

Note

Temporal restriction of salt inducibility in expression of salinity-stress related gene by the circadian clock in *Solanum lycopersicum*

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Abstract Exposure to salinity causes plants to trigger transcriptional induction of a particular set of genes for initiating salinity-stress responses. Recent transcriptome analyses reveal that expression of a population of salinity-inducible genes also exhibits circadian rhythms. However, since the analyses were performed independently from those with salinity stress, it is unclear whether the observed circadian rhythms simply represent their basal expression levels independently from their induction by salinity, or these rhythms demonstrate the function of the circadian clock to actively limit the timing of occurrence of the salinity induction to particular times in the day. Here, by using tomato, we demonstrate that salt inducibility in expression of particular salinity-stress related genes is temporally controlled in the day. Occurrence of salinity induction in expression of *SISOS2* and *P5CS*, encoding a sodium/hydrogen antiporter and an enzyme for proline biosynthesis, is limited specifically to the morning, whereas that of *SIDREB2*, which encodes a transcription factor involved in tomato responses to several abiotic stresses such as salinity and drought, is restricted specifically to the evening. Our findings not only demonstrate potential importance in further investigating the basis and significance of circadian gated salinity stress responses under fluctuating day/night conditions, but also provide the potential to exploit an effective way for improving performance of salinity resistance in tomato.

Key words: circadian rhythms, salinity stress, tomato.

Abiotic stress is the largest cause of crop loss across the planet, causing decreases in major crop yields by up to 50% (Wang et al. 2003). In particular, salinity stress is one of the most limiting factors in plant growth, with salt produced and washed into groundwater supplies from farming irrigation contributing 45 million hectares to the 77 million hectares of global salt affected land (Alian et al. 2000; Munns and Tester 2008). Simultaneously, global food production is estimated to need to increase by 50 to 100% before 2050 in order to sustain the world's population (Godfray et al. 2010). It is therefore important to first understand the impact of salinity stress on plant growth and its interactions within the plant before seeking to solve the issue in a sustainable way. Salinity stress has two main effects in plants: short term osmotic stress, and long term ionic buildup (Munns and Tester 2008). Osmotic stress is the immediate stress on the plant caused by decrease in water potential in the soil. Ionic stress is the accumulation of ions, usually sodium

and chlorine, to toxic levels (Maggio et al. 2007). Plant responses seek to assuage these impacts on plant growth and proliferation.

Recent analyses for understanding molecular-genetic mechanisms that confer salinity tolerance in plants provided information on genes involved in these mechanisms (Ouyang et al. 2007; Singh and Laxmi 2015). Plant mechanisms for combating osmotic stress upon increased salinity include accumulating proline, an amino acid that counteracts osmotic stress and “scavenges” hydroxyl radicals, which protects macromolecules from destabilization (Zhang and Blumwald 2001). Under salinity and drought stress, proline is known to accumulate (Hmida-Sayari et al. 2005; Peng et al. 1996). PYRROLINE-5-CARBOXYLATE SYNTHASE (*P5CS*) is an enzyme necessary for converting L-glutamate to glutamate semialdehyde. It acts as the rate-limiting step in the pathway that synthesizes proline from glutamate, which occurs under osmotic

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stress conditions (Delauney et al. 1993; Hmida-Sayari et al. 2005; Hu et al. 1992). Expression of the gene encoding this enzyme has been reported to be induced by salinity in plants including *Lotus japonicus*, tomato, and potato (Fujita et al. 1998; Hmida-Sayari et al. 2005; Signorelli and Monza 2017).

