

# Dissecting the transcriptional networks underlying the gibberellin response in *Nicotiana tabacum*

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## Abstract

The present work examines global transcriptional and limited metabolic profiling to dissect the signalling pathways associated with gibberellin A<sub>3</sub> (GA<sub>3</sub>) induced alterations in tobacco (*Nicotiana tabacum* L.) under field conditions. Our microarray analysis revealed that GA<sub>3</sub> foliar application elicited 7 032 differentially expressed genes (DEGs) with 3 507 and 3 525 genes displaying up- and down-regulation, respectively. These DEGs broadly belong to diverse metabolic pathways: GA signalling and homeostasis, ethylene and auxin biosyntheses, saccharide metabolism, glycolysis-tricarboxylic acid cycle, terpenoid biosynthesis, shikimate-phenylpropanoid pathway, S-adenosyl methionine cycle, fatty acid biosynthesis, cell wall metabolism, oxidative stress, DNA repair, and stress responses. Transcription validation of representative DEGs was confirmed by real-time quantitative PCR. A relative enhancement in cellular acetyl-CoA pool corroborated with a concomitant increase and decrease in transcriptions as well as activities of histone acetyltransferases and histone deacetylases, respectively. Phytochemical analysis shows an enhanced content of total sugars, reducing sugars, total flavonoids, and phenolics with a concomitant decrease of the content of starch, chlorophylls *a* and *b*, and carotenoids. Gas chromatography with mass spectrometry analysis revealed the presence of 30 differential metabolites, mostly belonging to terpenoid biosynthesis pathway.

*Additional key words:* differentially expressed genes, GC-MS, HPLC, microarray, *Nicotiana tabacum*.

## Introduction

Plants are usually adapted to complex and ever-changing environmental stresses by adjusting their biochemical and cellular machineries at numerous levels to maintain their survival and growth. Phytohormones, a diverse group of signalling molecules produced endogenously in very low concentrations, mediate these responses (reviewed by Verma *et al.* 2016). Phyto-hormones act either locally at their site of synthesis or systemically following their transport (Verma *et al.* 2016). These include auxin –

indoleacetic acid (IAA), cytokinins (CKs), abscisic acid (ABA), ethylene (ET), gibberellins (GAs), salicylic acid (SA), brassinosteroids (BRs), jasmonates (JAs), and strigolactone (SL). Among these, the importance of plant-specific GAs in vegetative and reproductive development has been well documented (Sun and Gubler 2004).

Gibberellins, constituting a large family of plant-specific tetracyclic diterpenoid compounds, control plant growth and development throughout their life cycle under

*Submitted* 8 August 2017, *last revision* 4 February 2018, *accepted* 12 February 2018.

**Abbreviations:** ABA - abscisic acid, BRs - brassinosteroids; CDP-ME - 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol; CK - cytokinin; DEG - differentially expressed gene; DDR - DNA damage response; DOX - 1-deoxy-D-xylulose-5-phosphate; ET - ethylene; GA - gibberellin; GC-MS - gas chromatography with mass spectrometry; GO - gene ontology; HAT - histone acetyltransferase; HDAC - histone deacetylase; HPLC - high-performance liquid chromatography; IAA - indoleacetic acid; JA - jasmonic acid; MEP - 2-methyl-D-erythritol-4-phosphate; MVA - mevalonate; NtDNMT - *N. tabacum* DNA methyltransferase; RT-qPCR - quantitative real time PCR; PEPC - phosphoenolpyruvate carboxylase; PFK - phosphofructokinase; PGK - phosphoglycerate kinase; PGM - phosphoglyceromutase; PK - pyruvate kinase; PTM - post-translational modification; RFC - relative fold-change; ROS - reactive oxygen species; SA - salicylic acid; SAM - S-adenosyl methionine; SL - strigolactone; TCA - tricarboxylic acid cycle; TF - transcription factor; TFC - total flavonoid content; TPC - total phenolic content.

**Acknowledgments:** We would like to acknowledge Dr. C.C. Lakshmanan (the head of the ITC Life Science and Technology Centre) for his consistent support. Genotypic Technology, Bengaluru, India is acknowledged for performing microarray processing and data analysis. We also acknowledge field-workers at Northern Light Soil region, Rajahmundry, Andhra Pradesh, India, for providing their consistent help and support during field experiment and sample collection.

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non-stress conditions and play a pivotal role in abiotic stress tolerance (reviewed by Richards *et al.* 2001). Even though, a hundred of GAs were identified in the plant kingdom, only a few are known to be bioactive (*e.g.*, GA<sub>1</sub>, GA<sub>3</sub>, GA<sub>4</sub>, and GA<sub>7</sub>) (Hedden and Phillips 2000, Sun and Gubler 2004). Among them, GA<sub>3</sub> (the first GA structurally characterized) controls important aspects of plant development including seed germination, leaf expansion, stem elongation, flowering, and seed development (Richards *et al.* 2001).

Recent studies have also uncovered the new role of GA<sub>3</sub> in diverse physiological processes including leaf differentiation, photo-morphogenesis, and pollen-tube growth (Hauvermale *et al.* 2012). Significant contributions towards understanding GA-regulated morphogenesis including identification of its upstream receptors, elucidation of its signalling component functions, and isolation of downstream targets were reviewed by Thomas and Sun (2004). Genetic and biochemical analyses revealed that GA depresses its signalling by

destabilization of DELLA protein growth repressors *via* 26S proteasome pathway (Hedden and Phillips 2000, Sun and Gubler 2004). Recently, we demonstrated a novel epigenetic impact of GA<sub>3</sub> in global euchromatinization and DNA hypo-methylation in tobacco (Manoharlal *et al.* 2018).

Recently, modulation in transcription of 163 genes and metabolic adjustment in response to changes in plant growth in the presence of varying GA concentrations was evaluated in GA over-accumulated, wild-type, and GA down-regulated tobacco plants (Li *et al.* 2015). Nevertheless, aside from a circumstantial evidence, previous studies provide a very limited insight into the overall physiological role of GAs in *N. tabacum* and only under laboratory conditions. Hence, the aim of the present investigation was to identify GA-affected genes at the global level as well as to dissect the underlying transcriptional networks and metabolic pathways in *N. tabacum* grown under field conditions with GA<sub>3</sub> foliar application.

## Materials and methods

**Plants and growth conditions:** Tobacco (*Nicotiana tabacum* L.) cv. Kanchan was used in this study. The stock of 50 mg dm<sup>-3</sup> GA<sub>3</sub> in 25 % (v/v) ethanol was prepared freshly before use. Field trials were conducted at Northern Light Soil (NLS) region, Rajahmundry, Andhra Pradesh, India. Tobacco seedlings were transplanted from a nursery to the main-field (60 d after sowing) in 3 plot replicates. Foliar spray with GA<sub>3</sub> (50 mg dm<sup>-3</sup>) was done only once at 30 d after transplantation (DAT) in such a way that each plant leaf at all positions was fully drenched. A non-ionic surfactant APSA-80 (Amway, New Delhi, India) of 0.5 cm<sup>3</sup> dm<sup>-3</sup> in water was used as a spreading/wetting agent during foliar application. Control (GA<sub>3</sub>-untreated) plants were sprayed with GA<sub>3</sub> solvent, *i.e.*, 25 % ethanol. After GA<sub>3</sub> application, 10 uniform untreated (control) and GA<sub>3</sub> treated plants were selected. For microarray, gas chromatography with mass spectrometry (GC-MS), and biochemical analyses [*viz.* mRNA accumulation, estimation of chlorophylls *a* and *b*, carotenoids, soluble and reducing sugars, starch, total flavonoid content (TFC), total phenolic content (TPC), and enzymatic assays], leaf samples were harvested from the middle-position (generally the 10<sup>th</sup> - 11<sup>th</sup> leaf from the bottom of plant) of five representative plants at the indicated time of growth, immediately snap frozen in liquid nitrogen, ground to a fine powder, and stored at -80 °C till further use. Notably in the present study, an early-vegetative growth stage (13 d after GA<sub>3</sub> foliar application) was chosen for aforementioned analysis as a highly significant difference in global DNA hypo-methylation, and DNA methyltransferases activities were observed previously at this particular stage (Manoharlal *et al.* 2018).

**Gene transcription measurements:** The total RNA was extracted from leaves of control and GA<sub>3</sub>-treated plants using a TRIzol<sup>®</sup> reagent (Ambion, Bengaluru, India) as per manufacturer's instructions. The quality and integrity of the extracted RNA was checked with a bioanalyzer Agilent 2100 (Agilent Technologies, Palo Alto, CA, USA). The isolated RNA samples were stored at -80 °C till further use. A customized gene transcription tobacco 1×1 M array chip (AMADID: 043314) was designed based on the transcriptome data (high throughput RNA-sequencing) as per standard methodology used by Genotypic Technology Private (Bengaluru, India; <http://www.genotypic.co.in>). The 1 × 1 M array chip covers a total of 974 016 features with 6 001 Agilent control features and 968 015 available features to be filled in by probes. After a preliminary array analysis, a final array of 8 × 6 000 format (AMADID: 049727) was designed by selecting the subset of probes from 1 × 1 M array based on unique probes with gene transcripts with and without annotation. The 8 × 6 000 array covers 62 976 features including 1 319 Agilent control features.

