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Isolation of Rice Genes Possibly Involved in the Photoperiodic Control of Flowering by a Fluorescent Differential Display Method

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To better understand the molecular mechanisms of the photoperiodic regulation of rice, a short-day plant, we isolated 27 cDNAs that were differentially expressed in the photoperiod-insensitive *se5* mutant from approximately 8,400 independent mRNA species by the use of a fluorescent differential display (FDD). For this screening, we isolated mRNAs at five different time points during the night and compared their expression patterns between *se5* and the wild type. Of 27 cDNAs isolated, 12 showed diurnal expression patterns often associated with genes involved in the determination of the flowering time. In *se5*, expression of nine cDNAs was increased. Five of these cDNAs were up-regulated under SD, suggesting that they may promote flowering under SD. They included genes encoding a cDNA containing a putative NAC domain, the fructose-bisphosphate aldolase, and a protease inhibitor. Expression of three cDNAs was decreased in *se5* but not photoperiodically regulated. These cDNAs included a rice homolog of Arabidopsis *GIGANTEA* (*GI*), *lir1*, and a gene for *myo*-inositol 1-phosphate synthase, all of which were previously shown to be under the control of circadian clocks. The expression patterns of the rice homolog of *GI*, *OsGI*, were similar to those of the Arabidopsis *GI*, suggesting the conservation of some mechanisms for the photoperiodic regulation of flowering between these two species.

Key words: Circadian clock — FDD — Flowering time — Rice.

Abbreviations: *GI*, *GIGANTEA*; FDD, fluorescent differential display; LD, long day; SD, short day.

Introduction

The photoperiodic control of flowering has been long studied with many long-day (LD) and short-day (SD) plants (Thomas and Vince-Prue 1997). Molecular genetic studies with *Arabidopsis thaliana*, a LD plant, have recently identified a number of genes involved in the photoperiodic control of flowering. There are two primary classes: one includes those encoding photoreceptors, and the other includes those involved in circadian clock functions, inputs or outputs of circadian clocks. Major photoreceptors involved in the photoperiodic control of flowering in Arabidopsis are phytochromes and cryptochromes (Lin 2000). *PHYA* promotes flowering under LD conditions (Johnson et al. 1994), while *PHYB* inhibits flowering under both LD and SD conditions (Goto et al. 1991). *CRY2* promotes flowering in LD (Guo et al. 1998). Antagonistic interaction of *PHYB* and *CRY2* has been suggested based on the results obtained by the analysis of mutants (Guo et al. 1998, Lin 2000). In another LD plant, the pea, the *phyA* mutation causes late flowering under LD conditions; however, under SD conditions, the plant flowers normally (Weller et al. 1997).

The second class of genes influencing the day-length control of flowering in Arabidopsis includes *ELF3*, *LHY*, *CCA1*, *GI*, *TOC1*, *ZTL*, and *FKF1*, all of which exhibit circadian rhythms at the mRNA levels (Fowler et al. 1999, Park et al. 1999, Hicks et al. 1996, Schaffer et al. 1998, Wang and Tobin 1998, Somers et al. 2000, Nelson et al. 2000, Strayer et al. 2000, Alabadi et al. 2001). These genes are thought to act either as inputs or components of circadian clocks. On the other hand, *CO*, *FT*, and *SOC1* (Putterill et al. 1995, Kobayashi et al. 1999, Kardailsky et al. 1999, Samach et al. 2000) are most likely to be regulators of the photoperiodic control of flowering, acting downstream of circadian clocks. The mRNAs of *CO* and *FT* are under the control of circadian clocks (Suarez-Lopez et al. 2001), and *CO* has been suggested to be a mediator of circadian clocks and the control of flowering (Suarez-Lopez et al. 2001).

Despite extensive analysis of the day-length control of flowering in Arabidopsis, little is known regarding genes involved in the control of flowering time in SD plants although a large number of physiological studies have been carried out with several SD plants (Thomas and Vince-Prue 1997). It has been shown that the *phyB* mutation of sorghum causes early flowering in LD plants and decreases photoperiodic sensitivities (Childs et al. 1997). The rice *se5* mutant, which lacks functional phytochromes due to a mutation in an enzyme for chromophore biosynthesis, was shown to completely lack photoperiod sensitivity (Izawa et al. 2000). A mutation in a rice ortholog of *CO* termed *Hd1* has been shown to cause early flowering in LD plants, which decreases the photoperiod sensitivity (Yano et al. 2001). The rice *Hd6* gene identified by QTL analysis was shown to encode the α subunit of protein kinase CK2 (Takahashi et al. 2001). Another QTL locus, *Hd3a*, was

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shown to encode a homolog of the Arabidopsis *FT* gene (Kojima et al. 2001). Although a limited number of genes involved in the photoperiodic control of flowering have been isolated in rice, the results obtained so far seem to indicate a conservation of the flowering-time genes in LD and SD plants (Samach and Gover 2001).

Since rice is the most amenable system for molecular biological studies among SD plants and in order to understand the photoperiodic control of flowering in SD plants, we isolated genes that are possibly involved in the photoperiodic control of flowering by using an FDD method for mRNA. For this screening, we used an *se5* mutant that is completely photoperiod-insensitive and characterized cDNAs that are differentially regulated in *se5* during the night. Results of the analysis indicate that we isolated cDNAs that were photoperiodically regulated and those that showed diurnal expression patterns. Among the isolated cDNAs, we found a homolog of the Arabidopsis *GI* gene. The expression patterns of isolated genes suggest that they may function either as promoters or suppressors of flowering.

Results

A strategy for screening the genes of the rice flowering time by an FDD method using an early-flowering se5 mutant

To initiate the screening of rice genes involved in the photoperiodic control of flowering, we used *se5* mutants which are completely deficient in the photoperiodic response. The *SE5* gene encodes a heme oxygenase involved in the synthesis of phytochrome chromophore and is a rice counterpart of the Arabidopsis *HY1* gene (Izawa et al. 2000). Under LD conditions, which suppress flowering in rice, *se5* plants flowered extremely early, ca. 47 d after sowing, whereas wild-type plants flowered ca. 100 d after sowing (Izawa et al. 2000). Under SD conditions, *se5* plants flowered slightly earlier than the wild type. No alterations in the rhythmic expression of circadian clock-controlled genes were detected in the *se5* mutant, suggesting that the clock function in this mutant is not impaired (T. Izawa et al., unpublished results). We thought that genes that play roles in the photoperiodic control of flowering in rice may exhibit altered expression levels or patterns during the night since the length of the night is a critical determinant of flowering time (Thomas and Vince-Prue 1997). Therefore, we collected RNAs from the leaves of wild-type and *se5* plants at 0 h and four time points with 2 h intervals between 16 h and 22 h (Fig. 1A).

