Identification in lettuce seedlings of a catecholamine active in synergistically enhancing the gibberellin effect on lettuce hypocotyl elongation

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Abstract. The occurrence of catecholamines in lettuce seedlings was examined by bioassay and gas chromatography-mass spectrometry (GC-MS), since synthetic catecholamines can synergistically enhance the stimulating effect of gibberellic acid (GA_3) on hypocotyl elongation of decotylized lettuce seedlings. The catecholamine fraction on TLC obtained from lettuce seedlings synergistically enhanced the GA_3 effect on hypocotyl elongation. The analysis of the catecholamine fraction from lettuce seedlings by GC-MS demonstrated the occurrence of dopamine.

Introduction

The gibberellin enhancement of lettuce hypocotyl elongation was found to depend upon a growth regulating substance(s) supplied by the cotyledons [3]. This 'cotyledon factor' was identified as dihydroconiferyl alcohol (DCA) [9]. An assessment of the relationship between the structure of DCA and its physiological activity suggested that catecholamines might have a similar activity [10]. Thus, catecholamines such as dopamine, epinephrine and norepinephrine were tested and found to synergistically enhance the effect of gibberellic acid (GA_3) on lettuce hypocotyl elongation [4].

Preliminary screenings of catecholamine-like substances by the lettuce hypocotyl elongation test have revealed that the basic ethyl acetate (EtOAc) fraction prepared from lettuce seedlings synergizes the GA_3 effect on lettuce hypocotyl elongation [4]. Analysis of this basic EtOAc fraction by thin-layer chromatography (TLC) indicated that the active substance(s) has the same Rf value as that of dopamine and epinephrine in two different solvent systems [4]. Catecholamines are known to occur in some higher plants [2, 13, 14]. These facts suggested that catecholamines might function as endogenous plant growth regulators in lettuce seedlings.

In the present study, we have characterized by bioassay and gas chromatography-mass spectrometry (GC-MS) from lettuce seedlings catecholamines active in enhancing the GA_3 effect on hypocotyl elongation.

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Materials and methods

Hypocotyl elongation test

Lettuce seeds (*Lactuca sativa* L., cv. Grand Rapids) were germinated on filter paper moistened with distilled water at 25.0 ± 0.5 °C. After 48 h in continuous light (ca. 10 W $\cdot m^{-2}$ at plant level) seedlings were selected for size uniformity and the cotyledons were excised just below the cotyledon joint. Ten decotylized seedlings were transferred onto filter paper moistened with 4 ml of test solution in a Petri dish (9 cm dia.), and grown for 48 h as noted above. Hypocotyl elongation was determined to a precision of 0.5 mm.

Extraction of catecholamines

Catecholamine-like substances in lettuce seedlings were extracted and partially purified according to the method of Weil-Malherbe and Bone [15]. Four-day old lettuce seedlings (30 g fresh weight) grown in the light were homogenized with dry ice and extracted with 140 ml of 0.2 N perchloric acid at ca. 0 °C for 1 h. The homogenate was passed through two layers of filter paper (Toyo No. 3). To 150 ml of filtrate were added 2.4 g of activated alumina, 1.2 g of ethylenediamine tetraacetic acid and 60 mg of sodium hydrogen sulfite. The pH of the filtrate was then adjusted to 8.4–8.6 by adding 2 N NaOH at ca. 0 °C. Activated alumina was packed in a glass column and washed with water. Catechol-type compounds adsorbed on the alumina were eluted by passing 0.2 N acetic acid through the column. The acetic acid fraction was collected, then evaporated to dryness under reduced pressure at ca. 40 °C.

TLC of the acetic acid fraction

The acetic acid fraction was dissolved in methanol: H_2O (70:30, v/v), then streaked on SiO_2 TLC plates (Merck, 5 x 20 cm, 0.25 mm), and developed with chloroform:methanol (30:1, v/v). The 10 equal Rf bands were extracted with EtOAc. The solvent was evaporated at 50 °C by air-streaming, then the residue was dissolved in distilled water for the bioassay using decotylized lettuce seedlings [4].

GC-MS of the acetic acid fraction

For GC-MS of catecholamines, pentafluoropropionyl derivatives were formed according to the modified method of Änggård and Sedvall [1]. The acetic acid fraction was dissolved in 100 µl of acetonitrile, to which 100 µl of pentafluoropropionic anhydride was added. After 30 min at 60 °C, the reagent and solvent were removed with a stream of N_2, then the residue was dissolved in benzene and injected into the column of GC. GC-MS of the acetic acid fraction was carried out by the modified method of Koslow and Cattabeni [6] using a GC-MS, JOEL D-300, equipped with a mass data

analysis system (JMA-2000). A 2-m glass column (i.d., 3 mm) was packed with silicone OV-105 on Gas Chrom Q 60–80 mesh. The conditions of GC-MS were: Column temperature, 170 °C; carrier gas (He) flow rate, 20 ml/min; molecular separator temperature, 250 °C; ion source temperature, 200 °C; ionization voltage, 20 V and ionizing current, 300 μA.

