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Existence of differential epigenetic control in a pair of cultivated tobacco (*Nicotiana tabacum* L.) cultivars harbouring contrast nicotine levels

Raman Manoharlal* and GVS Saiprasad

ITC Limited, ITC Life Sciences and Technology Centre (LSTC), Bengaluru-560 058, Karnataka, India

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Nicotine (NIC) is a primary and characteristic alkaloid in tobacco (*Nicotiana* sp.). To date, there have been no comprehensive studies conducted to assess the prevalence of epigenetic phenomena related to tobacco NIC levels. Therefore, our present research seeks to investigate the potential prevalence of two significant epigenetic markers, specifically chromatin organization and genomic DNA (gDNA) methylation, in relation to physiological levels of NIC in tobacco. Our agronomical-field studies on the cultivated tobacco (*N. tabacum* L.) germplasm collection screening revealed a contrast pair of tobacco cv., GP150 and GP98,that exhibit consistent differences in their inherent NIC levels. GP150 harbors inherently high-NIC levels in its green- [~1.27% d.w. (pre-topping) and 2.87% d.w. (post-topping)] and cured-leaves (~3.01% d.w.). On the contrary, GP98 harbors inherently low-NIC levels in its green- [~0.04% d.w. (pre-topping) and 0.07% d.w. (post-topping)] and cured-leaves (~0.09% d.w.). A comparative evaluation of epigenetics phenomenon revealed that GP150 and GP98 differ significantly in terms of: (1) chromatin organization, (2) gDNA methylation status, (3) gDNA 5-methylcytosine (^{5m}C) levels and (4) gDNA methyltransferases activity. An induced physiological alteration in gDNA methylation corroborated with corresponding alterations in NIC level further substantiates an intriguing correlation between aforementioned epigenetics phenomenon(s) and NIC biosynthesis in tobacco. These results reinforce the need of future in-depth epigenetics studies on NIC biosynthesis and/ signalling pathways in tobacco.

Keywords: Chromatin organization and DNA methylation, Nicotine

In world's agriculture, cultivated tobacco (*N. tabacum* L.) is an annual herbaceous cash-crop with over 4 million hectares of cultivated land across 125 countries that corresponds to an annual production of 32.4 million tonnes (MT) and 6.48 MT of green- and cured-leaves, respectively[1]. Globally, the conventional uses of different tobacco formats mainly revolve around its signature molecule, NIC. In tobacco, NIC is the primary alkaloid accounting for 2-5% of cured leaf dry-weight (d.w.) or 90-95% of total alkaloids, whereas the remaining 5-10% of major alkaloids is attributed by anatabine, nornicotine and anabasine (herein mentioned in decreasing order of relative abundance)[2]. NIC biosynthesis takes place in tobacco roots, translocated to aerial-parts *via* xylem and accumulates in the tonoplast of mesophyll leaf-cells[3]. Thanks to the public available tobacco genome database and recent molecular and genomic studies, almost complete inventory of structural- and regulatory-genes of NIC biosynthetic pathway along with their expression, phylogenetic and other functional-properties has been elucidated[4]. It has been shown that NIC content is genetically controlled by two distinct, independent and synergistic acting loci, *NICOTINE 1* and *2* (*NIC1* and *NIC2*), originally termed as A and B, respectively[4,5]. The *NIC2* locus has been characterised by the presence of gene cluster that comprises a group of at least 7 transcription factors (TFs) from IXa ethylene response factor (ERF) subfamily[5]. Although complete structural- and functional-characterization of *NIC1* locus has not been fully elucidated yet, its semi-dominant impact on NIC content has been reported to be stronger (~2.4 folds) than *NIC2* loci[6].

NIC (a heterocyclic pyridine-pyrrolidine alkaloid) is formed enzymatically by condensation of nicotinic acid (pyridine ring) and N-methyl-Δ^1-pyrrolinium cation (pyrrolidine ring) *via* a series of structural-genes (that constitutes a regulon)[7]. Transcriptional control of NIC biosynthesis is inferred by frequency of conserved cognate *cis*-regulatory elements (P- and G-boxes) in the promoter of NIC regulon, controlled by respective orthologous pair of master TFs *viz. ERF189* and *ERF199* (clustered at *NIC2* loci) in tandem with basic helix-loop-helix (bHLH) family TFs *viz. MYC2a* and *MYC2b*[7]. These studies impart a deep understanding of genetic mechanisms underlying the

*Correspondence:
E-mail: ramanpdf02@gmail.com

NIC biosynthesis, wherein aforementioned NIC regulon, TFs and feedback regulation of different cross-talking metabolic modules constitutes the genetic determinants of NIC level[7]. Nevertheless, apart from genetics factors, *Nicotiana* sp. itself has garnered the attention of several plant-epigenetics investigations pertaining to various metabolites / metabolic pathways[8,9]. However, until now, despite a few circumstantial evidences, none of the previous studies provide substantial evidence of epigenetics phenomenon in particular context of NIC biosynthesis in tobacco. Biosynthesis and accumulation of tobacco alkaloids (NIC in particular) has been reported to be dynamically regulated[10]. For instance, NIC concentration in different tobacco organs (except seed) has been shown to be influenced by diverse external-factors *viz.* weather, high-temperature, flooding, salt-stress, land-type, agricultural-practices (*e.g*. nitrogen fertilization, planting density, topping, suckering and harvesting method *etc*.), plant growth regulators (PGRs), mechanical-wounding and pest-infestation *etc*., to name a few[11-16]. Our recent study involving two different weather factors and continental locations has also shown the influence of complex genotype-environmental interaction (G×E) on varying NIC level in tobacco[17]. Indeed, NIC as an inducible functional-readout exemplifies the adaptive phenotypic plasticity in a number of *Nicotiana* sp.[18]. In separate studies, recent emerging evidences indicates that environmental cues, PGRs and epigenetic mechanisms integrate across various signalling pathways to regulate various plant traits and plasticity, wherein global and gene-specific transcriptional response has been reported to predominantly governed by two key epigenetic marks *viz.* chromatin organization and DNA methylation status[7,19]. All these background studies prompted us to investigate the potential existence of underlying epigenetic mechanism(s) associated with NIC physiological levels in model tobacco, *N. tabacum* L.

In this study, we probe these questions by comparatively measuring the chromatin organization and gDNA methylation-associated epigenetic changes in contrast tobacco cv. GP150 and GP98, harbouring inherently high- and low-NIC respectively. To the best of our knowledge, this is the first comprehensive report that evaluates the existence and correlation of epigenetics phenomenon in context of physiological NIC levels in tobacco.

