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ABSTRACT OF DISSERTATION

Nita Sachan

The Graduate School
University of Kentucky

2004

**IDENTIFICATION OF SIGNALING FACTORS INVOLVED IN THE
REGULATION OF ALKALOID METABOLISM IN *N. TABACUM***

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture
at the University of Kentucky

By
Nita Sachan

Lexington, Kentucky

Co-Directors: Dr. Deane Falcone, Assistant Professor of Agronomy
and Dr. George Wagner, Professor of Agronomy
Lexington, Kentucky

2004

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ABSTRACT OF DISSERTATION

IDENTIFICATION OF SIGNALING FACTORS INVOLVED IN THE REGULATION OF ALKALOID METABOLISM IN *N. TABACUM*

To identify the signaling mechanisms and components that are involved in regulation of a promoter for a gene involved in a secondary pathway I studied the nicotinic alkaloid biosynthetic pathway using various *N. tabacum* tissues. Nicotine and tropane alkaloids are widely known to be synthesized predominantly in the roots of species that produce pyrrolinium ring containing alkaloids. Putrescine *N*-methyltransferase (*PMT*) catalyzes the first committed step in the biosynthesis of these alkaloid secondary products and earlier studies have indicated that *PMT* gene expression is restricted to root tissue in Solanaceae plants. To further elucidate the factors that govern the regulation of alkaloid synthesis, expression patterns dictated by the 5'-flanking region of one of the members of the *PMT* -gene family, *NsPMT3*, using the β -glucuronidase (*GUS*) reporter gene were examined. Various treatments were used to characterize the nature of signaling in various tissues of seedlings, whole plants and callus. High expression levels were detected in root tissue and no expression was detected in leaves, in agreement with previous studies. However, mechanically wounded leaves resulted in highly localized *PMT* expression. This wound-induced expression was transient, with maximum levels occurring immediately after wounding and diminishing after approximately 2–4 h. RT-PCR analysis of mRNA isolated from wild-type plants also indicated upregulation of *PMT* expression in leaves upon wounding as well as very low transcript levels in unwounded leaves. Low levels of *PMT* activity were detected in leaf tissue, and this activity did not increase significantly upon wounding.

Transgenic callus material showed strong repression of *PMT* promoter activity in the presence of light and auxin, whereas dark conditions and the absence of auxin upregulated *PMT* promoter activity. Reactive oxygen species have been implicated in signaling. When treated with the scavengers of reactive oxygen species (*ROS*), dimethylthiourea (*DMTU*) or catalase, tobacco callus tissue, which displays highly repressed alkaloid synthesis under normal culture conditions in the light, exhibited significant induction of *PMT* promoter activity and alkaloid accumulation. It is thought that light repression signals through an *ROS* intermediate to affect changes in alkaloid pathway gene expression.

Upregulation of *PMT*-promoter activity was observed upon treatment with JA (jasmonic acid) or darkness in roots of very young transgenic seedlings. Treatment with auxin, salicylic acid (SA) and H₂O₂, on the other hand, was found to highly repress *PMT* promoter activity. Action of other ROS such as nitric oxide and superoxide radicals on *PMT* expression is not clear but probably play less of a role, compared to H₂O₂. Consistent with this contention, treatment with light or glucose oxidase (GOX) and glucose to generate H₂O₂, also repressed alkaloid accumulation, and treatment of seedlings to dark conditions, the ROS scavenger DMTU, or jasmonic acid resulted in alkaloid accumulation. Long distance signaling from leaves to roots is also suspected to involve ROS, as leaves treated with GOX and glucose exhibited repressed *PMT* promoter activity in roots. The responses of the *PMT* promoter to auxin, salicylic acid and H₂O₂ treatments were conserved as shown by similar responses of the *N. tabacum* *PMT* promoter when examined in transgenic *Arabidopsis*, thereby suggesting that these molecules signal through a conserved mechanism. Thus, ROS is strongly implicated in acting as an intermediate in these signaling processes with H₂O₂ proposed as a major signaling component.

KEYWORDS: Alkaloids, putrescine *N*-methyltransferase, wounding, reactive oxygen species, signaling in different tissues.

Nita Sachan

07/27/04

IDENTIFICATION OF SIGNALING FACTORS INVOLVED IN THE
REGULATION OF ALKALOID METABOLISM IN *N. TABACUM*

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DISSERTATION

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ACKNOWLEDGEMENTS

First of all I would like to thank my family, who have been the rock of my life. This work would not have been possible without their love and sacrifice. My parents have been the guiding force throughout my education and have always encouraged me to reach for higher goals. My husband, Manish, for having made endless sacrifices to help me reach this stage. His research ideas and intellectual stimulation has helped me tremendously. Biggest thanks to my five month old daughter, Diya, who is now the shining light of my life, for her patience during writing of this thesis.

My advisor and mentor, Dr. Deane Falcone, for his constant encouragement, guidance and support throughout this thesis. This work would not have been possible without his relentless efforts to guide me through new areas. I would also like to thank Dr. George Wagner, for his constant encouragement and for constantly reminding me of the value of patience. Thanks are also due to Dr. Arthur Hunt, Dr. Robert Houtz and Dr. Said Ghabrial for agreeing to be on my Ph.D. committee and for their helpful suggestions. I am grateful to Dr. Harold Burton for agreeing to be my external examiner.

Thanks are also due to all the past and present Falcone lab members. They have been like my second family. This work would not have been possible without their support and encouragement. In particular, I would like to thank Dr. Jingxian Zhang, for her help in coming up with new ideas and Irinia Artiushin for her help in lab. I would also like to thank my other lab members, Dr. Kil-Young Yun, Dr. Suchada Sukrong and Gabriela Diniello. It goes without saying that I could not have survived my initial years at UK without the help of my dear friends Annaps and Vigyan and my brother and friend, Sunil, have helped me get over the worst of graduate school. There are so many others that I have not mentioned, to whom I am grateful for their help and kindness throughout my stay at Lexington.

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Chapter 1

Introduction

Plant secondary metabolism

About 100,000 different secondary metabolites have been isolated from plants to date (Verpoorte, 2000a; Nugroho et al., 2002; Verpoorte et al., 2002). Secondary metabolites or plant natural products can generally be defined as those small-molecule products that are not essential for normal growth and life of the producing organism but might play a role in enhancing their fitness. They are biosynthesized from one or more general metabolites by a wider variety of pathways than is typically available in “primary” metabolism (Kutchan, 1995; Facchini, 2001). Based on their biosynthetic origin, secondary metabolites can be classified into three main groups: terpenoids, phenylpropanoids and alkaloids.

For many years, secondary metabolites were considered to be more or less waste products, with no apparent use to the plant. However, increasing knowledge of secondary metabolism has led to the greater acceptance of their involvement in the relationship of an organism with its environment (Verpoorte, 2000b). Also, some essential growth regulators are still considered to be plant secondary metabolites such as gibberellins, abscisic acid, ethylene, indole acetic acid and kinetin (Seigler, 1998).

Plant secondary metabolites are of commercial importance. They are used in the form of drugs, flavors, fragrances, insecticides and dyes. Twenty-five percent of prescribed drugs in western medicine are derived from plants (Verpoorte, 2000b). Due to the huge diversity of organisms in nature, there is at least an equally enormous variety of secondary metabolites, and these has potential to be screened for new activities for drug development.

Diversity of secondary metabolites provides the plants with the capability to improve defenses against microbial attack or insect/ animal predators. The amounts of secondary compounds found in an organism is a result of an equilibrium between synthesis, storage and degradation. The developmental stage of the organism is also often a determinant in triggering the onset of secondary metabolite accumulation (Haslam, 1986). It was earlier thought that

secondary metabolites arose spontaneously or via the action of non-specific enzymes. However, it is now generally accepted that highly specific enzymes are involved in the biosynthesis of the vast majority of secondary metabolite products (Wink, 1999).

Water soluble compounds are normally stored in plant vacuoles (Boller and Weimken, 1986) while lipophilic substances are sequestered to resin ducts, lactifers, trichomes or cuticles (Wiermann, 1981). The site of biosynthesis can be restricted to a single organ and accumulation might occur in different plant tissue. Long distance transport of metabolites can take place via xylem or phloem to the site of storage (Wink, 1999).

Plant Alkaloids

The term alkaloid is derived from Arabic word al-qali, the plant from which “soda” was first obtained (Kutchan, 1995). Alkaloids are a group of naturally occurring low-molecular weight nitrogen-containing compounds, found in 20% of plant species. This nitrogen is derived from amino acids, is incorporated into a heterocyclic ring, and is basic (Pelletier, 1983). The classification of alkaloids is based on their carbon-nitrogen skeletons. Amongst the basic ring structures are pyridine, isoquinoline, pyrrole, indole, piperidine, and pyrrolidine (Pettersen et al., 1981). Alkaloids have potent biological activity, which makes them suitable for use as pharmaceuticals, stimulants, narcotics and poisons. In nature, alkaloids are mainly found in higher plants, but they can also be found in club mosses (*Lycopodium* spp.), horsetails (*Equisetum* spp.), and fungi (Robinson, 1981). Alkaloids are also found in microorganisms and animals. Alkaloids occur in 34 of the 60 Orders in higher plants, about 40% of all plant families (Pettersen et al., 1981).

The majority of alkaloids in plants are derived from amino acids, such as tyrosine, phenylalanine, ornithine/ arginine, lysine etc. However, in insects and microorganisms, increasing numbers of alkaloids have been found to be of terpenoid or polyketide origin (Roberts and Strack, 1999). The discovery of morphine in 1806 began the field of plant alkaloid biochemistry. However, the structure of morphine was not determined until 1952 due to its stereochemical complexity. The fact that morphine is still derived from plant sources today dramatically illustrates the challenges of chemical synthesis and the importance of natural sources. Major technical advances have occurred in this field that allowed the elucidation of the

alkaloid biosynthetic pathways. Among these were the introduction of radiolabeled precursors and the use of plant cell cultures as an abundant source of enzymes that could be isolated, purified and characterized. Molecular techniques made it possible to isolate genes involved in alkaloid secondary pathways. More recent studies have addressed topics regarding the events of signal perception, pathways of signal transduction and the function of gene promoters in relation to the regulation of alkaloid biosynthesis (Facchini, 2001).

Nicotinic alkaloids

Nicotine and related alkaloids are found in genus *Nicotiana*. *Nicotiana tabacum* is cultivated throughout the world for preparation of cigars, cigarettes and chewing tobacco. *Nicotiana tabacum* was indigenous to tropical America. Native Indians smoked the dried leaves when Columbus discovered the New World. The history of chemical identification of nicotine has been well described (Holmstedt, 1988; Domino, 1999). In 1807, Cerioli and Vauquelin in 1808 isolated the 'essential oil' or 'essence of tobacco'. Posselt and Reimann in 1828 isolated nicotine from tobacco and in 1843 Melsens described its empirical formula. Pictet and Crepieux synthesized nicotine in 1893 and in 1904, Pictet and Rotschy described the chemical isomerism of nicotine. In 1978, Pitner et al. identified the stereoisomeric orientation of natural (S)-nicotine (Domino, 1999).

Nicotine is also found in other plant species such as *Erythoxylon coca* (coca), *Lycopersicon esculentum* (tomato), *Atropa belladonna* (deadly nightshade) and *Asclepia syriaca* (milkweed). Other tobacco alkaloids include nornicotine, anatabine and anabasine and are important because of their chemical properties that confer physiological stimulant activities in the brain, making them addictive products (Wink, 1998). Nicotine constitutes about 2-8% of the total dry weight of the cured commercial tobacco leaf, although a much larger range exists in different *Nicotiana* species. Nicotine is the most abundant alkaloid in a majority of *Nicotiana* species, constituting about 90% of the total alkaloid fraction. Related alkaloids occur in much lower concentrations. For example anabasine, anatabine and nornicotine constitute less than 5% of the total alkaloid fraction. The amount of alkaloids in different tobacco species varies. In *N. glauca* for example, anabasine is the major alkaloid (Sisson and Severson, 1990). Nornicotine is produced mainly during the curing or aging of tobacco leaves. High levels of nornicotine yield a

tobacco product that has undesirable smoking quality. Many minor alkaloids are not produced by specific enzymes, but are probably produced after the tobacco leaves are harvested. The alkaloids formed nonenzymatically from nicotine and nornicotine include myosmine, cotinine, nicotyrine, nicotine-1'-N-oxide, N'-methylmyosmine and 2,3'-bipyridine. Other species besides *Solanaceae* genera containing nicotine include *Duboisia*, *Equisetum* (horsetails), *Lycopersicum* (tomatoes), *Lycopodium* (club mosses), *Sedum* (succulent plants) and *Solanum* (potatoes).

Efficient synthesis of complex alkaloids would benefit from the physical association of a series of complex enzymes or subcellular compartmentation of some of the enzymes so that pathway intermediates do not inefficiently diffuse into the cytoplasm. No data has yet been obtained to suggest metabolic channeling in alkaloid biosynthesis. Electron micrographs of *Berberis* spp. cells show spherical vesicles corresponding to isolated alkaloid vesicles as clusters in small vacuoles (Amann et al. 1986). Nicotine production can be elicited in tobacco BY-2 cell cultures (Imanishi et al., 1998). Goossens et al., (Goossens et al., 2003) used pleiotropic drug resistance (PDR)-type ATP-binding cassette (ABC) transporters of yeast (*Saccharomyces cerevisiae*) to study the secretion of both exogenously applied and endogenously synthesized secondary metabolites. Yeast strains deficient for one of the multidrug resistance-associated protein-type transporters was used to assess sensitivity to hyosyamine and scopolamine. *pdr5* mutant strain showed substrate specificity for tropane alkaloids. Thus, it was shown that Pdr5p is the only ABC transporter involved in transport of these alkaloids in yeast. Involvement of Pdr5p in nicotine transport is unclear because *pdr5* mutant strains showed only a weak tolerance to nicotine. Numerous approaches have been used to increase the production of alkaloids (Fecker et al., 1993; Bird et al., 2003). Problems related to obtaining useful metabolites from whole plant sources can be circumvented using cell cultures (Kutchan, 1995).

Biochemistry of nicotinic alkaloids

The four principal tobacco alkaloids all have a pyridine ring in common (see figure 1.1 and 1.2). Production of both nicotine and anabasine involves the condensation of the primary metabolite nicotinic acid and another nitrogen-containing metabolite. In the case of nicotine, it is N-methyl pyrrolinium (Feth et al., 1986). The pyridine ring of these alkaloids is derived from nicotinic acid via the pyridine nucleotide cycle. The rate-limiting step in this cycle is catalyzed

by quinolinic acid phosphoribosyltransferase (QPRTase). QPRTase activity is found in roots of tobacco, but not in leaves. QPRTase activity in roots is proportional to the increase in its activity upon decapitation of the plant to increase the amount of nicotine (Wagner et al., 1986). QPRTase seems to have less influence on the nicotine biosynthesis, since the nicotinamide adenine dinucleotide (NAD) formed by this cycle, is mainly required by primary metabolic pathways (Wagner et al., 1986).

The pyrrolidine ring of nicotine is derived from putrescine. Nicotine is synthesized in tobacco roots from ornithine and/or arginine by the way of putrescine (Leete and Liu, 1973). Formation of putrescine, which is possible from either of the enzymes ornithine decarboxylase (ODC) or arginine decarboxylase (ADC) has been shown to occur mostly via ADC. This was determined experimentally using inhibitors of the decarboxylases and by monitoring changes in enzyme activities during the induction of alkaloid biosynthesis (Tiburcio and Galston, 1986). At the whole plant level, roots of decapitated plants show a 4.5-fold higher ADC activity than ODC, along with increased alkaloids levels (Yang et al., 1984).

Putrescine is either metabolized to higher polyamines, such as spermidine or spermine, or conjugated with cinnamic acid derivatives or fatty acid conjugates in higher plants (Smith, 1981). It is also converted to *N*-methylputrescine in plants producing nicotine to tropane alkaloids. Putrescine *N*-methyltransferase (PMT; EC 2.1.1.53) catalyzes the first committed step in the biosynthesis of pyrrolinium ring-containing

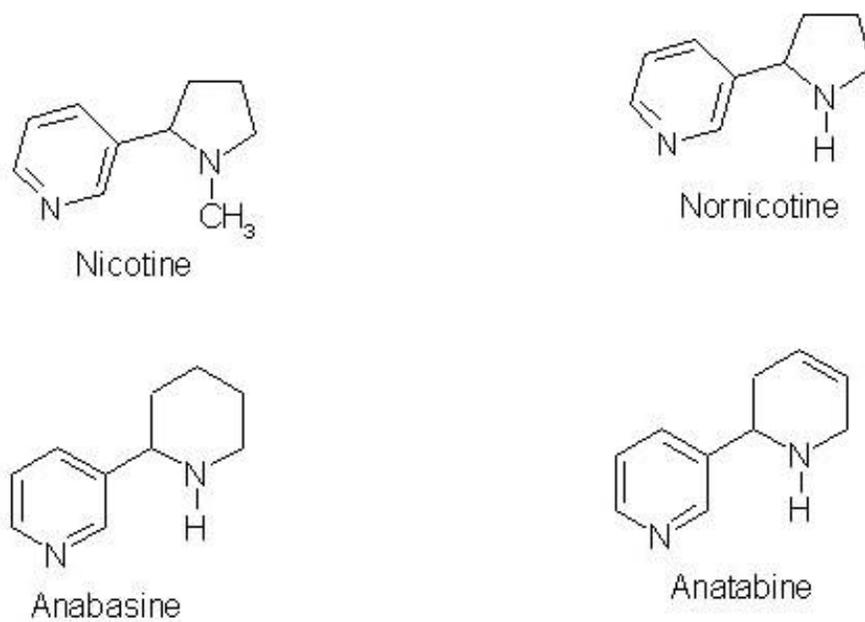


Figure 1.1 Structures of the main nicotinic alkaloids present in *N. tabacum*

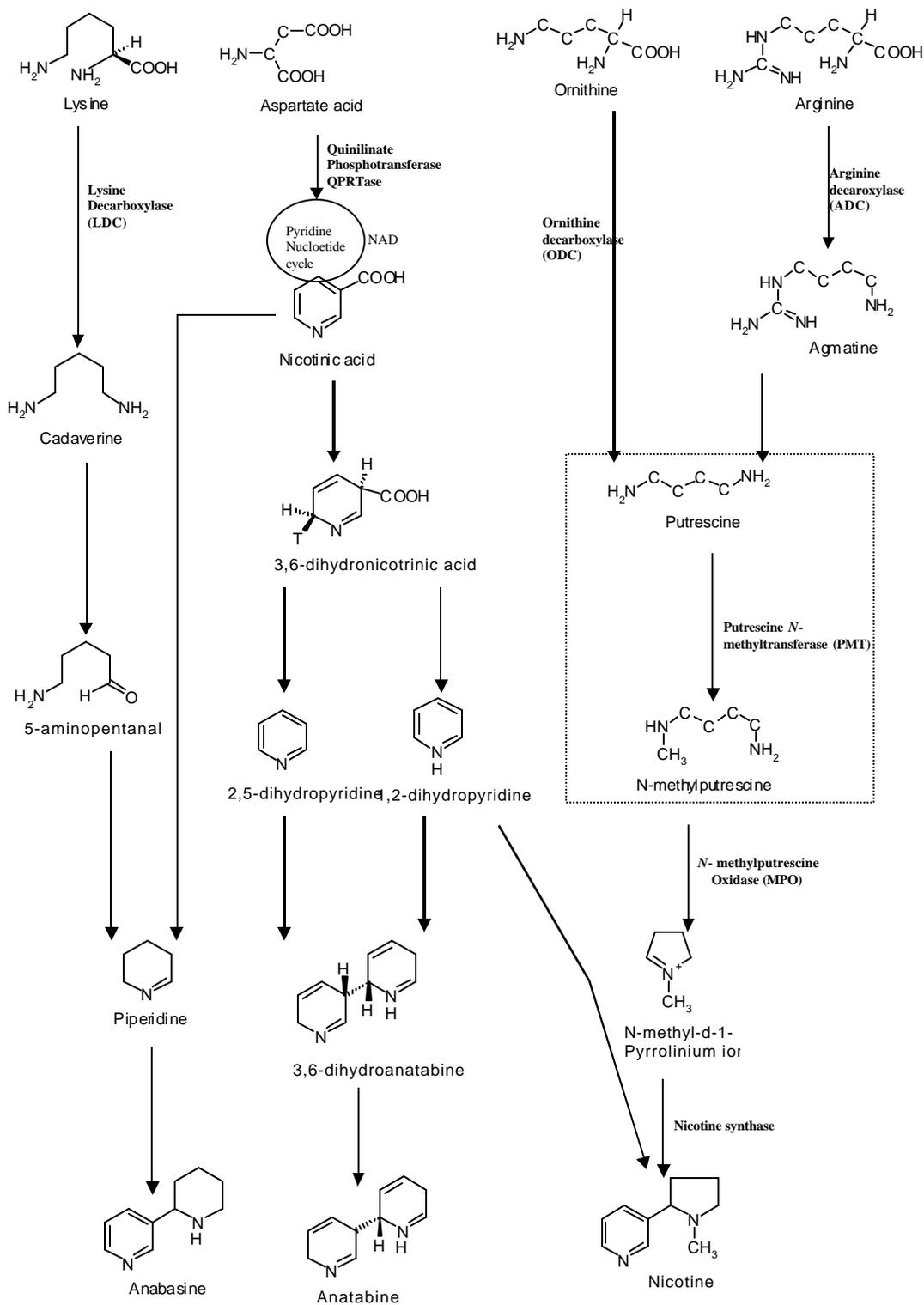


Figure 1.2 Biosynthetic pathways leading to the major alkaloids in *N. tabacum* with the enzymes catalyzing the major steps.

alkaloids (Hibi et al 1992) by incorporating the methyl group donated by S-adenosyl-methionine (SAM). *N*-methylputrescine is oxidized by *N*-methylputrescine oxidase (MPO; EC 1.4.3.6) to form the *N*-methyl- Δ^1 -pyrrolinium cation. Activity of MPO in roots is not proportional to nicotine accumulation in tobacco (Saunders and Bush, 1979). Nicotine synthase catalyzes the last step in nicotine biosynthesis, the condensation of *N*-methyl- Δ^1 -pyrrolinium ion with nicotinic acid to form nicotine. Nicotine synthase was reported to have been isolated by (Friesen and leete, 1990), but others have not been successful in reproducing this report (Bush et al., 1999).

Because of the central position of putrescine *N*-methyltransferase (*PMT*) as the catalyst for the committed step in the nicotine biosynthetic pathway, this study employs the expression of *PMT* as an indicator of the entire nicotine pathway expression.

Genetics involved in alkaloid biosynthesis

In the early 1930s, Cuban cigar varieties were found that had low nicotine content. Backcrossing these varieties lead to the identification of two semidominant mutant genes, *nic1* and *nic2*, that controlled the low-alkaloid phenotype (Legg et al., 1969). *Nic* genes are expressed in plant roots and the resulting alkaloid is found in leaves. The semidominant nature of *nic1* and *nic2* is shown by their effects on alkaloid accumulation. *nic1* locus is ~2.4 times stronger than *nic2* (Legg and Collins, 1971), and *nic1* has stronger effect on *PMT* expression than the *nic2* mutation. In tobacco plants containing both *nic1 nic2*, *PMT* transcripts are detected only in roots, but at levels much lower than in wild-type roots (wild type>*nic1*>*nic2*>*nic1nic2*) (Legg et al., 1969; Legg and Collins, 1971). Enzyme activities of *PMT* and quinolinic acid phosphoribosyltransferase are reported to be simultaneously reduced by either of the two mutations. Both mutations caused a decrease in *PMT* activity and simultaneous increase in polyamine content, indicating that for putrescine formation, arginine or ornithine is much less affected by the *Nic* genes than nicotine synthesis. This suggests that *Nic* genes may be regulatory in nature that control the multiple biosynthetic enzymes required for nicotine synthesis (Saunders and Bush, 1979). *Nic1* and *Nic2* maybe duplicate genes originating from one of the two tobacco progenitors. The stronger allele, *nic1* might be a null mutation while the weaker *nic 2* might be a

non-null mutation with residual *Nic* function (Hibi et al., 1994). Although no information is available on the nature of their encoded products, it has been speculated that *Nic1* and *Nic2* likely encode transcriptional regulators controlling genes involved in the alkaloid biosynthesis (Hibi et al., 1994).

The N-terminal amino acid sequence of PMT is highly homologous to mammalian spermidine synthase (SPDS), which transfers the aminopropyl moiety of decarboxylated S-adenosylmethionine to putrescine, producing spermidine (Hibi et al., 1994). The *PMT* gene is thought to originate from *SPDS* gene during diversification of Solanaceae because of this homology (Hashimoto et al., 1998).

N. tabacum is a natural amphidiploid and is thought to have originated from two wild progenitors (Gerstel, 1960, 1963). Kenton et al. (1993), based on cytological evidence, have shown that a portion of the *N. tabacum* genome originated from *N. sylvestris* and an introgressed hybrid between *N. tomentosiformis* and *N. otophora*. Five genes encoding *PMT* exist in *N. tabacum* whose primary structure supports its origin from *N. tabacum* as proposed by Kenton et al. (1993) (Riechers and Timko, 1999). Riechers et al. (1999) have also shown that the five *PMT* genes are expressed in roots of wild-type *N. tabacum*. Root pericycle of *Atropa Belladonna* shows expression of the *AbPMT1* 5' gene flanking region fused to the GUS reporter gene (Suzuki et al., 1999). However, we have shown wound-induced expression of *NtPMT3* in leaves (chapter 2; (Sachan and Falcone, 2002).

Hashimoto et al. (1998), based on a molecular analysis of the exon1 region, has shown that *N. sylvestris* contributes three genes and *N. tomentosiformis* contributes one gene to the *PMT* family in the *N. tabacum* genome. *NtPMT2*, *NtPMT3* and *NtPMT4* of *N. tabacum* are likely derived from *NsPMT1*, *NtPMT2* and *NtPMT3* genes of *N. sylvestris*. These *NtPMT1a* and *NtPMT1b* genes are thought to be derived from *PMT* genes in *N. tomentosiformis* and *N. otophora*, respectively (Riechers and Timko, 1999). Promoter analysis of the *PMT* genes (Riechers and Timko, 1999) did not reveal any similarity to reported sequence motifs for methyl jasmonate responsiveness (Mason et al., 1995; Rouster et al., 1997) or auxin response elements reported in other plant genes (Ottoline, 2002). Amongst all *PMT* genes, *NtPMT1a* is the most highly expressed gene in *N. tabacum* (Riechers and Timko, 1999).

