Regulatory mechanism of stress-induced flowering
（ストレス応答花成の制御機構）

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Abbreviations

ACC: 1-aminocyclopropane-1-carboxylic acid

AOA: aminooxyacetic acid

AOPP: L-2-aminooxy-3-phenylpropionic acid

d_4-SA: deuterium-labeled 2-hydroxybenzoic-3,4,5,6-d_4 acid

FT: *FLOWERING LOCUS T*

HPLC: high performance liquid chromatograph

IAA: indole-3-acetic acid

LC-MS: liquid chromatography-mass spectrometry

LD: long days

PAL: phenylalanine ammonia-lyase

PnPAL: *PAL* gene of *P. nil*

RT-PCR: reverse transcription-polymerase chain reaction

SA: salicylic acid

SD: short days

UV: ultraviolet

要旨

アサガオ、*Pharbitis nil* (シノニム *Ipomoea nil*)とシソ、*Perilla frutescens* var. *crispa* は絶対的短日植物であるが、ストレスを負荷されると長日条件下でも花成を誘導された。このようなストレス応答花成の基礎的な性質と制御機構を明らかにすることを本研究の目的とした。

アサガオ、品種ムラサキおよびテンダンを長日条件下、希釈した培養液または栄養塩を含まない培地で 20 日間培養した。ムラサキは花成を誘導され、栄養生長を抑制された。栄養生長を抑制されたことから、貧栄養条件での培養はストレスとして機能し、貧栄養ストレスは花成を誘導することがわかった。一方、品種テンダンは栄養生長を抑制されたが花成は誘導されなかった。貧栄養ストレスで花成を誘導されたムラサキは正常に開花して結実し、形成された種子は発芽し、正常に生育した。葉を切除したムラサキをムラサキまたはテンダンに接木して貧栄養ストレスを負荷したところ、接穂ムラサキは花成を誘導された。このことから、ストレス応答花成には輸送可能な花成刺激が関与することが明らかになった。テンダンをムラサキに接木したときには接穂テンダンは花成を誘導されなかった。これらのことは、テンダンは花成刺激を生成するものの、自身はこれに応答しないことを示唆する。貧栄養ストレス応答花成はフェニルアラニンアンモニアリアーゼ(PAL)阻害剤であるアミノオキシ酢酸で阻害され、この阻害はサリチル酸(SA)でほぼ完全に回復した。

アカジソは弱光(30 μmol m^{-2} s^{-1})下で培養すると長日条件下でも花成を誘導された。弱光下では通常光(120 μmol m^{-2} s^{-1})下で培養した場合と比較して栄養生長が抑制されたことから、弱光はストレスとして機能したと考えられる。

アカジソは４週間の弱光処理で花成を誘導された。子葉展開直後から弱光に応答し、花成反応は齢とともに低下した。弱光で花成を誘導されたアカジソの葉は緑色になった。これは、葉のアントシアニン含量の低下によるものであった。葉のアントシアニン含量と花成率との間には負の相関があった。PAL 阻害剤であるL-2-アミノオキシ-3-フェニルプロピオン酸は非誘導条件下で花成を誘導せず、弱光下での花成誘導を阻害した。この阻害は同時に処理した SA で部分的に回復した。これらのことから、シソの弱光花成には、アサガオと同様に、PALで生合成を制御されるSAが関与することが示唆された。

アサガオとアカジソのストレス応答花成には共通の制御機構が働いていると考えられたので、アサガオに注目して、*PAL* 遺伝子発現、PAL 酵素活性、内生 SA 量を調べた。アサガオ品種ムラサキを 1/100 希釈した栄養塩培養液で培養して花成を誘導した。貧栄養ストレス処理終了時に子葉を採取して、RNA、蛋白質、SA を抽出し、逆転写ポリメラーゼ連鎖反応によって *PAL* 発現解析を行い、PAL 酵素反応産物である *t*-桂皮酸を定量することで PAL 酵素活性を測定し、高速液体クロマトグラフィ-質量分析によって SA を定量した。貧栄養ストレスで花成を誘導されたとき、子葉の *PAL* 発現、PAL 活性、SA 含量はどれも上昇した。これらのことから、アサガオの貧栄養ストレス応答花成はPALで生合成を制御される SA によって引き起こされることが示唆された。非ストレス条件下のアサガオに SA を処理したところ、花成は誘導されなかった。これらの結果から、SA はストレス応答花成の必要条件ではあるが十分条件ではないと考えられる。ストレスは SA 生合成とともに他の必須条件を誘導するのであろう。

Abstract

The obligatory short-day plants *Pharbitis nil* (synonym *Ipomoea nil*) and *Perilla frutescens* var. *crispa* were found to be induced to flower when stressed under non-inductive long-day conditions. The characteristics and regulatory mechanism of such stress-induced flowering were studied.

P. nil cultivar Violet was grown in a diluted nutrient solution or water without nutrition under long-day conditions for 20 days. Violet plants were induced to flower and vegetative growth was inhibited, showing that Violet plants were stressed and induced to flower. *P. nil* cultivar Tendan was not induced to flower under these conditions, although vegetative growth was inhibited. The Violet plants induced to flower by poor-nutrition stress produced fertile seeds and the progeny developed normally. Defoliated Violet scions grafted onto the rootstocks of Violet or Tendan were induced to flower under poor-nutrition stress conditions, but Tendan scions grafted onto the Violet rootstocks were not induced to flower. These results indicate that a transmissible flowering stimulus is involved in the induction of flowering by poor-nutrition stress. It was indicated that Tendan produces a transmissible flowering stimulus but does not respond to it. The poor-nutrition stress-induced flowering was inhibited by aminooxyacetic acid, a phenylalanine ammonia-lyase (PAL) inhibitor, and this inhibition was almost completely reversed by salicylic acid (SA).

P. frutescens var. *crispa* was induced to flower under long-day conditions when grown under low-intensity light (30 μmol m^{-2} s^{-1}). Plant size was smaller under low light intensity than under normal light intensity (120 μmol m^{-2} s^{-1}),

indicating that the low-intensity light acted as a stress factor. Low-intensity light treatment for 4 weeks induced 100% flowering. The plants responded to low-intensity light immediately after the cotyledons expanded, and the flowering response decreased with increasing plant age. The induced plants produced fertile seeds, and the progeny developed normally. The plants that flowered under low-intensity light had greener leaves. This greening was because of the decrease in anthocyanin content, and there was a negative correlation between the anthocyanin content and percent flowering. Treatment with L-2-aminooxy-3-phenylpropionic acid, an inhibitor of PAL, did not induce flowering under non-inductive light conditions and inhibited flowering under inductive low-intensity light conditions. The metabolic pathway regulated by PAL may be involved in the flowering induced by low-intensity light as in *P. nil*.

Cotyledons were collected from *P. nil* cultivar Violet which was induced to flower by culturing in 1/100-strength mineral nutrient solution. RNAs, proteins and SA were extracted from the cotyledons, and expression of *PAL* gene, enzyme activity of PAL and content of SA were analyzed by reverse transcription-polymerase chain reaction, quantification of *t*-cinnamic acid as the product of enzyme reaction and high performance liquid chromatography-mass spectrometry, respectively. All of the *PAL* expression, PAL activity and SA content in the cotyledons increased when flowering was induced by poor-nutrition stress. These results suggest that poor-nutrition stress-induced flowering in *P. nil* is induced by SA of which synthesis is promoted by PAL. However, exogenously applied SA did not induce flowering under non-stress conditions, suggesting that SA may be necessary but not sufficient to induce flowering. Stress may induce the production of not only SA but also other factors

necessary to induce flowering.

General introduction

Plants switch growth mode from vegetative growth to reproductive growth after the vegetative growth of a certain period, then bloom and produce the next generation. This switching from vegetative growth to reproductive growth is called flowering. Flowering is regulated by both endogenous and environmental factors. One endogenous factor is the autonomous pathway of flowering regulation in *Arabidopsis thaliana* (Simpson 2004). Day-neutral plants switch from vegetative to reproductive growth in response to endogenous signals after a certain period of time (McDaniel 1996). Environmental factors that regulate flowering include the duration of the day and night periods in photoperiodic flowering and temperature in vernalization (Thomas and Vince-Prue 1997). The flowering stimulus which is produced in leaves under adequate photoperiodic conditions, transferred to shoot apical meristem, and changes the shoot apical meristem to floral meristem is called florigen. Isolation and identification of such substance has been considered as an important research subject in plant physiology for a long time. Recently, the proteins derived from the floral pathway integrator gene of *A. thaliana*, *FLOWERING LOCUS T* (*FT*), and its homologs have been reported to be florigens in several species (Corbesier et al. 2007, Lin et al. 2007, Tamaki et al. 2007). In the vernalization that is flowering induced by exposure to low temperature of 0 to 5 °C for a few weeks, it was revealed that epigenetics such as DNA demethylation is involved in its regulatory mechanism (Michaels and Amasino 2000, 2001). On the other hand, the short-day (SD) plant *Pharbitis nil* (synonym *Ipomoea nil*) can be induced to flower under long days (LD) when grown under poor-nutrition, low-temperature

or high-intensity light conditions (Hirai et al. 1993, 1994, Shinozaki 1985, Shinozaki and Takimoto 1982, Shinozaki et al. 1982, 1988a, b, 1994, Swe et al. 1985). The flowering induced by these conditions is accompanied by an increase in phenylalanine ammonia-lyase (PAL) activity (Hirai et al. 1995). This fact suggests that the flowering induced by these conditions might be regulated by a common mechanism. Poor nutrition, low temperature and high-intensity light can be regarded as stress factors, and PAL activity increases under stress conditions (Dixon and Paiva 1995). Accordingly, we assumed that such LD flowering in *P. nil* might be induced by stress. Non-photoperiodic flowering has been sporadically reported in several plant species other than *P. nil.* However, such unusual flowering has not been studied systematically. We surveyed the flowering behavior reported in the literature, and found that most of the factors responsible for such flowering could be regarded as stress. These factors include nitrogen deficiency, high- or low-intensity light, high or low temperature, drought, root removal, girdling, mechanical stimulation and others. Some examples of these factors are summarized in Table 1. In the last decade, a few papers mentioned that flowering was induced by stress (Hatayama and Takeno 2003, Kolár and Senková 2008, Martínez et al. 2004). Thus, the evidence for stress-mediated flowering is accumulating. Based on this, this flowering is called 'stress-induced flowering' (Hatayama and Takeno 2003, Takeno 2012, Wada and Takeno 2010). Plants can modify their development processes to adopt to stress conditions, and the stressed plants may flower as an emergency response, to produce the next generation. Thus, stress-induced flowering is an important phenomenon. However, the regulatory mechanism is still unclear. Accordingly, this study aimed to clarify the characteristics of stress-induced

Table 1. Some cases of stress-induced flowering.

Stress factor	Species	Flowering response	Reference
high-intensity light	*Pharbitis nil*	induction	Shinozaki et al. 1994
	Arabidopsis thaliana	promotion	Marín et al. 2010
low-intensity light	*Lemna perpusilla*	induction	Takimoto 1973
	Lemna paucicostata	induction	Tanaka et al. 1989
	Perilla frutescens var. *crispa*	induction	De Zeeuw 1953, Gaillochet et al. 1962, Wada et al. 2010a
	Arabidopsis thaliana	promotion	Halliday et al. 1994, Smith and Whitelam 1997, Dorn et al. 2000
continuous light	*Arabidopsis thaliana*	promotion	Marín et al. 2010
ultraviolet C	*Arabidopsis thaliana*	promotion	Martínez et al. 2004
ultraviolet	duckweeds	induction	Hicks 1932
salt	*Mesembryanthemum crystallinum*	promotion	Adams et al. 1998
drought	Douglas-fir	induction	Ebell 1967
	tropical pasture legumes	induction	Hopkinson 1977
	lemon	induction	Casella 1935, Monselise and Halevy 1964, Nir et al. 1972, Monselise et al. 1981
	Citrus spp.	promotion	Monselise 1985
	Ipomoea batatas	promotion	Jones 1980
	Brachypodium distachyon	promotion	Aronson et al. 1992
	Lemnaceae	induction	Krajncic et al. 2006
poor nutrition	*Pharbitis nil*	induction	Shinozaki et al. 1988a, Wada et al. 2010b
	Macroptilium atropurpureum	promotion	Imrie 1973
	Cyclamen persicum	promotion	Bussler 1969
	Ipomoea batatas	promotion	Jones 1980
	Arabidopsis thaliana	promotion	Kolár and Senková 2008
poor oxygen	*Pharbitis nil*	induction	Shinozaki et al. 1982
high temperature	*Arabidopsis thaliana*	promotion	Marín et al. 2010
	Chenopodium polyspermum	promotion	Chamont et al. 1982
low temperature	*Pharbitis nil*	induction	Hirai et al. 1994, Hatayama and Takeno 2003
	Chenopodium polyspermum	promotion	Chamont et al. 1982
photochilling	*Arabidopsis thaliana*	promotion	Marín et al. 2010
high conc. $GA_{4/7}$	Douglas-fir	promotion	McMullan 1980
crowdedness	*Lemna perpusilla*	induction	Landolt 1957
girdling	Douglas-fir	induction	Ebell 1971
root pruning	*Citrus* sp.	induction	Iwasaki et al. 1959
	Pharbitis nil	induction	Wada 1974
mechanical stimulation	*Ananas comosus*	induction	Metzger 1995
suppression of root elongation	*Pharbitis nil*	induction	Swe et al. 1985

flowering and its biological meanings and to analyze the regulatory mechanism of it.

Chapter I

Stress-induced flowering in *Pharbitis nil*

Introduction

The short-day (SD) plant *Pharbitis nil* (synonym *Ipomoea nil*) can be induced to flower under long days (LD) when grown in tap water (poor-nutrition conditions), at 12 to 15 °C (low-temperature conditions) or under 15,000 to 20,000 lux light (high-intensity light conditions) (Hirai et al. 1994, Shinozaki and Takimoto 1982, Shinozaki et al. 1994). Phenylalanine ammonia-lyase (PAL) activity increases when flowering is induced under these growth conditions (Hirai et al. 1995). These results suggest that such conditions may stimulate flowering through a common mechanism. Poor nutrition, low temperature and high-intensity light generally act as stress factors, and PAL activity increases when plants are stressed (Dixon and Paiva 1995). Accordingly, we predicted that such flowering might be induced by stress.

Plants can modify their development to adapt to stress conditions. Stressed plants may flower to produce the next generation as an emergency response. In this way, plants can preserve the species even in an unfavorable environment. In order for this to be a biologically advantageous response, plants induced to flower by stresses must produce fertile seeds and the progeny must develop normally. In this study, we tested the ability of *P. nil* plants grown under continuous stress to produce normal progeny.

Stress-induced flowering is inhibited by aminooxyacetic acid (AOA) which functions as PAL inhibitor (Kessmann et al. 1990), and therefore it is hypothesized that some compound or compounds in the metabolic pathway regulated by PAL act as flowering stimuli (Hatayama and Takeno 2003, Hirai et al. 1995). However, a transmissible flowering stimulus like florigen, which is

involved in photoperiodic flowering, has not been reported in stress-induced flowering. To investigate this possibility, we performed grafting experiments to detect the transmission of stress-induced flowering stimuli.

Among the metabolic intermediates in the pathway regulated by PAL, *t*-cinnamic acid and benzoic acid were shown to negate the inhibitory effect of AOA in low-temperature-induced flowering, while *p*-coumaric acid and caffeic acid did not (Hatayama and Takeno 2003). Stress promotes the metabolism of *t*-cinnamic acid to SA via benzoic acid (Gidrol et al. 1996, Mauch-Mani and Slusarenko 1996). Therefore, SA may be a direct factor involved in stress-induced flowering. Accordingly, it was examined whether SA is involved in the stress-induced flowering of *P. nil*.

Materials and methods

Plant materials and growth conditions

The cultivars Violet and Tendan of the SD plant morning glory [*Pharbitis nil* (L.) Chois., synonym *Ipomoea nil* (L.) Roth] were used. The seeds of Violet were purchased from Marutane Co., Kyoto, Japan, and those of Tendan were originally from the National Institute of Genetics, Mishima, Japan. The seeds were treated with conc. H_2SO_4 for 40 minutes, washed with running tap water for 10 minutes, 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 germinated at 25 °C 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 °C under LD conditions

for 5 days. Then, the seedlings were planted in vermiculite in plastic pots 50 mm in diameter or transferred to glass tubes (18 mm in diameter × 180 mm high) containing a mineral nutrient solution (Kondo et al. 2006, Table 2) and grown under the same conditions. The plants in vermiculite were watered daily with tap water and given nutrient solution once a week. White light (55–90 μmol m^{-2} s^{-1}) was provided from fluorescent lamps (FL20SW or FL40SSW/37, Toshiba Corporation, Tokyo, Japan).

Stress treatment

Five-day-old seedlings were cultured in 1/10- or 1/100-strength mineral nutrient solution or tap water without any mineral nutrients (instead of the mineral nutrient solution) for 20 days as the stress treatment. We considered that the plant was stressed if its vegetative growth was inhibited by any external factors (Hatayama and Takeno 2003). After the stress treatment, the seedlings were transferred to nutrient solution and grown further for 2 weeks until the flowering response was scored. In some experiments, the plants were not transferred to the nutrient solution until they produced seeds.

Treatment with chemicals

Aminooxyacetic acid (AOA) and salicylic acid (SA) were purchased from Wako Pure Chemicals Industries, Osaka, Japan. AOA and/or SA were dissolved in the culture solution, and the 5-day-old seedlings were grown in the solution for the designated periods. After the treatment, the plants were transferred to fresh nutrient solution without chemical(s), and grown under LD conditions for 2 weeks.

Table 2. The composition of the inorganic nutrient solution used in the present work.

The composition is based on Nakayama and Hashimoto (1973) and partly modified (Kondo et al. 2006).

Nutrient	mg/L
NH_4NO_3	250
KNO_3	250
$MgSO_4 \cdot 7H_2O$	250
$Ca(H_2PO_4)_2 \cdot H_2O$	250
KH_2PO_4	250
Fe(III)-EDTA	6
H_3BO_3	2
$MnSO_4 \cdot 4H_2O$	1
$ZnSO_4 \cdot 7H_2O$	0.2
$Na_2MoO_4 \cdot 2H_2O$	0.2
$CuSO_4 \cdot 5H_2O$	0.1

Quantification of anthocyanin

Anthocyanin was extracted and quantified according to the method described by Gong et al. (1997). The roots, stem, cotyledons and true leaves were harvested from five plants in each treatment. Each tissue sample was incubated in methanol containing 1% (v/v) HCl (2.0 g fresh weight/50 ml) in the dark at 4 °C overnight. The extract was filtered through a filter paper and then through a membrane filter (pore size 0.45 μm) (Advantec Toyo Kaisha, Ltd., Tokyo, Japan). Absorbance at 529.5 nm ($A_{529.5}$) was measured by a photometer (UVmini-1240, Shimadzu Corporation, Kyoto, Japan) as the level of anthocyanin.

Grafting

Plants grown on agar medium for 10 days were used as both donor stocks and receptor scions, and they were grafted as previously reported (Takeno 1991). A scion with a 1- to 2-cm hypocotyl was cut from the plant and the basal end was trimmed with a razor blade, leaving a wedge-shaped cut end. Cotyledons and expanded leaves were removed from the scions. The stock plant with epicotyl removed was cut longitudinally about 1 cm deep between cotyledons, the scion was inserted into the cleft of the stock, and the graft joint was fixed with a silicon rubber tube. The grafted plants were covered with a plastic bag to maintain high humidity during the first 4 days after grafting. The grafted plants were grown in tap water for 20 days and then moved to the nutrient solution and grown for a further 2 weeks until the flowering response was scored.

