DOI: 10.32615/bp.2019.040 BIOLOGIA PLANTARUM 63: 343-353, 2019

This is an open access article distributed under the terms of the Creative Commons BY-NC-ND Licence

Gibberellin A_3 mediated decreased transcriptional rate, mRNA stability and non-competitive inhibition of DNA methyltransferases in tobacco

R. MANOHARLAL*, G.V.S. SAIPRASAD, and V. KAIKALA

Corporate R&D (Agrisciences), ITC Limited, ITC Life Science and Technology Centre (LSTC), Peenya Industrial Area, Bengaluru-560058, Karnataka, India

Abstract

The present work aims to examine the underlying molecular mechanism(s) for the reduced transcriptions and activities of DNA methyltransferases (NtDNMTs) by gibberellin A_3 (GA_3) in tobacco (*Nicotiana tabacum* L). Nuclear run-on and cordycepin chase assays demonstrated a significant reduction in the transcriptional rate and transcript half-lives of *NtMET1* and *NtCMT3* by GA_3. Lineweaver-Burk plot analysis revealed a non-competitive mode of inhibition of NtDNMTs activity by GA_3. Taken together, both *in vivo* and *in vitro* studies clearly demonstrated the altered transcriptional and post-transcriptional regulation as a mechanism of inhibition in expression and activity of NtDNMTs by GA_3.

Additional key words: *Nicotiana tabacum*, nuclear run-on, transcriptional and post-transcriptional regulation.

Introduction

Gibberellins (GAs), a large family of plant-specific tetracyclic diterpenoid compounds, control important aspects of plant growth and development. Among the hundreds of GAs identified in the plant kingdom, GA_3 controls seed germination, leaf expansion, stem elongation, flowering, and seed development (Richards *et al.* 2001). Significant contribution towards understanding of GA-regulated morphogenesis includes identification and functional elucidation of its upstream receptors, signaling components and downstream targets (Thomas and Sun 2004). Genetic and biochemical analysis revealed that GA represses its signaling by destabilization of DELLA protein growth repressors *via* 26S proteasome pathway (Hedden and Phillips 2000, Sun and Gubler 2004). Recent genetic finding uncovered the novel role of GAs in diverse physiological processes including leaf differentiation, photo-morphogenesis and pollen-tube growth (Hauvermale *et al.* 2012). However, the epigenetic role of GAs is only starting to emerge.

Among five major epigenetic determinants [DNA methylation patterns, histone variants, histone modification signatures, chromatin conformation characteristics, and non-coding RNAs (ncRNAs)], DNA methylation is the most commonly studied epigenetic mark that plays an important role in different stages of plant growth and development (Bartels *et al.* 2018). There is evidence demonstrating a cross-talk between DNA methylation, endogenous GAs content, GA-signalling pathway and plant physiological processes. An indirect relationship between global DNA methylation and GAs during flowering of non-heading Chinese cabbage and azalea has been established (Meilan *et al.* 2002, Meijón *et al.* 2011). Grafting-induced DNA methylation and expression changes in GA response-

Submitted 7 May 2018, *last revision* 18 October 2018, *accepted* 22 October 2018.

Abbreviations: COR - cordycepin; DNMTs - DNA methyltransferases; GA_3 - gibberellin A_3; GEN - genistein; NRO - nuclear run-on, RFC - relative fold-changes; T - GA_3 treated samples; U - untreated samples.

Acknowledgments: We would like to acknowledge Dr. C.C. Lakshmanan [Head, Corporate R&D, ITC Limited, ITC Life Science and Technology Centre (LSTC)] for his consistent support. We also extend our appreciation to Naveen Duhan (School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana, Punjab) for his valuable assistance in nuclear run-on, mRNA stability, enzyme kinetics, and inhibition assays. Editorial and useful tips from the *In house* manuscript committee of ITC-LSTC are well appreciated. Overall support from team Agrisciences, ITC-LSTC is greatly acknowledged. A very special thanks to Dr. Yashwanth Radhakrishnan (ITC-LSTC) and Dr. Deepa Murali (ITC-LSTC) for their assistance in technical and English corrections. We also acknowledge field-workers at Northern Light Soils (NLS) region, Rajahmundry, Andhra Pradesh (A.P.) for providing their consistent help and support during field experiments and sample collection.

* Corresponding author; e-mail: ramanpdf01@gmail.com

related genes contributing to phenotypic variations in chimeric progenies of *Brassica juncea* and *B. oleracea* have been also proposed (Cao *et al.* 2016). Differential DNA methylation and expression of GA-receptor and GA-regulated proteins (GASR4 and GASR7) have been associated with *Arabidopsis* germination (Narsai *et al.* 2017) and root vascularization in *Sorghum bicolor* (Turco *et al.* 2017). Differential DNA methylation and expression of genes *OsGA2ox3* and *OsGA2ox4* leading to abortive ovule development in female-sterile line of rice has been reported (Liu *et al.* 2017). Recently, we demonstrated GA_3 mediated morphometric alterations in tobacco were associated with global euchromatinization and DNA hypo-methylation (Manoharlal *et al.* 2018a).

In tobacco, the process of cytosine methylation is catalysed by three main DNA (cytosine-5) methyl-transferases (DNMTs): methyltransferase 1 (*NtMET1*; AB280788.1), domains rearranged methyltransferase 1 (*NtDRM1*; AB087883.1), and chromomethylase 3 (*NtCMT3*; AB032538.1), catalysing the methylation at 'CG', 'non-CG', and 'CHG' motifs, respectively (Kyo *et al.* 2003, Wada *et al.* 2003, Kim *et al.* 2007). Modification in cytosine methylation frequency by synthetic DNMTs inhibitors leads to development of novel and improved trait(s) in plants (Arase *et al.* 2012). However, the existence of plants DNMTs inhibitors is not yet explored. Hence, there is a great need for the understanding, identification and development of effective, specific, and non-toxic DNMTs inhibitors in plants.

In our previous study, GA_3 as a natural NtDNMTs inhibitor has been demonstrated in *N. tabacum* (Manoharlal *et al.* 2018a). However, the underlying molecular mechanism(s) for the same has not been explored yet. In this study, we have addressed the effect of GA_3 on: *1*) NtDNMTs transcriptional rate, *2*) NtDNMTs mRNA stability, and *3*) mode of inhibition of NtDNMTs activity under *in vivo* and *in vitro* conditions.

Materials and methods

Plants and growth conditions: Seeds of tobacco (*Nicotiana tabacum* L.) cv. Kanchan were used in this study. Working stock of 50 mg dm^{-3} GA_3 (in 25 % ethanol) was prepared freshly before use. Field trials were conducted at Northern Light Soil (NLS) region, Rajahmundry (17.00 °N, 81.80 °E), Andhra Pradesh, India, during October 2015 - March 2016. Tobacco seedlings 60-d-old were transplanted from nursery to main-field in three plots replicates with s density of two plants per m^2. The GA_3 (50 mg dm^{-3}) solution with a non-ionic surfactant *APSA-80* (0.5 cm^3 dm^{-3}; *Amway*, Bengaluru, India) was sprayed on leaves only once at 30 d after transplantation in such a way that each plant leaf was fully drenched. On day 13 after GA_3 application, 10 uniform untreated ('U') and GA_3 treated ('T') plants were selected. For biochemical studies (NRO, NtDNMTs activity, and inhibition analysis), leaf samples were harvested from middle-position (generally 10^{th} - 11^{th} leaf from bottom of plant) of 5 plants and immediately frozen in liquid nitrogen, ground to a fine powder, and stored at -80 °C till further use. An early-vegetative growth stage was chosen for the aforementioned analysis as most significant difference in chromatin alteration, global DNA hypo-methylation, and *NtDNMTs* gene expressions were previously observed at this particular stage (Manoharlal *et al.* 2018a).

