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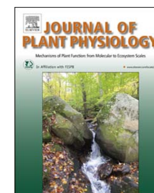

Research Paper

Transcriptome-wide analysis of jasmonate-treated BY-2 cells reveals new transcriptional regulators associated with alkaloid formation in tobacco

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ABSTRACT

Jasmonates (JAs) are well-known regulators of stress, defence, and secondary metabolism in plants, with JA perception triggering extensive transcriptional reprogramming, including both activation and/or repression of entire metabolic pathways. We performed RNA sequencing based transcriptomic profiling of tobacco BY-2 cells before and after treatment with methyl jasmonate (MeJA) to identify novel transcriptional regulators associated with alkaloid formation. A total of 107,140 unigenes were obtained through *de novo* assembly, and at least 33,213 transcripts (31%) encode proteins, in which 3419 transcription factors (TFs) were identified, representing 72 gene families, as well as 840 transcriptional regulators (TRs) distributed among 19 gene families. After MeJA treatment BY-2 cells, 7260 differentially expressed transcripts were characterised, which include 4443 MeJA-upregulated and 2817 MeJA-downregulated genes. Of these, 227 TFs/TRs in 36 families were specifically upregulated, and 102 TFs/TRs in 38 families were downregulated in MeJA-treated BY-2 cells. We further showed that the expression of 12 ethylene response factors and four basic helix-loop-helix factors increased at the transcriptional level after MeJA treatment in BY-2 cells and displayed specific expression patterns in *nic* mutants with or without MeJA treatments. Our data provide a catalogue of transcripts of tobacco BY-2 cells and benefit future study of JA-modulated regulation of secondary metabolism in tobacco.

1. Introduction

Plants are capable of synthesising a multitude of small compounds, called secondary metabolites, which function in the attraction of pollinating insects and defence against infection by microorganisms and predation by herbivores (Ersek and Kiraly, 1986). Currently, about 100,000 secondary metabolites have been identified in plants, and over 2500 compounds, of which alkaloids and terpenoids are major contributors, have been isolated from tobacco (Nugroho and Verpoorte, 2002). Studies on the metabolic regulation of alkaloids in tobacco cover a wide spectrum due to their important roles in defensive function as insecticides (Steppuhn et al., 2004), in addiction through recreational euphoric effects (Laviolette and van der Kooy, 2004), and potential therapeutic uses (Powledge, 2004). The biosynthesis of alkaloids is under strict transcriptional control, which allows plants to precisely regulate their formation in response to biotic and abiotic stress. However, the limited genomic information remains a problem for the study of transcriptional regulation of alkaloid biosynthesis in tobacco.

Jasmonates (JAs), including jasmonic acid and its derivatives, are oxylipin-derived phytohormones that regulate a wide variety of plant

Abbreviations: JAs, jasmonates; MeJA, methyl jasmonate; BY-2, *Nicotiana tabacum* L. cv. Bright Yellow-2; RNA-seq, RNA sequencing; TFs, transcription factors; TRs, transcriptional regulators; ERF, ethylene response factors; bHLH, basic helix-loop-helix factors; PMT, putrescine N-methyltransferase; QPT, quinolinate phosphoribosyltransferase; MPO, N-methylputrescine oxidase; EST, expressed sequence tag; AFLP, cDNA-amplified fragment length polymorphism; JAZ, JA ZIM-domain protein; KEGG, kyoto encyclopaedia of genes and genomes; GO, gene ontology; FDR, false discovery rate; PlnTFDB, plant transcription factor database; TOBFAC, tobacco transcription factors; FPKM, fragments per kilobase of transcript per million fragments mapped; DEGs, differentially expressed genes; qPCR, quantitative real-time PCR

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physiological processes, ranging from growth and development to reproduction and defence (De Geyter et al., 2012). In addition, JAs function as the central elicitor to activate the secondary metabolism in plants (Goossens et al., 2003; Shoji et al., 2008; van der Fits and Memelink, 2000). Previous studies showed that there exists a conserved module for JA perception and subsequent signal transduction to regulate the formation of defensive metabolites, including the terpenoids, alkaloids, and phenylpropanoids in tobacco and other plants (Gundlach et al., 1992; Oh et al., 2012; Pauwels et al., 2009). The transcription factors (TFs) transcriptionally activated early during JA elicitation were considered as the master regulators to boost the production of specific secondary metabolites. In *Catharanthus roseus*, the APETALA 2/ethylene response factor (AP2/ERF)-like transcription factors ORCA2 and ORCA3 have been proved to regulate primary and secondary metabolism during jasmonate responses (Menke et al., 1999; van der Fits and Memelink, 2000; van der Fits and Memelink, 2001). Combinatorial action of TFs has been demonstrated in the JA-mediated elicitation of tobacco nicotine biosynthesis, which requires the concerted action of AP2/ERF and basic helix-loop-helix (bHLH) family TFs (De Boer et al., 2011b). The JA-responsive ERFs, NtORC1, and NtJAP1 are able to positively regulate the JA-inducible gene putrescine N-methyltransferase (PMT), which plays a key role in alkaloid metabolism (De Sutter et al., 2005). At least seven NIC2-locus ERFs, ERF189, ERF115, ERF221, ERF104, ERF179, ERF17, and ERF168, were demonstrated to bind a GCC-box element in the promoters of various nicotine biosynthetic pathway genes and regulate JA-induced nicotine formation in tobacco (Shoji and Hashimoto, 2012; Shoji et al., 2010). A non-NIC2 locus AP2/ERF TF, NtERF32, was also characterised to be required for methyl jasmonate (MeJA)-induced transcription of NtPMT1a and nicotine biosynthesis in tobacco (Sears et al., 2014). Todd et al. have characterised two bHLH TFs, NbbHLH1 and NbbHLH2, as the positive regulators in the JA-activation of nicotine biosynthesis in *Nicotiana benthamiana* (Todd et al., 2010). We and others previously reported that the bHLH transcription factors NtMYC2a, b, and c were induced rapidly by JA and specifically activate the expression of *NtPMT1a* and *NtPMT2* by binding a G-box motif within the *NtPMT1a* and *NtPMT2* promoters to regulate nicotine biosynthesis in tobacco (Shoji and Hashimoto, 2011; Zhang et al., 2012). Moreover, researchers have used transcript analysis tools, such as expressed sequence tag (EST) databases (Ghannam et al., 2005; Todd et al., 2010), microarrays (Heidel and Baldwin, 2004; Hui et al., 2003), cDNA-amplified fragment length polymorphism (AFLP) methods (Goossens et al., 2003), and transcriptome analysis (Bombarely et al., 2012; Lu et al., 2012) to find that JA treatment triggers an extensive transcriptional reprogramming of metabolism and typically shifts the balance from growth to defence-related cellular processes through inhibition of expression of genes involved in cell cycle progression and photosynthesis, and activation of defence-related genes in plants (Pauwels et al., 2008; Zhang et al., 2008; Zhang and Turner, 2008).

