Materials and methods

Micrococcal Nuclease (MNase I) assay: MNase I assay was performed as described earlier (Zhao *et al.* 2001) with some modifications. Chromatin was extracted from untreated and corresponding GA_3 treated tobacco leaves at early-vegetative growth stage (13 d after GA_3 application) using *EpiQuik ChromaFlash*™ plant chromatin extraction kit (*Epigentek,* Farmingdale, NY, USA), following the manufacturer's instructions. Equal amounts of chromatin (~8 µg) from untreated and GA_3 treated tobacco leaves were washed and resuspended in 200 mm^3 of reaction mixture containing 1× MNase I digestion buffer (50 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$). A range (titration) of MNase I digestion (*NEB*, Hitchin, UK) for different duration was performed to the isolated chromatin DNA to identify the extent of digestion desired (data not shown). Subsequently, an optimised MNase I concentration (0.5 gel units per reaction) was added and digestions were carried out for 0, 2, 4, and 6 min. The reaction was stopped by adding equal volume of stop buffer (100 mM EDTA and 10 mM EGTA, pH 7.5), followed by overnight digestion at 37 °C with proteinase K. The samples were treated with RNase A (10 mg cm^{-3}, room temperature, 25 min), extracted once with phenol + chloroform + isoamyl alcohol (25:24:1, v/v/v) and precipitated with 100 % ethanol. The dried DNA pellet was resuspended in 30 mm^3 of $T_{10}E_1$ (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Finally, MNase I digestion products were resolved on 2 % (m/v) agarose gels and stained with ethidium bromide. The signal intensity of each undigested and MNase I digested chromatin DNA bands (nucleosome ladders) from untreated and GA_3 treated tobacco leaves was measured by densitometry scanning using *ImageQuant* (*GE Healthcare Life Sciences*, Pittsburgh, PA, USA) software.

MNase I Southern blot assay: In order to analyse the chromatin configuration at two specific genomic loci (*SYL2* and 26S rDNA), equal amounts of isolated chromatin were partially digested with MNase I for an optimized time of 6 min. Chromatin isolation, MNase I digestion, DNA extraction and precipitation were performed exactly as described above. RNase A (20 µg cm^{-3}, room temperature, 25 min) treated DNA samples were resuspended in 30 mm^3 of $T_{10}E_1$. MNase I digestion products were resolved on 0.9 % (m/v) agarose gels, alkali blotted onto *Hybond-XL* membranes (*GE Healthcare*, Little Chalfont, UK) and hybridised with α-[^{32}P] dCTP-labelled DNA probes (*DekaLabel* kit*, MBI, Fermentas*, Vilnius, Lithuania) according to protocols described in Kovařík *et al.* (1997). The probes were cloned repeats mapping to sub-telomeric heterochromatin *SYL2*, (Koukalova *et al.* 2010) and a 26S rDNA probe (Lim *et al.* 2000). After washing (2 × 5 min with 2× SSC, 0.1 % sodium dodecylsulphate (SDS) at room temperature followed by 2 × 15 min with 0.6× SSC, 0.1 % SDS, 65 °C, the hybridisation bands were visualised with a *PhosphorImager* (*Typhoon 9410, GE Healthcare*, PA, USA).

Table 1 Suppl. List of primers used in this study (F - forward primer, R - reverse primer).

Sample	Primer	Sequence (5'-3')	Target gene	Tm [°C]	Primer length (No. of bases)	Amplicon size [bp]
1	IME1079F	CGCGCTACACTGATGTATTC	*18S rRNA*	52	20	170
2	IME1079R	GTACAAAGGGCAGGGACGTA	*18S rRNA*	54	20	170
3	IMN1078F	AAGCCCATGGTTGTTGAGAC	*EF-1 α*	51.8	20	80
4	IMN1078R	GTCAACGTTCTTGATAACAC	*EF-1 α*	47.7	20	80
5	IMN1282F	TGAACCAGAAGACAAGCCGTAGT	*NtMET1*	59	23	73
6	IMN1282R	ATCTCATCCTCATTGATCTTGATGTAA	*NtMET1*	58	27	73
7	IMN1285F	AGACCGCTTGGAGCAACTGATA	*NtDRM1*	60	22	66
8	IMN1285R	CACGGGCTTCCACCAATTA	*NtDRM1*	58	19	66
9	IMN1286F	CCAGAGACTGCGGTAAGAAATGA	*NtCMT3*	59	23	65
10	IMN1286R	TGCCTCCACTCCCTCAAAAG	*NtCMT3*	59	20	65
11	IME2157F	GAACAAGATGGATTGCACGC	*nptII*	52	20	108
12	IMnp101R	CCGGAACACGGCGGCATCAG	*nptII*	60	20	108

Table 2 Suppl. Quantification of digested chromatin after MNase I digestion of control and GA_3 treated nuclei. The signal intensity [%] of each undigested and MNase I digested chromatin DNA bands (nucleosome ladders *r2* - *r5*') from untreated and GA_3 treated tobacco leaves were measured by densitometry scanning. (n.d. - not determined).

