PERIODICO di MINERALOGIA Volume 88, No. 1, 2019

An Epigenetic Study of Bacterial NPTII Gene Expression in A*grobacterium Tumefaciens* and A. *Rhizogenes* Mediated Potato Transformants

NATALIA CUCU

University of Bucharest, Institute of Genetics, 1-3 Aleea Portocalelor, sector 6, Bucuresti

Abstract

A bacterial nptII gene expression in two different potato (Solanum tuberosum) transformation systems has been studied. The variabiliy of the gene expression observed in Agrobacterium tumefaciens mediated transformants, as compared with A.rhizogenes mediated ones, has been ascribed to the involevement of the DNA methylation processes. The already established inverse correlation between the distribution of the critical 5-methyl cytosine (5mC) into the gene sequence and its expresssion has been demonstrated using specific DNA methylation sensitive methods („in vitro" treatements with the known DNA methyltransferase inhibitor 5-aza-cytidine and the specific DNA methylation sensitive restriction with the isoschisomeric pair MspI/HpaII). A possible explanation linked with the influence of the intergenic interractions introduced by the two different transformation systems upon the nptII alogene in the transformed potato genome is discussed.

Keywords: marker alogenes, mediated genetic transformation, transgenes, minor bases, epigenetic information, DNA methylation, isoschisomeric enzymes

Introduction

The DNA methylation processes have been extensively claimed to influence the gene expression in eukaryotes. An already established general rule is referred to as the *inverse relation between the concentration or, more precisely, the distribution of the methyl groups on the critical cytosine residues into the gene sequence and the transcription status of this particular gene*.

The actual common *term of "DNA methylation*" is referring to as a domain dealing with a **biochemical reaction** catalyzed by an enzyme, named DNA methylase (E.C.). It specifically transfers a methyl group from its donor to the purine or pyrimydine ring of the DNA bases. S-adenosyl-methionine or SAM is frequently claimed to be the universal methyl donor for all organisms, while the preferentially targeted base is cytosine (C). However, the above mentioned transcription function of DNA is generally linked all the minor bases resulted from their modification by the methyl group insertion into their rings, the common key minor base in regulation processes of the gene expression is 5mC (CUCU et al, 1994; CUCU, 2000 and 2001).

It is to b thate noted the methylation of DNA bases can be perfomed by two distinct processes: one which is referred to as „native" or „**biochemical**", implying the organisms' DNA methylase or DNA methyltransferase (DNMTase) activity and the other one, which

results from a **chemical reaction** performed by the external, environmental methylation agents (CUCU, 2000a). The last one, however, involves the biochemical activity of the DNA **reparatory enzymes** (specific glycosylases), based on the excision of the transformed bases (usually claimed as O- methylguanine or O- methyladenine) (LAIRD and YAENISCH, 1998; SZYF, 2001).

The study of the native DNA methylation processes linked to a particular gene expression in eukaryotes may be therefore facilitated by treatements with a specific DNMTase inhibitor, 5-aza-cytidine (5azaC). These usually entail an "in vitro" approach which ascertains both an enhanced rate of DNA replication by the increased cell division and suppressed DNA methylation by the strong covalent bonds between the inhibitor and the targeted DNA C residues. The resulted demethylated DNA has frequently proved to assure the reactivation of certain initially silenced genes (JONES, 1984 a,b).

The DNA methylation domain has had enormous gains also from the analysis of *genetically transformed or transgenic organisms*, especially plants, well known for their genome flexibility towards DNA methylation dynamics. The transferred *alogenes* (referred to as *„transgenes*" when stably inserted and expressed in the host genome) into the endogenes of a given host genome determine the so called „insertional mutagenesis". This process does not have the classical known *mutagenic effect* either upon the alogene or the background genome: both of these keep their DNA sequences, however certain bases (preferentially C) from their sequence undergo minor modification such as methylation (CUCU, 2000b).

The stable genetic background shows a particular reactivity towards the newly inserted alogenes which depends essentially on the newly emerged intergenic interactions. The stabilisation of the affected host genome is performed through a specific re-distribution of the methylation marks on the critical C residues. This affects the alogenes' expression drammatically and depends on numerous factors: (i) copy number of the alogenes, (ii) structure of chromatin and DNA methylation status inside the genome region of insertion or (iii) the turnover of the corresponding RNA (CUCU and GAVRILA, 1997; GAVRILA and CUCU, 1996).

