

Investigation of Protein – Protein Interactions among Nicotine Biosynthetic Enzymes and
Characterization of a Nicotine Transporter

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ABSTRACT

Alkaloids are a class of plant secondary metabolites produced in about 20% of plant families. Domesticated tobacco, *Nicotiana tabacum* produces nicotine as the predominant alkaloid. The biosynthesis of nicotine occurs exclusively in the roots of tobacco, yet accumulates in the leaves of tobacco where it acts as a defense compound to deter insect herbivory. The research detailed in this dissertation addresses two aspects of nicotine physiology in tobacco: 1) an investigation of hypothesized protein-protein interactions among nicotine biosynthetic enzymes and 2) the characterization of a novel nicotine transporter.

A hypothesized metabolic channel including the two nicotine biosynthetic enzymes putrescine N-methyltransferase (PMT), N-methylputrescine Oxidase (MPO) and the S-adenosylmethionine (SAM) recycling enzyme S-adenosylhomocysteine hydrolase (SAHH) has been proposed. To further explore this hypothesis, protein-protein interactions among nicotine biosynthetic enzymes PMT, MPO and SAHH were investigated using yeast two-hybrid assays and co-immunoprecipitation experiments. The yeast two-hybrid was conducted as both a directed screen to detect interactions between the hypothesized metabolic channel members and as a library screen to detect interactions between hypothesized metabolic channel members and proteins from a tobacco root cDNA library. Co-immunoprecipitation experiments were conducted using proteins produced in an *in vitro* transcription/ translation system and using native proteins from a tobacco root extract. The outcome of these experiments provided no further evidence of a

nicotine metabolic channel and a discussion of the methods and outcomes of the experiments conducted is presented.

The nicotine uptake permease, NUP1, was identified in tobacco roots and was shown to preferentially transport nicotine when expressed in *Schizosaccharomyces pombe*. This report presents the characterization of tobacco plants and hairy roots with diminished *NUP1* transcripts created by using RNAi. The *NUP1-RNAi* hairy roots and plants showed a decreased level of nicotine and the hairy root cultures displayed an altered distribution of nicotine from the root to the culture medium. Additionally NUP1-GFP was used to determine that NUP1 localized to the plasma membrane of tobacco BY-2 protoplasts. Potential models for the role of NUP1 in nicotine physiology will be discussed.

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Chapter I

General Introduction and Overview of Research

Nicotine biosynthesis and regulation

Plants produce a wide variety of chemical compounds or metabolites including those compounds necessary for sustaining life and additional compounds that are not necessary for survival but give the plant an ecological advantage. Primary metabolites are those compounds essential for fundamental cellular processes such as cell growth and division. In addition to these essential metabolic processes, plants produce a wide variety of so-called secondary or specialized metabolites that are not evenly dispersed cell types or among plant species. These secondary compounds are not essential for basic cellular function, but nevertheless confer advantages that increase plant fitness and vigor. In addition to providing an ecological advantage to the plant, many secondary metabolites are used as dyes, fragrances, flavorings, nutritional supplements, and medicines. Plant secondary metabolites include alkaloids, flavonoids, terpenes, and glucosinolates. Alkaloids are a particularly rich and diverse class of secondary metabolites and are the focus of this literature review.

Alkaloids

Alkaloids are defined structurally as heterocyclic compounds with nitrogen as a member of one or more rings (Ziegler and Facchini, 2008). In most cases, alkaloids are derived from amino acids and a characteristic feature of alkaloids is their pharmacologic

affects on animals. The affects of alkaloid consumption can range from paralysis to toxicity in insects while in animals the effects are often dosage dependant and can range from stimulants to depressants to toxicity depending upon the compound consumed. Due to the biological activity of alkaloids in animal species, these compounds are generally considered to function in plant defense, although humans have exploited many alkaloids for these same pharmacological properties.

Alkaloids are a particularly rich source of pharmacologically-active compounds and are used in a variety of cultural and medicinal applications. Caffeine and nicotine are common alkaloids that act as stimulants in humans and are typically self-administered through the direct use of the leaf products. Morphine and codeine produced in the opium poppy are used as pain relievers and the use of these compounds is typically in a highly purified form of the drug administered by professionals (Facchini and Luca, 2008). Vincristine and vinblastine are alkaloids purified from *Catharanthus roseus* that are used in the treatment of childhood leukemias and non-Hodgkin's lymphomas (vincristine), and Hodgkin's disease (vinblastine). These compounds cannot be chemically synthesized economically and therefore must be derived as plant natural products (Facchini and Luca, 2008; Zhou et al., 2009). These examples highlight only a few of the many alkaloids desired for their pharmacological properties and the variety of medicinal applications in which alkaloids are used.

Alkaloid production tends to be concentrated into certain families with about 20% of plant species known to accumulate alkaloids (De Luca and St Pierre, 2000). The Solanaceae, also known as the deadly nightshades, produce a variety of pharmacologically-active alkaloids including pyridine, tropane, and steroidal

glycoalkaloids. Nicotine, a pyridine alkaloid, is the predominant alkaloid produced in *Nicotiana tabacum* and most other *Nicotiana* species (Saitoh et al., 1985). *In planta*, nicotine deters insect feeding thereby increasing plant fitness (Baldwin, 1998; Voelckel et al., 2001; Steppuhn et al., 2004). Nicotine in humans is rapidly absorbed, transported and sensed by acetylcholine receptors in the brain (Silvette et al., 1962). The pleasure derived from nicotine consumption has been the principle motivation for the domestication and agricultural demand for tobacco products.

Nicotine production in *Nicotiana* species

Nicotiana species are members of the Solanaceae, a large plant family that produces several categories of alkaloids. *Nicotiana tabacum* is the agriculturally domesticated member of the genus *Nicotiana*. *N. tabacum* is derived from a hybrid complex that includes the wild progenitor species *Nicotiana tomentosiformis*, *Nicotiana otophora*, and *Nicotiana sylvestris* (Goodspeed and Clausen, 1928; Ren and Timko, 2001). Domesticated tobacco (*N. tabacum*) is an amphidiploid, containing two distinct sets of diploid chromosomes with one diploid set derived from *N. sylvestris* and one diploid set derived from an apparent hybrid of *N. tomentosiformis* and *N. otophora* (Hashimoto et al., 1998; Riechers and Timko, 1999; Ren and Timko, 2001). Goodspeed (1954) noted that naturally-occurring *N. tabacum* could not be found in the same geographical region of South America as the overlapping wild progenitor species. Moreover, in all cases where he found untended *N. tabacum* they were always associated with prior human habitation. Thus, Goodspeed conjectured that *N. tabacum* may have

originated from an unusual (man-made?) hybrid between the aforementioned wild *Nicotiana* species and subsequently cultivated as a domesticated crop (Goodspeed, 1954).

Nicotine is produced exclusively in the roots (Dawson, 1942; Dawson, 1942; Solt, 1957; Dawson and Solt, 1959) and accumulates throughout the plant in both aerial and root tissues (Ohnmeiss and Baldwin, 2000). Nicotine production in roots was initially demonstrated through the grafting of tomato scions onto tobacco rootstocks, resulting in the accumulation of nicotine in tomato leaves, whereas tobacco scions grafted to tomato rootstocks did not show significant increases of nicotine accumulation in the tobacco leaves (Dawson, 1942). Nicotine produced in the roots is translocated to the aerial portions of the plant through xylem transport (Baldwin, 1989). Nicotine accumulation occurs primarily in young leaf tissue, although nicotine is also distributed in the root, stem and flowers.

In addition to nicotine, *Nicotiana* species also produce other pyridine alkaloids that are often referred to as nicotine-related alkaloids. These compounds are generally found in several-fold lower concentrations than nicotine and their presence and relative concentrations vary depending upon the specific *Nicotiana* species. Nornicotine is the demethylated form of nicotine and is converted from nicotine only in leaves (Dawson, 1942; Siminszky et al., 2005). The alkaloids anatabine, anabasine, and anatalline all contain the nicotinic acid derived pyridyl moiety and differ from nicotine by replacing N-methylpyrrolidine with one or more five- or six-membered N-containing rings.

Hormonal control of nicotine biosynthesis

Nicotine biosynthesis is an inducible chemical defense regulated in response to a variety of hormonal signals. The plant hormone auxin has been shown to suppress nicotine biosynthesis in whole plants (Yasumatsu, 1967) and in cell culture (Tabata et al., 1971). Auxin is produced at the shoot apical meristem and removal of the shoot apical meristem disrupts basipetal auxin flow. Disruption of auxin flow in tobacco results in increased nicotine production as well as increased expression of some nicotine biosynthetic genes, while application of auxin to root cultures decreases expression of the same nicotine biosynthetic genes (Hibi et al., 1994). Many herbivores favor grazing on the youngest tissues because they are more tender. Because of the nature of plant development these same young tissues include the apical meristem, the primary source of auxin biosynthesis. Removal of the palatable apical meristem disrupts auxin biosynthesis and subsequent basipetal flow. Thus, the roots are able to sense above ground herbivory by integrating changes in auxin homeostasis and respond with an increase in production of the defense compound nicotine.

Nicotine biosynthesis is also inducible by the plant defense hormone, methyl jasmonate (MeJA). MeJA is rapidly produced in damaged leaves in response to foliar wounding. MeJA production levels are proportional to the amount of foliar wounding and there are strong positive correlations between the amount of MeJA produced and the amount of nicotine that is produced (Baldwin et al., 1997; Ohnmeiss et al., 1997). MeJA is subsequently transported from the site of biosynthesis to roots (Zhang and Baldwin, 1997). Thus, MeJA is a long-distance signal carried via the phloem to the root resulting in a strong increase in nicotine biosynthesis (Baldwin et al., 1994; Baldwin et al., 1997; Imanishi et al., 1998; Shoji et al., 2000; Shoji et al., 2000; Jiang et al., 2009). The

application of MeJA to the roots or shoots results in an induction of nicotine biosynthesis (Baldwin et al., 1994).

Nicotine is an effective chemical defense against insect herbivores

There are several lines of indirect and direct evidence that demonstrate that inducible nicotine biosynthesis is an effective chemical defense against herbivores. First of all the pharmacological and neurotoxic properties of purified nicotine implicate nicotine as a possible defensive chemical. Secondly, the inducible character of nicotine biosynthesis is consistent with a costly chemical defense that is mounted only when herbivory is encountered. Consistent with this idea, *Nicotiana* species that are stimulated for nicotine biosynthesis show reduced growth and seed production (Baldwin and Callahan, 1993). These aforementioned lines of evidence are correlative and are consistent with the expectations of Optimal Defense Theory (Ohnmeiss and Baldwin, 2000). The Optimal Defense Theory predicts that metabolically costly defense mechanisms will be induced only when they are needed and their localization restricted to only those tissues that contribute the most to plant evolutionary fitness. There is also more direct evidence that support the hypothesis that nicotine is a chemical defense. Plants that are either subject to natural herbivory and/or are artificially induced for nicotine biosynthesis show increased fitness during moderate herbivory pressure (Baldwin, 1998). *Nicotiana* plants with dramatically reduced nicotine levels (Voelckel et al., 2001; Jackson et al., 2002; Chintapakorn and Hamill, 2003; Steppuhn et al., 2004)

show significantly increased herbivory levels. Thus, nicotine is an effective natural chemical defense against herbivory.

Biosynthesis of Nicotine

Nicotine is comprised of two asymmetric rings, a pyridine ring and an N-methylpyrrolinium ring that are joined together by a carbon-carbon bond. Figure 1.1 shows the current model of the nicotine biosynthetic pathway. The N-methylpyrrolinium moiety is derived from the diamine putrescine while the biosynthetic origin of the pyridine ring is not well understood. Biosynthesis of the pyridine ring is derived from quinolinic acid. However, the exact intermediates between quinolinic acid and nicotinic acid have not been established, but are assumed to include intermediates present in the biosynthesis of nicotinamide adenine dinucleotide (NAD⁺).

Biosynthesis of the N-methylpyrrolidine ring is derived from the diamine putrescine (Figure 1.1). Putrescine is present throughout the plant and functions in a variety of physiological and developmental processes (Kusano et al., 2008). Putrescine can be derived from the amino acids arginine or ornithine through independent pathways, with both pathways contributing to the production of putrescine for nicotine biosynthesis (Yoshida, 1969). The conversion of ornithine to putrescine is direct and catalyzed by the enzyme ornithine decarboxylase (ODC). ODC enzyme levels increase with the removal of the shoot apical meristem (Mizusaki et al., 1973). Transgenic hairy roots expressing recombinant yeast ODC show a modest increase in nicotine levels (Hamill et al., 1990). *ODC* expression in the root is coordinated with known nicotine biosynthetic genes (Shoji et al., 2000; Reed and Jelesko, 2004). For example, *ODC* transcript levels increase in

response to MeJA(Shoji et al., 2000) and auxin removal treatments (Reed and Jelesko, 2004).

The synthesis of putrescine from arginine is indirect and includes the intermediates agmatine and N-carbomylputrescine. The conversion of arginine to agmatine is catalyzed by arginine decarboxylase (ADC). *ADC* mRNA levels increase in response to auxin removal (Reed and Jelesko, 2004) but not to MeJA treatment (Imanishi et al., 1998).

The first committed step in the biosynthesis of the N-methylpyrrolinium ring is the N-methylation of the diamine putrescine, resulting in the production of N-methylputrescine (Mizusaki et al., 1971; Hibi et al., 1994). The methylation of putrescine is catalyzed by putrescine N-methyltransferase (PMT) using S-adenosylmethionine (SAM) as the methyl donor (Mizusaki et al., 1971). *PMT* transcripts and PMT enzyme activity are exclusively associated with the root (Mizusaki et al., 1972; Hibi et al., 1994). *PMT* transcript levels as well as PMT enzyme activity increase in response to plant decapitation or removal of exogenous auxin (Mizusaki et al., 1971; Hibi et al., 1994; Hashimoto et al., 1998; Riechers and Timko, 1999; Reed and Jelesko, 2004; Heim et al., 2006). Additionally *PMT* transcript levels increase with increases in endogenous MeJA production and with exogenously applied MeJA (Shoji et al., 2000; Shoji et al., 2002; Chintapakorn and Hamill, 2003; Heim et al., 2006; Kidd et al., 2006). *PMT* is thought to have evolved from spermidine synthase and appears in the genome of *N. tabacum* in at least five copies (Hibi et al., 1994; Hashimoto et al., 1998; Riechers and Timko, 1999). Each copy has been correlated with one of the progenitor species of *N. tabacum* with three copies being derived from *Nicotiana sylvestris*, and one copy each from *Nicotiana*

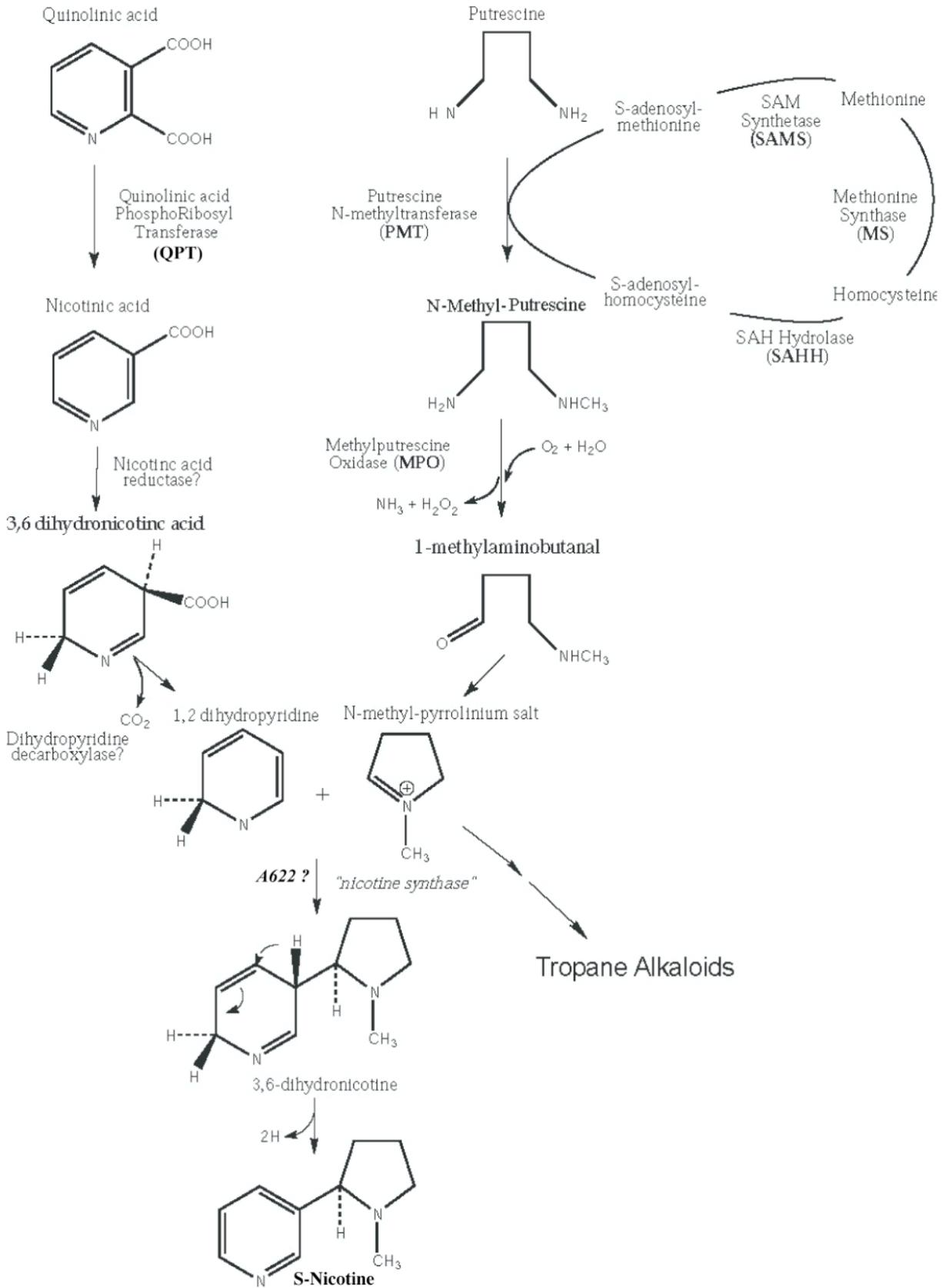


Figure 1.1: The current nicotine biosynthetic pathway

otphoria and *Nicotiana tomentosiformis*. Gene silencing of *PMT* transcripts in tobacco result in plants with drastically reduced nicotine levels (Chintapakorn and Hamill, 2003; Steppuhn et al., 2004), demonstrating that *PMT* is an essential nicotine biosynthetic gene.

The second step in the biosynthesis of the N-methylpyrrolinium ring is oxidative deamination of N-methylputrescine to 4-aminobutinal by the enzyme N-methylputrescine oxidase (MPO) (Mizusaki et al., 1972). MPO has been partially purified from tobacco roots (Mizusaki et al., 1972; Feth et al., 1985) and tropane alkaloid-producing *Hyoscyamus niger* roots (Hashimoto et al., 1990). MPO activity has been identified in tobacco root extracts and increases after plant decapitation (Mizusaki et al., 1972; Saunders and Bush, 1979; Davies et al., 1989), suggesting it is regulated in part by auxin levels. MPO has been cloned from *N. tabacum* and shown to be part of a small multi-gene family (Heim et al., 2007; Katoh et al., 2007). *MPO* transcripts are found exclusively in roots and transcript levels increase with auxin removal from root cultures (Heim et al., 2007) and with MeJA elicitation in plantlets (Katoh et al., 2007). MPO oxidizes a range of aliphatic diamines, but the preferred substrate is N-methylputrescine (Mizusaki et al., 1972; Hashimoto et al., 1990; Walton and McLauchlan, 1990; Heim et al., 2007; Katoh et al., 2007). MPO requires copper as a cofactor (Mizusaki et al., 1972) and displays characteristics of a quinoprotein (Davies et al., 1989). The predicted size of the MPO polypeptide is 89 kDa and MPO was empirically estimated at between 130 - 172 kDa, corresponding to MPO existing as a functional dimer (Heim et al., 2007; Katoh et al., 2007). The product from MPO 4-aminobutinal spontaneously cyclizes to form the N-methylpyrrolinium ring *in vitro* and it is assumed this is also the case *in vivo*.

The biosynthesis of the pyridine ring moiety of nicotine has not been completely resolved. Nicotinic acid fed to tobacco roots was incorporated into nicotine, but it is not known if nicotinic acid directly or a metabolite derived from nicotinic acid is the species conjugated to N-methylpyrrolidine (Dawson, 1960; Leete and Liu, 1973). Nicotinic acid is a derivative of quinolinic acid. Quinolate is derived from the amino acid aspartate through activity of the consecutive enzymes: aspartate oxidase (AO), quinolate synthase (QS), and quinolate phospho-ribosyltransferase (QPT). *QS* mRNA levels increase with MeJA treatment, indicating coordinate expression with other nicotine biosynthetic genes (Kidd et al., 2006). QPT from tobacco shows increased activity with foliar wounding (Mizusaki et al., 1973; Saunders and Bush, 1979) as do other nicotine biosynthetic enzymes, but unlike PMT and MPO that are specific to nicotine biosynthesis, QPT is also involved in production of NAD⁺ for primary metabolism. There are two *QPT* genes in tobacco, only one of which is coordinately regulated by hormonal signals that also regulate nicotine biosynthesis (Sinclair et al., 2004). Tobacco plants with antisense *QPT* transgenes show reduced *QPT* mRNA levels and reduced nicotine accumulation levels, demonstrating that *QPT* is also an essential nicotine biosynthetic gene in tobacco (Xie et al., 2004).

Analysis of tobacco BY2 cell cultures has provided insights into the balance between nicotine and anatabine biosynthesis. BY2 cell cultures produce only a small amount of nicotine and instead predominately accumulate the related alkaloid anatabine (Goossens et al., 2003). Anatabine is formed by the conjugation of two pyridine rings, and is independent of the N-methylpyrrolidine intermediate. Analysis of the BY2 cells has shown low expression of MPO is responsible for the shift of alkaloid levels from

nicotine to anatabine (Shoji and Hashimoto, 2008) and that MPO has a direct role in nicotine biosynthesis. The fact that the alkaloid composition of BY2 cells is substantially different than that of intact plants emphasizes the importance of cell-type specific developmental cues in the proper regulation of pyridine alkaloid biosynthetic gene expression in tobacco.

The conjugation of pyridine and N-methylpyrrolinium salt remains a very poorly understood step in nicotine biosynthesis. A putative nicotine synthase activity was reported in cell-free extracts that conjugated nicotinic acid to the N-methylpyrrolinium salt producing nicotine (Friesen and Leete, 1990). However, multiple investigators have been unsuccessful in reproducing these results (J. Jelesko, personal communication). However, important insights about conjugation of the pyridine and N-methylpyrrolidine rings have come from investigations into a gene initially named *A622*. *A622* was identified in the same differential expression screen as *PMT* (Hibi et al., 1992; Goossens et al., 2003; Kidd et al., 2006; Jiang et al., 2009). *A622* tissue- and cell-specific expression profiles are very similar to those of *PMT*, implicating a role for *A622* in nicotine biosynthesis. The *A622* coding sequence predicts a protein with similarity to NADPH reductases (Hibi et al., 1994; Deboer et al., 2009; Kajikawa et al., 2009) although attempts to assign a specific biochemical activity to recombinant *A622* protein have not yielded any conclusive results (Hibi et al., 1994; Shoji et al., 2002). Despite the current lack of knowledge regarding the biochemical activity of *A622*, two groups recently demonstrated that reduced *A622* expression levels are correlated with both reduced nicotine accumulation and reduced accumulation of nicotine-related pyridine alkaloids (Deboer et al., 2009; Kajikawa et al., 2009). Studies in tree tobacco (*N. glauca*)

correlated the expression of A622-RNAi in plants and transgenic roots with the reduction in production of nicotine as well as the related alkaloids, anatabine and anabasine (Deboer et al., 2009). Studies with hairy root cultures and transgenic plants both showed a reduction in alkaloid accumulation in A622-RNAi lines. Thus, A622 appears to play some role in the condensation of pyridine and the N-methylpyrrolidine ring of nicotine and pyridine and other five- or six-membered rings of related alkaloids. Whether this role is direct (i.e. biosynthetic) or indirect (i.e. regulatory) still needs to be resolved.

Additional N-methylpyrrolinium derived alkaloids found in the *Solanaceae*

The tropane alkaloids are an important class of plant-derived anticholinergic compounds derived from the basic structure of the tropane ring produced in the Solanaceae species *Hyoscyamus*, *Atropa* and *Datura* (Ziegler and Facchini, 2008). Scopolamine and hyoscyamine are medicinal tropane alkaloids used in the treatment of motion sickness and various gastrointestinal disorders (Roberts and Wink, 1998; Schmeller and Wink, 1998). Calystegines, or nortropane alkaloids, are also derivatives of the tropane ring are more widely distributed, but found primarily in the Solanaceae and Convolvulaceae (Drager, 2004). Calystegines were first identified in 1990 and have demonstrated selective glucosidase inhibition activity (Drager, 2004).

Biosynthesis of the tropane ring from which tropane and nortropane alkaloids are derived involves the intermediate N-methylpyrrolinium. Therefore, both tropane alkaloid and calystegine biosynthesis have common biosynthetic steps with that of nicotine biosynthesis. The tropane ring is derived from putrescine and proceeds with the

enzymatic reactions of PMT and MPO. Therefore, early steps in the tropane biosynthetic pathway have common intermediates with the production of the N-methylpyrrolinium ring of nicotine. PMT, the first dedicated enzyme in N-methylpyrrolinium biosynthesis, has been cloned and characterized from *Hyoscyamus niger*, *Atropa belladonna*, and *Solanum tuberosum* in addition to tobacco (Suzuki et al., 1999; Stenzel et al., 2006; Teuber et al., 2007). MPO has not been cloned from tropane- or calystegine-producing species, but MPO enzymatic activity has been characterized in *H. niger* (Hashimoto et al., 1990). The N-methylpyrrolinium ring is purportedly condensed with acetoacetic acid to form hygrine, the precursor to tropane alkaloids and calystegines (Ziegler and Facchini, 2008).

Genetic Control of Nicotine Biosynthesis

Nicotine biosynthesis is also under genetic control. Low-nicotine varieties of Cuban tobaccos discovered in the 1930's were used in breeding programs to establish low nicotine varieties of domesticated tobacco (Heggstad, 1966; Legg et al., 1970). Genetic studies of near isogenic lines of a commercial burley tobacco Burley 21 (B21) and a low alkaloid Burley 21 (LA21) cultivar revealed two non-linked, semi-dominant genetic loci (*A* and *B*) are responsible for the reduced nicotine content observed in LA21 (Legg et al., 1969; Legg and Collins, 1971). The genetic loci, *A* and *B*, account for the two largest quantitative trait loci affecting nicotine levels; however, the *A* locus has a larger quantitative effect than the *B* locus. The original *a* and *b* mutant alleles are semidominant with synergistic effects on nicotine accumulation levels. However, the

double mutant *aabb* genotype does not influence other agronomic traits such as height, leaf number, or days to flower (Legg et al., 1970). The one exception is that mutant *aabb* plants are highly susceptible to insect damage, presumably due to decreased nicotine accumulation levels (Legg et al., 1970; Jackson et al., 2002). The association of the *A* and *B* loci with nicotine biosynthesis led one group to rename the *A* and *B* loci *NIC1* and *NIC2*, respectively (Hibi et al., 1994). The decreased nicotine in *aabb* is correlated with decreases in enzyme activity of PMT, MPO and QPT (Saunders and Bush, 1979; Hibi et al., 1994) and decreased mRNA transcript accumulation for *PMT*, *A622*, *QPT*, and *MPO* (Hibi et al., 1994; Riechers and Timko, 1999; Reed and Jelesko, 2004; Cane et al., 2005; Kidd et al., 2006; Katoh et al., 2007). These results suggest that decreases in nicotine biosynthesis correlated with the *A* and *B* loci are not due to mutations in individual nicotine biosynthetic genes but rather results from the coordinate down-regulation of nicotine biosynthetic genes.

Coordinate regulation of nicotine biosynthetic genes has proven to be of great value to researchers, allowing the use of molecular tools leading to the cloning and characterization of several nicotine biosynthetic genes. Comparative molecular analysis of *AABB* and *aabb* genotypes has been used to clone nicotine biosynthetic genes *PMT*, *A622* and *MPO* (Hibi et al., 1994; Katoh et al., 2007). Original observations of mutants at the *A* and *B* loci identified only affects nicotine biosynthesis while other agronomic traits were unaffected; however, at a molecular level the *A* and *B* loci affect the expression of many stress response genes in addition to nicotine biosynthetic genes (Kidd et al., 2006). Nevertheless, the *A* and *B* loci are useful tools for gene discovery purposes

in *N. tabacum*, which has limited resources in forward genetic screens. To date, the identity of the genes at the *A* and *B* loci are unknown.

Tissue and cell culture methods used to investigate nicotine physiology

Because nicotine biosynthesis is confined to roots, there are several methods focused on propagating tobacco roots in culture that are particularly effective for investigating nicotine biosynthesis. Root cultures provide a well-controlled environment to manipulate the nicotine-producing organ and experimentally determine the resulting changes in nicotine physiology. Root cultures also provide an economic advantage over growing whole plants as they require much less space, do not have specific light requirements, and can be cultured quickly.

The classical method of obtaining root tissue cultures involves the establishment of primary root cultures from the excision of roots from tobacco seedlings. These root cultures are dependent on the addition of the hormone auxin to the growth medium and are typically sufficient for studies in which a few short-term passages are needed (Solt, 1957). The excised root cultures retain the nicotine physiology of the parent plant, including responding to an induction of nicotine biosynthesis by hormonal elicitation (Solt, 1957; Haslam and Young, 1992; Kidd et al., 2006).

More recently, transgenic hairy root cultures (McLauchlan et al., 1993; Shoji et al., 2002) have become widely used for system in the study of nicotine biosynthesis. Hairy root cultures are transgenic roots resulting from the inoculation of tobacco leaves with *Agrobacterium rhizogenes* (Hamill et al., 1986; Hamill et al., 1990). The bacterial

transformation of tobacco leaf cells results in a metabolic shift within the transformed plant cell, initiating the production of hairy roots. These hairy roots contain a genetic copy of the parent plant with the inserted *Agrobacterium* Ri-DNA. Additionally, *Agrobacterium rhizogenes* can be genetically manipulated to co-transform a gene of interest harbored plant on a T-DNA binary plasmid that is also inserted into the plant genome along with the genes necessary to induce hairy root formation. Once established, these transgenic hairy roots can be excised from the leaf and will grow independent of original plant on culture medium. Tobacco hairy root cultures are genetically stable and grow independent of exogenous hormones, conduct nicotine biosynthesis, and respond to the elicitation of nicotine by the application of exogenous MeJA (Hamill et al., 1986; Hamill et al., 1990).

