Plant Biotechnology 24, 201–207 (2007)

Original Paper

Isolation and characterization of novel genes controlled by short-day treatment in *Pharbitis nil*

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Received October 12, 2006; accepted October 30, 2006 (Edited by M. Umeda)

Abstract The short-day plant *Pharbitis* [*Ipomoea*] *nil* has been a model plant in physiological studies of photoperiodic control of floral initiation. However, molecular mechanisms underlying photoperiodic responses, including the induction of flowering in *P. nil*, are largely unknown. Here we identified and characterized cDNAs whose expression patterns were different in short- and long-day conditions, based on fluorescent differential display and RNA gel blot analysis, to gain insight into the molecular mechanisms of photoperiodic responses in *P. nil*. The cDNA clones included genes encoding ADP-glucose pyrophosphorylase, a putative protein kinase, eukaryotic initiation factor eIF-4, a putative aldehyde dehydrogenase, subtilisin-like proteinase, crooked neck (crn)-like protein, and cryptochrome 1. The possible roles of these genes in the photoperiodic response, including the control of flowering time in *P. nil*, are discussed.

Key words: Flowering, *Ipomoea nil*, *Pharbitis nil*, photoperiod, short-day plant.

Fluctuations in day length affect the developmental processes and behavior of many organisms. This phenomenon is called photoperiodism and allows detection of seasonal changes and anticipation of future environmental conditions. Photoperiodism was first described in detail by Garner and Allard (1920). They found that many plants flowered in response to changes in day length. Plants in which flowering requires, or is accelerated by, short-day (SD) or long-day (LD) photoperiods are referred to as short-day plants (SDPs) or long-day plants (LDPs), respectively. *Arabidopsis* is a facultative LDP, and rice is a facultative SDP. Recent molecular-genetic approaches have identified a number of genes that are required for the day-length response in *Arabidopsis* and rice, some of which encode regulatory proteins that are specifically involved in the regulation of flowering or are components of light input pathways or circadian clocks (Yanovsky and Kay 2003; Searle and Coupland 2004).

GIGANTEA (*GI*), *CONSTANS* (*CO*), and *FLOWERING LOCUS T* (*FT*) regulate the photoperiodic control of flowering downstream of the circadian clock (Fowler et al. 1999; Putterill et al. 1995; Kobayashi et al. 1999; Kardailsky et al. 1999). *GI* and *CO* exhibit diurnal rhythms in mRNA expression and have different phase shapes in plants grown under LD than plants grown under SD (Fowler et al. 1999; Suárez-López et al. 2001). The expression of the *FT* gene, which promotes flowering, is activated only under inductive LD conditions (Kobayashi et al. 1999; Kardailsky et al. 1999). The rice ortholog of *GI* (*OsGI*) was isolated by a differential-display method and was shown to play important roles in photoperiodic flowering (Hayama et al. 2002, 2003).

Recent studies have revealed several differences in the photoperiodic induction mechanisms of flowering in *Arabidopsis* and rice (Yano et al. 2000; Kojima et al. 2002; Izawa et al. 2002, 2003; Hayama et al. 2003; Doi et al. 2004). These differences include not only the regulation of orthologous genes, but also differences in genes and pathways. Therefore, other plant systems should be studied for further information on variations in the molecular mechanisms of this process.

Pharbitis nil Choisy cv. Violet, a typical SDP, is ideal for the study of the early events in the photoperiodic induction of flowering. Young, light-grown seedlings can be induced to flower by exposure to a single 16-h dark period (Vince-Prue and Gressel 1985). An experiment in which cotyledons were removed indicated that the floral stimulus sufficient for minimum flowering is transmitted from the leaves to the shoot apex between 14 and 16 h after the start of the inductive dark period (Zeevaart 1962). These observations suggest that changes in the expression levels of certain genes that occur specifically during the inductive dark period participate in the generation of the floral stimulus. Recently, a *P. nil* homolog of *CO*, *PnCO*, was isolated in a differential-display screen designed to identify genes with increased

