Gibberellin A₃ mediated decreased transcriptional rate, mRNA stability and non-competitive inhibition of DNA methyltransferases in tobacco

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Abstract

The present work aims to examine the underlying molecular mechanism(s) for the reduced transcriptions and activities of DNA methyltransferases (NtDNMTs) by gibberellin A₃ (GA₃) 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 GA₃. Lineweaver-Burk plot analysis revealed a non-competitive mode of inhibition of NtDNMTs activity by GA₃. 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 GA₃.

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, GA₃ 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-

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Abbreviations: COR - cordycepin; DNMTs - DNA methyltransferases; GA₃ - gibberellin A₃; GEN - genistein; NRO - nuclear run-on, RFC - relative fold-changes; T - GA₃ treated samples; U - untreated samples.

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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 GA₃ 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) methyltransferases (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, GA₃ 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 GA₃ 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 GA₃ (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 a density of two seedlings 60-d-old were transplanted from nursery to main-field in three plots replicates with a density of two plants per m². The GA₃ (50 mg dm⁻³) solution with a non-ionic surfactant *APSA-80* (0.5 cm² 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 GA₃ application, 10 uniform untreated (‘U’) and GA₃ treated (‘T’) plants were selected. For biochemical studies (NRO, NtDNMTs activity, and inhibition analysis), leaf samples were harvested from middle-position (generally 10⁻th - 11th 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 to nylon membranes and UV-crosslinked, 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 aRNA 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 aRNA, 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 performed 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 cm² ice-cold diethylether for 3 - 5 min and after washing they were resuspended in 40 cm³ of ice-cold H-buffer [10 mM Tris-HC1 (pH 7.6),...
1.14 M sucrose, 5 mM MgCl₂, 7 mM β-mercaptoethanol; [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 mm³ at 65 °C for 15 min, followed by the addition of 200 mm³ of ice-cold 20× saline-sodium citrate (SSC) buffer. The total content of each sample was transferred onto the charged nylon membrane (Hybond™-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 °C 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 °C 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 °C 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 sulphate (SDS) at room temperature followed by one wash in pre-warmed 0.2× SSC and 0.1 % SDS at 60 °C for 15 min. The semi-dried membrane was exposed to phosphoimage hyperscan 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 phosphoimagerr 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 NiDNMTs genes. Briefly, tobacco seeds were surface-sterilized in 0.05 % HgCl₂ (m/v) for 8 min, followed by 5 times washing with double-distilled water (ddH₂O). Thereafter, seeds were kept in water for around 30 min which facilitated better germination. Subsequently, seeds were germinated at a 16-h photoperiod, 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 GA₃ 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 phosphomager and normalized to constitutively expressed 18S rRNA. Normalized signal intensity of NtMET1, NtDRM1, and NtCMT3 transcripts with respect to their corresponding intensity at time T₀ 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 (t₁/₂) were calculated by fitting non-linear regression according to the following exponential decay formula, t₁/₂ = -0.693/k, where k is the slope of the best fit-line. The t₁/₂ 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 NtDNMTs assay kit (Epigeniek, Farmingdale, NY, USA). NtDNMTs activity [A₄₅₀ mg⁻¹ (protein) min⁻¹] was calculated by equation: A₄₅₀ × 1000/µg protein × t, where A₄₅₀ is absorbance and t is incubation time (min) of reaction assay. For in vitro inhibition, GA₃ (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:

\[
\text{Inhibition} = \left[1 - \frac{A_f/A_0}{A_t/A_0}\right] \times 100
\]

where A₀ and Aₜ 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 [³²P] 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 phenylmethysulfonyl fluoride (PMSF) and 20 mM β-mercaptoethanol (pH 7.4)]. For in vitro inhibition study, GA₃ (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 mm³ 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 mm³ of stop-solution [1 % (m/v) SDS, 3 % (m/v) 4-aminoalicylate, 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⁻² protease K]. To remove proteins, the resulting mixture was vortexed with 300 mm³ 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 mm³ of 0.3 M NaOH and labelled DNA substrate was spotted on Whatman filters, dried, and washed five times with 5 % (v/v) trichloroacetic acid (TCA), followed by a two washing with 70 % ethanol. Filters were placed in scintillation cocktail and NtDNMTs activity measured as ³H 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 [³²P] 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 (Kₘ and Vₘₜₐₓ) 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 GA₃ application in N. tabacum. Firstly, we assessed the differential mRNA accumulation by RNA dot-blotting (Fig. 1A). 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

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due to GA3 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. 1B,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 \textit{NtMET1} and \textit{NtCMT3} due to GA3 application could be caused either by affecting their promoter activity and/or mRNA stability. Therefore, further experiments were performed to elucidate the role of GA3 in mediating their transcriptional and/or post-transcriptional control mechanism(s).