This work is aiming at revealing the causes and mechanisms which may determine the variation of a well studied *nptII* bacterial gene when inserted into a plant (*Solanum tuberosum*) genome through two different mediated (indirect) genetic transformation systems: *A.tumefaciens* and *A.rhizogenes*, respectively. Usual methods of modern epigenetics, are proposed to explain the action of certain possible intergenic interactions induced by specific, Ti*)* or Ri T-DNA derived genes. Such a study is proposed for further genetic stability assays and risk assessment approach for genetic modified organisms (GMO) domain and its linked ecologic and bioethic subdomains.

Materials and Methods

Agrobacterium Strains

Ti (tumour inducing) -JIT and Ri (root inducing) -160 plasmids of the two modified *Agrobacterium* strains have been used for the bacterial *nptII* transfer into the plant genome. Noteworthy for the further discussion of the results, however, the two T-DNA provided by the different *Agrobacterium* strains had the same marker *nptII* or *neo* gene, this one was transferred at the same time into the plant genome with different accompanying genes: the *A.tumefaiens* derived T-DNA was disarmed, or *free of* the specific plant oncogenes, *cyt* (gene coding for the synthesis of cytokinin) and *aux* (two genes for the synthesis of auxin), while the *A.rhizogenes* derived T-DNA *kept* its specific *rol* (root inducing locus) oncogenes. The expression of *nptII* alogene into the plant genome is controlled by the *nos* promotor in both

cases (**Figure 1**). The strains were kindly offered by the University of Agronomic and Veterinary Sciences, Faculty of Biotechnologies, Bucharest.

Also the pJIT79 plasmid has been used for obtaining the labelled *nptII* or *neo* coding sequence probes which have been further used for molecular analysis of the inserted *nptII* gene into the plant genome DNA.

Potato Transformant Plants

The two lines of Ro potato (*S.tuberosum* var. Desiree) vitroplants selected as transiently transformants were kindly provided from the "in vitro" culture of Drd. A.Calin (Institute of Biology, Roumanian Academy of Sciences). The technique of obtaining different variants of potato transformed plants and their selection and maintaining as an „in vitro" culture have been performed by an indirect (*Agrobacterium* mediated) method described elsewhere (CALIN et al, 1996; CALIN A, personal communications).

The transformant variants under consideration contained the same marker *nptII* gene, coding for a corresponding NPTII protein, which confers kanamycin resistance to infected and regenerated plants. They differred however by the presence of the oncogenes. The obtaining of the *A.tumefaciens* mediated transformed plants needed a disarmed strain, which lacked the two wilde *aux* oncogenes (for auxin synthesis) and the *cyt* oncogene (for cytokinin synthesis), while the *A.rhizogenes* mediated ones were able to regenerate in spite of the presence of the wilde *rol* oncogenes (characterized by the so - called root inducing locus). The desarmation of *A.tumefaciens* strain was needed for the obtaining of a whole plant development, while the *rol* oncogenes from the *A.rhizogenes* system do not affect the regeneration processes and in the meantime have an interesting effect upon the development of the plant the genetic stability of the marker alogene under consideration.

Methods for Functional Genomic Analysis of the Putative Transformed Potato Vitroplants

A combination of genotype(DNA) and phenotype(protein) and proteomic analyses has been approached in order to assure the further discussion of the epigenetic mechanisms involved into genetic variation observed with certain transformed vitroplants.

Genotype analysis

Genomic DNA isolation

A CTAB (cethyltrimethyl ammonium bromide) cathionic detergent based protocol (DELLAPORTA, 1995) has been used for the extraction of plant DNA. This implies briefly: CTAB/NaCl/PVP (polyvinyl pyrrolidone, 50M) treatment at 65^{o}C of the plant tissue; protein and lipid removement by using a mixture of chlorophorm: isoamylic acid) (1:20); DNA precipitation in isopropylic alcohol; repeating the deproteinising steps and precipitation of DNA with cold absolute ethanol; rinsing the pellet with 70% ethanol. The DNA was dissolved in TEN, treated with Rnase and preserved at –20^{o}C.

The used solutions are as follows: *CTAB buffer* (2%- w/v- CTAB, 1.4M NaCl, PVP 1% and 20 mM EDTA, pH 8.0, in 100mM Tris; *conc CTAB solution* (10% CTAB in 0.7M NaCl); *ppt sol* (1% CTAB and 10mM EDTA in 50mM Tris, pH 8.0); *TEN* (1mM NaCl in 10mM Tris, pH 8.0); *TE* (0,1 mM EDTA in 1 mM Tris (pH 8,0)).

