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Transcriptome Profiling Identified Multiple Jasmonate ZIM-Domain Proteins Involved in the Regulation of Alkaloid Biosynthesis in Tobacco BY-2 Cells

Yuping Yang · Jing Guo · Pengcheng Yan · Yunshuang Li · Kun Liu · Ping Gao ·
Heping Zhao · Yubao Chen · Yingdian Wang · Michael P. Timko · Shengcheng Han

Abstract Jasmonate (JA) zinc-finger expressed in inflorescence meristem (ZIM)-domain (JAZ) proteins are key regulators of the JA response in plants. Transcriptome profiling of tobacco BY-2 cells was used to identify 17 members of the *NtJAZ* family, which were divided into 12 distinct groups based on their predicted amino acid sequences and conserved domains. Transcript levels of eight of the *NtJAZ* groups increased rapidly upon JA treatment, whereas the remaining members did not show a significant response. The majority of JA-induced NtJAZs formed homo- and heteromers and interacted with NtMYC2a (but not NtERF189) in yeast two-hybrid assays. NtJAZ1, NtJAZ3b, NtJAZ7 and NtJAZ10 were localised in the nucleus and degraded rapidly via the 26S proteasome pathway following the treatment of BY-2 with MeJA. RNAi-induced silencing of *NtJAZ1*, *NtJAZ3*, *NtJAZ7a* and *NtJAZ10* greatly reduced the levels of *NtPMT* transcripts and specifically decreased the nicotine content in the four RNAi transgenic BY-2 lines. The levels of transcripts encoding other nicotine biosynthesis enzymes, NtERF189 and NtMYC2a, and other NtJAZs exhibited different expression patterns in RNAi lines with or without MeJA treatment. Our results indicate that cross-talk occurs among different NtJAZs and forms a complex transcription regulatory scheme for JA-induced nicotine biosynthesis in tobacco.

Y. Yang · J. Guo · Y. Li · K. Liu · P. Gao · H. Zhao · Y. Wang ·
S. Han (✉)
Beijing Key Laboratory of Gene Resource and Molecular
Development, College of Life Sciences, Beijing Normal University,
Beijing 100875, China
e-mail: schan@bnu.edu.cn

Y. Yang
e-mail: yangyuping19860522@126.com

J. Guo
e-mail: guoj@mail.bnu.edu.cn

Y. Li
e-mail: liyunshuang629@126.com

K. Liu
e-mail: softtop@163.com

P. Gao
e-mail: gaoping1008@bnu.edu.cn

H. Zhao
e-mail: hpzhao@bnu.edu.cn

Y. Wang
e-mail: ydwang@bnu.edu.cn

P. Yan · Y. Chen
Department of Computational Biology, Beijing Computing Center,
Beijing 100094, China

P. Yan
e-mail: yanpc@bcc.ac.cn

Y. Chen
e-mail: chenyb@bcc.ac.cn

Y. Yang · M. P. Timko
Department of Biology, University of Virginia, Charlottesville,
VA 22904, USA

M. P. Timko
e-mail: mpt9g@virginia.edu

Introduction

Nicotine is the predominant alkaloid produced in many wild and cultivated *Nicotiana* species, where it is primarily used as a defence compound against insect herbivory. Nicotine is formed through the condensation of two transitory compounds, *N*-methyl-Δ1-pyrrolinium and nicotinic acid, mediated by the enzyme known as 'A622' (De Boer et al. 2009; Kajikawa et al. 2009). *N*-methyl-Δ1-pyrrolinium is synthesised from arginine and/or ornithine by putrescine *N*-methyltransferase (PMT), the key enzyme in diverting metabolism towards the biosynthesis of nicotine and other alkaloids (Hashimoto and Yamada 1994; Hibi et al. 1994). In cultivated tobacco, PMT is encoded by a small gene family (*NtPMT*) (Riechers and Timko 1999). Additionally, the co-suppression or antisense regulation of *NtPMT* expression leads to reduced levels of nicotine and total alkaloids but increased levels of anatabine and polyamines in transgenic tobacco plants, suggesting that NtPMT plays a key role in alkaloid formation (Sato et al. 2001; Voelckel et al. 2001). Nicotinic acid is synthesised from quinolinic acid via the pyridine nucleotide cycle and is controlled by the enzyme quinolinate phosphoribosyl transferase (QPT) (Sinclair et al. 2000). Reduced NtQPT levels also lead to a reduction in nicotine content (Xie et al. 2004).

Nicotine biosynthesis is affected by a variety of factors, including developmental age, phytohormones and biotic and abiotic stresses (Baldwin 1999; De Luca and St Pierre 2000; Facchini 2001). Endogenous levels of jasmonate (JA) have been shown to increase rapidly upon wounding and other stresses in plants (Creelman et al. 1992; Doares et al. 1995; Sembdner and Parthier 1993), and in tobacco, this increase is correlated with an increase in nicotine content (Baldwin et al. 1997). The levels of both nicotine and total leaf alkaloids increase following the removal of the floral meristem (topping) as a result of the decreased auxin availability and increased JA levels caused by wounding (Hashimoto and Yamada 1994). Topping-induced increases in *NtPMT* transcript abundance are observed in roots as early as 2 h and peak within 12–24 h post-topping (Riechers and Timko 1999). Exogenous methyl jasmonate (MeJA) treatment alone is sufficient to increase nicotine levels (Baldwin et al. 1994) and, concomitantly, the transcript levels of structural genes encoding enzymes and transporters involved in nicotine accumulation (Cane et al. 2005; Imanishi et al. 1998; Shoji et al. 2000; Winz and Baldwin 2001; Shoji and Hashimoto 2011a).

Previous studies have shown that COI1, the F-box protein subunit of the SCF^{COI1} complex, is the receptor for JA that mediates the JA signalling pathway in plants (Sheard et al. 2010; Yan et al. 2009). The cognate substrates of SCF^{COI1} have been identified as members of the JAZ repressor proteins (Chini et al. 2007; Thines et al. 2007; Yan et al. 2007). The degradation of JAZ proteins (JAZ1, JAZ3 and JAZ6) following MeJA treatment is dependent on the SCF^{COI1} component of E3 ubiquitin ligase and the 26S proteasome in *Arabidopsis*. Recent research has focused on the characterisation of protein–protein interactions in JAZ complexes, such as the binding of JA-Ile in the co-receptor structure (Melotto et al. 2008; Sheard et al. 2010; Yan et al. 2009), interactions among individual JAZ proteins and their splice forms (Chini et al. 2009; Chung et al. 2010; Chung and Howe 2009) and the interaction of JAZs with various TFs in *Arabidopsis* (Pauwels and Goossens 2011). JAZs also interfere with the degradation of other JAZs, causing the dominant JA-insensitive phenotypes in transgenic or mutant plants, including male sterility and resistance to JA in root-growth bioassays (Chini et al. 2009). Twelve members of the JAZ family have been identified in *Arabidopsis*, and similar numbers of the JAZ family have been found in tomato (Sun et al. 2011), rice (Seo et al. 2011) and *Nicotiana attenuata* (Oh et al. 2012).

However, how tobacco transcription factors are activated to induce the expression of nicotine-related structural genes after JA is perceived by the COI1–JAZ complex and the JAZ repressors are degraded is not fully understood. Shoji et al. (2008) have reported that homologues of three JAZ proteins identified in *N. attenuata* (NtJAZ1, NtJAZ2 and NtJAZ3) are present in *N. tabacum*, and the ectopic expression of a non-degradable form of NtJAZs inhibits JA-inducible alkaloid formation in tobacco BY-2 cells. MG132, a proteasome inhibitor, suppresses JA-induced expression of nicotine biosynthesis genes in tobacco, which suggests that NtJAZs are important regulatory components of JA-inducible alkaloid biosynthesis. We and others have previously reported that the *Arabidopsis* MYC2 orthologues NtMYC2a, NtMYC2b and NtMYC2c in tobacco encode basic helix–loop–helix transcription factors. Their expression is induced rapidly by JA and can specifically activate *NtPMT1a* and *NtPMT2* expressions by binding a G-box motif within the *NtPMT1a* and *NtPMT2* promoter to regulate nicotine biosynthesis (Shoji and Hashimoto 2011c; Zhang et al. 2012). The NIC2 locus has been characterised and shown to be a cluster of at least seven ERF TFs originating in the cultivated tobacco from its diploid progenitor, *Nicotania tomentosiformis* (Shoji et al. 2010). The NIC2 locus ERFs (e.g. ERF189 and ORC1/ERF221) bind the GCC-box element in the promoters of various nicotine biosynthetic pathway genes to activate their expression (Shoji and Hashimoto 2011b, 2012; Shoji et al. 2010).

