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Determination in Plant Cells

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Abstract. The possibility that some of the variation in callus cultures involves epigenetic changes is examined in cultures established from the hypocotyls and roots of *Euphorbia heterophylla*. It is shown that the responses of the cultures are affected by the light regimes under which they are grown and that in the dark and under short photoperiods, there are differences between the two types of culture with respect to pigmentation, auxin requirement, capacity to regenerate buds and roots and in certain isozyme patterns, whereas the two cultures are similar from the first passage under continuous light. However, these differences are only maintained for 2—3 passages, after which the root callus becomes similar to the hypocotyl callus. Evidence is presented that these differences between cultures are epigenetic. Callus cultures established from the apical meristems of shoots and roots of *E. heterophylla* show similar differences to those observed between hypocotyl and root cultures and these differences are also lost after 3 passages. These results indicate that the cells of apical meristems are not totally uncommitted, but are determined as 'shoot' and 'root' meristem cells, respectively. The practical importance of a better understanding of epigenetic effects in plant cells is stressed.

It is a central dogma in plant science that plant cells are totipotent. It is difficult to prove the generality of this dogma, since altho ugh there are many demonstrations of totipotency in plant cells, there are many species in which regeneration of whole plants from cell cultures has not yet been achieved. However, even if we accept the dogma in principle, it is well-known that it is easier to regenerate buds, roots and embryos from cultures of certain tissue than from others. The reasons for these differences are not known, but it is commonly assumed that they reflect differences in the nature and the degree of differentiation between various tissues of the plant. It has been known for 50 years, since the pioneer work on plant tissue culture, that considerable variation in pigmentation, texture and regenerative capacity occurs within callus cultures established from different tissues of the same plant. Some of this variation is known to be genetic, since chromosomal changes involving polyploidy, aneuploidy, deletions and point mutations are known to be relatively frequent in callus cultures, and there is considerable current interest in the possibility of using this genetic variation in cultures in breeding programmes. However, I wish to discuss aspects of variation in cell cultures which is not genetic in origin.

Now, before cultures can be established from mature, differentiated tissue, it is necessary that the cells undergo "de-differentiation" De-differentiation involves two distinct types of change:

(1) the resumption of cell division in mature cells,
(2) the removal of any specialized metabolism or developmental commitment which the cells may have acquired.

The fact that there are differences between calluses established from various tissues suggests that division can be induced in mature cells before there has necessarily been complete de-differentiation in their metabolic specialization.

If we assume that differentiation involves selective gene expression without any changes in the genome, then it would appear that differences in patterns of gene expression between mature cells can, to some extent, be retained after renewed cell division has been induced. We do not yet know how selective gene expression is controlled in eucaryotes, but it would appear that differences in the 'programming' of gene expression can be transmitted through repeated cell divisions. Persistent differences in gene expression, without changes in the genome, which are transmitted through cell division have been called epigenetic. Epigenetic variation in cell cultures has received little attention and has only recently started to be studied systematically.

Habituation as an Epigenetic Change

We know very little about the nature of epigenetic variation, but in recent years MEINS has produced much experimental evidence that the well-known phenomenon of habituation in cell cultures probably involves epigenetic changes (MEINS 1982, 1983). MEINS has pointed out that epigenetic changes differ from genetic changes involving mutation in the following respects:

1. Epigenetic change is directed — it occurs regularly in response to specific inducing conditions. The rate of change is higher than 10^{-5} per cell generation and is thus greater than the normal rate of mutation.
2. The variant phenotype, though stable, is reversible. Reversal is directed and at a high rate.
3. By definition epigenetic changes are not transmitted through meiosis.

MEINS has shown that the phenomenon of habituation in cultures of tobacco pith shows the characteristics of epigenetic variation, on the following evidence:

1. Clones established from single cells may show habituation.
2. The rate of habituation is 100—1000 times higher than the normal rate of mutation.
3. The habituated state is reversible. If plants are regenerated from cultures which show habituation for cytokinins, then fresh cultures established from the pith of such cells are found to be cytokinin-dependent.

