

Effects of 9,10-ketol-octadecadienoic acid (KODA) application on single and marginal short-day induction of flowering in *Pharbitis nil* cv. Violet

Michiyuki Ono^{1,*}, Mayuko Kataoka¹, Mineyuki Yokoyama², Ohji Ifuku³,
Masayuki Ohta¹, Shoko Arai¹, Hiroshi Kamada¹, Kimiyo Sage-Ono¹

¹Gene Research Center, Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan; ²Technology & Engineering Center, Shiseido Co., Ltd., Yokohama, Kanagawa 236-8643, Japan; ³Shiseido Research Center, Shiseido Co., Ltd., Yokohama, Kanagawa 236-8643, Japan

*E-mail: ono.michiyuki.fm@u.tsukuba.ac.jp Tel: +81-29-853-7759 Fax: +81-29-853-7746

Received October 31, 2012; accepted November 5, 2012 (Edited by T. Mizoguchi)

Abstract Seedlings of the Japanese morning glory (*Pharbitis nil*, formerly *Ipomoea nil*) cv. Violet are induced to flower by a single short day treatment, and endogenous levels of 9,10-ketol-octadecadienoic acid (KODA) in cotyledons were found to correlate with this mode of short-day induction. When 100 μ M KODA solution was sprayed on 7-day-old seedlings before and after a marginal short-day induction, the seedlings bore approximately 4 flower buds, a one-bud increase compared with control seedlings. In treated seedlings, the second node most commonly bore the first flower bud, one node lower than in the control seedlings. We then used RT-PCR to determine expression levels of 10 genes related to photoperiodic induction of flowering. In the cotyledons, no differences were observed in expression of any genes, including *P. nil* FLOWERING TIME LOCUS T (*PnFT1* and *PnFT2*), between the KODA-treated and the control seedlings. In the apical buds, *P. nil* APETALA1 (*PnAPI*) was expressed earlier in the KODA-treated seedlings than in the control seedlings. A decrease in the expression of *P. nil* TERMINAL FLOWER1b (*PnTFL1b*) was also observed in the KODA-treated seedlings. These results suggest that KODA acts as a weak enhancer of flower bud formation.

Key words: Flowering, *Ipomoea nil*, 9,10-ketol-octadecadienoic acid (KODA), *Pharbitis nil*, *PnAPI*.

The transition from vegetative to reproductive growth is a significant phase change in higher plants, and the floral stimulus, florigen, plays a central role. Recent molecular and genetic studies in *Arabidopsis thaliana* have revealed the molecular identity of florigen, which is a product of the FLOWERING LOCUS T (FT) (Corbesier et al. 2007; Jaeger and Wigge 2007; Kardailsky et al. 1999; Kobayashi et al. 1999; Turck et al. 2008). In *A. thaliana*, when the appropriate photoperiodic signal for flowering is perceived in a leaf, the leaf produces FT as a graft-transmissible signal substance. FT then moves to the meristem of the active growing bud and up-regulates flower bud identity genes like APETALA1 (*API*) by activating the bZIP transcription factor, FD (Abe et al. 2005; Wigge et al. 2005). In *Oryza sativa*, the FT homologue Hd3a plays essentially the same role (Kojima et al. 2002; Tamaki et al. 2007; Tsuji et al. 2011). In other photoperiodic plant species, the homologues of FT/Hd3a are largely considered to function as florigens (Lifschitz et al. 2006; Lin et al. 2007).

The Japanese morning glory (*Pharbitis nil*, formerly

Ipomoea nil) is a model ornamental plant. Libraries of expression sequence tags (ESTs) and bacterial artificial chromosomes have been created, genetic and molecular maps have subsequently been made available, transformation systems have been established, and whole genome sequencing is in progress. These experimental results are available through the National BioResource Project (NBRP) in Japan (Yamazaki et al. 2010). The Japanese cv. Violet is recognized as an absolute short-day (SD) plant for the study of flowering response because seedlings can be induced to change from the vegetative to the reproductive phase by a single SD treatment (Higuchi et al. 2011; Imamura 1967). Several genes associated with SD induction have been isolated and characterized (Hayama et al. 2007; Higuchi et al. 2007, 2011; Liu et al. 2001; O'Neill et al. 1994; Ono et al. 1996; Sage-Ono et al. 1998). Among them, a pair of cDNAs encoding PnFT1 and PnFT2 were isolated and it was demonstrated that products of these function as florigens in *P. nil* (Hayama et al. 2007).

Several floral stimuli in addition to florigen have been

reported in *P. nil* (Hatayama and Takeno 2003; Ishioka et al. 1990; Kong et al. 2005; Shinozaki and Takimoto 1983; Suzuki et al. 2003; Wada et al. 2010). Among them, 9,10-ketol-octadecadienoic acid (KODA) is appropriate because endogenous levels of KODA in cotyledons are highly correlated with the mode of photoperiodic induction of flowering (Suzuki et al. 2003). Endogenous levels of KODA are elevated during the latter half of flower-inductive darkness, and floral induction can be canceled by the night-break treatment, that is a light exposure during the 8th hour of inductive darkness (Suzuki et al. 2003). KODA was originally isolated from *Lemna paucicostata* during experiments related to flowering and it showed flower-inducing activity when applied exogenously (Yamaguchi et al. 2001; Yokoyama et al. 2000). However, exogenous application of KODA to *P. nil* seedlings under long day non-inductive conditions showed no effects on flowering. The enhancing effects were observed only when KODA was sprayed on marginally induced seedlings; it then produced an increase in the number of flower buds (Yokoyama 2005).