Scoring of flowering response

All nodes of the plants were dissected under a binocular microscope 2 weeks after the end of the stress treatment to determine whether floral buds or vegetative buds had been formed. The length of the main stem was also measured. The percentage of plants with at least one flower bud (% flowering out of all plants in the treatment condition) and the number of flower buds per plant were determined. The number of nodes on which either flower buds or vegetative buds were formed per plant was presented as an indicator of vegetative growth along with the average length of the main stem. Twenty plants were used for each treatment. Each experiment was repeated two to four times. The means and standard errors of the most typical experiment are shown in each table.

Results

Flower induction by poor nutrition

Two cultivars of *P. nil* were subjected to poor-nutrition stress by growing in 1/10- or 1/100-strength nutrient solution or tap water (designated 0) under LD conditions for 20 days. Violet was induced to flower when grown in diluted nutrient solution or tap water whereas no flowering occurred in the nutrient solution (Table 3). The vegetative growth of the plants grown in poor-nutrition conditions was significantly inhibited. The flowering response was weaker under the weaker stress condition (1/10-strength nutrient solution) than under the stronger stress condition (1/100-strength nutrient solution). The flowering

Table 3. Flowering of *Pharbitis nil* by poor-nutrition stress.

The seedlings of two cultivars of *P. nil* were grown on vermiculite and fed with mineral nutrient solution (×1), diluted nutrient solution (×1/10 and ×1/100) or tap water (0) at 25 ℃ under 16-hour light long-day (LD) conditions for 20 days. The plants were then transferred to nutrient solution and grown at 25 ℃ under LD conditions for two weeks and the flowering response was scored. The data are means with standard errors. Values followed by the same superscript symbol do not differ significantly at the 5% level in a *t*-test within the same cultivar.

Cultivar	Strength of nurtient solution	% flowering	Floral buds /plant	Nodes /plant	Stem length (mm)
Violet	×1	0	0 ± 0^{a}	14 ± 0.65^{a}	765 ± 37.5^{a}
	×1/10	70	1.2 ± 0.25^{b}	11 ± 0.27^{b}	645 ± 26.5^{b}
	×1/100	100	2.1 ± 0.18^{c}	12 ± 0.33^{c}	622 ± 28.6^{b}
	0	95	1.5 ± 0.14^{b}	13 ± 0.32^{a}	577 ± 33.6^{b}
Tendan	×1	0	0±0	15 ± 0.49^{a}	842 ± 50.7^{a}
	×1/10	0	0±0	12 ± 0.30^{b}	642 ± 23.0^{b}
	×1/100	0	0±0	13 ± 0.32^{c}	594 ± 16.3^{b}
	0	0	0±0	14 ± 0.24^{a}	528 ± 14.3^{c}

response was also reduced when the plants were given the strongest stress by growing in tap water. Tendan was not induced to flower even when grown in tap water, although vegetative growth was significantly inhibited.

Production of progeny by plants induced to flower by poor-nutrition stress

Violet was grown in 1/10-strength nutrient solution or tap water throughout the plant life. The plants that were induced to flower by poor-nutrition conditions reached anthesis, fruited and produced seeds. Eleven seeds were obtained from 10 plants grown in 1/10-strength nutrient solution, and two seeds were obtained from 10 plants grown in tap water (Fig. 1A). The seeds produced by the stressed plants were the same size as or slightly smaller than the control seeds produced by plants flowering under SD conditions. The fresh weight of the seeds produced on the plants grown in nutrient solution, 1/10-strength nutrient solution and tap water were 55±2.0 (n =31), 51±3.3 (n =11) and 34±9.2 (n =2), respectively. All these seeds germinated and the progeny of the stressed plants developed normally, although they were slightly shorter than the control plants (Fig. 1B). Furthermore, they responded to SD treatment and formed floral buds (Fig. 1C).

The third generation of the stressed plants also developed normally. There is no difference in fresh weight between the seeds of the third generation and the seeds from the same lot of the parent generation, and flowering response and growth of the seedlings were better in the third generations (Table 4).

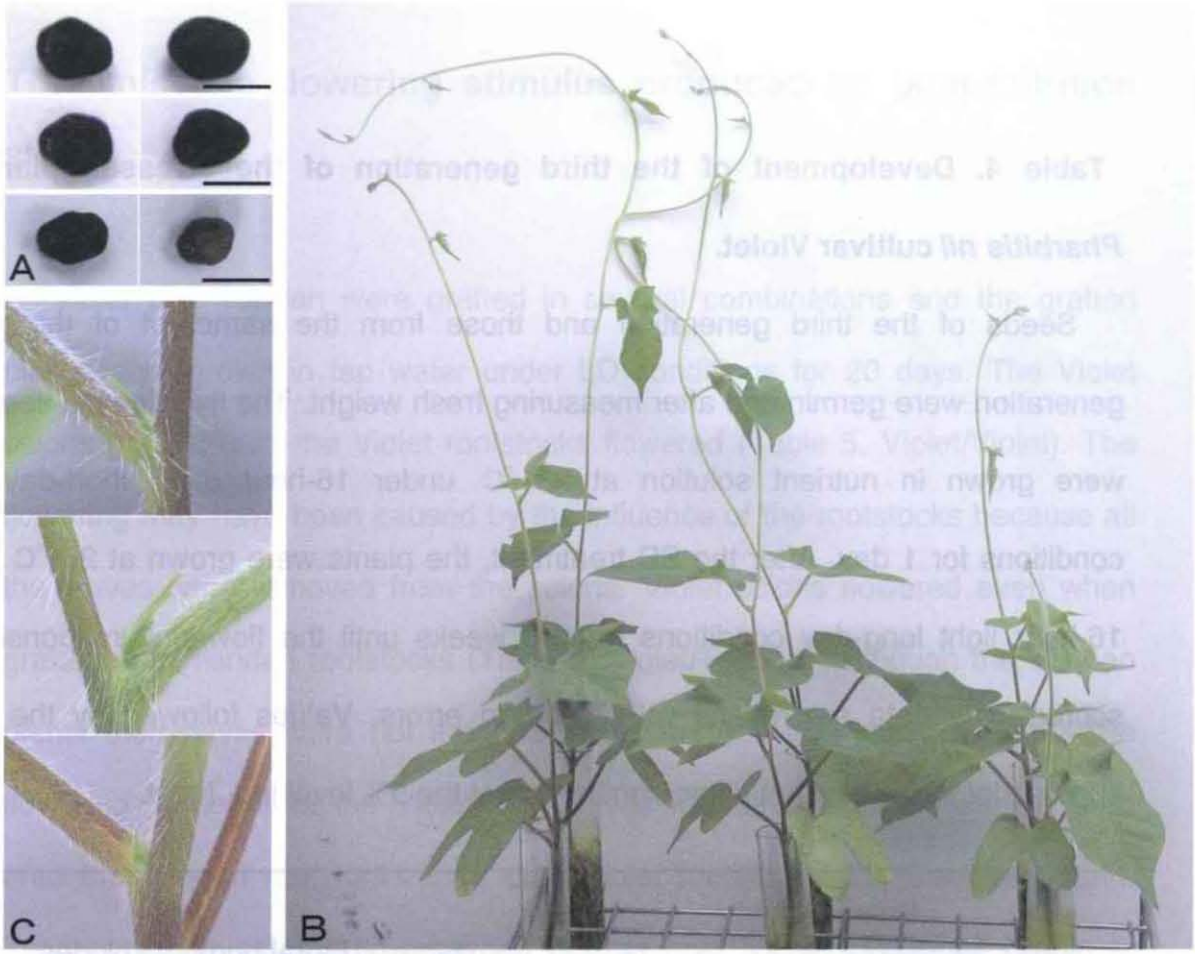

Fig. 1. The progeny of *Pharbitis nil* Violet induced to flower by poor-nutrition stress.

(A): Seeds produced by plants induced to flower photoperiodically as a control (upper), by plants grown in 1/10-strength nutrient solution (middle), and by plants grown in tap water (lower). Scale bars are 5 mm. (B): Seedlings developed from the seeds produced by plants induced to flower photoperiodically as a control (left), from seeds produced by plants grown in 1/10-strength nutrient solution (middle), and from seeds produced by plants grown in tap water (right). (C): Floral buds formed by control seedlings (upper), by the progeny of plants grown in 1/10-strength nutrient solution (middle), and by the progeny of plants grown in tap water (lower) in response to a short-day treatment.

Table 4. Development of the third generation of the stressed plants in *Pharbitis nil* cultivar Violet.

Seeds of the third generation and those from the same lot of the parent generation were germinated after measuring fresh weight. The five-day-old seedlings were grown in nutrient solution at 25 ℃ under 16-hour dark short-day (SD) conditions for 1 day. After the SD treatment, the plants were grown at 25 ℃ under 16-hour light long-day conditions for two weeks until the flowering response was scored. The data are means with standard errors. Values followed by the same superscript symbol do not differ significantly at the 5% level in a *t*-test.

	Fresh weight of seeds (mg/seed)	% germination	% flowering	Floral buds /plant	Nodes /plant	Stem length (mm)
Parent generation	62.4±2.20[a]	87	19	0.19±0.065[a]	10±0.39[a]	262±16
The third generation of the stressed plants	60.6±2.98[a]	74	57	0.88±0.16[b]	11±0.32[a]	354±18

Transmissible flowering stimulus produced by poor-nutrition stress

Violet and Tendan were grafted in several combinations and the grafted plants were grown in tap water under LD conditions for 20 days. The Violet scions grafted onto the Violet rootstocks flowered (Table 5, Violet/Violet). The flowering may have been caused by the influence of the rootstocks because all the leaves were removed from the scions. Violet scions flowered even when grafted onto Tendan rootstocks (Table 5, Violet/Tendan), although the Tendan plants themselves were not induced to flower by the stress treatment. The flowering response of the Violet scions was weaker when they were grafted onto the Tendan rootstocks than onto Violet rootstocks. On the other hand, Tendan scions did not flower when grafted onto Violet rootstocks (Table 5, Tendan/Violet). The vegetative growth of the Tendan scions was reduced compared to that of the Violet scions. The reason for this growth retardation is unknown, but these results were reproduced in several repeated experiments.

Involvement of the metabolic pathway regulated by PAL in stress-induced flowering

When *P. nil* was induced to flower by responding to poor-nutrition stress, the roots of the stressed plants turned into red (Fig. 2, A). Accordingly, we determined the anthocyanin content of roots, stem, cotyledons and true leaves that were harvested from Violet grown in nutrient solution or tap water for 20 days. Anthocyanin content increased in the roots and stem of Violet grown in

Table 5. Flowering of grafted plants under poor-nutrition stress conditions in *Pharbitis nil*.

Two cultivars of *P. nil* were grafted in the combinations shown in the table and grown in tap water at 25 ℃ under 16-hour light long-day conditions for 20 days. Cotyledons were removed from the scions and newly expanded true leaves of the scions were cut off. After the poor-nutrition stress treatment, the plants were transferred to nutrient solution and grown for two additional weeks until the flowering response of the scions was scored. The data are means with standard errors. Values followed by the same superscript symbol do not differ significantly at the 1% level in a *t*-test.

Cultivar (scion/stock)	% flowering	Floral buds /plant	Nodes /plant	Stem length (mm)
Violet/Violet	68	1.3 ± 0.22^{a}	7.9 ± 0.49^{a}	84 ± 10^{a}
Violet/Tendan	41	0.93 ± 0.27^{ab}	8.0 ± 0.27^{a}	80 ± 8.8^{a}
Tendan/Violet	0	0 ± 0^{b}	4.6 ± 0.60^{b}	18 ± 4.4^{b}

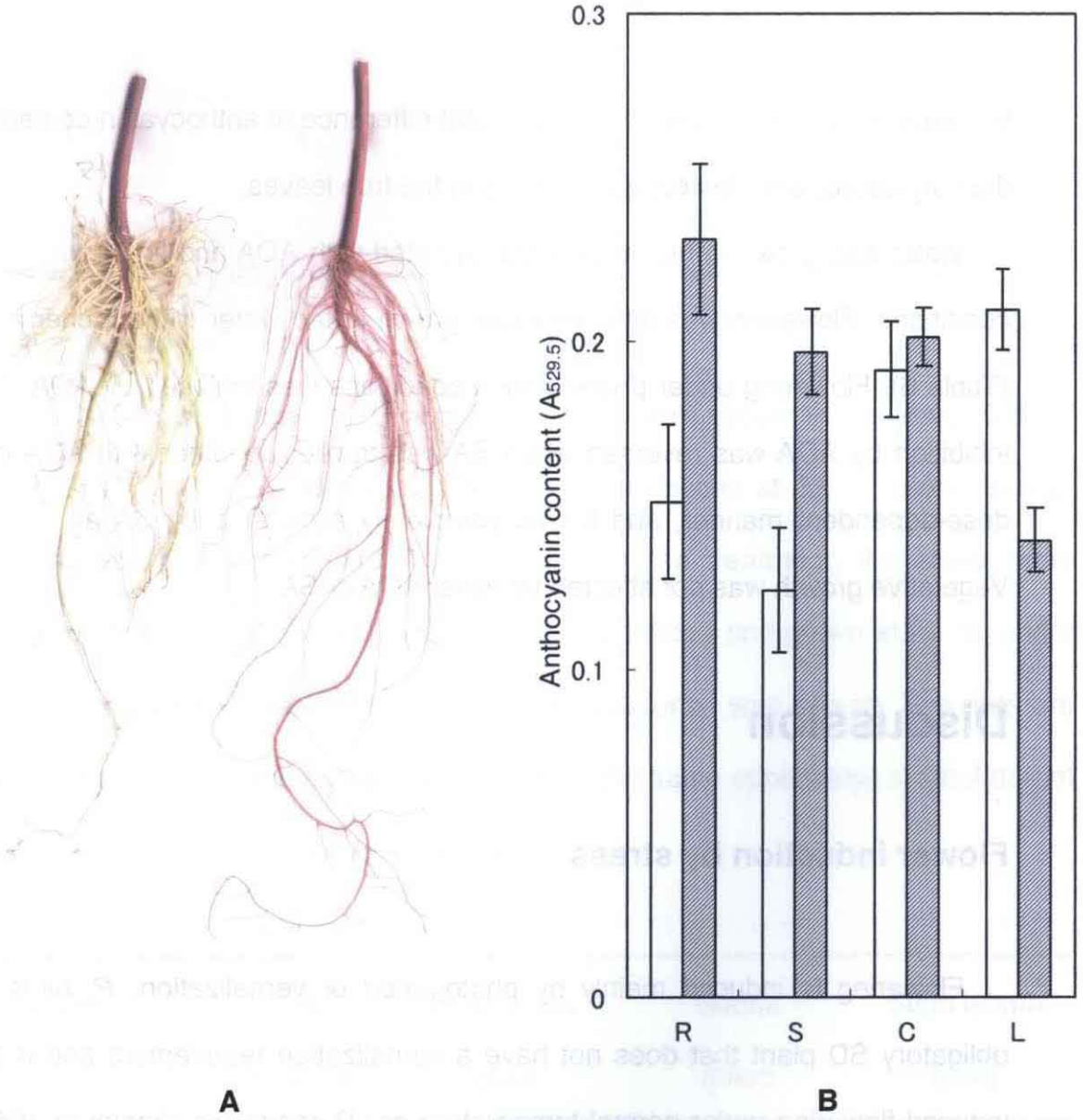

Fig. 2. Color of roots (A) and anthocyanin content (B) of *Pharbitis nil* cultivar Violet grown in poor-nutrition conditions.

(A): Roots of *P. nil* Violet grown in nutrient solution (left) or tap water (right) under long-day conditions for 20 days were photographed. (B): The roots (R), stem (S), cotyledons (C) and true leaves (L) were harvested from the plants grown in nutrient solution (open column) or tap water (shaded column) and anthocyanin was extracted. Anthocyanin content was measured as absorbance at 529.5 nm ($A_{529.5}$).

tap water (Fig. 2, B). There is no significant difference of anthocyanin content in the cotyledons, and decreased by stress in the true leaves.

Violet was grown in tap water supplemented with AOA and/or SA under LD conditions. Flowering was induced when grown in tap water without chemicals (Table 6). Flowering under poor-nutrition conditions was inhibited by AOA. The inhibition by AOA was reversed when SA was applied together with AOA in a dose-dependent manner, and it was completely eliminated by SA at 10^{-4} M. Vegetative growth was not affected by either AOA or SA.

Discussion

Flower induction by stress

Flowering is induced mainly by photoperiod or vernalization. *P. nil* is an obligatory SD plant that does not have a vernalization requirement and is not induced flowering under normal temperature or LD conditions (Imamura 1967). However, *P. nil* cultivar Violet was induced to flower when grown in diluted mineral nutrient solution or tap water at 25 °C under LD conditions (Table 3). The vegetative growth of the plants grown in these poor-nutrition conditions was significantly inhibited. Since the suppression of vegetative growth indicated that the plants were stressed (Hatayama and Takeno 2003), this flowering can be considered to be stress-induced flowering. The flowering response was weaker under the weaker stress condition (1/10-strength nutrient solution). The other cultivar, Tendan, was not induced to flower even when grown in tap water,

Table 6. Effects of aminooxyacetic acid (AOA) and salicylic acid (SA) on poor-nutrition stress-induced flowering of *Pharbitis nil*.

Five-day-old seedlings of *P. nil* cultivar Violet were grown in tap water with or without 3×10^{-5} M AOA and SA at different concentrations at 25 ℃ under 16-hour light long-day (LD) conditions for 20 days. After the treatment, the plants were transferred to fresh nutrient solution without the chemicals and grown at 25 ℃ under LD conditions for two weeks until the flowering response was scored. The data are means with standard errors. Values followed by the same superscript symbol do not differ significantly at the 5% level in a *t*-test.

Treatment	% flowering	Floral buds /plant	Nodes /plant	Stem length (mm)
Without AOA/SA	89	1.5 ± 0.20^{a}	9.4 ± 0.63^{a}	309 ± 36.7^{a}
AOA alone	21	0.53 ± 0.27^{b}	10 ± 0.37^{a}	279 ± 36.6^{a}
AOA+10^{-6} M SA	24	0.53 ± 0.27^{b}	9.3 ± 0.44^{a}	211 ± 38.1^{a}
AOA+10^{-5} M SA	67	1.4 ± 0.32^{a}	10 ± 0.54^{a}	297 ± 38.1^{a}
AOA+10^{-4} M SA	78	1.5 ± 0.25^{a}	10 ± 0.49^{a}	292 ± 32.8^{a}

although vegetative growth was inhibited. Thus, nutrient stress does not induce flowering in all cultivars.

Progeny of the plant flowered by stress

Plants were grown in poor-nutrition conditions for 20 days and then moved to normal-nutrition conditions, as in previous studies. This temporal stress treatment induced flowering, and the subsequent normal growth conditions allowed the induced floral primordia to develop rapidly (Shinozaki and Takimoto 1982, Swe et al. 1985). Whether or not any external factors have flower-inducing activity can be determined in this way. However, it has never been determined whether normal progeny can be produced by stress-induced flowering. We hypothesized that stressed plants may flower to produce the next generation as an emergency response if they cannot adapt to unfavorable environmental conditions. If this is the case, the plants induced to flower by stress must produce progeny under the continuous stress conditions. Violet plants grown under poor-nutrition conditions throughout the plant's life were induced to flower, produced seeds, and the next generation developed normally (Fig. 1). This indicates that the stressed plants do not need to await the arrival of a season when photoperiodic conditions are suitable for flowering, and such precocious flowering may assist in species preservation. Therefore, stress-induced flowering may have a biological benefit, and it should be considered to be as important as photoperiodic flowering and vernalization.

