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DNA methylation of embryogenic carrot cell cultures and its variations as caused by mutation, differentiation, hormones and hypomethylating drugs

F. LoSchiavo [1], L. Pitto [2], G. Giuliano [2], G. Torti *, V. Nuti-Ronchi [2], D. Marazziti [2], R. Vergara [2], S. Orselli [2] and M. Terzi [3]

[1] Istituto Internazionale di Genetica e Biofisica, CNR, C.P.3061, I-80100 Napoli, Italy
[2] Istituto di Mutagenesi e Differenziamento, CNR, Pisa, Italy
[3] Dipartimento di Genetica, Biologia Generale e Molecolare, Università di Napoli, Italy

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Summary. The level of auxin – both natural and synthetic – in the medium has a strong effect on the level of 5-methyl-cytosine in the DNA of carrot cells in culture. This level may vary from approximately 15% to 70% of total cytosine without apparent effects on growth rate and cell morphology. No effect was seen with cytokinin. During somatic embryogenesis, in the absence of hormones, variations were seen in the level of methylation according to a characteristic pattern. If hypomethylation is induced with drugs such as azacytidine, ethionine or ethoxy-carbonyl-pyrimidine, embryogenesis is immediately blocked. A mutant was isolated which is resistant to the action of hypomethylating drugs. It shows variations in the methylation pattern and variations in indole-acetic acid metabolism. In addition its regeneration is often associated with the production of tumors.

Key words: Methylation – Somatic embryogenesis – Auxin – Tumors – Developmental mutants

Introduction

DNA methylation is abundant in plants, but its role is still to be elucidated. There is much more information from animal systems, but differences in DNA methylation between plants and animals are certainly present; e.g. 5-methyl-cytosine(MC) is always adjacent to guanine in animal systems but not necessarily so (CXG is a definite possibility) in plant systems (Vanyushin 1984).

As for the biological meaning of methylation, a correlation has been made in animal systems between hypomethylation and gene expression (Van der Ploeg and Flavell 1979). There is some controversy on the general validity of this correlation (Cooper 1983). One of the difficulties is that we cannot pre-determine the level of methylation, we can only lower it with hypomethylating drugs. Among these are azacytidine (aza-C) (Jones and Taylor 1980), ethionine (Cox and Irving 1977) and 2-amino5-ethoxy-carbonyl-pyrimidine-4(3H)one (ECP) (Raugei et al. 1981).

Carrot embryogenesis is one of the best-characterized differentiative systems in vitro: a proliferating cell culture can be made to differentiate by simply removing the hormones from the medium and, similarly, embryogenesis, after its start, can be blocked by the addition of auxin (for general reference, see Sung et al. 1984). We have studied the level of methylation in this system during the course of somatic embryogenesis and have determined how this level is influenced by natural and synthetic auxins. Moreover we have isolated and characterized a mutant line that does not respond to hypomethylating treatment and that shows a different indole-acetic acid (IAA) metabolism.

Materials and methods

Carrot cells (established from seedlings of *Daucus carota* var. S. Valery) were grown in suspension in Gamborg's B5 medium (commercially available from Flow). The medium for proliferation was supplemented with 0.5 mg/l 2,4-dichloro-phenoxy acetic acid (2,4-D) (from BDH) and 0.25 mg/l 6-benzyl-aminopurine (BAP) (Sigma). For additional details see Pitto et al. (1985).

Embryogenesis was started by diluting and transferring the cells to B5 medium devoid of hormones.

Purification of embryonic stages was performed according to our published procedure (Giuliano et al. 1983).

Mutagenesis was performed with ethyl-methane sulfonate (EMS) 0.8% (v/v) for 1 h at room temperature in a screw-cap

* Deceased

tube. The culture was then centrifuged and washed three times with fresh medium. This treatment kills about 80% of the colony-formers. Selection for resistant mutations was applied at the onset of embryogenesis and developing variants were scored and picked manually under the dissecting microscope.

