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Gibberellin A_3 as an epigenetic determinant of global DNA hypo-methylation in tobacco

R. MANOHARLAL[1]*, G.V.S. SAIPRASAD[1], C. ULLAGADDI[1], and A. KOVAŘÍK[2]

ITC Life Science and Technology Centre, Peenya Industrial Area, Bengaluru-560058, Karnataka, India[1]
Institute of Biophysics, Academy of Sciences of the Czech Republic, CZ-61265 Brno, Czech Republic[2]

Abstract

Gibberellins (GAs) are a large family of tetracyclic diterpenoids, controlling important aspects of growth and development throughout the plant life cycle. To explore the possibility that gibberellin A_3 (GA_3) signalling induces epigenetic alteration(s), we carried out a field experiment study using *Nicotiana tabacum* as a model system. The GA_3 application on leaves resulted in increased plant-height, foliage density, leaf cell area, and trichome density. The plants exposed to GA_3 also exhibited: *1*) increased chromatin de-condensation, *2*) reduced global DNA methylation, *3*) reduced DNA methyltransferases (NtDNMTs) activities accompanied by decreased amounts of *NtMET1* and *NtCMT3* transcripts, and *4*) partial restoration of phenotype and expression of epigenetically silenced reporter transgene. Based on these observations, we propose that GA_3 application induces complex epigenetic re-programming, which may lead to distinct developmental phenotypes. These results could provide an important insight for future studies on epigenetic mechanism(s) in other important crops.

Additional key words: chromatin de-condensation, DNA methyltransferase, *Nicotiana tabacum*, leaf cell area, plant height, trichome density.

Introduction

Phytohormones gibberellins (GAs) are a large family of tetracyclic diterpenoids, controlling important aspects of growth and development throughout the plant life cycle under non-stress conditions. Its significance has been demonstrated by the fact that embryos synthesize endogenous GAs, releasing it into the endosperm during germination (Atzorn and Weiler 1983). GAs are mostly used in laboratory and greenhouse settings to trigger the seed germination that would otherwise remain dormant. Under field conditions, GA application has been reported to promote early growth, flowering, and fruit and grain settings. GAs were also reported to play a pivotal role in abiotic stress tolerance of plants (Richards *et al.* 2001). Diverse roles of GAs suggest the existence of multiple receptors and signal transduction pathways (Olszewski *et al.* 2002). Significant contributions to understanding of GAs-regulated morphogenesis include the identification of upstream regulators of GA biosynthesis, the elucidation of function of GA signalling components and the isolation of downstream targets (Thomas and Sun 2004, Bar and Ori 2014). Present understanding of roles of GAs in plant physiology is largely based on analyses in model plants such as *Arabidopsis thaliana* and *Nicotiana tobacum*. Genetic and biochemical analysis revealed that GA depresses its signalling by destabilization of DELLA protein growth repressors *via* 26S proteasome pathway (Hedden and Phillips 2000, Olszewski *et al.* 2002). The continued use of ever-improving forward and reverse genetic techniques further lead to the discovery of novel roles for GAs in plant development. Although these studies have increased our understanding of the effect of GAs in specific plant

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Abbreviations: Ado-Met - S-adenosyl-methionine; 5-AZA -C - 5-azacytidine; DNMT - DNA methyltransferase; DSMO - dimethyl sulfoxide; GA_3 - gibberellin A_3; Gen - genistein; PTGS - post-transcriptional gene silencing; TGS - transcriptional gene silencing; TSA - trichostatin A.
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*Corresponding author; fax: (+91) 80 42787604, e-mail: ramanpdf01@gmail.com

developmental phases, however, the role of GAs in precise epigenetic molecular mechanism(s) remain unexplored.

DNA cytosine methylation, histone modifications, and RNA interference (RNAi) are most common molecular epigenetic mechanisms, causing epi-genome alteration and they play essential role in gene regulation. Among this, DNA cytosine methylation constitutes up to 30 % of the total cytosine residues (Finnegan and Dennis 1993). Both asymmetric (^{m}CpHpH)-methylation and symmetric (^{m}CpG and ^{m}CpHpG)-methylation (where H = A, C, or T) are important epigenetic markers in plants and they are involved in chromatin remodelling, regulation of the transcriptional genome output as well as direction of the deposition of other epigenetic markers (Lewis and Bird 1991, Razin and Cedar 1991, Bird 2002).

The process of cytosine methylation is catalysed by an enzyme, DNA (cytosine-5) methyltransferase (MET), transferring a methyl group from S-adenosyl-methionine (AdoMet) to the fifth position of a cytosine residue. So far, the plant DNA methyltransferases (DNMTs) have been identified and grouped into four main families based on their linear domain arrangement involving a variable N-terminal domain and the catalytic C-terminal domain: *1*) methyltransferase 1 (MET1), *2*) domains rearranged methyltransferase (DRM), *3*) chromomethyltransferases 3 (CMT3), and *4*) DNA methyltransferase 2 (DNMT2). All these DNMTs utilize a common catalytic mechanism and employ AdoMet co-factor as the source of the methyl group (Finnegan and Kovac 2000, Pavlopoulou and Kossida 2007).

Modification in the frequency of DNA cytosine methylation by DNMTs inhibitors can be a feasible tool to engineer novel and improved trait(s) in plants. Indeed, this point has been demonstrated by studies with well-known DNMT inhibitors that inhibit hyper-methylation, restore suppressor gene expression, and exert anti-tumor effects in *in vitro* and *in vivo* laboratory models (Colot and Rossignol 1999, Baylin and Herman 2000). Although in *Arabidopsis*, a population of recombinant inbred lines with epigenetically mosaic chromosomes consisting of wild-type and CG methylation-depleted segments (epiRILs) shows the impact of cytosine DNA methylation on various observed altered phenotypes (Reinders *et al.* 2009). However, the practical implication of DNMT inhibitors in plants is not well understood. Thereby, there is a great need for the understanding, identification and development of effective, specific and non-toxic DNMT inhibitors in plants. In this study, for the first time we provide evidences that GA_3 also acts as DNMT inhibitor in plants and can be used as an epigenetic inducer for attaining desirable traits.

Our current study was focused on the role of cytosine DNA methylation in GA_3 mediated morphogenic and biochemical changes in tobacco. We tested the role of GA_3 application on morphometric observations, chromatin alteration, global DNA methylation and 5-methylcytosine (5-mC) status, NtDNMTs activity, their respective genes expression, and partial restoration of DNA methylation dependent epigenetic-silenced transcriptional and post-transcriptional *npt*II reporter gene expression.

Materials and methods

Plants, growth, and treatments: Seeds of tobacco (*Nicotiana tabacum* L.) cv. Kanchan were used in this study. The GA_3 (*Sigma*, St. Louis, USA) was either added into Murashige and Skoog (MS) medium during *in vitro* cultivation or sprayed on leaves under field conditions. During *in vitro* cultivation, plants were cultured in a growth chamber at a temperature of 26 °C or in a greenhouse at a temperature of 28 °C, 16-h photoperiod, day/night relative humidities of 70/80 %, and irradiance of 80 μmol m^{-2} s^{-1}. Stock of 50 mg dm^{-3} GA_3 (in ethanol) was prepared freshly before use. For GA_3 foliar application under field condition, tobacco seedlings transplanted from nursery to main-field in three plot replicates 60 d after sowing at Northern Light Soil (NLS) region, Rajahmundry, Andhra Pradesh, India were sprayed only once with 50 mg dm^{-3} GA_3 at 90 d after sowing (*i.e.* 30 d after transplantation; DAT). Foliar spray was done in such a way that each plant leaves at all positions gets fully drenched with GA_3 solution (~30 cm^3 $plant^{-1}$). A non-ionic surfactant *APSA-80* (*Amway*, India) at concentration 0.5 cm^3 dm^{-3} of water was used as spreading/wetting agent. Control plants were sprayed with solvent (Fig. 1 Suppl.). After GA_3 application, 10 uniform plants for each untreated and treated variants were selected and tagged. Among these, morphometric measurements were done from 5 representative biological replicates. For microscopy (leaf cell-area and leaf trichome density measurement) and biochemical (chromatin accessibility, global DNA methylation, NtDNMT activities, and mRNA accumulation) analysis, leaf samples were harvested from middle-position (generally 10^{th} - 11^{th} leaf from bottom of a plant) of these 5 plants at indicated time of growth, immediately snap frozen in liquid nitrogen, ground to a fine powder in mortar and pestle and stored at -80 °C till further use.

