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Molecules and Cells

Rhythmic Expression of Mitogen Activated Protein Kinase Activity in Rice

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Mitogen activated protein kinase (MAPK) are known to get activated during various stress signals and transduce the message from the cell membrane to the nucleus for appropriate cellular reorganization. Though, a certain basal activity of MAPK is often observed in the control plants. Prolonged exposure of rice plants to lowered or elevated temperature exhibited a rhythm in the activation of MAPKs. We analyzed existence of a possible endogenous rhythm in the activity of MAPKs in rice plants. The plants growing at constant temperature entrained in 16/8 h day-night cycle showed diurnal rhythm in activity. When the activation of MAPK was tested under continuous conditions by shifting plants to continuous darkness for a period of 72 h, the periodic rhythm persisted and followed a circadian pattern. Analysis of the transcripts of group A, B and C members of MAPKs under above conditions by quantitative real time PCR revealed that the members of group C exhibit periodic rhythm. Our data indicates that the MAP kinase activity in rice follows rhythmic expression in a circadian manner.

INTRODUCTION

Almost all organisms from prokaryotes to humans have circadian rhythms (Pittendrigh, 1993). These are the subset of biological rhythms with period defined as the time to complete one cycle of ~24 h (Dunlap, 2004). Traditionally the circadian clock system has been depicted to consist of three components: a circadian oscillator, an input pathway responsible for the entrainment of the oscillator, and an output pathway mediating the time signal transmission. In general circadian rhythms are driven by an endogenous biological clock(s) that regulates many biochemical, physiological and behavioral process in a wide variety of organisms (Dunlap, 1999). In higher plants too there are wide range of biological processes that are controlled through such a circadian clock. They include movement of organs such as leaves and petals, opening of stomata, daily fluctuations of metabolic activities such as respiration, photosynthesis and gene expression (Harmer et al., 2000; Schaffer et al., 2001). Circadian clock pathways have been well studied in animals, fungi and bacteria. Although a growing number of genes either regulated by the clock or affecting the clock function have been identified in plants, a full picture has yet to emerge.

Mitogen activated protein kinases (MAPKs) are an important signal transducing enzymes that connects diverse receptors/sensors to a wide range of cellular responses in mammals, yeasts and plants (Jonak et al., 2002). MAPK is the last component of the MAPKKK(MEKK)-MAPKK(MKK)-MAPK(MPK) cascade that plays crucial roles in signal transduction of extracellular stimuli in eukaryotes (Zhang et al., 2006). MAPK gets activated upon phosphorylation of threonine and tyorosine residue in Thr-x-Tyr (where x is either Asp or Glu) motif by a MAPKK. Molecular and biochemical studies have revealed that plant MAPKs play important role in response to a broad variety of biotic and abiotic stresses including wounding, pathogen infection, temperature, drought, salinity as well as in the signaling of plant hormones and the cell division (Mishra et al., 2006; Tena et al., 2001). MAPK is a multi gene family consisting of 20 members in *Arabidopsis* and 15 members in rice that are further divided in four subgroups A-D. Rice genome consists of 2 members in group A, 1 in group B, 2 in group C while 10 members in group D (Hamel et al., 2006).

There are few reports from mammalian system where tissues such as mouse suprachiasmatic nucleus (Obrietan et al., 1998), chicken pineal gland (Sanada et al., 2000) and bull frog retina (Harada et al., 2000) exhibit circadian activation/deactivation cycles of mitogen activated protein kinases under constant darkness. In plants constant mining and characterization of clock components is in progress with respect to light signaling in *Arabidopsis*. To date there is no report of MAP kinase showing circadian regulation in plants. A certain basal activity of MAP kinase was frequently observed in control samples that showed fast and transient activation when exposed to various biotic and abiotic stresses. When these basal activities was analyzed for a period of 24 and 72 h, an existence of rhythm in the activities of MAPK was observed which followed the pattern of circadian regulation.

