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Original Paper

Immediate induction of *APETALA1*-like gene expression following a single short-day in *Pharbitis nil*

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Abstract *Pharbitis* [*Ipomoea*] *nil* cv. Violet is an excellent model plant for the study of photoperiodic induction of flowering because it can be induced flowering by a single short-day. However there are few molecular-level studies of the induction of flowering in *Pharbitis*. To gain insight into the photoperiodic induction of flowering, we isolated an *APETALA1*-like gene (*PnAP1*), which showed high similarity to *SQUAMOSA* (*SQUA*) and *AP1*. *PnAP1* expression at the shoot apex was induced only under flowering-inductive conditions, and *PnAP1* expression was induced from 24 h after the start of the inductive dark period. Both night-break and low ambient temperature during the dark period effectively repressed *PnAP1* expression and flowering. Timing of *PnAP1* expression assessed through induced cotyledon removal indicated that the floral stimulus began to move from cotyledons 14 h after the start of the inductive dark period. The results indicate that floral transitions begin immediately after the inductive dark period and that *PnAP1* is a good molecular marker of floral transition.

Key words: *APETALA1*, flowering, *Pharbitis nil*, photoperiod, short-day plant.

Regulation of flowering is crucial in plants because mistiming of flowering may result in reproductive failure. The flowering mechanism is well known in *Arabidopsis* (a facultative long-day plant) and rice (a facultative short-day plant), and many genes involved in flowering have been isolated (Hayama and Coupland 2004; Yanovsky and Kay 2003). However flowering responses are different among plant species, and for deeper understanding of flowering it is important to analyze and compare the flowering mechanism among several plants. In nature, many plants regulate flowering based on environmental signals, such as photoperiod and temperature. In photoperiodic flowering, a light signal is received by a photoreceptor, such as phytochrome and cryptochrome (Hayama and Coupland 2004). The plant's circadian clock receives inputs from these photoreceptors, allowing it to react to the light and dark cycles. When the photoperiod is suitable for the induction of flowering, a floral stimulus is synthesized at the induced leaf and is transferred to the shoot apex, where the signal triggers the transition from vegetative to reproductive growth.

It is known that photoperiodic responses differ among species (Garner and Allard 1922), and that plants induce flowering based on short-day (SD) or long-day (LD). *Pharbitis* [*Ipomoea*] *nil* cv. Violet (*Pharbitis*) is an absolute SD plant, and a single 16 h dark period (1 SD) can induce flowering, whereas it will not form flowers for months under continuous light (LL) or LD. Moreover induction of flowering is abolished by a night-break (NB), a 10-min irradiation with red light in the middle of 16 h of darkness (Imamura 1967). The sensitivity of *Pharbitis* to photoperiod makes this species useful in the study of photoperiodic induction of flowering. However, the molecular mechanism of *Pharbitis* flowering is not well known.

In *Arabidopsis*, the circadian clock-controlled flowering pathway comprising the genes *GIGANTEA* (*GI*), *CONSTANS* (*CO*), and *FLOWERING LOCUS T* (*FT*) promotes flowering (Fowler et al. 1999; Martin-Tryon et al. 2007; Mizoguchi et al. 2005; Park et al. 1999; Putterill et al. 1995; Samach et al. 2000). The *FT* gene encodes a 20-kD protein with homology to a phosphatidylethanolamine binding protein (PEBP) and the Raf kinase inhibitor protein (Kardailsky et al. 1999; Kobayashi et al. 1999). Recently, it was reported that FT fused with green fluorescent protein could move from leaf to shoot apex and accelerate flowering in *Arabidopsis* and rice (Corbesier et al. 2007; Tamaki et al. 2007). It was also reported that FT can interact with FD (a bZIP transcriptional factor), and induce floral identity genes such as *APETALA1* (*AP1*) at the shoot apex in

Abbreviations: SD, short day; LD, long day; LL, continuous light; NB, night break.
This article can be found at http://www.jspcmb.jp/

Arabidopsis (Abe et al. 2005; Wigge et al. 2005). An FT-FD induction system has also been suggested in wheat, however, it does not work in the shoot apex. Instead, TaFT (an *FT* homolog in wheat) interacts with TaFDL2 (an *FD homolog*) and may induce *VRN1* (an *AP1* homolog) in the leaf (Li and Dubcovsky 2008). This also suggests that the *AP1* gene is an early target of *FT* genes.