Researchers have also utilized a tobacco cell culture system for the study of tobacco alkaloids. Tobacco BY2 cells were derived from the Bright Yellow variety of *Nicotiana tabacum* and have been used as a model system to study many aspects of plant cell physiology. With that said, the appropriateness of BY2 cells for the study of nicotine biosynthesis is currently unclear. Unlike whole *N. tabacum* plants, BY2 cell cultures predominantly accumulate the alkaloid anatabine (Goossens et al., 2003; Shoji and Hashimoto, 2008). However, BY2 cells do respond to MeJA elicitation by increasing production of anatabine, suggesting they share some physiology with nicotine biosynthesis (Goossens et al., 2003). As mentioned above, the apparent shift from nicotine to anatabine production seems to be associated with insufficient production of the N-methylpyrrolinium intermediate due to inadequate expression of *MPO* enzyme activity (Shoji and Hashimoto, 2008). The shift in alkaloid composition in the

undifferentiated BY2 cells highlights the importance of developmental cues associated with differentiated cell types in determining the production of specific secondary metabolites.

Alkaloid and Nicotine Transport

Importance of alkaloid transport for mounting a chemical defense

Vascular plants are comprised of asynchronous tissues and organs that must communicate in order to form a functioning organism. Transport mechanisms within the plant are essential to the proper distribution of small molecules such as hormones, metabolites, nutrients and defense compounds. These methods include long-distance xylem transport from the roots to the shoots and source-sink transport from photosynthetic tissues through the phloem. In addition to these long-distance transport mechanisms, cell-to-cell movement of small molecules is required for local distribution and for the loading and unloading of the xylem and phloem (Buchanan et al., 2000). Local movement of small molecules can occur through both apoplastic and symplastic transport mechanisms (Robert and Friml, 2009). Movement of small molecules between adjacent cells can occur through symplastic transport, utilizing the plasmodesmata that exist between mature plant cells. Plasmodesmata are small channels linking the cytoplasm of one cell with the cytoplasm of adjacent cells, therefore allowing small molecules to pass directly from one cell to the next. In addition to the symplastic mode of transport, intracellular or apoplastic transport is also used to selectively move small molecules from one cell to another. Apoplastic transport involves the export of small molecules from one cell into the apoplastic space between cell walls. These molecules are

then selectively removed from the apoplastic space by transporter proteins present on the surface of the destination cell. These independent transport mechanisms are used collectively for the proper distribution of small molecules throughout the plant.

Several aspects of whole plant physiology suggest the long-distance transport of alkaloids requires multiple, independent transport steps. Both pyridine and tropane alkaloids are exclusively synthesized in the roots, yet these compounds accumulate in leaves and flowers where they function as a chemical defense. In *Nicotiana* species, nicotine is transported from the roots to the shoot via the xylem. While nicotine accumulates throughout the plant, it is not evenly distributed in all tissues. During the course of plant development nicotine accumulation levels change both between tissues and also within a given tissue over time (Ohnmeiss et al., 1997; Ohnmeiss and Baldwin, 2000). Nicotine preferentially accumulates in those tissues that at any given ontological stage will contribute the most to eventual plant fitness (i.e. seed production) and this distribution is in concert with the Optimal Defense Theory (Ohnmeiss and Baldwin, 2000). For example, young leaves accumulate relatively higher nicotine levels than do older leaves that are nearing senescence. However, as those young leaves mature they show decreased nicotine accumulation levels (Ohnmeiss et al., 1997; Ohnmeiss and Baldwin, 2000), suggesting that nicotine is moved away from those tissues into younger leaves or flowers. Thus, throughout plant development nicotine is mobilized from one tissue to another according to the Optimal Defense Theory. In the case of leaf mesophyll cells nicotine is predominately localized in the vacuole (Saunders and Bush, 1979). To achieve this dynamic redistribution of nicotine throughout the plant one must infer multiple nicotine transport steps including export from the biosynthetic cell(s) in the root,

loading of nicotine into root xylem, nicotine uptake into leaf or flower cells, and import into the vacuole.

Another important theme in alkaloid physiology is the apparent essential role of the trafficking of alkaloid biosynthetic intermediates between non-adjacent cell types in order to complete the biosynthesis of many plant alkaloids. Studies investigating the tissue-specific localization of several alkaloid biosynthetic genes indicate that many of these alkaloid biosynthetic pathways are not confined to any single cell type (De Luca and St Pierre, 2000; Facchini and Luca, 2008; Ziegler and Facchini, 2008). Instead, specific alkaloid biosynthetic enzymes are expressed in different, and often non-adjacent, cell types. Thus, these alkaloid biosynthetic pathways are cell non-autonomous processes in which intermediates produced in one cell type are released to the apoplastic space and then move to other differentiated cell types where they are taken up and metabolized to the next intermediate in the pathway.

An example of this cell type-specific gene expression is demonstrated in tropane alkaloid biosynthesis in *H. niger* (Nakajima and Hashimoto, 1999). Immunohistochemical analysis of *H. niger* roots shows that three enzymes responsible for biosynthesis of tropane alkaloids have distinct cellular expression patterns. Tropinone reductase I (TR-I) and tropinone reductase II (TR-II) modify tropinone into the stereoisomers tropine and pseudotropine, respectively. Hyoscyamine 6 β -hydroxylase (H6H) is responsible for conversion of hyoscyamine, the downstream product of TR-1, to scopolamine. TR-1 was localized to the endodermis and the outer cortex. TR-2 localized to the pericycle, endodermis, and inner cortical layers. H6H was found exclusively in the pericycle. The absence of TR-I from the pericycle where the downstream H6H is

exclusively found suggests trafficking of intermediates across cell layers is required for production of scopolamine (Nakajima and Hashimoto, 1999). Localization of earlier biosynthetic steps of tropane alkaloid biosynthesis has not been performed in *H. niger*. However, *Atropa belladonna* also produces tropane alkaloids and analysis of *PMT promoter:GUS* fusions in transgenic *A. belladonna* roots indicate that *PMT* expression localizes exclusively to the pericycle of *Atropa belladonna* (Suzuki et al., 1999). Assuming the same is true for the closely related *H. niger*, then one or more intermediates between N-methylputrescine and tropine must be trafficked between the pericycle and the endodermis or outer cortex.

Cell non-autonomous alkaloid biosynthesis is not restricted to tropane alkaloid biosynthesis. Similar differential cell type-specific localization of alkaloid biosynthetic enzymes involved in monoterpenoid indole alkaloid biosynthesis has been observed in *Catharanthus roseus* (St-Pierre et al., 1999). Thus, intercellular transport of alkaloid intermediates appears to be a common theme in the biosynthesis of diverse plant alkaloids. At this time there is still a poor understanding of which alkaloid intermediates are moving between cells and the properties of specific membrane transporters that are facilitating intermediate trafficking between cells.

Current knowledge of alkaloid transporters

Plants use two major categories of transporters to move metabolites across membranes, primary transporters and secondary transporters. Primary transporters require energy for transfer while secondary transporters utilize an established

electrochemical gradient. The major class of plant primary transporters is the ATP-Binding Cassette (ABC) transporters. Plant ABC transporters belong to a single, large superfamily composed of multiple subfamilies. For example, the Arabidopsis genome encodes over 120 predicted ABC members, comprising 12 subfamilies (Sanchez-Fernandez et al., 2001). Plant ABC transporters have been implicated in the transport of diverse phytochemicals such as terpenoids (Jasinski et al., 2001), auxins (Terasaka et al., 2005), flavones (Markus Klein et al., 2000), and alkaloids (Goossens et al., 2003a; Shitan et al., 2003; Otani, 2005). Thus, plant ABC transporters comprise a large and diverse superfamily of transporters that are implicated in the movement of small molecules throughout the plant. The large size of the ABC transporter family suggests ABC transporters may participate in the movement of many diverse plant metabolites, but currently the *in planta* substrates for only a small fraction of plant ABC transporters have been identified.

The general movement of alkaloids from the site of synthesis to the site of storage is well understood. However, the molecular mechanisms facilitating intercellular transport are poorly understood. The first alkaloid ABC transporter identified was in *Coptis japonica*, a producer of the isoquinoline alkaloid berberine, which is widely used as an antifungal drug. Initially, *C. japonica* cell cultures were characterized to have an ABC transporter mediated berberine uptake (Sakai et al., 2002). A gene encoding an ABC transporter of the multidrug resistance (MDR) subclass, CjMDR1, was cloned from *C. japonica* (Yazaki et al., 2001) and its expression profile was associated with the possible import of berberine (Yazaki et al., 2001; Shitan et al., 2003). Recombinant CjMDR1 has ATP-dependant berberine uptake activity when expressed in *Xenopus*

oocytes (Shitan et al., 2003). *In planta*, CjMDR1 immunolocalized specifically to the rhizome, the organ of concentrated berberine accumulation and was associated with the plasma membrane microsomal fraction (Shitan et al., 2003). Thus an ABC transporter, CjMDR1, is responsible for the uptake of berberine in the rhizome of *C. japonica*.

Secondary transporters use an established electrochemical gradient of protons or sodium ions to move other organic molecules across cellular membranes. ATPases in the plasma membrane (P-type ATPases) establish the electrochemical gradient between the apoplast and the cytoplasm and ATPases (V-type ATPases) and pyrophosphatases in the tonoplast establish the electrochemical gradient between the cytoplasm and the interior of the vacuole (Gaxiola et al., 2007). Secondary transporters have been shown to participate in the movement of primary and secondary metabolites such as flavonoids (Debeaujon et al., 2001; Marinova et al., 2007), purines (Gillissen et al., 2000), cytokinins (Burkle et al., 2003), and alkaloids (Otani, 2005; Morita et al., 2009). A representative family of secondary transporters is the multidrug and toxin extrusion (MATE) transporter family that was first identified in prokaryotes and subsequently found in all living organisms characterized. MATE transporters in prokaryotes are well studied and named for their ability to excrete multiple drugs and toxins. Characterization of MATEs in eukaryotes has trailed behind. Prokaryotic MATEs display a relatively low degree of specificity in the compounds they extrude and are considered general efflux pumps to remove toxins. The MATE family in *Arabidopsis* contains a relatively large gene family containing 56 members, while the human genome only contains two *MATEs*. In humans, *MATE1* is expressed in the kidney and liver and *MATE1* and *MATE2* are thought to function in the excretion of toxic organic cations from renal tubular cells and hepatocytes (Terada and

Inui, 2007). Characterization of MATE transporters from plants has only recently begun to elucidate the function of some of these transporters, exposing a wide diversity of substrates. MATEs have been associated with metal transport, detoxification, and aluminum tolerance in plasma membrane-localized transporters and in vacuolar accumulation of secondary metabolites such as flavonoids and alkaloids in tonoplast-localized MATE transporters (Yazaki et al., 2008). The diversity of compounds transported by the few MATEs that have been characterized make this class of transporters interesting with respect to metabolite transport in plants.

Recently, two different tobacco MATE-type transporters were shown to move nicotine from the cytoplasm into the vacuole (Shoji et al., 2008; Morita et al., 2009). Jasmonate Induced Alkaloid Transporter 1 (NtJAT1) was identified as having higher steady state expression levels in MeJA induced tobacco BY-2 cells compared to non-induced controls. NtJAT1 expression was observed in leaf, roots and stem tissue and NtJAT1 associated with the vacuolar membrane. NtJAT1 was shown to export nicotine when heterologously expressed in yeast. In proteoliposomes NtJAT1 transports the tobacco alkaloids, nicotine and anabasine, the tropane alkaloid, hyoscyamine, and the isoquiniline alkaloid, berberine, but not the flavonols, kaempferol and quercetin. Physiological studies of NtJAT1 *in planta* were not reported. The broad substrate recognition profiles together with vacuolar localization suggest that NtJAT1 may function in general toxic organic cation sequestration into the vacuole.

The second recently-identified MATE nicotine transporter is Multidrug and Toxic Compound Extrusion 1 (and NtMATE2). *NtMATE1* was identified in a fluorescent differential display screen where it exhibited higher mRNA expression levels in wild-

type tobacco roots than in low alkaloid-producing tobacco roots (Shoji et al., 2008). *NtMATE1* expression was observed only in roots and expression patterns mirrored those of the nicotine biosynthetic gene *PMT*, including increased expression with MeJA application. *NtMATE1 promoter:GUS* fusions displayed GUS staining only in the root, with the root tip staining strongly. Longitudinal and cross sections of the root showed GUS expression to be associated with the root cortex. Physiological studies with RNAi and overexpression mutants were performed. Overexpression of MATE1 in BY2 cells supplemented with either nicotine in the culture medium or with MeJA to induce endogenous alkaloid production resulted in an abnormal acidification of the cytoplasm, presumably due to the antiport of protons associated with the uptake of nicotine into the vacuole. Exogenous nicotine in the growth medium is known to retard tobacco seedling root growth (Baldwin and Callahan, 1993) and *NtMATE1/2-RNAi* seedling root growth was inhibited by exogenous nicotine in culture medium. Neither the reduction nor the overexpression of *NtMATE1* resulted in alterations in nicotine or related alkaloid accumulation. In addition to the transport of nicotine, yeast heterologously expressing NtMATE1 also transported the tropane alkaloids, hyoscyamine and scopolamine.

These two tobacco MATE transporters are the first reported cloned plant nicotine transporters. Both are located in the tonoplast and are thought to function in the import of nicotine from the cytoplasm into the vacuole. As neither of these transporters display a high degree of selectivity for nicotine, it is likely they act not in the specific sequestration of nicotine, but more as general transporters functioning in the sequestration of potentially inhibitory cations to the vacuole as part of a generalized detoxification physiology.

PUP family of transporters

Another family of secondary transporters is the Purine Uptake Permease (PUP) family. The PUP transporters were first identified and characterized in Arabidopsis and appear to be a plant-specific class of proton symporters. Arabidopsis contains at least 15 transporters of the *PUP* family, three of which have been characterized (Gillissen et al., 2000; Burkle et al., 2003; Cedzich et al., 2008). *AtPUP1* was the first PUP family transporter described in any species and was identified by the ability of AtPUP1 to complement growth of a yeast strain deficient in both adenine biosynthesis and uptake. Recombinant AtPUP1 protein expressed in yeast conferred adenine and cytosine uptake activity. Further biochemical characterization of AtPUP1 demonstrated the additional ability to also transport cytokinins, albeit at a lower affinity than adenine. AtPUP1 expression is localized to the hydathodes and the stigma of siliques (Gillissen et al., 2000; Burkle et al., 2003). AtPUP2 displays a lower affinity of adenine transport than AtPUP1 when expressed in yeast and also shows significant inhibition of adenine transport by cytokinins (Burkle et al., 2003). *AtPUP2* expression is closely correlated with the phloem of leaves. A third PUP transporter, AtPUP3, was localized to anthers, but was not shown to actively transport purines or cytokinins (Burkle et al., 2003). The exact physiological substrates and roles for AtPUP1, 2, and 3 have not been established.

The Jelesko laboratory previously identified a cDNA fragment encoding a PUP-like transporter from tobacco roots that was coordinately regulated with several known nicotine biosynthetic genes (Kidd et al., 2006). During the biochemical characterization

of AtPUP1, it was observed that this protein weakly recognized nicotine as a substrate (Gillissen et al., 2000). This led us to consider the possibility that the tobacco PUP-like homolog might encode a nicotine-specific uptake permease (NUP1). Recombinant NUP1 expressed in yeast conferred nicotine uptake activity (unpublished results, collaborator Angus Murphy). Moreover, this nicotine uptake activity was not efficiently competed by tropane or other pyridine alkaloids, nor was it competed by the cytokinin kinetin. Thus, NtNUP1 is hypothesized to participate in some role of nicotine uptake in tobacco roots.

Overview of Research

The objectives of this dissertation research were to investigate protein - protein interactions predicted by a hypothesized nicotine metabolic channel and to further characterize the potential role of NUP1 in nicotine transport. These objectives were approached by the following specific aims:

- I. Identify tobacco proteins that interact in the yeast two-hybrid assay with known nicotine biosynthetic enzymes PMT, MPO and SAHH (Chapter 2).

- II. Use the complementary method of co-immunoprecipitation to explore the interaction between PMT, MPO and SAHH enzymes in both a native tobacco root extract in an *in vitro* cell-free system (Chapter 2).

III. Characterize nicotine accumulation in *NUP1-RNAi* plants and hairy root cultures
(Chapter 3).

IV. Determine the subcellular localization of NUP1 in tobacco BY-2 protoplasts
(Chapter 3).

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CHAPTER II

Investigation of Protein - Protein Interactions Among N-methylpyrrolidine Biosynthetic Enzymes in *Nicotiana tabacum*.

Abstract

The predominant alkaloid of *Nicotiana tabacum* is the pharmacologically-active compound nicotine. Nicotine is comprised of two asymmetrical rings, a quinolate-derived pyridine ring and a putrescine-derived N-methylpyrrolidine ring. The first committed step in N-methylpyrrolidine ring biosynthesis is the enzymatic methylation of putrescine to N-methylputrescine by putrescine N-methyltransferase (PMT). N-methylputrescine is then enzymatically oxidized by N-methylputrescine oxidase (MPO) to form 4-aminobutanal. This intermediate is believed to spontaneously cyclize to form the N-methylpyrrolidine ring of nicotine. Previous results in the Jelesko laboratory indicate that the nicotine biosynthetic enzyme MPO, either directly or indirectly associates with the SAM cycle enzyme S-adenosylhomocysteine hydrolase (SAHH) in tobacco root extracts. The discovery of an interaction between MPO and SAHH led to the hypothesis that a multienzyme complex mediates the biosynthesis of the N-methylpyrrolidine moiety of nicotine. This hypothesized complex was believed to include the enzymes SAHH, MPO, and PMT that together would form a metabolic complex. The hypothesized role of the SAHH association with the nicotine biosynthetic enzymes PMT and MPO is to detoxify the methyltransferase inhibitor S-adenosylhomocysteine (SAH) that is produced by the SAM-dependent PMT activity.

In order to experimentally corroborate these initial findings, several independent experimental methods were used to investigate the hypothesized protein - protein interactions between SAHH, MPO, and PMT. Co-immunoprecipitation experiments were performed using both native tobacco root cell free extracts and *in vitro* produced recombinant SAHH, MPO, and PMT proteins. In addition, yeast two hybrid (Y2H) assays were carried out in an effort to obtain evidence for the hypothesized metabolic channel. Y2H assays were performed both as directed assays to detect interactions between SAHH, PMT, and/or MPO and also as an open-ended library screen to find additional tobacco root proteins interacting with the hypothesized multienzyme complex. This report details the results of these studies that together did not provide further evidence supporting a nicotine metabolic channel. A discussion of the limitations of these experimental approaches and of the challenges inherent to the investigation of metabolic channels is presented.

Introduction

Nicotiana tabacum is an important agricultural crop in the south central United States, grown primarily for its nicotine-containing leaves used for their mood-altering properties. In 2008, tobacco crops in the United States were valued at over 1.4 billion dollars, with 90% of production occurring in the states of North Carolina, Kentucky, Tennessee, Virginia and South Carolina (United States Department of Agriculture, 2009).

Nicotine is a small molecule chemical categorized as an alkaloid. Alkaloids are a large and diverse class of cyclic nitrogen-containing chemical compounds that are predominantly found in plants, many of which have potent biological activities when ingested by animals. Thus, the accumulation of alkaloids *in planta* can serve as chemical defenses against herbivory. Like nicotine, several plant-derived alkaloids are physiologically-active when consumed by humans. Alkaloids such as caffeine, cocaine, opium, strychnine, and berberine are used throughout the world for their pharmacological properties (Schmeller and Wink, 1998). The altered physiological effects corresponding to alkaloid use are wide ranging and dependent on the specific alkaloid consumed. Medicinal plant-derived alkaloids currently in use include quinine (anti-malaria), vinblastine and vincristine (white-blood-cell cancer), morphine (pain relief), atropine (pupil dilation) and scopolamine (motion sickness) (Roberts and Wink, 1998; Schmeller and Wink, 1998; Wink, 1998).

Nicotine accumulation in tobacco acts as a defense against herbivory by affecting the nervous system of insects and other animals (Silvette et al., 1962; Yamamoto, 1970). In humans nicotine has effects that are dose-dependent, with consumption of small doses resulting in addictive mood-altering effects while ingestion of large doses is lethal (Silvette et al., 1962). Nicotine is produced exclusively below ground in the roots of tobacco (Dawson, 1942; Dawson,

1942; Tso, 1972) and is subsequently translocated through the xylem (Baldwin, 1989) and delivered to the leaves and flowers (Baldwin and Karb, 1995; Ohnmeiss and Baldwin, 2000).

Nicotine is comprised of two asymmetric rings, the five-member N-methylpyrrolinium ring, and the six-member pyridine ring. The N-methylpyrrolinium intermediate is not exclusive to nicotine biosynthesis. In some other members within the Solanaceae, N-methylpyrrolinium can be acetoacetylated to form tropinone. Tropinone then gives rise to both the classes of the nortropane (calystigenes) alkaloids and the tropane alkaloids. Tropane alkaloids such as scopolamine and hyoscyamine are important medicinal pharmaceuticals for the treatment of heart attack and motion sickness, and for use in pupil dilation (Wink, 1998). In addition, atropine is the only available antidote to accidental organophosphate fertilizer poisoning and Sarin nerve gas poisoning during unconventional warfare (Wills, 1955; Cullumbine, 1957; Orma and Middleton, 1992; Kulling and Persson, 1997; Weinbroum, 2004; Lynch, 2005). Characterization of N-methylpyrrolinium ring biosynthesis in tobacco will not only increase our knowledge of nicotine biosynthesis but will likely also increase our understanding about the early steps of tropane alkaloid biosynthesis as well. Thus, further elucidation of the molecular details and mechanisms of N-methylpyrrolinium biosynthesis should broaden the capability for metabolic engineering of these important medicinal alkaloids.

Enzymes of the N-methylpyrrolinium biosynthetic pathway

The first committed step in the biosynthesis of the N-methylpyrrolinium ring is the N-methylation of the diamine putrescine resulting in the production of N-methylputrescine (Figure 1.1). This reaction is catalyzed by putrescine N-methyltransferase (PMT) and involves S-

adenosylmethionine (SAM) as the methyl donor (Mizusaki et al., 1971). *PMT* is thought to have evolved from spermidine synthase and appears in the genome of *N. tabacum* in at least five copies (Hibi et al., 1994; Hashimoto et al., 1998; Riechers and Timko, 1999). *PMT* transcripts and *PMT* enzyme activity is associated exclusively with the root (Hibi et al., 1994; Walton et al., 1994). Gene silencing of *PMT* in tobacco results in plants with drastically-reduced nicotine levels (Chintapakorn and Hamill, 2003; Steppuhn et al., 2004), demonstrating that *PMT* is an essential nicotine biosynthetic gene.

The methylation of putrescine by *PMT* requires the methyl donor SAM. Upon *PMT* catalyzed transfer of the methyl group, SAM is converted to S-adenosylhomocysteine (SAH). SAH is chemically similar to SAM and will readily bind to the active site of most SAM-dependent methyltransferases functioning as a competitive inhibitor. Characterization of *PMT* from *Hyoscyamus albus* and *Datura stramonium* established SAH inhibited *PMT* activity (Hibi et al., 1992; Walton et al., 1994) and in *Hyoscyamus albus* SAH was shown to be a competitive inhibitor of SAM but not putrescine (Hibi et al., 1992). The hydrolysis of SAH to homocysteine and adenine by S-adenosylhomocysteine hydrolase (SAHH) mitigates the potential accumulation of inhibitory intracellular SAH pools. This establishes SAHH as a potential accessory enzyme for nicotine biosynthesis, even though it is not directly responsible for the structural synthesis of nicotine.

Adequate intra-cellular SAM pools are required for SAM-dependent methyltransferase activity. Intracellular SAM pools are regenerated through a series of enzymatic reactions known collectively as the SAM cycle (Figure 1.1). In the first step of the SAM cycle, SAHH hydrolyzes SAH into homocysteine and adenine. SAHH was mentioned previously as the enzyme responsible for hydrolyzing SAH, the competitive inhibitor of all SAM-dependent methylation

reactions. Thus SAHH plays a dual role as both a SAM recycling enzyme and a SAH “detoxifying” enzyme. Following the conversion of SAH to homocysteine and adenine, methionine synthase (MS) converts homocysteine to methionine. Then methionine is converted to SAM by SAM synthetase (SAMS). It should be mentioned that the SAM cycle is not exclusive to N-methylpyrrolinium biosynthesis, but rather is an important conserved cellular process.

The second step of N-methylpyrrolinium biosynthesis involves the oxidative deamination of N-methylputrescine producing 4-methylaminobutanal (Figure 1.1). MPO was recently cloned from *N. tabacum* and shown to be part of a small multi-gene family, with five to six MPO-like genes identified (Heim et al., 2007; Katoh et al., 2007). *MPO* transcripts are found exclusively in roots and transcript levels increase with auxin removal from root cultures (Heim et al., 2007) and with MeJA elicitation in plantlets (Katoh et al., 2007). MPO can oxidize a range of diamines, although the preferred substrate is N-methylputrescine (Mizusaki et al., 1972; Hashimoto et al., 1990; Walton and McLauchlan, 1990; Heim et al., 2006; Katoh et al., 2007). MPO requires a copper cofactor (Mizusaki et al., 1972) and displays the characteristics of a quinoprotein (Davies et al., 1989). The predicted size of the MPO polypeptide is 89 kDa and recombinant MPO was estimated at between 130-172 kDa, corresponding to a functional dimer (Heim and Jelesko, 2004; Katoh et al., 2007).

MPO was purportedly purified to homogeneity from tobacco root extracts as a 54 kDa subunit protein for production of MPO specific antiserum (McLauchlan et al., 1993). The resulting antiserum was subsequently shown to specifically bind to the SAM recycling enzyme SAHH (Heim and Jelesko, 2004) rather than the expected MPO. Nevertheless, immunoprecipitation experiments show depletion of MPO enzymatic activity by

immunodepleting SAHH from tobacco root extracts (McLauchlan et al., 1993; Heim and Jelesko, 2004). These results suggest a physical association between the enzymes SAHH and MPO. The association of SAHH with MPO does not need to be a direct interaction, but could be part of a larger nicotine metabolic complex comprised of associating nicotine biosynthetic enzymes. The association of SAHH with a nicotine metabolic complex is potentially advantageous when one considers that a methyltransferase is involved in the formation of the substrate used by the MPO enzyme.

Multienzyme complexing in nicotine biosynthesis

The complexing of related enzymes into functional metabolic channels is a phenomenon that has been demonstrated in plant metabolic pathways such as glycolysis and flavonoid biosynthesis (Ovadi, 1991; Spivey and Ovadi, 1999; Winkel, 2004). The association of metabolic enzymes within a particular metabolic pathway creates an efficient means to hand metabolic intermediates from one enzyme to the next in amounting to a “molecular bucket brigade.” A multienzyme complex effectively reduces the potential inefficiencies related to the random diffusion of metabolic intermediates when moving from one dispersed enzyme to the next. Likewise, metabolic channeling also provides a mechanism to minimize the availability of potentially competing substrates to enzymes.

The inclusion of PMT in a theoretical nicotine metabolic complex including MPO and SAHH could be physiologically beneficial to the plant. PMT would present the metabolic intermediate N-methylputrescine directly to MPO, thereby increasing the efficiency of these combined biosynthetic steps. However there is also a potential disadvantage to the inclusion of

PMT into a metabolic channel. SAH is a methyltransferase inhibitory intermediate produced during all SAM-dependent methylations, including the methylation of putrescine by PMT. The accumulation of SAH to high intra-cellular concentrations or high local concentrations within a metabolic channel will result in competitive binding of SAH to the SAM binding site of PMT. The binding of SAH to PMT would inhibit further methylation of putrescine and greatly reduce the capacity for nicotine biosynthesis. However, the inclusion of SAHH into a nicotine multienzyme complex would provide a means to locally hydrolyze the SAH produced from the methylation of putrescine.

There is documented evidence that SAHH from other organisms participates in protein - protein interactions. Tobacco SAHH participates in a protein complex that binds cytokinins (Mitsui and Sugiura, 1993). Because of this, SAHH was first classified in tobacco as a cytokinin binding protein. SAHH in *Xenopus* eggs is nuclear localized and interacts *in vivo* and *in vitro* with a methyltransferase responsible for methylation of the 5' guanine cap of mRNA (Radomski et al., 2002). The association of SAHH with the *Xenopus* methyltransferase suggests that SAHH may facilitate other methylation reactions by reducing localized SAH concentrations. Additionally, yeast two hybrid assays using a library of rice genes aimed at identifying cell cycle interacting proteins showed a truncated SAHH interacting with three other proteins, a receptor kinase, a putative transcription factor, and a KNOX family homeobox domain containing protein (Cooper et al., 2003).

Experimental approaches to identify and characterize protein - protein interactions

Protein - protein interactions occur in many cellular processes and can range from identical polypeptide subunits associating to form a functional enzyme to large multi-protein complexes possessing multiple enzymatic processes cooperating as a molecular machine. The stability of these interactions also has great variability. Some protein complexes form very stable structures while others are transient and appear to form in relation to specific intra-cellular conditions (An et al., 2008; Kurepa and Smalle, 2008).

Protein - protein interactions have been shown to be integral to many cellular processes. The 26S proteasome is a large multisubunit, multicatalytic protease involved in protein degradation. The individual protein subunits comprising the proteasome interact to form a stable functional complex of over 2000 kilodaltons (kDa) (Kurepa and Smalle, 2008). Multiple biosynthetic enzyme complexes are known to form and are thought to increase metabolic efficiency by decreasing the diffusion of intermediates, regulating branch point competition for substrates, and sequestering potentially harmful intermediates (Winkel, 2004). These metabolic channels are comprised of independent enzymes functioning in the coordinated biosynthesis of an important metabolite. Characterized metabolic channels in plants have demonstrated this type of enzymatic organization contributing to both primary and secondary metabolism. An example of metabolic channeling in primary metabolism is among the enzymes of the Calvin cycle (Anderson et al., 1995; Anderson et al., 1996; Wang et al., 1996). Examples of metabolic channels that contribute to secondary metabolism include the enzymes of phenylpropanoid biosynthesis (Burbulis and Winkel-Shirley, 1999; Rasmussen and Dixon, 1999; Winkel, 2004).

The variability inherent in the size, shape, and kinetic behavior of individual proteins and protein associations combined with the limitations of current *in vivo* and *in vitro* methodologies make the detection of protein - protein interactions challenging. At this time, no one approach is sufficient for unequivocally demonstrating specific protein - protein interactions, instead results from complementary methods are used to provide evidence for the existence of protein assemblies. Some of the more widely used techniques are briefly discussed here.

Co-immunoprecipitation

Co-immunoprecipitation is a biochemical approach for detecting of protein - protein interactions. This method involves using antigen-specific antibodies (usually attached to an insoluble matrix) to precipitate a protein of interest from a complex mixture of soluble proteins in cell free extracts generated from disrupted cells (Ausubel, 1989). The precipitated material is then analyzed for associated or “co-immunoprecipitating” proteins. This method is often used when evidence of protein interaction in the native tissue is desired. The method requires the availability of antibodies specific to the desired immunoprecipitated protein and is thereby labor intensive, but the advantage is the detection of proteins that have been produced and post-translationally processed in the native system.

