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DNA METHYLATION AS A KEY PROCESS IN REGULATION OF ORGANOGENIC TOTIPOTENCY AND PLANT NEOPLASTIC PROGRESSION?

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SUMMARY

Progressive loss of organogenic totipotency appears to be a common event in long-term plant tissue culture. This loss of totipotency, which has been proposed to be a typical trait of plant neoplastic progression, is compared to some mechanisms that occur during the establishment of animal differentiation-resistant cancer lines *in vitro*. Evidence is presented that alteration in DNA methylation patterns and expression of genes occur during long-term callus culture. An effect of the auxin, 2,4-dichlorophenoxyacetic acid, in the progressive methylation, is moreover suggested. Methylation of genes relevant to cell differentiation and progressive elimination of cells capable of differentiation is proposed as being responsible for this progressive loss of organogenic potential. Finally, the epigenetic alteration (DNA methylation) that occurs during prolonged periods of culture may induce other irreversible genetic alterations that ultimately make the loss of totipotency irreversible.

Key words: in vitro cultures; totipotency; habituation; neoplastic progression; DNA methylation; 2,4-D.

INTRODUCTION

In eukaryotic organisms, DNA is frequently modified by methylation of cytosine residues into 5-methylcytosine. In DNA of higher plants, up to 30% of cytosine residues are methylated (Gruenbaum et al., 1981) and the level of DNA methylation is known to be modulated during plant development and organ or tissue differentiation (Hepburn et al., 1987; Vergara et al., 1990). Cytosine methylation has been shown to be negatively correlated with transcriptional activity of several endogenous genes such as those encoding storage proteins (Bianchi and Viotti, 1988), photosynthetic proteins (Ngernprasirtsiri et al., 1989), rRNA (Flavell et al., 1988), or several transgenes (Linn et al., 1990; Ottaviani et al., 1993; Lambé et al., 1995). These genes are usually undermethylated in cells in which they are expressed in comparison with cells in which they are inactive or slightly active.

In *in vitro* plant cultures, many differences in the methylation status have been observed between different cell types (Palmgren et al., 1991). Moreover, investigations with isoschizomeric restriction endonucleases differentially sensitive to methylation of recognition sites have demonstrated frequent methylation changes after tissue culture of somatic cells (Brown, 1989; Kaeppler and Phillips, 1993; Phillips et al., 1994).

In the present study, we postulate that hypermethylation of DNA during tissue culture could be involved in the appearance of many aberrant phenotypes such as habituated callus and nondifferentiating callus. The progressive methylation of genes relevant to cell differentiation during the phase of undifferentiated-cell division and multiplication and the continuous elimination of the cells capable of differentiation are proposed to be responsible for the progressive loss of totipotency during callus culture. In the animal kingdom, hypermethylation has been proposed to be an initial event associated with immortalization and onset of differentiation-resistant cell lines *in vitro* (Rideout et al., 1994). We present here evidence for a similarity between the mechanism observed during the occurrence of immortalized and nondifferentiated animal cell lines and some modifications that occur during plant tissue cultures. Some basic considerations are first recalled.

The concept of callus, organogenic totipotency, habituation, and neoplastic progression. A primary callus is a cellular mass generated at a wounded surface of a plant organ generally in the presence of an auxin and a cytokinin at similar concentrations (for dicotyledon) or of auxin alone at a high concentration (for monocotyledon). Growth of the primary callus after excision can be maintained through regular subcultures in the same hormonal conditions. Classically, a callus results from the anarchic proliferation of undifferentiated cells. This concept has, however, to be modified, first, because most of the calluses contain differentiated cells, tracheary elements for instance (Yeoman and Street, 1977; Crèvecoeur et al., 1987; Fransz and Schel, 1990), although they lack the signals to organize into authentic tissues or organs; and second, because most of them grow through meristematic centers (Floh and Handro, 1985; Fransz and Schel, 1990). Changing the auxin/cytokinin ratio of callus culture of dicot plants, or removal (or reduction) of the auxin for monocots provokes organogenesis (formation of adventitious buds or roots) or embryogenesis. This illustrates the totipotency of the normal callus cells.