Differential transcript assessment: Total RNA was isolated from 'U' and 'T' leaves with *TRIzol*® reagent (*Ambion*®, *Invitrogen Bioservices India*, Bengaluru, India). The content of mRNA *NtMET1*, *NtDRM1*, and *NtCMT3* genes was quantified by RNA dot-blotting (Jones *et al.* 1996) on the charged nylon membrane (*Hybond*™-*N*$^+$; *GE Healthcare Life Sciences*, Pittsburgh, PA, USA) using *Bio-Dot* microfiltration apparatus (*Bio-Rad*, Hercules, CA, USA) by applying a gentle vacuum as per manufacturer's instructions. For hybridization, blots were UV-crosslinked followed by probing with gene specific probes of *NtMET1*, *NtDRM1*, and *NtCMT3* (Table 1 Suppl.). Northern-blot analysis was carried out essentially by standard protocols as described earlier (Kalantidis *et al.* 2002). Approximately 20 µg of total RNA from 'U' and 'T' was fractionated on a 1 % (m/v) agarose gel with 2.2 M formaldehyde. Equal loading of RNA was assessed by rRNA bands. For hybridization, RNA was blotted on to nylon membranes and UV-cross-linked, followed by probing with gene specific probes (Table 1 Suppl.). The membranes were subsequently de-probed and rehybridized with the *18S* rRNA and *EF-1 α*RNA probes to monitor equal RNA loading and transfer. Hybridization signals were quantified using a high resolution phosphoimager (*FLA-5000*, *Fujifilm Co.,* Tokyo, Japan). The steady state transcription of each gene was quantified using densitometry scanning, normalized to *18S* rRNA and *EF-1 α*RNA, and expressed as relative fold-changes (RFC) with respect to corresponding 'U' sample. The standardised real-time qPCR (conditions and primers are in Table 1 Suppl.) was applied using *Power SYBR® Green PCR Master Mix* (*Applied Biosystems*, Carlsbad, CA, USA) and *7500* real-time PCR system (Manoharlal *et al.* 2018a).

Nuclear run-on (NRO) analysis was performed as described by Kanazawa *et al.* (2000) with the certain modifications. Homogenized leaves (5 g) from 'U' and 'T' plants were suspended in 15 cm^3 ice-cold diethylether for 3 - 5 min and after washing they were resuspended in 40 cm^3 of ice-cold H-buffer [10 mM Tris-HC1 (pH 7.6),

1.14 M sucrose, 5 mM $MgC1_2$, 7 mM β-mercaptoethnol; Hamilton *et al.* 1972]. The resuspension was gently stirred and filtered through 2 - 3 layers of cheesecloth (pre-wetted with H-buffer). The homogenate was slowly decanted through the cheesecloth filter in a 50-cm^3 culture tubes placed on ice. The resulting extract was centrifuged at 1 000 *g* and 4 °C for 10 min. The supernatant was discarded, pellet was resuspended completely in 25 cm^3 of cold lysis buffer (H-buffer + 0.15 %, v/v, *Triton X-100*) and centrifuged at 1 000 *g* and 4 °C for 10 min. This step was repeated twice. The resulting nuclei pellet was resuspended in 1 cm^3 of transcription buffer [50 mM Tris-HC1 (pH 8), 5 mM $MgC1_2$, and 5 mM KC1], transferred to 1.5-cm^3 Eppendorf tube and centrifuged at 1 000 *g* and 4 °C for 2 min. The final pellet was washed again in 1 cm^3 of transcription buffer and suspended in 500 mm^3 of transcription buffer. *In vivo* transcription was reinitiated by resuspending the 92 mm^3 of nuclear-extract in 6 mm^3 of AGC mix (15 mM ATP, 10 mM GTP, and 10 mM CTP, a final working concentrations of 0.75, 0.5, and 0.5 mM, respectively) together with 1 U mm^{-3} RNase inhibitor and 5 mm^3 of α-[^{32}P] UTP (*Perkin Elmer*, Hopkinton, MA, USA). Reaction mixture was maintained on ice at all times. The final volume was adjusted to 120 mm^3 with a pre-warmed diethyl pyrocarbonate (DEPC) treated water and the mix was incubated at 30 °C with gentle shaking for 40 min to allow transcription elongation. For *in vitro* transcription inhibition, GA_3 (in ethanol) and a reference transcriptional inhibitor, cordycepin (COR; in dimethyl sulfoxide, DMSO) was also added to nuclear fraction at a working concentration of 50 mg dm^{-3} and 200 µg cm^{-3}, respectively. Notably, the solvent concentration in reaction mixture was reduced to < 0.01 % (v/v) of the final reaction volume.

The reaction was stopped by adding 10 U of RNase-free DNase I (*Thermo Fisher Scientific*, Wilmington DE, USA) and incubated at 30 ºC for 10 min followed by addition of 1 cm^3 of ice cold DEPC treated water to the mix. Nuclei fraction was recovered by centrifugation at 5 000 *g* to remove non-incorporated radioactive nucleotides. Isolated total labeled RNA (*TRIzol®* reagent; *Ambion*) was again precipitated by adding 2.5 M NH_4-acetate and an equal volume of isopropanol. The mixture was stored at -20 °C overnight. To pellet the RNA, tubes were centrifuged at 16 000 *g* for 15 min. The labelled RNA pellet was washed twice with 70 % (v/v) ethanol, dried, and resuspended in 50 mm^3 of 50 % (v/v) formamide in DEPC treated water. The isolated RNA was stored at -80 ºC for further use in hybridization assay. An aliquot was used for specific radioactivity determination in a *Tri-CARB 2900 TR* liquid scintillation analyzer (*Packard Instrument Co.*, USA).

The complementary DNA (cDNA) fragments of *NtDNMTs* genes; *NtMET1* (nucleotides: 1069-1142 from the start codon), *NtDRM1* (nucleotides: 1642-1708 from the start codon), *NtCMT3* (nucleotides: 669-734 from the start codon) and reference genes, *18S* RNA nucleotides: [1467-1638 from the transcription start point (TSP)] and *EF-1α* (nucleotides: 1185-1290 from the start codon) were PCR amplified (Table 1 Suppl.), column purified (PCR purification kit, *Qiagen*, Germantown, MD, USA), and quantified using *NanoDrop 8000* (*Thermo Fischer Scientific*). Approximately 1 - 2 µg of PCR amplicons were denatured in 0.4 M NaOH in a total volume of 100 mm^3 at 65 ºC for 15 min, followed by the addition of 200 mm^3 of ice-cold 20× saline-sodium citrate (SSC) buffer. The total content of each sample was transferred onto the charged nylon membrane (*Hybond*^{TM}-N^+; *GE Healthcare*) using *Bio-Dot* microfiltration apparatus (*Bio-Rad*) by applying a gentle vacuum. To ensure the equal spotting of DNA on the membrane, similar blots were also stained with 0.02 % (m/v) methylene blue in 0.3 M sodium acetate (pH 5.2). Dot-blotting was followed by rinsing in 2× SSC buffer, drying, and fixing of the membrane by UV-crosslinking (UV dose 120 mJ cm^{-2}). For hybridization, membranes with the immobilized DNA were incubated at 60 ºC for 2 - 3 h in pre-hybridization buffer (*Miracle-Hyb* buffer, *Stratagene,* San Diego, USA). *In vivo* labelled RNA of each extract was denatured by heating at 95 ºC for 5 min, immediately placed on ice for 5 min, and subsequently used for reverse Northern hybridization by adding to the pre-hybridization solution containing the membranes. Hybridization was carried out at 60 ºC for 16 h in hybridization oven/shaker *RPN2512E* (*GE Healthcare*). For washing, hybridization solution was discarded and membranes were washed twice with 2× SSC and 0.1 % (m/v) sodiumdodecyl suphate (SDS) at room temperature followed by one wash in pre-warmed 0.2× SSC and 0.1 % SDS at 60 ºC for 15 min. The semi-dried membrane was exposed to phosphorimage hyper-screen in a cassette and was kept at room temperature for 1 - 12 h (depending on radioactivity counts). Radioactive signals were detected with the help of a high resolution phosphoimager scanner. Signal intensity of hybridized nuclear RNA was quantified and normalized to the corresponding *18S* rRNA intensity using densitometry scanning.

