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# Jasmonate-Responsive Regions in a *Nicotiana sylvestris PMT* Gene Involved in Nicotine Biosynthesis

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## Abstract

Putrescine *N*-methyltransferase (PMT) catalyzes the first committed step of nicotine biosynthesis in tobacco. Tobacco *PMT* genes are activated in the root after wounding or by jasmonate treatment. In this study, a dual luciferase transient expression assay was used to show that the promoter of *Nicotiana sylvestris NsPMT2* gene is induced by methyljasmonate in tobacco BY-2 protoplasts. An 80-bp TATA-proximal region was necessary and sufficient for the jasmonate response. We further demonstrated that a tetramer of the 24-bp sequence including a T/G-box within the region confers jasmonate responsiveness to a cauliflower mosaic virus 35S minimal promoter.

**Key words**: nicotine, jasmonate, promoter, tobbaco.

## Introduction

Plants profoundly alter gene expression patterns in response to insect attack and mechanical wounding. Jasmonic acid and its methyl ester (MeJA), collectively called jasmonates, are established signaling molecules mediating wounding and hervivory responses (Ryan, 2000). Exogenous applications of jasmonates have been reported to induce defense-related genes and enzyme genes involved in secondary metabolism in several plant species (Creelman and Mullet, 1997; Reymond and Farmer, 1998; Gundlach *et al.*, 1992; Menke *et al.*, 1999).

Nicotine is an insecticidal alkaloid produced in the root of *Nicotiana* species. The pyridine ring of nicotine is supplied from the pyridine nucleotide pathway whereas its pyrrolidin ring is derived from a diamine putrescine (Hashimoto and Yamada, 1994). The first committed step of nicotine biosynthesis is catalyzed by putrescine *N*-metyltransferase (PMT). *PMT* genes have been isolated from several Solanaceae species producing nicotine or biosynthetically related tropane alkaloids (Hibi *et al.*, 1994; Suzuki *et al.*, 1999). In *Nicotiana sylvestris*, three *PMT* genes (*NsPMT1*, *NsPMT2*, and *NsPMT3*) are found in the genome, encode highly conserved proteins, and are activated coordinately by jasmonate treatment in the root (Hashimoto *et al.*, 1998). Our previous studies showed that the conserved 0.25-kb 5′-flanking regions of these *NsPMT* genes are sufficient for jasmonate response (Shoji *et al.*, 2000a). Here, by using a tobacco protoplast transient expression assay, we further narrowed down the *NsPMT2* promoter region necessary for the response, and identified a 24-bp sequence that confers jasmonte response to a minimal promoter when fused as a tandem tetramer.

## Materials and Methods

*Reporter plasmid construction*

The internal reference plasmid CaMV35S::R-luc was obtained from K. Hiratsuka (Matsuo *et al.*, 2001). Various truncated versions of the *NsPMT2* promoter were generated by PCR and, after sequence confirmation, were cloned into an F-luc plasmid at the combinations of *Sal*I-*Nco*I, *Cla*I-*Nco*I, or *Bgl*II-*Xho*I sites. Detailed description of the plasmid construction will be supplied upon request.

Primers used for 5′-deletion constructs were as follows: (restriction enzyme sites are underlined)
PMTrv: 5′-CGTCTAGAATTCCATGGTCGAGC-CATTTGTGTTGG-3′
-236PMTf: 5′-GCGTCGACACTTTACTAATAA-TTGC-3′,
-206PMTf: 5′-GCATCGATTATATTTTAGTTC-CAAAATGAC-3′,

-184PMTf: 5′-GCATCGATCAGTCCAACCATG-C-3′
-150PMTf: 5′-GCATCGATCTCTATTATATCG-AGTTCCG-3′
-120PMTf: 5′-GCATCGATCTCGGTGTCCAAA-TTGTAT-3′

Primers used for 3′-deletion constructs were as follows:
-184PMTf: 5′-GCATCGATCAGTCCAACCATG-C-3′
-105PMTrv: 5′-CCGCTCGAGCAATTTGGACA-CCGAGGAGT-3′
-125PMTrv: 5′-CCGCTCGAGGGAGGGCGGA-ACTCGATATAATAG-3′
-137PMTrv: 5′-CCGCTCGAGTCGATATAATA-GAGTTAAAAAATCATTAC-3′
-151PMTrv: 5′-CCGCTCGAGTTAAAAAATCA-TTACAACGTGCATGG-3′

The 6-bp scanning mutations were introduced into the -184-bp 5′-deletion construct shown in Fig. 1 by inverse PCR using KOD plus taq polymerase (Toyobo). In each mutant, six adjacent nucleotides were changed into their transversion nucleotides (i.e. changing A to C and G to T).

