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GA_3 mediated enhanced transcriptional rate and mRNA stability of 3-hydroxy-3-methylglutaryl coenzyme a reductase 1 (*NtHMGR1*) in *Nicotiana tabacum* L.

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Abstract

Our present study evaluated the underlying molecular-mechanism(s) associated with the observed enhanced transcript levels and concomitant functional activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase 1 (*NtHMGR1*), a rate-limiting enzyme of cytosolic mevalonate (MVA) pathway of terpenoids biosynthesis, by gibberellin A_3 (GA_3) treatment in model cultivated tobacco, *Nicotiana tabacum* L. Based on the transcription run-on and cordycepin chase assays, our results demonstrated that tobacco seeds-priming with GA_3 causes a relative and significantly enhanced transcriptional rate and mRNA stability of *NtHMGR1*. Taken together, our study established that GA_3 mediated transcriptional and post-transcriptional regulatory control as one of the mechanisms for the observed enhanced transcript-levels and consequently enhanced functional activity of *NtHMGR1*.

Keywords: GA_3; *HMGR1*; *Nicotiana tabacum*; transcription run-on; transcriptional rate and mRNA stability

Abbreviations: GA_3, Gibberellin A_3; *HMGR1*, 3-hydroxy-3-methylglutaryl coenzyme A reductase 1; TRO, Transcription Run-On; COR Cordycepin

Introduction

Owing to their biological significance and practical utilization, there has been growing interest in secondary plant metabolites (SPMs) in recent times. Until now, >100,000 species specific SPMs have been identified (Wink, 2010). Broadly, SPMs can be categorised into 3 chemically distinguish groups: isoprenoids / terpenoids, nitrogen (N) or sulphur (S) containing compounds and phenolics (Pagare *et al*., 2015). Among these, terpenoids represents a large (>40,000), diverse and most celebrated group of SPMs having implications in both basic- and applied-sciences (e.g. nutraceuticals, perfumeries, pesticides, agrochemicals, disinfectants,

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colorants, rubber and pharmaceuticals drugs, etc) (Abbas *et al*., 2017). Although predominantly categorized as SPMs, terpenoids are involved in various fundamental biological processes in all living organisms (Brahmkshatriya and Brahmkshatriya, 2013). In plants, terpenoids are must for a wide-range of primary (e.g. growth, cell-division, photosynthesis and respiration etc.) as well as secondary physiological functions (e.g. plants defense responses towards biotic- and abiotic-stresses) (Tholl, 2015; Pichersky and Raguso, 2018). In animals, all terpenoids (e.g. squalene, lanosterol, cholesterol and steroid hormones) are derived from the cytosolic / endoplasmic-reticulum / mitochondrial / peroxisomal mevalonic acid (MVA) pathway, that serves as a direct precursor of active five carbon isoprene monomeric-unit i.e. isopentenyl diphosphate (IPP) and/ its allylic isomer, dimethylallyl pyrophosphate (DMAPP) (Lichtenthaler *et al*., 1997; Leivar *et al*., 2005; Kuzuyama and Seto, 2012). On the contrary, plants terpenoid pathway is relatively more complicated, wherein apart from 'classical' MVA pathway, an additional 'alternative' plastidial non-MVA pathway (*a.k.a.* 2-*C*-methyl-D-erythritol 4-phosphate, MEP) derived from the endo-symbiotic ancestor of plastids, do also exists (Lichtenthaler *et al*., 1997).

A key and rate-limiting enzyme of MVA pathway is 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (*HMGR*, E.C. 1.1.1.34) catalyses the irreversible reaction of one molecule of HMG-CoA and two molecules of triphosphopyridine nucleotide (NADPH) into MVA (Chappell *et al*., 1995). A positive correlation between *HMGR* expression and terpenoid content do exists in various plant systems (Aquil *et al.*, 2009; Bansal *et al*., 2018; Wei *et al*., 2019). On the contrary to mammalian *HMGR* which is encoded by a single gene, plant *HMGRs* are encoded by a gene-family (generally encompasses 2-4 genes) and each *HMGR* isoform respond specifically by regulating the biosynthesis of specific functional MVA metabolites (Campos and Boronat, 1995; Loguercio *et al*., 1999; Suzuki *et al*., 2009). Like other members of *Solanaceae* family, cultivated tobacco (*N. tabacum* L.) also do possesses two *HMGR* isoforms (namely *NtHMGR1* and *NtHMGR2*), with certain difference in intracellular-localization and cellular-functions. Although not clearly defined, it has been shown that *NtHMGR1* and *NtHMGR2* serves as a house-keeping and stress-responsive gene, respectively (Hemmerlin *et al*., 2004; Merret *et al*., 2007).

The unique feature of plant *HMGR* isoforms is their specific spatio-temporal responsiveness towards myriad of cellular- and environmental-signals e.g. development, light, calcium, calmodulin, chemical-challenges, plant growth regulators (PGRs), pathogens and wounding (Stermer *et al*., 1994; Bach, 1995; Chappell *et al*., 1995; Learned and Connolly, 1997; Korth *et al*., 2000; Antolín-Llovera *et al*., 2011; Diarra *et al*., 2013; Shaikh *et al*., 2020). Among these, PGRs (a diverse group of endogenously signalling bio-molecules) and/ their complex interactions have been studied extensively (Depuydt and Hardtke, 2011; Manoharlal *et al*., 2018b). In certain independent studies, it has been shown that exogenous application of abscisic acid (ABA), ethylene (ET), gibberellins (GAs), salicylic acid (SA) and methyl jasmonates (MeJA) induces MVA metabolites mainly *via* up-regulation of specific *HMGR* isoform (Wang *et al*., 2007; Dai *et al*., 2011; Mansouri *et al*., 2011; Manoharlal and Saiprasad, 2019; Wei *et al*., 2019). Influenced by internal- and/ external-factors, there are circumstantial evidences indicating that *HMGR* is under multi-level regulation (transcriptional, post-transcriptional, translational and/ post-translational levels) (Brooker and Russell, 1979; Wong *et al*., 1982; Russell *et al*., 1985; Bach, 1986; Goldstein and Brown, 1990; Hemmerlin and Bach, 2000; Korth *et al*., 2000; Nieto *et al*., 2009; Hemmerlin, 2013), however no clear consequences have been made specifically about *NtHMGR1*.

