ABSTRACT

CHEN, HONGXIA. Toward Understanding the Molecular Regulation of Nicotine Biosynthesis. (Under the direction of Dr. Rongda Qu).

Tobacco *(Nicotiana tabacum* L.) is an important non-food crop widely grown worldwide due to its great economic value through tobacco industry (Layten Davis and Nielsen, 1999). Tobacco plant is also a key tool for fundamental biology study due to its feasibility for tissue culture and genetic transformation (Gelvin, 2003). Alkaloids are major secondary metabolites in tobacco and nicotine is the predominant alkaloid in tobacco leaves (Saitoh, et al., 1985; Sisson and Severson, 1990). Nicotine is naturally synthesized in tobacco roots and accumulates in leaves as a defense compound against herbivory attack (Baldwin, et al., 2001). Nicotine biosynthesis pathway has been extensively studied with major genes and enzymes being isolated and functionally characterized (Dewey and Xie, 2013). However, the molecular regulation of nicotine synthesis has not been fully understood. A transcription factor *NtMYC2a* gene was previously cloned in our lab and identified as a positive regulator of nicotine biosynthesis (Wang, 2011; Wang, et al., 2015). The phytohormone jasmonic acid (JA) mediates many aspects of plant defense responses including nicotine biosynthesis (Wasternack and Hause, 2013). Major genes and enzymes of JA biosynthesis and metabolism have been well characterized in Arabidopsis (Wasternack and Hause, 2013). JA signal perception, transduction and responses are dependent on the master regulator MYC2 and the repressor complex composed of JAZ repressors, a NINJA adaptor and a general repressor TPL (Pauwels et al., 2010; Kazan and Manners, 2013).

This dissertation intends to study molecular regulatory mechanisms of nicotine biosynthesis in tobacco, and includes three projects. In the first project, we hypothesize that an interactor of the master regulator NtMYC2a may exist in order to mediate root specific expression of the nicotine biosynthesis pathway genes. A yeast two hybrid (Y2H) based library screening was performed to identify proteins interacting with NtMYC2a. Several candidate proteins were isolated in the screening, only a JAZ3 protein was further confirmed by a Bimolecular Fluorescence Complementation (BiFC) assay. In the second project, five key genes (*AtLOX2*, *AtAOS*, *AtAOC2*, *AtOPR3*, *AtJAR1*) involved in JA biosynthesis were overexpressed and a JA-Ile degradation related *NtJIH1* gene was suppressed by RNAi approach. Interestingly, overexpression of *AtLOX2*, *AtAOC2*, *AtOPR3* and *AtJAR1* increased the transcript levels of nicotine biosynthetic genes but not nicotine content. Overexpression of *AtAOS* and RNAi-mediated knockdown of *NtJIH1* didn't increase nicotine synthesis gene expression or nicotine content. In the third project, a *NINJA* RNAi construct was expressed in tobacco plants in an attempt to reduce the repressor complex activity. *NINJA* knockdown induced nicotine synthesis gene expression but paradoxically had a negative effect on nicotine accumulation. The data seem to point to an unknown missing factor which either controls translation of *PMT1a* and/or *QPT2* transcripts or activation of the translated enzymes (e.g., by phosphorylation, or dimerization and de-dimerization). The project revealed a very complicated regulatory network for nicotine synthesis modulation, which involves signaling by JA and other phytohormones, and is also affected by the cellular toxicity of nicotine.

Toward Understanding the Molecular Regulation of Nicotine Biosynthesis

by
Hongxia Chen

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Crop Science

Raleigh, North Carolina

2017

APPROVED BY:

_____
Dr. Rongda Qu
Chair of Advisory Committee

_____ _____
Dr. Ralph E. Dewey Dr. Ramsey S. Lewis

_____ _____
Dr. Heike W. Sederoff Dr. Deyu Xie

BIOGRAPHY

Hongxia Chen was born on March 5, 1987, in Dangyang, Hubei Province, China. After she finished her high school studies in Dangyang No.1 middle school, she was admitted to China Agriculture University at Beijing in August 2006 and graduated with Bachelor's degree in Seed Science and Engineering in June 2010. From August 2010 to May 2011, she worked in Dr. Andrea Cardinal's laboratory at North Carolina State University. In June 2011, she started her Ph.D. study under the supervision of Dr. Rongda Qu in Department of Crop and Soil Science, North Carolina State University, Raleigh, US. Her work about the molecular regulation of nicotine biosynthesis in tobacco is described in this thesis.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my advisor Prof. Rongda Qu, for his advice, guidance and continuous support of my Ph.D. study and research. I also appreciate my committee members: Drs. Ralph E. Dewey, Ramsey S. Lewis, Heike W. Sederoff, and Deyu Xie, for their help and guidance throughout my graduate study.

I would like to thank all my colleagues, past and present members of Dr. Qu's laboratory, Drs. Bingwu Wang, Minesh Patel, Ruyu Li, Qian Wu, Jyoti Dalal, Zhaohui Hu, Jing Xi, Binbin Zhou, Wanjun Zhang, Sanyong Lee for all their kind help and encouragement. I would like to thank Harry Lopez, Naresh Vasani, Keshalben Bhadiyadara, Hang Zhong, Tiangao Lin, Dr. Huiping Zhou, Chesa Wang, Siddu Chowdary, Risigan Logendran for their technical support in laboratory and greenhouse. I appreciate Tyler Steede, Karen Andres, Albert Buck for their great help with alkaloids analysis. I would like to thank Dr. Arellano Consuelo for her consulting in statistical data analysis. I would like to thank Dr. Sixue Chen and Dr. Sisi Geng for their hard work in jasmonates quantification. I would like to thank all my dear friends Drs. Lan Luo, Lingjiao Qi, Xiaoxian Tang, Huixuan Liang, Zelan Xiao, Yi Chao, Leichen Zhang and Jianxun Cui for the fun and happiness they bring to me during my Ph. D study. I would like to acknowledge my parents, Xiu Chen and Kaiping Chen, my sister, Xiaoyun Chen, for their support and encouragement over all these years.

I would like to thank China Scholarship Council and Yunnan Academy of Tobacco Agricultural Science for their fund support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. Introduction

1.1 Tobacco plant and nicotine

Tobacco *(Nicotiana tabacum* L.) is an important non-food crop widely grown across the world due to its great economic value because of the widespread usage of tobacco products (Layten Davis and Nielsen, 1999). Tobacco is a naturally occurring allotetraploid (2n=4x=48), evolved from the interspecific hybridization of two wild progenitors, *N. sylvestris* (2n=24), the maternal donor, and *N. tomentosiformis* (2n=24), the paternal donor (Bogani, et al., 1997; Murad, et al., 2002). The whole genome of three major tobacco market types (K326, TN90 and Basma Xanthi) and the two progenitors have been sequenced by a whole-genome shotgun sequencing approach (Sierro, et al., 2013; Sierro, et al., 2014). Alkaloids are important secondary metabolites in tobacco plants. Nicotine is the predominant alkaloid in tobacco plants, representing approximately 90% of total alkaloid content in commercial tobacco cultivar. Minor alkaloids, including nornicotine, anatabine and anabasine, accounts for the remaining 10% (Saitoh, et al., 1985; Sisson and Severson, 1990). Nicotine is a defensive compound against insect herbivores because it causes a continual excitation of neurons and even paralysis or death of insects (Baldwin, et al., 2001).

1.2 The importance of developing tobacco plants with high nicotine level

In tobacco products, nicotine is the addictive component that causes pleasure for smokers (Silvette, et al., 1962). Nicotine is also the precursor in producing carcinogenic tobacco-specific nitrosamine (TSNA) when tobacco leaves are cured for producing tobacco products (Bush, et al., 2001). Developing plants with higher nicotine content has many practical

applications. If tobacco leaves contain a higher level of nicotine, the smokers will consume fewer cigarettes to satisfy nicotine effect, leading to overall reduced exposure to carcinogenic compounds such as TSNA, harmful tars and carbon monoxide (Russell et al., 1973). Nicotine is the essential component of smoking reduction devices, such as the electronic cigarette. In addition, nicotine can be used as a type of insecticide in agriculture and horticulture practice (Layten Davis and Nielsen, 1999). Moreover, nicotine can be pharmaceutically used after industrial purification, such as treating Parkinson's disease and alleviating inflammatory bowel syndrome (Quik, et al., 2008; Polosa, et al., 2013). Therefore, in addition to reducing carcinogenic effect of cigarettes, developing tobacco plants with increased nicotine content might be valuable for commercial nicotine extraction facilities for insecticidal and pharmaceutical purposes.

1.3 Nicotine biosynthesis pathway and key genes

Nicotine is synthesized by the condensation of a nicotinic acid derivation and N-methylpyrroline. These two crucial precursors are produced by two separate primary metabolic pathways respectively: the methylpyrroline pathway and the pyridine-nucleotide cycle pathway (Figure 1.1). The methylpyrroline pathway can either start from ornithine or arginine. Ornithine directly produces putrescine by ornithine decarboxylase (ODC). Arginine converts to agmatine first by arginine decarboxylation (ADC) and subsequently yields putrescine. Putrescine is a ubiquitous plant metabolite and methylated to produce N-methylputrescine by putrescine N-methytransferase (PMT). Through oxidative deamination, N-methylputrescine is subsequently converted to N-methylaminobutanal, catalyzes by methylputrescine oxidase (MPO). N-methylaminobutanal spontaneously cyclizes to produce

N-methyl-Δ1-pyrrolinium cation which is the direct precursor for nicotine biosynthesis. The pyridine-nucleotide cycle is initiated from oxidization of L-aspartic acid by L-aspartate oxidase to form alpha-iminosuccinic acid which then condenses with the glyceraldehyde 3-phosphate by quinolinate synthetase, producing quinolinic acid. In the following step, quinolinic acid is converted to NAMN by a quinolinic acid phosphoribosyltransferase (QPT). NAMN goes through the pyridine nucleotide cycle to produce nicotinic acid either directly by a NAMA glycohydrolase, or through a multi-step process involving the synthesis and degradation of the ubiquitous coenzyme NAD. The product, nicotinic acid, is reduced to 3, 6-dihydronicotinic acid. In order to synthesize the final product nicotine, 3, 6-dihydronicotinic acid is believed to be decarboxylated and condensed with the N-methyl-Δ1-pyrrolinium cation. Two putative candidate enzymes, A622 (a member of the PIP family of NADPH-dependent reductases) and BBL (berberine bridge-like enzyme), are predicted to be involved in the nicotinic acid reduction, decarboxylation of 3, 6-dihydronicotinic acid, and condensation of pyridine and pyrrolidine rings. Nicotine can be converted to nornicotine by nicotine demethylase (NDM) (Dewey and Xie, 2013).

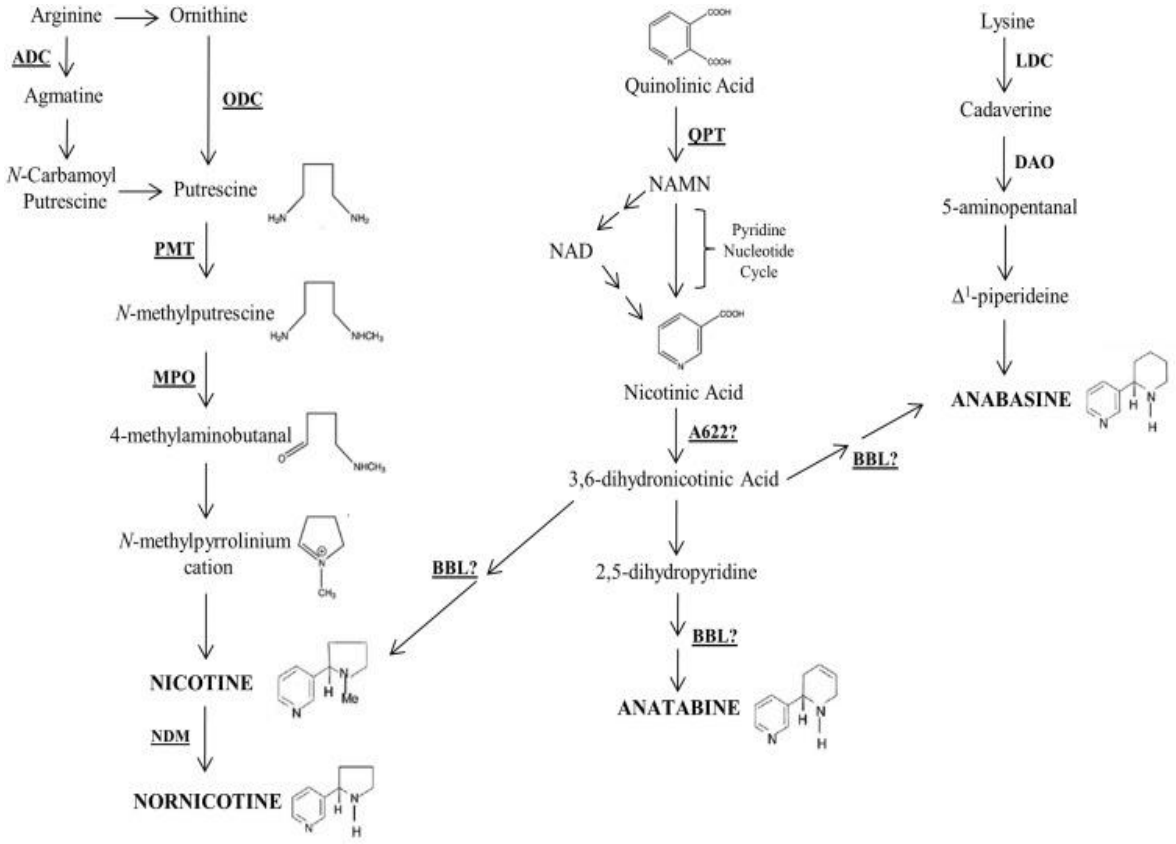

Figure 1. 1 Scheme of Nicotine biosynthesis pathway.

From Lewis, et al. (Lewis, et al., 2015). ADC: arginine decarboxylase, ODC: ornithine decarboxylase, PMT: putrescine methyltransferase, MPO: N-methylputrescine oxidase, QPT: quinolinate phosphoribosyltransferase, A622: isoflavone reductase-like protein, BBL: berberine bridge enzyme-like, DAO: diamine oxidase, LDC: lysine decarboxylase, NDM: nicotine demethylase, NAMN: niacin mononucleotide, NAD: nicotinamide adenine dinucleotide.

ODC and ADC

Both ODC and ADC can catalyze putrescine formation, but from different substrates, ornithine and arginine, respectively, for alkaloids synthesis (Malmberg, et al., 1998). The *ODC* transcript is exclusively expressed in roots, whereas *ADC* transcript levels are very low in roots but high in flowers (Wang, et al., 2000). Both of these *ADC* and *ODC* are induced by topping (Wang, et al., 2000). Southern blot analysis demonstrated that both of them are encoded by small gene families with multiple isoforms in tobacco genome (Wang, et al., 2000; Xu, et al., 2004). Down regulation of *ADC* in transgenic tobacco did not decrease nicotine levels but did increase anatabine (Chintapakorn and Hamill, 2007). In contrast, suppression of ODC caused a significant reduction of nicotine content and increase of anatabine, similar to *PMT* and *MPO* suppressed plants (DeBoer, et al., 2011). Therefore, it is speculated that ODC route is more preferred than ADC for producing putrescine dedicated to alkaloid biosynthesis.

PMT

PMT is the first dedicated step for the pyrrolidine synthesis branch. *NtPMT1a* was isolated by subtractive hybridization using *nic1nic2* double mutant cultured roots (Hibi, et al., 1994). Five *PMT* isoforms, *NtPMT1a*, *NtPMT1b*, *NtPMT2*, *NtPMT3*, and *NtPMT4*, have been found in tobacco genome (Hashimoto, et al., 1998; Riechers and Timko, 1999). All five *NtPMT* genes are specifically expressed in roots (Riechers and Timko, 1999). *PMT* gene expression is upregulated by wounding, topping, and JA treatment but downregulated by auxin (Hibi, et al., 1994; Imanishi, et al., 1998; Winz and Baldwin, 2001). Topping triggers root *NtPMT1a*

transcript increase as early as 30 minutes after the treatment and peaks one hour after topping (Hibi, et al., 1994). Earlier studies reported that the PMT-catalyzed step is a rate limiting and thus an important regulatory point (Saunders and Bush, 1979; Wagner and Wagner, 1985; Feth, et al., 1986). Reduced *PMT* expression level by RNAi significantly suppressed nicotine accumulation in tobacco leaves (Chintapakorn and Hamill, 2003; Wang, et al., 2008). Overexpression of *NtPMT1a* under the constitutive CaMV 35S promoter increased leaf nicotine content by 40% in *N. sylvestris* plants growing in a greenhouse (Sato, et al., 2001). However, research in our lab showed that overexpression of *NtPMT1a* driven by a strong root-specific promoter enhanced its transcript level but didn't significantly change the nicotine content of the commercial tobacco variety NC95 grown in the field (Wang, 2011).

MPO

The deamination of N-methylputrescine is catalyzed by MPO. The *MPO1* gene was simultaneously identified by two research groups via reverse transcription PCR using degenerated oligonucleotide derived from MPO amino acid sequence and differential expression profiling of *nic1nic1*/*nic2nic2* respectively (Heim, et al., 2007; Katoh, et al., 2007). *MPO* is exclusively expressed in roots, induced by JA elicitation and repressed by auxin (Heim, et al., 2007). *MPO* suppression resulted in a decreased nicotine level and increased anatabine level in tobacco root hairs because anatabine formation is independent of the N-methylpyrroline branch (Shoji and Hashimoto, 2008). Southern blot analysis predicted six *MPO* genes within the tobacco genome (Heim, et al., 2007). MPO2 is 88% identical to MPO1 in amino acid sequence (Katoh, et al., 2007). MPO is a copper-containing amino oxidase family protein and oxidizes multiple diamines, but N-methylputrescine is the most

preferred substrate (Walton and McLauchlan, 1990; Heim, et al., 2007; Katoh, et al., 2007). The predicted size of the MPO1 polypeptide is 89 kDa, but the molecular weight of recombinant MPO protein expressed in E. coli was around 172 KDa, therefore, the authors suggested homodimer formation (Katoh, et al., 2007). However, further testing of MPO-MPO interactions using a Y2H assay failed to support his conclusion (Hildreth, 2009).

QPT2

QPT2 was isolated and characterized by two research groups independently (Song, 1997; Sinclair, et al., 2000). *QPT2* transcripts were specifically found in roots, especially in the root cortex of fully differentiated region rather than in the root tip (Song, 1997). *QPT2* was strongly induced 4 hours post topping and continued to be elicited after 24 hours but was inhibited by auxin treatment (Song, 1997). Leaf wounding and JA treatment triggered *QPT2* expression (Sinclair, et al., 2000). *QPT1* shares 94% nucleotide identity to *QPT2* in the coding region. *QPT1* is ubiquitously expressed in all tissues at a basal level and not responsive to topping, wounding, plant hormone signaling and thus QPT1 may not be involved in nicotine biosynthesis (Shoji and Hashimoto, 2011a). Suppression of *NtQPT2* mediated by antisense driven by a root-specific endogenous *QPT2* promoter causes a severely reduced leaf nicotine level in transgenic Burley 21 (Xie et al. 2004). However, similar to the observation with PMT, overexpression of *QPT2* driven by the same root-specific *QPT2* promoter didn't cause an increase of nicotine levels (Wang, 2011).

A622 and BBL

A622 was first identified in the same study where *NtPMT1a* was discovered (Hibi, et al., 1994). *A622* has an expression pattern similar to *PMT* (Shoji, et al., 2002; Cane, et al., 2005; Kidd, et al., 2006). The A622 protein is an NADPH-dependent isoflavone reductase-like homolog (Hibi, et al., 1994; Shoji, et al., 2002), however, in vitro enzymatic assay failed to support the reductase activity as substrates were not reduced when recombinant A622 protein and NADPH were added (Shoji, et al., 2002). RNAi mediated suppression of A622 caused a substantial reduction of anabasine, nicotine and anatabine in *N. glauca* (DeBoer, et al., 2009). Based on the increase of intermediate nicotinic acid β-N-glucoside (NaNG) in A622 suppressed tobacco plants, NaNG was suggested to be a reversible product of nicotinic acid (Kajikawa, et al., 2009). It is hypothesized that A622 enzyme could catalyze the formation of nicotinic acid-derived pyridine precursor or the final condensation of the pyridine ring and methyl-pyrrolidine ring in nicotine biosynthesis.

Four cDNAs encoding Berberine Bridge Enzyme-Like proteins (BBLs) were isolated by differential expression profiling of WT versus *nic1nic2* double mutant (Kajikawa, et al., 2011). The authors found *BBL* transcripts are present primarily in roots in a JA-inducible manner, and BBL proteins are localized in the vacuole. Protein structure alignments indicated a flavin-containing oxidase structure within the BBLs (Kajikawa, et al., 2011). BBLs contribute to nicotine biosynthesis as shown by the fact that leaf nicotine content was dramatically decreased in BBL-suppressed plants (Kajikawa, et al., 2011; Lewis, et al., 2015). A novel metabolite, dihydrometanicotine (DMN), was found to be increased in BBL suppressed plants (Kajikawa, et al., 2011). DMN is composed of a straight chain N-

(methylamino) butyl moiety linked with the pyridine ring. Based on these two facts, BBL was suggested to be placed after the initial condensation of pyridine and pyrroline rings. However, in vitro enzyme assay failed to approve the oxidization of DMN by BBLa protein.

1.4 Tissue and cellular localization of nicotine biosynthetic enzymes

The tissue and cellular specific localization of enzymes involved in nicotine biosynthesis are listed in Table 1.1. The early enzymes involved in the NAD cycle, like AO and QS, are localized in the chloroplast, as demonstrated in the analysis of GFP-fused proteins in Arabidopsis (Katoh, et al., 2006). The motif prediction program predicted a mitochondrial motif in *QPT2* cDNA sequences, suggesting that the QPT2 protein is probably localized to the mitochondria (Sinclair et al., 2000). GUS reporter driven by the QPT2 promoter was activated in the elongation zone of root tips, outermost cortex layer (Shoji and Hashimoto, 2011a). For the methylpyrroline pathway, immunoblotting showed that ADC proteins occur within the chloroplast (Borrell, et al., 1995). MPO1 proteins are targeted to the peroxisome, demonstrated by the observation of the MPO1-YFP fusion protein in *N. benthamiana* (Naconsie et al., 2013). PMT and A622 proteins are localized in the same cell type and the same sub-cellular compartments. Immuno- histochemical detection demonstrated that PMT and A622 proteins are expressed in epidermis and cortex cells of the root tips (Shoji, et al., 2002). The BBL proteins are localized within the vacuole, as the BBL-GFP fusion proteins were detected in the central vacuoles in BY-2 cells (Kajikawa, et al., 2011).

Table 1. 1 Tissue and subcellular localization of nicotine biosynthetic enzymes.

Enzyme	Subcellular localization	Tissue localization
AS, QS	Chloroplast	All types of tissues
QPT2	Mitochondria	Outmost layer of cortex, root tip
ADC	Chloroplast	Root tips
PMT	Cytoplasm	Epidermis and cortex, root tips
MPO1	Peroxisome	Root tips
A622	Cytoplasm	Epidermis and cortex, root tip
BBL	Vacuole	Root tips

1.5 Nicotine transport

Nicotine is transported from roots to leaf tissues via the xylem and accumulates in leaf cell vacuoles (Steppuhn, et al., 2004; Katoh, et al., 2005). A total of four nicotine transporters has been identified so far. Three multidrug and toxic compound extrusion (MATE) protein family transporters, NtJAT1, NtMATE1 and NtMATE2, localized on the vacuolar membranes and are responsible for transporting alkaloids into the vacuole from the cytoplasm (Shoji, et al., 2009; Morita, et al., 2009). NtMATE1 and NtMATE2 are primarily expressed in roots, responsible for transporting alkaloid from the cytoplasm into the vacuole in the alkaloid-synthesizing root cells. NtJAT1 is expressed in the leaf, stem and root, contributing to transporting alkaloid from cytoplasm to vacuole in aerial parts. Sequestering alkaloid into the vacuole protects cells from alkaloid toxicity. Another nicotine transporter,

nicotine uptake permease 1 (NtNUP1), belongs to a class of purine uptake permease-like transporter. Nicotine transport assay conducted in yeast cells demonstrated that NUP1 transport nicotine and other alkaloids. GFP-NUP1 fusion proteins are localized to the plasma membrane. They proposed that NUP1 transport nicotine from the apoplast into the cytoplasm in order to keep a preferred nicotine balance between root cells and the rhizosphere by releasing and reabsorbing of nicotine (Hildreth, et al., 2011). In contrast to MATE-type transporters which has broad specificity to various alkaloid species, NtNUP1 is highly specific for nicotine transportation in tobacco plants (Hildreth, et al., 2011). Recently *NtNUP1* was found to positively regulate the expression of a key ERF gene (*ERF189*) expression in nicotine biosynthesis (Kato, et al., 2014). So far, long distance alkaloid transportation from root to shoot, including the uploading of alkaloids from the alkaloid-synthesizing root cells into the xylem and downloading nicotine from xylem to mesophyll cells, remains unclear.

1.6 Factors affecting nicotine biosynthesis

Nicotine biosynthesis is regulated by multiple factors and various levels, including wounding, topping, transcription factors such as bHLHs and ERFs, micro RNAs, post-translational phosphorylation, and negative feedback by nicotine. Topping and wounding regulate nicotine biosynthesis through modulating plant hormones.

Wounding-induced JA signaling regulates nicotine biosynthesis

Mechanical or herbivory mediated wounding of tobacco leaves can significantly promote root nicotine synthesis and the whole plant nicotine accumulation (Baldwin, et al., 1994; Shoji, et al., 2000). JA treatment induced expression of a large number of genes involved in

alkaloid biosynthesis, transportation, and alkaloid synthesis regulation (Major and Constabel, 2006; Wasternack and Hause, 2013).

Wounding triggers a rapid increase of JA in both local and systemic tissues in Arabidopsis and tobacco. The first detectable increases in JA content were reported to occur 30-45 seconds after wounding in the local leaf and 2 minutes in a systemic leaf sharing vascular connections in Arabidopsis (Glauser, et al., 2009; Koo and Howe, 2009). In *N. sylvestris*, JA pool within local leaves increased 10-fold within 90 minutes after wounding treatment, and increased 3.5-fold in roots within 180 minutes (Baldwin, et al., 1994). Many studies have been conducted to explore what is the long-distance wounding signal leading to local and systemic JA increase. By external application of radioactive labeled JA to leaves, Baldwin and coworkers demonstrated that wounding-induced JA was transported from local leaves to younger systematic leaves and to roots through the phloem in *N. sylvestris* (Zhang and Baldwin, 1997). They proposed that direct JA transportation from local leaves to roots is the transmissible signal which triggers de novo nicotine synthesis. However, they didn't rule out the possibility that an 18-amino acid polypeptide called systemin is transported from the wound site and serves as wounding signal, which is the case in tomato (Ryan and Pearce, 1998; Schilmiller and Howe, 2005). Kubigsteltig et al. argued that JA or its derivatives are not the direct long distance wounding signal because they are quantitatively insufficient to induce a detectable response in systemic tissue (Kubigsteltig, et al., 1999). Others think *Glutamate Receptor-like (GLP)* genes related electrical activity is responsible for the leaf to leaf wound signaling in Arabidopsis (Mousavi, et al., 2013). So far, there is no agreement on

which is the true long-distance wounding signal. It is nevertheless clear that endogenous JA levels are increased after wounding in both local and systemic tissues.

Nicotine biosynthesis is induced by topping

Decapitation of the apical meristem (topping) at an early stage of flowering is an important practice to stimulate nicotine accumulation in tobacco leaves. The developmental stage when the topping is applied affects total alkaloid accumulation. The highest total alkaloids production occurred when topping was applied before the first flower bud opened (early flowering stage) and the alkaloids decreased when topping was applied later after this stage (Elliot, 1966). For flue-cured tobacco, nicotine levels decreased when the topping height increased from 12 to 20 leaves (Brown and Terrill, 1973).

The mechanism of topping induced nicotine biosynthesis has not been fully clarified yet. Both JA and auxin seem to play a role. Removal of the apical meristem not only increase auxin level in roots (Dai, et al., 2009; Fu, et al., 2013) but also causes mechanical wounding which can induce JA synthesis in the wounded area and distal areas (Baldwin, et al., 1997). Auxin treatment suppresses *NtPMT* expression, NtPMT enzyme activity and nicotine content (Yasumatsu, 1967; Feth, et al., 1986; Hibi et al., 1994)). External MeJA treatment induces expression of all the key genes involved in the nicotine biosynthesis pathway and nicotine levels (Wasternack, 2007). In summary, topping triggers both JA and auxin increase in root tips and both plant hormones have the ability to affect nicotine biosynthesis. Therefore, it is hard to discern which single hormone causes topping-induced nicotine synthesis. It is most likely an outcome of the two.

Another topping effect related to nicotine biosynthesis involves microRNA. Topping caused differential expression of a number of regulatory small RNAs which might target nicotine biosynthesis genes (Qi, et al., 2012; Tang, et al., 2012; Li, et al., 2015). As an independent regulator, microRNAs are described in detail later in this chapter.

The bHLH family transcription factors regulate nicotine biosynthesis

The basic helix-loop-helix (bHLH) transcription factors play a key role in regulating nicotine biosynthesis in tobacco. Three MeJA induced transcription factor genes *NbbHLH1*, *NbbHLH2 and NbbHLH3* were isolated in a functional genetic screening and were found to regulate alkaloid biosynthesis (Todd, et al., 2010). Based on the *NbbHLH1* and *NbbHLH2* nucleotide sequence, four homologs were cloned from *Nicotiana tabacum*, named *NtMYC1b* (GQ859159), *NtMYC1a* (GQ859158), *NtMYC2a* (GQ859160), and *NtMYC2b* (GQ859161) respectively (Todd, et al., 2010). However, these *NtMYC2*s were not functionally characterized in this study. In another study, using the same sequence, Shoji and Hashimoto (2011b) demonstrated that *NtMYC2b* caused transient activation of *NtPMT2* and *NtQPT2* promoter when co-expressed in BY-2 cells. Electrophoresis mobility shift assay (EMSA) indicated that NtMYC2b binds to a G-box-related promoter region of *NtPMT2* and *NtQPT2* with great affinity. Moreover, the G-box-related cis-element was predicted to exist in the proximal promoter regions of other nicotine synthesis genes, such as *A622* and *MATE1*. RNAi-mediated NtMYC2 knockdown lead to significant reduction in *PMT*, *QPT*, *MPO*, *A622*, *BBL* transcript levels and nicotine levels (Shoji and Hashimoto, 2011b), suggesting NtMYC2 be an overall positive transcriptional activator for nicotine biosynthesis.

Almost at the same time, our group cloned NtMYC2a and NtMYC2b, using a Yeast One Hybrid screen o identify proteins binding to the *NtQPT2* promoter (Wang, 2011). *NtMYC2a* and *NtMYC2b* share 96% nucleotide identity and their amino acid sequences are identical to those reported by Todd et al. (2010). Overexpression of *NtMYC2a* in tobacco plants increased nicotine content approximately by 1-1.5 fold, and RNAi-induced knockdown of *NtMYC2* decreased the nicotine level to 25% of the WT. The high nicotine phenotype in *NtMYC2a* overexpression lines was consistent throughout T_0, T_1, T_2, and T_3 generation in field tests (Wang, et al., 2015). Unlike *NtMYC2a*, overexpression of *NtMYC2b* only slightly increased nicotine level.

Three more NtMYC2 isoforms were identified in *N. tabacum* (cv. Burley 21) through reverse transcription-PCR with gene specific primers based on the *bHLH207* cDNA sequence, the closest tobacco homolog of *AtMYC2*. These three genes were also named *NtMYC2a* (GenBank No. HM466974), *NtMYC2b* (GenBank No. HM466974) and *NtMYC2c* (GenBank No. HM466976) (Zhang, et al., 2012), although they are more homologous to *NbbHLH1*, *NtMYC1a* and *NtMYC1b*, nomenclatures given by Todd et al. (2010). These three proteins are isoforms of NtMYC2 because they belong to the same clade with the *Arabidopsis* MYC2 transcription factor, together with the previously identified NtMYC2a and NtMYC2b proteins (Todd, et al., 2010; Wang, 2011).

NtMYC2 transcripts are present in all the organs with more abundance in the roots (Wang 2011; Shoji and Hashimoto, 2011b). Both studies showed a biphasic expression pattern of

NtMYC2; *NtMYC2* transcripts rapidly increased within 30 minutes, decreased slightly afterward and then increased again after 4 hours.

Recently, ubiquitination was reported to affect MYC2 turnover in Arabidopsis (Jung, et al., 2015). MYC2 was found to physically interact with a U-BOX Protein 10 (PUB10) in Y2H and in vitro pull-down assays. PUB10 is an E3 ligase which can poly-ubiquitinate MYC2 in vitro with the help of ubiquitin-conjugating enzymes. Mutated PUB10 stabilized MYC2 and extended its half-life. Pub10 mutant phenocopied Arabidopsis plants overexpressing *MYC2* in terms of primary root length inhibition and JA-responsive genes activation (Jung, et al., 2015). Therefore, the authors suggest that ubiquitination inhibits MYC2 activity.

ERF transcription factors regulate nicotine biosynthesis

At least seven ERF (ethylene responsive factors) TF genes were identified as being clustered at the *NIC2* loci in the studies characterizing the *nic1nic2* low nicotine mutant (Shoji, et al., 2010). The two unlinked *NIC1* and *NIC2* loci have long been known to positively impact nicotine biosynthesis (Legg and Collins, 1971; Hibi, et al., 1994; Reed and Jelesko, 2004). Based on differential transcript profiling of *nic1nic2* double mutant versus WT plants, *ERF189* was found to be the most severely reduced gene among the top 200 downregulated genes in *nic1nic2* individuals. Through genetic analysis of different *NIC* genotypes (WT, *nic1*, *nic2*, *nic1nic2*), *ERF189*, *ERF115*, *ERF221*, *ERF104*, *ERF179*, *ERF17* and *ERF168* were amplified in WT and *nic1* but not in *nic2* lines, indicating the deficient *nic2* locus represents a deletion of these *ERF* genes (Shoji, et al., 2010).

NIC2-locus *ERF* genes were mostly expressed in tobacco roots and had low expression in other organs (Shoji and Hashimoto, 2011b). All the *NIC2*-locus ERFs are functional redundancies, with ERF189 being the most effective one (Shoji, et al., 2010). ERF189 activated *PMT, QPT2, ODC, MPO, AO (Aspartate oxidase)* and *QS (Quinolinic acid synthase)* promoters by binding to the GCC box as shown by EMSA (Shoji, et al., 2010). Overexpression of ERF189 led to nicotine increase to approximately 6-fold in transgenic tobacco hairy roots either with or without MeJA treatment compared to the control (Shoji, et al., 2010). ERF221, also named NtORC1, upregulates *NtPMT*, *NtQPT2*. Overexpression of *ERF221* increased nicotine level by 1.8 fold (De Boer, et al., 2011). A non-*NIC2* locus AP2/ERF transcription factor, NtERF32, was identified in a yeast one-hybrid screen using the GAG motif of *NtPMT1a* as bait (Sears, et al., 2014). *NtERF32* gene expression is independent of the NtMYC2 transcription factor. *NtPMT1a, NtQPT2, NtODC1 and NtA622* expression were up-regulated in *NtERF32* overexpressing BY-2 cell lines whether treated with MeJA or not, and alkaloid accumulation increased up to 6-fold (Sears, et al., 2014). However, all these results were observed from BY-2 cells which have very low level of nicotine and there is no result reported from transgenic plants.

ERF family TFs and *bHLH* family TFs function collaboratively to regulate nicotine synthesis. In tobacco hairy root lines, RNAi-mediated suppression of *NtMYC2* strongly down-regulated several *ERF* genes including *ERF189* (Shoji and Hashimoto, 2011b). The highest transactivation by NtORC1/ERF221 on *PMT* promoters requires both GCC-box and G-box motifs within the proximal promoter region, indicating NtORC1/ERF221 and bHLH TF have a synergistic effect on *NtPMT* promoter activation (Shoji and Hashimoto, 2011b; De

Boer, et al., 2011). All of these pieces of evidence suggest a collaborative function of ERF TFs and bHLH TFs.

MicroRNAs and endogenous target mimicry regulate nicotine biosynthesis

MicroRNAs are a type of noncoding regulatory small RNA with typically 21 nucleotides which can regulate gene expression by binding to its complementary sequences, resulting in degradation of target transcripts or blocked translation (Wu, et al., 2013). It is reported that microRNAs regulated plant stress responses and secondary metabolite synthesis (Zhang, et al., 2006; Khraiwesh, et al., 2012), including benzylisoquinoline alkaloid biosynthesis in opium poppy and nicotine biosynthesis in tobacco (Boke, et al., 2015; Li, et al., 2015). The corresponding noncoding endogenous target mimicry (eTM) which could be targeted by microRNAs are very recently discovered mechanisms for gene expression regulation (Xie and Fan, 2016). Plant eTM competes with microRNA's authentic targets for binding to microRNAs, and thus attenuating microRNAs inhibited gene expression.

A microRNA nta-miRX27 and an eTM called nta-eTMX27 were identified in tobacco by small-RNA deep sequencing combined with the recently available tobacco reference genome (Li, et al., 2015). The nta-miRX27 was predicted to be able to bind to *QPT2* transcripts. nta-miRX27 transcript levels were decreased upon topping treatment and negatively correlated with *QPT2* transcript level. Overexpression of nta-miRX27 decreased *QPT2* transcript by 50%, and nicotine decreased by approximately 50%. Silencing of nta-miRX27 increased *QPT2* transcripts by 8-folds and doubled nicotine content as compared to vector control.

These results suggest nta-miRX27 is a negative regulator of nicotine biosynthesis (Li, et al., 2015).

Tobacco nta-eTMX27 is a 1213 bp long non-coding RNA containing a nta-miRX27 target region (Li, et al., 2015). Overexpression of nta-eTMX27 led to suppressed nta-miRX27 expression level, elevated *QPT2* expression level and increased nicotine levels. The nta-eTMX27 silenced plants had higher nta-miRX27 expression levels, lower *QPT2* transcripts and lower nicotine content as compared to the vector control. In summary, nta-eTMX27 positively regulates nicotine biosynthesis. The nta-eTMX27 can up-regulate nicotine biosynthesis because it prevents nta-miRX27 from targeting *QPT2* transcripts (Li, et al., 2015).

Protein phosphorylation affects nicotine biosynthesis

The bHLH and ERF TFs may need a mitogen-activated protein kinase kinase (MAPKK1) dependent phosphorylation to enhance their positive effects on nicotine biosynthesis. This *MAPKK1*, now renamed as *JAM1* (DeBoer, et al., 2011), was induced by MeJA treatment (Goossens, et al., 2003). Together with MAPKK1, NbbHLH1 enhanced transient transactivation of *QPT2* and *PMT1* promoters by approximately 10 fold in the presence of MeJA as compared to NbbHLH1 alone. Similarly, ERF221 with MAPKK1 co-expressed caused a 2-fold increase of *NtPMT1* promoter activity as compared to ERF221 alone (De Boer, et al., 2011). These studies suggest that post-translational phosphorylation could upregulate nicotine biosynthesis by modulating bHLH and ERF TF functions.

