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ORIGINAL ARTICLE

Diurnal and Circadian Regulation of Salt Tolerance in Arabidopsis

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Abstract Most living organisms have a circadian clock, which coordinates their internal biological events with external environmental signals. Recent work showed that the Arabidopsis circadian clock regulates the plant's responses to stresses such as drought, cold, pathogens, and wounding. However, the link between the circadian clock and the plant's response to salinity, which retards plant growth and reduces crop yields, has not yet been investigated. In this study, we showed that tolerance to salinity stress is regulated by the diurnal cycle in Arabidopsis. The salt-induced expression of the salt- and drought-responsive transcription factor gene *RD29A* depends on the time of day and the transcription of the Na^+/H^+ antiporter gene *SOS1* is under the control of the circadian clock. Furthermore, accumulation of SOS1 protein upon salt stress in transgenic plants that constitutively overexpress *SOS1* (*SOS1ox*) appears to occur in a diurnal cycle. These findings suggest that during the salinity stress response, the expression of *RD29A* and *SOS1* is modulated by diurnal cycles and the circadian clock, which allows the plant to anticipate and respond effectively to daytime transpiration-triggered dehydration, drought, and salinity stress.

Keywords: Arabidopsis, Circadian clock, NaCl, RD29A, Salinity, SOS1

Introduction

Salinity is a growing threat to agriculture worldwide. According to the Food and Agriculture Organization of the United Nations (FAO) Land and Plant Nutrition Management Service (2008) (htpp://www.fao.org/ag/agl/agll/spush), over 800 million hectares of land are affected by salt stress, a number that is rising very quickly. Salinity has become a major obstacle to agricultural production, as it limits crop yields and new farmland cultivation. Sodium chloride (NaCl), the most soluble and widespread salt on earth, accounts for most of the harmful effects of salts on plant growth and development. Excessive [Na^+] in soil causes osmotic stress, resulting in reduced available water and deficiencies in other nutrients from the soil, such as potassium (K^+). The osmotic effect of salt significantly reduces shoot growth rates (Munns and Tester 2008). Under salinity stress, high concentrations of Na^+ accumulate in plant cells, and when they reach a certain level, ion toxicity causes secondary damage, which hastens senescence in leaves (Munns and Tester 2008).

In *Arabidopsis thaliana*, osmotic stress is more harmful than sodium ion toxicity (Essah et al. 2003). Plant cells accumulate osmolytes, which help lower the cytosolic osmotic potential and maintain cell turgor. Osmolytes are also thought to function as low-molecular-weight chaperones (Hasegawa et al. 2000). Many genes involved in osmolyte biosynthesis are upregulated upon salt stress. For example, the expression of *P5CS* (*DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE 1*), which is required for proline accumulation under osmotic stress (Székely et al. 2008), is induced upon salt stress (Abraham et al. 2003). The promoters of salt stress-responsive genes contain some common regulatory *cis*-elements, such as the DEHYDRATION RESPONSIVE ELEMENT (DRE) and ABA RESPONSE ELEMENT (ABRE). Salt stress induces abscisic acid (ABA) accumulation, as well as transcription factors that specifically bind to these *cis*-elements in the promoters of *DREB2A* (*DEHYDRATION RESPONSIVE ELEMENT-BINDING FACTOR2A*), *DREB2B*, *RD29A/COR28* (*RESPONSIVE TO DESICCATION29/COLD REGULATED78*) and *RD29B*, and genes encoding bZIP (basic leucine zipper) transcription factors, which activate downstream genes that are required for plant salt tolerance (Mahajan and Tuteja

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2005). Many abiotic stress responses, such as cold and drought stress responses, share *cis*-elements and transcription factors with salt stress adaptive responses (Mahajan and Tuteja 2005).

One way that plants respond to [Na^+] involves the SOS (SALT OVERLY SENSITIVE) pathway. The transmembrane Na^+/H^+ antiporter SOS1 is maintained in a resting state by its C-terminal auto-inhibitory domain under normal conditions (Quintero et al. 2002). Upon salt stress, root-specific SOS3, an EF-hand calcium binding protein, senses the changes in cellular Ca^{2+} levels (Liu and Zhu 1998). The binding of calcium to SOS3 facilitates the dimerization of SOS3 and its subsequent interaction with the serine/threonine protein kinase SOS2 (Halfter et al. 2000; Liu et al. 2000; Sánchez-Barrena et al. 2005). The SOS2-SOS3 complex targets the cell membrane through the N-myristoylation of SOS3 and subsequently phosphorylates SOS1. This phosphorylation relieves SOS1 from auto-inhibition, and activated SOS1 starts to pump Na^+ out of the cell (Shi et al. 2000; Quintero et al. 2002; Qiu et al. 2002; Qiu et al. 2003).