Taking a cue from these studies and other similar supporting observations from literature impelled us to examine the mechanistic effect of GA_3 application on altered expression patterns of *NtHMGR1*. Henceforth in this study, we sought to explore the *NtHMGR1* specific transcriptional and post-transcriptional control by GA_3 in model tobacco plant, *N. tabacum* L. We addressed these questions by evaluating the transcriptional rate

and mRNA stability of *NtHMGR1* in the absence and presence of GA_3 application under controlled growth-conditions of tobacco.

Materials and Methods

Plant material and growth-conditions

A primary and globally grown FCV tobacco (*N. tabacum* L.) cv. 'K326' *a.k.a.* 'Kanchan' was used in this study. Following surface-sterilization (0.05% $HgCl_2$ for 7 min), washing with double-distilled water (ddH_2O), approximately 0.5 g seeds (~450-500 seeds in numbers) were washed in 70% ethanol for 10 min. Subsequently, the seeds were washed 5 times with sterile ddH_2O and incubated either in 5 ml of water (untreated, 'U') or 50 mg L^{-1} GA_3 (treated, 'T') for 24 h with a continuous shaking at 10 rpm, followed by three times washing in ddH_2O and drainage of excess-water. A 250 mM GA_3 stock solution (in ethanol) was prepared freshly before use. The chosen working concentration of GA_3 (50 mg L^{-1}) was determined empirically in our previous study (Manoharlal *et al*., 2019). Approximately 15 seeds of 'U' and 'T' were seeded on the moistened surface of bio-tech grade Soilrite (a mix of expanded perlite, Irish peat moss and exfoliated vermiculite in equal ratio i.e. 1/3:1/3:1/3; Keltech Energies Ltd., Bengaluru) in plastic-tray (30 cm length × 20 cm width × 7 cm height) and allowed to germinate in green-house for 4 weeks (temperature: 25 ± 3 °C, relative humidity: 60-70%, photoperiod: 16 h-light/dark alternate cycle and irradiance: 80 µmol m^{-2} s^{-1}). An equivalent volume of diluted ethanol was also used for seed-priming (*solvent-control*). Each treatment was done in triplicate (*n=3*). Visual and quantitative difference in plantlets growth for 'U' and 'T' were monitored and recorded until the end of test-period. Following 4 weeks of growth under prescribed growth conditions, uniform, representative and homogenous grown 'U' and 'T' plantlets were collected, snap-frozen in liquid N_2, grounded to fine-powder and stored at -80 °C until further use.

RNA dot-blotting

Total RNAs was isolated from fine-grounded 'U' and 'T' plantlets using GeneJET Plant RNA Purification Mini Kit (*Thermo Fisher Scientific*, Inc.). Purified RNAs (~2 µg dot^{-1}) from each sample was subjected to RNA dot-blotting on positively-charged nylon membrane (Hybond^{TM}-N^+; *GE Healthcare Life Sciences*, Pittsburgh, PA, USA) as described earlier (Manoharlal *et al.* 2019). For hybridization, blots were UV-cross-linked, followed by probing with α-[^{32}P]dATP (3000 Ci $mmol^{-1}$; *PerkinElmer*, Hopkinton, MA, USA) labelled gene-specific probes (i.e. *NtHMGR1* and constitutively expressed house-keeping gene, 18S rDNA, Table 1). Hybridization signals were quantified by densitometry-scanning using a phosphoimager (FLA-5000, Fuji phosphoimager). The steady-state transcript levels of *NtHMGR1* from 'U' and 'T' was normalized to their corresponding 18S rRNA and plotted as relative *NtHMGR1* transcript level.

NtHMGR activity measurement

NtHMGR (total proteins encoded by both *HMGRs* isoforms of *N. tabacum*, namely *NtHMGR1* and *NtHMGR2*) activity was determined in a cell-free assay system using spectrophotometric-based HMG-CoA reductase assay kit (Sigma-Aldrich, *St. Louis, USA*) following the manufacturer's instructions. Briefly, crude cellular-extract was prepared from fine-grounded 'U' and 'T' plantlets in freshly prepared buffer [25 mM Tris-HCl pH 7.5, 10 mM NaCl, 10 mM $MgCl_2$, 5 mM EDTA, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonylfluoride (PMSF) and protease inhibitor cocktail (*Sigma-Aldrich,* Sigma Chemical Co., St. Louis, USA)] @ 1 ml 100 ml^{-1} of homogenisation-buffer. The homogenate was cleared by centrifugation (15,000 × g, 5 min and 4° C) and an aliquot was used for protein determination by bicinchoninic acid (BCA) protein assay kit (Pierce^{TM}, *ThermoFisher^{TM} Scientific*, *San Jose, USA*) using bovine serum albumin (BSA) as

standard. Approximately 50 µg (~12.5 µl) of crude-extract from 'U' and 'T' was incubated in reaction containing 4 µl of NADPH (400 µM) and 12 µl of HMG-CoA substrate (400 µM) in a reaction volume of 0.2 ml of 100 mM potassium phosphate buffer, pH 7.4 harbouring 120 mM KCl, 1 mM EDTA and 5 mM DTT) in the absence or presence of 2.5 µl (~30 µM) of reference statin drug, pravastatin (*Sigma*), an inhibitor of HMGR activity. Reaction was initiated (time 0) by incubating the reaction-mix at 37 °C. The NADPH consumption rate was monitored at every 20 s for 10 min by monitoring a decrease in absorbance at 340 nm. Recorded absorbance was base-corrected to corresponding blank (without crude-extract addition) for both the samples. Result was expressed as specific activity [µmol (NADPH oxidized) mg^{-1} (protein) min^{-1}]. One unit of HMGR activity has been defined as amount of enzyme that oxidizes 1 µmol NADPH to $NADP^+$ min^{-1} at 37 °C.

Transcription Run-On (TRO) analysis

TRO experiment was performed as described earlier (Manoharlal *et al*., 2019) with specific modifications. Approximately 100 ng of PCR amplified complimentary DNA (cDNA) fragments of target-(*NtHMGR1*; nucleotides: 308-412 from start-codon) and reference-gene (18S rDNA; nucleotides: 1467-1638 from transcription start site, TSS) (Table 1) were blotted onto the positively charged nylon membrane (Hybond™-N^+; *GE Healthcare*) using microfiltration apparatus (*Biorad*). Equal spotting of DNA on the membrane was ensured by staining the similar kind of blots with 0.02% methylene blue stain. Nuclear fractions were prepared from 'U' and 'T' plantlets as described earlier (Manoharlal *et al*., 2019) and subjected to *in vivo* transcription reaction either in the absence or presence of an optimised concentration (200 µg ml^{-1}) of transcriptional inhibitor, cordycepin (COR). Notably, COR solvent (dimethyl sulfoxide, DMSO) concentration in the reaction mixture was reduced to <0.01% (*v/v*) of the final reaction volume. *In vivo* labelled transcripts prepared from permeabilized nuclear-fractions for each sample was subjected to reverse Northern hybridization with dot-blotted DNA. The hybridized signals were detected by phosphoimager-scanner. The signal-intensity of hybridized nuclear RNA of *NtHMGR1* was quantified, base-corrected to corresponding signal-intensity of 18S rRNA using densitometry scanning and plotted as relative *NtHMGR1* transcript level.

