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Instability of Anthocyanin Accumulation in *Vitis vinifera* L. var. Gamay Fréaux Suspension Cultures

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Abstract The inherent instability of metabolite production in plant cell culture-based bioprocessing is a major problem hindering its commercialization. To understand the extent and causes of this instability, this study was aimed at understanding the variability of anthocyanin accumulation during long-term subcultures, as well as within subculture batches, in *Vitis vinifera* cell cultures. Therefore, four cell line suspensions of *Vitis vinifera* L. var. Gamay Fréaux, A, B, C and D, originated from the same callus by cell-aggregate cloning, were established with starting anthocyanin contents of 2.73 ± 0.15, 1.45 ± 0.04, 0.77 ± 0.024 and 0.27 ± 0.04 CV (Color Value)/g-FCW (fresh cell weight), respectively. During weekly subculturing of 33 batches over 8 months, the anthocyanin biosynthetic capacity was gradually lost at various rates, for all four cell lines, regardless of the significant difference in the starting anthocyanin content. Contrary to this general trend, a significant fluctuation in the anthocyanin content was observed, but with an irregular cyclic pattern. The variabilities in the anthocyanin content between the subcultures for the 33 batches, as represented by the variation coefficient (VC), were 58, 57, 54, and 84% for *V. vinifera* cell lines A, B, C and D, respectively. Within one subculture, the VCs from 12 replicate flasks for each of 12 independent subcultures were averaged, and found to be 9.7%, ranging from 4 to17%. High- and low-producing cell lines, VV05 and VV06, with 1.8-fold differences in their basal anthocyanin contents, exhibited different inducibilities to L-phenylalanine feeding, methyl jasmonate and light irradiation. The low-producing cell line showed greater potential in enhanced the anthocyanin production.

Keywords: plant cell culture, subculture, secondary metabolite, anthocyanin, instability, *Vitis vinifera*

INTRODUCTION

Plants may represent one of the best chemical factories in nature, with over 100,000 compounds already having been identified, with about 4,000 new discoveries added every year [1]. Many of these compounds have been used as pharmaceuticals, as exemplified in the USA where 25% of the pharmaceutical market is based on plant-derived chemicals [2]. Plant cell cultures are an enabling alternative technology for the production of these bioactive compounds, and have many prominent advantages compared with directly extraction from field-growing plant materials [1-6]. However, at present there are very few commercial plant cell culture bioprocesses with only a handful of products such as shikonin, ginseng saponins and paclitaxel, being produced [1,7]. One of the ubiquitous and major impediments is the metabolic variability in plant cell cultures, resulting in the highly unstable production of secondary metabolites to those of interest [8]. From a bioprocess operation point of view, any attempt at commercialization would be prohibitive if this instability problem can not be resolved before scale-up.

Metabolic variability or instability in plant cell cultures has been widely reported [9-16]. Two types of biosynthetic instability may be encountered: those in both yield and composition. Ketchum *et al.* [15] reported monthly variabilities in the yield of paclitaxel as well as in the composition of taxoids. They claimed that variability remains the single most problematic issue in the use of cell cultures of *Taxus* for the production of paclitaxel. Variability and loss of anthocyanin production have been reported by Vogelien *et al.* [10] and Hirasuma *et al.* [11] during repeated subcultures. Several studies have also documented the change in anthocyanin composition in cell cultures of *Daucus carota* [17], strawberries [18], *Vitis vinifera* [19] and *Ajuga reptans* [13]. In contrast to the well-documented phenomena of metabolic instability in plant cell culture, however the causes and mechanisms

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responsible remain to be properly addressed. It is generally regarded that the biosynthetic instability in plant cells is caused by their inherent genetic and epigenetic instabilities [20]. The lack of experimental evidences and mechanistic understanding has prevented the development of improved strategies and further commercialization.

In order to understand the production instability of secondary metabolites in plant cell culture, a systematic investigation of anthocyanin accumulation in suspension cultures of *Vitis vinifera*, as a model system, has been initiated in our laboratory. Anthocyanins are visible pigments, with applications in food, nutriceutical and potential pharmaceutical components for treatment of cancer and heart disease [8]. Anthocyanin production in a *Vitis vinifera* cell culture system has proved to be a good model system, as the variability can be visibly observed and measured by HPLC. In addition, anthocyanin biosynthetic pathways have been well studied which allows the delineating of metabolic instability at the molecular level. Here, our preliminary studies in the understanding of anthocyanin biosynthetic instability between subculture batches and within subcultures, during long-term subcultures, are reported. The effects of cell lines with different biosynthetic capacities were investigated, and their responses to an integrated process strategy of precursor feeding, elicitation and light irradiation discussed.