AP1 and it' homolog *SQUAMOSA* (*SQUA*) are MADS box genes and belong to the A class of genes in the ABC model of floral patterning (Bowman et al. 1991; Coen and Meyerowitz 1991; Huijser et al. 1992). *AP1* plays a key regulatory role in specifying floral meristem identity in *Arabidopsis*, and *ap1* mutations often results in partial flower transformation into inflorescence with alterations in sepal and petal organ identity (Gustafson-Brown et al. 1994; Mandel et al. 1992; Weigel and Meyerowitz 1993; Bowman et al. 1993). In addition, constitutive expression of *AP1* genes accelerate flowering in several species (Berbel et al. 2001; Chen et al. 2008; Mandel and Yanofsky 1995; Pena et al. 2001), indicating that A class genes play an important role during floral transition.

Recently it was reported that an *FT* homolog could also induce flowering in *Pharbitis*. (Hayama et al. 2007 and our unpublished data). However, *Pharbitis* genes that are involved in the induction of flowering at the shoot apex have not been established. To investigate the induction of flowering at the shoot apex, we isolated an *AP1*-like gene which showed coupled expression with floral transition at the shoot apex. Moreover, we predicted the floral stimulus transport timing from the cotyledons to the shoot apex by induction of *PnAP1* expression.

Materials and methods

Plant materials and growth conditions

Seeds of *Pharbitis nil* Choisy cv. Violet (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. Seeds were then soaked in distilled water for 16 h and sown on wet vermiculite to germinate. After germination, seedlings were grown at 24°C with illumination by continuous cool-white fluorescent light (60 μmol m^{-2} s^{-1}, FL 40SS W/37 lamps; Matsushita Electronics Co., Tokyo, Japan) for 6 d. Seedlings with fully expanded cotyledons were used in each experiment. In the NB experiment, red (660±20 nm) LED lamps (EYELA, Tokyo, Japan) were used to provide red light. Temperature during the dark period was controlled. Tissues were harvested during dark periods and in complete darkness. Samples were frozen in liquid nitrogen, and stored at −80°C. To determine the extent of flower induction, plants were assessed 3 weeks after photoperiodic treatments for the presence of terminal and axillary flower or vegetative buds.

Isolation of the full-length PnAP1 gene

A partial sequence of *PnAP1* was isolated from the EST library of *Pharbitis nil* cv. Tokyo Kokei Standard. The 5′-upstream region of the partially cloned *PnAP1* was isolated by 5′RACE using the Marathon cDNA Amplification Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. The gene-specific primer *PnAP1*-5′RACE: 5′-TCAGAGTTATTAGCAAGCAAGCGTCTC-3′ was used for PCR. The PCR conditions were 94°C for 30 s; 5 cycles at 94°C for 5 s and 72°C for 3 min; 5 cycles at 94°C for 5 s and 70°C for 3 min; and 25 cycles at 94°C for 5 s and 68°C for 3 min. The resulting cDNAs were sequenced using a CEQ8000 automated DNA sequencer and a DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA, USA). *PnAP1*-Full (5′-ATGGGGAGAGGAAAGGTGCA-3′ and 5′-AACACGGAG-GGCAGATGAAA-3′) primers were used for the amplification of the full length cDNA of *PnAP1*.