To further explore the role of NtJAZs in the regulation of JA-inducible alkaloid formation in tobacco, we performed a transcriptome analysis of tobacco BY-2 cells and were able to identify 17 members of the NtJAZ family in tobacco. A detailed examination of their interactions and expression patterns after MeJA treatment indicated that these proteins are part of a complex regulatory network that participates in the regulation of nicotine and total alkaloid formation in tobacco BY-2 cells.

Materials and Methods

Plant Cell Cultures and Transformation

Nicotiana tabacum L. cv. Bright Yellow-2 suspension cultures were grown and transformed with *Agrobacterium tumefaciens* strain LBA4404, as described previously (Zhang et al. 2012). For MeJA treatment, a 4-day cell suspension was subcultured in fresh medium lacking 2,4-D and grown for 1 day. MeJA was added to a final concentration of 100 μM. To determine the effect of cycloheximide (CHX) on MeJA-induced gene expression, BY-2 cells were pre-treated with 40 μM CHX for 20 min prior to the addition of MeJA. Control cells were pre-treated with dimethyl sulphoxide as a mock treatment. The cells from the treated and untreated cultures were collected by vacuum filtration at the times indicated for further analyses and alkaloid measurements.

RNA Extraction

Total RNA was extracted from the sample frozen in liquid nitrogen using the TRIzol reagent (Invitrogen, USA) and purified with the PureLink RNA Mini Kit (Invitrogen) combined with PureLink DNase kit (Invitrogen), according to the manufacturer's protocol. The quality of 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).

Transcriptome Sequencing and De Novo Assembly

Solexa sequencing 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. 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).

Identification of NtJAZs and Construction of a Phylogenetic Tree

We used 12 *Arabidopsis* JAZs to perform a TBLASTN search of the tobacco transcriptome database and obtained tobacco-specific *JAZ*-related sequences containing only ZIM and Jas motifs. Following the removal of redundant sequences, protein sequences were manually annotated for the presence of ZIM and Jas motifs. Phylogenetic trees were created using MEGA (ver. 4) (http://www.megasoftware.net/mega4/mega.html). The conserved sequences of the ZIM and Jas motifs were determined using BioEdit (ver. 7.1.3) software (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

RT-PCR and Quantitative Real-Time PCR

Total RNA (5 μg) was used for cDNA synthesis with the Revert-Aid First-Strand cDNA Synthesis Kit (Fermentas, Canada) according to the manufacturer's instructions. PCR reactions were carried out in a MyCycler™ thermal cycler (Bio-Rad, USA) using the extension cycling conditions of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s. The amplification was performed at 25 cycles of *NtJAZ1*, *NtJAZ3*, *NtJAZ3b* and *NtJAZ7*, 27 cycles for *NtJAZ2b.2*, and 30 cycles for *NtJAZ5*, *NtJAZ10*, *NtJAZ11* and *NtJAZ12b*. Each PCR contained 0.2 μg of cDNA and the gene-specific primers listed in Table S2. The *NtPMT1a* gene was included as a positive control and *actin* (GenBank accession numbers, EU938079) as a non-MeJA-induced control. PCR products were separated in 2 % agarose gels.

Quantitative real-time PCR (qRT-PCR) was performed on an ABI 7500 Real-time Detection System with Power SYBR Green PCR Master Mix (ABI, USA), according to the manufacturer's protocol. The primer sequences are provided in Table S3. The thermal program was 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. The melting curve technique was used to ensure that single reaction products were detected, and these data were confirmed by electrophoresis in 2 % agarose gels. *Actin* was used as a reference gene. The experiments were repeated at least three times. Analysis was performed using the Data Processing System by one-way ANOVA, taking $p < 0.05$ as significant according to Tukey's multiple range test (Tang and Zhang 2013).

JA-Inducible NtJAZ Gene Cloning

To clone NtJAZ genes, we first designed nine pairs of primers to clone the corresponding full-length sequences according to

the tobacco transcriptome sequence. In specific cases, a variation of the standard protocol was used. A total of eight NtJAZ genes were cloned by direct PCR, whereas NtJAZ7 was cloned using the 5′-RACE method (SMART™ RACE cDNA Amplification Kit, Clontech). Full-length protein-coding regions of *NtJAZs* were amplified with high-fidelity Pfu polymerase using the Gateway-compatible primers listed in Table S1, ligated into the pENTR/D-TOPO vector (Invitrogen) and confirmed by sequencing. At least three or more independent clones were fully sequenced for each gene.

The Construction of NtJAZ-RNAi Vectors

Primers were designed to PCR amplify a short fragment of each gene (*NtJAZ1*, 538 bp; *NtJAZ3*, 499 bp; *NtJAZ7a*, 678 bp; and *NtJAZ10*, 513 bp) (see Table S3 for primer sequences). PCR products were inserted into the RNAi vector *pHZPRi-Hyg* using the Gateway LR II kit (Invitrogen) to obtain the *NtJAZ-RNAi* vectors expressing the inverted fragment repeats of *NtJAZ* with a ~400-bp GUS fragment as a spacer (Zhang et al. 2012). Plasmid constructs were confirmed by sequencing. Plasmids were electroporated into *A. tumefaciens* and used for BY-2 cell transformation.

Subcellular Localisation of NtJAZs

To generate the construct 35S::NtJAZs-GFP, the respective NtJAZ-coding regions minus the stop codon were recombined into the destination vector pMDC83 (http://www.arabidopsis.org/) using the Gateway LR II kit. For the subcellular localisation experiments, vectors containing GFP and NtJAZ-GFP were transformed into BY-2 cells, as described above. GFP fluorescence was visualised using a Zeiss LSM 700 confocal microscope. The nucleus was identified by 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) staining as a control.

Yeast Two-Hybrid Assays

pENTR-D/TOPO constructs containing nine NtJAZs were used in combination with the destination vectors pDEST-GADT7 and pDEST-GBKT7 (http://www.arabidopsis.org/) with the Gateway LR II kit to generate all vectors used in this study. To assess protein interactions, the corresponding plasmids were co-transformed into *Saccharomyces cerevisiae* AH109, as described in the yeast protocols handbook (TaKaRa, Japan). Transformed colonies were identified on yeast SD medium containing Leu and Trp drop-out supplementation and grown in liquid media lacking Leu and Trp until $OD_{600}=1$. Then, 6-μL of undiluted and 100-fold diluted cell suspensions were plated on yeast SD medium lacking His, Leu and Trp to identify protein interactions (Chini et al. 2009).

The plates were incubated at 28 °C for 72 h. The empty vector pGADT7 was co-transformed with pGBKT7-NtJAZ as a control to examine NtJAZ self-activation.

To detect the interaction of NtJAZs with NtMYC2a and NtERF189, full-length *NtMYC2a* (HM466974.1) and *NtERF189* (EB447203.1) were amplified using primers (Table S4), cloned into pENTR-D/TOPO, recombined into the vector pDEST-GADT7 and confirmed by sequencing. The yeast transformation and interaction assay was performed as described above.

Bimolecular Fluorescence Complementation

The full-length coding sequences of NtJAZ1, NtJAZ3, NtJAZ3b, NtJAZ7 and NtJAZ10 (minus the stop codons) were recombined into pDEST-n(1–174)EYFP-N1, pDEST-n(1–174)EYFP-C1, pDEST-n(175-end)EYFP-N1 and pDEST-n(175-end) EYFP-C1 vectors to generate plasmids NtJAZ-nYFP, nYFP-NtJAZ, NtJAZ-cYFP and cYFP-NtJAZ, respectively, using the Gateway system. Mixtures of two constructs (four possible combinations of constructs) were co-transformed into tobacco BY-2 cell protoplasts as described previously (Kovtun et al. 2000). The transformed cells were analysed for fluorescence, which is indicative of protein–protein interactions using a microscope.

