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Salt overly sensitive pathway members are influenced by diurnal rhythm in rice

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Abbreviations: d, days; LD, light/dark cycles; h, hours; min, minutes

The diurnal rhythm controls many aspects of plant physiology such as flowering, photosynthesis and growth. Rice is one of the staple foods for world's population. Abiotic stresses such as salinity, drought, heat and cold severely affect rice production. Under salinity stress, maintenance of ion homeostasis is a major challenge, which also defines the tolerance level of a given genotype. Salt overly sensitive (SOS) pathway is well documented to play a key role in maintaining the Na^+ homeostasis in plant cell. However, it is not reported yet whether the transcriptional regulation of genes of this pathway are influenced by diurnal rhythm. In the present work, we have studied the diurnal pattern of transcript of SOS pathway genes in rice at seedling stage. To rule out the effect of temperature fluctuations on the expression patterns of these genes, the seedlings were grown under constant temperature. We found that *OsSOS3* and *OsSOS2* exhibited a rhythmic and diurnal expression pattern, while *OsSOS1* did not have any specific pattern of expression. This analysis establishes a cross-link between diurnal rhythm and SOS pathway and suggests that SOS pathway is influenced by diurnal rhythm in rice.

Introduction

Soil salinity is one of the most severe abiotic stresses for crops worldwide, affecting several million hectares of agricultural land.[1] Therefore, characterization of Na^+ transport and its distribution in plants has been a prime area of research for decades. High concentration of sodium ion (Na^+) is toxic to plants primarily due to its adverse effects on cellular metabolism and ion homeostasis.[2,3] Therefore, maintenance of low level of Na^+ in cells is essential for plants.[3,4] Plants remove Na^+ from the cytoplasm by using vacuolar and plasma membrane localized Na^+/H^+ transporters.[4,5] Na^+/H^+ transporters are membranous proteins that transport protons (H^+) across the membrane in exchange for Na^+.[6,7] This exchange activity requires H^+ electrochemical gradient across the membrane which is generated by the H^+ pumps such as H^+-ATPase present on plasma membrane or vacuole and H^+-pyrophosphatase.[5] In plants, the exchange activity of the plant vacuolar Na^+/H^+ transporters has been well studied.[5,8,9] In addition, enhanced salinity tolerance has been reported by overexpression of a vacuolar Na^+/H^+ antiporter in *Arabidopsis*.[10]

In plants, three salt overly sensitive genes (*SOS1, SOS2* and *SOS3*) have been found to function in a common pathway that contributes to salt tolerance.[11-14] *SOS1* gene encodes a membrane protein containing 12 putative trans-membrane domains and a long hydrophilic tail at the C-terminal end.[15] SOS1 transports sodium ions across the plasma membrane. Expression of *SOS1* gene is induced significantly in roots and to a much lesser extent in shoots in seedlings by exposure to high levels of NaCl.[15] SOS2 is a Ser/Thr protein kinase, which contains an auto-inhibitory domain.[16] SOS3 is a Ca^{2+} binding protein with strong similarity with the regulatory β subunit of the protein phosphatase calcineurin and with related proteins of the neuronal Ca^{2+} sensor family.[13] Hence, it has been hypothesized that SOS3 perceives the Ca^{2+} transients elicited by salt stress and activates SOS2 by relieving auto-inhibition.[16] These SOS pathway genes are later grouped into large protein families of calcineurin B-like proteins (CBL) and CBL-interacting protein kinases (CIPK), therefore SOS2 and SOS3 are also known as CIPK24 and CBL4, respectively.[17] Furthermore, the orthologous-*OsSOS1*, *OsSOS2* (*OsCIPK24*) and *OsSOS3* (*OsCBL4*) have also been isolated from rice and it has been demonstrated that all OsSOS proteins could coordinately function with AtSOS proteins and nicely complemented mutations in the corresponding *sos* mutant of *Arabidopsis* plants.[18] Recent work from our lab has also reported various SOS orthologs from Brassica species involved in maintenance of Na^+

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homeostasis in Brassica.[19] Together, these results suggested that the SOS-like salinity tolerance mechanism is conserved in plants.

Plant diurnal oscillation is a 24 h period cycle. Genes of multiple pathways have been implicated in stress responses and regulated by diurnal rhythm.[20-24] Time course transcriptome analysis has revealed that genes involved in several biological pathways like lipid and carbohydrate metabolic processes, photosynthesis, nucleotide binding, translation, amino acid and nucleotide metabolism, nitrogen metabolism and hormone biosynthesis, etc., show rhythmicity.[25-28]

Rice, a salt sensitive crop, is the staple food for about half of the world's population. Hence, in order to improve salinity tolerance in this crop plant, it is imperative to develop a "thorough understanding" of the complex molecular mechanisms and gene regulatory networks operative under stress conditions.[22] Recently, proteomics approach has revealed that a set of 91 proteins, involved in diverse processes including stress response, is controlled by diurnal cycles in developing endosperm of rice.[28] However, there is still no report about the diurnal transcriptional regulation of salt stress related genes like *SOS1, SOS2* and *SOS3*. Rice has been reported to be most sensitive to salinity stress at seedling stage.[29,30] The aim of the present study was to see if the expression of SOS pathway members is influenced by the diurnal rhythm in seedlings of rice.