Protoplasts were prepared according to Cella et al. (1983). Protoplast fusion was performed according to Schieder (1978): 5.10^5 protoplasts of the mutant line E9 inactivated for 20 min with 0.3 m*M* iodoacetate (lithium salt, Calbiochem) at 25 °C were fused with equal amounts of wild type (wt) protoplasts and plated in 5 ml protoplast medium containing 0.4% Sea-Plaque Agarose (Marine Colloids). After 1 week, 5 ml of embryogenic B5 medium were overlaid on the agarose. After one additional week, the agarose "bead" was transferred in 10 ml of B5, disrupted by vigorous pipetting, and selection (ECP, 400 µg/ml) was applied.

DNA isolation and MC determination. Freeze-dried cells and embryos were ground with quartz sand, treated with pronase and extracted with chloroform: octanol according to Murray and Thompson (1980). DNA was hydrolysed in sealed glass vials with formic acid and run on C8 reverse-phase column in high-performance liquid chromatography (HPLC) according to Citti et al. (1983).

Tyrosinase activity was determined spectrophotometrically according to Romeo and Preston (1984).

Labelling of cells and embryos and analysis of tryptophan and IAA metabolic products. Cells and purified embryos were labelled for 2 h with 16 µCi/ml of 5-^{3}H-tryptophan (specific activity 28 Ci/m*M*, Amersham) or, in other cases, with 7.5 µCi/ml of 3-[5(*n*)-^{3}H-Indolyl-acetic acid] (specific acitvity 25 Ci/m*M*). In the case of IAA, this compound, bought from Amersham, was re-purified before use. In some of the experiments, after labelling with IAA, radioactivity was chased with cold IAA (1 m*M*) for 4.5 and 15 h, as stated in the text. The labelled cells or embryos were freeze-dried and extracted overnight with 70% acetone. The solid was rinsed with 70% acetone, counted and discarded. The acetone was evaporated at 30 °C under a nitrogen stream and the resulting water phase was loaded onto an anion exchange and a cation exchange column connected in series (Supelchem Supelclean SCX and SAX). The eluates and the washings of the columns were freeze-dried and checked for alkali-labile IAA-conjugates. The anion exchange (SAX) column was eluted with 4 ml of 4% formic acid. The eluate was made 0.05 *M* with KH_2PO_4 buffer (pH 2.5) and loaded on a Sep-PAK_{18} Cartridge (Waters Associates). After washing with 4 ml of 0.05 *M* KH_2PO_4 (pH 2.5), the cartridge was eluted with 5 ml of diethyl-ether. The ether was evaporated under a nitrogen stream. This sample represents the "Acid fraction". The cation exchange (SCX) column was eluted with methanol. Methanol was evaporated under a nitrogen stream. This sample represents the "basic fraction".

The samples after drying under nitrogen were resuspended in methanol and analysed by silica gel (Merck 60 F_{254}) thin-layer chromatography (TLC) using the following developing system: $CHCl_3$,AcOH,H_2O (90:9.5:0.5) in the tryptophan labelling experiment. In the IAA labelling experiment, two different systems were used: for the "acid fraction" CH_3CN:AcOH:H_2O (84:15:1) and for the "basic fraction-butanol: AcOH: H_2O (90:10:20).

Results

The auxin level influences methylation

The ratio of MC to total cytosine in the DNA of our wild type (wt) line, grown in the usual level (0.5 mg/l) of 2,4-D is 16%. If however, the level of 2,4-D is raised to 2 mg/l or 5 mg/l, after 5 days, the level of MC reaches 40% and 45%, respectively.

