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Anthocyanin accumulation and changes in activities of phenylalanine ammonia-lyase and chalcone synthase in roselle (*Hibiscus sabdariffa* L.) callus cultures

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Abstract Time-course changes in anthocyanin accumulation, phenylalanine ammonia-lyase activity and chalcone synthase activity were examined in roselle callus tissues incubated under different culture conditions. Phenylalanine ammonia-lyase activity was not affected by either the kind of auxin supplemented to the medium or light regime. In contrast, chalcone synthase activity was markedly suppressed when the callus was cultured with a medium containing indole-3-acetic acid instead of 2,4-dichlorophenoxyacetic acid (2,4-D) or in the dark. The results imply that in roselle callus cultures chalcone synthase plays a more important role in anthocyanin biosynthesis regulated by 2,4-D and light irradiation than phenylalanine ammonia-lyase.

Abbreviations: LS, Linsmaier and Skoog; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indole-3-acetic acid; PAL, phenylalanine ammonia-lyase; CHS, chalcone synthase

INTRODUCTION

Important factors influencing anthocyanin biosynthesis in roselle (Hibiscus sabdariffa L.) callus cultures are 2,4-D and light irradiation, both of which remarkably enhanced the anthocyanin production (Mizukami et al. 1988). Both PAL and CHS are important enzymes regulating flavonoid biosynthesis (Hinderer and Seitz 1988; and references cited therein). In the present investigation, we examine the regulation of PAL and CHS activities in roselle callus tissues under different culture conditions to determine which enzyme plays a more important role in the anthocyanin biosynthesis regulated by 2,4-D and light irradiation.

MATERIALS and METHODS

Chemicals

A Sephadex PD-10 column was purchased from Pharmacia. Dowex 50Wx2 resin was from Dow Chemicals. Naringenin and bovine serum albumin were from Sigma, and the dye-reagent for protein assay from Bio-Rad Laboratories. [2-^{14}C]Malonyl CoA was purchased from Amersham, Japan. 4-Coumaroyl CoA was synthesized by the method of Stöckigt and Zenk (1975). All other chemicals were of reagent grade.

Callus cultures

Callus tissues derived from germinating roselle seeds were subcultured on LS agar medium (Linsmaier and Skoog 1965) supplemented with 1 μM 2,4-D and 1 μM kinetin at 25°C under light (16 h/day) with white fluorescent lamps (about 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 1-month intervals. For time-course experiments callus tissue (about 0.2 g) was inoculated onto 20 ml LS agar medium supplemented with 2,4-D or IAA in 50 ml Erlenmeyer flasks and cultured under light or in the dark for appropriate periods before harvest.

Preparation of enzyme extract

All operations were carried out at 4°C. Callus tissues (typically 1 g) were homogenized in a pre-chilled mortar with polyvinylpolypyrrolidone (0.5 g) in 100 mM potassium phosphate buffer, pH 8.0, containing 1.4 mM 2-mercaptoethanol. After being filtered through three layers of gauze, the homogenate was centrifuged at 15,000 x g for 20 min. Dowex 50Wx2 resin (0.2 g) was added to the supernatant, which was then allowed to stand for 20 min with occasional stirring. The mixture was centrifuged again at 1,100 x g for 1 min and the supernatant was used for the CHS assay as an enzyme preparation. For the PAL assay, the extract was buffer-exchanged with 50 mM borate buffer, pH 8.8, containing 1.4 mM 2-mercaptoethanol using a Sephadex PD-10 column.

The protein concentration in the enzyme extract was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Enzyme assay

PAL activity was assayed spectrophotometrically as reported by Tanaka et al.(1974). The assay mixture (1.2 ml) contained 50 mM sodium borate, pH 8.8, 1.4 mM 2-mercaptoethanol, 20 mM L-phenylalanine and the enzyme extract. After 30 min incubation, the reaction was terminated by adding 0.5 ml 2 M perchloric acid. After centrifugation at 1,100 x g for 30 min, the absorbance of the supernatant was measured at 280 nm. The blank consisted of the assay mixture without the enzyme extract but with perchloric acid added. The amount of trans-cinnamic acid formed was calculated from a standard calibration line.

