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Autor: Mink, Mátyás

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Faster growth and altered transcription induced transiently by 5-azacytidine in tobacco

Mátyás Mink *

Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, P.O. Box 521, H-6701 Szeged, Hungary

* Present address: Department of Genetics, Attila József University, Középfasor 52, H-6726 Szeged, Hungary. Tel./Fax: 00-36-62-432485. E-mail: Mink@biocom. bio.u-szeged.hu

Dedicated to the late Z. J. H.

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Abstract

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Seedlings of *Nicotiana tabaccum* SR1 were treated with 5-azacytidine (5-azaC), a potent inhibitor of DNA methylation *in vivo*, resulting in retarded growth or death in some of the exposed plantlets. Following growth on drugless medium and later in soil under greenhouse conditions the treated plants developed faster and initiated flowering significantly earlier than the untreated controls. Among the F_1 progeny of the self-pollinated, treated plants a continuous transition was observed regarding flowering times: About 10% of the plants flowered as early as the F_0. Another 10% grew slower than the untreated controls and 80% of them was similar to the untreated plants or were slightly earlier or later flowering than the untreated plants, but faster growth was not as evident as in the F_0 or in the fastest developing 10% of the F_1 generation. In the F_2 progeny of either self pollinated plants or of various crosses, flowering times of the mature plants remained undistinguishable from untreated samples. Northern analysis revealed a higher level of *Nt-ran* transcripts in the F_0 plants than in controls, which remained elevated in F_1, but dropped to physiological values in the F_2 generation. These results strengthen our view suggesting an inverse relationship between DNA methylation and gene expression, since demethylation of DNA, as a consequence of 5-azaC exposure, may have resulted in the overexpression of *Nt-ran* loci.

Key words: 5-azaC – Transitional phenotype – Temporarily altered transcription.

Introduction

A growing body of evidence suggests a role of DNA methylation in the regulation of gene expression in mammals (Cedar 1988, Cedar and Razin 1990). Non-tissue-specific genes are apparently hypermethylated except in tissues where the genes are expressed (Shemer et al. 1990, Waalwijk and Flawell 1978). Moreover, spatial and temporal changes in DNA methylation patterns were reported during mouse embryonic development, demonstrating its role in tissue differentiation (Kafri et al. 1992, Monk et al. 1987).

5-azaC can effectively replace cytidine in replicating DNA and in the incorporated drug forms covalent bonds with DNA methyltransferase, thus preventing nucleotide substitution; the hypomethylated DNA, lacking one level of transcriptional control, allows constitutive expression of methylation sensitive loci (Shin et al. 1992).

The biological effect of 5-azaC is rather bizarre. The drug can induce conversion of fibroblast cells to myocytes due to overexpression of a single gene, *myoD* (Lasser et al. 1986, Davis et al. 1987). Heritable dwarfism and reduced expression of the *rpg1* gene, encoding a monomeric GTP-binding protein of the *ras* superfamily were reported in the monocotyledonous rice, *Oryza sativa* (Sano and Youssefian 1991). Demethylation was induced in a late flowering ecotype of *Arabidopsis thaliana* by vernalization or 5-azaC treatment, leading to a non-heritable early flowering phenotype, whereas late flowering mutants, which are insensitive to vernalization did not respond to the drug (Burn et al. 1993). This suggests that demethylation of specific genes involved in floral initiation may result in early flowering. These phenotypes appear to originate from altered transcription, i.e. perturbation of mRNA synthesis. Two mechanisms may explain this phenomenon: The hypomethylation of nuclear DNA in the promoter regions of transcriptionally active genes, or the action of a transcription factor (Shin et al. 1992).

Ras related nuclear (Ran) proteins characterised in yeasts and mammals appear to have diverse cellular functions including maintenance of cell cycle coordination, RNA metabolism and transport, or protein import into the nuclei (Merkle et al. 1994 and references therein). Tobacco cDNAs encoding homologous proteins were isolated and shown to suppress the *pim1-46ts* lesion in the fission yeast, demonstrating an evolutionary conserved function of this protein (Merkle et al. 1994).