The tetramer constructs in which 4 copies of the test DNA sequences were ligated in tandem were produced according to the procedure of Rushton *et al.* (2002). The *Bam*HI-*Bgl*II junctions were used here instead of *Spe*I-*Xba*I junctions in the original protocol. The upper and lower strand oligonucleotides used are listed below:
(M3-M6)f: 5′-AGCTTGGATCCGCACGTTGTA-ATGATTTTTTAACTA-3′
(M3-M6)rv: 5′-GATCTAGTTAAAAAATCATT-ACAACGTGCGGATCCA-3′
(M3mut-M6)f: 5′-AGCTTGGATCCTACATGTG-TAATGATTTTTTAACTA-3′
(M3mut-M6)rv: 5′-GATCTAGTTAAAAAATCA-TTACACATGTAGGATCCA-3′
(M8-M10)f: 5′-AGCTTGGATCCTATCGAGTT-CCGCCCTCCA-3′
(M8-M10)rv: 5′-GATCTGGAGGGCGGAACTC-GATAGGATCCA-3′
(M8M9mutM10)f: 5′-AGCTTGGATCCTATCGA-TGGAATCCCTCCA-3′
(M8M9mutM10)rv: 5′-GATCTGGAGGGATTCC-ATCGATAGGATCCA-3′

*BY-2 suspension cultures*

Tobacco BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow-2) cells were cultured at 27℃ in a modified Linsmaier and Skoog medium as described previously (Setiady *et al.*, 1995).

*Protoplast preparation and electroporation*

Protoplasts were isolated according to the procedure of Dansako *et al.* (2003). Three-day-old BY-2 cells were treated with 1% cellulase Onozuka RS, 0.1% pectolyase Y-23 in a 0.4 M mannitol (pH 5.5) at 30℃ for 2 hours. The protoplasts were washed twice with 0.4 M mannitol and once with the electroporation buffer (0.3 M mannnitol, 5 mM MES, 70 mM KCl, pH 5.8), and then were resuspended in the electroporation buffer at the density of 1.5 x $10^6$ protoplasts per 0.5 ml.
The test plasmid 10 $\mu$g and the internal reference plasmid CaMV35S::R-luc 1 $\mu$g were mixed with the 0.5 ml of the protoplasts and transferred to the 0.4-cm electroporation cuvette (BioRad). Electroporation was carried out using the BioRad gene pulser (500V/cm and 250 $\mu$F). After electroporation, the protoplast suspension was supplemented with 5 ml of the protoplast medium in which 30 $gl^{-1}$ sucrose of the modified Linsmaier and Skoog medium was replaced with 10 $gl^{-1}$ sucrose and 72.4 $gl^{-1}$ mannitol, and 2,4-D was omitted.

*Methyl jasmonate treatment*

Electroporated protoplasts were divided into two fractions (for MeJA treatment and control) and each fraction (1.5 ml) was transferred to 24-well microplates. To one protoplast fraction, MeJA was added at a final concentration of 20 $\mu$M. Both treated and non-treated protoplasts were cultured at 27℃ for 16 hours in the dark.

*Dual luciferase assay*

The dual luciferase assay was performed using Dual-Luciferase® Reporter Assay System (Promega). After centrifugation, pelleted protoplasts were resuspended in a passive lysis buffer, and then centrifuged. The supernatant was used for the enzyme assay according to the manufacture's protocol.

## Results

We have previously demonstrated that approximately 0.25-kb 5'-flanking regions of three *NsPMT* genes are sufficient to confer the jasmonate responsive expression to a downstream reporter gene in transgenic *N. sylvestris* roots (Shoji *et al.*, 2000a). Since *NsPMT* genes have highly conserved 5'-upstream sequences up to 0.25 kb (Hashimoto *et al.*, 1998) and since *NsPMT2* is expressed most strongly among three *NsPMT* genes in wild-type *N. sylvestris* roots (Shoji *et al.*, 2000a), we started our promoter deletion analysis with the 0.25-kb upstream region of *NsPMT2*.

For the promoter assay, we employed a dual

luciferase assay (Matsuo *et al.*, 2001) in protoplasts prepared from cultured tobacco BY-2 cells. Briefly, various *NsPMT2* promoter regions were fused to firefly luciferase (F-luc) and introduced into BY-2 protoplasts by electroporation, together with an internal reference plasmid CaMV35S::R-luc in which *Renilla* luciferase was fused to cauliflower mosaic virus 35S RNA promoter. After introduction of two plasmids, protoplasts were divided into two equal populations, and one population was then treated with 20 $\mu$M MeJA for 16 hours. The F-luc and R-luc activities were sequentially measured in protoplast protein extracts and the *NsPMT2* promoter activity was expressed as a ratio of the F-luc activity to the R-luc activity in each measurement.