MATERIALS AND METHODS

Plant growth conditions

Rice (*Oryza sativa* L. indica cultivar var Pusa Basmti-1) seedlings were grown in growth chamber (SCILAB instruments, Taiwan) at 28°C with 16 h light/8 h dark cycle. Two-three weeks old rice plants were used for all the experimental purposes. To observe the native rhythms, rice leaves were sampled at an interval of 2 h for a period of 24 h from the plants growing in growth chamber.

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Table 1. List of MAPK primers used in qRT PCR analysis

Name of the MAPK	NCBI accession number	Primer code	Sequence
OsMPK3	DQ826422	OsMPK3-F	5′ GCTCCAACCAAGAACTGTC 3′
		OsMPK3-R	5′ AGTCGCAGATCTTGAGG 3′
OsMPK4	FJ621301	OsMPK4-F	5′ CGAGGTCTCCTCCAAGTACG 3′
		OsMPK4-R	5′ GCGAAGCAGCTTGATTTCTC 3′
OsMPK6	EU675863	OsMPK6-F	5′ AGGTCACCGCCAAGTACAAG 3′
		OsMPK6-R	5′ AGCAGCTTGATCTCCCTGAG 3′
OsMPK7	DQ826424	OsMPK7-F	5′ GCTCGCACAAACAACAC 3′
		OsMPK7-R	5′ GCCAAGAAGCTCAGCAA 3′
OsMPK14	EU675864	OsMPK14-F	5′ TCCTGAGTTGCTCCTTTGCT 3′
		OsMPK14-R	5′ CGAGCTTTTGGGTTGTCAAT 3′

To examine the free running circadian rhythms plants grown under normal conditions were transferred to continuous dark and leaves were sampled at appropriate time intervals for a period of 72 h. Rice plants were exposed to cold and heat stress by moving the plants to 4°C and 42°C respectively.

Protein extraction and in-gel kinase activity assay

Rice leaf tissue were ground in liquid nitrogen and homogenized in extraction buffer containing 50 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 50 mM β glycerol phosphate, 1 mM phenylmethylsulfonyl fluoride, 10 mM DTT, 10 mM Na_3VO_4, 10 mM NaF, 10% glycerol and 0.74 g ml^{-1} Polyvinyl polypyrrolidone (PVPP). After centrifugation at 16,000 × *g*, aliquots of supernatant were used for in-gel kinase assay. The protein concentration was determined by Bradford method using BSA as standard. The in-gel kinase assay was performed as described previously (Link et al., 2002a; Zhang and Klessig, 1997) with some modifications. Briefly, 50 µg of total protein was fractionated on a 12% polyacrylamide gel containing 0.1% SDS and 0.5 mg ml^{-1} bovine brain myelin basic protein (MBP) (Sigma Aldrich, USA). After electrophoresis, the SDS was removed by washing the gel three times (30 min each) at room temperature with buffer containing 25 mM Tris, pH 7.5, 0.5 mM DTT, 0.1 mM Na_3VO_4 and 5 mM NaF, 0.5 mg ml^{-1} BSA and 0.1% Triton X-100. The kinases were allowed to renature overnight at 4°C with three changes of renaturing buffer (25 mM Tris, pH 7.5, 0.5 mM DTT, 5 mM NaF and 0.1 mM Na_3VO_4) 1.5 h, overnight, 2 h interval. The phosphorylation of MBP was performed in 20 ml of reaction buffer (25 mM Tris, pH 7.5, 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT, 0.1 mM Na_3VO_4, 1 µM ATP and 50 µCi of γ^{32}P-ATP (3,000 Ci $mmol^{-1}$) at room temperature for 60 min. The gel was transferred into washing buffer (5% tricholoroacetic acid and 1% sodium pyrophosphate) at room temperature for 15 min with 3-4 changes of the washing buffer. Finally the gel was dried on filter paper and autoradiographed in phosphorimager (Typhoon, GE Health Care, UK). Relative MAPK active was measured by quantifying the intensity of activity band by phosphorimager software.

