ABSTRACT

NONAVINAKERE CHANDRAKANTH, NIHARIKA. Artificial Positive Feedback Loop for Maximizing Alkaloid Content in *Nicotiana tabacum*. (Under the direction of Dr. Ralph Dewey).

Nicotiana tabacum, a cultivated tobacco species, is commercially grown for its leaves. A hallmark of tobacco plants is their production of nitrogenous alkaloids that possess insecticidal properties and help in defense against herbivore attack. Nicotine accounts for approximately 90% of the alkaloid pool. The remaining 10% includes nornicotine, anabasine, and anatabine. Nicotine is produced in the roots and then transported and stored in leaf vacuoles. Nicotine is composed of a pyridine and a pyrrolidine ring, which are synthesized by two distinct metabolic pathways. Studies have shown that transcription factors designated MYC2a and ERF189 serve as master regulators of alkaloid biosynthesis in tobacco. Work conducted in the lab of Dr. Dominique Loqué described a simple, innovative strategy for maximizing the production of secondary metabolites by creating an artificial positive feedback loop (APFL). An APFL is created by placing a master regulatory transcription factor under the transcriptional control of one of its own downstream gene targets. In this study, we established a similar APFL strategy in an attempt to maximize alkaloid production in tobacco. Previous studies have shown that the genes *PMT1a* and *A622S* are highly expressed in roots via activation by MYC2a and ERF189. Our APFL constructs were designed such that the promoter elements of either *PMT1* or *A622S* were placed upstream of *MYC2a* and *ERF189* (giving rise to four combinations). The primary question addressed in this study is whether the APFL constructs can induce a higher level of nicotine in comparison to merely constitutively overexpressing these transcription factors using a strong constitutively active promoter such as the 35S promoter of Cauliflower Mosaic Virus (CaMV). This study also includes the introduction of the APFL constructs in TN86 PMT RNAi plants which have previously been shown to redirect the alkaloid pathway away

from nicotine and into anatabine. Anatabine is an alkaloid of interest due to its ability to alleviate the symptoms of Alzheimer's and Parkinson's diseases in animal systems. The expression of the MYC2a-based APFL constructs (pPMT1:MYC2a and pA622S:MYC2a) in the popular commercial variety K326 increased leaf nicotine levels by approximately 2-fold in T_0 transgenic greenhouse-grown tobacco plants. Interestingly, significant changes in anatabine levels were not observed using either an APFL or 35S promoter-driven overexpression strategy in the TN86 RNAi PMT background, suggesting that the process of anatabine production in these plants has encountered a bottleneck that the manipulation of regulatory transcription factors cannot overcome. The results of this preliminary greenhouse-based analysis of T_0 generation plants are supportive of the conclusion that the APFL strategy is an effective means for maximizing nicotine, but not anatabine production in tobacco. An analysis of the progeny of the most promising of these materials in a field environment should more definitively establish the degree by which this strategy can enhance alkaloid accumulation when grown under standard conditions of commercial tobacco production.

Artificial Positive Feedback Loop for Maximizing Alkaloid Content in *Nicotiana tabacum*

by
Niharika Nonavinakere Chandrakanth

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APPROVED BY:

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Dr. Rongda Qu	Dr. Ramsey S. Lewis

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Dr. Ralph E. Dewey
Committee Chair

DEDICATION

I dedicate this thesis to my parents Chandrakanth Nonavinakere Chikkanna Setty and Jyothi Mamani Gurubasavaraj, pets Cleo and Bhairu, friends Sunil Narsimhamurthy and Raghuveer Sarma MS and all my dear ones.

Assume I'm a plant cell, and I can recruit my organelles. Then my mother would be chloroplasts, and my father would be mitochondria. That is how important they are to me! I'm a blend of my Mother's love, care, creativity, and righteousness, and my Father's warmth, dexterity, vigilance, and audacity. They have sacrificed a lot of things in their life to ensure my happiness. Without their love and support, I couldn't have reached so far. Love you, Mom and Dad. Cleo and Bhairu, my two Pomeranian dogs, who showered me with their unconditional love, were and are the best part of my life. I'm indebted to Raghuveer, without whom I couldn't have made it to the USA. Finally, Sunil, who made me feel like at home away from home, thank you for bearing and supporting me throughout my grad life.

BIOGRAPHY

Niharika Nonavinakere Chandrakanth was born in Tumkur, Karnataka, India on February 17th, 1994. As a young girl, the idea of lactic acid bacteria which turns milk into curd fascinated her. A mini microscope was her favorite toy. Dolly happens to be Niharika's nickname. She came to know about the Sheep Dolly, the first mammal to be cloned. The idea of cloning sounded like a miracle and made her believe that she could make multiple clones of her beloved pets and be with them forever! All these reasons spurred her to choose Biotechnology as her major.

Niharika has her undergraduate degree in Biotechnology from India. She graduated with a silver medal from B.V. Bhoomaraddi College of Engineering and Technology, Karnataka, India.

She joined NC State in the Fall of 2016. She finished two semesters of Master of Microbial Biotechnology (MMB), a non-thesis professional science master's program. MMB is a blend of science and business studies. She has completed industry practicum projects with Novozymes and Advanced Animal Diagnostics and an Internship on Market Research on NC Craft Breweries. Then she got a transfer from MMB to Crop Science Thesis Program in the Fall of 2017, to gain research experience. Dr. Ralph Dewey, Philip Morris Professor, Department of Crop and Soil Sciences, is her Principal Investigator. Currently, she is working on her graduate research project, which aims at maximizing alkaloids in *Nicotiana tabacum* by an artificial positive feedback loop mechanism. After graduation, Niharika will continue working in Dr. Dewey's lab as a Research Technician.

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Firstly, I would like to thank my Principal Investigator, Dr. Ralph Dewey for accepting me as a graduate student, for letting me work in his lab, and for imparting knowledge. He has supervised, guided, supported, and encouraged me throughout this process. Secondly, I would like to thank Dr. Ramsey Lewis and Dr. Ron Qu for accepting to be on my committee and for their guidance. I would like to thank Dr. Ramsey Lewis again, for providing additional greenhouse space for this project.

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I would like to mention and thank Dr. Paul Hamilton, Director of Master of Microbial Biotechnology Program and Dr. Jason Cramer, Director, Accelerate to Industry Program for accepting me as a Master of Microbial Biotechnology (MMB) graduate student. I pursued MMB, a professional master's program for a year and took a transfer to Crop Science. I learned a lot of soft skills during my MMB year. I would like to thank Dr. Deborah Thompson, Director of Partnerships, NCARS for accepting me as an intern and teaching essential business writing skills.

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CHAPTER 1

Tobacco: Past, Present, and Future

1.1. History of Tobacco

Christopher Columbus wrote about receiving pungent dried leaves as a gift from the Native Americans in his journal. The leaves were thrown away, but later Columbus found that they were considered to be of high value. Tobacco is native to the Americas, and the indigenous populations typically consumed crude aqueous extracts or chewed on green leaves. The smoking of tobacco was innovated by two Europeans, Rodrigo de Jerez and Luis Torres. Rodrigo de Jerez is known as the first European smoker. In the 16th century, as sea routes became established, there was a rapid spread of tobacco seeds by sailors. Tobacco was introduced in Portugal, Spain, Japan, France, Turkey, and England, among others. Tobacco was initially utilized as a medicine and was even compulsory for a time at the elite Eton College, as people believed that it helped ward off infections. In the 1580s, however, a German writer gave the first recorded caution about using tobacco by calling it a "violent herb". [1]

In terms of tobacco usage, the 17th century could aptly be referred to as the age of pipes, due to the rising popularity of pipes as the means of consumption. During this period, large-scale cultivation of tobacco expanded across many regions of North and South America. In places such as Virginia, tobacco was even used as currency. In 1753, Carolus Linnaeus designated the genus of tobacco as *Nicotiana*, with the initial two recognized species being *Nicotiana rustica* and *Nicotiana tabacum*. Suspicions concerning negative health consequences also began to appear, as John Hill from England reported in 1761 that the excessive use of snuff made people vulnerable to nose cancer. [1]

The 19th and 20th century could be called the age of cigarettes, as this form of consumption grew to predominate. [1][2] In the early 1800's Gaspare Cerioli and Louis Vanquelin (independently) isolated nicotine from smoke, which was called the essence of tobacco. Later, Wilhelm Heinrich Posselt and Karl Ludwig Reimann purified nicotine directly from the tobacco plant. In 1889, Langley and Dickinson conducted revolutionary studies on the effects of nicotine on the ganglia. A. Pictet and P. Crepieux first synthesized pure nicotine in 1893. In 1965, the Federal Cigarette Labeling and Advertising Act required that the Surgeon General's warning, "Caution: Cigarette Smoking May Be Hazardous to Your Health," the first of what would become many warning labels, be placed on each cigarette package.

Recent landmarks include the discovery and marketing of the nicotine patch and nicotine gums in the mid-1980s, products that have been utilized by millions of people to help them quit smoking. [3] In 2003, electronic cigarettes were first commercialized. [4] Currently, a broad array of tobacco and tobacco-derived products are available to consumers; for example, cigarettes, cigars, little cigars, cigarillos, bidis, kreteks (containing cloves), dissolvable products, nicotine patches, chewable gums, various smokeless tobacco products, electronic cigarettes, and water pipes. [5]

1.2. Tobacco Markets

1.2.1. Global tobacco market

The giants of the global tobacco industry include Philip Morris International (PMI), British American Tobacco (BAT), Imperial Tobacco Company, Altria Group, Reynolds American, Hongyunhonghe Tobacco, Japan Tobacco, Gudang Garam Tobacco, and Korea Tobacco and Ginseng Corporation. In 2018, PMI was ranked number one worldwide, based on net sales. [6] [7]

Currently, cigarettes command the largest share of the global tobacco market. According to Grand View Research, Inc., the global tobacco market size is expected to reach approximately US $700 billion by 2021, with a 2.8% compound annual growth rate (CAGR) from 2016 to 2021. [6] Recent estimates by BAT, however, placed the current global tobacco market as being worth approximately US $760 billion. It is important to note that these estimates exclude China, the world's largest producer and consumer of tobacco products. Of BAT's estimated global market valuation of US $760, US $680 billion is attributed to the sale of conventional cigarettes. In 2016, the global sales of what has been termed Next Generation Products (NGPs) like e-cigarettes, vaping and heat-not-burned (HNB) accounted for approximately US $12.3 billion. The market share of NGPs is expected to increase substantially in the future. [8]

1.2.2. US Tobacco market

According to an Ibis World report on the US cigarette and tobacco manufacturing industry, cigarette sales have been declining in recent years due to a decrease in the number of smokers, rising public scrutiny, stringent regulations, and increased excise taxes. The tobacco industries in the US are thus focusing on developing alternative products like e-cigarettes that have lower health risks. Increased demand for e-cigarettes and other noncombustible tobacco products are balancing some of the decrease in sales of traditional cigarettes. [9] In the US, cigarette and tobacco manufacturing generates approximately $46.3 billion annually. Traditional cigarettes account for 70.7% of that revenue. E-vapor products, smokeless tobacco, cigars and other products like pipe tobacco, and alternative products such as clove cigarettes, homogenized and reconstituted tobacco, tobacco extracts and essences accounts for 13.8%, 9.8%, 1.8%, and 3.9% respectively. [9]

The biggest players of the US cigarette and tobacco manufacturing industry include Altria Group, Reynolds American, Imperial brands, Vector Group and others, accounting for 54.4%, 28.5%, 8.1%, 2.4%, and 6.6% of the market share, respectively. [9] In 2003, Philip Morris Companies Inc. changed its name to Altria Group. The Altria family of companies includes Philip Morris USA, US Smokeless Tobacco Company, John Middleton (cigars), Nat Sherman, Nu Mark (an innovation company that makes e-vapor product), Ste. Michelle Wine Estates, and Philip Morris Capital Corporation. [10] Recently, Altria acquired a 35% stake in Juul Labs, which is the most popular e-cigarette brand in the United States. [9] Ibis World predicts that US cigarette and tobacco manufacturing industry annual revenues will decrease to $44.2 billion by 2023. [9]

1.3. Tobacco Products

Tobacco products can be broadly divided into three categories: (1) combustible tobacco products; (2) smokeless tobacco products; (3) and E-vapor products.

1.3.1. Combustible tobacco products

Tobacco products that require combustion during use include cigarettes, cigars, cigarillos, roll your own tobacco, pipe tobacco, and other minor products. Cigarettes are defined as any form of tobacco that is wrapped in a paper or substance that is not made from tobacco. Small cigarettes are defined as cigarettes weighing no more than three pounds per thousand, and large cigarettes are those weighing more than three pounds per thousand. [11] It has been estimated that currently around 5,500 billion cigarettes are being consumed in the US each year. [7] Popular cigarette brands in the US include: Marlboro, Newport, Camel, Pall Mall, Maverick, Santa Fe, Winston and Kool. Based on 2017 sales data, Marlboro (Altria Group) was the leading brand, followed by Newport and Camel (Reynolds American). [12] Cigarettes are often flavored with menthol and other flavoring agents to reduce the harshness, bitterness, and astringency of the

tobacco smoke. The 2009 Family Smoking Prevention and Tobacco Control Act, however, banned the sale of cigarettes with flavors other than menthol.

Cigars are any roll of tobacco wrapped in leaf tobacco or other matrix containing tobacco. Similar to the classification of cigarettes, small cigars are defined as those that weigh less than three pounds per thousand, with large cigars being those that weigh more than three pounds per thousand. [11] Based on 2015 sales data, Swisher little (a small cigar type) was the leading cigar brand in the US. Swisher little was followed by Swisher sweets (large cigars and cigarillos) and Black & Mild (large cigars and cigarillos). [12] Cigarillos are small and narrow cigars that fundamentally differ from cigarettes in that they are wrapped in tobacco leaf, as opposed to paper. [9] Roll your own tobaccos are the loose tobacco filler used by consumers who prefer to hand roll their cigarettes. Hand-rolled cigarettes are less expensive and are often called rollies, roll-ups, burns, and rolls. [13] Finally, pipe tobacco is loose tobacco designed to be smoked out of a pipe, a once popular form of tobacco usage that now represents a very small portion of the market.

Nicotine is the agent that causes people to become addicted to smoking cigarettes. However, nicotine is not a direct chemical carcinogen. Tobacco smoke contains more than 7,000 chemicals, of which at least 69 of these have been associated with causing cancer. [14] In addition to carcinogens, other classes of toxicants are also present in tobacco smoke that contribute to different smoking-related pathologies. Among the more notable harmful and potentially harmful chemicals (HPHCs) found in tobacco smoke are lead, cadmium, acrolein, acetaldehyde, benzene, ammonia, carbon monoxide, 1,3 Butadiene, tobacco-specific nitrosamines (TSNAs), and polycyclic aromatic hydrocarbons. The FDA has listed 93 known HPHCs present in tobacco smoke on their website. [15]

Tar is the term used to collectively describe the chemical substances produced when tobacco is burned. [16] In addition to the compounds listed above, studies have shown that the tars in cigarette smoke contain free radicals that damage DNA. [17] When tobacco smoke is inhaled, the tar can form a sticky layer on the inside of the lungs. Continued exposure to cigarette smoke may lead to lung cancer, emphysema, or non-lung related problems such as heart disease. Depending on how the cigarettes are made, different amounts of tar are produced [16].

In the 1950s, the use of cigarette filters was initiated in an attempt to trap harmful tar residues. Tobacco companies started manufacturing cigarettes with filters and advertised them as high tar, mild tar, low tar, and ultra-low tar cigarettes. In the US, the 2009 Family Smoking Prevention and Tobacco Control Act prohibited companies from advertising cigarettes as "light," "low," or "mild". This was done to prevent smokers from believing that low or mild cigarettes are less harmful than standard cigarettes. [18][19]

Mainstream smoke is the tobacco smoke directly inhaled by the smoker and exhaled into the environment. Sidestream smoke is the smoke that is released from the burning end of a cigarette, cigar or pipe. Sidestream smoke is inherently more dangerous as it contains higher concentrations of toxic carcinogens than mainstream smoke. The particles in the sidestream smoke are smaller in size, and are easily absorbed by cells. Secondhand smoke (SHS), which is also referred to as environmental tobacco smoke, is a mixture of the mainstream smoke exhaled by the smoker into the environment and the sidestream smoke. Passive or involuntary smoking are the terms used when non-smokers are exposed to SHS. SHS contains the same amount of HPHCs as the mainstream smoke inhaled by smokers. SHS can cause lung cancer, even if the person has never smoked. [20] [21] [22] Hence, most governments worldwide have banned smoking in public places.

1.3.2. Smokeless tobacco products

Smokeless tobacco products include chewing tobacco, snuff (moist, and dry), snus, and dissolvable tobacco products. Chewing tobacco is cured tobacco packaged in different forms, like loose leaves, plugs (compressed small brick-shaped leaves) and twists (braids of leaves). Chewing tobacco is placed in the mouth between the cheek and gum, and the saliva that builds up is generally spit out or swallowed. Chewing tobacco can also be flavored. [23] [24]

Tobacco that is finely cut or ground and packaged in small tins or teabag-like pouches is used as snuff. It is available in both dry and moist forms. A small amount of snuff is placed either along the gumline, behind the lip, or between the gum and the cheek. An act of using snuff is also called dipping. Dry snuff is often sniffed through the nose, and like chewing tobacco can also be flavored. [23] [24] Snus is a flavored tobacco product that originated in Sweden. Snus is like snuff, except that the tobacco is pasteurized during the manufacturing process to kill the bacteria that can produce harmful metabolites, particularly TSNAs. [23] [24] Levi Garrett Plug, Day's work, Red Man, Grizzly, and Copenhagen are some of the leading smokeless tobacco brands. [12] Smokeless tobaccos are often promoted as less harmful products. However, they have still been associated with increased risk of oral cancer, esophageal cancer, pancreatic cancer, stillbirth or perinatal death, and other diseases. [14] [24]

Dissolvable tobacco products are generally flavored and are sold as lozenges, strips, sticks, and orbs/pellets that dissolve in the mouth and do not require spitting out the product. These products look like candies and are marketed for therapeutic purposes such as aids to help people quit smoking. Nicotine gels, patches and gums are noncombustible tobacco products. The nicotine gels and transdermal patches are applied to the skin where the nicotine becomes absorbed and directed to the bloodstream. The nicotine gums release nicotine as they are being

chewed. These products are mainly marketed to people who are attempting to quit smoking and are collectively referred to as nicotine replacement therapy (NRT) products. [24]

1.3.3. E – vapor products

A wide variety of electronic nicotine delivery systems (ENDS) are being manufactured and are termed as vapes, vaporizers, vape pens, hookah pens, electronic cigarettes (e-cigarettes or e-cigs), and e-pipes. These products are noncombustible tobacco products. In the case of ENDS, the e–liquid is heated to create an aerosol, which is inhaled by the user. E-liquids are produced with varying concentrations of nicotine, and can also include flavoring agents, propylene glycol, vegetable glycerin, and other ingredients as described below. [9] [24]

1.3.3.1. E-cigarettes

A patent granted to Joseph Robinson in 1931 is the first documented reference to an electronic cigarette model. [25] In 1965, Herbert A. Gilbert from Beaver Falls, PA patented the first smokeless, non-tobacco cigarette device. Around 1979-80, Phil Ray with Norman Jacobson created the first commercialized variation of an e-cigarette. However, this device proved to be unsuccessful. Norman Jacobson attributed the failure of the device to be due to an inherently faulty design. Later in the 20th century, RJ Reynolds Tobacco and Philip Morris USA introduced Eclipse and Accord, respectively. Both products were "heat-not-burned" devices, meaning that the tobacco in them is not exposed to temperatures high enough to initiate combustion. Both Eclipse and Accord failed to establish a sizeable consumer base. In 2003, Chinese pharmacist Hon Lik created the first commercially successful e-cigarette. Hon Lik commercialized e-cigarettes in China through the company Golden Dragon Holdings. This company later changed its name to Ruyan meaning "like smoke." In 2006-2007 electronic cigarettes were introduced in Europe and the US. [26] [27]

E-cigarette devices contain the following components: a battery, a reservoir for holding an e-liquid (generally containing nicotine), a heating element or an atomizer, and a mouthpiece through which the user inhales. The device heats the e-liquid, also called e- juice, into an aerosol that is inhaled. E-liquid is mainly composed of propylene glycol and/or vegetable glycerin as the solvent for the nicotine. They are most often flavored. According to a study conducted by Zhu et al., in 2014, over 7,500 different flavors had been incorporated into these products. Among the most popular flavors were those tasting like various fruits, candies, mint, coffee, bubble-gum, as well as novel flavors such as Belgium waffle and those mimicking alcoholic drinks. Custom flavors can also be made based on the customer's preference. These flavors are playing a key role in attracting young consumers. **[28]**

The first-generation of e-cigarette devices were designed to look like conventional cigarettes and were often called cigalikes. Some were also manufactured in the shape of cigars and pipes. Newer models were designed with a cartridge to hold the e-liquid, which was either prefilled and disposable, or could be used then refilled by the user. Second-generation devices included pen-shaped products referred to as "tank systems" and are larger in shape. Third- and fourth-generation devices included products with even different shapes, such as USB-like (rectangular), square, or cylindrical. E-cigarette devices can also be built by customers on their own using individual parts and are referred to as "mods." The various e-cigarette brands differ in terms of design, amount of aerosol particles, nicotine content, flavoring agents, and solvents used. **[28]**

In 2016, the global e-cigarette and vaping markets were valued at US $7.19 billion. **[29]** According to "Research and Market's Report on E-Cigarette Market by Product - Global Size, Share, Development, Growth, and Demand Forecast, 2013-2023", the e-cigarette market is

estimated to reach $44.6 billion by 2023. The US is currently the largest e-cigarette market, followed by the UK and China. [30] E-cigarette product manufacture, however, is largely localized in Shenzhen, Guangdong Province, China. [28]

The major industry players in the e-cigarette market include: BAT, Altria Group, Japan Tobacco, Imperial Brands, PMI, VMR Products, NJOY Inc, International Vapor Group, Vapor Hub International, and FIN Branding Group. [30] Altria owns the brands MarkTen and Green Smoke. Recently, Altria purchased a 35% stake in e-cigarette maker Juul, worth $12.8 billion. [32] Juul represented more than two-thirds of the e-cigarette market at the time of this purchase. [31] VUSE (R.J. Reynolds Vapor Company), Puritane (Formerly Ruyan, later acquired by Imperial Tobacco), blu (Lorillard Vapor Company until 2015, later acquired by Imperial Tobacco), VYPE (BAT), E- Swisher (Swisher), E-lites, and Ploom (Japan Tobacco International) are other popular e-cigarette brands. [28] E-cigarettes are available for sale in convenience stores, tobacco stores, pharmacies, Costco (and other countrywide chains), online retailers, and shops dedicated to e-cigarette products called “vape shops”. E-cigarettes can also be used as marijuana delivery systems. Ploom PAX (Japan Tobacco International) is one such product. [28]

PMI has been the leader in the resurgence of heat-not-burned tobacco products. IQOS is the name of their first and most successful heat-not-burned commercialized product. PMI has claimed that 6.6 million smokers have quit smoking conventional cigarettes and switched to IQOS. IQOS heats the tobacco to comparatively low temperatures (up to 350°C), sufficiently high to release nicotine and aromatic components associated with flavor, but without producing combustion, fire, ash, or smoke. In contrast, traditional cigarettes burn at temperatures over 600°C, generating a plethora of harmful chemicals that are the products of combustion. As the

tobacco in IQOS is merely heated and not burned, the levels of harmful chemicals are significantly reduced. TEEPS is another PMI product, similar to IQOS but using an alternative heating source. In 2014, PMI acquired Nicocigs, a leading e-cigarette company in the UK that produces disposables, rechargeables, and tank systems. Also, Altria Group has made its e-cigarette products available to PMI for commercialization outside the US. PMI's next generation e- vapor product is called IQOS MESH. Another product called STEEM is still under development. STEEM generates a nicotine-containing vapor in the form of a nicotine salt which can be more easily inhaled by the consumer. [33]

E-cigarettes, and more recently heat-not-burned products, are manufactured as a better alternative to conventional cigarettes. E-cigarettes produce fewer harmful chemicals when compared to traditional tobacco smoke. Even toxicants that are found in the unburned tobacco tend to be greatly reduced in e-cigarettes and other vaping products. TSNAs, for example, are potent carcinogenic agents generated primarily during the curing of the tobacco leaves and are thus present in cigarettes prior to combustion. These compounds are filtered during the manufacturing of the e-liquids, however, and studies have shown that the e-liquid and its corresponding aerosol contain only minimal amounts of TSNAs. [34] [35] [36]

E-cigarettes have both advantages and disadvantages. Their usage is clearly advantageous to existing adult smokers. In addition to reducing toxicant exposure, switching to e-cigarettes may also help adult smokers quit smoking. However, definitive data in this regard is still lacking. E-cigarettes are not considered safe for children, young adults, pregnant woman, and for first-time users of tobacco products. Unfortunately, youth have become highly attracted to e-cigarettes. The flavoring agents in the e-liquid play a key role in attracting young users. In 2018, approximately 3.6 million US middle school (4.9%) and high school students (20.8%) reported

using e-cigarettes. [36] As a result, the FDA is currently considering the implementation of regulations that would ban or severely restrict the addition of flavorings in vaping products, or at least the sale of these products in stores accessible to minors.

1.4. Tobacco varieties and cigarette blends [37]

Tobacco plants are transplanted to the field when they are around 15-20 cm high. It takes roughly two months for a tobacco seed to grow into a plant of this height. After transplant, the tobacco plants are grown in the field for another two to three months and are harvested differently based on the plant variety. The three main tobacco types used in cigarette manufacturing are Virginia, burley and oriental. These varieties are grown in over thirty countries including Argentina, Brazil, China, and the United States. Virginia and oriental tobaccos are harvested leaf by leaf, whereas the whole plant is harvested in the case of burley tobaccos. An additional market type known as dark tobaccos are used in the production of smokeless tobacco products.

Curing is the next major step post-harvest. It refers to the drying of the mature leaf and largely defines the character and ultimate leaf quality. The curing processes differ depending on the variety and the desired end product. For example, cigarettes produced according to what is referred to as the "American Blend" are made using a combination of the three major tobacco types. For this blend the burley tobacco is air-cured, the Virginia tobacco is flue-cured, and the oriental plants are sun-cured. In the case of flue-curing, the leaves are hung in a barn and heated air is circulated to dry the leaves. Air-curing involves hanging the leaves in a well-ventilated barn and allowing them to dry for up to eight weeks. Finally, sun-curing refers to exposing the leaves to the sun for about two weeks. In each case, the leaves gradually lose moisture and develop a distinct aroma, texture, and color.

After curing the leaves are typically sorted by stalk position and quality grade. The sorted leaves are then packed in bales for evaluation by leaf buyers. The leaves are separated from the stem in the case of burley and Virginia tobaccos. Finally, the leaves undergo processing to remove sand and non-tobacco matter. The processed, dried leaves are then blended and incorporated into cigarettes. Sugars like sucrose are sometimes added to make up for the sugars lost during the curing process; other ingredients that impact flavor such as menthol can also be added.

Virginia tobacco is also known as bright tobacco as it turns golden-yellow to deep-orange during curing. Burley tobacco is generally light to dark brown after curing. Oriental tobaccos are best known for their highly aromatic properties. The two most commonly used cigarettes blends are the American Blend (mentioned above) that combines portions of all three tobacco types, and the Virginia Blend, which is produced only using Virginia style tobaccos. American blend cigarettes predominate the markets in the US, Japan, most of Europe, South America and Africa. Virginia blend cigarettes are the most popular cigarette type in the UK, Canada, Australia and China.

1.5. Regulation of tobacco products

Globally, approximately 5.4 million people die due to tobacco-related illnesses every year. The death rate is expected to increase to more than 8 million/year by 2030. **[38]** Among adults in the US, cigarette smoking has declined from 42% in 1965 to 18% in 2012. **[14]** In spite of this decrease, nearly 40 million US adults still smoke cigarettes. In 2018, about 4.7 million middle and high school students were reported to use at least one form of tobacco product, 3.6 million of who were e-cigarette users. In the US, cigarette smoking is responsible for more than 480,000 deaths per year. This includes more than 41,000 deaths resulting from SHS exposure.

Smoking is responsible for more deaths each year than the total number of deaths caused by HIV, illegal drug use, alcohol use, motor vehicle injuries, and firearm-related incidents combined. About 90% of all lung cancer deaths are caused by smoking tobacco. The negative effects of tobacco cost approximately $300 billion every year, of which nearly $170 billion is spent on medical care to treat smoking-related disease in adults, and more than $156 billion in lost productivity due to premature death and exposure to SHS. [39] On average, it has been estimated that every cigarette takes 11 minutes off the smoker's life. [40]

To reduce the adverse effects of tobacco and to create a healthier future for Americans, on June 22, 2009, President Obama signed the Family Smoking Prevention and Tobacco Control Act into law. This law granted the FDA authority to regulate the manufacture, import, packaging, labeling, advertising, promotion, sale, and distribution of cigarettes, roll your own tobacco, the tobacco rolling paper, and smokeless tobacco products like chewable tobaccos, snus and some dissolvables. [19] [41] [42] In 2016, the FDA extended their regulatory authority to include cigars, pipe tobacco, all dissolvables, nicotine gels, hookah tobacco, electronic nicotine delivery systems (ENDS) and their components. Dissolvables and nicotine gels are marketed for therapeutic purposes and are regulated by the FDA through the Center for Drug Evaluation and Research (CDER) as drugs.

The 2009 Tobacco Control Act restricted tobacco marketing and sales to youth, and mandated warning labels on smokeless tobacco products. Smokeless tobacco packaging must contain at least one of the following warning labels: "this product can cause mouth cancer"; "this product can cause gum disease and tooth loss"; "this product is not a safe alternative to cigarettes"; or "smokeless tobacco is addictive". These warnings were aimed to increase the awareness of the health risks associated with smokeless tobacco. With respect to cigarettes, the

Tobacco Control Act prohibited tobacco companies from making reduced harm claims like "light," "low," or "mild," without having first applied for and received approval of the product being officially designated as a modified risk tobacco product. This step was taken to prevent the consumers to falsely believe that products labeled "light," "low," or "mild," are safer than regular cigarettes. The Tobacco Control Act mandated that tobacco companies provide the FDA with detailed information about the ingredients in their products. This law also preserved the authority of state, local, and tribal governments to regulate tobacco products in specific respects. What the 2009 Tobacco Control Act cannot do is require the following: (1) a prescription to purchase tobacco products; (2) the reduction of nicotine yields to zero; (3) ban face-to-face sales in a particular category of retail outlets; and (4) ban specific classes of tobacco products. **[19]**

In 1994, Benowitz and Henningfield proposed the idea of reducing the nicotine content in cigarettes over time, with the rationale that this would lead to lowering nicotine dependence. As nicotine is the addictive agent, decreasing its content in cigarettes would arguably make them less addictive. This would theoretically lead to fewer young people becoming addicted adult smokers, and for previously addicted smokers, potentially make the act of quitting easier. **[43]** The nicotine content in most commercial cigarettes ranges from 10 – 15 mg/cigarette. Smokers systemically absorb 10% of the nicotine on average, i.e., 1–2 mg of nicotine per cigarette. The amount of nicotine required to produce reinforcing effects and to sustain addiction differs from person to person. Thus, it would be difficult for the FDA to decide on a threshold nicotine content in cigarettes that could prevent all new smokers from becoming addicted.

Tobacco harm reduction is the term used to refer to a public health strategy to lower the health risks associated with tobacco. Around more than half of the adult smokers in the US (~22 million) are interested in less harmful tobacco products. **[44] [45]** In July 2017, the FDA announced

a comprehensive plan to lower the health risks associated with tobacco. Pursuing lowering the nicotine to non-addictive levels in cigarettes was one of the key aspects of this plan. [41] Specifically, the FDA has proposed that future cigarettes be produced that limits the nicotine content to 0.5 mg/g (dry weight) in the cut filler tobacco. Producing tobacco plants that can routinely produce nicotine levels this low will be a challenge. Some of the ways in which the genetics of the plant can be altered to lower overall nicotine content, and thus help in meeting this ambitious goal, are discussed in Chapter 2.

In addition to regulating the allowable levels of nicotine in future tobacco products, the FDA has also been granted the authority to mandate the lowering of specific toxicants that are found in these products. One class of toxicant that has received particular attention is the TSNAs, which unlike most of the carcinogens associated with cigarette smoke are typically not the products of combustion, as they are present in the unburned leaf. As a result, TSNAs have been associated with cancers in users of both cigarettes and smokeless tobacco products. One of the most troublesome TSNAs is called *N*-nitrosonornicotine (NNN), a carcinogen that has been strongly implicated in cancers of the esophagus, and oral and nasal cavities in both smokers and users of chewing tobaccos and snuff. As its first action in the area of potentially regulating toxicant levels in tobacco products, in January of 2017 the FDA submitted a proposal that would restrict the levels of NNN that can be present in smokeless tobacco products to no greater than 1 µg/gram dry weight. Similar to their low nicotine proposal, achieving levels of NNN that are this low is ambitious, and it will not be easy for the industry to meet this standard. Among the more promising strategies that could be implemented to meet such a standard involves mutating the genes in tobacco that are responsible for producing the nornicotine precursor of NNN. This strategy is discussed in more detail in Chapter 2.

1.6. Conclusion

Tobacco is native to the Americas and was spread rapidly to many parts of the world in the 16th century by seafarers. Since then, various forms of tobacco usage have captivated a substantial proportion of the human population. Manufacturing industries currently produce a variety of tobacco products, such as cigarettes, cigars, cigarillos, bidis, kreteks, dissolvables, nicotine patches, nicotine gels, chewable gums, smokeless tobacco products, electronic cigarettes and water pipes. The FDA is now authorized to regulate of all these products under the Tobacco Control Act. Fortunately, cigarette sales are declining due to the decrease in the number of smokers, rising public scrutiny, stringent regulations, and increased excise taxes. Nevertheless, cigarette smoking is still believed to be responsible for more than 480,000 deaths per year in the US alone. The FDA's mission is to reduce the health risks caused by tobacco. One avenue the FDA is pursuing to achieve this goal is a proposed lowering of the nicotine content in cigarettes to non-addictive levels.

Tobacco combustible products are more harmful than smokeless tobacco products, due to the fact that the majority of the characterized toxicants found in the smoke are the products of combustion. Nicotine is not carcinogenic, but some of the nitrosated derivates of nicotine and other alkaloids, referred to as TSNAs, are carcinogens. E-cigarettes were commercialized as an alternative nicotine delivery system for adult smokers. They contain a minimal amount of TSNAs and other carcinogenic agents and are generally considered to be a far safer alternative source of nicotine for adult smokers. However, e-cigarettes cannot be considered entirely harmless, and concern is growing over the rapid increase in the number of youth and young adults who are using e-cigarettes. The FDA is currently making efforts to regulate the type of

flavorings permissible in e-cigarettes and vaping products**,** as well as limit their access as a means of curbing this trend.

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CHAPTER 2

Alkaloid Biosynthesis in *Nicotiana tabacum*

2.1. ***Nicotiana tabacum***

Nicotiana tabacum is a cultivated tobacco species, commercially grown for its leaves. *N. tabacum* belongs to the Solanaceae/Nightshade family that consists of more than 90 genera and around 3000 species. Many species of this family produce toxic alkaloids possessing insecticidal activity. *N. tabacum* (2n = 48) is an amphidiploid, meaning this organism contains a diploid number of chromosomes originating from two different species. It is believed that *N. tabacum* was formed either through the union of two unreduced gametes, or through a chance hybridization between two progenitor species (both being 2n = 24) followed by chromosome doubling (Lewis et al. 2007). The progenitor species are *Nicotiana sylvestris* and possibly an introgressive hybrid of *Nicotiana tomentosiformis* and *Nicotiana otophora*. (Kenton et al. 1993) *N. tabacum's* component genomes are commonly referred to as S and T, with the S genome derived from *N. sylvestris* and the T genome originating from the *N. tomentosiformis* and *N. otophora* hybrid. (Bindler et al. 2011) However, many questions related to the genetic variability of modern *N. tabacum* remain unanswered.