Expression analysis of AP1-like genes

Total RNA was extracted by using a Get Pure RNA Extraction kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. For RT-PCR analysis, first-strand cDNA synthesis was performed on 1 μg RNA by using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) with an oligo dT. RT-PCR was performed with Ex *Taq* DNA Polymerase (TaKaRa, Otsu, Japan). *PnAP1* (5′-AGTGGGCTTGATTGTCTTCT-3′ and 5′-ACTCTTCTCCTTCTCCTTGA-3′), *PnSAH1* (5′-GCAGGA-CCAAATATCAGCTTTCTAA-3′ and 5′-AGTTTCCGCATAT-TTGTTCAAAGTA-3′), *PnSAH2* (5′-AAAGAGATGGCTGA-ACAGCCTAACT-3′ and 5′-ATAACTAGGAGGCAAATGTG-GATAC-3′), *PnSAH3* (5′-CACAGGTCTAAAGCGAATAAG-AACA-3′ and 5′-TAACAAGATGATTATTATTTGGATAC-3′), *PnACTIN3* (5′-TCCATTATGAAGTGTGATGT-3′ and 5′-GGGCCAGACTCCTCATACTC-3′) primers were use for PCR. These primers were used to amplify each genes using 35 (*PnAP1*, *PnSAH1*, *PnSHA2*, *PnSAH3*) and 24 cycles (*PnACTIN3*). The specificity of the amplifications was verified by sequence.

Quantitative PCR was performed on a Roche LightCycler using the Fast-start DNA Master SYBG Green I Kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's protocol. Quantitative RT-PCR was performed by using same primers with RT-PCR. For PCR, 1.5 μl of cDNA was used as a template in a 15 μl reaction mix. The PCR program consisted of denaturation, followed by Taq activation (95°C for 10 min), then by 40 cycles of denaturation (94°C for 10 s), annealing (57°C for 10 s) and extension (72°C for 10 s). At the end, amplified products were denatured (95°C), renatured (65°C) and progressively denatured (stepped from 65 to 95°C over 30 min for melting curve analysis). The specificity of the amplifications was verified by analysis of the PCR products on agarose gels and by melting curve analysis. The plasmid solution containing cDNAs (*PnAP1*) were serially diluted, and used for establishment of a standard curve for quantification. The amount of cDNA was calculated using LightCycler 3.1 (Roche Applied Science) Expression level of *PnAP1* was normalized with the expression level of *PnACTIN3*.

