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Gene Expression in Tobacco Low-Nicotine Mutants

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Two nuclear genes, *Nic1* and *Nic2*, regulate nicotine levels in tobacco. *nic1* and *nic2* are semidominant mutations in Burley 21 that reduce leaf nicotine levels and the activities of multiple enzymes in the nicotine pathway and simultaneously increase polyamine levels in cultured roots. Cultured roots homozygous for both mutations were used to isolate two cDNAs by subtraction hybridization; the transcript levels of these two cDNAs were much lower in the mutant roots than in the wild-type roots. The *A411* gene encodes a 41-kD protein with considerable homology to mammalian spermidine synthase, whereas the *A622* gene encodes a 35-kD protein with high homology to isoflavone reductase. When these genes were expressed in *Escherichia coli*, A411 had no spermidine synthase activity but did show putrescine *N*-methyltransferase activity, which is the first enzyme committed to the nicotine biosynthetic pathway, and A622 did not show isoflavone reductase activity. Both the methyltransferase and *A622* genes are predominantly expressed in the root, and their expression levels in cultured roots are coordinately decreased by the *nic* mutations in the order of wild type > *nic2* > *nic1* > *nic1 nic2*. Removal of tobacco flower heads and young leaves rapidly and coordinately induced both genes in the root. Further, exogenous supply of auxin down-regulated both genes in cultured tobacco roots. These results suggest that *Nic1* and *Nic2* are regulatory genes for nicotine biosynthesis.

INTRODUCTION

Defined mutants are valuable tools in elucidating biosynthetic pathways and the regulation of secondary metabolite synthesis. In particular, genetic studies of flavonoid pigments have identified ~35 loci in petunia, ~12 loci in Antirrhinum, and ~10 loci in maize; these loci influence flavonoid biosynthesis (van Tunen and Mol, 1991). Most of these loci control only one step in the flavonoid biosynthetic pathway, and many have been identified as structural genes encoding biosynthetic enzymes. Some of the loci have been found to be regulatory genes that control multiple steps in the pathway and encode transcriptional factors (Dooner and Robbins, 1991). In contrast to current advances in our understanding of flavonoid biosynthesis, similar studies on other secondary products are limited. Because many secondary products are colorless, extensive screening programs by chemical analysis or pure luck is needed to identify rare mutants that display altered accumulation patterns of such invisible compounds (Hashimoto and Yamada, 1994).

Nicotine is the predominant alkaloid in most commercial varieties of tobacco. In the early 1930s, certain Cuban cigar varieties were found to have very low nicotine contents, and their low-alkaloid genes were subsequently incorporated into cigarette varieties through a series of backcrosses to meet the expected demand for low-nicotine cigarettes (Valleau, 1949). LA Burley 21 was thus developed as a genetically stable breeding line with a very low-alkaloid content. This breeding line is not different from parental Burley 21 in days to flower, number of leaves, leaf size, and plant height, but is more susceptible to insect damage, probably due to its low nicotine content (Legg et al., 1970). Thorough genetic studies have demonstrated that the nicotine level is controlled by two nonlinked loci (Legg et al., 1969). In this article, the two loci, originally called A and B, will be referred to as *Nic1* and *Nic2*, respectively, and the mutant alleles in LA Burley 21 will be referred to as *nic1* and *nic2*. *nic1* and *nic2* are semidominant and act synergistically. The dosage effect of *nic1* on leaf nicotine levels is ~2.4 times stronger than that of *nic2* (Legg and Collins, 1971).

Nicotine is synthesized in the tobacco root from ornithine and/or arginine by way of putrescine (Leete, 1980). Putrescine is either metabolized to higher polyamines, such as spermidine and spermine, or conjugated with cinnamic acid derivatives or fatty acids in all higher plants (Smith, 1981); it is also converted to *N*-methylputrescine in plants that produce nicotine or tropane alkaloids (Figure 1). Thus, putrescine *N*-methyltransferase (PMT; EC 2.1.1.53) participates in the first committed step in alkaloid biosynthesis (Hibi et al., 1992). *N*-Methylputrescine is then oxidized by a diamine oxidase (EC 1.4.3.6) and cyclized spontaneously to the 1-methyl-Δ^1-pyrrolinium cation, which is condensed with nicotinic acid or its derivative by an unknown enzyme to form nicotine (Hashimoto and Yamada, 1993). Quinolinic acid phosphoribosyltransferase (QPT; EC 2.4.2.19) serves as the entry-point enzyme into the pyridine nucleotide cycle that supplies nicotinic acid (Wagner and Wagner, 1985).