Nicotine feedback regulation

High levels of nicotine are toxic to tobacco cells. Shoji et al. (2009) found that root growth of tobacco seedlings was inhibited by supplementation with 2 mM nicotine. Wang (2011) discovered that *NtPMT1a* and *NtQPT2* transcripts were down-regulated in *NtMYC2a* overexpression lines, although the nicotine level was substantially increased. Later, he demonstrated that external application of 0.4 mM nicotine to tobacco seedlings significantly repressed nicotine biosynthetic gene expressions two hours after the treatment (Wang, et al., 2015). They concluded that high levels of nicotine can suppress the expression of its own biosynthetic genes (Wang, et al., 2015).

1.7 JA biosynthesis and metabolism: pathway and key genes

The JA biosynthesis pathway has been well established with major pathway components being functionally characterized as shown in Figure 1.2 (Wasternack, 2007; Wasternack and Hause, 2013; Goossens, et al., 2016). JA biosynthesis starts from α-linolenic acid (18:3) which is released from glycolipids of chloroplast membrane, catalyzed by two lipases DEFECTIVE IN ANTHER DEHISCENCE1 (DAD1) and DONGLE (DGL) (Ishiguro, et al., 2001). Free α-linolenic acid is oxidized by lipoxygenases (LOXs) to form 13 (S) – hydroperoxy-octadecatrienoic acid (13-HPOT) or (9S)-hydroperoxy-octadecatrienoic acid (9-HPOT). Only 13-HPOT is involved in JA biosynthesis. 13-HPOT is an intermediate shared by several xylidines synthesis pathway, but its conversion to 12,13 (S)-epoxy-octadecatrienoic acid [12,13 (S)-EOT] by allene oxide synthase (AOS) commits 13-HPOT to JA biosynthesis. The unstable allene oxide of 12,13 (S)-EOT is immediately cyclized by allene oxide cyclase (AOC) to produce cis-(+)-12-oxophytodienoic acid (OPDA). Up to OPDA, all the reactions and enzymes are located to the plastid. Thereafter, OPDA is

translocated from the chloroplasts into the peroxisomes where the peroxisomal OPDA reductase (OPR) reduces OPDA. The reduction product goes through three rounds of beta-oxidative side-chain shortening to yield jasmonoyl-CoA which is then converted to (+)-7-*iso*-JA. (+)-7-*iso*-JA equilibrates to the more stable (-)-JA, the predominant form of JA in plants. JA is released into the cytosol where it is metabolized to various derivatives, including JA-Ile, the most active form of jasmonates. JA-Ile is generated by conjugating isoleucine to JA, catalyzed by the JA amino acid synthetase (JAR1) (Suza and Staswick, 2008). JA can be methylated by JA carboxyl methyltransferase (JMT) to form MeJA (Seo, et al., 2001). For JA-Ile catabolism, JA-Ile is hydroxylated by the cytochrome P450 enzyme CYP94B3 to produce an inactive form 12-OH-JA-Ile which subsequently undergoes carboxylation to yield 12-COOH-JA-Ile by the p450 enzyme CYP94C1 (Heitz, et al., 2012). JA-Ile can be hydrolyzed to form JA by a jasmonoyl-L-isoleucine hydrolase 1 (JIH1) in *N. attenuata* (Woldemariam, et al., 2012). The formation of inactive compound 12-OH-JA-Ile, 12-COOH-JA-Ile, and the hydrolysis of JA-Ile contribute to partial switch off of JA signaling (Miersch, et al., 2008).

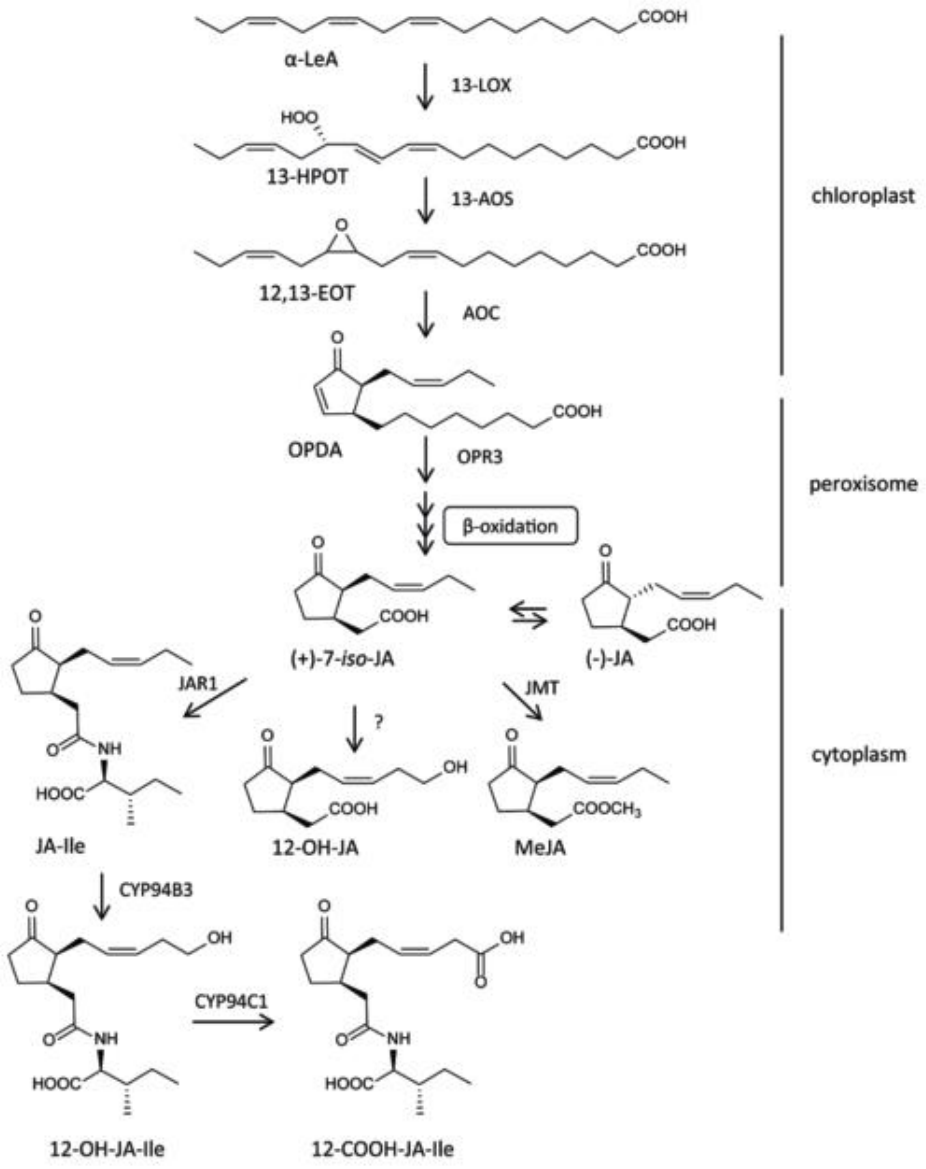

Figure 1. 2 Scheme of Jasmonate biosynthesis pathway in Arabidopsis.

From Zhang (Zhang, 2016). 13-LOX: 13-lipoxygenase, 13-AOS: 13-allene oxide synthase, AOC: allene oxide cyclase, OPR3: OPDA reductase, JAR1: jasmonate resistant 1, JMP: Jasmonates carboxyl methyltransferase; α-LeA: α-linolenic acid, 13-HPOT: 13-hydroperoxylinolenic acid, 12,13-EOT: 12,13-epoxyoctadeca-trienoic acid, OPDA: 12-oxo-phytodienoic acid, (+)-7-iso-JA and (-)-JA for jasmonic acid, JA-Ile: jasmonoyl-isoleucine, 12-OH-JA for 12-hydroxyjasmonic acid, MeJA: methyl jasmonate, 12-OH-JA-Ile and 12-COOH-JA-Ile for oxidized 12-hydroxy-JA-Ile.

LOX

LOX catalyzes oxidization of α-linolenic acid by inserting oxygen at the C-13 position. LOX2, LOX3, LOX4, and LOX6 contribute to wound-induced JA biosynthesis. The LOX6 promoter is not active at the seedling stage (Vellosillo, et al., 2007), but is remarkably strong in and near xylem cells of young apical tissues of 35-days old rosettes, so LOX6 was suggested to play a major role in early wound-induced JA production (Caldelari, et al., 2011; Chauvin, et al., 2013). LOX2 promoter is remarkably active in all the leaf tissues of Arabidopsis seedlings, whereas LOX3 and LOX4 promoters have activity constrained in the vascular tissue of Arabidopsis seedlings (Vellosillo, et al., 2007). LOX2 is believed to contribute to the bulk of JA production1-4 hours post wounding (Bell, et al., 1995; Glauser, et al., 2009). Antisense suppression of *LOX2* demonstrated that *LOX2* didn't affect JA-dependent flower development (Bell, et al., 1995), whereas *lox3lox4* is male sterile (Caldelari, et al., 2011).

AOS

AOS is a cytochrome P450 enzyme of the CYP74 family, encoded by a single gene in the Arabidopsis genome (Laudert, et al., 1996). *AOS* was preferentially expressed in mature leaves and floral organs (Laudert and Weiler, 1998). *AOS* is activated by JA, wounding, OPDA and salicylic acid (Laudert and Weiler, 1998; Kubigsteltig, et al., 1999). Ziegler and co-workers demonstrated that at least two *AOS* isoforms exist in the *N. attenuata* genome and they are constitutively expressed in stems, leaves and flowers (Ziegler, et al., 2001). They found JA accumulated earlier than *AOS* transcripts increased and JA induction attenuated faster than AOS transcript reduction (Ziegler, et al., 2001). They, therefore, proposed that

AOS gene expression may not be responsible for the initial wound induced-JA synthesis in wounded leaves but contributed to the sustaining JA burst (Ziegler, et al., 2001).

AOC

The AOC and AOS steps are believed to be functionally connected because the unstable intermediate necessitates that these two steps happen almost simultaneously (Stenzel et al., 2012). Four *AOCs* isoforms have been identified in Arabidopsis (Stenzel, et al., 2003) and these isoforms have functional redundancies (Stenzel, et a., 2012). *AOC1* and *AOC2* transcripts are more abundantly found in leaves than roots, whereas *AOC3* and *AOC4* are more abundantly found in roots rather than in leaves. *AOC1*, *AOC2*, and *AOC3* promoters show high activity in all types of leaf tissue, whereas the *AOC4* promoter has preferentially strong activity in main veins of fully developed leaves and root tips (Stenzel, et a., 2012). In tomato, there is only one *AOC* gene which is strongly expressed in the flower buds, flower stalks, and roots but just moderately expressed in the stem and leaf. Tomato AOC protein is confined to vascular bundles and the sieve elements and was speculated to be induced in tomato by systemin transported from the wound site through the phloem (Hause, et al., 2000; Hause, et al., 2003).

OPR3

There are six OPR enzymes in *Arabidopsis*, but OPR3 is the only one that is involved in JA biosynthesis (Breithaupt, et al., 2006). The crystal structure of OPR3 and two other mutants in tomato suggested that OPR3 exists as a dimer for self-inhibition of enzyme activity, which is critical for fine-tuning the regulation of JA biosynthesis (Breithaupt, et al., 2006). *OPR3*

carries a peroxisomal target sequence specific for β-oxidation which occurs in peroxisomes (Stintzi and Browse, 2000) while LOXs, AOSs, and AOCs have putative chloroplast localization sequences and were reported to localize on the chloroplast membrane (Bell, et al., 1995; Harms, et al., 1995; Ziegler, et al., 2000; Stenzel, et al., 2003).

JAR1

JAR1 is an ATP-dependent JA-amino synthetase (Staswick and Tiryaki, 2004). JAR1 is able to catalyze JA conjugation to several amino acids including Ile, Leu, Val, Phe, but with stronger preference to Ile than other amino acids (Suza and Staswick, 2008). In Arabidopsis, a single copy of *JAR1* is found. *JAR4* and *JAR6* are two homologs identified in *N. attenuata* and they have similar functions (Wang, et al., 2007). Their transcripts were highly induced by wounding and insect attack. Independent silencing of *JAR4* or *JAR6* in N. *attenuata* significantly reduced JA-Ile, JA-Leu, and JA-Val, however, both the basal levels or herbivory-induced levels of nicotine in transgenic lines remained unchanged compared to the control (Wang, et al., 2007).

JA methylation: JA methyl transferase (JMT)

The JA methyltransferase (JMT) enzyme catalyzes JA methylation to form MeJA (Seo, et al., 2001). MeJA was believed to be the active form of JA signaling before identification of the JA signaling receptor complex composed of JA-Ile, JAZs, and COI1. Overexpression of the JA methyltransferase (JMT) gene negatively affected JA-Ile production in N. *attenuata* and the following JA response, indicating MeJA itself doesn't activate JA signaling, but only if it is converted to JA followed by conjugation to form JA-Ile (Stitz, et al., 2011).

JA-Ile catabolism: JA-Ile hydrolase (JIH1)

A jasmonoyl-L-isoleucine hydrolase 1 (JIH1) was isolated and demonstrated to hydrolyze JA-Ile to form JA in *N. attenuata* (Woldemariam, et al., 2012), a mechanism to inactivate JA-Ile. This NaJIH1 is a homolog of indoleacetic acid alanine resistant 3 (IRA3) in *Arabidopsis* (LeClere, et al., 2002). *NaJIH1* gene expression was induced by both mechanical wounding and herbivory attack in both local and systemic leaves. *NaJIH1* RNAi transgenic lines showed an herbivore-induced increase of JA-Ile. As a result, the subsequent direct and indirect herbivore defense responses were strongly induced, including nicotine accumulation (Woldemariam, et al., 2012).

1.8 JA manipulation by engineering JA biosynthesis pathway genes

Several research efforts have been conducted to genetically modify JA biosynthetic genes, in an attempt to elevate JA biosynthesis. Overexpression of the chloroplast localized flax *AOS* gene driven by the strong constitutive CaMV 35S promoter caused a 6 to 12-fold increase of basal jasmonates level in transgenic potato plants (Harms, et al., 1995). In contrast, overexpression of cytoplasm-localized flax *AOS* driven by a tetracycline- inducible promoter increased the wounding induced JA levels in tobacco wounded tissues but didn't alter the basal JA level (Wang, et al., 1999). The discrepancy may be caused by the higher enzyme activity of chloroplast-located AOS than cytoplasm-located AOS, given the fact that JA biosynthesis substrates and enzymes are co-localized in the cytoplasm. When the Arabidopsis *AOS* gene was constitutively overexpressed in both Arabidopsis and tobacco, the basal level of JA did not change. However, wounding induced jasmonates were more quickly and highly accumulated, and the maximum JA levels achieved doubled in both *Arabidopsis* and tobacco, as compared to wild type (Laudert, et al., 2000). Constitutive overexpression of *AOC* in

tomato elevated jasmonates and octadecanoids in floral organs but not in leaves of unwounded transgenic plants, suggesting a different regulation of JA biosynthesis in leaves and flowers of tomato (Miersch, et al., 2004). Constitutively overexpression of the *Hyoscyamus niger* L. *AOC* gene using the CaMV 35S promoter in tobacco resulted in a notable 4 to 8-fold increase of *NtPMT* transcripts and a slight increase of *NtQPT* transcripts and a 4.8-fold increase of nicotine as compared to WT (Jiang, et al., 2009). Unfortunately, endogenous JA levels were not determined in this study. Tomato plants constitutively overexpressing the *AOC* gene exhibited unchanged basal JA level but increased levels of JA, OPDA, and a defense-related *PIN2* transcript upon wounding treatment (Stenzel, et a., 2003). In summary, almost all these efforts to overexpress JA biosynthetic genes lead to increased endogenous JA only when wounded, indicating JA biosynthesis needs the supply of the fatty acid precursor generated by wounding stimuli.

1.9 JA biosynthesis regulation

Positive feedback

JA biosynthesis is regulated by the positive feedback loop. All genes encoding enzymes of JA biosynthesis are induced by the end product JA in Arabidopsis and other species (Wasternack, 2007). Mutants with constitutively raised JA levels exhibited a JA-induced alike phenotype (Ellis, et al., 2002). The JA-deficient *opr3* or *coi1* mutant displayed decreased *AOC* expression (Stenzel, et a., 2003). All these evidence suggest that JA biosynthesis is regulated by positive feedback.

Substrate availability

Substrate (fatty acid precursor) availability is suggested to be another essential determinant of JA biosynthesis. This conclusion is based on some experimental observations. First, the wound-induced JA synthesis is instant and appears before expression of *LOX*, *AOS*, and *AOC* (Wasternack, 2007). Second, JA biosynthetic enzymes are abundant in pre-wounded leaf tissues, but JA accumulation occurs only after wounding (Stenzel, et al., 2003). Moreover, although plants over-expressing *AOS* or *AOC* constitutively didn't show increased basal levels of JA, wounding or herbivory attack induced JA levels were elevated in the *AOS* or *AOC* overexpression lines (Laudert, et al., 2000; Stenzel, et al., 2003). These experimental observations lead to the speculation that the reason why JA biosynthesis is constrained before wounding treatment, even if JA enzymes are abundantly available, is because the fatty acid precursor is limited in absence of wounding. A fact supporting this speculation is that enhanced fatty acid release correlates with the JA production upon wounding (Conconi, et al., 1996). Later on, by studying plant-herbivory interaction in *N. attenuata*, it was demonstrated that wound and herbivory attack induced SIPK (Salicylic acid-induced protein kinase) and NPR1 (Non expression of PR-1), which upregulate de novo JA synthesis by positively activating GLA1 (Galactolipase A1), an essential early enzyme for releasing fatty acids from membrane lipids (Kallenbach, et al., 2010; Wang and Wu, 2013). Collectively, substrate (fatty acid precursor) availability is a critical regulator of JA biosynthesis.

Enzyme self-control by dimer formation

The crystal structure of tomato OPR3 suggested homodimer formation and in vitro enzymatic assay showed that the dimerization caused loss of enzymatic activity (Breithaupt, et al.,

2006). BiFC performed in protoplasts of Arabidopsis showed both homo and heterodimer formation by AOCs, but there is limited evidence to show that enzyme activity or JA biosynthesis were affected by the dimerization. Nevertheless, the author speculates AOC dimerization could regulate JA biosynthesis and JA responses (Stenzel, et al., 2012).

MAPK cascade

MAPK (mitogen-activated protein kinases) cascades can either induce or attenuate JA signaling. Overexpression of MAPK KINASE 3(MKK3)-MAPK6 (MPK6) cascade upregulated *LOX2 in* Arabidopsis (Takahashi, et al., 2007). Overexpression of a tobacco *MPK3* homolog called *WIPK (wound-induced protein kinase)* increased JA level and JA-inducible *proteinase inhibitor II* transcripts (Seo, et al., 1999*)*. In *N. attenuata*, two herbivory-induced MAPKs, salicylic acid -induced protein kinase (SIPK) and WIPK, were reported to positively regulate JA signaling. Silencing of *SIPK* and *WIPK* repressed *LOX3* and *AOS* and caused a notable decrease of both wounding and herbivory induced-JA level (Wu, et al., 2007). On the other hand, MAPK cascade attenuates JA signaling by negatively regulating *MYC2*. The gain of function and loss of function MKK3-MPK6 mutant analysis demonstrated that MKK3 and MPK6 were activated by JA treatment and caused the suppression of *MYC2* (Takahashi, et al., 2007).

1.10 JA perception and signal transduction

JA perception and signal transduction are dependent on several components, including SCF^{COI1} ubiquitin-ligase complex, JAZ repressors, MYC2 transcription factors, Topless (TPL) proteins and Novel Interactor of JAZ (NINJA). As shown in Figure 1.3, the SCF^{COI1} ubiquitin-ligase complex is assembled by CUL1, SKP1 (ASK1 and ASK2) and F-box protein

COI1 (CORONATINE INSENSITIVE 1) (Xu, et al., 2002). CUL1 bridges COI1 and a RING -box (RBX1) protein at the N-terminus and C-terminus respectively (Hua and Vierstra, 2011). According to a prevailing JA perception and transduction model in Arabidopsis (Figure 1.4), in the absence of JA, JAZs bind to MYC2 and recruit co-repressor NINJA (Novel Interactor of JAZ) and TPL (Groucho/Tup1-type co- repressor TOPLESS), to form a complex and consequently repress MYC2-induced transcriptional activation. In response to JA signaling, (+)-7-iso-JA-Ile forms a complex with COI1 and JAZs, and RBX1 recruits a ubiquitin-conjugating enzyme E2 which catalyzes the addition of ubiquitin moiety to JAZs. Ubiquitin tagged JAZ proteins are then degraded by the 26S proteasome, releasing MYC2 transcription factor from JAZ repressor and JA-responsive genes are then activated by free MYC2 (Pauwels and Goossens, 2011; Kazan and Manners, 2013; Wasternack and Hause, 2013). COI1, MYC2, and JAZs orthologues have been identified in tobacco and similar roles have been confirmed to mediate JA-elicited nicotine biosynthesis in tobacco (Shoji, et al., 2008).

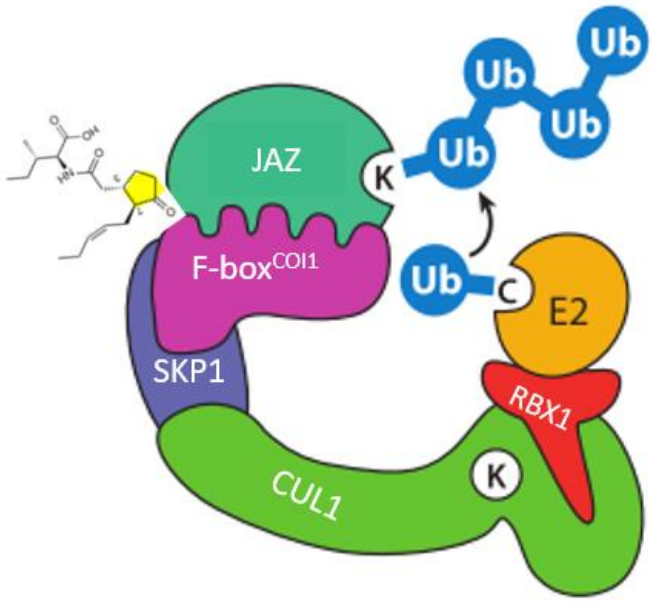

Figure 1. 3 SCF E3 ligase complex in planta.

Adapted from Hua and Vierstra (Hua and Vierstra, 2011). CUL1 bridges a F-box protein COI1 and a RING-box protein RBX1. SKP1 function as an adaptor between CUL1 and COI1. Upon JA perception, JA-Ile mediates the interaction between COI1 and JAZ repressors. RBX1 recruits an E2 enzyme which catalyzes ubiquitination of JAZ repressors.

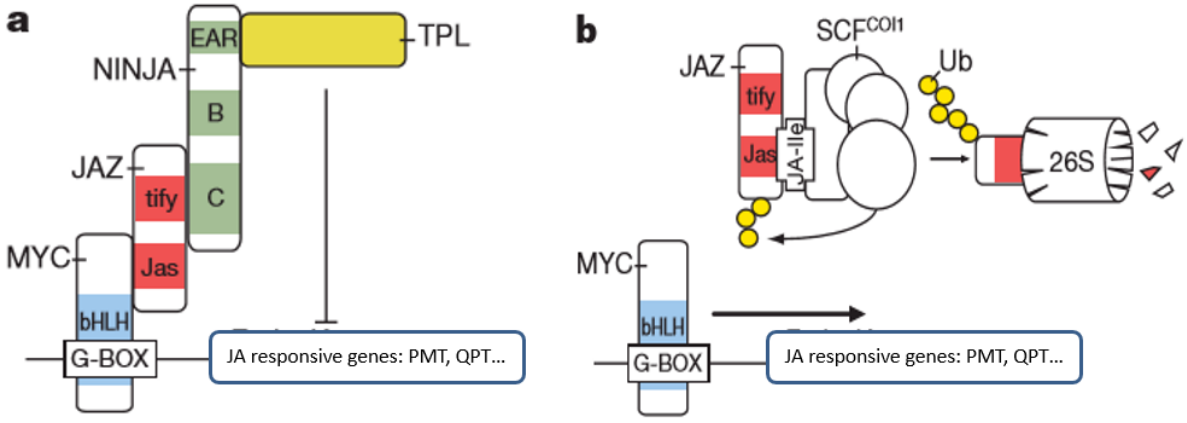

Figure 1. 4 JA signal perception and transduction model.

From Pauwels and Goossens (2011). a. In absence of JA, repressed state, b. In presence of JA, induced state.

MYC2 transcription factor

MYC2 is a key component in JA signaling and a master regulator of various JA responses (Kazan and Manners, 2013). MYC2 has a putative transcription activation domain (TAD) near its N-terminus, responsible for recruiting a general mediator to initiate transcription (Kazan and Manners, 2008). MED25, a subunit of the mediator complex, is reported to interact with MYC2 and subsequently initiate transcription by recruiting RNA polymerase II (Chen, et al., 2012). A JAZ interaction domain (JID), located just upstream of TAD, is required for interacting with JAZ proteins (Chini, et al., 2007; Fernandez-Calvo, et al., 2011). Binding with JAZs also requires the TAD region (Zhang et al., 2015). The C-terminal helix loop helix structure is a conserved feature of bHLH family transcription factors. This structure contributes to dimerization with other TFs such as MYC3 and MYC4 (Fernandez-Calvo, et al., 2011). The basic region adjacent to the HLH structure, containing 5-20 mostly basic amino acids, is a DNA-binding region (Carretero-Paulet, et al., 2010). Mutation of the conserved Asp (D105) to Asn within JID attenuated the MYC2 binding with JAZ proteins and lead to MYC2 hyperactivity in the presences of JAZs (Goossens et al., 2015).

JAZ (jasmonate ZIM- domain) repressor

Thirteen *JAZ* genes were identified in the Arabidopsis genome (Thines, et al., 2007; Chini, et al., 2007; Thireault et al., 2015). JAZ proteins have a conserved C-terminus Jas domain and a conserved ZIM domain in the middle. Some JAZ proteins have an additional EAR motif at the very beginning of the N-terminus (Pauwels and Goossens, 2011). Outside of the conserved ZIM and Jas regions, JAZ proteins share little sequence similarity.

The Jas domain is essential for binding both MYC2 and COI1 to JAZs (Chini, et al., 2007; Katsir, et al., 2008; Pauwels, et al., 2010). Y2H assays and crystal structure determination of protein complexes JAZ9-MYC3 and JAZ1-COI1 demonstrated that two neighboring but independent regions within the Jas domain are responsible for binding with the two proteins respectively. The core of the Jas motif which contains highly conserved 17 amino acids is sufficient for JAZ9 interaction with MYC3 (Zhang, et al., 2015). The minimal amino acid fragment LPIARR (called JAZ degron) with two non-conserved amino acids, located upstream of the core of Jas, is essential for JAZs interacting with COI1 (Sheard, et al., 2010). All JAZs interact with MYC2 but only half of them, including JAZ1, 2, 3, 6, 9, 10, interact with COI1 in presence of JA-Ile (Pauwels and Goossens, 2011). As a result of interaction with COI1, JAZ1, and JAZ3 protein are degraded in a 26S proteasome-dependent manner (Thines, et al., 2007; Chini, et al., 2007). Artificial JAZs deleted in the Jas domain or JAZ naturally lacking the JAZ degron, such as JAZ8, are unable to bind to COI1 and are thus degradation resistant (Shyu, et al., 2012). JAZs adopt conformational changes in order to switch between co-repressor function in absence of JA and hormone receptor function in presence of JA-Ile (Zhang, et al., 2015).

The ZIM domain mediates formation of homodimers or heterodimers between most JAZs. The conserved TIFY motif within the ZIM domain is essential to form homo- or hetero-dimers (Chini et al., 2009; Chung and Howe, 2009) and for interaction with the co-repressor NINJA (Pouwels and Goossens, 2011). A natural occurring non-TIFY JAZ13 doesn't interact with NINJA nor form dimers with other JAZs (Thireault, et al., 2015). Some JAZs,

such as JAZ5, JAZ6, JAZ7, JAZ8, and JAZ13, have an extra EAR motif, allowing direct recruiting of the TPL co-repressor without NINJA adaptor (Goossens, et al., 2016). JAZs negatively modulate JA responses by dual repression mechanisms. First, JAZs bind to the NINJA adaptor which subsequently recruits the TPL co-repressor, inducing a closed state of the target gene chromatin structure (Pauwels, et al., 2010). Second, JAZ directly competes with the MED25 for binding to MYC and thus inhibits MYC-MED25 dependent transcription initiation (Chen, et al., 2012; Zhang, et al., 2015).

A total of 17 NtJAZs were identified in tobacco by transcriptome profiling of tobacco BY-2 cells after MeJA treatment (Yang, et al., 2015). Unlike the prevailing model that the JAZs inhibit MYC2-dependent transcription of JA-responsive genes, Yang et al. (2015) found that suppression of *NtJAZ1*, *NtJAZ3*, *NtJAZ7a* and *NtJAZ10* individually decreased *NtPMT1a* transcripts level and nicotine content in BY-2 cell lines (Yang, et al., 2015). Interestingly, both MeJA induced and basal *NtMYC2a* transcripts level increased in NtJAZ1, NtJAZ3, NtJAZ7a and NtJAZ10 RNAi lines compared to vector control. Moreover, *NtJAZ7A* transcript level increased in the NtJAZ1, NtJAZ3, NtJAZ10-RNAi lines. These results indicate a complex transcriptional regulation by JAZs, and JAZs might have overlap and interplay functions in tobacco (Yang, et al., 2015).

NINJA repressor

NINJA was first isolated in tobacco BY-2 cells by cDNA-amplified fragment length polymorphism (AFLP)-based transcript profiling, in an experiment whose purpose was to discover genes involved in JA-induced secondary metabolite synthesis (Goossens, et al.,

2003). *NINJA* function was revealed in Arabidopsis when NINJA protein was identified as an interactor of JAZ1 by tandem affinity purification (TAP) (Pauwels, et al., 2010). In addition to JAZ1, NINJA also interacted with the TPL protein both in yeast-based and in BiFC assays (Pauwels, et al., 2010). NINJA binds to JAZs with the C-terminal conserved domain and interacts with TPL through the N-terminal EAR motif, a distinguishing characteristic of transcriptional repressors (Pauwels, et al., 2010).

Similar to the JA-inducible *JAZ* genes, *NINJA* expression was induced by MeJA within 1 hour and last for at least 12 hours after JA treatment in 7 days old Arabidopsis seedlings (Pauwels, et al., 2010). Unlike JAZ repressor, NINJA is resistant to protease-dependent proteolysis upon JA treatment (Pauwels, et al., 2010). Overexpression of NINJA driven by the CaMV35S promoter significantly impaired JA-induced inhibition of root elongation. RNAi-mediated NINJA knockdown resulted in more sensitive JA responses, as reflected by reduced root length after JA treatment and early expression of JA-responsive genes either with or without JA treatment (Pauwels, et al., 2010). These results demonstrated that NINJA is a co-repressor in JA signaling. Based on these studies, a model has proposed that JAZ proteins recruit TPL through NINJA for repression of MYC2 dependent transcription (Pauwels, et al., 2010). The NINJA loss-of-function mutants have constitutive expression of a JAZ10 reporter gene in roots and hypocotyls of unwounded seedlings. NINJA mutants showed 30% reduction in root length compared to WT without MeJA treatment. However, no root length differences between the NINJA mutant and WT were observed when 25 μM MeJA was applied (Acosta, et al., 2013). These results indicate that the role of NINJA in roots is to suppress JA signaling and allow normal cell elongation.

1.11 Outline of thesis

This dissertation study intends to gain more insights on molecular regulation mechanisms of nicotine synthesis in tobacco, mainly focusing on the transcription factor NtMYC2a and the phytohormone JA.

In the first project, a yeast two-hybrid (Y2H) approach was used to identify proteins interacting with the master regulator NtMYC2a. We hypothesized that additional factors may exist and regulate root-specificity of nicotine biosynthesis through forming a complex with NtMYC2a. In the second project, JA biosynthetic genes, *AtLOX2*, *AtAOS*, *AtAOC2*, *AtOPR3*, and *AtJAR1*, were individually overexpressed and a *JIH1* gene was knocked down via RNAi. At T_0 generation, transgene expression levels and total alkaloids levels were measured. Two transgenic plants of each gene construct with high transgene expression level and total alkaloids level were analyzed at the T_1 generation for their nicotine levels, transcript levels of nicotine biosynthetic genes, and JA levels. In the third project, a *NINJA* RNAi construct was expressed in tobacco plants in an attempt to impair the repressor complex of JA signal transduction and to evaluate how it affects nicotine accumulation.

II. Materials and Methods

2.1 Construction of a cDNA library

Make Your Own "Mate & Plate" library system (Clontech, Mountain View, CA) was used to make a cDNA library of tobacco roots after topping. Total RNA was isolated from root tissues collected from greenhouse-grown tobacco plants (cv. NC95) 15 minutes after topping. The TRIzol Reagent (Invitrogen, Carlsbad, CA) was used to isolate RNA according to the manufacturer's instruction. RNA integrity was verified by running an aliquot of RNA on 1% agarose gel. 28S ribosomal RNA band should be approximately twice the intensity of the 18S ribosomal RNA band. 565 ng RNA was used for cDNA synthesis. A CDS III oligo dT primer with a specific 25-mer sequence fused at the 5' end of oligo dT and a modified SMART III oligo were used to synthesize the first strand cDNA, using the SMART^{TM} technology kit (Clontech). The resulting first strand cDNA is flanked by two ends which could serve as a universal priming site in the subsequent amplification. The long-distance PCR (LD-PCR) was carried out to synthesize the double-stranded cDNA using two primers provided by the kit. The double-strand cDNA was purified by HROM SPIN TM TE-400 Columns (Clontech) to select DNA molecules longer than 200 base pair. Twenty μl of cDNA was mixed with 6 μl linearized prey vector pGADT7-Rec. The mixture was transformed into Y187 yeast strain according to the library-scale transformation protocol in the Yeastmaker^{TM} yeast transformation system 2 user manual (Clontech). Homologous recombination took inside yeast cells and the cDNAs were cloned into the linearized pGADT7-Rec vector. The transformed Y187 cells was spread on 150 mm SD/-leu plates. After four days of incubation at 30 °C, all the colonies were harvested and suspended in 5 ml of freezing medium (YPDA medium with 25% glycerol). One ml aliquot was used for library screening.

2.2 Bait construction

Matchmaker Gold Yeast Two-Hybrid System (Clontech) was employed to screen for proteins that interact with NtMYC2a. Full-length *NtMYC2a* complementary DNA (cDNA) has been isolated and cloned into pTOPO_NtMyc2a construct in our lab previously. The coding sequence of *NtMYC2a* was amplified from the pTOPO_NtMYC2a construct through Polymerase Chain Reaction (PCR) using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) with appropriate primers containing specific homologous sequence to bait vector pGBKT7. Primers are listed in Appendix Table A1. The resultant PCR product was column purified and inserted between the BamH1 and *Eco*R1 sites of bait vector pGBKT7 by In-Fusion HD Cloning (Clontech) reaction. A restriction digestion followed by DNA sequencing was carried out to verify the insertion. Competent Y2HGlod strain yeast cells were prepared and transformed with bait construct using the lithium acetate -mediated yeast transformation method according to Yeast Transformation System 2 user manual (Clontech). The transformants were plated on SD/-Trp solid medium and incubated at 30 °C for five days. NtMYC2a is expressed as a fusion protein to GAL4 DNA-binding domain in Y2HGlod yeast cells.

In order to determine whether the bait autonomously activates reporter genes in the absence of a prey protein, yeast strains co-transformed with bait construct and an empty prey vector were plated on DDO/X-α-Gal/AbA and DDO/-His/-Ade/X-α-Gal/AbA medium and colony growth were recorded.

2.3 Identification of NtMYC2a activation domain

In order to determine which part of NtMYC2A activates reporter gene transcription, a series of truncated NtMYC2a fragments were tested for their transactivation. Fragments 1-164AA, 1-166AA, 1-203AA, 1-261AA, 261-482AA, 261-329AA, 261-375AA, 483-659AA of NtMYC2a were generated by PCR and cloned into pGBKT7 vector by In-Fusion HD Cloning (Clontech) reaction. Phusion® High-Fidelity DNA Polymerase (New England Biolabs) was used for all the PCR reactions. Yeast transformants co-expressing NtMyc2a fragments and empty prey vector pGADT7 were plated on SD/-Leu/-Trp/-His/-Ade medium. After determining the transactivation region, the region was deleted from NtMYC2a by performing three-primer PCR reactions according to a PCR-based precise gene fusion method developed by Yon and Fried (Yon and Fried, 1989). This new bait was used in the second round of library screening.

2.4 cDNA library screening, colony selection, and characterization

Library screening was performed according to the user's manual of Make Your Own "Mate & Plate" library system. Bait strain was cultured in 4 ml SD/-Trp liquid medium until OD_{600} reading reached 0.8. Next, 4 ml bait strain culture was mixed with 1 ml cDNA library strain and the mixture was cultured in 45 ml nutritious YPDA medium with 50 μg/ml kanamycin for 24 hours until zygotes were present. The mated culture was centrifuged at 700 g for 5 minutes to pellet cells and discard supernatant. The pelleted cells were suspended in 10 ml of 0.5X YPDA liquid medium with 50 μg/ml kanamycin. Two hundred μl of the mated cell culture was plated on appropriate selection medium and incubated at 30 °C, generating 50 plates in total for each screening procedure. After five days' incubation, colonies appeared on

selective medium. Colony PCR was performed on the yeast colonies individually in order to verify cDNA insertions in prey vectors. Five μl of PCR products was analyzed by electrophoresis on a 1% agarose gel. Yeast colonies which contained more than one prey plasmid, indicated by more than one DNA band on agarose gels, were subjected to successive re-streaking until only one prey plasmid was left in the colony. In order to identify the cDNA insertions, plasmids were isolated from positive yeast colonies using an alkaline lysis based bacterium plasmid isolation kit (Qiagen, Valencia, CA). The plasmids isolated from the positive colonies were transformed into *E. coli* for propagation. Plasmids were purified from *E. coli* and sequenced. To characterize cDNA insertions in prey vectors, the obtained cDNA sequences, and deduced peptides were compared with reference sequences in NCBI database by Basic Local Alignment Search tool (BLAST).

A total of three screenings of cDNA library were performed using full or truncated NtMyc2a coding sequences as bait with various selection stringency. In the first screening, full-length NtMYC2a was used as bait to screen cDNA library against SD/-Leu/-Trp/-His/-Ade (QDO, low stringency) selection medium, and then all colonies were re-streaked onto SD/-Leu/-Trp/-His/-Ade/AbA/X-α-Gal (QDO/A/X, high stringency). In the second screening, truncated NtMyc2a without activation domains was used as a bait to screen library against QDO selective medium. In the third screening, full-length NtMYC2a was used as bait to screen library directly on highest stringency selective medium QDO/AbA/ X-α-Gal. The darker blue colonies and/or colonies that appeared earlier were used for further analysis.