Abbreviations: DD, continuous dark; FDD, fluorescent differential display; LD, long day; LL, continuous light; NB, night break; SD, short day.

expression under SD conditions (Liu et al. 2001). Over-expression of *PnCO* rescued the late-flowering phenotype of the *co* mutant in *Arabidopsis*, suggesting that *PnCO* may be a key regulator of flowering in *P. nil* (Liu et al. 2001). Although several attempts have been made to identify the genes involved in photoperiodic responses, including the induction of flowering in *P. nil* (Lay-Yee et al. 1987; Ono et al. 1993, 1996; Zheng et al. 1993; O'Neill et al. 1994; Sage-Ono et al. 1998; Kim et al. 2003), molecular mechanisms underlying these processes are largely unknown.

In this study, we isolated novel genes in *P. nil* whose expression patterns differed in SD and LD using FDD. Our results suggest that these genes may play key roles in photoperiodic responses, including the induction of flowering in *P. nil*.

Materials and methods

Plant materials and growth conditions

Seeds of *Pharbitis nil* Choisy cv. Violet (purchased from Marutane Co., Kyoto, Japan) were soaked in concentrated sulfuric acid for 30 min, with occasional stirring, and then rinsed in running tap water for 1 h. The seeds were then soaked in distilled water for 16 h and sown on wet vermiculite for germination. The growth conditions were a temperature of 24±1°C with continuous illumination of cool-white fluorescent light (60 μmol s^{-1} m^{-2}, FL 40SS W/37 lamps, Matsushita Electronics Co., Tokyo, Japan). Once the cotyledons were fully opened (6 d after the sulfuric-acid treatment), the seedlings were subjected to photoperiodic treatments. The treatments began with a dark period, and the elapsed time was counted from the beginning of the dark period. LED panels (EYELA, Tokyo, Japan) were used to provide a 10-min red light exposure for the night break (NB) treatment in the middle of the 16-h dark period. Cotyledons and other organs were excised, frozen in liquid nitrogen, and stored at −80°C. Harvesting of tissues during the dark period was performed in complete darkness. To determine the extent of flower induction, the plants were scored 3 wk after the photoperiodic treatments for the presence of terminal and axillary flowers or vegetative buds.

Fluorescent differential display (FDD)

Total RNA was isolated using the phenol/SDS method (Ausubel et al. 1987). For FDD analysis, total RNA (50 μg) was treated with RNase-free DNase I using the Message Clean kit (GenHunter Corporation, Nashville, TN) for 1 h at 37°C to eliminate contaminating DNA. RT-PCR was carried out with a Fluorescence Differential Display Kit (rhodamine version; TaKaRa, Kyoto, Japan) according to the manufacturer's instructions. First-strand cDNAs were synthesized from total RNA (300 ng per downstream primer) using nine different rhodamine-labeled downstream primers and AMV Reverse Transcriptase XL (Life Sciences, St. Petersburg, FL). Each of the nine cDNA samples was amplified by PCR in combination with 24 arbitrary upstream primers and the nine downstream primers that were used in the reverse transcription reaction. Thus, a total of 216 PCR operations (24×9) were performed. The PCR conditions were as follows: 94°C for 2 min, 40°C for 5 min, and 72°C for 5 min, followed by 34 cycles of 94°C for 30 sec, 40°C for 2 min, and 72°C for 1 min, and then an additional extension step at 72°C for 5 min. The amplified cDNA fragments were separated on 6% polyacrylamide gels containing 8 M urea and visualized using a Molecular Imager FX (Bio-Rad, Hercules, CA, USA). Candidate bands were excised from the gel, eluted into distilled water, and reamplified in a second round of PCR using the same primer sets as in the RT-PCR. These PCR products were resolved on 3% NuSieve 3 : 1 agarose gels (TaKaRa) containing H.A.-Yellow (GeneScan Europe AG, Freiburg, Germany), a ligand specific for A/T bases in DNA (Müller et al. 1997). Bands that showed enhanced intensities were eluted, reamplified in a third round of PCR, and then resolved on NuSieve 3 : 1 agarose gels containing H.A.-Red (GeneScan), a ligand specific for G/C bases in DNA (Müller et al. 1997). Bands with enhanced intensities were eluted and subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA).

