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Selection of a High and Stable Pigment-producing Strain in Cultured *Euphorbia millii* Cells

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Summary. A high pigment-producing strain of cultured *Euphorbia millii* cells was isolated by clonal selection. The pigment obtained was red and consisted mainly of anthocyanin. The amount of this pigment obtained after 24 selections was seven times that found in the original cells. Statistical and cell-pedigree analyses proved that this cell strain has stable productivity for this red pigment.

Key words: Clonal selection – *Euphorbia millii* – Anthocyanin – Cell-pedigree – Cultured cells

Introduction

Usually plant cell cultures produce only small amounts of secondary metabolites. Recently, however, cell strains containing amounts of secondary metabolites greater than those found in intact plants have been isolated by clonal selection. These are high vitamin B_6-producing strains (Yamada and Watanabe 1980), high alkaloid-producing strains (Zenk et al. 1977; Ogino et al. 1978) and high ubiquinone 10-producing strains (Matsumoto et al. 1980).

There are two questions in respect to clonal selection: 1) How can we determine whether the high productivity for secondary metabolites in selected cell strains is stable? 2) How long must we continue clonal selection in order to obtain stable cell strains that produce secondary metabolites?

We continuously selected cell strains that showed high contents of a red pigment, an anthocyanin from cultured *Euphorbia millii* cells (Yamamoto et al. 1981), and studied the stability of their productivity for this pigment with statistical and cell-pedigree analyses.

Materials and Methods

Selection and Culture

The outline of the selection method, cell-aggregate cloning, is shown in Fig. 1. Original *Euphorbia millii* calluses which produced a red pigment, cyanidin monoglucoside, were induced from leaves. The color of the induced calluses was mottled red and white. These calluses were cut into 128 segments (cell mass, ca. 3 mm) with a scalpel. Each segment was coded and placed on agar-medium (25 ml) in a sectioned Petri dish 9 cm in diameter. The agar-medium consisted of Murashige and Skoog's basal solution (Murashige and Skoog 1962), 10^{-6} M 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2% (w/v) malt extract, 2% (w/v) sucrose and 0.8% (w/v) agar. The segments were cultured at 28 °C under fluorescent light of 6000 lux for

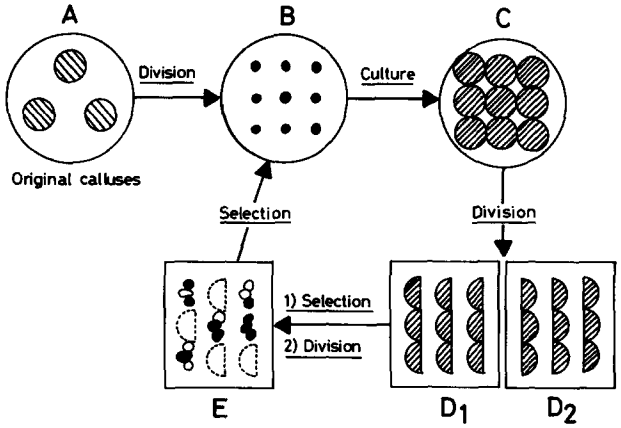

Fig. 1. Outline of the selection method. *A* The original *E. millii* calluses were divided into 128 segments, and a segment was placed on agar-medium in one section of a 9-section Petri dish and coded. *B* Segments were cultured at 28 °C under light (6000 lux) for 10 days. *C* Each grown segment was then divided into two cell-aggregates; one (D_1) for subculture and the other (D_2) for quantitative analysis of the pigment. D_1 The reddest of the nine aggregates were removed and placed in an empty Petri dish (*E*). *E* Each of these red aggregates was divided into several segments, of which the reddest pieces were removed, then coded and placed on agar-medium (*B*). ○ = unselected segment; ● = selected segment; ◌ = unselected aggregate; ⊘ = grown aggregate

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10 days. Each of the 9 segments on a Petri dish was cut into two cell-aggregates; one (D_1) for subculture and the other (D_2) for quantitative analysis of the pigment. From the analysis of D_2, we selected the reddest D_1 cell-aggregate from each Petri dish. These selected cell-aggregates were cut into several segments (cell mass, ca. 3 mm). All these segments were coded and transplanted onto fresh medium in a 9-section Petri dish. The code of each segment was put into the data file of a computer together with the code of the mother cell-aggregate. Segments selected at each transplantation were cultured on the same medium under the above conditions. This selection procedure was repeated 28 times.

