Inhibition by Cytokinin of the Accumulation of Betacyanin in Suspension Cultures of *Phytolacca americana*

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Z. Naturforsch. **47c**, 705–710 (1992); received June 29/August 17, 1992

Betacyanin, Cytokinin, *Phytolacca americana*, Suspension Culture, Tyrosine

The accumulation of betacyanin was reduced by the addition of various cytokinins to suspension cultures of *Phytolacca americana*. The decrease in the accumulation of betacyanin was overcome by exogenously supplied tyrosine which is a precursor of betacyanin. Benzylaminopurine (BAP) decreased the level of free tyrosine in the cells. Feeding experiments using labeled tyrosine revealed that BAP reduced the incorporation of labeled tyrosine into betacyanins (to about 50% of the control rate). These results suggest that both the availability of tyrosine and the biosynthetic activity of the pathway from tyrosine to the betacyanins are involved in the inhibition of the accumulation of betacyanins by cytokinins in *Phytolacca americana* cells.

Introduction

Betacyanin, red-violet pigment synthesized from tyrosine *via* 3,4-dihydroxyphenylalanine (DOPA), is a characteristic secondary metabolite in most species of Centrospermae. Production of betacyanins is affected markedly by environmental factors (plant growth regulators, light, nutrients, etc., [1]). Light-stimulated synthesis of betacyanin in *Amaranthus* seedlings, mediated by the red/far-red-reversible reaction of phytochrome, has been reported [2–4]. Plant growth regulators also affect the accumulation of betacyanin [5–9]. In particular, it is well known that cytokinins markedly promote the synthesis of betacyanin in dark-grown etiolated seedlings of *Amaranthus*, exogenously supplied cytokinins strongly promote the accumulation of betacyanin in the dark [10]. The actions of both light and kinetin on the synthesis of betacyanin have been analyzed [11, 12]. Kochhar *et al.* [13] reported that light and cytokinin affect the production of betacyanin independently of one another. A bioassay for cytokinins, using the accumulation of betacyanin in *Amaranthus* seedlings was reported by Biddington and Thomas [14] and the variability of this method was discussed by Elliott [15–18]. However, the details of the way in which cytokinins affect the accumulation of betacyanin are still poorly understood.

In suspension cultures of *Phytolacca americana*, a close correlation has been reported between growth and accumulation of betacyanin [19]. Effects of phosphate and the sources of carbon and nitrogen on the accumulation of betacyanin have also been reported [19–21]. Accumulation of betacyanin shows clearly a positive correlation with cell division in *Phytolacca americana* cells, whereas most of other secondary metabolites such as anthocyanin show a negative correlation with cell division [22].

In the present study, we reported that cytokinins showed inhibitory, but no promotive effects on the accumulation of betacyanin in suspension cultures of *Phytolacca americana*, and the mode of inhibition by cytokinins was investigated with respect to the size of the endogenous pool of tyrosine, a precursor of betacyanin, and the biosynthetic activity of the pathway from tyrosine to betacyanin.

Materials and Methods

Suspension culture

The suspension cultures used in present experiments were prepared from callus initiated from

Abbreviations: BAP, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; DOPA, 3,4-dihydroxyphenylalanine; HPLC, high performance liquid chromatography; IPA, isopentenylaminopurine; 4-PU, 4-pyridylurea.

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Verlag der Zeitschrift für Naturforschung, D-W-7400 Tübingen
0939–5075/92/0900–0705 $ 01.30/0

stem explants *Phytolacca americana*. This cell line acquired the ability to produce betacyanin spontaneously during subculture in dark. Cells were maintained in the medium of Murashige and Skoog [23] that contained 3% (w/v) sucrose and 5×10^{-6} M 2,4-dichlorophenoxyacetic acid (2,4-D), as described previously [19].

Determination of cell growth and betacyanin content

Cells were harvested 6 days after transfer by filtration and weighed (fresh weight). Cell number was estimated by counting protoplasts in a haemocytometer after enzymatic maceration of cell clusters. Cells (100 mg fresh weight) were suspended in 2 ml of a solution of enzymes [2% Driselase (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan), 0.1% Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Noda, Japan) in 0.7 M mannitol] and incubated for 2 h at 27 °C on a shaker at 60 strokes/min for maceration. Levels of betacyanin were determined from absorbance at 535 nm of a solution obtained by extraction of 100 mg frozen cells with 5 ml of 80% methanol.