RNA gel blot analysis

Total RNA (20 μg) was subjected to electrophoresis on formaldehyde-agarose gels and transferred to a Biodyne B nylon filter (Nippon Genetics, Tokyo, Japan). RNA on the filter was allowed to hybridize with ^{32}P-labeled cDNA in a hybridization solution containing 50% formamide, 5X SSPE, 5X Denhardt's solution (1X Denhardt's: 0.01% Ficoll, 0.02% PVP, 0.02% BSA), 0.1% SDS, and 150 μg mL^{-1} herring sperm DNA at 42°C for 16 h. The filter was washed twice with 2X SSC +0.1% SDS for 10 min at 42°C and then twice with 0.1X SSPE +0.1% SSC for 10 min at 42°C. A Bioimage analyzer (Fuji) was used for visualization. To provide an internal control, the same blot was rehybridized with the *PnrRNA* cDNA, which encodes the *P. nil* 16S rRNA (Sage-Ono, unpublished data). The RNA samples used in Figure 1 and Figure 2 are biologically independent. The RNA gel blot analyses in Figure 3 were performed at least twice with independent RNA samples, with similar results.

5′-RACE (rapid amplification of cDNA ends), DNA sequencing, and sequence analyses

5′-RACE was performed using a SMART RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA, USA), according to the manufacturer's instructions. The resulting cDNAs were sequenced using a CEQ8000 automated DNA sequencer and a DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA, USA). Homology searches were performed using BLASTX, available at the DNA Data Bank of Japan (http://spiral.genes.nig.ac.jp/homology/) (Altschul et al. 1997).

Results and discussion

To identify novel genes involved in photoperiodic induction of flowering in *P. nil*, we used fluorescent differential display (FDD) to isolate cDNAs whose