Leaf epidermal cell area and trichome density measurements: Imaging of tobacco leaves was carried out as described by Wuyts *et al.* (2010) with certain modifications. Untreated and GA_3 treated tobacco leaves at early vegetative growth stage (13 d after GA_3 application), were fixed in 5 cm^3 of a mixture of ethanol + acetic acid (3:1, v/v) with a drop of *Tween-20*. Leaf samples were put under vacuum for 1 h and kept on a shaker at 4 °C for 48 h. Subsequently, they were rinsed in 50 and 70 % (v/v) ethanol. Leaf samples were conserved in 70 % ethanol at 4 °C till further use. The procedure for

clearing and staining included the following steps: *1*) chloroform treatment for 10 min, rinsing in 70 % ethanol, progressive rehydration, clearing in sodium dodecylsulphate (SDS)/NaOH for 15 min, rinsing in water, and amylase treatment at 37 °C overnight; *2*) rinsing in water, periodic acid treatment for 40 min, rinsing in water, and staining in pseudo-Schiff-propidium iodide for 6 h followed by an overnight rinsing in water; 3) clearing in chloral hydrate for 4 h and montage in Hoyer's solution, if required. Leaf samples were positioned on cover slips and then turned and mounted on microscope slides. This was done to limit the distance between the sample and cover slip, thereby optimizing for the limitation imposed by the working distance of the objective and the path of the laser and emitted light. Samples were imaged on a fluorescent microscope (*Leica DM1000*, *Leica Microsystems*, Buffalo Grove, IL, USA) with a 40× optical magnification used for snaps and measurements. *Qwin* software was used for cell-area measurement (standard frame size for cell size measurement was 22424.4 mm^2). All measurements were done in triplicates for a minimum of 20 cells for each replication.

For trichome counting, standardized safranin staining technique was used as described by Zajączkowska *et al.* (2015). Briefly, leaf sections were placed in safranin dye for 4 h, followed by 70 % ethanol wash for 1 min. Leaf sections were finally rinsed with tap water and were observed under microscope (*Leica DM1000*). Both adaxial as well as abaxial surfaces were used for trichome scoring with at least 6 biological replicates.

Micrococcal nuclease (MNase I) assay: MNase I assay was performed as described by Zhao *et al.* (2001) with slight modifications. Chromatin was extracted from untreated and corresponding GA_3 treated tobacco leaves at early- to late-vegetative growth stages (0, 4, 8, 24, 48, 72 h and 13, 25, 35, 50 d after GA_3 treatment) using *EpiQuik ChromaFlash*™ extraction kit (*Epigentek,* New York, USA), following the manufacturer's instructions. Equal amounts of chromatin (~2 µg) from untreated and corresponding GA_3 treated tobacco leaves at each indicated growth stages were washed and resuspended in 50 mm^3 of 1 × MNase I digestion buffer (50 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$). and digestions were carried out for 6 min. The reaction was stopped by adding equal volume of stop buffer (100 mM EDTA and 10 mM EGTA mix, pH 7.5) followed by digestion with proteinase K at 37 °C overnight. Following digestion, nucleosomal-associated DNA was extracted once with phenol + chloroform + isoamyl alcohol (25:24:1, v/v/v) and DNA was precipitated by adding 1 cm^3 of 100 % ethanol, followed by centrifugation at 13 400 *g*. The dried DNA pellet was resuspended in 30 mm^3 of $T_{10}E_1$ (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), treated with RNase A (10 mg cm^{-3}, at room temperature for 25 min). Finally, the MNase I digestion products were resolved on 2 % (m/v) agarose gels and stained with ethidium bromide. The signal intensity (% of each undigested and MNase I digested chromatin DNA bands; nucleosome ladders) from untreated and corresponding GA_3 treated tobacco leaves was measured by densitometry scanning using *ImageQuant* software.

Global DNA methylation assay: For the analysis of global DNA methylation, total genomic DNA was extracted from the untreated or GA_3 treated tobacco leaves at 0, 4, 8, 24, 48, 72 h and 13, 25, 35, 50 d after GA_3 treatment using the DNeasy kit (*Qiagen Sciences*, Germantown, MD, USA) following the manufacturer's protocol. Concentration of extracted DNA was estimated using a *NanoDrop 8000* (*Thermo Fischer Scientific*, Wilmington DE, USA). The genomic DNA was normalized to a concentration of 40 ng mm^{-3}. The global DNA methylation was determined from 200 ng of genomic DNA using *Imprint*® methylated DNA quantification kit (*Sigma*), following the manufacturer's instructions. The methylated fraction of DNA is recognized by a 5-methylcytosine (5-mC) antibody. The amount of methylated DNA, which is proportional to the absorbance, was quantified colorimetrically. Notably, this analysis provides the level of global DNA methylation and is not specific to any particular gene.

Analysis of 5-methylcytosine (5-mC) in genomic DNA by dot-blot assay: Total genomic DNA was extracted from the untreated and GA_3 treated tobacco leaves at 0, 4, 8, 24, 48, 72 h and 13, 25, 35, 50 d after GA_3 treatment using the DNeasy kit (*Qiagen Sciences*), following the manufacturer's protocol (as described above). Dot-blot analysis was performed according to Clement and Benhattar (2005) and Wang *et al.* (2012). Briefly, genomic DNA (10 ng) was denatured and then blotted onto a neutral nylon membrane (*Hybond*™*-N*; *GE Healthcare, Life Sciences*, Pittsburgh, PA, USA) using a *Bio-Dot* microfiltration apparatus (*Bio-Rad*, Hercules, CA, USA) according to the manufacturer's instructions. This was followed by rinsing the membrane in 2× saline-sodium citrate buffer, dried and subsequently fixing by UV-crosslinking (120 mJ cm^{-2} on both sides of the membrane). After blocking the membrane in freshly prepared Tris-buffered saline (TBS) with 0.1 % *Tween-20* containing 5 % (m/v) non-fat dry milk, the membrane was incubated at 4 °C overnight with a mouse monoclonal antibody specific to 5-methylcytosine (5-mC) at a dilution of 1:250 (v/v), followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG secondary antibody at a dilution of 1:5,000 (v/v) at room-temperature for 3 - 4 h. The membrane was then treated with *ECL* detection reagents and exposed to *Molecular Imager* chemiluminescence detection system (*ChemiDoc XRS+ system*, *Bio-Rad*). The intensity of each dot was measured by densitometry scanning using *ImageQuant* software, normalized to corresponding positive control dot-blotted methylated DNA. To ensure equal spotting of total genomic DNA on the membrane, the same blot was then stained with 0.02 % methylene blue in 0.3 M sodium acetate (pH 5.2).

NtDNMTs activity assay: For NtDNMTs activity measurement, untreated or GA_3 treated tobacco leaves were harvested at the same developmental stage as mentined above. Nuclear extracts were prepared using *CelLytic*™ *PN* plant nuclei isolation/extraction kit (*Sigma*) following the manufacturer's instructions. NtDNMTs activity was determined from the verified nuclear-extracts using *EpiQuik* DNMT activity/inhibition assay ultra kit (*Epigentek*) by the standard equation following the manufacturer's protocol.

