Derivation of Non-Anthocyanin-Producing Grape Cell Culture from a Stable Anthocyanin-Producing Grape Cell Culture: an Example of Reverse Engineering

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Abstract

Production instability in plant cell culture is a major obstacle to commercial production of valuable secondary metabolites, such as, new drugs, or natural pigments. The production of anthocyanin in grape cell culture was used as a model to understand the physiological basis for instability of secondary metabolites production in plant cell culture. The study began with a stable anthocyanin-producing grape cell line. In order to compare the differences between anthocyanin-producing cell and non-anthocyanin-producing cell in physiological and genetic level, a stable non-anthocyanin-producing cell line with same genetic background was derived from the anthocyanin-producing grape cell line. The results show that reducing sucrose concentration to 10 g/l in solid B5 medium can help to significantly lower the anthocyanin production in grape callus and form white callus. In addition to the low sucrose level in the medium, restriction of gas exchange was a critical stress factor in the formation of non-anthocyanin-producing suspension cultures. The anthocyanin content was 0.1 mg/ml and 2.9 mg/ml in the non-anthocyanin-producing suspension culture and the original anthocyanin-producing suspension culture respectively. For enhancement of production of anthocyanin by plant cell cultures, many efforts in creating a more supportive environment such as increasing sucrose level, lights, oxygen supply have been studied and outstanding results have been reported. On the contrary, this research suggests that environmental stress, such as reducing the level of carbon source such as sucrose concentration and restriction of gas exchange, could be methods to derive a

non-anthocyanin-producing cell line with same genetic background from a stable anthocyanin-producing cell line.

Background

Plant cell culture is viewed as a potential means of producing useful plant products such that conventional agriculture, with all its attendant problems and variables, can be circumvented. Plant cell culture also offers the potential advantage of assured and expandable supply when desired compounds extracted from plant tissue are limited due to the rareness, the geological isolation, or seasonal characteristics of the plants. However, production instability of secondary metabolites in plant cell culture is still a major problem in both research and in development of commercial processes. In order to study the anthocyanin production instability in grape cell culture, a non-anthocyanin-producing cell line with same genetic background from a stable anthocyanin-producing cell line is needed.

Materials and methods

Callus cultures of Bailey Alicant A grape cells (*Vitis hybrid*) were from Hirasuna (1991) of School of Chemical Engineering, Cornell University, Ithaca, NY. Their original source was from Dr. R. Ilker of General foods Corporation, Tarrytown, NY. The callus were first established from vines in 1985. Vines were surface sterilized and placed on solid GB5 medium for callus induction. This hybrid has the lineage of (*V. lincocumii* x *V. labrusca* x *V. vinifera*) x (*V. vinifera* x *V. vinifera*), according to Yamakawa et al. (1983).

Suspension cultures were obtained from this callus by inoculating 2-3 g of callus into 50 ml of medium contained in 250 ml flasks and agitating for 2 – 3 weeks on a shaker at 125 rev./min. Both suspension and callus cultures were maintained in Gamborg's B-5 medium (Gamborg, 1968), which were

prepared by Gibco/BRL, with 2% (w/v) sucrose and 0.5 mg/l 2,4-D. The initial pH of the medium was adjusted to 5.8 with KOH or HCl, and pH was not regulated during culturing. Callus medium contained 0.5% (w/v) Phytagar (Gibco/BRL) in sealed petri dishes. Callus was transferred to new medium every 3 - 4 weeks depending on the development of white callus on the red callus. The suspension cultures were transferred every 3 weeks by transferring 10 ml of cell suspension into 50 ml of fresh medium. Both callus and suspension cultures were kept in dark at 23 – 25 ^{o}C. except otherwise indicated.

Because the purpose of the experiment in this section was to derive a stable non-anthocyanin-producing grape cell culture and it can be simply discriminated from anthocyanin-producing grape cell culture by their color difference, the quantitative methods in measuring the contents of anthocyanin and growth were not applied in this section of experiments. The color changes in this section will be described as purple, dark red, red, pink, and white.

Results and Discussions

The most distinguished phenomenon in the study of instability of anthocyanin production by plant cell culture is the color changes during culture. The spectrum of color changes could vary from purple, dark red, red, pink, and white. For the most significant comparison in the purple cell cultures (highly anthocyanin-producing cell cultures), white cell cultures (non-anthocyanin-producing cell cultures) with common genetic background, were needed. The callus from previous study conducted by Hirasuna in 1991 was a highly selected cell line with as high as 2.9 g/l production of anthocyanin in production medium (Figure 1). However, the other extreme cell line, the white, non-anthocyanin-producing cell culture, was not available when this research began. This non-producing cell line must be then derived from the purple callus that has a history of stable high anthocyanin production for many years.

Almost all the efforts in anthocyanin production before this research were directing at enhancing the production of anthocyanin in cell lines. No previous studies in how to reduce the production of anthocyanin in plant cell culture were found. Several experiments were conducted by reversing the procedure applied in promoting anthocyanin production in plant cell culture in order to obtain the non-anthocyanin-producing cell line.

Figure 1. The anthocyanin-producing (purple) grape callus.