The RNA from three biological replicates of each sample was used to perform the microarray experiments. The total RNA was reverse transcribed using an oligodT primer tagged to a T7 polymerase promoter and converted to double stranded cDNA, which was subsequently converted to cRNA by *in vitro* transcription and labeled with dye Cy3 CTP using an Agilent Quick-Amp labeling kit (Agilent Technologies, Santa Clara, USA). The labeled cRNA was cleaned up using RNeasy columns (Qiagen, Valencia, CA, USA). The labeled cRNA sample (600 ng) was fragmented at 60 °C and hybridized onto customized gene transcription tobacco

8 × 6 000 arrays. Fragmentation of labeled cRNA and hybridization were done using a gene expression hybridization kit (*Agilent Technologies*). Hybridization was carried out in *Agilent Surehyb* chambers at 65 °C for 16 h. The hybridized slides were washed using *Agilent* gene expression wash buffers and scanned using an *Agilent* microarray scanner.

Image analysis and quantification were performed for the washed and scanned array using the feature extraction software (*Agilent*, v. 11.5). Feature extracted raw data were normalized by the 75<sup>th</sup> percentile shift method and analyzed using *GeneSpring GX* (*Agilent*, v. 12). Transcripts with a relative fold change of  $\geq 2$  ( $\log_2FC \geq 1$ ) were marked as being significantly differentially expressed between the two groups compared, and hierarchical clustering analysis was performed to identify significant gene transcription patterns. Gene ontologies (GOs) for the annotated transcripts were retrieved from the *UniProt* database (<http://www.uniprot.org/>). The functional classification of differentially regulated transcripts was performed based on their GOs.

**Real-time quantitative PCR:** The total RNA was extracted using a *GeneJET* plant RNA purification mini kit (*Thermo Fisher Scientific*, San Jose, CA, USA) following the manufacturer's protocol. The RNA was treated with RNase free DNase I (*Thermo Fisher Scientific*) and reverse-transcribed by a high capacity cDNA reverse transcription kit (*Applied Biosystems*). The cDNA synthesized was amplified under standard PCR conditions followed by melt-curve analysis using *Power SYBR® Green PCR Master Mix* (*Applied Biosystems*) with a *7500 Real-Time PCR* system as per manufacturer's instructions (*Applied Biosystems*). A list of primers is mentioned in Table 1 Suppl. A  $\Delta C_T$ -value for each sample was calculated using the cycle threshold ( $C_T$ ) method. The amount of individual mRNA was normalized to transcription of a constitutively expressed endogenous house-keeping gene encoding 18S rRNA and presented as relative-fold changes with respect to the control sample.

**Measurement of biochemical traits:** Biochemical analyses were performed from snap-frozen and fine grounded control and GA<sub>3</sub>-treated leaf samples, based on spectrophotometric measurements as described earlier (Laware *et al.* 2015). Sample analysis was performed by a sequential (2 steps) extraction of metabolites. Each extract was prepared freshly for the analysis to prevent any degradation.

Step 1: Exactly, 0.5 g of ground material was used for extraction of photosynthetic pigments by 80 % (v/v) acetone. Chlorophyll (Chl) a, b, a+b, and carotenoids were estimated by measuring the absorbance at 645, 663, and 470 nm according to Lichtenthaler and Welburn (1983). Step 2: The pellet obtained in step 1 was extracted twice with 2 cm<sup>3</sup> of 80 % (v/v) methanol in a

boiling water bath for 30 min. The supernatant obtained in step-1 (chlorophyll extract) and step-2 were pooled and used for estimation of soluble and reducing sugars, TFC, and TPC. Soluble sugars content were estimated with anthrone reagent by method of Hansen and Møller (1975). Reducing sugars were estimated with 3,5-dinitro-salicylic acid (DNSA) reagent according to Miller (1959) method. The aluminium chloride method was used for determination of TFC (Silva *et al.* 2015). TPC was estimated by using Folin-Ciocalteu reagent (Kim *et al.* 2003). The residue obtained after step-2 was used for extraction of starch (Laware *et al.* 2015). Starch was estimated with anthrone reagent according to Hansen and Møller (1975).

#### High performance liquid chromatography (HPLC)

**analysis:** The CoA derivatives in samples were extracted and analyzed using an HPLC method and conditions as described by Tumaney *et al.* (2004). The identities of putative CoA esters were also confirmed by their sensitivity to a mild alkali. For this, aliquots (150 mm<sup>3</sup>) of trichloroacetic acid soluble extracts were also treated with concentrated (14.5 M) NH<sub>4</sub>OH (25 mm<sup>3</sup>) at room temperature for 15 min prior to HPLC analysis. Under these conditions, the acyl-CoAs were hydrolyzed and free CoA-SH released.

#### Gas chromatography-mass spectrometry (GC-MS)

**analysis:** The sample preparation protocol was based mainly on the method described by Mabiki *et al.* (2013) with certain modifications. Briefly, the metabolites were extracted using 1.0 g of fine grounded (40-mesh sieve) fresh mass using a 40 cm<sup>3</sup> of dichloromethane (DCM) at 4 °C for 48 h. The filtered and air dried extracts were reconstituted in 2 cm<sup>3</sup> of DCM, centrifuged at 6 000 g, and transferred to glass vials, from which 1 mm<sup>3</sup> was used for injection into the GC-MS. Chromatography was performed with a *Shimadzu* (Kyoto, Japan) *GCMS TQ 8030* equipment harboring a *20is* automated sample injection system, split/splitless injector, 30 m length, 0.25 mm inner diameter, and 0.25 µm film thickness *DB-5MS* fused silica capillary column coated with 5 % (m/v) phenylmethyl siloxane (*J & W Scientific*, Folsom, CA, USA). An oven temperature was held at 60 °C with a 3 min hold and a ramp up of 3 °C min<sup>-1</sup> up to 300 °C with a 7 min hold with a total run time of 90 min. The injection port and detector temperature were maintained at 220 °C and 250 °C, respectively, on a split less mode with an injection volume of 1 mm<sup>3</sup> and a helium flow rate of 1 cm<sup>3</sup> min<sup>-1</sup>. All data were obtained by collecting the full-scan mass spectra within a scan range of 40 - 550 atomic mass unit. The percentage composition of crude extract constituents was estimated by percentage of peak area. The identification and characterization of individual compounds in crude extracts was based on GC retention time by comparing the obtained mass spectra with those of computer matched standards available in mass

spectrum libraries, the National Institute of Standards and Technology (Gaithersburg, MD, USA).

**Measurement of enzyme activities:** Nuclear extracts were prepared from control and GA<sub>3</sub>-treated leaf samples using a *CellLytic™* PN plant nuclei isolation/extraction kit (Sigma, St. Louis, USA) following the manufacturer's instructions. Briefly, 5 µg of verified nuclear protein extract was used for ELISA based colorimetric assay.

Activity of DNA methyltransferases (NtDNMTs) was determined using an *EpiQuik DNMT* activity/inhibition assay ultra kit (Epigentek, Farmingdale, USA). A reference DNMT inhibitor, genistein (25 µM), was used as a positive control.

Activity of histone acetyltransferases (HATs) was

measured using an *EpiQuik™* HAT activity/inhibition assay kit (Epigentek). A reference HAT inhibitor, anacardic acid (50 µM), was used as a positive control.

Activity of histone deacetylases (HDACs) was measured using an *Epigenase™* HDAC activity/inhibition direct assay kit (Epigentek). A reference HDAC inhibitor, trichostatin A (TSA; 5 µM), was used as a positive control.

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

## Results and discussion

In order to get a global insight of transcriptional response as well as to identify the molecular events following GA<sub>3</sub> foliar application in the *N. tabacum* transcriptome, we performed a microarray experiment using a customised 60 K DNA microarray chip of *N. tabacum* on the Agilent platform. Analyses of DEGs revealed 7 032 genes, among which 3 507 (49.87 %) and 3 525 (50.12 %) were significantly ( $\geq 2$  fold) induced or repressed, respectively (Fig. 1). DEGs modulated by GA<sub>3</sub> application were

analyzed for GO term enrichment to identify the cellular processes affected by GA<sub>3</sub> application as manifested by a relative difference ( $\geq 2$  fold) at the transcript level, DEGs were categorized (according to their annotated and putative functions) into 29 biological functional categories (Fig. 1). Real-time qPCR was used to validate the results obtained from microarray data. For this, representative genes from six diverse functional categories [epigenetics, hormone signalling (including GA,

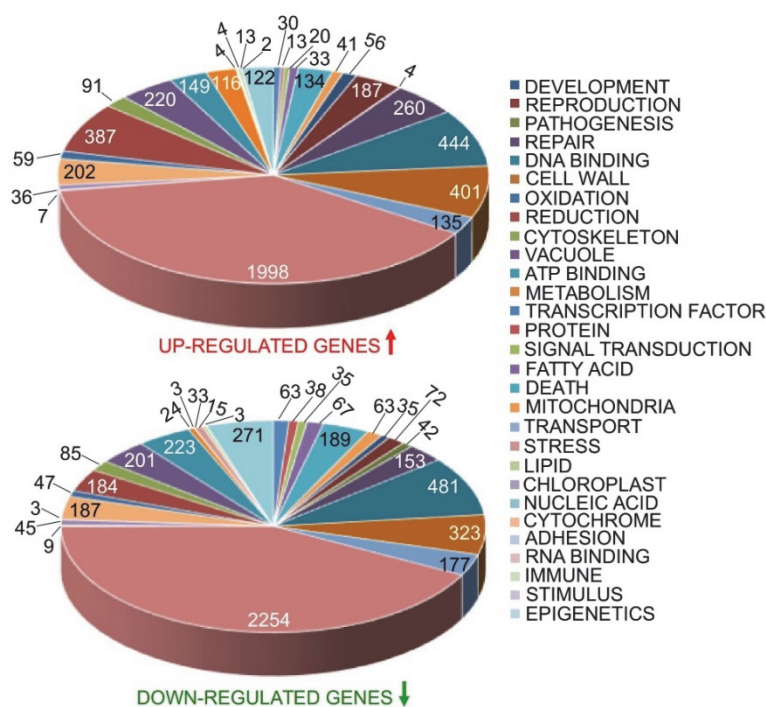


Fig. 1. Genome-wide transcriptome profiling *Nicotiana tabacum* following GA<sub>3</sub> foliar application performed on a 60 K customized DNA microarray chip. A total of 7 032 differentially expressed genes (DEGs) with 3 507 (49.87 %) and 3 525 (50.12 %) displaying up- and down-regulation, respectively, were identified. Functional categories of DEGs are represented as the numbers of total genes that were up- (top) and down- (bottom) regulated.

ethylene and auxin), sugar metabolism, terpenoid mevalonate/2-methyl-D-erythritol-4-phosphate (MVA/MEP) biosynthesis pathway, shikimate-phenylpropanoid pathway, and cell wall metabolism] were evaluated. The steady-state mRNA content of selected genes was quantified relative to mRNA content of constitutively expressed house-keeping gene *18S rRNA*. The differential mRNA transcription pattern observed was largely in accordance with each other in all the cases with a high degree of positive correlation ( $r \sim 90.73\%$ ) between microarray and qPCR results (Fig. 2 A-F). The high degree of consistency obtained by two independent methods indicates the reliability of our results and corroborates the accuracy and robustness of the high-density microarray experimental system.