DNA sample purity assays have been performed as follows: diluted (1: 10) samples of DNA preparation in water were used for absorbance spectra registrations in the wave length domain between 200-300 nm and A260/A280 ratio based purity estimation. The measurements were performed on Spectronic Genesys UV-VIS spectrophotometer. Genomic DNA was tested by its

electrophoretic behavior in 0.8% agarose gels, in TBE (89 mM Na Borate and 2,5 mM EDTA in Tris, pH 8.0). The DNA bands were coloured in 0.1% Ethidium Bromide and photographed in a gel scanning dark room (UVP Biological Instruments).

NptII gene analysis in plant genome

A PCR method has been used for the detection of the *nos* (nopaline synthase) promoter which controls the expression of the *nptII* or *neo* alogene.The primer pair sequences were as follows: 5'CGGGGGGTGGGCGAAGAACTCCAG3' and 5'CCCCTCGGGTATCCCAATTAGAG3' (Meyer et al, 1995).

The reaction mixture contained 21 µl (1.0 µL dNTP/0.5 µL reaction buffer/2.5 µL each primer/14 µL water; 0.5 SIGMA Taq polymerase is added after a previous 5 min denaturation step in termocycler (Technix); 40 cycles including the following steps were performed: 95 ^{o}C – 45 sec; 58 ^{o}C – 40 sec and 72 ^{o}C – 2 min; a final polymerisation step of 72 ^{o}C was also included.

Common protein analysis

The expre ssed NPTII protein has been detected as a 59kDa (representing the active, stable dimer) or as a 28 kDa band (representing the inactive monomer) in (12,5%) SDS-PAGE of the plant total protein extract (COLBERE-GARRAPIN et al, 1981). A protein mixture marker representing the domain of 29-205 kDa have been used.The plant tissues used for this assay were represented by whole regenerated and selected plantlets and by the callus obtained for the in vitro maintaining of the already transformed plantlets (CALIN et al, 1996).

The total protein extract has been obtained following a method described by HERRERA-ESTRELLA et al, 1994, which briefly included: 100mg plant material treated with cca 50 µl extraction buffer (1% β-mercaptoetanol - 0,13% leupeptine - 0,1% ascorbic acid in 2x 50mM Tris-HCl, pH 6,8) is extracted on ice; the further centrifugation pellet is assayed for the protein concentration and for the electrophoretic fractionation.

The enzyme NPT II activity has been estimated by „in vitro" methods for the selection of the transformed plant tissues on kanamycin containing media (CALIN et al, 1996). Such analyses have been performed periodically, during 0.5, 1 and 2 years. The rooting capacity criterium has been used for the estimation of the NPTII detoxification capacity of the kanamycin containing media. Also different tissue types (whole plantlets, callus) have been approached in order to estimate the involvement of certain endogenous factors affecting the *nptII* gene expression (CALIN et al., 1998).

Epigenetic analysis

The accordance between the genomic/proteomic results have been periodically verified in R0 transformant potato vitrolines. The variation relevant cases were supposed to involve epigenetic modifications which determined certain negative proteomic results. The reversibility ratio of cca 25% of the relevant cases after in vitro treatments with 5azaC suggested the futher study of the DNA methylation processes in *nptII* inserts.

In situ analysis of the nptII gene methylation

Restriction genomic DNA with the isoschisomeric pair MspI/HpaII has been performed for the two different *Agrobacterium* mediated transformant lines. The digestion reaction was performed briefly as follows: 10µg DNA in 10µl TE was treated with 50U (5-10µl) restriction enzyme in 10µl 10xenzyme buffer (distilled water added up to 100µl); the 4h digestion at 37 ^{o}C, was followed by deproteination and DNA precipitation in ethanol; aqueous

DNA solution obtained afterwards was used for electrophoretic fractionation on 2% agarose (20 x 20cm) gel, in TBE buffer.

The subsequent Southern hybridisation was performed on the nylon (Hybond N^+ , Amersham) membrane, containing the DNA restriction fragments after a previous alcaline transfer. The *nptII* radiolabelled probes were kindly provided by. Dr. N.Barbacar from Institute of Genetics, Moldavian Academy of Sciences, Chisinau, where the Southern hybridisation has also been performed and the *nptII* insertion autoradiography obtained.