Recently, fast and cost-effective approaches for generating large expression data profiles have become available, enabling more precise quantification of genome-wide transcript levels than was previously obtainable through microarray-based methods. We reported previously the characterisation of multiple JA ZIM-domain (JAZ) proteins involved in the regulation of alkaloid biosynthesis (Yang et al., 2015) based on analysis of the transcriptome profile of MeJA-treated tobacco BY-2 cells. Here, we report the results of RNA sequencing (RNA-seq)-based transcriptomic profiling of tobacco BY-2 cells before and after treatment with MeJA and the identification of a set of novel JA-responsible TFs and transcriptionally active proteins associated with JA biosynthesis, signalling, and regulation of secondary metabolic pathways in tobacco.

2. Materials and methods

2.1. Plant material and JA treatment

Nicotiana tabacum L. cv. Bright Yellow-2 (BY-2) suspension cultures were grown and maintained as described previously (Yang et al., 2015). For MeJA treatment, a four-day cell suspension was subcultured in fresh medium without 2,4-dichlorophenoxyacetic acid (2,4-D) and grown for one day; then MeJA was added to a final concentration of 100 μM. The cells were treated with dimethyl sulfoxide (DMSO) as a mock control. The cells from the treated cultures were collected by vacuum filtration at the times indicated for further analyses.

Tobacco plants (*N. tabacum* L.) were germinated and grown to maturity under 16/8-h light/dark illumination in pots in the greenhouse. The wild-type, *nic1*, *nic2*, and *nic1nic2* genotypes of the Burley 21 cultivar were used in the experiments. For MeJA treatment, a four-week-old seedling was immersed into 100 μM MeJA solution, and the materials were collected at the indicated times and frozen in liquid nitrogen for further analyses.

2.2. RNA extraction

Total RNA was prepared using TRIzol (Invitrogen, USA) and purified with a PureLink RNA Mini Kit (Invitrogen) combined with a PureLink DNase kit (Invitrogen) according to the manufacturer's protocol. The quality of the RNA was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) with a readout RNA integrity number (RIN) value of 9.2, and the quantity of RNA was measured using a Nanodrop ND-1000 spectrophotometer (LabTech, USA).

2.3. Transcriptome sequencing and de novo assembly

BY-2 cells were collected for three replications after treatment with MeJA and DMSO as control for 2 h, and total RNA was separately extracted from these samples and combined to construct the MeJA-untreated and MeJA-treated transcriptome libraries. Solexa sequencing of these two libraries was performed as a commercial service using an Illumina HiSeq 2000 at the Beijing Genomic Institute. The sequencing data were deposited in the NCBI Sequence Read Archive (SRA, http://www.ncbi.blm.nih.gov/Traces/sra) under accession number SRA091805. Low-quality bases ($Q < 20$) at the end of sequencing reads were trimmed using the SolexaQA software (Cox et al., 2010) (ver. 1.10, parameters: −b. −h 20). After trimming, read lengths greater than 24 bases were retained for further analysis. MeJA-untreated and MeJA-treated RNA-seq data were pooled and used to conduct *de novo* transcriptome assembly using the trinity software (Grabherr et al., 2011) (ver. r2012-01-25, parameters: −edge-thr = 0.26 −compatible_path_extension −min_contig_length 300). The transcriptome was validated by comparison with tobacco EST sequences (http://www.ncbi.nlm.nih.gov/nucest/?term=tobacco) using BLASTN (version: 2.2.25, parameters: −e 1e-10).

2.4. Coding sequence extraction and functional annotation of the tobacco transcriptome

The coding regions were extracted from transcripts using Trinity (http://trinityrnaseq.sourceforge.net/analysis/extract_proteins_from_trinity_transcripts.html). The functions of each transcript were identified by searching against the National Centre for Biotechnology Information non-redundant databases using BLASTX (version: 2.2.25, parameters: −e 1e-5–v 1–b 1), and ≥50% sequence overlap with the target gene was required for functional identification. Using the same strategy, the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/) and SWISS-PROT plant database (http://www.uniprot.org/) were also searched. Gene Ontology (GO) term association information was extracted from BLAST results

against the SWISS-PROT database. GO enrichment analysis with Plant GO Slim was carried out using BinGO (version 2.44; (Maere et al., 2005). False discovery rate (FDR) correction was used to control the false positive rate. If a GO term had a FDR value < 0.01, the GO term was determined to be significantly overrepresented in the test gene set.

2.5. Characterisation of transcription regulators in the tobacco transcriptome

The general transcription regulators in the tobacco transcriptome, including two kinds of factors, transcription factors (TFs) containing a DNA binding domain (BD) and transcriptional regulators (TRs) having no BD, were identified by searching against the Plant Transcription Factor database (PlnTFDB; http://plntfdb.bio.uni-potsdam.de/v3.0/) and the TOBFAC database (The database of tobacco transcription factors, http://compsysbio.achs.virginia.edu/tobfac/) using BLASTX (version: 2.2.25, parameters: −e 1e-5–v 1–b 1), and ≥50% sequence overlap with the target TF was required for functional identification. The TFs and TRs were named according to their family and phylogenic relationship among them. After comparing the results from these searches, some repeating results were removed manually.

2.6. Gene expression estimation and differential analysis

Expression levels were measured as the number of fragments per kilobase of transcript per million fragments mapped (FPKM). For paired-end sequence data, one fragment contained two short reads. Following the Trinity user guide, the RSEM software was used to estimate expression levels in the context of transcripts assembled by Trinity (version: 1.1.13, with default parameters (Li and Dewey, 2011). RSEM used BOWTIE to generate read alignments with transcripts as reference sequences. According to the recommendations from the Trinity group, the FPKM value of each transcript was computed within a given component resulting from trinity assembler. By using the edgeR package (version: 2.2.5; (Robinson et al., 2010), the FPKM values from two samples were normalised using the quantile method and differential expression analysis was conducted. All significantly differentially expressed genes (DEGs) had at least a two-fold change and a FDR value of 0.01 between two samples.

2.7. Quantitative real-time PCR (qPCR)

Total RNA (5 μg) was used for cDNA synthesis with a Revert-Aid First Strand cDNA Synthesis Kit (Fermentas, Canada) according to the manufacturer's instructions. qPCR was performed as described previously (Yang et al., 2015). The gene-specific primer sequences are provided in Table S3. *Actin* was used as a reference gene. The experiments were repeated at least three times. Data were analysed using the Data Processing System (Tang and Zhang, 2013) by one-way analysis of variance (ANOVA) and Tukey's multiple range test, with $P < 0.05$ considered to indicate significance.