Overexpression of *A. belladonna* *PMT* cDNA driven by an enhanced cauliflower mosaic virus (CaMV) 35S promoter caused the *PMT* transcript level to increase 1.3 to 3.3-fold, however,

no change in alkaloid level was seen. When *PMT* was overexpressed in *N. sylvestris*, *PMT* transcript levels went up 4- to 8-fold. These plants had 40% higher nicotine accumulation. Co-suppression of *PMT* caused the nicotine levels to be highly reduced, to about 2% of control (Sato et al., 2000). These plants had lower *PMT* transcript level in roots, but higher polyamines in leaves. When an *N. tabacum* *PMT* gene was overexpressed using a CaMV 35S promoter in *Duboisia* plants, a 2-4-fold increase in *N*-methylputrescine levels was seen compared to wild-type, but no significant changes in tropane or pyridine-type alkaloids resulted (Moyano et al., 2002). Another report on antisense-*PMT* manipulation showed a reduction in the nicotine content at the same time an increase occurred in the anatabine content. It was hypothesized that the increase in nicotinic acid accumulation in the antisense-*PMT* lines resulted from a decrease in the 1-methyl- Δ^1 -pyrrolinium cation, which resulted an increased synthesis of anatabine (Chintapakorn and Hamill, 2003). These studies show widely differing results in different alkaloid producing species.

Regulation of PMT during alkaloid biosynthesis

PMT gene expression is primarily restricted to root tissue in Solanaceae plants. However, *PMT* expression has also been shown to occur upon wounding in leaves (Sachan and Falcone, 2002). *PMT* expression is known to be repressed by auxin and stimulated by jasmonic acid (Hibi et al., 1994; Imanishi et al., 1998). It was also shown that *PMT* enzyme activities and nicotine content in the roots increase several-fold 1 day after decapitation (Mizusaki et al., 1973). *PMT* mRNA increases within 30 minutes after decapitation, peaks after 1 hr, and then declines (Hibi et al., 1994). Cultured roots of tobacco when grown in the presence of 3 μ M indolebutyric acid (IBA) and then shifted to auxin-free medium, had increased *PMT* mRNA levels in less than 30 minutes, which peaked at 1 hr, and then slowly decreased. Mizusaki et al. (1973) have shown that decapitation of tobacco shoots causes a rapid stimulation of nicotine biosynthesis 24 hours after decapitation and then a decline after 3 days to basal levels of non-decapitated control plants. Activity of the ODC, *PMT* and MPO enzymes have been shown to increase 24 hrs after decapitation. The transient nature of the enzyme activities might be due to decreased auxin synthesis and transport in the shoot apex and therefore reduced basipetal auxin movement (Goldsmith, 1968; Mizusaki et al., 1973).

Tobacco callus cultures derived from petioles showed lower alkaloid contents when grown in the presence of high α -naphthalene acetic acid (11.5 μ M α -NAA). In contrast, when the tissue was placed in a lower auxin concentration (1.5 μ M α -NAA), the amounts of nornicotine and nicotine increased (Tiburcino et al., 1985). Polyamine levels have been shown to be affected by auxin treatment within the same time frames (Tiburcino et al., 1985). Yang et al. (1984) have reported a decrease in putrescine, spermine and spermidine by 50 to 80 % after 2 days and 12 days, respectively, after decapitation of the tobacco plant.

Nicotine and related alkaloids play an important role in protecting the plant against insects and herbivores, and also as a regulatory substance for plant growth. Nicotine is synthesized in roots and is transported via the xylem to the aerial parts where it is stored in vacuoles (Wink, 1998). Nicotine is present in most tobacco tissues. In the tobacco leaf, nicotine content is higher around the edges and at the tip (Hashimoto and Yamada, 1995). (Karban and Baldwin, 1997) proposed that the reason for production of nicotine in roots and then its subsequent translocation to the shoots is that nicotine protects roots from herbivory. If the leaves were the site of nicotine production then increased removal by herbivory would diminish the alkaloidal response to the damage. But with roots as the site of nicotine synthesis, even if majority of leaves are grazed, there is a continuous supply of nicotine to the remaining leaves, thus increasing overall alkaloid concentration. Alkaloids other than nicotine may be produced in organs other than roots. A large number of endogenous and exogenous factors are known to affect gene expression leading to alkaloid formation in plants including developmental stage, phytohormones, and various biotic and abiotic stresses (Kutchan, 1995). Tobacco farmers traditionally remove the flowering parts (a process called 'topping') of commercial *Nicotiana tabacum* before seed set, thereby removing the principal source of auxin and breaking apical dominance. This results in increase of leaf mass and also a rise in alkaloid levels. An increase in alkaloid levels in leaves is due to a decrease in auxin flow from the apex to the roots, the site of alkaloid biosynthesis (Bush et al., 1999). Auxin has been shown to decrease nicotine synthesis in cultured tobacco callus (Takahashi and Yamada, 1973; Tiburcino et al., 1985) and also reduce the enzyme activities in the nicotine biosynthetic pathway (Mizusaki et al., 1973). Alkaloids have also been shown to accumulate in the aerial parts of *Nicotiana* species not as a result of removal of apical dominance but as a result of damage to leaf. Such an increase is thought to

confer a survival advantage on the plant against herbivory (Baldwin, 1994; Karban and Baldwin, 1997; Bush et al., 1999).

Plants have evolved several defense strategies against herbivores. Accumulation of defense proteins and secondary metabolites in damaged tissues is thought to prevent excessive herbivory (Baldwin and Preston, 1999). Herbivore attack results in the induction of both local and systemic defense proteins and chemicals. Studies on tomato proteinase inhibitor (PI) genes have shown their induction upon insect feeding or mechanical damage via signal transduction pathways involving systemin, oligosaccharides and jasmonates (Koiwa et al., 1997). Jasmonic acid (JA) and its methyl ester (MeJA) are fatty acid derivatives of linolenic acid via the octadecanoid pathway (Creelman and Mullet, 1997). JA inhibits wound-induced expression of proteinase inhibitor genes (Doares et al., 1995; Pena-Cortes et al., 1995) and at the same time induces accumulation of defense related compounds such as phenolics, flavonoids and alkaloids (Gundlach et al., 1992; Muller et al., 1993). Upon leaf injury by insect or mechanical wounding, the signal, which is most probably jasmonate, is transmitted to the roots where the genes for nicotine synthesis are activated. Baldwin et al., (Baldwin et al., 1997; Baldwin, 2001) have done whole plant research into the relationship between herbivory in plants and increases in the endogenous JA pools and nicotine levels.

Accumulation of JA and nicotine was inhibited by application of salicylic acid (SA) directly to the wounded tissue. SA is thought to be an important signal that activates plant defense genes after pathogen attack. However, application of SA to the tissue adjacent to wounded tissue does not affect nicotine accumulation (Baldwin, 1999). So it is thought that SA may inhibit the JA signal cascade (Baldwin et al., 1997; Preston et al., 1999). However, (Nugroho et al., 2002) have shown that the leaves from transgenic tobacco that express constitutive salicylic acid (CSA) do not accumulate enough SA to inhibit the production of nicotine, suggesting that the levels of SA in the CSA plants were not enough to inhibit the JA signal cascade.

Hydrogen peroxide (H_2O_2) was viewed as a toxic byproduct, until it was shown to be a signaling molecule in both plants and animals (Neil et al., 2002). High intracellular concentrations of H_2O_2 concentrations are balanced by activity of efficient antioxidant systems (Orozco-Cardenas and Ryan, 2002). Increasing evidence indicates that H_2O_2 functions as a transient signaling molecule in plants. H_2O_2 generation during the oxidative burst is one of the

earliest cellular responses to potential pathogens and elicitor molecules (Lamb and Dixon, 1997). H_2O_2 induces the expression of defense-related genes such as glutathione S-transferase and phenylalanine ammonia lyase (Levine et al., 1994). H_2O_2 also activates mitogen-activated protein kinases (MAPKs), which are conserved signaling kinases that modulate gene expression and transduce cellular responses to extracellular stimuli (Desikan et al., 1999). Transgenic potato plants overexpressing a fungal glucose oxidase exhibit constitutive production of sub-lethal levels of H_2O_2 and these plants exhibit enhanced disease resistance (Wu et al., 1997).

Recently an ornamental tobacco *NEC5* (Nectarin 5) gene, which encodes a flavin-containing berberine bridge enzyme (BBE)-like protein, has been shown to possess glucose oxidase (GOX) activity (Carter and Thornburg, 2004). BBE catalyzes the conversion of *s*-reticuline into *s*-scoulerine and is a major branch point in benzophenanthridine alkaloid biosynthesis in plants (Bird and Facchini, 2001). However, most BBE-like proteins have unknown functions. The nectar of ornamental tobacco lacks alkaloids, hence it is thought that *NEC5* may not play a role in alkaloid metabolism (Carter and Thornburg, 2004). Tobacco nectar has a high concentration of sugars (35% [w/v]), and *NEC5* GOX activity might contribute to the antimicrobial levels of hydrogen peroxide. Honey is known for its antimicrobial nature and has been used in wound dressings. It is thought that GOX is secreted from the hypopharyngeal gland of worker honeybees (*Apis mellifera*) into the nectar. Studies have demonstrated that the hydrogen peroxide present in honey might be responsible for its antiseptic powers (Bang et al., 2003; Carter and Thornburg, 2004). Thus, analogous systems may exist in tobacco nicotine biosynthesis and the *Helicoverpa zea* (corn earworm) caterpillar which has GOX activity in its saliva (Musser et al., 2002). Caterpillar feeding on the "*Nicotiana*" leaves causes degradation of nicotine by production of H_2O_2 .

Studies involving the overexpression of *Vitreoscilla hemoglobin* (VHb) in tobacco resulted in higher chlorophyll content and increased accumulation of nicotine (up to ~34%) while anabasine was decreased (Holmberg et al., 1997). It was proposed that nicotine synthase, which is oxygen dependent and one of the least studied enzymes in the nicotine biosynthetic pathway, might be involved. VHb is known to increase oxygen availability, and has also been shown to scavenge oxygen radicals, act as terminal oxidase and could reduce peroxidase (Holmberg et al., 1997). Higher oxygenation during germination results in an enhanced respiration rate and reduced toxic byproducts (Holmberg et al., 1997).

Apart from H₂O₂, salicylic acid (SA) and nitric oxide (NO) also play important roles in the hypersensitive responses or programmed cell death in plants, and in systemic acquired resistance (Shirasu et al., 1997; Delledonne et al., 1998). Salicylic acid can inhibit catalase activity in vitro and cause increased H₂O₂ concentration in vivo (Conrath et al., 1995; Durner and Klessing, 1996). Nitric oxide (NO) and reactive oxygen species (ROS) play important roles in the activation of defense responses against pathogen attacks (Durner and Klessing, 1999). NO activates or inhibits certain heme-containing enzymes (Durner and Klessing, 1999) and it has been shown to stimulate plant defense responses through cGMP-dependent signaling cascade involving generation of cADPR and activation of mitogen-activated protein kinases (Klessing et al., 2000; Kumar and Klessing, 2000; Orozco-Cardenas and Ryan, 2002).

In the work described here regulation of alkaloid metabolism in *N. tabacum* is further studied in the context of intact plants and also undifferentiated callus tissue. New findings that indicate a role of ROS in alkaloid biosynthesis is presented and discussed in terms of the role of ROS in alkaloid regulation.

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Chapter 2

Wound-induced gene expression of putrescine-*N*-methyltransferase in leaves of *Nicotiana tabacum*

Introduction

Plants are continually exposed to diverse environmental challenges, including attacks from a wide range of herbivores. Signals elicited by such attacks arise from a number of specific perturbations associated with tissue injury. Among these, mechanical wounding may be one of the most critical signals because of its immediacy, causing the initiation of further plant responses. Thus, mechanical injury, pathogen attack, and damage from herbivores and insects can all set into motion defined self-defense systems, with some wound signals being transmitted from damaged tissues to the whole plant systemically or some acting locally, thus inducing changes only in the vicinity of the wound site. These responses enable the precise elaboration of defense reactions to counter specific challenges at the plant's first line of defense (Baldwin et al., 1994; Baldwin et al., 1997; Walling, 2000).

Nicotine and related alkaloids are predominant secondary metabolites produced by a number of *Nicotiana* species and may function as defensive toxins by acting at specific receptors in the nervous system of herbivores (Wink, 1998; Shoji et al., 2000). Pyrrolinium alkaloids, including nicotine, are synthesized in the tobacco root from arginine and/or ornithine by the way of putrescine (Hashimoto and Yamada, 1994). Putrescine *N*-methyltransferase (PMT; EC 2.1.1.53) catalyzes the *S*-adenosyl methionine-dependent *N*-methylation of putrescine and is the first committed step in the biosynthetic pathways that lead to nicotine and tropane alkaloids. Elevated nicotine levels in leaves of transgenic *pmt*-overexpressing lines of *N. sylvestris* confirm its role as a key regulatory step in the pathway (Sato et al., 2001). Consistent with the location of the pathway based on precursor labeling studies, tobacco PMT enzyme activity and gene

expression is detected mainly in root tissues, where it has been established to be negatively regulated by auxin (Saunders and Bush, 1979; Hashimoto and Yamada, 1994; Hibi et al., 1994). After synthesis, alkaloids are translocated from the roots through the xylem to the leaves, where they accumulate. In other alkaloid-producing species, including *Atropa belladonna*, *Hyoscyamus niger* and *Datura stramonium*, PMT enzyme activity has been detected in the root, but not in the leaf, stem or flower tissues (Hibi et al., 1992). Histochemical studies of *A. belladonna* plants containing *PMT*-promoter GUS fusions indicated specific *PMT* gene expression in the root pericycle. Unlike the response in *N. tabacum*, the expression of *PMT* in *A. belladonna* roots was not responsive to treatment with MeJA (Suzuki et al., 1999).

Alkaloid synthesis is increased in tobacco roots when auxin production is diminished by removal of the floral meristem. Alkaloid production is also stimulated by JA, implicating the involvement of JA in wound-induced signaling. Baldwin et al. (1997) have clearly shown the role of JA signaling in alkaloid accumulation after leaf damage by insects or wounding.

Quinolinic acid phosphoribosyltransferase (QPRTase) is another enzyme that plays a key role in pyridine alkaloid metabolism in *Nicotiana* species. QPRTase ensures the availability of the primary metabolite, nicotinic acid, which is used as an intermediate in the synthesis of alkaloids, since it is responsible for the biosynthesis of nicotinamide adenine dinucleotide (NAD). QPRTase gene expression has been examined in *N. tabacum* and *N. sylvestris*, where nicotine is the most abundant alkaloid. In these species, QPRTase transcripts are detected in roots, but not in leaves, and are found to increase sharply in roots within 12-24 hrs after damage to aerial tissue along with increases in *pmt* transcript levels (Sinclair et al., 2000). In *N. glauca*, where anabasine is the most abundant alkaloid, QPRTase expression occurs in both leaves and roots. However, 12-24 hr after mechanical damage, an increase in QPRTase transcripts was observed only in leaf tissues (Sinclair et al., 2000).

N. tabacum has five *PMT*-encoding genes: *NtPMT1*, *NtPMT2*, *NtPMT3*, *NtPMT4* and *NtPMT1a* (Riechers and Timko, 1999). Many studies have documented the rapid induction of *PMT*-encoding genes by herbivore attack, wounding, or exogenous applications of the endogenous wound hormone, JA, or its methyl ester, MeJA, in roots of the plant (Baldwin et al., 1997; Hashimoto et al., 1998; Riechers and Timko, 1999; Hara et al., 2000). During the course of our study of the regulation of alkaloid biosynthesis in *Nicotiana*, we obtained evidence for a

low level of *PMT* expression in leaves the transcripts of which are abruptly upregulated upon wounding. This upregulated expression was found to be highly localized and short-lived.

Materials and Methods

Plant material

N. tabacum cv. SR1 was used in all studies (Maliga et al., 1973). All plants were grown either in sterile Magenta boxes in a growth room at 22°C on Murashige and Skoog (Sigma) medium containing 1% sucrose, or in standard Fafard (Conrad Fafard, Inc., Agawam, MA) potting mix in a greenhouse.

Promoter isolation, DNA constructs, plant transformation

A *N. tabacum* genomic library (Clontech, Palo Alto, CA) was screened using a probe prepared by PCR from SR1 genomic DNA using published cDNA sequences of the *N. tabacum* *NtPMT1* cDNA (Hibi et al., 1994). DNA was labeled with ³²P using a random primer DNA labeling system (Life Technologies, Inc.). Eight distinct genomic λ plaques were obtained and the clone possessing the longest insert was chosen for further studies. A DNA fragment possessing 2.6-kb upstream of *PMT* was derived from the λ clone and a fragment encompassing 627-bp immediately upstream of the *PMT* coding region was subcloned into the pBlueScript KS vector (Stratagene, Inc.). The 627-bp insert was sequenced by automated capillary sequencing using an ABI prism 310 sequencer and used for further studies. Upon comparison to published *PMT* sequences, the 627 bp *PMT* clone was shown to be similar to *NtPMT3* (GenBank accession number AF126811, Riechers and Timko, 1999). The 627-bp upstream region of *NtPMT3* was fused upstream of a GUS coding sequence (derived from pBI121) construct in a final construct using pZP211 as the vector (Hajdukiewicz et al., 1994). The resulting construct was transferred into *Agrobacterium tumefaciens* strain GV3101 by electroporation (Biorad Micropulser) and used for transformation of *N. tabacum* by the leaf-disc method performed according to (Horsch et al., 1985). Resulting callus material was selected for kanamycin-resistance (300 μ g/ml) and transferred onto fresh MS kanamycin-containing plates every 2 wk. Transgenic plants were regenerated from calli derived from leaf discs and transferred to Magenta boxes containing MS media plus kanamycin (50 μ g/ml). After a period of 5 wk, plants were transferred to a greenhouse.

The *PMT*-promoter GUS fusion construct was also transformed into *Arabidopsis* plants by the “floral-dip” method (Clough and Bent, 1998).

Wounding treatments and histochemical assays

Approximately two-month-old greenhouse-grown plants were used for the wound treatments. For a given experiment, leaf discs were taken from a single leaf to ensure uniformity. A cork borer (1 cm diameter) was used to excise uniform-sized leaf discs. Discs were taken over a 24 hr period of time at following intervals: 24, 12, 8, 4, 2, 1 and 0 hr. The 24-hr disc was removed first and floated in 1 X MS medium for 24 hr before staining for GUS activity. Similarly, 12-hr discs were floated in 1 X MS medium for 12 hr before undergoing the staining regime, while a 0-hr leaf disc was removed and put directly into the X-gluc solution for 12 hr. Histochemical staining for GUS activity was performed according to (Jefferson et al., 1987)). Plant tissue was infiltrated for 15 min in a solution of (1 mg/ml) 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-Gluc), 100 mM K-phosphate buffer (pH 7.0) and incubated at 37°C overnight. After staining, samples were treated with 70% ethanol for 24 hr to enhance tissue transparency.

Fluorometric GUS assays were conducted by taking one-cm diameter leaf sections from a one-month-old plant, each punched with a needle (varying from 0 to 4-5 punches to heavily smashed) to induce wounding. GUS enzyme activity assays were performed according to the 96-well plate determination as described by (He and Gan, 2001). Individual leaf discs were placed into wells of a Costar 96-well plate (with opaque walls) containing 10 μ l of distilled water and 50 μ l of GUS extraction buffer (50 mM NaHPO₄ pH 7.0, 10 mM Na₂EDTA, 0.1% sarcosyl, 0.1% Triton X-100) and were incubated at 37°C for 5 min. Fifteen μ l of 5 mM 4-MUB- β -D-glucuronide (MUG) in extraction buffer was added and further incubated for 1 hr at 37°C. The reaction was stopped by adding Na₂CO₃ to a concentration of 180 mM to the wells. The leaf discs were removed from the wells and fluorescence was measured at 365 nm excitation and 455 nm emission using a Perkin Elmer luminescence spectrophotometer LS50B (Perkin-Elmer, Beaconsfield, UK). Since grinding the tissue to extract proteins would have influenced the injury response, the GUS activity was calculated as fluorescent units per mg fresh weight of the tissue instead of per total soluble protein.

RNA isolation, RT-PCR

Leaf and root tissue samples were frozen in liquid nitrogen at different time points after injury. RNA was isolated using a kit (RNeasy, QIAGEN, Inc., Valencia, CA) according to the manufacturers instructions and the final elution was done with DEPC-treated water. RT-PCR was performed using the One-Step RT-PCR kit (QIAGEN). Fifty-six day-old plant leaves were injured over a time period using a sharp forceps and tissue was frozen in liquid nitrogen. Leaf (200 µg) and root total RNA (20 µg) was amplified using two *PMT*-specific sense and antisense primers:

5'-CCTCGAGATGAACGGCCACCAAAT-3' and

5'-GGGGATCCGCCGATGATCAAAACCTT-3'.

Following DNAase treatment for 30 min at 37°C and inactivation for 10 min at 75°C, RT-PCR was conducted with a reverse transcription cycle consisting of 50°C, 30 min. Then a PCR cycle was performed with the following program: 95°C for 15 min, denaturation at 94°C for 30 sec, annealing at 52°C for 1 min and extension at 72°C for 1 min. Final extension occurred for 10 min at 72°C and end at 4°C. Internal standards used were 18 S rRNA PCR probes included in the kit (QuantumRNA 18S internal standards, Ambion, Austin, TX). Five µL of the total reaction mix was loaded onto a 1% agarose gel. SYBR Green I nucleic acid gel stain (Molecular Probes, Eugene, Oregon, USA) was used for staining the gel for 30 min at a 1:10,000 dilution in TAE buffer in a light proof container. Fluorescing bands were visualized using a Fuji phosphorimager.

PMT enzyme assay

At 13 weeks of growth, flowering greenhouse-grown transgenic plants were decapitated to promote induction of root *PMT* activity. Tissue was harvested 3 d after topping and frozen in liquid nitrogen. Extracts were prepared from roots and both wounded and unwounded leaves according to Mizusaki et al., (1973). One and a half g of tissue was ground in 5 ml extraction buffer with a mortar and pestle. Homogenates were filtered through 4 layers of cheesecloth and clarified by centrifugation at 12,000×g for 15 minutes. Supernatants were applied to Centriplus YM-10 centrifugal concentrators (Fisher scientific) to concentrate proteins. All operations were

performed at 4°C. Total protein content in plant extracts were determined by dye binding according to Bradford (Bradford, 1976) using a kit provided by Sigma.

Putrescine *N*-methyltransferase activity was determined by measuring the radioactivity of ¹⁴C-labeled *N*-methylputrescine formed from putrescine and *S*-adenosyl-*L*-methionine-¹⁴CH₃. Reaction mixtures contained 50µM of Tris-HCl (pH 8.3), 2.5 µM of β-mercaptoethanol, 2 µM of putrescine, 680 nmoles of *S*-adenosyl-*L*-methionine-¹⁴CH₃ (0.04 µCi) and 0.31 ml of enzyme extract in a final volume of 0.355 ml. After 30 min incubation at room temperature, the reaction was stopped by adding 0.145 ml of 20% NaOH saturated with NaCl. The labeled *N*-methylputrescine reaction product was extracted with 0.98 ml of chloroform. The chloroform extract was evaporated using a gentle stream of nitrogen gas and its radioactivity then measured by counting in a liquid scintillation counter (Packard Tri-carb 2100TR) after the addition of liquid scintillation cocktail.

Cycloheximide/ JA/ auxin/ SA/ cellulase treatment

As a control, leaf discs were excised from a whole leaf using a 10-mm diameter cork borer and left unwounded, or injury was inflicted by puncturing them with sharp forceps at various time points. All discs were vacuum-infiltrated for 15 min with MS solution containing the chemical or additive under a vacuum pressure of 15-mm Hg and then floated in the same solution for various times. At the end of the treatments, leaf discs were vacuum infiltrated for 15 min at 15- mm Hg in X-Gluc solution (1 mg/ml X-gluc in 100 mM potassium phosphate buffer pH 7.0) followed by incubation at 37°C overnight for staining. The final concentrations of substances used were: jasmonic acid (100 µM, 400 µM), cycloheximide (50µg/ml), auxin (0.1 µg/ml) cellulase (1.5%), and SA (100 mM).

Results and Discussion

A 627-bp region upstream from the ATG start site of the *pmt* gene was isolated from an *N. tabacum* genomic library and fused to a GUS reporter gene. The isolated sequence was identical to the *NtPMT3* published sequence (GenBank accession number AF126811, Riechers and Timko, 1999). Previous studies with *N. tabacum* cv. Burley 21 showed the expression of the five *PMT* genes to be localized mainly to the roots (Riechers and Timko, 1999).

