Constitutive expression of the GIGANTEA Ortholog Affects Circadian Rhythms and Suppresses One-shot Induction of Flowering in Pharbitis nil, a Typical Short-day Plant

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GIGANTEA (GI) is a key regulator of flowering time, which is closely related to the circadian clock function in Arabidopsis. Mutations in the GI gene cause photoperiod-insensitive flowering and altered circadian rhythms. We isolated the GI ortholog PnGI from Pharbitis (Ipomoea) nil, an absolute short-day (SD) plant. PnGI mRNA expression showed diurnal rhythms that peaked at dusk under SD and long-day (LD) conditions, and also showed robust circadian rhythms under continuous dark (DD) and continuous light (LL) conditions. Short irradiation with red light during the flower-inductive dark period did not change PnGI expression levels, suggesting that such a night break does not abolish flowering by affecting the expression of PnGI. In Pharbitis, although a single dusk signal is sufficient to induce expression of the ortholog of FLOWERING LOCUS T (PnFT1), PnGI mRNA expression was not reset by single lights-off signals. Constitutive expression of PnGI (PnGI-OX) in transgenic plants altered period length in leaf-movement rhythms under LL and affected circadian rhythms of PnFT mRNA expression under DD. PnGI-OX plants formed fewer flower buds than the wild type when one-shot darkness was given. In PnGI-OX plants, expression of PnFT1 was down-regulated, suggesting that PnGI functions as a suppressor of flowering, possibly in part through down-regulation of PnFT1.

Keywords: Circadian rhythm • Flowering • GIGANTEA • Pharbitis nil • Photoperiodism • Short-day plant.

Abbreviations: CO, CONSTANS; DD, continuous dark; EOD-FR, end of day far-red; EST, expressed sequence tag; FT, FLOWERING LOCUS T; GI, GIGANTEA; LD, long day; LDP, long-day plant; LL, continuous light; NB, night break; SD, short day; SDP, short-day plant.

The nucleotide sequences reported in this paper have been submitted to DDBJ under the accession numbers: PnGI, AB265781; PnLHY, AB607848; UBQ, AB265782.

Introduction

Plants adjust their flowering time by measuring day length, which changes depending on the time of the year. This phenomenon, called photoperiodism, allows anticipation of future environmental conditions and enables plants to reproduce at favorable times of the year (Garner and Allard 1920, Thomas and Vince-Prue 1997). Plants are classified into three main categories based on photoperiodic response: short-day plants (SDPs), long-day plants (LDPs) and day-neutral plants (DNPs). In addition to the classical model based on physiological studies on various photoperiodic responses, recent molecular-genetic approaches have identified a number of genes that are required for the flowering response in Arabidopsis (LDP) and rice (SDP), and several models have been proposed to explain the molecular basis of photoperiodic flowering (Hayama and Coupland 2004, Imaizumi and Kay 2006).

The ‘external coincidence model’ is currently the most consistent with the physiological and molecular-genetic evidence for explaining day-length measurement (Bünning 1936, Pittendrigh and Minis 1964, Yanovsky and Kay 2003, Hayama and Coupland 2004). This model proposes that day length is measured through a circadian clock that controls the expression of some light-sensitive regulatory product. The photoperiodic responses are triggered only when the external (light) signals coincide with the light-sensitive phase of the circadian rhythms. In Arabidopsis, the regulation of FLOWERING LOCUS T (FT) gene expression by CONSTANS (CO) can be explained using this model. CO encodes a B-box type zinc-finger
transcriptional activator that induces expression of the floral inducer FT gene (Putterill et al. 1995, Kardailsky et al. 1999, Kobayashi et al. 1999, Samach et al. 2000). The expression of CO mRNA is set by the circadian clock in the afternoon in LD, and CO protein is stabilized and activated by light signals perceived by phytochrome and cryptochrome photoreceptors (Yanovsky and Kay 2002, Valverde et al. 2004). In this model, an endogenous circadian oscillator as well as external light signals are essential for measuring day length.

In Arabidopsis, circadian rhythms are generated by a central oscillator that consists of three interlocking negative feedback loops. LATE ELONGATED HYPOCOTYL (LHY), CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and TIMING OF CAB EXPRESSION 1/PSEUDO-RESPONSE REGULATOR 1 (TOC1/PRR1) are thought to be components of the core loop of the circadian oscillator (Schaffer et al. 1998, Wang and Tobin 1998, Strayer et al. 2000, Makino et al. 2000, Alabadi et al. 2001, Mizoguchi et al. 2002). GIGANTEA (GI) is another clock-associated protein that plays an important role in circadian oscillation and flowering-time regulation. Mutations in the GI gene cause delayed flowering under long day conditions and altered period length of circadian rhythms (Fowler et al. 1999, Park et al. 1999), and overexpression of GI causes extreme early flowering and changes in the phase and period of circadian rhythms (Mizoguchi et al. 2005). The role of GI as a component of the interlocked oscillator loop is further supported by mathematical simulations (Locke et al. 2005, 2006). GI is thought to act upstream of CO because the expression levels of CO mRNA are reduced in gi mutants, and the overexpression of CO in gi mutants suppresses the late-flowering phenotype (Suarez-Lopez et al. 2001). Recent studies have demonstrated that GI regulates circadian rhythms and flowering time by forming a complex with ZTL/FKF1/LKP2 family proteins to control the protein stability of TOC1/PRR1, an oscillator component, and CYCLING DOF FACTOR 1 (CDF1), a transcriptional repressor of CO, respectively (Kim et al. 2007, Sawa et al. 2007).