Immunokinase assay of activated MAPK

Immunokinase assay was performed by immunoprecipitation of the activated MAPK followed by an in-gel kinase assay. For the immunoprecipitation 100 µg of total protein in a crude extract was brought to 150 mM NaCl and 0.1% (v/v) Nonidet P40. After addition of 1 µg of the phospho-Tyr specific monoclonal antibody 4G10 (Upstate biotechnology) the assay was shaken at 4°C for 2 h and after the subsequent addition of 15 µl of protein A-Sepharose (GE Healthcare), for another 4 h. The protein A-Sepharose with the bound antigen was pelleted at 20,000 *g* for 2 min and washed with 500 µl of 100 mM HEPES/KOH, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 10 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerol phosphate, 10% (w/v) glycerol, 1 µg ml^{-1} antipain, 0.1 mM phenyl methyl sulfonyl fluoride, 1 mm benzamidine, 150 mm NaCl, and 1% (v/v) Nonidet P40. The pellet was resuspended in 40 µl of SDS-loading buffer and incubated at 37°C for 15 min and at 65°C for another 15 min. After centrifugation, the supernatant was analyzed by an in-gel kinase assay with MBP as substrate.

Quantitative real time PCR (qRT-PCR) analysis

Specific primers' pairs were designed for five OsMPK genes namely OsMPK3, OsMPK6 belonging to group A, OsMPK4 belonging to group B and OsMPK7 and OsMPK14 belonging to group C (Table 1). The specificities of the primers were confirmed by running the RT-PCR product in agarose gel, before they were used for qRT-PCR analysis. The amplicon product of each of them was around 200 bp. Each PCR reaction consisted of 10 µl of SYBR Green PCR master mix (SYBR Green mix, Applied Biosystems,USA), 0.9 µl of each primer (20 mM), 6.2 µl of water and 2 µl of each reverse transcribed cDNA product. Quantitative real time PCR was carried out using Step one Real time PCR systems (Applied Biosystems, USA). The thermocycler program had an initial 95°C for 10 min followed by 40 cycles consisting of 15 s denaturation at 95°C, 60 s annealing and extension at 60°C. At the end of each reaction, a melting curve was created using a single cycle consisting of 15 s denaturation at 95°C and a 60sec annealing at 60°C. This was followed by a slow temperature increase to 95°C at the rate of 0.3°C/s. The melting curve was used to detect the presence of primer dimer or other unwanted amplified products that would produce a detectable CT value and negatively skew the results.

RESULTS

Cold and heat activated MAP kinase exhibit a rhythm

While analyzing the activation of MAP kinase in response to cold and heat for a period of 24 h we observed a certain rhythm in the activity of MAPK in an in-gel kinase assay system. The plants growing at 28°C were moved either at 4°C for cold treatment or to 42°C for heat treatment and leaves were sampled at different time points to analyze the activation of MAPK activity using MBP as substrate. As shown in Fig. 1, a fast activation of MAPK could be observed even five minutes after shifting the plants to lowered or elevated temperatures. The activated state of MAPK in response to heat remains high up to 1 h while it goes down at 3 h and again showed elevated activity