Table 1. List of primers used in this study

Primer	Sequence (5'-3')	Accession No.	Target gene	Purpose	Primer (F/R)	Tm (°C)	Primer length (bases)	Amplicon size (bp)
IME1079F	CGCGCTACACTGATGTATTC	AJ236016	*Nt18S rRNA*	RNA dot-blotting, qRT-PCR, TRO	F	52	20	170
IME1079R	GTACAAAGGGCAGGGACGTA				R	54	20	
IMN1078F	AAGCCCATGGTTGTTGAGAC	XM_009784954	*NtEF-1α*	qRT-PCR	F	51.8	20	105
IMN1078R	GTCAACGTTCTTGATAACAC				R	47.7	20	
HMGR1-F	TTGGCATCGGATTTGTTCAG	U60452	*NtHMGR1*	RNA dot-blotting, qRT-PCR, TRO	F	49.73	20	105
HMGR1-R	GGCGGCTATCTTCCTCAAT				R	51.09	19	
EXPA2-F	CCTAAATGGTGTAGAAAAG	AF049351.1	*NtEXPA2a*	RNA dot-blotting	F	45	19	171
EXPA2-R	AATGCCACCTCTGTAAAT				R	43	18	

F - forward, R - reverse
qRT-PCR - quantitative real-time polymerase chain reaction
TRO - transcription run-on

Cordycepin (COR) chase assay

COR (Cayman Chemical Co.), a potent *in vivo* global transcriptional-inhibitor, was used to evaluate the mRNA half-life of *NtHMGR1* as described previously (Manoharlal *et al*., 2019). Four-weeks old 'U' and 'T' plantlets (10 in numbers per time-point of chase) were kept in an incubation buffer (1 mM PIPES pH 6.25, 1 mM KCl, 1 mM sodium citrate and 15 mM sucrose) for 30 min, followed by incubation with or without addition of an optimized concentration (200 µg ml^{-1}) of COR for transcriptional shut-off. This mixture was swirled slowly on a rotary-shaker and chased for the indicated time (0, 1, 2, 3 and 4 h). At indicated time-point of chase, plantlets were collected, blotted dry, frozen in liquid N_2 and stored at -80 °C until further use. For RNA dot-blotting and hybridization, ~2 µg of total RNAs isolated from collected plantlets at indicated time-point of chase were dot-blotted and hybridized with specific PCR amplified DNA probes of *NtHMGR1* and 18S rDNA. The signal-intensity of hybridized *NtHMGR1* transcript at each time-point of chase was quantified with phosphoimager-scanner by densitometry scanning and normalized to 18S rRNA. Normalized signal-intensity of *NtHMGR1* transcript with respect to their corresponding time T_0 was plotted as a line-graph (%). A semi-log plot of mRNA remaining (%) *versus* time of chase allows for the assessment of mRNA $t_{1/2}$ (the time-point at which the amount of steady-state mRNA declines to 50% of the initial T_0 value). *NtHMGR1* mRNA $t_{1/2}$ was calculated by fitting the non-linear regression according to the following exponential decay formula, $t_{1/2} = -0.693\ k^{-1}$, wherein k is the slope of best fit-line and $t_{1/2}$ is half-life.

Data analysis

The results were expressed as means ± standard deviations (SDs). One-way analysis of variance (ANOVA) was used to analyse the statistically significant difference ($p \leq 0.05$) between groups.

Results

GA_3 improves the tobacco growth-parameters

Since the last seven decades (Skoog and Miller, 1957), cultivated tobacco (*N. tabacum* L.) has been used as a model plant for various laboratory investigations in basic- as well as applied-science. In few separate studies, GAs and in particular GA_3 has been reported to promote the seed germination in tobacco (Leubner-Metzger, 2001; Li *et al*., 2018). Considering these facts, thereby in this study, cultivated tobacco cv. 'K326' was selected. Following priming with either water alone i.e., untreated ('U') or an optimized dose (50 mg L^{-1}) of purified GA_3 ('T') under the prescribed germination-conditions, four-weeks old, healthy, and uniformly grown tobacco plantlets were harvested. As a control, tobacco seeds were also treated with an equivalent volume (<0.1% *v/v*) of GA_3 solvent, ethanol (solvent-control). As depicted in Figure 1A, a remarkable visual-difference in growth attributes of 'U' and 'T', characterized by a relative improved growth of 'T' plantlets was observed. A significant quantitative difference in the seedling bio-mass was also observed (Figure 1B), wherein 'T' plantlets were having relatively higher fresh-weight (~74 mg per 50 seedlings) over its 'U' counterpart (~48 mg per 50 seedlings). Notably, no difference in fresh-weight was observed in tobacco plantlets in solvent-control, validating the GA_3 specific-effect (Figure S1 Suppl.). The aforesaid established working tobacco system comprising the homogenous and healthy 'U' and 'T' plantlets were collected at the end of test-period (28^{th} day) and their comparative ('U' versus 'T') molecular -evaluation was carried out in our subsequent study.

