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Differential Methylation Pattern of *RolA*, *B* and *C* Genes of *Agrobacterium rhizogenes* in *Nicotiana glauca* and its Hybrid

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Abstract: The present study tends to investigate the role of DNA methylation in silencing and expression of *rolA*, *B* and *C* genes in *Nicotiana glauca* and its hybrid, respectively. Exposure of the hybrid seed to 5-azacytidine, a DNA hypomethylating agent resulted severe alterations in morphology, early tumor induction and loss of phytohormone independent growth. On the contrary, DNA hypermethylation caused by cefotaxime treatment resulted in enhanced rate of tumorigenesis and callus growth. The above results convey that changes in DNA methylation in the hybrid plants possess a significant effect on tumorigenesis. Therefore, DNA isolated from the parents, hybrid and tumor were restriction digested with cytosine methylation specific isoschizomers MspI and HpaII; and Southern analysis was carried out using *rolA*, *B* and *C* genes as probes. The size of the bands differed in MspI and HpaII digests of *N. glauca*, hybrid and tumor whereas no signals were observed in *Nicotiana langsdorffii*. Results were discussed in terms of silencing of *rolA*, *B* and *C* genes in *N. glauca* and their expression in the hybrid. Hence, it could be concluded that DNA hypermethylation of *rol* genes in *N. glauca* resulted in their silencing causing normal morphology and behavior of the plant, whereas DNA hypomethylation of *rol* genes was responsible for their expression in the hybrid causing morphological abnormalities and tumorigensis.

Key words: 5-azacytidine, cefotaxime, DNA methylation, phytohormone independent growth, tumorigenesis

INTRODUCTION

The presence of cellular T_L-DNA genes in tobacco plants indicate ancient horizontal transfer of genes between plants and an ancestor *Agrobacterium rhizogenes* (Suzuki *et al*., 2002; Aoki, 2004). In general, the transgenic plants with *rol* genes show a dwarfed phenotype, reduced apical dominance, smaller, wrinkled leaves, increased rooting, altered flowering and reduced fertility (Cosanova *et al*., 2005). Expression of *rol* genes in *Kalanchoe blossfeldiana* resulted in a compact plant with several morphological changes with respect to ornamental value (Christensen *et al*., 2008). Individual and combined expression of *rolA*, *B* and *C* genes resulted in increased biosynthesis of anthraquinones in transformed calli of *Rubia cordifolia* (Shkryl *et al*., 2008). They are also potential activators of secondary metabolism (Bulgakov, 2008).

Ri T_L-DNA homologues (*rol* genes) of *A. rhizogenes* are present in the genome of *N. glauca* and in *N. glauca* x *N. langsdorffii* hybrid (Udagawa *et al*., 2004). *Nicotiana glauca* x *N. langsdorffii* hybrid showed several morphological variations, tumorigenesis and phytohormone independent growth but the parents remained normal. From earlier investigations it is evident that DNA methylation is a potent suppressor of gene activity (Jones and Larid, 1999) and changes in DNA methylation could result in cancers (Ehrlich, 2006). The present investigation was undertaken to study whether the changes in DNA methylation pattern play a role in silencing of *rol* gene homologues in *N. glauca* and their consecutive expression in the *N. glauca*×*N. langsdorffii* hybrid. As a part of it, the morphology and tumorigenesis of the hybrid were studied. Experiments were conducted with 5-azacytidine and cefotoxime treatments of the parent and hybrid seeds. Molecular analysis for changes in DNA methylation was carried out by doing Southern analysis. Changes in DNA methylation pattern of *rolA*, *B* and *C* genes of *A. rhizogenes* in parents, hybrid and tumor were studied.

MATERIALS AND METHODS

Plant material: Seeds of *N. glauca*, *N. langsdorffii* and their hybrid (provided by Dr. GRK Sastry, University of Leeds, UK) were grown in pots at experimental farm. The

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study was carried out during 2006-07 in the laboratories of Dept. of Botany Andhra University and Department of Genetics, Osmania University, India.

Phytohormone independent growth of the leaf discs: The leaf discs of the parents and the hybrid were surface sterilized and cut with cork borer (0.5 mm diameter) and cultured on MS basal medium (Murashige and Skoog, 1962) at 25±2°C and 16 h light/8 h dark photoperiod.

