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Pseudodirected variation in the requirement of cultured plant cells for cell-division factors

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SUMMARY

Cells cultured from explants of tobacco leaf require exogenous cell-division factors such as the cytokinin kinetin for sustained proliferation. When cytokinin-requiring (C^-) cells are cultured on medium containing 1/100 the optimum cytokinin concentration they rapidly give rise to cytokinin-autotrophic (C^+) variants. Some of these variants result from a meiotically transmitted change at the *Habituated leaf-2* locus. We measured the rate of phenotypic variation by a simple, quantitative method and found that cultured tobacco cells alternate between the C^- and C^+ states at extremely high rates of approx. 10^{-2} per cell generation, which is 10^2- to 10^3-fold more rapid than most somatic mutations in tobacco. These changes are so rapid that the classical distinction between random and induced events is blurred. Selection of alternate phenotypes arising by rapid, reversible cellular variation results in changes that appear to be directed at the tissue level. This phenomenon, called *pseudodirected* variation, is of particular interest because it suggests novel stochastic mechanisms of cytokinin action and a plausible explanation for the directed, but plastic nature of development in plants.

Key words: cell proliferation models, cell division factors, cytokinins, epigenetic selection, growth factors, phenovariation, tobacco

INTRODUCTION

The formation of organs in plants is an orderly process. Cells or groups of cells become determined to form predictable structures. Nevertheless, the morphogenetic pathways leading to these structures are remarkably plastic: the size and shape of organs is strongly influenced by environmental factors and plants have a pronounced capacity for regeneration (Sachs, 1988). This plasticity is also apparent in cultures established from plant tissues. Cells cultured in vitro often show high rates of variation in capacity for differentiation and morphogenesis (Meins, 1983). In principle, this variation could arise *preadaptively* by random cell-heritable changes followed by environmental selection for the variant phenotype or *postadaptively* by directed cell-heritable changes in response to specific environmental signals (Hayes, 1968). To assess the contribution of pre- and postadaptive processes, we measured variation of a specific character, requirement for the cell-division factor cytokinin, which alters the selective advantage of cells cultured in media containing different concentrations of the growth factor. This character is of particular interest because cell heritable changes in cytokinin requirement occur during normal plant development and the shift from a cytokinin requiring (C^-) to a cytokinin autotrophic (C^+) phenotype is a key event in neoplastic transformation (Meins and Wenzler, 1986; Braun, 1978).

Cultures established from different parts of the tobacco plant differ in their requirement for the cytokinin kinetin, e.g. tissues of leaf origin are C^- whereas tissues from the cortex of the stem are C^+ (Meins and Foster, 1986a). These phenotypes persist when the cells are cloned, indicating that the tissue-specific C^- and C^+ states can be inherited at the cell level. By altering the hormonal constitution of the culture medium, complete plants can be regenerated from cloned lines of both cell types. Regardless of the cytokinin phenotype of the clones, tissues cultured from the regenerated plants exhibit the cytokinin requirement of comparable tissues from seed-grown plants. This observation and the fact that the changes in phenotype occur at rates 10^2 to 10^3 faster than well-characterized, spontaneous gametic mutations provide compelling evidence that tissue specific states of cytokinin requirement result from epigenetic changes (Meins and Binns, 1982).

An important exception is C^+ variants obtained by subculturing cloned C^- leaf cells on medium containing reduced concentrations of cytokinin. Leaf tissues of plants regenerated from these variants exhibit a C^+ phenotype in culture. This new phenotype, which is regulated at the *Habituated leaf-2* (*Hl-2*) locus, is inherited meiotically as a dominant trait (Meins and Foster, 1986b). The high incidence of these genetic variants suggested that they might arise postadaptively as a result of the low-cytokinin treatment. Here we report the use of indirect selection and kinetic analyses of cell populations to test this hypothesis. The results lead to the unexpected conclusion that cultured leaf cells alternate between the C^+ and C^- states at extremely high rates. Lowering the cytokinin concentration in the culture medium results in changes that appear to be directed at the tissue level, but reflect selection for C^+ cells. Pseudodirected variation of this type suggests novel stochastic mecha-

nisms of cytokinin action and provides a plausible explanation for the directed, but plastic nature of development in plants.