MATERIALS AND METHODS

Plant Cell Line and Suspension Cultures

The cell line used in this study was developed by Cormier *et al.* [21], originating from a callus established in 1978 from *Vitis vinifera* L. cv. *Gamay Fréaux* var. teinturier berry pulp. This cell line, capable of anthocyanin accumulation in the dark, was a gift from Dr. Francois Cormier's group (Quebec, Canada). The suspension cell lines, A, B, C, D, VV05 and VV06 with different anthocyanin contents were established by screening callus, with different pigment intensities, by cell-aggregate cloning, which were initially originated from the callus in one dish. Suspension cultures of these cell lines were established by transferring the selected callus into suspension. Subcultures were conducted weekly, in the dark, in 500-mL Erlenmeyer flasks, enclosed with aluminum foil, containing 150 mL B5 medium [22] supplemented with 30 g/L sucrose, 250 mg/L casein hydrolysate, 0.1 mg/L α-naphthaleneacetic acid (NAA) and 0.2 mg/L kinetin (K). The pH was adjusted to 5.7~5.8 before autoclaving. The inoculum size was approximately 10 g wet cells per 150 mL medium, which was prepared by filtering precultured 7-day-old suspension cells with a 50-μm mesh. The subcultures were maintained on a reciprocating shaker at 100 rpm and 25 ± 1°C.

Anthocyanin Biosynthetic Instability during Long-term Subcultures

The four suspension cell lines: A, B, C and D were subcultured weekly under the conditions described above. For the data presented in this paper, cell line A was subcultured in suspension for 50 generations, and cell lines B, C and D in suspensions for 60 generations. At the time of each subsequent subculture, triplicate samples were taken, and their anthocyanin contents analyzed. The long-term subculture experiment was carried out for 33 batches over 231 days.

Anthocyanin Biosynthetic Instability in Replicate Flasks within Subculture

The experiments were carried out in 250-mL Erlenmeyer flasks containing 50 mL B5 medium supplemented with 30 g/L sucrose, 250 mg/L casein hydrolysate, 0.1 mg/L α-naphthaleneacetic acid and 0.2 mg/L kinetin, with an inoculum of 5.0 g of wet cells. For each of the four cell lines: A, B, C and D, 12 flasks were inoculated from the same parental culture and cultivated under the same subculture conditions detailed above. The cells were harvested 8 days after inoculation, and the anthocyanin content analyzed for all replicate flasks.

Response to an Integrated Process Strategy of Precursor Feeding, Elicitation and Light Irradiation of High- and Low-producing Cell Lines

At the time of these experiments, the high- and low-producing cell lines, VV05 and VV06, were chosen to examine their responses to treatment with 5 mg/L L-phenylalanine feeding, 218 μM methyl jasmonate elicitation and light irradiation. This integrated treatment has been proved to have a synergistic effect on anthocyanin production in our laboratory. The cultures were maintained in 100-mL Erlenmeyer flasks, containing 20 mL medium, with an inoculum size of 2.00 g wet cells, at 25 ± 1°C, on a reciprocating shaker at 100 rpm, under continuous white fluorescent light irradiation of 3,000~4,000 lx. Four days after inoculation, 5 mg/L phenylalanine and 218 μM methyl jasmonate were added, and 7 days after inoculation the cells from triplicate flasks harvested for analysis of cell growth and anthocyanin content.