Leaf pmt expression: Localized and transient

Numerous studies have established that *PMT* gene expression in tobacco is restricted to the roots (Leete and Liu, 1973; Hibi et al., 1994; Riechers and Timko, 1999; Wang et al., 2000). Sequences upstream of the *PMT* gene were isolated by screening an *N. tabacum* genomic library with a probe derived by PCR using a published cDNA sequence of *NtPMT1* (GenBank accession number D28506; Hibi et al., 1994). To confirm that this *PMT* promoter region exhibited root-specific expression, 13 independently derived transgenic *N. tabacum* plants harboring the *PMT*-promoter GUS reporter construct were examined. GUS histochemical staining indicated that *PMT* was expressed strongly in roots as it was detectable after 1 hr of exposure to the X-gluc substrate (Fig. 2.1 A, B); intense staining was observed in roots and root hairs after 12 hr (Fig. 2.1 C, D). To verify that *PMT*-promoter activity from this construct was also consistent with established expression patterns in leaf tissue, leaves were examined by taking small leaf sections and incubating them for 12 hr in the X-gluc solution. In general, GUS staining was not evident on surfaces of most leaves (Fig. 2.1 E to H). However, histochemical staining was apparent in leaves as a narrow area around the perimeter of sections that had been cut or punctured. Thus it appeared that *pmt* promoter activity was induced in leaf tissue by wounding, and GUS staining was observed only 2 to 3 mm beyond the wound site (Fig. 2.1 E). Even though the solution was vacuum infiltrated into the leaf sections, one explanation for the localized staining pattern could be due to inadequate access of the X-gluc substrate to sites distal from the primary wound. Substrate accessibility was partly addressed by examining the staining pattern obtained in wounded leaves from transgenic plants that harbored a strong, constitutive promoter from the Figwort mosaic virus fused to a GUS reporter gene (Bhattacharyya et al., 2002). In 3-4 week-old

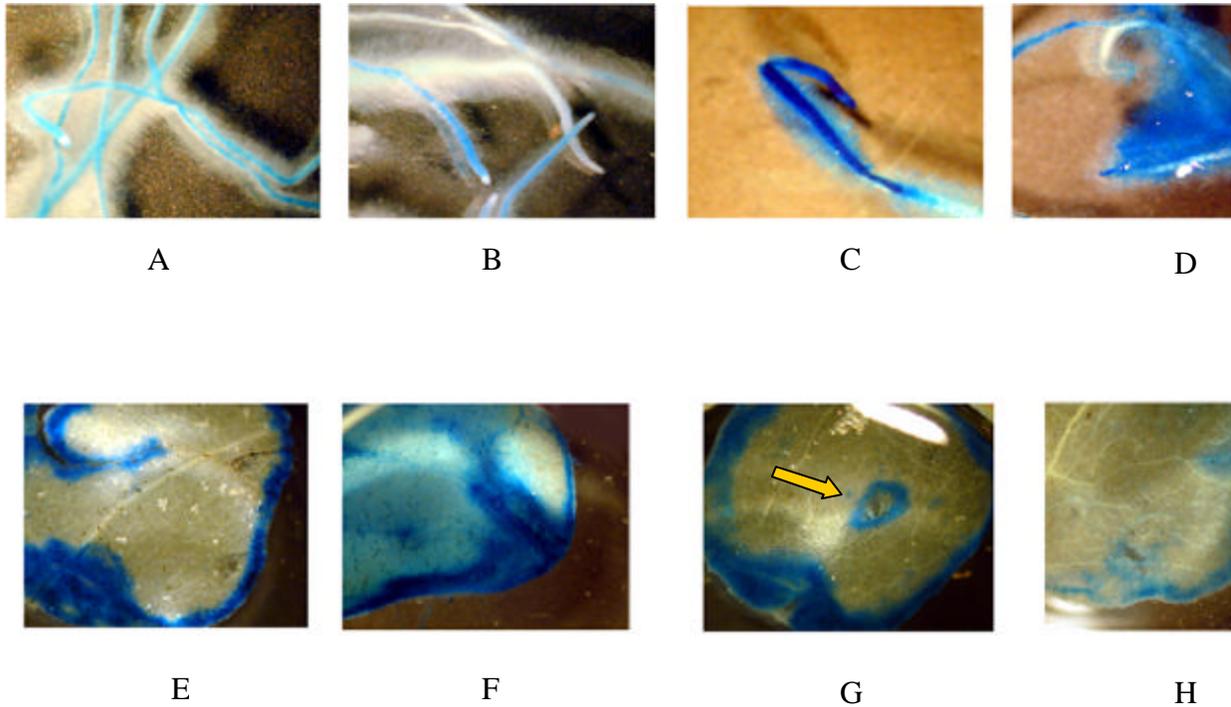


Figure 2.1 Transgenic tobacco lines expressing *PMT* promoter GUS fusions after staining with X-Gluc. (A–D) Histochemical reaction of GUS activity in roots after 1 h staining in X-Gluc solution (A, B) or after 12 h staining (C, D). (E–H) Histochemical GUS localization in leaf discs from four independently derived transgenic lines showing activity in tissues upon injury, line 1 (E), line 3 (F), line 4 (G) and line 5 (H) after 12 h staining. Control transgenics containing no *PMT* promoter sequences showed no staining (not shown). Arrow in (G) indicates the staining pattern surrounding a puncture wound within a leaf section, illustrating the presence of GUS activity only in a zone of intact cells close to the wound.

plants, GUS staining was evident at the wound site and throughout the leaves and persisted for extended periods (> 12 hr) after wounding (data not shown). Thus these findings indicate that the methods used to infiltrate the X-gluc substrate into the leaf sections is sufficient to visualize GUS activity beyond the wound site and that the pattern observed in the *PMT*-promoter GUS transgenic lines upon wounding is distinct.

To examine the temporal nature of this wound-induced expression, single leaf sections were punctured with a needle in a time-course fashion. As shown in figure 2.2 A, leaf discs were cut out from a single leaf of a two-month-old transgenic tobacco plant, punctured, and incubated in 1X MS liquid media for various times before being subjected to GUS histochemical staining. The staining pattern indicated that *PMT* expression was activated immediately after wounding, persisted for several hours and then visibly tapered off at 4 hr. Leaf discs punctured and stained after about 4 hr showed essentially no GUS staining around the puncture or cut sites (Fig. 2.2 A). It should be noted that the histochemical staining pattern shown in the time course experiments reflects an additional 12 hr incubation period in the X-gluc solution after the wound punctures were made. Therefore, it is possible that additional expression of the GUS gene driven by the *PMT* promoter could also have occurred during this staining period.

Since the histochemical staining was evident only around the wounded sites in excised leaf discs, we wished to obtain evidence for wound-induced signaling of *PMT* expression within an intact leaf still attached to the whole plant. A wounding experiment was conducted by cutting leaf discs out of a single leaf over a 24-hr period. Following the removal of the final leaf disc (0-hr wounding), the leaf was detached from the plant and stained for GUS expression (Fig. 2.2, B). GUS staining here was observed only in cells within a narrow band a few mm beyond the area of injury. Thus, in the aerial tissues examined, wound activation of the *PMT* promoter was restricted to cells adjacent to the wound site, including the last injury made by cutting the leaf from the stem. Injury-induced expression of *PMT* in isolated leaf discs followed a time course similar to that obtained for *PMT* promoter activity upon wounding in intact leaves. Maximum staining was evident in leaf discs wounded at 0-hr and the signal gradually faded away after 2-4 hr (Fig. 2.2 A, B). Staining at the 8-hr wound site was likely due to pressure applied to the leaf surface, as we have observed that additional handling of wounded tissue tended to prolong the wound response. This result also reflected the highly localized nature of the wound-induced expression since the GUS staining was not influenced by prior leaf injuries located close to the

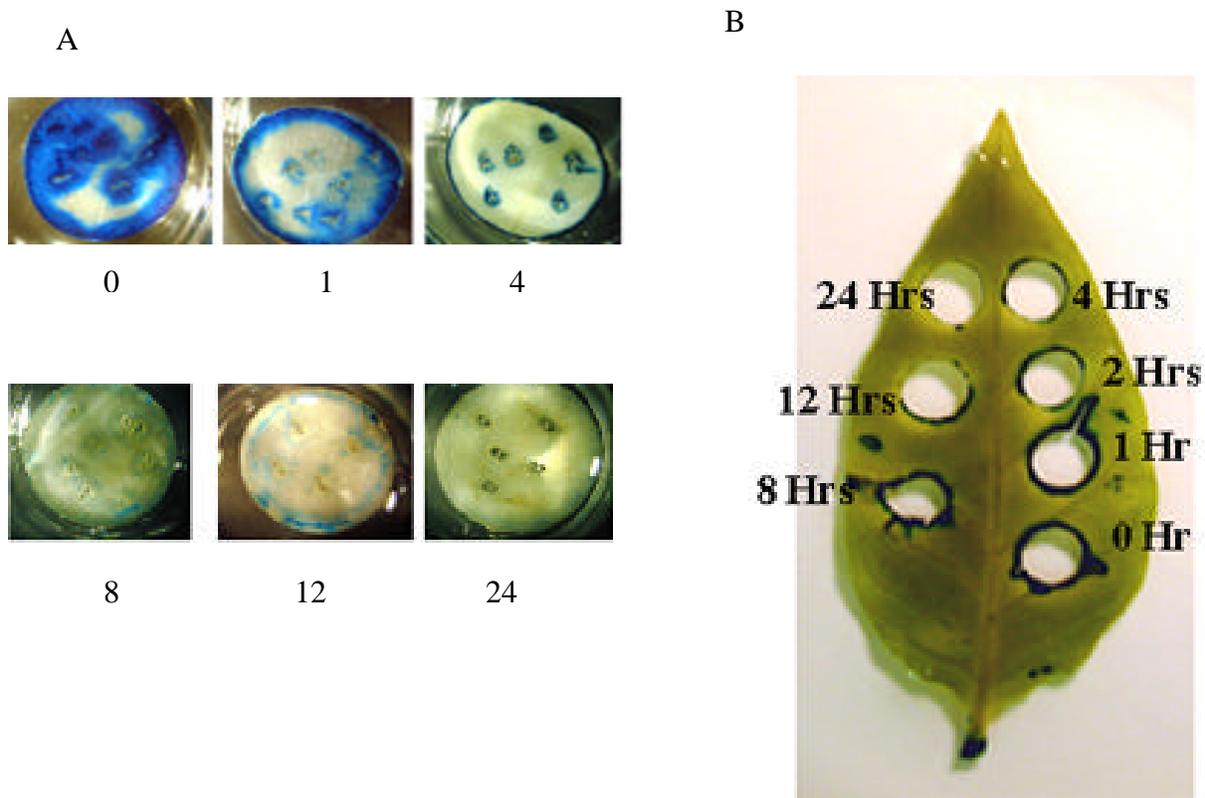


Figure 2.2 (A) Time course of wound induction of *PMT* promoter activity by GUS histochemical staining in leaf tissue. Leaf sections were made with a cork borer and punctured with sharp forceps. Numbers below the panels indicate the time in hours after puncturing each disc. Punctured discs were placed on MS media during the times indicated and then subjected to GUS histochemical staining for 12 h for each disc. Results were typical in at least four independently derived transgenic lines (4×magnification). **(B)** Localization of wound-induced *PMT* expression in an intact leaf. Injuries were made to a whole leaf still attached to the plant by cutting discs at various time points, then removing the entire leaf from the plant and staining for GUS histochemical activity.

second injury site. Fig. 2.2 C shows the relationship between the number of puncture wounds in a leaf disc and the relative fluorometric GUS activity. These data correlate well to the visible histochemical GUS staining observed.

RT-PCR expression analysis in wild-type Nicotiana

The results presented thus far are based on GUS histochemical or fluorometric determinations conducted with several independently-derived transgenic plants harboring the *PMT* -promoter GUS constructs. The highly localized and short-lived nature of the apparent wound-induced *PMT* expression in leaves suggests that the *PMT* messages might be below the limit of detection for Northern blot analysis. Therefore, quantitative RT-PCR was employed to detect expression of *PMT* in wounded and unwounded leaves (Fig. 2.3). RNA extracted from leaves of wild-type plants after wounding at various time points indicated the same basic expression pattern observed in the *PMT* -promoter GUS transgenic plants. However, a very low level of expression was consistently detected in unwounded leaves and, after a lag of approximately 15 min, an increase in *PMT* RT-PCR product was detected. Maximum expression was seen at the 30-min and 1-hr time points, which then sharply diminished 2 hr after wounding. Quantitative data for the expression levels are shown (Fig. 2.3 C) where the level of *PMT* product is expressed relative to that of the 18S rRNA product, used as an internal control. Transcripts detected by RT-PCR are likely to more accurately reflect the time course of wound-activation of *PMT* expression compared to the histochemical data because the time points for histochemical staining also include the incubation time required for the GUS-catalyzed reaction to occur. GUS enzymatic activity might also be expected to turn over more slowly than the message detected in RT-PCR. Taking this into account, the histochemical analysis and RT-PCR expression data appear to correlate relatively well. On the other hand, the level of *PMT* expression in roots was very high; the detected transcripts in this tissue were highly abundant even after a 10-fold dilution of the RT-PCR product loaded into the agarose gel (Fig. 2.3A). Therefore, the high expression level of *PMT* in root tissue is consistent with previous studies (Mizusaki et al., 1973; Hibi et al., 1994; Riechers and Timko, 1999) and these results indicate that the relative level of *PMT* expression in leaf tissue is approximately 50-fold lower than that in root tissue.

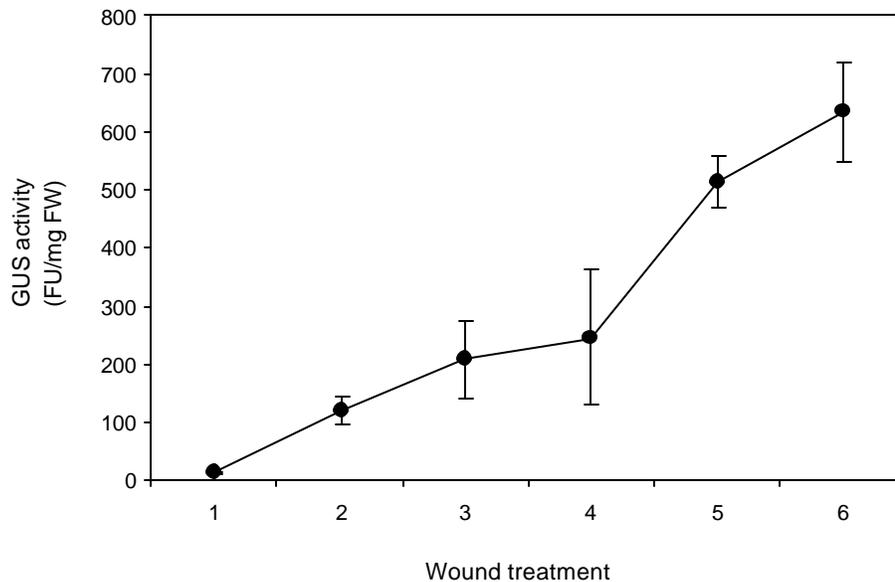


Figure 2.2 (C) Quantification of the wound induced response by GUS fluorometric assay. Leaf disc samples were taken and injured using a sharp forceps. GUS activity was measured using a luminescence spectrophotometer. Values indicated are the means and SE for each of 10 samples. X-axis: 1, wild type; 2, one puncture wound; 3, two-to-three puncture wounds; 4, four-to-five puncture wounds; 5, five-to-six puncture wounds; 6, heavily smashed. GUS activity is presented as FU/mg fresh weight.

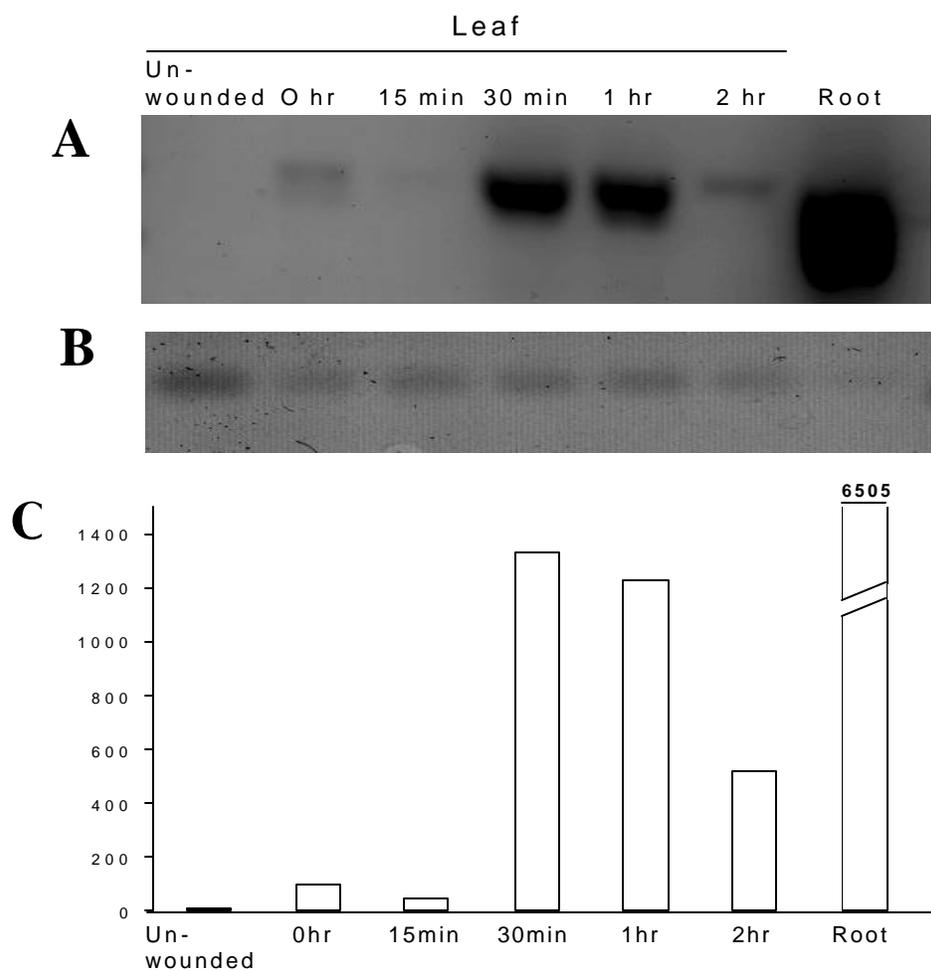


Figure 2.3. RT-PCR analysis of *PMT* expression at various time points after wounding. (A) *PMT* RT-PCR products derived from RNA prepared from: unwounded leaves, lane 1, or leaves wounded and used to prepare RNA after the times indicated. Root lane indicates RT-PCR product derived from root RNA of unwounded plants. RT-PCR products derived from the root RNA template was diluted 10-fold before loading relative to product derived from the leaf RNA, which were loaded undiluted. (B) RT-PCR products obtained using primers specific to 18S rRNA, used as an internal standard. (C) Quantification of the *PMT* RT-PCR product levels expressed as the ratio of *PMT* product to that of the 18S rRNA internal control.

In order to fully confirm that the RT-PCR product obtained was at least one of the known *PMT* genes, we cloned the wound-induced RT-PCR product from leaf tissue into a pSTSBlue-1 vector (Novagen, Inc.) and sequenced it. Alignment results indicated that the RT-PCR product was identical to *PMT* sequences, including *NtPMT1* and *NtPMT4* (A411, (Hibi et al., 1994; Riechers and Timko, 1999) from tobacco (data not shown). Since the five *PMT* genes in tobacco share extensive homology, it is possible that they could be amplified with the specific primers used in this RT-PCR experiment (Riechers and Timko, 1999). Therefore, the detection of these specific *PMT* members does not imply that they are the only members whose expression is upregulated; it does, however, substantiate that *PMT* is indeed expressed in tobacco leaves and is transiently upregulated upon wounding. In addition, the GUS analyses performed in this study employed transgenic plants that harbored *PMT* -promoter fusions using the region derived upstream of *NtPMT3*. The RT-PCR result also reveals why earlier studies (Sinclair et al., 2000) examining *PMT* expression in leaves were unable to detect any *PMT* transcripts since extremely low levels are expressed in leaves and only a transient and localized induction occurs upon wounding.

PMT enzyme activity is detectable in leaves

To determine if putrescine *N*-methyltransferase activity could be detected in wounded and unwounded leaf tissue, enzyme assays were conducted. In several separate experiments, low levels of *PMT* enzymatic activity were detected in leaf tissue (Table 2.1). However, a significant increase in *PMT* activity in leaves upon wounding was not consistently obtained. Typically, leaves wounded and assayed 1 hr later showed a slight increase in activity when compared to the unwounded tissues. *PMT* enzyme activity determined in leaves 2 hr after wounding dropped to levels below those obtained in unwounded leaves; this might be a consequence of inactivation of the enzyme after prolonged exposure within the damaged tissues. The absence of a clear correlation between increased *PMT* transcript levels and significantly elevated *PMT* activity levels may be the result of assaying at time points that do not coincide with a short-lived activity. It may also be possible that only highly localized *PMT* increases occur, and any increase in activity may be masked by the large amounts of tissue required to prepare the extracts. Detection of low levels of *PMT* enzyme activity in unwounded leaves however, does coincide

with the low transcript levels detected in leaves. Further study of this low level of *PMT* expression in leaves is required to determine its metabolic significance.

Further characterizations of leaf pmt expression

Wounding is known to cause an increase in *PMT* transcripts involved in alkaloid biosynthesis in roots of mature plants in a JA-dependent manner (Baldwin et al., 1997). These responses are thought to fulfill the need to upregulate the synthesis of alkaloids to deter leaf-eating insects. Therefore, the effects of JA, as well as other chemicals implicated as components in wound- or pathogenesis-responsive signaling pathways were investigated to determine if these influence *PMT* expression in leaves. Leaf discs were treated with JA by infiltrating 100 μ M or 400 μ M JA in MS medium, puncturing, and after various periods of time, subjecting them to GUS histochemical staining. Treatment with JA did not influence the wound induction pattern shown previously to any degree (data not shown). Therefore, it appears that the wound-induced stimulation of *PMT* promoter activity in leaves occurs by way of a jasmonate-independent pathway. Leaf discs were also treated in this manner with several other substances known to mediate wound or pathogen-induced signaling. Treatment with auxin, salicylic acid and cellulase did not show any effects on leaf *PMT* expression based on histochemical GUS staining (data not shown) and did not influence the observed wound-induction. *PMT* expression in leaves therefore does not seem to be affected by JA or other factors previously shown to affect its expression in roots or that are known to function as general elicitors (Winz and Baldwin, 2001).

To further characterize the *PMT* wound responsiveness in leaves, leaf discs were treated with cycloheximide (CHX), to assess the requirement of protein synthesis during the wound-induced response. Somewhat unexpectedly, the response to mechanical wounding was not inhibited and instead appeared to cause the wound-induced expression of *PMT* to persist (data not shown). The ability of cycloheximide to cause a normally transient transcriptional signal to persist has been observed in a number of instances (Usami et al., 1995; Hara et al., 2000). Therefore, its effect on *PMT* expression suggests a similar response. A possible explanation for how CHX could eliminate the abatement of *PMT* transcription during extended periods following wounding is that new protein synthesis is required for the rapid decrease of *PMT* - promoter activity. An additional explanation is that the decrease in *PMT* expression observed

Table 2. 1. PMT activities in root and wounded leaf tissues.

Roots	Leaves		
	Unwounded	1-hr wounded	2-hr wounded
1.87 ± 0.18	0.16 ± 0.06	0.20 ± 0.01	0.10 ± 0.02

Putrescine *N*-methyltransferase (PMT) enzyme activity (nmoles/ μ g protein) was determined in roots and wounded leaves. Leaves were wounded by puncturing the entire surface of a leaf with a wire brush and extracts were prepared from the indicated tissues as described in Experimental Protocols. Values are the average of three determinations \pm SE.

after wounding coincides with wound healing that occurs to repair tissues after injury. Inhibition of protein synthesis at this stage would prevent healing, allowing *PMT* expression to persist.

NtPMT promoter responses in Arabidopsis

Evaluation of the *PMT* promoter activity in *Arabidopsis*, a non-alkaloid producing plant, was performed to determine its expression characteristics in a heterologous species. The same *NtPMT3* promoter-GUS construct used in tobacco was introduced into *Arabidopsis* to obtain stable transgenic plants. GUS histochemical staining revealed *PMT*-promoter activity in the resulting lines in roots and trichomes of *Arabidopsis* leaves but displayed no evidence of wound induction (data not shown). The lack of a similar wound-induced expression in a different species suggests that the *PMT*-transcriptional response to wounding is a specific phenomenon in tobacco leaves.

Conclusions

The spatial expression patterns of many defensive genes are affected by wounding (Chang et al., 1995; Grantz et al., 1995; McGurl et al., 1995; Nishiuchi et al., 1997) and the assessment described by Nishiuchi (1997) pertains to the situation we have observed for *PMT* expression in *N. tabacum* leaves. Nishiuchi (1997) points out that these defensive genes are constitutively expressed at high levels in roots but at lower levels in leaves. One type of wound-regulated expression is the induction of those genes in tissues in which the genes are normally not highly expressed under non-induced conditions (Nishiuchi et al., 1997). Such is the situation described here with the highly localized *PMT* expression in leaves. The enhanced *PMT* expression seen in response to wounding in root tissues corresponds to the type of wound response pattern for those genes that are constitutively expressed at higher levels (Hibi et al., 1994; Riechers and Timko, 1999; Winz and Baldwin, 2001).

The observations made here of wounding inducing a transient, highly localized upregulation of *PMT* transcription in leaves, but with little evidence of major increases in *PMT* activity, are not fully understood. The low level of *PMT* transcript and enzyme activity in unwounded leaves suggests the possibility that it may play an additional, subtle role in alkaloid metabolism in leaf tissue. In this regard, Sato, et al. (2001) observed that transgenic *N. sylvestris* lines overexpressing *PMT* using the 35S CaMV promoter not only exhibited increased leaf nicotine contents but also increases in accumulated methylputrescine along with slight decreases in the level of spermidine and spermine in leaves. On the other hand, in a line containing cosuppressed *PMT*, an elevation of putrescine and spermidine levels was observed in leaves, which was postulated to be a result of transport of these polyamines from the root to the leaves (Sato et al., 2001). The low levels of *PMT* expression and activity in leaves described in our work suggests an additional possibility to explain the polyamine increases observed by Sato (2001). Specifically, putrescine and spermidine accumulate directly in leaves as a result of cosuppression of the low level of *PMT* activity that is present in leaves.

Another possible function for low levels of leaf-based *PMT* expression in *Nicotiana* may be related to the diversity of alkaloid metabolism in distinct species and in distinct cell types. For example in *N. repanda*, the presence of a specific *N*-acylated nornicotine derivative has been

detected, and the site of accumulation as well as synthesis is exclusive to trichomes on the leaf surface (Laue et al., 2000). This suggests that further metabolism of alkaloids can occur after their synthesis in roots and translocation to the leaf. In this regard, the low level of *PMT* expression and its transient upregulation in leaves may provide an additional means to alter the diversity of alkaloidal components in different species. Future studies on establishing *PMT* expression profiles in leaves of different alkaloid-accumulating species of *Nicotiana* may shed light on this possibility.