For FDD, we followed a published protocol (Kuno et al. 2000) and used four anchored oligo (dT) primers, each in combination with 80 arbitrary primers, and screened ca. 32,000 cDNA bands. The representative results of such screening are shown in Fig. 1B. Some bands clearly showed changes in their intensity during the night, as indicated by arrowheads in Fig. 1B. Among those bands whose abundance changed with time, some exhibited changes in patterns of expression or intensity in

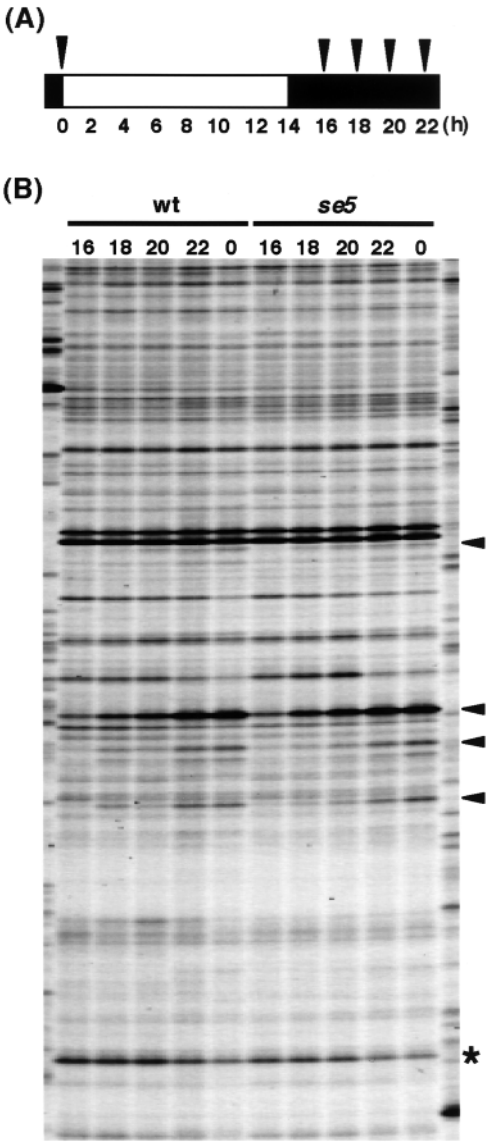

Fig. 1 Screening of *SE5*-regulated genes by the FDD method. (A) Time schedule for sampling of leaves from wild-type and *se5* plants. (B) Acrylamide gel electrophoresis of PCR-products. The arrows indicate bands for which signal intensities were altered during the time course. The asterisks indicate bands for which the signal intensity was altered in *se5* relative to the wild type.

se5 mutants compared with wild type. They are indicated by asterisks in Fig. 1B. We identified a total of 10 cDNA bands whose expression was up-regulated in *se5* mutants and 17 cDNA bands which were down-regulated in *se5* (Table 1). We confirmed the expression patterns of these cDNA bands by experiments using RNase protection assays.

In our FDD screening, the same cDNAs were repeatedly detected in separate experiments using different primer combinations. Therefore, we excluded redundant cDNA clones by subjecting the RT-PCR products to Southern blot analysis with

Table 1 Summary of the FDD screening

	Total		Genes showing diurnal expression	
	No. of bands	No. of non-redundant clones	No. of bands	No. of non-redundant clones
Up-regulated in *se*5	32	10	31	9
Down-regulated in *se*5	67	17	18	3
Unchanged	~32,000		~3,200 [a]	

[a] Bands showing changes in signal intensity during night as indicated in Fig. 1.

cloned cDNAs as probes. Results of Southern blot analyses indicated that 27 cDNA clones, which were finally identified as differentially regulated cDNAs, came from 99 PCR-amplified bands, suggesting that those screened 32,000 cDNA bands may have originated from ca. 8,400 distinct mRNA species. Our results also indicate that ca. 0.3% of mRNAs expressed in leaves of the *se5* plants under LD conditions were differentially regulated in the night.

Identification of differentially regulated cDNA clones that showed diurnal expression patterns

The photoperiodic control of flowering is thought to be regulated by the rhythmic expression of genes over 24 h in a day. A number of Arabidopsis genes involved in the photoperiodic control of flowering have been demonstrated to be under the control of circadian clocks (Hicks et al. 1996, Schaffer et al. 1998, Wang and Tobin 1998, Fowler et al. 1999, Park et al. 1999, Somers et al. 2000, Nelson et al. 2000, Strayer et al. 2000, Harmer et al. 2000, Suarez-Lopez et al. 2001). Therefore, to further screen for those cDNA clones that are more likely to be involved in the photoperiodic control of flowering, we screened those cDNAs whose expression showed diurnal changes in a day by using RNase protection assays. For these experiments, we collected leaf RNAs from wild-type and *se5* plants every 4 h for a 24 h period and subjected them to RNase protection assays. Of 27 non-redundant cDNA clones examined, 12 exhibited diurnal expression patterns (Fig. 2 and Table 1). Peaks of their expressions varied from clone to clone, and

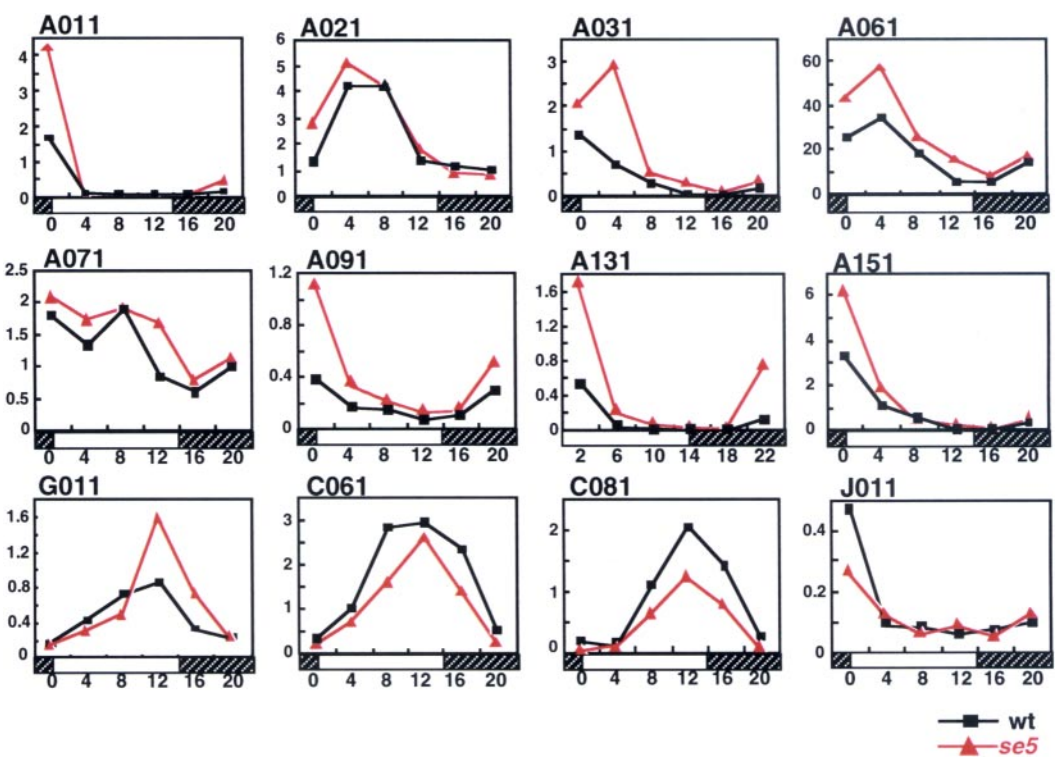

Fig. 2 Expression patterns of 12 isolated genes which showed diurnal expression. Wild-type (wt) and *se5* plants were grown for 28 d under LD conditions. RNA was isolated from their leaves and used for an RNase protection assay. The vertical line indicates signal intensity normalized by the ubiquitin signal. The horizontal axis indicates the time during a day.

Table 2 Sequence characteristics of diurnally expressed genes

Gene name	Best homology: accession number
Up-regulated in *se5*	
A011	Arabidopsis unknown protein: AAF24535
A021	Arabidopsis putative B-box zinc-finger protein: CAB38816
A031	Rice putative NAC-domain protein: CAB55403
A061	Rice fructose-bisphosphate aldorase (AldP): Q40677
A071	Arabidopsis putative O-GlcNAc transferase: AAC25927
A091	— [a]
A131	Rice proteinase inhibitor: AAB17095
A151	Rice putative pyruvate dehydrogenase kinase: AAG46146
G011	— [a]
Down-regulated in *se5*	
C061	*GIGANTEA* homolog of rice: CAB56058
C081	Rice *lir1* gene: S33632
J011	Rice *myo*-inositol phosphate synthase (RINO1): AB012107

[a] Showed no significant homology.

seven clones (A011, A031, A071, A091, A131, A151, and J011) were shown to peak at dawn, two clones (A021 and A061) at midday, and three clones (G011, C061, and C081) at dusk. The peak positions of these genes were not greatly altered in *se5* mutants compared with the wild type except for A031, whose expression in *se5* showed a clear peak at midday, while, in the wild type, it peaked at dawn.

