Planta 149, 292–297 (1980)

Planta

A Clonal Analysis of Anthocyanin Accumulation by Cell Cultures of Wild Carrot

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Abstract. The accumulation of anthocyanin by clones and subclones from a cell suspension culture of wild carrot (*Daucus carota* L.) has been measured under standard conditions. Clones which accumulate low amounts of anthocyanin were shown, by recloning after maintenance by serial passage, to have become heterogenous and to contain cells with increased accumulation of anthocyanin. There appears to be a maximum amount of anthocyanin that clones can accumulate. Clones which accumulate the maximum amount of anthocyanin were shown by recloning after maintenance by serial passaging, to have become heterogenous and to contain many cells which accumulate less than the maximum possible amount of anthocyanin. When clones which accumulate the maximum amount of anthocyanin are maintained by serial passage, the decline in anthocyanin accumulation is different in different media. The results indicate that the changes in the ability of cells to accumulate anthocyanin involve no qualitative change in the genetic information of the cells, i.e., the changes are not the consequence of mutations.

Key words: Anthocyanin – Cell cultures (variability) – *Daucus*.

Introduction

In recent years, it has become increasingly clear that plant cell cultures may produce chemicals characteristic of the species of origin in amounts approximating those found in the whole plant (for reviews, see Zenk 1978 and Dougall 1979a, b). Plant cell cultures which produce chemicals yield clones which accumulate different amounts of the chemical. Thus Tabata and Hiraoka (1976) found that the nicotine content ranged from 0.0035 to 0.0866% of dry weight in clones obtained from callus cultures of *Nicotiana rustica* and that in some but not all clones, it had fallen to low levels after 40–50 serials transfers. Zenk et al. (1977) showed that the range of yields of ajmalicine and serpentine were 0–0.85% and 0–1.4% dry weight, respectively, in different clones from cell cultures of *Catharanthus roseus*. Ogino et al. (1978) showed that in clones from a callus culture from *Nicotiana tabacum* the nicotine content ranged from 0.1 to 3.0% dry weight and that by recloning the high-yielding clones, subclones with a nicotine content of 2.5% dry weight could be obtained.

In their study, Ogino et al. (1978) only sought high-yielding cell lines, however. They did not describe the variability in or the stability of the nicotine content of cells from clones which had low yields of nicotine. A study of variability in cells from low-yielding clones from a plant cell culture would show whether or not the changes in yields were reversible.

In order to determine whether or not the cells in a plant cell culture could undergo reversible changes in their ability to produce secondary products, we have examined the anthocyanin accumulation by cells from high- and low-accumulating clones from a wild-carrot cell suspension culture. This was done by recloning the clones. We conclude that the ability of the cells to accumulate anthocyanin does change reversibly during culture.

Materials and Methods

Cells, Media, Culture Conditions, and Anthocyanin and Growth Measurements. The cell line WC-63 used in this study was derived by subculturing anthocyanin-containing portions of a culture established from a petiole explant of wild carrot by Dr. D.F. Wetherell (University of Connecticut, Storrs, Conn., USA). Line WC-63 and

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0032-0935/80/0149/0292/$ 01.20

the clones generated from it in this study were maintained by the methods described by Dougall and Weyrauch (1980a, b). The medium used for maintenance of cultures was described by Wetherell (1969) and is referred to here as "stock-culture" medium. The antocyanin accumulation by each clones was measured after growth for 14 d in the medium referred to as W.C. Imp. (Kinnersley and Dougall 1980). At an initial pH of 4.5, W.C. Imp. gives a four- to six-fold increase in anthocyanin accumulation by WC-63 over that found in stock-culture medium. The pH of all media was adjusted with NaOH or HCl prior to autoclaving at 1.05 kg/s·cm^2 for 15 min. The methods used for the determination of anthocyanin and growth in cultures have been described in Dougall and Weyrauch (1980a, b).

The anthocyanins contained in the wild-carrot cells used here are cyanidin derivatives (J.C. Hemmingson and R.P. Collins, University of Connecticut, Storrs, Conn., USA, unpublished data). The factor of 10.8 can be derived from the molar extinction coefficients for cyanidin derivatives in methanol containing 1% concentrated HCl (Harborne 1967) to convert absorbance at 530 nm (A_{530}) into mg cyanidin/l.