The functions of GI, CO and FT are likely to be universal, because their orthologs [namely, OsGI, Heading-date 1 (Hd1) and Hd3a, respectively] in rice, a facultative SDP, play important roles in photoperiodic flowering (Yano et al. 2000, Kojima et al. 2002, Hayama et al. 2003). Overexpression of OsGI causes late flowering under both SD and LD conditions (Hayama et al. 2003). The expression of Hd1 increases in plants overexpressing OsGI, whereas the expression of Hd3a is suppressed. These results indicate that the regulation of FT by CO is reversed in rice (SDP) relative to Arabidopsis (LDP) (Hayama et al. 2003). In addition to this GI-CO-FT (OsGI–Hd1–Hd3a) pathway, rice contains an alternative pathway that functions independently of Hd1. Early heading date 1 (Ehd1), encoding a B-type response regulator, activates Hd3a expression under SD conditions, independent of Hd1 (Doi et al. 2004, Itoh et al. 2010). Ghd7 (for Grain number, plant height and heading date 7), which encodes a CCT domain protein, acts to suppress Ehd1 and Hd3a expression independently of Hd1 under LD (Xue et al. 2008, Itoh et al. 2010). Both Ehd1 and Ghd7 are evolutionarily unique genes in rice, with no counterparts in Arabidopsis.

In Arabidopsis and rice, even though flowering is accelerated under an appropriate photoperiod, flowering finally occurs under a non-appropriate photoperiod. However, some species require an appropriate photoperiod to induce flowering. Pharbitis nil Choisy cv. ‘Violet,’ an absolute SDP, is ideal for the study of early events in the photoperiodic induction of flowering. Young, light-grown seedlings can be fully induced to flower by exposure to a single dark period of 16 h (one-shot flowering), whereas under continuous light (LL) or LD conditions, they do not form flowers for months. Moreover, this induction is completely abolished by 10 min irradiation with red light provided in the middle of 16 h of darkness [night break (NB); Imamura 1967, Vince-Prue and Gressel 1985]. An experiment that removed cotyledons 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 (Zeewaart 1962, Sasaki et al. 2008). These observations suggest that changes in the expression levels of some genes that occur specifically during the single inductive dark period may participate in the generation of the floral stimulus. Thus, several attempts have been made to identify the genes involved in the photoperiodic induction of flowering in Pharbitis (Lay-Yee et al. 1987, Ono et al. 1993, 1996, Zheng et al. 1993, O’Neill et al. 1994, Sage-Ono et al. 1998, Higuchi et al. 2007). However, none of these genes has been shown to directly participate in the photoperiodic induction of flowering. For example, an ortholog of CO, PnCO1, was isolated using a differential display screen to identify genes with increased expression under SD conditions (Liu et al. 2001a). Constitutive expression of PnCO by the Cauliflower mosaic virus (CaMV) 35S promoter complemented the late-flowering phenotype of the Arabidopsis co mutant (Liu et al. 2001a). Although these results strongly support the idea that PnCO may be a key regulator of flowering in this species, functional analyses of PnCO by generating transgenic Pharbitis plants have not been reported, possibly because of difficulties in generating transgenic Pharbitis plants. Recently, two orthologs of FT (PnFT1 and PnFT2) were identified and shown to exhibit expression patterns specific to a flower-inductive dark period (Hayama et al. 2007). Moreover, the expression of PnFT was set by a single dusk signal, whereas expression of PnCO was not. These results suggest that the mechanism for measuring day length in Pharbitis is quite different from those of Arabidopsis and rice.

Because the flowering response of Pharbitis strongly depends on the length of a single dark period, some timekeeping mechanism was expected to measure the length of night precisely from the beginning of darkness. However, the relationship between circadian rhythms and flowering response of Pharbitis has not been verified at the molecular level. Here we isolated the Pharbitis ortholog of GI, which is closely related to circadian clock and flowering-time regulation in Arabidopsis and rice. We succeeded in generating transgenic plants constitutively
expressing \textit{PnGI} and investigated its function in the regulation of circadian rhythms and the photoperiodic induction of flowering in \textit{Pharbitis}.

### Results

#### Identification of a \textit{GI} ortholog from \textit{Pharbitis}

Full-length cDNA of the putative \textit{GI} ortholog was isolated from a cDNA library prepared from \textit{Pharbitis} flower buds. Sequence analysis revealed that the cDNA encodes a putative polypeptide of 1166 amino acids with a molecular mass of 127 kDa. The deduced amino acid sequence of this cDNA shares 70% and 67% identity with the Arabidopsis and rice proteins, respectively (Supplementary Fig. S1). Database searches using BLAST showed that the GI protein of \textit{Pharbitis}, like other GI homologs, has no significant homology to proteins of known biochemical function. The regions containing four clusters of basic amino acids, which were identified because of their nuclear localization (Huq et al. 2000), were highly conserved among GI proteins of \textit{Pharbitis}, Arabidopsis and rice (Supplementary Fig. S1). The cloned cDNA is henceforth referred to as \textit{PnGI}.

