Gene regulation of stress-induced flowering in Japanese morning glory

（アサガオにおけるストレス応答花成の遺伝子制御）

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Contents

Abbreviations

ACC: 1-aminocyclopropane-1-carboxylate

ACT4: actin 4

AGL20: *AGAMOUS-LIKE 2*

AOA: aminooxyacetic acid

AP1: *APETALA1*

BMV: brome mosaic virus

CaMV: cauliflower mosaic virus

CO: *CONSTANS*

COL: *CONSTANS-LIKE*

EMG medium: embryoid maturation and germination medium

FLC: *FLOWERING LOCUS C*

FRI: *FRIGIDA*

FT: *FLOWERING LOCUS T*

IAA: indole-3-acetic acid

LD: long-day

LFY: *LEAFY*

PAL: phenylalanine ammonia-lyase

PCR: polymerase chain reaction

RNAi: RNA interference

RT-PCR: reverse transcription-polymerase chain reaction

SA: salicylic acid

SAM: *S*-adenosylmetionine

SD: short-day

SOC1: *SUPPRESSOR OF OVEREXPRESSION OF CO1*

TCOL3: *Tomato CONSTANS-Like 3*

UV-C: ultraviolet-C

WT: wild type

要旨

貧栄養、低温ストレス処理はアサガオ（*Pharbitis nil*; synonym *Ipomoea nil*）品種ムラサキの花成を誘導した。ストレス処理はシロイヌナズナの花成経路統合遺伝子 *FLOWERING LOCUS T*（*FT*）の 2 つのホモログの 1 つである *PnFT2* の発現を誘導し、1 回の 16 時間暗処理は *PnFT1* と *PnFT2* の両方の発現を誘導した。別の品種テンダンは貧栄養ストレスで花成も *PnFT2* 発現も誘導されず、低温ストレスでは両者が誘導された。これらのことから、アサガオはストレスによって花成を誘導され、このとき *PnFT2* の発現が誘導されること、光周的花成とストレス応答花成では異なる *PnFT* 発現制御がなされていることが示唆された。

別の花成経路統合遺伝子 *SUPPRESSOR OF OVEREXPRESSION OF CO1*、*FT* の上流遺伝子 *CONSTANS*、*FCA* は花成の有無にかかわらず常に発現し、*FLOWERING LOCUS C*、*FRIGIDA*、*FVE* の発現は常に見られなかった。また、*FT* の下流遺伝子では *LEAFY* のホモログは常に発現し、*FD* のホモログの発現は検出されなかった。一方、花成のトリガー遺伝子 *APETALA1*（*AP1*）のホモログ *PnAP1* の発現はストレス処理で誘導され、*PnFT2* 発現、花成反応と正の相関が見られた。

PnFT は葉で特異的に発現し、*PnAP1* 発現は茎頂で認められた。この結果は、葉で発現した *PnFT2* が茎頂で *PnAP1* の発現を誘導することによって花成が誘導されるという仮説と矛盾しない。

PnFT2 をアサガオ、シロイヌナズナで過剰発現させたところ、いずれも早期花成を誘導された。このことから、*PnFT2* には花成誘導活性があることが示された。RNA interference による *PnFT1* 発現抑制は短日処理による光周的花成を完全に抑制したのに対し、*PnFT2* の発現抑制は光周的花成を部分的にのみ抑制した。このことから、光周的花成では両方の *PnFT* が発現するものの、*PnFT1* が主要な役割を果たしていることが示唆された。

ストレス応答花成にはサリチル酸（SA）が関与している。SA 生合成の鍵酵素であるフェニルアラニンアンモニアリアーゼを阻害するアミノオキシ酢酸（AOA）は貧栄養ストレスによっ

て誘導された花成と *PnFT2* 発現を阻害し、この AOA による阻害効果は外生 SA によって回復した。また、外生 SA は貧栄養ストレス条件下で *PnFT2* 発現を促進した。しかし、非ストレス条件下で発現を促進することはなかった。これらの結果は、ストレスによって生合成された SA が *PnFT2* 発現を誘導し、それによって花成が誘導されること、しかし SA は必要条件であっても十分条件ではないことを示唆する。他の未知の要因が関与している可能性が考えられる。

本研究の結果を総合すると、アサガオのストレス応答花成では、ストレスによって生合成された SA と他の未知のストレス物質が葉で *PnFT2* 発現を誘導し、*PnFT2* が茎頂で *PnAP1* の発現を誘導することによって花成が誘導されるということが示唆された。

ABSTRACT

Poor-nutrition and low-temperature stress treatments induced the flowering in *Pharbitis nil* (synonym *Ipomoea nil*) cv. Violet. The stress treatment induced the expression of *PnFT2*, one of two homologs of flowering pathway integrator gene *FLOWERING LOCUS T* (*FT*) of *Arabidopsis thaliana*, whereas the single 16-hour dark treatment induced the expression of both *PnFT1* and *PnFT2*. Another cultivar Tendan was not induced flowering and the expression of *PnFT2* by the poor-nutrition stress, whereas the low-temperature stress induced both of them. These results suggest that the expression of *PnFT2* is induced when *P. nil* flowered by stress, and *PnFT* expression is regulated differently between stress-induced and photoperiodic flowering.

Another flowering pathway integrator gene, *SUPPRESSOR OF OVEREXPRESSION OF CO1* and genes in upstream of *FT*, *CONSTANS* and *FCA* express regardless of flowering status, and the expression of *FLOWERING LOCUS C*, *FRIGIDA* and *FVE* was not detected. Among genes in downstream of *FT*, *LEAFY* homolog expressed constitutively and the expression of *FD* homolog was not detected. The expression of *PnAP1*, a homolog of *APETALA1* (*AP1*) which is the trigger gene of flowering, was induced by stress treatment, and there was the positive correlation among the expression level of *PnFT2* and *PnAP1* and intensity of flowering response.

PnFT expressed specifically in leaves, and the expression of *PnAP1* was observed in shoot apices. This result is consistent with an assumption that *PnFT2* induces *PnAP1* expression to induce flowering.

Overexpression of *PnFT2* induces early flowering in *P. nil* and *A.*

thaliana, indicating that *PnFT2* has the activity to induce flowering. The suppression of *PnFT1* expression by RNA interference suppressed the photoperiodic flowering completely, whereas the suppression of *PnFT2* expression suppressed it only partially. This suggests that both *PnFT* express in photoperiodic flowering, but *PnFT1* plays a main role in it.

Salicylic acid (SA) is involved in stress-induced flowering. Aminooxyacetic acid (AOA), the inhibitor of phenylalanine ammonia-lyase, the key enzyme of SA biosynthesis, inhibited the flowering and the *PnFT2* expression induced by the poor-nutrition stress, and this inhibition was overcome by the exogenous SA. The exogenous SA enhanced the *PnFT2* expression under the poor-nutrition stress conditions, but did not induce it under non-stress conditions. These results suggest that SA of which biosynthesis is induced by stress induces the expression of *PnFT2*, which induces flowering, and SA is necessary but not sufficient for the *PnFT2* expression. It is possible that other unknown factor(s) are involved in stress-induced flowering.

Taken together, the present study suggests that SA and other unknown factor which are synthesized by stress induces *PnFT2* expression in leaves, *PnFT2* induces *PnAP1* expression in shoot apices, and then *PnAP1* induces flowering in *P. nil*.

General Introduction

Flowering, the transition from vegetative growth to reproductive growth, is critical in plant development. The proper timing of flowering ensures the success of plant reproduction. Flowering in many plants is regulated by environmental cues, such as night length in photoperiodic flowering and temperature in vernalization (Thomas and Vince-Prue, 1997). Recently, stress has also been recognized as a cue to induce flowering. The short-day (SD) plant *Pharbitis nil* (synonym *Ipomoea nil*) flowered under long-day (LD) conditions when grown under poor-nutrition or low-temperature stress conditions (Shinozaki et al., 1988; Hirai et al., 1994; Hatayama and Takeno, 2003; Wada et al.,2010b). The SD plants *Lemna paucicostata* (synonym *Lemna aepuinoctialis*) and *Perilla frutescens* var. *crispa* flowered under poor-nutrition and low-intensity light stresses (Wada et al., 2010a; Shimakawa et al., 2012). Ultraviolet-C (UV-C) light (Martínetz et al., 2004), poor-nutrition (Kolář and Seňková, 2008), drought (Riboni et al., 2013) and low-temperature (Xu et al., 2014) stresses induced early flowering in the LD plant *Arabidopsis thaliana*. Similar non-photoperiodic flowering has been sporadically reported in various plant species other than those mentioned above, although the authers did not argue that the flowering was induced by stress. A review of those reports suggested that most of the factors responsible for flowering regarded as stress (Wada and Takeno, 2010; Takeno, 2012). The plants that were induced to flower by stress produced fertile seeds, and the progeny developed normally (Wada et al., 2010a, b). Plants can modify processes to adapt to stress conditions. Stress-induced flowering is one such adaptation to stress. Stressed plants

do not need to wait for the arrival of a season when photoperiodic conditions are suitable for flowering. Plants flower as an emergency response if stressed, ensuring their ability to produce the next generation. Through this mechanism, plants can preserve the species even under unfavorable environmental conditions. Therefore, stress-induced flowering can be considered as universal and important as photoperiodic flowering and vernalization (Wada and Takeno, 2010; Takeno, 2012).

The regulatory mechanism of stress-induced flowering, especially the gene regulation of it is poorly understood. Accordingly, this study aimed to clarify the genes which are involved in the regulation of stress-induced flowering using *P. nil* in which the knowledge on stress-induced flowering has been most intensively accumulated. The gene focused as the main object to be studied for this purpose is *FLOWERING LOCUS T* (*FT*). *FT* is known as the flowering pathway integrator gene in *A. thaliana* (Boss et al., 2004), and FT protein behaves as florigen, the transmissible signal molecule to induce photoperiodic flowering (Jaeger and Wigge, 2007; Tamaki et al., 2007). The *FT* gene encodes a small protein similar to mammalian phosphatidyl ethanolamine-binding protein (Schoentgen et al., 1987). The *FT*-like genes belonging to the same gene family are conserved in the wide range of plant species, and some of them are involved in the regulation of flowering. These information lead the author to suppose that *FT* homolog may be also involved in stress-induced flowering of *P. nil*.

Chapter I

Flowering genes that express in stress-induced flowering

Introduction

The molecular basis of the regulation of stress-induced flowering is not well understood. The flowering of *A. thaliana* is induced by LD conditions, vernalization, autonomous cues and gibberellins, and these factors operate through a common pathway integrator gene *FT* (Boss et al., 2004). UV-C induced the expression of *FT*, indicating that UV-C stress-induced flowering is also mediated by *FT* in *A. thaliana* (Martínez et al., 2004; Segarra et al., 2010). Thus, all of the factors known to induce the flowering of *A. thaliana* function through the activation of *FT* expression, suggesting that the *FT* homolog could also be involved in the stress-induced flowering of *P. nil*. Two homologs of *FT*, *PnFT1* and *PnFT2*, have been identified in *P. nil*, and these genes are expressed under inductive SD conditions to promote flowering (Hayama et al., 2007). It was reported that poor-nutrition stress induced the expression of *PnFT2* but not that of *PnFT1* in *P. nil* cv. Violet (Wada et al., 2010b). In the present study, the expression of *PnFT1* and *PnFT2* was examined more intensively in two cultivars of *P. nil* which were treated with poor-nutrition and low-temperature stresses.

SUPPERSSOR OF OVEREXPRESSION OF CO1 (*SOC1*) (alias *AGAMOUS-LIKE 20; AGL20*) functions as another floral pathway integrator gene in *A. thaliana* (Lee and Lee, 2010). Therefore, it was expected that *SOC1* may also be involved in the stress-induced flowering of *P. nil*. Although a homolog of *SOC1* has not been reported in *P. nil*, a *SOC1* homolog *IbAGL20* is known in *Ipomoea batatas*, which is a close relative of *P. nil* (Kim et al., 2005). The inductive LD conditions induce the expression of *CONSTANS* (*CO*), whose product directly induces the transcription of *FT* in *A.*

thaliana (Suárez-López et al., 2001; Valverde et al., 2004). The CO-FT module is conserved in various plant species (Ballerini and Kramer, 2011). Therefore, *CO* is possibly involved in the regulation of the stress-induced flowering where *FT* is also involved. The *P. nil* homolog of *CO*, *PnCO*, is known to complement the *co* mutation in *A. thaliana* (Liu et al., 2001). Furthermore, several genes are known to act in the upstream of *FT* in the regulation of flowering in *A. thaliana*. *FLOWERING LOCUS C* (*FLC*) suppresses the expression of *FT* and *SOC1* and acts as a major repressor of flowering. Vernalization and autonomous cues promote flowering by activating *FT* and *SOC1* through the suppression of *FLC* (Michaels and Amasino, 1999; Sheldon et al., 1999; Searle et al., 2006). *FLC* is also reported to be involved in the stress-induced flowering of *A. thaliana* (Xu et al., 2014). *FCA*, *FRIGIDA* (*FRI*) and *FVE* regulate the expression of *FLC* (Michaels and Amasino, 1999; Rouse et al., 2002). *FCA* and *FVE* are floral promoter in the autonomous pathway (Veley and Michaels, 2008). The present study examined whether the expression of the homologs of these genes is induced in response to the stress treatment in *P. nil*.

FT requires the other gene *FD* to induce flowering in *A. thaliana*. *FD* encodes the transcription factor which binds to the promoters of florally expressed genes such as *APETALA1* (*AP1*) (Wigge et al., 2005). The expression of *FT* and *FD* is spacially restricted in leaves and shoot apices, respectively (Takada and Goto, 2003; Abe et al., 2005). FT protein is transported from the leaves to the shoot apices through the vascular system (Corbesier et al., 2007; Jaeger and Wigge, 2007; Notaguchi et al., 2008), and both FT and FD proteins form a complex in the shoot apices (Abe et al.,

2005). This complex binds to DNA of downstream genes, such as *AP1* and activates those transcriptions. *AP1* triggers flowering and initiates flower development in *A. thaliana* (Kaufmann et al., 2010). *FT* also regulates the expression of *SOC1* and *LEAFY* (*LFY*), which integrate the flowering pathway or regulate the flower development (Moon et al., 2005; Yoo et al., 2005; Lee and Lee, 2010). The homologs of *AP1* and *LFY* of *P. nil*, *PnAP1* and *PnLFY* were reported (Parfitt et al., 2004; Kikuchi et al., 2008; Sasaki et al., 2008), but the relationship with *PnFT* is unknown. These downstream genes of *FT* were examined whether the expression was induced when flowering was induced by stress in *P. nil*.

Materials and methods

Plant materials and growth conditions

The SD plant Japanese morning glory [*Pharbitis nil* (L.) Chois., synonym *Ipomoea nil* (L.) Roth] cultivars Violet and Tendan were used. Violet and Tendan seeds were originally provided by Marutane Co. (Kyoto, Japan) and National Institute of Genetics (Mishima, Japan), respectively. The seeds were treated with concentrated H_2SO_4 for 25 to 40 minutes, washed with running tap water for 1 hour, and then soaked in tap water overnight. The swollen seeds were placed on moist filter paper in a Petri dish 90 mm in diameter and were germinated at 25 ℃ under 16-hour light and 8-hour dark LD conditions for 1 day. The germinated seeds were planted on 0.6% plain agar medium in a plastic box (240 mm × 330 mm × 90 mm in depth) and grown at 25 ℃ under the LD conditions for 5 days. The seedlings were then transferred to glass tubes (15 mm in diameter × 150 mm high)

containing a mineral nutrient solution (Kondo et al., 2006) and were grown under the same conditions as before. White light (55-90 μmol m^{-2} s^{-1}) was provided by fluorescent lamps (FL20SW or FL40SSW/37, Toshiba Corporation, Tokyo, Japan). For SD treatment, 5-day-old seedlings were given a single 16-hour dark treatment. After the SD treatment, the seedlings were transferred to the LD conditions and grown for 2 weeks until the flowering response was scored.

Stress treatments

Five-day-old seedlings were grown in 1/10- or 1/100-strength mineral nutrient solution instead of full-strength mineral nutrient solution as the poor-nutrition stress treatment. Five-day-old seedlings were also cultured at 15 ℃ instead of 25 ℃ as the low-temperature stress treatment. Plant was considered to be stressed if its vegetative growth was suppressed by any external factor (Hatayama and Takeno, 2003). After the stress treatment, the seedlings were transferred to the normal growth conditions and grown for 2 weeks until the flowering response was scored.