Cloning Procedures. a) Plating. The inocula for plating were obtained from cultures which were at or near the end of their growth phase. These were screened through a 120 µm stainless-steel screen and then through a 44-µm stainless-steel screen (S.G.A. Scientific, Bloomfield, N.J., USA). The material retained on each screen was washed with stock-culture medium and the washings added to the fraction which passed through each screen. The suspension which passed through both screens was centrifuged at 200·*g* for 5 min, 90% of the medium was removed and the cells resuspended in the remainder. The concentration of single cells and multi-cellular units in the suspension was determined by counting in a Spiers-Levy Eosinophil Counter (Arthur H. Thomas & Co., Philadelphia, Pa., USA). Single cells and clumps of cells with common cell walls were counted as single cellular units. The inoculum for plates was prepared by diluting the suspension with stock-culture medium so that the required number of cellular units for a plate (25, 100 and 500 units) were contained in 1 ml. The plating medium was the W.C. Imp. supplemented with 10 mM *myo*-inositol and 0.5% agar purified according to Binns and Meins (1979). The pH was adjusted to 5.8. One ml of inoculum was mixed with 10 ml of melted plating medium at 40° C and allowed to set in a 100-mm diameter platic tissue-culture dish (Falcon No. 3003; Fisher Scientific Co., Pittsburgh, Pa., USA). The plates were kept in gas-impermeable plastic bags ("anaerobags"; Cedanco, Wellesley, Mass., USA) at 25° C in the dark. When the colonies were approx. 1 mm in diameter, well separated colonies were transferred to screw-caps tubes, 16 mm diameter, 150 mm high, containing 2 ml of stock-culture medium supplemented with 10 mM *myo*-inositiol at pH 5.8. The tubes were placed ina roller drum at 10–20 rpm.

b) Microcultures. Suspension cultures of cell line WC-63 and its clones WC-63-1 and WC-63-2, after 10–21 d of growth in stock-culture medium, were screened through stainless-steel screens of mesh sizes of 125, 63 and 44 µm, without the addition of any fresh medium. The material passing through the 44-µm screen was a population consisting of single cells and of cell aggregates ranging up to 15 cells. The sizes of the single cells ranged for 15 to 30 µm in isodiametric cells, and up to 40 µm wide and 150 µm long in enlarged cells. The screened suspension was diluted with W.C. Imp. medium containing 250 mg/l enzymatically hydrolyzed casein (Sigma Chemical Co., St. Louis, Mo., USA) and adjusted to pH 5.8, to facilitate observation and handling of cells and clumps. Cells and clumps for establishing clones were selected by examination at a maximum magnification of 120×. Using a lower magnification, the selected cells and clumps were picked up with a fine-drawn Pasteur pipette. Each selected cellular unit was cultured in a well of 96-well platic tissue culture plate (Falcon Platics, Oxnard, Cal., USA). About 0.05 ml of diluting medium was added and covered with one or two drops of sterile mineral oil (Saybolt Viscosity 335/350; Fisher Scientific Co., Pittsburgh, Pa., USA). The plates were incubated in the dark at 25 °C. Observations of the completed microchambers were made at 40–200-fold magnification using an inverted microscope (Model M/S; Nikon, Garden City, N.Y., USA).

After the cells had grown to colonies estimated to be from 500 to 2,000 cells, they were removed from the wells using long, bent-tip Pasteur pipettes. The colonies were placed in tubes contain-