In our previous study, we observed that exogenous GA<sub>3</sub> application stimulates alterations in chromatin architecture by its transition from a condensed or ‘closed’ (heterochromatin) to relatively de-condensed or ‘open’ (euchromatin) form (Manoharlal *et al.* 2018). This finding is further supported by a concomitant decrease in transcription of a switch/sucrose non-fermentable (*SWI/SNF*) nucleosome remodeling complex subunit *SMARCC2* (relative fold change, RFC = -2.06), a regulator of chromosome condensation (*RCC1*, RFC = -5.21), and chromatin assembly factor 1 subunit A (*CHAF1A*, RFC = -2.50) that were reported to be involved in chromatin remodeling by alteration of DNA-nucleosome topology (Speranzini *et al.* 2016). Methylation of DNA, histone acetylation, and methylation of histone H3 at lysine 9 (H3K9) are three best-characterized covalent modifications associated with an altered chromatin state (Fuks 2005). Specific DEGs identified to be involved in cellular DNA histone methylation/acetylation have been sub-categorized under an ‘epigenetics’ category. Among these, some of the genes that contribute either directly or indirectly to previously observed global DNA hypomethylation by GA<sub>3</sub>, for example methyl transferases 1 (*MET1*), chromomethylase (*CMT3*), and repressor of silencing 1 (*ROS1*), were identified. Transcriptions of *MET1* (RFC = -2.55) and *CMT3* (RFC = -7.67) were found to be significantly reduced, whereas that of their antagonist *ROS1* significantly increased (RFC = 4.76). Among four groups of functional DNA methyl transferases [*DNMTs*: *MET1*, chromomethyltransferases 3 (*CMT3*), domains rearranged (cytosine-5) DNA methyltransferase (*DRM2*), and (cytosine-5) DNA methyltransferase 2 (*DNMT2*)] in plants, *MET1* is considered to play a major role in maintaining symmetric ‘mCpG’ methylation patterns (Finnegan and Kovac 2000). The *CMT* family, unique to plants, was reported to be involved in methylation cytosine residues at symmetric non-CG sites ‘mCpHpG’ (where H = A, C, or T) (Lindroth *et al.* 2001). A *ROS1* is a bi-functional DNA glycosylase/lyase (a DNA demethylases, *DEMETEER* family of DNA glycosylases) that participates in transcriptional gene silencing by active de-methylation of

target promoter DNA. Overexpression of *ROS1* leads to a reduced level of mC and to a subsequent increase in transcription of target genes in *A. thaliana* (Agius *et al.* 2006). Notably, *DNMTs* alone do not act in methylating DNA. It is probable that active or passive DNA demethylation is also linked with underlying patterns of histone post-translational modifications (PTMs). There is evidence that DNA methylation and histone PTMs are directly inter-connected, dependent on one another, and can be mediated by biochemical interactions between *DNMTs*, a su(var)3-9, enhancer-of-zeste and trithorax (SET) domain of histone methyltransferases (*HMTs*), and a histone acetyltransferases (*HATs*)/histone deacetylases (*HDACs*) ratio. For example, in plants and fungi, DNA methylation machinery teams up with histone deacetylation and H3K9 methylation to generate a self-propagating cycle that promotes a long-term transcriptional repression (Fuks 2005). In our microarray data, a reduced transcription of *HDAC1* (RFC = -7.01) and H3K9 specific suppressor of variegation 3-9 homolog protein 1 (*SUVH1*) (RFC = -4.56) were also observed following GA<sub>3</sub> foliar application. Differential transcription analyses of *NtDNMTs* genes *NtMET1*, *NtDRM1*, and *NtCMT3*, *HAT1*, and *HD2a/b* were also confirmed by real-time qPCR (Fig. 3A). Consistency in a differential mRNA accumulation of *NtDNMTs* genes was also validated with respect to transcription of another constitutively expressed endogenous house-keeping gene encoding elongation factor 1- $\alpha$  (*EF1- $\alpha$* ) mRNA (Fig. 1 Suppl.). Of note, no significant difference in *NtDRM1* mRNA accumulation was observed in our study.

To verify whether GA<sub>3</sub> affected up- or down-regulation of transcriptions of identified ‘epigenetic’ genes (which also reflects the corresponding alteration in their functional activities), we further determined the enzymatic activities of DNMTs, HATs, and HDACs in leaf extracts from control and GA<sub>3</sub>-treated plants. Prior to enzyme activity measurement, the extract quality was verified using an anti-histone H4 antibody (Fig. 2 Suppl.). We observed that in contrast to the control, GA<sub>3</sub> application decreased the DNMTs and HDACs activities by ~39 and ~22 %, respectively, whereas HATs activities increased by ~35 % (Fig. 3B-D). We further evaluated the specific inhibition/activation effect of GA<sub>3</sub> on DNMTs, HDACs, and HATs activities under *in vitro* conditions. For this, GA<sub>3</sub> (20 mg dm<sup>-3</sup>) was added to the tested extracts prior to the analysis of each enzyme activity. Genistein (Gen; 25  $\mu$ M), trichostatin A (TSA; 5  $\mu$ M), and anacardic acid (AA; 50  $\mu$ M), well-known inhibitors of DNA methylation, histone de-acetylation, and histone acetylation, respectively, were used as a reference positive control for *in vitro* inhibition of corresponding DNMTs, HDACs, and HATs activities. The solvent controls of GA<sub>3</sub> (in ethanol) and Gen, TSA, and AA (in dimethyl sulfoxide, DMSO) established that *in vitro* inhibition/stimulation of enzyme activities by GA<sub>3</sub> as well as by Gen, TSA, and AA, were highly specific and

not due to the solvent addition (Fig. 3 Suppl.). As shown in Fig. 3B-D, *in vitro* GA<sub>3</sub> supplementation inhibited the DNMTs and HDACs activities, whereas increased those of HATs. Notably, the declines in DNMTs and HDACs activities by GA<sub>3</sub> addition to the extracts were higher in the control (~48 and ~28 %) compared to the corresponding extract from GA<sub>3</sub>-treated plants (~37 and ~12 %), respectively. Similarly, stimulation of HATs activity by GA<sub>3</sub> was relatively higher in the extract from control (~42 %) in comparison to the extract from GA<sub>3</sub>-treated plants (~21 %). Moreover, unlike GA<sub>3</sub>, the presence of Gen and TSA significantly decreased the corresponding DNMTs (~86 vs. ~80 %) and HDACs (~92 v.s. ~91 %) activities in both control and GA<sub>3</sub>-treated extracts suggesting that GA<sub>3</sub> is compa-

ratively less potent *in vitro* inhibitor of DNMTs and HDACs activities than Gen and TSA. Taken together, altered transcriptions and functions of *NtDNMTs*, *NtHDACs*, and *NtHATs* could be one of the major potential reason for previously observed global chromatin de-condensation and DNA hypo-methylation in the epigenome of GA<sub>3</sub>-treated plants (Manoharlal *et al.* 2018). However, the possibility of other epigenetic mechanism(s) influenced by GA<sub>3</sub> application cannot be ruled out at this stage.

