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A possible role of an anthocyanin filter in low-intensity light stress-induced flowering in *Perilla frutescens* var. *crispa*

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ABSTRACT

The red-leaved form of *Perilla frutescens* var. *crispa* was induced to flower by low-intensity light stress. The leaves of this form are normally red, but turned green under low-intensity light due to anthocyanin depletion in the epidermis. Flowering did not occur when plants were grown under light passed through a red-colored cellophane paper, which has an absorption spectrum similar to that of anthocyanins. High-concentration anthocyanins may play the role of a red-colored optical filter under normal light conditions, and this filter effect may be lost under low-intensity light, causing a change in the wavelength characteristics of the light with which the mesophyll cells are irradiated. This change may induce a photobiological effect leading to flowering. The gene expression and enzyme activity of phenylalanine ammonia-lyase (PAL), the key enzyme for anthocyanin biosynthesis, decreased under low-intensity light. L-2-aminooxy-3-phenylpropionic acid (AOPP), which is widely used as a PAL inhibitor, inhibited low-intensity light stress-induced flowering and increased PAL activity and anthocyanin content. The inhibition of flowering by AOPP in *P. frutescens* may be through different mechanisms than PAL inhibition.

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Introduction

Flowering in many plants is regulated by environmental cues such as night length in photoperiodic flowering and temperature in vernalization. Stress also acts as a cue to induce flowering: pharbitis (*Ipomoea nil*, formerly *Pharbitis nil*) flowers when grown under poor-nutrition and low-temperature stresses (Shinozaki et al., 1988; Hirai et al., 1994; Hatayama and Takeno, 2003; Wada et al., 2010b) and *Lemna paucicostata* (synonym *Lemna aepuinoctialis*) flowers in response to poor-nutrition stress (Shimakawa et al., 2012). Ultraviolet-C (UV-C) light, poor-nutrition, drought and low-temperature stresses induce early flowering in *Arabidopsis thaliana* (Martínez et al., 2004; Kolář and Seňková, 2008; Riboni et al., 2013; Xu et al., 2014). Similar non-photoperiodic flowering has been sporadically reported in various plant species, and most of the factors responsible for flowering can be regarded as stressors (Wada and Takeno, 2010; Takeno, 2012). However, those flowering responses had not been studied systematically. Plants that are induced to flower by stress produce fertile seeds, and the progeny develop normally (Wada et al., 2010a, b). Stressed plants do not need to wait for the arrival of a particular season when photoperiodic conditions are suitable for flowering, and flower as an emergency response. This precocious flowering ensures their ability to produce the next generation, and they can preserve the species even under unfavorable environmental conditions. Therefore, stress-induced flowering is considered to be as important as photoperiodic flowering and vernalization (Wada and Takeno, 2010; Takeno, 2012).

Perilla frutescens var. *crispa*, an obligatory short-day (SD) plant, flowers under long-day (LD) conditions with weak irradiance because low-intensity light acts as a stress factor (Wada et al., 2010a). A few reports indicated that flowering occurred under low-intensity light (De Zeeuw, 1953; Gaillochet et al., 1962; Takimoto, 1973), but the phenomena were not analyzed in detail. Early flowering under low-intensity light resembles the shade-avoidance response that is observed in plants shaded by neighbors. The major phenotypes of the response are etiolation and rapid stem elongation growth (Adams et al., 1998; Lorrain et al., 2008), and early flowering is also a part of this syndrome (Smith and Whitelam,

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AOA, aminooxyacetic acid; AOPPL, L-2-aminooxy-3-phenylpropionic acid; CHS, chalcone synthase; IAA, indole-3-acetic acid; LD, long-day; PA, polyamine; PAL, phenylalanine ammonia-lyase; RT–PCR, reverse transcription–polymerase chain reaction; SA, salicylic acid; SAMS, *S*-adenosylmethionine; SD, short-day; UV-C, ultraviolet-C.

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1997; Franklin, 2008). However, the leaves of *P. frutescens* under low-intensity light are green and the stem length is short. Therefore, the response of *P. frutescens* to low-intensity light is different from the shade-avoidance response. Photosynthetic activity may decrease under low-intensity light, but the photosynthetic deficiency may not be the cause of early flowering. The photoassimilate is a flower-inducing factor (Bernier and Périlleux, 2005), and *P. frutescens* flowers *in vitro* when sucrose was added to the culture medium (Purse, 1984). There are two forms of *P. frutescens*: a red-leaved form, in which the leaf epidermis contains a large amount of anthocyanins, and a green-leaved form, which contains only a trace amount of anthocyanins (Yamazaki et al., 2003b). The key enzyme of anthocyanin biosynthesis is phenylalanine ammonia-lyase (PAL), and PAL activity is induced by light (Gong et al., 1997). The flowering response caused by low-intensity light is stronger in the red-leaved form than in the green-leaved one. These facts suggest that the quality and quantity of light may be modified by anthocyanins when light passes through the leaf epidermis, and this modification of incident irradiance may affect processes in the mesophyll cells, leading to flowering.