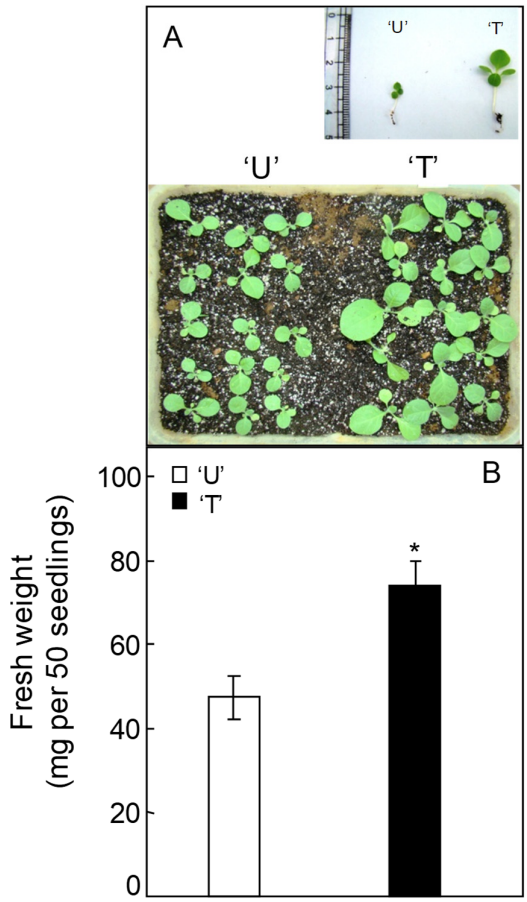

Figure 1. Tobacco seedlings growth measurement

A. Representative image of 'U' and 'T' at the end of test-period (28th day post-treatment), wherein *inset* depicts the individual 'U' and 'T' plantlet. B. Fresh weight of 'U' and 'T' at the end of test-period. For seedlings weight measurement, 50 seedlings per biological replicate were used. Means ± SDs, $n = 3$, wherein * indicate the significant differences ($p \leq 0.05$). 'U'- Untreated and 'T' - GA_3 treated plantlets.

GA_3 increases the steady-state levels of NtHMGR1 transcript

Previous independent studies in various plants demonstrated a positive-correlation between PGRs and terpenoids, wherein *HMGR1*, being a rate-limiting enzyme of MVA pathway (Figure 2A) has been exploited as functional read-out (Wang *et al*., 2007; Pu *et al*., 2009; Mansouri *et al*., 2011; Diarra *et al*., 2013; Wei *et al*., 2013). Henceforth, with an objective to uncover the underlying molecular mechanism(s) related with GA_3 mediated differential *NtHMGR1* expression changes (if any), firstly we investigated the altered expression patterns (in terms of mRNA) of *NtHMGR1* in 'U' versus 'T' system. In order to assess, we estimated the mRNA accumulation of *NtHMGR1* by RNA dot-blotting. As depicted in Figure 2B, the steady-state mRNA level of *NtHMGR1* from 'T' showed significant increment (relative fold changes, RFC ~4.23) as compared to 'U'. The observed difference in transcriptional pattern of *NtHMGR1* was further validated by qRT-PCR using specific-primers corresponding to *Nt18S rRNA* as well as *NtEF-1 α* (Figure S2 Suppl.). A high and positive-correlation (r ~95%) between these two independent methods i.e., RNA dot-blotting vs qRT-PCR corroborated the accuracy of RNA dot-blotting, which will be further used in our subsequent study (mRNA chase assay, *described ahead*). These results indicated and further established GA_3 as a generic and positive modulator of *NtHMGR1* expression.

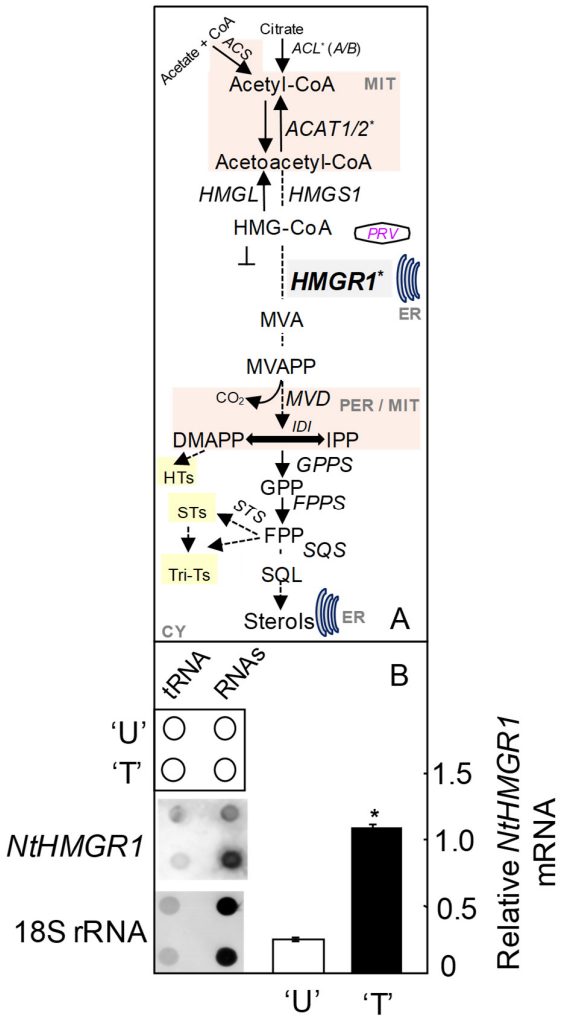

Figure 2. Differential *NtHMGR1* mRNA analysis.

A. A simplified schematic representation of MVA biosynthesis pathway. *Solid-* and *dashed-arrows* indicate single- and multi-step reactions, respectively. The studied *HMGR1* gene is highlighted in *bold*.

Genes abbreviations (*in order of appearance*): *ACS* - acetyl-CoA synthetase, *ACL** (*A/B*) - ATP-citrate lyase A or B subunit, *ACAT1/2** - acetyl-coenzyme A acetyltransferases 1 or 2 isoform, *HMGS* - 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase, *HMGL* - HMG-CoA lyase, *HMGR1** - HMG-CoA reductase 1, *MVD* - mevalonate-5-pyrophosphate decaroboxylase, *IDI* - isopentenyl diphosphate isomerase, *GPPS* - geranyl pyrophoshate synthase, *FPPS* - farnesyl pyrophoshate synthase, *STS* - sesquiterpene synthase and *SQS* - squalene synthase. *Asterisks* indicates the rate-limiting enzymes. Metabolites abbreviations (*in order of appearance*): HMG-CoA - 3-hydroxy-3-methylglutaryl-CoA, MVA - mevalonic acid, MVAPP - mevalonic acid diphosphate, DMAPP - dimethylallyl diphosphate; IPP - isopentenyl diphosphate, GPP - geranyl diphoshate, FPP - farnesyl diphosphate, SQL - squalene, HTs - hemiterpenes, STs - sesquiterpenes and Tri-Ts - Tri-terpenes. Other abbreviations: PRV - pravastatin, CY - cytoplasm, ER - endoplasmic-reticulum, PER - Peroxisomes and MIT - Mitochondria.

B. Total RNAs (~2 µg dot^{-1}) from 'U' and 'T' was subjected to dot-blot hybridization. The position of individual dot-blot RNA and recognition of dotted RNA by α-[^{32}P]dATP-labelled *NtHMGR1* and 18S rDNA probes are shown in the *upper-*, *middle-* and *bottom*-panel, respectively. The individual *NtHMGR1* mRNA amount with respect to constitutively expressed 18S rRNA is shown in *y-axis*. Means ± SDs, $n = 2$, wherein * indicate the significant differences ($p \leq 0.05$). 'U'- Untreated and 'T' - GA_3 treated plantlets.