Aside from this evidence related to flowering, the mechanisms of KODA function are unknown. In particular, the effects of KODA on genes related to flowering are poorly understood. Kittikorn et al. (2011) showed the expression levels of the *TFL1* homologue, *MdTFL1* mRNA, are decreased by KODA application in apple. To the best of our knowledge, this is the only report on changes in gene expression caused by KODA. In this paper, we precisely observed the enhancer activity of KODA using one-shot and marginal SD treatments in *P. nil*, and studied changes in the expression of genes related to photoperiodic flowering induced by KODA treatment.

Materials and methods

Plant materials and growth conditions

Seeds of *Pharbitis nil* cv. Violet (provided by Dr. Nitasaka) were slightly abraded on a part of their surface with a file, and then soaked in distilled water overnight. Swollen seeds were sown on wet vermiculite in trays and covered with Saran Wrap to maintain high humidity. Culture conditions were set at $24 \pm 1^\circ\text{C}$ with illumination from continuous cool-white fluorescent lights ($60 \mu\text{mol m}^{-2} \text{s}^{-1}$, FL40SW lamps; NEC Lighting Ltd., Tokyo Japan). When the primary roots were approximately 3 cm long, the seed coats of seedlings in similar stages of development were removed and the seedlings were planted in fresh vermiculite in trays ($11 \times 15 \times 5$ cm). Six seedlings were planted per tray and they were watered once a day. The flower inductive dark period was given in a black box for 12 h. From the 8th day, a 0.1% (v/v) nutrient solution (Liquid Hyponex™ 5-10-5; Hyponex Japan Co. Ltd., Tokyo, Japan) was applied.

KODA application

KODA solution was prepared and treated as described previously (Yokoyama 2005; Yokoyama et al. 2005). KODA solution (100 μM) containing Triton X-100 [0.1% (w/v)] and ethanol [0.6% (v/v)] was sprayed on all above-ground organs at 12 h before the beginning of the 12-h dark period and just after the dark period. All controls were sprayed with a Mock solution containing an equivalent concentration of Triton X-100 [0.1% (w/v)] and ethanol [0.6% (v/v)].

Measurement of flowering response

Plants were assessed about 3 weeks after exposure to the inductive dark period, and the number of flower buds per plant and the nodal position of the flower buds on each plant were recorded. The nodal number was counted from the cotyledonal node. Data were presented as the means \pm SD of 40 plants, and the Student's *t*-test was used to test for significance.

Isolation of PnTFL1 cDNAs

Partial genomic fragments of *PnTFL1s* were isolated using degenerate primers and total genomic DNA of *P. nil* cv. Violet. The degenerate primers containing inosine (I) used for the initial isolation were as follows: TFL-1S 5'-AAC/TGGICAC/TGAA/GC/TTITTC/TCC-3' and TFL-3A 5'-CG/TC/TTGIGCA/GTTA/GAAA/GAAIAC-3' (provided by Dr. Wada; Kotoda and Wada 2005). PCR was carried out under standard conditions using a thermal cycler (TP600, Takara Bio Inc., Ohtsu, Japan), with an initial denaturation step at 94°C for 12 min, followed by 40 cycles at 94°C for 60 s, 50°C for 60 s, and 72°C for 120 s, using AmpliTaq Gold DNA polymerase (Takara Bio, Inc.). The 5'-upstream and 3'-downstream regions of the partially cloned *PnTFL1s* were isolated by RACE using a Marathon cDNA Amplification Kit (Clontech Laboratories, Inc., Mountain View, CA, USA) according to the manufacturer's protocol. The resulting DNA sequence information was analyzed with GENETYX MAC software (Software Development Co. Ltd., Tokyo Japan). Databases were searched using BLAST (Altschul et al. 1997; DNA Data Bank of Japan, Mishima, Japan). The phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987) with ClustalW (Thompson et al. 1994; DNA Data Bank of Japan). The resulting phenogram was drawn using the NJplot program (Perrière and Gouy 1996).

Reverse transcription polymerase chain reaction (RT-PCR)

For the RNA extraction, apical buds (the plumule excluding the first leaf) and cotyledons were harvested at the time indicated in Figure 3, frozen in liquid nitrogen, and stored at -80°C . Total RNAs were isolated from apical buds and cotyledons using a Get pure RNA Kit (Dojindo, Kumamoto, Japan). First-strand cDNA was synthesized from 1 μg of each RNA sample in a 20- μl reaction solution using a SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. PCR was carried out under

Table 1. PCR primers used in this study.

| Gene | Sense primer | Antisense primer | Annealing temperature (°C) | Product length (bp) | Accession number |
|--------------------|-------------------------------------|------------------------------------|----------------------------|---------------------|----------------------|
| <i>PnGI</i> | 5'-GGTGGTGTAGTGGTGTAAAGGG-3' | 5'-TTGGAGTTGAAGGATATAGTTG-3' | 55 | 111 | AB265781 |
| <i>PnCO</i> | 5'-ACAATGGGTTCTTGACTCAGGATGC-3' | 5'-GAGGCTCTTGGTGGAGATTGAGG-3' | 55 | 514 | AF300700 |
| <i>PnFT1/PnFT2</i> | 5'-TCTGATGCTCCAAGTCCAAC-3' | 5'-CGGAAAAGCACAAACACAAAA-3' | 55 | 165 | EU178859 AB182478 |
| <i>PnAPI</i> | 5'-AGTGGGCTTGATGTTCTTCT-3' | 5'-ACTTCTCTCTCTCTCTTGA-3' | 55 | 409 | AB302848 |
| <i>PnSAH1</i> | 5'-GCAGGACCAATATCAGCTTCTTAA-3' | 5'-AGTTTCCGCATATTTGTTCAAAGTA-3' | 55 | 196 | AB013105 |
| <i>PnSAH2</i> | 5'-AAAGAGATGGCTGAACAGCCTAACT-3' | 5'-ATAACTAGGAGGCAATGTGGATAC-3' | 55 | 288 | AB013106 |
| <i>PnSAH3</i> | 5'-CACAGGTTAAAGCGAATAAGACA-3' | 5'-TAACAAAGATGATATTTGGATAC-3' | 55 | 196 | AB302847 |
| <i>PnTFL1a</i> | 5'-TTTCCCTCTGCTGTCAACTTAG-3' | 5'-GGGGTAGGTAGCTGGTTAGCGACG-3' | 55 | 419 | AB219403 |
| <i>PnTFL1b</i> | 5'-TTCACCCCACTTCCATAAGAAT-3' | 5'-TGCGTGTGTTGAAAGAGAATA-3' | 55 | 328 | AB219404 |
| <i>PnMADS1</i> | 5'-CGGATGTCATGACAAGAGACAAGATTCAG-3' | 5'-GGAGCTCGAGCTCTCAATAATCTTTGTG-3' | 55 | 196 | D84512 |
| <i>PnUBQ</i> | 5'-CTCCATCTCGTCTCCGTTTGA-3' | 5'-GCCCTCCTTGCTCCGTAATCTTG-3' | 55 | 363 | AB265782 |