Analysis

The anthocyanin content was analyzed according to the protocol reported by Zhang *et al.* [19]. At the time of sampling, the cultures were harvested by vacuum filtration through filter paper, washed with MilliQ water and weighed to obtain the fresh cell weight. To analyze the anthocyanin content, 0.2 g of fresh cells were sampled after vacuum filtration and extraction with 50% acetic acid solution, with a volume equivalent to 20 times the fresh cell weight, for 1-h period at room temperature. After filtering through a 0.22-μm filter, 3-mL of the filtrate was mixed with 9 mL of McIlvaine's buffer (14.7 g/L $Na_2HPO_4 \cdot 12H_2O$ and 16.7 g/L anhydrous citric acid) and the pH adjusted to 3.0. The absorbance of the resulting solution was measured at 535 nm with 50% acetic acid: McIlvaine's buffer (1:3) was used as the

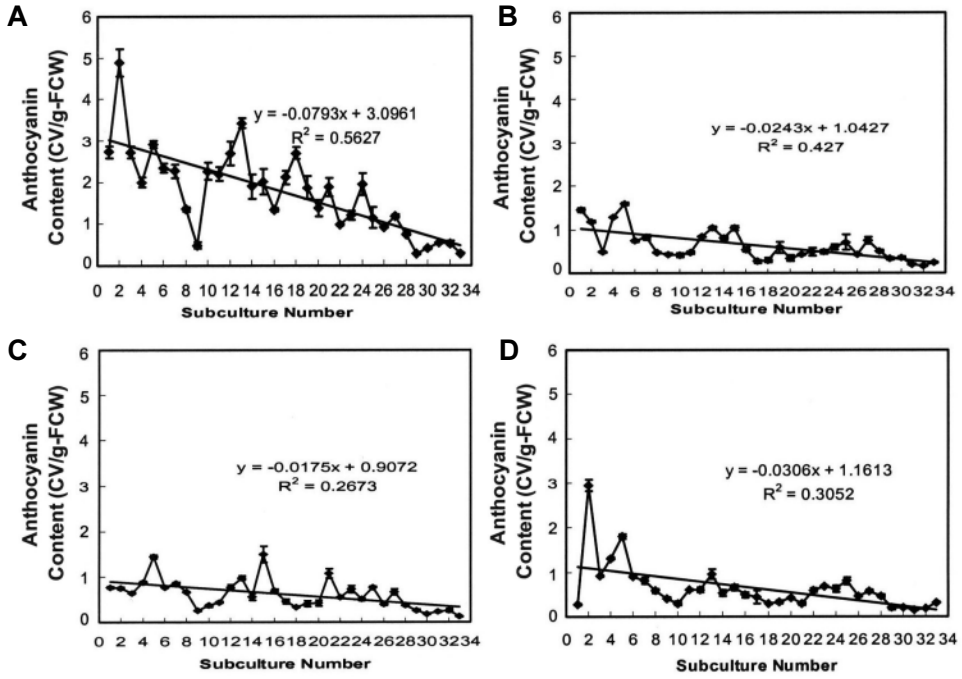

Fig. 1. Long-term analysis of the anthocyanin accumulation in four cell line suspensions: A, B, C and D, of *Vitis vinifera* originated from the same callus material, in continuous weekly subcultures of 33 batches. The subcultures were carried out in B5 medium [16], supplemented with 30 g/L sucrose, 250 mg/L casein hydrolysate, 0.1 mg/L α-naphthaleneacetic acid (NAA) and 0.2 mg/L kinetin (*K*), on a reciprocating shaker (100 rpm) at 25°C in darkness. The inoculum size was 10 g of wet cells per 150 mL of medium. The data are presented as the mean ± standard deviation of three replicate samples.

blank control. The color value (CV) of the pigment extract, a commercial indicator of anthocyanin, was calculated by the following formula [19]:

$$CV = 0.1 \times \text{Absorbance} \times \text{Dilution factor} \quad (CV/g - FCW)$$

In the above-described procedure, the dilution factor was 80. The CV allows for the accurate and comparative quantification of anthocyanins produced from a mixture of different pigments, as is the case for many cell cultures.

