Control of Flowering by Phloem Exudate from Cotyledons of *Ipomoea tricolor* II. Low Molecular Weight Flower-inhibiting Substance(s)

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The flower-inhibiting activity of phloem exudate prepared from cotyledons of *Ipomoea tricolor* strain Heavenly Blue seedlings cultivated to continuous light conditions was examined, using apex cultures *in vitro* as a bioassay system. The phloem exudate inhibited flowering in apices excised from seedlings exposed to a single 16 h dark period to induce flowering. When the phloem exudate was dialyzed and separated to 3 fractions such as low (<1,000), middle (1,000–10,000) and high (>10,000) molecular weight, low molecular weight fraction had flower-inhibiting activity, but not middle and high molecular weight fractions. The low molecular weight fraction was separated and examined the nature of the flower-inhibiting substance(s). The flower-inhibiting activity appeared to be heat-stable. The low molecular weight fraction was extracted by CHCl_3 and ethyl acetate. The fraction with activity from solvent participation was further fractionated by ion-exchange chromatography. The active fractions were applied to a Sep-Pak C18 cartridge. From the results of fractionation, the flower-inhibiting substance(s) was low molecular weight, high polar, seemed to be basic, and the flower-inhibiting activity was increased about 10-fold.

Keywords : apex culture, flower-inhibiting activity, *Ipomoea tricolor*, phloem exudate

INTRODUCTION

The control of flowering is very useful in agriculture, and many studies are performed. Many investigators demonstrated that photoperiodic stimuli on flowering were received in leaves in short-day (SD) plants (Garner and Allard, 1925; Hammer and Bonner, 1938; Zeevaart, 1958; King, 1972). From facts that the receivable organ is leaf and flower is initiated in apex, Knott (1934) postulated that any substances produced in leaves transported to apex and induced floral buds. Although this substance was termed as florigen in 1937 (Chailakhyan, 1937), it has not been isolated and identified up to the present. Harada (1967) reported that *in vitro* culture was very significant for flowering research as bioassay system. In *Pharbitis* (= *Ipomoea*) *nil*, the phloem exudate (PE) taken from the cotyledons of seedlings grown under short-day had flower-inducing activity in apex cultures (Ishioka et al., 1990), and was elucidated some of the nature of this active substance(s) (Ishioka et al., 1991). In addition, PE of *Pharbitis nil* that was purified by solvent participation, dialysis, ion-exchange resin and Sep-Pak C18 cartridge was able to purify up to about 10 to 30 times compared to the flower-inducing activity of crude PE before purification (Kondoh et al., 1999b).

On the other hand, flower inhibition by non-induced leaves was reported in many plant species, such as *Kalanchoe* (Hander et al., 1949), *Xanthium* (Lincoln et al., 1956), *Lolium* (Evans, 1960), *Pharbitis nil* (Imamura,

1961), and *Perilla* (King and Zeevaart, 1973). In *Perilla* (King and Zeevaart, 1973) and *Xanthium* (Zeevaart et al., 1977), the amount of assimilates transported from induced leaves was remarkably decreased at flower inhibition. In *Pharbitis nil*, by varying the areas of the non-induced cotyledon, parallel inhibitory effects were shown on the export of flower-inducing substance and of assimilate to the apex from the induced cotyledon (Ogawa and King, 1990). Thus, the interpretation that flower inhibition was by interference with assimilate/substance co-transport in the phloem was generally accepted.

However, there are many reports suggesting that flower-inhibiting substance is actually generated in the non-induced leaves. In *Xanthium* and *Kalanchoe*, Papafotiou and Schwabe (1990) revealed that the inhibitory effect on flowering was not reduced by inhibiting photosynthesis in non-induced leaf, and that the amount of photosynthesis in the induced leaf did not affect the floral induction. From these results, they concluded that inhibition of flowering occurred for inhibitor(s) generated by the non-induced leaf (Papafotiou and Schwabe, 1990). In SD species of *Fragaria*, flower-inhibiting substance was produced in the leaves under long day condition, transmitted along the stolon, and acted at the growing regions (Guttridge, 1959; Vince-Prue and Guttridge, 1973). The inhibition of flowering by the graft or extract of non-induced cotyledons or leaves have been well documented (Jacobs, 1980). In *Pharbitis nil*, the PE from cotyledons of seedlings grown under continuous light had flower-inhibiting activity on the apices cultured under SD condi-

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tion (Ishioka et al., 1990). PE prepared from cotyledons of *Pharbitis nil* seedlings grown under continuous light inhibited flowering in *Ipomoea tricolor* that is a different species. And PE similarly prepared from cotyledons of *Ipomoea tricolor* also inhibited flowering in *Pharbitis nil* (Data not shown). These results suggested that the PE prepared from cotyledons of seedlings grown under continuous light contain substance(s) that cause the floral inhibition across the species. In addition, it was reported that flower formation was controlled by quantitative balance between flower-inducing and -inhibiting substances in PE (Kondoh et al., 1999a). In this study, we have partially purified the flower-inhibiting substance in PE prepared from the cotyledons of seedlings grown under continuous light condition of *Ipomoea tricolor* of the species that is different from *Pharbitis nil*, using the apex culture as assay system.