For *in vitro* inhibition of NtDNMTs activity, GA_3 (dissolved in ethanol) and genistein (Gen; dissolved in dimethyl sulfoxide, DMSO), as a positive control, were added to nuclear extracts of untreated and corresponding GA_3 treated tobacco (13 d after GA_3 application) at a final working concentration of 20 mg dm^{-3} and 25 µM, respectively, so that the original solvent can be reduced to 0.01 % (v/v) of the reaction solution or less. NtDNMTs activity inhibition was measured by the standard equation, following the manufacturer's protocol. All assays were performed with three technical replicates for each sample.

***NtDNMT* mRNA expression analysis using real-time PCR:** For the mRNA analysis, the total RNA was extracted from the untreated and corresponding GA_3 treated tobacco (13 d after GA_3 application) leaves using *GeneJET* plant RNA purification mini kit (*Thermo Fisher Scientific*), following the manufacturer's protocol. The RNA was treated with RNase free DNaseI (*Thermo Fischer Scientific*) and reverse-transcribed by the high capacity cDNA reverse transcription kit (*Applied Biosystems*). The synthesized cDNA was amplified using Power SYBR® *Green* PCR master mix (*Applied Biosystems*) with *7500 Real-Time* PCR system (*Applied Biosystems*). Manufacturer-supplied standardized primer-pairs were designed by *Primer 3* program (http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to measure the expression of *N. tabacum* DNMT genes *NtMET1*, *NtCMT3*, and *NtDRM1* (Table 1 Suppl.). The standard PCR conditions were: 50 °C for 2 min, 95 °C for 10 min, and then 40 cycles at 95 °C for 15 s, 60 °C for 1 min followed by melt-curve analysis, as recommended by the manufacturer's instructions (*Applied Biosystems*). The negative control (without reverse transcriptase) established that the PCR products generated were not due to genomic DNA contamination (data not shown). The mRNA expressions were normalized to the constitutively expressing house-keeping gene, *18S rRNA* and relative to the average of all ΔC_T-values in each sample using the cycle threshold (C_T) method. All samples were run in triplicate to ensure amplification integrity.

***In planta* epigenetically silenced reporter assay system as a readout of DNA cytosine methylation status:** For the study of GA_3 driven epigenetic activation of silenced transgene loci, we used transgenic tobacco lines hemizygous for post-transcriptional locus 1, *Lo1* (PTGS, designated as *HeLo1*) and it's transcriptional silenced locus variant, *Lo1E* (TGS, designated as *HeLo1E*). The transgenic cassette contains the neomycin phospho-transferase reporter gene (*npt*II) whose transcription is controlled by cauliflower mosaic virus (CaMV) 35S promoter. In PTGS *Lo1*, the *npt*II coding region is methylated whereas in TGS *Lo1E*, the CaMV 35S promoter is hyper-methylated (Van Houdt *et al.* 2000, Fojtova *et al.* 2003). Seeds were surface-sterilized in 0.05 % (m/v) $HgCl_2$ for 8 min and washed with doubled-distilled water (ddH_2O). Thereafter, the seeds were forwarded to wash in 70 % ethanol. After rotating for 10 min on a rotospin, the seeds were washed 5 times with sterile ddH_2O and kept in water for around 30 min which facilitated better germination. Subsequently surface-sterilized tobacco seeds were germinated on Murashige and Skoog (MS) medium (0.46 % MS salts and 3 % sucrose). The pH of the medium was adjusted to 5.8 before autoclaving. Media was solidified with 0.7 % (m/v) agar, amended with 50 µg cm^{-3} of kanamycin (final concentration) either alone or along with 50 mg dm^{-3} GA_3 just before plates were poured. Then 10 - 20 seeds per plate were sown on MS media and plates were incubated at a 16-h photoperiod, an irradiance of 80 µmol m^{-2} s^{-1}, and a temperature of 26 °C for two weeks. The resultant plantlets were grown for 1 month in kanamycin selection medium (50 µg cm^{-3}) either alone or in the presence of 50 mg dm^{-3} of GA_3. As a positive control, seedlings were also grown on MS-kanamycin selection media containing known DNA de-methylating agents *i.e.* 20 µM 5-azacytidine (5-azaC), 50 µM genistein (Gen), and 6 µM trichostatin A (TSA). Difference in developmental and growth phenotypes on MS-kanamycin selection media alone and in the presence of 50 mg dm^{-3} of GA_3 were observed. Each treatment was done in triplicate.

For the mRNA analysis, the total RNA was extracted from young transgenic TGS (*HeLo1E*) and PTGS (*HeLo1*) seedlings (as described above) grown on MS-kanamycin selection media using *GeneJET* plant RNA purification mini kit (*Thermo Fisher Scientific*) following the manufacturer's protocol. Real time qPCR reaction was performed as described above. Manufacturer-supplied standardized primer-pair used to measure the *npt*II reporter gene expression were designed by *Primer 3* program (Table 1 Suppl.).

Total crude cell extract was prepared from the untreated and corresponding GA_3 treated tobacco leaves (early- vegetative growth stage) using plant total protein extraction kit (*Sigma*) following the manufacturer's instructions. Proteins (~50 µg) were electrophoresed on 15 % (m/v) acrylamide gels and then transferred onto *Hybond-P* (*Amersham*) PVDF membranes. After blocking the membrane in freshly prepared TBS with 0.1 % *Tween-20* containing 5 % non-fat dry milk, the membranes were incubated with polyclonal rabbit anti-*npt*II antibody at a dilution of 1:1 000 at 4 °C with agitation overnight, followed by incubation with an affinity purified donkey anti-rabbit HRP conjugated IgG antibody (*GE Healthcare, Life Sciences*) at a dilution of

1:5 000 with agitation at room temperature for 1 h. Immunoreactivity was detected and exposed to X-ray *Hyperfilm*™ ECL (*GE Healthcare, Life Sciences*) using an *ECL* detection reagent system (*Thermo Scientific*) and visualized on *Molecular Imager* chemiluminescence detection system (*Bio-Rad*). The equal loading of proteins was verified by *Ponceau S* staining of membrane as well as by anti-histone H4 antibody probing. The immuno-reactive band intensity of NPTII protein was measured by densitometry scanning using *ImageQuant* software, normalized to corresponding positive control immune-reactive band intensity of anti-histone H4 for each treatment.

Data analysis: The results are expressed as means ± SDs. One-way analysis of variance (*ANOVA*) was used to analyse statistical significance between groups. $P < 0.05$ level was considered as statistically significant.

Results

For GA_3 application, tobacco seedlings were transplanted after 60 d from nursery to main-field and their leaves were sprayed with 50 mg dm^{-3} of GA_3 at 90 d after sowing (30 d after transplantation; DAT). The tobacco plants sprayed with GA_3 exhibited dramatic alteration in phenotypic appearance and development as compared to corresponding untreated control plants (Fig. 1*A*). Untreated control plants were smaller and had less and smaller leaves as compared to corresponding GA_3 plants (Fig. 1*A* and Fig. 1 Suppl.). Morphometric analysis of the mature and fully grown plants revealed increased leaf thickness and internode length in GA_3 treated plants (Table 1). The increased internode length contributed to the overall height difference of GA_3 treated plants as compared to untreated control plants. Also image analysis of epidermal tissue revealed increased cell area (~62 %) together with decreased intercellular spaces in GA_3 treated tobacco (Fig. 1*B*). Moreover, GA_3 treated tobacco leaves shows enhanced trichome density (~31 and ~43 % for abaxial and adaxial surfaces, respectively) in comparison to untreated plants (Fig. 1*C*).