Results

***OsSOS3* and *OsSOS2* show diurnal rhythmicity.** The diurnal expression profiles of *OsSOS* genes were observed under entraining conditions of 12 h light/12 h dark (LD) with a constant temperature 28 ± 2°C during day and night in IR-64 genotype of rice at seedling stage. *OsTOC1* has been reported to be expressed rhythmically during 12 h light and 12 h dark cycle in rice.[31] Level of mRNA of *OsTOC1* oscillates with light-dark cycles and shows peak in the late day (**Fig. 1A**). Changes in *OsTOC1* levels coincide with dusk transition as reported earlier.[31] *OsTOC1* expresses with a period of about 24 h and an evening specific phase. This result is consistent with the prior published reports, which in turn validates the experimental conditions used by us.

The nucleotide sequences of the cDNA clones used for probe preparation were taken as query in BLASTN search in Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu/) which revealed that regions of clones used for probe preparation were specific to the corresponding genes, thus giving a clear signal on northern blots. In case of *OsSOS* genes, we found that *OsSOS2* and *OsSOS3* were expressed in diurnal manner. Diurnal rhythms of these two genes have a characteristic waveform, described by peaks and troughs. *OsSOS3*, with a phase of 24 h show higher expression level during night. Its expression shoots up in night and goes down during day, creating rhythmicity (**Fig. 1B**). Similarly, the transcript of *OsSOS2* also shows a clear oscillation profile. MRNA levels of *OsSOS2* cycles in light-dark periods, showing higher expression around the transition period of light to dark with a period of 24 h (**Fig. 1C**). The amplitude of *OsSOS2* diurnal expression was higher in comparison to that of *OsSOS3.* mRNA level of *OsSOS1* transcript fluctuates with time but do not show a clear rhythmicity. Its expression level drops before dawn (**Fig. 1D**).

Discussion

The biological clock is an endogenous timing mechanism present in almost all organisms examined to date. This time-keeping mechanism is able to generate 24 h rhythms in a wide variety of biological processes. Regulation of cellular ion homeostasis during stresses is critical for plant survival. One of the responses of plant cell to stresses is the generation of a cytosolic Ca^{2+} transient and the subsequent activation of Ca^{2+} sensor protein expression and/or activity.[32] The components (SOS1, SOS2 and SOS3) of SOS pathway transduce a salt stress induced Ca^{2+} signal to reinstate cellular ion homeostasis.[33] Although, these three members (proteins) coordinate in sequential manner but do not show diurnal co-expression as *OsSOS3* and *OsSOS2* have expression peaks at different times of 24 h cycle; during night and dusk transition respectively, while *OsSOS1* does not have a clear rhythmicity. It has been reported that changes in mRNA levels may not be correlated with changes in protein or enzyme activity levels.[34] It has also been suggested that the clock-regulated proteins in rice are modulated at not only transcriptional but also at post-transcriptional and/or post-translational levels.[26] Whether SOS2 and SOS3 show diurnal rhythm at protein level and whether specific activities of these proteins also oscillate during the 24 h day-night cycle, these are the important questions to be answered. Nevertheless, expression profiles do provide a useful starting point for more in depth analyses. For instance, in the present study, *SOS2* and *SOS3* represent good candidates to study the relationship between salinity stress and diurnal rhythm. Plants are always changing and adapting to their changing environments. Thus, experimental changes are always being observed in a background of uncertain variation. Although clocks are temperature compensated, which means that overall clock period is constant over a range of temperatures and temperature changes can act to entrain or reset the clock patterns.

It has also been shown in *Brassica juncea* that *BjSOS3* mRNA is downregulated in presence of calcium chelator EGTA.[35] It indicates that Ca^{2+} has a dual role as signaling agent, controlling the expression of SOS pathway genes at transcript level and initiating signaling at protein level. Diurnal oscillations of cytosolic and chloroplastic Ca^{2+} have been reported in *A. thaliana* and *Nicotiana plumbaginifolia*.[36] The relationship between this diurnally regulated Ca^{2+} and *SOS* genes is still unexplored. The purpose of the diurnal oscillations of Ca^{2+} is not known but it has been reportedly involved in regulating numerous signaling events.[37] Calcium is suggested to be a part of the light signal transduction chain regulating the rhythm as well as gene expression.[37] These reports are in support to our study, as we are also reporting a diurnal expression pattern of genes of SOS pathway which is related to Ca^{2+} signaling.