For measurement of transcriptional rate of \textit{NtDNMTs} genes, an optimized NRO assay was performed (Fig. 2A). The GA3 treated plants exhibited a significant reduction in transcriptional rate of \textit{NtMET1} and \textit{NtCMT3}. In corroboration with the result of differential mRNA accumulation, no significant difference in transcription of \textit{NtDRM1} was observed. The specific inhibitory effect of GA3 on \textit{NtMET1} and \textit{NtCMT3} transcription was further evaluated under \textit{in vitro} condition by adding GA3 (50 mg dm$^{-3}$) to the nuclear-extract prior to the NRO analysis. The solvent control of GA3 (in ethanol) established the specific transcriptional inhibition by GA3 (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, \textit{in vitro} GA3 supplementation inhibited the \textit{NtMET1} and \textit{NtCMT3} transcription in both ‘U’ and ‘T’ plants, without affecting the transcription of
NiDRM1 and constitutively expressed 18S rDNA and EF1-α (Fig. 2A). In comparison to in vivo conditions, the decline in transcription of NiMET1 and NiCMT3 was relatively higher under in vitro GA3 supplementation. However, unlike GA3, COR decreased the transcription of NiMET1, NiDRM1, NiCMT3, 18S rDNA, and EF1-α in both ‘U’ and ‘T’ plants, suggesting that GA3 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 NiMET1, NiDRM1, and NiCMT3 genes were immobilized on charged nylon membranes. The NRO analysis of aforementioned transcripts after in vitro addition of GA3 (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-[32P] 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 NiMET1, NiDRM1, and NiCMT3 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 NiMET1 and NiCMT3 after GA3 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 NtEXPA2a (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. 3A). Fig. 3B depicts a typical mRNA decay profile of NiDNMTs genes. NiMET1 and NiCMT3 mRNA could be detected at time T0 and the signal intensity diminished more progressively with time in ‘T’ plants with a corresponding mRNA half-life (t1/2) of 0.85 and 0.95 h, respectively. In contrast, the mRNA turnover of NiMET1 and NiCMT3 occurred much more slowly in the ‘U’, with a corresponding t1/2 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 t1/2 of ~0.5 and ~2.2 - 2.4 h, respectively (Fig. 3B 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 GA3 as a specific transcriptional inhibitor in tobacco. It is pertinent to mention here that despite different modes of GA3 application under experimental field and laboratory conditions (foliar application vs supplementation in MS culture media), no discrepancy in GA3 effect was observed (Manoharlal et al. 2018a).

Further, we tried to verify whether GA3 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 GA3 under in vivo condition was observed (Fig. 4). We further evaluated the specific inhibitory effect of GA3 on NtDNMTs activity under in vitro condition. For this, GA3 (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 GA3 (in ethanol) and GEN (in DMSO) established that in vitro inhibition of NtDNMTs activity by GA3 and GEN was highly specific (Fig. 6 Suppl.). As shown in Fig. 4, in vitro GA3 supplementation inhibited the NtDNMTs activity in a dose dependent manner for both ‘U’ and ‘T’ plants, with a respective IC50 value of ~56 and ~50 mg dm⁻³. Notably, at a sub-saturated concentration of GA3 (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 GA3, the presence of GEN significantly and comparably decreased the NtDNMTs activity in both ‘U’ and ‘T’ (~7.27 vs ~4.92 fold), further corroborating GA3 as relative less potent NtDNMTs inhibitor.

Further, we were interested to determine whether GA3 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 GA3 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 GA3, Vmax was 0.059 and 0.035 Bq μg⁻¹ (protein) min⁻¹ with a corresponding Km of 0.56 and 0.53 μg in ‘U’ and ‘T’ plants, respectively. However, in the
presence of GA3 (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 GA3 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 GA3.

Fig. 5. NtDNMTs activities in nuclear-extract from ‘U’ and ‘T’ plants, along with in vitro addition of GA3 (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 GA3 on chromatin de-condensation and global DNA hypo-methylation in tobacco has been demonstrated recently (Manoharlal et al. 2018a). Furthermore, GA3 mediates transcriptional modulation of 393 genes (Manoharlal et al. 2018b). Albeit, GA3 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 GA3 mediated epigenetic changes in tobacco.

We observed that GA3 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 GA3 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 GA3, which is consistent with its ubiquitous transcription in tobacco (Wada et al. 2003). Since NtDRM1 is involved in RNA-directed methylation, smRNA pathways controlling the transposons expression is not influenced by GA3. 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, GA3 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 GA3 treatment. In our experiments, a marked decrease in NtDNMTs activity was observed at 13 d after GA3 application. It is likely that tobacco epigenetic re-programming in response to GA3 requires some time and multiple cell divisions.

Numerous reports demonstrate the role of GA3 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 GA3 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 GA3 application caused reduced mRNA half-lives of NtMET1 and NtCMT3 (Fig. 3). Notably, GA3 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 GA3 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).

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 GA3, conversely GA3 stimulates the DNMTs activity in wheat embryo nuclear-extract (Vlasova et al. 1995). In our study, GA3 mediated significant decline in NtDNMTs activity was observed (Fig. 4). Lineweaver-Burk plot analysis demonstrated GA3 as a non-competitive inhibitor of NtDNMTs activity (Fig. 5). Previous studies have also reported the role of GA3 as non-competitive inhibitor of herbicidas barban [4-chloro-2-butynyl N-(3-chlorophenyl) carbamate], phenylurethanes, as well as sodium butyrate (Yung and Mann 1967, Kumar et al. 1985). Similarly, GA3 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 GA3 also cannot be ruled out. Indeed, our earlier observations also demonstrated the altered activities of histone deacetylases (NtHDACs) and histone acetyltransferases (NtHATs) by GA3 in N. tabacum (Manoharlal et al. 2018b). Recently, GA3 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 GA3-induced global DNA hypomethylation 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 GA3 as ‘green’ natural alternative to otherwise toxic and synthetic DNMTs inhibitors. Future studies on GA3 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**


