

Differential expression of anthocyanin biosynthesis genes in suspension culture cells of *Rosa hybrida* cv. Charleston

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Abstract A suspension cultured cell line was established from the cultivar of *Rosa hybrida* ‘Charleston’ as a study model to understand the response of the anthocyanin biosynthesis pathway to environmental cues. The major identified anthocyanin in cell cultures was cyanidin 3-glucoside (chrysanthemins). The anthocyanin yield was enhanced by culturing cells in the EM medium with added sucrose at high concentration under additional UV-B radiation to white light. Three cDNA fragments were cloned with degenerate primers by RT-PCR and the obtained sequences shared high homology with putative key enzymes (DFR, ANS, and UF3GT) of other species. The expression levels of these three genes were promoted under optimum conditions for anthocyanin accumulation. These results suggest that expression levels of these genes were closely correlated with a temporal buildup of anthocyanins in response to environmental factors.

Key words: Anthocyanin, chrysanthemins, cyanin, *Rosa hybrida*, suspension cell culture.

Anthocyanins are a class of flavonoids that are the most widespread secondary metabolites in higher plants. Anthocyanins are the pigments responsible for changing colors from pale pink to flaming red and from pale blue to bright violet in plant tissues. Anthocyanins act for the survival of plants as attractors for pollinators and protectors against attacks from microbes, insects, herbivores and UV irradiation. Their pharmacological activities in mammals have been utilized for food additives and beverages to enhance health by antioxidant properties (Jackman et al. 1993; Li et al. 1993; Jackman and Smith 1996; Mol et al. 1998; Gantet and Memelink 2002). Therefore, much effort has been directed toward understanding the regulatory mechanisms of anthocyanin biosynthesis.

Anthocyanin production in cell culture systems has been well studied in many plant species such as grape (Zhang et al. 2002), *Perilla* (Wang et al. 2004), strawberry (Sato et al. 1996; Edahiro et al. 2005), and sweet potato (Konczak et al. 2005) and the general biosynthetic pathway has been well explained in a review (Schwinn and Dadies 2004). Figure 1 illustrates the putative anthocyanin biosynthesis pathway in rose (*Rosa hybrida*). Three molecules of malonyl-CoA and 4-coumaroyl-CoA are the precursors catalyzed to tetrahydroxychalcone by chalcone synthase (CHS) to yield yellow color. Then, chalcone isomerase (CHI)

catalyzes tetrahydroxychalcone to colorless naringenin. The subsequent hydroxylation of flavanone by flavanone 3-hydroxylase (F3H) leads to the formation of dihydrokaempferol. The subsequent B-ring hydroxylation of dihydrokaempferol by flavonoid 3'-hydroxylase (F3'H) has a key impact on anthocyanin color and generates dihydroquercetin. Reduction of the dihydroquercetin by dihydroflavonol 4-reductase (DFR) leads to production of leucoanthocyanidin. Then, anthocyanidin synthase (ANS) converts colorless leucoanthocyanidin to the unstable colored anthocyanidin. The 3-position of the C-ring in anthocyanidin is glycosylated by UDP glucose. In case of rose cultivar ‘Charleston’, UDP-glucose:flavonoid 3-O-glucosyltransferase (UF3GT) produces the stable anthocyanin pigment, cyanidin 3-monoglucoside (chrysanthemins; Hennayake et al. 2006). Further modification may occur at the 5-position of the A-ring that is catalyzed by UDP-glucose:flavonoid 5-O-glucosyltransferase (UF5GT) for formation of cyanidin 3,5-diglucoside (cyanin) (Yamazaki et al. 1999; Mikanagi et al. 2000; Yoshihara et al. 2005; Hennayake et al. 2006). For centuries, rose has been the most important cut flower in the floricultural industry worldwide and highly valued for its form, fragrance and endless variety of new colors. The red coloration of rose petals is derived from chemical components of