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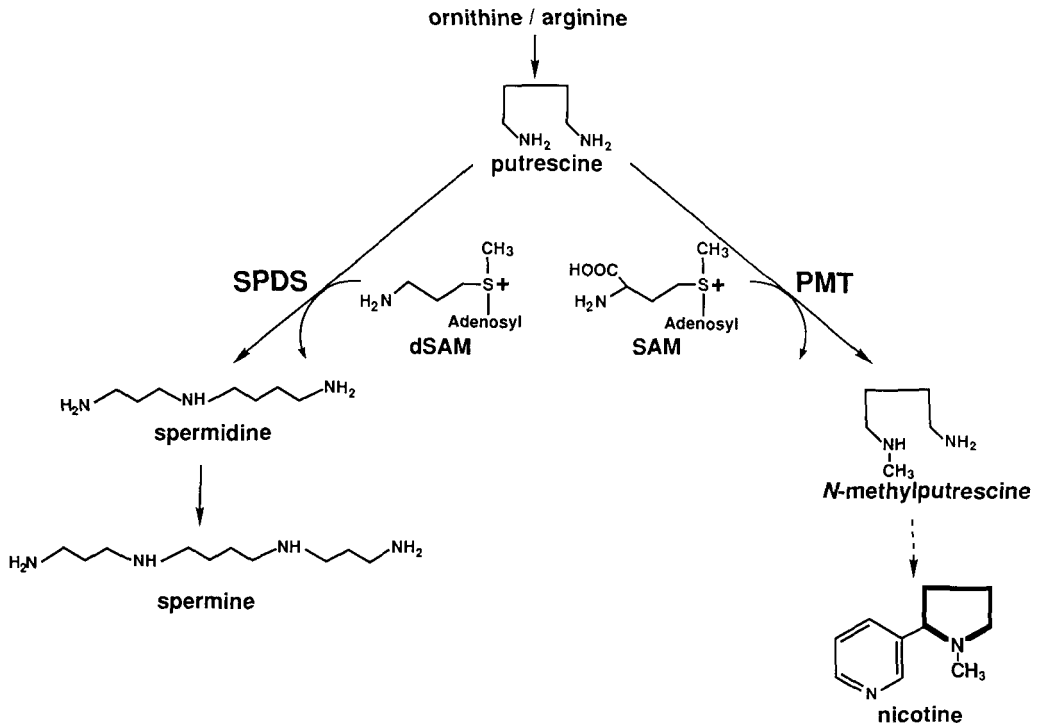

Figure 1. Biosynthesis of Polyamines and Nicotine.

The diamine putrescine is metabolized to either polyamines or nicotine in tobacco. Spermidine synthase (SPDS) transfers the aminopropyl moiety of decarboxylated *S*-adenosylmethionine (dSAM) to putrescine, thus forming spermidine. In contrast, putrescine *N*-methyltransferase (PMT) transfers the methyl moiety of *S*-adenosylmethionine (SAM) to putrescine, thus forming *N*-methylputrescine, which serves as a precursor of nicotine.

The four genotypes of tobacco Burley 21 that have different *Nic1* or *Nic2* loci have been biochemically characterized to some extent. The activities of PMT and QPT in the root decrease in the order of wild type > *nic2* > *nic1* > *nic1 nic2* as do the leaf nicotine contents of the respective genotypes, whereas the diamine oxidase activity in the root does not closely parallel the nicotine contents (Saunders and Bush, 1979). Several labeled nicotine precursors were incorporated into nicotine at reduced rates in hydroponically grown tobacco plants having *nic1* or *nic2* mutations (Hall, 1975). Apparently, multiple steps in nicotine biosynthesis are affected by the *Nic* loci. In this study, we employed differential screening to isolate genes that are under control of the *Nic* genes. In an analogous study, Martin et al. (1991) screened an Antirrhinum flower cDNA library for transcripts that differed in abundance between the wild type and the regulatory mutant *del* and identified previously uncloned biosynthetic genes in the flavonoid pathway that are regulated by *Del*. The two genes isolated in our study are regulated by *Nic1* and *Nic2*: one encodes PMT, whereas the other may also be involved in nicotine biosynthesis.

RESULTS

Biochemical Characterization of Mutant Cultured Roots

Because nicotine is synthesized in the root, we established root cultures from four homozygous tobacco genotypes with Burley 21 background: wild type, *nic1*, *nic2*, and *nic1 nic2*. These cultured roots grew well in vitro, but the growth of *nic1 nic2* roots was somewhat slower than that of the others (Table 1). Nicotine was the main alkaloid in the root cultures; nornicotine was the second most abundant alkaloid found. As much as 53% of nicotine and as much as 29% of nornicotine in the flasks were excreted into the culture media. The total alkaloid contents in the cultures are shown in Table 1. The combined contents of nicotine and nornicotine decreased stepwise in the order of wild type > *nic2* > *nic1* > *nic1 nic2*. The nornicotine content in the *nic2* roots was significantly higher than that in the wild-type roots. Putrescine was the predominant polyamine in the wild-type cultured roots, followed by *N*-methylputrescine, spermidine, and spermine. These amines were present in both

bound, conjugated forms and the free, soluble form: typical proportions of the bound forms in total cellular amines were 90% for putrescine, 50% for *N*-methylputrescine, 76% for spermidine, and 51% for spermine in the cultured roots used. Total *N*-methylputrescine contents decreased in the order of wild type > *nic2* > *nic1* > *nic1 nic2*, whereas the total cellular contents of putrescine, spermidine, and spermine increased in contrast to the changes in the alkaloids and *N*-methylputrescine (Table 1).

