Changes in Catecholamine Levels in Short Day-Induced Cotyledons of *Pharbitis nil*

Masayuki Suzuki[a], Masaya Mizoguchi[b], Fumihiko Yano[b], Utako Hara[b], Mineyuki Yokoyama[c], and Naoharu Watanabe[b*]

[a] United Graduate School of Agricultural Science, Gifu University (Shizuoka University), 836 Ohya, Shizuoka 422–8529, Japan
[b] Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, Ohya, Shizuoka 422–8529, Japan. Fax: +81-54-238-4870.
E-mail: acnwata@agr.shizuoka.ac.jp
[c] Basic Resarch Center, Shiseido Co. Ltd., Yokohama 224-8558, Japan

* Author for correspnodence and reprint requests

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We investigated the effects of catecholamine on flower-induction in *P. nil* (cv. Violet). GC-SIM analysis identified dopamine for the first time in *P. nil* seedlings. Dopamine levels in the cotyledons did not show a significant change during the inducing dark treatment. The dopamine content of cotyledons exposed to various durations of darkness were 0.1–0.2 nmol/g fresh weight. The same content was found when cotyledons were exposed to continuous light.

Key words: Pharbitis nil, Dark Treatment, Catecholamine

Introduction

There have been numerous reports on substances that induce flowering in *Pharbitis nil* (Kondoh *et al.*, 1999a, 1999b). However, neither the presence of substances related to flowerinduction, nor their physiological functions are well clarified. A water extract of *Lemna paucicostata* incubated under aerobic conditions induced flowering activity (Takimoto *et al.*, 1989). (–)-Norepinephrine, which was found in the supernatant after centrifugation of the water extract, should be involved in flower induction (Takimoto *et al.*, 1991). In addition, Yokoyama *et al.*, (2000) identified 9-hydroxy-10-oxo-12(*Z*),15(*Z*)-octadecadienoic acid (α-ketol of a-linolenic acid: KODA), which was released from of *L. paucicostata* exposed to drought, heat or osmotic stress. KODA demonstrated strong flower-inducing activity in the presence of (–)-norepinephrine. Furthermore, reaction products of (–)-norepinephrine with KODA under alkaline conditions also showed strong flower-inducing activity in *Lemna* (Yamaguchi *et al.*, 2001).

P. nil is a typical short-day plant; in which flowering is induced when day length becomes shorter than a critical value. This process is very sensitive and flowering can be induced by exposing a seedling cultivated under continuous light to a single 16-h dark period (Imamura and Takimoto, 1955). Flowerinducing activity was observed in *P. nil* seedlings after exposure to phloem exudate from a *P. nil* cotyledon that was exposed to darkness (Ishida *et al.*, 1991). Based on above results, we expected that similar factors are involved in flowerinduction in both *Lemna* and *P. nil*.

We previously focused on the presence of endogenous KODA, and have recently identified KODA in the cotyledon of *P. nil* after a dark treatment for 16 h (Suzuki *et al.*, 2002). KODA content in the cotyledon increased only after dark treatment for 14, 15 or 16 h. However, the KODA content failed to increase upon interruption of this dark period with a 10-min light period, and the flower budding was not induced. Based on these observations, it could be assumed that KODA is associated with flower induction in *P. nil.* In contrast, KODA did not induce flowering in *P. nil*

Abbreviations: MSFTA, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide; TMCS, trichloromethylsilane; dopamine, 3,4-dihydroxyphenethylamine; DOPA, 3,4-dihydroxyphenylalanine; DOPS, 3,4-dihydroxyphenylserine; GC-MS, gas chromatography-mass spectrometry; SIM, selected ion monitoring.

grown under long-day conditions. These results indicate that other factors are involved in flower induction in *P. nil*.

Changes in cotyledon components under conditions of flower induction in *P. nil* seedlings have previously been reported. Under conditions of flower induction, quantitative changes in ascorbic acid or phenylpropanoid levels in the cotyledons are observed (Shinozaki *et al.*, 1988, 1994; Hirai *et al.*, 1993, 1994, 1995). However, the relationship between such changes and flower induction has not yet been elucidated.

Inhibitors of catecholamine biosynthesis also inhibited flower induction in *P. nil.* However, when norepinephrine was exogenously fed in the presence of inhibitors of catecholamine biosynthesis, flower induction was recovered. (Ueno and Shinozaki, 1999). This suggests that catecholamines may be involved in *P. nil* flower induction. Shinozaki *et al.* (1999) conducted HPLC analysis and did not detect catecholamines in the cotyledon of *P. nil.* To the best of our knowledge, no reports have yet documented the presence of catecholamines in the *P. nil* cotyledon.