Bands	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8
r1	100	100	100	96.29	100	100	60.05	63.24
r2	0	0	0	0.47	0	0	18.53	15.76
r3	0	0	0	1.52	0	0	9.66	11.79
r4	0	0	0	1.73	0	0	11.77	9.21
r5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table 3 Suppl. A quantitative representation of the relative percentage release of nucleosomes as a function of percentage of MNase I digestion from untreated and corresponding GA_3 treated tobacco chromatin. The signal intensity [%] of each undigested and MNase I digested chromatin DNA bands (nucleosome ladders; '*r2* - *r5*') from untreated and corresponding GA_3 treated tobacco leaves was measured by densitometry scanning.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
r1	100	100	100	100	100	100	100	98.05	71.93	58.13	100	100	100	100	61.25	63.68	55.05	65.48	60.24	54.62
r2	0	0	0	0	0	0	0	1.18	7.42	14.08	0	0	0	0	16.43	10.32	15.48	10.29	9.34	9.25
r3	0	0	0	0	0	0	0	0.15	3.89	6.33	0	0	0	0	0.96	4.37	5.65	5.47	4.79	6.31
r4	0	0	0	0	0	0	0	0.56	5.23	5.19	0		0		1.93	5.30	5.71	4.77	5.57	5.86
r5	0	0	0	0	0	0	0	0.07	11.54	16.25	0	0	0		19.43	16.33	18.11	13.99	20.07	23.96

Table 4 Suppl. Statistical evaluation of kanamycin sensitivity assay of transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) transgenic plantlets to GA_3 and DNA de-methylating agents *viz.* 5-azacytidine (5-azaC), genistein (Gen), and trichostatin A (TSA). In each variant 10 seeds were tested and number of survived seedlings was evaluated. No seedlings were survived when they are treated with kanamysin + GA_3 in ethanol or by kanamycin + dimethyl sulfoxide (DMSO). Means ± SDs, $n = 3$ (from three independent experiments); *asterisks* indicate significant differences at $P < 0.05$ when compared with untreated control.

Treatments	TGS line	PTGS line
MS only	10	10
MS + kanamycin	0	0
MS + kanamycin + GA_2	9.00 ± 1.00	8.66 ± 0.57*
MS + kanamycin + 5-azaC	7.33 ± 0.57	6.66 ± 0.57*
MS + kanamycin + Gen	8.00 ± 1.00	8.00 ± 1.00*
MS + kanamycin + TSA	7.66 ± 0.57	8.00 ± 1..00*

Fig. 1 Suppl. Experimental field with control (*left panel*) and GA_3 treated (*right panel*) tobacco plants.

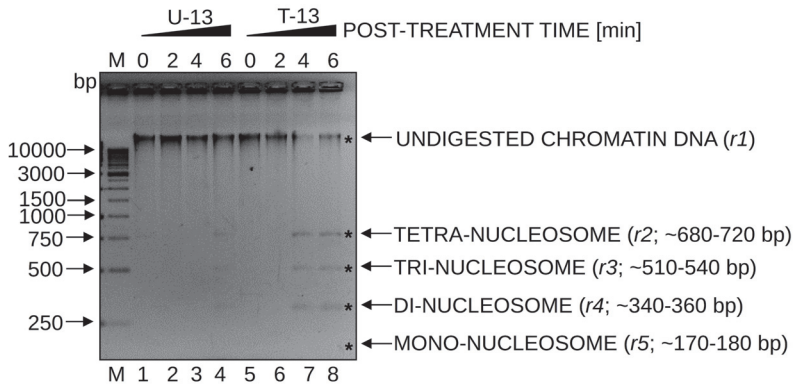

Fig. 2 Suppl. Time kinetics of MNase I digestion of control and GA_3 treated nuclei. Chromatin extracted from the untreated (U-13) and corresponding GA_3 treated (T-13) tobacco leaf nuclei at early-vegetative growth stage (13 d after GA_3 application) were MNase I digested for an indicated amount of time, purified, and subsequently resolved on 2 % (m/v) agarose gel. *Lanes* 1 - 4 and 5 - 8 indicate MNase I digested chromatin for a time-period of 0, 2, 4, and 6 min from untreated and GA_3 treated tobacco leaf samples, respectively. M - DNA size marker of 250 bp. Notably, under our experimental conditions, electrophoretic mobility of second uppermost band (*r2*) in MNase I digested nucleosome ladder corresponds to a size of ~680 - 720 bp indicating its high probability as "tetra-nucleosome". The signal intensity of each undigested and MNase I digested chromatin DNA bands (nucleosome ladders; *r2* - *r5*) from untreated and corresponding GA_3 treated tobacco leaves as measured by densitometry scanning is shown in Table 2 Suppl. Notably, the normalized mean signal intensity of mono-nucleosome band (*r5*) was too low to be detected in this particular gel.