Materials and Methods

Plant material and growth conditions

Our *In house* tobacco seed-bank harboring 580 germplasms (procured from US National Plant Germplasm System, NPGS) were screened for various qualitative- and quantitative-traits. Based on two different seasonal-data (2011-12 and 2012-13) conducted at Northern Light Soil (NLS) region at ITC-ABD research station, Rajahmundry (Latitude: 17.004874° N, Longitude: 81.8040° E), a pair of Flue-cured Virginia (FCV) tobacco cultivars, namely GP150 and GP98 that differed consistently in terms of morphometric-characterization, cured leaf-yield, flavor, metabolite-profiling (*data not shown*) and NIC content (*present study*) were selected. For this study, field-trials were conducted for GP150 and GP98 cv. at NLS region (2017-18), as per common agricultural practices implemented in that region. After 2 months of growth in nursery-bed, seedlings from GP150 and GP98 were transplanted to main-field in three plot replicates (n=3) with each replicate plot harboring 200 plants. Approximately 20 uniform, homogenous and healthy plants from each plot were selected and tagged. For molecular- and biochemical-analysis, disease-free, green leaves before-topping (45 d after transplantation (DAT)] and after-topping (55-60 DAT) from the middle-position (10^{th}-11^{th} leaf from bottom of a plant) of representative tagged plants (~5-6 in numbers) were harvested, immediately snap-frozen in liquid N_2, grounded to a fine-powder and stored at -80°C till further use. Three technical replicates for each biological sample were used for analysis. Remaining plants from each plot were further allowed to grow until they attained full maturity (~65-70 DAT) and subsequently subjected to pick-wise leaf harvesting and flue-curing as per industrially standardized protocol followed by Central Tobacco Research Institute (CTRI), Rajahmundry, A.P., India (*https://ctri.icar.gov.in/for_curing.php*).

Estimation of NIC levels

Middle-positioned green- (before-topping, BT and after-topping, AT) and cured-leaves from GP150 and GP98 cv. were used for estimation of NIC. Approximately 250 mg dry weight (d.w.) leaves were used for sample preparation and analysis using continuous flow auto analyzer III by following the classical CORESTA recommended method no. 35 (CRM35)/ISO 15152[20]. This method is based on the measurement of the color-complex at 460 nm

produced by the reaction of NIC with *in-situ* generated cyanogen chloride. Notably, results expressed in terms of major alkaloid, NIC (in % d.w.) also represents the presence of other minor alkaloids (*viz.* anabasine, anatabine and nornicotine).

Tobacco-specific N-nitrosamines (TSNAs) estimation

A quantitative estimation method for the extraction of individual TSNA namely. *N'*-nitrosoanabasine (NAB), *N'*-nitrosoanatabine (NAT), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N'*-nitrosonornicotine (NNN) was performed by liquid chromatography tandem mass spectrometry (LC-MS/MS) as outlined earlier[21,22], with certain modifications. *Sample preparation.* Approximately 1.0 g of fine-grounded cured-leaves from GP150 and GP98 were extracted with 50 mL of 100% methanol by shaking at 200 rpm for 1 h in an orbital shaker at room-temperature (RT, ~ 25-27°C). The extracted solution was subjected to centrifugation at 4600 rpm for 5 min to remove the debris. The resulting supernatant was filtered with 0.45 µm PVDF syringe-filter and subjected to analytical estimation by LC-MS/MS. *LC-MS/MS conditions and instrumentation.* TSNA level was measured by LC-MS/MS using an API 6460 triple quadrupole MS/MS (Applied Biosystems) operated in positive electrospray ionization (ESI+) mode. The deuterated internal standards: 5 ng/mL d_4-NAB and 10 ng/mL each for d_4-NAT, d_4-NNN and d_4-NNK were added to aforementioned methanol-extracted supernatants. The 5 µL of extractant was automatic injected onto a LC system (Agilent Technologies, model series 1290), equipped with a quaternary pump, auto-sampler, vacuum-degasser and a stationary-phase column (C18 column; length: 150 mm, diameter: 4.6 mm and thickness: 0.35 µm). The column temperature was maintained at 40°C. Pre-equilibration of analytical column was carried out following the sample-extraction. Two mobile phases were used to elute the analyte: solvent A (20% methanol and 0.31% formic acid in deionized water with pH 4.0, adjusted with ammonia) and solvent B (100% methanol). The mobile-phase was run in a binary-gradient manner (mobile phase A: 20% and mobile phase B: 80%) with a defined flow-rate of 0.5 mL/min and total run-time of 10 min. The protonated parent / daughter-ion pair *m/z* 192-162, *m/z* 190-160, *m/z* 208-122 and *m/z* 178-148 transition was monitored for NAB, NAT, NNK and NNN, respectively. Machine detection limits (10 µg/L) was calculated for each of the analysed TSNAs. Results were expressed in mg/L on d.w. basis (dwb). All the reagents used in the analysis were of analytical grade (*Sigma-Aldrich*).

Chromatin accessibility assay

Chromatin accessibility was performed by measuring the micrococcal nuclease (MNase I) digestion of chromatin as described earlier[9]. Purified chromatin was extracted from the green-leaves of GP150 and GP98 using *EpiQuik ChromaFlash*™ extraction kit (*Epigentek Inc*) as per manufacturer's instructions. ~2 µg of isolated chromatin per reaction from each sample was resuspended in 50 µL of reaction-mixture harbouring 1×MNase I buffer (50 mM Tris-HCl, pH 8.0 and 5 mM $CaCl_2$). An optimised concentration (0.5 gel units/reaction) of MNase I (*New England Biolabs*) was used for chromatin digestion for an indicated time-period of 0, 5 and 10 min. Reaction was stopped by adding an equivalent volume of stop-buffer (100 mM EDTA and 10 mM EGTA mix, pH 7.5), followed by overnight digestion of reaction-mix with proteinase K at 37°C. The nucleosomal-DNA was extracted by phenol: chloroform: isoamyl alcohol (25:24:1, *v/v/v*), followed by precipitated with 1 mL of absolute ethanol and subsequent centrifugation at 12,000 g. The dried DNA pellet was resuspended in 30 µL of $T_{10}E_1$ (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), followed by treatment with 10 mg/mL RNase A at RT for 25 min. The resulting MNase I digested products were resolved on 2% (*w/v*) agarose gels and visualised after staining with ethidium-bromide. The signal intensity of undigested chromatin DNA and nucleosome-ladders (MNase I digested chromatin DNA) from each tested sample was measured by densitometry-scanning (*ImageQuant* software, *GE Healthcare Life Sciences*) and expressed as %.

