Table 1 Primers used in RT-qPCR

Gene	Primer sequence (5'->3')	Amplicon size (bp)
OsCCA1/LHY (LOC_Os08g06110)	F: TGGTTCCAACACACCGTCAA R: ACCGGCTGAAGAGTTACTGC	112
OsPRR1/TOC1 (LOC_Os02g40510)	F: AGGCACACCAGAGGGTTTAC R: AGCAGAAGACTCAGCAACCC	115
OsGI (LOC_Os01g08700)	F: AACCACGATAGCCCAGAAGC R: GACTGCTCTGGCGGTTACTT	141
OsFKF1 (LOC_Os11g34460)	F: GTCCAACCACAGACCCAACA R: TGTGCGCCAGAACTTCATCT	119

Data analysis

The experiment was arranged as a completely randomized design (CRD) with three replicates. To study the pattern of expression, relative expression levels of selected genes were plotted against sampling times and were fitted to nonlinear equation using GraphPad Prism 5.0 (GraphPad Software, Inc., USA)

Results

Effects of drought and salt stresses on leaf water potential

The water potential in flag leaf was diurnal regulated with a period about 24 hours. In Figure 1, drought and salt stresses lowered the baseline from -0.63 MPa in control group to -0.94 and -1.00 MPa in drought and salt stressed plants, respectively. Moreover, flag leaf of salt stressed plants had slightly lower amplitude of cosine curve than those of control and drought stress plants (Figure 1).

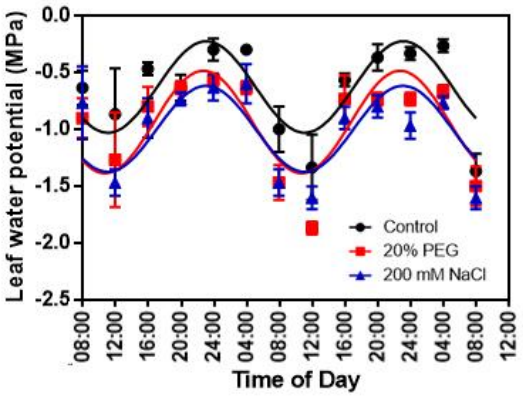

Figure 1 Diurnal changes of water potential in flag leaves of KDML 105 rice plants grown under control, drought and salt stress conditions for 48 hours. Bars indicate mean ± SD.

Effects of drought and salt stresses on expression levels of circadian clock-associated genes

The analysis of gene expression in flag leaves of KDML 105 rice plants grown under greenhouse condition revealed that circadian clock genes show a diurnal pattern of expression under control and stress conditions except *OsPRR1/TOC1* (Figure 2). *OsCCA1/LHY* peaked at 04:00 (Figure 2A), followed by *OsPRR1/TOC1* at noon (Figure 2B), and *OsGI* and *OsFKF1* at 20:00 (Figure 2C and D). Flag leaves of rice plants subjected to drought and salt stress conditions had higher levels of *OsCCA1/LHY* transcript than control plant at 04:00 (Figure 2A). Oscillations of *OsPRR1/TOC1* expression were disrupted by drought and salt stresses (Figure 2B). At 20:00, drought and salt stress conditions decreased the amplitude levels of *OsGI* (Figure 2C). The similar effect of drought stress on the expression of *OsFKF1* was observed (Figure 2D). Furthermore, salt stress advanced the phase of *OsFKF1* expression compared with control condition (Figure 2D).