The mechanism for preventing accumulation of sodium ion in cells involves *SALT-OVERLY-SENSITIVE 1 (SOS1)* and *SALT-OVERLY-SENSITIVE 2 (SOS2)*, which encode a hydrogen-sodium antiporter and a serine/threonine kinase, respectively. Expression of these genes are upregulated under salinity stress in *Arabidopsis thaliana* (Gao et al. 2012). Apart from induction of expression of these genes by salinity stress, activity of these proteins are also controlled by exposure to salinity in *A. thaliana*. Under saline conditions, *SALT-OVERLY-SENSITIVE 3 (SOS3)*, a calcium binding protein, reacts to the cellular changes in calcium concentration and activates *SALT-OVERLY-SENSITIVE 2 (SOS2)* (Du et al. 2011). *SOS2* then phosphorylates *SALT-OVERLY-SENSITIVE 1 (SOS1)* to mediate sodium/hydrogen ion exchange at the cell membrane in *A. thaliana* (Olías et al. 2009). Due to *SOS1*'s localization to the leaf vasculature junctures, it is thought to pump sodium ions that accumulate in the leaves into the stem (Ji et al. 2013; Olías et al. 2009). *SOS1* under-expressing *Arabidopsis* mutants exhibit reduced growth under saline conditions, which suggests a role in plant robustness against salinity stress (Shi et al. 2000). *SOS1* and *SOS2* have been reported to confer salt tolerance in tomato as well. Silencing of tomato *SOS1 (SISOS1)* also exhibit reduced growth under saline condition (Olías et al. 2009). Also, overexpression of tomato *SOS2 (SISOS2)* causes increased tolerance against salinity. Expression of both of these genes have been reported to be induced by salinity treatment (Huertas et al. 2012; Olías et al. 2009).

Due to cross-talk between drought, salinity stress, temperature, and biotic stress regulatory pathways, transcription of a significant number of genes involved in these pathways are commonly affected in response to a stress (Singh and Laxmi 2015). Another potential interacting factor is the circadian clock, an endogenous oscillator that generates timing information to allow organisms to anticipate daily changes in light and temperature by triggering behavior and physiology at the appropriate time of the day (Boikoglou et al. 2011; Dalchau et al. 2010; Millar 2004). While disruption of circadian rhythms does not strongly affect viability of *Arabidopsis*, it significantly impacts growth of the plants (Dodd et al. 2005; Green et al. 2002; Grundy et al. 2015). Also, mutations in *Arabidopsis* clock genes generally affect the magnitude of tolerance against various stressors, suggesting involvement of the circadian clock in controlling stress responses (Greenham and McClung 2015). Consistent with this observation, based

on microarray analyses of circadian-regulated genes in *Arabidopsis*, transcription of 50% of salt, heat, drought, and osmotic-stress-induced genes also oscillate under constant conditions (Covington et al. 2008; Grundy et al. 2015).

Several reports have demonstrated that the circadian clock determines the timing of stress-mediated induction in gene expression by temporally restricting or “gating” the activity of the induction mechanisms (Grundy et al. 2015). For example, in *Arabidopsis*, expression of genes that encode DEHYDRATION-RESPONSIVE ELEMENT B1/C-REPEAT-BINDING FACTORS (DREB1/CBF) are most strongly induced by morning cold treatment, whereas the same treatment during the evening does not induce the same level of response (Fowler et al. 2005; Kidokoro et al. 2017; Nakamichi et al. 2009). Similar circadian responses to cold treatment have been reported to be observed in CBFs in *Prunus persica*, the peach (Artlip et al. 2013). Similarly in *Vitis vinifera*, the grapevine, significant differences were observed in the transcriptional induction of heat stress-related genes, depending on whether the heat treatment was during the day or night (Rienth et al. 2014). These results clearly indicate that circadian gating of stress-mediated induction of gene expression confers a selective advantage, though little is known about exactly how this system contributes to plant fitness.