Identity of differentially regulated genes showing diurnal expression patterns

Since results of the expression analysis suggested that 12 selected cDNAs that showed diurnal changes in their expression patterns may be involved in the photoperiodic control of flowering in rice, we determined the complete sequences of these cDNAs and found their identities based on the database (Table 2). The sequences of five cDNA clones (A061, A151, C061, C081, and J011) completely matched those of the known rice cDNA sequences in the database. For the cDNAs that showed no homologies with the known sequences, we applied 5′ and 3′ RACE methods to obtain full-length cDNAs and determined their complete DNA sequences (A011, A021, A031, A091, A131, and G011). For A071, no full-length cDNA was isolated from this clone.

A021—This cDNA has homology with an Arabidopsis protein in the database having a putative B-box type zinc-finger. A B-box type zinc-finger was first identified in some oncogenes and transcription factors of mammals (Borden 1998) and is known to contain many cysteine residues. Although the Arabidopsis CO protein contains this type of zinc-finger motif (Putterill et al. 1995) and since the deduced amino acid sequences of A021 lacked a C-terminal motif highly conserved among the *CO* gene family (Ledger et al. 2001), its function in the photoperiodic control of flowering in rice remains to be studied.

A031—The deduced amino acid sequence of A031 has high homology with a rice gene in the database that has an NAC domain. The NAC domain of A031 showed high homology with those of other NAC gene families (data not shown). The NAC domain has been found in diverse groups of plant proteins with a variety of functions, such as meristem fate determination, elongation of the flower organ, and auxin-regulated lateral root formation (Souer et al. 1996, Aida et al. 1997). Although the gene family of rice that has an NAC domain has been previously studied, this clone was not included (Kikuchi et al. 2000). Comparison of the A031 sequence with other known plant genes with NAC domains revealed that it has little homology with other plant NAC gene families except for the NAC domain (data not shown).

A061—This cDNA encodes the rice chloroplastic fructose-bisphosphate aldolase, which is an important enzyme for CO_2 fixation (Tsutsumi et al. 1994). Studies with transgenic potato plants expressing an antisense construct indicate that plants with lower levels of this enzyme had decreased photosynthetic ability, starch accumulation, and sucrose contents (Haake et al. 1998). More recently, it was shown that an Arabidopsis gene for this enzyme is circadian-controlled and that its mRNA expression has a peak at evening (Harmer et al. 2000), suggesting a possible link of sugar metabolism with the determination of flowering time.

A071—This is a partial clone of 414 bp in length, and the 131 bp of the 5′ region has a significant homology with the 3′ region of a predicted Arabidopsis O-GlcNAc transferase gene. This enzyme is involved in the transfer of *N*-acetylglucosamine to various polypeptides. Sugar modification of proteins by this enzyme is considered to be one of the mechanisms of signal transduction (Wells et al. 2001). In Arabidopsis, the *SPY* gene

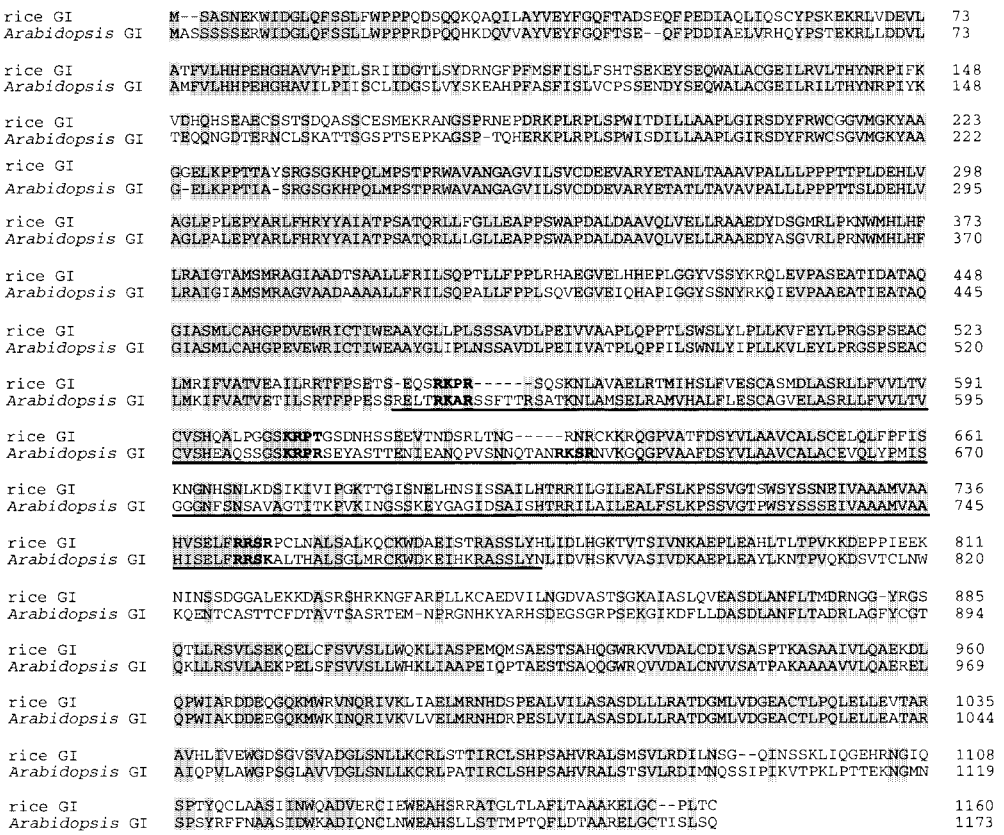

Fig. 3 Comparison of amino acid sequences between Arabidopsis and rice *GI*. Amino acids similar to Arabidopsis *GI* are shaded. The region for the nuclear localization of the GI protein is underlined. The clusters of basic amino acids are indicated by bold letters.

involved in gibberellin signaling has been shown to encode the O-GlcNAc transferase protein (Jacobsen et al. 1996).

A131—The deduced amino acid sequence of A131 showed a high homology with a protease inhibitor of rice. This rice protease inhibitor has a high identity with γ-thionin, which was shown to have antifungal activity (Florack and Stiekema 1994, Epple et al. 1997).

A151—This cDNA encodes pyruvate dehydrogenase kinase (PDHK), which is a negative regulator of the mitochondrial pyruvate dehydrogenase complex (mtPDC). PDC catalyzes the conversion of pyruvate to acetyl-CoA, and PDHK phosphorylates PDC, leading to the inactivation of the enzyme. Thus, mtPDC acts as a link between the glycolytic carbon metabolism and the TCA cycle. Genes for PDHK have been isolated from maize (Thelen et al. 1998a) and Arabidopsis (Thelen et al. 1998b). Transgenic Arabidopsis plants expressing an antisense PDHK gene caused alteration of vegetative growth and early flowering (Zou et al. 1999). Increased mtPDC activity as well as the increased activities of the TCA cycle enzymes in the antisense plants led the authors to propose that the altered phenotypes resulted from an increased TCA cycle activity.