Table 1. 5-methyl cytosine to total cytosine %

Hormone	Concentration (mg/l)	% MC
2,4-D	0.5	16
	2	40
	5	45
NAA	2	19
	5	23
IAA	2	19
	5	21
BAP	2	16

The first row constitutes the control in representing the usual growth conditions. Its precise methylation level is 15.7 ± 0.8. The stated concentrations represent the final one. To that, 0.25 mg/l BAP or, last row, 0.5 mg/l 2,4-D should be added. The cells were kept 5 days in these different types of media and then were freeze-dried, the DNA extracted and HPLC run according to the procedure described under "Materials and methods"

This increase in methylation is not paralleled by obvious changes in the cells that keep dividing regularly, following the same growth curve, and keep their embryogenic potential (see below).

The hypermethylating activity is not restricted to 2,4-D, because when other auxins such as naphthylacetic acid (NAA) or IAA were used instead of 2,4-D in the medium for 5 days at concentrations of 2 and 5 mg/l, they also caused an increase in the level of methylation. Instead, cytokinins such as 6-BAP, from 0 to 2 mg/l did not cause appreciable variations in the level of methylation (Table 1).

Demethylation is also very fast: from the highest level of methylation we were able to obtain (70%, after 22 days of growth in 5 mg/l 2,4-D), the cells went back to 15% after 1 week of growth in the usual concentration of 2,4-D (0.5 mg/l).

Methylation in the course of somatic embryogenesis

When 2,4-D is removed from the medium and, at the same time, the cell concentration is lowered, embryogenesis starts and the level of methylation shows an initial drop followed by a raise at later stages according to a characteristic pattern (Table 2).

Hypomethylating drugs

If ECP is added at 500 µg/ml to our wt cell culture just induced to differentiate, formation of normal embryos is $\geq 90\%$ inhibited. (The same effect is produced by aza-C and ethionine at concentrations of 1 and 3 µg/ml, respectively). The few forms that develop are mostly aberrant:

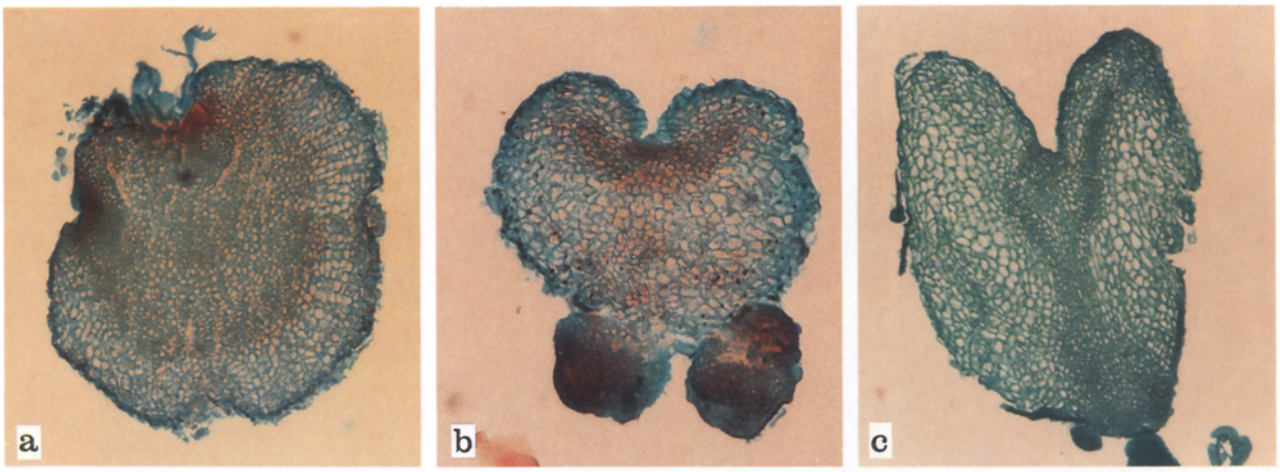

Fig. 1a–c. Sections of embryonal forms arrested by ECP. **a** globular; **b** heart-shaped, with arrested secondary globular embryos; **c** torpedo-shaped embryos. Paraffin-embedded material was cut in a rotary microtome and stained with safranin, fast green

Table 2. Changes in % of 5-methylcytosine during embryogenesis

Stages	% MC/total cytosine
Cells	16
Early embryos (pre-globular)	14
Late embryos (heart-torpedo)	19
Plantlets	20