Differences in Cultures from Hypocotyl and Root

Apart from the phenomenon of habituation, the question arises whether some of the other variation observed in tissue cultures is epigenetic, and whether there is evidence that cultures set up from different tissues show epigenetic differences which persist in culture. We have investigated this

TABLE 1
Callus cultures from hypocotyl and roots of *Euphorbia heterophylla*

Hormone	Dark		Short day		Continuous light	
	H	R	H	R	H	R
BAP	C B+ [R]	No C	Green C	No C	Green C B [R]	No C
NAA	Small C R	Small C [R]	Cream C R	Small brown C	Cream C [R]	Small brown C
BAP+ NAA	Large C B+ R	Cream C	Cream C B [R]	*Cream C	Green C B+	Cream- green C B

C — callus, B — buds, B+ — many buds, R — roots, [R] — few roots.
* First two passages, later becoming green with buds.

problem in cultures of *Euphorbia heterophylla*. Sections of hypocotyl and roots of young seedlings were placed on Murashige and Skoog medium, with various hormone supplements as follows: (1) no hormone; (2) benzylaminopurine (BAP) 10^{-6}M; (3) naphthalene acetic acid (NAA) 10^{-6}M; (4) BAP 10^{-6}M + NAA 10^{-6}M. The results are summarized in Table 1. Neither hypocotyl nor root sections produced callus without hormone, but with BAP alone abundant callus and buds were produced by hypocotyl sections, especially in the dark, whereas the root sections produced neither callus nor buds with BAP alone. Thus, it appears that the hypocotyl tissue is auxin-independent, whereas the root tissue is auxin dependent. There was slow growth of callus, with some root formation, with NAA alone, indicating a low degree of habituation for cytokinins. There was active callus production from both hypocotyl and root sections with BAP + NAA, but the responses were affected by the light regimes. In the dark, the hypocotyl callus produced both buds and roots, whereas the root callus produced only roots. Under short days, hypocotyl callus on BAP + NAA was green and produced buds in the first passage, whereas the callus from the root sections was white and without buds and only slowly became greenish and produced buds in the third passage. Under continuous light, both hypocotyl and root sections produced green callus with buds and roots in the first passage.

Thus, in the dark there were clear differences in the ability of callus from hypocotyl and root to form buds with BAP + NAA, and under short days there were differences between the two types of callus in pigmentation and texture, and in the timing of bud regeneration, which only occurred in the root tissue in the third passage.

It seems likely that the differences between the hypocotyl and root callus in the first 2—3 passages were epigenetic and not due to random genetic variation, from the following evidence:

(1) Characteristic differences shown by the calluses developed from all the hypocotyl and root sections cultured in several experiments. That is to say, the differences were regular and directed, not random.

(2) Although the differences were persistent through many cell divisions, the root callus ultimately came to resemble the hypocotyl callus by becoming green and producing buds, under both short days and continuous light.
(3) If whole plants are regenerated from buds on root callus and roots are taken from regenerated plants, the callus from such roots shows the same characteristics as the callus from the original seedling roots, so that the loss of the latter characteristics is reversed during regeneration.

Protein and Isozyme Studies

It remains possible that even where apparently there are not gross differences between calluses established from different organs and tissues, there may well be finer and more subtle differences. Indeed, RAFF *et al.* (1979) examined calluses established from stems, leaves, pistils and anthers of *Prunus avium* by immunological methods and found that there were differences in antigenic determinants and that specific parental organ antigens were still present in the callus cells after four subcultures. This observation provides striking evidence for the continued transmission of certain tissue- or organ-specific characteristics when cell division is induced in mature, differentiated cells.

A different approach has been adopted by other workers, who have studied isozyme patterns in cultures from different tissues. ARNISON and BOLL (1974) made electrophoretic analyses of isoenzymes of callus cultures from roots, hypocotyl and cotyledons of *Phaseolus vulgaris*. They found that the isozyme patterns of the three cultures were very similar but there were also persistent differences between them. Similar studies on *Phaseolus vulgaris* were also carried out by BASSIRI and CARLSON (1978) and they obtained similar results to those of ARNISON and BOLL (1974).