Scoring of the flowering response

All of the plant nodes were dissected under a binocular microscope to determine whether flower buds or vegetative buds formed. The percentage of plants with at least one flower bud out of all the plants in a treatment (% flowering) and the number of flower buds per plant were determined. The number of nodes, that is the total number of flower buds and vegetative buds per plant, was used as indicator of vegetative growth along with the

average length of the main stem. Twenty to 30 plants were used for each treatment. Each experiment was repeated at least three times. The means with standard errors of the most representative experiment are shown in each table or figure.

Gene expression analysis using reverse transcription-polymerase chain reaction (RT-PCR)

The cotyledons, true leaves and other tissues of plants were collected 16 hours after the start of the dark period (8 hours after the end of the dark period of 16-hour light LD conditions, or the end of the dark period of 8-hour light SD conditions). The harvested plant tissues were frozen in liquid nitrogen and stored at -80 ℃ prior to analysis. The total RNA was isolated from the tissues using the Plant RNA Purification Reagent (Invitrogen Corporation, Carlsbad, CA, U.S.A.) and Fruit-mate (Takara Biotechnology Co. Ltd., Shiga, Japan). The isolated RNA samples were treated by DNase I (Sigma-Aldrich Inc., Saint Louis, MO, U.S.A.) to digest contaminated genomic DNA, and then cDNAs were synthesized from each RNA sample using ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan). The gene amplification was performed by nested PCR using KOD -Plus- Neo (Toyobo Co. Ltd., Osaka, Japan). *PnFT1* and *PnFT2* were amplified from the cDNAs with 2 pairs of nested primers designed referring to a previous report (Hayama et al., 2007). The reactions were carried out on a thermal cycler, PC320 (Astec Co. Ltd., Fukuoka, Japan) or i Cycler (Bio-Rad Laboratories, Hercules, CA, U.S.A.), with 20 + 20 cycles; the annealing temperature was 62 ℃ and the extension time was 20 seconds. The products of the *PnFT1* and *PnFT2*

amplification were separated on an agarose gel with *PnACT4* (which encodes actin) as a loading control. The images on the gel were visualized using EDAS 290 (Invitrogen Corporation, Carlsbad, CA, U.S.A.) or Bio-Pyramid (MeCan Imaging Inc., Saitama, Japan). The PCR products corresponding to *PnFT1* and *PnFT2* were extracted from the agarose gel, and the nucleotide sequences were determined using GenomeLab DTCS Quick Start Kit (Beckman Coulter Inc., Fullerton, CA, U.S.A.). Each sequence was consistent with that reported previously (Hayama et al., 2007). The homologs of flowering genes of *P. nil* were searched referring the sequences of flowering genes of *A. thaliana*. The RT-PCR analyses for the homologs of *SOC1*, *CO* (*PnCO*), *FLC*, *FCA*, *FRI*, *FVE* (*PnFVE*), *AP1* (*PnAP1*), *LFY* (*PnLFY*) and *FD* were also performed in the same manner as above (but not nested PCR) with some modifications. Some degenerated primers were also used for the *FD* homolog. The expression of each gene was normalized to that of *PnACT4* and shown as a relative value. The nucleotide sequences of the primers used are shown in Tables 1 and 2.

Results

Induction of flowering and *PnFT* gene expression by stress treatments

The flowering response to stress was studied in *P.nil* cv. Violet. Plants were grown in nutrient solution of different strength at 25 ℃ under the LD conditions for 3 weeks. Other plants were grown in nutrient solution of full strength and given a single 16-hour dark SD treatment or continuously grown under the LD conditions as a positive and a negative control,

Table 1. Primer sequences used for RT-PCR analysis of the expression of *PnFT*.

gene			sequence
PnFT1	1st PCR	forward	5'-CAGAGAAAGGTTAGTTTTGATCGAG-3'
		reverse	5'-CTTCATTGCCATATATAAAGGG-3'
	2nd PCR	forward	5'-GCTAGGATGCGAAGGGGAACAGTAG-3'
		reverse	5'-CATGAAATTAACGGGAAGGAG-3'
PnFT2	1st PCR	forward	5'-ATGTCGGGAGGAAGAGAC-3'
		reverse	5'-TACATATAGGACGATACATATGAC-3'
	2nd PCR	forward	5'-AGGGTTGAGATTGGCGGAGATG-3'
		reverse	5'-GAGAGAGGTTAGGGTGCGTCATTAC-3'

Table 2. Primer sequences used for RT-PCR analysis of the expression of several flowering genes.

gene		sequence
PnCO	forward	5'-ACCTCAATCTCCCACTCAAGAGC-3'
	reverse	5'-CGTATCCACTTTCTGGCATCAATG-3'
SOC1 homolog	forward	5'-CACAAGCAGGCAGGTGACTTTC-3'
	reverse	5'-GGGAGAGAAGATGATAAGAGCAACC-3'
FLC homolog	forward	5'-TTTCTGTTCTCTGTGACGCATCC-3'
	reverse	5'-TCTTGACCAGGTTATCGCCG-3'
FCA homolog	forward	5'-GCTACAGAAGAAGAAATCCGTCCC-3'
	reverse	5'-GGCTTGCTTGTTTAGTGAACCAAC-3'
FRI homolog	forward	5'-TCTGTAGAAACCACCGTCACTGTG-3'
	reverse	5'-ACAAACTTCGCTGGCTCCTTG-3'
PnFVE	forward	5'-ACCTGCTGGTTTCATCAAGCC-3'
	reverse	5'-TATGGACTGGTGAGCCAACTCC-3'
PnAP1	forward	5'-ATTTCCGTCCTCTGCGATGC-3'
	reverse	5'-CTTTGTCCTTTTTCTGGAGCACAG-3'
PnLFY	forward	5'-CTGCATTGCCTTGACGAG-3'
	reverse	5'-GGGGGTGGGCATTGAAGAT-3'
FD homolog	forward	5'-TCTTTGAACCAGGAACCAGCAC-3'
	reverse	5'-TGAGCGTTTGAGAGGTGATGG-3'
PnACT4	forward	5'-CGGTATTGCGGATAGAATGAGC-3'
	reverse	5'-ATCTGTTGGAATGTGCTGAGGG-3'

Table 3. Flowering response of *Pharbitis nil* cv. Violet treated with poor-nutrition stress. *P. nil* cv. Violet was grown in nutrient solution (Normal nutrition; ×1N) for 5 weeks or in 1/100- or 1/10-strength nutrient solution (Poor nutrition; ×1/100 N, ×1/10 N) for 3 weeks under long-day (LD) conditions. Control plants were grown in the normal nutrition conditions and either given a short-day (SD) treatment or not (LD). Values followed by the different superscript letters differ significantly at the 5% level, according to a *t*-test.

	% flowering	Floral buds /plant	Nodes /plant	Stem length (mm)
SD	100	6.4 ± 0.23^{a}	7.5 ± 0.19^{d}	118 ± 16.0^{d}
LD	0	0 ± 0^{d}	12 ± 0.44^{c}	150 ± 20.4^{d}
×1/100 N	100	1.7 ± 0.13^{b}	13 ± 0.31^{c}	260 ± 33.5^{c}
×1/10 N	40	0.45 ± 0.14^{c}	15 ± 0.33^{b}	533 ± 27.4^{b}
×1 N	0	0 ± 0^{d}	18 ± 0.82^{a}	681 ± 67.1^{a}

respectively. The SD treatment induced flowering, whereas the LD-grown plants did not flower (Table 3). The plants grown in 1/10- or 1/100-strength nutrient solution were induced flowering, and those grown in full strength nutrient solution were not. The number of nodes per plant and the main stem length of the plants grown in 1/10- or 1/100-strength nutrient solution were less than those of the plants grown in normal nutrient solution.

A time-course study on the induction of flowering was performed and the expression of *PnFT* was examined. Violet plants were grown in 1/100-strength or normal nutrient solution for 1 to 3 weeks. The plants grown under the poor nutrition conditions for 3 weeks flowered, whereas the plants grown under the normal nutrition conditions never flowered (Fig. 1). The *PnFT2* expression was detected in the cotyledons of the plants stressed for 2 weeks or longer. The *PnFT1* expression was almost undetectable, even when the plants were stressed for 3 weeks. Expression of *PnFT1* and *PnFT2* was induced by the SD treatment; by contrast, neither gene was expressed at detectable levels under the LD conditions. The *PnFT2* expression induced by stress was weaker than the expression induced by the SD treatment.

Violet plants were grown at 15 ℃ in full-strength nutrient solution under the LD conditions for 4 weeks. Flowering was induced when the plants were grown at 15 ℃, whereas those grown at 25 ℃ did not flower (Table 4). The vegetative growth was suppressed at 15 ℃.

A time-course study on the induction of flowering and the expression of *PnFT* were then performed. Flowering was induced when the low-temperature stress was applied for 2 weeks or longer, and the expression of *PnFT2* was detected in the cotyledons of the plants grown at

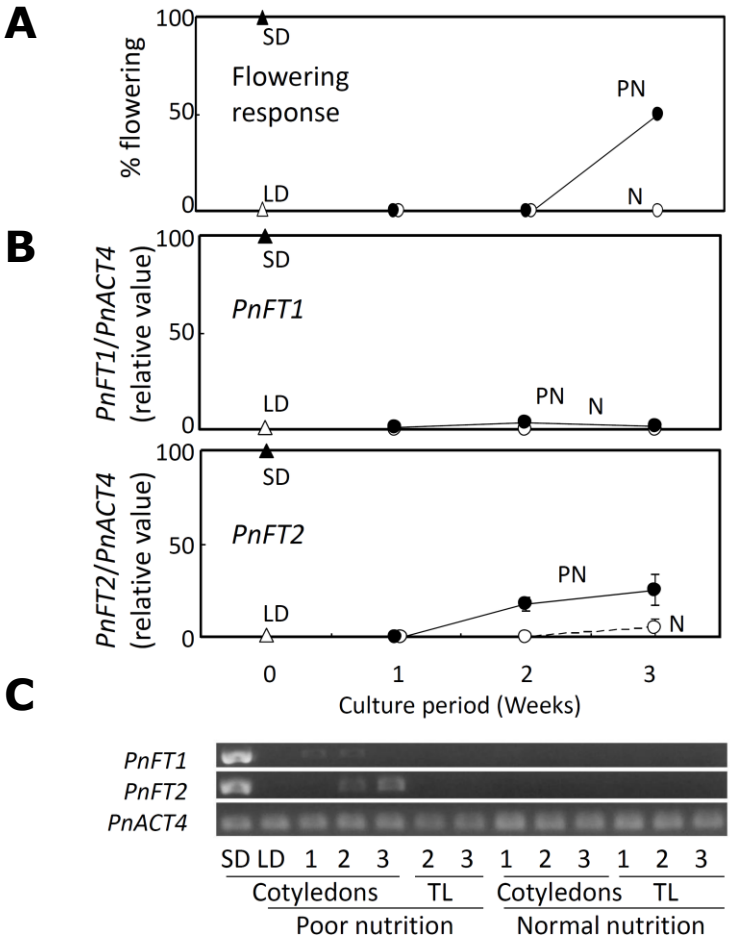

Fig. 1. Time-course of flowering response and *PnFT* expression in *Pharbitis nil* cv. Violet treated with poor-nutrition stress. Plants were grown in nutrient solution [Normal nutrition (N); open circles] or 1/100-strength nutrient solution [Poor nutrition (PN); closed circles] under long-day (LD) conditions for 1–3 weeks. Control plants were grown in nutrient solution, and given a short-day treatment (SD, closed triangles) or not (LD, open triangles). (A) After the stress or SD treatment, flowering response was scored. (C) The cotyledons and expanded true leaves (TL) were collected for RT-PCR analysis of *PnFT1* and *PnFT2*. (B) The mRNA abundance of each gene was normalized to that of *PnACT4*, and the data from the cotyledons are shown as relative values.

Table 4. Flowering response of *Pharbitis nil* cv. Violet treated with low-temperature stress. *P. nil* cv. Violet was grown at 25 ℃ for 6 weeks or at 15 ℃ for 4 weeks under long-day (LD) conditions. Control plants were grown at 25 ℃, and either given a short-day (SD) treatment or not (LD). Values followed by the different superscript letters differ significantly at the 5% level, according to a *t*-test.

	% flowering	Floral buds /plant	Nodes /plant	Stem length (mm)
SD	90	1.0 ± 0.10^{b}	13 ± 0.38^{c}	302 ± 23^{c}
LD	0	0 ± 0^{c}	13 ± 0.34^{c}	318 ± 21^{c}
25℃	0	0 ± 0^{c}	24 ± 1.7^{a}	1262 ± 140^{a}
15℃	91	3.2 ± 0.57^{a}	20 ± 0.58^{b}	545 ± 40^{b}

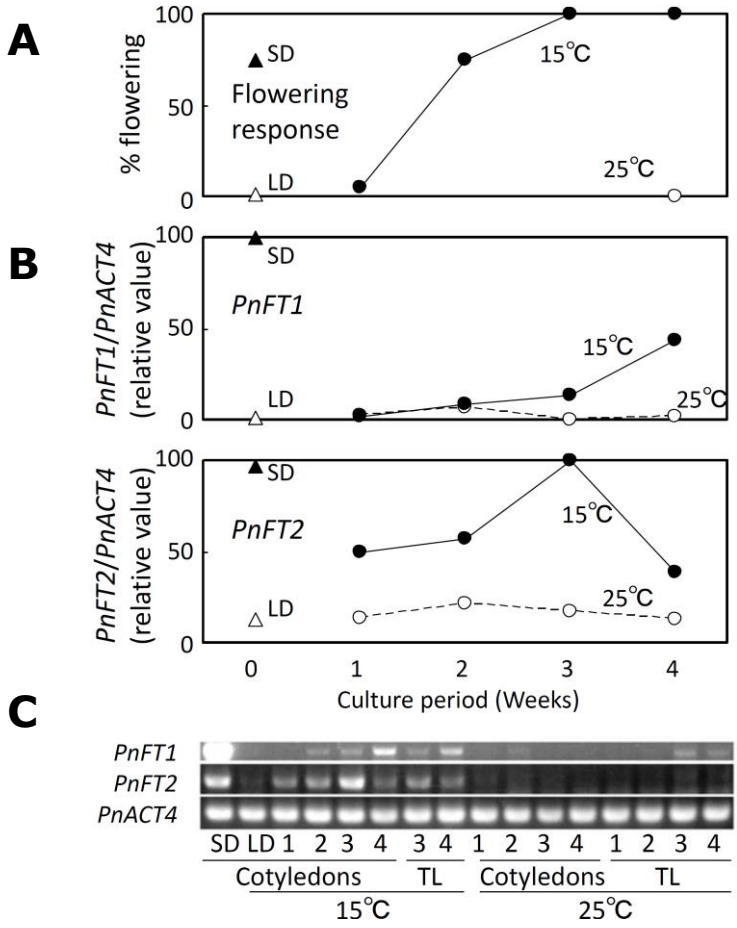

Fig. 2. Time-course of flowering response and *PnFT* expression in *Pharbitis nil* cv. Violet treated with low-temperature stress. Plants were grown at 15 ℃ (closed circles) or 25 ℃ (open circles) under long-day (LD) conditions for 1–4 weeks. Control plants were grown at 25 ℃, and given a short-day treatment (SD, closed triangles) or not (LD, open triangles). (A) After the stress or SD treatment, flowering response was scored. (C) The cotyledons and expanded true leaves (TL) were collected for RT-PCR analysis of *PnFT1* and *PnFT2*. Because the true leaves had not expanded at 15 ℃ until after the third week, *PnFT* expression was examined only at the stages following three weeks of growth. (B) The mRNA abundance of each gene was normalized to that of *PnACT4*, and the data of cotyledons are shown as relative values.

15 ℃ from the first week of the stress treatment, reaching the same level as that induced by the SD treatment on the third week (Fig. 2). The *PnFT1* expression was detected in the stressed plants, although a significant increase was detected only from the fourth week of the stress treatment, and the expression level was lower than that induced by the SD treatment. The results in the true leaves were similar to those in the cotyledons, but the expression levels in the true leaves were lower than that in the cotyledons.

