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Methyl jasmonate induced expression of the tobacco putrescine *N*-methyltransferase genes requires both G-box and GCC-motif elements

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Abstract

Putrescine *N*-methyltransferase (PMT) catalyzes the first committed step of nicotine biosynthesis, converting putrescine into *N*-methylputrescine. A variety of chemical, environmental, and developmental cues have been implicated in its regulation. Here we have examined the differential expression of β-glucuronidase (GUS) transgenes under the control of the transcriptional regulatory sequences of four distinct members of the *NtPMT* gene family from tobacco (*Nicotiana tabacum* L.). BY-2 cell cultures expressing various *NtPMT* promoter-*GUS* constructs were examined for their response to treatment with various combinations of methyl jasmonate (MeJA), auxin (AUX), and ethylene (ETH). All four *NtPMT* gene promoters examined were inducible by MeJA, although the extent of the induction varied dramatically, with the *NtPMT1a* promoter being the most responsive. High AUX levels in the cell growth media repressed *NtPMT::GUS* transgene expression and inhibited their MeJA-induced transcription. Treatment of BY-2 cells with ETH alone did not result in a significant alteration in *NtPMT::GUS* expression. However, similar to AUX, ETH treatment led to the suppression of MeJA-induced transcription. Detailed deletion analysis of the *NtPMT1a* gene promoter showed that as little as 111 bp upstream of the transcriptional start site were sufficient to confer MeJA-responsiveness. Deletion of a conserved G-box element (GCACGTTG) at −103 to −96 bp completely abolished MeJA-responsiveness. Further mutagenesis studies revealed that in addition to a functional G-box, MeJA-responsiveness of the *NtPMT1a* promoter also required a TA-rich region and a GCC-motif (TGCGCCC) located at −80 to −69 bp and −62 to −56 bp relative to the start site, respectively. A synthetic G-box tetramer (*4 X syn G-box*) fused to a −83 bp fragment from the *NtPMT1a* promoter (containing the TA-rich region, GCC-box, and TATA-box) displayed a 30-fold induction by MeJA treatment, whereas when the *4 X syn G-box* was fused to a minimal (−46 bp) promoter fragment derived from the CaMV 35S gene, no induction by MeJA treatment was detected. Our results indicate that multiple intersecting signal transduction pathways and different transcriptional regulatory factors are involved in mediating JA-responsiveness of *NtPMT* expression in tobacco.

Introduction

Nicotine is the major alkaloid present in wild and cultivated tobacco (*Nicotiana* spp.) and its biosynthesis is controlled by a variety of factors including developmental age, phytohormones, and biotic and abiotic stresses (Kutchan, 1998; Waterman, 1998; Wink, 1998; Baldwin, 1999; De Luca and St. Pierre, 2000; Facchini, 2001). In nature, nicotine and related alkaloid play an important role in plant defense against herbivore and insect attack (Baldwin *et al.*, 1994; Ohnmeiss

et al., 1997; Baldwin and Prestin, 1999). The biosynthesis of nicotine and related alkaloids begins with the polyamine, putrescine, formed by one of two pathways in plants (Chattopadhyay and Ghosh, 1998; Chou and Kutchan, 1998; Malmberg *et al*., 1998). Putrescine can be synthesized directly from ornithine, in a reaction catalyzed by the enzyme ornithine decarboxylase (ODC, EC 4.1.1.17), or formed indirectly from arginine in a reaction sequence initiated by arginine decarboxylase (ADC, EC 4.1.1.19). The relative contribution of the two pathways to putrescine formation and subsequent nicotine biosynthesis are not known (Malmberg *et al*., 1998). Putrescine is then converted to *N*-methylputrescine through the action of an *S*-adenosylmethionine-dependent enzyme known as putrescine *N*-methyltransferase (PMT) (Chou and Kutchan, 1998; Chattopadhyay and Ghosh, 1998; Malmberg *et al*., 1998). *N*-methyl putrescine then is oxidized by a diamine oxidase and cyclized to form a 1-methyl-Δ^1-pyrrolium cation that is condensed with nicotinic acid or its derivative to form nicotine (Chattopadhyay and Ghosh, 1998).

Nicotine biosynthesis occurs exclusively in the roots of *N. tabacum* (Baldwin, 1999) and following their synthesis, nicotine and other tobacco alkaloids are translocated from the roots through the xylem to the leaves, where they accumulate, often to very elevated levels. Consistent with this, root tissues are the predominant site of *PMT* transcript accumulation and PMT enzyme activity in *N. tabacum* and other alkaloid producing plant species, such as *Atropa belladonna*, *Hyoscyamus niger* and *Datura stramonium* (Hibi *et al*., 1992; Hibi *et al*., 1994; Riechers and Timko, 1999). Analysis of transgenic *A. belladonna* and tobacco plants expressing β-glucuronidase (GUS) reporter gene constructs under the control of the *A. belladonna PMT* gene promoters showed that *PMT* gene expression was localized to the pericycle (Suzuki *et al*., 1999).

Changes in phytohormone (e.g. auxin, cytokinin) levels and/or ratios as a consequence of plant development (Mizusaki *et al*., 1973; Akehurst, 1981; Hashimoto and Yamada, 1994; Kutchan, 1998) or as the result of direct manipulation of plant cell culture media have been shown to affect the synthesis and accumulation of nicotine and its derivatives (Eilbert, 1998; Hashimoto and Yamada, 1994; Hibi *et al*., 1994; Imanishi *et al*., 1998a, b, 2000). For example, both nicotine levels and levels of total leaf alkaloids increase in tobacco following removal of the floral meristem (e.g. decapitation or topping) as a result of decreased auxin production and availability (Saunders and Bush,1979; Hashimoto and Yamada, 1994; Hibi *et al*., 1994). Topping induced increases in nicotine in *N. tabacum* plants are correlated with a marked rise in *PMT* transcript abundance in roots within 24 h (Hibi *et al*., 1994; Riechers and Timko, 1999; Sinclair *et al*., 2000). Similarly, PMT gene expression is repressed by auxin (Hibi *et al*., 1994; Imanishi *et al*., 1998a, b), with its removal or reduction in cell culture media resulting in increased PMT expression. Various abiotic factors including wounding, drought stress, and pH imbalance (Hashimoto and Yamada, 1994; Kutchan, 1998; Waterman, 1998; Baldwin, 1999), as well as biotic factors, such as herbivory, insect feeding, and attack by various microbial and fungal pathogens, are also known to elicit increased production of nicotine and other alkaloids in the leaves of wild and cultivated tobacco species (Baldwin *et al*., 1994, 1996, 1997; Ohnmeiss *et al*., 1997; Saito and Murakoishi, 1998; Baldwin and Prestin, 1999). Convincing evidence has been produced that wound-induced increases in jasmonate are associated with increased nicotine content of damaged *Nicotiana* plants (Baldwin *et al*., 1994, 1997). Consistent with these observations, application of methyl jasmonate (MeJA) to cell cultures of *N. tabacum* produced rapid and substantial increases in *PMT* transcript levels (Imanishi *et al*., 1998; Shoji *et al*., 2000a; Winz and Baldwin, 2001), as well as increases in transcripts encoding ornithine decarboxylase (ODC), *S*-adenosylmethionine synthase (SAMS), and quinolinate phosphoribosyltransferase (QPTase) (Imanishi *et al*., 1998a). Subsequently, it has been shown that the MeJA-induced expression of PMT in *N. sylvestris* could be repressed by ethylene (Shoji *et al*., 2000a,b; Winz and Baldwin 2001). In addition, experiments using *PMT* gene cosuppression and antisense regulation in transgenic tobacco plants have shown that reduction of *PMT* transcript levels leads to reduced nicotine and total alkaloid levels (Sato *et al*., 2001; Voelckel *et al*., 2001) and increases in polyamine content (Sato *et al*., 2001), further indicating the important role of PMT.

At least two independent genetic loci (Legg *et al*., 1969; Legg and Collins, 1971), designated *A (Nic1)* and B (*Nic2)* (Hibi *et al*., 1994) operate synergistically to control plant alkaloid content, with mutations within these genes resulting in plants with reduced levels of nicotine and total leaf alkaloids (wild-type > *nic1* mutant > *nic2* mutant > *nic1 nic2* double mutant) (Legg *et al*., 1969; Legg and Collins, 1971). That these loci directly control PMT expression is indicated by the fact that topping-induced increases in PMT gene expression occur in wild type (AABB) plants, but not in *nic1 nic2* (aabb) double mutant in (Hibi *et al*., 1994; Riechers and Timko, 1999).