Under certain conditions (Bednar and Linsmaier-Bednar, 1971; Syono and Fujita, 1994) or after prolonged periods of time in culture,

TABLE 1

CELL LINES THAT LACK SPECIFIC ENZYMES AND THAT REGAIN A NORMAL PHENOTYPE AFTER GROWTH IN PRESENCE OF 5-AZACYTIDINE (ACCORDING TO HOLLIDAY, 1987)

Cell Line and Organism	Altered Phenotype Gene	Enzyme Reactived	Reference
W7 Mouse	MT⁻ Cadmium sensitive	Metallothionein	Compere and Palmiter, 1981
CHO Hamster	MT⁻ Cadmium sensitive	Metallothionein	Stallings et al., 1986
V79 Hamster	tk⁻ Bromodeoxyuridine resistant	Thymidine kinase	Harris, 1982
L5178Y Mouse	tk⁻ Bromodeoxyuridine resistant	Thymidine kinase	Nakamura and Okada, 1983
L61M Mouse	tk⁻ Bromodeoxyuridine resistant	Thymidine kinase	Liteplo et al., 1984
FEL Mouse	tk⁻ Bromodeoxyuridine resistant	Thymidine kinase	Hickey et al., 1986
CHO Hamster	**ODC⁻ Requires putrescine**	**Ornithine decarboxylase**	Steglich et al., 1985
FA32 Rat	OCT⁻ Requires arginine	Ornithine carbamyltransferase	Delers et al., 1984
JR45 Rat	asp⁻ Requires asparagine	Asparagine synthase	Sugiyama et al., 1983
CHO hamster	asp⁻ Requires asparagine	Asparagine synthase	Harris, 1986
CHO hamster	pro⁻ Requires proline	Pyrroline-5-carboxylate synthase and ornithine aminotransferase	Harris, 1984a
V79 Hamster	glu⁻ Requires glutamine	Glutamine synthase	Harris, 1984b
GH3 Rat	rPRL⁻ Prolactin deficient	Prolactin	Ivarie and Morris, 1982

some callus acquires the capability to grow in the absence of auxin and/or cytokinin. They become habituated (Gautheret, 1942; Meins, 1989). In most cases (but not always), the process of habituation is reversible and in most cases habituated cells keep their totipotency, so that they can regenerate roots, buds, or somatic embryos. Habituation is thus regarded as an epigenetic phenomenon (Meins, 1989). This latter statement is supported by strong experimental evidence such as the observation that the conversion of normal, hormone-requiring cells to the habituated phenotype is gradual and progressive and, unlike mutation, is strongly influenced by the physiological state of the cells. Moreover, induction of habituated cells occurs at much higher rates than those of mutation and reversal from habituated cells to normal cells is also dependent on the physiological conditions. Habituated tissue bears a striking resemblance to crown gall tissue, which grows independently of exogenous hormones. Habituation has thus been considered as a step of neoplastic progression that can be initiated in *in vitro* cultures (Gaspar et al., 1991; Gaspar, 1995). This process implicates progressive reduction of cell-to-cell adhesion, which renders callus friable and cells dissociable (Liners et al., 1994), a loss of the capability to organize meristematic centers, many morphological abnormalities (Hagège et al., 1992), biochemical deviations (Le Dily et al., 1993), and irreversible loss of organogenic totipotency at the terminal stage.

Apparently, irreversible loss of totipotency is also frequently reported during culture of monocot callus. The regeneration potential can be maintained only for a few months or at most 1 to 2 yr depending on the species, cultivar, and culture conditions (Ziauddin and Kasha, 1990; Kishor et al., 1992; Lambé, 1995). In many species, *in vitro* culture is also accompanied by a progression from compact and nodular meristematic callus to friable and watery callus and a reduction of meristematic centers. Friable callus was correlated with a less differentiated state than compact callus (Fransz and Schel, 1990) and the proportion of friable calli was shown to increase with increased subcultures (Lambé, 1995). In many species, such calluses regenerate only roots (King et al., 1978; Mott and Cure, 1978) or are nonregenerating.