MATERIALS AND METHODS

Plant tissues and tissue culture

Leaf and pith parenchyma tissues were isolated from *Nicotiana tabacum* L. cv. Havana 425 plants grown from seed in a greenhouse. Methods for starting and maintaining tissue cultures, cloning cells, and regenerating plants were as described (Meins and Binns, 1977; Meins and Lutz, 1979, 1980). In brief, tissue lines were subcultured at 3-week intervals starting with a 10 mg inoculum of approx. 1.4×10^3 cells. C^+ tissues were cultured on a basal medium containing the salts, thiamine, *myo*-inositol, and sucrose concentrations of Linsmaier and Skoog (Linsmaier and Skoog, 1965), solidified with 1% (w/v) purified agar and supplemented with 5 mg/l of the pH indicator chlorophenol red (Eastman) and 2 mg/l of the auxin α-naphthalene acetic acid. C^- tissues were cultured on kinetin medium, the basal medium supplemented with 0.3 mg/l of the cytokinin kinetin.

Measurement of cytokinin requirement

The cytokinin requirement of tissues was measured by subculturing 25-50 explants twice on basal medium and scoring the fraction of growing colonies. Alternatively, the relative growth rate (R) was measured. Two sets of explants were subcultured twice, one set on kinetin medium, the other set on basal medium. R was calculated from the expression

$$\ln(W/W_o)_{-\text{kinetin}}/\ln(W/W_o)_{+\text{kinetin}}$$

where W_o and W are the fresh weights of the inoculum and the tissue after 3 weeks, respectively. Under these conditions, C^- pith tissues exhibit R values < 0.4 and C^+ tissues exhibit R values > 0.4 (Meins and Binns, 1977). Sampling error in distributions of C^- and C^+ clones was estimated by the binomial proportions test (Simpson et al., 1960).

RESULTS

Indirect selection

Earlier we showed that a high incidence of C^+ variants could be obtained by serially culturing cloned C^- leaf tissues on medium containing suboptimal concentrations of kinetin (Meins and Foster, 1986b). Indirect selection, i.e. subjecting siblings of the cells being tested to selection, was used to distinguish between pre- and postadaptive mechanisms for this variation. We started 50 lines of cells from the same cloned C^- line, L253N, maintained on standard, kinetin-containing medium. After 3 weeks incubation, explants from each line were transferred for the same interval of time onto standard medium and onto K/100 medium containing 1% of the standard kinetin concentration to give sets of control and treated cultures, respectively. After incubation, the set of control cultures was subcultured again on standard medium. The set of treated cultures was assayed for the C^+ phenotype by subculturing on basal, kinetin-free medium and then weighing. The control culture corresponding to the treated culture giving the highest growth rate on basal medium was designed the ‘fast’ line and was used for the next cycle of indirect selection.

In a similar way, 2 to 3 control lines corresponding to treated lines giving low growth rates on basal medium were designated ‘slow’ lines and were used for the next cycle of selection. This scheme was repeated 9 times over a period of 54 weeks. Each

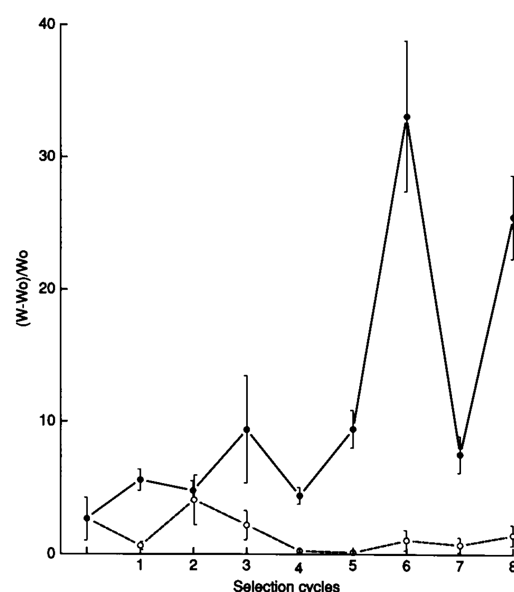

Fig. 1. Indirect selection with cloned C^- leaf tissue subcultured on K/100 medium. Growth of tissues from the ‘fast’ line (●) and ‘slow’ line (○) on basal medium. Error bars: ± s.e.m. for 10-25 samples.

time, untreated cultures of the fast and slow lines identified in the previous transfer were selected for fast and slow growth on basal medium, respectively.