gDNA methylation analysis

gDNA was extracted from green-leaves of GP150 and GP98 using DNeasy® plant mini kit (*Qiagen Sciences Inc.*) as described earlier[9]. The purified gDNA concentration was estimated by NanoDrop 8000 (*Thermo Fischer Scientific*) for each sample. The gDNA methylation level of each sample (~200 ng) was evaluated using ELISA based Imprint® methylated DNA quantification kit (*Sigma-Aldrich*) as per manufacturer's instructions. The methylated gDNA recognized by antibody specific to ^{5m}C, which is directly proportional to the absorbance at 450 nm (A_{450}), was quantified calorimetrically and expressed in terms of A_{450}.

gDNA ^{5m}C measurement

gDNA ^{5m}C level was evaluated from green-leaves of GP150 and GP98 by dot-blot DNA hybridization using α-^{5m}C antibody as described previously[9]. Approximately 10 ng of alkali-denatured purified gDNA from each sample was blotted onto positive-charged nylon membrane (*GE Healthcare Life Sciences*) using Bio-Dot Microfiltration apparatus (*Bio-Rad Laboratories, Inc*.). An empty plasmid DNA vector, pUC19 (negative-control) was also dot-blotted along with in order to evaluate the non-specific binding of random non-methylated DNA fragment to α-^{5m}C. Equal dot-blotting of DNA on membrane was validated by staining the membrane with 0.02% (*v/v*) methylene blue in 0.3 M sodium acetate (pH 5.2) in a parallel set of experiment. The DNA blotted-membrane was UV-crosslinked (120 mJ/cm^2 on both sides of the membrane), followed by blocking in tris-buffered saline (TBS) with 0.1% tween-20 (TBS-T) containing 5% (*w/v*) non-fat dry milk. Subsequently, the membrane was incubated overnight with specific mouse monoclonal α-^{5m}C antibody (Epigentek, *Inc*.) at a dilution of 1:200 (*v/v*) at 4°C, followed by incubation with 1:5000 (*v/v*) horseradish peroxidase-conjugated sheep α-mouse IgG secondary antibody (*GE Healthcare Life Sciences*) at RT for 3 h. The membrane was exposed to *ECL* detection reagents and hybridization-signals were detected using *Molecular Imager* chemiluminescence detection system (*Bio-Rad*). The hybridized signal-intensity from each dot-blotted DNA was measured by densitometry-scanning (*ImageQuant* software), background corrected from corresponding negative-control and expressed as ratio relative to that of GP150.

DNA methyltransferases (NtDNMTs) activity measurement

Crude nuclear-extract prior (CNE) was prepared from green-leaves of GP150 and GP98 using CelLytic^{TM} PN plant nuclei isolation / extraction kit (*Sigma-Aldrich*) as described previously[9]. Briefly, 5 µg of verified CNE was used for NtDNMTs activity measurement using EpiQuik DNMT activity /inhibition assay ultra kit (*Epigentek*) as per manufacturer's instructions. NtDNMTs activity [A_{450}/mg (protein)/min] was calculated by following the standard equation: $A_{450} \times 1000 \times$ protein (µg) / t (min), where A_{450} is net absorbance at 450 nm and t is incubation time (min) of reaction. For *in vitro* inhibition of NtDNMTs activity, 10 µM genistein (GEN, *Sigma-Aldrich*) was added exogenously in CNE of each sample. Stock solution of GEN (200 µM) in dimethyl sulfoxide (DMSO) was prepared freshly before use. NtDNMTs activity activation (%) was measured by equation: $[(A_{GP98}/A_{GP150}\text{-}1)] \times 100$, wherein A_{GP150} and A_{GP98} corresponds to absorbance at 450 nm for GP150 and GP98, respectively.

Induced alterations in epigenetics marks and corresponding measurement of physiological NIC levels

K326 *a.k.a.* Kanchan, a leading FCV tobacco cv. grown globally, was used to evaluate the effect of altered chromatin organization and gDNA methylation associated epigenetic changes on phenotypes and physiological level of NIC. *Phenotypic evaluation*. Surface-sterilized K326 seeds (0.05% $HgCl_2$ for 7 min, followed by extensive washing with doubled-distilled water) were kept in water for around 30 min to facilitate the better germination. Approximately 10-15 these seeds were seeded on Murashige and Skoog (MS) agar [0.46% MS salts, 3% sucrose; 1% (*w/v*) agar, pH 5.8] and incubated for 1 week in dark at 28°C. The resultant plantlets were further grown either on MS media alone and/ supplemented with 1 mM S-adenosyl methionine (SAM, methyl donor to DNMTs[23], *New England Biolabs Ltd.*) or 50 µM GEN (a DNA de-methylating agent) for another 3-4 weeks at 28°C under NIC-inducing culture condition [*viz.* 16 h photoperiod (10 µmol photons/m^2/s)] as described earlier[24]. Stock solutions of 32 mM SAM (in buffer containing 0.005 M sulphuric acid and 10% ethanol) and 200 µM GEN (in DMSO) was prepared freshly before use. As a solvent-control, an equivalent volume of SAM buffer and DMSO were also added to MS media. Media (with and without supplements) were refreshed over a period of one week. Each treatment was done in triplicate (n=3). Visual difference in plantlets growth and developmental phenotypes in the absence and presence of SAM and GEN supplementation along with their corresponding solvent-controls were recorded. *Molecular- and biochemical-characterisation*. Approximately four weeks old untreated ('U'), SAM and GEN supplemented bulked young leaves (both supplementations hereafter collectively referred as 'T') were used for chromatin accessibility, gDNA methylation, ^{5m}C levels, NtDNMTs activity and physiological NIC estimation (*as described in preceding section*). NtDNMTs activity activation and/ inhibition (%) was measured by equations: $[(A_T/A_U\text{-}1)] \times 100$ and/ $[(1\text{-}A_T/A_U)] \times 100$ respectively, wherein A_U and A_T corresponds to absorbance at 450 nm for 'U' and 'T', respectively.