2.2. Alkaloids

Plants are naturally occurring factories producing a wide variety of chemical products. These products can be divided mainly into two classes, primary and secondary metabolites. Primary metabolites like carbohydrates, amino acids, proteins and lipids are directly involved in plant growth and metabolism. Secondary metabolites are compounds that are not essential for plant growth, but have specialized functions such as defense against herbivores and other pathogens. Major classes of secondary plant metabolites include terpenoids (e.g. terpenes and

sterols), phenolics (e.g. flavonoids, tannins, lignin and coumarins) and nitrogen-containing compounds (alkaloids and glucosinolates – nitrogen and sulfur-containing compounds). Alkaloids can be defined as nitrogen-containing plant secondary metabolites and are classified based on the heterocyclic ring they possess. Many alkaloids have pharmaceutical uses, such as quinines as antimalarial and antipyretic agents, vinblastine as antitumor agents, codeine and morphine as analgesics and many more (Robinson et al. 1974; Babbar 2015). Some alkaloids can be toxic to the plant itself at high concentration. Colchicine, for example, inhibits mitosis (Robinson et al. 1974). Plants belonging to the genus *Nicotiana* produce alkaloids that contain a pyridine ring, the most prevalent of these being nicotine, nornicotine, anatabine, and anabasine (Zhang et al. 2007). Pyridine alkaloids in tobacco are strictly produced within the roots, followed by transport through the xylem to the aerial portions of the plant (Shitan et al. 2015).

2.3. Pharmacological Effects of Tobacco Alkaloids

In human pharmacology, nicotine is universally known for its addictive properties. When a person smokes, the nicotine in the cigarette smoke enters the bloodstream and binds to nicotinic acetylcholine receptors (nAChRs) found in the brain. This binding further facilitates the release of various neurotransmitters, including dopamine, which is known for its rewarding effects (Benowitz 1996). The desire to perpetuate reward stimulation is the main reason people become addicted to tobacco products containing nicotine. Interestingly, nicotine is also commonly used as a medication for smoking cessation in gums and patches (and more recently in vaping products) as a means of stimulating the brains reward centers while minimizing exposure to the toxic compounds found in cigarette smoke (Silagy et al. 1994; Abakay et al. 2017).

Nicotine also has pharmacological properties with potential medical applications aside from its use in smoking cessation therapies. Nicotine acts as an anti-inflammatory agent and is used in alleviating the symptoms of ulcerative colitis (Lakhan et al. 2011; Pullan et al. 1994; Gomes et al. 2018). nAChRs play a vital role in the functioning of the central nervous system (CNS), and many studies have identified their involvement in neurodegenerative diseases (Posadas et al. 2013; Picciotto et al. 2008). Nicotinic agonists like nicotine modulate nAChRs and act as neuroprotective agents that can alleviate the symptoms of disorders like Alzheimer's disease (Picciotto et al. 2008), Parkinson's disease (Picciotto et al. 2008; Quik et al. 2012), schizophrenia (Lucatch et al. 2018), depression (Tizabi et al. 1999), and HIV-associated neurocognitive disorders (Han et al. 2018).

Similar to nicotine, anatabine can also passage the blood-brain barrier, and bind to high-affinity nicotinic receptors, eliciting neuro transmitter secretion. As a result, there is evidence suggesting that anatabine has potential in the treatment of neurodegenerative diseases (Lippiello et al. 1994). Alzheimer’s disease (AD) is associated with pathological Aβ deposits, neuroinflammation, and behavioral deficits. Studies have shown that anatabine treatment lowers levels of Aβ deposits, thus alleviating the symptoms of AD in mouse models (Verma et al. 2015; Lippiello et al. 1994; Paris et al. 2011; Paris et al. 2014). Anatabine acts as an anti-inflammatory agent and ameliorates experimental autoimmune thyroiditis (De Remigis et al. 2012) and autoimmune encephalomyelitis (Paris et al. 2013) in mouse models. Anatabine in nutraceutical compositions with yerba mate extract may be efficacious for temporarily reducing the desire to smoke and lessen smoke withdrawal symptoms (Williams et al. 2010). Finally, although less has been reported concerning potential medical applications for nornicotine and anabasine, the latter

compound was shown to prevent chronic seizures in mouse models and might have potential in the treatment of human schizophrenia (Mastropaolo et al. 2004).

Within its native plant background, nicotine and other related pyridine alkaloids possess insecticidal properties and help in defense against herbivore attack. Nicotine accounts for approximately 90% of the alkaloid pool in *N. tabacum*. The remaining 10% is mainly comprised of nornicotine, anabasine, and anatabine (Dewey and Xie 2013). Alkaloid production in tobacco is stimulated in response to the physical damage of the plant and accumulates within leaf vacuoles to prevent auto-toxicity. When insects feed on tobacco leaves, the nicotine enters the insect's bloodstream and interacts with the acetylcholine receptors present in the insect brain. The insect's acetylcholine receptors cannot distinguish between acetylcholine and nicotine. Nicotine competes with acetylcholine for binding to the receptors and thus damages the proper functioning of the CNS, ultimately resulting in paralysis and then death of the insect (Rattan et al. 2010; Mcindoo et al. 1937). Because of these properties, has been a long history of nicotine use as an insecticide. Anabasine is also a very toxic alkaloid, showing strong insecticidal activity similar to nicotine (Zammit et al. 2014).

2.4. Structures of Major Tobacco Pyridine Alkaloids

Chemical structures of the four major alkaloids produced in *Nicotiana* species are shown in Figure 2.1. Each compound contains two ring structures, one of which is a pyridine ring. Nicotine and nornicotine are comprised of a pyridine and a pyrrolidine ring. Anatabine is characterized as having a pyridine ring that is coupled to another pyridine-derived ring, while anabasine contains a pyridine ring coupled to a piperidine moiety. Due to the chiral nature of the bonds joining the two rings, each of the major tobacco alkaloids can exist as optical isomers, or enantiomers. The optical variants for nicotine are depicted in Figure 2.2. Nicotine has a chiral

carbon in the 2'- position of the pyrrolidine ring (Fig. 2a) (Gorrod et al. 1999; Jasiewicz et al. 2014). The chiral carbon binds to an amine group, the pyridine ring, a methylene group and a hydrogen atom, and exists in enantiomeric forms referred to as S and R (Fig. 2b). Enantiomers are pairs molecules that are mirror images of one another. The S enantiomer rotates plane-polarized light to the left, and the letter 'S' is derived from the Latin word sinister, meaning left. The R enantiomer rotates plane-polarized light to the right and the letter 'R' is derived from the Latin word rectus, meaning right. Based on the RS System/Cahn-Ingold-Perlog priority rules, the R groups of the chiral carbon atoms have been numbered. Since the amine group has the highest atomic number, it is numbered as '1'. Hydrogen has the lowest atomic number and hence is numbered as '4'. Numbering the R groups helps in understanding the rotation of the enantiomer (Fig 2b). In *N. tabacum,* S-nicotine occurs abundantly in nature, whereas R-nicotine accounts for only ~0.5% of the total nicotine pool. The potency of S-nicotine, in terms of interacting with acetylcholine receptors, is far greater than that observed with R-nicotine.

A study conducted by Cai et al. (2013) investigated scion/rootstock grafts to observe the accumulation of R- and S-nicotine and R- and S-nornicotine in root and leaf tissue separately. Nornicotine is a demethylated form of nicotine that is produced via the *N*-demethylation of nicotine by a small family of nicotine demethylase enzymes designated CYP82E4, CYP82E5 and CYP82E10. According to this study, in normal tobacco plants nicotine is synthesized within the root at an initial ratio of approximately 4% R-nicotine and 96% S-nicotine. Because the nicotine demethylase enzymes possess a strong specificity for the R-enantiomer (particularly CYP82E5 and CYP82E10), most of the R-nicotine is converted into R-nornicotine both within the root and even later after translocation to the leaf. Hence a very low amount of R-nicotine is ultimately observed in leaf tissue.

2.5. Pyridine Alkaloid Biosynthesis

Alkaloid biosynthesis in tobacco has been extensively studied for many years. However, there are still important gaps to fill. A low alkaloid trait from a Cuban cigar type tobacco was discovered in Germany and subsequently introduced into other lines including Burley 21. Crossing and selection studies confirmed that two semi-dominant, non-linked loci named the A and B loci genetically control the alkaloid content to a significant degree. LA Burley 21 (aabb) is a low alkaloid derivative of Burley 21(AABB). The A and B loci are also designated as NIC1 and NIC2, respectively, in much of the recent tobacco literature (Dewey and Xie 2013; Shoji and Hashimoto 2013).

The dosage effect of a/nic1 on leaf nicotine levels is 2.4 times greater than the b/nic2 mutant locus. (Gorrod et al. 1999) The NIC loci have been shown to control the expression of several of the genes encoding enzymes responsible for alkaloid biosynthesis, including ornithine decarboxylase (ODC), putrescine methyltransferase (PMT), *N*-methylputrescine oxidase (MPO), aspartate oxidase (AO), quinolate synthase (QS), quinolate phosphoribosyltransferase (QPT), an isoflavone reductase-like enzyme (A622), berberine bridge enzyme-like protein (BBL), as well as genes encoding the MATE1/2 and NUP 1 nicotine transporters. The B/NIC 2 locus has been shown to be defined by a large deletion containing at least seven genes encoding ethylene response factor (ERF) transcription factors. (Shoji and Hashimoto 2011) These transcription factors bind to GCC boxes within the promoters of nicotine biosynthetic genes and upregulate their expression (Shoji and Hashimoto 2013).

2.5.1. Nicotine Biosynthesis

The six-member pyridine and a five-member pyrrolidine heterocyclic rings that define nicotine are synthesized by two distinct metabolic pathways. Figure 2.3 shows a schematic

representation of our current understanding of the alkaloid biosynthetic pathway (derived from Dalton et al. 2016).

2.5.1.1. Pyrrolidine ring synthesis:

Pyrrolidine ring synthesis can either be initiated by arginine or ornithine via the enzymatic actions of ADC and ODC, respectively (Dewey and Xie 2013; Shoji and Hashimoto 2013; Gorrod et al. 1999). In addition to its function as an amino acid in protein synthesis, arginine is a major component of the urea cycle, which is involved in the removal of toxic ammonia in the form of urea. Ornithine is also an amino acid, though not one that becomes incorporated into proteins. Ornithine is a by-product of the urea cycle (Gad et al. 2010). Putrescine is directly formed by the decarboxylation of ornithine via ODC. In contrast, the formation of putrescine from arginine involves multiple steps. Arginine is decarboxylated by ADC to form agmatine. Agmatine is subsequently converted to putrescine through an additional N-carbamoyl putrescine intermediate. The contribution of arginine derived putrescine in nicotine biosynthesis, however, appears to be minor (Dewey and Xie 2013; Shoji and Hashimoto 2013). Suggesting that ODC is the most important of the enzyme involved in initiation of pyrrolidine ring formation. Putrescine is a polyamine that can also serve as a precursor for the synthesis of higher polyamines such as spermidine and spermine. These polyamines play important roles in various physiological processes including plant stress tolerance to environmental stimuli (Gill et al. 2010). RNAi-mediated silencing of *ODC* in *Nicotiana tabacum* resulted in the decrease of the alkaloids nicotine and nornicotine, and an increase in the anatabine concentrations (an alkaloid that does not contain a pyrrolidine ring). Downregulation of *ODC* also resulted in a decrease of polyamine synthesis (Dalton et al. 2016; DeBoer et al. 2011).

S-adenosylmethionine-dependent N-methylation of putrescine is catalyzed by the enzyme PMT, which converts putrescine to N-methyl putrescine. This is the first committed step in pyrrolidine ring biosynthesis. Five isoforms of *PMT,* namely *NtPMT1a*, *NtPMT1b* (partial cDNA reported), *NtPMT2, NtPMT3*, and *NtPMT4* have been described in tobacco. The four completely characterized *NtPMT* isoforms contain 8 exons with 7 introns. These isoforms differ the most in the exon 1 region. Exon 1 contains an amino acid repeat motif 'NGHQNGTSEHQ' that is repeated 4, 4, 2, 5, and 8 times in *NtPMT1a*, *NtPMT1b*, *NtPMT2*, *NtPMT3*, and *NtPMT4*, respectively. Studies have shown that these repeats do not affect PMT enzyme activity. *NtPMT2*, *NtPMT3*, and *NtPMT4* originated from *N. sylvestris*; *NtPMT1a* and *NtPMT1b* were proposed to have been derived from an introgressive hybrid of *N. tomentosiformis* and *N. otophora*. *NtPMT1a* and *N. tomentosiformis PMT*, and *NtPMT1b* and *N. otophora PMT* share greatest sequence similarities, respectively (Dewey and Xie 2013).

NtPMT gene expression analysis has shown that all five *NtPMT* isoforms are expressed specifically in roots. PMT promoter - GUS reporter gene investigations have also confirmed its root specificity (Dewey and Xie 2013). A study conducted by Shoji et al. (2000) showed that *N. sylvestris PMT* expression is confined to the root cortex, epidermis, and xylem, as determined by GUS expression analysis. An antisense approach was used by Chintapakorn et al. (2003) to downregulate the activity of the *PMT* in *N. tabacum.* This resulted in a decrease in nicotine along with a substantial increase in anatabine levels. Decreased nicotine levels were also observed when the *PMT* expression was suppressed in *N. sylvestris*. Furthermore, the suppression of *PMT* activity resulted in the increased accumulation of spermidine in the leaf (Sato et al. 2000). Overexpression of *PMT* by the strong constitutive CaMV 35S promoter in *N. sylvestris* resulted in an approximately 40% increase in the leaf nicotine levels when compared to wild type plants

(Sato et al. 2000). In contrast, no significant change in the nicotine content was observed when *PMT1a* was constitutively overexpressed in commercial tobacco variety NC95 and grown in the field (Wang 2011).

The oxidative deamination of *N*-methyl putrescine forms 4-methylaminobutanal, a reaction catalyzed by the MPO enzyme. 4-methylaminobutanal spontaneously cyclizes to form *N*-methyl-Δ^1- pyrrolinium cation, which further condenses with a nicotinic acid-derived metabolite (possibly 3,6 dihydro nicotinic acid) to form nicotine (Dewey and Xie 2013; Shoji and Hashimoto 2013).

In 1968, a researcher at the Hatano Tobacco Experimental Station in Japan established the BY-2 cell line from the callus induced from a seedling of *N. tabacum* cultivar Bright Yellow No. 2. A study conducted by Kato et al. (1972) demonstrated that the BY-2 cells were the most proliferative when compared cultures derived from 40 other *Nicotiana* species and 3 *Populus* species. These cells have been distributed globally for research purposes. The characteristics of tobacco BY-2 cells include: (1) rapid growth in culture with a 13-hour doubling time; (2) well separated cells, only forming small aggregates; and (3) easy and efficient synchronization. Because of these characteristics, BY-2 cells have been widely used as a model system for higher plants. These cells have been used to study a wide range of higher plant cellular activities such as cell division, plant hormone signaling, and metabolism. Thus, tobacco BY-2 cells have been likened to HeLa cells used in human research (Shoji and Hashimoto 2008; Nagata et al. 1992).

Elicitation of BY-2 cells with methyl jasmonate results in the accumulation of anatabine, but not nicotine. Shoji et al. (2008) reported that the cultured tobacco BY-2 cells are severely deficient in *MPO* expression. The overexpression of *MPO* in tobacco BY-2 cells resulted in a dramatic increase of nicotine, accompanied by a concomitant decrease in anatabine levels. The

same research group suppressed *MPO* expression using RNA interference in tobacco hairy roots, a tissue system that normally accumulates nicotine. The *MPO* suppression in the hairy roots resulted in the decrease of nicotine, with a marked increase in anatabine levels.

2.5.1.2. Pyridine ring synthesis:

As shown in Figure 2.3, nicotinic acid and its derivatives are utilized in the formation of the pyridine ring of tobacco alkaloids. Nicotinic acid is an intermediate of the nicotinamide adenine dinucleotide (NAD) biosynthetic pathway (Dewey and Xie 2013; Shoji and Hashimoto 2013). NAD is an essential cellular cofactor, ubiquitously involved in redox reactions. It is also involved in signaling processes. NAD can lose and accept electrons, and as a result, exists in two forms. NAD^+ and NADH are the oxidized and the reduced states of NAD, respectively. In dicotyledonous plants, NAD synthesis originates with aspartate. Aspartate oxidase (AO) converts aspartate to α-iminosuccinic acid. Subsequently, quinolinic acid is formed by the condensation of α-iminosuccinic acid and glyceraldehyde-3-phosphate, the latter being an important intermediate of glycolysis. Quinolinate synthase (QS) catalyzes the condensation and the cyclization processes involved. Quinolinate phosphoribosyl transferase (QPT) converts quinolinic acid to nicotinic acid mononucleotide (NAMN) (Shoji and Hashimoto 2013). A study conducted by Katoh et al. (2006) showed that the Arabidopsis AO, QS and QPT enzymes are localized to plastids. This suggests that the early steps of the *de novo* synthesis of NAD via aspartate occurs within the plastid.

NAMN formation marks the entry into the pyridine nucleotide cycle of NAD biosynthesis through its adenylation to form nicotinate adenine dinucleotide (NAAD). The subsequent conversion of NAAD to NAD via NAMN/NMN adenylyltransferase (NMNAT) completes the *de novo* synthesis of NAD (Hashida et al. 2009). The pyridine ring donor,

nicotinic acid, can be formed by either of two routes: (1) directly from NAMN by the action of NAMN glycohydrolase; or (2) by the degradation of NAD. NAD can be degraded to nicotinamide mononucleotide (NMN), which is further converted to nicotinamide, then finally nicotinic acid (Ashihara 2006).

The enzyme QPT serves as the entry point into the pyridine nucleotide cycle that leads to the production of nicotinic acid and has been shown to be rate-limiting in the production of the pyridine ring during nicotine biosynthesis. Select tobacco varieties such as NC95 and K326 possess *QPT* genomic contributions derived only from the *N. tomentosiformis*-like progenitor, while other tobacco varieties like Xanthi, Samsun, Petite Havana SR1, and SC 58 possess *QPT* genomic contributions from both *N. sylvestris* and *N. tomentosiformis* (Ryan et al. 2012; Dewey and Xie 2013).

The tobacco genome contains duplicated *QPT* genes, designated *QPT1* and *QPT2*. *QPT1* is constitutively expressed at a basal level, with comparatively stronger expression in apical meristem, while *QPT2* is strongly expressed in the root tissue and is regulated coordinately with the other nicotine biosynthetic genes. *In vitro* DNA binding assays have shown that the *QPT2* promoter contains three ERF189 transcription factor-binding sites, motifs that are absent within the promoter region of *QPT1*. Analysis of the promoter activities of *QPT1*-GUS and *QPT2*-GUS fusion constructs, including mutant versions of *QPT2* promoters which contained only two or no ERF-binding sites were studied in the tobacco hairy root system. *QPT1* promoter activity was much weaker when compared to the wild-type *QPT2* promoter. Mutant *QPT2* promoters containing only two or no ERF-binding sites showed reduced and residual activity, respectively, when compared to wild type *QPT2* promoter-GUS fusions, suggesting that the ERF-binding sites are essential for the high levels of expression of *QPT2* within the root. Collectively, these studies

suggest that *QPT1* is mainly involved in the primarily metabolism of the essential cofactor NAD that is required throughout the plant, and *QPT2* is largely dedicated to the production of secondary metabolites (pyridine alkaloids) specifically within the root. It has been proposed that an original ancestral *QPT* gene dedicated to NAD synthesis became duplicated and neofunctionalized during the evolution of the *Nicotiana* genus, resulting in a specializes isoform dedicated to nicotine biosynthesis (Shoji and Hashimoto, 2011).

Although QPT is believed to represent a rate-limiting step in nicotine biosynthesis, its constitutive overexpression in the flue-cured variety NC95 failed to enhance nicotine accumulation in field grown plants (Wang 2011). Enhanced nicotine biosynthesis was observed with up-regulation of *QPT2* with a functional endogenous target mimicry (eTM) nta-eTMX27, which mediated inhibition of the expression and functions of nta-miRX27 that target *QPT2* (Li et al. 2015). Antisense-mediated down-regulation of *QPT2* within the LA Burley 21(aabb) background lead to the development of Vector 21-41, a low nicotine variety that has been used to produce low nicotine cigarettes (Xie et al. 2004).

2.5.1.3. Pyrrolidine and pyridine ring coupling:

The explicit mechanism by which the pyrrolidine and pyridine rings become joined has yet to be elucidated. Friesen et al. (1990) used *in vitro* enzyme assays with purified protein fractions to demonstrate the existence of a nicotine synthase enzyme that catalyzes the formation of S-nicotine from nicotinic acid and 1-methyl-Δ^1- pyrrolinium chloride. Other researchers, however, failed to replicate these results. More recently, several studies have shown that the proteins A622 (an isoflavone reductase-like enzyme) and BBL (berberine bridge-like enzyme) are involved in the late steps of nicotine biosynthesis. The pathway shown in Figure 2.3 suggests that A622 may catalyze the decarboxylation and reduction of nicotinic acid to 3,6-dihydro

nicotinic acid, and that the condensation of 3,6-dihydro nicotinic acid with *N*-methyl-Δ^1-pyrrolinium cation to form nicotine may be mediated by BBL. To date, however, there have been no reports substantiating this conjecture.

Initially, Hibi et al. (1994) showed that the expression of *A622* and *PMT* was greatly decreased in tobacco plants possessing mutant *NIC* loci, or in plants provided with an exogenous supply of auxin. Also, the removal of tobacco flower heads and young leaves, a process known as topping in the commercial production of tobacco, resulted in the upregulation of *A622* and *PMT* gene expression (Hibi et al. 1994). Shoji et al. (2002) analyzed the tissue specificity of *A622* expression by coupling the *N. sylvestris A622* promoter with the GUS gene reporter in *N. sylvestris* transgenic seedlings and hairy roots. These results showed that the expression pattern of *A622* is highly root specific and greatly mimics that of *PMT*. These results suggested the involvement of A622 in nicotine biosynthesis.

In 2009, DeBoer et al. analyzed the RNAi mediated down-regulation of *A622* in *N. glauca*, a wild *Nicotiana* species that produces anabasine as the principal alkaloid. Down-regulation of *A622* resulted in a reduction of leaf anabasine and other pyridine alkaloid levels. A similar analysis in transgenic hairy roots also showed a significant decrease in both anabasine and nicotine levels. When nicotinic acid (NA) was fed to wild type *N. glauca* hairy roots, anatabine levels increased. In contrast, when transgenic hairy roots with the *A622* RNAi construct were supplied with NA, no increases in anatabine levels were observed (DeBoer et al. 2008).

Kajikawa et al. (2009) studied *A622S* and its close homolog *A622L* in *N. tabacum.* RNAi mediated suppression of *A622S* and *A622L* resulted in the inhibition of cell growth, a decrease in alkaloid levels, and a concomitant accumulation of nicotinic acid β-*N*-glucoside in transgenic

hairy roots and cultured cells. Because excess NA is toxic to plant cells, the increase in nicotinic acid β-*N*-glucoside in these materials was interpreted as a detoxification response to the over-accumulation of NA. Finally, *N*-methyl-Δ^1- pyrrolinium cation accumulation was also observed in transgenic hairy roots containing an *A622L* RNAi construct (Kajikawa et al. 2009). Collectively, the results of the studies conducted by DeBoer et al. (2009) and Kajikawa et al. (2009) present compelling evidence that *A622* is involved in a step of the nicotine biosynthetic pathway that lies after the formation of NA and before the joining of the pyridine and pyrrolidine rings.

Kajikawa et al. (2011) were the first to directly implicate BBL enzymes in the nicotine biosynthetic pathway. BBLs are FAD-containing oxidases of that are highly expressed in roots, are induced by jasmonate, and regulated by the *NIC* loci. Four *BBL* isoforms, *BBLa, BBLb, BBLc,* and *BBLd* were identified in the tobacco genome; *BBLa* and *BBLc* likely originated from *N. sylvestris*, and *BBLb* and *BBLd* from the *N. tomentosiformis*-like progenitor. The RNAi-mediated down-regulation of *BBL* in tobacco hairy roots and plants resulted in a decrease in nicotine with subsequent accumulation of dihydromethanicotine (DMN). DMN had not been previously reported in tobacco plants but had been identified as a metabolite of nicotine in rats, and had also been detected in cigarette smoke. In the RNAi *BBL* transgenic plants, DMN accumulated only in the roots. It was not found in leaves, suggesting that DMN cannot be transported efficiently from roots to leaves like nicotine (Kajikawa et al. 2011).

As described in section 2.5.1.1, jasmonate-elicited cultured tobacco BY-2 cells accumulate anatabine as the predominant alkaloid (Shoji and Hashimoto, 2008). Kajikawa et al. (2011) suppressed *BBL* expression in jasmonate-elicited BY-2 cells, which resulted in the inhibition of anatabine and other pyridine alkaloid accumulation. They also showed that BBL

proteins are localized in the vacuole, using subcellular fractionation experiments as well as confocal microscopy analysis of GFP-tagged BBL fusion proteins. The results of this study suggested that BBL enzymes are involved in the catalysis of a very late step of alkaloid synthesis, probably after the initial condensation of the pyridine and pyrrolidine rings has occurred (Kajikawa et al. 2011).

In a study conducted by Lewis et al. (2015) transgenic doubled haploid lines of tobacco cultivar K326 carrying an RNAi construct designed to reduce expression of the *BBL* gene family were produced. *BBLa* and *BBLd* are the highest and the lowest expressed isoforms, respectively (Kajikawa et al. 2011). *BBLa* is 94%, 83% and 63% identical to *BBLb, BBLc,* and *BBLd,* respectively. Hence, the *BBLa* sequence was selected to generate the anti-*BBL* RNAi construct. Reduced nicotine contents were observed in field-grown transgenic lines carrying functional RNAi constructs (0.68% of total dry weight) when compared to untransformed control K326 plants (2.45% total dry weight). Furthermore, a mutation breeding approach was also pursued to overcome the barriers associated with the commercialization of transgenic crops. EMS (Ethyl Methyl Sulfonate) was used to induce mutations in *BBLa, BBLb,* and *BBLc.* The triple homozygous knockout genotype exhibited a greater than 13-fold reduction in percent total alkaloids.

2.5.1.4. Nicotine transport

As early as 1941, Ray F. Dawson showed that nicotine is produced in the roots of tobacco plants using reciprocal grafts between tobacco and tomato. No noticeable accumulation of nicotine was observed in newly formed tobacco leaves when tobacco scions were grown upon tomato stocks. In contrast, a substantial amount of nicotine was found in tomato leaves when tomato scions were grown upon tobacco stocks (Dawson 1942). *N. alata* is an interesting

Nicotiana species in that it produces alkaloids in the roots but fails to transport them to the aerial portion of the plant (Shoji and Hashimoto 2013). Hence, accumulation of alkaloids in the leaf tissues is negligible in these plants. Pakdeechanuan et al. (2002) conducted genetic studies involving interspecific hybrids between *N. alata* and *N. langsdorffii*, two closely related *Nicotiana* species. The results of the genetic studies showed that the non-translocation phenotype in *N. alata* is dominant over the translocation phenotype found in *N. langsdorffii*. The authors proposed that the mechanism by which *N. alata* retains alkaloids within root tissues might be utilized to limit the accumulation of alkaloids in other *Nicotiana* species when such a phenotype is desired.

A model summarizing our current knowledge of nicotine transport is shown in Figure 2.4. Nicotine produced in root tissues is loaded into the xylem, a vascular tissue that connects the root and the shoot systems. Leaf damage results in the increase of nicotine concentration in xylem, where it is then transported through the transpiration stream to the leaves (Baldwin 1989). The transporters involved in the loading and the unloading of nicotine into and out of the xylem, as well as the transporters involved in the efflux of nicotine from the root cell and influx into the leaf cell through the plasma membrane have yet to be identified (Shoji and Hashimoto 2013).

Once nicotine is transported to the leaf, it is primarily stored in the vacuoles (although some nicotine can also be exuded to the leaf surface). High concentrations of nicotine are cytotoxic, so vacuolar localization enables to plant to accumulate nicotine in high quantities while avoiding its cytotoxic effects. When nicotine enters the plasma membrane of the leaf cell (through an unknown transport mechanism) the slightly alkaline environment of the cytosol favors the uncharged lipophilic state of the molecule, a condition by which nicotine can freely diffuse through the tonoplast and enter the vacuole. Vacuoles are slightly acidic, an environment favoring a protonated state. Protonated nicotine is hydrophilic and gets trapped inside the

vacuole as it can no longer permeate the tonoplast membrane. This process is called the ion-trap mechanism (Shoji and Hashimoto 2013) (Fig. 2.4).

Despite the presumed ability of nicotine to be capable of accumulating within vacuoles in the absence of a protein-based transport system, recent research has shown that the active vacuolar sequestration of nicotine via multidrug and toxic compound extrusion (MATE)-type transporters does indeed play a significant role in this process (Shoji and Hashimoto 2013). *NtMATE1* and *NtMATE2* encode two closely related proteins (MATE1 and MATE2, respectively) that are expressed in root tissues and are coordinately regulated with several characterized nicotine biosynthetic genes (Shoji et al. 2008). They are regulated by the *NIC* loci and are induced by methyl jasmonate treatment. Tobacco MATE1 and MATE2 proteins are phylogenetically related to Arabidopsis and tomato proteins involved in the vacuolar sequestration of flavonoids (Shoji and Hashimoto 2013). MATE transporters generally transport molecules in exchange for H^+ or Na^+ and are thus classified as antiport transporters (Omote et al. 2006). MATE1 and MATE2 were shown to be localized to the vacuolar membrane, and knockdown of *NtMATE1/2* expression resulted in the increased sensitivity of the tobacco plants to exogenously applied nicotine. Overexpression of *NtMATE1* by jasmonate elicitation or by exogenous addition of nicotine resulted in the acidification of the cytoplasm in tobacco BY-2 cultured cells, possibly due to over-accumulation of H^+ in the cytoplasm in exchange for the nicotine transferred into the vacuoles by NtMATE1 proteins. Paradoxically, expression of *NtMATE1* in yeast cells resulted in the slower and reduced accumulation of exogenously supplied nicotine into the yeast cells when compared to the wild type controls. Further investigation showed that NtMATE1 became localized on the plasma membrane in this heterologous system and functioned to transport cytosolic nicotine into the culture medium, thus

reversing the NtMATE1-independent uptake of nicotine into yeast cells. Despite the unexpected results observed in yeast, the authors proposed that MATE1/2 transports nicotine from the cytosol into the vacuole in exchange for protons, specifically within tobacco root cells (Shoji et al. 2008).

Morita et al. (2009) characterized a different jasmonate-inducible alkaloid transporter, designated NtJAT1, which also belongs to the MATE family of transporters. In methyl jasmonate-treated tobacco cells, the expression of *NtJAT1* was co-regulated with that of nicotine biosynthetic genes. However, it is not regulated by the *NIC* loci (Shoji and Hashimoto 2013). *NtJAT1* is expressed in roots, stems and leaves, and localizes to the tonoplast of leaf cells. Like NtMATE1, when produced in yeast cells, NtJAT1 was found mainly in the plasma membrane and showed nicotine efflux activity. Within the plant cell, it is thought that NtJAT1 is like NtMATE1/2, being involved in the vacuolar sequestration of nicotine. Because NtJAT1 is found in the aerial parts of the plants, however, it is believed to primarily function in sequestering nicotine after it has been transported from the root and unloaded from the xylem into leaf tissue. In a separate study, Shitan et al. (2014) described a jasmonate-inducible alkaloid transporter 2 gene (*NtJAT2*), which encodes a protein that is 32% identical to NtJAT1. NtJAT2 shares properties similar to NtJAT1 and is believed to be involved in the vacuolar sequestration of nicotine in the leaf.

Nicotine uptake permease (NUP1) represents a different class of transport protein and belongs to a class of purine uptake permease (PUP)-like transporters (Hildreth et al. 2011). Arabidopsis PUP1 and PUP2 are responsible for proton-mediated uptake of purines and cytokinins, and function as symporters rather than antiporters. Expression of *NUP1* is co-regulated with that of nicotine biosynthetic genes with respect to JA responsiveness, tissue

specificity and regulation by *NIC* loci (Hildreth et al. 2011). When expressed in yeast cells, NUP1 preferentially transported nicotine, relative to other pyridine alkaloids, from the extracellular media into the cytoplasm. NUP1 proteins localized to the plasma membrane of tobacco BY-2 protoplasts. In roots, *NUP1* transcripts preferentially accumulated in the root tips. Downregulation of *NUP1* in transgenic hairy roots cultures resulted in the accumulation of nicotine in the culture medium. Transgenic *NUP1*-RNAi plants showed reduced leaf and root nicotine levels, along with an increased root growth phenotype. Studies have shown that some of the nicotine produced in roots is released to the rhizosphere and later reabsorbed by the plant to maintain an optimal root nicotine content. Based on the results of Hildreth et al. (2011) it is believed that NUP1 maintains nicotine homeostasis by regulating the release and reabsorption of apoplastic nicotine between the root cells and the rhizosphere.

2.5.2. Novel Aspects of Nornicotine Biosynthesis

Nornicotine is formed by the *N*-demethylation of nicotine, catalyzed by nicotine *N*-demethylase (NND) enzymes, a process commonly referred to in the literature as nicotine conversion. In tobacco plants known to produce atypically high levels of nornicotine, nicotine conversion mostly occurs during leaf senescence and curing. In most *N. tabacum* plants, nornicotine accounts for approximately 2 – 4 % of the total alkaloids. Plants that have a high capacity for nicotine demethylation to nornicotine are called converters, and a single major locus that has been associated with this phenomenon is called the converter locus (Siminsky et al., 2005). The converter locus found in *N. tabacum*, designated C_t, originated from *N. tomentosiformis*; a similar locus in the *N. sylvestris* progenitor is called C_s. This observation was not expected, as *N. tomentosiformis* accumulates nornicotine both in green and senescing leaves, while *N. sylvestris* only accumulates nornicotine in senescing leaves, which is phenotypically

similar to a *N. tabacum* converter plant (Dewey and Xie 2013). The reason why most *N. tabacum* plants are nonconverters, despite possessing a converter locus originating from *N. tomentosiformis*, was deduced by Gavilano et al. (2006). *CYP82E4* encodes a cytochrome P450 monooxygenase that functions as nicotine *N*-demethylase gene but is active only in converter plants and only during senescence. *CYP82E3*, a closely related homolog of *CYP82E4*, was also discovered, but shown to exhibit no nicotine *N*-demethylase activity. Examination of the *N. tomentosiformis* orthologs of *CYP82E4* and *CYP82E3*, however, reveal that both isoforms are functional nicotine *N*-demethylase genes in this background. From domain swapping and site-directed mutagenesis studies, it was found that the tobacco *CYP82E3* gene is non-functional due to a mutation that changed a single amino acid in comparison to the functional version of *CYP83E3* found in *N. tomentosiformis* (Gavilano et al. 2006).

Nitrosation of nornicotine results in the formation of N-nitrosonornicotine (NNN). NNN belongs to a group of compounds called tobacco-specific nitrosamines (TSNAs). Numerous studies in animal systems have shown that NNN is a strong carcinogen. Thus, efforts have been made to lower the levels of NNN that accumulate within the cured leaf. One strategy for lowering NNN involves reducing the levels of the nornicotine precursor. In a study conducted by Lewis et al. (2008), transgenic lines of burley tobacco plants carrying an RNAi construct designed to inhibit the expression of NND genes was evaluated. A six-fold decrease in nornicotine compared to untransformed controls and a corresponding decrease in NNN was observed.

After the characterization of *CYP82E4* as the major nicotine demethylase gene of tobacco that defines the converter locus, two "minor" nicotine demethylase genes were discovered, designated *CYP82E5* (Gavilano et al. 2007) and CYP82E10 (Lewis et al. 2010).

Using a chemical mutagenesis approach, knockout mutations in all three tobacco NND genes (*CYP82E4, CYP82E5 and CYP82E10*) were identified and pyramided together. Plants that possessed knockout mutations in all three genes displayed significantly lower nornicotine content (~ 0.5% of the total alkaloids) compared to conventional tobacco cultivars. Subsequently, dCAPS (derived cleaved amplified polymorphic sequence) and CAPS (cleaved amplified polymorphic sequence) markers were developed for the easy identification of *CYP82E4, CYP82E5v2* and *CYP82E10* gene mutants (Li et al. 2012). These markers are co-dominant and can easily differentiate between heterozygous and homozygous plants and thus can serve as important tools in the transfer of the low nornicotine/NNN trait into elite tobacco varieties.

