Original Paper

Effects of Salicylic Acid on the Production of Procyanidin and Anthocyanin in Cultured Grape Cells

Noe OBINATA[1], Takashi YAMAKAWA[1,a]*, Mikiko TAKAMIYA[2], Norie TANAKA[2], Kanji ISHIMARU[2] and Tohru KODAMA[3]

[1]*Department of Biotechnology, The University of Tokyo, Bunkyo–ku, Tokyo 113–8657, Japan*
[2]*Department of Applied Biological Sciences, Faculty of Agriculture, Saga University, Honjo, Saga 840–8502, Japan*
[3]*Department of Bioscience, Tokyo University of Agriculture, Sakuragaoka, Setagaya–ku, Tokyo 156–8502, Japan*
Present address: [a]Department of Global Agricultural Sciences, The University of Tokyo, Bunkyo–ku, Tokyo 113–8657, Japan
Corresponding author E–mail address: ayama@mail.ecc.u–tokyo.ac.jp

Abstract

The production of procyanidin and anthocyanin in grape cells is markedly increased by treatment with salicylic acid. The activity of phenylalanine ammonia–lyase and chalcone–flavanone isomerase increased in close relation to the production of these phenolics. Salicylic acid–induced elevation of these enzyme activities occurred more rapidly than elevation induced by light irradiation. In the cells, the phenolics were degraded in a different manner by salicylic acid treatment than that by light irradiation. Salicylic acid might be used as a simple and effective stimulant for the production of procyanidin as well as anthocyanin in large scale cultures of plant cells.

Introduction

Anthocyanin is frequently used as food additives (Hong et al., 1990) and it has been recognized that procyanidin shows anticarcinogenic activity (Hayatsu et al., 1993) and antioxidant activity (Okuda et al., 1983). They are plant–specific phenolic compounds and are produced in the phenylpropanoid pathway, a secondary metabolic pathway in plants. Lignin and coumarins are also synthesized in the same pathway, which is important secondary metabolic pathway for generating useful plant metabolites.

The biosynthesis of anthocyanin has been studied using tissue culture in a variety of plants including carrot (Ozeki et al., 1985; Takeda, 1990; Gläßgen et al., 1998), petunia (Hagendoorn et al., 1991), and grape (Yamakawa et al., 1983; Hirose et al., 1990). The relationship between anthocyanin accumulation and cell division (Ozeki et al., 1986; Sakuta et al., 1994) and differentiation (Ozeki et al., 1981) in these plant cells has been investigated. Recently, much attention has also been paid to the production

of tannin in green tea (Hayatsu et al., 1993), because of its anti–carcinogenic activity. Alain et al. reported the production of tannin in grape cultures (Alain et al., 1996). Other than the effect of NH_4NO_3 in *Sapium sebiferum* (Neera et al., 1992), *Liquidambar styraciflva* (Ishimaru et al., 1992), and *Cornus kousa* (Ishimaru et al., 1993), the conditions that stimulate tannin production are poorly understood.

Phenylalanine ammonia–lyase (PAL) and chalcone–flavanone isomerase (CHFI) are involved in the synthesis of anthocyanin and tannin (particularly procyanidin). Procyanidins are synthesized by the condensation of flavan–3–ol and flavan–3,4–diol, which synthesized from chalchone through flavanone. PAL catalyzes the reaction from phenylalanine to cinnamic acid, the first step in the phenylpropanoid pathway (Hahlbrock et al., 1976), and is regarded as a key enzyme in this pathway. CHFI catalyzes the step from chalcone to naringenin. Takeda reported that CHFI's activity changes in the carrot cells were parallel to those of chalcone synthase (CHS), which is the first enzyme to branch off from phenylpropanoid metabolism to flavonoid

metabolism and is supported to be a key enzyme of this system (Takeda, 1990). Previous studies demonstrated that stimulation with UV-light or elicitors enhanced the activity of PAL in parsley (Hahlbrock et al., 1981) and carrot (Takeda, 1990; Gläßgen et al., 1998), and the levels of both PAL mRNA and PAL protein also increased in the UV-light-irradiated plants (Chappell et al., 1984; Takeda et al., 1993; Gläßgen et al., 1998). Therefore, increased PAL activity seems to be regulated at the transcriptional level. In the light-induced production of plant metabolites, photo-receptor(s) must be activated initially.