The apparent permanent loss of totipotency led us to implicate, in this case, irreversible genetic alterations. Alterations, such as single gene mutations, chromosome breakages, polyploidisations, or transposable element activation, are in fact commonly observed after prolonged tissue culture and have been shown to be responsible for a variety of morphological and biochemical modifications (Brettel et al., 1986; Lee and Phillips, 1988; Brown, 1989) known as somaclonal variations. Some observations indicate, however, that the first step in the progressive loss of totipotency during callus culture may be related to an epigenetic modification known as variation of DNA methylation.

Oncogenic transformation during the development of permanent animal cell lines. In animal cells, it has been shown that the transformation of normal diploid somatic cells to permanent (cancer) cell lines is accompanied by the activation of a *de novo* methylase activity that is capable of methylating the 5′-position of cytosine residues which were previously not methylated (Holliday, 1991). When tissue cells are first introduced in culture, there is a rapid general loss of methyl moieties but, as immortal cells emerge, the methylated DNA content appears to increase (Wilson and Jones, 1983; Mann et al., 1985). These authors have suggested that freshly *in vitro* cultured cells activate the transcription of a certain number of genes to adapt to the new culture conditions and to proliferate. This establishment in culture that needs a general reorganization of the cellular activity is first manifested by a general activation of genes (demethylation). Similar modifications of the DNA methylation status is also observed during certain developmental changes *in vivo* (Cedar and Razin, 1990), indicating that it can be a general mechanism in the reorientation of a developmental pathway.

However, the cells certainly do not use all their genes during cell culture and some that were active in the primary cells (but not necessary during the culture) may become progressively inactivated. This observation is supported by several examples of enzymatic activity present in the initial cells that were absent in the cultured lines (Table 1). In all instances, the lost enzymatic activity could be restored after culture in medium supplemented with 5-azacytidine, which competes DNA hypomethylation. This latter observation confirms that the loss of the enzymatic activity results from transcriptional inactivation by DNA methylation and is not the result of a genetic alteration. Such phenotypic alteration, which can be reversed by 5-azacytidine, has been called epi-mutation (Holliday, 1987).

Further evidence that this type of process occurs during the production of an animal cell line comes from studies by Antequera et al. (1990) in which a large number of CpG islands (target sequence for cytosine methylation in animal) was examined. Although island DNA is generally nonmethylated in all tissues of the intact organism, it was found that CpG islands of many nonessential genes are highly methylated in cultured cells. These changes presumably occur progressively over a long time scale under growth-selective conditions (Shorderet et al., 1988; Wise and Harris, 1988). Moreover, it has been demonstrated that the expression of the DNA methyltransferase enzyme is low in normal human cells, is significantly increased in virally transformed cells, and is strikingly elevated (several hundredfold) in cultured cancer cells (El-Deiry et al., 1991).

Inactivation of differentiating genes by methylation during establishment of undifferentiated animal cell lines. Animal cell line initiation and oncogenic transformation are associated with a failure of cells to differentiate (differentiation-resistant cells). These failures may also be the consequence of epigenetic changes during cell line initiation and maintenance.

An example of this process at the level of single gene has been well documented for mice MyoD gene (determination gene whose activation induces a new developmental pathway leading finally to the differentiation of some embryogenic cells into myoblast cells) (Jones et al., 1990; Rideout et al., 1994). This gene has a large intronic CpG island that is fully unmethylated in all murine tissues. By monitoring the methylation pattern of this gene during the establishment of a cell line, it was shown that the sequence undergoes a progressive process of *de novo* methylation culminating in the emergence of the immortal line. Moreover, after exposure of the permanent line (which is incapable of myoblast differentiation) to 5-azacytidine, the potential to differentiate is restored at high frequency and it has been demonstrated that this effect is mediated by demethylation and activation of the MyoD gene.