Cordycepin chase assay: COR (*Cayman Chemical Co.*, Ann Arbor, MI, USA) a potent *in vivo* transcriptional inhibitor (Park *et al.* 2012) was used to measure the mRNA half-life of *NtDNMTs* genes. Briefly, tobacco seeds were surface-sterilized in 0.05 % $HgCl_2$ (m/v) for 8 min, followed by 5 times washing with double-distilled water (ddH_2O). Thereafter, seeds were kept in water for around 30 min which facilitated better germination. Subsequently, seeds were germinated at a 16-h photo-period, irradiance of 80 µmol m^{-2} s^{-1}, a temperature of 26 °C, and a relative humidity of 75 - 80 % on Murashige and Skoog (MS) medium with 0.46 % (m/v) agar and 3 % (m/v) sucrose (pH 5.8), either alone or supplemented with 50 mg dm^{-3} GA_3 for two weeks. The seedlings were transferred to a flask containing incubation buffer (1 mM

PIPES, pH 6.25, 1 mM sodium citrate, 1 mM KCl and 15 mM sucrose) for 30 min, followed by incubation with or without addition of 200 µg cm^{-3} COR on a rotary shaker for the indicated time. At harvest, seedlings were blotted dry by paper towels, frozen in liquid nitrogen and stored at -80 °C till further use. For RNA dot-blotting and hybridization, approximately 2 - 4 µg of total RNA isolated from the above mentioned samples were dot-blotted and hybridized with specific PCR amplified DNA probes of *NtDNMTs* genes (Table 1 Suppl.). Hybridization signal intensity was quantified with a phosphoimager and normalized to constitutively expressed *18S* rRNA. Normalized signal intensity of *NtMET1*, *NtDRM1*, and *NtCMT3* transcripts with respect to their corresponding intensity at time T_0 was plotted as a line graph. A semi-log plot of percentage of mRNA *versus* time allows for the assessment of mRNA half-life. The mRNA half-lives ($t_{1/2}$) were calculated by fitting non-linear regression according to the following exponential decay formula, $t_{1/2} = -0.693/k$, where k is the slope of the best fit-line. The $t_{1/2}$ was defined as the time-point at which the amount of steady-state mRNA declines to 50 % of the initial value.

NtDNMTs activity assay: Briefly, nuclear-extract (5 µg) prepared from 'U' and 'T' samples (*CelLytic*™ PN plant nuclei isolation/extraction kit, *Sigma-Aldrich*) was used for ELISA based colorimetric assay using *EpiQuik* DNMT activity/inhibition assay ultra kit (*Epigentek*, Farmingdale, NY, USA). NtDNMTs activity [A_{450} mg^{-1} (protein) min^{-1}] was calculated by equation: A_{450} × 1000/protein (µg) × t, where A_{450} is absorbance and t is incubation time (min) of reaction assay. For *in vitro* inhibition, GA_3 (in ethanol) and reference DNMT inhibitor, genistein (GEN; in dimethyl sulfoxide, DMSO) were added at a final working concentrations of 0 - 100 mg dm^{-3} and 25 µM, respectively, to the reaction mixture 5 min before the addition of nuclear-extract and allowed to equilibrate. Notably, the solvent concentration in reaction mixture was reduced to < 0.01% (v/v) of the final reaction volume. NtDNMTs activity inhibition (%) was calculated by following standard equation: $[(1-A_T/A_U)] \times 100$, where A_U and A_T are the absorbances of 'U' and 'T', respectively.

Inhibitory kinetics of NtDNMTs: Radiolabelled NtDNMTs assay was performed as described previously (Adams *et al.* 1991, Belinsky *et al.* 1996) with certain modifications. In brief, 5 µg of nuclear-extract prepared from 'U' and 'T' plants was incubated with varying concentrations (0 - 3 µg dm^{-3}) of *poly(dI-dC).poly(dI-dC)* substrate and 2 µM of [^{3}H] *AdoMet* (15.54 µBq; *PerkinElmer*, Hopkinton, MA, USA) as a labelled methyl donor in a 1× DNA methylation assay buffer [20 mM Tris-HCl, 25 % glycerol (v/v), 10 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 20 mM β-mercaptoethanol (pH 7.4)]. For *in vitro* inhibition study, GA_3 (0 - 100 mg dm^{-3}) and GEN (25 µM) was added to the reaction mixture 5 min before the addition of nuclear-extract and allowed to equilibrate. The final reaction volume was adjusted to 30 mm^3 with *MilliQ* water and mix was incubated at 37 °C for 90 min. The reaction was initiated by the addition of nuclear-extract and stopped by addition of 300 mm^3 of stop-solution [1 % (m/v) SDS, 3 % (m/v) 4-aminosalicylate, 5 % (v/v) butanol, 2 mM EDTA, 125 mM NaCl, 0.25 mg cm^{-3} of carrier ssDNA salmon sperm testes and 1 mg cm^{-3} protease K). To remove proteins, the resulting mixture was vortexed with 300 mm^3 of a solution containing 88 % (v/v) phenol, 12 % (v/v) *m*-cresol and 0.1 % (m/v) 8-hydroxyquinoline and centrifuged at 9 300 *g* for 5 min. The labelled methylated DNA substrate was recovered from the aqueous phase by ethanol precipitation and washed three times with 70 % ethanol. RNA was removed by resuspension of the precipitates in 30 mm^3 of 0.3 M NaOH and labelled DNA substrate was spotted on *Whatman* filters, dried, and washed five times with 5 % (m/v) trichloroacetic acid (TCA), followed by a two washing with 70 % ethanol. Filters were placed in scintillation cocktail and NtDNMTs activity measured as ^{3}H incorporation, was determined in a *Tri-CARB 2900 TR* liquid scintillation analyzer (*Packard Instrument Co.*). The amount of the labelled methyl group transferred was calculated based on the specific activity of [^{3}H] *AdoMet* (2.59 Bq $fmol^{-1}$). Results were expressed as Bq μg^{-1} (protein) min^{-1}. Background levels were determined in reaction mixture without addition of *poly(dI-dC)-poly(dI-dC)* substrate. The limit of detection (LOD) was ≥ 0.42 Bq above the background levels. Enzyme kinetic parameters (K_m and V_{max}) were evaluated using the non-linear regression method based on Michaelis-Menten equation and inhibition mode was determined by double-reciprocal Lineweaver-Burk plot analysis according to Michaelis-Menten kinetics (Fang *et al.* 2003).

Data analysis: The results are expressed as means ± standard deviations (SDs). One-way analysis of variance (*ANOVA*) was used to analyze the statistical significant difference ($P < 0.05$) between groups.