GA_3 enhances the NtHMGR activity

To investigate, if an increase in *NtHMGR1* transcript levels by GA_3 also displays a concomitant alteration in its functional activity, we further evaluated the total enzymatic activity of NtHMGR in a cell-free assay system. For this, cellular-extracts from 'U' and 'T' were subjected to total NtHMGR activity evaluation (based on spectrophotometric measurement of NADPH consumption rate). As a positive-control, purified *HMGR* representing the catalytic domain of human HsHMGR (expressed in *E. coli* as recombinant GST fusion protein) was also used. Notably, statins are effective and competitive-inhibitors of HMGR that are known to comprehensively inhibits the HMGR activity (da Costa *et al*., 2012). Thereby, reference statin drug, pravastatin (PRV, 30 µM) was also employed as an inhibition-control in all cases. The solvent-control of PRV (i.e., DMSO) established the specific inhibition of NtHMGR activity by PRV only (Figure S3 Suppl.). In alignment with the gene-expression result, our study also showed a relative enhanced (RFC ~2.33) NtHMGR activity in 'T' as compared to 'U' (Figure 3), further corroborating GA_3 as a positive modulator of NtHMGR.

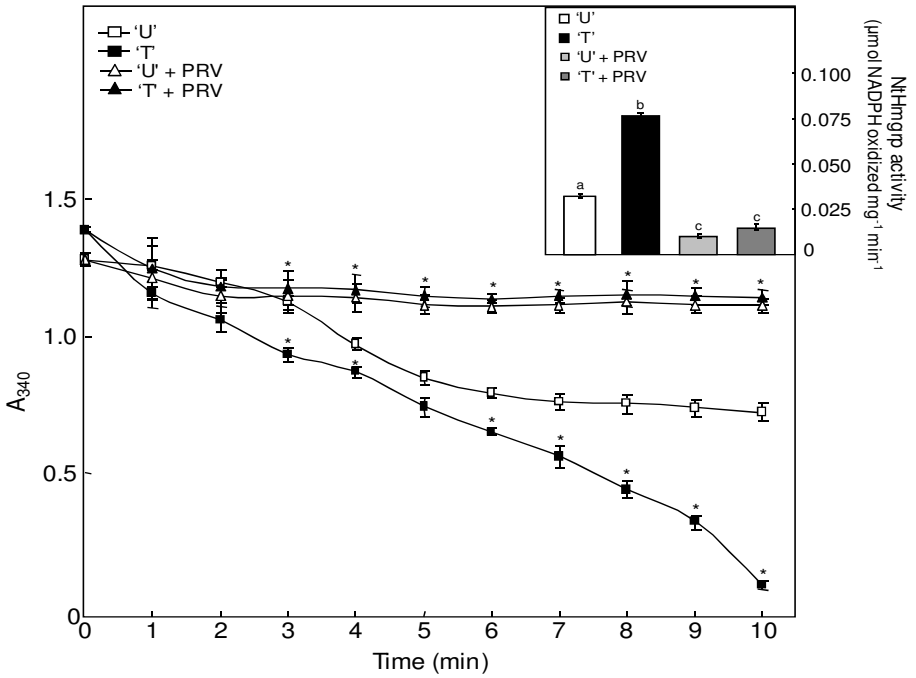

Figure 3. NtHMGR activity measurement

Differential NtHMGR activity from 'U' and 'T' cellular-extract (~50 µg) in the absence or presence of reference statin drug, PRV (~30 µM) was measured. NADPH consumption rate was monitored at 37 °C at 340 nm by spectrophotometric-scanning for 10 min. The specific activity of 'U' and 'T' calculated between 60-600 s was observed to be 0.03 and 0.08 µmol NADPH oxidized mg^{-1} (protein) min^{-1}, respectively. The inset depicts HMGR specific activity of each sample at the end of test period (10 min). Means ± SDs, n = 3, wherein * indicate the significant differences ($p \leq 0.05$). PRV - Pravastatin, 'U'- Untreated and 'T' - GA_3 treated plantlets.

GA_3 enhances the transcriptional rate of NtHMGR1

Based on the aforementioned observations, we anticipated that GA_3 mediated enhanced *NtHMGR1* transcript level concomitant with its enhanced functional activity could be attributed to its altered promoter activity. Henceforth, *NtHMGR1* promoter activity was evaluated by measuring the transcriptional rate of *NtHMGR1*. For this, an optimized TRO assay was performed (Manoharlal *et al*., 2019). Our results demonstrated a relative and significant induction (RFC ~3.06) in transcriptional rate of *NtHMGR1* in 'T' as compared to 'U', without having a significant impact on transcriptional rate of constitutive-expressed 18S rDNA (Figure 4A and B). The validity of TRO assay was confirmed by pre-incubating the permeabilized

nuclear-fractions of 'U' and 'T' with an effective and potent *de novo* transcriptional inhibitor, cordycepin (COR, 200 µg ml^{-1}) (Figure 4A and B). The solvent-control of COR (in DMSO) established the specific transcriptional inhibition by COR (Figure S4 Suppl.). Overall, these results showed GA_3 as a specific transcriptional inducer of *NtHMGR1*.