To illustrate the effects of GA<sub>3</sub> application on GA biosynthesis and signalling, we investigated the changes in transcriptions of genes participated in GA<sub>3</sub> metabolic and signalling pathways. We observed that a pool of GA biosynthetic pathway related genes were strongly affected

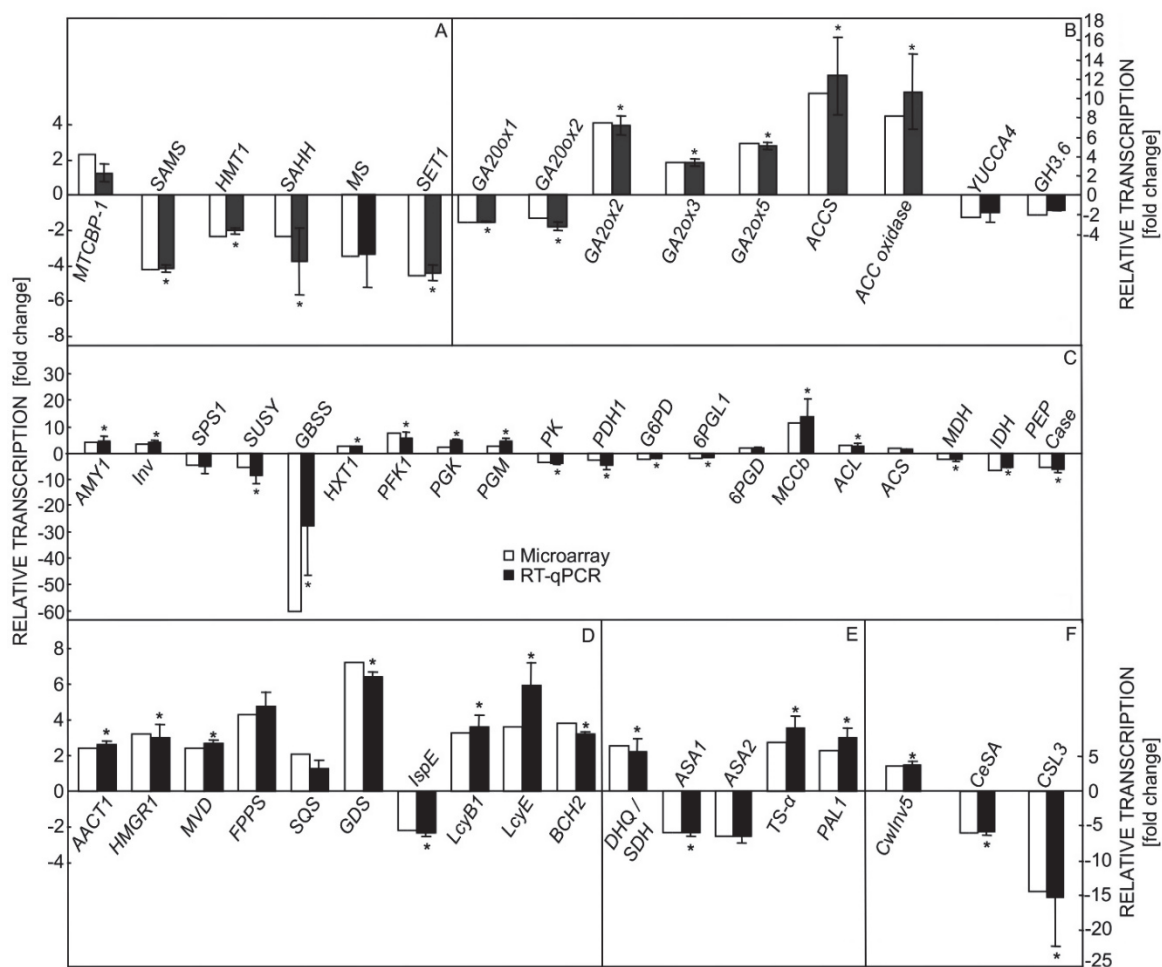


Fig. 2. Real-time quantitative (q) PCR confirmation of genes showing significant changes using microarray technique. Representative genes are from six diverse functional categories: epigenetics (A), hormone (including GA, ethylene, and auxin) biosynthesis and signalling (B), saccharides and energy metabolism (C), terpenoid biosynthesis mevalonate/2-methyl-D-erythritol-4-phosphate (MVA/MEP) pathway (D), shikimate/phenylpropanoid pathway (E), and cell wall metabolism (F). The data are depicted as a relative fold-change (RFC) in the gene transcription. Validation as well as comparison of microarray (empty bars) and qPCR analysis (filled bars) of differentially expressed genes (up- and/down-regulated) corroborated significant gene transcription changes following gibberellin A<sub>3</sub> (GA<sub>3</sub>) treatment. For qPCR, the y-axis shows the amount of individual mRNA in RFC to 18S rRNA transcription. The value of each corresponding gene for control sample was set to 1. If RFC was < 1, then the reciprocal value was taken, otherwise the original value was taken. Means  $\pm$  SDs,  $n = 3$ , asterisks indicate significant differences at  $P < 0.05$  when compared with the control.



by GA<sub>3</sub> application. Transcriptions of GA catabolic genes *viz.* *GA2ox1* (RFC = 5.31), *GA2ox2* (RFC = 7.36), *GA2ox3* (RFC = 3.39), *GA2ox4* (RFC = 12.64), and *GA2ox5* (RFC = 5.28) significantly increased. On the contrary, transcriptions of GA anabolic genes: *GA20ox1* (rate-limiting enzyme of GA biosynthesis, RFC = -2.81), *GA20ox2* (RFC = -2.43), and *GA20ox3* (RFC = -2.33) were found to be significantly reduced. Notably, *GA20oxs* and *GA20oxs* were reported to be involved in maintaining cellular homeostasis of endogenous bioactive GA level (reviewed in Colebrook *et al.* 2014). Moreover, an altered transcription of GA receptor, gibberellin-insensitive dwarf 1 (*GID1*, RFC = 2.5), acting DELLA protein components (*RGL1*, RFC = -2.73), and a downstream readout ( $\alpha$ -amylase, RFC = 4.2) also indicate suppression of GA signalling pathway upon GA<sub>3</sub> application. This was further corroborated by an observed up-regulation of apetala 2/ethylene responsive factor (*AP2/ERF*, RFC=2.71) family transcription factors (TFs), which are known to regulate the transcriptions of *GA2ox* and repressor of GA (*RGA*) like 3 (*RGL3*), thereby leading to a reduction in bioactive GAs and GA

signalling under abiotic stresses (Colebrook *et al.* 2014). Our results corroborate well with the previous findings (Li *et al.* 2015, Wang *et al.* 2015), that exogenously applied GA<sub>3</sub> attributes to a negative feedback regulation of GA biosynthesis.

Gibberellins and ethylene are known to influence plant growth by a mutual reciprocal cross-talk as well as by interaction with other hormones (Corbineau *et al.* 2014). Previous meta-analysis of transcripts also suggested that GA<sub>3</sub> application induces the transcription of some ethylene biosynthesis genes (Dugardeyn *et al.* 2008). In leaf tissue, we observed that GA<sub>3</sub> application up-regulated the transcription of 1-amino-cyclopropane-1-carboxylic acid synthase (*ACCS*; RFC = 10.48) as well as of 1-amino-cyclopropane-1-carboxylic acid oxidase (*ACO1/ACO2*; RFC = 8.17), a further proof that GA<sub>3</sub> application induced ethylene biosynthesis in plants. Moreover, our results also indicate that an enhanced transcription of these ethylene biosynthetic genes probably exerted a negative feedback as observed by down-regulation of following ethylene precursor genes from the S-adenosyl methionine (SAM) cycle: SAM-

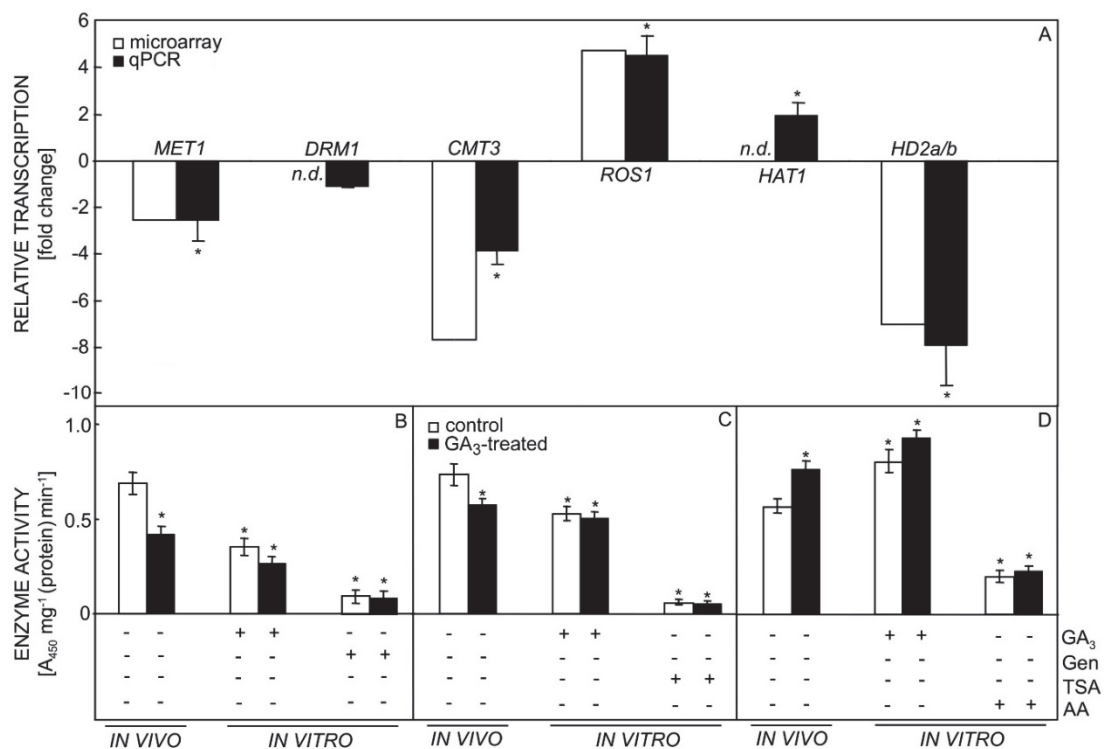


Fig. 3. Effect of GA<sub>3</sub> application on the transcription and activity of epigenetics associated genes. *A* - Differential transcription of genes *NtMET1*, *NtDRM1*, *NtCMT3*, *NtROS1*, *NtHD2a/b*, and *NtHAT1* was measured by real-time qPCR (filled bar) or microarray (empty bar). For qPCR, the y-axis shows the amount of individual mRNA in relative-fold changes (RFC) to *18S rRNA* transcription. The value of each corresponding gene for control sample was set to 1. If the RFC is < 1, then reciprocal was taken, otherwise the original value was taken. Notably, transcription of *NtDRM1* and *NtHAT1* was not detected (*n.d.*) in microarray experiment. Means  $\pm$  SDs,  $n = 3$ , asterisks indicate the significant difference at  $P < 0.05$ . Activities of DNMTs (*B*), HDACs (*C*), and HATs (*D*). Enzyme activities after *in vitro* addition of GA<sub>3</sub> was also measured. As a positive control activity inhibitors Gen (25  $\mu$ M), AA (50  $\mu$ M), and TSA (5  $\mu$ M) were also added. Means  $\pm$  SDs,  $n = 3$ . Empty and filled bars indicate control and GA<sub>3</sub>-treated samples, respectively. Asterisks indicate the significant difference at  $P < 0.05$ .