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Chapter 3

Identification of signaling factors that impact the regulation of alkaloid biosynthesis in *N. tabacum* callus

Introduction

Overview of alkaloid biosynthesis in intact plants

Nicotine and related alkaloids are predominant secondary metabolites synthesized by a number of *Nicotiana* species and may function as defensive toxins by acting on the nervous system of herbivores (Wink, 1998; Shoji et al., 2000). Pyrrolinium alkaloids, including nicotine, are synthesized in the tobacco root from arginine and/or ornithine by the way of putrescine (Hashimoto and Yamada, 1994). Putrescine *N*-methyltransferase (PMT; EC 2.1.1.53) is a key enzyme catalyzing the committed step for the biosynthesis of tropane alkaloids and nicotine. PMT catalyzes the *S*-adenosyl methionine-dependent *N*-methylation of putrescine directing the conversion of a primary metabolite into the biosynthetic pathways that leads to the secondary metabolic products nicotine and tropane alkaloids (as shown in fig. 1.1, chapter 1). Putrescine is therefore a precursor not only of polyamines but also to important alkaloids such as the pyrrolidine alkaloids of tobacco (nicotine and nornicotine), tropane alkaloids of *Datura*, *Hyoscyamus* (hyoscyamine, hyoscyne, and meteloidine), pyrrolizidine alkaloids (retronecine), and possibly phenanthroindolizidines (tylophorine) (Tiburcino, 1985; Leete, 1980).

Nicotine and tropane alkaloids are formed in the roots and transported to the aerial parts of the plant. *PMT* transcripts are found exclusively in roots of tobacco and are localized specifically in the cortex, endodermis and xylem of young roots of *N. sylvestris* (Shoji et al., 2000). In *A. belladonna*, a tropane alkaloid producing plant, *PMT* genes are exclusively expressed in root pericycle adjacent to xylem tissue (Suzuki et al., 1999).

N. tabacum has five *PMT*-encoding genes: *NtPMT1*, *NtPMT2*, *NtPMT3*, *NtPMT4* and *NtPMT1a* (Riechers and Timko, 1999). Many studies have also documented a rapid induction of *PMT*-encoding genes by herbivore attack, wounding, or exogenous applications of the

endogenous wound hormone, jasmonic acid (JA), or its methyl ester, MeJA, to roots of the plant (Baldwin et al., 1997; Hashimoto et al., 1998; Riechers and Timko, 1999; Hara et al., 2000).

Alkaloid regulation in undifferentiated tissues

Regulation of alkaloid biosynthesis in whole plants is different from its regulation in callus cultures. Some studies have shown that light stimulated the production of a number of secondary metabolites in plant cell cultures. In tobacco cell cultures, there have been reports that light both enhances (Tabata et al., 1971) and suppresses nicotine accumulation (Hobbs et al., 1991; Ohta and Yatazawa, 1978). Cell suspension cultures derived from *N. tabacum* cv. Wisconsin accumulated less total alkaloids than the intact plant (Hobbs, 1989). In *N. tabacum*, nicotine accounted for two-thirds of the total alkaloid content, and the ratio of nornicotine to nicotine was 0.2 (Hobbs, 1989) whereas in cell cultures, nicotine accounted for one-third of the alkaloid content and ratio of nornicotine to nicotine was found to be 1.0 (Holden et al., 1988). The amount of nicotine was reduced by 80% when tobacco callus tissues previously grown in darkness were shifted to light for three weeks (Ohta and Yatazawa, 1978). Callus cultures in general have been reported to have lower alkaloid content compared to intact tobacco plants (Ohta et al., 1978). Thus, the range of alkaloid levels found in different studies is likely to be due to various culturing methods and environmental conditions used to propagate undifferentiated callus.

Several reports have indicated that auxin levels are involved in regulation of nicotine production in callus and root cultures (Takahashi, 1973; Solt, 1957). Tiburcio et al., (1985) have shown that callus tissue obtained from petiolar explants of *N. tabacum* showed increased alkaloid levels when grown on high α -naphthalene acetic acid (α -NAA) medium, then were transferred to a medium containing lower concentrations of auxin. However, some inconsistencies have been reported in the ability to synthesize alkaloids in undifferentiated callus tissue derived from different sources. Callus cultures derived from shoots showed lower alkaloid production when compared to those derived from roots of *N. tabacum* (Hobbs and Yeoman, 1991). Nicotine, nornicotine, anabasine and traces of anatabine were detected in callus. Shoot cultures of *N. tabacum* had only small amounts of nicotine and nornicotine, indicating that they may be able to synthesize alkaloids (Dawson and Solt, 1959). More recently, gene expression studies have

shown that *PMT* expression levels are reduced when tobacco callus cultures are treated with auxin (Hibi et al., 1994; Imanishi et al., 1998).

As indicated above, the biosynthesis of secondary metabolites in plants is complex and highly variable results have been obtained using tissue culture systems. In addition, few studies have focused on determining the nature of signaling components involved in the regulation of secondary metabolite biosynthesis. Undifferentiated tissue from plants would represent a convenient and economical means to obtain useful plant metabolites, if the production of these metabolites was predictable. Therefore the studies described in this chapter were undertaken to begin to decipher the major signaling components that regulate a secondary pathway in callus tissue. Using transgenic plant material that enables an efficient means to report pathway-specific gene expression, a number of conditions were tested. The results obtained enabled the identification of a primary signaling factor that seems to be involved in governing pathway expression.

MATERIALS AND METHODS

Plant Material

N. tabacum cv. SR1 was used in all studies (Maliga et al., 1973). All plants were grown in sterile Magenta boxes in a growth room at 22°C with continuous light (80 $\mu\text{mol m}^{-2} \text{s}^{-1}$) on Murashige and Skoog (Sigma) medium containing 1% sucrose.

Promoter isolation and plant transformation

A *N. tabacum* genomic library (Clontech, Palo Alto, CA) was screened using a probe prepared by PCR from SR1 genomic DNA using published cDNA sequences of the *N. tabacum NtPMT1* cDNA (Hibi et al., 1994). A 4.5-kb *NtPMT3* clone was isolated as described previously (chapter 2, Sachan and Falcone, 2002). A sequence containing 627 bp of the *NtPMT3* promoter region was fused to the GUS reporter gene. The resulting construct was introduced into *Agrobacterium tumefaciens* GV3101 strain and used for transformation of *N. tabacum* as described previously (chapter 2, Sachan and Falcone, 2002).

Protoplast isolation and callus preparation

N. tabacum plants were grown in sterile Magenta boxes on solid 1X MS medium for 5-6 weeks. Leaves were excised from the plants and cut into sections of 1 cm^2 using a sharp razor blade. Approximately 3-5 g of leaf tissue was added to 20-ml filter-sterilized enzyme solution (1.5% cellulase and 0.5% pectinase (Sigma) in 0.4 M K-3 medium and incubated in dark at 26°C overnight. The digested leaf enzyme solution was passed through a 100 μM sieve and 12 ml was transferred to 17×100 mm polystyrene snap cap culture tubes. The tubes were centrifuged at approximately 2000 rpm in a Jouan-MR22 swing-out bucket centrifuge for 6 minutes (0 brake; 0 acceleration). After centrifugation, tubes were carefully removed from the centrifuge so as not to disturb the intact protoplasts that floated on top of the medium, cell debris was pelleted to the bottom of

the tube. The cell debris pellet and the solution were removed with a 1.5-mm diameter capillary tube connected to a peristaltic pump. Medium was carefully withdrawn until approximately two ml of medium containing intact protoplasts remained. The tube was refilled with fresh 0.4 M K-3 medium (Fritze and Walden, 1995), mixed gently by inversion and centrifuged again. This was repeated at least 2-3 times to completely remove the enzyme solution. In the last wash, the medium was removed until three ml remained and the protoplasts from all tubes were mixed together. The amount of protoplasts in the final pooled medium was estimated by counting under a microscope. The final concentration of protoplasts transferred to a 10×60-mm petri dish was adjusted to 1 million cells per 10 ml of 0.4 M K-3 medium (1 mg/ml or 5.3 μM final concentration of α-naphthalene acetic acid (α-NAA) and 0.2 mg/ml or final concentration of 0.93 μM kinetin). Sealed dishes containing the fresh protoplasts were incubated in dark for 4 days at 26°C for recovery after isolation.

Protoplast washing

Nine days after cutting leaves, protoplasts were washed in W-5 medium. Five milliliters of W-5 medium (a medium containing, NaCl, CaCl₂, KCl and 100 mM glucose) was added to polystyrene snap cap culture tubes and to this 5 ml of protoplasts in K-3 medium was slowly added. Tubes were centrifuged in a Jouan swing-out bucket rotor at 1,000 rpm for 3 min at 24°C (acceleration-9, brake-3, ΔG-157). The protoplast pellet at the bottom of the tube was washed with W-5 medium, and the wash was repeated twice. In the last wash, a 5-ml pipette was used to remove the majority of the media and leaving washed cells in a small volume of media. Protoplasts are then resuspended in 0.4 M K-4 medium, (a medium containing asparagine, arginine and glutamine as nitrogenous sources instead of NH₄NO₃ and NaNO₃ as the nitrogen source in K-3 medium). Two tubes of protoplasts were combined to give protoplasts in 10 ml of 0.4 M K-4 medium, which was transferred to 60×15-mm plates. When viewed under the microscope at this stage, protoplasts float on top, have elongated shapes, and are ready to divide.

Embedding of protoplasts to generate callus

On day 14 after the isolation, protoplasts were embedded in low-melting agarose (Seaplaque agarose, FMC bioproducts; melting temperature $\leq 65^{\circ}\text{C}$, gelling temperature $26^{\circ}\text{-}30^{\circ}\text{C}$). Each $60\times 15\text{-mm}$ petri dish of protoplasts was embedded to give one $100\times 15\text{-mm}$ petri dish of protoplasts. To embed protoplasts in 0.3 M K-4 medium, a final concentration of 0.3% low melting agarose was added to protoplasts and the suspension was quickly poured into a $100\times 15\text{-mm}$ petri dish and allowed to solidify for one hour. Subsequently 10 ml of 0.3 M K-4 medium was added and dishes were placed at 26°C in light.

After one week, the medium containing the embedded protoplasts was changed to 0.2 M K-4 medium. The following week, the medium was changed to 0.1 M K-4 medium. After incubation in 0.1 M K-4 medium for another 2 weeks, the microcalli appear and are ready to be surface embedded. These media changes were undertaken to decrease the osmolytes to induce callus formation. All media contained $\alpha\text{-NAA}$ and kinetin at the same concentrations mentioned above.

Surface embedding of microcalli

Microcalli embedded within agarose from a single plate were pooled together and divided into approximately 16-18 parts for surface embedding on $100\times 15\text{-mm}$ -sized petri plates. To each part, 0.6% of low-melting agarose and 0.1 M K-4 medium was added and quickly stirred. This was poured over 0.1 M K-4 medium solidified with 0.2% phytigel (Sigma) in 150-mm petri dishes. The microcalli were allowed to embed on the surface of the media in these plates for 3 hrs. Ten millilitres of 0.1 M K-4 liquid medium was added on top of the embedded material on each plate. Plates were sealed and incubated at 26°C .

Callus treatments

Two weeks after surface embedding microcalli, now grown larger callus material was used for various treatments. All callus tissue was vacuum-infiltrated with 0.1 M K-4 medium containing the chemical under a vacuum pressure of 10-mm hg for 15 min. The final concentrations of the substances used were 5 mM DMTU (1, 3-dimethyl 2-thiourea, Aldrich), 200 units/ml catalase (Sigma), 40 μM jasmonic acid, 10 mM tiron (4,5-

dihydroxy 1,3-benzene disulfonic acid, Sigma), 30 mM ascorbate and 10 mM N-acetyl cysteine. For normal growth of callus, light was provided continuously at $15\text{-}20\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$, while “high light” was $100\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$. For dark treatments, callus plates were wrapped in aluminum foil.

GUS fluorometric assay

GUS enzyme activity assays were performed in 96-well plates as described by (He and Gan, 2001) with modifications. Callus material was taken and removed of all media and the tissue was ground in 200 μl GUS extraction buffer (50 mM NaHPO_4 pH 7.0, 10 mM Na_2EDTA , 0.1% sarcosyl, 0.1% Triton X-100). One-hundred microlitres of the extract was taken for the assay to which 30 μl of 5 mM 4-MUB- β -D-glucuronide (MUG) in extraction buffer was added and further incubated for 20 minutes at 37°C . The reaction was stopped by adding Na_2CO_3 to a concentration of 180 mM to the wells. Fluorescence was measured at 365 nm excitation and 455 nm emission using a Perkin Elmer luminescence spectrophotometer model LS50B (Perkin-Elmer, Beaconsfield, UK). Protein estimation was done colorimetrically by dye binding according to a kit provided by BioRad.

Measurement of H_2O_2 activity

Measurement of H_2O_2 activity made using callus treated with varying concentrations of auxin (as indicated in table 1) for 2 weeks. A kit provided by Molecular Probes- Amplex[®] red hydrogen peroxide/ peroxidase assay kit (A-22188) was used. In a 96-well plates 50 μl of the medium, in which the callus was maintained, was diluted with 50 μl horseradish peroxidase (HRP) solution. The reaction was incubated at room temperature for 30 minutes. Fluorescence was measured at 530 nm excitation and 590 nm emission using a Perkin Elmer luminescence spectrophotometer LS50B (Perkin-Elmer, Beaconsfield, UK).

Extract preparation for alkaloid displacement assay

Approximately 50 mg of callus material was removed with a pair of scoop ring forceps for preparing the callus extracts. Callus was ground in eppendorf tubes containing 500 μl water using a small pestle connected to a drill press for one minute. An additional

500 μl of water was added afterwards. Extracts were centrifuged at 14,000 rpm for 10 minutes. Supernatants were used for conducting the alkaloid displacement assay.

Alkaloid displacement assay

Radioligand binding displacement were conducted in 96-well filter plates (350 μL well volume) at room temperature (unless otherwise noted), using methods modified after those described elsewhere (Houghtling et al., 1995; Mahanandeeshwar et al., 1995). Briefly, competitor plant tissue sample extract, or unlabelled ligand for non-specific binding measurement was pre-incubated with a homogenized brain tissue preparation prepared by Dr. Trent Rogers in Prof. John Littleton's laboratory. Following appropriate pre-incubation of competitor, radiolabelled ligand (168 pM [^3H]-epibatidine, S.A. 66.6 Ci/mmmole, New England Nuclear) was added to each well and incubated further. Following incubation, samples were harvested onto 96-well GF/B filter plates and rapidly washed three times with 350 μL 50 mM Tris-HCl buffer (pH 7.4). After overnight drying, scintillation fluid was added to each filter and the filterplate was counted using a 96-well plate scintillation counter. Specific binding in the presence of competitor was converted to percentage of total specific binding of radioligand alone. Non-specific binding was calculated based upon the amount of radioligand binding in the presence of excess competitor ligand (300 μM nicotine). Significant differences between individual samples were calculated from radioactivity remaining in counts per minute (CPM) average and from triplicate measurements per sample using Students 2-tailed t-tests.

Extract preparation and GC-MS analysis of callus alkaloids

Callus was maintained in light conditions (15-20 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and in the presence of auxin. The media in which the callus was maintained was removed and the callus was macerated with a drill machine and then centrifuged. The supernatant was subsequently lyophilized in a vacuum drier. 1.5 g of the lyophilized tissue was added to 10 ml of acidified methanol (pH 1.5 with HCl). Quinaldine (2-methyl quinoline) 100 ng/ml was added as an internal standard and the suspension was shaken on an orbital shaker overnight. Two ml of the above suspension was mixed with an equal volume of a 50:50 mixture of hexane:diethyl ether. This was centrifuged at 5000 g for 20 min in a sorvall centrifuge. The aqueous layer was collected and the pH adjusted to 11 with NH_4OH . An

equal amount of CH_2Cl_2 solution was vortexed and then centrifuged. The CH_2Cl_2 layer is collected in a separate tube. While the upper aqueous layer is again treated with equal volume of CH_2Cl_2 and centrifuged. The above process was repeated twice; total of three times. All the CH_2Cl_2 layers were combined and dried under argon gas. The dried residue was dissolved in 100 μl methanol. An Agilent 6890 GC with 5973 mass spectrometer fitted with a HP5- MS 30 x 25 x 25 column was used for the analysis. Injection volumes were 1 μl .

RESULTS

NtPMT3 gene promoter-GUS fusion constructs and preparation of transgenic *N. tabacum* plants were described in chapter 2. Stable transgenic line number nine was chosen based on its reproducible GUS expression characteristics. Seeds from this line were germinated on MS plates with kanamycin selection. Seedlings were then transferred to Magenta boxes to obtain larger plants, and these were used to isolate protoplasts and subsequently to prepare undifferentiated callus.

Fresh protoplasts were isolated from leaf tissue by Irina Artiushin. Callus cultures derived from freshly isolated leaf protoplasts were then grown by surface plating. Callus tissue prepared in this way was actively growing and healthy as indicated by its vigorous growth on agar surfaces and its green appearance. Surface plating was done to maintain regenerable tissue and to provide a supply of equivalently grown tissue to ensure consistent responses. Alkaloid production has been shown to be tissue specific. In this study callus tissue was used to evaluate the expression characteristics of the alkaloid pathway at the transcriptional level and at the level of alkaloid accumulation.

Effect of light and auxin on alkaloid pathway expression

Because the earlier literature has described variable results of alkaloid accumulation in *N. tabacum* callus tissue we conducted experiments to evaluate the effect of light and auxin on *PMT*-promoter expression to provide initial information regarding regulation of the pathway at the transcriptional level. All callus materials were grown in the presence of normal concentrations of auxin and kinetin (1 mg/ml or 5.3 μmol final concentration of α -NAA and 0.2 mg/ml or final concentration of 0.93 μM of kinetin) unless otherwise indicated. Callus materials were maintained in continuous light conditions (15-20 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Five-week-old agarose embedded callus pieces were then transferred to fresh medium containing different auxin concentrations (indicated in table 3.1) for 2 weeks in light or dark conditions. Callus material that was placed in the light for 2 weeks with normal auxin concentrations showed little or no *PMT* expression based on GUS

histochemical and fluorometric data (figure 3.1, 3.2 and 3.3 respectively). *PMT* expression levels are indicative of nicotine biosynthesis and total alkaloid levels as has been shown previously (Yun, Littleton and Falcone, unpublished results). This is consistent with the conclusion that *PMT* enzyme catalyzes the first committed step of nicotine production. This low background of *PMT* expression indicates that there is no or low alkaloid pathway expression as well as alkaloid synthesis in tissue-cultured callus material derived from isolated leaf protoplasts. Earlier studies involving shoot cultures of *N. tabacum*, *N. glauca* and *N. rustica* found lower alkaloid in light conditions when compared to dark (Hobbs and Yeoman, 1991). We used different auxin concentrations in both light and dark to directly examine their effects on alkaloid pathway expression. When the callus was maintained in dark conditions with reduced concentrations of auxin (0.79 μ M - 0.15-fold auxin), a marked increase in *PMT* expression was observed (figure 3.2). However, when the microcalli were plated a little more densely and at a later stage of development, the callus material with no auxin in dark conditions showed greater *PMT* expression based on histochemical analysis (figure 3.1). In the experiment of figure 3.1 callus tissue was transferred as agarose pieces and surface-embedded while in figure 3.2 the tissue was much younger and were not surface embedded. Even with differing concentrations of auxin little differences in *PMT* expression as indicated by *PMT* promoter activity was observed in presence of light based on GUS histochemical data and fluorometric analysis (figure 3.1 and 3.3). However, when placed in the dark conditions with reduced auxin concentrations, dramatic upregulation of *PMT* expression based on GUS histochemical data was apparent in the callus tissue (figure 3.2). Under the light levels used for normal propagation of the tissue, *PMT* expression was very low, as shown by low levels of GUS activity (figure 3.5). Higher light causes expression to be reduced further (figure 3.5). In dark conditions, even in the presence of typical levels of auxin, *PMT* expression was higher compared to the normal growth condition of callus. This was not that evident in the histochemical analysis, presumably because the fluorometric method detection is more sensitive to changes in experimental conditions.

Table 3.1 Different concentrations of α -naphthalene acetic acid (α -NAA) used for maintenance of callus cultures

Auxin conc.	α -Naphthalene acetic acid (α -NAA) (stock conc. 5.3 mM)	Kinetin (stock conc. 0.93 mM)
1X	5.3 μ M	0.93 μ M
0.15 X	0.79 μ M	0.93 μ M
5 X	26.5 μ M	0.93 μ M
No auxin	-	0.93 μ M

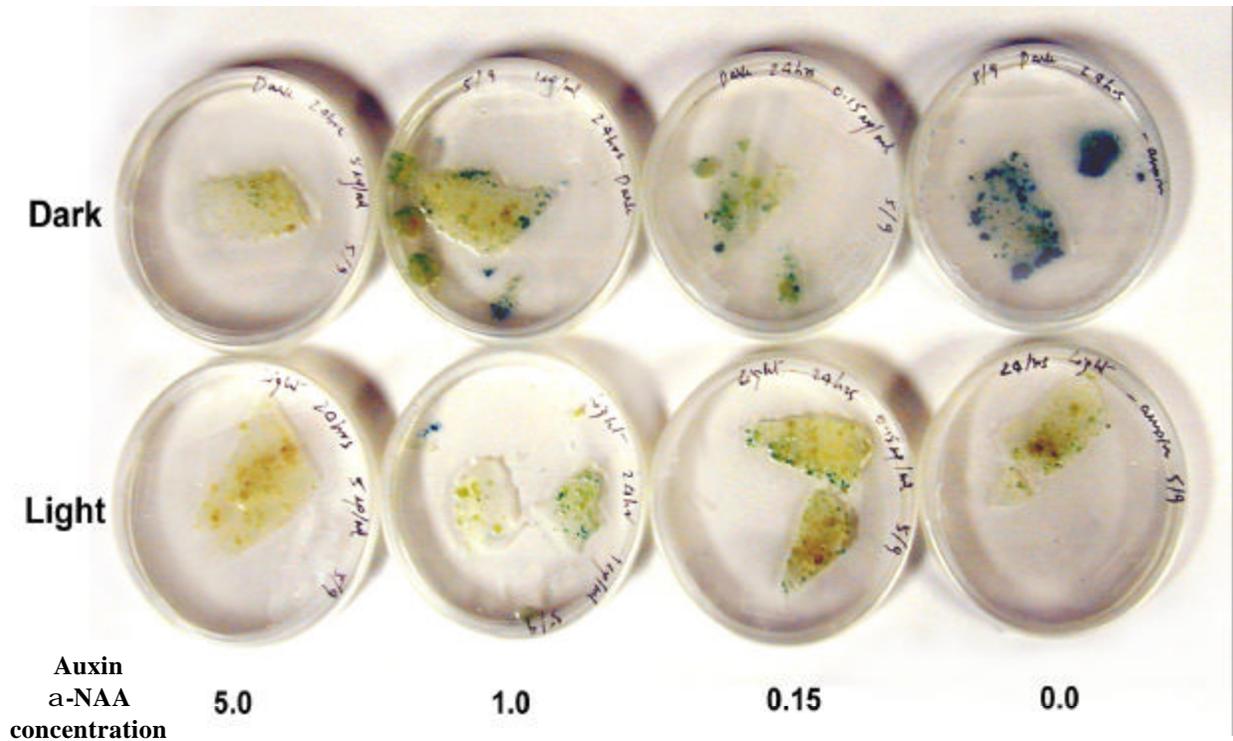


Figure 3.1 GUS histochemical analysis of surface-embedded transgenic callus. The callus material generated from protoplasts isolated from transgenic tobacco leaves containing the *NtPMT 3* promoter-GUS fusion. Five-week-old callus embedded in agarose was treated for one week and stained with X-gluc to assess *PMT* promoter activity. The surface-embedded callus was cut out as sections to ensure equivalent tissue for all treatments. Callus were treated to varying concentrations of α -NAA (for details see table 3.1).