Results

Physiological activity of the acetic acid fraction

The fraction eluted with 0.2 N acetic acid from the column of activated alumina was dried, then dissolved in distilled water to test its physiological activity in enhancing the effect of GA_3 on lettuce hypocotyl elongation. An amount equivalent to 1 g fresh weight of lettuce seedlings was dissolved in 1 ml of distilled water; this was taken as 1 unit. Dectotylized lettuce seedlings were grown in the light for 2 days with or without 10^{-5} M GA_3, together with varying units of the acetic acid fraction. As shown in Figure 1, the acetic acid fraction synergistically enhanced the effect of GA_3 on hypocotyl elongation of decotylized lettuce seedlings, but it showed no effect on hypocotyl elongation when given alone.

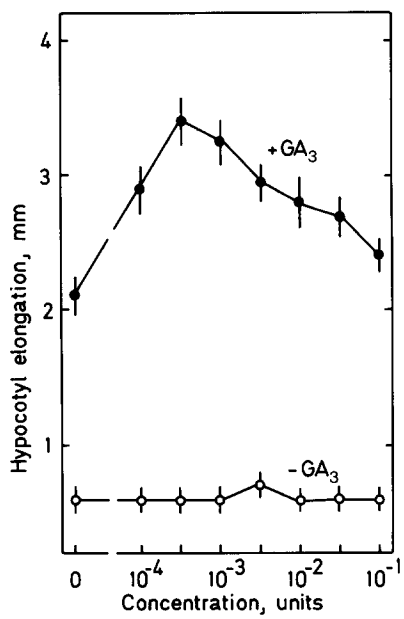

Figure 1. Effect of the acetic acid fraction of a lettuce seedling extract on GA_3-induced hypocotyl elongation of decotylized lettuce seedlings. Decotylized seedlings were grown in light for 2 days in the presence or absence of 10^{-5} M GA_3, and with or without varying concentrations of the acetic acid fraction. An amount equivalent to 1 g fresh weight of lettuce seedlings was dissolved in 1 ml of distilled water; this was taken as 1 unit. The initial length of the hypocotyl was 2.7 mm. Vertical lines represent standard error (n = 10).

Next, the acetic acid fraction was developed on a SiO_2 TLC plate using methanol:chloroform (30:1, v/v). The chromatogram was bioassayed as described above. Physiological activity was detected in the area of Rf 0–0.2, Rf 0.4–0.5 and Rf 0.7–0.8 (Fig. 2A). To examine whether this physiological activity was due to basic substance(s), an attempt to remove basic substances in the acetic acid fraction was made by passing the fraction, dissolved in distilled water, through a cation exchange resin (Dowex 50W, Na^+ type) column. The water eluate from the column was dried at 40 °C under reduced pressure. The residue was dissolved in 70% aqueous methanol and fractionated by TLC, then bioassayed (Fig. 2B). The physiological activity in the area of Rf 0–0.2 had disappeared, while that in Rf 0.4–0.5 and Rf 0.7–0.8 zone remained unchanged. The Rf value of dopamine and epinephrine in this TLC system was near zero. This suggested that the physiological activity of Rf 0–0.2 area is due to catecholamine-like substances in lettuce seedlings.

GC-MS of the acetic acid fraction

The gas chromatogram and mass fragmentogram of a standard mixture of pentafluoropropionyl derivatives of dopamine, epinephrine and nore-