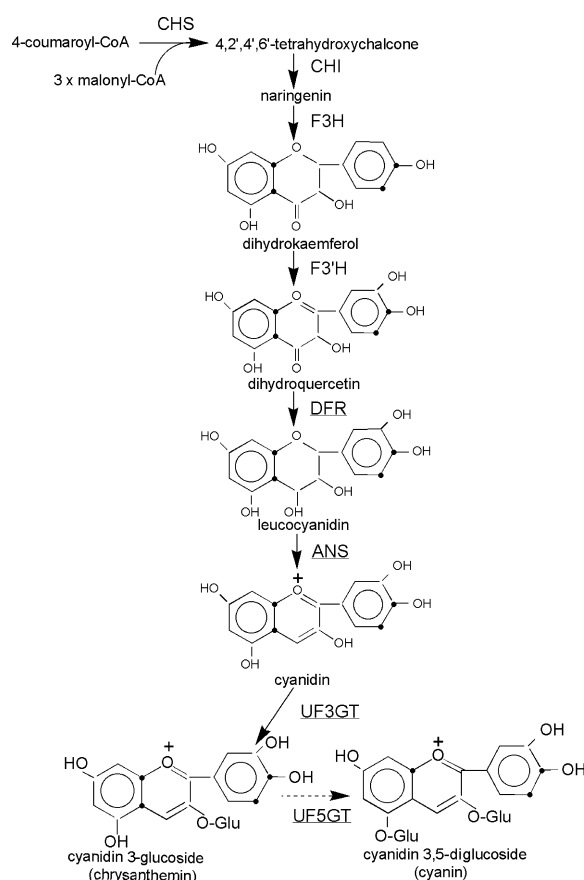


Figure 1. Putative biosynthetic pathway of anthocyanin in *Rosa hybrida* cultivar 'Charleston'. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase, F3'H, flavonoid 3'-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UF3GT, UDP-glucose: flavonoid 3-O-glucosyltransferase; UF5GT, UDP-glucose: flavonoid 5-O-glucosyltransferase. Underlining shows the enzymes whose putative gene probes were used for Northern blot analyses. No detectable cyanin was accumulated in the suspension cells.

pelargonidin, cyanidin and peonidin type anthocyanins (Biolley et al. 1994). Recently, tissue culturing has become an important biotechnological technique to produce various secondary metabolites including anthocyanins on a mass scale for industrial and academic interest (Hirner et al. 2001; Gantet and Memelink 2002). Despite the fact that some plant species have been shown to produce anthocyanins in cell cultures, little information of the cell culture techniques that are capable of producing *in vitro* anthocyanins has been reported in rose.

The biosynthesis of anthocyanins in plant cell culture is stimulated by not only genetic factors but also environmental factors such as light, temperature, growth regulators and nutrition (Sato et al. 1996; Zhang et al. 1997; Zhang et al. 2002). The identity of the environmental stimuli that lead to the accumulation of anthocyanins in rose is of considerable interest. To date, Sato et al. (1996) has examined the intensity of white

light that induces anthocyanin production in strawberry cell culture lines; however, no information was obtained about the effect of light quality on the production of anthocyanins in cultural cells. Therefore, the originality of this research is to investigate anthocyanin biosynthesis in cultural cells exposed to UV-B (290–320 nm) radiation.

In this study, we used *Rosa hybrida* cultivar 'Charleston', a climbing type, originated in Australia. The open flowers of 'Charleston' undergo a striking color change from yellow to red over 10–12 days under natural daylight. We established a suspension cultured cell line from this cultivar of rose. Anthocyanin content was quantified by HPLC to determine the most efficient conditions, such as light including UV-B radiation and media, for continuous production of the pigments in the rose cell suspension. The expression levels of three genes encoding putative key enzymes (DFR, ANS, and UF3GT) for anthocyanin biosynthesis were analyzed by Northern blot. The correlations between the accumulation of genes and the final products were discussed to understand a possible biosynthetic pathway of anthocyanin in rose.