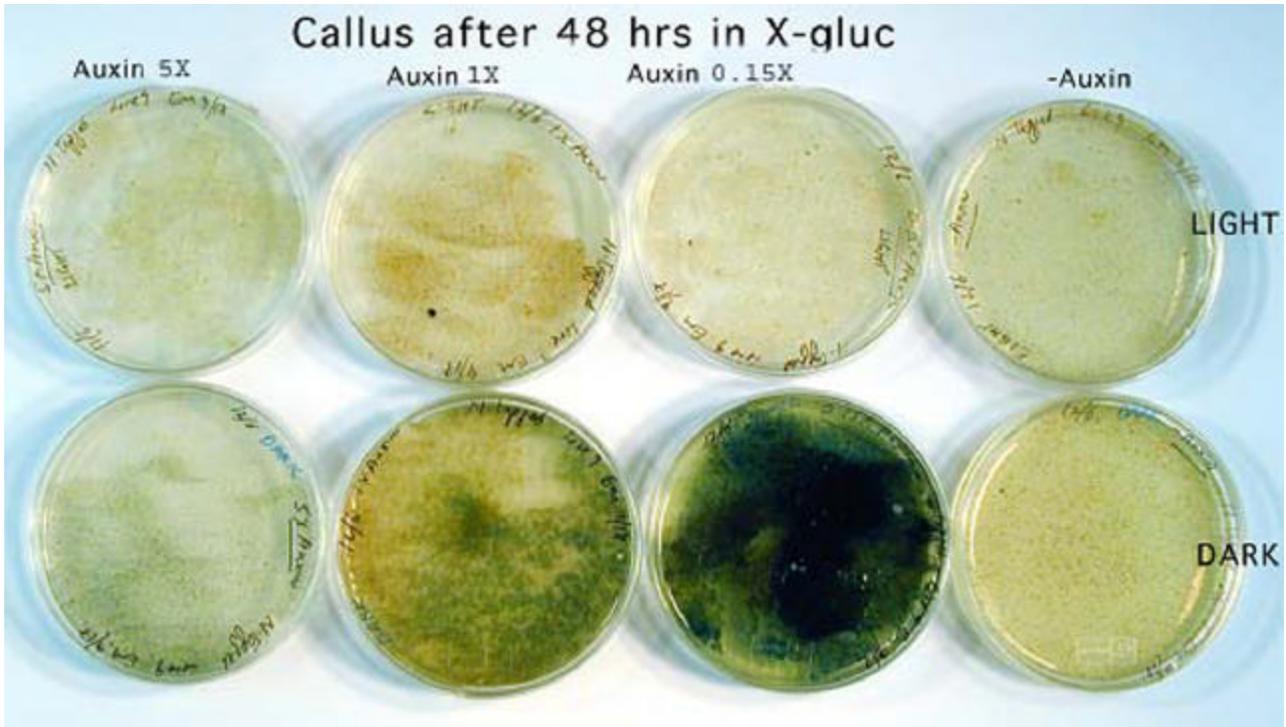


Figure 3.2 GUS histochemical analysis microcalli derived from protoplasts. Callus material was generated from protoplasts isolated from transgenic tobacco leaves containing the *NtPMT 3* promoter-GUS fusion. Three-week-old callus embedded in agarose was treated for one week and stained with X-gluc to assess *PMT* promoter activity. This callus tissue was not surface-embedded to avoid handling of the tissue and thereby affecting *PMT* expression. Callus were treated to varying concentrations of α -NAA (for details see table 3.1)

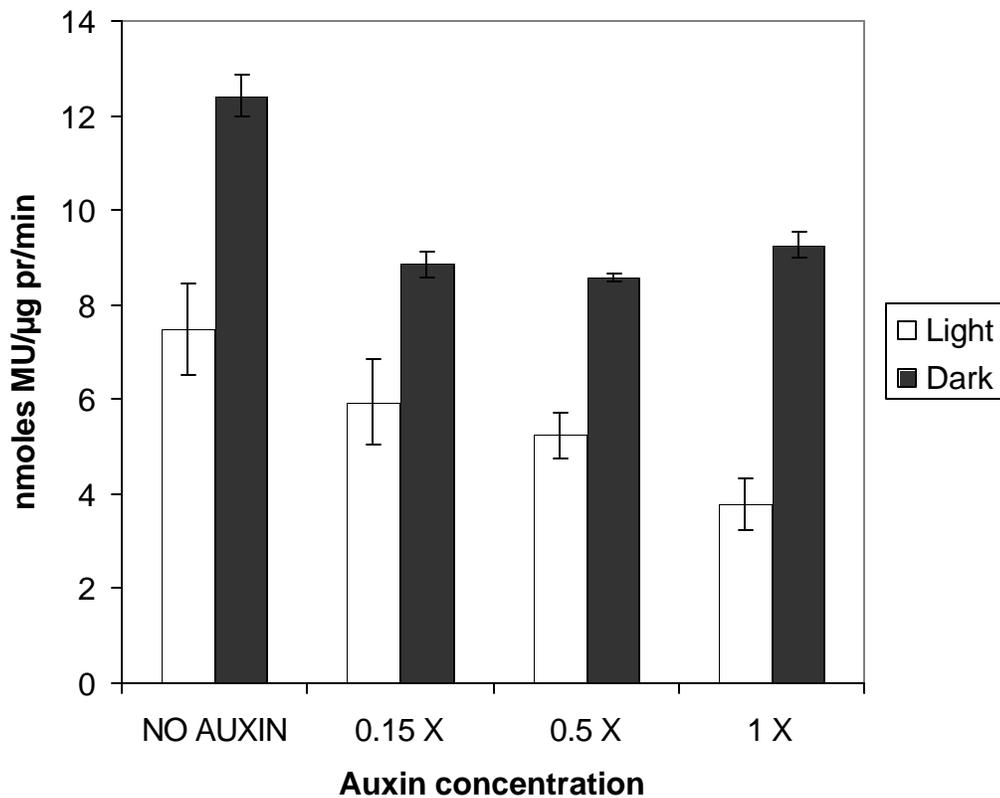


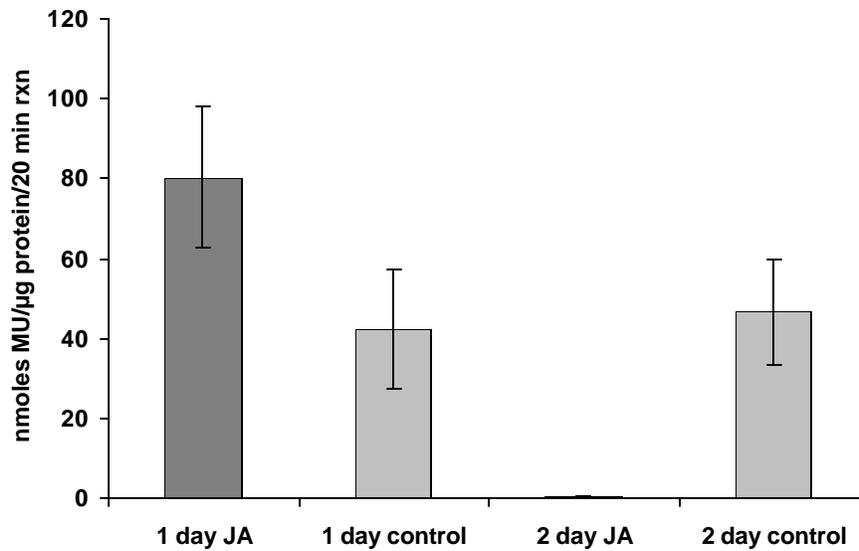
Figure 3.3 Treatment of callus material to different auxin concentrations in light and dark conditions. Callus material was generated from protoplasts isolated from transgenic tobacco leaves containing the *NtPMT 3* promoter GUS fusion were examined for β -glucuronidase activity after treatment with varying concentrations of auxin (as indicated in table 3.1) in light and dark conditions after one week of treatment. Dark gray bars, dark treated callus; white bars callus kept in light conditions. All treatments were conducted by vacuum infiltrating the callus material at 10 mm Hg for 15 minutes.

Response of alkaloid expression in callus to jasmonic acid

The responses of alkaloid pathway expression in callus tissue to jasmonic acid (JA), which is known to cause upregulation of alkaloid synthesis in intact plants, was examined. When callus tissue was treated with jasmonic acid in the presence of light and auxin, upregulation of the *NtPMT 3* promoter occurred, based on GUS fluorometric expression. However, this expression was transient. After one day of treatment, an increase in *PMT* promoter activity was seen in callus treated with JA (figure 3.4). This activity dropped to below that of non-treated levels after 2 days. This transient effect is similar to the *NtPMT 3* transient expression in wounded leaves (chapter 2).

Involvement of light in expression of *NtPMT* in callus tissue

Surface-embedded callus material was treated for two weeks in the presence of auxin and low light conditions ($15\text{-}20 \mu\text{mol m}^{-2} \text{s}^{-1}$) normally used for growth, high light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$), or darkness. Earlier studies using light to repress alkaloid biosynthesis did not use varying light intensity and dark for the experiments. Here we utilized differing light conditions to examine its effect on callus cultures derived from leaf protoplasts, and used GUS fluorometric analysis to measure *NtPMT 3* promoter activity. Hobbs et al., (Hobbs and Yeoman, 1991) determined the effect of light and dark on *N. tabacum* callus cultures. The alkaloid content in that study of green illuminated callus cultures was greatly reduced compared to non-green cultures grown in the dark (Hobbs and Yeoman, 1991). In our work callus grown in low (“normal”) illumination showed extremely low expression based on the GUS fluorometric analysis (figure 3.5). This low *PMT* expression was further repressed (or inhibited) when cultures were exposed to high light conditions ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$) (figure 3.5). All the cultures were grown in presence of auxin ($5.3 \mu\text{M}$), which is known to have some repressive effect on *PMT* expression and also alkaloid accumulation (Hibi et al., 1994). When these cultures were placed in the dark for a week, both GUS histochemical and fluorometric analysis indicated that *PMT* promoter expression increased (figure 3.1 and 3.5 respectively).



Figures 3.4 Treatment of transgenic callus to jasmonic acid causes transient upregulation. Callus material was generated from protoplasts isolated from transgenic tobacco leaves containing the *NtPMT 3* promoter GUS fusion were examined for β -glucuronidase activity after treatment with 40 μ M JA on day 1 and day 2. Dark gray bars, JA treated; light gray bars non-treated control. All treatments were conducted by vacuum infiltrating the callus material at 10 mm Hg for 15 minutes.

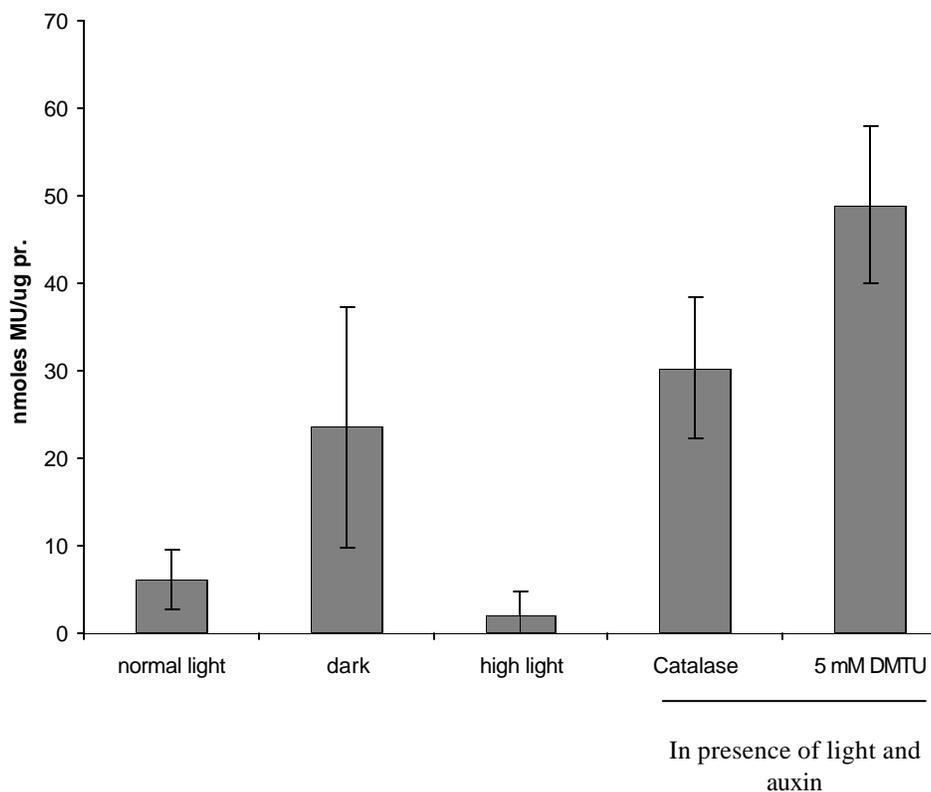


Figure 3.5 PMT promoter activity in different light conditions and ROS scavengers. Tobacco callus tissue, which displays highly repressed alkaloid synthesis under normal culture conditions in light, exhibits significant induction of *PMT* promoter activity and alkaloid accumulation when treated with an ROS scavenger, DMTU and catalase. Transgenic tobacco containing the *PMT 3* promoter-GUS fusion was used to obtain callus material and were treated in the following ways 1) low light (normal) conditions ($15\text{-}20 \mu\text{mol m}^{-2} \text{s}^{-1}$); 2) in darkness; 3) high-light conditions ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$); 4) 100 units/ml catalase.; 5) in the presence of 5 mM dimethylthiourea (DMTU). Callus treated with catalase and DMTU were maintained in low-light conditions in presence of auxin. All treatments were conducted by vacuum infiltrating the callus material at 10 mm Hg for 15 minutes.

Involvement of reactive oxygen species in alkaloid pathway regulation

We hypothesized that light may elicit its effect on alkaloid pathway expression through reactive oxygen species (ROS), such as H_2O_2 , superoxide radicals, etc. and these might function as signals, ROS are known to be produced during normal metabolism such as photorespiration and photosynthesis. Thus we tested the involvement of ROS on pathway expression by utilizing ROS scavenging chemicals such as DMTU (1,3 dimethyl 2-thiourea, an ROS scavenger for OH^\cdot and $\text{O}_2^\cdot^-$ radicals), and a H_2O_2 degrading enzyme, catalase. DMTU is a well-known scavenger of ROS. DMTU also traps H_2O_2 (Jiang and Zhang, 2002). Tobacco callus cultures, which display highly repressed alkaloid synthesis under normal conditions in the light, exhibited significant (8-fold) induction of *PMT* promoter activity when treated with 5 mM DMTU (figure 3.5). These cultures were maintained in the presence of light and auxin, which are known repressors of *PMT* expression. However, the presence of DMTU apparently effectively overcame this light and auxin-mediated repression. Quantification of β -glucuronidase activity using GUS fluorometric analysis on the DMTU-treated callus showed activity higher than that observed when callus was maintained dark (figure 3.5).

An additional means of lowering ROS levels, specifically H_2O_2 , was applied by treating with 200 units/ml of catalase, an enzyme which catalyzes the breakdown of H_2O_2 . This treatment might provide evidence as to the effectiveness of H_2O_2 as potential ROS species involved in signaling. Upon infiltrating the soft callus tissue with medium containing catalase, the repression due to the presence of light and auxin was, like the DMTU effect, overcome (approximately 6-fold) as indicated by GUS fluorometric analysis (figure 3.5).

To test the hypothesis that the external media in which the callus material was maintained had caused leaching of H_2O_2 from tissue, perhaps via by porin channels, we tested for the H_2O_2 in the medium.

Other chemicals such as 10 mM Tiron (4,5 dihydroxy 1,3 benzene disulfonic acid, an ROS scavenger) and 100 μM DPI (diphenylene iodonium chloride, a well known inhibitor of NAD(P)H oxidase) and 10 μM Rose Bengal diacetate (a superoxide anion generator), were used to attempt to differentiate among distinct reactive oxygen species as potential signals. However, treatment with these agents for 1 week killed the callus

tissue. Also 10 mM N-acetyl cysteine along with 30 mM ascorbic acid was used as a reactive oxygen species scavenger, but these agents also did not effect *PMT* promoter activity in callus tissue (data not shown).

Effect of ROS on alkaloid biosynthesis

To determine if a correlation existed between *PMT* expression after ROS scavenging and alkaloid accumulation, the sensitive epibatidine binding displacement assay was used to detect the presence of alkaloids.

In low-light conditions, alkaloid accumulation was very low, averaging approximately 3 nM in a five-week-old callus tissue that was surface embedded (figure 3.6). In high light the amount of alkaloids detected in the callus was lower. However in dark conditions, the alkaloid content increased even in presence of auxin (figure 3.6). When the callus tissue was infiltrated with catalase, the alkaloid content was also elevated (figure 3.6). However, when DMTU was added the increase in alkaloid levels was the same as in untreated callus (data not shown). This might be because of the fact that *NtPMT3* might not be able to contribute a sufficient level of activity to increase alkaloid synthesis. GC-MS analysis of callus tissue treated with DMTU and catalase was done to confirm the epibatidine displacement data. An initial gas chromatographic (GC) examination of the alkaloids produced in callus tissue was undertaken and are shown for untreated callus samples in Table 3.2. The identification of these alkaloids supports their detection by epibatidine-displacement assays. GC-MS analysis of DMTU and catalase-treated callus tissues are in progress.

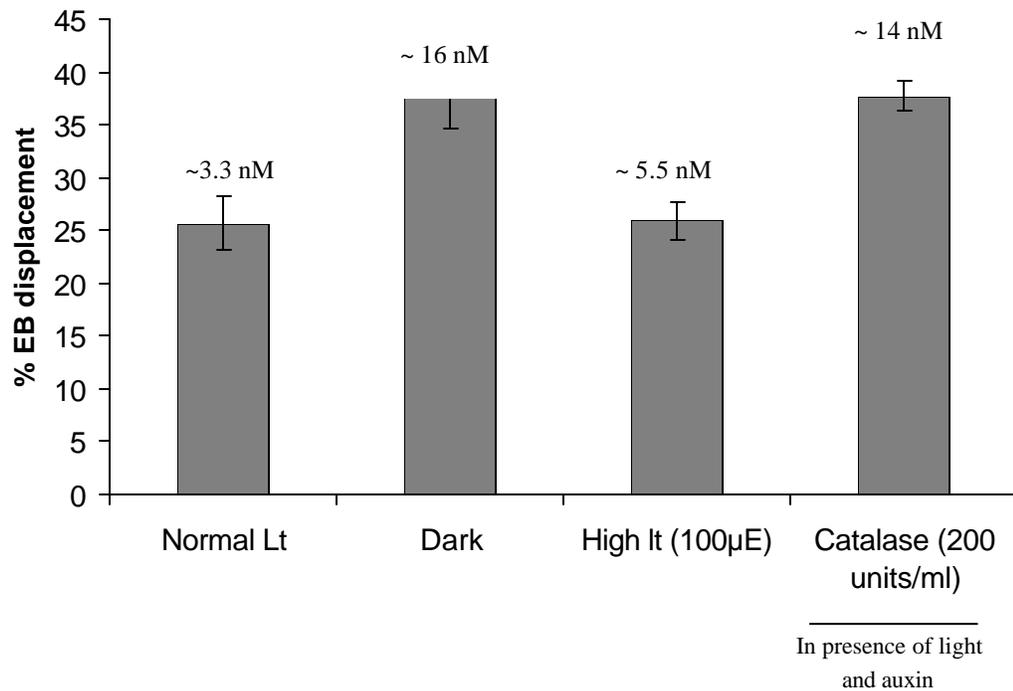


Figure 3.6 Alkaloid activity levels in callus tissue is affected by treatments that impact reactive oxygen species levels. Callus material was generated from protoplasts isolated from transgenic tobacco leaves containing *NtPMT 3* promoter-GUS fusion construct. Five-week-old callus grown on agar plates was treated by infiltrating tissue with catalase and analyzed for alkaloid activity two weeks later. Callus extracts were prepared by grinding the tissue in water and using the supernatant for the displacement assay. Number above the bar indicates the estimated concentration of “nicotine equivalents” derived from curves generated using pure nicotine in the assay. Concentration values therefore indicate the amount of displacement activity that is equivalent to the given concentration of nicotine.

Table 3.2 Top six alkaloids detected in GC-MS profile conducted on untreated callus material. Callus was maintained in light conditions (15-20 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and in presence of auxin. Callus was macerated with a drill machine and the centrifuged. The supernatant was subsequently lyophilized and alkaloids extracted for conducting GC-MS analysis. The values indicated are based on 1 gram of lyophilized tissue.

Alkaloids	ng/g dry wt
Nicotine	51.55
Nornicotine	1.1
Myosmine	0.26
Anabasine	0.4
Anatabine	6.34
Bipyridine	0.26

DISCUSSION

Treatment with factors known to affect the regulation of alkaloid biosynthesis were first tested with our undifferentiated callus system. Auxin and jasmonic acid are known to affect *PMT* expression in *N. tabacum* (Hibi et al., 1994; Imanishi et al., 1998). Treatments with jasmonic acid resulted in an increase in alkaloid levels as studied earlier in callus cultures (Imanishi et al., 1998) and in intact plants (Baldwin et al., 1994). Light and auxin have been also shown to have a negative affect on alkaloid accumulation in callus (Hobbs and Yeoman, 1991; Hibi et al., 1994) and intact plants (Mizusaki et al., 1973; Hobbs and Yeoman, 1991; Hibi et al., 1994).

In our studies here with undifferentiated tobacco callus, we established that the repressive effect of light and auxin on alkaloid expression occurred at the level of *PMT* promoter activity. GUS histochemical and quantitative fluorometric data revealed a strong repression of *PMT* promoter activity in the presence of both light and auxin (figure 3.1 and 3.3). Growing the callus material in the dark and in the absence of auxin gave higher levels of *PMT* promoter expression. Collectively, these results show that light is the primary factor in alkaloid pathway expression because a lower degree of change was seen by reducing auxin levels, whereas the absence of light consistently gave the strongest inductive effect, even in the presence of auxin. In some experiments, such as with younger, plated callus tissue, it appeared that darkness plus a low level of auxin provides the maximum *PMT* promoter expression, probably because a low level of auxin was required to maintain growth.

Also there was an upregulation of *PMT* promoter activity in presence of JA, but this was shortlived in that elevated *PMT* expression lasted only 24 hours (figure 3.4). This transient effect has not been noted in other studies, which focused particularly on studies with *NtPMT1* (Imanishi et al., 1998) using tobacco BY-2 cells. The cells showed an increase in *PMT* transcripts 6 h after treatment with MeJA (methyl jasmonate), which lasted up to 24 h. However, the cells were not examined further. In the same report a transient induction of *ODC* was seen upon treatment with MeJA (Imanishi et al., 1998).

One major difference between these studies and the results described here is that Iminashi et al., used transformed BY-2 cells which are dissimilar to the freshly prepared callus cultures used here. The protoplast-derived callus cultures are fully regenerable whereas BY-2 cells are not. Since a different *PMT* gene belonging to *N. slyvestris* was examined by Iminashi et al., and also the time period during which the *PMT* expression was examined was different, being only 24 hours, results might be expected to differ from those found here. A transient expression of *NtPMT3* has been reported in leaves upon wounding (Sachan and Falcone, 2002).

The conditions used to promote the formation of undifferentiated tissue from single leaf cells, namely the presence of the growth regulators cytokinin and auxin, results in a tissue dissimilar to either root or leaf tissue in regard to expression of alkaloid metabolism. Therefore, the regulation of alkaloid biosynthesis is probably also unique to this tissue. The identification of signaling intermediates that play a role in alkaloid biosynthesis in undifferentiated tissue may have relevance in production of secondary metabolites in plant cell cultures.

During a plant's life cycle, it is subjected to a range of internal and external stresses. Its ability to adapt to metabolic and environmental changes is essential for its survival. Production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide ($\text{O}^{\cdot -}_2$) and its toxic derivatives, hydroxyl radicals (OH^{\cdot}) and singlet oxygen ($^1\text{O}_2$) occurs at all times during plant growth and development, but when the plant is exposed to biotic and abiotic stresses production of these ROS increases (Navabpour, 2003). Several studies have indicated the production of ROS in presence of increased light conditions (Karpinski et al., 1999). Prolonged exposure to conditions that cause excess excitation energy can result in an increase in the generation of ROS. ROS are an integral part of intracellular signaling in essentially all biological systems (Finkel, 1998). Treatments of callus tissue with light causes a decrease in alkaloid accumulation (figure 3.6). It is possible that elevated levels of reactive oxygen species produced in the presence of light causes repression of *PMT* gene expression (figure 3.5). This repression in turn causes a decrease in alkaloid accumulation. Thus ROS, H_2O_2 , might act as intermediates in light signaling by changing levels of *PMT* expression. The use of the ROS scavenger, DMTU, resulted in increased expression of *PMT* based on GUS

fluorometric analysis and an increase in alkaloid accumulation (fig. 3.5 and 3.6). This increase could be a result of scavenging the ROS released in the media through the various porins. It is hypothesized that free radicals are in equilibrium between the interior and exterior of the cells and hence DMTU is able to scavenge the ones in apoplast and the media in which the callus is maintained. It should be kept in mind that the epibatidine assay is able to detect all nicotine related alkaloids. Hence the GUS expression data for *PMT* gene expression generally correlate with the alkaloid accumulation data. Catalase is not as effective as DMTU in causing an upregulation of promoter activity perhaps because catalase cannot cross the cell wall and plasma membrane barriers, but with callus tissue being basically heterogenous it might be able to penetrate a poorly developed cell wall. H_2O_2 may also rapidly move into and out of cells. Whereas H_2O_2 is generally considered to be capable of crossing the plasma membrane, peroxiporins or aquaporins have also been recently implicated in such transmembrane transport of H_2O_2 (Henzler and Steudle, 2000; Neill et al., 2002). Thus we hypothesize that there is an equilibrium between the levels of H_2O_2 inside and outside the cell and catalase is able to scavenge H_2O_2 which is present in the external medium, thereby lowering the overall concentration of H_2O_2 . The decreased H_2O_2 level then results in relieving the repression of *PMT* gene expression. These results indicate a strong presence of ROS as intermediates in signaling involved in alkaloid biosynthesis.

Another explanation for the greater *PMT* promoter activity in the presence of DMTU compared to the catalase treatment might be that catalase only reduces H_2O_2 levels and DMTU is capable of scavenging a broader range of ROS. However, other general chemical scavengers did not show equivalent effects on *PMT* promoter activity. This might be due to highly specific scavenging of ROS. Also use of the H_2O_2 generating enzyme, glucose oxidase, caused death of callus tissue probably because H_2O_2 is lethal for long-term maintenance of tissues and callus is a very fragile tissue that survives poorly in adverse conditions.

Tobacco callus tissue, which displays highly repressed alkaloid synthesis under our typical laboratory conditions in the light, exhibited significant induction of *PMT* promoter activity and alkaloid accumulation when treated with an ROS scavenger-dimethylthiourea (DMTU) or with the H_2O_2 degrading enzyme, catalase. Based on GUS

fluorometric data, it was determined that treatment with these compounds relieves the suppression caused by light and auxin in the callus tissue. Indeed, the level of *PMT* promoter induction is even greater than that observed when callus tissue is incubated in complete darkness. This perhaps suggests that a low level of ROS formation even in dark-grown tissue limits the full extent of *PMT* promoter activity.

Earlier reports have reported very low levels of alkaloids in callus tissue derived from shoots (Hobbs and Yeoman, 1991). Callus which was treated to the different light conditions for changes in expression of *PMT* was also used to determine if the levels of alkaloids are affected. For this, a very sensitive methodology was used to determine various alkaloids in *N. tabacum* callus tissue. Thus the epibatidine displacement assay was used to measure the very low levels of alkaloids present in callus cultures. This assay is very useful since it is highly sensitive and able to detect slight changes in alkaloid levels in extremely small amounts of tissue. The assay is also able to detect most of the nicotine-related alkaloids present in *N. tabacum* (Rogers and Littleton, unpublished). Callus exposed to low-light or high-light conditions did not show appreciably higher alkaloid contents. However, when under dark conditions or in the presence of the H₂O₂ degrading enzyme, catalase, an increase in alkaloid accumulation resulted (figure 3.6).