Treatment of seeds with 5-azacytidine: The seeds of the parents and the hybrid were surface sterilized and imbibed in the solutions of 0, 50 and 150 μM concentrations of 5-azacytidine for one week at 25±2°C in dark. The filter paper soaked in 5-azacytidine solution was changed every day for each concentration. Then the plantlets were shifted to MS basal medium and the observations of morphology and tumorigenesis were recorded (Table 1).

Treatment of seeds with cefotaxime: The surface sterilized seeds of the parents and the hybrid were placed on sterile filter papers soaked with 0 and 500 mg mL^{-1} concentrations of cefotaxime. They were kept at 25±2°C in dark for 5 days and then transferred to 16 h light/8 h dark photoperiod. The seeds were transferred every day to a fresh filter paper containing cefotaxime solution. After germination the seedlings were transferred to 250 mL conical flasks containing 50 mL of MS basal medium supplemented with cefotaxime. The plantlets were shifted to a fresh medium every week and the observations were recorded (Table 2).

DNA extraction protocol according to Dellaporta *et al.* (1983): Fresh tender leaves of 1-3 g were harvested and ground to very fine powder with liquid N_2. The powder was transferred to a 30 mL autoclaved polypropylene tube, 15 mL of extraction buffer (1 M Tris HC) l (pH 8.0), 0.5 M EDTA (pH 8.0), 5 M NaCl, 10 μM β-mercaptoethanol, 1% Polyvinyl pyrrolidone) was added and mixed. One milliliter of 20% SDS was added, mixed by inverting and incubated at 65°C for 10 min. Five milliliter of 5 M potassium acetate was added, mixed gently and incubated at 0°C for at least 20 min. The tubes were then centrifuged at 12,000 rpm for 20 min at 4°C in a refrigerated high speed centrifuge (Remi C_{24}). The supernatant was transferred into a tube containing 10 mL of iso-propanol and incubated at-20°C for 20 min. Then, the tubes were centrifuged at 12,000 rpm for 15 min, the supernatant was discarded and the crude DNA pellet was dissolved in 0.7 mL of 10 mM EDTA and 50 mM Tris HCl.

Purification of DNA: The contents of the crude extract were transferred into sterile 1.5 mL eppendorff tubes. To this DNA solution 4 μL of RNase was added and incubated for one hour at room temperature. Equal volume of phenol:chloroform (1:1) was added to the supernatant, mixed and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was recovered. Then equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added to the supernatant, mixed and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was recovered. To the supernatant 75 μL of 3 M sodium acetate and 500 μL of chilled ethanol were added, mixed by inverting and incubated at -20°C for 30 min. The precipitate was washed with 70% ethanol and left to air dry. Then it was resuspended in 100 μL of 1 mM EDTA and 10 mM Tris HCl. The concentration and purity of DNA were estimated using UV-VIS spectrophotometer (Systronics, Model-117,

Table 1: Effect of 5-Azacytidine on tumor induction of *N. glauca, N. langsdorffii* and hybrid

		No. of seed treated with 5-azaC			Plants grown under culture conditions				Plants grown under field conditions		
Name of the plant	5-azaC conc. (μM)	Sown	Germinated	Total	[A]NT	[B]T	C%T	Total	[A]NT	[B]T	%T
N. glauca	0	300	250	125	125	Nil	0	125	125	Nil	0
N. glauca	50	300	260	130	130	Nil	0	130	130	Nil	0
N. glauca	150	300	220	110	110	Nil	0	110	110	Nil	0
N. langsdorfii	0	300	280	140	140	Nil	0	140	140	Nil	0
N. langsdorfii	50	300	270	135	135	Nil	0	135	135	Nil	0
N. langsdorfii	150	300	270	135	135	Nil	0	135	135	Nil	0
Hybrid	0	300	210	105	91	14	13	105	95	10	10
Hybrid	50	300	200	100	4	96	96	100	6	94	94
Hybrid	150	300	230	115	0	115	100	115	1	114	99

[A]NT: No. of non-tumorous plants; [B]T: No. of tumorous plants; %T: Percentage of tumorous plants

Table 2: Effect of Cefotaxime on tumor expression of *N. glauca, N. langsdorffii* and hybrid

	Number of seed treated with cefotaxime (500 mg L^{-1})				Plants grown under culture conditions			Plants grown under field conditions		
Name of the plant	Sown	Germinated	Total	[A]NT	[B]T	%T	Total	[A]NT	[B]T	%T
N. glauca	300	240	120	120	Nil	0	120	120	Nil	0
N. langsdorfii	300	280	140	140	Nil	0	140	140	Nil	0
Hybrid	300	210	105	95	10	90	105	79	26	76

[A]NT: No. of non-tumorous plants; [B]T: No. of tumorous plants; %T: Percentage of tumorous plants

India). To store for longer time the DNA fibers were left in ethanol at -20°C.

