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THESIS

EXPRESSION ANALYSIS OF PHOTOPERIOD RESPONSIVE GENES IN RICE (*Oryza sativa* L.) KDML 105

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A Thesis Submitted in Partial Fulfillment of
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GeneChip DNA microarray was used to identify photoperiod responsive genes of rice (*Oryza sativa* L.) KDML 105. A comparative analysis of gene expression changes between short day (SD) and long day (LD) conditions was performed with three independent biological replications. A total of 184 probe sets were selected as differentially expressed genes on the basis of their expression ratios with t-test p value (fold changes ≥ 2, $p \leq 0.05$) and were verified with RT-PCR and real-time PCR. Among them, 79 genes were up-regulated while 105 genes were down-regulated. Those genes were classified into nine classes from their putative functions, i.e., unknown, transcription factor, defense/stress, metabolism, growth/structure, processing, signaling, transport and energy transduction. Pathway analysis revealed that the photoperiod response is involved in photosynthesis, carbohydrate metabolism, circadian clock, phytochrome signaling, hormone signaling and miRNA synthesis pathways. Several flowering time related genes were also associated with those pathways; the floral inducers were up-regulated while the floral repressors were down-regulated, including the targets of miRNAs such as *AP2* floral repressor. Expression analysis of miRNA of *AP2, miRNA172a,* showed that its expression was induced by SD light. This indicated that *miRNA172a* was involved in the regulation of photoperiodic flowering time in rice via the down-regulation of *AP2*. Monitoring expression of photoperiodic flowering time genes during SD induction revealed that floral inducers, i.e., *Hd3a* and *AP1 like* were induced in day 6 and day 10, respectively, while floral repressor, *AP2* was suppressed in day 4. This showed that *AP1 like* was a downstream of *Hd3a* and their expression were probably induced by the down-regulation of *AP2*.

To elucidate the regulatory mechanism of photoperiod response, the *cis*-regulatory elements of photoperiod responsive promoters were analyzed. The results showed that the transcription of photoperiod responsive genes is controlled by light in coordination with hormones and stress responses. GARE motif and G-box are the coordinated motifs integrating gibberellins to photoperiod while MBS and G-box are the coordinated motifs integrating ethylene or abscisic acid and stresses to photoperiod. Phytochrome A (phyA) regulated genes were further identified using the specific organization of *cis*-regulatory elements. The data showed that phyA is involved in the transcriptional regulation of flowering time genes either by activation of floral inducers or suppression of floral repressors. So far, two novel *cis*-regulatory elements which are specific to daylength were identified. The novel A-rich element is specific to LD light involved in the regulation of phyA and circadian rhythm to inhibit flowering whereas the novel GC element is specific to SD light involved in the regulation of gibberellins signaling to promote flowering. Taken altogether, the photoperiodic flowering pathway of KDML 105 was proposed. The flowering transition is controlled by phyA and circadian rhythm in coordination with hormone signaling, stress responses and metabolic state. The floral development is switched by down-regulated *AP2* by *miRNA172a* and up-regulated *Hd3a* leading to activation of floral meristem identity gene, *AP1 like*, *CAL* and *OsMADS1,* via the meristem maintenance CLAVATA pathway.

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Chareerat Mongkolsiriwatana

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LIST OF ABBREVIATIONS

ABA	abscisic acid
AP1	APETALA 1
AP2	APETALA 2
CAL	CALIFLOWER
CCA1	CIRCADIAN ASSOCIATED 1
CLV	CLAVATA
CO	CONSTANS
COP1	CONSTITUTIVELY PHOTOMORPHOGENIC 1
FAR1	far-red impaired 1
FT	FLOWERING LOCUS T
GA	gibberellic acid
GI	GIGANTEA
Hd1	Heading date 1
Hd3a	Heading date 3a
LD	long day
LFY	LEAFY
LHY	LONG HYPOCOTYL
LRE	light responsive element
PHY A	phytochrome A
PHY B	phytochrome B
RISC	RNAi silencing complex
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SAM	shoot apical meristem
SD	short day
SOC1	SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1
TOC1	TIMING OF CAB 1
WUS	WUSCHEL

EXPRESSION ANALYSIS OF PHOTOPERIOD RESPONSIVE GENES IN RICE (*Oryza sativa* L.) KDML 105

INTRODUCTION

Flowering transition is one of the most important period in the life cycle of plants. To achive reproductive success, plant needs the most favorable environmental condition to initiate their reproductive development. Furthermore, in crop species, flowering initiation and flower and fruit developmental decisions can determine the quality and quantity of crop production (Boss *et al*., 2004).

Rice, a short day plant, has adjusted the floral transition to coincide with the rainy season, which also happens to be the part of the year with the shortest photoperiod. Therefore decreasing the day length will promote the floral transition whereas increasing day length will prolong the vegetative growth phase (Izawa, 2007).

Thai jasmin rice (Khao Dok Mali 105; KDML105) cultivar, an *indica* rice, is the most popular cultivar due to its pleasant aroma, having soft and tender texture after cooking. However, its production is limited due to photoperiod-sensitive trait generally grown only one crop per year. Thus, the mechanism of flowering photoperiod response is attractive for rice researchers to improve the crop production.

Extensive genetic and molecular analysis of flowering in photoperiod response were performed in the facultative long day (LD) plant *Arabidopsis thaliana* L., generating a complex genetic model explaining how the plant integrated environmental signals (mainly light and temperature) to regulate the expression of genes controlling flowering time (Ausin *et al*., 2005). In contrast, the regulation of flowering transition in rice is little known. Although, previous genetic analysis in rice revealed that several genes are involved in response to photoperiod, but how these genes regulated photoperiodic flowering at molecular level is unclear.

Due to its complexity, a full understanding of the control of flowering in rice is very difficult. A recent technological advance, microarray analysis, is likely to help in analyzing complex pathways including flowering time. The survey of expression levels of large

numbers of genes can link the expression profiles with the transition to flowering, and perhaps uncover crosstalk between the different pathways.

In this thesis, GeneChip DNA microarray technique was used to investigate global gene expression in response to photoperiod in KDML 105, to dissect of floral induction pathways that response to photoperiod. This study provides not only a new insight in response to photoperiod at the whole genome level but also provides a basic knowledge on the floral signaling mechanism and the floral transition genetic network of KDML 105 and extend to other SD plants.

OBJECTIVES

1. To study global gene expression and regulation in response to photoperiod in rice (*Oryza sativa* L.) KDML 105.

2. To dissect photoperiod responsive pathway of KDML 105.

3. To identify photoperiodic flowering time genes of KDML 105.

LITERATURE REVIEW

1. Rice Information

Rice is a grass (Gramineae) belonging to the genus *Oryza* L. of which two species are cultivated, *O. sativa* L. and *O. glaberrima* Steud. *O. sativa* can be further divided into 3 groups: *indica*, *japonica*, and *javanica.* Rice other than these two species is classified as wild rice (Grist, 1983).

Rice can be classified by photoperiod-sensitive into two groups including: 1) photoperiod-sensitive variety and 2) non-photoperiod-sensitive varitiety. The first group has uncertain harvest day because it flowers only in short day (SD) light, while the latter group has a certain harvest day because its flowering depends on vegetative growth phase (The International Rice Research Institue [IRRI], 1985).

2. Growth phases of Rice

The growth of the rice plant can be divided into three stages: 1) the vegetative growth phase, from germination to panicle initiation; 2) the reproductive phase, from panicle initiation to flowering; and 3) the ripening phase, from flowering to full development of grain. In the tropics, the reproductive phase is about 35 days while the ripening phase ranges from 30 to 35 days. Both phases are relatively constant, although low temperatures have been known to prolong them and high temperatures to shorten them. The ripening phase may be prolonged to as much as 60 days. However, it is the vegetative growth phase whose duration generally varies greatly and which largely determines the growth duration of a cultivar, especially in the tropics (IRRI, 1985).

The vegetative growth phase can be further divided into the basic vegetative phase (BVP) and the photoperiod-sensitive phase (PSP).The BVP refers to the juvenile growth stage of the plant, which is not affected by photoperiod. It is only after the BVP has been completed that the plant is able to show its response to the photoperiodic stimulus for flowering — this is the PSP of the plant (Yoshida, 1981).

3. Characteristics of KDML 105 Rice Cultivars

KDML 105 is the local aromatic rice derived from the 199 ears collection of grains from Bangkhla District, Chachoengsao Province. They were grown and compared to each other until on May 25, 1959 KDML 4-2-105, also called KDML105, was introduced to farmers. KDML 105 is 140-150 centimeters in height. It is photoperiod-sensitive rice that will flower around October, 20th thus it can be grown only in rainy season. Stem and leaf are quite small, the leaf is long and pale green, seed dormancy is about 8 weeks and seed length is 7.5 millimeters. Good characteristics are drought and acid soil tolerance, cooked rice quality, fragrance and softness. Poor characteristics are the susceptibilities to yellow orange leaf disease, bacterial leaf blight disease, bacterial blight disease, brown plant hopper, green leaf hopper, rice stem borer, and rice gall midge (Rice Research Institute, 2003).

4. Floral transition in *Arabidopsis* and rice

In the plant life cycle, the transition from vegetative phase to reproductive phase (flowering) is one of the most important decisions in order to guarantee the success to produce progeny. Rice has adjusted the floral transition to coincide with the rainy season which also happens to be the part of the year with the shortest photoperiod. Therefore, decreasing the day length will promote the floral transition whereas increasing day length will prolong the vegetative growth phase (Izawa, 2007). In contrast to rice*, Arabidopsis* contains mechanism to prevent precocious flowering during winter. The nature of these mechanisms resulted in vernalization requirement for floral transition (Michael *et al*., 2004).

The switch from vegetative to reproductive growth can be observed as morphological changes in the vegetative plant tissues. The shoot apical meristem (SAM), which consists of indeterminate stem cells, produces leaves and branches during vegetative growth. In response to external and internal signals, the SAM will change its identity to a floral meristem and start the production of the flower primordia. In *Arabidopsis,* these primordia later give rise to the four unique organs, petals, sepals, stamens and carpels, which constitute the mature flower (Simpson *et al*., 1999).

Environmental signals (temperature and light) and developmental stages (size, age) act in concert to trigger floral transition. Investigations in the model plant *Arabidopsis*

thaliana have provided the basic knowledge on floral signaling mechanisms and floral transition genetic network. Four major pathways have been described, the vernalization pathway, the photoperiod pathway, the autonomous pathway and the gibberellic acid (GA) pathway (Levy and Dean, 1998; Mouradov *et al*., 2002; Putterill *et al*., 2004). In addition to these four well-characterized pathways, other factors such as carbohydrate availability, ambient temperature and salicylic acid have also been shown to influence the floral transition (Zhou *et al*., 1998; Blazquez *et al*., 2003; Marti′nez *et al*., 2004).

In the following part, the *Arabidopsis* floral signaling mechanisms, the interplay between them, the pathways and their components will be discussed. It will be attempted to provide the essential advances in monocots research in this field. In particular, focus will be placed on the photoperiod signaling pathway and the integration of the photoperiod signal through *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*).

4.1. Floral transition

In *Arabidopsis*, the floral transition occurs in response to environmental and developmental cues. Genetic and molecular analysis have shown that three genes *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) and *LEAFY* (*LFY*) control the switch from vegetative to reproductive growth. Upregulation of these genes marks the floral transition and will promote the activation of floral meristem identity genes such as *APETALA 1* (*AP1*), *CAULIFLOWER* (*CAL*), *LFY* itself, and subsequently the floral organ identity genes. These genes have been characterized as floral integrators (Nilsson *et al*., 1998; Blazquez and Weigel, 2000) since the actions of all four promotive pathways are directed into the upregulation of these genes either directly or indirectly. Direct activation happens both via the long day pathway through *CO* and via the GA pathway. Indirect activation happens through the autonomous and the vernalization pathways which act in concert to downregulate *FLOWERING LOCUS C* (*FLC*), a MADS-box protein encoding gene, which is a repressor of *FT* and *SOC1*. Thus, reduction of *FLC* expression will allow transcription of *FT* and *SOC1*, which is further stimulated by photoperiod pathway and by endogenous GA (Ratcliffe and Riechmann, 2002).

4.2 Photoperiodic regulation of flowering time in *Arabidopsis*

Light provides seasonal information to the plants. Photoperiod variation in particular promotes flowering in temperate regions. *Arabidopsis* is a facultative LD plant, which means that LD light promotes floral transition but is not a prerequisite for flowering. Photoperiodic induction of floral transition starts at the photoreceptors, which persieve the duration, intensity and quality of the light. The photoreceptors produce a dual signal to entrain the circadian clock and to induce floral transition through activation of *GIGANTEA* (*GI*), *CO* and finally *FT*, three key genes of the signaling photoperiod pathway (Mouradov *et al*., 2002).

The *CO* gene plays a central role in the photoperiod floral induction pathway and promotes flowering in inducible conditions. The *co* mutant was identified as a late flowering phenotype only in LD conditions (Putterill *et al*., 1995). The CO protein is a zinc finger protein that harbors two B box type domains and a CCT motif, which are believed to mediate protein-protein interaction. Over expression of *CO* in *Arabidopsis* leads to an early flowering phenotype in LD and in non-inductive SD conditions (Suarez-Lopez *et al*., 2001). In the wild type, the *CO* transcripts level follows a diurnal pattern with a similar biphasic peak in both LD and SD. In LD, the level of *CO* expression increases at the end of the light period, then reaches a first maximum at dusk. In SD, it occurs during the dark period (Suarez-Lopez *et al*., 2001). The transcription level of *CO* is entrained by the circadian clock, it continues to oscillate with a 24 hours rhythm in constant light after the plants are grown and entrained in LD and then moved to constant light (Suarez-Lopez *et al*., 2001). Spatial expression of *CO* is detected mainly in vascular tissues (Takada and Goto, 2003). Recent data indicates that the CO protein localizes in the phloem companion cells (An *et al.*, 2004). CO protein abundance follows a diurnal rhythm in LD and a maximum of abundance was detected the late afternoon in the day. Under blue light conditions, the abundance of CO protein is stabilized in the nuclei of the stomatal guard cells. In 35S::*CO* plant, the level of CO proteins still follows a diurnal pattern, which indicates that *CO* is subjected also to posttranscriptional regulation in order to trigger activation of its target gene, *FT* (Valverde *et al*., 2004).

FT, which encodes a Raf-kinase-inhibitor-like protein promotes flowering and was identified from a mutational screen as a downstream gene of *CO*. The early flowering phenotype of 35S::*CO Arabidopsis* plants could be suppressed by a mutation in the *FT* gene

(Onouchi *et al*., 2000). Although in the late flowering *co* mutants, no *FT* transcripts were detected, overexpression of *FT* was able to restore the wild type phenotype (Kobayashi *et al*., 1999). Acting downstream of *CO,* the transcriptional regulation of *FT* also follows a diurnal rhythm and peaks at dusk. In LD condition, flowering is correlated to a high expression level of *FT*, whereas in SD condition, no *FT* was detected (Suarez-Lopez *et al*., 2001). Furthermore, elevated *CO* expression in light was suggested to promote flowering by activating expression of *FT* (Onouchi *et al*., 2000).

The activity of *CO* is mainly controlled by two components of the photoperiod pathway; the circadian clock output genes, which act at the transcriptional level and the photoreceptors, which are suggested to modulate the protein activity of *CO* to enhance *FT* transcription and floral transition.

4.2.1 Circadian clock

The Circadian clock is an autoregulatory endogenous mechanism that allows organisms, from bacteria to humans, to advantageously time a wide range of activities within 24 hours cycles (Young and Kay, 2001). The photoperiod control of flowering in *Arabidopsis* is a circadian clock controlled process. The circadian system is commonly divided into three components, the input, which perceives the daily cycles of light and temperature, the central oscillator, which generates the 24 hours rhythm and the output which regulates developmental processes. The circadian clock is entrained by the light spectrum, which is sensed by photoreceptors. In the *phytochrome A* (*phyA*), *phytochrome B* (*phyB*) and *cryptochrome 2* (*cry2*) mutants, periods of circadian rhythm are lengthened at low fluence of both red and blue light (Somers *et al*., 1998).

Although, the exact molecular mechanism of the interface between light perception and the circadian clock remains unclear, candidate genes have been suggested to act in this process. The ZEITLUPE (ZTL) protein harbors a LOV domain, involved in blue light sensing, a kelch repeat domain for protein-protein interactions and an F-Box domain for protein degradation. Similar to *phya*, *phyb* and *cry2* mutants, the *ztl* mutation also lengthens the circadian period that is dependent of light intensity (Somers *et al*., 2000). ZTL, which interacts with PHYB and CRY1 was suggested to mediate the light signaling to the circadian clock (Jarillo *et al*., 2001). Recently, ZTL has been proposed to recruit TIMING OF CAB1 (TOC1), a central component of the circadian clock, for degradation by

the proteasome during the night (Mas *et al*., 2003). The EARLY FLOWERING 3 (ELF3) is circadian clock regulated and has been proposed to act as an input to the clock by gating light in interaction with phytochromes (Hicks *et al*., 2001; Liu *et al*., 2001). The *elf3* mutant has reduced transcripts level of two components of the circadian clock, *LONG HYPOCOTYL* (*LHY*) and *CIRCADIAN CLOCK ASSOCIATED* (*CCA1*).

The central oscillator, which is the 24 hour pace keeper, is based on a feedback loop between three proteins, the two myb-like DNA binding proteins CCA1 (Wang and Tobin, 1998), the LHY (Schaffer *et al*., 1998) and the TOC1 protein (Millar *et al*., 1995). TOC1 acts as a positive regulator of CCA1 and LHY, which in turn are negative regulators of TOC1 (Alabadi *et al*., 2001). In the morning, TOC1 activates expression of *LHY* and *CCA1* genes. Accumulation of LHY and CCA1 proteins bind to the *TOC1* promoter and repress *TOC1* expression. Since *LHY* and *CCA1* expression are enhanced by TOC1, levels of LHY and CCA1 proteins decrease. Thus, repression of *LHY* and *CCA1* releases expression on the *TOC1* promoter and allows TOC1 to accumulate in the evening.

The plant clock regulates the floral transition through *GIGANTEA* (*GI*) and *FLAVIN BINDING, KELCH REPEAT, F-BOX1* (*FKF1*), which mediate the output circadian rhythm and regulate *CO* expression. The *GI* gene transcription follows a diurnal regulation that peaks 8–12 hours after dawn and *GI* expression is altered in the *elf3*, *lhy* and *cca1* mutants. In contrast, the expression of *LHY* and *CCA1* is reduced in the *gi* mutant, suggesting a reciprocal regulation (Fowler *et al*., 1999). However, the *gi* mutant, in which the cycle length is shortened, causes a severe late flowering and *CO* expression is altered (Devlin and Kay, 2000). *FKF1* transcription also shows diurnal oscillations and the peak of expression, which is observed during the day may generate the peak of *CO* expression. In the late flowering *fkf1* mutant, which does not affect expression of the central oscillator genes, the first peak of *CO* expression was lacking and no *FT* transcripts were detected (Imaizumi *et al*., 2003).

4.2.2 Photoperiod posttranscriptional regulation

Several photoreceptor mutations lead to early or late flowering and are involved in the regulation of the photoperiod pathway in the floral transition and the regulation of the posttranscriptional level of *CO*. In *Arabidopsis,* two main classes of photoreceptors perceive the light. The red/far-red (600-700 nm) is sensed by the

phytochromes and the blue (400-500 nm)/UV-A by the cryptochromes (Devlin and Kay, 2000). The far-red or red light activates translocation of PHYA and PHYB, respectively to the nucleus. The far-red light sensor *phyA* mutant is late flowering in LD but flowers similar to the wild type in SD (Bagnall *et al*., 1995). The late-flowering phenotype observed in LD was shown to be correlated to a reduction of *FT* transcripts level, indicating that PHYA promotes flowering (Johnson *et al*., 1994). In this mutant, the *CO* expression level is slightly reduced; however, the authors suggested that *CO* transcription has a minor effect on the severely reduced *FT* expression level (Yanovsky and Kay, 2002). The red light sensor *phyB* mutant is early flowering under both LD and SD conditions (Goto *et al*., 1991). The *phyB* early flowering phenotype mutant is associated with increasing *FT* transcript level; however the observed *CO* expression level was unchanged (Halliday *et al*., 2003). PHYA activates *FT* expression and promotes flowering whereas PHYB suppresses *FT* expression to repress flowering.

The CRY2 photoreceptors are soluble flavoproteins and are activated by blue light. The *cry2* mutant flowers much later than the wild type in LD but not in SD, indicating that CRY2 mediates the LD photoperiod signal in the control of flowering (Guo *et al*., 1998). In the *cry2* mutant, *CO* expression level was unchanged compared to wild type, whereas *FT* mRNA level was significantly reduced. However, mutations of *CO* suppressed the early flowering caused by the functional *CRY2* allele, indicating that *CRY2* is dependant on *CO* to be active (Yanovsky and Kay, 2002). In summary, the photoreceptors PHYA, PHYB and CRY2 regulate floral transition through CO *by* regulating FT expression.

Recent experiments have shown that photoreceptors may be regulated by light-induced factors and act as complexes to induce flowering. PHYTOCHROME AND FLOWERING TIME 1 (PFT1) modulates the expression of *FT* independently of *CO*. PFT1 is a nuclear protein involved in the "shade-avoidance syndrome", which is correlated to the altered ratios of red and far-red light mediated principally by the PHYB. The *pft1* mutant is late flowering under LD conditions and when crossing with the *phyB* mutant completely suppresses the early flowering phenotype of the *phyB* mutant (Cerdan and Chory, 2003). PFT1 induces flowering downstream of PHYB in response to suboptimal light conditions. The nuclear transcription factor PHYTOCHROME INTERACTING FACTOR 3 (PIF3), which is also required for light-induced flowering, was able to bind to PHYB *in vitro* (Ni *et al*., 1998). Antisense suppression of *PIF3* induced upregulation of *CO* and *FT* transcripts

without affecting the expression of circadian clocks *LHY*, *CCA1* and *GI* genes, suggesting that *PIF3* acts as repressor of *CO* and *FT* by activating PHYB (Oda *et al*., 2003).

Photoreceptors promote floral transition through *CO* and trigger CO posttranscriptional regulation. Expression studies of *CO* and *FT* in different photoreceptor mutants showed that *FT* mRNA levels were greatly reduced in *cry2* mutants (Yanovsky and Kay, 2002) and upregulated in *phyB* mutants (Halliday *et al*., 2003), whereas the levels or oscillation of *CO* expression were the same as in wild type (Suarez-Lopez *et al*., 2001). In fact, an increase of CO protein was observed in the *phyB* mutant under red light and associated to the observed elevated *FT* transcript level. In contrast, under blue light, in *cry1* and *cry2* mutants, both CO protein level and *FT* expression were reduced. Moreover, CO protein was shown to be ubiquinitated and susceptible to degradation by the proteasome in the *cry1, cry2* mutants (Valverde *et al*., 2004). Early in the day, red light photoreceptors PHYB, either repress CO activity or the protein is degraded by the proteasome. Later in the evening, blue light receptors, CRY1 and CRY2, stabilize the CO protein and allow CO activation and thereby expression of *FT* (Klejnot and Lin, 2004). However, the mechanism of induction of *FT* is still unknown, although *FT* appears to be a direct target of CO.

4.3 Photoperiodic regulation of flowering in rice

The functions and components of the circadian clock and the way they affect flowering are not well studied in monocots. However, in rice, orthologs of *LHY* and *GI, OsLHY* and *OsGI* and one homolog of *ZTL, ELF3* and *FKF1* have been identified, suggesting that similar components of the circadian clock exist in the two species (Izawa *et al*., 2003). *OsGI* expression is clock regulated and expression level peaks after dawn in both LD and SD conditions, similar to *GI* in *Arabidopsis*. Light perception also influences the levels of *OsGI* transcripts, which was illustrated by the observed low expression level in the *photoperiod sensitivity5* (*se5*) mutant, which has a defect in the biosynthesis of the phytochrome chromophore (Hayama *et al*., 2002). Downregulation of *OsGI* by *OsGI*-RNAi leads to reduced transcript levels of *Hd1*, the rice *CO* ortholog, both in SD and LD, and consequently the *OsGI*-RNAi plants flower later than wild type under SD and earlier under LD (Hayama *et al*., 2003). However, quite unexpectedly, overexpression of *OsGI* in rice leads to the same late flowering phenotype under SD and early flowering phenotype under LD, even though *Hd1* transcript levels were increased under both conditions (Hayama *et al*.,

2003). Thus *GI* and *OsGI* genes are regulated similarly and may mediate the circadian clock output to floral transition, although with different results.