Extraction and analysis of free amino acids

One g of frozen cells (harvested 6 days after transfer) was homogenized in a Potter-Elvehjem glass homogenizer after the addition of a known amount of 6-amino-caproic acid (internal standard) and 80% ethanol. The homogenate was centrifuged at 10,000 × *g* for 15 min. The supernatant was evaporated to dryness and the residue was dissolved in 10 ml of distilled water. The solution was diluted with an equal volume of 0.04 M HCl after washing with ethyl ether to remove lipophilic substances. Amino acids were analyzed in an automatic amino acid analyzer (Hitachi-835) with a series of lithium citrate buffer [24].

Tracer experiments

L-[U-^{14}C]tyrosine (74 kBq; specific activity, 16.65 TBq/mol; ICN, Irvine, U.S.A.) was added to a 4-day-old suspension of cells in a small vial with a center well. The cells were then incubated for 4 h at 27 °C and respiratory CO_2 was absorbed by 0.2 ml of 1 M KOH that had been placed in the center well (CO_2 fraction). After incubation, cells were collected by filtration, washed with an aliquot of distilled water, weighed and frozen. Two ml of 80% methanol were added to frozen cells and the mixture was centrifuged at 10,000 × *g* for 15 min. The pellet was washed with 80% methanol. Supernatants were combined and evaporated to dryness. The residue was dissolved in 0.5 ml of distilled water (80% methanol-soluble fraction). The protein fraction was extracted from the pellet by boiling with 2 ml of 1 M NaOH in a water bath (1 M NaOH-soluble fraction). The radioactivity of each fraction was determined in an LKB 1216 RACKBETA II liquid scintillation counter using Scintisol 500 (Dojindo Laboratories, Kumamoto, Japan) after neutralization (in the case of the 1 M NaOH-soluble fraction and CO_2 fraction).

Separation of betacyanins by HPLC and measurement of radioactivity

The soluble fraction in 80% methanol was further analyzed by HPLC [Shimadzu LC-6A HPLC system, equipped with detectors of both visible and UV light (Shimadzu D-2PD-2A), a Shimadzu C-R2AX integrator and an Aloka RLC-551 analyzer of radioactivity]. Two hundred µl of sample were injected onto an HPLC column, 250 mm × 4.6 mm, M & S pack C18 (M & S Instruments, Inc., Osaka, Japan) and developed with a linear gradient system: 0–3 min, 78% A, 22% B; 3–43 min, the concentration of B was increased at the rate of 1%/min; 43–45 min, A 38%, B 62%; where A was 10 mM KH_2PO_4 (pH 2.1) and B was 40% CH_3CN in 10 mM KH_2PO_4 (pH 2.1). Radioactivity was calculated from the area under each peak. Various betacyanins were identified by comparison of absorption spectra with authentic sample of betacyanin prepared from beet roots (*Beta vulgaris*) according to von Elbe *et al.* [25].

Results

Effects of various cytokinins on cell growth and the accumulation of betacyanin

Cytokinins inhibited the accumulation of betacyanin in suspension cultures of *Phytolacca americana*, but no promotion by any cytokinin was observed at any concentrations. The effects of kinetin, BAP and 4-pyridylurea (4-PU) on the accumulation of betacyanin and cell growth are shown in Fig. 1. More than 50% inhibition was