Table 3. Effects of ECP on proliferating cell cultures; **A** cell growth (grams of fresh weight/50 ml of culture); **B** DNA and protein synthesis (TCA-precipitable counts/100 mg cells) [1]

Cells/time (days)	0	8	18
A			
Control	2	6.7	29.7
ECP-treated	2	3.8	21.1
B			
		DNA	Protein
Control		5,071	93,872
ECP-treated		5,402	97,318

[1] A final concentration of 1 μCi/ml of tritiated thymidine (Amersham, 10 Ci/mmol) or 1 μCi/ml ^{14}C-leucine (150 Ci/mmol, Amersham) were added to 8-day-old cultures for 5 h

Table 4. Effect of ECP-pre-treatment on embryogenesis

Cells	Embryogenic efficiency
Control	3.2%
ECP-treated	0.3%
Treated and rescued	3.5%

Cells were set to differentiate in basal medium at time zero. Control untreated (first row) or after biweekly cycles in the presence of ECP (second row) or treated and left to recover for 1 week in growth medium before being shifted to basal medium. The embryogenic efficiency is the ratio of embryos scored 3 weeks after the induction of differentiation to the number of cell clumps inoculated at time zero

globular forms show hypertrophy and lack of polarity, heart-shaped embryos lack hypocotyl and root primordia, but a well-developed cotyledonary procambium is already apparent – torpedo-shaped embryos lack the root but hypocotyl is present; secondary arrested embryos are of frequent occurrence (Fig. 1).

If embryos of different stages are purified according to the technique routinely used in our laboratory (Giuliano et al. 1983) and treated with ECP, a sudden arrest of development is seen, followed by hypertrophic growth and, oddly enough, by greening.

Cells in non-embryogenic conditions instead, when treated with ECP, undergo only a transient inhibition in volume growth, and general toxicity effects can be excluded by the lack of inhibition of DNA synthesis (Table 3). A similar transient inhibition of volume growth is also exhibited by ethionine. Interestingly enough, however, pre-treatment with ECP of undifferentiated cells reduces their embryogenic potential in a reversible way (Table 4).

The embryo-associated functions described in carrot are few. Among these, tyrosinase induction is one of the first to be expressed and best characterized (Pitto et al. 1985). The block caused by ECP involved also this finer level: if added at time zero (time of hormone removal and cell dilution, ECP prevents the induction of tyrosinase which, in the control, goes from 12 to 55 units/mg protein in 48 h. If ECP is added 12 h after the onset of embryogenesis, the induction of tyrosinase has already occurred (Table 5).