A cDNA for *PMT* was first isolated by differential screening between the cultured roots of high- and low-alkaloid varieties of *N. tabacum*, Burley 21 and LA Burley 21, respectively (Hibi *et al*., 1994). As part of a broader study to determine the factors regulating PMT expression in tobacco, we previously reported the characterization of the nuclear gene family encoding PMT in cultivated tobacco, *N. tabacum* and described the differential expression of its five members designated *NtPMT1a*, *NtPMT1b*, *NtPMT2*, *NtPMT3*, and *NtPMT4* (Riechers and Timko, 1999). Transgenic expression studies involving the three *N. sylvestris* PMT genes showed that as little as 0.25 kb of the *NsPMT1*, *NsPMT2* or *NsPMT3* gene promoter region is sufficient to confer cell-type specific expression in *N. sylvestris* roots and MeJA responsiveness (Shoji *et al*., 2000a). Recently, Sachan and Falcone (2002) reported that a 627-bp region upstream from the ATG start site of the *NtPMT3* genes was capable of directing high levels of GUS expression in the roots of transgenic tobacco plants, and low levels of GUS expression at wound sites in tobacco leaves. However, in both of these studies no specific transcriptional elements were identified. Here, we report a detailed characterization of the transcription regulatory properties of four different members of the *N. tabacum PMT* (*NtPMT*) gene family and analyze the differential response of each of the *NtPMT* gene promoters to various phytohormone signals (i.e., MeJA, AUX, and ETH). In addition, we identify a MeJA responsive elements in the *NtPMT1a* promoter and show that synthetic elements based upon its core sequence function in directing phytohormone response. Our results are discussed in terms of cross-talk among competing signal transduction pathways regulating alkaloid biosynthesis pathways in tobacco.

Materials and methods

Plant cell culture

N. tabacum cv. Bright Yellow-2 (BY-2) cell suspension cultures were grown in Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose and 0.2 mg/l 2,4-dichlorophenyoxyacetic acid (2,4-D), pH 5.8, as described by An (1985). Cell suspensions were subcultured into fresh media every 7 days or at times noted otherwise in the text.

GUS reporter gene construction

To ascertain the transcriptional regulatory activities of the various tobacco *NtPMT* gene promoters, chimeric transgenes were constructed in which various lengths of the transcriptional regulatory regions 5′ to the coding sequences of the *NtPMT1a, NtPMT2, NtPMT3, and NtPMT4* (Riechers and Timko, 1999) were fused to the coding sequences of the *β*-glucuronidase (*GUS)* gene (Jefferson *et al*., 1987) contained in the binary vector pBI101. PCR amplification was carried out using standard protocols and sequence-specific primers for each *NtPMT* promoter into which *Hin*dIII and *Xba*I restriction sites were introduced at the 5′- and 3′-ends, respectively, to facilitate subsequent cloning. Unless otherwise noted, the complete 5′- untranslated region (UTR) of the respective *NtPMT* transcripts were included in the promoter-GUS constructs. Constructs are named based upon the distance of the 5′ -end-point nucleotide.

For detailed analysis of the transcriptional regulatory regions in the *NtPMT1a* promoter, DNA fragments encompassing nucleotides −201 to −36 bp and −163 to −90 bp of the *NtPMT1a* gene promoter were generated by PCR amplification using sequence-specific primers. The resulting amplification products were fused to the −46 bp CaMV 35S gene promoter and the 5′ -UTR of Tobacco Etch Virus (TEV) contained in the binary vector pBI101 (Skinner and Timko, 1999) to form the transgenes designated Nt*PMT1a* -201/-36 and Nt*PMT1a* -163/-90. *E. coli* DH5α cells containing the

various *NtPMT* promoter-GUS transgenes were grown at 37°C in LB medium.

Construction of a synthetic G-box tetramer and site-directed mutagenesis of NtPMT1a *promoter*

A single-stranded synthetic oligonucleotide (5′-AAGTCGACTGCACGTTGTAATATGCA-CGTTGTAATATGCACGTTGTAATATGCA-CGTTGTAATAAAGCTTCG-3′) containing four tandem copies of the G-box sequence (TGCA-CGTTGT) found in the *NtPMT1a* gene was synthesized chemically. Two 17 bp synthetic oligonucleotides (5′-end: 5′-CAACGTGCAGT-CGACTT-3′; 3′-end: 5′-CGAAGCTTTATTA-CAAC-3′) complementary to the 5′ and 3′ ends of the above single-stranded DNA were annealed to it and the partially double-stranded fragment ligated into the *Sma*I site of pUC19. The resulting plasmid was transformed into *E. coli* DH5α to allow for the natural repair system of the cells to create a complete double-stranded DNA fragment. The tetrameric G-box containing fragment (*4 X syn G-box*) was excised using *Xba*I and *Hind*III and ligated to the 5′-end of the CaMV 35S −46 bp promoter or *NtPMT1a* -83 promoter fragment in the plant binary vector pBI101.

For site-directed mutagenesis, the −111 bp fragment of *NtPMT1a* was cloned into pBSK + vector. For each mutant constructed, two complimentary oligonucleotides containing the mutation at the target sites and flanked by unmodified nucleotide sequences were designed and synthesized. Mutagenesis was carried out using the Quick Change Site-directed Mutagenesis kit (Stratagene) and *pfu* Turbo DNA polymerase according to the manufacturer's protocols. The mutagenized DNA templates were digested with *Dpn*I and the circular nicked dsDNA transformed into DH5α cells. The modified promoter fragments were cut from the pBSK-based plasmids and cloned into pBI101.

Agrobacterium*-mediated transformation of BY-2 and phytohormone induction experiments*

Plasmids encoding the *NtPMT* promoter-GUS transgenes were transformed into *Agrobacterium tumefaciens* strain EH105 and the transformed cells grown at 28 °C in YEB medium as previously described (An, 1985). *Agrobacterium*-mediated transformation of BY-2 cells was carried out as described by An (1985). Transformants were selected on the solid MS medium containing 250 mg/l kanamycin sulfate (kan) and 500 mg/l cefotaxime (cef). Several hundred individual resistant calli were selected for each construct through at least two round of replating, and ultimately about 50 individual calli were re-introduced into MS liquid medium with 100 mg/l kan and 100 mg/l cef.

MeJA treatment was carried out on 4-day-old cultures of transformed BY-2 cells. MeJA was added at a final concentration of 100 μM as an aqueous suspension. ETH treatment was carried out by adding 100 μM ethaphon into 4-day-old suspension cultures. In experiments testing the interaction between MeJA and ETH, the phytohormones were added to the 4-day-old cultures simultaneously.

To observe the effect of AUX on *NtPMT* gene expression, transgenic BY-2 cells were grown on MS medium containing a low level of 2,4-D (0.2 mg/l) for 3 days. The cells were then transferred into either 2,4-D free medium or medium containing a high concentration (1 mg/l) of 2,4-D. In experiments testing the interaction between MeJA and AUX, MeJA was added to the medium 24 h after transfer to 2,4-D free medium or medium containing 1 mg/l 2,4-D. The BY-2 cells were harvested and analyzed for GUS expression 24 h later.

Treated and untreated transgenic BY-2 cells were harvested at various times after phytohormone treatment by vacuum filtration and the cells were immediately assayed for GUS activity according to the methods described by Jefferson *et al.* (1987). For statistical analysis, each experiment was repeated a minimum of three times.

Nucleic acid sequencing and analysis

Nucleotide sequencing was carried out using an ABI 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA) using double-stranded plasmid DNA templates prepared utilizing the Qiaprep Spin Plasmid Kit (Qiagen USA, Valencia, CA). Sequence alignments were carried out using the PILEUP program (Genetics Computer Group Sequence Analysis package, Version 9.0 (GCG, University of Wisconsin, Madison, WI). Analysis of *cis*-acting regulatory elements was carried out

using PlantCARE and other available databases capable of identifying potential *cis*-acting regulatory elements in plant promoters (Hehl and Windgender, 2001).

Real time PCR

Four independently derived transgenic BY-2 cell lines expressing the various *NtPMT* promoter-GUS constructs were selected and used in these experiments. Control (untreated) and phytohormone- treated cell cultures were harvested as described above and total RNA was isolated using the Qiagen Plant RNeasy kit according to the manufacturer's protocol. RNase-Free DNase was added to remove any possible genomic DNA contamination. RNA quantities were measured with Molecular Probes RiboGreen^{TM} RNA Quantitation Kit. Synthesis of first-strand cDNA was performed by using 1 μg total RNA in each reaction. Quantification of gene transcript levels was performed on a Bio-Rad iCycler iQ^{TM} Real-time Detection System. The melt curve technique was used to ensure that single reaction products were being detected and these data were confirmed by agarose gel electrophoretic analysis. Because an unspecific DNA dye, SYBR-Green, was used as the reporter, a melt curve technique was adopted to analyze the specificity of PCR products (as described in the manufacturer's handbook).

