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

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Abstract

Putrescine *N*-methyltransferase (PMT) catalyzes the first committed step in the biosynthesis of pyrrolinium ring-containing alkaloids. Earlier studies have indicated that *PMT* gene expression is restricted to root tissue in Solanaceus plant species. During the analysis to further elucidate factors that govern the regulation of alkaloid synthesis, evidence was found for a novel expression pattern dictated by the 5′-flanking region of at least two members of the *PMT*-gene family. A 627-bp DNA fragment upstream of the *NtPMT3* gene was fused to the β-glucuronidase (GUS) reporter gene and used to produce stable transgenic lines of *Nicotiana tabacum*. Fluorometric and histochemical assays conducted on transgenic plants indicated high expression levels in root tissue and, in agreement with previous studies, no expression was detected in leaves. However, expression was observed in leaves when they were mechanically wounded. This expression was highly localized around the wound site and showed little evidence of long distance signaling, including lack of responsiveness to jasmonic acid. Expression was transient, with maximum levels 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, which did not increase significantly upon wounding.

Keywords: *Nicotiana tabacum*; Solonaccae; Tobbacco; Alkaloids; Mechanical wounding; Putrescine *N*-methyltransferase regulation; Jasmonate-independent; Leaf expression

1. Introduction

Plants are continually exposed to diverse environmental challenges, including attacks from a wide range of herbivores. Signals elicited by such attacks arise from specific perturbations associated with tissue injury. Thus, mechanical injury, pathogen attack, and damage from herbivores all set into motion defined self-defense systems, with 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, 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 tobacco root from arginine and/or ornithine by 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 *Nicotiana 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

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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 methyl jasmonate (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 jasmonic acid (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 *Nicotiana 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 h after damage to aerial tissue along with increases in *PMT* transcript levels (Sinclair et al., 2000). In *Nicotiana glauca,* where anabasine is the most abundant alkaloid, QPRTase expression occurs in both leaves and roots. However, 12–24 h 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 JA, or, 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 studying the regulation of alkaloid biosynthesis in *Nicotiana*, evidence was obtained for low *PMT* expression levels in leaves, which are abruptly upregulated upon wounding. This upregulated expression was found to be highly localized and short-lived.

2. Results and discussion

2.1. 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). Among these sequences, a 627-bp region upstream from the ATG start site of the *PMT* gene was identical to the published *Ntpmt3* sequence (GenBank accession number AF126811, Riechers and Timko, 1999). 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 its signal was detectable after 1 h exposure to the X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) substrate (Fig. 1A, B); intense staining was observed in roots and root hairs after 12 h (Fig. 1C, 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 h in the X-gluc solution. In general, GUS staining was not evident on surfaces of most leaves (Fig. 1E–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 2–3 mm beyond the wound site (Fig. 1E). To check whether the localized staining pattern could be due to inadequate access of the X-gluc substrate to sites distal from the primary wound the staining pattern in wounded leaves from 3–4 week-old transgenic plants that harbored a strong, constitutive promoter from the Figwort mosaic virus fused to a GUS reporter gene (Bhattacharyya et al., in press) was examined. In the latter, GUS staining was evident at the wound site and throughout the leaves which persisted for extended periods (>12 h) after wounding (data not shown). Thus these findings indicate that X-gluc substrate infiltration into the leaf sections is sufficient to visualize GUS activity beyond the wound site, and that the *PMT*-promoter activity is localized in the latter.

To examine the temporal nature of this wound-induced expression, 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

