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An investigation into the role of 5-Azacytidine in tissue culture

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Summary. A major problem associated with cereal biotechnology remains the extreme difficulty of reliably and efficiently regenerating plants from protoplasts. Because of the assumed inverse correlation between levels of the modified nucleotide 5-methylcytosine in a gene and the degree of transcription, we report here on experiments to determine whether exposure of maize and tobacco cultures to the 5-methylcytosine analogue 5-Azacytidine (5-Azt) induces gene de-methylation and, as such, enhances tissue culture response, for example by increasing protoplast division frequency. The results show that whilst 5-Azt may be of use in expanding leaf areas capable of producing callus as well as increasing the amount of callus produced, in all other aspects 5-Azt is strongly inhibitory to growth at all but the lowest concentrations. Molecular analysis shows that no readily discernible changes in gene methylation status can be found, regardless of 5-Azt concentration or the gene probe used. Differences can, however, be found in methylation status between callus and developmentally determined tissues, irrespective of 5-Azt treatment. The results suggest that, apart from a very limited role, 5-Azt has no obvious use in tissue culture.

Key words: Gene methylation – Tissue culture – 5-Azacytidine

Introduction

Whilst the regeneration of rice plants from protoplasts has been a significant advance in the field of cereal tissue culture (review Vasil 1987), this has only emphasized the inability to obtain a corresponding result from other graminaceous species. This low efficiency of regeneration from cereal protoplasts remains a considerable obstacle in the application of modern methods of single gene transfer to these important crop species. Even in systems where tissue culture and plant regeneration are regarded as standard techniques (as in many members of the Solanaceae), problems such as somaclonal variation and habituation are now regarded as being an integral, though often unwanted characteristic. In cereals, regeneration via callus and other multicellular explants remains the main tool for the geneticist, with all the associated problems, such as explant source and medium composition. In view of these problems, attempts are being made to investigate more thoroughly the molecular aspects of tissue culture and, thereby, to reduce these limitations.

As part of this work, the role of gene methylation in tissue culture and, in particular, the role of the anti-methylation analogue 5-Azacytidine (5-Azt) was investigated. Alterations in the level of 5-methylcytosine residues in DNA have been shown to be closely related to the degree of gene expression (for review, see Doerfler 1983). Sellem et al. (1985) were able to show that the entire 5′ region in transcriptionally active mouse albumin genes was unmethylated, whilst in non-active cells this sequence was heavily methylated. Despite recognition that DNA methylation can inhibit gene expression, the mechanism of this inhibition is not understood, though it is assumed that such modifications are related to the binding of specific regulatory proteins or other elements of transcriptional control. The pattern of DNA methylation also changes during development. There are many similarities in methylation between plants and animals, although significant differences do exist, not the least

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being that in mammalian systems, 5-methylcytosine is the sole modified base appearing exclusively as CpG dinucleotides. Plant DNA is exceptional in that methylated residues can exist as CpG or CpXpG sequences, and also that the level of methylated residues is significantly higher than that found in mammals (Gruenbaum et al. 1981).

The amount of work relating to plant methylation is limited, though examples confirm a pattern similar to that found in animals. Watson et al. (1987) found that in young seedlings of *Pisum sativum*, methylation levels were generally low, whilst DNA from apical buds was highly methylated. However, as development progressed, the methylation level of sequences such as the rDNA genes decreased. As it is now generally accepted that a correlation exists between gene methylation and expression, the possibility of a general activation of genes, including those that may be associated with tissue culture (Hodges et al. 1986), by use of the anti-methylation agent 5-Azacytidine (and by definition gene activator), raised intriguing possibilities. Although the mode of action is unknown, it is proposed that incorporation of 5-Azt into DNA inhibits the progress of a non-specific DNA methyltransferase (Taylor and Jones 1982). However, the paradox is that if the analogue acts as a general enzyme inhibitor, then a general increase in gene expression would be expected, rather than the selective increases usually found (Jones 1985). Experiments using 5-Azt in plant systems are limited and relate predominantly to induction of T-DNA genes in transformed plants. Hepburn et al. (1983) found that treatment of a flax tumour line with 5-Azt resulted in the demethylation of, on average, one copy of the nopaline synthase gene per cell. Amasino et al. (1984) found that 5-Azt treatment of a heavily methylated tobacco line in which T-DNA was not expressed resulted in subsequent expression and phytohormone-independent growth. We report here on a series of experiments, the purpose of which was to investigate whether exposure of tissue cultures to the anti-methylation agent 5-Azacytidine would result in the demethylation and subsequent activation of genes and an associated enhancement of tissue culture response.

Materials and methods

Plant growth conditions

Seeds of *Nicotiana tabacum* L. cv Xanthi, were surface-sterilised by immersing briefly in 70% ethanol, followed by 1% NaOCl for 10 min. The seeds were then washed five times in sterile distilled water, placed on hormone-free half-strength MS medium containing 1.5% sucrose and 0.8% agar in petri dishes and allowed to germinate at 26 °C. The seedlings were transferred to the same medium in glass containers and kept under a 16-h photoperiod. Axenic shoot cultures were maintained by repeatedly excising shoot tips and transferring them once a month to fresh medium.