Flowering stimulus produced by stress

The presence of cotyledons is necessary for flowering of *P. nil* seedlings in response to poor nutrition or low temperature (Shinozaki 1985, Shinozaki and Takimoto 1982). This suggests that a flowering stimulus like florigen, which is involved in photoperiodic flowering, is also involved in stress-induced flowering, and that it is produced in cotyledons. If the stress-induced flowering stimulus is transmissible, defoliated scions may flower when grafted onto rootstocks with cotyledons and grown under poor-nutrition conditions. In the present experiment, defoliated Violet scions grafted onto Violet rootstocks with cotyledons were induced to flower (Table 5, Violet/Violet). Therefore, we conclude that a transmissible flowering stimulus is involved in the stress-induced flowering of *P. nil*.

We predicted that Tendan would not produce such a flowering stimulus because Tendan did not flower in response to the poor-nutrition stress conditions (Table 3). If this were the case, Violet would not be expected to flower when grafted onto Tendan rootstocks. However, defoliated Violet scions grafted onto Tendan rootstocks with cotyledons were induced to flower (Table 5, Violet/Tendan). The flowering response of the scions grafted onto Tendan was slightly weaker than those grafted onto Violet, but the difference was not statistically significant. Therefore, Tendan may produce almost the same amount of the flowering stimulus as Violet. Conversely, the Tendan scions grafted onto Violet rootstocks were not induced to flower (Table 5, Tendan/Violet). These results indicate that Tendan produces a transmissible flowering stimulus but does not respond to it. The growth of the Tendan scions was always suppressed in comparison to Violet scions. The cause of this

growth suppression is unclear, and it should be further examined in future studies to clarify why Tendan does not respond to its own flowering stimulus.

Involvement of the metabolic pathway regulated by PAL

The flowering in Violet induced by poor-nutrition stress was inhibited by the PAL inhibitor AOA. Among the metabolic intermediates in the pathways regulated by PAL (Fig. 3), SA almost completely reversed this inhibition by AOA (Table 6). In the low-temprature stress-induced flowering of Violet, it has been reported that *t*-cinnamic acid and benzoic acid were shown to negate the inhibitory effect of AOA while *p*-coumaric and caffeic acids did not (Hatayama and Takeno 2003). These suggest that SA is involved in the stress-induced flowering of *P. nil*. Stress promotes the metabolism of *t*-cinnamic acid to SA via benzoic acid (Gidrol et al. 1996, Mauch-Mani and Slusarenko 1996). SA plays several physiological roles in plant development (Raskin 1992). The treatment of *P. nil* with benzoic acid, SA or some benzoic acid derivatives prior to low-temperature treatment enhances the flower-inducing effect of low temperature (Shinozaki 1985, Shinozaki et al. 1985). In addition, several derivatives of benzoic acid induce flowering in *P. nil* (Shinozaki and Takimoto 1983), and benzoic acid enhances the flowering of cultured plumules excised from photoinduced *P. nil* seedlings (Ishioka et al. 1990). SA induces flowering in many species belonging to the Lemnaceae (Cleland et al. 1982), and it has been implicated in the stress-induced flowering of *A. thaliana* (Corbesier and Coupland 2005, Martínez et al. 2004). These data support the conclusions described above.

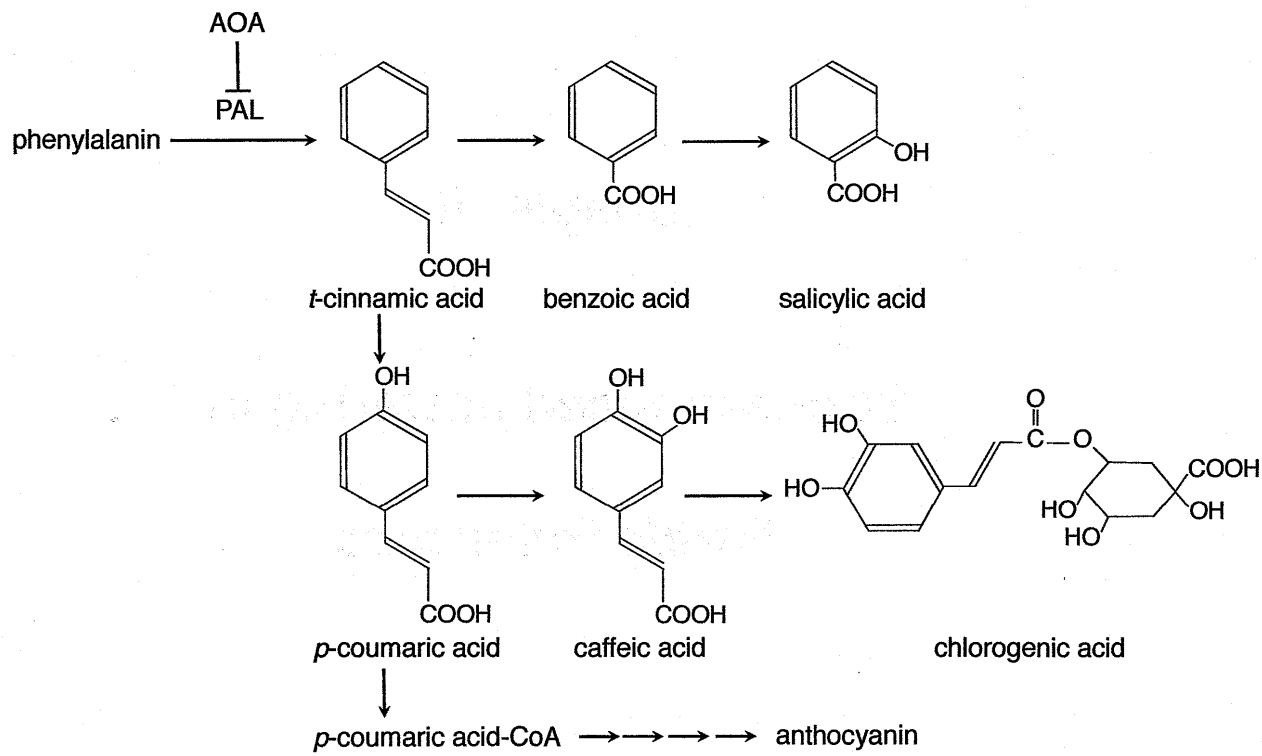

Fig. 3. The metabolic pathway regurated by phenylalanine ammonia-lyase (PAL).

The aminooxyacetic acid (AOA) inhibits PAL.

Chapter II

Stress-induced flowering in

Perilla frutescens

Introduction

In the preliminary experiment, we found that *Perilla frutescens* var. *crispa,* an obligatory short-day (SD) plant (Jacobs 1982), flowered under long-day (LD) conditions if the light intensity was low. Flowering under low-intensity light accompanied a reduction in vegetative growth. The reduction of vegetative growth results from stress (Hatayama and Takeno 2003), and therefore the flowering of *P. frutescens* under low-intensity light may be another example of stress-induced flowering. If this flowering response in *P. frutescens* was stress-induced flowering, it suggests that stress-induced flowering is a general phenomenon among plant species. If stress-induced flowering can be generalized, stress can be considered as a factor to regulate flowering other than photoperiod or low temperature, providing us a new viewpoint in the study on flowering. So far stress-induced flowering has been mostly studied in *P.nil. P. frutescens* may become another good experimental material, because many knowledge have been accumulated about photoperiodic flowering in this species (Zeevaart 1986). Accordingly, the flowering response of *P. frutescens* under low-intensity light was analyzed in detail.

Materials and methods

Plant materials and growth conditions

Red- and green-leaved forms of *P. frutescens* (L.) Britton var. *crispa* (Thunb. ex Murray) Decne. ex L. H. Bailey were used as the experimental materials. The seeds were placed on moist filter paper in a Petri dish (90 mm in diameter) and

germinated at 25 °C under 16-hour light and 8-hour dark (LD) conditions. The germinated seedlings were planted in vermiculite in a plastic pot (50 mm in diameter) when the cotyledons expanded 5–10 days after seed planting and were grown under the same conditions. White light was provided from fluorescent lamps (FL20SW or FL40SSW/37, Toshiba Corporation, Tokyo, Japan). The light intensity was 120 μmol m^{-2} s^{-1} in the normal conditions. The plants were watered daily with tap water and fertilized with a mineral nutrient solution (Kondo et al. 2006, Table 2) once a week.

Stress treatments

As the low-intensity light stress treatment, plants were grown under the light conditions of 30 μmol m^{-2} s^{-1} for 4 weeks. Plants were grown in tap water without any mineral nutrients for 6 weeks as the poor-nutrition stress treatment. When the low-temperature stress was given, plants were grown at 5-15 °C for 5 or 7 weeks. As the salt stress, nutrient solution containing NaCl at the concentration of 50-400 mM was added to vermiculite once or twice a week. Plants exposed to the drought stress were limited watering so that the tips of the cotyledons showed slight browning. Except for the cold stress, each stress treatment was given at 25 °C under LD conditions.

Treatment with chemicals

L-2-aminooxy-3-phenylpropionic acid (AOPP) and aminooxyacetic acid (AOA) (both from Wako Pure Chemical Industries, Ltd., Osaka, Japan) were used as phenylalanine ammonia-liase (PAL) inhibitors. AOPP or AOA was

dissolved in nutrient solution. The nutrient solution supplemented with the chemical was added to the vermiculite once a week for 4 weeks.

Scoring of flowering response

P. frutescens flowers form as solitary flowers or inflorescences at leaf axils or as a terminal inflorescence at the shoot apex (Fig. 4). All of the leaf axils and the shoot apex of the main stem for each treated plant were dissected under a binocular microscope to determine whether they formed flowers and/or inflorescences. We recorded the percentage of plants with at least one flower or inflorescence among all the treated plants (% flowering), the number of solitary flowers per plant, and the number of inflorescences per plant. The average length of the main stem was presented as an indicator of vegetative growth. Twenty plants were used for each treatment. Each experiment was repeated at least three times.

Seed germination test

P. frutescens forms mericarp as its dispersal unit, and the mericarp is denoted as a seed in this study. *P. frutescens* seed is in a dormant state for 4 months after the harvest. Therefore, newly harvested seeds were treated at about 5 °C in wet conditions for 30 days to break the dormancy. The seeds that had been stored over 4 months were not cold-treated. To score the germination rate, we placed the seeds on moist filter paper in a Petri dish at 25 °C, LD conditions (120 μmol m^{-2} s^{-1}) for 10 or 11 days.

Quantification of anthocyanin

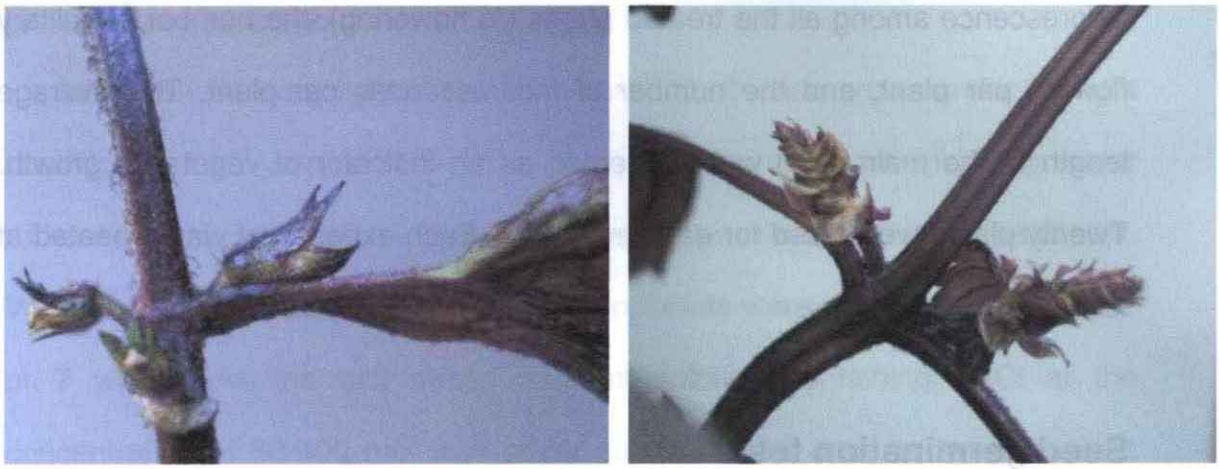

Fig. 4. Solitary flowers (left) and inflorescences (right) of *Perilla frutescens*.

Anthocyanin was extracted and quantified according to the method described by Gong et al. (1997). The leaves were harvested from five plants in each treatment. Each tissue sample was incubated in methanol containing 1% (v/v) HCl (0.2 g fresh weight/50 ml) in the dark at 4 °C overnight. The extract was filtered through a filter paper and then through a membrane filter (pore size 0.45 μm, Advantec Toyo Kaisha, Ltd., Tokyo, Japan). Absorbance at 529.5 nm ($A_{529.5}$) and 652.5 nm ($A_{652.5}$) was measured by a photometer (UVmini-1240, Shimadzu Corporation, Kyoto, Japan) as the levels of anthocyanin and chlorophyll, respectively.

Results

Flower induction by low-intensity light

Two forms of *P. frutescens* were planted on vermiculite when the cotyledons expanded and were grown under LD conditions with different light intensities. All of the red-leaved plants grown under 30 μmol m^{-2} s^{-1} flowered, whereas the plants grown under 60 or 120 μmol m^{-2} s^{-1} did not (Table 7). The green-leaved form was also induced to flower under 30 μmol m^{-2} s^{-1}, although the flowering response was lower than that of the red-leaved form. The stem length was shorter under lower light intensity in both forms.

If the sensitivity to light is low and therefore low-intensity light is recognized as darkness in *P. frutescens*, the LD conditions with low-intensity light may have been recognized as SD conditions. Therefore, we examined this possibility. The red-leaved form of *P. frutescens* was grown under 8-hour light (normal light

Table 7. Flower induction by low-intensity light in *Perilla frutescens.*

Two forms of *P. frutescens* were planted in vermiculite when the cotyledons expanded. Plants were then grown under 16-hour light long-day conditions with different light intensities for 6 weeks, and the flowering response was scored. The data are means with standard errors. Values followed by the same superscript letters do not differ significantly at the 1% level, as determined by *t*-test performed among individuals of the same form.

Light intensity (μmol m^{-2} s^{-1})	% Flowering	Solitary flowers /plant	Inflorescences /plant	Stem length (mm)
Red-leaved form				
120	0	0±0	0±0	101±6.3[a]
60	0	0±0	0±0	68±4.8[b]
30	100	4.0±0.32	7.4±0.37	21±1.1[c]
Green-leaved form				
120	0	0±0	0±0	124±4.8[a]
60	0	0±0	0±0	100±3.5[b]
30	14	0.6±0.4	0.4±0.4	29±1.3[c]

intensity of 120 μmol m^{-2} s^{-1}) and 16-hour dark conditions or 8-hour light (normal light intensity of 120 μmol m^{-2} s^{-1}) and 16-hour low-intensity light (30 μmol m^{-2} s^{-1}) conditions for 3 weeks.

P. frutescens was not induced to flower under the 8-hour light and 16-hour low-intensity light conditions, whereas about 90% flowering occurred under the 8-hour light and 16-hour dark conditions (Table 8). Therefore, the possibility that low-intensity light is recognized as darkness can be excluded.

Kinetics of the flowering induced by low-intensity light

We determined the length of low-intensity light treatment required to induce flowering. The red-leaved *P. frutescens* that was exposed to low-intensity light when their cotyledons had just expanded were induced to flower by the 3-week treatment, and 100% flowering was obtained by a 4-week treatment (Table 9). Flowers even formed at the cotyledonal nodes (Fig. 5). Prolonged treatment over 5 weeks did not increase the numbers of flowers or inflorescences.

Next, the plants were exposed to low-intensity light for 4 weeks. Treatment was begun at various stages of plant development. The plants could respond to low-intensity light immediately after the cotyledons expanded (Table 10). The flowering response decreased with increase in plant age, and flowering was not induced when the low-intensity light treatment started 2 weeks after the cotyledons expanded or later.

We examined the flower-inducing effect of stress factors other than low-intensity light. First, we examined the effect of poor-nutrition stress by culturing in tap water for 6 weeks. Flowering did not occur under the poor-nutrition stress

Table 8. Flowering response of *Perilla frutescens* grown under short-day conditions the dark period of which was replaced by low-intensity light irradiation.

The seedlings of the red-leaved form of *P. frutescens* was grown on vermiculite under 8-hour light (normal light intensity of 120 μmol m^{-2} s^{-1}) and 16-hour dark conditions or 8-hour light (normal light intensity of 120 μmol m^{-2} s^{-1}) and 16-hour low-intensity light (30 μmol m^{-2} s^{-1}) conditions for 3 weeks, and the flowering response was scored. The data are means with standard errors. Values followed by the same superscript symbol do not differ significantly at the 1% level, as determined by the *t*-test.

Light conditions	% flowering	Solitary flowers /plant	Inflorescences /plant	Stem length (mm)
8-hour light and 16-hour dark	89	17±1.7	3.7±0.42	103±8.47[a]
8-hour light and 16-hour low-intensity light	0	0±0	0±0	95±5.2[a]

Table 9. Length of low-intensity light stress treatment and flowering response in red-leaved *Perilla frutescens*.

Red-leaved *P. frutescens* was planted in vermiculite when the cotyledons expanded and was grown under 16-hour light long day (LD) conditions with low-intensity light (30 μmol m^{-2} s^{-1}) for 0–6 weeks. After the low-intensity light treatment, the plants were moved to LD conditions with normal intensity light (120 μmol m^{-2} s^{-1}). The flowering response was scored 7 weeks after the start of low-intensity light treatment. The data are means with standard errors. Values followed by the same superscript letters do not differ significantly at the 1% level, as determined by the *t*-test.