We therefore focused our research on detecting catecholamines in the cotyledon of *P. nil* and recording changes in their levels during inductive dark treatment. GC-MS was used in order to identify catecholamine and quantify concentration changes in the cotyledons during exposure to darkness.

Experimental

Reagents

Norepinephrine and epinephrine were purchased from Wako Pure Chemical. (Osaka, Japan) Dopamine, DOPA, DOPS, and methoxyamine hydrochloride were purchased from Aldrich (St. Louis, MO, USA). MSTFA, TMCS, and pyridine were purchased from Wako Pure Chemical. Methanol was purchased from Kanto Chemical (Tokyo, Japan) or Dojin Chemical (Tokyo, Japan). 2-(3,4-Dihydroxyphenyl)ethyl-1,1,2,2,-d_4 amine (dopamine-d_4) HCl (98.4 atom% D) for internal standard was purchased from CDN Isotope (Quebec, Canada).

Plant materials

Seedlings of *P. nil* (cv. Violet), purchased from Marutane (Kyoto, Japan), were germinated as described by Kaihara *et al.* (1989). Briefly, seeds of *P. nil* (cv. Violet) were immersed in sulfuric acid (96.0%) for 20 min at 35 °C, washed in running tap water overnight, placed on moistened sea sand, and then transplanted into wet vermiculite. They were grown under continuous light at 26 °C for a further 4 days, which included the completion of cotyledon unfolding. Seedlings were then transplanted into Nakayama's culture solution (Nakayama and Hoshino, 1973), and subjected to a single dark period. Darkness was induced by covering plants with a black craft box in a chamber at 26 °C. An equal number of plants were incubated under continuous light irradiation as controls. After dark treatment, plants were cultivated for 2 weeks under continuous light provided by cool-white fluorescent tubes at 26 °C; the irradiance level varied from 5000 lx to 6000 lx, as evaluated with an optical power meter (Topcon, Tokyo, Japan).

Sample preparation for catecholamine analysis

Aerial parts of *P. nil* were harvested at each interval (shown in Fig. 3), powdered in liquid nitrogen and stored at – 80 °C until use. A portion of each sample (*ca.* 200 mg) was sonicated in methanol at 0 °C for 1 min with 50 µl of dopamine-d_4 (4 µM in methanol) as an internal standard. The sonicated mixture was centrifuged at $1500 \times g$ for 10 sec. Extraction was repeated 3 times. After combining the supernatant of each extraction, the solvent was removed under reduced pressure and below 25 °C. Residues were further dried under reduced pressure in the presence of P_2O_5 at 30 °C for 8 h. For identification of DOPA and DOPS, the previously reported procedure (Roessner *et al.*, 2000; Szopa *et al.*, 2001) was slightly modified and used. Methoxyamine hydrochloride (25 µl) in pyridine (stock 20 mg/ml solution) was added to dried sample and incubated at 40 °C for 60 min. The samples were then derivatized with 25 µl of MSTFA for 30 min at 40 °C, 1 µl was subjected to GC-SIM analysis. For identification and quantification of catecholamines, dried samples were added with 50 µl of reagent mixture (MSTFA-TMCS = 10:1 v/v) and 50 µl of pyridine,

and incubated for 30 min at 40 °C, 1 µl was subjected to GC-SIM analysis. Recovery samples were spiked with dopamine, DOPA, norepinephrine, epinephrine and DOPS.

GC-MS (SIM) analysis

A gas chromatograph-mass spectrometer QP-5000 and QP-5050 (Shimadzu, Japan), equipped with a TC-1 fused silica capillary column (30 m × 0.25 mm; film thickness, 0.25 µm; GL Science, Tokyo, Japan) was used. The following ions were used for quantification: internal standard (I. S., dopamine-d_4), *m/z* 176; dopamine, *m/z* 174; DOPA, *m/z* 218, norepinephrine, *m/z* 174, epinephrine, *m/z* 116 and DOPS, *m/z* 355. The amount of catecholamine was determined from the ratio of the catecholamine peak height to internal standard (dopamine-d_4) peak height, and this was plotted against the amount ratios using calibration curves. All the data are the mean of 3 independent experiments (in each experiment 3 plants were used) with the SD.