Statistical Analysis (Determination of Pigment Contents: the Mean and Maximum Values)

A random sample of 30 from the D_2 population was prepared for quantitative analysis of the red pigment. Each segment of the sample was soaked in MeOH (4 ml) containing 2% HCl for 1 hr. The absorbance of the methanolic solution was measured at 530 nm, which was the λ_{max} of this pigment, then the pigment content (absorbance at 530 nm/ fresh weight (mg) of the cell-aggregate) was calculated. A frequency distribution of cell-aggregates with various pigment contents was made (class-width was fixed at 0.5). Two characteristic terms; the mean ($\bar{C}$) and maximum (C^{max}) values were calculated.

Cell-pedigree Analysis (Determinations of the Distribution Rate, the Frequency Distribution and the Mean Value of the Red Pigment in the Specific Cell Line)

All codes for the 1588 cell-aggregates from the 8th to 29th passages were put into the data file. The pedigree of the cell-aggregate was graphed, and a cell line (a group of cell-aggregates derived from one ancestor) was tabled by the computer. The distribution rate (%) [(the number of cell-aggregates of a specific cell line/ the number of total cell-aggregates) × 100] for each line was calculated for each generation. A frequency distribution then was made, and its mean value was calculated. This is marked in the histogram of the total cell-aggregates.

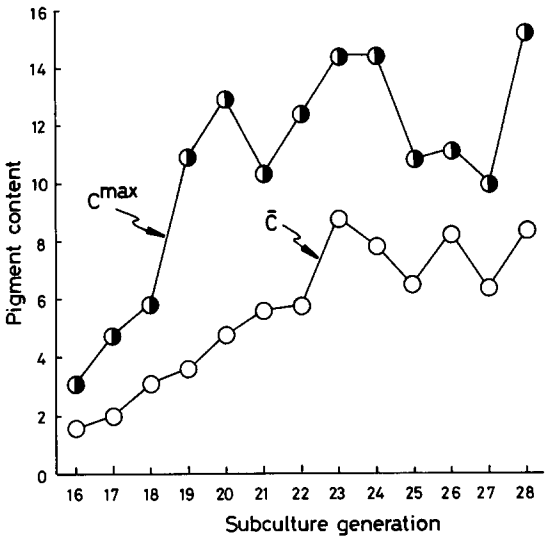

Fig. 2. Trends of the two characteristic terms; the mean value ($\bar{C}$) and maximum value (C^{max}) in the frequency distribution of cell-aggregates with various pigment contents from the 16th to 28th passages. Cell-aggregates were subcultured on Murashige and Skoog's agar-medium with 2,4-D (10^{-6} M), malt extract (0.2% w/v) and sucrose (2% w/v) at 28 °C under light (6000 lux) every 10 days

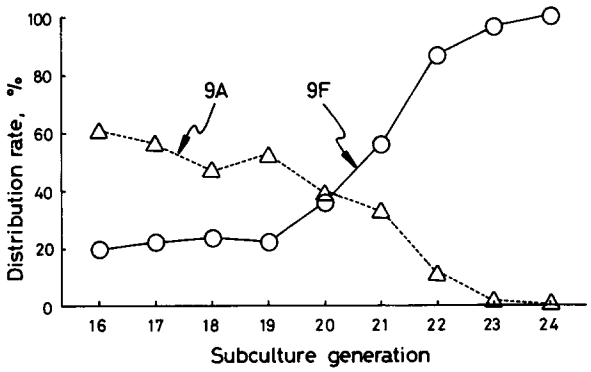

Fig. 3. Distribution rates of the 9A (△) and 9F (○) cell lines in the population of cell-aggregates from the 16th to 22nd passages. The 9A and 9F cell lines were derived from the 9A and 9F cell-aggregates of the 9th subculture. Distribution rate = (the number of cell-aggregates of a specific cell line/ the number of total cell-aggregates) × 100

Results

The reddest cell-aggregates were selected continuously from each of the 28 subcultures of the original mottled-red, *Euphorbia millii* callus. Two characteristic terms ($\bar{C}$ and C^{max}) in the frequency distribution of cell-aggregates from the 16th to 28th passages were plotted against the pigment content in Fig. 2.

The mean value ($\bar{C}$) for the pigment content increased nearly threefold from the 16th to 22nd passages. It came within the limit of 7.46 ± 0.56 after the 23rd passage, which was seven times higher than the pigment content (1.05) of the original callus. The maximum value (C^{max}) increased nearly threefold from the 16th to 19th passages, then was within the limit of 12.96 ± 2.36 after the 20th passage.

The 9A and 9F cell lines originated from the 9A and 9F cell-aggregates of the 9th subculture. In this subculture, the 9A and 9F lines produced pigment-rich descendants at high frequencies. The distribution rates of these two cell lines in the population of cell-aggregates from the 16th to 24th passages is shown in Fig. 3.