C061—This cDNA was shown to encode a rice homolog of the Arabidopsis *GIGANTEA* gene (*GI*), *OsGI*. The rice EST sequence covering most of *OsGI* has been previously published (Fowler et al. 1999), and we determined the complete sequence of *OsGI*. In Arabidopsis, loss of *GI* causes late flowering under LD conditions but has a negligible effect on flowering under SD conditions (Fowler et al. 1999). Therefore, *GI* is one of the key regulatory genes involved in the photoperiodic control of flowering in Arabidopsis. Its mRNA expression is under the control of the circadian clock (Fowler et al. 1999, Park et al. 1999) and was recently shown to activate *CO* mRNA expression (Suarez-Lopez et al. 2001).

Comparison of the deduced amino acid sequences of *GI* and *OsGI* revealed 67% identity, and regions identified for their nuclear localization (Huq et al. 2000) were conserved between Arabidopsis and rice *GI* genes (Fig. 3).

C081—This cDNA is identical with a previously identified rice *lir1* gene whose expression is activated by light and circadian clock-controlled (Reimmann and Dudler 1993). It was previously shown that the mRNA level of this gene reached its maximum at the end of the light period and its minimum at the end of the dark period. The oscillations of *lir1* mRNA persisted after the plants were transferred to continuous light and dark (Reimmann and Dudler 1993). The function

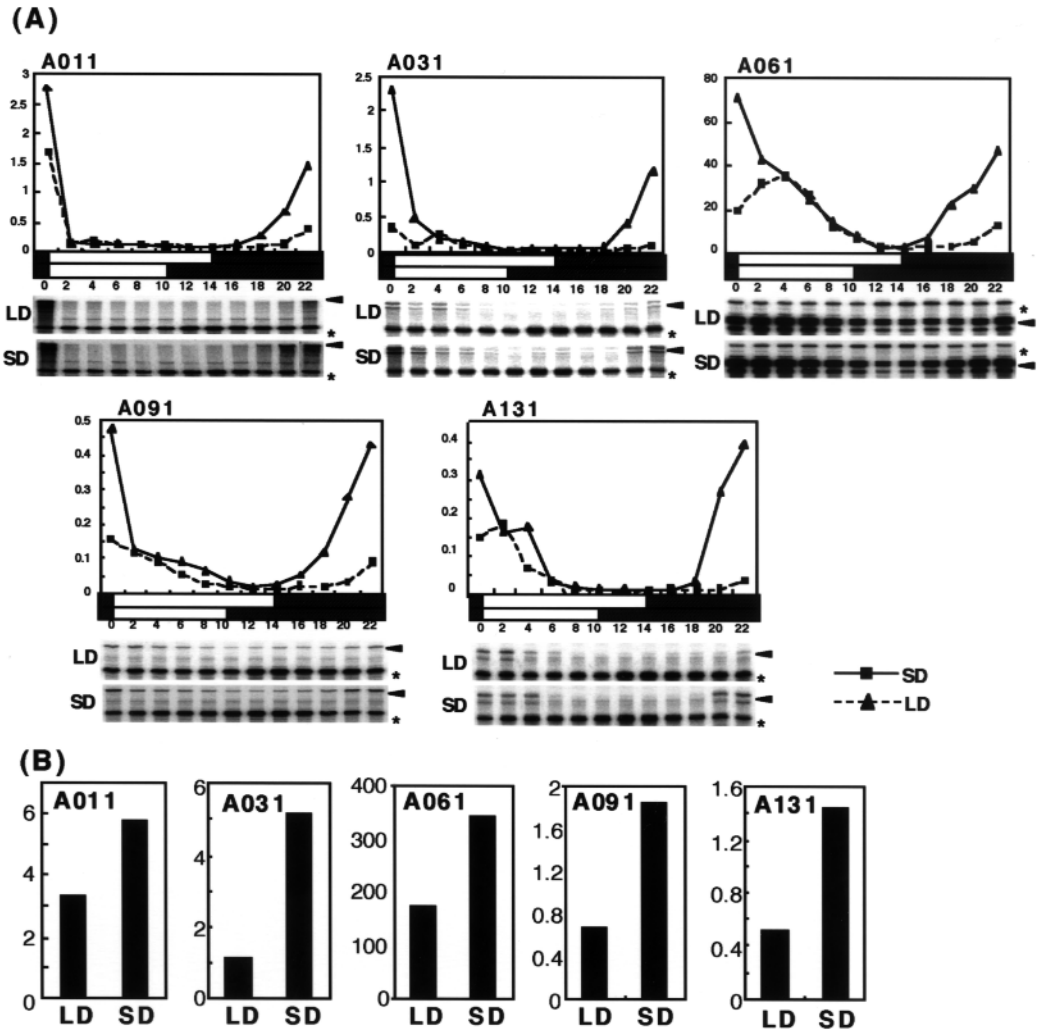

Fig. 4 Five isolated genes showing higher mRNA levels under SD conditions. Wild-type plants were grown for 47 d under LD and SD conditions. RNA was isolated from their leaves and used for the RNase protection assay. Signals of isolated genes are indicated by arrows, and the control ubiquitin signal is shown by an asterisk. The vertical line indicates the signal intensity normalized by the ubiquitin signal. The horizontal axis indicates the time during a day. (A) Diurnal expression patterns of the five genes under LD and SD conditions. (B) Total sum of the signal intensities of all time points analyzed under LD and SD conditions.

of *lir1* is not known.

J011—This cDNA encodes *myo*-inositol 1-phosphate synthase, which catalyzes the first step of *myo*-inositol synthesis by producing *myo*-inositol 1-phosphate from glucose-6-phosphate. cDNAs for this enzyme have been isolated from citrus, Arabidopsis, the ice plant, and rice (Abu-Abied and Holland 1994, Ishitani et al. 1996, Yoshida et al. 1999). *Myo*-inositol 1-phosphate is used to generate a variety of compounds, including phosphoinositides, which are involved in various types of cellular signaling, and various saccharides (Bohnert et al. 1995). The diurnal expression pattern of the *myo*-inositol 1-phosphate synthase gene has been shown for Arabidopsis and the ice plant (Abu-Abied and Holland 1994, Ishitani et al. 1996), and its expression was induced by salt stress (Ishitani et al. 1996).

Identification of cDNA clones whose expression patterns were photoperiodically regulated

Since photoperiodism is thought to be controlled by changes in the expression patterns of certain genes, it was expected that some rice cDNA clones identified by the FDD method would show changes in their expression patterns by photoperiods. In Arabidopsis, expression of flowering time genes such as *FT* and *SOC1* is increased under inductive LD conditions (Kobayashi et al. 1999, Kardailsky et al. 1999, Lee et al. 2000), and the expression patterns of *GI* and *CO* are different between LD and SD conditions (Fowler et al. 1999, Suarez-Lopez et al. 2001). Therefore, we examined the expres-

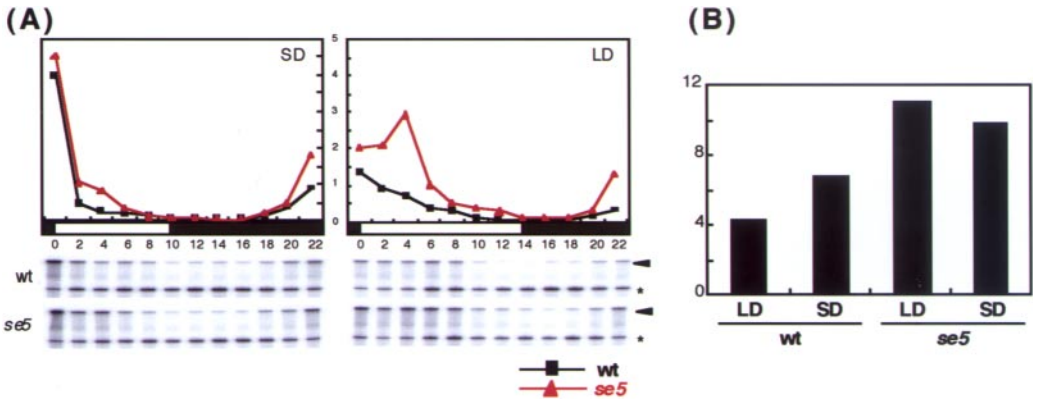

Fig. 5 Expression patterns of A031 under SD and LD conditions. Wild-type (wt) and *se5* plants were grown for 28 d under LD and SD conditions. RNA was isolated from their leaves and used for the RNase protection assay. The A031 signal is indicated by an arrow, and the ubiquitin signal is shown by an asterisk. The vertical line indicates the signal intensity of A031 normalized by the ubiquitin signal. The horizontal axis indicates the time during a day. (A) Diurnal pattern of A031 expression under SD and LD conditions. (B) Total sum of the signal intensities of all time points analyzed under LD and SD conditions for the wild-type and the *se5* mutant.

sion profiles of isolated cDNA clones over 24 h by RNase protection assays under SD and LD conditions.