Length of treatment (weeks)	% flowering	Solitary flowers /plant	Inflorescences /plant	Stem length (mm)
0	0	0 ± 0^{a}	0 ± 0^{a}	65 ± 2.5^{a}
3	81	5.0 ± 1.3^{bc}	4.0 ± 0.78^{b}	51 ± 2.1^{b}
4	100	4.2 ± 0.39^{c}	6.3 ± 0.28^{c}	37 ± 1.4^{c}
5	100	3.6 ± 0.22^{bc}	5.6 ± 0.24^{bc}	42 ± 2.1^{d}
6	100	3.2 ± 0.22^{b}	5.2 ± 0.24^{b}	34 ± 1.5^{c}

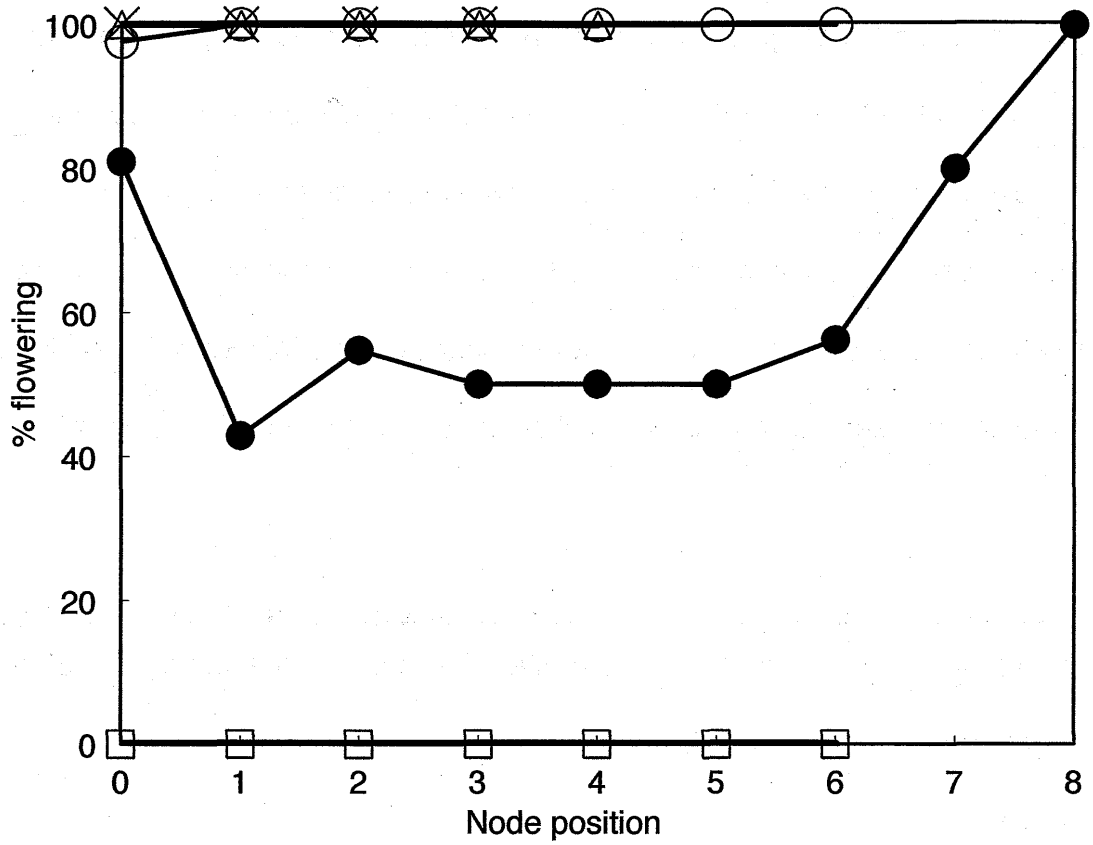

Fig. 5. Flowering response at each node in *Perilla frutescens* grown under low-intensity light stress conditions.

The flowering percentage at each node [0, cotyledonal node; 1, first (the lowest) node; 2, second node; ・・・8, eighth (the highest) node] was calculated for the plants in the experiment shown in Table 9. Low-intensity light stress was given for 0 (□), 3 (●), 4 (○), 5 (△) or 6 (×) weeks.

Table 10. Age-dependency of the flowering response to low-intensity light in red-leaved *Perilla frutescens.*

Red-leaved *P. frutescens* was planted in vermiculite when the cotyledons expanded and was grown under 16-hour light long-day conditions with normal light intensity (120 μmol m^{-2} s^{-1}) for 0–4 weeks. After preincubation under the normal light conditions, the plants were exposed to low-intensity light (30 μmol m^{-2} s^{-1}) stress and grown for 4 weeks. Then the plants were returned to the normal light conditions, and the flowering response was scored 11 weeks after planting. The data are means with standard errors. Values followed by the same superscript letters do not differ significantly at the 1% level, as determined by the *t*-test.

Age at the start of stress treatment (weeks after cotyledon expansion)	% flowering	Solitary flowers /plant	Inflorescences /plant	Stem length (mm)
0	95	13 ± 1.3^{a}	3.3 ± 0.44^{a}	309 ± 12.6^{a}
1	52	4.2 ± 1.1^{b}	1.2 ± 0.46^{b}	294 ± 17.4^{a}
2	0	0 ± 0^{c}	0 ± 0^{c}	292 ± 17.7^{a}
3	0	0 ± 0^{c}	0 ± 0^{c}	230 ± 20.8^{b}
4	0	0 ± 0^{c}	0 ± 0^{c}	241 ± 15.4^{b}

conditions (Table 11). Since the vegetative growth was inhibited as the same extent as in the low-intensity light conditions, poor-nutrition acted as a stress factor. We also examined the effect of nutrient solution which was diluted to 1/100- or 1/10-strength but flowering did not occur. Next, we examined the effect of low-temperature stress by culturing at 15 °C. Flowering did not occur although the vegetative growth was suppressed. Flowering did not occur also at 5 or 10 °C. Additionally, we examined the effect of 50 mM NaCl, finding that flowering did not occur. Flowering did not occur even when treated with 100-400 mM NaCl. A lot of the treated plants died by the treatment with 50 mM NaCl, showing that *P. frutescens* was very weak against salt stress. However, the plants did not show any sign of flowering. We also examined the effect of drought stress by limiting the water supply. Flowering was not induced under the drought conditions. Since the vegetative growth was not inhibited significantly by drought, it is difficult to consider that the drought treatment acted as stress.

Progeny of the plants induced to flower by low-intensity light

Red-leaved *P. frutescens* plants were grown under LD conditions with low-intensity light from the stage when the cotyledons expanded. Plants were then continuously grown under the same conditions. The plants induced to flower under these conditions reached anthesis and formed seeds (Fig. 6). Anthesis and seed formation continued for 7 months until the experiment terminated. There were four seeds per flower as in the normal plants (Fig. 6). The seeds produced under low-intensity light (designated stress progeny) were heavier

Table 11. Effect of several stress factors on flowering in *Perilla frutescens*.

Red-leaved *P. frutescens* was planted in vermiculite when the cotyledons expanded and was given stress factors under 16-hour light long-day conditions. Plants were grown in tap water for 6 weeks (poor-nutrition stress), at 15 °C for 5 weeks (low-temperature stress), in nutrient solution containing 50 mM NaCl (salt stress), or were limited watering so that the tips of the cotyledons showed slight browning (drought stress). Except for the cold stress, each stress treatment was given at 25 °C. After the stress treatment, the flowering response was scored. The data are means with standard errors. Values followed by the same superscript letters do not differ significantly at the 1% level, as determined by the *t*-test.

Stress factors	% flowering	Solitary flowers + inflorescences /plant	Stem length (mm)
Nutrient solution (control)	0	0±0	64±3.0[a]
Tap water (poor-nutrition)	0	0±0	19±0.44[b]
25 °C (control)	0	0±0	109±4.6[a]
15 °C (low-temperature)	0	0±0	57±2.6[b]
0 mM NaCl (control)	0	0±0	80±3.4[a]
50 mM NaCl (salt)	0	0±0	40±4.5[b]
Watering (control)	0	0±0	111±5.60[a]
Limited watering (drought)	0	0±0	106±5.72[a]

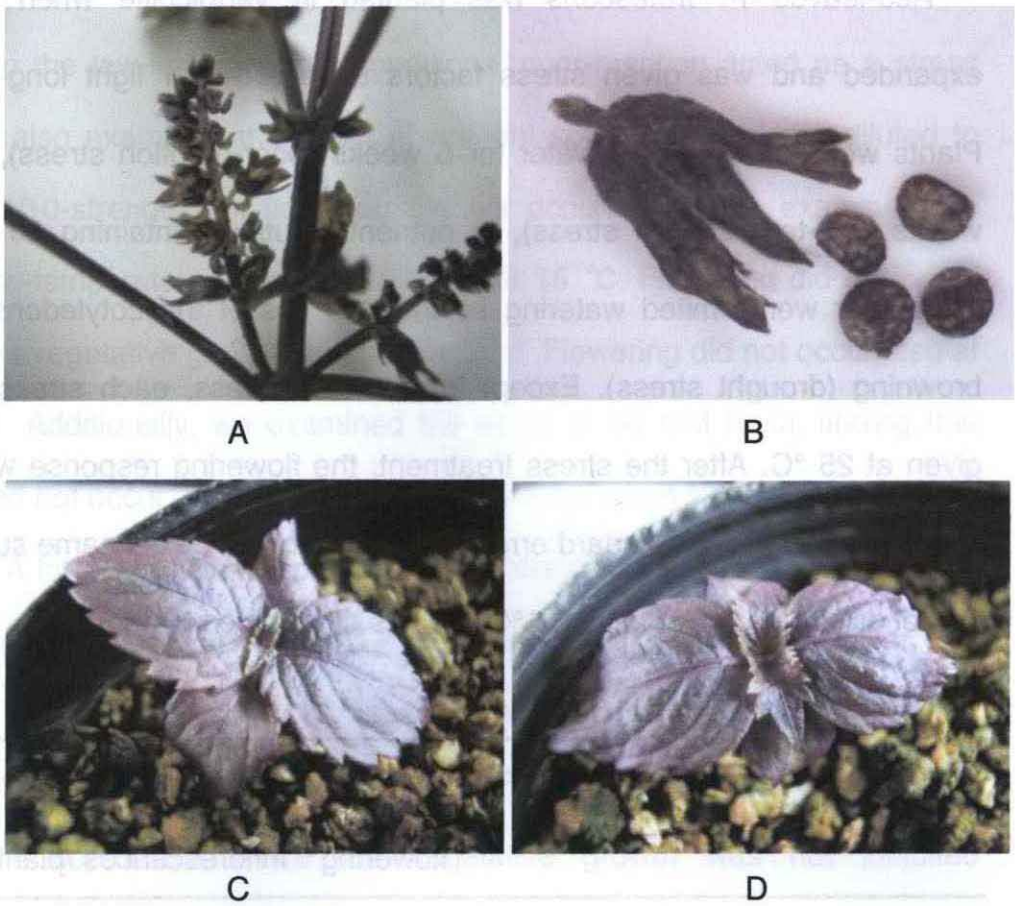

Fig. 6. Anthesis, seed germination and growth of the progeny in *Perilla frutescens* flowering of which was induced by low-intensity light stress.

(A): Red-leaved *P. frutescens* was grown under 16-hour light long-day (LD) conditions with low-intensity light (30 μmol m^{-2} s^{-1}). The floral buds reached anthesis. (B): A flower of the stressed plant formed four normal seeds (*P. frutescens* forms mericarp as dispersal unit, and the mericarp is denoted as a seed in this study). The structure on the left side is sepal which exists even after the anthesis. (C): The seed formed under low-intensity light stress conditions (B) was sown and grown under LD conditions with normal light intensity (120 μmol m^{-2} s^{-1}). It grew normally as did the control plant derived from the seed formed under short-day conditions (D).

than the control seeds produced under usual SD conditions (Table 12). They germinated, grew normally (Fig.6) and were induced to flower in response to SD treatments.

Effect of PAL inhibitors on the flowering induced by low-intensity light

It was observed that the size of plants was small and the leaves of red-leaved plants were deep green under low-intensity light (Fig. 7). Although the leaf color change was not observed in green-leaved *P. frutescens* since their leaves were originally green, the size of plants was smaller under lower-intensity light also in the green-leaved form. Accordingly, we extracted the leaves with methanol and obtained absorption spectra for the extract (Fig. 8). Red color of the leaves of red-leaved *P. frutescens* plants was due to anthocyanin that has an absorption peak at 529.5 nm. With decreased light intensity, the absorbance at 529.5 nm ($A_{529.5}$) decreased and that at 652.5 nm increased (Table 13), indicating that the greening of the leaves was because of the decrease in anthocyanin content and the increase in chlorophyll content. The relationship between anthocyanin content ($A_{529.5}$) and percent flowering was examined using the data of three experiments that studied the effect of different intensities of light on flowering (Fig. 9). There was an apparent negative correlation between these variables ($r = -0.71$). It also seemed like a threshold reaction indicating that flowering did not occur when $A_{529.5}$ was over *ca.* 0.8.

Table 12. Growth and development of the progeny of red-leaved *Perilla frutescens* of which flowering was induced by low-intensity light stress.

Red-leaved *P. frutescens* was induced to flower by growing under 16-hour light long-day (LD) conditions with low-intensity light (30 μmol m^{-2} s^{-1}). Plants were grown under the same conditions until plants reached anthesis and formed seeds. These seeds were harvested and used as stress progeny. Seeds from the same lot as the maternal plants were used as a control. Seed germination was tested, and the germinated seeds were planted in vermiculite and grown under LD conditions (120 μmol m^{-2} s^{-1}). When four pairs of the leaves expanded, they were exposed to short days with 16-hour darkness for 10 days and were then grown under LD conditions for 6 weeks until the flowering response was scored. The data are means with standard errors. Values followed by the same superscript letters do not differ significantly at the 1% level, as determined by the *t*-test.

	Seed weight (mg)	% seed germination	% flowering	Solitary flowers /plant	Inflorescences /plant	Stem length (mm)
Control	0.7 ± 0.03^{a}	47	100	1.0 ± 0.28^{a}	6.1 ± 1.5^{a}	35 ± 1.6^{a}
Stress progeny	1.0 ± 0.06^{b}	30	100	0.67 ± 0.33^{a}	7.6 ± 0.29^{b}	41 ± 3.2^{a}

Fig. 7. The red-leaved *Perilla frutescens* grown under long-day (LD) conditions with various light intensity.

Red-leaved *P. frutescens* was planted in vermiculite when the cotyledons expanded and grown under 16-hour light LD conditions with 30 (left) or 60 (middle), 120 (right) μmol m^{-2} s^{-1} for 5 weeks.

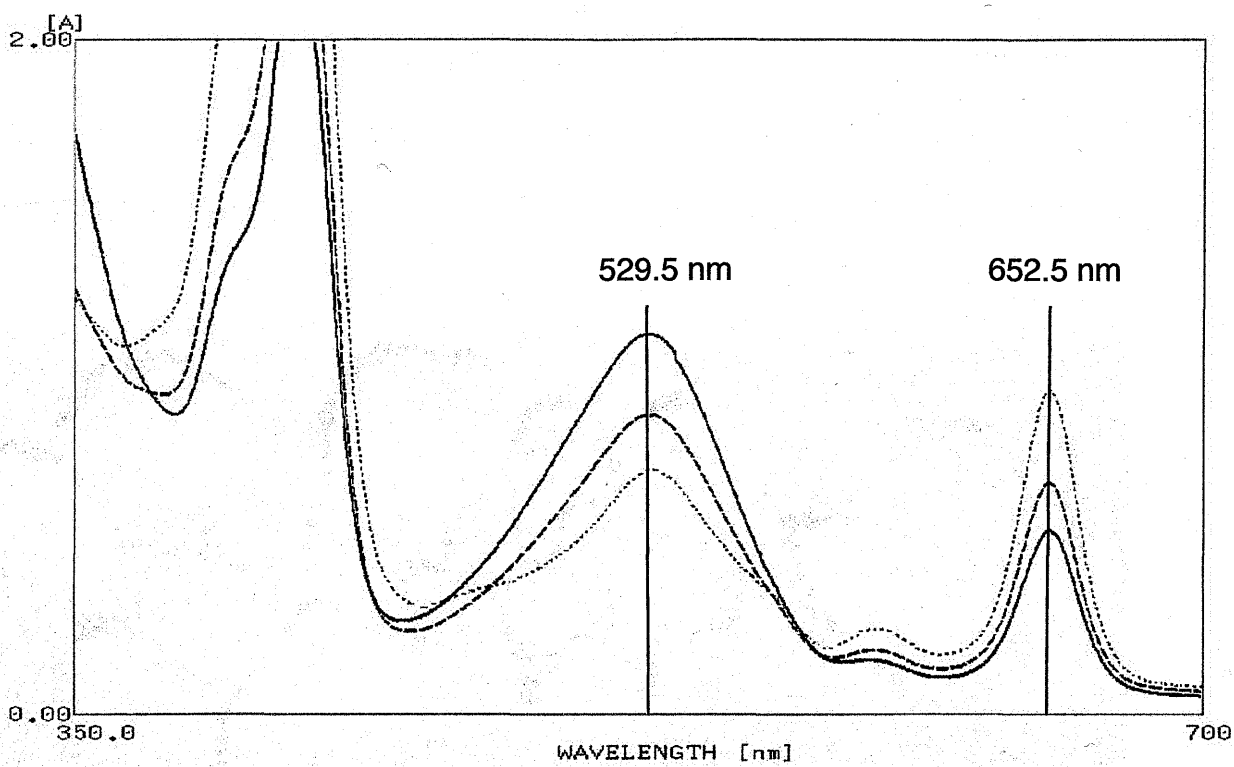

Fig. 8. Absorption spectra of the extract of the leaves of red-leaved *Perilla frutescens*.

The leaves were harvested from red-leaved *P. frutescens* grown under 16-hour light long-day conditions with 30 (·····), 60 (- - -) or 120 (——) μmol m^{-2} s^{-1}, extracted with methanol containing 1% (v/v) HCl, and measured the absorbance between 350 and 700 nm. The absorption spectra show the peaks at 529.5 and 652.5 nm which represents the presence of anthocyanin and chlorophyll, respectively.

Table 13. Contents of anthocyanin and chlorophyll in the leaves of *Perilla frutescens*.

Red-leaved *P. frutescens* was planted in vermiculite when the cotyledons expanded, and then grown under 16-hour light long-day conditions with different light intensities for 6 weeks. The leaves were harvested and incubated in methanol containing 1% (v/v) HCl overnight. Absorbance of the extract at 529.5 nm ($A_{529.5}$) and 652.5 nm ($A_{652.5}$) was measured as the content of anthocyanin and chlorophyll, respectively. Values are means with standard errors. Values followed by the same superscript letters do not differ significantly at the 1% level, as determined by the *t*-test.

Light intensity ($\mu mol\ m^{-2}\ s^{-1}$)	Anthocyanin content ($A_{529.5}$)	Chlorophyll content ($A_{652.5}$)
120	1.16 ± 0.0685^{a}	0.557 ± 0.0276^{a}
60	0.916 ± 0.0333^{b}	0.605 ± 0.0428^{a}
30	0.755 ± 0.0354^{c}	0.841 ± 0.0419^{b}

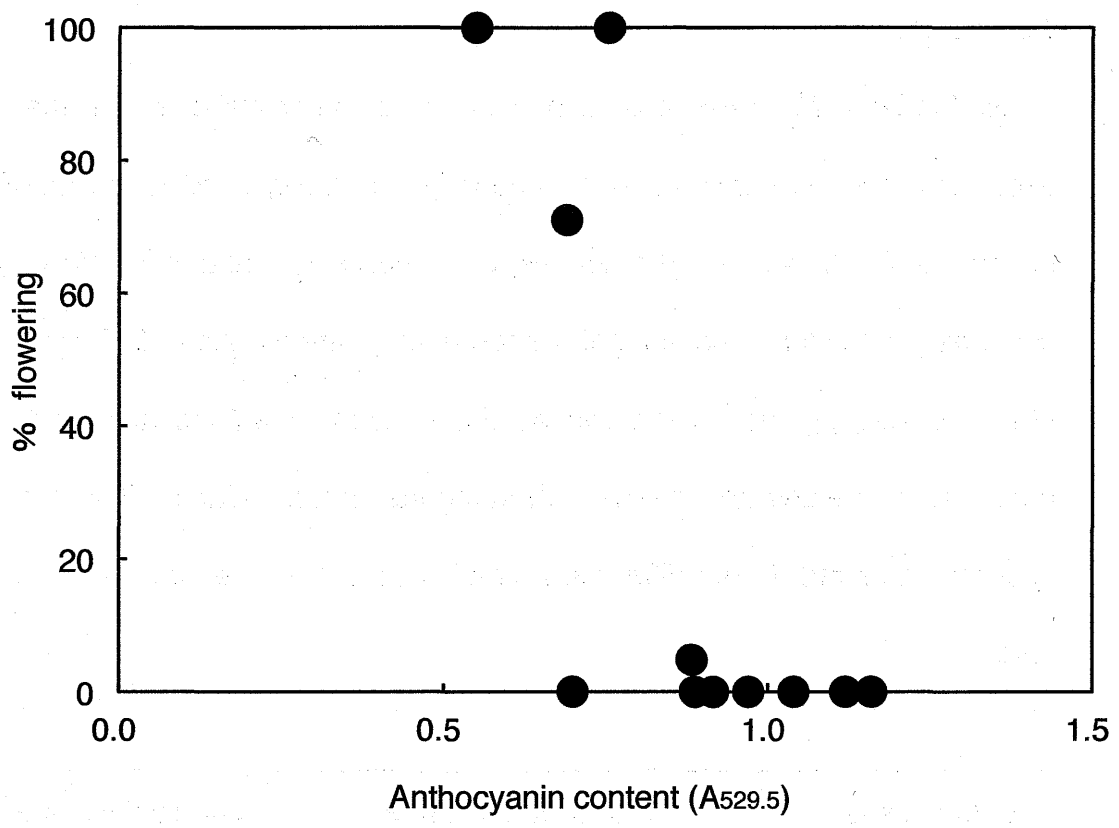

Fig. 9. The relationship between anthocyanin content and percent flowering in red-leaved *Perilla frutescens* grown under different light intensities.