A modification of the traditional co-immunoprecipitation procedure uses epitope tagged recombinant proteins produced in native or *in vitro* expression systems. Development of epitope tags, or short protein sequences to which commercially available specific antibodies are easily obtainable offer the possibility to probe the epitope tagged recombinant proteins expressed in the native system. Using an epitope tagged approach negates the need to develop a protein of interest specific antiserum. An additional aspect of this method often employed by researchers is

to express the protein of interest in the native system with constitutive promoters increasing the levels of the proteins of interest. Increasing the expression levels of the specific protein allows for easier detection of the proteins of interest. The disadvantage of this approach is that the intracellular protein homeostasis has been altered by the increased production of the protein of interest. Co-immunoprecipitations from *in vitro* systems couple the production of proteins in a cell-free extract with targeted immunoprecipitation. This method provides a simple matrix and relatively high concentrations of the proteins of interest.

Physical methods for assessing protein – protein interactions

In vitro methods for the identification and characterization of protein - protein interactions have also been developed. For example, surface plasmon resonance (SPR) allows for the direct measurement of protein - protein binding *in vitro* (Smith and Corn, 2003). In this method a protein or peptide is immobilized on a solid surface and the potential binding partner is carried across the surface of the immobilized protein in a mobile phase solution. Conformational changes in the immobilized protein occurring from interaction with the binding partner will alter the resonance of the plasmon, thereby indicating a protein - protein interaction. Slight changes in the plasmon can be detected; therefore SPR is a sensitive method for detection of protein - protein interactions and can also provide dynamic information on interaction kinetics and strength.

Microcalorimetry is another *in vitro* technique used to characterize protein - protein interactions by measuring changes in heat energy associated with protein - protein interactions (Leavitt and Freire, 2001). The advantage of both SPR and microcalorimetry is that kinetic

details such as binding constants and reaction stoichiometry can be assessed. The downside to these *in vitro* methods is that they are dependent on large amounts of pure protein in a native confirmation. The purification of sufficient amounts of native protein is time consuming and purification conditions must be determined empirically for each protein of interest. These methods allow for measurements based upon interactions between isolated proteins (or protein domains), but do not replicate the complex nature of intracellular dynamics.

Reporter gene methods to evaluate protein – protein interactions

Heterologous expression systems are often employed for the detection of specific protein - protein interactions. These systems use a non-native organism to produce a recombinant protein of interest and then couple specific protein - protein interactions with the expression of a reporter gene. Heterologous two-hybrid transcriptional activators were originally developed in yeast (*S. cerevisiae*) (Chien et al., 1991) with a comparable *E. coli* system (Joung et al., 2000) now available. In general these systems provide a means for establishing evidence of interaction between heterologous proteins. These systems can be used to explore direct relationships between two proteins of interest or to use one protein of interest to screen a specific cDNA library for interactions. The choice of yeast or *E. coli* is dependent upon the research being conducted. *E. coli* based systems provide a greater efficiency for library screens due to a shorter life cycle and higher transformation efficiency, but are limited to the protein processing machinery of a prokaryotic system (Joung et al., 2000). Yeast-based systems are more time consuming and tedious to use, but provide a eukaryotic system for protein processing (Fields and Song, 1989).

Fluorescence based methods to evaluate protein – protein interactions

Recently developed *in vivo* methods based on the use of fluorescence proteins are opening new avenues for the detection of protein - protein interactions. In comparison to *in vitro* methods and heterologous systems, fluorescent technologies allow for the viewing of fluorescently-tagged proteins in living cells that often provides subcellular resolution. Fluorescent Resonance Energy Transfer (FRET) is a method used *in vivo* to confirm close spatial relationship between two proteins (dos Remedios et al., 1987). FRET is used in the study of protein - protein interactions providing evidence that two proteins of interest are physically near one another. The methodology of FRET uses a set of fusion proteins, whereby protein of interest A is fused with a donor fluorophore (typically cyan fluorescent protein) and the hypothesized interacting protein of interest B is fused with an acceptor fluorophore (typically yellow fluorescent protein) (Vogel et al., 2006). The complementary donor/acceptor fluorophores are chosen for their spectral qualities; specifically that the donor can be excited independently of the acceptor and that the donor emits light at a wavelength that can excite the acceptor. The donor molecule is excited by a specific wavelength of light and will emit light at a predetermined wavelength. If the acceptor is in close proximity to the excited donor, the acceptor fluorophore will become excited and emit light at a second predetermined wavelength. However, if the acceptor is not in close proximity to the donor it will not become excited and will not emit light at the second wavelength. In this method, visualization of light at the wavelength corresponding to the emission spectrum of the acceptor fluorophore demonstrates the proteins of interest are physically near one another in the living cell. Although this method does not guarantee proteins

are interacting, the sensitivity of the procedure allows for detection of FRET pairs residing between 1-10 nm of one another.

Variations of the original FRET method have been developed to further assist in the detection of protein - protein interactions. Bimolecular fluorescence complementation (BiFC) or split-fluorescent protein analysis is based upon the separation of a single fluorescent protein into two domains. Neither domain is capable of producing a fluorescent signal alone, but when the independent domains are attached to one of a pair of interacting proteins the fluorescent protein will reconstitute (Walter et al., 2004). BiFC requires an interaction between the proteins of interest to reconstitute a functional reporter and the viewing of a single fluorescent protein alleviates the complementary spectral requirements required by the acceptor and donor fluorophores of FRET. Another variation on FRET is Bioluminescence Resonance Energy Transfer (BRET). BRET uses luciferase, a biological enzyme, to emit light and excite an acceptor fluorescent protein (Subramanian et al., 2004). As with FRET, luciferase is fused to one member of the interacting protein pair and an acceptor fluorophore (GFP or YFP) is fused to the other hypothesized interacting protein. When the proteins are in close proximity, the bioluminescent photons emitted from the luciferase enzyme will excite the acceptor fluorophore. The advantage of BRET is that no external light is needed for activation of the fluorophore, thereby eliminating known problems with fluorescent microscopy such as photobleaching, fluorescence bleed-through, and sample autofluorescence.

Research objectives and rationale for experiments described in Chapter 2

The objectives of this dissertation project were to explore protein - protein interactions among nicotine biosynthetic enzymes hypothesized to form a nicotine metabolic channel. The supposition of a metabolic channel was based upon findings that an antiserum specific for the SAM recycling enzyme SAHH is capable of immunodepleting MPO enzyme activity from a cell free tobacco root extract. The hypothesized metabolic channel would include not only SAHH and MPO, but also the first committed nicotine biosynthetic enzyme, PMT. The physiological role of a metabolic channel would be to increase the metabolic efficiency of the defense compound nicotine by bringing the biosynthetic enzymes PMT and MPO as well as the SAM recycling enzyme SAHH into close proximity in order to both detoxify the potentially harmful intermediate SAH and to decrease the diffusion of N-methylputrescine between subsequent pathway enzymes PMT and MPO.

The immunodepletion of MPO by an SAHH antiserum suggests an association between MPO and SAHH, but does not give further indication of how these enzymes may be interacting. The interaction could be direct with MPO and SAHH forming an enzyme complex, or the interaction could be indirect with structural proteins or additional enzymes acting as bridging proteins. To further characterize the MPO and SAHH relationship and to explore the expanded hypothesis of a nicotine multienzymatic complex, we chose to use both the yeast two hybrid assay and co-immunoprecipitation assays. As the predominant goal of this research was to characterize protein - protein interactions in a system where only indirect evidence existed, we chose methods that required little specific knowledge regarding the nature of the interactions. The yeast two hybrid allows for both direct testing of our hypothesized metabolic channel members and an open-ended screen of potential interactors from a specific cDNA library. Co-IP experiments were chosen as a complementary method to both replicate and expand upon the

original immunodepletion results. Additionally, preparation for co-IP experiments will result in the production of specific antisera to SAHH, MPO and PMT.

Using a combination of both the yeast two hybrid assay and the co-immunoprecipitation technique, I did not obtain convincing evidence for either direct or indirect protein - protein interactions among SAHH, MPO, and/or PMT. These experiments were well controlled and suggest that under the conditions tested, these three proteins do not interact. Technical and theoretical challenges to the investigation of presumed metabolic channels will be discussed.

Materials and Methods

Media and Growth Conditions

Media used for yeast growth was prepared as described in the Yeast Protocols Handbook (Clontech Laboratories, 2001). Yeast Peptone Dextrose (YPD) and Yeast Peptone Dextrose Adenine (YPDA) were used for growth of yeast under non-selecting conditions. Synthetic dropout (SD) minimal medium was prepared with appropriate dropout solutions to obtain selection media. SD media lacking tryptophan SD(-Trp) was used for selection of yeast containing plasmid pLP-GBKT7. SD lacking leucine, SD(-Leu), was used for selection of yeast containing plasmid pLP-GADT7 or pDNR-GADT7. SD(-Leu,-Trp) was used for selection of mated yeast containing both bait and prey plasmids. SD/-Leu,-Trp,-Histidine (SD-(Leu,-Trp,-His) and SD/-Leu,-Trp,-His-,Adenine (SD-(Leu,-Trp,-His,-Ade) was used to select for yeast in interaction screening assays.

Liquid cultures were grown at 30°C and 250 rpm except for yeast mating cultures grown at 30°C and 50 rpm. Liquid cultures were typically grown overnight, but if sufficient growth was not observed the growth time would be extended until dense growth was observed. Growth on solid media was done at 30°C with all plates wrapped in Parafilm (Pechiney Plastic Company, Chicago, IL) to avoid excessive dehydration of the solid media. Library screens were allowed to grow 21 days after plating and pair-wise screens were allowed to grow 14 days after plating.

Yeast two-hybrid bait constructs and prey library

Bait and prey constructs for SAHH, MPO and PMT were prepared previously by John Jelesko. The bait constructs were prepared as GAL4-binding domain fusions in plasmid pLP-GBKT7 (Clontech, Mountain View, CA) and transformed into haploid yeast strain AH109. The resulting yeast strains, including an empty vector control, were selected on SD(-Trp) and will be referred to as the protein of interest-binding domain (SAHH-BD, PMT-BD, MPO-BD, Empty-BD). The prey constructs were prepared as GAL4-activation domain fusions in plasmid pLP-GADT7 (Clontech, Mountain View, CA) transformed into yeast haploid strain Y187. The resulting yeast strains, including an empty vector control, were selected for on SD(-Leu) and will be referred to as the protein of interest-activation domain (SAHH-AD, PMT-AD, MPO-AD, Empty-AD).

Bait constructs were tested for the ability to auto-activate reporter genes by plating transformed yeast on SD(-Trp,-His,-Ade). The yeast containing bait constructs did not display intrinsic transcriptional activation properties verifying the constructs were acceptable for use as bait in the Y2H screen.

To address the common problem of basal *HIS3* reporter gene leakiness; transformed Y187 yeast strains were plated on SD(-Leu,-His) media containing various concentrations of 3-amino-1,2,4-triazole (3-AT) (Sigma #A-8056, Sigma-Aldrich, St. Louis, MO). 3-AT is a competitive inhibitor of the *HIS3* gene product and serves to inhibit basal yeast growth due to low basal levels of *HIS3* gene expression. The addition of 2.5 mM 3-AT was found to be adequate for all of our transformed AD constructs to inhibit yeast growth on selection media.

A custom yeast GAL4-activation domain library was manufactured at Clontech (Mountain View, CA) using mRNA extracted from *Nicotiana tabacum* cv. B21 roots grown in culture. The library was cloned into plasmid pDNR-GADT7-Rec (Clontech, Mountain View, CA) and

transformed into haploid yeast strain Y187 containing 2.4×10^8 cfu/ml. The library was received in 1 ml aliquots and stored at -80°C .

Pair-wise Screens of SAHH, PMT and MPO

Yeast two-hybrid screens were performed in all pair-wise combinations including yeast strains containing empty vectors (i.e. SAHH-BD was mated with SAHH-AD, PMT-AD, MPO-AD and Empty-AD). Yeast harboring both the desired BD and AD constructs were generated by yeast mating (Yeast Protocols Handbook), initial selection for on SD(-Leu,-Trp), subsequent streak isolation on SD(-Leu,-Trp), after which a single colony was chosen as a representative.

All pair-wise combinations were investigated for protein - protein interaction by growing mated cultures in SD(-Leu,-Trp) to a density of $\text{OD}_{600}=1.0$. Five serial dilutions at 10x each were prepared from the normalized cultures and $10\mu\text{l}$ of each dilution was spotted onto SD(-Leu,-Trp,-His,-Ade) for detection of interaction and SD(-Leu,-Trp) for detection of viability.

Screens of the yeast two-hybrid library

Library screens to identify proteins interacting with SAHH, PMT and MPO were performed basically as directed in the Clontech Custom Library Handbook (Clontech Laboratories, 2001). In brief, the haploid yeast strain containing the desired bait construct was grown in 100 mL of SD(-Leu,-Trp) media overnight, centrifuged to pellet and resuspended in the residual liquid. Yeast cells were counted to ensure cell density of $> 1.0 \times 10^9$. A 1 mL aliquot of the yeast cDNA library was added to the bait culture and grown in approximately 50 ml 2x YPDA + Kanamycin ($50\mu\text{g}/\text{ml}$) overnight at 30°C at 30 rpm. This mating mixture was

centrifuged at 1000 x g for 10 minutes and the pellet was resuspended in 10 ml 0.5x YPDA + Kan. The entire mating mixture was plated at 200 µl per 150 mm Petri plate on SD(-Leu,-Trp,-His,-Ade) and plates were wrapped with Parafilm and incubated at 30°C for 21 days.

Yeast colonies appearing on selection plates were streaked on SD(-Leu,-Trp,-His,-Ade) and colonies isolated to ensure a reproducible growth phenotype. Liquid cultures grown in SD(-Leu,-Trp,-His,-Ade) were used for plasmid rescue from positive colonies. Plasmid rescue was performed by crude yeast plasmid preps carried out by the method of Robzyk and Kassir. To characterize the cDNA insert responsible for the positive yeast growth, yeast plasmid preps were amplified by pDNR-GADT7-Rec specific PCR using primers oJGJ197 (CCATGGAGTACCCATACGACGTACCAGATTACGCTCATA) and oJGJ198 (TGGTGAGAATCCAAGCACTAGTCATACCAGGATCTCCTA). Amplification products were then subjected to restriction enzyme digests using *AluI* and the digest pattern was analyzed by TAE buffer agarose gel electrophoresis (Ausubel, 1989). Positives were grouped together based upon restriction enzyme digestion pattern and a single representative clone of each pattern was chosen for further characterization.

Plasmids of interest were transformed into *E. coli* strain DH5α to obtain enough plasmid DNA for yeast transformation and DNA sequencing. Transformed cells were plated on LB (Ausubel, 1989) Chloramphenicol (34µg/ml) and incubated at 37°C overnight. A single colony of transformed *E. coli* was selected and grown in LB Chloramphenicol at 37°C overnight and plasmid DNA was isolated and purified using QIAprep Spin Miniprep Kit (QIAGEN Inc., Valencia, CA).

Recovered plasmids were transformed into yeast strain Y187 to test for auto-activation and repeated interaction. Plasmid transformation into yeast was performed as described in (Gietz and Schiestl, 2007) and selection performed on SD(-Leu). Yeast mating was performed as described above with both the original bait and with empty-BD. The mating mixtures were plated on SD(-Leu,-Trp) and SD(-Leu,-Trp,-His,-Ade), incubated at 30°C and observed for growth. Growth on SD(-Leu,-Trp) signified the viability of the mating cultures. Growth on SD(-Leu,-Trp,-His,-Ade) when mated with both empty-BD and bait-BD signified auto-activation. Growth on SD(-Leu,-Trp,-His,-Ade) only when mated with bait-BD signified true interaction, whereas the absence of growth on SD(-Leu,-Trp,-His,-Ade) with bait-BD signified a false-positive.

Isolated plasmids capable of recapitulating the yeast two-hybrid interaction were sequenced using primers oJGJ197 and oJGJ198 at the Virginia Bioinformatics Institute, Blacksburg, VA. Sequence files were analyzed using the LaserGene software package (v 7.2.1, DNASTAR, Inc, Madison, WI). The resulting sequence was entered in a Basic Local Alignment Search Tool X (BLASTX) (Altschul et al., 1997) for comparison against the non-redundant protein sequences (nr) database at NCBI for determination of gene homology.

Screening for DC1 domain containing proteins

Yeast two-hybrid screens are known to be prone to producing false positive interactions. As screens progressed a class of proteins containing a DC1 domain was identified as being responsible for the majority of the false positives. Sequences from four isolated positive plasmids identified as having homology to DC1 domain containing proteins were aligned and

primers were designed to amplify 300 bp region of the inserts. Primers SBH001 (GATCTYCATGTGATCTGTGC) and SBH002 (MATAGKWGSCTGCTGGAATTG) were used in screening for DC1-like clones using PCR. PCR conditions using 1 μ l yeast plasmid prep as template were 94°C 2:00 for 1 cycle; 94°C 0:30 sec; 55° 0:30 sec; 72°C 1 min for 35 cycles; and 72°C 2 min for 1 cycle. Positive yeast colonies identified in the yeast two-hybrid library screen were first screened for homology to DC1 before any further analysis was done; thereby reducing the amount of time and materials consumed by characterizing these positives.

In vitro transcription and translation

Protein production in cell free extracts was accomplished using the Promega TnT T7 Quick Coupled Transcription/Translation System (Promega Corporation, Madison, WI). DNA template was supplied from Y2H constructs in pLP-GADT7 or pLP-GBKT7 containing SAHH, PMT, and MPO under the control of the T7 promoter. The MATCHMAKER system 3 plasmids contain an in-frame epitope tag fusion on the N-terminus of the *in vitro* produced proteins. pLP-GADT7 provides an HA epitope tag and pLP-GBKT7 supplies a c-MYC epitope tag. Constructs in pLP-GADT7 were first digested with *NcoI* restriction enzyme due to lack of T7 transcriptional terminator.

All *in vitro* transcription/ translation reactions were carried out according to the TnT instruction manual with the addition of FluoroTect™ Green_{Lys} tRNA (Promega Corporation, Madison, WI) for *in vitro* translated protein detection. *In vitro* reactions were carried out for 1.5 hours and the *in vitro* produced proteins were analyzed by SDS-PAGE and visualized using the Typhoon® Trio (GE Healthcare, Piscataway, NJ) with a 532 nm excitation and 526 nm filter.

Co-immunoprecipitations were performed using the *in vitro* FluoroTect™ labeled proteins. Ten μ l of the desired *in vitro* reactions were combined and the total volume diluted to 100 μ l with Phosphate Buffered Saline (PBS), pH 7.4 (8 g NaCl, 0.2 g KCL, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄/L), protease inhibitors leupeptin and PMSF were added at 0.4 mg/ml and 10 μ l of the desired antisera was added. Reactions were incubated at room temperature for 1 hour while rotating at 8 rpm. Pre-washed Protein-A Sepharose beads (Sigma, St. Louis, MO) were added and the reactions proceeded at 8 rpm at room temperature for 1 hour. Beads and immunoprecipitated material were pelleted by centrifugation at 1,000 rpm for 30 seconds. The supernatant was removed and retained for analysis; the pellet was washed in 100 μ l Phosphate Buffered Saline with Tween-20 (PBST) (PBS w/ 0.05% Tween 20 (Sigma). Immunoprecipitated and immunodepleted extracts were analyzed by SDS-PAGE and fluorescent proteins were imaged with the Typhoon® Trio (GE Healthcare).

Antisera Production

Polyclonal antisera specific for recombinant tobacco proteins PMT, MPO and SAHH were generated for use in immunoprecipitation experiments. The antigens necessary for antisera production were produced as recombinant thioredoxin fusion proteins from genes cloned into the pET32b vector (Novagen, San Diego, CA) and subsequently transformed into *E. coli* strain BL21. The *E. coli* strains containing plasmid constructs for recombinant protein production of SAHH, PMT and MPO were previously prepared by the Jelesko laboratory prior to my joining the laboratory.

NtSAHH2 (Heim and Jelesko, 2004) was subcloned into pET32b+ (Novagen, San Diego, CA) creating plasmid pTBL1 and subsequently transformed into *E. coli* strain BL21(DE3). pTBL1 provides Isopropyl β -D-1-thiogalactopyranoside (IPTG) inducible production of NtSAHH2 as a fusion protein containing a N-terminal thioredoxin (TRX) and poly-histidine tag. A helper plasmid, pRIL (Stratagene, LaJolla, CA) was co-transformed into *E. coli* to assist with translation of rare codons. *E. coli* cultures containing pTBL1 were grown at 37°C 250 rpm in LB Ampicillin (Amp) 100 μ g Chloramphenicol 30 μ g to an OD₆₀₀ of ~0.6. Cultures were induced for recombinant protein production by addition of IPTG to a concentration of 1.0 mM and grown at 25°C 250 rpm for 4 hours. Cellular material was harvested by centrifuging at 3220 x g. Cells were lysed per manufacturer's instructions (Qiagen Inc. Valencia, CA) using a denaturing guanidine hydrochloride (Fisher Scientific, Atlanta, GA) buffer. The cellular lysate was applied to a column packed with nickel-NTA Superflow resin (Qiagen Inc, Valencia, CA) for purification of the recombinant polyhistidine-tagged protein. The recombinant protein was eluted from the nickel-NTA (NiNTA) column using a low-pH denaturing buffer as per manufacturer instructions.

The purified lysate was analyzed by SDS-PAGE (Shapiro et al., 1967), stained using Coomassie brilliant blue (Bio-Rad, Hercules, CA) and the protein band corresponding to 71 kDa, the expected size of the fusion protein, was excised from a SDS-PAGE gel and eluted from the gel fragment into solution using the Bio-Rad (Hercules, CA) electro-eluter. Protein concentration was estimated using Bio-Rad DC (detergent compatible) protein assay (Hercules, CA). The 71 kDa protein band was trypsin digested, and the corresponding protein fragments were analyzed by mass spectrometry analysis (Helm laboratory, Blacksburg, VA) revealing the desired TRX-SAHH fusion protein was present in the purified fraction (data not shown). The

purification procedure was repeated to obtain approximately 2 mg of TRX-SAHH protein. Purified TRX-SAHH was sent to Co-Calico Biologicals (Reamstown, PA) for immunization of two rabbits and two chickens.

To determine specific animals for suitable antibody production, pre-immune serum samples were received for six chickens and six rabbits. Serum from each animal was analyzed against tobacco root protein extract using western blot analysis to determine if animals had background immunoreactivity to proteins in tobacco roots. The antisera tested did not display immunoreactivity to proteins in tobacco root extracts, and two individuals from each species were immunized. The production of antisera was contracted through Cocalico Biologicals (Reamstown, PA)..

NtPMT1 was subcloned into pET32a+ (Novagen, San Diego, CA) creating plasmid pJGJ388 and subsequently transformed into *E. coli* strain BL21(DE3). pJGJ388 provides Isopropyl β -D-1-thiogalactopyranoside (IPTG) inducible production of NtPMT1 as a fusion protein containing a N-terminal thioredoxin (TRX) and poly-histidine tag. The preparation of antisera for TRX-PMT was performed as described for SAHH with the following exceptions, *E. coli* cultures were induced with 5 mM IPTG and incubated for one hour at 28° before harvesting. Protein identification by Mass Spectrometry analysis (Helm Lab, Virginia Tech, VA) validated TRX-PMT in the purified protein fraction. Antisera to TRX-PMT were produced at Cocalico Biologicals by immunization of three chickens and three rabbits prescreened for immunoreactivity to tobacco root proteins.

NtMPO (Heim et al., 2007) was subcloned into pET32a+ (Novagen, San Diego, CA) creating plasmid pJGJ389 and subsequently transformed into *E. coli* strain BL21(DE3). pTBL1

provides Isopropyl β -D-1-thiogalactopyranoside (IPTG) inducible production of NtMPO as a fusion protein containing a N-terminal thioredoxin (TRX) and poly-histidine tag. The preparation of antisera for TRX-MPO was performed as described for SAHH with the following exceptions. *E. coli* cultures were induced with 1 mM IPTG and incubated for 24 hours at 18° C before harvesting. Bacterial pellets corresponding to 1L of culture were resuspended in 20 ml denaturing buffer and purified using a NiTA column. Protein identification by Mass Spectrometry analysis (Helm Lab, Virginia Tech, VA) validated TRX-MPO in the purified protein fraction. Antisera were generated in prescreened chickens and rabbits.

Co-Immunoprecipitation

Tobacco hairy root cultures (cv. Xanthi) were grown in Gamborg's B-5 media + Gamborg's B-5 vitamins (B-5) (Research Products International Corp., Mt. Prospect, IL). Liquid hairy root cultures were initiated using 3-4 excised hairy root tips into 60 mL B-5 in a sterile 250-mL erylenmeyer flask. The flask was capped with a silicone cap with a foam insert (Sigma, St. Louis, MO) to allow for gas exchange and the flasks were placed on a rotary shaker at approximately 90 rpm for 14 days. The root mass was harvested, blotted of residual media and immediately frozen in liquid nitrogen. Frozen roots were ground into a fine powder and the powder was extracted as previously described (Heim and Jelesko, 2004). Protease inhibitors leupeptin and PMSF (Sigma-Aldrich, St. Louis, MO) were added at the final concentration of 0.4 mg/ml to the desalted extract and the extract was used immediately or frozen in 1 ml aliquots at -20°C. Protein concentrations were estimated using the BioRad (Hercules, CA) Protein Assay with IgG (Bio-Rad Hercules CA) as the protein reference standard.

Immunoprecipitation and immunodepletion experiments were performed with rabbit antisera bound to Protein-A agarose beads as previously described (Heim and Jelesko, 2004). Pre-immune serum corresponding to the same animal as the immune serum was used as a negative control. Immunoprecipitated material was resuspended in 1x SDS-PAGE sample buffer (10% glycerol/62.5 mM Tris-HCl, pH 6.8, 2% SDS, 0.01 mg/ml bromophenol blue, 5% β -mercaptoethanol) and analyzed by SDS-PAGE. Protein was transferred to a 0.45 pore size nitrocellulose membrane (GE Osmonics, Minnetonka, MN) using a semi-dry blotting system (Hoefer, Inc. Holliston, MA). Western blots were performed using primary chicken antibodies and a goat anti-chicken secondary antibody conjugated to HRP and immunodepleted of cross-reactivity to IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) to probe for co-immunoprecipitating proteins. Western blots were developed using the Immobilon HRP substrate (Millipore, Billerica, MA) and were visualized using x-ray film (Research Products International Corporation, Mt. Prospect, IL). Immunodepleted material was analyzed for MPO activity as described in Heim and Jelesko (2004) using putrescine (Sigma-Aldrich, St. Louis, MO) as the substrate.

Immunoprecipitations were performed using antisera to SAHH, MPO and PMT. Co-immunoprecipitation was assessed by western blot using antisera specific to the hypothesized metabolic channel members (i.e. if anti-PMT was used to immunoprecipitate; MPO and SAHH would be probed on the western blot).

RESULTS

Yeast two hybrid directed screen

To evaluate the hypothesized interactions among nicotine metabolic enzymes PMT, MPO, and the SAM recycling enzyme SAHH, were used in a directed yeast two-hybrid assay. This heterologous-based system utilizes a GAL-4 based transcriptional activation assay with proteins of interest fused to the GAL-4 DNA binding domain (bait) and proteins of interest (prey) fused to the GAL-4 transcriptional activation domain. Protein - protein interactions between bait and prey proteins of interest results in the GAL-4 transcriptional activator to be functionally reconstituted by bringing the DNA binding and transcriptional activation domains together, resulting in the expression of as many as four reporter genes regulated by a GAL-4 promoter. The Clontech MATCHMAKER3 GAL-4 system has three reporter genes controlled by a *GAL-4* responsive promoter *ADE2*, *HIS3*, *lacZ*, and *MEL1*. The yeast strains used in the MATCHMAKER3 Y2H are auxotrophic for adenine and histidine under normal conditions, but when the reporter genes *ADE2* and *HIS3* are expressed by GAL-4 responsive promoters, adenine and histidine prototrophy are recovered. Recovery of prototrophy is assessed by the ability of the yeast to grow in the absence of adenine and/or histidine in the growth media. *LacZ* and *MEL1* encode for the respective enzymes β -galactosidase and α -galactosidase and expression of these reporters is detected through the application of respective substrates. *MEL1* can be utilized as a reporter in this system by including X- α -gal in the media, but this method was cost prohibitive due to the large numbers of yeast screened. *LacZ* in the MATCHMAKER3 is not used for screening purposes, but is useful for quantitative assays of protein – protein interactions.

The *lacZ* and *MEL1* reporters were not used as part of this study. The reporter genes are present in varying copy number and the corresponding promoters contain regulatory sequence variations altering the strength of the GAL4 mediated transcriptional response. Sensitivity and stringency in the GAL4 system is controlled by the selection of the nutritional reporter genes.

The *HIS3* reporter gene is both the least stringent and most sensitive of the nutritional reporter genes because of a low-level activation by an endogenous transcriptional activator that functions in addition to the reconstituted GAL4 transcriptional activator. The low level of endogenous *HIS3* expression can be controlled by the addition of 3-aminotriazole to suppress background growth when the *HIS3* reporter is used as the only selection criteria. On the other hand, the low level of *HIS3* expression adds sensitivity when combined with the *ADE2* reporters. In contrast, *ADE2* and *lacZ* transcription is tightly regulated providing a more stringent screen, with the disadvantage of decreasing the sensitivity and biasing against the detection of weak protein – protein interactions. The directed screens were conducted under high stringency using the *ADE2* and *HIS3* reporters, and then repeated with low stringency conditions using only the *HIS3* reporter.

Some proteins have the inherent ability to activate transcription in the Y2H system. Thus an important step in conducting a Y2H assay is to ensure that the bait constructs do not auto-activate the reporter genes in the absence of an activation domain containing protein fusion partner. All three bait constructs were independently plated on SD(-Trp,-His) and SD(Trp,-His,-Ade) and did not complement the nutritional auxotrophies.

All possible pair-wise combinations of PMT, MPO, SAHH and empty vector were generated by mating haploid yeast strains containing the respective bait and prey plasmids. The

resulting diploid yeast were plated on selection media and assayed for reporter gene activation of both *ADE2* and *HIS3* on SD(-Trp,-Leu,-His,-Ade). The yeast were allowed to grow for 21 days on selection media followed by visual inspection for colony growth. This constituted a stringent screen for all pairwise protein – protein interactions. Colony formation was observed only on plates corresponding to SAHH-bait and SAHH-prey diploids (data not shown). No other combinations produced adenine and histidine prototrophic diploid colonies.