Data analysis

Each experiment was evaluated in biological triplicates and repeated at least twice. Results were expressed as means ± standard deviations (SDs). One-way analysis of variance (ANOVA) was performed to evaluate the statistically significant difference ($P \leq 0.05$) between comparative groups.

Results

Biochemical characterization of GP150 and GP98

To analyze the association of epigenetic makers and physiological NIC levels (*if any*) in tobacco, we employed a pair of contrast FCV cv. GP150 and GP98 differs significantly in terms of morphometric-characterization, cured leaf-yield, flavor and metabolite-profiling (*data not shown*). Firstly, we compared the physiological NIC levels from the middle-positioned green-leaves of GP150 and GP98 before topping (BT). We observed that GP150 displayed a NIC content of 1.27% (d.w.). On contrary, NIC level was not detectable in GP98 (below the limit of quantitation, BLQ < 0.04%) (Fig. 1A, *left bars*). Notably, topping (i.e. decapitation of the apical meristem harboring terminal panicle inflorescence) practice in FCV tobacco is associated with significant higher accumulation of NIC in leaves[25]. Henceforth, we were tempted to instigate the NIC level from the similar middle-positioned green-leaves of GP150 and GP98 after topping (AT). As expected, topping mediated relative fold change (RFC) increment in NIC level was evident in both GP150 (RFC = 2.27; AT *v/s* BT) and GP98 (RFC = 0.07; AT *v/s* BT) (Fig. 1A, *middle bars*). Comparative analysis within AT demonstrated a significant observed difference in NIC level of GP150 and GP98 (2.87% *v/s* 0.07%; Fig. 1A, *middle bars*). In order to further validate the NIC associated biochemical demarcation between GP150 and GP98, we specifically quantified the NIC as well as descendants of NIC (and related alkaloids) in the form of individual TSNAs *viz.* NNN, NAT, NAB and NNK from the similar middle-positioned cured leaves of GP150 and GP98. Our results demonstrated that cured-leaves of GP150 and GP98 possesses NIC content of ~3.01% and 0.09%, respectively (Fig. 1A, *right bars*). For GP150, the NNN, NAT, NAB and NNK level were quantified at a detectable level of 1.46, 2.59, 0.19 and 0.42 mg/L, respectively (Fig. 1B). In contrast, none of the individual TSNA was detected (BLQ <0.001 mg/L) in GP98 (Fig. 1B). All these results established the consistent differentiated physiological levels of NIC (as well as its corresponding derived TSNAs) in GP150 and GP98.

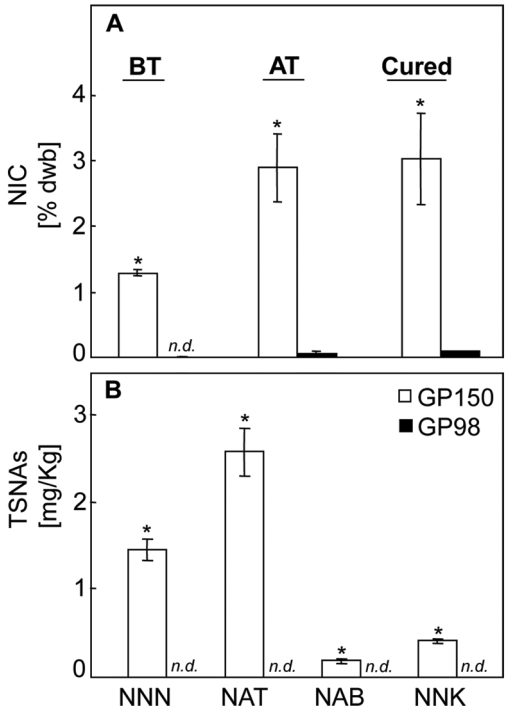

Fig. 1 — Quantitative estimation of NIC and TSNAs levels. (A) NIC and (B) TSNA estimation. NIC (mainly as total alkaolids) was determined from green- (BT and AT) as well as cured-leaves of GP150 and GP98. Notably, NIC (BLQ < 0.04%) in BT green-leaves and any of the tested individual TSNA (BLQ < 0.001 mg/L) in cured-leaves of GP98 was not detected (*n.d.*). Means ± SDs, *n*=3, wherein a*sterisks* indicate the significant difference ($P \leq 0.05$) between GP150 and GP98. NIC - nicotine, dwb - dry weight basis, TSNAs - Tobacco specific N-nitrosamines, NNN - N-Nitrosonornicotine, NAT - N'-nitrosoanatabine, NAB - N'-Nitrosoanabasine, NNK - 4-(metylnitrosamino)-1-(3-pyridyl)-1-butanon, BT - Before topping, AT - After topping and BLQ - Below the limit of quantitation

Differential chromatin organization of GP150 and GP98

Chromatin-remodeling, a dynamic spatiotemporal conversion of chromatin organization from a condensed (closed / restrictive / heterochromatin) to de-condensed (open / permissive / euchromatin) state and *vice-versa*, is a prominent epigenetic-mark linked with diverse array of metabolic processes[26]. To explore the possibility of differential chromatin organization in GP150 and GP98, a preliminary time-scale digestion analysis (0, 2, 5 and 10 min) using an optimised concentration (0.5 gel units/reaction) of MNase I was performed to identify the extent of chromatin DNA digestion required (Suppl. Fig. 1 and Suppl. Table 1). Subsequently, MNase I accessibility of leaf-chromatin from GP150 and GP98 over a time-

period of 0, 5 and 10 min was performed. Our results demonstrated an identical nucleosomal periodicity in MNase I digested chromatins for both GP150 and GP98 (Fig. 2: *lanes* 2-3 *v/s* 5-6), characterised by nucleosome-ladder ('*n2-n5'*) with a repeat mono-nucleosome length of ~170 bp. Interestingly, GP98 chromatin displayed a relative reduced MNase I sensitivity at 5 and 10 min, wherein a visible appearance of nucleosomal-ladder ('*n2-n5*') was less evident than GP150 (Fig. 2: *lane* 2 *v/s* 5; *lane* 3 *v/s* 6 and Suppl. Table 2). These results advocated that GP98 chromatin inherently exists in relatively condensed form in contrast to its GP150 counterpart.