Southern hybridization: DNA (20 μg) isolated from each of the parent, hybrid and the tumor was digested separately with methylation specific isoschizomers-MspI and HpaII (Banglore Genei, India). MspI can cut C↓^{m}CGG and HpaII is blocked by CG methylation. The bands were separated by gel electrophoresis at 15 volts for 8 h. The gel was treated with denaturation solution (1.5 M NaCl and 0.5 M NaOH), neutralization solution (0.5 M Tris HCl pH 7.5, 3 M NaCl) and transferred onto nitrocellulose membrane by capillary transfer method (Southern, 1975) using 20× SSC buffer. The DNA fragments were fixed onto the membrane by UV crosslinking.

Preparation of *rolA*, *B* and *C* probes: PCR amplification of *rolA*, *B* and *C* genes was done using the pPCV002 plasmid containing *rolABC* genes (provided by Dr. Czaba Koncz, Germany) as template and *rolA*, *B* and *C* sets of primers (Biotech Desk, India).

The primer sequences are as follows:

***rolA* primers:**

- Forward: 5'-GGAATTAGCCGGACTAAA-3'
- Reverse: 5'-AGGTCTGAATTTTCACGT-3'

***rolB* primers:**

- Forward: 5'-CAAATTGCTATTCCTTCC-3'
- Reverse: 5'-TTACTGCAGCAGGCTTCA-3'

***rolC* primers:**

- Forward: 5'-GTCGAGGATGTGACAAGC-3'
- Reverse: 5'-GCCGATTGCAAACTTGCA-3'

PCR programme:

Amplification was carried out in Appendorf's master cycler. Thermal profile was as follows:

- Initial denaturation at 94°C for 4 min
- Denaturation at 94°C for 1 min, Annealing at 50°C for 1 min and Extension at 72°C for 1.5 min-this reaction was repeated for 30 cycles
- Final extension at 72°C for 10 min

Amplified fragments of DNA were separated by gel electrophoresis using 1.2% agarose gel. 100 bp ladder was used to calculate the base pair length of *rol* genes. Specific amplified fragments of *rolA* (~260 bp), *rolB* (~750 bp) and *rolC* (~540 bp) were observed on gel electrophoresis, which were equivalent to the sizes of the respective gene sequences of *A. rhizogenes*. These fragments were recovered from the gel using gel elution method and used as probes.

Hybridization was done using *rolA*, *B* and *C* genes as probes by following the instructions of Alkphos direct labeling and detection system (Amersham Biosciences, UK).

Hybridization with probe: Preparation of labeled probe: One microgram of probe DNA was taken into a 0.5 mL eppendorf tube and denatured by heating in a boiling water bath for 5 min. Then cooled on ice for 5 min and spun to collect the contents at the bottom of the tube.

Labeling: Ten microgram of reaction buffer was added to the cooled DNA and mixed thoroughly but gently. Two microgram of labeling reagent was added and mixed gently. Ten microgram of working concentration of cross linker solution was added spun briefly to collect the contents at the bottom of the tube. The reaction was incubated for 30 min at 37°C.

Hybridization: The hybridization buffer was preheated to 55°C, blots were prehybridized in buffer for 15 min at 55°C with shaking. The labeled probe was added to hybridization buffer and left overnight for hybridization in hybridization oven at 65°C with gentle shaking. After hybridization the blots were carefully removed from hybridization buffer and washed in primary wash buffer (2 M Urea, 0.1% SDS, 0.5 M Sodium dihydrogen phosphate (pH 7.0) twice for 10 min at 55°C with gentle shaking. The blots were then washed with secondary wash buffer (1 M Tris base, 2 M Sodium chloride, 2 mM Magnesium chloride (pH 10.0)) twice for 5 min with gentle shaking at room temperature.