In these experiments, we identified five cDNA clones that showed differential expression patterns under SD and LD conditions (Fig. 4). Interestingly, the expression patterns of these five genes were similar; in other words, they all peaked at dawn or midday, and their expression began to increase during the night. Their expression levels, however, varied greatly among different cDNAs (Fig. 4A). In A061 and A131, their expression patterns under LD were 4 and 2 h delayed relative to those in SD, respectively. In contrast, the peaks of A011, A031, and A091 coincided with the lights-on period and remained low during the day. Their expression patterns did not change under SD or LD conditions. The total mRNA abundance of these five genes was higher under SD than LD conditions (Fig. 4B), suggesting that they might represent genes whose role in flowering is to promote flowering under SD.

Since these three genes showed peaks of expression at dawn and had similar expression under SD or LD conditions, we thought that light might suppress their expression. Thus, if the *SE5* gene is involved in the light suppression of their gene expression, we may see differences in the expression patterns in the *se5* mutants relative to the wild type. To perform such experiments, we chose A031, whose expression was high under SD conditions. The results of these experiments indicated that, in *se5* mutants, the expression pattern of A031 under LD conditions was delayed 4 h relative to the wild type, whereas it was not different in the mutants and the wild type under SD conditions (Fig. 5). These results suggested that the functional *SE5* gene suppresses A031 expression during the day. Furthermore, when the total sum of mRNA expression was compared between the two conditions, it was lower under SD than LD conditions in the wild type, as expected. Interestingly, in *se5* mutants under LD conditions, it was slightly higher than under SD conditions, which corresponded well with the flowering time of *se5* mutants under LD and SD conditions.

OsGI mRNA expression was controlled by photoperiods, and it showed circadian rhythms

Arabidopsis *GI* mRNA was previously shown to peak toward the end of the day and when grown under LD conditions, and its peak was delayed toward the night (Fowler et al. 1999). To see if the expression patterns of *OsGI* were similar to those of Arabidopsis *GI*, we examined the mRNA abundance of *OsGI* over 24 h under SD and LD conditions. The results of the experiments showed that its expression patterns were very similar in Arabidopsis and rice (Fig. 6A), suggesting that similar mechanisms for the photoperiodic regulation of *GI* mRNA expression may operate in rice and Arabidopsis.

We also examined whether stable circadian rhythms were observed in *OsGI* mRNA levels (Fig. 6B). Results showed that robust circadian rhythms were observed in *OsGI* mRNA levels under both constant light and constant dark after entrainment with light/dark cycles for 5 d. The observed circadian oscillations of *OsGI* mRNA were similar to those of the *CAB1R*::*luc* gene in rice previously analyzed by bioluminescence imaging (Sugiyama et al. 2001).

Discussion

The se5 mutation affects the expression patterns of a subset of light-regulated genes

The expression of many plant genes is controlled by circadian clocks. A recent study using microarrays showed that 6% of the Arabidopsis genes examined exhibited circadian changes in mRNA levels (Harmer et al. 2000). In our study, approximately 10% (3,200/32,000) of the transcripts isolated from rice leaves showed diurnal expression patterns (Table 1). In contrast, only 1.5% (49/3,200) of the total cDNAs showed altera-

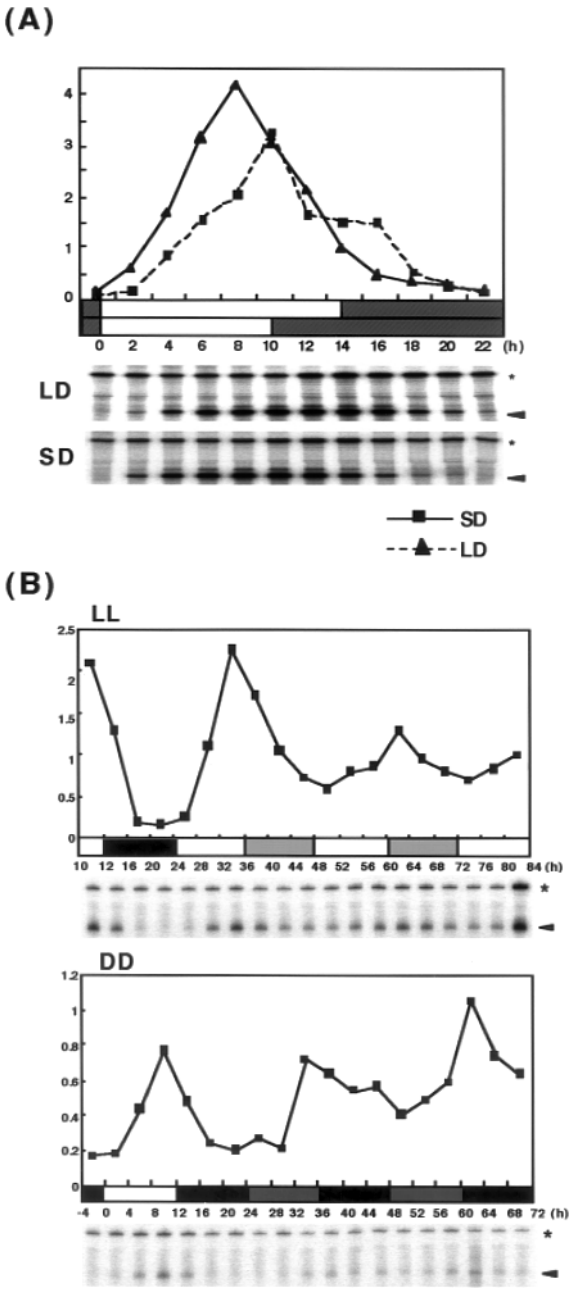

Fig. 6 Expression patterns of C61 (*OsGI*) under SD and LD and circadian rhythms of *OsGI* mRNA. Wild-type plants were grown for 47 d under LD and SD conditions. RNA was isolated from their leaves and used for the RNase protection assay. The rice *GI* signal is indicated by an arrow, and the ubiquitin signal is shown by an asterisk. The vertical line indicates the signal intensity of rice *GI* normalized by the ubiquitin signal. The horizontal axis indicates the time. (A) Expression patterns of C61 (*OsGI*) under SD and LD. (B) Circadian rhythms of *OsGI* mRNA. LL, constant light after L/D entrainment; DD, constant dark after L/D entrainment.