Seeds of *Zea mays* L. inbred line A188 were immersed in running water overnight and sterilised by washing with 70% ethanol for 1 min, followed by stirring in 1% NaOCl for 40 min. The sterilised seeds were washed five times in sterile distilled water and kept under the conditions described above. For callus induction experiments, seeds were germinated and grown under dark conditions. After 8 days growth, some of the etiolated seedlings were moved to 16-h light conditions.

Tobacco protoplast isolation

For mesophyll protoplast isolation, young leaves (third and fourth leaves) were placed in a 6-cm petri dish, to which was added a few drops of a 0.3 *M* mannitol solution before cutting the leaves into 3–5 mm^2 pieces. These pieces were then washed by shaking in 10 ml of 0.3 *M* mannitol solution before removing the solution with a pipette. The shredded leaf material was then transferred into an enzyme solution (5 ml/leaf) which consisted of 1% Yakult R10 cellulase and 0.6 *M* mannitol (pH 5.5), and then incubated at 27 °C for 2–3 h with agitation at 60 rpm. After digestion, the leaf material and enzyme solution were filtered through a 100 μm wire mesh sieve. The filtrate was diluted with an equal volume of sterilised sea water adjusted to 700 mOsm and centrifuged for 3 min at 800 rpm. The pellets were washed once by suspending and centrifuging in sea water and resuspended in a 20% sucrose solution followed by transfer to a centrifuge tube. One to 2 milliliters of 10% mannitol solution were then layered gently on top of the suspension. After centrifugation at 1,000 rpm for 10 min, the intact protoplasts present at the interface were removed, washed once with sea water and counted with a haemocytometer.

Protoplast culture

The procedure of regeneration from tobacco mesophyll protoplasts to plants was carried out according to the feeder method of Firoozabady (1986) with a modification of the culture medium. Protoplasts were suspended at a density of 10^5 protoplasts per ml in V47As medium (V47 supplemented with 1.7% sucrose, 1% glucose, 0.4 mg/l BAP, 1.5 mg/l NAA, 877 mg/l glutamic acid, 266 mg/l aspartic acid and 174 mg/l arginine, pH 5.5). Two milliliters of the protoplast suspension was incubated in a 6-cm petri dish at 26 °C for 1 week under dark conditions. Subsequently, an equal volume of MS2 medium (MS salts and vitamins supplemented with 0.5 mg/l of thiamine-HCl, 1 mg/l NAA, 0.2 mg/l BAP, 4% glucose and 3% sucrose, pH 5.5) was added weekly to the cultures until colonies grew to 0.2–0.5 mm. These colonies were then transferred to double-filter feeder plates, which were layered on top of *N. plumbaginifolia* suspension cells in SMPi medium (Firoozabady 1986) (MS salts and vitamins, 3% sucrose, 7.5 mg/l Zip [6-(88-dimethylallylamino)-purine], 0.1 mg/l pCPA and 0.7% agar, pH 5.5). After 1 week incubation under light conditions, the transfer disk was removed and placed on SMPi medium until the first colonies developed. The green globular colonies were again transferred individually onto new SMPi medium for shoot development. Subsequently, shoots were cut and transferred onto hormone-free MS medium supplemented with 3% sucrose and 0.7% agar (pH 5.5), and grown under 16-h light conditions at 26 °C.

Callus induction from maize leaves

Maize leaves were harvested from 9- and 14-day-old seedlings. Regular sections were cut beginning approximately 0.5 mm above the apical meristem proceeding upwards in sections of 1.0 mm to obtain 8–10 sections (Wernicke et al. 1986). The rolled leaf sections were separated and placed on MS medium containing 3% sucrose and 2 mg/l Dicamba, in an order corre-

sponding to their position in the leaf. The segments were then cultured in the dark at 26 °C. After 1 month incubation, the callus induction response of the leaf explants was assessed.

Preparation and analysis of DNA

DNA isolation from all plant material was carried out according to the modified CTAB procedure as described by Taylor and Powell (1985). All restriction enzymes were used according to the manufacturer's specifications. DNA restriction fragments were separated by electrophoresis on 1% agarose gels and transferred to Hybond TM (Amersham) using the manufacturer's protocol. The filters were pre-hybridised for 4–6 h at 45 °C in prehybridisation buffer consisting of 50% formamide, 5 × Denharts solution (1 × Denharts solution contains 0.02% Ficoll, 0.02% BSA, 0.02% PVP), 5 × SSPE (1 × SSPE contains 0.18 *M* NaCl, 10 *M* $Na_2HPO_4 \cdot 2H_2O$, 1 m*M* EDTA, pH 7.0), 0.1% SDS and 100 µg/ml denatured herring sperm DNA. The probes used were the 3.0-kb HindIII actin fragment of pSAC3 from soybean (courtesy of Dr. B. Baker), the 1.2-kb Pst 1 ATP/ADP translocator gene (courtesy of Prof. C. Leaver) and the 4.0-kb BamHI/Sph I promoter and 4.4-kb HindIII coding sequence of the sucrose synthase gene (courtesy of Dr. W. Werr). These were labelled with alpha ^{32}P dCTP by nick translation (Rigby et al. 1977) to a high specific activity ($0.5–2.0 \times 10^9$ cpm/µg) and then hybridised to the filters overnight at 42 °C. The filters were then washed once in 2 × SSC buffer and 0.5% SDS at 60° or 65 °C for 15 min, two times in 2 × SSC and once in 1 × SSC and 0.5 × SSC each for 10 min. The washed filters were then placed against Kodak XAR-5 X-ray film at −70 °C for 24–48 h using Dr. Goos cassettes and intensifying screens.