When efforts in altering external environmental factors could not induce formation of a non-anthocyanin-producing cell line, changing the medium content to acquire such a cell line expected became necessary. The first ingredient we considered to adjust in the medium was the carbon source, sucrose. Carbon source is known to have an effect on anthocyanin and other plant metabolites produced by cell cultures. Eight sugars as carbon source were examined in detail for carrot callus cultures and glucose and sucrose, especially sucrose alone, were found helpful in anthocyanin accumulation (Narayan and Venkataraman, 2002). The addition of sucrose to the culture medium resulted in the major sugars accumulated in grape cells to become glucose and fructose, reaching 40% of the dry weight. The increase in the level of these hexoses closely coincided with the increase in anthocyanin accumulation in grape cells (Larronde, 1998). The

enhancement of anthocyanin accumulation by increased sucrose level could be due to the effect of sucrose as a nutrient and also the alteration in osmolarity (Hirasuna et al., 1991; Tholakalabavi et al., 1997). The previous study involving the cell line used in this research showed that sucrose concentration increased from 2% to 8%, the yield of anthocyanin in grape cell culture increase about 14 fold from 139 ml/l to 1513 mg/l (Hirasuma et al., 1991). Therefore, reducing the sucrose level in the medium could be considered as a method to decrease the anthocyanin production and might further result in the formation of non-anthocyanin-producing cell line. The sucrose concentration in the solid B-5 medium was reduced from the original level, 20 g/l, to 3 levels, 5 g/l, 10 g/l, and 15 g/l. The anthocyanin-producing callus were then transferred to the sucrose-reduced solid medium. After cultivation in the dark for three weeks, the callus on the solid medium with 15 g/l sucrose showed no difference from the control medium, sucrose concentration at 20 g/l. The callus on the solid medium with sucrose concentration at 5 g/l showed no growth and the color of the callus turned brown. On the solid medium of 10 g/l sucrose content, the callus grew well and the color became paler than the ones on the medium with 20 and 15 g/l sucrose. In addition, some tiny colorless, transparent callus particle formed on the edge of the dark red callus. The colorless, transparent callus should accumulate less or even no anthocyanin in the cell.

These callus were collected and transferred to the solid medium containing 10 g/l sucrose for further cultivation. The size of the collected colorless callus was less than 0.5 cm in diameter. After placing on the solid medium as described above, the callus turned to pink and then red in the first week,

then turn dark red after cultured for three weeks. However, again, there was a small amount of colorless, transparent callus found at the edge of the dark red callus. Since the colorless callus might result from the shortage of sucrose content in the medium, a longer cultivation time that reduced sucrose levels could result in more formation of colorless callus. The results were as expected. At week four, more colorless callus had formed on the dark red callus.

Prolonging the culture time seemed to be a method to gain more colorless callus, but it could lead to the death of the callus, too. Subsequently, the callus were transferred every four weeks for the following experiments. The collection and transfer continued for more than 20 transfers, approximately 20 months, then a stable white callus of the size about 5 cm in diameter was formed (Figure 2).

During the procedures of transfer, in order to maintain the stability in color change, a large portion of the white callus must be transferred to the new solid medium unlike traditional transfer. If only a small part of the white callus was transferred, it quickly turned back to red in a short period of time. Such a response might be related to the ratio between callus size and the sucrose level of the medium.

Though the white, non-anthocyanin production callus was successfully formed, the callus grew too slowly to fit our needs in further experiments. A relatively fast growing non-anthocyanin production suspension culture was still needed.

Approximately 2-3 g white callus were transferred to 50 ml B-5 medium with a sucrose level at 10 g/l in 250 ml flask. The suspension culture quickly turned to pink in the first 2-3 days, and then turned to red by the end of first week and turned to dark red after culturing for 3 weeks. The same callus-sucrose level hypothesis could be applied to explain the color change. While the callus was introduced into the liquid medium, the single grape cell exposed to a relatively high sucrose level around them that could offer higher energy for the cells to produce anthocyanin, while the cells in the large congregation of callus could not produce anthocyanin due to the relatively low sucrose around them. Thus, in order to obtain a stable non-anthocyanin producing suspension culture, additional alteration in environment was taken into consideration.

Figure 2. Stable white grape callus formed after 20 transfers

Restriction of gas exchange was applied after some other attempts in formation of a stable non-anthocyanin production suspension culture failed (data not show). Parafilm was put on the top of the 250 ml flasks containing suspension cultures to prevent gas exchange. The restriction of gas exchange would lead to the decrease of oxygen level and increase of carbon dioxide level and some other gas levels such as ethylene in the flask. All these changes in gas levels would generate stresses that directly affect the primary metabolism and consequently production of secondary metabolites or directly affect the enzymes of secondary metabolism.

After the application of restriction of gas exchange, most of the cultures derived from the white callus turned to pink in the first week, and color faded after that. At week 3, the white, non-anthocyanin production suspension culture was formed. However, there were still a few cultures that maintained pink after cultivation for three weeks in spite of the restriction of gas exchange (Figure 3). Approximately 1 ml of the suspension culture was placed on the plates and observed under microscope. Some red cells can still be found for the pink suspension culture; in contrast, in the white suspensions only a few red cells could be found. At this point, the white cells were the dominant type in the suspension culture, and a stable non-anthocyanin-accumulation cell line was formed (Figure 4).