#### Expression patterns of \textit{PnGI} mRNA under various photoperiods

In most plant species studied to date, \textit{GI} expression shows diurnal changes under both SD and LD that peak at dusk (Fowler et al. 1999, Hayama et al. 2002, Dunford et al. 2005, Zhao et al. 2005, Miwa et al. 2006, Hecht et al. 2007). To test the expression patterns of \textit{GI} in \textit{Pharbitis}, we performed reverse transcriptase–polymerase chain reaction (RT–PCR). Seedlings were grown for 6 d under LL and then transferred to SD or LD conditions. The expression of \textit{PnGI} mRNA in cotyledons was monitored with RT–PCR from just after the beginning of the first dark period (Fig. 1). \textit{PnGI} expression showed clear diurnal rhythms that peaked at dusk under SD conditions (Fig. 1A). Under LD conditions, \textit{PnGI} expression also showed diurnal rhythms peaking at dusk (Fig. 1B). These expression patterns of \textit{PnGI} under SD and LD conditions were very similar to those of Arabidopsis and rice \textit{GI} (Fowler et al. 1999, Hayama et al. 2002).

We also examined whether stable circadian rhythms occur in \textit{PnGI} mRNA levels (Fig. 1C, D). Seedlings were grown for 6 d under LL and then transferred to DD or a single dark period followed by LL. Under DD conditions, \textit{PnGI} expression exhibited robust circadian rhythms (Fig. 1C). This circadian oscillation was also observed under LL conditions after a single 16-h dark period (Fig. 1D). These results indicate that \textit{PnGI} expression is controlled by the endogenous circadian clock. Interestingly, a robust circadian oscillation of \textit{PnGI} mRNA was observed even when the LL-grown seedlings were simply shifted to DD without any entrainment to a light–dark cycle (Fig. 1C). In addition, \textit{PnGI} expression showed clear diurnal or circadian rhythms from the first cycle of photoperiodic treatment (Fig. 1A–D).

In \textit{Pharbitis}, the induction of flowering by 16 h of darkness is completely abolished by 10 min irradiation with red light 8 h after the beginning of darkness. To elucidate the point of action of this light pulse, we analyzed changes in \textit{PnGI} mRNA after NB treatment (Fig. 1E). Seedlings were grown for 6 d under LL and then transferred to a 16-h dark period. Eight hours after the beginning of darkness, seedlings were exposed to red light for 10 min. Dramatic changes in \textit{PnGI} expression levels were not observed with NB treatment, compared with treatment with 16 h of darkness (Fig. 1E). These results indicate that changes in the levels or timing of \textit{PnGI} mRNA expression are not the main cause of the inhibitory effect of NB.

#### Effect of a lights-off signal on the phase setting of \textit{PnGI} expression

Because \textit{Pharbitis} flowering is induced by exposure to a single period of darkness, it is expected that some timekeeping mechanisms may measure the night length precisely from the beginning of darkness. Such timekeeping mechanisms should be reset by lights-off signals, rather than lights-on signals. Interestingly, as predicted in physiological studies, it was recently shown that a single dusk signal is sufficient to reset the expression of \textit{PnFT1} and \textit{PnFT2} (Hayama et al. 2007). Although robust circadian oscillation of \textit{PnGI} mRNA was observed even when the LL-grown seedlings were simply shifted to DD (Fig. 1C), its peak phase during the first inductive dark period sometimes differed under different experimental conditions (Fig. 1, data not shown). To test whether transcriptional regulation of the \textit{GI–CO–FT} pathway is reset by lights-off signals, expression of \textit{PnGI}, \textit{PnCO} and \textit{PnFT1} was examined using quantitative RT–PCR. Plants were grown for 6 d under LL and then exposed to 8 h of darkness followed by 16, 20, 24 or 28 h of light before the inductive 24 h of darkness (Fig. 2). Cotyledons were harvested every 4 h during the photoperiodic treatments and were used for RNA extraction and quantitative RT–PCR (Fig. 2A–C). As reported by Hayama et al. (2007), \textit{PnFT1} mRNA expression specifically increased during the inductive dark period, with almost the same phase under each photoperiod condition, suggesting that expression of \textit{PnFT1} is strongly reset by a single dusk signal (Fig. 2C). However, unlike \textit{PnFT1}, the timing of \textit{PnGI} mRNA expression was different under each photoperiod condition (Fig. 2A). Expression of \textit{PnGI} was set by the lights-off and lights-on signals of non-inductive 8-h darkness, so the phase of \textit{PnGI} mRNA expression during inductive darkness was mainly affected by the length of the light period preceding the inductive darkness (Fig. 2A). Similarly, the timing of \textit{PnCO} mRNA expression during inductive darkness was altered under different photoperiod conditions, depending on the length of the light period before the inductive darkness (Fig. 2B, Hayama et al. 2007). Therefore, the expression of these two clock-controlled genes, especially that of \textit{PnGI}, is entrained mainly by lights-on signals; a single lights-off signal appears to be insufficient to reset the expression of these genes.
Constitutive expression of PnGI affects the period length of leaf-movement rhythm and PnFT mRNA expression in Pharbitis

To examine the function of PnGI, cDNA of PnGI was fused to the CaMV 3S promoter and introduced into P. nil (cv. 'Violet') using Agrobacterium-mediated transformation. Several regenerated plants were obtained, and the integration of the transgene in the genomes of T1 plants was confirmed using PCR. The expression levels of PnGI for each T1 line were tested using RT–PCR, and three representative lines were used for further analyses (Supplementary Fig. S2).