Grape cells are useful for investigating the production of secondary metabolites because they can be cultured on plates or in suspension on a large scale. Grape cell strains have been established that produce anthocyanin, and that are unable to produce anthocyanin (Yamakawa et al., 1983). In this study, we used anthocyanin producing strains for all experiments. Anthocyanin production in grape cell cultures depends on the culture conditions, especially the conditions that produce environmental treatment such as light-irradiation-induced anthocyanin synthesis (Yamakawa et al., 1983).

In this study, we further examined the effects of environmental treatment caused by salicylic acid, one of the elicitors, on the production of procyanidins and anthocyanin in grape cell cultures. We also measured both PAL and CHFI activity with salicylic acid stimulation, which efficiently enhanced the production of these phenolic constituents.

Materials and Methods

Chemicals

Chalcone (4, 2', 4', 6'-tetrahydroxy chalcone) was synthesized according to the method of Moustafa and Wong (1967). Naringenin (4', 5, 7-trihydroxyflavanone) was purchased from Nakarai Tesque (Kyoto, Japan).

Cell cultures

A suspension culture of Vitis hybrid (Bailey Alicante A) cells was carried out in a modified Murashige and Skoog's (MS) medium (Murashige et al, 1962), supplemented with 3% sucrose, 0.05 mg l^{-1} 2.4-D, and 0.2 mg g^{-1} of kinetin, in test tubes (21 mm d.) on a reciprocal shaker at 300 strokes per min transferring 10% of culture broth. Cells were maintained in the dark at 30 ℃ and subcultured every 14 days. Time course experiment was carried out by the following method. Culture broth of 5-7 test tubes (20-40 mg fresh weight cells

per tube) were combined and mixed with ten times of volume of fresh medium in a sterile Erlenmyer flask and transferred to each test tube (10 ml) again for the start culture. They were cultured on the reciprocal shaker (150 rpm) at 30 ℃. After the treatment of salicylic acid addition or light irradiation, cultured cells were harvested by the pipette from each test tube at the sampling time aseptically. To extract enzymes, cells collected by vacuum filtration were weighed, frozen quickly in liquid nitrogen and stored at -80 ℃.

Measurement of anthocyanin content

The anthocyanin content was measured using the methods of Ozeki and Komamine (Ozeki et al., 1987) and Yamakawa et al. (Yamakawa et al., 1983). Briefly, anthocyanin was extracted from 0.1 g (fr. wt) of harvested cells with 1% HCl-methanol overnight at room temperature, and then the absorbance at 525 nm was measured. Content was calculated from the calculation curve of authentic sample as an cyanidin-3-monoglucoside.

Measurement of procyanidin content

A part of harvested cells were lyophilized and 20 mg of lyophilized cells were ground with a mortar and pestle and the procyanidin was extracted with 2 ml of 80% Me_2CO overnight at room temperature. The extract was filtered with filter paper and subjected to HPLC analysis in a TSK-gel ODS 80Ts (4.6 mm i.d. 250 mm) column at 40 ℃. The solvent for the mobile phase was a mixture of 1 mM tetrabutylammonium (pH 2.9, adjusted with CH_3COOH) and CH_3CN; initially, the ratio was 19:1 and it changed linearly to 2:3 over 30 min at a flow rate of 0.7 ml min^{-1}. Rt (min): (+)-catechin (20.3), (-)-epicatechin (22.5), procyanidin B-2 (21.5). The procyanidin and related compounds were identified from UV spectra measured using a photodiode array detector (Shimadzu SPD-M10AVP).

Enzyme assay

PAL and CHFI activity was assayed spectrophotometrically as described (Zucker, 1965; Moustafa et al., 1967; Ozeki et al., 1987). Enzymes were extracted at 4 ℃ by homogenizing the harvested frozen cells (0.4 g) in 1 ml of 0.1 M borate buffer (pH 8.8) containing 1 mM 2-mercaptoethanol with a homogenizer (Polytron). The homogenates were treated with 0.1 g of Dowex 1x4 (Cl⁻ form) for 10 min and the cell debris and resin were removed by centrifugation at 15,000 rpm for 10 min. Dowex 1x4 resin, 0.2 g, was added to the supernatant and treated for another 20 min. Then the resin was removed by centrifugation at 15,000 rpm for 15 min.