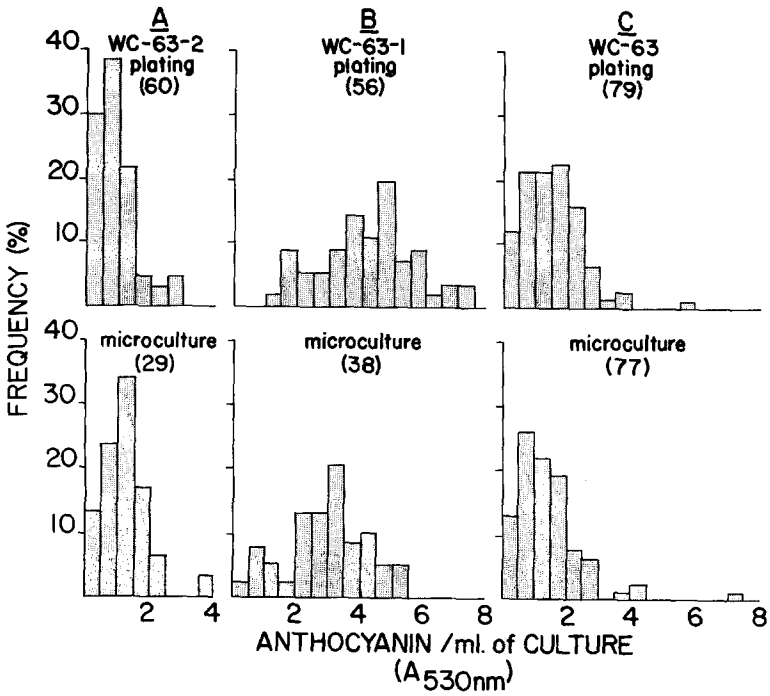

Fig. 1 A–C. Comparison of anthocyanin accumulation by wild-carrot cell clones obtained by plating and by microculture from WC-63 (**C**), WC-63-1 (**B**), WC-63-2 (**A**). The number of clones examined is given in parentheses. See Materials and Methods for details of methods

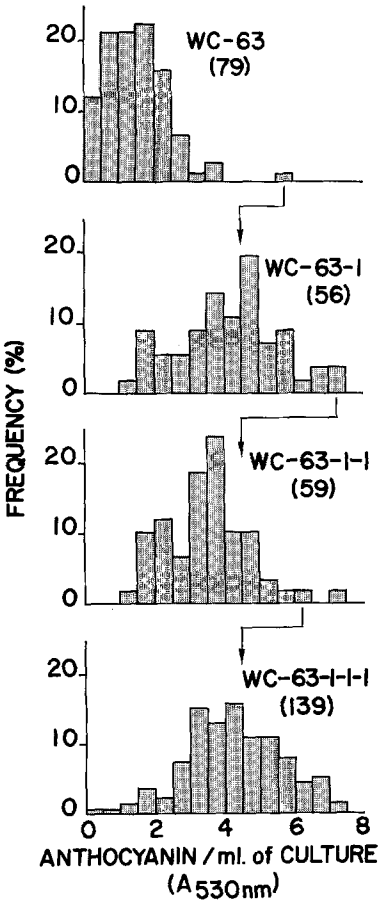

Fig. 2. The distribution of anthocyanin production by subclones from the high accumulating clone WC-63-1 from wild-carrot cell suspension culture. In addition, a subclone with the highest accumulation of anthocyanin was selected from each cloning and then recloned. The number of clones examined at each clone is given in parentheses. The origin of each clone is given by the origin of each arrow. The details of the methods used are given in Materials and Methods

ing 2 ml of stock-culture medium supplemented with 10 mM *myo*-inositol and adjusted to pH 5.8. The tubes were then placed in a roller drum (10–20 rpm) for further growth.

Measurement of Abilities of Clones to Accumulate Anthocyanin. When the clones had grown to a density approximating that of a stationary-phase culture (3–4 weeks), the entire 2-ml culture was transferred to 25 ml of W.C. Imp. medium at pH 4.5. After 2 weeks, the accumulated anthocyanin and the growth of the tissue were measured.

Results

Methods of Obtaining Clones. When the pH of the plating medium was 5.8, the number of visible colonies on the plates equaled the number of cellular units plated. Colonies were obtained from 80 to 90% of the cellular units at pH 5.5 and from 60 to 70% of the cellular units at pH 6.1. In microcultures, clones were obtained in more than 80% of attempts when groups of four or more connected cells were used. Clones were obtained from 70% of the microwells in which groups of three connected cells $\leqq 30$ μm in length were placed, from 56% of the wells in which pairs of cells $\leqq 30$ μm length were placed, from 38% of the wells containing single cells $\leqq 30$ μm, and from 3% of the wells containing single cells $\leqq 50$ μm in length. The percentage of success when single cells or pairs of cells were grown in microchambers was less than the percentage of colonies obtained on plates. This difference indicated that a series of clones obtained by microculture and a series of clones obtained by plating from the same population might show different distribution of anthocyanin accumulation. To test this possibility, clones were obtained from three populations using both methods. The yield of anthocyanin of each clones was measured. As shown in Fig. 1 there is no major difference in the distribution of anthocyanin accumulation among clones obtained by the two methods from each population.