Results

We investigated the possibility of altered transcriptional patterns of *NtMET1*, *NtDRM1*, and *NtCMT3* following GA_3 application in *N. tabacum*. Firstly, we assessed the differential mRNA accumulation by RNA dot-blotting (Fig. 1*A*). The steady state mRNA levels of *NtMET1* and *NtCMT3* from 'T' plants were significantly lower as compared to those from 'U' plant. However, no significant difference in *NtDRM1* mRNA accumulation

due to GA_3 application was observed. The observed differential transcriptional pattern was further corroborated by RT-qPCR and Northern blotting, wherein a high degree of positive correlation (r = 95 - 99 %) was observed in all cases (Fig. 1*B,C*). Consistency in differential mRNA accumulation determined by three independent methods indicate the reliability of our results and corroborated the accuracy and robustness of RNA dot-blotting. The observed decrease in transcriptions of *NtMET1* and *NtCMT3* due to GA_3 application could be caused either by affecting their promoter activity and/or mRNA stability. Therefore, further experiments were performed to elucidate the role of GA_3 in mediating their transcriptional and/or post-transcriptional control mechanism(s).

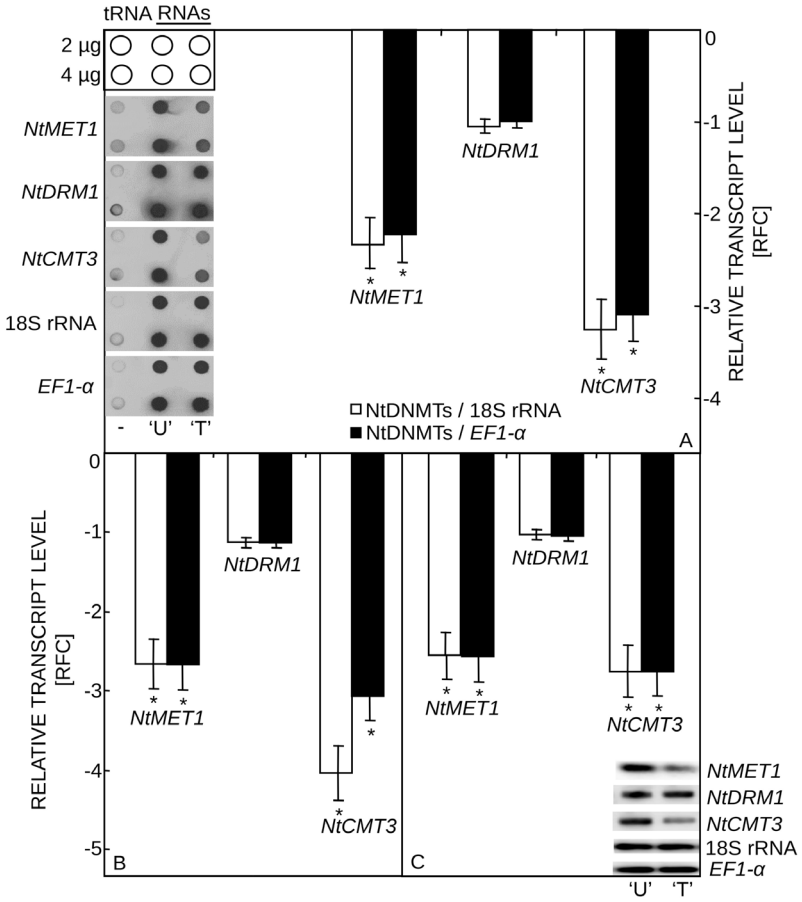

Fig. 1. Differential transcription of *NtDNMTs* genes. *A* - Total RNA (2 - 4 µg dot^{-1}) from untreated ('U') and GA_3 treated ('T') tobacco leaves were subjected to dot-blot hybridization. Relative position of individual dot-blot and recognition of dotted RNA by hybridized α-[^{32}P] ATP-labelled probes of *NtMET1*, *NtDRM1*, *NtCMT3*, *18S* rRNA, and *EF1-α* are shown. *B* - Data analyzed by $2^{-\Delta\Delta}C_T$ was depicted as relative fold-changes (RFC) in the gene transcription with respect to constitutively expressed *18S rRNA* and *EF1*-α mRNA. The y-axis shows the amount of individual mRNA (in RFC), wherein the value of 'U' was set at 1. Since the RFC was < 1, thereby reciprocal was taken. *C* - Inset depicts the Northern blots hybridized with α-[^{32}P] dATP-labelled gene specific probes. Hybridization signal intensity was quantified using densitometry scanning of phosphoimages. Means ± SDs, n = 3, *asterisks* indicate significant differences ($P < 0.05$) between 'T' and 'U'.

For measurement of transcriptional rate of *NtDNMTs* genes, an optimized NRO assay was performed. (Fig. 2*A*). The GA_3 treated plants exhibited a significant reduction in transcriptional rate of *NtMET1* and *NtCMT3*. In corroboration with the result of differential mRNA accumulation, no significant difference in transcription of *NtDRM1* was observed. The specific inhibitory effect of GA_3 on *NtMET1* and *NtCMT3* transcription was further evaluated under *in vitro* condition by adding GA_3 (50 mg dm^{-3}) to the nuclear-extract prior to the NRO analysis. The solvent control of GA_3 (in ethanol) established the specific transcriptional inhibition by GA_3 (Fig. 1 Suppl.). The validity of NRO assay was also confirmed by optimized dose of cordycepin (COR, 200 µg cm^{-3}), a well-known transcriptional inhibitor (Fig. 2 Suppl.). The solvent control of COR (in DMSO) further established the specific transcriptional inhibition by COR (Fig. 1 Suppl.). Notably, *in vitro* GA_3 supplementation inhibited the *NtMET1* and *NtCMT3* transcription in both 'U' and 'T' plants, without affecting the transcription of

NtDRM1 and constitutively expressed *18S rDNA* and *EF1-α* (Fig. 2*A*). In comparison to *in vivo* conditions, the decline in transcription of *NtMET1* and *NtCMT3* was relatively higher under *in vitro* GA_3 supplementation. However, unlike GA_3, COR decreased the transcription of *NtMET1*, *NtDRM1*, *NtCMT3*, *18S rDNA*, and *EF1-α* in both 'U' and 'T' plants, suggesting that GA_3 is a more specific and relatively less potent *in vitro* transcriptional inhibitor.