The resultant supernatant was used for the PAL and CHFI assays. To assay PAL activity, 0.5 ml of 0.1 M phenylalanine in 0.1 M borate buffer pH 8.8 was added to 1 ml of enzyme extract that was preincubated at 30 ℃ for 5 min. The mixture was incubated for 30 min. The reaction was terminated by adding 2 N perchloric acid (PCA) and then the absorbance at 290 nm was measured. To assay CHFI activity, 100 μl of enzyme extract was mixed with 1.89 ml of 50 mM Tris-HCl buffer, pH 7.6, containing 10 mM KCN. The enzyme reaction was allowed to proceed for 1 min at 30 ℃ after adding 10 mg chalcone in 10 μl ethylene glycol monomethylether. The activity was determined by measuring the absorbance at 370 nm.

Protein assay

Protein concentrations were determined by the Bradford method as described (Bradford, 1976).

The light irradiation treatment

White fluorescent tubes were used as the white light source. The light intensity was about 26 μE m^{-2} s^{-1}. In the experiments in the dark, the test tubes for culture were covered with two layers of aluminum foil.

Results and Discussion

Chemicals and light - induced anthocyanin production

In order to clarify the effects of various treatments on anthocyanin production, cultured grape cells were subjected to different stimulant addition, and the anthocyanin content was determined in each case after 2 weeks culture (**Fig. 1**). When exposed to white light from white fluorescent tubes at an intensity of 26 μE m^{-2} s^{-1}, anthocyanin production was markedly enhanced (**Fig. 1A**), as reported previously (Yamakawa et al., 1983). In addition, we found that treatment with chemical agents was also very effective in stimulating anthocyanin production. Salicylic acid, a typical elicitor that induces a defensive response in plants (Malamy et al., 1990), was particularly effective. When salicylic acid was added to the culture medium (0.1 mM in final concentration), anthocyanin production in grape cells increased approximately 3-fold (**Fig. 1B**). Yeast extract also enhanced anthocyanin production (**Fig. 1C**), while chitin and glutathione (GSH) were not effective (data not shown) (Furze et al., 1991; Robbins et al., 1991). When the cells were cultured in a phosphate-deficient medium, anthocyanin production was also increased in inverse proportion to the phosphate concentration (**Fig. 1D**), indicating

that phosphate deficiency might stimulate anthocyanin induction (Yamakawa et al., 1983).

Time course for the salicylic acid or light-induced production of procyanidin and anthocyanin