26S Proteasome Degradation Assays

Tobacco BY-2 cells expressing NtJAZ-GFP fusion proteins were subcultured and transferred to a 12-well culture plate for MeJA treatment. The cells were incubated with 100 μM MeJA at 24 °C for 2 h. To assess the effect of a proteasome inhibitor on protein stability, the cells were pre-treated with water or 30 μM MG132 (Sigma-Aldrich, USA) for 2 h prior to 100 μM MeJA treatment. Fluorescence was visualised using a microscope.

Alkaloid Analysis

Wild-type or transgenic BY-2 cells were collected 72 h after treatment with MeJA and weighed to 0.15–0.20 g fresh weight. The samples were homogenised in liquid nitrogen and soaked in 2 ml of 1.5 M sodium hydroxide, and quinoline was added as an internal standard. Alkaloids were extracted from the samples and measured using Bruker 320 gas chromatography mass spectrometry with a VF-5 ms capillary column (30 m×0.25 mm, 0.25 μm) using a split-sampling mode, as described previously (Zhang et al. 2012), with minor modifications. The injection was set to direct injection mode, and the MSD was operated with electron impact ionisation in the full scan mode. The experiments were repeated at least three times.

Results

Transcriptome Profiling of *Nicotiana tabacum*

In the absence of a sequenced genome, de novo assembly of RNA-seq data was the only viable option to study the tobacco transcriptome. Using Illumina HiSeq 2000 sequencing technology, a total of 131 million 90 bp pair-end RNA-seq reads were obtained for a total of ~11.8 Gb nucleotide bases. After trimming reads with a quality score of less than 20, ~129 million reads remained with an average length of ~84 bp, generated using the 'trinity' de novo transcriptome assembly program (Grabherr et al. 2011). By overlapping reads into longer fragments, 107,140 transcripts with a mean length of 707 bp were combined (Table 1). The sequence length with the highest percentage ranged from 200 to 600 bp (61.02 %), followed, in succession, by 601-2,000 bp (35.30 %), 2,001-3,000 bp (2.76 %) and longer than 3,000 bp (0.92 %).

Characterisation of NtJAZs in Tobacco

To identify transcripts encoding JAZ proteins in tobacco, we BLAST-searched the tobacco transcriptome using the *Arabidopsis* JAZ protein-coding sequence. A total of 17 putative NtJAZs were identified and clustered into 12 groups according to their conserved domains: an N-terminal domain, a TIFY-containing domain and a C-terminal Jas domain. To compare the evolutionary relationship of the JAZs in *Nicotiana* plants, a phylogenetic tree was generated using the predicted amino acid sequences of the 17 NtJAZs and 12 NaJAZs, which were characterised in *N. attenuata* (Oh et al. 2012) (Fig. 1a). The 29 proteins were classified into two major groups. One group contained the subgroup NtJAZ11a and NtJAZ11b, which are similar to NaJAZm, and the subgroup NtJAZ12a and NtJAZ12b, which are related to NaJAZf and NaJAZj. The other group contained the remaining JAZ members. According to the phylogenetic analysis, each NaJAZ has one or two orthologues in tobacco, excluding NaJAZb and NtJAZ8, which represented a separate branch in the phylogenetic tree. We found that some NtJAZs contain another orthologues, such as NtJAZ2, NtJAZ3, NtJAZ7, NtJAZ10,

Table 1 Summary of the tobacco transcriptome

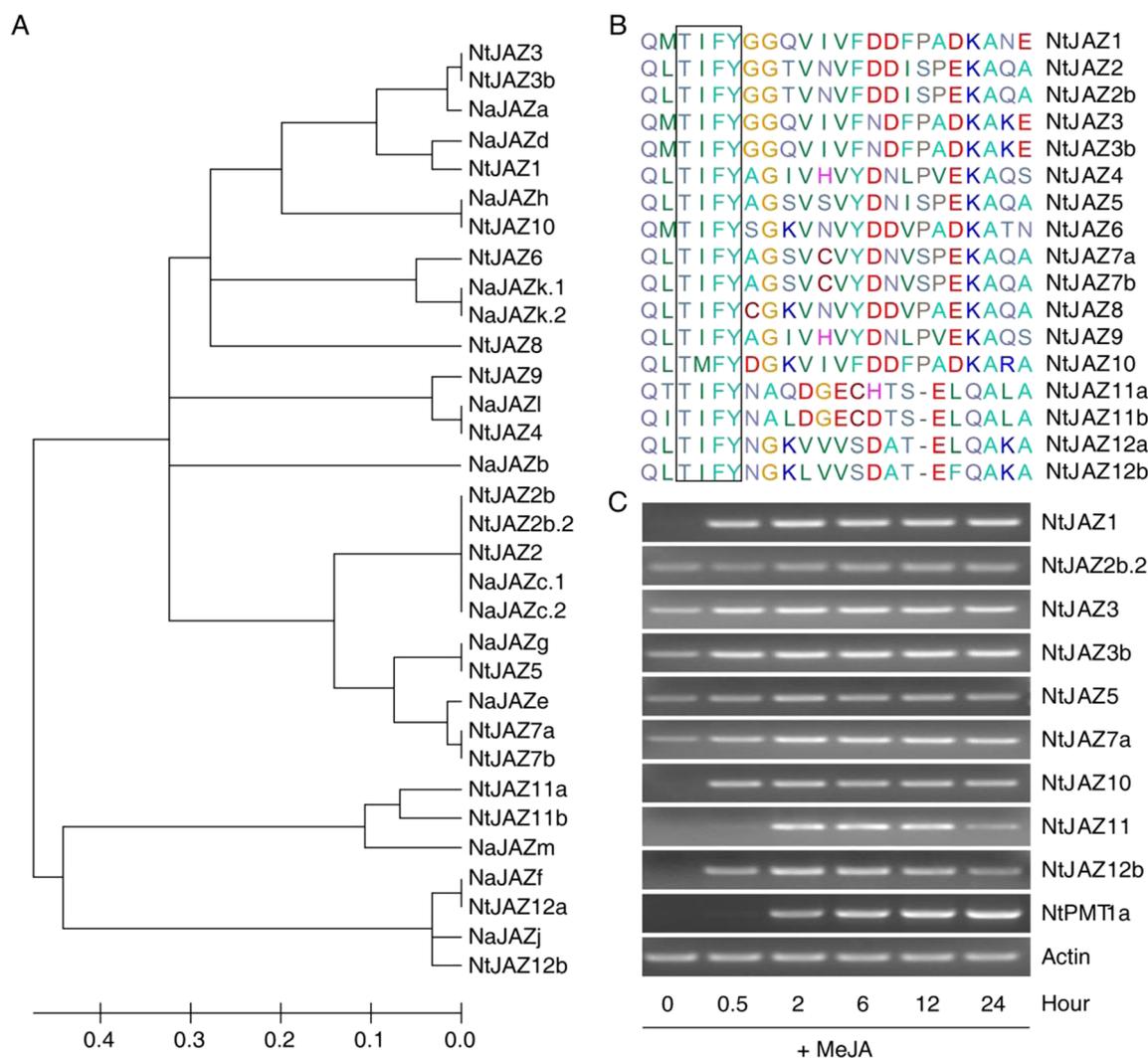|	
Total raw reads	131,333,340
Total raw nucleotides (nt)	11,820,000,600
Total high-quality reads (trimmed with Q20)	129,881,424
Total high-quality nucleotides (trimmed with Q20) (nt)	11,006,300,659
Total transcript number	107,140
Total transcript length (nt)	75,776,502
Mean transcript length (bp)	707

NtJAZ11 and NtJAZ12. Both the TIFY and Jas domains were highly conserved among all NtJAZs (Fig. 1b; Fig. S1).

