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Characterization of cDNA for *PMT*: a Partial Nicotine Biosynthesis-Related Gene Isolated from Indonesian Local Tobacco (*Nicotiana tabacum* cv. Sindoro1)

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Nicotine is the major alkaloid compound in cultivated tobacco (*Nicotiana tabacum*) that could potentially be converted into carcinogenic compound (nor-nicotine). The *PMT* gene encoding putrescine N-methyltransferase (PMT) is one of the two key genes that play a prominent role in nicotine biosynthesis. The aimed of this study was to isolate and characterize the cDNA sequence originated from Indonesian local tobacco cv. Sindoro1 (*Ntpmt_Sindoro1*). The results showed that the *Ntpmt_Sindoro1* was 1124 bp in length. This cDNA fragment encodes for 374 amino acid residues. The predicted polypeptide from the cDNA is a hidrophilic protein, and has a predicted molecular weight of 40.95 kDa. The predicted amino acids sequence also showed high similarity to the *PMT* gene product *Nicotiana* sp. available in the GenBank data base. The amino acid sequences also exert conserved residues specifically exhibited only by PMT gene originated from *N. tabacum*. Clustering analysis revealed that *Ntpmt_Sindoro1* belongs to the same clade as the *PMT3* gene, a member of the *N. tabacum PMT* gene family. The *Ntpmt_Sindoro1* cDNA sequence covering exon1-exon8 of the *PMT* gene fragment has been registered in the GenBank data base, under the accession number JX978277.

Keywords: Indonesian local tobacco (*Nicotiana tabacum*), *PMT* gene, nicotine biosynthesis, gene characterization

INTRODUCTION

Alkaloid, a group of plant secondary metabolic compounds, is low-molecular-weight nitrogenous bases derived from an amino acid (Faccini 2001). Over 12.000 alkaloids have been described, in which most of them were isolated from tobacco plants (*Nicotiana tabacum*) (Nugroho & Veerporte 2002). The main alkaloid (85-95%) that commonly found in commercial tobacco (*Nicotiana tabacum*) is nicotine (Cordell 2013). Several research suggest that nicotine at low level acts as a mild-stimulant (Goodsell 2004). However, at moderate to high level, nicotine has a potential to be converted into nor-nicotine during senescence and curing of tobacco leaves (Gavilano *et al*. 2006; Lewis *et al*. 2008) which was proved to be associated with carcinogenesis in mammals (Hecht 2003).

Indonesian local tobaccos, the main raw material utilized in tobacco industry, have high nicotine content (5-8%) (Rochman *et al*. 2007). Attempt to reduce the local tobacco's nicotine content has been carried out through conventional breeding approach. However, the disanvantages of this technique include linkage drag, a simultaneous transfer of other deleterious genes linked to the gene of interest (Morandini & Salamini 2003). Recent studies suggest that metabolic engineering related to the nicotine biosynthesis can be used as an alternative method to reduce the tobacco nicotine content (Chintapakorn & Hamill 2007; Wang *et al*. 2008).

Nicotine is synthesized specifically in the root, then translocated through xylem vessels (De Luca & St. Pierre 2000), and finally accumulated in the tobacco leaf vacuoles (Shitan *et al*. 2009). Studies on nicotine biosynthesis in the *Solanaceae* family have been reported (Walton *et al*. 1994; Suzuki *et al*. 1999; Winz & Baldwin 2001; Liu *et al*. 2005; Stenzel *et al*. 2006). The intensive studies on nicotine biosynthesis in tobacco plants showed that nicotine is synthesized by two separate pathways (Katoh *et al*. 2005). Enzymes and their encoding genes involved in the biochemical reactions of nicotine biosynthesis have been widely studied (Hakkinen *et al*. 2007). Putrescine N-methyltransferase (PMT) is one of the two key enzymes in the biosynthesis that play an important role in the early step of

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the reaction. It converts the N-metilpirolin into N-metilputresin (Biastoff *et al*. 2009a), resulting in nicotine.

Nicotianatabacum putrescine N-methiltransferase is encoded by four genes family, namely *PMT1*, *PMT2*, *PMT3*, and *PMT4* (Hashimoto *et al*. 1998). Genes encoding putrescine N-methyltransferase from tobacco plants (*Nicotiana tabacum*, cv. Xanthie) have been isolated and characterized. The functional analysis of these genes showed that each *PMT* gene has an open reading frame composed of eight exons (Riechers & Timko 1999). Nevertheless, characterization of the *PMT* gene isolated from Indonesian local tobacco has not been reported. Therefore, the study to characterize and analyze cDNA sequence isolated from local tobacco need to be carried out. Here, we report the characterization and sequence analysis of cDNA isolated from an Indonesian local tobacco cv. Sindoro1.

MATERIALS AND METHODS

Plant Material, Total RNA Isolation, and cDNA Synthesis. Genetic material used in this study was a local tobacco variety Sindoro1. Total RNA was isolated from the root samples of 30 days old tobacco seedlings using Trizol reagent (Invitrogen) according to the manufacturer's protocol. The concentration and purity of RNA were assayed by spectrophotometer at 260 and 280 nm. The first strand cDNA was synthesized from 1 µg RNA template using Super script III Reverse Transcriptase (Invitrogen), according to the manufacturer's protocol.

