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Stress and salicylic acid induce the expression of *PnFT2* in the regulation of the stress-induced flowering of *Pharbitis nil*

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ABSTRACT

Poor nutrition and low temperature stress treatments induced flowering in the Japanese morning glory *Pharbitis nil* (synonym *Ipomoea nil*) cv. Violet. The expression of *PnFT2*, one of two homologs of the floral pathway integrator gene *FLOWERING LOCUS T* (*FT*), was induced by stress, whereas the expression of both *PnFT1* and *PnFT2* was induced by a short-day treatment. There was no positive correlation between the flowering response and the homolog expression of another floral pathway integrator gene *SUPPRESSOR OF OVEREXPRESSION OF CO1* and genes upstream of *PnFT*, such as *CONSTANS*. In another cultivar, Tendan, flowering and *PnFT2* expression were not induced by poor nutrition stress. Aminooxyacetic acid (AOA), a phenylalanine ammonia-lyase inhibitor, inhibited the flowering and *PnFT2* expression induced by poor nutrition stress in Violet. Salicylic acid (SA) eliminated the inhibitory effects of AOA. SA enhanced *PnFT2* expression under the poor nutrition stress but not under non-stress conditions. These results suggest that SA induces *PnFT2* expression, which in turn induces flowering; SA on its own, however, may not be sufficient for induction.

Introduction

Flowering in many plants is regulated by environmental cues, such as night length in photoperiodic flowering and temperature in vernalization (Thomas and Vince-Prue, 1997). Recently, stress has also been recognized as a cue to induce flowering. The short-day (SD) plant Japanese morning glory *Pharbitis nil* (synonym *Ipomoea nil*) flowered under long-day (LD) conditions when grown under poor nutrition or low temperature stress conditions (Shinozaki et al., 1988; Hirai et al., 1994; Hatayama and Takeno, 2003; Wada et al., 2010b). The SD plants *Lemna paucicostata* (synonym *Lemna aequinoctialis*) and *Perilla frutescens* var. *crispa* flowered under poor nutrition and low intensity light stress conditions, respectively (Wada et al., 2010a; Shimakawa et al., 2012). Ultraviolet (UV) light (Martínez et al., 2004) and poor nutrition (Kolář and Seňková, 2008) stresses induced early flowering in the LD plant *Arabidopsis thaliana*. Similar non-photoperiodic flowering has been sporadically reported in various plant species, although the authors of these studies did not claim that the flowering was induced by stress. A review of those reports suggested that most of the factors responsible for flowering can be regarded as stress (Wada and Takeno, 2010; Takeno, 2012). The plants that were induced to flower by stress produced fertile seeds, and the progeny developed normally (Wada et al., 2010a,b). Plants can modify their developmental processes to adapt to stress conditions, and stress-induced flowering is one such adaptation. Stressed plants are not dependent on seasons in which photoperiodic conditions are suitable for flowering. Plants tend to flower as an emergency response when stressed, thereby ensuring their ability to produce offspring. Through this mechanism the preservation of plant species, even under unfavorable environmental conditions, is ensured. Therefore, stress-induced flowering can be considered as universal and important as photoperiodic flowering and vernalization (Wada and Takeno, 2010; Takeno, 2012).

The molecular basis of the regulation of stress-induced flowering is not well understood. The flowering of *A. thaliana* is induced by LD, vernalization, autonomous cues and gibberellins, and these factors operate through a common pathway integrated by the floral pathway integrator gene *FLOWERING LOCUS T* (*FT*) (Boss et al., 2004). UV-C induced the expression of *FT*, indicating that UV-C stress-induced flowering is also mediated by *FT* (Martínez et al., 2004). Thus, all of the factors known to induce flowering of *A. thaliana* function by activating *FT* expression, suggesting that the *FT* homolog could also be involved in the stress-induced flowering of *P. nil*. Two orthologs of *FT*, *PnFT1* and *PnFT2*, have been identified

Abbreviations: AOA, aminooxyacetic acid; *CO*, *CONSTANS*; *FLC*, *FLOWERING LOCUS C*; *FRI*, *FRIGIDA*; *FT*, *FLOWERING LOCUS T*; LD, long-day; PAL, phenylalanine ammonia-lyase; RT-PCR, reverse transcription-polymerase chain reaction; SA, salicylic acid; SD, short-day; *SOC1*, *SUPPRESSOR OF OVEREXPRESSION OF CO1*; UV, ultraviolet.

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in *P. nil*, and these genes are expressed under inductive SD conditions to promote flowering (Hayama et al., 2007). We reported that poor nutrition stress induced the expression of *PnFT2* but not *PnFT1* (Wada et al., 2010b). *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*) functions as another floral pathway integrator gene in *A. thaliana* (Lee and Lee, 2010). *SOC1* may also be involved in the stress-induced flowering of *P. nil*. Although no *SOC1* homologs have been reported in *P. nil*, one such homolog, *IbAGL20*, is known in *Ipomoea batatas*, a close relative of *P. nil* (Kim et al., 2005). The inductive LD conditions induce the expression of *CONSTANS* (*CO*), whose product directly induces the transcription of *FT* in *A. thaliana* (Suárez-López et al., 2001; Valverde et al., 2004). Therefore, *CO* may be involved in the regulation of stress-induced flowering where *FT* is involved. The *P. nil* homolog of *CO*, *PnCO*, is known to complement the *co* mutation in *A. thaliana* (Liu et al., 2001). Furthermore, several genes are known to regulate the *FT* transcription upstream of *FT* in the flowering gene regulatory network in *A. thaliana* (Bernier and Périlleux, 2005; Amasino, 2010).