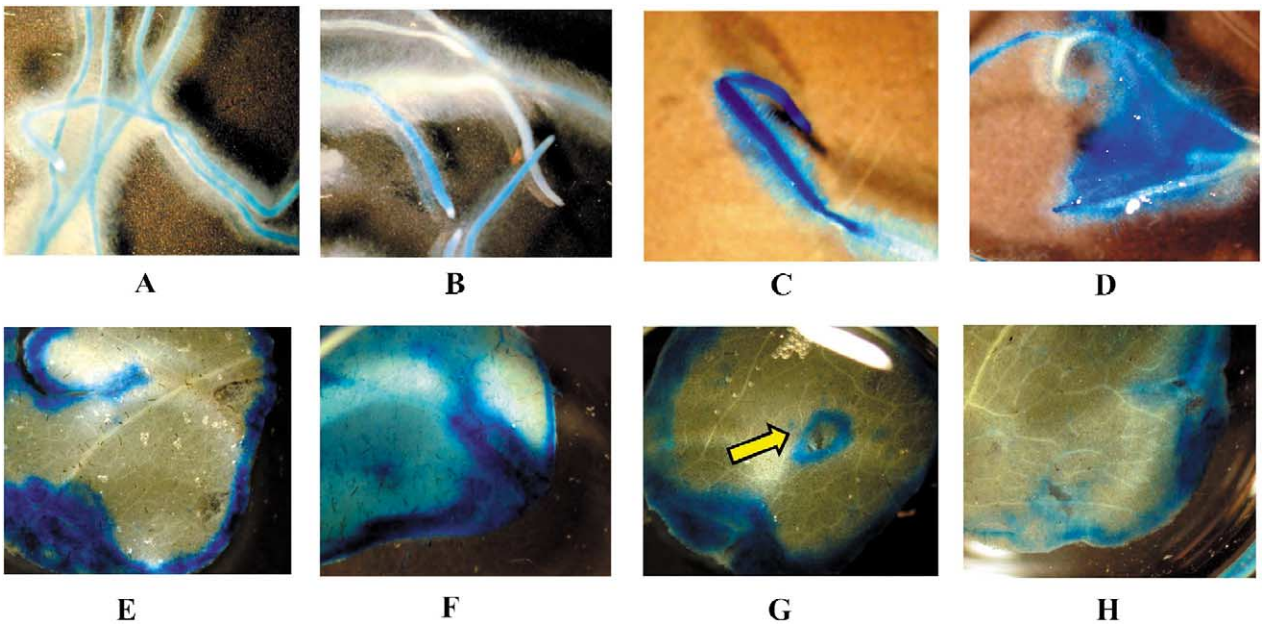

Fig. 1. Transgenic tobacco lines expressing *PMT* promoter GUS fusions after staining with X-Gluc. (A–D) Histochemical reaction of GUS activity in roots after 1 h staining in X-Gluc solution (A, B) or after 12 h staining (C, D). (E–H) Histochemical GUS localization in leaf discs from four independently 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.

staining (Fig. 2). The staining pattern indicated that *PMT* expression was activated immediately after wounding, persisted for several hours and then visibly tapered off at 4 h. Leaf discs punctured and stained after about 4 h showed essentially no GUS staining around the puncture or cut sites (Fig. 2A). In control experiments using the Figwort mosaic virus promoter, no decrease in GUS histochemical staining was observed even after several hours of staining (data not shown). Therefore, this pattern of transient expression after wounding is representative of the *PMT* promoter activity. It should be noted, however, that within the 12 h incubation period in X-gluc solution, additional expression of the GUS gene driven by the *PMT* promoter may have occurred.

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-h period. Following the removal of the final leaf disc (0-h wounding), the leaf was detached from the plant and stained for GUS expression (Fig. 2B). GUS staining here was observed only within a narrow band of cells a few millimeters 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-h and the signal gradually faded away after 2–4 h (Fig. 2A, B). Staining at the 8-h wound site was likely due to pressure applied to the leaf surface, as 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 GUS staining was not influenced by prior leaf injuries. Fig. 2C 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.

2.2. RT-PCR expression analysis in wild-type Nicotiana

The highly localized and short-lived nature of the apparent wound-induced *PMT* expression in leaves suggests that *PMT* messages might be below the limit of detection for Northern blot analysis. Therefore, RT-PCR was employed to detect expression of *PMT* in wounded and unwounded leaves (Fig. 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-h time points, which then