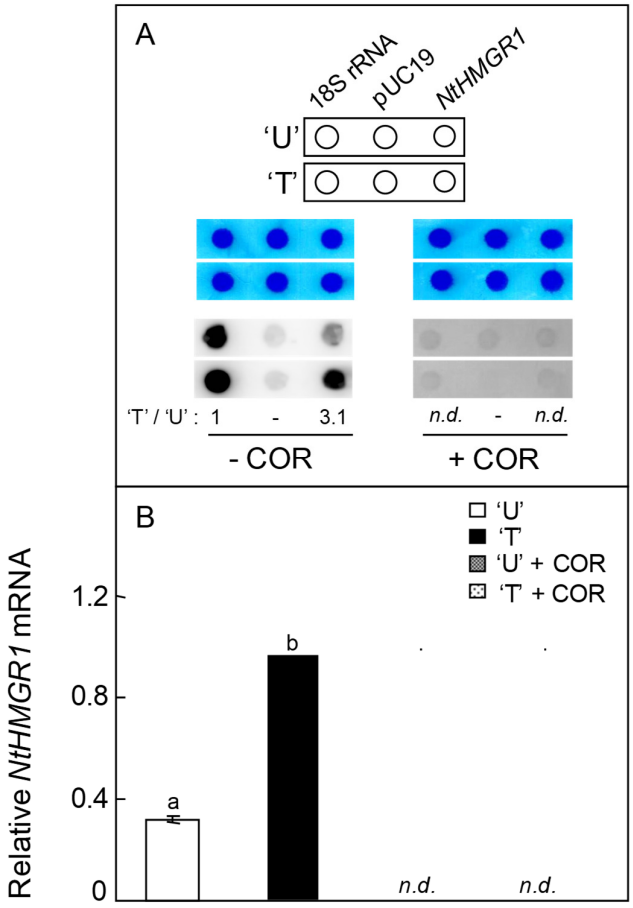

Figure 4. Measurement of *NtHMGR1* transcriptional rate

A. Approximately 100 ng of empty-vector DNA, pUC19 (negative-control), 18S rDNA (positive-control) and *NtHMGR1* PCR amplicon were dot-blotted on nylon membranes. The blots were probed with α-[^{32}P] UTP-labelled nascent TRO transcripts derived from permeabilized nuclear-fractions of 'U' and 'T'. As a control for nascent transcript synthesis inhibition, a pre-treatment of isolated nuclear-fraction of 'U' and 'T' with an optimized concentration (200 µg ml^{-1}) of COR was also performed. The position of individual dot-blot DNA, methylene-blue stained dotted DNA (loading-control) and recognition of dotted DNA by hybridized labelled nuclear RNA of 'U' and 'T' are shown in the *upper-*, *middle-* and *bottom*-panel, respectively. The signal-intensity of hybridized nuclear RNA of *NtHMGR1* for each sample was quantified by densitometry scanning of phosphoimages. The indicated ratio under the blot signifies the base-corrected nuclear RNA intensity between the indicated samples. B. Bar-graph indicates the relative signal-intensity of *NtHMGR1* transcript with respect to corresponding 18S rRNA. Means ± SDs, n = 2, wherein data marked with different letters indicates the significant differences ($p \leq 0.05$). *n.d.* - not detected, COR - Cordycepin, TRO - Transcription Run-On, '- COR' - without COR and '+ COR' - with COR addition, 'U'- Untreated and 'T' - GA_3 treated plantlets.

GA_3 enhances the mRNA stability of NtHMGR1

To test that along with transcriptional control, if post-transcriptional control (mRNA stability, in specific) also contributes to the observed enhanced accumulation of *NtHMGR1* transcripts, we measured its

mRNA stability in 'U' and 'T'. For the same, COR chase assay was performed. Our previous study has demonstrated that 200 µg ml^{-1} COR inhibited (~90%) *de novo* transcription of a very unstable transcript encoded by expansin-like protein 2 (*NtEXPA2*) in tobacco under normal growth-conditions (Manoharlal *et al*., 2019). Herein, we performed the COR chase assay using this optimised concentration of COR over a period of 0-4 h. Figure 5 *left-panel* shows the representative RNA dot-blotting of *NtHMGR*1 mRNA decay experiment. Figure 5 *right-panel* shows the normalized (with respect to corresponding 18S rRNA at each time-point of chase) quantitative decay profile of *NtHMGR1* mRNA. Notably, our results demonstrated a consistent differential *NtHMGR1* mRNA level at time T_0 in both 'U' and 'T'. However, in 'U', the normalized signal-intensity of *NtHMGR1* mRNA diminished much progressively with time with a corresponding mRNA half-life ($t_{1/2}$) of ~0.82 h (Figure 5). Conversely, the *NtHMGR1* mRNA turnover occurred relatively slower in 'T', with a corresponding $t_{1/2}$ of ~1.21 h. Notably in 'U' and 'T', the mRNA turnover of *NtEXPA2a* occurred almost at equal rate, with a characteristic $t_{1/2}$ of ~0.5 h (Figure S5 Suppl.). These results clearly demonstrated that *NtHMGR1* mRNA stability differs significantly between 'U' and 'T' and pointing out towards the specific role of GA_3 in mRNA stability of *NtHMGR1*.

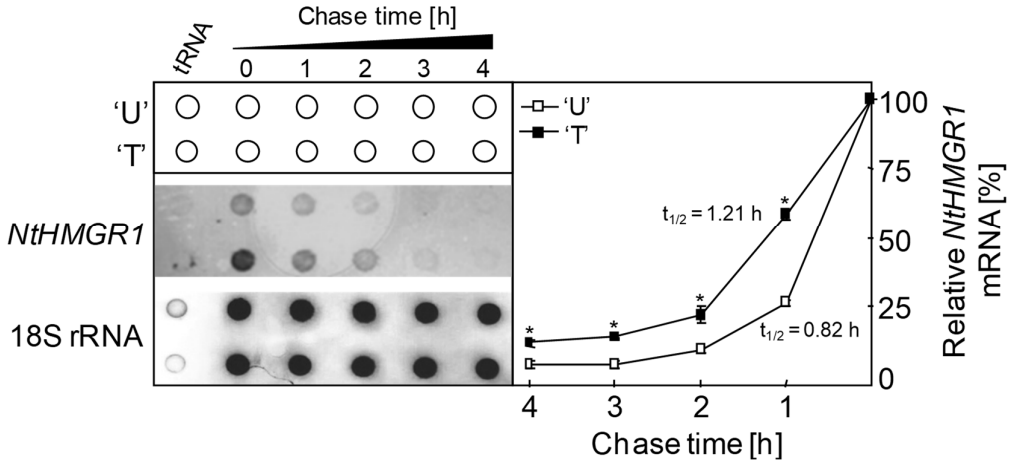

Figure 5. Measurement of *NtHMGR1* mRNA decay

Post-addition of an optimized concentration (200 µg ml^{-1}) of COR, an *in vivo* transcriptional-inhibitor, total RNAs was isolated from 'U' and 'T' at the indicated time of chases (0, 1, 2, 3 and 4 h) and thereafter subjected to dot-blot hybridization (~2 µg dot^{-1}). The relative position of individual dot-blot and recognition of dotted RNA by hybridized α-[^{32}P]dATP-labelled DNA probes of *NtHMGR1* and 18S rDNA are shown in *upper*-, *middle*- and *bottom*-panel, respectively (*left-panel*). The signal-intensity for hybridized *NtHMGR1* transcripts from 'U' and 'T' at each time-point was quantified by densitometry-scanning and normalized to the corresponding constitutively expressed 18S rRNA. Normalized signal-intensity of *NtHMGR1* transcript for 'U' and 'T' with respect to their corresponding time T_0 was plotted as line-graph and expressed in % (*right-panel*). *NtHMGR1* mRNA $t_{1/2}$ was calculated by fitting non-linear regression. Means ± SDs, $n = 2$, * indicate the significant differences ($p \leq 0.05$). COR - Cordycepin, $t_{1/2}$ - half-life, 'U'- Untreated and 'T' - GA_3 treated plantlets