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CHS activity was assayed according to the method described by Kreuzaler and Hahlbrock (1975) with slight modifications. The reaction mixture (200 µl) containing 100 mM potassium phosphate, pH 8.0, 1.4 mM 2-mercaptoethanol, 0.08 mM 4-coumaroyl CoA and 0.014 mM [2-^{14}C]malonyl CoA (1.5 GBq/mmol) was incubated at 30°C. After 60 min, 200 µl ethanol containing 0.4 µmol naringenin was added to the reaction mixture and then 100 µl of the mixture was applied onto a Silica gel 60F (Merck) thin-layer plate, which was developed using toluene-ethyl acetate-methanol-petroleum ether (6:4:1:3). The band corresponding to naringenin was detected under UV-light (254 nm), scraped off and the radioactivity was measured in a scintillation counter.

Extraction and quantitative determination of anthocyanin

Anthocyanin was extracted and spectrophotometrically determined by a method described elsewhere (Mizukami et al. 1988).

RESULTS

Figure 1 shows the time-course changes in callus fresh weight, anthocyanin accumulation and activities of PAL and CHS over a culture period of 35 days when roselle callus was cultured in LS medium containing 1 µM 2,4-D and 1 µM kinetin (2,4-D medium) under light irradiation. The callus tissues started to accumulate anthocyanin after a lag period of 7-10 days, and the anthocyanin accumulation increased in parallel with cell growth until the culture reached the stationary phase of cell growth (28 days after cell inoculation). PAL activity started to increase after a lag period of 3 days and reached a maximum on day 10, when the anthocyanin accumulation had just started. A slight increase of PAL activity was also observed during the stationary phase of cell growth. CHS activity began to increase after a lag period of 7 days and reached a maximum on day 14, when anthocyanin was most actively synthesized.

The time-course changes in the anthocyanin biosynthesis were compared between the callus tissues cultured with 1 µM 2,4-D and with 1 µM IAA instead of 2,4-D as shown in Fig.2. Cell growth and anthocyanin accumulation in IAA medium decreased by 60% and 90%, respectively, compared to that of cells cultured in 2,4-D medium. PAL activity in IAA medium did not show any significant difference from

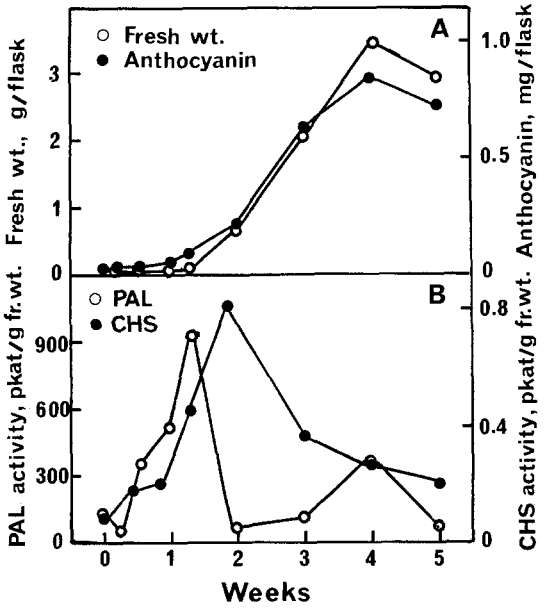

Figure 1 Time-course of changes in (A) callus fresh weight (open circles) and anthocyanin accumulation (closed circles), and (B) activities of PAL (open circles) and CHS (closed circles) in roselle callus cultures. The roselle callus was cultured with the medium containing 1 µM 2,4-D and 1 µM kinetin under light over a period of 35 days. Each data point represents an average of two separate experiments.