Because it is probable that expression of the MyoD gene in culture leads to the differentiation process and a cessation of cell division, it has been postulated that the cells expressing this gene will be eliminated in the course of the culture (no more division of such cells) (Jones et al., 1990). On the contrary, cells in which the CpG island has undergone fortuitous methylation will be amplified and selected to become progressively predominant. This may explain why the overall level of methylation of this gene progressively increases and culminates at the time of the emergence of actively dividing cell lines. In this system, there will therefore be a progressive selection of the cells that are incapable of differentiation (only capable of cell division).

Numerous other data in the animal kingdom indicate that the methylation of genes during culture may be responsive for the blockade of a differentiation process. First, it was shown that the human MyoD homologue, Myf 3, is also methylated in cultured human cell tumors (Rideout et al., 1994). Second, treatment of erythroleukemia cells with dimethyl sulfoxide (DMSO) induces the differentiation into globin cells and this process was associated with hypomethylation of the DNA (Christman et al., 1977, 1980; Bestor et al., 1984). Similar induction was obtained with ethionine, which is a competitive inhibitor of the methyl donor, 5-adenosylmethionine. In the same way, retinoic acids induce F9 teratocarcinoma cells to differentiate (Bestor et al., 1984) and it has been shown that this treatment induces specific activation of the collagen IV gene and is associated with a loss of methylation in this gene (Burbelo et al., 1990). Another DNA hypomethylating agent (5-azadeoxycytidine) was also able to induce differentiation in human monoblastic leukemia cells (Attadia, 1993).

From these examples, it appears that the permanent silencing of the expression of genes relevant to cell differentiation occurs commonly during immortalization of animal cells.

Methylation changes during callus initiation and culture of plant cells. As in animal cell cultures, the overall level of 5-methylcytosine content seems to decrease at the time of the initiation of plant callus cultures. This was demonstrated in *Pennisetum purpureum* by measuring the total amount of methylcytosine by High Phase liquid chromatography (HPLC) (Morrish and Vasil, 1989). The level of 5-methylcytosine appears to be lower in callus (34% of total cytosine) than in the explant (38–40%). However, this decline of methyl moieties seems to be lower than in animal cells. However, it must be remembered that the 5-methylcytosine content is considerably higher in plants (15–40%) than in animals (1%) (Wagner and Capesius, 1981). Discrete changes of methylation may be sufficient for adaptation of the metabolism to the new environment. The observation of Morrish and Vasil (1989) is moreover supported by analysis of specific gene methylation patterns during culture initiation. Grisvard (1985), who examined the satellite sequences from *Cucumis melo* with isoschizomeric enzymes responding differentially to the methylation of the restriction site, found that methylation of these sequences was higher in explants (hypocotyls) than in callus tissues. Quemada et al. (1987), working with the 5S RNA gene of soybean, also showed that DNA from recently cultured tissues was exceedingly cleaved by enzymes sensitive to methylated cytosines in the target sequences as compared to DNA from explant tissues.

As in animal cell cultures, however, some observations indicate a progressive remethylation during prolonged *in vitro* cultures of plant cells. For example, in the continuation of the work described above, Quemada et al. (1987) observed that upon prolonged culture, some cell lines regained resistance to methylation-sensitive restriction enzymes. Other evidence, that progressive methylation occurs during long-term culture, comes from the analysis of transgenic cultures. In crown gall tumors of *Nicotiana tabacum*, progressive methylation of the transferred T-DNA was observed during culture simultaneously with a loss of the hormone-independent phenotype conferred by T-DNA transcript (Amasino et al., 1984). In transgenic pearl millet (*Pennisetum glaucum*) containing the GUS gene, we have observed a progressive loss of the GUS expression during callus maintenance on media containing 2,4-dichlorophenoxyacetic acid (2,4-D), which in most calluses, led to a total suppression of the enzymatic activity after 12 to 18 mo. of culture (Lambé et al., 1995). Similar results were obtained in transgenic rice callus (*Oryza sativa*) containing the GUS gene or the human growth hormone gene (Mutambel, unpublished results). Reactivation of GUS expression after exposure to 5-azacytidine and analysis of the restriction pattern of the GUS sequence with *Sau*3A1 and *Mbo*I restriction enzymes have indicated that instability resulted from transgene methylation (Lambé et al., 1995). Such phenomenon observed with transfected DNA may probably also occur with endogenous sequences (at least some) during culture.