Sequencing of the rice genome has revealed that rice contains a subset of three phytochromes, *PHYA*, *PHYB* and *PHYC* found in *Arabidopsis*. So far none of them have been related to control floral transition. However, the *se5* mutant is deficient in photoperiodic response and exhibits a very early flowering phenotype (Izawa *et al*., 2000). The expression of circadian related rice genes were not altered in the *se5* mutant, suggesting that, like in *Arabidopsis*, the floral transition is enhanced by light spectra and that the signal is propagated not only through the circadian clock but also directly by photoreceptors to downstream floral genes (Izawa *et al*., 2002).

Responses to photoperiod have been investigated in several monocot species such as rice, wheat, barley and ryegrass. Homology of the *Arabidopsis* photoperiod genes *CO* and *FT* have been identified in rice. Rice *CO* and *FT* were originally discovered as two QTLs for daylength sensitivity called *Heading date 1* (*Hd1*) and *Hd3a* genes that encode ortholog genes of *Arabidopsis CO* and *FT*, respectively (Yano *et al.*, 2000; Kojima *et al.*, 2002; Takahashi *et al.*, 2001).

The *Hd1,* rice *CO* ortholog, promotes flowering in inductive SD conditions. The *se1* mutant, which corresponds to a deletion in the *Hd1* gene, has as expected a late flowering phenotype in SD. However, in contrast to *Arabidopsis CO*, *Hd1* represses floral transition in non-inductive LD (Yano *et al*., 2001). The *Hd1* is circadian clock regulated and has a diurnal rhythm similar to *CO* under SD or LD conditions (Izawa *et al*., 2002). *Hd1*acts upstream of *Hd3a,* the rice *FT* ortholog, that enhances flowering in SD. In the *se1* mutant, reduced *Hd3a* expression level is observed in SD suggesting that *Hd1* regulates *Hd3a* by a similar manner as *CO* and *FT* (Izawa *et al*., 2002; Kojima *et al*., 2002). This suggestion is further strengthened by the observation that *Hd3a* expression is low, both in the *se1* mutant and the wild type under non-inductive LD. However, although these results suggest a regulation of *Hd3a* which is similar to that of *FT,* overexpression studies of *OsGI* in rice do not. Plants constitutively expressing *GI* from the maize ubiquitin promoter shows elevated levels of *Hd1* expression irrespective of day length and late flowering in both LD and SD conditions, an effect which is opposite to that in *Arabidopsis* 35S::*CO* (Hayama *et al*., 2003).

The rice *Hd3a* belongs to a family of ten known rice *FT-like* genes that are mainly expressed in leaves (Izawa *et al.,* 2003). In rice, overexpression of *Hd3a*, leads to an early flowering phenotype. Similar to *FT* in *Arabidopsis, Hd3a* was strictly up-regulated under inductive conditions and promotes flowering (Kojima *et al*., 2002; Izawa *et al.,* 2002). In LD, low *Hd3a* expression level is observed in the *se1* mutant and wild type suggesting that *Hd1* regulates *Hd3a*, as *CO* does with *FT*.

In summary, under inductive conditions, both *CO* and *Hd1* genes enhance *FT* and *Hd3a* transcription, respectively, and promote flowering. In contrast, under non-inductive conditions, *Hd1* represses *Hd3a* expression whereas *CO* has no action on *FT* expression. Thus, genes involved in the signaling photoperiod pathway are conserved and act in the same order between dicots and monocots even though the daylength induction is opposite.

5. Influence of GA on flowering time

Physiological studies examining the effect of GA on flowering time and study of mutants defective in either GA biosynthesis or signalling have revealed GA as a promoter of flowering, with this role being predominant in SD where the photoperiodic pathway is inactive (Langridge, 1957; Wilson *et al*., 1992). Genes involved in GA biosynthesis include *GA1* encoding ent-copalyl diphosphate synthase (entkaurene synthase) and *GA5* encoding GA 20-oxidase that catalyse the first committed step and some of the final steps in GA biosynthesis, respectively (Sun and Kamiya, 1994).

The *ga1-3*, GA biosynthesis null mutant is incapable to flower in SD and in LD flowers slightly later than wild type plants (Wilson *et al*, 1992). The slight delay in flowering in LD indicates some redundancy of the function of GA1. Genetically manipulated plants designed to overexpress GA 20-oxidase flower early in both SD and LD, and plants carrying reduced GA 20-oxidase levels because of antisense suppression exhibit delayed flowering in SD (Huang *et al*, 1998; Coles *et al*, 1999). Promotion of flowering in wildtype plants overexpressing GA suggests that GA levels appear to be naturally limiting for control of flowering time.

SPINDLY (SPY), a homolog of animal O-linked N-acetyl glucosamine transferase (OGT), has been shown to exhibit OGT activity and is a negative regulator of GA signaling.

OGTs act as post-translational regulators in signal transduction pathways by transferring a GlcNAc monosaccharide in O-linkage to a serine or threonine residue within proteins. The *spy* mutants exhibit pleiotropic phenotypes, one of which is early flowering (Silverstone *et al.*, 2007).

Other genes involved in GA signalling include *GAINSENSITIVE (GAI), REPRESSOR OF GA1-3* (*RGA*) and *RGA-LIKE 1* (*RGL1*) that share homology and encode nuclear-localized proteins (Wen and Chang, 2002). They are DELLA family members, which form part of a larger family of GRAS transcription factors, and are partially redundant negative regulators of GA signaling responses (Alvey and Haberd, 2005).

DELLA proteins can be considered as repressors of plant growth and development being involved in a number of plant developmental processes. GA regulates flowering, as it does in other plant developmental processes, through suppression of the repressive activity of the DELLA proteins. This mechanism, at least for some of the DELLA proteins, involves targeting of the proteins for destruction in the 26S proteosome (Alvey and Harberd, 2005).

6. Flowering Time and microRNA (miRNA)

miRNAs have emerged as important regulators of developmental processes in a range of multi-cellular organisms. miRNAs are small non-coding regulatory RNAs, about 21 nucleotides in length, produced from the processing of longer precursor transcripts. Plant miRNAs identified to date display near-complete matches with a complementary sequence in their target mRNAs and this often produces the cleavage of the mRNA at this site (Bartel, 2004).

At least 92 *Arabidopsis thaliana* miRNAs have been described, with most targeting mRNAs coding for transcription factors involved in different developmental processes (Dugas and Bartel, 2004). The biological function of several *A. thaliana* miRNAs has been established. Three *A. thaliana* miRNAs, *miRNA172*, *miRNA159* and *miRNA156* are known to be involved in the regulation of flowering time (Rhoades *et al*., 2002; Emery *et al*., 2003; Palatnik *et al*., 2003).

The *Arabidopsis* genome encodes four precursors of *miRNA172*: *miRNA172a-1, miRNA172a-2, miRNA172b* and *miRNA172c, miRNA172* exhibits complementary with the

floral meristem and floral organ identity gene, *APETALA2 (AP2)* and a group of genes related to *AP2*. This group includes *TARGET OF EAT1* (*TOE1*), *TARGET OF EAT2* (*TOE2*), *SCHLAFMUTZE* (*SMZ*), *SCHNARCHZAPFEN* (*SNZ*) and *At5g67190*. *TOE1*, *TOE2*, *SMZ* and *SNZ* are all repressors of *A. thaliana* flowering (Aukerman and Sakai, 2003; Schmid *et al*., 2003). The assignment of these repressors to particular flowering time pathways is currently unclear. Microarray analysis revealed that all four were down-regulated upon flowering and that this down-regulation was dependent on *FT* and *CONSTANS* (*CO*), indicating that they were repressors ultimately targeted by the photoperiod pathway (Schmid *et al*., 2003).

7. DNA microarray

DNA microarray is one the latest breakthough in experimental molecular biology, which allows monitoring of gene expression for ten of thousands of genes in parallel and is already producing large amounts of valuable data (Brazma and Vilo, 2000). DNA microarray uses between hundreds and hundreds of thousands of DNA probes arrayed on a solid surface to interrogate the abundance and/or binding ability of DNA or RNA target molecules.The DNA probes that are used in a DNA microarray could be amplified cDNA fragments or synthesized DNA oligonucleotides having sequences complementary to the target sequences (Lockhart *et al*., 2000). Thus, DNA microarrays are categorized into cDNA microarray and DNA oligonucleotide probe microarrays. These DNA oligomers are either directly synthesized on the surface of the microarray or deposited onto the suface mechanically and covalently immobilized on the surface (Schena *et al*., 1995). The samples to be interrogated are labeled fluorescently or radioactively before the detection. When samples are applied to the DNA microarray, the DNA probes will capture the nucleic-acid target molecules through sequence complementarity. The strength of the fluorescent or radioactive signals from the captured targets reflects the abundance of the target molecules and/or the binding compatibility between the probe and target molecules. A charge-coupled device (CCD) or laser scanner can then be used to record these fluorescent or radioactive signals quantitatively. Because at least hundreds and possibly thousands of DNA probes are arrayed in a single microarray, one microarray could be used to analyze many different targets in parallel, significantly improving the efficiency of this detection strategy (Zhu, 2003).

GeneChip is a specialized DNA microarray that uses *in-silico* synthesized DNA oligonucleotides as probes to detect the sequence similarity and abundance of target-DNA or -RNA molecules through complementary-sequence binding (Lipshultz *et al*., 1999). The standardized fabrication process and data-processing techniques used in this technology not only ensure data quality but also make data mining across a normalized database feasible (Zhu and Wang, 2000; Finkelstein *et al*., 2002). Because of their broad gene coverage, these microarrays have become increasingly used for genome-wide analysis of gene expression. GeneChip microarrays have been developed for *Arabidopsis* (Town *et al*., 2002; Zhu and Wang, 2000; Zhu *et al*., 2002), maize (Hunter *et al*., 2002), rice (Zhu *et al., 2003*) and barley (Wise *et al*., 2003).

GeneChip microarrays have already been applied to characterize transcript abundance, complexity, stability; to identify novel target genes and pathways that are associated with biological process or applied treatments (Cheong *et at*., 2002; Kreps *et al*., 2002; Menges *et al*., 2002; Fowler and Thomashow, 2005).

Cheong *et al.* (2002) surveyed the transcriptional response of wounding in *Arabidopsis* plants using *Arabidopsis* Genome GeneChip arrays. Studies of expression patterns of these genes provided new information on the interactions between wounding and other signals, including pathogen attack, abiotic stress factors, and plant hormones. These results further dissected the nature of mechanical wounding as a stress signal and identified new genes that may play a role in wounding and other signal transduction pathways.

Kreps *et al.* (2002) used *Arabidopsis* GeneChip Microarray (Affymetrix, Santa Clara, CA) to identify genes potentially important to cold, salt, and drought tolerance in *Arabidopsis.* Combined results from all three stresses identified 2,409 genes with a greater than 2-fold change over control suggested that about 30% of the transcriptome was sensitive to regulation by common stress conditions. A stress response was observed for 306 (68 %) of the known circadian controlled genes, supporting the hypothesis that an important function of the circadian clock is to “anticipate” predictable stresses such as cold nights. Although these results identify hundreds of potentially important transcriptome changes, the biochemical functions of many stress-regulated genes remain unknown.

The GeneChip Rice genome array was developed by Affymetrix Company that contains probe sets to detect transcripts from all the high-quality expressed sequence from the entire rice genome which includes 51,279 transcripts representing two rice cultivars. This unique design was created within the Affymetrix GeneChip Consortia Program and provides scientists with a single array that can be used for the study of rice genomics. Sequence information for this array includes public content from UniGeneBuild, GenBank mRNAs, and gene predictions from TIGR's osa1version 2.0 (Affymetrix, Santa Clara, CA).

Affymetrix rice genome array was used to explore the transcriptome of the salt-tolerant and salt-sensitive genotypes under control and salinity-stressed condition during vegetative growth. Cell wall-related genes were responsive in both genotypes, suggesting that cell wall restructuring is a general adaptive mechanism (Walia *et al*., 2005).

Jain *et al*. (2007b) used Affymetrix GeneChip Rice Genome Array analyzing the expression profile of all the two-component signaling elements in rice at various stages of vegetative and reproductive development. They found that most of the components were expressed in all the developmental stages analyzed. A few of these were found to be specifically expressed during certain stages of seed development, suggesting their role in embryo and endosperm development. Some of these components express differentially under various abiotic stress conditions, indicating their involvement at various levels of hierarchy in abiotic stress signaling.

Yang *et al*. (2006) identified *Os8N3*, a susceptibility gene to *Xanthomonas oryzae* pv. *Oryzae* strain PXO99A using GeneChip Rice Genome Array (Affymetrix, Sata Clara, CA). Silencing of *Os8N3* by inhibitory RNA produced plants that were resistant to infection by strain PXO99A but remained susceptible to other strains of the pathogen.

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MATERIALS AND METHODS

1. Rice Cultivation and Photoperiodic Control

Rice seeds, KDML 105 were soaked in water for 1 day, and grown in the tray containing soil for 7 days then transferred the rice seedlings into the plant pots. The rice pots were placed in the long day (LD) condition (13 hr light /11 hr dark per day) for vegetative growth for 50 days and then separated into 2 groups. The first group was exposed to short day (SD) light (11 hours per day) for 15 days, while the other group was continued under LD condition. Four independent biological replications were performed, 3 replications for DNA microarray analysis and 1 replication for RT-PCR analysis. An aerial part of plants were harvested at day 0, 3, 4, 5, 6, 7, 8, 9, 10, 12, and 15, by collecting at 0, 4, 8, 11 hours after dawn, from both SD and LD treatments. The tissue samples were immediately frozen in liquid nitrogen for RNA extraction.

2. RNA extraction

RNA samples were processed as recommended by Affymetrix (Affymetrix GeneChip Expression Analysis Technical manual; Affymetrix Santa Clara, CA). Total RNAs were initially extracted from frozen tissue using TRIzol reagent (Invitrogen Life Technologies) and quantified by spectrophotometer.

Frozen tissue was ground to powder in liquid nitrogen. 0.1 gram of powder was put into a 1.5 ml microcentrifuge tube and then added with 1 ml Trizol reagent, mixed thoroughly and incubated at room temperature for 5 minutes. After incubation, 200 µl of chloroform was added to the homogenate, inverted and mixed well, and incubated at room temperature for 5 minutes. To get phase separation, the sample was centrifuged at 10,000 ×g for 15 minutes at room temperature. The supernatant was transferred to new tube and then added with 500 µl of isopropyl alcohol to precipitate the total RNA. The RNA pellet was collected by centrifugation at 10,000 ×g for 15 minutes at room temperature, washed twice with 75 % ethanol and air-dried. The air-dried pellet was dissolved in RNase-free water. The total RNA was quantified by measuring the absorbance at 260 nm and the quality of the total RNA was determined by A260/A280 ratio.

2.1 RNA for DNA microarray analysis

The RNA samples from KDML 105 were used for DNA microarray analysis. The equal amount of each RNA sample from day 3-10 in the same treatment were pooled, treated with DNase and purified using RNeasy spin column (Qiagen). Eluted total RNAs were quantified and checked quality on NanoDrop ND-100 and adjusted to a final concentration of 1.0 μg/μl. The RNAs of LD condition (LD RNA) were used as control and the RNAs of SD condition (SD RNA) were used as experiment.

2.2 RNA for RT-PCR analysis

To monitor gene expression during the photoperiod treatment, RNA of several time points were prepared. The equal amount of each total RNA sample from 4 collected-times in the same day were pooled, representing RNA from day 0, 3, 4, 5, 6, 8, 10, 12 and 15.

3. DNA microarray analysis

Affymetrix GeneChip rice genome arrays were used for DNA microarray expression analysis in this experiment. Labeling, hybridization and scanning were performed according to the manufacture's instructions (Affymetrix, Sata Clara, CA). In brief, total RNA was reverse transcribed using a T7-oligo (dT) promoter primer in the first stand synthesis reaction. Following RNase H-mediated second-strand cDNA synthesis, the double stand cDNA was purified and served as a template in the subsequent *in vitro* transcription (IVT) reaction. The IVT reaction was carried out in the presence of T7 RNA polymerase and a biotinylated nucleotide analog/ribonucleotide mix for complementary RNA (cRNA) amplification and biotin labeling. The biotinylated cRNA targets were then cleaned up, fragmented, and hybridized to GeneChip expression array.

3.1 Target labeling

One-cycle target labeling and control reagents, Affymetrix, P/N 900493 were used in this experiment.

3.1.1 Preparation of poly-A RNA controls for one-cycle cDNA synthesis (Spike-in Control): Eukaryotic GeneChip probe array contains probe sets for several *B. subtilis* genes that are absent in eukaryotic samples (*lys, phe, thr,* and *dap*). These poly-A RNA controls are *in vitro* synthesized, and the polyadenylated transcripts for these *B. subtilis* genes are pre-mixed at staggered dilutions.

The concentrated poly-A control stock was diluted with the polyA control dilution buffer and spiked directly into the RNA samples to achieve the final dilutions (referred to as a ratio of copy number) summarized in table 1. The controls were amplified and labeled together with the samples. Examining the hybridization intensities of these controls on GeneChip arrays helps to monitor the labeling process independently from the quality of the starting RNA samples.

3.1.2 First-stand cDNA synthesis

2 µg of total RNA sample, 2µl of appropriately diluted poly-A RNA controls and 2 µl of 50 µM T7-Oligo (dT) primer were mixed with RNase-free water to a final volume of 11 µl. The reaction was incubated at 50ºC for 10 minutes and then cooled at 4º for at least 2 minutes. The reaction tube was centrifuged briefly to collect the sample at the bottom and added with first-strand mixture containing 4 µl of 5X first-strand reaction mix, 2 µl of 0.1M DTT and 1 µl of 10 mM dNTP. The reaction was incubated at 50º C for 2 minute and added 2 µl SuperScript III reverse transcriptase and continued incubation at 50ºC for 1 hour.

Table 1 Final concentration of poly-A RNA control.

Poly-A RNA spike	Final concentration (ratio of copy number)
Lys	1: 100,000
Phe	1:50,000
Thr	1:25,000
Dap	1: 6,667

3.1.3 Second-strand cDNA synthesis

The second-strand reaction mix was prepared; 30 µl of 5X second-strand reaction mix, 3 µl of 10 mM dNTP, 1 µl *E.coli* DNA ligase, 4 µl *E.coli* DNA polymerase I, 1µl of RNaseH and RNase-free water to a final volume of 130 µl, and transferred into first-strand cDNA sample tube in step 3.1.2. The reaction was incubated at 16 ºC for 2 minutes and then added with 2 µl of T4 DNA polymerase, continued incubation at 16 ºC for 5 minutes. After incubation, 10 µl of 0.5M EDTA was added in the reaction to stop the activity of T4 DNA polymerase enzyme.

3.1.4 Synthesis of Biotin-labeled cRNA

GeneChip IVT labeling kit (MEGAscript T7 kit, Ambion, Inc) was used for generating biotin-labeled cRNA. 12 µl of second-strand cDNA from step 3.1.3, 4 µl of 10X IVT labeling buffer, 12 µl of IVT labeling NTP mix, 4 µl of IVT labeling enzyme mix and RNase-free water to a final volume of 40 µl were carefully mixed together and incubated at 37 ºC for 18 hours.

3.1.5 Fragmenting cRNA

Fragmentation of cRNA target before hybridization onto GeneChip probe array are recommended by Affymetrix. This technique has been shown to be critical in obtaining optimal assay sensitivity. The fragmentation buffer from Affymetrix has been optimized to break down full-length cRNA to 200 base fragmented by metal-induced hydrolysis. 20 µg of cRNA, 8 µl of 5X fragmentation buffer and RNase-free water to a final volume of 40 µl were mixed and incubated at 94 ºC for 35 minutes and then put on ice for 5 minutes. RNA fragment size was determined by Bioanalyzer (should be 35 to 200 bases).

3.2 Target hybridization

Hybridization cocktail was compost of 15 µg of fragment cRNA, 5µl of control oligonucleotide B2 (3 nM), 15 µl of 20X Eukaryotic Hybridization Controls (*bioB*, *bioC*, *bioD*, *cre*), 150 µl of 2X hybridization mix, 30 µl of DMSO, and nuclease-free water to final volume of 300 µl.

GeneChip probe array was equilibrated at room temperature and then wet the array with an appropriate volume of pre-hybridization mix by filling it through one of the septa and incubated at 45°C for 10 minutes with rotation.

The hybridization cocktail was heated at 99 °C for 5 minutes, transferred to 45°C for 5 minutes and then centrifuged at 13,000×g for 5 minutes to remove insoluble material from the hybridization mixture.

The pre-hybridization buffer was removed from array with micropipettor and then refilled with appropriate volume of the clarified hybridization cocktail, avoiding any insoluble matter at the bottom of the tube, and incubated at 45°C for 16 hours.

3.3 Washing, Staining

GeneChip Hybridization kit, washing and staining kit: Affymetrix P/N 900720 was used in this step. Washing and staining were worked on the Fluidics station 450 (FS450). After 16 hours of hybridization, hybridization cocktail was removed from array and refilled with the appropriate volume (~250 µl) of Wash buffer A.

Wash buffer A, wash buffer B, stain cocktail 1 and 2 contained streptavidin phycoerythrin (SAPE) and array holding buffer were prepared and put on FS450 system. The GeneChip array was inserted into the fluidics station modules and run with appropriate protocol (Table 2). When it had finished, the GeneChip probe array was removed from the FS450 station.

3.4 Scanning

GeneChip® Scanner 3000 was used in this experiment. The scanner is controlled by Affymetrix® Microarray Suite 5. The probe array was scanned after the wash protocol was complete. The laser was warmed up prior to scanning by turning it on at least 15 minutes and inserted the array(s) into the scanner and tested the autofocus to ensure that the Tough-Spots did not interfere with the focus and run the scanner.

Table 2 FS450 protocol.

Step	Program cycle
Post Hyb Wash #1	10 cycles of 2 mixes/cycle with Wash Buffer A at 25°C
Post Hyb Wash #2	4 cycles of 15 mixes/cycle with Wash Buffer B at 50°C
1st Stain	Stain the probe array for 10 minutes with Stain Cocktail 1 at 25°C
Post Stain Wash	10 cycles of 4 mixes/cycle with Wash Buffer A at 25°C
2nd Stain	Stain the probe array for 10 minutes with Stain Cocktail 2 at 25°C
3rd Stain	Stain the probe array for 10 minutes with Stain Cocktail 3 at 25°C
Final Wash	15 cycles of 4 mixes/cycle with Wash Buffer A at 30°C. The holding temperature is 25°C

3.5 Data analysis

Each GeneChip array was scanned and data image was interpreted by Affymetrix® Microarray Suite 5.0 (MAS5) software with scaling factor of 500, a normalization value of 1, and default parameter for the rice genome array.The Affymetrix MAS5 report provided statistics for each chip that can be used in quality control purposes.

MAS5 "absolute" analysis was performed. For each probe set, absolute analysis generated signal value (expression level), a detection call of absent, present, or marginal, and a p-value associated with detection call. A text file of all data was generated and exported to Microsoft Excel

The "detection filter method" (McClintick 2003; McClintick and Edenberg, 2006) was used to cut off probe sets. Probe sets were considered to further analysis only if, in at least one of the treatment groups, probe set was called "present" in at least two of the three replicates.

The mean, standard deviation and coefficient of variation of the signal for each probe set within each treatment group (SD and LD treatments) were calculated. The ratio and $\log_2$ ratio (fold change) of the mean signals for SD versus LD treatment groups were calculated and welch's t-test was used to evaluate the statistical significance of the difference of the expression data values used for comparisons (McClintick, 2003).

Expression changes between SD and LD groups were assessed as significant if the probe set passed the "detection call filter" and the p-value was less than 0.05 for the t-test using either the original sinal or log transformed signal. This probe set was designated as differentially expressed gene.

4. Gene annotation

The transcript sequence of differentially expressed probe sets were downloaded from NCBI (http://www.ncbi.nlm.nih.gov) and TIGR rice genome project (http://www.-tigr.org).The transcript sequences were then searched using BLASTN, BLASTX and BLASTP against the TIGR rice gene annotation and *Arabidopsis* genome annotation (http://www.tigr.org/tdb/2k1/osa1)

5. Functional classifications

Differentially expressed genes were classified according to positive function. The MIPS MATDB database (http://mips.gsf.de/proj/thal/db/index.html), TIGR rice and TAIR (http://www.arabidopsis.org) web database were consulted. All classifications are based on information available at the time of analysis, and may have been revised and updated. These following criteria were used in this experiment:

Unclassified: no putative function or similarity to other genes was determined.