When BY-2 protoplasts were treated with MeJA, the *NsPMT2* promoter encompassing from the translational start ATG to 236 bp 5′-upstream region showed 2.7-fold increased F-luc/R-luc activity compared to the non-treated protoplasts (**Fig. 1**). The same *NsPMT2* promoter region was activated by MeJA treatment by 2- to 3.5-fold in stably transformed *N. sylvestris* roots (Shoji *et al.*, 2000a), demonstrating that the transient expression assay using tobacco BY-2 protoplasts can be used to study gene activation by MeJA. *NsPMT2* promoters truncated to -206 bp and -184 bp (counting from the translational initiation ATG) still showed MeJA induction (2.5 times and 2.1 times, respectively), but the promoters further deleted to -150 bp and -120 bp were not induced by MeJA treatment (**Fig. 1**).

To further delineate the region required for MeJA induction, the 80-bp *NsPMT2* promoter region from just upstream of the putative TATA-box (-105 bp) to -184 bp was fused to the 46-bp TATA-box region of the CaMV35S promoter (35S TATA). This chimeric promoter still showed a modest but reproducible activation by MeJA (**Fig. 2**). The attenuation of MeJA responsiveness indicates that the -104/+1 region of *NsPMT2* may contain additional regulatory element(s) for the MeJA response. It should be noted that the 35S TATA alone showed a modest but reproducible "reduction" in the F-luc/R-luc ratio after MeJA treatment in our assay system, possibly because the full-length CaMV35S promoter was slightly activated by MeJA treatment and increased the R-luc control activity. The 3'-deletions to -125 bp, -137 bp, and -149 bp completely abolished the modest MeJA induction observed in the -105-bp deletion construct (Fig. 2). To test whether the modest MeJA activation of the *PMT2* (-184/-105)::35S TATA construct is statistically significant, we analyzed all the data in Fig. 2 with the Student t-test. Only the *PMT2* (-184/-105)::35S TATA data was statistically different from the 35S TATA data ($P<0.01$). Therefore, the 20-bp region between -105 bp and -125 bp is required for the MeJA induction of the *NsPMT2* promoter.

To precisely identify the regions required for the MeJA response, 6-bp scanning substitutions were introduced into the -184-bp 5'-deletion construct shown in Fig. 1. In total, 14 test promoters (mR1 to mR14) which contained six successive transversion mutations (A to C, C to A, G to T, or T to G) in each promoter were generated and tested for the MeJA induction (**Fig. 3A**). The results shown in Fig. 3B indicate that the mutations in R3, R5, R6, R8, R9, and R10 abolished the MeJA induction whereas

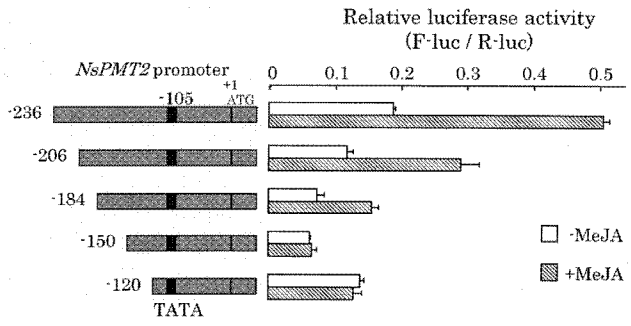


**Fig. 1** Deletion analysis of MeJA-responsive expression of the *NsPMT2* promoter from the 5'-upstream end. Truncated *NsPMT2* promoters were fused to the F-luc reporter gene as shown on the left. The promoter position was numbered with the first adenine in the translational initiation ATG as +1. Relative luciferase activity was caluculated as the F-luc activity divided by the R-luc activity, and shown on the right. Open bars; control without MeJA treatment, and shaded bars; MeJA treatment.