To date, expression of a handful of salinity-inducible genes have been demonstrated to exhibit circadian rhythms, including soybean proline-rich protein (SbPRP), cyclophilin 2 (CYP2) in *Oryza sativa*, Universal stress protein (USP) in *S. lycopersicum*, *Solanum* double B-box zinc finger (DBBX24), and *S. lycopersicum* ERD15 (He et al. 2002; Kiełbowicz-Matuk et al. 2014; Loukehaich et al. 2012; Ruan et al. 2011; Ziaf et al. 2016). However, in general, it is not known whether the oscillation of mRNA accumulation of salinity-inducible genes represents the effect of the circadian clock on their basal expression levels independent of their induction by salinity stress, or demonstrates the presence of the clock function to actively limit inducibility of gene expression by salinity to particular times in the day. In order to understand how the circadian clock contributes to the salinity-stress signalling pathway in tomato, we checked daily responses in expression of tomato genes to salinity. In this study we analyzed *SISOS1*, *SISOS2*, and *P5CS* since expression of these genes have been reported to be up-regulated by salinity treatment in tomato.

We treated tomato plants grown in 12 h of darkness/12 h of light (LD) with salinity in a hydroponic setup continuously for 3 days. On the third day, we harvested plants every 4 h over 24 h in the LD cycle and performed expression analyses. All of these genes were found to be upregulated by salinity treatment as compared to the non-treatment control (Figure 1A–C).

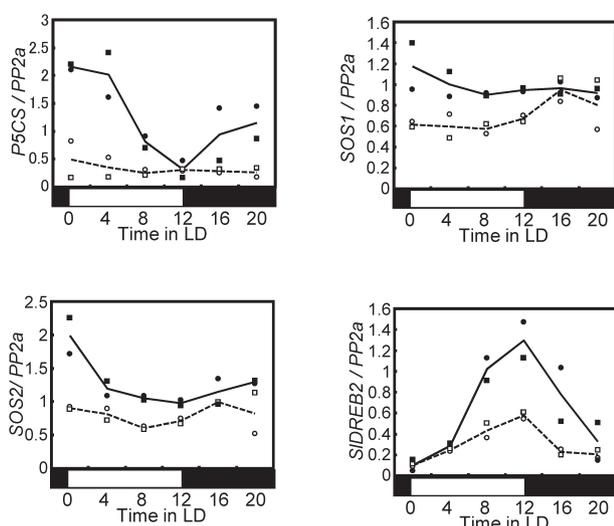


Figure 1. Effect of salinity treatment on expression of tomato salinity-stress inducible genes in LD. Plants grown in 12h light/12h dark were treated with 150mM NaCl or kept in the hydroponic condition. On the 3rd day of the treatment plants were harvested every 4h over 24h and subjected to expression analysis. Expression of *P5CS* (A), *SOS1* (B), *SOS2* (C) and *SIDREB2* (D) are shown. Circle and square dots represent expression levels of genes in two biological replicates. Filled and opened dots represent expression levels of genes in samples with and without NaCl treatment, respectively. Solid and dotted lines, representing average levels of gene expression among two replicates, indicate gene expression in plants treated with or without NaCl. White and black bars under the graphs indicate the day and the night, respectively. Error bars indicate SE for two biological replicates.

At the same time, despite constant salinity treatment over 24h, salinity-induced transcript levels of *PC5S* and *SISOS2* exhibited diurnal patterns with peaking in the morning (Figure 1A, C). In the non-stressed condition, expression of these genes did not exhibit a clear diurnal pattern. Though the diurnal induction pattern of *SISOS2* observed under salinity-stress condition was relatively mild, that of *P5CS* was stronger and clearer. While in non-treatment control *P5CS* expression did not show a diurnal induction pattern, in salinity-stressed plants its expression exhibited a strong diurnal rhythm, with its lowest level observed in the evening almost identical to that of non-stressed plants (Figure 1A). These results indicate that salinity-mediated transcriptional induction of *P5CS* and *SISOS2*, encoding a proline synthetic enzyme and sodium/hydrogen antiporter respectively, is restricted to the morning in a light/dark cycle. On the other hand, expression of *SISOS1*, which encodes a putative serine/threonine kinase, did not exhibit a clear induction pattern with a diurnal rhythm (Figure 1B).