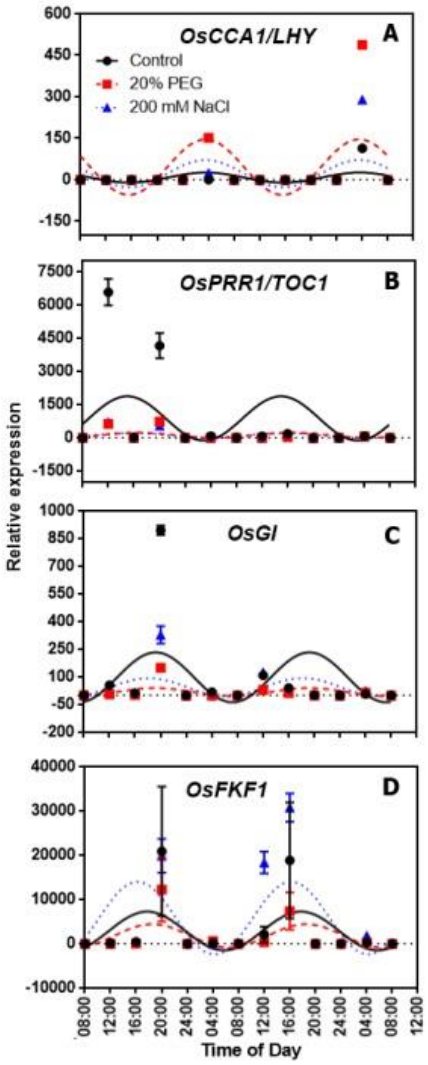

Figure 2 Expression patterns of *OsCCA1/LHY* (A), *OsPRR1/TOC1* (B), *OsGI* (C) and *OsFKF1* (D) in flag leaves of KDML 105 rice plants grown under control, drought and salt stress conditions for 48 hours. Bars indicate mean ± SD.

Discussion and Conclusion

Salt and drought stress conditions inhibited water uptake of plants resulting in the reduction in water potential of KDML105 flag leaf. High salt stress lowered the baseline and amplitude of leaf water potential cosine curve. The leaf water potential of rice plant under salt stress was lower than that of drought stress, and the difference increased after midday. Habte et al. (2014) reported that osmotic stress applied at the barley roots affected osmotic potential, stomatal conductance, transpiration and expression of clock and stress responsive genes in the shoot.

Transcript levels of *CCA1/LHY*, showed a peak at 04:00, followed by *PRR1/TOC1* at 12:00. Salt and drought stress also affected expression levels of *PRR1/TOC1* by reducing transcripts. There is some evolutionary conservation between rice and *Arabidopsis* in the circadian clock that consists of interlocked subloops. It has been reported that overexpression of *OsLHY* repressed the rhythmic expression of *OsPRR1* in rice cells as well as *Arabidopsis* (Ogiso et al., 2010). Therefore, Yang et al. (2013) speculated that the central negative feedback loop consists of *OsLHY* and *OsPRR1*, and *OsLHY* and some members of the *OsPRRs* constitute the morning loop.

Kwon et al. (2014) study on alternative splicing and nonsense-mediated decay of circadian clock genes under environmental stress conditions in *Arabidopsis*. It appeared that expression of *CCA1* was not influenced by high salinity, while *TOC1* was suppressed. High salinity resulted in lengthening of the circadian period of clock genes and advanced their phase of expression in barley (Habte et al., 2014). However, the effect of high salinity in lengthening the circadian period of clock genes was not observed in this study. Similarly, drought stress reduced the expression of evening-specific components of the clock (*TOC1, LUX*, and *ELF4* genes) in soybean, and this led to disruption of the circadian system (Marcolino-Gomes et al., 2014).

OsGI and *OsPRR1/TOC1* transcripts showed peak at 12:00. Flag leaves of control plants had higher *OsGI* and *OsPRR1/TOC1* expression levels than stressed plants. *GI* was found to regulate the expression of genes involved in drought and cold stress responses, this effect of *GI* was dependent on CYCLING DOF FACTORs (CDFs), a family of rhythmically expressed transcriptional repressors known for their roles in the photoperiodic regulation of flowering time (Fornara et al., 2015)

The maintenance of the daily expression oscillation of *OsFKF1* was changed under salt stress condition. FKF1 interact with GI to control the light-dependent degradation of CDF proteins, these observations provide independent evidence for a role of the LKP2/GI/CDF regulatory module in drought tolerance (Imaizumi et al., 2005).

Based on the results, it can be concluded that water potential and expression of circadian clock-associated genes are diurnal regulated in rice flag leaves. High salt concentration lowered the baseline and amplitude of leaf water potential cosine curve. Salt and drought stresses affected expression of *CCA1/LHY*, *PRR1/TOC1*, *GI* and *FKF1*. Salt stress disturbed the maintenance of the daily expression oscillation of *FKF1*.

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