Since the only observed interaction in the stringent Y2H screen was SAHH-SAHH, a more sensitive assay capable of identifying less strong protein – protein interactions was performed. Mated diploid yeast cultures were normalized and plated as a dilution series not only to qualify an interaction but also to gauge the relative strength of the interactions by assessing growth based only upon activation of *HIS3*. After 14 days of incubation the plates were evaluated for diploid growth. The only yeast combination displaying growth was again SAHH-BD with SAHH-AD (Figure 2.1). All other pair-wise combinations (e.g. SAHH-MPO, SAHH-PMT, MPO-PMT, PMT-PMT, and MPO-MPO) did not result in growth of the corresponding diploid yeast strains. This lack of evidence for direct interactions between these three hypothesized metabolic channel members suggested there was not a direct interaction between these enzymes in the Y2H assay. However, these results did not rule out the possibility that these three enzymes might interact with other tobacco root proteins that might be necessary to facilitate a nicotine biosynthetic channel.

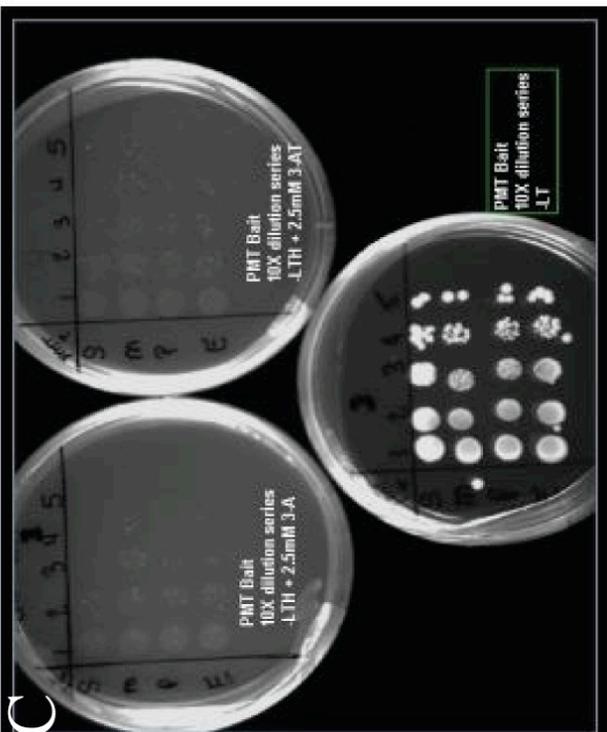
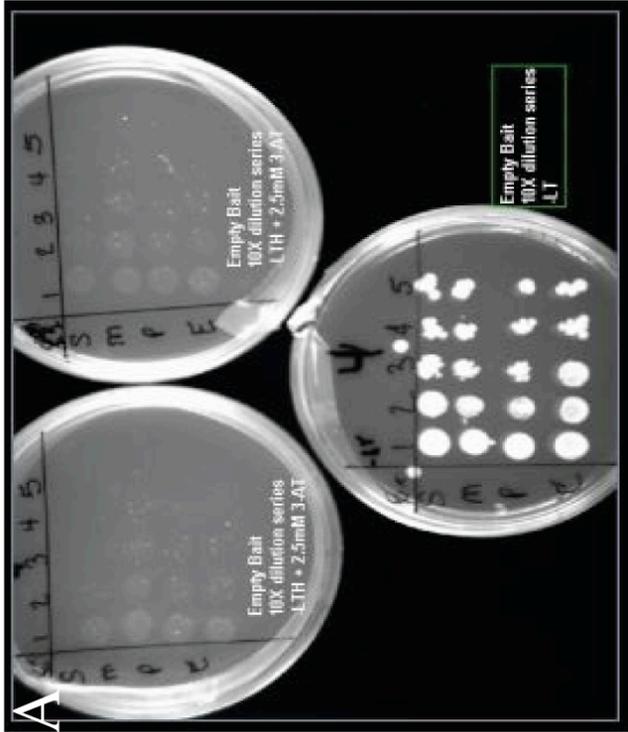
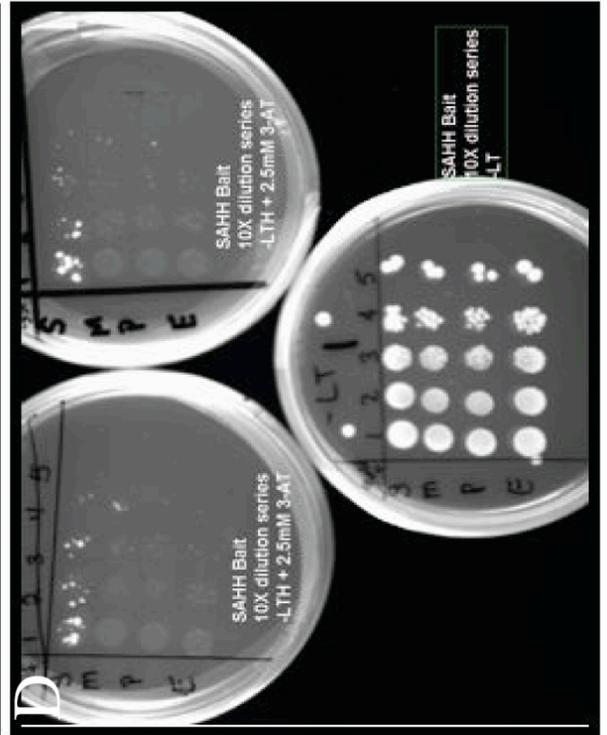
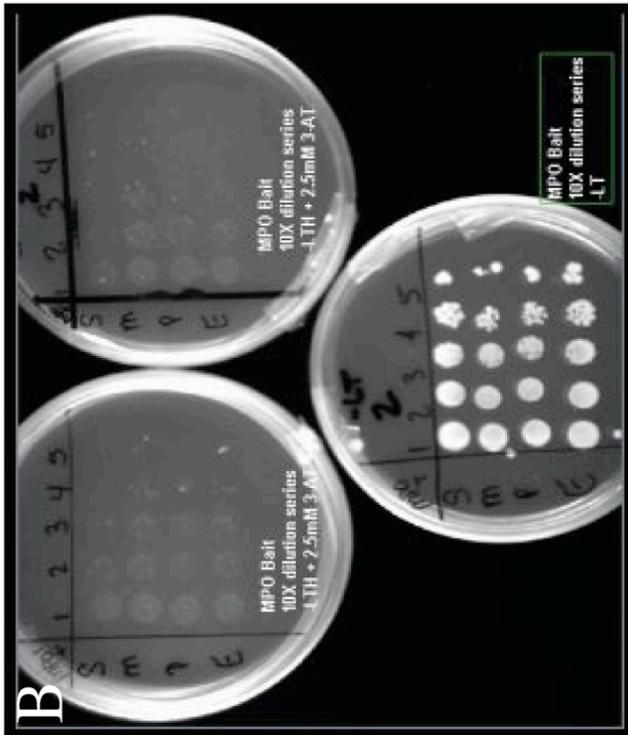


Figure 2.1: Directed Y2H Screen

Aliquots from yeast cultures containing the pairwise combinations were plated on SD(-Leu,-Trp,-His) to test for interaction and on SD(-Leu,-Trp) to test for viability. Yeast harboring all pair-wise combinations of SAHH (S)/ PMT (P)/ MPO (M) and Empty vector (E) bait (BD) and prey (AD) are shown after a 14-day incubation. Plates in the upper portion of each field correspond to SD (-Leu,-Trp,-His) while the plate in the lower portion of each field corresponds to SD (-Leu,-Trp). Each panel corresponds to a single bait and the prey is represented on the Y axis of each individual plate. The X-axis contains a dilution series of the yeast, each aliquot corresponds to a 10x dilution of the previous culture.

Panel A shows E-BD paired with S-AD (first row), M-AD (second row), P-AD (third row), and E-AD (fourth row).

Panel B shows M-BD paired with S-AD (first row), M-AD (second row), P-AD (third row), and E-AD (fourth row).

Panel C shows P-BD paired with S-AD (first row), M-AD (second row), P-AD (third row), and E-AD (fourth row).

Panel D shows S-BD paired with S-AD (first row), M-AD (second row), P-AD (third row), and E-AD (fourth row).

Yeast two hybrid library screen

The original evidence of an association between MPO and SAHH did not indicate if this apparent association is directly between the two proteins or facilitated by other proteins. Therefore, an Y2H library screen was conducted to individually screen SAHH/MPO/PMT for respective interacting proteins using a cDNA library of genes expressed in tobacco roots.

The Y2H library screen is a high-throughput open-ended method for identifying interacting proteins. As employed in this project, the Y2H was used to identify proteins from a tobacco root expression library that interacted with the individual hypothesized metabolic channel members SAHH, PMT, and MPO. Screens were conducted independently with each of the three bait proteins and screening was continued until the full complexity of the Y2H cDNA library was sampled at least 1X (minimum of 15×10^6 independent diploids).

All Y2H cDNA library screens used the recovery of both histidine and adenine prototrophies in order to reduce the amount of background growth leading to potential false positives. The potential disadvantage of using the higher stringency screen was somewhat compensated for by allowing the plates to incubate for 21 days to accommodate slow growing colonies (i.e. those containing weakly interacting proteins). Initial screens produced poor mating efficiency and thus the number of clones screened was relatively low. Screen efficiency was increased using denser cultures of bait sufficient to be in 100-fold excess of the viable prey in a 1 mL library aliquot (2.4×10^8). Table 2.1 summarizes the results of extensive sampling of the tobacco root Y2H cDNA library with the three nicotine biosynthetic enzyme bait constructs.

Table 2.1: Overview of Y2H Library Screen

Bait Protein	Independent Clones Screened	Initial Positives (% of screened)	Reproducible Interactions	Confirmed Interactions
SAHH	42.3 x 10 ⁶	64 (0.00015%)	4	2
PMT	125.5 x 10 ⁶	200 (0.00016%)	2	1
MPO	21.2 x 10 ⁶	35 (0.00017%)	1	0

The yeast two-hybrid screen is prone to a variety of artifacts. Thus, one must evaluate each initial positive for these well-known artifacts. In the following section, I will outline the results for the SAHH Y2H cDNA library screen and how I systematically eliminated all false positives.

Screens using SAHH as bait were conducted as four independent screens resulting in 64 initial apparent adenine and histidine prototrophic diploid colonies. For initial identification of false positives, the yeast colonies were first streaked on fresh SD(-Ade,-His) media to reproduce the prototrophic growth phenotype. This initial screen identified that approximately 20% of the apparent prototrophic colonies were in fact auxotrophic and thus represented false positives. These false positives were not further evaluated.

Identification of the prey plasmid responsible for the prototrophic growth phenotype was the next step. It is technically challenging to recover large quantities of the prey plasmids from the prototrophic diploid yeast colonies thereby making simple whole plasmid restriction enzyme digests difficult. Instead, the cDNA inserts from the prey plasmids conferring adenine and histidine prototrophic growth were first subjected to plasmid recovery from the yeast, followed by PCR amplification of the cDNA insert, and then subsequent restriction enzyme digest of the amplified DNA products. The digestion products were analyzed by agarose gel electrophoresis to view the *AluI* restriction fragment banding patterns. Inserts with similar DNA fragment banding patterns were grouped together and a representative clone was chosen for further investigation. Figure 2.2 shows the results of a restriction enzyme digest of amplified PCR products obtained from the cDNA insert region of 15 library plasmids providing positive results in the Y2H. Initial grouping of representative unique prey plasmids eliminated approximately 25% of the remaining positives.

Spontaneous suppressor mutations in yeast resulting in the activation of reporters are a common cause of false positive interactions. In these false positives, a mutation in the yeast has resulted in the transcription of one or more reporter genes independent of a protein interaction induced reconstitution of the GAL4 transcriptional activator. To rule out suppressor mutations as the cause of the positive growth phenotype, the isolated plasmid was transformed back into a haploid yeast strain and was subsequently mated with a compatible haploid strain harboring the original bait construct. Recapitulation of the prototrophic growth phenotype effectively ruled out suppressor mutations as a source of reporter gene activation. Another source of artifactual restoration of prototrophic growth is the situation in which the prey plasmid contains a cDNA clone that encodes a protein that has both DNA binding and transcriptional activation activity



Figure 2.2: Restriction digest of Y2H inserts conferring positive yeast growth

Y2H inserts were amplified by PCR and the amplification product was digested with the restriction enzyme, Alu1. Positives with corresponding digestion patterns were grouped together and a representative clone was chosen for further characterization.

Lanes 1 and 2 are molecular weight markers. Lane 1 - 500 bp marker and Lane 2 - 1 kb marker.

Lanes 3, 4, 5, 6, 12, 14, 16 and 17 were determined to be unique positives.

Plasmids corresponding to the patterns in lanes 7, 11, and 15 were grouped.

Plasmids corresponding to the pattern in lanes 8, 9, 10 and 13 were grouped.

independent of the bait construct. To identify such artifactual “positives” it was necessary to mate haploid yeast containing each positive prey plasmid with haploid yeast containing empty-BD to rule out intrinsic autoactivation of reporter genes by the prey. Thus, diploid yeast capable of growing on selection media independent of the bait protein were categorized as auto-activators and were not investigated further. These initial tests were performed to identify known artifactual Y2H positives, leaving the remaining clones as candidates encoding possible interacting proteins with the SAHH-BD protein.

Plasmids corresponding to facilitated activation of the reporter genes in yeast were sequenced to determine the identity of the interacting protein. In total four of the initial 64 positive SAHH interactors were sequenced to determine their identity. The DNA sequence of individual cDNAs were compared to sequences in the Genbank nonredundant database to identify either identical or homologous genes. Y2H_012-01 and Y2H_408-01 were shown to be homologous to *N. tabacum* SAHH. This was an anticipated result because SAHH is known to function as a homo-dimer and was also shown in the directed screen to interact. Thus, this result from the unbiased cDNA library screen using SAHH as bait serves as a positive control indicating that the Y2H screen was capable of identifying SAHH interacting proteins.

Putative SAHH interacting clone Y2H_028-02 was shown to be homologous (e-value = 2×10^{-156}) to *N. tabacum* phosphoribosylaminoimidazole carboxylase (ADE2). Nutritional selection in this screen was based upon increased expression from the yeast ADE2 and clone Y2H-028-02 encoded a *N. tabacum* ortholog that could potentially enzymatically complement the yeast ADE2 prototrophy. Analysis of interaction of ADE2 and SAHH was performed independent of ADE2 reporter selection and the analysis revealed the HIS3 reporter gene was not activated. The absence of HIS3 reporter gene expression suggested that ADE2 and SAHH did

not interact and *NtADE2* was likely complementing the yeast ADE2 auxotrophy directly. These results suggest ADE2 was not likely an SAHH interacting protein.

Putative SAHH interacting clone Y2H_006-01 encoded a protein predicted to be a tobacco homolog of a polyadenolate-binding protein originally identified in *Arabidopsis thaliana*. Further investigation of this cDNA sequence showed an in-frame stop codon in the linker region between the activation domain fusion and the inserted cDNA sequence. Because the stop codon was encountered in the cDNA sequence prior to the start of the poly-adenolate-binding protein, it was categorized as a probable false positive and was not investigated further. Thus, the analysis of 42.3×10^6 independent members of the tobacco root cDNA library provided 64 SAHH-interacting candidates. The majority of these initial positives were ruled out as artifacts of the Y2H screen. However two of the positives were identified as containing a cDNA encoding SAHH proteins and recreated a SAHH bait-dependent interaction, consistent with SAHH forming a functional homotetrameric enzyme.

PMT yeast two hybrid cDNA library screen

In total two primary screens were conducted with PMT as bait with a total of 125.5×10^6 diploid colonies screened. Characterization of 200 initial adenine and histidine prototrophic colonies was performed in the same manner as with the SAHH positives with the addition of an extra screening step. During the initial screening process for artifacts, it quickly became apparent that many of these initial positive prey plasmids conferred prototrophic adenine and histidine growth in the absence of the PMT-bait plasmid; demonstrating the prey-AD plasmids were causing autoactivation of the reporter genes independent of the PMT-BD bait plasmid.

Therefore, in an effort to streamline the characterization process, I sequenced several inserts from proven autoactivator prey-AD plasmids were sequenced. The data revealed that the majority of the autoactivator sequences belonged to a family of unknown function proteins each containing a DC1 domain (Pfam PF03107). The DC1 Domain containing sequences were aligned and areas of conserved sequence were used to design oligonucleotide primers suitable for a rapid PCR-based screen for clones containing DC1-like sequences (Figure 2.3). To detect positive colonies harboring a DC1 domain containing cDNA, yeast plasmid preparations were screened by PCR using primers SBH001 and SBH002. The appearance of an amplification product indicated the presence of a DC1 domain and those autoactivation artifacts were not further characterized.

The PMT screen identified two unique reproducible interactors. Plasmid Y2H_529-01 encoded another *N. tabacum ADE2* clone capable of directly functionally complementing the yeast *ADE2* auxotrophy. The second positive was plasmid Y2H_270-06 that encoded a partial cDNA encoding a predicted PMT protein. The tobacco PMT enzyme is a functional homodimer, so this latter interaction was an expected finding and thereby increased the level of confidence in this Y2H assay. It is noteworthy that the cDNA corresponding to PMT identified in the Y2H screen was a 5' truncated *PMT* cDNA. Interestingly, the PMT-PMT interaction was not detected in the directed Y2H screen when two identical full-length cDNAs were used. Lastly, the PMT-PMT interaction in the cDNA library screen only appeared once in 125.5×10^6 diploid colonies.

MPO screens were conducted as three separate screens assaying a total of 21.2×10^6 diploid colonies, representing 1.4 times the size of the cDNA library. MPO produced only one



Figure 2.3 : PCR screen to identify cDNA inserts containing a member of the DC1 domain containing family.

Crude plasmid preps were performed from yeast displaying a positive phenotype in the Y2H screen. Lanes 1 and 15 correspond to a 100-bp marker. Lane 2 is a no-template control. Lanes 16 and 17 are previously verified DC1 domain containing inserts. Lane 18 displays the empty plasmid.. Lanes 3-14 represent positive colonies from the PMT-bait screen. Plasmids corresponding to lanes 4 and 7 were explored further as potential positives, while the plasmids corresponding to the remaining positive colonies were disregarded as false-positives.

reproducible apparent interacting clone (Y2H_312-01) and this cDNA corresponded again to the complementing *NtADE2* gene.

Both directed and cDNA library Y2H screens were expected to provide details regarding interacting proteins that form a postulated nicotine metabolic channel. While expected SAHH-SAHH and PMT-PMT interactions were identified in the open-ended cDNA library screens, no direct evidence for either protein – protein interactions amongst the three enzymes or with any other potential “bridging” proteins were discovered. Thus, the Y2H assays and screens did not provide any direct evidence supporting our hypothesized nicotine metabolic channel. One caveat of the Y2H is that the use of GAL4-domain protein fusions can disrupt the natural folding properties of the protein fusion partners and result in an altered shape of the protein of interest. An advantage of the MATCHMAKER3 system is the ability to directly utilize the Y2H plasmid constructs to synthesize the proteins of interest *in vitro* without the added GAL4 fusion domains. Therefore, *in vitro* transcription/translation was employed to produce SAHH/PMT/MPO in a cell free system for use in co-immunoprecipitation reactions.

In vitro transcription and translation coupled with co-immunoprecipitation

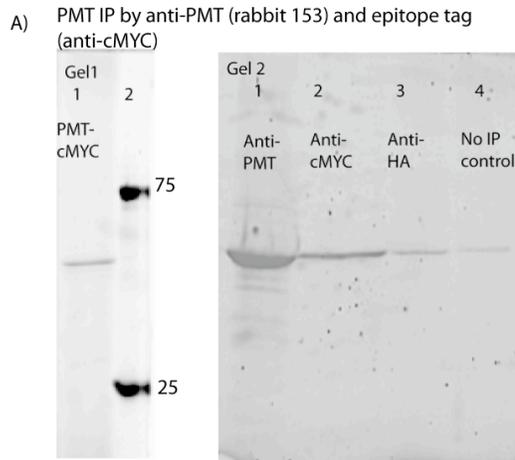
A feature of the MATCHMAKER3 Y2H system is the potential for multifunctional use of the plasmids designed for the Y2H screens. The plasmids in addition to providing functioning yeast promoter sequences also contain T7 promoter sequences in the linker regions between the GAL4 and protein of interest domains, allowing the proteins of interest to be produced as recombinant proteins without the constraints of the GAL4 fusion proteins present in the Y2H assay system. An additional feature of the MATCHMAKER3 system is the inclusion of epitope

tags (hemagglutinin (HA), and c-MYC) in the linker region to assist in the co-immunoprecipitation of *in vitro* produced recombinant proteins of interest.

The plasmids constructed for the Y2H assays were used as templates for the production of *in vitro* recombinant proteins followed by subsequent co-immunoprecipitation analysis using antiserum to the epitope tags. This method allows for the detection of direct interactions between proteins synthesized from plasmid DNA in a eukaryotic protein expression system. In addition to the standard *in vitro* transcription/translation method, the FluoroTect protein labeling system was used. The FluoroTect reagent provides fluorescently charged lysine tRNAs that will be incorporated into the *in vitro* translated proteins. Labeling of the lysine residues allows for detection of fluorescently labeled proteins directly within a SDS-PAGE gel, thereby eliminating the need for western blotting. Another advantage of the FluoroTect detection system is a very high degree of sensitivity when using a fluorescent scanner for detection. However, implementation of the FluoroTect protein labeling was not without challenges. The fluorescently labeled proteins were subject to non-immuno associated protein precipitation during the course of the experiment. To minimize non-immuno precipitation of labeled proteins, co-immunoprecipitation experiments were performed immediately after *in vitro* transcription/translation procedures. Likewise, the amount of Fluorotect reagent used was decreased and the centrifugation of protein aliquots prior to the addition of antibody were found to decrease the non-specific fluorescently labeled protein precipitation. Added controls of non-immunoprecipitated controls were performed alongside of co-immunoprecipitation experiments to gauge the level of non-specific fluorescently-labeled protein precipitation.

The fluorophore labeled lysine residues proved to be problematic with immunoprecipitations using the c-MYC epitope antibody, as the c-MYC epitope tag

(EQKLISEEDL) contains a lysine. The c-MYC antibody was unable to efficiently bind to the epitope tag and immunoprecipitate the fluorescently labeled c-MYC tagged proteins. As protein specific antisera was generated for use in native co-immunoprecipitation (results presented below), protein targeted polyclonal antisera was used for the co-immunoprecipitations. Figure 2.4.A shows *in vitro* produced c-MYC-PMT in lane 1 of gel 1 and immunoprecipitations of cMYC-PMT are shown in gel 2. As demonstrated in gel 2 (lane 1) the PMT-specific antiserum provides a more robust immunoprecipitation of cMYC-PMT than the anti-c-MYC epitope tag antibodies shown in lane 2. Lanes 3 and 4 display the general background associated with this screen with lane 3 demonstrating the non-specific immunoprecipitation when using anti-HA and lane 4 showing general background as no antibodies were added to the reaction. Figure 2.4.B demonstrates the immunoprecipitation of SAHH by anti-SAHH (lane 3), but not by the SAHH pre-immune serum. Therefore for all *in vitro* co-immunoprecipitations, protein specific immune serum was used. *In vitro* produced fluorescently labeled SAHH and PMT were shown to be effectively immunoprecipitated by protein specific antisera, yet were not precipitated by non-specific antisera (i.e. SAHH antisera immunoprecipitated SAHH, but not PMT) (data not shown). MPO antisera did not show effective immunoprecipitation of MPO (Figure 2.5), so co-immunoprecipitations using anti-MPO serum were not attempted. Using PMT- and SAHH- specific antisera as the immunoprecipitating antibodies, protein solutions containing *in vitro* recombinant MPO, PMT and SAHH were probed. Fluorescent scanning of SDS-PAGE gels showed immunoprecipitation of the targeted protein, as expected, but did not show evidence for co-immunoprecipitation of the hypothesized nicotine metabolic complex members (Figure 2.5).



B) SAHH IP by anti-SAHH (rabbit 150)

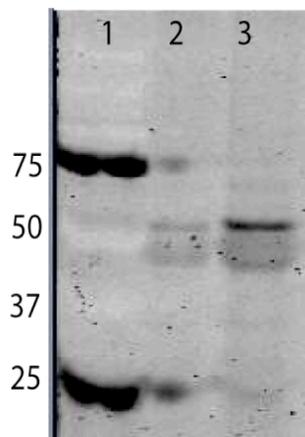


Figure 2.4 : PMT and SAHH immunoprecipitation by protein specific antisera

A) PMT immunoprecipitation by anti-PMT and anti-cMYC (12% gels)

Gel 1: 1) c-MYC PMT

2) Molecular Weight Marker

Gel 2: 1) Immunoprecipitation by anti-PMT (rabbit 153)

2) Immunoprecipitation by anti-cMYC

3) Immunoprecipitation by anti-HA

4) Non-immunoprecipitation

B) SAHH immunoprecipitation by anti-SAHH (10% gel)

1) Molecular weight markers

2) Pre-immuned anti-SAHH (Rabbit 150)

3) Anti-SAHH (Rabbit 150)

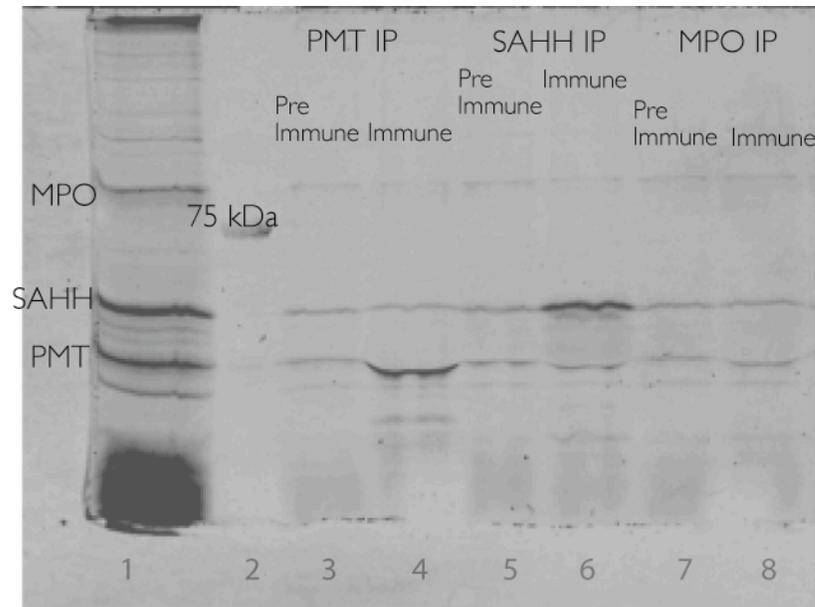


Figure 2. 5: Co-Immunoprecipitation in the in vitro system

In vitro produced MPO, SAHH and PMT were combined and incubated with the indicated antisera to determine if co-immunoprecipitation was occurring between MPO/SAHH/PMT.

- 1) Identification of MPO/SAHH/PMT in in-vitro reaction
- 2) Molecular Weight Marker
- 3) Co-IP using pre-immune serum from Rabbit 153 (Anti-PMT)
- 4) Co-IP using immune serum from Rabbit 153 (Anti-PMT)
- 5) Co-IP using pre-immune serum from Rabbit 150 (Anti-SAHH)
- 6) Co-IP using immune serum from Rabbit 150 (Anti-SAHH)
- 7) Co-IP using pre-immune serum from Rabbit 160 (Anti-MPO)
- 8) Co-IP using immune serum from Rabbit 160 (Anti-MPO)

The co-immunoprecipitation experiments using *in vitro* synthesized recombinant PMT, SAHH, and MPO did not provide direct evidence for an association between these hypothesized members of a nicotine metabolic complex. The *in vitro* system produces proteins in a cell-free wheat germ extract, a simple matrix containing the necessary enzymes for T7 promoter transcription and eukaryotic translation. However, this cell-free system does not provide the necessary machinery for post-translational modification of the proteins. Thus, co-immunoprecipitation of proteins processed in the native tissue may be an important factor for the original observation of the immunodepletion of MPO activity using anti-SAHH antiserum (McLauchlan et al., 1993; Heim and Jelesko, 2004).

Co-Immunoprecipitation using cell free tobacco root extracts

In order to determine if interactions between SAHH, PMT and/or MPO could be detected in a native tobacco extract, co-immunoprecipitation experiments using cell-free tobacco root extracts were conducted. The investigation of interactions between proteins produced in native, nicotine-producing cells is a crucial aspect of this work. Co-immunoprecipitation experiments target the desired protein through protein-specific antibodies. The antibodies, attached to an insoluble matrix, bind specifically to the protein of interest and are selectively removed from the extract by sedimentation of the attached insoluble matrix. Any additional proteins associating with the target protein should sediment with the antibody complex. Proteins co-immunoprecipitating with the target protein are detected by assaying the immunoprecipitated material by western blot analysis. Thus, co-immunoprecipitation requires an antiserum specific

to the targeted protein and another antiserum for detection of the associated (co-immunoprecipitated) proteins by western blot analysis.

Specific antisera are required not only for each protein (targeted and co-immunoprecipitated), but additional care must be taken to prepare antisera from different animal species in order to avoid cross-reactivity of the secondary antibodies with the primary antibodies used in the initial immunoprecipitation. Detection of co-immunoprecipitation by western blot requires use of a species-specific secondary antibody to detect the primary antibody specifically bound to the co-immunoprecipitated plant protein of interest. If the same animal species is used for immunoprecipitation and for the detection of co-immunoprecipitating proteins on a western blot, the secondary antibody will bind to both populations of antibodies and obscure visualization of the blot. Therefore, antisera to SAHH, PMT and MPO were individually produced both in chickens and rabbits.

SAHH, PMT, and MPO antigens necessary for induction of an animal immune response was prepared by purification of recombinant protein produced in *E. coli*. The protein of interest was produced as a fusion protein containing the bacterial protein thioredoxin to promote protein solubility and a six-histidine tag to aid in purification. The antigen was purified from a denatured bacterial lysate by Nickel-nitrilotriacetic acid NiNTA resin binding of the histidine tag and subsequent elution from the matrix using a low pH buffer. The NiNTA purification produced a protein fraction highly enriched for the desired recombinant protein, but other contaminating proteins were also present (Figure 2.6). SDS-PAGE gels were used to separate the NiNTA purified TRX-HIS⁶-recombinant protein from the majority of contaminating *E. coli* proteins. Subsequently, the band corresponding to the desired recombinant protein was excised. The protein contained in the excised gel slice was electro-eluted to obtain a highly purified

fraction of the TRX-HIS⁶-recombinant protein of interest for use as antigen. The animal immune response was monitored throughout the production schedule and antisera immunoreactivity was gauged by antisera binding to protein species of the expected size from tobacco root extracts.