Although light-induced anthocyanin production has been reported (Yamakawa et al., 1983), little is known about the effects of salicylic acid on the production of plant phenolic metabolites generated in the phenylpropanoid pathway of cultured grape cells. Therefore, we examined the salicylic acid-induced phenolics in detail, focusing on anthocyanin and procyanidin and related compounds [(+)-catechin, (-)-epicatechin, and procyanidin B-2] produced in the cultures. The time course for the salicylic acid-induced production of these phenolics in the dark is shown in **Fig. 2**. Grape cells were cultured for 7 days until they entered the logarithmic growth phase, and then they were stimulated by salicylic acid treatment. Cell growth was barely affected by this treatment (**Fig. 2A**). Anthocyanin
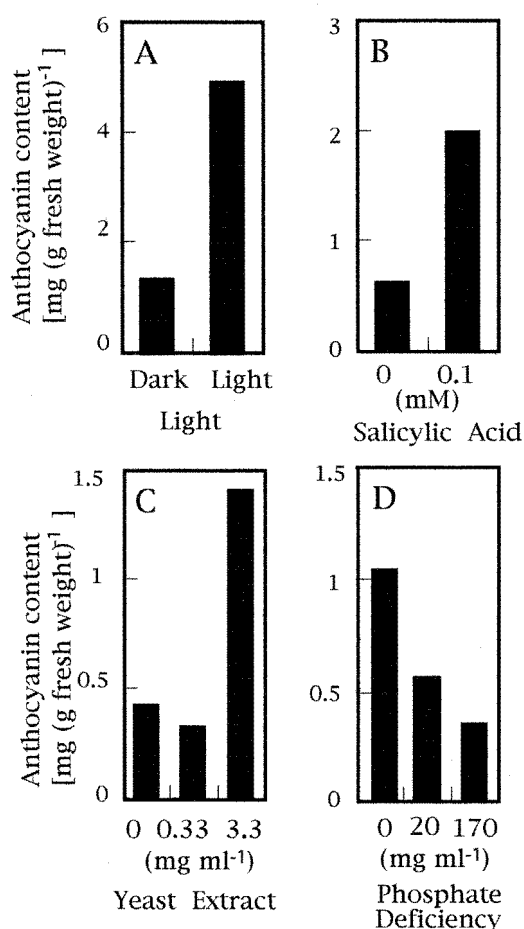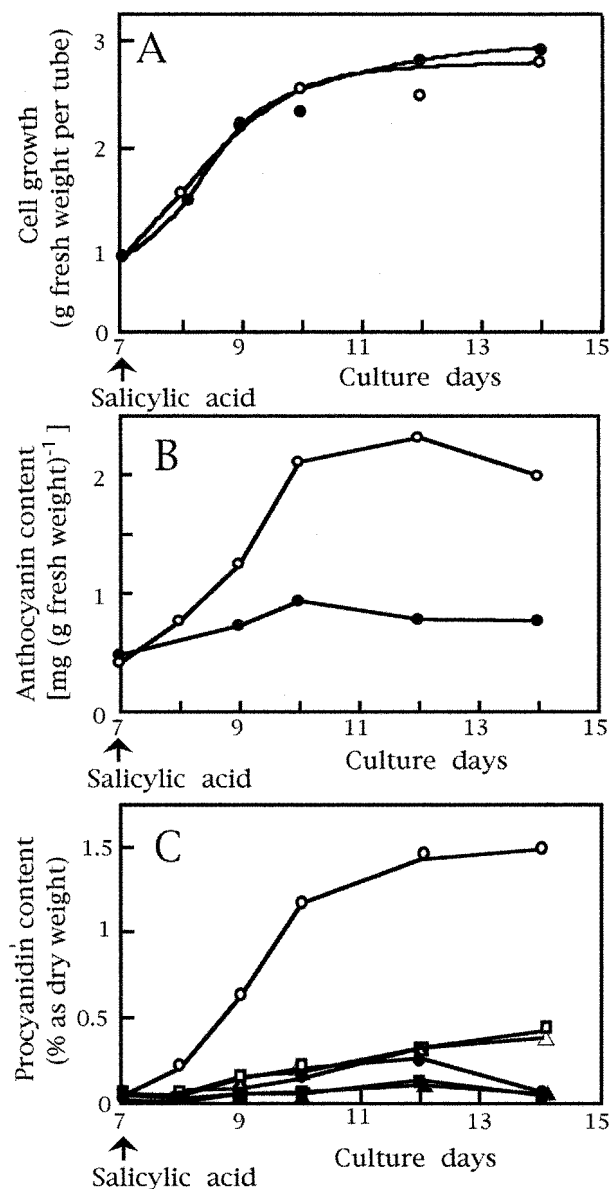

Fig. 1 Anthocyanin production in cultured grape cells stimulated with light irradiation and chemical compounds. Grape cells were cultured under dark or light conditions (A), in the presence or absence of salicylic acid (B), yeast extract (C), or phosphate (D) at the concentrations shown for 12-14 days.
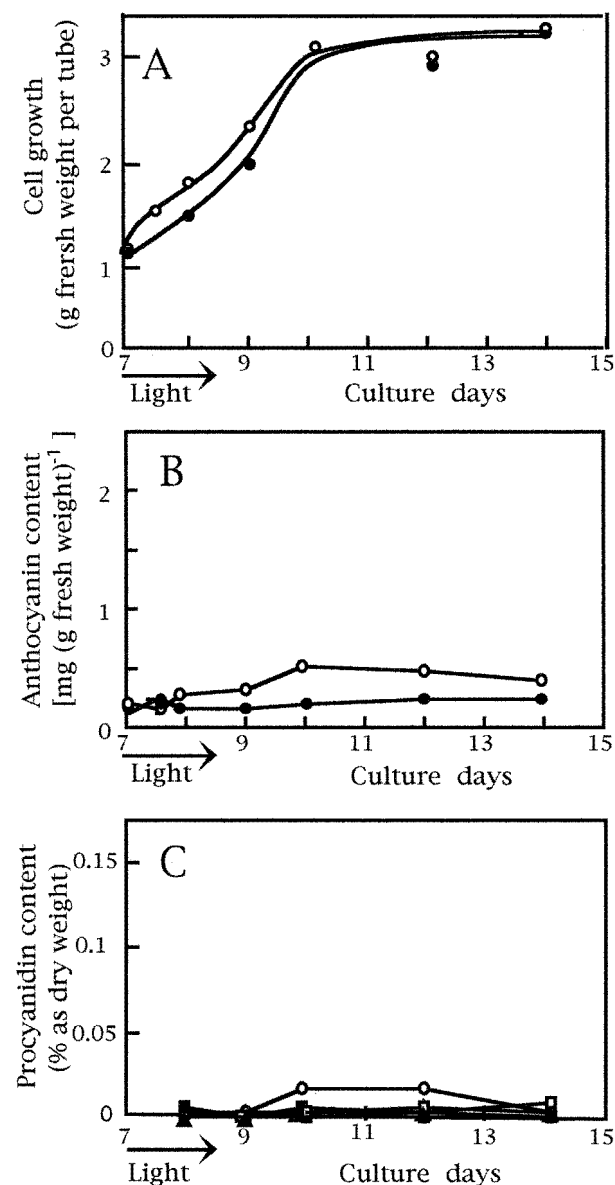
production started to increase soon after the addition of the salicylic acid (**Fig. 2B**). The anthocyanin content of the cells cultured in the presence of salicylic acid continued to increase until the fifth day of stimulation and then remained at a high level (**Fig. 2B**). We also investigated the effect of light irradiation on growth and phenolic production in grape cells. Grape cells were cultured for 7 days until they entered the logarithmic growth phase, and