synthase/methionine adenosyltransferase (*MAT*, RFC = -4.26), L-homocysteine-S-methyltransferase (*HMT*, RFC = -2.31), S-adenosyl-homocysteine hydrolase (*SAH hydrolase*, RFC = -2.31), and methionine synthase (*MS*, RFC = -3.48). Notably, apart from ethylene, SAM also participates in polyamine biosynthetic pathways providing the methyl group for the majority of cellular methylation reactions required for plant growth and development (Sauter *et al.* 2013). The multiple essential roles of SAM require regulation of its synthesis, recycling, and distribution to sustain these different pathways. The observed reduced transcriptions of SAM cycle enzymes and cellular methyl pool were further supported by our previously observed global DNA hypomethylation (Manoharlal *et al.* 2018) as well as by the fact that there was a significant increase in transcription

of 1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase 1 (*MTCBP-1*, RFC = 2.35) involved in ‘methionine salvage/Yang cycle’ (reviewed by Sauter *et al.* 2013). Activation of ethylene signalling was also evident by an observed increase in transcription of an ethylene responsive sensor (*ERS1a*, RFC = 2.77) and ethylene-responsive-element-binding factors: *ERF1* (RFC = 3.25), *ERF3* (RFC = 2.87), *ERF5* (RFC = 2.38), *ERF6* (RFC = 4.26), and *ERF13* (RFC = 4.17). Consistent with this, a reduced transcription of a negative regulator of the ethylene response pathway, constitutive triple response 1 (*CTR1*, RFC = -2.01), was also observed. All these observations reflect that GA<sub>3</sub> treatment mediated a part of its action passively through induced endogenous ethylene biosynthesis and signalling pathways.

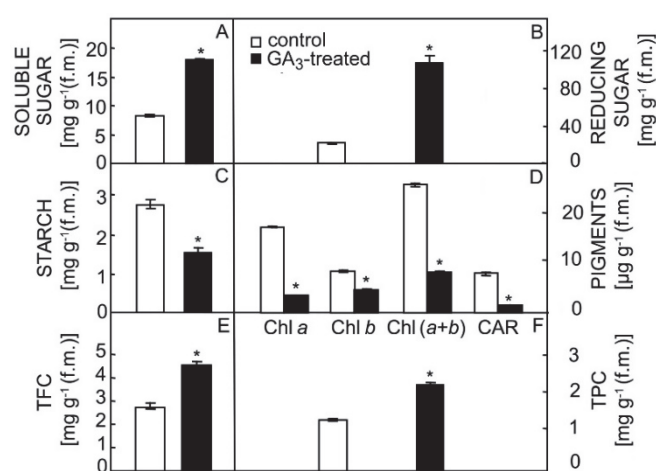


Fig. 4. Effects of gibberellin A<sub>3</sub> (GA<sub>3</sub>) application on content of soluble sugars (A), reducing sugars (B), starch (C), chlorophylls [Chl *a*, Chl *b*, Chl (*a+b*)] and carotenoids (CAR) (D), total flavonoids (TFC) (E), and total phenolics (TPC) (F). Means ± SDs, *n* = 3. Empty and filled bars indicate control and GA<sub>3</sub>-treated samples, respectively. Asterisks indicate significant differences at *P* < 0.05.

Auxin positively affects GA signalling by regulating the expression of GA biosynthetic genes (Nemhauser *et al.* 2006). Decapitation of pea and tobacco shoot apices has been shown to reduce content of active GAs in stems and this effect is reversed by auxin application (Ross *et al.* 2000, Wolbang and Ross 2001). Auxin also induces the expression of *GA20ox* in tobacco and *Arabidopsis*, whereas in pea, it induces and suppresses the expression of *GA3ox* and *GA2ox*, respectively (O'Neill and Ross 2002, Frigerio *et al.* 2006). In this study, we also observed a significant increase and decrease in transcription of *GA2ox* and *GA20ox*, respectively (as described above), indicating probable reduced auxin signalling pathways upon GA<sub>3</sub> foliar application. The physiological relevance of this regulation was further supported by the observation that transcriptions of auxin response factors (*ARFs*) viz. *ARF2* (RFC = -3.10), *ARF5* (RFC = -2.79), *ARF8* (RFC = -2.97), *ARF10* (RFC = -2.33), *ARF12* (RFC = -4.06), and *ARF13* (RFC = -2.27) were significantly reduced. Interestingly, we also observed a significant decrease in transcription of

upstream precursor (tryptophan) of the auxin biosynthetic pathway: anthranilate synthase component II (*ASII*, RFC = -2.85), rate-limiting enzyme anthranilate synthase α-1 (*ASAI*, RFC = -2.60), and feedback-insensitive anthranilate synthase α-2 chain (*ASA2*, RFC = -2.55) (Inaba *et al.* 2007). Along with this, transcription of indole-3-acetic acid-amido synthetase (*GH3.5*, RFC = -2.01) as well as *YUCCA* family genes, encoding a flavin monooxygenase-like enzyme *YUCCA3* (RFC = -2.23) involved in a downstream rate-limiting step of tryptophan dependent auxin biosynthesis pathway (Zhao 2010), were also down-regulated suggesting a probable reduced endogenous auxin content following GA<sub>3</sub> foliar application. Taken together, all these observations reflect that GA<sub>3</sub> application also mediates a part of its action through an endogenous homeostatic balance between GA and auxin biosynthesis and signalling pathways. However, the probability of GA<sub>3</sub> interaction(s) with other phytohormones cannot be ignored. In this context, it is worth mentioning that exogenous GA<sub>3</sub> also alters morphology, anatomy, and transcriptional regulatory



networks associated with other hormones in carrot roots and shoots (Wang *et al.* 2015) indicating that GA<sub>3</sub> in general interacts with other phytohormones to regulate plant growth through various cross-talk mechanisms.

Significant changes in primary metabolism are induced by GA<sub>3</sub> (Gupta and Chakrabarty 2013), and saccharides are the main source of energy. We observed that transcriptions of sucrose catabolism genes encoding invertase (RFC = 3.41) and  $\beta$ -fructosidase (RFC = 3.41) significantly increased. Conversely, transcriptions of genes encoding sucrose synthase (*SUS*, RFC = -5.28) and sucrose-phosphate synthase 1 (*SPS1*, RFC = -4.63) were significantly reduced. Starch serves as an important reserve and GA-induced  $\alpha$ -amylase gene expression has been reported as a useful model to study GA response pathway (Sun and Gubler 2004). Transcription of  $\alpha$ -amylase (RFC = 4.2) was significantly up-regulated after GA<sub>3</sub>-application. In contrast, transcriptions of starch biosynthesis genes, phosphoglucosyltransferase (*PGT*, RFC = -4.47), ADP-glucose pyrophosphorylase (*AGPase*, RFC = -3.58), and starch synthase (*SS*, RFC = -60.13) showed significant down-regulations. The altered transcriptions of sucrose and starch biosynthetic genes were also supported by biochemical analyses, wherein relative increases in content of total soluble sugars (~2.16-folds) and reducing sugars (~4.94-folds), and a decrease in starch content (~1.8-folds) were observed in GA<sub>3</sub> treated leaf extracts (Fig. 4A-C).

Raffinose and trehalose metabolism is directly linked to sucrose, starch, and glycolysis. Transcripts encoded by raffinose biosynthesis genes: galactinol synthase (*GolS*, RFC = 13.83), raffinose synthase (*Rafsynt*, RFC = 25.11), and  $\alpha$ -galactosidase 1 (*AGAL*, RFC = 25.11) were relatively more abundant following GA<sub>3</sub> application indicating a probable enhanced content of raffinose family oligosaccharides raffinose and stachyose. Trehalose biosynthetic genes showed contrasting results, with up-regulation of UTP-glucose-1-phosphate uridylyltransferase (*UGP*, RFC = 2.5) and trehalose pyrophosphatase (*TPP*, RFC = 2.33), and down-regulation of trehalase (RFC = -2.35). An observed increase and decrease in transcriptions of trehalose-6-phosphate synthase (*TPS*) and *AGPase*, respectively, further confirm the previous notion that trehalose-6-phosphate catalyzed by *TPS* is implicated in the redox activation of *AGPase*, the enzyme that catalyzes the first committed step of starch synthesis (Kolbe *et al.* 2005). Besides the activation of sugar metabolism, up-regulation of a plasma membrane hexose transporter (*HXT*, RFC = 2.46) was also observed. Taken together, observed key changes in saccharide metabolism supports a link between GA signalling, sucrose, starch, raffinose, and trehalose metabolisms that could alter source-sink relations in the plant.

Glycolysis and the tricarboxylic acid (TCA) pathway is an important backbone of saccharide metabolism fuelling the energy required for primary metabolism.