We have carried out preliminary isozyme analysis of callus cultures of hypocotyl and root of *E. heterophylla*. The callus samples were taken from cultures in the second passage on BAP 10^{-6}M + NAA 10^{-6}M and incubated under both short days and continuous light. The enzymes studied included phosphoglucoisomerase (PGI), 6-phosphogluconate dehydrogenase (6-PGDH) and phosphoglucomutase (PGM). The isozyme patterns were affected by the light regimes under which the cultures were incubated. Thus, there were differences between root and hypocotyl cultures in the isozyme patterns for PGI and 6-PGDH under continuous light, but not under short days, while the reverse was true for the isozymes of PGM.

The cultures of hypocotyl and root under continuous light were both becoming green, so that the results of these preliminary isozyme studies would suggest that there may still be isozyme differences, even when the visible differences between the calluses have been lost.

Determination in Shoot Apices

The occurrence of stable cell differences which are transmitted through cell division has been well-known in normal plant development for many years. Thus, in vernalization, certain normally irreversible changes occur in the embryos of rye or wheat during the chilling period which control the flowering of plants at a later stage. We know that the changes which occur during vernalization affect the cells of the shoot meristems (since excised

TABLE 2
Callus from apical meristems (under short days)

Hormone	Callus from shoot apical meristem	Callus from root apical meristem
BAP 10^{-6}M + NAA 10^{-6}M (Two passages)	Cream and light green callus. Few buds	Light brown callus. Roots
No hormone	No buds or roots	Roots
BAP 10^{-6} M	Buds	Buds
NAA 10^{-6}M	Few roots	Roots
BAP + NAA (Third passage)	Buds	Buds, roots

shoot meristems can be vernalized, (PURVIS 1940)), and that the changes are transmitted through many cell generations before they are finally expressed in flower initiation (WAREING and PHILLIPS 1982). Thus, the apical meristem cells of the embryo become determined, so that they become committed to a particular pathway of development.

Another example of determination in shoot apices is seen in the phenomenon of phase change in woody plants. Many woody plants show a distinct 'juvenile' phase, during which they are unable to initiate flowers, but ultimately they attain the mature or 'adult' condition in which flowers are initiated if other conditions are favourable. Although the presence or absence of the ability to flower is the primary distinguishing characteristic of the two phases, a number of differences in vegetative characters may also be recognized.

The two phases show great stability, as shown by the ability of rooted cuttings of the adult part of the vine of ivy (*Hedera helix*) to retain their adult character for many years. However, reversion of adult cuttings to the juvenile state can be induced by relatively high temperatures or by treatment with gibberellic acid (ROBBINS 1957). It appears that the meristematic cells of the apices are determined as either 'juvenile' or 'adult' cells, since very small pieces of the adult shoot apex of *Citrus*, bearing only three small leaf primordia, can be successfully grafted on to seedling stocks and are then found to retain their adult character (NAVARRO *et al.* 1975).

Callus cultures derived from juvenile and adult stem or petiole tissues of *Hedera* grown in the same nutrient medium show differences in their rates of growth and in the fact that the 'juvenile' calluses show abundant regeneration of adventitious roots, while 'adult' calluses show little capacity for root regeneration (STOUTEMEYER and BRITT 1965). Thus, it is clear that although renewed cell division is stimulated in the juvenile and adult tissues to establish callus cultures, the cells are not completely "de-differentiated" in all respects. Since the adult parts of the ivy vine produce seeds which give rise to juvenile seedlings, it is clear that there is phase-reversal during sexual reproduction, indicating that the differences between the two phases are epigenetic.

Differences in Cultures from Shoot and Root Apical Meristems

In the experiments described above callus cultures were established from mature tissues of hypocotyl and root; that is, from differentiated cells in

which renewal of cell division had been stimulated. The question arises whether the cells of the apical meristems of shoots and roots show similar differences to those shown by calluses from older tissues.

Root tips and shoot apices, consisting only of the apical dome and only 1 or 2 leaf primordia, of size about 0.5 mm were excised and cultured on Murashige & Skoog medium with BAP 10^{-6}M + NAA 10^{-6}M. The cultures were incubated under short days and continuous light. The calluses formed from the two types of meristem showed several characteristic differences from each other under short days, for the first two passages. The callus from the shoot apical meristems under short days formed a light-green callus, with a few buds and shoots, but no roots, while the root callus under the same conditions formed a light brown callus, with roots. On the other hand, under continuous light, both shoot and root meristems formed light green calluses, which differed in texture, but both formed buds, and the 'root' callus also formed roots. Thus, the differences were reduced under continuous light.