Results

Isolation of AP1-like gene from Pharbitis

A fragment of *AP1*-like gene (*PnAP1*) was isolated from

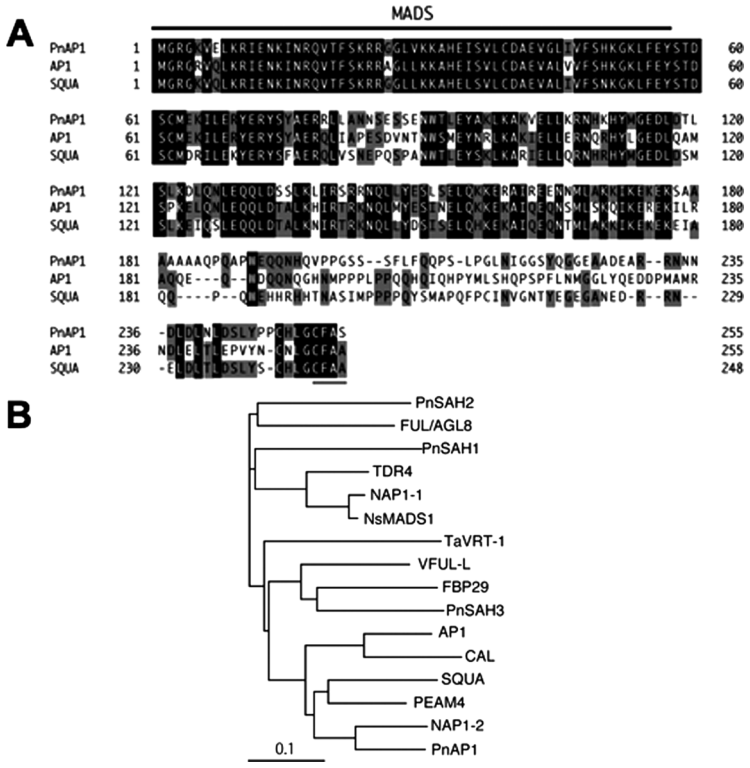

Figure 1. Sequence analysis of *PnAP1*. (A) Comparison of the predicted amino acid sequences of AP1 proteins. Predicted amino acid sequence of PnAP1 is compared with those of *Arabidopsis* AP1 (Z16421) and *Antirrhinum* SQUA (X63701). A black line indicates the conserved MADS box. The prenylation motif (CaaX) is underlined at the carboxy-terminal end of the AP1 and SQUA sequence. (B) Phylogenic tree of AP1 related proteins. Tree includes AP1 homologs from *Antirrhinum* SQUA (X63701), *Arabidopsis* AP1, CAL, FUL/AGL8 (Z16421, L36925, AY072463), *Nicotiana tabacum* NAP1-1, NAP1-2, NsMADS1 (AF009126, AF009127, AF068725), *Petunia hybrida* FBP29 (AF335245), *Pharbitis nil* PnSAH1, PnSHA2, PnSAH3, PnAP1 (AB013105, AB013106, AB302847, AB302848), *Pisum sativum* PEAM4 (AF461740), *Solanum lycopersicum* TDR4 (AY098732), *Triticum aestivum* TaVRT-1 (AY280870), and *Vitis vinifera* VFUL-L (AY538747). The ClustalW program was used for the construction of phylogenic tree.

an expressed sequence tag (EST) library of *P. nil* cv. Tokyo Kokei Standard from the National Bio Resource Project for Japanese morning glory (http://www.nbrp.jp/index.jsp). Contig1749 was a 671-bp of 3′-cDNA, which showed high similarity to *SQUA* and *AP1*. To obtain the missing 5′-cDNA sequence, we performed 5′RACE and PCR using a cDNA library of *P. nil* cv. Violet. The isolated full-length cDNA from *P. nil* cv. Violet encoded a 29 kD protein of 255 amino acids that showed 63 and 60% amino acid identity with SQUA (*Antirrhinum*) and AP1 (*Arabidopsis*), respectively, and the N-terminal MADS domain was well conserved (Figure 1A). In C-terminal of PnAP1 protein the prenylation motif (CaaX) was conserved (Figure 1A). A phylogenic tree was constructed using amino acids of the *AP1* or *SQUA*-like genes in several species (Figure 1B). The tree indicated that the isolated cDNA belonged to a clade of *AP1* and *SQUA*, and that *FRUITFULL* (*FUL*) and *AGL8* were in a different clade (Figure 1B). Other *SQUA*/*AP1*-like genes previously isolated in *Pharbitis* (*PnSAH1*, *PnSAH2*, and *PnSAH3*, Ono et al., unpublished) belonged to different clade of *AP1* and *SQUA* (Figure 1B).

Expression of AP1-like genes in floral organs

In *Arabidopsis*, *AP1* specifies the identity of the floral meristem and determines sepal and petal development. The A class genes are mainly expressed in the sepal and petal flower primordia (Mandel et al. 1992). To investigate the expression pattern of *PnAP1* in developing floral organs, we examined their expression analysis by RT-PCR. In *AP1*-like genes *PnAP1* expression showed strong expression in sepal and petal (Figure 2). *PnSAH1* and *PnSAH2* expression were equally detected in all organs of the flower bud, but *PnSHA3* expression was not detected (Figure 2).

PnAP1 expression is induced by the induction of flowering only at the shoot apex

Pharbitis can be induced to flower by 1 SD at the juve*nil*e stage. To investigate the patterns of *AP1*-like genes expression in different organs under 1 SD (inductive) or LL (non-inductive) conditions, we performed RT-PCR by using different seedling organs at 48 h after the inductive dark period. In *AP1*-like genes *PnAP1* expression was detected only in the 1 SD shoot apex and not detected in other organs (Figure 3A). *PnSAH1* and *PnSAH2* expressions were increased under

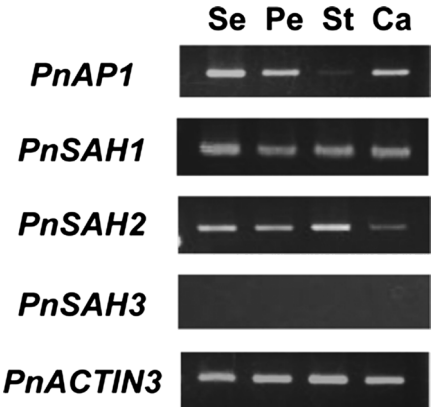

Figure 2. Expression patterns of *AP1*-like genes in floral organs. Sepal (Se), petal (Pe), stamen (St) and carpel (Ca) of developing flower buds (1.5 cm) were used for RT-PCR analysis. *PnACTIN3* was used as the control.