3. Results

Based on the RNA-seq data of MeJA-untreated and MeJA-treated BY-2 cells, we have constructed three transcriptome libraries of MeJA-untreated, MeJA-treated, and the combination of both samples by *de novo* assembly (Table S1). Sequence-based alignments of a total of 107,140 transcripts identified from the combination library were performed against the NCBI NR database, the KEGG database, the SWISS-PROT plant database, and the EuKaryotic Orthologous Groups (KOG) database using the BLASTX algorithm with an E-value threshold of 1e-5, and against the NCBI Nucleotide (NT) database using BLASTN with an E-value threshold of 1e-10. Using this approach, 66,010 distinct sequences (61.61%) returned valid BLAST results (Table S2). Further BLAST searches against the publicly available tobacco EST databases revealed that 67,408 (62.9%) of our unigenes were homologous to known tobacco homologs and 39,732 (37.1%) unigenes were new transcripts, whereas when we used BLAST to query the EST database against our data, 67,825 EST sequences (88.3%) had hits with ≥50% overlap and only 9038 (11.7%) ESTs had no hits (Fig. S1A). We also found that 47% of the transcripts had coding regions, including complete coding DNA sequences (CDSs) (10%), 3′ partial CDSs (8%), 5′ partial CDSs (13%), and internal CDSs (16%), which suggests that at least 33,213 transcripts (31%) encode proteins (Fig. S1B). These results indicate that the tobacco transcriptome we have generated is of high quality and provides an invaluable resource for studies of the transcriptional regulation of tobacco secondary metabolism.

3.1. Functional annotation of unigenes

To predict and classify gene functions, the unigenes of the tobacco transcriptome were annotated according to two functional annotation conventions, GO and KOG. The unigenes were classified into the three GO categories: molecular function, cellular component, and biological process, with 51 subcategories. Because multiple GO functions can be assigned to a single unigene in some cases, 75,744 GO terms were associated with the 20,744 unigenes. The cellular component, molecular function, and biological process categories were represented by 58,687, 17,384, and 7601 terms, respectively (Fig. 1). The most frequently occurring cellular component GO terms were cell (24.93%), intracellular (20.73%), and cytoplasm (15.15%). In the molecular function category, binding (88.54%) was the most abundant class. In the biological process category, classes related to cellular process (33.13%) and metabolic process (27.41%) occurred most frequently. We also found that a total of 36,325 unigenes had hits in the KOG database and grouped into the 25 categories (Fig. S2). The largest KOG group was "general function prediction only" (7641, 21.04%), followed by "signal transduction mechanisms" (2603, 7.17%), "posttranslational modification, protein turnover, chaperones" (2184, 6.01%), "transcription" (1232, 3.39%), "function unknown" (1204, 3.31%), "intracellular trafficking, secretion, and vesicular transport" (1274, 3.51%), and "carbohydrate transport and metabolism" (1211, 3.33%). The groups "cell motility" (7, 0.02%), "extracellular structure" (64, 0.18%) and "nuclear structure" (160, 0.44%) were the smallest groups. Taken together, these annotations will be helpful for further studies of specific processes, structures, functions, and pathways involved in JA-responsive transcriptional regulation in tobacco BY-2 cells.

3.2. Genes involved in JA-responsive stress responses and secondary metabolism

To better understand the scope of JA-induced transcriptional reprogramming in tobacco, we compared the gene expression profiles of JA-treated and untreated BY-2 cells. As shown in Table S1 and Fig. 2a, 93,919 transcripts (87.7%) were found in both MeJA-untreated and MeJA-treated BY-2 cells, while 6896 transcripts (6.4%) were present only in untreated BY-2 cells and 6325 transcripts (5.9%) were present only in JA-treated cells. The DEGs based on FPKM were analysed using a cut-off of a two-fold change and a FDR value of 0.01 between two samples. A total of 4443 unigenes were upregulated in the JA-treated BY-2 cells, 1222 of which were new transcripts that were not present in untreated cells. There were 2817 downregulated unigenes in the JA-treated sample, 751 of which were expressed only in the untreated sample (Fig. 2b). Enrichment analysis was performed with GO annotation to further characterise the DEGs. Of the GO categories, the transcripts were enriched in the metabolic pathway class, especially in the cellular amino acid and derivative metabolic process and the secondary metabolic process subcategories (Fig. 2c). The enriched DEGs were further focused on nine pathways, in which the enriched map00592 and map00591 represented α-linolenic acid metabolism

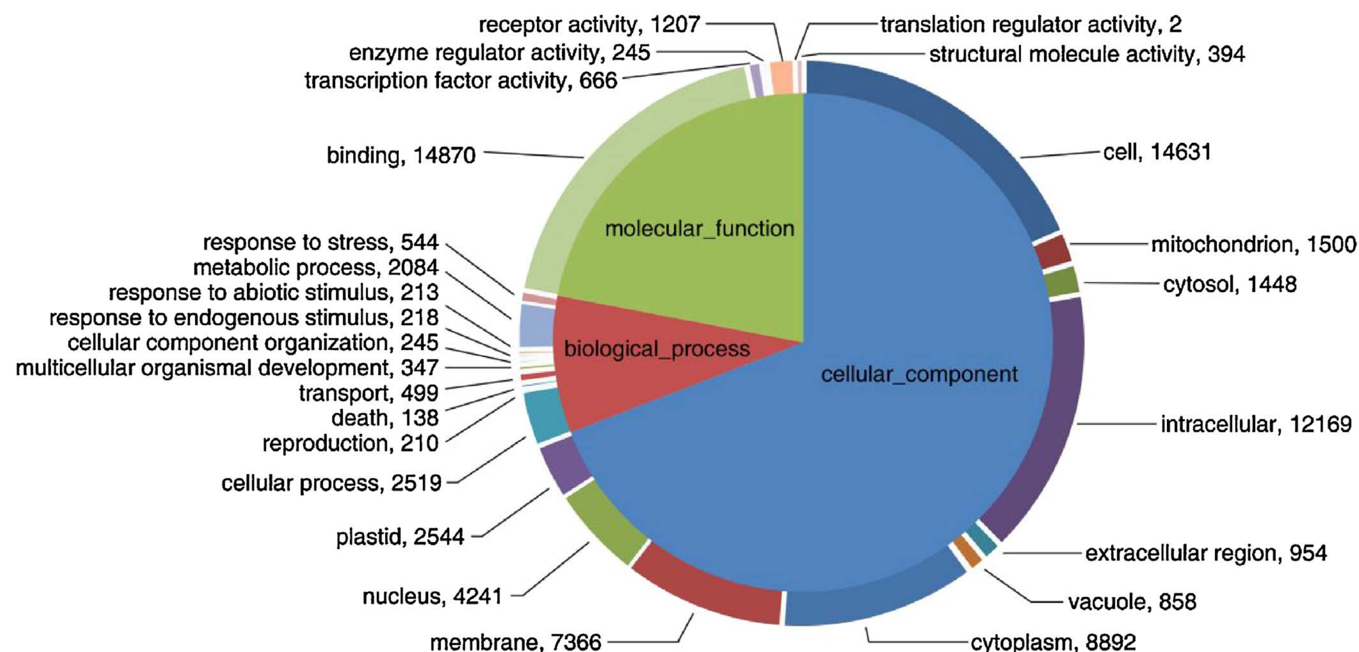

Fig. 1. GO classification of the transcriptome sequencing data. GO terms were derived based on a similarity search of the Swiss-Prot plant database. The top 10 GO terms in the cellular component, molecular function, and biological process classes are displayed.