If low-kinetin treatment induces variation, then tissues from the fast and slow lines should continue to grow at the same low rate on basal medium because these cells have never been subjected to the inductive treatment. On the other hand, if C^+ cells arise spontaneously and have a selective advantage on the low-kinetin medium, then cells in the slow line should remain slow growing and cells in the fast line should grow more rapidly on basal medium.

The results in Fig. 1 show that the growth rate of the slow line on basal medium declined with the number of selection cycles. The growth rate of the fast line fluctuated widely, but increased markedly with the number of selection cycles. Therefore, using growth on basal medium as a criterion, the C^+ variants arise preadaptively and do not result from inductive effects of low-kinetin treatment.

Indirect selection was also performed omitting the low-kinetin treatment and using a more stringent criterion for the C^+ phenotype. Selection was made on the basis of R value, the relative growth rate of tissues on media with and without kinetin. Under these conditions C^+ tissues capable of sustained growth on basal medium exhibit R values greater than 0.4 (Meins and Binns, 1977). The results in Fig. 2 show that lines selected for low R value remained C^- with increasing number of selection cycles. In contrast, lines selected for high R value gradually increased in R value into the range typical of cloned, C^+ cells. These results show that C^+ variants can also arise spontaneously when cells are cultured on standard, kinetin-containing medium.

Interconversion of C^- and C^+ cells in equilibrium populations

The quantitative model used to estimate the rates at which cells shift between the C^- and C^+ states is outlined in the Appendix.

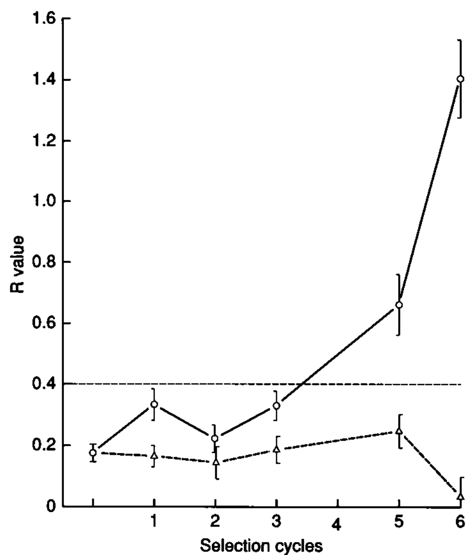

Fig. 2. Indirect selection with cloned C^- leaf tissue subcultured on standard kinetin-containing medium. The *R* values of tissues cultured from lines selected for high *R* value (○) and for low *R* value (△). Error bars: ± s.e.m. for 10-25 samples.