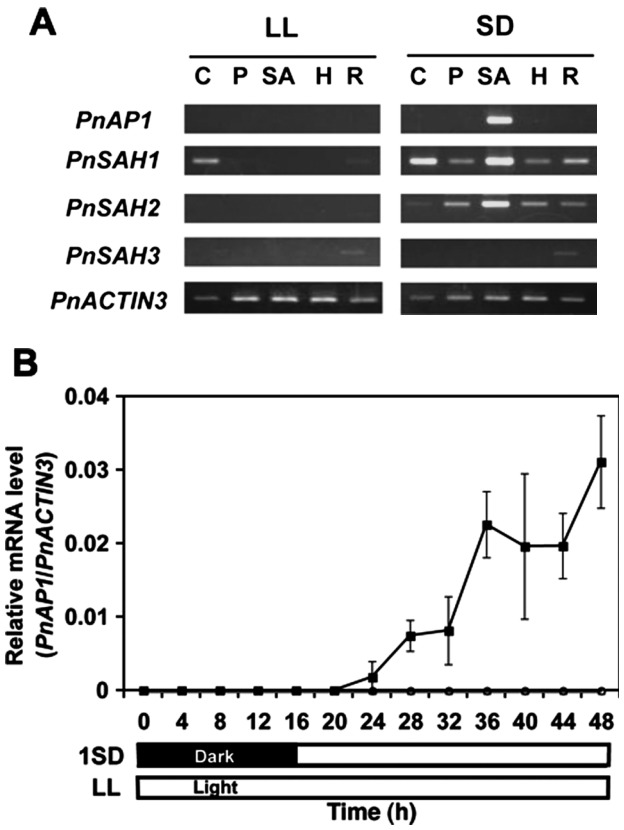

Figure 3. Induction of *PnAP1* expression by 1 SD. (A) Expression of *AP1*-like genes in cotyledons (C), shoot apex (SA), petiole (P), hypocotyls (H) and roots (R). Fifteen seedlings from 1 SD (48 h after a single inductive dark period) or LL conditions were used for analysis. (B) *PnAP1* expression under 1 SD and LL. Expression levels were determined by quantitative RT-PCR and normalized to *PnACTIN3* expression levels. Plants were grown under 1 SD and LL for 2 d. Shoot apices were harvested every 4 h. Error bars show SD of three PCR experiments.

1 SD in all organs (Figure 3A). *PnSAH3* expression was detected in root, but there was no change between 1 SD and LL (Figure 3A). Thus *PnAP1* showed most ideal expression patterns with floral transition at shoot apex.

Table 1. Effect of NB and ambient temperature on the number of flower buds

Treatment	Average number of flower buds (mean±SD)
1SD	5.9±0.54
NB[a]	N.D.
24°C[b]	5.5±0.32
20°C	5.3±0.43
18°C	0.9±0.65
15°C	N.D.

[a] The 16 h inductive dark period was interrupted after 8 h by 5 min of red light (15 μmol m^{-2}s^{-1}) from LED panels.

[b] Plants were exposed to each temperature during 16 h inductive dark period and returned to 24°C under LL condition. The number of flower buds were assessed 3 weeks after treatments.

To investigate detailed expression timing of *PnAP1* after the inductive dark period, we performed expression analyses every 4 h for 2 d by quantitative RT-PCR. *PnAP1* expression was not detected under LL condition, but *PnAP1* expression was induced 24 h after the start of the inductive dark period and increased gradually (Figure 3B).

NB and low ambient temperature inhibit flowering and PnAP1 expression

To investigate the NB effect on *PnAP1* expression, we compared *PnAP1* expression under SD and NB condition by quantitative RT-PCR. Flowering was not induced following NB treatment (Table 1). Expression of *PnAP1* was detected under SD condition, but was repressed under NB conditions (Figure 4A). Thus, NB inhibited the induction of *PnAP1* expression. In addition to NB, it has been reported that low ambient temperature during the dark period inhibits flowering (Imamura 1967). To investigate the effect of low ambient temperature on the expression of *PnAP1*, we exposed seedlings to low ambient temperatures during a 16 h dark period and examined *PnAP1* expression 48 h from the start of the dark period. Flowering was repressed by an ambient temperature of 18°C, while at 15°C flowering did not occur (Table 1). Under low ambient temperatures, *PnAP1* expression was also repressed (Figure 4B). Thus, low ambient temperature inhibited the induction of *PnAP1* expression.