and linoleic acid metabolism, respectively, fatty acids from which jasmonic acid is synthesised (Dataset S1). This is consistent with previous reports that JA autoregulates its own biosynthesis through direct activation of genes encoding the relevant biosynthetic enzymes (Baldwin et al., 1994). In addition, MeJA treatment resulted in the activation of pathways that are directly or indirectly involved in the plant defence system, including pathways leading to phenylpropanoid biosynthesis, gingerol biosynthesis, flavonoid biosynthesis, and biosynthesis of alkaloids and other secondary metabolites. These results indicated that the expression of JA-responsive genes was mainly correlated with stress signalling and secondary metabolic pathways in tobacco.

The 7260 DEGs were subjected to KOG analysis to assign their functions within known biological pathways. Among the 25 categories in KOG classification, the DEGs were mapped in the following pathways: “secondary metabolites biosynthesis, transport, and catabolism” (1.74%), “signal transduction mechanisms” (1.85%), “amino acid transport and metabolism” (1.02%), “lipid transport and metabolism” (1.07%), and “carbohydrate transport and metabolism” (1.02%) (Fig. S3). In the KEGG enrichment analysis, the most represented pathways that were inhibited or activated after JA treatment included “amino acid metabolism” (1.25%), “biosynthesis of the other secondary metabolites” (0.65%), “lipid metabolism” (0.76%), and “carbohydrate metabolism” (0.61%) (Fig. S4). These results further demonstrated that an extensive JA-mediated transcriptional reprogramming of metabolism is correlated with the biosynthesis of secondary metabolites in tobacco.

3.3. Identification of JA-induced TFs and TRs in tobacco

To characterise TFs and TRs in tobacco, we BLAST-queried the transcriptome against the PlnTFDB and TOBFAC database. Using this approach, we found 3419 TFs from 72 families and 840 TRs from 19 families in the tobacco transcriptome (Table 1 and Dataset S2). The largest gene family was the bHLH family, which had 239 members in our transcriptome data. There were also 236 members of the C3H genes. Among the other large TF families in tobacco were the ERF (207), homeodomain (186), PHD (184), MYB-related (161), C2H2 (161), MADS (159), bZIP (156), WRKY (154), and FAR (151) gene families. In contrast, the C2C2-YABBY, LFY, S1Fa-like, ULT, GIF, and IWS1 families were each only represented by a single gene in our dataset.

In tobacco, 329 TFs and TRs were further characterised as JA-responsive and possibly involved in JA-regulated secondary metabolism (Dataset S3 and Table 1). Among these were 227 distributed among 36 families that were significantly (two-fold or greater) upregulated and 102 from 38 families that were downregulated in JA-treated BY-2 cells. Many of these upregulated TFs were in the bHLH, ERF, WRKY, and NAC families, which are well-known TF families involved in plant growth and development and biotic and abiotic stress responses. Twenty-four families contained both upregulated and downregulated TFs, indicating the existence of a complex regulatory network in which JA regulates different branches of intersecting defence-related and secondary metabolism pathways.

3.4. Expression of ERF and bHLH TFs in JA-treated BY-2 cells

Previous studies have demonstrated that ERF and bHLH TFs play a key role in mediating JA signal transduction and regulating nicotine formation in tobacco. In our transcriptome data, 34 members of the ERF family and 27 members of the bHLH family were upregulated after JA treatment. To further validate these results, we detected the transcriptional expression of eight members of ERF group IX, four members of group X, and four members of the bHLH family under MeJA treatment in BY-2 cells. Based on their patterns of transcript accumulation, JA-induced TFs could be classified into three categories: for the bHLH family, all four members exhibited similar expression patterns and reached maximum levels at ~2 h or 6 h after JA treatment. For the ERF family, the first category included group IX members ERF19, ERF123, ERF127, and ERF66 and group X members ERF142, ERF43, ERF211, and ERF42; these TFs rapidly increased and peaked by 0.5 h, and then sharply declined. The second category contained group IX members ERF29, ERF115, ERF163, and ERF91; these TFs rapidly increased, reaching peak levels at 2 h or 6 h, and then steadily decreased (Fig. 3). The expressions of *NtPMT1a* and *NtQPT2* were used as the positive controls for MeJA treatment in BY-2 cells (Fig. S5). The various expression patterns of these JA-inducible *ERF* and *bHLH* genes suggest their diverse roles in JA-regulated alkaloid metabolism in BY-2 cells.