Mapping the transcription start site using 5′-RACE and RNase protection assays

To map the 5′-end of the PMT transcript, 5′ -RACE was carried out using total RNA isolated from BY-2 cells 12 h after treatment with MeJA using the Qiagen Plant RNeasy kit according to the manufacturer's protocol. Total RNA (1 μg) was reverse-transcribed with Superscript II RNase H$^-$ reverse transcriptase at 42–48 °C using a synthetic primer (GSP1; 5′-AATAGTAACTTCTC-AAC-3′), that recognizes exon 2 of the various *PMT* genes. After first-strand cDNA synthesis, the template RNA was removed by RNase H treatment, and the cDNA was purified using a Qiagen PCR Clean kit. The cDNA was then tailed at the 3′-end using a poly(dC) TdT tailing reaction. Nested PCR was then performed using GSP1 and AAP (5′ -RACE Abridged Anchor Primer, Invitrogen) in the first amplification reaction and GSP2 (5′-CAACCTTAAGTGAGAATGC-3′), a PMT exon 2 specific primer, and AUAP (Abridged Universal Amplification Primer, Invitrogen) in the second amplification reaction. The products of the nested PCR reactions were separated by agarose gel electrophoresis, purified from the gel using the Qiagen gel extraction kit, and their nucleotide sequence determined.

RNase protection assays (RPA) were performed using the Ambion RNA probe synthesis kit and RPA Ø kit (Ambion, Austin, TX). A radiolabeled antisense RNA probe was synthesized by *in vitro* transcription using the *NtPMT1a* 5′ UTR sequence ranging from -181 to -13 nucleotides upstream of the AUG as the template, which was previously cloned into pBSK. The labeled probes were hybridized with total RNA from MeJA treated BY-2 cells. Then digestion with RNase was performed followed by analysis of the protected RNAs on denaturing acrylamide gels. Mouse α-actin gene and the mouse RNA provided by RPA Ø kit served as the positive control. Decade^{TM} Markers (10–100 nucleotides in length) were used to determine the size of the products.

Results

MeJA induction of PMT gene expression in BY-2 cells

We have previously shown that *NtPMT* gene expression is up-regulated in *N. tabacum* roots in response to decapitation (removal of the top 1/3 of the plant) and that the expression kinetics and levels of transcript accumulation differs for each of the five *N. tabacum NtPMT* gene family members (Riechers and Timko, 1999). It has also been shown that *NtPMT* gene expression is induced in BY-2 cells by MeJA-treatment (Hibi *et al.*, 1994; Imanishi *et al.*, 1998a, b), with the maximum levels of *NtPMT* transcript observed approximately 5–12 h after treatment with a decrease in the expression level thereafter. To further explore the nature of the transcriptional regulatory sequences and factors controlling *NtPMT* gene expression in tobacco, a series of reporter gene constructs were generated in which the 5′-flanking regions and complete 5′-UTR of the *NtPMT1a*, *NtPMT2*, *NtPMT3*, and *NtPMT4* genes were fused to the

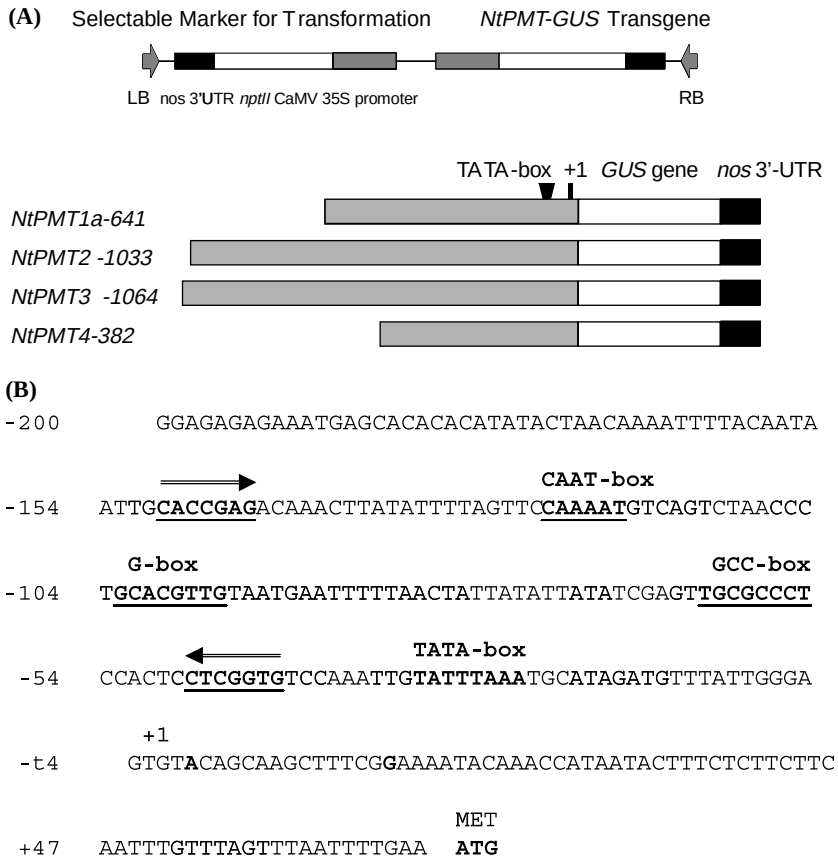


Figure 1. Analysis of the transcriptional control regions of the tobacco *NtPMT* genes. (A) Schematic representation of the *NtPMT::GUS* transgene constructs used for *Agrobacterium*-mediated transformation of tobacco BY-2 cells. The general structure of the relevant portion of the binary vector used for cell transformation is shown. The numbers to the left indicate the position relative to the start site of transcription (designated as +1). The TATA-box is indicated. GUS, *β*-glucuronidase gene coding region; LB, left border; nptII, neomycin phosphotransferase; nos, nopaline synthase; RB, right border; UTR, untranslated region. (B) Partial nucleotide sequence of the *NtPMT1a* gene promoter showing the location of the various *cis*-acting regulatory elements investigated in these studies. The sequences of interest are given in bold and underlined. The numbers to the left indicate the position relative to the start site of transcription (designated as +1). Arrows indicate the left (IR-L) and right (IR-R) halves of an inverted repeat.

coding region of the *β*-glucuronidase (GUS) gene in the binary transformation vector pBI101 (Figure 1A). The length of the 5′-flanking region available for each of the four *NtPMT* gene promoters differs (Riechers and Timko, 1999), with the *NtPMT1a*, *NtPMT2*, *NtPMT3*, and *NtPMT4* gene promoters extending 711, 1120, 1134 and 469 bp, respectively, upstream of the ATG codon used for translation initiation.

Comparison of the genomic DNA sequences to the longest available cDNA sequence previously set the transcription initiation site 57 bp upstream of the ATG start codon in *NtPMT1a* (Riechers and Timko, 1999). Prior to the construction of *NtPMT* promoter::GUS transgenes used in this present study, 5′-RACE and RNase protection experiments were carried out to confirm the start site of transcription. To facilitate mapping of the start site, BY-2 cells were subjected to MeJA treatment in order to enrich for transcripts encoding PMT used in our analysis. Consistently, a single size fragment was observed following agarose gel electrophoresis of the 5′-RACE amplification products. DNA sequence analysis of these fragments showed that they were all derived from the *NtPMT1a* gene. This is not unexpected, since the *NtPMT1a* gene is by far the most highly expressed of the *NtPMT* genes (Riechers and Timko, 1999). Comparison of the nucleotide sequence of the 5′-RACE product with the previously available

cDNA sequence showed that the 5′-RACE product contained an additional 13 bp at the 5′ end, placing the start site for transcription 70 bp upstream of the ATG translation initiation codon. Conducting the first-stand cDNA synthesis at higher temperatures or altering the source of enzyme used for reverse transcription to overcome the possibility of pre-terminated reverse transcripts caused by RNA secondary structure, failed to yield amplification products of different lengths. RNase protection assays, conducted using a radioactively-labeled probe extending from 13 to 181 bp upstream of the ATG codon in *NtPMT1a* yielded two protected fragments following RNase treatment, one 57 bp in length and the other 53 bp in length. The longest of these fragments is consistent with a transcription start site 70 bp upstream of the ATG codon. The radioactively-labeled probe used in these studies was based on the *NtPMT1a* sequence and capable of cross-hybridizing with the transcripts derived from the other *PMT* genes (i.e., *NtPMT2, NtPMT3,* and *NtPMT4*). However, because of multiple mismatches, these transcripts would only have produced protected fragments less than 30 bp in length. Based on these results, the start site for transcription in the *NtPMT1a* gene is located 70 bp upstream of the ATG translation initiation codon. Consistent with our previous predictions (Riechers and Timko, 1999) the sequence element "TATTTAAA" located 32 bp upstream of the defined transcription initiation site is likely the functional TATA box (Figure 1B). A second potential TATA-box is observed in a TA-rich region located between nucleotides −80 and −68 bp. We found no evidence indicating that this element sets transcription initiation, but as shown below, this region is necessary for MeJA-responsiveness.