then they were irradiated with continuous light. Again, cell growth was barely affected by this treatment (**Fig. 3A**). Phenolics accumulated in the cells soon after the irradiation started, and the anthocyanin maximum was reached on the 10 day, and that of (+)-catechin almost on the 10 day. After reaching a maximum, both the anthocyanin and (+)-catechin levels gradually decreased.

The procyanidin content of the cells was also stimulated with salicylic acid treatment (**Fig. 2C**). In particular, the (+)-catechin content increased rapidly to maximum levels almost four times larger than those of (-)-epicatechin and procyanidin B-2. Therefore, salicylic acid treatment seems to promote

Fig. 2 Time course for cell growth and the production of anthocyanin and procyanidins in the presence or absence of salicylic acid (0.1 mM). Cell growth (A) in the presence (○) or absence (●) of salicylic acid, anthocyanin content (B) in the presence (○) or absence (●) of salicylic acid, and procyanidin content (C) were examined during different culture periods in the presence [(+)-catechin: ○ , (-)-epicatechin: □ , procyanidin B-2: △] or absence [(+)-catechin: ● , (-)-epicatechin: ■ , procyanidin B-2: ▲] of salicylic acid.

Fig. 3 Cell growth (A) and anthocyanin content (B) under light (○) or dark (●) conditions. Procyanidin content (C) were examined during different culture periods under light [(+)-catechin: ○ , (-)-epicatechin: □ , procyanidin B-2: △] or dark [(+)-catechin: ● , (-)-epicatechin: ■ , procyanidin B-2: ▲] conditions.

the biosynthetic pathway for (+)-catechin and to a lesser extent those for (-)-epicatechin [epimerization from (+)-catechin] and procyanidin B-2 (oxidative polymerization of catechins).

The (+)-catechin content also increased with light irradiation(**Fig. 3C**). The high (+)-catechin content continued for a few days (culture days 10 to 12). In contrast to the continuous increase in the procyanidin content of cells treated with salicylic acid, the (+)-catechin content decreased suddenly late in the culture period under light illumination.

Effects of light and salicylic acid on the activity of enzymes involved in the synthesis of phenolic metabolites

When salicylic acid was added to the grape cell cultures (0.1 mM in final concentration), the activity of phenylalanine ammonia-lyase (PAL) and chalcone-flavanone isomerase (CHFI) increased quite quickly and reached a maximum on the first day of stimulation (**Fig. 4A, B**). Production of anthocyanin and (+)-catechin was clearly induced on days 1 and 2 of the treatment with salicylic acid, as shown in **Fig. 2B, C**. Thus, the activation of PAL and CHFI preceded the increase in the level of both products by 1 - 2 days. Two days after the administration of

salicylic acid, the enzyme activity gradually decreased (**Fig. 4A, B**).