Fig. 1 Diurnal and circadian expression patterns of PnGI mRNA under various photoperiods. Seedlings were grown under LL for 6 d and then transferred to (A) SD, (B) LD, (C) DD or (D) LL after a single 16-h dark period conditions. (E) Effect of NB treatment on PnGI mRNA expression. Seedlings were grown under LL for 6 d and then exposed to 16 h of darkness. After 8 h of darkness, seedlings were exposed to red light (NB; 8 μmol m$^{-2}$ s$^{-1}$) for 10 min or kept in the dark (SD). Cotyledons were harvested every 4 h during the photoperiodic treatments and used for RNA extraction. The expression of PnGI was analyzed using RT–PCR. PnGI mRNA was quantified with UBQ mRNA. The black and white bars at the bottom represent the dark and light periods, respectively. The horizontal axis indicates time (h) from the first dusk. Each experiment was done at least twice with similar results.
In Arabidopsis, mutations in the GI gene shorten the period length in circadian rhythms in leaf movements and alter circadian rhythms in the expression of the CHLOROPHYLL a/b BINDING PROTEIN (CAB) gene (Park et al. 1999). Overexpression of GI also shortens the free-running rhythms in COLD CIRCADIAN REGULATED2 (CCR2) expression under LL (Mizoguchi et al. 2005). To test whether PnGI affects circadian rhythms in Pharbitis, we used rhythmic movement of cotyledons as a rhythm marker. Seedlings were grown for 5 d under LL, followed by two cycles of LD (16 h L/8 h D) to entrain the circadian clock, and then transferred to LL to monitor cotyledon movement (Fig. 3A, B). In both wild-type and PnGI-OX plants, robust rhythmicity in cotyledon movement was observed under LL for up to 4 d (Fig. 3A). However, the mean period of the leaf-movement rhythm in the PnGI-OX plant was significantly longer than that in the wild type: 31.8 ± 0.6 h (SE) for PnGI-OX line #9, 29.3 ± 0.6 h for PnGI-OX line #15 and 26.2 ± 0.4 h for wild type (Fig. 3B).

We also tested the free-running rhythms of the Pharbitis ortholog of LHY (PnLHY), PnCO, PnFT1 and PnFT2 mRNA expression in the PnGI-OX (line #9 and #14) plants under DD using quantitative RT–PCR (Fig. 3C–G, Supplementary Fig. S3). Plants were grown for 6 d under LL and then shifted to DD. At each time point, PnGI mRNA was more abundant in PnGI-OX plants than in wild-type plants (Fig. 3C). In PnGI-OX plants, rhythmic expression of PnLHY and PnCO was slightly reduced in amplitude compared with that in wild type, but no remarkable change in phase or period length was observed (Fig. 3D, E). Expression of PnFT1 in wild-type plants showed robust circadian rhythms that peaked at 16 and 40 h after dusk; however, the phase of the second peak was delayed in PnGI-OX plants, resulting in a longer period length compared with that in wild type (Fig. 3F). Moreover, expression of PnFT2 in PnGI-OX plants was slightly delayed in peak phase and greatly reduced in amplitude compared with that in the wild type (Fig. 3G). Taken together, these results suggest that PnGl may play a general role in the regulation of circadian rhythms in Pharbitis.

Constitutive expression of PnGI inhibits one-shot flowering and suppresses PnFT1 expression

Pharbitis is an absolute SD plant that forms flower buds after a single exposure to an inductive dark period of >10 h. Providing 14–16 h of darkness leads to the maximum number of flower buds (six to seven) with a terminal flower form and no further floral or vegetative primordia. The number of flower buds increases in proportion to the length of the inductive dark period within the range one to six (or seven). Therefore, counting the number of flower buds per plant is the most appropriate way to evaluate the strength of the Pharbitis flowering response. To test the flowering response of PnGI-OX plants, plants were grown for 6 d under LL and then subjected to a single dark period of 12 h, which is a moderate inductive condition. Wild-type plants formed two or three flower buds per plant, whereas PnGI-OX plants formed fewer flower buds (Fig. 4A). When a single 14 h of darkness was provided, all of the wild-type plants formed a maximum number of flower buds with a terminal flower, whereas 11–67% of PnGI-OX plants did not form a terminal flower (Fig. 4B). These results suggest that

Fig. 2 Effect of lights-on and lights-off signals on PnGI, PnCO and PnFT1 mRNA expression. Relative mRNA abundance of (A) PnGI, (B) PnCO and (C) PnFT1 was determined by quantitative RT–PCR. Plants were grown for 6 d under LL and then exposed to 8 h of darkness followed by 16, 20, 24 or 28 h of light before the inductive 24 h of darkness (SD1–4). Cotyledons were harvested every 4 h during the photoperiodic treatments and were used for RNA extraction and quantitative RT–PCR. Plants in each photoperiod developed five to seven flower buds with a terminal flower after 3 weeks in LL. Black bars at the bottom represent the dark period. Average values and standard deviations from three PCR experiments are shown. The maximum value in each experiment was set to 1. Error bars, when not evident, were smaller than the symbols used. A biological replicate provided a similar result.