Serial Recloning of the Clone with the Highest Anthocyanin Accumulation. From the plating of WC-63, the clone (WC-63-1) with the highest accumulation of anthocyanin/ml of culture ($A_{530}=5.5$–6.0) (see Fig. 2), was recloned. Subclones gave anthocyanin/ml of culture ranging from A_{530} of 1.0–7.5. One subclone from the highest anthocyanin-accumulating class was selected. The recloning and selection was repeated as shown in Fig. 2. In each of the three reclonings and selections, the highest class of anthocyanin accumulation in which clones were found was the same. This result indicates that there is an upper limit of anthocyanin accumulation in the cells. In two reclonings, clones were found in all the classes of anthocyanin accumulation from 1.0 to 7.5. In the third recloning there were clones in all classes of anthocyanin accumulation from 0 to 7.5. This is probably a result of the larger number of clones examined. It is clear from the data in Fig. 2 that clones from the highest class of anthocyanin accumulation were not stable and continuously produced cells which accumulate less than maximum amounts of anthocyanin.

In view of the appearance of cells which accumulate less than the maximum amount of anthocyanin, one would expect that cultures of high-yielding clones which were maintained by serial passage would decline in ability to accumulate anthocyanin. The decline should increase with increased number of passages. The decrease in anthocyanin/ml culture on serial passage is shown in Table 1. In addition, the data in Table 1 show that the decline in anthocyanin/ml of culture was greater when the clones were grown

Table 1. Decrease in anthocyanin/ml culture ($A_{530\ nm}$) by clones from wild-carrot cell cultures on serial passage in two media.

Two flasks of each clone were carried in each medium from the beginning of the experiment. Cultures carried in stock-culture medium were transferred to W.C. Imp. medium and anthocyanin measured at the end of the second serial passage in the latter medium. The values are the mean ± standard deviation of four samples from each flask

Clone No.	Anthocyanin/ml culture ($A_{530\ nm}$)				
	Number of passages				
	0	in W.C. Imp. medium		in stock-culture medium	
		7	18	5	16
WC-63-1-1-1	6.23	3.07 ± 0.10 2.37 ± 0.05	1.29 ± 0.06 1.45 ± 0.07	4.83 ± 0.22 5.05 ± 0.05	3.95 ± 0.14 3.97 ± 0.19
WC-63-1-2-1	5.77	3.33 ± 0.16 3.06 ± 0.14	2.27 ± 0.03 2.31 ± 0.12	3.25 ± 0.10 3.40 ± 0.05	3.41 ± 0.04 3.12 ± 0.11
	0	6	17	4	15
WC63-1-7	7.48	3.00 ± 0.10 2.77 ± 0.05	1.13 ± 0.03 1.22 ± 0.06	4.98 ± 0.02 5.02 ± 0.18	4.03 ± 0.07 3.85 ± 0.13
WC-63-1-8	7.77	1.91 ± 0.10 2.00 ± 0.04	1.81 ± 0.09 1.84 ± 0.04	3.59 ± 0.33 3.62 ± 0.21	3.28 ± 0.12 3.18 ± 0.11
WC-63-1-9	7.56	1.59 ± 0.07 2.24 ± 0.07	1.24 ± 0.03 1.23 ± 0.01	4.18 ± 0.02 3.31 ± 0.14	3.44 ± 0.10 3.50 ± 0.17

Table 2. Comparison of anthocyanin accumulation by several low-yielding wild-carrot cell clones when it was first measured for each clone with the anthocyanin accumulation of subclones obtained from each.

Recloning of each clone was initiated one or two passages after the first measurement of anthocyanin. Clones WC-63-2 and WC-63-5 were obtained from WC-63; WC-63-2-1 and WC-63-2-2 were subclones from WC-63-2

Clone No.	Anthocyanin/ml culture ($A_{530\ nm}$)											
	At time of first measurement	Range										
		0–0.1	0.1–0.25	0.25–0.5	0.5–1.0	1.0–1.5	1.5–2.0	2.0–2.5	2.5–3.0	3.0–3.5	3.5–4.0	4.0–4.5
		Frequency (%) on recloning										
WC-63-2	0.086	3.3	3.3	23.3	36.7	21.7	5.0	3.3	5.0	0	0	0
WC-63-5	0.183	6.6	5.0	0	5.0	16.7	20.0	21.7	16.7	5.0	3.3	1.7
WC-63-2-1	0.102	5.0	60	28.3	6.7	0	0	0	0	0	0	0
WC-63-2-2	0.072	55.4	28.6	12.5	3.6	0	0	0	0	0	0	0

in the W.C. Imp. medium than when they were grown in stock-culture medium.