Primer Design and PCR Analysis. Degenerate primers used in this study were designed and evaluated by Basuki *et al*. (2011). Primers were synthesized by Genetika Science of Indonesia. Three pairs of primers used in this study are listed in Table 1. PCR reactions were prepared in a total volume of 25 mL containing 1x PCR buffer, 25 mM MgCl2, 10 mM dNTPs, 1 unit of Taq DNA polymerase, 5 µM of forward and reverse degenerate primers, and 30 ng template cDNA. The cycling profile of PCR was as follows: an initial denaturation step at 94 °C for 3 min, followed by 30 cycles consisted of 94 °C for 2 min, 52-57 °C for 1 min, 72 °C for 1 minute, and a final extension step at 72 °C for 5 min. The PCR products were then examined by electrophoresis on 1% agarose gels, visualized following ethidium bromide staining, and photographed using an UV illumination (Bio-Rad, Herculas, CA, USA).

Cloning and cDNA Sequencing. The amplification product using PMT-5 primer was retrieved and purified by gel extraction kit (Geneaid, USA) prior to sub-cloning. The gel was purified then ligated into the pGEM-T easy-vector (Promega). The ligated products were transformed into *Escherichia coli* DH5α, and subsequently screened by blue-white colony selection technique (Sambrook *et al*. 1989). The recombinant plasmids that carried positive *PMT* gene fragment were sent for sequencing to 1st Base Pte. Ltd. Malaysia.

Nucleotide and Amino Acids Sequence Analysis. Forward and reverse primers position in the sequence were determined using DNA analysis program. Furthermore, the nucleotide and amino acid of *Ntpmt_Sindoro1* sequence were compared to the *PMT* gene sequences from the gene bank database NCBI (http://www.ncbi.nlm.nih.gov.id), and analyzed using BlastN and BlastP programs (McGinnis & Madden 2004). Amino acid residues were retrieved by translating nucleotides using virtual ribosome software (Wernersson 2006). Pairwise and multiple sequence alignment were performed using Clustal W 1.83 (Thompson *et al*. 1994). The gene accessions names used in this study are listed in Table 2. Protein and amino acid property of the candidate gene were analyzed using the DNAMAN program (ver.5.2.2. Lynnon Biosoft, USA).

Phylogenetic Analysis. Phylogenetic analysis was performed to assess the relationship between *Ntpmt_Sindoro1* amino acid sequence and the other *PMT* amino acid sequences deposited in the NCBI

Table 1. Degenerate primers synthesized and used to isolate the candidate *PMT* genes

Primer	Primer sequences (5' -3')	Degeneracy	Interval of exon (F - R)	Tm (°C)	% GC	Estimated fragment size (bp)
PMt-4	F1: ATG GAA GTC ATA TCT ACC A	4	E1 – E6	57.5	47	1568-1623
	R2: CCA AGC RTA GTT GAC AGA RC					
PMt-5	F1: ATG GAA GTC ATA TCT ACC A	2	E1 – E8	59.0	40	2013-2409
	R3: GCG AAA GAT GGY AAA ATG AA					
PMt-7	F2: TCT GAY TAC CAA GAT GTC A	4	E2 – E8	59.5	42	1406-1755
	R3: GCG AAA GAT GGY AAA ATG AA					

gene bank database. The phylogenetic relationship between sequences was inferred by genetic distance between pairs of sequences using the pairwise and multiple sequence alignment analysis (http://www.genebee.msu.su/services/malign_reduced.html). The resulting tree was constructed using the neighbour-joining method (Saitou & Nei 1987) on the program MEGA3 (Kumar *et al*. 2004). The branching order reliability was evaluated by bootstrap analysis of 1000 replicates.

Table 2. Accessions name of the PMT protein used in the multiple sequence alignment (Figure 5) and the phylogenetic analysis (Figure 6)

Accessions name	Accsessions number	Gene
Anisodus acutungulus (Aacut)	ACF21005.1	*PMT1*
Anisodus acutungulus (Aacut)	ACF21006.1	*PMT2*
Atropa belladonna (Abella)	BAA82264.1	*PMT1*
Atropa belladonna (Abella)	BAA82262.1	*PMT2*
Datura inoxia (Dinox)	CAJ46253.1	*PMT1*
Datura inoxia (Dinox)	CAJ46254.1	*PMT2*
Nicotiana attenuata (Natt)	AAK49870.1	*PMT1*
Nicotiana attenuata (Natt)	AAK49871.1	*PMT2*
Nicotiana sylvestris (Ns)	BAA74542.1	*PMT1*
Nicotiana sylvestris (Ns)	BAA74543.1	*PMT2*
Nicotiana sylvestris (Ns)	BAA74544.1	*PMT3*
Nicotiana tabacum (Nt)	AAF14879.1	*PMT1*
Nicotiana tabacum (Nt)	AAF14878.1	*PMT2*
Nicotiana tabacum (Nt)	AAF14880.1	*PMT3*
Nicotiana tabacum (Nt)	AAF14881.1	*PMT4*
Nicotiana benthamiana (Nbentha)	ABY25273.1	*PMT*
Nicotiana othopora (Nothop)	AAGO9273.1	*PMT*
Nicotiana tomentosiformis (Ntom)	AAGO9272.1	*PMT*

RESULTS

PCR Amplification and *Ntpmt_Sindoro1* Sequence Analysis. Three pairs of degenerate primers (PMT-4, PMT-5, and PMT-7) were used to amplify *Ntpmt_Sindoro1* fragment resulted in a single fragment amplified products. These primers generated amplicon size of ~ 900 bp (PMT-4), ~ 1100 bp (PMT-5), and ~ 800 bp (PMT-7) respectively (Figure 1). The amplicons generated by cDNA template would be shorter in size than the amplicons generated by genomic DNA (gDNA) template (Table 3). As the intron segment will be disposed from genomic DNA fragment during cDNA synthesis, thus only the exon segment existed.