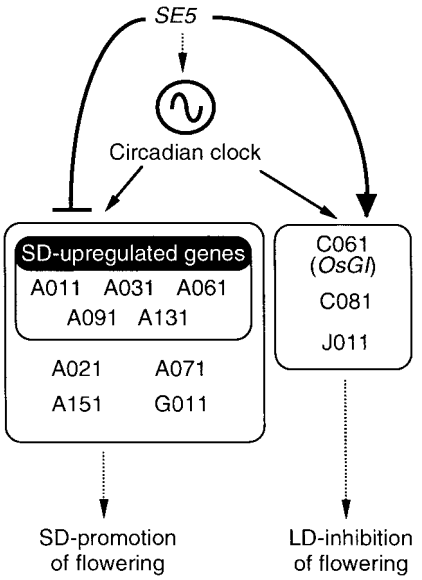

Fig. 7 Possible roles of isolated genes in the photoperiodic regulation of flowering in rice. Nine genes for which expression of mRNA was increased and three genes for which expression of mRNA was decreased in the early-flowering *se5* mutant were isolated. The former genes may function to promote flowering under SD conditions. The expression patterns of the five SD-up-regulated genes are consistent with the model that they promote flowering under SD conditions. In contrast, the second group of genes, including *OsGI*, may function to inhibit flowering under LD conditions.

tions in their expression patterns by the *se5* mutation (Table 1). These results suggest that the *se5* mutation affects only a subset of circadian clock-controlled genes. This is consistent with our unpublished results showing that circadian regulation of several rice genes is not altered in the *se5* mutant.

It has been shown that the expression of many genes involved in photosynthesis, such as *cab*, *rbcS*, and *psbA*, is light-inducible and shows circadian rhythms (Harmer et al. 2000). It is also well established that phytochromes are required for the greening of etiolated tissues. However, we did not detect any photosynthesis-related genes in our FDD analysis. This finding suggests that the expression patterns of those genes were not greatly altered in the *se5* mutant. In fact, although *se5* plants are pale when they are young, they become fully green when they mature (Yokoo and Okuno 1993, Izawa et al. 2000). Therefore, photosynthetic genes are normally expressed in the *se5* mutants. Similar observations are made with the Arabidopsis *hy1* mutant, in which the expression levels of *cab*, *rbcS*, and *psbA* are essentially the same as those of the wild type (Chory et al. 1989).

SE5-regulated genes showing diurnal expression patterns

Three cDNAs identified in this study encode enzymes involved in normal metabolism of the cell. A061 encodes the chloroplastic fructose-biphosphate aldolase. Analysis of transgenic potato plants expressing the antisense fructose-biphosphate aldolase gene showed that plants with lowered fructose-biphosphate aldolase activity exhibited a decrease in their

starch content as well as reduced sugars in addition to several intermediates in the Calvin cycle (Haake et al. 1998). Previous observations that treatments of plants with sucrose and glucose influence flowering time in Arabidopsis (Zhou et al. 1998, Ohto et al. 2001) suggest the possibility that alterations in carbohydrate metabolism caused by changes in the expression patterns of these genes may cause some effects on the photoperiodic control of flowering in the rice *se5* mutant.

A151 encodes pyruvate dehydrogenase kinase, which regulates the TCA cycle and respiration. J011 encodes *myo*-inositol 1-phosphate synthase, which converts glucose-6-phosphate to *myo*-inositol-1-phosphate, which is used in turn to produce a variety of inositol compounds for cellular signaling as well as saccharides. However, no molecular evidence for the involvement of these genes in the photoperiodic regulation of flowering has been obtained yet.

Rice homolog of the Arabidopsis GI gene

The fact that we were able to isolate the rice homolog of the Arabidopsis *GI* gene showed the validity of our FDD screen for genes involved in the photoperiodic control of flowering in rice, since *GI* is one of the key regulators of flowering time in Arabidopsis (Fowler et al. 1999). Interestingly, the expression patterns of rice and Arabidopsis *GI* genes are both photoperiodically regulated and are very similar under LD and SD conditions (Fig. 7, Fowler et al. 1999), suggesting the conservation of molecular mechanisms for regulation of *GI* gene expression in these two species. However, because rice *GI* was up-regulated in the *se5* mutant its role in photoperiodic control of flowering in rice may be opposite to that in Arabidopsis. The fact that the rice *GI* expression was altered in the *se5* mutant indicates that it is regulated by phytochromes. *GI* may function to regulate the light input of circadian clocks (Fowler et al. 1999, Park et al. 1999). In this respect, the recent finding that the Arabidopsis *gi* mutations affect phytochrome signaling (Huq et al. 2000) is of interest. The temporal pattern of the rice *GI* gene expression in *se5* was not drastically altered relative to the wild type in LD conditions, under which flowering is extremely early in *se5* plants, suggesting that the expression pattern of *GI* does not cause early flowering in *se5*. It has been shown that *CO* and *FT* genes act downstream of *GI* in the floral induction of Arabidopsis under LD conditions. Thus, the interaction of *GI* and those downstream factors may be critical in controlling the photoperiodic regulation of flowering both in rice and Arabidopsis. Recent isolation of *CO* and *FT* homologs as regulators of the photoperiodic control of flowering in rice (Yano et al. 2001, Kojima et al. 2001) provides exciting possibilities for the comparative genomics of flowering time regulation in these two model species.

Possible roles of isolated genes in the photoperiodic regulation of flowering in rice

Based on their expression patterns, the 12 isolated genes may be categorized into two groups. Although all of these genes showed diurnal expression patterns, changes in their expression patterns are not caused by a general impairment of the clock function in the *se5* mutant, which is not altered in the mutant (T. Izawa et al., unpublished results). The first group includes nine genes whose expression is up-regulated in the early-flowering *se5* mutant. These genes potentially promote flowering. Among those, the expression of five genes was up-regulated under SD conditions, suggesting that they are possibly involved in the photoperiodic induction of flowering under SD conditions. They included genes encoding a cDNA containing a putative NAC domain (A031), fructose-bisphosphate aldolase (A061), and a protease inhibitor (A131).

The second group includes three genes whose expression was down-regulated in the *se5* mutant. Thus, they may encode inhibitors of flowering, although none of them was up-regulated under LD conditions. It is also possible that they affect processes which are independent of flower induction. These three genes were *OsGI*, *lir1*, and a gene for *myo*-inositol phosphate synthase, all of which were shown to be under the control of circadian clocks. The functions of these isolated genes should be identified by the analysis of transgenic plants either over-expressing or inhibiting these genes. Such an analysis is in progress.

Materials and Methods

Plant materials and growth conditions

Norin 8 and *se5* (Yokoo and Okuno 1993, Izawa et al. 2000) were grown in climate chambers with 24-h temperature cycles (12 h, 30°C for the day/12 h, 25°C for the subjective night). Plants at 28 d after germination were used for RNA extraction. The fluence rates of light measured with a spectroradiometer (model LI-1800, Licor, Lincoln, NE, U.S.A.) were ≈300 μmol m^{-2} s^{-1} (400–750 nm) under LD and SD conditions.

Fluorescent differential display

FDD was performed as described previously (Kuno et al. 2000) with some modifications. Total RNA was treated with DNase I (in Message Clean™ Kit, GenHunter, Nashville, TN, U.S.A.) to remove genomic DNA. First-strand cDNAs were synthesized from each total RNA (2.5 μg) using four different oligo(dT) primers (5′-GT_{15}VA-3′, 5′-GT_{15}VT-3′, 5′-GT_{15}VC-3′, 5′-GT_{15}VG-3′, V = G, C, and A, Takara, Kyoto, Japan) and a SuperScript Preamplification System (GIBCO-BRL, Gaithersburg, MD, U.S.A.). cDNAs produced from 25 ng of total RNA were amplified by PCR using combinations of four Texas Red-labeled 3′-anchored oligo(dT) primers and arbitrary 10-mer primers (Operon Technologies, Alameda, CA, U.S.A.). The conditions for PCR were as follows: 94°C for 3 min, 40°C for 5 min, and 72°C for 5 min, followed by 25 cycles of 95°C for 15 s, 40°C for 2 min, and 72°C for 1 min, with an additional extension step at 72°C for 10 min. PCR products were electrophoresed on 4% polyacrylamide gel. The detection of signals was performed with FMBIO II Multi-View (Takara, Kyoto, Japan).