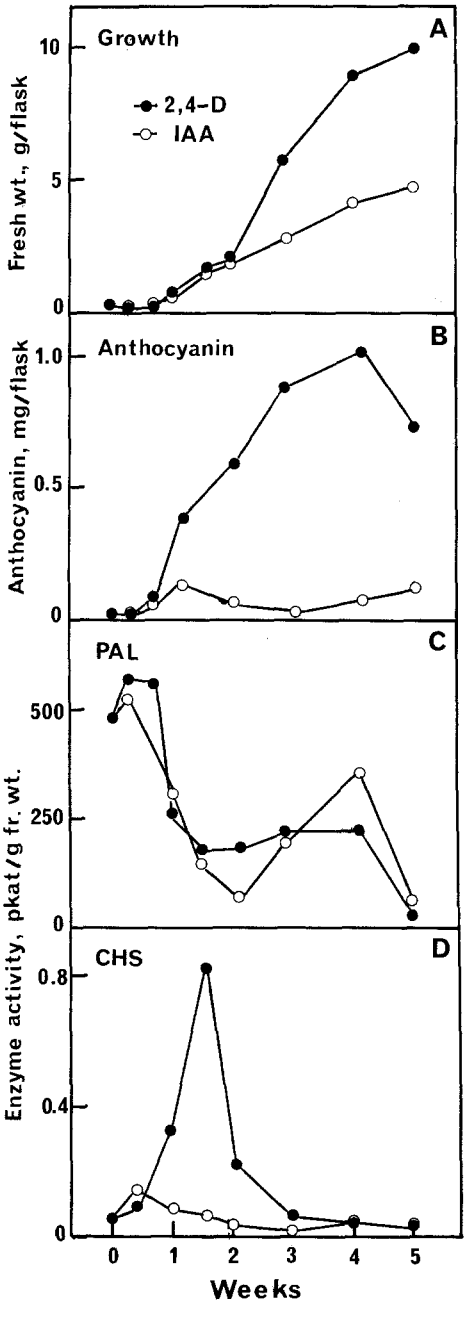

Figure 2 Time-course of changes in (A) callus fresh weight, (B) anthocyanin accumulation, (C) PAL activities and (D) CHS activities in roselle callus cultured with the medium containing 2,4-D (closed circles) or IAA (open circles). Both medium contained 1 µM kinetin. The incubation was carried out under light for 35 days. Each data point represents an average of two separate experiments.

that in 2,4-D medium over the time course. In contrast, a sharp increase in CHS activity observed during a period of day 7 to day 10 in 2,4-D medium was not observed at all in the IAA medium.

Figure 3 shows a comparison of anthocyanin biosynthesis in light- and dark-treated callus tissues over time. Cell growth and anthocyanin production were markedly suppressed when the callus was cultured in the dark. PAL activity was unaffected by the absence of light irradiation, whereas a rapid induction of CHS activity during the exponential phase of growth was not observed in the dark-grown culture.