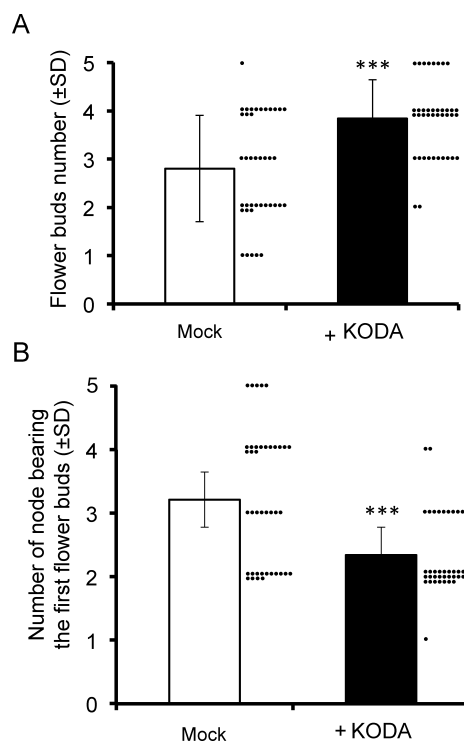


Figure 1. Effects of KODA application on flowering in marginally induced seedlings. The number of flower buds per plant (A) and the number of the first node bearing a flower bud (B) were examined in *P. nil* seedlings exposed to 12h of darkness. KODA solution (100 μ M, +KODA) or the control solution (Mock) was sprayed at 12h before the 12-h darkness and immediately after the 12-h darkness. Data are the means \pm SD ($n=40$, *** $p<0.001$). The number of flower bud in (A) and node in (B) was shown by dots.

standard conditions using a thermal cycler (TP600, Takara Bio Inc., Ohtsu, Japan), with an initial denaturation step at 94°C for 2 min, followed by 27–37 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s. The primer sequences, annealing temperature, product length and accession numbers of all genes analyzed are listed in Table 1. The PCR products were separated on 1.5% agarose gels. After staining with ethidium bromide, DNA bands were photographed under ultraviolet light. Only the portion of the bands showing the amplified fragments is presented. For the time course experiments using *PnAPI* (Figure 5) and *PnTFL1b* (Figure 6), the intensity of DNA bands was quantitated using a Molecular Imager FX (Bio-Rad Laboratories, Hercules, CA, USA) and the data was normalized to *PnUBIQUITIN* (*PnUBQ*) expression levels. All RT-PCR analyses were performed three times with independent RNA samples. For Figures 5 and 6, data were averaged and graphed as the means \pm SE of three experiments. For Figures 5 and 6, one representative gel image of three independent experiments is also shown.

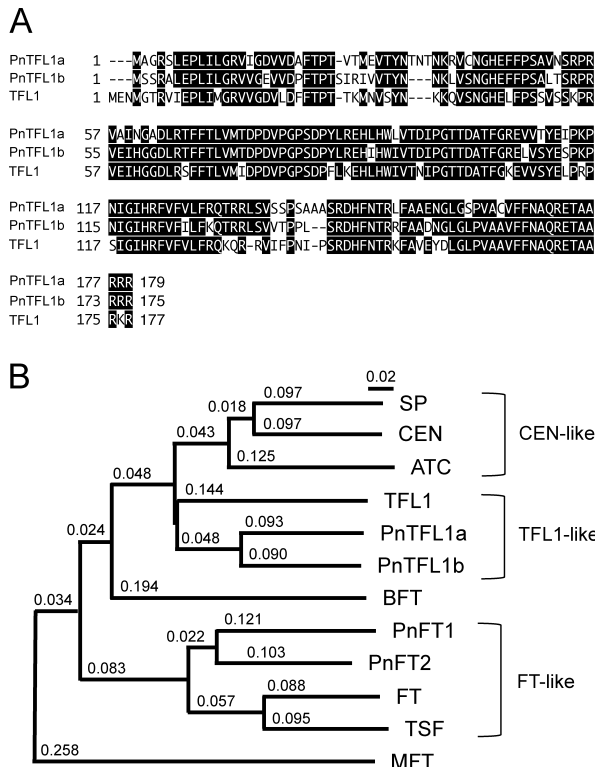


Figure 2. Phylogenetic analysis of PnTFL1a and PnTFL1b. (A) Comparison of the predicted amino acid sequences of TFL1 proteins. The predicted amino acid sequences of PnTFL1a and PnTFL1b are compared with those of *A. thaliana* TFL1. Conserved amino acid residues are indicated by outlined characters. (B) A phylogenetic tree analysis of PnTFL1a and PnTFL1b. The tree includes TFL1-related proteins from *Solanum lycopersicum* SP (U84140), *Antirrhinum majus* CEN (S81193), *A. thaliana* ATC, TFL1, BFT, FT, TSF, and MFT (AT2G27550, AT5G03840, AT5G62040, AT1G65480, AT4G20370, and AT1G18100, respectively) and *P. nil* PnTFL1a, PnTFL1b, PnFT1, PnFT2 (numbers of database accessions are shown in Table 1). The three major clades are indicated as CEN-, TFL1- and FT-like. The horizontal branch length is proportional to the estimated number of amino acid substitutions per residue (bar=0.02 aa substitution per residue). The program ClustalW was used for construction of phylogenetic tree.