When the cells were irradiated by continuous light, the PAL activity increased and reached a maximum on the third day of stimulation (**Fig. 5A**). The CHFI activity also increased and reached a maximum on the second day (**Fig. 5B**).

After salicylic acid treatment, the extractable total protein content in the cultured cells decreased gradually by further cultivation and it decreased more rapidly (more than 10 % per day) by light irradiation. However, the stimulation pattern of the enzymes did not change significantly by the enzyme activities per g fresh cell weight. Since the levels of anthocyanin and (+)-catechin reached high levels on days 3 to 4 (**Fig. 3B**), there was a lag of approximately 1 day between activation of the enzymes and the increase of the levels of anthocyanin and (+)-catechin. Therefore, PAL and CHFI were more rapidly stimulated by salicylic acid than by light.

When grape cells in the stationary growth phase or at the confluent state, on the 10th day of subculture or later, were treated with light or salicylic acid, PAL and CHFI were not activated and no increase in the levels of anthocyanin or procyanidin
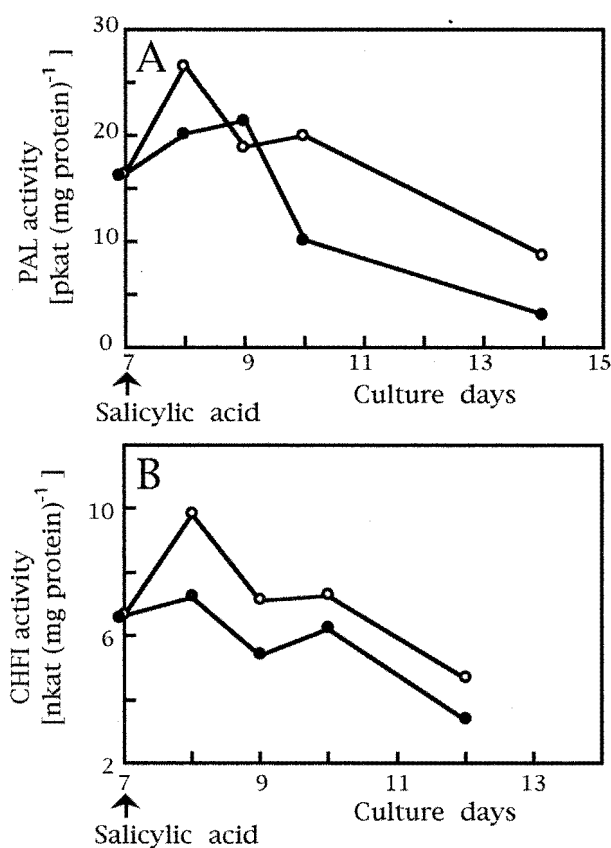
Fig. 4 Effects of salicylic acid on PAL (A) and CHFI (B) activity in cultured grape cells in the logarithmic growth phase. PAL and CHFI activity was measured in cells cultured in the presence (○) or absence (●) of salicylic acid.
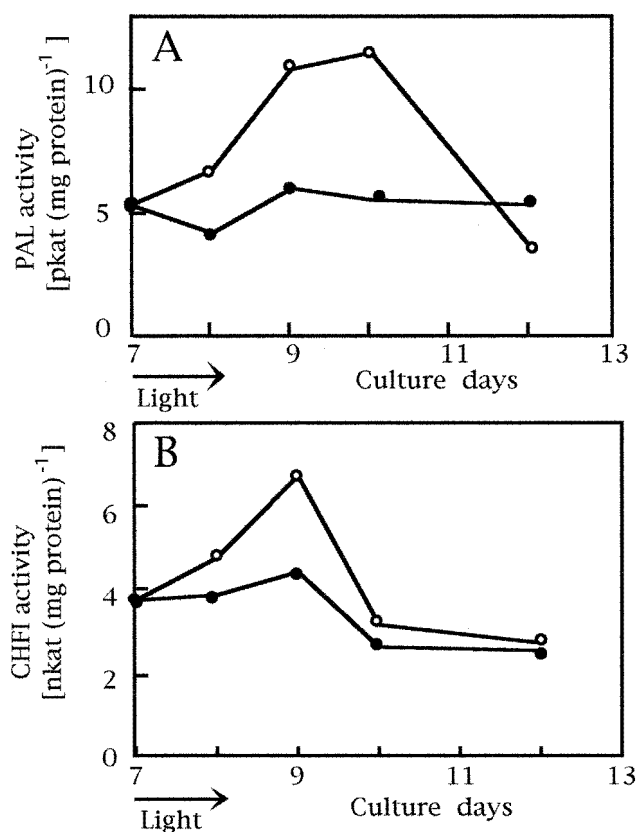
Fig. 5 Effects of light irradiation on PAL (A) and CHFI (B) activity in cultured grape cells in the logarithmic growth phase. PAL (A) and CHFI (B) activity was measured in cells cultured under dark (●) or light (○) conditions.