Transcription factor: genes encoding putative DNA-binding proteins – such as transcription factors, DNA repair proteins, and mRNA-processing proteins – but not including genes encoding proteins likely involved with cell cycle/DNA synthesis.

Transport/ion homeostasis: genes encoding transporters (ion, sugar, amino acid, etc.) and ion-binding proteins, excluding those genes encoding proteins likely involved in energy-related transport and signaling.

Metabolism: includes genes encoding proteins that are putatively involved in metabolic processes such as biosynthesis and other enzymatic reactions but not including energy-related enzymes or structure-related metabolites.

Translation: includes genes encoding proteins associated with ribosomal complexes and other key enzymes involved in protein synthesis.

Signaling: genes encoding those proteins involved in signal transduction and perception such as kinases, phosphatases, and calcium-binding proteins.

Energy transduction: includes genes encoding proteins involved in energy transduction within the plant such as those associated with photosynthesis, respiration, and general redox reactions. Also includes genes encoded by either the chloroplast or mitochondrial genomes.

Processing: genes encoding proteins involved in the modification, transport, and/or degradation of proteins; for example; vesicular trafficking proteins, chaperones, and proteases.

Growth/structure: genes encoding proteins with the primary function of regulating and promoting cell division and genes encoding proteins that make up the cell structure, including cytoskeletal elements and cell wall components.

Defense: includes genes encoding proteins most closely associated with the plant response to disease.

6. Pathway analysis

For the biological pathway analysis, differentially expressed genes were analyzed to associate biochemical pathway(s) based on their putative functions using the Omics Viewer (http://pathway.gramene.org/expression.html) and KEGG Pathway (http://www-.genome.jp /kegg/pathway.html)

7. Promoter analysis

The goal of promoter analysis is to investigate the regulatory mechanism of the transcriptional control of photoperiod responsive genes. Differentially expressed genes supported by available full-length cDNA sequence were used to analyze and their orthologous genes in *Arabidopsis* were identified using rice nucleotide sequences searched

against *Arabidopsis* genes in TAIR database (http://www.arabidopsis.org). Based on the gene location from Rice TIGR or TAIR database, 1.5-kb sequence upstream of the 5′ UTR was extracted as a putative promoter region. The data sets consisted of the promoter sequences of rice from induced or suppressed genes under SD light, which were named SD and LD promoter, respectively, as well as those of SD and LD orthologous genes in *Arabidopsis*.

7.1 Search for known *cis*-regulatory elements

The putative promoter sequences were searched against known *cis*-regulatory elements in the collection of PlantCARE database using Search for Care progam (http://bioinformaticssoftware.psb.ugent.be/webtools/ plantcare/html). The *cis*-regulatory elements were listed and the occurrence number on each promoter was recorded. The percentage of these specific elements relative to the total number in each data set was calculated.

7.2 Analysis of over-representative motif from co-expressed genes

Each promoter data set of co-expressed genes was used to detect the over-representative sequence using Oligo-analysis program. The consensus sequence was converted to logo picture by Gibbs program. The over-representative sequences were analyzed for positional bias using Position-analysis program. These programs are available in the Regulatory Sequence Analysis Tool (RSAT) (http://rsat.scmbb.ulb.ac.be/rs.at/).
The over-represented motifs were searched against with known plant motifs in both the PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) and PLACE databases (http://www.dna. affrc.go.jp/PLACE/fasta.html).

8. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was used to verify the reliability of the DNA microarray expression data and further study gene expression patterns using Superscript III™ One Step RT- PCR with Platinum® Taq (Invitrogen™ Life Technology, USA) with gene-specific primers designed by Primer 5.0 . RT-PCR results were inspected on 1.5% agarose gel eletrophoresis system.

9. Quantitative RT-PCR (qRT-PCR)

qRT-PCR was used to verify the reliability of the DNA microarray expression data and further study some interesting genes expression pattern. SuperScript™ III platinum® SYB green one-Step qRT-PCR and gene specific primers were used to perform with a Mastercycler® ep *Realplex* according to the manufacture's instruction (Eppendorf AG, Hamburg). The relative quantification method ($\Delta\Delta C_T$ method) was used to evaluate the expression level using *Actin1* as a housekeeping gene for normalization. .

10. Detection and quantification of microRNA (miRNA)

miRNA172a was examined in this experiment. NCode™ miRNA first-strand cDNA synthesis and qRT-PCR kits were used to detect and quantify miRNA expression according to the instruction manual (Invitrogen™ Life Technology, USA).

Unlike mRNAs, miRNAs are not polyadenylated. Before the reverse transcription step, miRNAs are polyadenylated by poly(A) polymerase. Reverse transcriptase converts RNA including miRNA, other small noncoding RNA to cDNA using oligo-dT primer. The oligo-dT primer has a universal tag sequence on the 5′ end. This universal tag allows amplification in the real-time PCR step.

10.1 Poly(A) tailing of miRNA

1 µg of total RNA was polyadenylated. The reaction contained 1X miRNA buffer, 2.5 mM $MnCl_2$, 1 mM ATP and 0.5 µl of poly A polymerase in a total volume of 25 µl and incubated at 37º C for 15 minutes. After incubation, proceed immediately to first-strand cDNA synthesis.

10.2 First-strand cDNA synthesis

4 µl of Polyadenylated RNA (from step 10.1), 1 µl of annealing buffer and 3 µl of 25 mM universal RT primer were mixed well and then incubated at 65 º C for 5 minutes and placed on ice for 1 minute. The reaction were added with 10 µl of 2X First-strand reaction mix and 2 µl of SuperScript™ III RT/RNaseOUT™ enzyme mix and incubated at 50 ºC for 30 minutes and 85 ºC for 5 minutes to stop the reaction.

10.3 Quantitative RT-PCR

Quantification of specific miRNA was performed using platinum® SYBR green qPCR supermix with specific miRNA primer and universal qPCR primer. The reaction was run on Mastercycler® ep *Realplex* at 50º C for 2 minutes, 95º for 2 minutes and 40 cycles of : 95 ºC for 15 seconds, 60 ºC for 30 seconds. The relative quantification method ($\Delta\Delta C_T$ method) was used to evaluate the expression level using 18S rRNA as a housekeeping gene for normalization.

RESULTS

1. Data set filtering and analysis

KDML 105, a photoperiod sensitive rice, enable to flower only when their tilling stage, competence to floral induction, are exposed to SD light for at least 8-9 days which is sufficient to initiate panicle formation. Therefore, to investigate photoperiod regulated genes during floral induction phase, aerial part of plant samples were collected at 3-10 days after photoperiod treatment to detect differential gene expression between SD and LD light using Affymetrix rice genome arrays.

After whole-chip data processing by MAS5, generated signal values and detection calls associated with detection call p-value were reported and exported to Microsoft Excel for further analysis. Signal mean and signal mean of $\log_2$ value of each probe set from three replicates were calculated both in control (LD) and experiment (SD) as shown in supplement data (available online).

For each probe set on each array, MAS5 provided a detection call as Absent (A), Present (P) or Marginal (M), which indicated whether the specific mRNA was detectable. For filter probe sets, the detection call filter method was used to cut off probe sets. A total of "Present call" in all triplicates of control or experiment were counted and a maximum "present call" of each probe set in one treatment was calculated. The total number of probe sets with "Absent" or "Present" are shown in table 3.

Table 3 Number of probe sets called present or absent.

Count of probe sets	Control	Experiment	Maximum
All absent	28291	28858	26373
Present ≥ 1	28267	27750	30056
Present ≥ 2	24872	24542	25749
All present	22878	22639	23596

The probe sets meeting cut off maximum present at least 2 arrays in either SD or LD groups were selected. The results showed that 25,749 probe sets were passed the criteria indicating detectable transcripts. A global representation of the changes in the expression of all selected probe sets is depicted in figure 1.

2. Differentially expressed genes

Statistically significant differentially expressed genes were identified from probe sets which passed detection call filter and expression ratio at least two fold changes with t-test p-value ≤ 0.05. A total of 79 probe sets were up-regulated while 105 probe sets were down regulated under SD condition and these probe sets were assigned as differentially expressed genes. These genes were considered as photoperiod responsive genes and were further analyzed using public access databases. Thus, transcript accumulation was altered for at least 184 genes of rice in response to photoperiod treatment; 79 genes were induced and 105 genes were repressed.

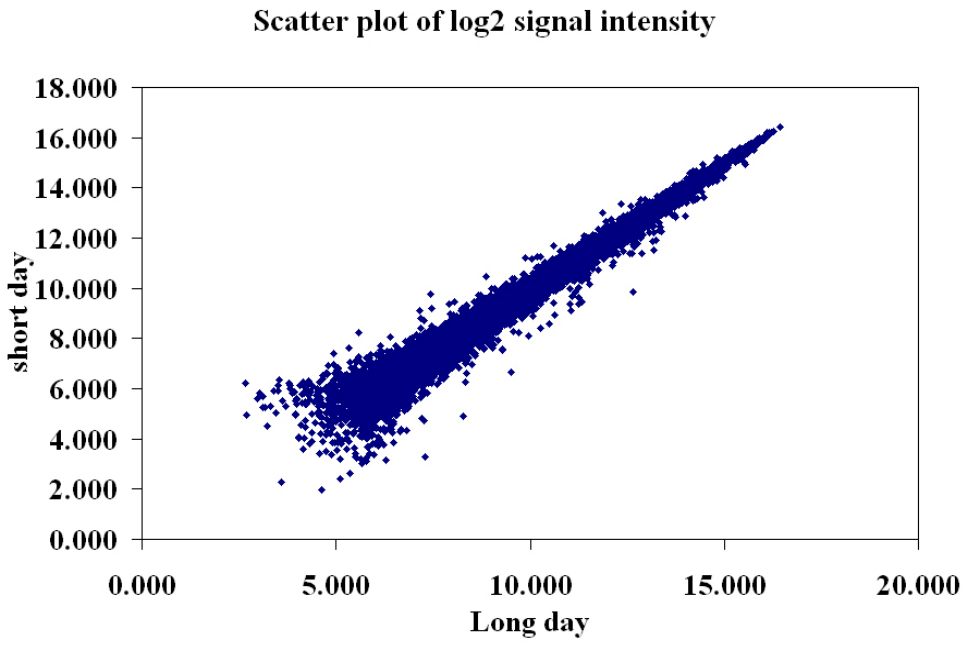


Figure 1 A representative of scatter plot of signal intensities for all selected probe set.

3. Functional classification

To gain the overall view of the photoperiod response, differentially up- and down-regulated genes were functionally categorized based on the most likely role of the predicted gene products into 9 classes (Table 4 and 5, Figure 2). As expected, a major class of both up- and down-regulated genes was unknown function (24 % and 25 % respectively). Among the known function of up-regulated genes, the largest class was transcription factor (18%) followed by defense/stress-related (15%), metabolism (11%), processing equal to growth and structure (8%), transport (6%), signaling (3%) and energy (1%).

Among the down-regulated genes, the largest class was again transcription factor (21%), followed by signaling (11%), defense/stress (10%), metabolism equaling to processing (9%), energy transduction (8%), growth/structure (3%) and processing (3%) as shown in figure 2.

4. Overview of global expression

4.1 Transcription factor

SD light altered expression of a large number of transcription factors. Among them of increase expression are members of basic helix loop helix (bHLH) (*9630.m05489*, *AK058561*), MADS-box (*L34271*, *AK069331, AY332478*), WRKY *(AY31842*) and MYB families. There were three genes encoding RNA metabolism protein, i.e., two RNA binding protein (*9639.m03552*, *AK067428*), and argonaute-like1 (*AGO1*; *AK069685*) (Table 4).

Transcription factors which decreased expression are members of AP2 (*AK062882*, *AY345234*, *9636.m03709*, *AY345233)*, Zinc finger (*NM_197656*, *AK108997*, *AY579411*), WRKY (*AK106282, AK058733*, *9630.m00802*), bHLH (*AB040744, AK071734* and *AK121198*), NAC (*AT1G01720*, *AK071274*) and MYB families. There are two genes encoding RNA metabolism protein (*AK070782*, *9631.m00675*) (Table 5).

Three of the up-regulated genes are involved in floral transition and development, i.e., *AK069331* encoding protein similar to MADS box transcription factor14, *AY332478* encoding MADS-box protein14 and *L34271* encoding OsMADS1, *Oryza* transcription factor controlling flowering time. Moreover, the expression of *Hd3a*, a key

Table 4 Differentially up-regulated genes under SD light in KDML 105.

Functional classification	Representative ID	Description	Organism	Fold change	t-test P-value
Unclassification	9639.m03143	hypothetical protein	Rice	2.05	0.03
	AK101374	Hypothetical protein	Rice	2.06	0.00
	CF318727	Retrotransposon protein	Rice	2.08	0.02
	NM_190971	Expressed	Rice	2.08	0.01
	NM_192881	leucine-rich repeat family protein	*Arabidopsis*	2.16	0.05
	AK059202	Expressed protein	Rice	2.17	0.05
	9629.m05429	Uncharacterized protein family	Rice	2.34	0.02
	9631.m00677	Retrotransposon protein, putative, unclassified,putative reverse transcriptase	rice, *Arabidopsis*	2.35	0.05
	9640.m00101	Hypothetical protein	rice, *Arabidopsis*	2.36	0.00
	NM_195063.1	Retrotransposon protein, putative	rice, *Arabidopsis*	2.39	0.01
	AK099709	unknown protein	rice, *Arabidopsis*	2.46	0.02
	AK073678	Retrotransposon,reverse transcriptase	rice, *Arabidopsis*	2.56	0.05
	9636.m03485	Hypothetical protein	rice, *Arabidopsis*	2.64	0.02
	9639.m00375	Retrotransposon protein, reverse transcriptase	Rice	2.68	0.01
	9638.m02863	Hypothetical	rice, *Arabidopsis*	2.71	0.03
	AK063190	Expressed protein	Rice	2.75	0.03
	AK063193	Hypothetical protein	Rice	2.76	0.02
	AK108236	leucine-rich repeat	Rice	2.80	0.01
	9631.m02338	Expressed protein	rice, *Arabidopsis*	3.07	0.00
Transcription	NM_190298	predicted mRNA,indole-3-acetate beta-glucosyltransferase,	Rice	3.40	0.01
	9630.m05489	Helix-loop-helix DNA-binding domain containing protein	rice, *Arabidopsis*	2.01	0.03
	AK112056	Myb-related protein Myb4, Member of the R2R3 factor gene /ATMYB9	rice, *Arabidopsis*	2.01	0.02
	AK067428	RNA-binding region-containing protein 1,putative, expressed	rice, *Arabidopsis*	2.05	0.01
	AK069685	Argonaute-like protein	rice, *Arabidopsis*	2.06	0.03
	AK058561	Helix-loop-helix DNA-binding domain containing protein	Rice	2.20	0.02
	9639.m03552	RNA-binding protein,UBP1 interacting protein 2a (UBA2a)	rice, *Arabidopsis*	2.30	0.05

Table 4 (Continued).

Functional classification	Representative ID	Description	Organism	Fold change	t-test P-value
Transcription factor	L34271	MADS1,Oryza sativa box protein (MADS1),transcription factor controlling flowering time	rice, *Arabidopsis*	2.54	0.05
	AY341842	OsWRKY17 - Superfamily of rice TFs having WRKY and zinc finger domains	rice, *Arabidopsis*	2.55	0.00
	NM_185579	WRKY family transcription factor,ATWRKY10, similar to SPF1	rice, *Arabidopsis*	2.59	0.03
	AK069331	Floral homeotic protein,MADS-box transcription factor 14	rice, *Arabidopsis*	2.65	0.00
	CR289442	Endonuclease V, putative, expressed	rice	2.65	0.05
	AY332478	MADS-box transcription factor 14, MADS box protein (AGL10) mRNA	rice, *Arabidopsis*	3.32	0.00
	AY332478	MADS box protein (AGL10)	rice, *Arabidopsis*	4.53	0.01
Transporter	AK108963	ALMT1, putative, expressed,malate transporter	rice, *Arabidopsis*	2.44	0.00
	9636.m04060	Heavy metal-associated domain containing protein,copper-binding family protein	rice, *Arabidopsis*	2.51	0.02
	AK102086	putativeplant lipid transfer	rice, *Arabidopsis*	4.08	0.01
	AK101194	ABC transporte,rMDR-like ABC transporter /multidrug resistance	rice, *Arabidopsis*	5.58	0.01
	AK062381	RCc3 protein// Plant lipid transfer/seed storage	rice, *Arabidopsis*	5.97	0.01
Metabolism	AK119922	3-ketoacyl-CoA synthase, putative, expressed	rice, *Arabidopsis*	2.05	0.03
	9629.m01582	UTP-glucose-1-phosphate uridylyltransferase family protein	rice, *Arabidopsis*	2.09	0.03
	AK062595	Carboxy-lyase, putative, expressed	Rice	2.16	0.03
	AK070289	von Willebrand factor type A domain	Rice	2.23	0.02
	AK070289	von Willebrand factor type A domain containing protein, expressed	Rice	2.23	0.01
	9629.m03761	hypothetical protein	Rice	2.23	0.00
	AK106630	Cone SR1 sucrose-regulated	Rice	2.65	0.04
	9630.m01390	1,3-beta-glucan synthase component family protein	rice, *Arabidopsis*	2.88	0.02
	AB052943	Hd3a	rice, *Arabidopsis*	3.95	0.03
Signaling	AK067894	NPGR2, putative, expressed,encodes a calmodulin-binding protein		2.19	0.05
	AK068816	SHR5-receptor-like kinase	rice, *Arabidopsis*	2.81	0.04
	9637.m00612	Casein kinase II alpha chain 2	*Arabidopsis*	2.03	0.05
	9636.m04555	Calreticulin-like protein,calreticulin 2 (CRT2)	*Arabidopsis*	3.65	0.01
	CF297681	similar to PFT1 (PHYTOCHROME AND FLOWERING TIME 1)	*Arabidopsis*	5.51	0.05
	9636.m01883	Calcineurin-like phosphoeste...	*Arabidopsis*	2.06	0.05

Table 4 (Continued).

Functional classification	Representative ID	Description	Organism	Fold change	t-test P-value
Energy/ Transduction	BQ907184	Photosystem II 10 kDa polypeptide, chloroplast precursor, putative, expressed	rice, *Arabidopsis*	2.08	0.04
Processing	AK100128	PA domain containing protein, subtilase family protein	rice, *Arabidopsis*	2.02	0.05
	AK065631	protein binding protein, zinc finger (C3HC4-type RING finger) family protein	rice, *Arabidopsis*	2.14	0.01
	AK107044	F-box protein interaction domain	rice, *Arabidopsis*	2.76	0.02
	9630.m05386	F-box domain containing protein,SLEEPY 1 protein	rice, *Arabidopsis*	3.13	0.05
	NM_186259	F-box domain containing protein, expressed,hypothetical protein	rice	3.88	0.05
	9640.m02992	F-box domain containing protein	rice	4.26	0.03
	9629.m06682	circadian clock coupling factor, putative,//EDL3, EID1-LIKE 3,circadian clock coupling factor ZGT like protein (F-box protein)	*Arabidopsis*	2.72	0.03
Growth/ Structure	U77297	Transmembrane protein,PLASMA MEMBRANE INTRINSIC PROTEIN 1	rice, *Arabidopsis*	2.01	0.02
	9634.m04977	storage protein-like	rice	2.02	0.05
	BI798695	prolamin, putative, expressed	rice	2.66	0.02
	9633.m02656	fiber protein Fb2, putative	rice	2.77	0.01
	U30479	Expansin	rice, *Arabidopsis*	6.19	0.01
	CA761467	Cytoplasmic membrane-bound vesicle	rice,	2.13	0.02
Stress/defense	9635.m00956	disease resistance protein RPM1 homolog	Rice	2.04	0.02
	NM_184482	Verticillium wilt disease resistance protein, disease resistance family protein	rice, *Arabidopsis*	2.11	0.02
	AK067524	Mla1, putative, expressed	rice	2.12	0.05
	9631.m04608	disease resistance protein RPM1 (CC-NBS-LRR class)	rice, *Arabidopsis*	2.20	0.03
	NM_190345	Hypothetical protein	rice	2.32	0.01
	AK069503	Peroxidase 47 precursor	rice	2.53	0.00
	AY256682	Putative brown planthopper-inducible	rice	2.72	0.05
	AB017914	L-zip+NBS+LRR	rice, *Arabidopsis*	2.99	0.01
	AK065474	WD40-like Beta Propeller ,TolB protein-related	rice, *Arabidopsis*	2.99	0.02
	AK101991	Jasmonate-induced protein,jacalin lectin family protein	rice, *Arabidopsis*	3.25	0.00
	AK107926	pathogenesis-related protein PRB1-2 precursor, putative, expressed	rice, *Arabidopsis*	3.87	0.05
	AK108159	ENOD18 protein / USP family protein	rice, *Arabidopsis*	2.32	0.00

Table 5 Differentially down-regulated genes under SD light in KDML 105.

Functional classification	Representative ID	Description	Organism	Fold change	t-test P-value
Unclassification	AK111335	Expresse protein	rice, *Arabidopsis*	-10.50	0.04
	AK063042	Expressed protein	rice	-9.59	0.04
	9629.m00079	hypothetical protein	rice	-4.14	0.05
	AK111059	Expressed protein	rice	-3.93	0.02
	AK072262	Expressed protein	rice	-3.64	0.04
	NM_191716	hypothetical protein	rice	-3.58	0.01
	9633.m03408	Ovate protein, putative	*Arabidopsis*	-3.41	0.03
	AT003735	Transcribe locus	rice	-3.17	0.04
	9630.m00919	hypothetical protein	rice	-2.80	0.04
	AK106968	Expressed protein	rice	-2.76	0.01
	9634.m01150	Expressed protein	*Arabidopsis*	-2.73	0.01
	9639.m01629	Expressed protein	rice	-2.47	0.01
	AK121627	Expressed protein	rice	-2.46	0.01
	9639.m01688	hypothetical protein	rice	-2.46	0.01
	AK110739	hypothetical protein	rice	-2.40	0.00
	AK119435	Cystatin-1 precursor, putative	rice, *Arabidopsis*	-2.31	0.01
	AK120328	Expressed protein	rice	-2.29	0.03
	9634.m02654	Expressed protein/	rice	-2.24	0.01
	9629.m03028	hypothetical protein	rice	-2.19	0.04
	AK099048	Expressed protein	rice	-2.19	0.05
	AK067400	Expressed protein	rice	-2.18	0.01
	AK059847	Expressed protein	rice	-2.10	0.05
	AK109030	Expressed protein	rice	-2.05	0.04
	9634.m03045	hypothetical protein	rice	-2.03	0.03
	9633.m03901	Expressed protein	rice	-2.01	0.05
	9638.m02289	hypothetical protein	rice	-2.01	0.01

Table 5 (Contined).

Functional classification	Representative ID	Description	Organism	Fold change	t-test P-value
Transcription	AK070782	XRN 5'-3' exonucleas	rice, *Arabidopsis*	-4.74	0.05
	AK106282	WRKY28 protein	Rice	-4.50	0.04
	9635.m02514	Similar to myb-related protein	rice, *Arabidopsis*	-3.28	0.01
	AB040744	Oryza sativa RERJ1 mRNA for Transcription Factor,PIF3	rice, *Arabidopsis*	-3.24	0.05
	AK111571	Myb-like DNA-binding domain	rice, *Arabidopsis*	-3.19	0.05
	AK058455	tRNA-intron endonuclease activity	Rice	-3.16	0.03
	AK062882	AP2 domain containing protein,the ERF (ethylene response factor) subfamily B-1 of ERF/AP2 transcription factor family (ATERF-9)	rice, *Arabidopsis*	-3.15	0.02
	AY345234	transcription factor RCBF2,dehydration and cold-relative ,DREB ts factor	*Arabidopsis*	-2.82	0.02
	9636.m03709	AP2 domain, putative	rice, *Arabidopsis*	-2.79	0.05
	AK108997	zinc finger (C2H2 type) family protein	rice, *Arabidopsis*	-2.63	0.02
	AY345233	Oryza sativa transcription factor RCBF3 mRNA	rice, *Arabidopsis*	-2.61	0.05
	AK058773	OsWRKY71 - Superfamily of rice TFs having WRKY and zinc finger domains, expressed	rice, *Arabidopsis*	-2.58	0.04
	AK068115	Harpin-induced protein 1 containing protein	rice, *Arabidopsis*	-2.48	0.05
	AY581256	ARR1 protein-like, putative, expressed or LUX	rice, *Arabidopsis*	-2.45	0.01
	AK107424	putative myb-related transcription factor	rice, *Arabidopsis*	-2.42	0.02
	AK107746	NAC23, putative, expressed,NAC domain-containing protein48, putative, (TIGR)	rice, *Arabidopsis*	-2.39	0.05
	AK071734	Helix-loop-helix DNA-binding domain containing protein, expressed	rice, *Arabidopsis*	-2.38	0.05
	AK121198	Helix-loop-helix DNA-binding domain containing protein	Rice	-2.23	0.01
	CR278953	hypothetical protein,RNA recognition motif familyprotein	Rice	-2.23	0.01
	AK071274	Putative NAC-domain protein	rice, *Arabidopsis*	-2.12	0.02
	AY579411	RING/C3HC4/PHD zinc finger-like protein mRNA, complete cds.	rice, *Arabidopsis*	-2.02	0.01
	9631.m00675	putative eribonuclease P, putative, expressed	rice, *Arabidopsis*	-2.01	0.05
Transporter	AK120542	heavy metal-associated domain containing protein	rice, *Arabidopsis*	-2.53	0.02
	AK067594	MATE efflux family protein	rice, *Arabidopsis*	-2.30	0.02
	AK067836	Sodium/dicarboxylate cotransporter, putative, expressed	rice, *Arabidopsis*	-2.14	0.02

Table 5 (Continued).