Results

The model system for these experiment was the well-characterised regeneration of tobacco plants from mesophyll protoplasts. The effects of 5-Aza on protoplast and tissue development at five different stages of regeneration were tested as shown in Fig. 1.

Effect of 5-Aza on tobacco protoplast development. In order to determine whether 5-Azt had any effect on protoplast growth or division frequency, freshly isolated protoplasts were incubated in V47As medium for 1 week, followed by addition of an equal volume of MS2 medium containing 5-Azt (Fig. 1 A). The addition of 5-Azt, even at concentrations as low as 5 µ*M*, strongly suppressed micro-callus formation. When micro-calli, which developed from cultures containing only 1 µ*M* 5-Azt, were isolated and transferred to fresh medium without 5-Azt, further development was unaffected. However, addition of 5-Azt at concentrations higher than 10 µ*M* resulted in a permanent inhibition of growth, so much so that protoplasts isolated from these calli were incapable of division. Protoplast experiments were extended (Fig. 1 B) by embedding 1-week-old cell clusters into 1.2% agarose sections, which were then incubated in fresh V47As medium as a bead-type culture. This allowed easy transfer of cells, as well as allowing the media containing 5-Azt to be changed more readily, ensuring a steady concentration of

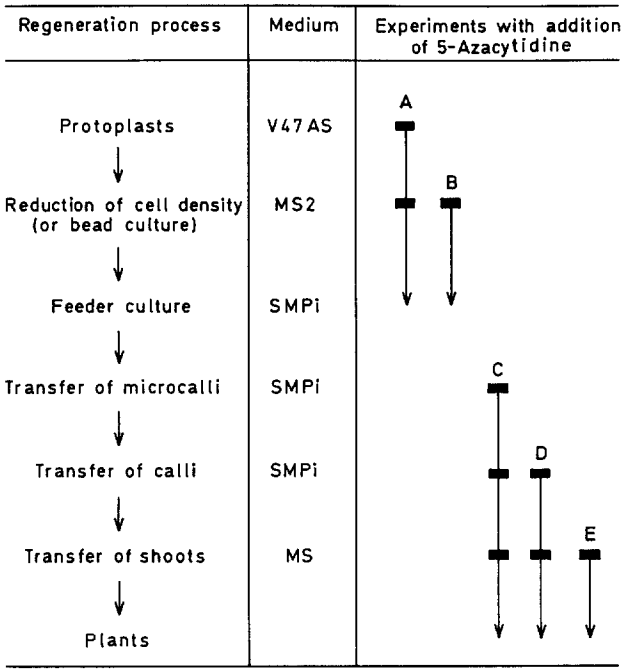

Fig. 1. Representation of the experiments subjecting *N. tabacum* at five different developmental stages to differing concentrations of 5-Azacytidine. ■: Transfer to media containing different concentration of 5-Azacytidine; experiments A–E: developmental period incubated in the presence of 5-Azacytidine

Table 1. Concentration of 5-Azacytidine (µ*M*). Inhibitory effect of 5-Azacytidine on tobacco colony formation in bead type culture (numbers are derived from an average colony number per petri dish from 12 plates)

5-Azacytidine concentration (µ*M*)	0	1	5	10	25	50	100
Percentage growth of colonies	100	74.0	28.5	6.4	0	0	0

5-Azt for the duration of the experiment. Once again, 5-Azt was shown to be highly inhibitory to growth (Table 1), 5 µ*M* being the critical level at which significant growth reduction occurred (Fig. 2).

Only approximately 25% of the cells at a concentration of 5 µ*M* were able to form micro-calli compared to the controls. Increasing levels of 5-Azt to 10 µ*M* resulted in an inhibition of protoplast growth of approximately 90%.

