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Endogenous factors regulating poor-nutrition stress-induced flowering in pharbitis: The involvement of metabolic pathways regulated by aminooxyacetic acid

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ABSTRACT

The short-day plant pharbitis (also called Japanese morning glory), *Ipomoea nil* (formerly *Pharbitis nil*), was induced to flower by poor-nutrition stress. This stress-induced flowering was inhibited by aminooxyacetic acid (AOA), which is a known inhibitor of phenylalanine ammonia-lyase (PAL) and the synthesis of indole-3-acetic acid (IAA) and 1-aminocyclopropane-1-carboxylic acid (ACC) and thus regulates endogenous levels of salicylic acid (SA), IAA and polyamine (PA). Stress treatment increased PAL activity in cotyledons, and AOA suppressed this increase. The observed PAL activity and flowering response correlate positively, indicating that AOA functions as a PAL inhibitor. The inhibition of stress-induced flowering by AOA was also overcome by IAA. An antiauxin, **4**-chlorophenoxy isobutyric acid, inhibited stress-induced flowering. Both SA and IAA promoted flowering induced by stress. PA also promoted flowering, and the effective PA was found to be putrescine (Put). These results suggest that all of the pathways leading to the synthesis of SA, IAA and Put are responsive to the flowering inhibition by AOA and that these endogenous factors may be involved in the regulation of stress-induced flowering. However, as none of them induced flowering under non-stress conditions, they may function cooperatively to promote flowering.

Introduction

Flowering is regulated mainly by environmental cues, such as night length in photoperiodic flowering and temperature in vernalization, and stress also regulates flowering (Hatayama and Takeno, 2003; Segarra et al., 2010; Wada and Takeno, 2010; Yaish et al., 2011; Rivas-San Vicente and Plasencia, 2011; Takeno, 2012). The short-day (SD) plant pharbitis (also called Japanese morning glory), *Ipomoea nil* (formerly *Pharbitis nil*), flowers under long-day (LD) conditions when grown under poor-nutrition, low-temperature or high-irradiance conditions (Hirai et al., 1993, 1994; Shinozaki et al., 1994; Ishimaru et al., 1996; Hatayama and Takeno, 2003; Wada et al., 2010b). Similar non-photoperiodic flowering has been sporadically 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, though it has not been studied systematically. We found that plants that were induced to flower by stress produced fertile seeds, and the resulting pharbitis and *Perilla frutescens* var. *crispa* progeny developed normally (Wada et al., 2010a,b). To enable plant species to persist during unfavorable environmental conditions, they flower as an emergency response when stressed, thereby ensuring the ability to produce the next generation. Therefore, stress-induced flowering can be considered as important as photoperiodic flowering and vernalization (Wada and Takeno, 2010, 2013; Takeno, 2012; Wada et al., 2013).

The expression of *PnFT2*, one of the two pharbitis homologs of the floral pathway integrator gene *FLOWERING LOCUS T* (*FT*), is induced by stress; in contrast, the expression of both *PnFT1* and *PnFT2* is induced by SD treatment (Wada et al., 2010b; Yamada and Takeno, 2014). Additionally, a positive correlation between the degree of the flowering response and the expression level of *PnFT2* was reported. These results suggest that *PnFT2*, but not *PnFT1*, is the major regulatory gene involved in stress-induced flowering in

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACS, 1-aminocyclopropane-1-carboxylic acid synthetase; AOA, aminooxyacetic acid; AOPP, L-2-aminooxy-3-phenylpropionic acid; *AP1*, *APETALA1*; *FT*, *FLOWERING LOCUS T*; IAA, indole-3-acetic acid; LD, long-day; PA, polyamine; PAL, phenylalanine ammonia-lyase; PCIB, **4**-chlorophenoxy isobutyric acid; Put, putrescine; SA, salicylic acid; SAM, *S*-adenosylmethionine; SD, short-day; Spd, spermidine; Spm, spermine.

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pharbitis. The overexpression of *PnFT2* induced early flowering, and suppression of *PnFT2* by RNA interference inhibited flowering in pharbitis, indicating that *PnFT2* has the ability to induce flowering (Yamada et al., unpublished data). Among the genes downstream of *PnFT*, *PnAP1*, a homolog of *APETALA1* (*AP1*), which is the trigger gene of flowering in *Arabidopsis thaliana*, was induced to express by stress treatment. *PnFT* was found to be expressed specifically in leaves, and the expression of *PnAP1* was observed in shoot apices (Yamada et al., unpublished data). These results suggest that stress induces the expression of *PnFT2*, which then induces *PnAP1* expression to induce flowering in pharbitis. The application of salicylic acid (SA) enhanced *PnFT2* expression (Yamada and Takeno, 2014) and induced the expression of *A. thaliana FT* and sunflower *HAFT*, an ortholog of *FT* (Martínez et al., 2004; Dezar et al., 2011), indicating that SA may play an important role as an endogenous regulatory factor in flowering.