The PAL activity increases when plants are stressed (Dixon and Paiva, 1995; Neuenschwander et al., 1995; Borsani et al., 2001; Scott et al., 2004). PAL catalyzes the conversion of phenylalanine to *t*-cinnamic acid from which salicylic acid (SA) is derived. Aminooxyacetic acid (AOA) and L-2-aminooxy-3-phenylpropionic acid (AOPP), PAL inhibitors (Kessmann et al., 1990; Appert et al., 2003), inhibit stress-induced flowering in *L. paucicostata* and pharbitis, and the inhibitory effect of AOA is negated by SA (Wada et al., 2010b; Shimakawa et al., 2012). The endogenous SA content increases in the plants flowered by stress (Shimakawa et al., 2012; Wada et al., 2014). Furthermore, the early flowering response induced by UV-C light stress in *A. thaliana* is 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 stress-induced flowering. Low-intensity light stress-induced flowering in *P. frutescens* is also inhibited by AOA and AOPP (Wada et al., 2010a). Therefore, it is thought that PAL activity may increase when *P. frutescens* is induced to flower by low-intensity light stress. However, PAL activity may decrease when the plants are grown under low-intensity light because PAL activity is induced by light. These two contradictory observations make it difficult to predict the involvement of PAL in the stress-induced flowering in *P. frutescens*.

The present study examined the possibility that anthocyanins play a role in the regulation of low-intensity light stress-induced flowering in *P. frutescens*. It was also examined whether PAL activity would increase or decrease when low-intensity light functions as a stress factor by studying the gene expression and enzyme activity of PAL under the stress and AOPP treatments.

Materials and methods

Plant materials and growth conditions

The red-leaved form of *P. frutescens* (L.) Britton var. *crispa* (Thunb. ex Murray) Decne. ex L. H. Bailey was used as the experimental material. The seeds were placed on moist filter paper in a Petri dish (90 mm in diameter) and germinated at 25 °C under 16-h light and 8-h dark LD conditions. When the cotyledons expanded (5–10 days after the seed planting), the seedlings were planted in vermiculite in a plastic pot (50 mm in diameter) and were grown under the same LD conditions as during germination. White light was generated by fluorescent lamps (FL20SW or FL40SSW/37, Toshiba Corporation, Tokyo, Japan). The fluence rate of light at the plant level was 120 μmol m^{-2} s^{-1} in normal conditions. The plants were watered daily with tap water and fertilized with a mineral nutrient solution (Kondo et al., 2006) once a week.

Light treatments

For the low-intensity light stress treatment, plants were grown under LD conditions with white florescent light of 30 μmol m^{-2} s^{-1} for 4 weeks starting on the day when the cotyledons expanded.

Red-colored cellophane paper (Toyo Corporation, Tokyo, Japan) was used to mimic anthocyanins. Colorless transparent cellophane paper was used as a control. The cellophane paper was placed between the light source and the plants. The quantity of light that passed through the cellophane paper was adjusted to 30 μmol m^{-2} s^{-1} at the plant level.

Chemical treatment

AOPP (Wako Pure Chemical Industries, Ltd, Osaka, Japan) was dissolved in nutrient solution, and the solution was added to the vermiculite once a week for 4 weeks during cultivation.

Scoring of the flowering response

P. frutescens flowers as solitary flowers, inflorescences at leaf axils or a terminal inflorescence at the shoot apex. For each treated plant, all of the leaf axils and the shoot apex of the main stem were dissected under a binocular microscope to determine whether they formed flowers and/or inflorescences. The percentage of plants with at least one flower or inflorescence among all of the treated plants (% flowering), the number of solitary flowers per plant and the number of inflorescences per plant were recorded. 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. Means with standard errors for the most representative experiment are presented.

Quantification of anthocyanins

Anthocyanins were extracted and quantified according to the method described by Gong et al. (1997). The leaves were harvested, and each tissue sample was incubated in 1% HCl–methanol (v/v) (1.0 g fresh weight/100 mL) in the dark at 4 °C overnight. The extract's absorbance at 350–700 nm was measured by a photometer (UVmini-1240, Shimadzu Corporation, Kyoto, Japan). The absorbance at 529 nm (A_{529}) was determined to be the level of anthocyanins. The red-colored cellophane paper was stuck on a cuvette to determine the absorption spectrum by the photometer.

Analysis of gene expression by reverse transcription–polymerase chain reaction (RT–PCR)

The expression of *P. frutescens PAL* and its downstream gene *chalcone synthase* (*CHS*) was studied by RT–PCR. Whole aerial vegetative tissues were harvested, frozen in liquid nitrogen and stored at −80 °C prior to analysis. Total RNA was isolated from the tissues using Plant RNA Purification Reagent (Invitrogen Corporation, Carlsbad, CA, USA), and cDNA was synthesized from each RNA sample using ReverTra Ace (Toyobo Co. Ltd, Osaka, Japan). The National Center for Biotechnology Information database was used to design the primers: 5′-CGTTGATGAAGCCGAAGCAG-3′ and 5′-GGAAGGAAGCCCGTTGTTGTAG-3′ for *PAL* and 5′-AGGACCTTGCTGAGAACAACGC-3′ and 5′-TCCAATCCGAAATCCCCAAC-3′ for *CHS*. The reactions were carried out in a thermal cycler using 24–30 cycles of 90 °C for 30 s, 60 °C for 30 s and 72 °C for 15 s. The product for each gene was separated on a 1.2% agarose gel. The images

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