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BRIEF COMMUNICATION

Cross talk between phytohormones in the regulation of flower induction in *Pharbitis nil*

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Abstract

Application of gibberellic acid (GA_3) on the cotyledons of 5-d-old *Pharbitis nil* reversed the inhibitory effect of both abscisic acid (ABA) and ethylene on flowering. Application of GA_3 slightly decreased ethylene production and did not affect the endogenous ABA content in the cotyledons during the night. However, it reversed the stimulating effect of ABA on ethylene production.

Additional key words: abscisic acid, ethylene, gibberellic acid, photoperiod.

Studies conducted both on long-day plants (LDPs) and short-day plants (SDPs) indicate that various phytohormones play a significant role in the regulation of flowering. Some of them stimulate while others inhibit the flowering (Vince-Prue and Gressel 1985, King *et al*. 2001, Kęsy *et al.* 2003). A proper balance between them decides the direction of plant differentiation. There are only a handful of papers concerning the role of hormone interactions in flower induction (Wijayanti *et al*. 1997, Kęsy *et al*. 2008, 2010, Wilmowicz *et al*. 2008).

Auxins and ethylene are among the most efficient compounds inhibiting flowering of the model SDP *Pharbitis nil* (Amagasa and Suge 1987, Kulikowska-Gulewska *et al*. 1995). Research indicates that flower inhibition by indole-3-acetic acid (IAA) occurs indirectly by increasing both the content of ethylene (Kęsy *et al*. 2008) and the transcriptional activity of genes encoding enzymes involved in its biosynthesis (Frankowski *et al*. 2009, Kęsy *et al*. 2010). Ethylene, in turn, inhibits *P. nil* flowering by lowering the content of endogenous abscisic acid in the cotyledons (Wilmowicz *et al*. 2008).

As yet, no hormone has been found that could replace the inductive photoperiod completely, but there are substances which stimulate flowering in *P. nil* cultivated under sub-inductive conditions, among them gibberellic acid (GA_3) and to some extent also abscisic acid (ABA). ABA applied on the cotyledons and/or shoot apices under sub-inductive conditions resulted in a minor stimulation of flowering (Wilmowicz *et al*. 2008). Similar results were obtained when GA_3 was applied (Kulikowska-Gulewska *et al*. 2000).

It has been shown many times that GA_3, ABA and ethylene interacts in the regulation of numerous developmental processes, *e.g*., the plant transition from the embryonic stage to the vegetative stage, the sprouting of seeds, or shoot growth (Razem *et al*. 2006, Weiss and Ori 2007). Therefore we decided to investigate whether such an interaction exists also in the regulation of flower induction in a model SDP *Pharbitis nil*.

Seeds of *Pharbitis nil* Chois (syn *Ipomoea nil*) cv. Violet (*Marutane Seed Co*., Kyoto, Japan) were soaked in concentrated sulfuric acid for 45 min, washed under running tap water for 2 h and soaked for 24 h in water (26 ± 1 °C). The swollen seeds were sown in pots (15 seeds in each pot) filled with

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Abbreviations: ABA - abscisic acid; GA_3 - gibberellic acid; IAA – indole-3-acetic acid; LDP - long day plant; SDP - short day plant.
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Vermiculite and sand (1:1). The seedlings were grown in a growth chamber at temperature of 26 ± 1° C and continuous irradiance of 130 μmol m^{-2} s^{-1} (cool white fluorescent tubes, *Polam*, Warsaw, Poland) for 5 d. Then the seedlings were exposed to 16-h-long darkness. The control plants were treated with 0.05 % *Tween 20* (v/v) solution. The second group of plants was treated with ABA at a concentration of 1 mM in 0.05 % *Tween 20* (v/v) and the third group with 1 mM GA_3. The solution was applied to the cotyledons (about 0.05 cm^3 per plant) at 0, 2, 4, 6, 8, 10, 12, 14 or 16 h of the darkness. All manipulations during the dark period were performed under dim green safe light. After the completion of treatments, the plants were grown in a growth chamber under continuous irradiance (130 μmol m^{-2} s^{-1}) and 26 ± 1 °C for 14 d. The number of floral buds per plant were then determined using a stereoscopic microscope. At least 15 plants were used in each treatment, and each experiment was repeated at least three times. Student's *t*-test was used to calculate the significant differences compared with the control.