Effect of 5-Azt on tobacco callus development. In order to determine what effect 5-Azt had on later stages of tobacco development (Fig. 1 C), micro-calli were incubated by a double feeder system and transferred to SMPi agar medium containing various concentrations of 5-Azt. In the presence of 5-Azt concentrations greater than 10 µ*M*,

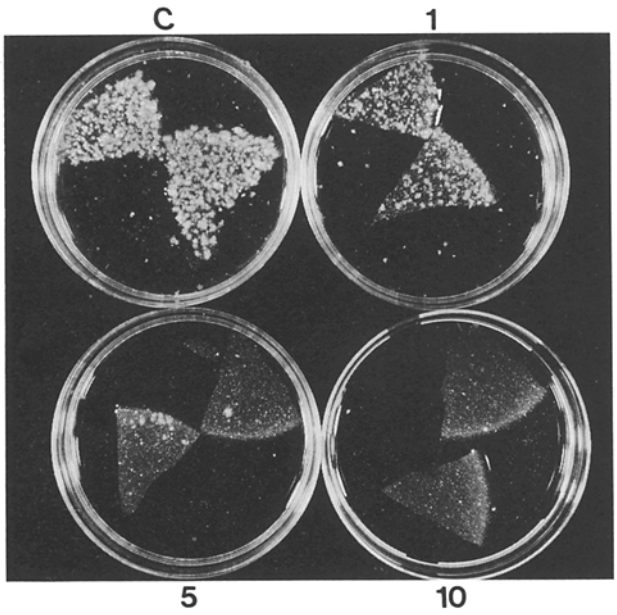

Fig. 2. Inhibition of micro-callus development in bead-type cultures. Protoplasts of *N. tabacum* were subjected to increasing concentrations of 5-Azacytidine 1 (1 μM), 5 (5 μM) and 10 (10 μM). The results demonstrate that compared to controls (C), 5-Azacytidine induces significant inhibition of micro-callus development

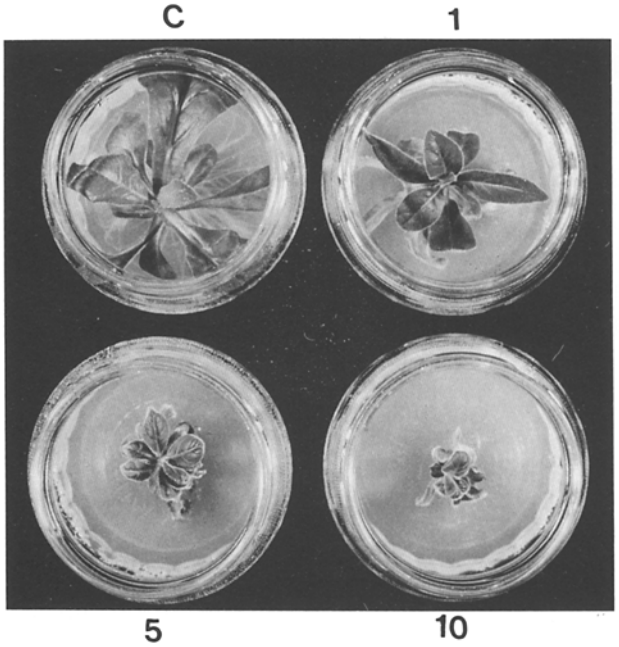

Fig. 3. Developmental inhibition of *N. tabacum* shoot growth. Shoots developing from callus were excised and placed in medium without 5-Azacytidine (C) or at concentrations of 1 (1 μM), 5 (5 μM) and 10 (10 μM). Increasing levels of 5-Azacytidine clearly result in greater shoot inhibition

phenotypic alterations occurred, the majority of the micro-calli changing colour from green to brown. These calli subsequently showed no further sign of development. However, of the remaining micro-calli which showed no colour change, development proceeded as normal, producing callus and shoot bearing callus even at 5-Azt concentrations as high as 50 μM. When callus which was beginning to develop shoots was transferred to media (Fig. 1 D) with 5-Azt concentrations of 10 μM and above, further development was again inhibited, which may be a reflection of greater tolerance in developmentally determined tissues. However, when inhibition did occur, developing shoots began to grow more as tightly packed, cluster-like structures rather than as normal shoots. At higher concentrations, growth inhibition was total.

Effect of 5-Azt on tobacco shoot development. When shoots from developing callus were excised and placed in media containing 5-Azt (Fig. 1 E), shoot growth was considerably inhibited (Fig. 3). The higher the concentration of 5-Azt, the greater was the inhibition of shoot development. Root formation was particularly inhibited by 5-Azt, and apparently more permanently than that of shoot inhibition, as transfer of inhibited shoots to media without 5-Azt led to their subsequent development at rates comparable to that of controls, whilst further root development was considerably delayed in re-starting, and roots were phenotypically abnormal.

Callus induction from maize leaves. When explants from different maize leaves were cultured without 5-Azt, it was found that the greatest response was produced by the second leaf from 9-day-old etiolated seedlings and the third leaf of 14-day-old seedlings (Fig. 4), suggesting that callus induction in maize leaves depends on a specific stage of leaf development and differentiation.

Incubation of etiolated leaf explants in the presence of 5-Azt produced no discernible difference in callus formation, neither stimulation, nor suprisingly, in view of the previous experiments, any inhibition. However, in the case of green leaves, the area capable of callus induction was significantly stimulated by the presence of 5-Azt. Although the response of each leaf to callus induction was, as expected, related closely to leaf age, induction was greatly stimulated at 5-Azt concentrations as low as 1–10 μM. At higher levels (25 μM), the degree of callus induction was reduced compared to the lower 5-Azt concentrations, though still greater than controls. In addition to increasing the areas of the leaf capable of callus induction, 5-Azt also induced significantly greater quantities of callus than the controls.