was observed (data not shown). When the cells were irradiated by continuous light starting on the first day of the subculture, the anthocyanin and procyanidin levels only started to increase on the 9th day of the subculture, two days after the growth phase started (data not shown). This result indicates that the stimulation of (+)-catechin production by light-irradiation, only occurs early in the culture period.

These observations indicate that how cultured grape cells respond to salicylic acid and light depends greatly on their phase of growth. Cells in the stationary growth phase cannot respond to salicylic acid and light. Under our culture conditions, the 7th day of the subculture, when the cells started the logarithmic growth phase, was the optimal time to induce a rapid response in the production of secondary metabolites. We suggest that there is some specific switching point(s) in the grape cells, from an insensitive phase to a sensitive phase or vice versa, with regard to their responsiveness to salicylic acid and light.

Although there was considerable variation on the productivity of anthocyanin (300%) among the cell lines we used in the experiments, the response pattern of the cultured cells did not change. In conclusion, although the regulatory systems for the production of secondary metabolites in grape cells are complicated, we found that: 1) Salicylic acid is an effective stimulus to induce plant secondary metabolites; 2) the production of procyanidins and related compounds, especially (+)-catechin, can be enhanced in grape cells by salicylic acid and light; and 3) there might be more than two response systems against salicylic acid and light in grape cells that function in a growth phase-dependent manner.

Acknowledgments

References

Alain, D., Kishan, G. R., Pierre, W., Gerard, D., Alain, B., Michel, M., 1996. Anthocyanins, catechins, condensed tannins and piceid production in *Vitis vinifera* cell bioreactor cultures. Biotechnol. Lett., **18**: 659-662.

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., **72**: 248-254.

Chappell, J., Hahlbrock, K., 1984. Transcription of plant defense genes in response to UV light or fungal elicitor. Nature, **311**: 76-78.

Furze, J. M., Rhodes, M. J. C., Parr, A. J., Robins, R. J., Whitehead, I. M., Threlfall, D. R., 1991. Abiotic factors elicit sesquiterpenoid phytoalexin production but not alkaloid production in transformed root cultures of *Datura stramonium*. Plant Cell Rep., **10**: 111-114.

Gläßgen, W. E., Rose, A., Madlung, J., Koch, W., Gleitz, J., Seitz, H. U., 1998. Regulation of enzymes involved in anthocyanin biosynthesis in carrot cell cultures in response to treatment with ultraviolet light and fungal elicitors. Planta, **204**: 490-498.

Hagendoorn, M. J. M., Zethof, J. L. M., van Hunnik, E., van der Plas L. H. W., 1991. Regulation of anthocyanin and lignin synthesis in *Petunia hybrida* cell suspensions. Plant Cell, Tissue Organ Cult., **27**: 141-147.

Hahlbrock, K., Knobloch, K. H., Kreuzaler, F., Potts, J. R. M., Wellman, E., 1976. Coordinated induction and subsequent activity changes of two groups of metabolically interrelated enzymes. Eur. J. Biochem., **61**: 199-206.

Hahlbrock, K., Lamb, C. J., Purwin, C., Ebel, J., Fautz, E., Schafer, E., 1981. Rapid response of suspension-cultured parsley cells to the elicitor from *Phytophthora megasperma* var. *sojae*. Plant Physiol., **67**: 768-773.

Hayatsu, H., Negishi, T., Arimoto, S., 1993. Dietary inhibitors against mutagenesis and carcinogenesis. In: Bronzetti, G. *et al.* (Eds.): Antimutagenesis and Anticarcinogenesis Mechanism III, pp. 387-418. Plenum, New York.

Hirose, M., Yamakawa, T., Kodama, T., Komamine, A., 1990. Accumulation of betacyanin in *Phytolacca americana* cells and of anthocyanin in *Vitis* sp. cells in relation to cell division in suspension cultures. Plant Cell Physiol., **31**: 267-271.

