Expression of Circadian Clock-related Genes in Flag Leaves of KDML 105 Rice Cultivar under Osmotic Stress Conditions

การแสดงออกของยีนที่เกี่ยวข้องกับนาฬิกาชีวภาพในใบธงของข้าวพันธุ์ขาวดอกมะลิ 105 ภายใต้สภาวะเครียดออสโมซิส

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ABSTRACT

The objective of this research was to investigate the expression patterns of circadian clock-associated genes under normal, drought and salt stress conditions. The KDML 105 rice plants were subjected to salt or drought stressed conditions by adding 200 mM NaCl or 20% polyethylene glycol 6000 to the nutrient solution during heading stage. Flag leaves were collected every four hours for 48 hours. Changes in relative transcript levels were determined using reverse transcription quantitative PCR. The results showed that, water potential and expressions of circadian clock-associated genes in flag leave were diurnal regulated. High salt and drought stresses lowered leaf water potential. Salt and drought stresses affected expression levels of *CCA1/LHY*, *PRR1/TOC1* and *GI* by changing their amplitudes. Moreover the daily expression oscillation of *FKF1* was slightly shifted under salt stress condition.

บทคัดย่อ

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษารูปแบบการแสดงออกของยีนที่เกี่ยวข้องกับนาฬิกาชีวภาพในใบธงของข้าวที่ปลูกในสภาวะปกติ และสภาวะที่ได้รับความเครียดเกลือและความเครียดแล้ง โดยชักนำให้ข้าวขาวดอกมะลิ105 เกิดความเครียดเกลือหรือเครียดแล้งในระยะออกดอกด้วยการเติมโซเดียมคลอไรด์ (NaCl) ความเข้มข้น 200 mM หรือ พอลิเอทิลีนไกลคอล 6000 ความเข้มข้น 20% ลงในสารละลายธาตุอาหาร เก็บตัวอย่างเนื้อเยื่อใบธงทุก 4 ชั่วโมง เป็นเวลา 48 ชั่วโมง วิเคราะห์การเปลี่ยนแปลงระดับทรานสคริปต์ด้วยวิธี reverse transcription quantitative PCR ผลการทดลองพบว่าค่าชลศักย์ และการแสดงออกของยีนที่เกี่ยวข้องกับนาฬิกาชีวภาพในใบธงเปลี่ยนแปลงตามจังหวะรอบวัน ความเครียดเกลือและแล้งทำให้ค่าชลศักย์ลดลง และส่งผลกระทบต่อแอมพลิจูดการแสดงออกของยีน *CCA1/LHY*, *PRR1/TOC1* และ *GI* นอกจากนั้นยังพบว่า ยีน *FKF1* มีการแสดงออกเปลี่ยนแปลงไปเล็กน้อยภายใต้เสภาวะเครียดเกลือ

Keywords: Circadian rhythms, Osmotic stress, Rice
คำสำคัญ: จังหวะรอบวัน ความเครียดออสโมซิส ข้าว

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Introduction

Abiotic stresses such as drought and salinity cause widespread crop losses throughout the world and impose severe limitations on the amount of land that can be used for agricultural purposes (Grundy et al., 2015). Data from FAO world soil resources report 2000 showed that approximately 64% and 6% of global land area were affected by drought and salinity, respectively (Land and Water Development Division, 2000). As the world population is rising exponentially, there is an urgent need for development of crop varieties with improved tolerance to abiotic stresses. However, the development of such improved varieties will require a thorough understanding of the mechanisms by which plants are affected by these conditions, and how they can tolerate them (Grundy et al., 2015). Osmotic stress can affect cellular homeostasis via a variety of mechanisms. Salt stress produces toxic salt ions that directly damage plant cells and further inhibit the water use efficiency in the plant while drought directly interferes with the water-use system in plants. Both conditions lead to the reduction of plant water potential and osmotic pressure of the soil (Hasthanasombut et al., 2011). Recent evidence indicate that circadian clock contributes to plant's ability to tolerate different types of abiotic stress (Legnaioli et al., 2009; Nakamichi et al., 2009; Kim et al., 2013; Grundy et al., 2015)