In experiments examining the influence of ABA and GA_3 on ethylene production, ABA (first variant) or ABA and GA_3 (second variant) at a concentration of 1 mM were applied on the cotyledons at the beginning of the inductive night. The pots with 5-d-old seedlings were enclosed in a glass container with a capacity of 9 dm^3.

The collection time of plant material is indicated in the tables. Cotyledons (2 g) were frozen in liquid N_2 and homogenized in a chilled mortar with a pestle. Free ABA was extracted with 80 % methanol (v/v) in two parts of 15 cm^3 each. [6-$^2H_3$]ABA (100 ng) was added to the extract as an internal standard. The extract was reduced to the aqueous phase, acidified to pH 2.0 with 12 M HCl and centrifuged at 10 000 *g* for 30 min to remove chlorophyll. The supernatant was partitioned three times against ethyl acetate, and dried under vacuum. The pellet was dissolved in 3 cm^3 of 80 % methanol (v/v) and applied to a silica gel solid-phase extraction column (*Backer-bound SPE* silica gel, 500 mg, 3 cm^3; *J.T. Backer*, Philipsburg, NJ, USA). The eluate was evaporated and further purified by HPLC using a *SUPELCOSIL ABZ+PLUS* column (250 × 4.5 mm, 5 μm particle size; *Supelco*, Park Bellefonte, PA, USA). The samples were dissolved in 0.2 cm^3 of 20 % methanol (v/v) and chromatographed with a linear gradient of 20 - 80 % methanol in 1 % formic acid (v/v) for 20 min at flow rate 1.0 cm^3 min^{-1} and temperature of 22 °C. The fractions collected at 12.57 ± 0.5 min were evaporated to dryness, methylated with diazomethane, dissolved in 0.1 cm^3 of methanol and analysed by *GC-MS-SIM* (*Auto-System XL* coupled to a *Turbo Mass*, *Perkin-Elmer*, Norwalk, USA) using a *MDN-5* column (30 m × 0.25 mm, 0.25 μm phase thickness, *Supelco*). The temperature programme was 120 °C for 1 min, 120 - 250 °C at 10 °C min^{-1}, flow rate 1.5 cm^3 min, injection port was 280 °C, electron potential 70 eV. The retention times of ABA and [6-$^2H_3$]ABA were 14.07 and 14.3 min, respectively. *GC-MS-SIM* was performed by monitoring m/z 162 and 190 for endogenous ABA and 166 and 194 for [6-$^2H_3$]ABA according to the method described by Vine *et al*. (1987).

Ethylene production of seedlings was measured every 2 h during the 16-h-long dark period by enclosing pots with 15 plants for 30 min in 9 dm^3 jars. After that time, 100 cm^3 of air was taken through the septa and ethylene from the sample was trapped for 5 min in 2 cm^3 of 0.25 M mercuric perchlorate in 2 M perchloric acid according to Abeles (1973). The trapping solution (1.8 cm^3) was transferred to a 4.6 cm^3 vial and an equal volume of 4 M LiCl was added. The vial was tightly closed and shaken for 2 min. The released ethylene was taken through the septa and determined by gas chromatography on an *RTX-5 Q-PLOT* column (*RESTEK* Corp., Bellefonte, PA, USA) at 40° C, using a flame ionizing detector. Injector and detector temperatures were 60 and 105 °C, respectively.

We showed that 1 mM ABA applied to the cotyledons of *P. nil* seedlings during the inductive night decreased the number of flower buds developed by the plants (Table 1). Simultaneous treatment of plants with both ABA and GA_3 restored the inductive effect of the long night on flowering (Table 1). GA_3 applied alone slightly reduced the content of endogenous ABA in later hours of the inductive night (Table 2) and did not affect the ability of *P. nil* to flower (data not shown). On the other hand, flower formation in *P. nil* was completely inhibited when plants are exposed to ethylene during the second phase of the 16-h inductive dark period and this effect was not observed when ethylene was applied to plants earlier treated with GA_3 (data not shown). The inhibitory effect of ethylene on the flowering of *P. nil* has been frequently reported (Amagasa and Suge 1987, Suge 1972, Kęsy *et al*. 2008), but the exact role of that hormone in the mechanism of photoperiodic induction of flowering is still not completely clear. It cannot be excluded that, like in seeds of *Fagus sylvatica* (Calvo *et al*. 2004), ethylene inhibits the expression of gibberellin 20-oxidase, contributing to the lowering of the endogenous gibberellin content. An increased content of gibberellins in the cotyledons of *P. nil* after induction has been observed (Yang 1995). Moreover, Kulikowska-Gulewska *et al*. (2000) showed that application of chlormequat (an inhibitor of gibberellin biosynthesis) to plants subjected to full photoperiodic induction inhibited flowering, while treating seedlings subjected to incomplete induction with gibberellin GA_3 stimulated that process.