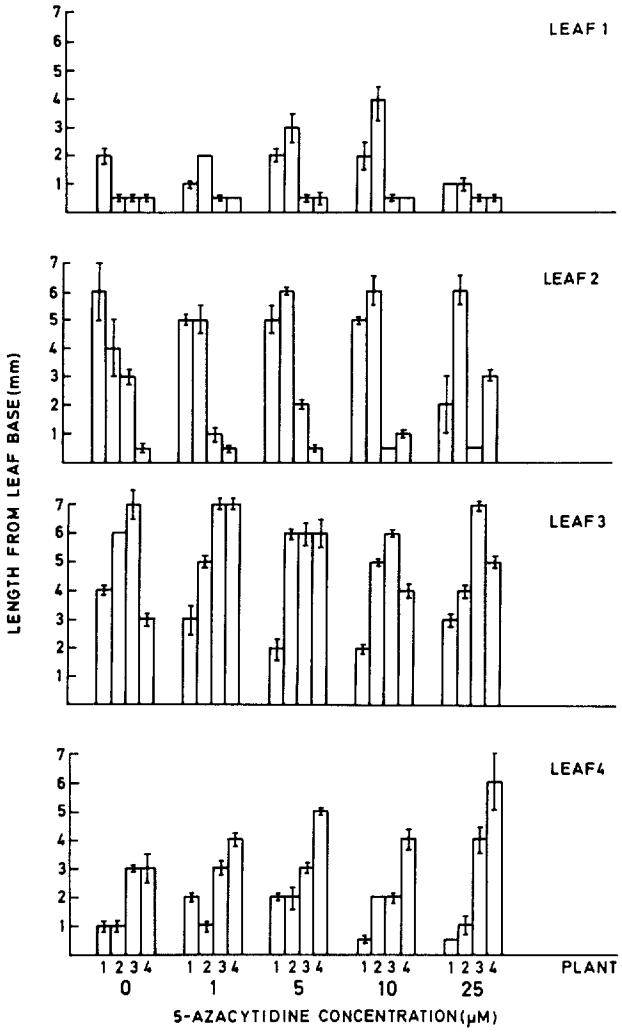

Fig. 4. Effect of varying concentrations of 5-Azacytidine on callus induction in leaf sections of *Zea mays* grown under different conditions and for different times. Plant 1 etiolated seedlings 9 days old; plant 2 green seedlings 9 days old; plant 3 etiolated seedlings 14 days old; plant 4 green seedlings 14 days old

Effect of 5-Azt on maize germination. Sterilised maize seeds were allowed to germinate on media with and without 5-Azt, in an attempt to determine, firstly, whether 5-Azt would affect the rate at which seeds germinated and, secondly, whether the developing seedlings would be affected. No observable difference between the rates of seed germination could be found, although a strong post-germination effect was seen (Fig. 5). The subsequent inhibition of seedling development was in direct proportion to the concentration of 5-Azt in the medium, so that at concentrations of 50 μ*M*, 5-Azt growth was only approximately half that of controls.

Molecular analysis

Effect of 5-Azt on maize seedling gene methylation. In an attempt to determine whether the observed phenotypic changes could be changes in the degree of gene methylation due to exposure to 5-Azt, genomic DNA from seedlings grown at different 5-Azt concentration was digested with the methylation-sensitive isoschizomer enzymes Msp 1 and Hpa II. Probing first with the structural gene for actin (Fig. 6 A) showed very little molecular alteration, although there was evidence that at 5-Azt concentrations above 10 μ*M*, there was demethylation of the gene. In view of the gross phenotypic distortions, this was regarded as surprising. Similar results were, however, found when the filter was subsequently re-probed with the ATP/ADP translocator housekeeping gene (Fig. 6 B), or with the promoter or coding sequence for the sucrose synthase gene (results not shown).

Effect of 5-Azt on tobacco shoot gene methylation. When shoots from differentiating tobacco callus were transferred to media with 5-Azt, an inhibition of growth similar to that exhibited by germinating maize seeds was seen. Isolation of genomic DNA and subsequent digestion with methylation-sensitive enzymes as a means of

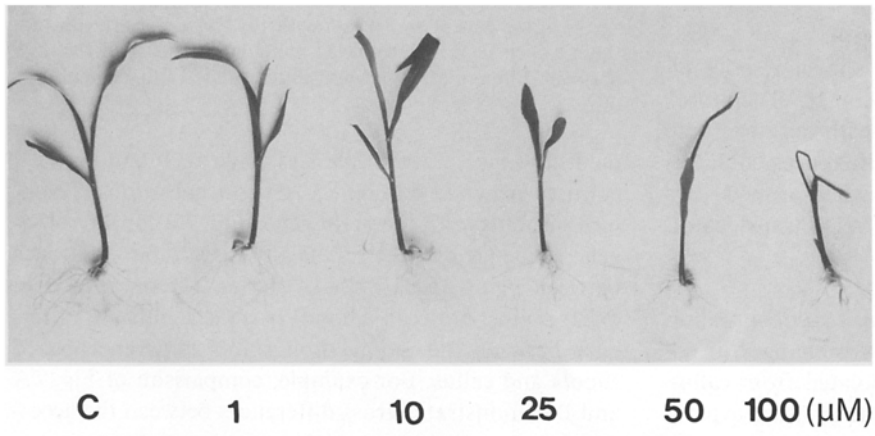

Fig. 5. Effects of 5-Azacytidine on post-germination development of *Zea mays* seedlings. As the concentration of the compound increases, the degree of growth inhibition is also increased