We also checked expression of the *SOLANUM LYCOPERSICUM DEHYDRATION RESPONSIVE ELEMENT BINDING 2* (*SIDREB2*) gene, the tomato homolog of the Arabidopsis *DREB2* gene (Li et al. 2012). *DREB2* encodes an AP2/ERF transcription factor whose expression is induced by several abiotic stresses, such as drought and salinity, and is involved in stress

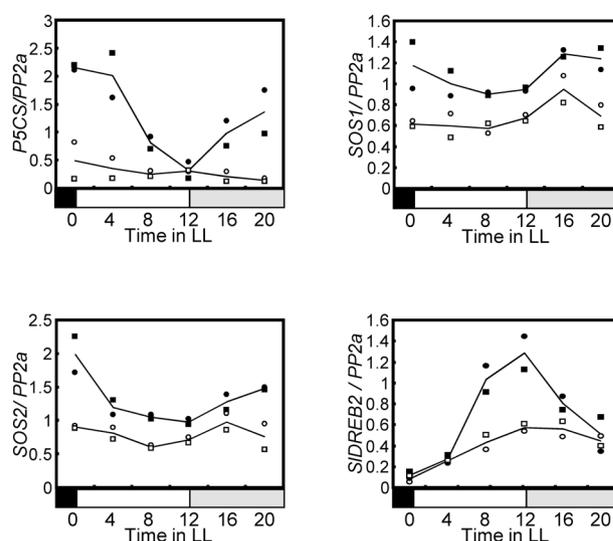


Figure 2. Effect of salinity treatment on expression of tomato salinity-stress inducible genes in LL. Experiments were carried out with samples of time 0, 4, 8, 12 in Fig. 1 combining with those newly harvested at 16 and 20 from plants that were sequentially kept in the light from the time 12. Expression of *P5CS* (A), *SOS1* (B), *SOS2* (C) and *SIDREB2* (D) are shown. Circle and square dots represent expression levels of genes in two biological replicates. Filled and opened dots represent expression levels of genes in samples with and without NaCl treatment, respectively. Solid and dotted lines, representing average levels of gene expression among two replicates, indicate gene expression in plants treated with or without NaCl. White, black and gray bars under the graphs indicate the day, the night and the subjective night in constant light, respectively. Error bars indicate SE for two biological replicates.

signaling pathways, including slowing plant growth by downregulating gibberellin biosynthesis (Agarwal et al. 2017; Li et al. 2012). Expression of *SIDREB2* is also induced by several abiotic stresses such as drought and salinity, with its overexpression increasing tolerance against salinity but creating dwarf plants (Agarwal et al. 2006, 2017; Li et al. 2015). We found that its expression exhibits a clear diurnal pattern with peaking in the evening even under the non-salinity treatment condition (Figure 1D). Under the salinity stress condition, its expression was increased in the evening alone, indicating that, in contrast to the timing of salinity-mediated induction of *P5CS* and *SISOS2*, *SIDREB2* induction is restricted to the evening.

The observed diurnal patterns in *P5CS*, *SISOS2* and *SIDREB2* expression under the salinity stress condition could be due to the circadian clock, whose activity autonomously fluctuates in the day, or by the direct effect of the transition from light to dark in the LD cycle. To determine the underlying mechanism, we performed expression analysis with salinity-treated and non-treated plants grown in constant light for 24h (LL). Since in LL no environmental light cues are present, we are able to directly check whether the circadian clock is involved in generation of the observed diurnal patterns in gene expression under the salinity stress condition. In this

experiment we used plants harvested at time 0, 4, 8, 12 in the experiment in LD as samples for the subjective day, combining with plants continuously kept in the light after time 12 and harvested at time 16 and 20 as samples for the subjective night. We found that, despite continuous light exposure during the subjective night, expression of *P5CS* and *SISOS2* clearly rose during these times in response to salinity treatment, like those in LD (Figure 2A, C). These results show that salinity-induced patterns in *P5CS* and *SOS2* expression observed in LL are well consistent with circadian rhythms. We also checked the salinity-induced pattern of *SIDREB2* expression in LL. Its expression also fell during the subjective night despite continuous exposure to light, additionally implying involvement of the circadian clock in producing its salinity-induced diurnal pattern (Figure 2D).