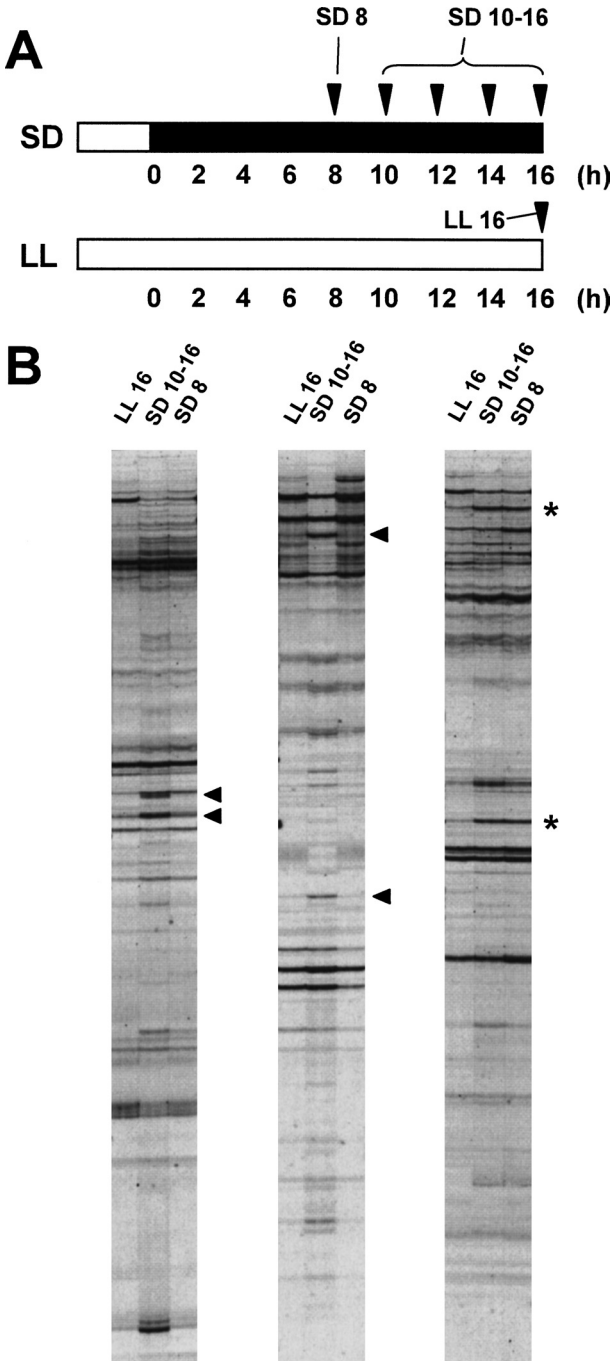

Figure 1. FDD screen for short-day-specific genes. (A) Schedule for the sampling of cotyledons under SD and LL conditions. (B) Acrylamide gel electrophoresis of PCR products. Arrowheads indicate bands for which the signal intensities were high only in the second half of the dark period. Asterisks indicate bands for which the signal intensity was high throughout the dark period.

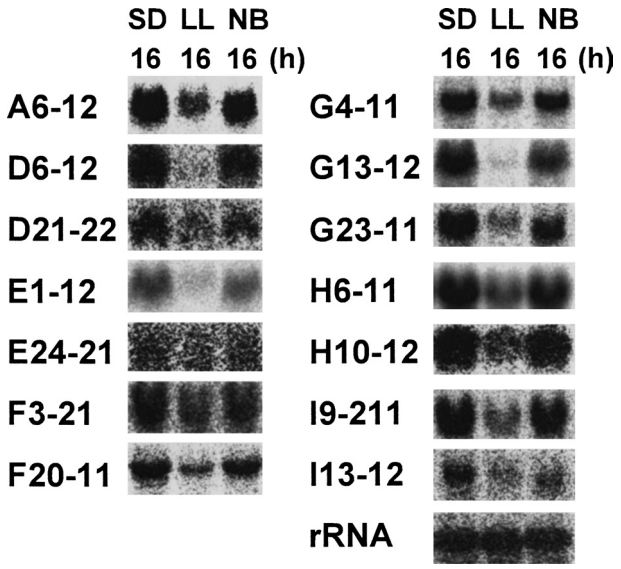

Figure 2. Effects of night-break exposure to red light. Seedlings of *Pharbitis nil* cv. Violet were grown under LL for 6 d and then subjected to photoperiodic treatments of SD (16 h of darkness), LL (continuous light), or NB (a 10-min exposure to red light after 8 h of darkness). The cotyledons of 15 plants were harvested at the indicated times, as measured from the beginning of the photoperiodic treatment, and used for RNA extraction. Total RNA (20 μg per lane) was fractionated by gel electrophoresis and allowed to hybridize with each cDNA probe.

transcripts transiently increased during a 16-h flower-inductive dark photoperiod. We compared the mRNAs from cotyledons harvested after 8, 10, 12, 14, or 16 h under SD conditions, and after 16 h under LL, as measured from the beginning of photoperiodic treatment (Figure 1A). We used nine anchored oligo(dT) primers, each in combination with 24 arbitrary primers, and screened approximately 20,000 cDNA bands. Representative results of the screen are shown in Figure 1B. Among these bands, 140 cDNA fragments that exhibited expression differences were excised and reamplified in a second round of PCR using the same primer sets as in the RT-PCR. Approximately 85% of the bands (118 bands) amplified in the second PCR were reamplified in a third round of PCR, fused into the pGEM-T Easy vector, and sequenced. Homology searches were performed against the DDBJ database using BLASTX (Altschul et al. 1997). The sequences of 57 cDNA clones showed similarities to known genes. As expected, *PnC401*, which we isolated previously, was one of the 57 clones (Sage-Ono et al. 1998). In contrast, 61 clones did not correspond to any known gene in the database (data not shown), perhaps because many were fragments corresponding to the 3′ ends of mRNAs.