Role of auxin in the maintenance of the undifferentiated state. LoSchiavo et al. (1989) found that, in *Daucus carota* cultures, the level of 5-methylcytosine was greatly dependent on the concentrations of natural or synthetic auxin in the medium. After 5 d on initiation medium, the level of 5-methylcytosine varied from 16 to 45% depending on the type and on the concentrations of auxin. Higher 5-

methylcytosine contents were observed in the presence of 2,4-D than in the presence of naphthaleneacetic acid (NAA) or indol-3-acetic acid acid (IAA). No methylation changes were observed in the presence of other hormones such as cytokinins. Moreover, continuous methylation occurred during culture in the presence of auxin since on medium supplemented with 5 mg/l of 2,4-D, the 5-methylcytosine content of callus increased from 45% after 5 d to 70% after 22 d.

Thus, culture in the presence of auxin seems to be accompanied by the acquisition of a *de novo* methyl transferase activity (LoSchiavo et al., 1989). Moreover, this activity seems to be lost in the absence of the causative hormone since the 5-methylcytosine content in callus maintained on medium containing 5 mg/l of 2,4-D (70%) decreased to 15% after 1 wk of culture on lower concentration (0.5 mg/l) (LoSchiavo et al., 1989). Moreover, in the transgenic pearl millet lines described above, some GUS activity is restored when 2,4-D is removed (Lambé, unpublished result). Transfer onto medium without 2,4-D generally induces a cessation of active cell division and the initiation of the embryo developmental process. The results of LoSchiavo and coworkers that show higher methylation rate in the presence of 2,4-D compared to other auxins may perhaps explain why 2,4-D is superior in inducing callus proliferation as compared to other auxins (at least in monocot species). Similarly, the results indicating higher methylation in the presence of high concentrations of this growth regulator may explain why high concentrations of this auxin are generally required (Vasil, 1987; Bhaskaran and Smith, 1990).

As previously described, it appears that in animal cells, the acquisition of such a *de novo* methyl transferase activity seems to be concomitant with the initiation of actively dividing cell lines by methylation of genes important for the differentiation process. May a similar phenomenon occur in plant cells cultured in the presence of auxin? The formal observation exposed above that auxin (at least 2,4-D) induces a hypermethylation and division and that its removal (or reduction of concentration) induces hypomethylation and initiation of cell differentiation indicated that auxin (2,4-D) may be necessary to maintain the undifferentiated state by methylation of differentiating genes. This might explain why auxin is always necessary in numerous phenomena involving cell proliferation (induction of calli, tumor formation, induction of adventitious root).

The relationship between hypermethylation (at least of some regions of the genome) and maintenance of an undifferentiated state is, moreover, supported by results of Watson et al. (1987), which were able to demonstrate that in *Pisum sativum*, DNA from the apical bud is highly methylated and as the buds begin to develop, the methylation level falls. Moreover, LoSchiavo et al. (1989) have isolated a mutant carrot line that is resistant to hypomethylation. This mutant is difficult to regenerate and is prone to tumor formation.

However, the maintenance of an undifferentiated state in the presence of an auxin does not concern all cells in a callus. In fact, as already stated above, the classical concept that a callus is a mass of undifferentiated cells has to be modified. It is now known that in dicotyledonous species, normal callus contains both meristematic centers and differentiated cells (Yeoman and Street, 1977; Floh and Handro, 1985; Crèvecoeur et al., 1987). Some cells are thus able to differentiate in conditions that promote active growth. May these ones represent cells in which genes relevant to differentiation were not inactivated by methylation? This is supported by observations by Palmgren et al. (1991) who have shown that, in *Daucus carota* suspension cells, the cell population that is the precursor of somatic embryos contains a lower level of DNA methylation than the suspension as a whole.