The circadian rhythm is a temporal oscillation of genetic, metabolic, and physiological processes based on a 24-hour cycle, which allows organisms to anticipate day-night changes in the environment (Nakamichi 2011). Many circadian clock-regulated processes in plants are observable in the natural environment, such as rhythmic hypocotyl elongation, flower opening, and leaf movements (Millar et al. 1995; Dowson-Day and Millar 1999). The biochemical processes of plant cells that are affected by the circadian rhythm include oscillations in Ca^{2+} level (Johnson et al. 1995), hormone biosynthesis and responses (Thain et al. 2004; Covington and Harmer, 2007), and water uptake (Takase et al. 2011). *CCA1* (*CIRCADIAN CLOCK ASSOCIATED1*), *LHY* (*LATE ELONGATED HYPOCOTYL*), and *TOC1* (*TIMING OF CAB EXPRESSION1*) constitute the central oscillation loop in the plant circadian clock. CCA1 and LHY are morning-expressed MYB-like DNA binding transcription factors with partially redundant functions (Wang and Tobin 1998; Schaffer et al. 1998; Mizoguchi et al. 2002). TOC1 (also referred to as PRR1 [PSEUDO-RESPONSE REGULATOR1]) is an evening-expressed DNA binding transcription factor (Strayer et al. 2000; Gendron et al. 2012). Recently, several studies have shown that the plant circadian clock is involved in adaptive responses to stress (Hotta et al. 2007; Grundy et al. 2015; Seo and Mas 2015), including drought, cold, pathogens, and wounding by insect herbivory (Fowler et al. 2005; Legnaioli et al. 2009; Wang et al. 2011; Goodspeed et al. 2012). For instance, TOC1 regulates plant responses to drought (Legnaioli et al. 2009), and the induction of *CBF/DREBs* (*C-REPEAT BINDING FACTOR/DEHYDRATION RESPONSIVE ELEMENT BINDING FACTOR*) under low temperatures is controlled by the circadian clock (Fowler et al. 2005). The circadian clock "remembers" the 24-hour light/dark cycle and anticipates temporal environmental changes, which has given plants an advantage during evolution. Plants with a clock period matched to the environment contain more chlorophyll, fix more carbon, and grow faster than those with circadian periods differing from their environment (Dodd et al. 2005). However, the relationship between the plant circadian clock and salt stress responses has not yet been clarified.

We previously showed that a clock-regulated protein, GIGANTEA (GI), negatively regulates plant salt tolerance by blocking formation of the SOS2-SOS3 complex and thus preventing SOS1 phosphorylation (Fowler et al. 1999; Kim et al. 2013; Park et al. 2013). In this study, to fully elucidate the relationship between the plant circadian clock and salt responses, we investigated the expression of two salt stress-responsive genes, *RD29A* and *SOS1*. Salt-induced expression of *RD29A* and *SOS1* was much higher during the day than at night, suggesting that their salt-induced expression patterns are gated by the clock. Furthermore, the oscillations in SOS1 protein abundance appear to be under circadian regulation upon salt stress. The results of this study indicate that the diurnal cycling of SOS1 levels is one of the mechanisms that help plants effectively respond to salt stress.

Results

Arabidopsis is More Sensitive to Salt Stress During the Day Than at Night

To investigate how diurnal cycles and/or the circadian clock affect salt tolerance in plants, we initially tested the effects of diurnal cycles on salinity stress tolerance in Arabidopsis. Arabidopsis plants were grown under 12L/12D conditions in inert soil pots (Fig. 1B, C) or in hydroponic devices (Fig. 1D, E). The latter method allowed us to quickly and efficiently rinse salt off of the plants, for short-term salt treatments. Two groups of plants were treated with salt stress at different times. The first group of plants, the "Day" group, was treated with 200 mM NaCl only during the day under the light period. The second group of plants, the "Night" group, was treated with 200 mM NaCl only at night, from ZT12 to ZT24 under darkness (Fig. 1A). Two additional groups of plants were treated with either water or 100 mM NaCl for 24 hours per day to serve as the negative and positive controls, respectively. After a 10-d salt treatment, the Day group exhibited inhibited growth compared to the Night group (Fig. 1B, D), and the fresh weight of Day group plants was less than that of Night group plants (Fig. 1C, E). The results of both the hydroponic and inert soil experiments show that tolerance to salt stress differs in the day versus the night.