Expression of *PnFT* in stress-induced flowering in other cultivar

The response to stress in other cultivar, Tendan, was examined with Violet for the comparison. Plants were grown in 1/100-strength nutrient solution under the LD conditions for 3 weeks. Tendan did not flower under the poor nutrition conditions, although the number of nodes per plant and the main stem length of the plants grown under the poor nutrition conditions were less than those of the plants grown under the normal nutrition conditions (Table 5). These plants were examined for the expression of *PnFT1* and *PnFT2* in the cotyledons and true leaves at the end of the poor-nutrition stress treatment. The expression of these genes in the cotyledons of the plants given a single 16-hour dark SD treatment or those grown under the LD conditions were also examined as controls. The expression of *PnFT1* and *PnFT2* were induced by the SD treatment, and the expression of both genes were quite weak under the LD conditions in Violet (Fig. 3). Tendan expressed *PnFT1* and *PnFT2* under the SD conditions as did Violet and expressed *PnFT2* even under the LD conditions. The *PnFT2* expression in the true leaves was higher in the stressed plants than in the

Table 5. Flowering response of *Pharbitis nil* treated with poor-nutrition stress. *P. nil* cvs. Violet and Tendan were grown in nutrient solution (Normal nutrition) for 5 weeks or in 1/100-strength nutrient solution (Poor nutrition) for 3 weeks under long-day (LD) conditions. Control plants were grown in the normal nutrition conditions and either given a short-day (SD) treatment or not (LD). Values followed by the different superscript letters differ significantly at the 5% level, according to a *t*-test within the same cultivar.

Cultivar	Conditions	% flowering	Floral buds /plant	Nodes /plant	Stem length (mm)
Violet	SD	55	0.60 ± 0.13^{b}	12 ± 0.47^{c}	280 ± 26^{c}
	LD	0	0 ± 0^{c}	11 ± 0.47^{c}	280 ± 24^{c}
	Normal nutrition	0	0 ± 0^{c}	20 ± 0.67^{a}	1022 ± 58^{a}
	Poor nutrition	83	1.1 ± 0.16^{a}	17 ± 0.24^{b}	512 ± 20^{b}
Tendan	SD	20	0.20 ± 0.092^{a}	11 ± 0.32^{b}	361 ± 26^{c}
	LD	0	0 ± 0^{b}	11 ± 0.30^{b}	360 ± 21^{c}
	Normal nutrition	0	0 ± 0^{b}	17 ± 0.55^{a}	903 ± 47^{a}
	Poor nutrition	0	0 ± 0^{b}	11 ± 0.24^{b}	448 ± 19^{b}

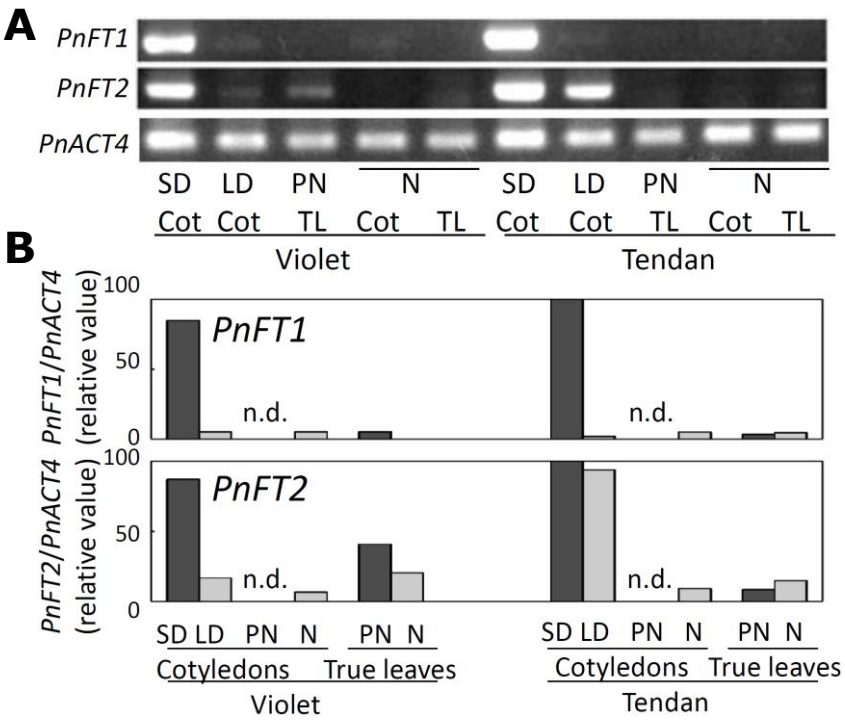

Fig. 3. Expression of *PnFT* under poor-nutrition stress in *Pharbitis nil*. (A) The cotyledons (Cot) and expanded true leaves (TL) of cvs. Violet and Tendan grown in poor nutrition (PN) or normal nutrition (N), or given a short-day treatment (SD) or not (LD) (Table 5) were collected for RT-PCR analysis of *PnFT1* and *PnFT2*. (B) The mRNA abundance of each gene was normalized to that of *PnACT4*, and shown as a relative value. The cotyledons of stressed plants (PN) were not analyzed because they had withered by the end of the stress treatment (n.d., not determined).

non-stressed plants in Violet. The expression of *PnFT2* in the true leaves of Tendan was almost the same under both stress and non-stress conditions and was extremely lower than that in the SD-treated cotyledons. The *PnFT2* expression in the stressed true leaves was weaker in Tendan than in Violet. The expression of *PnFT1* in the stressed plants was negligible in the both cultivars.

Tendan flowered when grown at 15 ℃ as did Violet (Table 6). The vegetative growth was suppressed at 15 ℃ in both cultivars. The flowering response induced by the low-temperature stress was weaker than that induced by the SD treatment in Violet, while the trend was reversed in Tendan. The flowering response induced by the low-temperature stress was stronger in Tendan than in Violet. These plants were analyzed for the expression of *PnFT*. The low-temperature stress treatment induced the expression of *PnFT2*, and the expression of *PnFT1* was weak in both cultivars (Fig. 4). The expression level of *PnFT2* in the cotyledons was almost the same between the plants grown under the stress conditions and those given the SD treatment in both cultivars, while the expression was stronger in Tendan than in Violet.

Expression of some other flowering genes in stress-induced flowering

The expression of homologs of another floral pathway integrator gene, *SOC1*, and the regulatory gene of the *FT* gene expression, *CO*, was also examined in the cotyledons and true leaves of Violet and Tendan which were treated with poor-nutrition or low-temperature stress. The *SOC1* homolog

Table 6. Flowering response of *Pharbitis nil* treated with low-temperature stress. *P. nil* cvs. Violet and Tendan were grown at 25 °C for 5 weeks or at 15 °C for 3 weeks under long-day (LD) conditions. Control plants were grown at 25 °C, and given a short-day (SD) treatment or not (LD). Values followed by the different superscript letters differ significantly at the 5% level, according to a *t*-test within the same cultivar.

Cultivar	Conditions	% flowering	Floral buds /plant	Nodes /plant	Stem length (mm)
Violet	SD	70	0.80 ± 0.14^{a}	12 ± 0.44^{c}	304 ± 28^{c}
	LD	0	0 ± 0^{b}	12 ± 0.43^{c}	346 ± 26^{c}
	25 ℃	0	0 ± 0^{b}	22 ± 0.83^{a}	1180 ± 78^{a}
	15 ℃	21	0.21 ± 0.11^{b}	15 ± 0.61^{b}	416 ± 40^{b}
Tendan	SD	20	0.20 ± 0.092^{b}	11 ± 0.48^{c}	359 ± 34^{b}
	LD	0	0 ± 0^{c}	12 ± 0.29^{c}	399 ± 24^{b}
	25 ℃	0	0 ± 0^{c}	17 ± 1.1^{a}	826 ± 61^{a}
	15 ℃	47	1.1 ± 0.30^{a}	14 ± 0.77^{b}	488 ± 63^{b}

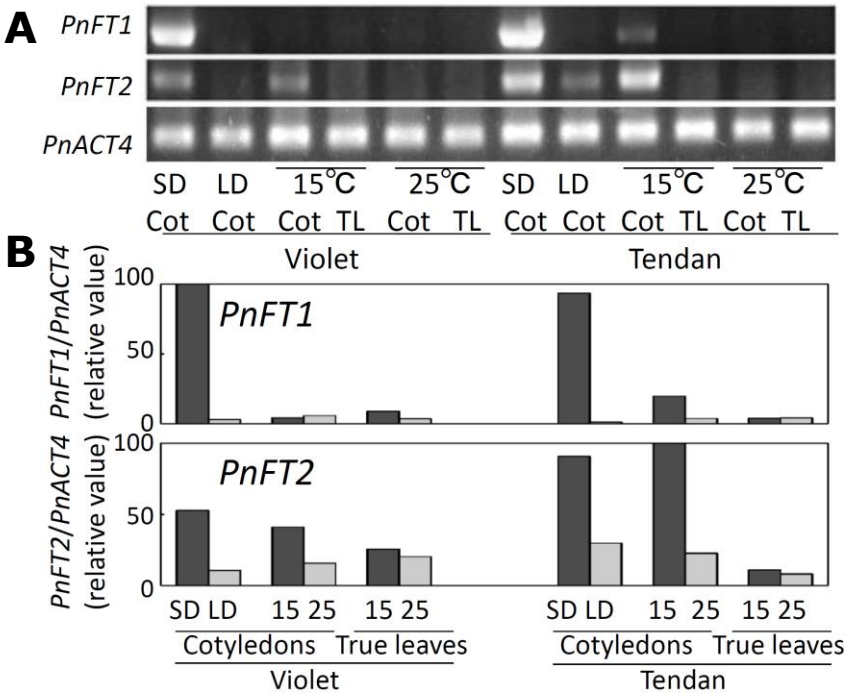

Fig. 4. Expression of *PnFT* under low-temperature stress in *Pharbitis nil*. (A) The cotyledons (Cot) and expanded true leaves (TL) of cvs. Violet and Tendan grown at either 15 ℃ or 25 ℃, or given a short-day treatment (SD) or not (LD) (Table 6) were collected for RT-PCR of *PnFT1* and *PnFT2*. (B) The mRNA abundance of each gene was normalized to that of *PnACT4*, and shown as a relative value.

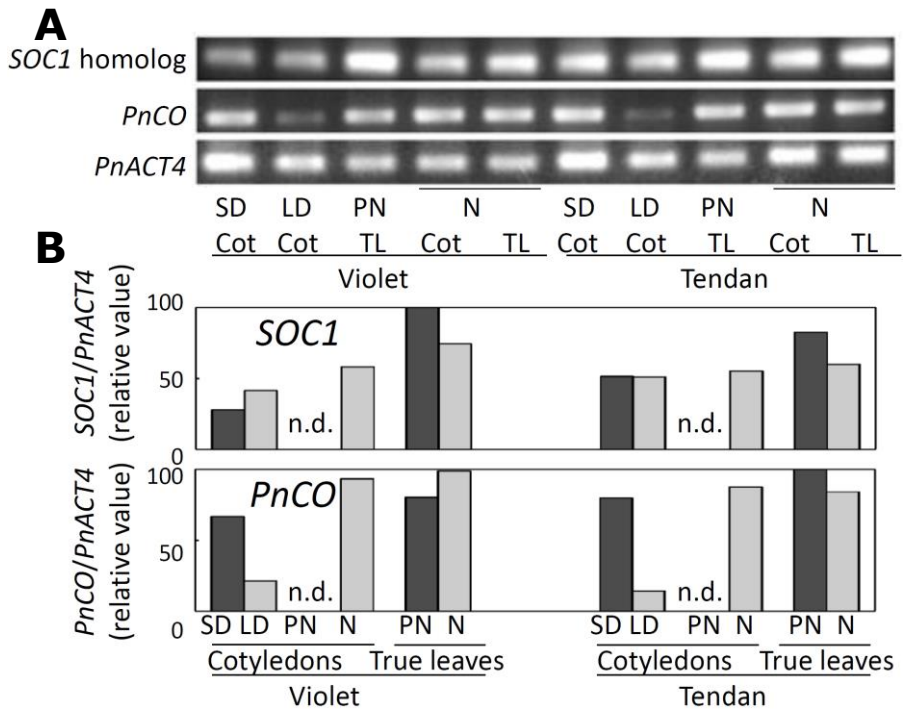

Fig. 5. Expression of *SOC1* homolog and *PnCO* under poor-nutrition stress in *Pharbitis nil*. (A) The cotyledons (Cot) and expanded true leaves (TL) of cvs. Violet and Tendan grown in poor nutrition (PN) or normal nutrition (N), or given a short-day treatment (SD) or not (LD) (Table 5) were collected for RT-PCR analysis of *SOC1* homolog and *PnCO*. (B) The mRNA abundance of each gene was normalized to that of *PnACT4*, and shown as a relative value. The cotyledons of stressed plants (PN) were not analyzed because they had withered by the end of the stress treatment (n.d., not determined).

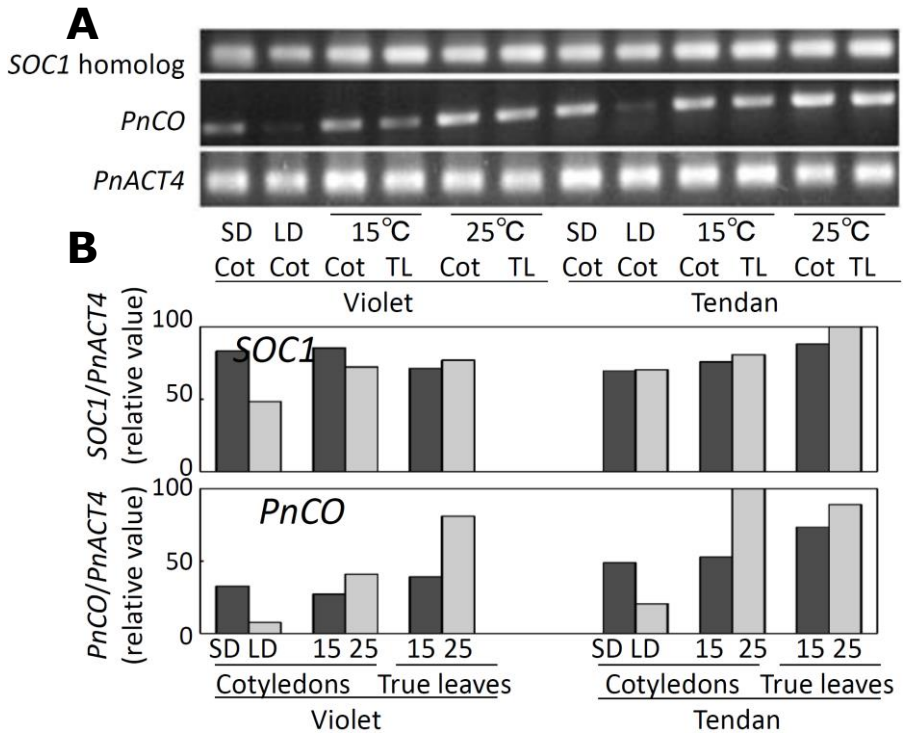

Fig. 6. Expression of *SOC1* homolog and *PnCO* under low-temperature stress in *Pharbitis nil*. (A) The cotyledons (Cot) and expanded true leaves (TL) of cvs. Violet and Tendan grown at either 15 ℃ or 25 ℃, or given a short-day treatment (SD) or not (LD) (Table 6) were collected for RT-PCR of *SOC1* homolog and *PnCO*. (B) The mRNA abundance of each gene was normalized to that of *PnACT4*, and shown as a relative value.

was expressed regardless of the growth conditions (stressed and non-stressed, SD and LD) in both cultivars (Figs. 5 and 6). *PnCO* was also expressed regardless of the growth conditions with the exception of the LD conditions in both cultivars.

The expression of homologs of *FLC*, *FCA*, *FRI* and *FVE* (*PnFVE*) was analyzed in the cotyledons and true leaves of Violet which was given the poor-nutrition stress treatment. The *FCA* homolog was expressed under all conditions examined, and the expression of the homologs of *FLC*, *FRI* and *FVE* was not detected under any conditions (Fig. 7).

The flowering genes that function in shoot apex; the homologs of *AP1*, *SOC1*, *LFY* and *FD* were examined for their expressions in Violet. The *PnAP1* expression was detected in the shoot apices of the plants given the SD treatment or grown under the poor-nutrition stress conditions (Fig. 8). The *SOC1* homolog and *PnLFY* expressed regardless of the growth conditions. The expression of *FD* homolog was not detected. The *FD* homolog was further analyzed by degenerated PCR, but its expression was never detected (data not shown).