To determine the transcriptional regulatory properties of the various *NtPMT* gene promoters, the various *NtPMT* promoter::GUS transgenes

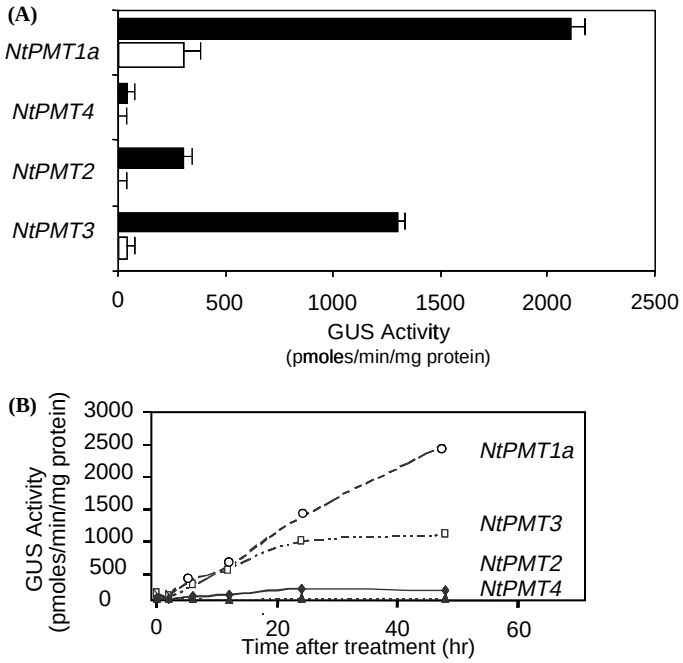


Figure 2. Expression analysis of *NtPMT::GUS* transgenes in transformed BY-2 cells. Cell suspensions of transformed BY-2 cells were grown in liquid MS medium for 4 days as described in the Materials and Methods at which point MeJA (100 μM) was added. Treated and untreated transgenic BY-2 cells were harvested at various times after phytohormone treatment by vacuum filtration and the cells were immediately assayed fluorometrically for GUS activity. (A) Relative MeJA-responsiveness of the four different *NtPMT::GUS* constructs in BY-2 cells harvested 24 h after treatment with MeJA (100 μM). For statistical analysis, each experiment was repeated a minimum of three times with independently derived transformed lines. Differences between treated and untreated samples were significant at $P \leq 0.05$. (B) Time course of GUS expression in MeJA-treated transgenic BY-2 cells lines expressing the four different *NtPMT::GUS* constructs. Points represent the mean of two experiments, using pooled samples of independently derived transformed lines expressing each construct.

were transformed into BY-2 cell using *Agrobacterium*-mediated cell transformation, and the resulting transgenic cell lines analyzed for GUS activity before and after treatment with various phytohormones. As shown in Figure 2A, all four *NtPMT* gene promoters were capable of directing MeJA-induced GUS expression. Consistent with our previous studies (Riechers and Timko, 1999), the extent of the induction varied dramatically among gene family members. On average, the *NtPMT2::GUS* and *NtPMT4::GUS* transgenes showed a 2- to 3-fold increase in GUS activity 24 h after MeJA treatment, whereas the *NtPMT3::GUS* and *NtPMT1a::GUS* transgenes showed a 10- to 15-fold increase in GUS activity. Interestingly, while GUS activity directed by the *NtPMT2::GUS* and *NtPMT4::GUS* transgenes appeared to plateau 12–24 h after MeJA treatment, GUS activity directed by the *NtPMT3::GUS* and NtPMT1a*::GUS* transgenes was still increasing 48 h post-treatment (Figure 2B). Carrying out MeJA treatment in the presence of 1% DMSO to increase absorption of the phytohormone by the BY-2 cells did not significantly affect *NtPMT* gene expression.

To determine whether GUS enzyme levels accurately reflected the levels of GUS transcripts arising from each transgene and induction of endogenous *PMT* gene expression, quantitative real-time PCR was performed using total RNA isolated from the various transgenic BY-2 cell lines. As shown in Figure 3A, prior to MeJA-treatment, transcripts encoding PMT were barely detectable in the cells. However, following MeJA treatment, the levels of total *PMT* transcripts present increased significantly and accumulated to approximately the same level in each transgenic line by 24 h post-treatment. Similarly, prior to MeJA treatment, only low copy numbers of GUS transcripts derived from the various *NtPMT::GUS* transgenes were present in the different transgenic BY-2 cell lines. Following MeJA treatment, the number of GUS transcripts detected in the various transformed cell lines increased significantly, with the number of transcripts detected reflecting the previously observed increases in GUS

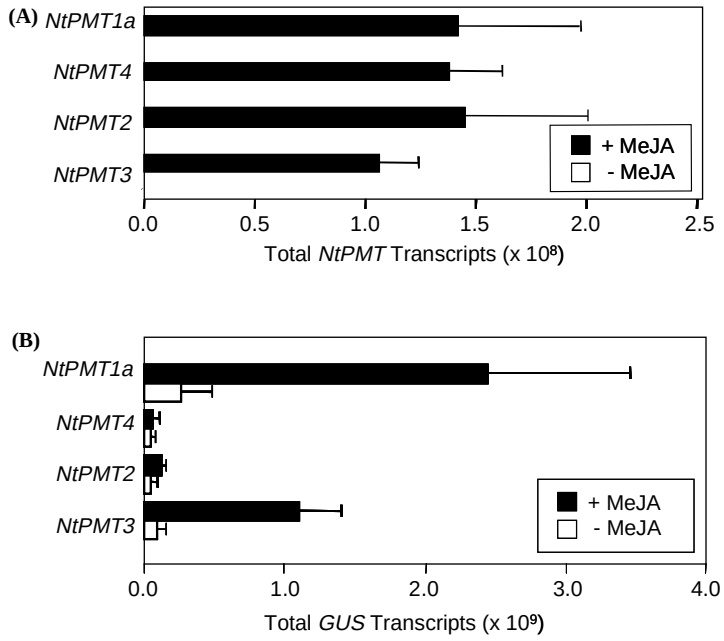


Figure 3. Real time-PCR analysis of endogenous *NtPMT* and *GUS* transcript levels in transformed BY-2 cells lines. Total RNA was isolated from control (untreated) and MeJA-treated BY-2 cell cultures 24 h after treatment and quantification of gene transcript levels in 30 ng of RNA was carried out using a Bio-Rad iCycler iQ^{TM} Real-time Detection System as described in the Materials and Methods. A melt curve technique was adopted to analyze the specificity of PCR products as described in the manufacturer's handbook. (A) Total number of *NtPMT* transcripts present in transgenic BY-2 cells before and following MeJA treatment. Transcript levels in control (minus MeJA treatment) were below 10^7. (B) Total number of *GUS* transcripts present in transgenic BY−2 cells expressing the various *NtPMT::GUS* transgene constructs before and following MeJA treatment. Differences between treated and untreated samples were significant at $P \leq 0.05$, except for *NtPMT4* which was significant at $P \leq 0.1$.

activity directed by the respective *NtPMT* promoters (Figure 3B).

Response of the various NtPMT *promoters to other phytohormones*

Previous investigators have shown that both ETH and AUX affect nicotine formation and gene expression in tobacco plants and cultured cells (Hibi *et al*., 1994; Imanishi *et al*., 2000a; Shoji *et al*., 2000a, b). To gain further insight into the interaction among different phytohormones in controlling the expression of the various *NtPMT* genes, the effects of ETH and AUX treatment on the expression of the various *NtPMT::GUS* transgenes and the influence of these phytohormones on MeJA-induced gene expression were examined. As shown in Figure 4A, transgenic BY-2 cells expressing the various *NtPMT::GUS* transgenes show low levels of GUS activity directed by the various *NtPMT* promoters. Treatment of these transgenic BY-2 cells lines with ethephon did not result in a significant change in GUS activity, indicating that ETH alone does not affect *NtPMT* gene expression. In contrast, the simultaneous application of ethephon and MeJA resulted in an ETH induced suppression of the MeJA-induced expression of all four *NtPMT::GUS* transgenes. The inhibitory effect of ethephon treatment disappeared in the presence of $AgNO_3$ (Figure 4B), consistent with the previously reported role of silver cations as inhibitors of ethylene perception.