Sequence analysis showed that the amplified *Ntpmt_Sindoro1* has 1124 bases in length, and encoded a protein that consisted of 374 amino acids (Figure 2). Nucleotide and amino acid sequences of the gene were then analyzed to compare the level of similarity of the *Ntpmt_Sindoro1* to those deposited in NCBI gene bank database. BlastN analysis

Table 3. Amplicon size comparison between cDNA and genomic DNA template generated from Sindoro1 variety using three pairs of degenerate primers

Primer	Interval of exon (F – R)	Amplicon size (bp)	
		gDNA	cDNA
PMT-4	E1 – E6	~1500	~900
PMT-5	E1 – E8	~2000	~1100
PMT-7	E2 – E8	~1200	~800

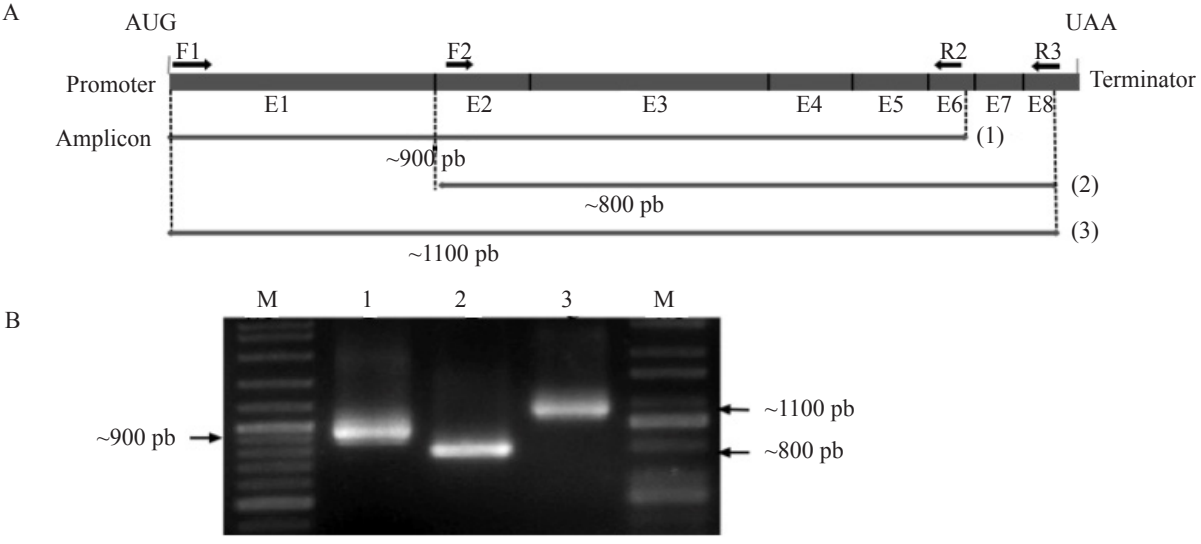

Figure 1. Amplification product using cDNA template. (A) Representation of the primer position in the exonic region, and amplicon size yielded using degenerate primers: 1, 2, and 3; (B) electrophoregram of *Ntpmt_Sindoro1* PCR products which were amplified by degenerate primers: 1, 2, and 3. 1: PMT-4 (F1R2), 2: PMT-7 (F2R3), and 3: PMT-5 (F1R3). M = 100 bp DNA ladder: 400, 500, 600, 700, 800, 900, 1000; 1200; 1500; 2000; 2500; 3000. E = exon; bp = base pairs, F = forward primer, and R = reverse primer.

revealed that the *Ntpmt_Sindoro1* sequence has high similarity (94-100%) to *Nicotiana PMT* gene, accessions AF126809.1, AF126810.1, AF126811.1, and AF126812.1 (Table 4). While BlastP analysis showed that *Ntpmt_Sindoro1* amino acid sequence has high similarity (88-99%) to the PMT protein, accessions Q42963.1, Q9SEH7.1, Q9SEH5.1, and Q9SEH4.1 (Table 5).