Spacial expression pattern of *PnFT* in poor-nutrition stress-induced flowering

The spacial expression pattern of *PnFT* was examined in Violet which was induced flowering by SD or poor-nutrition stress treatment. The expression of *PnFT1* and *PnFT2* induced by the SD treatment was detected only in the cotyledons and the expression of *PnFT2* induced by the stress treatment was detected only in the cotyledons and true leaves (Fig. 9). The

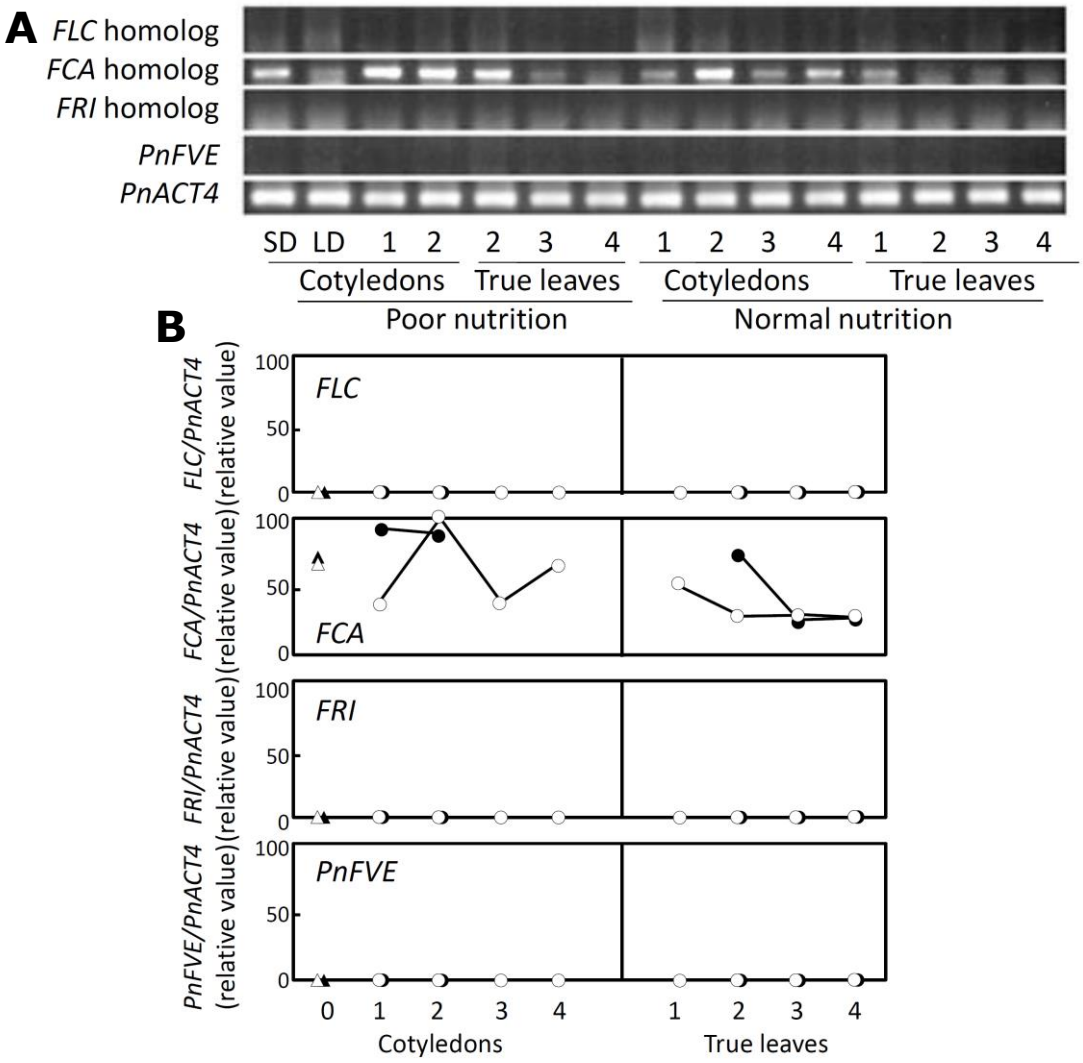

Fig. 7. Time-course of expression of homologs of *FLC*, *FCA*, *FRI* and *PnFVE* in *Pharbitis nil* cv. Violet treated with poor-nutrition stress. Plants were grown in nutrient solution(Normal nutrition; open circles) or 1/100-strength nutrient solution (Poor nutrition; closed circles) under long-day (LD) conditions for 1–4 weeks. Control plants were grown in nutrient solution, and given a short-day treatment (SD, closed triangles) or not (LD, open triangles). (A) The cotyledons and expanded true leaves were collected for RT-PCR analysis of homologs of *FLC*, *FCA*, *FRI* and *PnFVE*. (B) The mRNA abundance of each gene was normalized to that of *PnACT4*, and the data from the cotyledons are shown as relative values.

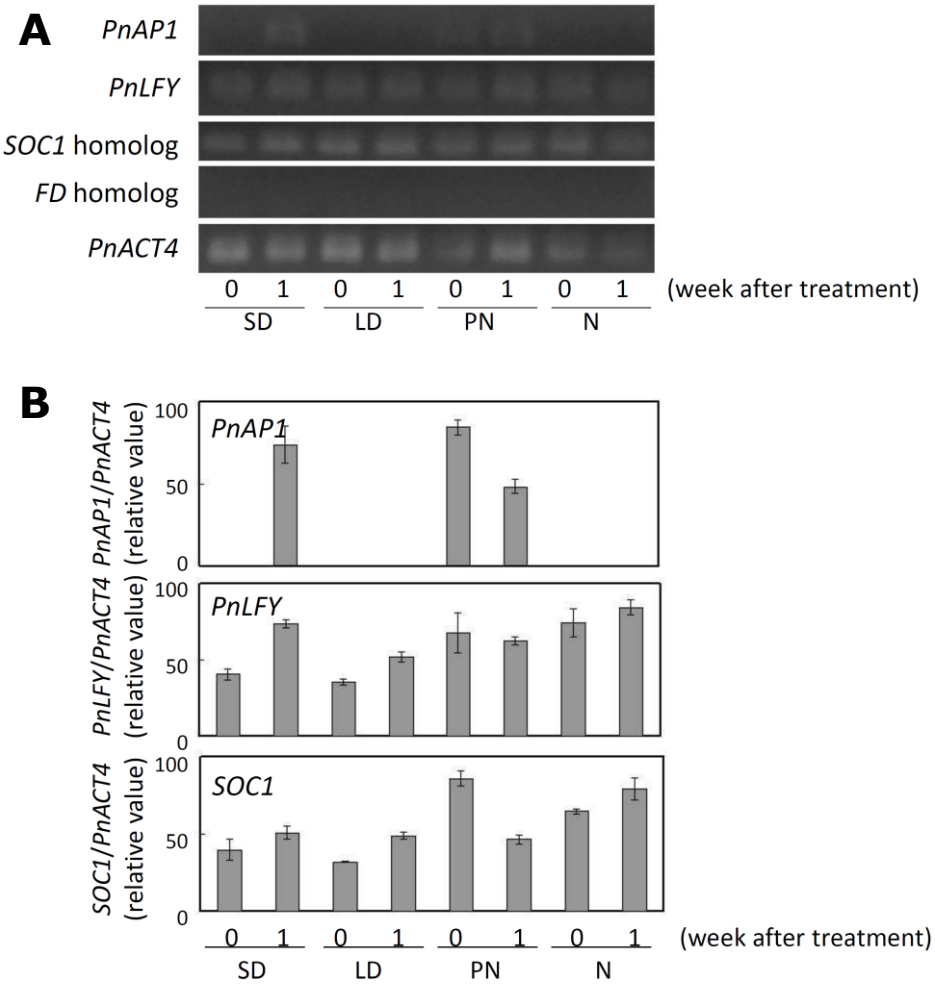

Fig. 8. Expression of *PnAP1*, *PnLFY*, *SOC1* homolog and *FD* homolog in *Pharbitis nil* cv. Violet treated with short day or poor-nutrition stress. Plants were grown in nutrient solution (N) or 1/100-strength nutrient solution (PN) under long-day conditions for 3 weeks or given a short-day treatment (SD) or not (LD). (A) Immediately after (0) or 1 week after (1) the stress or SD treatment, the shoot apices were collected for RT-PCR analysis of *PnAP1*, *PnLFY*, *SOC1* homolog and *FD* homolog. (B) The mRNA abundance of each gene (except for *FD* homolog) was normalized to that of *PnACT4*, and the data are shown as relative values. The RT-PCR procedure was repeated three times and the means with standard errors (B) and a representative gel image (A) are shown.

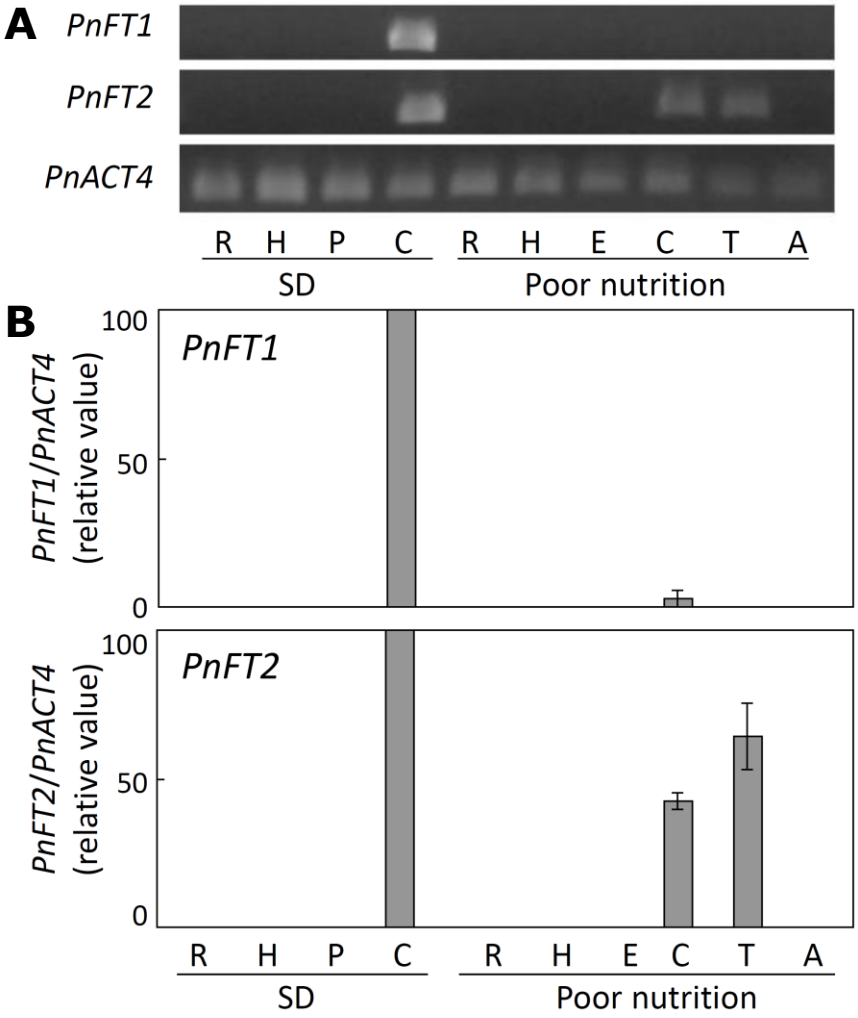

Fig. 9. Spacial pattern of *PnFT* expression in *Pharbitis nil* cv. Violet treated with short-day or poor-nutrition stress. Plants were grown in 1/100-strength nutrient solution (Poor nutrition) under long-day conditions for 3 weeks or given a short-day treatment (SD). (A) After the stress or SD treatment, the roots (R), hypocotyls (H), plumules (P), cotyledons (C), epicotyls (E), expanded true leaves (T) and shoot apices (A) were collected for RT-PCR analysis of *PnFT1* and *PnFT2*. (B) The mRNA abundance of each gene was normalized to that of *PnACT4*, and the data are shown as relative values. The RT-PCR procedure was repeated three times and the means with standard errors (B) and a representative gel image (A) are shown.

expression of neither *PnFT* was detected in other tissues such as roots, hypocotyls, plumules, epicotyls and shoot apices.

Time-course of *PnFT2*, *PnAP1* expression and flowering response

The temporal expression pattern of *PnFT2* and *PnAP1*, and time-course of flowering responses in poor-nutrition stress-induced flowering were examined in Violet. The *PnFT2* expression in the cotyledons became detectable first and was followed by the *PnAP1* expression in the shoot apices (Fig. 10). The stress treatment for 12 days induced the apparent expression of *PnFT2* and the slight expression of *PnAP1*, and did not induce flowering. The stress treatment for 16 days induced the expression of both genes and flowering.

Discussion

When *P. nil* was given a SD treatment under the non-stress conditions, it was induced flowering, whereas the plants grown under the LD conditions did not flower (Tables 3 to 6), indicating that *P. nil* is an absolute SD plant. *P. nil* cv. Violet flowered even under the LD conditions when grown under the poor-nutrition or low-temperature conditions (Tables 3 to 6). The number of nodes and the stem length in these flowered plants were reduced, indicating that poor nutrition and low temperature functioned as stress factors (Hatayama and Takeno, 2003). Therefore, such flowering is considered as stress-induced flowering. Cv. Tendan was induced flowering by low-temperature stress treatment, but did not flower under the poor-nutrition conditions although the vegetative growth was suppressed

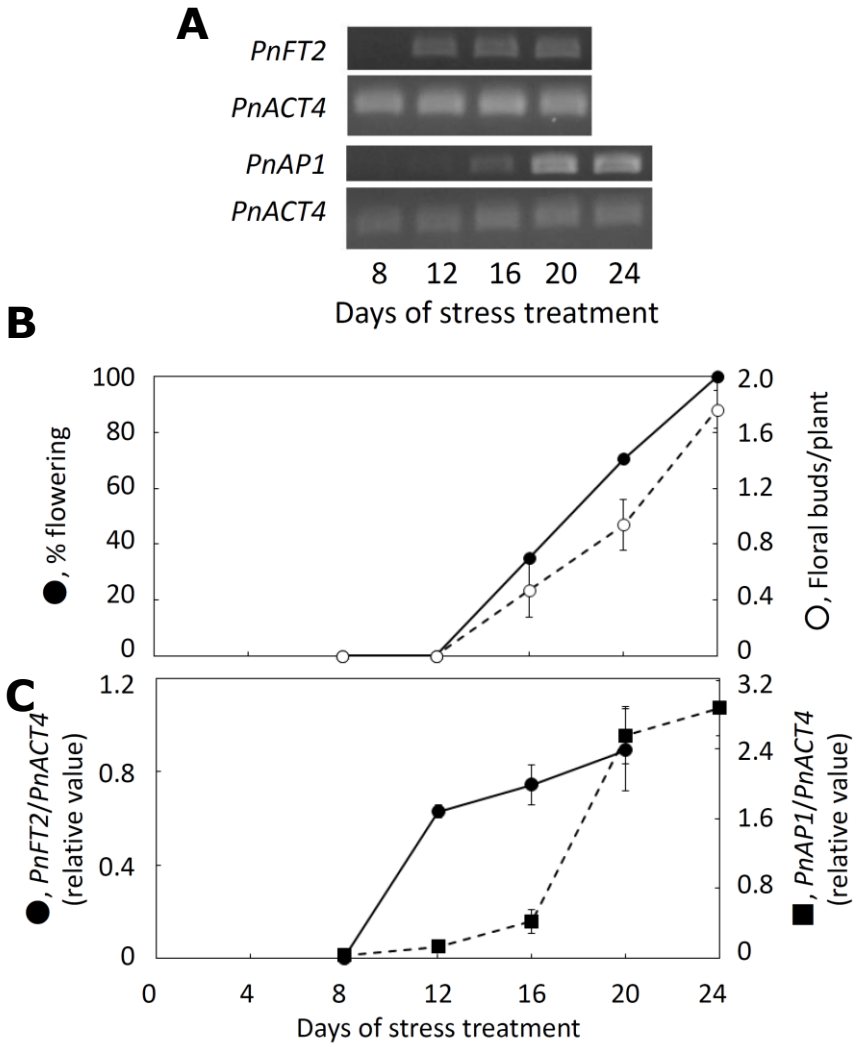

Fig. 10. Time-course of flowering response and expression of *PnFT2* and *PnAP1* in *Pharbitis nil* cv. Violet treated with poor-nutrition stress. Plants were grown in 1/100-strength nutrient solution under long-day conditions for 8–24 days. (B) After the poor-nutrition stress treatment, the plants were grown under the normal nutrition conditions for 2 weeks to score the flowering response. (A) The cotyledons and the shoot apices were collected after the stress treatment for RT-PCR analysis of *PnFT2* and *PnAP1*, respectively. (C) The mRNA abundance of each gene was normalized to that of *PnACT4*, and the data are shown as relative values. The RT-PCR procedure was repeated three times and the means with standard errors (B) and a representative gel image (A) are shown.

(Tables 5 and 6). The reason why Tendan does not respond to the poor-nutrition stress was not clarified (Mizuuchi, 2013).