The relationship between anthocyanin content ($A_{529.5}$) of the leaves and the percent flowering was examined using the data of three experiments that studied the effect of different light intensities (30 to 120 μmol m^{-2} s^{-1}) on flowering.

The decrease in the anthocyanin content suggests that PAL activity decreased under the low-intensity light conditions. On the other hand, the suppression of the vegetative growth indicates that the low-intensity light conditions acts as stress suggesting that PAL activity increased. To verify which is correct, we examined the effect of AOPP, a PAL inhibitor, on the flowering of red-leaved *P. frutescens*. AOPP did not induce flowering when applied under non-inductive normal-intensity light, and inhibited flowering when applied under inductive low-intensity light (Table 14). Treatment with another PAL inhibitor, AOA, gave the same results (Table 15).

Red-leaved *P. frutescens* was grown under low-intensity light conditions and treated with AOPP and/or SA. Flowering was induced by low-intensity light, this flowering was inhibited by AOPP and the inhibition was partly reversed by SA (Table 16).

Discussion

Flower induction by stress

The red-leaved *P. frutescens* grown under 30 μmol m^{-2} s^{-1} flowered, and the green-leaved form was also induced to flower under 30 μmol m^{-2} s^{-1}, although the flowering response was lower than that of the red-leaved form (Table 7). Flowering under low-intensity light accompanied a reduction in stem length. Because the suppression of vegetative growth means that the plants were stressed (Hatayama and Takeno 2003), the flowering under low-intensity light can be considered stress-induced flowering.

Table 14. Effects of L-2-aminooxy-3-phenylpropionic acid (AOPP) on flowering in red-leaved *Perilla frutescens* grown under non-stress light conditions and low-intensity light stress conditions.

Red-leaved *P. frutescens* was planted in vermiculite when the cotyledons expanded, grown under 16-hour light long-day conditions with normal light intensity (120 μmol m^{-2} s^{-1}) or low-intensity light (30 μmol m^{-2} s^{-1}), and treated with AOPP for 4 weeks. The treated plants were moved to normal light conditions and grown for 3 (120 μmol m^{-2} s^{-1}) or 5 (30 μmol m^{-2} s^{-1}) weeks until the flowering response was scored. Values are means with standard errors. Values followed by the same superscript letters do not differ significantly at the 1% level, as determined by *t*-test for plants under the same light-intensity conditions.

Light intensity (μmol m^{-2} s^{-1})	AOPP (M)	% flowering	Solitary flowers /plant	Inflorescences /plant	Stem length (mm)
120	0	0	0±0	0±0	95 ± 3.0^{ab}
	10^{-7}	0	0±0	0±0	109 ± 6.19^{a}
	10^{-6}	0	0±0	0±0	92 ± 0.20^{b}
	10^{-5}	0	0±0	0±0	29 ± 2.2^{c}
30	0	56	1.7 ± 0.70^{a}	2.1 ± 0.54^{a}	74 ± 2.9^{a}
	10^{-7}	17	0 ± 0^{b}	0.56 ± 0.32^{b}	62 ± 3.5^{b}
	10^{-6}	12	0 ± 0^{b}	0.24 ± 0.16^{b}	69 ± 2.3^{ab}
	10^{-5}	0	0 ± 0^{b}	0 ± 0^{b}	30 ± 3.1^{c}

Table 15. Effects of aminooxyacetic acid (AOA) on flowering in red-leaved *Perilla frutescens* grown under non-stress light conditions and low-intensity light stress conditions.

Red-leaved *P. frutescens* was planted in vermiculite when the cotyledons expanded, grown under 16-hour light long-day conditions with normal light intensity (120 μmol m^{-2} s^{-1}) or low-intensity light (30 μmol m^{-2} s^{-1}), and treated with AOA for 4 weeks. The treated plants were moved to normal light conditions and grown for 3 weeks until the flowering response was scored. The data are means with standard errors. Values followed by the same superscript letters do not differ significantly at the 5% level, as determined by *t*-test for plants under the same light-intensity conditions.

Light intensity (μmol m^{-2} s^{-1})	AOA (M)	% flowering	Solitary flowers /plant	Inflorescences /plan	Stem length (mm)
120	0	0	0±0	0±0	65 ± 5.4^{a}
	3×10^{-6}	0	0±0	0±0	60 ± 5.6^{a}
	10^{-5}	0	0±0	0±0	24 ± 0.98^{b}
	3×10^{-5}	0	0±0	0±0	7.7 ± 2.9^{c}
	10^{-4}	0	0±0	0±0	0.5
30	0	30	2.3 ± 0.96^{a}	2.1 ± 0.78^{a}	52 ± 3.8^{bc}
	3×10^{-7}	57	2.9 ± 0.78^{a}	3.0 ± 0.79^{a}	61 ± 2.3^{ac}
	10^{-6}	38	1.7 ± 0.71^{a}	1.3 ± 0.57^{a}	50 ± 2.0^{b}
	3×10^{-6}	48	1.6 ± 0.58^{a}	2.0 ± 0.62^{a}	53 ± 1.7^{b}
	10^{-5}	0	0 ± 0^{b}	0 ± 0^{b}	62 ± 2.1^{a}

Table 16. Effects of salicylic acid (SA) and L-2-aminooxy-3-phenylpropionic acid (AOPP) on flowering in red-leaved *Perilla frutescens* grown under low-intensity light stress conditions.

Red-leaved *P. frutescens* was planted in vermiculite when the cotyledons expanded, grown under 16-hour light low-intensity light (30 μmol m^{-2} s^{-1}), and treated with AOPP and/or SA for 5 weeks. The treated plants were moved to normal light conditions and grown for 1 week until the flowering response was scored. The data are means with standard errors. Values followed by the same superscript letters do not differ significantly at the 1% level, as determined by the *t*-test.

AOPP (M)	SA (M)	% flowering	Solitary flowers /plant	Inflorescences /plant	Stem length (mm)
0	0	100	4.0 ± 0.069^{a}	4.7 ± 0.16^{a}	14 ± 0.79^{a}
3×10^{-5}	0	40	0.27 ± 0.18^{b}	0.53 ± 0.22^{c}	2.1 ± 0.19^{c}
3×10^{-5}	10^{-6}	68	0.11 ± 0.11^{b}	0.74 ± 0.13^{b}	2.4 ± 0.23^{bc}
3×10^{-5}	10^{-5}	93	0.27 ± 0.18^{b}	1.2 ± 0.20^{b}	2.9 ± 0.28^{b}
3×10^{-5}	10^{-4}	92	0.42 ± 0.29^{b}	1.1 ± 0.19^{bc}	3.3 ± 0.46^{b}

Epidermis of red-leaved *P. frutescens* contains large amount of anthocyanin which may play a role of light filter reducing the light intensity in mesophyll cells. Therefore, low-intensity light could be recognized as darkness. However, the red-leaved *P. frutescens* was not induced to flower when 16-hour darkness of the SD conditions was replaced by the low-intensity light irradiation (Table 8). Therefore, the possibility that low-intensity light was recognized as darkness can be excluded. In this experiment, flowering did not occur under the low-intensity light conditions. It is because the flowering response was scored after 3-weeks treatment as same as scoring of the flowering response by SD treatment, whereas 6 weeks are needed to recognize the occurrence of flowering induced by low-intensity light (Table 7). *P. frutescens* is an obligatory SD plant (Jacobs 1982) that does not have a vernalization requirement. Therefore, the flowering under LD conditions found in this study is flowering independent of photoperiodism and vernalization. Generally, the plants grown under low-intensity light conditions are etiolated and elongated (Lorrains et al. 2008). In the present study, however, the stem length of *P. frutescens* was shortened and the leaves became green under low-intensity light (Tables 7 and 13, Fig. 7). Therefore, the response of *P. frutescens* to low-intensity light was different from the general photomorphogenetic response. The response to low-intensity light seems like a shade avoidance response. However, it is not the case because the shade avoidance response is wavelength-dependent phenomenon (Lorrains et al. 2008) whereas white light was used in both of the normal- and weak-intensity light conditions in the present experiments. Photosynthetic activity may have decreased under low-intensity light conditions. However, it is unlikely that the photosynthetic deficiency induced flowering

because the photoassimilate is a flower-inducing factor (Bernier and Périlleux 2005, Seo et al. 2011). In fact, sucrose induced flowering of *P. frutescens* cultured *in vitro* under LD conditions (Purse 1984).

P. frutescens is reportedly induced to flower by poor nutrition (Wada and Totsuka 1982) or low temperature (Zeevaart 1969). Accordingly, we treated the red-leaved *P. frutescens* with several stress factors other than low-intensity light. None of these factors induced flowering, although they retarded vegetative growth. This indicates that not all kinds of stress can induce flowering. Although high-intensity light has been well studied as a stress factor (Chalker-Scott 1999), there are only a few reports on low-intensity light as a stress factor. Red-leaved *P. frutescens* (De Zeeuw 1953, Gaillochet et al. 1962), *Lemna perpusilla* (Takimoto 1973) and *A. thaliana* (Smith and Whitelam 1997) flowered under low-intensity light. Low-intensity light of 0.3 to 5 lux induced flowering of *P. nil* cultivar Violet (Ogawa 1961). However, these phenomena were not analyzed to be induced by stress, and not analyzed in detail.

We analyzed the basic nature of the low-intensity light-induced flowering in red-leaved *P. frutescens*. Treatment for over 3 weeks was required to induce flowering (Table 9). The plants did not produce new flowers when treatment was prolonged over 4 weeks. Vegetative growth was suppressed, and the shoot apex changed into a terminal inflorescence under low-intensity light. Therefore, a lack of space may have prevented the formation of new flowers. The plants responded at a very early stage, immediately after the cotyledons expanded (Table 10). This is consistent with the fact that the flowers were formed from the cotyledonal nodes (Fig. 5). Plants that were 2 weeks old or older were not induced to flower (Table 10). Stress treatment for 4 weeks may have not been

long enough to induce flowering among older plants. These plants may have been induced to flower if the treatment were prolonged. In fact, the plants continued to flower for 7 months under continuous low-intensity light conditions as in the experiment shown in Fig. 6.

Progeny of the plants flowered by stress

Stress-induced flowering may be an emergency response to produce the next generation under unsuitable growth conditions. In this respect, the plants induced to flower by stress must produce fertile seeds, and the progeny must develop normally. In this study, red-leaved *P. frutescens* plants grown under low-intensity light conditions produced normal seeds (Fig. 6). The stress progeny germinated, grew normally and were induced to flower in response to SD treatments (Table 12). These results indicate that the stressed plants do not need to await the arrival of a season when photoperiodic conditions are suitable for flowering, and such precocious flowering might assist in species preservation. This conclusion supports the previous one in the study on *P. nil* (Chapter I). Therefore, stress-induced flowering might have a biological benefit, and it should be considered to be as important as photoperiodic flowering and vernalization.

Involvement of the metabolic pathway regulated by PAL in the stress-induced flowering

Under low-intensity light, the plants were smaller and the leaves were deep green (Table 13, Fig. 7). The small plant size and/or greening may be linked with the stress-induced flowering. The growth of green-leaved plants under low-intensity light was retarded to the same extent as observed in red-leaved plants, but the flowering response in green-leaved plants was quite weak. Growth was more severely retarded when the plants were treated at an older age, but there was no positive relationship between growth retardation and flowering response (Table 10). Stress factors other than low-intensity light also retarded growth but did not induce flowering (Table 11). Therefore, it is unlikely that the growth retardation itself induced flowering.

Although greening of the leaves was partially because of the increase in chlorophyll content (Table 13), chlorophyll content may not directly relate to flowering because the increase in chlorophyll content under low-intensity light was observed even in the green-leaved plants, which exhibited only a slight flowering response. On the other hand, there was an apparent negative correlation between anthocyanin content ($A_{529.5}$) and percent flowering (Fig. 9). Therefore, the metabolic pathway related to anthocyanin synthesis may be involved in the regulation of flowering. Namely, low-intensity light stress may have decreased anthocyanin synthesis and induced flowering. However, this conflicts with previous reports. Stress generally promotes anthocyanin biosynthesis by increasing PAL activity (Chalker-Scott 1999, Christie et al. 1994, Dixon and Paiva 1995), and stress-induced flowering in *P. nil* was accompanied by an increase in PAL activity (Chapter I). Thus, two hyposes are possible; low-intensity light stress may decrease or increase PAL activity in *P. frutescens*. Therefore, we examined whether the PAL inhibitor could induce flowering under

normal-intensity light conditions in *P. frutescens* and whether the PAL inhibitor could inhibit its low-intensity light-induced flowering. AOPP which functions as PAL inhibitor (Appert et al. 2003) did not induce flowering when applied under non-inductive normal-intensity light conditions and inhibited flowering when applied under inductive low-intensity light conditions (Table 14). The treatment with another PAL inhibitor, AOA, gave the same results (Table 15). These results suggest that the stress increased PAL activity in *P. frutescens* as in *P. nil*, suggesting that the same mechanism is involved in the flowering induced by low-intensity light in *P. frutescens* and by poor-nutrition stress in *P. nil*. However, the fact that the anthocyanin content decreased under low-intensity light in *P. frutescens* can not be explained.

The preliminary analysis by reverse transcription-polymerase chain reaction suggested that expression of the *PAL* gene was suppressed under low-intensity light conditions in red-leaved *P. frutescens* (Wada 2007). If this is the case, we have to consider that AOPP and AOA inhibited flowering not by inhibiting PAL activity but rather by some unknown mechanism. Although AOPP was known as specific inhibitor of PAL (Kessmann et al. 1990, Mavandad et al. 1990, Ni et al. 1996, Orr et al.1993), it was recently reported that AOPP inhibited also the synthesis of indole-3-acetic acid (IAA) (Ishii et al. 2010). We consider a possibility that AOPP inhibited flowering by inhibiting IAA synthesis. Further investigation will be required to determine what regulates the flowering induced by low light intensity.

Chapter III

Involvement of salicylic acid in stress-induced flowering

Introduction

The studies in the previous chapters revealed that stress can induce flowering. Namely, the short-day (SD) plant *Pharbitis nil* (synonym *Ipomoea nil*) can be induced to flower under long-day (LD) conditions when grown under poor-nutrition stress conditions, and the SD plant *Perilla frutescens* var. *crispa* can flower under LD conditions when grown under low-intensity light stress conditions. The activity of phenylalanine ammonia-lyase (PAL) and the salicylic acid (SA) content increase when plants are stressed (Borsani et al. 2001, Dixon and Paiva 1995, Larkindale et al. 2005, Mateo et al. 2006, Scott et al. 2004). PAL catalyzes the conversion of phenylalanine to *t*-cinnamic acid, and SA is a metabolic intermediate derived from *t*-cinnamic acid. Stress also promotes the metabolism of *t*-cinnamic acid to SA via benzoic acid (Gidrol et al. 1996, Mauch-Mani and Slusarenko 1996). Aminooxyacetic acid (AOA) and L-2-aminooxy-3-phenylpropionic acid (AOPP), which function as PAL inhibitors (Appert et al. 2003, Kessmann et al. 1990), inhibited stress-induced flowering in *P. nil* and *P. frutescens* (Chapters I and II). The inhibitory effects of AOA and AOPP were negated by SA in *P. nil* and *P. frutescens* under stress conditions. It was recently found in our laboratory that the SD plant *Lemna paucicostata* can be induced to flower by poor-nutrition stress and that the endogenous content of SA increased in the plants that flowered under the stress conditions (Shimakawa et al. submitted). Further, ultraviolet (UV) light stress induced an early flowering response in *Arabidopsis thaliana*, and this flowering response

was weaker in SA-deficient *NahG* transgenic lines than in wild-type plants (Martínez et al. 2004). These facts suggest that SA, the synthesis of which is regulated by PAL, is involved in the regulatory mechanism of stress-induced flowering. However, no evidence has yet indicated that endogenous SA levels increase when *P. nil* plants are induced to flower through the application of stress factors. Accordingly, it was examined whether the gene expression and enzyme activity of PAL and the endogenous SA content increase in *P. nil* when flowering is induced by poor-nutrition stress.

Materials and methods

Plant materials and growth conditions

The SD plant morning glory [*Pharbitis nil* (L.) Chois., synonym *Ipomoea nil* (L.) Roth] cultivar Violet was used. The seeds of Violet were provided by the Morning Glory Stock Center of Kyushu University with support in part by the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan (managed by Dr. E. Nitasaka, Kyushu University, Fukuoka, Japan). The seeds were treated with conc. H_2SO_4 for 40 minutes, washed with running tap water for 10 minutes, 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 °C 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 °C under LD conditions for 5 days. Then, the seedlings were

transferred to glass tubes (18 mm in diameter × 180 mm high) containing a mineral nutrient solution (Kondo et al. 2006, Table 2) and grown under the same conditions. White light (55-90 μmol $m^{-2} s^{-1}$) was provided from fluorescent lamps (FL20SW or FL40SSW/37, Toshiba Corporation, Tokyo, Japan).

Stress treatment

Five-day-old seedlings were cultured in 1/100-strength mineral nutrient solution instead of full-strength mineral nutrient solution as the stress treatment. We considered that the plant 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 nutrient solution and grown for 2 weeks until the flowering response was scored.

Treatment with chemical

SA (Wako Pure Chemicals Industries, Osaka, Japan) was dissolved in the culture solution, and the seedlings were grown in the solution for the designated periods. After the treatment, the plants were transferred to fresh nutrient solution without chemical, and grown under LD conditions for 2 weeks.

Scoring of flowering response

All nodes of the plants were dissected under a binocular microscope to determine whether floral buds or vegetative buds were formed. The percentage of plants with at least one flower bud out of all 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 presented as an indicator of vegetative growth along with the average length of the main stem. Twenty plants were used for each treatment. Each experiment was repeated at least twice. The means with standard errors for the most representative experiment are shown in each table or figure.