2.5.3. Novel Aspects of Anatabine Biosynthesis

Anatabine is unique among the four major tobacco pyridine alkaloids in that the synthesis of both rings is dependent only on the pyridine metabolic pathway (Fig. 2.3). Although commonly found in all *Nicotiana* species, anatabine is never the predominant alkaloid, and its function has been questioned, given that it has been shown to perform poorly as an insecticide. One possibility involves a potential role in NA detoxification. As shown in Figure 2.3, NA serves as an intermediate in the synthesis of all tobacco pyridine alkaloids. Excess NA, however, is toxic to plant cells. Anatabine might be produced in the plant to serve as an NA detoxifying metabolite. When pyrrolidine ring synthesis is suppressed it likely results in the accumulation of NA, or a related derivative, as it cannot become coupled with *N*-methyl-Δ^1- pyrrolinium to form nicotine. Channeling these compounds into the non-toxic compound anatabine might ameliorate the toxic effects of NA/NA derivatives (Dewey and Xie 2013). This model provides an explanation of why the levels of anatabine dramatically increase in the case of downregulation of

the *ODC* (Dalton et al. 2016; DeBoer et al. 2011), *PMT* (Chintapakorn et al. 2003) and *MPO* (Shoji and Hashimoto, 2008) genes dedicated to pyrrolidine ring synthesis.

The complete set of enzymes involved in the synthesis of anatabine is still unclear. Similar to nicotine biosynthesis, the production of anatabine also requires A622 and BBL activity, though the specific substrates and products of these enzymes have yet to be elucidated. One possibility is that A622 converts the nicotinic acid to 3,6 dihydro nicotinic acid, as depicted in Figure 2.3. Early studies using radiolabeled tracers suggested that 3,6 dihydro nicotinic acid can be decarboxylated to 1,2-dihydropyridine and 2,5-dihydropyridine. It was also proposed that the 1,2-dihydropyridine could react with 2,5-dihydropyridine to yield 3,6-dihydroanatabine, which could then be aromatized to produce anatabine (Leete and Mueller 1982; Gorrod et al. 1999). Identification of the enzymes and genes responsible for these reactions, however, is currently lacking.

2.5.4. Novel Aspects of Anabasine Biosynthesis

Anabasine is a minor alkaloid in the great majority of *Nicotiana* species, including *N. tabacum.* The exception is *Nicotiana glauca,* which predominantly accumulates anabasine rather than nicotine (Solt et al. 1960). Anabasine is composed of a pyridine and a piperidine ring. Similar to other tobacco alkaloids, NA derived from the aspartate pathway serves as the precursor for the pyridine ring. The piperidine ring is synthesized from lysine, which is decarboxylated to cadaverine by the enzyme lysine decarboxylase (LDC). Cadaverine undergoes oxidative deamination to form 5-aminopentanal, which cyclizes spontaneously to form Δ' – piperidine (Dewey and Xie 2013). LDC might act as a rate-limiting enzyme in the synthesis of the piperidine ring, as a positive correlation between LDC activity and the levels of cadaverine and anabasine has been observed (Gorrod et al. 1999). There are still many gaps in our

understanding of anabasine biosynthesis in *Nicotiana* species. As just one example, the role of BBL enzymes in anabasine biosynthesis is unclear. Although anabasine levels do decrease in plants whose *BBL* gene function has been compromised, its reduction is very modest compared to that observed with nicotine or anatabine (Lewis et al. 2015).

2.6. Hormonal Regulation of Alkaloid Biosynthesis

Jasmonic acid (JA) levels in the leaf have been shown to increase upon herbivore attack or physical damage to the leaf. JA is an oxylipin and its methylated derivative methyl jasmonate (MeJA) are derived from the polyunsaturated fatty acid linolenic acid, which is released from the galactolipids of the chloroplast membranes when the plant is wounded (Baldwin et al. 1994). JA may be transported to the root tissues directly, or alternatively, a systemin-like protein signal may be transmitted to increase JA production directly within the root tissues (Shoji and Hashimoto 2013). JA signaling recruits a basic helix-loop-helix transcription factor called MYC2. MYC2 binds to the G-box of the promoters of genes involved in nicotine biosynthesis, as well as to the promotor of *Nic2* loci ERF genes and activates their transcription. The combined action of the master transcription factors MYC2 and specific ERFs found at the *NIC2* locus upregulate all of the genes involved in nicotine biosynthesis (Shoji and Hashimoto 2011).

Takashi Hashimoto and his colleagues have proposed a model that describes how JA signaling can lead to the transcriptional activation of nicotine biosynthetic genes. Figure 2.5 is a representation of this model. In the absence of JA, or the ground state when there is no JA signaling, the jasmonate-ZIM domain protein JAZ binds to a MYC2 transcription factor as it is bound to the G-box of the promoters of nicotine biosynthetic genes. Furthermore, JAZ proteins may recruit chromatin remodeling factors like Groucho/Tup1-type, including TOPLESS, as co-repressors through an interaction with an adaptor protein NINJA which inhibits transcription.

JAZ proteins can also bind to free MYC2 proteins to prevent them from binding the promoters of ERF genes and thus prevent their activation (Shoji and Hashimoto 2011).

The conjugation of JA to the amino acid isoleucine by the enzyme jasmonic acid-amido synthetase (JAR1) results in the production of JA-Ile, which is believed to be the bioactive form of JA (Katsir et al. 2008). In the presence of JA, CORONATINE INSENSITIVE 1 (COI1) proteins recruit JAZ proteins, and when the COI1-JAZ complex perceives JA-Ile, JAZs are believed to be presented to the SCF-type E3 ubiquitin ligase complex where they become ubiquitinated and then degraded by the 26S proteasome. This results in the removal of JAZ proteins, which allows the MYC2 to activate both the nicotine biosynthetic and *ERF* genes (Shoji and Hashimoto 2011). Finally, a study has shown that salt stress induces the biosynthesis of JA in tobacco, which in turn can trigger nicotine synthesis (Chen et al. 2016).

Ethylene and its natural precursor 1-aminocyclopropane-1-carboxylic acid (ACC) suppress JA-induced nicotine biosynthesis. Ethylene exposure may also lead to transcriptional repression of the B/*NIC* 2 locus ERF transcription factors, though no significant influence of ethylene on MYC2 expression has been observed (Shoji and Hashimoto 2013). *Manduca sexta* is a nicotine-tolerant insect species. When *Manduca* larva feed on the leaves of *N. attenuata*, ethylene is released at the wound sites. In this case, however, insect-mediated wounding of the leaf did not lead to an increase in the nicotine levels. It has been proposed that plant might actively repress the metabolically taxing induction of nicotine biosynthesis against a nicotine-tolerant herbivore to utilize its resources more effectively by channelizing them to a different defense mechanism (Shoji et al. 2000).

Shi et al. (2006) studied the effects of different kinds of mechanical wounding on nicotine production in tobacco plants. Higher levels of nicotine accumulation were observed in

the case of decapitation of the apical meristem (topping) in comparison to leaf wounding. Further increases in nicotine concentration were also observed when the lateral buds that emerged after the excision of shoot apex were removed. Apical meristems are the major source of auxin in the plant. Application of 1-naphthyl acetic acid (NAA), a plant hormone belonging to the auxin family, at the decapitation site completely inhibited the stimulation of nicotine production in the root. In addition, the application of the auxin transport inhibitor N-1-naphthylphthalamic acid (NPA) underneath the shoot apex of intact plants caused an increase in nicotine accumulation (Li et al. 2007). From these results, it can be concluded that auxin negatively affects nicotine biosynthesis. The mechanism by which auxin exerts this effect, however, remains unclear (Shoji and Hashimoto 2013).

2.7. Conclusion

Nicotiana tabacum, a cultivated tobacco species that is commercially grown for its leaves, belongs to the Solanaceae family. *N. tabacum* produces the pharmacologically important alkaloids nicotine, nornicotine, anatabine, and anabasine. The most notable property of nicotine involves its interaction with acetylcholine receptors, a phenomenon that leads to addiction in humans and in nature helps the plant in its defense against herbivore attack. Tobacco alkaloids have also shown promise in alleviating the effects of neurodegenerative diseases in animal models. Nicotine accounts for approximately 90% of the alkaloid population in commercial tobacco varieties and is found almost exclusively in the pharmacologically active S enantiomer within tobacco leaves. Although alkaloid biosynthesis has been extensively studied in tobacco, the enzymes and metabolic intermediates involved at the end of the pathway remain unclear. Information is also lacking with respect to identification of the proteins involved in the transport of alkaloids from root cells to the leaf. Hormone signaling plays a key role in regulating alkaloid

levels within the plant. JA signaling is of particular importance due to its function in triggering nicotine biosynthesis in tobacco root tissues. Hormones regulate the alkaloid pathway via the master regulatory transcription factor MYC2 and certain members of the ERF superfamily of transcription factors. The discovery and the concomitant genetic manipulation of the genes associated with nicotine biosynthesis via mutagenesis, overexpression, knockdown, and knockout have opened a gateway for the creative alteration of the alkaloid profiles for the common good.

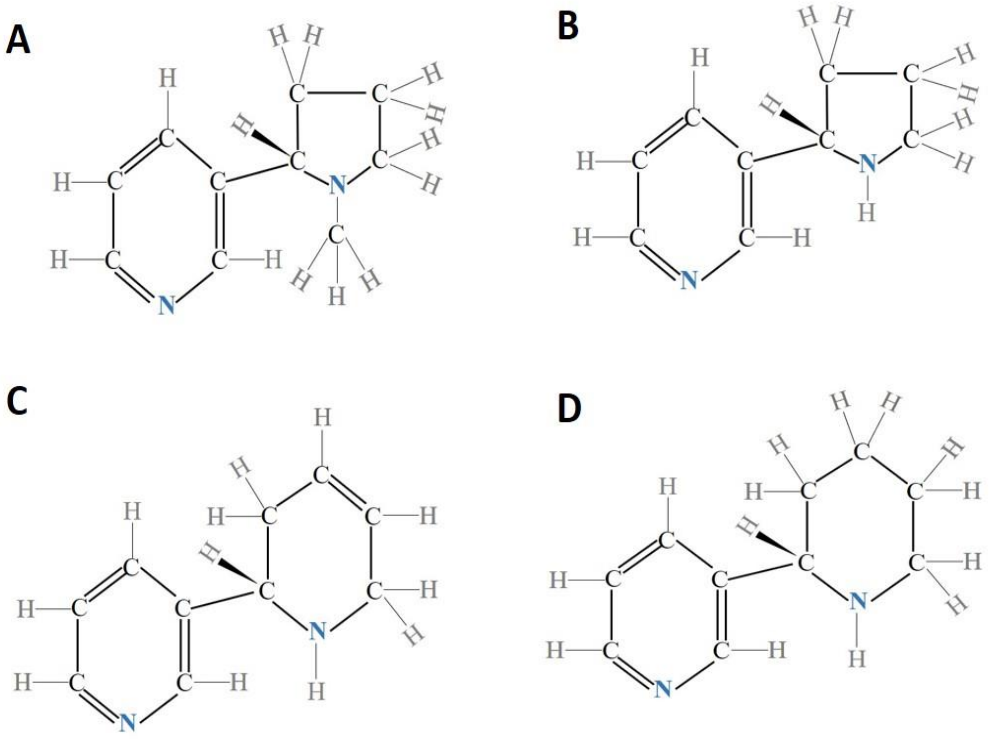

Figure 2.1. Chemical structures of nicotine (A), nornicotine (B), anatabine (C) and anabasine (D).

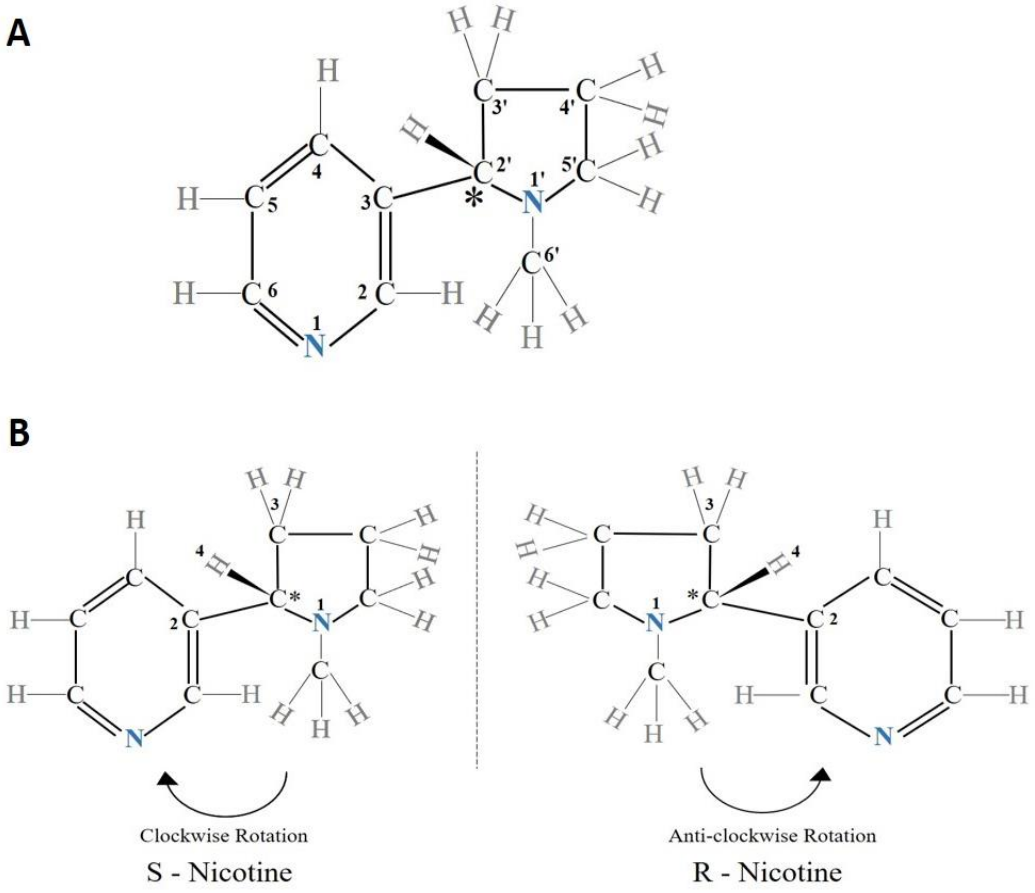

Figure 2.2 Nicotine structure and enantiomers. **A.** The structure of nicotine is shown with each carbon and nitrogen atom numbered according to standard convention. The chiral carbon atom at the 2' position of the pyrrolidine ring is highlighted with an asterisk. **B.** The S and R structural enantiomers of nicotine. The chiral carbon atom is bound to an amine (1), pyridine (2), methylene (3) and hydrogen (4).

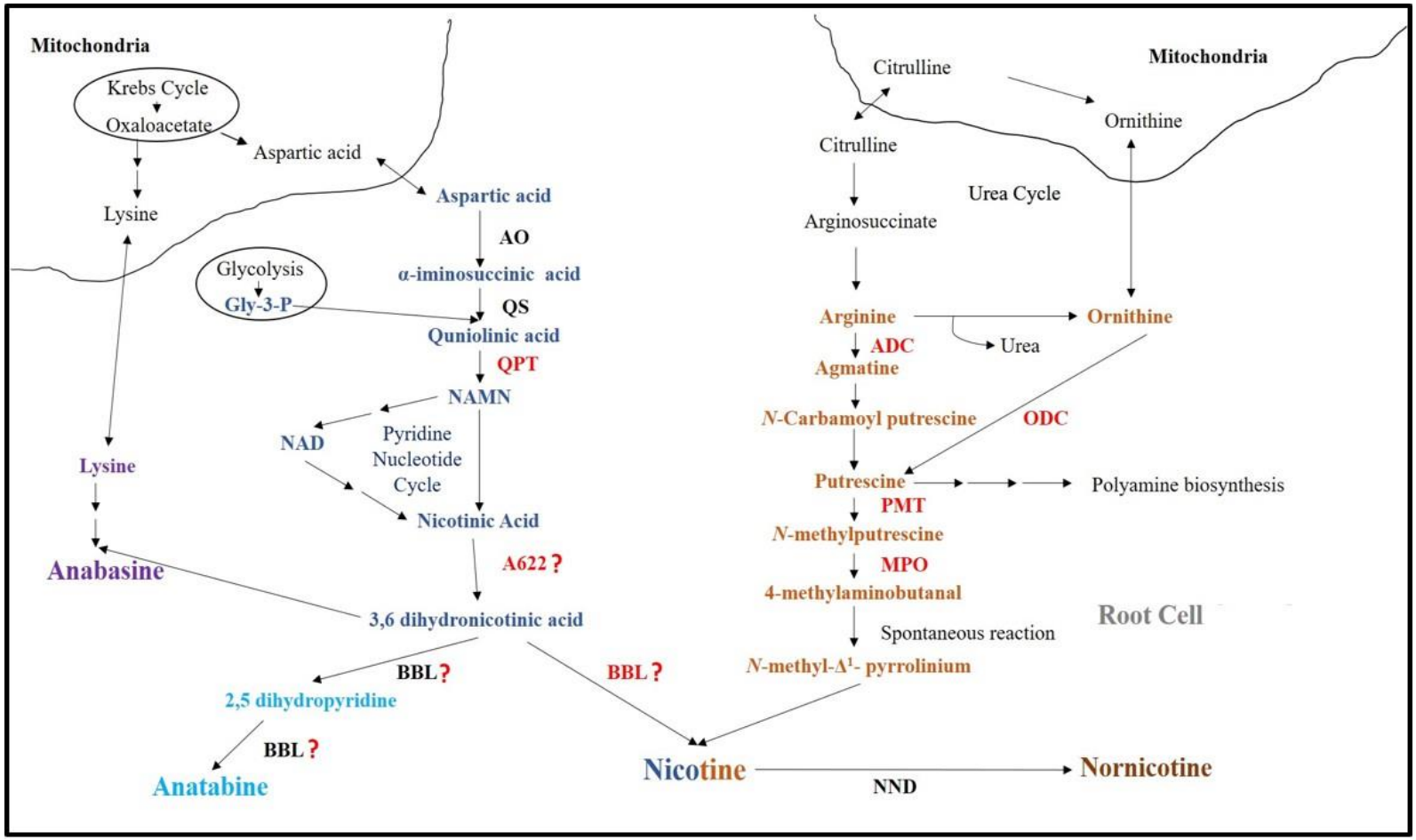

Figure 2.3. Current model of alkaloid biosynthesis in *Nicotiana* species. A622, isoflavone reductase-like protein; ADC, arginine decarboxylase; AO, aspartate oxidase; BBL, berberine bridge enzyme-like; MPO, *N*-methyl putrescine oxidase; PMT, putrescine methyltransferase; NND, nicotine *N*-demethylase; QS, quinolate synthase; QPT, quinolate phosphoribosyl transferase.

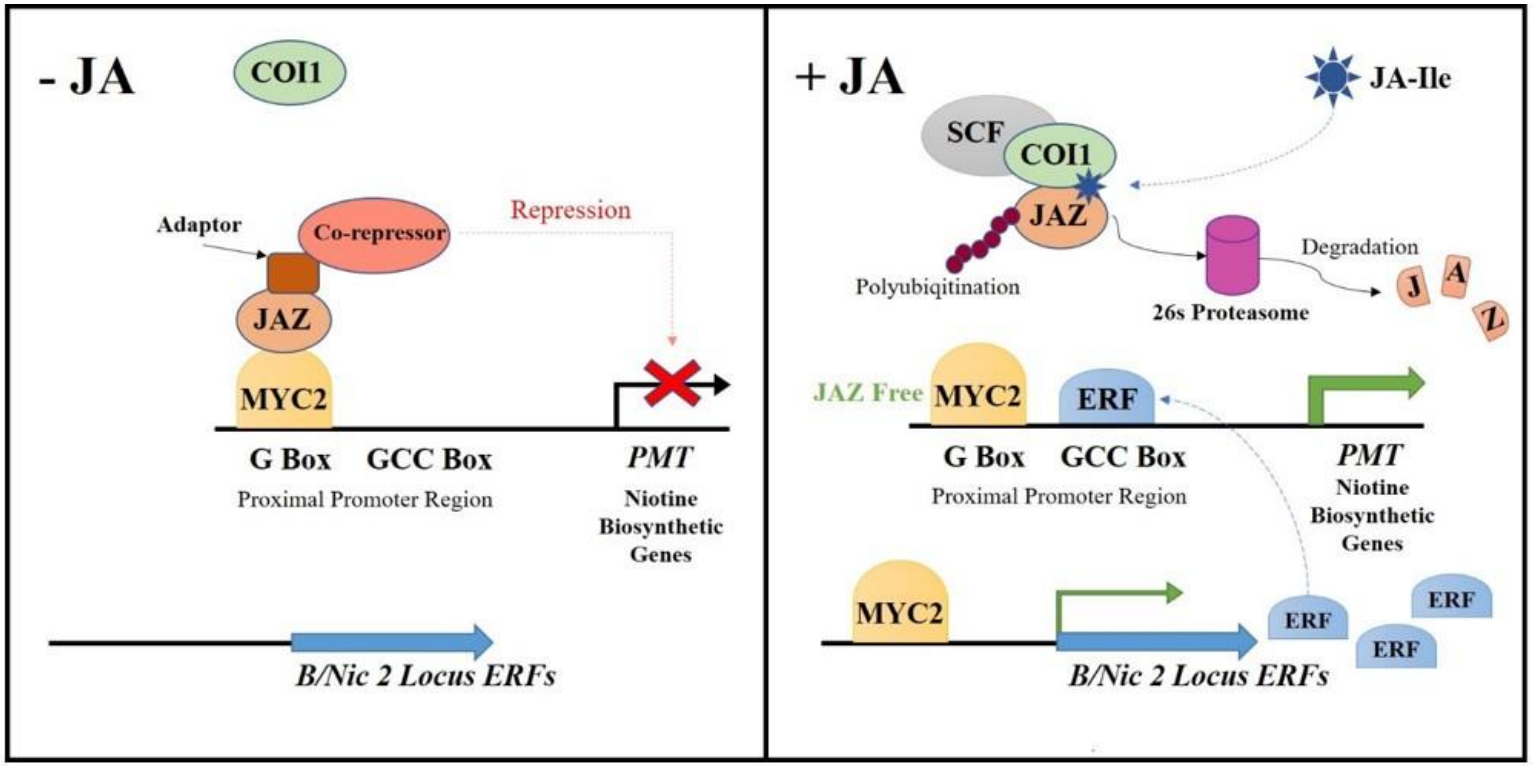

Figure 2.5. A model of jasmonate signaling leading to the transcriptional activation of nicotine biosynthetic genes. See text for abbreviations.

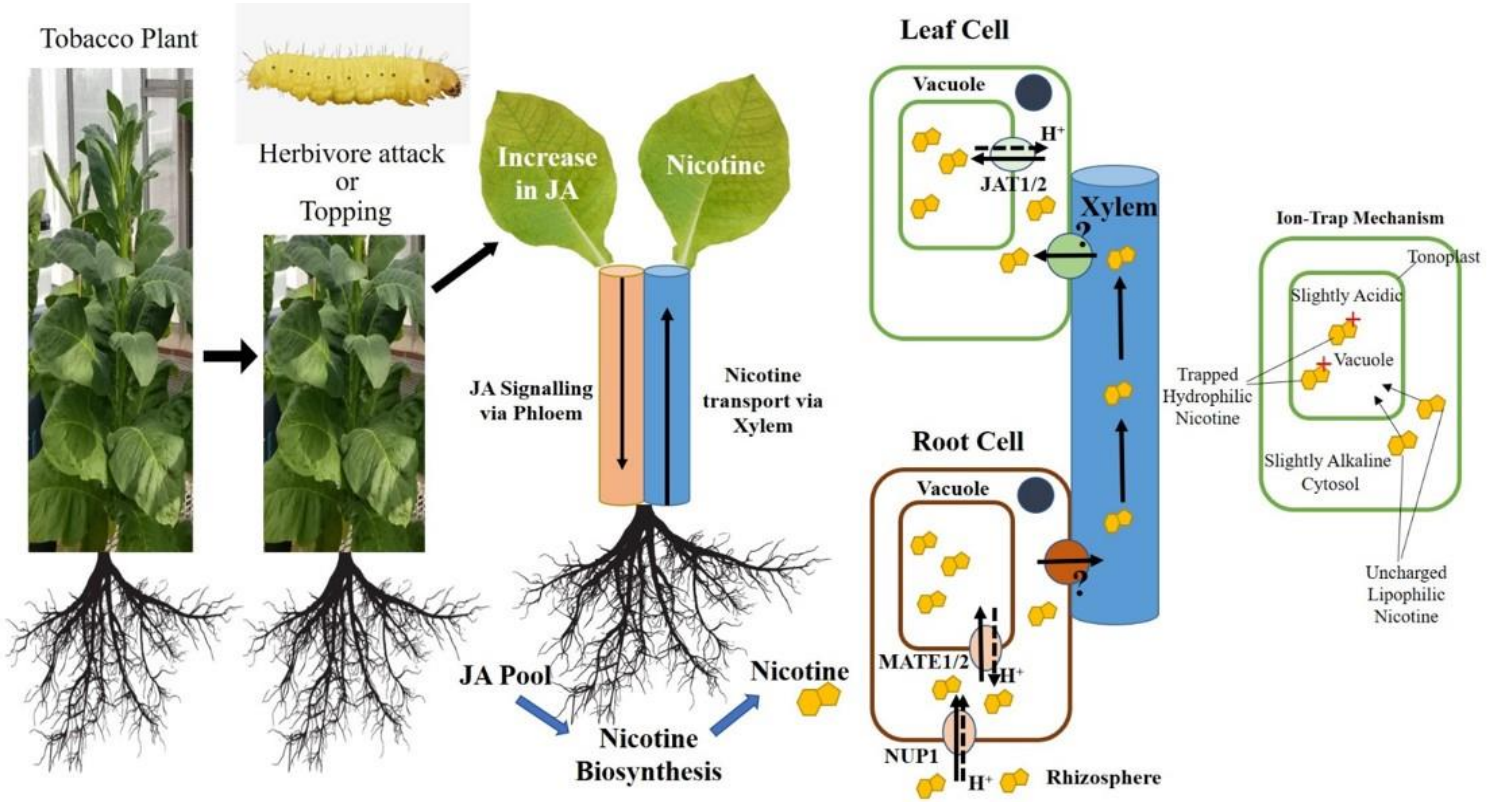

Figure 2.4. A model of current understating of the nicotine transport from root cells to leaf vacuoles.

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CHAPTER 3

Artificial Positive Feedback Loop for Maximizing Alkaloid Content in *Nicotiana tabacum*

3.1. Introduction

As discussed in Chapter 2, nicotine and related pyridine alkaloids have numerous pharmacological applications. The tobacco alkaloids nicotine, anatabine, and anabasine have been shown to function as neuroprotective agents and might help to alleviate the symptoms of disorders like Alzheimer's, Parkinson's, and other neurogenerative diseases. Also, with continued global awareness of the hazards of cigarette smoking, the demand for products that can help smokers quit is at an all-time high. Purified nicotine is the chief component of many smoking cessation products and smoking alternatives like e-cigarettes and other vaping devices. Thus, tobacco plants with the ability to accumulate very high levels of nicotine may have considerable commercial potential, especially considering that the chemical synthesis of nicotine is not cost-effective in comparison to nicotine derived and purified from tobacco plants (Farsalinos et al. 2014). For example, a company called Next Generation Labs has reported that the chemical synthesis of nicotine costs thirteen times as much as purifying it from its natural source (Next Generation Labs website, accessed 2019) (Zhang et al. 2016).

Recently, the laboratory of Dr. Dominique Loque of the Joint BioEnergy Institute in Berkeley, CA described a simple, yet clever system for maximizing the products of secondary metabolism in circumstances where a master regulatory transcription factor (TF) had been previously characterized. The Loque group hypothesized that by placing the master TF under the transcriptional control of one of its downstream targets (preferably one that is strongly induced), and introducing this construct back into the plant, one could create a situation where the overexpression of the entire metabolic pathway would be maximally enhanced, as the initial

stimulation of the pathway (by whatever natural signals control it) via the native master TF, will lead to even more production of the master TF as it promotes its own synthesis. This process was given the term Artificial Positive Feedback Loop (APFL) (Yang et al. 2013). As a proof-of-concept, it was demonstrated that engineering the secondary cell walls of Arabidopsis to provide more strength in mutant plants deficient in lignin (a valuable trait when plants are engineered as a biofuels feedstock) using an APFL-based strategy was much more successful than using what has been the more traditional approach of simply overexpressing the master TF using a strong constitutive promoter, such as the 35S promoter of Cauliflower Mosaic Virus (CaMV). It was proposed that the generation of an APFL would have the following two major advantages over the more conventional means of TF overexpression: (1) higher levels of the master TF would likely be produced, and thus the entire metabolic pathway be upregulated to a higher degree; and (2) pathway overexpression would be strictly confined to the cell/tissue types where that pathway has naturally evolved to function within. One common problem with the constitutive overexpression of a master TF is that the metabolic pathway becomes activated in cell types that normally are not the targets of the given pathway, leading to perturbations in normal plant growth and development. In the case of secondary cell wall biosynthesis in Arabidopsis, activating the pathway via a 35S promoter led to ectopic expression in non-target tissues and a reduction in plant growth and vigor. In contrast, when the same master TF was introduced as an APFL construct, plant growth and development was normal, and the secondary cell walls were reinforced only in the desired locations, such as the vessel and fiber cells (Yang et al., 2013).

The purpose of this thesis project is to test whether the upregulation of alkaloid biosynthesis in tobacco can be maximized if the plant is engineered using an APFL-based strategy. Previous studies in tobacco have shown that a MYC2 TF binds to the G-box(es) found

in promoters of the nicotine biosynthetic genes ornithine decarboxylase (*ODC*), putrescine methyltransferase (*PMT*), *N*-methylputrescine oxidase (*MPO*), aspartate oxidase (*AO*), quinolate synthase (*QS*), quinolate phosphoribosyltransferase (*QPT*), an isoflavone reductase-like gene (*A622*), and berberine bridge-like enzyme (*BBL*) genes, as well as binding to the promoters of certain Ethylene Response Factor (***ERF)*** genes, resulting in activation of their transcription. These ERF TFs in turn bind to GCC boxes, also found in the promoters of nicotine biosynthetic genes. The combined action of the master TFs MYC2 and select ERFs is believed to upregulate all of the genes involved in nicotine biosynthesis (Shoji et al. 2011).

MYC2 is a basic-helix-loop-helix (bHLH) TF that has been characterized in numerous plants systems. It exists as a homodimer with motifs that include two amphipathic helices connected by a variable loop. The amino-terminal region of MYC2 contains a basic domain, which binds the TF to DNA and its carboxy-terminal region contains the HLH domain, which facilitates interactions with another protein subunit to form the homodimer (Jones 2004). Figure 3.1a represents the MYC2 3D structure as predicted using Swiss-Model software. In tobacco, Wang et al. (2015) characterized two MYC2 TFs, encoded by genes designated *NtMYC2a* and *NtMYC2b*, which were discovered using a yeast one-hybrid approach with a *QPT* promoter region as the bait. To evaluate the effects of the two MYC2 TFs on nicotine biosynthesis, they created overexpression lines by transforming tobacco cultivar NC95 separately with *35S:NtMYC2a* and *35S:NtMYC2b*. Within a greenhouse environment, T_1 generation transgenic *NtMYC2a* and *NtMYC2b* overexpression lines displayed nicotine levels that were 134% and 35% higher than vector control plants, respectively. Subsequent field trials using T_2 and T_3 generation lines demonstrated that this strategy was successful in significantly increasing the nicotine content of tobacco grown under standard field conditions by as much as 58% (Wang et al. 2015).

A weakness of this strategy, however, was that the *35S:NtMYC2a* lines were also associated with an approximately 25% decrease in cured-leaf yield. Given that the *NtMYC2a* transgene in these plants was not only overexpressed in the root cells where nicotine biosynthesis naturally occurs, but also ectopically expressed throughout the entire plant, one possible explanation for the yield reduction may be related to undesired secondary consequences associated with overexpressing this master TF in tissues not accustomed to accommodating the upregulation of the alkaloid biosynthetic pathway.

The *A/NIC1* and *B/NIC2* loci genetically control the nicotine content in the tobacco plants. The mutant alleles of these genes, *nic1nic1* (*aa*) and *nic2nic2* (*bb*), mediate low nicotine phenotypes. ERF TFs were found to be clustered at the *B/NIC 2* locus, and at least seven ERF-encoding genes, *ERF17, ERF104, ERF115, ERF168, ERF179, ERF189,* and *ERF221* were found to be deleted in the nic2 mutant (Shoji et al. 2010). *ERF189* and its close homolog *ERF199* were found to be induced by jasmonic acid (JA) but not by salt stress, whereas both JA and salt stress were shown to induce the rest of the *ERF* genes within that cluster. ERF189 and ERF199 were shown to upregulate nicotine biosynthetic genes by binding to GC-rich P-box motifs that are located on the promoters of the many nicotine biosynthetic genes. When tobacco hairy root cultures were treated with methyl jasmonate (MeJA), *ERF189* transcript levels were higher than *ERF199* (Kajikawa et al. 2017). The ERF189 protein is a monomer consisting of three anti-parallel β-sheets in the N-terminal region that interacts with DNA and an α-helix motif at the C-terminus (Shoji et al. 2013). Figure 3.1b represents the ERF189 3D structure as predicted using Swiss-Model software.

Based on the aforementioned studies, MYC2a and ERF189 can be considered master regulatory TFs, and thus serve as prime candidate for incorporating into APFL constructs.

Although the promoters of any of the well characterized nicotine biosynthetic genes (*ODC, PMT, MPO, AO, QS, QPT, A622, BBL*) could theoretically be used for coupling with *MYC2a* or *ERF189* to create an APFL construct, the promoters that regulate the specific *PMT* isoform *PMT1a* (Xu and Timko 2004) and the specific *A622* isoform *A622S* (Shoji et al. 2002) have been the most comprehensively studied and are thus the promoters of choice for generating APFL constructs designed to maximize alkaloid production in tobacco.

The studies conducted for this thesis present compelling evidence that the implementation of an APFL-based strategy is effective in maximizing nicotine production when implemented in normal tobacco varieties. The APFL strategy was less successful, however, when employed in a tobacco background that had been previously engineered to produce anatabine, rather than nicotine as its primary alkaloid.

3.2. Materials and Methods

A flowchart detailing the various steps that were followed in generating and testing the APFL-based system for the purpose of enhancing alkaloid biosynthesis is shown in Figure 3.2.

3.2.1. Molecular Cloning

DNA sequence and the gene-specific primer data of the promoters and transcription factors incorporated into the APFL and control constructs are shown in Appendix A. Details of the cloning process used to generate these constructs are described Appendix B. Briefly, the entry vectors used in this study included pCR2.1 and pMiniT. Both confer ampicillin resistance, but, carbenicillin, an ampicillin analog, is the more stable of the two and thus was used for selection in bacteria. Colony PCR and restriction digestion assays were used for the selection of positive colonies. Insert integrity of the cloning steps was confirmed by Sanger sequence analysis. The promoter and TF cassettes developed were cloned into the destination vector

pCAMBIA1301, a vector that confers kanamycin resistance for selection in bacteria and hygromycin resistance for selection in plants. Colony PCR and restriction digestion assays were used for the selection of positive colonies. *E. coli* strain DH5-α, LB agar and broth with appropriate antibiotics were used for bacterial culture.

3.2.2. Protein modeling

SWISS-MODEL software was used to model the 3D protein structures of MYC2a and ERF189. It is a fully automated protein structure homology-modeling server, accessible via the ExPASy web server, or from the program DeepView (SWISS-MODEL Website, Accessed 2019).

3.2.3. Agrobacterium Transformation

Validated constructs within the pCAMBIA1301 vector backbone were transformed into *Agrobacterium tumefaciens* strain LBA4404. Colony PCR was used to confirm the presence of the destination vector in the *Agrobacterium* colonies. LB agar and broth with appropriate antibiotics were used for the *Agrobacterium* culture. All vectors utilized in this study were numbered for convenience and are listed in Table 3.1 and Figure 3.2.

