Gene 646 (2018) 169–180

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

# Transcriptomic analysis of topping-induced axillary shoot outgrowth in *Nicotiana tabacum*

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A R T I C L E  I N F O

*Keywords:*
*Nicotiana tabacum*
Axillary shoot outgrowth
Differentially expressed genes
Sugars
Hormone signal

A B S T R A C T

Topping is an important agronomic practice that significantly impacts the yield of various crop plants. Topping and the regulation of axillary shoot outgrowth are common agronomic practices in tobacco. However, the effects of topping on gene expression in tobacco remain unknown. We applied the Illumina HiSeq™ 2000 platform and analyzed differentially expressed genes (DEGs) from untopped and topped plants to study the global changes in gene expression in response to topping. We found that the number of DEGs varied from 7609 to 18,770 based on the reads per kilobase per million mapped reads (RPKM) values. The Gene Ontology (GO) enrichment analysis revealed that the cellular carbohydrate metabolic process and the disaccharide metabolic process, which may contribute to starch accumulation and stress/defense, were overrepresented terms for the DEGs. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed that many DEGs were involved in starch and sucrose metabolism, glycolysis/gluconeogenesis, pyruvate metabolism, and plant hormone signal transduction, among other processes. The knowledge gained will improve our understanding of the processes of axillary shoot formation and enlargement at the transcriptional level. This study lays a solid foundation for future studies on molecular mechanisms underlying the growth of axillary shoots.

## 1. Introduction

Apical dominance is a phenomenon of many plant species that governs shoot tip production and suppresses the outgrowth of axillary shoots. In response to the loss of their apical shoot buds, plants rapidly release axillary shoots. The regulation of axillary shoot outgrowth is an important aspect of plant architecture (Horvath et al., 2003; Leyser, 2003). Topping is an important agronomic practice in tobacco production used to maximize yield (Rao et al., 2003). However, dormant axillary shoots become active after topping (Taylor et al., 2008) and must be periodically removed to obtain the full benefits of topping.

Tobacco (*Nicotiana tabacum*) is among the most important economic crops. Topping and the regulation of axillary shoot outgrowth are common agronomic practices in tobacco (Tso, 1990). Topping causes tobacco plants to divert their energy and nutrients to the leaves rather than the reproductive organs (Rao et al., 2003), which improves the chemical and physical characteristics of the leaves. Strong interest in controlling axillary shoot formation exists.

Axillary shoot control in tobacco production is a time-consuming, labor-intensive and expensive process to control either with manual or chemical methods (Taylor et al., 2008; Mahmood et al., 2007). Currently, the development of biotechnology has led to increasing amounts of genetic engineering methods being applied to plant breeding, including the successful application of transgenic tobacco lines (Eltayeb

*Abbreviations:* DEGs, Differentially expressed genes; RPKM, Reads per kilobase per million mapped reads; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; IAA, Auxin; CK, Cytokinin; SSH, Suppression subtractive hybridization; *N. tabacum*, *Nicotiana tabacum*; M-MLV, Moloney murine leukemia virus; FDR, False discovery rate; NY, Untopped; TY, Topped; TPS, Trehalose phosphate synthase; TPP, Trehalose phosphate phosphatase; SPS, Sucrose phosphate synthase; SPP, Sucrose phosphate phosphatase; SS, Sucrose synthase; S6P, Sucrose-6-phosphate; SP, Starch phosphorylase; UGP, Uridine diphosphate-glucose pyrophosphorylase; 6-PFK, 6-phosphofructokinase; PK, Pyruvate kinase; PDC, Pyruvate dehydrogenase complex; GA, gibberellin; ABA, Abscisic acid; AUX1, Auxin influx carrier; TIR1, Transport inhibitor response 1; AUX/IAA, Auxin/indole-acetic acid inducible; ARF, Auxin response factor; GH3, Gretchen Hagen 3; SUAR, Small auxin up RNA; CRE1, Cytokinin response 1; AHP, Histidine-containing phosphotransfer protein; B-APP, ARR-B family; A-APP, ARR-A family

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https://doi.org/10.1016/j.gene.2017.12.053
Received 1 November 2017; Received in revised form 11 December 2017; Accepted 27 December 2017
Available online 29 December 2017
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et al., 2007; Lee et al., 2007). Therefore, the suppression of axillary bud differentiation by molecular genetics methods is our ideal goal.

Plants are constantly challenged by biotic (e.g., insects and herbivores) or abiotic (e.g., mechanical damage/topping) factors and have evolved relevant mechanisms to survive under adverse conditions. Tobacco exhibits complex physiological responses to topping, including axillary shoot development, stress tolerance and changes in signal transduction balance (Baldwin et al., 1994; Li et al., 2007). The elongation of axillary shoots is a typical plant response to topping. Hormones, especially auxin (IAA) and cytokinin (CK), are involved in the initiation and growth of axillary shoots. Topping affects the transduction of plant hormones and stimulates the growth of axillary shoots in the leaf axils (Ferguson and Beveridge, 2009; Domagalska and Leyser, 2011; Müller and Leyser, 2011). In addition, the depletion of IAA is not sufficient to induce bud release after topping during the earliest stages of bud release, and sugars are necessary for axillary shoot release from apical dominance (Michael and Mason, 2014). Although topping is a common agronomic practice, only a few studies have investigated the influences of topping on gene expression related to different metabolic and developmental processes. Researchers analyzing differential gene expression in response to topping and axillary shoot control chemicals using RNA sequencing in tobacco identified 179 differentially expressed genes (DEGs) that were largely related to wounding, phytohormone metabolism and secondary metabolite biosynthesis, under all conditions (Singh et al., 2015). Topping also affects the expression of miRNAs: 129 high-quality expressed sequence tags that were involved in secondary metabolism, hormone metabolism, signaling/transcription, stress/defense, protein metabolism, and carbon metabolism were identified before and after topping using a combination of suppression subtractive hybridization (SSH) and miRNA deep sequencing (Qi et al., 2012). However, the effects of topping on gene expression in tobacco remain unknown.