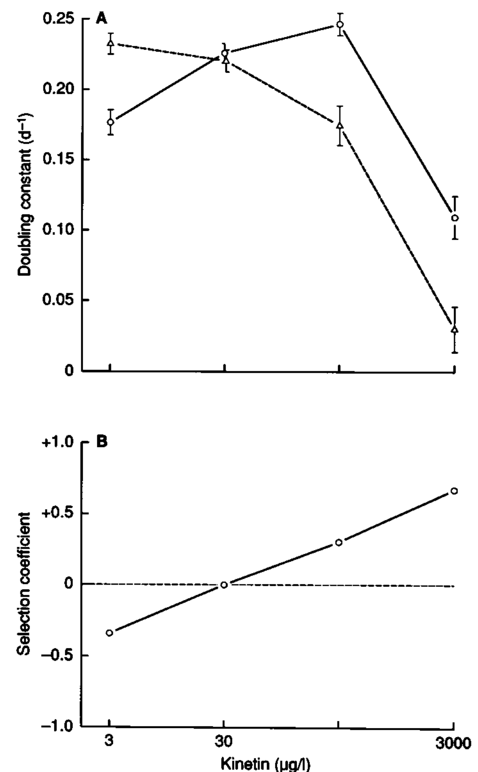

Fig. 3. Tissue doubling constants (A) and the selection coefficient (B) of cloned C^- and C^+ leaf tissue grown on media containing different concentrations of kinetin. Doubling constants were measured on a fresh-weight basis after 3 weeks incubation. Mean values for 7 C^- clones (○) and 5 C^+ clones (△). Selection coefficients (*S*) were calculated from the mean doubling constants of the C^- and C^+ lines, k_A and k_B respectively, from the expression S=1–(k_B/k_A). Error bars: ± s.e.m.

In brief, the method takes advantage of the fact that the frequency of C^+ cells in the population approaches a constant, equilibrium value when the rates of cell proliferation and rates of variation are balanced. Once equilibrium is reached, the rates of variation can be estimated from experimentally determined equilibrium frequencies and the selection coefficient.

Selection coefficients were estimated from the average doubling constants of 7 C^- leaf clones and 5 clones of C^+ variants grown on different concentrations of kinetin. The kinetin dose responses for growth and calculated selection coefficients are shown in Fig. 3. The selection coefficient was proportional to the logarithm of kinetin concentration over 4 orders of magnitude. As predicted by the preadaptation hypothesis, C^+ cells have a pronounced selective advantage over C^- cells, S=–0.35, when tissues are incubated on K/100 medium which gives a high frequency of C^+ variants.

The equilibrium frequencies of C^+ cells were measured in populations subjected to strong selection for and against the C^+ phenotype. Cloned lines of C^- and C^+ cells were serially subcultured for long periods of time on standard, kinetin-containing medium and on basal medium, respectively. The frequency of C^+ cells was measured at regular intervals by cell cloning. Cell lines in which the frequency of C^+ cells did not change beyond the limits expected by chance were judged to have reached equilibrium. This test was performed with the cloned C^- line L235N and a C^+ variant of L235N, L235H, obtained by culturing L235N for 6 transfers on K/100 medium and then cloning.

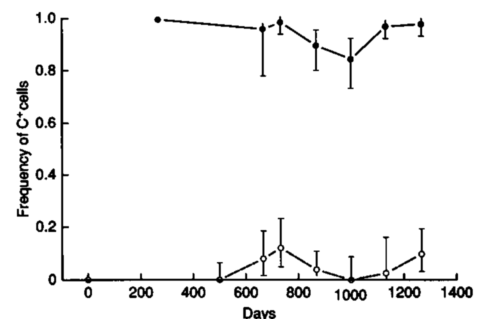

Fig. 4. The frequency of C^+ cells in serially propagated cloned lines of C^- and C^+ cells. Frequencies were estimated from the fraction of C^+ subclones obtained from line L235N cultured on standard kinetin-containing medium (○) and line L235H cultured on basal medium (●). Error bars: 95% confidence interval calculated for binomial proportions for 40-75 samples.

When cultured on standard medium, which selects strongly against C^+ cells, the first C^+ clones were recovered from the C^- line after approx. 650 days (Fig. 4). Thereafter the frequency of C^+ cells fluctuated within the sampling error (binomial proportions test, 95% level) indicating that equilibrium had been reached. The mean frequency was $6.1\pm1.9\times10^{-2}$ (± s.e.m.) for 6 measurements made over the period from 650 to 1260 days. Using the same criterion, the C^+ line maintained on basal medium, which selects strongly against C^- cells, also reached equilibrium. The mean frequency of C^+ cells was 0.944±0.023 (± s.e.m., 6 measurements). Similar frequencies were obtained with at least 11 different cloned lines of C^- and C^+ cells derived from pith and leaf tissues. The grand

averages for these lines including the results from Fig. 4 were $6.89\pm1.5\times10^{-2}$ for 13 C^- clones on kinetin medium and 0.926 ± 0.021 for 14 C^+ clones on basal medium.