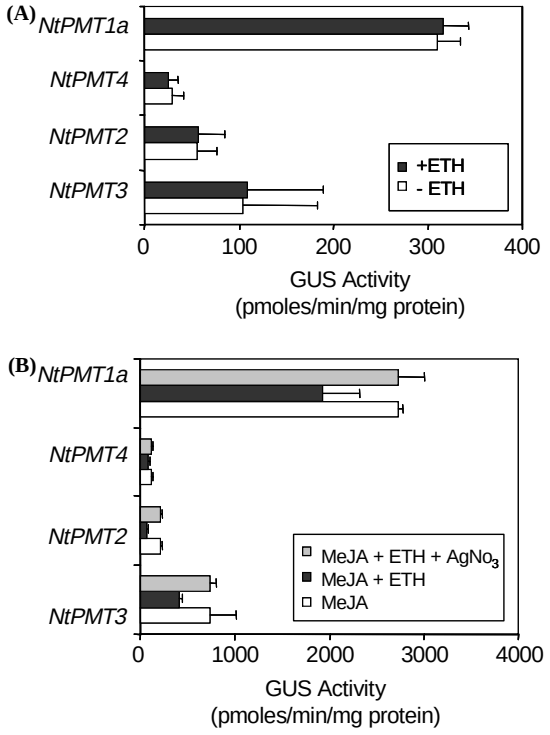


Figure 4. Effect of ethylene and ethylene inhibitors on the expression of *NtPMT::GUS* transgenes in transformed BY-2 cells with and without MeJA treatment. (A) Cell suspensions of transformed BY-2 cells were grown in MS medium for 4 days at which point 100 μM ethaphon was added. GUS activity was 24 h after treatment. Differences between treated and untreated samples were significant at $P \leq 0.05$. (B) To examine the effect of (ETH) and ethylene inhibitors on the MeJA-induced *NtPMT::GUS* genes expression, cell suspensions of transformed BY-2 cells were grown in MS medium for 4 days. The cells were then treated with either MeJA (100 μM), MeJA (100 μM) and ethaphon (100 μM), or MeJA (100 μM), ethaphon (100 μM) , and $AgNO_3$ (1.2 μM). as an aqueous suspension. Treated and untreated transgenic BY-2 cells were harvested 24 h after treatment and the cells were immediately assayed fluorometrically for GUS activity. For statistical analysis, each experiment was repeated a minimum of three times with independently derived transformed lines. Differences between ETH treated and untreated samples were significant at $P \leq 0.05$.

Hibi *et al*. (1994) reported that PMT expression was inhibited by high levels of AUX in cell culture medium. To observe the effects of AUX on the various *NtPMT::GUS* transgenes, transgenic BY-2 cells expressing the various constructs were grown on MS medium containing a low level of 2,4-D (0.2 mg/l) for 3 days. The cells were then transferred into either 2,4-D free medium or medium containing a high concentration (1 mg/l) of 2,4-D. As shown in Figure 5A, transfer to media containing high AUX concentration AUX resulted in an approximately 50% repression *NtPMT::GUS* expression.

In experiments testing the interaction between MeJA and AUX, MeJA was added to the medium 24 h after transfer to 2,4-D free medium or medium containing 1 mg/l 2,4-D. The BY-2 cells were harvested and analyzed for GUS expression 24 h later. As shown in Figure 5B, high AUX levels in the medium decreased the extent of stimulation of MeJA-induced *NtPMT::GUS* expression. Stimulation of ETH biosynthesis is a common response when AUX is applied to some sensitive species (Hansen and Grossmann, 2000). Thus, in principal, it is possible that elevated AUX triggered ETH biosynthesis leading to a down-regulation of *PMT* gene expression. To test this possibility, cells were

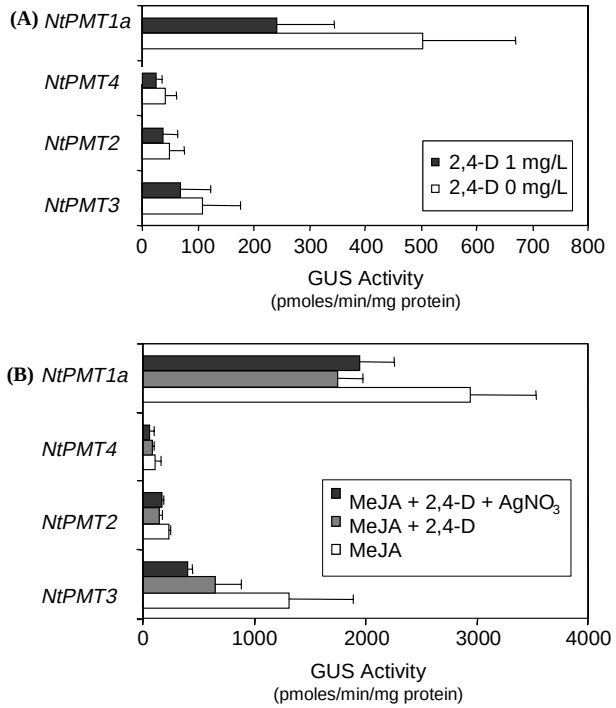


Figure 5. Effect of auxin on the expression of *NtPMT::GUS* transgenes in transformed BY-2 cells with and without MeJA treatment. (A) To observe the effect of AUX treatment on *NtPMT* gene expression, transgenic BY-2 cells were grown on MS medium containing 0.2 mg/l 2,4-D for 3 days. The cells were then transferred to 2,4-D free medium (0 mg/l) or medium containing a high concentration (1 mg/l) of 2,4-D. GUS activity was measured 24 h after transfer. Differences between samples were significant at $P \leq 0.05$, except for *NtPMT2* which was only significant at $P \leq 0.1$. (B) To examine the effect of auxin on the MeJA-induced *NtPMT::GUS* genes expression, transgenic BY-2 cells were grown on MS medium containing 0.2 mg/l 2,4-D for 3 days. The cells were then transferred to 2,4-D free medium (0 mg/l) or medium containing a high concentration (1 mg/l) of 2,4-D. After 24 h, MeJA (100 μM) or MeJA (100 μM) and $AgNO_3$ (1.2 μM) were added to the cultures. Treated and untreated cells were harvested 24 h after treatment and the cells were immediately assayed fluorometrically for GUS activity. For statistical analysis, each experiment was repeated a minimum of three times with independently derived transformed lines. Differences between samples were significant at $P \leq 0.05$.

treated with silver cation to block the ethylene signal pathway. As shown in Figure 5B, AUX had the same negative effect on *PMT* gene expression in the presence or absence of $AgNO_3$ indicating that AUX and ETH likely worked through separate pathways to bring about regulation of *PMT* gene expression. The observed AUX repression of MeJA-induced *PMT* expression measured using the various *NtPMT::GUS* transgenes in transformed BY-2 cultures was confirmed by carrying out real-time PCR analysis of endogenous *PMT* transcipt levels (data not shown).

Identification of the MeJA responsive element in the NtPMT1a *promoter*

To determine the regions of the *NtPMT1a* promoter involved in phytohormone regulated transcriptional control, a series of 5′-end deletion constructs were made (Figure 6A). Gross scale deletion analysis showed that as little as 200 bp upstream of the transcription initiation site are sufficient to confer MeJA-responsiveness, however, maximal expression is achieved when the full approximately 650 bp *NtPMT1a* promoter is used. Interestingly, the MeJA-induced GUS expression driven by the *NtPMT1A −201* promoter was only moderately suppressed by ETH ($P \leq 0.1$), and not significantly affected by AUX and ETH, whereas MeJA-induced expression of the *NtPMT1A −441::GUS* transgene was responsive to both phytohormones (Figure 6B). This suggests that the AUX regulatory site must lie in the promoter region between −400 and −200 bp, whereas the ETH regulatory site resides relatively close (within 200 bp) to the transcription start site (Figure 6B).