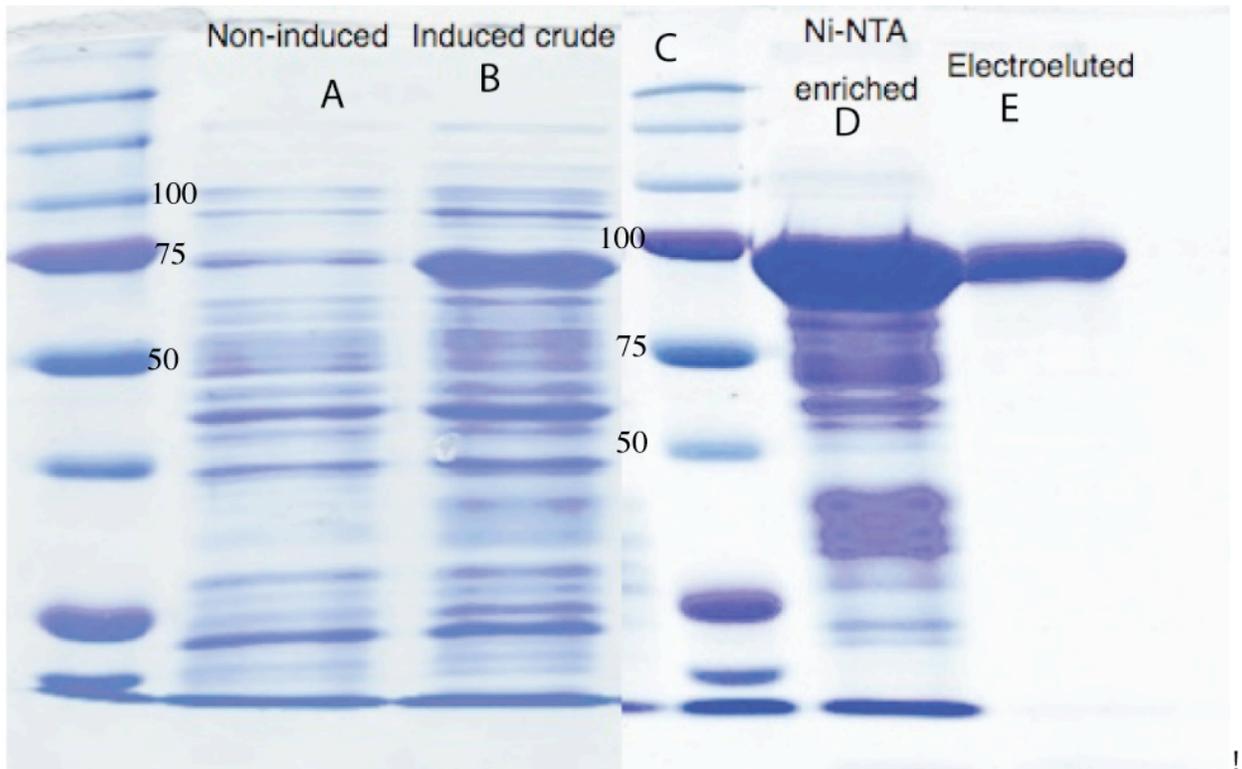


Figure 2.6: Purification of TRX-HIS-SAHH for use as antigen
 A) crude bacterial lysate not induced for TRX-HIS-SAHH expression
 B) crude bacterial lysate induced for TRX-HIS-SAHH production
 C) ladder
 D) NiNTA enriched fraction of bacterial lysate containing TRX-HIS-SAHH
 E) gel-excised and electro-eluted TRX-HIS-SAHH

Antisera characterization

The antiserum corresponding to SAHH, PMT and MPO was characterized by western blot prior to conducting the co-IP experiments. All antisera were first analyzed by western blotting to determine whether the sera contained antibodies specific to denatured native proteins and to determine the relative titer of these antigen-specific antibodies. Rabbit antisera were analyzed against tobacco root extract to find candidates that bound only the expected single protein species. Chicken antisera were analyzed against tobacco root extract to find candidates that bound only the expected single protein species and that produced a strong signal to background ratio. As summarized in Table 2.2, a total of 14 different antisera were produced and characterized by western blotting for binding properties to the initial antigen and to the expected protein species from a tobacco root extract. Appropriate antisera from chicken and rabbit were identified for SAHH and PMT, but not for MPO (Figure 2.7). MPO antisera in general produced a high background to signal ratio and of the 2 rabbits and 2 chickens immunized, only one rabbit was found to produce an interpretable image on a western blot. The MPO immunoreactive rabbit identified required at least 100 μg of tobacco extract to be loaded onto the western blot for detection, and at this high concentration non-specific background binding was also observed (Figure 2.7.C). These findings suggest the antisera had a low affinity for MPO and therefore, unsuitable for co-IP experiments. The failure to obtain a chicken antiserum specific for MPO and acceptable for a western blot, ruled out detection of MPO by western blot in co-IP experiments. Therefore, MPO activity assays were performed on the co-IP supernatant and the co-IP of MPO was gauged by immunodepletion assays.

Table 2.2 Summary of antisera produced for immunoprecipitation projects

Antigen	Species	Designation	Characterization
SAHH	Rabbit	R150	Western blot primary antibody 1:10,000; Immunoprecipitates
SAHH	Rabbit	R151	Western blot primary antibody 1:5000; Immunoprecipitates
SAHH	Chicken	C6	Western blot primary antibody 1: 5000
SAHH	Chicken	C7	Recognizes SAHH on western blot, optimization not carried out
PMT	Rabbit	R152	No affinity for native PMT discovered
PMT	Rabbit	R153	Western blot primary 1:5000; Immunoprecipitates
PMT	Rabbit	R154	No affinity for native PMT discovered
PMT	Chicken	C8	Western blot primary 1:2500
PMT	Chicken	C9	No affinity for native PMT discovered
PMT	Chicken	C10	No affinity for native PMT discovered
MPO	Rabbit	R159	Western blot primary 1:2500 (high background). Apparent poor affinity for the native protein.
MPO	Rabbit	R160	No affinity for native MPO discovered
MPO	Chicken	C11	No affinity for native MPO discovered
MPO	Chicken	C12	No affinity for native MPO discovered

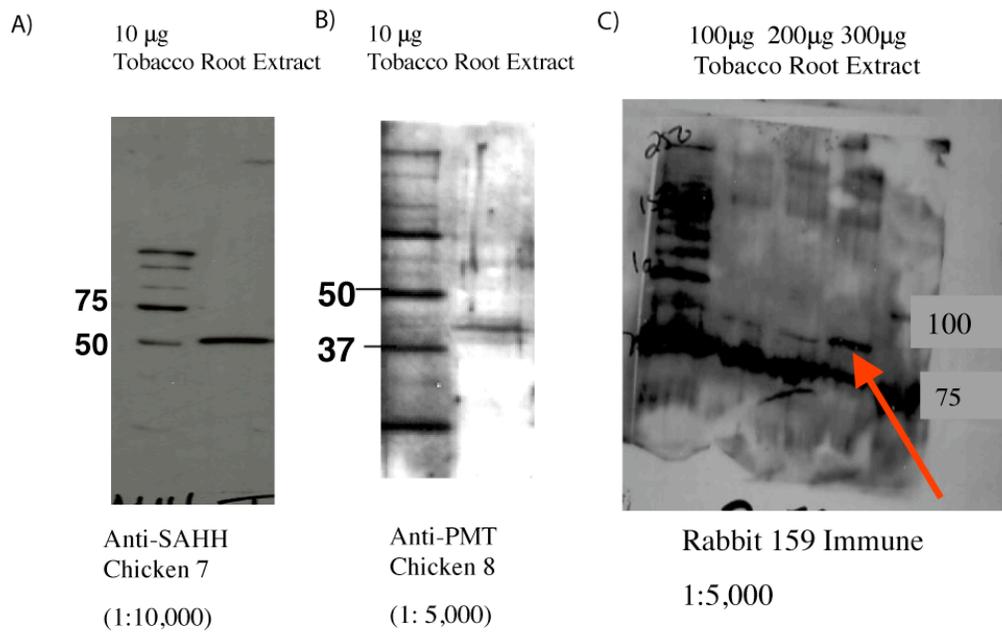


Figure 2.7: Characterization of immune serum for use in co-immunoprecipitations

- A) Western blot with 10 µg tobacco root extract, probed with anti-SAHH
- B) Western blot with 10 µg tobacco root extract, probed with anti-PMT
- C) Western blot with 100/200/300 µg tobacco root extract, probed with anti-MPO.
Arrow indicates MPO corresponding band.

Co-immunoprecipitation

Co-immunoprecipitation experiments were performed using cell-free tobacco root extracts and either PMT or SAHH as the initial immunoprecipitated protein. Figures 2.4 and 2.5 show that the R153 anti-PMT and R150 anti-SAHH immunosera immunoprecipitate their recombinant antigens when expressed in the *in vitro* transcription translation system and thus are immunoprecipitating antibodies. Figure 2.8 shows that the immunoprecipitation of PMT from tobacco root extract did not show immunodepletion of MPO activity from the root cell-free extracts. Similarly, the immunoprecipitation of SAHH did not show immunodepletion of MPO activity. Although anti-PMT and anti-SAHH antisera did not immunodeplete MPO activity, the anti-MPO antisera did show a 40% reduction in MPO activity. Since MPO antisera do detect MPO on a western blot, albeit inefficiently, it was likely the 40% reduction was due to a low-affinity immunoprecipitation of MPO, although non-immunoprecipitating antibodies that bound to MPO and inhibited the enzyme activity could also create the observed results. The McLauchlan antisera previously characterized to be immunoreactive to SAHH and to immunodeplete MPO, in a separate assay displayed a 75% decrease in MPO activity (data not shown) and proved this method could detect a depletion of MPO activity. Therefore, it was concluded antisera specific to PMT and SAHH were not able to immunodeplete MPO activity.

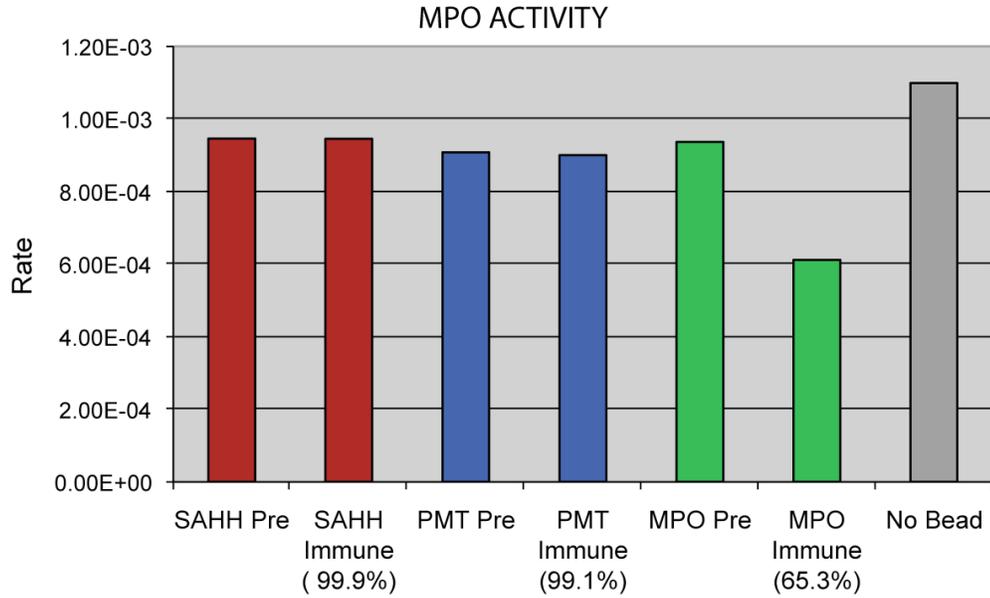


Figure 2.8: Immunodepletion results

Antisera specific to SAHH, PMT and MPO were used to selectively remove the targeted protein from a tobacco root extract. The resulting supernatant was then analyzed for MPO activity. The percentage of MPO activity depleted by the anti-serum is listed in parenthesis. Pre-immune serum from each animal obtained prior to immunization was used as a negative control.

Co-immunoprecipitation experiments were also performed to directly address SAHH and PMT co-immunoprecipitation. Anti-PMT was shown to be capable of immunoprecipitating PMT, but technical difficulties precluded the detection of SAHH and MPO on a western blot. SAHH is a predicted 53 kDa protein, and thus co-migrated with the heavy chain of IgG (which appears on western blots around 55 kDa) during SDS-PAGE. The relatively high proportion of IgG to SAHH in these experiments, masked the appearance/binding of SAHH on western blots and resulted in a non-specific background signal around 53 kDa. Therefore the presence or absence of SAHH in a co-immunoprecipitation could not be confirmed by western blot analysis directly, but were confirmed during immunoprecipitation experiments using *in vitro* produced recombinant SAHH proteins.

Discussion

The research objectives described in this chapter were designed to explore the predicted physical interactions of three tobacco enzymes (MPO, SAHH, and PMT) in a hypothesized nicotine metabolic channel. Previously, the laboratory obtained indirect evidence of an association between the SAM recycling enzyme SAHH and the nicotine biosynthetic enzyme MPO. The inferred association was discovered when an antiserum specific for SAHH had the demonstrated ability to immunodeplete MPO from a cell free tobacco root protein extract. The implied association between SAHH and MPO gave no indication of whether this association is due to a direct protein - protein interaction between the two enzymes or if other proteins were facilitating a larger multi-enzyme complex. To further address the possibility that MPO and SAHH interact we used the yeast two-hybrid assay (Y2H). The Y2H was approached from two directions, as a simple assay to detect pairwise interactions among SAHH, MPO and PMT and as an expression library screen to detect other tobacco root proteins capable of interacting with the three aforementioned enzymes. In addition to SAHH and MPO, the nicotine biosynthetic enzyme PMT was included in the Y2H screens because PMT provides a functional link between SAHH and MPO by producing substrates for both enzymes.

Yeast two-hybrid analysis

The directed Y2H assayed the relationship between SAHH, MPO and PMT in all pairwise combinations. The Y2H assay was conducted under both stringent conditions (dual

reporter) and under less stringent conditions (single reporter) for increased sensitivity. The results from each screen showed only a single positive pair, SAHH-SAHH. SAHH is known to function as a homooligomer and this result can be viewed as a positive control, confirming the SAHH-fusions were functioning as expected in the yeast heterologous system. On the other hand, both PMT and MPO were also expected to form homodimers, but neither displayed positive Y2H interactions when assayed as both bait and prey in the directed Y2H assay. The use of low stringency conditions did not reveal PMT-PMT and MPO-MPO interactions, suggesting the fusion proteins likely are not able to conform to the native active form. If PMT and MPO are unable to recapitulate dimerization when expressed as fusions in the Y2H system, then it is likely the proteins are not able to present the necessary epitopes required for protein – protein interaction. .

The lack of direct evidence of pairwise SAHH, PMT, and/or MPO interaction in the directed Y2H screen can be interpreted several ways and as a stand alone piece of evidence does not preclude a nicotine metabolic channel. The lack of a detectable interaction among the assayed proteins could signal a direct interaction does not occur specifically among these three enzymes and that additional proteins are required to facilitate a larger metabolic complex. Likewise, the lack of detectable MPO - MPO and PMT - PMT dimer formation could suggest an inherent problem with these particular bait and prey constructs in the Y2H assay as a whole. The Y2H system is efficient and relatively simple to use, yet cannot truly mimic the *in planta* conditions. The heterologous proteins produced in the Y2H are fusion proteins, localized to the nucleus and subject to the post-translational machinery present in yeast. The fact MPO and PMT were not detected as dimers in the low stringency screen might indicate that the conditions in yeast result in constraints on the heterologous proteins not present *in planta*.

The Y2H library screen provided the means to independently examine a large number of potential interactions with these three proteins in case the hypothesized multienzyme complex requires interactions with “bridging” proteins. The Y2H cDNA library-based screen allowed screen a large number of potential interactors without having to individually identify and clone the corresponding genes. The open ended Y2H cDNA library screens were conducted using each of the three proteins of interest as bait with a tobacco root library as prey. One frequent limitation of Y2H assays is insufficient sampling of the complexity of the Y2H cDNA library. However, because the tobacco cDNA library was propagated in a haploid yeast strain, I was able to mate this with a compatible haploid strain containing the bait construct. Thus, the mating efficiency of yeast is much greater than the plasmid transformation frequency, enabling full sampling of the Y2H cDNA library complexity in the Y2H cDNA library screens. Therefore, we could rule out insufficient sampling of the cDNA library as a reason for not identifying novel interacting clones in the Y2H library. Nevertheless, no reproducible novel protein - protein interactions were identified in these screens beyond the expected SAHH – SAHH and PMT – PMT heterodimers.

Differentiating between true and false positives in Y2H screen is time consuming and is the major disadvantage of this method. In general, the screen did not have a high background rate as only 0.00016% of clones screened produced an initial positive result. Nevertheless, when screening totals that range in the tens of millions of diploid colonies this still constituted a tremendous amount of work. From the total number of positives identified, further characterization revealed 1% of positives and 0.000001% of total clones screened could be attributed to a true protein - protein interaction. In the end, this method is high-throughput in

regards to the number of potential interacting proteins that can be screened, but the subsequent sorting of false positives was nevertheless quite labor intensive.

In contrast to the directed Y2H screen, the library screen identified the expected interaction between PMT-bait and a PMT clone. The PMT-prey cDNA identified from the library screen encoded a truncated PMT, lacking the N-terminus. This result was in contrast to the directed Y2H assays that used identical copies of a full length *PMT* cDNA that did display an interaction. The dichotomy between the full-length and truncated PMT suggests there may be constraints present in the full-length bait and prey that interfere with formation of the dimer. If PMT is unable to form a dimer in the yeast two-hybrid assay, it suggests that native epitopes that could be required for interaction with other proteins may also be absent/altered. It should also be considered that dimerization may not be required for proper protein - protein interactions, but constraints placed on the PMT subunit by the GAL4-domain fusions may inhibit the confirmation necessary for protein - protein interactions to form.

The lack of detectable MPO-bait interactions in both the directed and library screen should be considered. MPO as a protein subunit is over 80 kDa and is believed to function as a dimer. Large bulky proteins, such as MPO, may interfere with the mechanisms inherent to the Y2H reporter gene activation. Large proteins are suspected of hindering the Y2H by either not being unable to localize to the nucleus or inhibiting access of DNA transcription machinery to the promoter regions of the reporter gene (Clontech, technical support). Information regarding the structure of MPO is not available, so it is hard to predict if structural restraints from the fusion domain or if the large size of the protein actually interfered in this assay. It is particularly noteworthy that yeast harboring the MPO-bait plasmid grew slower than yeast harboring either

the SAHH-bait or PMT-bait constructs. Together these results suggest the Y2H assay may not be well suited for detecting MPO protein interactions.

In the end, the yeast two-hybrid did not further our knowledge of protein - protein interactions among PMT, MPO and/or SAHH. In previous characterizations of the SAHH-MPO association, gel filtration studies failed to show co-elution of SAHH and MPO in the same protein fraction (Heim and Jelesko, 2004), suggesting the interaction between SAHH and MPO was likely weak or dynamic. With this fact in mind, the directed Y2H screen was conducted using low stringency conditions to provide the maximum sensitivity for detecting weak interactions, yet no interactions were discovered. The library screen was conducted with the intention of identifying accessory proteins that may be interacting with SAHH, MPO and/or PMT and facilitating the formation of a larger complex. Again, the library screens did not identify any interacting proteins beyond the individual subunits of SAHH and PMT. In summary, the outlined Y2H experiments constituted an exhaustive effort for this experimental method. With that said, the failure of the Y2H system to identify full length PMT-PMT and MPO-MPO interactions demonstrates that this method was not an effective assay for detecting protein - protein interactions with these specific enzymes.

The MATCHMAKER3 system plasmids provided a complementary approach to assay protein - protein interactions using an *in vitro* system that couples DNA transcription with mRNA translation for the production of proteins of interest in a cell-free plant extract. The advantage of this approach is that *in vitro* proteins are expressed without the hindrance of bulky GAL4 fusion domains and the cell free extract provides plant-based translational machinery for protein production. The *in vitro* produced proteins can then be used in co-immunoprecipitation experiments can be performed using epitope-tagged recombinant proteins and specific antiserum.

The *in vitro* system also provides an advantage over co-IPs performed from extracts of the native tobacco root by providing relatively high concentrations of the proteins of interest in an otherwise simpler milieu. Nevertheless, the *in vitro* co-IP experiments did not provide evidence of protein - protein association between SAHH, PMT and MPO. Individual proteins were shown to be immunoprecipitated by specific antisera, but associating proteins were not detected.

The *in vitro* expression system has limitations worth considering. The *in vitro* system does not replicate internal cellular conditions and only provides the proteins of interest in a simple and relatively dilute matrix. The cell-free system does not produce nicotine and does not contain intracellular organelles that may be necessary to facilitate protein - protein interactions. The intracellular localization of nicotine biosynthetic enzymes has not been conclusively determined and protein sorting prediction algorithms predict the localization to be possibly cytoplasmic or peroxisomal. Therefore it is possible specific intracellular conditions in the peroxisome are necessary for SAHH, PMT, and/or MPO interactions and these conditions are not replicated in the cell-free system. Also absent from the *in vitro* system is the machinery necessary to emulate post-translational modifications and the structure provided by cytoskeletal network, both of which could be necessary for protein - protein interactions. Considering the limitations of the system together with the limited knowledge of how these proteins may interact, it is hard to draw firm conclusions from the negative results presented. However, within the limitations of this assay, the proteins do not appear to interact with one another in any direct combination. It is not possible to infer from these results whether the lack of interaction of nicotine biosynthetic enzymes in the *in vitro* system has any physiological relevance.

Co-Immunoprecipitation and immunodepletion

Co-immunoprecipitation of SAHH, PMT and MPO from tobacco extracts provided the advantages that the enzymes were processed in their native environment and were in the presence of the full accompaniment of structural and accessory proteins. Unlike the proteins in the yeast-two hybrid or those produced *in vitro*, the proteins in the tobacco extract were metabolically active and processed in a functioning nicotine biosynthetic environment. Thus, it was an important aspect of this work to investigate the hypothesized association using a cell-free extract derived from native tissue. Additionally, the co-immunoprecipitation experiments attempted to replicate the original immunodepletion of MPO by a SAHH-antiserum that served as the experimental basis of the hypothesis of a nicotine metabolic channel.

The co-immunoprecipitation results presented here did not provide further evidence of associations between SAHH, MPO, and PMT. One goal of this set of experiments was to recapitulate the results of SAHH antiserum immunodepleting MPO activity obtained using McLauchlan's SAHH antisera. Using two independent, newly-prepared SAHH antisera to immunodeplete SAHH from tobacco extract, MPO activity in the supernatant was unaltered. Thus, the original association of SAHH and MPO was specific to the original McLauchlan antisera and was not reproducible with the two newly-produced anti-SAHH sera.

The antigen used for anti-SAHH McLauchlan antiserum and the anti-SAHH sera prepared as a part of this study were produced by different methods. The SAHH antisera produced in this study utilized an antigen derived from a highly purified denatured recombinant TRX-HIS⁶-SAHH protein produced from a bacterial lysate. In contrast, the McLauchlan antigen was purified from tobacco root extract and the purified antigen fraction was believed to consist

primarily of MPO, due to the detection of MPO activity in the purified fraction. The McLauchlan antiserum was originally characterized as being specific for MPO as it appeared to bind a single constituent in a tobacco root extract by western blot and it also depleted MPO activity from the same extract (McLauchlan et al., 1993). Later analysis of the resulting antiserum (Heim and Jelesko, 2004) revealed the primary constituent of the antigen was SAHH instead of the expected MPO and the antiserum exclusively interacted with SAHH expressing clones from a tobacco root cDNA expression library.

Expectations of the co-immunoprecipitation/immunodepletion experiments presented here were that production of new SAHH antisera would reproduce the association between SAHH and MPO originally identified with the McLauchlan antiserum, but this was not the case. The TRX-HIS⁶-SAHH antiserum immunoprecipitated fluorescently labeled recombinant SAHH proteins, so the inability of this antiserum to recapitulate the immunodepletion of MPO activity from tobacco root cell-free extracts cannot be due to an inability to effectively immunoprecipitate SAHH proteins. Many differing aspects of these experiments should be considered when trying to understand these conflicting results. Differences in protein antigen from native versus recombinant sources could provide an explanation for the different specificities of the McLauchlan serum versus the serum reported here. In using native SAHH, the McLauchlan antigen was properly folded and in functional form including oligomerization and any post-translational modifications. Use of the native SAHH as antigen presents epitopes corresponding to the non-linear protein sequence that forms on the surface of the functional protein that are not present in a denatured antigen. Therefore, SAHH binding by the antiserum will occur at different epitopes when using the McLauchlan antiserum as opposed to the antiserum presented here. The differences in epitope binding sites could account for the

differences in the immunodepletion results. For example the McLauchlan antiserum may immunoprecipitate SAHH in the native conformation, while the anti-SAHH serum produced here induces a conformational change in the enzyme upon binding, thereby disrupting SAHH – MPO protein - protein interactions.

An additional consideration is the McLauchlan antiserum was prepared from a purified SAHH fraction that contained MPO activity, suggesting a small amount of the purified antigen was MPO. Therefore, the polyclonal antiserum produced in response to the presence of two antigens may have generated one population of antibodies specific to SAHH and another population to MPO. Use of this antiserum in immunodepletion would create the appearance of SAHH assisted MPO depletion without the need for physical interaction of the proteins. An experimentally proven explanation of how the McLauchlan antiserum is capable of depleting MPO by targeting SAHH may not be possible due to the limited amount of the original McLauchlan antiserum remaining.

Technical difficulties experienced with this set of experiments also limit our ability to fully interpret the results. MPO antisera produced was characterized to have a relatively low affinity for the antigen and subsequently provided a high background on western blots. Using the antiserum in immunodepletion assays, the antiserum immunodepleted much less native MPO activity. Additionally none of the MPO antisera could provide conclusive results for the detection of MPO on a western blot, complicating the detection of MPO in SAHH or PMT immunoprecipitated material. Although direct detection of MPO was not available, the indirect method of immunodepletion of MPO enzyme activity was used, as has been done previously (McLauchlan et al., 1993; Heim and Jelesko, 2004) to track the MPO containing fraction.

Another technical difficulty encountered in these experiments is that the size of SAHH is closely aligned with the size of the heavy chain of IgG complicating analysis of immunoprecipitates by SDS-PAGE and subsequent western blot. The interference of IgG and the relatively large concentration of IgG in comparison to SAHH obscured the detection of a SAHH-specific band when analyzing immunoprecipitated material by western blot. Alternate methods of detection such as using ELISA and IgG adsorbed secondary antiserum were investigated with some degree of success, but neither proved to assist in providing conclusive evidence of SAHH presence in an immunoprecipitated fraction.

Future directions in nicotine metabolic channel research

Three complementary experimental methods were used to investigate the hypothesis that nicotine biosynthesis involves a multienzyme complex including SAHH, PMT, and MPO. The choice of these experimental methods were based upon the complementary nature of their respective advantages and disadvantages, as well as the fact that the Jelesko lab was more familiar with these experimental approaches than other *in vivo* methods used to assess protein – protein interactions. Taken together these experiments did not produce additional evidence for protein - protein interaction between SAHH and MPO or of a larger nicotine metabolic channel.

The set of experiments described above suggest that if a nicotine metabolic complex is present in tobacco, it likely does not involve stable protein - protein interactions among SAHH, PMT and/or MPO. Weak or transient protein – protein interactions are inherently difficult to detect with Y2H and co-immunoprecipitation methods. The difficulty of obtaining evidence for a weak metabolic channel has been evidenced in research geared to establish the existence of

protein – protein interactions involved in the enzymes responsible for the production of the plant secondary metabolite dhurrin. Dhurrin biosynthesis was initially believed to involve channeling based upon lability and high toxicity of biosynthetic intermediates, a common theme for compounds typically sequestered by metabolic channeling. However, microsomal fractions contained the two cytochrome P450s necessary for dhurrin biosynthesis, but did not contain the final glucosyltransferase, suggesting a weak association of the pathway enzymes, at best. More recent studies utilizing the reconstituted dhurrin pathway in *Arabidopsis* and a combination of fluorescent-fusion proteins, enzyme activity assays and high-resolution laser confocal microscopy have provided a higher resolution view and evidence for the establishment of a dhurrin metabolon (Nielsen et al., 2008). Fluorescent-fusion proteins that retained individual enzyme activity were not able to produce the expected end product *in planta*. The abolished biosynthetic activity *in planta* was determined to result from an N-terminal fluorophore-fusion that did not abolish enzyme activity, but blocked the epitope for protein-protein interaction between the P450s. Additionally, the fluorophore reporters showed the P450's localized to a subset of the ER membrane and localization of the glucosyltransferase was also associated with this subset of the ER, but only in the presence of the P450s. These experiments provide interesting results as they do not directly rely on the interaction of proteins to activate a reporter, but simply show that the proteins are in close proximity when the end product is formed.

Similarly, a recent report investigating enzymes of purine biosynthesis in mammalian cell culture addressed the complexity of detecting protein - protein interactions in a metabolic channel (An et al., 2008). Previous attempts at establishing evidence for interaction of the enzymes of purine biosynthesis using standard protein - protein interaction methodologies did not support the hypothesis of a metabolic channel among purine biosynthetic enzymes.

However, using fluorescent reporter technologies to view proteins *in vivo* they were able to show the enzymes clustered in a manner that supported the hypothesis of a purine metabolic channel. Using pairwise combinations of the purine biosynthetic enzymes fused with either green fluorescent protein (GFP) or orange fluorescent protein (OFP) they showed the proteins clustered to the punctate positions within the cytoplasm. Moreover, they determined that this co-localization was not static, but rather dependent on the endogenous purine level in the culture media. During purine depleting conditions, which stimulated de novo purine biosynthesis, the enzymes were viewed in the same punctate pattern, but when purines were added to the culture media, the purine biosynthetic enzymes were dispersed throughout the cytoplasm.

These recent studies emphasize the importance and complexity of intracellular conditions in the formation and detection of metabolic channels. In these cases simply The finding in purine biosynthesis suggests that heterologous expression systems such as the Y2H system and *in vitro* co-immunoprecipitation from cell free extracts do not adequately recapitulate as yet undetermined intracellular conditions that are required for the formation of the purine multienzyme metabolic channel. Thus, future efforts to characterize a nicotine metabolic channel should be focused more on *in vivo* imaging systems to localize SAHH, MPO, and PMT. The localization of SAHH/MPO/PMT enzymes by fluorophore fusions in nicotine biosynthetic cells will allow the researcher to apply conditions known to induce nicotine biosynthesis and follow the localization of the enzymes under normal and induced conditions. Although this technique does not directly indicate that intimate protein - protein interactions are occurring, it is a powerful tool allowing for an overview of the proteins of interest in living cells and under relevant nicotine biosynthetic conditions.

It should also be considered that the toolbox of current available research methods along with our present understanding of both nicotine biosynthesis and protein-protein interactions is insufficient to provide the resolution necessary to evaluate the hypothesis of a nicotine metabolic channel. Together the research presented here suggests stable protein-protein interactions do not occur directly between SAHH/PMT/MPO, but these results also do further support the hypothesis that protein interactions are necessary for nicotine biosynthesis. As further research into nicotine biosynthesis provides details regarding the cell-type and sub-cellular localization of nicotine biosynthetic enzymes, structural models of the enzymes, identification of post-translational modifications and further elucidation of the enzymes involved in nicotine biosynthesis the hypothesis of a nicotine metabolic channel should be revisited. The aforementioned research areas will enlighten our knowledge regarding nicotine biosynthesis and provide clues as to if and how nicotine biosynthetic enzymes may form a channel.

