Inhibition of Betacyanin Accumulation by Abscisic Acid in Suspension Cultures of *Phytolacca americana*

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Growth of cells and the accumulation of betacyanin were suppressed by the addition of abscisic acid (ABA) 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. ABA decreased the level of free tyrosine in the cells. Feeding experiments using labeled tyrosine revealed that ABA 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 ABA in *Phytolacca americana* cells.

Introduction

It is well known that the production of many of secondary plant metabolites are markedly effected by various environmental factors such as plant growth regulators, light and nutrients. Suspension cell cultures are suitable for studying the regulation of secondary plant metabolites by environmental factors, because environmental conditions can be easily controlled for cultured cells.

Betacyanin, red-violet pigment synthesized from tyrosine **via** 3,4-dihydroxyphenylalanine (DOPA), is a characteristic secondary metabolite distributed in most spices of Centrospermae (Mabry, 1980). Sakuta *et al.* (1986) demonstrated that a maximum accumulation of betacyanin was observed during the logarithmic phase in suspension cultures of *Phytolacca americana*. A clear positive correlation of the accumulation of betacyanin with cell division was reported by Hirose *et al.* (1990). From results obtained in these reports, it is suggested that the accumulation of betacyanin is involved in the growth of cells. Thus, effects of factors affecting growth of cells on accumulation or biosynthesis of betacyanin have been investigated. Sakuta *et al.* (1987 a, b) showed that the effects of nutrients, which are one of the important factors regulating the growth of cells, on the accumulation of betacyanin and discussed in relation to growth of cells for suspension cultures of *Phytolacca americana* (Sakuta *et al.*, 1987). Many investigators have reported effects of plant growth regulators, another important factor in growth of cells, on the accumulation of betacyanin (Piattelli, 1976). However, the mode of regulation of the accumulation of betacyanin by plant growth regulators is still obscure. We have already studied the mode of promotion by auxin (Sakuta *et al.*, 1991) and that of inhibition by cytokinin (Hirano *et al.*, 1992) of the accumulation of betacyanin in suspension cultures of *Phytolacca*. In this study, we report that ABA showed inhibitory effects on both the growth of cells and the accumulation of betacyanin in suspension cultures of *Phytolacca americana*, and the mechanism of inhibition by ABA 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.

Abbreviations: ABA, abscisic acid; A_{535}, absorbance at 535nm; 2,4-D, 2,4-dichloro-phenoxyacetic acid; DOPA, 3,4-dihydroxy-phenylalanine; HPLC, high performance liquid chromatography.

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Materials and Methods

Suspension culture

The suspension cultures used in present experiments were maintained in the medium of Murashige and Skoog (1962) that contained 3% (w/v) sucrose and $5x10^{-6}$ M 2,4-dichlorophenoxyacetic acid (2,4-D), as described previously (Sakuta *et al.*, 1986).

Determination of cell growth and betacyanin content

Cells were harvested 6 days after transfer by filtration and fresh weights of them were weighed (fresh weight). Cell number was estimated by counting protoplasts in a haemocytometer after enzymatic maceration of cell clusters as described previously (Hirano *et al.*, 1992). 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 (Hirano *et al.*, 1992).

Extraction and analysis of free amino acids

Extraction and analysis of free amino acids were performed as described previously (Hirano *et al.*, 1992).

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 hours at 27° C and respiratory CO_2 was absorbed by 0.2 ml of 1N 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 10,000x*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).

The soluble fraction in 80% methanol was further analyzed by HPLC and estimated incorporation of radioactivity from tyrosine to betacyanin as described previously (Hirano *et al.*, 1992).

Chemicals

ABA ((±)*cis-trans* isomer) was purchased from Sigma (St. Louis, U.S.A).

Results and Discussion

The effect of ABA on the accumulation of betacyanin and growth of cells is shown in Fig. 1. Reduction of the accumulation of betacyanin was observed at concentration of ABA above 10^{-6}M. Some other investigators reported that inhibitory effect of ABA on the accumulation of betacyanin in *Amaranthus* seedlings (Biddington and Thomas,