MATERIALS AND METHODS

Plant material and apex cultures as bioassay system

Seeds of *Ipomoea tricolor* strain Heavenly Blue were obtained from Takii Seed Company (Kyoto, Japan). The seeds were soaked in concentrated sulfuric acid for 45 min with occasional stirring, and then rinsed well with water. These seeds were sterilized with a solution of NaOCl (available chlorine, 1%) for 20 min, rinsed well with sterilized water and then sown on Murashige and Skoog's medium (Murashige and Skoog, 1962; hereafter referred to as MS medium) containing 3% sucrose and 0.3% Gelrite (Merck, USA). They were placed in a incubation room at $25 \pm 2^\circ\text{C}$ under 16 h long-day (LD) conditions (daylight-type fluorescent lamps with $100 \mu\text{mol m}^{-2} \text{s}^{-1}$; Panasonic, Osaka, Japan). After 6 days, these seedlings were exposed to a single 16 h dark period to induce flowering. The apices for bioassay were obtained from these seedlings. Each apex (about 5 mm in length) was excised and cultured on 10 mL of MS medium with 5% sucrose, 0.3% Gelrite and PE or fraction of PE. In separation of dialysis, all 3 fractions (low, middle and high molecular weight) were filter-sterilized and added to the autoclaving medium. To examine the heat-stability of the flower-inhibiting activity of the PE, the PE was heated at 70°C or 100°C for 15 min, filter-sterilized and then added to the autoclaving assay medium. In another experiments, PE and/or fractions separated from PE were added to medium before autoclaving. Cultures were maintained under 16 h LD conditions at a constant temperature of $25 \pm 2^\circ\text{C}$. Formation of flower buds on the developed plantlet from cultured apices was observed after 4 weeks of culture. As described in the previous report, only the plantlet with a terminal floral bud was counted and the results were expressed as the percentage of plantlets with a terminal flower (Watanabe et al., 2015). Inhibitory effects of PE or fraction of PE on growth of plantlets from cultured apices may be conceivable. The height of plantlet was usually more than 3 cm therefore, if the height was less than 3 cm, suppression of flower inhibition effect was judged by growth inhibition. However, in all present experiments, the height of plantlets was more

than 3 cm.

Preparation of phloem exudate

Seeds of *I. tricolor* strain Heavenly Blue were soaked in concentrated sulfuric acid for 45 min with occasional stirring, and then rinsed well with water. These seeds were sown on wet vermiculite and had been cultivated under continuous light (CL) conditions at a constant temperature of $25 \pm 2^\circ\text{C}$ for 7 days. We used the method described in the previous report to collect PE from the petioles of cotyledons (Watanabe et al., 2015). Cotyledons were excised from the seedlings and placed into vials containing 20 mM EDTA. After incubation with EDTA for 1 h, the cotyledons were transferred to distilled water and incubated for 6 h. The resultant solution was designated crude CL-PE and used for subsequent experiments.

The crude CL-PE was lyophilized in a vacuum freeze dryer, the residue was dissolved in 10 mL distilled water, and then dialyzed with a dialysis membrane (Spectra/Por 7 MWCO 1,000; Spectrum Laboratories, Houston, USA) against 1 L of distilled water at 4°C for 12 h. The non-diffusate was concentrated by freeze-dried and re-dialyzed with a dialysis membrane (Spectra/Por 7 MWCO 10,000; Spectrum Laboratories, Houston, USA) against 1 L of distilled water at 4°C for 12 h. All of diffusate and non-diffusate fractions were freeze-dried; weight of the residue was measured (hereafter referred to as FDW) and dissolved in distilled water. Using the resultant 3 fractions such as low, middle and high molecular weight, flower-inhibiting activity was examined. The diffusate fraction from the first dialysis was used for subsequent experiments.