3.2.4. Tobacco Transformation

Transgenic plants were produced using standard *Agrobacterium*-based leaf disc transformation protocols. Validated *Agrobacterium* colonies were transformed into tobacco lines K326 and TN86 RNAi PMT, whose predominant alkaloids are nicotine and anatabine, respectively. A total of nine vectors were transformed into each of the two different tobacco backgrounds, necessitating a total of eighteen different transformation experiments. Due to the large number of transformation experiments needed for this project, limitations in both labor and growth chamber space prevented us from conducting all experiments at the same time. As an

alternative, the transformations were conducted as four different groups, as shown in Table 3.1. Leaf discs exposed to the *Agrobacterium* cultures were incubated on nursery plates, Murashige and Skoog (MS) media-based selection plates containing hygromycin, and then in rooting media in growth incubators set at a temperature of 23º C. PCR amplification using primers directed against the hygromycin resistance gene (*hptII*) was used as a marker for genotyping the transgenic T_0 plants once transferred to rooting media to verify the presence of the transgene. In addition, distally localized primers were designed for genotyping to ensure the presence of the entire transgene construct. A list of all junction primers and the sizes of the expected PCR products can be found in Appendix C.

Initially, 10 to 15 marker positive T_0 plants from each independent transformation experiment were transferred to soil and allowed to grow in a growth chamber for approximately one month, after which 10 to 12 healthy T_0 transgenic plants per genotype were transferred to a greenhouse. K326 pPMT1:ERF189 (experiment K-3) and K326 Empty Vector control (experiment K-9) experiments were the only exceptions, as only four healthy K-3 and eight healthy K-9 plants were recovered and transferred to the greenhouse. However, six additional K-3 T_0 plants were generated in a repeated transformation experiment and included with the genotypes that comprised Group 4. Group 1 and Group 2 plants were transferred to the greenhouse in July 2018 and October 2018, respectively. Group 3 and Group 4 plants were both transferred to the greenhouse in December 2018.

A standard greenhouse soil and fertilizer mix was used for all plants. Within each Group, the plants were arranged on greenhouse benches using a randomized design.

3.2.5. Phenotypic analysis

Phenotypic analysis was conducted on the T_0 greenhouse plants at the pre-flowering

(budding) stage, just prior to topping. The height of the plant, and length and width of the ninth leaf positioned from the bottom of each T_0 plant were recorded. Any notable growth abnormalities were also noted.

3.2.6. Alkaloid Analysis

When the majority of the T_0 greenhouse plants were on the verge of flowering, leaves 4 - 6 from the top were harvested for alkaloid analysis. After the leaf samples were collected, the plants were topped. Two weeks post-topping, three leaves from the top of each decapitated plant were also collected. Suckers were removed by hand during this period, as the auxin that is produced upon the re-establishment of an apical meristem would have negatively impacted alkaloid synthesis. The leaf samples collected before and after topping were dried to completeness at 65º C for three days in an incubating oven, then ground and submitted to the NCSU Tobacco Analytical Lab for alkaloid analysis. Gas chromatography with the flame ionization detector was used to analyze the alkaloid content in each sample. After the post-topping leaf sample collection, suckers were allowed to grow, flower, and set seed for subsequent analysis of T_1 plants in the field, if warranted.

3.2.7. Statistical Analysis

Due to the inherent variability that exists among T_0 plants transformed with identical constructs, data obtained from such plant are not typically subjected to statistical analysis. To obtain a preliminary indication of the effects of each construct on the alkaloid content of the tobacco, however, we conducted a simple statistical analysis to determent whether a significant difference was predicted among different genotypes within the same transformation Group using a two-tailed Student’s t-test. When multiple genotypes across different Groups were compared, a one-way ANOVA was conducted, assuming equal variances among the different Groups. Least

significant difference (LSD) calculation were determined using SAS software to test whether the genotypes were significantly different. Probability values of < 0.05 were considered statistically significant. However, a high error rate may be expected due to low sample numbers.

3.2.8. Gene Expression Analysis

Two weeks post-topping, root tissue was collected from all T_0 transgenic plants. With the exception of plants transformed with the pPMT1:GUS1 vector, the two plants of each transgene genotype represented in Group 1 (K-2, K-3, K-4 and K-5 of Table 1) that displayed the highest nicotine accumulation phenotype, along with the two plants of the Empty Vector control genotype from Group 4 (K-9 of Table 1) with the highest nicotine content were selected for RNA isolation. RNA was isolated from root tissue using the QIAGEN RNeasy Plant Mini Kit. First strand cDNA was synthesized using SuperScript™ III Reverse Transcriptase (Invitrogen) with an oligo (dT) primer. qRT-PCR was performed using FastStart Universal SYBR Green dye (Bio-Rad) on an Agilent Technology AriaMx Real-Time PCR System. An *N. tabacum* actin gene was used as a normalization control. Three technical replicates were performed on each RNA preparation using the following PCR program: 95 °C for 2 min; 40 cycles of 95 °C for 15 sec, followed by 63 °C for 30 sec, and 72 °C for 30 sec using the primers for nicotine biosynthetic genes previously described by Wang et al. (2015). The primer sequences, target gene isoform information, and the expected product lengths have been included in Appendix D.

3.2.9. Histochemical staining

Seeds collected from T_0 transgenic K326 plants K-1 (pPMT1:GUS-1), K-6 (pA622S: GUS-6), and K-2 (pPMT1:MYC2a, as a negative control) were germinated, and nine-day-old seedlings were utilized for GUS analysis. The seedlings were incubated in a GUS staining solution containing the X-Gluc substrate for 24 hours at 37º C, after which the solution was

replaced with 70% ethanol to remove chlorophyll from the leaf tissue. Photographs of the GUS stained seedlings were taken using a Leica MZ FLIII stereo dissecting microscope at the NCSU Cellular and Molecular Imaging Facility.

3.3. Results and Discussion

To initiate an APFL strategy for alkaloid production in tobacco, master regulatory TFs such as *NtMYC2a* and *NtERF189* need to be coupled to the promoter of a gene that is highly induced by that TF. Previous studies have shown that the promoters driving the expression of the *PMT1a* and *A622S* genes of the alkaloid biosynthetic pathway are very strong, root-specific, and induced by MYC2a and ERF189 (Shoji and Hashimoto 2011; Shoji and Hashimoto 2013). Despite these previous reports, we considered it important to confirm this directly for the specific sequences that we either amplified (*pA622S*) or had synthesized (*pPMT1a*) for the purpose of incorporating into our APFL constructs. To test their root specificity, a GUS reporter gene was placed downstream of the presumed *PMT1a* and *A622-S* promoter regions and expressed in K326. As shown in Figure 3.3, GUS staining was observed in the root tissue of transgenic plants containing pPMT:GUS and pA622:GUS constructs, but not in leaf tissue, consistent with the results of prior studies.

A schematic representation of the proposed APFL strategy to enhance alkaloid synthesis and accumulation is shown in Figure 3.4. To test whether employing such a strategy could be effective for maximizing alkaloid production, constructs were generated in which the MYC2a and ERF189 master TFs were placed individually under the transcriptional control of the *PMT1a* and *A622S* promoters. In theory, the upregulation of a metabolic pathway should be greater when an APFL is formed, in comparison to simply placing the master TF under the regulatory control of a strong constitutive promoter. Therefore, additional constructs were generated in which the

MYC2a and ERF189 TFs were cloned downstream of an enhanced CaMV 35S promoter. The former construct, designated 35S:MYC2a, is essentially a re-creation of the vector used by Wang et al. (2015) to enhance nicotine production in tobacco. As an additional control, plants were also transformed with an "empty" pCAMBIA1301 vector that was used as the backbone for the various vectors generated in this study. Finally, in addition to testing whether the APFL strategy could be effective in maximizing nicotine accumulation in a typical commercial tobacco variety (K326), we also wanted to determine whether such a strategy could be used to generate tobacco plants capable of producing exceptionally high amounts of the alternative, pharmacologically interesting alkaloid anatabine. To test this possibility, all constructs generated in this study were also introduced in a tobacco line generated by Dr. Ramsey Lewis (North Carolina State University) in which an anti-*PMT* RNAi construct had been introduced into burley variety TN86 (referred to herein as TN86 RNAi PMT). Previous research studies have shown that the RNAi-mediated inhibition of *PMT* gene expression results in tobacco plants that accumulate anatabine, rather than nicotine, as the predominant alkaloid (Chintapakorn et al. 2003).

Including the pPMT1:GUS and pA622:GUS constructs described in the first paragraph of this section, a total of nine genetic constructs where used in this study. A diagram of each construct is shown in Figure 3.5. Transforming nine constructs into two different tobacco backgrounds is exceptionally labor intensive and demanding of growth chamber space. It was therefore considered impractical to be conduct all of the transformations experiments simultaneously. Instead, it was decided to stagger the eighteen transformations experiments required into four groups, with each group of transformations separated in time as described in Materials and Methods. Of necessity, plants from different groups were grown in growth chambers, then in a greenhouse at different times and thus the environmental conditions for each

group cannot be considered equal. As a result, caution must be taken when comparing results between genotypes represented in different groups. Combined with the fact that phenotypic data from T_0 generation plants is inherently less reliable than that obtained in subsequent generations (particularly after transgene copy number has been fixed), the data presented below is to be considered preliminary. Nevertheless, the analysis of these materials should give an initial indication whether the APFL strategy is effective, and which of the numerous materials generated in this study should be forwarded for subsequent analysis in the field. A listing of the individual constructs, the plant backgrounds, the coding designation for each transformation experiment, and the grouping of the individual transformation experiments is shown in Table 3.1.

3.3.1. Phenotypic Analysis

With the exception of the transformation experiments using the GUS reporter construct driven by the *PMT1a* and *A622S* promoters (which were generated solely for the purpose of validating the root specificity of these promoters), the goal was to evaluate 10 T_0 plants from each transformation experiment. As a preliminary indicator of whether the expression of any given transgene is negatively impacting plant growth, the height of each plant was measured at the time of topping. The results of this analysis are presented according to group in Figure 3.6. From even a cursory examination of the data, it is apparent that the plants represented in Groups 1 and 2 are taller on average than those in Groups 3 and 4. The likely explanation for this is the differences in environmental conditions among the groups, as Group 1 and Group 2 plants were grown in the greenhouse during late summer and fall, and thus received greater light intensity and duration than the plants in Groups 3 and 4 that were grown during the winter months. The single most compelling result of Figure 3.6 is that the average height of the plants in experiment K-5 (K326 plants containing 35S:ERF189 transgene) was significantly shorter than the other

genotypes within that same group (K-2, K-3, and K-4). Interestingly, when this same construct was expressed in the TN86 RNAi PMT background (T-5), no significant difference in average height was observed in comparison to the other genotypes of that group (T-2, T-3 and T-4).

3.3.2. Alkaloid Analysis

Once a substantial proportion of the plants in the greenhouse for any given group began to initiate flowering, all plants of that group were topped. Just prior to topping, however, leaves 4 - 6 from the top were excised, the mid-ribs removed, pooled, then dried and ground for alkaloid analysis. Topped plants were grown for an additional two weeks (with suckers removed as needed) after which the top three leaves from each plant were similarly processed and submitted to the NCSU Tobacco Analytical Lab for alkaloid analysis. Although the alkaloid assays determined the content of all four of the most prevalent pyridine alkaloids of tobacco (nicotine, nornicotine, anatabine and anabasine) for each sample, nothing unexpected or revealing was noted for any of the "minor" alkaloids within a given parental background (data not shown). Therefore, for the sake of ease of interpretation, the results of the alkaloid assays are only presented for the alkaloid of primary interest within the given parental background; i.e., nicotine in case of the transgenic plants in variety K326, and anatabine in case of the transgenic plants in the TN86 RNAi PMT background. Similar to the analysis of plant height, the most legitimate comparisons are among different genotypes within the same group, as in these cases all of the plants were grown at the same time and under the same environmental conditions.

3.3.2.1. Nicotine Analysis: Group 1 and Group 4 plants

As shown in Figure 3.7, for Group 1 plants the APFL line pPMT1:MYC2a clearly accumulated the highest levels of nicotine, both before and after topping. None of the other three genotypes included in Group 1 were significantly different from each other based on a two-tailed

Student's t-test ($\alpha = 0.05$). Of particular interest is a comparison of plants containing the pPMT1:MYC2a construct with those transformed with the 35S:MYC2a vector, as plants containing the latter construct represent the strategy that to date has been shown to result in the highest yields of nicotine using any type of transgene-based approach (Wang et al., 2015). Almost twice the nicotine concentration was observed among plants possessing the PMT1:MYC2a APFL construct compared to those constitutively overexpressing the MYC2a TF using the strong constitutive enhanced CaMV 35S promoter.

The genotypes of Group 4 included the two APFL constructs driven by the *A622S* promoter (as opposed to the *PMT1a* promoter), and the empty vector pCAMBIA1301 control. Similar to the results of Group 1, the plants containing an APFL construct where the MYC2a TF (and not ERF189) was driven by the root-specific *A622*S promoter clearly accumulated the highest levels of nicotine, both before and after topping (Fig 3.8).

Analysis and comparisons of data derived from T_0 plants are complicated by the fact great variation can exist among independent T_0 plants possessing the same construct, ranging from plants that express the transgene at a high level, to those that do not express it at all. For these reasons, pooling results from different T_0 individuals containing the same transgene construct (as was done in Figs. 3.6 - 3.8) has the tendency to underestimate the true impact of a given transgene construct. One means of controlling for this phenomenon would be to conduct an expression analysis (e.g. quantitative real time PCR or Northern blotting) of each T_0 plant, then "normalize" the data accordingly. For this project, conducting expression analyses of this nature for plants with APFL constructs is complicated by the difficulties associated with isolating high-quality RNA from soil-grown root tissue from dozens of plants. In addition, were such a sampling be made prior to the final leaf harvest, the wounding damage imposed on the

plants by extracting a portion of the roots would likely impact the alkaloid profile, as well as general plant growth.

As an alternative means of potentially controlling for the some of the impact that low expressing T_0 individuals may have imposed on the results shown in Figures 3.7 and 3.8, the five plants of each genotype from both Group 1 and Group 4 that contained the highest nicotine content were compared in a separate analysis. These results are shown in Figure 3.9. As expected, plants possessing the two APFL constructs with the MYC2a TF show the highest nicotine levels. In this case, however, the differential between the APFL construct and its relevant "control" are even greater. In Group 4, for example, the pA622:MYC2a individuals on average accumulated 59% more nicotine than the empty vector control plants when all 10 T_0 individuals from genotype were compared (Fig. 3.8), but this increases to a 116% increase when only the top 5 T_0 individuals for each genotype are considered (Fig. 3.9). Furthermore, when all individuals are given equal consideration, the Group 4 plants with the pA622S:ERF189 construct are not considered statistically different from the empty vector control plants (Fig. 3.8). When only the top 5 T_0 individuals are considered, however, the difference is statistically significant, with the pA622S:ERF189 individuals averaging 51% more nicotine than the empty vector controls (Fig. 3.9). The nicotine levels for each the individual plant for all genotypes from both Group 1 and Group 4 included in the end of this chapter.

Overall, the results of the alkaloid analysis of the plants included in Groups 1 and 4 strongly suggest that the APFL lines generated using a strong root-specific promoter driving the expression of the MYC2a master TF accumulate nicotine to much higher concentrations than not only the empty vector controls, but in comparison to 35S:MYC2a overexpression lines as well, at least when grown in a greenhouse environment.

3.3.2.2. Anatabine Analysis: Group 2 and Group 3 plants

As expected, when placed in a parental background possessing an RNAi construct targeting the *PMT* gene family, all plants in Groups 2 and 3 accumulated anatabine as the predominant alkaloid (data not shown). Group 2 was comprised of individuals containing the pPMT1:MYC2a, pPMT1:ERF189, 35S:MYC2a or 35S:ERF189 constructs). Although genotypes pPMT1:MYC2a and pPMT1:ERF189 were deemed statistically different from each other, it is clear that as a whole the APFL constructs were no more effective in altering the anatabine content than were the constructs that simply used an enhanced CaMV 35S promoter to drive the expression of the TFs (Fig. 3.10). When the three genotypes included in Group 3 were compared, the two lines possessing *A622S* promoter-driven APFL constructs showed slightly higher mean levels of anatabine both before and after topping, but these differences were not considered statistically significant (Fig. 3.11).

Similar to the materials generated in the K326 background, the five plants of each genotype from both Group 2 and Group 3 that displaying the highest anatabine content were compared in an attempt to alleviate potential biases imposed by T_0 individuals that may not be expressing the transgene effectively. These results are presented in Figure 3.12. When analyzed in this manner, the pPMT1:MYC2a genotype displays the lowest levels of anatabine accumulation, a phenotype considered statistically significant in comparison to four of the other transgene constructs. Nevertheless, no anatabine accumulation phenotype was considered statistically different than the vector only control plants. The anatabine levels of each individual plant for all of the genotypes in both Group 2 and Group 3 are included in Appendix E.

As a whole, alkaloid analysis of the Group 2 and 3 genotypes suggests that the overexpression of the MYC2a or ERF189 TFs, whether it be through an APFL strategy or

merely using an enhanced CaMV 35S promoter does not enhance the plant's ability to produce and/or accumulate anatabine beyond that which is mediated by the anti-*PMT* RNAi construct. One possible explanation may relate to the function of anatabine within the plant, and the fact that in all characterized *Nicotiana* species found in nature, anatabine is always considered a minor alkaloid. Anatabine has not been shown to have strong insecticidal properties (Funayama et al, 2015) and as such there may have been minimal evolutionary pressure that would favor plants that accumulate high levels of this alkaloid. Instead, it has been speculated that anatabine may function mainly as a nicotinic acid detoxification metabolite (Dewey and Xie 2013). Therefore, it is not unreasonable to speculate that the metabolic pathways that have evolved in the plant to produce nicotine, nornicotine and even anabasine, do not fully apply to anatabine. The steps in anatabine biosynthesis that differ from the general metabolic pathway responsible for nicotine production are unknown (see Fig. 2.3 of Chapter 2). Our results suggest that those "missing steps" are not regulated by the MYC2a or ERF189 TFs, as their overexpression, whether creating via an APFL or through the use of a 35S promoter, does not appear to influence anatabine levels within an anti-*PMT* RNAi background. Instead, it appears that a bottleneck may be present in the synthesis of anatabine that is not affected by the overexpression of either of these two regulatory TFs. This hypothetical bottleneck may also explain why the levels of anatabine in anti-*PMT* or anti-*ODC* RNAi lines always appears to fall short of what the nicotine content was in the parental genotype (Chintapakorn et al. 2003; Dalton et al. 2016; DeBoer et al. 2011; Shoji and Hashimoto 2008).

3.3.3. qRT-PCR Analysis

One of the most interesting, and unexpected, results reported by Wang et al. (2015) in their investigation of mechanism by which the overexpression of MYC2a using the CaMV 35S

promoter leads to enhanced nicotine accumulation was the observation that most of the genes encoding enzymes of the nicotine biosynthetic pathway were actually down regulated in the plants displaying the highest levels of nicotine. In that study, data was presented suggesting that high levels of nicotine can be toxic to the tobacco root cells, and once they become too high, a negative feedback inhibition mechanism may be triggered that results in the repression of the genes which are involved in nicotine biosynthesis. To investigate this phenomenon within the context of MYC2a and ERF189 overexpression mediated through an APFL strategy, RNA was isolated from the roots of the two T_0 individuals that displayed the highest nicotine accumulation phenotypes for each of the following constructs: pPMT1:MYC2a, pPMT1:ERF189, 35S:MYC2a, 35S:ERF189, and the pCAMBIA1301 empty vector control. Quantitative Real-Time PCR (qRT-PCR) was used to measure the steady-state transcript levels of the same set of nicotine biosynthetic pathway assayed in this same manner by Wang et al. (2015). Genes that were assayed include those encoding *ADC*, *ODC*, *PMT*, *MPO*, *QPT2*, *A622* and *BBL*.

In Figure 3.13, the qRT-PCR expression results for the plants containing pPMT1:MYC2a, pPMT1:ERF189, 35S:MYC2a, and 35S:ERF189 constructs are presented relative to that observed in the empty vector control plants. Thus, if transcript accumulation for a given gene is equal in both control and transgenic lines, the "fold-change" value on the graph in Figure 3.13 will be one. Genes expressed more highly in a line containing a TF transgene will show fold-changes greater than one, and genes expressed at a lower level in transgenic TF line will show values less than one. The data in Figure 3.13 represent the averages of two independent plants per genotype, subjected to three technical replications for each qRT-PCR assay. The fold-change observed in transcript levels for each individual plant, replicate, and specific gene relative to the empty vector controls have been included in Appendix F. Given that

the four TF expressing transgenic lines included in this assay were from plants in Group 1 and the empty vector control plants were from Group 4, these results should be interpreted with this caveat in mind, and therefore must be regarded as preliminary.

Similar to the results reported by Wang et al. (2015), the transcripts corresponding to most of the alkaloid biosynthetic genes in plants possessing the 35S:MYC2a construct are reduced in comparison to the empty vector control, with transcript reduction being the most pronounced for the *MPO* and *A622* genes. Similarly, transcript accumulation for most of the alkaloid biosynthetic genes was also reduced in the plants possessing a pPMT1:ERF189 APFL construct. In contrast, most of the transcript levels were roughly equal to, or even somewhat elevated, in plants with a constitutively active 35S:ERF189 construct. Interestingly, the plants that displayed the severest reductions in alkaloid biosynthetic gene transcript accumulation were those possessing the pPMT1:MYC2a APFL construct. Relative to the empty vector controls, the levels of *ADC, ODC, MPO, A622* and *BBL* transcript accumulation was reduced to the greatest extent in the plants containing pPMT1:MYC2a (Fig. 3.13). These results both confirm and even extend the somewhat paradoxical observation of Wang et al. (2015) that the plants that accumulate the greatest amounts of nicotine in the leaf show the lowest levels of transcript accumulation for the genes encoding the enzymes responsible for nicotine biosynthesis. The proposed explanation that a negative feedback inhibition mechanism exists within the tobacco roots in response to excessive nicotine production remains a plausible one to explain this phenomenon.

3.4. Summary

In summary, we successfully designed four APFL constructs by placing the promoter elements from both the *PMT1a* and *A622S* genes upstream of the master regulatory TFs *MYC2a*

and *ERF189* giving rise to four combinations, namely pPMT1:MYC2a, pPMT1:ERF189, pA622S:MYC2a, and pA622-S:ERF189. Subsequently, the APFL constructs along with both 35S promoter-driven and empty vector controls were transformed into K326 and TN86 RNAi PMT plants in an attempt to maximize nicotine and anatabine production, respectively. The primary question addressed in this study was whether the APFL constructs could induce a higher level of alkaloid accumulation in comparison to merely constitutively overexpressing these transcription factors using the CaMV 35S promoter, which up to this time had been the most effective means for enhancing nicotine production in tobacco reported in the literature. Results from the T_0 generation greenhouse-grown plants presented very compelling evidence that our hypothesis was correct, as plants possessing the MYC2a APFL lines, using either pPMT1- or pA622S-driven constructs, averaged nearly twice the nicotine production in K326 plants compared to any other construct tested in this experiment. From our results it can also be concluded that the MYC2a master TF is far superior to ERF189 in mediating enhanced nicotine production via an APFL strategy. One possible explanation for this result may be related to observations from past studies that although MYC2a is capable of inducing expression of *NtERF* genes (as depicted in Fig. 4), the reciprocal is not true (i.e. ERF TFs cannot bind to and increase expression of *NtMYC* genes). Thus, MYC2a abundance may ultimately prove to be rate-limiting when implementing an APFL using ERF TFs, as both MYC2 and ERF TFs need to bind to the promoters of alkaloid biosynthetic genes for maximal induction of the pathway (Fig. 3.4). Consistent with previously published results using the strong constitutive CaMV 35S promoter to drive MYC2a expression, we observed an inverse relationship between the ability of an APFL construct to enhance nicotine accumulation in the leaf, and the steady-state transcript accumulation of nicotine biosynthetic genes in the root. A negative feedback inhibition model in

response to nicotine overproduction appears to provide the best explanation for this phenomenon. Finally, despite the very encouraging results observed in the K326 background, we did not observe any significant changes in anatabine levels when the same APFL or enhanced 35S-driven constructs were introduced in TN86 RNAi PMT plants. This suggests that there is a rate-limiting step in anatabine production in these plants that is not influenced by regulatory cascades that are controlled by either MYC2a or ERF189.

3.5. Future Studies

As stated previously, due to logistical issues that prevented the simultaneous generation and analysis of the extensive materials generated in this study at the same time, combined with inherent weaknesses associated with drawing conclusions based on results derived from T_0 generation transgenic plants, the observations reported in this chapter must be viewed as preliminary. T_1 generation plants derived from the most promising T_0 plants possessing APFL constructs containing pPMT1 as the promoter, in addition to all relevant enhanced 35S-based and WT controls lines, are scheduled to be tested in field trials this summer (2019). Seeds stocks were not available in time to include APFL lines contain the pA622S promoter element but will be included in subsequent years if warranted. It is the performance of these materials in the field that will ultimately determine the value of the APFL technology in maximizing alkaloid production in tobacco.

Table 3.1 List of all constructs, tobacco lines and transformation experiment codes used in the project. Also indicated are groupings of experiments according to when the transformation experiments were initiated.

Transform-ation #	Construct Name	Plant Background	Code Given Each Transformation Experiment	Experimental Group No.
01	pPMT1:GUS – 1	K326	K-1	Group 1
02	pPMT1:MYC2a – 2	K326	K-2	Group 1
03	pPMT1: ERF189 – 3	K326	K-3	Group 1
04	35S: MYC2a – 4	K326	K-4	Group 1
05	35S: ERF189 – 5	K326	K-5	Group 1
06	pPMT1:GUS – 1	TN86 RNAi PMT	T-1	Group 2
07	pPMT1:MYC2a – 2	TN86 RNAi PMT	T-2	Group 2
08	pPMT1: ERF189 – 3	TN86 RNAi PMT	T-3	Group 2
09	35S: MYC2a – 4	TN86 RNAi PMT	T-4	Group 2
10	35S: ERF189 – 5	TN86 RNAi PMT	T-5	Group 2
11	pA622S: GUS – 6	TN86 RNAi PMT	T-6	Group 3
12	pA622S: MYC2a – 7	TN86 RNAi PMT	T-7	Group 3
13	pA622S: ERF189 – 8	TN86 RNAi PMT	T-8	Group 3
14	pCAMBIA1301– 9	TN86 RNAi PMT	T-9	Group 3
15	pA622S: GUS – 6	K326	K-6	Group 4
16	pA622S: MYC2a – 7	K326	K-7	Group 4
17	pA622S: ERF189 – 8	K326	K-8	Group 4
18	pCAMBIA1301 – 9	K326	K-9	Group 4

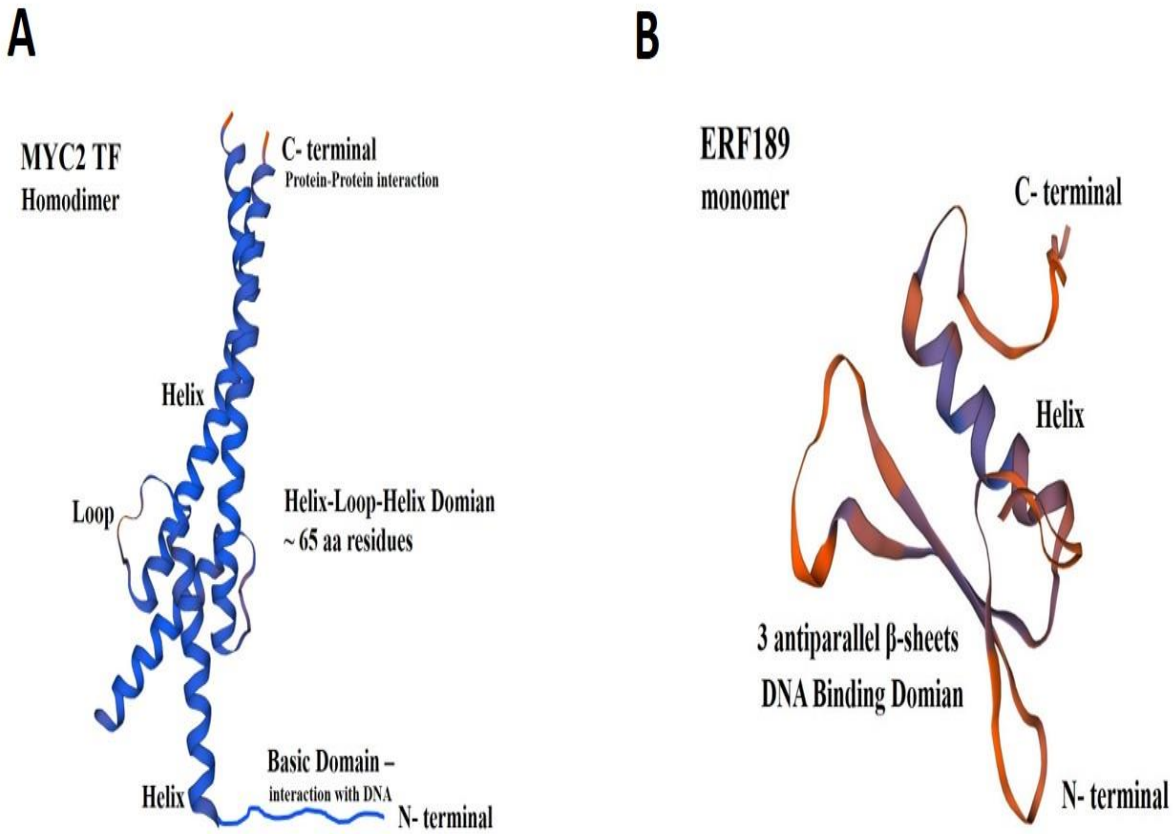


Figure 3.1. Three-dimensional structures of MYC2 (A) and ERF189 (B) as predicted using Swiss-Model software.

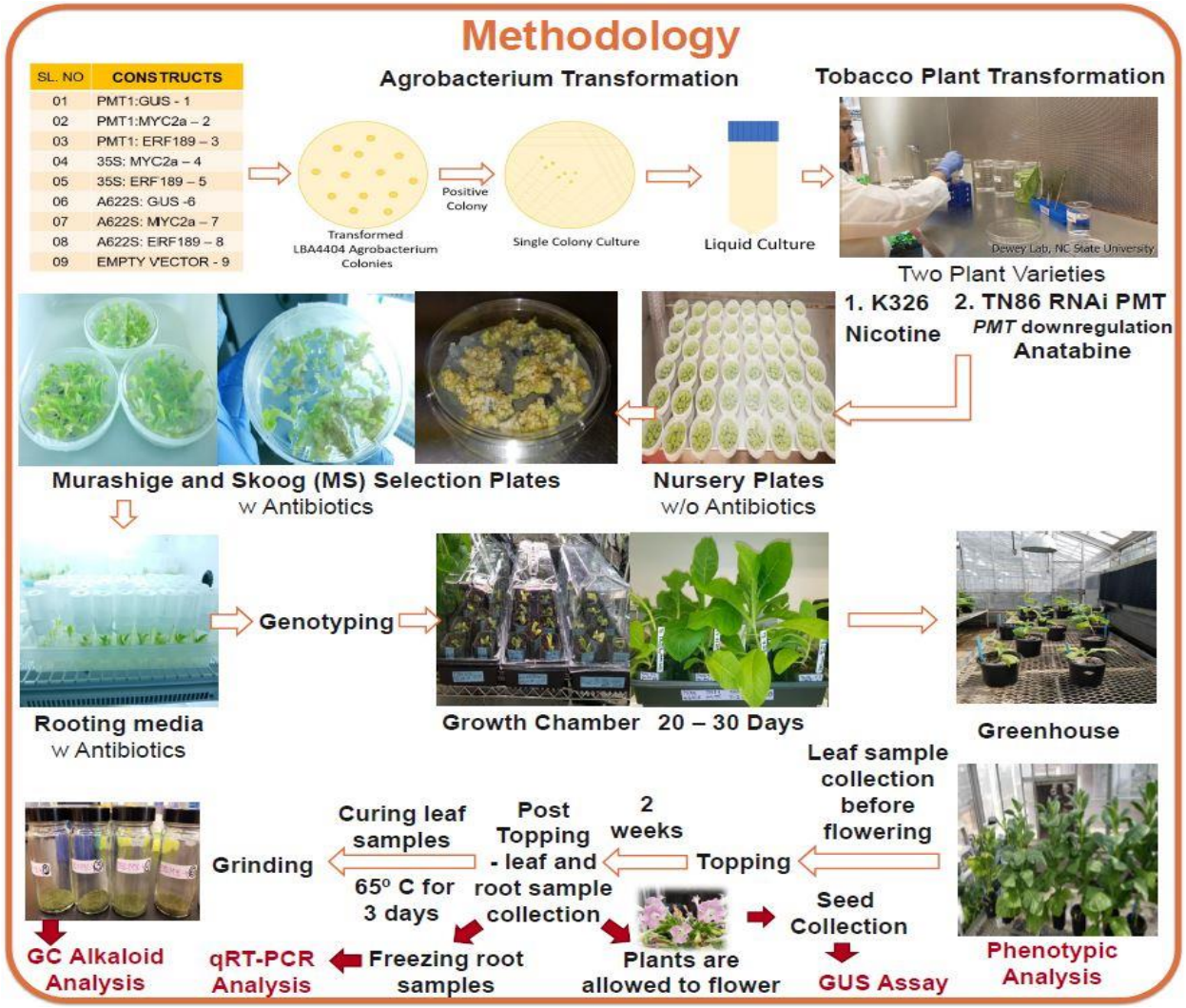


Figure 3.2. Flowchart of the overall methodology followed in the APFL project for maximizing alkaloid production.

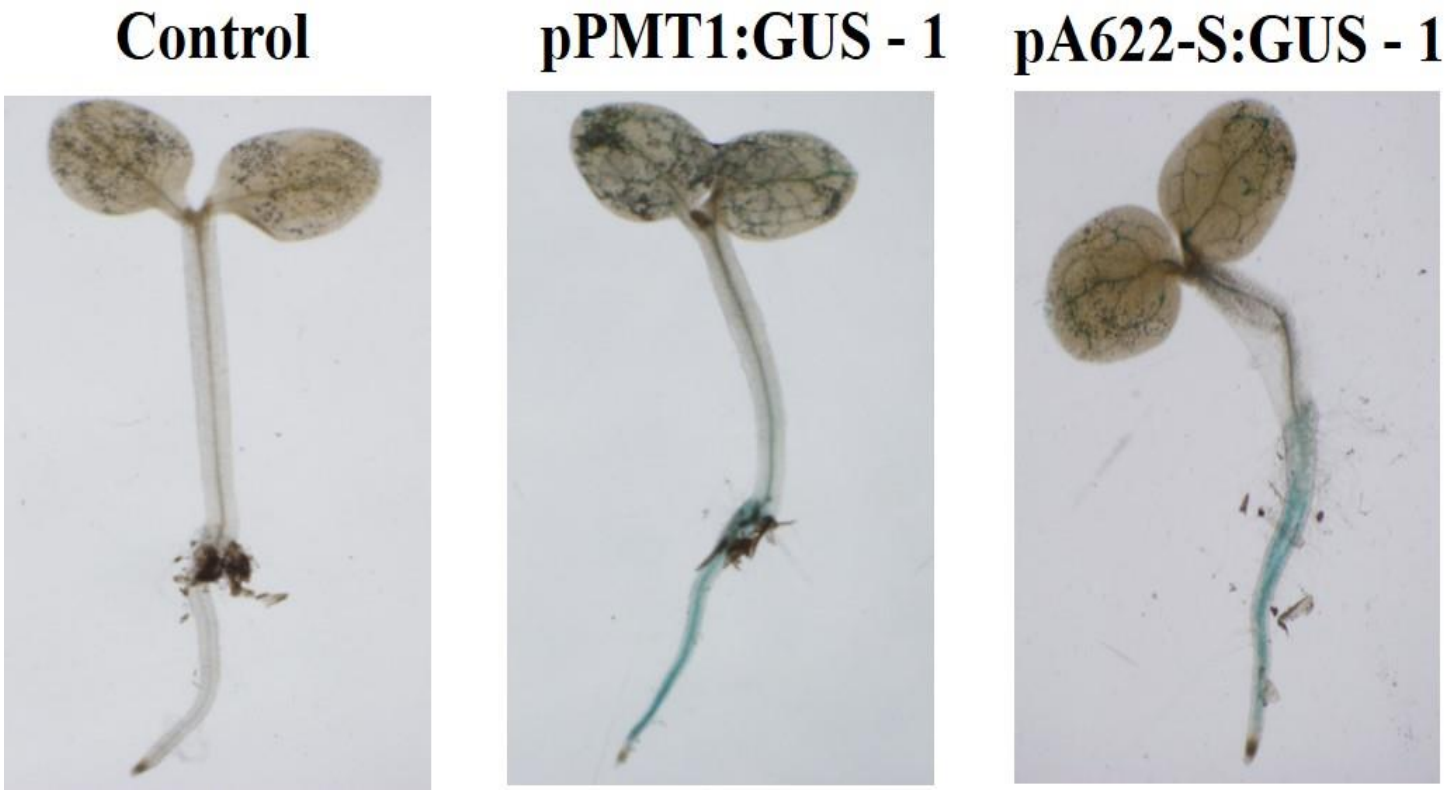


Figure 3.3. GUS stained K326 seedlings possessing either a vector control, or a GUS reporter gene under the transcriptional control of a *PMT1a* or *A622-S* promoter.