Functional classification	Representative ID	Description	Organism	Fold change	t-test P-value
Metabolism	AK061204	Sulfotransferase domain containing protein	rice, *Arabidopsis*	-4.60	0.04
	AK119315	5'-3' exonuclease, N-terminal resolvase-like,putative DNA polymerase I	rice, *Arabidopsis*	-3.86	0.03
	NM_197656.1	Zinc finger, C3HC4 type family protein,F-box domain containing protein,CK 2 site	rice, *Arabidopsis*	-3.50	0.02
	9634.m01544	Aldose 1-epimerase family protein//protein angel, putative	rice	-3.44	0.02
	AK062293	Cytochrome P450 family	rice, *Arabidopsis*	-2.85	0.05
	AK070485	Glutamate synthase	rice, *Arabidopsis*	-2.53	0.05
	AK069008	oxidoreductase, 2OG-Fe oxygenase family protein, leucoanthocyanidin dioxygenase,	rice, *Arabidopsis*	-2.15	0.04
	NM_184529	Calcineurin-like phosphoesterase family protein, putative, expressed	rice	-2.12	0.02
	CA755805	glyceraldehyde-3-phosphate dehydrogenase	rice, *Arabidopsis*	-2.03	0.04
Signaling	NM_184360	Auxin-independent growth promoter-like,	rice, *Arabidopsis*	-2.43	0.01
	NM_190760	Protein kinase domain containing protein	*Arabidopsis*	-2.34	0.01
	9635.m00169	NB-ARC domain containing protein	rice	-5.49	0.02
	AK058518	protein kinase,NPK1-related protein kinase-like	rice, *Arabidopsis*	-2.65	0.05
	9635.m03511	putative serine/threonine-specific protein kinase	rice, *Arabidopsis*	-2.48	0.05
	AK069654	protein kinase family	Rice	-2.45	0.05
	CB629347	putative receptor-like protein kinase (Arabidopsis thaliana)	rice, *Arabidopsis*	-2.42	0.01
	AK062671	EF hand family protein, expressed// calmodulin-like protein 41, putative, expressed	rice, *Arabidopsis*	-2.29	0.05
	9632.m04031	Protein phosphatase 2C containing protein, expressed	rice, *Arabidopsis*	-2.21	0.03
	AK108588	far-red impaired responsive protein,transposon protein	rice, *Arabidopsis*	-2.14	0.03
	9634.m04625	Protein kinase domain containing protein	rice, *Arabidopsis*	-2.11	0.05
	BP184627	far-red impared	rice, *Arabidopsis*	-2.05	0.05
Energy transduction	X15901	NADH dehydrogenase subunit 6, Chloroplast	rice, *Arabidopsis*	-2.18	0.03
	AB004865	AOX1b mRNA for alternative oxidase	rice, *Arabidopsis*	-2.75	0.05
	AK068266	Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit mRNA	rice, *Arabidopsis*	-2.63	0.00
	9630.m02292	NADH dehydrogenase ND2	rice, *Arabidopsis*	-2.41	0.01
	NM_195998	putative NADH dehydrogenase 49kDa protein	rice, *Arabidopsis*	-2.29	0.02
	NM_185254	Chloroplast NADH dehydrogenase ND4L	rice, *Arabidopsis*	-2.11	0.05
	AK110782	apocytochrome f precursor, putative	rice, *Arabidopsis*	-2.10	0.05

Table 5 (Continuted).

Functional classification	Representative ID	Description	Organism	Fold change	t-test P-value
Energy transduction	X15901	NADH dehydrogenase subunit 6, Chloroplast	rice, *Arabidopsis*	-2.08	0.03
Processing	AK111402	Eceriferum3 protein, putative	rice, *Arabidopsis*	-3.86	0.04
	AK110032	UNUSUAL FLORAL ORGANS protein, putative/F-box	*Arabidopsis*	-2.11	0.01
	AK109161	U-box domain containing protein, photoperiod responsive protein	rice, *Arabidopsis*	-2.15	0.01
	AK110825	Subtilisin N-terminal Region family protein	rice, *Arabidopsis*	-3.54	0.02
	AK072975	COP1 interacting protein	*Arabidopsis*	-2.97	0.05
	AK070773	Hydrolase, alpha/beta fold family protein,sigma factor sigB regulation protein rsbQ	rice, *Arabidopsis*	-2.11	0.03
	9631.m00907	Serine O-acetyltransferase, putative ,satase isoform II, putative, response to stress	rice, *Arabidopsis*	-2.03	0.03
	9629.m04985	Nucleosome assembly protein (NAP),	rice, *Arabidopsis*	-2.02	0.04
	AK108494.1	ubiquitin-protein ligase, putative, expressed	rice	-2.01	0.00
Growth/ Structure	9636.m00295	CLE40, putative, CLAVATA3/ESR-Related 40 (CLE40)	*Arabidopsis*	-2.40	0.03
	AK107930	Late embryogenesis abundant group 1 family protein,	rice, *Arabidopsis*	-2.41	0.05
	CA998377	Ovule development protein aintegumenta (ANT)-like, protein BABY BOOM 2	rice, *Arabidopsis*	-2.35	0.01
	AK063145	putative membrane-associated protein	rice, *Arabidopsis*	-2.12	0.05
	NM_184884	putative ankyrin, TPR Domain containing protein	rice, *Arabidopsis*	-2.33	0.02
Defense/stress	9632.m03516	Lipoxygenase 5	rice, *Arabidopsis*	-2.10	0.02
	AK064754	Endochitinase PR4 precursor, putative, expressed,putative class IV chitinase (CHIV)	rice, *Arabidopsis*	-2.28	0.03
	CB622792	Neoxanthin cleavage enzyme	rice, *Arabidopsis*	-3.30	0.02
	CA753125	Expressed protein/drought stress	rice, *Arabidopsis*	-2.95	0.01
	AK119780	Viviparous-14 or neoxanthin cleavage enzyme	rice, *Arabidopsis*	-2.83	0.05
	AK109382	NADP-dependent oxidoreductase P1	rice, *Arabidopsis*	-2.71	0.01
	AK104585	putative dehydration-responsive protein, BURP domain	rice, *Arabidopsis*	-2.67	0.04
	AK064627	Glutathione S-transferase, putative,expressed	rice, *Arabidopsis*	-2.03	0.04
	AK060639	pectinesterase inhibitor domain , putative stress-responsive protein	Rice	-2.48	0.02
	BI306222	BURP domain containing protein	rice, *Arabidopsis*	-2.19	0.05
	AK106094	BURP domain containing protein	Rice, *Arabidopsis*	-2.19	0.02

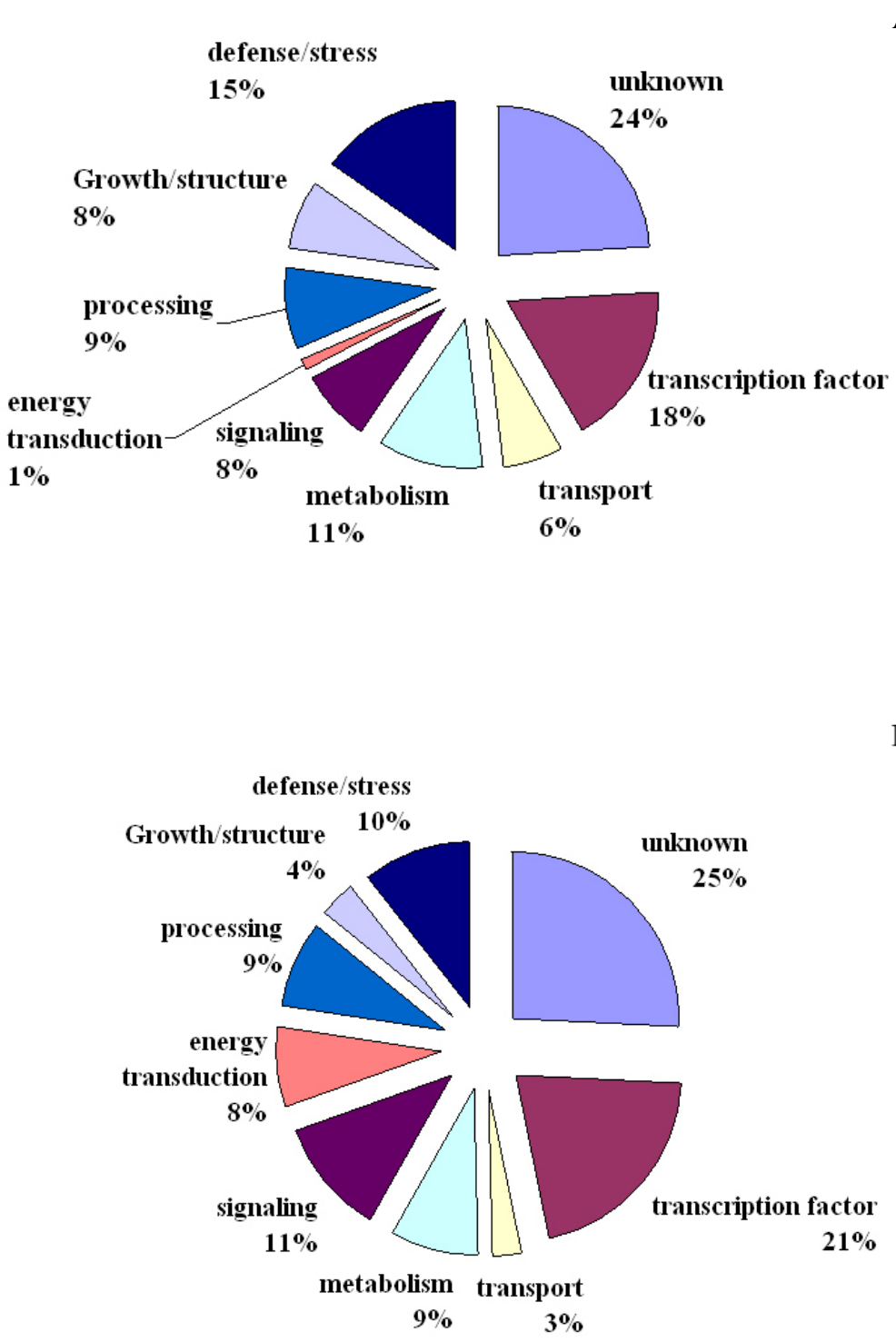


Figure 2 Functional distribution of differentially expressed genes: Functional distribution analysis of up-regulated genes (A), down-regulated genes (B).

gene of flowering time in rice, was increased in this condition. It is noted that *Hd3a* is not a transcription factor**.** In contrast, five of the down-regulated genes are invoved in floral repressors, i.e. four genes of *AP2* family (*AK062882, AY345234, 9636.m03709* and *AY345233*) and *XRN4* encoding XRN 5'-3' exonuclease in ethylene signaling.

In addition to those of flowering reported genes, *AGO1* which is a DICER in miRNA synthesis (Vaucheret *et al.* 2004) was found in up-regulated genes. miRNA has been reported to be involved in regulation of flowering time in *Arabidopsis*, *miRNA172* (Aukerman and Sasaki, 2003). In this experiment, the target genes of *miRNA172*, *AP2* family, were down-regulated. It suggested that the expression of *miRNA172* was probably up-regulated and involved in the regulation of flowering time in rice. Thus, its expression was further investigated.

Moreover, circadian rhythm related genes, i.e., *AB040744* encoding phytochrome interacting factor 3 (PIF3) and *AY581256* encoding ARR1 protein like or LUX protein were found among the down-regulated transcription factors.

4.2 Defense/stress-related

Expression of defense/stress-related genes was altered by SD light. Among the up-regulated genes, a large fraction of them are known pathogen-responsive genes or postulated to play a role in pathogen resistance, i.e. disease resistance protein RPM1 homolog (*9635.m00956),* Mla1 (*AK067524*), SHR5-receptor-like kinase (*AK068816)***,** L-zip+NBS+LRR (*AB017914*), WD40-like Beta Propeller (*AK065474*), jasmonate-induced protein (*AK101991*), pathogenesis-related protein PRB1-2 precursor (*AK107926*) and verticillium wilt disease resistance protein (*NM_184482*).

On the other hand, gene encoding dehydration responsive protein (*AK106094, AK104585*), Harpin-induced protein 1 (*AK068115*), protein containing pectin esterase inhibitor domain (*AK060639*), viviparous-14 or neoxanthin cleavage enzyme in abscisic acid biosynthesis (*CB622792, AK119780*) were down-regulated. All of these down-regulated genes have been reported as the genes response to abiotic stress such as cold and drought, (Zhou *et al*., 2007; Walia *et al*., 2005; Kreps *et al.,* 2002).

4.3 Metabolism

The metabolism-associated genes including gene involved in sugar metabolism, such as UTP-glucose-1-phosphate uridyltransferase (*9629.m01582*), Cone SR1sucrose-regulated(*AK106630*), 3, beta-glucan synthase (*9630.m1390*), and lipid metabolism such as 3-ketoacyl-CoA synthase (*AK119922*) were induced in SD light while genes involved in glutamate biosynthesis (*AK070485*), Calvin cycle (*CA755805*), flavonoid biosynthesis (*AK069008*) were repressed.

4.4 signal transduction

Gene encoding protein involved in signal transduction was also up- and down regulated in short day light. Gene encoding protein casein kinaseII α chain (*9637.m00612*), SHR-5 receptor-like kinase (*AK068816)*, NPGR2 (*AK067894*) Calreticulin like protein (*9636.m0455*) were up-regulated. Among the down-regulated signaling genes, some protein kinases (*AK05818, 9635.m03511, AK069654*, *9634.m04625*), EF hand family protein (*AK062671*) or calmodulin-like protein showed a significant decrease in expression. In addition to signal transduction genes, gene encoding protein involved in phytochrome signaling including far red impaired or *FAR1* (*AK108588, BP184627*), were down-regulated .

4.5 Energy transduction

The expression of the photosynthesis related-genes were changed by short day light. Gene encoding photosynthetic protein in light reaction, photosystem II chloroplast precursor (*BQ907184*) was up-regulated. while genes encoding photosynthetic protein in dark reaction, i.e., ribulose 1,5 bisphosphate carboxylase (*AK068266*), and other genes involved in energy transduction, i.e., NADH dehydrogenase (*X1590,9630.m02292*, *NM_195998* and *NM_18524*) and *AOX1b* for alternative oxidase were down-regulated. This result was further considered and discussed in the discussion part.

4.6 Processing

The expression of the translation associated-genes involved in protein degradation was altered in photoperiod response. Six genes encoding F-box domain containing proteins (*AK065631, AK107044, 9630.m05386, NM_186259, 9640.m-02992--,9629.m 06682*), one gene encoding subtilase family protein were up-regulated. Two of them (*9630.m05386*, *9629.m06682*) are involved in phytohormone signaling. *9630.m05386* encoding F-box protein homolog to SLEEPY protein in *Arabidopsis* is involved in gibberellin (GA) signaling (Dill *et al.,* 2004), while *9629.m06682* encoding circadian clock coupling factor, EDL3, is involved in circadian clock control (Dieterle *et.al*., 2001)

The expression of gene encoding unusual floral organ (UFO) protein, U-box containing protein, COP1 interacting protein and ubiquitin-protein ligase were down-regulated. One of them, COP1 interacting protein, is involved in phytochrome signaling. In addition, translation associated-genes involved in protein modification were also down-regulated in short day light, i.e., eceriferum3 protein, alpha/beta fold family protein and serine O-acetyl transferase.

4.7 Transport

Five genes encoding transporters were significantly up-regulated in short day light. These genes encode a heavy metal-associated binding protein similar to a copper homeostasis factor (*9636.m04060*), malate transporter *ALMT* (*AK108963*), ABC transporter (*AK101194*) and plant lipid transfer proteins (*AK102086*, *AK06238*). Two genes encoding MATE efflux family protein (*AK067594*) and sodium/dicarboxylate cotransporter were down-regulated. Although these transporters have not been reported their linking to photoperiod response but each of these protein may play an important role in the photoperiod signaling.

4.8 Growth and structure

Genes whose products are involved in growth and cell expansion such as fiber protein Fb2 (*9633.m02656*) and expansin (*U30479*) were up-regulated in short day light. Storage protein, i.e., storage protein like (*9634.m04977*) and prolamin (*B1798695*) were found in up-regulated genes. Gene whose products are involved in the transition of somatic

apical meristem (SAM), i.e., *CLAVATA3/ESR-related 40* (*CLE40*) (*9636m00295*), and ovule development protein aintegumenta (ANT)-like (*CA998377*) were found in down-regulated genes. These genes were further considered and discussed.

As mentioned above, those results showed that photoperiod respone in KDML 105 are involved in several biological processes, i.e., phytochrome signaling and circadian rhythm, hormone signaling (GA, ABA and ethylene), signal transduction, protein degradation stress/ defense processes.

5. Pathway analysis

To investigate the biochemical pathways influenced by short day light, up- and down-regulated genes were mapped to known pathways in the database using Omics Viewer (http://pathway.gramene.org/expression.html) and KEGG pathway (http://www.genome.jp/kegg/pathway.html). Four proteins of up-regulated genes were mapped on three pathways, i.e., CK2α subunit and expansin associated with circadian rhythm; subunit of PSII associated with light reaction of photosynthesis pathway; UTP-glucose-1-phosphate uridyltransferase associated with starch/sucrose metabolism. In down regulated genes, seven genes were mapped on 7 pathways, i.e., phytochrome interacting factor (PIF3) associated with circadian rhythm; glyceraldehyde-3-phosphate dehydrogenase associated with glycolysis/ gluconeogenesis; NADH dependent glutamate synthase associated with glutamate metabolism; chitinase class IV associated with aminosugar metabolism, lipoxygenase associated with alpha-linolinic acid metabolism; rubisco small subunit associated with carbon fixation in photosynthesis pathway and 9-cis-expoxycarotenoid dioxygenase associated with carotenoid biosynthesis.

Interestingly, two of these pathways contained both up- and down-regulated genes, i.e., circadian rhythm and photosynthesis which were considered and discussed in discussion part. In addition to these two pathways, the pathway related to flowering transition was also focused.

5.1 Circadian rhythm

One of the up-regulated genes (*CK2α*) and one of the down-regulated genes, *PIF3*, were mapped on the circadian rhythm pathway (Figure 3).The other protein involved

in the circadian rhythm, ARR1 or LUX, was not mapped on the pathway in this data (pathway database have not been updated). LUX is one of the proteins involved in core feed back loop (oscillator) of circadian rhythm.

The circadian rhythm pathway can be simply divided into three parts, light input, circadian clock oscillator and output pathway. Input of pathway includes phytochrome (phyA, phyB) photoreceptor perceived light signal and changed conformation into active form (Pfr). PIF3 interacts with both active phyA and phyB and recognize G-box in the promoter of *CCA 1* (circadian associated1) and *LHY* (late elongated hypocotyl) genes (MYB transcription factor) in the core oscillator to activate transcription in the morning. CCA1 and LHY inhibit evening expressed genes including TOC1 and LUX, through binding to an evening element of their promoters. Both TOC1 and LUX form feed back loop by activating transcription of CCA1 and LHY. They also require PIF3 to recognize G-box on the promoters of CCA1 and LHY. Output partway is to activate transcription of CCA1 target genes. Thus, the results in this experiment indicated that the regulation of photoperiod response in rice is involved in altered expression of circadian clock genes.

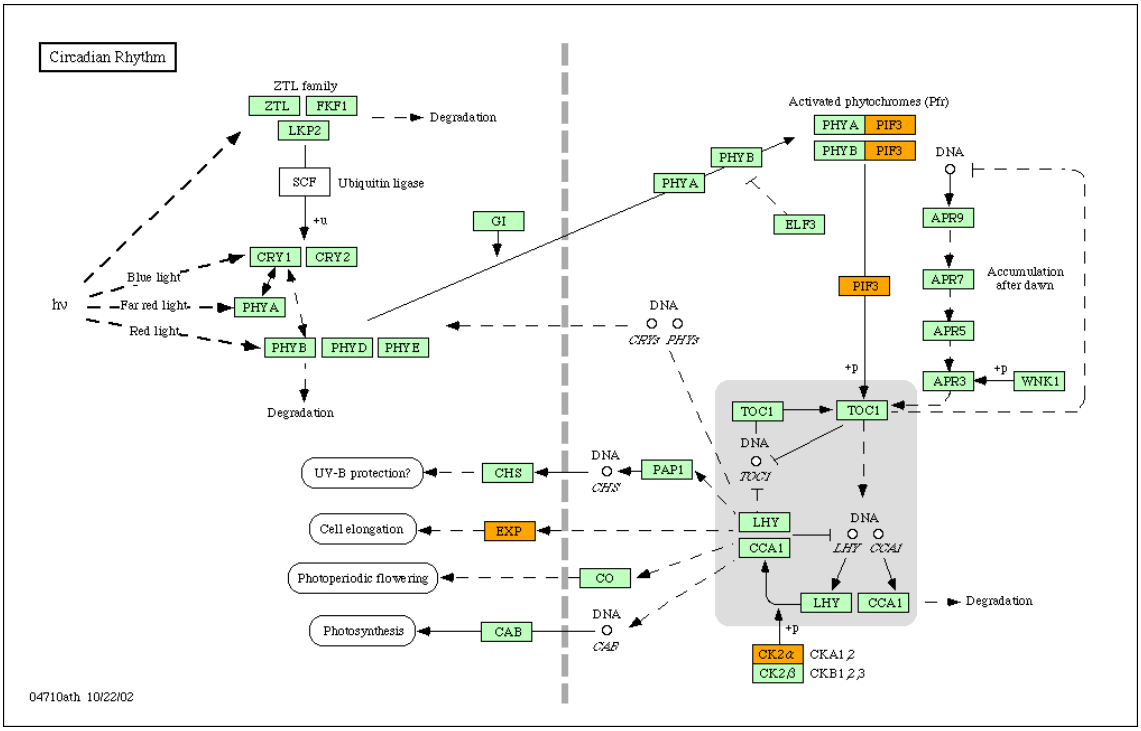


Figure 3 Circadian rhythm pathway. Orange color indicate differentially expressed genes mapped on the pathway.

5.2 Photosynthesis

From figure 4, *PsbR* (encoding Photosystem II or PS II) was mapped on the light reaction pathway. PS II are composted of several subunits encoded by *Psb* genes. Among the *psb* genes, *Psb*R gene was up-regulated in short day light. The transcripts levels of other genes that encode small subunits of PS II such as *PsbA, PsbB* etc. and PS I were not altered in this experiment. However, increase *PsbR* expression may be induced an activity of Photosystem II.