The distribution rate of the 9F cell line was lower than that of the 9A cell line at the 16th subculture, but equalled it at the 20th subculture. This rate gradually increased and reached 100% at the 24th subculture. The trends of two mean values ($\bar{C}_A$ and $\bar{C}_F$) for the pigment contents in cell-aggregates of the 9A and 9F cell lines

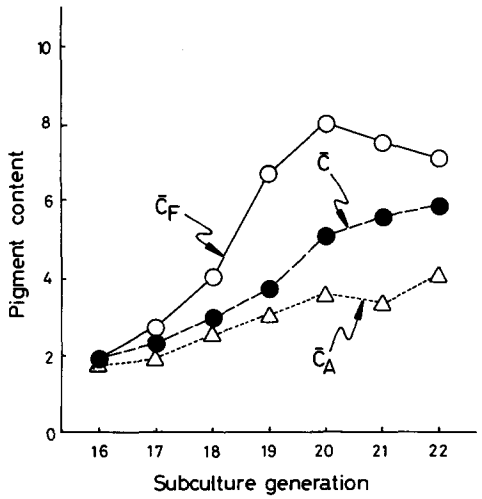

Fig. 4. Trends of the three mean values ($\bar{C}$, $\bar{C}_A$ and $\bar{C}_F$) for the pigment contents in cell-aggregates from the 16th to 22nd passages. ● = mean value ($\bar{C}$) in total cell-aggregates; △ = mean value ($\bar{C}_A$) in the 9A cell line; ○ = mean value ($\bar{C}_F$) in the 9F cell line

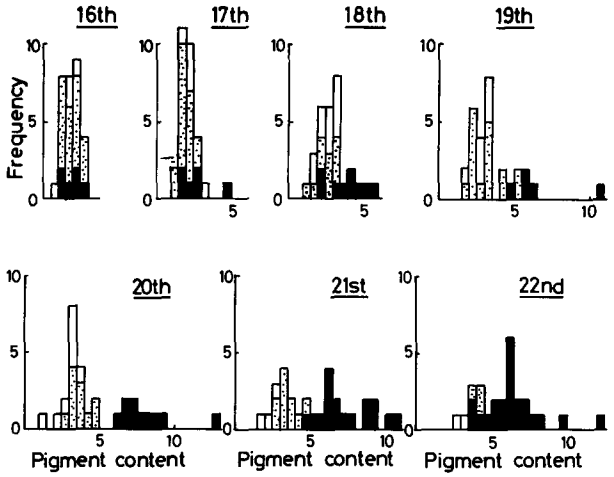

Fig. 5. Frequency distributions of cell-aggregates of the 9A (▨) and 9F (■) cell lines in histograms of the population of cell-aggregates with various pigment contents from the 16th to 22nd passages. The class-width of the frequency distribution was fixed at 0.5. □ = cell-aggregates other than the 9A and 9F cell lines

are shown in Fig. 4. The mean value ($\bar{C}_F$) was about twice the value ($\bar{C}_A$) from the 19th to 21st passages; $\bar{C}_F$ was 8.02 and $\bar{C}_A$ was 3.67 at the 20th subculture. Figure 5 shows the histogram for the frequency distributions of cell-aggregates from the 16th to 22nd passages.

Discussion

We found that the mean value ($\bar{C}$) for the pigment content in cell-aggregates of *Euphorbia millii* became stable after 24 clonal selections (Fig. 2). In addition, the distribution rate for cell-aggregates of the 9F cell line reached 100% in the population after the 24th passage (Fig. 3). All the aggregates after the 24th passage were derived from the 9F cell-aggregate. If the mean value for the content of secondary metabolites is stable in a population of cell-aggregates chosen by successive clonal selection, and if all the cell-aggregates are derived from one cell-aggregate, they should consist of cells with high and stable productivity for secondary metabolites. These results show that we could isolate and culture a cell strain containing a high and stable pigment content from *Euphorbia millii* callus after 24 successive clonal selections.

We also showed that the mean value ($\bar{C}_F$) in the 9F cell line was higher than the value ($\bar{C}_A$) in the 9A cell line from the 17th to 22nd passages (Fig. 4), and that the 9F cell line was comprised of the group of cell-aggregates that showed high pigment contents in the histograms after the 18th passage (Fig. 5). We believe that during early passages cultured cells are heterogeneous in their production of pigments and that they contain high pigment-producing variant cells. Results show that we could select more cell-aggregates of the 9F cell line than of the 9A cell line at each subculture (Fig. 4), and that the 9F cell-aggregate had high pigment-producing variant cells.

In preliminary experiments, we failed to obtain a high pigment-producing strain of cultured *E. millii* cells by single cell cloning because culture of a single cell was too difficult. The cell-aggregate cloning used in this study is superior to single cell cloning.

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