Analysis of *PAL* gene expression

The expression of the *PAL* gene of *P. nil* (*PnPAL*) was studied by reverse transcription-polymerase chain reaction (RT-PCR) using primers that were designed according to a previous report (Nakazawa et al. 2001). The cotyledons of *P. nil* plants were harvested, frozen in liquid nitrogen, and stored at -80°C prior to analysis. The total RNA was isolated from the cotyledons using the Plant RNA Purification Reagent (Invitrogen Corporation, Carlsbad, CA, U.S.A.), and cDNA was synthesized from each RNA sample using ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan). *PnPAL* was amplified from the cDNA. The primers used were 5'-TCTGGAGTTATGCTACTTGG-3' and 5'-TCAACTTGTAGGGTCATTGG-3'. The reactions were carried out in a thermal cycler using 24 to 30 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 45 seconds. The products for *PnPAL* and *PnACT4*, which encodes actin as a control, were separated on an agarose gel. The images were visualized using EDAS 290 imaging system (Invitrogen Corporation, Carlsbad, CA, U.S.A.). The PCR product corresponding to *PnPAL* was extracted from the agarose gel, and the nucleotide sequence was determined by Operon Biotechnologies (Tokyo, Japan). The sequence was coincident with that previously reported (Fig. 10).

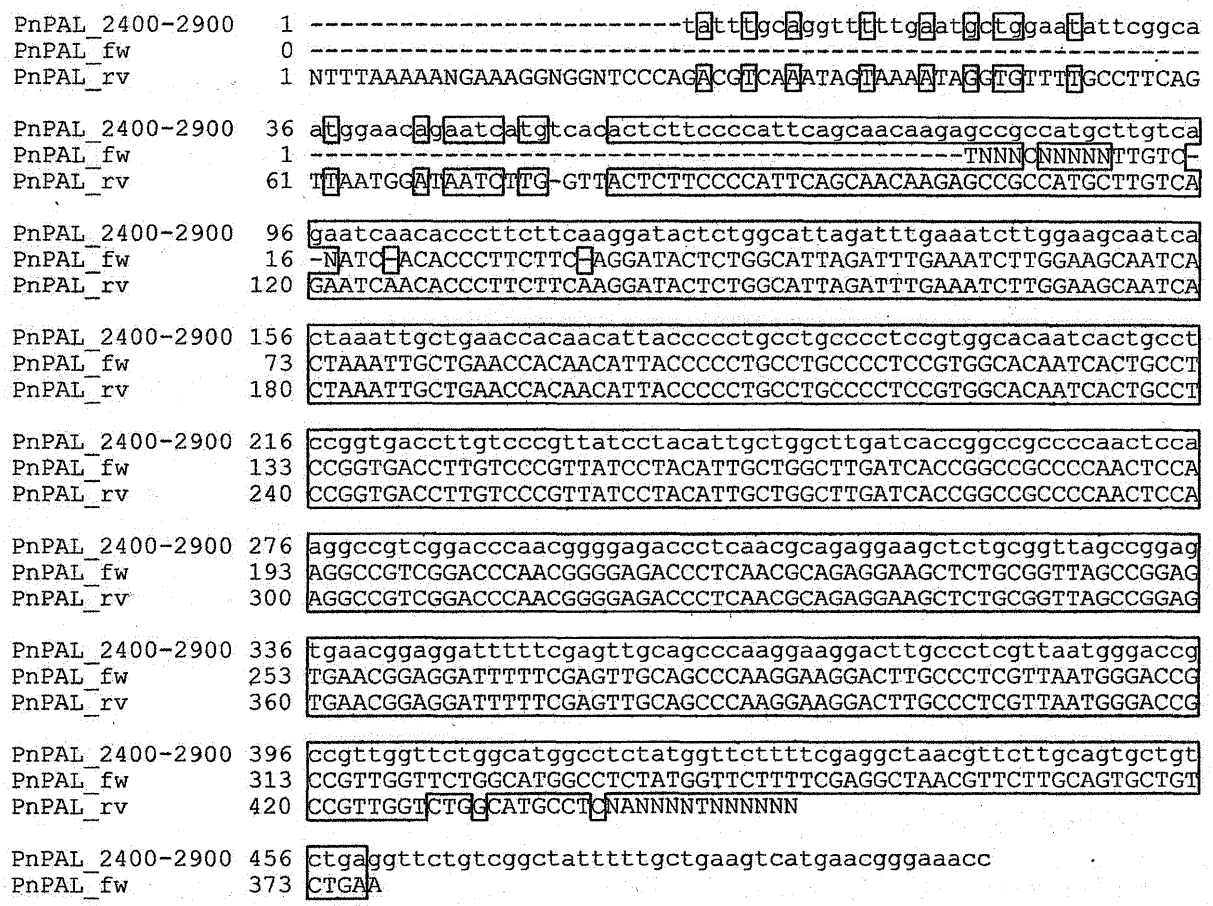

Fig. 10. Alignment of nucleotide sequences of *PnPAL* and the RT-PCR product.

The sequences of *PnPAL* (upper) and the RT-PCR product determined using the forward primer (middle) and the reverse primer (lower), respectively, are aligned. The numbers on the left side of the sequences show the base numbers from the 5' end. Boxed letter indicate identities.

Extraction of PAL and measurement of its activity

The PAL activity was measured basically as described previously (Ferrarese et al. 2000) with some modifications. The cotyledons of *P. nil* seedlings were harvested, frozen in liquid nitrogen, and stored at -80 °C prior to analysis. The frozen tissues were homogenized in 100 mM $Na_2B_4O_7$ buffer, pH 8.8, (2 ml/g fresh weight of tissue) on ice using a mortar and pestle. The homogenate was centrifuged at 4 °C and 12,000 × g for 15 minutes. The supernatant was diluted with the same volume of 100 mM $Na_2B_4O_7$ buffer, and 2 ml of the diluted supernatant was passed through a Sep-Pak Plus C18 cartridge (Waters Corporation, Milford, Massachusetts, U.S.A.) to remove highly water-soluble contaminants (Fig. 11). The amounts of proteins in the sample solutions before and after the Sep-Pak treatment were measured using the method by Bradford (1976) indicating the recovery of proteins was about 90% (Table 17). The eluate (700 µg protein eq.) was added to 100 µM $Na_2B_4O_7$ buffer, pH 8.7, and incubated at 40 °C for 5 minutes. Then, 7.5 µmol L-phenylalanine as a substrate or 75 µl of distilled water as a control was added (final volume, 2 ml), and the reaction mixture was incubated at 40 °C for 1 hour. The reaction was stopped by adding 5 N HCl. The reaction solution was injected into a high performance liquid chromatograph (HPLC) system (Hitachi L-6200, Hitachi Co., Tokyo, Japan) equipped with an ODS column (3 mm × 250 mm, Imtakt Cadenza CD-C18, Imtakt Corp., Kyoto, Japan), and the components were separated with 70% methanol/20 mM sodium acetate, pH 2.5, at a flow rate of 0.15 ml min^{-1}. The column oven temperature was 40 °C. The amount of *t*-cinnamic acid was determined using the A_{290} value based on a quantification line (Fig. 12), and the

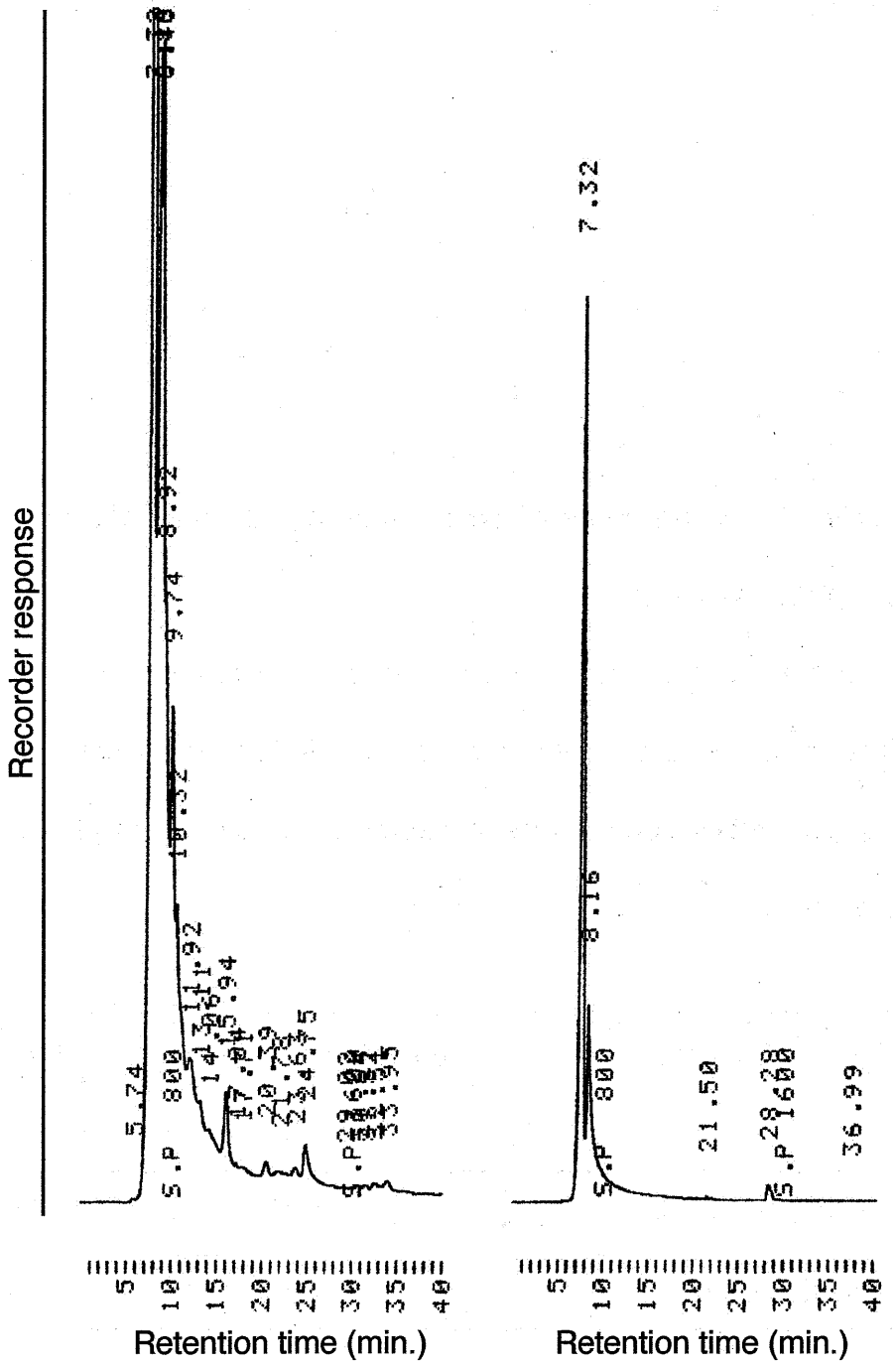

Fig. 11. Purification of the crude enzyme extract by Sep-Pak procedure.

Proteins were extracted from leaves of *Pharbitis nil*, the extracted sample was filtrated by membrane filter and then passed through a Sep-Pak cartridge (right) or without such a treatment (left). Each sample was analyzed by high performance liquid chromatography. The retention time of authentic *t*-cinnamic acid was 15.94 minutes.

Table 17. The amounts of proteins in the sample solutions before and after the Sep-Pak treatment.

Proteins were extracted from leaves of *Pharbitis nil*, the extracted sample was filtrated by membrane filter and then passed through a Sep-Pak cartridge or not as in Fig. 11. Protein content was measured in the samples with and without the Sep-Pak treatment.

Sample treatment	Content of proteins (mg/ml)	Recovery of proteins (%)
Without Sep-Pak	0.74	100
With Sep-Pak	0.67	91

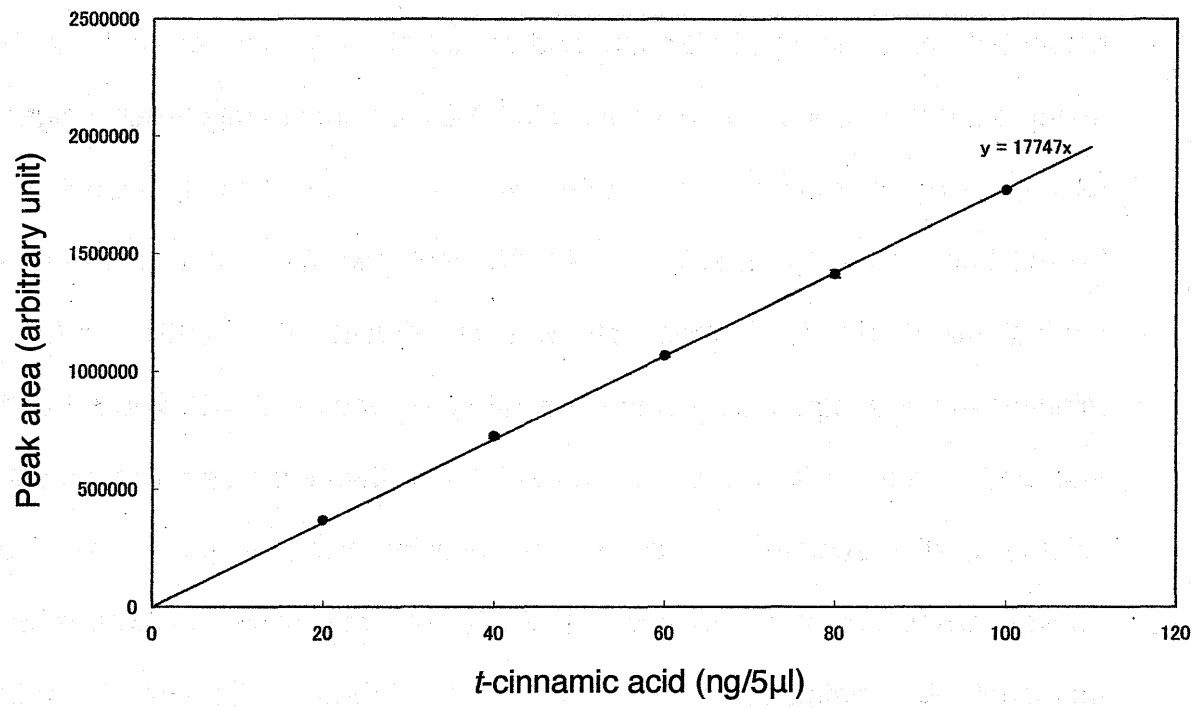

Fig. 12. The quantification line of *t*-cinnamic acid.

Authentic *t*-cinnamic acid at different concentrations was analyzed by high performance liquid chromatography obtaining the relationship between amount of *t*-cinnamic acid and peak area.

PAL activity was estimated as the nanomoles of *t*-cinnamic acid produced per hour per milligram of protein (nmol *t*-cinnamic acid h^{-1} mg^{-1} protein).

Extraction and purification of SA

The SA content was measured basically as described previously (Ogawa et al. 2007) with some modifications. The cotyledons of *P. nil* seedlings were harvested, frozen in liquid nitrogen, and stored at -80°C prior to analysis. The frozen plant tissues were homogenized in 70% methanol (5 ml/g fresh weight of tissue) using a mortar and pestle with 10 µg of deuterium-labeled 2-hydroxybenzoic-3,4,5,6-d_4 acid (d_4-SA, CDN Isotopes, Quebec, Canada) as an internal standard. The homogenate was centrifuged at 12,000 × g for 15 minutes, and the supernatant was evaporated *in vacuo* at 37 °C. The pH of the resulting aqueous phase was adjusted to 3.0 and then extrtacted with an equal volume of ethyl acetate three times. The resulting acidic ethyl acetate-soluble fraction was concentrated *in vacuo* at 37 °C and redissolved in 2 ml of 60% methanol. The methanol solution was passed through a Sep-Pak Plus C18 cartridge (Waters Corporation, Milford, Massachusetts, U.S.A.). The eluate was concentrated *in vacuo* at 37 °C to *ca.* 50 µl. The concentrated sample solution was injected into the HPLC system (Hitachi L-6200, Hitachi Co., Tokyo, Japan) equipped with an ODS column (3 mm × 250 mm, Imtakt Cadenza CD-C18, Imtakt Corp., Kyoto, Japan), and the reaction components were separated using 30% acetonitrile/ 0.1% formic acid at a flow rate of 0.15 ml min^{-1}. The column oven temperature was 40 °C. The absorbance at 285 nm was monitored. The eluate fraction with the same retention time as authentic SA was collected as the SA fraction (Fig. 13).

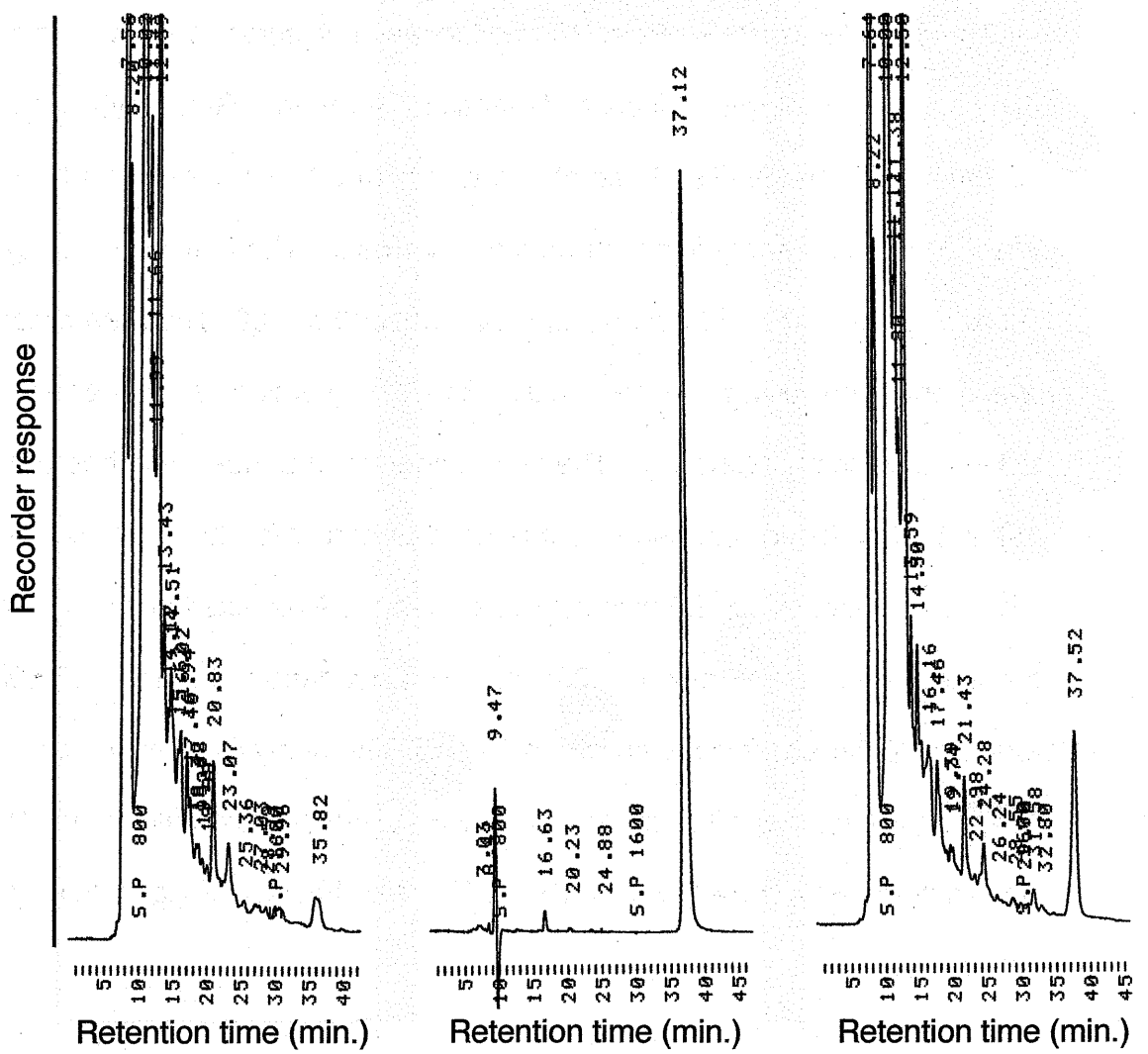

Fig. 13. High performance liquid chromatography (HPLC) of *Pharbitis nil* extract and salicylic acid (SA).