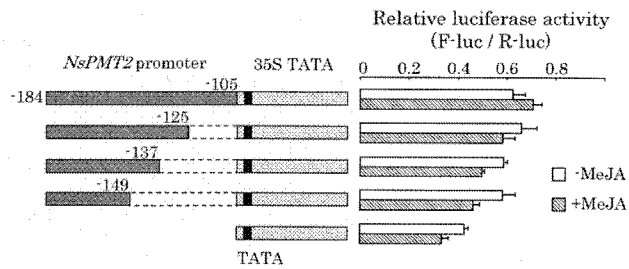


**Fig. 2** Deletion analysis of MeJA-responsive expression of the *NsPMT2* promoter from its putative TATA box. The CaMV35S TATA region (46 bp) was fused to various *NsPMT2* promoter sequences (without own TATA) truncated from the 3'-end. The dotted boxes indicate the regions deleted from the original promoter. See the Fig. 1 legend for other details. Open bars; control without MeJA treatment, and shaded bars; MeJA treatment.

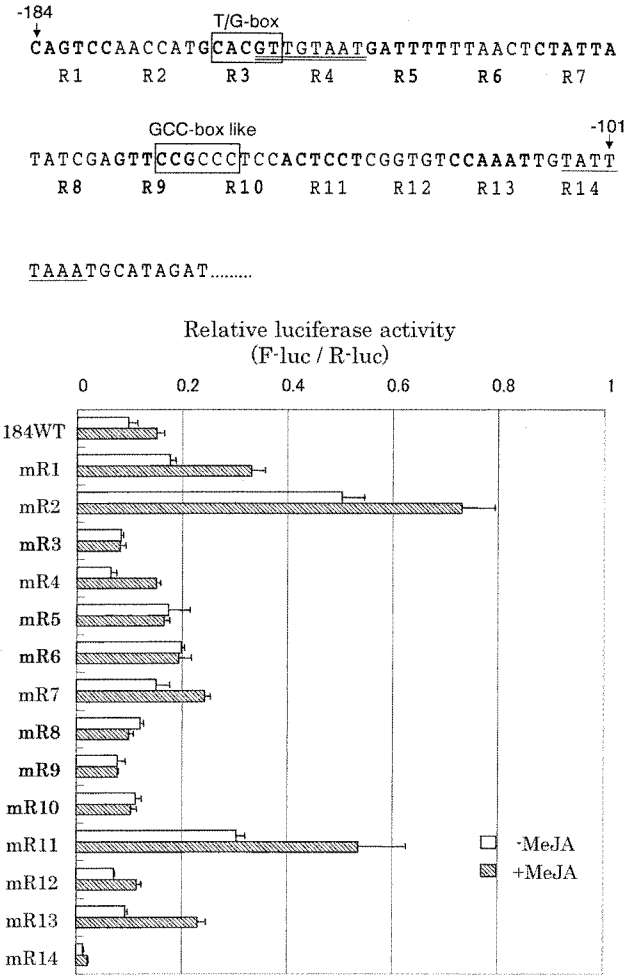


**Fig. 3** Scanning mutagenesis analysis of the *NsPMT2* promoter. (A) DNA sequence of the *NsPMT2* proximal promoter region. The 84-bp promoter region between -101 (within a putative TATA) and -184 was divided into 14 sub-regions (R1 to R14) and transversion mutations were introduced into each sub-region. The sub-regions required for the MeJA response are shown in bold letters. T/G-box and GCC-box-like sequences are boxed, and a putative TATA-box is underlined. The sequence conserved in the *A622* gene promoter is double underlined. (B) MeJA responsiveness of the *NsPMT2* mutant promoter-driven constructs in transiently expressed tobacco protoplasts. Mutations were introduced to the -184 promoter (-184 WT) shown in **Fig. 1**. Open bars; control without MeJA treatment, and shaded bars; MeJA treatment.

mutations in other regions did not significantly affect the response although the expression level *per se* varied among the test promoters. Increase in basal activities in some constructs (e.g., mR2 and mR11) indicate possible presence of negatively acting elements in these regions. Since the R14 region contained a part of the putative TATA

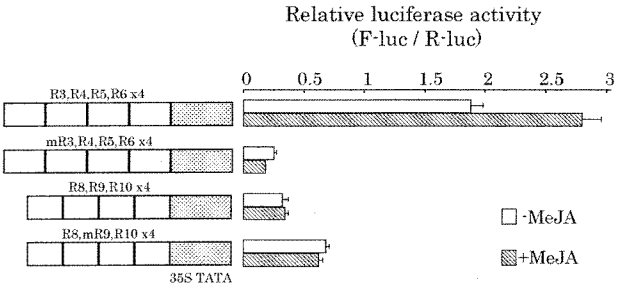


**Fig. 4** MeJA responsiveness of the constructs containing tetramers of the selected *NsPMT2* promoter regions in transiently expressed tobacco protoplasts. Four copies of wild-type sequences and their mutant versions (shown in open boxes) were fused to the 46-bp CaMV35S TATA region (shown in shaded boxes). Open bars; control without MeJA treatment, and shaded bars; MeJA treatment.

sequence (Shoji *et al.*, 2000a), it is reasonable that the mR14 construct was expressed very weakly while the MeJA response was still observed in this mutant.