Transcriptional Expression of NtJAZ with MeJA Treatment

Given the complexity of the gene family, we examined whether different expression patterns and perhaps unique physiological roles existed for the various NtJAZ members. Transcript levels of eight *NtJAZ* group members showed rapid increases following MeJA treatment by RT-PCR (Fig. 1c). Based on their patterns of transcript accumulation, the JA-responsive NtJAZs could be classified into three categories. The first, which includes *NtJAZ1*, *NtJAZ10* and *NtJAZ12b*, showed an undetectable basal level of expression in untreated cells and a rapid increase in transcript levels within 0.5 h after MeJA treatment. The second category, including *NtJAZ2b*, *NtJAZ3*, *NtJAZ3b*, *NtJAZ5* and *NtJAZ7a*, had a high basal level of expression in untreated BY-2 cells and relatively weak induction after MeJA treatment. The third category, which includes *NtJAZ11*, showed a delayed but strongly induced expression pattern (similar to that of *NtPMT1a*) in MeJA-treated BY2 cells. All JA-induced *NtJAZs* displayed an increasing trend of long duration, excluding *NtJAZ11* and *NtJAZ12b*, which showed significant reductions at later time points. We also found that *NtJAZ4*, *NtJAZ6*, *NtJAZ8* and *NtJAZ9* showed no response to JA treatment. These results suggest that the NtJAZs have diverse functions in JA signalling and the regulation of defence processing in tobacco plants.

To further characterise whether *NtJAZs* are the primary response genes for JA treatment, we used CHX (a protein synthesis inhibitor) to assess the expression of JA-induced *NtJAZs* (Fig. S2). After pre-treatment with CHX for 20 min, *NtJAZ1*, *NtJAZ10* and *NtJAZ12b* showed marked increases, *NtJAZ2b* showed a slight decrease and the other *NtJAZs* presented no obvious changes. Comparing the transcription levels of *NtJAZs* after MeJA treatment alone with that after CHX pre-treatment, *NtJAZ1, NtJAZ5, NtJAZ10* and *NtJAZ12b* showed higher levels after treatment with both MeJA and CHX than with MeJA alone, indicating that these *NtJAZs* were primary response genes and could be transcribed in the absence of de novo protein synthesis. *NtJAZ3, NtJAZ3b* and *NtJAZ11b* showed a smaller decrease after treatment with CHX and MeJA for 2 h than after treatment with MeJA alone. The expression of *NtJAZ7a* at 2 h did not differ between treatment with CHX and MeJA and MeJA alone, but it showed a marked increase at 6 h after treatment with MeJA and CHX. The MeJA-induced expression of NtJAZ2b and NtPMT1a showed a similar pattern, which was blocked by CHX. These results suggest that the various NtJAZs may play unique roles in the JA signalling pathway and in JA-induced nicotine formation in tobacco.

A

B

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QMTIFYGGQVIVFDDFPADKANE  NtJAZ1
QLTIFYGGTVNVFDDISPEKAQA  NtJAZ2
QLTIFYGGTVNVFDDISPEKAQA  NtJAZ2b
QMTIFYGGQVIVFNDFPADKAKE  NtJAZ3
QMTIFYGGQVIVFNDFPADKAKE  NtJAZ3b
QLTIFYAGIVHVYDNLPVEKAQS  NtJAZ4
QLTIFYAGSVSVYDNISPEKAQA  NtJAZ5
QMTIFYSGKVNVYDDVPADKATN  NtJAZ6
QLTIFYAGSVCVYDNVSPEKAQA  NtJAZ7a
QLTIFYAGSVCVYDNVSPEKAQA  NtJAZ7b
QLTIFYCGKVNVYDDVPAEKAQA  NtJAZ8
QLTIFYAGIVHVYDNLPVEKAQS  NtJAZ9
QLTMFYDGKVIVFDDFPADKARA  NtJAZ10
QTTIFYNAQDGECHTS-ELQALA  NtJAZ11a
QITIFYNALDGECDTS-ELQALA  NtJAZ11b
QLTIFYNGKVVVSDAT-ELQAKA  NtJAZ12a
QLTIFYNGKLVVSDAT-EFQAKA  NtJAZ12b
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C

Fig. 1 Phylogenetic analysis and MeJA-induced expression of NtJAZs. **a** Phylogenetic tree constructed using the predicted amino acid sequences of the 17 NtJAZs and 12 NaJAZs. The tree was generated using the MEGA (ver. 4) software with the neighbour-joining algorithm. The *scale bar* indicates the number of amino acid substitutions per site. **b** Amino acid sequence alignment of the TIFY motif in the 17 NtJAZs. The highly conserved TIFY motif is *boxed*. The in-colour alignment depicting amino acids with similar hydrophobicity was generated using the BioEdit software (ver. 7.0.9). **c** Time course of the accumulation of NtJAZ transcripts in BY-2 cells after JA treatment. Treated and untreated BY-2 cells were harvested at the times indicated after JA treatment and analysed for gene expression by semi-quantitative RT-PCR. *NtPMT1a* was used as a positive marker of JA induction, and actin was used as a control

Homo- and Heteromeric Interactions Among JA-Induced NtJAZ Proteins

The full-length coding region of each JA-induced NtJAZ was inserted into the yeast vectors pDEST-GADT7 and pDEST-GBKT7, and their ability to form homo- and heterodimers was assessed. As shown in Figs. 2 and S3, of the 81 possible BD/AD combinations tested, four NtJAZs (NtJAZ1, NtJAZ2b, NtJAZ3b and NtJAZ12b) were found to strongly interact as homodimers, and NtJAZ7a showed a relatively weaker homodimer interaction. However, the remaining NtJAZs did not interact with each other. Thirty BD/AD combinations showed heterodimeric interactions, in which nine were detected in both BD/AD orientations: NtJAZ1-NtJAZ2b, NtJAZ1-NtJAZ3, NtJAZ1-NtJAZ3b, NtJAZ1-NtJAZ12b, NtJAZ2b-NtJAZ3, NtJAZ2b-NtJAZ7a, NtJAZ3-NtJAZ7a, NtJAZ3-NtJAZ12b and NtJAZ3b-NtJAZ12b. NtJAZ10 only interacted with NtJAZ2 in the AD/BD orientation, and NtJAZ11b did not form homo- or heterodimers with the other NtJAZs.

To confirm these interactions, we conducted bimolecular fluorescence complementation (BiFC) assays in BY-2 cells using several NtJAZs (NtJAZ1, NtJAZ3, NtJAZ3b, NtJAZ7 and NtJAZ10), which exhibited strong, moderate and weak interactions with other NtJAZs in the yeast two-hybrid assay. N- and C-terminal fragments of yellow fluorescent protein (nYFP and cYFP, respectively) were fused to either the N or C terminus of each NtJAZ to produce four different