In order to confirm interactions with full-length NtMYC2a and avoid self-activation of the full-length NtMYC2a, full-length MYC2a cDNA was inserted in the prey vector and the candidate cDNA inserted into the bait vector. These new sets of switched prey and bait constructs were co-transformed into yeast. Interactions were evaluated on QDO, DDO/A/X, QDO/X and QDO/A/X selective medium. Colonies were considered promising and worth for further pursuing when at least two reporter genes were activated.

2.5 Bimolecular fluorescence complementation (BiFC) vector construction

Bimolecular fluorescence complementation (BiFC) is a widely used method for testing protein-protein interaction. This technique relies on two halves of a fluorescence protein brought together by two interacting proteins each fused to a half of the fluorescence protein. The advantage of this assay is it enables us to visualize protein-protein interactions in live plant cells (Walter, et al., 2004; Fang and Spector, 2010).

The vector sets developed by Walter et al. (Walter, et al., 2004) was used for BiFC analysis in this study. The vector map is shown in Figure 2.1. Full-length *NtMYC2a* coding sequence was cloned in frame with C-terminal half of YFP in pUC-SPYCE. Candidate cDNAs were inserted into pUC-SPYNE vector in frame *with* N-terminal half of *YFP* using In-Fusion HD Cloning kit (Clontech). Primers are shown in Appendix Table A2. Cloning events were verified by restriction digestions followed by sequencing analysis.

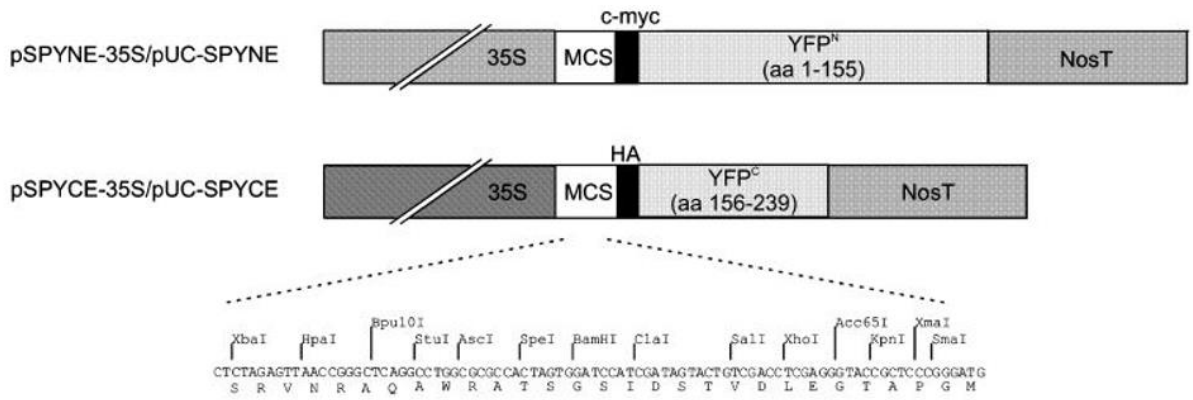

Figure 2. 1 Vector map of BiFC constructs.

Adapted from Walter, et al., (2004). Full-length NtMYC2a coding sequence was cloned into pUC-SPYCE between XbaI and SalI. Candidate coding sequence was inserted into pUC-SPYNE vector between XbaI and Xho1. YFP^N and YFP^C are coding sequences of N-terminal half and C-terminal half of the yellow fluorescence protein. Multiple cloning site (MCS), HA, and c-myc tags are also shown in the figure.

2.6 Biolistic bombardment of tobacco BY-2 suspension cells

BY-2 cells were sub-cultured every 3 days in liquid MS medium when the cell growth is in log phase. BY-2 cells are harvested on the third day after sub-culture, collected through a filter cloth and plated on MS medium. For biolistic bombardment, two constructs, encoding C-terminal half of YFP and N-terminal half of YFP fusion proteins were mixed at a 1:1 (w/w) ratio, 2.5 μg of each DNA was coated onto 30 mg of gold particles (1.0 μm in diameter) for 6 repeats according to the manufacturer's instruction (Bio-Rad, Hercules, CA). The particles were bombarded into the BY-2 suspension cells at a pressure of 1100 psi using a biolistic particle delivery system model PDS-1000/He (Bio-Rad). The transformed BY-2 cells were incubated for 24 hours at 25 °C in darkness for transgene expression.

2.7 Fluorescence detection

The confocal microscopy is an ideal tool for detecting fluorescence in living cells with high resolution. A Zeiss LSM710 laser scanning confocal microscope (Zeiss, Ontario, CA) was used for visualizing fluorescently labeled proteins in this study. The excitation wavelength was 488 nm, and emission wavelength ranges from 507 nm to 574 nm for detection of YFP. The objective Plan-Apochromatic 20x/0.8 M27 was used for cell viewing and photo-taking.

2.8 Generation of overexpression constructs

Binary vector pBI121 was used as a backbone vector to generate overexpression constructs (Figure 2.2). Vector pBI121 was digested with BamHI and EcoRI and separated via 1% agarose gel electrophoresis. The desired fragments were recovered from gel as described before. *AOS (*AY128733*), AOC2 (*AY054131*), OPR3* (AY097367) and *JAR1* (AY15043) cDNAs were acquired from Arabidopsis biological resource center (ABRC). *LOX2* cDNA

(L23968) was kindly provided by Dr. Carmen Castresana (Spain National Research Council, Madrid, Spain). The coding sequences of these genes were amplified by HiFi PCR premix (Clontech) and inserted into the pBI121 to replace the *GUS* gene by homologous recombination using In-Fusion HD cloning system (Clontech). Primers are listed in Appendix Table A3.

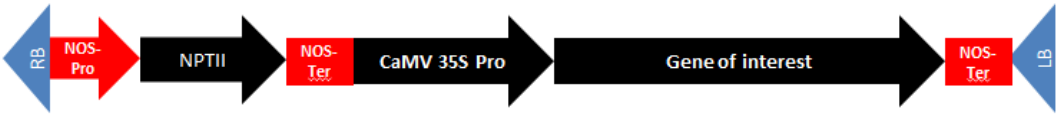

Figure 2. 2 T-DNA region of pBI121.

Binary vector pBI121 was used to deliver and express genes in tobacco plants. Gene of interest was cloned into pBI121. RB: right border. NOS-Pro: NOS promoter. NPTII: Neomycin phosphotransferase gene. CaMV 35S Pro: CaMV 35S promoter. NOS-Ter: NOS terminator.

2.9 Generation of JIH RNAi construct

RNA interference (RNAi) can be mediated by double-stranded RNA (dsRNA) in plants (McGinnis, 2010). Inverted repeats are used to produce dsRNA. A Gateway binary RNAi vector pB7GWIWG2(II) was used to generate inverted repeats and stably express dsRNA in tobacco plants (Figure 2.3; http://www.psb.ugent.be/gateway/). Leaf total RNA was extracted as described before. RNA was treated with RQ1 DNase (Promega, Madison, WI) to remove genomic DNA. 180 ng of treated RNA is used for cDNA synthesis by iScript™ cDNA Synthesis Kit according to the manufacturer's instructions (Bio-Rad). Two copies of *NtJIH1* gene were found in tobacco genome database, a conserved 453 bp *NtJIH1* fragment (shown Figure A1, A2) was targeted. The specific primers flanked by gateway attL sites were used for gateway LR reaction (Karimi, et al., 2002) (Figure A4). HiFi PCR premix (Clontech) was used for PCR reactions. PCR product was separated by agarose gel electrophoresis and purified with ZymoClean gel DNA recovery kit (Zymo Research, Irvine, CA). An LR reaction was carried out to assemble the fragment into pB7GWIWG2 (II) gateway vector by Gateway LR Clonase II Enzyme mix (Invitrogen, Carlsbad, CA).

Figure 2. 3 T-DNA of pB7GWIWG2(II) vector for RNAi.

A Gateway binary vector pB7GWIWG2(II) was used to generate inverted repeats and induce RNAi-mediated gene silencing in tobacco plants. RB: right border. NOS-Pro: NOS promoter. NPTII: Neomycin phosphotransferase gene. CaMV 35S Pro: CaMV 35S promoter. NOS-Ter: NOS terminator.

2.10 Generation of NINJA RNAi construct

Two copies of *NINJA* were found in tobacco genome. A conserved region located within the N-terminus were chosen to be the target site for RNAi (Figures A3, A4). The conserved nucleotide sequence flanked by gateway attL sites were commercially synthesized (GenScript, Piscataway, NJ) and cloned into pB7GWIWG2 (II) gateway vector as described before.

2.11 Generation of transgenic tobacco plants

Transgenic tobacco plants were developed by *Agrobacterium*-mediated leaf disc transformation as described (Horsch, et al., 1989). LBA4404 *Agrobacterium* strain harboring gene construct was inoculated into 30 ml of YM medium (0.04% yeast extract, 1.0% mannitol, 1.7 mM NaCl, 0.08 mM $MgSO_4 \cdot 7H_2O$, 2.2 mM $K_2HPO_4 \cdot 3H_2O$) with 100 μg/ml kanamycin for overexpression construct selection or 100 μg/ml spectinomycin for RNAi construct selection. The bacterial culture grew at 28˚C with shaking for 48 hours until OD value reached 0.6. Young fully expanded leaves were collected from 6 weeks old tobacco (*N. tabacum* cv. NC95) and surface sterilized first with 70% ethanol for 30 seconds and then with 10% Clorox (Oakland, CA) or 10 minutes, followed by five rinses in sterile distilled water. Sterilized leaves were cut into 1 cm x 1 cm discs, which were subsequently incubated with the bacterial culture for 10 minutes. The leaf discs were removed from bacteria suspension, gently blotted on sterile filter paper and then placed upside down on co-culture medium (4.43 g/l MS with vitamin, 3% sucrose, 0.1% 6-benzylaminopurine, 0.1 mg/l naphthaleneacetic acid, pH 5.7). After co-cultivation for 48 hours, the transformed tissues were sub-cultured on corresponding selective medium (4.43 g/l MS with vitamin, 3%

sucrose, 0.1% 6-benzylaminopurine, 0.1 mg/l naphthaleneacetic acid, 200 mg/l timentin, pH 5.7). Due to the vector difference, for overexpression construct, the selective medium was supplemented with 100 mg/l Kanamycin for NPTII gene selection. For RNAi constructs, the selective medium was supplemented with 3 mg/l phosphinothricin for *bar* gene selection. The transformed leaf discs were sub-cultured on selection medium every two weeks until the emergence of shoots. Approximately 5 weeks later, shoots were moved to rooting medium (2.16 g/l MS with vitamins, 3% sucrose, 200 mg/l timentin, pH 5.7) supplemented with 100 μg/ml kanamycin for overexpression construct selection or 10 mg/l phosphinothricin for RNAi construct with *bar* gene selection. After roots formation, the plantlets were transplanted to greenhouse approximately 3 weeks later. PCR was performed on T_0 plants to confirm the existence of the transgene(s). PCR positive plants were grown to the pre-flowering stage and sampled for gene expression level, nicotine level, and JA content. After sampling, T_0 transgenic plants were self-pollinated to produce T_1 seeds.

2.12 PCR analysis of the putative transgenic plants

Genomic DNA of putative transgenic plants were extracted by a quick method using Shorty Buffer (0.2 M Tris-HCl with pH 9.0, 0.4 M LiCl, 25 mM EDTA with pH 8.0, 1% SDS) as described (Edwards, et al., 1991). PCR was performed to detect the transgene using AccuPower ® PCR premix (Bioneer Inc, San Francisco, CA). The amplified DNA fragments were resolved in 1% agarose gel electrophoresis.

2.13 Real-time quantitative RT-PCR (qRT-PCR)

Tobacco root tissue was collected from plants without topping treatment or 3 hours post topping respectively. Root samples were immediately frozen in liquid nitrogen. Total RNA

was isolated and genomic DNA was removed as described before. To ensure genomic DNA is completely removed, PCR were performed with *actin*-specific primers. No amplification indicates no genomic DNA contamination in the RNA samples. 500 ng treated template RNA was used to synthesize cDNA with a blend of oligo (dT) and random hexamer primers provided in the kit as described before. 25 ng of cDNA were used for quantitative PCR on a Stratagene Mx3005P quantitative real-time PCR system (Agilent Technologies, Santa Clara, CA) with iTaq™ Universal SYBR® Green Supermix (Bio-Rad) according to the manufacturer's instructions. The thermal cycling program was 30 seconds at 95 °C, followed by 40 cycles of 5 seconds at 95 °C and 30 seconds at 57 ºC. The melting curve analysis was set following amplification to ensure single PCR product was detected in a reaction. Three technical repeats were performed for each transgene. A tobacco *actin* gene (GenBank: U60490.1) was used as a reference gene for normalization. The gene-specific primers are listed in Appendix Table A5.

2.14 JA extraction and quantification

A 1.5-inch diameter leaf circle was collected at the third fully expanded leaf from the top by a paper punch. The samples were immediately frozen in liquid nitrogen and then stored in -80℃ until using. An adapted non-targeted approach for HPLC-MS profiling of jasmonates was performed as described (Glauser and Wolfender, 2013). The frozen sample was ground into fine powder and 100 mg powder was quickly weighed before it thaws. One ml cold extraction buffer I (80% methanol spiked with 10 μl lidocaine as an internal control) was added to the 100 mg sample. After well mixing, the mixture was vortexed in a 4℃ cold room for 15 minutes, followed by sonication for 15 minutes in ice water. After centrifugation at

15700 g at 4℃ for 15 minutes, the supernatant was transferred into a glass tube on ice. The extraction steps were repeated one more time with 1 ml of cold extraction buffer II (Acetonitrile: Isopropanol: H_2O = 3:3:2) and then cold extraction buffer III (Acetonitrile: H_2O = 1: 1). The supernatants of three times extraction were combined in a glass tube. The total extracts were dried by a rotary evaporator at 30-40℃ for approximately 15 minutes. The dry extracts were stored at -80˚C until delivery. Samples were shipped to Dr. Sixue Chen' lab (University of Florida) for quantification. The residue was dissolved in 100 μl of sterile distilled water and centrifuged at 15700 g at 4℃ for 15 minutes, cleared supernatant was transferred to glass vials for HPLC-MRM (high-performance liquid chromatography with multiple reactions monitoring).

Determination of JA and derivatives was performed by Dr. Sixue Chen's lab at the University of Florida. HPLC-MRM-MS was performed using an Agilent 1100 HPLC (Agilent) coupled with an AB Sciex 4000 QTRAP^{TM} (AB Sciex, Framingham, MA). Optimized detection conditions including precursor ion, product ion, declustering potential (DP), collision energy (EP) and cell exit potential (CXP) were established for quantification of JA, MeJA, JA-Ile, OPDA. A reverse-phase C18 column (Agilent, Eclipse XDB-C18, 4.6 X 250 mm, 5 μm) was used for metabolite separation with 0.1 % formic acid in water as solvent A and 0.1 % formic acid in acetonitrile as solvent B. The LC gradient was:

1% solvent B for 5 minutes

A linear gradient from 1% B to 99.5% B over 41.5 minutes

99.5% B for 4.5 minutes

return to 1% B

The flow rate was 0.5 ml/min, and the total analysis time was one hour.

The mass spectrometer conditions were: 30 psi curtain gas, 50 psi GS1, 55 psi GS2, ion source voltage at + or – 4500 V, with the Turbo ElctroSpray Ionization (ESI) interface temperature at 350℃. A multiple period (segment) method was followed as previously described (Chen, et al., 2011).

2.15 Nicotine quantification

The Tobacco Analytical Services Lab (TASL) at North Carolina State University kindly helped us quantify nicotine, nornicotine, anatabine, anabasine and total alkaloids in tobacco leaves. Leaves were dried at 65 °C for 72 hours and then ground into fine powder. 0.2000 ± 0.0010 g of the leaf powder were weighted and recorded for total alkaloid extraction. 2.0 mL 2N NaOH solution was added to each sample in a 50 ml Erlenmeyer flask. After 15 minutes' incubation, 10.0 ml quinoline working solution (0.4 g quinoline/ml methyl-tert-butyl ether) were added to the flask and shaken for 2.5 hours. The mixture was incubated overnight for separation. Approximately 1.0 ml of the top methyl-tert-butyl ether (MTBE) layer was transferred into a labeled GC vial. Quantification was performed on an Agilent HP 6890 chromatograph using a DB-5MS column (30 m x 0.53 mm x 1.50 μm) and a deactivated guard column (0.53 mm x 1 m). 1 μl sample from the GC vial was injected. The carrier gas helium was set at an average velocity of approximately 38 cm/sec. The injector and detector were both set at 250 °C. The oven temperature program was set as 110 °C held for 1 minute, 200 °C at a rate of 10 °C/min, 300 °C at 25 °C/min and hold for 10 minutes. Data were collected and analyzed using Agilent Chemstation software. Pure nicotine (Sigma Aldrich, St. Louis, MO, US), nornicotine (Toronto Research Chemicals, Toronto, Canada), anabasine

(Alfa Aesar, Haverhill, MA) and anatabine (Toronto Research Chemicals) were purchased and used as internal standards to make a calibration table. The curves for each compound are listed as follows. X represents peak area, R^2 represents coefficient of determination.

Nicotine: Y = 0.79469396*X + 0.0083449 R^2=0.99873

Nornicotine: Y = 0.92352769*X - 0.0010492 R^2=0.99948

Anabasine: Y =0.88199877*X - 0.0001522 R^2: 0.99993

Anatabine: Y = 0.73706851*X - 0.0002168 R^2: 0.99980

2.16 Quantification of total alkaloids

Total alkaloids were determined by the segmented-flow colorimetric method in the Tobacco Analytical Chemistry Laboratory at NCSU as previously described (Collins et al., 1969; Davis et al., 1976). The top 12 leaves were collected from tobacco plants at the pre-flowering stage. Leaves were dried for 72 hours and then were ground. Approximately 250 mg leaves were weighed in a 50 ml dry conical flask. 25 ml distilled water was added to the flask and the flask was shaken for 30 minutes. The aqueous extract was filtered through a Whatman filter paper. The first few ml of the filtrate was rejected, and then the following filtrate was collected. The collected aqueous extract was reacted with the sulphanilic acid buffer and cyanogen chloride buffer. A colored complex was formed as a result of the reaction. The developed color was measured at 460 nm by a colorimeter. A graph of peak height against equivalent nicotine concentration was plotted using all the calibration standards. The nicotine concentrations of samples were obtained from the calibration curve based on the read of peak height from colorimeter.

III. Results

Chapter 1: Isolation of NtMYC2a Interacting Proteins

3.1.1 Overview

The Y2H assay was performed in this study to identify proteins interacting with NtMYC2a. *NtMYC2a* coding sequence was inserted into the pGBKT7 vector and tobacco cDNA library was cloned into the pGADT7-Rec vector. When the two constructs were co-transformed into yeast, NtMYC2a protein is expressed as a fusion to the Gal4 DNA binding domain and library proteins are expressed as a fusion to the Gal4 activation domain. The interaction between NtMYC2a and a prey protein reconstitutes a functional Gal4 transcription factor which is able to activate the transcription of independent reporter genes in yeast nucleus, enables yeast colony growing on synthetic dropout medium or dropout medium containing toxic drug AbA with the blue color when α-galactosidase is added.

Three screening experiments were performed to isolate the interacting proteins from the cDNA library. More than 1.5×10^6 diploids were screened and the mating efficiency is approximately 2% in each screening, indicating enough amount of diploids were available for screening. In the first and third screening, full-length *NtMYC2a* coding sequence was used as the bait, while the second screening used the truncated *NtMYC2a* with the activation domains depleted. In the first screening, more than 2000 colonies appeared on SD/-Leu/-Trp/-His/-Ade medium (QDO, moderate stringency) and all of them turned blue on SD/-Leu/-Trp/-His/-Ade/X-α-Gal/ AbA (QDO/X/A, high stringency). 200 normal size (approximately 2 mm in diameter) colonies were randomly selected for prey plasmid purification, sequence analysis, and annotation. In the second screening, truncated NtMyc2a

was used as bait, 209 colonies appeared on SD/-Leu/-Trp/-His/-Ade (moderate stringency), all of them were subjected to plasmid isolation, sequence analysis, and annotation. In third screening, 20 early-appeared blue colonies grow on QDO/X/A (high stringency) were subjected to plasmid isolation, sequence analysis and annotation.

In summary, a total of 429 (200+209+20) colonies generated from Y2H screening were subjected to plasmid isolation and cDNA insertion sequencing. We obtained 285 (114+153+18) sets of cDNA sequence data that have good sequencing quality and long open reading frames (more than 200 bp). Sequences that have no unambiguous hit to reference peptides in NCBI database or without any supporting evidence for their biological relevance to MYC2a protein or nicotine accumulation were considered to be less interesting and were not subjected to further validation. After this cleaning procedure, nine candidate proteins NtJAZ3, NtUBC, NtRBX-1, NtRNF185, NtARF9, NtbZIP-Like, NtSTPK, NtSNF-1, NtEPL were re-examined in switched vectors against higher stringency DDO/X-α-gal/AbA and QDO/X-α-gal/AbA in order to further confirm their interaction with full-length NtMYC2a. Interactions are judged to be worth for further studies when expression of at least two reporter genes are activated. Results were summarized in Table 3.1.

In order to further validate the interactions and narrow down candidate pool, a split YFP-based BiFC experiment (Walter, et al., 2004) was performed to test *in vivo* interaction between NtMYC2a and the nine candidate proteins NtJAZ3, NtUBC, NtRBX-1, NtRNF185, NtARF9, NtbZIP-Like, NtSTPK, NtSNF-1, and NtEPL. Full-length NtMYC2a cDNA was inserted into the pUC-SPYCE vector. Candidate cDNAs were inserted into the pUC-SPYNE

vector. The construct sets were introduced into BY-2 suspension cells by particle bombardment. Fluorescence was visualized under the confocal microscope. Results were listed in Table 3.1.

Table 3. 1 Evaluation of interactions in both Y2H and BiFC.

NtMYC2a protein is expressed in yeast cells, along with each candidate proteins in switched vector, and their interactions were examined on DDO/X-α-gal/AbA and QDO/ X-α-gal/AbA. The protein pairs were co-expressed in BY-2 cells and fluorescence was examined for protein interaction (n.t.: not tested).

Candidate proteins	Isolated in which screening	How many colonies has this gene	Y2H DDO/X-α-gal/AbA	Y2H QDO/ X-α-gal/AbA	BiFC
NtJAZ3	1st	1	Positive	Positive	Positive
NtUBC	2nd	3	Negative	n.t.	Positive, not repeatable
NtRBX-1	2nd	3	Negative	n.t.	Positive, not repeatable
NtSNF-1	2nd	1	Positive	Negative	Negative
NtRNF185	2nd	1	Positive	Negative	Negative
NtbZIP-Like	2nd	1	Positive	Negative	Negative
NtSTPK	2nd	1	Negative	n.t.	Negative
NtARF9	3rd	1	Positive	Negative	(see text)
NtEPL	3rd	1	Positive	Negative	n.t.

3.1.2 NtMYC2a has two independent activation domains

NtMYC2a is a bHLH family transcription factor with a typical activation domain and has autonomous activation in Y2H system that can generate false positive background (Figure 3.1.1). One possible solution for eliminating false positive is using the truncated TF devoid of activation domain as bait to screen library. However, the limitation is the conformational change caused by truncation may affect its interactions with other proteins. In order to identify which region is the activation domain, the NtMYC2a peptide fragment AA1-166, AA 1-203, AA 1-261, AA 261-329, AA 261-376, AA 261-483, AA 483-659 was expressed as a fusion to the GAL4 DNA binding domain, and the GAL4 activation domain protein was co-expressed into yeast. It turned out transactivation activity was detected when fragment AA 203-261AA and fragment AA 261-329 were expressed in the test, indicating the two adjacent fragments are activation domains (Figure 3.1.1). The truncated bait protein, NtMYC2a devoid of AA203-329, didn't show transactivation (Figure 3.1.1).

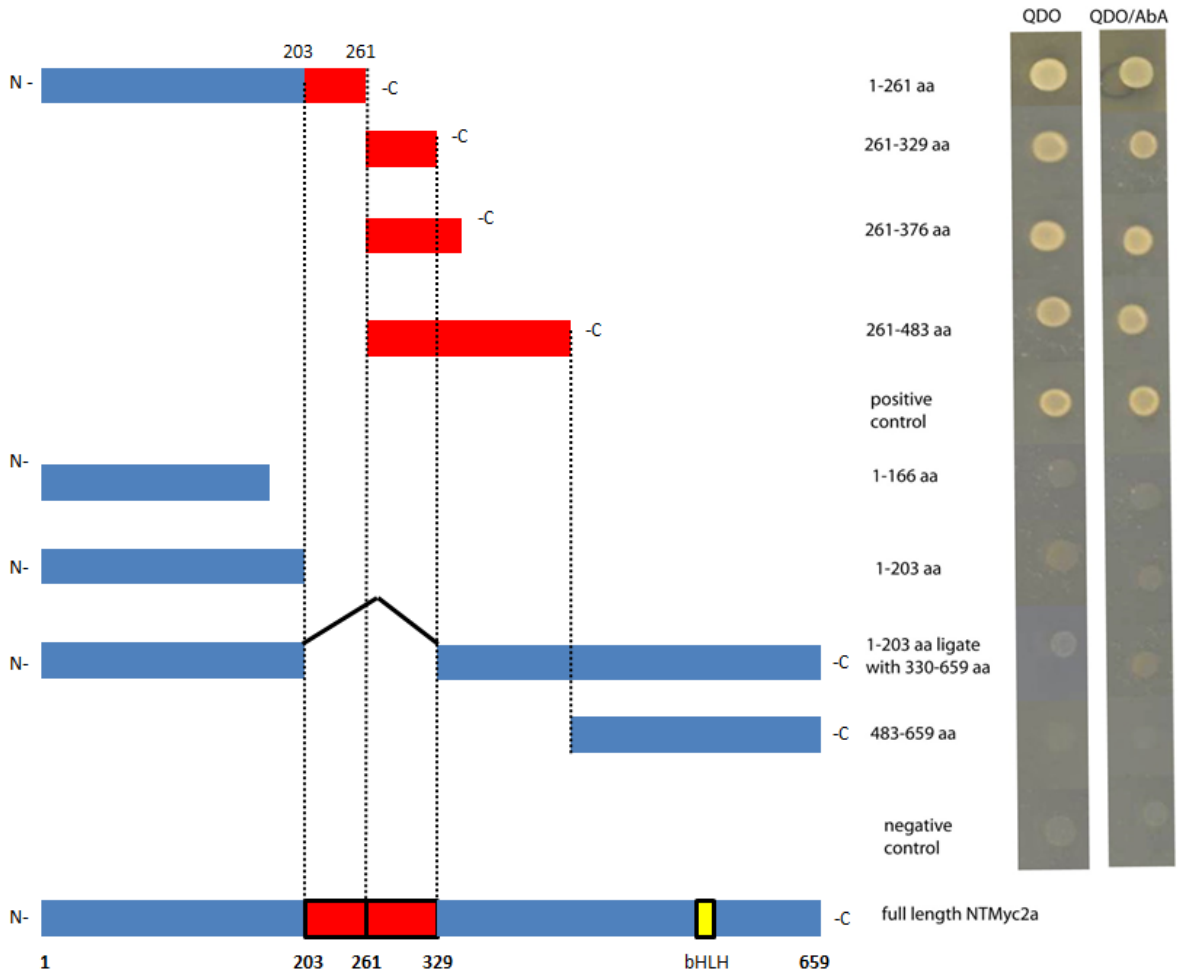

Figure 3. 1. 1 Identification of activation domains within NtMYC2a.

BD vector containing different fragments of NtMYC2a coding sequence was introduced into yeast cells, together with empty AD vector. The transformants were plated on SD/-Leu/-Trp/-His/-Ade (QDO) and SD/-Leu/-Trp/-His/-Ade/AbA(QDO/AbA) selective medium to evaluate their transactivation. On the left of the figure is a diagram to show various coding fragments used in the test. On the right of the figure are two photo columns showing yeast growth on QDO (left) and QDO/AbA (right). Yeast growth on the two selective medium indicated that the corresponding NtMYC2a fragment contains activation domain (shown in red color). The blue color fragments didn't contain activation domain. Fragment 1-261 has transactivation but 1-203 didn't, so the transactivation domain is within 203-261. Fragment 261-483 has transactivation but fragment 330-659 didn't, so the transactivation domain is within 261-329. Therefore, both fragment AA 203-261 and AA 261-329 (shown in red color) had transactivation. The new bait (AA1-203 ligated with AA 330-659) didn't confer transactivation. The yellow color represents bHLH structure.

3.1.3 JAZ3 protein interacted with NtMYC2a

A full-length *JAZ3* cDNA was isolated in the Y2H screening. *JAZ3* encodes the same protein identified by Shoji and coworkers (Shoji, et al., 2008). *JAZ3* was re-cloned into pGBKT7 and was co-expressed with NtMYC2a_pGADT7 in yeast cells. Transformants were plated on SD/-Leu/-Trp (DDO) medium to select for both constructs. Then the transformants were re-streaked on QDO, DDO/X/A, and QDO/X/A selective medium to investigate the interaction. Transformants grew on QDO, exhibited blue color on DDO/X/A and QDO/X/A, indicating a strong interaction between NtJAZ3 and NtMYC2a (upper panel, Figure 3.1.2). Expression of JAZ3 or NtMYC2a in yeast cells was not able to trigger reporter genes (middle and bottom panel, Figure 3.1.2). In BiFC assay, a strong fluorescence was detected in several BY-2 cells expressing NtJAZ3-nYFP and NtMYC2a-cYFP (Figure 3.1.3 upper panel). This experiment was repeated three times and strong fluorescence signal was detected every time in three to five cells, confirming the interaction between NtMYC2a and NtJAZ3.

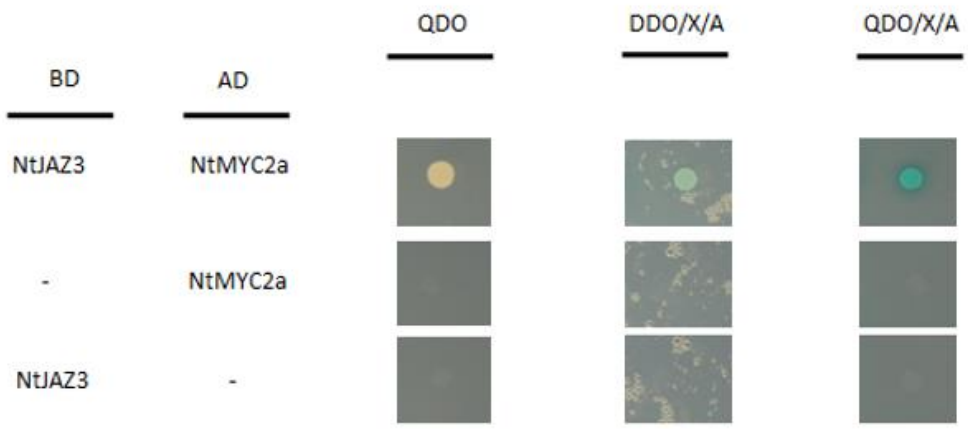

Figure 3. 1. 2 JAZ3 interacted with NtMYC2a in yeast.

NtJAZ3_pGBKT7 and NtMYC2a_pGADT7 were co-transformed into yeast cells. The yeast colonies grown on DDO medium were re-suspended. 5 μl of the cell suspension was plated on QDO, DDO/X/A and QDO/X/A subsequently to evaluate the interactions. Transformants can grow on QDO, exhibited blue color on DDO/X/A and QDO/X/A, indicating a strong interaction between NtJAZ3 and NtMYC2a (upper panel). Yeast cells harboring empty pGBKT7 and NtMYC2a_pGADT7, or NtJAZ3_pGBKT7 and empty pGADT7 did not grow (middle and bottom panel), indicating no background interference. BD: prey plasmid. AD: bait plasmid. DDO: double dropout medium without tryptophan and leucine. QDO: quadruple dropout medium without tryptophan, leucine, histidine and adenine. DDO/X/A: double dropout medium supplemented with α-galactosidase and Aureobasidin A.

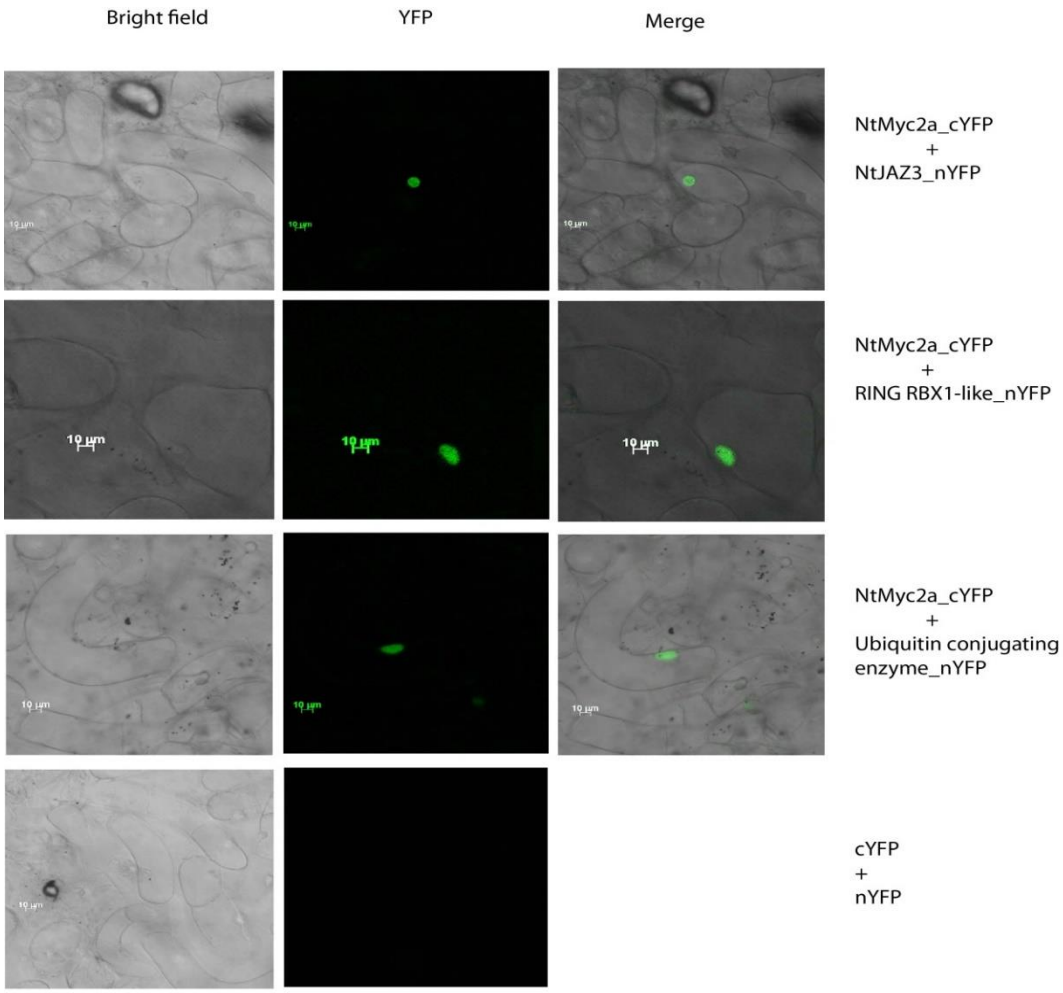

Figure 3. 1. 3 NtMYC2a interacted with NtJAZ3, Nt RBX1 and NtUBC in BiFC.

The indicated vector pair were introduced into BY-2 suspension cells by biolistic bombardment. The transformed BY-2 cells were cultured in darkness for 24 hours for gene expression, and then they are visualized under the confocal microscope. The detected fluorescence emitted from YFP in BY-2 cell is an indication of interaction. This figure suggests all of these three proteins interact with NtMYC2a. The cYFP and nYFP indicate the C-terminal and N-terminal part of YFP protein, respectively. NtJAZ3_nYFP, NtMYC2a_cYFP, RBX1 like_nYFP, and Ubiquitin conjugating enzyme_nYFP indicate vectors expressing these fusion proteins. Merge is digital merge of bright field and fluorescent images. The empty cYFP and nYFP vector pair served as a negative control.

3.1.4 NtUBC and NtRBX1 interacted with NtMYC2a

The *NtUBC* and *NtRBX1* cDNA were isolated in Y2H screening when truncated NtMYC2a was the bait. The NtUBC has 152 amino acids and the peptide sequence is identical to the predicted ubiquitin-conjugating (UBC) enzyme E2 (NCBI accession: XP_016498009.1) in tobacco. NtRBX1, with 120 amino acids, is identical to the predicted RING-box (RBX) protein 1a (NCBI accession: XP_009768569.1) in *Nicotiana sylvestris.* NtUBC and NtRBX1 were re-examined in switched vectors in Y2H. Vector NtUBC_ pGBKT7 and NtRBX1_ pGBKT7 were transformed into yeast cells respectively, along with full-length NtMYC2a_pGADT7. After growing up on DDO medium, the transformants were re-streaked on DDO/X/A and QDO/X/A subsequently. As demonstrated in Figure 3.1.4, colonies exhibited white color on DDO/X/A, indicating that NtMYC2 interacts with neither NtUBC nor NtRBX1. In BiFC assay, when the NtUBC-nYFP and NtMYC2a-cYFP vectors were co-expressed in BY-2 cells, strong fluorescence was detected in only one of the BY-2 cell (Figure 3.1.3 bottom panel). This fluorescence signal did not show up when the experiments were repeated twice more. Similarly, only one BY-2 cell exhibited fluorescence signal when NtRBX1-nYFP and NtMYC2a-cYFP were co-expressed in BY-2 cells (Figure 3.1.3 middle panel). The signal was not observed when the experiments were repeated twice more. With current data, we cannot claim with confidence that NtMYC2a interacts with NtUBC or NtRBX1. However, it seems we cannot completely exclude these two proteins at the moment, considering BY2 cells were used for BiFC assays and the introduced fusion proteins may have more opportunities to interact with the endogenous target proteins.

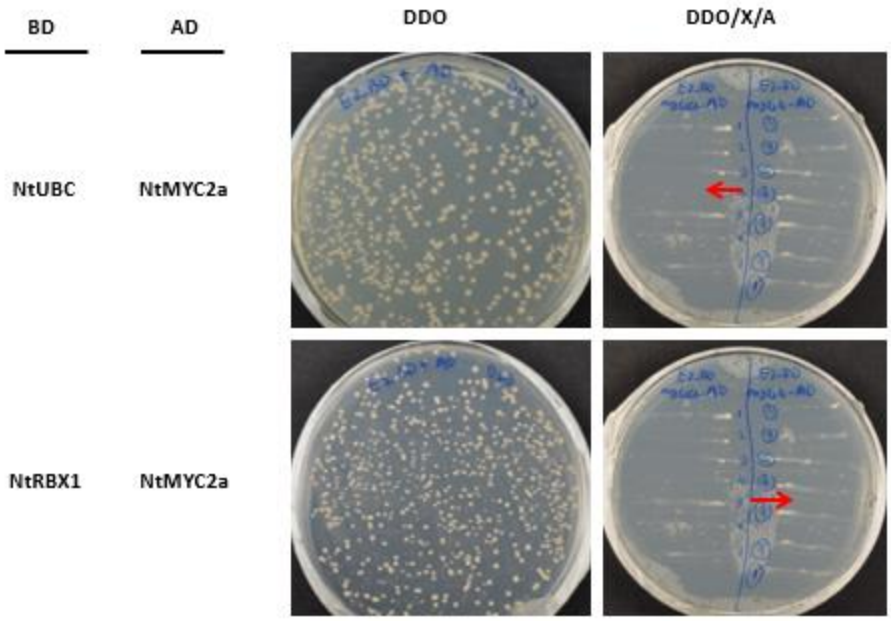

Figure 3. 1. 4 NtUBC and NtRBX1 interacted with NtMYC2a in yeast.

