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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