Differential gDNA methylation and ^{5m}C levels in GP150 and GP98

One of the most studied epigenetic mark is DNA methylation, characterised by existence of methyl group to the 5-carbon of cytosine residue to form ^{5m}C, which generally works in tandem with chromatin-remodeling in modulating the chromatin accessibility to TFs and transcription-machinery[26]. Thereby, we were intrigued to determine the potential difference in gDNA methylation level of GP150 and GP98. Comparative quantitative analysis revealed a significant increment (RFC = 2.13) in gDNA methylation level of GP98 over GP150 (Fig. 3A). Further, in order to evaluate the specific difference in gDNA ^{5m}C level, purified gDNA from GP150 and GP98 were also subjected to dot-blot analysis (Fig. 3B). The specificity of α-^{5m}C antibody towards

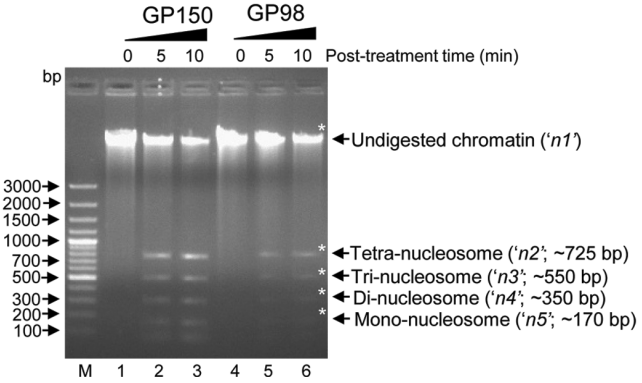

Fig. 2 — Chromatin accessibility measurement. Chromatin DNA extracted from GP150 and GP98 was subjected to MNase I digestion for an indicated time (0, 5 and 10 min), purified and subsequently resolved on 2% (*w/v*) agarose gel. *Lanes* 1-3 and 4-6 showed the MNase I digested chromatin for a time-period of 0, 5 and 10 min from GP150 and GP98, respectively. The signal-intensity of undigested ('*n1'*) and MNase I digested nucleosome ladders ('*n2-n5'*) from GP150 and GP98 was measured by densitometry-scanning, background-corrected and expressed as % (Table S2 Suppl.). M - 100 bp DNA size marker

^{5m}C oligonucleotide was also validated (*data not shown*). Notably, the observed non-specific binding of α-^{5m}C to random non-methylated plasmid DNA, pUC19 was normalized (background-correction) for each sample. We observed that GP98 also possess significantly enhanced gDNA ^{5m}C levels (Fig. 3B). Quantitative analysis of densitometry-scanning of hybridised DNA dot-blot demonstrated a significant increase (RFC = 2.5) in gDNA ^{5m}C level of GP98 in contrast to GP150 (Fig. 3B). These results further corroborated the relative gDNA hyper-methylation in GP98 in comparison to GP150.

Differential NtDNMTs activity in GP150 and GP98

Aberrant chromatin organization and gDNA methylation pattern is a typical feature of altered epigenetic machinery, that is characterised by a corresponding change in DNMTs activity and/ expression[9]. Henceforth to explore this possibility, we measured the NtDNMTs activity from the purified (CNE) of GP150 and GP98. CNE quality of GP150 and GP98 was verified by immune-blotting using α-histone H4 specific antibody (Suppl. Fig. 2). Our results showed that NtDNMTs activity was significantly enhanced (RFC = 1.78) in GP98 than its GP150 counterpart (Fig. 4). These results further substantiate the aforementioned gDNA and gDNA ^{5m}C hyper-methylation levels of GP98 (Fig. 3A & B). As a control, CNE of GP150 and GP98 were also pre-conditioned for 5 min with 10 µM GEN (a well-known DNMT inhibitor) prior to measurement of NtDNMTs activity. We observed that in contrast to

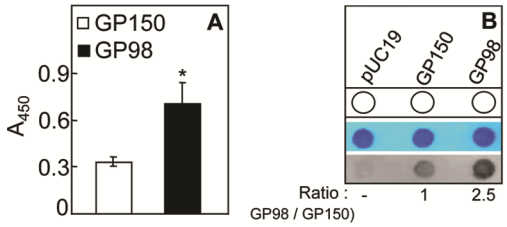

Fig. 3 — gDNA methylation and ^{5m}C levels. (A) gDNA methylation level of GP150 and GP98 was determined calorimetrically by measuring the absorbance at 450 nm (A_{450}). Means ± SDs, *n*=3, wherein *asterisk* indicate the significant difference ($P \leq 0.05$) between GP150 and GP98. (B) Approximately 10 ng of purified gDNA from GP150 and GP98 along with plasmid pUC19 (negative-control) were blotted and immobilized on nylon-membrane. The position of individual dot-blot is depicted by *upper-panel*, loading control (methylene-blue staining) by *middle-panel* and recognition of gDNA by α-^{5m}C antibody by *bottom-panel*. Hybridized signal-intensity of each dot-blotted DNA was quantified by densitometry-scanning and base-corrected. The GP150 or GP98 / GP150 ratio underneath the blot indicates the normalized signal-intensity relative to GP150

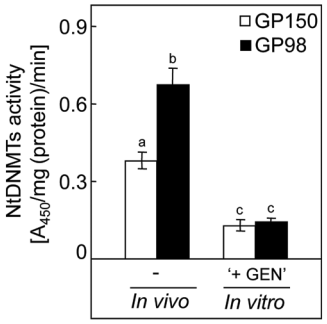

Fig. 4 — NtDNMTs activity measurement. NtDNMTs activity from the CNE of GP150 and GP98 was calorimetrically by measuring the absorbance at 450 nm (A_{450}) and expressed as per mg per min [A_{450}/mg (protein)/min]. *In vitro* effect of exogenous addition of reference DNMT inhibitor, GEN (10 µM) on NtDNMTs activity was also measured. Means ± SDs, n=3. Data marked with different letters are significantly ($P \leq 0.05$) different. GEN - Genistein

their corresponding untreated counterpart, *in vitro* supplementation of GEN resulted in a significant inhibition of NtDNMTs activity in both GP150 (RFC = -3.0) and GP98 (RFC = -4.8) (Fig. 4). *In vitro* supplementation of GEN solvent ('+ DMSO') in CNE of GP150 and GP98 further validated the GEN specific impact on NtDNMTs activity (Suppl. Fig. 3).