The plasmid pairs were co-transformed into yeast cells. Transformants were plated on DDO medium to select for both plasmids. Then the transformants were re-streaked on DDO/X/A medium to evaluate interactions. After incubation for three days, white colonies grew on the DDO/X/A plates, indicating NtMYC2 didn't interact with either NtUBC or NtRBX1. BD: prey plasmid. AD: bait plasmid. DDO: double dropout medium without tryptophan and leucine. QDO: quadruple dropout medium without tryptophan, leucine, histidine and adenine. DDO/X/A: double dropout medium supplemented with α-galactosidase and Aureobasidin A.

3.1.5 NtARF9 interacted with NtMYC2a in Y2H

A cDNA encoding for a 671 amino acid long protein was isolated in the third library screening. Peptide sequence alignment to NCBI database showed that this deduced peptide is 99% identical to an auxin response factor 9 (ARF9) isoform in *Nicotiana tomentosiformis* (XP_009590537.1), so that this protein is designated as NtARF9. *NtARF9* was re-cloned into pGBKT7 and co-transformed with full-length NtMYC2a_pGADT7 in yeast cells. Transformants were plated on DDO medium to select for both constructs. Then the transformants were re-streaked on DDO/X/A and QDO/X/A subsequently for evaluating the interaction. As shown in Figure 3.1.5, transformants exhibited blue color on DDO/X/A but no growth on QDO/X/A, indicating a moderate interaction between NtARF9 and NtMYC2a. Yeast cells harboring empty pGBKT7 and NtMYC2a_pGADT7, NtARF9_pGBKT7 and empty pGADT7 served as negative control. A BiFC test was performed using another pair of YFP vectors, which gives strong fluorescence background when empty vectors were used (data not shown). Thus, although we observed fluorescence in this test, we were not sure where it is background or caused by AFP9 interaction with MYC2a.

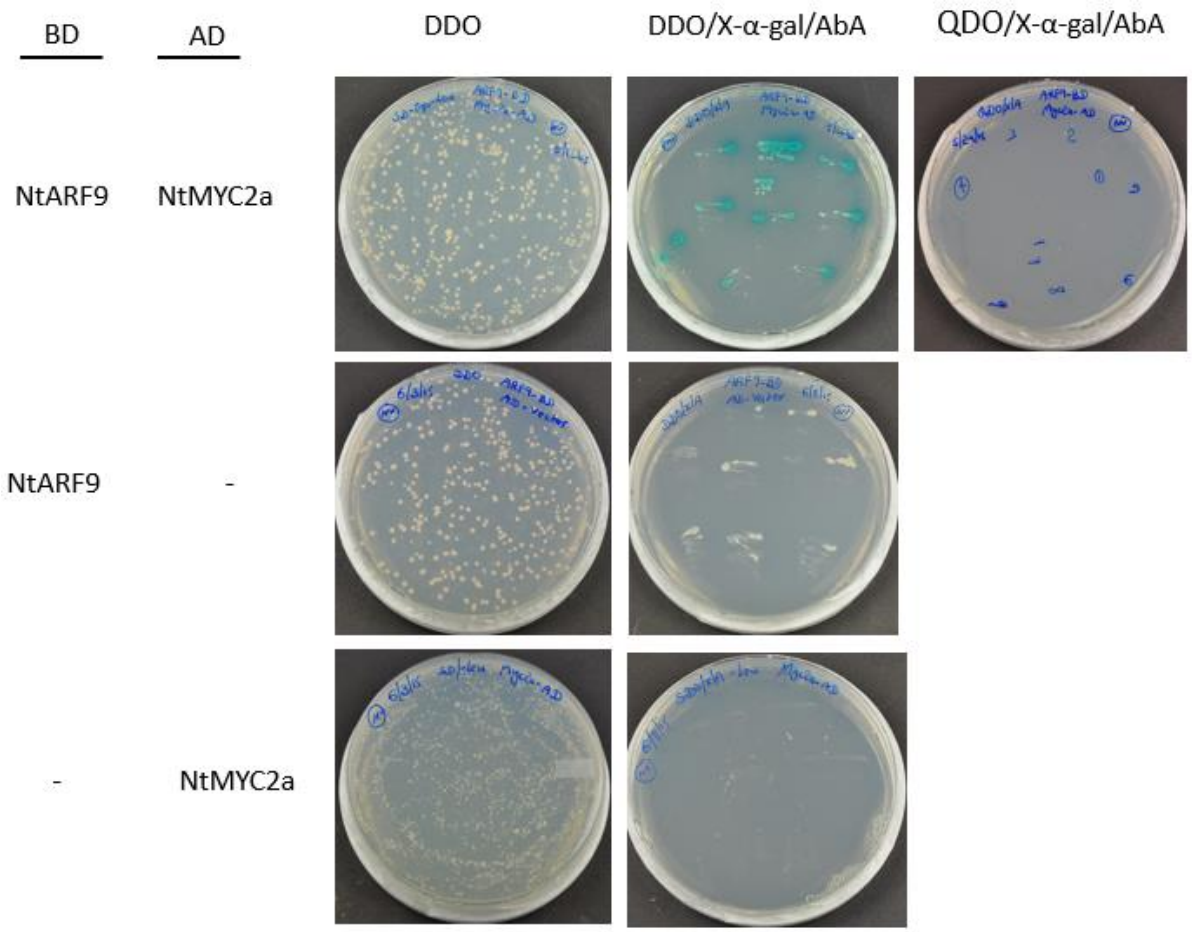

Figure 3. 1. 5 NtARF9 interacted with NtMYC2a in yeast.

NtARF9_pGBKT7 and NtMYC2a_pGADT7 were co-transformed into yeast cells. Transformants were plated on DDO medium to select for both constructs. Then the transformants were re-streaked on DDO/X/A and QDO/X/A subsequently to evaluate the interactions. Transformants exhibited blue color on DDO/X/A but no growth on QDO/X/A, indicating a moderate interaction between NtJAZ3 and NtMYC2a (up panel). Yeast cells harboring NtARF9_pGBKT7 and empty pGADT7served serves negative control (middle panel). Yeast cells harboring empty pGBKT7 and NtMYC2a_pGADT7 is another negative control (bottom panel).

3.1.6 Summary of results of chapter 2

Nine candidate proteins NtJAZ3, NtUBC, NtRBX-1, NtRNF185, NtARF9, NtbZIP-Like, NtSTPK, NtSNF-1, NtEPL were isolated as interactors of NtMYC2a in the Y2H library screening. The interactions were re-examined in switched vectors in Y2H and BiFC in tobacco BY2 cells. NtJAZ3 passed both examinations (Figure 3.1.2, 3.1.3). NtUBC and NtRBX1 did not interact with NtMYC2a in Y2H assay when vectors were switched, however, they interacted with NtMYC2a in BiFC (Figure 3.1.3, 3.4). NtARF9 interacted with NtMYC2a in Y2H (Figure 3.1.5), however, the interaction was inconclusive in BiFC and would need to be redone with the pair of BiFC vectors without background. NtSTPK did not interact with NtMYC2a in both examinations (Figures were not shown). The other four proteins NtRNF185, NtbZIP-Like, NtSNF-1, and NtEPL didn't interact with NtMYC2a in BiFC assays in BY2 cells but seem to have weak interactions with NtMYC2 in Y2H, as indicated by the yeast growth on moderate stringency medium DDO/X-α-gal/AbA (Figures were not shown). In summary, we demonstrated that NtJAZ3 interacted with NtMYC2a. Other candidate proteins seem to have ambiguous interaction with NtMYC2a and further validations are needed.

Chapter 2: Overexpression of JA Biosynthesis Genes and Knockdown of a JA Catabolism Gene

3.2.1 Overview

Since we do not know which enzyme controls the rate-limiting step of JA biosynthesis, we intended to investigate all the major genes. However, none of the JA biosynthetic genes were functionally characterized in tobacco and the gene sequences are not available in the database at the time when we started this project. Thus, the cDNA clones from Arabidopsis, *LOX2* (*13-lipoxygenase 2), AOS* (*allene oxide synthase*), *AOC2* (*allene oxide cyclase 2*), *OPR3* (*OPDA reductase 3)*, and *JAR1* (*JA-amino acid synthetase 1*), were individually introduced into tobacco genome and were constitutively overexpressed under the CaMV 35S promoter. In addition, RNAi-mediated gene silencing was used to knock down *NtJIH1*, which hydrolyzes JA-Ile, with an intention to reduce degradation of JA-Ile. Sequences of the two *NtJIH1* cDNAs are available in the database (Figure A2). The transgenic plants were developed by agrobacterium-mediated leaf disc transformation. Transgenic plants were grown in the growth chamber until roots were developed and then were transplanted into the greenhouse. All the transgenic plants looked phenotypically normal in terms of height, leaf numbers, and flowering time.

During the pre-flowering stage (approximately 2 months after transplanting), plants were topped (decapitation of the apical meristem) as a routine practice to stimulate nicotine accumulation. At T_0 generation, before topping, the third fully expanded leaf from the top was collected for transgene expression analysis. Seven days after topping, twelve leaves from the top were collected for total alkaloids measurement. We measured total alkaloids content

rather than nicotine content because the GC-MS facility at the Tobacco Analytical Chemistry Laboratory for nicotine analysis was not available at the time. Since more than 90% of the total alkaloids are composed by nicotine, total alkaloid contents are largely positively correlated with the nicotine content and total alkaloid content can be used as a good indicator of nicotine content. The transgenic plants overexpressing *AOS* gene and the plants expressing *JIH1RNAi* constructs showed lower nicotine content than the vector control at T_0 generation. Thus, they were not further investigated in the next generation. The rest four genes showed positive effects on total alkaloid accumulation at T_0 generation and they were further analyzed in the T1 generation.

Topping triggered sucker growth, flowers on suckers were self-pollinated and seeds were harvested from the suckers. Seeds of two transgenic plants of each gene construct with higher transgene transcript levels and nicotine levels were germinated. Due to limited space in the greenhouse, twelve PCR positive T_1 plants of each transgenic line were grown. In the T_1 generation, six of the twelve plants were subjected to topping treatment at the pre-flowering stage and the remaining six plants were “non-topped”. Root tissues were collected on three topped plants three hours after topping. Meanwhile, root tissues were also collected from three “non-topped” plants. For jasmonates quantification, leaf tissues were collected on the third fully expanded leaves from three topped plants three hours after topping, and from three “non-topped” plants. All the remaining leaves from these plants were collected for alkaloids analysis seven days later.

Jasmonic acid and its major derivatives including OPDA, MeJA, JA-Ile, and JA content were determined by HPLC-MS. However, the data points were randomly scattered without a comprehensible pattern, reflecting a high degree of variations of JAs among individual plants, and probably our inexperience on JA extraction. A few examples are presented in Appendix Figures A5 to A8. To help solve this problem, we developed an alternative method to qualitatively determine JA content in an indirect way, by measuring root growth during seed germination period, based on the knowledge that primary root growth is inhibited by higher jasmonate levels in Arabidopsis (Acosta, et al., 2013), *Nicotiana attenuata* (Hummel, et al., 2007) and tobacco (Shoji, et al., 2008). This assay was used for isolation of JA-insensitive *opr3* mutants (Stintzi and Browse, 2000), JA-hypersensitive *ninja* mutant (Acosta, et al., 2013) and *myc2-322B* gain of function mutant (Gasperini, et al., 2015).

Two transgenic lines, one from *AOC2* construct, and the other from *JAR1* construct, still had relatively higher transgene expression and nicotine levels in the T_1 generation tests. Considering the very limited number of samples collected from these experiments, which may have skewed the statistical analysis results, we used a small-scale, completely randomized block design to grow more PCR-positive T_1 plants in the greenhouse, mimic to the field test, for more accurate analysis. In this experimental design, each transgenic line and WT line has four blocks, and each block includes four plants. Plant leaves from each block were harvested and mixed together for nicotine determination as a replicate.

3.2.2. Overexpression of *LOX2* in tobacco plants

LOX (13-lipoxygenase) catalyzes the oxidation of α-linolenic acid, which is the first step of JA biosynthesis. It is suggested that LOX2 was responsible for the bulk production of JA during 1-4 hours post wounding (Bell, et al., 1995; Glauser, et al., 2009), therefore, we chose to overexpress *LOX2* rather other isoforms. Eight *LOX2* overexpression plants were regenerated through *Agrobacterium*-mediated leaf disc transformation. At T_0 generation, RNA was isolated from the third fully expanded leaf collected before topping and the qRT-PCR was performed to quantify transgene expression levels. The qRT-PCR result showed that the vector control plant didn't have detectable *AtLOX2* transcript level but all of the transgenic plants have detectable *AtLOX2* transcript levels. As shown in Figure 3.2.1 where the *AtLOX2* transcript level of the LOX2-4*2* plant was arbitrarily set at 1, the plant LOX2-3 and plant LOX2-33 had higher *AtLOX2* transcript level than the rest transgenic plants. Seven days after topping, twelve leaves were collected for total alkaloids measurement. Six transgenic plants had higher total alkaloid content as compared to vector control and the plant LOX2-3 and plant LOX2-33 had higher total alkaloid content that the other transgenic plants (Figure 3.2.2). The total alkaloid increase ranges from 8% to 47%.

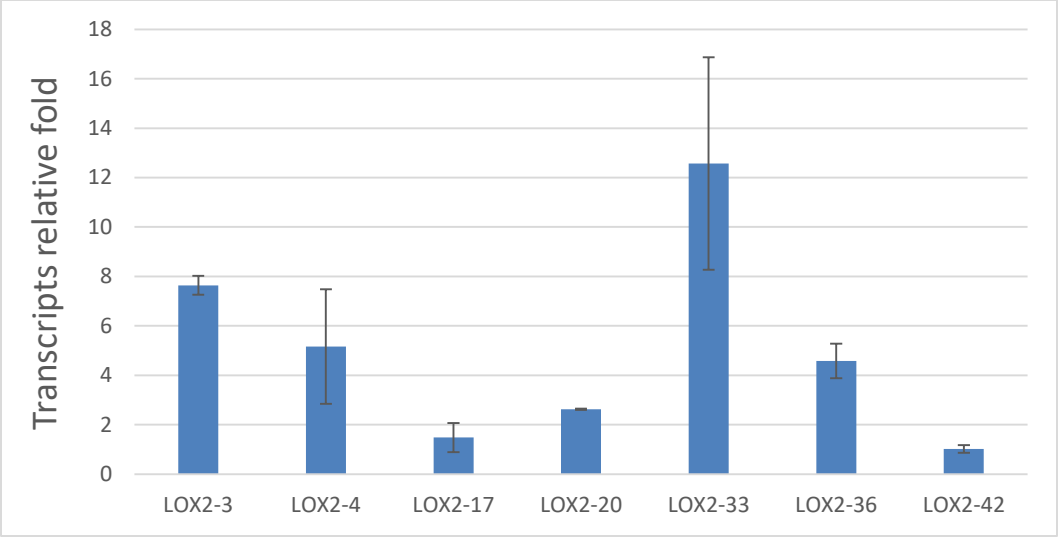

Figure 3. 2. 1 Relative *AtLOX2* transcript levels in LOX2 T_0 plants.

RNA was isolated from leaf tissues collected before topping. Transcript levels were determined by qRT-PCR. Values are means from 3 technical replicates. Error bar on the column represents standard error. *AtLOX2* transcript levels were normalized to *actin* and displayed relative to the *AtLOX2* level of the LOX2-4*2* plant which is arbitrarily set at 1.

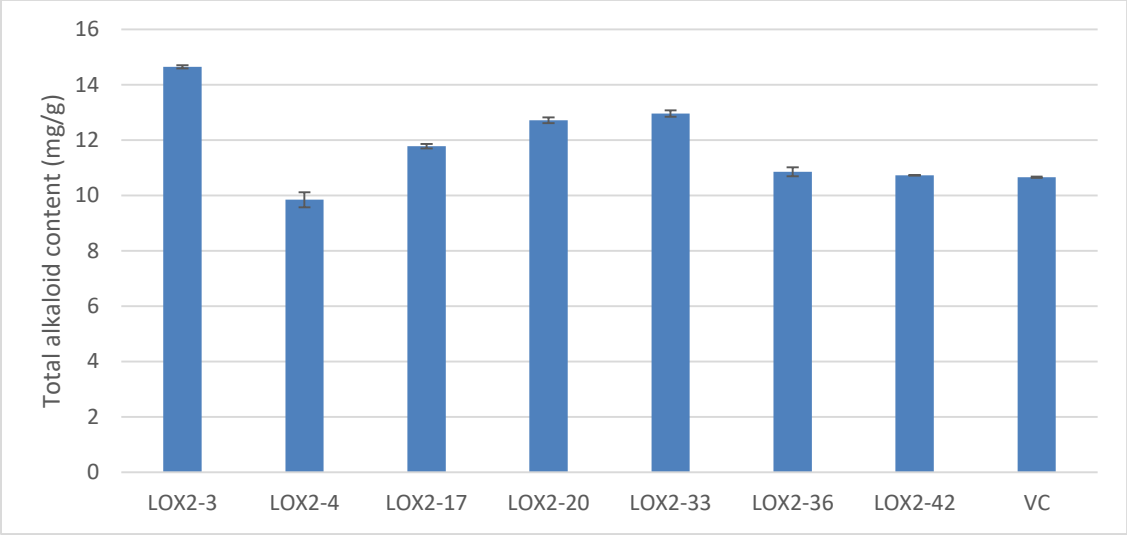

Figure 3. 2. 2 Total alkaloids levels of LOX2 T_0 plants.

The top twelve leaves were collected seven days after topping, dried and ground. 250 mg leaf tissues were used for total alkaloids extraction and quantification as described in the material and method section. Each sample was measured three times. The values shown are the average of three technical replicates. Error bar stands for standard error.

To further investigate whether the observed elevated nicotine levels in T_0 generation is statistically significant, two LOX2 overexpression plants (LOX2-20 and LOX2-33) with higher total alkaloid levels and transgene expression levels were self-pollinated and investigated in the next generation (T_1). Twelve T_1 LOX2 transgenic plants (PCR positive) of LOX2_20 and LOX2_33 lines were grown in the greenhouse for approximately two months. Root tissues were collected three hours after topping for nicotine biosynthetic genes analysis. Tissues were collected from the third leaf 1.5 hours post topping. Seven days after topping, the twelve leaves were collected for nicotine level quantification.

In absence of topping, *NtPMT1*, *NtQPT2*, *NtMYC2a*, *NtBBL* and *NtA622* transcript levels in *LOX2-20* T_1 line were comparable to, or slightly lower than that in vector control. In LOX2-33 line, however, the *NtPMT1*, *NtQPT2*, and *NtMYC2a* transcripts were doubled as compared to the vector control (Figure 3.2.3, left). With topping, the topping induced slightly higher *NtPMT1*, *NtQPT2*, and *A622* transcript levels in both transgenic lines than that in the vector control (Figure 3.2.3, right). The means of nicotine content of the two transgenic lines are slightly higher than that of the treatment-matched vector control (Figure 3.2.4), however, the difference is not statistically significant (ANOVA Dunnett comparison; α=0.05).

As mentioned previously, JA data (Figure A1) were not reliable. Based on the above results, we conclude that overexpression of *LOX2* slightly enhances nicotine biosynthesis pathway gene expression, but has little effect on nicotine content.

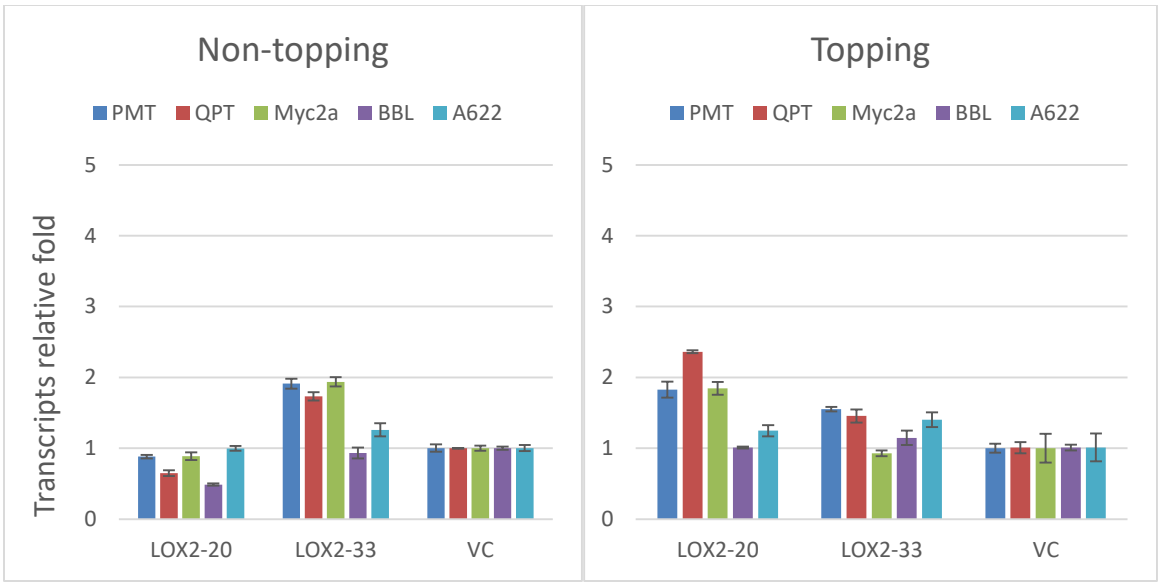

Figure 3. 2. 3 Transcript levels of nicotine synthetic genes in LOX2 T_1 lines.

Root tissues were collected 3 hours post topping. RNA were isolated from there replicate plants and were mixed. Transcripts were detected by qRT-PCR with non-topping (left) and topping (right) treatments. Values are average of 3 technical replicates. Error bar on the column represents standard error. Transcript levels were normalized to *actin* and displayed relative to the corresponding gene transcript levels of the vector control which were arbitrarily set at1. VC: vector control.

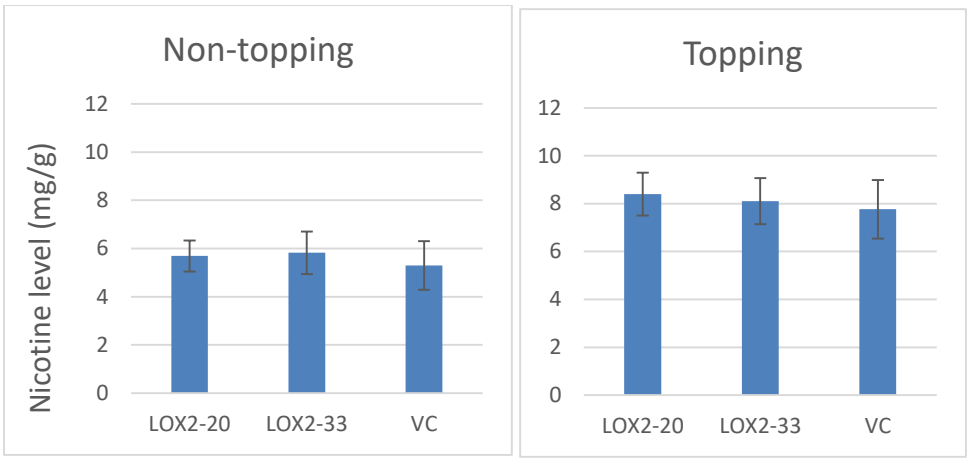

Figure 3. 2. 4 Nicotine content in two LOX2 T_1 lines.

Twelve leaves were collected from tobacco plants seven days after topping. The leaf tissues were dried and ground. 200 mg leaf tissue were used for nicotine quantification by GC-MS. The three minor alkaloids were measured at the same run. Non-topping treatment is shown on the left and topping treatment is on the right. Values shown are means of 3 replicate plants. Error bar represents standard error. ANOVA with Dunnett's test (α=0.05) was performed and no significant difference was observed. VC: vector control.

3.2.3 Overexpression of *AOS* in tobacco plants

AOS is the first dedicated enzyme in JA biosynthesis pathway. Arabidopsis has only one *AOS* gene in its genome (Kubigsteltig, et al., 1999). Six *AOS* overexpression T_0 plants were regenerated. At T_0 generation, before topping, the third leaf was collected for transgene transcript quantification. Seven days after topping, twelve leaves were collected for nicotine analysis. Although *AtAOS* transcript levels were detected by qRT-PCR (Figure 3.2.5) in transgenic plants, the nicotine content of transgenic plants was similar to, or even slightly lower than that of WT (Figure 3.2.6), suggesting overexpression of *AOS* had little impact on nicotine biosynthesis. Therefore, AOS transgenic plants were not examined in the next generation.

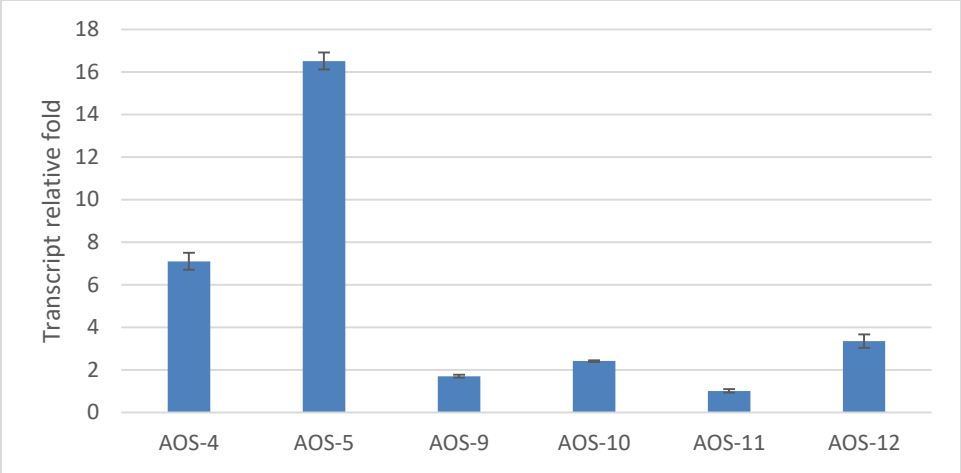

Figure 3. 2. 5 Relative *AtAOS* transcript levels in AOS T_0 plants.

RNA was isolated from tobacco leaves collected before topping. Transcript level was determined by qRT-PCR. Values shown are means of 3 technical replicates. Error bar on the column represents standard error. Transcript levels were normalized to *actin* and displayed relative to the *AtAOS* transcript level of AOS-11 plant which is arbitrarily set at1.

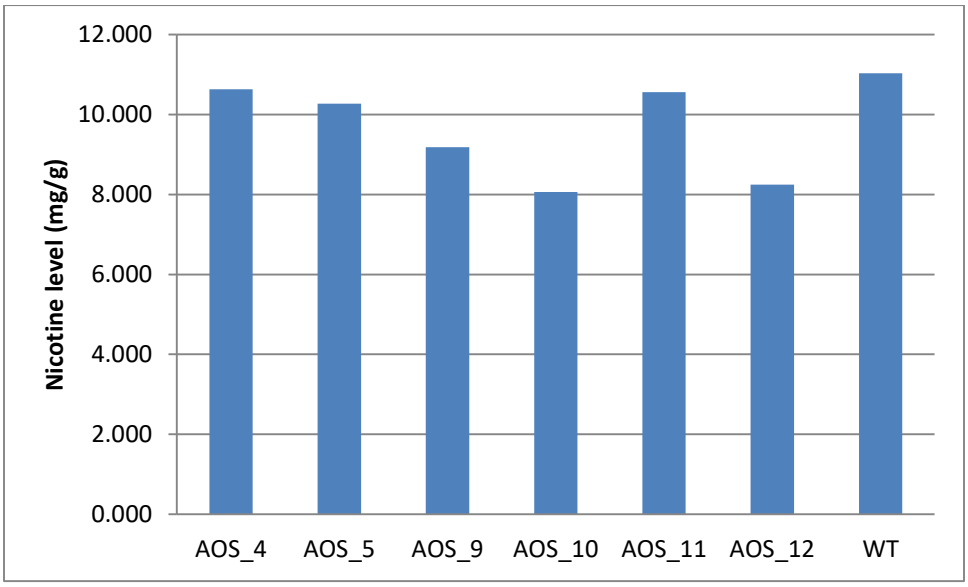

Figure 3. 2. 6 Nicotine levels of AOS T_0 plants.

Seven days after topping, the top twelve leaves were collected, dried and ground. Total alkaloids were extracted from approximately 200 mg leaf tissues and individual alkaloid were quantified by HPLC-MS as described in the material and method section. The columns represent values of nicotine content. WT: wild type NC95.

3.2.4 Overexpression of *AOC2* in tobacco plants

AOC (allene oxide cyclase) catalyzes the formation of OPDA. Four *AOC* isoforms were identified in Arabidopsis with AOC2 abundantly expressed in leaves and roots (Stenzel, et al., 2003). Eleven *AOC2* overexpression T_0 plants were regenerated and grown in the greenhouse for approximately two months. As shown in Figure 3.2.7, all of the transgenic plants had detectable *AtAOC2* transcripts (Figure 3.2.7) whereas the vector control plants showed non-detectable *AtAOC2* transcripts (data not shown). Six transgenic plants had obviously higher total alkaloids contents as compared to vector control (Figure 3.2.8). The AOC2_49 line and AOC2_50 line, with higher alkaloid level and transgene transcript level, were chosen for further analysis at T_1 generation.

Twelve PCR-positive T_1 AOC2 transgenic plants of AOC2_49 line and AOC2_50 line were grown in the greenhouse for approximately two months. The transcript levels of nicotine synthetic genes, nicotine levels, and JA levels were determined. The basal (non-topping) levels of *NtPMT1a, NtQPT2, NtMyc2a, NtBBL* and *NtA622* transcript in these transgenic plants were similar to vector control (Figure 3.2.9, left). Transcripts of all these five key genes were induced higher by topping with *NtPMT1*a being increased by 4-fold as compared to vector control (3.2.9, right). The means of nicotine content in the AOC2_49 and AOC2_50 transgenic plants were 10-20% higher than the mean of nicotine content in the treatment-matched vector control (Figure 3.2.10). However, the difference was not statistically significant (ANOVA with Dunnett comparison; $\alpha = 0.05$).

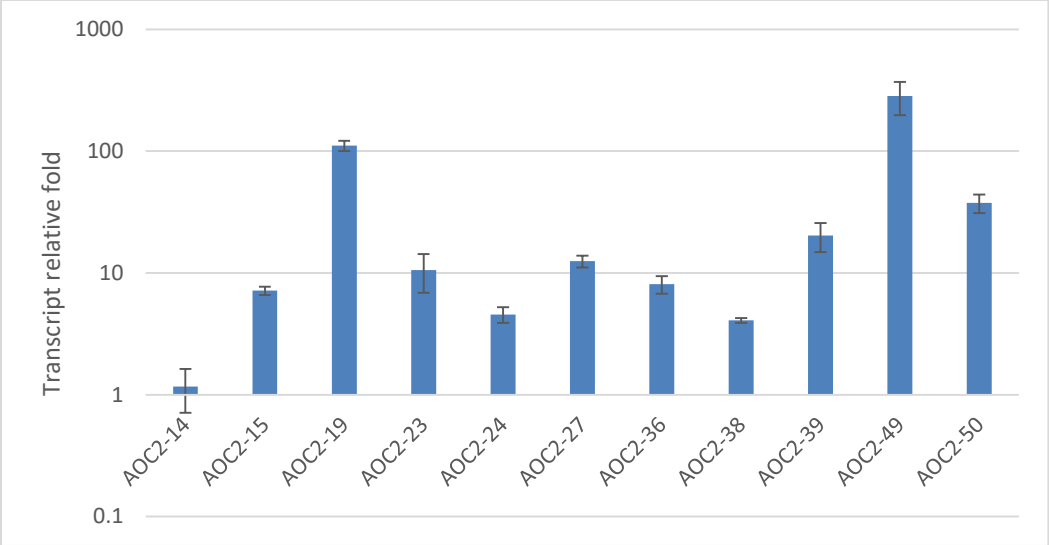

Figure 3. 2. 7 Relative *AtAOC2* transcript levels in AOC2 T_0 plants.

RNA was isolated from tobacco leaves before topping. Transcript levels were detected by qRT-PCR. Values shown are means from 3 technical replicates. Error bar on the column represents standard error. *AtAOC2* transcript levels were normalized to *actin* and displayed relative to the *AtAOC2* transcript level of AOC2-14 plant, which is arbitrarily set at 1.

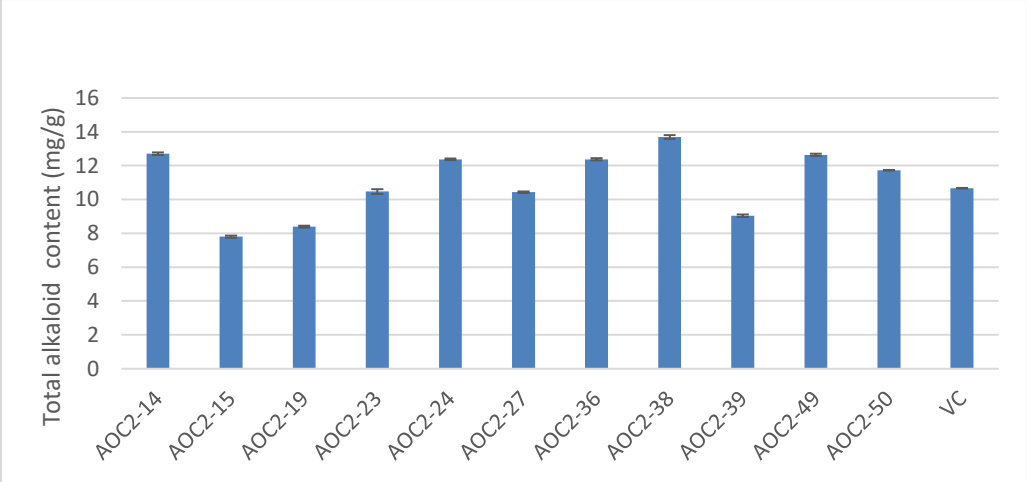

Figure 3. 2. 8 Total alkaloids levels of AOC2 T_0 plants.

Seven days after topping, the top twelve leaves were collected, dried and ground. Total alkaloids were extracted from approximately 250 mg leaf tissues and were quantified as described in the material and method section. The quantification was repeated three times. The values shown are average of three technical replicates. Error bar stands for standard error.

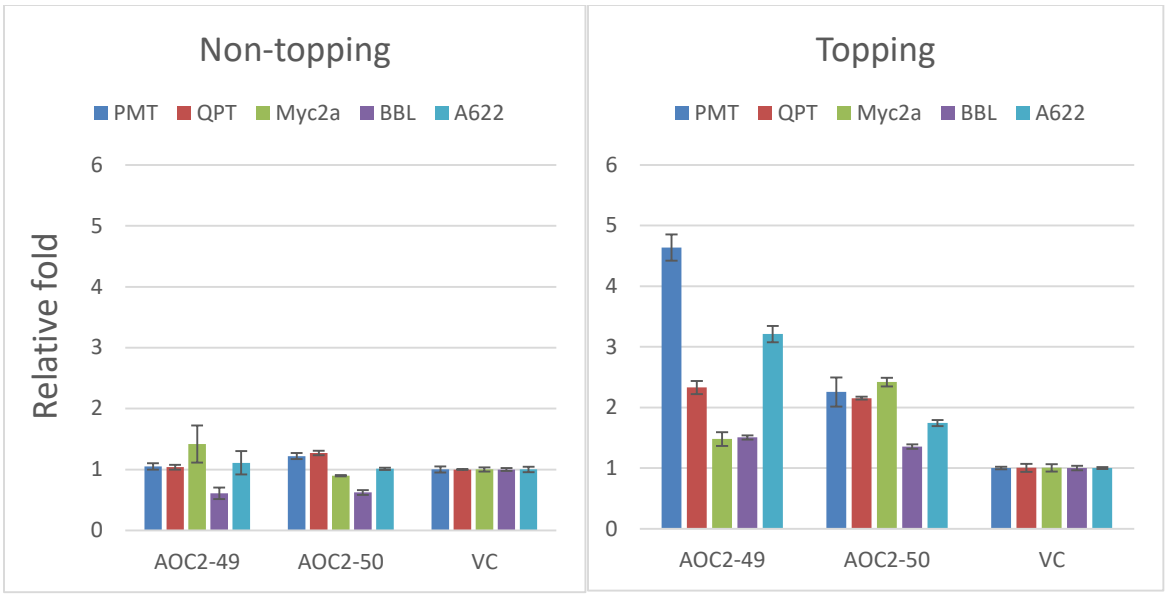

Figure 3. 2. 9 Transcript levels of Nicotine synthetic genes in AOC T_1 lines.

RNA was isolated from root tissues collected 3 hours after topping. The RNA of three plants were mixed. Transcripts were detected by qRT-PCR with non-topping (left) and topping treatment (right). Transcript levels were normalized to *actin* and displayed relative to the corresponding gene transcript levels of vector control that was arbitrarily set at 1. Values shown are mean of 3 technical replicates. Error bar on the column represents standard error.

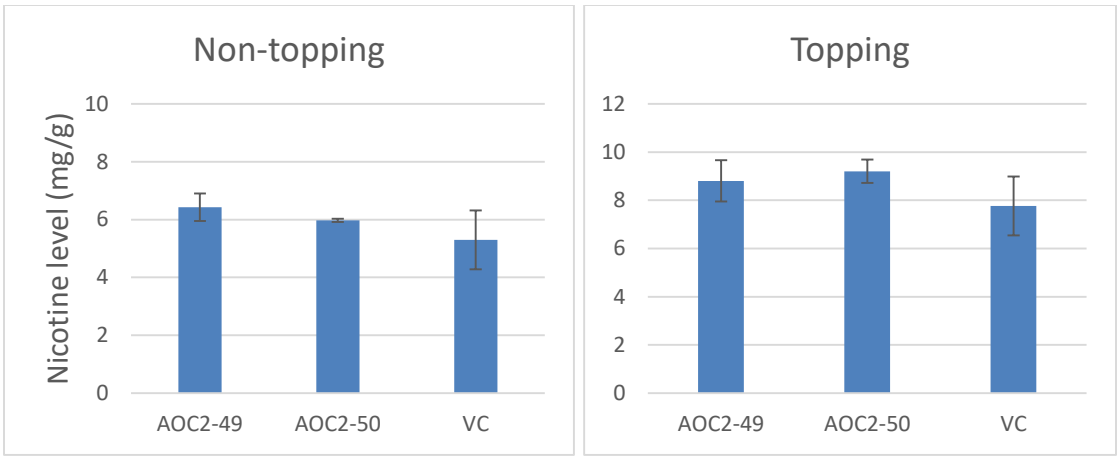

Figure 3. 2. 10 Nicotine content levels of two AOC2 T_1 plants.