Interestingly, genes involved in both energy investment steps (phosphofructokinase, *PFK*, a rate-limiting step, RFC = 7.67) and an energy payoff phase of the glycolytic pathway (phosphoglycerate kinase, *PGK*, RFC = 2.39) and phosphoglyceromutase (*PGM*, RFC = 2.5) were up-regulated indicating the activation of glycolysis pathway by GA<sub>3</sub>. On the contrary, the transcription of another glycolytic pathway gene, pyruvate kinase (*PK*) was significantly reduced (RFC = -3.48). Plant *PK* is believed to provide a bottom-up control of glycolytic flux and is subjected to a feedback allosteric inhibition by ATP and inorganic pyrophosphate-dependent *PFKs* (Dennis and Greyson 1987). Thus, an increased content of *PFK* and cellular ATP generated in glycolysis might be the probable reason for reduced transcription of *PK*. Within the TCA cycle, the initial and last steps of TCA cycle pathway genes, *i.e.*, isocitrate dehydrogenase (*Isocitrate D'Hase*, RFC = -6.36) and malate dehydrogenase (*Malate D'Hase*, RFC = -2.36) were significantly down-regulated. Phosphoenolpyruvate carboxylase (*PEPC*) catalyzing the conversion of phosphoenolpyruvate to oxaloacetate, thereby regulating flux through the TCA cycle, is another key regulatory point of the glycolytic pathway. We also observed a reduced transcription of *PEPC* (RFC = -5.24) further indicating that the TCA cycle was repressed by exogenous GA<sub>3</sub>. Interestingly, transcription of mitochondrial respiratory genes involved in oxidative phosphorylation, NADH:ubiquinone oxidoreductase belonging to complex I (RFC = -2.6), and cytochrome *c* oxidase assembly protein (*COX15*, RFC = -2.81), and ATP synthesis, *i.e.*, ATP synthase [ $\alpha$ -,  $\beta$ - (RFC = -3.58) and  $\gamma$ - (RFC = -2.46) chain subunits] were significantly repressed. Notably, the observed increase and decrease in transcription of *PFK* and *ATP synthase* ( $\beta$ -/ $\gamma$ -chain subunits), respectively, corroborates the fact that ATP produced by mitochondrial *ATP synthase* during oxidative phosphorylation allosterically inhibited the *PFK1* (Mustroph *et al.* 2007). Taken together, our results hypothesize that a remarkable enhancement and suppression of transcriptions of glycolytic (with exception of allosterically regulated *PK*) and TCA pathway genes causes a relative abundance of ATP and/or metabolic intermediate(s) of the secondary metabolism. However, at this stage, it may be premature to conclude that alterations in the transcription of genes involved in primary metabolism would diminish the energy available for growth and development. Nevertheless, metabolic adjustment in sugar and glycolysis-TCA cycle pathways could also affect the other key pathways of primary metabolism, which are not mentioned in our current study.

Acetyl-CoA links glycolysis to the TCA pathway, and it is a central metabolite in many processes, either being further metabolized as an energy source or used as a building block for biosynthesis of lipids, cholesterol, and a myriad of secondary metabolites. We observed that relative to the control, a cellular acetyl-CoA pool

increased dramatically in GA<sub>3</sub> treated leaf-tissue (~1.83-folds) (Fig. 5A). The identity of putative CoA esters was also confirmed by their sensitivity to mild alkali, wherein the acyl-CoAs were hydrolyzed and free CoA-SH was released (Fig. 5B). A typical chromatographic separation of CoA standards and TCA soluble extracts is shown in Fig. 4 Suppl. To further elucidate its expression dynamics, the mRNA content of acetyl-CoA biosynthetic genes was evaluated. Transcriptions of acetyl-CoA synthetase (*ACS*, RFC = 2.01) and ATP-citrate lyase1 (*ACLI1*, RFC = 2.95) were up-regulated, corroborating a probable reason for an enhanced acetyl-CoA pool after GA<sub>3</sub>-treatment. A number of recent studies have suggested that acetyl-CoA serves as an acetyl donor in protein acetylation reactions, linking metabolism to protein PTMs. A reduced transcription of acetyl-CoA carboxylase 1 (*ACCI*, a rate-limiting cytosolic enzyme of fatty acid synthesis) that competes with HATs for acetyl-CoA caused an increase in bulk histone acetylation (Galdieri and Vancura 2012). In support of this, we also observed an enhanced global histone acetylation (as explained earlier), most probably caused by a corresponding increase and decrease in transcription of *ATP-citrate lyase 1* (RFC = 2.35) and *l-amino-cyclopropane-1-carboxylic acid synthase* (RFC = -2.17), respectively. A pyruvate dehydrogenase complex (a PDHC-E3 subunit, dihydrolipoyl dehydrogenase) catalyzes the conversion of pyruvate to acetyl-CoA by pyruvate decarboxylation (Swanson conversion), thereby paving the entry of pyruvate into the TCA cycle, was observed to be significantly down-regulated (RFC = -3.81). This can be explained by the fact that pyruvate dehydrogenase complex was allosterically inhibited by an increased acetyl-CoA/CoA pool. In the same line, the enhanced content of the acetyl-CoA pool was further evident by down-regulation of a rate-limiting gene of pentose phosphate pathway, *glucose-6-phosphate dehydrogenase* (*G6PDH*, RFC = -2.2), wherein an increased content of acetyl-CoA as well as palmitoyl-CoA are known to inhibit *glucose-6-phosphate dehydrogenase* (Kawaguchi and Bloch 1974). Taken together, all these observations indicate that exogenous GA<sub>3</sub> application causes a relative abundance of cellular acetyl-CoA pool, thereby prompting histone hyper-acetylation (and probably in conjunction with DNA hypo-methylation) to cause subsequent global chromatin de-condensation as well as transcriptomic alterations.

Apart from regulating all aspects of plant growth and development, phytohormones and/or their complex interactions have also been reported as efficient elicitors in stimulating the production of secondary metabolites (Baenas *et al.* 2014). Broadly, plant secondary metabolites can be divided into three chemically distinct groups: terpenes/terpenoids, phenolics, and nitrogen or sulphur containing compounds. Among them, terpenoids constitute the largest class of secondary metabolites.

Recent studies have shown GA<sub>3</sub> as an efficient

inducer of terpenoid biosynthesis in plants (Mansouri *et al.* 2011). To identify the effect of GA<sub>3</sub> on quality and quantity of terpenoids, extracts of *N. tabacum* leaf tissue were analyzed by GC-MS. The GC-MS chromatogram showed 30 differential metabolite peaks in a GA<sub>3</sub> treated leaf extract (Fig. 5 Suppl. and Table 2 Suppl.) Those were identified according to their retention time on a fused silica capillary column. These compounds mainly

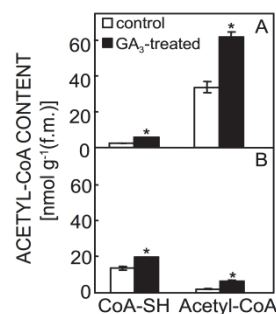


Fig. 5. Application of GA<sub>3</sub> affects a cellular acetyl-CoA pool. Bar graphs depict quantitative estimation of CoA-SH and acetyl-CoA content in control and GA<sub>3</sub>-treated plants before (A) and after (B) NH<sub>4</sub>OH treatment. Means  $\pm$  SDs,  $n=3$ . Asterisks indicate significant differences at  $P < 0.05$ .

comprised alkaloids, sesqui-, mono-, di-, and tetra-terpenes, and saturated fatty acids. Whereas the role of GA<sub>3</sub> in terpenoid biosynthesis has been well explored, relatively less is known about its effect on regulation of other secondary metabolic pathways. Precursors for the enhanced biosynthesis of pigments, squalene, sterols, *etc.*, are generated by sugar metabolism. In higher plants, the five-carbon building blocks of all terpenoids, isopentenyl diphosphate, and dimethylallyl diphosphate (Lange *et al.* 2000), are synthesized by cytosolic/mitochondrial mevalonate (MVA) and plastidal 1-deoxy-D-xylulose-5-phosphate/2-methyl-D-erythritol-4-phosphate (DOX/MEP) pathways (Disch *et al.* 1998, Rohmer 1999). Taking a clue from this, transcriptions of genes implicated in MVA and MEP pathways were evaluated. A total of nine genes (including one regulatory and one sensor protein) belonging to the MVA pathway (involved in biosynthesis of sterols, ubiquinones, sesqui-, hemi-, and tri-terpenes predominantly) were observed to be modulated. In particular, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (*HMGR*, RFC = 3.16) catalyzing the rate-limiting step of isopentenyl diphosphate biosynthesis (Rodwell *et al.* 2000), acetyl-coenzyme A acetyltransferases (*ACAT*, RFC = 2.43), mevalonate-5-pyrophosphate decarboxylase (*MVD*, RFC = 2.43), *cis*-prenyltransferases (*CPTs*, RFC = 10.63), farnesyl diphosphate synthase (*F-PP synthase*, RFC = 4.29), squalene synthase (*SQS*, RFC = 2.1), and intracellular sensor sterol regulatory element-binding protein 2 proteases (*SREBP site 2 proteases*, RFC = 2.07) were significantly up-regulated. All these genes were

annotated to be involved in phytosterol biosynthetic pathway (Laule *et al.* 2003). The activation of the MVA pathway, encoding phytosterols, by GA<sub>3</sub> application is corroborated by an observed increased content of a sterol intermediate precursor squalene as well as downstream phytosterols (*i.e.*, campesterol, stigmasterol,  $\beta$ -sitosterol, and cholesterol, Table 2 Suppl.). Consistent with the previous observation in *Cannabis sativa* (Mansouri *et al.* 2011), our study also confirmed that *in vitro* addition of 50 mg dm<sup>-3</sup> GA<sub>3</sub> caused an enhanced 3-hydroxy-3-methylglutaryl coenzyme A reductase activity (~2 folds, Fig. 6 Suppl.) indicating that in general GA<sub>3</sub> acts as a positive modulator of the MVA pathway in plants. Notably, probability of accumulated acetyl-CoA (a starting substrate of the MVA pathway) acting as a driving force for indirect activation of the MVA pathway also cannot be ruled out.