Results and Discussion

Identification of catecholamines in P. nil cotyledon

We focused whether 3 catecholamines (norepinephrine, epinephrine, dopamine) and the biosynthetic precursors (DOPA and DOPS) were present in the cotyledons of *P. nil.* One-hundred micrograms of the above catecholamine standards were derivatized with methoxyamine and MSTFA. Derivatives were analyzed by GC-MS. The ion at *m/z* 426 was observed as a molecular ion in the dopamine spectrum, which suggests that dopamine was converted into a tetra TMS derivative. Norepinephrine and epinephrine were detected as tetra TMS (TMS_4) and penta TMS (TMS_5) derivatives, respectively. The ion at *m/z* 485 was observed as a molecular ion in the DOPA spectrum. The presence of this ion suggests that DOPA was converted into a TMS_4 derivative rather than an oxime-TMS derivative. Although it was thought that DOPA and DOPS would convert to TMS derivatives after conversion to oxime-derivatives, oxime-derivatization did not proceed and instead they were converted to the TMS derivatives. The characteristic ions of the derivatives for determination are indicated in Table I.

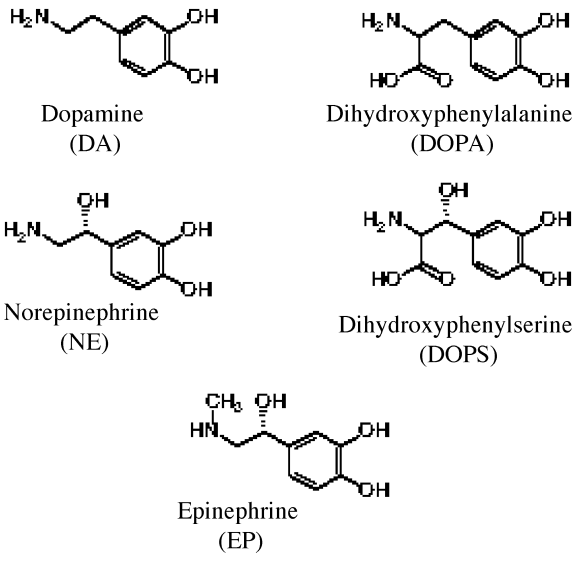

Fig. 1. Structures of catecholamine derivatives.

For identification of the catecholamines in the *P. nil* cotyledon, cotyledons subjected to a 16-h dark treatment were extracted and derivatized, and then analyzed by GC-MS (SIM). As shown in Fig. 2A, a peak of authentic TMS_4-dopamine at *m/z* 174 was observed 0.05 minute after a peak of TMS_4-dopamine-d_4 (I. S.) at *m/z* 176. The ratio of each peak intensity in the chromatogram at *m/z* 430 (M^+ for TMS_4-dopamine-d_4) and *m/z* 176 varied from 1:35 to 1:58 due to instability of the ion at *m/z* 430. The ratio for *m/z* 426 (M^+ for TMS_4-dopamine) and *m/z* 174 also varied 1:41 to 1:61. As shown in Fig. 2B, in each trace at *m/z* 426 and *m/z* 174 of cotyledon extract, a peak was detected in the ratio of 1:44 at the same retention time (28.1 min) as standard TMS_4-dopamine. This ratio corresponded well with the ratio of ion intensity of the ions at *m/z* 426 and *m/z* 174 in the SIM chromatogram of authentic TMS_4-dopamine. Based on the above-mentioned result, endogenous dopamine was identified in cotyledons that were subjected to a 16-h dark treatment. Other catecholamines were not detected in this sample or any other sample that was exposed to darkness.

Changes in dopamine content of P. nil cotyledons during exposure to darkness

As shown in Fig. 3A, cotyledons were harvested at 0, 4, 8, 12, 16, 17, 20, and 36 h after the com-

Table. I Retention times and characteristic ions of catecholamines.

	t_R[min]	M^+	Fragment ions (relative abundance [%])		
TMS_5-Norepinephrine	29.13	529	355 (2.6)	174 (100)	73 (42)
TMS_4-Epinephrine	25.42	471	355 (1.5)	116 (100)	73 (51)
TMS_4-Dopamine	28.35	441	426 [M-15](1.6)	174 (100)	73 (35)
TMS_4-Dopamine-d_4(I. S.)	28.30	445	430 [M-15](1.5)	176 (100)	73 (34)
TMS_4-DOPA	28.73	485	267 (50)218 (100)	73 (81)	
TMS_5-DOPS	29.95	511	355 (100)	291 (8.6)	73 (80)
TMS_5-DOPS	30.07	511	355 (100)	291 (11)	73 (75)