In this study, we focused on the identification of DEGs involved in axillary shoot growth in both untopped and topped *N. tabacum* plants that were not chemically treated. We applied the Illumina HiSeq™ 2000 platform and analyzed the DEGs to study the global changes in gene expression in response to topping in order to advance our understanding of how to control axillary shoot growth. The knowledge gained will improve our understanding of the processes of axillary shoot formation and enlargement at the transcriptional level.

## 2. Materials and methods

### 2.1. Plant materials, tissues and RNA isolation

K326 tobacco (*N. tabacum*) plants were grown at Qingdao Jimo (N36°45′17.01″, E120°59′13.05″), Shandong Province, China. All plants were planted in the experimental plots of Jimo under the same cultivation conditions on 8 April 2015. The flowering head and two leaves below the flag leaf were removed by topping. The axillary shoot samples were collected from control (untopped) and topped plants after 1, 3 and 5 days, after which the samples were immediately frozen in liquid nitrogen and stored at − 80 °C until RNA extraction.

Total RNA was isolated from 100 mg of shoot tissue using a reagent kit (Invitrogen, Germany) following the manufacturer's instructions. RNA quantity and purity were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). First-strand cDNA was generated by reverse-transcription of 5 mg of total RNA (50-μl reaction volume) using AMV reverse transcriptase (Takara Biotechnology, Japan) at 42 °C for 1 h.

### 2.2. RNA−Seq library construction and sequencing

A total amount of 3 μg of RNA per sample was used as input material for constructing the sequencing library. The RNA−Seq library was constructed using a NEBNext® Ultra™ RNA Library Prep Kit (Illumina, NEB, USA) following the manufacturer's recommendations, and index cards were added to attribute sequences to different samples.

Briefly, mRNAs were enriched from 3 μg of RNA using poly-T oligo-attached magnetic beads. cDNA was synthesized using both oligo (dT) primers and Moloney murine leukemia virus (M-MLV) reverse transcriptase (RNase H-) followed by the addition of DNA polymerase I and RNase H. The 3′ ends of the cDNA fragments were adenylated, and NEBNext adaptors with hairpin loop structure were ligated to the cDNA fragments. The AMPure XP system (Beckman Coulter, Beverly, MA, USA) was then used to purify and select cDNA fragments of preferentially 150–200 bp in length. Three microliters of USER Enzyme (NEB, USA) was then used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. PCR products generated using Phusion High-Fidelity DNA polymerase, universal PCR primers and Index (X) Primer were then purified with the AMPure XP system and quantified using an Agilent Bioanalyzer 2100 system.

The clustering of the index-coded samples was performed using a cBot Cluster Generation System using a TruSeq PE Cluster Kit v3-cBot-HS (Illumina) in accordance with the manufacturer's instructions. The RNA−Seq libraries were sequenced on an Illumina HiSeq platform, and 125-bp/150-bp paired-end reads were generated.

### 2.3. Data analysis

Raw data (raw reads) in fastq format were cleaned by removing adaptor sequences, reads containing poly-N sequences, and low-quality reads. The clean reads were mapped directly to the reference tobacco genome available from the genome website using TopHat v2.0.12 (Trapnell et al., 2009). The expected number of fragments per kilobase of transcript sequence per million base pairs sequenced (FPKM) was calculated based on the length of the gene and read counts mapped to the gene (Trapnell et al., 2010). DEGs of both conditions were identified by the DEGSeq R package (v1.20.0). The *P*-values were adjusted using the Benjamini and Hochberg method. A corrected *P*-value of 0.005 and a log2(fold change) of 1 were set as the threshold for significant differential expression.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used for the main functional enrichment analyses. The GOseq R package was used to analyze the GO enrichment of DEGs, in which gene length bias was corrected (Young et al., 2010). The GO terms with a corrected $P$-value $\leq 0.05$ were considered significantly enriched by DEGs. The KEGG pathway enrichment analysis for the DEGs revealed a database resource for understanding high-level functions and utilities of the biological system. We used KOBAS software to test the statistical enrichment of DEGs in the KEGG pathways (Mao et al., 2005). Those pathways with a false discovery rate (FDR) value less than 0.05 were defined as those with genes that were significantly differentially expressed.

### 2.4. qRT-PCR

Ten DEGs involved in axillary shoot development were selected for validation using qRT-PCR. Gene-specific primers for qRT-PCR were designed using Primer 5 software. qRT-PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's protocol. Each reaction mixture contained 6 μl of DNase/RNase-free water, 10 μl of 2 × Real-Time SYBR Premix ExTaqII, 0.4 μl of 50 × ROX Reference Dye II, 2 μl of diluted cDNA product from reverse-transcription PCR, and 0.8 μl of primers, and each reaction was technically repeated three times. The thermal cycle employed was as follows: 95 °C for 2 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 60 °C for 45 s. Fluorescence was measured at the end of each cycle. Gene expression was presented as relative units after their normalization with the expression of the ubiquitin-conjugating enzyme E2 gene (AB026056) using the $2^{-\Delta\Delta CT}$ method (Schmidt and Delaney, 2010).

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