Cotyledon removal effect on flowering and PnAP1 expression

In intact seedlings, a 14 h dark period is sufficient to induce flowering. However, when cotyledons are removed 14 h after the start of an inductive dark period, flowering was not observed (Table 2). On the other hand, when cotyledons are removed 16 h after the start of the inductive dark period flowering was induced. Furthermore, when cotyledons were removed after more

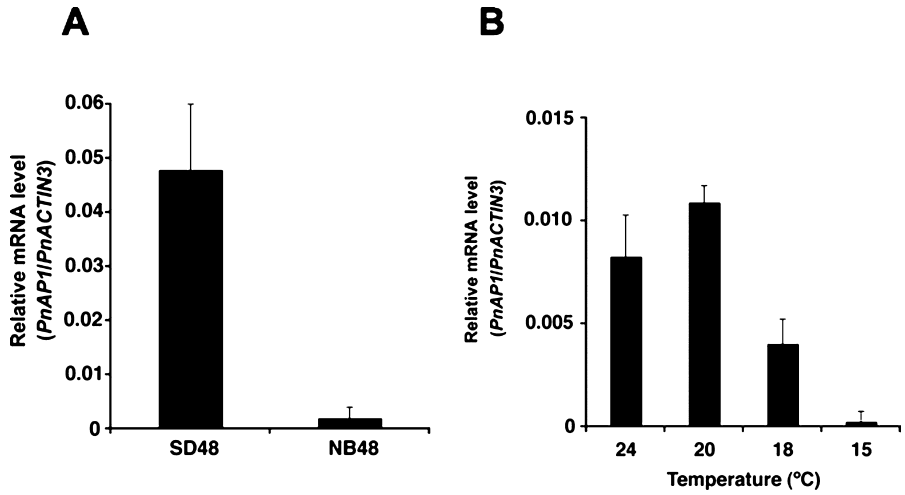

Figure 4. Night break (NB) and low ambient temperature repress *PnAP1* expression. (A) The effect of NB on *PnAP1* expression. A 16 h inductive dark period was interrupted after 8 h by 5 min of red light in NB plants; shoot apices were harvested 48 h after the start of dark period. (B) Effect of low ambient temperature on *PnAP1* expression. Seedlings were exposed to each temperature during a 16 h dark period and then returned to 24°C LL conditions. Shoot apices were harvested 48 h after the start of dark period. Expression levels were determined by quantitative real-time PCR and normalized to *PnACTIN3* expression levels. Error bars show SD of three PCR experiments.

Table 2. The number of flower buds when cotyledons were removed during 16 h dark period

Removal time of cotyledons (h)	Average number of flower buds (mean±SD)
0	N.D.
12	N.D.
14	N.D.
16	2.1±0.25
20	5.4±0.32
24	5.2±0.15

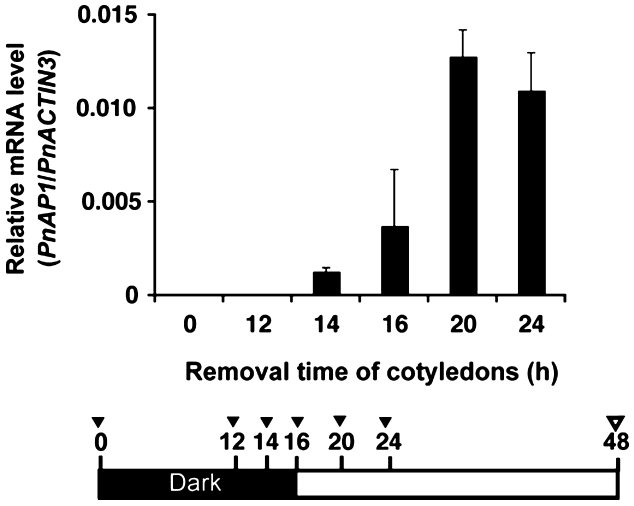

Figure 5. Effect of cotyledon removal on *PnAP1* expression. Cotyledons were removed after the start of the dark period (16 h) at indicated time, and shoot apices were harvested at 48 h. Expression levels were quantified by quantitative real-time PCR and normalized to *PnACTIN3* expression levels. Error bars show SD of three PCR experiments.