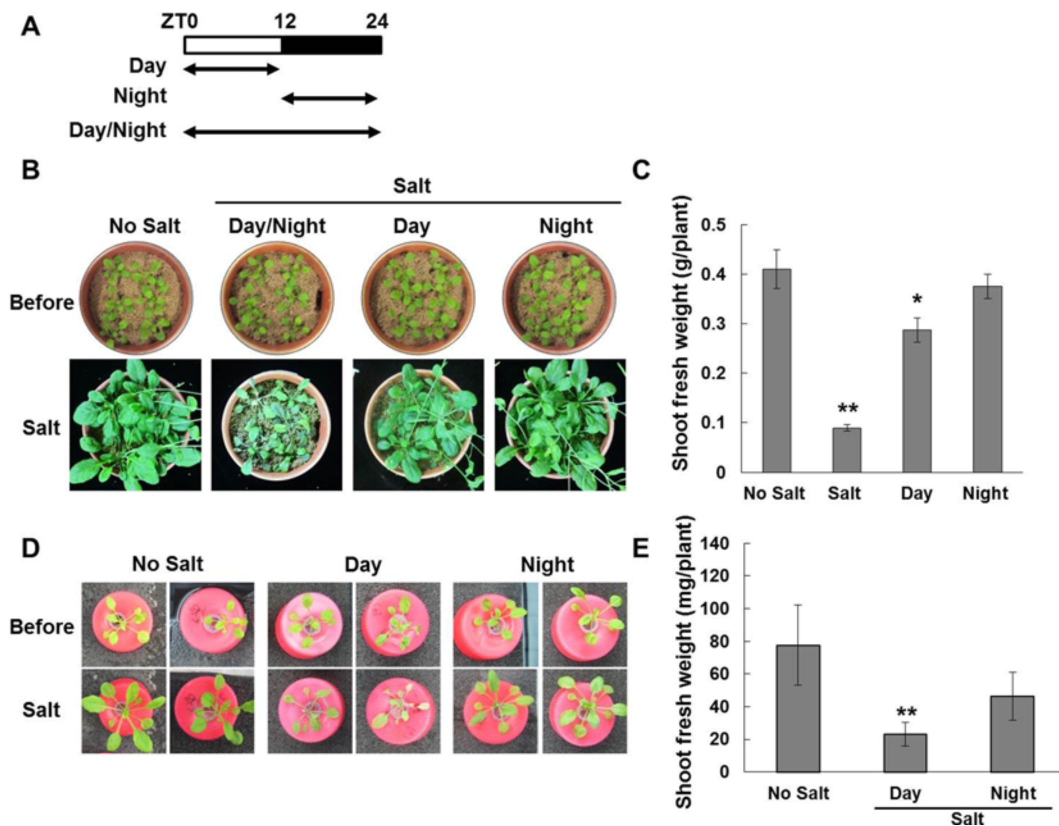

Fig. 1. Salt stress sensitivity differs during the day and night. (A) Strategy used for salt treatment. Three-week-old Arabidopsis wild-type Col-gl1 plants grown under 12L/12D conditions on inert soil (B, C) or in a hydroponic device (D, E) were exposed to 200 mM NaCl for 10 d. The "Day" group plants were treated with 200 mM NaCl only during the daytime (from ZT0 to ZT12) for 10 d. The "Night group" plants were treated with 200 mM NaCl only at night (from ZT12 to ZT24) for 10 d. For the control group, the "No Salt" plants were treated with 0 mM NaCl, and the "Day/Night" group was treated with 200 mM NaCl continuously for 10 d. After salt treatment, the plants were washed with distilled water twice and placed into basal nutrient medium. (B, C) Salt treatment in inert soil pots. The shoot fresh weight of plants from Figure (B) was measured and the data are shown in (C). The error bars represent standard error of 15 plants. (D, E) Salt treatment in the hydroponic devices. The shoot fresh weight of plants from Figure (D) was measured and the data are shown in (E). The error bars represent standard error of 15 plants. Significant differences compared with No Salt control group were determined by Student *t* test (Asterisk (*), $P<0.05$, Double asterisk (**), $P<0.01$ to No salt control).

Salt-induced Expression of *RD29A* is Affected by the Clock

The transcription of *RD29A*, a stress-responsive gene, is induced quickly and strongly upon drought stress (Yamaguchi-Shinozaki and Shinozaki 1993). Transcript levels of this salt-induced gene oscillate, with a peak at ZT8-10 in basal medium (Kreps et al. 2002; Dong et al. 2011). We previously showed that the salt-induced expression of *RD29A* is affected by the diurnal light/dark cycle and that the clock modulates *RD29A* expression (Kim et al. 2013). To determine the time of day at which *RD29* is the most highly induced upon salinity stress and whether salt-induced *RD29A* expression is affected by the clock, we treated wild-type plants (Col-0) under 12L/12D and LL conditions with 100 mM NaCl for 1 hour at different time points (ZT0, 4, 8, 12, 16, and 20) and measured *RD29A* transcript levels in the plants by qRT-PCR (Fig. 2). The difference between transcript levels in plants subjected to 0 mM and 100 mM NaCl treatment represents salt-induced *RD29A* transcript levels at each time point. The induction of *RD29A* expression was higher during the day (ZT0-1, ZT4-5, and ZT8-9) than at night (ZT12-13, ZT16-17, and ZT20-21) in 12L/12D conditions (Fig. 2A). A similar trend was observed under LL conditions. The increase in *RD29A* levels was higher during subjective day than during subjective night, indicating that *RD29A* was more highly induced by salt stress in subjective day than in subjective night (Fig. 2B). These results indicate that plants respond to salt stress more strongly during the day than at night, and they suggest that the circadian clock is involved in salt stress responses through regulating *RD29A* expression.

Salt-induced Expression of *SOS1* is Affected by the Clock

Next, we wondered whether *SOS1* transcription is under diurnal and circadian regulation, because SOS1 is critical for the salt stress response (Wu et al. 1996; Oh et al. 2010).