Results

Effects of KODA application on number of flower buds and node of flowering

When 100 μ M KODA solution was sprayed on 7-day-old seedlings 12 h before and just after a 12-h dark period, the average number of flower buds per plant was 3.8 ± 0.8 (Figure 1A). There was an increase of one bud compared with the average number of flower buds per plant in mock-treated control plants (2.8 ± 1.1). We also found that KODA application produced a decrease in the node of the first flower of nearly one node (Figure 1B). The average node number of the first flower bud in KODA-sprayed plants was 2.3 ± 0.6 , whereas the average number of the first flowering node in mock-treated control plants was 3.2 ± 1.0 . To gain insight into the molecular mechanisms of the effects of KODA application, we studied changes in expression of the genes related to

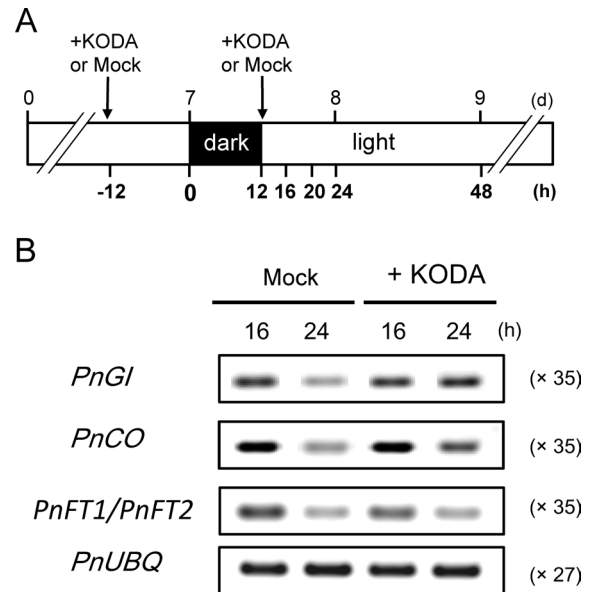


Figure 3. Effects of KODA application on mRNA level of the homologues of photoperiodic related genes. (A) Timing of KODA application and harvest of apical buds and cotyledons. On the seventh day, a 12-h period of darkness was given to seedlings grown under continuous light. KODA solution (100 μ M) or the mock control solution was sprayed 12-h before and just after the period of darkness (indicated by vertical arrows). Cotyledons and apical buds were harvested at the time indicated after the beginning of the darkness. (B) Results of RT-PCR. Transcript levels of *P. nil* GIGANTEA (*PnGI*), *P. nil* CONSTANS (*PnCO*), *P. nil* FLOWERING LOCUS T1, and *PnFT2* (*PnFT1/PnFT2*) in cotyledons were determined by RT-PCR. Total RNA was extracted from cotyledons and RT-PCR was performed as described in the Materials and methods. PCR products were electrophoresed in agarose gels and ethidium bromide-stained bands were photographed under ultraviolet light. Only the portions of the bands showing amplified fragments are presented. PCR primers used and the sizes of amplified fragments are shown in Table 1. The number of cycles used for PCR amplification was indicated in parenthesis. *PnUBQ* was used as an internal control.

photoperiodic induction of flowering.

Isolation of TFL1 homologues and phylogenetic analysis

Because TFL1 homologues had not been isolated from *P. nil*, we isolated two TFL1 homologues from *P. nil*, namely, PnTFL1a and PnTFL1b. The deduced amino acid sequence of PnTFL1a and PnTFL1b showed strong similarity to that of TFL1 of *A. thaliana* (Figure 2A). We performed a phylogenetic analysis that included all six phosphatidyl ethanolamine-binding proteins (PEBP) of *A. thaliana*, CEN of *Antirrhinum*, SP of tomato, and all the currently isolated PEBPs from *P. nil* (Figure 2B). The resultant phylogenetic tree indicated that both PnTFL1a and PnTFL1b proteins do not belong to either the CEN- or FT-clade, but clearly fall within the TFL1-like clade (Foucher et al. 2003; Mimida et al. 2001). We then studied changes in expression of PnTFL1a and PnTFL1b caused by KODA application.