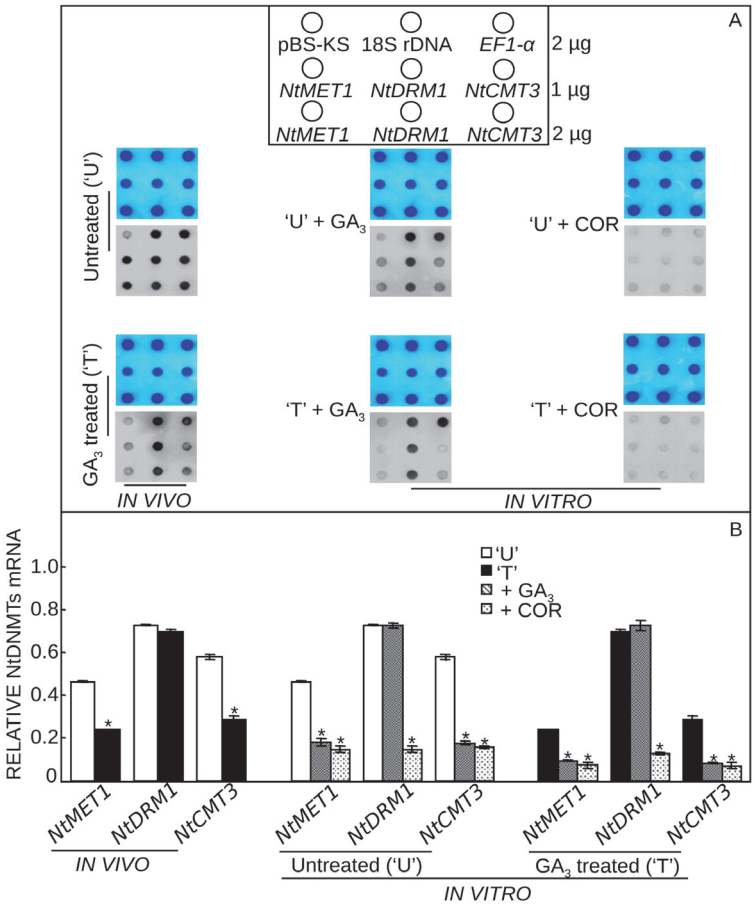

Fig 2. Nuclear run-on (NRO) analysis. *A* - Approximately 1 - 2 µg of empty vector pBS-KS(+) DNA (negative control), *18S* rDNA, *EF1-α* (positive controls) and *NtMET1*, *NtDRM1*, and *NtCMT3* genes were immobilized on charged nylon membranes. The NRO analysis of aforementioned transcripts after *in vitro* addition of GA_3 (50 mg dm^{-3}) was also measured. As a positive control of nascent transcription inhibition, an addition of 200 µg cm^{-3} COR was also tested. The blots were probed with α-[^{32}P] UTP-labelled nascent NRO transcripts derived from 'U' and 'T' samples. Relative position of individual dot-blot, loading control (methylene-blue stain) and recognition of dotted DNA by hybridized labelled nuclear RNA are shown in the *upper*, *middle,* and *bottom* panel, respectively. Hybridization signal of each nuclear RNA was quantified using densitometry scanning of phosphoimages. Ratio under each blot indicates the normalised nuclear RNA intensity between indicated samples after background correction. *B* - Relative signal intensity of *NtMET1*, *NtDRM1*, and *NtCMT3* transcripts (corresponding to 2 µg of dotted DNA) with respect to corresponding *18S* rRNA was plotted as *bar graph* with *asterisks* depicting the significant difference ($P < 0.05$) between 'T' and 'U' samples. Data are representative of two independent experiments.

To investigate if post-transcriptional events also contribute to the lower expression of *NtMET1* and *NtCMT3* after GA_3 application, we determined its mRNA stability in 'U' and 'T' plants. We used COR transcription inhibitor that blocks the *de novo* transcription by causing chain termination (Wolfraim *et al.* 1993) and 200 µg cm^{-3} COR inhibited ~90 % of *de novo* transcription of *NtEXPA2*a (expansin-like protein 2, a tobacco homologue of corresponding *Arabidopsis thaliana* gene; Gutiérrez *et al.* 2002, Lidder *et al.* 2005, Xu and Chua 2009; Figs. 2 and 3 Suppl.). COR chase assay was performed over a period of 0 - 5 h (Fig. 3*A*). Fig. 3*B* depicts a typical mRNA decay profile of *NtDNMTs* genes. *NtMET1* and *NtCMT3* mRNA could be detected at time T_0 and the signal intensity diminished more progressively with time in 'T' plants with a corresponding mRNA half-life ($t_{1/2}$) of 0.85 and 0.95 h, respectively. In contrast, the mRNA turnover of *NtMET1* and *NtCMT3* occurred much more slowly in the 'U', with a corresponding $t_{1/2}$ of ~1.9 h and 2.3 h, respectively.

Notably, the mRNA turnover of *NtDRM1* as well as *NtEXPA2* transcripts occurred almost equally in 'U' and 'T' plants, with a $t_{1/2}$ of ~0.5 and ~2.2 - 2.4 h, respectively (Fig. 3*B* and Fig. 4 Suppl.). These results clearly demonstrated that mRNAs stability of *NtMET1* and *NtCMT3* differs significantly between 'U' and 'T' plants, indicating the role of GA_3 as a specific transcriptional inhibitor in tobacco. It is pertinent to mention here that despite different modes of GA_3 application under experimental field and laboratory conditions (foliar application *vs* supplementation in MS culture media), no discrepancy in GA_3 effect was observed (Manoharlal *et al.* 2018a).

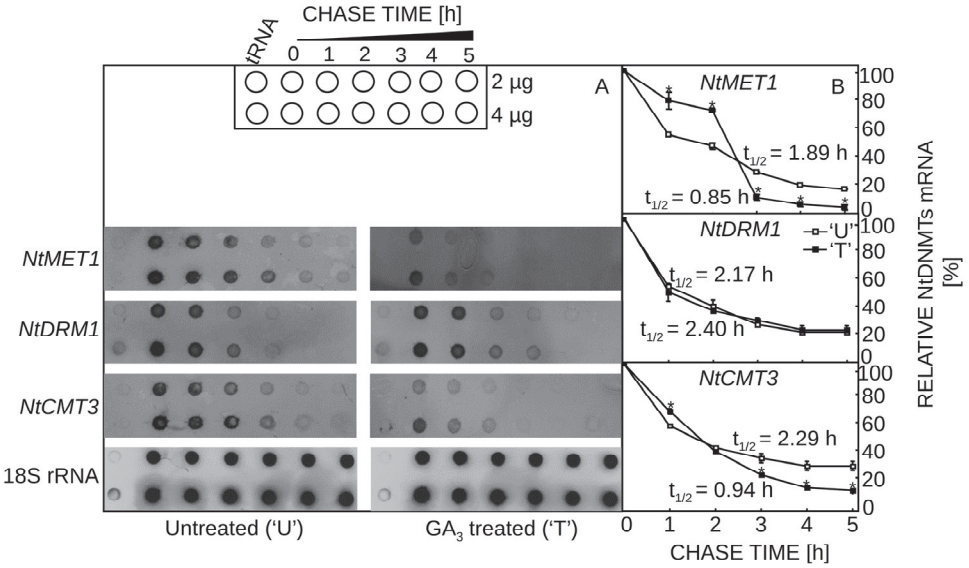

Fig. 3. Cordycepin (COR) chase assay. Two-week old tobacco seedlings grown in absence or presence of 50 mg dm^{-3} GA_3, were pre-incubated for 30 min in incubation buffer, followed by addition of 200 µg cm^{-3} COR to inhibit the ongoing *in vivo* transcription. *A* - Total RNAs (2 - 4 µg dot^{-1}) was isolated at the indicated times and subjected to dot-blot hybridization. Relative position of individual dot-blot and recognition of dotted RNA by hybridized α-[^{32}P] ATP-labelled DNA probes of *NtMET1*, *NtDRM1*, and *NtCMT3* are shown in the *upper* and *bottom* panels, respectively. Time-points of chase are indicated above the schematic representation of dot-blot. *B* - The hybridization signal intensity for each *NtDNMTs* transcripts at each time-point was quantified by densitometry scanning and normalized to the corresponding constitutively expressed *18S* rRNA. Normalized signal intensity of *NtMET1*, *NtDRM1*, and *NtCMT3* transcripts with respect to their corresponding intensity at time T_0 was plotted as line-graph. *Asterisks* depicted the significant difference ($P < 0.05$) between 'T' and 'U' plants. Data are representative of three independent experiments. The mRNA half-lives ($t_{1/2}$) was calculated by fitting non-linear regression.

Further, we tried to verify whether GA_3 also impacted the corresponding NtDNMTs activities. Prior to NtDNMTs activities measurements, the nuclear-extract quality was determined using an anti-histone H4 antibody (Fig. 5 Suppl.). A ~1.65 fold decline in NtDNMTs activity by GA_3 under *in vivo* condition was observed (Fig. 4). We further evaluated the specific inhibitory effect of GA_3 on NtDNMTs activity under *in vitro* condition. For this, GA_3 (0 - 100 mg dm^{-3}) was added to the nuclear-extract prior to the analysis of NtDNMTs activity. The 25 µM genistein (GEN, a well-known DNMT inhibitor) was also used as a reference positive control. The solvent controls of GA_3 (in ethanol) and GEN (in DMSO) established that *in vitro* inhibition of NtDNMTs activity by GA_3 and GEN was highly specific (Fig. 6 Suppl.). As shown in Fig. 4, *in vitro* GA_3 supplementation inhibited the NtDNMTs activity in a dose dependent manner for both 'U' and 'T' plants, with a respective IC_{50} value of ~56 and ~50 mg dm^{-3}. Notably, at a sub-saturated concentration of GA_3 (25 mg dm^{-3}), the decline in NtDNMTs activity was relatively higher in 'U' plants as compared to the corresponding 'T' plants (~1.94- *vs* ~1.6-fold). However unlike GA_3, the presence of GEN significantly and comparably decreased the NtDNMTs activity in both 'U' and 'T' (~7.27 *vs* ~4.92 fold), further corroborating GA_3 as relative less potent NtDNMTs inhibitor.