Stress induces phenylalanine ammonia-lyase (PAL) activity, resulting in the accumulation of salicylic acid (SA) (Dixon and Paiva, 1995; Scott et al., 2004). The PAL inhibitor aminooxyacetic acid (AOA) suppressed the stress-induced flowering in *P. nil*, and this inhibition was overcome by SA (Wada et al., 2010b). Poor nutrition stress-induced flowering in *L. paucicostata* was inhibited by AOA and another PAL inhibitor L-2-aminooxy-3-phenylpropionic acid, and a higher amount of SA was detected in the plants that flowered due to stress than in the vegetative plants (Shimakawa et al., 2012). Exogenously applied SA induces flowering in *L. paucicostata* under non-inductive photoperiodic conditions (Cleland and Ajami, 1974; Kandeler, 1985). UV-C light stress promoted flowering in *A. thaliana*, and the flowering response was weaker in the SA-deficient *NahG* transgenic lines than in the wild type, suggesting that SA is involved in this flowering response (Martínez et al., 2004). SA induced expression of *FT* in *A. thaliana* (Martínez et al., 2004) and of *HAFT*, an ortholog of *FT*, in sunflower (Dezar et al., 2011), indicating that *FT* and SA may interact to regulate flowering.

We hypothesized that stress-induced flowering is regulated by *PnFT* whose expression is induced by SA. Accordingly, we examined whether *PnFT1*, *PnFT2*, a *P. nil* homolog of *SOC1*, *PnCO* and several genes upstream of *FT* were expressed when *P. nil* flowered due to stress. Furthermore, we examined the influences of both a PAL inhibitor and SA on *PnFT* gene expression in *P. nil*.

Materials and methods

Plant materials and growth conditions

The short-day (SD) plant Japanese morning glory [*Pharbitis nil* (L.) Chois., synonym *Ipomoea nil* (L.) Roth)] cvs. Violet and Tendan were used. Violet and Tendan seeds were originally provided by Marutane Co. (Kyoto, Japan) and National Institute of Genetics (Mishima, Japan), respectively. The seeds were treated with concentrated H_2SO_4 for 25–40 min, washed with running tap water for 1 h and then soaked in tap water overnight. The swollen seeds were placed on moist filter paper in a Petri dish and were germinated at 25 °C under 16-h light and 8-h dark long-day (LD) conditions for 1 d. The germinated seeds were planted on 0.6% plain agar medium and grown for 5 d. The seedlings were then transferred to glass tubes (15 mm in diameter × 150 mm high) containing a mineral nutrient solution (Kondo et al., 2006), where they were grown under the same conditions described above. White light (55–90 μmol m^{-2} s^{-1}) was provided by fluorescent lamps (FL20SW or FL40SSW/37, Toshiba Corporation, Tokyo, Japan). For SD treatment, 5-day-old seedlings were given a single 16-h dark treatment. After the SD treatment, the seedlings were transferred to the LD conditions and grown for 2 weeks until the flowering response was scored.

Stress treatment

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

Treatment with chemicals

Aminooxyacetic acid (AOA) and/or salicylic acid (SA) (both Wako Pure Chemicals Industries, Osaka, Japan) were dissolved in the culture solution, and 5-day-old seedlings were grown in these solutions for the designated time periods. After the treatment, seedlings were transferred to the nutrient solution without chemicals, and in this solution, they were grown for 2 weeks until the flowering response was scored.

Scoring of the flowering response

All of the plant nodes were dissected under a binocular microscope to determine whether floral buds or vegetative buds formed. The percentage of plants, of all the plants in a treatment, with at least one flower bud (% flowering), and the number of flower buds per plant were determined. The number of nodes (i.e., the total number of flower buds and vegetative buds per plant), as well as the average length of the main stem, were used as indicators of vegetative growth. Twenty plants were used for each treatment. Each experiment was repeated at least three times. Similar results were obtained from all independent biological replicates, and the means with standard errors from one representative experiment are presented.

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

Plants were collected at the end of each treatment, frozen in liquid nitrogen, and stored at −80 °C prior to analysis. Total RNA was isolated from plant tissues using the Plant RNA Purification Reagent (Invitrogen Corporation, Carlsbad, CA, USA), and cDNAs were synthesized from each RNA sample using ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan). *PnFT1* and *PnFT2* were amplified from the cDNAs using primers designed according to a previous report (Hayama et al., 2007). The products of *PnFT1* and *PnFT2* amplification were separated on an agarose gel with the gene encoding actin, *PnACT4*, used as a loading control. The gels were visualized using EDAS 290 (Invitrogen Corporation, Carlsbad, CA, USA). The expression of each gene was normalized to that of *PnACT4* and shown as a relative value. The RT-PCR procedure was repeated three times in some analyses, and the means with standard errors, as well as a representative gel image, are shown. The PCR products corresponding to *PnFT1* and *PnFT2* were extracted from the agarose gel, and the nucleotide sequences were determined. All sequences were consistent with previously reported gene sequences (Hayama et al., 2007). RT-PCR analyses of the homologs of *SUPPRESSOR OF OVEREXPRESSION OF CO1*, *CO* (*PnCO*), *FLOWERING LOCUS C* (*FLC*), *FCA*, *FRIGIDA* (*FRI*) and *FVE* (*PnFVE*) were also performed in a manner similar to that described above, with some modification. The nucleotide sequences of the primers used are shown in Table 1.