Table 1. Morphometric characterization of untreated and GA_3 treated tobacco at 60 - 70 d after transplantation from nursery to main-field (B - bottom, M - middle, and T - top position of plant). Means ± SDs of 5 biological replicates. *Asterisks* indicate the significant difference at $P < 0.05$ when compared with untreated control.

Traits		Untreated	GA_3 treated
Plant height [cm]		94.2 ± 3.96	105.2 ± 3.3*
Number of leaves		19.6 ± 0.577	20.8 ± 1.3
Leaf length [cm]	T	74.0 ± 4.63	71.8 ± 7.15
	M	74.8 ± 5.58	73.2 ± 6.37
	B	66.0 ± 3.53	64.2 ± 7.42
Leaf width [cm]	T	29.0 ± 5.91	23.8 ± 4.08
	M	34.0 ± 7.84	29.4 ± 3.36
	B	34.4 ± 1.81	32.2 ± 2.58
Leaf area [cm^2]	T	1931.4 ± 452.1	1538.0 ± 412.9
	M	2288.9 ± 705.4	1936.9 ± 284.8
	B	2044.8 ± 179.6	1859.9 ± 243.4
Stem diameter [cm]	T	6.2 ± 0.83	7.1 ± 0.54
	M	9.8 ± 1.30	10.3 ± 0.44
	B	12.2 ± 0.83	12.5 ± 0.93
Leaf thickness [mm]	T	0.381 ± 0.01	0.47 ± 0.08*
	M	0.334 ± 0.03	0.37 ± 0.05
	B	0.332 ± 0.02	0.39 ± 0.04*
Internodal length [cm]	T	5.4 ± 0.82	6.84 ± 0.50*
	M	5.2 ± 0.57	6.10 ± 0.41*
	B	4.1 ± 0.41	5.01 ± 0.53
Leaf morphology		smooth, thin, broad, and less trichomes	serrated, thick, narrow and more trichomes

Epigenetic processes play an important role in maintaining the chromatin architecture by dynamically altering the chromatin transition from de-condensed or 'open' (euchromatin) interphase chromatin to condensed or 'closed' (heterochromatin) metaphase chromosome and *vice-versa* (Razin and Cedar 1977, Finnegan and Kovac 2000, Zhao *et al.* 2001, Pavlopoulou and Kossida 2007). Thereby, to explore the possibility that GA_3 application induces changes in chromatin condensation, we analyzed sensitivity of tobacco chromatin to MNase I in untreated and GA_3 treated plants at various growth stages. MNase I digestion with an incubation period of 6 min yielded the typical nucleosome ladders (*r2-r5*) (Fig. 2 Suppl. and Table 2 Suppl.), with a repeat mono-nucleosome length of about 170 bp. We observed that chromatin extracted from untreated tobacco was highly resistant to MNase I digestion and this resistance declined during subsequent developmental stages (Fig. 2 and Table 3 Suppl.). In contrast, chromatin isolated from GA_3 treated tobacco leaves showed relative enhanced sensitivity to MNase I digestion at early developmental stages as observed by the more visible appearance of nucleosomal bands (*r2-r5*), suggesting that tobacco cells responded to GA_3 by transiently remodeling their chromatin architecture from condensed to relatively de-condensed form. The observed nucleosomal periodicity was, however, identical in untreated and GA_3 treated chromatin demonstrating a typical nucleosome ladder with a repeat mono-nucleosome length of about 170 bp. Of note, possibility of observed transient chromatin transition as an indirect secondary effect related to observed growth changes (Fig. 1*A* and Table 1) following GA_3 foliar application cannot be ruled out.

By using MNase I assay, we further evaluated the chromatin configuration at two specific genomic loci (*SYL2* and 26S rDNA; Fig. 3 Suppl.). Chromatin extracted from the untreated and corresponding GA_3 treated tobacco leaves at early vegetative growth stage

(13 d after GA_3 application) was MNase I digested for 6 min. Southern blot analysis of this MNase I digested chromatin probed with [^{32}P]-labelled *SYL2* (Koukalova *et al.* 2010) and 26S rDNA (Lim *et al.* 2000) revealed that the heterochromatic *SYL2* probe mapping to sub-telomeric regions hybridized to high molecular mass DNA (*r1*) in both samples. However, a ladder of low molecular mass fragments was visible in GA_3-treated chromatin only (Fig. 3*B* Suppl.). Similar pattern was observed after the hybridization of a blot with the 26S rDNA labelled probe (Fig. 3*C* Suppl.). It is evident that as compared to untreated sample, these two loci were relatively less protected in GA_3 treated sample. These results suggest that both *SYL2* and 26S rDNA loci occurred in a condensed chromatin configuration while a part of each locus underwent relative de-condensation

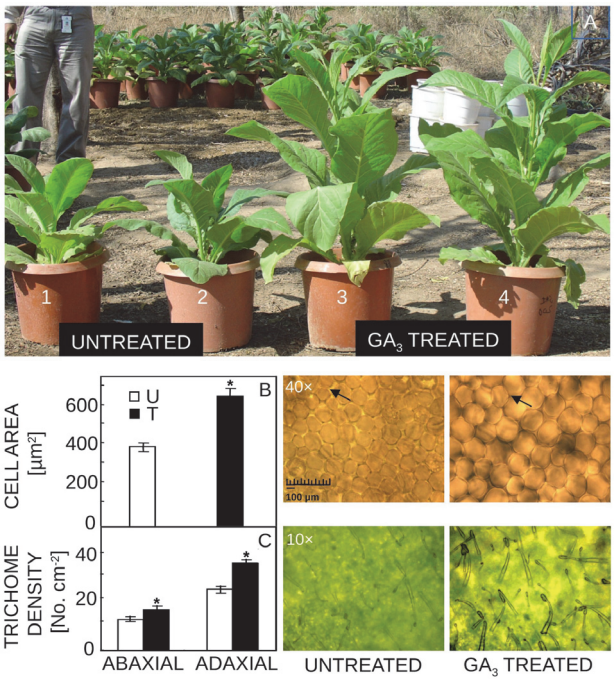

Fig. 1. GA_3 application causes morphometric and anatomical alteration in tobacco. *A* - Phenotype of untreated and GA_3 treated tobacco. *B* - Inverted microscope (*Leica DM1000*) with a 40× optical magnification was used for microscopy images of tobacco leaf mesophyll cells anatomy and cell area measurements. In each focus, approximately 20 cells were selected randomly for measurement. *C* - Inverted microscope with a 10× optical magnification was used for trichome density scoring. Standard safranin staining of 1 cm^2 -sized leaf section of both abaxial and adaxial surfaces were used with at least 3 - 6 biological replicates. Means ± SDs. *Asterisks* indicate significant differences at $P < 0.05$ when compared with untreated control (U - untreated, T - GA_3 treated tobacco).