Methods

Sterilised seeds of *Nicotiana tabacum* SR1 were imbided on solid MS medium, then transferred to MS medium containing 0.2, 0.3, 0.6, 1.0 mM 5-azaC at the age of 3, 6, 9, 12 or 15 days. After a three-day-exposure to the drug, total RNA was isolated from part of the seedlings either immediately or after several days or growth on drugless medium. The rest of the one-month-old seedlings were planted into soil and kept under greenhouse conditions.

Twenty µg of total RNA were fractionated in 1.2% formaldehyde-agarose denaturing gels, blotted on nylon membranes and UV-crosslinked. cDNA probes for *Nt-ran* (Merkle et al. 1994) were labelled with [α-^{32}P] dATP by random priming and separated from non-incorported radioactivity on Sephadex G-50 spin-columns. Hybridization was carried out as described (Dallmann et al. 1992). In order to avoid cross-hybridization to other transcripts of GTP-binding proteins, filters were washed stringently (0.1×SSC, 0.1% SDS, 68 °C), leading to weakening or loss of signals in control lanes.

Root tips were collected, treated with colchicine (0.5%, 3 h), stained by the standard acetocarmine method and chromosomes were observed in a light microscope.

Results

The effects of 5-azaC on the growth and flowering of tobacco plants

The growth of both germinating seeds and plantlets was retarded on medium containing 5-azaC. At the higher concentrations (0.6–1 mM) some of them were even killed by the treatment. Following transfer to drugless medium and later into soil all of the plantlets developed faster (Fig. 1). The time needed for flowering of the control plants in the greenhouse was ap-

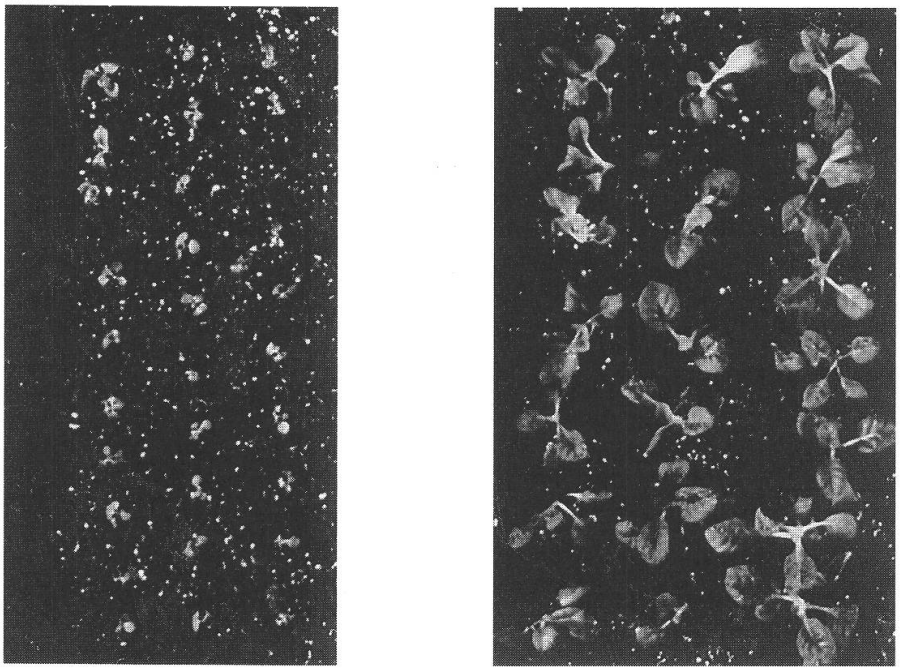

Fig. 1. Four-week-old tobacco seedlings germinated three days on RM medium, exposed to 0.6 mM 5-azaC and grown on drugless medium until transfer into soil (right panel); untreated control plants (left panel).