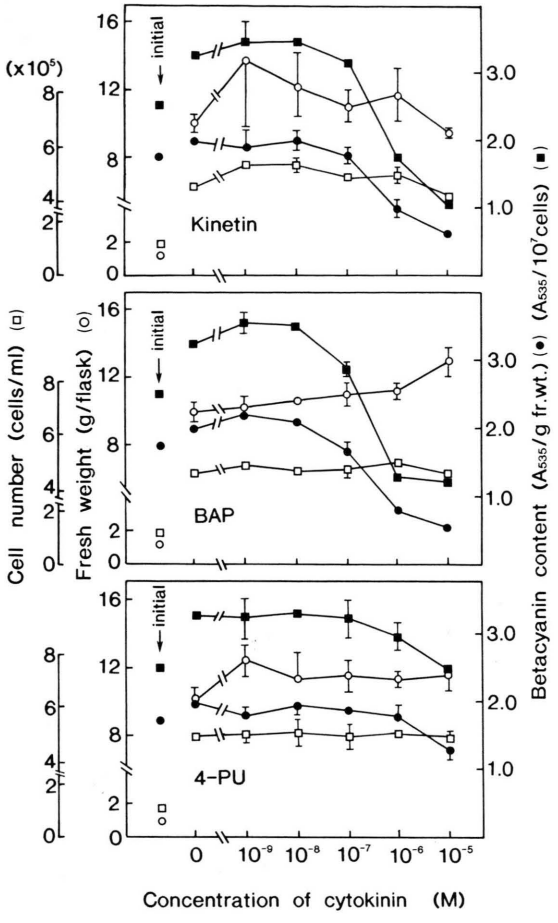

Fig. 1. Effects of kinetin, BAP and 4-PU on the accumulation of betacyanin, fresh weight and cell number. Fresh weight, cell number and A_{535} were measured 6 days after transfer. Vertical lines indicate S.D. (n = 3). "Initial" indicates the values of fresh weight, cell number and A_{535} at the start of culture.

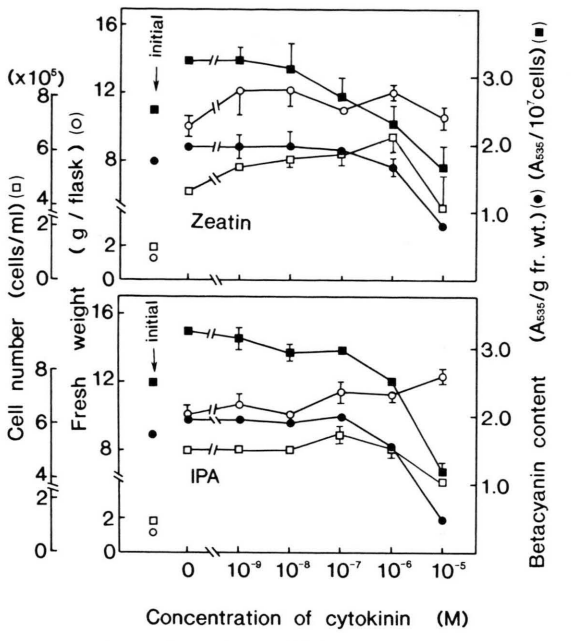

Fig. 2. Effects of zeatin and IPA on the accumulation of betacyanin, fresh weight and cell number. Fresh weight, cell number and A_{535} were measured 6 days after transfer. Vertical lines indicate S.D. (n = 3). "Initial" indicates the values of fresh weight, cell number and A_{535} at the start of culture.

observed at concentrations of kinetin above 10^{-7} M. BAP also inhibited the accumulation of betacyanin, while less inhibitory effect of 4-PU, another synthetic cytokinin, on the accumulation of betacyanin was observed. Two naturally occurring cytokinins, zeatin and isopentenylaminopurine (IPA), had the same effects on the accumulation of betacyanin as kinetin and BAP (Fig. 2). None of the cytokinins had any major effect on cell growth (as determined from the fresh weight and number of cells, measured 6 days after transfer). Since BAP caused the most dramatic inhibition of the accumulation of betacyanin, we used BAP as the cytokinin of choice in subsequent experiments.

Since it is possible that reduction of the amount of accumulated betacyanin by cytokinins is caused by reduction of the size of the pool of precursors of betacyanin, we examined whether or not a supply of tyrosine, a precursor of betacyanin, might reverse the reduction of the amount of accumulated betacyanin. The accumulation of betacyanin reduced by BAP was restored to 87% (on a fresh weight basis) or 83% (on a cell number basis) of control levels when 10^{-3} M tyrosine was included in the growth medium (Table I).

Changes caused by BAP in the levels of free amino acids

We next investigated the effects of BAP on the sizes of pools of free amino acids, as shown in Table II. BAP decreased the size of the total amino acid pool to 80% of control values and the size of pools of most individual amino acids, except for

Table I. Effect of 6-benzylaminopurine and tyrosine on the accumulation of betacyanin. Fresh weight, cell number and A_{535} were measured 6 days after transfer.

Additions	Betacyanin content [A_{535}/g fr. wt.]	[$A_{535}/10^7$ cells]
Control	2.49 ± 0.02*	3.21 ± 0.10
BAP 10^{-6} M	1.36 ± 0.04	1.47 ± 0.03
BAP 10^{-6} M+ Tyrosine 10^{-3} M	2.16 ± 0.14	2.67 ± 0.14
Tyrosine 10^{-3} M	3.12 ± 0.10	3.75 ± 0.13

BAP, 6-benzylaminopurine.
* S.D. (n = 3).