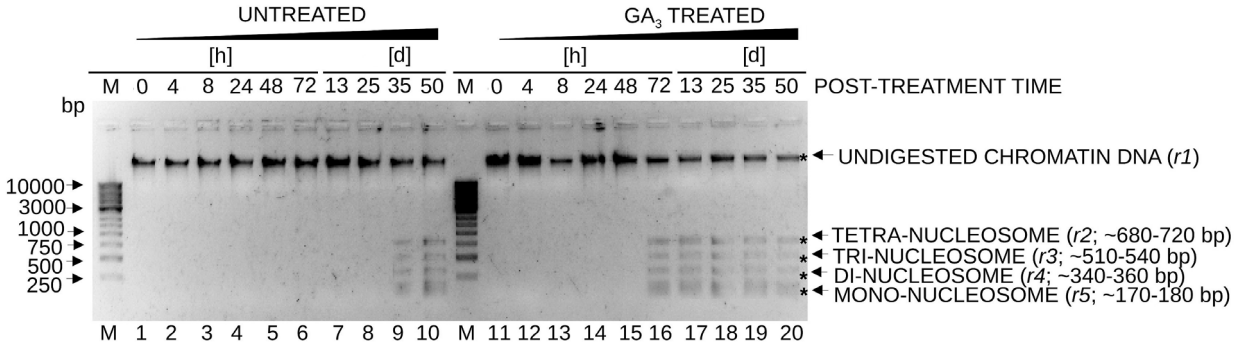

Fig. 2. GA_3 induced global de-condensation of tobacco chromatin. Chromatin extracted from untreated and GA_3 treated tobacco leaves at 0, 4, 8, 24, 48, 72 h, and 13, 25, 35, 50 d after GA_3 treatment was MNase I digested for 6 min, purified, and resolved on 2 % agarose gel. *Lanes* 1 - 10 and 11 - 20 indicate MNase I digested chromatin from untreated and GA_3 treated tocacco plants. M - DNA size markers. The signal intensity (*in percentage*) of each undigested and MNase I digested chromatin DNA bands (nucleosome ladders; '*r2-r5*') from untreated and corresponding GA_3 treated tobacco leaves as measured by densitometry scanning is shown in Table 3 Suppl.

following GA_3 foliar application. Thus, increased sensitivity of chromatin after the GA_3 application seems to be a global phenomenon.

DNA methylation patterns are distinguished by their close association with chromatin structure. Active regions of the chromatin, which enable gene expression, are associated with hypo-methylated DNA, whereas hyper-methylated DNA is packaged in inactive chromatin (Razin and Cedar 1977, Lewis and Bird 1991). Purified genomic DNA was isolated from untreated control and corresponding GA_3 treated tobacco leaves harvested at different growth stages and subjected to the analysis of global DNA methylation using global DNA methylation kit (*Sigma*). Global DNA methylation in both plants declined progressively with growth stage (up to 31 and 42 % in untreated control and corresponding GA_3 treated plants, respectively, Fig. 3) and also difference in global DNA methylation were observed between untreated

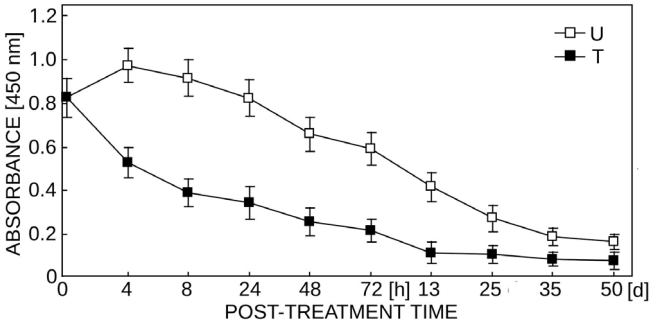

Fig. 3. Global DNA methylation levels in untreated (U) and GA_3-treated (T) tobacco leaves at 0, 4, 8, 24, 48, 72 h, and 13, 25, 35, 50 d after GA_3 treatment. Data are presented in terms of net absorbance at 450 nm, which is proportional to the amount of methylated DNA. Means ± SDs of two independent experiments.

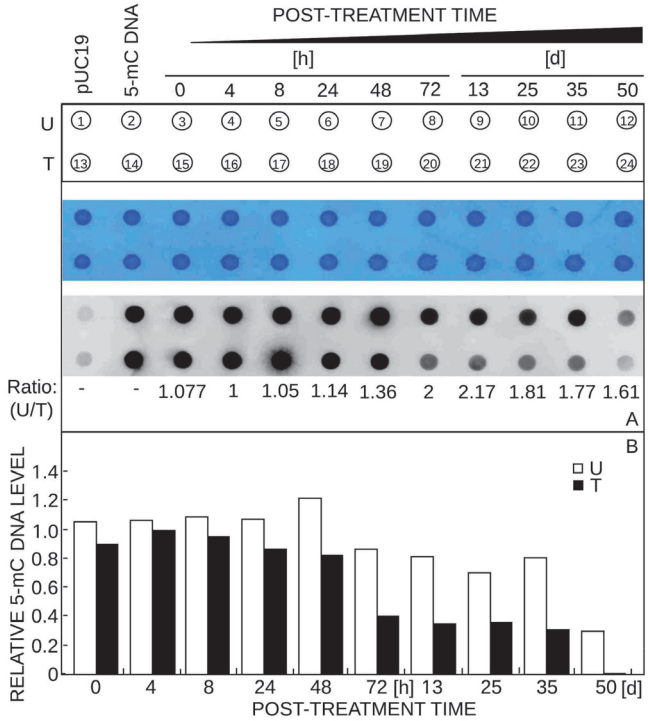

Fig. 4. Dot-blot analysis for genomic DNA 5-mC in untreated (U) and GA_3-treated (T) tobacco leaves. *A* - Approximately 10 ng each of empty vector pUC19 (negative control), fully methylated human Jurkat cell line DNA (positive control), and total purified genomic DNA from the U and T tobacco leaves at 0, 4, 8, 24, 48, 72 h, and 13, 25, 35, 50 d after GA_3 treatment were blotted and immobilized on nylon membrane (*Hybond*^{TM}-*N*). Relative position of individual dot-blot, loading control (methylene-blue stain) and recognition of genomic DNA by the anti-5-mC antibody are shown in the upper-, middle- and bottom-panel, respectively. Hybridization signal intensity of each blotted sample was quantified by densitometry scanning. U/T ratio indicates the normalized methylated DNA intensity ratio between untreated and corresponding GA_3-treated tobacco at each indicated time-point. *B* - Relative signal intensity of total purified genomic DNA from untreated and GA_3-treated tobacco at each indicated time-point with respect to corresponding positive-control methylated DNA.

control and corresponding GA_3 treated plants. The global DNA methylation in GA_3 treated leaves was always lower (15 - 40 %) than in those of untreated counterparts at all growth stages but the difference was the most profound in early-vegetative growth stage and then declined.

To examine the more specific effect of GA_3 on 5-mC in genomic DNA, purified total genomic DNA isolated from the untreated control and corresponding GA_3 treated tobacco leaf at various growth stages was also subjected to dot-blot assay using an antibody specific to 5-mC. The anti-5-mC antibody only recognized the 5-mC oligonucleotide but not the deoxycytidine triphosphate (dCTP; data not shown). In line with quantitative measurement of global DNA methylation (Fig. 3), GA_3 application also significantly decreased the level of 5-mC in total genomic DNA in a growth dependent manner (Fig. 4*A*). This is evident from the reduced signal intensities of the dot-blots (measured by densitometry scanning) from total genomic DNA of GA_3 treated tobacco. The intensities of DNA dot-blot of 5-mC of GA_3 treated tobacco declined by 14, 36, 100, 117, 81, 77, and 61 % respectively, as compared to corresponding untreated tobacco. The levels of 5-mC relative to corresponding total methylated DNA levels (positive control) at each vegetative growth-stage was also plotted for both untreated and GA_3 treated tobacco (Fig. 4*B*). The normalized 5-mC signal intensities at 3, 13, 25, 35, and 50 d after the GA_3 treatment were significantly (> 50 %) lower than those of the untreated controls.