To remove false-positive clones, we confirmed the expression patterns of these cDNAs by RNA gel blot analysis using another set of RNA samples. We ultimately selected 14 clones whose expression levels were higher in SD than in LL (Figure 2). With a night break (NB) irradiation treatment with red light, which inhibits flowering (Vince-Prue and Gressel 1985), the expression levels of these cDNAs were slightly reduced (Figure 2). It has been reported that the expression of *PnCO* is not affected by NB treatment (Liu et al. 2001). Recently, it was reported that the suppression of the *Hd3a* [FT] mRNA, and not the *OsGI* or *Hd1* [CO] mRNAs, is the principal cause of the NB effect on flowering in rice (Ishikawa et al. 2005). The

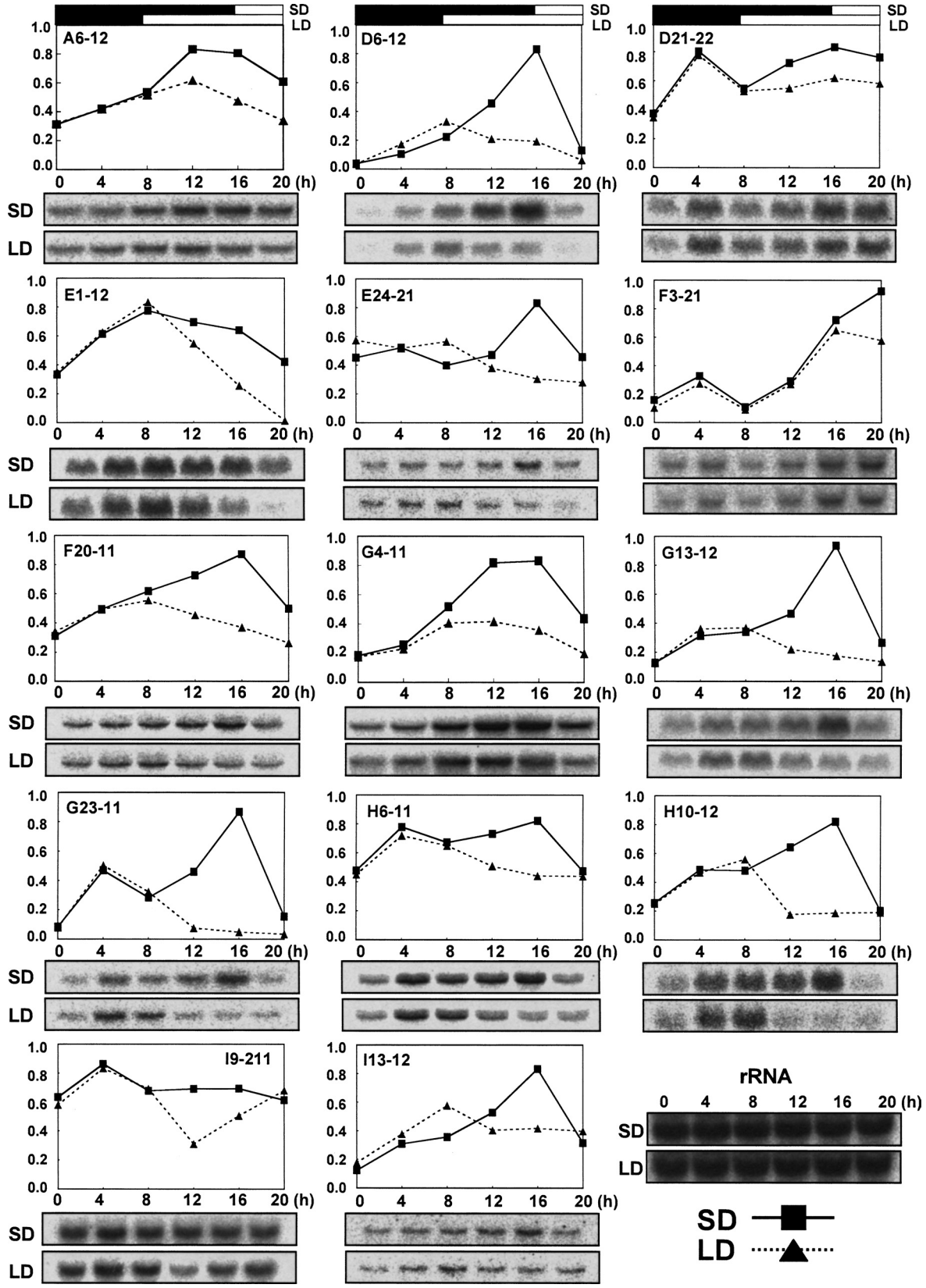

Figure 3. Expression patterns of the isolated genes under SD and LD conditions. Seedlings of *P. nil* cv. Violet were grown under LL for 6 d and then transferred to SD or LD conditions. The cotyledons of 15 plants were harvested every 4 h during the photoperiodic treatments and used for RNA extraction. Total RNA (20 μg per lane) was fractionated by gel electrophoresis and allowed to hybridize with each cDNA probe. The vertical line indicates signal intensities normalized by the *P. nil* 16S *rRNA* signal, as determined using an imaging plate. The horizontal axis indicates the time during the day.

Table 1. Sequence characteristics of cDNAs isolated by FDD method

Clone name	Length (bp)*	Accession number	Best homology: accession number	Score (S): E-value (E)**
A6-12	2099 (F)	AB277189	protein kinase domain containing protein [*Oryza sativa*]: ABA93633	S=477, E=e-133
D6-12	522(P)	AB277190	ADP-glucose pyrophosphorylase [*Ipomoea batatas*]: CAB55495	S=353, E=1e-96
D21-22	584 (P)	AB277191	putative preRNA processing ribonucleoprotein [*Arabidopsis thaliana*]: AB024033	S=199, E=5e-50
E1-12	339 (P)	AB277192	RPT2 (ROOT PHOTOTROPISM2); protein binding [*Arabidopsis thaliana*]: AF181683	S=77.0, E=2e-13