Table 1. Size and flowering times of treated F_0 and control in four independent experiments involving 109 treated and 59 control plants.

Treatment	Height (cm)	Floration (after days)
0.6 mM 5-azaC	62	39±3
–	59±2	66±4

proximately two months in summer and three in winter. However, the treated plants flowered within a time period which was ca. 40% shorter than the untreated controls (Table 1). Mature exposed and control plants were similar in size. No characteristic phenotype other than faster growth was observed in four independent series of experiments.

The self-pollinated F_1 generation of the plants exposed formerly to 5-azaC displayed a wide range of flowering times. Solely based on the heights of the self-pollinated F_1 plants measured when the first flowers appeared on the fastest developing mature individuals of the population, they could be distributed into five categories (Table 2, Fig. 2). Although the plants in the category V remained smaller than the untreated ones, the terminal phenotype (i.e. the height at flowering) of the other categories did not vary remarkably (data not shown). However, the plants in category I, which resembled the 5-azaC exposed parents, still initiated flowering remarkably earlier, whereas the categories in between did not show dramatic differences when their flowering times were compared with that of unexposed controls (Table 2, Fig. 2).

Plants of the F_2 generation were produced either by self-pollination within a single category (Table 2, Fig. 2), or in various crosses between category members and controls. The flowering times in all of the above combinations were comparable to the untreated control plants and were similar in size at maturity.

Table 2. Distribution of size and floration times in the self-polinated F_1 generation of the 5-azaC treated (0.6 mM, three days) and control tobacco plants in four independent experiments, involving 132 treated F_1 and 47 control plants. Height were measured at floration time of the fastest growing (category I) plants.

Category	Height (cm)	Probability of appearance (%)	Floration (after days)	Nr. of plants
I	59±1	8	48	11
II	47±3	24	70±2	32
III	28±3	36	78±5	47
IV	9.8±3	20	99±5	26
V	2	12	113±5	16
Control	11±2		85±4	47