than 16 h, the number of flower buds increased as removal time prolonged (Table 2). Thus, the induction of flowering depends on the timing of cotyledon removal after the start of an inductive dark period (Imamura 1967). To examine the effect of cotyledon removal on *PnAP1* expression, cotyledons were removed during inductive dark period at successive 4 h intervals, and *PnAP1* expression was examined 48 h after the start of an inductive dark period by quantitative RT-PCR. *PnAP1* expression was detected when cotyledons were removed at (14, 16, 20 and 24) h after the start of an inductive dark period, whereas it was not detected when cotyledons were removed at 12 h (Figure 5). Moreover, the expression of *PnAP1* increased as removal time prolonged (Figure 5). Thus, the expression of *PnAP1* depends on cotyledon removal timing, and responds to the strength of the induction of flowering.

Discussion

To investigate the induction of flowering at the shoot apex, we isolated an *AP1*-like gene from *Pharbitis*. *PnAP1* is similar to *SQUA* (*Antirrhinum*) and *AP1* (*Arabidopsis*) genes (Figure 1), and the prenylation motif (CaaX) which is typical in C-terminal of AP1 and SQUA proteins (Rodriguez-Concepcion et al. 1999) but is absent in FUL-like proteins, was conserved in PnAP1 (Figure 1A). Moreover in *AP1*-like genes only *PnAP1* expression showed ideal pattern with floral transition at shoot apex (Figure 3), indicating that *PnAP1* is involved in floral transition in *Pharbitis*.

We also assessed transport timing of the floral stimulus from cotyledons to shoot apex by induction of *PnAP1* expression. When cotyledons were removed at 14 h, flowering was not induced, but when removed at 16 h, flowering was induced (Table 2); thereby indicating that

a floral stimulus sufficient for the induction of flowering is transported from the cotyledons to the shoot apex between 14 and 16 h after the inductive dark period. However, the expression of *PnAP1* was induced when the cotyledons were removed at 14 h (Figure 5), indicating that at 14 h some floral stimuli have already been transported but the amount is insufficient for the induction of flowering. When cotyledons were removed later than 14 h, the number of flower buds and the *PnAP1* expression increased as removal time increased, indicating that the magnitude of the transported floral stimulus also increased. When cotyledons were removed at 12 h *PnAP1* expression was not detected (Figure 5), indicating that floral stimulus transport starts between 12 and 14 h after the inductive dark period. Also in *Pharbitis*, expression of an *FT* homolog, considered to be a floral stimulus, was induced at 14 h, increased rapidly, and showed maximum expression at 18 h (Hayama et al. 2007), indicating that after synthesis, a floral stimulus can transport immediately to the shoot apex from the cotyledons. Thus, in *Pharbitis* the process of flowering from the synthesis of the floral stimulus to floral transition at the shoot apex occurs within a few hours.

Our results reveal that *PnAP1* expression is coupled with floral transition. This indicates that *PnAP1* expression is a useful molecular marker for floral stimulus assays. Recent research indicates that the FT protein is a floral stimulus (Abe et al. 2005; Corbesier et al. 2007; Jaeger and Wigge 2007; Mathieu et al. 2007; Tamaki et al. 2007; Wigge et al. 2005). Although it has not been shown that the FT protein can induce flowering, a substance extracted from SD treated *Pharbitis* leaf in a previous study induced flowering (Ishioka et al. 1990), indicating that *Pharbitis* may be a good plant for identification of floral stimulus substances. By monitoring the detection of *PnAP1* expression a sensitive assay system to detect the activities of chemical substances, including the FT protein or the products of *FT*, may be established. Establishment of such an assay system in *Pharbitis* may provide novel insights into florigens.

Acknowledgements

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