Cloning of candidate PCR fragments

PCR products from FDD were electrophoresed, and the candidate bands were excised from the gel. The PCR fragments were eluted into distilled water, reamplified by PCR under the conditions described above, and then subcloned into a pGEM-T vector (Promega, Madison,

WI, U.S.A.). For each PCR fragment, several independent *E. coli* colonies were chosen, and their plasmid vectors were isolated; then, their inserts were used as a probe for Southern-blot analysis of the original products from FDD to select the vector with an insert corresponding to the target band. RNase protection assays were performed in order to remove false positive clones as described below.

Expression analyses

RNase protection assays were performed with an RPA III Kit (Ambion, Austin, TX, U.S.A.). RNA probes were synthesized with T7 or SP6 RNA polymerase (Promega, Madison, WI, U.S.A.). The relative radioactivity of those of A011, A031, A091, and A131 was increased by raising the concentration of [α-^{32}P]UTP and reducing the concentration of cold UTP in their reaction cocktails. RNA probes were purified by electrophoresing their reactants on 4% polyacrylamide gel, excising corresponding bands from the gel, and then eluting them in a Probe Elution Buffer (in RPA III Kit). 30,000 cpm of the purified RNA probe was added in a reaction buffer containing 5 μg of total RNA and annealed with RNA overnight, and protected RNA probes were detected on 4% polyacrylamide gel with BAS 2000 (Fuji Photo Film, Tokyo, Japan). Gel images were analyzed using the MacBAS ver. 2.52 analyzing software to calibrate the signal intensity of each clone. The sizes of the protected RNA were 245 bp for UBQ (accession no. D12629), 634 bp for A011, 501 bp for A021, 383 bp (in Fig. 2 and Fig. 3) or 516 bp (in Fig. 4) for A031, 178 bp for A061, 414 bp for A071, 531 bp for A091, 355 bp for A131, 105 bp for A151, 84 bp for C061 (rice *GI*), 220 bp for C081, 145 bp for G011, and 214 bp for J011.

5′- and 3′-RACE analyses

3′-RACE was performed with a Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA, U.S.A.). 5′-RACE was performed with a 5′ RACE System for the Rapid Amplification of cDNA Ends ver. 2.0 (GIBCO-BRL, Gaithersburg, MD, U.S.A.).

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References

Abu-Abied, M. and Holland, D. (1994) Two newly isolated genes from citrus exhibit a different pattern of diurnal expression and light response. *Plant Mol. Biol.* 26: 165–173.

Aida, M., Ishida, T., Fukaki, H., Fujisawa, H. and Tasaka, M. (1997) Genes involved in organ separation in Arabidopsis: An analysis of the *cup-shaped cotyledon* mutant. *Plant Cell* 9: 841–857.

Alabadi, D., Oyama, T., Yanovsky, M.J., Harmon, F.G., Mas, P. and Kay, S.A. (2001) Reciprocal regulation between *TOC1* and *LHY/CCA1* within the *Arabidopsis* circadian clock. *Science* 293: 880–883.

Bohnert, H.J., Nelson, D.E. and Jensen, R.G. (1995) Adaptations to environmental stresses. *Plant Cell* 7: 1099–1111.

Borden, K.L. (1998) RING fingers and B-boxes: Zinc-binding protein-protein interaction domains. *Biochem. Cell Biol.* 76: 351–358.

Childs, K.L., Miller, F.R., Cordonnier-Pratt, M.M., Pratt, L.H., Morgan, P.W. and Mullet, J.E. (1997) The sorghum photoperiod sensitivity gene, Ma_3, encodes a phytochrome B. *Plant Physiol.* 113: 611–619.

Chory, J., Peto, C.A., Ashbaugh, M., Saganich, R., Pratt, L. and Ausubel, F. (1989) Different roles for phytochrome in etiolated and green plants deduced from the characterization of *Arabidopsis thaliana* mutants. *Plant Cell* 1: 867–880.

Epple, P., Apel, K. and Bohlmann, H. (1997) Overexpression of an endogenous thionin enhances resistance of Arabidopsis against *Fusarium oxysporum*. *Plant Cell* 9: 509–520.

Florack, D.E. and Stiekema, W.J. (1994) Thionins: Properties, possible biological roles, and mechanisms of action. *Plant Mol. Biol.* 26: 25–37.

Fowler, S., Lee, K., Onouchi, H., Samach, A., Richardson, K., Morris, B., Coupland, G. and Putterill, J. (1999) *GIGANTEA*: A circadian clock-controlled gene that regulates photoperiodic flowering in *Arabidopsis* and encodes a protein with several possible membrane-spanning domains. *EMBO J.* 18: 4679–4688.

Goto, N., Kumagai, T. and Koornneef, M. (1991) Flowering responses to light-break in photomorphogenic mutants of Arabidopsis thaliana, a long-day plant. *Physiol. Plant.* 83: 209–215.

Guo, H., Yang, H., Mockler, T.C. and Lin, C. (1998) Regulation of flowering time by *Arabidopsis* photoreceptors. *Science* 279: 1360–1363.

Haake, V., Zrenner, R., Sonnewald, U. and Stitt, M. (1998) A moderate decrease of plastid aldolase activity inhibits photosynthesis, alters the levels of sugars and starch, and inhibits growth of potato plants. *Plant J.* 14: 147–157.

Harmer, S.L., Hogenesch, J.B., Straume, M., Chang, H.S., Han, B., Zhu, T., Wang, X., Kreps, J.A. and Kay, S.A. (2000) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. *Science* 290: 2110–2113.

Hicks, K.A., Millar, A.J., Carre, I.A., Somers, D.E., Straume, M., Meeks-Wagner, D.R. and Kay, S.A. (1996) Conditional circadian dysfunction of the *Arabidopsis early-flowering 3* mutant. *Science* 274: 790–792.

Huq, E., Tepperman, J.M. and Quail, P.H. (2000) GIGANTEA is a nuclear protein involved in phytochrome signaling in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* 97: 9789–9794.

Ishitani, M., Mjumder, A.L., Bornhouser, A., Michalowski, C.B., Jensen, R.G. and Bohnert, H.J. (1996) Coordinate transcriptional induction of *myo*-inositol metabolism during environmental stress. *Plant J.* 9: 537–548.

Izawa, T., Oikawa, T., Tokutomi, S., Okuno, K. and Shimamoto, K. (2000) Phytochromes confer the photoperiodic control of flowering in rice (a short-day plant). *Plant J.* 22: 391–399.

Jacobsen, S.E., Binkowski, K.A. and Olszewski, N.E. (1996) SPINDLY, a tetratricopeptide repeat protein involved in gibberellin signal transduction in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* 93: 9292–9296.

Johnson, E., Bradley, M., Harberd, N.P. and Whitelam, G.C. (1994) Photoresponses of light-grown *phyA* mutants of Arabidopsis. *Plant Physiol.* 105: 141–149.

Kardailsky, I., Shukla, V.K., Ahn, J.H., Dagenais, N., Christensen, S.K., Nguyen, J.T., Chory, J., Harrison, M.J. and Weigel, D. (1999) Activation tagging of the floral inducer *FT*. *Science* 286: 1962–1965.