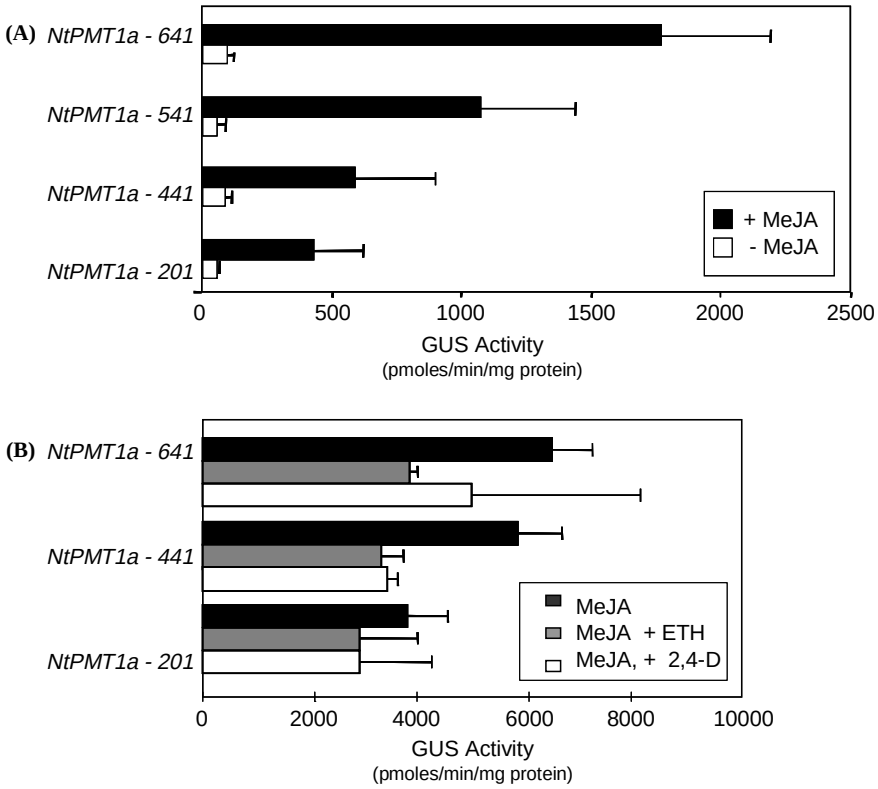
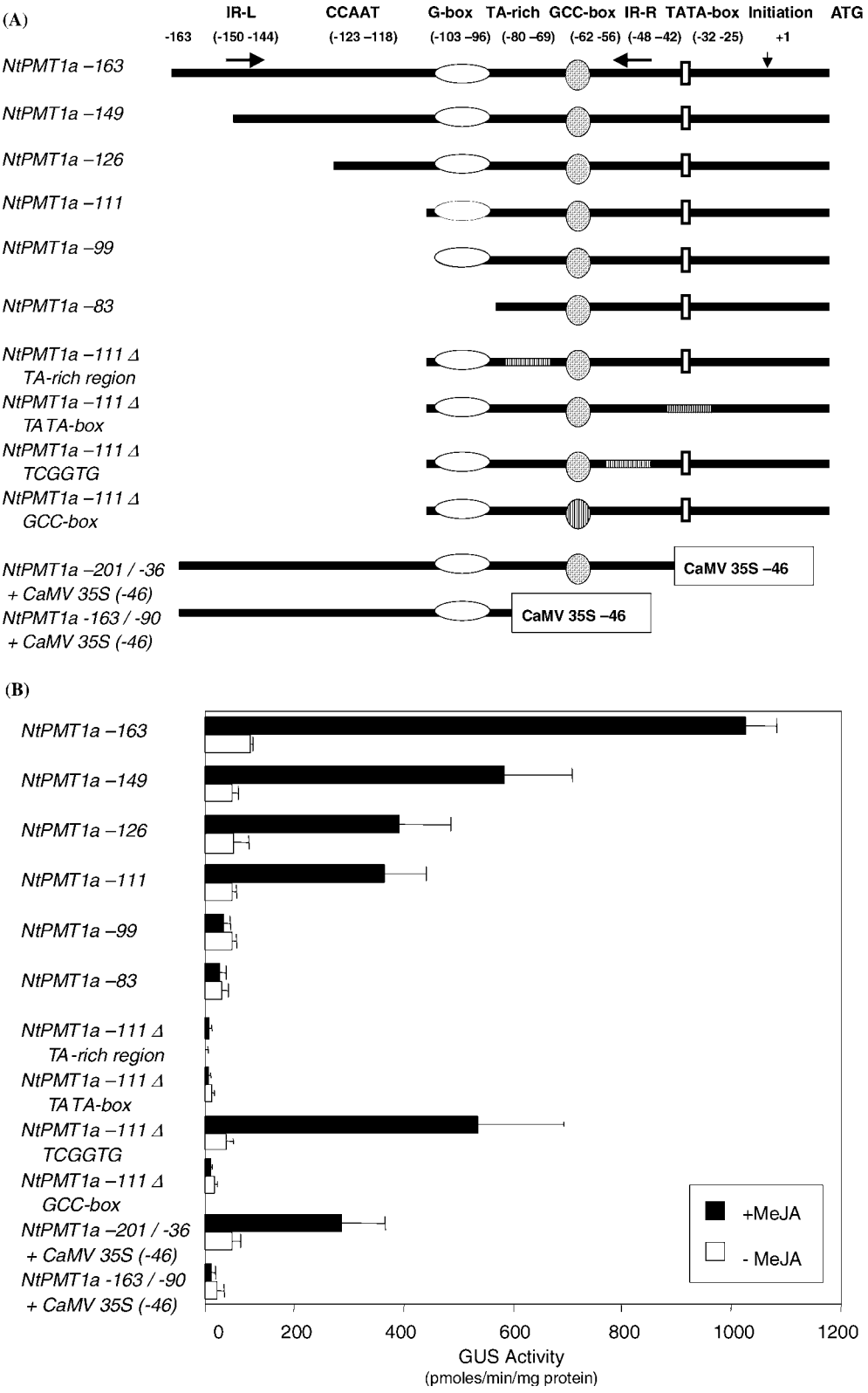


Figure 6. Deletion analysis of the *NtPMT1a* promoter and analysis of phytohormone responsiveness. The effects of deletions at the 5'-end of the *NtPMT1a* promoter were assayed for their ability to direct MeJA-inducible GUS expression in the presence or absence of other phytohormones. Treated and untreated transgenic BY-2 cells were harvested at various times after phytohormone treatment by vacuum filtration and the cells were immediately assayed fluorometrically for GUS activity. For statistical analysis, each experiment was repeated a minimum of three times with independently derived transformed lines. (A) Relative MeJA-responsiveness of four different *NtPMT1a::GUS* promoter deletion constructs in BY-2 cells harvested 24 h after treatment with MeJA (100 μM). The numbers to the left indicate the 5′-end of the promoter relative to the start site of transcription (designated as +1). Differences between treated and untreated samples were significant at $P \leq 0.05$. (B) To examine the effect of AUX or ETH treatment on the MeJA-induced expression of the various *NtPMT1a::GUS* promoter deletion constructs, transgenic BY-2 cells were grown on MS medium containing 0.2 mg/l 2,4-D for 4 days. The cells were transferred to fresh medium containing MeJA (100 μM), MeJA (100 μM) and 2,4-D (1 mg/l), or MeJA (100 μM) and ethaphon (100 μM). BY-2 cells were harvested 24 h after treatment and the cells were immediately assayed fluorometrically for GUS activity. For statistical analysis, each experiment was repeated a minimum of three times with independently derived transformed lines. The response to ETH treatment was significant at $P \leq 0.05$, except for *NtPMT1a -201*. The response to AUX treatment was not significant for *NtPMT1a -201*.

To identify which particular nucleotide sequences are responsible for this regulation, a more discrete series of 5′-end deletions were generated and analyzed for their ability to direct MeJA-induced expression (Figure 7). These studies revealed that deletion beyond −111 bp resulted in loss of MeJA-induced GUS expression and that a G-box (GCACGTTG) located at −103 to −96 bp was essential for MeJA-induced expression in the *NtPMT1a* promoter. Within the *NtPMT1a −111* promoter, two other sequence elements were also observed, a GCC-motif (TGCGCCT) similar to the JA- and elicitor responsive element (JERE) identified by Menke *et al*. (1999) involved in the regulation of the terpenoid indole alkaloid (TIA) biosynthetic gene strictosidine synthase (*STR*) and an inverted repeat sequence (CACC-GAG.....CTCGGTG). To analyze the importance of these sequences in *NtPMT1a* regulation, site-directed mutagenesis was carried out to change or delete 4–6 bp in the core sequence of each motifs found within the *NtPMT1A −111* promoter and the resulting constructs analyzed for their ability to direct MeJA-inducible GUS expression. As shown in Figure 7B, deletion of the GCC-motif (TGCGCC) resulted in a complete loss of GUS


(A)
IR-L
CCAAT
G-box
TA-rich
GCC-box
IR-R
TATA-box
Initiation
ATG
-163
(-150 -144)
(-123 –118)
(-103 –96)
(-80 –69)
(-62 -56)
(-48 –42)
(-32 -25)
+1
NtPMT1a –163
NtPMT1a –149
NtPMT1a –126
NtPMT1a –111
NtPMT1a –99
NtPMT1a –83
NtPMT1a –111 Δ TA-rich region
NtPMT1a –111 Δ TA TA-box
NtPMT1a –111 Δ TCGGTG
NtPMT1a –111 Δ GCC-box
NtPMT1a –201 / -36 + CaMV 35S (-46)
NtPMT1a -163 / -90 + CaMV 35S (-46)
CaMV 35S –46
CaMV 35S –46
(B)
NtPMT1a –163
NtPMT1a –149
NtPMT1a –126
NtPMT1a –111
NtPMT1a –99
NtPMT1a –83
NtPMT1a –111 Δ TA-rich region
NtPMT1a –111 Δ TA TA-box
NtPMT1a –111 Δ TCGGTG
NtPMT1a –111 Δ GCC-box
NtPMT1a –201 / -36 + CaMV 35S (-46)
NtPMT1a -163 / -90 + CaMV 35S (-46)
+MeJA
- MeJA
0
200
400
600
800
1000
1200
GUS Activity
(pmoles/min/mg protein)

expression, even though the G-box remained intact. Similarly, mutation of the sequence TATATT -> GACCG at positions −78 to −73 bp within the TA-rich region located just upstream of the GCC-motif also resulted in a loss of MeJA-inducible expression. Mutation of the inverted repeat region (IR-R) (CTCGG -> AGT) had no effect on MeJA-inducible GUS expression. As might be expected, mutations within the functional TATA-box located at −32 bp (TATTA -> ACGT) completely abolished *GUS* gene expression.

