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Clonal variability and light induction of betalain synthesis in red beet cell cultures

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ABSTRACT

When calli from a green habituated cell culture of red beet (Beta vulgaris L.) are transferred from dim-light to high-light intensity, red colored spots appear on the surface. Not all cells express the pigmented phenotype, giving rise to variegated patches. The pattern of patch formation is different from one callus to another, although all calluses stem from the same original clone. This clonal variability is an intrinsic property of the tissue and is not affected by light which is only needed for the induction of pigment synthesis, and therefore acts as a revealing factor.

INTRODUCTION

Spontaneous changes in the characteristics of plant cell culture are known to occur from time to time. A number of physiological and morphological changes have been reported in tissue culture including loss of exogenous growth factor requirement (MEINS and BINNS, 1977), variation in alkaloid content in Catharanthus roseus (ZENK et al., 1977), anthocyanin content in corn endosperm (STRAUS, 1958) and carrot cell cultures (DOUGALL et al., 1980), variability in organogenesis (SKIRVIN, 1978).

Mechanisms by which such phenotypic changes occur are not known. The fact that cells with the same genotype can inherit different characters; even if cells from an individual organism are thought to be genetically equivalent suggest the presence of stable phenotypic changes (positive feedback), mutable genes or transposable elements. Host of these changes have an epigenetic origin as the genome is not permanently altered. They are limited by the genetic potential of the cells, stable, transmitted to the daughter cells when they divide and regularly reversible (MEINS, 1983).

This report deals with a change in the competence for light-induced betalains accumulation of a non-pigmented line of red beet tissue. The study of the variability in cells from a non-colored phenotype mould show whether or not the changes are reversible.

Red beet tissue cultures require light for pigment biosynthesis. The ability of cells to accumulate pigments is not stable and not all cells in a culture do accumulate alkaloids.

MATERIAL AND METHODS

The green stable strain used in this study was derived by subculturing non-betalain containing portions of a culture established in our laboratory from a cotyledon explant of red table beet seedling (Beta vulgaris var. Bikores monogerm).

The calli were maintained under dim-light condition (6 W/m2, Gro-Lux wide Spectrum, Sylvania), 16 h light/8 h dark, and subcultured every three weeks on a modified LS medium (LINSMAIER and SKOOG, 1965) containing half strength salts, NH_4NO_3 being replaced by KNO_3}, 10 g/1 sucrose, 5 g/1 mannitol and 1,5 mg/1 thiamine-HCl, adjusted at pH 5.8 prior to autoclaving.

Pieces of callus (about 15 mg) were incubated for 6 days in the same culture condition in 65 mm x 40 mm petri dishes containing about 20 ml of medium. After this time, each piece was fractionated into two subcalli and randomly distributed in groups of 20 to 40 calli into new dishes (time 0), then incubated under normal culture condition for different periods of time. At the end of each period, groups were induced for betalains synthesis by transferring them to high-light intensity (18 W/m2). Fresh weight (W) and pigmented spots (isolated cells and/or groups of cells) were scored every 3 days starting from time 0.

To measure the number of cells per mg of fresh weight, 20 mg of tissue were incubated for 40 min in 10% CrO_3 at 60°C, then dispersed with a Baird & Taitlock shaker for 10 min and the resultant suspension counted in an haemocytometer.

Plotted fresh weight (w^1) values against time fit an exponential. To express individual growth of each callus, we calculated generation number from time 0 according to the following relation:

(1) $GN=\ln(W/W_0)/\ln 2$

As only the surface of the calli could be investigated, an "equivalent surface weight" [S(W)], based on a half sphere model was calculated according to the following relations:

$W=2\pi R^3/3$

Therefore $R=(3W/2\pi)^{1/3}$

as $S=2\pi R^2$

then (2) $S(W)=2\pi(3W/2\pi)^{2/3}$

The variegation intensity (VI) was obtained by dividing the number of pigmented spots by the corresponding S(W).

The variation frequency (VF) was determined as the probability of a cell to express the pigmented phenotype per cell generation and was calculated from the slope vr (variation rate) of the following regression:

(3) $VI=VI_0+vrGN$

where VI_0 is the intersect of the straight-line with the VI axis.

Then (4) $VF\ (\%)=vr/2.72$

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where 2.72 represented the number of cells per equivalent surface weight unit [S(W)] divided per 100, assuming that 1 mg fresh weight contained an average of 4'500 cells.

RESULTS

1. EFFECT OF EXPLANT AGE

If variation occurs randomly in a tissue, a large callus will show more colored spots than a small one. The relation between size and number of spots is expected to be linear. When a dim-light to high-light transition occurs after different periods of incubation (0, 6, 9, 12 and 15 days resp.), the appearance of pigmented spots is more rapid in calli that have been at least preincubated for two generations in dim light (Fig.1). Only the 3 first values, used to calculate the initial rate of variation, are represented.