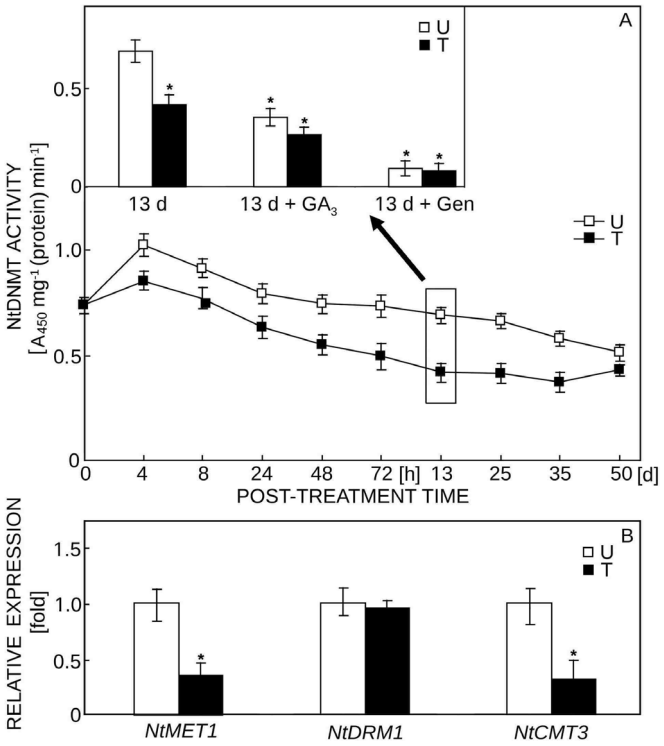

Fig. 5. Effect of GA_3 application on DNA methyltranferase activities and *NtDNMTs* gene expressions. *A* - Total NtDNMTs activity from untreated (U) and corresponding GA_3 treated (T) tobacco leaves at 0, 4, 8, 24, 48, 72 h, and 13, 25, 35, 50 d after GA_3 treatment. The *inset* depicts the effect of *in vitro* GA_3 (20 mg dm^{-3}) or Gen (25 μM) addition on total NtDNMTs activity after 13 d. *B* - Quantitative real-time PCR analyses of *NtMET1*, *NtDRM1*, and *NtCMT3* mRNA levels in U and T tobacco (13 d after GA_3 application). The results are presented as the individual *NtDNMT* mRNA levels normalized to *18S rRNA*. The value for control untreated plantlets was set at 1. Means ± SDs of triplicate measurements from three independent experiments. *Asterisks* indicate the significant difference at $P<0.05$ when compared with untreated control.

Plant DNA methyltransferases are essential components of DNA methylation system (Pavlopoulou and Kossida 2007). Therefore, we determined the *in vitro* activity of NtDNMTs in untreated control and GA_3 treated tobacco leaf at different growth stages. Prior to NtDNMTs activity measurement, the nuclear-extracts quality was verified using an anti-histone H4 antibody (Fig. 4 Suppl.) GA_3 application decreased NtDNMTs activity (15 - 35 %) in comparison with corresponding untreated controls and this effect of GA_3 was also growth stage dependent (Fig. 5*A*). The lowest NtDNMTs activity was observed in nuclear extracts isolated from plants at day 13 after the GA_3 application (Fig. 5*A*), consistent with significant reduction of global methylation and 5-mC DNA level at this particular stage.

We further evaluated the specific inhibitory effect of GA_3 on NtDNMTs activity in tobacco under *in vitro* conditions. For this purpose, GA_3 (20 mg dm^{-3}) was added to the nuclear protein extracts prior to the analysis of NtDNMTs activity. Gen is a well-known inhibitor of

DNA methylation and was also used as a positive control for *in vitro* inhibition of NtDNMTs activity. The solvent controls of GA_3 (in ethanol) and Gen (in DMSO) established that *in vitro* inhibition of NtDNMTs activity was specifically due to GA_3 and Gen addition only. The GA_3 addition inhibited the NtDNMTs activity in nuclear protein extracts from both untreated and GA_3 treated tobacco. Notably, the decrease of NtDNMT activity was relatively higher (48 %) in untreated control extract as compared to extract from GA_3 treated tobacco (37 %) (Fig. 5*A* inset). Of note, *in vitro* inhibitory effect of Gen addition (25 µM) on NtDNMTs activity was relatively greater (86 %) in control extracts than in extracts isolated from GA_3-treated plants (80 %). Moreover unlike to GA_3, the presence of Gen significantly decreased the NtDNMTs activity in extracts from both control and GA_3-treated cells (Fig. 5*A* inset), suggesting that GA_3 is relatively less potent *in vitro* inhibitor of NtDNMTs activity in tobacco than Gen. However, the possibility of other epigenetic mechanism(s) influenced by GA_3 application also can not be ruled out at this stage.

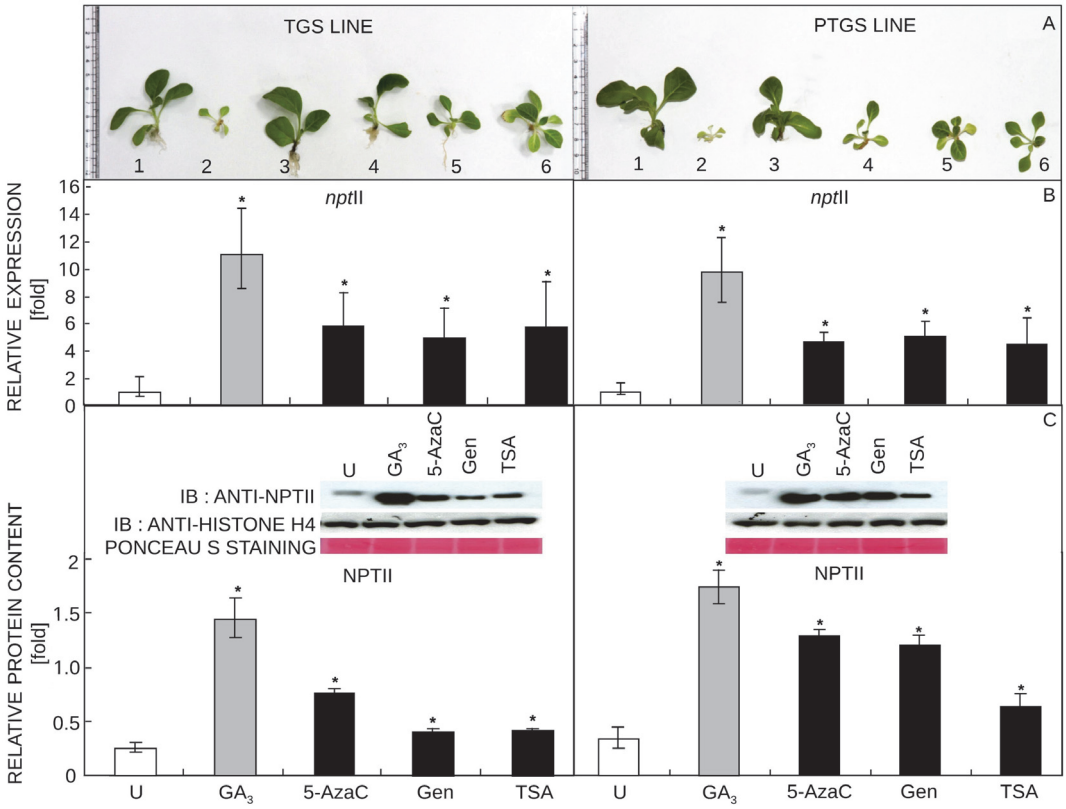

Fig. 6. GA_3 treatment induces re-expression of silenced transgenes. *A* - Phenotypes of plantlets harbouring locus 1E (*HeLo1E*, *left-panel*) and locus 1 (*HeLo1*, *right-panel*) transgenes. Plantlets were grown in kanamycin selection medium (50 µg cm^{-3}) supplemented with GA_3 (50 mg dm^{-3}) or with DNA de-methylating agents: 5-azaC (20 µM), Gen (50 µM), and TSA (6 µM). Control plantlets (U) were grown without supplements. *A* - representative images of plantlets for each treatment is shown: 1 - MS alone, 2 - MS + kanamycin, 3 - MS + kanamycin + GA_3, 4 - MS + kanamycin + 5-azaC, 5 - MS + kanamycin + Gen. and 6 - MS + kanamycin + TSA. Quantitative real-time RT-PCR analyses of *npt*II mRNA relative to endogenous *18S rRNA* (*B*) and immunoblotting analyses of NPTII protein relative to endogenous histone H4 (*C*). Means ± SDs of triplicate measurements from three independent experiments. *Asterisks* indicate the significant difference at $P < 0.05$ when compared with untreated control. IB - immunoblotted.