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Fig. 3 Effects of PnGI-OX on cotyledon-movement rhythm and gene-expression rhythm. (A) Cotyledon movement under continuous white light (LL). Seedlings were grown for 5 d under LL, followed by two cycles of 16 h light/8 h dark, and released into LL. Time in LL shows h after the transfer to LL. Representative traces of vertical leaf position (pixel position) from images of 17 wild-type, nine PnGI-OX line #9 and nine PnGI-OX line #15 leaves. Hatched bars indicate subjective night. (B) Plots showing the FFT-NLLS analysis of the leaf-movement data plotted in (A). (C–G) Temporal expression patterns of (C) PnGI, (D) PnLHY, (E) PnCO, (F) PnFT1 and (G) PnFT2 mRNAs in PnGI-OX plants (line #9) over 60 h of darkness. Seedlings were grown for 6 d under LL and then subjected to 60 h of darkness. Cotyledons were harvested every 4 h under DD and were used for RNA extraction and quantitative RT–PCR. Black bars at the bottom represent the dark period. Average values and standard deviations from three PCR experiments are shown. The maximum value in each experiment was set to 1. Error bars, when not evident, were smaller than the symbols used. A biological replicate using another transgenic line (PnGI-OX #14) provided a similar result and is shown as Supplementary Fig. S3.
**PnGI** has an inhibitory function in the induction of flowering in *Pharbitis*.

Because GI functions as an activator of CO transcription (Suarez-Lopez et al. 2001, Mizoguchi et al. 2005) and CO acts as an activator of FT (Samach et al. 2000), the expression patterns of PnCO, PnFT1 and PnFT2 were analyzed in PnGI-OX (line #9 and #15) plants when one-shot 12-h darkness was given (Fig. 4C–F).

Plants were grown for 6 d under LL conditions and then subjected to a single 12-h period of darkness. Cotyledons were harvested every 4 h, and the abundance of mRNA of each of PnGI, PnCO, PnFT1 and PnFT2 was analyzed using quantitative RT–PCR (Fig. 4C–F). In wild-type plants, the abundance of PnGI mRNA peaked 20 h after dusk, and was very low in the morning. However, in PnGI-OX plants, high accumulation of PnGl/UBQ was observed. The average number of flower buds per plant (A) and the ratio of terminal flower formation in the wild type and three independent transgenic lines (B) were scored. The data in (A) represent mean ± SE of 10–12 seedlings of each transgenic line. Each experiment was done at least twice with similar results. (C–F) Expression levels of (C) PnGl, (D) PnCO, (E) PnFT1 and (F) PnFT2 in PnGI-OX plants (lines #9 and #15) during one-shot induction of flowering. Plants were grown for 6 d under LL and then subjected to a single 12-h dark period. After induction, plants were grown for 3 weeks under LL, and the number of flower buds was scored. The data in (A) represent mean ± SE of 10–12 seedlings of each transgenic line. Each experiment was done at least twice with similar results. (C–F) Expression levels of PnFT2 (F) were represented relative to the maximum value of PnFT1 (E). The error bars, when not evident, were smaller than the symbols used. Each experiment was done at least twice, with similar results.
Phenylalanine ammonia-lyase (PnCO) transcripts was observed even 8, 12 or 16 h after dusk (Fig. 4C). The expression level of PnCO was not dramatically changed in PnGI-OX lines compared with the wild type (Fig. 4D). The expression of PnFT1 was increased after 12 h in darkness and peaked at 16 h after dusk in the wild type, whereas PnFT1 was expressed at lower amplitude in PnGI-OX plants (Fig. 4E). In contrast, expression of PnFT2 was not induced by a single 12-h period of darkness in either wild-type or PnGI-OX plants (Fig. 4F). Given that one-shot 12-h darkness was sufficient to induce flowering (Fig. 4A), PnGI may act to suppress flowering, possibly in part through down-regulation of PnFT1 in Pharbitis.

**Discussion**

**Expression of PnGI is regulated by photoperiod and an endogenous circadian clock**

To gain insight into the molecular mechanisms of photoperiodic induction of flowering in absolute SDPs, we isolated the *Pharbitis* homolog of GI, PnGI. In sequence comparisons, PnGI showed very high homology to the Arabidopsis and rice GI proteins (Supplementary Fig. S1). Previous studies have reported the nuclear localization of the GI protein (Huq et al. 2000, Mizoguchi et al. 2005), and PnGI also shows nuclear localization signals (Supplementary Fig. S1), indicating the importance of the action of GI in the nucleus. Recently, the FKF1–GI–CDF1 complex was detected on the promoter region of CO using ChIP–PCR analysis, suggesting that GI may regulate transcription indirectly by associating with a transcription factor (Sawa et al. 2007).

Expression analyses have revealed that the expression of PnGI mRNA is under control of the endogenous circadian clock (Fig. 1C, D). Interestingly, the expression patterns of *Pharbitis*, rice and Arabidopsis GI genes are all regulated photoperiodically and are very similar under LD and SD conditions (Fig. 1A, B, Fowler et al. 1999, Hayama et al. 2002). Moreover, homologs of GI from barley, wheat, *Lemna* and pea have recently been isolated, and their mRNA expression also peaks at dusk (Dunford et al. 2005, Zhao et al. 2005, Miwa et al. 2006, Hecht et al. 2007). These results suggest conservation of the molecular mechanisms that regulate GI gene expression in various plant species.