We then divided the *NsPMT2* promoter regions necessary for the MeJA response into two sub-regions: a 24-bp region containing R3, R4, R5 and R6, and an 18-bp region containing R8, R9, and R10. Tetramers of these sub-regions as well as mutant versions containing mutated R3 or R9 (mR3 or mR9) were fused to the 35S TATA sequence and tested (**Fig. 4**). Significant activation by MeJA was observed in the wild-type sub-region R3-R6, whereas the corresponding mutant tetramer was not activated by MeJA. The wild-type and mutant tetramers of the sub-region R8-R9, however, were not sufficient to confer the MeJA responsiveness to the 35S TATA.

## Discussion

The DNA *cis*-regions important for jasmonate-induced gene expression have been studied in several jasmonate-responsive genes which are involved in defense-related functions in plants (Kim *et al.*, 1992, Brown *et al.*, 2003, Boter *et al.*, 2004). Studies on enzyme genes for alkaloid biosynthesis are rather limited but include strictosidine synthase gene of *Catharanthus roseus* (Menke *et al.*, 1999). A G-box sequence has been identified as an essential *cis*-acting element for the jasmonate response in several jasmonate-inducible genes (Kim *et al.*, 1992, Mason *et al.*, 1993). In the strictosidine synthase promoter, the G-box sequence does not contribute to the jasmonate induction (Pasquali *et al.*, 1999) but a GCC-box-like element,

which is bound by the AP2-domain-containing transcriptional factors, has been shown to mediate jasmonate- and elicitor-responsiveness (Menke *et al.*, 1999).

In this study, we identified several short DNA sequences (R3, R5, R6, R8, R9, and R10) that are necessary for the induction by MeJA in the TATA proximal region of *NsPMT2*. These regions are mostly conserved in highly homologous *NsPMT1* and *NsPMT3* promoter regions (Shoji *et al.*, 2000a). The R9 and R10 sequences together contain a GCC-box-like sequence (5′-CCGCCC). The region containing the GCC-box-like sequence (R8-R10) was necessary but was not sufficient for the MeJA response, indicating other *cis*-elements are also required.

The R3 sequence is necessary for the MeJA response and contains a T/G box (5′-CACGT) and a part of the sequence (5′-GTTGTAAT) conserved between *NsPMT* and *NsA622* promoters (Shoji *et al.*, 2002)(**Fig. 3A**). The *A622* gene encodes a reductase that is postulated to be involved in nicotine biosynthesis (Hibi *et al.*, 1994) and is regulated in the same way as *Nicotiana PMT* genes with respect to root cell type-specific expression, jasmonate inducibility, repression by ethylene, and dependence on the *NIC* regulatory loci (Shoji *et al.*, 2000b; Shoji *et al.*, 2002), indicating involvement of common *cis*-element(s) in these genes. Most significantly, a multimerized R3-R6 region was sufficient to confer the MeJA responsiveness to the minimal TATA element and the intact R3 region was necessary for this response. Thus, the T/G-box element in the TATA-proximal region of *NsPMT* genes may be critical for the induction by jasmonates. Detailed analysis of the *NsA622* gene is now underway and is expected to provide further information on the importance of the T/G-box. We also note that the R5-R6 region comprising mostly T/A residues contributes the *NsPMT* gene expression.

The T/G-box (5′-AACGTG) in the tomato leucine aminopeptidase promoter is largely required for induction by jasmonates and the basic helix-loop-helix leucine-zipper transcriptinal factors JA-MYCs have been identified as the cognate *trans*-factors for the T/B-box (Boter *et al.*, 2004). A related factor CrMYC1 also binds to the G-box element (a variation of the T/G-box) of the strictosidine synthase gene promoter in *C. roseus*, and has been proposed to be mediate the jasmonate response (Chatel *et al.*, 2003). Interestingly, G-box and GCC-box motifs are found in close proximity in several jasmonate-responsive gene promoters (Sessa *et al.*, 1995; Menke *et al.*, 1999; Brown *et al.*, 2003). In the *NsPMT2* promoter, a T/G-box-like element and a GCC-box-like element, which are both required for the MeJA induction, are located 33 bp apart. Two different types of *cis*-elements (such as T/G- or G-box and GCC-box) might be required in combinations for optimal induction by jasmonates in many jasmonate-responsive genes in plants.

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