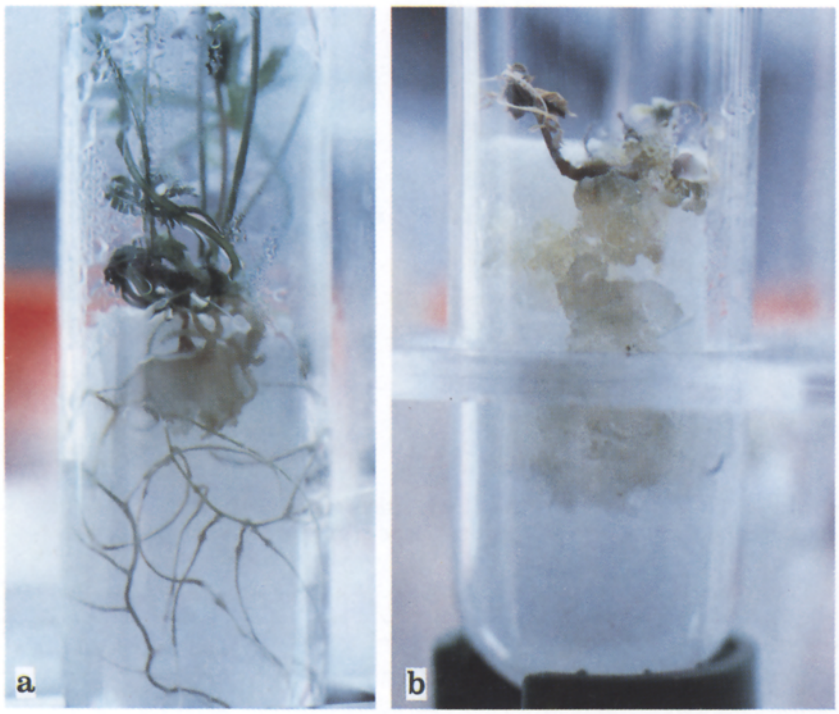

Fig. 2. **a** A regenerated plant from E9. Notice the root-associated tumors; **b** another regenerated plant from the same line, where the callus outgrowth completely abolished normal plant morphology

Table 5. Tyrosinase activity (units/mg protein)

Cells/time (days)	0	0.5	2	8
Control (no ECP)	12	12	55	45
ECP added at time zero		12	20	22
ECP added at time 0.5				50

Table 6. Percent MC in ECP treated cells percent of untreated controls, made equal to 100, reported in brackets. Values are given for wt and the variant line E9

	wt	E9
Proliferating cells	13 (79)	18 (89)
Cells, high density, no 2,4-D	10 (66)	18 (100)
Embryos	13 (64)	20 (100)

In order to correlate the biological and biochemical effects of ECP, DNA of cells or embryos, untreated or treated for 15 days with the drug, was extracted, hydrolysed and its MC content analysed by HPLC. The loss in methyl groups caused by the drug treatment was 21% in the case of proliferating cells, 34% in cells kept at high density without auxin, and 36% in the embryos, showing that it is the lack of hormones, rather than the differentiative state of the cells, that enhances the hypomethylating effect. (It is recalled that cells without auxin start embryogenesis only after their dilution) (Table 6).

Isolation and characterization of an ECP-resistant variant

A cell line capable of producing plantlets in the presence of ECP was isolated after EMS mutagenesis. An embryo developing in the presence of the drug was picked, submitted to a cycle of dedifferentiation-regeneration and analysed for the stability of the resistance trait. The resulting line, designated E9, showed cross-resistance to azacytidine but not to ethionine.

This resistant line is capable of regenerating in hormone-free medium, but its efficiency is lower and more cell-density-dependent than wt. Moreover, the formation of embryos was accompanied by cell growth, and further dilution of the embryogenic culture was, in general, necessary. The embryonal forms look normal but plantlets show (particularly associated with roots) numerous tumors which, in extreme cases, completely abolish normal plant morphology (Fig. 2).

In order to test the dominance of the character, E9 protoplasts – inactivated with iodoacetate – were hybridized with wt. As the hybrid was capable of regenerating in the presence of ECP, we concluded that the character is dominant.

The DNA of E9 was analysed by HPLC. The results, presented in Table 6 indicate that E9 DNA is less subject to variations, its methylation level being rather constant, with or without auxin and with or without ECP.

Studies on auxin metabolism

The E9 line is capable of regenerating but the constant presence, in embryogenic conditions, of tumors as well as

that of growing cells are indications of a hormonal imbalance. In order to characterize this imbalance, we studied the incorporation of labelled tryptophan to analyse IAA synthesis, and studied the incorporation of labelled IAA (in pulse-chase experiments) to study its catabolism.

When tritiated tryptophan was given, it went into the acetone-soluble fraction in wt embryos but not E9 (Table 7). This was not entirely due to the simultaneous presence, in E9 regenerating cultures, of embryos and cells, because hand-picked plantlets (see last row of Table 7) were still high in precipitable counts.

Differences in metabolism were also seen in the methanol fraction (not presented) and in the "acid fraction" eluted with ether, which shows that a band corresponding to cold IAA (marked by an arrow in Fig. 3) is present in E9 plantlets, much less in E9 cells (from purified plantlets) and is undetectable in wt plantlets.