Materials and methods

Plant materials and callus initiation

Young leaves (15–20 mm long) of *Rosa hybrida* cultivar 'Charleston' (Keihan Nursery, Osaka, Japan) were obtained during the spring season from growing rose plants in an experimental field of the Faculty of Agriculture, Kobe University, Japan. Plants were grown under natural environmental conditions with a 12–13 h photoperiod. Leaves were surface sterilized for a few seconds with 70% (v/v) ethanol, followed by 20 min with 1% (v/v) NaClO solution and 2% (v/v) Tween 20, and rinsed four times with sterilized distilled water. The leaf discs (5×5 mm) were prepared and placed abaxial side down on Woody Plant Medium (Sigma-Aldrich K.K., Tokyo, Japan) consisting of MS vitamins (Murashige and Skoog 1962), 3% (w/v) sucrose, 1 mg l⁻¹ indole-3-butyric acid (IBA), and 1 mg l⁻¹ 6-furfurylamino purine (kinetin) in 90 mm diameter and 15 mm deep petri-dishes. A 0.2% (w/v) gellan-gum (Merck Ltd., Tokyo, Japan) was added as a gelling agent before adjusting the pH to 5.8 and media were autoclaved for 20 min at 120°C at 1.1 kgf cm⁻². The explants were maintained at 24±1°C with a 14/10 h (light/dark) photoperiod supplied by a cool-white fluorescent lamp (35 μmol m⁻² s⁻¹, FL20SSD/18-B, Hitachi, Tokyo, Japan). After 30 days of culture, leaf discs with callus were subcultured under the same experimental conditions in glass tubes (120×20 cm) supplemented with either 10 mg l⁻¹ *p*-chloro-phenoxyacetic acid (CPA) and 5 mg l⁻¹ kinetin or 5 mg l⁻¹ IBA and 5 mg l⁻¹ kinetin.

Suspension culture and media composition

Suspension cultures were initiated by transferring about 0.2 g fresh weight of callus sieving through 1 mm size stainless mesh

into a 300 ml Erlenmeyer flask containing 50 ml of EM medium (Yamamoto *et al.* 1981) supplemented with 0.1 mg l^{-1} IBA and 1 mg l^{-1} kinetin. Then, cultures were shaken at 80 rpm at $24 \pm 1^\circ\text{C}$ under 14/10 h (light/dark) photoperiod supplied by a cool-white fluorescent lamp and subcultured every three weeks. After three weeks of subculturing, cells sized between 1 mm and 0.13 mm were collected by passing them through a 1 mm mesh sieve and a 0.13 mm mesh sieve and transferred onto the media solidified with 1% (w/v) gellan-gum in 90×15 mm petri-dishes. To investigate the anthocyanin biosynthesis in culture cells, two different concentrations of sucrose (3% and 6%) were used in two different types of media (EM and MS). Proliferated cells were harvested to measure the anthocyanin content during the cell growth cycle after 4, 7, 10, and 14 days of culture.

Light treatment

Petri-dishes were incubated under weak white light conditions ($< 20 \mu\text{mol m}^{-2} \text{ s}^{-1}$ with a fluorescent lamp) for the first three days followed by exposing them to different light conditions for one week to stimulate anthocyanin biosynthesis. To investigate the effect of different light conditions, white light (WL: 400–700 nm, $60 \mu\text{mol m}^{-2} \text{ s}^{-1}$) was provided by a cool-white fluorescent tube (FL20SSD/18-B, Hitachi, Tokyo, Japan) and UV-B radiation (WL+UV-B: 290–320 nm, 0.2 W m^{-2}) was supplemented by using a UV-B fluorescent tube (FL 20SE 20W, Toshiba, Tokyo, Japan) in addition to WL. The cell cultures maintained in the dark were used as a control.