These results confirm, by cell cloning, that cells in culture can shift reversibly between the C^- and C^+ states. The equilibrium frequency of C^+ cells ($\hat{q}$) depended on the conditions of culture, but not on the origin of the numerous independent clones tested. As predicted from our model, $\hat{q}$ was high when proliferation of C^+ cells was favored (i.e. basal medium, $S=-1$) and q was low when proliferation of C^- cells was favored (i.e. kinetin medium, $S=0.34$).

Estimating rates of variation

Rough estimates of the rates at which C^- cells convert to C^+ cells (μ) and C^+ cells convert to C^- cells (ν) were obtained in two ways. The first method takes advantage of the fact that $\hat{q}$ is essentially independent of ν when there is strong selection for C^- cells and essentially independent of μ when there is strong selection for C^+ cells (see Appendix). Under these conditions μ and ν can be estimated directly from $\hat{q}$ and the selection coefficient. The values calculated from average equilibrium frequencies of 13 C^- clones on kinetin medium and 14 C^+ clones on basal medium were $\mu \approx 2.1\times10^{-2}$ and $\nu \approx 2.8\times 10^{-2}$.

In the second method, rates were estimated by fitting the model (see equation 3, Appendix) to the q values obtained with a wide range of S values. The cloned C^- line L253N was serially transferred on media containing different kinetin concentrations. After 702 days, when it was likely that equilibrium had been reached, 50-75 clones were isolated from each line and scored for the C^+ phenotype. Fig. 5 shows the values of q obtained and the fit of the data to the model. The rates estimated, $\mu \approx 2.3\times10^{-2}$ and $\nu \approx 4.6\times10^{-2}$, were similar to those obtained with different cloned lines by the strong selection method. These results provide compelling evidence that cells alternate at high rates between the C^- and C^+ phenotypes in cultured leaf and pith tissues.

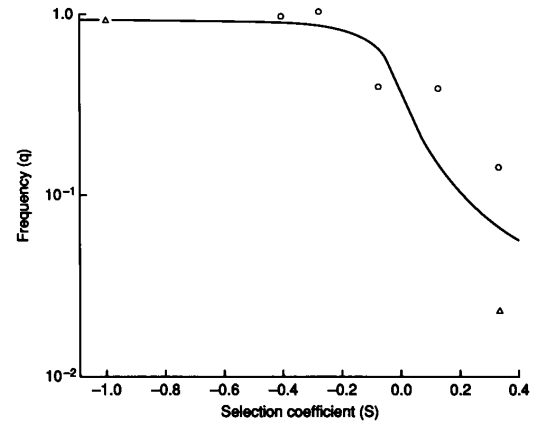

Fig. 5. The frequency of C^+ cells in L235N tissues as a function of selection coefficient. The fitted curve was obtained from the model (Appendix, equation 3) corresponding to $\mu=2.3\times10^{-2}$ and $\nu=4.6\times10^{-2}$.

DISCUSSION

It is commonly assumed that heritable cellular variation is either a rare, random event or the result of an inductive process. The major conclusion from our work is that cultured tobacco cells shift so rapidly in their requirement for cytokinins that the distinction between random and induced events is blurred. Rapid, reversible cellular variation followed by selection results in changes at the tissue level that appear to be directed. We call this phenomenon *pseudodirected* variation.

Very high frequencies of C^+ variants were recovered from C^- tobacco cells cultured on low-cytokinin medium. Indirect selection and cell cloning experiments showed that low-cytokinin treatment acts selectively rather than inductively to give C^+ variants. The rates of cellular variation cannot be measured directly because cell proliferation on different media is used as the test to distinguish phenotypes. Our approach, which can be applied to cell cultures generally, was to estimate rates from considerations of cell population dynamics.

Several key assumptions underlying the model we used (see Appendix) were verified experimentally. First, we established that the cell cloning method provides a reliable estimate of the frequency of C^- and C^+ cells in the cultures (Meins and Binns, 1977). Second, we verified that C^- and C^+ cells proliferate exponentially under the culture conditions used (Binns and Meins, 1979; Mohnen, 1985). Third, we showed that cells interconvert between the C^- and C^+ states and that the frequency of C^+ cells approaches an equilibrium value.