RESULTS AND DISCUSSION

Anthocyanin Biosynthetic Instability during Long-term Subcultures

To investigate the anthocyanin biosynthetic instability between subcultures, four suspension cell lines: A, B, C and D, of *Vitis vinifera* were established from the callus in one dish. After several months of subculturing in suspension, the four cell lines were used for further experiments, with different initial anthocyanin contents: 2.73 ± 0.15, 1.45 ± 0.04, 0.77 ± 0.024 and 0.27 ± 0.04 CV/g-FCW (fresh cell weight) (Table 1). The anthocyanin contents for all four cell lines during the subsequent 33 weekly subcultures are shown in Fig. 1. Gradual losses in anthocyanin contents were observed in all four cell lines, but with various rates. These losses were not continuous, but fluctuated significantly. Furthermore, the fluctuations had characteristically irregular cyclic patterns, with the anthocyanin content being higher in one subculture and becoming lower in adjacent subcultures. For example, the anthocyanin contents in the 2nd, 13th, 18th, 21st, 24th and 27th subcultures in cell line A were greater than in their adjacent batches. During the 33 subcultures, the maximum anthocyanin contents of cell lines A, B, C and D were 4.89, 1.59, 1.49 and 2.94 CV/g-FCW at the 2nd, 5th, 15th and 2nd of their respective subcultures; the minima were 0.28, 0.16, 0.12 and 0.15 CV/g-FCW at the 29th, 32nd, 33rd and 31st of their respective subcultures (Table 1). The ratios between the maxima and minima were 17.6, 9.7, 12 and 20-fold, respectively, indicating a high instability of anthocyanins accumulation in all cell lines examined. The variation coefficient (VC), defined as the standard deviation divided by the mean anthocyanin content over the 33 batches, is a quantitative indicator of the instability of anthocyanin accumulation between subcultures. The VC for cell lines A, B, C and D were 58, 57, 54 and 84%, respectively (Table 1).

Anthocyanin Biosynthetic Instability within Subculture

The variability in the anthocyanin accumulation within one given subculture, in 12 replicate flasks, originating from the same parental culture are presented in Table 2 for the four suspension cell lines: A, B, C, and D, of *Vitis*

Table 1. Variability in the anthocyanin accumulation in 33 subcultured batches of the four cell line suspensions of *Vitis vinifera* L. var. Gamay Fréaux: A, B, C and D, originated from the same callus

Cell lines	Anthocyanin content (CV/g-FCW)						
	Average ± SD*	Maximum	Minimum	Variation coefficient (VC)**	S-1 ± SD ***	S-33 ± SD ***	S-1/S-33
A	1.75 ± 1.02	4.89	0.28	0.58	2.73 ± 0.15	0.29 ± 0.015	9.4
B	0.63 ± 0.36	1.59	0.16	0.57	1.45 ± 0.04	0.25 ± 0.09	5.8
C	0.61 ± 0.33	1.49	0.12	0.54	0.77 ± 0.024	0.12 ± 0.007	6.4
D	0.64 ± 0.54	2.94	0.15	0.84	0.27 ± 0.04	0.32 ± 0.009	0.84

* SD: Standard deviation;

** VC represents the Variation coefficient, defined as the standard deviation divided by the mean anthocyanin content value. All subcultures were carried out under darkness, for a period of 7 days, in 500-mL shake flasks containing 150-mL B5 medium [22], supplemented with 30 g/L sucrose, 250 mg/L casein hydrolysate, 0.1 mg/L α-naphthaleneacetic acid (NAA) and 0.2 mg/L kinetin (*K*), on a reciprocating shaker (100 strokes/min) at 25 ± 1°C. The inoculum size was 10 g of wet cells per flask. Samples from three replicate flasks were analyzed each week for their anthocyanin content prior to the subsequent subculture.

*** S-1 and S-33 represents the 1st and 33rd subcultures, respectively.

Table 2. Variability of the anthocyanin accumulation in 12 replicate flasks within the same subculture for the four cell line suspensions of *Vitis vinifera* L. var. Gamay Fréaux: A, B, C and D, originated from the same callus

Subcultures	Average anthocyanin content ± SD (CV/g-FCW)	Variation Coefficient (VC)*
A-51	6.33 ± 0.90	0.14
A-52	4.26 ± 0.53	0.12
A-54	4.76 ± 0.18	0.04
A-55	10.46 ± 0.78	0.075
A-56	8.20 ± 1.27	0.16
B-64	1.99 ± 0.087	0.04
B-65	3.27 ± 0.13	0.04
B-67	2.35 ± 0.18	0.078
C-66	1.33 ± 0.10	0.076
C-67	1.84 ± 0.15	0.082
D-63	2.51 ± 0.42	0.17
D-64	2.01 ± 0.28	0.14

* VC represents the Variation coefficient, defined as the standard deviation divided by the mean anthocyanin content value. All subcultures were carried out under darkness, for a period of 8 days, in 250-mL shake flasks containing 50-mL B5 medium [22], supplemented with 30 g/L sucrose, 250 mg/L casein hydrolysate, 0.1 mg/L α-naphthaleneacetic acid (NAA) and 0.2 mg/L kinetin (K), on a reciprocating shaker (100 strokes/min) at 25 ± 1°C. The inoculum size was 5 g of wet cells per flask. 12 replicate flasks were inoculated from each parental culture. The cells were harvested for 8 days after inoculation, and the anthocyanin contents analyzed.