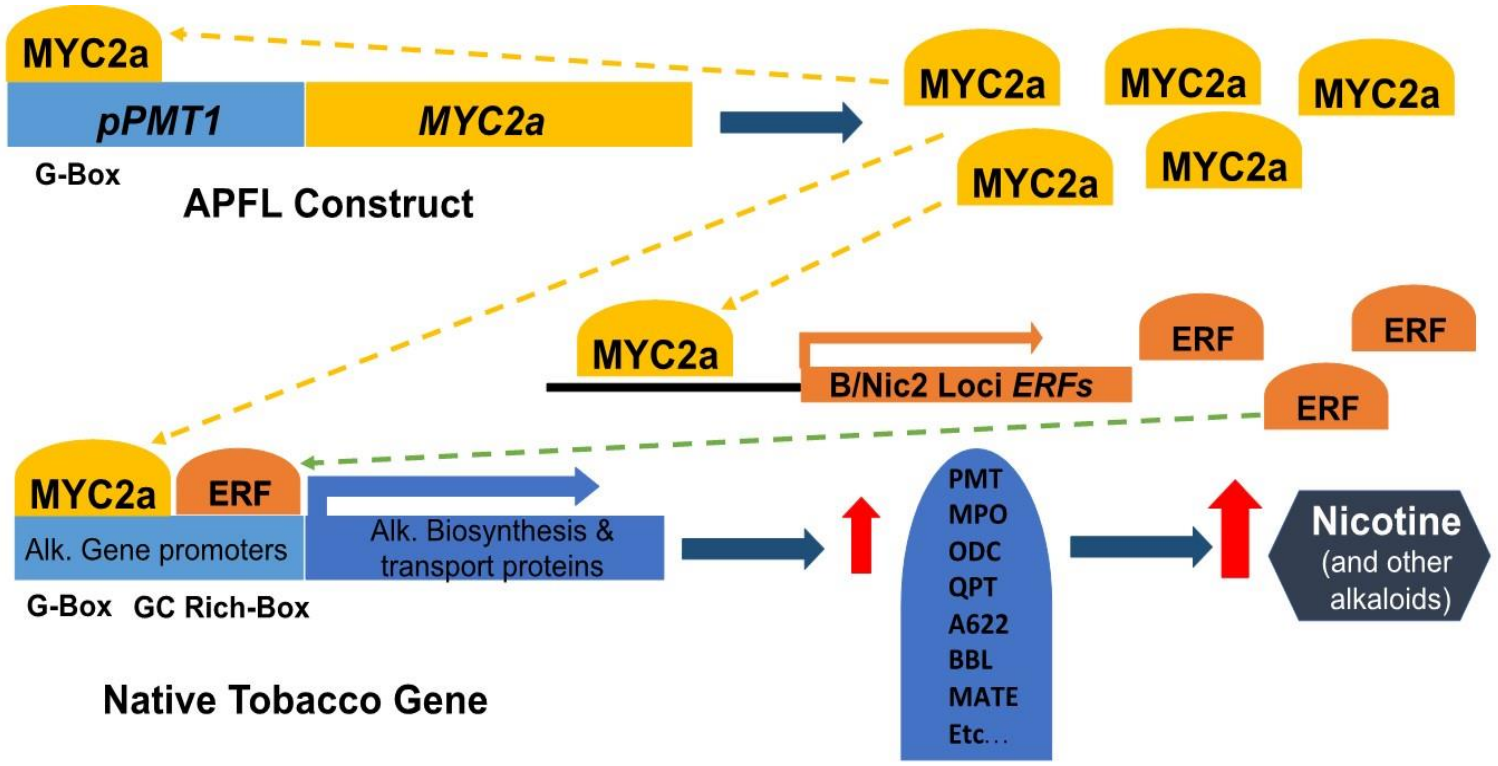


Figure 3.4. Creating an APFL to maximize alkaloid production in tobacco. An APFL construct is designed by placing a root specific promoter element (e.g. pPMT1) upstream of a master transcription factor (e.g. *MYC2a*). Conditions that initially stimulate *MYC2a* expression lead to the induction of the pPMT1 promoter, resulting in even more MYC2a production, thus creating an APFL. The excessive MYC2a production results in the increased production of MYC2a TFs, which in turn upregulates the native *ERF* TFs and the promoters of the all nicotine biosynthetic genes via binding to the G-Box. Creating an APFL using the ERF189 TF differs from the above scenario in that this TF is only known to upregulate genes involved in alkaloid biosynthesis and transport, and not *MYC2a.*

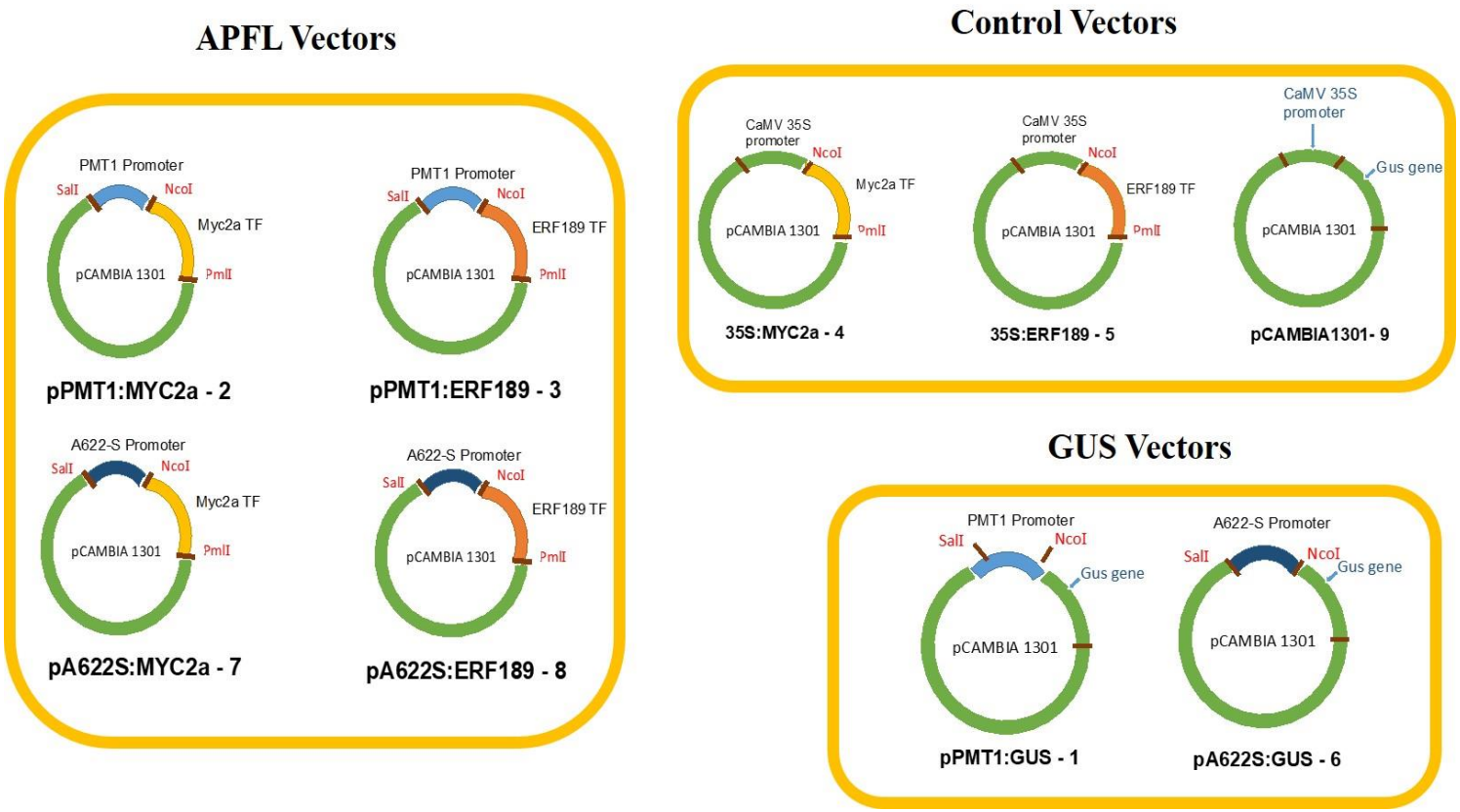


Figure 3.5. Final APFL, Control and GUS vectors utilized for tobacco transformation. Restriction sites utilized to facilitate cloning are indicated in red.

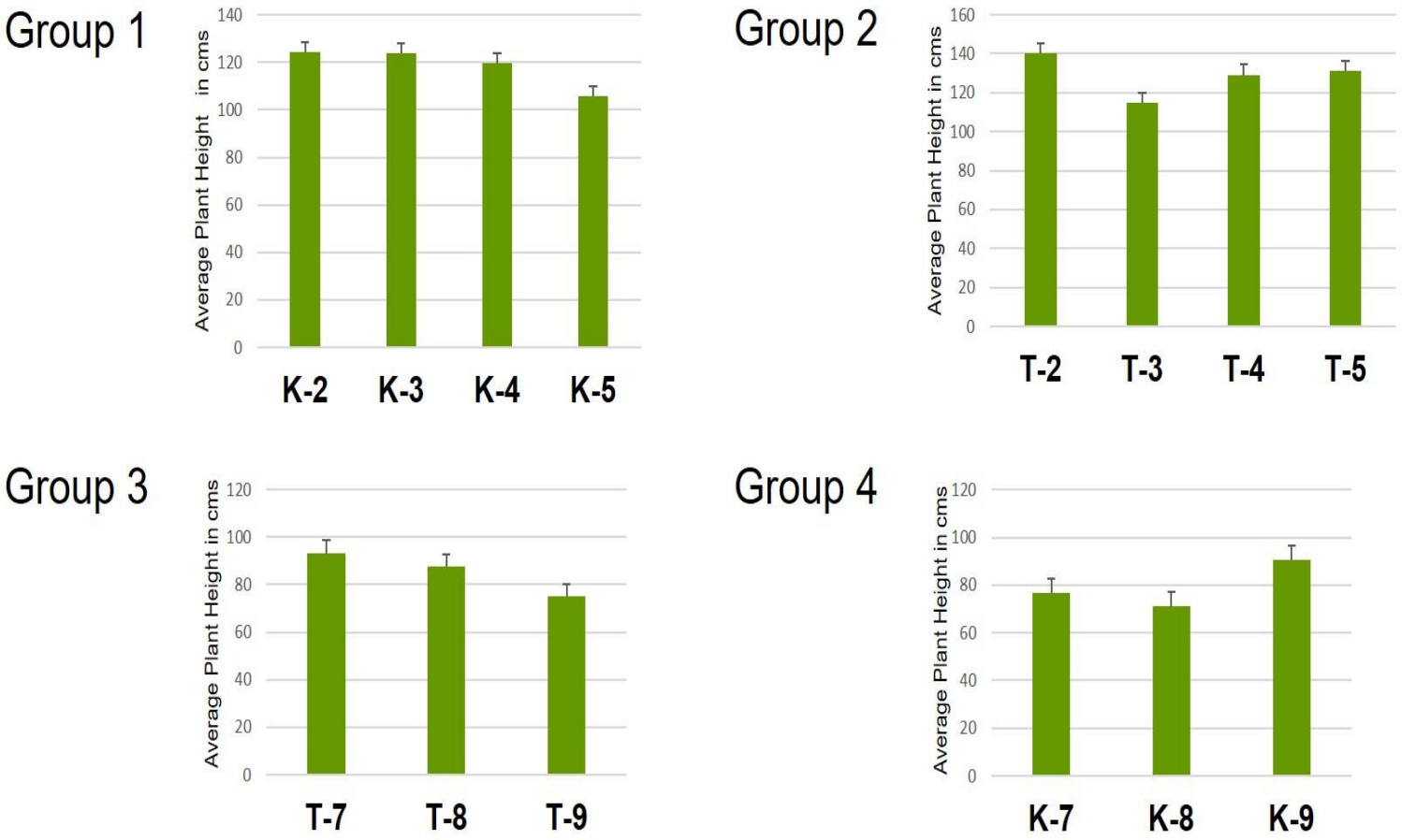


Figure 3.6. Average plant height at the time of topping. Individual transformation experiments are placed in groups according to when the experiment was initiated, as described in the text. See Table 1 for the definition of the experimental code names shown on the x-axis. Error bars show the standard error.

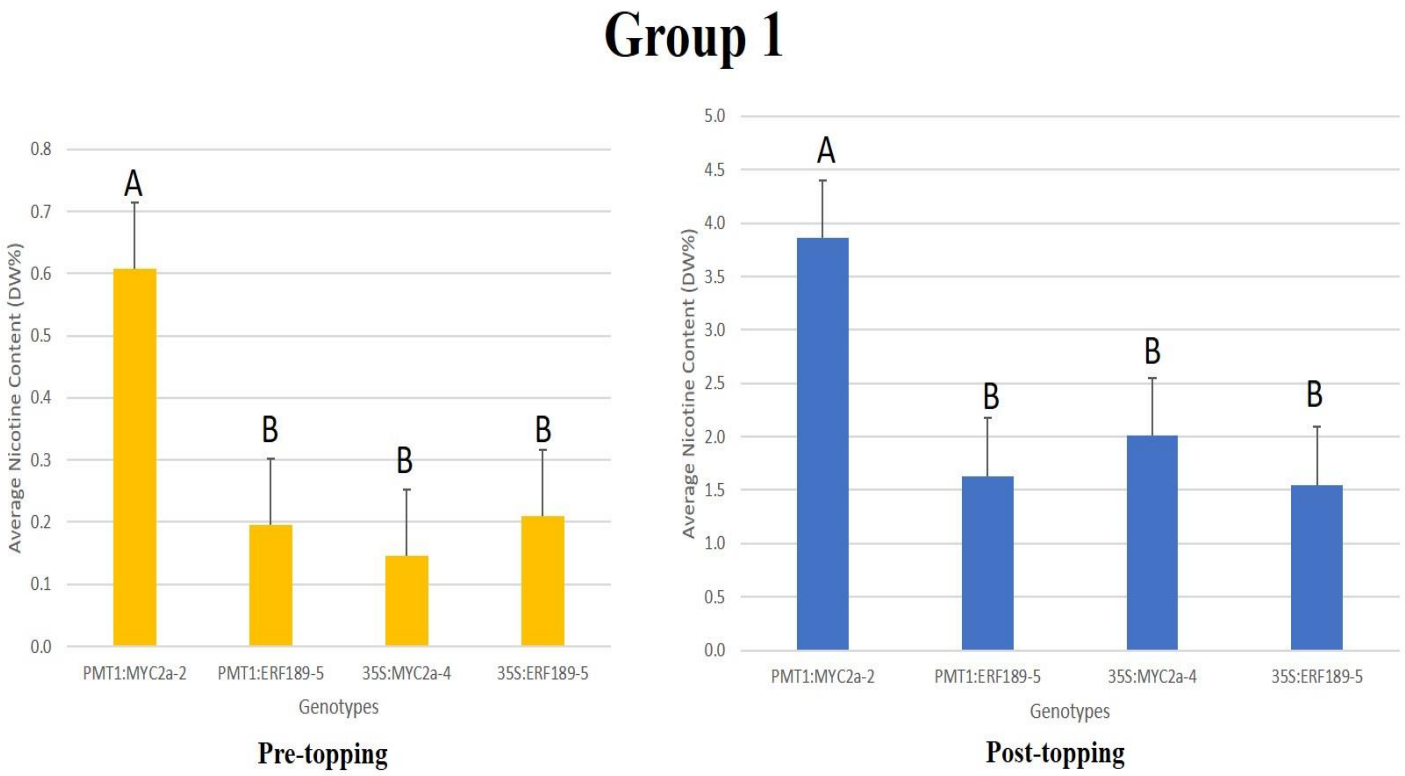
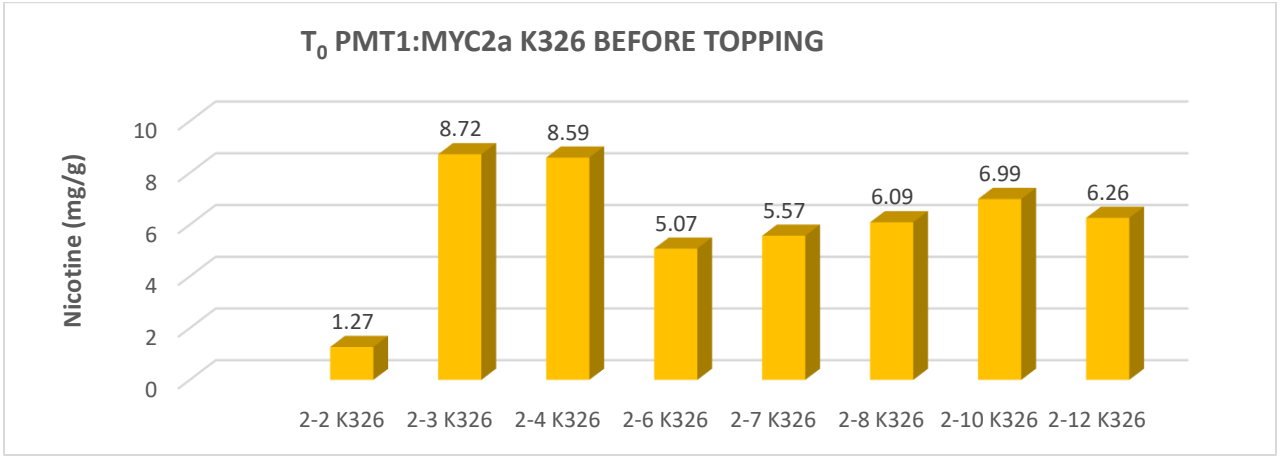
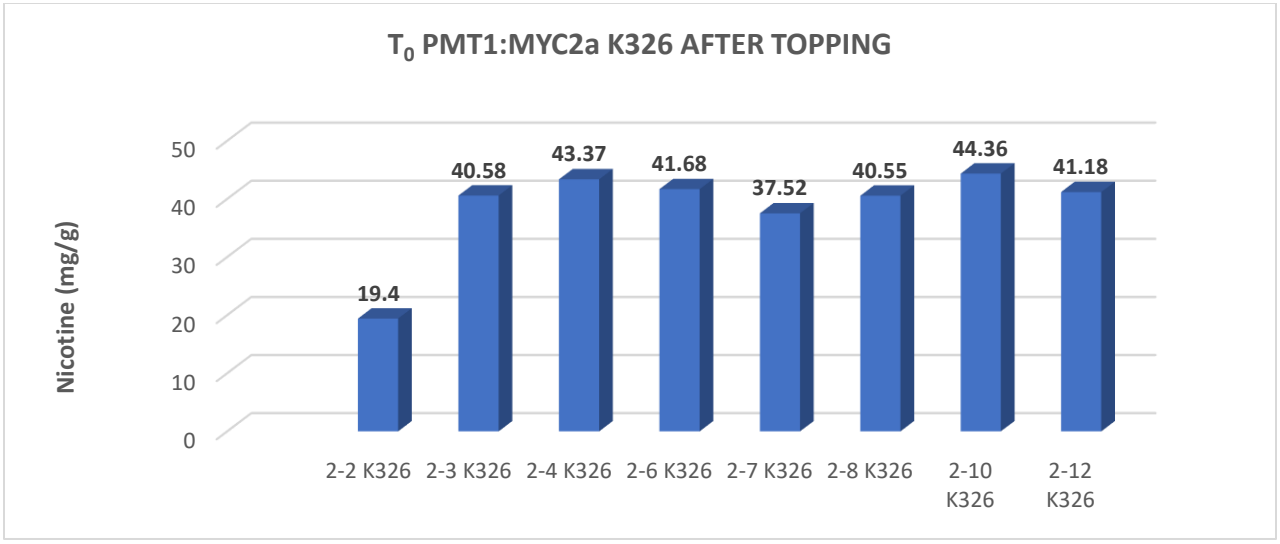
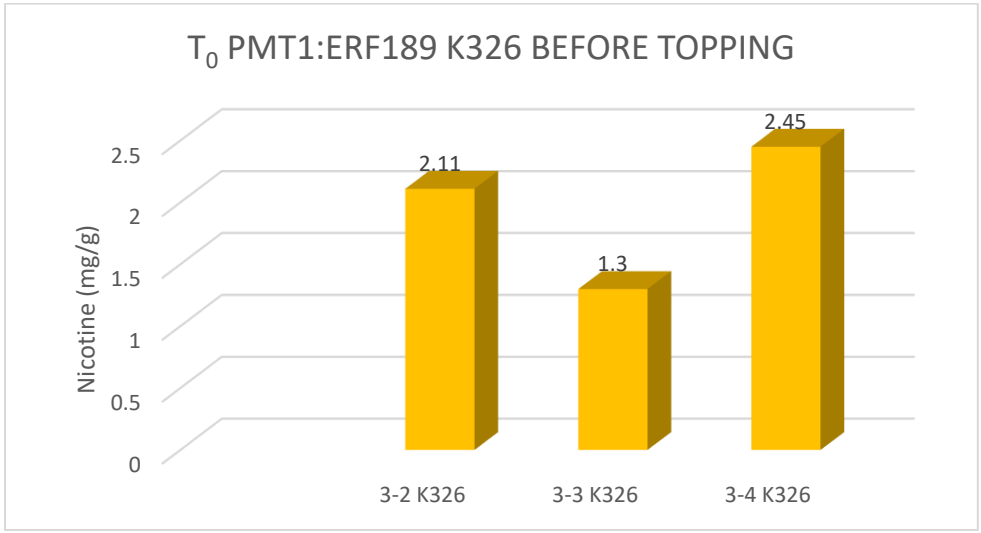


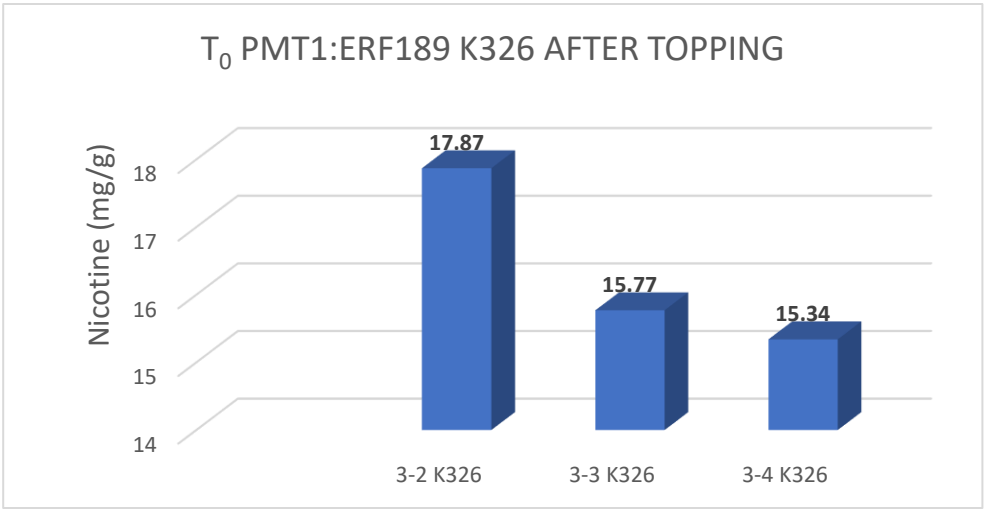
Figure 3.7. Nicotine content of Group 1 plants (% dry weight). Different letters indicate significant difference (LSD, α=0.05)

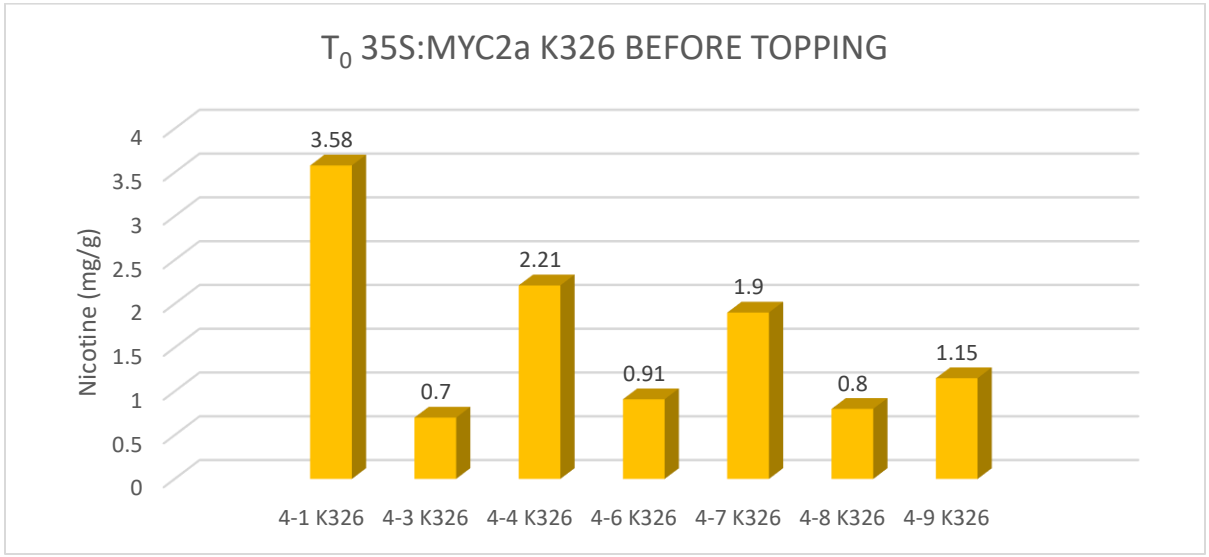
Group 1

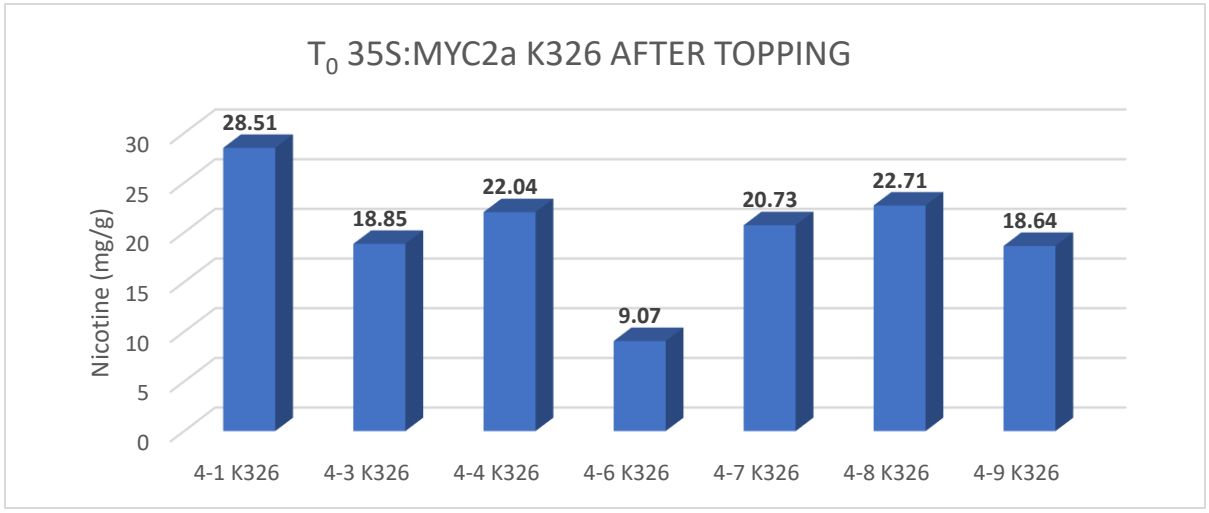

T0 PMT1:MYC2a K326 BEFORE TOPPING
Nicotine (mg/g)
0
2
4
6
8
10
1.27
8.72
8.59
5.07
5.57
6.09
6.99
6.26
2-2 K326
2-3 K326
2-4 K326
2-6 K326
2-7 K326
2-8 K326
2-10 K326
2-12 K326


T0 PMT1:MYC2a K326 AFTER TOPPING
Nicotine (mg/g)
0
10
20
30
40
50
19.4
40.58
43.37
41.68
37.52
40.55
44.36
41.18
2-2 K326
2-3 K326
2-4 K326
2-6 K326
2-7 K326
2-8 K326
2-10 K326
2-12 K326


T0 PMT1:ERF189 K326 BEFORE TOPPING
Nicotine (mg/g)
2.5
2
1.5
1
0.5
0
2.11
1.3
2.45
3-2 K326
3-3 K326
3-4 K326


T0 PMT1:ERF189 K326 AFTER TOPPING
Nicotine (mg/g)
18
17
16
15
14
17.87
15.77
15.34
3-2 K326
3-3 K326
3-4 K326


T0 35S:MYC2a K326 BEFORE TOPPING
Nicotine (mg/g)
4
3.5
3
2.5
2
1.5
1
0.5
0
3.58
0.7
2.21
0.91
1.9
0.8
1.15
4-1 K326
4-3 K326
4-4 K326
4-6 K326
4-7 K326
4-8 K326
4-9 K326


T0 35S:MYC2a K326 AFTER TOPPING
Nicotine (mg/g)
30
25
20
15
10
5
0
28.51
18.85
22.04
9.07
20.73
22.71
18.64
4-1 K326
4-3 K326
4-4 K326
4-6 K326
4-7 K326
4-8 K326
4-9 K326

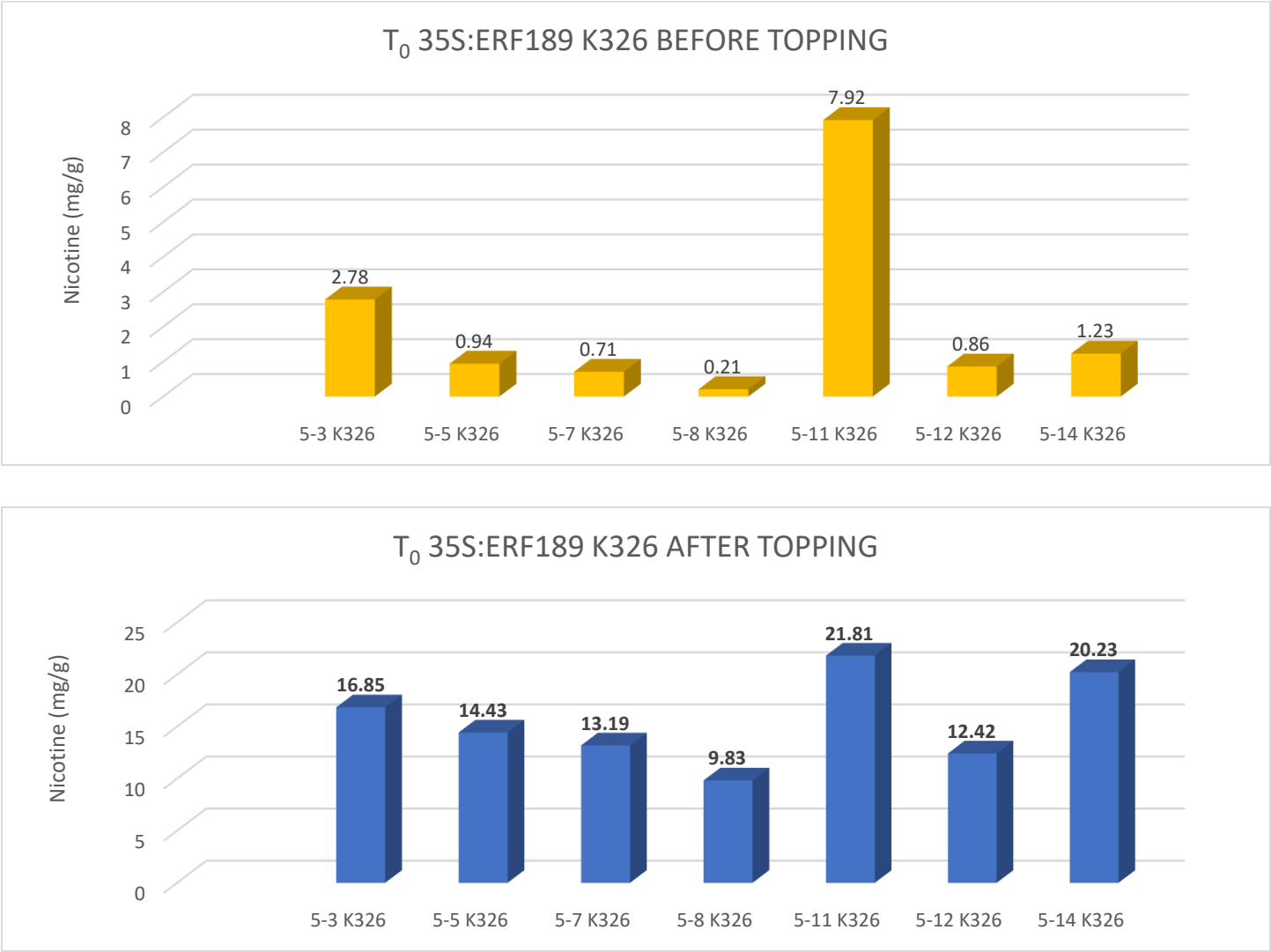


Figure 3.8. Nicotine content of Group 1 plants (dry weight)