Twelve leaves were collected from tobacco plants seven days after topping. The leaf tissues were dried and ground. 200 mg leaf tissue were used for nicotine quantification as described in material and method. The three minor alkaloids were measured at the same run. Topping treatment results are shown on the right and non-topping left. Values shown are means of 3 replicate plants. Error bar represents standard error. ANOVA with Dunnett's test (α=0.05) revealed no significant difference.

3.2.5 Overexpression of *OPR3* in tobacco plants

Although there are six *OPR* genes in Arabidopsis genome, only *OPR3* is involved in JA biosynthesis (Breithaupt, et al., 2006). Nine transgenic plants overexpressing *OPR3* were regenerated. All of them had a detectable *OPR3* transcript (Figure 3.2.11), and four had total alkaloid content increase in T_0 generation, ranging from 14% to 43% as compared to vector control (Figure 3.2.12). Two transgenic line, the OPR3-21 and OPR3-40, with highest alkaloid levels and highest transgene expression levels, were chosen to be further studied at T_1 generation.

Twelve PCR-positive T_1 plants from OPR3_21 and OPR3_40 line were grown in the greenhouse for transgene, nicotine pathway genes, nicotine level and JA level analysis. Without topping, in both *OPR3_21* and *OPR3_40* lines, *NtPMT1a* and *NtQPT2* transcript levels were doubled that in vector control (Figure 3.2.13, left). In OPR3_21 line, *NtBBL* and *NtA622* transcript levels have nearly 1.5 fold increase as compared to vector control. Topping further induced *NtPMT1*, *NtQPT2*, and *NtA622* transcripts to 2.5 to 3.5 fold of that in vector control (Figure 3.2.14, right). The mean of nicotine content in transgenic plants is similar to that of the treatment-matched vector control from a statistical point of view (Figure 3.2.14). Samples of OPR3-40 after topping was lost. Root length inhibition assay revealed that primary root length of the OPR3-21 and OPR3-40 line was similar to that of vector control (data not shown), indicating JA level may not substantially increase in these two lines. Based on these data, we conclude that overexpression of *OPR3* generally enhanced nicotine pathway gene transcript levels, but had little effect on nicotine accumulation.

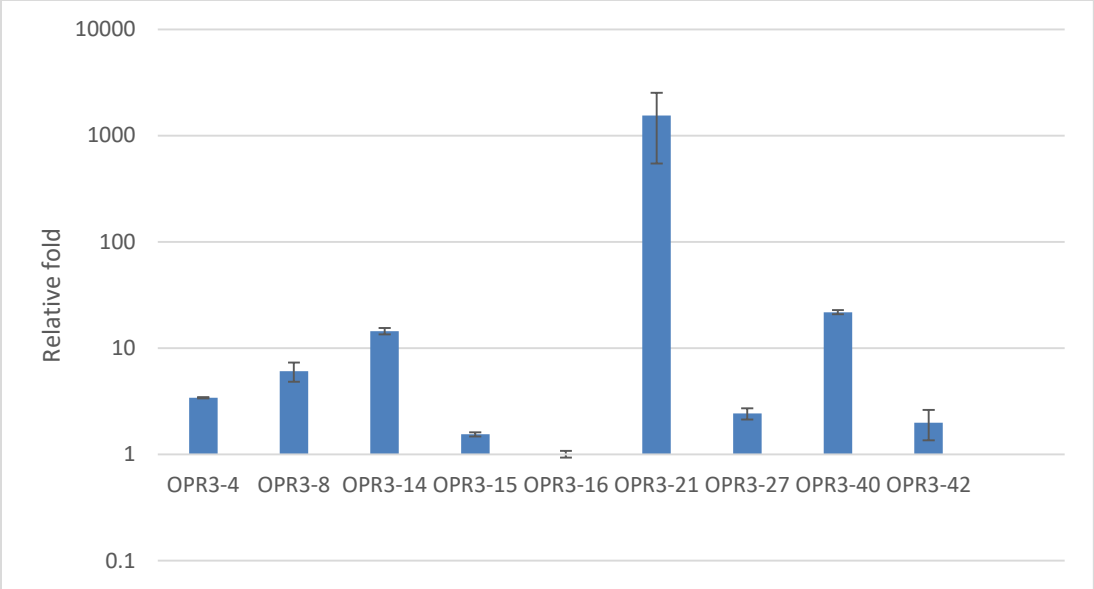

Figure 3. 2. 11 Relative *AtOPR3* transcript levels in OPR3 T_0 plants.

RNA was isolated from the third leaf collected before topping. *AtOPR3* transcripts level were detected by qRT-PCR. Values shown are means from 3 technical replicates. Error bar on the column represents standard error. Transcript levels were normalized to *actin* and displayed relative to *AtOPR3* transcript level of OPR3-16 plant which is arbitrarily set at1.

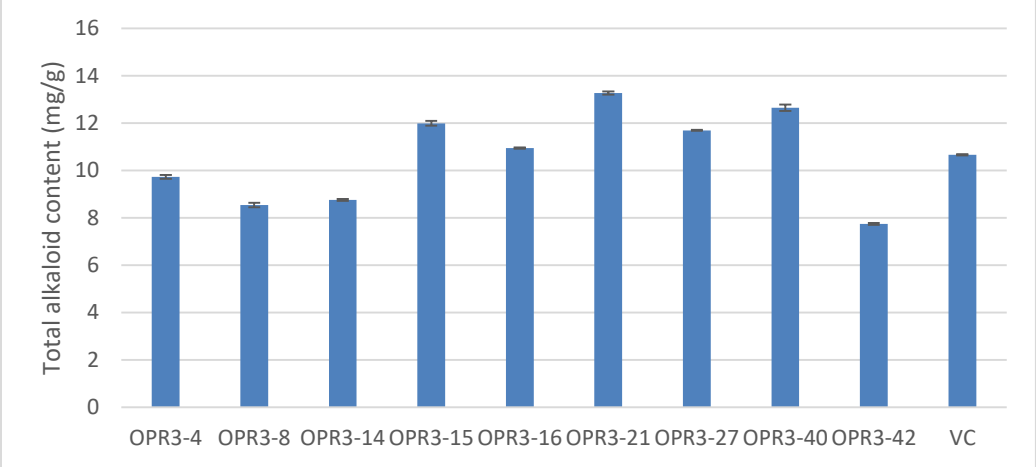

Figure 3. 2. 12 Total alkaloids levels of OPR3 T_0 plants.

The top twelve leaves were collected, dried and ground. Total alkaloids were extracted from approximately 250 mg leaf tissues and were measured as described in the material and method section. Each sample were measured three times. The values shown are average of three technical repeats. The error bar stands for standard error.

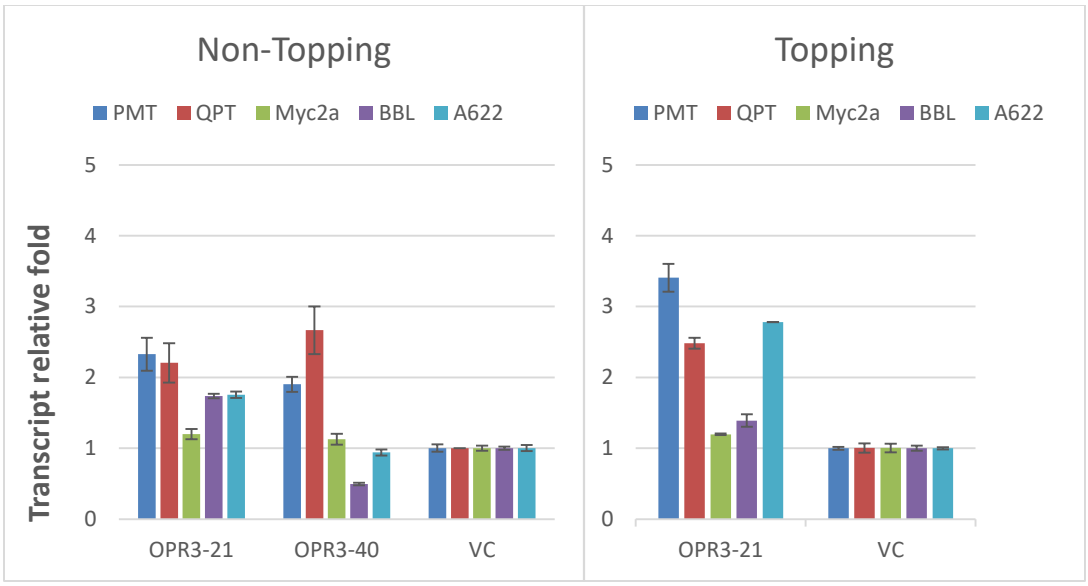

Figure 3. 2. 13 Nicotine pathway gene expression levels in OPR3 T_1 lines.

Root tissues were collected 3 hours post topping. RNA were isolated from there replicate plants and were mixed. Transcripts were detected by qRT-PCR with non-topping (left) and topping (right) treatments. Values are average of 3 technical replicates. Error bar on the column represents standard error. Transcript levels were normalized to *actin* and displayed relative to the corresponding gene transcript levels of the vector control which were arbitrarily set at1. Samples of the topped OPR3-40 was lost.

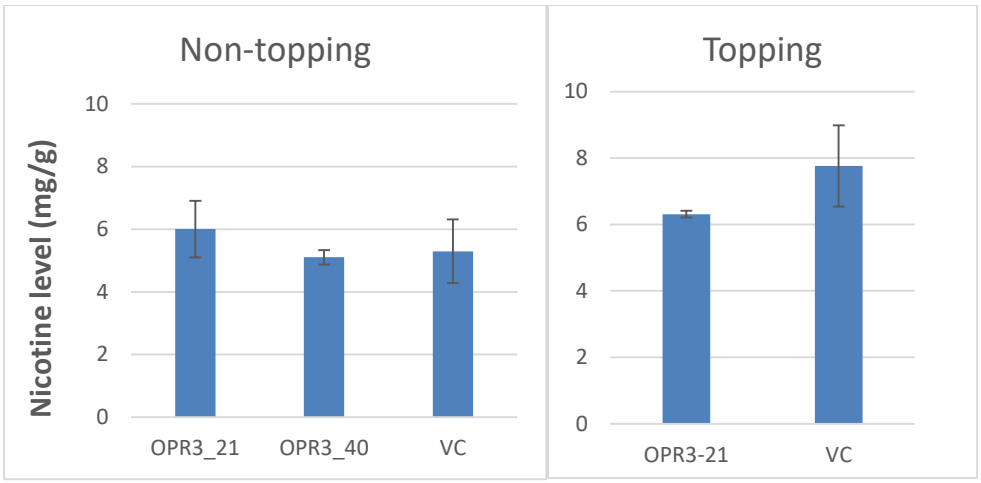

Figure 3. 2. 14 Nicotine content levels in OPR3 T_1 lines.

Twelve leaves were collected from tobacco plants seven days after topping. The leaf tissues were dried and ground. 200 mg leaf tissue were used for nicotine quantification as described in material and method. The three minor alkaloids were measured at the same run. Topping treatment is shown on the right and non-topping left. Values shown are means of 3 replicate plants. Error bar represents standard error. ANOVA with Dunnett's test (α=0.05) suggests no significant difference. Note: Topped OPR3-40 sample was lost.

3.2.6 Overexpression of *JAR1* in tobacco plants

JAR encodes the enzyme JA-amino acid synthetase and catalyzes the formation of JA-amino acid conjugates, including the bioactive JA-Ile (Staswick and Tiryaki, 2004; Suza and Staswick, 2008). Six *JAR1* overexpression plants were regenerated. All of the T_0 plants expressed the *JAR1* transgene detected at the transcript level (Figure 3.2.15). Five of them had increased total alkaloid content as compared to vector control (Figure 3.2.16). For example, JAR1-20 had 70% higher total alkaloids content than vector control. Two *JAR1* overexpression plants (*JAR1_20 and JAR1_60*) with higher total alkaloids level and transgene expression level were chosen for analysis at T_1 generation. Twelve each T_1 PCR positive plants of JAR1-20 and JAR1-60 lines were grown in the greenhouse. Transgene expression, nicotine pathway gene expression, nicotine level and JA level were analyzed in T_1 generation.

In T_1 generation, without topping, the basal level of *NtPMT1*, *NtQPT2*, *NtBBL* and *NtA622* transcript didn't change much as compared to vector control, only with MYC2a being somewhat higher (Figure 3.2.17, left). Some, but not all the nicotine pathway genes were induced more than doubled the control levels by topping (Figure 3.2.17, right). However, no significant changes in the nicotine levels were detected between the two transgenic lines and the vector control (Figure 3.2.18). Primary root length was not reduced in JAR1-20 and JAR1-60 seedlings, as compared to vector control (data not shown), indicating JA level may not substantially increase in these two lines. Collectively, overexpression of *JAR1* generally enhanced nicotine pathway gene expression but did not significantly change nicotine level.

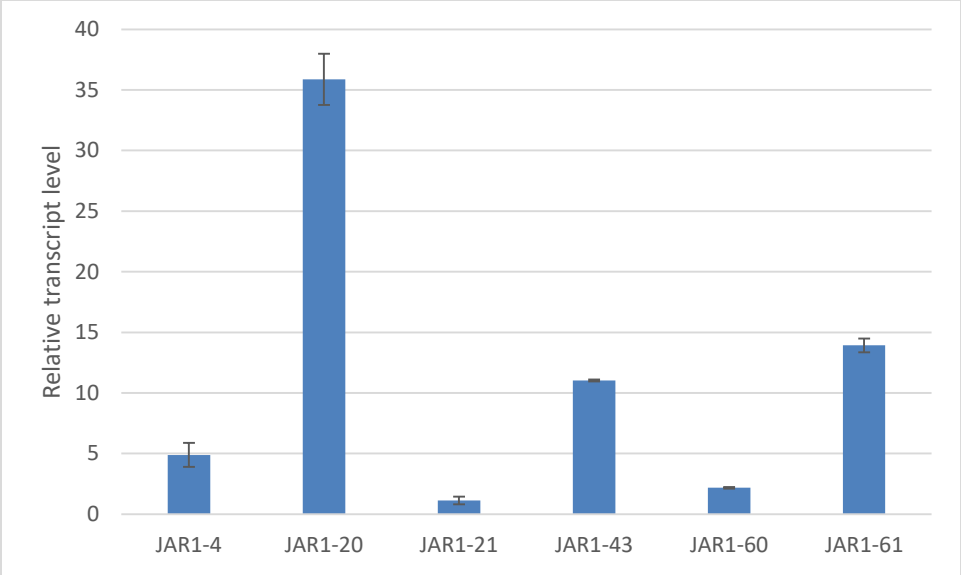

Figure 3. 2. 15 Relative *AtJAR1* transcript levels in JAR1 T_0 plants.

RNA was isolated from the third leaf collected before topping. *AtJAR1* transcript levels were detected by qRT-PCR. Values shown are means from 3 technical replicates. Error bar on the column represents standard error. Transcript levels were normalized to *actin* and displayed relative to the *AtJAR1* transcript level of *JAR1-21* plant which was arbitrarily set at 1.

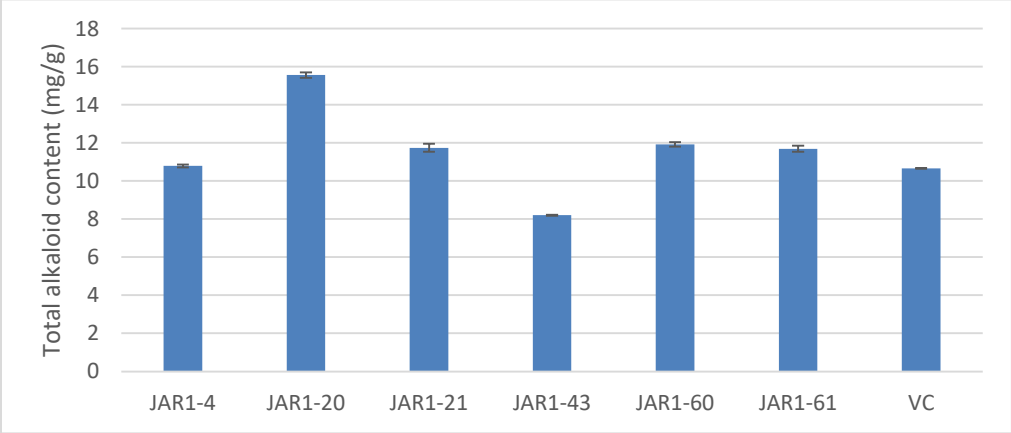

Figure 3. 2. 16 Total alkaloids levels of JAR1 T_0 plants.

Seven days after topping, the top twelve leaves were collected, dried and ground. Total alkaloids were extracted from approximately 250 mg leaf tissues and were quantified as described in the material and method section. The quantification of each sample was repeated three times. The values shown are the means of three technical replicates. The error bar stands for the standard error.

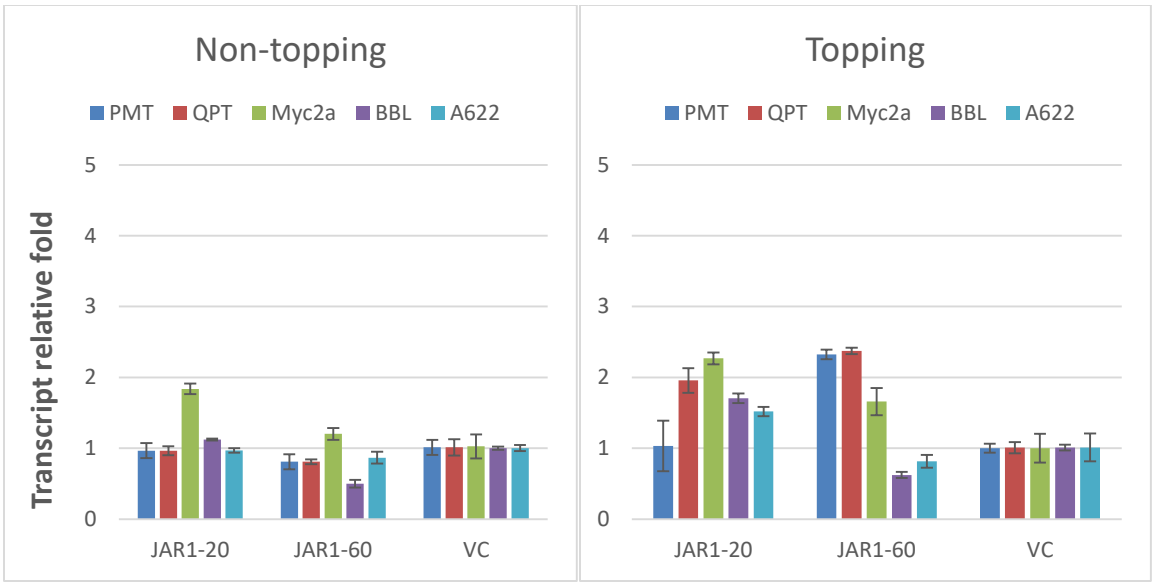

Figure 3. 2. 17 Nicotine pathway gene expression levels in JAR1 T_1 lines.

Root tissues were collected 3 hours post topping. RNA were isolated from there replicate plants and were mixed. Transcripts were detected by qRT-PCR with non-topping (left) and topping (right) treatments. Values are average of 3 technical replicates. Error bar on the column represents standard error. Transcript levels were normalized to *actin* and displayed relative to the corresponding gene transcript levels of the vector control which were arbitrarily set at1.

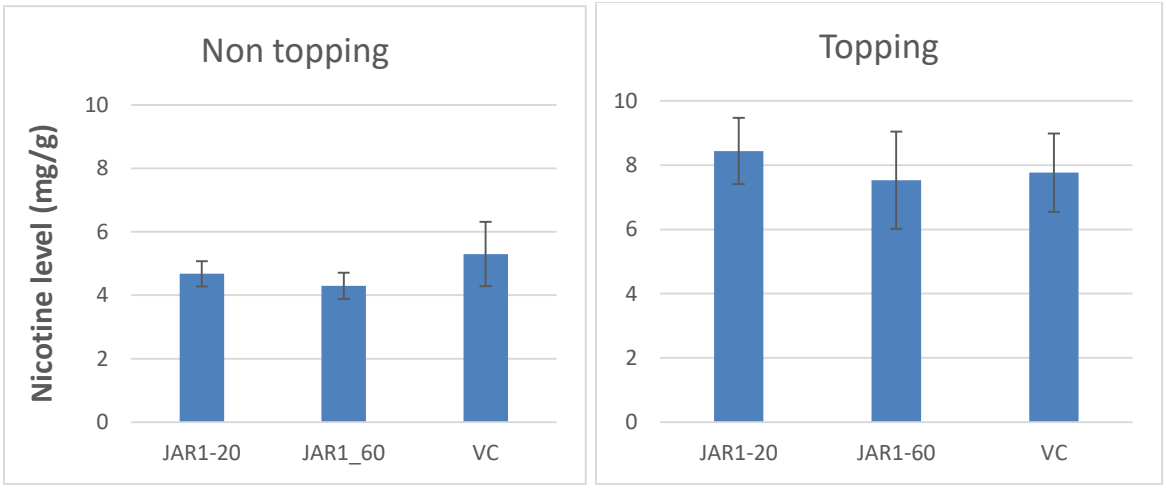

Figure 3. 2. 18 Nicotine content levels of JAR1 T_1 lines.

Twelve leaves were collected from tobacco plants seven days after topping. The leaf tissues were dried and ground. 200 mg leaf tissue were used for nicotine quantification as described in material and method. The three minor alkaloids were measured at the same run. Topping treatment is shown on the right, no topping treatment on the left. Data shown are means of 3 biology replicates. Error bar on the column represents standard error. ANOVA with Dunnett's test ($P<0.05$) suggested no significant difference.

3.2.7 Repression of *JIH1* in tobacco plants by RNAi

JIH1 encodes jasmonate-isoleucine hydrolase 1, which hydrolyzes JA-Ile. In an attempt to maintain higher JA-Ile for more active alkaloids biosynthesis, a partial tobacco JIH coding sequence was used to make an RNAi construct (Appendix Figure A1 and A2). Seven *JIH1* RNAi plants were generated. Total alkaloid levels and *JIH1* transcript levels of these plants were determined. *JIH1* transcript levels were reduced in transgenic lines to only 8 to 37% of that in WT (Figure 3.2.19). However, nicotine content of transgenic plants was very similar or slightly lower than WT (Figure 3.2.20). These results suggest that *JIH*1 downregulation does not affect nicotine synthesis. Therefore, *JIH1* RNAi transgenic plants were not examined further.

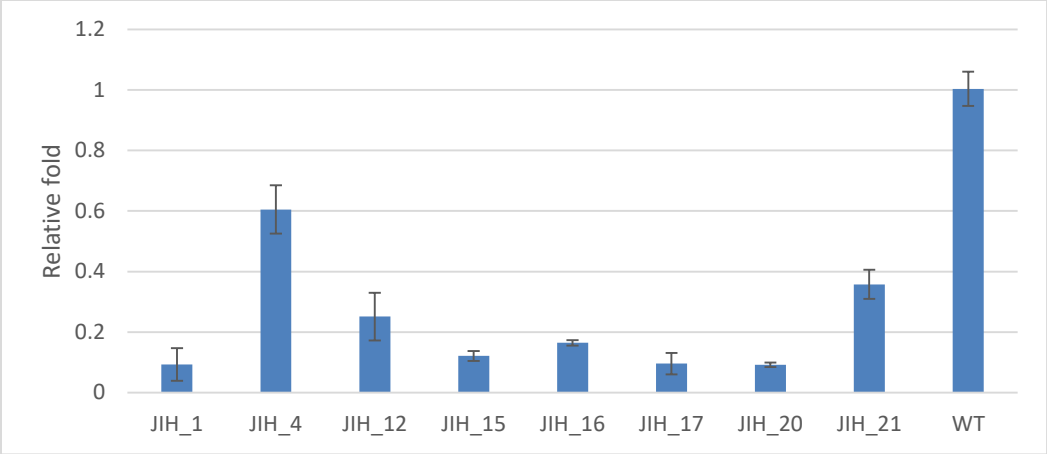

Figure 3. 2. 19 Relative *NtJIH1* transcript levels in T_0 JIH1 RNAi plants.

RNA was isolated from tobacco leaves collected before topping. *NtJIH1* transcripts were detected by qRT-PCR. Values shown are means from 3 technical replicates. Error bar on the column represents standard error. Transcript levels were normalized to *actin* and displayed relative to the transcript level of vector control which was arbitrarily set at1.

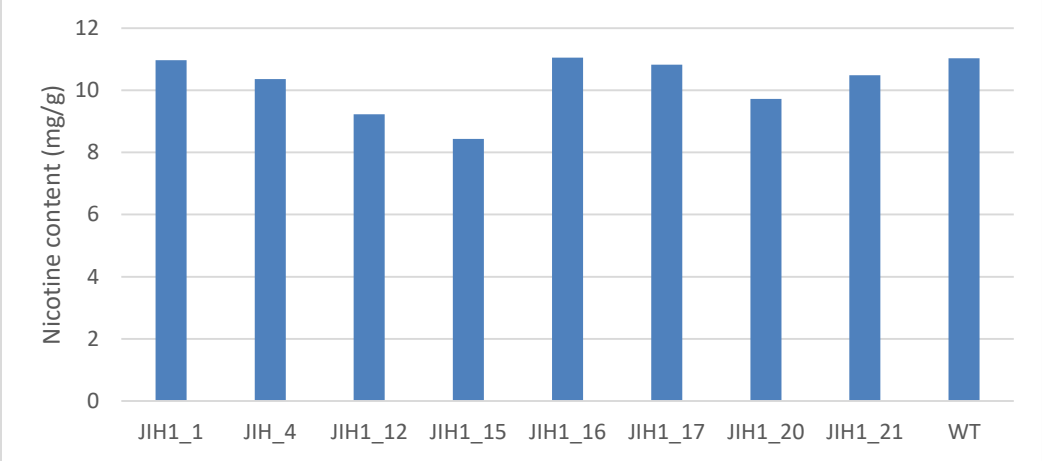

Figure 3. 2. 20 Nicotine levels of JIH1 RNAi T_0 plants.

Seven days after topping, the top twelve leaves were collected, dried and ground. Total alkaloids were extracted from approximately 200 mg leaf tissues and individual alkaloid was quantified as described in the material and method section. The columns represent the value of nicotine content.

3.2.8 An effort to improve statistical analysis

Since AOC2-49 line and JAR1-20 line looked more promising in terms of nicotine content (see sections 3.2.4 and 3.2.6) and the sensitivity of statistical analysis could have been reduced by the limited sample size, we constructed a randomized block design (RBD) for a higher resolution data analysis. RBD could help control location effect and reduce variance among the replicates. Based on RBD, PCR positive T_1 plants of the AOC2-49 line, JAR1_20 line and WT were randomly assigned to four blocks in the greenhouse. Each block contained four plants of a line. Leaves were pooled by blocks for nicotine analysis (Figure 3.2.21). Analysis of variance of randomized block design suggested that nicotine level in the AOC2_49 and JAR1_20 are not changed significantly as compared to WT (P-value = 0.1145, Table 3.2), despite that three out of the four blocks of the AOC2-49 had nicotine contents 45% to 114% higher than WT. The Tukey's Studentized Range (HSD) test was used to make multiple comparisons and statistical test, the calculated Minimum Significant Difference is 3.7454, so the nicotine levels of the AOC2_49 and JAR1_20 are not significantly different from WT (Table 3.2).

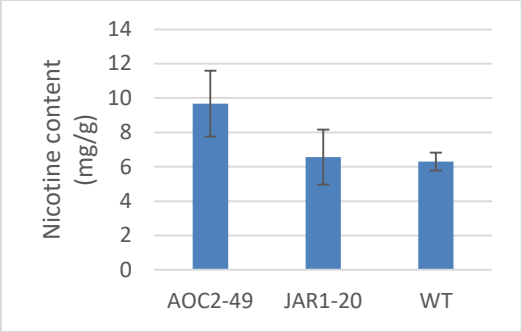

Figure 3. 2. 21 Nicotine levels of T_1 plants of AOC2-49, JAR1-20 and WT.

The third leaf from the top was collected from each plants seven days after topping. Leave were pooled by blocks and nicotine levels were determined by GC-MS. The columns represent average nicotine content of 4 blocks. Error bar on the column represents standard error.

Table 3. 2 Analysis of variance in RBD.

Source of variation	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	60.28889672	12.05777934	2.27	0.1275
Error	6	26.59059696	4.43176616		
Location	3	32.12215774	10.70738591	2.42	0.1648
Treatment effect	2	28.16673898	14.08336949	3.18	0.1145
Corrected Total	11	86.87949368			

Table 3. 3 Tukey's Studentized Range (HSD) test of nicotine level in RBD.

Means with the same letter are not significantly different. The calculated Minimum Significant Difference is 3.7454.

Tukey Grouping	Mean	N	Treatment
A	9.676	4	AOC2_49
A	6.564	4	JAR1_20
A	6.303	4	WT

3.2.9 Analysis of individual alkaloid levels of T_1 plants

At T_1 generation, tobacco tissues were collected from the top 12 leaves for alkaloids quantification. Four types of alkaloid in tobacco leaves, including nicotine, nor nicotine, anabasine, and anatabine, were measured in the same HPLC run. As shown in previous sections, nicotine content didn't change in the transgenic lines, as compared to vector control line. We further investigated whether the levels of the rest alkaloids changed in the transgenic lines (Tables 3.4, 3.5). Student t-test showed no significant difference of minor alkaloid levels between the transgenic lines and vector control lines.

Table 3. 4 Individual alkaloid levels of T_1 plants in absence of topping.

Tobacco tissues were collected from the top 12 leaves in absence of topping. All the four types of alkaloids were quantified in the same HPLC procedure. Values shown in this tables are means of three replicate plants and standard errors. Unit: mg/g dry weight. Student's t test (α=0.05) suggested no significant difference between VC and any of the transgenic lines. VC: vector control.

	Nicotine	Nornicotine	Anabasine	Anatabine
VC	5.298 ± 1.013	0.138 ± 0.013	0.028 ± 0.003	0.206 ± 0.032
LOX2_20	5.688 ± 0.648	0.138 ±0.009	0.028 ± 0.002	0.214 ± 0.014
LOX2_33	5.826 ± 0.882	0.141 ± 0.012	0.033 ± 0.005	0.222 ± 0.066
AOC2_50	5.974 ± 0.058	0.142 ± 0.004	0.029 ± 0.001	0.209 ± 0.007
AOC2_49	6.431 ± 0.476	0.152 ± 0.006	0.030 ± 0.002	0.224 ± 0.005
JAR2_60	4.740 ± 0.532	0.185 ± 0.058	0.027 ± 0.002	0.218 ± 0.020
JAR2_20	4.678 ± 0.400	0.122 ± 0.006	0.029 ± 0.002	0.215 ± 0.017
OPR3_21	6.005 ± 0.903	0.138 ± 0.010	0.026 ± 0.0004	0.190 ± 0.006
OPR3_40	5.178 ± 0.178	0.132 ± 0.002	0.026 ± 0.001	0.168 ± 0.006

Table 3. 5 Individual alkaloid levels of T_1 plants with topping.

Tobacco tissues were collected from the top 12 leaves 7 days after topping. All the four types of alkaloids were quantified in the same HPLC procedure as described in the material and method section. Values shown in this tables are means of three replicate plants and standard errors. Unit: mg/g dry weight. Student's t test (α=0.05) suggested no significant difference between VC and any of the transgenic line. VC: vector control.

	Nicotine	Nornicotine	Anabasine	Anatabine
VC	7.765 ± 1.223	0.167 ± 0.014	0.033 ± 0.002	0.285 ± 0.032
LOX2_20	8.396 ± 0.894	0.181+ 0.017	0.034 ± 0.005	0.310 ± 0.066
LOX2_33	8.109 ± 0.963	0.166 ± 0.012	0.030 ± 0.003	0.255 ± 0.041
AOC2_50	9.202± 0.490	0.174 ± 0.008	0.040 ± 0.002	0.387 ± 0.005
AOC2_49	8.560 ± 0.651	0.169 ± 0.007	0.036 ± 0.001	0.298 ± 0.016
JAR2_60	7.528 ± 1.517	0.162 + 0.013	0.038 ± 0.005	0.359 ± 0.063
JAR2_20	8.441 ± 1.033	0.181 ± 0.003	0.038 ± 0.001	0.306 ± 0.008
OPR3_21	6.310 ± 0.102	0.140 ± 0.003	0.029 ± 0.002	0.229 ± 0.013

3.2.10 Analysis of endogenous gene expression in transgenic tobacco plants

When a gene was overexpressed in plants, both the introduced and endogenous forms of the gene may be knocked down to differing degrees (Eamens, et al., 2008). Because of the homology between transgene-derived mRNA and endogenous mRNA, the transgene derived RNA could induce suppression of the endogenous gene by pairing with the complementary regions of endogenous mRNA (Flavell, 1994). This co-suppression phenomenon was reported in a few cases (Napoli, et al., 1990; van der Krol, et al., 1990; Flavell, 1994; Matzke and Matzke, 1995). To determine whether the endogenous gene might have been impaired in our transgenic plants overexpressing an Arabidopsis counterpart, we measured the transcript level of the corresponding endogenous genes by qRT-PCR.

BLAST analysis was performed to identify tobacco homologous proteins using the amino acid sequence of AtLOX2, AtAOS, AtAOC2, AtOPR3 and AtJAR1 as queries. The first hit protein sequences, with the highest score, query cover, identity percentage and lowest E-value, are determined as the corresponding tobacco homologs (Table 3.6). The cDNA sequences encoding these proteins were found in NCBI tobacco genome database. The "endogenous" cDNA in transgenic plants was quantified by qRT-PCR. The cDNA sequences were used as the query to search tobacco genome database via BLAST. Several cDNA isoforms were pulled out, indicating each gene has a small gene family in tobacco.

For each transgene construct, we focused on the two lines studied most, together with a vector control plant. The RNA was isolated from the third leaf which was collected from T_0 plants after topping treatment. The qRT-PCR was performed to measure the corresponding

endogenous gene expression in transgenic tobacco and vector control plants with three technical repeats. The sequence specific primers were used and the primer specificity was tested via the disassociation curve in the qRT-PCR procedure. The qRT-PCR primers were listed in Appendix A5.

Table 3. 6 Endogenous JA biosynthetic enzymes and genes in tobacco plants.

First hit peptide	E-value	Amino acid identity	Peptide accession number	cDNA accession number	Nucleotide identity	Number of isoforms
A predicated NtLOX2, chloroplastic-like	0	56%	XP_016510888.1	XM_016655402	61.3%	7
A predicted NtAOS, chloroplastic-like	0	68%	XP_016441462.1	XM_016585976.1	65%	5
NtAOC4, chloroplastic-like	5E-84	65%	NP_001311907.1	AJ308487.1 or NM_001324978.1	56.1%	2
A predicted NtOPR3	0	74%	XP_016448046.1	XM_016592560	68.3%	4
A predicated NtJAR1- like isoform X3	0	66%	XP_016444474.1	XM_016588990.1	65.5%	6

The transcript levels of the endogenous *NtLOX2*, *NtAOS*, *NtAOC2*, *NtOPR3*, *NtJAR1* were measured in LOX2 transgenic plants (LOX2-20 and LOX2-33), AOS transgenic plants (AOS-4 and AOS-10), AOC transgenic plants (AOC2-49 and AOC2-50), OPR3 transgenic plants (OPR3-21 and OPR3-40), and JAR1 transgenic plants (JAR1-20 and JAR1-60), respectively. As shown in Figure 3.2.22 (A), the transcript levels of *NtLOX2* in LOX2-20 and LOX2-33 plant were reduced to 45% and 59% of that in WT, respectively, suggesting that the transgene triggered a suppression of the endogenous gene. As demonstrated in Figure 3.2.22 B, C, D, the transcript levels of endogenous *NtAOS*, *NtAOC2*, *NtJAR1* were unchanged in the transgenic plants as compared to the vector control. In the OPR3-21 line (Figure 3.2.22 E), however, the *NtOPR3* transcript level increased by approximately 5 fold as compared to the vector control, which could be resulted from the elevated JA level and the JA positive feedback regulation. In the OPR3-40 line, however, the *NtOPR3* transcript level is unchanged as compared to vector control (Figure 3.2.22 E).

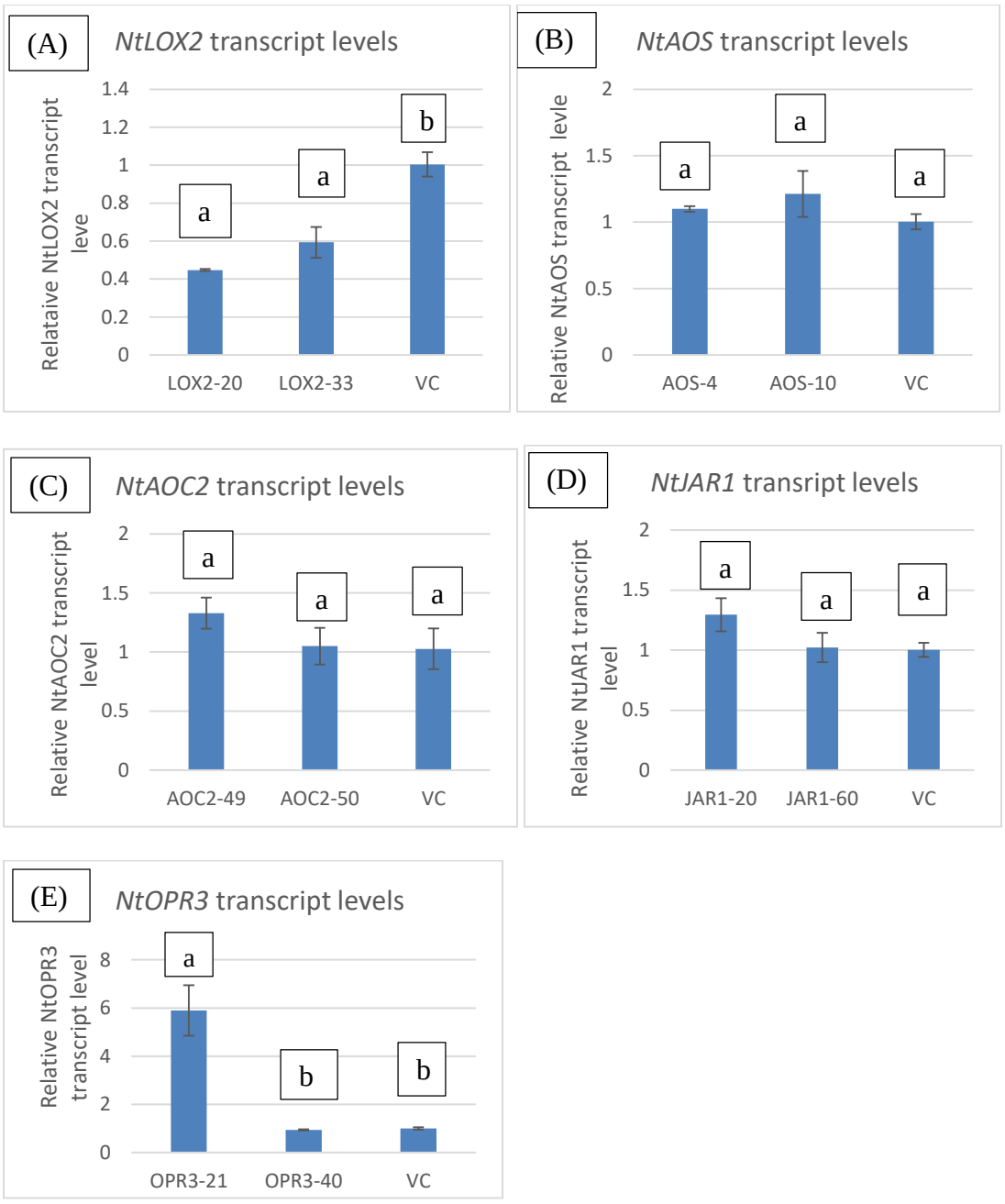

Figure 3. 2. 22 Endogenous transcript levels of JA synthetic genes.