Fig. 2 Interactions among MeJA-induced NtJAZs. Each of the nine full-length NtJAZs was tested in both the AD (*top row*) and BD (*left column*) orientations (in total, 81 combinations). Yeast cells were co-transformed with nine pGBKT7-NtJAZ proteins (bait), and all nine pGADT7-NtJAZ proteins (prey) were grown on yeast SD drop-out medium lacking His, Leu and Trp (−3). To test the auto-activation ability of NtJAZs, pGBKT7-NtJAZ (bait) constructs and the pGADT7 empty vector were co-transformed into yeast and selected on drop-out medium lacking His, Leu and Trp (−3)
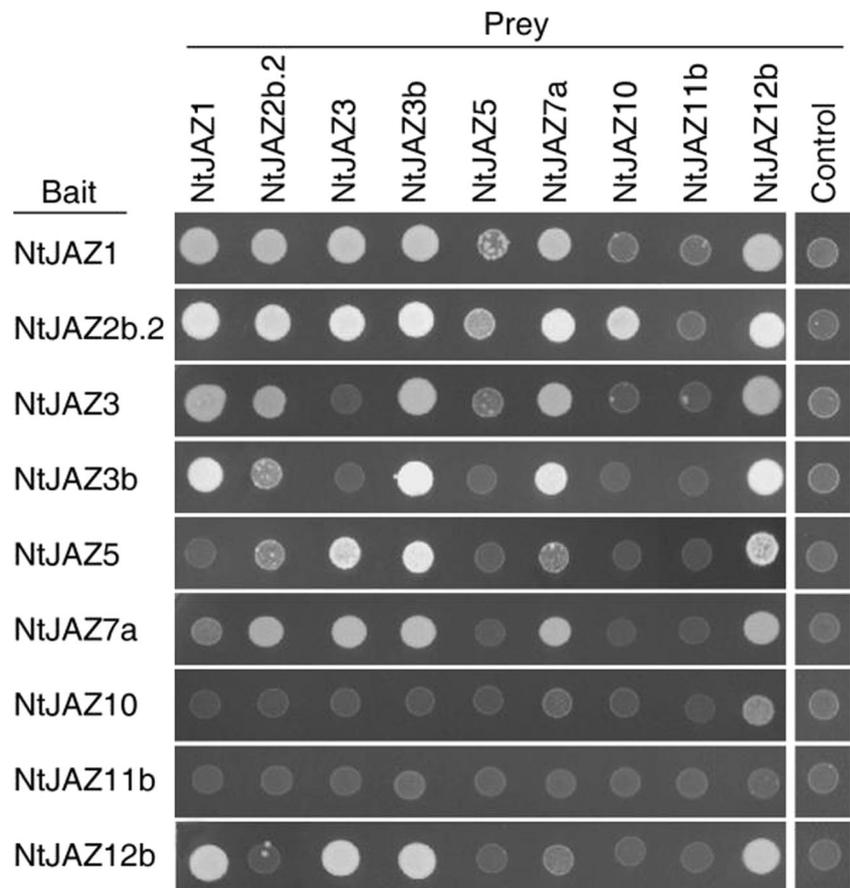
constructs. A protoplast transient transformation system was used to co-express each of the four possible combinations in BY-2 cells. Homodimeric combinations of nYFP-NtJAZ1/cYFP-NtJAZ1 and NtJAZ3b-nYFP/NtJAZ3b-cYFP yielded an YFP signal within the nucleus (Fig. S4), as did the hetero-dimer combinations of nYFP-NtJAZ1/cYFP-NtJAZ3, nYFP-NtJAZ3/cYFP-NtJAZ3b, nYFP-NtJAZ3b/cYFP-NtJAZ3 and nYFP-NtJAZ7/cYFP-NtJAZ3 (Fig. S5). We did not detect a fluorescent signal in the combinations between NtJAZ10 and itself or any of the four other NtJAZs. These findings further confirm that NtJAZs form homo- and/or heterodimers that play a role in the JA signalling pathway.

### Interactions of JA-Induced NtJAZs with NtMYC2a and NtERF189

Because NtJAZs may interact with specific transcription factors (TFs) to regulate JA response activity in plants, yeast two-hybrid assays were performed to analyse the interactions between two tobacco TFs, NtMYC2a and NtERF189, which are known to be involved in JA responsiveness, and nine JA-induced NtJAZs. NtMYC2a and NtERF189 were inserted into pDEST-GADT7 and co-transformed with nine NtJAZs (NtJAZ1, NtJAZ2b, NtJAZ3, NtJAZ3b, NtJAZ5, NtJAZ7a, NtJAZ10, NtJAZ11b and NtJAZ12b) into yeast. The results

revealed interactions between NtMYC2a and all nine NtJAZs, with little differences in the strength of the interactions among the various NtJAZs and NtMYC2a (Fig. 3). No interaction between NtERF189 and the nine NtJAZ proteins was detected (Fig. S6). These results indicated that NtMYC2a, but not NtERF189, is the common downstream interaction partner of NtJAZs in the JA signalling pathway in tobacco plants.

Degradation of NtJAZ Proteins Through the JA-Induced 26S Proteasome Pathway

To investigate whether NtJAZs are degraded by the 26S proteasome following JA treatment, we generated transgenic BY-2 cell lines overexpressing four NtJAZs (NtJAZ1, NtJAZ3, NtJAZ7a and NtJAZ10) as GFP fusion proteins under the control of the CaMV 35S promoter. All four NtJAZ-GFP fusion proteins were localised to the nucleus in BY-2 cells prior to JA treatment (Fig. 4a). The GFP fluorescence in the four NtJAZ-GFP transgenic lines disappeared after JA treatment for 2 h. However, application of a 26S proteasome-specific inhibitor, MG132, to pre-treat the transgenic lines for 2 h prior to the JA treatment impaired the JA-induced degradation of the NtJAZ-GFP proteins, supporting the interpretation that the JA-mediated NtJAZ removal occurred via the 26S proteasome pathway (Fig. 4b).

Fig. 3 Yeast two-hybrid assays of NtJAZs with NtMYC2a. Yeast cells co-transformed with nine pGBKT7-NtJAZ and pGADT7-NtMYC2a were grown on agar plates containing SD medium lacking Leu and Trp (−2) to evaluate transformation or drop-out medium lacking His, Leu and Trp (−3) to test for interactions. The yeast cultures were diluted 10-, 100- or 1,000-fold
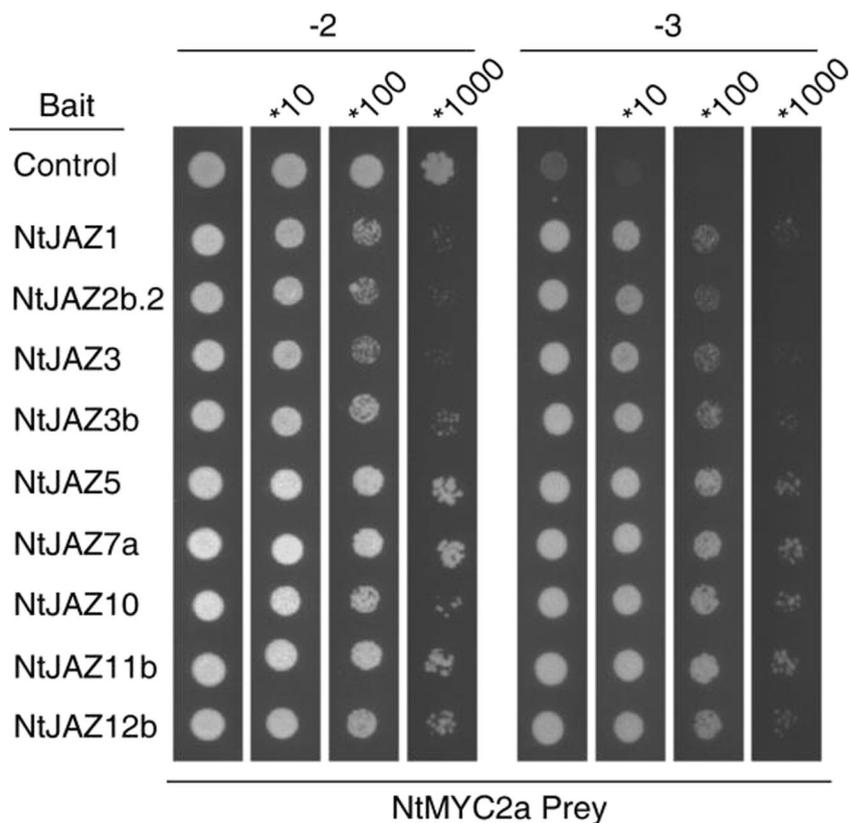
Down-Regulated NtPMT Was Found in All Four Independently NtJAZ-RNAi Lines

To identify the role of NtJAZs in regulating transcript levels of various enzymes involved in nicotine biosynthesis, the independently derived transgenic lines, in which *NtJAZ1*, *NtJAZ3*, *NtJAZ7a* and *NtJAZ10* were silenced separately using RNAi, were generated and treated with 100 μM MeJA, and gene expression at 12 h post-treatment was determined. To ensure the effect of RNAi, we selected about 500 bp 5′-end fragments in *NtJAZ* coding sequence, which include the TIFY domain. Thus, we used the alignment program to comare the cDNA sequence of TIFY domain in NtJAZs to confirm the co-suppression is not happen (see Fig. S7 for sequence similiarity analysis). As shown in Fig. 5, the expression of the corresponding *NtJAZ* was greatly reduced in the RNAi lines at 0 and 12 h post-treatment. We also found that the levels of three other *NtJAZs* present the different expression pattern in the four *NtJAZ-RNAi* lines. Compared with the control line, the expression of *NtJAZ1* showed a slight decrease in the three other lines without MeJA treatment and an increase after MeJA treatment. *NtJAZ3* showed no obvious variations in other three *NtJAZ-RNAi* cell lines with and without MeJA treatment. *NtJAZ7* showed an increase with or without MeJA treatment, whereas *NtJAZ10* showed a decrease in the *NtJAZ-RNAi* cell lines with and without MeJA treatment. The yeast two hybrid assays in this paper proved that NtJAZ10 has

no interaction with itself and other NtJAZs, but NtJAZ2. These results suggested that the expression of *NtJAZ* is regulated by JA signalling pathway but not co-suppressed in other *NtJAZ-RNAi* cell lines.