Fractionation of flower-inhibiting activity

To examine the polarity of flower-inhibiting substance in CL-PE, the diffusate fraction was fractionated by CHCl_3 and ethyl acetate participations as described in the previous report (Watanabe et al., 2015). All of fractions obtained by solvent participation were dried *in vacuo*, and dissolved in distilled water or MeOH and flower-inhibiting activity was examined.

To examine whether the flower-inhibiting substance in CL-PE is acidic, the fraction with flower-inhibiting activity was applied to a cation exchange column (CM Cellulose, $1.5 \times 10 \text{ cm}$, 20 mL bed volume; Whatman, Kent, UK), which was rinsed well with 10 mM phosphate buffer (pH 6.0). The flow-through (FT) fraction was collected. And the absorbed fraction was eluted with 10 mM phosphate buffer (pH 6.0) containing 1 M NaCl, and collected each 5 mL fraction. To examine whether the flower-inhibiting substance in CL-PE is basic, the FT fraction from cation exchange column was applied to an anion exchange column (DEAE Sephacel™, $1.5 \times 10 \text{ cm}$, 20 mL bed volume; GE Healthcare Bio-Sciences AB, Uppsala, Sweden), which was rinsed well with 10 mM Tris-HCl buffer (pH 8.0). The absorbed fraction was eluted with 10 mM Tris-HCl buffer (pH 8.0) containing 1 M NaCl, and collected each 5 mL fraction. The active fractions from anion exchange column (Fraction 4 and 5) were separated on a Sep-Pak C18 cartridge (Waters, MA, USA), previously equilibrated with distilled water. The cartridge was eluted with successive 100 mL solution of 0, 5, 10, 15, 20, 25, 30, 50, 75 and

100% MeOH with 0.1% acetic acid. Each fraction was dried *in vacuo*, dissolved in distilled water and added to the assay medium.

To examine the correlation between flower-inhibiting activity and concentration of CL-PE, various concentrations of the crude CL-PE and final 15% MeOH-eluted fraction from Sep-Pak C18 cartridge were added to the assay medium, and flower-inhibiting activity was examined.

Date analysis

All experimental data were statistically analyzed by one-way analysis of variance (ANOVA) followed by mean separation using least significant difference (LSD) test. The term significant has been used to indicate difference for which $P=0.05$ levels, the means from at least three times with thirteen replicates were compared by the Duncan's test.

RESULTS AND DISCUSSION

In *Pharbitis nil*, the PE from cotyledons of seedlings grown under CL conditions had flower-inhibiting activity (Ishioka et al., 1990). In this study, the flower-inhibiting activity of PE prepared from non-inducing cotyledons of *Ipomoea tricolor* of the species that was different from *Pharbitis nil* was examined.

The CL-PE was dialyzed with a dialysis membrane (MWCO 1,000) against distilled water. The non-diffusate was concentrated by freeze-dried and re-dialyzed with a dialysis membrane (MWCO 10,000) against distilled water. Using the resultant 3 fractions such as low (<1,000), middle (1,000–10,000) and high (>10,000) molecular weight, flower-inhibiting activity was examined. Low molecular weight (LMW) fraction had flower-inhibiting activity, but not high and middle molecular weight fractions (Fig. 1). From this result, molecular weight of flower-inhibiting

Fig. 1 Flower-inhibiting activities of fractions separated by dialysis. The phloem exudate (PE) was collected from seedlings grown under continuous light (CL) conditions. Using 30 µg FDW mL⁻¹ of the crude CL-PE and 10 or 30 µg FDW mL⁻¹ of low (L), middle (M) and high (H) molecular weight fraction, flower-inhibiting activity was examined. Culture medium without CL-PE or fraction separated from CL-PE was used as a control. FDW means freeze-dry weight. Means ± SE, $n=13$. The same letters represent no significant difference at $P=0.05$ level.

substance(s) was suggested to be less than 1,000. In *Pharbitis*, when the partially purified PE was dialyzed (MWCO 1,000), the flower-inhibiting activity was also located in the diffusate fraction (Kondoh et al., 1999b).

To examine the heat-stability of the flower-inhibiting activity of the LMW fraction, the fraction was heated at various temperatures. As shown in Fig. 2, heat treatment, and even autoclaving, did not alter the flower-inhibiting activity of the fraction. In *Pharbitis*, flower-inhibiting activity was also heat-stable (Kondoh et al., 1999b).