Ntpmt_Sindoro1 Protein Sequence Analysis. The Ntpmt_Sindoro1 protein encoded by 374 amino acids and had a predicted molecular weight of 40.95 kDa. The protein contained amino acids that were 60% soluble (hydrophilic), 30% insoluble (hydrophobic), and 10% neutral (Table 6). Further analysis showed that 75% of the total amino acids were hidrophilic, and present in N-terminal of the protein (the first exon).

```
M  E  V  I  S  T  N  T  N  G  S  T  I  F  K  N  G  A  I  P  M  N  G  Y  Q  N  G  T  S  K
ATGGAAGTCATATCTACCAACACAAATGGCTCTACTATCTTCAAGAATGGTGCCATTCCCATGAACGGTTACCAGAATGGCACTTCCAAA 90
  Forward primer

 H  Q  N  G  H  Q  N  G  T  S  E  H  R  N  G  H  Q  N  G  I  S  E  H  Q  N  G  H  Q  N  G
CACCAAAACGGCCACCAGAATGGCACTTCCGAACATCGGAACGGCCACCAGAATGGGATTTCCGAACACCAAAACGGCCACCAGAATGGC 180

 T  S  E  H  Q  N  G  H  Q  N  G  T  I  S  H  D  N  G  N  E  L  Q  L  L  G  S  S  N  S  I
ACTTCCGAGCATCAGAACGGCCATCAGAATGGGACAATCAGCCATGACAACGGCAACGAGCTACAGCTACTGGGAAGCTCCAACTCTATT 270

 K  P  G  W  F  S  E  F  S  A  L  W  P  G  E  A  S  S  L  K  V  E  K  L  L  F  Q  G  K  S
AAGCCTGGTTGGTTTTCAGAGTTTAGCGCATTATGGCCAGGTGAAGCATCCTCACTTAAGGTTGAGAAGTTACTATTCCAGGGGAAGTCT 360

 D  Y  Q  D  V  M  L  F  E  S  A  T  Y  G  K  V  L  T  L  D  G  A  I  Q  H  T  E  N  G  G
GATTACCAAGATGTCATGCTCTTTGAGTCAGCAACATATGGGAAGGTTCTGACTTTGGATGGAGCAATTCAACACACAGAGAATGGTGGA 450

 F  P  Y  T  E  M  I  V  H  L  P  L  G  S  I  P  N  P  K  K  V  L  I  I  G  G  G  I  G  F
TTTCCATACACTGAAATGATTGTTCATCTTCCACTTGGTTCCATCCCAAACCCTAAAAAGGTTTTGATCATCGGCGGAGGAATTGGTTTT 540

 T  L  F  E  M  L  R  Y  P  T  I  E  K  I  D  I  V  E  I  D  D  V  V  V  D  V  S  R  K  F
ACATTATTCGAAATGCTTCGTTATCCTACAATCGAAAAAATTGACATTGTTGAGATCGATGACGTGGTAGTTGATGTATCTAGAAAATTT 630

  F  P  Y  L  A  A  N  F  S  D  P  R  V  T  L  V  L  G  D  G  A  A  F  V  K  A  A  Q  A  G
TTCCCTTATCTTGCTGCTAATTTTAGCGATCCTCGTGTAACCCTAGTCCTTGGAGATGGGGCTGCATTTGTAAAGGCCGCACAAGCAGGA 720

 Y  Y  D  A  I  I  V  D  S  S  D  P  I  G  P  A  K  D  L  F  E  R  P  F  F  E  A  V  A  K
TATTATGATGCTATTATAGTGGACTCTTCTGATCCCATTGGTCCAGCAAAAGACTTGTTTGAGAGGCCATTCTTTGAGGCAGTAGCCAAA 810

 A  L  R  P  G  G  V  V  C  T  Q  A  E  S  I  W  L  H  M  H  I  I  K  Q  I  I  A  N  C  R
GCCCTAAGGCCAGGAGGAGTTGTATGCACACAGGCTGAAAGCATTTGGCTTCATATGCATATTATTAAGCAAATCATTGCTAACTGTCGT 900

 Q  V  F  K  G  S  V  N  Y  A  W  T  T  V  P  T  Y  P  T  G  V  I  G  Y  M  L  C  S  T  E
CAAGTCTTTAAGGGCTCTGTCAACTATGCTTGGACTACTGTTCCAACATATCCAACCGGTGTGATTGGTTATATGCTCTGTTCTACTGAA 990

 G  P  E  V  D  F  K  N  P  V  N  P  I  D  K  E  T  T  Q  V  K  S  K  L  A  P  L  K  F  Y
GGACCAGAAGTTGACTTCAAGAATCCAGTAAATCCAATTGACAAAGAGACAACTCAAGTCAAGTCCAAATTAGCACCTCTCAAGTTCTAC1080

 N  S  D  I  H  K  A  A  F  I  L  P  S  F
AACTCTGATATTCACAAAGCAGCATTCATTTTGCCATCTTTCGC  1124
                  Reverse primer
```

Figure 2. Nucleotide sequence (1124 bases) and amino acid sequence (374 aa) of *Ntpmt_ Sindoro1* gene fragment. Nucleotide sequence is written with a capital letter. Amino acid residues are written in capital letters above the nucleotide sequence.

Table 4. Homology comparison between the *Ntpmt_Sindoro1* nucleotide sequence and nucleotide sequences of *PMT* accessions that have been deposited in the NCBI gene bank database

Accession number	Species	Gene	Maximum score	Total score	Query coverage (%)	Homology (%)
AF126811.1	*Nicotiana tabacum*	*PMT3*	2128	2128	100	100
AF126809.1	*Nicotiana tabacum*	*PMT2*	1792	1792	100	94
AF126812.1	*Nicotiana tabacum*	*PMT4*	1258	1485	88	94
AF126810.1	*Nicotiana tabacum*	*PMT1*	1024	1274	88	97
AB004322.2	*Nicotiana sylvestris*	*PMT1*	1821	1821	100	95
AB004324.2	*Nicotiana sylvestris*	*PMT3*	1260	1486	88	94
AB004323.2	*Nicotiana sylvestris*	*PMT2*	1583	1583	74	99

Phylogenetic Analysis. The close relationship between Ntpmt_Sindoro1 protein sequence and the other protein sequences of the *PMT* gene family was studied by constructing phylogenetic trees. In this study, phylogenetic tree was constructed based on repetitive amino acids sequence that existed in the N-terminal of the PMT protein. The analysis showed that the Ntpmt_Sindoro1 protein sequence clustered together with the PMT protein sequences from genus *Nicotiana*, and clumped into a small group with *PMT3* gene from *Nicotiana tabacum* and *PMT2 Nicotiana sylvestris* gene (Figure 3).