Further, we were interested to determine whether GA_3 showed any selectivity as a modulator of NtDNMTs activity. With nuclear-extract from 'U' and 'T' plants as an enzyme source and *poly(dI-dC).poly(dI-dC)* as a substrate under our assay conditions, a dose-dependent inhibition in NtDNMTs activity by GA_3 was observed, with a linear production of methylated *poly(dI-dC).poly(dI-dC)* for 3 h (Fig. 5, *insets*). The inhibition mode was analyzed by double-reciprocal Lineweaver-Burk plot according to Michaelis-Menten kinetics. In the absence of GA_3, V_{max} was 0.059 and 0.035 Bq $µg^{-1}$ (protein) min^{-1} with a corresponding K_m of 0.56 and 0.53 µg in 'U' and 'T'plants, respectively. However, in the

presence of GA_3 (50 mg dm^{-3}), V_{max} in 'U' and 'T'was 0.04 and 0.025 Bq μg^{-1} (protein) min^{-1} with a corresponding K_m of 0.55 and 0.52 µg, respectively (Fig. 5). These observations demonstrated that GA_3 significantly decreased the V_{max} without affecting the apparent K_m of NtDNMTs activity under both *in vivo* and *in vitro* conditions, thereby suggesting a non-competitive mode of inhibition by GA_3.

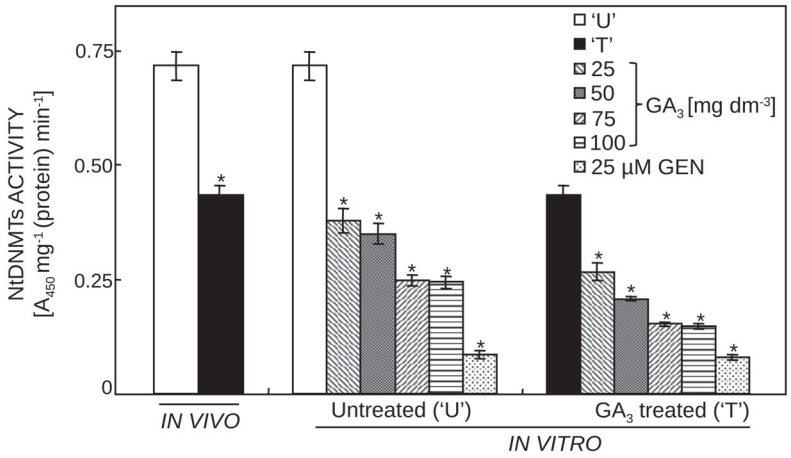

Fig. 4. NtDNMTs activities in nuclear-extract from 'U' and 'T' plants, along with *in vitro* addition of GA_3 (0 - 100 mg dm^{-3}), and 25 µM genistein (GEN) as positive control were presented. Means ± SDs, $n = 3$, *asterisks* indicate significant differences ($P < 0.05$) as compared to corresponding 'U' control.

Discussion

An epigenetic role of GA_3 on chromatin de-condensation and global DNA hypo-methylation in tobacco has been demonstrated recently (Manoharlal *et al.* 2018a). Furthermore, GA_3 mediates transcriptional modulation of 393 genes (Manoharlal *et al.* 2018b). Albeit, GA_3 induced changes could be related to the genomic DNA hypo-methylation, the precise mechanism still remains elusive. Hence, the aim of our study was to investigate the GA_3 mediated epigenetic changes in tobacco.

We observed that GA_3 markedly declined expression of two *NtDNMTs* genes, *NtMET1* and *NtCMT3* (Fig. 1). However, a direct correlation between global DNA methylation and altered DNMTs expression does not always exist. Thereby, the aforementioned expression changes by GA_3 could also be hypothesised as a negative feedback response of global DNA methylation (Vanyushin and Ashapkin 2011). On the contrary, *NtDRM1* expression was not influenced by GA_3, which is consistent with its ubiquitous transcripion in tobacco (Wada *et al.* 2003). Since *NtDRM1* is involved in RNA-directed methylation, smRNA pathways controlling the transposons expression is not influenced by GA_3. Thus, it seems that symmetrical methylated CpG and methylated CpHpG (where H = A, C, or T) catalysed by *NtMET1* and *NtCMT3* are primary targets of GA signalling. Notably in rice, GA_3 does not induce significant changes in *OrDNMTs* expression (Ahmad *et al.* 2014). However, in that study, *OrDNMTs* expression was traced for relatively short time-interval (up to 12 h) following GA_3 treatment. In our experiments, a marked decrease in NtDNMTs activity was observed at 13 d after GA_3 application. It is likely that tobacco epigenetic re-programming in response to GA_3 requires some time and multiple cell divisions.

Numerous reports demonstrate the role of GA_3 in transcriptional and post-transcriptional control of gene regulation (Shi and Olszewski 1998, Gubler *et al.* 2002, Gao *et al.* 2013). Therefore, our further study was focused on dissecting the transcriptional rate and mRNA stability of *NtDNMTs* genes. The NRO analysis demonstrated that apparent reduced transcriptions of *NtMET1* and *NtCMT3* could be partially explained by transcriptional silencing of their corresponding promoters (Fig. 2). The observed transcriptional suppression by GA_3 was relatively lower than corresponding steady-state mRNA levels (*NtMET1*; ~1.9- *vs* ~2-8 fold and *NtCMT3*; ~2- *vs* ~3-fold), indicating the prevalence of additional unknown factor(s). To evaluate this possibility, COR chase assay was performed to evaluate the differential mRNA stability of *NtMET1* and *NtCMT3*. Our observations revealed that GA_3 application caused reduced mRNA half-lives of *NtMET1* and *NtCMT3* (Fig. 3). Notably, GA_3 specifically and significantly accelerated the transcriptional suppression and mRNA decay of *NtMET1* and *NtCMT3*, indicating that active suppression and degradation of these mRNA required either *de novo* synthesis or inhibition of certain specific proteins. In support to this hypothesis, transcriptional activation of α-amylase by GA_3 has been reported to be dependent on continuous RNA and protein synthesis in

Hordeum vulgare (Nolan and Ho 1988). A similar mechanism has been also proposed in human cell lines, wherein altered *hDNMTs* expression is linked to its mRNA stability (López de Silanes *et al.* 2009).