Stress-induced flowering is accompanied by the accumulation of anthocyanin in pharbitis, 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 activity of which increases when plants are stressed (Dixon and Paiva, 1995; Scott et al., 2004). PAL catalyzes the conversion of phenylalanine to *t*-cinnamic acid, and SA is derived from the latter (Yalpani et al., 1993). Aminooxyacetic acid (AOA) and L-2-aminooxy-3-phenylpropionic acid (AOPP), which function as PAL inhibitors (Havir, 1981; Kessmann et al., 1990; Appert et al., 2003), inhibit stress-induced flowering, and this inhibitory effect is negated by *t*-cinnamic acid and SA in pharbitis (Hatayama and Takeno, 2003; Wada et al., 2010b). The increase in PAL activity and flowering inhibition by AOA have been reported in pharbitis under LD conditions (Hirai et al., 1995). Gene expression for the synthesis of and enzyme activity of PAL and endogenous SA levels increase when flowering is induced by stress in pharbitis and *Lemna paucicostata*, synonym *Lemna aequinoctialis* (Shimakawa et al., 2012; Wada et al., 2014). These facts suggest that SA, the synthesis of which is regulated by PAL, is involved in the regulatory mechanism of stress-induced flowering. The involvement of SA was also suggested in stress-induced flowering in *A. thaliana* (Martínez et al., 2004; Corbesier and Coupland, 2005). However, exogenous SA does not induce flowering under non-stress conditions in pharbitis. Thus, SA may not be directly involved in stress-induced flowering or may be necessary but not sufficient to induce flowering. In the latter case, stress may induce the production of not only SA but also other factor(s), and they may act together to induce flowering.

Although a strong piece of evidence suggesting the involvement of SA in stress-induced flowering is the inhibition of flowering by PAL inhibitors, as mentioned above, AOA and AOPP are not specific inhibitors for PAL. Indeed, AOA and AOPP were reported to inhibit the biosynthesis of indole-3-acetic acid (IAA), an auxin (Soeno et al., 2010). They are also known as inhibitors of 1-aminocycropropane-1-carboxylic acid synthetase (ACS), which converts *S*-adenosylmethionine (SAM) to 1-aminocycropropane-1-carboxylic acid (ACC; Amrhein and Wenker, 1979). The inhibition of ACC synthesis results in the accumulation of its precursor, SAM, promoting polyamine (PA) metabolism (Wimalasekera et al., 2011). Therefore, it is possible that the inhibition of stress-induced flowering by AOA and AOPP is not only due to PAL inhibition but also due to IAA and/or ACS inhibition, and IAA and/or PA may also be involved in stress-induced flowering. However, in the previous experiments on stress-induced flowering in pharbitis, it was not examined whether AOA and AOPP actually inhibit PAL activity. Furthermore, it has never been studied whether IAA and PA are involved in stress-induced flowering. Accordingly, we measured PAL activity in stressed pharbitis plants treated with AOA, examined whether IAA is involved in the flowering inhibition induced by AOA and studied the flowering inductive/promoting effects of SA, IAA and PA in pharbitis.

Materials and methods

Plant materials and growth conditions

The short-day (SD) plant pharbitis (also called Japanese morning glory), *Ipomoea nil* (L.) Roth (formerly *Pharbitis nil* (L.) Chois.), cv. Violet was used. Pharbitis seeds (Q0079) were provided by Marutane Co. (Kyoto, Japan) and Morning Glory Stock Center of Kyushu University (Fukuoka, Japan), which is supported, in part, by the National Bio-Resource Project of the Ministry of the Education, Culture, Sports, Science and Technology in Japan. The seeds were treated with concentrated H_2SO_4 for 40–70 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 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 under the same conditions. The seedlings were then transferred to glass tubes (15 mm in diameter × 150 mm high) containing a mineral nutrient solution (Kondo et al., 2006) and 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).

Stress treatment

Five-d-old seedlings were grown in diluted nutrient solution instead of full-strength nutrient solution for a poor-nutrition stress treatment. After the stress treatment, the seedlings were transferred to the normal full-strength nutrient solution and grown for 2 weeks until the 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

All the chemicals used were purchased from Wako Pure Chemicals Industries (Osaka, Japan) and were dissolved in nutrient solution. Because the addition of polyamine (PA) basifies the nutrient solution, the pH was adjusted to the original value (7.5) of the solution before the addition of PA. Five-d-old seedlings were grown in the nutrient solution supplemented with chemicals for the designated periods. After treatment, the seedlings were transferred to nutrient solution without chemicals and grown for 2 weeks until the flowering response was scored.

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. The number of nodes, that is, the total number of floral and vegetative buds per plant, and the average length of the main stem are presented as indicators of vegetative growth. Twenty plants were used for each treatment, and each experiment was repeated at least three times. The means with standard errors for the most representative experiment are shown in each table or figure.

Extraction of PAL and measurement of its activity

The activity of phenylalanine ammonia-lyase (PAL) was measured as described previously (Ferrarese et al., 2000), with some