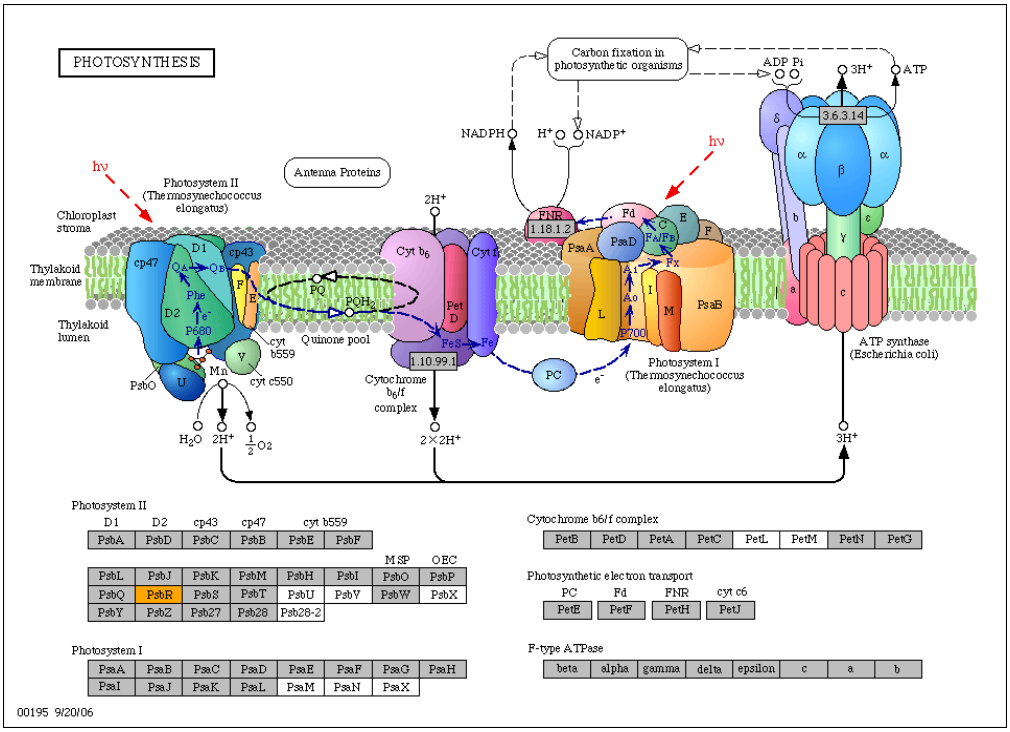


Figure 4 Light reaction of photosynthesis pathway; orange color indicates putative protein mapped on the pathway.

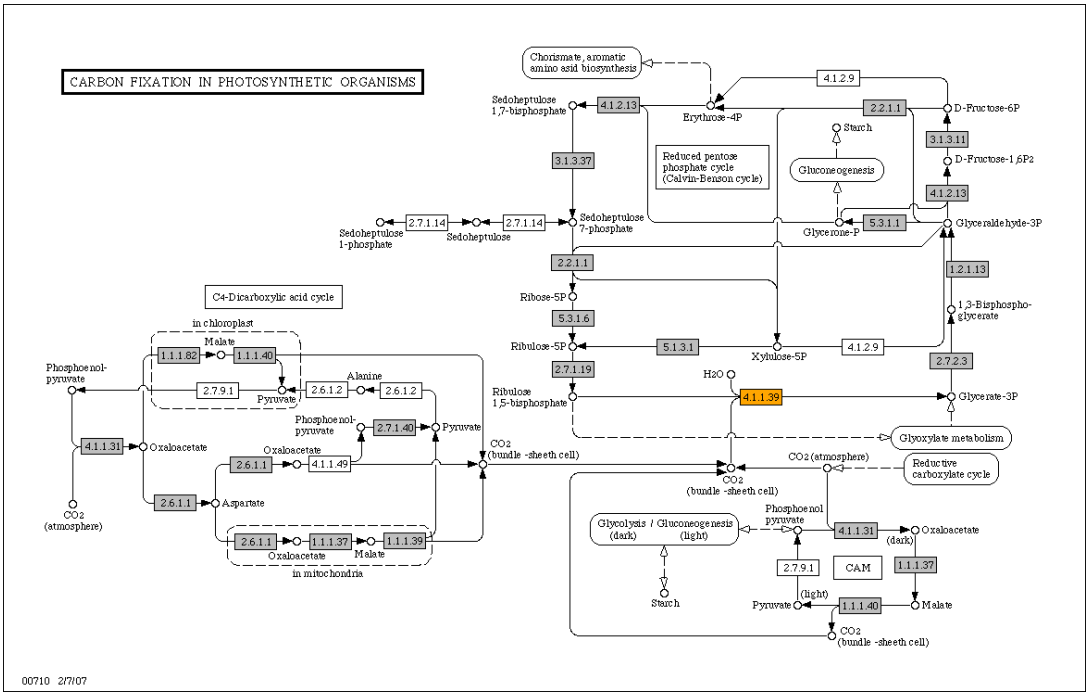


Figure 5 Carbon fixation in dark-reaction of photosynthesis pathway. Orange color indicates ribulose-1, 5-bisphosphate carboxylase enzyme.

From figure 5, gene encoding ribulose-1, 5-bisphosphate carboxylase was mapped on carbon fixation of dark reaction in photosynthesis pathway. This gene was down-regulation in SD light. Thus, this result suggested that carbon fixation pathway was suppressed under SD condition.

6. Common feature of photoperiod responsive promoters

To characterize the general feature of the promoter regions of photoperiod responsive genes, the 1.5-kb sequences upstream from the start site of the up- and down-regulated genes were used to search against known *cis*-regulatory elements in the PlantCARE database using Search for Care program. The specific elements presented in the promoter regions of coding strands were counted and listed in table 6. The data shows that these promoter regions contain several *cis*-regulatory elements, 839 in SD promoters and 1,230 in LD promoters. These elements were classified into three groups according to their responsive functions, i.e., hormone responsive element (HRE), light responsive element (LRE) and stress responsive element (SRE). The HREs are composed of five subgroups: abscisic acid (ABA) responsive elements, gibberellins (GA) responsive

elements, auxin responsive elements, jasmonic acid (JA) responsive elements and ethylene responsive elements as shown in table 6.

To compare the occurrence of elements in subset of genome, the percentage of individual element in each set of promoters was calculated and depicted in figure 6. The graph shows that light responsive elements (LREs) were enriched both in two sets of co-regulated genes. Among LREs themselves, G-box, Sp1 and Box-4 were predominantly found in both SD and LD promoters. Interstingly, G-box, a target site of phytochrome interacting factors (PIFs), which is required for phytochrome-regulated transcription in photoperiod response (Menkens *et al*., 1995) as well as stress and defense response (Aris *et al*., 1993) was the highest enrich one. In addition, circadian elements were found in both SD and LD promoters.

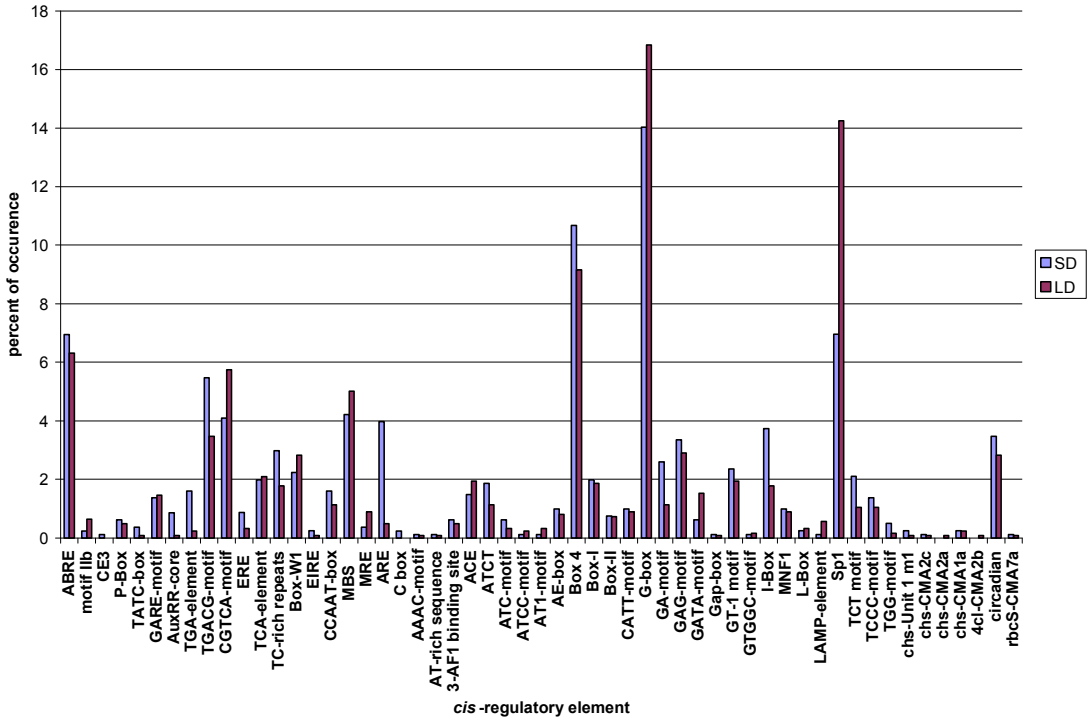


Figure 6 The distribution occurrence of *cis*-regulatory elements in the promoter of photoperiod responsive genes.

Table 6 The collection of *cis*-regulatory elements in the promoter of photoperiod responsive genes.

Type of motif	Sequence	Function	Total number of occurrence	
			SD	LD
Abscisic acid responsive element				
ABRE	TACGTG	cis-acting element involved in the abscisic acid responsiveness	56	78
motif IIb	CCGCCGCGCT	Abscisic acid responsive element	2	8
CE3		ABA and VP1 responsive	1	0
Gibberellins responsive element				
P-Box	CCTTTTG	gibberellin-responsive element	5	6
TATC-box	TATCCCA	Cis-acting element involved in gibberellin-responsiveness	3	1
GARE-motif	AAACAGA	gibberellin-responsive element	11	18
Auxin responsive element				
AuxRR-core	GGTCCAT	cis-acting regulatory element involved in auxin responsiveness	7	1
TGA-element	AACGAC	Auxin-responsive element	13	3
Jasmonic acid responsive element				
TGACG-motif	TGACG	cis-acting regulatory element involved in the MeJA-responsiveness	44	43
CGTCA-motif	CGTCA	cis-acting regulatory element involved in the MeJA-responsiveness	33	71
Ethylene responsive element				
ERE	ATTTCAAA	Ethylene-Responsive Element	7	4
TCA-element	CCATCTTTTT	cis-acting element involved in salicylic acid responsiveness	16	26
TC-rich repeats	ATTCTCTAAC	cis-acting element involved in defense and stress responsiveness	24	22
Box-W1	TTGACC	fungal elicitor responsive element	18	35
EIRE	TTCGACC	Elicitor-responsive element	2	1
Stress responsive element				
CCAAT-box	CAACGG	MYBHv1 binding site	13	14
MBS	TAACTG	MYB binding site involved in drought-inducibility	34	62
ARE	TGGTTT	Cis-acting regulatory element essential for the anaerobic induction	32	6
Light responsive element				
MRE	AACCTAA	MYB binding site involved in light responsiveness	3	11
C box	CTGACGTCAG	Cis-acting regulatory element involved in light responsiveness	0	3
AAAC-motif	CAATCAAAACCT	light responsive element	1	1
AT-rich sequence	ATTAATTTTACA	part of a light responsive module	1	1
3-AF1 binding site	TAAGAGAGGAA	light responsive element	5	6
ACE	CTAACGTATT	Cis-acting element involved in light responsiveness	12	24
ATCT	AATCTAATCT	part of a conserved DNA module involved in light responsiveness	15	14
ATC-motif	AGCTATCCA	part of a conserved DNA module involved in light responsiveness	5	4
ATCC-motif	CAATCCTC	part of a conserved DNA module involved in light responsiveness	1	3

Table 6 (Continue).

Type of motif	Sequence	Function	Total Number of occurrence	
			SD	LD
AT1-motif	AATTATTTTTTATT	part of a light responsive module	1	4
AE-box	AGAAACAT	part of a module for light response	8	10
Box 4	ATTAAT	part of a conserved DNA module involved in light responsiveness	86	113
Box-I	TTTCAAA	Light responsive element	16	23
Box-II	TGGTAATAA	part of a light responsive element	6	9
CATT-motif	GCATTC	part of a light responsive element	8	11
G-box	CACGTA	Cis-acting regulatory element involved in light responsiveness	113	208
GA-motif	AAAGATGA	part of a light responsive element	21	14
GAG-motif	AGAGATG	part of a light responsive element	27	36
GATA-motif	GATAGGA	part of a light responsive element	5	19
Gap-box	CAAATGAA(A/G)A	part of a light responsive element	1	1
GT-1 motif	GGTTAAT	Light responsive element	19	24
GTGGC-motif	GATTCTGTGGC	part of a light responsive element	1	2
I-Box	GATATGG	part of a light responsive element	30	22
MNF1	GTGCCC(A/T)(A/T)	Light responsive element	8	11
L-Box	TCTCACCAACC	part of a light responsive element	2	4
LAMP-element	CCAAAACCA	part of a light responsive element	1	7
Sp1	CC(G/A)CCC	Light responsive element	56	176
TCT motif	TCTTAC	part of a light responsive element	17	13
TCCC-motif	TCTCCCT	part of a light responsive element	11	13
TGG-motif	GGTTGCCA	part of a light responsive element	4	2
chs-Unit 1 m1	ACCTAACCTCC	part of a light responsive element	2	1
chs-CMA2c	ATATACGTGAAGG	part of a light responsive element	1	1
chs-CMA2a	TCACTTGA	part of a light responsive element	0	1
chs-CMA1a	TTACTTAA	part of a light responsive element	2	3
4cl-CMA2b	TCTCACCAACC	Light responsive element	0	1
rbcS-CMA7a	GGCGATAAGG	part of a light responsive element	1	1
Circadian	CAANNNNATC	cis-acting regulatory element involved in circadian control	28	35
		Total of regulatory elements	**839**	**1231**

7. Identification of coordinated motifs integrating hormones and stresses to photoperiod response

To further understand the coordinated regulation between photoperiod and hormones, the promoters of hormone signaling genes obtained from microarray data were analyzed. These genes were *SLEEPY 1* (*SLY 1*) in GA signaling with up-regulation, *XRN 4* in ethylene signaling and *Viviparous 14* (*Vip 14*) in ABA signaling with down-regulations. All *cis*-regulatory elements occurred on the promoter regions of these genes were mapped as shown in figure 7. Several LREs were identified in each of the three promoters but with different combinations. Interestingly, GA responsive element (GARE motif) was found in the promoter of *SLY 1* gene, supporting that the transcription of *SLY 1* was induced by GA in coordination with light responses. The expression of *SLY1* is known to control by GA level (Hirano *et al*., 2008). This evidence suggested that GA level was increased by SD light leading to the activation of *SLY1* transcription via GARE motif on its promoter. G-box which is a recognition site of PIFs was found in *SLY1* promoter. This showed that phytochromes are also involved in the regulation of *SLY1* expression in GA signaling. Thus, these data indicate that the expression of *SLY1* is an integator linking GA hormone to photoperiod via the specific organization of G-box and GARE. Thus, G-box and GARE are the coordinated motifs which integrate GA hormone to photoperiod response.

On the other hand, MBS motif which is the target site of myb transcription factor responded to abiotic stresses and G-box were found in the promoters of *XRN 4* and *Vip 14* (Figure 7). This showed that ethylene and ABA signaling are controlled by phytochrome in photoperiod and stress responses. It is known that both ethylene and ABA signaling were involved in abiotic stresses (Rabbani *et al*., 2003; Achard *et al*., 2006; Zhou *et al*., 2007; Hunang *et al*., 2008) and in photoperiod responses (Welling *et al*., 1997; Kesy *et al*., 2008). These data indicated that the expression of *XRN4* and *Vip14* are integrators linking stress responsive genes to photoperiod regulation. Thus, MBS and G-box are coordinated motifs which integrate ethylene or ABA hormones and stresses to photoperiod responses.

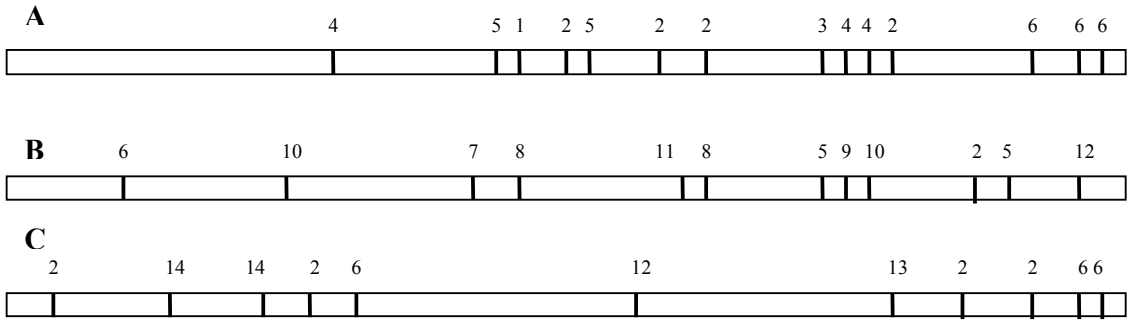


Figure 7 The feature map of *cis*-regulatory elements on the 1.5 kb promoter region of *SLY 1* (A), *XRN 4* (B) and *Vip 14* (C). The Arabic number 1 to 14 mean *cis*- regulatory elements; 1= ACE element, 2= G-box, 3 = GARE, 4 = LTR, 5 = MRE, 6= Sp1, 7 = AT1 motif, 8 = Box 4, 9 = CCGTCC-box, 10 = GAG motif, 11 = GT-1 motif, 12 = MBS, 13 = AE-box and 14 = GATA. The function of *cis*-regulatory elements is shown in table 6.

8. Identification of phytochrome A-regulated genes using specific motifs in their promoter regions

Phytochrome A (phyA) signaling genes, i.e., *PIF3*, *FAR1*, *COP1* were found in photoperiod responsive genes, showing that phyA must be involved in photoperiod response in rice. Thus, it is interesting to further identify the phyA regulated genes to elucidate the influence of phyA in photoperiod responsive pathway. The promoters of phyA regulated genes are known to contain G-box and GT-1 motif and/or GATA motif, while the G-box itself was found to locate at the upstream of GT-1 motif and/or GATA motif (Hudson and Quail, 2003; Jiao *et al*., 2007). Thus, these criteria were used to detect the phyA regulated genes from the members of SD induced and SD suppressed genes. These genes are listed in table 7 and Table 8. Interestingly, the flowering time regulated genes, i.e, floral inducers (*Hd3a*, *MADS box transcription factor 1* and *MADS box transcription factor 14*) in up-regulated genes and floral repressors (*AP2*, *FAR1* and *Vip14*) in down-regulated genes were regulated by phyA.

9. Identification of novel *cis*-regulatory elements specific to daylength

To identify the novel *cis*-regulatory elements that are specific response to daylength in rice, the promoter region of co-expressed genes in each set was used to identify a common over-representative motif using Oligo-analysis and subsequently converted to logo picture by Gibbs programs. The results showed that GCCGGCGCCC and AAAAAAAAAA elements were over-represented in the promoter of SD and LD genes, respectively (Figure 8). The GCCGGCGCCC was assigned as GC element while AAAAAAAAAA was assigned as A-rich element. Whether or not these over-represented sequences were positionally biased in the promoter of SD or LD genes, the distribution of each element in the promoter of these genes was determined using Position-analysis program from RSAT. The data revealed that the GC element was predominantly found in SD promoters while A-rich element was only enriched in LD promoters (data not shown), indicating the specificity of each element in each subset genome.

To further investigate whether the novel *cis*-regulatory motifs specific to daylength in rice (an SD plant) are conserved to *Arabidopsis thaliana* motifs (an LD plant), over-representative motifs in the promoter regions of orthologous genes in *Arabidopsis* were analyzed. The data are shown in figure 8. Interestingly, the consensus sequence of the over-representative sequences of LD promoters from rice and *Arabidopsis* is highly homology.

To examine the role of A-rich element, the LD responsive genes containing this element were identified and listed in table 9. Proteins encoded by these predicted genes appear to have diverse biological functions; transcription, metabolism, signal transduction, energy metabolism and protein processes (Table 9). In addition, the A-rich sequence was searched against known cis-regulatory elements in the database. The result showed that its sequence is similar to the CCA1 motif 2 binding site, AAAAAAAATCTATGA (RiceCis-elementSearcher, http://hpc.irri.cgiar.org/ tool/nias//ces) which is required for circadian rhythm controlled genes (McClung, 2006).

On the other hand, the over-representative motif (GC element) obtained from SD promoters of rice is not homology to SD promoters of *Arabidopsis*. It is interesting to further investigate whether the GC element has a specific role in SD response in rice. The SD responsive genes containing GC elements were identified as shown in table 10.

Table 7 Putative phytochrome A– regulated genes induced by SD light.

Representative ID	TIGR locus	Description
AK101991	LOC_Os12g09700	Jasmonate-induced protein
AK069331	LOC_Os03g54160	MADS-box transcription factor 14
AK067894	LOC_Os10g33290	NPGR2, putative, expressed
AK068816	LOC_Os08g10310	SHR5-receptor-like kinase
L34271	LOC_Os03g11614	MADS-box transcription factor 1
AK099709	LOC_Os03g61160	Expressed protein
U30479	LOC_Os05g19570	alpha-expansin 1 precursor
AK107926	LOC_Os01g28450	Pathogenesis-related protein PRB1-2
AK102086	LOC_Os10g40440	Cortical cell-delineating protein precursor,
AK065631	LOC_Os11g45990	Protein binding protein, putative
AB052943	LOC_Os06g06320	OsFTL2 – Rice FT-Like2 (Hd3a)
AK107044	LOC_Os04g11660	F-box protein interaction domain
AB017914	LOC_Os11g42090	Leucine Rich Repeat family protein
BI798695	LOC_Os03g55734	Prolamin, putative, expressed
AK100128	LOC_Os04g03796	peptidase/ subtilase, putative, expressed
AK059202	LOC_Os11g10590	Expressed protein
9629.m06682	LOC_Os01g67280	hypothetical protein
9630.m05489	LOC_Os03g23040	hypothetical protein
9639.m03143	LOC_Os11g34990	hypothetical protein
9640.m02992	LOC_Os12g30940	F-box domain containing protein
AK067428	LOC_Os02g48340	RNA-binding region-containing protein
NM_190345	LOC_Os01g60510	hypothetical protein
9631.m02338	LOC_Os03g23940	Expressed protein
9631.m04608	LOC_Os01g07180	hypothetical protein
9640.m00101	LOC_Os12g01990	Expressed protein

Table 8 Putative phytochrome A– regulated genes suppressed by SD light.