Detection: Thrity to forty microgram of detection reagent was pipette onto the blot cm^{-2} and left for 5 min. Excess of detection reagent was drained and the blots were wrapped in saran wrap. A sheet of X-ray film was placed on the top of the blot in hypercasette in a dark room and exposed for 2 h. The film was then developed and photographed.

RESULTS

Nicotiana glauca×*N. langsdorffii* hybrid plants showed many morphological variations like plagiotropic bulbus roots above the ground, dwarfism, very short

swollen internodes, distorted leaves and flowers. Similarly, physiological variations like sudden wilting of the whole plant, necrosis of terminal leaf, development of lateral buds into multiple shoots and partial to complete sterility resulting in poor or no seed set were observed. Among the five consecutive generations studied, the amount of variation was not to the same extent in all the plants and the degree to which these abnormalities were observed differed from plant to plant. Tumor development was triggered by damage caused by wounding (Fig. 1a), insect bite and mechanical injury. External stress like over crowding of the seedlings and high temperature also enhanced tumor expression. Teratomatous tumors (Fig. 1b) were developed on almost all parts of the plant (Fig. 1c) like hypocotyl region, stem, axil, petiole, leaf and inflorescence (Fig. 1d). Teratomatous callus growth (Fig. 1e) was observed from the leaf discs of the hybrid plants when cultured on hormone free MS medium. Parents neither showed tumorigenesis nor growth on hormone free MS medium.

The following results were observed on treatment of the parents and the hybrid with 5-azacytidine (a DNA hypomethylating agent (Kovarik *et al*., 2000) and cefotaxime (a DNA hypermethylating agent (Schmitt *et al*., 1997). Hybrid plants grown from 5-azacytidine treated seed showed poor plant organization with severe morphological and physiological variations (Fig. 2a). Tumor development was much earlier (from 7th day after germination) and within 40 days 94-99% of the treated plants turned tumorous whereas, only 10-14% of the control plants (Fig. 2b) were tumorous (Table 1). In the hybrid seed treated with cefotaxime, tumor development was observed from the 15th day onwards and most of the plants developed massive white root tumors (Fig. 2c). Ninty percent of cefotaxime treated plants (only 18% of their controls) turned tumorous within 60 days of germination (Table 2). The parents showed neither tumorigenesis nor morphological variations upon 5-azacytidine and cefotaxime treatments (Table 1, 2).

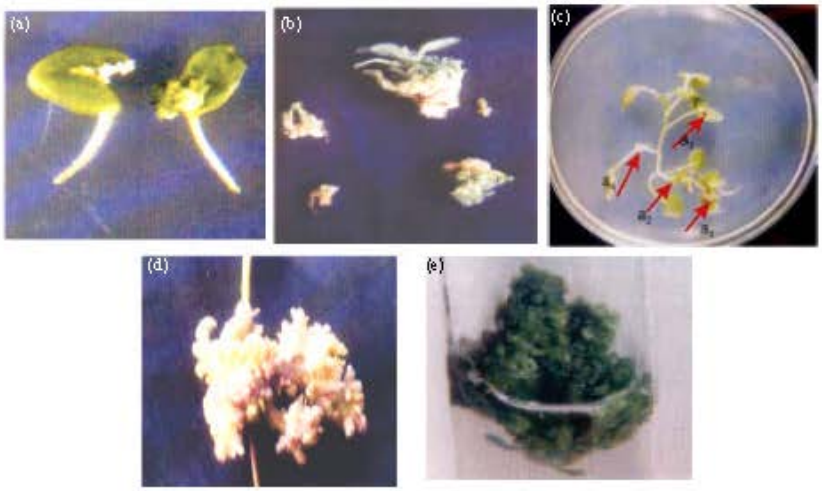

Fig. 1: Tumors of *N. glauce* x *N. Langsdorffi* hybrid. (a) Wound induced tumors, (b) teratomatous tumors, (c) tumors grown on different parts of the plant (a1) roots, (a2) hypocotyls region, (a3) leaf lamina, (4a) petiole, (d) inflorescence modified into a tumor medium and (e) Teratomatous callus grown from leaf disc on MS basal