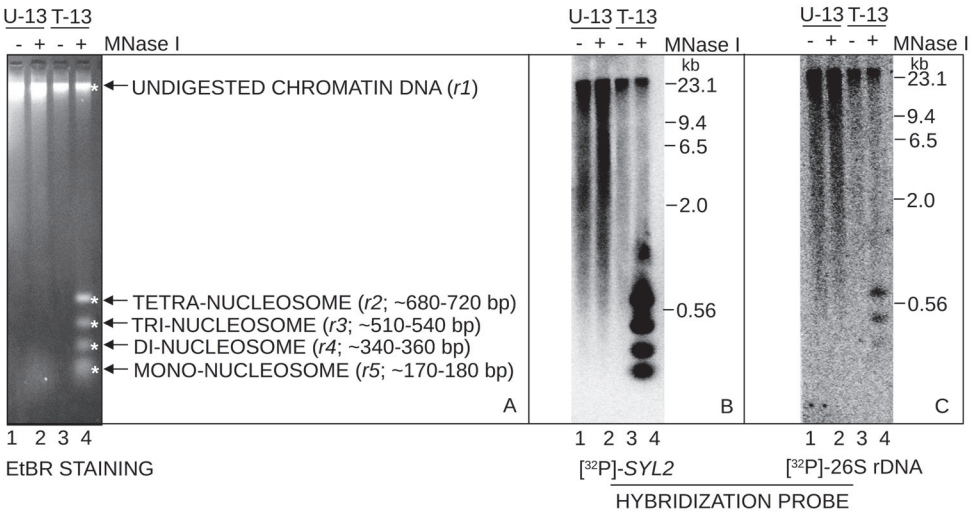

Fig. 3 Suppl. Assessment of chromatin configuration at *SYL2* and 26S rDNA loci following GA_3 application. *A* - Chromatin extracted from the untreated (U-13) and corresponding GA_3 treated (T-13) tobacco leaf nuclei at early-vegetative growth stage (13 d after GA_3 application) were MNase I digested for 6 min, purified, resolved on 0.9 % (m/v) agarose gels, and subsequently stained with ethidium bromide. *Lanes* 1,3 and 2,4 indicate DNA sampled from undigested and MNase I digested chromatin, respectively. Assessment of chromatin configuration at *SYL2* and 26S rDNA loci was performed by MNase I-Southern blotting. DNA products from MNase I treated chromatin were resolved on agarose gels as in *A*, blotted onto a nylon membrane and hybridised with [^{32}P]-labelled *SYL2* and 26S rDNA probes. *B* - DNA fragmentation at *SYL2* genomic loci. *C* - DNA fragmentation at 26S rDNA genomic loci. The exposure times of individual blots were approximately the same. Notably in all three analyses, oligonucleosomal bands of 170 - 180 (smear in EtBr but not after hybridisation), 340 - 360, 510 - 540 and 680 - 720 bp corresponding to mono-, di-, tri- and tetra-nucleosomes (nucleosome ladder; *r2* - *r5*) were revealed only in GA_3 treated sample.

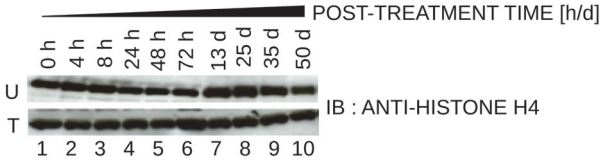

Fig. 4 Suppl. Immunoblotting of untreated (U) and GA_3 treated (T) nuclear-extracts using an anti-histone H4 antibody. The quality of the nuclear extracts was assessed by Western blot analysis using anti-histone antibody directed against the histone H4. Approximately 50 µg of nuclear extracts from untreated (U; *upper panel*) and corresponding GA_3 treated (T; *lower panel*) tobacco leaves at various growth-stages (0, 4, 8, 24, 48, 72 h and 13, 25, 35, 50 d after GA3 treatment) were separated by 15 % SDS-polyacrylamide gel electrophoresis and probed with anti-histone H4 antibody.