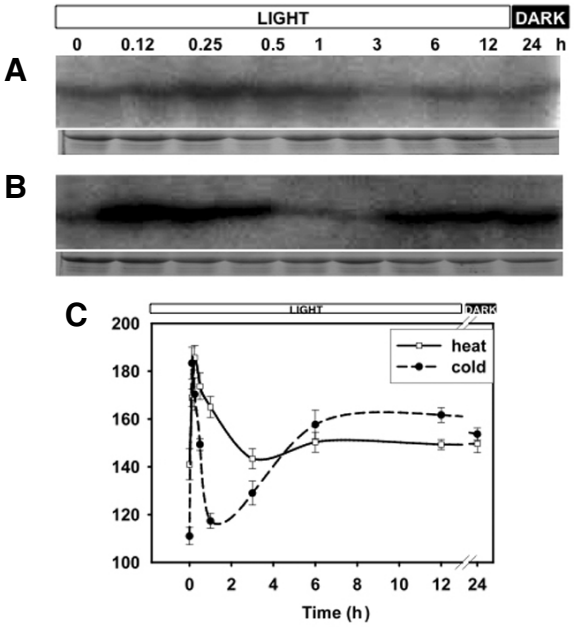

Fig. 1. Rhythm in temperature responsive activation of MAP kinase. Two week old rice plants variety Pusa Basmati-1 Indica cultivar group were subjected to either (A) elevated (heat stress) or (B) lowered (cold stress) temperature for a period of 24 h. Plants were harvested at respective time points and MAP kinase activity was assayed in an in-gel kinase assay using MBP as a substrate. Lower panel of in-gel kinase assay is the silver stained protein gel of the same samples indicating equal protein loading. The activity bands were quantified by phosphorimager and depicted in the form of graph (C). The experiments were repeated three times with similar results.

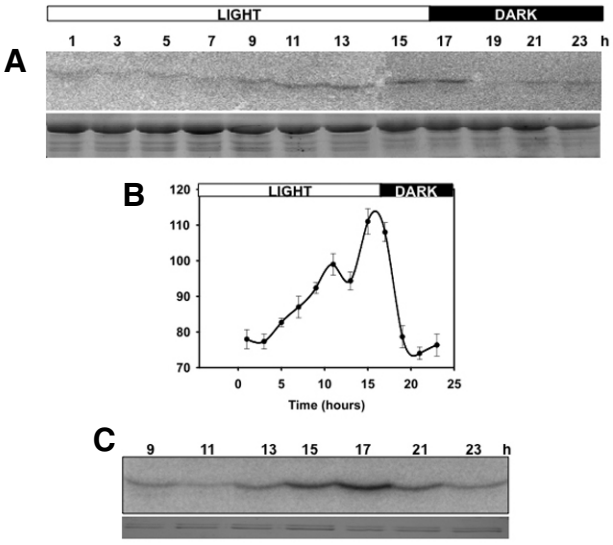

Fig. 2. Diurnal regulation of MAP kinase activity. Two weeks old rice plants variety Pusa Basmati-1 Indica cultivar group grown under 16 h light/8 h dark conditions at constant temperature. (A) Plants were harvested at a regular interval of 2 h for a period of 24 h and analyzed for MAP kinase activity in an in-gel kinase assay using MBP as substrate. (B) The activity bands were quantified by phosphor imager and depicted in the form of graph. (C) Samples from a select few time points from diurnal studies were immunoprecipitated with anti-phospho tyrosine, 4G10 antibody and subsequently analyzed for MAPK activity in an in-gel kinase assay with MBP as substrate. Lower panel of in-gel kinase assay (A) and (B) is the silver stained protein gel of the same samples indicating equal protein loading. The experiments were repeated three times with similar results.

level at 6, 12 and 24 h of shifting the plants to high temperature (Fig. 1A). When the rice plants were moved to 4°C from 28°C the fast activation of MAPK could be observed only up to 30 min, followed by deactivation at 1 and 3 h and again showing elevated activity at 6, 12 and 24 h (Fig. 1B). Quantifying the band intensity by phosphorimager showed that the cold and heat activated MAPK showed an increase in activity after the initial dip (Fig. 1C). The increase in activation state of MAPK after getting deactivated under low and high temperature observed during a course of 24 h indicated a sort of internal rhythm in the process of activation/deactivation of MAPKs.

Diurnal rhythm in MAP kinase activity

To get a better insight into the rhythm observed during prolonged temperature stress, we investigated for the existence of any diurnal rhythm in the basal activity of MAP kinase. And most of the time while analyzing the activation of MAP kinase in response to certain stress, a basal level of activity is observed in the control samples. Two week old rice plants growing under 16 h light /8 h dark cycles at 28°C constant temperature were sampled at a regular interval of 2 h. The samples were collected starting from 1 h after light and continued up to 24 h in regular intervals. The in-gel kinase assay using MBP as substrate revealed that MAPK activity increases constantly during light period attaining its peak at 15 h (Fig. 2A). This elevated state of activity is maintained even one hour after onset of darkness, which then decreases to a pre dawn value. Figure 2B is the graphical representation of the band intensity at each time points as determined by phosphorimager.