The sample extracted from *P. nil* leaves and purified by the Sep-Pak treatment (left), authentic SA (middle) and the mixture of them (right) were analysed by HPLC. The eluate fraction of which retention time as the same as SA was collected as the SA flaction to be analyzed by liquid chromatography-mass spectrometry.

Liquid chromatography-mass spectrometry (LC-MS)

The collected sample of the SA fraction was subjected to LC-MS using LTQ Orbitrap XL (Thermo Fischer Scientific, Bremen, Germany) with 30% acetonitrile as the mobile phase at a flow rate of 5 to 10 µl min^{-1} for 3 minutes. The ESI electron spray injection method was used. The ionization voltage was set to 5 kV, and the MS scan range was m/z 200 to 400. The data processing was performed using Xcalibur Qual Browser (version 2.1, Thermo Fisher Scientific, Bremen, Germany). The chromatogram and mass spectrogram of the authentic SA and d_4-SA used as internal standard are shown in Fig. 14. The amount of endogenous SA was estimated using the detected peak intensities of the internal standard (m/z 141.05) and the endogenous SA (m/z 137.02). An example of the analyses is shown in Fig. 15. The analysis of each sample was repeated 3 times, and the mean and standard errors were calculated. The LC-MS analysis was carried out by Dr. K. Kaneko, Faculty of Agriculture, Niigata University.

Results

Induction of flowering by poor-nutrition stress

P. nil seedlings were subjected to poor-nutrition stress by growing in 1/100-strength nutrient solution under non-inductive LD conditions for 1 to 3 weeks. Flowering started when the plants had been grown under the poor-nutrition

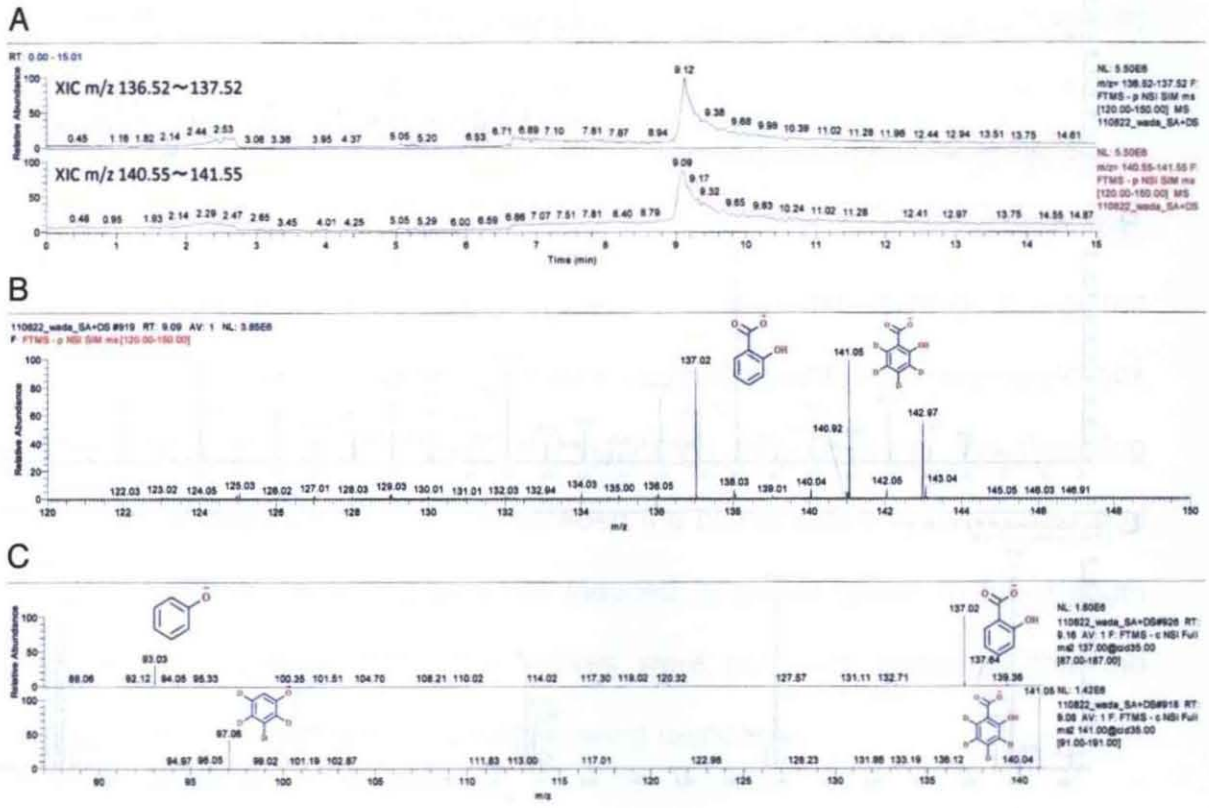

Fig. 14. Chlomatogram (A), full-mass spectrogram (B) and mass-mass spectrogram (C) of the mixture of authentic salicylic acid (SA) and duterium-labeled 2-hydroxybenzoic-3,4,5,6-d_4 acid (d_4-SA) that analyzed by liquid chromatography-mass spectrometry.

The anionic peak of SA and d_4-SA is m/z 137.02 and 141.05, respectively (B). The major fragment of each is m/z 93.03 and m/z 97.06, respectively (C).

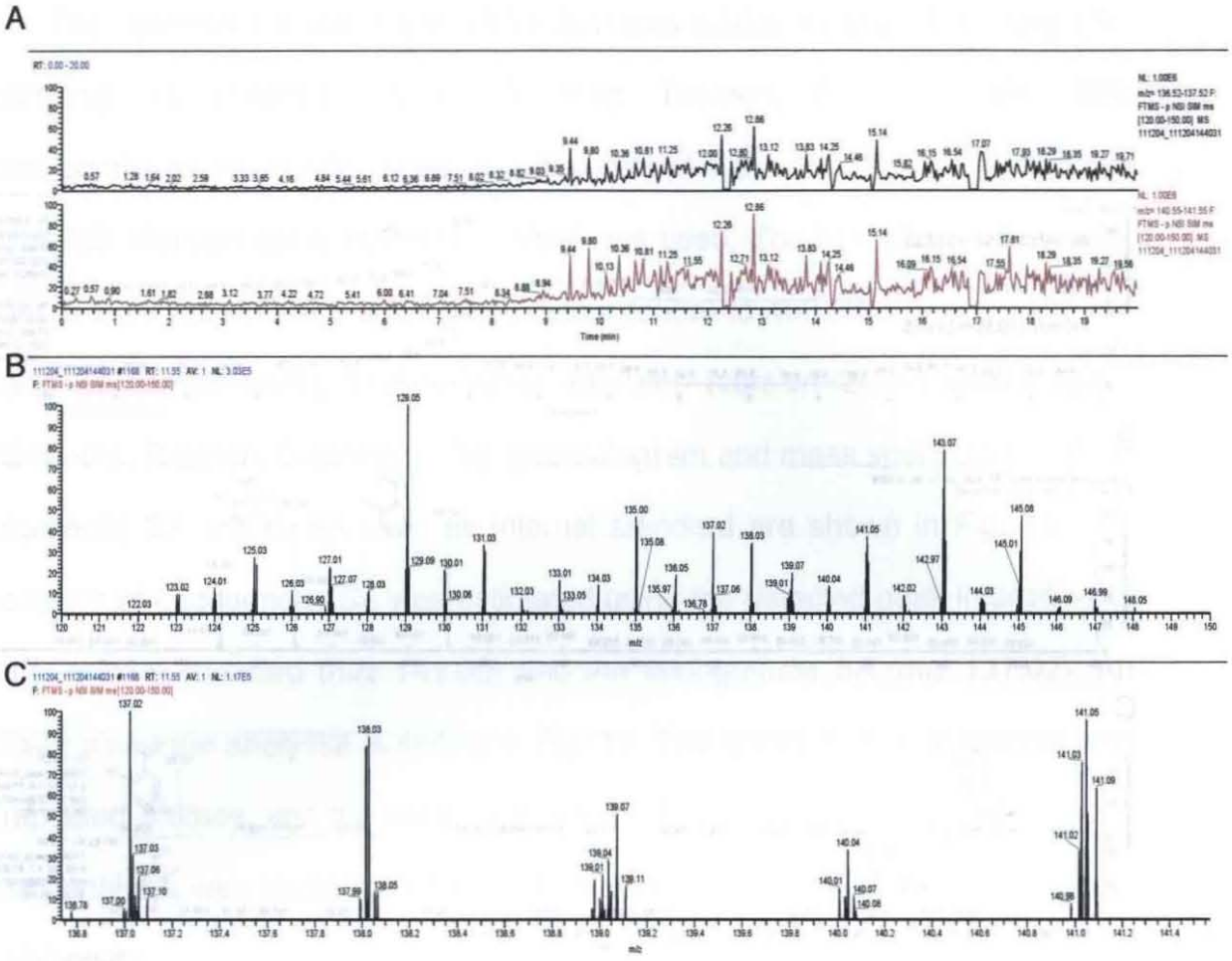

Fig. 15. Quantification of salicylic acid (SA) extracted from cotyledon of *Pharbitis nil* by liquid chromatography-mass spectrometry (LC-MS).

The sample extracted from cotyledons of *P. nil* containing 10μg of duterium-labeled 2-hydroxybenzoic-3,4,5,6-d_4 acid (d_4-SA) as internal standard was purifyed and analyzed by LC-MS; chlomatogram (A), full-mass spectrogram (B) and its enlarged view (C) are shown. The peak of m/z 137.02 and m/z 141.05 (C) is SA and d_4-SA, respectively.

conditions for 2 weeks, and all of the plants flowered when grown for 3 weeks (Table 18). The vegetative growth of the plants grown under poor-nutrition conditions was inhibited. Flowering never occurred for plants grown in full-strength nutrient solution even when they were grown for 3 weeks. Next, seedlings were grown as above, but newly formed true leaves were removed when fully expanded, leaving only the cotyledons on those seedlings. These plants were examined to determine whether they could respond to stress to flower. The plants with only cotyledons were induced to flower (Table 19). Flowering under the poor-nutrition conditions occurred starting during the second week, and almost all of the plants—both the plants with only cotyledons and the intact plants—flowered. No significant differences in the flowering response intensity were observed between the plants with only cotyledons and the intact plants. Flowering was not induced in plants grown in full-strength nutrient solution even if the true leaves were removed, indicating that the surgical treatment did not affect the flowering response.

PAL gene expression, PAL activity and SA content

P. nil plants were induced to flower by growing them in 1/100-strength nutrient solution under LD conditions for 10 to 20 days. The newly formed true leaves were removed when fully expanded, and the cotyledons were harvested at the end of the stress treatment to analyze *PAL* expression. The *PAL* expression was stronger in the plants that were induced to flower by poor-nutrition stress than in the control plants grown in standard nutrient solution (Fig. 16). The *PAL* expression in the plants grown in the poor-nutrient stress

Table 18. Flowering of *Pharbitis nil* cultivar Violet induced by poor-nutrition stress.

Seedlings of *P. nil* cultivar Violet were grown in 1/100-strength mineral nutrient solution (×1/100) or full-strength mineral nutrient solution (×1) under 16-hour light and 8-hour dark conditions for 1 to 3 weeks, and then transferred to full strength mineral nutrient solution, and grown further for 2 weeks. The data are means with standard errors. Values followed by the same superscript symbol do not differ significantly at the 5% level in a *t*-test within the same growth period.

Growth period (weeks)	Strength of nutrient solution	% flowering	Flower buds /plant	Nodes /plant	Stem length (mm)
1	×1	0	0±0	12±0.28[a]	464±23.7[a]
	×1/100	0	0±0	9.9±0.27[b]	303±19.3[b]
2	×1	0	0±0[a]	13±0.35[a]	528±25.9[a]
	×1/100	40	0.65±0.23[b]	11±0.21[b]	339±20.4[b]
3	×1	0	0±0[a]	13±0.60[a]	613±47.3[a]
	×1/100	100	1.7±0.13[b]	11±0.22[a]	360±25.4[b]

Table 19. Response of cotyledons to poor-nutrition stress to induce flowering in *Pharbitis nil* cultivar Violet.

Seedlings of *P. nil* cultivar Violet were grown in 1/100-strength mineral nutrient solution (×1/100) or full-strength mineral nutrient solution (1×) under 16-hour light and 8-hour dark conditions for 1 to 3 weeks. During the stress treatment, true leaves were removed when fully expanded, leaving only the cotyledons (Cotyledons only), or the seedlings were kept intact (Intact). After the stress treatment, the plants were transferred to full-strength mineral nutrient solution and grown for 2 more weeks. The data are means with standard errors. Values followed by the same superscript symbol do not differ significantly at the 5% level in a *t*-test within the same growth period.

Growth period (weeks)	Seedlings	Strength of nutrient solution	% flowering	Flower buds /plant	Nodes /plant	Stem length (mm)
1	Intact	×1	0	0±0	11 ± 0.40^{a}	442 ± 35.1^{ab}
		×1/100	0	0±0	10 ± 0.40^{b}	462 ± 19.3^{a}
	Cotyledons	×1	0	0±0	12 ± 0.38^{a}	445 ± 30.0^{ab}
	only	×1/100	0	0±0	10 ± 0.13^{b}	376 ± 18.0^{b}
2	Intact	×1	0	0 ± 0^{a}	12 ± 0.44^{ac}	503 ± 43.3^{ac}
		×1/100	90	1.1 ± 0.12^{b}	11 ± 0.24^{a}	440 ± 24.1^{bc}
	Cotyledons	×1	0	0 ± 0^{a}	15 ± 0.40^{b}	552 ± 29.7^{a}
	only	×1/100	90	1.5 ± 0.18^{b}	12 ± 0.22^{c}	377 ± 21.1^{b}
3	Intact	×1	0	0 ± 0^{a}	15 ± 0.61^{a}	728 ± 55.3^{a}
		×1/100	95	1.5 ± 0.14^{b}	12 ± 0.22^{b}	553 ± 20.2^{b}
	Cotyledons	×1	0	0 ± 0^{a}	15 ± 0.69^{a}	611 ± 45.2^{ab}
	only	×1/100	85	1.2 ± 0.16^{b}	12 ± 0.32^{b}	337 ± 25.1^{c}

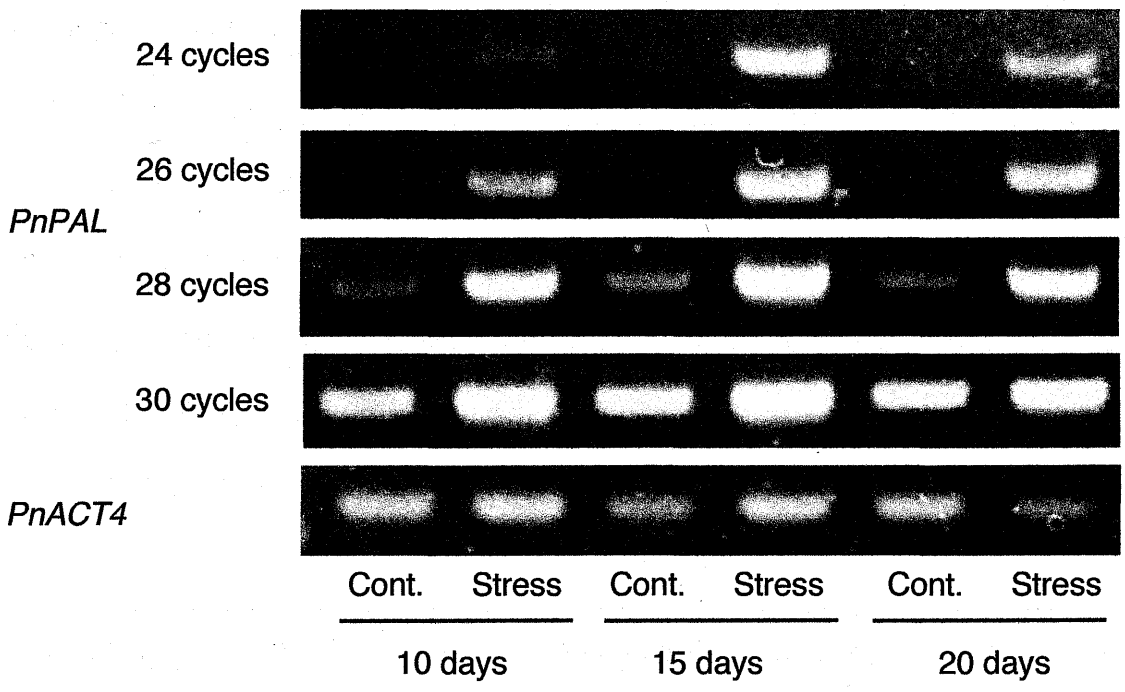

Fig. 16. Expression of the *PnPAL* gene in the cotyledons of *Pharbitis nil* cultivar Violet that flowered in response to poor-nutrition stress.

The seedlings of *P. nil* cultivar Violet were grown in 1/100-strength mineral nutrient solution (Stress) or full-strength mineral nutrient solution (Cont.) under 16-hour light and 8-hour dark conditions for 10, 15 or 20 days. The cotyledons were harvested after the stress treatment, and the level of *PnPAL* expression was determined by reverse transcription-polymerase chain reaction (24 to 30 cycles) with *PnACT4* as a control.

conditions increased from the 10th day to the 15th day and then decreased slightly. The expression level of *PAL* in the plants grown in the standard nutrient solution was almost the same regardless of the growth period.

Next, *P. nil* plants were induced to flower in the same manner as mentioned above except that the plants were grown for 1 to 3 weeks; then the PAL activity in the cotyledons was measured. The PAL activity was higher in the stressed plants than in the control plants (Fig. 17). The PAL activity increased with an increase in the growth period in the stressed plants, and the PAL activity in the control plants remained low throughout the growth period.

Finally, *P. nil* plants were induced to flower as above but were grown for 1 or 2 weeks, and the SA content in the cotyledons was measured. The SA content was higher in the stressed plants than in the control plants (Fig. 18). The SA content increased from the first week to the second week in the stressed plants.

Flower-inducing effect of exogenously applied SA

The effect of SA on the flowering of *P. nil* grown under non-stress conditions was examined under LD conditions. SA did not induce flowering of plants grown in mineral nutrient solution (Table 20). When the plants were given a weak stress by growing in tap water for only 16 days, the flowering response was enhanced a little by SA at 10^{-6} M.