RNA was isolated from leaf tissues collected from T_0 plants 1.5 hours after topping. *NtLOX2*, *NtAOS*, *NtAOC2*, *NtOPR3*, and *NtJAR1* transcript levels were determined by qRT-PCR. Values are means from 3 technical replicates. Error bar on the column represents standard error. Transcript levels of endogenous JA synthetic genes were normalized to *actin* and displayed relative to the transcript levels of vector control plant which is arbitrarily set at 1. The t test was conducted to compare transcript levels among transgenic lines and the vector control. Same letter indicates no difference. Different letters indicate significant difference.

3.2.11 Summary of results of chapter 2

In summary, six genes involved in biosynthesis and catabolism were studied. Overexpression of *AOS* and RNAi-mediated suppression of *JIH* didn't increase total alkaloid at T_0 generation. Therefore, *AOS* and *JIH* gene construct were not studied in the T_1 generation. For *LOX2, AOC2, JAR1, OPR3* transgenic plants at T_0 generation, two transgenic plants with the higher total alkaloids levels and transgene expression levels were further studied in T_1 generation. T_1 generation data suggested that overexpression of these four genes increased the transcript levels of the nicotine biosynthetic genes but not nicotine levels.

Chapter 3: Repression of *NtNINJA* in Tobacco Plants

3.3.1 Overview

NINJA is a co-repressor of JA signaling pathway and forms a repressor complex with JAZ and TPL to negatively regulate JA responses in Arabidopsis (Pauwels, et al., 2010). One copy of tobacco *NINJA* (AJ966363) cDNA was cloned in tobacco BY-2 cells (Goossens, et al., 2003). Tobacco whole genome sequence (WGS) database has two copies of *NtNINJA*, termed *NtNINJA1* and *NtNINJA2*. A conserved sequence was used to make RNAi construct (Appendix Figure A3). Sequences alignment is shown in Appendix Figure A4. NtNINJA1 shares 97 % of the nucleotide sequence with *NtNINJA* (AJ966363) while *NtNINJA2* shares 100 % of nucleotide sequence with *NtNINJA* (AJ966363), shown in Figure A4. In order to understand its function in regulating JA signaling and nicotine synthesis, RNAi construct was made and introduced into tobacco plants for *NtNINJA* suppression. A 483 bp of cDNA fragment from *NtNINJA* (AJ966363), as demonstrated in the appendix Figure A3 and red box in Figure A4, was used to create an RNAi construct to repress expression of both *NtNINJA* genes. Four *NINJA* RNAi tobacco plants were obtained. In order to collect fine root samples for gene expression studies, plants were grown in pots with holes on the bottom, from where root tips would appear two months later. Plants were topped when they started flowering and approximately 200 mg root tissues were collected per plant.

3.3.2 Analysis of NINJA RNAi transgenic plants

The *NtNINJA1/2, NtMYC2a, NtPMT1a, NtQPT2, NtBBL, NtA622, NtJAZ3 and NtJAZ12* transcript levels were determined from root tissues by qRT-PCR. Primers were listed in Appendix Table A4. The reason *NtJAZ3* and *NtJAZ12* are analyzed is because NtJAZ12a/b is different from most other JAZs in tobacco: NtJAZ12 does not have a degron region within

the Jas motif as most JAZ proteins do (see NtJAZs alignment in appendix Figure A8). Instead, it has a NINJA EAR domain at its N-terminus for directly recruiting NINJA co-repressor (See NtJAZs alignment in Appendix Figure A9). Therefore, NtJAZ12 is supposed to be non-degradable, just like JAZ8 in Arabidopsis (Shyu, et al., 2012). It is speculated that while most JAZs forming a co-repressor complex with NINJA and TPL to negatively regulate JA signaling, JAZ with an EAR domain may directly interact with MYC2 and TPL to form an NINJA-independent repressor complex (Pauwels et al., 2010; Pauwels and Goossens, 2011; Shyu, et al., 2012). Due to the time limit, only T_0 plants were analyzed.

As shown in Figure 3.3.1, *NINJA* transcripts declined to approximately 20% in the NINJA_5 plant and to 40% in the NINJA_9 plant, respectively, as compared to WT, whereas no obvious changes of *NINJA* transcript levels were observed in NINJA_7 and _8 plants. It appears the plants were quite sensitive to *NINJA* RNAi, changes in nicotine pathway gene expression levels were observed even in NINJA_7 and NINJA _8 plants (Figure 3.3.2). As shown in Figures 3.3.2 and 3.3.3, in all the four transgenic plants, *MYC2a* and *JAZ3* expression was substantially increased. *MYC2a* transcript levels were enhanced by one fold (as in NINJA_7) to 3 folds (as in NINJA_8), while *JAZ3* transcript levels increased by one fold (as in NINJA_8) to 3.5 folds (as in NINJA_5). In addition, in NINJA_5 plants, where *NINJA* transcript reduced the most, both *NtPMT1a* and *NtQPT2* transcript levels increased to 7 folds (Figure 3.2.2). *JAZ12* expression slightly increased in three out of the four transgenic plants (NINJA_8, _9, and _5), by 38% to 87% (Figure 3.3.3). Moreover, it is noticeable that both *BBL* and *A622* transcript levels declined in three out of the four *NINJA* RNAi plants (NINJA_7, _8, and _9), as shown in Figure 3.3.2.

The expectation of this approach was to increase nicotine content by reducing the repressor complex activity. To our surprise, nicotine contents in all the four transgenic plants were substantially reduced, even in the plants we could not tell *NINJA* transcripts were altered (Figure 3.3.4). Although we do not understand the mechanism of this decrease, reduction of *BBL* and *A622* expression in all the four *NINJA* RNAi plants may play a role. The results strongly stress the complexity of regulation of JA signaling and its effects on modulation of nicotine biosynthesis.

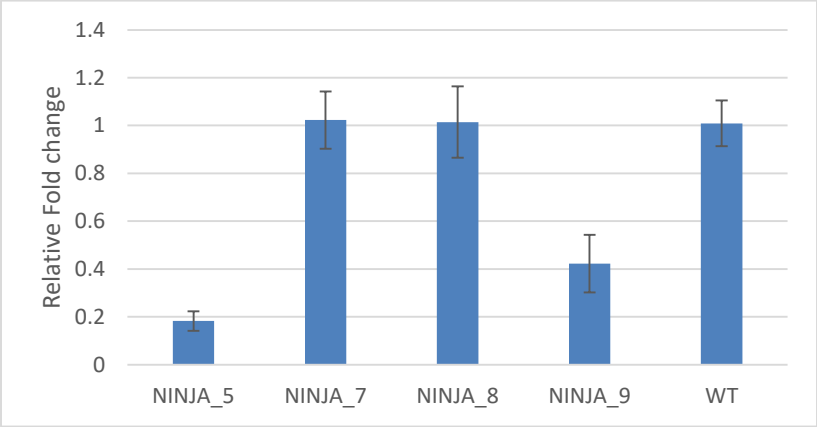

Figure 3. 3. 1 Relative NtNINJA transcript levels in T_0 NINJA RNAi plants.

Transcripts were detected by qRT-PCR. Values shown are means from 3 technical replicates. Standard error is presented. Transcript levels were normalized to *actin* and displayed relative to the transcript level of wild type, which was set at 1.

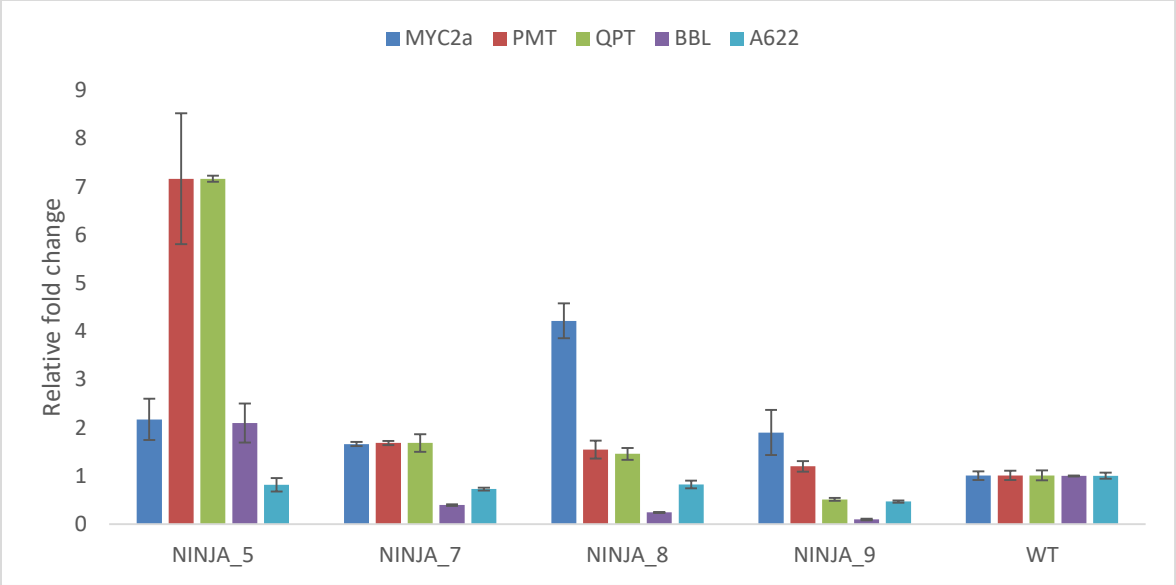

Figure 3. 3. 2 Transcript levels of nicotine pathway genes in NINJA RNAi plants.

Root samples were collected 3 hours after topping treatment. Transcripts were detected by qRT-PCR. Values shown are means from 3 technical replicates. Standard error is presented. Transcript levels were normalized to *actin* and displayed relative to the transcript level of WT which is arbitrarily set at 1.

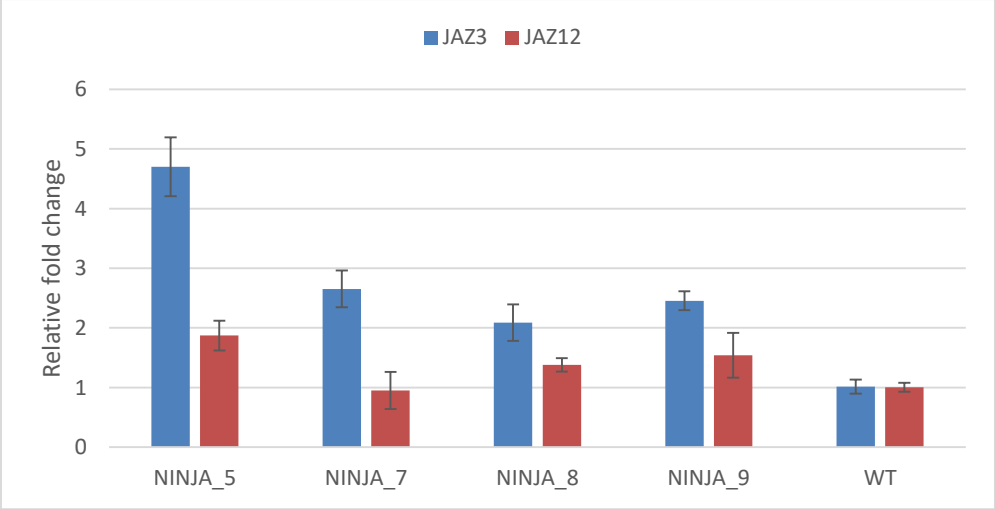

Figure 3. 3. 3 *NtJAZ3* and *NtJAZ12* transcript levels.

Root samples were collected 3 hours after topping treatment. Transcripts were detected by qRT-PCR. Values shown are means from 3 technical replicates. Standard error is presented. Transcript levels were normalized to *actin* and displayed relative to the transcript level of WT which is arbitrarily set at1.

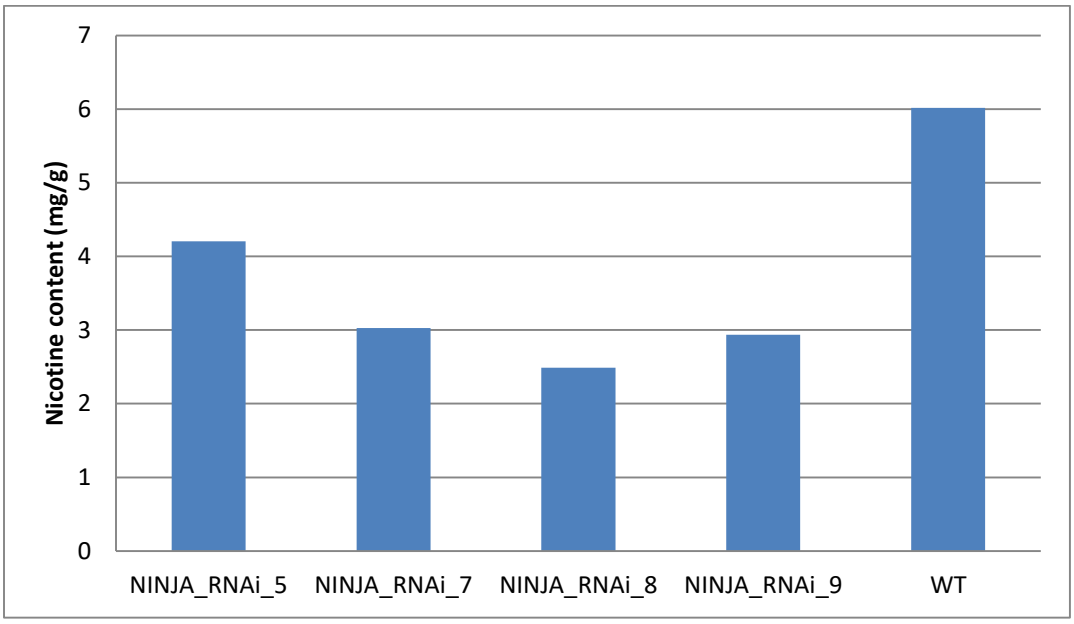

Figure 3. 3. 4 Nicotine levels of *NINJA* RNAi T_0 plants.

Seven days after topping, the top twelve leaves were collected, dried and ground. Total alkaloids were extracted from approximately 200 mg leaf tissues and individual alkaloid was quantified as described in the material and method section. The columns represent the values of nicotine content.

IV. Discussion

4.1 NtJAZ3 interacted with NtMYC2a

In our research on *NtMYC2a* and *NtMYC2b*, we noticed that the *MYC2* genes are expressed in all the major tissues (leaf, stem, root and flower) tested. However, all the nicotine synthesis pathway genes are only expressed in roots. That prompted us to hypothesize that there may be a protein that helps to coordinate nicotine pathway gene expression specifically in roots. In that case, this protein is likely to interact with NtMYC2a proteins, and we set out to pursue it by Y2H approaches.

In the first root cDNA library screening using full-length NtMYC2a as bait, we isolated a NtJAZ3 protein interacting with NtMYC2a, which confirmed the previous discovery that an AtJAZ interacted with the AtMYC2 in Arabidopsis (Chini, et al., 2007). It is in consistent with the observation that NtJAZ1 interacted with two other NtMYC2 isoforms (NCBI accession: HM466974 and HM466975) in BY-2 cells in BiFC assay (Zhang et al., 2012). In addition, Yang et al., (2015) found that nine NtJAZs, including NtJAZ1 and NtJAZ3, interacted with a NtMYC2 isoform (NCBI accession: HM466974) with various binding strength in Y2H. However, although it interacts with NtMYC2a, NtJAZ3 is not the protein that coordinates root-specific nicotine biosynthesis because NtJAZ3 is expressed in all the major tissues and JAZ proteins are involved in many aspects of JA-regulated responses as shown in recent reports (Shoji et al., 2008; Yang et al., 2015). JAZ proteins were proposed to be negative regulators in JA signaling (Chini, et al., 2007; Pauwels and Goossens, 2011). However, Yang et al., (2015) demonstrated that silencing of *NtJAZ1*, *NtJAZ3*, *NtJAZ7* and *NtJAZ10* genes greatly reduced *NtPMT* transcript levels and nicotine contents in BY-2 cells,

indicating a contradicting regulatory role of NtJAZ proteins to that suggested by the prevailing JA signaling model. However, we need to be cautious that the de-differentiated BY-2 cell used by Yang et al. is a different system which produces very low level of alkaloids and the major type of alkaloid is anatabine rather than nicotine due to repressed MPO gene expression and enzyme activity in BY-2 cells (Goossens, et al., 2003; Shoji and Hashimoto, 2008). Therefore, in actual tobacco plants, NtJAZs proteins may have various functions and further validations are needed to gain further insights into the regulatory functions of JAZ proteins in nicotine biosynthesis.

4.2 Overexpression of JA biosynthetic genes increased the transcript levels of *NtPMT1* and *NtQPT2* but nicotine content didn't increase

In this research, we studied six genes involved in JA biosynthesis or catabolism. Overexpression of at least four of them (*LOX2, AOC2, OPR3,* and *JAR1*) enhanced JA signaling after topping as indicated by fold increases of transcripts of nicotine synthesis pathway genes (Figures 3.2.3, 3.2.9, 3.2.13, 3.2.17). In some cases, e.g., LOX2-33 and OPR3-21, the transcript levels of nicotine synthetic genes were even induced without topping (Figures 3.2.3 and 3.2.13). Among all the tested lines, *AOC2-49* overexpression line exhibited the highest nicotine synthetic gene expression levels induced by topping, with 4.5 fold of *NtPMT1a*, 2.5 fold of *NtQPT2* and 3 fold of *NtA622* transcripts relative to vector control. However, the enhanced *NtPMT1* and *NtQPT2* transcript levels did not lead to nicotine increase in this commercial tobacco variety (cv. NC95) grown in the greenhouse. Similarly, in the previous study in our lab, direct overexpression of *NtPMT1a* and/or *NtQPT2* by the *NtQPT2* promoter didn't alter the nicotine content in the same variety grown in the field (Wang, 2011). A possible explanation why high transcript levels of *NtPMT1* and

NtQPT2 did not result in higher nicotine content is that the NtPMT1 and NtQPT2 enzyme activity may not have increased in the transgenic plants. We hypothesize that there could be some missing factors that regulate nicotine biosynthesis at translational and/or post-translational level, such as factors involved in protein degradation, protein modification or protein de-activation. To test this hypothesis, the enzymatic activity of NtPMT1 and NtQPT2 should be measured in both transgenic plants and vector control plants.

In contrast to our results, another study demonstrated that overexpression of *NtPMT1a* under a constitutive CaMV 35S promoter increased leaf nicotine content by 40% in N. sylvestris plants growing in the greenhouse (Sato, et al., 2001). The discrepancy is likely due to different plant species and growth environment in the two studies. Sato et al. (2001) employed the maternal donor of tobacco, *N. sylvestris*, which is the contributor of *NtPMT2*, *NtPMT3*, and *NtPMT4*, but doesn't comprise a *NtPMT1* sequence counterpart (Hashimoto et al., 1998; Riechers and Timko, 1999). Therefore, overexpression of an additional *NtPMT1* gene copy could significantly enhance nicotine biosynthesis in *N. sylvestris*. In addition, the *N. sylvestris* variety had a much lower baseline of nicotine content than the tobacco variety did in our study. In the molecular study of nicotine biosynthesis, we need to be cautious about what plant materials are used. Various tobacco-related species, "lab strains", and cell line were used in the study of nicotine biosynthesis, such as *N. sylvestris*, *N. benthamiana*, *N. attenuata*, *N. glauca*, and BY-2 cells. They are different in their genome and alkaloids composition, therefore, inconsistent results were obtained when the different plant materials were used.

Among all the tested lines, AOC2-49 line exhibited the highest transcript levels of the nicotine synthetic genes induced by topping, but the nicotine content of this line didn't increase as compared to the VC (Figures 3.2.9, 3.2.10). The randomized block design re-confirmed the result that nicotine didn't increase in AOC2 transgenic lines (Figure 3.2.21, Tables 3.2, 3.3). Unlike the observations in our study, another study demonstrated that constitutive overexpression of an *AOC* gene from a Solanaceae species, *Hyoscyamus niger* L., led to a 4.8-fold increase in nicotine yield in tobacco plants (Jiang, et al., 2009). The inconsistency may be caused by two reasons. First, Jiang et al. studied the tobacco "lab strain" (cv. Petit Havana), which had a lower baseline level of nicotine content than the commercial variety NC95 we used. Second, the AOC2 from *Hyoscyamus niger* L. may have higher enzyme activity in Jiang's study. The HnAOC protein shares 92% amino acids homology with the endogenous NtAOC, while the AtAOC we used shares only 65% amino acid homology with the NtAOC. It is likely that the AOC enzyme activities from these two species are different.

4.3 Could BBL and A622 be overexpressed for increasing nicotine content?

In the *LOX2, AOC2,* and *JAR1*overexpression lines, the transcript levels of *BBL* and *A622* slightly increased and not as much as *PMT1* and *QPT2* did (Figure 3.2.3, 3.2.9, 3.2.17). A similar result was observed in another study that *PMT* transcript level was induced to 6.4-fold but *A622* transcript level increased to 4.7-fold in response to 2.5 μM MeJA treatment in hairy roots of tobacco (NC95) (Cane et a., 2005). It seems that *BBL* and *A622* are not as sensitive as other nicotine biosynthetic genes in response to JA stimulation. In addition, tobacco root transcriptome sequencing showed that there were more fragment reads of *PMT*

and *QPT2* than *BBL* (Sierro, et al., 2014). These experiments indicate that *BBL* and *A622* transcript levels may be limited for nicotine biosynthesis. Moreover, BBL and A622 play positive roles in the nicotine biosynthesis as shown in the study that suppression of *BBL* and *A622* by RNAi reduced alkaloid content in *N. tabacum* plants and *N. glauca* plants respectively (Deboer et al. 2009, Lewis, et al., 2015). Based on these two reasons, *BBL* and *A622* genes could be possible targets for efforts designated to increase nicotine biosynthesis by overexpressing these two genes. However, BBL enzymes may need post-translational modification for its activation (Kajikawa et al., 2011). If this is the case, overexpression of *BBL* and *A622* gene would not be effective in increasing nicotine accumulation. In addition, we need to consider whether BBL and A622 are the rate limiting enzymes. Early studies reported that the PMT and QPT2 are the rate-limiting enzyme in the pyrrolidine branch and pyridine branch respectively, because they had much lower enzyme activity than other enzymes in the two branches (Saunders and Bush, 1979; Wagner and Wagner, 1985; Feth, et al., 1986). However, the biochemical properties of BBL and A622 are not elucidated since the *in vitro* enzymatic assays failed to detect BBL and A622 enzyme activity when substrates and the recombinant enzyme proteins were provided. Therefore, it's not clear whether BBL and A622 are the rate-limiting enzymes in the nicotine biosynthesis pathway. If BBL and A622 are not the rate-limiting enzymes, overexpression of *BBL* or *A622* would be less effective in increasing nicotine biosynthesis.

4.4 Expression of endogenous JA synthetic genes may or may not be affected by corresponding transgenes

In this study, the foreign Arabidopsis JA synthetic genes were introduced and overexpressed into tobacco genome in an attempt to enhance JA signaling and nicotine biosynthesis. Since

the nicotine levels were not significantly increased, we hypothesized that the expression of the corresponding endogenous JA synthetic genes may be affected due to homology between transgene-derived mRNA and endogenous mRNA. To test this hypothesis, we conducted the qRT-PCR to investigate the transcript levels of the corresponding endogenous JA synthetic genes in the two most studied plants for each gene construct in T_0 generation. A total of ten plants were examined.

We found, as compared to vector control, all the tested transgenic plants had similar transcript levels of the corresponding endogenous genes as vector control plant, except two LOX2 overexpression plants and one OPR3 overexpression plant. In the two LOX2 overexpression plants (LOX2-20, LOX2-33), the endogenous *NtLOX2* transcript level reduced to approximately half of that in the WT plant, indicating a co-suppression caused by the transgene. This endogenous gene suppression could be a source for the unchanged nicotine in the LOX2-20 and LOX2-33 transgenic plants. In one of the OPR3 overexpression plant (OPR3-21), however, the endogenous *NtOPR3* transcript level increased to approximately 6- fold, probably due to JA positive feedback which upregulates its own synthetic gene expression. Correspondingly, this transgenic plant (OPR3-21) has the highest total alkaloids level in the T_0 generation. For the other seven transgenic plants (OPR3-40, AOS-4, AOS-10, AOC2-49, AOC2-50, JAR1-20, JAR1-60), the transcript levels of the corresponding endogenous genes are similar to that in WT. However, the unchanged transcript level of endogenous gene didn't necessarily mean the endogenous are not co-suppressed because the elevated JA levels could induce the endogenous gene expression.

4.5 Why transgene expression level did not correlate with total alkaloid content?

In this research, we tried to overexpress JA biosynthesis pathway genes in an attempt to increase nicotine contents. At the T_0 generation, the transgene expression levels and the total alkaloids levels were measured, and no correlation was observed. Overall, the research revealed that the molecular regulation of nicotine biosynthesis is complicated. Although we saw that nicotine synthesis genes were often upregulated by the transgene expression, no nicotine content increase was observed. As we discussed in this discussion section, there may be an unknown factor or factors working at translational or post-translational level that regulates the enzyme activities of the nicotine biosynthesis pathway. As for the T_0 generation, the variation of transgene transcript levels among the transgenic plants (as shown in Figures 3.2.1, 3.2.5, 3.2.7, 3.2.11, and 3.2.15) should be mainly due to position effects. The overall total alkaloid content is the results of the "endogenous" nicotine synthesis plus the transgene stimulation effect. Since the transgene effect is minimum, if any, the measured total alkaloid level is mainly the results of the "endogenous" nicotine synthesis pathway. That is largely the reason why transgene expression, no matter high or low, did not have much effect on alkaloid content of the transgenic plants from each transgene construct (as shown in Figures 3.2.2, 3.2.6, 3.2.8, 3.2.12, and 3.2.16). There are some variations among the transgenic plants, which could be caused by the environmental factors, and is often more obvious at T_0 generation, due to tissue culture effect and lack of replicated design.

4.6 JA signaling is tightly controlled in plants

JA is a plant hormone. Its biosynthesis and signal transduction are the plant responses to environmental stresses. JA signaling competes for resources with plant growth and development. For instance, it is well documented that JA signaling negatively affects root

cell division and thus root length (Wasternack and Hause, 2013). To regulate JA signaling, plant developed fine tuning and JA attenuation mechanisms at many levels. Some examples are described below.

MYC2 has a negative feedback loop

MYC2 is a positive, "master" TF at the center of JA signaling (Kazan and Manners, 2013) and is most responsible for nicotine synthesis gene expression. However, it was shown that *MYC2* promoter has an MYC2 binding site, and *MYC2* was able to directly, negatively regulates its own transcript level (Dombrecht, et al., 2007), suggesting a potential attenuation step for JA signaling.

JA induces JAZ degradation and JAZ expression

JAZ is considered the major repressor for MYC2. After JA signal perception, JAZ is rapidly degraded by 26S proteasome so the MYC2 is released and the plants initiate the JA signaling mode. However, jasmonate treatment also induces the formation of new JAZ proteins within an hour, most likely to attenuate JA effects (Thines et al., 2007). In tobacco, expression of eight (out of 17) JAZ genes are induced by MeJA treatment, but not the other nine, indicating a complex transcription regulation scheme of JAZ genes (Yang, et al., 2015).

Redundancy and diversity of JAZ proteins and JAZ transcripts

There are thirteen JAZ proteins in Arabidopsis and seventeen in tobacco (Pauwels and Goossens et al., 2011; Yang et al., 2015). Many tobacco JAZ proteins have potential to form homodimers and/or heteromers with various degrees of interaction strength (Yang, et al.,

2015) to serve in a complicated regulatory network. In addition, although most of them seem to have functional redundancy, diversity of JAZ family members is observed. For example, most JAZ proteins have a conserved Jas domain, which is essential for interaction with COI and the subsequent JAZ degradation in the presence of JA-Ile. However, both JAZ7 and JAZ8 in Arabidopsis lack the two conserved basic amino acids at positions 205 and 206 within Jas domain, which are required for COI interaction (Pauwels and Goossens, 2011; Shyu, et al., 2012), and could affect the degradation of JAZ7 and JAZ8 in JA signaling. Moreover, several *JAZ* pre-mRNAs were involved in alternative splicing, resulting in dominant JA-insensitive variants. In the case of JAZ10 alternative splicing, the C-terminal degron motif in the Jas domain is spliced out, which affects COI1 binding, leading to JA-insensitive phenotypes (Chung and Howe, 2009; Moreno, et al., 2013).

NINJA co-repressor is induced by JA

NINJA is a co-repressor for JA signaling (Pouwels et al., 2010). It binds to JAZ and TPL to form a repressor complex. However, like JAZ, NINJA expression is also induced by JA, thus forming another loop for auto-regulation of JA signaling.

Dual repression mechanism of JAZs

JAZ3 represses JA-responsive genes not only through recruiting the NINJA-TPL repressor complex but also through competing with the mediator subunit MED25 for binding with MYC2 (Pouwels et al., 2010; Chen et al., 2012; Zhang et al., 2015).

Post-translational level regulation

The crystal structure of tomato OPR3 is a self-inhibited dimer (Breithaupt, et al., 2006). The authors suggested a strong and reversible dimerization in vivo involving phosphorylation of OPR3 as a regulatory step in JA biosynthesis. Furthermore, any two of the four functional AOC polypeptides, AOC1, 2, 3, and 4, were able to interact each other in BiFC experiments, prompting the authors to propose a layer of regulatory mechanism in JA biosynthesis through various combination of heterodimerization of the four AOCs (Stenzel et al., 2012). I believe the tight control mechanism plant has on JA signaling is the main reason that manipulating JA synthesis genes, as done in this thesis study, had little effect on nicotine content. In addition, phosphorylation of some key proteins in this pathway also plays an important role in the regulation (see below).

4.7 Nicotine has a strong negative feedback to its own biosynthesis

The regulation of JA signaling and nicotine biosynthesis is further complicated by the fact that nicotine is a toxic end product to tobacco cells. Shoji et al. (2009) observed that root growth of 7d tobacco seedlings reduced by ~50% after being cultured in 2 mM nicotine for another 7 d. They also showed that RNAi repression of MATE gene, which encodes a nicotine transporter to remove it from cytosol and transport to vacuoles, further reduced root growth in the same experiment to demonstrate cellular toxicity of nicotine (Shoji, et al., 2009). In addition, Wang et al. (2015) revealed that nicotine has a negative feedback effects: transcript levels of all major nicotine synthesis genes in tobacco seedlings were reduced by about 50% two hours after 0.4 mM nicotine treatment (Wang, et al., 2015).

In our early quest on nicotine synthesis regulation, we observed that overexpression of two key synthesis genes, *NtPMT1a* and *NtQPT2*, led to higher transcript levels, but nicotine contents did not increase (Wang, 2011). We were puzzled by the results. Now we observed a similar phenomenon in overexpressing JA biosynthesis genes: nicotine content did not increase when transcript levels of nicotine synthesis genes were higher. It seems the enhanced transcript levels of these genes are essential but not sufficient for increased nicotine accumulation. Some other factor(s) are missing in the system, and regulate nicotine biosynthesis at post-transcriptional, translational, and/or post-translational levels. Very recently, it was reported that *NtQPT2* gene is also regulated by miRNA (nta-miRX27) and its endogenous target mimicry (*nta-eTMX27*), a long, non-coding RNA bearing a nta-miRX27 binding site. Topping reduced *nta-miRX27* expression and enhanced both *NtQPT2* and *nta-eTMX27*, with clear negative correlations (Li et al., 2015; Xie and Fan, 2016). In addition to regulation at transcription level and post-transcription level (e.g., alternative splicing of pre-mRNAs, miRNA and its target mimicry), modulation at protein levels may be also important. De Boar et al. (2011) reported that in a transient expression assay using BY2 cells, JAM1, an MAPK kinase, enhanced the NtMYC2 (*NbbHLH1*) activity by ~10 fold, and an ERF221 (ORC1) activity by a few fold, suggesting that phosphorylation of key TFs (probably pathway genes as well) plays a pivotal role in the post-translational level regulation of nicotine biosynthesis.

4.8 NINJA is a co-repressor for MYC2 activity

The performance of the *NINJA* knockdown plants was complicated. Careful sort out of the data showed that knockdown of *NINJA* expression, in general, enhanced transcript levels of early genes in JA signaling (*MYC2a*) and nicotine synthesis (*PMT1a and QPT2*), indicating

that *NINJA* is a negative regulator of JA signaling. It is particularly clear in the NINJA_5 plant, where the *NINJA* gene was knocked down the most. However, somehow the high *NtPMT1a* and *NtQPT2* transcripts did not seem to "translate" into higher nicotine levels.

The original idea to look at expression of *NtJAZ3* and *NtJAZ12* was to see whether knockdown of *NINJA* gene makes the plants to switch to NINJA-independent mode by using EAR-containing JAZ proteins (NtJAZ12 in tobacco) to directly bind to TPL as it does in Arabidopsis (Pouwels et al., 2010; Pouwels and Goossens, 2011; Shyu, et al., 2012). The results are inconclusive since both JAZ3 and JAZ12 expression went up with JAZ3 expression increasing more. There are four EAR-containing JAZ proteins in Arabidopsis (JAZ5, JAZ6, JAZ7 and JAZ8) and they are speculated to form NINJA-independent pathway for JA signaling regulation (Pouwels and Goossens, 2011; Shyu et al., 2012; Goossens et al., 2016). We only identified two such proteins in tobacco (NtJAZ12a and NtJAZ12b), their functions in JA signaling and nicotine synthesis in tobacco remain to be elucidated.

As for NtJAZ3 is concerned, its high expression in the *NINJA* RNAi plants could be explained by the fact that JAZ3 inhibits transcription of JA-responsive genes by competing with the mediator subunit MED25 for binding to MYC3, independent of NINJA co-repressor, as shown in Arabidopsis (Zhang et al., 2015). A recent crystal structure study of JAZ9-MYC3 protein complex demonstrated that JAZ proteins (JAZ1 and JAZ9) and MED25 share the same binding surface of MYC3 and overexpression of JAZ proteins destabilized MYC3-MED25 complex in Arabidopsis (Zhang et al., 2015). The same mutually exclusive binding of MYC2 between JAZ3 and MED25 should also exist. Another explanation for

NtJAZ3 expression going so high could be: In the repressor complex, NINJA interaction with JAZ (such as JAZ3) may help to stabilize JAZ as a repressor to MYC2. JA-Ile helps removal of JAZ from this complex for its degradation. Reduced NINJA level could further destabilize JAZ, which in turn, triggers replenishment of JAZ to attenuate JA signaling (Thines et al., 2007). It may not be coincident that repression of *JAZ* genes by RNAi also led to transgenic tobacco plants with less nicotine accumulation (Yang et al., 2015), indicating a complicated relationship between JA signaling and its attenuation.

Another interesting phenomenon observed in this project is that even we cannot tell the target gene transcript level is altered, as shown in NINJA_7 and NINJA_8 lines (Figure 3.3.1), the downstream gene expression and nicotine levels were still impacted as the lines with reduced *NINJA* expression (Figures 3.3.2 and 3.3.3), indicating the power of the RNA interference. It is not clear whether it is the case specific for *NINJA* gene or it is a more general case that we didn't realize before.

4.9 Conclusions

In this thesis study, using Y2H, I isolated a clone encoding NtJAZ3 as an interacting protein with NtMYC2a, confirming previous observations (Shoji et al., 2008; Zhang et al., 2012). A few other promising clones were also isolated and partially characterized. More experiments are needed to determine whether they do interact with MYC2a *in vivo*. In addition, we overexpressed five key JA biosynthesis pathway genes to see whether they increase JA (especially its bioactive form, JA-Ile) content, expression of key nicotine biosynthesis pathway genes, and nicotine levels. Although JA content data did not look reliable, in most cases, topping did enhance expression of nicotine pathway genes substantially (often

doubled) in transgenic plants. However, nicotine levels were not significantly changed. In addition, two RNAi constructs were also introduced into tobacco plants in attempts to reduce degradation of JA-Ile, or to reduce the cellular level of a JA signaling co-repressor, NINJA. Nicotine levels in these plants were not affected or were even unexpectedly reduced in the case of *NINJA* RNAi. Our results and previous results in overexpressing *PMT1a* and *QPT2* (Wang, 2011) showed that many approaches could enhance *PMT1a* and *QPT2* transcript levels but not nicotine content. It seems to point to a missing factor or factors, which either controls translation of *PMT1a* and/or *QPT2* transcripts, or activation of the translated enzymes (e.g., by phosphorylation, or dimerization and de-dimerization). Alternatively, it may control the availability of the key substrates of nicotine synthesis pathway. In addition, expression of this factor(s) has to be tightly controlled by a threshold of MYC2: Only high concentration of MYC2 can turn on the pathway. The results indicate that molecular regulation of nicotine biosynthesis in tobacco is more complicated than we originally thought. It is tightly regulated due to the involvement of at least three major phytohormones, JA, auxin, and ethylene, and a toxic major product, nicotine.