The assay for MAP kinase activity is based on the ability of this enzyme to phosphorylate a protein containing a consensus sequence, P-x-S/T-P (proline-x-serine/threonine-proline). Myelin basic protein (MBP) is the widely used substrate for MAP kinase assay (Eichberg and Srinivas, 1996). It is interesting to note that MBP is a substrate for many other protein kinases such as protein kinase C, cAMP-dependent protein kinase and calmodulin-dependent protein kinase II (Harauz and Ishiyama, 2000). A positive MBP phosphorylation only indicates a putative MAPK and requires further confirmatory experiments like immunoprecipitation using antibodies specific to the MAP kinase or to an epitope tag introduced into the recombinant kinase (Reuter et al., 1995). MAP kinases are known to be activated by phosphorylation on the conserved sequence motif TXY, both at Tyr and Thr residues (Canagarajah et al., 1997). To support that the basal level of activity of a MBP phosphorylating protein observed diurnally is the activity of MAP kinase, we performed immunoprecipitation with an anti phospho-tyrosine specific antibody (4G10) at selective time points. The subsequent in-gel kinase assay results revealed that activated kinase is recognized by 4G10 antibody and is indeed a member of MAP kinase family (Fig. 2C).

Persistence of the rhythm in MAP kinase activity follows circadian regulation

To identify rhythmic activation of MAP kinase, their existence was tested under constant external cues, to implicate their role in circadian regulation. When plants are deprived of environmental time cues (e.g. light/dark cycle and temperature cycle) and placed in constant ("free-running") environmental condi-

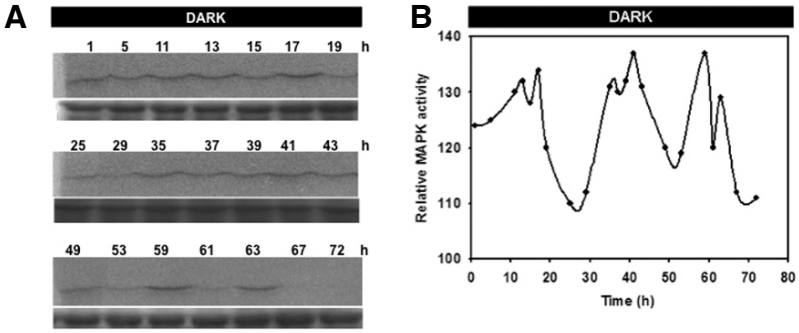

Fig. 3. Regulation of MAP kinase activity follows circadian pattern. Two week old rice plants variety Pusa Basmati-1 Indica cultivar group entrained under 16 h light/8 h dark conditions at constant temperature. Rice plants were transferred to (A) continuous dark and plants were harvested at specific time points for a period of 72 h. MAP kinase activity was analyzed in an in-gel kinase assay using MBP as substrate. Lower panel of in-gel kinase assays is the silver stained protein gel of the same samples indicating equal protein loading. (B) The activity bands were quantified by phosphor imager and depicted in the form of graph. The experiment has been repeated three times with similar results and a representative data has been presented.

tions, circadian rhythms must persist with a period of around 24 h, often for many days. Two week old plants entrained in 16 h light/8 h dark were transferred to continuous dark, samples were collected at different intervals up to next 72 h. In-gel kinase assay of MAP kinase revealed that the activation profile of MAP kinase was continued even in the absence of external entraining stimuli (Figs. 3A and 3B). The data observed indicates that the activation of MAPK may be under the control of circadian clock. The findings were in accordance with the reports from mammalian system that several circadian clock containing tissues exhibit circadian activation/deactivation cycles of mitogen activated protein kinases under constant darkness (Harada et al., 2000; Obrietan et al., 1998; Sanada et al., 2000).