Because *FT* integrates all known flowering pathways in *A. thaliana* (Boss et al., 2004), it was expected that *FT* may also be involved in the newly discovered stress-induced flowering. As expected, the expression of *PnFT2*, a homolog of *FT* in *P. nil* (Hayama et al., 2007), was induced when flowering was induced by stress treatments in the two cultivars of *P. nil*, as summarized in Table 7. There was a positive correlation between the degree of the flowering response and the expression level of *PnFT2* (Figs. 1 to 4; Tables 5 and 6). These results suggest an involvement of *PnFT2* in the stress-induced flowering of *P. nil*. It was previously reported that *PnFT2* might be involved in the poor-nutrition stress-induced flowering in Violet (Wada et al., 2010b). The present data reinforce this hypothesis and further suggest that *PnFT2* is also involved in low-temperature stress-induced flowering in both cultivars. These results are consistent with those in the previous report indicating that *FT* is involved in UV-C stress-induced flowering in *A. thaliana* (Martínez et al., 2004).

Among the two orthologs of *FT*, *PnFT1* and *PnFT2*, *PnFT2* was the gene primarily expressed during stress-induced flowering, whereas both *PnFT1* and *PnFT2* were expressed in SD-induced flowering (Table 7). These results suggest that *PnFT2*, but not *PnFT1*, is the major regulatory gene involved in the stress-induced flowering of *P. nil*. *PnFT2* is involved in both photoperiodic flowering and stress-induced flowering, whereas *PnFT1* is involved only in the former. The two *PnFT* genes may play different roles in the regulation of flowering depending on the inductive cue. *FT* is widely conserved in many

Table 7. Flowering response and expression of *PnFT* genes in the two cultivars of *Pharbitis nil* treated with short day or stress. +, flowering induced; −, flowering not induced. Parentheses indicate weak expression.

Cultivar	Short-day treatment		Stress treatment			
			Poor nutrition		Low temperature	
	Flowering	Genes	Flowering	Genes	Flowering	Genes
Violet	+	*PnFT1*, *PnFT2*	+	*PnFT2*	+	(*PnFT1*), *PnFT2*
Tendan	+	*PnFT1*, *PnFT2*	−	−	+	(*PnFT1*), *PnFT2*

plant species, and some plants have two or more *FT* homologs. However, not every *FT* homolog has a function to induce flowering. One of *FT* homologs induces flowering while other one does not or inhibits it in *A. thaliana* (Kobayashi et al., 1999), *Chenopodium rubrum* (Cháb et al., 2008) and *Beta vulgaris* (Pin et al., 2010). In contrast to these cases, both of the two *FT* homologs of *P. nil* induce flowering, but each gene uses different cue to express. Such a case has not been known before.

Expression of *PnFT2* was unexpectedly detected in the cotyledons of the LD-grown control plants of Tendan, but was not detected in the true leaves of the plants grown under the normal nutrition conditions (Fig. 3). Although the former plants acted as the control for the SD treatment, and the latter acted as the control for the stress treatment, both groups were grown under the same conditions (i.e., non-stress LD conditions). Sampling for the RT-PCR of *PnFT2* expression, however, was performed 3 weeks earlier in the former group than in the later group. The *PnFT2* expression under the non-inductive conditions was detected in young plants. This suggests that *PnFT2* is expressed preferentially in young cotyledons and that expression may cease in aged leaves.

Many genes are involved in the regulatory network of flowering in *A. thaliana*, and *SOC1* is thought to play the role as floral pathway integrator gene as does *FT* (Bernier and Périlleux, 2005; Amasino and Michaels, 2010). Therefore, it was expected that *SOC1* expression may also be induced when flowering was induced by stress in *P. nil*. However, the *P. nil SOC1* homolog was constitutively expressed in the leaves (Figs. 5 and 6). UV-C stress, which induced flowering, did not induce *SOC1* expression in *A. thaliana*

(Martínez et al., 2004). These results suggest that *SOC1* is not involved in the regulation of stress-induced flowering. The expression level of the *SOC1* homolog under the LD conditions was as high as the expression level under the SD conditions (Figs. 5 and 6). This result suggests that *SOC1* is also not involved in the regulation of photoperiodic flowering of *P. nil*.

CO is an important gene involved in the regulation of photoperiodic flowering in many plants (Valverde, 2011). The CO protein directly induces transcription of *FT* in *A. thaliana* and *Oryza sativa* (Suárez-López et al., 2001; Izawa et al., 2002; Valverde et al., 2004). It has been proposed that the *CO*/*FT* regulatory module (i.e., CO protein activates *FT* transcription) is highly conserved in both dicot and monocot plants (Matsoukas et al., 2012). Expression of *CO* was also moderately induced in UV-C stress-induced flowering in *A. thaliana* (Martínez et al., 2004). In contrast to these reports, however, *PnCO* was constitutively expressed regardless of the flowering status in *P. nil* (Figs. 5 and 6). These results suggest that *PnCO* may not be involved in the regulation of stress-induced or photoperiodic flowering of *P. nil*, which is consistent with the previous report that *PnFT* mRNA abundance was not related to *PnCO* expression; therefore, *PnFT* may not be regulated by the PnCO protein in *P. nil* (Hayama et al., 2007; Higuchi et al., 2011). A night-break treatment inhibited flowering, but did not influence *PnCO* expression (Liu et al., 2001). Flowering of tomato is accelerated by the tomato ortholog of *FT*, *SINGLE FLOWER TRUSS* (Lifschitz et al., 2006). Overexpression of the tomato homolog of *CO*, *Tomato CONSTANS-Like 3* (*TCOL3*), did not affect the flowering time of tomato, whereas the flowering of *A. thaliana* was affected by *TCOL3* (Ben-Naim et al., 2006). Based on

these results, it was suggested that the tomato *FT* ortholog has lost the promoter motifs required for recognition by *COL* transcriptional complexes. The *CO*/*FT* module may not be widely conserved.

The genes that regulate the expression of *FT* were also searched. *FLC* is an important gene that regulates the expression of *FT* and *SOC1* in vernalization-mediated flowering of *A. thaliana* (Seo et al., 2009; Lee and Lee, 2010; Deng et al., 2011). Because *P. nil* does not have a vernalization requirement, functional *FLC* was not expected to exist in *P. nil*. *FLC* is, however, also involved in the autonomous pathway of flowering regulation in *A. thaliana* (Veley and Michaels, 2008), and therefore, we examined whether the *P. nil* homolog of *FLC* was expressed in poor-nutrition stress-induced flowering. The expression of the *FLC* homolog was not detected (Fig. 7). The other genes that act upstream of *FT* were also examined. Expression of the homologs of *FRI* and *FVE* was not detected by poor-nutrition stress, and the *FCA* homolog was expressed even in the absence of stress treatment (Fig. 7). Thus, candidate genes that act upstream of *FT* and regulate *FT* expression were not identified among the known flowering genes.

The spacial expression pattern of *PnFT* was examined. *PnFT1* and *PnFT2* expressed only in the cotyledons by the SD treatment, and *PnFT2* expressed only in the cotyledons and true leaves by the poor-nutrition stress treatment (Fig. 9). *FT* expresses specifically in leaves in photoperiodic flowering of *A. thaliana* (Takada and Goto, 2003), and all previously characterized *FT*-like genes were mainly detected in leaves (Pin et al., 2010). The consistency in the spacial expression pattern of *FT* in *A. thaliana* and *PnFT* in *P. nil* suggests that the roles of these genes in flowering may be

similar between *A. thaliana* and *P. nil*.

Expression of *PnAP1*, the *P. nil* homolog of *AP1* was detected in the shoot apices which were treated with SD or poor-nutrition stress (Fig. 8). In the SD-treated shoot apices, the expression of *PnAP1* was not detected immediately after the SD treatment, and detected a week after the SD treatment. This is consistent with an assumption that the expression of *PnAP1* was induced by some floral stimuli which have been generated in leaves and moved to shoot apices in photoperiodic flowering of *P. nil* as in *A. thaliana*. The *PnAP1* expression was also detected in the stress-treated shoot apices. The expression of *PnAP1* was detected immediately after the end of stress treatment for 3 weeks. It was reported that floral stimuli which move from leaves to shoot apices exist in stress-induced flowering (Wada et al., 2010b). The floral stimuli generated by stress in the leaves may have moved to the shoot apices during the stress treatment to induce the *PnAP1* expression. The expression of *FD* homolog was not detected in the shoot apices treated by poor-nutrition stress or SD (Fig. 8). It has not been reported so far that *P. nil* has a homolog of *FD*. Therefore, the model established in *A. thaliana* and *O. sativa* that FT protein forms a complex with FD protein in shoot apices to induce flowering (Abe et al., 2005; Taoka et al., 2011) can not be applied to *P. nil*. *FT* induces also *SOC1* and *LFY* in *A. thaliana* (Moon et al., 2005; Yoo et al., 2005; Lee and Lee, 2010). The expressions of *SOC1* homolog and *PnLFY* were not related to the flowering status in *P. nil* (Fig. 8). This suggests that the expression of these genes may not relate to the expression of *PnFT*. The present results suggest that the role of *AP1* in flowering may be common between *P. nil* and *A. thaliana*, but

the gene regulation cascades of stress-induced flowering of *P. nil* are different from those of *A. thaliana*.

The results described above suggest that *PnFT2* expressed in the leaves and *PnAP1* expressed in the shoot apices are involved in stress-induced flowering of *P. nil*. Then, the time-courses of expression of *PnFT2* and *PnAP1* were compared to see the relationship between these genes in the regulation of flowering. The *PnFT2* expression in the cotyledons occurred first and then the *PnAP1* expression in the shoot apices followed (Fig. 10). The stress treatment for 12 days was enough to induce the detectable level of *PnFT2* expression, but not enough to induce flowering. The stress treatment for 16 days induced the expression of both *PnFT2* and *PnAP1* and also flowering response. Such sequential events are consistent with an assumption that *PnFT2* induces *PnAP1*, and then *PnAP1* induces flowering.

Chapter II

Flowering inducing activity of *PnFT2*

Introduction

The results of the Chapter I suggest that *PnFT2* is involved in the regulation of stress-induced flowering of *P. nil*. However, it is unknown whether *PnFT2* has the activity to induce flowering. The other *FT* homolog, *PnFT1* was reported to complement the *ft* mutation of *A. thaliana* and to induce early flowering in *P. nil* when overexpressed using *35S* promoter (Hayama et al., 2007), but the transformation with *PnFT2* of *A. thaliana* and *P. nil* was not performed. Accordingly, the flowering inducing activity of *PnFT2* was examined by the transformation experiments in which *A. thaliana* and *P. nil* were transformed to overexpress *PnFT2* or suppress its expression by RNA interference (RNAi).

Materials and methods

Plasmid construction and genetic transformation

Plasmid constructs were prepared to overexpress *PnFT1* and *PnFT2* in *P. nil* and *A. thaliana* and to suppress the expression of these genes in *P. nil*. For the construct to overexpress *PnFT1*, the full-length coding sequence (552 bp) of *PnFT1* was amplified by PCR from cDNA sample with the *PnFT1*-specific primers containing the adaptor sequence for pENTR using Phusion High-Fidelity DNA Polymerase (Finnzymes Oy, Espoo, Finland). The PCR product was separated on agarose gel, and then extracted from the agarose gel using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The nucleotide sequences were determined, and subcloned into the entry vector pENTR/D-TOPO (Invitrogen Corporation, Carlsbad, CA, U.S.A.). The pENTR plasmid was amplified by *Escherichia coli*

strain DH5α and purified by Labo Pass Mini (Cosmo Genetech Co. Ltd., Seoul, South Korea). This purified plasmid was amplified by the PCR with *PnFT1*-specific primers added with BMV RNA4 sequence, translation activation sequence (Skuzeski et al., 1990), with the restriction enzyme sites 5'*Xba*I and 3'*Sac*I. The PCR product was purified and digested with the restriction endonucleases *Xba*I and *Sac*I, and then the *PnFT1* fragment was subcloned into pBI121. The plasmid was amplified by *E. coli* strain DH5α and purified by Qiagen Plasmid Midi Kit (Qiagen N. V., Hilden, Germany). The construction of the plasmid for the *PnFT2* overexpression was performed in the same manner as above with some modifications. The nucleotide sequences of the primers used are shown in Table 8.

The *PnFT1*-RNAi construct was prepared as follows. The *PnFT1* PCR product containing a fragment of 3' end region (224 bp) was amplified from cDNA sample with the *PnFT1*-specific primers using Phusion High-Fidelity DNA Polymerase (Finnzymes Oy, Espoo, Finland). The PCR product was separated on agarose gel, extracted from the agarose gel using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel GmbH & Co. KG, Düren, Germany), and the nucleotide sequence was determined. The purified fragment was cloned into pRNAi with *Eco*RV and T4 DNA ligase (New England Biolabs, Ipswich, MA, U.S.A.). The pRNAi plasmid was amplified by *E. coli* strain DH5α and purified by Labo Pass Mini (Cosmo Genetech Co. Ltd., Seoul, South Korea). To construct the inverted repeat, PCR for pRNAi was performed with reverse primer and forward primer (containing the adaptor sequence for pENTR); concentration of the later was 10-fold higher than the former. The PCR product was purified and subcloned into entry vector pENTR/D-TOPO

Table 8. Primer sequences used for plasmid construction.

			sequence
PnFT1 ox	from cDNA to pENTR	forward	5'-CACCATGCGAAGGGGAACAGTAG-3'
		reverse	5'-GGAAGGAGCAGGGTAATTAATCGG-3'
	from pENTR to pBI121	forward	5'-GTATCTAGAGTATTAATA ATGCGAAGGGGAACAGTAG-3'
		reverse	5'-GATGAGCTC GGAAGGAGCAGGGTAATTAATCGG-3'
PnFT2 ox	from cDNA to pENTR	forward	5'-CACCATGTCGGGAGGAAGAGAC-3'
		reverse	5'-AGGGTGCTCATTACGCATT-3'
	from pENTR to pBI121	forward	5'-GTATCTAGAGTATTAATA ATGTCGGGAGGAAGAGAC-3'
		reverse	5'-GATCCCGGG AGGGTGCTCATTACGCATT-3'
PnFT1-RNAi	from cDNA to pRNAi	forward	5'-CGATCTCCGGCAGAGCCCTGG-3'
		reverse	5'-CTTCATTGCCATATATAAAGGG-3'
PnFT2-RNAi	from cDNA to pRNAi	forward	5'-CGGAGGCCGGAGACGATGAAT-3'
		reverse	5'-TACATATAGGACGATACATATGAC-3'
inverted repeat sequence with pRNAi		forward	5'-CACCCCTCGAGGTCGACGGTATCG ATAAGCTTGAT-3'
		reverse	5'-GATTGAGATATTACCTGCAGGTAC TCACCCGGGTG-3'

(Invitrogen Corporation, Carlsbad, CA, U.S.A.). Transfer of the insert sequence from the pENTR to the destination vector pH35GA was carried out using the Gateway system with Gateway LR Clonase Enzyme Mix (Invitrogen Corporation, Carlsbad, CA, U.S.A.). The plasmid was amplified by *E. coli* strain DH5α and purified by Labo Pass Mini (Cosmo Genetech Co. Ltd., Seoul, South Korea). The construction for *PnFT2*-RNAi plasmid was performed in the same manner as that of *PnFT1*-RNAi plasmid with some modifications.

Bacterial strain and plasmid

Agrobacterium tumefaciens strain EHA105 (Hood et al., 1993) was used. The binary vector was a modified pBI121 vector, from which the *GUS* gene was removed. The binary vector pH35GA was also used. These two binary vectors have cauliflower mosaic virus (CaMV) *35S* promoter that constitutively overexpresses genes in plants. Both pBI121-(-)*GUS* and pH35GA (Kubo et al., 2005) vector were introduced into *A. tumefaciens*.

Plant materials for transformation

A. thaliana ecotype Columbia was used. The plants were grown at 22 ℃ under continuous white light.

P. nil cv. Violet was also used. Violet plants were grown in a green house at 25 °C under natural light conditions for 7 months. Immature fruits were harvested from the plants about 2 weeks after flower-opening. The immature fruits were surface sterilized with 70% ethanol for 4 minutes and then with a 0.25% solution of sodium hypochlorite for 50 minutes. They were washed four times in a large volume of sterilized water and dissected to

isolate immature embryos, which were 2 to 8 mm in length. The isolated immature embryos were cultured on an embryoid induction medium [MS medium containing 3 mg/l α–naphthaleneacetic acid and 6% sucrose; Jia and Chua (1992)] to induce somatic embryos.

Transformation and regeneration of plants

In the transformation of *A. thaliana*, transformation was carried out by floral dip method described by Clough and Bent (1998). *A. tumefaciens* to be used for the transformation (*PnFT1* or *PnFT2* overexpression) was grown on an 2×YT medium containing antibiotics at 28 ℃ overnight. After the overnight culture, *A. tumefaciens* was washed and suspended in inoculation medium [5% sucrose and 0.05% silwet L-77 (Bio Medical Science Inc., Tokyo, Japan)]. Inflorescences with flowers of *A. thaliana* were dipped into the inoculation medium for 1 minute, the plants were incubated at 4 ℃ overnight and returned to 21 ℃ conditions. The plants were grown until seed maturation to harvest the transformed seeds.