E24-21	2239 (F)	AB277193	putative protein kinase [*Arabidopsis thaliana*]: AAG52471	S=796, E=0.0
F3-21	327 (P)	AB277194	no homology	—
F20-11	1774 (P)	AB277195	eukaryotic initiation factor elF-4 gamma [*Medicago truncatula*]: ABE79486	S=632, E=0.0
G4-11	663 (P)	AB277196	Cryptochrome 1 [*Nicotiana sylvestris*]: ABB36796	S=86.7, E=8e-16
G13-12	397 (P)	AB277197	putative aldehyde dehydrogenase [*Arabidopsis thaliana*]: CAE48164	S=95.5, E=7e-19
G23-11	391 (P)	AB277198	subtilisin-like ptoteinase [*Lycopersicon esculentum*]: CAA07001	S=93.6, E=5e-35
H6-11	407 (P)	AB277199	GMFP5 (*Glycine max* farnesylated protein 5) [*Glycine max*]: AAD09514	S=106, E=4e-22
H10-12	1460 (F)	AB277200	putative protein [*Arabidopsis thaliana*]: CAB52468	S=211, E=9e-53
I9-211	343 (P)	AB277201	no homology	—
I13-12	1313 (P)	AB277202	cell cycle control crn (crooked neck) protein-like [*Arabidopsis thaliana*]: AAN72051	S=105, E=9e-28

* Full-length (F) and partial fragment (P). ** BLAST scores (S) and expect value (E) determined using BLASTX program.

downregulation by the NB treatment may not be an essential characteristic for genes that participate in the photoperiodic induction of flowering.

We examined the expression patterns of the isolated cDNA clones over a 24-h period by RNA gel blot analysis under SD and LD conditions (Figure 3). The expression of all 14 cDNA clones showed significant differences under SD and LD conditions. These results were reproducible, as we obtained essentially similar results with at least two sets of RNA samples (data not shown). The expression of the 14 cDNA clones increased during the dark period under SD conditions. However, the expression of these cDNA clones peaked at 8 h of darkness and decreased upon light exposure in LD conditions. These results suggest that light suppresses or resets the expression of these genes. Seven of the cDNA clones (D6-12, E24-21, F20-11, G13-12, G23-11, H10-12, and I13-12) were strongly induced in the second half of the 16-h dark period (Figure 3).