Group C MAP kinase shows rhythmic expression at transcript level

In order to identify the MAPKs involved in rhythmic regulation, we analyzed the transcript regulation of five MAP kinases belonging to group A, B, and C. These MAP kinase were OsMPK3 and OsMPK6, belonging to Group A, OsMPK4 belonging to Group B, OsMPK7 and OsMPK14, belonging to Group C. All these MAP kinases showed a calculated molecular mass around 46 kDa, a size we observed the signals from MAP kinases during in-gel kinase and immunokinase assay. MAP kinase belonging to Group D had calculated molecular mass higher than 50 kDa, hence were not included for the transcript profile analysis. Rhythm in transcript level was analyzed by quantitative real time PCR (qRT-PCR). Two week old rice plants entrained in 16 h light/ 8 h dark conditions were sampled at selected time points up to 24 h, qRT-PCR of respective samples was carried out with gene specific primers of *OsMPK3*, *OsMPK4*, *OsMPK6*, *OsMPK7* and *OsMPK14*. Analysis of the results revealed that among the five MAPKs analyzed, a diurnal rhythm in expression pattern of only *OsMPK7* and *OsMPK14* transcripts was observed (Fig. 4A). To find out the existence of rhythm in continuous conditions, rice plants were shifted to dark conditions and samples were collected at selective time points up to 72 h. Interestingly rhythmic expression of *OsMPK7* and *OsMPK14* were also observed in continuous darkness with equal magnitude for first two days and reducing gradually on third day for *OsMPK7* (Fig. 4B) while *OsMPK14* transcripts showed a continuous reduction through out. The data indicates that group C MAPKs are the probable candidates for the circadian regulation of MAPK activity in rice.

DISCUSSION

Life occurs in an ever-changing environment. Some of the most striking and predictable changes are the daily rhythms of light and temperature. To cope up with these rhythmic changes, plants use an endogenous circadian clock to adjust their growth and physiology and anticipate daily environmental changes. As sessile organisms, plants have evolved a complex signaling network that mediates the perception and responses to different environmental cues. Recent studies have shown that MAPK cascades are evolutionarily conserved signaling modules that play a pivotal role in plant responses to multiple biotic and abiotic stresses (Jonak et al., 2002; Zhang et al., 2006). A number of studies have demonstrated that MAPKs are activated by a variety of abiotic stresses like wounding, heavy metals, hormones, during plant pathogen interaction and cell division (Mishra et al., 2006). Also a number of plant MAPK cascade genes have been characterized functionally and the importance of the three kinase module in signal transduction has been supported by numerous experimental studies (Nakagami et al., 2005). Among abiotic stresses, activation of MAPK by temperature responses has been reported earlier (Jonak et al., 1996; Link et al., 2002b; Sangwan et al., 2002). While checking the activation of MAPK by heat and cold stresses we observed a specific rhythm when the plants were continuously exposed to elevated or lowered temperature for a period of 24 h. When plants were subjected to heat shock for 5 min at 42°C and moved back to normal conditions (28°C), the rhythm in MAP kinase activation profile continued for 24 h (data not shown). These observations lead us to believe the existence of an internal rhythm in the activity of MAPKs. One of the common practices of analyzing the MAPK activation is to compare the activity of the control samples with the elicited/stressed samples. However a certain basal level of MAPK activity was observed most of the time in the control samples. First we analyzed the existence of any diurnal rhythm in theses basal activities of MAPK by an in-gel kinase assay. We indeed observed a rhythm in the plants entrained under 16 h light/8 h dark cycle, growing at a constant temperature of 28°C. Immunoprecipition with an anti-phosphotyrosine antibody, 4G10 of selected time point followed by immunokinase assay revealed that the MBP phosphorylating kinase is indeed a MAP kinase (Link et al., 2002a).