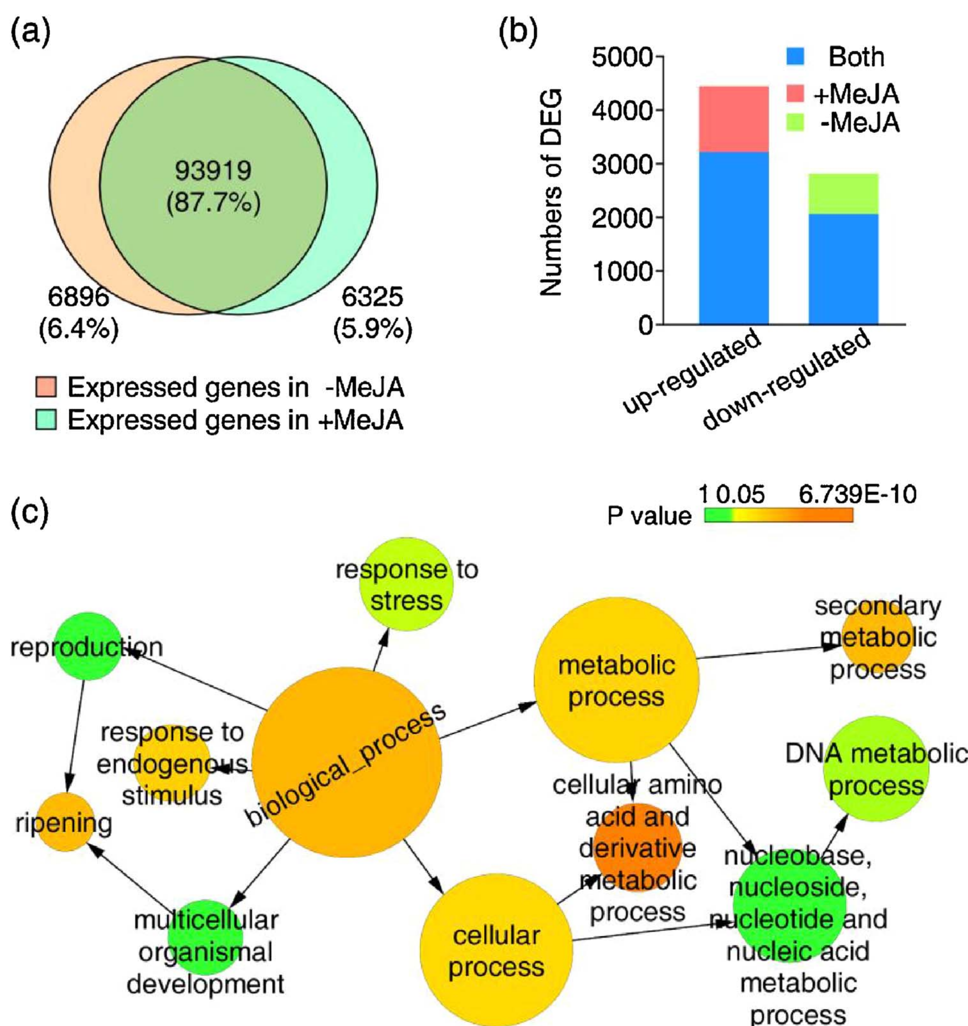

Fig. 2. The differentially expressed genes induced by JA in BY-2 cells transcriptomes. (a) Changes in gene expression profiles of BY-2 cell transcriptomes with JA treatment. (b) Comparison of unigene expression between treated and untreated BY-2 cells. The numbers of upregulated and downregulated genes two hours after JA treatment. (c) GO enrichment of differentially expressed genes.

Table 1
Size distribution of the TF families found in the transcriptome sequencing data. The tobacco TFs and TRs were identified from transcriptome sequencing libraries of tobacco BY-2 cells by BLAST searching against PlnTFDB and TOBFAC database. The first number shown indicates the number of members of each of the 72 TF families (black) and 19 TR families (blue). The numbers followed by arrows indicate the number of family members that were downregulated (↓) or upregulated (↑) after MeJA treatment in BY-2 cells.

ABI (29)	ABI3VP1 (117)(2↓)(10↑)	Alfin/Alfin-like (7/10)	AP2 (7)(1↓)(2↑)	ARF (63)(2↓)(1↑)
ARID (17)(1↓)	ARR-B (13)	AS2 (60)(6↓)	AUX-IAA (48)(1↓)	BBR-BPC (9)(1↓)
BES (30)	bHLH (239)(3↓)(27↑)	BSD (13)(1↓)	bZIP (156)(3↓)(3↑)	C2C2-CO-like (2)(1↑)
C2C2-Dof (17)	C2C2-YABBY (1)	C2H2 (161)(4↓)(14↑)	C2H2-GATA (47)(5↓)	C3H (236)(5↓)(5↑)
CAMTA (15)	CCAAT (21)	CCAAT-HAP2 (4)	CCAAT-HAP3 (15)	CCAAT-HAP5 (4)
Coactivator (3)	CONATANS (27)(2↑)	CPP (8)	DBP (20)(1↑)	DDT (18)(1↓)
Dof (14)(2↑)	E2F-DP (12)	EIL (17)(2↑)	ERF (207)(7↓)(34↑)	family (1)
FAR1 (151)(6↓)(5↑)	FHA (57)(3↓)	G2-like (47)	GARP-ARR-B (3)	GARP-G2 (15)
GeBP (9)	GIF (1)	GNAT (49)(4↑)	GRAS (84)(5↓)	GRF (10)
HMG (8)	Homeodomain (186)(2↓)(6↑)	HRT (4)	HAS (36)(2↑)	IWS1 (1)
JAZ (17)(13↑)	Jumonji (53)(1↓)(2↑)	LFY (1)	LIM (15)	LUG (17)
MADS (159)(2↓)(6↑)	MBF1 (10)(1↓)	MED6 (4)	mTERF (54)(1↓)(2↑)	MYB (84)(2↓)(15↓)
MYB-related (161)(1↓)(7↑)	NAC (138)(6↓)(19↑)	Nin (33)(1↓)(1↑)	NOZZLE (3)	OFP (8)(2↑)
Orphans (131)(3↓)(5↑)	PBF-2-like (5)	PcG (14)	PHD (184)(4↓)(3↑)	PLATZ (14)(1↓)(3↑)
Pseudo (11)	R2R3-MYB (45)(4↑)	RB (3)	Rcd1-like (5)	S1Fa-like (1)
SBP (37)(2↓)	SET (106)(1↑)	Sigma70-like (12)	SNF2 (104)	SRS (10)
SWI/SNP-BAF60b (29)(1↓)	SWI/SNF-SWI3 (22)(1↓)(1↑)	TAZ (14)(1↑)	TCP (38)(3↓)	TRAF (138)(5↓)(6↑)
Trihelix (62)(3↓)(1↑)	TUB (31)(2↓)	ULT (1)	VOZ (5)	WRKY (154)(1↓)(20↑)
Zf-HD (9)(1↓)				