The characteristics of the cDNA sequences are shown in Table 1. As the initial database searches for seven of the clones (A6-12, E24-21, F3-21, F20-11, H10-12, I9-211, and I13-12) revealed no similarities to known genes, we applied 5′ RACE (rapid amplification of cDNA ends) to determine more of the cDNA sequences. Two clones (F3-21 and I9-211) failed to amplify further, and these genes remain uncharacterized.

The database searches provided some information. D6-12 showed significant homology to the ADP-glucose pyrophosphorylase (AGPase) large subunit of *Ipomoea batatas*. ADP-glucose pyrophosphorylase (AGPase) is an important regulatory enzyme in the starch biosynthetic pathway in both photosynthetic and non-photosynthetic plant tissues. It catalyzes the first step of starch biosynthesis, generating ADP-glucose and pyrophosphate from glucose-1-phosphate and ATP. In *Arabidopsis*, the starch metabolic mutant *adg1* (for ADP-glucose pyrophosphorylase) does not accumulate starch in its leaves and shows a late-flowering phenotype (Eimert et al. 1995). The leaves of the late-flowering mutant *gigantea* (*gi*) have elevated starch levels, suggesting that a common regulatory pathway between starch metabolism and floral induction exists (Eimert et al. 1995). Our findings suggest the existence of similar mechanisms in *P. nil*.

G4-11, which encodes a *P. nil* homolog of the *Arabidopsis* blue-light receptor cryptochrome 1 (cry1), showed dark-induced expression patterns (Figure 3). In *Arabidopsis*, cry1 and cry2 are implicated in numerous blue light-dependent responses, including the inhibition of hypocotyl elongation, leaf and cotyledon expansion, pigment biosynthesis, entrainment of the circadian clock, and control of flowering time (Lin 2002). Cry1 and cry2 act redundantly to mediate the promotion of flowering by blue light (Koornneef et al. 1991; Guo et al. 1998; Mockler et al. 2003). *CRY1* also shows a diurnal expression pattern, but its expression peaks during the light phase (Harmer et al. 2000; Tóth et al. 2001). The activity of the *CRY1* promoter was three-fold higher in the light than in darkness (Tóth et al. 2001). These observations suggest that different mechanisms for the photoperiodic regulation of *CRY1* mRNA expression may operate in *P. nil* and *Arabidopsis*. Although the basis for the disparity between the expression of the *Arabidopsis* and *P. nil* genes is unclear, it may be due to differences between SD and LD plants, and may influence the sensitivity of plants to day length in photoperiodism.

We cannot exclude the possibility that we have identified genes more likely to be involved in other light-regulated processes rather than photoperiodic flowering. For instance, E1-12 showed high homology with

Arabidopsis ROOT PHOTOTROPISM 2 (*RPT2*). This gene encodes a protein with putative phosphorylation sites, a BTB/POZ domain, and a coiled-coil domain, and is required for phototropic responses and stomatal opening mediated by phototropin 1 (phot1), a blue-light receptor (Motchoulski and Liscum 1999; Sakai et al. 2000; Inada et al. 2004). The *RPT2* gene is light- and clock-regulated (Sakai et al. 2000; Harmer et al. 2000).

The other sequences resembled genes encoding a putative protein kinase (E24-21), eukaryotic initiation factor eIF-4 (F20-11), a putative aldehyde dehydrogenase (G13-12), subtilisin-like proteinase (G23-11), and crooked neck (crn)-like protein (I13-12; Table 1). To date, no molecular evidence has been presented for the involvement of these genes in photoperiodic responses, including the induction of flowering.

In this study, we isolated 14 genes whose expression levels were higher in SD than in LD conditions. At present, there is no evidence to support the hypothesis that the transcript levels of these genes are directly involved in photoperiodic responses, including the induction of flowering in *P. nil*. The functions of these genes should be verified through the analysis of transgenic plants that either over- or underexpress the genes. Such analyses are in progress.

Acknowledgements

This work was supported in part by the Program for the Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN).

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