Plant growth is normally governed not just by the clock, but also by the interaction of endogenous rhythms and external cues. To better understand the relationship between circadian and diurnal rhythms, it is helpful to examine the relationship between activity profiles in these two environments. While 11% of *Arabidopsis* mRNAs were found to be diurnally regulated, only a subset of these showed circadian rhythms in continuous conditions (Sachffer et al., 2001). To be under circadian clock control, a particular rhythm should comply with the following three criteria: persist under constant conditions with a period of

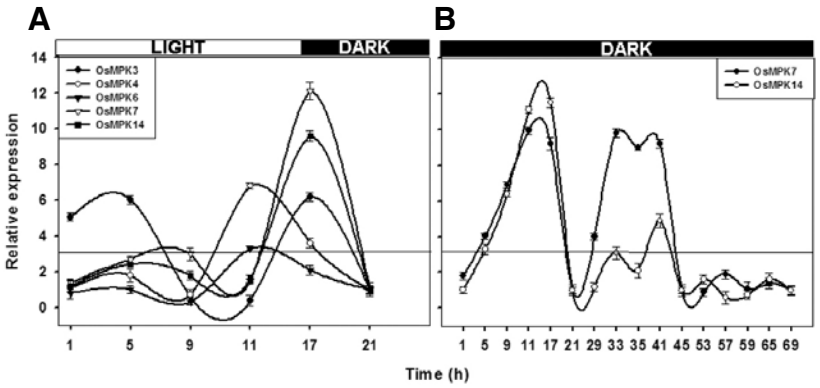

Fig. 4. Rhythmic expression of *OsMPKs* analyzed by qRT-PCR. Two weeks old rice plants variety Pusa Basmati-1 Indica cultivar group grown under 16 h light/8 h dark conditions at constant temperature. For diurnal regulation plants were harvested at specific time points, and for circadian plants were shifted to constant dark conditions and sampled at different time points up to 72 h. (A) Transcriptional regulation of *OsMPK3*, *OsMPK4*, *OsMPK6*, *OsMPK7* and *OsMPK14* in diurnal conditions, (B) transcriptional regulation of *OsMPK7* and *OsMPK14* under constant darkness. qRT-PCR data was normalized using rice actin gene. The data represent an average of three independent experiments.

about 24 h; be entrained to 24 h by environmental cues (such as day and night shifts or cycles of varying temperatures); and display the same period (Dunlap, 1999; Mittag et al., 2005). Most studies of circadian functions in plants have been performed under continuous conditions. Rice plants entrained under 16 h light/8 h dark conditions when transferred to continuous dark the rhythm continued up to 72 h. Based on the consistency of the rhythm in continuous dark suggests that rhythm in MAPK activities are endogenously generated rhythms and may be under the regulation of circadian clock. Recently Lee et al. (2008) reported that transcription of OsSIPK, which encodes a rice group B MAPK is repetitively induced during the dark period of a 12 h/12 h (day/night) cycle but in continuous conditions this rhythm disappeared. Analysis of transcript regulation of all the MAPKs from group A, B and group C revealed that group C members in rice are exhibiting rhythmic expression under diurnal as well as continuous dark conditions. Further rhythmic expression of rice homologue in *Arabidopsis MPK7* a group C MAPK as reported by Schaffer et al. (2001) suggests the involvement of group C members in rhythmic regulation is conserved across orthologues. Since MAP kinases are basically signaling molecules they may involve in transferring the signals between input pathways and central oscillator or mediating the feed back of the output signal to the input forming an interconnected loop, and this loop is likely to contribute to maintenance of circadian clock oscillation. MAP kinases are also reported to be activated by internal cues like hormones (Mishra et al., 2006) and sugars (Ehness et al., 1997), it can be speculated that changes in the internal metabolites status may result in the activation of this important protein kinase cascade required for appropriate cellular adjustment.

The involvement of MAP kinases in various metabolic processes in plant cells might have general implications. The overall progress of research on MAP kinases in plant systems has been slow compared to other systems. However, the increasing numbers of reports describing plant MAP kinase signaling components reflect their central role in plant growth and development. So far the role of these kinases in stress and hormonal signaling has been dissected. Our current report here that rhythmic expression of MAP kinase activity will add a new dimension to the plant MAP kinase research. A lot of effort is still required to uncover in detail each MAP kinase module and to understand the complexity of the signal transduction pathways.

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