Group 4

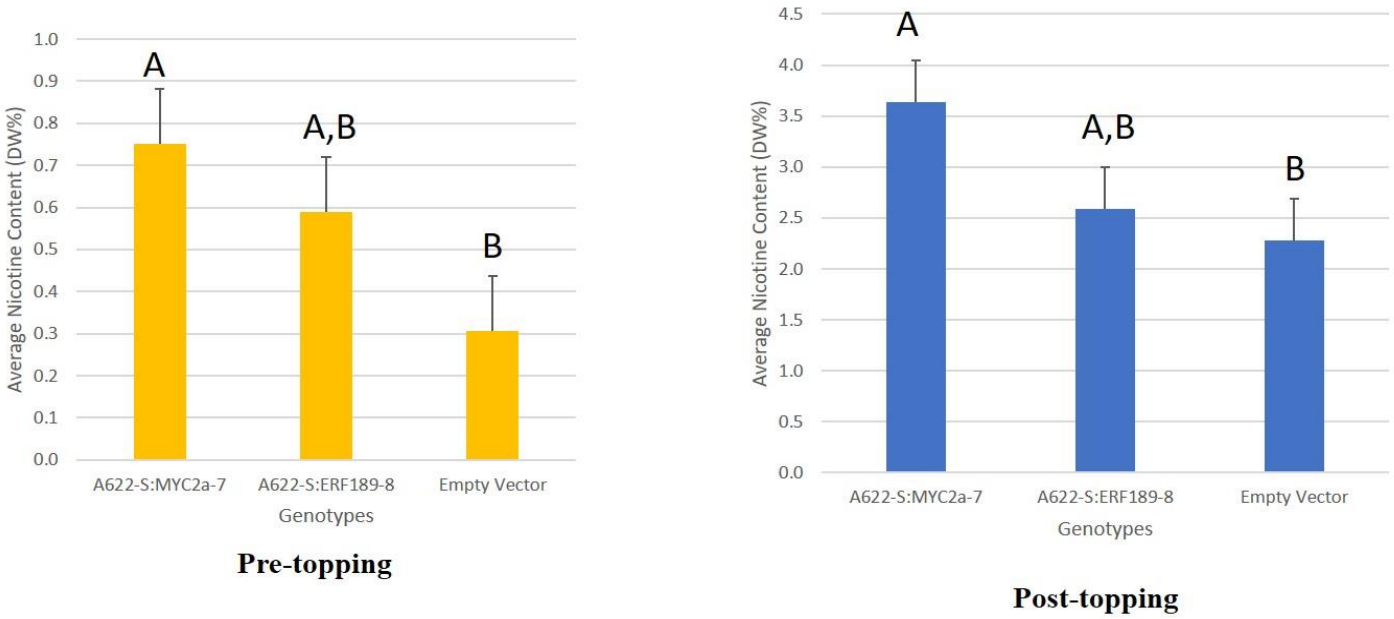
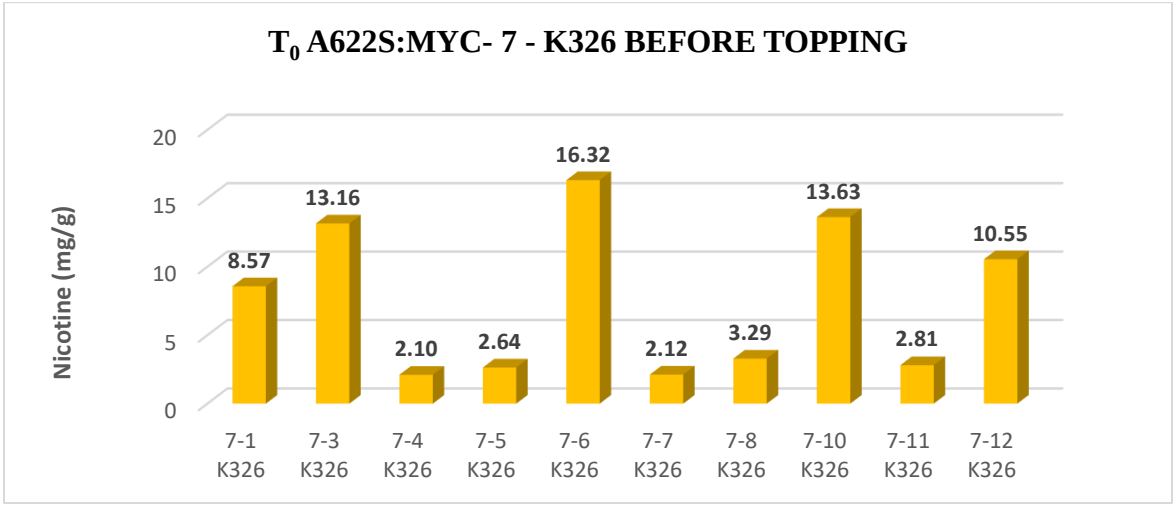
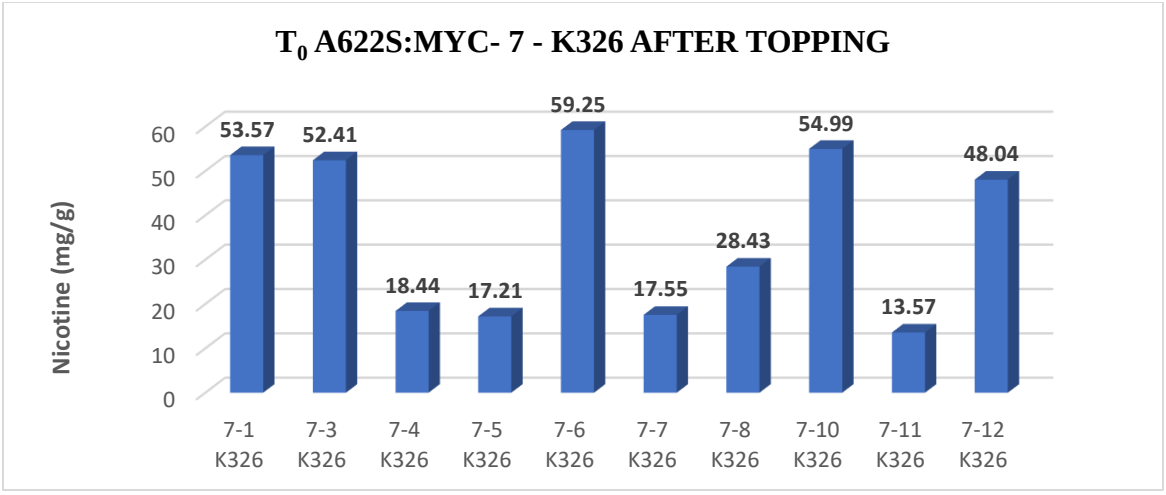
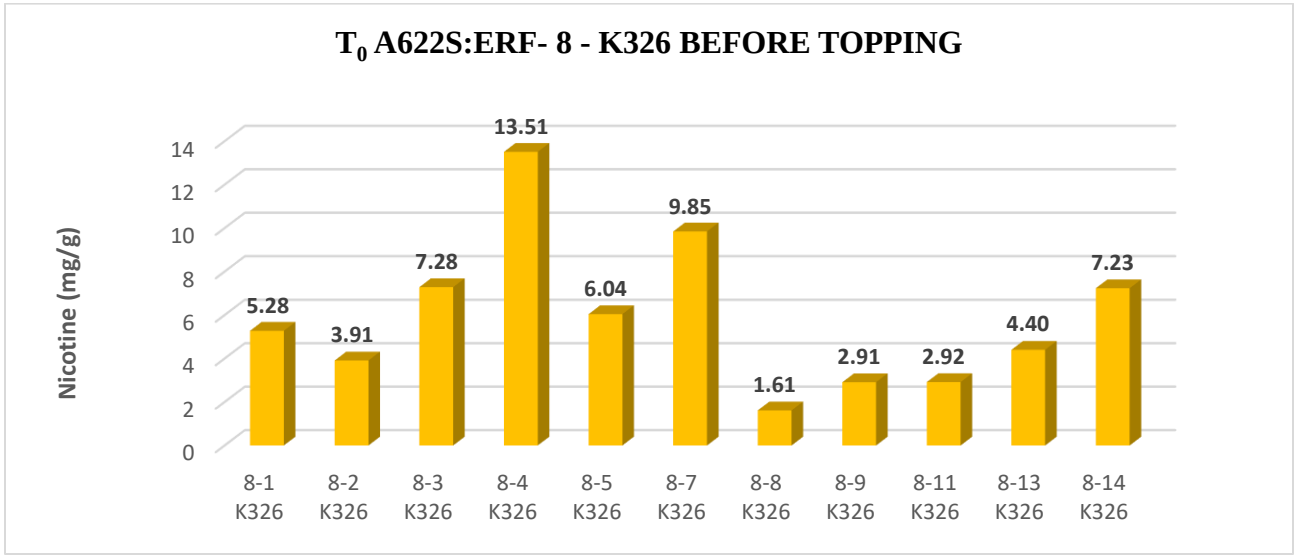


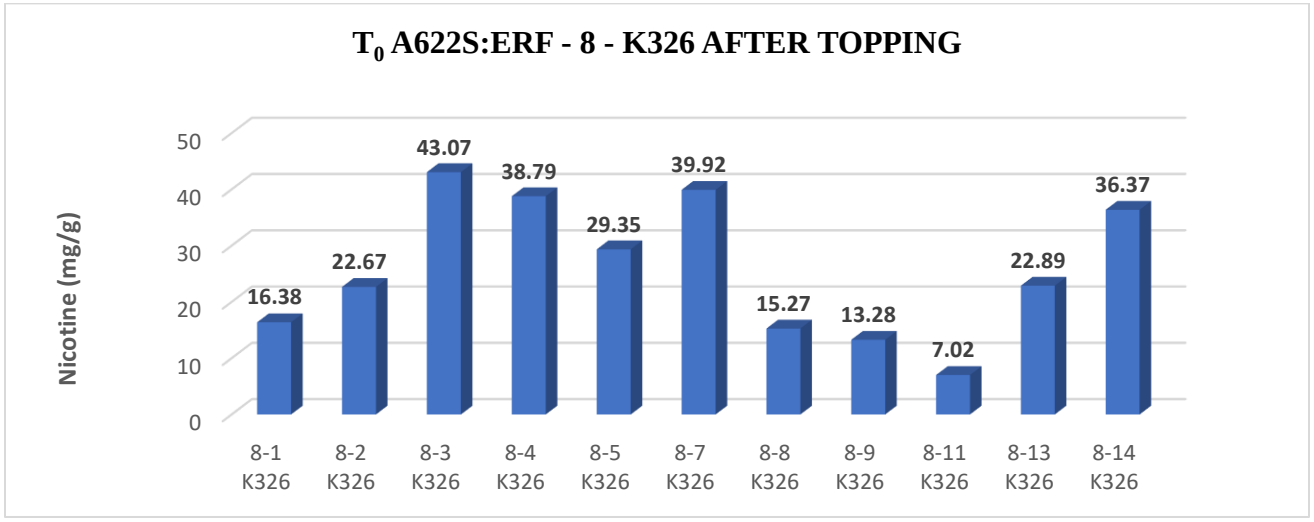
Figure 3.9. Nicotine content of Group 4 plants (% dry weight). Different letters indicate significant difference (LSD, α=0.05).

Group 4


T0 A622S:MYC- 7 - K326 BEFORE TOPPING
Nicotine (mg/g)
0
5
10
15
20
8.57
13.16
2.10
2.64
16.32
2.12
3.29
13.63
2.81
10.55
7-1 K326
7-3 K326
7-4 K326
7-5 K326
7-6 K326
7-7 K326
7-8 K326
7-10 K326
7-11 K326
7-12 K326


T0 A622S:MYC- 7 - K326 AFTER TOPPING
Nicotine (mg/g)
0
10
20
30
40
50
60
53.57
52.41
18.44
17.21
59.25
17.55
28.43
54.99
13.57
48.04
7-1 K326
7-3 K326
7-4 K326
7-5 K326
7-6 K326
7-7 K326
7-8 K326
7-10 K326
7-11 K326
7-12 K326


T0 A622S:ERF- 8 - K326 BEFORE TOPPING
Nicotine (mg/g)
0
2
4
6
8
10
12
14
5.28
3.91
7.28
13.51
6.04
9.85
1.61
2.91
2.92
4.40
7.23
8-1 K326
8-2 K326
8-3 K326
8-4 K326
8-5 K326
8-7 K326
8-8 K326
8-9 K326
8-11 K326
8-13 K326
8-14 K326


T0 A622S:ERF - 8 - K326 AFTER TOPPING
Nicotine (mg/g)
0
10
20
30
40
50
16.38
22.67
43.07
38.79
29.35
39.92
15.27
13.28
7.02
22.89
36.37
8-1 K326
8-2 K326
8-3 K326
8-4 K326
8-5 K326
8-7 K326
8-8 K326
8-9 K326
8-11 K326
8-13 K326
8-14 K326

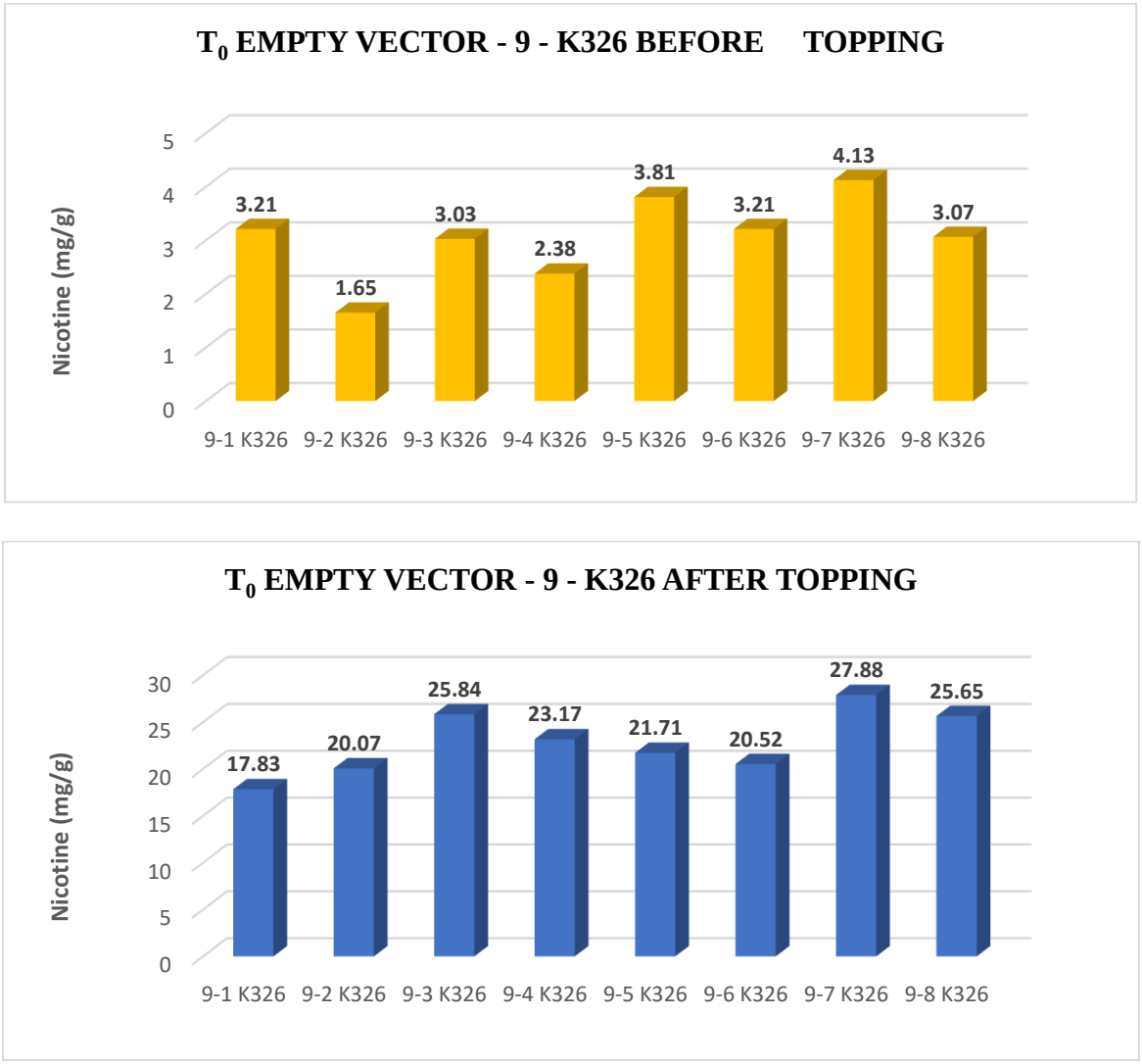


Figure 3.10. Nicotine content of Group 4 plants (dry weight)

Alpha	0.05
Error Degrees of Freedom	28
Error Mean Square	31.24938
Critical Value of t	2.04841
Least Significant Difference	7.2421

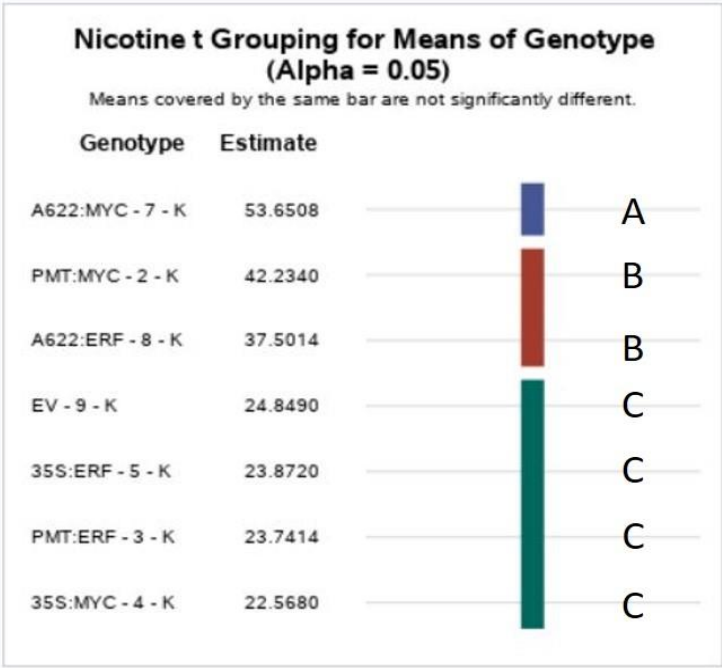


Figure 3.11. SAS output comparing the top five plants (post-topping) of each genotype from both Group 1 and Group 4. Data shown under Estimate is presented as mg nicotine/g dry weight. Different letters indicate a significant statistical difference (LSD, α=0.05)

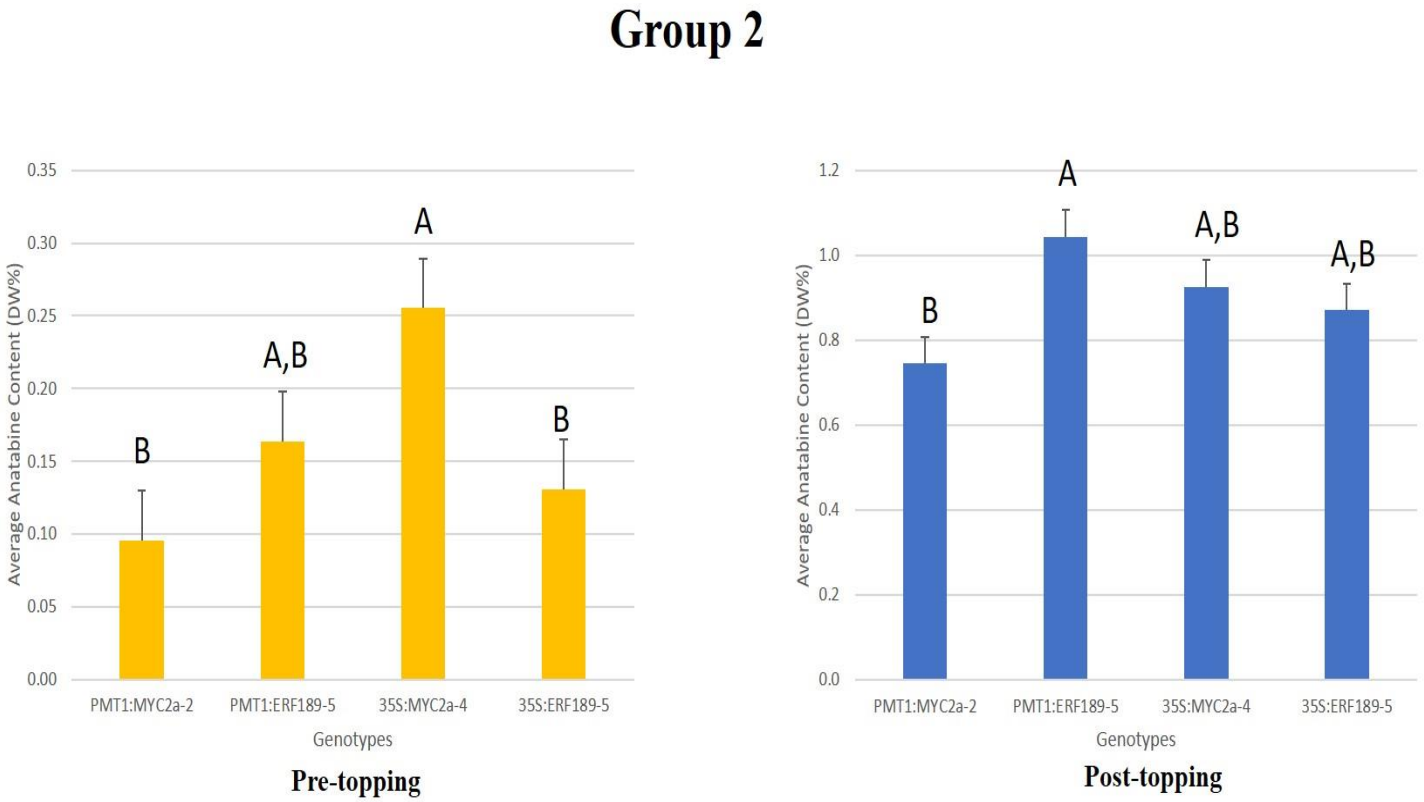


Figure 3.12. Anatabine content of Group 2 plants (% dry weight). Different letters indicate significant difference (LSD, α=0.05)

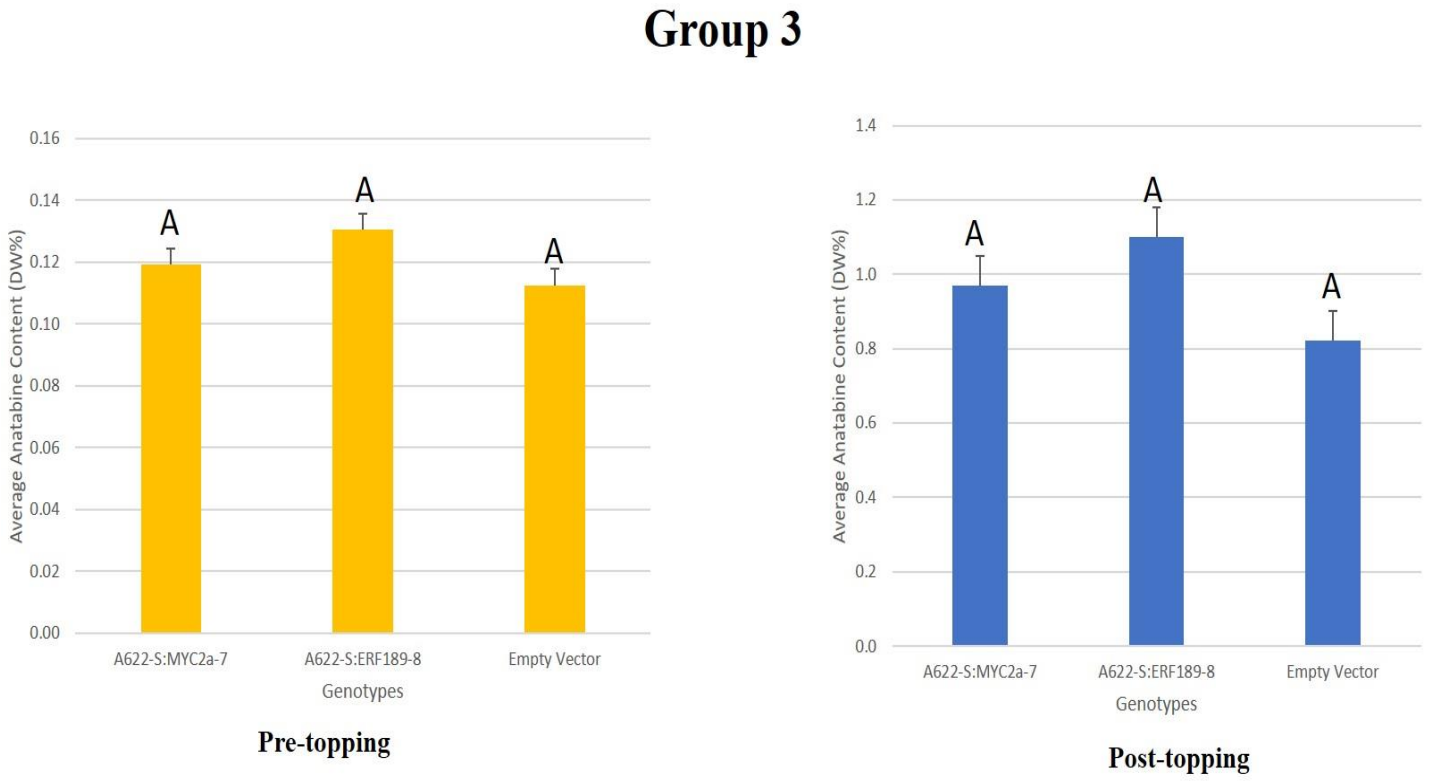


Figure 3.13. Anatabine content of Group 3 plants (% dry weight). The same letter (A) above each genotype indicates no significant difference observed (LSD, α=0.05)

The GLM Procedure

t Tests (LSD) for Anatabine

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	28
Error Mean Square	6.945732
Critical Value of t	2.04841
Least Significant Difference	3.4143

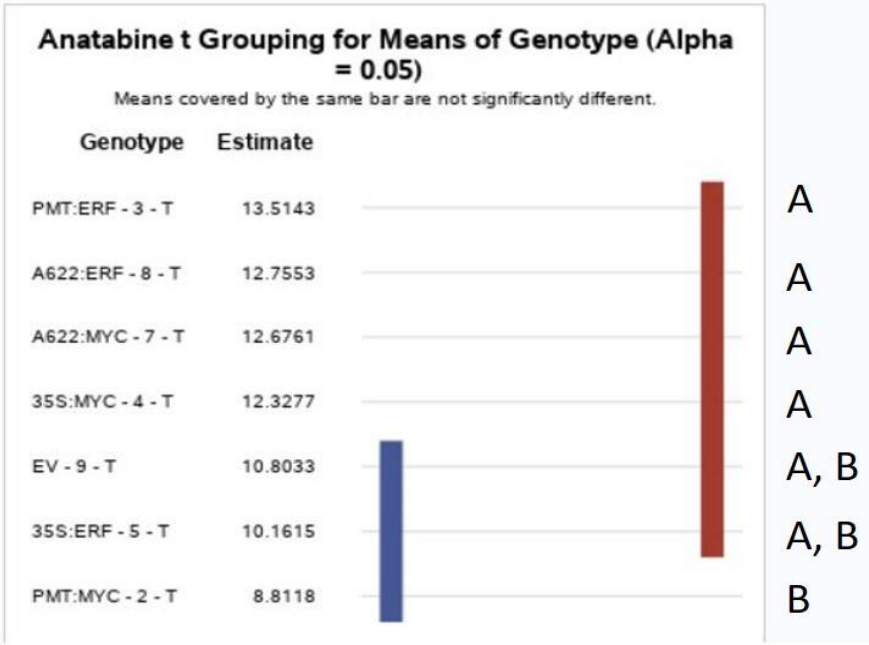


Figure 3.14. SAS output comparing the top five plants (post-topping) of each genotype from both Group 2 and Group 3. Data shown under Estimate is presented as mg anatabine/g dry weight. Different letters indicate significant difference (LSD, α=0.05)

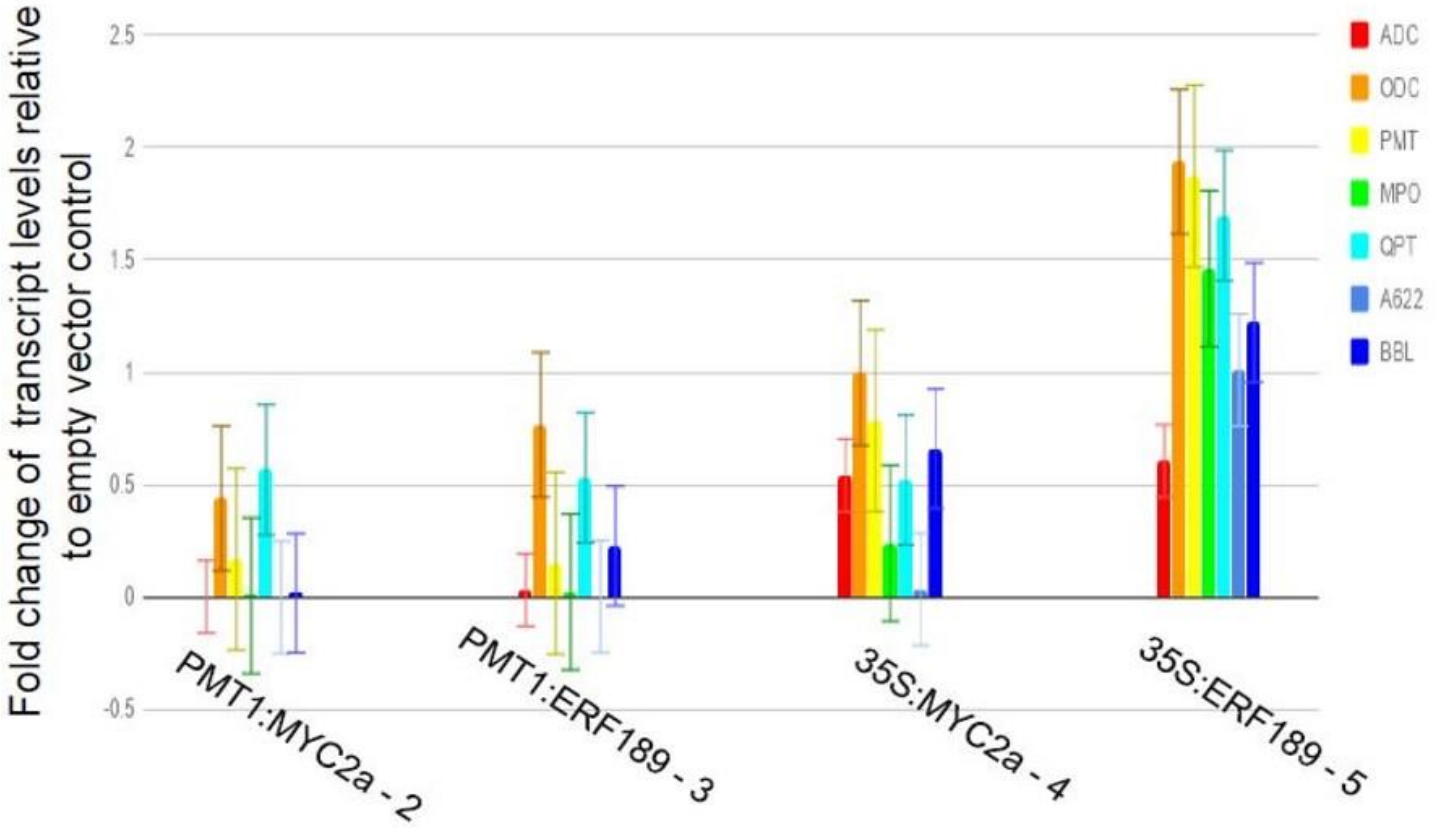


Figure 3.15. qRT-PCR Analysis of nicotine biosynthetis genes. Fold-change is indicated in the transcript levels of the genes *ADC, ODC, PMT, MPO, QPT, A622*, and *BBL* in the genotypes pPMT1:MYC2a-2, pPMT1:ERF189-3, 35S:MYC2a-4, and 35S:ERF189-5 relative to the empty vector control. Error bars show the standard error.

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APPENDICES

Appendix A

APFL promoters and transcription factors – gene sequence and primer sequence data

Appendix B

Creating an APFL construct for maximizing alkaloid production

Appendix C

Junction primers used for genotyping – sequence data

Appendix D

qRT-PCR primers – sequence, target isoforms, and product length data

Appendix E

Alkaloid analysis additional graphs

Appendix F

qRT-PCR additional graphs

APPENDIX A

1. *PMT1*

Size – 720 bp

GenBank Accession# AF126810

The sequences in red differ from WT and are added to facilitate cloning by placing a SalI site at the 5' end and NcoI site at the start ATG which is indicated in large bold type:

GTCGACAATTCAATGGAGAAGGAAAATATTTCCAGTGTAAACACAAGTGAATGAAGAGAAGCCAAAATAATCTCTAT
CATTCAAGCCTTAGGTGGAGATTAAAAAAATTATTTACTTTCTTATCAAAGTAATAGGTGATCAACAGCTTTCGTAA
AACGTCATTAGGAGAATATTATAATCTCTTTTATGCTGAAGAACCCACATAAGGAAGATCATAAAATACATGACTTT
CAGATGACTTCTTGGAGCTTTATTTTTAAAGAGTGGCTAGCTGGTCAGCAAAGAGGTGCTCGTCAGATATCATAAAA
TTTTACTATTATTTGTTTTAAGAGGGAGATGGGGCACACATGCTTGTGACAAAAGTAAGAGGAAGAAAGGAGACAGA
AGAGGAAATAGATTTGGGGGGGGGGGGGGGGTTTCACAATCAAAGAAAATTTTTAAAATGGAGAGAGAAATGAGCA
CACACATATACTAACAAAATTTTACTAATAATTGCACCGAGACAAACTTATATTTTAGTTCCAAAATGTCAGTCTAA
CCCTGCACGTTGTAATGAATTTTTAACTATTATATTATATCGAGTTGCGCCCTCCACTCCTCGGTGTCCAAATTGTA
TTTAAATGCATAGATGTTTATTGGGAGTGTACAGCAAGCTTTCGGAAAATACAAACCATAATACTTTCTCTTCTTCA
ATTTGTTTAGTTTAATTTTGA**CC****ATG**G

2. *A622-S*

Size – 1,492 bp

Reference Sequence:

A622-S Genomic DNA (from TN90)

- Start codons is indicated in bold, capitalized type
- The region of the 5' flanking region shown by Hashimoto lab to confer strong, root-specific expression is highlighted in yellow
- Sequences around the "n" residue (highlighted in green) are variable among the different tobacco types; otherwise, the rest of the sequence identity is very high

GATATCTGGTAGATATGACTCACAAGATTTTTGGAGTCCTCAGGACTTCAGGATTTGCAAGCAAACGAGAGTTTAAC
TATGTGTAGGACTCCCAAAGATTTCGAAGCATTGAGAAGTTAAATACTTTGCCTATGGACTGTCGAAGTTTATCCTT
TTGCACATCAACTAAAGAACATAGAACAACATTTTCAGTTCAACACTCAATTCTCTCATCAAGTGTGTTCTGAAAGT
CATTTTCTTGAGTCTGTTTAGTTTGTGTGTATTAGGAGATTGAGTTGTAATCAAAATATTAGTTGGAGTATTGTCTG
AGGGTGCAAGTTAGAGTAACTTGTAAATTCATAAAACAAGTAGAAGTATGAGAAGTGCGAGGGCATTAGGTTACATG
TGTTGTAATCAGTACATTTGGCTATGCTGTTCTAGTGAAATTAGAGTTAAAATCCTATGTGGTAGGTCGTGGTTTAT
GCACCTTTGAGCCAGGTGATTTTCTACGTAAAAATTAATGTTTTTACTTTACTGTTTATTTTATTTATGCGTAAGGA
GTAAGACAAAGAACCAAATTTTTCAATAATTGAGAAAGACACTCAAATTAAGAACTTGCATATTGTAAGTAGGGATG
TGCATTTGGATCGGATATTCGAAATTCGAACCGATCCGCTCAATTTCAAATTTCGAAATTTAAATTTTTGATATTTT
GATCGGATTTTAGATTATGTTTATTAAAATTTGATATATCCGGATCAGATACGGATTGGTATAATTTTAACCCGACC
CGGTCCGAAATGCGAAATTATTAGGGCATGTATTAACACTAAACTTTAATTTTGGATAGCTAGTACTTCCTTTACAT
CTTCCTCCAAGTTATTACCTTTACAATTGCTGGATTAAACTACATTGACACCATAGTACCCTCATAATTGCAGAAAC
GTGAATTTTTCTTGTTGTATTGCCTCTTCTAGAAAGAAAATATAAGTATGAGTTTTTCAACTTAGAGGCATAACAAC
TCAAACCTCCGGAGAAATATTTTCTTAGAGATGCAATGCTGATAAAAGGGATGAAAAAGCAAAGGGATATTTGTATT
AGCATAGAACACAGAAGAAGCAGTAAGGCAGATGCATTAGCCGATTAGATTATCAATAGCCTTAATAATATGTATAA
TTCGATCCGAAAATTCAAAATATGTATCCAATCCAAACTTTAAAATTCGATCAGATCCGATATTATTTCGGATCGGA
TTCGAATTGCATTTTGTAGAATCCGAAATTCAAAATTTTAATCTGAAATGTGCTAAATCCGATCCGATTTGATCCAA