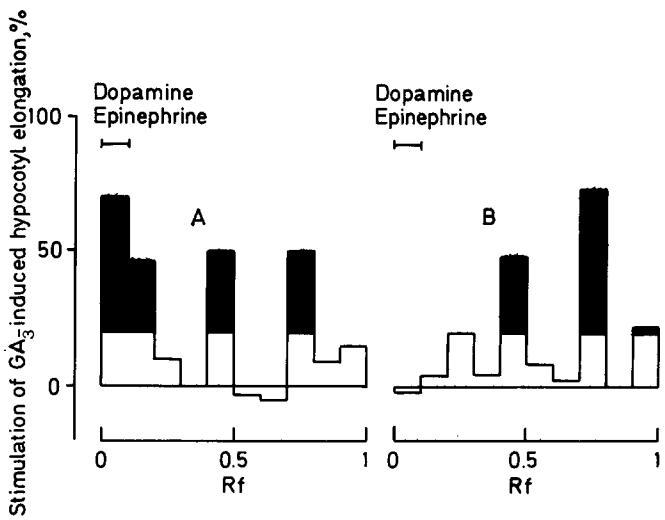

Figure 2. Bioassay of a chromatographed acetic acid fraction of a lettuce seedling extract. A. The acetic acid fraction, dissolved in 70% aqueous solution of methanol, was streaked on SiO_2 TLC plate, then developed with a mixture of chloroform:methanol (30:1, v/v). Decotylized lettuce seedlings for bioassay were grown in light for 2 days in the presence or absence of 10^{-5} M GA_3 and/or each fraction, the latter being at the concentration of 3×10^{-4} unit. The shaded area of the histogram represents physiological activity significantly different from the controls at the 5% level. B. Bioassay of the acetic acid fraction after dissolving in distilled water and passing through a column of Dowex 50W (Na^+ type) to remove basic substances. Chromatography and bioassay as per Figure 2A. The shaded area of the histogram represents physiological activity significantly different from the controls at the 5% level.

pinephrine are shown in Figure 3, and for the acetic acid fraction obtained from lettuce seedlings are shown in Figure 4. The total ion trace (TIM) of the acetic acid fraction showed one prominent peak (peak 3) and two small peaks (peak 1 and 2) at the retention time of ca. 2 to 5 min. Peak 3 at the retention time of ca. 3.3 min was found to have molecular ions of m/e 428, 281 and 176, which are characteristic of the dopamine pentafluoropropionyl derivative [7]. As shown in Figure 5, mass spectrum analysis of the peak 3 in Figure 4 demonstrated the presence of a molecular peak (m/e 591) together with major peaks (m/e 176, 281 and 428) which are characteristic of the authentic dopamine pentafluoropropionyl derivative. The area of the GC peak of dopamine was compared with a GC calibration-curve of authentic dopamine, and the endogenous content of dopamine was estimated to be 44.3 ng per g fresh weight of seedlings. Molecular ions of m/e 190 and 590, which are characteristic of the pentafluoropropionyl derivatives of epinephrine and norepinephrine [7], were not detected at the retention time of 2 to 5 min, suggesting that these catecholamines are not present in the acetic acid fraction obtained from lettuce seedlings.

Discussion

The occurrence of dopamine in lettuce seedlings was demonstrated by GC-MS. Dopamine has been isolated from various species of higher plants [12], and is considered to be an important intermediate in the biosynthesis

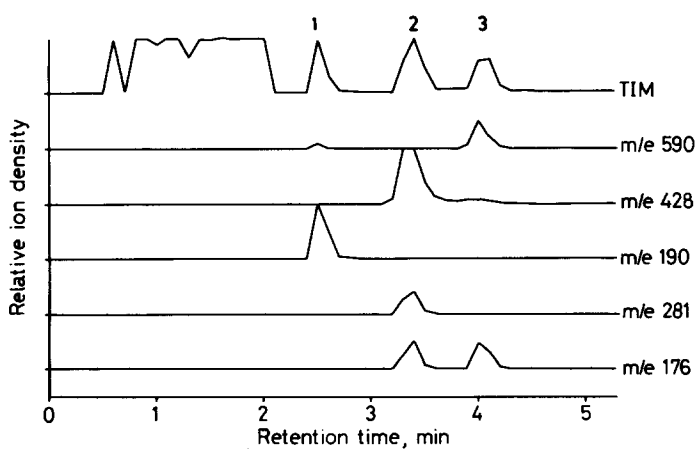

Figure 3. Gas chromatogram and mass fragmentogram of standard catecholamine-pentafluoropropionyl derivative mixture. The derivatives dissolved in benzene were injected into the GC-MS, then total ion density and each characteristic fragments (m/e) of the pentafluoropropionyl derivative of dopamine, epinephrine and norepinephrine were recorded. Peak 1, epinephrine; peak 2, dopamine peak 3, norepinephrine. TIM, total ion monitor.