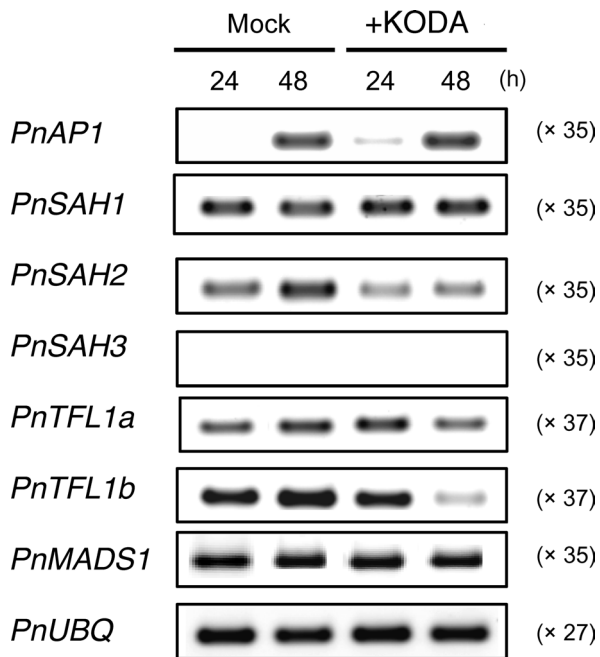


Figure 4. Effects of KODA application on mRNA levels of the homologues of photoperiod-related genes in apical buds. Transcript levels of *P. nil* homologues namely, *APETALA1* (*PnAP1*), *SEQUAMOSA-APETALA1* HOMOLOG (*PnSAH1*, *PnSAH2*, and *PnSAH3*), *TERMINAL FLOWER1* (*PnTFL1a* and *PnTFL1b*), and *PnMADS1* in apical buds were determined using RT-PCR. Total RNA was extracted from apical buds harvested at the time indicated in Figure 3 and RT-PCR was performed as described in the Materials and methods. PCR products were electrophoresed in agarose gels and ethidium bromide-stained bands were photographed under ultraviolet light. Only the portions of the bands showing amplified fragments are presented. The primers used and the sizes of amplified fragments are shown in Table 1. The number of cycles used for PCR amplification is indicated in parenthesis. *PnUBQ* was used as an internal control.

Effects of KODA application on the expression of flowering-related genes

In the SD-induced cotyledon of *P. nil*, changes in mRNA levels of *PnCO*, *PnFT1*, *PnFT2* and *PnGI* have been reported (Hayama et al. 2007; Higuchi et al. 2011; Liu et al. 2001). We first observed changes in expression of these genes using RT-PCR between KODA- and mock-treated cotyledons, because the internal level of KODA is dramatically changed during the SD dark period (Suzuki et al. 2003). However, none of these genes showed changes compared with the mock-treated cotyledons (Figure 3).

In apical buds, we studied changes in mRNA levels of the four *API* gene family members, namely *PnAP1*, *PnSAH1*, *PnSAH2*, and *PnSAH3* (Sasaki et al. 2008), two newly isolated *TFL1* homologues, namely *PnTFL1a* and *PnTFL1b* (Figure 2), and *PnMADS1* (Kikuchi et al. 2008). Only *PnAP1* up-regulation and *PnTFL1b* down-regulation were reproducibly detected (Figure 4). We then studied the timing of expression of *PnAP1* and *PnTFL1b*. Earlier up-regulation of *PnAP1* was reproducibly observed in the KODA-treated seedlings

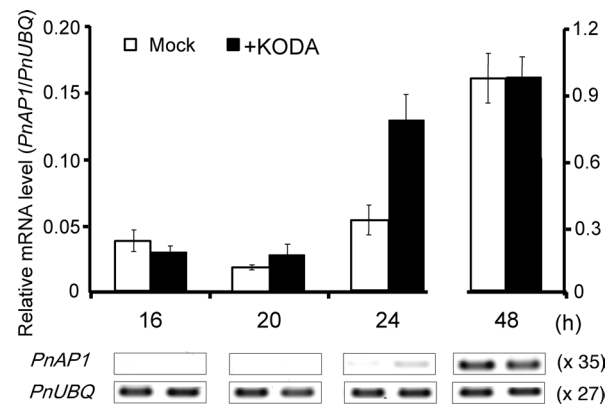


Figure 5. Effects of KODA application on the mRNA level of *PnAP1* in apical buds. The timing of the KODA application and of apical bud harvest are indicated in Figure 3. RT-PCR was performed as described in Figure 4. After the gel electrophoresis, the intensity of DNA bands was quantitated using a Molecular Imager FX (Bio-Rad Laboratories, Hercules, CA, USA) and the data was normalized to *PnUBQ* expression levels. RT-PCR analyses were performed three times with independent RNA samples. Data are the means \pm SE of three replicates. Only the portion of the bands showing amplified fragments of one representative analysis is presented.

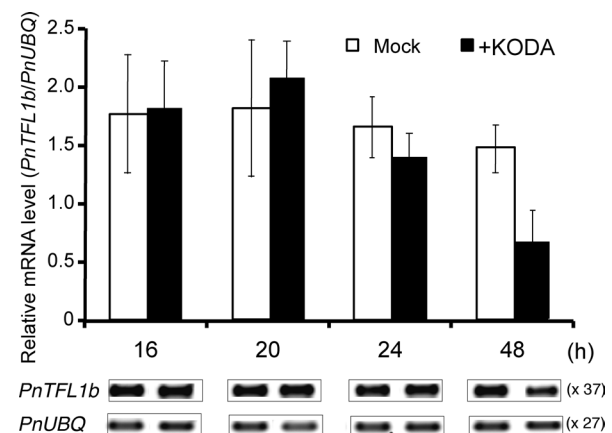


Figure 6. Effects of KODA application on the mRNA level of *PnTFL1b* in apical buds. The timing of the KODA application and of the harvest of apical buds are indicated in Figure 3. RT-PCR was performed as described in Figure 4. After the gel electrophoresis, the intensity of DNA bands was quantitated using a Molecular Imager FX (Bio-Rad Laboratories, Hercules, CA, USA) and the data was normalized to *PnUBQ* expression levels. RT-PCR analyses were performed three times with independent RNA samples. Data are the means \pm SE of three replicates. Only the portion of the bands showing amplified fragments of one representative analysis is presented.

compared with the mock-treated seedlings. Up-regulation began 20–24h after the beginning of the period of darkness (Figure 5). Earlier down-regulation of *PnTFL1* was reproducibly observed in the KODA-treated seedlings compared with the mock-treated seedlings. Down-regulation began 24–48h after the beginning of the period of darkness (Figure 6). Up-regulation of *PnAP1* began earlier than down-regulation of *PnTFL1b*. Furthermore, we also studied changes in *PnAP1* and *PnTFL1b* expression without SD treatment. KODA

treatment did not increase or decrease the *PnAPI* and *PnTFL1b* mRNA levels (Figure 7).