The used solutions were generally as follows: *denaturation buffer (*1,5 M NaCl - 0,5 M.NaOH); *neutralizing buffer* (1,5M NaCl– 1 mM EDTA in 0,5M Tris-HCl pH 7,2); *transfer buffer*: 10 x SSC (1 x SSC: 0,15 M NaCl; 0,015 M Na citrate); *fixative alcaline solution* of the radiolabelled probe (0,4M NaOH); *prehybridation solution* (2 mM EDTA pH 8,0 - 7% SDS in 0,25M Na_2HPO_4 pH 7,4); *membrane rinsing solution* (2 x SSC – 0,1% SDS si 0,2 x SSC - 0,1%SDS). The isotope treated membrane is introduced with the photographic plaque in a Roentgen casette; after 4 days exposure at dark and - 80^{o}C, the autoradiography may be analysed for the nptII spots on specific restriction DNA fragments.

Results and Discussions

The *nptII* (*neo* or *aph*) gene codes for an aminoglycoside phosphotransferase which catalyzes the phosphate group transfer from ATP on the aminoglycoside type antibiotic neomycine or kanamycine. The substitution of the antibiotic 3'OH group affects correspondigly its capacity to bond ribosome, which is actually its basic toxic activity in plants.Therefore, the expression of the marker gene, the protein NPT(or APH)II, has an enzyme activity which explains the kanamycin resistance of the transformed and actively expressing potato plants. The choice of this enzyme as a marker protein was based on the slight similar activities of endogenous plant kinases. This fact favoured the "in vitro" selection of the transformed plant cells or tissues.

This work is based on a complex experimental model which includes two different transgenesis systems implying *A.tumefaciens* and *A.rhizogenes* mediators for the transfer of the bacterial *nptII* gene into the potato vitroplants. The functional genomic analysis of the *nptII* alogene in transformant lines has been performed by approaching *genomic* (DNA) and *proteomic* (protein) analyses respectively. The variation of the accordance between the two types analyses suggested the *epigenetic* approach of the factors involved into the regulation of the gene expression. Usual processes linked with the epigenetic information are claimed as *DNA methylation* ones. These are known to detremine the repression of the alogenes not by mutagenesis, but by the apparently minor modification of C residues in DNA. The distribution of the resulting 5mC may reflect therefore a certain gene expression status. Specific DNA methylation methods such as HPLC have initially been used to study the relative concentration of 5mC towards all methylable C in genomic DNA. Such a general approach has not revealed notable DNA methylation dynamics (CUCU et al., 1998). Rather an "in situ" analysis of the interest gene was able to demonstrate a correlation with the variation of its expression. The experimental model described here is aiming at the study of the *nptII* DNA methylation level in the two different lines of *Agrobacterium* mediated potato transformants.

The variation of the rooting or callus development on kanamycin containing media observed with the two lines depended on the type of *Agrobacterium* used as a mediator in the *nptII* transfer process: the *A. rhizogenes* mediated transformants (Ar) manifested an interesting genetic stability as compared to those *A. tumefaciens* (At) transformants. The lack of concord between the genomic and proteomic results suggested the approach of the

reversability test with 5azaC, the DNMT ase inhibitor. The 25% percentage of reactivation of the rooting ability with the relevant At cases indicated a possible involvement of the DNA methylation processes in the At *nptII* expression variation (CALIN et al., 1998).

Figure 1 represents the pJIT79 gene map containing the *neo (nptII)* gene into the T-DNA, which is similar for both Ti and Ri plasmids used for the analysis of the same bacterial gene into the transformant DNA. The marker gene is controlled by the *Pnos* promotor.

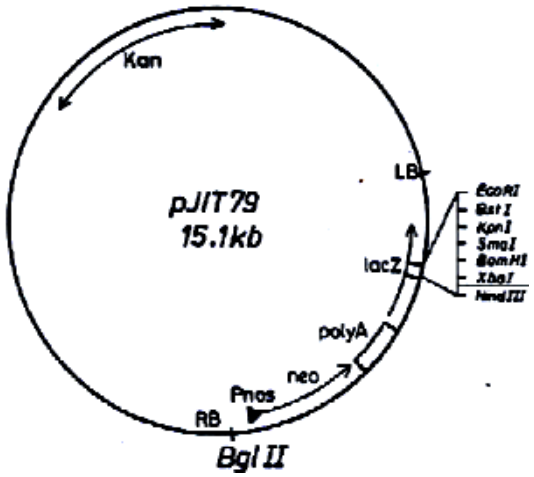

Figure 1. The gene map of the pJIT79. T-DNA is represented between right and left borders (RB and LB respectively). *Neo* or *nptII* gene is cloned into this T-DNA and is controlled by *Pnos* promoter.