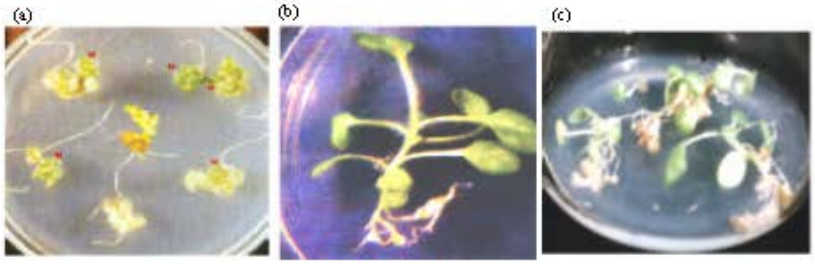

Fig. 2: Treatment of hybrid seed with 5-azacytidine and cefotaxime. (a) Plants grown from 5-azacytidine treated seed showing multiple shoots, (b) control plants and (c) plant grown from cefotaxime treated seed showing root tumous

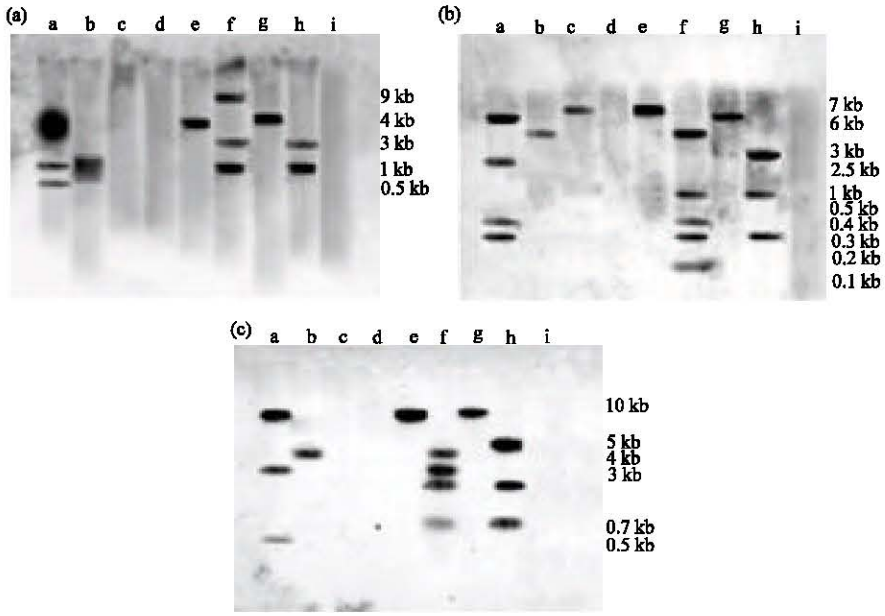

Fig. 3: Southern bolt analysis. (a) *rolA* gene as a probe, (b) *rolB* gene as a probe and (c) *rolC* gene as a probe. Lanes a, c, e, g: *N. glauca*, *N. Langgsdorffii*, hybrid and tumor DNA digested with Mspl. Lanes b, d, f, h: *N. glauca*, *N. Langgsdorffii*, hybrid and tumor DNA digested with HpaII. Lanei: DNA ladder

The results of Southern analysis further proved the effect of DNA methylation on tumorigenesis. Figure 3a shows the position of the bands when *rolA* gene was used as a probe. In the lanes cut with MspI, a band was observed at 4 kb region of *N. glauca*, hybrid and the tumor (lanes: a, e, g). Apart from that, two extra bands (1 and 0.5 kb) were present in the MspI digest of *N. glauca*. In the lanes cut with HpaII, a 1 kb band in *N. glauca*, hybrid and the tumor (lanes: b, f, h), a band at 3 kb region in hybrid and tumor (lanes: f, h) and one more band at 9 kb in hybrid (lane f) were identified. No signal was observed in the MspI and HpaII digests of *N. langsdorffii* (lanes: c, d).

Figure 3b shows the position of the bands when *rolB* gene was used as a probe. In the lanes cut with MspI, a 7 kb band in *N. langsdorffii* (lane c) and a 6 kb band in *N. glauca* and tumor (lanes: a, g) were observed. Two very close bands were identified in 6 and 7 kb region of hybrid (lane e) which were corresponding to the bands of *N. glauca*, tumor and *N. langsdorffii*. Three more bands (2.5, 0.4 and 0.3 kb) were identified in *N. glauca* (lane a). In the lanes cut with HpaII, a band at 4 kb region in *N. glauca* and hybrid (lanes: b, f) and 2 bands (1 and 0.3 kb) in hybrid and its tumor (lanes: f, h) were present. Two more bands (0.4 and 0.1 kb) in the hybrid (lane f) and a 3 kb band in the tumor (lane h) were identified. No signal was observed in the HpaII digest of *N. langsdorffii* (lane d).