Next, we examined the transcript levels of various enzymes involved in nicotine biosynthesis. MeJA treatment increased the transcript levels of all nicotine biosynthesis genes by several fold in the vector control lines, with the exception of *NtADC*. However, a significant reduction of the *NtPMT1a* transcript level and a relatively weaker reduction of A622 were identified in all four independent *NtJAZ-RNAi* lines (compared with the untransformed lines), regardless of whether they were treated with MeJA. By contrast, the expression levels of *NtODC* and *NtQPT2* showed slight increases in the transgenic *NtJAZ-RNAi* lines compared with the untransformed lines, and *NtADC* and *NtMPO1* showed different expression patterns among the four RNAi lines. Thus, under both the untreated and MeJA-treated conditions, knockdown of *NtJAZ* inhibited mainly the transcription of *NtPMT1a*, which is involved in nicotine biosynthesis but showed a generally weaker influence on the expression of the other genes.

From the yeast two hybrid assay, we found that NtMYC2a, but not NtERF189, can interact with NtJAZs, so we determined whether the levels of *NtERF189* and *NtMYC2a* were affected in the four independent *NtJAZ-RNAi* lines. As shown in Fig. 5, the expression of *NtERF189* increased in the
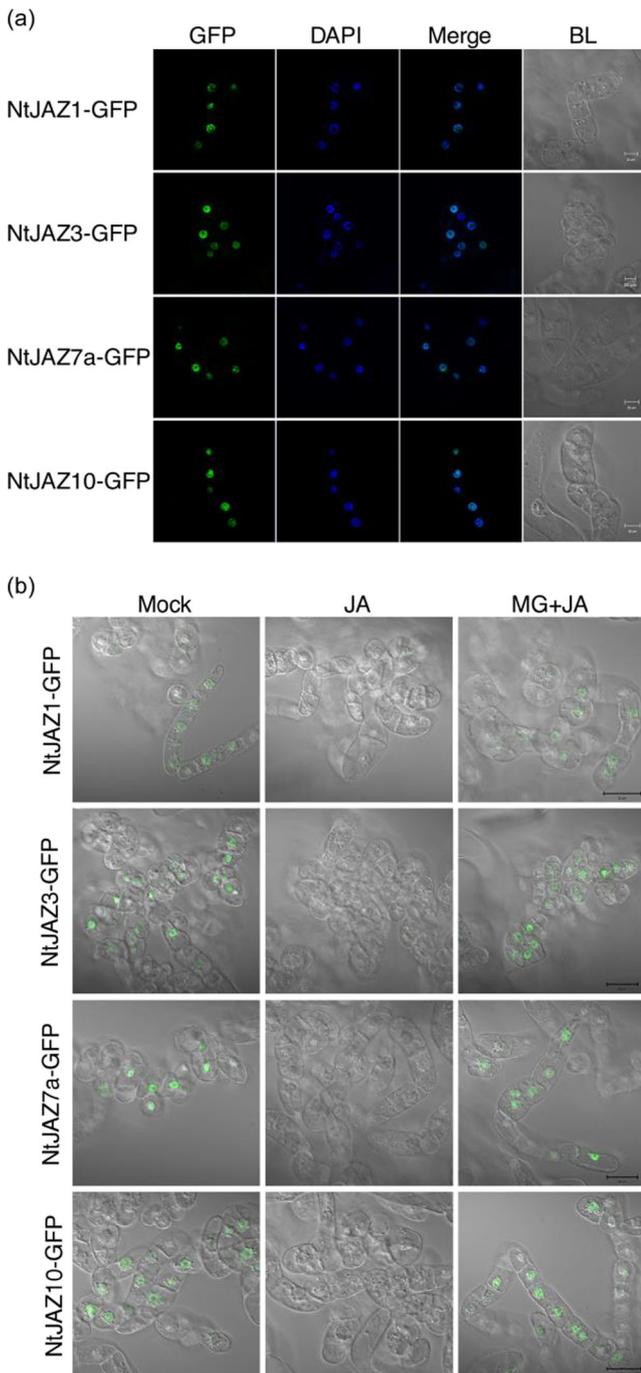
(a)

(b)

Fig. 4 Proteasome-mediated degradation of NtJAZs following JA treatment in BY-2 cells. **a** Visualisation of the nuclear-localised NtJAZ-GFP fusion protein in the 35S::NtJAZ-GFP transgenic BY-2 cell lines. GFP fluorescence was detected with a confocal laser scanning microscope. DAPI fluorescence was used as a marker for the nucleus. *Scale bar*: 50 μm. **b** Proteasome-dependent degradation of NtJAZs. BY-2 cells expressing the 35S::NtJAZ-GFP were pre-treated with water or the 26S proteasome-specific inhibitor MG132 (30 μM) for 2 h, and then the cells were treated with either MeJA (50 μM) or water (Mock) for 2 h. GFP signalling in BY-2 cells was visualised by fluorescence microscopy

NtJAZ1-RNAi, *NtJAZ7-RNAi* and *NtJAZ10-RNAi* lines with or without MeJA treatment but was decreased in the *NtJAZ3-*

RNAi line. The *NtMYC2a* transcript levels were increased in only the *NtJAZ1-RNAi* line. These results further illustrate the complexity of the transcriptional regulation of the NtJAZ-mediated JA signalling pathway involved in the control of tobacco alkaloid biosynthesis.

Nicotine Content Decreased in Four NtJAZs-RNAi Transgenic BY-2 Cell Lines

To further understand the role of NtJAZs in the control of the formation of nicotine and other alkaloids, the GC-MS analysis of the nicotine and other alkaloid contents in these RNAi lines 72 h post-treatment were showed that the nicotine content was reduced by more than 50 % in the RNAi lines compared with the control (Fig. 6a). No significant change was observed in the level of anabasine or anatalline in all four RNAi lines, except for a higher content of anatabine in the *NtJAZ7a*-RNAi and *NtJAZ10*-RNAi lines (Fig. 6b). These findings suggesting that the critical regulatory step of NtPMT mediating the conversion of putrescine to *N*-methyl putrescine was restrained, leading ultimately to inhibition of nicotine formation in *NtJAZs-RNAi* lines.

Discussion

When confronted with a variety of pests and pathogens in the natural environment, plants use a wide array of constitutive and induced defence responses to protect themselves. In tobacco, the rapid accumulation of nicotine and other alkaloids has proven to be a successful strategy for protection against herbivory. However, because of the energy cost for synthesis, the production of nicotine is highly regulated and under control of the JA signal transduction pathway (Gundlach et al. 1992; Howe and Jander 2008). How JA controls defence- and non-defence-related cellular responses had been under intensive investigation for some time. Here, we show that members of the NtJAZ gene family play a significant and complex role in regulating the formation of nicotine and other alkaloids in tobacco.