Representative ID	TIGR locus	Description
9632.m04031	LOC_Os04g42260	Protein phosphatase 2C isoform gamma
9635.m03511	LOC_Os07g35540	Protein kinase, putative, expressed
AK058773	LOC_Os02g08440	OsWRKY71 – Superfamily of rice TFs
AK060639	LOC_Os10g36500	Pectinesterase inhibitor domain
AK063042	LOC_Os03g08520	Expressed protein
AK064287	LOC_Os02g54590	Serine threonine kinase, putative
AK067836	LOC_Os09g31130	Tonoplast dicarboxylate transporter
AK069654	LOC_Os02g18064	expressed protein
AK101750	LOC_Os01g43740	Cytochrome P450 72A1, putative,
AK109161	LOC_Os03g13740	U-box domain containing protein,
AK110739	LOC_Os10g36520	expressed protein
AK111335	LOC_Os02g50110	expressed protein
AK111571	LOC_Os01g64360	Myb-like DNA-binding domain
AK119780	LOC_Os07g05940	viviparous-14, putative, expressed
AY345234	LOC_Os01g73770	Transcription factor RCBF2
BP184627	LOC_Os01g19050	far-red impared
CB621890	LOC_Os02g52390	Neoxanthin cleavage enzyme
NM_188146	LOC_Os01g17396	expressed protein
AK108588	LOC_Os06g49550	far-red impaired responsive protein

Among known functions, these genes have diverse biological functions, i.e., stress and defense response, metabolism, protein processing, transcription and signaling. Interestingly, the promoter of *SLY 1* was found to contain GC element. In addition, the sequence of GC element was searched against known *cis*-regulatory elements in the databases. There is no homology to all elements in three databases (PlantCare, PLACE and RiceCis-element databases)

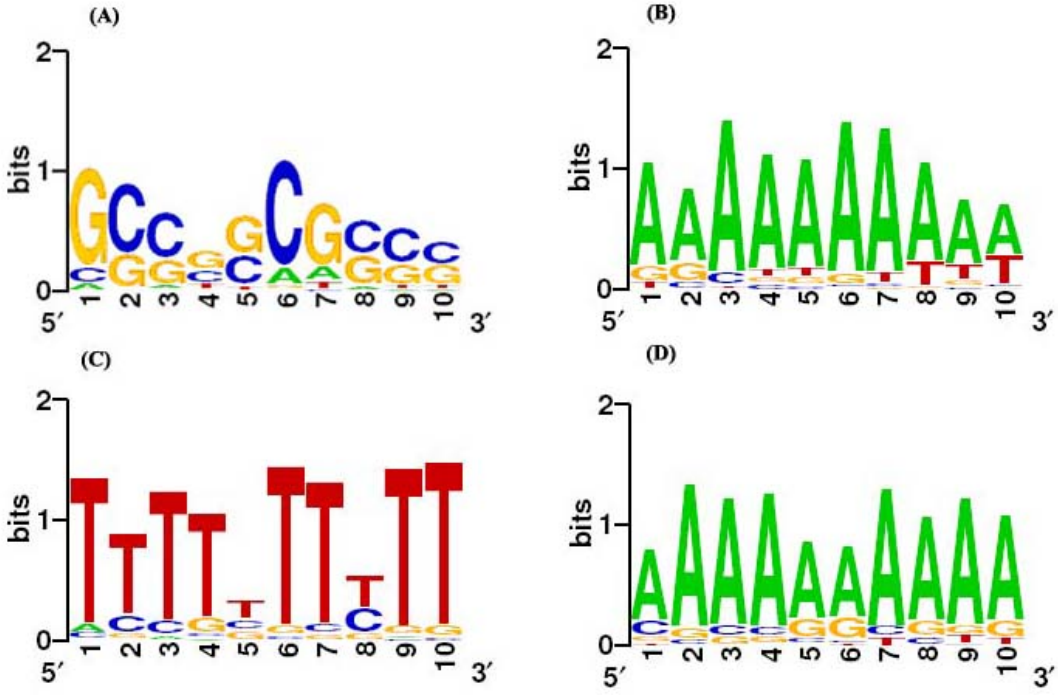


Figure 8 Over-representative *cis*-regulatory elements specific to daylength response. Over-representative *cis*-regulatory element of SD (A) and LD (B) promoters in rice. Over-representative *cis*-regulatory element of SD orthologous (C) and LD orthologous (D) in *Arabidopsis*. The overall height of each stack indicates the sequence conservation at that position (measured in bits), whereas the height of symbols within the stack reflect the relative frequencies of corresponding nucleic acid at that position.

Table 9 List of SD induced genes containing A-rich element in the promoter regions.

TIGR Gene Target	Rice annotation	Functional categories *
LOC_Os01g58310	expressed protein	Unknown
AK106968	expressed protein	Unknown
LOC_Os01g68269	expressed protein	Unknown
LOC_Os08g45200	expressed protein	Unknown
LOC_Os02g10030	hypothetical protein	Unknown
LOC_Os03g02110	expressed protein	Unknown
LOC_Os03g08520	expressed protein	Unknown
LOC_Os05g49630	expressed protein	Unknown
LOC_Os02g50110	expressed protein	Unknown
LOC_Os02g21269	hypothetical protein	Unknown
LOC_Os05g36970	expressed protein	Unknown
LOC_Os05g41630	expressed protein	Unknown
LOC_Os06g11760	expressed protein	Unknown
LOC_Os06g32540	hypothetical protein	Unknown
LOC_Os08g07860	hypothetical protein	Unknown
LOC_Os01g72990	hypothetical protein	Unknown
LOC_Os06g27860	expressed protein	Unknown
LOC_Os04g02880	expressed protein	Unknown
LOC_Os07g02624	expressed protein	Unknown
LOC_Os10g40280	expressed protein	Unknown
LOC_Os08g37660	expressed protein	unknown
LOC_Os02g52210	RING/C3HC4/PHD zinc finger-like protein	Transcription factor
LOC_Os02g08440	OsWRKY71	Transcription factor
LOC_Os01g64470	Harpin-induced protein 1 containing protein	Transcription factor
LOC_Os08g36920	**ERF (ethylene response factor) subfamily B-1 of ERF/AP2 transcription factor family**	Transcription factor
LOC_Os05g12640	BURP domain	Transcription factor
LOC_Os01g03720	putative myb-related transcription factor	Transcription factor
LOC_Os01g73770	**Transcription factor RCBF2/dehydration and cold-relative**	Transcription factor
LOC_Os10g25230	ZIM motif family protein	Transcription factor
LOC_Os03g28940	pnFL-2, putative, expressed, ZIM motif family protein	Transcription factor
LOC_Os01g74020	**ARR1 protein-like, putative, expressed (LUX)****	Transcription factor
LOC_Os06g44010	WRKY28 protein	Transcription factor
LOC_Os02g36530	helix-loop-helix DNA-binding domain containing protein	Transcription factor
LOC_Os03g07440	putative eribonuclease P, putative, expressed	Transcription factor
LOC_Os03g32220	Zinc finger	Transcription factor
LOC_Os04g23550	**Oryza sativa RERJ1 mRNA for Transcription Factor, PIF 3****	Transcription factor
LOC_Os03g53020	Helix-loop-helix DNA-binding domain containing protein	Transcription factor
LOC_Os01g64310	putative NAC-domain protein	Transcription factor
LOC_Os02g39330	Endochitinase PR4 precursor	Metabolism
LOC_Os10g34840	ripening-related protein 3 precursor	Metabolism
LOC_Os01g43740	cytochrome P450	Metabolism
LOC_Os08g14190	Sulfotransferase domain containing protein	Metabolism
LOC_Os05g43910	Cytochrome P450 family	Metabolism
LOC_Os06g15410	Aldose 1-epimerase family protein	Metabolism
LOC_04g37430	Lipoxygenase 5	Metabolism

Table 9 (Continue).

TIGR Gene Target	Rice annotation	Functional categories *
LOC_Os01g72530	EF hand family protein, calmodulin-like protein 41,	Signaling
LOC_Os07g44290	CBL-interacting serine/threonine-protein kinase 1	Signaling
LOC_Os05g07420	putative receptor-like protein kinase	Signaling
LOC_Os06g49550	**far-red impaired responsive protein** (FAR 1)**	Signaling
LOC_Os01g19050	**far-red impared** (FAR1)**	Signaling
LOC_Os02g54590	Kinase	Signaling
LOC_Os04g42260	Protein phosphatase 2C containing protein, expressed	Signaling
LOC_Os07g35540	putative serine/threonine-specific protein kinase	Signaling
LOC_Os12g19470	Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	Energy
LOC_Os07g41300	putative NADH dehydrogenase 49kDa protein	Energy
LOC_Os04g51160	AOX1b mRNA for alternative oxidase	Energy
LOC_Os07g02970	**COP 1 interacting protein**	Processing
LOC_Os01g72160	Glutathione S-transferase, putative,expressed	Processing
LOC_Os02g33590	Ubiquitin-protein ligase, putative, expressed	Processing
LOC_Os03g10050	serine O-acetyltransferase, putative	Processing
LOC_Os02g17000	Subtilisin N-terminal Region family protein	Processing
LOC_Os01g51450	nucleosome assembly protein (NAP),	Processing
LOC_Os10g35090	putative membrane-associated protein	Growth/structure
LOC_Os08g23870	Late embryogenesis abundant group 1 family protein	Growth/structure
LOC_Os09g27940	Avr9 elicitor response protein, putative, expressed	Stress/defense
LOC_Os01g03340	Bowman-Birk type bran trypsin inhibitor precursor	Stress/defense
LOC_Os07g05940	**Viviparous-14, neoxanthin cleavage enzyme**	Stress/defense
LOC_Os10g36500	Pectinesterase inhibitor domain	Stress/defense

Note The flowering time associated genes are highlighted by **bold font**.

***** Function categories were classified according to their putative function and consulted with MIPS MATDB database (http://mips.gsf.de/proj/thal/db/index.html).

Table 10 List of SD suppressed genes containing GC element in the promoter regions.

TIGR Gene Target	Rice annotation	Functional categories*
LOC_Os01g34970	Multidrugresistance protein 8, putative, expressed	Stress and defense
LOC_Os01g63010	USP family protein, putative, expressed	Stress and defense
LOC_Os12g11990	expressed protein	Unknown
LOC_Os03g54150	expressed protein	Unknown
LOC_Os07g43604	expressed protein	Unknown
LOC_Os06g29730	Expressed protein	Unknown
LOC_Os03g61160	Expressed protein	Unknown
LOC_Os10g22510	mla1, putative, expressed	Stress and defense
LOC_Os10g42040	expressed protein	Unknown
LOC_Os04g02640	3-ketoacyl-CoA synthase, putative, expressed	Metabolism
LOC_Os11g20239	Expressed protein	Unknown
LOC_Os11g35340	S-adenosylmethionine-dependent methyltransferase	Metabolism
LOC_Os12g04770	Conserved hypothetical protein	Unknown
LOC_Os04g11660	F-box protein interaction domain containing protein	Protein processing
LOC_Os09g13440	Expressed protein	Unknown
LOC_Os11g10590	Expressed protein	Unknown
LOC_Os01g15910	UTP--glucose-1-phosphateuridylyltransferase	Metabolism
LOC_Os01g39490	hypothetical protein	Unknown
LOC_Os02g54330	F-box domain containing protein , SLEEPY 1	Protein processing
LOC_Os03g47430	hypothetical protein	Unknown
LOC_Os03g47430	hypothetical protein	Unknown
LOC_Os09g08410	hypothetical protein	Unknown
LOC_Os11g34990	hypothetical protein	Unknown
LOC_Os12g30940	F-box domain containing protein	Protein processing
LOC_Os01g60510	hypothetical protein	Unknown
LOC_Os01g41960	Retrotransposon protein, putative, unclassified	Unknown
LOC_Os01g72500	Retrotransposon protein, putative, unclassified	Unknown
LOC_Os03g07460	Retrotransposon protein, putative, unclassified	Unknown
LOC_Os05g40460	Retrotransposon protein, putative, unclassified	Unknown
LOC_Os09g36410	Retrotransposon protein, putative, unclassified	Unknown
LOC_Os10g28250	retrotransposon protein, putative, LINE subclass	Unknown
LOC_Os02g55250	bHLH transcription factor, putative, expressed	Transcription factors
LOC_Os01g07180	hypothetical protein	Unknown
LOC_Os06g42240	Conserved hypothetical protein	Unknown
LOC_Os08g19190	Conserved hypothetical protein	Unknown
LOC_Os11g04630	hypothetical protein	Unknown
LOC_Os12g01990	Expressed protein	Unknown
LOC_Os08g44670	Calreticulin precursor, putative	Signaling
LOC_Os11g12540	hypothetical protein	Unknown

Note * Function categories were classified according to their putative function and consulted with MIPS MATDB database (http://mips.gsf.de/proj/thal/db/index.html).

10. Verification of DNA microarray data

Verification of microarray data is necessary because the cross-hybridization of probe sets on array might be occurred between the related gene family members. (Girke *et al.,* 2000). Gene expression changes might not come from the representative transcripts on array. RT-PCR was used for verification of this gene expression with gene specific primers. Thirty of both up- and down-regulated genes were selected and were listed in table 11. Some of them were gene family members. The results of RT-PCR were correlated with their results obtained from microarray (Figure 9). It was likely that the cross-hybridization between some members of a gene family had not occurred in our selected genes. It implied the efficiency of probe set designed by Affymetrix and high stringency of selected probe sets method.

Furthermore, the expression level of flowering related genes were verified using quantitative realtime-PCR. The expression levels from both techniques were shown in table 12. Altogether, the two methods provided correlating data as in up- and down-regulated genes, but the expression ratios from real-time PCR technique was slightly-higher than those obtained from DNA microarray due to high sensitivity of the real-time PCR.

11. Analysis of flowering time genes

The flowering related genes were identified from the microarray results and were listed in table 13. These genes included both floral inducer and floral repressor putative genes. *Hd3a*, *AP1 like* (*AY332478)*, *AP2* (*AK06288*), were monitored their expression changes during SD treatment using RT-PCR. The results showed that *Hd3a* and *AP1 like* were increased in day 6 and day 10 respectively (Figure 10 and 11) while *AP2* was decreased in day 4 (Figure 12).

12. Detection and quantification of miRNA

The microarray data showed that *AGO1* was induced by SD light. It has been reported that its role involved in miRNA synthesis. In addition, several target genes of microRNA were also detected in our experiment including *AP2* (target of *miRNA172*), *MYB* (target of *miRNA156*). To determine whether miRNAs might be induced in SD light, we detected

miRNA172a as described in materials and methods. We found that *miRNA172* was up-regulated in as shown in figure 13 and table 14.

Table 11 Differentially expressed genes verified by RT-PCR.

Representative Public ID	Gene products	Fold Change
AY332478	AP1 like	4.53
AB052943	Hd3a	3.95
AK065474	TolB	2.99
AB017914	disease resistance protein	2.99
AK107044	F-box	2.76
AK069331	MADS box	2.65
AY341842	WRKY transcription factor	2.55
L34271.1	OsMADS1	2.54
AK070289	Zn finger protein	2.23
AK065631	Zn finger protein	2.14
AK069685	Argonaute protein	2.06
AK067428	RNA binding protein	2.05
AK112056	MYB transcription factor	2.01
BP184627	Far-red impared	-2.05
AK101023	Kinase	-2.07
AK071274	NAC transcription factor	-2.12
AK108588	Far-red impaired responsive protein	-2.14
AK071734	bhlh transcription factor	-2.38
AK107746	NAC transcription factor	-2.39
AK107424	MYB transcription factor	-2.42
AY581256	MYB transcription factor	-2.45
AK069654	Kinase	-2.45
AK058773	WRKY transcription factor	-2.58
AK108997	Zn finger	-2.63
AK058518	Kinase	-2.65
AY345234	DREB transcription factor	-2.82
AK072975	COP1 interacting	-2.97
AK062882	AP2 transcription	-3.15
AK111571	MYB transcription factor	-3.19
AB040744	PIF3 transcription factor	-3.24
AK106282	WRKY transcription factor	-4.50

Table 12 Comparison of expression ratio between data obtained from GeneChip microarray and real-time PCR.

Representative ID	Gene name	Expression ratio	
		Microarray	Real-time PCR
AB052943	*Hd3a*	3.95	5.98
AY332478	MADS box protein (*AGL10*) or *AP1 like*	3.32	4.98
AK062882	*AP2*	-3.15	-6.08
AK108588.1	far-red impaired responsive protein (*FAR1*)	-2.14	-3.54
AY581256	ARR1 protein-like, putative, (*LUX*)	-2.45	-4.02

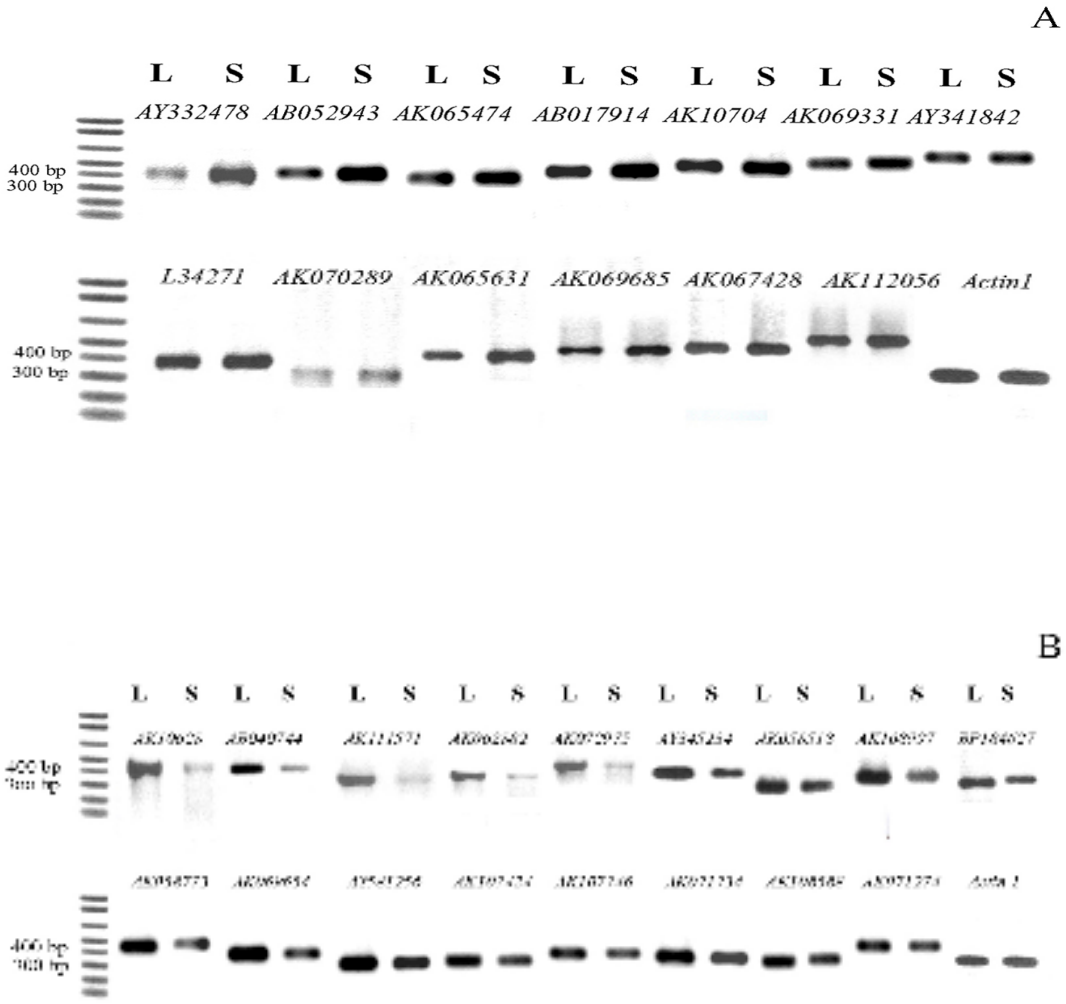


Figure 9 The expression pattern of the 30 randomly selected differentially expressed genes by RT-PCR.

Table 13 Putative flowering time associated genes in KDML 105.

	Gene product	Pathway/Role	Expression changes
Floral repressor			
AK062882	AP2 domain containing protein,the ERF (ethylene response factor) subfamily B-1 of ERF/AP2 transcription factor family (ATERF-9)	photoperiod	-3.15
AY345234	Transcription factor RCBF2, DREB transcription factor	Photoperiod	-2.82
9636.m03709	AP2 domain, putative	Photoperiod	-2.79
AK070782	XRN 5'-3' exonucleas	ethylene regulation	-4.74
AB040744	Oryza sativa RERJ1 mRNA for Transcription Factor,PIF3	phoreceptor signal transduction/circadian clock	-3.24
AY581256	ARR1 protein-like, putative, (LUX)	circadian clock	-2.45
AK072975	COP1 interacting protein	Phytochrome signaltransduction	-2.97
CB622792	viviparous-14, neoxanthin cleavage enzyme	Abscisic acid regulation	-2.83
AK119780	neoxanthin cleavage enzyme	Abscisic acid regulation	-3.30
AK108588.1	far-red impaired responsive protein (FAR1)	Phytochrome signal transduction	-2.14
BP184627	far-red impared responsive protein (FAR1)	Phytochrome signal transduction	-2.11
AK072975	COP1 interacting protein	Phytochrome signal transduction	-2.97
Floral inducer			
AK069685	Agronaute-like protein	floral transition	2.06
AB052943.1	Heading date 3a (Hd3a) , phosphotidylethanolamine binding protein	Photoperiod	3.95
L34271.1	MADS1,Oryza sativa box protein (MADS1),transcription factor controlling flowering	floral transition and development	2.54
AY332478	MADS box protein (AGL10)	floral transition and development	3.32
AK069331.1	MADS-box transcription factor 14	floral transition and development	2.65
9637.m00612	casein kinase II alpha chain 2	circadian clock	2.03
9630.m05386	SLEEPY 1 protein	GA regulator	3.13

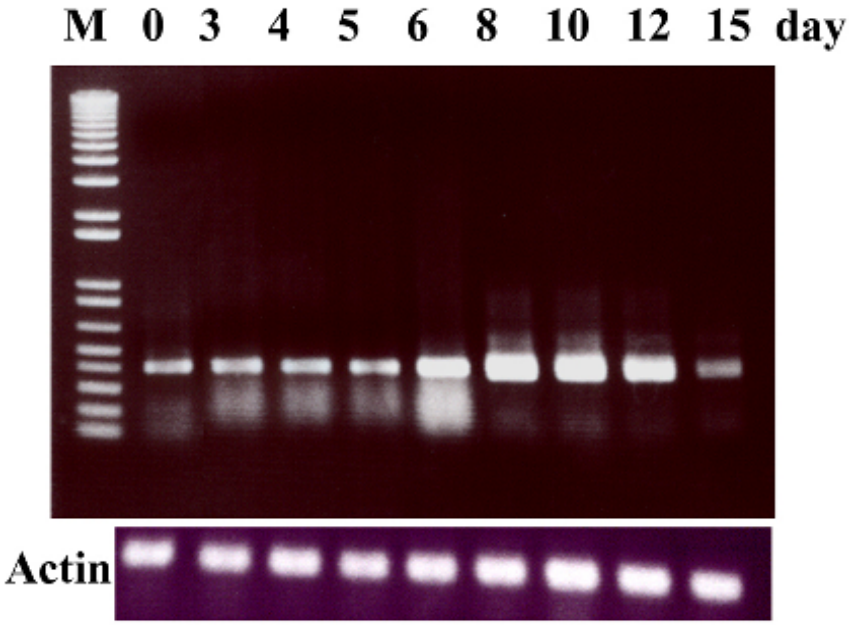


Figure 10 Expression pattern of *Hd3a* during SD light by RT-PCR

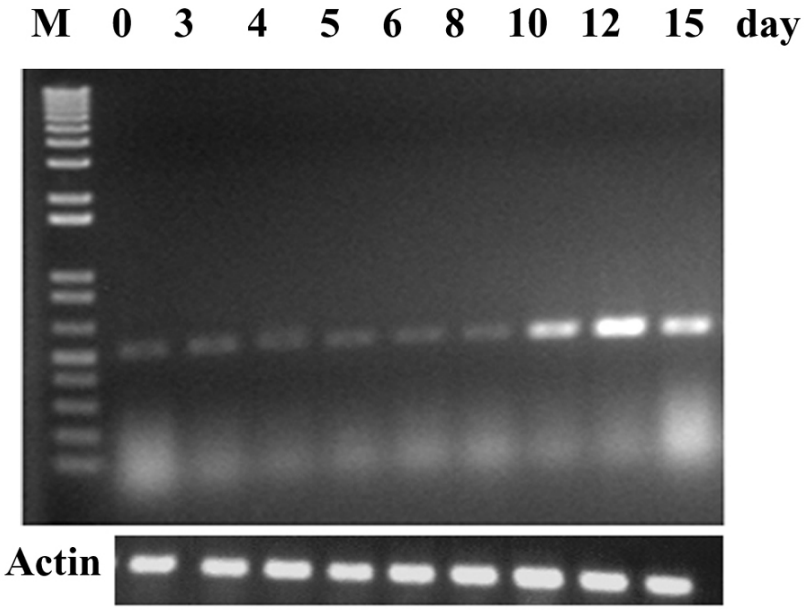


Figure 11 Expression pattern of *AP1 like* during SD light by RT-PCR.