As, the expression of SOS pathway genes and Ca^{2+} ion level show rhythmic oscillation, it would be interesting to study whether the diurnal changes in Ca^{2+} signatures mediate cross-talk

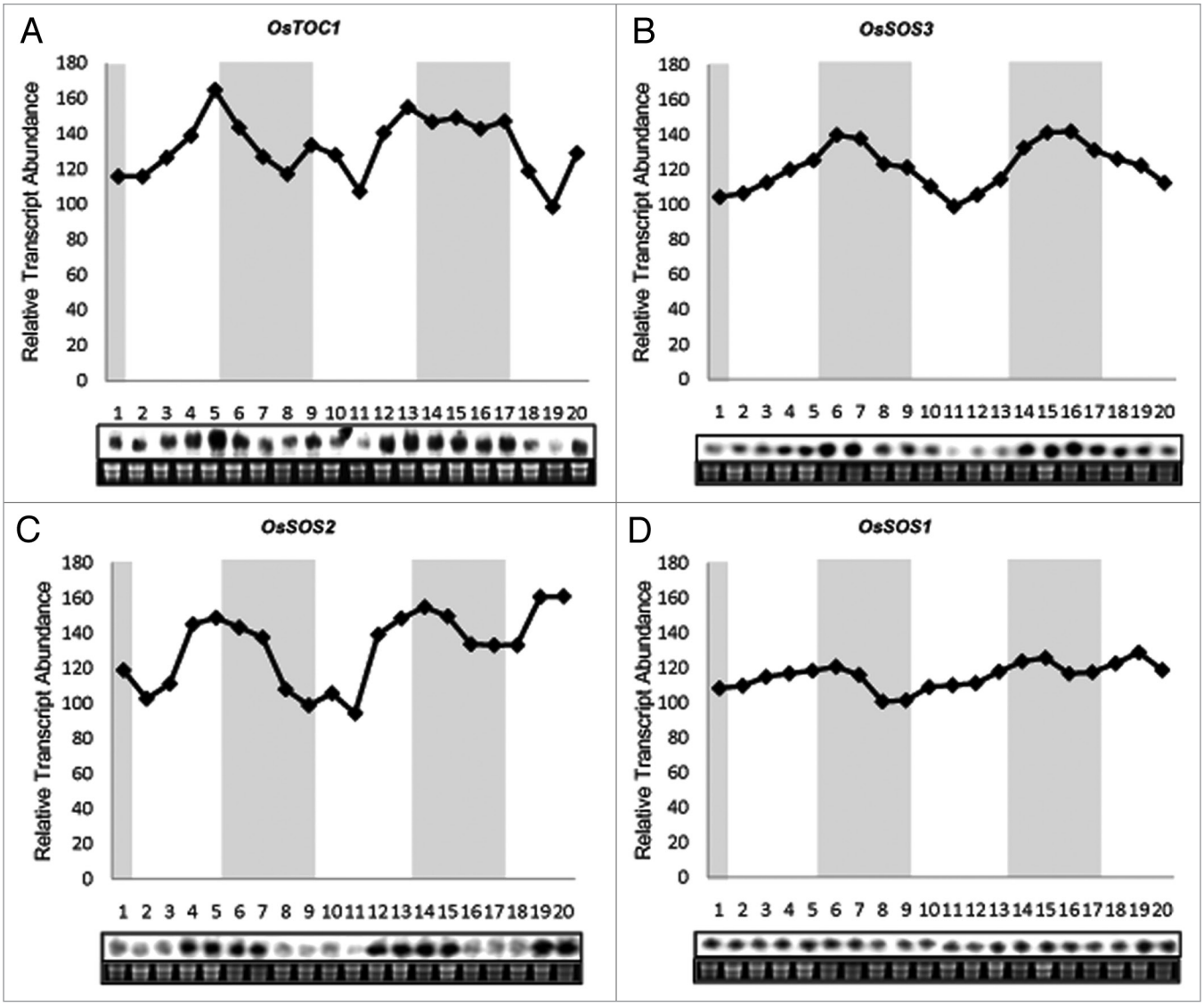

Figure 1. RNA gel blot analyses showing diurnal rhythm of OsSOS genes in shoots of IR64 rice seedlings. Seedlings were grown under the 12 h light/12 h dark cycle for 14 d. Shoot samples were harvested at 3 h intervals for 2.5 d under the 12 h light/12 h dark. Twenty μg RNA samples was used for northern blot hybridization. Ethidium bromide (EtBr) stained RNA-gel has been shown as the loading control. Y-axis shows relative transcript abundance of genes while numbers on X-axis shows different time points. Shaded area indicates dark period and white area indicates light period. (**A**) OsTOC1 shows rhythmic expression and has a peak during light to dark transition. (**B**) OsSOS3 expression peaks during night. (**C**) mRNA level of OsSOS2 show rhythmicity in 24 h cycles and show higher expression during the transition period of light to dark. (**D**) Transcript level of OsSOS1 doesn't show any clear rhythmicity.