Extraction of anthocyanins and HPLC analysis

Culture cells (1 g fresh weight) were ground to a fine suspension in 2 ml of acetic acid : methanol (1 : 1) solution with a glass motor and pestle. The extracts were centrifuged at 14,000 rpm for 5 min at 20°C and the supernatant was filtered through a micro-filter ($0.45 \mu\text{m}$ DISMIC-13HP, Advantec Toyo, Tokyo, Japan). HPLC analysis coupled with photodiode array detection was carried out with an HPLC system (Waters 600E Controller, Milford, USA) by modifying the procedures of Graham (1991). Ten micro-liters of the filtrated supernatant was injected into a C_{18} reversed-phase column (Mightysil RP-18GP (ODS), $5 \mu\text{m}$, $250 \times 4.6 \text{ mm}$, Kanto Chemical Co., Tokyo, Japan) and the separation of anthocyanins was done by using a 10 min elution of 1.5% (v/v) phosphoric acid in water, followed by a 20 min linear gradient increase elution from 30% to 50% of 1.5% (v/v) phosphoric acid, 20% (v/v) acetic acid and 25% (v/v) acetonitrile in water. The column chromatography was performed at 35°C and samples were eluted at a flow rate of 1 ml min^{-1} . Anthocyanins were monitored at 520 nm with a Photodiode Array Detector (Waters 996, Milford, USA). The identification of anthocyanins and their concentrations were determined by co-injecting cyanidin 3,5-di-*O*-glucoside chloride and cyanidin 3-*O*-glucoside chloride as standards (Extrasynthese, France).

Cloning of partial homologous cDNAs

Total RNA from leaves of 'Charleston' was used for primary-strand cDNA synthesis with ReverTra Ace[®] (Toyobo, Osaka, Japan). The first strand cDNA reaction was subjected to PCR with sets of primers directed against conserved sequence motifs (Hennayake *et al.* manuscripts in preparation). Obtained cDNA

fragments encoding putative DFR, ANS, UF3GT, and actin were cloned into pGEM[®]-T Easy vector using TA cloning system (Promega, Madison, USA) according to the manufacturer's protocol. Positive identification was done by the dye-primer cycle sequencing method using a DNA sequencer (model 3100, Applied Biosystems, California, USA). Sequence comparisons with databases were performed at the US NCBI GenInfo Network BLAST server.

Northern blot analysis

Northern blot analysis was accomplished with total RNA isolated from 10 g of culture cells exposed to different environmental factors. One hundred micrograms of total RNA was fractionated by using electrophoresis in 1% agarose gel with 5% formaldehyde and blotted on to a Hybond-N⁺ membrane (Amersham Biosciences, Buckingham, UK). For DNA probes, cDNAs of *Rosa hybrida* cultivar 'Charleston' *rhcDFR*, *rhcANS*, *rhcUF3GT* and *rhcACT* (DDBJ accession numbers AB239785, AB239787, AB239786 and AB239789, respectively) were digested with *EcoRI* from each clone and labeled using AlkPhos direct Labeling and Detection system kit (Amersham Biosciences, Buckingham, UK) according to the manufacture's instructions. Hybridization of the Northern blots was performed overnight at 55°C and blots were washed under stringent conditions, and then exposed to Hyperfilm[™] MP (Amersham Biosciences, Buckinghamshire, UK).

Results and discussion

Cell growth and anthocyanin production during a cell growth cycle

In our preliminary experiments, callus was induced from rose leaves. Since the balance of endogenous and exogenous auxin and cytokinin is essential for callus formation, we examined different combinations and concentrations of these growth regulators. A combination of 1 mg l^{-1} IBA (auxin) and 1 mg l^{-1} kinetin (cytokinin) was capable of producing calli successfully (data not shown). A suspension cultured cell line was established by transferring calli from solid to liquid medium. Either MS or EM medium was attempted for the production of anthocyanins in the cultured cells according to the report from Ooba (2004). This cell line produced anthocyanins with both media in the light but not under dark conditions (data not shown). Figure 2 shows anthocyanin production and the cell growth in terms of fresh cell weight of *Rosa hybrida* cultivar 'Charleston' cell suspension cultures. Cell growth was characterized as 4 days lag phase, followed by an exponential phase. Continuous cell growth notably increased the fresh weight, and maximum fresh weight was reached at day 14. Accumulation of anthocyanin followed the cell growth similarly as shown by the rapid increase after 7 days. This parallel correlation is clear since about 100 mg g^{-1} fresh weight of anthocyanin was maintained during the culture period. It was not possible to keep up the cell suspension culture more than 14 days