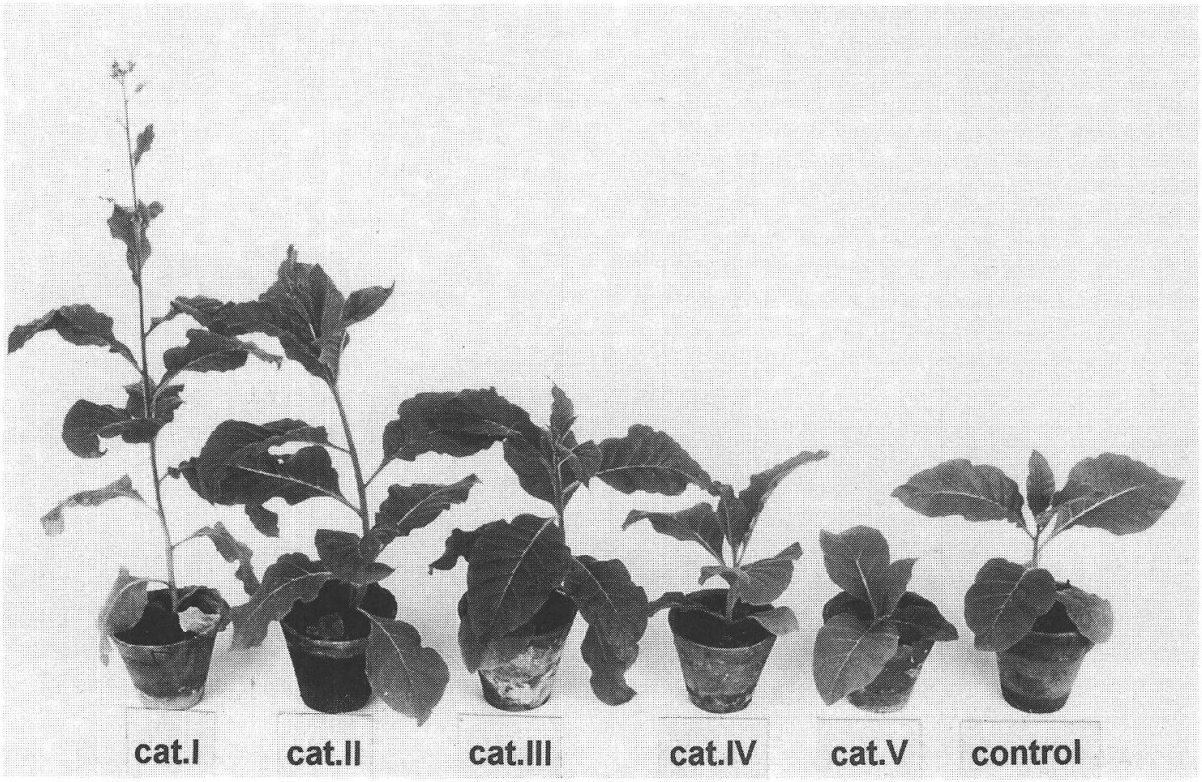

Fig. 2. The size distribution of the five categories of F_1 plants at the time of the earliest flowering (48 days).

Elevated transcription in 5-azaC treated tobacco

Reduced expression of *rpg1*, a gene encoding a small monomeric GTP binding protein, was reported in rice (*Oriza sativa*) induced by 5-azaC treatment. Furthermore, heritable dwarfism was observed in three subsequent generations (Sano and Youssefian 1991). These results suggest that genes for GTP binding proteins may serve as markers to follow alterations in gene activity induced by 5-azaC. Due to its pleiotropic effects and conserved function in fission yeast *Nt-ran* was chosen as a probe in Northern analyses (Merkle et al. 1994). Load-

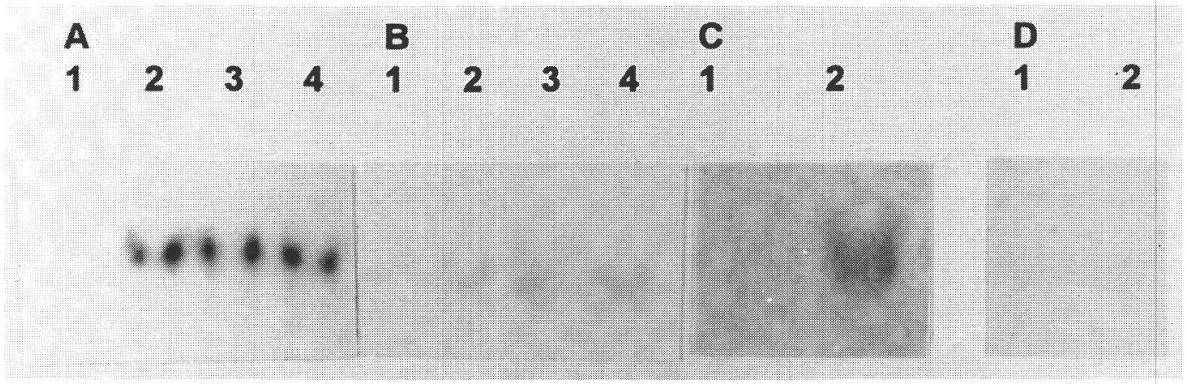

Fig. 3. *Nt-ran* transcript levels in 5-azaC induced and control 6-day-old tobacco seedling. Panel A, 1: Untreated control; 2: Three-day-old seedlings exposed to 0.2 mM, 3: 0.4 mM, 4: 0.6 mM 5-azaC for three days. Panel B: Transcripts in 18-day-old tobacco seedlings imbided for 15 days on RM medium before exposure to 5-azaC; order, treatment like in panel A. Panel C, 1: Untreated control; 2: F_1 progeny of self-pollinated plants exposed formerly to 0.6 mM 5-azaC; RNA was isolated from 6-day-old seedlings. Panel D, 1: Untreated control; F_2 progeny of self pollinated F_1, category I plant; RNA was isolated from 6-day-old seedlings.

ing of equal amounts of RNA of identical quality was controlled by ethidium bromide staining of the renatured agarose gels before blotting (not shown). cDNAs for other GTP binding proteins (*Nt-ypt2*, *Nt-rab1*, Merkle et al. 1994) were also used as probes in Northern analysis (not shown).