Conclusions

Callus tissue is good model for studying alkaloid regulation even though it is distinct from other plant tissues. Undifferentiated plant tissues can be highly useful as a vehicle for natural product synthesis. In callus tissue, light plays a major role in regulating both *PMT* gene expression and total alkaloid biosynthesis. Auxin and light completely repress *PMT* gene expression in callus cultures. ROS scavengers, such as DMTU and catalase, were able to overcome the repressive effect of light and auxin. Thus, ROS is strongly implicated as an intermediate in signaling *PMT* gene response. Also, treatment with catalase resulted in increased alkaloid production even in the presence of light and auxin. We thus infer that H_2O_2 acts downstream of auxin and light in this signaling pathway, which in turn negatively regulates *PMT* gene expression. A model showing this ROS involvement in regulating alkaloid expression in tobacco undifferentiated tissue is shown in figure 3.7.

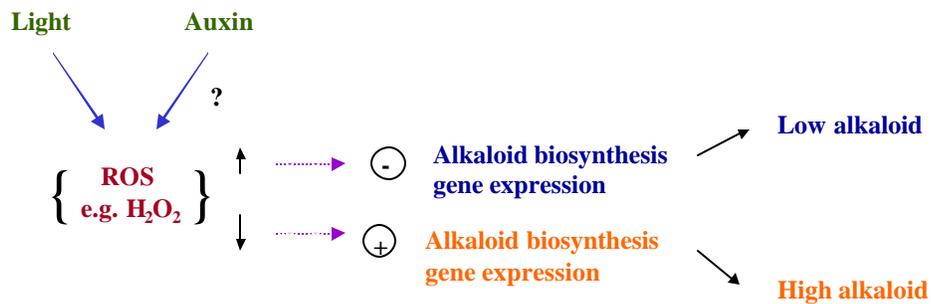


Figure 3.7 A hypothetical model based on results presented in this chapter showing the involvement of ROS (mainly H₂O₂) in regulating alkaloid pathway expression in undifferentiated tobacco tissue. Alkaloid biosynthesis is repressed by light and auxin via ROS such as H₂O₂. This repression is removed upon treatment with JA, which results in a decrease of ROS levels thereby increasing alkaloid biosynthesis.

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Chapter 4

Characterization of signaling involved in regulation of alkaloid biosynthesis in young *N. tabacum* seedlings

Introduction

Survival of young seedlings is critical for the propagation of individuals of the next generation. Extensive herbivory at the seedling stage seriously restrict survival. Synthesis of nicotine in especially young, small seedlings has not been evaluated previously. Thus we decided to study the regulation of nicotine production in young seedlings to determine whether significant alkaloid production occurs. Based on the elucidation of signaling factors that impacted alkaloid biosynthesis in *N. tabacum* callus tissue, we decided to evaluate if similar factors also play a role in roots of young seedlings. In chapter 3 it was found that the expression of the alkaloid biosynthetic pathway was strongly influenced by the levels of reactive oxygen species (ROS). Also, recent studies by Musser et al. (Musser et al., 2002), showed that a reduction in nicotine levels in tobacco occurred upon *Helicoverpa zea* caterpillar feeding. This stimulated our investigation of factors that might affect regulation at the gene level.

Removal of tobacco flower initials and young leaves is a common practice during tobacco cultivation. Decapitation in this way eliminates apical dominance and increases root growth and nicotine content (Akehurst, 1981). Auxin synthesized in young meristematic tissue is transported down the stem to the roots (Phillips, 1975), hence removal of the tip results in a reduced flow of auxin to the roots and thereby may cause activation or repression of genes involved in nicotine biosynthesis (Hibi et al., 1994). Repression has been shown to function at the level of transcription in roots of whole plants. Seedlings were used to determine if similar responses occurred in roots, thus implicating the presence of nicotine and related alkaloids for survival of such a young tissue. Details regarding synthesis of nicotine in young seedlings are unknown. In more mature plants several studies conducted by Baldwin et al. (Baldwin et al., 1994; Baldwin et al., 1997; Preston et al., 1999) report an upregulation of *PMT* expression along

with alkaloid accumulation in *N. sylvestris* upon treatment with jasmonic acid (JA). Baldwin et al. (Baldwin et al., 1994) hypothesize that upon injury to leaves the signal which is transmitted to the root for increased nicotine accumulation is most probably JA.

In this chapter the characterization of signals is conducted using the findings obtained in chapter 3 as a starting point to determine if ROS are also involved in alkaloid pathway expression in differentiated tobacco seedling roots.

Materials and Methods

The transgenic plants harboring the *PMT* promoter-GUS fusion construct described in chapter 2 (Sachan and Falcone, 2002) were used to generate stable transgenic *N. tabacum* plants, and these were used as a source of seeds. These seeds were germinated to yield young seedlings.

Plant material

N. tabacum SR1 was transformed with a *PMT* promoter-GUS fusion that included 627 bp of genomic sequence of the *NtPMT 3* gene. Details of the plasmid construction and plant transformation were as described in chapter 2. Tobacco seeds were sterilized by a two minute treatment in 70% ethanol, followed by two 20-minute treatments with 30% (v/v) household bleach (active ingredient 6% sodium hypochlorite) and 0.02% tritonX-100 with continuous rocking. Seeds were then rinsed 5 times with sterile water, followed by resuspension in 0.15% agar.

Treatment of seedlings

Seedlings were grown on solid MS medium plates in the light ($80 \mu\text{mol m}^{-2} \text{s}^{-1}$) for fourteen days. Subsequently seedlings were removed from the agar medium and transferred to liquid MS medium containing various chemicals for treatment. All seedlings were vacuum-infiltrated with MS medium containing the chemical under a vacuum pressure of 10-mm hg for 15 min. The final concentrations of the substances used were 40 μM jasmonic acid (Sigma), 5 mM hydrogen peroxide, glucose oxidase, glucose (all Sigma), salicylic acid, 5 mM DMTU (1, 3-dimethyl 2-thiourea, Aldrich), 200 units/ml catalase (Sigma), 10 mM tiron (4,5-dihydroxy 1,3-benzene disulfonic acid, Sigma), 30 mM ascorbate and 10 mM N-acetylcysteine.

GUS fluorometric assay

GUS enzyme activity assays were performed in 96-well plates as described in chapter 3. Seedling roots were excised from whole seedlings and the tissue was ground in 100 μl GUS

extraction buffer (50 mM NaHPO₄ pH 7.0, 10 mM Na₂EDTA, 0.1% sarcosyl, 0.1% Triton X-100). Five microliters of the extract was diluted with 95 µl of GUS extraction buffer and to this, 30 µl of 5 mM 4-MUB-β-D-glucuronide (MUG) in extraction buffer was added and incubated for 20 minutes at 37°C. The reaction was stopped by adding Na₂CO₃ to a concentration of 180 mM. Fluorescence was measured at 365 nm excitation and 455 nm emission using a Perkin Elmer luminescence spectrophotometer model LS50B (Perkin-Elmer, Beaconsfield, UK). Protein estimation was done colorimetrically by dye binding using a kit provided by BioRad (Bradford, 1976).

Alkaloid displacement assay and extract preparation

Equal amounts of leaf material were removed from seedlings for preparation of the extracts. Leaf material was ground for one minute in eppendorf tubes containing 500 µl water using a drill press and micropestle for one minute. An additional 500 µl of water was added afterwards. Extracts were centrifuged at 14,000 rpm for 10 minutes and supernatants were used for conducting the alkaloid displacement assays and radioligand binding displacement assays as described in chapter 3.

RESULTS

Primary transgenic plants harboring the *PMT* promoter-GUS fusion construct described in chapter 2 were used to generate T₁ seeds. Seeds were germinated on MS medium agar plates with kanamycin selection to yield young seedlings. Agar was removed from the roots and seedlings were subsequently transferred to MS liquid medium for conducting further experiments.

Treatment with auxin, jasmonic acid

Various plant hormones have been known to affect the production of nicotine in leaves of whole *Nicotiana tabacum* plants (Hibi et al., 1994; Baldwin, 1998), and these effects have been shown to impact key enzyme activities in the nicotine biosynthetic pathway (Mizusaki et al., 1973; Feth et al., 1986). To determine if these hormone-mediated effects occur at the level of transcription, we treated young seedlings with auxin and jasmonic acid and monitored *PMT* promoter activity. Treatment of fourteen-day-old seedlings with α -naphthalene acetic acid (auxin) for 12 hours in light conditions resulted in a decrease in *PMT* promoter activity based on GUS fluorometric analysis (figure 4.1). However there was no decrease in activity upon treatment for five hours (data not shown).

Fourteen-day-old seedlings were treated with jasmonic acid in the same way they were treated with auxin. Seedlings treated with jasmonic acid showed some variation in *PMT* promoter activity after 12 hours following treatment based on GUS fluorometric activity but showed pronounced upregulation at 24 hours after the treatment (figure 4.2). This *PMT* promoter activity was then decreased 48 hours after treatment. Thus, the upregulation of *NtPMT 3* expression is transient, similar to the effect of wounding leaf tissues in chapter 2. A difference in *PMT* promoter activity at the zero time between control and JA-treated seedlings is clear. This may be due to removal of agar from the tissue, thereby causing minor injury to the roots, and subsequently upregulating *PMT* expression.

Salicylic acid downregulates PMT promoter activity

Since salicylic acid (SA) and jasmonic acid are antagonistic to each other, we also examined the effect of salicylic acid on *PMT* promoter activity in roots of young seedling. Five

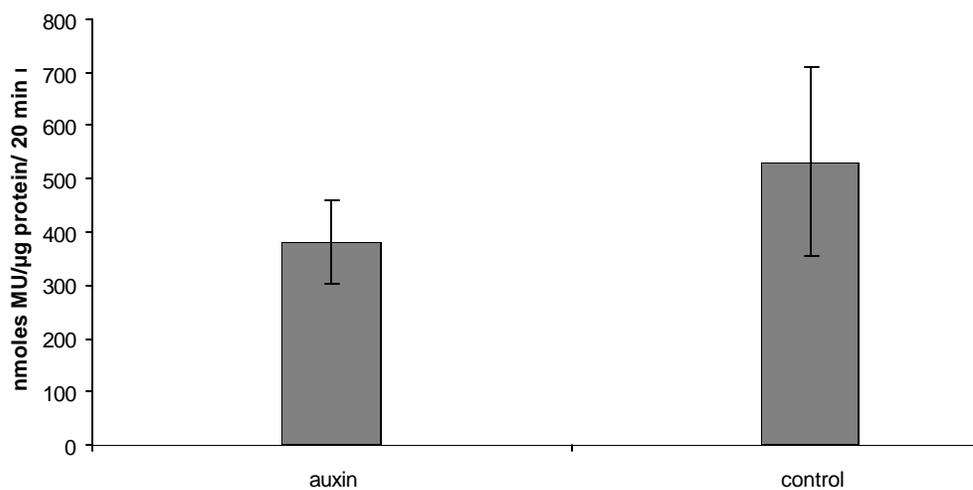


Figure 4.1 *PMT* promoter activity in roots in response to auxin treatment. Roots of nineteen-day-old transgenic seedlings containing the *PMT*-promoter GUS fusion were examined for β -glucuronidase activity after treatment with 30 μ M α -naphthalene acetic acid, (α -NAA, auxin) for 12 h.

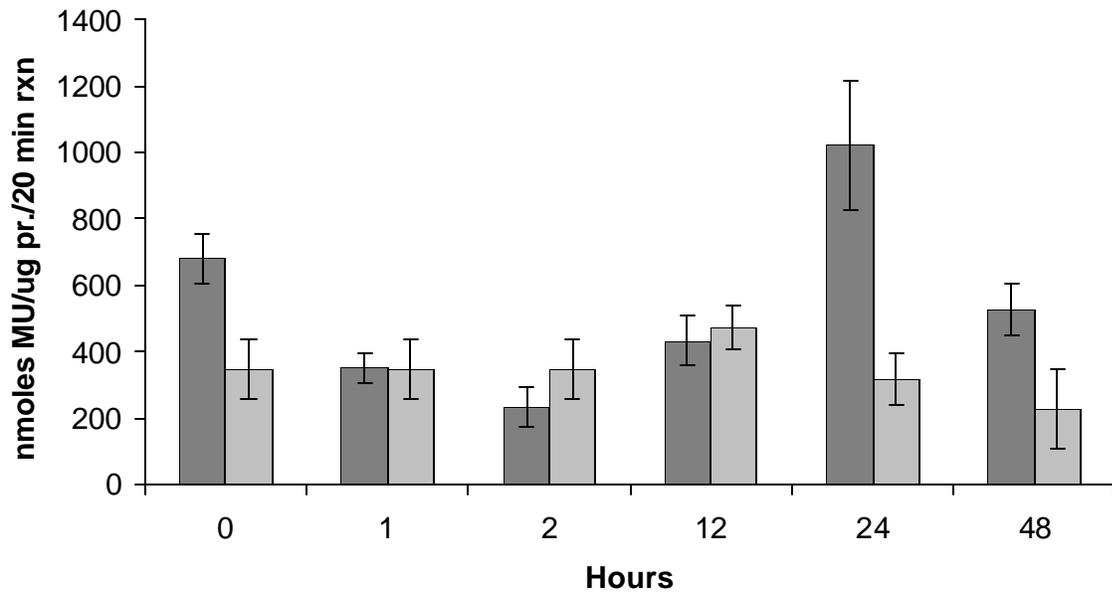


Figure 4.2 *PMT* promoter activity in roots in response to jasmonic acid treatment. Roots of nineteen-day-old transgenic seedlings containing the *NtPMT* 3 promoter GUS fusion were examined for β -glucuronidase activity after treatment with 40 μ M JA for various times. Dark gray bars, JA treated; light gray bars non-treated control.

hours after treatment with 1 mM salicylic acid a high degree of suppression of *PMT* promoter activity was found (Figure 4.3). Lower levels of salicylic acid (0.5 and 0.25 mM) correspondingly also diminished *NtPMT3* promoter activity with approximately 25% of the β -glucuronidase activity detectable after treatment with 0.25 mM salicylic acid.

Hydrogen peroxide downregulates PMT promoter activity

An earlier report on reduction/ degradation of tobacco leaf nicotine contents after insect feeding postulated that ROS was an active component of insect regurgitant (Musser et al., 2002). To examine whether ROS has a direct effect on *PMT* expression, 50 mM H₂O₂ was added to the incubation medium containing transgenic tobacco seedlings. Extracts prepared from roots of the seedlings indicated no detectable β -glucuronidase activity after one hour of treatment (data not shown). Five mM H₂O₂ also inhibited *NtPMT 3* promoter activity as measured by β -glucuronidase activity to approximately two-thirds the level of untreated controls and this inhibition was found to persist for several hours (figure 4.4).

The half-life of exogenously added H₂O₂ has been reported to be 2 min in plant cell cultures, and after only 5 min, no H₂O₂ is detectable (Desikan et al., 1998). Hence, we provided a continuous supply of H₂O₂, using glucose oxidase and glucose to generate it catalytically. Addition of the H₂O₂-generating system, glucose (0.5 mM)/glucose oxidase (0.5 U/ml), to *P. syringae* pv. *glycinea* cell suspension cultures gives a steady state accumulation of ~6 μ M for approximately 3 hours (Delledonne et al., 1998b). Treatment of seedlings with 50 units/ml of glucose oxidase in presence of 25 mM glucose caused a strong repression of *PMT* promoter expression based on GUS fluorometric analysis conducted on extracts prepared from root tissues (figure 4.5). This amount of glucose oxidase activity gave almost the same degree of *NtPMT3* promoter repression as treatment with 20 mM H₂O₂ in seedling roots (figure 4.5).

Effect of ROS scavengers

Based on the effects of ROS scavengers on *PMT* promoter activity observed with *N. tabacum* callus tissue (chapter 3), dimethylthiourea (DMTU) was utilized as a scavenging molecule to reduce the levels of ROS, such as H₂O₂. Seedling roots were treated with MS medium containing DMTU, which was vacuum infiltrated. After treatment of the seedling roots

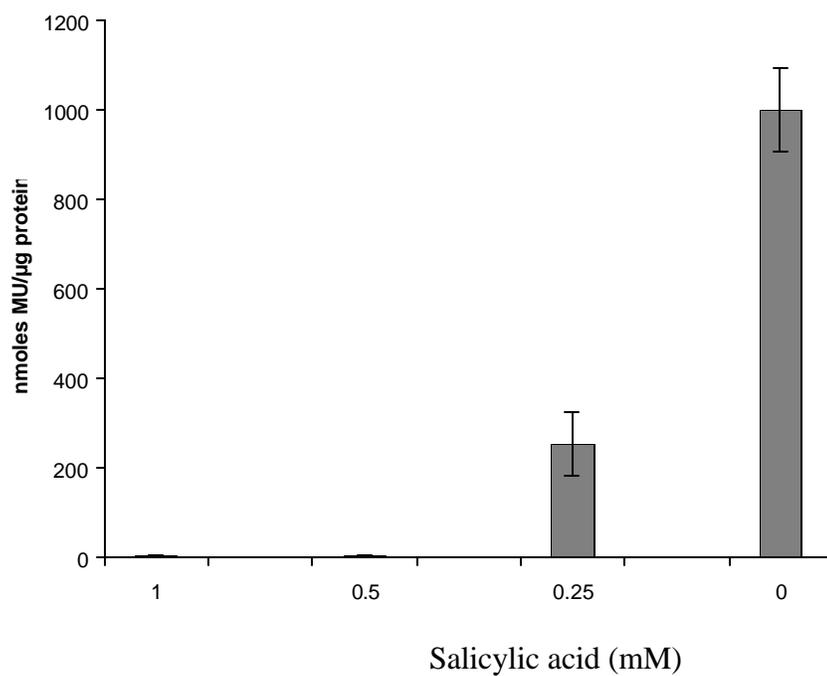


Figure 4.3 *PMT* promoter activity in roots in response to salicylic acid treatment. Roots of nineteen-day-old transgenic seedlings containing the *NtPMT* 3 promoter GUS fusion were examined for β -glucuronidase activity 5 hours after treatment with differing concentrations of salicylic acid.

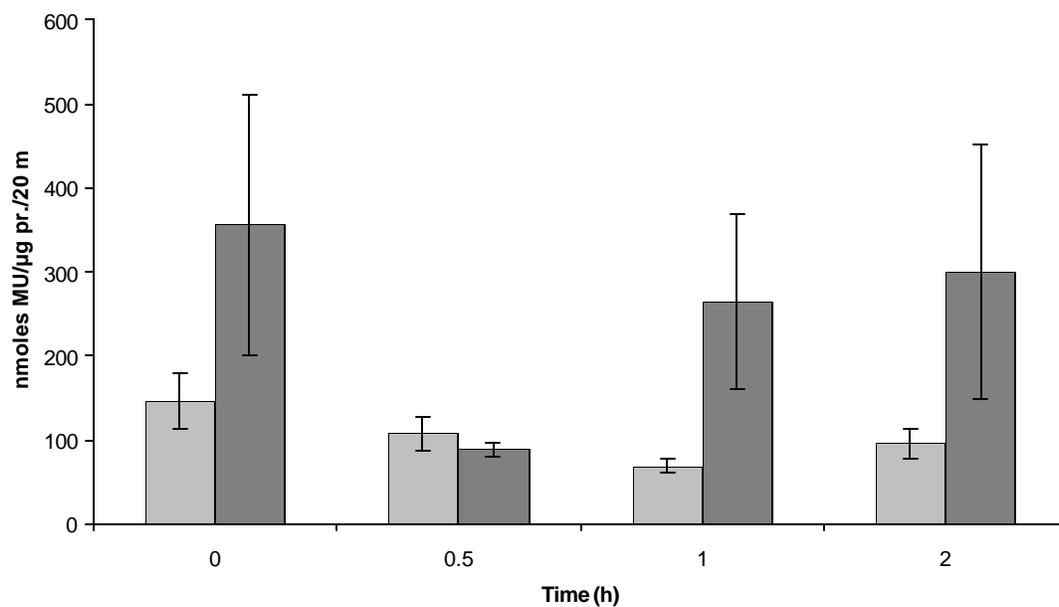


Figure 4.4 Hydrogen peroxide downregulates PMT promoter activity in roots. Roots of nineteen-day-old transgenic seedlings containing the *NiPMT* 3 promoter GUS fusion were examined for β -glucuronidase activity after treatment with 5 mM H₂O₂ for various times (in hours). Light gray bars, H₂O₂ treated; dark gray bars non-treated control.

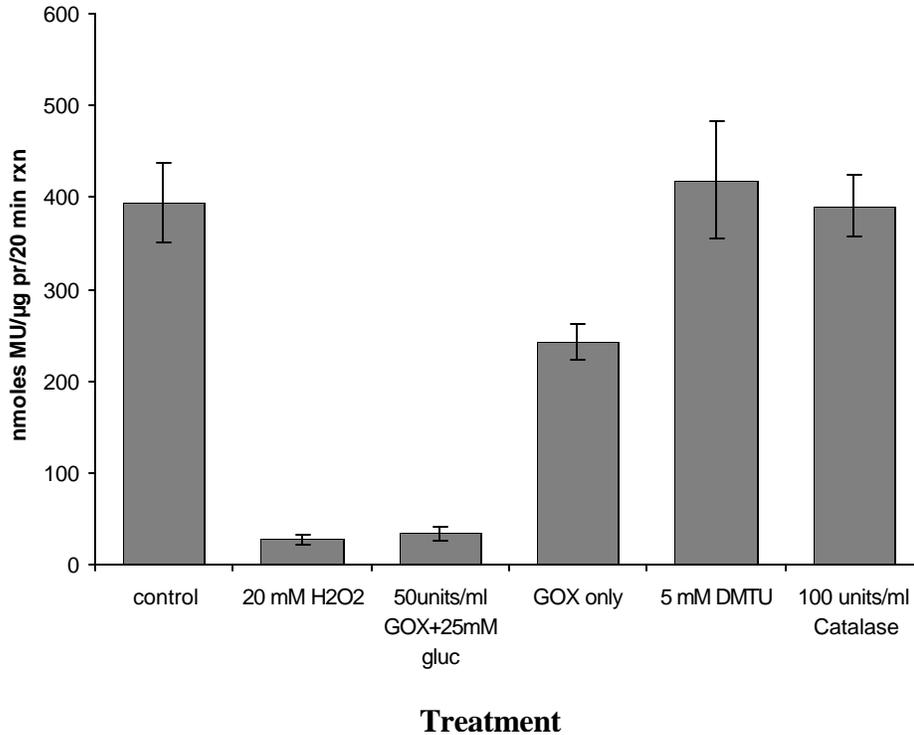


Figure 4.5 *PMT* promoter activity responds to treatments that elevate or diminish hydrogen peroxide levels. Roots of 21-day-old transgenic tobacco seedlings containing the *PMT* 3 promoter-GUS fusion were treated with the following: 1) control (no treatment); 2) in the presence of 20 mM hydrogen peroxide; 3) in the presence of 50 units/ml glucose oxidase (GOX) and 25 mM glucose; 4) 50 units/ml glucose oxidase only; 5) in the presence of 5 mM dimethylthiourea (DMTU); 6) 100 units/ml catalase. All treatments were conducted by vacuum infiltrating the seedlings in MS liquid medium followed by a 5-h incubation.

for 5 hours in light conditions, a slight increase in *NtPMT3* promoter activity based on GUS fluorometric activity was observed over control untreated seedlings (figure 4.5). Catalase was also used to test for the scavenging effect. Treatment of transgenic seedlings with catalase also did not result in dramatic changes in activity compared to control untreated seedlings (figure 4.5). N-acetyl cysteine (NAC), known to modulate ROS levels in plant systems (Costet et al., 2002), did not cause any upregulation or otherwise affect *NtPMT3* promoter activity in roots (data not shown). The catalase inhibitor, 3-amino-1, 2, 4-triazole (AT), that causes oxidative stress by generating endogenous H₂O₂ (May and Leaver, 1993), at 50 µM did not affect the *NtPMT3* promoter activity (data not shown).

Effect of Nitric oxide and superoxide radicals

Nitric oxide (NO) and ROS have important roles in the activation of plant defenses against pathogen attack (Bolwell, 1999; Durner and Klessing, 1999; Beligni and Lamattina, 2001). Soybean cell suspension cultures inoculated with *Pseudomonas syringae* when treated with 0.5 mM of sodium nitroprusside (SNP) is able to generate a steady-state concentration of ~2 µM nitric oxide (Delledonne et al., 1998b). In animals, NO is generated by the conversion of arginine to citrulline and nitric oxide. This reaction is catalyzed by NO synthase (NOS) (Schmidt and Walter, 1994). Usage of S-nitroprusside to determine the involvement of NO on *PMT* gene expression showed only a slight decrease in expression when compared to control untreated seedlings (figure 4.6). NO can potentiate the induction of hypersensitive cell death by reactive oxygen intermediates to induce genes involved in synthesis of protective natural products (Delledonne et al., 1998a). Sodium nitroprusside (SNP) is a NO donor. SNP caused a slight reduction in *PMT* promoter activity when compared to untreated controls. We used utilized S-nitroprusside in combination with glucose oxidase to see if its effect is more pronounced. However, since the *PMT* promoter activity was already low when treated with GOX, as seen in figure 4.6, it was not possible to evaluate the treatment with S-nitroprusside. Thus the effect of SNP on alkaloid biosynthesis via the *PMT* promoter remains unknown.