Agrobacterium-mediated transformation of *P. nil* was carried out as described by Kikuchi et al. (2005). *A. tumefaciens* which was transformed as mentioned above was grown at 28 ℃ on an 2×YT medium containing antibiotics overnight. After the overnight culture, *A. tumefaciens* was washed and suspended in suspension medium [MS medium containing 10% sucrose and 19.62 mg/l acetosyringone]. The *P. nil* somatic embryos induced from the immature embryo culture were inoculated with the *A. tumefaciens* suspension for 5 minutes and transferred to plates of co-cultivation medium [MS medium containing 10% sucrose, 19.62 mg/l

acetosyringone and 0.32% gellan gum]. After the co-cultivation for 2 days, the *P. nil* somatic embryos were transferred to a selection medium containing antibiotics (geneticin for pBI121 or hygromycin for pH35GA) and Augmentin (125 mg/l potassium clavulanate and 250 mg/l amoxicillin; Glaxo SmithKline K.K., Uxbridge, UK). After the cultivation on the selection medium for one month, the *P. nil* somatic embryos were transferred to embryoid maturation and germination (EMG) medium [MS medium containing 0.2 mg/l indole-3-acetic acid (IAA), 2 mg/l 6-benzylaminopurine, 3% sucrose and 1% agar; Ono et al. (2000)]. When the shoots were regenerated, they were transferred to rooting medium [a hormone-free 1/2MS medium with 1% agar]. When the roots were induced on the regenerated shoots, the plantlets were transplanted to moist vermiculite (Fujimi, Shizuoka, Japan) for acclimatization. When the shoots began to grow, the plantlets were transplanted to moist soil. The plants were grown at 25 ℃ under green house conditions and T1 seeds were harvested.

Scoring of the flowering response of *A. thaliana*

The transformed T1 seeds and wild type (WT) seeds of *A. thaliana* were surface sterilized and planted in a square Petri dish containing 1.5% agar medium with kanamycin, as described by Okada and Shimura (1992). The seeds were kept at 4 ℃ for 3 days and then incubated at 22 ℃ under continuous white light. After 1 week, the seedlings were transplanted to moist vermiculite and were acclimated. The seedlings were grown under the same conditions until flowers were formed.

The *A. thaliana* plants were observed under a binocular microscope to

determine whether flower buds were formed. The flowering time in days from the seed sowing to the detection of the first flower bud and number of the rosette leaves were scored as indicator of flowering response. Twenty to 27 plants per line were used for each experiment. The means with standard errors for the representative experiment are shown .

Scoring of the flowering response of *P. nil*

The transformed T1 or WT *P. nil* seeds whose seed coats were scraped soaked in tap water overnight. The swollen seeds were placed on moist filter paper in a Petri dish 90 mm in diameter and were germinated at 25 ℃ under 16-hour light and 8-hour dark LD conditions for 1 day. The germinated seeds were planted on 0.6% plain agar medium in a plastic box (240 mm × 330 mm × 90 mm in depth) and grown at 25 ℃ under the LD conditions for 5 days. The seedlings were then transferred to glass tubes (15 mm in diameter × 150 mm high) containing a mineral nutrient solution (Kondo et al., 2006) or 1/100-strength mineral nutrient solution as the poor-nutrition stress treatment. The seedlings were grown under the same conditions mentioned above. White light (55-90 μmol m^{-2} s^{-1}) was provided by fluorescent lamps (FL20SW or FL40SSW/37, Toshiba Corporation, Tokyo, Japan). For SD treatment, 5-day-old seedlings were given a single 16-hour dark treatment. After the stress treatment or SD treatment, the plants were transferred to the LD non-stress conditions and grown for 2 weeks until the flowering response was scored.

All of the plant nodes were dissected under a binocular microscope to determine whether flower buds or vegetative buds formed. The percentage

of plants with at least one flower bud out of all the plants in a treatment (% flowering) and the number of flower buds per plant were determined. The number of nodes, that is the total number of flower buds and vegetative buds per plant, was used as indicator of vegetative growth along with the average length of the main stem. Twenty to 30 plants were used for each treatment. Each experiment was repeated at least three times. The means with standard errors of the most representative experiment are shown in each table.

Gene expression analysis using RT-PCR

One of the two cotyledons and/or an expanded true leaf of *P. nil* plant were collected 16 hours after the start of the dark period (8 hours after the end of the dark period of 16-hour light LD conditions, or the end of the dark period of 8-hour light SD conditions). The harvested plant leaves were frozen in liquid nitrogen and stored at -80 ℃ prior to analysis. The total RNA was isolated from the tissues using the Plant RNA Purification Reagent (Invitrogen Corporation, Carlsbad, CA, U.S.A.) and Fruit-mate (Takara Biotechnology Co. Ltd., Shiga, Japan). The isolated RNA samples were treated by DNase I (Sigma-Aldrich Inc., Saint Louis, MO, U.S.A.) to digest contaminated genomic DNA, and then cDNAs were synthesized from each RNA sample using ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan). The gene amplification was performed by nested PCR using KOD -Plus- Neo (Toyobo Co. Ltd., Osaka, Japan). *PnFT1* and *PnFT2* were amplified from the cDNAs with 2 pairs of nested primers designed referring to a previous report (Hayama et al., 2007). The reactions were carried out on a thermal cycler,

PC320 (Astec Co. Ltd., Fukuoka, Japan) or i Cycler (Bio-Rad Laboratories, Hercules, CA, U.S.A.), with 20 + 20 cycles; the annealing temperature was 62 ℃ and the extension time was 20 seconds. The products of *PnFT1* and *PnFT2* amplification were separated on an agarose gel with *PnACT4* (which encodes actin) as a loading control. The images on the gel were visualized using EDAS 290 (Invitrogen Corporation, Carlsbad, CA, U.S.A.) or Bio-Pyramid (MeCan Imaging Inc., Saitama, Japan).

Results

Induction of flowering by overexpression of *PnFT2*

A. thaliana transformed with *35S::PnFT2* was induced early flowering as was the plant transformed with *35S::PnFT1* (Fig. 11, Table 9). Some *P. nil* embryoids transformed with *35S::PnFT2* formed flower on the EMG medium without the process of regeneration of vegetative shoot (Fig. 12, left). Some of the shoots regenerated on the rooting medium also flowered (Fig. 12, right). The genomic DNA was isolated from the regenerated shoots of *P. nil* and the transgene insertion was detected by the genomic PCR (Fig. 13). The regenerated T0 plants were further grown and the T1 seeds were obtained.

The T1 plants obtained from the *P. nil* plants transformed with *PnFT1* or *PnFT2* were grown under the non-inductive LD and non-stress conditions. The plants of any lines did not flower (Table 10).

Inhibition of flowering by suppression of *PnFT* expression

PnFT-RNAi was introduced to *P. nil* to suppress the expression of each *PnFT*. The transgene insertion in the transgenic T0 plants was detected by

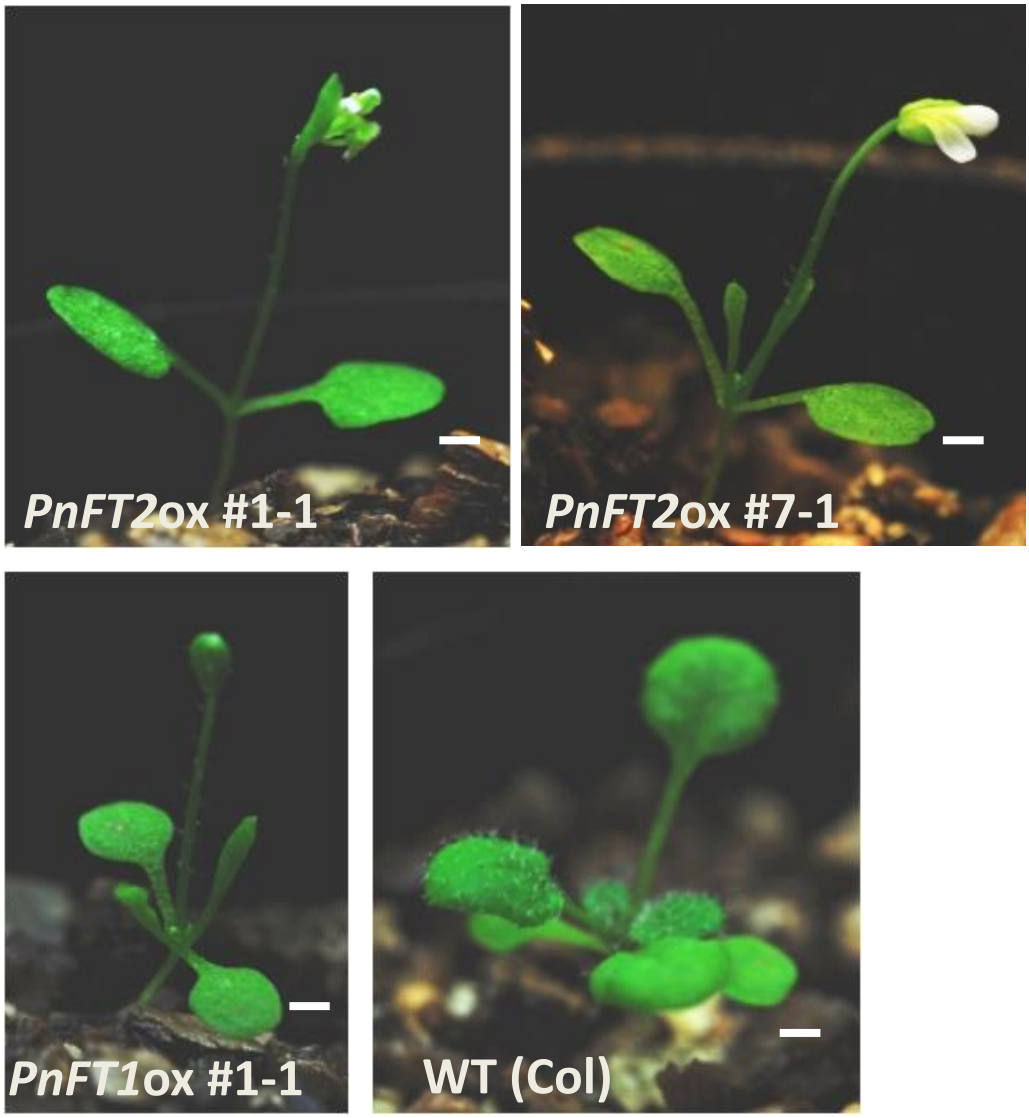

Fig. 11. Early flowering in the T1 plants of *Arabidopsis thaliana* ecotype Columbia transformed with *35S::PnFT*. The transgenic *A. thaliana* (*PnFT1* ox and *PnFT2* ox) and wild type (WT) as control were grown under continuous light. Photograph was taken 20 days after the seed sowing. Scale bar is 1mm.

Table 9. Flowering response of the T1 plants of *Arabidopsis thaliana* ecotype Columbia transformed with *35S::PnFT*. The transgenic *A. thaliana* (*PnFT* ox) and wild type as control were grown under continuous light until flowered. Time from the seed sowing to the appearance of the first flower was observed and is shown as flowering time in days from sowing to flowering. Number of the rosette leaves was scored after flowering was recognized and is shown as rosette leaf number. Values followed by the different superscript letters differ significantly at the 5% level, according to a *t*-test.

	Rosette leaf number	Flowering time (days from sowing to flowering)	Number of plants
WT (Columbia)	17±0.51[a]	41±1.1[a]	20
PnFT1 ox #1-1	3.0±0.16[b]	20±0.25[b]	27
PnFT2 ox #1-1	1.8±0.11[d]	16±0.33[c]	25
PnFT2 ox #7-1	2.1±0.069[c]	17±0.51[c]	20

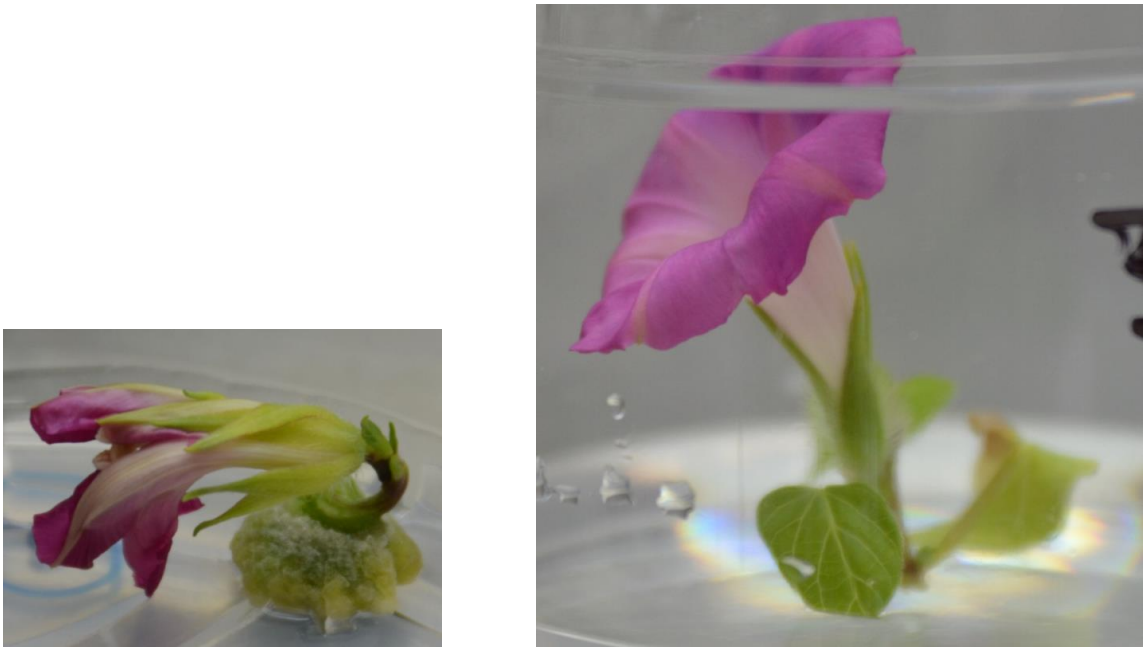

Fig. 12. Early flowering in *Pharbitis nil* transformed with *35S::PnFT2*. Flower was formed on the embryoid grown on the embryoid maturation and germination medium (left) and the regenerated shoot grown on the rooting medium (right). They had been grown at 25 ℃ under long-day conditions.

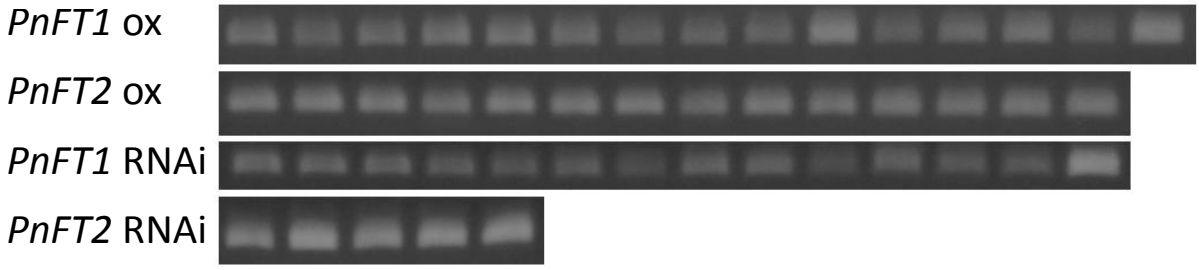

Fig. 13. Genomic PCR of the transgenes in the transgenic *Pharbitis nil* plants. Genome DNA was isolated individually from 4 to 14 plants which were transformed with *35S::PnFT* (*PnFT* ox) or *PnFT*-RNAi (*PnFT* RNAi), and PCR was performed with primers each of which anneals to *35S* promoter and *PnFT*, respectively. The rightmost band represents each construct used for the transformation and loaded as control.

Table 10. Flowering response of the T1 plants of *Pharbitis nil* cv. Violet transformed with *35S::PnFT*. The transgenic *P. nil* (*PnFT* ox) and wild type plants were grown in nutrient solution under long-day conditions for 2weeks. Values followed by the different superscript letters differ significantly at the 5% level, according to a *t*-test.