Kikuchi, K., Ueguchi-Tanaka, M., Yoshida, K.T., Nagato, Y., Matsusoka, M. and Hirano, H.Y. (2000) Molecular analysis of the NAC gene family in rice. *Mol. Gen. Genet.* 262: 1047–1051.

Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M. and Araki, T. (1999) A pair of related genes with antagonistic roles in mediating flowering signals. *Science* 286: 1960–1962.

Kojima, S., Monna, L., Fuse, T., Sasaki, T. and Yano, M. (2001) Map-based cloning of the rice photoperiod sensitivity QTL, *Hd3a*. *In* Plant & Animal Genome IX Conference 2001. pp. 116 (abstract).

Kuno, N., Muramatsu, T., Hamazato, F. and Furuya, M. (2000) Identification by large-scale screening of phytochrome-regulated genes in etiolated seedlings of Arabidopsis using a fluorescent differential display technique. *Plant Physiol.* 122: 15–24.

Ledger, S., Strayer, C., Ashton, F., Kay, S.A. and Putterill, J. (2001) Analysis of the function of two circadian-regulated *CONSTANS-LIKE* genes. *Plant J.* 26: 15–22.

Lee, H., Suh, S.S., Park, E., Cho, E., Ahn, J.H., Kim, S.G., Lee, J.S., Kwon, Y.M. and Lee, I. (2000) The AGAMOUS-LIKE 20 MADS domain protein integrates floral inductive pathways in *Arabidopsis*. *Genes Dev.* 14: 2366–2376.

Lin, C. (2000) Photoreceptors and regulation of flowering time. *Plant Physiol.* 123: 39–50.

Nelson, D.C., Lasswell, J., Rogg, L.E., Cohen, M.A. and Bartel, B. (2000) *FKF1*, a clock-controlled gene that regulates the transition to flowering in *Arabidopsis*. *Cell* 101: 331–340.

Ohto, M., Onai, K., Furukawa, Y., Aoki, E., Araki, T. and Nakamura, K. (2001)

Effects of sugar on vegetative development and floral transition in Arabidopsis. *Plant Physiol.* 127: 252–261.

Park, D.H., Somers, D.E., Kim, Y.S., Choy, Y.H., Lim, H.K., Soh, M.S., Kim, H.J., Kay, S.A. and Nam, H.G. (1999) Control of circadian rhythms and photoperiodic flowering by the *Arabidopsis GIGANTEA* gene. *Science* 285: 1579–1582.

Putterill, J., Robson, F., Lee, K., Simon, R. and Coupland, G. (1995) The *CONSTANS* gene of Arabidopsis promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. *Cell* 80: 847–857.

Reimmann, C. and Dudler, R. (1993) Circadian rhythmicity in the expression of a novel light-regulated rice gene. *Plant Mol. Biol.* 22: 165–170.

Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F. and Coupland, G. (2000) Distinct roles of CONSTANS target genes in the reproductive development of *Arabidopsis*. *Science* 288: 1613–1616.

Samach, A. and Gover, A. (2001) Photoperiodism: The consistent use of CONSTANS. *Curr. Biol.* 11: 651–654.

Schaffer, R., Ramsay, N., Samach, A., Corden, S., Putterill, J., Carre, I.A. and Coupland, G. (1998) The late elongated hypocotyl mutation of *Arabidopsis* disrupts circadian rhythms and the photoperiodic control of flowering. *Cell* 93: 1219–1229.

Somers, D.E., Schultz, T.F., Milnamow, M. and Kay, S.A. (2000) *ZEITLUPE* encodes a novel clock-associated PAS protein from *Arabidopsis*. *Cell* 101: 319–329.

Souer, E., van Houwelingen, A., Kloos, D., Mol, J. and Koes, R. (1996) The *no apical meristem* gene of Petunia is required for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. *Cell* 85: 159–170.

Strayer, C., Oyama, T., Schultz, T.F., Raman, R., Somers, D.E., Mas, P., Panda, S., Kreps, J.A. and Kay, S.A. (2000) Cloning of the *Arabidopsis* clock gene *TOC1*, an autoregulatory response regulator homolog. *Science* 289: 768–771.

Suarez-Lopez, P., Wheatley, K., Robson, F., Onouchi, H., Valverde, F. and Coupland, G. (2001) *CONSTANS* mediates between the circadian clock and the control of flowering in *Arabidopsis*. *Nature* 410: 1116–1120.

Sugiyama, N., Izawa, T., Oikawa, T. and Shimamoto, K. (2001) Light regulation of circadian clock-controlled gene expression in rice. *Plant J.* 26: 607–615

Takahashi, Y., Shomura, A., Sasaki, T. and Yano, M. (2001) *Hd6*, a rice quantitative trait locus involved in photoperiod sensitivity, encodes the α subunit of protein kinase CK2. *Proc. Natl. Acad. Sci. USA* 98: 7922–7927.

Thelen, J.J., Mienyk, J.A. and Randall, D.D. (1998b) Nucleotide and deduced amino acid sequences of the pyruvate dehydrogenase kinase from *Arabidopsis thaliana*. *Plant Physiol.* 118: 1533.

Thelen, J.J., Muszynski, M.G., Mienyk, J.A. and Randall, D.D. (1998a) Molecular analysis of two pyruvate dehydrogenase kinase from maize. *J. Biol. Chem.* 273: 26618–26623.

Thomas, B. and Vince-Prue, D. (1997) *Photoperiodism in Plants*. Academic Press, London.

Tsutsumi, K., Kagaya, Y., Hidaka, S., Suzuki, J., Tokairin, Y., Hirai, T., Hu, D.L., Ishikawa, K. and Ejiri, S. (1994) Structural analysis of the chloroplastic and cytoplasmic aldolase-encoding genes implicated in the occurrence of multiple loci in rice. *Gene* 141: 215–220.

Wang, Z.Y. and Tobin, E.M. (1998) Constitutive expression of the *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) gene disrupts circadian rhythms and suppresses its own expression. *Cell* 93: 1207–1217.

Weller, J.L., Murfet, I.C. and Reid, J.B. (1997) Pea mutants with reduced sensitivity to far-red light define an important role for phytochrome A in day-length detection. *Plant Physiol.* 114: 1225–1236.

Wells, L., Vosseller, K. and Hart, G.W. (2001) Glycosylation of nucleocytoplasmic proteins: Signal transduction and O-GlcNAc. *Science* 291: 2376–1278.

Yano, M., Katayose, Y., Ashikari, M., Yamanouchi, U., Monna, L., Fuse, T., Baba, T., Yamamoto, K., Umehara, Y., Nagamura, Y. and Sasaki, T. (2001) *Hd1*, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the Arabidopsis flowering time gene *CONSTANS*. *Plant Cell* 12: 2473–2484.

Yokoo, M. and Okuno, K. (1993) Genetic analysis of earliness mutations induced in the rice cultivar Norin 8. *Jpn. J. Breed.* 43: 1–11.

Yoshida, K.T., Wada, T., Koyama, H., Mizobuchi-Fukuoka, R. and Naito, S. (1999) Temporal and spatial patterns of accumulation of the transcript of *myo*-inositol-1-phosphate synthase and phytin-containing particles during seed development in rice. *Plant Physiol.* 119: 65–72.

Zhou, L., Jang, J.C., Jones, T.L. and Sheen, J. (1998) Glucose and ethylene signal transduction crosstalk revealed by an *Arabidopsis* glucose-insensitive mutant. *Proc. Natl. Acad. Sci. USA* 95: 10294–10299.

Zou, J., Qi, Q., Katavic, V., Marillia, E.F. and Taylor, D.C. (1999) Effects of antisense repression of an Arabidopsis thaliana pyruvate dehydrogenase kinase cDNA on plant development. *Plant Mol. Biol.* 41: 837–849.

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