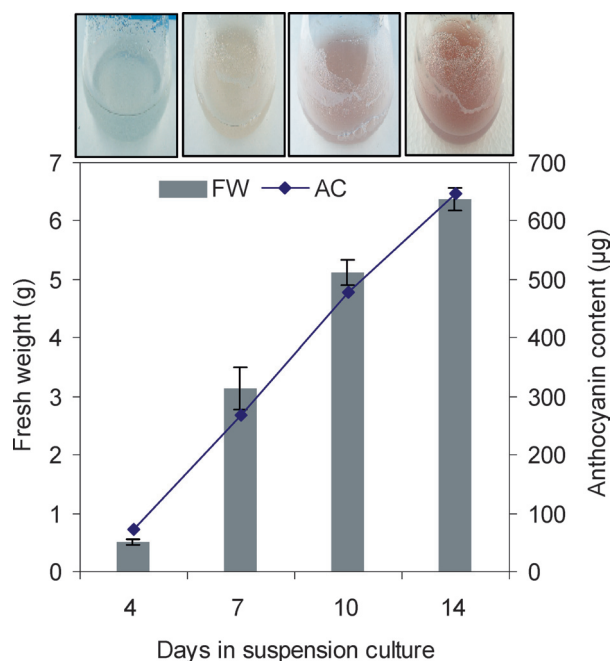


Figure 2. Anthocyanin accumulation and cell growth of suspension cultures after 4, 7, 10, and 14 days in *Rosa hybrida* cultivar 'Charleston'. The cells were inoculated in EM liquid medium supplemented with 3% sucrose and cultured on a reciprocal shaker at 80 rpm, $24 \pm 1^\circ\text{C}$ under white light conditions of 14/10h (light/dark) photoperiod. The pictures on the top show color of the cells at each stage of the culture period.

since most of the cells attained the stationary growth phase, turned brown in color, and no longer accumulated anthocyanins. These results are in general agreement with other reports that show cell growth is correlated with anthocyanin biosynthesis in cell suspension cultures of several species, such as *Daucus carota* (Takeda 1990) and *Perilla frutescens* (Zhong et al. 1993).

Our previous study revealed that the major anthocyanin pigments in flower petals of rose cultivar 'Charleston' were chrysanthemins and cyanin as shown in Figure 1 (Hennayake et al. 2006). In this study, the analysis of anthocyanin content by using HPLC showed chrysanthemins as the only predominant pigment and no detectable cyanin. On the other hand, flower petals produce 4–5 times more chrysanthemins than those of the culture cells (Hennayake et al. 2006). Thus, a possible explanation for this is that the level of chrysanthemins as a precursor substrate of cyanin is not high enough at the later stages of cell proliferation, resulting in lack of internal/external signals to stimulate the expression and/or the activity of *UF5GT* that produces cyanin. As another explanation, it is possible that there is a different regulatory pathway to promote *UF5GT* gene expression between flower and cultured cells induced from leaves.