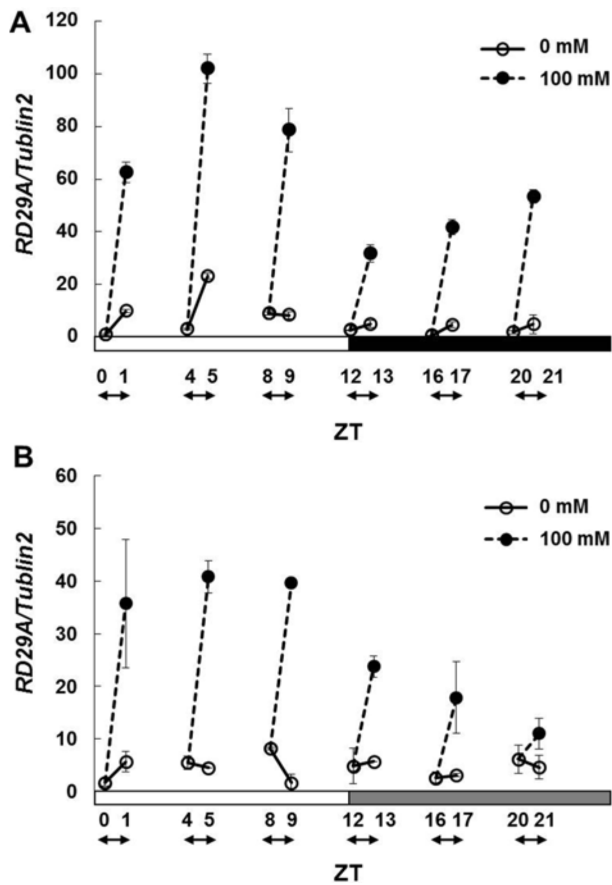

Fig. 2. Salt-induced *RD29A* expression is under the control of the circadian clock. (A) Salt-induced *RD29A* transcript levels under 12L/12D conditions. Two-week-old wild-type (Col-0) plants in 12L/12D conditions were treated with 0 mM (open circles) or 100 mM NaCl (filled circles) for 1 hr at the indicated time points. *RD29A* transcript levels were normalized to that of *TUBULIN2*. The results represent average values from three independent experiments, with three replicates per experiment. Bars indicate the standard deviation. (B) Salt-induced *RD29A* transcript levels under LL conditions. Twelve-day-old wild-type (Col-0) plants in 12L/12D were transferred to LL conditions. On the second day, the plants were treated with 100 mM NaCl (filled circles) for 1 hour at the indicated time points. *RD29A* transcript levels were normalized to that of *TUBULIN2*. The results represent average values from three independent experiments, with three replicates per experiment. Bars indicate the standard deviation.

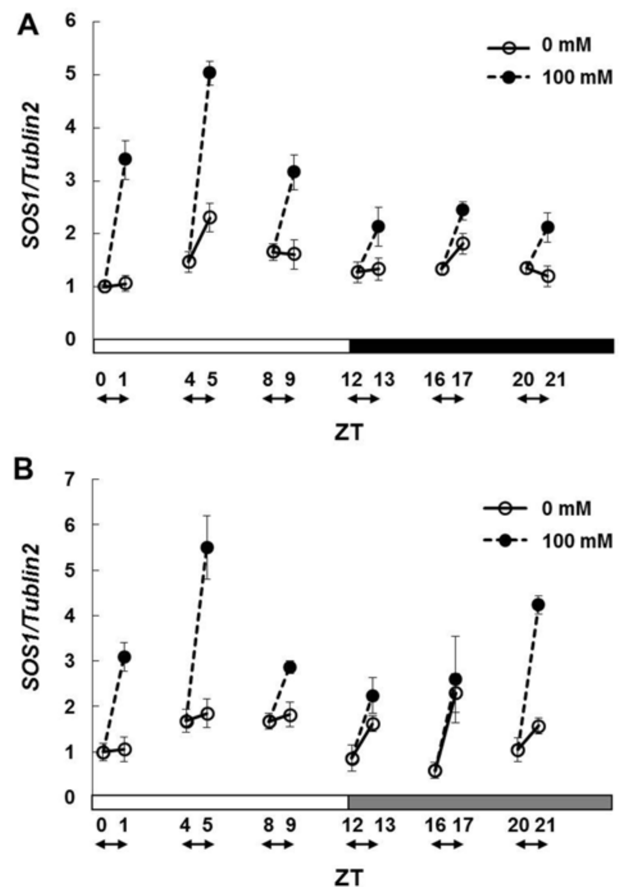

Fig. 3. Salt-induced *SOS1* expression is under the control of the circadian clock. (A) Salt-induced *SOS1* transcript levels under 12L/12D conditions. Two-week-old wild-type (Col-0) plants in 12L/12D conditions were treated with 0 mM (open circles) or 100 mM NaCl (filled circles) for 1 hr at the indicated time points. *SOS1* transcript levels were normalized to that of *TUBULIN2*. The results represent average values from three independent experiments, with three replicates per experiment. Bars indicate the standard deviation. (B) Salt-induced *SOS1* transcript levels under LL conditions. Twelve-day-old wild-type (Col-0) plants in 12L/12D conditions were transferred to LL conditions. On the second day of LL, the plants were treated with 100 mM NaCl for 1 hr at the indicated time points. *SOS1* transcript levels were normalized to that of *TUBULIN2*. The results represent average values from three independent experiments, with three replicates per experiment. Bars indicate the standard deviation.

Wild-type (Col-gl1) plants grown under 12L/12D or LL were harvested every 4 hours and *SOS1* transcript levels were measured by qRT-PCR. *SOS1* transcription oscillated approximately every 24 hours under both 12L/12D (Fig. S1A) and LL (Fig. S1B) conditions, but the amplitudes of the oscillations were not robust, with peaks around ZT8–10.

Next, we investigated whether the induction of *SOS1* transcription by salinity stress is under the control of diurnal cycles and the circadian clock (Fig. 3). We treated wild-type plants grown under 12L/12D or LL conditions with 100 mM NaCl for 1 hour at the indicated time points and measured *SOS1* transcript levels by qRT-PCR. *SOS1* transcription was significantly induced by one hour of NaCl treatment in the daytime, but *SOS1* expression was not affected in plants treated with 100 mM salt at night (Fig. 3A). Similarly, salt-induced *SOS1* expression was higher in the subjective day than at subjective night (Fig. 3B). These results suggest that the salt-induced expression of *SOS1* is modulated by the circadian clock.