In *Pharbitis*, the induction of flowering by a single period of darkness is completely cancelled by 10 min irradiation with red light. PnGI expression level was not dramatically altered by NB treatment (Fig. 1E), and previous results have shown that the expression of PnCO mRNA is not affected by NB treatment (Liu et al. 2001a). In addition, the expression of PnFT1 mRNA is completely suppressed by NB treatment (Hayama et al. 2007). An NB effect on flowering was reported in rice, and it was suggested that the suppression of Hd3a (FT) mRNA, and not OsGI or Hd1 (CO) mRNA, is the principal cause of this effect on flowering (Ishikawa et al. 2005). Therefore, the NB effect on flowering seems very similar in these two SDPs: light signals during NB may directly affect floral regulator activity without any phase shift in the circadian clock.

**Possible function of PnGI in the regulation of one-shot flowering and circadian rhythms**

PnGI-OX plants formed fewer flowers than the wild type when 12 or 14 h of darkness was provided (Fig. 4), suggesting that PnGI acts as a floral suppressor in *Pharbitis*. In PnGI-OX plants, although the expression of PnCO was not dramatically changed (Fig. 4D), the expression of PnFT1 was reduced (Fig. 4E). Interestingly, expression of PnFT2 was not induced by a single 12-h period of darkness, whereas flowering was moderately induced (Fig. 4A, F). Induction of PnFT2 occurs after 12–16 h in darkness, which occurs later than PnFT1 (Fig. 3F, G, Supplementary Fig. S3), also indicating that PnFT1 may play dominant roles in one-shot flowering by darkness. A recent study reported that expression of PnFT2 correlates with poor-nutrition stress-induced flowering response in *Pharbitis* (Wada et al. 2010), suggesting that PnFT1 and PnFT2 may act to promote flowering in response to different environmental cues. The relationship between PnGI and PnCO remains unclear because the expression level of PnCO was slightly reduced in amplitude in PnGI-OX plants under DD (Fig. 3E, Supplementary Fig. S3), but was not remarkably changed during one-shot 12-h darkness (Fig. 4D). In garden pea (*Pisum sativum*), a loss-of-function mutation of the GI ortholog (*late1*) greatly reduced expression levels of the FT gene (FTL) under LD, but caused only a minor alteration in rhythmic expression of the CO homolog (*COLA*) (Hecht et al. 2007). In rice, OsGI activates Hd1 (CO), which in turn represses Hd3a (FT) under LD conditions (Hayama et al. 2003). The relationship between PnGI and PnFT1 seems similar to that in rice, but the function of PnCO remains unknown. CO-independent photoperiodic pathways that are required for the regulation of flowering have recently been reported. For example, in Arabidopsis, a microRNA called miR172 promotes photoperiodic flowering through a CO-independent genetic pathway downstream of the GI (Jung et al. 2007). In rice, *Ehd1* activates *Hd3a* expression under LD conditions, independently of *Hd1* (Doi et al. 2004). Another flowering-time gene, *Ghd7*, acts to suppress *Ehd1* and *Hd3a* expression under LD (Xue et al. 2008). Very recently, *Ehd1* and *Ghd7* were shown to be required to set critical day length for *Hd3a* expression, acting downstream of OsGI (Itoh et al. 2010). Therefore, it is reasonable to assume that such an activator (or repressor) of PnFT1, which acts downstream of PnGI and independently of PnCO, may also exist, and similar machinery for day-length recognition might operate in *Pharbitis* (Fig. S5). Furthermore, in rice, both over-expression and loss of function of OsGI result in a late-flowering phenotype under inductive SD conditions (Hayama et al. 2003, Itoh et al. 2010). Although we suggest that PnGI acts as a suppressor of flowering based on the flowering phenotype of PnGI-OX plants in this study, more careful consideration of PnGI...
function will be needed. Loss-of-function analysis of PnGI will provide further information.

Although GI has been shown to be involved in the regulation of circadian rhythms in some LDPs including Arabidopsis, *Lemna* and pea (Park et al. 1999, Serikawa et al. 2008, Liew et al. 2009), its role in SDPs remains unclear. *PnGI*-OX plants exhibited an increased period length in cotyledon movement under LL, and the rhythmic expression of clock-controlled genes was affected under DD (Fig. 3), suggesting that PnGI plays a general role in the regulation of circadian rhythms in *Pharbitis*, an SDP. In *PnGI*-OX plants, circadian expression of PnFT1 was slightly reduced in amplitude, and its peak phase was delayed. One possible explanation for the flowering and circadian phenotype of *PnGI*-OX plants is that the constitutive expression of PnGI slowed down the clock that controls expression of PnFT1 and gave plants incorrect information that the night was shorter than it really was. In Arabidopsis, the early-flowering phenotype of the toc1-1 mutant under SD can be explained by their short-period phenotype and phase advance in CO mRNA expression (Yanovsky and Kay 2002). However, recent studies have suggested that GI regulates photoperiodic flowering independently of its role in the circadian clock (Mizoguchi et al. 2005, Martin-Tryon et al. 2007). The relationship between the function of GI in flower induction and circadian rhythms should also be clarified in *Pharbitis*.