To determine if superoxide anions are involved in signaling alkaloid biosynthesis by influencing *PMT* promoter activity, we used xanthine oxidase which converts xanthine and produces superoxide radicals. However, since xanthine must be dissolved in sodium hydroxide and NaOH itself reduces the *PMT* activity (figure 4.7.A and 4.7.B), we could not determine if

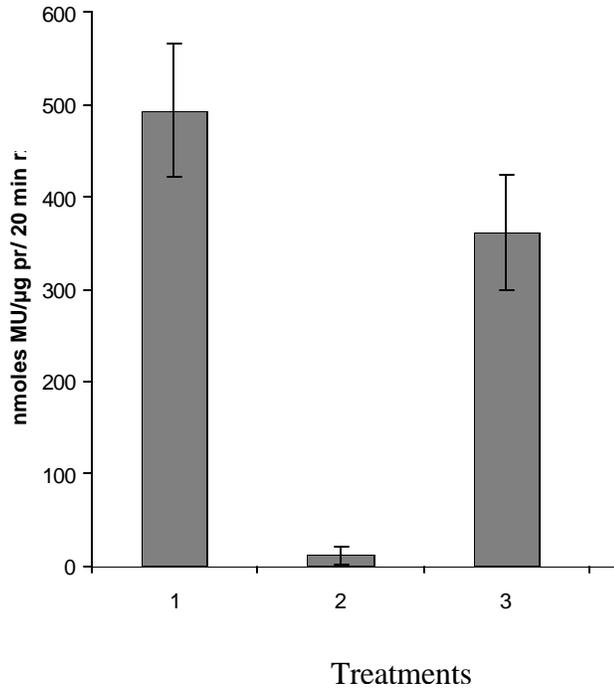


Figure 4.6 *PMT* promoter activity is not responsive to treatments that affect nitric oxide levels. Roots of 21-day-old transgenic tobacco seedling containing *PMT 3* promoter-GUS fusion were treated with the following: 1) control (no treatment); 2) in the presence of 15 units/ml glucose oxidase (GOX) and 12.5 mM glucose ; 3) in the presence of 5 mM sodium nitroprusside (SNP); 4) in the presence of 5 mM SNP, 15 units/ml GOX and 12.5 mM glucose. All treatments were conducted by vacuum infiltrating the seedlings in MS liquid medium followed by a 5-hour incubation.

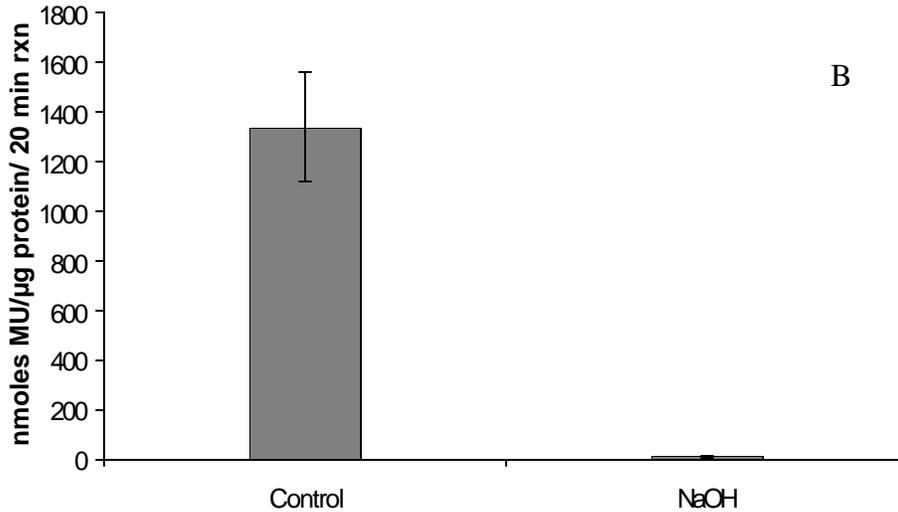
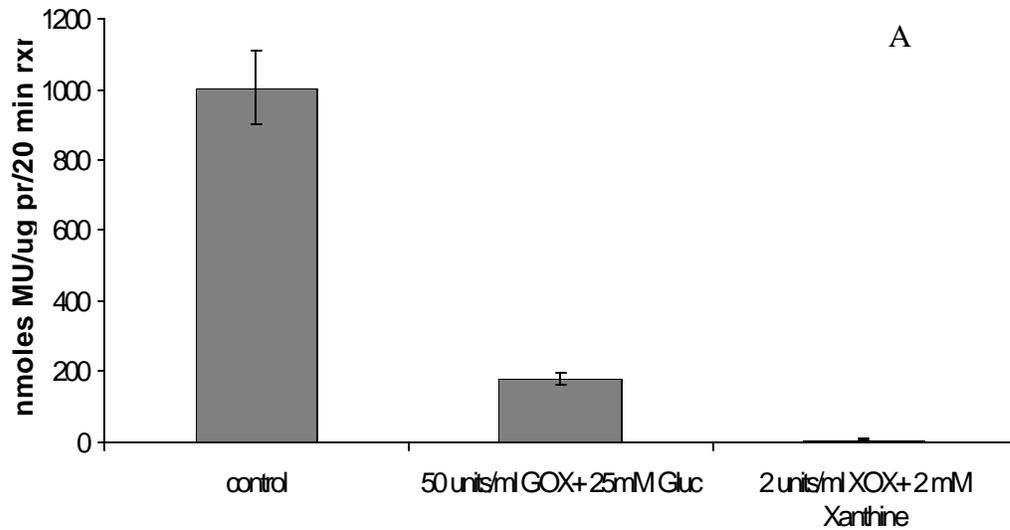


Figure 4.7.A *PMT* promoter activity is not responsive to treatments that affect nitric oxide levels. Roots of 21-day-old transgenic tobacco seedling containing *PMT* 3 promoter-GUS fusion were treated with the following: 1) control (no treatment); 2) in the presence of 50 units/ml glucose oxidase (GOX) and 25 mM glucose ; 3) in the presence of 2 units/ml xanthine oxidase (XOX) and 2 mM xanthine. All treatments were conducted by vacuum infiltrating the seedlings in MS liquid medium followed by 5-hour incubation.

Figure 4.7.B. Treatment with NaOH (same dilution as that used to prepare XOX) to show that pH changes can effect *PMT* expression levels

superoxide anions are involved in alkaloid biosynthesis by this means (figure 4.7). However, when diphenylene iodonium (DPI), an inhibitor of membrane bound NADP(H) oxidase, which has been shown to block the oxidative burst in different cell culture systems (Levine et al., 1994; Desikan et al., 1996), was applied to transgenic seedlings, no effect on *PMT* promoter expression was observed (data not shown). Although, the data are incomplete, thus far we have not obtained evidence showing that superoxide radicals are involved in the modulation of alkaloid biosynthesis.

Effect of auxin and DMTU in signaling

Because we had previously shown that reactive oxygen species, most likely hydrogen peroxide, may be a component of signaling alkaloid production at the level of gene expression, an experiment was conducted to examine if scavenging could affect auxin-mediated repression of *PMT* promoter activity. Transgenic tobacco plants grown on MS medium in standard Magenta boxes for 5 weeks were treated with auxin and DMTU both separately and together for 12 hours. Treatment with auxin caused a slight reduction of *PMT* promoter activity, but the presence of ROS scavenger DMTU had essentially no effect (figure 4.8). The presence of both auxin and DMTU did not appear to block the slight auxin-induced decrease in *PMT* promoter activity. This suggests that DMTU-scavenged ROS does not likely influence auxin repression.

Long distance signaling in ROS signaling

Most of the treatments used thus far for examining the response of *PMT* promoter activity in roots were based on the direct treatment of roots with various ROS-generating or scavenging compounds. Earlier reports had implicated long-distance signaling to cause reduction/degradation of nicotine by insect regurgitants in tobacco (Musser et al., 2002). To investigate whether signals generated on aerial plant parts can be sensed in roots and alter the level of expression of the *PMT* promoter, an experiment using seven-week-old transgenic tobacco plants grown in Magenta boxes was conducted. We infiltrated the leaves of intact plants with a solution of glucose oxidase and glucose in MS medium. After 5 hours, a portion of the roots was collected to perform GUS fluorometric analysis. As a control, MS medium containing glucose oxidase in the absence of glucose (GOX) was infiltrated into leaf tissue. As seen in figure 4.9,

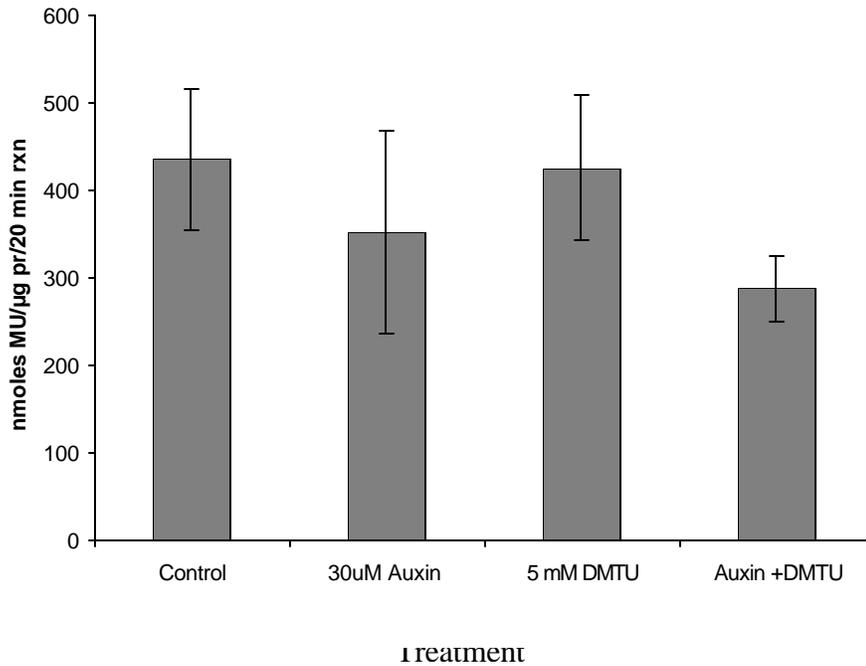


Figure 4.8 Reactive oxygen scavenging shows little effect on *PMT* promoter activity in six-week-old tobacco roots. Roots of Magenta box-grown transgenic tobacco containing *PMT 3* promoter-GUS fusion were removed from agar and treated with MS liquid medium containing the following treatments 1) control (no treatment); 2) in the presence of 30 µM α-NAA (auxin); 3) in the presence of 5 mM dimethylthiurea (DMTU); 4) in the presence of 30 µM α-NAA (auxin) and 5 mM dimethylthiurea (DMTU). All treatments were conducted by vacuum infiltrating the plants in MS liquid medium followed by a 5-hour incubation period.

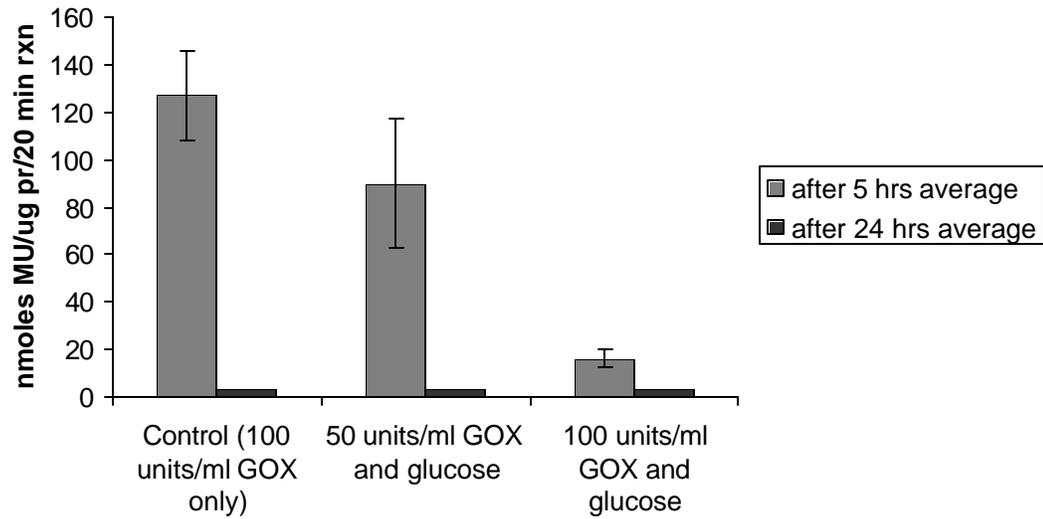


Figure 4.9 PMT promoter activity in roots can respond to ROS signals that originate in leaf tissue. Magenta box-grown leaves of transgenic tobacco containing the *PMT 3* promoter-GUS fusion were infiltrated with a syringe containing MS medium the following: 1) control (100 units/ml glucose oxidase (GOX)); 2) 50 units/ml glucose GOX and 25 mM glucose; 3) 100 units/ml glucose GOX and 50 mM glucose. Treatments incubated for 5 hours (light gray) and 24 hours (dark gray).

after just 5 h, *PMT* promoter activity was reduced by approximately 30% after treatment with 50 units/ml of GOX. Treatment of leaves with a higher GOX concentration (100 units/ml) led to further repression of *PMT* promoter activity within 5 h of treatment. After 24 h of leaf treatment, the level of *PMT* promoter activity was reduced to below detectable levels suggesting that the signal persisted and resulted in a further reduction of *PMT* promoter activity. Treatment with glucose oxidase only also resulted in a complete repression of *PMT* promoter activity after 24 h, perhaps due to availability of its substrate. Thus, it appears that alkaloid biosynthetic genes in the root can respond to signals generated within leaves. This signaling may be mediated by H₂O₂ or some other signaling molecule.

Alkaloid levels respond to treatments that lower or raise ROS

To determine whether the *PMT* promoter activities observed in response to treatments that affect ROS levels, correlation with the levels of alkaloids synthesized was determined using radioligand binding-displacement assays (described in chapter 3). The alkaloid activity was determined in extracts prepared from seedling leaves, the site of alkaloid deposition after synthesis in roots. Treatment of seven-day-old seedlings with jasmonic acid for four days caused an ~10-fold increase in the alkaloid levels. However, treatment with salicylic acid also caused higher (~2.5-fold) accumulation of alkaloids (figure 4.10) which was unlike the response of *PMT* promoter activity to salicylic acid treatment (figure 4.3).

Treatment of seven-day-old seedlings to different light conditions caused the alkaloid accumulation to be lower when compared to dark treatments (figure 4.11). Seedlings were treated to light conditions normally used during their propagation on synthetic medium (60 $\mu\text{mol m}^{-2} \text{s}^{-1}$), or “high light” (150 $\mu\text{mol m}^{-2} \text{s}^{-1}$) or darkness for four days. Under dark conditions, alkaloids accumulated ~ 2 fold higher in leaves than in leaves of plants treated to continuous light, as determined by alkaloid displacement activity. Seedlings treated to higher light conditions showed a slight decrease in alkaloid activity levels (figure 4.11). A similar treatment experiment was also conducted on three-week-old seedlings grown in soil. In seedlings treated to dark conditions, there was higher alkaloid accumulation. However, light did not influence alkaloid accumulation in these older plants (data not shown).

Treatment of seedlings to varying concentrations of ROS as generated by glucose oxidase and glucose caused lower accumulation of alkaloid activity in leaf extracts. Four days after treatment with higher concentrations of GOX, the seedlings were stunted, indicating that the treatment used was deleterious to seedling growth. Thus, equal amounts of seedling leaves were taken to prepare the extracts. Seedlings treated with DMTU exhibited a higher (~1.5-fold) accumulation of alkaloids (figure 4.12). The DMTU treated seedlings were similar in appearance to control-treated seedlings.

PMT-promoter regulation in transgenic Arabidopsis

To determine if the regulatory responses of the *PMT* promoter observed in *N. tabacum* is similar in a different plant species, we introduced the *PMT* promoter-GUS fusion construct into *A. thaliana*. Root extracts from treated seedlings were used for the GUS fluorometric assay. When fourteen-day-old *Arabidopsis* seedlings were treated with auxin, the response was similar to that seen in *N. tabacum*. A decrease in *PMT* promoter- GUS activity was seen upon treatment with auxin for 12 hours in light conditions (figure 4.13 A).

When *Arabidopsis* seedling roots were treated with SA for five hours, it resulted in the repression of *PMT* promoter expression (figure 4.13 B). Unlike the expression seen in *N. tabacum*, *Arabidopsis* seedlings did not respond to JA treatments (data not shown). *PMT* promoter activity in *Arabidopsis* responded to ROS treatments in a manner similar to that seen in tobacco. Treatment with H₂O₂ or glucose oxidase caused repression of *PMT* promoter expression. However like tobacco seedlings, treatment of *Arabidopsis* seedlings with DMTU or catalase did not seem to cause an upregulation but rather a slight downregulation (figure 4.14).

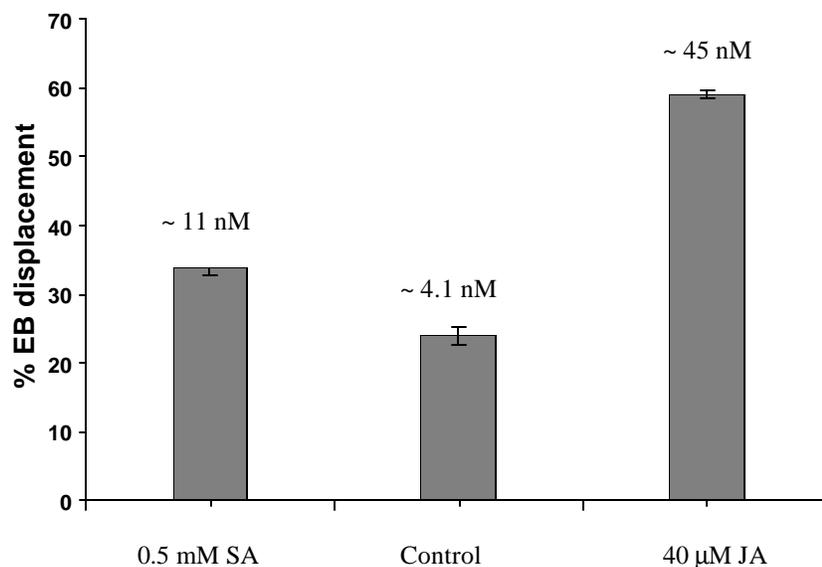


Figure 4.10 Alkaloid displacement activity levels in leaves respond to treatments that alter reactive oxygen oxygen levels. Seven-day-old tobacco seedlings were transferred to fresh MS plates and treated for 4 days. Aqueous extracts were prepared from equally weighed leaf tissue and used for the binding assay with 0.5 mM salicylic acid (SA); no treatment (control); or 40 μM jasmonic acid (JA). Numbers above the bar indicate the estimated concentration of “nicotine equivalents” derived from curves generated using pure nicotine in the assay. Concentration values therefore indicate the amount of displacement activity that is equivalent to the given concentration of nicotine.

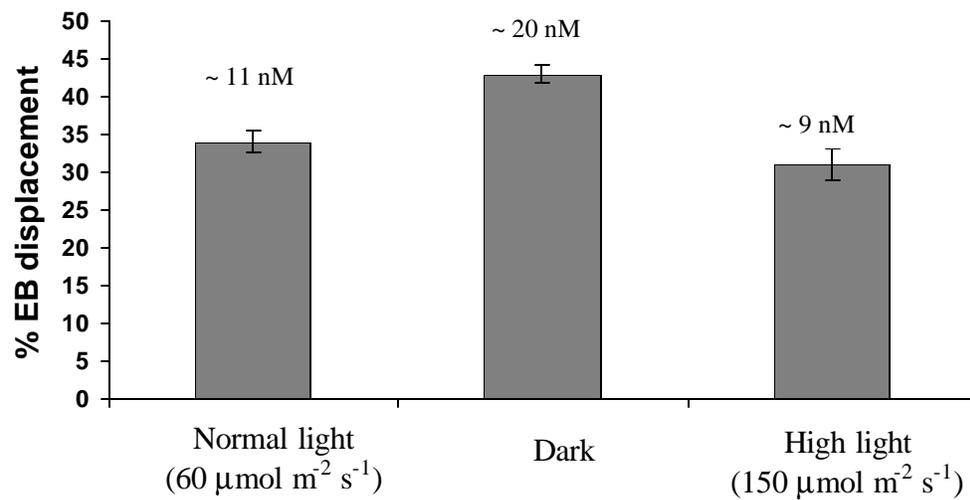


Figure 4.11 Seven-day-old tobacco seedlings were transferred to fresh MS medium plates and treated for 4 days. Extracts were prepared from equally weighed leaf tissue and the supernatant used for the binding assay. Numbers above the bars indicate “nicotine equivalents” as described in fig. 4.10.

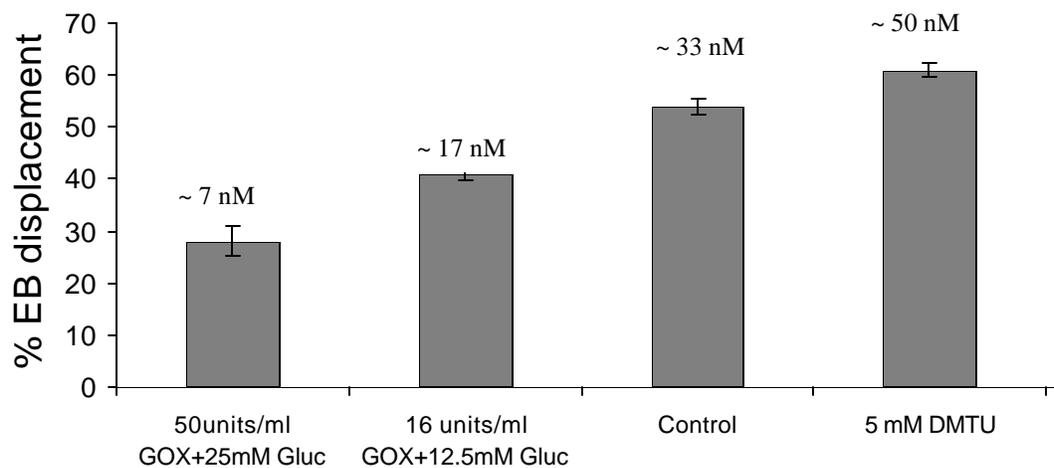


Figure 4.12 Seven-day-old tobacco seedlings were transferred to fresh MS medium plates and treated for 4 days. Extracts were prepared from equally weighed leaf tissue and the supernatant used for the binding assay. Numbers above the bars indicate “nicotine equivalents” as described in fig. 4.10.

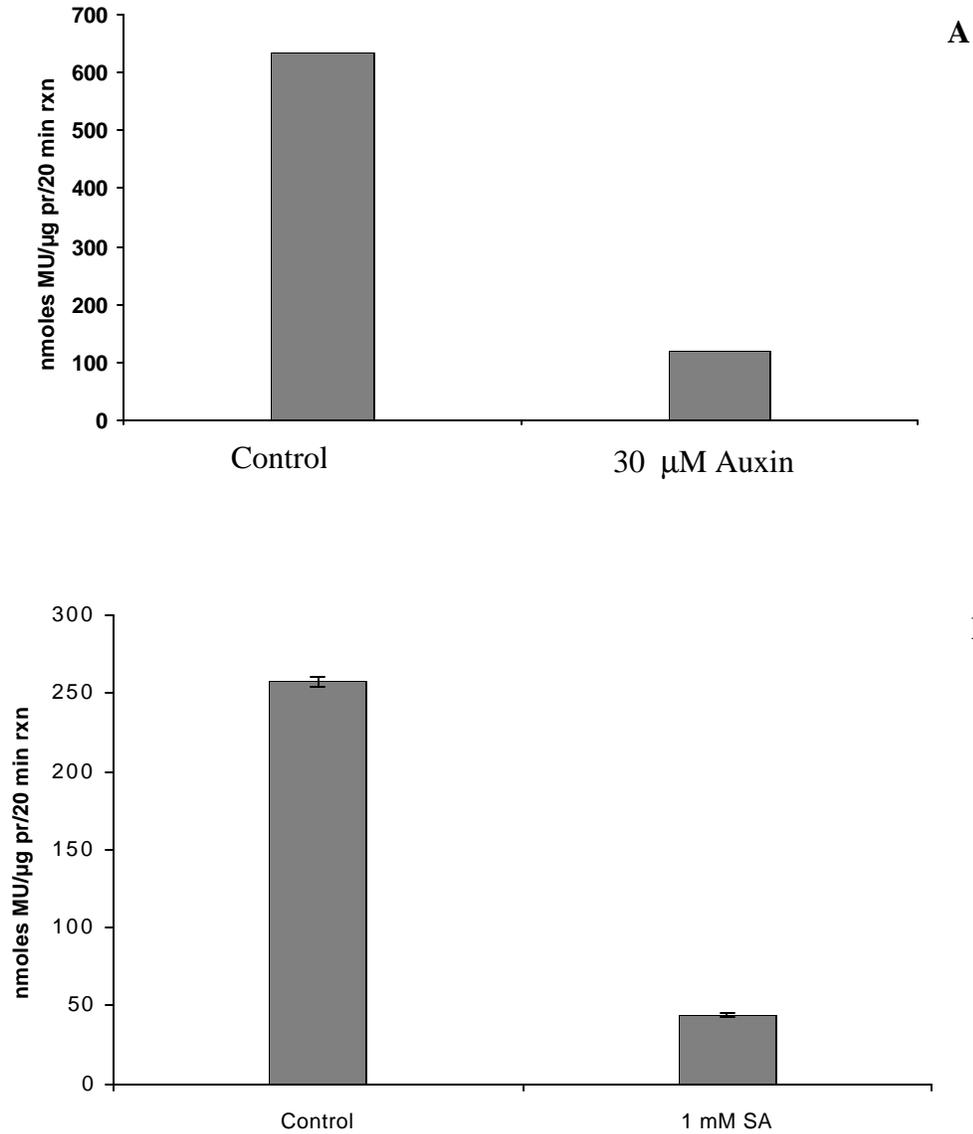


Figure 4.13 *PMT* promoter activity responds similarly in a heterologous host background, *Arabidopsis thaliana*. **(A)** Transgenic *Arabidopsis* seedlings grown on MS medium were removed and the seedlings treated in liquid MS medium with and without auxin overnight. **(B)** Transgenic seedlings grown on MS medium were removed and the seedlings treated in liquid MS medium with and without salicylic acid (SA) for five hours. GUS fluorometric analysis was conducted on extracts prepared from roots.