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CHAPTER III

Characterization of the NUP1 transporter from *Nicotiana tabacum*

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Abstract

Nicotine accumulates in tobacco leaves where it acts as a chemical defense against insect herbivory. Nicotine biosynthesis in tobacco occurs in the plant root, although nicotine accumulation occurs throughout the plant. The transport of nicotine from the site of underground synthesis to the aerial tissues occurs through the xylem. The molecular mechanisms of nicotine transport are poorly understood. I report here additional characterization of the Nicotine Uptake Permease (NUP1), a nicotine transporter. NUP1 subcellular localization was performed utilizing a NUP-GFP fusion protein where NUP1 was localized to the plasma membrane. Additionally nicotine levels were measured in *NUP1-RNAi* plants and hairy roots. The down regulation of *NUP1* mRNA levels was correlated with reduced nicotine concentrations in *NUP1-RNAi* plants and hairy roots. Additionally, *NUP1-RNAi* hairy roots displayed a shift of nicotine from the root into the

culture medium. The impact of the identification and characterization of NUP1 to the field of alkaloid transport is discussed.

Introduction

Nicotiana tabacum is a domesticated member of the *Nicotiana* genera and is currently grown as an important cash crop in the southern United States (United States Department of Agriculture, 2009). Nicotine is an alkaloid that accumulates to high concentrations in leaves of domesticated tobacco plants. The increase of nicotine in leaves was the predominant trait selected for during the domestication of tobacco and the nicotine containing leaves are the agricultural commodity of tobacco crops. Commercial use of tobacco is primarily in the production of tobacco products for non-nutritive human consumption for mood-altering effects.

The production of small molecule chemical compounds is the primary mechanism that plants use to defend themselves against herbivores. Alkaloids are a large class of nitrogen-containing plant secondary molecules that are implicated as plant defense compounds (Wink, 1998). Nicotine, the predominant tobacco alkaloid, is associated with higher plant fitness values and is believed to function as an insect deterrent (Steppuhn et al., 2004). Thus, the same compound exploited by humans for mood altering affects is in fact an ecological defense mechanism against insects. Given that insect herbivory entails wounding it is not surprising that nicotine production is increased in response to foliar wounding (Baldwin, 1989; Baldwin et al., 1997; Chintapakorn and Hamill, 2003), the

application of insect regurgitates (McCloud and Baldwin, 1997; Halitschke et al., 2003; Hui et al., 2003), and removal of the plant apical meristem (Hibi et al., 1994).

Nicotine biosynthesis in tobacco is correlated with two distinct genetic loci originally referred to as *A* and *B* (Legg et al., 1970) and later additionally named *NIC1* and *NIC2*, respectively (Hibi et al., 1994). Jasmonic acid and the volatile form methyljasmonate (MeJA) are defense hormones produced in response to foliar wounding (Creelman and Mullet, 1997), and their increased production results in a sharp increase in the transcription of nicotine biosynthetic genes (Imanishi et al., 1998; Shoji et al., 2000; Shoji et al., 2000) and in whole-plant nicotine levels (Baldwin et al., 1994; Ohnmeiss et al., 1997; Baldwin, 1999). Auxin is a plant hormone controlling many aspects of growth and development and disruption of the normal auxin supply results in an increase in nicotine biosynthetic gene expression levels (Hibi et al., 1994; Shoji et al., 2000; Chintapakorn and Hamill, 2003; Heim et al., 2006; Kidd et al., 2006). Near isogenic tobacco lines containing mutations at the *A* and *B* loci (Legg et al., 1969; Legg et al., 1970; Legg and Collins, 1971) combined with the elicitation of nicotine biosynthesis by altering hormonal levels has been used as a tool to identify the nicotine related genes by differential expression patterns. The use of transcript profiling from tissues with different nicotine biosynthetic capacity has led to the cloning of genes participating in nicotine biosynthesis (*PMT*, *MPO* and *A622*), and nicotine transport (*JAT1* and *NtMATE1*) (Hibi et al., 1994; Katoh et al., 2007; Shoji et al., 2008; Morita et al., 2009).

Nicotine biosynthetic genes are expressed in the root (Hibi et al., 1994; Reed and Jelesko, 2004; Sinclair et al., 2004; Cane et al., 2005; Heim et al., 2007; Katoh et al., 2007) and proteins with the respective activities have been isolated from root extracts

(Mizusaki et al., 1971; Mizusaki et al., 1972; Mizusaki et al., 1973; Saunders and Bush, 1979; Davies et al., 1989). However the exact location of nicotine biosynthesis within the root is not well defined. This is in large part because interpretation of the information currently available is complicated by the use of different *Nicotiana* species and both wild type and transgenic roots in different stages of development. *PMT* and *A622* transcript and protein localization studies were conducted with both *promoter:GUS* fusions in *N. sylvestris* and using immunolocalization in *N. tabacum* root tissue. GUS staining corresponding to expression of both proteins occurred along the length of the root of 14-day old seedlings, but staining was not observed in the root tip (most apical 0.5 mm) (Shoji et al., 2000; Shoji et al., 2002). On the other hand, GUS staining was observed primarily in the tips of transgenic hairy roots (Shoji et al., 2000; Shoji et al., 2002). Interpretation of localization based upon reporter gene expression should be viewed with caution, as stability and turnover of GUS may be different than the normal protein product. Therefore, experiments designed to evaluate protein accumulation along the root are a better gauge of the localization of nicotine biosynthetic enzymes. To this end *PMT* and *A622* enzyme localization was examined by western blot using protein extracts prepared from consecutive 5 mm root sections of 6 week old *N. tabacum* primary roots. Both *PMT* and *A622* protein levels were most abundant in the apical portions of the root with the majority of the protein present in the first 1 cm from the root tip (Shoji et al., 2002). In concert with *PMT* and *A622* being more abundant in the apical regions, MPO activity is also more concentrated in the bottom 1 cm of hairy roots in comparison to the activity associated with an entire root extract (Sherry Hildreth, unpublished results). The localization of the expression of nicotine biosynthetic genes and the localization of the

respective enzyme activity correlate with early tobacco root physiological studies showing nicotine production is associated with the growing tips of tobacco root cultures (Solt, 1957). The data available regarding longitudinal localization of nicotine biosynthesis suggests a subset of cells in the root apical region are the predominant site for nicotine biosynthesis.

The determination of the cell-type specific expression patterns of nicotine biosynthetic genes *PMT* and *A622* has been addressed utilizing both *promoter:GUS* fusions and immunohistochemistry. *PMT-promoter:GUS* and *A622-promoter:GUS* expression was observed in the xylem, endodermis and outer cortex of fully differentiated (root hairs present) hairy root sections (Shoji et al., 2000; Shoji et al., 2002). In contrast, immunolocalization of *PMT* and *A622* determined protein was present in all layers of the cortex and in the endodermis (Shoji et al., 2000; Shoji et al., 2002) of primary roots. The observed difference in localization patterns could be related to the region of the root from which the longitudinal section was obtained. In spite of the aforementioned differences in localization, a section from the apical region of the *A622 promoter::GUS* that had not differentiated (no root hairs present) displayed a cell-specific pattern identical to that of the *A622* and *PMT* immunolocalized section. As mentioned in the discussion of the longitudinal localization of nicotine, *GUS* reporter assays report where transcription has occurred and since the enzyme is highly stable the staining pattern may not accurately represent the current location of gene expression. Therefore, we can conclude from the immunological and immunohistochemical studies *A622* and *PMT* accumulate in the cortex and endodermis of the apical root, but further studies involving the differentiated

root are needed to determine if the observed GUS patterns are relevant to actual enzyme accumulation.

Nicotine is only produced in the roots of *Nicotiana tabacum* (Dawson, 1942; Dawson, 1942; Tso, 1972). Nicotine moves from the roots to the shoots via the xylem, where concentrations as high as 1 mM (Baldwin, 1989) can be found. Although the biosynthesis is localized exclusively to the root, nicotine is distributed in all above ground tissues (leaves, stems and flowers), where it accumulates within the cell vacuole (Saunders, 1979). While nicotine is found in all plant tissues, nicotine is not uniformly distributed among these tissues and its distribution correlates well with the Optimal Defense Theory (OD) (Ohnmeiss and Baldwin, 2000). The OD theory predicts that the allocation of defensive secondary metabolites within the plant will be positively correlated with the fitness value of the particular plant parts (McKey, 1974; Rhoades, 1979). Nicotine distribution patterns *in planta* follow the OD theory with nicotine accumulating in tissues with a higher assessed fitness value (Ohnmeiss and Baldwin, 2000). Studies of within-plant nicotine levels show that the distribution of nicotine throughout the ontology of the plant is not static, but changes as the associated fitness values of the plant parts change (Ohnmeiss and Baldwin, 2000). Moreover, whole plant nicotine levels are kept at a constant proportion relative to whole-plant mass (Ohnmeiss and Baldwin, 1994) in spite of differential accumulation in younger tissues.

Nicotine movement from the site of biosynthesis in the root to aerial tissues necessitates several independent transport events. At a minimum, we can assume nicotine is exported from the biosynthetic cell, loaded into the xylem, imported into individual cells, and imported from the cytoplasm into the vacuole. Since old leaf tissue

contains less nicotine than leaves at younger developmental stages, one can also predict transport processes from the vacuole to the cytoplasm and then into either the symplastic or apoplastic space. Thus, several types of transmembrane biochemical transport activities are necessary for the observed distribution and accumulation of this inducible defense chemical.

The first detailed studies of how transporters are involved in alkaloid movement within plants were performed in *Coptis japonica* in which the alkaloid berberine is synthesized in the root yet accumulates preferentially in the storage organ the rhizome. Identification of an ABC transporter of the MDR subfamily from *C. japonica* (Yazaki et al., 2001) provided the first in-depth characterization of an alkaloid transporter. CjMDR1 is a rhizome-localized transporter with demonstrated berberine transport activity when expressed in *Xenopus* oocytes (Shitan et al., 2003). CjMDR1 is localized to the plasma membrane and is believed to function in berberine uptake into the rhizome for storage (Shitan et al., 2003). In addition to rhizome uptake of berberine, *C. japonica* cultured cells also accumulate berberine in the vacuole. Studies to identify the mode of transport into the vacuole revealed an H⁺ antiporter of the MATE class was likely involved (Otani, 2005). Thus, two independent transport mechanisms appear to be used in the same cell type to properly sequester the alkaloid berberine.

While there is a lot of physiological data regarding nicotine movement in whole plants, much less is known about the hypothesized transporters required to move nicotine into and out of specific cell types and subcellular organelles. The first reports directed at understanding nicotine transport in tobacco began with the cloning of two genes encoding MATE transporters. *Nicotiana tabacum* Multidrug and Toxin Extrusion (*NtMATE1*), and

subsequently *NtMATE2*, were identified as having higher mRNA expression levels in the *AABB* genotype relative to the *aabb* genotype of domesticated tobacco, suggesting the transporter was coordinately regulated with known nicotine biosynthetic genes (Shoji et al., 2008). *NtMATE1* appears to be root specific with some expression in the flower and *NtMATE1-promoter:GUS* fusions showed a GUS staining pattern in the root that was MeJA responsive and similar to that of known nicotine. Heterologous expression of *NtMATE1* in yeast confirmed that it transports nicotine as well as the tropane alkaloids hyoscyamine and scopolamine, albeit at slightly reduced efficiencies. Plants with reduced *NtMATE1* expression levels did not display a reduction in plant nicotine or an altered root to shoot distribution of nicotine. However, *NtMATE1* reduced-expression plants were more sensitive to root growth inhibition in response to high levels of exogenous nicotine. *NtMATE* is located on the tonoplast of the vacuole suggesting that *NtMATE1* may be responsible for sequestering cytotoxic compounds that include but are not limited to nicotine as part of a generalized defense against toxic small molecules in the cytoplasm.

Nicotiana tabacum Jasmonate-inducible Alkaloid Transporter 1 (*NtJAT1*) was identified in jasmonate elicited *Nicotiana tabacum* BY-2 cell cultures (Goossens et al., 2003; Morita et al., 2009). *NtJAT1* is expressed in all tissues examined (leaf, stem, root) and expression in aerial portions of the plant is increased in response to MeJA as well as to other known defense and stress response elicitors. Heterologous expression in yeast demonstrated the ability of *NtJAT1* to transport nicotine and expression of *NtJAT1* in proteoliposomes facilitated transport of the alkaloids nicotine, hyoscyamine, berberine and anabasine, but did not facilitate transport of the flavonols, kaempferol and quercetin.

The relationship of NtJAT1 to nicotine physiology has yet to be examined and at this time it is not known if NtJAT1 is a transporter with *in vivo* specificity for nicotine sequestration in the vacuole or is part of a general small compound detoxification mechanism.

Materials and Methods

Creation of *NUP1*-RNAi transgenic plants and corresponding hairy root cultures

Oligonucleotides oEAT24 (TCTAGACTCGAGTTGGCATATGTGGTGGTCC TCTA) and oEAT25 (ATCGATGGTACCTTTACTTGTCACGCCCTCTGGTC) were used in standard Taq PCR reactions with pEAT1 as template to amplify a 448 bp PCR fragment internal to the *NUP1* coding region. The resulting PCR fragment resulted in the addition of 5' nested *XhoI* and *XbaI* sites as well as 3' *ClaI* and *KpnI* sites. These additional restriction sites were used to first subclone a *XhoI*-*KpnI* and then a subsequent *ClaI*-*XbaI* fragment into pHANNIBAL (Wesley et al., 2001), resulting in plasmid pEAT12. A 2.5 Kb *NcoI* fragment containing the *NUP1*-RNAi construct was filled in using Klenow Fragment and subcloned into pCAMBIA2300 cut with *SmaI*. This resulted in two plasmids (i.e. pEAT13 and pEAT14) that differed only in the orientation of the *NUP1*-RNAi fragment in the binary vector. Plasmids pEAT13 and pEAT14 were conjugated from *E. coli* into *Agrobacterium tumefaciens* LBA4404 using the pRK2013 helper plasmid (Figurski and Helinski, 1979). LBA4404 containing either pEAT13 or pEAT14 was used to transform axenic *Nicotiana tabacum* cultivar Xanthi plants using a rapid petiole inoculation method (Medina-Bolivar et al., 2003). Transgenic T1 shootlets were regenerated into whole plants and T2 seed was collected. T3 seed was screened on 0.5X MS plates with 50 μ g Kanamycin sulfate to identify T3 lines that were homozygous for the transgene. During the axenic cultivation/regeneration of T1 transgenic plants,

single leaves were removed from the transgenic shootlets/plantlets and were inoculated in the midrib with *Agrobacterium rhizogenes* strain ATCC15834 to generate corresponding hairy root cultures of each initial T1 transgenic plant (Medina-Bolivar et al.). Hairy root cultures that were cleared of *Agrobacterium* strains were propagated on Gamborgs B5 medium with B5 vitamins and 2% sucrose.

Growth conditions for NUP1-RNAi transgenic plants and corresponding Hairy Root Cultures

Liquid hairy root cultures were propagated in 60 ml of Gamborgs B5 medium (3.2 g Gamborgs B5 salts; 5% sucrose; 1 mL Gamborgs B5 Vitamins/ L) within sterile 250 ml Erlenmeyer flasks fitted with plastic foam stoppers rotating at 90 rpm at room temperature in the light. To induce alkaloid biosynthesis twelve-day old hairy root cultures were washed three times with 25 ml of fresh Gamborgs B5 and then resuspended in 60 ml of Gamborgs B5 liquid medium supplemented with either 100 μ M MeJA (treatment) and 0.02% DMSO or Gamborgs B5 medium with 0.02% DMSO alone (control). Treated hairy roots were grown for 24 hours, harvested, patted dry, flash frozen in liquid nitrogen, and stored at -80°C. Culture medium from the twelve-day old hairy roots and from the subsequent 24-hr induction period were collected and stored at -20°C. Complete experimental replicates were performed on three different days.

Plants for analysis of leaf nicotine were grown in soil for 20 days. At 20 days, all aerial tissues of the plant were removed, harvested, flash frozen and stored at -80°C. Biological replicates were performed on three different days. The fresh weight of the day 12-13

roots was recorded and converted to a dry weight using the empirically-derived conversion factor.

Quantification of nicotine levels

Hairy root tissues stored at -80°C were ground to a fine powder using a liquid nitrogen cooled mortar and pestle. A sample of the frozen tissue was lyophilized and stored in screw cap tubes at room temperature in a desiccated chamber. The dried plant material was extracted and analyzed for nicotine levels using a simple aqueous extraction procedure and HPLC method (Saunders and Blume, 1981). HPLC was performed on a Waters 2695 Separation unit a Waters 996 photodiode array detector. A Zorbax Eclipse XDB-C18 4.6 x 250 mm x 5 micron column (Agilent Technologies, Santa Clara, CA) was used for separation of extracted compounds. Nicotine (Sigma, St. Louis, Mo) was used to generate a standard curve and the amount of nicotine in each extract or medium sample was quantified using the Waters Empower Pro software build 1154 (Waters Corporation, Milford, MA). As an instrument control, duplicate injections for each sample were analyzed and then averaged to obtain the nicotine levels in each extract or medium sample. Statistical analysis of nicotine levels was performed using a General Linear Model (GLM) ANOVA with Tukey-correction using Minitab version 14.13 (Minitab Inc, State College, PA). Strong statistical support was defined with $P \leq 0.0500$ and moderate support was defined as $P - \text{values} \geq 0.05 \leq 0.1$. Foliar tissue nicotine analysis was performed in the same method as the hairy root tissue. All aerial parts of the

plant were included in the homogenization and the resulting powder was a combination of leaf and stem tissue.

NUP1 localization

The NUP1 translation termination codon was replaced with a serine codon followed by an in-frame ATG codon embedded within a *NcoI* recognition site by using pEAT1 (Kidd et al., 2006) as template DNA and oligonucleotide primers oEAT1 (5'-CAGTTTTGCTCCCCGTTAC-3') and oEAT9 (5'-TCTCGAGGTCACCTACCATG GAAACAGATTCAGTATGTG-3') in PCR reactions that generated a modified fragment. This PCR fragment was subcloned into pEAT1 as a *BsmBI* – *XhoI* fragment. A *GFP* gene was excised as a *NcoI* – *XhoI* fragment from pCAMBIA1302 (Cambia, Canberra, Australia) and subcloned into the modified pEAT1 plasmid described above to yield plasmid pEAT11 (*NUP1:GFP* gene fusion). The NUP1 coding region was excised and subcloned into plasmid pGFP-MRC (Rodriguez-Concepcion et al., 1999) similarly cut to create plasmid pJGJ402. Plasmids pGFP-MRC and pJGJ402 were used to transform BY-2 protoplasts were prepared by the method of Merkle, et al.(1996) and the transformation was performed by the method of Negrutiu, et al. (1987). The transformed BY-2 protoplasts were imaged 24 hours post-transformation using a Zeiss LSM 510 Laser Scanning Microscope with 488 nm laser excitation (Zeiss, Thornwood, NY).

Nicotine transport assays

A *NcoI* site was introduced into the *NUP1* ATG codon by PCR using oligonucleotide primers oJGJ199 (GTCTAGACCATGGAACATCCAGGATTAAGCA)

and oJGJ200 (GCAAGAGGTATAAGGGTGAGTG) to create a PCR fragment with a modified ATG codon. This PCR fragment subcloned into pCR2.1 to create pJGJ391, which was sequenced. A *Xba*I – *Nde*I fragment was cut from pJGJ391 and subcloned into pEAT1 digested with the same enzymes to create pJGJ392. A full length *NUP1* region was removed from pJGJ392 and subcloned into pTM1 similarly digested to create pJGJ393. A *Nco*I - *Sal*I *NUP1* fragment was cut from pJGJ393 and ligated into *Nde*I - *Sal*I sites in the *Schizosaccharomyces pombe* expression vector pREP41 after the *Nco*I and *Nde*I ends in the polylinker were blunted. *NUP1* was placed under the control of the *nmt41* inducible promoter (Basi et al., 1993). Empty vector pREP41 and the construct pREP41-NUP1 were transfected into the *Schizosaccharomyces pombe* wild-type strain YF016 (*h-*, *leu1-32*, *ura4-C190T*, *ade7::ura4*). Four separate positive transformants were selected in Edinburgh minimal medium (EEB) without Leu and confirmed with PCR. Pombe cells were grown to $OD_{600} = 2.0$ in Edinburgh minimal medium (EMM) containing 15- μ M thiamine. The thiamine was removed by washing twice with EMM, and cells were transferred to fresh EMM and incubated for 19 hours (final $OD_{600} = 1-2$) to induce the expression of the NUP1 protein. Cells were spun at 6,000g for 30 seconds. Pellets were washed once and resuspended in EMM pH 5.6. Cell density was determined again to ensure the $OD_{600} = 2$. Cell samples were kept at 4 °C in all following steps except 30 °C incubation. One μ l 14 C-nicotine (0.1 μ Ci, final concentration ~ 20 nM) (specific activity 54 mCi mmol^{-1} , Moravek Biochemicals) was added into 100 μ l cells ($OD_{600}=2$), and incubated at 30 °C for 0, 100, 200, and 300 seconds. Cells were washed twice with EMM, and resuspended in 0.5 ml EMM. 250 μ l cell aliquots were taken and retained radioactivity was quantified by scintillation counting. In the inhibitor

experiments, one μl of 2 μM inhibitor (final concentration 200nM) was added together with one μl ^{14}C -nicotine in 100 μl cells ($\text{OD}_{600} = 2$), and incubated at 30 °C for 300 seconds. Cells were washed twice with EMM, and resuspended in 0.5 ml EMM. 250 μl cell aliquots were taken and retained radioactivity was quantified by scintillation counting.

Quantification of gene-specific mRNA levels in hairy root cultures

RNA isolation, reverse transcription, and QRT-PCR conditions were performed as previously described (Kidd et al., 2006). *QPT*, *QS*, and purine permease-like (*PUP/NUPI* oligonucleotide primers and control plasmids were previously described (Kidd et al., 2006). Plasmid pDGR58 and oligonucleotide pair oJGJ203 (GTTTACATCCGGCCACCAGTT) and oJGJ204 (AAAAAACCGACCCGGTTCAG) were used to assay *NtODC* mRNA levels. Plasmid pWGH15 (Heim et al., 2007) and oligonucleotide pair oJGJ205 (TCACACATGTTTCCTCGGTTGG) and oJGJ206 (TCAACAGCCGGAGAGCAGTTA) were used to assay *MPOI* mRNA levels. *PMT* mRNA levels were assayed by QRT-PCR as previously described (Reed and Jelesko, 2004).

RESULTS

Alkaloid Analysis of *NUPI-RNAi* Hairy Root Cultures

In order to examine the physiological effects of reduced *NUPI* expression *NUPI-RNAi* plants and corresponding hairy root cultures were constructed by Dr. Rong-He Lu in the Jelesko laboratory. *NUPI-RNAi* T-DNA binary plasmids pEAT13 and pEAT14 contain inverted repeats of a *NUPI* cDNA fragment linked by a short intron. Expression of the *NUPI-RNAi* gene resulted in a *NUPI* mRNA transcript that will produce a double stranded *NUPI* mRNA hairpin structure. The *NUPI* mRNA hairpin will in turn induce the RNA interference pathway to degrade endogenous *NUPI* mRNAs. Plasmids pEAT13 and pEAT14 differ only in their relative orientation of the *NUPI-RNAi* insert within the pHANNIBAL plasmid (Wesley et al., 2001). Initial transgenic plants were transformed with *Agrobacterium tumefaciens* strains containing either pEAT13 or pEAT14. Hairy root lines were created from these primary transformed shootlets by excising sterile leaves and inoculating these with a strain of *Agrobacterium rhizogenes* to establish individual hairy root lines, each corresponding to the original transformed line. In total, four independent transgenic plant lines containing the *NUPI-RNAi* derived from plasmid pEAT13 were generated: NtEAT13-8, NtEAT13-10, NtEAT13-22, and NtEAT13-55 (referred hereafter collectively as “NtEAT13-x” lines). Another transgenic plant line containing the same *NUPI-RNAi* construct, but in the opposite orientation in the T-DNA binary vector was also created and called NtEAT14-1. As shown in Figure 3.1 endogenous steady state *NUPI* mRNA levels in the RNAi hairy roots were significantly decreased in comparison with a wild type Xanthi hairy root line (ANOVA

p-value < 0.05). The four NtEAT13-x lines consistently showed the lowest levels of *NUPI* mRNA accumulation, while NtEAT14-1 showed significantly reduced *NUPI* mRNA accumulation levels that were intermediate between the NtEAT13-x lines and the Xanthi-1 control.

Hairy root cultures were grown for 12 days (12 day cultures), the media harvested, and replaced with either fresh media supplemented either with 0.02% DMSO (control) or fresh media supplemented with 100 μ M MeJA. After 24 hours of additional growth (day 12-13 cultures) both the culture medium and the hairy roots were independently collected. Aqueous extractions from day 12-13 hairy roots were analyzed to determine the effect of reduced *NUPI* gene expression levels on alkaloid accumulation. Extracts from hairy root cultures were analyzed for nicotine as well as the related pyridine alkaloids nornicotine, anabasine, and anatabine. Nicotine was the only alkaloid detected at quantifiable levels and is the only alkaloid value presented here.

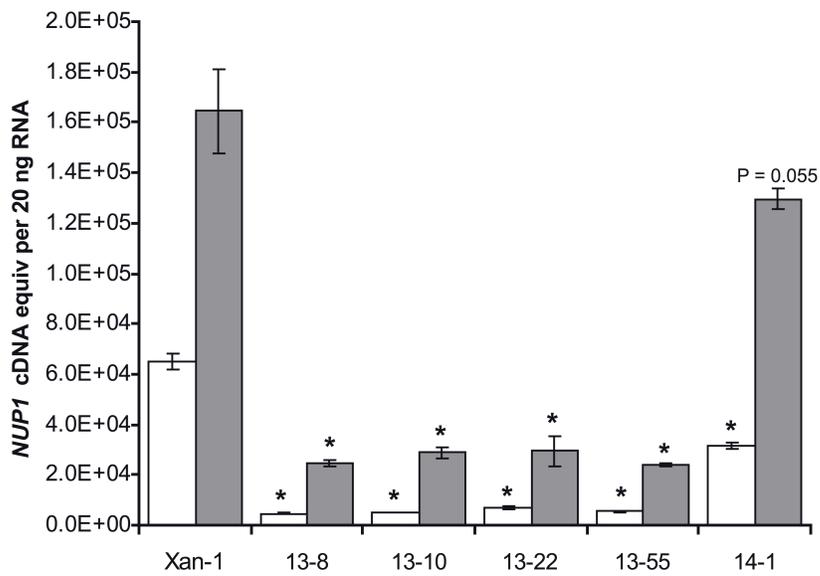
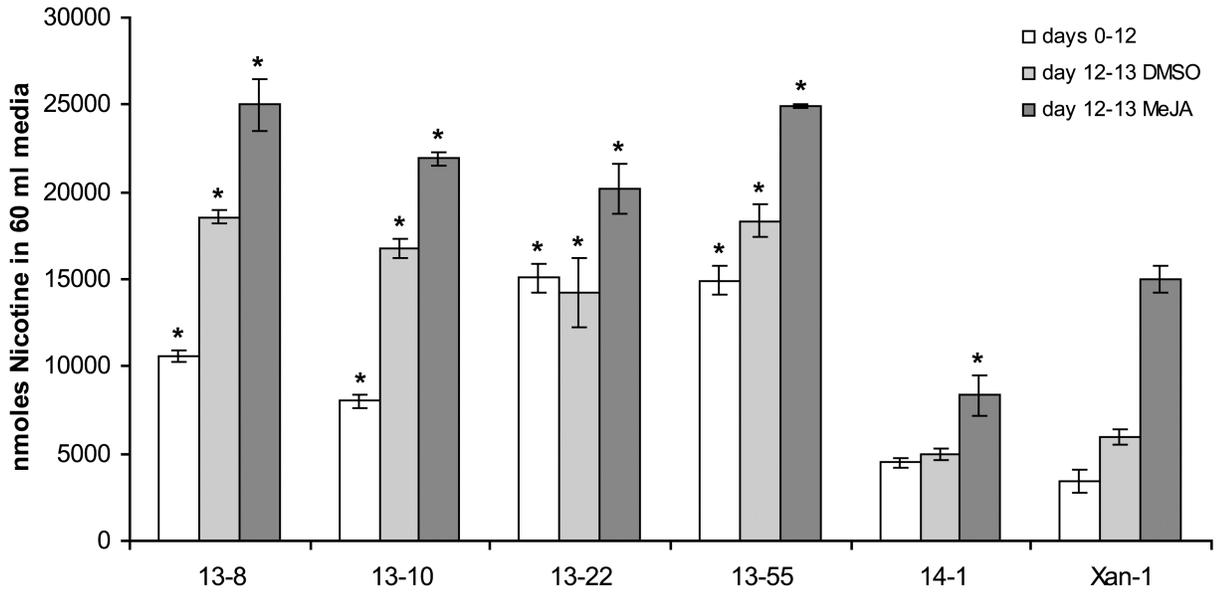


Figure 3.1: QRT-PCR estimates of *NUP1* mRNA expression levels. Asterisks indicate GLM ANOVA with Tukey correction P-value ≤ 0.05 ; numbers above bar indicate moderate statistical support with P-values between 0.05 – 0.10 relative to Xan-1 control values.

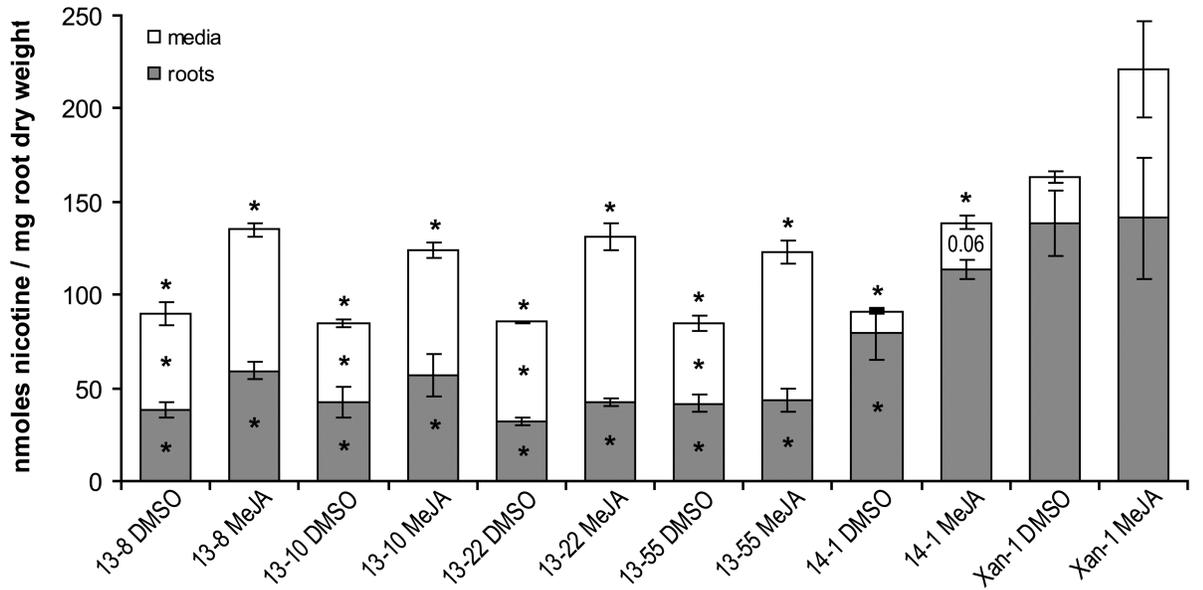
Hairy roots of *Nicotiana* species grown in culture accumulate nicotine in the roots and in the culture media (Hamill et al., 1986). Therefore, the nicotine content of the hairy root culture medium corresponding to both the 0-12 treatment and day 12-13 treatment (Figure 3.2.A) was analyzed. Since the root material at day 12 was not harvested, the nicotine levels could not be normalized to total mass and the values shown in Figure 3.2.A refer to the total nicotine content in 60 ml of culture medium. Consistent with the effect of MeJA in stimulating nicotine biosynthesis, the medium from the day 12-13 Xanthi-1 root culture contained more nicotine than did the non-induced roots subjected to DMSO treatment. Additionally, the day 12-13 Xanthi-1 cultures contained as much, or more, nicotine in the medium than had accumulated during the prior 12 days. These data show that nicotine accumulation in the medium is an on-going process and measurement of the nicotine associated with the medium is a necessary component when analyzing nicotine physiology of hairy root cultures.