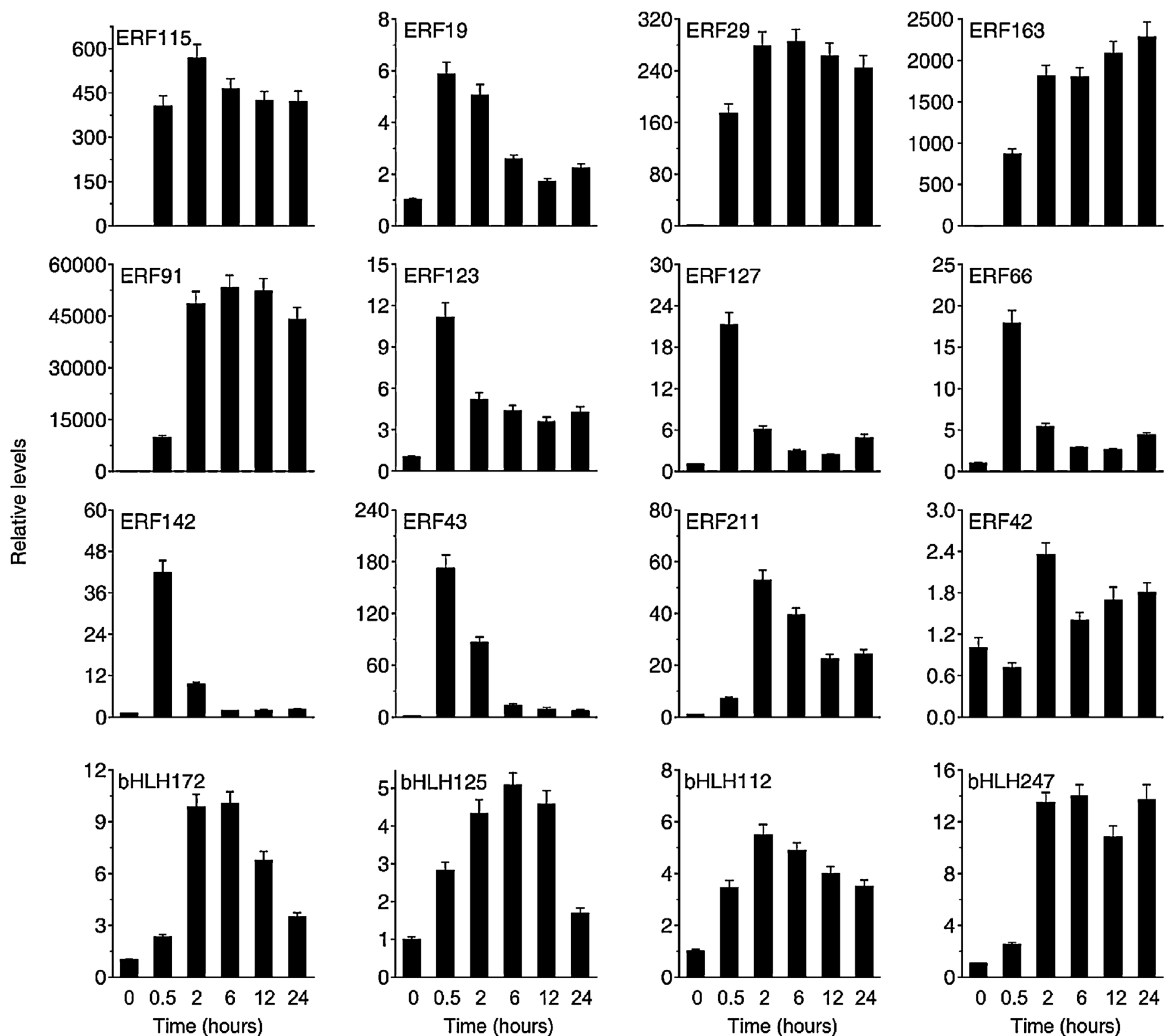

Fig. 3. Relative expression of the selected *ERFs* and *bHLHs* in JA-induced BY-2 cells. Treated and untreated BY-2 cells were harvested at the times indicated after JA treatment and analysed for gene expression by qPCR. *NtPMT1a* and *NtQPT2* were used as positive markers of JA induction (Fig. S5). Error bars indicate the standard deviations of three biological replicates.

3.5. Expression of ERF and bHLH TFs in nic mutant plants

In tobacco plants, two genetic loci, *nicotine1* and *nicotine2* (*nic1* and *nic2*), specifically control the nicotine levels, and the mutant alleles *nic1* and *nic2* have been used to confer a low-nicotine trait in commercial tobacco cultivars (Hibi et al., 1994). NIC2 was recently shown to be a cluster of seven ERF genes in group IX of the tobacco AP2/ERF family that regulates JA-inducible nicotine formation in tobacco (Shoji et al., 2010). Nicotine is exclusively synthesised in the roots (Dawson, 1942) and transported and accumulated in the vacuoles of leaf mesophyll cells (Saunders, 1979). Therefore, we detected the expression patterns of 12 *ERFs* and four *bHLHs* in the roots of the wild type (WT) and the *nic1*, *nic2*, and *nic1nic2* mutants (Fig. 4). First, we observed that *ERF19* in group IX had an expression profile similar to *ERF115*, which is a member of the NIC2 cluster of seven *ERF* genes and is highly expressed in WT and *nic1* plants, and almost undetectable in *nic2* and *nic1nic2* plants. Group IX members *ERF29*, *ERF163*, and *ERF91*, group X members *ERF142* and *ERF43*, and *bHLH125* and *bHLH112* had relatively higher expression levels in *nic2* plants than in WT and *nic1nic2* plants, and lower expression levels in *nic1* mutants. Meanwhile, group IX member *ERF123* and group X members *ERF211* and *ERF42* had higher expression levels in *nic1* plants than in WT and *nic1nic2* plants, and lower expression levels in *nic2* mutants. Group IX members *ERF127* and *ERF66*, and *bHLH172*, showed similar expression levels in the WT and three mutant plants. The expression level of *bHLH247* in *nic1nic2* was lower than that in WT, *nic1*, and *nic2* plants.

We further determined whether these 16 TFs were differentially expressed after JA treatment in WT, *nic1*, *nic2*, and *nic1nic2* plants (Fig. 5). The transcription of *bHLH112* and *bHLH172* was strongly induced and showed similar patterns in the WT and three mutants, while *bHLH125* and *bHLH247* were slightly affected in all four plants. The expression of *ERF16* and *ERF19* was very similar and only induced in the WT and *nic1* plants. *ERF29*, *ERF163*, *ERF91*, *ERF142*, *ERF43*, *ERF211*, and *ERF42* could be induced with MeJA in the WT and three mutant plants; however, their expression patterns varied among the plants. *ERF123*, *ERF127*, and *ERF66* showed no obvious induction in the