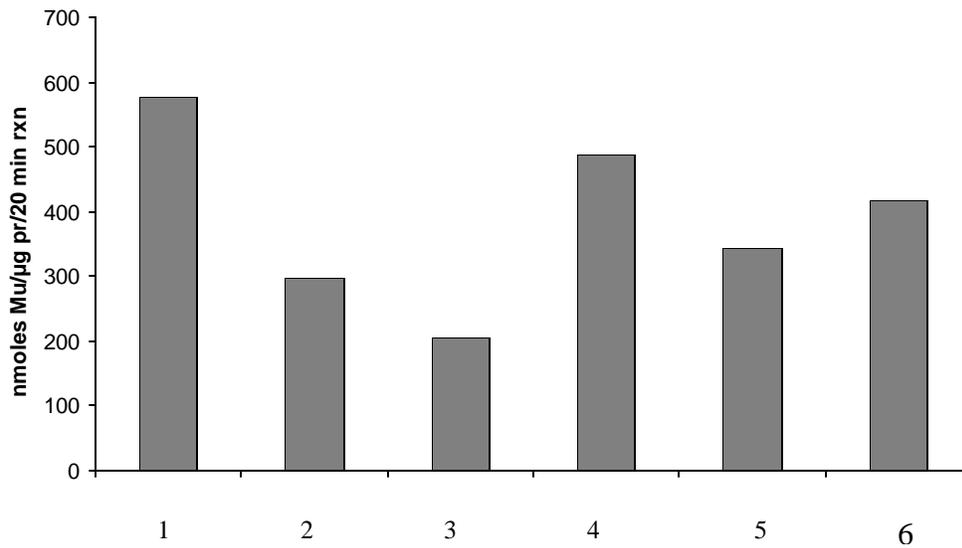


Figure 4.14 Transgenic *Arabidopsis* seedlings grown on MS media were removed and the seedlings treated in liquid MS media for five hours. GUS fluorometric analysis was conducted on extracts prepared from roots. Roots of 21-day-old transgenic *Arabidopsis* seedlings containing the *NtPMT3* promoter-GUS fusion were treated with the following:

1) control (no treatment); 2) in the presence of 20 mM hydrogen peroxide; 3) in the presence of 50 units/ml glucose oxidase (GOX) and 25 mM glucose; 4) in presence of 5 mM dimethylthiourea (DMTU); 5) 100 units/ml catalase 6) 10 mM Tiron. All treatments were conducted by vacuum infiltrating the seedlings in MS liquid medium followed by a 5-hour incubation.

Discussion

Treatment with known effectors of alkaloid biosynthesis

Earlier studies showed a repression of *NtPMT 1* expression upon treatment with auxin in cultured tobacco roots (Hibi et al., 1994). Decapitation of tobacco plants cause an increase in *PMT* enzyme activity and in nicotine content (Mizusaki et al., 1973). The effect of auxin on *NtPMT3* expression, as determined here, is also repressive, but it takes 12 hours to detect this decrease in the root tissues. Thus a slight delay seems to occur in the response to auxin, which may indicate an additional intermediary step(s) or signal(s) for the auxin-mediated regulation of *PMT*. In addition, the downregulation observed in the presence of auxin is only partial, with some *PMT* promoter expression still occurring, consistent with other studies (Hibi et al., 1994).

Several studies have been conducted to indicate the responsiveness of alkaloid synthesis or *PMT* expression to JA treatments (Baldwin, 1994, 1998; Imanishi et al., 1998; Shoji et al., 2000). A rapid increase in *PMT* transcript levels and nicotine contents are typically observed. Most of these studies were done with *Nt PMT1*. However, in our studies we observed a transient upregulation of *NtPMT3* upon treatment with 40 μ M of JA. But this is inconsistent with *PMT* expression in *N. sylvestris* in which treatment with JA does not result in a transient but apparently continuous expression (Shoji et al., 2000). Shoji et al., (1998) have shown the levels of *NsPMT* transcripts remain elevated up to 72 h after treatment in a gel blot analysis using the *NtPMT 1* as a probe. Jasmonic acid (JA) has been implicated as a wounding hormone and the JA levels are thought to increase rapidly and transiently, and with pools waxing and waning within minutes upon wounding (Baldwin, 1998). However, several studies investigating the JA effect on wounding have examined JA levels in plants up to 24 h but not beyond (Baldwin et al., 1997; Preston et al., 1999). It is possible that the transient upregulation seen with *Nt PMT 3* upon treatment with JA, could also be true for other *PMT* clones. Only one report, Shoji et al. (2002) reports that the *NsPMT* expression does not wane even after 72 hours of JA application (Shoji et al., 2000). Another factor could be that different species of *Nicotiana* are involved i.e., *N. tabacum* and *N. sylvestris*. *NtPMT3* transient expression when induced by JA is similar to transient effect of *NtPMT3* expression in leaves upon wounding (Sachan and Falcone, 2002).

Involvement of ROS molecules in alkaloid regulation

An earlier study reported on the reduction or degradation of nicotine in tobacco leaf tissue by the action of components in insect regurgitants that are released upon insect feeding (Musser et al., 2002). We tested whether the effect of ROS contributed directly to the reduction of nicotine and related alkaloids by acting at the level of gene expression. Treatment with 5 mM H₂O₂ was found to repress *NtPMT3* promoter expression. Similar repression of *NtPMT3* occurs upon treatment with glucose oxidase. Glucose oxidase is the principal salivary enzyme in *Helicoverpa zea* (the common, corn earworm), and converts D-glucose and molecular oxygen to D-gluconic acid and H₂O₂ (Eichenseer et al., 1999; Felton and Eichenseer, 1999). H₂O₂ is continually generated from various sources during normal plant metabolism. A wide range of steady-state H₂O₂ concentrations (e. g. 60 µM-7mM in *Arabidopsis* (Karpinski et al., 1999; D. et al., 2001) and 1-2 mM in maize and rice has been reported in plants (Jiang and Zhang, 2001; Lin and Kao, 2001). We verified that the pronounced reduction of the *NtPMT3* gene promoter, one of the five *PMT* gene family members in tobacco, actually reflected whole alkaloid pathway expression by measuring total alkaloid activity levels after the ROS treatments. The effect of H₂O₂ on alkaloid accumulation was also inhibitory (figure 4.12), confirming the use of *NtPMT3* gene as a useful indicator of alkaloid pathway expression.

Salicylic acid (SA) is antagonistic to JA. Application of SA at the site of wounding reduced the amount of JA and nicotine produced (Baldwin, 1999). However application of SA adjacent to the site of wounding does not inhibit nicotine production (Baldwin, 1999). However, no correlation was seen between alkaloid accumulation and SA levels in constitutive salicylic acid producing transgenic tobacco plants (Nugroho et al., 2002). This finding might have been due to the fact that the SA was produced in chloroplastic tissue where the substrate, chorismate, is synthesized, while the alkaloid is synthesized in roots which had little evidence of occurrence of chloroplasts (Nugroho et al., 2002).

Based on GUS fluorometric analysis, *NtPMT3* promoter-GUS fusion transgenic seedlings reproducibly showed a high degree of promoter repression upon SA application (figure 4.3). However, repression did not correlate to the alkaloid levels in SA untreated seedlings, and an opposite response occurred i.e., alkaloid activity actually increasing (figure 4.10). This lack of a correlation between *NtPMT3* promoter activity and alkaloid levels thus uncouples alkaloid production from at least expression of the *PMT 3* promoter. This result is in contrast to the

results of previous experiments employing ROS generators or scavengers. We added a similar final concentration of SA to the β -glucuronidase enzyme assay mixture to make sure that this effect is not simply due to addition of SA on β -glucuronidase enzyme activity. Northern analysis of seedlings treated with SA similarly did not show any difference compared to the untreated seedlings. The probe used for the analysis was prepared by PCR of genomic DNA used *NtPMT1* primers. Thus it appears that SA acts by a distinct mechanism that causes a reduction in *PMT3* expression. It is to be determined if it does effect other *PMT* family members. Another possibility may be that SA treatment downregulates all *PMT* gene expression but mechanisms of transport and/or alkaloid degradation are not effected thereby leading to alkaloid levels that are relatively unchanged. It is possible that SA does not directly affect the *NtPMT3*, but might be targeting a regulatory protein interacting with the *NtPMT3* promoter. Also, SA may be suppressing *PMT* gene expression by suppression of ascorbate peroxidase and catalase that are responsible for degradation of H_2O_2 . SA has been shown to inhibit catalases (Willenkens et al., 1994).

A related study employed transgenic potato that was transformed with fungal glucose oxidase to produce constitutively sub-lethal levels of H_2O_2 . The plants showed increased disease resistance and a several-fold increase in SA levels (Wu et al., 1997). GOX-transgenic plants show broad spectrum disease resistance to *Phytophthora infestans* late blight, *Alternaria solani* early blight and *Verticillium dahliae* wilt (Wu et al., 1997). Thus, intracellularly generated H_2O_2 is implicated in elevating SA levels (Slaymaker et al., 2002). On the other hand SA's involvement in generating H_2O_2 cannot be ruled out (Conrath et al., 1995; Durner and Klessing, 1996).

Dimethylthiourea was utilized as a scavenging molecule to reduce the levels of ROS such as H_2O_2 , as it functions as a trap for H_2O_2 (Levine et al., 1994) as well as other ROS species such as superoxide anions and hydroxyl radicals. Catalase was also used to decrease specifically H_2O_2 . Because catalases are enzymes they cannot cross cell wall or plasma membranes, we hypothesize that ROS such as H_2O_2 leach out into the media and thereby can be reduced by catalase to remove the repression of the *PMT* promoter. However, the amount of ROS production in roots may be very low to begin with, or that a distinct type of regulation occurs in tobacco callus. Hence we do not see a high degree of *PMT* overexpression as seen in callus tissue in response to ROS scavengers (Chapter 3). In addition the level of *PMT* expression in roots is already

relatively high. In regard to sources of ROS in seedling roots, peroxisomes are known to be a main source of ROS, and most of them occur in leaves. Peroxisomes play a key role in photorespiration that occurs in leaves of C₃ plants such as tobacco. Because photorespiration is an inevitable consequence of photosynthesis, leaf peroxisomes are large and abundant in photosynthetic tissues (Masters and Crane, 1995; Olsen and Harada, 1995). Mitochondria, on the other hand, are also abundant in roots and ROS production from these during oxidative electron transport may generate a very small and localized amount of ROS, such that a minimal effect is seen on the *PMT* promoter.

Nitric oxide (NO) and ROS have important roles in the activation of defenses against pathogen attack (Bolwell, 1999; Durner and Klessing, 1999; Beligni and Lamattina, 2001). When the plant recognizes an avirulent pathogen it triggers a rapid production of ROS such as H₂O₂ and O₂⁻, which results in initiation of several defense responses in the plant (Delledonne et al., 1998b). However, Delledonne et al., (1998) have shown that to produce a strong disease-resistance response, nitric oxide is also required as a signal. It has been shown to act independently of these intermediates to induce genes involved in production of protective natural products. It is not clear if NO has a role in alkaloid signaling since S-nitroprusside affects *PMT* only slightly (figure 4.6). Also SNP along with GOX did not potentiate the effect of GOX on *NtPMT3* promoter expression.

No evidence was obtained to suggest that superoxides affect *NtPMT3* promoter expression based on the effect of DPI and xanthine oxidase (figure 4.7). The evidence thus far suggests that H₂O₂ might be the primary signal affecting alkaloid regulation.

Long distance transport of signal

Based on the experiments involving infiltration of intact plant leaves with glucose oxidase and glucose (figure 4.9) it is clear that a signal can be transferred from leaves to roots, the site of alkaloid biosynthesis, after just 5 hours of treatment. It is not known if this signal is H₂O₂ or if it acts via other means. H₂O₂ is not generally known to be a long distance signaling molecule, thus additional signaling mechanisms are likely to be involved. However, one speculation is that ROS initiates a cell-to-cell signaling cascade, in which case a means to propagate the signal must also be involved. This aerial-to-root signaling however, is significant

in that the signal generated within a single leaf resulted in a potent reduction of *PMT* promoter activity in roots.

Downstream signals

In callus tissue, it is thought that auxin, JA and light act via an ROS signaling intermediate, such as H₂O₂ to regulate alkaloid synthesis (chapter 3). However, in whole plants where auxin was applied in combination with DMTU (figure 4.8), it did not appear that results were consistent with auxin acting via an ROS intermediate. Results of this experiment suggests that ROS might not act downstream of the auxin-mediated downregulation of *PMT* expression or that ROS is not involved in this process. Another explanation is that earlier studies of treatments with DMTU were done for five hours while the time required for auxin to elicit an effect on the plants to be lower (overnight). Thus the duration for the two studies differed. Also, as observed with GUS fluorometric assays conducted on young seedlings treated with DMTU (figure 4.5), little difference was seen between controls. Thus, DMTU does not affect *PMT* expression in seedling roots. From these experiments, it does not appear that the partial repression of *PMT* promoter activity by auxin occurs via an ROS intermediate.

Expression in A. thaliana

To determine if the same regulatory responses of the *PMT* promoter we have observed might occur in different plant species, we introduced the *PMT* promoter-GUS fusion construct into *A. thaliana*. The responses of the *PMT* promoter to auxin, salicylic acid and H₂O₂ treatments were similar to the inhibitory responses observed in tobacco seedlings as determined by GUS fluorometric assays, suggesting that these molecules signal through a conserved mechanism in these two unrelated plants.

Alkaloid accumulation

Alkaloid displacement activity assays in extracts derived from very small tissue samples of seedling leaves was used to detect very low (i. e., picogram) levels of alkaloids. The assay is based on radioligand binding-displacement, using an alkaloid drug with high affinity for specific

receptor proteins (nicotine acetylcholine receptors) in the mammalian brain. The assay utilizes tritium-labeled epibatidine, a highly specific alkaloid that binds with high affinity to these receptors. By comparing the amount of binding displaced to the displacement produced by standard concentrations of nicotine, one can get an estimate of the concentration of the alkaloids within the extract. This assay is able to recognize nicotine and nicotine-like compounds in the extract, as essentially all nicotine alkaloids tested in pure form show similar displacement activities.

Treatment with JA and darkness resulted in increased alkaloid levels. However, a reduction in accumulation was seen when the seedlings were treated with varying amounts of H_2O_2 generated catalytically with glucose oxidase and glucose. In addition, high light also caused a decrease in alkaloid accumulation in young (11-day-old) seedlings (figure 4.11). However, this apparent light-induced decrease in alkaloid accumulation was not found reproducibly in older plants, suggesting that if this represents a genuine physiological response, it is occurring in relatively in young seedlings. Thus, the role of ROS in signaling alkaloid expression in differentiated seedling tissue is not as straightforward as was found in undifferentiated callus tissue. It is clear that GOX-generated H_2O_2 can result in a decreased alkaloid accumulation in young seedlings but it is unknown how this might play a role in the life of the plant. The finding that leaf application of an ROS generating signal has a pronounced effect on *PMT* promoter activity certainly shows that some aspects of ROS are involved in alkaloid pathway expression repression.

Conclusions

Study of young seedling tissue was made to address whether significant alkaloid production occurred and also to study alkaloid regulation at the level of *PMT* gene expression in a differentiated tissue. *PMT* gene expression is observed in roots at a very early stage along with alkaloid accumulation. Unlike the callus tissue, in young seedlings light does not seem to play a major role in regulating *PMT* expression and alkaloid accumulation. However, ROS, particularly H_2O_2 , is implicated in signaling *PMT* gene expression and also alkaloid production. Low-level H_2O_2 treatment generated enzymatically by the GOX-glucose reaction was found to reduce alkaloid production in very young seedlings. Seedlings treated to a dark period were able to produce increased alkaloid levels. In seedlings, H_2O_2 might act downstream of auxin, SA and light in signaling, thereby acting as an intermediate in negatively regulating *PMT* gene expression. Also signal generated in the aerial part of plants by application of glucose oxidase and glucose (thereby generating H_2O_2) results in altering *PMT* expression in roots

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Chapter 5

Conclusions

This work was conducted to investigate various factors involved in the regulation of the alkaloid metabolism. Alkaloid biosynthesis and gene expression occurs only in specific tissues and cells and often in response to specific developmental cues. We wished to improve our understanding of the regulation of alkaloid biosynthesis and did this study by investigating signaling factors that might regulate pathway expression. For this we monitored the gene that is responsible for catalyzing the first committed step in nicotine biosynthesis, putrescine *N*-methyltransferase (*PMT*). The *PMT* gene was isolated from a genomic DNA library of *N. tabacum*. One of the clones was *NtPMT 3*, which was not previously isolated, was sequenced in its entirety including the upstream promoter region. A 627-bp fragment encompassing the promoter of *NtPMT 3* was fused to the GUS reporter gene to obtain transgenic *N. tabacum* plants that could be used to study the regulation of alkaloid biosynthesis in *N. tabacum* plants and tissues.

Earlier studies indicated that expression of *PMT* genes is restricted to roots (Hibi et al., 1992). During the course of our analysis of the transgenic *N. tabacum* plants, we found a novel upregulation of the *NtPMT 3* promoter in leaves in response to mechanical wounding. This expression was transient i.e., the expression declined 2-4 h after wounding. RT-PCR analysis of this phenomenon indicated that wounding of leaves upregulates the *PMT* transcripts within 15 minutes of injury and reaches a maximum levels at 30 minutes. Cloning and subsequent sequencing of the RT-PCR product revealed the presence of other *PMT* clones, indicating that wound-inducible expression in leaves is not just limited to *NtPMT 3*. A low level of *PMT* enzyme activity was seen in unwounded leaves. Efforts to analyze nicotine biosynthesis in leaves were not conclusive, since extensive wounding was required to see a slight increase in the level of nicotine biosynthesis. From these results we discovered that a trace amount of constitutive *PMT* activity exists in leaves. This may afford young leaves a slight advantage against insect herbivory.

Callus prepared from protoplasts freshly isolated from transgenic *N. tabacum* plants was used to study *PMT* gene regulation in an undifferentiated tissue. Light and auxin have a strong repressive effect on *PMT* promoter activity. However, growing callus in the dark and with lowered concentrations of auxin gave high levels of *PMT* promoter expression. Based on several studies showing the production of ROS when plants are exposed to high light conditions (Salin, 1987; Holmberg et al., 1997; Karpinski et al., 1997; Karpinski et al., 1999), we tested the effect of oxidants and ROS scavengers directly on callus tissue to evaluate if these treatments impacted both alkaloid accumulation and *PMT* promoter activity. Tobacco callus tissue, which displays highly repressed alkaloid synthesis under typical laboratory conditions in the light, exhibited significant induction of *PMT* promoter activity and alkaloid accumulation (indirectly measured) when treated with an ROS scavenger- dimethylthiourea (DMTU) or with the H₂O₂ degrading enzyme, catalase.

Most previous studies examining the regulation of alkaloid biosynthesis and accumulation employed whole plants or callus cell suspension cultures (Baldwin et al., 1997; Imanishi et al., 1998; Baldwin, 2001). Nicotine assists in enhancing the plants fitness by deterring insect herbivory, however, how early the biosynthesis of nicotine begins to confer a survival advantage to young seedlings is a question that has not been addressed thus far. Hence, we analyzed fourteen-day-old seedling roots to study the regulation of alkaloid biosynthesis. All the factors regulating the expression in fully-grown plants were conserved in young seedling roots. Auxin has been shown to have a repressive effect on *NtPMT3* promoter and jasmonic acid upregulates the *NtPMT3* promoter in roots, although transiently. In a recent report (Musser et al., 2002), products of glucose oxidase were proposed to act in suppressing the synthesis of insecticidal alkaloids such as nicotine in tobacco. That study proposed that hydrogen peroxide might interfere with the jasmonic acid induced wound signal that enhances nicotine accumulation in leaves. The studies presented here confirm this result and extend it by establishing that ROS levels have a direct influence on pathway expression, at the level of transcription, at least for *NtPMT3*. We propose that ROS constitute part of the signaling that represses the *PMT* gene and thereby nicotine production. The inability of ROS scavengers to result in detectable effects in roots may be due to low levels of H₂O₂. It is not clear from our experiments if other ROS such as nitric oxide or superoxide anions are involved in the regulation of *PMT*. Treatment of seedlings to dark conditions, resulted in increased alkaloid accumulation.

H₂O₂ has been clearly shown to play a key role in alkaloid regulation. We postulate that the mechanism of signaling involved in *N. tabacum* seedlings utilizes H₂O₂ as a downstream intermediate to SA and light, thereby acting in negatively regulating *PMT* gene expression. We were able to show that within hours of application glucose oxidase-generated H₂O₂ to the leaf tissue, the *PMT* promoter activity was reduced in roots. H₂O₂, produced by glucose oxidase and glucose treatments, also reduces alkaloid accumulation. Thus, we have shown that ROS is implicated in alkaloid biosynthesis by acting as an intermediate in the metabolism of alkaloid biosynthesis and that this control operates at the level of gene expression. A model depicting the essential features of these results are shown in figure 5.1.

To determine if the same regulatory responses of the *PMT* promoter we have uncovered were held in common with expression in a heterologous species, we introduced the *PMT* promoter-GUS fusion construct into *A. thaliana*. The responses of the *PMT* promoter to auxin, salicylic acid and H₂O₂ treatments were similar to the inhibitory responses observed in tobacco seedlings as determined by GUS fluorometric assays, thereby suggesting that these molecules signal through a conserved mechanism.

From our studies of *PMT* regulation in different *N. tabacum* tissues, we have unveiled several novel characteristics. In leaves that normally do not show any *PMT* expression, we found a transient increase in *PMT* expression upon mechanical wounding. Also, we have shown that ROS is an important signaling intermediate in alkaloid synthesis in undifferentiated callus tissue. Increased concentrations of ROS that might be produced in presence of light and auxin causes downregulation of *PMT* and, in turn, decreased alkaloid accumulation. This is strongly suggested from experiments using ROS scavengers, which were found to remove the repression of *PMT* gene expression in the presence of light and auxin. Treatment with catalase also resulted in increased alkaloid accumulation. In seedling roots, ROS were observed to decrease *PMT* expression as well as alkaloid accumulation. Long distance transduction of this signal from leaves to roots has also been implicated. A possibility is that this ROS signal might be H₂O₂. It is unknown whether ROS species such as H₂O₂ themselves, or other signaling molecules elicited by ROS in leaves are responsible for the potent effect on root *PMT* expression.

Involvement of ROS in signaling in alkaloid metabolism is further suggested by a report on transgenic tobacco overexpressing *Vitreoscilla* hemoglobin (VHb), which results in increased nicotine accumulation. An increased accumulation was attributed to higher oxygenation for

nicotine synthase, an oxygen-requiring enzyme, and also scavenging of oxygen radicals by the Vhb (Holmberg et al., 1997). The results of this work by Holmberg, et al., can now be considered in light of the findings presented here, which show that modulation of ROS can have dramatic effects on alkaloid pathway expressions.

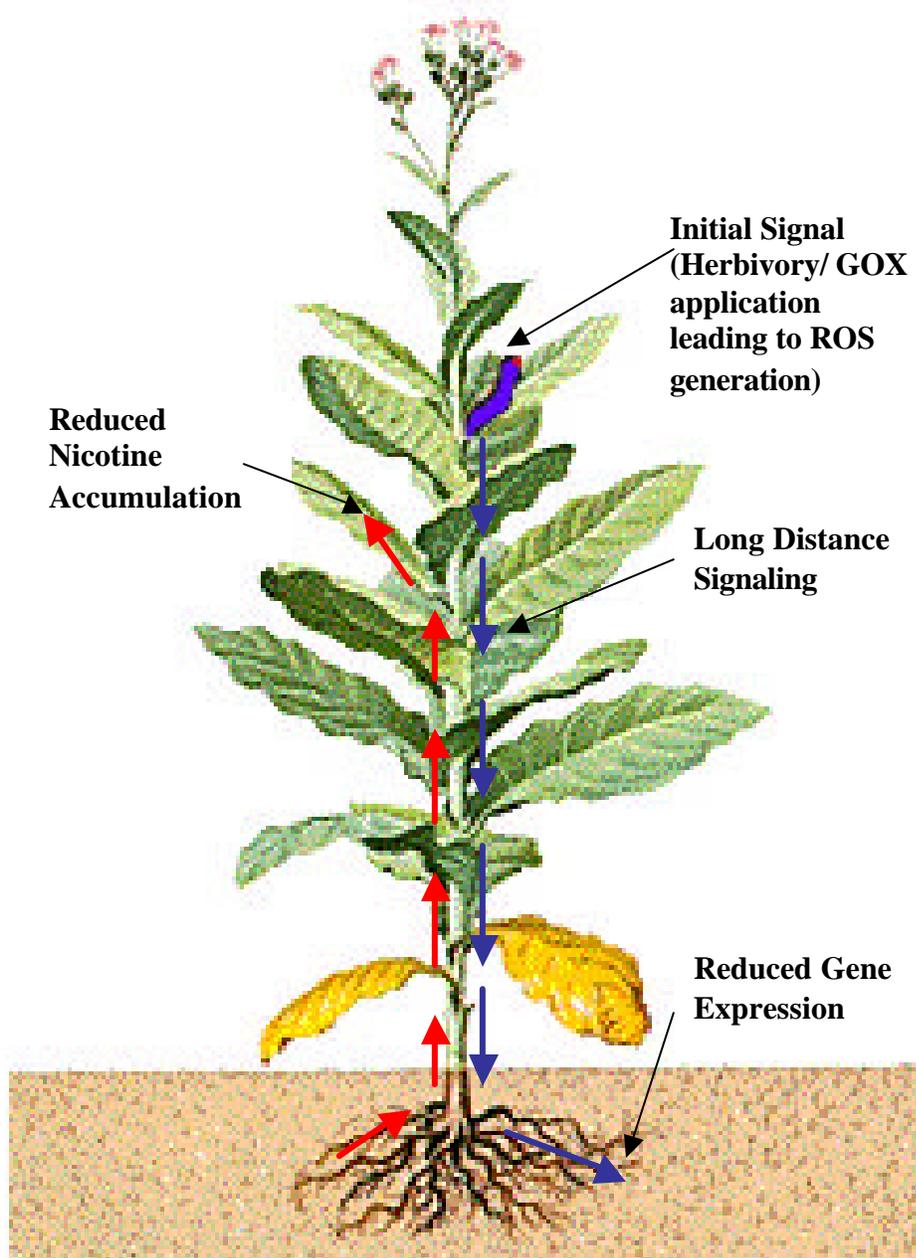


Figure 5.1 A model depicting ROS generated in leaves affecting gene expression in roots and nicotine accumulation. Signal generated in the aerial part of plants by application of glucose oxidase (GOX)-glucose generated H_2O_2 leads to reduced expression of *PMT* in roots. Reduced accumulation of nicotine has been shown Musser et al., (2002) by herbivory on leaves by *Helicoverpa zea*.

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