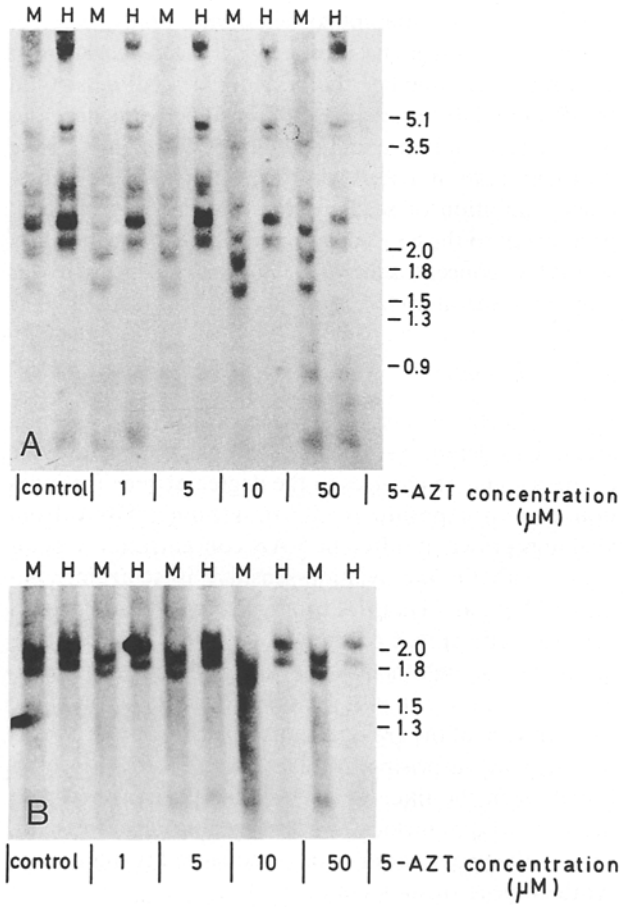

Fig. 6. **A** Genomic DNA from *Zea mays* seedlings grown under different concentrations of 5-Azacytidine was isolated and digested with the methylation-sensitive restriction enzymes MsPI (M) or HpaII (H). The gene probe was the 3.0 kb actin gene. Only at 5-Azacytidine concentrations above 10 μ*M* was there some evidence for gene demethylation. **B** Genomic DNA from *Zea mays* seedlings grown under different concentrations of 5-Azacytidine was isolated and digested with the methylation-sensitive restriction enzymes MspI (M) or HpaII (H). The gene probe was the 1.2-kb ATP/ADP translocator gene. No discernible difference in the methylation status of this gene could be found, regardless of the concentration of 5-Azacytidine used