Literature Cited

Acosta IF, Gasperini D, Chetelat A, Stolz S, Santuari L, Farmer EE (2013) Role of NINJA in root jasmonate signaling. Proc Natl Acad Sci U S A **110:** 15473-15478

Baldwin IT, Halitschke R, Kessler A, Schittko U (2001) Merging molecular and ecological approaches in plant–insect interactions. Curr Opin Plant Biol **4:** 351-358

Baldwin IT, Schmelz EA, Ohnmeiss TE (1994) Wound-induced changes in root and shoot jasmonic acid pools correlate with induced nicotine synthesis inNicotiana sylvestris spegazzini and comes. J Chem Ecol **20:** 2139-2157

Baldwin IT, Zhang Z, Diab N, Ohnmeiss TE, McCloud ES, Lynds GY, Schmelz EA (1997) Quantification, correlations and manipulations of wound-induced changes in jasmonic acid and nicotine in Nicotiana sylvestris. Planta **201:** 397-404

Bell E, Creelman RA, Mullet JE (1995) A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in Arabidopsis. Proc Natl Acad Sci U S A **92:** 8675-8679

Bogani P, Liò P, Intrieri MC, Buiatti M (1997) A Physiological and Molecular Analysis of the Genus< i> Nicotiana. Mol Phylogenet Evol **7:** 62-70

Boke H, Ozhuner E, Turktas M, Parmaksiz I, Ozcan S, Unver T (2015) Regulation of the alkaloid biosynthesis by miRNA in opium poppy. Plant biotechnology journal **13:** 409-420

Borrell A, Culianez-Macia FA, Altabella T, Besford RT, Flores D, Tiburcio AF (1995) Arginine Decarboxylase Is Localized in Chloroplasts. Plant Physiol **109:** 771-776

Breithaupt C, Kurzbauer R, Lilie H, Schaller A, Strassner J, Huber R, Macheroux P, Clausen T (2006) Crystal structure of 12-oxophytodienoate reductase 3 from tomato: self-inhibition by dimerization. Proc Natl Acad Sci U S A **103:** 14337-14342

Brown G, Terrill T (1973) Effects of Method of Harvest on Flue-Cured Tobacco Part II. Chemical Components. Agron J **65:** 268-273

Caldelari D, Wang G, Farmer EE, Dong X (2011) Arabidopsis lox3 lox4 double mutants are male sterile and defective in global proliferative arrest. Plant Mol Biol **75:** 25-33

Cane KA, Mayer M, Lidgett AJ, Michael AJ, Hamill JD (2005) Molecular analysis of alkaloid metabolism in AABB v. aabb genotype Nicotiana tabacum in response to wounding of aerial tissues and methyl jasmonate treatment of cultured roots. Functional Plant Biology **32:** 305-320

Carretero-Paulet L, Galstyan A, Roig-Villanova I, Martinez-Garcia JF, Bilbao-Castro JR, Robertson DL (2010) Genome-wide classification and evolutionary analysis of the bHLH family of transcription factors in Arabidopsis, poplar, rice, moss, and algae. Plant Physiol **153:** 1398-1412

Chauvin A, Caldelari D, Wolfender J, Farmer EE (2013) Four 13-lipoxygenases contribute to rapid jasmonate synthesis in wounded Arabidopsis thaliana leaves: a role for lipoxygenase 6 in responses to long-distance wound signals. New Phytol **197:** 566-575

Chen Q, Sun J, Zhai Q, Zhou W, Qi L, Xu L, Wang B, Chen R, Jiang H, Qi J, Li X, Palme K, Li C (2011) The basic helix-loop-helix transcription factor MYC2 directly represses PLETHORA expression during jasmonate-mediated modulation of the root stem cell niche in Arabidopsis. Plant Cell **23:** 3335-3352

Chen R, Jiang H, Li L, Zhai Q, Qi L, Zhou W, Liu X, Li H, Zheng W, Sun J, Li C (2012) The Arabidopsis mediator subunit MED25 differentially regulates jasmonate and abscisic acid signaling through interacting with the MYC2 and ABI5 transcription factors. Plant Cell **24:** 2898-2916

Chini A, Fonseca S, Fernandez G, Adie B, Chico J, Lorenzo O, Garcia-Casado G, Lopez-Vidriero I, Lozano F, Ponce M (2007) The JAZ family of repressors is the missing link in jasmonate signalling. Nature **448:** 666-671

Chini A, Fonseca S, Chico JM, Fernandez-Calvo P, Solano R (2009) The ZIM domain mediates homo- and heteromeric interactions between Arabidopsis JAZ proteins. Plant Journal **59:** 77-87

Chintapakorn Y, Hamill JD (2007) Antisense-mediated reduction in ADC activity causes minor alterations in the alkaloid profile of cultured hairy roots and regenerated transgenic plants of Nicotiana tabacum. Phytochemistry **68:** 2465-2479

Chintapakorn Y, Hamill JD (2003) Antisense-mediated down-regulation of putrescine N-methyltransferase activity in transgenic Nicotiana tabacum L. can lead to elevated levels of anatabine at the expense of nicotine. Plant Mol Biol **53:** 87-105

Chung HS, Howe GA (2009) A critical role for the TIFY motif in repression of jasmonate signaling by a stabilized splice variant of the JASMONATE ZIM-domain protein JAZ10 in Arabidopsis. Plant Cell **21:** 131-145

Conconi A, Miquel M, Browse JA, Ryan CA (1996) Intracellular Levels of Free Linolenic and Linoleic Acids Increase in Tomato Leaves in Response to Wounding. Plant Physiol **111:** 797-803

Collins, P., Sarji, N., Williams, J. (1969). Determination of nicotine alkaloids in tobacco using the autoanalyzer. Tob Sci, **13**: 79-81

Dai XY, Su YR, Wei WX, Wu JS, Fan YK (2009) Effects of top excision on the potassium accumulation and expression of potassium channel genes in tobacco. J Exp Bot **60:** 279-289

Davis, R. E (1976). A combined automated procedure for the determination of reducing sugars and nicotine alkaloids in tobacco products using a new reducing sugar method. Tob.Sci, **20**: 139-144

De Boer K, Tilleman S, Pauwels L, Vanden Bossche R, De Sutter V, Vanderhaeghen R, Hilson P, Hamill JD, Goossens A (2011) APETALA2/ETHYLENE RESPONSE FACTOR and basic helix–loop–helix tobacco transcription factors cooperatively mediate jasmonate-elicited nicotine biosynthesis. The Plant Journal **66:** 1053-1065

DeBoer KD, Dalton HL, Edward FJ, Hamill JD (2011) RNAi-mediated down-regulation of ornithine decarboxylase (ODC) leads to reduced nicotine and increased anatabine levels in transgenic< i> Nicotiana tabacum L. Phytochemistry **72:** 344-355

DeBoer KD, Lye JC, Aitken CD, Su AK, Hamill JD (2009) The A622 gene in Nicotiana glauca (tree tobacco): evidence for a functional role in pyridine alkaloid synthesis. Plant Mol Biol **69:** 299-312

Dewey RE, Xie J (2013) Molecular genetics of alkaloid biosynthesis in Nicotiana tabacum. Phytochemistry **94:** 10-27

Dombrecht B, Xue GP, Sprague SJ, Kirkegaard JA, Ross JJ, Reid JB, Fitt GP, Sewelam N, Schenk PM, Manners JM, Kazan K (2007) MYC2 differentially modulates diverse jasmonate-dependent functions in Arabidopsis. Plant Cell **19:** 2225-2245

Eamens A, Wang MB, Smith NA, Waterhouse PM (2008) RNA silencing in plants: yesterday, today, and tomorrow. Plant Physiol **147:** 456-468

Edwards K, Johnstone C, Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res **19:** 1349

Elliot J (1966) Some effects of topping five flue-cured tobacco varieties at three stages of floral development. Tobacco Science **10:** 100-104

Flavell RB (1994) Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc Natl Acad Sci U S A **91:** 3490-3496

Ellis C, Karafyllidis I, Wasternack C, Turner JG (2002) The Arabidopsis mutant cev1 links cell wall signaling to jasmonate and ethylene responses. Plant Cell **14:** 1557-1566

Fang Y, Spector DL (2010) BiFC imaging assay for plant protein-protein interactions. Cold Spring Harbor Protocols **2010:** pdb. prot5380

Fernandez-Calvo P, Chini A, Fernandez-Barbero G, Chico J, Gimenez-Ibanez S, Geerinck J, Eeckhout D, Schweizer F, Godoy M, Manuel Franco-Zorrilla J, Pauwels L, Witters E, Isabel Puga M, Paz-Ares J, Goossens A, Reymond P, De Jaeger G, Solano R (2011) The Arabidopsis bHLH Transcription Factors MYC3 and MYC4 Are Targets of JAZ Repressors and Act Additively with MYC2 in the Activation of Jasmonate Responses. Plant Cell **23:** 701-715

Feth F, Wagner R, Wagner K (1986) Regulation in tobacco callus of enzyme activities of the nicotine pathway. Planta **168:** 402-407

Fu Y, Guo H, Cheng Z, Wang R, Li G, Huo G, Liu W (2013) NtNAC-R1, a novel NAC transcription factor gene in tobacco roots, responds to mechanical damage of shoot meristem. Plant physiology and biochemistry **69:** 74-81

Gasperini D, Chételat A, Acosta IF, Goossens J, Pauwels L, Goossens A, Dreos R, Alfonso E, Farmer EE (2015) Multilayered organization of jasmonate signalling in the regulation of root growth. PLoS Genet **11:** e1005300

Gelvin SB (2003) Agrobacterium-mediated plant transformation: the biology behind the "gene-jockeying" tool. Microbiol Mol Biol Rev **67:** 16-37

Glauser G, Wolfender J (2013) A non-targeted approach for extended liquid chromatography-mass spectrometry profiling of free and esterified jasmonates after wounding. Jasmonate Signaling: Methods and Protocols 123-134

Glauser G, Dubugnon L, Mousavi SA, Rudaz S, Wolfender JL, Farmer EE (2009) Velocity estimates for signal propagation leading to systemic jasmonic acid accumulation in wounded Arabidopsis. J Biol Chem **284:** 34506-34513

Goossens J, Swinnen G, Vanden Bossche R, Pauwels L, Goossens A (2015) Change of a conserved amino acid in the MYC2 and MYC3 transcription factors leads to release of JAZ repression and increased activity. New Phytol **206:** 1229-1237

Goossens J, Fernández-Calvo P, Schweizer F, Goossens A (2016) Jasmonates: signal transduction components and their roles in environmental stress responses. Plant Mol Biol 1-17

Goossens A, Hakkinen ST, Laakso I, Seppanen-Laakso T, Biondi S, De Sutter V, Lammertyn F, Nuutila AM, Soderlund H, Zabeau M, Inze D, Oksman-Caldentey KM (2003) A functional genomics approach toward the understanding of secondary metabolism in plant cells. Proc Natl Acad Sci U S A **100:** 8595-8600

Harms K, Atzorn R, Brash A, Kuhn H, Wasternack C, Willmitzer L, Pena-Cortes H (1995) Expression of a Flax Allene Oxide Synthase cDNA Leads to Increased Endogenous

Jasmonic Acid (JA) Levels in Transgenic Potato Plants but Not to a Corresponding Activation of JA-Responding Genes. Plant Cell **7:** 1645-1654

Hashimoto T, Shoji T, Mihara T, Oguri H, Tamaki K, Suzuki K, Yamada Y (1998) Intraspecific variability of the tandem repeats in Nicotiana putrescine N-methyltransferases. Plant Mol Biol **37:** 25-37

Hause B, Stenzel I, Miersch O, Maucher H, Kramell R, Ziegler J, Wasternack C (2000) Tissue-specific oxylipin signature of tomato flowers: allene oxide cyclase is highly expressed in distinct flower organs and vascular bundles. The Plant Journal **24:** 113-126

Hause B, Hause G, Kutter C, Miersch O, Wasternack C (2003) Enzymes of jasmonate biosynthesis occur in tomato sieve elements. Plant Cell Physiol **44:** 643-648

Heim WG, Sykes KA, Hildreth SB, Sun J, Lu R, Jelesko JG (2007) Cloning and characterization of a< i> Nicotiana tabacum methylputrescine oxidase transcript. Phytochemistry **68:** 454-463

Heitz T, Widemann E, Lugan R, Miesch L, Ullmann P, Desaubry L, Holder E, Grausem B, Kandel S, Miesch M, Werck-Reichhart D, Pinot F (2012) Cytochromes P450 CYP94C1 and CYP94B3 catalyze two successive oxidation steps of plant hormone Jasmonoyl-isoleucine for catabolic turnover. J Biol Chem **287:** 6296-6306

Hibi N, Higashiguchi S, Hashimoto T, Yamada Y (1994) Gene expression in tobacco low-nicotine mutants. Plant Cell **6:** 723-735

Hildreth SB (2009) Investigation of Protein â Protein Interactions among Nicotine Biosynthetic Enzymes and Characterization of a Nicotine Transporter.

Horsch RB, Fry J, Hoffmann N, Neidermeyer J, Rogers SG, Fraley RT (1989) Leaf disc transformation. Plant molecular biology manual. Springer, , pp 63-71

Hua Z, Vierstra RD (2011) The cullin-RING ubiquitin-protein ligases. Annual review of plant biology **62:** 299-334

Hummel GM, Naumann M, Schurr U, Walter A (2007) Root growth dynamics of Nicotiana attenuata seedlings are affected by simulated herbivore attack. Plant, Cell Environ **30:** 1326-1336

Imanishi S, Hashizume K, Nakakita M, Kojima H, Matsubayashi Y, Hashimoto T, Sakagami Y, Yamada Y, Nakamura K (1998) Differential induction by methyl jasmonate of genes encoding ornithine decarboxylase and other enzymes involved in nicotine biosynthesis in tobacco cell cultures. Plant Mol Biol **38:** 1101-1111

Ishiguro S, Kawai-Oda A, Ueda J, Nishida I, Okada K (2001) The DEFECTIVE IN ANTHER DEHISCIENCE gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in Arabidopsis. Plant Cell **13:** 2191-2209

Jiang K, Liao Z, Pi Y, Huang Z, Hou R, Cao Y, Wang Q, Sun X, Tang K (2008) Molecular cloning and expression profile of a jasmonate biosynthetic pathway gene for allene oxide cyclase from Hyoscyamus niger. Mol Biol (NY) **42:** 381-390

Jiang K, Pi Y, Hou R, Jiang L, Sun X, Tang K (2009) Promotion of nicotine biosynthesis in transgenic tobacco by overexpressing allene oxide cyclase from Hyoscyamus niger. Planta **229:** 1057-1063

Riechers DE, Timko MP (1999) Structure and expression of the gene family encoding putrescine N-methyltransferase in Nicotiana tabacum: new clues to the evolutionary origin of cultivated tobacco. Plant Mol Biol **41:** 387-401

Jung C, Zhao P, Seo JS, Mitsuda N, Deng S, Chua NH (2015) PLANT U-BOX PROTEIN10 Regulates MYC2 Stability in Arabidopsis. Plant Cell **27:** 2016-2031

Kajikawa M, Hirai N, Hashimoto T (2009) A PIP-family protein is required for biosynthesis of tobacco alkaloids. Plant Mol Biol **69:** 287-298

Kajikawa M, Shoji T, Kato A, Hashimoto T (2011) Vacuole-localized berberine bridge enzyme-like proteins are required for a late step of nicotine biosynthesis in tobacco. Plant Physiol **155:** 2010-2022

Kallenbach M, Alagna F, Baldwin IT, Bonaventure G (2010) Nicotiana attenuata SIPK, WIPK, NPR1, and fatty acid-amino acid conjugates participate in the induction of jasmonic acid biosynthesis by affecting early enzymatic steps in the pathway. Plant Physiol **152:** 96-106

Karimi M, Inzé D, Depicker A (2002) GATEWAY™ vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci **7:** 193-195

Kato K, Shoji T, Hashimoto T (2014) Tobacco nicotine uptake permease regulates the expression of a key transcription factor gene in the nicotine biosynthesis pathway. Plant Physiol **166:** 2195-U1500

Katoh A, Shoji T, Hashimoto T (2007) Molecular cloning of N-methylputrescine oxidase from tobacco. Plant Cell Physiol **48:** 550-554

Katoh A, Uenohara K, Akita M, Hashimoto T (2006) Early steps in the biosynthesis of NAD in Arabidopsis start with aspartate and occur in the plastid. Plant Physiol **141:** 851-857

Katsir L, Schilmiller AL, Staswick PE, He SY, Howe GA (2008) COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. Proceedings of the National Academy of Sciences **105:** 7100-7105

Kazan K, Manners JM (2008) Jasmonate signaling: toward an integrated view. Plant Physiol **146:** 1459-1468

Kazan K, Manners JM (2013) MYC2: The Master in Action. Molecular Plant **6:** 686-703

Khraiwesh B, Zhu J, Zhu J (2012) Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants. Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms **1819:** 137-148

Kidd SK, Melillo AA, Lu R, Reed DG, Kuno N, Uchida K, Furuya M, Jelesko JG (2006) The A and B loci in tobacco regulate a network of stress response genes, few of which are associated with nicotine biosynthesis. Plant Mol Biol **60:** 699-716

Koo AJ, Howe GA (2009) The wound hormone jasmonate. Phytochemistry **70:** 1571-1580

Kubigsteltig I, Laudert D, Weiler E (1999) Structure and regulation of the Arabidopsis thaliana allene oxide synthase gene. Planta **208:** 463-471

Laudert D, Pfannschmidt U, Lottspeich F, Holländer-Czytko H, Weiler EW (1996) Cloning, molecular and functional characterization of Arabidopsis thaliana allene oxide synthase (CYP 74), the first enzyme of the octadecanoid pathway to jasmonates. Plant Mol Biol **31:** 323-335

Laudert D, Schaller F, Weiler EW (2000) Transgenic Nicotiana tabacum and Arabidopsis thaliana plants overexpressing allene oxide synthase. Planta **211:** 163-165

Laudert D, Weiler EW (1998) Allene oxide synthase: a major control point in Arabidopsis thaliana octadecanoid signalling. The Plant Journal **15:** 675-684

Layten Davis D, Nielsen MT (1999) Tobacco: production, chemistry and technology. Blackwell Science Ltd,

LeClere S, Tellez R, Rampey RA, Matsuda SP, Bartel B (2002) Characterization of a family of IAA-amino acid conjugate hydrolases from Arabidopsis. J Biol Chem **277:** 20446-20452

Legg PD, Collins G (1971) Inheritance of per cent total alkaloids in Nicotiana tabacum L. II. Genetic effects of two loci in burley 21× LA burley 21 populations. Canadian Journal of Genetics and Cytology **13:** 287-291

Lewis RS, Lopez HO, Bowen SW, Andres KR, Steede WT, Dewey RE (2015) Transgenic and Mutation-Based Suppression of a Berberine Bridge Enzyme-Like (BBL) Gene Family Reduces Alkaloid Content in Field-Grown Tobacco. PloS one **10:** e0117273

Li F, Wang W, Zhao N, Xiao B, Cao P, Wu X, Ye C, Shen E, Qiu J, Zhu QH, Xie J, Zhou X, Fan L (2015) Regulation of Nicotine Biosynthesis by an Endogenous Target Mimicry of MicroRNA in Tobacco. Plant Physiol **169:** 1062-1071

Major IT, Constabel CP (2006) Molecular analysis of poplar defense against herbivory: comparison of wound-and insect elicitor-induced gene expression. New Phytol **172:** 617-635

Malmberg RL, Watson MB, Galloway GL, Yu W (1998) Molecular genetic analyses of plant polyamines. Crit Rev Plant Sci **17:** 199-224

Matzke MA, Matzke A (1995) How and Why Do Plants Inactivate Homologous (Trans)genes? Plant Physiol **107:** 679-685

McGinnis KM (2010) RNAi for functional genomics in plants. Brief Funct Genomics **9:** 111-117

Miersch O, Neumerkel J, Dippe M, Stenzel I, Wasternack C (2008) Hydroxylated jasmonates are commonly occurring metabolites of jasmonic acid and contribute to a partial switch-off in jasmonate signaling. New Phytol **177:** 114-127

Miersch O, Weichert H, Stenzel I, Hause B, Maucher H, Feussner I, Wasternack C (2004) Constitutive overexpression of allene oxide cyclase in tomato (Lycopersicon esculentum cv. Lukullus) elevates levels of some jasmonates and octadecanoids in flower organs but not in leaves. Phytochemistry **65:** 847-856

Moreno JE, Shyu C, Campos ML, Patel LC, Chung HS, Yao J, He SY, Howe GA (2013) Negative Feedback Control of Jasmonate Signaling by an Alternative Splice Variant of JAZ10. Plant Physiol **162:** 1006-1017

Murad L, Lim KY, Christopodulou V, Matyasek R, Lichtenstein CP, Kovarik A, Leitch AR (2002) The origin of tobacco's T genome is traced to a particular lineage within Nicotiana tomentosiformis (Solanaceae). Am J Bot **89:** 921-928

Naconsie M, Kato K, Shoji T, Hashimoto T (2014) Molecular evolution of N-methylputrescine oxidase in tobacco. Plant Cell Physiol **55:** 436-444

Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. Plant Cell **2:** 279-289

Pauwels L, Barbero GF, Geerinck J, Tilleman S, Grunewald W, Pérez AC, Chico JM, Bossche RV, Sewell J, Gil E (2010) NINJA connects the co-repressor TOPLESS to jasmonate signalling. Nature **464:** 788-791

Pauwels L, Goossens A (2011) The JAZ proteins: a crucial interface in the jasmonate signaling cascade. Plant Cell **23:** 3089-3100

Polosa R, Rodu B, Caponnetto P, Maglia M, Raciti C (2013) A fresh look at tobacco harm reduction: the case for the electronic cigarette. Harm Reduct J **10:** 19

Qi Y, Guo H, Li K, Liu W (2012) Comprehensive analysis of differential genes and miRNA profiles for discovery of topping-responsive genes in flue-cured tobacco roots. FEBS Journal **279:** 1054-1070

Quik M, O'Leary K, Tanner CM (2008) Nicotine and Parkinson's disease: implications for therapy. Movement Disorders **23:** 1641-1652

Reed DG, Jelesko JG (2004) The A and B loci of Nicotiana tabacum have non-equivalent effects on the mRNA levels of four alkaloid biosynthetic genes. Plant Science **167:** 1123-1130

Riechers DE, Timko MP (1999) Structure and expression of the gene family encoding putrescine N-methyltransferase in Nicotiana tabacum: new clues to the evolutionary origin of cultivated tobacco. Plant Mol Biol **41:** 387-401

Ryan CA, Pearce G (1998) Systemin: a polypeptide signal for plant defensive genes. Annu Rev Cell Dev Biol **14:** 1-17

Saitoh F, Noma M, Kawashima N (1985) The alkaloid contents of sixty< i> Nicotiana species. Phytochemistry **24:** 477-480

Sato F, Hashimoto T, Hachiya A, Tamura K, Choi K, Morishige T, Fujimoto H, Yamada Y (2001) Metabolic engineering of plant alkaloid biosynthesis. Proceedings of the National Academy of Sciences **98:** 367-372

Saunders JW, Bush LP (1979) Nicotine Biosynthetic Enzyme Activities in Nicotiana tabacum L. Genotypes with Different Alkaloid Levels. Plant Physiol **64:** 236-240

Schilmiller AL, Howe GA (2005) Systemic signaling in the wound response. Curr Opin Plant Biol **8:** 369-377

Sears MT, Zhang H, Rushton PJ, Wu M, Han S, Spano AJ, Timko MP (2014) NtERF32: a non-NIC2 locus AP2/ERF transcription factor required in jasmonate-inducible nicotine biosynthesis in tobacco. Plant Mol Biol **84:** 49-66

Seo HS, Song JT, Cheong JJ, Lee YH, Lee YW, Hwang I, Lee JS, Choi YD (2001) Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. Proc Natl Acad Sci U S A **98:** 4788-4793

Seo S, Sano H, Ohashi Y (1999) Jasmonate-based wound signal transduction requires activation of WIPK, a tobacco mitogen-activated protein kinase. Plant Cell **11:** 289-298

Sheard LB, Tan X, Mao H, Withers J, Ben-Nissan G, Hinds TR, Kobayashi Y, Hsu F, Sharon M, Browse J (2010) Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. Nature **468:** 400-405

Shiraishi F, Suzuki Y (2008) Method for determination of the main bottleneck enzyme in a metabolic reaction network by dynamic sensitivity analysis. Ind Eng Chem Res **48:** 415-423

Shoji T, Hashimoto T (2011a) Recruitment of a duplicated primary metabolism gene into the nicotine biosynthesis regulon in tobacco. The Plant Journal **67:** 949-959

Shoji T, Hashimoto T (2011b) Tobacco MYC2 regulates jasmonate-inducible nicotine biosynthesis genes directly and by way of the NIC2-locus ERF genes. Plant Cell Physiol **52:** 1117-1130

Shoji T, Hashimoto T (2008) Why does anatabine, but not nicotine, accumulate in jasmonate-elicited cultured tobacco BY-2 cells? Plant Cell Physiol **49:** 1209-1216

Shoji T, Inai K, Yazaki Y, Sato Y, Takase H, Shitan N, Yazaki K, Goto Y, Toyooka K, Matsuoka K, Hashimoto T (2009) Multidrug and toxic compound extrusion-type transporters implicated in vacuolar sequestration of nicotine in tobacco roots. Plant Physiol **149:** 708-718

Shoji T, Kajikawa M, Hashimoto T (2010) Clustered transcription factor genes regulate nicotine biosynthesis in tobacco. Plant Cell **22:** 3390-3409

Shoji T, Ogawa T, Hashimoto T (2008) Jasmonate-induced nicotine formation in tobacco is mediated by tobacco COI1 and JAZ genes. Plant Cell Physiol **49:** 1003-1012

Shoji T, Winz R, Iwase T, Nakajima K, Yamada Y, Hashimoto T (2002) Expression patterns of two tobacco isoflavone reductase-like genes and their possible roles in secondary metabolism in tobacco. Plant Mol Biol **50:** 427-440

Shoji T, Yamada Y, Hashimoto T (2000) Jasmonate induction of putrescine N-methyltransferase genes in the root of Nicotiana sylvestris. Plant Cell Physiol **41:** 831-839

Shyu C, Figueroa P, Depew CL, Cooke TF, Sheard LB, Moreno JE, Katsir L, Zheng N, Browse J, Howe GA (2012) JAZ8 lacks a canonical degron and has an EAR motif that

mediates transcriptional repression of jasmonate responses in Arabidopsis. Plant Cell **24:** 536-550

Sierro N, Battey JN, Ouadi S, Bakaher N, Bovet L, Willig A, Goepfert S, Peitsch MC, Ivanov NV (2014) The tobacco genome sequence and its comparison with those of tomato and potato. Nature communications **5:**

Sierro N, Battey J, Ouadi S, Bovet L, Goepfert S, Bakaher N, Peitsch MC, Ivanov NV (2013) Reference genomes and transcriptomes of Nicotiana sylvestris and Nicotiana tomentosiformis. Genome Biol **14:** R60

SILVETTE H, HOFF EC, LARSON PS, HAAG HB (1962) The actions of nicotine on central nervous system functions. Pharmacol Rev **14:** 137-173

Sinclair SJ, Murphy KJ, Birch CD, Hamill JD (2000) Molecular characterization of quinolinate phosphoribosyltransferase (QPRTase) in Nicotiana. Plant Mol Biol **44:** 603-617

Sisson V, Severson R (1990) Alkaloid composition of the Nicotiana species. Beiträge zur Tabakforschung International **14:** 327-339

Song W (1997) Molecular characterizations of two tobacco root-specific genes: TobRB7 and NtQPT1. PhD thesis. 1997. North Carolina State University, Raleigh, North Carolina

Staswick PE, Tiryaki I (2004) The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in Arabidopsis. Plant Cell **16:** 2117-2127

Stenzel I, Hause B, Maucher H, Pitzschke A, Miersch O, Ziegler J, Ryan CA, Wasternack C (2003) Allene oxide cyclase dependence of the wound response and vascular bundle-specific generation of jasmonates in tomato–amplification in wound signalling. The Plant Journal **33:** 577-589

Stenzel I, Otto M, Delker C, Kirmse N, Schmidt D, Miersch O, Hause B, Wasternack C (2012) ALLENE OXIDE CYCLASE (AOC) gene family members of Arabidopsis thaliana: tissue- and organ-specific promoter activities and in vivo heteromerization. J Exp Bot **63**: 6125-6138

Stintzi A, Browse J (2000) The Arabidopsis male-sterile mutant, opr3, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. Proc Natl Acad Sci USA **97:** 10625-10630

Stitz M, Gase K, Baldwin IT, Gaquerel E (2011) Ectopic expression of AtJMT in Nicotiana attenuata: creating a metabolic sink has tissue-specific consequences for the jasmonate metabolic network and silences downstream gene expression. Plant Physiol **157:** 341-354

Suza WP, Staswick PE (2008) The role of JAR1 in jasmonoyl-L-isoleucine production during Arabidopsis wound response. Planta **227:** 1221-1232

Takahashi F, Yoshida R, Ichimura K, Mizoguchi T, Seo S, Yonezawa M, Maruyama K, Yamaguchi-Shinozaki K, Shinozaki K (2007) The mitogen-activated protein kinase cascade MKK3-MPK6 is an important part of the jasmonate signal transduction pathway in Arabidopsis. Plant Cell **19:** 805-818

Tang S, Wang Y, Li Z, Gui Y, Xiao B, Xie J, Zhu Q, Fan L (2012) Identification of wounding and topping responsive small RNAs in tobacco (Nicotiana tabacum). BMC plant biology **12:** 1

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J (2007) JAZ repressor proteins are targets of the SCFCOI1 complex during jasmonate signalling. Nature **448:** 661-665

Thireault C, Shyu C, Yoshida Y, St Aubin B, Campos ML, Howe GA (2015) Repression of jasmonate signaling by a non-TIFY JAZ protein in Arabidopsis. The Plant Journal **82:** 669-679

Todd AT, Liu E, Polvi SL, Pammett RT, Page JE (2010) A functional genomics screen identifies diverse transcription factors that regulate alkaloid biosynthesis in Nicotiana benthamiana. Plant Journal **62:** 589-600

Vellosillo T, Martinez M, Lopez MA, Vicente J, Cascon T, Dolan L, Hamberg M, Castresana C (2007) Oxylipins produced by the 9-lipoxygenase pathway in Arabidopsis regulate lateral root development and defense responses through a specific signaling cascade. Plant Cell **19:** 831-846

van der Krol AR, Mur LA, Beld M, Mol JN, Stuitje AR (1990) Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell **2:** 291-29

Wagner R, Wagner K (1985) The pyridine-nucleotide cycle in tobacco Enzyme activities for the de-novo synthesis of NAD. Planta **165:** 532-537

Walter M, Chaban C, Schütze K, Batistic O, Weckermann K, Näke C, Blazevic D, Grefen C, Schumacher K, Oecking C (2004) Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. The Plant Journal **40:** 428-438

Walton NJ, McLauchlan WR (1990) Diamine oxidation and alkaloid production in transformed root cultures of Nicotiana tabacum. Phytochemistry **29:** 1455-1457

Wang B. (2011) Factors in Nicotine Biosynthesis in Tobacco. PhD thesis. North Carolina State University, Raleigh, North Carolina

Wang B, Lewis RS, Shi J, Song Z, Gao Y, Li W, Chen H, Qu R (2015) Genetic Factors for Enhancement of Nicotine Levels in Cultivated Tobacco. Sci Rep **5:** 17360

Wang C, Avdiushko S, Hildebrand DF (1999) Overexpression of a cytoplasm-localized allene oxide synthase promotes the wound-induced accumulation of jasmonic acid in transgenic tobacco. Plant Mol Biol **40:** 783-793

Wang J, Sheehan M, Brookman H, Timko MP (2000) Characterization of cDNAs differentially expressed in roots of tobacco (Nicotiana tabacum cv Burley 21) during the early stages of alkaloid biosynthesis. Plant Science **158:** 19-32

Wang L, Halitschke R, Kang J, Berg A, Harnisch F, Baldwin IT (2007) Independently silencing two JAR family members impairs levels of trypsin proteinase inhibitors but not nicotine. Planta **226:** 159-167

Wang L, Wu J (2013) The Essential Role of Jasmonic Acid in Plant–Herbivore Interactions–Using the Wild Tobacco Nicotiana attenuata as a Model. Journal of Genetics and Genomics **40:** 597-606

Wang P, Liang Z, Zeng J, Li W, Sun X, Miao Z, Tang K (2008) Generation of tobacco lines with widely different reduction in nicotine levels via RNA silencing approaches. J Biosci **33:** 177-184

Wang B, Lewis RS, Shi J, Song Z, Gao Y, Li W, Chen H, Qu R (2015) Genetic Factors for Enhancement of Nicotine Levels in Cultivated Tobacco. Sci Rep **5:** 17360

Wasternack C (2007) Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. Ann Bot **100:** 681-697

Wasternack C, Hause B (2013) Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. Ann Bot **111:** 1021-1058

Winz RA, Baldwin IT (2001) Molecular interactions between the specialist herbivore Manduca sexta (Lepidoptera, Sphingidae) and its natural host Nicotiana attenuata. IV. Insect-Induced ethylene reduces jasmonate-induced nicotine accumulation by regulating putrescine N-methyltransferase transcripts. Plant Physiol **125:** 2189-2202

Withers J, Yao J, Mecey C, Howe GA, Melotto M, He SY (2012) Transcription factor-dependent nuclear localization of a transcriptional repressor in jasmonate hormone signaling. Proc Natl Acad Sci U S A **109:** 20148-20153

Woldemariam MG, Onkokesung N, Baldwin IT, Galis I (2012) Jasmonoyl-l-isoleucine hydrolase 1 (JIH1) regulates jasmonoyl-l-isoleucine levels and attenuates plant defenses against herbivores. The Plant Journal **72:** 758-767

Wu HJ, Wang ZM, Wang M, Wang XJ (2013) Widespread long noncoding RNAs as endogenous target mimics for microRNAs in plants. Plant Physiol **161:** 1875-1884

Wu J, Hettenhausen C, Meldau S, Baldwin IT (2007) Herbivory rapidly activates MAPK signaling in attacked and unattacked leaf regions but not between leaves of Nicotiana attenuata. Plant Cell **19:** 1096-1122

Xie J, Song W, Maksymowicz W, Jin W, Cheah K, Chen W, Carnes C, Ke J, Conkling M (2004) Biotechnology: a tool for reduced risk tobacco products–the nicotine experience from test tube to cigarette pack. Rev Adv Tob Sci **30:** 17-37

Xie J, Fan L (2016) Nicotine biosynthesis is regulated by two more layers: small and long non-protein-coding RNAs. Plant signaling & behavior **169:** 00-00

Xu B, Sheehan MJ, Timko MP (2004) Differential induction of ornithine decarboxylase (ODC) gene family members in transgenic tobacco (Nicotiana tabacum L. cv. Bright Yellow 2) cell suspensions by methyl-jasmonate treatment. Plant growth regulation **44:** 101-116

Xu L, Liu F, Lechner E, Genschik P, Crosby WL, Ma H, Peng W, Huang D, Xie D (2002) The SCF(COI1) ubiquitin-ligase complexes are required for jasmonate response in Arabidopsis. Plant Cell **14:** 1919-1935

Yang Y, Guo J, Yan P, Li Y, Liu K, Gao P, Zhao H, Chen Y, Wang Y, Timko MP (2015) Transcriptome Profiling Identified Multiple Jasmonate ZIM-Domain Proteins Involved in the Regulation of Alkaloid Biosynthesis in Tobacco BY-2 Cells. Plant Mol Biol Rep 33: 153-166

Yasumatsu N (1967) Studies on the Chemical Regulation of Alkaloid Biosynthesis in Tobacco Plants: Part II. Inhibition of Alkaloid Biosynthesis by Exogenous Auxins. Agric Biol Chem **31:** 1441-1447

Yon J, Fried M (1989) Precise gene fusion by PCR. Nucleic Acids Res **17:** 4895

Zhang B, Pan X, Cobb GP, Anderson TA (2006) Plant microRNA: a small regulatory molecule with big impact. Dev Biol **289:** 3-16

Zhang F, Yao J, Ke J, Zhang L, Lam VQ, Xin X, Zhou XE, Chen J, Brunzelle J, Griffin PR (2015) Structural basis of JAZ repression of MYC transcription factors in jasmonate signalling. Nature **525:** 269-273

Zhang K (2016) MYC transcription factors: masters in the regulation of jasmonate biosynthesis in Arabidopsis thaliana. PhD thesis. Leiden University, Leiden, Netherlands.

Zhang Z, Baldwin IT (1997) Transport of [2-14C] jasmonic acid from leaves to roots mimics wound-induced changes in endogenous jasmonic acid pools in Nicotiana sylvestris. Planta **203:** 436-441

Zhang HB, Bokowiec MT, Rushton PJ, Han SC, Timko MP (2012) Tobacco transcription factors NtMYC2a and NtMYC2b form nuclear complexes with the NtJAZ1 repressor and regulate multiple jasmonate-inducible steps in nicotine biosynthesis. Mol Plant **5:** 73-84

Ziegler J, Keinänen M, Baldwin IT (2001) Herbivore-induced allene oxide synthase transcripts and jasmonic acid in Nicotiana attenuata. Phytochemistry **58:** 729-738

Ziegler J, Stenzel I, Hause B, Maucher H, Hamberg M, Grimm R, Ganal M, Wasternack C (2000) Molecular cloning of allene oxide cyclase. The enzyme establishing the stereochemistry of octadecanoids and jasmonates. J Biol Chem **275:** 19132-19138

APPENDICES

Table A 1. Primers used in Y2H.

Primers used for cDNA library and bait construction	
SMART III oligo	5’-AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGG-3’
CDS III primer	5’-ATTCTAGAGGCCGAGGCGGCGGACATG-d (T_{30})-3’
5’ PCR primer	5’-TTCCACCCAAGCAGTGGTATCAACGCAGAGTGG-3’
3’ PCR primer	5’-GTATCGATGCCCACCCTCTAGAGGCCGAGGCCGAGGCGGCCGACA-3’
MYC2a_BD_FP	5’-CATGGAGGCCGAATTCATGACGGATTATAGAATACCAACG-3’
MYC2a_BD_RP	5’-GCAGGTCGACGGATCCAGTAGCGCTAAGTCGTTAAGACCT-3’

Table A2. Primers used in BiFC

Myc2a_CE_FP	5’-AAAGGATCC ATGACGGATTATAGAATACCAAC-3’
Myc2a_CE_RP	5’-AAAGTCGAC TCGCGATTCAGCAATT-3’
JAZ3_NE_FP	5’-AAATCTAGAATGGCATCATCGGAGATTGT-3’
JAZ3_NE_RP	5’-AAACTCGAGGAATTGCTCAGCTTTCACTGG-3’
UBC_NE_FP	5’-AAATCTAGAATGTCGACTCCGGCTAG-3’
UBC_NE_RP	5’-AAACTCGAGGTCTGCAGTCCAGCTCT-3’
RBX1_NE_FP	5’-AAATCTAGAATGGCATCTGTCGACACC-3’
RBX1_NE_RP	5’-AAACTCGAGGTGACCATATTTCTGAAACTCC-3’

Table A3. Primers for making gene overexpression constructs

AtAOS_FP	5’- GGACTCTAGAGGATCCATGGCTTCTATTTCAACCCCTT -3’
AtAOS_RP	5’- GACGGCCAGTGAATTCCTAAAAGCTAGCTTTCCTTAACGAC-3’
AtAOC2_FP	5’-GGACTCTAGAGGATCCATGGCTTCTTCAGCAGTGT -3’
AtAOC2_RP	5’-GACGGCCAGTGAATTCTTAGTTGGTATAGTTACTTATAACTCCGC -3’
AtJAR1_FP	5’-GGACTCTAGAGGATCCATGTTGGAGAAGGTTGAAACTTTCG-3’
AtJAR1_RP	5’- GACGGCCAGTGAATTCTCAAAACGCTGTGCTGAAGTAG-3’
AtOPR3_FP	5’-GGACTCTAGAGGATCCATGACGGCGGCACAAGG -3’
AtOPR3_RP	5’- GACGGCCAGTGAATTCTCAGAGGCGGGAAAAAGGAG -3’
AtLOX2_FP	5’- GGACTCTAGAGGATCCATGTATTGTAGAGAGTCCTTGTCGA -3’
AtLOX2_RP	5’-GACGGCCAGTGAATTCTCAAATAGAAATACTATAAGGAACACCCATT -3’

Table A4. Primers used for making JIH1 RNAi construct.