Salt-induced Accumulation of SOS1 Protein is Regulated by Day/night Diurnal Cycles

SOS1 is not significantly induced by salt stress in wild-type plants (Dinneny et al. 2008). However, *SOS1* is highly and rapidly induced upon salt treatment in *SOS1*-overexpressing transgenic plants (*SOS1ox*), where *SOS1* is constitutively expressed under the control of the *35S* promoter (Chung et al. 2008), probably through salt-induced increases in mRNA stability. SOS1 is activated by the SOS2-SOS3 kinase complex upon salt stress. This phosphorylation leads to an increase in the Na^+/H^+ exchange rate of the SOS1 transporter (Shi et al. 2000; Quintero et al. 2002; Qiu et al. 2002; Qiu et al. 2003). Thus, we next investigated whether the protein stability of SOS1 is also under a diurnal cycle using the *SOS1* overexpressor *SOS1ox* (*35S::SOS1-HA* transgenic line) and performing immunoblotting with HA antibody (Fig. 4; Fig. 5). While the protein abundance of SOS1 in *SOS1ox* transgenic plants did not display an obvious diurnal cycle in basal medium without salt treatment, a 2-hour salt treatment with 100 mM NaCl from ZT0 to ZT2 increased the accumulation of SOS1, which reached a peak in 8 to 12 hours. Salt-induced SOS1 levels exhibited robust cycling, with a period of nearly 24 hours (Fig. 4; Fig. S2). However, salt stress treatment given at night (from ZT12 to ZT14) failed to induce the accumulation of SOS1 (Fig. 5). Instead, 8 to 12 hours after the 2-hour salt treatment and at dawn on the following day, salt-induced accumulation of SOS1 began, but the levels of this protein increased only approximately 2-fold at their peak, exhibiting very weak oscillation (Fig. 5C). These results suggest that the diurnal cycle affects SOS1 accumulation under salinity stress, which might represent an adaptive stress response.

Discussion

Our findings demonstrate that plants have different levels of sensitivity to salt stress in the day versus at night, as plants exposed to salinity stress during the day grew much more poorly than those treated at night (Fig. 1). This temporal variation in the susceptibility to high salinity is not unexpected, because the rate of transpiration, which facilitates Na^+ transport from root to shoot, as well as the accumulation of salt in plant cells, is much higher during the day than at night.

The transcription of almost one-third of Arabidopsis genes

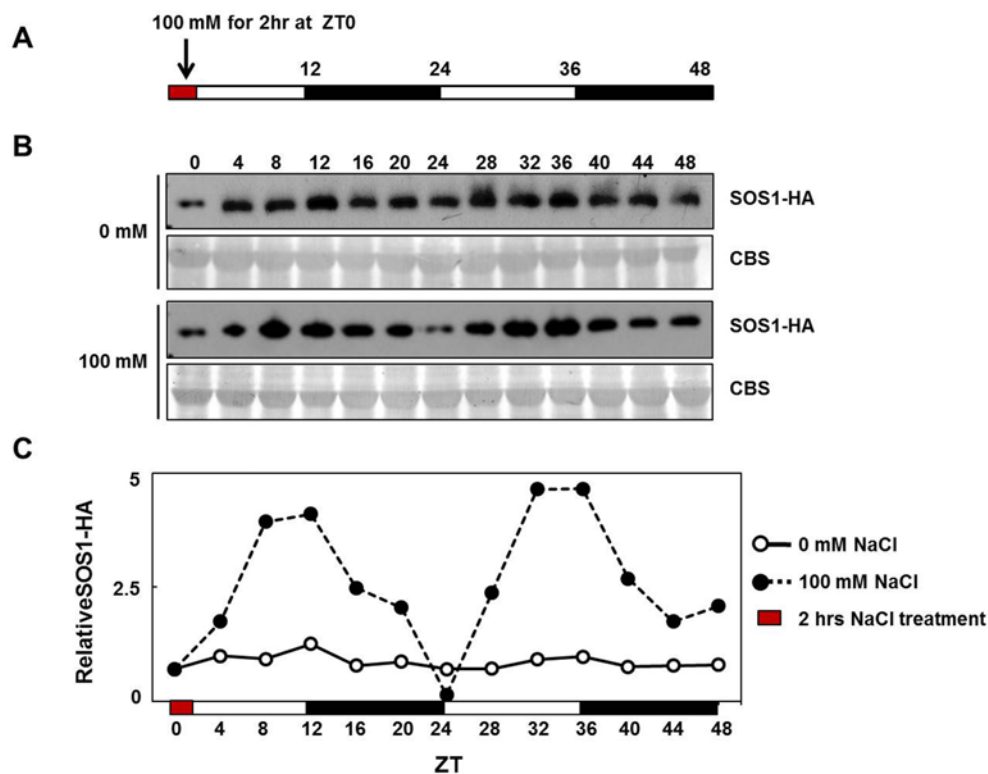

Fig. 4. Salt-induced SOS1 protein accumulation during the daytime exhibits a diurnal cycle. (A) Strategy of salt treatment. *35S::SOS1-HA* transgenic plants under 12L/12D conditions were treated with 0 mM or 100 mM NaCl for only 2 hours from ZT0 to ZT2. After salt treatment, the plants were moved back to normal MS medium and harvested at the indicated time points. (B) Total proteins were extracted from plants treated with 100 mM NaCl for 2 hours from ZT0 to ZT2 in panel (A). SOS1-HA protein levels were analyzed by immunoblotting with anti-HA. Relative amounts of SOS1-HA were measured based on the band intensity of SOS1-HA relative to Coomassie Blue staining (CBS). (C) The graph shows the standardized quantification of SOS1-HA levels from one representative blot. Open and filled circles indicate 0 mM and 100 mM NaCl treatment, respectively.