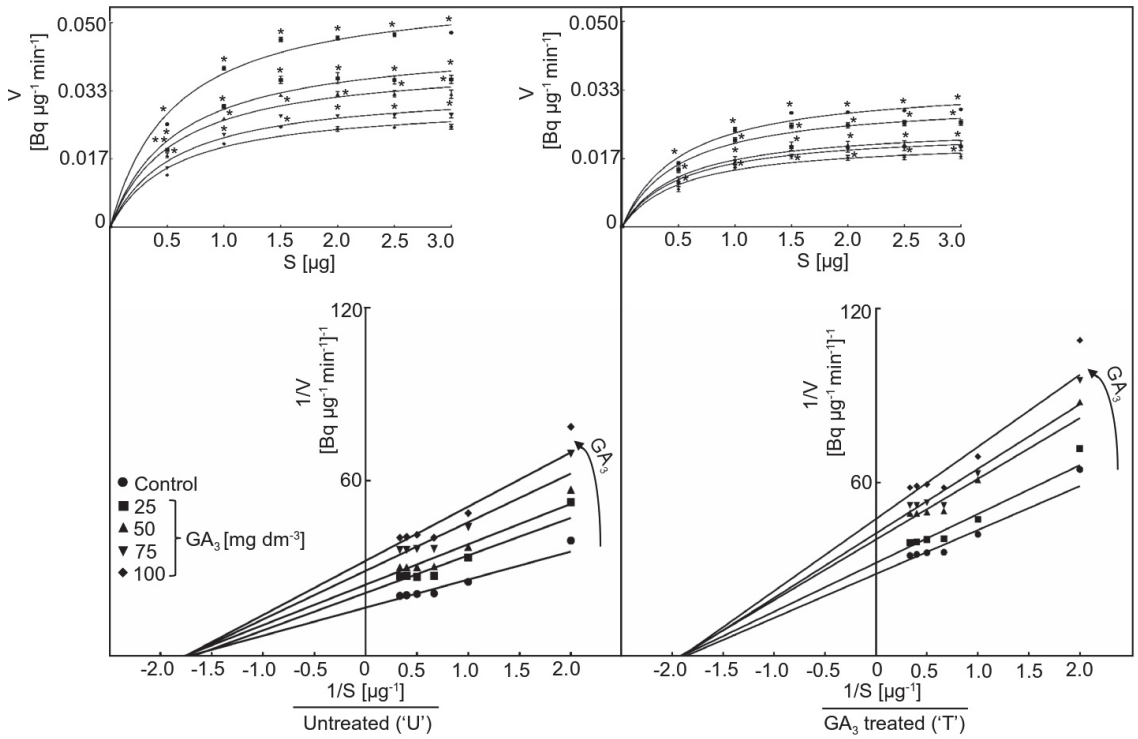

Fig. 5. Lineweaver-Burk plot analysis of NtDNMTs activity. The reaction mixture harbouring 5 µg of nuclear-extract from 'U' (left-panel) and 'T' (right-panel) plants was incubated with 2 µM of labelled precursor, [^{3}H] *AdoMet*, varying concentrations (0 - 3 µg dm^{-3}) of *poly(dI-dC).poly(dI-dC)* substrate, along with varying concentrations (0 - 100 mg dm^{-3}) of GA_3 at 37 °C for 90 min (described in Materials and methods). Inset depicts the corresponding Michaelis-Menten plot. Means ± SDs, n = 3, *asterisks* indicate significant differences ($P < 0.05$) as compared to control. Data was analysed by *GraphPad Prism v. 7.03*.

There is no clear consensus about the impact of GAs on plant DNMTs activity. While the activity of purified DNMTs from wheat seedlings and germinating embryos is inhibited by GA_3, conversely GA_3 stimulates the DNMTs activity in wheat embryo nuclear-extract (Vlasova *et al.* 1995). In our study, GA_3 mediated significant decline in NtDNMTs activity was observed (Fig. 4). Lineweaver-Burk plot analysis demonstrated GA_3 as a non-competitive inhibitor of NtDNMTs activity (Fig. 5). Previous studies have also reported the role of GA_3 as non-competitive inhibitor of herbicides barban [4-chloro-2-butynyl N-(3-chlorophenyl) carbamate], phenylurethanes, as well as sodium butyrate (Yung and Mann 1967, Kumar *et al.* 1985). Similarly, GA_3 as a specific non-competitive antagonist to indolyl-acetic acid and an ethyl acetate-soluble acidic fraction (extracts from sporophytes *Ecklonia radiata*) has been also proposed (Brian *et al.* 1960, Jennings 1969). It is pertinent to mention here that a probable passive role of GA_3 also cannot be ruled out. Indeed, our earlier observations also demonstrated the altered activities of histone deacetylases (*NtHDACs*) and histone acetyltransferases (*NtHATs*) by GA_3 in *N. tabacum* (Manoharlal *et al.* 2018b). Recently, GA_3 mediated cross-talk of endogenous phytohormones (GAs, ethylene, and auxin) has been also established (Manoharlal *et al.* 2018b). Notably, the question about the impact of GA_3-induced global DNA hypo-methylation on endogenous expression still persists. Indeed, our time-kinetics based genome-wide transcriptome and methylated DNA immuno-precipitation-sequencing (MeDIP-Seq) studies are underway to identify the responsive epialleles and to decipher complex epigenetic signalling by GAs.

Taken together, our current findings supported a novel biological function of GA_3 as 'green' natural alternative to otherwise toxic and synthetic DNMTs inhibitors. Future studies on GA_3 regulated epigenome and its potential use as a DNA de-methylation agent, will be of immense use for better understanding of plant development and metabolic engineering.

References

Adams, R., Rinaldi, A., Seivwright, C.: Microassay for DNA methyltransferase. - J. biochem. biophys. Methods **22**: 19-22, 1991.

Ahmad, F., Huang, X., Lan, H.X., Huma, T., Bao, Y.M., Huang, J., Zhang, H.S.: Comprehensive gene expression analysis of the DNA (cytosine-5) methyltransferase family in rice (*Oryza sativa* L.). - Genet. mol. Res. **13**: 5159-5172, 2014.

Arase, S., Kasai, M., Kanazawa, A.: *In planta* assays involving epigenetically silenced genes reveal inhibition of cytosine methylation by genistein. - Plant Methods **8**: 10. 2012.

Bartels, A., Han, Q., Nair, P., Stacey, L., Gaynier, H., Mosley, M., Huang, Q., Pearson, J., Hsieh, T., An, Y., Xiao, W.: Dynamic DNA methylation in plant growth and development. - Int. J. mol. Sci. **19**: 21-44, 2018.

Belinsky, S.A., Nikula, K.J., Baylin, S.B., Issa, J.: Increased cytosine DNA-methyltransferase activity is target-cell-specific and an early event in lung cancer. - Proc. nat. Acad. Sci. USA. **93**: 4045-4050, 1996.

Brian, P.W., Hemming, H.G., Lowe, D.: Inhibition of rooting of cuttings by gibberellic acid. - Ann. Bot. **24**: 407-419, 1960.

Cao, L., Yu, N., Li, J., Qi, Z., Wang, D., Chen, L.: Heritability and reversibility of DNA methylation induced by *in vitro* grafting between *Brassica juncea* and *B. oleracea*. - Sci. Rep. **6**: 27233, 2016.

Fang, M.Z., Wang, Y., Ai, N., Hou, Z., Sun, Y., Lu, H., Welsh, W., Yang, C.S.: Tea polyphenol (-)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. - Cancer Res. **63**: 7563-7570, 2003.

Gao, Z., Meng, C., Gao, H., Li, Y., Zhang, X., Xu, D., Zhou, S., Liu, B., Su, Y., Ye, N.: Carotenoid genes transcriptional regulation for astaxanthin accumulation in fresh water unicellular alga *Haematococcus pluvialis* by gibberellin A_3 (GA_3). - Ind. J. Biochem. Biophys. **50**: 548-553, 2013.

Gubler, F., Chandler, P.M., White, R.G., Llewellyn, D.J., Jacobsen, J.V.: Gibberellin signaling in barley aleurone cells. Control of *SLN1* and *GAMYB* expression. - Plant Physiol. **129**: 191-200, 2002.

Gutiérrez, R.A., Ewing, R.M., Cherry, J.M., Green, P.J.: Identification of unstable transcripts in *Arabidopsis* by cDNA microarray analysis: rapid decay is associated with a group of touch-and specific clock-controlled genes. - Proc. nat. Acad. Sci. USA. **99**: 11513-11518, 2002.