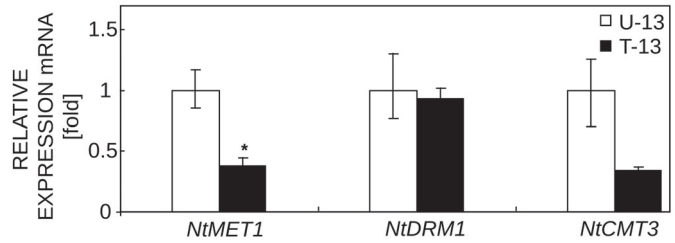

Fig. 5 Suppl. Effect of GA_3 application on *NtDNMT's* steady-state mRNA. Real-time qPCR analyses of *NtMET1*, *NtDRM1*, and *NtCMT3* mRNA in untreated (U) and GA_3 treated (T) tobacco at early growth stage (13 d after GA_3 application). The results are presented as the expression of the individual mRNA normalized to corresponding endogenous *EF1-α* rRNA. The value for control untreated plantlets was set at 1. Means ± SDs, $n = 3$ (triplicate measurements from three independent experiments). *Asterisks* indicate the significant difference at $P < 0.05$ when compared with corresponding untreated control.

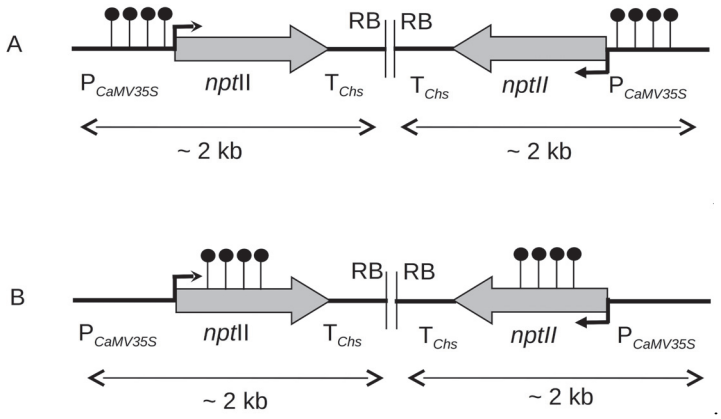

Fig. 6 Suppl. Schematic outline of inverted repeat T-DNA harboring the epigenetically silenced *npt*II reporter transgene under the control of cauliflower mosaic virus (CaMV) 35S promoter at hemizygous locus 1 (*HeLo1*). The genomic organization of transgene insertion is an inverted repeat of two T-DNA copies (Fojtova *et al.* 2003). Each T-DNA copy (~2 kb) contains *npt*II reporter gene (~900 bp) which is under control of the promoter fromCaMV 35S (~300 bp, symbolized by the *bent arrow*) in transcriptional gene silencing (TGS; *A*) and post-transcriptional gene silencing (PTGS;*B*) tobacco lines. The chimeric *npt*II reporter genes (*grey arrows*) in locus 1 contain 287 bp of the 3′-termination signalling sequence of the chalcone synthase gene (T_{chs}) of *Antirrhinum majus* for securing more stable transcription. The region between both right borders (RB) is an unknown sequence of probably less than 100 bp. DNA methylation sites are represented by the *filled circle* and upstream and downstream regions flanking the *npt*II reporter genes are represented by *solid lines*.

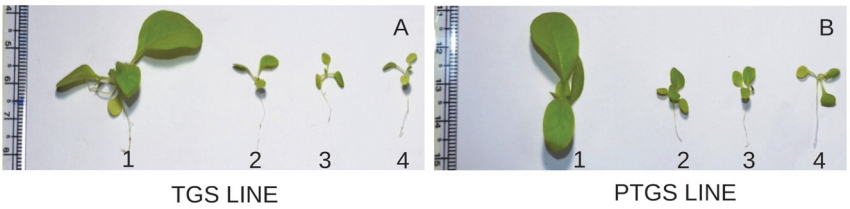

Fig. 7 Suppl. Phenotypic sensitivity of transcriptional gene silencing (TGS; *A*) and post-transcriptional gene silencing (PTGS;*B*) transgenic plantlets grown for 1 month in kanamycin (50 μg cm^{-3}) selection medium either alone or with 50 mm^3 of GA_3 solvent (ethanol) or DNA de-methylating agents solvent (DMSO). Control plantlets were also grown in a kanamycin selection medium with no supplement. A representative image of plantlets grown on: 1 - MS medium alone, 2 - MS medium + kanamycin, 3 - MS medium + kanamycin + GA_3, and 4 - MS medium + kanamycin + DMSO. Statistical evaluation of kanamycin sensitivity assay is shown in Table 5 Suppl.

References

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