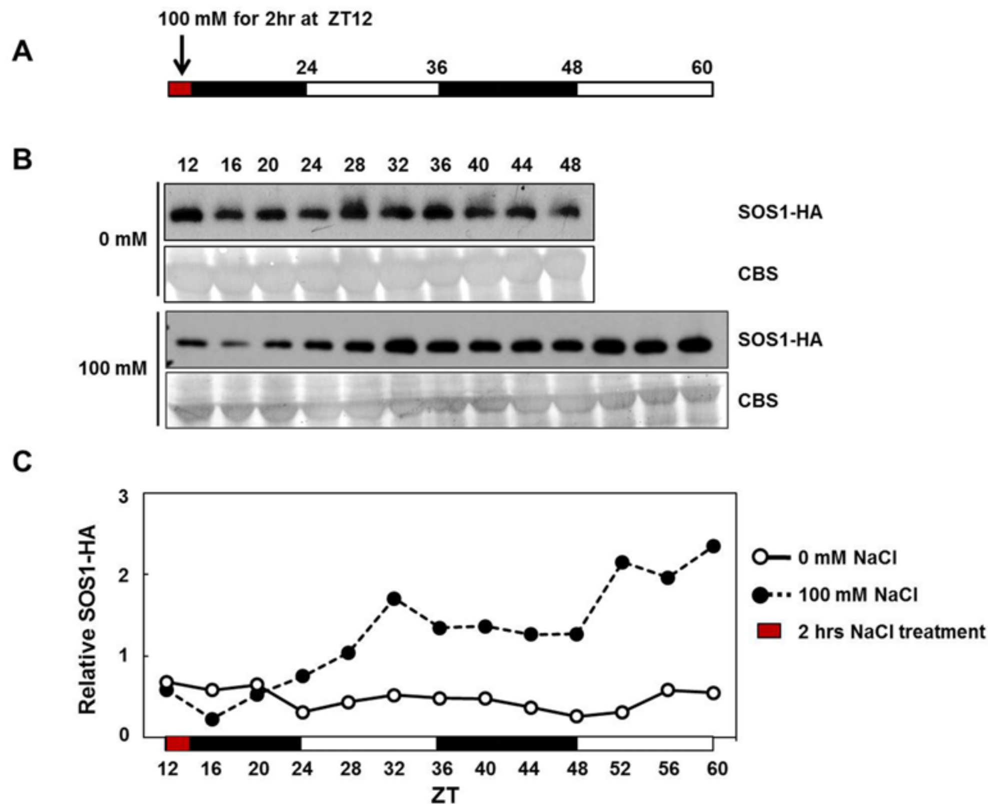

Fig. 5. Salt treatment at night does not induce diurnal cycling of SOS1 protein abundance. (A) Strategy of salt treatment. *35S::SOS1-HA* transgenic plants under 12L/12D conditions were treated with 0 mM or 100 mM NaCl for only 2 hours from ZT12 to ZT14. After salt treatment, the plants were moved back to normal MS medium and harvested at the indicated time points. (B) Total proteins were extracted from plants treated with 100 mM NaCl for 2 hours from ZT12 to ZT14 in panel (A). SOS1-HA protein levels were analyzed by immunoblotting with anti-HA. Relative amounts of SOS1-HA were measured based on the band intensity of SOS1-HA relative to Coomassie Blue staining (CBS). (C) The graph shows the standardized quantification of SOS1-HA levels from one representative blot. Open and filled circles indicate 0 mM and 100 mM NaCl treatment, respectively.

is regulated diurnally and/or by the circadian clock, including stress-responsive genes such as *RD29A*, *CBF*/*DREB*, and ABA biosynthesis and signaling pathway genes (Harmer et al. 2000; Fowler et al. 2005; Nováková et al. 2005; Covington et al. 2008; Michael et al. 2008; Lee and Thomashow 2012). A range of transporter genes are also regulated, such as genes encoding nutrient and ion transporters including sucrose transporters (*SUC2* and *SUC5*), nitrate transporters (*NRT1.1* and *NRT2.7*), potassium transporters (K^+ uptake permeases [*KUP3*, *KUP5*, *KUP6*, and *KUP11*] and Arabidopsis Potassium Transport 2/3 [*AKT2*]), vacuolar Na^+/H^+ antiporter (*NHX1*), copper transporters (*CORT1* and *CORT2*), and zinc transporter precursors (*ZIP11* and *ZTP29*) (Haydon et al. 2011). A recent report indicates that *COPPER TRANSPORTER2* (*CORT2*) and *IRON SUPEROXIDE DISMUTASE* (*FSD1*), the targets of the SQUAMOSA PROMOTER BINDING PROTEIN-LIKE7 (SPL7) transcription factor, are under circadian and diurnal regulation (Perea-García et al. 2016a; Perea-García et al. 2016b). Here, we showed experimentally that *SOS1* expression is modulated by the circadian clock, because the basal transcript levels of this plasma membrane-localized Na^+/H^+ antiporter oscillate even under normal growth conditions and in constant light (Fig. S1).

