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Stress enhances the gene expression and enzyme activity of phenylalanine ammonia-lyase and the endogenous content of salicylic acid to induce flowering in pharbitis

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ABSTRACT

The involvement of salicylic acid (SA) in the regulation of stress-induced flowering in the short-day plant pharbitis (also called Japanese morning glory) *Ipomoea nil* (formerly *Pharbitis nil*) was studied. Pharbitis cv. Violet was induced to flower when grown in 1/100-strength mineral nutrient solution under non-inductive long-day conditions. All fully expanded true leaves were removed from seedlings, leaving only the cotyledons, and flowering was induced under poor-nutrition stress conditions. This indicates that cotyledons can play a role in the regulation of poor-nutrition stress-induced flowering. The expression of the pharbitis homolog of *PHENYLALANINE AMMONIA-LYASE*, the enzyme activity of phenylalanine ammonia-lyase (PAL; E.C. 4.3.1.5) and the content of SA in the cotyledons were all up-regulated by the stress treatment. The Violet was also induced to flower by low-temperature stress, DNA demethylation and short-day treatment. Low-temperature stress enhanced PAL activity, whereas non-stress factors such as DNA demethylation and short-day treatment decreased the activity. The PAL enzyme activity was also examined in another cultivar, Tendan, obtaining similar results to Violet. The exogenously applied SA did not induce flowering under non-stress conditions but did promote flowering under weak stress conditions in both cultivars. These results suggest that stress-induced flowering in pharbitis is induced, at least partly, by SA, and the synthesis of SA is promoted by PAL.

Introduction

Flowering is mainly regulated by environmental cues, such as night length in photoperiodic flowering and temperature in vernalization, but stress also regulates flowering. The short-day (SD) plant pharbitis (also called Japanese morning glory) *Ipomoea nil* (formerly *Pharbitis nil*) can be induced to flower under long-day (LD) conditions when grown under poor-nutrition or low-temperature stress conditions (Wada et al., 2010b; Yamada and Takeno, 2014). *Lemna paucicostata* (synonym *Lemna aequinoctialis*) is also induced to flower by poor-nutrition stress conditions (Shimakawa et al., 2012). *Perilla frutescens* var. *crispa* flowers under low-intensity light stress (Wada et al., 2010a). Ultraviolet-C (UV-C) light, poor-nutrition and drought stresses induce early flowering in *Arabidopsis thaliana* (Martínez et al., 2004; Kolář and Seňková, 2008; Riboni et al., 2013). Similar non-photoperiodic flowering has been reported in several plant species, and most of the factors responsible for such flowering can be regarded as stress (Wada and Takeno, 2010; Takeno, 2012). Thus, stress-induced flowering is widely conserved in flowering plants. The plants that were induced to flower by stress produced fertile seeds, and the resulting progeny developed normally (Wada et al., 2010a,b). If plants are stressed, they flower as an emergency response without waiting for the arrival of a season when photoperiodic conditions are suitable for flowering. This response ensures the ability to produce the next generation so that plant species persist through unfavorable

Abbreviations: AOA, aminooxyacetic acid; *CO*, *CONSTANS*; *FT*, *FLOWERING LOCUS T*; HPLC, high performance liquid chromatography; 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 CO 1*; UV-C, ultraviolet-C.

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environmental conditions. Therefore, stress-induced flowering can be considered as important as photoperiodic flowering and vernalization (Wada and Takeno, 2010; Takeno, 2012).

The expression of *PnFT2*, one of two pharbitis 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 (Wada et al., 2010b; Yamada and Takeno, 2014). There was a positive correlation between the degree of the flowering response and the expression level of *PnFT2*. These results suggest that *PnFT2*, but not *PnFT1*, is the major regulatory gene involved in stress-induced flowering of pharbitis. The expression of *FT* was induced by UV-C, indicating that UV-C stress-induced flowering is also mediated by *FT* (Martínez et al., 2004). There was no positive correlation between the flowering response and the homolog expression of another floral pathway integrator gene, *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) (Yamada and Takeno, 2014). The expression of *SOC1* was not induced by UV-C stress in *A. thaliana* (Martínez et al., 2004). These results suggest that *SOC1* is not involved in the regulation of stress-induced flowering. 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). It has been proposed that the CO/FT regulatory module (*i.e.*, CO protein activates *FT* transcription) is highly conserved in both dicot and monocot plants (Matsoukas et al., 2012). The expression of *CO* was also moderately induced in UV-C stress-induced flowering in *A. thaliana* (Martínez et al., 2004). In contrast to these reports, *PnCO*, the pharbitis homolog of *CO*, was constitutively expressed in pharbitis, regardless of flowering status (Yamada and Takeno, 2014). This result suggests that *PnCO* may not be involved in the regulation of stress-induced flowering of pharbitis, which is consistent with the previous report that *PnFT* mRNA abundance was not related to *PnCO* expression. Therefore, *PnFT* may not be regulated by the PnCO protein in pharbitis (Hayama et al., 2007; Higuchi et al., 2011).