To confirm that both an intact G-box and GCC-motif were necessary for MeJA-inducible expression, truncated promoter fragments, either containing both elements (*NtPMT1a* −201/−36) or missing the GCC-motif (*NtPMT1a*−163/−90), were fused to the minimal −46 bp promoter of the CaMV 35S gene and their expression analyzed. As found with the mutagenesis analysis, only the *NtPMT1a* −201/−36 promoter containing both regulatory elements was capable of directing MeJA-induced GUS expression.

To further explore the nature of the G-box in mediating MeJA responsiveness, a synthetic fragment (*4 X syn G-box*) was generated containing four identical repeats of the core sequence 5′-TGC**ACGT**TGT-3′. This synthetic fragment was fused to either the minimal *NtPMT1a* −83 promoter containing a GCC-motif and TATA-box, or to the minimal CaMV 35S −46 bp promoter that only contains a TATA-box, in order to test its ability to direct MeJA-responsiveness (Figure 8A). As shown in Figure 8B, the *4 X syn G-box* fragment conferred high level MeJA-inducibility onto the *NtPMT1a* −83 promoter, but not the −46 bp CaMV 35S promoter. Furthermore, the level of induction observed in the *NtPMT1a −83::GUS* construct was approximately 30-fold higher in MeJA treated cells than untreated cells, and approximately 10-fold higher than that observed with the *NtPMT1a −111::GUS* construct, the transgene having the minimal sized MeJA responsive *NtPMT* promoter. These results also indicate that the G-box requires the presence of the GCC-motif for function.

Discussion

The role of jasmonates in the inducible expression of genes involved in the biosynthesis of nicotine in tobacco and alkaloids in other plant species has been well documented. Previous studies of the *Nicotiana sylvestris NsPMT* gene promoters showed that a conserved 0.25 kb fragment derived from either of the three *NsPMTs* genes (*NsPMT1*, *NsPMT2*, or *NsPMT3*) is sufficient to confer cell-specific pattern of expression on *GUS* transgenes in *N. sylvestris* hairy root culture cells and to respond positively to MeJA treatment (Shoji *et al.*, 2000a). Similarly, Sachan and Falcone (2002) reported that a 627 bp region upstream from the ATG start site of the *NtPMT3* gene was capable of directing high levels of GUS expression in the roots of transgenic tobacco plants. A 1.7 kb fragment of the *NsPMT2* gene promoter fused to the *GUS* gene was reported to contain all of the regulatory information necessary to respond to MeJA and ETH (Shoji *et al.*, 2000b). However, none of these earlier studies identified specific regulatory elements responsible for mediating these effects. Here, we have demonstrated that the MeJA-induced expression of the *NtPMT* genes in tobacco involves two distinct *cis*-acting regulatory elements, a G-box (GCACGTTG) and a GCC-motif element homolog. Furthermore, we show a co-requirement for these elements in mediating the MeJA-inducible expression of the *NtPMT* genes of tobacco. We also demonstrate that a TA-rich region separating these elements is also important to allow their interaction.

Figure 7. Analysis of the transcriptional regulatory regions of the tobacco *NtPMT*1a gene promoter. (A) Shown is a schematic representation of the various promoter-*GUS* transgene constructs used to identify critical regulatory regions in the *NtPMT1a* promoter. The general structure of the relevant portion of the trangenes used for cell transformation is shown. The numbers indicate the position of the various regulatory elements relative to the start site of transcription (designated as +1). *NtPMT* promoter constructs are designated by the location of the terminal nucleotide. The location of mutations introduced into the various regulatory elements present in the *NTPMT1a -111* promoter are indicated by the stripes. Two constructs *NtPMT1a-201/-36 + CaMV 35S* (-46) and *NtPMT1a-163/-90 + CaMV 35S* (-46) are made using the minimal promoter fragment of the *CaMV 35S* gene which ends at −46 bp. (B) Relative MeJA-responsiveness of the various *NtPMT1a::GUS* and *NtPMT1a/CaMV 35S (-46)::GUS* transgenes assayed 24 h following treatment with 100 μM MeJA.

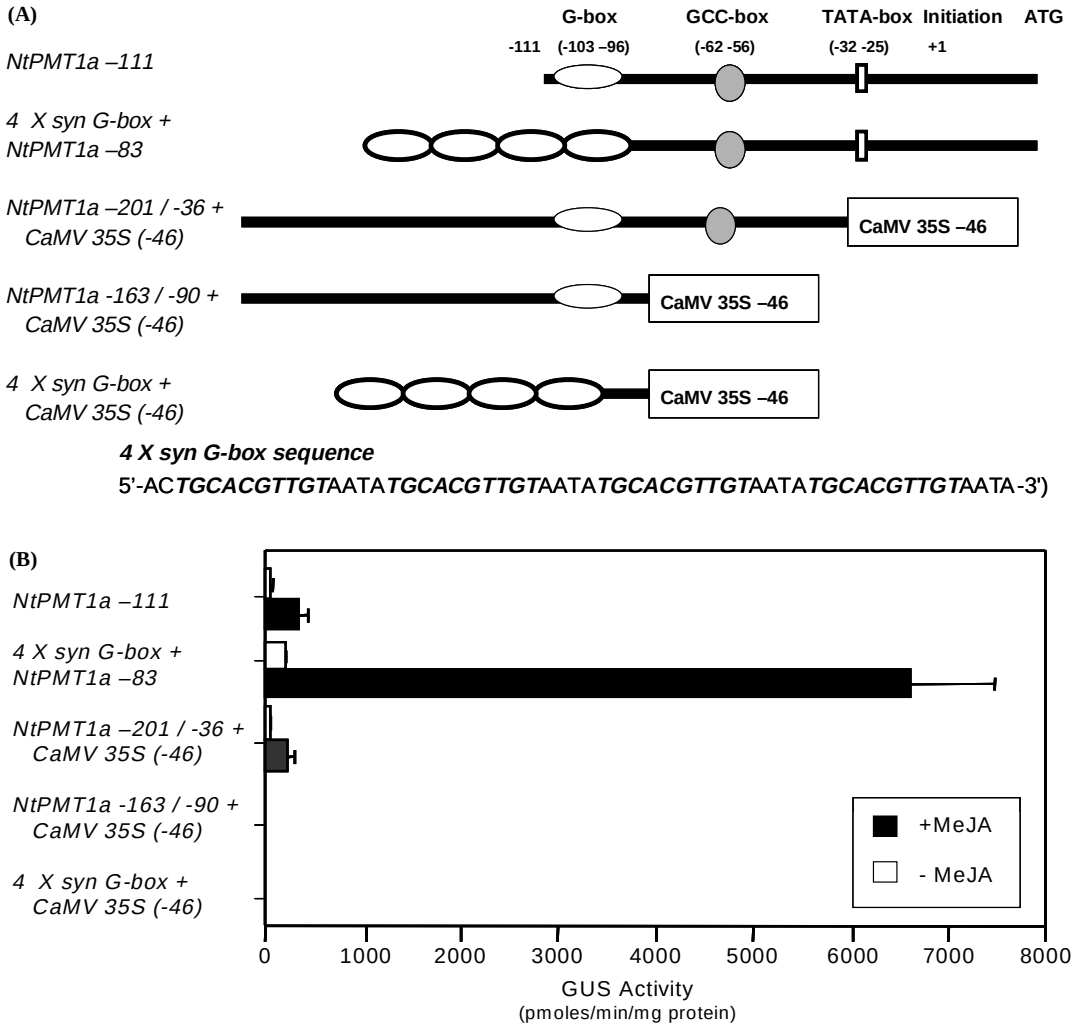


Figure 8. Interaction of G-box and other transcriptional regulatory regions of the tobacco *NtPMT*1a gene promoter. (A) Shown is a schematic representation of the various promoter-*GUS* transgene constructs used to examine the interaction of the G-box and other regulatory regions in the *NtPMT1a* promoter and the activity of a synthetic G-box tetramer. The general structure of the relevant portion of the trangenes used for cell transformation is shown. The numbers indicate the position of the various regulatory elements relative to the start site of transcription (designated as +1). *NtPMT* promoter constructs are designated by the location of the terminal nucleotide. The structure of the tetrameric sequence containing the G-box tetramer (large ovals; *4 X syn G-box*) is shown. The tetramer was fused to the 5′-end of the promoter in the *NTPMT1a -83::*GUS construct and the minimal promoter fragment of the *CaMV 35S* gene which ends at −46 bp. (B) Relative MeJA-responsiveness of the various *NtPMT1a::GUS* and *NtPMT1a/-46 CaMV 35S::GUS* transgenes containing the *4 X syn G-box* assayed 24 h following treatment with 100 μM MeJA.