The most important assumption is that selection coefficients calculated from the doubling constants of C^+ and C^- cells cultured separately reflects the selection coefficient of mixed cultures. C^+ cells produce cell-division factors which can promote the proliferation of neighboring C^- cells in the same tissue and in adjacent tissue explants cultured in the same Petri dish (Tandeau de Marsac and Jouanneau, 1972; Binns and Meins, 1979). Therefore, cross-feeding probably affects the selection coefficient of mixed populations of cells propagated on basal medium. The selection coefficient estimated is likely to be higher than the actual values and, hence, the estimates of the back rate ν are undoubtedly high. On the other hand, the concentration of cytokinin in the standard medium is optimal for C^- cells. Unless C^- cells inhibit proliferation of C^+ cells in the same culture, the estimates of the forward rate μ are likely to be more reliable. Even assuming a large, i.e. 10-fold, error in selection coefficients, computer simulations based on our model indicate that provided the frequency of the cell type selected against is in the order of 10^{-2}, the rates of variation will be very high relative to classical types of spontaneous variation.

The equilibrium frequencies of the cell type selected against were consistently in the range 10^{-1} to 10^{-2} and gave estimates of $\mu \approx 2\times10^{-2}$ for the forward rate and $\nu \approx 3\text{-}5\times10^{-2}$ for the reversion rate. The forward and backward rates were roughly equal and extremely rapid, at least 10^2 to 10^3 times faster than the rate of somatic mutation in *Nicotiana* (Sand et al., 1960; Carlson, 1974; Dulieu, 1974, 1975). Therefore, cultured tobacco tissues consist of rapidly interconverting populations of C^+ and C^- cells. Cytokinins act in a pseudodirected fashion by altering the selection coefficient to give tissues in which either C^- cells or C^+ cells predominate.

The variation in cytokinin requirement described here has features similar to a broad range of developmental and genetic phenomena loosely classified as epigenetic changes or epimu-

tations (Meins, 1983; Jorgensen, 1993). In the case of paramutation (Brink, 1973), variation in transposable-element activity (Fedoroff et al., 1989), genomic imprinting (Matzke and Matzke, 1993), and some forms of gene silencing in transgenic plants (Jorgensen, 1993), different stable states can be meiotically inherited. At present the genetic basis for rapid variation in cytokinin requirement is not known. The cytokinin requirement of cultured tobacco tissues is regulated by at least two, unlinked genes, *Hl-1* and *Hl-2* (Meins and Foster, 1986b). So far only 4 plants regenerated from independently isolated C^+ variants have been analyzed genetically. Although the C^+ phenotype was inherited in each case as a dominant monogenic trait (Meins and Foster, 1986b), more extensive crosses of numerous C^+ variants and their revertants are needed to establish whether or not alternative C^+ and C^- states are regularly transmitted through meiosis.

Several molecular mechanisms could, in principle, account for the variation we observed (Meins, 1989a). For example, the well-documented 'flip-flop' of bacteria cells between alternative states results from a site-specific recombinational switch (Silverman et al., 1980). DNA methylation has been implicated in stable variation reported for the cytokinin requirement of Ti-plasmid transformed tobacco cells (van Slogteren et al., 1984; Amasino and John, 1989) and cells cultured from tumor-prone *Nicotiana* genetic hybrids (Durante et al., 1989). Finally, there is strong evidence that rapid, cell-heritable switching in the cytokinin requirement of cultured tobacco pith cells involves self-perpetuating regulatory circuits that result in alternative stable states without genetic modification (Meins, 1989b).

Our findings bear on the problem of developmental plasticity in plants. To account for the pronounced environmental effects on plant development, Sachs (1988) has rejected the commonly accepted notion of a detailed developmental program in favor of nondeterministic mechanisms. He proposes that mature structures are formed by epigenetic selection, i.e. internal and environmental factors act to select a suitable developmental event from a repertoire of alternative possibilities.