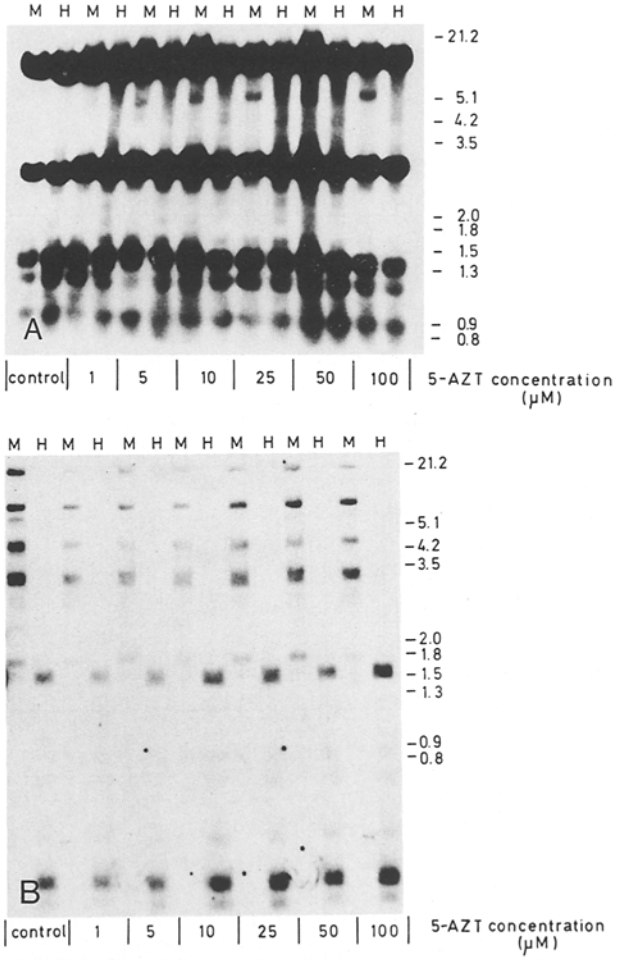

Fig. 7. **A** Shoots of *N. tabacum*, isolated from callus cultures, were transplanted into medium containing different concentrations of 5-Azacytidine. Genomic DNA was subsequently isolated from these shoots and digested with the methylation sensitive-restriction enzymes MspI (M) or HpaII (H). The gene probe was the 3.0-kb actin gene. Regardless of the 5-Azacytidine concentration used, no difference in the methylation status of this gene could be found. **B** Callus cultures of *N. tabacum* were grown under different concentrations of 5-Azacytidine. Genomic DNA was subsequently isolated and digested with the methylation-sensitive restriction enzymes MspI (M) and HpaII (H). The gene probe was the 3.0-kb actin gene. Although the different levels of 5-Azacytidine had no effect on the methylation status of the gene, comparison with Fig. 7A demonstrates that there is a clear difference in the methylation status of the gene between differentiated and non-differentiated tobacco cultures

determining gene methylation status shows a series of results similar to those found previously. Therefore, when filter-bound DNA was probed with the structural actin gene (Fig. 7A), no detectable differences could be found. This result was also the same when probed with a housekeeping gene such as the ATP/ADP translocator (results not shown).

Effect of 5-Azt on tobacco callus gene methylation. Callus cultures also showed significant growth changes in response to 5-Azt. When DNA was isolated from callus and digested with Msp1 and HpaII, as in other experiments, no molecular differences could be found between cultures grown at different 5-Azt concentrations. Therefore, probing with the actin gene (Fig. 7B) or any other gene sequence did not reveal any discernible difference between plants, regardless of the 5-Azt concentration. What is most noticeable however is the significant difference between the methylation status between tobacco shoots and callus. For example, comparison of Fig. 7A and B demonstrates gross differences between the acces-

sibility of methylation-sensitive sites between the developmentally determined shoot tissues and the developmentally undifferentiated callus cultures.

Discussion

Although significant advances in cereal tissue culture have been made, severe limitations and problems associated with such cultures remain. Therefore, in an attempt to overcome some of these problems, we have examined the possibility of using the anti-methylation agent 5-Azacytidine during tissue culture. The rationale for this was the possibility that genes that have been reported to be associated with tissue culture response (Hodges et al. 1986), as well as other gene sequences, may be extensively methylated and hence limited in their expression, thereby reducing the efficiency of tissue culture response.

As the specific mode of action of 5-Azt is assumed to be an inhibition of 5-methyltransferase, the effect would be expected to be a general rather than a specific gene activation. However, the effect appears to be predominantly selective (Jones 1985), activating some previously inactive genes as well as increasing, sometimes dramatically, the expression of other genes. For example, Harris (1983) was able to demonstrate that expression of the thymidine kinase gene is increased by 10^5-10^6-fold following exposure to 5-Azt. The compound is also known to induce a wide range of phenotypic changes, an effect amply reflected in the results from these present experiments. For example, as well as inhibiting growth of germinating maize seedlings, high concentrations of 5-Azt induce abnormal leaf morphology, whilst in tobacco shoot cultures, root growth was severely reduced and phenotypically abnormal.

It is strange that in the majority of the systems examined here, the main effect of 5-Azt was to inhibit growth and development, reducing protoplast division frequency, callus growth and shoot and root development from callus. Also of interest is the differential effect of 5-Azt on maize, having no discernible effect on the rate of seed germination, whilst subsequent development was severely reduced and the plants phenotypically abnormal. One possible explanation may be that protoplasts and callus were in direct contact with the 5-Azt containing medium and hence immediately susceptible to its effects, whilst germinating maize seeds were reliant only on endosperm reserves and, as such, the effects of 5-Azt were mitigated, only subsequently being revealed with the increasing reliance on media substrates.

The greatest paradox is, however, that a compound which is ostensibly an anti-methylation agent and, hence, by definition a gene activator should be responsible for such a general growth inhibition. Certainly previous work has shown only evidence for transcriptional activation. Van Slogteren et al. (1984) were able to show that the genes for octopine, agropine and mannopine in a non-expressing tobacco line became, on addition of 5-Azt, active. The key, therefore, to the growth inhibition exhibited here may be that the genes activated by the 5-Azt are inhibitory to growth. Certainly if tissue culture response in maize is related to the presence of nuclear genes (Hodges et al. 1986), then the fact that there is a differential effect by 5-Azt, dependent on whether the tissue is green or not, may be relevant. Hodges states that these factors are nuclear-encoded but the fact that they are not obviously active in etiolated plants would suggest that chloroplast-encoded factors may also be involved in tissue culture response.