between diurnal rhythm of SOS pathway genes and salinity stress tolerance. In *Arabidopsis,* elimination of *SOS1* leads to the changes in expression of genes related to circadian rhythm.[38] It suggests that though *SOS1* does not show any diurnal rhythm but it regulates diurnal rhythm of several other genes. It also indicates toward unknown and unexplored connection between SOS pathway and circadian clock.

It is also interesting to find out whether the activities of downstream components of SOS2-SOS3 complex oscillates and coincides with diurnally regulated *SOS2* and *SOS3* transcripts. One of the downstream components, regulated by this complex is CCR1 (cold-circadian rhythm-RNA binding1) which encodes a Gly-rich RNA-binding protein. CCR1 has similar expression profiles in *sos1*, *sos2* and *sos3* mutants implicating this as transcriptional output requiring all components of the SOS pathway.[39] Expression of CCR1 as well as its homolog CCR2 is regulated by diurnal rhythm.[40]

Not only downstream genes of SOS pathway, but also the interacting partners of SOS pathway show the diurnal rhythm. It has been reported that SOS2 interacts with catalase 2 (CAT2) and catalase 3 (CAT3) connecting SOS2 to H_2O_2 metabolism and signaling. Expression of CAT2 is under circadian control, with the highest expression during the light period, consistent with a primary role in detoxifying H_2O_2 derived from photosynthesis or photorespiration.[41] Interestingly, CAT3 is also circadian regulated, but in the opposite manner as CAT2. CAT3

Table 1. List of primers used in the present study

OsTOC1 forward primer	5'-CGG AAT TCA TGG TGG GCG CCG GCG AG-3'
OsTOC1 reverse primer	5'-ACG CGT CGA CCT ACT CTG GAG AAG AAA CCA TC-3'
OsSOS1 forward primer	5'-ATG TGA CTG GAA GGG TTT GC-3'
OsSOS1 reverse primer	5'-TCT AGC CTC CTC TCC CTC AG-3'
OsSOS2 forward primer	5'-ATG GGA GGG GAG GAG GGA ATG-3'
OsSOS2 reverse primer	5'-CTA GCA TGT GGC TGT CCT CAG-3'
OsSOS3 reverse primer	5'-TCA GTC ATG GGC TTC TGA ATG-3'

expression is highest in the dark period.[41] Circadian oscillation of interacting members and downstream components of SOS pathway revalidate our results.

Our data demonstrates that *OsSOS3, OsSOS2* genes show rhythmic expression profile under diurnal condition at the transcription level. This study also strongly establishes a possible molecular link of diurnal rhythm with SOS pathway.

Materials and Methods

Plant materials and growth conditions. Seeds of *Oryza sativa* L. cv "IR-64" were germinated in half Yoshida medium under hydroponic system for 48 h in dark and then grown for 14 d under control conditions (28 ± 2°C, 12 h light and 12 h dark cycle) in plant growth room.[42] Shoot samples were harvested for 2.5 d at an interval of 3 h starting from the dawn of 15 d.

RNA extraction and northern blot analysis. Total RNA was extracted from tissue using TRIzol method as per the manufacturer's instructions (T9424; Sigma-Aldrich). Northern blots were prepared using 20 μg total RNA. Appropriate probes [partial for *OsSOS1*(AK065608) corresponding to its N-terminal part and full length for other genes; *OsSOS2* (AK102270); *OsSOS3* (AK101368); *OsTOC1* (AK111828)] were amplified using the primers listed in **Table 1** and radiolabeled using the DecaLabel™ DNA labeling kit, (K0621; Fermentas). To avoid any error due to unequal loading of RNA samples, same blot was re-probed each time with different gene probes after washing. Northern blots were hybridized at 65°C in 5 × SSC, 5 × Denhardt's reagent, 0.1% SDS and 100 μg/ml denatured salmon sperm DNA for 16–18 h. Membrane was washed twice in 0.5 × SSC, 0.1% SDS and 0.1 × SSC, 0.1% SDS for 15 min each at 65°C and scanned on phosphorimager using the software Fujifilm Image Reader. The relative transcript abundance was calculated using the Image Gauge (Fuji Photofilm Co. Ltd.).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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