NIC as a functional-readout of physiological alteration in epigenetics marks

Next, we sought to determine whether the induced alterations in aforementioned epigenetics marks could be linked with corresponding physiological NIC levels or not. Henceforth, we conducted a laboratory investigation to evaluate the phenotypic, molecular and biochemical effects of exogenous application of an epigenetic modifier, SAM (cofactor for trans-methylation reactions[23]) in tobacco growing culture media. Seeds of tobacco cv. K326 were surface-sterilized and grown on MS media for 1 week in dark at 28°C, following which the resultant plantlets were transferred to MS media supplemented with 1 mM SAM and grown under NIC-inducing culture condition[24]. As a control, tobacco plantlets were grown on either MS media alone or supplemented with 50 µM GEN. Notably, GEN exposure has been reported to induce the epigenetically silenced T-DNA loci and/ other endogenous genes[27]. We observed that four weeks old plantlets grown on MS media supplemented with SAM (referred as '+ SAM') showed relative slow growth and semi-dwarf phenotype in contrast to corresponding untreated ('U') counterpart (Fig. 5A). Conversely, plantlets on MS media supplemented with GEN (referred as '+ GEN') grew relatively faster than 'U' plantlets (Fig. 5A). Of note, MS media supplementation with solvent of SAM (referred as '+ SAM buffer') and GEN (referred as '+ DMSO') did not have any impact on the growth, viability as well as NIC content (Suppl. Fig. 4A & B). Molecular- and biochemical-characterization of 'U', '+ SAM' and '+ GEN' supplemented (collectively referred as 'T') plantlets were evaluated by measuring the chromatin accessibility, gDNA methylation, ^{5m}C levels, NtDNMTs activity and NIC level. Prior to NtDNMTs activity measurement, CNE quality of 'U', '+ SAM' and '+ GEN' was also verified by immune-blotting using α-histone H4 specific antibody (Suppl. Fig. 5). Our results demonstrated that relative to 'U', '+ SAM' plantlets displayed relatively reduced MNase I accessibility (Fig. 5B and Suppl. Table 3) in concomitant with an enhanced gDNA methylation (RFC = 2.2; Fig. 5C), ^{5m}C levels (RFC = 3.1; Fig. 5D), NtDNMTs activity (RFC = 2.0; Fig. 5E) and reduced NIC level (RFC = -1.6; Fig. 5F). Conversely, '+ GEN' plantlets displayed a relatively enhanced MNase I accessibility (Fig. 5B and Table S3 Suppl.) concomitant with a decline in gDNA methylation (RFC = -2.01; Fig. 5C), ^{5m}C levels (RFC = -1.96; Fig. 5D), NtDNMTs activity (RFC = -2.07; Fig. 5E) and enhanced NIC levels (RFC = 1.36; Fig. 5F) relative to 'U'.

Discussion

Albeit the genetic basis of NIC biosynthesis in tobacco has been elucidated to a great extent, possible epigenetics involvement has been largely overlooked so far. Recent research studies on fungi and to certain extent in plant demonstrated an emerging relationship between secondary metabolites/ metabolism and epigenetics[28,29]. Notably, *N. tabacum* L., being a natural and classic allotetraploid, exhibits the wide range of ecological adaptation and adaptive speciation as a result of many years of extensive artificial cross-breeding mainly through epigenetic regulation[30]. Additionally, there are also certain clues supporting the positive correlation between ploidy status, epigenetics (gDNA methylation in particular) and secondary metabolites accumulation[31,32]. Drawing inspiration from the findings of these studies, our current research aims to explore the prevalence (*if any*) of two critical epigenetic markers (*viz.* chromatin organization and genomic DNA methylation) in context of varying NIC accumulation in two contrasting FCV tobacco