Stress-induced flowering is accompanied by the accumulation of anthocyanin, which may indicate the involvement of a metabolic pathway relating to anthocyanin synthesis in flowering. The key enzyme that regulates anthocyanin synthesis is phenylalanine ammonia-lyase (PAL; E.C. 4.3.1.5). The PAL activity increases when plants are stressed (Dixon and Paiva, 1995; Scott et al., 2004). Furthermore, PAL catalyzes the conversion of phenylalanine to *t*-cinnamic acid, and salicylic acid (SA) is derived from *t*-cinnamic acid (Yalpani et al., 1993). We found that aminooxyacetic acid (AOA) and L-2-aminooxy-3-phenylpropionic acid, which function as PAL inhibitors (Kessmann et al., 1990; Appert et al., 2003), inhibited stress-induced flowering. This inhibitory effect was negated by *t*-cinnamic acid and SA in pharbitis (Hatayama and Takeno, 2003; Wada et al., 2010b). These facts suggest that SA, the synthesis of which is regulated by PAL, is involved in the regulatory mechanism of stress-induced flowering in pharbitis (Wada et al., 2013). However, no evidence has yet indicated that PAL activity and endogenous SA levels increase when pharbitis plants are induced to flower by stress. Accordingly, this study examined whether the gene expression and enzyme activity of PAL and the endogenous SA content increase when flowering is induced by stresses. We also examined whether SA induces flowering when exogenously applied to pharbitis.

Materials and methods

Plant materials and growth conditions

The SD plant pharbitis (also called Japanese morning glory) *Ipomoea nil* (L.) Roth (formerly *Pharbitis nil* (L.) Chois.) cvs. Violet and Tendan were used. Violet seeds (Q0079) were provided by Marutane Co. (Kyoto, Japan) and the Morning Glory Stock Center of Kyushu University (Fukuoka, Japan) with support, in part, by the National Bio-Resource Project of the Ministry of the Education, Culture, Sports, Science and Technology in Japan. The Tendan seeds were originally obtained from National Institute of Genetics (Mishima, Japan). The seeds were treated with concentrated H_2SO_4 for 35–70 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 LD conditions for 1 d. The germinated seeds were planted on 0.6% plain agar medium and grown for 5 d. Then, the seedlings were transferred to glass tubes (15 mm in diameter × 150 mm high) containing a mineral nutrient solution (Kondo et al., 2006) and grown under the same conditions. 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-d-old seedlings were given a single 16-h dark treatment. After the SD treatment, the seedlings were transferred to LD and grown for 2 weeks until flowering response was scored.

Stress treatments

Five-d-old seedlings were grown in 1/10- or 1/100-strength nutrient solution instead of full-strength nutrient solution for poor-nutrition stress treatment. For low-temperature treatment, the 5-d-old seedlings were moved into a growth cabinet at 15 °C and grown for 2 weeks. After the stress treatment, the seedlings were transferred to the normal full-strength nutrient solution and grown at 25 °C for 2 weeks until flowering response was scored. We considered a plant to be stressed if its vegetative growth was suppressed by any external factor (Hatayama and Takeno, 2003).

Treatment with chemicals

SA (Wako Pure Chemicals Industries, Osaka, Japan) was dissolved in culture solution, and 5-d-old seedlings were grown in the solution for 3 weeks. After the treatment, the seedlings were transferred to a nutrient solution without chemicals and grown for 2 weeks until flowering response was scored. The DNA demethylating reagent zebularine [1-(β-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one] (Wako Pure Chemicals Industries, Osaka, Japan) was dissolved in 30% ethanol, and 10 μL of the solution was dropped onto the shoot apical meristem of 5-d-old seedlings using a microsyringe. The same amount of 30% ethanol was given to the control plants in the same manner.

Scoring of flowering response

All plant nodes were dissected under a binocular microscope to determine whether flower 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 floral buds per plant were determined. Number of nodes, that is, the total number of floral and vegetative buds per plant, and the average length of the main stem were presented as indicators of vegetative growth. Twenty plants were used for each treatment, and each experiment was repeated at least three times. Means with standard errors for the most representative experiment are shown in each table or figure.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Cotyledons were harvested, frozen in liquid nitrogen, and stored at −80 °C prior to analysis. Total RNA was isolated from plant tissues using Plant RNA Purification Reagent (Invitrogen