Hamilton, R.H., Künsch, U., Temperli, A.: Simple rapid procedures for isolation of tobacco leaf nuclei. - Anal. Biochem. **49**: 48-57, 1972.

Hauvermale, A.L., Ariizumi, T., Steber, C.M.: Gibberellin Signaling: A theme and variations on DELLA repression. - Plant Physiol. **160**: 83-92, 2012.

Hedden, P., Phillips, A.L.: Gibberellin metabolism: new insights revealed by the genes. - Trends Plant Sci. **5**: 523-530, 2000.

Jennings, R.: Gibberellin antagonism by material from a brown alga. - New Phytol. **68**: 683-688, 1969.

Jones, W.K., Grupp, I.L., Doetschman, T., Grupp, G., Osinska, H., Hewett, T.E., Boivin, G., Gulick, J., Ng, W.A., Robbins, J.: Ablation of the murine α myosin heavy chain gene leads to dosage effects and functional deficits in the heart. - J. clin. Invest. **98**: 1906, 1996.

Kalantidis, K., Psaradakis, S., Tabler, M., Tsagris, M.: The Occurrence of CMV-specific short rnas in transgenic tobacco expressing virus-derived double-stranded RNA is indicative of resistance to the virus. - Mol. Plant-Microbe Interact. **15**: 826-833, 2002.

Kanazawa, A., O'Dell, M., Hellens, R.P., Hitchin, E., Metzlaff, M.: Mini-scale method for nuclear run-on transcription assay in plants. - Plant mol. Biol. Rep. **18**: 377-383, 2000.

Kim, H., Yano, A., Wada, Y., Sano, H.: Properties of a tobacco DNA methyltransferase, *NtMET1* and its involvement in chromatin movement during cell division. - Ann. Bot. **99**: 845-856, 2007.

Kumar, S., Chandra, G.R., Albaugh, G.P., Muthukrishnan, S.: Regulation of the expression of α-amylase gene by sodium butyrate. - Plant mol. Biol. **5**: 269-279, 1985.

Kyo, M., Hattori, S., Yamaji, N., Pechan, P., Fukui, H.: Cloning and characterization of cDNAs associated with the embryogenic dedifferentiation of tobacco immature pollen grains. - Plant Sci. **164**: 1057-1066, 2003.

Lidder, P., Gutiérrez, R.A., Salomé, P.A., McClung, C.R., Green, P.J.: Circadian control of messenger RNA stability. Association with a sequence-specific messenger RNA decay pathway. - Plant Physiol. **138**: 2374-2385, 2005.

Liu, H., Wu, Y., Cao, A., Mao, B., Zhao, B., Wang, J.: Genome-wide analysis of DNA methylation during ovule development of female-sterile rice *fsv1*. - G3: Genes Genomes Genet. **7**: 3621-3635, 2017.

López de Silanes, I., Gorospe, M., Taniguchi, H., Abdelmohsen, K., Srikantan, S., Alaminos, M., Berdasco, M., Urdinguio, R.G., Fraga, M.F., Jacinto, F.V., Esteller, M.: The RNA-binding protein HuR regulates DNA methylation through stabilization of DNMT3b mRNA. - Nucl. Acids Res. **37**: 2658-2671, 2009.

Manoharlal, R., Saiprasad, G.V.S., Thambrahalli, A., Madhavakrishna, K.: Dissecting the transcriptional networks underlying the gibberellin response in *Nicotiana tabacum*. - Biol. Plant. **62**: 647-662, 2018b.

Manoharlal, R., Saiprasad, G.V.S., Ullagaddi, C., Kovařík, A.: Gibberellin A_3 as an epigenetic determinant of global DNA hypo-methylation in tobacco. - Biol. Plant. **62**: 11-23, 2018a.

Meijón, M., Jesús Cañal, M., Valledor, L., Rodríguez, R., Feito, I.: Epigenetic and physiological effects of gibberellin inhibitors and chemical pruners on the floral transition of azalea. - Physiol. Plant. **141**: 276-288, 2011.

Meilan, L., Qiaomei, W., Zhujun, Z., Guangwen, Z.: Studies on the changes of DNA methylation level, GA content and protein in non-heading Chinese cabbage during vernalization. - Acta hort. sin. **29**: 353-357, 2002.

Narsai, R., Gouil, Q., Secco, D., Srivastava, A., Karpievitch, Y.V., Liew, L.C., Lister, R., Lewsey, M.G., Whelan, J.: Extensive transcriptomic and epigenomic remodelling occurs during *Arabidopsis thaliana* germination. - Genome Biol. **18**: 172, 2017.

Nolan, R.C., Ho, T.D.: Hormonal regulation of α-amylase expression in barley aleurone layers the effects of gibberellic acid removal and abscisic acid and phaseic acid treatments. - Plant Physiol. **88**: 588-593, 1988.

Park, S., Chung, P.J., Juntawong, P., Bailey-Serres, J., Kim, Y.S., Jung, H., Bang, S.W., Kim, Y., Do Choi, Y., Kim, J.: Posttranscriptional control of photosynthetic mRNA decay under stress conditions requires 3' and 5' untranslated regions and correlates with differential polysome association in rice. - Plant Physiol. **159**: 1111-1124, 2012.

Richards, D.E., King, K.E., Ait-Ali, T., Harberd, N.P.: How gibberellin regulates plant growth and development: a

molecular genetic analysis of gibberellin signaling. - Annu. Rev. Plant Physiol. Plant mol. Biol. **52**: 67-88, 2001.

Shi, L., Olszewski, N.E.: Gibberellin and abscisic acid regulate GAST1 expression at the level of transcription. - Plant mol. Biol. **38**: 1053-1060, 1998.

Sun, T., Gubler, F.: Molecular mechanism of gibberellin signaling in plants. - Annu. Rev. Plant Biol. **55**: 197-223, 2004.

Thomas, S.G., Sun, T.P.: Update on gibberellin signaling. A tale of the tall and the short. - Plant Physiol. **135**: 668-676, 2004.

Turco, G.M., Kajala, K., Kunde-Ramamoorthy, G., Ngan, C., Olson, A., Deshphande, S., Tolkunov, D., Waring, B., Stelpflug, S., Klein, P.: DNA methylation and gene expression regulation associated with vascularization in *Sorghum bicolor*. - New Phytol. **214**: 1213-1229, 2017.

Vanyushin, B.F., Ashapkin, V.V.: DNA methylation in higher plants: past, present and future. - Biochim. biophys. Acta. **1809**: 360-368, 2011.

Vlasova, T.I., Demidenko, Z.N., Kirnos, M.D., Vanyushin, B.F.: *In vitro* DNA methylation by wheat nuclear cytosine DNA methyltransferase: effect of phytohormones. - Gene **157**: 279-281, 1995.

Wada, Y., Ohya, H., Yamaguchi, Y., Koizumi, N., Sano, H.: Preferential de novo methylation of cytosine residues in non-CpG sequences by a domains rearranged DNA methyltransferase from tobacco plants. - J. biol. Chem. **278**: 42386-42393, 2003.

Wolfraim, L.A., Langis, R., Tyson, H., Dhindsa, R.S.: cDNA sequence, expression, and transcript stability of a cold acclimation-specific gene, *cas18*, of alfalfa (*Medicago falcata*) cells. - Plant Physiol. **101**: 1275-1282, 1993.

Xu, J., Chua, N.: *Arabidopsis* decapping 5 is required for mRNA decapping, P-body formation, and translational repression during postembryonic development. - Plant Cell **21**: 3270-3279, 2009.

Yung, K.H., Mann, J.D.: Inhibition of early steps in the gibberellin-activated synthesis of α-amylase. - Plant Physiol. **42**: 195-200, 1967.