Hong, V., Wrolstad, R. E., 1990. Characterization of anthocyanin-containing colorants and fruit juices by HPLC/photodiode array detection. J. Agric. Food Chem., **38**: 698-708.

Ishimaru, K., Arakawa, H., Neera, S., 1992. Tannin production in *Liquidambar styraciflua* callus cultures. Plant Tissue Cult. Lett., **9**: 196-201.

Ishimaru, K., Arakawa, H., Neera, S., 1993. Polyphenol production in cell cultures of *Cornus kousa*. Phytochemistry, **32**: 1193-1197.

Malamy, J., Carr, J. P., Klessing, D. F., Raskin, I., 1990. Salicylic acid: A likely endogenous signal in the resistance response to tobacco to viral infection. Science, **250**: 1002-1004.

Moustafa, E., Wong, E., 1967. Purification and properties of chalcone-flavanone isomerase from soya bean seed. Phytochemistry, **6**: 625-632.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant., **15**: 473-497.

Neera, S., Arakawa, H., Ishimaru, K., 1992. Tannin production in *Sapium sebiferum* callus cultures. Phytochemistry, **31**: 4143-4149.

Okuda, T., Kimura, Y., Yoshida, T., Hatano, T., Okuda, H., Arichi, S., 1983. Studies on the activities of tannins and related compounds from medicinal plants and drugs. I. inhibitory effects on lipid peroxidation in mitochondria and microsomes of liver. Chem. Pham. Bull., **31**: 1652–1631.

Ozeki, Y., Komamine, A., 1981. Induction of acthocyanin synthesis in relation to embryogenesis in a carrot suspension culture: Correlation of metabolic differentiation with morphological differentiation. Physiol. Plant., **53**: 570–577.

Ozeki, Y., Komamine, A., 1985. Effects of inoculum density, zeatin and sucrose on anthocyanin accumulation in a carrot suspension culture. Plant Cell, Tissue Organ Cult., **5**: 45–53.

Ozeki, Y., Komamine, A., 1986. Effects of growth regulators on the induction of anthocyanin synthesis in carrot suspension cultures. Plant Cell Physiol., **27**: 1361–1368.

Ozeki, Y., Komamine, A., Noguchi, H., Sankawa, U., 1987. Changes in activities of enzymes involved in flavonoid metabolism during the initiation and suppression of anthocyanin synthesis in carrot suspension cultures regulated by 2,4–dichlorophenoxyacetic acid. Physiol. Plant., **69**: 123–128.

Robbins, M. P., Hartnoll, J., Morris, P., 1991. Phenylpropanoid defence responses in transgenic *Lotus cornic-ulatus* 1. Glutathione elicitation of isoflavan phytoalexins in transformed root cultures. Plant Cell Rep., **10**: 59–62.

Sakuta, M., Hirano, H., Kakegawa, K., Suda, J., Hirose, M., Richard, W. J. lv Sugiyama, M., Komamine, A., 1994. Regulatory mechanisms of biosynthesis of betacyanin and anthocyanin in relation to cell division activity in suspension cultures. Plant Cell, Tissue Organ Cult., **38**: 167–169.

Takeda, J., 1990. Light–induced synthesis of anthocyanin in carrot cells in suspension. J. Exp. Bot., **41**: 749–755.

Takeda, J., Abe, S., Hirose, Y., Ozeki, Y., 1993. Effect of light and 2,4–dichlorophenoxyacetic acid on the level of mRNAs for phenylalanine ammonia–lyase and chalcone synthase in carrot cells cultured in suspension. Physiol. Plant., **89**: 4–10.

Yamakawa, T., Ishida. K., Kato, S., Kodama, T., Minoda, Y., 1983. Formation and identification of anthocyanins in cultured cells of *Vitis* sp. Agric. Biol. Chem., **47**: 997–1001.

Yamakawa, T., Kato, S., Ishida, K., Kodama, T., Minoda, Y., 1983. Production of anthocyanins by Vitis cells in suspension culture. Agric. Biol. Chem., **47**: 2185–2191.

Zucker, M., 1965. Induction of phenylalanine deaminase by light and its relation to chlorogenic acid synthesis in potato tuber tissue. Physiol. Plant., **40**: 779–784.