GCACAACCCTAATTGTAAGTATCTGAAACTGATTATTTCATTGATTTGCTTCAAAAAACAAAGTATTAGCAGAAAAT
GATATTTTTATATAGAAGTAATGTAAAGCTTTTAAAAGCCACCGTTCCTTTTTTTTTTCTTTTTTCTTTTTAAATGC
TTTTATTAATTTCGCTGCCACTCAATTTCAGTTCTAGTCCCAGATTTCTCTCACATGGCCCTATTTTCATAATGCAT
GAAATAAGTGTTATTATATGTAACACAATCACTATGTTATTATTAAGTTTAGGTAGGTACCATGCCTCCTCAGTAAT
GTTCCTTGCAATTGATTCCTCCGGCTAAATCTAGATCTCTCTTATGTTTCATGCACGTGGAAAAAAAAGAAGTTTGG
TTTTTTTTGGTTCTTATTGCTATTTAAATGTGGTAGGTGAGAAGACTGGATTAAATAAATAACAAACCGTATTCAAC
TTTTCCGTCCTGTATTATATTTATTATTACAGTAAATAGCAACAAAATATTAAAAGAAAATGAGAATGAGGATCTTT
TCAAAATTAAATTTCTTTAAAATAAACAGAATGTTGTTCCCTACAGGCTACAGCTACACGTAAGAAAAGTACAACAA
GTCTTTTCTTTTGTCCTTATCGATATCTGAACGCAATAGGTGGGAATATTGGATCGAAAAATTAAACCCACTATTGC
CTGAAAGATAAAAAATATAAAAATTCTCTAATAATTAGTTCTTTAATACAACACATGACAAAATGTAATTCTTTATA
TTATGGCAAGATTCGATTCCTTATACTAGCACCACTTTGGCAGCAAGAAATCAATATTTAATATTAAAACAAAATAT
GACACAGCATTATTTTTCTATATTATGGCAATATATAATTCTTAAAAATTAGTTCTTTAATACAACAATATATGACA
AAATGTAATTCTTTATATAATGACAAGATTCGATTTTTAGCACCACTTTGGCAGCAAGAGATACATATTTAATATTA
AAAAAATATGAAAAACAATTATATTTTATATTATGGCAATATATAATTCTTTATATTAGCACCACTTTCCAGCAAGA
TAAAATATTTAAAATCAATTTCTACTTTGAACCACTATCCCCTGCTCTAAAATAATTTGTTTATTTCAGGGGATTGG
AAAATGACTTTAAGTTATATATTGTTAGTGTAAAACGAATTTGATCACCTAAAGAATATTTACAAGTAAGCCTCTAT
AAAAAGTAATTAAGGTTTGTAATCTTAAAAGAGAGCATATTACATGTTGTAATTGGTAAATGTGACCTGATAGTGTA
CNTATATACCTTTTCTTTCATTAAATAATGTAATTTCCCGAGGAAGGATTCAACCCTCCCTTATATGCGGCTCGCTA
TATAATATTTTTTTTAATTATTTTCTTACATGCTCATCTAGTAGGTAATGACACAGCTAGACGCATGGATCAACATT
CTAAGACAGATCATCACAAATTTATTAATGGTATGATATCGTAATTCCATGTGTTAGGTTAATTGTGCAAAATTCCC
TAGCTTGAATTTGCCTATAAGTACCAGCGTTAATATCAGATTCTCCACAGCATTTCATACAAAAATCCGATTTAATT
CCTAGTTTCTAGCCCCTCCACCTTAACCCGAAGCTACTTTTTTTCTTCCCCTAGGAGTAAA**ATG**GTTGTATCAGAGA
AAAGCA

Primers for amplification and sequencing:

Primer name	Purpose	Sequence	Length (bp)	Tm (°C)	GC%
A622S_58_Rev	Amplification	5'TTCCTATGTAGCCTGTGCCTCC 3'	22	69	55
A622Sprom_NcoI_R	Amplification	5'ACAACCATGGTACTCCTAGGGG 3'	22	68	55
A622Sprom_SalI_F4	Amplification	5'GTCGACGCCTCCTCAGTAATGTTCCTTG 3'	28	73	54
A622Sprom_Seq673_F	Sequencing	5'CCACTTTGGCAGCAAGAGAT 3'	20	65	50
A622Sprom_Seq810_R	Sequencing	5'GCAGGGGATAGTGGTTCAAA 3'	20	65	50

Amplified Sequence:

The sequences in red differ from WT and are added to facilitate cloning by placing a SalI site at the 5' end and NcoI site at the start ATG which is indicated in large bold type:

- Mismatch highlighted in blue (G instead of A)
- TA repeats – 70 bp highlighted in yellow

GTCGACGCCTCCTCAGTAATGTTCCTTGCAATTGATTCCTCCGGCTAAATCTAGATCTCTCTTATGTTTCATGCACGTGGAAAAAAAAGAAGTTTGGTTTTTTTTGG
TTCTTATTGCTATTTAAATGTGGTAGGTGAGAAGACTGGATTAAATAAATAACAAACCGTATTCAACTTTTCCGTCCTGTATTATATTTATTATTACAGTAAATAGC
AACAAATATTAAAAGAAAATGAGAATGAGGATCTTTTCAAAATTAAATTTCTTTAAAATAAACAGAATGTTGTTCCCTACAGGCTACAGCTACACGTAAGAAAG[A→G]GTA
CAACAAGTCTTTTCTTTTGTCCTTATCGATATCTGAACGCAATAGGTGGGAATATTGGATCGAAAAATTAAACCCACTATTGCCTGAAAGATAAAAAATATAAAAAT
TCTCTAATAATTAGTTCTTTAATACAACACATGACAAAATGTAATTCTTTATATTATGGCAAGATTCGATTCCTTATACTAGCACCACTTTGGCAGCAAGAAATCAA
TATTTAATATTAAAACAAAATATGACACAGCATTATTTTTCTATATTATGGCAATATATAATTCTTAAAAATTAGTTCTTTAATACAACAATATATGACAAAATGTA
ATTCTTTATATAATGACAAGATTCGATTTTTAGCACCACTTTGGCAGCAAGAGATACATATTTAATATTAAAAAAATATGAAAAACAATTATATTTTATATTATGGC
AATATATAATTCTTTATATTAGCACCACTTTCCAGCAAGATAAAATATTTAAAATCAATTTCTACTTTGAACCACTATCCCCTGCTCTAAAATAATTTGTTTATTTC
AGGGGATTGGAAAATGACTTTAAGTTATATATTGTTAGTGTAAAACGAATTTGATCACCTAAAGAATATTTACAAGTAAGCCTCTATAAAAAGTAATTAAGGTTTGT
AATCTTAAAAGAGAGCATATTACATGTTGTAATTGGTAAATGTGACCTGATAGTGTACAAAATAATTTAAGCTAACCGTGTAACTAATTAAATT**TATATATATATAT**
ATATATATATATATATATATATATATATATATATATATATATATATATATATATACCTTTTCTTTCATTAAATAATGTAATTTCCCGAGGAAGGATTCAACCCTC
CCTTATATGCGGCTCGCTATATAATATTTTTTTTAATTATTTTCTTACATGCTCATCTAGTAGGTAATGACACAGCTAGACGCATGGATCAACATTCTAAGACAGAT
CATCACAAATTTATTAATGGTATGATATCGTAATTCCATGTGTTAGGTTAATTGTGCAAAATTCCCTAGCTTGAATTTGCCTATAAGTACCAGCGTTAATATCAGAT
TCTCCACAGCATTTCATACAAAAATCCGATTTAATTCCTAGTTTCTAGCCCCTCCACCTTAACCCGAAGCTACTTTTTTTCTTCCCCTAGGAGTA**CC**__**ATG**__G

3. *MYC2a*

Size – 720 bp

GenBank Accession# GQ859160

Primers for amplification and sequencing:

Primer name	Purpose	Sequence	Length (bp)	Tm (°C)	GC%
5UTR_MYC2a_F	Amplification	5'CACTCACTCCTTATCACCAAACA 3'	23	64	43
PmlI_3UTR_MYC2a_R	Amplification	5'CACGTGGAGGAAAGCTCAATCAACTTTCCAC 3'	31	73	48
NcoI_MYC2a_F	Amplification	5'CCATGGCTACGGATTATAGAATACCAACG 3'	29	68	45
MYC2aSeq282_F	Sequencing	5'TTGGCAATCGTCTGTTGTGG 3'	20	66	50
MYC2aSeq1168_R	Sequencing	5'ACAAGCTCCCATTTGCACTG 3'	20	66	50
MYC2aSeq1282_F	Sequencing	5'TCGGGTGTGATTTTGCCAAG 3'	20	66	50
MYC2aSeq1690_R	Sequencing	5'AGTTTGATCCCTTGTTGGCT 3'	20	64	45
MYC2aSeq814_F	Sequencing	5'TCGGGCTCATGTGCTATTCA 3'	20	66	50

The sequences in red differ from WT and are added to facilitate cloning by placing a NcoI site at the 5' end and PmlI site at the 3' end. 3' UTR (141 bp) (Italicized) is included to help ensure transcript stability.

CACTCACTCCTTATCACCAAACAATTCTTGGGTGTTTGAATATATACCCGAAATAATTTCCTCTCTGTATCAAGAATCAAACAGATCTGAATTGATTTGTCTGTTTT
*TTTTTCTTGATTTTGTTATATGGA*CC**ATG**GCTACGGATTATAGAATACCAACGATGACTAATATATGGAGCAATACTACATCCGATGATAATATGATGGAAGCTTTT
TTATCTTCTGATCCGTCGTCGTTTTGGCCCGGAACAACTACTACACCAACTCCCCGGAGTTCAGTTTCTCCAGCGCCGGCGCCGGTGACGGGGATTGCCGGAGACCC
ATTAAAGTCTATGCCATATTTCAACCAAGAGTCACTGCAACAGCGACTCCAGACTTTAATCGATGGGGCTCGCAAAGGGTGGACGTATGCCATATTTTGGCAATCGT
CTGTTGTGGATTTCGCGAGCCCCTCGGTTTTGGGGTGGGGAGATGGGTATTATAAAGGTGAAGAAGATAAAAATAAGCGTAAAACGGCGTCGTTTTCGCCTGACTTT
ATCACGGAACAAGCACACCGGAAAAAGGTTCTCCGGGAGCTGAATTCTTTAATTTCCGGCACACAAACCGGTGGTGAAAATGATGCTGTAGATGAAGAAGTAACTGA
TACTGAATGGTTTTTTCTGATTTCCATGACACAATCGTTTGTTAACGGAAGCGGGCTTCCGGGCCTGGCGATGTATAGTTCAAGCCCGATTTGGGTTACTGGAACAG
AGAGATTAGCTGTTTCTCACTGTGAACGGGCCCGACAGGCCCAAGGTTTCGGGCTTCAGACTATTGTTTGTATTCCTTCAGCTAATGGTGTTGTTGAGCTCGGGTCA
ACTGAGTTGATATTCCAGACTGCTGATTTAATGAACAAGGTTAAAGTTTTGTTTAATTTTAATATTGATATGGGTGCGACTACGGGCTCAGGATCGGGCTCATGTGC
TATTCAGGCCGAGCCCGATCCTTCAGCCCTTTGGCTGACTGATCCGGCTTCTTCAGTTGTGGAAGTCAAGGATTCGTCGAATACAGTTCCTTCAAGGAATACCAGTA
AGCAACTTGTGTTTGGAAATGAGAATTCTGAAAATGTTAATCAAAATTCTCAGCAAACACAAGGATTTTTCACTAGGGAGTTGAATTTTTCCGAATATGGATTTGAT
GGAAGTAATACTCGGTATGGAAATGGGAATGCGAATTCTTCGCGTTCTTGCAAGCCTGAGTCTGGTGAAATCTTGAATTTTGGTGATAGTACTAAGAGGAGTGCTTG
CAGTGCAAATGGGAGCTTGTTTTCGGGCCAATCACAGTTCGGGCCCGGGCCTGCGGAGGAGAACAAGAACAAGAACAAGAAAAGGTCACCTGCATCAAGAGGAAGCA

ACGATGAAGGAATCCTTTCATTTGTTTCGGGTGTGATTTTGCCAAGTTCAAACACGGGGAAGTCCGGTGGAGGTGGCGATTCGGATCAATCAGATCTCGAGGCTTCG
GTGGTGAAGGAGGCGGATAGTAGTAGAGTTGTAGACCCCGAGAAGAAGCCGAGGAAACGAGGGAGGAAACCGGCTAACGGGAGAGAGGAGCCATTGAATCATGTGGA
GGCAGAGAGACAAAGGAGGGAGAAATTGAATCAAAGATTCTATGCACTTAGAGCTGTTGTACCAAATGTGTCAAAAATGGATAAAGCATCACTTCTTGGTGATGCAA
TTGCATTTATCAATGAGTTGAAATCAAAGGTTCAGAATTCTGACTCAGATAAAGAGGACTTGAGGAACCAAATCGAATCTTTAAGGAATGAATTAGCCAACAAGGGA
TCAAACTATACCGGTCCTCCCCCGTCAAATCAAGAACTCAAGATTGTAGATATGGACATCGACGTTAAGGTGATCGGATGGGATGCTATGATTCGTATACAATCTAA
TAAAAAGAACCATCCAGCCGCGAGGTTAATGACCGCTCTCATGGAATTGGACTTAGATGTGCACCATGCTAGTGTTTCAGTTGTCAACGAGTTGATGATCCAACAAG
CGACTGTGAAAATGGGAAGCCGGCTTTACACGCAAGAACAACTTCGGATATCATTGACATCCAGAATTGCTGAATCGCGA**TGA***AGAGAAATACAGTAAATGGAAATT*
ATCATAGTGAGCTCTGAATAATGTTATCTTTCATTGAGCTATTTTAAGAGAATTTCTCCTATAGTTAGATCTTGAGATTAAGGCTACTTAAAGTGGAAAGTTGATTG
*AGCTTTCCTC***CACGTG**

4. *ERF189*

Size – 707 bp
GenBank Accession# AB827951

Primers for amplification and sequencing:

Primer name	Purpose	Sequence	Length (bp)	Tm (°C)	GC%
ERF189_PCR_FP	Amplification	5'CCATGGAAATGAATCTAGCTGACGAA 3'	26	67	42
ERF189_PCR_RP	Amplification	5'CACGTGTTAAGCTAATCTCTGTAAATTTAAGCTATG 3'	36	66	33
ERF189 _Seq_234_FP	Sequencing	5'TTCACATGAGAAGTGCTCCG 3'	20	65	50
ERF189 _ Seq_462_RP	Sequencing	5'TCGAGCCGCGGATTTTGAAA 3'	20	68	50

The sequences in red differ from WT and are added to facilitate cloning by placing a NcoI site at the 5' end and PmlI site at the 3' end.

CCATG**G**AAATGAATCTAGCTGACGAAACCTTGTTTTTCTCTGAGTCTCATCTCCTTGAATCGATAAAGCAACATCTTCTTGATGATTCAGATTTTTCTGAAATTTTT
TCGCCGATGAGTTCAAGCAACGAAATATTGCCTAACAGTCCTAGCTCAAGTTTTAGCAGCTTCGACTGCAGCTTCCTCAATTGGGATGAAAACTTTGAGGAAACATT
AATACCAACTGATCAAAATCCTTCACATGAGAAGTGCTCCGAGTCCGAGGAGCAAACCCAGGGCCCAGCGGTGGTGCGTGAGAAAAACGCGCCGCGAGATTGGACGC
GGTATATAGGAGTGAAACGGCGGCCGTGGGGGACGTTTTCGGCGGAGACAAGAGACCCAAGTAGGAAAGGTGAAGGTGCAAGGCTGTGGTTAGGAACTTACGAGACC
GCAGAGGATGCAGCGTTGGCTTACGATCAAGCCGCTTTCAAAATCCGCGGCTCGAGAGCTCGGCTCAATTTTCCCCACTTAATTGGCTCAAACATGCCTAAGCCGGC
TAGAGTAACAGCGAGGCGTAGTCGTACGCGCTCACCCGAGCCATCCTCTTCTTCATCCACTTCATCATCAGAAAATGTGCCAAGAAAAAGGAATATAGATGTGATAA
ATTCCATAGCCAAAGCCAAATTCCTTTGTCATAGCTTAAATTTACAGAGATTAGCTTAA**CACGTG**

APPENDIX B

Creating an APFL construct for maximizing alkaloid production

I. Isolating and characterizing alkaloid pathway-specific promoters;

a. *PMT1* promoter:

Amplifying *PMT1* promoter specifically is hard as the *PMT* gene exists in five isoforms. Hence a 720 bp fragment that represents the promoter and 5'-flanking regions of the *PMT1* gene as described by Reichers and Timko (1999) was ordered from GenScript. For the cloning purposes SalI and NcoI restriction sites were added at the 5' and the 3' end respectively. Since the NcoI site contains ATG (CCATGG), it was added at the *PMT1* gene start codon ATG. The sequence of the PMT1 promoter with the restriction sites is included in Appendix A. Further, the *PMT1* promoter element is digested with SalI and NcoI enzymes from the GenScript vector and cloned into the destination vector pCAMBIA1301 that has also been cut with these same enzymes (replacing a ~790 bp fragment containing the 35S promoter). This final vector: *PMT1: GUS* was used to validate the tissue specificity of the *PMT1* promoter.

b. *A622-S* promoter:

Initially, the plan was to amplify *A622-T* promoter. But we couldn't amplify the promoter region. Since *A622-T* and *A622-S* have similar activity, *A622-S* was chosen for amplification. PCR primers that can specifically amplify the promoter region of *A622-S* region was designed. Similar to *PMT1* promoter, the reverse primer to amplify *A622-S* promoter was designed in a manner such that it will create NcoI restriction site at the site of initiation of translation of *A622-S* gene. And the forward primer was designed such that it is located at least 1,500 bp 5' of the start ATG codon and have a SalI site introduced at the 5' end of the primer. Unfortunately, A622-S promoter region couldn't be amplified with high fidelity Phusion polymerase enzyme

with the exonuclease activity. However, we could amplify the promoter region with APEX Taq polymerase enzyme – master mix without the exonuclease activity, which is generally used for colony PCR.

The amplified sticky- PCR product was cloned into the entry vector pCR2.1 by TA cloning. The insert integrity was confirmed by DNA sequence analysis. Universal primers specific to pCR2.1 (M13 F and M13 R) and specifically designed internal primers were used for sanger sequencing. The sequence was analyzed by Genewiz. After the sequence analysis it was evident that the A622-S promoter region contains TA repeats of length 70 bp. The amplified sequence had one mismatch at 313th position (G instead of an A) when compared to the reference sequence. Also, the reference sequence included "n" region which is variable for different cultivars and this region included the TA repeats. The reference sequence, amplified sequence and the primer sequences are included in Appendix A.

Further, the promoter fragment in pCR2.1 was digested with SalI and NcoI and cloned into the destination vector pCAMBIA1301 that has also been cut with the same enzymes (replacing a ~790 bp fragment containing the 35S promoter). This final vector, *A622-S: GUS* was used to validate the tissue specificity of the *A622-S* promoter.

II. Cloning the tobacco MYC2a and ERF189 transcription factors;

a) MYC2a TF

The sequence of the *NtMYC2a* cDNA that was used by Wang et al. (2015) to increase nicotine production was used as the reference. The primers were designed against the 5' and 3' UTR regions to amplify a copy of the *NtMYC2a*. The reverse primer was placed far enough to include a substantial amount of the 3' UTR (>100 bp) to help ensure transcript stability. PCR was used to introduce restriction sites to facilitate cloning into pCAMBIA1301. PmlI restriction

site (CACGTG) was introduced by the reverse primer. The forward primer was designed to introduce NcoI site at the start codon. This required adding an additional alanine codon (GCT) at the #2 position since the existing Thr codon (ACG) was incompatible with generating NcoI site. The sequence around the start codon ga**ATG**acggat was changed to cc**ATG**gctacggat (the new bp are shown in red).

Genomic DNA (this gene does not contain introns) and the high fidelity Phusion polymerase were used for the amplification. The amplified blunt-PCR product was cloned into the NEB's entry vector pMiniT with the help of NEB PCR cloning kit for both blunt and sticky PCR products. The insert integrity was confirmed by DNA sequence analysis. NEB primers specific to pMiniT vector and specifically designed internal primers were used for sanger sequencing. The sequence was analyzed by Genewiz. The *MYC2a* sequence and the primer sequences are included in Appendix A.

Further, the positive pMiniT vector with *MYC2a* construct was digested with NcoI and PmlI enzymes and cloned into destination vector pCAMBIA1301 that has also been cut with the same enzymes (substituting the GUS gene – 2048 bp of pCAMBIA1301 with *MYC2a*). This created a *35S:MYC2a* construct similar to that used by Wang et al. (2015) to increase alkaloid content.

b) ERF189 TF

Similar to *MYC2a* amplification, PCR was used to introduce NcoI and PmlI sties at the 5' and 3' end respectively. In this case, only CC bp are newly added (CC**ATG**GAA - the new bp are shown in red), as the Glu codon (GAA) is compatible with generating NcoI site. Tobacco cDNA and the high fidelity Phusion polymerase were used for the amplification. The amplified blunt-PCR product was cloned into the NEB's entry vector pMiniT with the help of NEB PCR

cloning kit for both blunt and sticky PCR products. The insert integrity was confirmed by DNA sequence analysis. NEB primers specific to pMiniT vector and specifically designed internal primers were used for sanger sequencing. The sequence was analyzed by Genewiz. The *ERF189* sequence and the primer sequences are included in Appendix A.

Further, the positive pMiniT vector with *ERF189* construct was digested with NcoI and PmlI enzymes and cloned into destination vector pCAMBIA1301 that has also been cut with the same enzymes (substituting the GUS gene – 2048 bp of pCAMBIA1301 with *ERF189*). This created a *35S:ERF189* control construct.

III. Cloning and transforming the APFL and Control Constructs

The four APFL constructs by were created by digesting *35S:MYC2a* and *35S:ERF189* with SalI and NcoI, removing the 35S promoter region, and substituting it with the SalI – NcoI digested fragments containing the *PMT1* and *A622-S* promoters described in section I. Thus, creating the final APFL constructs *PMT1:MYC2a, PMT1:ERF189, A622-T: MYC2a*, and *A622-S: ERF189* that have the potential of creating an artificial positive feedback loop.

Finally, the four APFL constructs, two control constructs (*35S:MYC2a* and *35S:ERF189*), two GUS constructs (*PMT1: GUS, A622-S: GUS*) and empty vector pCAMBIA1301, nine constructs in total were transformed into two plant varieties; K326 and TN86 RNAi PMT plants. Alkaloid of interest in K326 and TN86 RNAi PMT were nicotine and anatabine respectively. Resulting in eighteen transformations. ~10 T_0 plants per construct were obtained using hygromycin selection. Figure 5 includes all the final APFL, control and GUS vectors.

Tobacco cultivar K326 is flue-cured tobacco developed by Novartis Seeds Inc. This cultivar is known for its high quality and ease of processing. It has a low level of resistance to the

blank shank and Granville wilt diseases, susceptible to mosaic and is resistant to root-knot nematodes. TN86 is an air-cured Burley tobacco cultivar, which was developed by Tobacco Experiment Station in Greeneville, Tennessee. It is the first Burley variety to produce normal trichome secretions and not to exhibit the insect susceptibility. TN86 is resistant to TVMV, TEV, PVY, black shank, black root rot, wildfire diseases. TN86 RNAi PMT involves the RNAi mediated downregulation of PMT gene that results in decreased nicotine and increased anatabine levels. (Sierro et al. Supplementary Information, 2014)

The specificity of the *PMT1* and *A622-S* promoters was established by histochemical staining. The standard alkaloid assays were conducted on the leaf samples collected from the greenhouse plants before flowering and two weeks after topping. The alkaloid analysis data were used to determine whether higher levels of nicotine can be realized by expressing *MYC2a* and *ERF189* under the control of their inducible promoters *PMT1* and *A622-S* in comparison to the standard 35S promoter. Also, qRT-PCR experiment was conducted using the standard known genes of the alkaloid biosynthetic pathway on RNA isolated from root tissues at the two-week post-topping date.

APPENDIX C

PMT – F - 5’TGTCAGTCTAACCCTGCACG 3’

A622S – F - 5’GCTAGACGCATGGATCAACA 3’

35S – F - 5’ CAAATGCCATCATTGCGATA 3’

MYC – R - 5’ TTTCACCACCGGTTTGTGTG 3’ – RC - CACACAAACCGGTGGTGAAA

GUS – R - 5’ TCTGCCAGTTCAGTTCGTTG 3’ – RC - CAACGAACTGAACTGGCAGA

ERF – R - 5’ GAAAGCGGCTTGATCGTAAG 3’ – RC - CTTACGATCAAGCCGCTTTC

Sl No	Construct	Total Length - BP
1	PMT: MYC	**670**
2	PMT: ERF	**657**
3	PMT: GUS	**830**
4	A622S: MYC	**773**
5	A622S: ERF	**760**
6	A622S: GUS	**933**
7	35S: MYC	**735**
8	35S: ERF	**895**
9	35S: GUS	**722**

APPENDIX D

qRT-PCR Primers

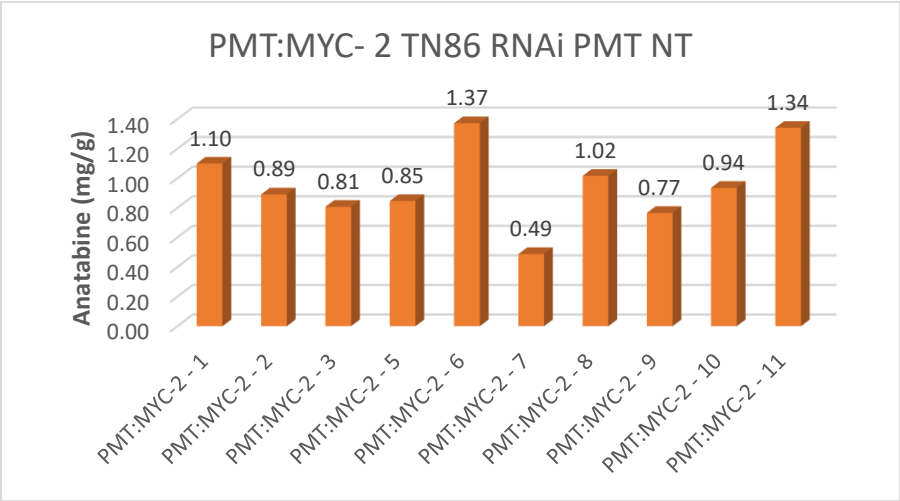
Nicotine Biosynthetic Gene	Primers	Product length in bp	Additional Info.
PMT	*NtPMT_F* 5' GCTATTATAGTGGACTCTTCTG 3' *NtPMT_R* 5' TGTGTGCATACAACTCCTCCT 3' RC of *NtPMT_R* 5' AGGAGGAGTTGTATGCACACA 3'	**113**	Primers bind to all *PMT* isoforms
QPT	*NtQPT_F* 5' GTTGAGGTTGAAACCAGGAC 3' *NtQPT_R* 5' GAACAACCATATTGTCCAGCA 3' RC of *NtQPT_R* 5' TGCTGGACAATATGGTTGTTC 3'	**106**	Primers bind to *Nicotiana tabacum QPT* Accession number. NM_001326216
ODC	*NtODC_F* 5' CTTGTGATGCTCTTGATACTG 3' *NtODC_R* 5' GCGAGGTGAGTAACAATGG 3' RC of *NtODC_R* 5' CCATTGTTACTCACCTCGC 3'	**154**	Primers bind to *Nicotiana tabacum ODC* Accession number. NM_001325697
ADC	*NtADC_F* 5' GTGTCTGCTTCTAGTCACTC 3'	**134**	Primers bind to

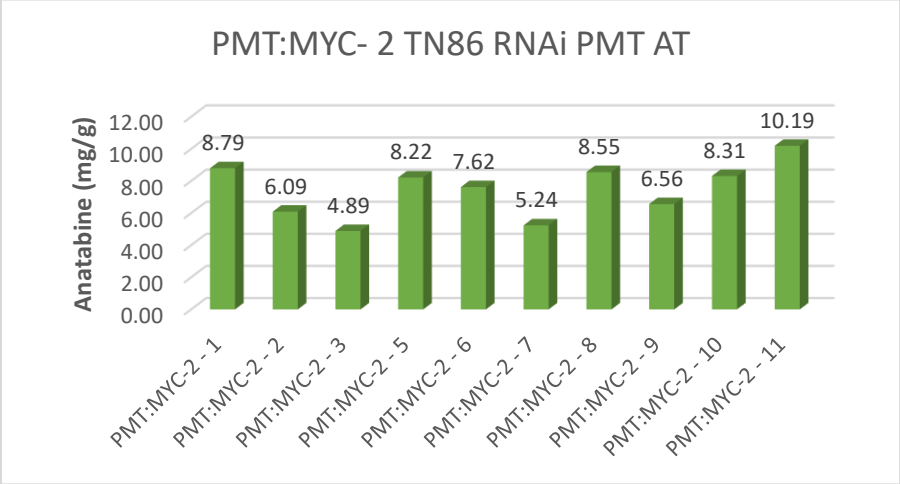
	NtADC_R 5’ TACTCTCCACGAACTGCAGC 3’ RC of *NtADC_R* 5’ GCTGCAGTTCGTGGAGAGTA 3’		*ADC1* and *ADC2* isoforms
MPO	*NtMPO_F* 5’ CCTACTAAGCTTCCTCCAAG 3’ *NtMPO_R* 5’ TCGAGCAGCAGCATGTACTT 3’ RC of *NtMPO_R* 5’ AAGTACATGCTGCTGCTCGA 3’	**108**	Primers bind to *MPO1* and *MPO2* isoforms
A622	*NtA622_F* 5’ GGATGATAGAGGCAGAAGGA 3’ *NtA622_R* 5’ TGACAACTTTGTCTCTAGGAG 3’ RC of *NtA622_R* 5’ CTCCTAGAGACAAAGTTGTCA 3’	**120**	Primers bind to *A622S* and *A622T* isoforms
BBL	*NtBBL_F* 5’ GTATCTAGCACAGTGGAATG 3’ *NtBBL_R* 5’ CAAAGGGCGTCATTGTATTG 3’ RC of *NtBBL_R* 5’ CAATACAATGACGCCCTTTG 3’	**92**	Primers bind to only *BBLa* isoform
Control - *Actin*	*NtActin_F* 5’ CTGAGGTCCTTTTCCAACCA 3’ *NtActin_R* 5’ TACCCGGGAACATGGTAGAG 3’ RC of *NtActin_R* 5’ CTCTACCATGTTCCCGGGTA 3’	**153**	Primers bind to *Nicotiana tabacum Actin 97- like gene*

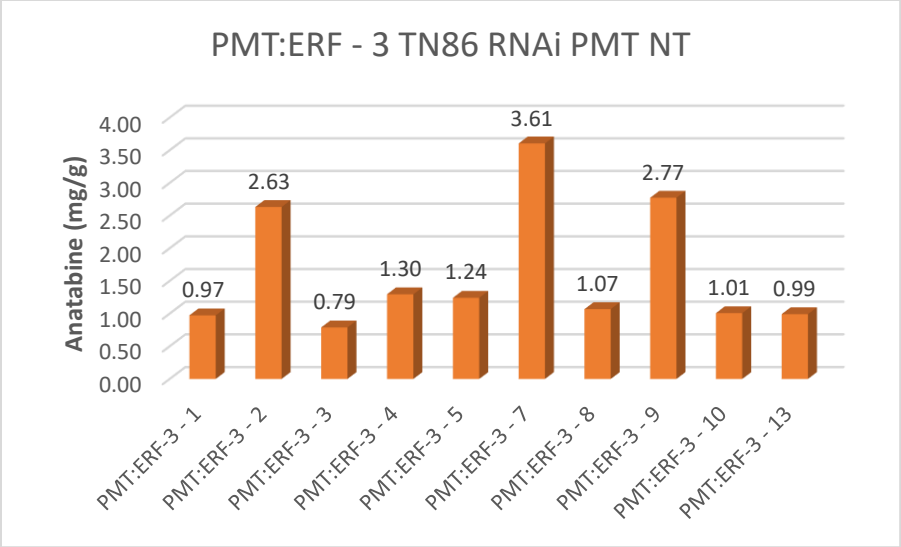
APPENDIX E

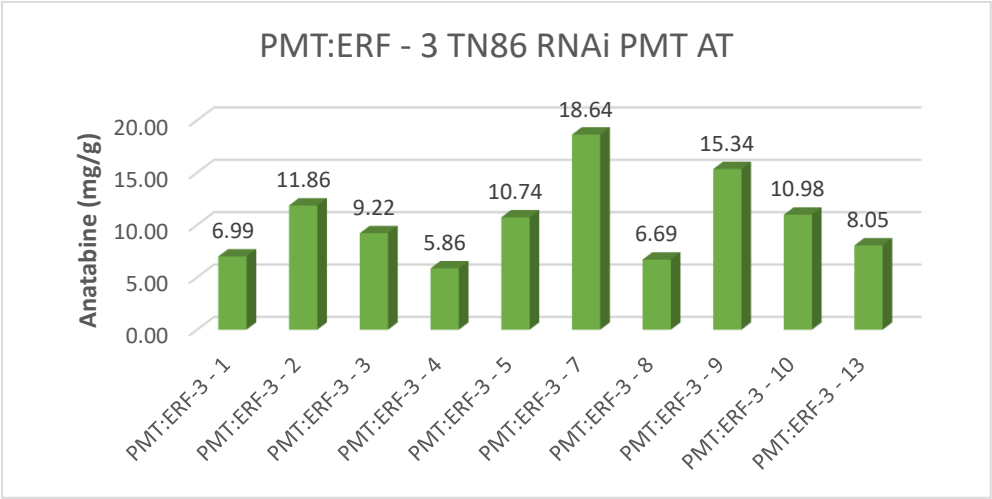
Alkaloid analysis additional graphs

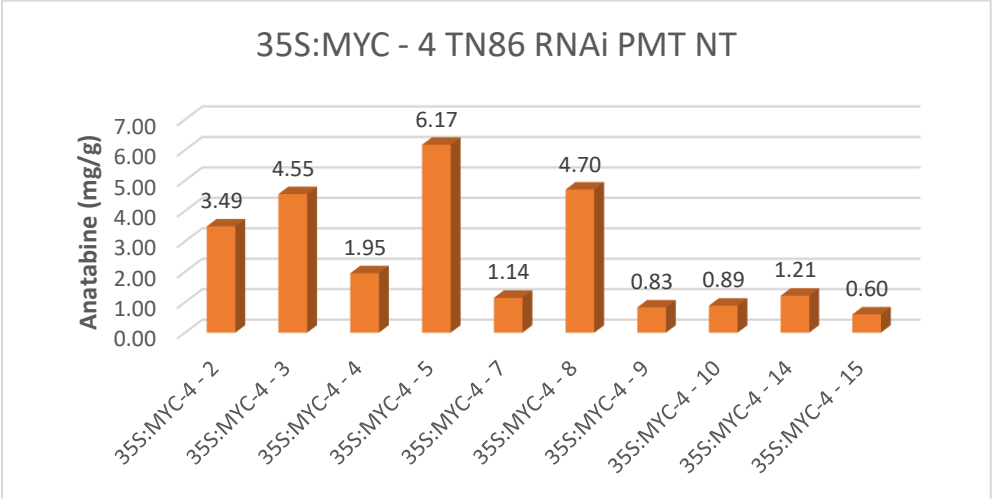
Group 2 (NT – Pre-Topping, AT- Post-Topping)