To date, a number of genes whose expression is induced by salinity stress have been successfully identified (Agarwal et al. 2006; Ashrafi-Dehkordi et al. 2018; Munns and Tester 2008; Zhang and Blumwald 2001). The fact that expression of a set of the genes was demonstrated to exhibit circadian rhythms, and that mutations in and overexpression of Arabidopsis clock genes generally affect the magnitude of tolerance against various stressors including salinity, imply significance in regulation of salinity tolerance by the circadian clock (Greenham and McClung 2015; He et al. 2002; Kielbowicz-Matuk et al. 2014; Loukehaich et al. 2012; Ruan et al. 2011; Ziaf et al. 2016). In this study, we mainly focused on diurnal expression of tomato genes involved in proline synthesis and sodium-ion exclusion, which are both important physiological responses to salinity. We found that expression of tomato salinity tolerance-related genes *SISOS2*, *P5CS*, and *SIDREB2* is induced by salinity treatment, with the levels clearly involving diurnal rhythms in both LD and LL cycles. This is despite the fact that salinity treatment was provided constantly over 24 h. We propose that occurrence of salinity induction of *P5CS* and *SISOS2* expression is strongly restricted to particular times in light/dark cycles.

Of the four tomato genes tested, induction of *SOS2* and *P5CS* expression by salinity was mainly limited to the subjective morning. Restriction of *P5CS* induction to the morning was especially clear; despite constant salinity treatment, its expression exhibited diurnal patterns with the lowest level observed in the evening almost identical to that of the non-salinity condition. *P5CS* encodes an enzyme involved in synthesizing proline, which counteracts osmotic stress, suggesting that salinity-induced proline synthesis occurs selectively at these times. We also observed that *SOS2* induction in response to salinity is restricted to the morning, also suggesting that sodium-ion exclusion from cells takes place mainly at this time.

Though temporal restriction of stress responses

in the day is likely to provide a survival advantage in tomato, we currently don't know how this confers environmental fitness to the tomato. Expression of *P5CS* was dramatically reduced during the evening, which evokes the hypothesis that accumulation of proline during the evening might be harmful for maintaining cellular functions. Further molecular and physiological approaches may be needed to understand the significance of restricting the salinity stress responses to the morning. Though induction of *P5CS* and *SOS2* expression by the salinity treatment occurred mainly in the morning, induction of *SIDREB2* expression took place in the evening. Since *SIDREB2* expression is induced by several abiotic stresses including drought and salinity (Argawal et al. 2017; Hichri et al. 2016; Li et al. 2012), its induction in the evening may have been evolved to control other aspects of stress responses whose functionally optimal timing is different from that mediated by *P5CS* and *SOS2*.

This study provided a novel finding that inducibility in expression of salt stress-related gene by salinity is temporally controlled in the day. This finding not only demonstrates a novel insight into regulation of salinity responses coordinated with daily environmental fluctuation, but also provides the potential to exploit an effective way for improving performance of salinity resistance in tomato. One general way of improving crops by genetic manipulation is to introduce and express a master regulator constitutively at a high level, but such a modification often causes pleiotropic effects including inefficient growth and reduced yield (Hussain et al. 2011). Our data demonstrating that salinity stress responses are coordinated with daily light/dark cycle, is also consistent with the idea that using a technique with constitutive overexpression is not necessarily efficient. Inducing the activity of a particular salinity-stress responsive gene only at times when that naturally occurs would help increase performance of crops more effectively. Such genetic modification may still enable the plants to maintain fitness of endogenous systems to natural day/night cycles.

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