Previous studies have implicated a number of different types of regulatory elements in conferring MeJA-responsivenss on plant promoters. One group shares sequences containing the motif TGACG (or its inverse CGTCA) as in the case of the *as*-1-type element in the glutathione S-transferase gene (Xiang *et al.*, 1996) or JASE1 and JASE2 elements in the *OPR1* (12-0xo-phytodienoic acid-10,11-reductase) gene (He and Gan, 2001). In some cases the CGTCA....TGACG palindromic motif is part of a longer inverted repeats, as in the case of the lipoxygenase 1 (*Lox*l) gene (Rouster *et al.*, 1997), potato cathepsin D inhibitor gene (Ishikawa *et al.*, 1994) and nopaline synthase (*nos*) gene (Kim *et al.*, 1993). Such TGACG containing elements have been previously identified as a binding site for the bZIP-typ of transactivating factors (Schindler *et al.*, 1992a).

Another common feature of MeJA-responsive promoters is the presence of a G-box (CACGTGG) or G-box-like motifs. For example, G-boxes have been identified in JA-responsive *pin2* gene promoter of potato (Kim *et al*., 1992), the *vspB* gene promoter of soybean (Mason *et al*., 1993), and the *Str* gene promoter of *Catharanthus roseus* (Ouwerkerk and Memelink, 1999). G-boxes are not unique to MeJA responsiveness. These elements have been identified in a large number and variety of plant gene promoters and their role in regulation of gene expression in response to light, anaerobiosis, and various phytohormones have been heavily investigated (Ishige *et al*., 1999; Siberil *et al*., 2001). At least two different classes of transcription factors bind specifically to G-boxes, basic leucine zipper (bZIP)-type proteins (Schindler *et al*., 1992b) and basic helix-loop-helix (bHLH) proteins (Buck and Atchley, 2003; Heim *et al*., 2003). G-box binding factors (GBFs) specifically implicated in mediating MeJA responsiveness have been identified (Menkens *et al*., 1995; Pasquali *et al*., 1999; Pré *et al*., 2000; Chatel *et al*., 2003). For example, studies of the *C. roseus Str* gene promoter led to the identification of several cDNAs encoding bHLH-type transcription factors that specifically bind a tetramerized 18 bp G-box containing fragment based on the G-box present in the *Str* gene promoter (Pré *et al*., 2000). One of these bHLH type factors, termed CrMYC1, was further analyzed for its binding activity and expression in *C. roseus* suspension cells (Chatel *et al*., 2003). Although CrMYC1 binds G-box sequences specifically and *Crmyc1* transcript levels are upregulated by elicitor and MeJA treatment, the increase in *Crmyc1* transcripts temporally trails observed increases in *Str* transcripts leading these investigators to suggest that CrMYC1 may not be directly involved in regulation of *Str* gene expression in response to MeJA.

The final class of elements found to mediate JA-responsiveness are the GCC-motif containing elements, like the JERE present in the *Catharanthus roseus Str* gene promoter (Menke *et al*., 1999; Ouwerkerk and Memelink, 1999). The *str* JERE is recognized by elicitor- and MeJA-responsive AP2/ERF-domain transcription factors (termed ORCAs) that specifically activate *Str* gene expression in response to these signals (Menke *et al*., 1999; van der Fits and Memelink, 2000; Memelink *et al*., 2001). ORCA3 regulates MeJA-responsive expression of the *Str* gene via direct interaction with the JERE. The activating activities of ORCA proteins do not seem to depend on MeJA-induced *de novo* protein synthesis, but presumably occur via modification of pre-existing ORCA proteins (van der Fits and Memelink, 2001).

Our findings that the G-box (GCACGTTG) as well as the GCC-element are both required to bring about MeJA-inducible expression of the *NtPMT* genes is interesting in that it indicates that some level of interaction exists between these elements and the transcriptional factors that bind them. The G-box (located at −103 to −96 bp relative to the start of transcription) and GCC-element (at −62 to −53 bp) required for MeJA-responsiveness in the *NtPMT1a* promoter are positioned almost identically to a functional G-box (located at −108 to −103 relative to the start of transcription) and the JERE element (located at −94 to −70) in the *C. roseus Str* gene promoter (Menke *et al*., 1999; Ouwerkerk and Memelink, 1999). While these earlier studies did not find a specific requirement for a functional G-box to bring about either the elicitor- or MeJA- responsiveness, Ouwerkerk and Memelink (1999) observed that a *Str* G-box tetrameric element interacted with the *as-1* enhancer of the CaMV 35S promoter in GUS transgene constructs to bring about expression in leaves, whereas in the absence of the *as-1* enhancer element, transgene expression directed by the −47 bp CaMV 35S promoter was more organ-specific (seed only) and developmentally restricted (Ouwerkerk and Memelink, 1999).

It is likely that two distinct classes of MeJA-activated transcription factors bind at the G-box and GCC-motif to bring about MeJA-responsiveness in the *NtPMT1a* gene. Whether this binding is cooperative or independent remains to be determined. Other groups have reported cooperation between G-box elements and other functional elements in bringing about regulation and it has been clearly demonstrated that GBFs are capable of homo-and heterodimerization, allowing for a high degree of specificity in regulation of gene expression (Siberil *et al*., 2001). It has also been shown that GBFs are also capable of heterodimerization with other classes of transcription factors (Shindler *et al*., 1992b). For example, Buttner and Singh (1997) reported that GCC-motif

binding EREBP transcription factors can couple with bZIP proteins bound at either TGACG motifs or G-boxes, and Cheong *et al.* (1998) demonstrated that the zinc-finger protein STF1 from soybean heterodimerizes with the GBFs GmGBF-1 and GmGBF-2.

The role of the TA-rich region (located at −80 to −69 relative to the start site of transcription) containing a TATA-box element remains unclear. Its location between the G-box and GCC-motif suggests that this region could simply play a role by providing proper spacing between important binding sites, however, the results of our mutagenesis studies indicated that plays a role beyond simple spacing. Studies aimed at mapping the transcript initiation site of the *NtPMT* genes in MeJA-treated BY-2 cells did not find any direct evidence that the TATA-box element present in this region was operational. We can not rule out a possible role in regulating basal level transcription. It has been reported that transcriptional co-activators such as the multiprotein bridging factor 1 (MBF1), mediate transcriptional activation by bridging between a basic region/leucine zipper (bZIP)-type transcriptional activator and TATA-binding proteins TBP (Godoy *et al.*, 2001; Matsushita *et al.*, 2002). In plants, it has been shown that *ER24* gene, a tomato counterpart of MBF1 (LeMBF1), is immediately and transiently induced in ethylene-treated late immature fruit (Zegzouti *et al.*, 1999). Transcription of potato *MBF1* (*StMBF1*) is also up-regulated during fungal attack and upon wounding (Godoy *et al.*, 2001). Evidence also exists that the three AtMBF1s identified in Arabidopsis have distinct tissue-specific expression patterns and may have distinct interacting partners that mediate different responses to phytohormones and stress (Tsuda *et al.*, 2004).

Consistent with the earlier studies (Hibi *et al.*, 1994; Imanishi *et al.*, 1998; Shoji *et al.*, 2000a, b), we demonstrated that high AUX levels repress *NtPMT* gene expression and that MeJA-induced *NtPMT* expression can be partially repressed by high AUX and ETH. We also showed that the effect of AUX on MeJA-induced *NtPMT* gene expression was not linked to an ETH response (Hansen and Grossmann, 2000), since $AgNO_3$ treatment had no effect on the AUX mediated reduction in *NtPMT* expression. How cross-talk among various phytohormone signal-transduction pathways regulating *NtPMT* gene expression is brought about is still an open question. Our studies clearly demonstrated that both the G-box and GCC-motif are required for MeJA-responsiveness. No obvious elements bearing similarity to known auxin-responsive elements were found in the −400 to −200 bp region proximal to the start site of transcription of the *NtPMT1a* promoter, despite the fact that this region of the promoter was capable of responding to the phytohormone. GCC-motifs are also well known binding sites for ethylene-responsive element binding proteins or EREBPs (Park *et al.*, 2001), including the ethylene-responsive transcription factor EREBP1 from tobacco (Ohme-Takagi *et al.*, 2000). Despite the presence of an apparent EREBP1 binding site (i.e., the GCC-motif) in the *NtPMT1a* promoter, expression of the *NtPMT1a::GUS* transgene in BY-2 cells was not altered by ethephon treatment alone. Members of the AP-2 domain family of transcription factors including ORCA2 from *C. roseus* mediating elicitor and JA-responsiveness (Menke *et al.*, 1999) and EREBP1 mediating ETH responsiveness (Ohme-Takagi *et al.*, 2000) have similar binding sites containing GCC-motifs. Whether competition for occupancy of the GCC-motif binding site by distinct transcription factors responding to MeJA and ETH stimulation underlie the suppression of MeJA-induced *NtPMT* expression by ethephon treatment remains to be determined. Clearly, additional characterization of the *NtPMT* promoters and further analysis of the specific transcription factors involved in binding at the various *cis*-acting elements identified in this study are necessary to resolve this question.

Acknowledgements

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