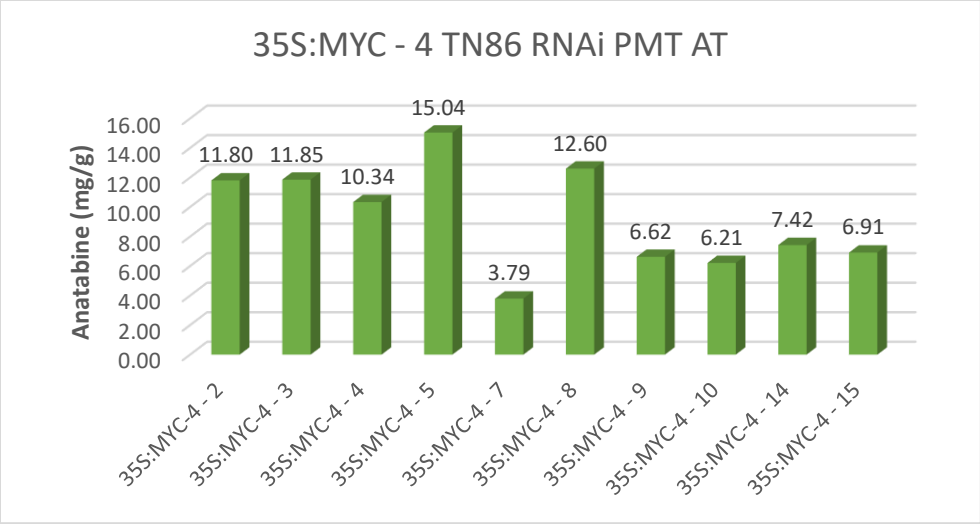


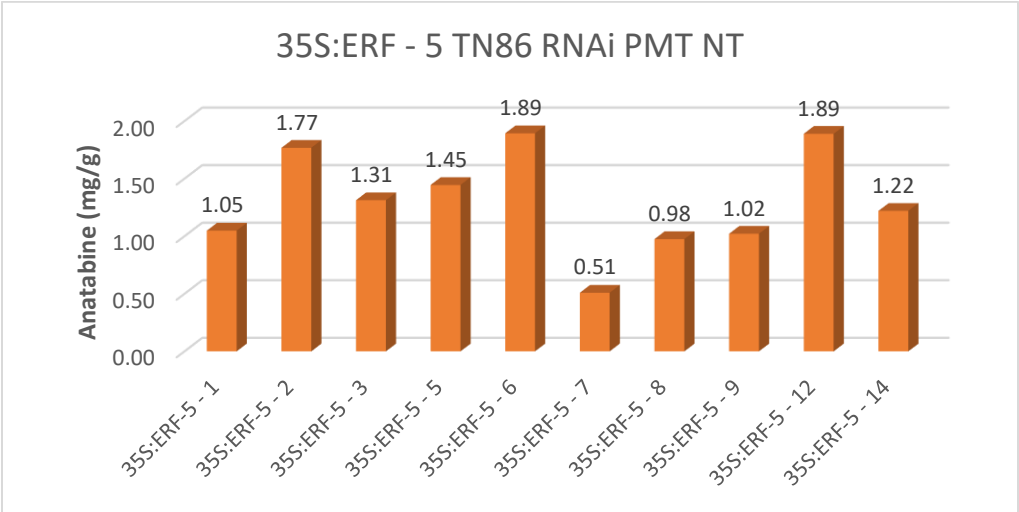


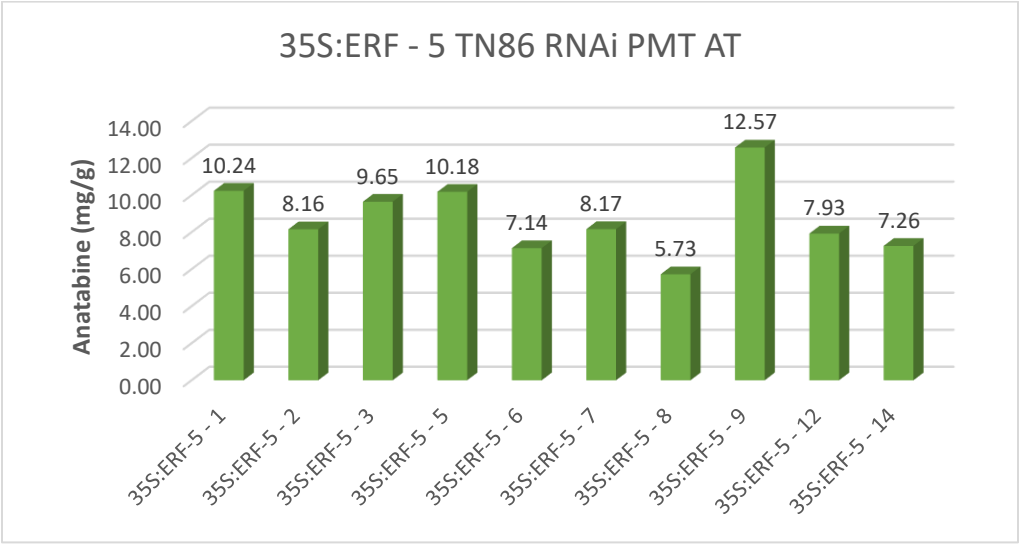

PMT:ERF - 3 TN86 RNAi PMT NT
Anatabine (mg/g)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0.97
2.63
0.79
1.30
1.24
3.61
1.07
2.77
1.01
0.99
PMT:ERF-3 - 1
PMT:ERF-3 - 2
PMT:ERF-3 - 3
PMT:ERF-3 - 4
PMT:ERF-3 - 5
PMT:ERF-3 - 7
PMT:ERF-3 - 8
PMT:ERF-3 - 9
PMT:ERF-3 - 10
PMT:ERF-3 - 13


PMT:ERF - 3 TN86 RNAi PMT AT
Anatabine (mg/g)
20.00
15.00
10.00
5.00
0.00
6.99
11.86
9.22
5.86
10.74
18.64
6.69
15.34
10.98
8.05
PMT:ERF-3 - 1
PMT:ERF-3 - 2
PMT:ERF-3 - 3
PMT:ERF-3 - 4
PMT:ERF-3 - 5
PMT:ERF-3 - 7
PMT:ERF-3 - 8
PMT:ERF-3 - 9
PMT:ERF-3 - 10
PMT:ERF-3 - 13

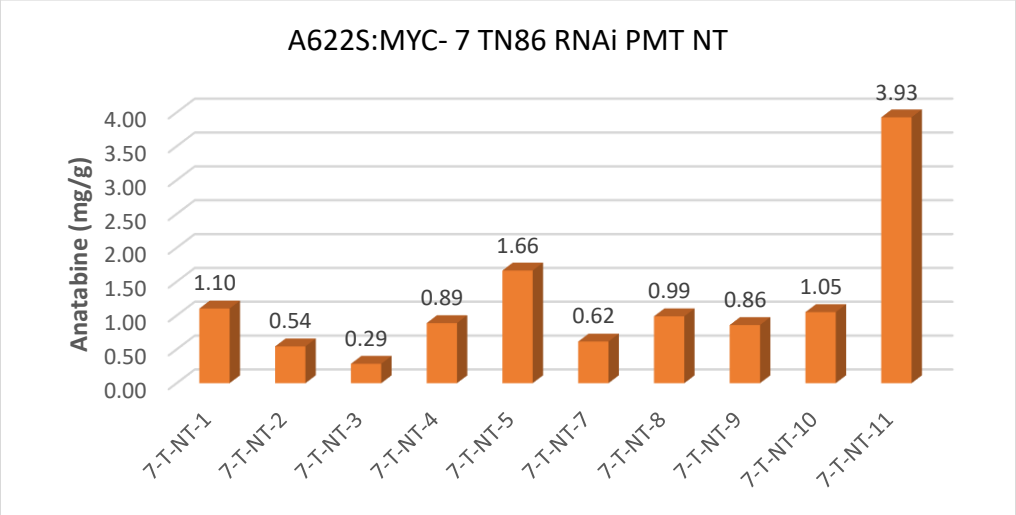

35S:MYC - 4 TN86 RNAi PMT NT
Anatabine (mg/g)
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
3.49
4.55
1.95
6.17
1.14
4.70
0.83
0.89
1.21
0.60
35S:MYC-4 - 2
35S:MYC-4 - 3
35S:MYC-4 - 4
35S:MYC-4 - 5
35S:MYC-4 - 7
35S:MYC-4 - 8
35S:MYC-4 - 9
35S:MYC-4 - 10
35S:MYC-4 - 14
35S:MYC-4 - 15

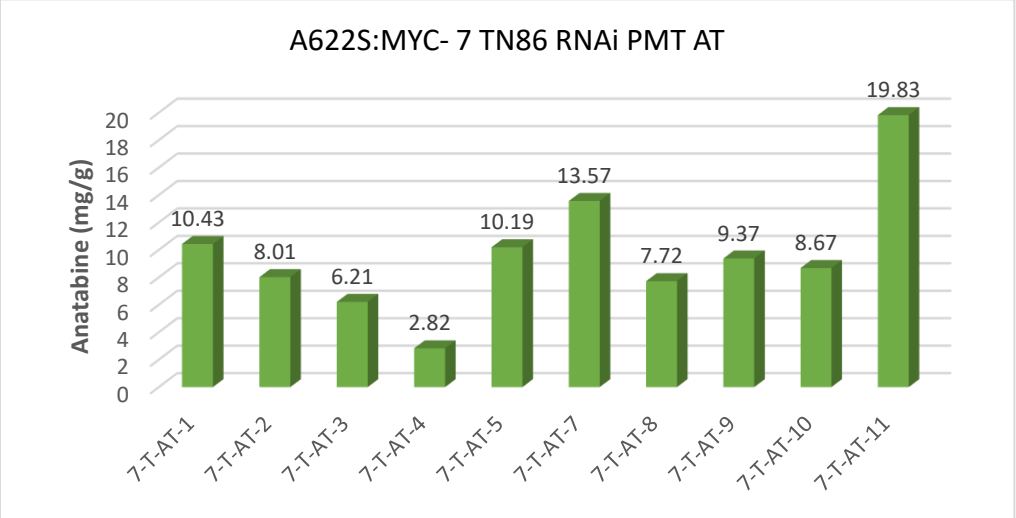

35S:MYC - 4 TN86 RNAi PMT AT
Anatabine (mg/g)
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
11.80
11.85
10.34
15.04
3.79
12.60
6.62
6.21
7.42
6.91
35S:MYC-4 - 2
35S:MYC-4 - 3
35S:MYC-4 - 4
35S:MYC-4 - 5
35S:MYC-4 - 7
35S:MYC-4 - 8
35S:MYC-4 - 9
35S:MYC-4 - 10
35S:MYC-4 - 14
35S:MYC-4 - 15

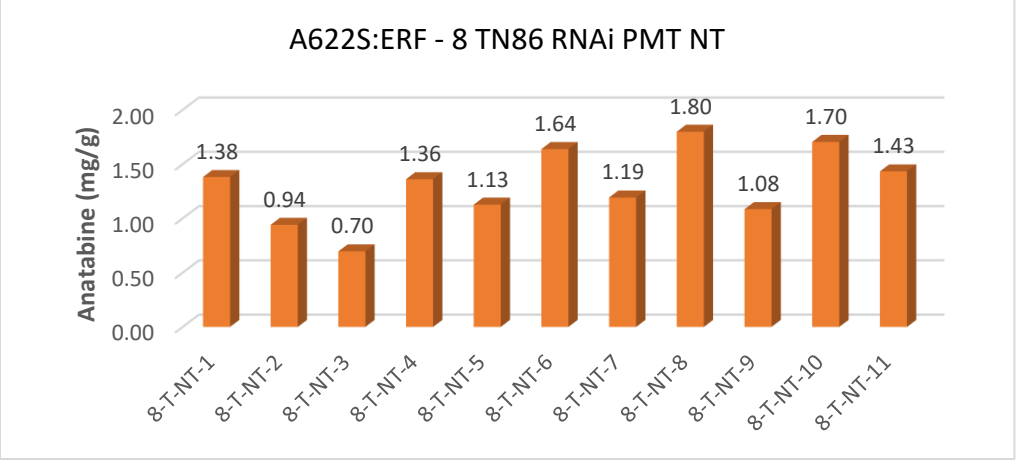

35S:ERF - 5 TN86 RNAi PMT NT
Anatabine (mg/g)
2.00
1.50
1.00
0.50
0.00
1.05
1.77
1.31
1.45
1.89
0.51
0.98
1.02
1.89
1.22
35S:ERF-5 - 1
35S:ERF-5 - 2
35S:ERF-5 - 3
35S:ERF-5 - 5
35S:ERF-5 - 6
35S:ERF-5 - 7
35S:ERF-5 - 8
35S:ERF-5 - 9
35S:ERF-5 - 12
35S:ERF-5 - 14

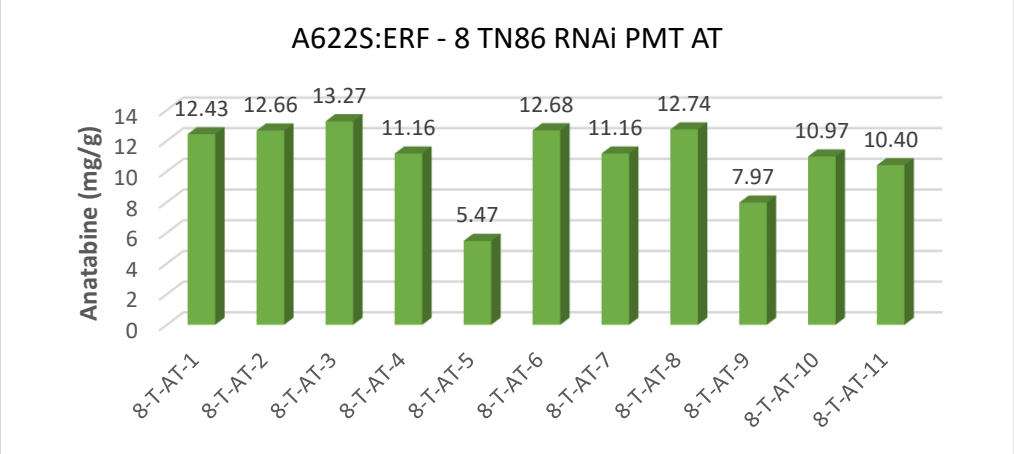

35S:ERF - 5 TN86 RNAi PMT AT
Anatabine (mg/g)
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
10.24
8.16
9.65
10.18
7.14
8.17
5.73
12.57
7.93
7.26
35S:ERF-5 - 1
35S:ERF-5 - 2
35S:ERF-5 - 3
35S:ERF-5 - 5
35S:ERF-5 - 6
35S:ERF-5 - 7
35S:ERF-5 - 8
35S:ERF-5 - 9
35S:ERF-5 - 12
35S:ERF-5 - 14

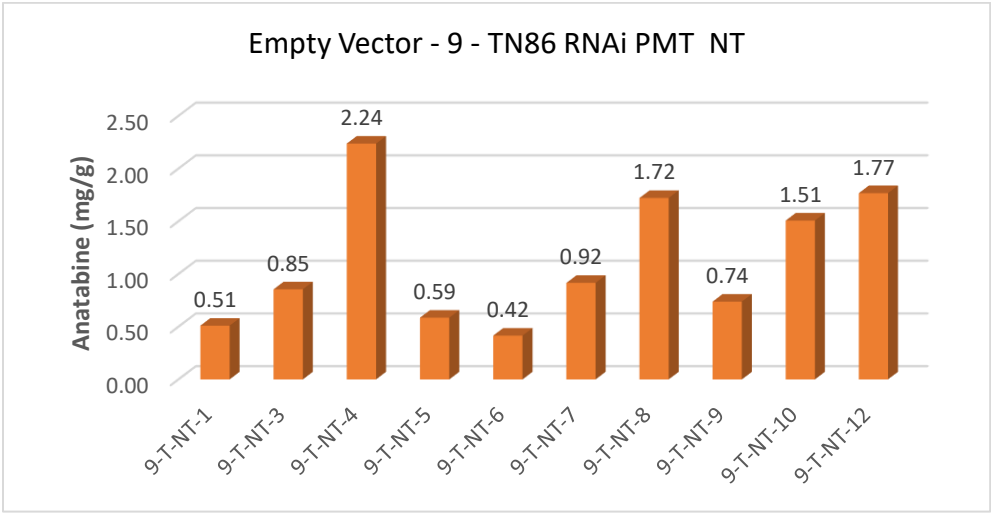
Group 3 (NT – Pre-Topping, AT- Post-Topping)

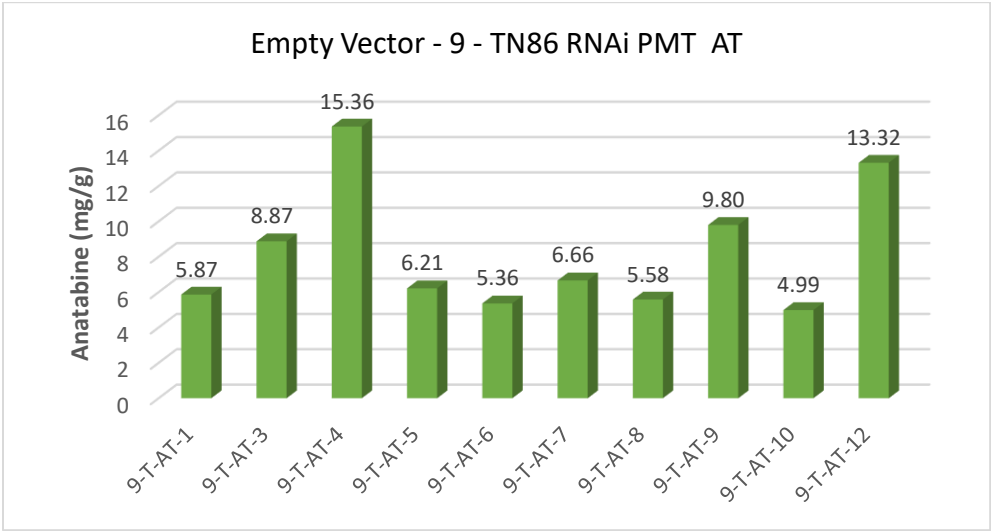






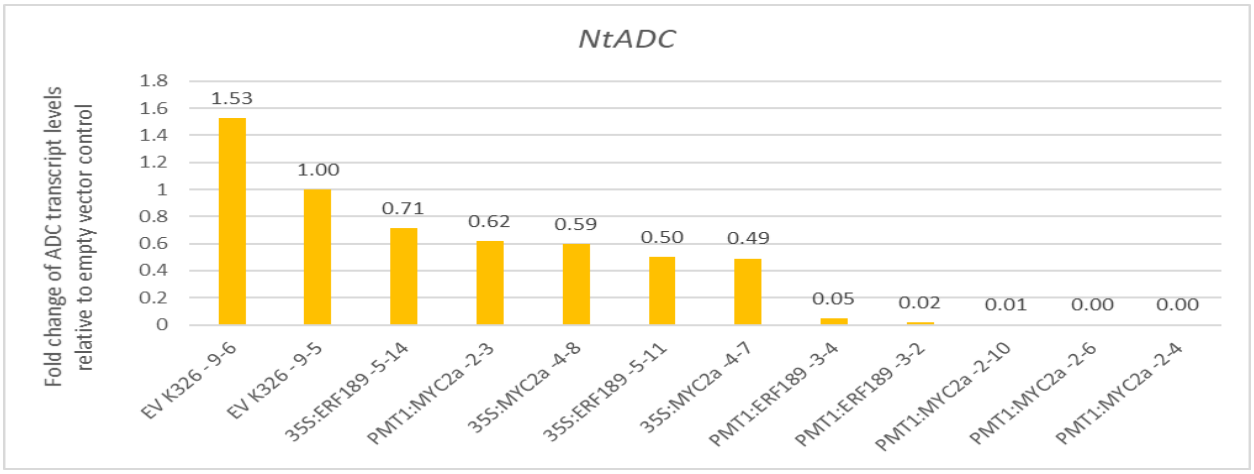

A622S:ERF - 8 TN86 RNAi PMT AT
Anatabine (mg/g)
0
2
4
6
8
10
12
14
12.43
12.66
13.27
11.16
5.47
12.68
11.16
12.74
7.97
10.97
10.40
8-T-AT-1
8-T-AT-2
8-T-AT-3
8-T-AT-4
8-T-AT-5
8-T-AT-6
8-T-AT-7
8-T-AT-8
8-T-AT-9
8-T-AT-10
8-T-AT-11

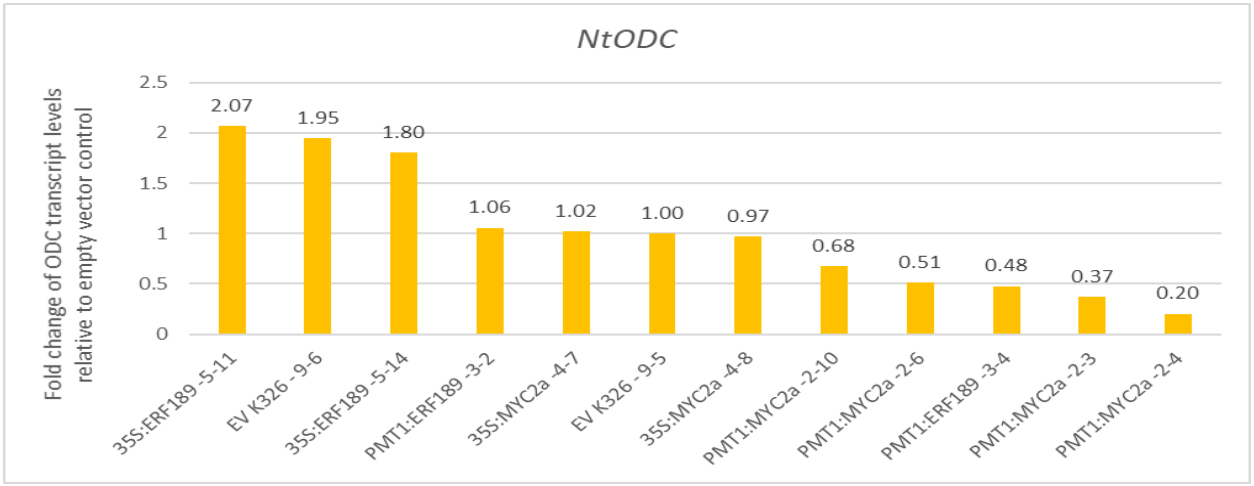

Empty Vector - 9 - TN86 RNAi PMT NT
Anatabine (mg/g)
0.00
0.50
1.00
1.50
2.00
2.50
0.51
0.85
2.24
0.59
0.42
0.92
1.72
0.74
1.51
1.77
9-T-NT-1
9-T-NT-3
9-T-NT-4
9-T-NT-5
9-T-NT-6
9-T-NT-7
9-T-NT-8
9-T-NT-9
9-T-NT-10
9-T-NT-12

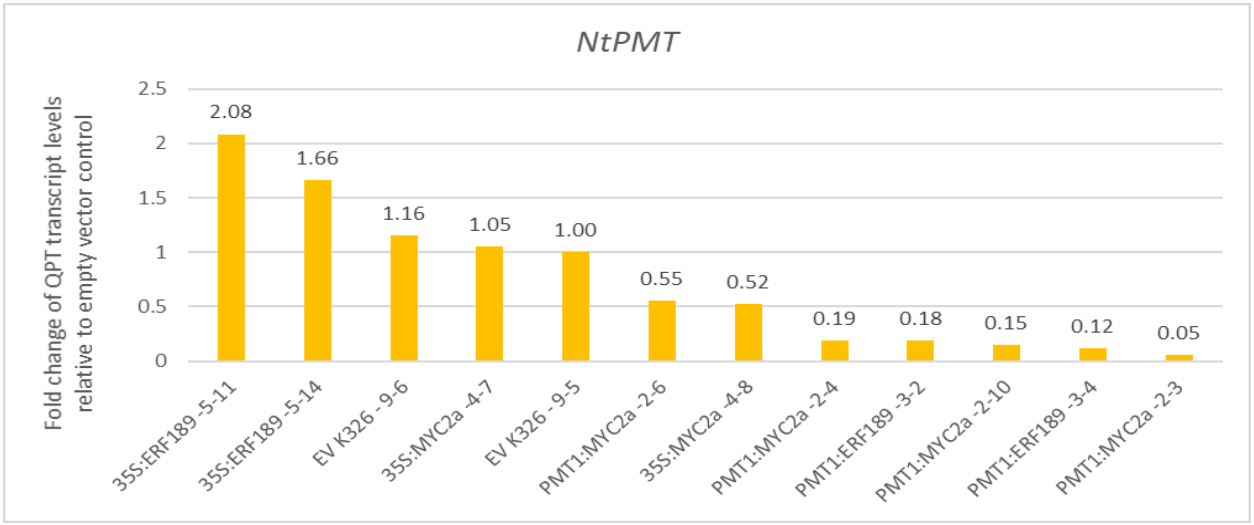

Empty Vector - 9 - TN86 RNAi PMT AT
Anatabine (mg/g)
0
2
4
6
8
10
12
14
16
5.87
8.87
15.36
6.21
5.36
6.66
5.58
9.80
4.99
13.32
9-T-AT-1
9-T-AT-3
9-T-AT-4
9-T-AT-5
9-T-AT-6
9-T-AT-7
9-T-AT-8
9-T-AT-9
9-T-AT-10
9-T-AT-12

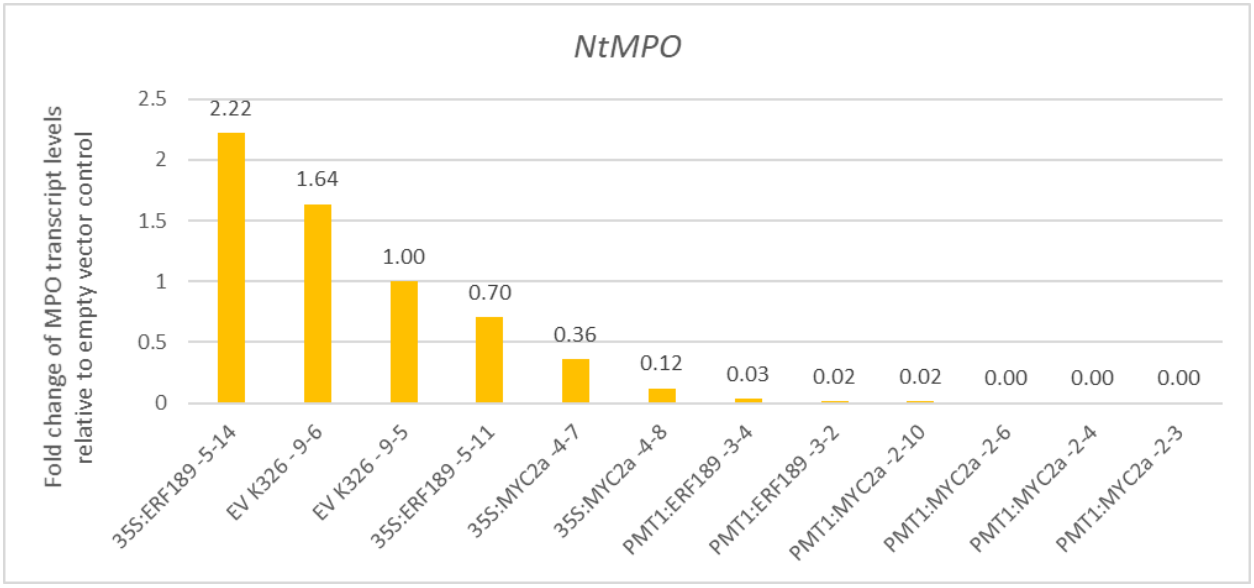
APPENDIX F

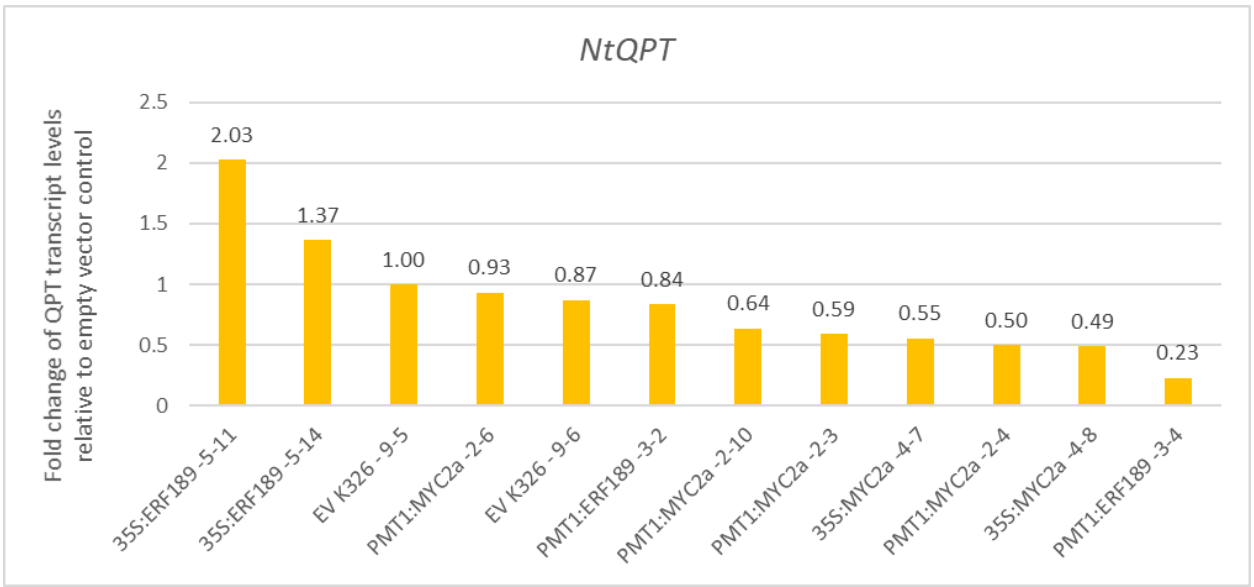
qRT-PCR Additional Graphs

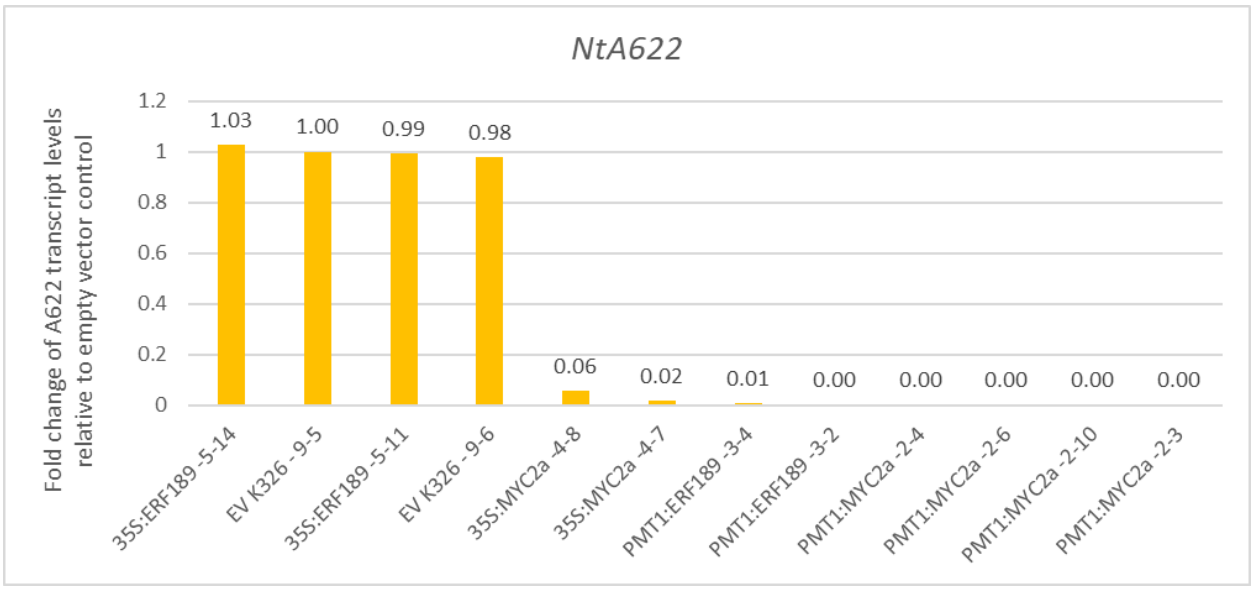


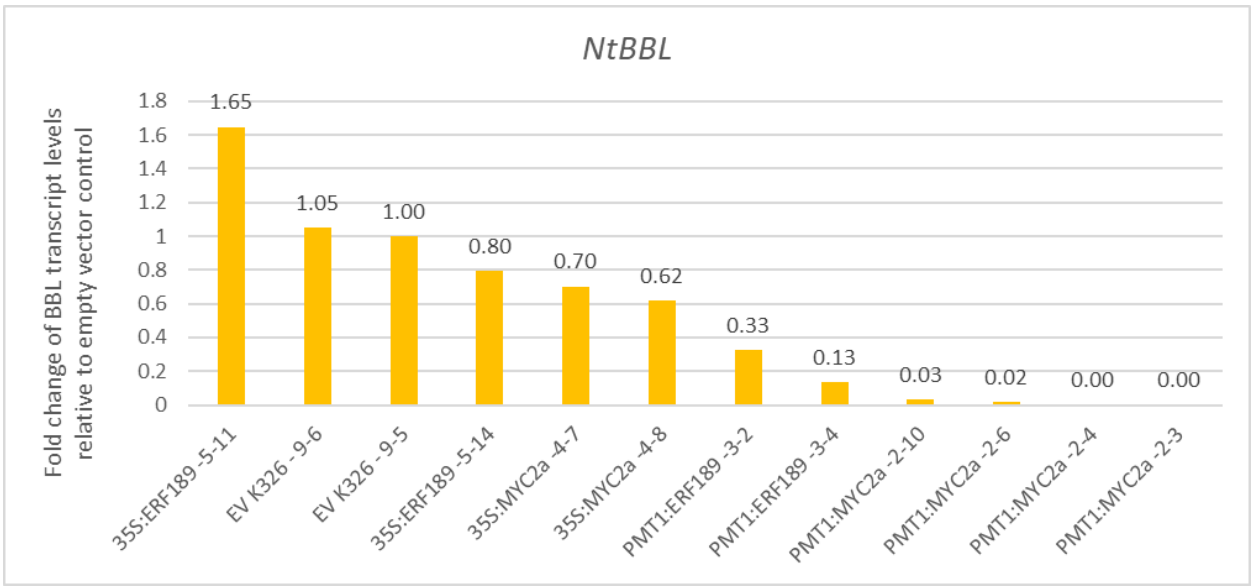





NtMPO
Fold change of MPO transcript levels relative to empty vector control
2.5
2
1.5
1
0.5
0
2.22
1.64
1.00
0.70
0.36
0.12
0.03
0.02
0.02
0.00
0.00
0.00
35S:ERF189 -5-14
EV K326 - 9-6
EV K326 - 9-5
35S:ERF189 -5-11
35S:MYC2a -4-7
35S:MYC2a -4-8
PMT1:ERF189 -3-4
PMT1:ERF189 -3-2
PMT1:MYC2a -2-10
PMT1:MYC2a -2-6
PMT1:MYC2a -2-4
PMT1:MYC2a -2-3


NtQPT
Fold change of QPT transcript levels relative to empty vector control
2.5
2
1.5
1
0.5
0
2.03
1.37
1.00
0.93
0.87
0.84
0.64
0.59
0.55
0.50
0.49
0.23
35S:ERF189 -5-11
35S:ERF189 -5-14
EV K326 - 9-5
PMT1:MYC2a -2-6
EV K326 - 9-6
PMT1:ERF189 -3-2
PMT1:MYC2a -2-10
PMT1:MYC2a -2-3
35S:MYC2a -4-7
PMT1:MYC2a -2-4
35S:MYC2a -4-8
PMT1:ERF189 -3-4


NtA622
Fold change of A622 transcript levels relative to empty vector control
1.2
1
0.8
0.6
0.4
0.2
0
1.03
1.00
0.99
0.98
0.06
0.02
0.01
0.00
0.00
0.00
0.00
0.00
35S:ERF189 -5-14
EV K326 - 9-5
35S:ERF189 -5-11
EV K326 - 9-6
35S:MYC2a -4-8
35S:MYC2a -4-7
PMT1:ERF189 -3-4
PMT1:ERF189 -3-2
PMT1:MYC2a -2-4
PMT1:MYC2a -2-6
PMT1:MYC2a -2-10
PMT1:MYC2a -2-3


NtBBL
Fold change of BBL transcript levels relative to empty vector control
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1.65
1.05
1.00
0.80
0.70
0.62
0.33
0.13
0.03
0.02
0.00
0.00
35S:ERF189 -5-11
EV K326 - 9-6
EV K326 - 9-5
35S:ERF189 -5-14
35S:MYC2a -4-7
35S:MYC2a -4-8
PMT1:ERF189 -3-2
PMT1:ERF189 -3-4
PMT1:MYC2a -2-10
PMT1:MYC2a -2-6
PMT1:MYC2a -2-4
PMT1:MYC2a -2-3