DISCUSSION

Ntpmt_Sindoro1 had an average of 97% identical sequence to *Nicotiana tabacum PMT* (Table 5), suggesting that *Ntpmt_ Sindoro1* is associated with the *PMT* gene sequences originated from *Nicotiana tabacum*. This finding supports allignment analysis

Table 5. Homology comparison between *Ntpmt_Sindoro1* amino acid sequence and *PMT* amino acid sequences that have been deposited in the NCBI gene bank database

Accession number	Species	Gene	Maximum score	Total score	Query coverage (%)	Homology (%)
Q9SEH5.1	*Nicotiana tabacum*	*PMT3*	774	774	100	99
Q42963.1	*Nicotiana tabacum*	*PMT1*	691	691	100	94
Q9SEH7.1	*Nicotiana tabacum*	*PMT2*	635	635	100	89
Q9SEH4.1	*Nicotiana tabacum*	*PMT4*	698	698	100	88
BAA74543.1	*Nicotiana sylvestris*	*PMT2*	640	640	100	89
BAA74544.1	*Nicotiana sylvestris*	*PMT3*	691	691	100	86
BAA74542.1	*Nicotiana sylvestris*	*PMT1*	660	660	85	99

Table 6. Specific amino acid motif in the C-terminus of the Ntpmt_Sindoro1 protein

AA motif	AA position		Interval of exon	Base length (bp)
	Start	Stop		
NGGFPYTEMIV	148	166	Exon3	33
NGGGIGFTLFE	180	192	Exon3	33
DVSRKFFPYLAANF	212	226	Exon3-Exon4	149
DSSDPIGPAKDL	247	259	Exon5-Exon6	125

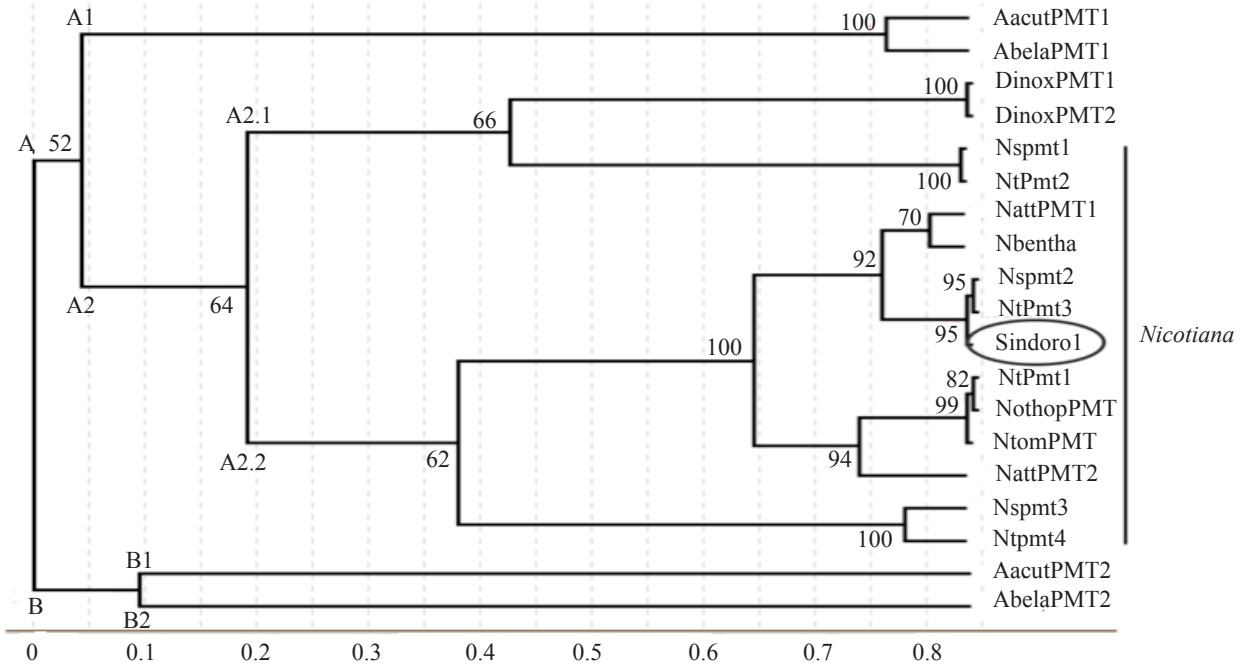

Figure 3. Phylogenetic tree, based on N-terminus of the PMT protein, showing evolutionary relationship between *Ntpmt_Sindoro1* amino acids sequence and the PMT genes sequences within family *Solanaceae* deposited in NCBI database. Value in the x-axis is the rates of nucleotide substitution between two or more sequences aligned. Bootstrap values based on 1000 replicates are indicated at the branches.