When the cultures under short days were further subcultured (third passage) to various hormone treatments, the 'root' callus formed more roots than the 'shoot' callus on media without hormone and on NAA 10^{-6}M, but both produced buds on BAP + NAA (Table 2). Thus, as with the cultures from mature root tissue, the calluses from root tips ultimately began to green and produce buds on BAP + NAA under short days. Nevertheless, these results are of considerable interest in showing that for the first two passages there were marked differences between the cultures from shoot and root meristems, particularly in their capacities to regenerate buds and roots. The differences observed in the first two passages indicate that the commonly held view that meristem cells of both shoots and roots are totally uncommitted and both equally totipotent, is not valid. Since root and shoot meristems are very stable entities and in higher plants root meristems are only very rarely converted directly into shoot meristems or *vice versa*, it has been suggested (WAREING 1978, 1979) that they may show determination into the shoot and root pathways of development, respectively, and the results of the present experiment seem to support this conclusion.

CONCLUSIONS

The experiments with callus cultures from hypocotyl and root tissue show that in the first two passages the two types of callus differ markedly in their pigmentation, requirement for auxin and capacity for regenerating buds. The evidence that these differences are probably epigenetic is summarized on pp. 243—244. Clearly, the epigenetic differences between hypocotyl and root cultures are not as stable as those shown, for example, by cultures of 'juvenile' and 'adult' callus of *Hedera*. However, we have found marked differences (including differences in isozymes) established from young male and female flowers of gourd (*Cucurbita pepo*), which have persisted through several passages (AL-ANI and WAREING, unpubl.). Thus, it is probable that other stable epigenetic differences between cultures established from different organs and tissues may be found.

It would appear that even cultures which are similar in appearance and regenerative abilities may still show epigenetic differences in proteins or

isozymes, as indicated by the studies on *Prunus avium* (p. 244) and the isozyme studies on hypocotyl and root callus (p. 244).

The experiments with callus derived from shoot and root apical meristems showed that the cells of these two meristems gave rise to calluses which differed significantly, particularly in their respective abilities to regenerate buds and roots. The results are important, even though the differences were lost in the third passage, since they indicate that the cells of these meristems are not totally uncommitted but are 'programmed' differently. Moreover, although the differences gradually disappeared *in vitro*, these differences are clearly maintained when the meristems remain *in vivo*. This conclusion is consistent with the results of other studies. Thus, FELDMAN and TORREY (1976) isolated the quiescent centres of maize roots and found that when these were cultured on indol-3-ylacetic acid (IAA) and kinetin, organized root tissues were formed after a few initial unorganized cell proliferations, suggesting that the cells of the quiescent centre are programmed as root cells. On the other hand, SMITH and MURASHIGE (1970) showed that excised shoot apical meristems less than 0.1 mm in height can grow and develop into complete plants on a nutrient medium with IAA alone.

Effectively nothing is known about the molecular basis of epigenetic changes which, by definition, are not transmitted through meiosis and sexual reproduction. However, the possibility exists that the DNA of somatic cells may be modified during development. Thus, it is known that a high proportion of the cytosine in the DNA of higher plants is methylated to form 5-methyl cytosine. It has been suggested that this methylation process may be involved in the control of gene expression, since there appears to be a correlation between low methylation and activity of specific DNA sequencies. A mechanism has been proposed whereby a methylation pattern, once established in somatic cells, could be inherited by progeny cells (HOLLIDAY and PUGH 1975). Thus, we cannot exclude the possibility that stable epigenetic effects may involve modification of the DNA in somatic cells.

It is of great importance that we should have a better understanding of the molecular basis of epigenetic effects, for at least two good practical reasons. On the one hand, totipotency may be more effectively attained in species which are difficult to regenerate from cultures, if more complete de-differentiation of somatic cells can be achieved. On the other hand, if we knew more about the molecular basis of epigenetic effects, we might be able to delay or prevent the loss by cell cultures of the capacity to produce secondary plant products.

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