The MEP pathway comprised of lower and upper (feeding) modules, mainly involved in biosynthesis of mono-, di-, and tetra-terpenes along with carotenoids and a phytol chain of chlorophyll; 4 genes specifically belonging to MEP module were also observed to be coherently modulated. In particular, transcriptions of three genes encoding key enzymes of carotenoid biosynthesis pathway,  $\beta$ -carotene hydroxylase (*BCH*, RFC = 3.78), lycopene  $\beta$ -cyclase (*LCYB*, RFC = 3.25), and lycopene  $\epsilon$ -cyclase (*LCYE*, RFC = 3.61) were significantly up-regulated. Additionally, a gene encoding chloroplastic magnesium-protoporphyrin IX monomethyl ester cyclase (*CRD1*) catalyzing the formation of isocyclic ring in chlorophyll biosynthesis was also up-regulated (RFC = 2.5). The altered transcriptions of these genes were also in agreement with the increased content of phytol (Table 2 Suppl.) indicating that accumulation of carotenoids and chlorophylls was similarly controlled by GA<sub>3</sub>. Notably, our observation of pigment accumulation suppression by GA<sub>3</sub> (Fig. 4D) is also in line with previous findings (Mansouri *et al.* 2011) indicating a most probable positive feedback mechanism on the genes underlying in MEP module. On the contrary, in feeding module, a reduced transcription of a gene encoding 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase (*CDP-ME kinase*, RFC = -2.22) was observed. The CDP-ME kinase acts downstream of an initial and rate-limiting step of MEP pathway, 1-deoxy-D-xylulose 5-phosphate synthase (Cordoba *et al.* 2009). Its activity and abundance is under the feedback control of MVA/MEP intermediates, isopentenyl diphosphate and dimethylallyl diphosphate (Banerjee *et al.* 2013) as well as under its own post-transcriptional regulation (Guevara-García *et al.* 2005, Cordoba *et al.* 2009). These observations prompted us to study the effect of *in vitro* GA<sub>3</sub> application on activities of enzymes involved in feeding module. For this purpose, a commercially available *CDP-ME synthase* kit was used as a readout of feeding module. Our data show that *in vitro* addition of 50 mg dm<sup>-3</sup> GA<sub>3</sub> resulted in a significant decrease (~5.2-folds) in *CDP-ME*

*synthase* activity (Fig. 6 Suppl.). These results are also in good agreement with a previous observation (Mansouri *et al.* 2011), wherein a decrease in 1-deoxy-D-xylulose 5-phosphate synthase activity by exogenous GA<sub>3</sub> application has been reported in *Cannabis sativa*. These observations suggest that GA<sub>3</sub> in general acts as a negative modulator of upper MEP pathway. Notably, a reduced activity of feeding module may also be due to a probable limited supply of feeding module precursors pyruvate and glyceraldehyde-3-phosphate, which could be either due to reduced activity of pentose phosphate pathway (as explained above) or due to channelization of acetyl-CoA towards MVA pathway. Briefly, all these observations suggest that GA<sub>3</sub> application in *N. tabacum* causes an activation of MVA and MEP pathways through an induced 3-hydroxy-3-methylglutaryl coenzyme A reductase activity. On the contrary, GA<sub>3</sub> treatment causes the suppression of feeding module through a probable compensatory decrease in *CDP-ME synthase* activity. Taken together, our data confirm previously reported effects of exogenous GA<sub>3</sub> on MVA and MEP pathways.

In plants, one of the most widespread secondary metabolic pathways involves the biosynthesis of phenylpropanoids through the shikimic acid pathway (Herrmann 1995). Activation of the phenylpropanoid biosynthesis pathway is by a complex hormonal cross-talk, thereby creating a flexible signalling network that enables them to fine-tune its defence response to the exogenous stimuli (Kant *et al.* 2015, Kasote *et al.* 2015). Thereby, to look for a possible correlation between phenylpropanoid biosynthesis and GA<sub>3</sub> treatment, transcriptional analysis of shikimic acid-phenylpropanoid pathway biosynthesis genes was done. Our data shows that GA<sub>3</sub> treatment elicited the transcription of eight key genes involved in this pathway. In particular, transcriptions of shikimic acid pathway gene, a 3-dehydroquinate dehydratase/shikimate dehydrogenase isoform (*DHQ dehydratase/SDH*, RFC = 2.91), general phenylpropanoid pathway genes, phenylalanine ammonia lyase (*PAL1*, RFC = 2.62), cinnamate-4-hydroxylase (*C4H*, RFC = 4.72), 4-coumarate-CoA ligase 2 (*4CL2*, RFC = 2.19), and flavonoid biosynthesis genes, chalcone isomerase (*CHI*, RFC = 2.6), flavonone 3-hydroxylase (*F3H*, RFC = 2.55), dihydroflavonol-4-reductase 1 (*DFR1*, RFC = 2.23), and UDP-glucose:flavonoid 3-O-glucosyltransferase (*UFGT*, RFC = 14.72) showed a significant up-regulation indicating an activation of flavonoid biosynthetic pathway. Activation of the flavonoid biosynthesis branch of phenylpropanoid pathway is further evident from a relative enhanced accumulation of TFC (~1.65-folds) in GA<sub>3</sub> treated leaf-extracts (Fig. 4E). In addition, malonyl-CoA, a substrate required for synthesis of flavonoids, related polyketides, as well as for elongation of fatty acids, is the product of acetyl-CoA carboxylation (Fatland *et al.* 2005), further indicating a role of GA<sub>3</sub>-induced secondary metabolism through enhanced

accumulation of the acetyl-CoA pool (as described above).

In context of increased biosynthesis of phytosterols, it is worth mentioning that fatty acid biosynthetic pathway also seemed to be activated, as evident from a relative increase in amount of saturated fatty acids: tetradecanoic (myristic acid), pentadecanoic, hexadecanoic (palmitic acid), and octadecanoic acid (stearic acid) (Table 2 Suppl.). Regarding fatty acid biosynthetic genes, transcriptions of two genes involved in the citrate shuttle of fatty acid biosynthesis: *ACLI* (RFC = 2.35) and NADP-dependent malic enzyme (*ME1*, RFC = 3.78) were up-regulated. Similarly, transcriptions of malonyltransferase (*MaT*, RFC = 2.38) and fatty acid desaturase (RFC = 2.71), which plays a key role in the maintenance of the proper structure and functioning biological membranes, were significantly up-regulated. Conversely, a reduced transcription of *ACCI* (RFC = -2.17), a rate-limiting cytosolic enzyme of fatty acid synthesis, can be explained by the fact that it probably undergoes allosteric product inhibition by increased content of palmitoyl-CoA. Moreover, activation of *de novo* fatty acid biosynthesis can also be hypothesized by enhanced accumulation of its precursor acetyl-CoA, which is also known to inhibit the final catabolic step of  $\beta$ -oxidation of fatty acids, catalyzed by 3-ketoacyl thiolase (Poirier *et al.* 2006).

Gibberellins are known to play a role in cell wall expansion and as consequence in leaf cell expansion (Keyes *et al.* 1990). In our previous studies, we also observed that GA<sub>3</sub> treatment in *N. tabacum* causes a significant increase (~62 %) in leaf cell area concomitant with a decrease in intercellular spaces (Manoharlal *et al.* 2018). The above anatomy experiment prompted us to examine whether the transcription of cell wall related genes were also affected or not. Indeed, transcriptions of cell wall loosening or catabolic genes, acidic endochitinase Q (*AECCase Q*, RFC = 2.17), chitinase/lysozyme (*C/L*, RFC = 4.96), pectinesterase (*PEase*, RFC = 2.17), xyloglucan endotrans-glucosylase/hydrolases: *XTH5* (RFC = 3.34), *XTH6* (RFC = 4.06), *XTH7* (RFC = 2.38), and cell wall invertase (*INV5*, RFC = 3.66), endo-1,4- $\beta$ -glucanase/endo- $\beta$ -1,3-glucanase (*EGase*, RFC = 4.29/6.23), expansin like B1 (*ExpB1*, RFC = 45.57), and plasma-membrane proton pump (*PM-H<sup>+</sup>-ATPase*, *MHA*, RFC = 4.35), were co-ordinately up-regulated. These cell wall related proteins are believed to play a role in modulating cell wall extensibility, thereby mediating cell wall loosening and cell enlargement and expansion (Achard *et al.* 2006). Conversely, transcriptions of  $\beta$ -glucan-binding protein (*BGBP*, RFC = -3.51), callose synthase (*CS*, RFC = -6.23), and cellulose synthase A catalytic subunit 3 (*CeSA*, RFC = -6.06) were significantly reduced. Recently, transcriptions of these cell wall related genes have been reported to be correlated with global histone hyper-acetylation of histone H3 at lysine 9 (H3K9Ac) in maize roots, mediating their salt stress

induced growth response (Achard *et al.* 2006). Thereby, the probability of epigenetic regulation of these affected cell wall related genes with the observed GA<sub>3</sub> induced global histone hyper-acetylation also cannot be ruled out.