The possibility of growth inhibition by 5-Azt being an indirect effect, e.g. by being mutagenic, has been discussed by Jones (1985). The evidence shows that the induction of gene expression in systems such as the thymidine kinase gene (Harris 1983) is several orders of magnitude greater than would be expected of a mutagenic agent, though there is some evidence of an ability to alter chromosome morphology (Shafer and Priest 1984). A second possibility, perhaps given a greater credence as a result of the molecular analysis, is that 5-Azt may exert an inhibitory effect, perhaps by a toxic action. Certainly, the fact that in shoots and seedlings, e.g., no evidence for gene demethylation could be found, even at 5-Azt concentrations as high as 100 μM, may confirm this, even though this resulted in significant phenotypic distortion. This response also disproves the theory that 5-Azt was not taken up or metabolised by the plant. That 5-Azt also enhanced callus inducibility in maize leaves also confirms uptake and metabolism.

The fact that no significant molecular difference could be found between plants grown at different 5-Azt concentrations may well be a reflection of the limited number of genes that were probed. However, as these included both housekeeping and structural genes as well as promoter and coding sequences, it would have been expected that some differences would have emerged, especially for those plants grown at high 5-Azt concentrations. It should also be noted that the enzymes used here recognise only the CCGG tetrad. Nearest neighbour analysis of methylated sequences in plant DNA (Gruenbaum et al. 1981) revealed that, whilst CpG dinucleotides account for over 80% of methylatable sites, other sequences such as CpA and CpT may also be methylated, although only at much lower levels (approximately 19%). It would be expected, therefore, that the enzymes used here would encompass the majority of the potential methylation sites, revealing any change in methylation status.

One result of considerable interest was the difference in methylation status between genetically identical plants, one of which, as a developmentally determined organ

(shoots), was shown by molecular analysis to be in a much more highly methylated state than the same plant as a de-differentiated callus. This suggests that the status of uncontrolled proliferation found in callus leads to a highly significant demethylation of gene sequences, which can be revealed by an increased accessibility of methylation-sensitive restriction sites to such enzymes as Msp1 and HpaII. This is presumably a reflection of the higher metabolic activity of callus compared to shoots, with the corresponding necessity for gene expression and, hence, the decrease in methylation levels in callus.

The work here was an attempt to determine whether 5-Azt may be of use in tissue culture. It was hoped that the incorporation of such anti-methylation agents would be useful in overcoming some of the limitations at present encountered in cereal tissue culture. Whilst the compound showed some potential as a means of increasing leaf areas capable of sustaining callus development as well as producing greater amounts of callus, the general effect of 5-Azt appears to be one of severe growth inhibition. The reasons for this and our inability to find any molecular evidence of demethylation remain unknown.

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References

Amasino RM, Powell ALT, Gordon MP (1984) Changes in T-DNA methylation and expression are associated with phenotypic variation and plant regeneration in a crown gall tumour line. Mol Gen Genet 197:437–446

Doerfler W (1983) DNA methylation and gene activity. Annu Rev Biochem 52:93–124

Firoozabady E (1986) Rapid plant regeneration from *Nicotiana* mesophyll protoplasts. Plant Sci 46:127–131

Gruenbaum Y, Naveh-Many T, Cedar H, Razin A (1981) Sequence specificity of methylation in higher plant DNA. Nature 292:860–862

Harris M (1983) Induction of thymidine kinase in enzyme-deficient Chinese hamster cells. Cell 29:483–492

Hepburn AG, Clarke LE, Pearson L, White J (1983) The role of cytosine methylation in the control of nopaline synthase gene expression in a plant tumour. Mol Appl Genet 2:315–329

Hodges TK, Kamo KK, Imbrie CW, Becwar MR (1986) Genotype specificity of somatic embryogenesis and regeneration in maize. Biotechnology 4:219–223

Jones PA (1985) Altering gene expression with 5-azacytidine. Cell 40:485–486

Rigby PWJ, Dieckmann M, Rhodes C, Berg P (1977) Labelling DNA to high specific activity *in vitro* by nick translation with DNA polymerase I. J Mol Biol 113:237

Sellem CH, Weiss MC, Cassio D (1985) Activation of a silent gene is accompanied by its demethylation. J Mol Biol 181:363–371

Shafer DA, Priest JH (1984) Reversal of DNA methylation with replication pattern in human lymphocyte and fibroblast cultures. Am J Hum Genet 36:534–545

Taylor SM, Jones PA (1982) Mechanisms of action of eukaryotic DNA methyltransferase. Use of 5-azacytidine-containing DNA. J Mol Biol 162:679–692

Taylor B, Powell A (1985) Isolation of Plant DNA and RNA. Focus 4:4–6

Van Slogteren GMS, Hooykaas J, Schilperoort RA (1984) Silent T-DNA genes in plant lines transformed by *Agrobacterium tumefaciens* are activated by grafting and by 5-azacytidine treatment. Plant Mol Biol 3:333–336

Vasil IK (1987) Developing cell and tissue culture systems for the improvement of cereal and grass crops. J Plant Physiol 128:193–218

Watson JC, Kaufman LS, Thompson WF (1987) Developmental regulation of cytosine methylation in the nuclear ribosomal RNA genes of *Pisum sativum*. J Mol Biol 193:15–26

Wernicke W, Gorst J, Milkovits L (1986) The ambiguous role of 2,4 dichlorophenoxyacetic acid in wheat tissue culture. Physiol Plant 68:597–602