If the seedlings were exposed to 5-azaC at different concentrations (cf. Materials and Methods), elevated transcription of the *Nt-ran* loci could be observed, while *Nt-ran* transcripts were hardly detectable in unexpossed controls (Fig. 3). At a later stage of development (15-day-old seedlings) induction by 5-azaC dropped (Fig. 3B). Similarly, seedlings germinating for different lengths of time (6–15 days) on RM medium containing various concentrations of 5-azaC without later cultivation on drugless medium also displayed elevated levels of *Nt-ran* transcripts in comparison with the untreated controls (not shown).

Higher levels of *Nt-ran* RNA were detected when seedlings which were exposed to 5-azaC were transferred onto drugless medium for 14 days before RNA isolation. However, the results indicate that the most pronounced effect is observed when 3-day-old seedlings were treated with the drug for three days and processed immediately.

RNA was isolated from 6-day-old F_1 seedlings of selfed F_0 plants and Northern analysed. The level of *Nt-ran* transcripts was still higher than in the untreated controls (Fig. 3C). It seems worthwile mentioning that faster growing individuals still appeared in this generation (Table 2).

Northern analysis of RNA isolated from 6-day-old seedlings of the F_2 generation produced either by self-pollination or by different crosses, revealed that the levels of *Nt-ran* transcripts were identical with that of the untreated controls (Fig. 3D). Following a similar pattern, the F_2 and control plants were flowering within comparable periods of time (not shown).

The spi^+/spi^- heterozygous diploids of the fission yeast *S. pombe* undergo fast haploidization, demonstrating a dose dependent effect of this protein which is homologous to *Nt-ran* in this organism (Matsumoto and Beach 1991). Examination of the metaphase chromosomes in root tips of *Nt-ran* overexpressing plants induced by 5-azaC revealed no karyotypic change. Similarly, the cell morphology in the tips was the same in treated and untreated plants (not shown), suggesting that elevated transcript levels of *Nt-ran* do not influence chromosome number in tobacco.

Discussion

Similarly to other higher plants, 30–35% of the cytosine are methylated in *Nicotiana* (Klaas et al. 1989), whereas a mere 4.6% show this phenomenon in *A. thaliana*, a small genome species (Finnagan et al. 1993). The CpG dinucleotides which occur at a higher frequency in plants than in animals and the CNG trinucleotides are considered the targets of methylation *in vivo* (Finnegan et al. 1993). Methylation of cytosines in promotor regions hamper transcription due to alteration in the stereochemistry of DNA or methylated DNA forms an altered chromatin structure, which is less sensitive to DNaseI and not transcribed (Monk et al. 1987). Consequently, active chromatin is hypomethylated. An extreme example is the inactive X chromosome in female mammals (Holliday 1993). In many biological contexts studied so far 5-azaC has significantly reduced the amount of 5-methylcytosine in DNA (Holliday 1987), leading to a heritable alteration of gene activity at the transcriptional level.