When tritiated IAA was used as a precursor for 2 h and then chased for 4.5 or 15 h, the results presented in Table 8 were obtained: the neutral and basic fraction are more relevant in E9 than wt. The major (unidentified) component present in E9 plantlets is different from the one present in E9 cells and wt (Fig. 3b).

Table 7. Partition of radioactive counts from incorporated tryptophan. (Recovered counts in freeze-dried material expressed as % of total)

Extract	Fraction	
	Solid	Acetone-soluble
Wt cells	74	26
globular-heart	9	91
torpedo	10	90
plantlets	13	87
E9 cells	65	35
globular-heart	61	39
plantlets (unfraction.)	50	50
plantlets (purified)	31	69

When the unfractionated E9 plantlets were manually separated from the accompanying cells we obtained two fractions: the purified plantlets (last row) and another fraction which was indistinguishable from the previous E9 cells

Discussion

Our results show that in proliferating cell cultures of carrot, there is a positive correlation between exogenously-added auxin and methylation of cytosine: passing from 0.5 to 5 mg/l 2,4-D leads to an increase of MC from 16% up to 70% of total cytosine. These induced changes in the level of methylation are rather fast and reversible.

In the absence of auxin, embryogenesis takes place and shows, in its course, an initial decrease in methylation, followed by an increase during late embryogenesis. This "basal level" of methylation observable in the absence of hormones, with its temporal variation during embryogenesis, cannot be changed without impairing the process: both hypermethylation (as caused by auxin) and hypomethylation (as caused by hypomethylating drugs) immediately and irreversibly stop embryogenesis and the expression of embryo-related functions (one possible exception being greening).

If we pre-treat proliferating cells with ECP, we obtain a drop in the embryogenic potential. If however, the cells

Table 8. **A** Counts incorporated after a 2-h pulse of labelled IAA expressed as counts incorporated per μg freeze-dried material (first column) or as percentage (in the other columns); **B** same, pulse-chased

Extract	Total inc. counts	Acetone-insoluble	Fractions			Yield
			Acid	Neutral	Basic	
A						
Wt cells	155	1.3	77	0.69	10	0.89
E9 cells	114	1.2	64	5.6	19	0.89
Wt embryos	49	2.0	63	8.9	4.8	0.78
E9 embryos	62	0.7	54	6.0	9.3	0.70
B						
Wt cells, no chase	155	1.3	77	0.69	10	0.89
4.5-h-chase	86	2.2	53	1.5	23	0.79
15-h-chase	70	3.4	50	2.0	33	0.84
E9 cells, no chase	114	1.2	64	5.6	19	0.89
4.5-h-chase	84	1.0	43	4.8	48	0.96
15-h-chase	65	1.5	35	3.7	59	0.98

The counts of the acetone-insoluble fraction that could not be extracted with methanol were a negligible fraction (less than 1%)

