence-associated protein SAG29 regulates cell viability under high salinity. Planta 233, 189–200. https://doi.org/10.1007/s00425-010-1293-8.

Shavrukov, Y., 2013. Salt stress or salt shock: which genes are we studying? J. Exp. Bot. 64, 119–127. https://doi.org/10.1093/jxb/ers316.

Sümer, A., Zörb, C., Yan, F., Schubert, S., 2004. Evidence of sodium toxicity for the vegetative growth of maize (*Zea mays* L.) during the first phase of salt stress. J. Appl. Bot. 78, 135–139.

Thiel, G., Lynch, J., Läuchli, A., 1988. Short-term effects of salinity stress on the turgor and elongation of growing barley leaves. J. Plant Physiol. 132, 38–44. https://doi.org/10.1016/s0176-1617(88)80180-9.

Trenkamp, S., Martin, W., Tietjen, K., 2004. Specific and differential inhibition of very-long-chain fatty acid elongases from Arabidopsis thaliana by different herbicides. Proc. Natl. Acad. Sci. 101, 11903–11908. https://doi.org/10.1073/pnas.0404600101.

Vetterlein, D., Kuhn, K., Schubert, S., Jahn, R., 2004. Consequences of sodium exclusion for the osmotic potential in the rhizosphere-Comparison of two maize cultivars differing in Na^+ uptake. J. Plant Nutr. Soil Sci. 167, 337–344. https://doi.org/10.1002/jpln.200420407.

Westhoff, M., Reuss, R., Zimmermann, D., Netzer, Y., Gessner, A., Gessner, P., Zimmermann, G., Wegner, L.H., Bamberg, E., Schwartz, A., Zimmermann, U., 2009. A non-invasive probe for online-monitoring of turgor pressure changes under field conditions. Plant Biol. 11, 701–712. https://doi.org/10.1111/j.1438-8677.2008.00170.x.

Zimmermann, D., Reuss, R., Westhoff, M., Gessner, P., Bauer, W., Bamberg, E., Bentrup, F.W., Zimmermann, U., 2008. A novel, non-invasive, online-monitoring, versatile and easy plant-based probe for measuring leaf water status. J. Exp. Bot. 59, 3157–3167. https://doi.org/10.1093/jxb/ern171.