The LMW fraction was extracted with CHCl₃ and then ethyl acetate; flower-inhibiting activity of each fraction was examined. The LMW fraction was fractionated into fractions of 1–7 by solvent partition with CHCl₃. As shown in Fig. 3, the activity was localized in the Fraction 1. Then, the Fraction 1 was fractionated into fractions of 8–14 by solvent partition with ethyl acetate. As a result (Fig. 4), the

Fig. 2 Effect of heat treatment on the flower-inhibiting activity of phloem exudate (PE). The PE was collected from seedlings grown under continuous light (CL) conditions. The diffusate fraction from the first dialysis of the CL-PE was heated at 70°C (B), 100°C (C) or 121°C (D). A; No heat treatment. Means ± SE, $n=13$. The same letters represent no significant difference at $P=0.05$ level.

Fig. 3 Flower-inhibiting activities of fractions separated by CHCl₃ participation. The diffusate fraction (dialyzed with a dialysis membrane MWCO 1,000) was fractionated by CHCl₃ participation. Means ± SE, $n=13$. The same letters represent no significant difference at $P=0.05$ level.

Fraction 8 was shown flower-inhibiting activity. From these results, high flower-inhibiting activity was detected in the aqueous phase fraction; flower-inhibiting substance(s) was suggested to be highly polar substance.

The Fraction 8 was further fractionated by ion exchange chromatography. When the Fraction 8 was fractionated by cation (CM Cellulose) exchange resin, absorbed fractions were inactive, and the activity was located in FT fraction (Fig. 5). The FT fraction from cation exchange chromatography was further fractionated by anion (DEAE Sephacel) exchange resin. The activity was located in a fractions eluted from anion exchange resin (Fig. 6). Thus, this flower-inhibiting substance(s) seemed to be basic.

The fractions with flower-inhibiting activity from DEAE Sephacel were applied to a Sep-Pak C18 cartridge, and eluted successively with 0, 5, 10, 15, 20, 25, 30, 50, 75 and 100% MeOH containing 0.1% acetic acid. As shown in Fig. 7, the fraction eluted with 15% MeOH exhibited flower-inhibiting activity. From the results of fractiona-

tion, the nature of flower-inhibiting substance(s) in CL-PE was low molecular weight, heat-stable, high polar, and seemed to be basic. In *Pharbitis*, the nature of flower-inhibiting substance(s) seemed to be low molecular weight, heat-stable, highly polar and neutral one(s) (Kondoh et al., 1999b). The nature the flower-inhibiting substance(s) are similar in both species, and the PE prepared from cotyledons of seedlings grown under CL contained substance(s) that caused the floral inhibition of each other. The flower-inhibiting substance(s) in both species might be a similar one(s).

In order to determine how much the activity increased by the above partial purification, the correlation between flower-inhibiting activity and the concentrations of crude and partially purified CL-PE was examined. As shown in Fig. 8, the flower-inhibiting activity was observed when the crude CL-PE was added, and the highest activity was obtained with $30 \mu\text{g FDW mL}^{-1}$. The partially purified CL-PE at $3 \mu\text{g FDW mL}^{-1}$ expressed similar inhibitory activity

Fig. 4 Flower-inhibiting activities of fractions separated by ethyl acetate participation. The active phase for CHCl_3 participation (Fraction 1) was fractionated by ethyl acetate participation. Means $\pm$ SE, $n=13$. The same letters represent no significant difference at $P=0.05$ level.

Fig. 6 Flower-inhibiting activities of fractions separated by anion exchange column. The fraction with flower-inhibiting activity after cation exchange chromatography (flow-through fraction) was applied to anion exchange column. Means $\pm$ SE, $n=13$. The same letters represent no significant difference at $P=0.05$ level.

Fig. 5 Flower-inhibiting activities of fractions separated by cation exchange column. The active phase of ethyl acetate participation (Fraction 8) was applied to cation exchange column. Means $\pm$ SE, $n=13$. The same letters represent no significant difference at $P=0.05$ level.

Fig. 7 Flower-inhibiting activities of fractions separated by Sep-Pak C18 cartridge. The fractions with flower-inhibiting activities after anion exchange chromatography (Fraction 4 and 5) were applied to Sep-Pak C18 cartridge. Means $\pm$ SE, $n=13$. The same letters represent no significant difference at $P=0.05$ level.

Fig. 8 Correlation between flower-inhibiting activity and concentration of phloem exudate (PE). The PE was collected from seedlings grown under continuous light (CL) conditions. Various concentrations of crude CL-PE (○) and final 15% MeOH-eluted fraction from Sep-Pac C18 cartridge (●) were added to assay medium. FDW means freeze-dry weight. Means $\pm$ SE, $n=13$.

to the crude CL-PE at $30 \mu\text{g FDW mL}^{-1}$. Therefore, the activity was increased about 10-fold by the above purification steps.

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