Effects of medium components and light irradiation on anthocyanin production

In order to investigate the conditions for efficient production of anthocyanin, the established suspension culture cells were treated on solid agar plates under different media compositions (MS or EM), concentrations of sucrose (3% or 6%) and light illumination (white light \pm UV-B or dark). Anthocyanin production was considerably greater with EM medium than with MS medium at all sugar concentrations. We hypothesize that the ratio of NH_4^+ and NO_3^- may be one of the important factors in culture media because it has been reported that in several plant species a nitrogen source improves anthocyanin production by changing primary metabolism of the cells (Sakamoto et al. 1993, Sato et al. 1996). When comparing 3% to 6% sugar in EM medium, 6% sugar resulted in the highest anthocyanin production (Figure 3). The same phenomena have been reported in *Vitis vinifera* by Do and Cormier (1991) and Pasqua et al. (2005). It is considered that glycosylation of cyanidin might be promoted if cells took up much more sugar from the sugar rich medium because the level of *UF3GT* gene expression obviously increased with high sugar EM medium (Figure 4). However, it is possible that the high concentration of anthocyanin was generated as the result of the simple loss of cell fresh weight by osmotic cell dehydration. In fact, 25% of the cell fresh weight was lost by increased sugar concentration from 3% to 6% in medium (data not shown). Although sucrose is an indispensable nutrient, it also acts as an osmotic agent when used at high concentrations. Sato et al. (1996) pointed out that the decrease of cell growth in media containing a high concentration of sucrose might have been caused by inhibition of nutrient uptake in strawberry suspension culture due to an increase in the osmotic potential or the high viscosity of the medium.

Accumulation of anthocyanin was dependent on light irradiation as shown in Figure 3. Higher anthocyanin content was observed under white light, while little or no anthocyanin was produced in darkness. Furthermore, addition of UV-B irradiation to white light increased anthocyanin of the cultured cells more under the conditions of 6% sucrose in EM medium. This result suggests that UV-B could be an important factor for anthocyanin production in culture cells and stimulate the expression and/or the activity of the enzymes in the latter stages of the anthocyanin biosynthesis pathway. The findings that there was little or no anthocyanin production when cultured in darkness indicate that light is necessary for the pigmentation reported in several other *in vitro* studies on different species (Zhong and Yoshida 1995; Ramachandra and Ravishankar 2002; Zhang et al. 2002).

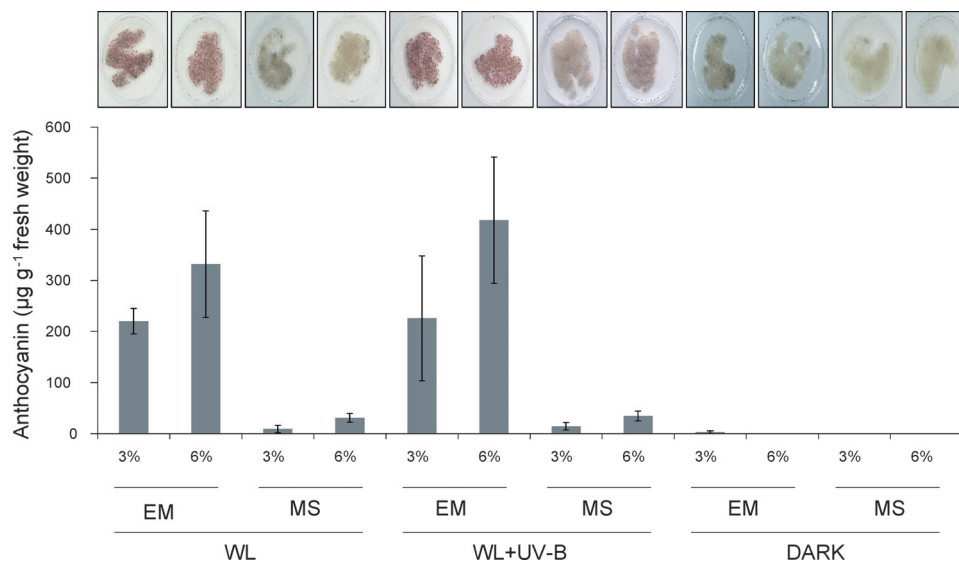


Figure 3. Effects of media components and sugar concentrations under different light conditions on the growth and anthocyanin biosynthesis in cell cultures of *Rosa hybrida* cultivar 'Charleston'. The cells were inoculated on the solid MS or EM medium supplemented with 3% or 6% sucrose. The cells in petri-dishes were incubated under weak white light conditions for the first three days followed by exposing them to different light conditions (WL, white light; WL+UV-B, white light with UV-B; and dark) for one week at $24 \pm 1^\circ\text{C}$ under 24 h photoperiod. The pictures on the top show color of the cells at each treatment.