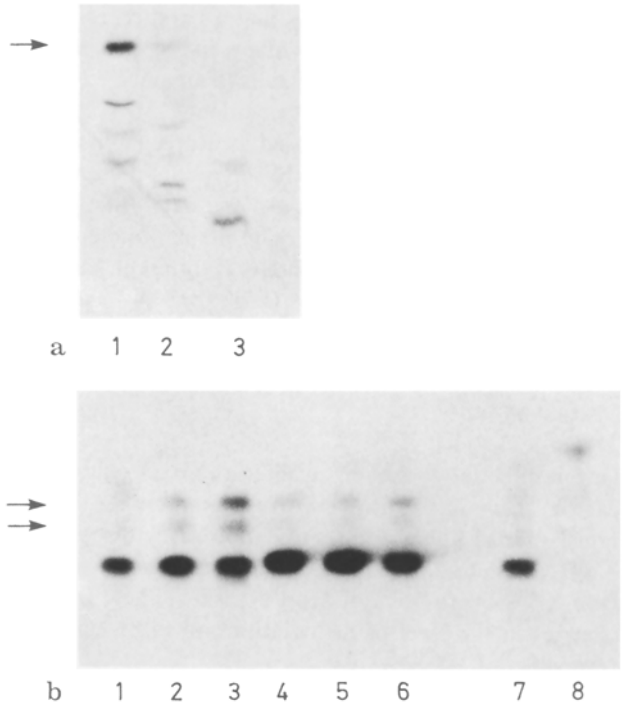

Fig. 3. **a** Autoradiogram of the TLC of the acid fraction eluted with ether of tryptophan-labelled extracts. *Lane 1* = E9 purified plantlets; *lane 2* = cells accompanying the regenerating plantlets of E9; *lane 3* = wt plantlets; **b** autoradiogram of a TLC of the basic fraction, eluted with methanol of IAA-labelled extracts. *Lane 1* = wt cells, pulsed, no chase; *lane 2* = same, chased 4.5 h; *lane 3* = same, chased 15 h; *lane 4* = E9 cells, pulsed, no chase; *lane 5* = same, chased 4.5 h; *lane 6* = same, chased 15 h; *lane 7* = wt plantlets, pulsed; *lane 8* = E9 plantlets pulsed.

are left to recover in the presence of auxin, the embryogenic potential is restored. In a specular way, hypermethylated cells, if left without hormones, lower their level of methylation and embryogenesis can occur.

In a purely formal sense, hypomethylating drugs and 2,4-D are antagonistic. This is shown by the fact that hypermethylated cells are more resistant to ECP and that an abortive differentiation starts when cells are left in the presence of ECP + 2,4-D (not shown).

Azacytidine and ECP do both cause hypomethylation but the target of their action is not the same. Azacytidine acts on DNA transmethylases (Jones and Taylor 1980). ECP in prokaryotes acts as a methyl acceptor (Raugei et al. 1981). In carrot this may not be the case, as the ECP action cannot be completed by methyl donors such as methionine or S-adenosyl-methionine. DNA methyl transferases were not inhibited, in an in vivo assay, by ECP (not shown).

The mutant line E9, which is resistant to ECP and azacytidine shows a higher internal level of IAA (at least in late embryonal stages) and a different metabolism and/or compartmentalization of IAA. At the level of methylation, E9 shows a somewhat higher level in proliferating cell cultures but, perhaps more meaningful, an almost constant level of MC, which does not vary during embryogenesis nor in the presence of ECP. This is at variance with out wt line but also with several other lines of carrot we tested which, in spite of the absolute level of MC which was somewhat variable, showed constancy in behavior in being hypomethylated by ECP and in following the characteristic curve of methylation during embryogenesis.

All these indications lead us to the inescapable conclusion that not all methylations are equivalent. What we would like to propose is the following: each and every tissue has its own level of methylation (we may say "pattern") which is necessary for the maintenance of the differentiative state. This is what we have called "basal state" and might be equivalent to the methylation level we encounter without auxin. The basal level would vary among different tissues and it does so during embryogenesis. If we induce a hypomethylation with drugs, this pattern is disorganized with serious consequences on, e.g., embryogenesis.

There is another type of methylation which occurs, more or less randomly, as a response to auxin. This type of methylation is not tissue-specific and is lost in the absence of its causative hormone. This methylation is, or occurs concomitantly with, a signal to divide, and disorganizes the differentiative patterns. In this light we can understand why in the phenomena involving a change of program, be it acquisition of embryogenic potential (Sung et al. 1984) or wound response, gall formation, tumor formation or adventitious root induction, the presence of auxin is always necessary (for general reference, see Kahl and Schell 1982).

Along these lines, E9, with its higher level of endogenous IAA, resistance to hypomethylation and proneness to tumors, difficulties in regeneration (always accompanied by cell growth) may prove very useful in helping to solve some of the outstanding problems concerning the active forms of IAA and its role in differentiation. We will gladly make it available to everybody willing to tackle these problems.

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