The number of spots is a guasi-linear function of tissue age. The slope vr which provides the variation frequency (VF) per cell generation reflects the random occurrence of the spots over the surface of the tissue. When the variation frequency (calculated from the slope vr) is plotted against generation number (Fig.2), we observe that calli must be above a threshold generation number of 1.8 to show a constant variation frequency independent of the duration of

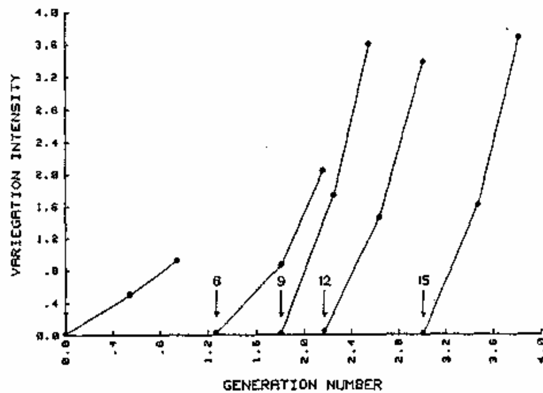

Fig.1

Evolution of number of spots per surface unit (VI) relatively to the age after transfer to high-light (arrows) following different times of incubation in dim-light (resp. 0, 6, 9, 12, and 15 days).

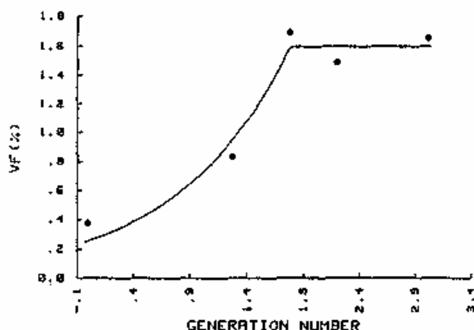

Fig. 2

Relationship between variation frequency (VF), calculated from initial slope (vr) from Fig.1, and age (GN) of calli at the time at which they were transfered to high-light intensity [fitting to Richards function (RICHARDS, 1959)].

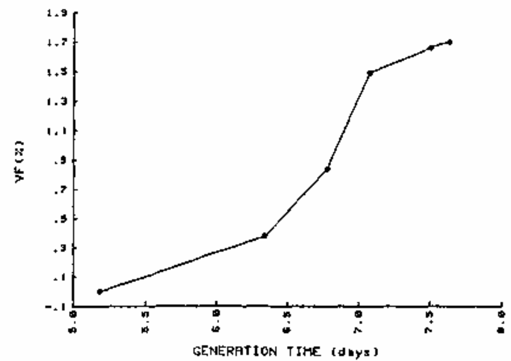

Fig. 3

Relationship between variation frequency (VF) and growth-rate (generation time)

pre-incubation in dim light. Below this threshold, the plot shows an increase in the competence of cells with age.

The variation frequency is related to the generation time (time/GN), as shown in Fig.3. Small calli just after transfer grow faster (VF = 0%, generation time = 6,3 d) than larger and older ones (VF = 1,7%, generation time = 7,5 d). The development of the colored phenotype is accompanied by a decrease of the growth rate. The generation time of 5.2 d for calli maintained in dim light reaches a value of 7 d and more for calli transferred to high-light intensity, indicating that complex physiological changes have occurred.

2. EFFECT OF LIGHT

Light plays a major role in the biosynthesis of betalains in red beet cultures as shown by the fact that the variegation intensity (VI) decreases when the variegated calli were transferred back to dim light (Fig.4).

However, the decrease of VI occurs more rapidly than it could be expected from a mere dilution of the spots over the surface during callus growth (Table 1). If we agree that no more pigments were synthesized after transfer to dim-light, diminution of VI is linear when plotted against GN, VI being equal to the variegation intensity of the calli at the time of dim-light transition divided by S(W). The fact that the measured slope is steeper than the calculated slope (dilution only) point toward an active degradation of the betalain-type pigments. In this case, the absolute variation frequency of disappearance is about half the value observed for appearance under high-light culture condition. As it has been shown previously (Fig.3), doubling time increased from 4.8 to 6.9 days (Table 1). This

	VF (%)	GN (d)
1° High light	0.317+0.176	4.8
2° Dim Light	-0.379+0.149	6.9
3° Dilution effect hypothesis	-0.226+0.046	6.9
2°-3° Absolute disapp. freq.	-0.153+0.090	6.9

Table 1

Variation frequency (VF) and growth-rate (GN) before (1°) and after (2°) high-/dim-light transition.