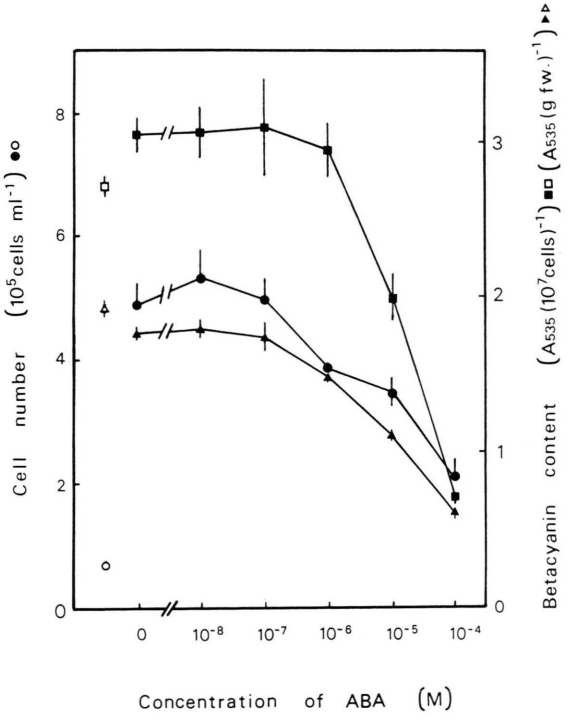

Fig. 1. Effects of various concentrations of abscisic acid on cell number (○, ●) and accumulation of betacyanin (A_{535} [g fw.]$^{-1}$, △, ▲; A_{535} [10^7 cells]$^{-1}$, □, ■). Cell number and A_{535} were measured at day 0 (open symbols) and day 6 (closed symbols) after transfer. Vertical lines indicate SD (n = 3).

Table I. Effects of ABA on pool size of free amino acids. Cells were cultured for 6 days.

Amino acids	Amino acids content [μmol (10^7 cells)$^{-1}$]			
	Control		with ABA (10^{-5} M) present	
Asp	0.82 ± 0.13*	(1.0)**	0.49 ± 0.23	(0.9)
Thr	0.79 ± 0.07	(1.0)	0.60 ± 0.09	(1.1)
Ser	5.71 ± 0.62	(7.0)	3.10 ± 0.45	(5.7)
Glu	6.39 ± 0.80	(7.9)	5.58 ± 0.39	(10.2)
Gln	15.03 ± 2.85	(18.5)	8.40 ± 1.34	(15.4)
Gly	1.36 ± 0.27	(1.7)	0.91 ± 0.19	(1.7)
Ala	24.40 ± 5.05	(30.0)	15.85 ± 4.93	(29.1)
Val	1.62 ± 0.49	(2.0)	1.17 ± 0.18	(2.1)
Cys	0.12 ± 0.08	(0.1)	–	–
Met	0.12 ± 0.04	(0.1)	0.07 ± 0.02	(0.1)
Ile	0.54 ± 0.09	(0.7)	0.70 ± 0.11	(1.3)
Leu	0.86 ± 0.14	(1.1)	0.85 ± 0.13	(1.6)
Tyr	1.28 ± 0.16	(1.6)	0.81 ± 0.15	(1.5)
Phe	0.55 ± 0.07	(0.7)	0.57 ± 0.07	(1.0)
GABA	7.04 ± 3.59	(8.7)	4.00 ± 1.05	(7.3)
Trp	0.20 ± 0.03	(0.2)	0.19 ± 0.02	(0.3)
Lys	0.59 ± 0.59	(0.7)	0.54 ± 0.11	(1.0)
His	0.64 ± 0.09	(0.8)	0.53 ± 0.12	(1.0)
Arg	2.15 ± 0.21	(2.6)	1.59 ± 0.24	(2.9)
Asn	10.32 ± 4.15	(12.7)	6.82 ± 1.55	(12.5)
Pro	0.81 ± 0.24	(1.0)	1.67 ± 0.28	(3.1)
Sum	81.34	(100.0)	54.45	(100.0)

* SD (n = 3); ** % of total amino acids in parentheses.

1977; Guruprasad and Laloraya, 1980; Ray *et al.*, 1983; Stobart *et al.*, 1970). Fig. 1 also showed inhibitory effect of ABA on the growth of cells (as determined from the number of cells, measured 6 days after transfer). Guruprasad and Laloraya (1980) reported that ABA inhibited both the growth of hypocotyl and the accumulation of betacyanin in *Amaranthus* seedings. We reported that the accumulation of betacyanin was prompted by auxin, which promote growth of cells (Sakuta *et al.*,1991). In our previous investigations, a close correlation has reported between the growth of cells and the accumulation of betacyanin (Sakuta *et al.*, 1986; Hirose *et al.*, 1990). Therefore, the reduction of the accumulation of betacyanin caused by ABA, which also inhibits the growth of cells, is understandable.

We previously reported that availability of tyrosine, precursor of betacyanin, was one of the regulatory factors of the accumulation of betacyanin (Sakuta *et al.*, 1991, Hirano *et al.*, 1992), therefore, the effect of ABA on the endogenous size of the pool of free amino acids was investigated. ABA decreased the size of the total amino acid pool to 67% of control values and the size of pools of most individual amino acids, except for Ile and Pro were decreased. The size of the pool of tyrosine was reduced (to 63% of control value) by ABA, though that of phenylalanine, an other aromatic amino acid, did not changed (Table I). We next examined that whether or not an exogenous supply of tyrosine might restore the reduction of accumulation of betacyanin by ABA. The accumulation of betacyanin reduced by ABA was restored by simultaneous addition of tyrosine with ABA to the medium up to 117% of control value (Table II). Those results suggest that reduction of accumulation of betacyanin by ABA depends on the lack of availability of tyrosine taken place by decrease of

Table II. Effect of ABA and tyrosine on the accumulation of betacyanin. Fresh weight, cell number and betacyanin content were measured 6 days after transfer.