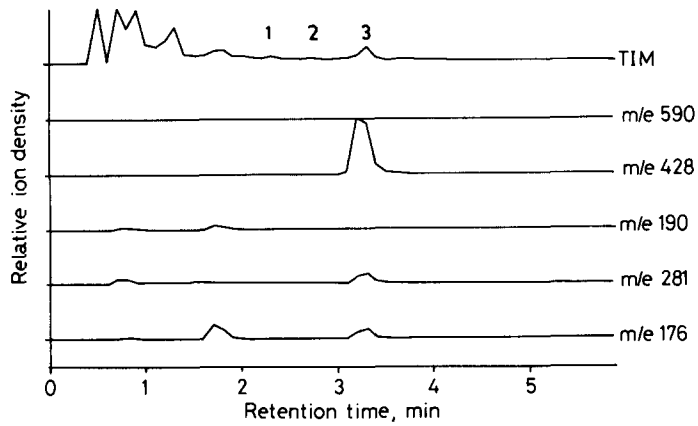

Figure 4. Gas chromatogram and mass fragmentogram of the acetic acid fraction obtained from lettuce seedlings. The acetic acid fraction derivatized with pentafluoropropionic anhydride was analyzed by GC-MS. The sample was dissolved in 10 μl of benzene and injected (2 μl) into the GC-MS. As to the conditions of GC-MS, see Materials and methods. Total ion density and each characteristic fragment (m/e) of catecholamine pentafluoropropionyl derivatives were recorded. The retention time of the peak 3 coincided with that of dopamine pentafluoropropionyl derivative. TIM, total ion monitor.

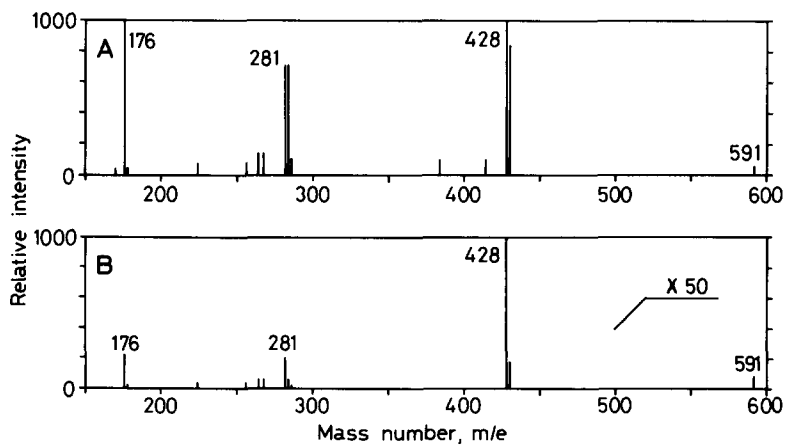

Figure 5. Mass spectra of authentic dopamine pentafluoropropionyl derivative and the peak 3 in Figure 4. A: authentic dopamine pentafluoropropionyl derivative; B: the peak 3 in Figure 4.

of some plant alkaloids [11]. Dopamine has been extensively studied as a neurotransmitter in animals. However, the physiological role of the amine in higher plants is basically unknown, although catecholamines have been reported to affect flowering in *Lemna* [8].

Our previous study [4] demonstrated that dopamine synergistically enhanced the GA_3 effect on lettuce hypocotyl elongation, as did DCA. Analysis of the interaction in the GA_3-induced hypocotyl elongation between catecholamines and an anticotyledon factor [5], *trans*-cinnamic acid, suggested that DCA and catecholamines bind to the same postulated receptor when they function [4]. These findings, together with the present results obtained by GC-MS, strongly suggest that dopamine acts as an endogenous growth regulator in lettuce seedlings.

As reported previously [4], the optimal concentration of dopamine to enhance the GA_3 effect on lettuce hypocotyl elongation was 10^{-6} M. The present study revealed that the endogenous level of dopamine in lettuce seedlings is 44.3 ng per g fresh weight, corresponding to 2.87×10^{-7} M. It is thus highly probable that changes in the endogenous level of dopamine in lettuce seedlings affects GA_3-induced hypocotyl elongation as an endogenous growth regulator.

DOPA is a possible precusor of dopamine [11]. The endogenous content of DOPA was reported to increase in parallel with epicotyl elongation of *Stizolobium hassjoo* [6]. But, DOPA did not enhance the GA_3 effect on lettuce hypocotyl elongation [4]. In order to further assess the physiological role of dopamine, the distribution of dopamine and DOPA in lettuce seedlings and the correlation between hypocotyl elongation and the endogenous level of dopamine are now under investigation.

In addition to the Rf zone corresponding to dopamine, the area of Rf 0.4–0.5 and Rf 0.7–0.8 showed cotyledon factor-like activity (Fig. 2A) which is not basic in nature. In the present chromatographic conditions, catechol-type compounds are known to be specifically adsorbed by the alumina column used and eluted by acetic acid. If so, the cotyledon factor-like activities detected in Rf 0.4–0.5 and Rf 0.7–0.8 may still be due to catechol-type compounds. However, the Rf value of authentic DCA is 0.42 in these TLC conditions. This suggests that DCA may be a contaminant in the acetic acid fraction. We are now attempting to isolate and identify these unknown cotyledon factor-like substances.

Acknowledgements

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