Pseudodirected variation in cytokinin requirement provides a plausible cellular mechanism for epigenetic selection. Interconversion of C^+ and C^- cells, or their committed progenitors, occurs during the development of tobacco plants (Meins and Wenzler, 1986). We suggest that cells rapidly alternate between the C^- and C^+ state within the shoot meristem. There is evidence that shoot meristems exhibit regional differences in cytokinin concentration (Sossountzov et al., 1988). This would alter the selection coefficient locally to generate regions greatly enriched in either C^- or C^+ cells. Assuming that the two cell types have different developmental potentialities, this would result in a spatial pattern of differentiation. According to our model variation depends on cell division, which occurs primarily in the meristem (Steeves and Sussex, 1988). Thus the patterns, once established, would be fixed in mature tissues. This heuristic model illustrates how a gradient in hormone concentration could act by selection rather than by induction to give predictable patterns at the tissue and organ level. Unlike a developmental program, this process depends on stochastic cellular variation and, hence, would be expected to be plastic and especially sensitive to environmental influences.

As yet there is no direct evidence implicating pseudodirected variation in organogenesis. Nevertheless, it is remarkable that combinations of auxins and cytokinins that initiate organogenesis in cultured tissues are also required for sustained cell proliferation (Skoog and Miller, 1957). Moreover, overproduction of these hormones by tumor cells causes autonomous growth as well as a profound disruption of normal morphogenesis to give tumors ranging from abnormal, but organized teratomas to unorganized masses (Braun, 1978). It is also of interest in this regard that the formation of shoots from cultured tobacco tissues in response to cytokinin treatment is a stochastic process (Meins et al., 1982). Only a small fraction of the cells - less than 10^{-3} - have the potential to form shoots. The incidence of cells competent for shoot formation increases when tissues are subcultured on media containing combinations of auxin and cytokinin that promote cell proliferation.

Pseudodirected variation provides an attractive paradigm for epigenetic selection. Elucidating the molecular basis for this variation and testing the hypothesis that variation in cytokinin requirement has a causal role in organogenesis will depend on identifying molecular and cellular markers suitable for distinguishing between C^+ and C^- cells.

APPENDIX

Define the rate at which C^- and C^+ cells interconvert by analogy to mutation rate as the fraction of cells in the population that convert to the other cell type per cell generation (Hayes, 1968). Let a = number of C^- cells, b = number of C^+ cells, μ = rate of C^- cells converting to C^+ cells and ν = the rate of C^+ cells converting to C^- cells. Assume: (1) C^- and C^+ cells proliferate exponentially with doubling constants k_A and k_B respectively. (2) There is minimal cell-cell interaction affecting proliferation, i.e. the parameters are independent of a and b. (3) Cellular variation is random, i.e. the rates μ and ν are independent of the conditions of culture. The equations of motion are:

$$\mathrm{d}a/\mathrm{d}t = k_A(1-\mu)a + k_B\nu b \quad (1a)$$

$$\mathrm{d}b/\mathrm{d}t = k_B(1-\nu)b + k_A\mu a. \quad (1b)$$

Combining equations 1a and 1b:

$$\mathrm{d}q/\mathrm{d}t = k_A[\mu + [S(\nu-1) - \nu - \mu]q + Sq^2], \quad (2)$$

where q is the frequency of C^+ cells in the population and S is the selection coefficient, $1-(k_B/k_A)$. At equilibrium, $\mathrm{d}q/\mathrm{d}t=0$ and equation 2 becomes:

$$\mu + [S(\nu-1) - \nu - \mu]\hat{q} + S\hat{q}^2 = 0, \quad (3)$$

where $\hat{q}$ is the equilibrium frequency.

When selection for C^- cells is strong, the effect of ν on $\hat{q}$ is small and equation 3 simplifies to:

$$\mu \approx \hat{q}S. \quad (4)$$

Conversely, when selection for C^+ cells is strong, the effect of μ on q is small and equation 3 simplifies to:

$$\nu \approx -S(1-\hat{q})/(1-S). \quad (5)$$

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