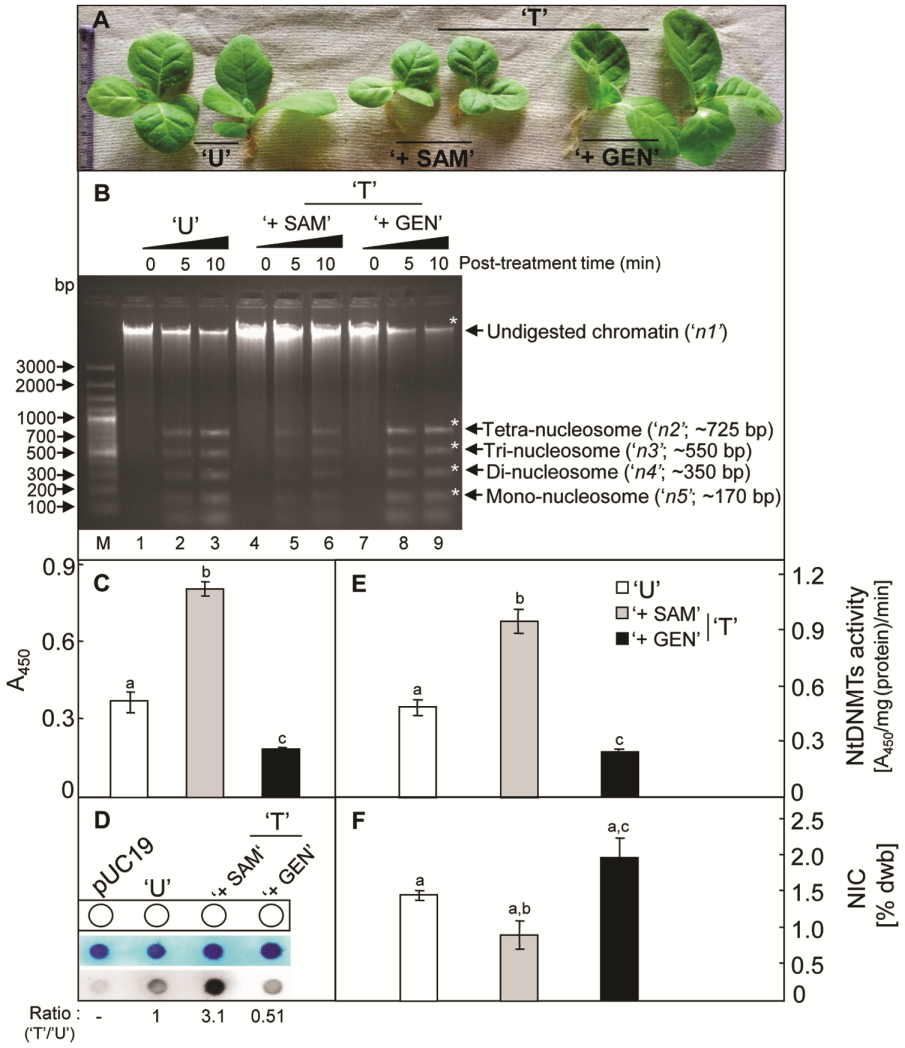

Fig. 5 — *In planta* chromatin organization and gDNA methylation alteration and physiological NIC levels. (A) Phenotypic evaluation of tobacco plantlets grown on MS media under NIC-inducing culture condition were supplemented with either 1 mM SAM ('+ SAM'), a universal methyl donor to DNMTs or 50 µM GEN ('+ GEN'), a universal DNA de-methylating agent / DNMT inhibitor (both supplementation collectively referred as 'T'). Untreated plantlets ('U') were grown on MS media without any supplements. Representative image of plantlets for each treatment is shown as: 'U', '+ SAM' and '+ GEN'. Molecular- and biochemical-assays; (B) chromatin accessibility; (C) gDNA methylation levels; (D) gDNA ^{5m}C levels; (E) NtDNMTs activity; and (F) physiological NIC levels, were evaluated from bulked 'U' and 'T' plantlets leaves after 4 weeks of growth on respective MS media. Means ± SDs, n=3. Data marked with different letters are significantly ($P \leq 0.05$) different. SAM - S-adenosyl methionine, GEN - genistein, 'U' - Untreated and 'T' - Treated K326 tobacco cv, wherein '+ SAM' and '+ GEN' corresponds to plantlets grown MS media supplemented with SAM and GEN, respectively. NIC - nicotine and dwb - dry weight basis

cv. GP150 and GP98. These cultivars are notably distinct in their morphometric-characteristics, cured leaf yield, flavor and metabolite-profiles (*data not shown*).

Our results showed that NIC level differs significantly in BT green-leaves of GP150 and GP98 even, indicating the prevalence of inherent (epi)genetic factors that could account for differential NIC level in GP150 and GP98 (Fig. 1A, *left bars*). In order to improve the leaf-quality, an important culture-practice followed for FCV tobacco is topping *a.k.a.* decapitation i.e. flowering-head and young-leaves removal upon emergence of first flower of inflorescence. '*Topping*' is marked by alteration in number of metabolic- and physiological-processes (*viz.* hormonal-imbalance, root-development, stress-tolerance/defense, source-sink relationship, protein-, carbon- and other-metabolisms)[33]. A global transcriptomics and metabolomics analysis in tobacco in particular response to topping revealed the involvement

of secondary-metabolites and secondary-metabolism related genes, wherein a prominent enhanced NIC biosynthesis and accumulation has been ascribed as most typical feature[33,34]. In corroboration with these studies, we also observed a significant amplification of NIC level in AT leaves of GP150 and GP98 (Fig. 1A, *right bars*). Like topping, one of most essential agronomical-practice followed in tobacco cultivation is post-harvest leaf-curing *a.k.a.* color-curing (a natural / artificial drying-process), wherein most of the moisture and chlorophyll content of harvested leaves are removed to produce dry, yellow and fragrant leaves with certain well-defined and desirable quality parameters[35]. During tobacco leaf-curing, NIC and other minor alkaloids undergoes spontaneous nitrosation leading to the formation of undesirable TSNAs, thus demonstrating a positive correlation between them[35]. In corroboration with the differential NIC level in green-leaves, we observed that NIC (and related alkaloids) derived TSNAs (*viz.* NNN, NAT, NAB and NNK) were also significantly higher in cured-leaves of GP150 than GP98 (Fig. 1B). Taken together, all these results establishes the existence of differential NIC level in leaves of GP150 and GP98 that remains consistent throughout their life-cycle.

To evaluate the probable correlation between epigenetics and observed differential NIC levels in GP150 and GP98, subsequently we investigated the two universal epigenetic mechanisms *viz.* chromatin modification and gDNA methylation[19]. Firstly, we measured the chromatin architecture of GP150 and GP98 using a standardized MNase I assay[9]. MNase (EC 3.1.31.1) preferentially cleaves the accessible double-strand linker DNA (~20-90 bp) between adjacent nucleosomes (~200 bp) resulting in the release of nucleosome cores DNA (~147 bp)[9]. Thereby, the chromatin DNA sensitivity to MNase I is an indicator of its accessibility extent towards chromatin. Our results demonstrated that in contrast to its GP150 counterpart, GP98 possesses a relative condensed chromatin as characterized by its less accessibility to MNase I (Fig. 2; *lanes 2-3 vs 5-6*). Of note, the almost identical nucleosomal periodicity observed for chromatin of both GP150 and GP98 ruled out the unlikely possibility of differential genome-wide chromatin (de)-condensation patterns in GP150 and GP98. These results were also in accordance with other studies in tobacco, wherein differential chromatin accessibility has been unlinked with nucleosomal periodicity of heterochromatic sequences[9].

Chromatin-remodelling has been reported to be orchestrated by multiple epigenetic marks: gDNA methylation, histone modification and non-coding RNAs (ncRNAs)[19]. Among these, the best and most studied one is gDNA methylation, that co-ordinately regulates the chromatin status *via* association with other epigenetic marks[19]. Henceforth, we measured the quantitative analysis of gDNA methylation and ^{5m}C status in GP150 and GP98. Our results showed a significant increment in gDNA methylation and ^{5m}C levels of GP98 in comparison to GP150 (Fig. 3A and B). Considering the close association between chromatin organization and gDNA methylation, we anticipate that the aforementioned relative decline in MNase I accessibility towards the chromatin for GP98 could be attributed to enhanced recruitment of docking sites for methyl-CpG binding protein (MeCP2), which are known to stimulate chromatin condensation[36].

In tobacco, gDNA methylation at 'CG', 'non-CG' and 'CHG' motifs are governed by 3 major families of NtDNMTs: methyltransferase 1 (*NtMET1*), domains rearranged methyltransferase 1 (*NtDRM1*) and chromomethylase 3 (*NtCMT3*), respectively[37]. Our comparative measurement of total NtDNMTs activity from the leaf CNE demonstrated a relative increment in GP98 over GP150 (Fig. 4), in consistent with it's correspondingly observed gDNA methylation and ^{5m}C levels (Fig. 3A and B). We anticipate that reduced NtDNMTs activity may account for corresponding gDNA hypo-methylation in GP98. However, a straight-forward correlation between gDNA methylation status and DNMTs activity / expression and does not always exist. Thereby, the differential NtDNMTs activity observed in GP150 and GP98 could also be attributed to its feedback regulation by their corresponding gDNA methylation status.