After an initial increase, ethylene production during the inductive night was maintained at the level about of 2 pmol g^{-1}(f.m.) min^{-1} (Table 2) and a clear decrease in ethylene production was observed when seedlings were treated with 1 mM GA_3 at the beginning of the 16-h long inductive night (Table 2). Application of 1 mM ABA to

Table 1. Effect of ABA and GA_3 on the flowering response of 5-d-old *Pharbitis nil* seedlings expressed as the number of flower buds per plant. GA_3 and ABA at a concentration of 1 mM were applied on the cotyledons at hour 0, 2, 4, 6, 8, 10, 12, 14 or 16 of the 16-h long inductive night. Means ± SE. In each treatment, at least 15 plants were used. Each experiment was repeated three times. Significant differences to the plants treated with ABA are indicated as ** - $P < 0.01$ and * - $P < 0.05$.

	0	2	4	6	8	10	12	14	16
ABA	2.2 ± 0.2	2.1 ± 0.1	2.4 ± 0.3	2.5 ± 0.2	2.8 ± 0.3	4.1 ± 0.4	4.9 ± 0.2	5.1 ± 0.1	5.4 ± 0.1
ABA + GA_3	6.2 ± 1.1**	6.1 ± 0.8**	6.2 ± 0.9**	6.1 ± 1.0**	5.9 ± 0.8**	6.1 ± 0.7**	6.1 ± 1.0*	5.9 ± 0.9*	5.7 ± 1.0*

Table 2. Effect of 1 mM GA_3 and 1 mM ABA (in 0.05 % *Tween*) applied at the beginning of the dark period on ethylene production [pmol g^{-1}(f.m.) min^{-1}] by 5-d-old *Pharbitis nil* seedlings subjected to the 16-h-long inductive night and changes in the endogenous ABA content [pmol g^{-1}(f.m.)] in the cotyledons of control and GA_3-treated plants. The control plants were treated with 0.05 % *Tween*. Means ± SE $n = 6$.

		0	2	4	6	8	10	12	14	16
Ethylene	control	0.12±0.01	1.68±0.02	2.25±0.03	2.13±0.21	1.91±0.04	2.15±0.05	1.32±0.13	1.13±0.05	2.09±0.28
	GA_3	0.12±0.02	1.33±0.09	0.73±0.08	1.24±0.09	1.10±0.08	0.73±0.09	0.54±0.03	0.95±0.10	1.10±0.03
	GA_3 + ABA	0.12±0.02	1.82±0.06	1.61±0.03	1.73±0.09	1.78±0.12	1.17±0.12	0.83±0.15	1.07±0.05	1.12±0.09
ABA	control	83.6 ±1.7	71.0 ±12.0	67.5 ±24.8	72.0 ±14.0	70.8 ±19.8	40.3 ±7.6	50.4 ±7.1	54.3 ±13.0	87.2 ±16.6
	GA_3	-	74.4 ±3.2	71.5 ±1.2	68.4 ±1.9	72.3 ±2.0	24.3 ±6.2	42.2 ±1.8	29.8 ±1.0	67.6 ±2.9

the cotyledons partially reversed the inhibitory effect of gibberellin on ethylene production (Table 2). This suggests that GA_3 can reverse the inhibitory effect of ABA on *P. nil* flowering indirectly, through decreasing ethylene production.

As GA_3 applied to plants cultivated under sub-inductive conditions stimulates flowering (Kulikowska-Gulewska *et al*. 2000), it is also possible that gibberellins independently stimulate flowering and the inhibitory effect of ethylene on *P. nil* flowering may be due to the inhibition of gibberellin biosynthesis or action. For a more precise explanation of interactions between ABA, GA_3 and ethylene, additional measurements of gibberellin content changes during the inductive night are necessary.

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