Monocot callus cultures also contain cells capable of differentiation in conditions that promote callus maintenance. In fact, such cultures contain small, isodiametric, meristematic cells and large elongated differentiated cells (Vasil, 1987; Fransz and Schel, 1990). Moreover, during the first subcultures, most of the callus contained somatic embryos that are arrested in the first step of the embryogenic developmental pathway (globular state) (Vasil and Vasil, 1981; Vasil, 1987; Lambé, 1995). Although in the presence of auxin (2,4-D) these embryos no longer continue their development, it appears that these cells are capable of initiating some differentiation events. In many cases, increasing the number of subcultures is accompanied by a decrease of the proportion of callus that shows globular embryos (Lambé, 1995). Because the development of the embryo is arrested at the globular stage in media containing 2,4-D, we can postulate that the cells capable of initiating the differentiation process will be progressively eliminated (no more division) from the culture. In these conditions, as in culture of animal cells, we observe a progressive increase of the number of cells incapable of differentiation.

In this view, normal (regenerating) callus may represent a population of cells in which some of them are capable of differentiation (cells in which genes important for differentiation are nonmethylated or cells in which the methylation of such genes may be reversible in the physiological conditions used for induction of regeneration).

Habituation and methylation change. Habituation has been considered to be induced spontaneously by prolonged subculturing. Moreover, in *N. tabaccum*, habituated callus was induced by high concentrations of synthetic auxin but not by low concentrations (Syono and Fujita, 1994). It is important to remember that these two conditions have been shown to increase the methylation status by LoSchiavo et al. (1989).

Habituated callus may perhaps be considered as abnormal callus in which the undifferentiated state may be maintained in the absence of auxin. These calluses might conserve (for an unknown reason) their methylated state in the absence of auxin. Perhaps, the methylation state becomes irreversible beyond a certain level of methylation of the genome. In this light, it must be mentioned that in tomato (*Lycopersicon esculentum*), the methylation level was shown to be higher in a habituated clone as compared to an auxotrophic callus (Karp, 1993). Moreover, in habituated nonregenerating callus of rice, some formation of embryogenesis can be restored after treatment with 5-azacytidine (Mutambel, unpublished results). The observations of a reversion of habituated callus into normal hormone-dependent callus (Hervagault et al., 1991; Syono and Fujita, 1994) further strengthens the hypothesis of an epigenetic basis in the control of the hormonal autonomy.

Although the formal similarities between habituation and variation of DNA methylation are striking, there is at present little data to indicate that there is a direct relation between these two phenomena. Therefore, it will be interesting to (a) compare the 5-methylcytosine content of habituated and nonhabituated cells (in the presence or after removal of auxin), (b) analyze if the habituated state may be reversed by agents that induce hypomethylation of DNA (e.g., 5-azacytidine), and (c) utilize marker genes that can be used to screen for differences in DNA sequences methylation in cells expressing different states of habituation.

Loss of totipotency. There is at present no direct proof that, as suggested above, the loss of totipotency during long-term callus cul-