The circadian clock facilitates predictive regulation of molecular and physiological processes in plants, with temporal changes over the 24 hour day/night cycle (Seung et al. 2011; Chow and Kay 2013; Seo and Mas 2015; Singh et al. 2015). A few studies have investigated the effects of circadian rhythms on stress responses (Greenham and McClung 2015), including shade avoidance (Salter et al. 2003), gibberellin (GA) signaling (Arana et al. 2011), jasmonate-mediated defense responses to fungal pathogens (Ingle et al. 2015) and herbivores (Goodspeed et al. 2012), ABA signaling and its downstream targets (Seung et al. 2011; Lee et al. 2016), and osmotic stress responses (Kiełbowicz-Matuk et al. 2014). For example, the response to ABA varies with the time of day, as ABA-induced stomatal closure is the most sensitive in the afternoon, indicating that plant defense against dehydration varies during the day (Correia et al. 1995). The resistance of a plant to pathogen infection is highest in the morning, when the environment is most favorable for pathogen infection due to high humidity levels (Bhardwaj et

al. 2011; Wang et al. 2011). Cold-responsive genes such as CBF transcription factor genes are more inducible during the day than at night, as cold-induced increases in *CBF* transcript levels are much higher in the morning (ZT4) than at night (ZT16), suggesting that the plant can deal with low temperatures more efficiently in the morning than in the evening or at night (Fowler et al. 2005). In the current study, we showed that Arabidopsis has different responses to salinity stress in the day and at night, using two salt stress-responsive genes, *RD29A* and *SOS1*. Salt-induced expression of *RD29A* and *SOS1* was much higher in the daytime than at night (Fig. 2 and 3), indicating that the plant apprehends dehydrating conditions and higher cellular sodium concentrations in the daytime over repeated 24 dark/night cycles and schedules its defensive processes or systems for temporal changes.

The molecular mechanism by which the circadian clock regulates stress responses, especially cold stress responses, involves transcriptional regulation. The promoter regions of *CBF* transcription factor genes include several Evening Elements (EE, AAATATCT) and CCA1 binding sites (CBS, AA[A/C]AATCT) (Harmer et al. 2000). The MYB transcription factors CCA1 and LHY, which are morning-expressed circadian components of the plant oscillator, directly bind to *CBF* promoter regions and regulate their expression. We therefore investigated the promoter region of *RD29A* using the Plant Promoter Analysis Navigator (http://PlantPAN2.itps.ncku.edu.tw) (Chang et al. 2008; Chow et al. 2016). The *RD29A* promoter region contains a putative CBS and two putative EEs, which might be subjected to regulation by CCA1 and LHY (Wang et al. 1997; Harmer and Kay 2005). The *SOS1* promoter contains two putative CBS (AACAATCA, -1731 bp and -1059 bp from the ATG start codon) and one putative EE (AAAAATCT, -1111 bp). However, further experiments are required to determine whether circadian components such as CCA and LHY associate with the promoter regions of *RD29A* and *SOS1*, consequently leading to transcriptional regulation.

Nonetheless, we found that the circadian cycling of basal *SOS1* transcription did not display great robustness. Salt enhances the stability of the *SOS1* transcript rather than promoting new transcription (Chung et al. 2008). However, SOS1 must be phosphorylated by SOS2, a Serine/Threonine protein kinase (Liu et al. 2000), after which activated SOS1 can export Na^+ out of the cell (Quintero et al. 2002). Thus, we decided to analyze whether the clock influences the abundance of SOS1 protein (Fig. 4; Fig. 5). The basal levels of SOS1 protein did not exhibit robust cycling. However, surprisingly, in plants treated in the morning, salt-induced SOS1 protein levels exhibited diurnal cycling (Fig. 4; Fig. S2). Salt treatment in the evening failed to induce the robust cycling of SOS1 protein abundance, and SOS1 protein abundance did not show clear dampening in the dark period, even though SOS1 protein levels increased during the day (Fig. 5). We employed *SOS1*-overexpressing transgenic plants (*SOS1ox*), which exhibited constitutively high *SOS1* transcription. The diurnal oscillation of SOS1 levels might arise from the diurnal cycling of the stability (and/or turnover) of *SOS1* transcripts and the resulting translation or diurnal oscillation of protein abundance. There are a few examples of posttranslational degradation and regulation of clock protein stability, such as the proteasomal degradation of GI, ZTL, TOC1, and PRR5 (Kim et al. 2003; Mas et al. 2003; Yu et al. 2008) and the phytochrome-mediated degradation of PHYTOCHROME-INTERACTING FACTOR (PIF3). PIF3, which promotes seedling growth, undergoes oscillations in protein abundance under diurnal short-day light/dark conditions. PIF3 degradation is mediated by photoactivated PhyB, leading to a peak in PIF3 accumulation before dawn, which results in the strong accumulation of bHLH (basic helix-loop-helix protein) during the dark period, as well as hypocotyl elongation (Soy et al. 2012). Whether the salt-induced oscillation in SOS1 levels occurs through ubiquitin-mediated proteasomal degradation or phytochrome-mediated degradation remains to be determined.