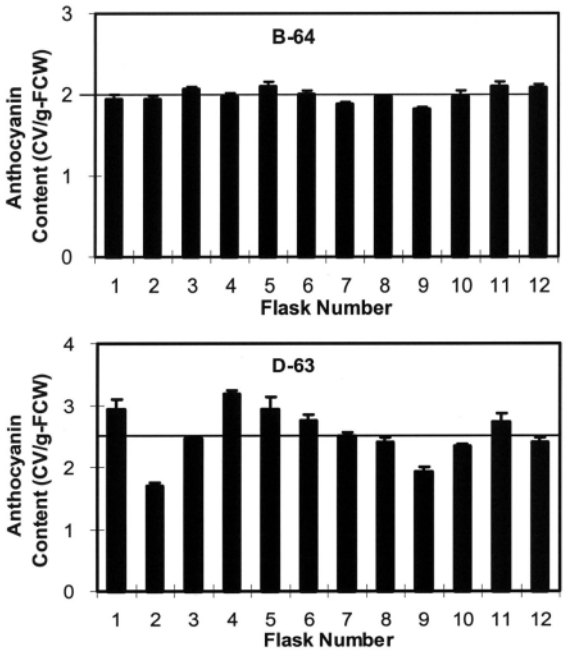

Fig. 2. Instability of the anthocyanin accumulation of *Vitis vinifera* in 12 replicate flasks, originated from the same parental culture, for the lowest subculture batch variability of VC=4% (B-64) and the highest subculture variability of VC=17% (D-63). The experiments were carried out in 250-mL flasks, containing 50 mL B5 medium, with a 5.0 g wet cells inoculum. Eight days after inoculation, the cells were harvested for analysis of their anthocyanin content. The data are presented as the mean ± standard deviation of three replicate samples.

vinifera. These experiments were carried out randomly during the long-term subcultures with a broad range of anthocyanin contents (from 1.33 to 10.46 CV/g-FCW) in the four cell lines. During the long-term subcultures, the numbers of experiments used to investigate the anthocyanin biosynthetic instability within subcultures were 5, 3, 2 and 2 for cell lines A, B, C and D, respectively. They showed similar trend, therefore only results from one experiment are presented. It was found that the anthocyanin biosynthetic instability within a subculture was relatively low, with an average variation coefficient (VC) of 9.7% in 12 subculture batches across the four cell lines (Table 2). The highest VC variability of 17% was observed for the subculture batch, D-63, and the lowest of 4% for subculture batches, A-54, B-64 and B-65. Fig. 2 shows the anthocyanin contents in 12 replicates flasks for the subcultures with the lowest (B-64) and highest (D-63) variabilities.

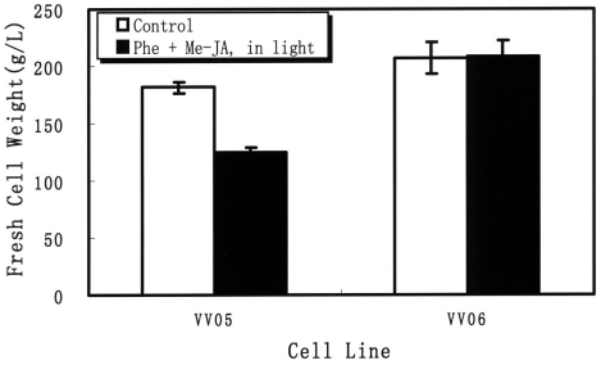

Fig. 3. Effects of 5 mg/L L-phenylalanine feeding, 218 μM methyl jasmonate elicitation and light irradiation on the cell growth (fresh cell weight) of the high- and low-producing cell lines, VV05 and VV06. The experiments were carried out in 100-mL flasks containing 20 mL B5 medium. On day 0, 2.00 g of wet cells were inoculated, and the cultures incubated at 25 ± 1°C on a reciprocating shaker at 100 rpm, either under darkness, as a control, or continuous white fluorescent light irradiation of 3,000~4,000 lx. On day 4, 5 mg/L phenylalanine and 218 μM methyl jasmonate were added and 7 days after inoculation the cells harvested for analysis. The data are presented as the mean ± standard deviation of three replicate samples.