Transcriptomic profiling of tobacco BY-2 cells identified 17 distinct members of the NtJAZ family, which could be classified into 12 groups according to their sequence similarity and orthology to JAZ proteins in *Arabidopsis*. We also found that cultivated tobacco contained a complement of JAZ genes to those found in wild tobacco, *N. attenuata* (Oh et al. 2012), with the exception of orthologs to NtJAZ8 and NaJAZb. It is also noteworthy that there are two alleles of NtJAZ2: NtJAZ2b, which shares 96 % cDNA and 95 % protein similarity with NtJAZ2, and NtJAZ2b.2, which has an extra 96-bp fragment and is an alternative splicing form of NtJAZ2b. In the same subgroup of the phylogenetic tree, there are also two members of NaJAZc.2 and NaJAZc.1 in *N. attenuata*, the
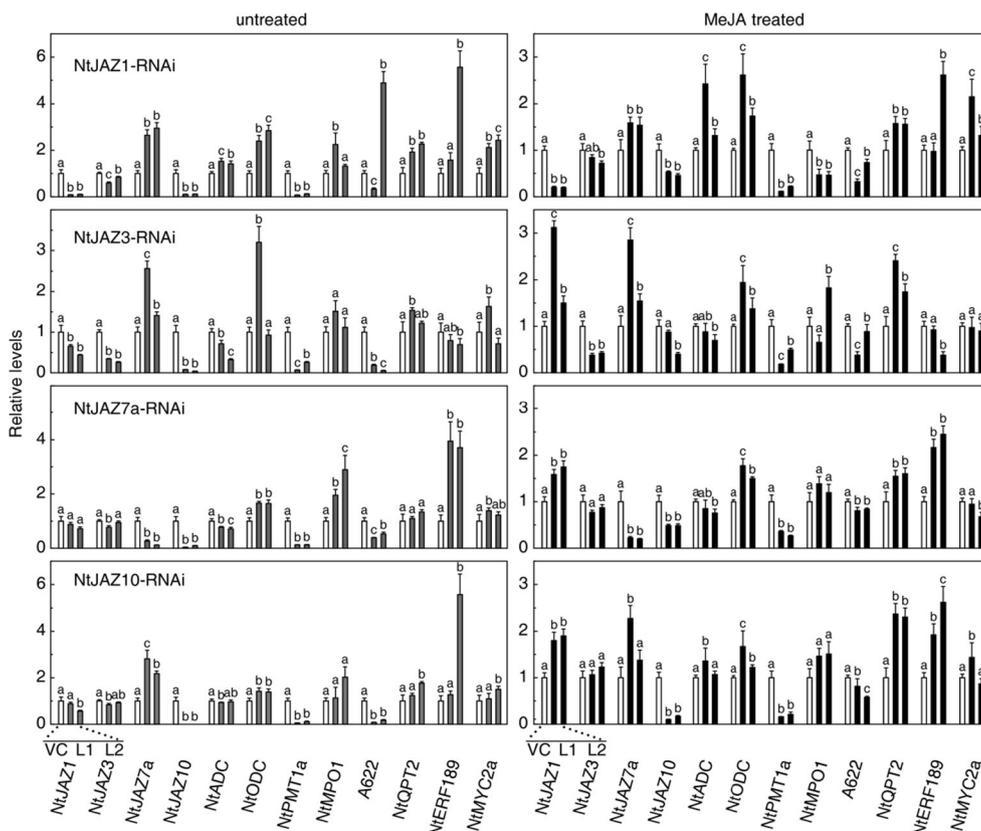
Fig. 5 Expression levels of nicotine biosynthetic enzymes and JA pathway-related genes in NtJAZ-RNAi-transformed BY-2 cell lines. Transgenic BY-2 cell lines were cultured in the absence or presence of 50 μM MeJA for 12 h and analysed for the expression of four *NtJAZs* (*NtJAZ1*, *NtJAZ3*, *NtJAZ7a* and *NtJAZ10*), nicotine biosynthesis genes (*NtADC*, *NTODC*, *NtMPO1*, *NtPMT*, *NtQPT* and *A622*) and JA signalling-related genes (*NtERF189* and *NtMYC2a*) by qRT-PCR. In the VC line, MeJA increased the transcript levels, as follows: *NtJAZ1*, 154.167-fold; *NtJAZ3*, 19.658-fold; *NtJAZ7a*, 22.951-fold; *NtJAZ10*, 94.197-fold; *NtADC*, 0.983-fold; *NtODC*, 4.151-fold; *NtMPO1*, 3.852-fold; *NtPMT*, 851.481-fold; *NtQPT*, 63.477-fold; *A622*, 2188-fold; *NtERF189*, 2.821-fold; and *NtMYC2a*, 2.905-fold. *White bars* indicate the vector control line, *grey bars* represent the transgenic lines without MeJA treatment and *black bars* with MeJA treatment. *Error bars* indicate the SD of three biological replicates. Different letters (*a–c*) indicate statistically significant differences (*p* < 0.05) according to Tukey's multiple range test

latter of which is the alternative splicing form of NaJAZc.2 and contains an extra 96-bp fragment with only a three-base difference between NtJAZ2b and NtJAZ2b.2. Additionally, only an 11-amino-acid difference is observed between NtJAZ2b and NaJAZc.2. We also showed that NtJAZ12a and NtJAZ12b, which showed 81 % similarity in their open reading frame and 77 % similarity in their amino acid sequence, are homologues of NaJAZf and NaJAZj, respectively.

The protein synthesis inhibitor CHX can be used to characterise whether JA-induced genes are the primary response

Fig. 6 Downregulation of *NtJAZs* inhibits MeJA-induced nicotine biosynthesis in transgenic tobacco BY-2 cells. The content of nicotine (**a**) and three other alkaloids (**b**) in four independently NtJAZ-RNAi-transgenic BY-2 cell lines was analysed with GC-MS 72 h after MeJA treatment. *Error bars* indicate the SD of three biological replicates. *FW* fresh weight
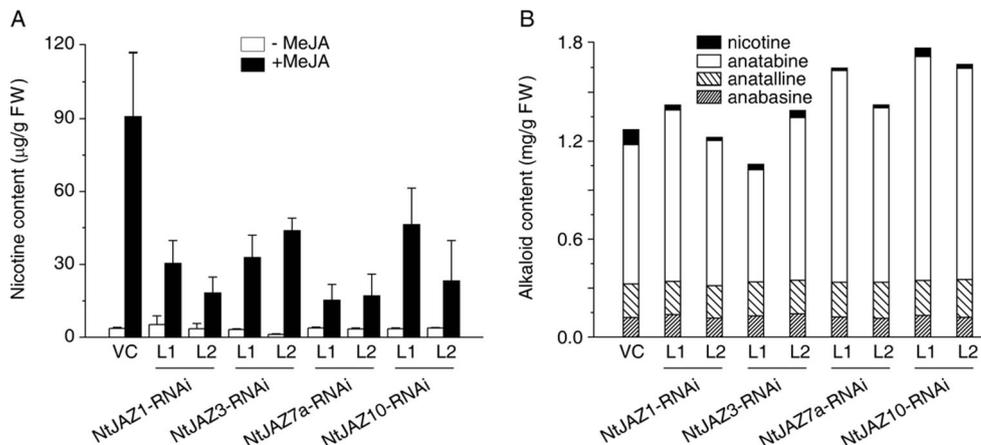
genes according to their expression pattern after JA treatment (Chung et al. 2008; Pauw and Memelink 2004). MeJA-induced expression of *NtPMT1a* was blocked by CHX, suggesting that *NtPMT1a* is not a primary response gene, and a complex transcriptional regulatory network may exist to modulate its expression. Members of the NtJAZs exhibit different patterns of expression following JA treatment and in the presence and absence of CHX. Some *NtJAZs* (e.g. *NtJAZ1*, *NtJAZ5*, *NtJAZ10* and *NtJAZ12b*) may function as primary response genes, while others (e.g. *NtJAZ2b*, *NtJAZ3*, *NtJAZ3b*, *NtJAZ7a* and *NtJAZ11b*) function as secondary response genes, which indicated s a different role for various NtJAZs in regulating the expression of PMT and other nicotine biosynthesis-related enzymes in tobacco.