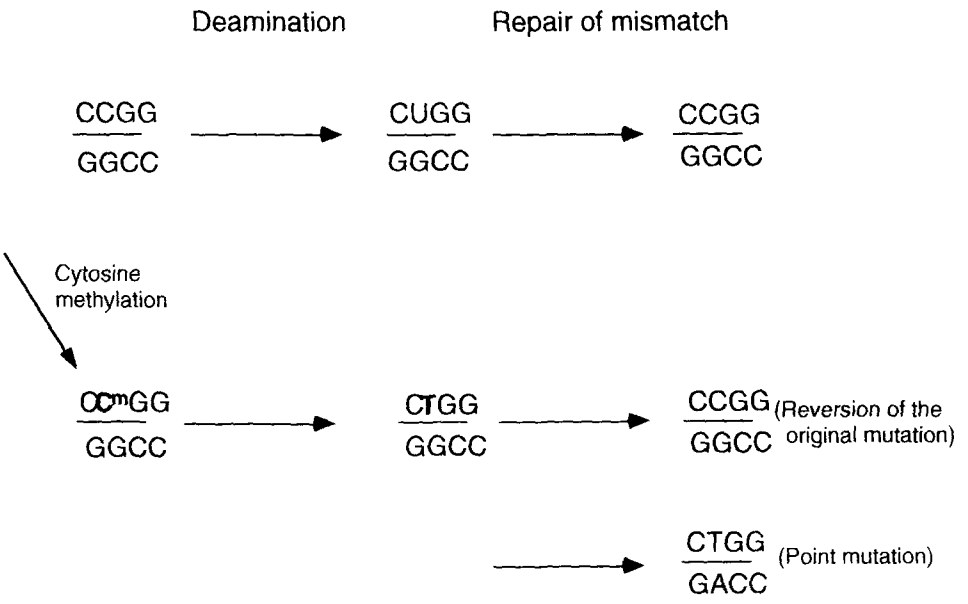

FIG. 1. Potential creation of mutation in 5-methylcytosine containing DNA during plant tissue cultures (according to Phillips et al., 1994).

ture may reflect the progressive elimination of cells capable of differentiating during the culture or the evolution to a permanently and irreversible silencing of differentiation genes. This mechanism has been proposed to occur in animals during immortalization and production of terminal-differentiation resistant cell lines (Jones et al., 1990; Razin and Cedar, 1991). However, some indirect observations also support this hypothesis in plant cell cultures.

It appears from numerous experimental systems that prior to each differentiative step in plants, the cellular level of some enzymes of the polyamine pathways such as ornithine decarboxylase (ODC) or arginine decarboxylase (ADC) increases. This has been observed prior to embryogenic development in *Daucus carota* (Montague et al., 1979; Feirer et al., 1984) and in bud differentiation in *Chrysanthemum morifolium* (Aribaud et al., 1994). This type of observation is also described in lower plants; for instance, during differentiation in fungi (Ruiz-Herrera, 1994) and in yeast (Guevara-Olvera et al., 1993). Moreover, application of inhibitors of ODC or ADC block several developmental processes in fungi (Ruiz-Herrera, 1994), yeast (Guevara-Olvera et al., 1993), and higher plants (Feirer et al., 1984; Fienberg et al., 1984; Mengoli et al., 1989; Martin-Tanguy and Carré, 1993). Moreover, it has been shown that the yeast-to-mycelium differentiation, which is blocked in the presence of ODC inhibitor, may be reversed by putrescine, or by growing the cells in the presence of 5-azacytidine (Guevara-Olvera et al., 1993).

The ODC and ADC enzymes seem to have a central role in redirecting a differentiation pathway. It has been reported that calluses which had lost their totipotency do not show a typical polyamine peak seen in totipotent cultures after subculturing to media that normally allow differentiation (Fienberg et al., 1984). All the previous observations may indicate that the gene coding for ODC (or other) may be silenced in callus cultures that have lost their differentiation potential. This is supported by the fact that this gene has been shown to become methylated in some animal-cultured cell lines (Table 1). Much support for our hypothesis comes from observations by Speranza and Bagni (1977), who have shown that crown-gall tumors (nondifferentiating cells) of *Scorzonera hispanica* showed altered ADC activity. Moreover, Bastola and Minocha (1995) have recently transformed *Daucus carota* cells with the cDNA of the mouse ornithine decarboxylase. The transgenic cell lines showing high ODC activity (which caused a significant increase in cellular putrescine and spermidine) always exhibited a higher degree of somatic embryogenesis compared to those cell lines that showed lower ODC activity.

In order to verify the above hypothesis, it will be interesting to (a) quantify ODC (ADC) activity in normal and nonregenerating callus in conditions that normally induce differentiation, (b) study the effect of 5-azacytidine on ODC activity in nonregenerating callus, (c) analyze if this hypomethylating agent restores some differentiation potential, and (d) determine the methylation status of the ODC gene in totipotent and nontotipotent culture.