We are also interested in understanding how plants anticipate and respond to the elevated cellular sodium levels that are likely driven by daytime transpiration. Transpiration in leaves leads to higher cellular sodium concentrations. This diurnal variation in cellular sodium levels might serve as an input to coordinate the circadian clock in the shoot and root under a certain threshold salt concentration in the plant cell. Sucrose transported from the shoot to root was proposed to serve as a signal that coordinates the circadian clock in the shoot and root or helps synchronize the internal clock to environmental factors (James et al. 2008). Transpiration from the shoot results in diurnal changes in root hydraulic conductivity and root-specific aquaporin expression (Sakurai-Ishikawa et al. 2011). In addition, exogenous copper (Cu) influences a few circadian clock components such as GI, leading to the proposal that dynamic oscillations in Cu levels are integrated into the clock to maximize metabolic efficiency under Cu-limited conditions (Perea-García et al. 2016a; Perea-García et al. 2016b). Thus, once the amount of Na^+ increases to a level not toxic to plant cells, diurnal fluctuations in cellular Na^+ levels could serve as a signal, which moves rapidly from shoot to root upon exposure to light, since roots are present underground and cannot usually sense the light signal.

In summary, plants modulate clock-dependent processes during the salinity stress response through regulating the

expression of salt stress-responsive genes such as *RD29A* and *SOS1*. This mechanism reflects the effective, elaborate way in which plants adapt to temporal environmental changes.

Materials and Methods

Plant Growth and Salt Stress Treatments

Seeds of wild-type (Col-0 or Col-gl1), *SOS1ox*, and *SOS1*-overexpressing (*35S::SOS1-HA* in Col-gl1) (Kim et al. 2013) *Arabidopsis thaliana* plants were surface-sterilized with 2.4% sodium hypochlorite (NaClO). The seeds were plated onto 1X Murashige and Skoog (MS) medium (sucrose 1.5%, pH 5.7), stratified at 4°C for 2–3 d before germination, and incubated in a growth chamber under a 12 h light/12 h dark (12L/12D) cycle (~120 μmol $m^{-2}s^{-1}$, 22–23°C). For plants in constant light conditions (LL), seedlings were entrained for 12 d under 12L/12D conditions before being introduced to the assay conditions. For salinity stress, the seedlings were treated with 100 mM NaCl as indicated in the figure legends.

Hydroponic experiments were performed using an in-house-made hydroponic device and inert soil (Isolite CG-1; Isolite Insulating Products, Osaka, Japan) (Ali et al. 2012). The plants were supplied with 1/8 liquid MS medium. Two-week-old wild-type Arabidopsis plants in the hydroponic device or artificial soil were treated with 200 mM NaCl as indicated in the figure legends. For salt treatment, hydroponic devices containing plants were transferred into MS solution supplemented with NaCl. After 12 h salt treatment, the entire hydroponic device and artificial soil pots were rinsed twice with fresh tap water before being transferred to standard MS solution to wash off the salt residue on the roots.

RNA Isolation and Expression Analysis

Plant tissue (100 mg) harvested at the appropriate times was used for RNA extraction. Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands). Total RNA (2 μg) was digested with DNase1 (SIGMA, St. Louis, USA) and reverse transcribed with SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, USA) for cDNA synthesis. The amplified products from quantitative reverse-transcription (qRT) PCR were detected using iQ SYBR Green Supermix (Bio-Rad) in a thermal cycler (CFX384 C1000TM Real time system, Bio-Rad, Hercules, USA). Primers used for RT-PCR include: Tubulin2-qRT-F (5′-AGCAAATGTGGGACTCCAAG-3′), Tubulin2-qRT-R (5′-CACCTTCTTCATCCGCAGTT-3′), SOS1-qRT-F (5′-CGCCAAACAACAACAAGAGA-3′), SOS1-qRT-R (5′-GGCT-GAAACGAGACCTTGAG-3′), RD29A-qRT-F (5′-ATCACTTGG-CTCCACTGTTG-3′), and RD29A-qRT-R (5′-ACAAAACACACA-TAAACATCCAAAGT-3′). The level of *TUBULIN2 (TUB2)* mRNA was used as an internal control. All qRT-PCRs were performed at least twice with three independent biological RNA samples.

Protein Extraction and Immunoblot Analysis

Plant tissue (100 mg) was extracted with 100 μL protein extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 3 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 5 g/mL Leupeptin, 1 g/mL Aprotinin, 1 g/mL Pepstatin, 5 g/mL Antipain, 5 g/mL Chymostatin, 2 mM Na_3VO_4, 2 mM NaF, 50 mM MG132). The total protein extract was separated by SDS-PAGE, followed by immunoblotting using anti-HA (clone 3F10; Roche, Penzberg, Germany) to detect SOS1-HA. Coomassie Brilliant Blue Staining (CBS) was used as the loading control. The graphs show standardized quantification of SOS1-HA levels from one representative blot.

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Author's Contributions

WYK and DJY initiated the project; HJP and ZQ performed the experiments; HJP wrote the paper with input from other authors. All authors discussed the results and approved the manuscript.

Supporting Information

Fig. S1. *SOS1* transcription is under the control of the circadian clock.
Fig. S2. Salt-induced protein accumulation of SOS1 is diurnal.
Fig. S3. *RD29A* and *SOS1* promoters contain a few putative CCA1 binding sites.

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