The measurement of nicotine in the medium (Figure 3.2.A) also showed significantly higher nicotine levels in the four NtEAT13-x *NUPI-RNAi* lines relative to the control Xanthi line (P-value ≤ 0.0500). These differences were observed in both the days 0-12 and day 12-13 cultures. In contrast, line NtEAT14-1 did not show significant

A.



B.



C.

Ratio of nicotine in media / total 0.57* 0.56 0.50* 0.55 0.63* 0.68 (P=0.053) 0.51* 0.65 (P=0.092) 0.13 0.18 (P=0.055) 0.16 0.37

Figure 3.2: Nicotine in NUP1-RNAi hairy roots.

A. Nicotine present in the 60 ml of culture media at day 12 and at day 13.

B. Total nicotine (nmol/mg dry wt.) present in the closed hairy root system, grey bars represent root nicotine, white bars represent nicotine in media.

C. The ratio of nicotine in the media to the total nicotine

(*) represents statistical significance between NUP1-mRNA lines and Xanthi ($P < 0.05$).

Near significance ($P < 0.10$) is represented as the (P-value).

differences in nicotine accumulation in the medium relative to the Xanthi-1 control during DMSO treatment. The only significant difference in nicotine accumulation observed in the medium of line NtEAT14-1 was in the day 12-13 MeJA treatment, in which line NtEAT14-1 accumulated less nicotine than the wild type control. These data show an association between reduced *NUPI* mRNA accumulation in the NtEAT13-x lines and increased amounts of nicotine present in the culture medium independent of the treatment.

In order to normalize for differences in root growth/mass at the time of harvest, the nicotine levels in the root tissue were normalized to root dry weight at the end of day 13. As shown in Figure 3.2.B, all four NtEAT13-x lines showed significantly less nicotine associated with the roots than did the Xanthi-1 line (ANOVA P-value ≤ 0.05). The reduction in root nicotine levels in the NtEAT13-x lines was independent of elicitation by MeJA treatment. During control conditions (DMSO treatment) NtEAT14-1 showed an intermediate root alkaloid phenotype with higher root nicotine levels relative to the NtEAT13-x lines, yet still significantly lower than wild type Xanthi-1 (P-value < 0.05). However, during MeJA treatment the NtEAT14-1 line did not show a statistically significant difference in root nicotine accumulation relative to the wild type Xanthi-1.

Since the increased nicotine levels in the culture medium of the *NUPI-RNAi* lines did not correlate with an increase in nicotine associated with the roots, we further examined the nicotine levels to determine if the total amount of nicotine in the entire hairy root system was altered. In order to determine the amount of nicotine in the total system, nicotine levels present in the medium were normalized to total root dry weight on day 13 (Figure 3.2.B, white bars). Similarly, the root nicotine levels were normalized to

root weight (Figure 3.2.B grey bars). These combined measurements reflect the total amount of nicotine (normalized to root mass) present in the culture at day 13. When the amount of nicotine in the medium was normalized to the total root mass, it became apparent that during DMSO control conditions the NtEAT13-x lines showed significantly more nicotine accumulating in the medium than the Xanthi-1 control (P-values < 0.05). This effect was not observed during MeJA treatment. Because the hairy root culture system is a closed system, the total amount of nicotine present can be calculated by combining the normalized nicotine levels in both the medium and the roots. When this analysis was performed all *NUPI-RNAi* cultures showed significantly less total nicotine (combined heights in Figure 3.2.B) in the closed system than did the Xanthi-1 control (P value \leq 0.05). This trend was independent of MeJA or DMSO treatment and these results indicate that total nicotine levels were lower in the *NUPI-RNAi* lines when normalized for total root mass.

The findings that both absolute and normalized nicotine levels in the medium were elevated despite the fact that total nicotine biosynthesis decreased suggested that nicotine was being redistributed from the roots into the medium. To confirm this assertion we calculated the ratio of nicotine present in the medium to nicotine present in the total system (Figure 3.2.C). This comparison showed all NtEAT13-x lines had a greater percentage of the total nicotine located in the medium than did the Xanthi-1 control line, while NtEAT14-1 was closer to the distribution seen in the control.

The mRNA levels of *NUPI-RNAi* line 14-1 accumulate to an intermediate level in between those of the NtEAT13-x lines and the wild type Xanthi-1 control. The nicotine levels associated with the NtEAT14-1 hairy roots (Figure 3.2.B, grey bars) were less than

the Xanthi control, yet greater than the levels observed in the NtEAT13-x lines. The nicotine present in the NtEAT14-1 medium showed levels similar to the Xanthi-1 control (Figure 3.2.B) while the total nicotine in NtEAT14-1 was significantly lower than the Xanthi-1 control (Figure 3.2.A). Additionally, the distribution of nicotine in the hairy root culture closed system was altered during MeJA conditions, but not when control conditions were used.

Together these results correlate the reduction in *NUPI* mRNA transcript levels with alterations in the nicotine physiology of hairy root cultures. The alkaloid accumulation phenotypes were altered most within the NtEAT13-x lines where significant reductions in *NUPI* mRNA accumulation, reduction in total nicotine, and an alteration of the distribution of nicotine between the medium and the roots were correlated. NtEAT14-1 displays an intermediate *NUPI* mRNA transcript level in comparison to the NtEAT13-x lines and the Xanthi-1 also displayed altered alkaloid phenotypes, but these were generally intermediate between the NtEAT13-x lines and the Xanthi-1 control.

Reduced *NUPI* expression resulted in decreased foliar nicotine levels

To determine the effect of reduced *NUPI* expression on foliar nicotine levels, the aerial portion of 20-day old wild type and homozygous T3 *NUPI-RNAi* plants were harvested and assayed for foliar nicotine accumulation levels. Figure 3.3 illustrates that all five *NUPI-RNAi* lines showed reduced foliar nicotine levels, with three of the five lines showing strong statistical support (ANOVA P-values less than 0.05). Thus, reduced

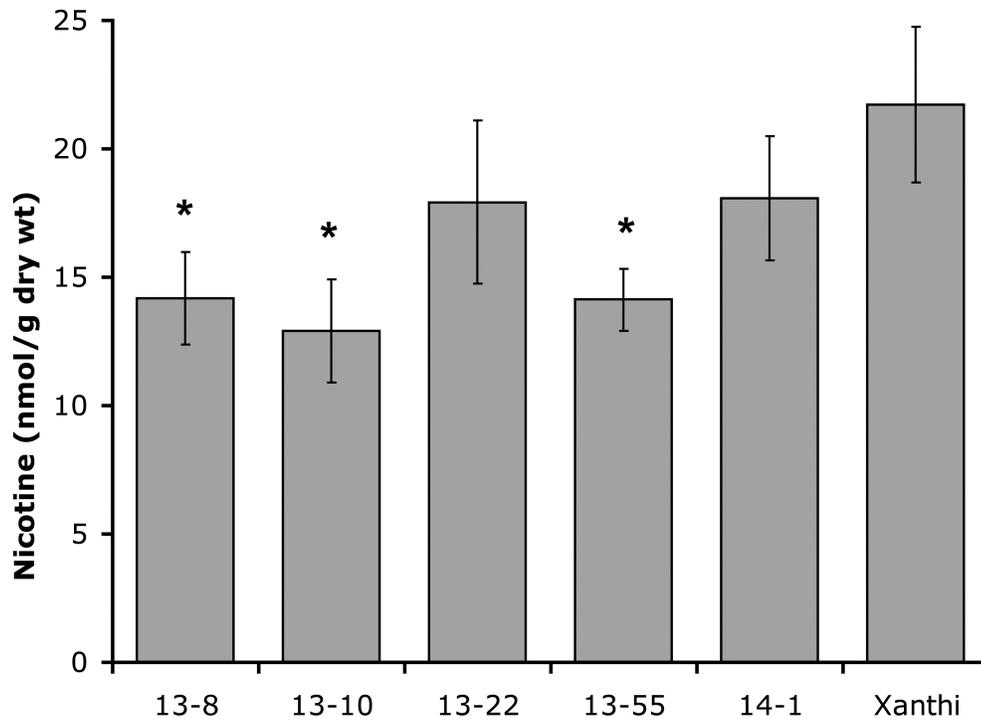


Figure 3.3 Nicotine in 20-day old plants

Nicotine levels from the aerial portions of NUP1-mRNA plants and Xanthi

(*)represents a statistical significance in nicotine levels between NUP1-mRNA and Xanthi at $P < 0.05$

total nicotine levels observed in the T1 *NUP1-RNAi* hairy root lines correlated with reduced foliar nicotine accumulation in 20 day-old homozygous T3 plants.

NUP1 is a nicotine uptake transporter

We successfully expressed a functional NtNUP1 protein in *Schizosaccharomyces pombe* cells. Figure 3.3.A demonstrates that *S. pombe* cells with an empty cloning vector control did not significantly uptake ^{14}C -nicotine. However, *S. pombe* cells containing a plasmid with the full length *NtNUP1* cDNA under the expression of an inducible promoter showed rapid ^{14}C -nicotine uptake activity. Figure 3.3.B shows that the NtNUP1-specific ^{14}C -nicotine uptake activity was not effectively competed with 10-fold excess of unlabeled kinetin (16.5 % inhibition of ^{14}C -nicotine uptake activity). Thus, NtNUP1 is not a cytokinin-uptake permease and therefore not an ortholog of AtPUP1. To determine the substrate specificity, competition experiments were performed with 10-fold excess of the tropane alkaloids atropine and scopolamine. Scopolamine did not show any significant inhibition of ^{14}C -nicotine uptake activity, whereas atropine showed only 21% reduction of ^{14}C -nicotine uptake activity. Thus, neither atropine nor its epoxide scopolamine showed strong inhibition of nicotine uptake by NUP1. Because nicotine is a pyridine alkaloid, we also investigated the competition of nicotine by two pyridine alkaloids, anatabine and anabasine. Anabasine is comprised of an N-methylpiperidine ring conjugated to a pyridine ring and 10-fold excess of anabasine showed 36 % inhibition of ^{14}C -nicotine uptake activity by NtNUP1. Anatabine is less closely related to nicotine as it is comprised of two conjugated pyridine rings and 10-fold excess of

anatabine inhibited ^{14}C -nicotine uptake activity by 26 %. Taken collectively, the results from the ^{14}C -nicotine uptake competition studies indicated that heterologously expressed NtNUP1 is a selective nicotine uptake permease.

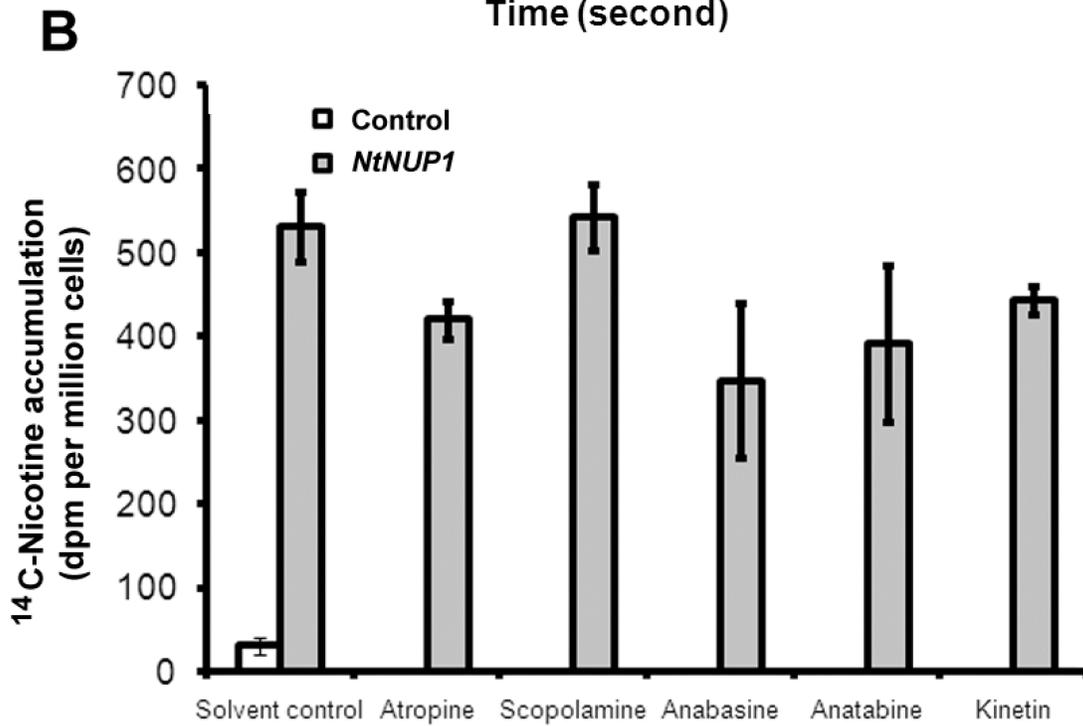
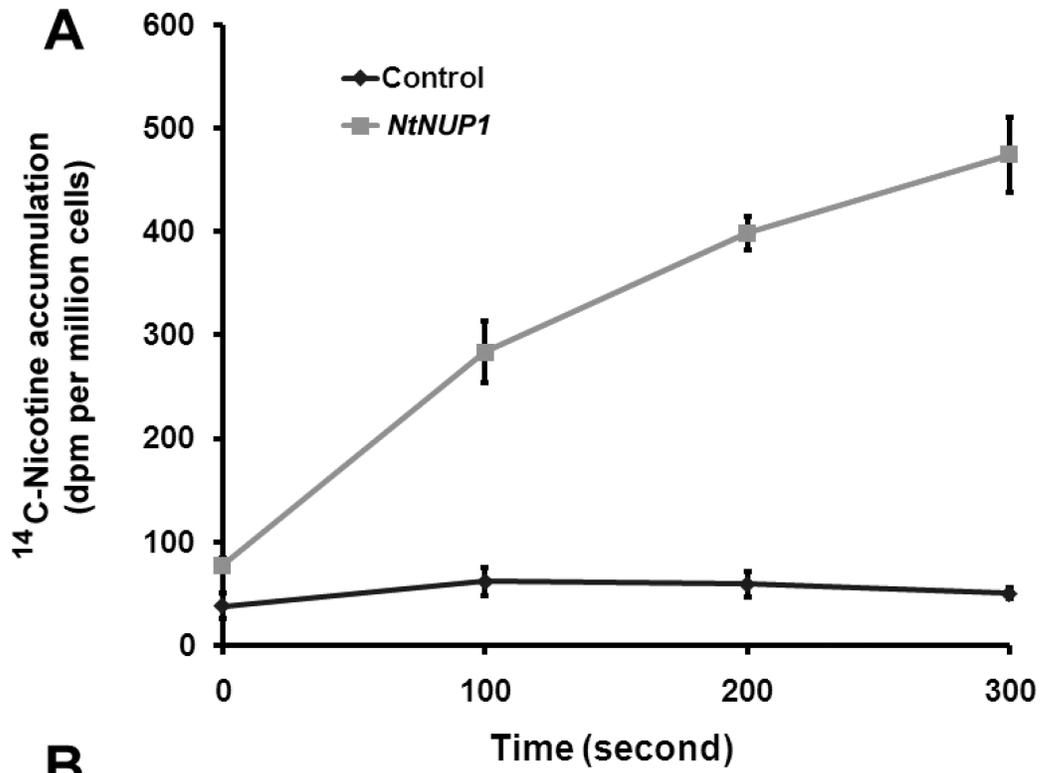


Figure 3.4: Nicotine uptake assays in *S. pombe*

- A.** ¹⁴C-nicotine uptake kinetics of *S. pombe* cells containing empty cloning vector control and vector expressing *NUP1*.
- B.** ¹⁴C-nicotine uptake of *S. pombe* cells expressing NUP1 in the presence of competitors. Solvent control indicates ¹⁴C-nicotine uptake of cells carrying empty vector control and *NUP1* in the presence of DMSO. All competitors were dissolved in DMSO and used at 10-fold excess relative to labeled nicotine. Asterisks indicate statistically significant (GLM ANOVA P-value ≤ 0.05; numbers above bar indicate moderate GLM ANOVA statistical support with P-values between 0.0500 – 0.1000) relative to corresponding Xan-1 controls.

NUP1 localizes to the plasma membrane

In order to gain a more detailed understanding of NUP1 function, the sub-cellular distribution of NUP1 was examined using a C-terminal NUP1 protein fusion to the green fluorescent protein (GFP). This was accomplished by the transient expression of an in-frame *NUP1-GFP* gene fusion in tobacco BY2 protoplasts controlled by the Cauliflower Mosaic virus 35S promoter (CaMV35S). The GFP fluorescence corresponding to the NUP1-GFP fusion protein was observed predominately at the plasma membrane (Figure 3.3.A, .B and .C), although fluorescence was also associated with the endomembrane system. Since plasma membrane proteins are processed through the endomembrane system, fluorescence associated with the endomembrane system is also consistent with NUP1 plasma membrane localization. In contrast, when a *CaMV35S-GFP* construct was similarly expressed in BY2 protoplasts (Figure 3.3.A) fluorescence was observed predominantly in the cytoplasm and nucleus, typical of previously described GFP localization in plants (Chiu et al., 1996; Grebenok et al., 1997; Haseloff et al., 1997). Therefore, NUP1 was determined to predominantly localize to the plasma membrane.

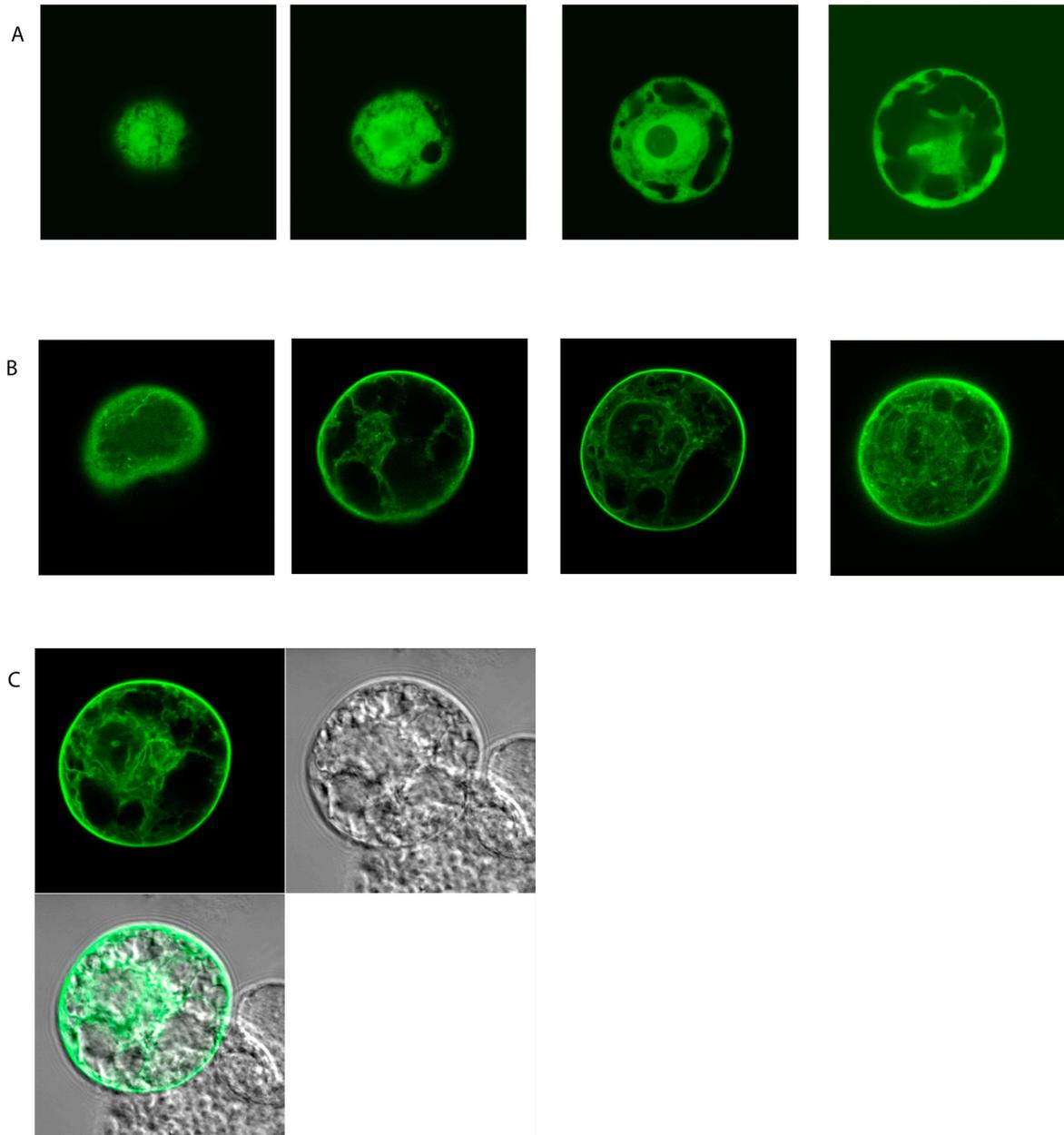


Figure 3.5: Laser Scanning Confocal Microscopy images of CMV35S-GFP and CMV35S-NUP1-GFP localization in BY-2 protoplasts.

A. CMV35S-GFP expressed in BY2 protoplasts. From left to right, images display a single protoplast viewed as laser sections in the Z-axis.

B. CMV35S-NUP1-GFP expressed in BY2 protoplasts. From left to right, images display a single protoplast viewed as laser sections in the Z-axis.

C. A fluorescent image (top left) and light image (top right) of a BY2 protoplast expressing CMV35S-NUP1-GFP. The bottom left shows an overlay of the light and fluorescent images.

Reduced alkaloid levels in *NtNUP1-RNAi* lines correlated with reduced nicotine biosynthetic gene expression levels during non-inducing conditions

Since *NtNUP1-RNAi* lines showed overall reduced nicotine biosynthesis on a normalized root mass basis, the mRNA accumulation levels of five nicotine biosynthetic genes were also assayed using QRT-PCR. The enzymes quinolinate phosphoribosyl transferase (QPT) and quinolinate synthase (QS) contribute to the biosynthesis of the pyridine ring, whereas the enzymes ornithine decarboxylase (ODC), putrescine methyltransferase (PMT), and methylputrescine oxidase (MPO) contribute to the formation of the N-methylpyrrolidine ring. Figure 3.6 shows that *ODC*, *PMT*, and *QS* mRNA levels in the DMSO-treated cultures showed a pattern of reduced mRNA accumulation levels, with three to four lines showing strong/moderate statistical support (Figure 3.5 A-C, respectively). Thus during DMSO treatment, reduced total alkaloid biosynthesis was correlated with significantly reduced gene expression levels of three genes that encode enzymes involved in the biosynthesis of both the pyridine (i.e. *QS*) and N-methylpyrrolidine (i.e. *ODC* and *PMT*) rings. However, not all of the nicotine biosynthetic genes tested showed significantly reduced expression levels. For example, both *QPT* and *MPO* transcripts were not reproducibly lower in any of the *NtNUP1-RNAi* lines (Figure 3.6 D-E). Similarly, the housekeeping β -*ATPase* transcripts did not show a consistent reduction in mRNA accumulation levels in the five *NtNUP1-RNAi* lines (Figure 3.6F).

Similar to DMSO-treatment, the MeJA-treated NtEAT13-x hairy root cultures also showed a significant reduction in total nicotine biosynthesis levels relative to MeJA treated controls (Figure 3.2.B). However, in contrast to the DMSO-treated cultures, the MeJA-treated cultures did not show any consistent pattern of reduced mRNA levels of any of these five nicotine biosynthetic genes (Figure 3.6 A–F). Therefore, during MeJA treatment the observed decreased nicotine accumulation levels in Figure 3.2.B could not solely account for the corresponding reduced mRNA expression levels of any one of these five nicotine biosynthetic enzymes. These gene expression studies indicate that NtNUP1 may be influencing nicotine biosynthesis at both a transcriptional level during non-inducing conditions, and perhaps also at the post-transcriptional level during MeJA treatment.

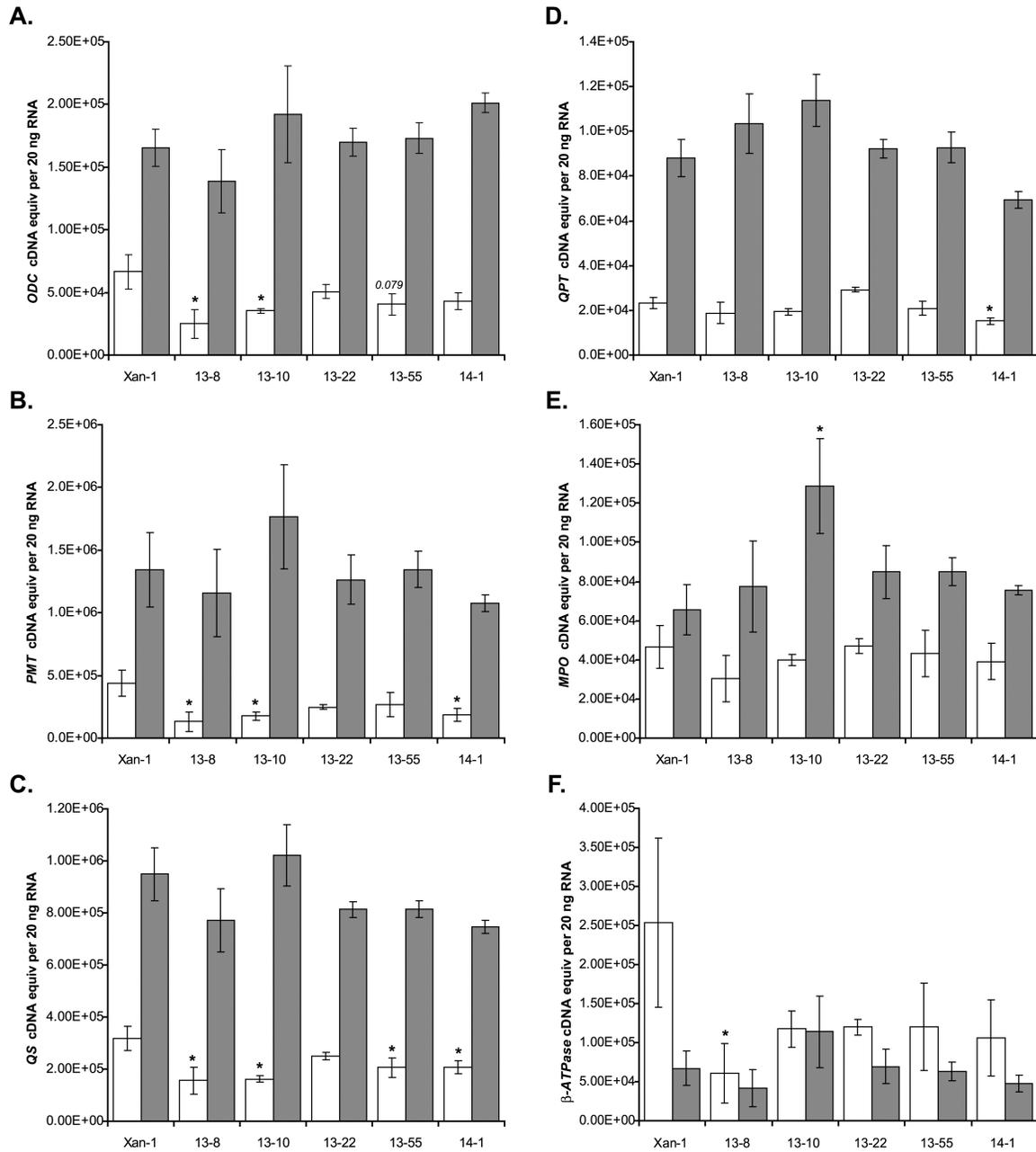


Figure 3.6 mRNA accumulation levels of nicotine biosynthetic genes.

A – F; *ODC*, *PMT*, *QS*, *QPT*, *MPO*, and β -*ATPase* mRNA levels respectively.

Discussion

NUP1 Localization and NUP1-RNAi Characterization

This chapter describes the characterization of the *Nicotiana tabacum* Nicotine Uptake Permease 1 (NUP1). NUP1 is homologous to a proton symporter of the Arabidopsis family of PUP transporters. The PUP family initially identified in Arabidopsis and the first characterized family member was AtPUP1, a plasma membrane localized cytokinin and purine uptake transporter (Gillissen et al., 2000). The *AtPUP* gene family in Arabidopsis contains approximately 20 members and to date PUP-type transporters have been identified exclusively in plants. AtPUP1 and AtPUP2 are cytokinin and purine uptake transporters, whereas AtPUP3 does not show the ability to transport these substrates (Gillissen et al., 2000; Burkle et al., 2003; Cedzich et al., 2008). *AtPUP1* and *AtPUP2* transcripts localize to leaves with vasculature-localized expression in the hydathode and phloem, respectively (Burkle et al., 2003; Cedzich et al., 2008). In contrast, AtPUP3 is expressed only in pollen. Thus, even with the limited characterization of three members within the *AtPUP* multigene family, there is evidence for neofunctionality of different *AtPUP* gene products in both tissue distribution and substrate recognition.

NUP1 was originally identified during a transcript profiling screen that identified gene fragments coordinately regulated with nicotine biosynthetic genes in tobacco roots (Kidd et al., 2006). Research by the collaborating laboratory of Angus Murphy at Purdue University used *Schizosaccharomyces pombe* to demonstrate that NUP1 is a nicotine transporter and that nicotine transport is not competed with a ten-fold excess of cytokinin,

tropane alkaloids, or related pyridine alkaloids (Figure 3.4). NUP1 is a paralog of Arabidopsis AtPUP1/2 proteins, as these proteins display different substrate preferences. Therefore, NUP1 possesses a novel transport profile and the focus of the experiments reported here were to examine the physiological effects of reduced *NUP1* mRNA levels *in planta* and to determine the subcellular localization of the NUP1 protein.