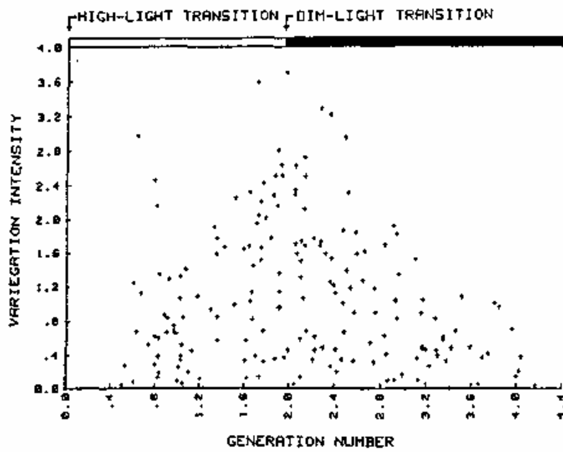

Fig. 4
The effect of high-light followed by a dim light irradiation on the number of pigmented spots covering the surface of the calli expressed as a function of the number of generation.

reduction of the growth-rate reflects the modification of the cell physiology following the light treatment. These changes persist in calli well after removal of the inducer.

3. CLONAL-VARIABILITY

From experiment 1, we selected calli expressing the highest and lowest variation frequencies. They were incubated for an additional period of one month in dim light. After this time, they were non pigmented and no obvious difference could be seen between them. Then, they were cut in pieces as described in Material and Methods but using only the 12 days preincubation period for the estimation of VF. One hundred subclones were divided in ten equal size classes (subclone number) according to VF and the mean value for each class was calculated.

Cell sensitivity for the trigger factor (high light intensity) is highly variable from subclone to subclone. As shown in Fig.5, recloning of calli with low variation frequencies yields some subclones, which have even lower variability. A few subclones were also obtained, which variability was greater. A reciprocal situation is shown in Fig.6. Here, calli with the highest variation frequencies produce also some subclones which have decreasing ability.

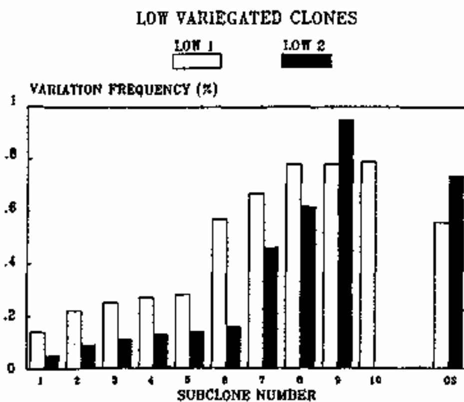

Fig.5
Distribution of the variation frequencies for subclones of two low variegated calli. Parental values are indicated by OS bars.

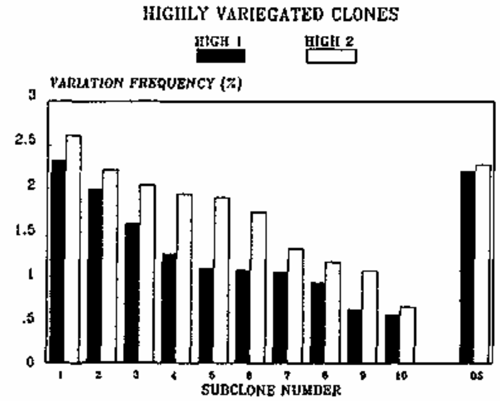

Fig.6
Distribution of the variation frequencies for subclones of 2 high variegated calli. Parental values are represented by the OS bars.

Changes in variability occur in both directions, reflecting the intrinsic heterogeneity of the clones. However, variation among the subclones population is restricted by the parental properties. For example, only a few subclones from a highly-variegated parental clone has a variability in the range of low-variegated clones (below 1%). Variation appears to be a tissue specific heritable property.

DISCUSSION

The colored spots arising over the surface of a callus are the expression of two sets of responses.

First, light induction is a prerequisite for pigment biosynthesis perhaps through the control of precursor formation (light sensitive enzymatic pathway). It has been shown that betalains biosynthesis is stimulated by light in seedlings of Amaranthus tricolor and Celosia plumosa (G. DE NICOLA et al., 1972, 1974) and recently, (BERLIN et al. 1986) reported a light requirement for betacyanin biosynthesis in Chenopodium rubrum cells suspension. The nature of the precursor could well be L-dihydroxy-phenylalanine (L-DOPA) whose production is strongly stimulated by light in Mucuna pruriens cells culture (HUIZING and WICHERS, 1985). Callus culture of the related species Stizolobium hassjoo does not accumulate L-DOPA, but instead catabolizes this metabolite into stizolobic acid (SAITO et al., 1982). This non-protein amino acid arises by extradiol ring cleavage of L-DOPA. Such ring-cleavage is also the key step in the betalains biosynthesis.