Discussion

Poor-nutrition conditions resulting from cultivation in 1/100-strength nutrient

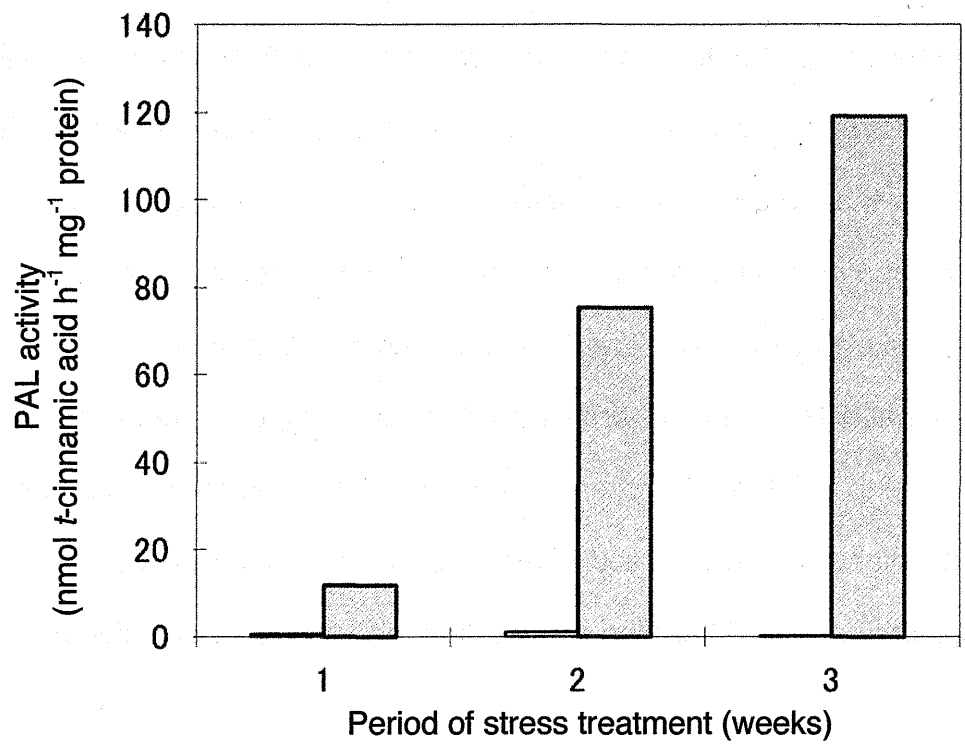

Fig. 17. The phenylalanine ammonia-lyase (PAL) activity in the cotyledons of *Pharbitis nil* cultivar Violet plants that flowered in response to poor-nutrition stress.

Seedlings of *P. nil* cultivar Violet were grown in 1/100-strength mineral nutrient solution (shaded column) or full-strength mineral nutrient solution (open column) under 16-hour light and 8-hour dark conditions for 1, 2 or 3 weeks. The cotyledons were harvested after the stress treatment, and the PAL activity was determined.

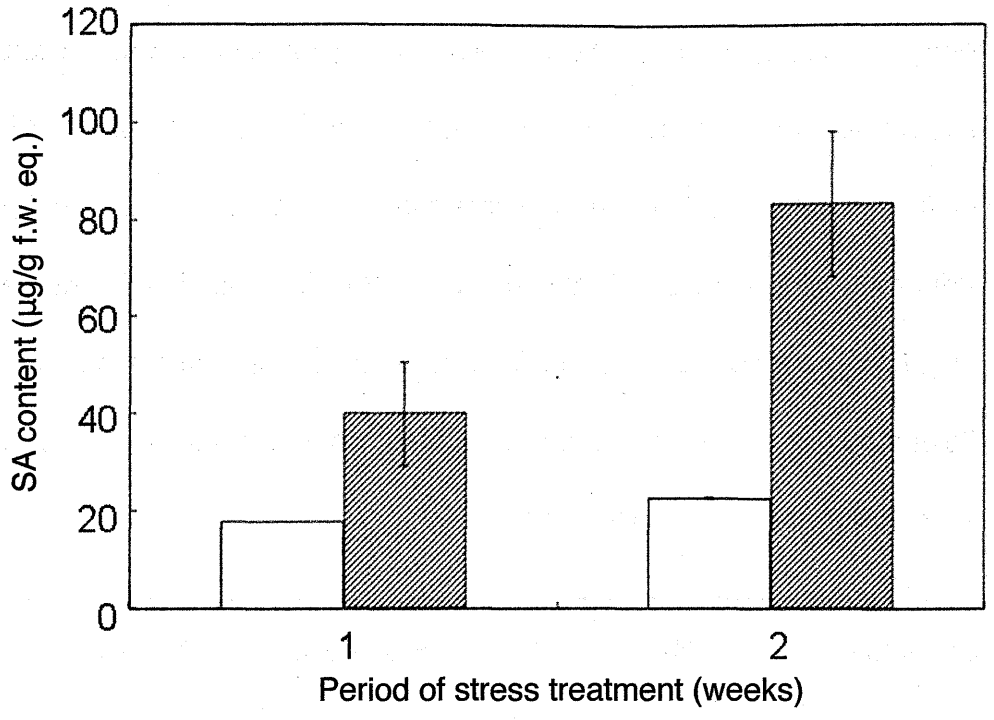

Fig. 18. The salicylic acid (SA) content in the cotyledons of *Pharbitis nil* cultivar Violet that flowered in response to poor-nutrition stress.

Seedlings of *P. nil* cultivar Violet were grown in 1/100-strength mineral nutrient solution (shaded column) or full-strength mineral nutrient solution (open column) under 16-hour light and 8-hour dark conditions for 1 or 2 weeks. The cotyledons were harvested after the stress treatment, and the SA content was determined by liquid chromatography-mass spectrometry. The data are means with standard errors.

Table 20. Effect of salicylic acid (SA) on flowering in *Pharbitis nil*.

Five-day-old seedlings of *P.nil* cultivar Violet were grown in nutrient solution (non-stress conditions) or in tap water (weak stress conditions) with SA added at different concentrations for 20 and 16 days, respectively, at 25 °C under 16-hour light long-day (LD) conditions. After the SA treatment, the plants were transferred to a fresh nutrient solution without SA and grown at 25 °C under LD for two weeks until the flowering responce was scored. The data are means with standard errors. Values followed by the same superscript symbol do not differ significantly at the 5 % level in a *t*-test within the same conditions.

Conditions	SA (M)	% flowering	Floral buds /plant	Nodes /plant	Stem length (mm)
non-stress	0	0	0±0	13±0.73^{a}	802±49.3^{a}
	10^{-6}	0	0±0	14±0.72^{a}	751±47.4^{a}
	10^{-5}	0	0±0	13±0.44^{a}	734±37.2^{a}
	10^{-4}	0	0±0	13±0.67^{a}	724±40.6^{a}
weak stress	0	100	1.8±0.14^{a}	11±0.27^{ab}	350±22.5^{ab}
	10^{-6}	95	2.6±0.25^{b}	11±0.22^{a}	317±21.6^{a}
	10^{-5}	100	2.1±0.18^{ab}	10±0.44^{b}	292±30.5^{a}
	10^{-4}	60	0.75±0.16^{c}	11±0.21^{b}	407±25.7^{b}

solution functioned as a stressor and induced flowering in *P. nil* cultivar Violet (Table 18), reproducing the previous results (Chapter I). The number of nodes per plant decreased under the poor-nutrition conditions, indicating that the development of true leaves was delayed in the stressed plants. A true leaf at the certain node position of the stressed plant may have been formed later than the corresponding leaf at the same node position of the control plant. Therefore, the developmental stages (or physiological ages) of these two leaves may be different even if they were harvested on the same day. In contrast, the developmental stages (or physiological ages) of the cotyledons were the same between the stressed and the control plants because uniformly grown seedlings were selected for use at the start of the stress treatment. Therefore, the cotyledons are more suitable experimental materials than true leaves for the physiological comparison of stressed and control plants. However, the cotyledons of the control plants withered and died earlier than those of the stressed plants because true leaves developed faster and became sources of photosynthetic assimilates instead of the cotyledons sooner in the control plants than in the stressed plants. Accordingly, we removed the true leaves so that cotyledons were kept functional for longer period. The plants with only cotyledons responded to stress and were induced to flower to the same extent as the intact plants (Table 19), indicating that cotyledons can play a regulatory role in stress-induced flowering. Therefore, the cotyledons were used as the experimental material for the analyses of *PAL* gene expression, PAL activity and SA content.

SA is synthesized from *t*-cinnamic acid, which is produced from phenylalanine by PAL in the majority of plant species (Yalpani et al. 1993). SA is

also derived from isochorismate in bacteria and some plant species, including *A. thaliana* (Chen et al. 2009). Generally, stress induces PAL activity, resulting in the accumulation of SA (Dixon and Paiva 1995, Scott et al. 2004). PAL inhibitors suppressed the stress-induced flowering in *P. nil* and *P. frutescens*, and this inhibition was overcome by SA (Chapters I and II). These results lead us to hypothesize that stress-induced flowering is regulated by SA that is synthesized in the pathway mediated by PAL. Therefore, we analyzed PAL and SA in *P. nil.*

The *PAL* gene expression, the PAL enzyme activity and the SA content in the cotyledons all increased when flowering was induced by poor-nutrition stress (Figs. 16 to 18). These results suggest that poor-nutrition stress-induced flowering in *P. nil* is induced by SA, the synthesis of which is promoted by PAL. Our results provide the first direct evidence revealing the involvement of SA in the regulatory mechanism of stress-induced flowering in *P. nil.*

Phenylpropanoids, such as chlorogenic acid, which are derived from *t*-cinnamic acid via *p*-coumaric acid have been reported to increase when *P. nil* was induced to flower under poor-nutrition or low-temprature conditions (Hirai et al. 1993, 1994, Shinozaki et al. 1988a, b, 1994). Dihydrokaempferol-7-*O*-D-glucoside which is derived from the pathway from *t*-cinnamic acid to anthocyanin via *p*-coumaric acid has been reported to promote the flowering of *P. nil* (Nakanishi et al. 1995). Among the metabolic intermediates derived from *t*-cinnamic acid, benzoic acid was shown to counteract the inhibitory effect of AOA under stress conditions, whereas *p*-coumaric acid did not (Hatayama and Takeno 2003). These results suggest that phenylpropanoids and dihydrokaempferol-7-*O*-D-glucoside are not involved in the stress-induced flowering. AOA also inhibits 1-aminocyclopropane-1-carboxylic acid (ACC)

synthase. ACC synthase catalyzes the conversion of S-adenosylmethionine to ACC, which is converted to ethylene. However, the involvement of ethylene in the stress-induced flowering is excluded, because flowering was completely inhibited in the presence of ACC (Hatayama and Takeno 2003). This is consistent with the observation that ethylene inhibits the photoperiodic flowering of *P. nil* (Suge 1972). Recentry, AOA and AOPP were reported to inhibit the biosynthesis of indole-3-acetic acid (IAA) (Soeno et al. 2010). However, IAA is known to inhibit flowering of *P. nil* (Takeno 1996).

Stress increases PAL activity and induces SA biosynthesis (Borsani et al. 2001, Larkindale et al. 2005, Mateo et al. 2006, Rasmussen et al. 1991, Scott et al. 2004, Wen et al. 2005). Stress also induces reactive oxygen species (Leon et al. 1995, Okuda et al. 1991), and reactive oxygen species promote the conversion of benzoic acid to SA (Gidrol et al. 1996, Leon et al. 1995, Mauch-Mani and Slusarenko 1996, Neuenschwander et al. 1995, Summermatter et al. 1995). SA has been the focus of intensive research due to its function as an endogenous signal (Rivas-San Vicente and Plasencia 2011). Flowering-inducing activity is one of the activities of SA. Cleland and Ajami (1974) found that *Lemna gibba* can be induced to flower by SA. It has been shown that SA and related compounds, including the SA precursor benzoic acid, induce the flowering of *L. gibba, L. paucicostata* and some other Lemnaceous species (Kandeler 1985). However, SA is not considered to be an endogenous flower-regulatory factor in *Lemna* spp. because the endogenous level of benzoic acid is not altered by photoperiodic conditions (Fujioka et al. 1983). In our laboratory, it was recently found that *L. paucicostata* is also induced to flower by poor-nutrition stress, and higher amounts of SA were detected in the flowered plants than in the control

plants (Shimakawa et al. submitted). SA may be the endogenous flower-regulatory factor of stress-induced flowering but not of photoperiodic flowering.

UV-C light stress promoted flowering in wild-type *A. thaliana* but not in SA-deficient *NahG* transgenic plants (Martínez et al. 2004). UV-C light irradiation increased the expression of the SA-responsive *PR1* gene in the wild-type plants but not in the *NahG* plants. The level of the transcript of the *SA induction deficient 2/isochorismate synthase 1* gene encoding the SA biosynthetic enzyme increased under UV-C light irradiation in the wild-type plants but not in the *NahG* plants. These results suggest the involvement of SA in the UV-C stress-induced flowering in *A. thaliana*, supporting our conclusion that SA is involved in the stress-induced flowering in *P. nil*.

The present result that flower-inducing stress treatment increased the endogenous SA level strongly suggests that the stress-induced flowering of *P. nil* is regulated by SA, a conclusion that is supported by the previous results showing that a reduction in the endogenous SA level by a PAL inhibitor inhibited stress-induced flowering and that this inhibition was overcome by SA (Chapter I). However, exogenously applied SA did not induce flowering of *P. nil* under non-stress conditions (Table 20). This experiment was carried out in the same manner as that shown in Table 6 of the Chapter I where SA effectively promoted flowering in the AOA-treated plants. Therefore, the possibilities that SA was not incorporated into the assay plants or was deactivated in the experimental system can be excluded. The decrease in the flowering response of the plants treated with 10^{-4} M SA under weak stress conditions (Table 20) may be due to the toxic effect of SA at high concentration. This argues against the possibility that the concentration of SA used in this experiment was too low to induce

flowering. Therefore, we conclude that exogenously applied SA does not induce flowering. SA may be necessary but not sufficient to induce flowering. Stress may induce the production of not only SA but also other factors necessary to induce flowering.

General discussion

It is apparent that plants can flower in response to several conditions. Stresses generally have negative effects on growth and development, and therefore plants have evolved to establish protection and adaptation strategies to minimize stress influences. However, the protection or adaptation mechanism may not be sufficient if the stress is too severe. Precocious flowering may assist in species preservation under such conditions. Thus, stress-induced flowering can be regarded as an ultimate adaptation to stress and should be considered a central component, along with tolerance, resistance and avoidance, of stress physiology. The concept of stress-induced flowering has not been proposed before Hatayama and Takeno (2003). The previous papers which reported flowering responses other than photoperiodic flowering and vernalization (Table 1) did not mention that such flowering was induced by stress although we can now consider the involvement of stress in these flowering response. Recently, the concept and the term of stress-induced flowering are becoming to be accepted in the area of flowering physiology (Blanvillain et al. 2011, Dezar et al. 2010, Marín et al. 2010, Rivas-San Vincente and Plasencia 2011, Segarra et al. 2010, Yaish et al. 2011). Therefore, stress-induced flowering may be a universal phenomenon in the plant kingdom.

Stress can be simply defined as a situation in which vegetative growth of plants is suppressed. Flowering is the change from vegetative growth to reproductive growth. Therefore, it is quite natural that flowering is accelerated by the suppression of vegetative growth by stress. The plants induced to flower by stresses produced fertile seeds and the progeny developed normally in both

of *P. nil* and *P. frutescens*. Plants can modify their developmental processes to adapt to stress conditions. Stress-induced flowering is one of such adaptation to stress. 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 conditions. The stressed plants do not need to await the arrival of a season when photoperiodic conditions are suitable for flowering, and such precocious flowering might assist in species preservation. Thus, stress-induced flowering might have a biological benefit and should be considered as important as photoperiodic flowering and vernalization. This idea is supported by the recent change in the understanding of shade avoidance responses. The most typical phenotype of the shade avoidance response is rapid stem elongation, but recent articles report that an important component of the shade avoidance syndrome is an acceleration of flowering observable in all shade-avoiding plants (Adams et al. 2009). Accelerated flowering and seed production under unfavorable environments increases the probability of the survival of the individual and therefore of the species. This is true also in flowering induced under stress conditions.

It was revealed that SA of which biosynthesis is regulated by PAL is involved in stress-induced flowering of *P. nil*. On the other hand, it is still unknown whether the flowering response correlated positively with *PAL* expression, PAL activity and SA content in *P. frutescens*. Further analyses in *P. frutescens* are necessary. One of the most important questions is why exogenous SA does not induce flowering in *P. nil*. As discussed above, SA may work together with some other factors to induce flowering. A possible candidate of such a factor is DNA demethylation. Low-temperature stress induced DNA demethylation in *Zea*

mays (Steward et al. 2002). Low temperature can be replaced by DNA demethylation to induce flowering in vernalization-requiring plants (Burn et al. 1993). Among the plants of which flowering is regulated by photoperiodism, *P. frutescens* and *P. nil* were induced to flower by DNA demethylation without SD treatment (Iwase et al. 2010, Kondo et al. 2006, 2007, 2010). Stress may induce not only SA synthesis but also DNA demethylation both of which may act together to induce flowering. Other stress substances are also possible candidate. Jasmonic acid is a stress hormone (Reymond and Farmer 1998), which induces flowering of *P. nil* (Yoshihara et al. 2000), *Spirodela polyrrhiza* (Krajncic and Nemec 1995), *Lemna minor* (Krajncic et al. 2006). Another stress substance abscisic acid (ABA) promotes floral evocation of *P. nil* (Takeno and Maeda 1996) and flowering of *L. minor* (Krajncic 1985). It is also conceivable that stress enhances the sensitivity to SA. In the induction of the systemic acquired resistance in potato, injury stress did not change the endogenous SA level but increased the sensitivity to SA (Yu et al. 1997). SA is not essential for seed germination of *A. thaliana*, but exhibited the promotive activity only under salt stress conditions (Lee et al. 2010). If SA acts only under stress conditions or SA sensitivity is enhanced by stress, it can explain the reason why exogenously applied SA did not induce flowering under non-stress conditions.

Interaction between SA and gene expression should be also considered. The proteins derived from the floral pathway integrator gene *FLOWERING LOCUS T* (*FT*) and its orthologs were reported to be florigens in *A. thaliana*, rice and cucurbits (Corbesier et al. 2007, Lin et al. 2007, Tamaki et al. 2007). Two orthologs 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). We already reported that the expression of *PnFT2* was induced in the plants that flowered under the poor-nutrition conditions, whereas *PnFT1* was not expressed regardless of the nutritional conditions (Wada et al. 2010b). These results suggest that *PnFT2*, but not *PnFT1*, is involved in the stress-induced flowering of *P. nil*. SA application induced the expression of *A. thaliana FT* and sunflower *HAFT*, an ortholog of *FT*, (Dezar et al. 2010, Martínez et al. 2004), indicating that *FT* and SA may collaborate to regulate flowering. In *P. nil*, stress may induce both SA biosynthesis and *PnFT2* expression, and SA and the PnFT2 protein may act together to induce flowering. The requirement of both SA and the PnFT2 protein can explain why SA application alone did not induce flowering under the non-stress conditions. The analyses of the interaction between *PnFT2* and SA are necessary.

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3. Wada KC, Kaneko K, Shiraya T, Mitsui T, Takeno K. Poor-nutrition stress enhances the enzyme activity and gene expression of phenylalanine ammonia-lyase and endogenous content of salicylic acid to induce flowering in *Pharbitis nil.* (submitted)

Related publications

1. Kondo H, Miura T, Wada KC, Takeno K (2007) Induction of flowering by 5-azacytidine in some plant species: Relationship between the stability of photoperiodically induced flowering and flower-inducing effect of DNA demethylation. Physiol Plant 131: 462-469
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