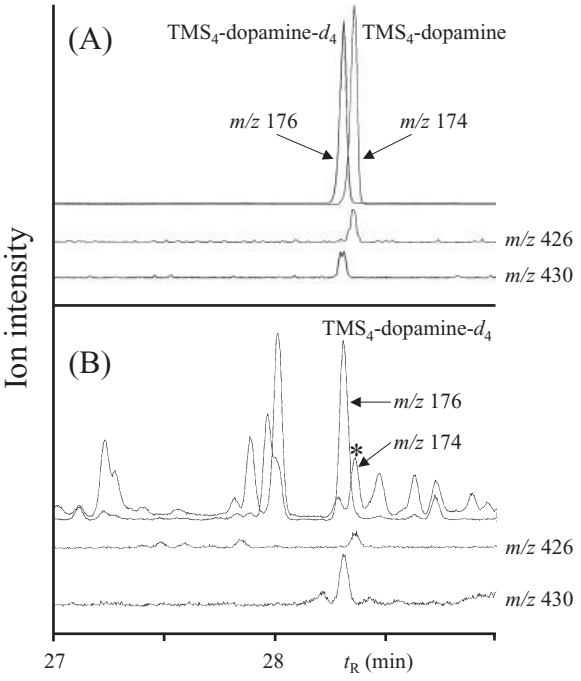

Fig. 2. GC-SIM trace at *m/z* 176, 430 (TMS_4-dopamine-d_4) and *m/z* 174, 426 (TMS_4-dopamine) of authentic samples (A) and extracts (B) from *P. nil* grown under the flower-inductive conditions (exposure to 16h darkness).
The peak with an asterisk in the plant extract shows same retention time (28.35 min) as authentic TMS_4-dopamine.

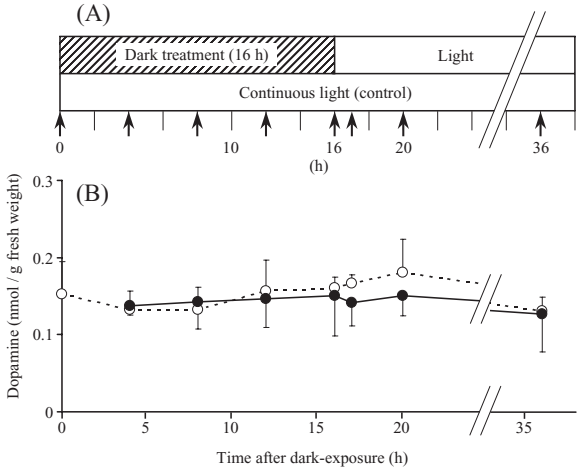

Fig. 3. Procedure for sample preparation to estimate the contents of dopamine under flower-inducing conditions, and endogenous dopamine changes induced by a single dark-exposure of *P. nil* (violet).
(A) Seedlings harvested at each intervals (↑ indicate sampling) were immediately crushed in a liquid N_2, then stored −80 °C until the analyses.
(B) Time course is shown of dopamine contents in aerial tissues of the seedlings exposed to darkness for 16 h (●,black dots) or to continuous light (○,white dots). Each value is the mean of 3 independent experiments with the SD (in each experiment, 3 plants used).

mencement of dark treatment. Immediately after dark treatment, cotyledons were exposed to light for 16 h. Three cotyledons were frozen and crushed immediately after photoperiodic treatment and kept for later analysis. To estimate the number of flower buds, the remaining seedlings (5 seedlings from each group) were cultivated under continuous light for 2–4 weeks and the number of the induced flower buds was counted. The peak number of flower buds in *P. nil.* was found to occur only after 14–16 h of a single dark treatment, whereas no flower buds were induced by exposure to a continuous light.

Dopamine levels in the sample cotyledons from each exposure time were 0.1–0.2 nmol/g fresh wt. As shown in Fig. 3B, no changes were observed in dopamine levels in the cotyledon due to dark treatment. Furthermore, dopamine content of the control cotyledons, which were cultivated under continuous light, was the same as those of plants exposed to darkness. Dopamine content of the

plant cotyledons that were harvested after 20 h of continuous light following 16 h of darkness was the same level as that of cotyledons exposed to darkness. These results indicate that changes in dopamine content of the cotyledon do not correlate with photoperiodic conditions.

Previous evidence suggested an important role for catecholamines in flower induction in *P. nil.* This evidence primarily includes observations that inhibitors of catecholamine biosynthesis also inhibit flower induction in *P. nil*, and that exogenously fed (–)-norepinephrine reverses this inhibition (Shinozaki *et al.*, 1999). While KODA concentration decreases rapidly during re-exposure to light after 16 h of dark treatment, dopamine content remains almost constant. Therefore, there is likely to be little direct interaction between KODA and dopamine during dark treatment.

In this research we could, for the first time, identify dopamine in the cotyledons of *P. nil.* But we could not obtain positive evidence for dopamine to be involved in the flower induction based on the data as shown in Fig. 3.

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