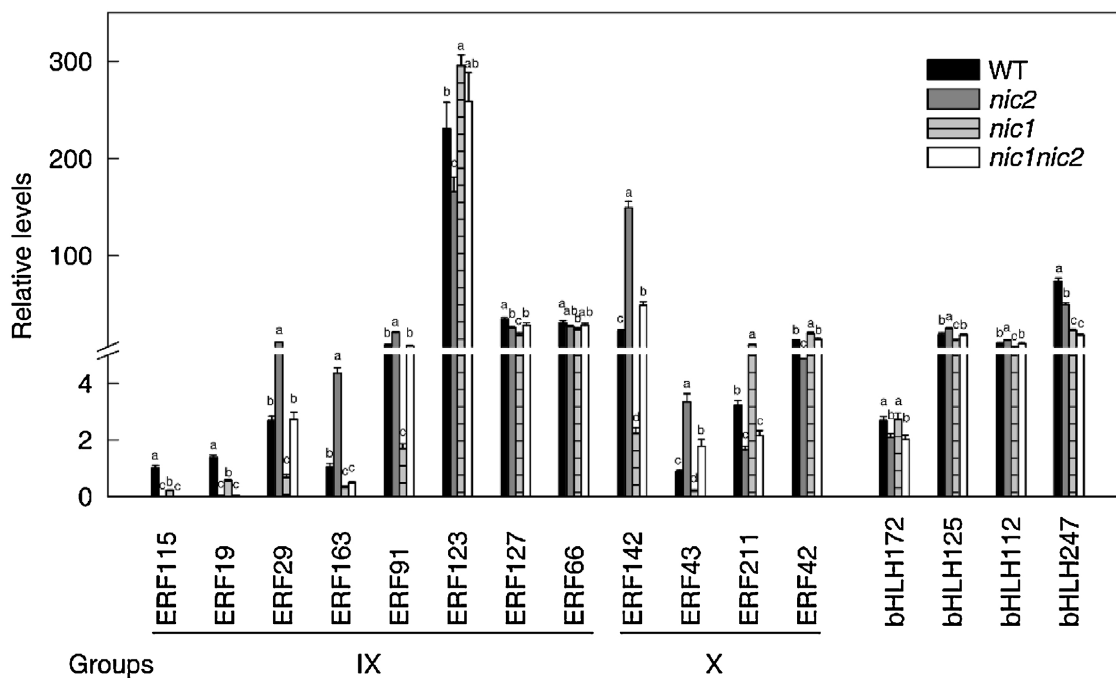

Fig. 4. Transcript levels of 12 upregulated *ERFs* and four *bHLHs* in the roots of wild-type (WT) and *nic*-mutant plants. Transcript levels of the 12 selected upregulated *ERFs* and four *bHLHs* in the tobacco roots of different *NIC* genotypes were measured by qPCR and are shown relative to the wild-type levels. Error bars indicate the standard deviations of three biological replicates. Different letters (a–c) indicate statistically significant differences ($P < 0.05$) according to Tukey's multiple range test.

WT or three mutants. *NtPMT1a* and *NtQPT2* were used as controls for JA treatment in the WT and three *nic* mutants (Fig. S6). These results suggest that different members of the ERF and bHLH gene families are indeed involved in JA responses and play diverse roles in regulating secondary metabolism in tobacco.

4. Discussion

JAs have an evolutionarily conserved role in activating the biosynthesis of secondary metabolites in response to developmental requirements and various environmental stimuli, in which the key aspect is the concerted transcriptional activation of genes encoding the enzymes that catalyse secondary metabolic reactions. For a better understanding of MeJA-dependent metabolic changes, and characterising the novel MeJA signal transduction components, transcriptome-wide studies are a powerful approach to evaluate the expression profiles of gene involved, either structurally or regulatorily, with secondary metabolism and to identify the potential TFs regulating secondary metabolism in tobacco. In this study, we employed next-generation sequencing technology to generate a transcriptome database of 107,140 transcripts and identified 3419 TFs and 840 transcriptional regulators, of which 329 JA-responsive differentially expressed TFs could be associated with tobacco secondary metabolism in BY-2 cells.

Previous studies have shown that JAs can modulate particular primary metabolic pathways to supply connected secondary metabolite pathways with the necessary substrates (Pauwels et al., 2009). Goossens et al. (Goossens et al., 2003) used cDNA-AFLP-based transcript profiling to demonstrate an extensive genetic reprogramming of metabolism that was well correlated with observed shifts in the biosynthesis of the secondary metabolites investigated. Gális et al. (Gális et al., 2006) identified 828 genes that were upregulated by MeJA treatment and involved in the accumulation of several phenylpropanoid–polyamine conjugates with a cDNA microarray of BY-2 cells. In this paper, we characterised 7260 differentially expressed unigenes in JA-treated BY-2 cells and found that the numerous differentially expressed genes were especially enriched in the cellular amino acid and derivative metabolic process and the secondary metabolic process categories according to GO enrichment analysis, providing a more detailed analysis of transcriptional machineries in JA-mediated secondary metabolism in tobacco.

The expression of key enzymes, such as ornithine decarboxylase (ODC) (De Boer et al., 2011a), PMT (Chintapakorn and Hamill, 2003), N-methylputrescine oxidase (MPO) (Naconsie et al., 2014), and quinolinate phosphoribosyltransferase (QPT) (Ryan et al., 2012), is involved in alkaloid biosynthesis and exhibited remarkable upregulation after JA treatment, leading to the recognition of so-called 'transcriptional regulons', which cooperatively regulate the transcription of these enzymes. Transcription factors (TFs) are the master regulators owing to the capacity of single TFs controlling the expression of many target downstream genes. Compared to the previous discovery of members from 64 gene families in the tobacco genome (Rushton et al., 2008), we have identified a total of 3419 TF genes from 72 families and 840 transcriptional regulators from 19 families, of which 227 TFs from 36 families were upregulated and 102 TFs from 38 families were downregulated after JA treatment in tobacco BY-2 cells. These results suggest that there is a sophisticated transcriptional network to regulate JA-triggered secondary metabolism in tobacco.

ERF and bHLH regulons are conserved and involved in the control of primary and secondary metabolism, growth and developmental programs, as well as responses to environmental stimuli in the plant kingdom. The AP2/ERF TFs are a large multigene family whose members play diverse roles in the regulation of plant developmental progress as well as responses to abiotic and biotic stresses (Kidd et al., 2006; Mizoi et al., 2012). They also play a prominent regulatory role in secondary metabolism leading to nicotine and related alkaloid formation in tobacco (Sears et al., 2014; Shoji et al., 2010) and terpenoid indole alkaloid synthesis in *C. roseus* (Memelink et al., 2001). In this study we found that the ERF family has 207 members in the tobacco transcriptome, of which 34 members were upregulated and seven were downregulated in response to MeJA treatment in BY-2 cells. We further confirmed that nine of the 34 ERFs, members of groups IX and X (Rushton et al., 2008), were induced by MeJA in BY-2 cells and tobacco plants. However, ERF123, ERF127, and ERF66 were not induced by MeJA in tobacco plants. More interestingly, ERF19, a non-NIC2 locus