This paper introduces a transcriptionally fast growing phenotype of tobacco plants as the result of 5-azaC treatment, most probably due to DNA hypomethylation. The phenotype inheritance was limited and was lost in the subsequent two generations. However, the transition to earlier flowering induced by 5-azaC in *Arabidopis* was reset in the progeny (Burn et al. 1993). These non-heritable changes in the phenotype suggest an epigenetic event induced by altered transcription. In contrast, the heritable dwarfism in rice reported as a result of 5-azaC treatment demonstrates that an induced phenotype or epimutation can be transmitted through germlines in plants (Sano and Yuossefian). The results presented here and that found for *Arabidopis* (Burn et al. 1993) suggest that loss of an induced phenotype may follow different mechanisms in the two plant species, which may be partially based on the significantly lower level of methylation of cytosine residues in *A. thaliana*. On the other hand, repeated and heavily methylated DNA replicates in late S phase, prolonging cell cycle (Finnegan et al. 1993). Demethylation may reduce the time needed for DNA synthesis and may accelerate cell cycle, leading to faster growth and earlier flowering in tobacco. Nevertheless, re-methylation of hypomethylated DNA seems to be a more effective process in dicotyledoneous plants than in monocots or mammals. The latter represent more advanced evolutionary stages, indicating an altered interspecific *de novo* DNA methylation ability.

Using *Nt-ran* as a marker, a similar event was observed at the transcriptional level which corresponded to the occurrence and loss of the early floral initiation (Fig. 3). The activity of *Nt-ran* alleles seems to be regulated transcriptionally (Merkle et al. 1994) and confined to organs with dividing cells. Thus, the elevated levels of *Nt-ran* transcripts in fast growing tobacco plants may be interpreted by its altered regulation, whereas reset to physiological behaviour may reflect indirectly a repair of the methylation pattern in the subsequent two generations. This repair may start immediately, since exposed plants which were later cultivated in the absence of the drug had reduced levels of *Nt-ran* transcripts than those kept on 5-azaC until RNA isolation. Furthermore, a 5-azaC sensitive developmental stage was observed: The post-embryonal 3-day-old seedlings expressed higher levels of *Nt-ran* transcripts than those which developed for fifteen days prior to the treatment (Fig. 3 A versus 3 B). Transgenic tobacco plants which overexpress *Nt-ran* do not have a clear-cut phenotype (Nagy, F., unpublished results), which may be explained by the supposed pleiotropic effect of this protein (Merkle et al. 1994). The faster growing phenotype and elevated transcript levels of *Nt-ran* in 5-azaC induced tobacco plants seem to be parallel events, although a linkage can not be excluded.

Nevertheless, cultured plants with significantly reduced generation time may consider to be of agricultural importance.

Zusammenfassung

Keimlinge von *Nicotiana tabaccum* SR1 wurden mit 5-Azacytidin (5-Aza-C), einem DNA-Methylierungshemmstoff behandelt, welcher das Wachstum z. T. stark inhibierte. Nachdem die Keimlinge ins Medium ohne Inhibitor bzw. in Erde übertragen wurden, zeigten sie eine raschere Entwicklung und Blüte als die unbehandelten Kontrollpflanzen. Von den F_1Nachkommen der behandelten, selbstbefruchteten Pflanzen entwickelten sich 10% langsamer, 10% wuchsen wie die F_0-Pflanzen und etwa 80% etwas rascher, wobei das Ausmaß des rascheren Wachstums nicht so ausgeprägt war wie in der F_0. Die durch Selbstbefruchtung oder Kreuzung hergestellten F_2-Pflanzen wiesen eine den Kontrollen ähnliche Entwicklung auf.

Eine erhöhte Transkriptionsrate des *Nt-ran*-Lokus wurde bei behandelten F_0-Pflanzen beobachtet, was auch bei F_1-, nicht aber bei F_2-Pflanzen erkennbar war. Diese Ergebnisse lassen vermuten, daß sich der durch 5-Aza-C induzierte Phänotyp auf Transkriptionsänderungen zurückführen läßt. Es kann nicht ausgeschlossen werden, daß die erhöhte Transkriptionsrate von *Nt-ran* am schnelleren Wachstum beteiligt ist. In jedem Fall könnten aber schneller wachsende Pflanzen von landwirtschaftlicher Bedeutung sein.

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