Expression analysis of anthocyanin biosynthesis related genes

To explain the effect of irradiation on producing anthocyanin in rose cultured cell suspension at the molecular level, the expression of anthocyanin biosynthesis-related gene homologues was analyzed on different days of culture (4, 7, 10, 14 days). Three partial PCR fragments were cloned by RT-PCR and encoded similar amino acids for DFR, ANS and UF3GT, respectively. These fragments shared high degrees of identity at the amino acid level, DFR; 97% with D85102 of *Rosa hybrida* cv. 'Kardinal' (Tanaka et al. 1995), ANS; 97% with AY695818 of *Fragaria ananassa* (Almeida et al. direct submission to Gen-Bank), UF3GT; 88% with AY695816 of *Fragaria ananassa* (Almeida et al. direct submission to Gen-Bank). Based on their sequence identity with other plant proteins, we termed those cDNA fragments *rhcDFR*, *rhcANS*, and *rhcUF3GT*. Figure 4A shows the comparison of *rhcDFR*, *rhcANS*, and *rhcUF3GT* gene expressions at various days of cell suspension culture. Although the level of gene expression was varied among the three genes, the highest expression of these genes was observed at 7–14 days of the culture period.

Higher expression of *rhcDFR* gene after 7 days of suspension culture was detected when most of the dihydroquercetin might be converted into leucocyanidin in the cell. The lower level of *rhcDFR* expression at 10 days of culture suggest that *rhcDFR* is probably deactivated and the pathway swiftly flows downstream, which leads to the production of the corresponding leucoanthocyanidin. Therefore, it is highly expected that

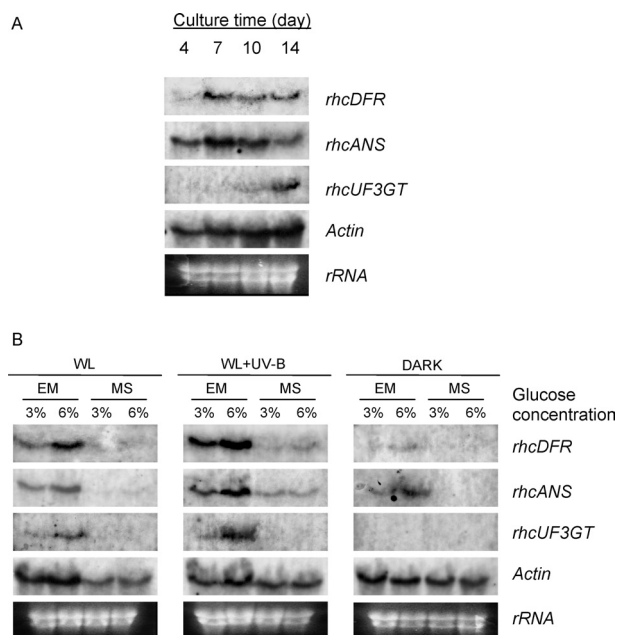


Figure 4. The expression of putative anthocyanin biosynthesis related genes (*rhcDFR*, *rhcANS*, and *rhcUF3GT*) in cultured cells of *Rosa hybrida* cultivar 'Charleston'. (A) Transient expression of genes on 4, 7, 10, and 14 days of suspension culture under white light. For details of culture conditions, see Figure 2 legends. (B) Light-induced expression of genes after one-week exposure to different light treatment: WL, white light; WL+UV-B, white light with UV-B; and dark conditions, respectively. Rose *actin* cDNA (*Actin*) probe was used as a control and ethidium bromide stained ribosomal RNA (rRNA) indicates equal loading of total RNA. For details of culture conditions, see Figure 3 legends.

a large amount of colorless dihydroflavonol and/or leucoanthocyanidin may be accumulated in the cells, waiting to be converted into colored anthocyanidin. In another rose cultivar, *DFR* mRNA accumulation in rose petals (cv. 'Kardinal') was developmentally regulated and paralleled anthocyanin production in petals (Tanaka et al. 1995). Also, in the rose cultivar 'Jaguar', the decreased anthocyanin concentration in flower buds under transient high-temperature conditions was due in part to reduced transcription of *DFR* (Dela et al. 2003). According to these reports and our results, *DFR* seems to be a key enzyme in anthocyanin biosynthesis pathway of rose plants.