We investigated the possibility that the decline of *NtDNMT's* activity after the GA_3 application was related to transcriptional down-regulation of respective genes. For this, we assessed mRNA accumulation of *MET1*, *DRM1*, and *CMT3* orthologs (termed as *NtMET1*, *NtDRM1*, and *NtCMT3,* respectively) by quantitative analysis using real-time PCR. The amount of *NtMET1* (~2.8 folds) and *NtCMT3* (~3 folds) mRNA declined significantly at day13 after GA_3 application compared to untreated plant. However, no significant difference in *NtDRM1* mRNA accumulation was observed. Thus, *NtMET1* and *NtCMT3* transcription (Fig. 5*B*) reflected the altered enzyme activities in nuclear extracts (Fig. 5*A*). Consistency in mRNA accumulation of *NtMET1*, *NtDRM1*, and *NtCMT3* was further validated with respect to mRNA of other constitutive gene *EF1-α* (Fig. 5 Suppl.).

In previous sections, we demonstrated the effect of GA_3 on epigenetic pathways. Next we studied the effect of GA_3 on expression of silenced tobacco transgenes. We used *N. tabacum* lines harboring the CaMV35S promoter driven *npt*II reporter transgenes, silenced either at the

transcriptional (TGS, designated as *HeLo1E*) or post-transcriptional (PTGS, designated as *HeLo1*) levels (Van Houdt *et al.* 2000, Fojtova *et al.* 2003). Notably in *HeLo1E* and *HeLo1* lines, silencing of the *npt*II reporter gene was closely correlated with DNA cytosine hyper-methylation of CaMV35S promoter and *npt*II coding region, respectively (Fig. 6 Suppl.). The advantage of this system was that positive kanamycin selection allowed us to detect the changes in *npt*II reporter gene expression. Plantlets of *HeLo1E* and *HeLo1* were transferred to MS-kanamycin selection media (50 μg cm^{-3}), either alone or supplemented with 50 mg dm^{-3} of GA_3. As a positive control, these transgenic tobacco seedlings (*HeLo1E* and *HeLo1*) were also transferred to MS-kanamycin selection media containing DNA de-methylating agents, *i.e.*, 5-azaC (20 μM), Gen (50 μM), and TSA (6 μM). The non-treated seedlings showed dwarf phenotypes, bleaching, and overall loss of viability (Fig. 6*A*) indicating extreme sensitivity to kanamycin. In contrast, the GA_3 and DNA de-methylating agents-treated plantlets grew normally like the untreated plantlets, indicating restoration of kanamycin resistance (Fig. 6*A*). Of note, solvent controls of both GA_3 (in ethanol) and DNA de-methylating agents (in DMSO) alone did not restore the growth and viability of plantlets in the presence of kanamycin (Fig. 7 Suppl.).

To determine *npt*II mRNA and protein content, we isolated total RNA and proteins from bulked plantlets of both *HeLo1E* and *HeLo1* lines after 1 month of growth on control medium and a medium supplemented with GA_3. Endogenous *18S rRNA* and H4 histone protein were used as internal reference controls for normalization, respectively. After the reverse transcription of cDNA, we determined the *npt*II mRNA by quantitative PCR (Fig. 6*B*). It is evident that plantlets grown on GA_3 containing medium showed increased (10 to 11-folds) *npt*II mRNA accumulation compared to controls. A similarly increased transcription was observed in plantlets treated with 5-azaC, Gen, or TSA (Fig. 6*B,C*). The NPTII protein content in *HeLo1E* and *HeLo1* lines was analyzed by immunoblotting (Fig. 6*C*). The chemiluminiscent signal was observed in ~30 kDa region, which is the expected size of the NPTII protein in recombinant plants (Zhang *et al.* 2012). The signal intensity of *npt*II antibody staining was much stronger in GA_3 treated plantlets (5 to 8-folds) than in control. Similarly, the signal intensity in lanes loaded with protein extracts from plantlets treated with DNA de-methylating drugs were stronger than with extracts from control plants, but weaker than those of GA_3 treated plants (Fig. 6*B*,*C*). Here, it is worth mentioning that a similar DNA hypo-methylation effect of GA_3 has been observed despite different modes of GA_3 application under experimental field and *in vitro* condition (foliar application *vs.* supplementation in MS culture media).

Discussion

Higher organisms including plants use three systems to initiate and sustain epigenetic gene regulation: DNA methylation, histone modification, and RNA-interference. These epigenetic tools are important for growth and development, polyploidization, ecological adaptation, and adaptive speciation (Lee and Chen 2001, Liu and Wendel 2003). Among these epigenetic marks, DNA methylation has an important role in different stages of plant development and also represent an efficient mean to modulate gene expression in response to internal as well as external stimuli. Recent discovery also reveal the importance of phytohormone, abscisic acid (ABA) in regulating the abiotic stress response through DNA methylation (Chinnusamy *et al.* 2008). Similarly potential role of an essential and commonly used phytohormone, GA_3 in epigenetic mechanism(s) cannot be ignored.

Taking a cue from this, our present study was designed to investigate the effect of GA_3 foliar application on obacco system under field conditions. We observed an increase in plant-height, density of foliage, leaf cell-area, and trichome density, following GA_3 application on *N. tabacum* leaves (Fig. 1, Table 1). DNA methylation also represents a central epigenetic mechanism for mediating the developmental and environmental stress induced gene regulation in *N. tabacum*. For example in tobacco cell-suspension culture, osmotic and salt stress induced a reversible and transient DNA hyper-methylation in two heterochromatic loci (Kovařík *et al.* 1997). Abiotic stresses involving aluminium, paraquat, salinity, and cold induce a DNA de-methylation in the coding sequence of the glycero-phosphodiesterase-like protein (*NtGPDL*) gene, correlated with its up-regulation in tobacco leaves (Choi and Sano 2007). In present study, we observed a decline trend of global DNA methylation during tobacco vegetative growth (Figs. 3, 4). This is in line with earlier reports of global DNA methylation drops after germination (Valledor *et al.* 2007, Santamaria *et al.* 2009, Meng *et al.* 2012, Brautigam *et al.* 2013). GA_3 application on tobacco also induced a similar trend of declining global DNA methylation from early- to late-vegetative growth stages (Figs. 3, 4). Notably, dormant tissues with higher DNA methylation usually exhibit lower transcription activity than metabolically active tissues (Michalak *et al.* 2013, Nonogaki 2014). Thereby, possibility of observed global DNA hypo-methylation as a secondary effect related to rapid growth following GA_3 foliar application cannot be ruled out.