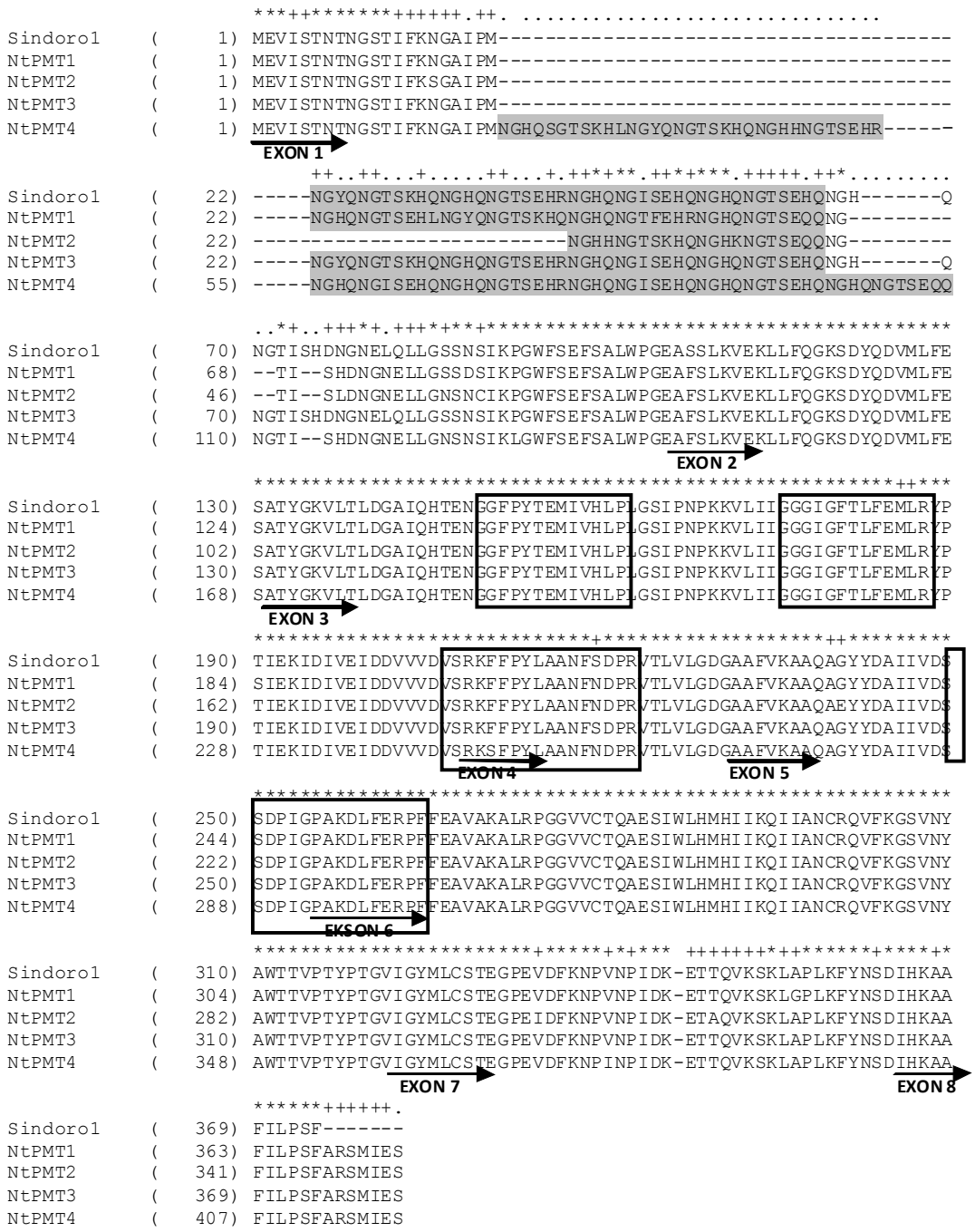

Figure 4. Amino acid sequences alignment of Ntpmt_Sindoro1 and amino acid sequences of *Nicotiana tabacum PMT* gene family that are available in the NCBI database. Repeated amino acid sequences are shaded in gray. Identical amino acids in the alignment are marked with an asterisk (*), while the non-identical amino acids are marked with a positive (+) or a period (.). Specific motifs of amino acid sequences at the C-terminus are boxed.