The *rhcANS* gene appeared from the lag phase of the culture and its expression level had a peak during 7 to 10 days of suspension culture. The expression of *rhcUF3GT* was evident after 10 days, where the first signs of pigmentation become visible and essentially parallel the accumulation of anthocyanin. The specific glycosylation of anthocyanidin (cyanidin for rose variety 'Charleston') is dependent on the addition of glycoside group to the 3-position of C-ring hydroxyl group and the 5-position of A-ring hydroxyl group. These glycosylations are essential for stable expression of color. Ogata et al. (2005) reported that the novel enzyme, which is specific to rose, cyanidin 5,3-*O*-glycosyltransferase (RhGT1) catalyzes either unglycosylated anthocyanidin or anthocyanidin 5-*O*-glucoside to produce stable cyanidin 3,5-*O*-diglucoside that determines the flower color. However, in the present study, we observed that only chrysanthemine (cyanidin 3-monoglucoside) was produced in suspension cells and there was no detectable level of *UF5GT* gene expression (data not shown) that causes production of cyanin (cyanidin 3,5-diglucoside). Based on these results, we assume that chrysanthemine is the final product in the anthocyanin biosynthesis pathway in cell culture of 'Charleston'.

Since we observed the effects of environmental factors on the accumulation of anthocyanin in cultured rose cell as shown in Figure 3, further analysis of the expression levels of *rhcDFR*, *rhcANS* and *rhcUF3GT* was carried out with the same treatment. The expression of all three genes was less or not detectable in cultured cells grown with MS medium but with EM medium (Figure 4B). The comparison of sugar concentration in the culture medium showed that the mRNAs corresponding to three genes accumulated with 6% sucrose more than with 3% sucrose. The expression levels of *rhcDFR*, *rhcANS* and *rhcUF3GT* were prominent with addition of UV-B to white light compared with white light alone. Under dark conditions, *rhcUF3GT* was not detected, however, *rhcANS* expression stayed very low. To confirm the results, RT-PCR analysis was carried out including the optimization of reaction conditions and the results obtained were consistent with the Northern blot analysis

(data not shown). Taken together, all these results suggest a positive regulation of genes related to anthocyanin biosynthesis could occur with UV irradiation, high concentration of sugar, and EM medium. Our data demonstrated that the induction of putative anthocyanin biosynthesis genes in 'Charleston' was strongly related to the productivity of anthocyanin.

In particular, the hyper-induction of specific gene expression by UV-B irradiation even with MS media was remarkable in the anthocyanin biosynthetic pathway. Similar responses were reported for *CHS*, *CHI* and *DFR* genes in radish (Song and Lee, 1998). It is possible that the light regulatory systems exist in rose as well as in several plants, as reviewed by Jenkins et al. (1995). Although further studies are needed to elucidate the mechanisms, light irradiation and culture conditions might act coordinately on the anthocyanin biosynthetic pathway of 'Charleston' cell culture.

Despite the fact that some species have been shown to produce anthocyanin in cell cultures (Zhang et al. 1997; Hirner and Seitz 2000; Zhang et al. 2002), little information was provided about optimum conditions for cell culture that are capable of producing *in vitro* anthocyanins in rose. The results from this study demonstrated the potential of developing integrated processes that rationally combine different enhancement strategies for further increase in anthocyanin production. Information about both gene resources and environmental cues such as UV-B and medium components will provide an efficient avenue for the development of integrated strategies to advance the plant cell culture process for commercial anthocyanin production.

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