Cys, Met, Phe, GABA and His, were decreased. The size of the pool of tyrosine was also reduced (to 61% of control values) by BAP. When cells were cultured with both BAP (10^{-6} M) and tyrosine (10^{-3} M), the size of the pool of tyrosine in cells were increased by about 5 times that in control cells (data not shown).

Tracer experiments

Incorporation of radioactivity into the 1 M NaOH-soluble fraction (which corresponds to the protein fraction), the 80% methanol-soluble fraction, the CO_2 fraction, and betacyanins from labeled tyrosine is shown in Table III. BAP had little effect on the rate of incorporation of radioactivity into the 1 M NaOH-soluble fraction, the 80% methanol-soluble fraction and the CO_2 fraction.

The 80% methanol-soluble fraction was fractionated by HPLC and the radioactivities of resultant tyrosine and peaks of betacyanins were monitored. Two peaks of radioactive betacyanins (BC 1, BC 2) were observed after a 4 h incubation, while radioactivity was detected in some other peaks of betacyanins after a 24 h incubation (Fig. 3). The retention times of BC 1 and BC 2 corresponded to those of betacyanins isolated from *Beta vulgaris*. In particular, BC 1 corresponded to the major betacyanin in *Beta vulgaris* which was

Table II. Effects of 6-benzylaminopurine on sizes of pools of free amino acids. Cells were cultured for 6 days.

Amino acids	Amino acids content [µmol/10^7 cells] Control	with BAP (10^{-6} M) present
Asp	0.82 (1.0)*	0.42 (0.6)
Thr	0.79 (1.0)	0.54 (0.8)
Ser	5.71 (7.0)	3.13 (4.8)
Glu	6.39 (7.9)	4.29 (6.6)
Gln	15.03 (18.5)	13.55 (21.0)
Gly	1.36 (1.7)	1.07 (1.7)
Ala	24.40 (30.0)	16.54 (25.6)
Val	1.62 (2.0)	1.06 (1.6)
Cys	0.12 (0.1)	0.13 (0.2)
Met	0.12 (0.1)	0.18 (0.3)
Ile	0.54 (0.7)	0.40 (0.6)
Leu	0.86 (1.1)	0.79 (1.2)
Tyr	1.28 (1.6)	0.78 (1.2)
Phe	0.55 (0.7)	0.59 (0.9)
GABA	7.04 (8.7)	9.75 (15.1)
Trp	0.20 (0.2)	0.14 (0.2)
Lys	0.59 (0.7)	0.47 (0.7)
His	0.64 (0.8)	0.64 (1.0)
Arg	2.15 (2.6)	1.21 (1.9)
Asn	10.32 (12.7)	8.69 (13.4)
Pro	0.81 (1.0)	0.30 (0.5)
Sum	81.34 (100.1)	64.67 (99.9)

BAP, 6-benzylaminopurine.
* % of total amino acids in parentheses.

Table III. Incorporation of radioactivity from labeled tyrosine into CO_2 fraction, 1 M NaOH-soluble fraction, 80% methanol-soluble fraction and betacyanins. Cells were incubated with labeled tyrosine for 4 h.

Control, treated culture	Radioactivity [kBq/10^6 cells] CO_2	1 M NaOH-soluble fraction	80% methanol-soluble fraction	Tyrosine	Betacyanins BC 1	BC 2	Total
Control	0.16 (0.56)*	14.5 (51.1)	11.0 (38.5)	6.65 (22.9)	0.17 (0.59)	0.17 (0.59)	0.34 (1.18)
BAP 10^{-6} M	0.14 (0.58)	11.5 (49.1)	8.29 (35.3)	5.73 (24.5)	0.08 (0.35)	0.07 (0.29)	0.15 (0.63)

BAP, 6-benzylaminopurine.
* % of total uptake in parentheses.