Discussion

KODA has a weak enhancer activity to marginal SD induction

The present study demonstrated that KODA application has a weak enhancer activity to marginal SD induction in seedlings of *P. nil* cv. Violet. Figure 1A shows a small but statistically significant increase in the average number of flower buds per plant. Previously, Yokoyama reported an approximately two-fold increase in an essentially similar experiment (Yokoyama 2005). The difference between the previous results and Figure 1A may be caused by differences in the methods used to cultivate the experimental seedlings. For example, seedlings were hydroponically cultivated with Nakayama's culture solution (Nakayama and Hashimoto 1973) in Yokoyama (2005), whereas the present study used vermiculite. Detailed comparison of different culture conditions will be important for the studies on the effects of KODA on flowering and also on expression of flowering-related genes. The mechanism of this enhancing effect of KODA remains unknown. However, we did find that application of KODA alters the node of the first flower (Figure 1B).

Application of KODA lowered the nodal position of the first flower bud

In seedlings of *P. nil*, it is well established that the timing of the SD treatment determines the node in which the first flower bud is induced. When SD treatment is administered later, the nodal position of the first flower bud is higher (Imamura 1967; King and Evans 1969; Owens and Paolillo 1986). As the main shoots grow, apical dominance causes the axillary buds of lower nodes to gradually stop growing, and to cease to respond to florigen. Therefore, these axillary buds of lower nodes remained vegetative after SD treatment (Kujirai and Imamura 1958; Ono et al 1993). Figure 1B shows a statistically significant decrease in the average number of the node of the first flower bud. This result indicates that KODA application mimics the appearance of accelerating the timing of the SD treatment. The mechanism by which KODA lowers the node of the first flower bud remains to be elucidated. Acceleration of PnFT expression and PnFT movement through sieve elements and apical cells, acceleration of the association of the PnFT to PnFD-like bZip transcriptional factors (Tsuji et al. 2011; Turck et al. 2008), retardation of the effects of apical dominance to make the axillary bud dormant (Ono et al. 1993) and others are candidates for the mechanism.

Effects of KODA application on the expression of flowering-related genes

Using RT-PCR, we compared the expression of *PnCO*, *PnFT1*, *PnFT2* and *PnGI* between KODA- and mock-treated cotyledons. However, none of these genes showed changes in expression levels between treated and mock-treated cotyledons, unless the internal level of KODA is dramatically changed during the SD dark period.

We then studied changes in gene expression in apical buds. In the apical bud, we observed *PnAPI* up-regulation and *PnTFL1b* down-regulation (Figure 4). In *A. thaliana*, it is well established that *API* down-regulates *TFL1* (Alvarez et al. 1992; Mandel and Yanofsky 1995; Shannon and Meeks-Wagner 1991). Although we did not study the pattern of expression nor the function of *PnTFL1b*, high homology in the amino acid sequence of PnTFL1b to TFL1 suggests that regulation of PnTFL1b is similar to regulation of TFL1. *PnAPI* may control the down-regulation of *PnTFL1b*. As shown in Figures 5 and 6, the up-regulation of *PnAPI* began earlier than the down-regulation of *PnTFL1b*. These results suggest that the initial target of KODA treatment is *PnAPI* enhancement and that *PnTFL1* is subsequently down-regulated by PnAPI. A more detailed analysis with narrower sampling intervals will be required for further discussion on the timing. Kittikorn et al. (2011) reported that KODA may be related to flower bud formation through its influence on *MdTFL1*, which is a *TFL1* homologue in apple. Our results for *PnTFL1b* may conflict with what was reported for *MdTFL1*. However, the results in apple were obtained through experiments conducted over several months, whereas our results in *P. nil* were obtained within a few hours. Therefore, we cannot compare these two results directly. In spite of orthologous gene pairs such as *CONSTANS* of *A. thaliana* and *Hd1* of *Oryza sativa*, highly homologous genes sometimes play antagonistic roles in different plant species (Yano et al. 2000). Therefore, we should study carefully on every homologous gene for further analysis in *P. nil*.

Additionally, we briefly examined the possibility that KODA alone can up-regulate *PnAPI* and down-regulate *PnTFL1b*, without SD treatment (data not presented). KODA application failed to change the expression of *PnAPI* and *PnTFL1b* under long day un-inductive conditions of continuous light. Similar to the results for flower bud number, expression of these genes required marginal SD treatment.

In the near future, whole genome sequencing in *P. nil* will reveal the existence of an *FD* homologue and many other genes related to flowering. Using such information, we will be able to study the effects of KODA application on flowering more extensively. However, the one-bud increase caused by the combination of KODA application and marginal SD treatment may be too

weak, and may cause difficulty in carrying out detailed analysis in further studies. In *A. thaliana*, KODA has not been discovered yet, and thus the flower bud inducing activity of KODA will be studied in plants such as *P. nil*, regardless of the difficulty.

Acknowledgements

We are grateful to Dr. E. Nitasaka (Kyushu University) and the National BioResource Project (NBRP) "Morning glory" by MEXT Japan for supplying seed of *P. nil* cv. Violet. We are also grateful to Dr. M. Wada (National Institute of Fruit Tree Science, Shimokuriyagawa, Morioka, Japan) for supplying DNA primers and the helpful advice on the isolation of *PnTFL1* homologues.