Expression of a *NUP1-GFP* gene fusion demonstrated that the fusion protein was predominantly localized to the plasma membrane. The localization of NUP1 to the plasma membrane together with the characterization of *NUP1*-dependent nicotine uptake activity in yeast indicates a role for NUP1 in moving nicotine from the apoplastic space into the cytoplasm of as yet undefined tobacco root cells. The plasma membrane localization of NUP1 is in agreement with the presumed localization of AtPUP1, although NUP1 is the first PUP family transporter to be experimentally localized. In order to assess the physiological importance of NUP1 in nicotine uptake, transgenic plants and hairy root cultures expressing *NUP1-RNAi* transgenes were created and used to examine the effects of reduced *NUP1* mRNA expression on nicotine metabolism.

Hairy root lines with reduced *NUP1* expression provided a closed system to address the affect of NUP1 on nicotine accumulation levels. Because nicotine is not further metabolized in the axenic root cultures, the hairy root system allows for quantification of the total amount of nicotine accumulating in both the root tissue and medium throughout the duration of the experiment. NUP1 as a plasma membrane localized nicotine importer was implicated in two aspects of nicotine metabolism: the regulation of nicotine biosynthesis and the altered distribution of nicotine between roots and the culture medium.

First, the implication NUP1 is involved in the regulation of nicotine biosynthesis is based on lower total nicotine levels in the *NtNUP1-RNAi* hairy root cultures during both inducing (MeJA) and non-inducing (DMSO) conditions. The reduction in nicotine accumulation during non-inducing conditions correlated with reduced mRNA levels of three nicotine biosynthetic genes (*ODC*, *PMT*, and *QS*) in the *NUP1-RNAi* hairy roots although mRNA levels of two other nicotine biosynthetic genes were not affected in the *NUP1-RNAi* hairy root cultures.

The observed decreases in mRNA levels of *PMT*, *QPT*, or *QS* (Figure 3.6) of *NUP1-RNAi* hairy roots were not also observed in the corresponding MeJA-induced cultures. *NUP1-RNAi* MeJA-induced cultures showed a lower level of total nicotine than did Xanthi, but the reduced nicotine level did not correspond with a decreased mRNA expression of any of the measured nicotine biosynthetic genes. Since tobacco produces nicotine both at a basal level and at a higher induced level in response to MeJA, it is likely different mechanisms are in place to regulate the biosynthesis of nicotine during these different physiological states. The MeJA induced hairy root cultures showed equivalent mRNA expression of nicotine biosynthetic genes in the *NUP1-RNAi* lines and in the Xanthi-1. The stimulation of the hairy root cultures with MeJA likely induced nicotine production in the *NUP1-RNAi* cultures and Xanthi in the same manner, yet lower nicotine levels observed in the *NUP1-RNAi* lines were due to a lower basal level of nicotine before the cultures were induced.

The observation that reduced *NUP1* mRNA levels correlated with a reduction in nicotine levels suggests that NUP1 may be directly integrated with a mechanism controlling nicotine production. Early physiological studies on nicotine production in

tobacco showed that the exogenous feeding of nicotine to roots of decapitated plants resulted in a 50% reduction in enzymatic activity of nicotine biosynthetic enzymes PMT, MPO and ODC (Mizusaki et al., 1973) although nicotine added to cell-free root extracts has no direct effects on enzyme activity (Mizusaki et al., 1971; Mizusaki et al., 1972). This unspecified internal control mechanism induced with nicotine feeding correlates with our current findings that *NUPI-RNAi* hairy roots have reduced nicotine. Since, NUP1 is located at the plasma membrane and removes nicotine from the apoplastic space, we expect *NUPI-RNAi* hairy roots will experience a higher level of apoplastic nicotine than the control hairy roots. In turn this high apoplastic nicotine may trigger a reduction in nicotine biosynthetic activity, as is observed in nicotine-fed plants. This mechanism is consistent with our findings of lower nicotine biosynthetic gene expression levels and a lower total nicotine level in the DMSO treated *NUPI-RNAi* hairy root cultures. In addition to the reduced nicotine levels *NUPI-RNAi* lines also displayed an altered distribution of nicotine during non-inducing conditions (i.e. DMSO treatments), demonstrating this phenotype is consistent in basal and induced conditions. During the non-inducing conditions the NtEAT13-x lines all showed significantly more nicotine in the medium, relative to the Xanthi-1 control. This phenotype was observed during alkaloid inducing conditions (i.e. MeJA treatment), but not at the level of statistical significance. It is noteworthy that the standard error of measurement of the Xanthi-1 MeJA induced cultures was exceptionally large due to one apparent outlier replicate that was a slow-growing culture and when compensated for mass displayed higher nicotine levels in the media, yet lower nicotine levels in the root than the other two replicates. Thus, it is formally possible that the *NUPI-RNAi* induced alteration in the media nicotine

levels was independent of treatment and the result obtained was due to an unidentified experimental variation rather than a treatment effect. Of course, more replicates of the experiment would clarify this issue. In summary, reduced *NUP1* mRNA accumulation levels resulted in both decreased total nicotine accumulation levels and, at least during non-inducing conditions, increased nicotine accumulation in the culture media.

Consistent with the observation that the *NUP1-RNAi* hairy roots displayed altered nicotine physiology, T3 *NUP1-RNAi* plants also displayed decreased nicotine levels in the aerial tissues of rosette stage plants. Thus, *NUP1-RNAi* affects normal nicotine physiology within isolated root cultures and in whole-plants.

Model for NUP1 *in planta*: xylem loading

The long distance transport of nicotine from the site of biosynthesis in the root to the aerial portions occurs through the xylem. An important aspect of nicotine physiology is the inducibility of nicotine production as a defense response. Induction of nicotine biosynthesis in response to foliar wounding and plant decapitation results in the increase of foliar nicotine concentrations within 24-hours. Under these induced conditions, *de novo* nicotine biosynthesis is stimulated and nicotine concentrations in the xylem can reach 1mM. To achieve such a concentration of nicotine within the xylem, plants must have a transport system in place to actively facilitate the loading of high concentrations of nicotine into the xylem.

Although the mechanisms of nicotine transport are poorly understood, the high concentrations of nicotine that occur in the xylem suggest that a method of diffusive transport is unlikely. Therefore, it can be assumed that one or more active transport

mechanisms are in place that can facilitate the loading of nicotine in the xylem. The understanding of nicotine movement within the root is hampered by the lack of knowledge regarding the site of nicotine biosynthesis in the root. Currently our best markers for localization of nicotine biosynthesis have resulted from the immunolocalization of the nicotine biosynthetic enzymes PMT and A622. These enzymes are concentrated in the apical region of the root and localize to the cortex and endodermis cell layers. Therefore, we can assume the movement of nicotine from the site of biosynthesis to the xylem involves the movement across cell layers and into the vascular bundle.

This study indicates that NUP1 is a nicotine specific proton symporter localized to the plasma membrane. The biochemical characterization of NUP1 in *S. pombe* demonstrated NUP1 co-transport a proton from the established proton gradient at the apoplastic surface of the plasma membrane along with a molecule of nicotine. Plasma membrane localized symporters utilize the energy provided by the proton gradient to move solutes against the concentration gradient, and the movement of nicotine from the apoplast into the cytoplasm by NUP1 results in a high internal nicotine concentration. The NUP1 facilitated movement of nicotine is characteristic of the required facilitated transport required to obtain high nicotine concentrations in the xylem. Therefore the demonstrated properties of NUP1 are characteristic of those expected for the facilitated transport of nicotine from the root.

The characteristics of NUP1 mentioned above make it a good candidate for a facilitator of long distance nicotine transport, but it should be clarified that NUP1 is likely not directly involved in the final xylem loading step. The direct loading of the

xylem requires a nicotine export activity from the cytoplasm of xylem parenchyma into the xylem proper and the proton gradient between the xylem parenchyma and the xylem lies on the side of the xylem. Therefore, some active transport mechanism must be responsible for moving cytoplasmic nicotine from the xylem parenchyma into the xylem. Two distinct types of transporters could serve this role. One possibility is a MATE-type antiporter that exchanges a proton from the xylem for the export of nicotine into the xylem. Alternatively, an ABC multidrug resistance type transporter could use cytoplasmic ATP to drive the active transport of nicotine from the cytoplasm into the xylem. Therefore NUP1 is likely involved in creating a high concentration of nicotine within the xylem parenchyma cytoplasm in concert with a separate active transport mechanism that facilitates the specific loading of nicotine in xylem.

The physiological data presented here demonstrated *NUP1-RNAi* plants have reduced nicotine in the aerial tissues in comparison to wild-type plants. This reduction is in agreement with NUP1 functioning in the xylem loading of nicotine for long distance transport. The reduced expression of a xylem parenchyma localized NUP1 would decrease the ability to concentrate nicotine in the xylem, therefore reducing the nicotine transported to the aerial tissues. Alterations to nicotine physiology were also observed in *NUP1-RNAi* hairy root cultures. Hairy root cultures contain only the isolated nicotine biosynthetic organ without the aerial tissues known to accumulate the xylem-transported nicotine. Therefore drawing parallels between the root cultures and whole-plant physiology is difficult. The fact alterations within root cultures were observed in the *NUP1-RNAi* cultures corroborates with NUP1 being involved in nicotine physiology, but

direct parallels between the cultures and the whole-plants cannot be established at this point.

The hypothesis of NUP1 facilitating nicotine transport is supported by the biochemistry, the localization and the physiological data presented here. In order to further support the evidence presented here for NUP1 as facilitator of long distance transport further investigation of NUP1 will be required. Studies designed to determine the localization of NUP1 within the root will provide a direct view of where NUP1 is present. The localization of the cell types expressing NUP in a root cross section will provide valuable insight into the *in planta* role of NUP1. In support of the hypothesis NUP1 is involved in long distance nicotine transport, the identification of NUP1 in the xylem parenchyma is expected. The expression of NUP1 in any area of the stele in addition to or instead of the xylem parenchyma would also support the hypothesis that NUP1 facilitates long distance nicotine transport since nicotine is expected to concentrate in the stele as it approaches the xylem.

The cell type expression patterns of NUP1 may alternately show NUP1 expression does not correlate with the pattern expected for long distance transport of nicotine. This result would suggest NUP1 functions in another aspect of root nicotine physiology and this result would also be interesting. In addition to nicotine accumulation in the aerial tissues, nicotine also accumulates in the root and the mechanisms leading to the accumulation of below ground nicotine have not been determined. Therefore the localization of NUP1 to non-vascular cell types could provide insight into the poorly understood accumulation of nicotine within the root.

Current knowledge of nicotine and alkaloid transport

In the emerging field of nicotine transport two distinct transporter families of nicotine transporters are currently known to be involved in moving nicotine into the cytoplasm and then into the vacuole. Both classes are energized by the proton gradient, yet each class displays a different sub cellular localization and specificity for nicotine. NUP1, a PUP family transporter, is localized to the plasma membrane and is a selective nicotine transporter that moves nicotine from the apoplast into the cytoplasm using the established proton gradient. The proton gradient is the driving force for NUP1 to symport (co-transport) a molecule of nicotine into the cytoplasm along with a proton. The general model for symporters is while the proton gradient drives the transport of protons from high concentrations to low concentrations, the substrate is moved from low concentrations to high concentrations.

In contrast to the transport of apoplastic nicotine by NUP1, the identified nicotine MATE transporters move nicotine from the cytoplasm into the vacuole. NtMATE1 and NtJAT1, are localized at the vacuolar membrane and are accepting of a range of alkaloids, including but not exclusive to nicotine. As antiporters, these transporters exchange one proton from the concentrated proton pool in the vacuole for a substrate present in the cytosol and thereby create a high substrate concentration in the vacuole. Since nicotine is known to accumulate in the vacuoles of leaf mesophyll cells (Saunders, 1979), the identification of two independent tonoplast localized nicotine transporters is in agreement with what is known regarding the subcellular localization of nicotine. The

expression patterns of NtMATE1 in the root and NtJAT1 in the roots, leaves and flowers correlate with areas of the plant known to accumulate nicotine. Studies examining the role of NtJAT1 *in-planta* have yet to be reported, but physiological studies characterizing *NtMATE1-RNAi* were reported. *NtMATE1-RNAi* plants did not display alterations in root or leaf nicotine levels or in the root/shoot distribution *in-planta*. The only observed physiological alteration of the *NtMATE1-RNAi* plants was the increased sensitivity of seedling root growth in the presence of exogenous nicotine. Exogenous nicotine slows root elongation in seedlings and the finding *NtMATE1-RNAi* plants have an increased sensitivity suggests NtMATE1 is involved in the tolerance of the root to exogenously applied nicotine. Additionally, NtMATE1 was shown to transport a range of alkaloids and alterations to nicotine physiology were not observed in the *NtMATE1-RNAi* plants. Thus the available information on NtMATE1 implicates a function in the sequestration of exogenous nicotine to prevent the deleterious effects of nicotine on root elongation.

The multiple classes of transporters identified in nicotine transport parallels what is currently known regarding the transport of other alkaloids. In *Coptis japonica*, the ABC transporter CjMDR1 is believed to be responsible for the uptake of the alkaloid berberine into the sink tissue the rhizome. CjMDR is an ATP-dependent transporter of the ABC class of transporters localized to the plasma membrane. Furthermore, a proton antiporter using the established proton gradient at the tonoplast is implicated in the import of berberine into the vacuole. These results show that alkaloid-producing plants contain multiple transport mechanisms to accumulate alkaloids in specific tissues and in specific subcellular organelles. The characterization of a PUP family transporter involved in nicotine transport provides a novel alkaloid transport mechanism and

characterization of additional PUP family members in tobacco may reveal additional PUP facilitated transport mechanisms.

AUTHOR CONTRIBUTIONS:

Sherry Hildreth contributed to the project design, drafting of the manuscript, localization of *NUP1-GFP*, alkaloid measurements and data analyses. Elizabeth Gehman isolated the full length *NUP* cDNAs, performed *NUP1* and *NUP1-GFP* expression studies in *Xenopus* oocytes, and created the *NUP1-RNAi* constructs. Haibing Yang created the *NUP1* yeast expression constructs and performed *NUP1* nicotine transport assays in yeast. Rong-He Lu generated transgenic *NUP1-RNAi* plants and hairy root cultures, contributed to alkaloid analyses, and RNA isolations. Jackson Sandoe performed extensive tissue homogenization for both the alkaloid analyses and RNA extractions. Sakiko Okumoto intellectually contributed to developing an optimal expression system for *NUP1-GFP* localization and performed BY2 protoplast transformations. Angus Murphy performed mammalian *NUP1* expression experiments, supervised nicotine transport assays in yeast, and contributed to manuscript preparation. John Jelesko supervised the overall project, executed foliar nicotine experiments, performed QRT-PCR experiments, statistical analyses, and contributed to drafting and editing of the manuscript.

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CHAPTER IV

Summary and Future Directions

The data presented in this dissertation addresses two aspects of plant specialized metabolism in domesticated tobacco, the biosynthesis and transport of nicotine. Several lines of experimental inquiry examined the existence of a potential multienzyme complex functioning in nicotine biosynthesis. Additionally nicotine transport was explored through the characterization of a novel nicotine transporter and by establishing a connection between nicotine transport, biosynthesis, and partitioning in root cultures.

Studies into a putative nicotine metabolic channel

The experimental inquiry into a nicotine metabolic channel was based on prior indirect evidence of an interaction between the nicotine biosynthetic enzyme MPO and the S-adenosylmethionine (SAM) recycling enzyme SAHH (Heim and Jelesko, 2004). This observation demonstrated that an antiserum specific to SAHH also immunodepleted MPO activity from a tobacco root extract and suggested an interaction was occurring between the two enzymes. The SAHH-specific antiserum was developed against a highly-purified protein fraction from tobacco roots that contained MPO activity and appeared as a single protein species at 53 kDa on SDS-PAGE (McLauchalan et al., 1993). This antiserum was assumed to be MPO-specific because it immunodepleted MPO activity from a tobacco root extract and also recognized a single protein species at 53 kDa on a western blot (McLauchalan et al., 1993). Further characterization of this

antiserum determined the immunoreactive 53 kDa species was actually SAHH instead of the expected MPO (Heim and Jelesko, 2004). However, this SAHH-specific antiserum immunodepleted MPO activity from a tobacco root extract (Heim and Jelesko, 2004). It is noteworthy that immunodepletion was defined as reduced MPO enzyme activity within the remaining cell-free extract and that attempts to identify MPO enzyme activity associated with the immunoprecipitated material were unsuccessful (Bill Heim, unpublished results). Thus, it was assumed that during the washing of the protein-A Sepharose beads the SAHH/MPO complex disassociated and was lost. Taken together, the fact that SAHH and MPO had seemingly co-purified, as observed by the MPO activity in the antigen fraction and the immunodepletion of MPO by SAHH-specific antisera, the evidence for a metabolic channel functioning in nicotine biosynthesis was established.

The interaction of SAHH and MPO as part of a larger nicotine metabolic channel was hypothesized based upon the fact that both SAHH and MPO utilize substrates produced by the nicotine biosynthetic enzyme PMT. Since the hypothesis of a metabolic channel was built upon indirect evidence for the interaction of SAHH and MPO, I used a two-pronged approach in order to obtain additional evidence for our hypothesis and to further characterize the interaction(s). I first used yeast two hybrid assays to directly test the hypothesized interactions between the three metabolic channel members and additionally I screened a tobacco root cDNA library for additional proteins interacting with SAHH, MPO and/or PMT. To complement the yeast two hybrid assays, co-immunoprecipitation was used with new anti-SAHH, anti-MPO and anti-PMT antisera in an attempt to reproduce the original immunodepletion results and to expand the

experiments by immunoprecipitating MPO and PMT in addition to SAHH. *In vitro* transcription/translation with co-immunoprecipitation was also used to analyze the relationship between these three proteins in a cell-free matrix.

The combined results from these independent approaches did not provide additional evidence for interactions between these three proteins, or any others. I repeated the original McLaughlan SAHH antiserum immunodepletion experiments and I was again able to deplete MPO activity, yet the SAHH antisera produced as part of my research did not deplete MPO activity from root extracts. Furthermore, co-immunoprecipitation of PMT showed no evidence of depleting MPO activity. Likewise, co-immunoprecipitation of *in vitro* produced SAHH and PMT with provided no evidence of interactions among the three proteins combined in a cell free matrix.

The inability to reproduce the immunodepletion of MPO by anti-SAHH antiserum when new antisera were produced was an unexpected result of this research. One interpretation of why the original anti-SAHH was capable of immunodepleting MPO is that the MPO purification procedure McLaughlan utilized resulted in the inadvertent copurification of SAHH with MPO. During the last purification step, the SAHH-enriched fraction may have been inadvertently chosen over the MPO fraction and SAHH used as antigen. The resulting SAHH antiserum would have been produced in response to native SAHH in contrast to the SAHH antisera produced in this study which was produced to denatured recombinant protein. These antisera may recognize different epitopes on SAHH, possibly explaining why we see evidence of a SAHH-MPO interaction with one serum and not with the other. It is possible that using antisera produced to denatured

proteins results in a conformational change in the target antigen and that this disrupts the SAHH-MPO interaction.

A second interpretation of why the newly-produced antiserum does not detect an interaction between MPO and SAHH is that the original antiserum contains antibodies to both SAHH and MPO. In what now appears to be the inadvertent purification of SAHH in an attempt to purify MPO, the final purification product was predominately SAHH as evidenced by the single 53 kDa species on an SDS-PAGE gel. Yet, this purified SAHH fraction also contained MPO activity suggesting a pooled group of protein fractions containing both MPO and SAHH could have been used as antigen for antiserum production. In this case the immunized animal was exposed to both SAHH and MPO as antigen. Western blots with the resulting antisera did not show the antiserum to be immunoreactive to any protein except for SAHH, but this information should be interpreted carefully. With SAHH at 53 kDa and MPO expected around 80 kDa the proteins should be easily resolved by western blotting, but my experiences working with tobacco root extract indicate SAHH is present in much higher quantities in the root than is MPO. As SAHH is a member of the SAM cycle, it is not limited to nicotine biosynthesis and it likely present in more cells than is MPO. SAHH was identified as the only immunoreactive constituent from a tobacco extract when 1 μ g of total protein was analyzed, while in my experience 100 μ g of total protein was required to identify MPO under these same conditions. The discrepancy of protein expression levels would make it difficult to observe both SAHH and MPO on the same blot, as the concentration of SAHH would be prohibitive for identifying MPO. Thus, we cannot rule out the original McLauchlan polyclonal anti-serum was immunoreactive to both SAHH and MPO,

resulting in the appearance of an interaction. If this were the case, then the anti-MPO antibodies were not capable of inhibiting MPO enzyme activity and were most likely present at a significantly lower titer than the anti-SAHH polyclonal antibodies. To my knowledge the original rabbit anti-SAHH serum produced by McLauchlan is no longer available and thus further analyses are not possible. Thus we may never have a definitive answer as to why the McLauchlan antisera immunoreacted with SAHH and immunodepleted MPO activity.

The negative results obtained from the Y2H and the co-IP experiments do not necessarily indicate that a nicotine metabolic channel does not exist. These results combined with previous fractionation of tobacco root extracts by gel filtration that showed SAHH and MPO in different fractions (Heim and Jelesko, 2004) suggest a stable interaction among protein domains is unlikely, although we cannot rule out that weak, transient, or conditional interactions exist in intact plant cells.

More sensitive methods of detecting protein-protein interactions are available that also offer the advantage allowing for analysis in intact living cells. These methods of live-cell imaging involve using stable, visible reporter molecules fused to the protein of interest and include fluorescence resonance energy transfer (FRET), bimolecular fluorescence complementation (BiFC) and bioluminescence resonance energy transfer (BRET). *In vivo* approaches utilizing sophisticated imagers to view reporters activated only when the proteins of interest interact provide high-sensitivity and can be performed in living plant cells, both distinct advantages over the yeast two hybrid assay and co-immunoprecipitations. Disadvantages inherent to these methods include being laborious to set-up, being technically demanding, producing high backgrounds, requiring the use of

fusion proteins, and being low throughput. Thus although the *in vivo* methods provide distinct advantages over the yeast two hybrid assay and co-immunoprecipitations, they also require a substantial commitment in time and resources to pursue and have their own drawbacks.

A recently reported study in mammalian cells provided the first evidence of a long hypothesized metabolic channel among purine biosynthetic enzymes. After attempts to use various *in vitro* and *in vivo* approaches, fluorescence microscopy of fusion proteins in living cell cultures uncovered reversible clustering between all six of the pathway enzymes (An et al., 2008). This clustering was not defined by intermolecular energy transfer between the fluorescent reporters, but rather by display of the intracellular clustering of distinct fluorescent foci. The clustering was observed under purine-depleting conditions while the enzymes dispersed when observed in a purine rich environment. Although this study did not directly demonstrate very close interactions, it provided insight into the intricacies of intracellular conditions that may be required for interactions among biosynthetic enzymes. In contrast to the aforementioned techniques of FRET, BRET and BiFC that require a directed interaction, this study used a broad approach of pair-wise tagging of the enzymes of interest with distinct fluorophores that made it possible to follow the localization of the proteins. This study provided evidence for a conditional interaction. This knowledge can now be applied to further studies into the mechanics of the interaction.

A co-localization approach using cultured cells should be considered for probing interactions among nicotine biosynthetic enzymes. Although this method does not directly detect protein-protein interactions, it does provide the means to address the

unresolved question of a nicotine metabolic complex, albeit at lower resolution. As we have conflicting evidence of an interaction between SAHH and MPO and we lack the evidence detailing if the perceived interaction is direct or indirect, preparing SAHH and MPO fluorophore fusions and examining the co-localization in the presence of stimuli that induce nicotine biosynthesis would provide a method to consider many factors potentially influencing or facilitating an interaction. Using tobacco BY-2 cells, which can be stimulated to produce nicotine, the fusion proteins can be viewed in an environment where active nicotine biosynthesis is occurring and their general spatial relationship established. Evidence of fluorophore clustering, especially if stimulated by MeJA, would provide sufficient preliminary evidence to warrant more laborious methods of detecting protein-protein interactions.

Although the co-immunoprecipitation experiments presented here did not uncover evidence of protein-protein interactions, the antisera specific to MPO, PMT and SAHH produced as part of this research can be used to address additional aspects of nicotine physiology. Antisera specific to the two enzymes necessary to produce the N-methylpyrrolidine ring of nicotine can be used to address many unanswered questions regarding nicotine biosynthesis. For example, a comprehensive study localizing nicotine biosynthetic enzymes within the root is needed. Immunolocalization of nicotine biosynthetic enzymes PMT and A622 in root sections and immunodetection in root extracts suggest overlapping expression of these enzymes in the apical region of the root (Shoji et al., 2002). The use of these antisera in immunohistochemistry can provide the first comprehensive spatial view of the N-methylpyrrolinium pathway. In addition to PMT and MPO activities in tobacco, these enzymes are also required for the production

of the tropane ring in related Solanaceae family members. Although, it has not yet been proven, the polyclonal antisera produced in response to tobacco alkaloid biosynthetic enzymes are also expected to specifically bind to the corresponding enzymes in tropane alkaloid producing species. A phylogenic analysis of 8 different PMT sequences from 5 different *Solanaceae* genera showed greater than 75% identity between the PMT protein sequences. A comparison of similarity in MPO enzymes is not currently available, as the MPO from *Nicotiana tabacum* is the only available MPO sequence. Localizing these enzymes in roots of tropane alkaloid producing species and in tobacco will help us determine if spatial expression is a conserved trait among the related plant family members.

An additional unanswered question in nicotine biosynthesis is the subcellular localization of nicotine biosynthetic enzymes. The only report of subcellular localization is from the immunolocalization of PMT and A622 in the root tip. In these studies staining was reportedly associated primarily with the cytoplasm and excluded from the nucleus (Shoji et al., 2002). Using subcellular prediction programs the predicted localization of some nicotine biosynthetic genes to be either cytoplasmic or peroxisomal. Therefore, high resolution studies localizing the cell type along with the subcellular compartment of nicotine biosynthetic enzymes are important to our understanding of how this compound is synthesized and transported.

An important aspect of the work presented here is the production of PMT and MPO specific antisera. The MPO antisera described in chapter 2 were characterized as having a low affinity for the native antigen. In anticipation of the need for an MPO-specific antiserum to address the outstanding issues in nicotine biosynthesis and a

nicotine metabolic channel, I produced additional MPO anti-sera during the last year of my laboratory research. The newly produced anti-MPO sera are from five animals and have not been fully characterized, but preliminary results suggest the quality of the serum is much better than that previously obtained. Preliminary results show the antisera is immunoreactive to a single species from a tobacco root extract at the expected size of approximately 80 kDa. Additionally the sera from the five animals show a diminished background staining allowing for clear interpretation of the results. Therefore the Jelesko lab now has the specific antisera at hand to address the questions regarding the localization of nicotine biosynthesis.

An emerging theme in alkaloid biosynthesis is that the pathway components responsible for the synthesis are often distributed across multiple cell types. An example of this asynchronous cell-type specific gene expression in alkaloid biosynthesis is among the biosynthetic enzymes responsible for production of tropane alkaloids in *Hyoscyamus niger* (Nakajima and Hashimoto, 1999). Therefore, it is possible that nicotine biosynthesis requires trafficking of intermediates between cell types. Using *in situ* hybridization to localize transcripts corresponding to nicotine biosynthetic genes will allow us to determine if nicotine biosynthesis is a cell autonomous or cell non-autonomous process.

The role of nicotine transport in nicotine biosynthesis and tissue distribution.

Understanding the cell-type expression patterns associated with nicotine biosynthesis will also clarify the importance of nicotine transport between and into

specific cell types. The importance of nicotine transport was highlighted by the physiological studies focusing on the novel nicotine uptake permease, NUP1, reported in Chapter 3.

Nicotine accumulation in individual leaves is not typified by a steady increase over the lifetime of the plant, but varies in relation to wounding and the stage of plant development. Nicotine accumulates preferentially in younger leaf tissue as the plant matures. The molecular mechanisms controlling differential nicotine distribution within the plant are completely unknown, but the results from *NUP1-RNAi* hairy root analysis demonstrated that NUP1 plays a significant role in both nicotine biosynthesis and localization. Future experiments to determine the tissue specific localization of NUP1 will uncover if this protein has a role in nicotine transport in the aerial portions of the plants as well as in the root.

Studies designed to determine the localization of NUP1 will provide valuable insight into the *in planta* role of NUP1. In support of the hypothesis that NUP1 is involved in long distance nicotine transport, the identification of *NUP1* by *in situ* hybridization in the xylem parenchyma is expected. The expression of *NUP1* in any area of the stele in addition to or instead of the xylem parenchyma would also support the hypothesis that NUP1 facilitates long distance nicotine transport since nicotine is expected to concentrate in the stele as it approaches the xylem.

The cell type expression patterns of *NUP1* may alternately show *NUP1* expression does not correlate with the pattern expected for long distance transport of nicotine. This result would suggest NUP1 functions in another aspect of root nicotine

physiology and this result would also be interesting. In addition to nicotine accumulation in the aerial tissues, nicotine also accumulates in the root and the mechanisms leading to the accumulation of below ground nicotine have not been determined. Therefore the localization of *NUP1* to non-vascular cell types could provide insight into the poorly understood accumulation of nicotine within the root.

The observation that decreased *NUP1* expression resulted in the accumulation of more nicotine in the root culture medium has important implications for increased bioprocessing efficiency of other alkaloids of commercial interest. Expanding this work in related *Solanaceae* species that produce medicinal alkaloids holds promise for the use of biological cultures as bioproduction systems. The tropane alkaloid scopolamine is used for the treatment of motion sickness and the related alkaloid atropine is used for pupil dilation and as an antidote for toxins such as organophosphates (nerve gas). Like nicotine, these medicinal tropane alkaloids are produced in the root and in hairy root cultures. Developing a bioreactor system in which root cultures selectively excrete the desired alkaloid through bioengineering of a tropane alkaloid transporting *NUP*-like paralog has considerable commercial potential. The use of hairy root cultures in contrast to whole-plants would provide substantial savings because the export of the alkaloid to the medium would obviate the need for chemical extraction of the alkaloid from harvested roots. Instead the alkaloids could be extracted directly from the growth medium with greatly reduced chemical complexity. This would greatly reduce the costs associated with current batch culture methods that require organic extraction of disrupted root tissues, producing extracts with thousands of organic small molecules. These chemically complex mixtures require extensive purification steps required to isolate low-

yield but high-value natural product pharmaceuticals from hundreds of other small molecules in the extract. However, by moving the low-yield high-value natural product pharmaceutical to the culture medium, the purification of one molecule from a mixture of only a few dozen chemicals comprising the synthetic culture medium becomes a very simple problem from a process engineering perspective.

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