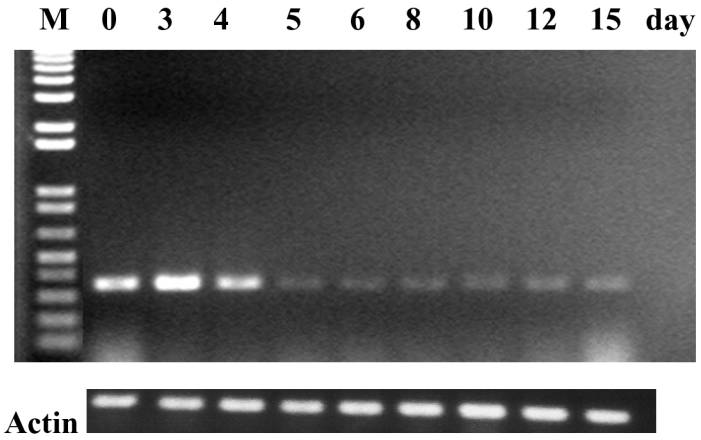
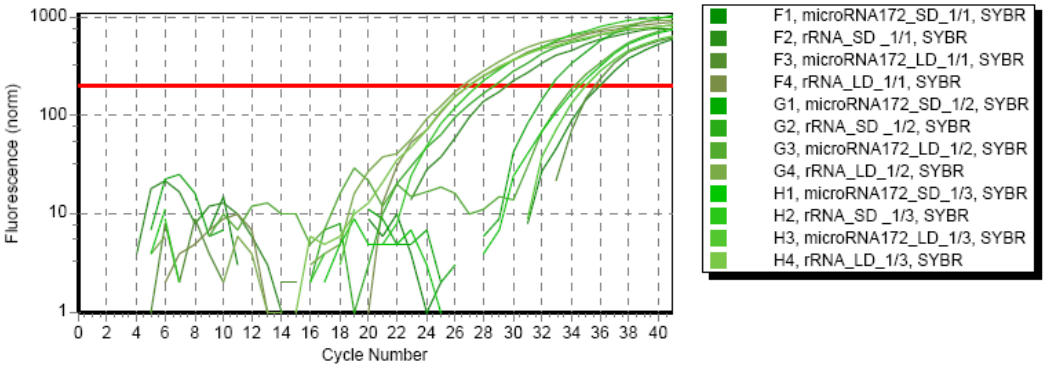


Figure 12 Expression pattern of *AP2* during SD light by RT-PCR.

Table 14 Expression data of *miRNA172a* obtained from real-time PCR.

	Ct SYBR	Ct Mean	SYBR Ct Dev.	Expression level
microRNA172_SD_1	35.97	34.42	1.65	5.07
rRNA_SD _1	29.61	28.64	1.02	
microRNA172_LD_1	35.65	34.96	0.72	
rRNA_LD_1	27.04	26.85	0.24	
microRNA172_SD_2	32.69	34.42	1.65	5.07
rRNA_SD _2	26.57	28.64	1.02	
microRNA172_LD_2	34.22	34.96	0.72	
rRNA_LD_2	26.57	26.85	0.24	
microRNA172_SD_3	34.59	34.42	1.65	5.07
rRNA_SD _3	27.58	28.64	1.02	
microRNA172_LD_3	35.02	34.96	0.72	
rRNA_LD_3	26.94	26.85	0.24	



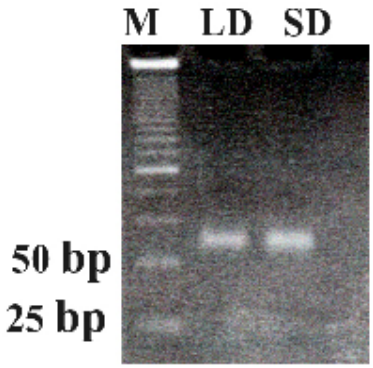


Figure 13 Amplification plot of *miRNA 172a* (A) and PCR product of mature *miRNA172a* on 4.5 % agarose gel.

DISCUSSION

GeneChip DNA microarray

Microarray has been shown to be a powerful tool for generating large amounts of data for parallel gene expression analyses. Affymetrix GeneChips are routinely used to measure relative amounts of mRNA transcripts on a genome scale. Here, we used GeneChip Rice genome array to study global expression of photoperiod responsive genes in KDML105 rice. Although the large number of probe sets (representing genes) available on the array gave us a wealth of information, but it could be raised a large number of false positive due to biological variation as well as cross-hybridization between related genes family members. To resolve those problems, three independent biological replications were used and non-reliable detection call probe sets were removed using detection call filter method before further analysis. High stringent criteria were also used for accepting differentially expressed probe sets (fold changes ≥ 2 with t-test p value ≤ 0.05). The microarray data was further verified with RT-PCR and real-time PCR which showed consistent results. Thus, our data from the GeneChip rice genome array was reliable. Although these data provided only a measure of relative steady-state transcripts levels, the gene expression profiles provided useful starting points for more in depth functional analyses.

Photosynthesis and carbohydrate metabolism involved in photoperiod response

Plant photosynthesis is composed of two processes, light reaction and dark reaction .The light reaction takes place in photosystem II and photosystem I where light energy is harvested and is used to split water into oxygen, hydrogen ions and free electrons which are transferred among a series of electron donors and accepters to produce the energy rich substrates, ATP, NADH. ATP and NADH generated from light reaction are used in calvin cycle (dark reaction) where free carbon dioxide (CO_2) are assimilated by ribulose-1,5-bisphosphate carboxylase in the process forming carbohydrate such as starch and sucrose (Archer and Barber, 2004). The photosynthetic products are transported, primarily as sucrose, to meristem and developing organs such as growing young leaves, roots, flowers, fruits and seeds (review in Pego *et al*., 2000).

This experiment showed that photosynthetic gene in light reaction, PSII, was up-regulated while ribulose-1,5-bisphosphate carboxylase, glyceraldehydes-3-phosphate dehydrogenase in calvin cycle and glutamate synthase were down-regulated. This result suggested that the activity of light reaction was increased during SD induction to provide the energy (ATP) for other metabolic processes as well as floral induction. Kandeler *et al.* (1975) reported that high levels of ATP promoted flowering and that the flower-inhibiting effect of treatment with high levels of CO_2 was caused by the lower ATP. Thus, to reduce the energy consumption from light reaction, genes involved in dark reaction; ribulose 1,5-bisphosphate carboxylase and glyceroldehyde-3-phosphate were suppressed. In addition, other enzyme involved in energy consuming such as glutamate synthase, NADH dehydrogenase were also down-regulated.

Genes encoding enzyme involved in sucrose degradation and sucrose metabolism were also induced under short day light. This result indicated that high concentration of sucrose in sink tissue inhibited sucrose formation process in Calvin cycle. Sugar sensing and signaling are involved in the control of growth and development during entire plant life cycle (Rolland *et al*., 2002). A recent report confirmed the effects of sugar on floral transition, depending on sugar concentration and vegetative growth phase (Ohto *et al*., 2001). Taken those previous reports together with this result, we proposed that SD light induced accumulation of sucrose in somatic apical meristem and then activated sucrose degradation to provide carbon skeletons for basic metabolic processes and to promote floral transition.

Phytochrome signaling involved in photoperiod response

Phytochromes are photoreceptors that monitor the incident red (R) and far-red (FR) light by switching reversibly between the R light-absorbing, biologically inactive Pr form and the FR light-absorbing, biologically active Pfr form (Lin, 2000). Upon photoactivation, phyA, the primary photoreceptor for FR light, is translocated from cytoplasm into nucleus to induce FR light-responsive gene expression that is required for various photoresponses, such as germination, seedling de-etiolation and flowering. PhyA signaling proteins identified in this study include FAR1 (far-red-impaired1), PIF3, COP1 and EID1. The primary photoreceptor for red light is phytochrome B and its identified signaling proteins include PIF and COP1 (Jiao *et al*., 2007).

In previous reports, *phyA* null mutants exhibited late flowering both in SD- and LD-conditions (Johnson *et al*., 1994) and overexpression of phyA in *Arabidopsis* plants exhibited early flowering (Bagnall *et al*., 1995). From both reports, thus, phyA was proposed to promote flowering in response to photoperiod in LD plants. In addition to *Arabidopsis*, *phyA* mutant of pea, another LD plant, also showed a phenotype similar to that of the *Arabidopsis phyA* mutant (Weller *et al*., 1997). Furthermore, the pea *phyA* mutant accumulated a graft-transmittable inhibitor that could delay the flowering of the grafting recipient plants, suggesting that phyA signaling may suppress the biosynthesis of floral repressor (Weller *et al*., 1997).

In this experiments, *BP184627* and *AK108588* encoding protein homology to FAR1 (far-red-impaired1) family in *Arabidopsis* were down-regulated under SD induction, suggesting that FAR1 might not be involved in flowering induction of phyA signaling pathway. But it might be involved in floral inhibition under LD condition.

FAR1 family, containing 12 related-genes, has been reported of having distinct roles in light control and development in *Arabidopsis*. To examine whether *BP184627* and *AK108588* can be grouped in the same clad as gene defined in *Arabidopsis FAR1*, phylogenetic analysis of rice and *Arabidopsis* FAR1 protein sequences was performed (data not shown). The phylogeny showed that *BP184627* and *AK108588* can be grouped in *FRS10* (far-red-related sequence 10). Interestingly, all of *FRS* genes, except *FRS10*, were expressed in all tissues such as rosette leaves, cauline leaves, inflorescence stem, and flower under LD light. Thus, the expression of *FRS10* was proposed to respond to specific condition (Lin and Wang *et al*., 2004). Although, *FRS10* has not been reported for its involvement in flowering, *FRS6* and *FRS8* mutants exhibited early flowering both in LD and SD conditions, suggesting that *FRS6* and *FRS8* regulated flowering time in *Arabidopsis* as the floral repressor. Thus, possibly, *FRS10* might be involved in specific photoperiod response and in regulation of flowering time as a floral repressor idependent from phyA signaling pathway.

From the study of phytochrome regulated genes, the genes involved in flowering time including *Hd3a* and MADS box transcription factor were belonged to this group, supporting that phyA promote flowering in SD light.

PIF3 and *COP1* involved in both phyA and phyB signaling were also down-regulated. PhyB has been proposed to inhibit flowering under SD light in *Arabidopsis* (Endo *et al*., 2005) and LD light in rice (Takano *et al*, 2005). Thus, phyB signaling in a flowering inhibition pathway might be suppressed by phyA (Hennig *et al*., 2001) but its mechanism is still unclear.

However, both *PIF3* and *COP1* were involved in the flowering regulation as a floral repressor (Oda *et al*., 2003; Nakagawa *et al*., 2004), but how their regulations were unclear.

Circadian rhythm and photoperiod response

In higher plant, the circadian clock orchestrates fundamental processes such as light signaling and the transition to flowering in response to photoperiod. Circadian rhythms persist with a period close to 24 hours in the absence of environmental time cues and are regulated by an internal timing mechanism (Young and Kay, 2001). In *Arabidopsis*, circadian clock consists of negative feed back loop comprised of two morning-expressed Myb transcription factors, *CIRCADIAN CLOCK ASSOCIATED 1*(*CCA1*) and *LATE ELONGATE HYPOCOTYL* (*LHY*) and the evening expressed, *TIMING OF CAB2 EXPRESSION* (*TOC1*) and *LUX ARRYTHMO*(*LUX,ARR1*). In this model, *TOC1* and *LUX* feed back either directly (or indirectly) regulated *CCA1* and *LHY*, which in turn suppress *TOC1* expression by binding to its regulatory region (G-box) (Alabadi *et al*., 2001). Interestingly, one of the key circadian rhythm genes, *LUX*, was down-regulated by SD-light. For further discussion, *lux* mutant phenotype of *Arabidopsis* in TAIR database was investigated. *lux* mutant exhibit arhythm expression of multiple clock-output genes and early flowering, which are part of the circadian clock regulation. In contrast, CK2, encoding essential protein for CCA1 function, was up-regulated, suggesting that CCA1 activity was increased and might induce or suppress their target genes. Taken results together, we proposed that photoperiod cause gene expression changes in circadian clock and these genes affected the expression of output genes controlling flowering time in KDML 105.

Phytohormone signaling in photoperiod response

The expression of phytohormone signaling-related genes were altered by SD-light, including *SLEEPY1* in GA signaling, *XRN 5′-3′ exonuclease* encoding protein involved in ethylene signaling and *viviparouse-14* encoding neoxanthin cleavage enzyme involved in

ABA biosynthesis pathway. These hormones involve in controlling of diverse growth and developmental processes. They have been reported to involve in flowering regulation, the role of GA is to promote whereas the role of ethylene and ABA are to inhibit flowering (Amagasa and Suge, 1987; Martinez-Zapater *et al*., 1994; Magome *et al*., 2004).

GA is a growth regulator to promote flowering of *Arabidopsis* in SD light where the photoperiodic pathway is inactive (Wilson *et al*., 1992). A role of GA in the control of flowering is suggested by studying genes involved in GA signaling, including *GIBBERLLIC ACID INSENSITIVE (GAI)*, *REPRESSOR OF GAI-3* (*RGA*) and *RGA-like1* (*RGL1*) (Wen and Change, 2002).They are DELLA family members known to inhibit GA response in the absence of active GA and GA relieves this function. (Willege *et al*., 2007). In *Arabidopsis*, GA derepressers its signaling pathway by inducing proteolysis of the DELLA proteins. *SLEEPY1* (*SLY1*) encodes an F-box-containing protein, recruits RGA to SCF^{SLY1} E3 ligase complex for ubiquitination and subsequent degradation by 26S proteasome (Guo and Ecker, 2003; Dill *et al*., 2004). The GA-DELLA system regulates the timing of floral transition via effects on the levels of transcripts identity genes *LEAFY* (*LFY*) and *SUPPRESSOR OF OVEREXPRESSION CONSTANS1* (*SOC1*). In this experiment, *SLY1* was up-regulated, suggesting SD light induced GA response in rice. The expression of *SLY1* is known to control by GA level (Hirano *et al*., 2008). Thus this result suggested that GA level was increased by SD ligt and leading to activation of *SLY1* transcription. This hypothesis was supported by the work of Kulikowska-Gulewska *et al*. (2000), showing that GA level was increased by SD light in *Pharbitis nil*. The *SLY1* promoter analysis revealed that its expression is controlled by GA in coordination with phytochrome. It is known that phytochromes are mainly involved in the regulation of photoperiod response in rice (Izawa *et al*., 2000). Thus, these data suggested that GA signaling is activated by SD light through the phytochrome function. This is in correlation to the expression of a transcriptional repressor of GA signaling, OsWRKY71, (Zhang *et al*., 2004) which was down-regulated by SD light in this experiment. Taken altogether, it can be concluded that photoperiodic flowering in rice is controlled, partially, by GA signaling.

In contrast, ethylene phytohormone enhances the DELLA accumulation in turn delay flowering (Achard *et al*., 2007). The positive transcriptional factors of the response to ethylene perception are Ethylene Insensitive 3(EIN3) and probably one of the EIN3-like proteins (Solano *et al*., 1998). EIN3 level is kept in low in the absence of ethylene but increase in response to ethylene hormone. EIN3 abundance is controlled by EIN3-binding F-

box1 (EBF1) or EBF2 degradation (Gagne *et al*., 2002; Potuschak *et al*., 2003). The EBF1/2 levels affect EIN3 accumulation, and, in turn, EBF1/2 mRNAs are substrates of Exoribonuclease 4 (XRN4) involved in the degradation of short-lived mRNA (Potuschak *et al*., 2006). From our experiment, *XRN 4* transcript was down-regulated in SD light. This results suggested that EBF1/2 abundance were increased in order to reduce EIN3 protein and leading to inhibition of ethylene response.

ABA was also involved in response to SD light in KDML105. As similar to other hormones, the response to ABA depends on its concentration within tissue and on the sensitivity of the tissue to hormone. ABA is synthesized from a carotenoid intermediate that takes place in chloroplast and other plastids (Hirchberg *et al*., 1997). The pathway begins with isopentenyl (IPP) leads to the synthesis of the C40 xanthophyll, violaxanthin. Violaxanthin is converted to the C40 compound 9-cis-neoxanthin then cleavage to C15 compound xanthoxal, called xanthoxin, a neutral growth inhibitor. The cleavage step is catalyzed by 9-cis-expoxy carotenoid dioxygenase or neoxanthin cleavage enzyme (Taiz and Zeiger, 2006). In this study, gene encoding 9-cis-expoxy carotenoid dioxygenase, a key step of ABA biosynthesis, was down-regulated. Thus, the result suggested that low concentration of ABA was found in rice tissue during SD light. Moreover, ABA enhances DELLA accumulation and delay flowering in a DELLA-dependent manner (Achard *et al*., 2007).

Furthermore, AP2 family members, NAC family members and BURP domain (or RD22) known as stress responses induced by ABA and ethylene hormone were also down-regulated (Rabbani *et al*., 2003), supporting the reduction of ABA hormone and ethylene signaling.

In addition to those hormones, jasmonic acid (JA), a plant defense hormone was also considered in this experiment. We found that the lipoxygenase in the first step of JA biosynthesis was down-regulated. Maciejewska *et al.* (2004) investigated the role of JA in the photoperiodic flower induction of SD plant *Pharbitis nil*, the results conferred that phytochrome system controlled both the photoperiodic flower induction and JA metabolism, and JA inhibited flowering in *P.nil.* Thus, this report supported our experiment, suggesting that JA hormone was involved in photoperiodic flowering response.

Photoperiod and stress/defense response

In response to photoperiod, several stress/defense related genes were up- and down-regulated. In up-regulated genes, a large fraction was pathogen/defense related genes. In the other hand, the large faction of down-regulated genes was abiotic stress response related genes such as cold and drought (*RD22*, *viviparourse14*, *AK10694*, *AK104585*). Moreover, some of transcription factors in down-regulated genes are involved in the abiotic stress responses including *AP2*, *NAC* and *WRKY* family members and all of them are also response to stress hormone ABA and ethylene (Rabbnai *et al.,* 2003) as described above.

Although, the correlation between stress/defense and photoperiod response have not been reported. We observed that most of genes in up-regulation, pathogen related genes, might be involved in flowering time regulation. Because of pathogen infection is known to accelerate flowering time, with the effect more pronounce in resistant interaction (Korves and Bergelson, 2003). Furthermore, increase production of salicylic acid, a chemical of plant defense and systemic immunity due to pathogen infection, accelerates flowering (Martinez *et al*., 2004).

In contrast, most of genes in down-regulation were involved in drought, cold, NaCl responses. These stresses cause delay flowering, prolong vegetative phase by accumulation of DELLA protein (Rabbani *et al*, 2003; Achard *et al*., 2006) that crosstalk with hormone response.

Thus, It was concluded that the photoperiod response crosstalk with stress and hormones responses.

Signal transduction and photoperiod response

Signal transduction genes were identified from up- and down-regulated genes by SD light. CK2α kinase, SHRP-receptor like kinase, calreticulin 2 (CRT2) and NPGR2 calmodulin-binding protein were up-regulated while five protein kinases, receptor like kinase and EF-hand or calmodulin like protein kinase 41 were down-regulated.

Protein kinases have been shown to be involved in many cellular processes including regulation of metabolism, transcription and response to variety of stimuli (Manning *et al*.,

2002). CK2 kinase is involved in circadian clock regulation in *Arabidopsis* as described in the section of circadian rhythm. SHRP-receptor like kinase is the member of receptor-like kinase (RLK) family. RLKs have been implicated in mechanisms of perception and transduction of extracellular signals into the cell (Shiu and Bleecker, 2001) Members of this family were know to play roles in diverse processes related to plant growth/development, stress, defense againt pathogen (Shiu *et al*., 2004). SHRP-RLK was involved in plant nitrogen fixing endophytic bacteria associated and hormone response (Vinagre *et al*., 2006). Thus, they probably perceived in diverse signals of internal cues as well as environmental stimuli to regulate the photoperiod response. However, their exact roles could not be concluded in this experiment.

In addition, calcium binding protein dependent-protein kinase including NPGR2 calmodulin-binding protein (CaM) was up-regulated while EF-hand or calmodulin like (CaM-like) protein kinase 41 was down-regulated. Both CaM and CaM-like are calcium sensors that specify the cellular response to different extracellular signals (Trewavas and Malho, 1998).

Calcium is a ubiquitous secondary messenger involved in the transduction of many environmental and developmental stimuli in plants (Trevalas, 1999). In response to diverse stimuli, cell generate transient increases in the concentration of cytosolic free Ca^{2++} (Ca^{2++}_{cyt}) that vary in amplitude, frequency, duration, and timing (Allen *et al*., 2001). The circadian oscillations in the concentration of cytosolic free Ca^{2++}_{cyt} are believed to be involved in signaling to or from the endogenous circadian clock (Gomez and Simon, 1995) but the mechanism remain unclear. However, it was considered in the signal transduction pathways of photoperiod response in this experiment. Friedman *et al.* (1989) showed that alteration of Ca^{2++}_{cyt} concentration was involved in floral transition in response to photoperiod changes. Although, our experiment did not detect the concentration changes of Ca^{2++}_{cyt}. The expression of calcium-dependent protein kinase acting as calcium sensor were altered, implying that the concentration of Ca^{2++}_{cyt} was changed in SD light. Thus, the results suggested that Ca^{2++} binding protein dependent-protein kinase activity involved in the photoperiod response acting as a calcium sensor.

In addition, it has recently been suggested that extracellular Ca^{2++} may have a role in regulating the switch to flowering through the action of a cell-surface Ca^{2++} sensor (Han *et al*., 2003).

Changing in the maintenance of the shoot apical meristem

The aerial part structure of higher plants derives from cells at the tip of the stem, the shoot apical meristem (SAM) (Carles and Fletcher, 2003). The SAM produces leaves and axillary meristem during vegetative phase and floral meristem during reproductive phase (Sharma and Fletcher, 2002). Once the SAM has been initiated, meristem maintenance is controlled by CLAVATA signal transduction pathway. Four genes encode key components pathway, i.e., *CLAVATA* 1 (*CLV1*), *CLV2*, *CLV3*, *WUSCHEL* (*WUS*) (Sharma and Fletcher, 2002). During the reproductive phase, the SAM produces floral meristems from its flanks, whereas its central pool of stem cells is maintained by CLV pathway. Signaling by CLV3 through CLV1 receptor complex limit the WUS expression. When it switches to floral transition, WUS is up-regulated to activate the expression of the floral meristem genes, MADS box *AGAMOUS* (*AG*) (Busch *et al*., 2000). WUS promotes stem cell proliferation in the floral meristem, and, together with the meristem identity gene *LEAFY* (*LFY*). In our experiment, *CLV3* gene was down-regulated, indicated that WUS gene was up-regulated in order to promote floral meristem genes. Consistently, floral meristem identity genes including *OsMADS box 14* or *AP like* were up-regulated. These results indicated that SD light induced floral transition via CLV signal pathway.

Post-translational control in photoperiod response

The ubiquitin proteasome system (UPS) is a key regulator of many biological processes in all eukaryotes. This mechanism employs several types of enzymes, the most important of which are the ubiquitin E3 ligase that catalyse the attachment of polyubiquitin chain to target proteins for their subsequent degradation by the 26S proteasome (Lechner *et al*., 2006). Among the E3 families, the SCF is the best understood; it consists of a multiple-protein complex in which the F-box protein plays a crucial role by recruiting the target substrate.

F-box proteins contain a conserved F-box domain (40-50 amino acid) at their N-terminus, which interacts with SUPPRESSOR OF KINETOCHORE PROTEIN1 (SKP1) while the C-terminus interacts with specific target protein (Jain *et al*., 2007a).

In our experiment, we found that expression of several F-box genes were altered by SD light (Table 4 and 5). Two of up-regulated F-box genes were known putative function

including *SLEEPY1* involved in GA signaling and *EID1-like3* or *ZGT like* involved in circadian clock. Two of down-regulated F-box genes were known putative function including *UNUSUAL FLORAL ORGAN* (*UFO*) involved in meristem identity and *CONSTITUTIVELY PHOTOMORPHOGENIC* (*COP1*) involved in circadian clock. Although, the others were unknown function, those may be involved in the photoperiod regulation.

ZGT like, or circadian coupling clock, is regulated by circadian clock and light. It is believed that ZGT plays a role in the endogenous circadian system. How it controls photoperiod response is unclear.

COP1 is a key repressor of photomorphogenesis in darkness by acting as E3 ubiquitin ligase in the nucleus, and is responsible for the targeted degradation of a number of photomorphogenesis-promoting factors, including phyA, HY5. Recently, Liu *et al.* (2008) reported that COP1 promoted CONSTANT (CO), a major regulator of photoperiodic flowering, degradation in darkness, whereas it is stabilized by cryptochrome-mediated signal in light. They proposed that COP1 acted as a repressor of flowering by promoting the unbiquitin-mediated proteolysis of CO in darkness and that CRY-mediated signal may negatively regulate COP1, thereby stabilizing CO, activating FT transcription, and inducing flowering.

The 'coincidence model' was proposed that LD can trigger flowering in *Arabidopsis* because the expression of CO coincidence with the exposure of plant to light (Roden *et al*., 2002; Yanovsky and Kay, 2002). CO protein is stabilized by light, while in darkness; it is rapidly degraded in the proteasome (Valerde *et al*., 2004). The combination of circadian regulation of CO transcript levels and light-induced stabilization of CO protein ensures that the CO protein accumulates exclusively under inductive LD conditions. But it is unclear to used the coincidence model to explain flowering time control in KDML105 (SD plant).