The circadian clock is an endogenous oscillator evolved for perceiving and responding to environmental stimuli by generating transcriptional, metabolic and physiological changes synchronized with the day and night cycle. A cycle or period of circadian rhythms is approximately 24 hours (Murakami et al., 2007; Pruneda-Paz, Kay, 2010). Circadian clock is an interconnection among several signaling networks. The simplest model describes the circadian clock as consisting of a central oscillator, which generates the rhythmic behavior; the input pathways, which carry environmental information to entrain the central oscillator; and the output pathways that regulate physiological processes. (Dunlap, 1999; Hotta et al., 2007) In *Arabidopsis thaliana*, a three-loop model for circadian clock has been proposed (Ueda, 2006; Yon et al., 2012). A central oscillation loop comprises of two MYB transcription factors, CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), and TIMING OF CAB EXPRESSION 1/PSEUDO-RESPONSE REGULATOR 1 (TOC1/PPR1) transcription factor. The second loop is established by TOC1 and the evening complex (EC) consisting of EARLY FLOWERING 3 (ELF3), ELF4, and LUX ARRHYTHMO (LUX). The third loop is formed by negative feedback between PRR7/PRR9 and CCA1/LHY (Alabadí et al., 2001; Farré et al., 2005; Salomé, McClung, 2005; Yon et al., 2012). ZEITLUPE (ZTL) and GIGANTEA (GI) are also play a crucial role in controlling a normal circadian period (Kim et al., 2013). Transcription feedback loops and oscillating changes in transcription generated by these clock components establish circadian rhythms in *A. thaliana*. Although the clock is conserved among plant species, little is known about its functions in cereal crop plants.

Rice (*Oryza sativa* L.) is one of the main staple crops for people worldwide. In Thailand, rice is the most important cereal crop for consumption and export. Forty one percentage of total rice production is obtained from the Northeastern region where the majority of rainfed areas and saline soil are presented.

Although this region produces the highest rice yield, the rice yield/growing area is low and fluctuated. (Chakhonkaen et al., 2012) Therefore, improvement of drought/salt tolerant rice varieties has become an urgent task and might increase actual rice yield in rainfed and saline soil areas. For crop species like rice, flowering is one of factors affecting seed productivity. The precise synchronization of circadian clock using abiotic stresses as environmental cues may useful for plants to survive and increase the yield under unfavorable conditions.

Objective of the study

The objective of this research was to investigate the effects of drought and salt stress conditions on expression patterns of circadian clock-associated genes in flag leaves of KDML 105 rice cultivar.

Materials and Methods

Plant material and treatments

Seeds of rice (*Oryza sativa* L. cv. KDML 105) were hydroponically grown in plastic plots containing Yoshida nutrient solution (Yoshida et al., 1976) on August 12, 2014 and were maintained under greenhouse condition at Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen. Ninety-day-old rice plants were subjected to drought or salt stresses by adding 20% polyethylene glycol (PEG) 6000 or 200 mM NaCl to the nutrient solution, respectively. Water potential was measured in flag leaves using pressure chamber model 3005 (Soil Moisture Equipment Corp, USA) before they were cut and kept at -80 ^{o}C for later gene expression analysis. Flag leaves were sampled every 4 hours for 48 hours by starting at 08:00 after rice plants were exposed to stress conditions. Non-stressed plants were grown concurrently and harvested at the same time.