	% flowering	Floral buds /plant	Nodes /plant	Stem length (mm)
WT (Violet)	0	0±0	11±0.36^{ab}	232±20.7^{a}
PnFT1 ox #36	0	0±0	11±0.54^{b}	126±15.8^{c}
PnFT1 ox #55	0	0±0	11±0.60^{b}	152±23.2^{bc}
PnFT1 ox #93	0	0±0	11±0.44^{b}	168±23.9^{bc}
PnFT2 ox #8	0	0±0	11±0.47^{b}	153±22.5^{bc}
PnFT2 ox #23	0	0±0	12±0.41^{a}	255±21.6^{a}
PnFT2 ox #54	0	0±0	12±0.53^{ab}	196±21.9^{ab}

the genomic PCR (Fig. 13). The regenerated T0 plants were further grown and the T1 seeds were obtained.

The T1 plants obtained from the *P. nil* plants transformed with *PnFT1*-RNAi or *PnFT2*-RNAi were given the SD treatment or the poor-nutrition stress. The treated plants in each line were individually examined whether *PnFT*-RNAi and *PnFT* expressed, and determined the flowering response.

The expression of *PnFT*-RNAi was detected in 5 out of 6 lines, and the rate of the plants in which the expression was detected in the whole plants in each line was close to 3/4 (Tables 11 and 12).

The plants which expressed *PnFT*-RNAi was selected, and the number of the plants which expressed each *PnFT* was determined among those plants expressed *PnFT*-RNAi (Tables 11 and 12). In the experiment where the plants were treated with SD, the *PnFT1* expression was not detected in all the plants expressed *PnFT1*-RNAi, but the *PnFT2* expression was detected in all these *PnFT1*-RNAi expressed plants (Table 11). The *PnFT2* expression was not detected in all the *PnFT2*-RNAi expressed plants, but the *PnFT1* expression was detected in almost all these *PnFT2*-RNAi expressed plants.

In the experiment with the poor-nutrition stress treatment, the *PnFT1* expression was not detected in all the *PnFT1*-RNAi expressed plants, but the *PnFT2* expression was detected in some of these *PnFT1*-RNAi expressed plants (Table 12). The *PnFT2* expression was not detected in all the *PnFT2*-RNAi expressed plants, but the *PnFT1* expression was detected in a few of these *PnFT2*-RNAi expressed plants.

The plants which expressed *PnFT*-RNAi were classified into those

Table 11. The expression of *PnFT* and flowering response in *Pharbitis nil* transformed with *PnFT*-RNAi and given a short-day treatment. The T1 plants of the *PnFT*-RNAi transgenic *P. nil* cv. Violet and wild type plants as control were given a short-day (SD) treatment. The cotyledons were collected after the SD treatment for RT-PCR analysis of *PnFT*-RNAi and *PnFT*. Flowering response was scored after the additional cultivation under the long-day conditions for 2 weeks. Number of the plants which expressed *PnFT*-RNAi in all the plants which had been transformed with *PnFT*-RNAi is shown as RNAi expression rate. Number of the plants which expressed *PnFT* in all the plants expressed *PnFT*-RNAi is shown as *PnFT* expression rate. The plants which expressed *PnFT*-RNAi were classified into those expressed neither of *PnFT*s (*ft1 ft2*), only *PnFT2* (*ft1 FT2*), only *PnFT1* (*FT1 ft2*) and both *PnFT*s (*FT1 FT2*), and number of the plants which flowered in all the plants in each class is shown as flowering rate.

	RNAi expression rate	*PnFT* expression rate		Flowering rate			
		PnFT1	*PnFT2*	*ft1 ft2*	*ft1 FT2*	*FT1 ft2*	*FT1 FT2*
WT				0/0	0/0	0/0	10/18
PnFT1-RNAi#24	6/12	0/6	6/6	0/0	0/6	0/0	0/0
PnFT1-RNAi#48	9/11	0/9	9/9	0/0	0/9	0/0	00
PnFT1-RNAi#70	0/18	-	-	-	-	-	-
Total		0/15	15/15	0/0	0/15	0/0	0/0
PnFT2-RNAi#13	13/16	13/13	0/13	0/0	0/0	7/13	0/0
PnFT2-RNAi#47	11/17	11/11	0/11	0/0	0/0	4/11	0/0
PnFT2-RNAi#48	10/12	8/10	010	0/2	0/0	0/8	0/0
Total		32/34	0/34	0/2	0/0	11/32	0/0

Table 12. The expression of *PnFT* and flowering response in *Pharbitis nil* transformed with *PnFT*-RNAi and given poor nutrition stress. The T1 plants of the *PnFT*-RNAi transgenic *P. nil* cv. Violet and wild type plants as control were grown in 1/100-strength nutrient solution under long-day (LD) conditions for 3 weeks. The true leaves were collected after the poor-nutrition stress treatment for RT-PCR analysis of *PnFT*-RNAi and *PnFT*. Flowering response was scored after the additional cultivation under the LD non-stress conditions for 2 weeks. Number of the plants which expressed *PnFT*-RNAi in all the plants which had been transformed with *PnFT*-RNAi is shown as RNAi expression rate. Number of the plants which expressed *PnFT* in all the plants expressed *PnFT*-RNAi is shown as *PnFT* expression rate. The plants which expressed *PnFT*-RNAi were classified into those expressed neither of *PnFT*s (*ft1 ft2*), only *PnFT2* (*ft1 FT2*), only *PnFT1* (*FT1 ft2*) and both *PnFT*s (*FT1 FT2*), and number of the plants which flowered in all the plants in each class is shown as flowering rate.

	RNAi expression rate	*PnFT* expression rate		Flowering rate			
		PnFT1	*PnFT2*	*ft1 ft2*	*ft1 FT2*	*FT1 ft2*	*FT1 FT2*
WT				3/3	3/4	1/2	3/3
PnFT1-RNAi#24	10/15	0/10	4/10	3/6	3/4	0/0	0/0
PnFT1-RNAi#48	9/13	0/9	2/9	6/7	1/2	0/0	0/0
PnFT1-RNAi#70	0/15	-	-	-	-	-	-
Total		0/19	6/19	9/13	4/6	0/0	0/0
PnFT2-RNAi#13	15/20	2/15	0/15	6/13	0/0	1/2	0/0
PnFT2-RNAi#47	13/16	1/13	0/13	7/12	0/0	1/1	0/0
PnFT2-RNAi#48	9/13	1/9	0/9	6/8	0/0	1/1	0/0
Total		4/37	0/37	19/33	0/0	3/4	0/0

expressed neither of *PnFT* (*ft1 ft2*), only *PnFT2* (*ft1 FT2*), only *PnFT1* (*FT1 ft2*) and both *PnFT*s (*FT1 FT2*), and number of the plants which flowered in all the plants in each class was determined (Tables 11 and 12). In the experiment with the SD treatment, all of the WT plants expressed both *PnFT*s (*FT1 FT2*), and many of those plants were induced flowering. All of the *PnFT1*-RNAi expressed plants expressed *PnFT2* (*ft1 FT2*), but flowering was not induced in those plants. Almost all of the *PnFT2*-RNAi expressed plants expressed *PnFT1* (*FT1 ft2*), and some of them were induced flowering.

In the experiment with the poor-nutrition stress treatment, the numbers of the plants which expressed both *PnFT*s (*FT1 FT2*), one of the two *PnFT*s (*FT1 ft2*, *ft1 FT2*) and neither *PnFT*s (*ft1 ft2*) were almost the same in WT plants (Table 12). The plants in each of these classes were induced flowering in the same extent. In the plants expressed *PnFT1*-RNAi, the plants which expressed *PnFT2* and those which did not express *PnFT2* were induced flowering in the same extent. In the plants expressed *PnFT2*-RNAi, the plants which expressed *PnFT1* and those which did not express *PnFT1* were induced flowering in the same extent.

Discussion

The transformation of *A. thaliana* and *P. nil* with *35S::PnFT2* induced early flowering (Figs. 11 and 12, Table 9). These results indicate that *PnFT2* has the activity to induce flowering. However, the T1 plants of the transgenic *P. nil* were not induced flowering under the non-inductive LD and non-stress conditions (Table 10). It is possible that the introduced *PnFT2* was silenced when the generation changed.

The expression of *PnFT*-RNAi was not detected in one out of 6 lines of the *PnFT*-RNAi plants (Tables 11 and 12); the T0 plant of this line #70 may have not been transformed. In the other 5 lines, *PnFT*-RNAi suppressed the expression of its target *PnFT* completely. The expression of *PnFT* which was not targeted by *PnFT*-RNAi was not suppressed in almost all the plants in the experiment with the SD treatment. This result was not reproduced in the experiment with the poor-nutrition stress treatment. That is, only a few of the *PnFT*-RNAi plants expressed another *PnFT*. However, this does not necessarily mean that the RNAi of one *PnFT* suppressed the expression of the other *PnFT*. The expression of *PnFT* induced by the poor-nutrition stress is weaker than that by the SD treatment (Figs. 1 and 3). Therefore, the absence of the *PnFT* expression is possibly because the stress was not enough strong to induce the expression.

All the *PnFT1*-RNAi plants expressed *PnFT2* by the SD treatment (*ft1 FT2*), but were not induced flowering (Table 11). On the other hand, the *PnFT2*-RNAi plants which expressed *PnFT1* (*FT1 ft2*) were induced flowering. These results suggest that the gene which play a main role in flowering by SD treatment is *PnFT1*. This is consistent with the conclusion obtained in the Chapter I.

In the experiment with the poor-nutrition stress treatment, the *PnFT1*-RNAi expressed plants which expressed *PnFT2* (*ft1 FT2*) flowered. However, even the plants which expressed neither *PnFT*s (*ft1 ft2*) were induced flowering. This is not consistent with the previous observations. The involvement of *PnFT2* in stress-induced flowering was not clarified by this RNAi experiment. The *FT* homolog, *BvFT2*, of vernalization-requiring sugar

beet (*Beta vulgaris*) is essential for flowering, but *BvFT2*-RNAi plants responded to vernalization and bolted (Pin et al., 2010). This was explained as additional mechanisms are acting to promote bolting in parallel to *BvFT2*. Similarly, it is possible that some additional mechanisms act to promote flowering, although *PnFT2* plays an inductive role in stress-induced flowering in *P. nil*.

Chapter III

Interaction between *PnFT2* and salicylic acid

Introduction

Stress induces phenyalanine ammonia-lyase (PAL) activity, resulting in the accumulation of salicylic acid (SA) (Dixon and Paiva, 1995; Scott et al., 2004). The PAL inhibitor aminooxyacetic acid (AOA) suppressed the stress-induced flowering in *P. nil*, and this inhibition was overcome by SA (Wada et al., 2010b). Poor-nutrition stress-induced flowering in *L. paucicostata* was inhibited by AOA and another PAL inhibitor L-2-aminooxy-3-phenylpropionic acid, and a higher amount of SA was detected in the plants that flowered under the poor nutrition conditions than in the vegetative plants cultured under normal nutrition conditions (Shimakawa et al., 2012). Exogenously applied SA can induce flowering in *L. paucicostata* and other species belonging to the Lemnaceae under non-inductive photoperiodic conditions (Cleland and Ajami, 1974; Kandeler, 1985). UV-C light stress promoted flowering in *A. thaliana*, and the flowering response was weaker in SA-deficient *NahG* transgenic lines than in the wild type, suggesting that SA is involved in this flowering (Martínez et al., 2004). SA application induced the expression of *A. thaliana FT* and sunflower *HAFT*, an ortholog of *FT*, indicating that *FT* and SA may interact to regulate flowering (Martínez et al., 2004; Dezar et al.,2011). These results lead to hypothesize that stress-induced flowering is regulated by *PnFT* whose expression is induced by SA. Accordingly, the influences of a PAL inhibitor and SA on the expression of *PnFT* were examined in *P. nil* grown under stress and non-stress conditions to test whether SA and *PnFT* interact to regulate flowering.

Materials and methods

Plant materials and growth conditions

The SD plant Japanese morning glory [*Pharbitis nil* (L.) Chois., synonym *Ipomoea nil* (L.) Roth] cv. Violet was used. Violet seeds were originally provided by Marutane Co. (Kyoto, Japan). The seeds were treated with concentrated H_2SO_4 for 25 to 40 minutes, washed with running tap water for 1 hour, and then soaked in tap water overnight. The swollen seeds were placed on moist filter paper in a Petri dish 90 mm in diameter and were germinated at 25 ℃ under 16-hour light and 8-hour dark LD conditions for 1 day. The germinated seeds were planted on 0.6% plain agar medium in a plastic box (240 mm × 330 mm × 90 mm in depth) and grown at 25 ℃ under the LD conditions for 5 days. The seedlings were then transferred to glass tubes (15 mm in diameter × 150 mm high) containing a mineral nutrient solution (Kondo et al., 2006) and were grown under the same conditions mentioned above. Five-day-old seedlings were grown in 1/100-strength mineral nutrient solution instead of full-strength mineral nutrient solution as the poor-nutrition stress treatment. After the stress treatment, the seedlings were transferred to the normal growth conditions and grown for 2 weeks until the flowering response was scored. White light (55-90 μmol m^{-2} s^{-1}) was provided by fluorescent lamps (FL20SW or FL40SSW/37, Toshiba Corporation, Tokyo, Japan). For SD treatment, 5-day-old seedlings were given a single 16-hour dark treatment. After the SD treatment, the seedlings were transferred to the LD conditions and grown for 2 weeks until the flowering response was scored.

Treatment with chemicals

AOA and/or SA (both were from Wako Pure Chemicals Industries, Osaka, Japan) were dissolved in the culture solution, and 5-day-old seedlings were grown in the solution with chemicals for 3 weeks. After the treatment, the seedlings were transferred to the nutrient solution without chemicals and grown for 2 weeks until the flowering response was scored.

Scoring of the flowering response

All of the plant nodes were dissected under a binocular microscope to determine whether flower buds or vegetative buds formed. The percentage of plants with at least one flower bud out of all the plants in a treatment (% flowering) and the number of flower buds per plant were determined. The number of nodes, that is the total number of flower buds and vegetative buds per plant, was used as indicator of vegetative growth along with the average length of the main stem. Twenty to 30 plants were used for each treatment. Each experiment was repeated at least three times. The means with standard errors of the most representative experiment are shown in each figure.

Gene expression analysis using RT-PCR

The cotyledons and true leaves of plants were collected 16 hours after the start of the dark period (8 hours after the end of the dark period of 16-hour light LD conditions, or the end of the dark period of 8-hour light SD conditions). The harvested leaves were frozen in liquid nitrogen and stored at -80 ℃ prior to analysis. The total RNA was isolated from the tissues using

the Plant RNA Purification Reagent (Invitrogen Corporation, Carlsbad, CA, U.S.A.) and Fruit-mate (Takara Biotechnology Co. Ltd., Shiga, Japan). The isolated RNA samples were treated by DNase I (Sigma-Aldrich Inc., Saint Louis, MO, U.S.A.) to digest contaminated genomic DNA, and then cDNAs were synthesized from each RNA sample using ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan). The gene amplification was performed by nested PCR using KOD -Plus- Neo (Toyobo Co. Ltd., Osaka, Japan). *PnFT1* and *PnFT2* were amplified from the cDNAs with 2 pairs of nested primers designed referring to a previous report (Hayama et al., 2007). The reactions were carried out on a thermal cycler, PC320 (Astec Co. Ltd., Fukuoka, Japan) or i Cycler (Bio-Rad Laboratories, Hercules, CA, U.S.A.), with 20 + 20 cycles; the annealing temperature was 62 ℃ and the extension time was 20 seconds. The products of *PnFT1* and *PnFT2* amplification were separated on an agarose gel with *PnACT4* (which encodes actin) as a loading control. The images on the gel were visualized using EDAS 290 (Invitrogen Corporation, Carlsbad, CA, U.S.A.) or Bio-Pyramid (MeCan Imaging Inc., Saitama, Japan). The expression of each gene was normalized to that of *PnACT4* and shown as a relative value. The nucleotide sequences of the primers used are shown in Table 1.

Results

Induction of the *PnFT2* expression by SA

The involvement of SA in the stress-induced flowering and the regulation of *PnFT2* expression was analyzed in *P. nil* cv. Violet. Violet plants were grown in 1/100-strength nutrient solution supplemented with AOA.

The poor-nutrition stress treatment induced flowering, and AOA inhibited this flowering at 3×10^{-5} M and higher (Fig. 14A). The stress treatment induced the expression of *PnFT2*, and this expression was nullified by 10^{-4} M AOA (Fig. 14B, C). Next, Violet plants were grown in 1/100-strength nutrient solution supplemented with 3×10^{-5} M AOA and SA of different concentrations. AOA inhibited the stress-induced flowering, and SA overcame this inhibition at concentrations of 10^{-5} M and higher(Fig. 14D). Although the induction of *PnFT2* expression by stress treatment was not inhibited by AOA in this experiment, its expression was enhanced by SA (Fig. 14E, F).