References

- Abe M, Kobayashi Y, Yamamoto S, Daimon Y, Yamaguchi A, Ikeda Y, Ichinoki H, Notaguchi M, Goto K, Araki T (2005) FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. *Science* 309: 1052–1056
- Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25: 3389–3402
- Alvarez J, Guli CL, Yu XH, Smyth DR (1992) *Terminal flower*: a gene affecting inflorescence development in *Arabidopsis thaliana*. *Plant J* 2: 103–116
- Corbesier L, Vincent C, Jang S, Fornara F, Fan Q, Searle I, Giakountis A, Farrona S, Gissot L, Turnbull C, Coupland G (2007) FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis*. *Science* 316: 1030–1033
- Foucher F, Morin J, Courtiade J, Cadioux S, Ellis N, Banfield MJ, Rameau C (2003) *DETERMINATE* and *LATE FLOWERING* are two *TERMINAL FLOWER1/CENTRORADIALIS* homologs that control two distinct phases of flowering initiation and development in pea. *Plant Cell* 15: 2742–2754
- Hatayama T, Takeno K (2003) The metabolic pathway of salicylic acid rather than of chlorogenic acid is involved in the stress-induced flowering of *Pharbitis nil*. *J Plant Physiol* 160: 461–467
- Hayama R, Agashe B, Luley E, King R, Coupland G (2007) A circadian rhythm set by dusk determines the expression of FT homologs and the short-day photoperiodic flowering response in *Pharbitis*. *Plant Cell* 19: 2988–3000
- Higuchi Y, Sage-Ono K, Kamada H, Ono M (2007) Isolation and characterization of novel genes controlled by short-day treatment in *Pharbitis nil*. *Plant Biotechnol* 24: 201–207
- Higuchi Y, Sage-Ono K, Sasaki R, Ohtsuki N, Hoshino A, Iida S, Kamada H, Ono M (2011) Constitutive expression of the *GIGANTEA* ortholog affects circadian rhythms and suppresses one-shot induction of flowering in *Pharbitis nil*, a typical short-day plant. *Plant Cell Physiol* 52: 638–650
- Imamura S (1967) *Physiology of Flowering in Pharbitis nil*. Japanese Society of Plant Physiologists, Tokyo
- Ishioka N, Tanimoto S, Harada H (1990) Flower-inducing activity of phloem exudate in cultured apices from *Pharbitis* seedlings. *Plant Cell Physiol* 31: 705–709
- Jaeger KE, Wigge PA (2007) FT protein acts as a long-range signal in *Arabidopsis*. *Curr Biol* 17: 1050–1054
- Kardailsky I, Shukla VK, Ahn JH, Dagenais N, Christensen SK, Nguyen JT, Chory J, Harrison MJ, Weigel D (1999) Activation tagging of the floral inducer FT. *Science* 286: 1962–1965
- Kikuchi R, Sage-Ono K, Kamada H, Handa H, Ono M (2008) *PnMADS1*, encoding an StMADS11-clade protein, acts as a repressor of flowering in *Pharbitis nil*. *Physiol Plant* 133: 786–793
- King RW, Evans LT (1969) Timing of evocation and development of flowers in *Pharbitis nil*. *Aust J Biol Sci* 22: 559–572
- Kittikorn M, Okawa K, Ohara H, Kotoda N, Wada M, Yokoyama M, Ifuku O, Yoshida S, Kondo S (2011) Effects of fruit load, shading, and 9,10-ketol-octadecadienoic acid (KODA) application on *MdTFL1* and *MdFT1* in apple buds. *Plant Growth Regul* 64: 75–81
- Kobayashi Y, Kaya H, Goto K, Iwabuchi M, Araki T (1999) A pair of related genes with antagonistic roles in mediating flowering signals. *Science* 286: 1960–1962
- Kojima S, Takahashi Y, Kobayashi Y, Monna L, Sasaki T, Araki T, Yano M (2002) *Hd3a*, a rice ortholog of the *Arabidopsis* FT gene, promotes transition to flowering downstream of *Hd1* under short-day conditions. *Plant Cell Physiol* 43: 1096–1105
- Kong F, Abe J, Takahashi K, Matsuura H, Yoshihara T, Nabeta K (2005) Allene oxide cyclase is essential for theobroxide-induced jasmonic acid biosynthesis in *Pharbitis nil*. *Biochem Biophys Res Commun* 336: 1150–1156
- Kotoda N, Wada M (2005) *MdTFL1*, a *TFL1*-like gene of apple, retards the transition from the vegetative to reproductive phase in transgenic *Arabidopsis*. *Plant Sci* 168: 95–104
- Kujirai C, Imamura S (1958) Über die photoperiodische Empfindlichkeit der Kotyledonen von *Pharbitis nil* Choisy. *Bot Mag (Tokyo)* 71: 408–416
- Lifschitz E, Eviatar T, Rozman A, Shalit A, Goldshmidt A, Amsellem Z, Alvarez JP, Eshed Y (2006) The tomato FT ortholog triggers systemic signals that regulate growth and flowering and substitute for diverse environmental stimuli. *Proc Natl Acad Sci USA* 103: 6398–6403
- Lin MK, Belanger H, Lee YJ, Varkonyi-Gasic E, Taoka K, Miura E, Xoconostle-Cázares B, Gendler K, Jorgensen RA, Phinney B, Lough TJ, Lucas WJ (2007) FLOWERING LOCUS T protein may act as the long-distance florigenic signal in the cucurbits. *Plant Cell* 19: 1488–1506
- Liu J, Yu J, McIntosh L, Kende H, Zeevaart JAD (2001) Isolation of a CONSTANS ortholog from *Pharbitis nil* and its role in flowering. *Plant Physiol* 125: 1821–1830
- Mandel MA, Yanofsky MF (1995) A gene triggering flower formation in *Arabidopsis*. *Nature* 377: 522–524
- Mimida N, Goto K, Kobayashi Y, Araki T, Ahn JH, Weigel D, Murata M, Motoyoshi F, Sakamoto W (2001) Functional divergence of the *TFL1*-like gene family in *Arabidopsis* revealed by characterization of a novel homologue. *Genes Cells* 6: 327–336
- Nakayama S, Hashimoto T (1973) Effects of abscisic acid on flowering in *Pharbitis nil*. *Plant Cell Physiol* 14: 419–422
- O'Neill SD, Zhang XS, Zheng CC (1994) Dark and circadian regulation of mRNA accumulation of mRNA accumulation in the short-day plant *Pharbitis nil*. *Plant Physiol* 104: 569–580
- Ono M, Sage-Ono K, Inoue M, Kamada H, Harada H (1996) Transient increase in the level of mRNA for a germin-like protein in leaves of the short-day plant *Pharbitis nil* during the photoperiodic induction of flowering. *Plant Cell Physiol* 37: 855–861
- Ono M, Sage-Ono K, Yamada K, Yasui M, Okazaki M (1993) Axillary bud flowering after apical decapitation in *Pharbitis* in relation to photoinduction. *Physiol Plant* 87: 1–6
- Owens V, Paolillo DJJ (1986) Effect of aging on flowering of the