JIH1_RNAi_FP	5’-CAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAAC AAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTACAAAAAAGC AGGCTCTGTTACCATCGGTGGCACTTTTCG-3’
JIH1_RNAi_FP	5’-CAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAAC AAATTGATAAGCAATGCTTTCTTATAATGCCAACTTTGTACAAGAAA GCTGGGTACGAAGGAACTTGTGGTTGTGCTTC-3’

Table A 5. Primers for real-time qRT-PCR

Ntactin_FP	5’- CTGAGGTCCTTTTCCAACCA-3’
Ntactin_RP	5’- GGCGACCACCTTAATCTTCA-3’
AtAOC2_FP	5’-ATATCGAAAACCCTAGACCAAGCAA-3’
AtAOC2_RP	5’-GAGATCTCCGAGACCGAACA-3’
AtJAR1_FP	5’-ACGGCTCATCAAGTCCAGAAACA-3’
AtJAR1_RP	5’-CAGGGTCAGTAGCGTTTCCA-3’
AtOPR3_FP	5’-AAACCCGGATTTGGTTTCGC-3’
AtOPR3_RP	5’-CCGTGTAGCCAACAACTGGA-3’
AtLOX2_FP	5’-TTTCTGGCCCGGGAAGTATG-3’
AtLOX2_RP	5’-AGCCAACCCCCTTTTGATGA-3’
AtAOS_FP	5’-CCGACGGTGGGGAATAAACA-3’
AtAOS_RP	5’-TAACGGAGCTTCCTAACGGC-3’
NtJIH_FP	5’- GAAAAACCCTTCTTCCCTCCA-3’
NtJIH_RP	5’- TCATCCTGCATTCCGAGTAGG-3’
NtNINJA_FP	5’- TTCAGGGCAAAAGACCCTCC-3’
NtNINJA_RP	5’- GGTCTCAACGACTGGTCCTG-3’
NtPMT1_FP	5’-CGTGTAACCCTAGTTCTCGGA-3’
NtPMT1_RP	5’- GCTACTGCCTCAAAGAATGGC-3’
NtQPT2_FP	5’-AGAGGTGAAACCACCAGCAC-3’
NtQPT2_RP	5’-TCCCGTCTTCCTTTGCTAGA-3’
NtMYC2a_FP	5’-GATGGGATGCTATGATTCGTATAC-3’
NtMYC2a_RP	5’-CTGAAACACTAGCATGGTGCACATC-3’
NtA622_FP	5’-GGATGATAGAGGCAGAAGGA-3’

NtA622_RP	5’-TGACAACTTTGTCTCTAGGAG-3’
NtBBL_FP	5’-CTGCTGATAATGTCGTTGATGCTC-3’
NtBBL_RP	5’-CACCTCTGATTGCCCAAAACAC-3’
NtJAZ12_FP	5’-CACCATGAGAAGAAACTGTAACTT-3’
NtJAZ12_RP	5’-GTGATGATAAGGAGAAGTTGCT-3’
NtJAZ3_FP	5’-GGTTCCTTTGGAGATCTCAGC-3’
NtJAZ3_RP	5’-GACCGCCGTAGAATATCGTC-3’
NtLOX2_FP	5’- GATTATTGATGGTAGAAATGCTGATTGCAATTTG-3’
NtLOX2_RP	5’-TGAGCATTTGCAAAAGAAAAAATAGCACATTT-3’
NtAOS_FP	5’-CGGAAAATGAAAGCCCGTCG-3’
NtAOS_RP	5’-ACCCAAAGGAGAAGCACCAA-3’
NtAOC_FP	5’-GATCTGCCGTCTGAGTTGCT-3’
NtAOC_RP	5’-CGGAAATGACCCCACGAAAA-3’
NtOPR3_FP	5’-TGGAAGCAACGTACCCTTGT-3’
NtOPR3_RP	5’-AGCATCAGCTACATCACAACCT-3’
NtJAR1_FP	5’-GGAGGTGCAGTTAGCCAGTT-3’
NtJAR1_RP	5’-AAGAGAAGAATGCCCAGCAG-3’

CTGTTACCATCGGTGGCACTTTTCGGGCCTTTTCAAAGGAGAGCTTTCAGCAGCT
TAGGCAGCGAATTGAGGAGGTTATTGTTGGGCAAGCTGCTGTACAGAGATGCAA
TGCAACTGTGGATTTTTTAACAAAAGAAAAACCCTTCTTCCCTCCAACCGTGAAC
GATAAAAACTTGCACAAACACTTCCAGAGAGTTGCAGGTGATATGCTTGGTAAC
GATCATGTAAAAGACATGGAACCACTAATGGGATCGGAGGATTTTGCGTTTTAC
CAAGAGGTTATTCCTGGTTACTTCTACCTACTCGGAATGCAGGATGAAACAAATG
AAAAACTTGTTTCAGTCCATTCACCTTATTTTAAAATCAACGAAGAAGCACTTCC
TATCGGTGCTGCACTTCAAGCATCTTTGGCTATCAGATATCTTCTCGAAGCACAA
CCACAAGTTCCTTCGT

Figure A 1. JIH1 RNAi fragment.

The most highly conserved 453 bp fragment was used to generate JIH1 RNAi construct.

```
NaJIH1          ATGGATTTCTCCAGATGGGTTTTCTTGATTTTGATTTTTGTTTCATTTTC
NtJIH1a         ATGGATTTTTCCAGATGGGTTTTCTTGATTTTGATTTTGATTTCATTTTC
NtJIH1b         ATGGATTTCTCCAGATGGGTTTTCTTGATTTTGATTTTTGTTTCATTTTT
                ******** ****************************  *********

NaJIH1          TGCCATACCCATTTGGTCAGACTCTTCATTATCAGAAATTCCTATTAATT
NtJIH1a         TGCCATACCCATTTGGTCAGACTCTTCATTATCAGAAATTCCTATTAATT
NtJIH1b         TGCCATACCCATTTGGTCAGACTCTTCATTATCAGAAATTCCTATTAATT
                **************************************************

NaJIH1          TCCTCAATTTTGCAAAGAAAGCTGAGGTTTTTGATTGGATTGTGGGGGTT
NtJIH1a         TCCTCAATTTTGCAAAGAAAGCTGAGGTTTTTGATTGGATTGTGGGGGTG
NtJIH1b         TTCTCAATTTTGCAAAGAAGGCTGAGGTTTTTGATTGGATTGTGGGGGTG
                * ***************** ****************************

NaJIH1          AGGAGAAGGATACATGAGAATCCTGAGCTGGGATATGAAGAATTTGAGAC
NtJIH1a         AGGAGAAGGATACATGAGAATCCTGAGCTGGGATATGAAGAATTTGAGAC
NtJIH1b         AGGAGAAGGATACATGAGAATCCAGAGCTGGGATATGAAGAATTTGAGAC
                ********************** *************************

NaJIH1          CAGTAAGATTATAAGGGAAGAATTGGATAAATTGGGGATTTCATACAAAT
NtJIH1a         CAGTAAGCTTATAAGGGAAGAATTGGATAAATTGGGGATTTCATACAAAT
NtJIH1b         CAGTAAGCTTATAAGGGAAGAATTGGATAAGTTGGGGATTTCATACAAAT
                ******* ********************** ******************

NaJIH1          ACCCTTTTGCTACTACTGGTATTGTTGGTTTTGTTGGTTCAGGAAAATCC
NtJIH1a         ACCCTTTTGCTACTACTGGTGTTGTTGGTTTTATTGGTTCAGGAAAATCC
NtJIH1b         ACCCTTTTGCTACTACTGGTGTTGTTGGTTTTATTGGTTCAGGAAAACCC
                ******************* *********** ************** **

NaJIH1          CCTTTTGTTGCAATCAGAGCTGATATGGATGCTCTCCCTATGCAGGAAAT
NtJIH1a         CCTTTTGTTGCAATCAGAGCTGATATGGATGCTCTTCCTATGCAGGAAAT
NtJIH1b         CCTTTTGTTGCAATTAGAGCTGATATGGATGCTCTCCCTATGCAGGAAAT
                ************** ******************* **************

NaJIH1          GGTGGACTGGGAGCACAAAAGTAAAAATGCTGGAAAAATGCATGCATGTG
NtJIH1a         GGTGGACTGGGAGCACAAAAGTAAAAATGCTGGAAAAATGCACGCGTGTG
NtJIH1b         GGTGGACTGGGCGCACAAAAGTAAAAACGCTGGGAAAATGCATGCATGTG
                *********** *************** ***** ******** ** ****

NaJIH1          GACATGATGCTCATGTTGCAATGCTTCTCGGTGCTGCAAAGATTCTTCAA
NtJIH1a         GCCATGATGCTCATATTGCTATGCTTCTCGGTGCTGCAAAAATTCTTCAA
NtJIH1b         GCCATGATGCTCATGTTGCAATGCTTCTTGGTGCTGCAAAGATTCTTCAA
                * ************ **** ******** *********** *********

NaJIH1          GAACATCGAGACATTTTGAAGGGAACGGTTGCTCTTGTTTTTCAACCAGC
NtJIH1a         GAACATCGAGACATTTTGAAGGGAACAGTTGCTCTTGTTTTTCAACCAGC
NtJIH1b         GAACATCCAGACATTTTGAAGGGAACAGTTGCTCTTGTTTTTCAACCAGC
                ******* ***************** ***********************

NaJIH1          AGAGGAGGGAGGTGGTGGGGCCAAGAAAATGATAGATGCTGGAGCACTAG
NtJIH1a         AGAGGAGGGAGGTGGTGGGGCGAAGAAAATGATAGATGCTGGAGCACTAG
NtJIH1b         AGAGGAGGGAGGTGGTGGGGCCAAAAAAATGATAGATGCTGGAGCACTAG
                ********************** ** ************************
```


NaJIH1 AAAACATAGAATCAATATTTGGTCTGCATGTCAATCCCCAGTTTCCTTTG
NtJIH1a AAAATATAGAAGCAATATTTGGTCTGCATGTCAATCCCCAGTTTCCTCTG
NtJIH1b AAAACATAGAAGCAATATTTGGTCTGCATGTCAATCCCCAGTTTCCTTTG
NaJIH1 GGTAAAGTTTCTTCAAGGCCTGGACCTTTTTTGGCTGGAAGTGGTTTTTT
NtJIH1a GGTAAAGTTTCTTCAAGGCCTGGACCTTTTTTGGCTGGAAGTGGTTTTTT
NtJIH1b GGTAAAGTTTCTTCAAGGCCTGGACCTTTTTTGGCTGGAAGTGGTTTTTT
NaJIH1 TGAAGCTGTAATTAGTGGAAAAGGAGGGCATGCCGCTATTCCACAACATT
NtJIH1a CGAAGCTGTAATTAGTGGAAAAGGGGGTCATGCTGCTATTCCACAACATT
NtJIH1b CGAAGCTGTAATAAGTGGAAAAGGGGGTCATGCCGCTATTCCACAGCATT
NaJIH1 CGATAGACCCAATTCTGGCAGCATCAAATGTAATTGTCAGCTTACAACAT
NtJIH1a CGATAGACCCAATTCTTGCAGCATCAAATGTAATTGTCAGCTTACAACAT
NtJIH1b CGATAGACCCAATTCTTGCAGCATCAAATGTAATTGTCAGCTTACAGCAT
NaJIH1 CTTGTTTCCCGAGAGGCTGATCCTCTGGATTCGCAGGTAGTCACAGTTGC
NtJIH1a CTTGTTTCCCGAGAGGCCGATCCTCTGGATTCGCAGGTAGTCACAGTTGC
NtJIH1b CTTGTTTCCCGAGAGGCTGATCCTCTGGATTCTCAGGTAGTCACAGTTGC
NaJIH1 TAAATTCCAAGGAGGTGGTGCATTTAACGTTATTCCAGACTCTGTTACCA
NtJIH1a TAAGTTCCAGGGAGGTGGTGCATTTAACGTTATTCCAGATTCTGTTACCA
NtJIH1b TAAATTCCAAGGAGGTGGTGCATTTAACGTTATTCCAGACTCTGTTACCA
NaJIH1 TCGGTGGCACTTTTCGGGCCTTTTCAAAGGAGAGCTTTCAGCAGCTTAGG
NtJIH1a TCGGTGGCACTTTTCGGGCCTTTTCAAAGGAGAGTTTTCAGCAGCTTAGG
NtJIH1b TCGGTGGCACTTTTCGGGCCTTTTCAAAGGAGAGCTTTCAGCAGCTTAGG
NaJIH1 CAGCGAATTGAGGAGGTTATTGTTGGGCAAGCTGCTGTACAGAGATGCAA
NtJIH1a CAGCGAATTGAGGAGGTTATTGTAGGGCAAGCTGCTGTACAGAGATGCAA
NtJIH1b CAGCGAATTGAGGAGGTTATTGTTGGGCAAGCTGCTGTACAGAGATGCAA
NaJIH1 TGCAACTGTGGATTTTCTTACAAAAGAGAAACCCTTCTTCCCTCCAACCG
NtJIH1a TGCAACTGTGGATTTTCTTACAAAAGAGAAACCCTTCTTCCCTCCAACCG
NtJIH1b TGCAACTGTGGATTTTTTAACAAAAGAAAAACCCTTCTTCCCTCCAACCG
NaJIH1 TGAACGATAAAAACTTGCACAAACACTTCCAGAGAGTTGCAGGTGATATG
NtJIH1a TGAACGATAAAAACTTGCACAAACACTTCCAGAGAGTTGCAGGTGATATG
NtJIH1b TGAACGATAAAAACTTGCACAAACACTTCCAGAGAGTTGCAGGTGATATG
NaJIH1 CTTGGTAACGATCATGTAAAAGACATGGAACCGCTAATGGGATCAGAGGA
NtJIH1a CTTGGTAACGATCATGTAAAAGACATGGAACCGCTAATGGGATCAGAGGA
NtJIH1b CTTGGTAACGATCATGTAAAAGACATGGAACCACTAATGGGATCGGAGGA

Figure A 2. NtJIH1 nucleotide sequences alignment.

A NaJIH1 cDNA sequence and two NtJIH1 cDNA sequences were used to do alignment using T-COFFEE multiple sequence alignment tool (http://tcoffee.org/Projects/tcoffee/). RNAi targeted region is shown in red box.

CCGCACAAGTCATCTGATTCTGTACAATATGATGGGAGGGCAATGGAGCATGTG
AAAGGCAATGGGAGACAGCATAAGGCAGAAGAAACTTCCAATTCTCGAGGGGA
GGAAAATGTGAAAGGAAGCAACATAAGCTTCAGGGCAAAAGACCCTCCTGACC
AGCCCAGAGCAGAAGCAGTTCCTTCTGAATTTTCAACTATTAGGCCAGGTCTTGC
TGCAGATTTGAAATTTGGAGGATCTGGTTCCTACCCGAATCTACCGTGGGTCTCA
ACGACTGGTCCTGGTCCAAATGGTAGAACAATATCTGGTGTTACTTACAGATACA
GTTCCACCCAAATCAGGATTGTCTGTGCTTGTCATGGGTCTCACATGTCACCAGA
TGACTTTGTGCGGCATGCAAGCGTAGAGCAAACTAGTCAAGAACCTGGCACTGG
GGTTTCATCATTTCCAAGTAGCAATCCGGCTGCCTCTGCCCAAAGCTGA

Figure A 3. NINJA RNAi fragment.

The mostly highly conserved 483 bp fragment was used to generate NINJA RNAi construct.

```
NtNINJA1            ATGGACGAAAACGGTCTTGATCTGAGCTTAGGTCTGCCCTGTGGTGGAGG
NtNINJA2            ATGGATGAAAACGGTCTTGATCTGAGCTTGGGTCTGCCCTGTGGTGGAGG
NtAJ966363          ATGGATGAAAACGGTCTTGATCTGAGCTTGGGTCTGCCCTGTGGTGGAGG
                    ***** ********************** ********************

NtNINJA1            GGCTGCCTCCGAGAAAAGTAAAAGTGGGAGCTCATCGGATTCTAAGGTTG
NtNINJA2            GGCTGCCTCCGAGAAAAGTAAAAGTGGGAGCTCATCGGATTCTAAGGTTG
NtAJ966363          GGCTGCCTCCGAGAAAAGTAAAAGTGGGAGCTCATCGGATTCTAAGGTTG
                    ************************************************

NtNINJA1            AGGAAGTTGATAGAGATGGCAAGGTGATCAATGATTTCAAGAACTTTCTG
NtNINJA2            AGGAAGTTGATAGAGATGGCAAGGTGATCAATGATTTCAAGAACTTTCTG
NtAJ966363          AGGAAGTTGATAGAGATGGCAAGGTGATCAATGATTTCAAGAACTTTCTG
                    ************************************************

NtNINJA1            GATGGAAGCACTAGCAGCCAAAAACATGATTCTGGTGTGGGTTCTCAGAG
NtNINJA2            GATGGAGGCACTAGCAGCCAAAAGCATGATTCTGGTGTGGGTTCTCAGAG
NtAJ966363          GATGGAGGCACTAGCAGCCAAAAGCATGATTCTGGTGTGGGTTCTCAGAG
                    ****** *************** *************************

NtNINJA1            AAGTGATTCAACAAAACATGAGGGGAACTTGCTCCCCAGCATTAGTGTTG
NtNINJA2            AAGTGATTCAACCAAACATGAGGGGAACTTGCTCTCCAGCATTAATGTGG
NtAJ966363          AAGTGATTCAACCAAACATGAGGGGAACTTGCTCTCCAGCATTAATGTGG
                    ************ ******************** ********* *** *

NtNINJA1            ACGTAGATGCTTCTAAAAAGTTAAATAGTGGAGGATTCTGGGTTCAAAAT
NtNINJA2            ACGTAGATGCTTCTAAAAAGTTAAATAGTGGAGGATTCTGGGTTCAAAAT
NtAJ966363          ACGTAGATGCTTCTAAAAAGTTAAATAGTGGAGGATTCTGGGTTCAAAAT
                    ************************************************

NtNINJA1            GATAGTAGACCTATAGAAGTTGAAGAAGATAGGAGAGCTGATGTGGGTGA
NtNINJA2            GATAGTAGACCAGTAGAAGTTGAAGAAGATAGGAGAGCTGATGTGGGTGA
NtAJ966363          GATAGTAGACCAGTAGAAGTTGAAGAAGATAGGAGAGCTGATGTGGGTGA
                    ***********  ************************************

NtNINJA1            CAAACGTAAAAATCTGTTCAGGGAGTCAAGTCAACAGAAGAAGCAAGAGA
NtNINJA2            CAAACGTAAAAATCTGTTCAGGGAGTCAAGTCAACAGAAGAAGCAAGAGA
NtAJ966363          CAAACGTAAAAATCTGTTCAGGGAGTCAAGTCAACAGAAGAAGCAAGAGA
                    ************************************************

NtNINJA1            GAGAAGGTCATCATGCTGATACGCATGACAAGACCAGGACATCACACATT
NtNINJA2            GAGAAGGTCATCATGCTGATACGCATGACAAGACCAGGACATCACACATT
NtAJ966363          GAGAAGGTCATCATGCTGATACGCATGACAAGACCAGGACATCACACATT
                    ************************************ ************

NtNINJA1            TCAATAACCACAGATGAAGGTTCGACTGCAGAAAATGAAGATGTAGCTGA
NtNINJA2            TCAATAACCACAGATGAGGGTTCGACTGCAGAAAATGAAGATGTAGCTGA
NtAJ966363          TCAATAACCACAGATGAGGGTTCGACTGCAGAAAATGAAGATGTAGCTGA
                    ***************** ********************************

NtNINJA1            TTCTGAAACTGTGGGTTCAACATCCAGGCAAATTTTGCAGCATGATGAGA
NtNINJA2            TTCTGAAACTGTAGGTTCAACATCCAGGCAGATTTTGCAGCATGATGAGA
NtAJ966363          TTCTGAAACTGTAGGTTCAACATCCAGGCAGATTTTGCAGCATGATGAGA
                    ************ ***************** ******************

NtNINJA1            GCTCCAAGAGATTTGTTGGAAGTAGTGGTTTAGCTGAGGTTCATAAGGAG
NtNINJA2            GCTCCAAGAGATTTGTTGGAAGTAGTGGTTTAGCTGAGGTTCATAAGGAG
NtAJ966363          GCTCCAAGAGATTTGTTGGAAGTAGTGGTTTAGCTGAGGTTCATAAGGAG
                    ************************************************

NtNINJA1            CTTCGCAGTGTTCCGTCTTCAAGTGGTGTAGAATTAATAGGACACAGAAG
NtNINJA2            CTTCGCAGTGTTCCTGCTTCAAGTGGTGTAGAATTAATAGGACAAAGAAG
```

```
NtAJ966363          CTTCGCAGTGTTCCTGCTTCAAGTGGTGTAGAATTAATAGGACAAAGAAG
                    **************  *************************** *****

NtNINJA1            GTTTACTATTTCTTCAGAGAAGGATGTTAAGTTTAGGAATATACCATATA
NtNINJA2            GTTTACTATCTCTTCAGAGAAGGATGTTAAGTTTGGGAATATACCATATA
NtAJ966363          GTTTACTATCTCTTCAGAGAAGGATGTTAAGTTTGGGAATATACCATATA
                    ********* ************************ ***************

NtNINJA1            CTATCCCATTCCAGGGCCAATCAATAAACATCATGAACCTGCCTTACTCT
NtNINJA2            CTATCCCATTCCAAGGCCAATCAATAAACATCATGAACCTGCCTTACTCT
NtAJ966363          CTATCCCATTCCAAGGCCAATCAATAAACATCATGAACCTGCCTTACTCT
                    ************* ************************************

NtNINJA1            ATGCCTCTGAATTCTAACACTGCTGGTACAACAAGTACGCCTAGTTACCC
NtNINJA2            ATGCCTCTGAATTCTAACACTGTTAGTACAACAAGTACGACTAGTTACCC
NtAJ966363          ATGCCTCTGAATTCTAACACTGTTAGTACAACAAGTACGACTAGTTACCC
                    ********************** * ************** **********

NtNINJA1            AATTCCTGGCATGATGCAAGTGATGGCTACCACAAGTGGAGATAGACCAG
NtNINJA2            AGTTCCTGGCGTGATGCAAGTGATGGCTACCACAAGTGGAGATAGACCAG
NtAJ966363          AGTTCCTGGCGTGATGCAACTGATGGCTACCACATGTGTAGATAGACCAC
                    * ******** ******** ************** *** **********

NtNINJA1            GAAGCCAGCCTGTCATACCTGCAAACTTGCCATTAATGTTTGGCTACTCT
NtNINJA2            GAAGCCAGCCTGTCATACCTGCAAACTTGCCATTAATGTTTGGCTACTCT
NtAJ966363          CAAGCCATCCTGTCATACCTGCATACTTGCCATTAATGTTTGGCTACTCT
                     ****** *************** **************************

NtNINJA1            TCTGTACAGCTTCCAACATTGGACAAGGATAACCTCCGCGGTGTGGCTTC
NtNINJA2            TCTGTACAGCTTCCAACATTGGACAAGGATAACCTCCGCGGTGTGGCTTC
NtAJ966363          TCTGTACAGCTTCCAACATTGGACAACGATAACCTCCACGGAGTGGCTTC
                    ************************ * ********** *** ********

NtNINJA1            TCATCTTCAACAGCTTCACCCTTCCCATGGAAGAGGTCCTCTGGGCTCAG
NtNINJA2            TCATCTTCAACAGCTTCACCCTTCCCATGGAAGAGGTCCTCTGGGTTCAG
NtAJ966363          TCATCTTCTACAGCTTCACCCTTCTCATGGAAGAGGTCCTCTGGGTTCAG
                    ******** *************** ******************** ****

NtNINJA1            ATATGCAGAAAGATGGACCAAATATTTCTCAAGCTACTACGTCAACTATT
NtNINJA2            ATATGCAGAAAGATGGACCAAATATTTCTCAAGCTACTACGTCATCTATT
NtAJ966363          ATAAGCAGAAAGATGGACCAAATATTTCTCAAGCTGCTGCGTCATCTATT
                    *** ****************************** ** ***** *****

NtNINJA1            CCGCACAAGTCATCTGATTCTGTACAATATGATGGGAGGGCAATGGAGCA
NtNINJA2            CCGCACAAGTCATCTGATTCTGTACAATATGATGGGAGGGCAATGGAGCA
NtAJ966363          CCGCACAAGTCATCTGATTCTGTACAATATGATGGGAGGGCAATGGAGCA
                    **************************************************

NtNINJA1            TGTGAAAGGCAATGGGAGACAGCATAAGGCAGAAGAAACTTCCACTTCTC
NtNINJA2            TGTGAAAGGCAATGGGAGACAGCATAAGGCAGAAGAAACTTCCAATTCTC
NtAJ966363          TGTGAAAGGCAATGGGAGACAGCATAAGGCAGAAGAAACTTCCAATTCTC
                    ****************************************** ******

NtNINJA1            GAGGGGAAGAAAATGTGAAAGGAAGCAACATAAGCTTCAGGGCAAAAGAC
NtNINJA2            GAGGGGAGGAAAATGTGAAAGGAAGCAACATAAGCTTCAGGGCAAAAGAC
NtAJ966363          GAGGGGAGGAAAATGTGAAAGGAAGCAACATAAGCTTCAGGGCAAAAGAC
                    ******* ****************************************

NtNINJA1            CCTCCTGAGCAGCCCAGAGCAGAAGCAGTTCCTTCTGAATTTTCAACTAT
NtNINJA2            CCTCCTGACCAGCCCAGAGCAGAAGCAGTTCCTTCTGAATTTTCAACTAT
NtAJ966363          CCTCCTGACCAGCCCAGAGCAGAAGCAGTTCCTTCTGAATTTTCAACTAT
                    ******** ****************************************
```

Figure A 4. *NtNINJA* nucleotide sequences alignment.

RNAi targeted region is boxed in red. Three *NtNINJA* cDNA sequences were isolated from tobacco genome database. Sequences alignment were performed by T-coffee DNA sequence alignment tool (http://tcoffee.org/Projects/tcoffee/). The sequences of NtNINJA2 and NtAJ966363 are identical, indicating there are two *NtNINJA* isoforms in the tobacco genome.

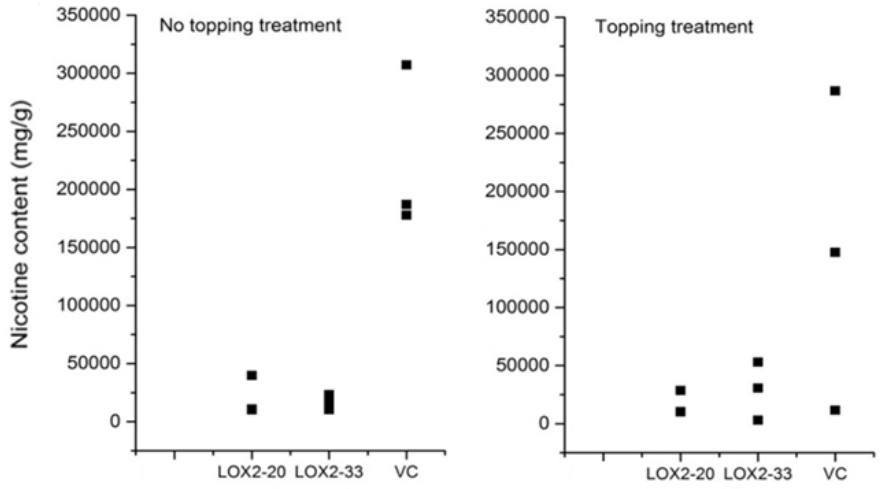

Figure A 5. JA-Ile levels in tobacco leaves.

Each dot represents a biology replicate. JA profile was determined by HPLC-MS. Left panel: basal JA-Ile levels. Right panel: topping induced JA-Ile levels. Values are normalized to internal controls.

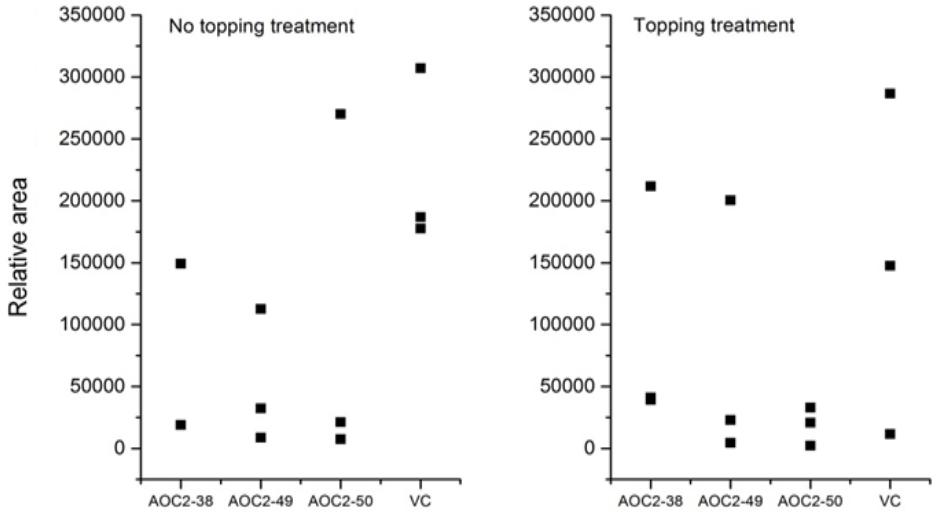

Figure A 6. JA-Ile levels in AOC2 tobacco leaves.

Each dot represents a biology replicate. JA profile was determined by HPLC-MS. Left panel: non-topping treated JA-Ile levels. Right panel: JA-Ile levels after topping. Values are normalized to internal controls. VC: Vector control.

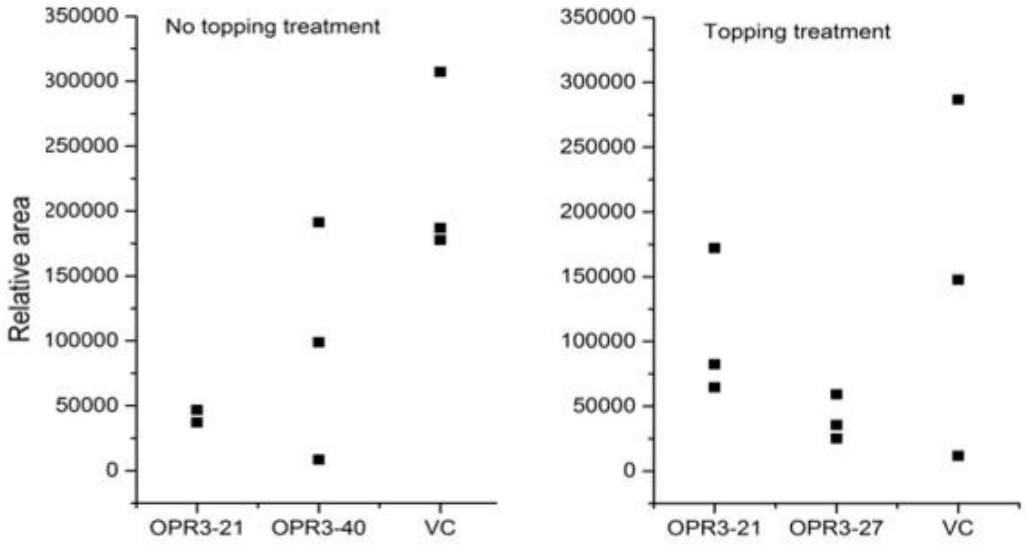

Figure A 7. JA-Ile levels in OPR3 tobacco leaves.

Each dot represents a biology replicate. JA profile was determined by HPLC-MS. Left panel: basal JA-Ile levels. Right panel: JA-Ile levels after topping. Values are normalized to internal controls. VC: Vector control.

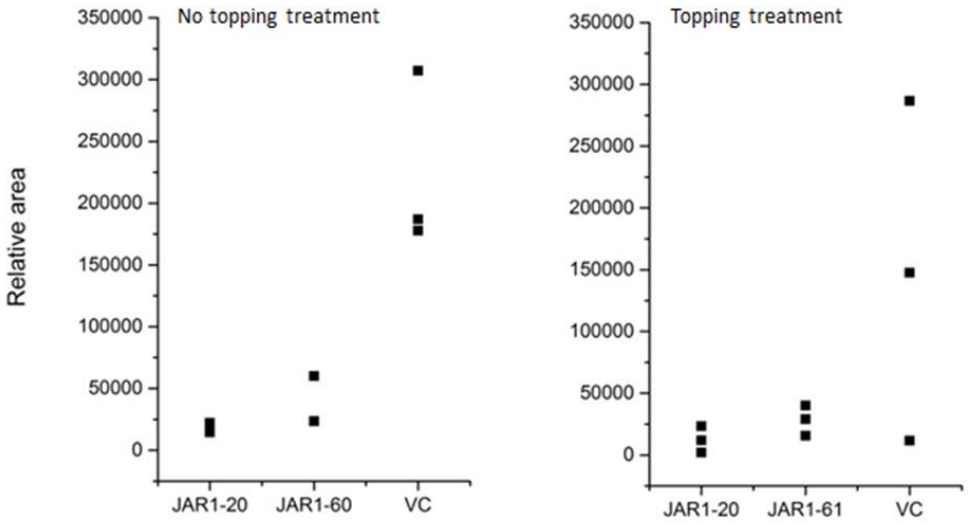

Figure A 8. JA-Ile levels in JAR1 tobacco leaves.

Each dot represents a biology replicate. JA profile was determined by HPLC-MS. Left panel: basal JA-Ile levels. Right panel: JA-Ile levels after topping. Values are normalized to internal controls. VC: Vector control.

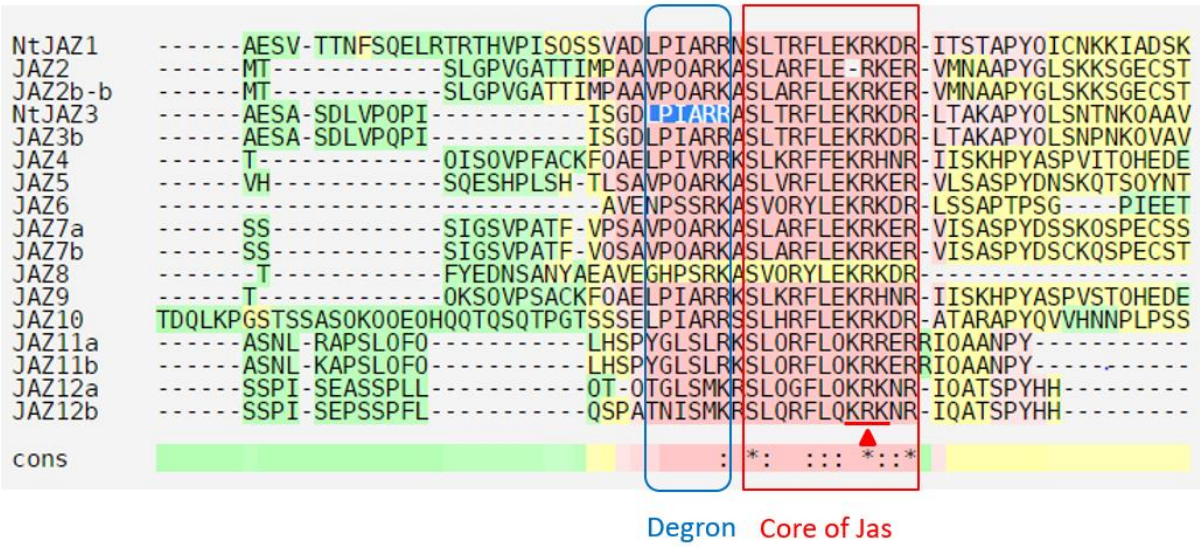

Figure A 9. Amino acid sequence alignment within the Jas motif of NtJAZs.

Seventeen NtJAZs amino acid sequences were used to generate sequence alignment using T-COFFEE multiple sequence alignment tool (http://tcoffee.org/Projects/tcoffee/). The highly conserved core of Jas motif, indicated in red box, is sufficient for interacting with MYC2. The central R residue, indicated by red triangle, within the tribasic cluster (underlined) is essential for interaction with MYC2 (Withers, et al., 2012). All the 17 NtJAZs contain the core of Jas motif. Y2H showed all the 17 NtJAZs interact with NtMYC2a (Yang, et al., 2015). The minimal AA region sufficient for COI1 binding (called JAZ degron) has two non-conserved amino acids within LPIARR region, indicated by red box (Sheard, et al., 2010).

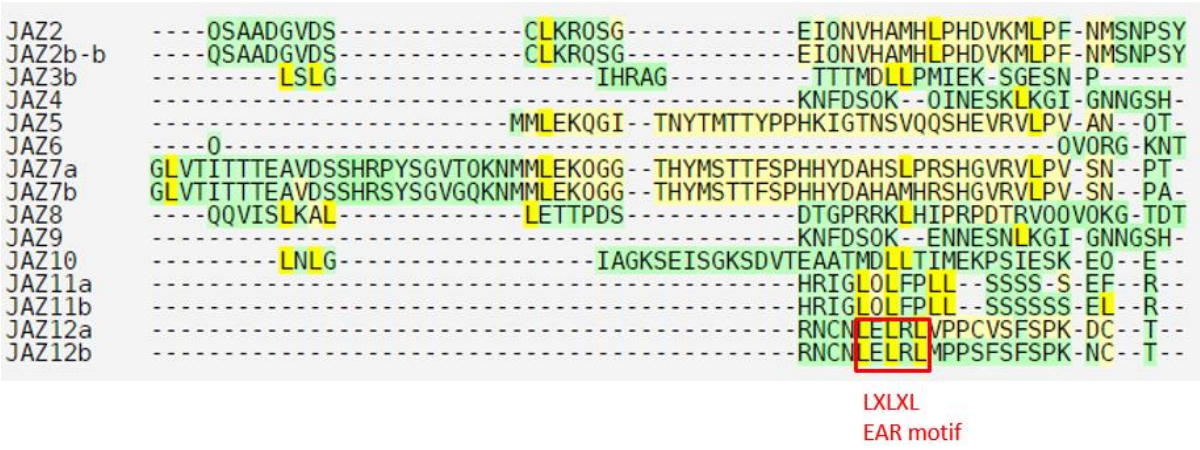

Figure A 10. Amino acid sequence alignment within the N-terminal region of NtJAZs.

16 NtJAZs amino acid sequences were used to generated sequence alignment. The highly conserved EAR motif (boxed), is responsible for binding with NINJA. Only NtJAZ12a and NtJAZ12b have EAR m**otif.**