Discussion

One of the most challenging aspects in the study of plant terpenoids or any other biosynthetic pathway *per se*, is the identification, functional-characterisation and regulation of key enzyme(s) that catalyses the rate-limiting step(s) in their corresponding pathway(s). The over-expression (either by genetic-engineering or elicitors) of endogenous key enzymes in a particular metabolic-pathway, however, mostly fails to enhance the yield of corresponding end-product(s) either due to negative-feedback or down-stream rate-limiting steps (Vranová *et al*., 2013; Soma *et al*., 2014). Thereby, the regulatory mechanism(s) of rate-liming enzyme need to

be characterized in more detail. In higher plants, *HMGR* is one of such essential, committed and first rate-limiting enzyme of MVA pathway that plays an important role in the regulation of whole terpenoid pathway (Friesen and Rodwell, 2004). *HMGRs* driven carbon-flow towards MVA pathway of terpenoid metabolism is central to the plethora of cellular- and physiological-processes (Wagner and Elmadfa, 2003; Brahmkshatriya and Brahmkshatriya, 2013; Pichersky and Raguso, 2018), therefore, its regulation is of critical importance. Cultivated tobacco (*N. tabaccum* L.) has been considered as an ideal model-system to study the biosynthesis and function of MVA (as well as MEP) pathway derived metabolites (Hemmerlin *et al*., 2004). Plant *HMGRs* expression has been reported to mediated by a variety of developmental and environmental signals including mechanical damages (e.g. wounding), abiotic (e.g. PGRs, light and calcium etc.) and biotic stresses (e.g. pathogen infection) (Stermer *et a*l., 1994; Shaikh *et al*., 2020). Our recent and extensive study on *N. tabaccum* L. have also shown that GA_3 foliar-application under agronomical field-conditions led to over-expression of *NtHMGR1* characterised by an enhanced production of MVA metabolites (*viz.* campesterol, stigmasterol, β-sitosterol, squalene and farnesol) (Manoharlal *et al*., 2018b). However, the precise mechansitic effect of GA_3 on *NtHMGR1* induction remains elusive. To answer this question, in this study, we were prompted to evaluate the transcriptional and post-transcriptional control of *NtHMGR1* by GA_3 in tobacco.

Firstly, we mimicked our previous agronomical field observations by establishing a system wherein tobacco plantlets respond (in terms of growth-parameters) significantly to exogenous treatment of GA_3 under prescribed growth-conditions. We observed that GA_3 primed tobacco plantlets grow relatively faster, characterised by corresponding and significant increase in plantlets fresh-weight in contrast to untreated counterpart (Figure 1A and B). Expression analysis revealed that GA_3 influences a marked increase in *NtHMGR1* mRNA level (Figure 2B). Measurement of total NtHMGR activity also revealed a significant enhancement in the presence of GA_3 (Figure 3). These results were also in agreement with previous studies, wherein. treatment of tobacco Bright Yellow-2 (TBY-2) cells with farnesol (an intermediate of MVA pathway), squalestatin [an inhibitor of a squalene synthase (*SQS*)] and terbinafine [an inhibitor of squalene epoxidase (SQE)] has been linked with the concomitant enhanced mRNA level of *NtHMGRs* and/ NtHMGR activity (Wentzinger *et al*., 2002). Likewise, it has been demonstrated that mevinolin (6α-methylcompactin *a.k.a.* lovastatin, a competitive inhibitor of HMGR) treatment of TBY-2 cells leads to its efficient growth inhibition, mainly by cell-cycle arrest in late G1 phase (Hemmerlin and Bach, 1998). Similarly, transgenic tobacco as well as tomato (*Lycopersicon esculentum* M.) overexpressing *Brassica juncea* HMG-CoA synthase 1 [*BjHMGS1*, EC 2.3.3.10, the second enzyme in MVA pathway) displaying an enhanced plant growth along with pod-size and seed yield, which has been credited to the feed-forward up-regulation of native *NtHMGR1* (Liao *et al*., 2014; Liao *et al*., 2018). Considering the exogenous GA_3 mediated 'cross-talk' between various endogenous PGRs (*viz.* GAs, ET and auxin) and between MEP-MVA pathways in tobacco (Manoharlal *et al*., 2018b), a probable indirect / passive role of GA_3 in regulating the *NtHMGR1* expression cannot be ruled out. Notably, irrespective of precise physiological role of GA_3 in tobacco, all these interpretations suggested that apparent observed early-growth and development induced by GA_3 (either directly or indirectly) are either the causes or consequences of enhanced *NtHMGR1* expression and NtHMGR functional activity *via* a diverse and complex-array of interacting pathways causing a host of molecular- and physiological-consequences.

Plant growth is co-ordinately regulated by metabolic-adjustment between various physiological- and biochemical-pathways (Tohge *et al*., 2015). Our previous study has also shown that tobacco plants exposed to GA_3 exhibited increased plant-height (coupled with dense-foliage, enhanced leaf cell-area and trichome density) in concomitant with an enhanced *NtHMGR1* expression, mainly by drifting the balance in favour of primary-metabolism [*viz.* glycolysis and tricarboxylic acid (TCA) pathways], characterised by significant enhanced intracellular accumulation of acetyl Co-A (Manoharlal *et al*., 2018b). It has been hypothesised that HMGR activity is primarily regulated at transcriptional-level, whereas post-transcriptional and/ post-translational mechanisms also pitches in for a finer and faster metabolic-adjustment (Chappell *et al*., 1995).