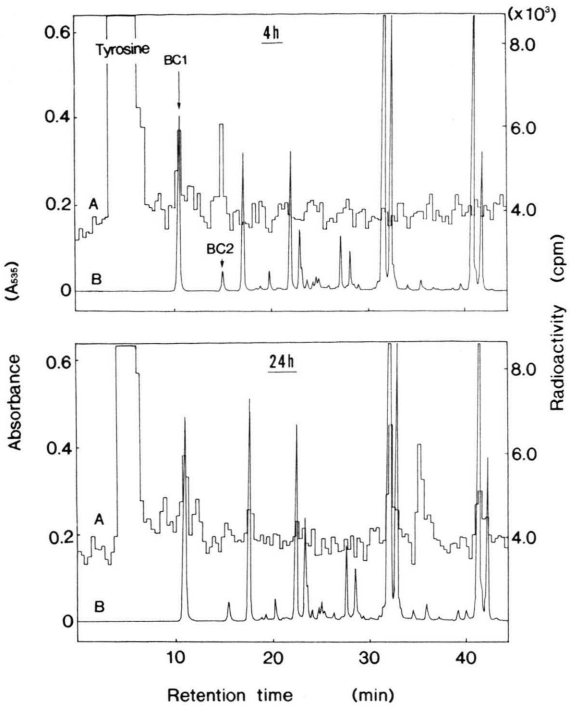

Fig. 3. Results of high performance liquid chromatography of the 80% methanol-soluble fraction, showing radioactivity (A) and absorbance at 535 nm (B). Cells were incubated for the indicated periods with labeled tyrosine. BC 1 and BC 2 are explained in Results.

identified as betanin (betanidin 5-O-β-glucoside) [26]. Incorporation from labeled tyrosine into peaks of betacyanin after 4 h incubation is shown in Table III. BAP decreased the rate of incorporation of radioactivity from labeled tyrosine into peaks of betacyanins (both BC 1 and BC 2) to less than 50% of control value.

Discussion

The promotive effects of cytokinins on the accumulation of betacyanin in *Amaranthus* seedlings have been reported by many authors [10, 14, 27]. By contrast, in the present study, it was demonstrated that cytokinins reduced the amount of accumulated betacyanin in suspension cultures of *Phytolacca americana* (Fig. 1 and 2). It is possible that the suspension cultures of *Phytolacca americana*, used in this study, contain higher level of endogenous cytokinins than do intact plants such as *Amaranthus* seedlings, or cultured cells of *Phytolacca americana* cells have a higher sensitivity to cytokinins, so that exogenously supplied cytokinins generate superoptimum conditions in *Phytolacca americana* cells. However, since cytokinins did not affect the growth of *Phytolacca americana* cells both on basis of cell number and fresh weight at lower concentration than 10^{-6} M at least, in which inhibition of accumulation of betacyanins was observed, exogenously supplied cytokinins seem not to generate superoptimum conditions for general metabolism in *Phytolacca americana* cells (Fig. 1 and 2). Furthermore, as shown in Table III, BAP reduced incorporation of radioactivity into betacyanin from [^{14}C]tyrosine to 53% of control, while it had little effect on the incorporation of radioactivity into CO_2, 80% methanol-soluble and protein fractions. These results indicate that the reduction of accumulation of betacyanin is not caused by inhibition of general metabolism, but apparently by inhibition of betacyanin biosynthesis from tyrosine.

The decrease in the amount of accumulated betacyanin caused by addition of BAP was reversed by addition of tyrosine (Table I). The size of the pool of endogenous tyrosine was reduced by BAP (Table II). These results suggest that the supply of tyrosine is an important factor in the inhibition of accumulation of betacyanin by BAP. The availability of tyrosine may not be enough to synthesize the maximum amount of betacyanin even in control cells, because the amount of accumulated betacyanin in these cells was increased by exogenously supplied tyrosine (Table I). BAP should reduce furthermore the availability of tyrosine as indicated by the reduction of the size of the pool of tyrosine by BAP. The reduction of the availability of tyrosine by BAP was not due to a change in the metabolic flow of tyrosine to protein synthesis, because BAP had little effect on the incorporation of labeled tyrosine into the protein fraction (Table III). Therefore, it might be considered that BAP affected the biosynthesis or degradation of tyrosine. However, it is rather improbable that the degradation of tyrosine is promoted by BAP, because the rate of incorporation of radioactivity from tyrosine into the CO_2 fraction was little affected by BAP.

In conclusion, the inhibitory effects of cytokinins on the accumulation of betacyanin in suspension cultures of *Phytolacca americana* cells are due to an insufficient supply of tyrosine, a precursor of the betacyanin; in the inhibitory effects are also due to inhibition of the activity of biosynthesis from tyrosine to betacyanins by cytokinins.

Acknowledgement

The authors are grateful to Mr. T. Takagi, Nishiki Laboratories of Kureha Chemical Industry Co. Ltd., Iwaki, Fukushima, for his valuable assistance for analysis of free amino acids and useful advice for this work.

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