result which was also revealed high similarity (88-99%). Multiple allignment analysis also revealed the variation of the *Ntpmt_ Sindoro1* amino acids sequence existed in the first exon (Figure 4). Hashimoto *et al*. (1998) reported that the *PMT* gene fragment of *Nicotiana* is characterized by the presence of repeated amino acid motif on the first exon that varies among *Nicotiana* species. These unique features were not found in the *PMT* gene sequences derived from species other than the *Nicotiana* species (Figure 5). Thus, variation of this motif that exclusively located in the first exon of *Ntpmt_ Sindoro1* confirm previous results (Hashimoto *et al*. 1998), and can be used as a molecular marker to distinguish *Nicotiana PMT* genes from the other species.

```
                    **++++++++++++...*****+++++...++..+.....+++..+.++*+**.++
Nspmt1      (     1) MEVISTNTNGSTI---FKSGAIPM----------------------NGHQNGTSK-----
Nspmt2      (     1) MEVISTNTNGSTI---FKNGAIPMNGYQNGTSKHQNGHQNGTSEHRNGHQNGISE-----
Nspmt3      (     1) MEVISTNTNGSTI---FKNGAIPMNGHQSGTSKHLNGYQNGTSKHQNGHHNGTSEhrngh
NtPmt1      (     1) MEVISTNTNGSTI---FKNGAIPMNGHQNGTSEHLNGYQNGTSKHQNGHQNGTFE-----
NtPmt2      (     1) MEVISTNTNGSTI---FKSGAIPM----------------------NGHHNGTSK-----
NtPmt3      (     1) MEVISTNTNGSTI---FKNGAIPMNGYQNGTSKHQNGHQNGTSEHRNGHQNGISE-----
NtPmt4      (     1) MEVISTNTNGSTI---FKNGAIPMNGHQSGTSKHLNGYQNGTSKHQNGHHNGTSE-----
NattPMT1    (     1) MEVISTNTNGSTI---FKNGAIPMNGYQNGTSKHQNGHQNGTSEHRNGHQNGISE-----
NattPMT2    (     1) MEVISTNTNGSTI---FKNGAIPMNGHQNGTSKH----QNGTSEHPNGHQNGTSE-----
Nbentha     (     1) MEVISTNKNGSNI---FKNGAIPMNGYKNGTSKHQNGHQNGTSEHRTGHQNGISE-----
NtomPMT     (     1) MEVISTNTNGSTI---FKNGAIPMNGHQNGTSEHLNGYQNGTSKHQNGHQNGTFE-----
NothopPMT   (     1) MEVISTNTNGSTI---FKNGAIPMNGHQNGTSEHLNGYQNGTSKHQNGHQNGTSE-----
Sindoro1    (     1) MEVISTNTNGSTI---FKNGAIPMNGYQNGTSKHQNGHQNGTSEHRNGHQNGISE-----
DinoxPMT2   (     1) MEVISTQTNGS---------AIIM----------------------NYSHHNGNSD-----
DinoxPMT1   (     1) MEVISTQTNGS---------AIIM----------------------NYSHHNGNSD-----
AacutPMT1   (     1) MEVISNHNNGSTTKIILKNGSI-------------------------------------
AacutPMT2   (     1) M-----------A---FRNGSI--------TT---------TTSTTINHLNGAST-----
AbelaPMT1   (     1) MEV--NHNNGSTTKIILKNGSI-------------------------------------
AbelaPMT2   (     1) M-----------A---FRNGS---------TT---------TTITTINHPNDAST-----

                         .*+***.+*++++++*+.....................................
Nspmt1      (    31) ------HQNGHKNGTSEEQNG-------------------------------------
Nspmt2      (    53) ------HQNGHQNGTSEHQNGHQNG---------------------------------
Nspmt3      (    58) qngisEHQNGHQNGTSEHRNGHQNGTSEHRNGHQNGISEHQNGHQNGTSEHQNGHQNGTS
NtPmt1      (    53) ------HRNGHQNGTSEQQNG-------------------------------------
NtPmt2      (    31) ------HQNGHKNGTSEQQNG-------------------------------------
NtPmt3      (    53) ------HQNGHQNGTSEHQNGHQNG---------------------------------
NtPmt4      (    53) ------HRNGHQNGISEHQNGHQNGTSEHRNGHQNGISEHQNGHQNGTSEHQNGHQNGTS
NattPMT1    (    53) ------HQNGHKNGTSEHQNGHQNGTSE-----------------------------
NattPMT2    (    49) ------HQNGHQNGTSEQQNG-------------------------------------
Nbentha     (    53) ------HQNDHQNGTSEHQNDHHNGRSE-----------------------------
NtomPMT     (    53) ------HRNGHQNGTSEQQNG-------------------------------------
NothopPMT   (    53) ------HRNGHQNGTSEQQNG-------------------------------------
Sindoro1    (    53) ------HQNGHQNGTSEHQNGHQNG---------------------------------
DinoxPMT2   (    26) ------HRNG---GATAH-----------------------------------------
DinoxPMT1   (    26) ------HRNG---GATAH-----------------------------------------
AacutPMT1   (    23) -------RNGNVNG-----NSH-------------------------------------
AacutPMT2   (    25) -----IPKNGNNIATT--RNI-------------------------------------
AbelaPMT1   (    21) -------CNGDVNG-----NSH-------------------------------------
AbelaPMT2   (    24) -----IPKNGTNITTTLLKNG-------------------------------------

                        .....+++***.**+..++*++*+**********+***
Nspmt1      (    46) -----TISHDN-GNE--LLGNSNCIKPGWFSEFSALWPX
Nspmt2      (    72) -----TISHDN-GNELQLLGSSNSIKPGWFSEFSALWPX
Nspmt3      (   118) EQQNGTISHDN-GNE--LLGNSNSIKLGWFSEFSALWPX
NtPmt1      (    68) -----TISHDN-GNE--LLGSSDSIKPGWFSEFSALWPX
NtPmt2      (    46) -----TISLDN-GNE--LLGNSNCIKPGWFSEFSALWPX
NtPmt3      (    72) -----TISHDN-GNELQLLGSSNSIKPGWFSEFSALWPX
NtPmt4      (   107) EQQNGTISHDN-GNE--LLGNSNSIKLGWFSEFSALWPX
NattPMT1    (    75) -QQNGTISHDN-GNELQLLGSSNSIKPGWFSEFSALWPX
NattPMT2    (    64) -----TISHDN-GNK--LVGNSNCIKPGWFSEFSALWPX
Nbentha     (    75) -QQNGTISHDN-GNELQLLGSSNSIKPGWFSEFSALWPX
NtomPMT     (    68) -----TISHDN-GNE--LLGSSNSIKPGWFSEFSALWPX
NothopPMT   (    68) -----TISHDN-GNE--LLGSSNSIKPGWFSEFSALWPX