**Timekeeping mechanisms in the one-shot induction of flowering**

Although constitutive expression of PnGI has been shown to affect circadian rhythms and suppress flowering (Figs. 3, 4), our results prompt us to question whether the GI–CO–FT pathway is also the main pathway of one-shot flowering in *Pharbitis*. We systematically varied the length of the light period preceding the flower-inductive dark period and demonstrated that mRNA expression of PnGI cannot be reset by a single inductive period of darkness (Fig. 2A). Induction of PnFT1 seems to be uncoupled from PnGI and PnCO mRNA behavior: expression of PnFT1 was precisely up-regulated between 16 and 20 h after lights off (Fig. 2, Hayama et al. 2007).

Several hypotheses can be proposed to explain the decoupling of the GI–CO cascade from PnFT1 expression. The protein abundance of PnGI or PnCO might be more important, rather than the timing of gene expression. A recent study reported that the protein abundance of GI oscillator under LD and SD, and is degraded by proteasomes in the dark (David et al. 2006). Moreover, CO protein is stabilized by light in the evening but degraded by proteasomes in darkness (Valverde et al. 2004). Thus, degradation of PnGI and/or PnCO protein in the inductive dark period might cause the de-repression of PnFT1 expression.

One might also speculate that another timekeeping mechanism, which is regulated by the lights-off signal, might occur in the regulation of one-shot flowering. In Arabidopsis, EARLY FLOWERING 3 (*ELF3*) and TIME FOR COFFEE (*TIC*) mediate circadian gating of light responses (Hicks et al. 2001, Covington et al. 2001, Liu et al. 2001a, McWatters et al. 2000, Hall et al. 2003). In *elf3* and *tic* mutants, the phase of CHLOROPHYLL a/b-BINDING PROTEIN (*CAB*) expression is reset by a single light-to-dark transition, whereas the phase of the oscillation in wild-type plants is reset by the previous dawn (McWatters et al. 2000, Hall et al. 2003). *CAB* expression in these gating-defect mutants is strikingly similar to the PnFT1 expression and flowering response rhythm of *Pharbitis* (Fig. 2C, Imamura 1967, Hayama et al. 2007). In rice, a recent study demonstrated that acute induction of *Hd3a* in response to critical day length is achieved by the interaction of two gating mechanisms: gating of *Ehd1*, an *Hd3a* activator, and gating of *Ghd7*, an *Hd3a* repressor (Itoh et al. 2010). The gate for *Ehd1* induction with blue light is always set around dawn, regardless of day-length conditions; however, the gate for *Ghd7* induction with red light has different openings, depending on day length (Itoh et al. 2010). Therefore, in *Pharbitis*, it seems reasonable to assume that at least two distinct timekeeping components with different gating mechanisms are involved in the regulation of PnGI and PnFT1 transcription (Fig. 5).

In the 1960s, the effects of phytochromes on timekeeping were studied extensively in many SDPs, including *Pharbitis*, in which an NB with red light inhibits flowering, and exposure to far-red light before inductive darkness (EOD-FR) inhibits flowering (Thomas and Vince-Prue 1997). In rice, phytochromes are...
absolutely required for photoperiodic flowering, because the loss of function of the PHOTOPERIOD SENSITIVITY 5 (SES) gene, which encodes a heme oxygenase for phytochrome chromophore biosynthesis, is completely deficient in photoperiodic response (Izawa et al. 2000, 2002). This conclusion was further supported by analysis of the phyAphyBphyC triple mutant, which completely lacks all phytochromes of rice (Takano et al. 2009). These results strongly suggest that phytochromes play a major role in the photoperiodic control of flowering in SDPs. Although the molecular function of phytochromes in one-shot flowering of *Pharbitis* remains unclear, it is of interest to test whether manipulation of phytochrome genes affects phase setting of *PnFT* genes during a single period of inductive darkness.

Recent efforts to establish an effective transformation system in *Pharbitis* have made it possible to analyze the functions of some genes of interest in this classic model plant for photoperiodic flowering (Ono et al. 2000, Kikuchi et al. 2005). Functional analysis of flowering-time genes such as *PHYs* and *PnCO* using transgenic plants will provide clues for obtaining a better understanding of the molecular basis of photoperiodic control of flowering in higher plants. Such an analysis is currently underway.

**Materials and Methods**

**Plant materials and growth conditions**

Seeds of *P. nil* cv. ‘Choisy’ (Marutane Co., Kyoto, Japan) were soaked in concentrated sulfuric acid for 30 min with occasional stirring, rinsed in running tap water for 1 h, then soaked in distilled water for 16 h and sown on wet vermiculite to germinate. Growth conditions were set at 24 ± 1°C with illumination from continuous cool-white fluorescent light (60 μmol m⁻² s⁻¹, FL 40SS W/37 lamps; Matsushita Electronics Co., Tokyo, Japan). Red (660 ± 20 nm) light-emitting diode (LED) lamps (LED-R; Eyela, Tokyo, Japan) were used to provide red light. Once the cotyledons were fully expanded (6 d after sulfuric acid treatment), the seedlings were subjected to photoperiodic treatments. The treatments began with a dark period, and the elapsed time was recorded from the beginning of this period. The cotyledons were excised, frozen in liquid nitrogen and stored at −80°C. Tissues were harvested during dark periods in complete darkness. To determine the extent of flower induction, plants were scored 3 weeks after photoperiodic treatments. The treatments began with a dark period, and the elapsed time was recorded from the beginning of this period. The cotyledons were excised, frozen in liquid nitrogen and stored at −80°C. Tissues were harvested during dark periods in complete darkness. To determine the extent of flower induction, plants were scored 3 weeks after photoperiodic treatments for the presence of terminal and axillary flowers or vegetative buds.