Gene expression analysis

Expression of circadian clock-associated genes (*OsCCA1 / LHY*, *OsPRR1 / TOC*, *OsGI* and *OsFKF1*) were analyzed using reverse transcription quantitative PCR (RT-qPCR). Total RNA was extracted from approximately 100 mg tissue powder with the GF-1 total RNA extraction kit (Vivantis, Malaysia) according to the manufacturer's instructions. Traces of contaminating DNA were removed from the extract by DNAse provided in the kit. RNA concentration and purity were determined using NanoDrop® spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA). A 1-µg RNA was primed with oligo dT primer and reverse transcribed using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Massachusetts, USA) Primers specific to the target genes were designed using Primer Express 2.0 software (PE Applied Biosystems, USA) and Primer blast (NCBI, USA) (Table 1). RT-qPCR was performed with SYBR® Green I dye in LightCycler® Real-Time PCR System (Roache Diagnostics, Thailand). A 16-µl of PCR reaction contained 10 µl of master mix, 2.6 µl of nuclease-free water, 1.25 nM each of forward and reverse primers and 3 µl of cDNA. Two independent analyses with three sub-replicates for each sample were performed. Relative expression levels of each gene was normalized against the reference *protein kinase* (*PK*) gene (Narsai et al., 2010) and calculated with $\Delta\Delta_{Ct}$ method (Livak, Schmittgen, 2001).

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).

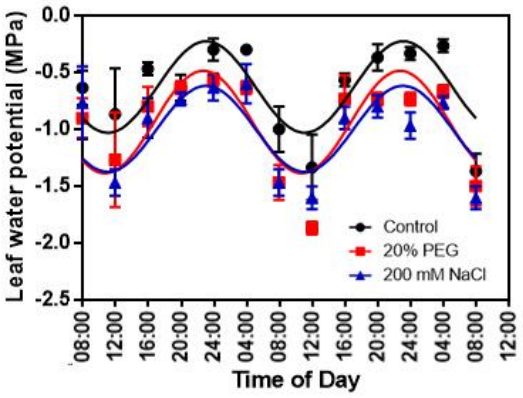

Figure 1 Diurnal changes of water potential in flag leaves of KDML 105 rice plants grown under control, drought and salt stress conditions for 48 hours. Bars indicate mean ± SD.

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).

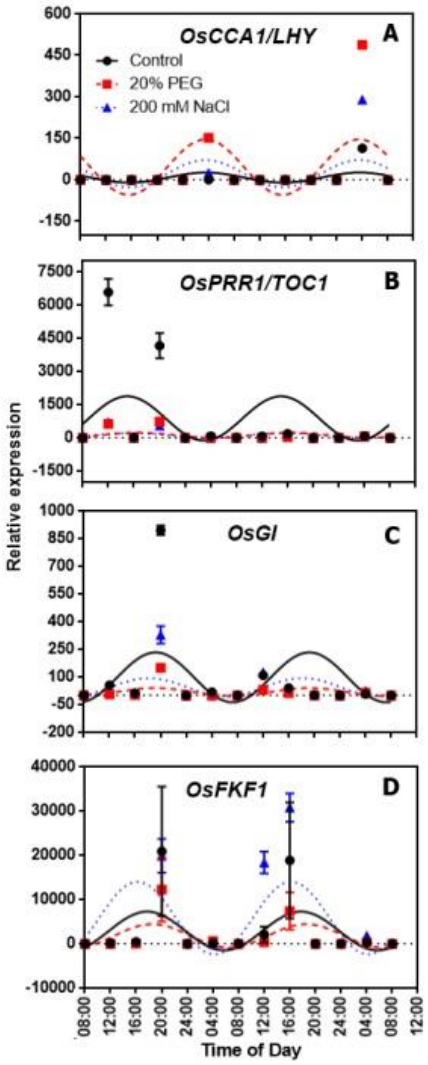

Figure 2 Expression patterns of *OsCCA1/LHY* (A), *OsPRR1/TOC1* (B), *OsGI* (C) and *OsFKF1* (D) in flag leaves of KDML 105 rice plants grown under control, drought and salt stress conditions for 48 hours. Bars indicate mean ± SD.

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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