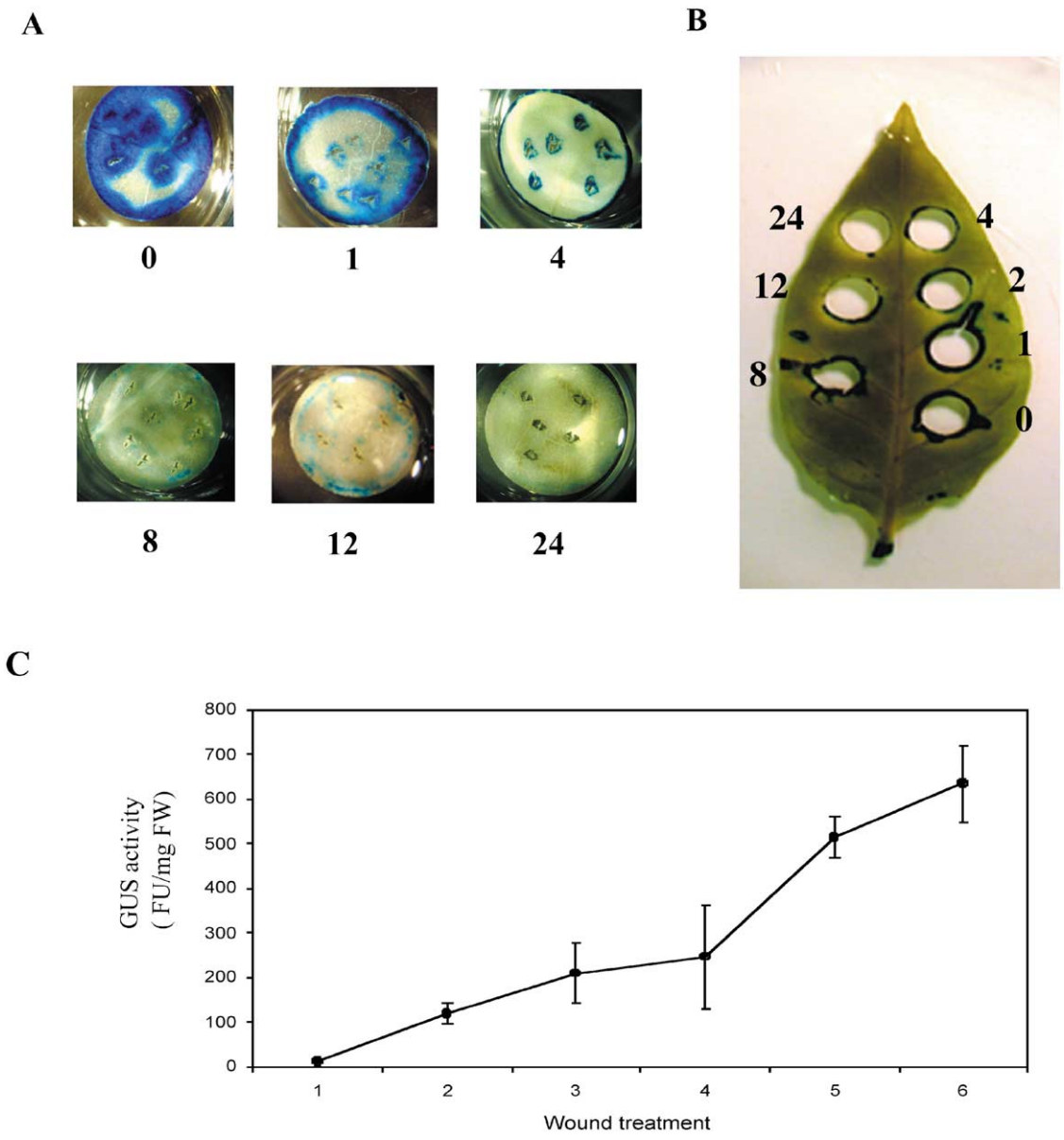

Fig. 2. (A) Time course of wound induction of *PMT* promoter activity by GUS histochemical staining in leaf tissue. Leaf sections were made with a cork borer and punctured with sharp forceps. Numbers below the panels indicate the time in hours after puncturing each disc. Punctured discs were placed on 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. (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.

sharply diminished 2 h after wounding. Quantitative data for the expression levels are shown (Fig. 3C) 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. Nevertheless, 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, consistent with previous studies (Mizusaki et al., 1973; Hibi et al., 1994; Riechers and Timko, 1999). 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. 3A), which indicate that *PMT* expression in leaf tissue is approximately 50-fold lower than that in root tissue.

In order to fully confirm that the RT-PCR product obtained was at least one of the known *PMT* genes, the wound-induced RT-PCR product from leaf tissue was cloned into a pSTSBlue-1 vector (Novagen, Inc.) and sequenced. Alignment results indicated that the RT-PCR product was identical to *PMT* sequences, including *Ntpmt*1 and *NtPMT*4 (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 were all amplified with the primers used in this RT-PCR experiment (Riechers and Timko, 1999). Therefore, detection of 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. 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.