Second, the ability of cells to accumulate pigments is conditioned by the competence of cell to respond to the light signal, competence which reaches a maximum 1.8 generations after the cells transfer. The incidence of variation (Fig.2) is then constant in a determined cell population propagated under defined culture conditions and occurs at a rate of $1.6 + 0.2 \times 10^{-2}$ events per cell generation (probably an underestimate). These values are 1'000 to 10'000 times higher than somatic mutations rates. The lower frequencies observed for the two first generations would depend on the physiological state of the cells at the time of transfer to high-light intensity. An effect of the same magnitude has been reported by MEINS et al. (1980) for the cytokinin habituation of pith tissue of Nicotiana tabacum.

Our results show that variation is the combined effect of two sequential events which are (1) acquisition of the competence and (2) expression of the specified phenotype, variation occurring at a constant frequency per cell generation. The lower frequencies observed from the first two cell generations indicate

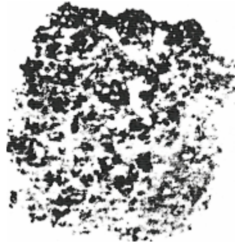

Fig.7

Variegation of the surface of a callus developped after 6 days culture under high light intensity.

that the development of the colored phenotype is conditioned by external influences others than light. Thus, only a competent cell (this competence being the result of a sequence of unknown events) would undergo a differentiation process. As differentiation is somehow delayed, cell division will give rise to a group of competent cells and we should observe patches of red differentiated cells. Cell differentiation processes could be controlled both by inherited factors and by extrinsic metabolites picked up from the neighboring cells and from the medium. The nature of these inducing factors is still unknown, but they will be needed for the acquisition of competence. In a certain sense, the existence of patches of competent cells would be a prerequisite for the initiation of differentiation (RIDEAU, 1985), as we observed that, after two cell generations, most spots were composed of more cells than expected by a simple clonal development (Fig.7).

The sequence of events can be schematically represented as in Fig.8, where tissue specific transdetermination is defined as a switch from one heritable state to another (KAUFFMAN, 1973). Thus, calli are typically mosaics of determined and differentiated (colored and not colored cells), transdetermination occurring continuously in a specific orientation and at a constant rate in the cell population. Unfortunately, we can not measure reverse frequency in our system, but we can expect it to occur with a lower frequency.

The physiological changes associated with the development of the pigmentation result in a decrease of the growth rate (Fig.3), reflecting that differentiation processes have occurred. Pigment synthesis is not restricted to the culture stationary growth phase, as it is the case for many secondary metabolites (BARZ and KOESTER, 1981), since differentiation is observed at all ages (Fig.1).

Experiment 3 shows that the cloning procedure itself results in an increase of the variability (MURPHY, 1981). This variability behave in some way like an heritable property. The question whether this variability involves a genetic element will remain open due to, the lack of knowledge of the genetic background of betalains synthesis. However, as secondary metabolism is restricted to certain developmental stage in the whole plant, variability exhibited by plant cells in culture could be a reminiscence of control circuits possessing stationary states. Homeotic genes could be involved in such regulation sequences.

It is encouraging to notice that in our case, as in other well studied cases, it can be shown that clonal selection allowed the isolation of high producing cultures [see for ex. anthocyanin synthesis in Euphorbia millii (YAMAMOTO and MIZUGUCHI, 1982)] which proved in fact heterogeneous and could be maintained by continuous selection (see MESTRE and PETIARD, 1985).

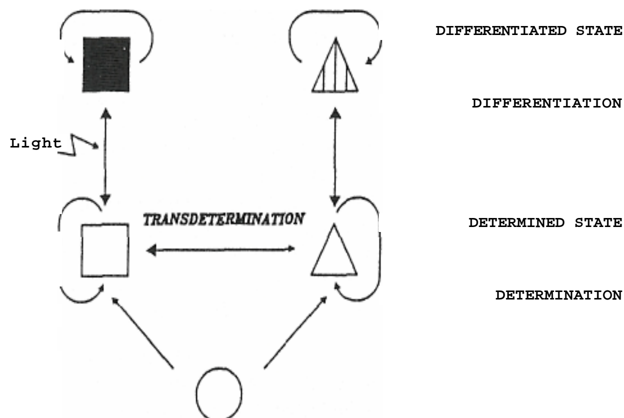

Fig. 8

Schematic representation of an hypothetical mechanism for the differentiation of non pigmented cells into colored cells. The determined state is a persistently regenerated, self-maintained state.

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