Reversibility-irreversibility of the process and relation to tumor progression in habituated callus. Together with tumors induced by pathogens, fully habituated calluses have been classified as neoplasms (Braun, 1978; Pengelly, 1989). However, the process of habituation does not normally implicate genetic alterations because, in most cases, this process is reversible. Because in numerous cases other irreversible alterations (irreversible loss of totipotency) occur, it has been postulated that progression to habituation may represent one step before irreversible alteration (mutation). Based on several data in the animal kingdom, one possibility is that the most important mutagen is endogenous. It could be the high 5-methylcytosine content of habituated callus or of callus maintained for a long time in the presence of auxin (at least 2,4-D).

It is well known that cytosine undergoes spontaneous deamination to uracil producing a mismatch that the cell can readily detect and repair because uracil is not a normal component of DNA. 5-Methylcytosine also deaminates spontaneously to produce, in this case, thiamine. In vertebrate genome, this conversion has been estimated to occur at the rate of 1 per 10 000 methylated cytosine per year

(Cooper and Krawczak, 1989). After this conversion, the DNA repair enzymes can also detect a GT mismatch. However, there are two ways to correct this mismatch: replace the T with a C (reverse the original mutation) or replace the G with an A (produce a GC to AT transition) (Fig. 1) (Phillips et al., 1994). Methylated cytosine may thus be regarded as a hotspot for GC to AT transition (mutation). The fact that 5-methylcytosine may act as an endogenous mutagen is moreover supported by the observation that although 5-methylcytosine represents less than 1% of the base in the human genome, it is estimated that 35% of point mutation occurs at CpG nucleotides (the most frequent sequence that shows cytosine methylation in mammalians) and more than 90% of these are GC to AT transition (Rideout et al., 1990). More specific evidence for the role of 5-methylcytosine as a mutagen comes from an analysis of sites of mutation in the p53 gene in humans (Nigro et al., 1989). Sequence data on 21 tumors that were hemizygotes at the p53 locus revealed three sites where a point mutation had occurred in more than 1 tumor and in each case a CpG sequence was involved. Forty-three percent of the point mutations were at one of these sites. All three sites were found to be methylated in all tissues tested (Rideout et al., 1990). This type of carcinogenesis, where the most important mutagen is endogenous, has been proposed to be an important factor in cancers found in some young children, where no environmental factors or pathogens have been detected (Buckley, 1992). In this view, it must be mentioned that the auxin 2,4-D has been shown to induce mutation in cultured mammalian cells (Pavlica et al., 1991). This may perhaps be correlated with the higher methylation rates found in plant cells in the presence of this growth regulator. Such hypothetical GC to AT transition in nonreversible, nonregenerating callus may probably be confirmed by Restriction Fragment Length Polymorphism (RFLP) analysis. Such a transition was identified in tissue-culture-induced mutants, which show resistance to the herbicide chlorsulfuron in *Arabidopsis thaliana* (Mazur et al., 1987).

Altered levels of DNA methylation during *in vitro* culture may also induce other irreversible alterations such as chromosome breakage, translocation, inversion, deletion, and duplication. In fact, several studies have associated increased methylation with heterochromatinization of chromatin (Ball et al., 1983; Keshet et al., 1986). These authors have shown, using antibodies against 5-methylcytosine, that the nucleosomes containing histone H1 contain more methylcytosine. Because H1 is involved in chromatin condensation, increased 5-methylcytosine content could affect the rate of DNA duplication. This delayed replication could cause anaphase bridges, chromosome breakage, and rearrangements (Phillips et al., 1994), which are common in callus cultures or in plants regenerated from tissue cultures (Lee and Phillips, 1988; Ziauddin and Kasha, 1990; Kaeppler and Phillips, 1993).

Conclusion

There is an increasing body of information indicating that plant tissue culture is clearly mutagenic. Specific genomic alterations associated with tissue culture variation have been well characterized, but the mechanism leading to these changes is not well understood. This review has concentrated on changes in DNA methylation as a basis for such variation. Our hypothesis contributes to a better understanding of how epigenetic alteration (DNA methylation change) during callus culture may be one step in the progression through irreversible modification (mutation) during *in vitro* maintenance of plant cells and progressive irreversible loss of organogenic totipotency at the terminal stage.

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