Additions	Betacyanin content		
	[A_{535} (g fw.)$^{-1}$]	[A_{535} (10^7 cells)$^{-1}$]	[A_{535} (10 ml of medium)$^{-1}$]
Control	2.49 ± 0.06*	3.21 ± 0.08 (2.84 ± 0.07)**	1.69 ± 0.12
ABA 10^{-5} M	1.79 ± 0.02	2.05 ± 0.02 (1.81 ± 0.02)	1.00 ± 0.10
ABA 10^{-5} M Tyrosine 10^{-3} M	2.56 ± 0.04	3.08 ± 0.05 (2.72 ± 0.04)	1.36 ± 0.07
Tyrosine 10^{-3} M	3.26 ± 0.04	4.25 ± 0.04 (3.75 ± 0.04)	2.27 ± 0.20

* SD (n = 3); ** The absolute amount of betacyanin (10^{-7} mol (10^7 cells)$^{-1}$) was calculated on the basis of the molar extinction coefficient of 5.66×10^4 at 537 nm (Piatelli *et al.*, 1969) in parentheses for reference.

Table III. Variations in percentages of radioactivity incorporated from labeled tyrosine into CO_2, 80% methanol and 1 M NaOH soluble fractions in total radioactivity taken up.

Control treatment culture	Relative radioactivity [% of total uptake]		
	CO_2	1 M NaOH-soluble fraction	80% methanol-soluble fraction
Control	0.55 ± 0.09*	51.1 ± 0.79	38.6 ± 2.82
ABA 10^{-5} M	0.52 ± 0.09	57.8 ± 0.68	31.3 ± 1.06

* SD (n = 3).

endogenous the size of the pool of tyrosine by ABA.

To elucidate the metabolic flow of tyrosine, labeled tyrosine was fed and the rate of incorporation of radioactivity into CO_2 fraction, 80% methanol-soluble fraction and 1M NaOH-soluble fraction was measured (Table III). Rate of incorporation of radioactivity from labeled tyrosine into 1M NaOH soluble fraction (corresponding to protein fraction) was increased by ABA. Obata-Sasamoto *et al.* (1981) reported that the shift of the metabolic flow of tyrosine from DOPA synthesis to protein synthesis can explain the suppression of DOPA accumulation during callus culture of *Mucuna hassjoo*. In the present study, decrease of the size of pool of tyrosine by ABA, resulting in reduction of the accumulation of betacyanin, may be due to the shift of the metabolic flow of tyrosine to protein synthesis .

The addition of tyrosine elevated the accumulation of betacyanin to 132% of control, whereas the addition of tyrosine with ABA restored the level of betacyanin but not completely (up to 96% of control), nevertheless tyrosine which was supplied exogenously to ABA-treated cells increased the size of the endogenous pool of tyrosine up to 5 fold of that of control, (data not shown). Those results suggest that the inhibition of accumulation of betacyanin by ABA may be not only due to reduction of the availability of tyrosine but also to inhibition of the activity of biosynthetic pathway from tyrosine to betacyanin. Enzymes involved in biosynthesis of betacyanin following tyrosine have not yet been identified in higher plant. Therefore, the activity of the biosynthetic pathway from tyrosine to betacyanin with or without ABA was estimated by tracer experiments using labeled tyrosine (Table IV). ABA reduced the incorporation of radioactivity from labeled tyrosine to betacyanin to less than 45% of control value, suggesting that ABA reduced the biosynthetic activity of the pathway from tyrosine to betacyanin.

The concentration of ABA (10^{-5}M) used in the present experiments is rather high compared with that in usual plant tissues. In the most plant tissues, the endogenous level of ABA is very low (10^{-8}-10^{-7}M), but it is known that the level of ABA reaches above 10^{-6}M in some kinds of tissues (e.g. water stressed leaf, developing seed, dormant bud or seed etc.; Wallon, 1987). Therefore, it should be possible that the betacyanin production is controlled by rather high endogenous concentration of ABA in intact plant tissues.

In the present experiments, it was confirmed that the inhibition of accumulation of betacyanin by ABA was due to both supply of tyrosine and the activity of the biosynthetic pathway from tyrosine to betacyanin, indicating that these two mechanisms are important for the regulation of accumulation of betacyanin in suspension cultures of *Phytolacca americana*.

Table IV. Incorporation of radioactivity from labeled tyrosine into betacyanin. Cells were incubated with labeled tyrosine for 4 hours.

Control treatment, culture	Radioactivity [kBq (10^7 cells)$^{-1}$]	
	Tyrosine	Betacyanin
Control	66.5 ± 0.21* (22.9 ± 0.07)**	3.41 ± 0.27) (1.18 ± 0.13)
ABA 10^{-5} M	38.8 ± 1.86 (19.9 ± 0.95)	1.54 ± 0.16 (0.79 ± 0.12)

* SD (n = 3); ** % of total uptake in parentheses.

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