Our present study also demonstrates that GA_3 foliar application in tobacco causes chromatin alteration (Fig. 2). Being identical nucleosomal periodicity in untreated and GA_3 treated chromatin (Fig. 2), the possibility of complete genome-wide opening of chromatin by GA_3 is unlikely. This is also in line with

earlier observation that experimentally induced hypo-methylation of tobacco cells with DNA de-methylating drugs (5-azaC and DL-ethionine) enhances the MNase I digestibility of chromatin without altering the nucleosomal periodicity of heterochromatic sequences (Fajkus *et al.* 1992). Thereby, observed increased accessibility of MNase I towards GA_3 treated chromatin could have been possibly caused by loss of docking site for methyl-CpG binding protein (MeCP2) that is known to drive chromatin condensation (Zemach and Grafi 2007). Alternatively, GA_3 may have indirect effect on DNA methylation by increasing the mobility and/or modification of linker histones (H1 and its variants) (Wierzbicki and Jerzmanowski 2005).

GA_3 induced morphometric and anatomical alteration(s) can be clearly related to these observed epigenetic alterations. However, we can only speculate about the mechanism of GA_3 induced global DNA hypo-methylation. For example, GA signalling may influence the expression and activity of DNA methyltransferases and perhaps expression of other genes as well. Indeed, we observed a consistent decrease in total NtDNMTs activity (except at 4 h) as well as expression of two *NtDNMT* genes *NtMET1* and *NtCMT3* after the GA_3 treatment (Fig. 5). Using molecular modeling, we also observed that GA_3 might directly interact with the catalytic domain of mammalian *DNMT1*, thus competitively inhibiting the binding of hemi-methylated DNA to the catalytic domain of *DNMT1* (data not shown). Whereas the activity of purified DNMT isolated from nuclei of wheat seedlings and germinating embryos is inhibited by GA_3, addition of GA_3 to nuclear extracts from wheat embryo paradoxically stimulates a DNMT activity (Vlasova *et al.* 1995). Meilan *et al*. (2002) suggested an indirect relationship between reduced DNA methylation and increased endogenous GAs content in shoot apical tissues during vernalization induced flowering of non-heading Chinese cabbage. In rice, GA_3 does not induce significant changes in *OrDNMT* expression (Ahmad *et al.* 2014). However in their study, *OrDNMT* expression was traced for relatively short time-interval (up to 12 h) following GA_3 treatment. In our experiments, the effect of GA_3 induced global DNA hypomethylation was relatively low (~20 % reduction) within several hours following GA_3 application while marked decrease of NtDNMTs activity (~40% reduction) and steady-state mRNA levels of *NtMET1* and *NtCMT3* were observed at day 13 d after GA_3 application (Fig. 5). It is likely that epigenetic re-programming in response to GA_3 requires more time and multiple cell divisions. In any case, there is no clear consensus about the effect of GAs on DNA methylation pathway(s) and further research is needed in this field. Notably, in our study GA_3 induced epigenetic changes were transient in nature and did not exhibit *trans*-generational epigenetic inheritance (data not shown).

Several studies showed variable expression of different classes of cytosine *DNMT's* (*MET*, *DRM*, and *CMT*) between different plant tissues (Nakano *et al.* 2000, Sharma *et al.* 2009, Ahmad *et al.* 2014, Lin *et al.* 2015). Possibly, the reduced expression of *NtMET1* and *NtCMT3* may account for enhanced genomic DNA hypo-methylation in GA_3-treated plants. However, a correlation between *DNMT* expression alterations and global DNA methylation is not always observed. Thereby, our observed decrease in expression of *NtMET1* and *NtCMT3* by GA_3 could also be related to a negative feedback regulation by global DNA hypo-methylation (Vanyushin and Ashapkin 2011). In contrast to *NtMET1* and *NtCMT3*, the *NtDRM1* expression does not seem to be influenced by GAs signalling consistent with ubiquitous accumulation of these transcripts in tobacco tissues (Wada *et al.* 2003). The *NtDRM1* class of MTase's is involved in RNA-directed methylation suggesting that small modulatory RNA (smRNA) pathways controlling expression of transposons may not be influenced by GA_3 treatment. It thus seems that symmetrical ^{m}CpG and ^{m}CpHpG (where H = A, C, or T) catalysed by NtMET1 and NtCMT3 are primary targets of GA signalling. Of note, possible role of GA_3 in regulating other epigenetic processes involving histone modification(s) and short interfering RNA (siRNA) pathways cannot be ruled out. Indeed our preliminary observation also indicates a concomitant reduced and enhanced functional activity of histone deacetylase (NtHDAC) and histone acetyl transferase (NtHAT) respectively, following GA_3 foliar application in *N. tabacum* (unpublished results). Thereby, altered functional activity of these enzymes could either be the cause or a consequence of observed chromatin alteration and associated global DNA hypo-methylation changes in GA_3 treated tobacco epigenome.

The relationship between DNA methylation and transcription regulation has been well documented in literature (Vanyushin and Ashapkin 2011). In line with this, we observed a partial restoration of silenced reporter transgene activity in GA_3 treated plantlets (Fig. 6). Although DNA methylation status of transgene loci in GA_3-treated plants needs to be determined yet, it is conceivable that the GA_3 induced DNA hypo-methylation of a CaMV35S promoter may lead to its activation of *npt*II reporter expression in the TGS epiallele. The stimulatory effect of GA_3 on the *npt*II expression in a PTGS epiallele is more difficult to explain. The PTGS locus 1 (*HeLo1*) harbours predominantly DNA methylation in a 3′-end of the coding region (Kovařík *et al*. 2000). However in most cases, DNA methylation in coding region inhibits gene expression (Zilberman *et al*. 2009). One of the explanations is that GA_3 influences PTGS phenomenon indirectly through the perturbation of double- stranded RNA-specific endoribonuclease (DICER; ribonuclease type III) and RNA-induced silencing complex I (RISCI) complexes. It is necessary to stress that *npt*II reporter expression is not restored to the level of non-silenced transgenes (Van Houdt *et al.* 2000), which may be explained by the presence of strong silencing RNA signals in transgenic loci. Of note, the stimulatory effect of GA_3 on reporter transgene expression was relatively higher than the effect of well known *DNMT* inhibitors (Fig. 6*A,B,C*), suggesting that

GA_3 can be potentially used to reactivate epigenetically silenced transgenes (and perhaps even endogenes). It may, thus, represent a convenient non-toxic alternative to otherwise toxic and synthetic *DNMT* inhibitors, which often display adverse effects on plant development. However, question still persists about the effect of GA_3-induced global DNA hypomethylation on expression of endogenes. Previous work showed stimulatory effect of GAs on major nucleolar organizer regions (NORs; rDNA) expression in onion (Karagiannis and Pappelis 1994). It is also known that GAs promote flowering in *Arabidopsis* through the activation of genes encoding the floral integrators *viz.* Suppressor of Overexpression of Constans 1 (*SOC1*), LEAFY (*LFY*) and Flowering locus T (*FT*) (Mutasa-Gottgens and Hedden 2009). Moreover a progeny of rice plant line produced by treatment with a DNA demethylating agent, conferring resistance against *Xanthomonas oryzae* is due to specific transcriptional up-regulation of *Xa21G* gene (Akimoto *et al.* 2007). In tobacco, seedling treatment with a DNA methylation inhibitor resulted in up-regulation and ectopic expression of flower-promoting homeotic genes (Fulnecek *et al.* 2011).

Taken together, genomic DNA hypomethylation induced by GA_3 may be a part of gibberellin signalling pathway. Further detailed exploration of DNA methylation changes in specific genomic regions in response to GA_3 is required to understand the mechanism(s) underlying practical applications of GA_3 as well as to identify candidate epigenetic marker(s) for the GA_3 responsiveness. Our genome-wide transcriptome and methylated DNA immunoprecipitation sequencing studies are underway to identify the responsive epi-alleles and to decipher complex epigenetic signalling by GAs. These future studies on GA_3 regulated epi-genome will be of immense use for better understanding of plant development, stress adaptation, stress memory and engineering of these processes for agricultural use.

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