Furthermore, CO protein accumulation in the light depends on the light quality because CO accumulated in FR and B light, but not in R light (Valverde *et al*., 2004). This correlated well with the knowledge that FR light-perceiving photoreceptor phyA and B light-responsive cry2 promote flowering in LD, while the R light- photoreceptor phyB inhibit flowering (Hayama and Coupland, 2004). Besides the photoreceptor, light signaling intermediates affect photoperiodic flowering and *cop-1* mutant showed no delay in

flowering under SD condition in *Arabidopsis* (Nakagawa *et al*., 2004), indicating that COP1 was required for suppression of flowering in non-inductive condition. Thus, our results implied that COP1 protein regulated photoperiodic flowering by degradation of floral inducer protein that might be *Hd1* (homolog of *CO*). Moreover, *Hd1* transcript was not significant differentially expressed between SD and LD conditions from the microarray data. Thus, probably, *Hd1* was regulated in the posttranslational level in photoperiod response.

miRNA pathway and photoperiod response

Regulation of RNA metabolism is an important role in flowering time control in plant. A group of genes encoding RNA binding proteins (*FPA*, *FVE*, *FCA* and *FLK)* regulates flowering initiation through the autonomous pathway (Ausin *et al*., 2004; Macknight *et al*., 1997; Schomburg *et al*., 2001; Lim *et al*., 2004; Quesada *et al*., 2003). Interestingly, genes encoding RNA binding protein were found both in up- and down-regulation groups (Table 4, 5). This result indicated that posttranscriptional regulation was involved in photoperiod response and those genes might be involved in photoperiodic flowering pathway.

One of interesting genes, *AGO1* is a family member in the regulation of gene expression via the RNAi Silencing Complex (RISC). AGO1 acts within the *Arabidopsis* miRNA pathway, within the miRNA-program RISC (Vaucheret *et al*., 2004). *AGO1* was up-regulated by SD-light, suggesting that miRNA might be involved in photoperiod response. Moreover, miRNA targets were found in down-regulated genes such as *NAC*, *MYB* and *AP2* which supported this idea.

Three miRNAs have been functionally characterized in flowering time control including *miRNA156* (Schwab *et al*., 2005), *miRNA159* (Achard *et al*., 2004) and *miRNA172* (Aukerman and Sakai, 2003; Chen, 2003). In our study, we investigated the expression of *miRNA 172a* in photoperiod change and found that its expression was up-regulated under SD light. Consistent with this result, *AP2* family members known as a target of *miRNA172a* and floral repressor in photoperiod pathway were down-regulated. Thus, these results indicated that *miRNA172* was up-regulated by SD light, resulting in down-regulation of *AP2* transcripts and leading to floral transition in rice.

Jung *et al.* (2007) showed that *miRNA172* mediated photoperiodic flowering by regulating *FT* in *Arabidopsis*. The functional role of *miRNA172* depended on *GI,* demonstrating that *miRNA172* belongs to a unique photoperiod flowering pathway. Their results supported our idea.

AGO1 is involved in miRNA pathway and their roles include control of flowering time. Kidner and Martienssen (2005) showed that *ago1* had no effect to flowering time, but *AGO1* was required for full expression of *LEAFY* (*LFY)*, *AP1* (*APETALA1*) and AGMOUS (*AG*).

The transcriptional regulation of photoperiod responsive genes

Transcriptional control is of critical importance in mediating the responses of plant to external stimuli including photoperiod response. The promoter of gene is important in determining if and when transcription will be initiated (Hudson *et al*., 2003). The organization of *cis*-regulatory elements on the promoter determines the recruitment of DNA-binding protein which regulates transcription of gene (Babu *et al*., 2004). Thus, *cis*-regulatory elements of photoperiod responsive promoters were further identified. The result showed that the common feature of them is the specific combination of LREs, HREs and SREs (Table 6). This indicated that the regulatory mechanism of photoperiod response is controlled by light in coordination with hormones and stresses. Thus, the transcription of photoperiod responsive genes was specifically influented not only by light but also by various hormones and stresses. This result supports the microarray data as described on above.

The comparison of those elements, light responsive elements (LREs) were enriched both in up- and down-regulated genes. LREs, which commonly occur in light-regulatory promoters, are essential for light controlled transcriptional activity (Jiao *et al*., 2007). Thus, this data showed that the expression of photoperiod responsive genes was mainly controlled by LREs in coordination with HREs and SREs. This agreed with the obtained from microarray data in which the expression of some hormone signaling and stress responsive genes were altered by SD light.

Among the LREs, G-box which is required in the function of phytochrome regulated promoter via the recognition of PIF3 (Martinez-Garcia *et al*., 2000), was prominent both in

up-and down-regulated genes. Thus, this observation supported that photoperiod responsive genes were mainly controlled by phytochrome. In addition, circadian elements were found in both two sets of promoters. These data indicated that some photoperiod responsive genes were controlled by circadian clock as also reported by Young and Kay (2001).

Obviously, no single element of LREs was found in any photoperiod regulated promoter, suggesting that a combination of light regulatory network is required for the photoperiod response. Thought, the specific combination of LREs could not be identified in this experiment, because of its complexity, this study is the first report to show the characteristic of the photoperiod responsive promoter.

By finding conserved regions between multiple promoters, motif may be identified with no prior knowledge of transcription factor-binding sites. The promoters of co-regulated genes are likely to be responsive to the same pathway and therefore to share commonly regulatory motifs, providing a potential way to discover new mechanism of transcriptional regulation. In this work, Oligo-analysis program was used to elucidate the novel *cis*-regulatory elements that are specific response to daylength by their over-representation in the promoter sets of SD induced and SD suppressed genes. The results showed that the novel GC-rich element was specific to SD induced (LD light condition) while the novel A-rich element was specific to SD suppressed genes (SD light condition).

Interesringly, the novel A-rich element is highly homology to the over-representative element obtained from LD orthologous genes in *Arabidopsis*. It suggested that rice and *Arabidopsis* might have some conserved regulatory mechanisms in response to LD light. The A-rich element was found to contain in the promoter of genes whose products to have diverse biological functions (Table 9), suggesting that novel A-rich element plays a role in coordination with various pathway during LD light response in rice. Moreover, the novel A-rich element was found in the promoter of the putative floral repressor genes, i.e., *AP2*, *Vip14*, *FAR1*, *COP1* and *ARR1 like*. *FAR1, COP1* and *ARR1 like* are also involved in phyA signaling and circadian rhythm (Hoecker and Quail, 2001; Lin and Wang, 2004; Hazen *et al*., 2005). In addition, the novel A-rich element is similar to the CCA1 motif 2 binding site which required for circadian rhythm controlled genes (McClung, 2006). These evidences supported that novel A-rich is involved in the regulation of phyA and circadian rhythm to inhibit flowering during non-inductive condition. Thus, we proposed that the novel A-rich

element is one of the key *cis*-regulatory elements in a specific daylength to control photoperiodic flowering in rice.

In contrast, the novel GC element is not homology to the over-representative element obtained from SD orthologous gene in *Arabidopsis*, indicating that this element is unique to rice. Interestingly, this element was found in the promoter of pathogen resistant genes (*mla* and *multidrung resistant protein 8*). Although, the correlation between plant pathogen and photoperiod has not been reported, pathogen infection is known to accelerate flowering (Korves and Bergelson, 2003). In addition, the promoter of *SLY1* was also found to contain GC element, suggesting that this element plays a positive role in the regulation of GA signaling. GA is known as a growth regulator to promote flowering. Taken altogether, this result implied that GC element is possibly involved in the regulation of photoperiodic flowering in rice.

It should be noted that the most of SD induced genes that contain GC elements are unknown genes (Table 10). These genes might have a specific role in response to SD light in rice. Their functions and regulatory mechanisms might clarify the mystery of different response to daylength shown by SD and LD plant.

Flowering time genes in photoperiod response

Several flowering time associated genes were identified from the microarray data including floral inducer and floral repressor genes. (Table 13) As expected, floral inducers were up-regulated and floral repressors were down-regulated. Moreover, *Hd3a,* photoperiodic flowering time genes, was also included in the inducer genes. Thus, this result supported that KDML 105 is induced to floral transition in response to SD light.

The rice *Hd3a*, ortholog of *Arabidopsis FT* gene, belongs to a family of ten known rice *FT-like* genes that are mainly expressed in leaves (Izawa *et at*., 2003). The previous report revealed that *Hd3a* promote flowering (heading) under SD condition and its function downstream of *Hd1,* a key component of photoperiod pathway. Hayama and Coupland (2004) showed that *Hd1* repressed *Hd3a* expression in LD condition but promoted *Hd3a* expression in SD, leading to flowering. Thus, *Hd1* and *Hd3a* became the central interest to photoperiodic regulation of flowering in rice. However, interpreting the exact role of *Hd3a* and *Hd1* was complicated, and *Hd1* transcript was not detected as differentially

expressed gene because the expression ratio was less than 2 fold changes (1.7). Thus, the key component of photoperiod regulation remained to be investigated in the future. In addition, *Hd6* encoding CKII α kinase, the other component of photoperiod pathway, was not detected as differentially expressed genes in our experiment. But the other locus of CK2 α subunit was up-regulated. Interestingly, *Hd6* was reported to inhibit flowering in rice under LD condition (Takahashi *et al*., 1998), but CK2 kinase in *Arabidopsis* promoted flowering under LD conditionn (Sugano et al., 1999). These results suggested the different role of phosphorylation in photoperiod response. Thus, further analysis will clarify the function of CK2 in both rice and *Arabidopsis*.

In addition to photoperiodic component pathway, the floral transition genes including three MADS boxes were up-regulated by SD light (Table 13). The phylogenetic tree analysis showed that *OsMADS1* was grouped to *Arabidopsis SEPALATA* (*SEP*) genes while two MADS box transcription factors (*AY332478* , *AK069331*) were grouped in *SQUA-like* genes (or *AP1*).

In *Arabidopsis*, *SEP* genes and *AP1* participate in flower meristem identity and organ identity (Ditta *et al*., 2004; Ferrandiz *et al*., 2000). Recent report showed that the expression of *AP1* in the floral meristem was directly activated by a complex of FT and FD proteins (Abe *et al*., 2005; Wigge *et al*., 2005). In our experiment, *Hd3a* was induced in day 6 while *AP1 like* was induced in day 10. This results suggested that *AP1 like* was downstream of *Hd3a*. Thus, by analogy to *Arabidopsis, AP1 like* genes were activated by *Hd3a* and induced floral transition.

In constrast, putative floral repressor *AP2* family members were down-regulated. These gene included *RCBF3* (*AY3345233*), *RCBF2* (*AY345234*) and AP2 containing protein (*AK062882* and *9636.m03709*) (Table 13).

AP2 (APETALA2) and EREBPs (ethylene-responsive element binding protein) are the prototype members of a family of transcription factors unique to plant whose distinguishing characteristic is that they contain AP2 DNA-binding domain. They play a variety of roles throughout the plant life cycle such as being regulators of several development procresses and responding to biotic and environmental stresses, and etc. These proteins contain either one (EREBP subfamily) or two (AP2 subfamily) copies of an

approximately 70 amino acid domain termed the AP2 repeat because of its initial description in the floral homeotic protein APETALA2 (AP2) (DRTF;http://drtf.cbi.pku.edu.cn).

Proteins that have a single AP2 repeat include EREBPs/ethylene response factors (ERFs), C-repeat/dehydration response element binding proteins (CBFs/DREBs), ABI4 and TINY (DRFT database). In our experiment, all AP2 family members are in EREBP subfamily. Although, they have not been reported for their function in floral repressor in rice, phylogenetic analysis showed that they fall within the same clade as *AP2* floral repressor of *Arabidopsis*, *TOE1, TOE2*, *SMZ* and *SNZ* (Data not shown). Furthermore, *RCBF3* (*AY3345233*) and *RCBF2* (*AY345234*) are high homology to *AT1g63030* and *AT4g25470*, DREB-subfamily A-1 of ERF/AP2 in *Arabidopsis* and overexpression of *AT1g63030* and *AT4g25470* showed late flowering phenotype, suggesting that they are floral repressor (TAIR; Magome *et al*., 2004). Thus, these results suggested that *AP2* family members in rice were putative floral repressors as in *Arabidopsis*.

Taken all together, the photoperiod controlling of flowering time pathway in KDML 105 has been proposed as shown in figure 14.

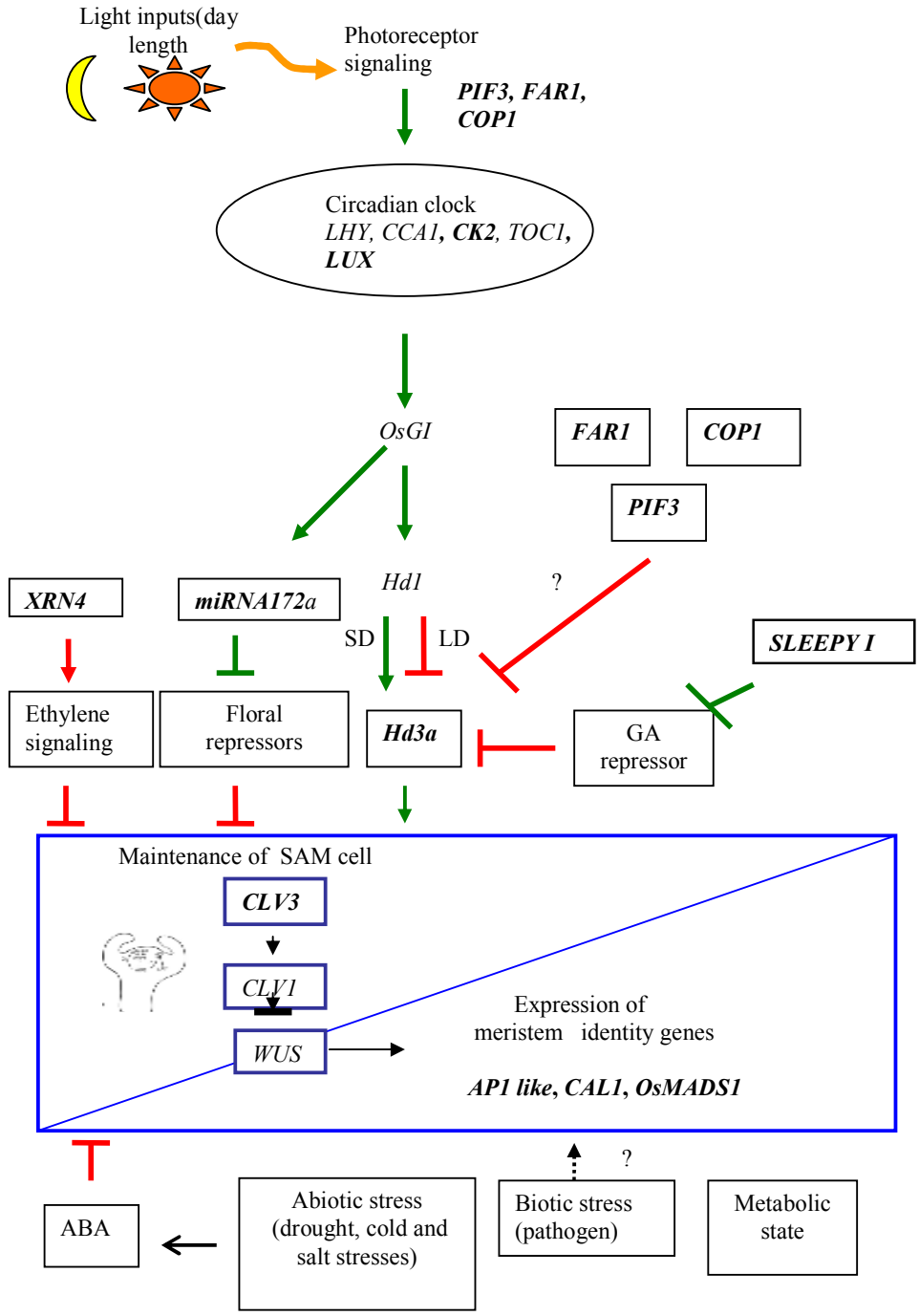


Figure 14 Putative genetic control of photoperiodic flowering time pathway in KDML 105 responding to daylength. Arrows indicate activation and T-bars depict inhibition. Green lines indicate positive regulation whereas red lines indicate negative regulation of flowering. Bold text indicates genes that were identified in this experiment. Question marks denote activities for which there is currently unclear.

CONCLUSION

GeneChip rice genome arrays from Affymetrix containing 55,515 probe sets were used to identify photoperiod responsive genes of rice (*Oryza sativa* L.) KDML 105. A comparative analysis of gene expression changes between SD and LD light was performed with three independent biological replications. A total of 184 probe sets were selected as differentially expressed genes on the basis of their expression ratios with t-test p value (≥ 2 fold changes, $p \leq 0.05$) and were verified with RT-PCR and real-time PCR. Among them, 79 genes were up-regulated while 105 were down-regulated. These genes were classified into nine classes from their putative functions, i.e., 24% unknown, 18% transcription factor, 15% defense/stress, 11% metabolism, 8% growth/structure, 9% processing, 8% signaling, 6% transport and 1% energy transduction of up-regulated genes; 25% unknown, 21% transcription factors, 11% signaling, 10% defense/stress, 9% processing, 8% energy transduction, 9% metabolism, 4% growth/structure and 3% transport of down-regulated genes. The pathway analysis showed that the photoperiod response was involved in photosynthesis, carbohydrate metabolism, circadian clock and phytochrome signaling, plant hormone signaling and miRNA synthesis pathways. Several flowering time related genes were also associated in those pathways; the floral inducers were up-regulated while the floral repressors were down-regulated including the targets of miRNAs such as *AP2* floral repressor. The expression of miRNA of *AP2*, *miRNA172a,* was further investigated. Its expression was induced by SD light, suggesting that *miRNA 172a* is involved in the regulation of photoperiodic floweing through the down-regulation of *AP2* floral repressor. The component of photoperiodic flowering pathway was analyzed. Monitoring expression of floral inducer genes (*Hd3a*, *AP1 like*) and floral repressor genes (*AP2*), during SD induction showed that *Hd3a* and *AP1* like were indued in day 6 and day 10, respectively, while *AP2* was repressed in day 4. This indicated that *AP1* was a downstream of *Hd3a* and their expression were possibly acrivated by the down-regulation of *AP2*.

In addition, the transcription control of photoperiod responsive genes was also analyzed. The *cis*-regulatory elements on their promoters were investigated using bioinformatics tools. The results revealed that the transcription of photoperiod responsive genes was controlled mainly by LREs in coordination of HREs and SREs. Analysis of LREs showed that photoperiod responsive genes were controlled by phytochrome and circadian clock. Moreover, phyA regulated genes were further identified using the specific organization of *cis*-regulatory elements on their promoters. The data showed that phyA is

involved in the transcription of flowering time genes either by activation of floral inducers or suppression of floral repressors. So far, the novel cis-regulatory elements which are specific to daylength in rice were identified in this experiment. The novel A-rich element is specific to LD light involved in the regulation of phyA and circadian rhythm to inhibit flowering whereas the novel GC element is specific to SD light involved in the regulation of GA signaling to promote flowering. Thus, photoperiodic flowering in rice, KDML 105 is controlled, partially, by phyA in coordination with A-rich and GC-elements.

In conclusion, the photoperiodic flowering pathway of rice, KDML 105 was proposed as showed in figure 14. The flowering transition is controlled by phyA and circadian clock in coordination with hormone signaling, stress responses and metabolic state. In SD induction, GA signaling is induced whereas ABA and ethylene signaling are suppressed. The floral development is switched by down-regulated *AP2* by *miRNA172a* and up-regulated *Hd3a* leading to activate floral meristem identity gene, *AP1 like*, *CAL* and *OsMADS1,* via the meristem maintenance CLAVATA pathway.

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APPENDIX

Appendix Figure 1 KDML 105 under SD (A) and LD conditions (B).

Guidelines for assessing data quality of GeneChip DNA microarray

The purpose of this section is to help researchers establish quality control processes for GeneChip expression analyses. To achieve this, Affymetrix has developed several controls which allow researchers to monitor assay data quality. The following are a series of quality control parameters associated with assay and hybridization performance.

Probe array image (.dat) inspection

Inspect for the presence of image artifacts (i.e., high/low intensity spots, scratches, high regional, or overall background, etc.) on the array

B2 Oligo performance

The boundaries of the probe area (viewed upon opening the .dat/.cel file) are easily identified by the hybridization of the B2 oligo, which is spiked into each hybridization cocktail.

B2 Oligo serves as a positive hybridization control and is used by the software to place a grid over the image. Variation in B2 hybridization intensities across the array is normal and does not indicate variation in hybridization efficiency. If the B2 intensities at the checkerboard corners are either too low or high, or are skewed due to image artifacts, the grid will not align automatically. The user must align the grid manually using the mouse to click and drag each grid corner to its appropriate checkerboard corner.

Average background and noise values

The Average Background and Noise (Raw Q) values can be found either in the Analysis Info tab of the Data Analysis (.chp) file, or in the Expression Report (.rpt) file. Although there are no official guidelines regarding background, Affymetrix has found that typical Average Background values range from 20 to 100 for arrays scanned with the GeneChip® Scanner 3000. Arrays being compared should ideally have comparable background values. Noise (Raw Q) is a measure of the pixel-to-pixel variation of probe cells on a GeneChip array. The two main factors that contribute to noise are:1) Electrical

noise of the scanner and 2) Sample quality.

Each scanner has a unique inherent electrical noise associated with its operation. Since a significant portion of Noise (Raw Q) is electrical noise, values among scanners will vary. Array data (especially those of replicates) acquired from the same scanner should ideally have comparable Noise values.

Poly-A Controls: *lys, phe, thr, dap*

Poly-A RNA controls can be used to monitor the entire target labeling process. *Dap, lys, phe,thr*, and *trp* are *B. subtilis* genes that have been modified by the addition of poly-A tails, and then cloned into pBluescript vectors, which contain T3 promoter sequences. Amplifying these poly-A controls with T3 RNA polymerase will yield sense RNAs, which can be spiked into a complex RNA sample, carried through the sample preparation process, and evaluated like internal control genes. The GeneChip® Poly-A RNA Control Kit (P/N 900433) contains a pre-synthesized mixture of *lys, phe, thr,* and *dap*. The final concentrations of the controls, relative to the total RNA population, are: 1:100,000; 1:50:000; 1:25,000; 1:7,500, respectively. All of the Poly-A controls should be called "Present" with increasing Signal values in the order of *lys, phe, thr, dap.*

Hybridization controls: *bioB, bioC, bioD,* and *cre*

BioB, bioC and *bioD* represent genes in the biotin synthesis pathway of *E. coli*. *Cre* is the recombinase gene from P1 bacteriophage. The GeneChip® Eukaryotic Hybridization Control Kit (P/N 900299 and 900362) contains 20x Eukaryotic Hybridization Controls that are composed of a mixture of biotin-labeled cRNA transcripts of *bioB, bioC, bioD*, and *cre,* prepared in staggered concentrations (1.5 pM, 5 pM, 25 pM, and 100 pM final concentrations for *bioB, bioC, bioD*, and *cre*, respectively).The 20x Eukaryotic Hybridization Controls are spiked into the hybridization cocktail,independent of RNA sample preparation, and are thus used to evaluate sample hybridization efficiency on eukaryotic gene expression arrays. *BioB* is at the level of assay sensitivity (1:100,000 complexity ratio) and should be called "Present" at least 50% of the time. *BioC,bioD*, and *cre* should always be called "Present" with increasing Signal values, reflecting their relative concentrations.

Internal control Genes

For the majority of GeneChip® expression arrays, β-actin and GAPDH are used to assess RNA sample and assay quality. Specifically, the Signal values of the 3' probe sets for actin and GAPDH are compared to the Signal values of the corresponding 5' probe sets. The ratio of the 3' probe set to the 5' probe set is generally no more than 3 for the 1-cycle assay. Since the Affymetrix eukaryotic expression assay has an inherent 3' bias (i.e., antisense cRNA is transcribed from the sense strand of the synthesized ds cDNA, via the incorporated T7 promoter), a high 3' to 5' ratio may indicate degraded RNA or inefficient transcription of ds cDNA or biotinylated cRNA. 3' to 5' ratios for internal controls are displayed in the Expression Report (.rpt) file. The 2-cycle assay typically gives higher 3' to 5'ratios than the 1-cycle assay, due to the additional cycle of amplification. There are occasions when the 3' to 5' ratio of one internal control gene is normal, but the 3' to 5' ratio of another internal control gene is high. This discrepancy in 3' to 5' ratios is most likely due to a specific transcript-related or image artifact issue and is not an indication of overall sample and assay quality.

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