SAM cycle, a fundamental metabolic pathway, provides a source of universal methyl donor, SAM to various cellular trans-methylation reactions[23]. DNA methylation catalysed by DNMTs involves the transfer of methyl group from SAM to the 5th position of a cytosine residue, generating S-adenosylhomocysteine (SAH) as a by-product, which also happens to be negative feedback inhibitor of DNMTs[38]. The intracellular SAM / SAH ratio *a.k.a.* '*methylation-index*', thus serves as an important determinant of cellular methylation reaction, including DNA hypo- and hyper-methylation[38]. A

long-standing notion posit that exogenous administration of SAM causes an enhanced '*methylation-index*', thereby stimulates DNMTs activity and gDNA hyper-methylation[38,39]. An alternative mode of action for SAM as an inhibitor of intracellular de-methylase activity and subsequent active DNA de-methylation, thereby resulting in gDNA hyper-methylation status, has been also proposed[40]. Taking a cue from these studies, our subsequent study was designed to investigate the effect of physiological attenuations of gDNA methylation (and probable chromatin organization too) on physiological NIC level in growing tobacco under standard laboratory conditions[9]. For the same, tobacco plantlets were grown on MS media supplemented with an optimized concentration (1 mM) of SAM as described earlier for *Arabidopsis*[39] under NIC-inducing culture condition[24]. We observed that SAM supplemented tobacco plantlets showed relative enhanced gDNA methylation, ^{5m}C levels and NtDNMTs activity in concomitant with relative reduced MNase I chromatin accessibility and physiological NIC level (Fig. 5B-F). Conversely, tobacco plantlets were also grown on MS media harboring 50 µM of universal DNA de-methylating agent / DNMT inhibitor, GEN, which has been known to erase gDNA methylation and causes the activation of epigenetically silenced T-DNA loci as well as certain endogenous genes[9,26]. As expected, GEN supplemented plants showed relative reduced gDNA methylation, ^{5m}C levels and NtDNMTs activity in concomitant with relative enhanced MNase I accessibility and physiological NIC level (Fig. 5B-F). Overall, all these observations provide the (in)direct evidences of chromatin organization and gDNA methylation associated epigenetic control of NIC biosynthesis in tobacco. However, the possibility of other epigenetic mechanism(s) influencing the NIC biosynthesis and accumulation also cannot be ruled out.

Notably, in concomitant with reduced physiological level of NIC, we also observed a relative slow growth and semi-dwarf phenotype in tobacco plantlets supplemented with SAM (Fig. 5A). The pathways that compete for SAM determine the effectiveness of SAM supplementation. We anticipated that DNMTs seems to have a competitive edge over *PMT* (a rate-limiting upstream enzyme of NIC biosynthesis pathway) in utilization of SAM as methylation source. Likewise in earlier studies, it has been also hypothesized for the polyamine recycling and ethylene metabolism, wherein polyamine (putrescine, spermidine and spermine) and ethylene biosynthesis pathway has been reported to be closely connected with trans-methylation pathways *via* SAM[41]. In certain separate studies on tobacco, mutually antagonistic interactions between alkaloids-polyamines, NIC-ethylene and polyamines-ethylene have been also demonstrated[41-43]. Considering the growth-defense trade-off in plants, physiological role of gDNA methylation (as well as chromatin remodelling) in plant-defense and metabolic function of polyamines / ethylene in growth, senescence, biotic- and abiotic-stresses[41,44,45], thereby, observed phenotypic alterations along with low physiological NIC level in SAM supplemented tobacco plantlets could also be attributed to altered cross-talks between NIC-polyamine-ethylene pathways, characterised by activation of polyamine and/ ethylene biosynthesis pathway(s). However, apart from ethylene, elicitation of NIC biosynthesis as a component of tobacco defense-response also involves complicated cross-talks with other PGRs (*viz.* jasmonic acid, salicylic acid, auxins, gibberllins and absicissic acid)[46]. Additionally, an intriguing relationship between epigenetic marks and PGRs do also exists[47]. Considering all these possibility, the probable '*domino effect*' as a cause or consequence of differential NIC level in GP150 and GP98 also cannot be denied.

In tobacco, NIC function as direct defensive molecule to keep pathogens and herbivores at bay[18]. In our study, physiological implication of enhanced NIC accumulation in GP150 was also highlighted by its characteristic enhanced resistant to agricultural pests (aphids, in particular) under field conditions (*visual observations*). Of note, a key component of plant defense response to pathogens is the involvement of epigenetic factors[47]. For instance, sulforaphane, a secondary metabolite identified in many crucifers, exhibits its defensive mode of action mainly through epigenetic modifications[48]. Likewise, growing number of evidences provides significant insight that NIC could also serves as an epigenetic modulator (either through chromatin organization or gDNA methylation or '*histone-code*' modifications)[49,50]. Thereby, a (in)direct regulatory effect (s) of NIC on observed chromatin organization and gDNA methylation associated epigenetic changes in GP150 also cannot be ruled out at this stage.

Considering these facts, we hypothesize the existence of growth-defense trade-off in GP150 (as observed by semi-dwarf phenotype and enhanced resistance to aphids), wherein high endogenous NIC level has its saying on transcriptional regulation of growth and defence related genes mainly through chromatin organization and gDNA methylation associated epigenetic mechanism(s) or *vice-versa*. However, in order to confirm these speculations, further laboratory tests coupled with field-studies needs to be performed to characterize the effect of NIC attenuation on genome-wide as well as gene-specific DNA methylation in tobacco. In this context, our ongoing integrated genome-wide and gene-specific studies on transcriptomics, methylomics, hormonomics and single nucleotide polymorphisms (SNPs) will be of immense value in understanding the epigenetic patterns of NIC biosynthesis and regulation in tobacco.

Conclusion

Our current study provides the evidences of differential chromatin organization, gDNA methylation and NtDNMTs activity in contrast NIC (and corresponding derived TSNA) harbouring FCV tobacco cv. GP150 and GP98. We postulate that enhanced NIC accumulation in GP150 could be either the cause and/ consequence of reduction in the epigenetic marks (gDNA methylation and NtDNMTs activity) of restrictive epigenomic state, leading to the priming of transcriptional permissive epigenomic environment for NIC biosynthesis and/ other growth and/ defense-related gene(s). To the best of our knowledge, this is the first study to provide the molecular rationale of the epigenetics phenomenon linked with physiological levels of NIC in tobacco.

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Conflict of interest

Both the authors declare no conflicts of interest.

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