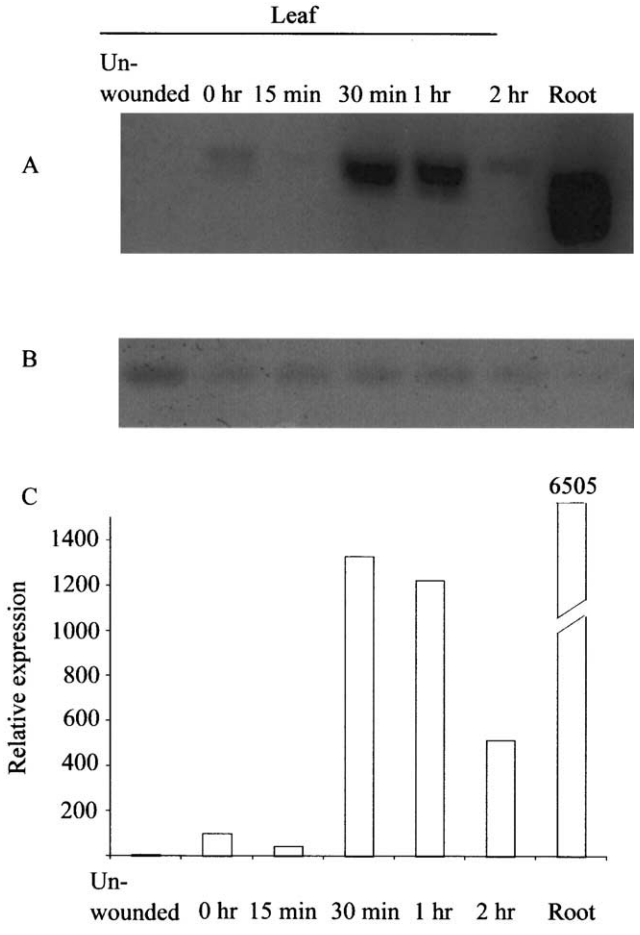

Fig. 3. RT-PCR analysis of *PMT* expression at various time points after wounding. (A) *PMT* RT-PCR products derived from RNA prepared from: unwounded leaves, lane 1, or leaves wounded and used to prepare RNA after the times indicated. Root lane indicates RT-PCR product derived from root RNA of unwounded 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.

2.3. PMT enzyme activity is detectable in leaves

In several separate experiments, low levels of PMT enzymatic activity were detected in leaf tissue (Table 1). However, a significant increase in PMT activity in leaves upon wounding was not consistently obtained. Typically, leaves wounded and assayed 1 h later showed a slight increase in activity when compared to unwounded tissues. PMT enzyme activity determined in leaves 2 h 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 enzyme activity. It may also be possible that only highly localized PMT increases occur, and any increase in activity may be masked by the large amount 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 enzyme activity in leaves is required to determine its metabolic significance.

2.4. Further characterization 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

Table 1
PMT activities in root and wounded leaf tissues

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

Putrescine *N*-methyltransferase (PMT) enzyme activity (nmoles/μg 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 Section 4. Values are the average of three determinations±SE.

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 or 400 μM 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 JA-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 act as general elicitors (Winz and Baldwin, 2001).

To further characterize the *PMT* wound response in leaves, leaf discs were treated with cycloheximide (CHX), to assess the requirement for protein synthesis. Somewhat unexpectedly, the response to mechanical wounding was not inhibited and CHX appeared to cause the wound-induced expression of *PMT* to persist (data not shown). The ability of CHX 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). A possible explanation 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 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.

2.5. *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.

3. Concluding remarks

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). Some defensive genes are constitutively expressed at high levels in roots but at lower levels in leaves Nishiuchi et al. (1997). 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 as described here for 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 content but also increased methylputrescine along with slight decreases in the level of spermidine and spermine. 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 et al. (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.

4. Experimental

4.1. 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 (ms, Sigma) medium containing 1% sucrose, or in standard Farfard (Conrad Fafard, Inc., Agawam, MA) potting mix in a greenhouse.