Violet plants were then grown in normal nutrient solution supplemented with SA. SA did not induce flowering under such non-stress conditions (Fig. 15A). The expression of *PnFT2* in the SA-treated plants was much lower than that in the SD-treated plants and almost the same as in the LD-grown plants (Fig. 15B, C). Next, Violet plants were treated with SA while grown in 1/100-strength nutrient solution. SA did not enhance the flowering response induced by the poor-nutrition stress treatment (Fig. 15D). SA enhanced the *PnFT2* expression induced by the poor-nutrition stress treatment in an almost concentration-dependent manner (Fig. 15E, F).

Discussion

Stress-induced flowering was inhibited by the PAL inhibitor AOA, and this inhibition was overcome by SA (Fig. 14A and D), as reported previously (Wada et al., 2010b). The poor-nutrition stress treatment induced *PnFT2* expression, and AOA suppressed this enhancement (Fig. 14B and C). The

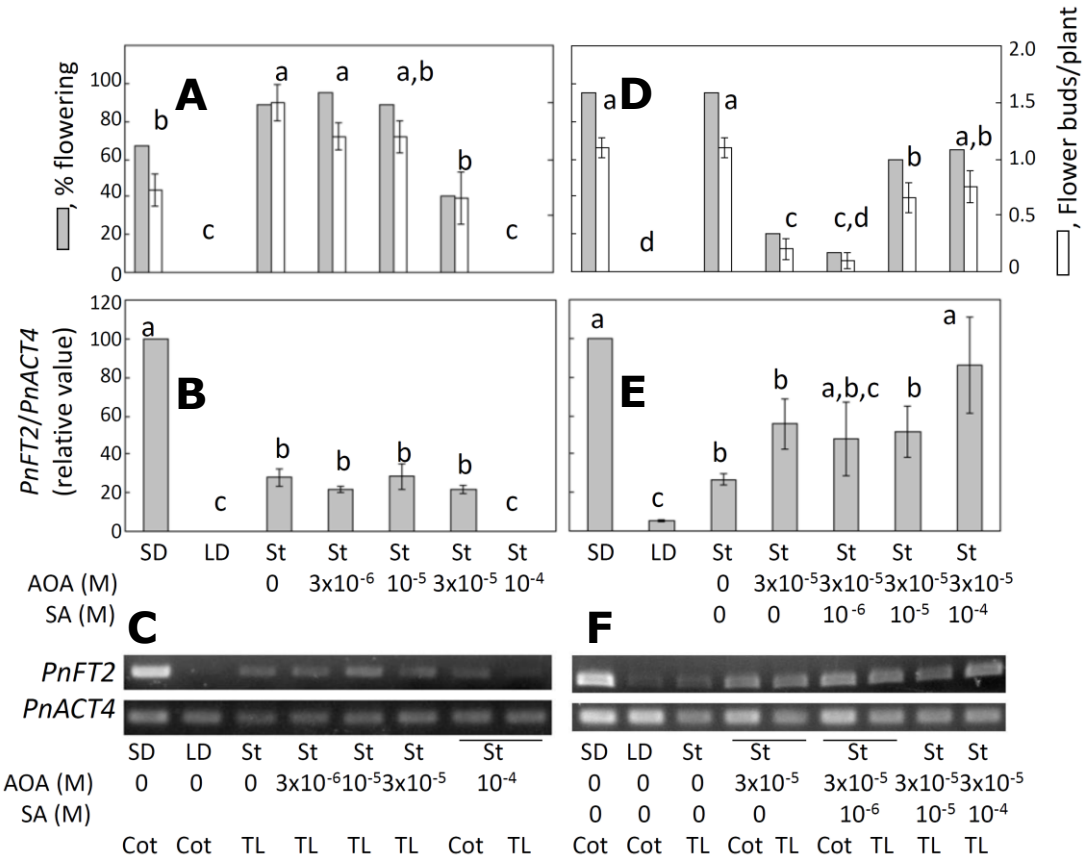

Fig. 14. Effects of aminooxyacetic acid (AOA) and salicylic acid (SA) on flowering and *PnFT2* expression under poor-nutrition stress in *Pharbitis nil* cv. Violet. Plants were grown in 1/100-strength nutrient solution (St) supplemented with AOA (A–C) or AOA plus SA (D–F) for 3 weeks. Control plants were grown in nutrient solution, and given a short-day treatment (SD) or not (LD). (A and D) After the treatment, the flowering response was scored. (C and F) The cotyledons (Cot) and expanded true leaves (TL) were collected for RT-PCR analysis of *PnFT2*. (B and E) The mRNA abundance of *PnFT2* was normalized to that of *PnACT4*, and shown as a relative value. The RT-PCR procedure was repeated three times and the means with standard errors (B and E) and a representative gel image (C and F) are shown. Different letters above the columns indicate statistically significant differences at the 5% level in a *t*-test.

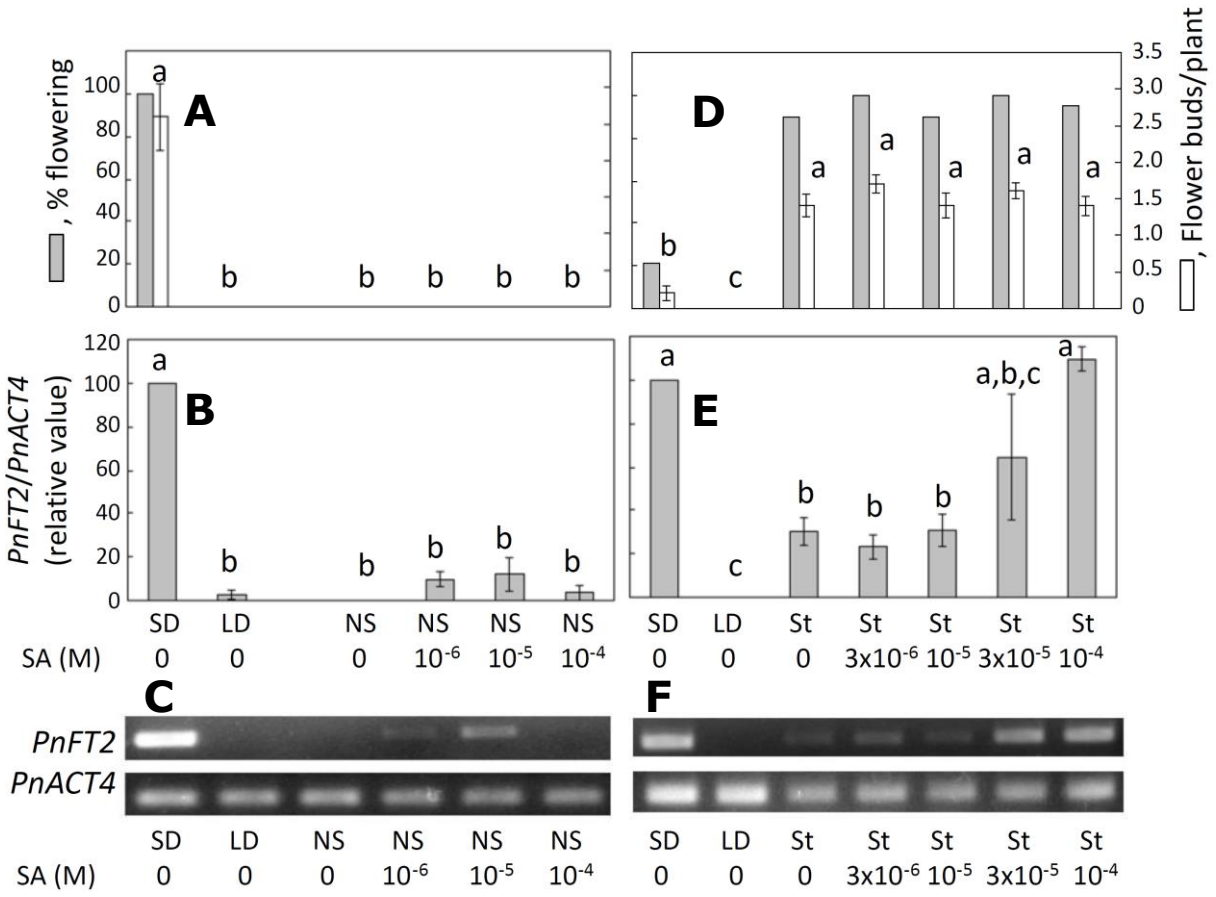

Fig. 15. Effect of salicylic acid (SA) on flowering and *PnFT2* expression in *Pharbitis nil* cv. Violet. Plants were grown in nutrient solution (A–C; NS) or 1/100-strength nutrient solution (D–F; St) supplemented with SA for 3 weeks. Control plants were grown in nutrient solution, and given a short-day treatment (SD) or not (LD). (A and D) Following the treatment, the flowering response was scored. (C and F) The cotyledons of the SD and LD plants and the expanded true leaves of the other plants were collected for RT-PCR analysis of *PnFT2*. (B and E) The mRNA abundance of *PnFT2* was normalized to that of *PnACT4*, and shown as a relative value. The RT-PCR procedure was repeated three times and the means with standard errors (B and E) and a representative gel image (C and F) are shown. Different letters above the columns indicate statistically significant differences at the 5% level in a *t*-test.

reduction in *PnFT2* expression level caused by AOA was reversed by SA (Fig. 14E and F). SA enhanced the *PnFT2* expression when applied under the poor-nutrition stress conditions (Fig. 15E and F). These results suggest that SA induces *PnFT2* expression to induce flowering. This is consistent with the results in *A. thaliana* and sunflower, in which SA induced the expression of *FT* (Martínez et al., 2004; Dezar et al., 2011). Here, however, SA did not induce *PnFT2* expression or flowering under the non-stress conditions (Fig. 15A–C), suggesting that SA alone may not be sufficient to induce *PnFT2* expression. Stress may induce the production of SA and other unknown factor(s), which may work in combination to induce *PnFT2* expression and flowering in *P. nil*.

It has been reported that exogenous SA does not promote flowering not only under non-stress conditions but also under stress conditions (Wada et al., 2010b). It is possible that the flowering response saturated under the stress conditions and therefore exogenous SA could not enhance the flowering response anymore. In fact, SA promoted flowering if applied under weak stress conditions by growing the plants in 1/30-strength nutrient solution instead of usual 1/100-strength nutrient solution (Wada et al., submitted). It should be supposed that some factors other than SA may be generated under the stress conditions and affect the flowering response together with SA. A possible candidate is IAA, an auxin. AOA reportedly inhibits biosynthesis of IAA (Soeno et al., 2010), and IAA regulates the initiation of flower primordia by inducing the expression of *LFY* (Yamaguchi et al., 2013). AOA inhibits also 1-aminocyclopropane-1-carboxylate (ACC) synthase that synthesize ACC from *S*-adenosylmethionine (SAM). SAM is aminopropyl group donor for polyamines. Polyamines induce flowering of *P.*

nil (Wada et al., 1994). SAM is also methyl group donor for DNA. DNA demethylation induces flowering in *P. nil* (Iwase et al., 2010). Those factors influenced by AOA could affect flowering. ACC is the precursor of ethylene, and therefore the inhibition of ACC synthase by AOA reduces ethylene level. Ethylene inhibits flowering in *P. nil* (Suge, 1972). AOA may affect flowering promotively through the inhibition of ethylene synthesis. Such a positive effect of AOA should be also considered because the regulatory mechanism of flowering seems too complex to be explained by a single inductive factor.

General Discussion

The present study revealed that *PnFT2* is involved in the regulation of stress-induced flowering of *P. nil*. The other *FT* homolog, *PnFT1* is mainly involved in the regulation of photoperiodic flowering. The gene downstream of *PnFT*s, *PnAP1* is involved in the regulation of both stress-induced and photoperiodic flowering. Thus, stress-induced and photoperiodic flowering are under the control of the basically same gene regulatory system, but partly different system may have been evolved for each flowering.

SA is required for stress-induced flowering, but exogenous SA did not induce the *PnFT2* expression under non-stress conditions. It is possible that the expression of *PnFT2* is regulated by SA and some unknown factor(s).

The results mentioned above are summarized in Fig. 16. *PnFT1* and *PnFT2* are both involved in flowering positively. *PnFT2* is involved in stress-induced flowering. Both *PnFT*s are involved in photoperiodic flowering, but *PnFT1* plays a main role. *PnFT2* can not induce photoperiodic flowering by itself. These *PnFT*s express in leaves, and may induce *PnAP1* in shoot apices to induce flowering.

This model is too simple to compare with the other model describing the gene regulation cascade in *A. thaliana* because the knowledge of gene regulation of stress-induced flowering in *P. nil* is still limited. However, it may be concluded that the regulation by *FT* and *AP1* is the main framework to regulate flowering which is common between *P. nil* and *A. thaliana*. On the other hand, the present study suggests some possible differences in the gene regulation cascade between *P. nil* and *A. thaliana*. Namely, the involvement of some important flowering genes in *A. thaliana* such as *SOC1*,

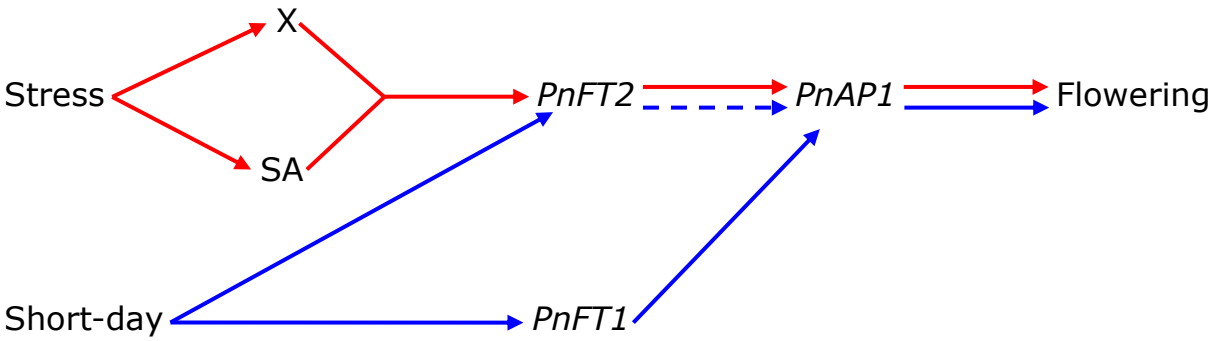

Fig. 16 A model showing the regulation of flowering in *Pharbitis nil*. The pathway of stress-induced flowering is indicated with red lines, and that of photoperiodic flowering is blue lines. Each line does not mean the direct link, but it may contain some unknown steps. The broken line indicates that *PnFT2* plays a minor role in photoperiodic flowering. "X" indicates the unknown stress factor that may induce the expression of *PnFT2* together with salicylic acid (SA).

LFY and *FD* was not found in *P. nil*. More detailed study is required to argue the differences between stress-induced and photoperiodic flowering, and between species. Especially, the homolog of *FD* should be studied further. FD protein is necessary for FT protein to act as the transcription factor to activate the promoter of *AP1* in *A. thaliana*, because the FT protein itself does not bind to DNA directly, and needs to form a complex with FD protein. Therefore, *FD* homolog or some proteins which substitute for FD must exist in *P. nil*.

The regulation of *FT* expression is possibly different between *P. nil* and *A. thaliana*. The involvement of CO which acts as the transcription factor to activate *FT* in *A. thaliana* was not found in *P. nil*. Instead of it, it was shown that SA may induce the expression of *PnFT2* in *P. nil*. However, SA alone does not induce the *PnFT2* expression and the involvements of some other factor(s) are supposed. It is important to search this unknown factor(s) in future study.

Stress-induced flowering is considered as universal and important strategy for plants to survive (Wada and Takeno, 2010; Takeno, 2012), and therefore this may be conserved widely in plant species. Recently the data on the gene regulation of stress-induced flowering in *A. thaliana* are accumulating (Segarra et al., 2010; Riboni et al., 2013). Therefore, a question whether the stress-induced flowering in different plant species is under the common gene regulatory system could be solved in near future.

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Publication

1. Yamada M, Takeno K. (2014) Stress and salicylic acid induce the expression of *PnFT2* in the regulation of the stress-induced flowering of *Pharbitis nil.* Journal of Plant Physiology 171: 205-212.

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1. 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*. Journal of Plant Physiology 167: 447-452.
2. Hasegawa H, Yamada M, Iwase Y, Wada KC, Takeno K. (2010) Reduction of critical dark length for flower induction during aging in the short-day plant *Pharbitis nil* var. Kidachi. Sexual Plant Reproduction 23: 291-300.
3. Wada KC, Yamada M, Takeno K. (2013) Stress-induced flowering in Pharbitis - a review. American Journal of Plant Science 4: 74-79.