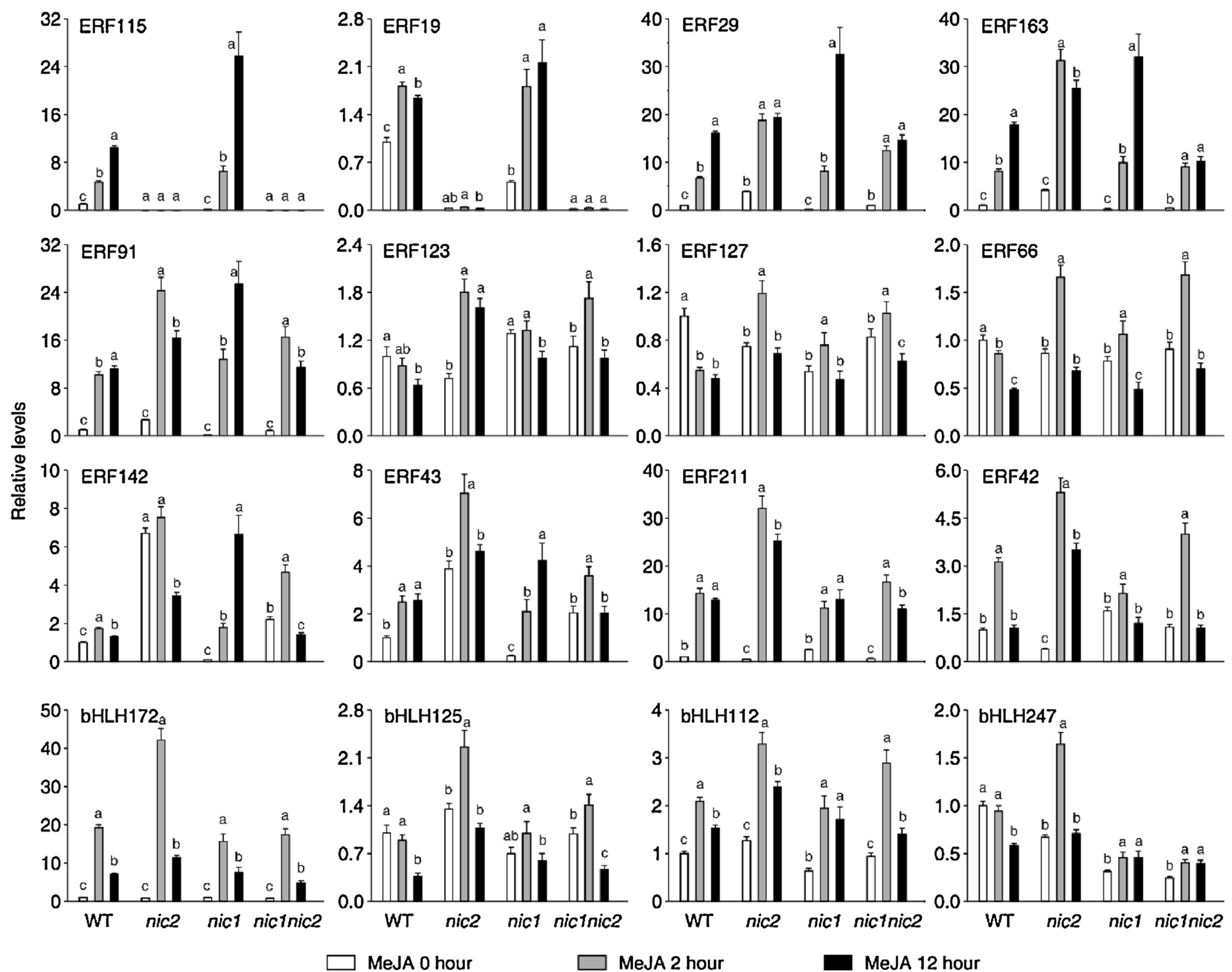

Fig. 5. Expression of the selected *ERFs* and *bHLHs* in JA-induced WT and *nic*-mutant roots. Transcript levels of the *ERFs* and *bHLHs* in the roots of WT and different *NIC* genotypes were measured by qPCR and are shown relative to the untreated wild-type levels (WT–0 h). *NtPMT1a* and *NtQPT2* were used as positive markers of JA induction in plants (Fig. S6). Error bars indicate the standard deviations of three biological replicates. Different letters (a–c) indicate statistically significant differences ($P < 0.05$) according to Tukey's multiple range test.

AP2/ERF, exhibited the same expression pattern as NIC2 locus AP2/ERFs, such as ERF115 (Shoji et al., 2010), and was only induced in wild type and *nic1* plants, but not in *nic2* and *nic1nic2* plants, suggesting that ERF19 may be a novel NIC2 locus AP2/ERF and associated with transcriptional regulation of nicotine and total alkaloid formation in tobacco.

In addition to ERFs, the bHLH family members were revealed to regulate nicotine formation via regulating the expression of *PMT* and *QPT* in tobacco. Ryan et al. showed that NtQPT2 transcript levels increase following wounding or methyl jasmonate treatment whilst QPT1 transcript levels remain largely unaltered by these treatments and MYC2-bHLH and specific ERF-type transcription factors interact with NtQPT2 promoter sequences in tobacco (Ryan et al., 2012). Previous studies had also shown that the perception of JA by the receptor F-box protein CORONATINE INSENSITIVE1 (COI1) leads to the formation of a stable COI1/JA complex that binds to JAZs proteins, resulting in the degradation of JAZ repressors by the 26S proteasome and releasing bHLH TFs to activate JA-responsive genes, such as *PMT*, *QPT*, and *MPO* (Shoji and Hashimoto, 2011; Todd et al., 2010; Zhang et al., 2012). We reported previously that 17 members of the NtJAZ family exist in the tobacco transcriptome, and the NtJAZs interact with NtMYC2a, but not NtERF189, to regulate JA-induced expression of nicotine biosynthesis genes in tobacco (Yang et al., 2015). Recent studies have shown that NUP1, a tobacco plasma membrane-localised nicotine uptake permease, acts downstream of the COI1 receptor and NtMYC2, but upstream of NtERF189 to control the expression of NtERF189 to regulate the biosynthesis of tobacco alkaloids (Kato et al., 2014). In this paper, 239 bHLH members were identified in the tobacco transcriptome, of which 27 members were upregulated and 3 were downregulated by MeJA treatment in BY-2 cells. We further detected that the expression of four bHLHs, bHLH172, bHLH125, bHLH112, and bHLH247, was induced by MeJA and exhibited similar expression patterns in BY-2 cells. However, the four bHLHs showed different expression levels in WT and *nic1*, *nic2*, and *nic1nic2* mutant plants. TFs bHLH125 and bHLH112 had relatively higher expression levels in *nic2* plants and a lower expression level in *nic1* mutants, and the expression of bHLH247 was inhibited more in *nic1nic2* double mutants than in *nic1* and *nic2* single mutants. We also found that bHLH112 and bHLH172 were strongly induced and showed a similar pattern in WT and the three mutants, while bHLH125 and bHLH247 were slightly affected in all four plants. These results indicate that the redundancy of highly homologous bHLH TFs provides a selective adaptation to ensure proper signal transduction in JA signalling pathways and precise regulation for nicotine biosynthesis in tobacco.

5. Conclusion

Alkaloid biosynthesis is under strict transcriptional control in tobacco, which allows plants to precisely regulate their formation in response to biotic and abiotic stress. However, the limited genomic information restricts this kind of research. In this study, we performed RNA sequencing based transcriptomic profiling of tobacco BY-2 cells before and after treatment with methyl jasmonate (MeJA) to identify novel transcriptional regulators (TRs) associated with alkaloid formation. 7260 differentially expressed transcripts were characterised in MeJA-treated BY-2 cells, of which 227 TRs in 36 families were specifically upregulated and 102 TRs in 38 families were downregulated. We further showed that the expression of 12 ethylene response factors and four basic helix-loop-helix factors increased at the transcriptional level after MeJA treatment in BY-2 cells and displayed specific expression patterns in *nic* mutants with or without MeJA treatments. Our data provide a catalogue of transcripts of tobacco BY-2 cells and benefit future study of JA-modulated regulation of secondary metabolism in tobacco.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2017.05.004.

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