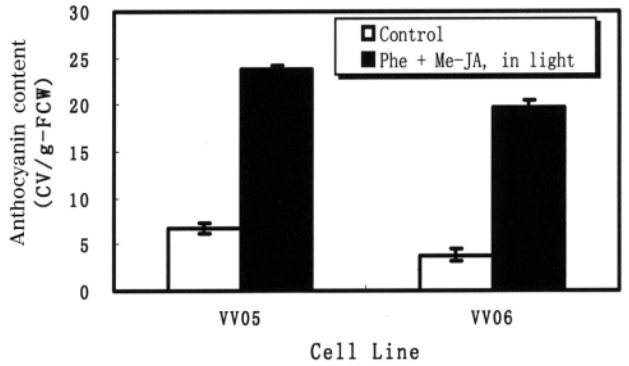

Fig. 4. Effects of 5 mg/L L-phenylalanine feeding, 218 μM methyl jasmonate elicitation and light irradiation on the anthocyanin content (CV/g-FCW) of the high- and low-producing cell lines, VV05 and VV06. The experiments were carried out in 100 mL flasks containing 20 mL B5 medium. On day 0, 2.00 g of wet cells were inoculated, and the cultures incubated at 25 ± 1°C on a reciprocating shaker at 100 rpm, either under darkness, as a control, or continuous white fluorescent light irradiation of 3,000~4,000 lx. On day 4, 5 mg/L phenylalanine and 218 μM methyl jasmonate were added, and 7 days after inoculation the cells harvested for analysis. The data are presented as the mean ± standard deviation of three replicate samples.

Response to an Integrated Process Strategy of Precursor Feeding, Elicitation and Light Irradiation of High- and Low-producing Cell Lines

In response to 5 mg/L phenylalanine feeding, 218 μM methyl jasmonate elicitation 4 days after inoculation, and with continuous light irradiation at 3,000~4,000 lx, the cell growth of the high-producing cell line, VV05, was inhibited, while that of the low-producing cell line VV06 was unaffected (Fig. 3). The fresh cell weight of VV05 was reduced by 30% of that of the control. The anthocyanin content of both the high- and low-producing cell lines were increased from 6.71 to 23.9 CV/g-FCW (3.6-fold of the control), and from 3.83 to 19.6 CV/g-FCW (5.12-fold of the control), respectively (Fig. 4). As a result, the anthocyanin production of VV05 was 2975 CV/L, and 2.45-fold that of the control, whereas that of VV06 was 4090 CV/L, 5.16-fold that of the control (Fig. 5).

DISCUSSION

Over many years, the variability in the metabolic output, which is the theoretical basis for cell line improvement by screening, has been identified as the single most problematic issue in the commercialization of plant cell cultures [8,15]. Variability induces instability of metabolite accumulation, which often leads to reduction in productivity and changes to the chemical composition in high-producing cell lines during extended culturing. A number of mechanisms have been proposed as the causes of this instability, including inherent heterogeneity of the culture populations, genetic and epigenetic instability, environmental stress and lack of tissue differentiation [14].

In this study, four cell lines of *Vitis vinifera*, with significantly different (up to 10-fold) anthocyanin contents were established from the same callus materials (Table 1). It was clearly shown that a cell culture is a heterogeneous system, consisting of cell populations with a diverse range of biosynthetic capabilities. Variability in metabolite accumulation could thus occur at any given time, as different cell populations may exhibit variable responses to their environments, which are often not completely and precisely controlled over long-term subcultures. During the long-term weekly subculture of 33 batches (over 231 days), the instability in anthocyanin accumulation between batches was very high, as characterized by the variation coefficient in the anthocyanin content, ranging from 54~84% for all four cell lines (Table 1). This high VC between batches was in sharp contrast with the very low VC within one subculture period, which 9.7% for 12 subcultures across four cell lines (Table 2). The results could simply reflect the fact that changes in the culture environments would be relatively small for any given subculture period: however, these are much greater over long period subcultures. In addition, these effects could be cumulative, or have a non-linear effect, causing the observed fluctuation in the anthocyanin accumulation (Fig. 1). A similar observation was also reported in callus cultures of *Ajuga reptans* flowers, which produced a complex mixture of cyanidin- and delphinidin-based pigments [13]. The anthocyanin composition varied little during one growth period: however, the difference in anthocyanin composition between the callus lines became pronounced during a time span of 5 years. To further under-