A common response to biotic and abiotic stresses is the generation of reactive oxygen species (ROS). It has been reported previously that GA<sub>3</sub> in conjunction with other phytohormones plays an important role in modulation of redox homeostasis in plants (Corbineau *et al.* 2014). We observed that GA<sub>3</sub> increased transcriptions of several genes involved in glycolytic pathway (*PFK*, *PGK*, and *PGM*) and fermentation [aldehyde dehydrogenases (RFC = 4.89) and alcohol dehydro-genases (RFC = 2.5)]. In contrast, some of the mitochondrial respiratory genes like NADH:ubiquinone oxidoreductase belonging to complex I, *cytochrome c oxidase assembly protein 15*, and *ATP synthase*  $\beta$ - and  $\gamma$ -chain subunits were significantly repressed. Over-expression of these glycolytic and fermentative genes with concomitant repression of mitochondrial respiratory genes in response to GA<sub>3</sub> application indicates a metabolic shift from aerobic respiration to fermentation. The conversion of pyruvate to lactate/ethanol during fermentation is typically induced by hypoxic conditions. Such a situation is relatively well known in the case of maize and rice, where an increased glycolysis due to limited oxygen supply (hypoxia) enables the plant cells to adapt to submergence (Tamang and Fukao 2015). Recently, activation of ethylene signalling capable of displaying such a metabolic shift has already been documented, thereby also indicating a probable passive role of GA<sub>3</sub> in such a metabolic drift (Nishiuchi *et al.* 2012). Mitochondrial respiration leads to the generation of free radicals, which have to be scavenged to maintain redox homeostasis. The absence of scavengers can result in the accumulation of ROS (Bailey-Serres and Mittler 2006) as evident from significantly increased transcription of a ROS producing gene encoding *NADPH oxidase*, a respiratory burst oxidase homolog (*Rboh*, RFC = 4.56). As mentioned above, four genes (NADH: ubiquinone oxidoreductase, *cytochrome c oxidase assembly protein 15*, and *ATP synthase*  $\beta$ - and  $\gamma$ -chain subunits) belonging to the mitochondrial electron transport chain were particularly down-regulated indicating the probable mitochondrial dysfunction, thereby leading to ROS generation. Since ROS accumulation is perceived as a stress by plants, GA<sub>3</sub>-treated cells displayed induced transcriptions of antioxidant enzymes encoded by respective genes *viz.* peroxidase (*PX*, RFC = 2.25), cell wall peroxidase (*CwPX*, RFC = 4.32), ascorbate peroxidase (*APX*, RFC = 2.55), and glutathione *S*-transferase (*GST*, RFC = 3.34). Antioxidant potential stimulated by GA<sub>3</sub> was further corroborated by an observed increased accumulation of natural antioxidants, TFC and vitamin E ( $\alpha$ - and  $\beta$ -tocopherols). Moreover, we also observed an increased accumulation of TPC (~1.64-folds, Fig. 4F), which is

also known to contribute to the cellular anti-oxidative potential. Notably, an enhanced cellular antioxidant potential due to phenylpropanoid pathway activation triggered by *PAL1* over-expression has also been reported in plants (Kim and Hwang 2014).

Accumulation of ROS can lead to damage of DNA, lipids, and other macromolecules. Conversely, various types of spontaneous and chemically induced DNA damage mediate an increase in intracellular ROS content (Manova and Gruszka 2015), which is likely to function in various signalling pathways. Indeed, we also observed an up-regulation of the following DNA damage response (DDR) genes: DNA photolyase (RFC = 12.82), DNA ligase (RFC = 2.62), DNA damage-binding protein 1 (*DDBP1*, RFC = 7.46), DNA-repair proteins *viz.* RAD5 (RFC = 2.36), RAD50 (RFC = 2.79), and UVH3 (RFC = 8.63), DNA repair helicase (UVH6, RFC = 2.06), DNA mismatch repair proteins Msh6-1 (RFC = 8.69) and Muts2 (RFC = 4.86), DNA-3-methyladenine glycosylase (3-MeAG, RFC = 2.14), X-ray repair cross complementing protein 2 (XRCC2, RFC = 2.51), and a bi-functional DNA glycosylase/lyase ROS1 (RFC = 4.76). Over-expressions of these DDR genes indicate that GA<sub>3</sub> application, either directly or indirectly *via* ROS content, exerts a genotoxic stress in *N. tabacum*. It is pertinent to mention here that a probable passive role of GA<sub>3</sub> induced DNA chromatin alteration and/or hypomethylation in mediating the observed genotoxic stress response cannot be ruled out. These observations indicate that enzymatic and non-enzymatic antioxidant systems along with the DDR system seems to be activated in response to GA<sub>3</sub> application in *N. tabacum* for the amelioration of the oxidative stress-associated damage in different cellular organelles.

Plant hormones are also considered as regulators of stress responses. A rapid increase in the rate of ROS production known as ‘oxidative burst’ also sometimes occurs in response to stress conditions (Miller *et al.* 2008). The GA-signalling pathways that regulate plant tolerance to various stresses are very complex. There is a relatively less understanding of relationship between GA signalling and stress tolerance beyond the well-established role of GA as a growth and ROS regulator (Achard *et al.* 2008b). However, evidence is accumulating that GA content and signalling are affected by plant growth under abiotic stresses (Achard *et al.* 2006, 2008b). In *A. thaliana*, GA<sub>3</sub> application reverses the inhibitory effect of salt and cold stresses by triggering a reduction in bioactive GAs and promoting DELLA accumulation, thereby resulting in a DELLA-mediated growth restriction (Achard *et al.* 2008a). In both cases, the up-regulation of specific gibberellin 2- $\beta$ -dioxxygenase (*GA2ox*) by the dehydration-responsive element binding protein/C-repeat binding factor (*DREB1/CBF*) family and a subfamily of the *AP2/ERF* group TFs appears to contribute to reduction in endogenous bioactive GA and subsequent downstream responses (Achard *et al.* 2008a,

Magome *et al.* 2008). Interestingly, we also observed increased transcriptions of *DREB1/CBF* (RFC = 2.75), *AP2/ERF* (RFC = 2.71) TFs as well as its downstream targets, *i.e.*, *GA2ox1*, *GA2ox2*, *GA2ox3*, *GA2ox4*, and *GA2ox5*. Application of GA<sub>3</sub> also induced the transcriptions of ERF subfamily TFs *viz.* *ERF1*, *ERF3*, *ERF5*, *ERF6*, and *ERF13* (as described above), SHAGGY-related protein kinase (*ASK*, RFC = 2.83), and calcineurin B-like protein (CBL) interacting with serine/threonine-protein kinase 24 (*CIPK24*, RFC=2.51), which are reported to implicate in the metabolic adaptation of plants to osmotic stress (Richard *et al.* 2005, Dubois *et al.* 2013). The *WRKY* family TFs have many regulatory roles in response to biotic and abiotic stresses (Phukan *et al.* 2016). We observed an up-regulation of several *WRKY* TFs: *WRKY2* (RFC = 9.38), *WRKY4* (RFC = 3.12), *WRKY5* (RFC = 4.47), *WRKY6* (RFC = 2.17), *WRKY10* (RFC = 2.14), *WRKY12* (RFC = 2.31), *WRKY16* (RFC = 2.25), *WRKY18* (RFC = 5.62), *WRKY19* (RFC = 3.46), *WRKY20* (RFC = 3.03), *WRKY21* (RFC = 7.11), *WRKY23* (RFC = 3.29), *WRKY29* (RFC = 2.71), *WRKY30* (RFC = 2.2), *WRKY34* (RFC = 3.1), *WRKY36* (RFC = 2.04), and *WRKY45* (RFC = 2.43). Interestingly, stress-inducible NAC [*NAM* (for no apical meristem), *ATAF-1* and -2, and *CUC2* (for cup-shaped cotyledon)] family TFs reported to be involved in abiotic stress tolerance (Fujita *et al.* 2004) were also up-regulated (*NAC*, RFC = 3.58). These observations indicate that GA<sub>3</sub> application mimics abiotic stress response networks in *N. tabacum*. Indeed, regulation of expression or activity of TFs that modulate expression of GA metabolic genes could represent one of mechanisms by which GA<sub>3</sub> signalling is integrated into wider stress response networks.

Absciscic acid is responsible for plant defence against abiotic stresses. Drought, salinity, cold, heat stress, and wounding trigger an increase in ABA content (Zhang *et al.* 2006, Lata and Prasad 2011). We observed that transcriptions of salt overly sensitive 1 (*SOS1*, RFC = 2.41) as well as ABA biosynthesis genes residing at a MEP module, such as zeaxanthin epoxidase (*ZEP*, RFC = 2.77) and ABA aldehyde oxidase (*AAO3*, RFC = 5.78), were significantly up-regulated by GA<sub>3</sub> application. The stress response networks induced by GA<sub>3</sub> may be generated either by hormonal cross-talk or by metabolic alterations resulting in activation of defence-related proteins/compounds (Kant *et al.* 2015). For example, observed changes in trehalose biosynthetic genes and/or enzymes but not necessarily trehalose content were also postulated to play a signalling or regulatory role in stress-response pathways. Similarly, accumulation of TFC, TPC, and ROS by plants is a response to different abiotic stresses (such as UV radiation, water stress, high or low temperature, and wounding) as well as biotic stresses (Kasote *et al.* 2015). Application of GA<sub>3</sub> thus induces several genes and metabolites involved in stress response, which is only one component of the response mechanism.

Taken together, current findings show that used GA<sub>3</sub>-concentration for foliar application in the field induces morphological, anatomical, and biochemical alterations in *N. tabacum*, which comprises very complex transcriptional networks and primary and secondary

metabolism pathways (Fig. 7 Suppl.). An understanding of the role of GA<sub>3</sub> signalling in stress responses would be an important step towards improving plant growth and development under adverse environmental conditions.

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