**Isolation of full-length cDNAs from *Pharbitis***

We screened >56,000 expressed sequence tags (ESTs) from *P. nil* cv. ‘TKS’ flower buds (Hoshino et al., unpublished data), the majority of which were deposited in the DDBJ database, and found three EST clones showing homology to the Arabidopsis *GIGANTEA* from *Pharbitis nil*.

**Gene expression analysis using RT–PCR**

For semiquantitative RT–PCR (Fig. 1, Supplementary Fig. S2), total RNA was extracted from *Pharbitis* cotyledons using the phenol/SDS method (Ausubel et al. 1987). For RT–PCR analysis, total RNA (10 μg) was treated with RNase-free DNase I (Roche, Basel, Switzerland) for 30 min at 37°C to eliminate contaminant DNA. RT–PCR was performed with 1 μg total RNA using a SuperScript™ first-strand synthesis system for RT–PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. A 1-μl aliquot of the 100-μl reaction mixture was used in a 50-μl PCR mixture. The PCR products were separated on 1.5% agarose gels and transferred to Biodyne B membranes (Nippon Genetics, Tokyo, Japan). The membranes were hybridized with 32P-labeled probe DNA in hybridization solution containing 5 × SSC, 0.1% SDS, 0.1% sarkosyl, 0.75% blocking reagent (Boehringer Mannheim, Mannheim, Germany) and 5% dextran sulfate sodium, at 65°C for 16 h. The blot was washed twice with 2 × SSC and 0.1% SDS for 10 min at 65°C, and then the hybridization signal was visualized using a Biomaging Analyzer (BAS 5000; Fuji Photo Film, Tokyo, Japan). All RT–PCR analyses were performed at least twice with independent RNA samples.

For quantitative real-time RT–PCR (Figs. 2–4, Supplementary Fig. S3), total RNA was extracted from *Pharbitis* cotyledons using the RNasy Plant Mini Kit (Qiagen) and treated with RNase-free DNase (Qiagen) in accordance with the manufacturer’s instructions. cDNA was synthesized from 1 μg total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). The cDNA was diluted 10-fold, and 2 μl were used in 20-μl quantitative real-time PCR reactions with the SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan) performed on a Thermal Cycler Dice Real-Time System (Takara Bio). The PCR conditions were as follows: primary denaturation at 95°C for 30 s followed by 40 amplification cycles of 5 s at 95°C and 30 s at 60°C. PCR products were quantified against a standard curve using a plasmid containing the gene of interest. Primer sequences used in these analyses are listed in Supplementary Table S1.

**Vector construction and analysis of transgenic *Pharbitis* plants**

A 35S: *PnGI* plasmid was constructed using the GATEWAY recombination system (Invitrogen) as follows. Full-length *PnGI* PCR product (containing a 108-bp fragment and a 155-bp fragment as 5′ and 3′ untranslated regions, respectively) was amplified using the primers 5′-CCACCATGATATCGGTCCGT-3′ and 5′-GGAGGAGCATGTTGTTGATT-3′, and cloned into the entry vector pENTR/D-TOPO (Invitrogen). The LR reaction was used to transfer the inserts from the entry vector to the destination vector pK2GW7 (Karimi et al. 2002), which was performed according to the manufacturer’s instructions. A 1-μl aliquot of the 100-μl reaction mixture was used in a 50-μl PCR mixture. The PCR products were separated on 1.5% agarose gels and transferred to Biodyne B membranes (Nippon Genetics, Tokyo, Japan). The membranes were hybridized with 32P-labeled probe DNA in hybridization solution containing 5 × SSC, 0.1% SDS, 0.1% sarkosyl, 0.75% blocking reagent (Boehringer Mannheim, Mannheim, Germany) and 5% dextran sulfate sodium, at 65°C for 16 h. The blot was washed twice with 2 × SSC and 0.1% SDS for 10 min at 65°C, and then the hybridization signal was visualized using a Biomaging Analyzer (BAS 5000; Fuji Photo Film, Tokyo, Japan). All RT–PCR analyses were performed at least twice with independent RNA samples.
instructions (Invitrogen). *Agrobacterium*-mediated transformation was performed as previously reported (Kikuchi et al. 2007).

**Cotyledon-movement assay**

Seedlings were grown for 5 d under LL, and entrained by two cycles of LD (16 h L/8 h D) photoperiod. On the eighth day, seedlings were released into continuous white light (58 μmol m⁻² s⁻¹) at 24°C, and cotyledon movement was recorded every 10 min over 4 d using a video camera (DCR-TRV30; Sony Corporation, Tokyo, Japan). The images were traced by plotting the coordinates of the cotyledon tips using the NIH image program (National Institutes of Health, Bethesda, MD, USA). In each case, rhythmic traces were analyzed by fast-Fourier transform-nonlinear least squares (FFT-NLLS; Plautz et al. 1997).

**Supplementary data**

Supplementary data are available at PCP online.

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**References**


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