Figure 3c shows the position of the bands when *rolC* gene was used as a probe. In the lanes cut with MspI, a band at 10 kb region was identified in *N. glauca*, hybrid and its tumor (lanes: a, e, g). Two extra bands (3, 0.5 kb) were present in *N. glauca* (lane a). In the lanes cut with HpaII, 4 kb bands in *N. glauca* and hybrid (lanes: b, f), two common bands (2.5 and 0.7 kb) in hybrid and tumor (lanes: f, h), a 3 kb band in hybrid (lane f) and a 5 kb band in tumor (lane h) were identified. No signal was observed in the MspI and HpaII digests of *N. langsdorffii* (lanes: c, d).

DISCUSSION

Tumor development and abnormalities observed on the hybrid plants could be due to the expression of *rol* genes of *Agrobacterium* because similar results were observed in *Nicotiana* and other plants when transformed with *rol* genes. The presence of cellular T_L-DNA genes in plants is because of ancient horizontal transfer of genes between plants and an ancestor *A. rhizogenes* (Furner *et al*., 1986; Aoki, 2004). The integration of T-DNA into the *Nicotiana* genome is through insertion at double stranded breaks by nonhomologous end joining (Chilton and Que, 2003). The tumorigenesis and behavioral changes observed in *N. glauca* x *N. langsdorffii* hybrid plants were considered to be due to the collective expression of *rolA, B* and *C* genes of

A. rhizogenes. The product of *rolA* interfered with the formation of polyamine conjugates (Sun *et al*., 1991), *rolB* protein has β-indole glucosidase activity responsible for the stimulation of auxin accumulation (Estruch *et al*., 1991a) and *rolC* encoded a polypeptide β-glucosidase that hydrolyses cytokinin N- and O-glucosidase releasing the active form of cytokinin from its inactive conjugates (Estruch *et al*., 1991b). As the products of *rolA*, *B* and *C* genes are involved in altering endogenous auxin/cytokinin balance; they interfere with all metabolic activities resulting in tumorigenesis, several abnormalities and phytohormone independent growth. Further, the treatment of both 5-azacytidine and cefotaxime triggered tumorigenesity and increase in 5-azacytidine concentration enhanced tumorigenesity.

The results of Southern analysis revealed changes in the levels DNA methylation of *rolA, B* and *C* genes in *N. glauca*, hybrid and tumor. More bands in MspI digest of *N. glauca* represent the hypermethylated state of these genes, one of the factors responsible for gene silencing. This might be the reason for normal morphology, absence of tumors and lack of phytohormone independent growth in cultures of *N. glauca*. No signals in *N. langsdorffii* represent the absence of these genes. In contrast, the hybrids show many cuts with HpaII. This may be due to DNA hypomethylation, resulting in gene activation. Methylation plays a decisive role in the activity of many genes (Popiela *et al*., 2004).

Though same set of *rol* genes are present in *N. glauca* and its hybrid, the hybrid is tumorous and the parent is normal. Expression of *rolA, B* and *C* genes made the hybrid tumorous. The previous studies of Furner *et al*. (1996) and Aoki (2004) could show the presence of *rol* genes in *N. glauca* and its hybrid but the present study clearly illustrates that DNA hypermethylation of *rol* genes is responsible for their silencing in *N. glauca* and their consequent hypomethylation in the hybrid caused their expression. The results of set of experiments carried out with 5-azacytidine and cefotaxime convey that methylation has a role in tumorigenesis. Further, Southern analysis proved the silencing of *rolA*, *B* and *C* genes in *N. glauca* which is due to an increase in DNA methylation. On the other hand, decrease in DNA methylation of these genes led to the expression of *rol* genes in the hybrid and tumor. It was reported for the first time that DNA methylation is responsible for silencing and expression of *rol* genes in *N. glauca* and its hybrid, respectively. Thus, it can be concluded that DNA methylation status is a promising biomarker for malignancy, it occurs at an early stage of tumor development and may precede morphological changes.

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