JAZ proteins are characterised by three conserved domains; an N-terminal domain, a TIFY-containing zinc-finger expressed in inflorescence meristem (ZIM) domain, and a C-terminal Jas domain in plants (Wager and Browse 2012). The N-terminal domain is not well characterised, although some studies have implicated this domain in a small set of protein–protein interactions (Hou et al. 2010; Zhu et al. 2011). Previous research showed that the ZIM domain with the TIF[F/Y]XG motif and its variants mediate the homo- and heteromeric interactions between JAZ proteins in *Arabidopsis* (Chini et al. 2009; Chung and Howe 2009; Pauwels et al. 2010; Vanholme et al. 2007). In this study, almost all 17 NtJAZ sequences contained a conserved TIF[F/Y]XG motif, except for NtJAZ10, which contained the variant-form TMF[F/Y]XG motif, and the NtJAZ11 group, which has the TIF[F/Y]XA motif. These findings may explain why NtJAZ10 and NtJAZ11 have few/no interactions with other JA-induced NtJAZs. In *Arabidopsis*, the C-terminal Jas domain is involved in a wide range of protein–protein interactions with MYC-related bHLH (Cheng et al. 2011; Chini et al. 2009), some MYB TFs (Song et al. 2011), DELLA proteins in GA signalling (Hou et al. 2010) and EIN3/EIL1 in Ethylene signalling (Zhu et al. 2011) and are responsible for the degradation of JAZs in the presence of JA–Ile (Chini et al. 2007; Melotto et al. 2008; Thines et al. 2007). In tobacco, Shoji and Hashimoto (2011c) showed that NtMYC2 induces, directly or indirectly, the transcription of the *NIC*-locus *ERF189* genes, then ERFs activate the *PMT* promoter in cooperation with NtMYC2 depending on their cognate binding sites. Using yeast two-hybrid assay they further proved that NtMYC2, but not ERF189, interacted with NtJAZ1, NtJAZ2 and NtJAZ3. In our present study, we also showed that nine JA-inducible NtJAZs interacted with NtMYC2a, but not with NtERF189 in a yeast two-hybrid assay, suggesting that NtERF plays a role in JA-mediated nicotine biosynthesis in a different pathway than NtMYC2-clade bHLH proteins in tobacco.

According to the prevailing model of the JA signalling pathway in plants, JAZ proteins serve as transcriptional repressors to control JA responsive gene expression in the absence of

the phytohormone. An increase in levels of JA–Ile allows the hormone receptor COI1 to bind and target JAZ repressors for ubiquitination and degradation via the 26S proteasome pathway, releasing transcription factors AtMYC2 to induce the expression of JA-responsive genes (Chini et al. 2007; Thines et al. 2007). We showed that MeJA treatment results in rapid activation of transcription of *NtPMT* and *NtJAZs* genes via 26S proteasome-dependent removal of pre-existing JAZ proteins and subsequent nicotine biosynthesis, whereas rapid synthesis of new JAZ proteins ensures attenuation of the JA response shortly after signal transmission. In this report, we examined the expression of another three *NtJAZs* in these four *NtJAZ*-RNAi lines. *NtJAZ7a* showed increased gene expression in the *NtJAZ1*-RNAi, *NtJAZ3*-RNAi and *NtJAZ10*-RNAi lines with or without MeJA treatment. By contrast, *NtJAZ10* showed reduced expression in all four *NtJAZ*-RNAi lines. We also showed that the expression of *NtERF189* increased in the *NtJAZ1*-RNAi, *NtJAZ7a*-RNAi and *NtJAZ10*-RNAi lines with or without MeJA treatment but decreased in the *NtJAZ3-RNAi* line, and the *NtMYC2a* transcript levels were increased in only the *NtJAZ1-RNAi* line. Therefore, *NtJAZ* silencing may have directly or indirectly affected the expression of other JAZ genes, which was not the result of a cross-silencing effect, but the inverse effect and cross-talk among different NtJAZs. These results proved that NtJAZs play the multi-feedback roles in JA signalling pathway to maintain the balance between normal growth and nicotine formation in tobacco.

Previous studies showed that NtJAZ1 and NtJAZ3, which lack the C-terminal Jas region, are resistant to COI1-dependent degradation after JA treatment and consequently repress JA-induced *PMT* expression and total alkaloid accumulation in tobacco hairy roots and BY-2 cells (Shoji et al. 2008), which suggested that NtJAZs (as repressors) play an important role in regulating *PMT* transcription and nicotine formation. Oh et al. showed that RNAi-mediated knockdown of *NaJAZh* (an ortholog of NtJAZ10) suppressed nicotine biosynthesis and significantly reduced nicotine levels in inverted-repeat (ir) JAZh *N. attenuata* plants. In addition, NaJAZh suppression alone had a multitude of direct and indirect effects, including altering the expression of other *NaJAZ* genes and transcriptional components, such as *AP2/ERFs* and *MYC2* (Oh et al. 2012). We also found that suppressing the expression of a single *NtJAZ* gene using RNAi technique leads to an unexpected reduction of *NtPMT1a* expression, and reduced nicotine accumulation by more than 50 % in all four independent *NtJAZ*-RNAi lines compared with the control after MeJA treatment. To further analyse the regulatory roles of different NtJAZs, we examined the expression of tobacco proteinase inhibitor II (NtPI-II) in these *NtJAZ7a*-RNAi and *NtJAZ10*-RNAi lines. Our results showed that the expression of *NtPI-II* was strongly increased in the *NtJAZ10*-RNAi lines with or without MeJA treatment, suggesting that NtJAZ10 negatively regulates the transcriptional expression of *NtPI-II* (Fig. S8). However, we

also found that the expression of *NtPI-II* decreased in the *NtJAZ7a*-RNAi line compared with the control line after MeJA treatment (Fig. S8). Thus, we can get the conclusion that NtJAZs is as a key regulator, not just a transcriptional repressor, to be involved in JA signalling pathway to regulate nicotine formation in tobacco.

Anatabine is the major alkaloid induced in BY-2 cells after MeJA treatment (Shoji and Hashimoto 2008). In the nicotinic acid branch of the pathway, this results in the formation of anatabine, anabasine, and anatalline. A higher concentration of anatabine was found in the *NtJAZ7a-RNAi* and *NtJAZ10-RNAi* lines but not in the *NtJAZ1-RNAi* or *NtJAZ3-RNAi* lines. Moreover, the anabasine and anatalline contents showed no obvious changes in any of the four *NtJAZ*-RNAi lines, possibly due to the lack of inhibition of *NtQPT2* transcription in RNAi lines. The above results are consistent with the previous conclusion that suppressed *NtPMT* expression reduces levels of nicotine and increases levels of anatabine and polyamines in transgenic tobacco plants (Sato et al. 2001; Voelckel et al. 2001; Wang et al. 2009).

Our findings indicate that a complex regulatory network scheme for JA-induced nicotine biosynthesis exists in tobacco, in which different JAZ proteins control different branches of intersecting defence-related and secondary metabolic pathways. These observations underscore the need for additional studies to explore the mechanism of this regulation and how interaction-distinct JAZ proteins can result in seemingly contradictory regulatory outcomes.

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Accession Numbers

Sequence data for the genes in this report can be found in the GenBank database under the following accession numbers: *N. tabacum* JAZ2b (KC246550), *N. tabacum* JAZ2b-2 (KC246551), *N. tabacum* JAZ3b (KC246552), *N. tabacum* JAZ4 (KC246553), *N. tabacum* JAZ5 (KC246554), *N. tabacum* JAZ6 (KC246555), *N. tabacum* JAZ7a (KC246556), *N. tabacum* JAZ7b (KC246557), *N. tabacum* JAZ8 (KC246558), *N. tabacum* JAZ9 (KC246559), *N. tabacum* JAZ10 (KC246560), *N. tabacum* JAZ11a (KC246561), *N. tabacum* JAZ11b (KC246562), *N. tabacum* JAZ12a (KC246563) and *N. tabacum* JAZ12b (KC246564).