Recloning of the Clones with the Lowest Anthocyanin Accumulation. The conclusion that the clones with the highest anthocyanin accumulation produce cells with decreased accumulation led us to examine the possibility that clones with the lowest anthocyanin accumulation could produce cells with increased amounts of anthocyanin. This possibility was examined by selecting from WC-63, clones with the lowest anthocyanin level in W.C. Imp. medium. These were then recloned. Two subclones with the lowest anthocyanin levels were also selected and recloned. The distribution of anthocyanin accumulation in these clones and in the subclones is shown in Table 2. It is clear from the data in Table 2 that at each cloning, subclones were obtained whose anthocyanin accumulation was greater than the initial value measured for the clone at the time of its selection. This result shows that clones which have low anthocyanin accumulation, produce cells with increased accumulation.

Discussion

All measurements of anthocyanin accumulation by clones and subclones were performed under the same conditions. The differences in the accumulation of anthocyanin between clones, therefore, reflects differences in the inherent capacity of the clones to accumulate anthocyanin. It is to be noted that the numerical value of the anthocyanin produced by a clone will be altered by the medium in which the clone is grown.

The results obtained lead to four conclusions concerning the abilities of the cells of the anthocyanin producing carrot cell culture to accumulate anthocyanin:

1. The ability to accumulate anthocyanin has an upper limit (Fig. 2).
2. The rate of loss of ability to accumulate anthocyanin from high ability clones is influenced by the culture medium (Table 1).
3. The ability to accumulate anthocyanin is heritable. As seen in Fig. 2, subclones with the maximum anthocyanin accumulation continue to remain in the population at a frequency of 1–2%. As shown in Table 2, recloning of clones with low ability to accumulate anthocyanin always yields some subclones which have the lowest anthocyanin accumulation. In each case these subclones must be progeny of the cell giving rise to the clone which have not undergone alteration in their ability to accumulate anthocyanin. Thus, the ability to accumulate anthocyanin is a heritable property of the cells.
4. Changes in ability to accumulate anthocyanin are reversible. The data in Fig. 2 shows that the clones with the highest ability to accumulate anthocyanin produce cells which have decreased ability. The data in Table 2 show that clones with the lowest ability to accumulate anthocyanin produce cells with increased ability. The rate of appearance of cells with increased ability to accumulate anthocyanin appears to be slower than the rate of appearance of cells with decreased ability. Changes in ability to accumulate anthocyanin occur in both directions. This latter fact indicates that the genetic information in the cells remains qualitatively unchanged, i.e., the changes do not involve mutations. The observation that the rate of loss of ability to accumulate anthocyanin from cells is different in different culture media is also not compatible with mutations as a mechanism of loss of yield.

The characteristics of the abilities of the cells in these carrot cultures to accumulate anthocyanin are not unique. There are striking parallels in the study of Alt et al. (1978) with methotrexate-resistant murine cells, of Devonshire and Sawicki (1979) with insecticide-resistant varieties of the aphid *Myzus persicae*, and of Strauss and Hoffman (1975) with *Salmonella typhimurium*. In these cases, gene amplification has been shown or proposed to be the mechanism of change. Heritable changes which occur at high frequency and are reversible, occur in albumin production by rat hepatoma cells (Peterson 1979), in cytokinin requirement by tobacco cells (Meins and Binns 1977, 1979) and in polypeptides produced by cytokinin-independent tobacco cells (Carlson 1979). Thus, there are a number of reports which show that cells from plants and animals as well as microorganisms can undergo changes which lead to heritable and reversibly altered cellular phenotypes. Mechanisms by which such changes can occur include gene amplification and changes in the rate of transcription and/or translation of genes and messengers. Knowledge of the mechanism by which the heritable changes in cellular phenotype occur should lead us to an understanding of the variation observed in cell cultures. Understanding the mechanisms may also allow control of these mechanisms and lead to methods to prevent decreases of the yields of secondary products in plant cell cultures.

This work was supported in part by the W. Alton Jones Foundation.

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Received 31 December 1979; accepted 16 May 1980

Note added in proof. All clones in W.C. Imp. gave extracted dry wt. of approximately 2 mg/ml after 14 days. Expressing anthocyanin produced by clones on a dry wt. basis, therefore, gives the same distributions as are shown here and does not alter the interpretations.