- axillary buds of *Pharbitis nil*. *Am J Bot* 73: 882–887
- Perrière G, Gouy M (1996) WWW-query: an on-line retrieval system for biological sequence banks. *Biochimie* 78: 364–369
- Sage-Ono K, Ono M, Harada H, Kamada H (1998) Accumulation of a clock-regulated transcript during flower-inductive darkness in *Pharbitis nil*. *Plant Physiol* 116: 1479–1485
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4: 406–425
- Sasaki R, Kikuchi R, Sage-Ono K, Kamada H, Ono M (2008) Immediate induction of *APETALA1*-like gene expression following a single short-day in *Pharbitis nil*. *Plant Biotechnol* 25: 565–571
- Shannon S, Meeks-Wagner DR (1991) A mutation in the *Arabidopsis TFL1* gene affects inflorescence meristem development. *Plant Cell* 3: 877–892
- Shinozaki M, Takimoto A (1983) Effects of some growth regulators and benzoic acid derivatives on flower initiation and root elongation of *Pharbitis nil*, strain Kidachi. *Plant Cell Physiol* 24: 433–439
- Suzuki M, Yamaguchi S, Iida T, Hashimoto I, Teranishi H, Mizoguchi M, Yano F, Todoroki Y, Watanabe N, Yokoyama M (2003) Endogenous α -ketol linolenic acid levels in short day-induced cotyledons are closely related to flower induction in *Pharbitis nil*. *Plant Cell Physiol* 44: 35–43
- Tamaki S, Matsuo S, Wong HL, Yokoi S, Shimamoto K (2007) Hd3a protein is a mobile flowering signal in rice. *Science* 316: 1033–1036
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22: 4673–4680
- Tsuji H, Taoka K, Shimamoto K (2011) Regulation of flowering in rice: two florigen genes, a complex gene network, and natural variation. *Curr Opin Plant Biol* 14: 45–52
- Turck F, Fornara F, Coupland G (2008) Regulation and identity of florigen: FLOWERING LOCUS T moves center stage. *Annu Rev Plant Biol* 59: 573–594
- Wada KC, Yamada M, Shiraya T, Takeno K (2010) Salicylic acid and the flowering gene *FLOWERING LOCUS T* homolog are involved in poor-nutrition stress-induced flowering of *Pharbitis nil*. *J Plant Physiol* 167: 447–452
- Wigge PA, Kim MC, Jaeger KE, Busch W, Schmid M, Lohmann JU, Weigel D (2005) Integration of spatial and temporal information during floral induction in *Arabidopsis*. *Science* 309: 1056–1059
- Yamaguchi S, Yokoyama M, Iida T, Okai M, Tanaka O, Takimoto A (2001) Identification of a component that induces flowering of *Lemna* among the reaction products of α -ketol linolenic acid (FIF) and norepinephrine. *Plant Cell Physiol* 42: 1201–1209
- Yamazaki Y, Akashi R, Banno Y, Endo T, Ezura H, Fukami-Kobayashi K, Inaba K, Isa T, Kamei K, Kasai F, Kobayashi M, Kurata N, Kusaba M, Matuzawa T, Mitani S, Nakamura T, Nakamura Y, Nakatsuji N, Naruse K, Niki H, Nitasaka E, Obata Y, Okamoto H, Okuma M, Sato K, Serikawa T, Shiroishi T, Sugawara H, Urushibara H, Yamamoto M, Yaoita Y, Yoshiki A, Kohara Y (2010) NBRP databases: databases of biological resources in Japan. *Nucleic Acids Res* 38 (Database issue): D26–D32
- Yano M, Katayose Y, Ashikari M, Yamanouchi U, Monna L, Fuse T, Baba T, Yamamoto K, Umehara Y, Nagamura Y, Sasaki T (2000) *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
- Yokoyama M (2005) Roles of 9,10- α -ketol linolenic acid (KODA), one of the oxylipins formed with 9-lipoxygenase in plant growth regulation. *Regul Plant Growth Dev* 40: 90–100 (in Japanese)
- Yokoyama M, Yamaguchi S, Iida T, Suda A, Saeda T, Miwa T, Ujihara K, Nihio J (2005) Transient accumulation of α -ketol linolenic acid (KODA) in immature flower buds of some ornamental plants. *Plant Biotechnol* 22: 201–205
- Yokoyama M, Yamaguchi S, Inomata S, Komatsu K, Yoshida S, Iida T, Yokokawa Y, Yamaguchi M, Kaihara S, Takimoto A (2000) Stress-induced factor involved in flower formation of *Lemna* is an α -ketol derivative of linolenic acid. *Plant Cell Physiol* 41: 110–113