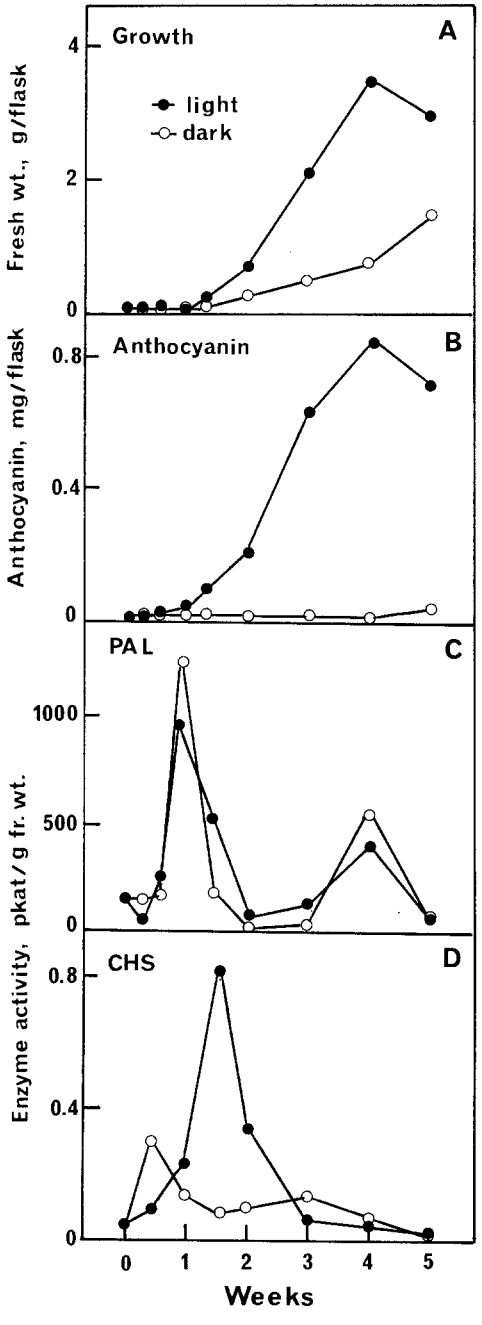

Figure 3 Time-course of changes in (A) callus fresh weight, (B) anthocyanin accumulation, (C) PAL activities and (D) CHS activities in roselle callus cultured with the medium containing 2,4-D and kinetin under light (closed circles) or in the dark (open circles) for 35 days. Each data point represents an average of two separate experiments.

DISCUSSION

Earlier studies (Mizukami et al. 1988) on roselle callus cultures showed that 2,4-D medium caused a 15-30 fold increase in anthocyanin production in comparison with cultures grown on other auxins such as indole-3-butyric acid (IBA), 1-naphthaleneacetic acid (NAA) and IAA. The stimulatory effect of 2,4-D on the anthocyanin formation correlates a marked induction of CHS activity during the exponential phase of cell growth. These results are different; in carrot cells (Ozeki and Komamine 1986; Ozeki et al. 1987), neither CHS nor anthocyanin accumulation could be detected in a medium containing 2,4-D. In those studies anthocyanin synthesis as well as the activities of PAL and CHS was induced only when the cells were transferred to medium lacking 2,4-D. In addition, it was also reported that in the carrot cell system, 2,4-D inhibits anthocyanin biosynthesis through repressing transcription of a CHS gene (Ozeki 1987). Because of the contrast in results of the present investigation and the results of research done in carrot cells, it is of interest to clarify the molecular mechanism of the stimulation of CHS activity by 2,4-D in cultured roselle cells.

In general, anthocyanin production in cultured plant cells depends on light (Seitz and Hinderer 1988) with some exceptions including carrot (Alfermann and Reinhard 1971) ,Strobilanthes dyeriana (Smith et al. 1981) and Vitis hybrida (Yamakawa et al. 1983). In callus cultures of Haplopappus gracilis, being completely devoid of anthocyanin when cultured in the dark, the anthocyanin synthesis was effected by blue- (Reinert et al. 1964) or UV-light (Wellmann et al. 1976), and drastic increases in activities of both PAL and CHS were observed prior to the anthocyanin accumulation induced by UV light irradiation. In flavonoid-producing cell cultures of parsley PAL plays a rate-limiting role in flavonoid biosynthesis induced by UV irradiation (Hahlbrock et al. 1976). In contrast to these results, a rapid increase of PAL activity during early exponential phase of cell growth was expressed even in the dark, and the time-course pattern of PAL activity in the dark-grown culture showed no difference from that in light-grown culture in our roselle callus system though the anthocyanin production was remarkably suppressed when the callus was cultured in the dark. The expression of CHS activity was strictly dependent on light irradiation. It has recently been clarified that light induced changes of PAL and CHS activities in parsley cells depend on mRNA transcription by using cDNA of PAL (Kuhn et al. 1984) and CHS (Kreuzaler et al. 1983), respectively. It remains to be investigated whether the light-induced expression of the CHS activity in roselle callus depends on the similar molecular mechanism.

In conclusion, the results presented in this paper lead to the conclusion that in roselle callus cultures not PAL but CHS plays an important role in the anthocyanin biosynthesis regulated by 2,4-D and light irradiation.

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