For instance, mammalian *HMGR* despite being encoded by a single gene is subjected to multi-level feedback regulation (either at the transcriptional, post-transcriptional and post-translational level) by intermediate and/ end-products of MVA biosynthetic pathway (Friesen and Rodwell, 2004; Espenshade and Hughes, 2007; Ness, 2015; Khan *et al*., 2018; Su *et al*., 2018; Khan *et al*., 2020). Plants being sessile in nature have to evolve a dynamic and more robust gene-regulatory system in a much better way to cope up the adverse environmental calamities throughout their life-cycle. For the same, higher plants have developed multiple *HMGRs* isoform (Campos and Boronat, 1995; Loguercio *et al*., 1999; Suzuki *et al*., 2009) so that *HMGRs* transcript isoform diversity could respond not only to myriad of external cues, but also to feed-back regulation from upstream precursors (e.g. acetyl-CoA, HMG Co-A and MVA etc.) and downstream-metabolites (e.g. sterols and PGRs etc.). So far, plant *HMGRs* regulatory mechanism has been studied more intensively at the transcriptional-level only (Stermer *et al*., 1994; Jelesko *et al*., 1999; Holmberg *et al*., 2002; Wentzinger *et al*., 2002; Atsumi *et al*., 2018; Tang *et al*., 2019; Wei *et al*., 2019). In these studies too, a stringent correlation between *HMGRs* expression (mRNA and protein levels) and HMGR activity has not been always observed, thus indicating the potential existence of multi-level regulation (apart from transcriptional control) do exists in plants too that can explain the gaps between its expression and activity (Hemmerlin and Bach, 1998; Korth *et al*., 2000). Numerous endeavours have also demonstrated the GA_3 role in transcriptional, post-transcriptional and/ post-translational control of gene expression (Shi and Olszewski, 1998; Gubler *et al*., 2002; Zhang *et al*., 2018; Manoharlal *et al*., 2019). In our previous study, we have also mentioned that GA_3 causes transcriptional suppression and reduced mRNA half-lives of tobacco DNA methyltransferases genes, *NtMET1* and *NtCMT3* (Manoharlal *et al*., 2019). Henceforth, we were prompted to evaluate the influence of GA_3 on transcriptional rate and mRNA-stability of *NtHMGR1*. Our TRO analysis demonstrated a significant transcriptional activation of *NtHMGR1* by GA_3 (Figure 4). Notably, GA_3 specifically causing the transcriptional activation of *NtHMGR1*, indicating that either *de novo* synthesis or inhibition of specific-factor(s) are required for the same. Thereby, the observed transcriptional activation of *NtHMGR1* by GA_3 could also be attributed to differential contribution of *cis*-acting element(s) and/ corresponding *trans*-acting regulatory-factor(s) within the *NtHMGR1* promoter. Moreover, an epigenetic role of GA_3 in genome-wide transcriptional modulation in tobacco has been advocated recently (Manoharlal *et al*., 2018a; Manoharlal *et al*., 2018b; Manoharlal *et al*., 2018c). Considering these facts, GA_3 mediated differential chromatin-architecture, DNA methylation and 'histone-code' status of *NtHMGR1* promoter attributing to its enhanced transcriptional rate also cannot be ruled out. However, these findings need to be supported by experimental evidences to make any substantial claim.

As stated above, apart from well-defined transcriptional modulation in plants, evidences of *HMGR* post-transcriptional control are still scarce. A few studies have advocated a probable existence of post-transcriptional and/ post-translational regulation of *HMGR* using TBY-2 cells (as well as *Arabidopsis*) treated with MEV, squalestatin and terbinafine *(*Hemmerlin and Bach, 2000; Wentzinger *et al*., 2002; Kobayashi *et al*., 2007; Nieto *et al*., 2009). Notably, steady-state mRNA levels represent a dynamic balance between transcription rate and mRNA degradation. In our study, we observed that transcriptional activation by GA_3 was relatively lower than corresponding induced steady-state mRNA level (RFC ~3.06 *vs* ~4.23; Figures 2B and 4), indicating that transcriptional activation of *NtHMGR1* could only partially explain the reason for its enhanced steady-state transcript levels by GA_3 and the prevalence of post-transcriptional control involving its altered mRNA stability cannot be ignored. To confirm this possibility, COR chase assay was performed to determine the differentially induced mRNA stability of *NtHMGR1* by GA_3. Our COR chase assay revealed that GA_3 causes a specific and relative enhanced mRNA half-life of *NtHMGR1* (Figure 5). Notably, COR by itself does not have any significant impact on HMGR activity (Brooker and Russell, 1979). A similar sort of tissue-specific mechanism has been also anticipated in tomato, wherein altered *LeHMGR1* expression in

ripened-fruit has been linked to its enhanced mRNA stability (Jelesko *et al*., 1999). In support to these observation, recently in an aromatic plant Kesum (*Persicaria minor*), characteristic involvement of microRNAs (miRNAs) as post-transcriptional regulator (controlling the mRNA turnover *via* miRNAs-induced silencing complex) of MVA pathway specific genes (e.g. *HMGR*, *MVK*, *MVD* and *FPPS*) in response to biotic-elicitor (pathogenic fungus, *Fusarium oxysporum*) has been demonstrated (Samad *et al*., 2019). The biological implication of such multi-level regulation of *NtHMGR1* seems to ensure a range of physiological benefits to tobacco in rapid response to GA_3 induced metabolic-fluctuations. It is worth mentioning here that various post-translational mechanisms (e.g., protein-degradation, inhibition by calcium and reversible phosphorylation at a conserved site of the catalytic-domain) regulating *HMGRs* have been also proposed (Russell *et al*., 1985; Dale *et al*., 1995; Korth *et al*., 2000). For instance, in tobacco, despite NtHMGR activity inhibition by MEV and farnesol, an apparently observed enhanced NtHMGR accumulation and activity has been hypothesised to its increased translation or decreased degradation through feed-back regulatory mechanism (Hemmerlin *et al*., 2003). Thereby, the possible post-translational control of NtHmgr1p by GA_3 also cannot be ruled out at this stage.

Conclusions

Overall, our study concluded that GA_3 (directly / indirectly) exerts a transcriptional and post-transcriptional control on *NtHMGR1* expression and NtHMGR activity, characterized by its enhanced transcriptional rate and mRNA stability. Our findings thus, sets a platform for future studies in dissecting the regulatory pathways of terpenoids biosynthesis that could have significant impact on yield of agronomical important crops and production of commercially important terpenoids. Notably, the precise molecular determinants of the observed phenomenon are still lacking. Henceforth, our future in-depth gene-specific studies involving epigenetics (to characterise the differential chromatin-architecture, DNA methylation and 'histone-code profiling), *NtHMGR1* promoter, 5'-UTR and 3'-UTR analysis [to identify and characterise the GA_3 responsive element and corresponding binding-factor(s)], NtHmgr1p stability and site-directed mutagenesis of NtHmgr1p [to identify and characterise its ubiquitination and/ phosphorylation site(s)] will be of enormous value in better understanding of terpenoid metabolism, its metabolic-engineering and to decode the complex signalling-pathways induced by GAs.

Authors' Contributions

RM and RP designed the experiments. RM drafted the manuscript. LD performed the experiments. GVSS guided and facilitated the research. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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