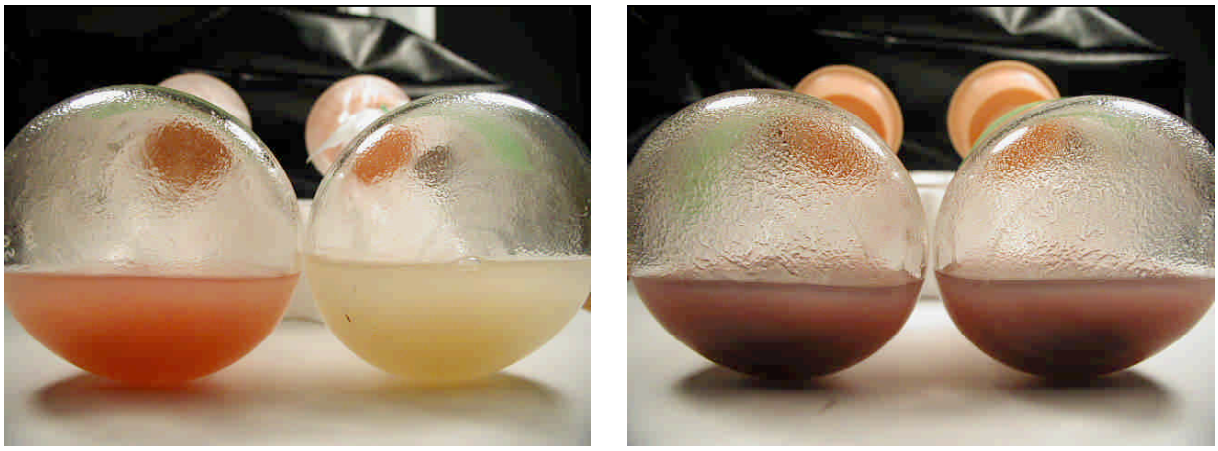

Figure 3. The white and pink suspension culture from the white callus (left), and the purple suspension cultures from the dark red callus (right).

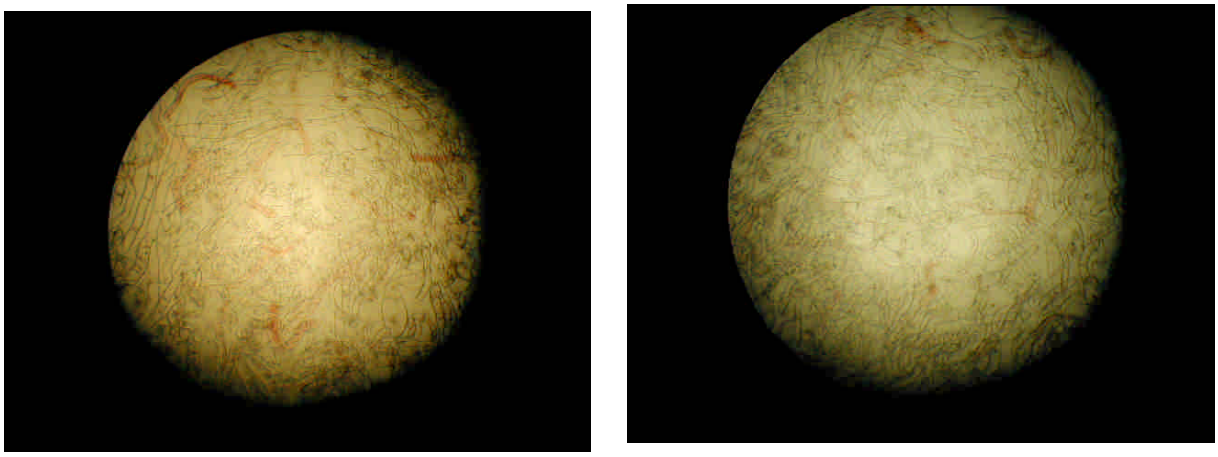

Figure 4. The cells from pink and white suspension culture as shown in figure 3 observed under microscope.

It took approximately two years to derive a stable non-anthocyanin-producing cell line from a stable highly selected anthocyanin-producing cell line. The reasons for this long journey could be: 1. The original anthocyanin-producing cell line was highly selected and had a long history of stable high anthocyanin accumulating rate that was difficult to reverse. As described above, the previous study with this cell line took about 11

months to recover the red callus from a very faded suspension culture. 2. As pioneers in this field of “reverse engineering” in reducing the production of secondary metabolites by plant cell culture, no previous study was available as a reference for the experiments.

For enhancement of production of anthocyanin by plant cell cultures, many efforts in creating a more supportive environment such as increasing sucrose level, lights, oxygen supply and optimizing culture temperature have been studied and outstanding results have been reported. For the contrast, this research suggests that environmental stress, such as reducing the level of arbon source such as sucrose concentration and restriction of gas exchange, could be useful to derive a non-anthocyanin-producing cell line from a stable anthocyanin-producing cell line.

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