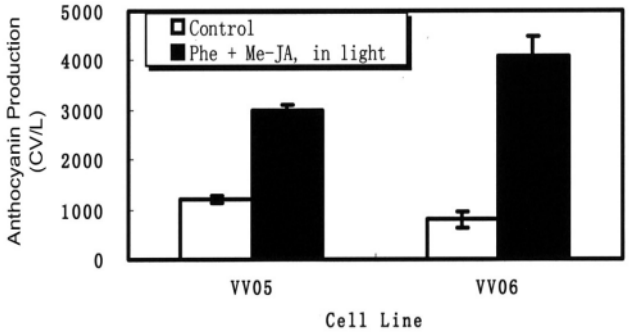

Fig. 5. Effects of 5 mg/L L-phenylalanine feeding, 218 μM methyl jasmonate elicitation and light irradiation on the anthocyanin production (CV/L) of the high- and low-producing cell lines, VV05 and VV06. The experiments were carried out in 100-mL flasks containing 20 mL B5 medium. On day 0, 2.00 g of wet cells were inoculated, and the cultures incubated at 25 ± 1°C on a reciprocating shaker at 100 rpm, either under darkness, as a control, or continuous white fluorescent light irradiation of 3,000~4,000 lx. On day 4, 5 mg/L phenylalanine and 218 μM methyl jasmonate were added, and 7 days after inoculation the cells harvested for analysis. The data are presented as the mean ± standard deviation of three replicate samples.

stand these effects, a set of variables characterizing the physiological states of the cell populations would need to be established and quantified to illustrate the underlying mechanisms.

One interesting aspect that should be noted as a general trend, the anthocyanin content decreased across all four cell lines, with various rates over the 33 subcultures (Fig. 1). Given that all four cell lines were originated from the same genetic callus materials, it may be logical easily to draw the conclusion that the cultures will ultimately completely lose their anthocyanin accumulation ability. However, this may not be true, as the anthocyanin content of cell line D actually increased, from 0.27 ± 0.04 CV/g-FCW for the 1st subculture to 0.32 ± 0.009 CV/g-FCW by the 33rd. To further clarify this trend, longer-term subculturing over years, would be very valuable to demonstrate whether the decrease in the biosynthetic capability could be reversed.

The combined use of precursor feeding or elicitation in plant cell cultures is an effective approach to improve the production of secondary metabolites [8,15,19,20]. Among the productivity enhancement techniques developed for anthocyanin biosynthesis, elicitation has been shown to be the most efficient strategy, leading to the highest enhancement of anthocyanin production [8]. Zhang *et al.* [19] recently demonstrated that an integrated process, combining elicitation and light irradiation could improve the anthocyanin production in *Vitis vinifera* cell suspension cultures. To understand the instability of anthocyanin accumulation, it would be very valuable to investigate how heterogeneous cell populations within a culture respond to elicitor, precursor or other environment factors. The high- and low-producing cell lines VV05 and VV06, with 1.8-fold differences in anthocyanin contents, had rather different responses to the combined induction of a precursor, elicitor and light irradiation (Figs. 3, 4 and 5). The low-producing cell line showed a higher responsive to the combined induction, which may suggest that different cell populations respond differently to variations in the culture conditions and inducers. Given the difficulty in strictly maintaining uniform culture conditions, the instability may be a result of both the heterogeneous cell populations in a culture and variations in the culture parameters. Therefore, the strict control of the culture parameters and selecting highly-responsive, uniform cell lines are equally important in establishing a stable plant cell culture bioprocess.

CONCLUSION

In summary, the *Vitis vinifera* cell suspension culture is a heterogeneous system, containing a range of cell populations, with variable anthocyanin biosynthetic capabilities. The anthocyanin content of a given culture can be very different, depending on the composition of the cell populations, which could be the source for instability in the anthocyanin accumulation. The instability between subculture batches is much greater than that within a subculture, illustrating the accumulating effect of variability during long-term subcultures, as well as the influences of variable culture environmental factors. Differentiated responses of high- and low-producing cell populations to precursor feeding, elicitation and light irradiation demonstrated the importance of selecting highly-responsive uniform cell lines and strict control process parameters.

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