Sindoro1    (    72) -----TISHDN-GNELQLLGSSNSIKPGWFSEFSALWPX
DinoxPMT2   (    35) -------DHDN-GRK--LLGNSNSIKPGWFSEFSALWPX
DinoxPMT1   (    35) -------DHDN-GRK--LLGNSNSIKPGWFSEFSALWPX
AacutPMT1   (    33) -------SHEKIENK--LVECTNSIKPGWFSEFSALWPX
AacutPMT2   (    39) ----ATTLLKN-GST-HEFG---CIKPGWFSEFSELWPX
AbelaPMT1   (    31) -------THKKIENK--LVECTNSIKPGWFSEFSALWPX
AbelaPMT2   (    40) ----------S-TNE---FG---CIKPGWFSEFSQLWPX
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Figure 5. Amino acid sequence alignment of Ntpmt_Sindoro1 N-terminus sequence and other *PMT* N-terminus sequences within the *Solanaceae* family. Conserved amino acids are marked with an asterisk (*), while the amino acids that are not conserved are marked with a positive (+) or a period (.).

PMT protein composed of N-terminal and C-terminus. N-terminal of the PMT protein in *Nicotiana* species is exon-1, while the C-terminus is a region that covers exon 2 up to exon 8, in which amino acids in this region are highly conserved (Riechers & Timko 1999; Biastoff *et al*. 2009a). The highly conserved amino acid sequence suggests that the PMT protein has a specific role for tobacco plant, so the function needs to be maintained from generation to generation. Our

results are in agreement to previous studies. The *Ntpmt_ Sindoro1* contains N-terminal which was located at the first exon and C-terminus which was covered exon 2 – exon 8 (Figure 4). Our result also showed that almost 75% of amino acids in the Ntpmt_ Sindoro1 protein are soluble and it mostly present in the first exon. Eventhough studies of PMT protein have been conducted intensively, the relationship between amino acid motif repeat and PMT enzyme activity has not yet been explained. However, the modeling study on the N-terminus of the PMT protein conducted by Biastoff *et al*. (2009a) showed that the PMT enzymes derived from *Nicotiana* species were more soluble than the PMT enzymes derived from species other than the genus *Nicotiana*. This was believed to be related to the 11 amino acids repeat at the N-terminal of the PMT protein. Research conducted by Biastoff *et al*. (2009b) confirmed that treatment by eliminating the 11 amino acid repeat in the first exon has reduced the total solubility of the PMT protein.

C-terminus analysis of the Ntpmt_Sindoro1 protein sequence revealed four specific amino acid motifs that exist in exons 3, 4, 5, and 6 (Table 6). The *Nicotiana tabacum* PMT protein shared the four motifs in their sequence (Figure 4). This result is in agreement with the study reported by Biastoff *et al*. (2009a), which indicated that the PMT protein of *Solanaceae* family is characterized by specific existence of the four motifs (11-14 amino acids). Three (NGGFPYTEMIV, DSSDPIGPAKDL, DVSRKFFPYLAANF) out of four specific amino acid motifs are similar to those of the spermidin synthase (SPDs) domain. SPDs is a polyamine group which has a wide range of biological activities in living organisms (Minguet 2008), while the PMT enzyme is only detected in plants with a specific function (Biastoff *et al*. 2009b). Previous study reported by Hashimoto *et al*. (1998) suggested that *PMT* genes were evolved from an ancient SPDs gene and then they changed their function. Thus, the feature to make PMT protein can be distinguished from other proteins (Teuber *et al*. 2007). The existence of four specific amino acid motifs in Ntpmt_Sindoro1 protein sequence suggested that the Ntpmt_Sindoro1 protein might had the same function as the *PMT* gene family proteins originated from *Nicotiana tabacum* encoding putrescine N-methyltransferase enzyme involved in nicotine biosynthesis.

Clustering analysis in this study supports Hashimoto *et al*. (1998) finding that *Nicotiana sylvestris* (the *Nicotiana tabacum* progenitor) contributed three genes (*NsPMT1*, *NsPMT2*, *NsPMT3*) to the existing *PMT* gene family in *Nicotiana tabacum* genome, whereas the other progenitors, *Nicotiana tomentosiformis* and *Nicotiana othophora*, contribute only one gene. The result also in agreement with Riechers and Timko (1999) who reported that *NtPMT2*, *NtPMT3*, and *NtPMT4* have similar sequences to *NsPMT1*, *NsPMT2*, *NsPMT3*; while *NtPMT1* has a similar sequence to the *PMT* gene which originated from *Nicotiana tomentosiformis* or *Nicotiana othophora*. Furthermore, the *PMT* gene family originated from *Nicotiana* species has an evolutionary relationships based on the number of repeats of 11 amino acids that exist in the first exon (Riechers & Timko 1999). It can be concluded that *Ntpmt_Sindoro1* is the candidate for *PMT3* gene related to nicotine biosynthesis, which derived from Indonesian local tobacco varieties Sindoro1. The gene has been registered to the NCBI gene bank with accession numbers JX978277.

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