4.2. Promoter isolation, DNA constructs, plant transformation

A *N. tabacum* genomic library (Clontech, Palo Alto, CA) was screened using a probe (580 base pairs) prepared by PCR from SR1 genomic DNA using published cDNA sequences of the *N. tabacum NtPMT*1 cDNA (Hibi et al., 1994). DNA was labeled with ^{32}P 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 with an ABI Prism 310 automated capillary sequencer and used for further studies. Upon comparison to published *PMT* sequences, the 627 bp *PMT* clone was shown to be identical 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 μg/ml) and transferred onto fresh MS kanamycin-containing plates every 2 weeks. Transgenic plants were regenerated from calli derived from leaf discs and transferred to Magenta boxes containing MS media plus kanamycin (50 μg/ml). After a 5 week period, 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).

4.3. Wounding treatments and histochemical assays

Approximately 2-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 h period at following intervals: 24, 12, 8, 4, 2, 1 and 0 h. The 24-h disc was removed first and floated in 1 X MS medium for 24 h before staining for GUS activity. Similarly, 12-h discs were floated in 1 X MS medium for 12 h before undergoing the staining regime, while a 0-h leaf disc was removed and put directly into GUS-staining solution for 12 h. Histochemical staining for GUS activity was performed according to Jefferson et al., (1987). Plant tissue was infiltrated for 15 min in a solution of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc, 1 mg/ml), 100 mM K-phosphate buffer (pH 7.0) and incubated at 37 °C for the overnight (12 h) staining. After staining, samples were treated with 70% EtOH for 24 h to enhance tissue transparency.

Fluorometric GUS assays were conducted by taking 1-cm diameter leaf sections from a 1-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 μl of distilled water and 50 μl of GUS extraction buffer (50 mM $NaHPO_4$ pH 7.0, 10 mM Na_2EDTA, 0.1% sarcosyl, 0.1% Triton X-100) and were incubated at 37°C for 5 min. Fifteen microliters of 5 mM 4-MUB-β-D-glucuronide (MUG) in extraction buffer was added and further incubated for 1 h at 37 °C. The reaction was stopped by adding Na_2CO_3 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.

4.4. RNA isolation, RT-PCR

Fifty-six day-old plant leaves were heavily injured by poking repeatedly with sharp forceps and incubated on MS media for given time periods and then tissue was frozen in liquid nitrogen. Each time point consisted of injured leaves from several plants to obtain sufficient tissue for RNA extraction. RNA was isolated using a kit (RNeasy, QIAGEN, Inc., Valencia, CA) according to the manufacturers instructions and the final elution used DEPC-treated water. RT-PCR was performed using the One-Step RT-PCR kit (QIAGEN). Leaf (200 μg) and root total RNA (20 μg) was amplified using two *PMT*-specific sense and antisense primers: 5′-CCTCGAGATGAACGGCCACCAAAAT-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 s, annealing at 52 °C for 1 min and extension at 72 °C for 1 min. The cycle was repeated 35 times. Final extension occurred for 10 min at 72 °C. Internal standards used were 18 S rRNA PCR probes included in the kit (QuantumRNA 18S internal standards, Ambion, Austin, TX). Five microliters 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.

4.5. 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 days after topping and frozen in liquid nitrogen. Extracts were prepared from roots and both wounded and unwounded leaves according to Mizusaki et al., (1973). Tissue (1.5 g) was ground in 5 ml extraction buffer with a mortar and pestle. Homogenates were filtered through four layers of cheesecloth and clarified by centrifugation at 12,000 ×g for 15 min. 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 was determined by dye binding according to Bradford (1976) using a kit provided by BioRad.

Putrescine *N*-methyltransferase activity was determined by measuring the radioactivity of ^{14}C-labeled *N*-methylputrescine formed from putrescine and *S*-adenosyl-L-methionine-$^{14}CH_3$. Reaction mixtures contained 50 μM of Tris–HCl (pH 8.3), 2.5 μM of β-mercaptoethanol, 2 μM of putrescine, 680 nmoles of *S*-adenosyl-L-methionine-$^{14}CH_3$ (0.04 μCi) and 0.31 ml of enzyme extract in a final volume of 0.355 ml. After 30 min incubation at room temp, the reaction was stopped by adding 20% NaOH (0.145 ml) saturated with NaCl. The labeled *N*-methylputrescine reaction product was extracted with $CHCl_3$ (~1 ml). The $CHCl_3$ 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.

4.6. 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 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 (50 μg/ml), auxin (0.1 μg/ml) cellulase (1.5%), and salicylic acid (100 mM).

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