The genomic analysis revealed the presence of the neo gene into the genomic plant DNA through the detection of the PCR (0.289kpb) amplicons of its *nos* promoter (**Figure 2b**) obtained by the PCR reaction performed on the plant DNA (**Figure 2a**).

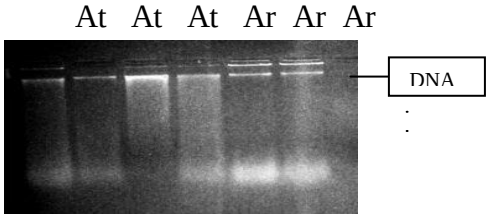

Figure 2a. Genomic DNA electrophoresis

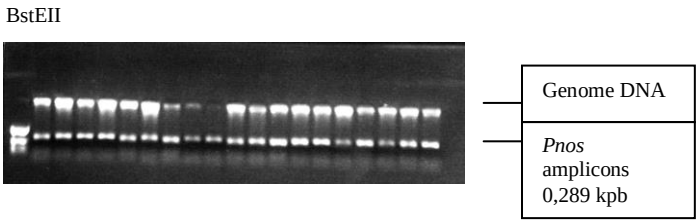

Figure 2b. The *Pnos* amplicons obtained by PCR reactions demonstrate the insertion of the *nptII* gene in plant DNA

Figure 3 represents the NPTII protein detected in the electrophoregrame towards the protein marker. An interesting intense band has been observed with the Ar transformant as compared to the At ones, which may be ascribed to a high *nptII* gene expression in the Ar transformants.

The already commented lack of concord between the two described analyses noticed after periodical assays suggested the epigenetic approach by MspI/HpaII (M/H) DNA restriction analysis and Southern blotting with radiolabelled *nptII* probes. **Figure 4a** presents the autoradiography of the *nptII* copy inserts in each pair of the isoschisomeric restriction DNA

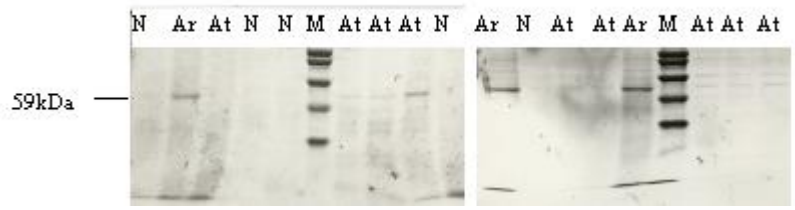

Figure 3. Electrophoregrams of the plant total proteins showing the NPTII band of 59kDa: N-untransformed; At-*A.tumefaciens* mediated transformant; Ar-*A.rhizogenes* mediate transformant; M-protein marker

fragments. As it is described in **Figure 4b** the DNA methylation status may be estimated by comparing the pair profiles: the identical ones indicate a demethylated DNA fragment, while the different ones, a methylated DNA fragment.

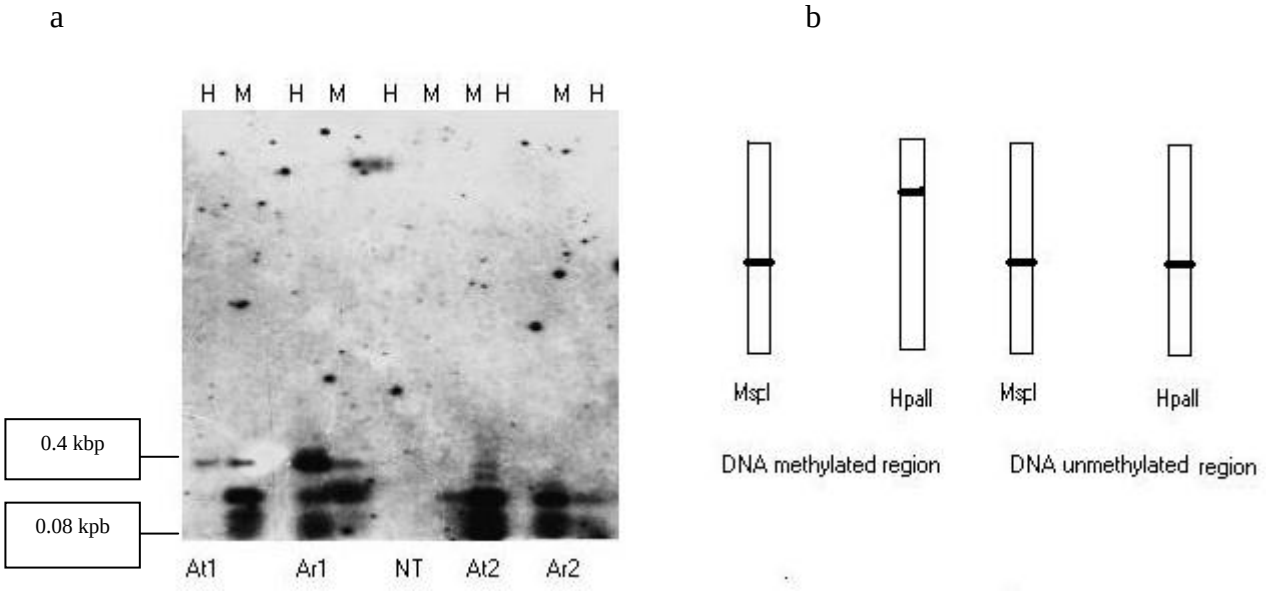

Figure 4. Autoradiography of the Southern hybridised nptII labelled probes on DNA restriction fragments obtained with the isoschisomeric pair MspI/HpaII.

As a conclusion, using the specific DNA methylation method for the „in situ" analysis of the *nptII* alogene in transformant DNA it was possible to verify the already established indirect correlation between the DNA methylation status and the considered gene transcriptional activity. Further experiments aiming at revealing the factors involved in the activation of the DNA methylation suppression with the Ar transformants are currently run.

Aknowledgements

This is a a work based on the updated Ph.D thesis dissertation presented in 2000. I thank for helpful advises Prof. Dr. Lucian Gavrila, the coordinator of my Ph.D. training program, and to my colleagues (Drd. A. Calin) for their technical support.

References

A. CALIN, N. CUCU, C. TESIO, *Biotechnol &Biotechnol Equip* **10** (2-3), 18-24 (1996).

F. COLBERE-GARAPIN, F. HORODNICEANU, P. KOURILSKY, A. C. GARAPIN, *J. Mol. Biol.*, **150,** 1-14 (1981).

N. CUCU, I. ANGHEL, A. BREZEANU, A. CALIN, G. COGALNICEANU *in „DNA methylation level as a possible biomarker in cytodifferentiation experiments"*, Proceedings of the 8th Nat Symp of Ind Microbiol and Biotechnol, I.Anghel ed., University of Bucharest Press, pp. 366-78, 1995.

N. CUCU, A. CALIN, Proceedings of the XXV Ann Meeting of European Society for New Methods in Agricultural Research (ESNA), Piacenza-Italy, p. 75, 1995.

N. CUCU, L. GAVRILA, *Roum Biotechnol Lett* **2**(3), 183-191 (1997).

N. CUCU, A. CALIN, M. STEFAN, M. MITRU, L. GAVRILA, *Rev roum Biochimie* **35**(1-2), 175 (1998).

N. CUCU, A. CALIN, D. ALEXANDRU, *Revue Roumaine de Biochimie*, **35**(1-2), 43 (1998).

N. CUCU, M. STEFAN, M. MITRU, L. GAVRILA, in Proceedings of the First National Symposium "Scientific and bioethic implications of genome analysis and manipulation" Bucharest, October 15-16 1998, Ed *Ars Docendi*, Bucuresti, p. 161, 2000.

N. CUCU, *Roum Biotechnol Lett*, **6**(6), 456-467 (2001).

DELLAPORTA, *Plant Mol Biol Rep,* **3**(6), 425 (1995).

L. GAVRILA, N. CUCU, L. GHETEA, *Roum Biotechnol Lett*, **1**(3), 163-170 (1996).

L. HERRERA-ESTRELLA, P. LEON, O. OLSSON, T. H. TEERI, *Plant Mol Biol Man* C2: 1-32 (1994).

P. A. JONES, *in DNA Methylation: "Biochemistry and Biological Significance",* A.Razin, H. Cedar and AD Riggs eds., Springer Verlag, pp. 165-187, 1984.

P. A. JONES, *Curr Top Microb Immunol*, **108,** 157-173 (1984).

P. W. LAIRD, R. JAENISCH, *Hum Mol Gen*, **3,** 1487-95 (1999).

P. MEYER, I. NIEDENHOF, M. TEN LOHUIS, *EMBO J,* **13**, 2084-2088 (1994).

M. SZYF, *Frontiers in Bioscience*, **6** (1), 599-609 (2001).