These changes in the contents of nitrogen-containing compounds suggest that the metabolism of putrescine in the alkaloid pathway is blocked by mutations in *Nic* genes, resulting in the accumulation of putrescine and in an increased flow of metabolites into the polyamine pathway. This was supported by the measurement of PMT activity in cultured roots (Figure 2). PMT activity changed in proportion to the alkaloid contents in the root cultures. Therefore, the biochemical characterization of the mutant root cultures shown here confirms previous findings that *nic1* affects nicotine biosynthesis more strongly than *nic2* and that *nic1* and *nic2* act synergistically (Legg and Collins, 1971).

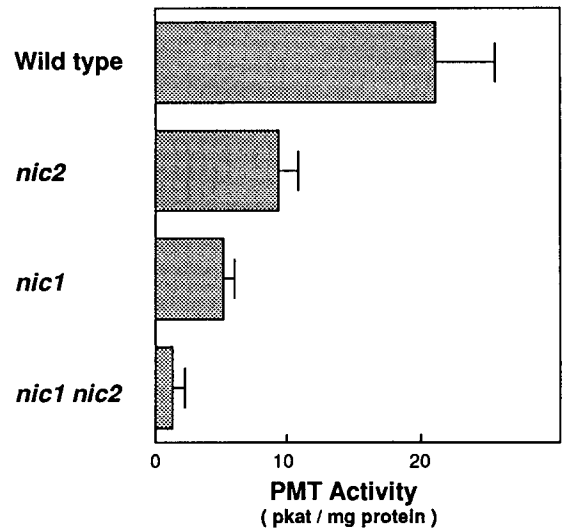

Figure 2. PMT Activities Are Reduced in *nic* Mutant Roots.

Cultured roots of four tobacco genotypes were grown as given in the legend to Table 1, and PMT activities in the crude enzyme preparations from the roots were measured. Error bars represent standard deviations.

Two Classes of Transcripts Are Underrepresented in *nic1 nic2* Roots

To identify genes that are regulated by the *Nic* loci, we looked for transcripts that were considerably less abundant in *nic1 nic2* roots than in wild-type roots. A cDNA library of $\sim 10^4$ clones was constructed from wild-type cultured roots and screened with both a subtracted cDNA probe enriched in wild-type specific sequences and a nonsubtracted cDNA probe from *nic1 nic2* roots. A total of 11 clones that hybridized consistently to the subtracted probe but not to the *nic1 nic2* probe was obtained. After analysis by cross-hybridization, these clones were classified into two groups in which *A411* and *A622* were the longest cDNAs. To gain insight into possible biochemical functions of the two cDNA groups, the sequences of *A411* and *A622* were determined.

A411 Is Homologous to Spermidine Synthase

A411 is 1395 bp long and contains one long open reading frame that encodes a polypeptide of 375 amino acids with a molecular weight of 41,084. The predicted protein has a very hydrophilic N terminus of ~80 amino acid residues; the N terminus contains four repeats of the heptapeptide Asn-Gly-His (or Tyr)-Gln-Asn-Gly-Thr and a total of six potential N-glycosylation sites (Figure 3). A search of the GenBank data base revealed similarity to spermidine synthase (SPDS; Figure 4). Over almost the entire length of the synthase, identity and homology (including conservative amino acid replacements) were 49 and 73% similar to human SPDS (Wahlfors et al., 1990), 46 and 70%

Table 1. Growth and Metabolite Contents in Cultured Tobacco Roots

Genotype	Growth (mg dry weight)	Alkaloids (nmol/mg dry weight)		Polyamines (nmol/mg dry weight)			
		Nicotine	Nornicotine	Putrescine	*N*-Methyl-putrescine	Spermidine	Spermine
Wild Type	235 ± 24[a]	120 ± 15	5.1 ± 1.1	30 ± 1	5.0 ± 1.3	2.4 ± 0.2	0.26 ± 0.05
nic2	225 ± 28	52 ± 8	31.9 ± 6.0	39 ± 5	3.5 ± 0.9	3.7 ± 0.5	0.26 ± 0.04
nic1	238 ± 27	39 ± 6	1.6 ± 0.4	45 ± 4	1.2 ± 0.1	3.7 ± 0.4	0.22 ± 0.02
nic1 nic2	195 ± 14	24 ± 4	1.1 ± 0.4	100 ± 14	0.5 ± 0.1	8.1 ± 1.4	0.41 ± 0.04

[a] Cultured roots of four tobacco genotypes (~60 mg dry weight of tissue) were grown in a liquid culture medium for 2 weeks in five replicates.

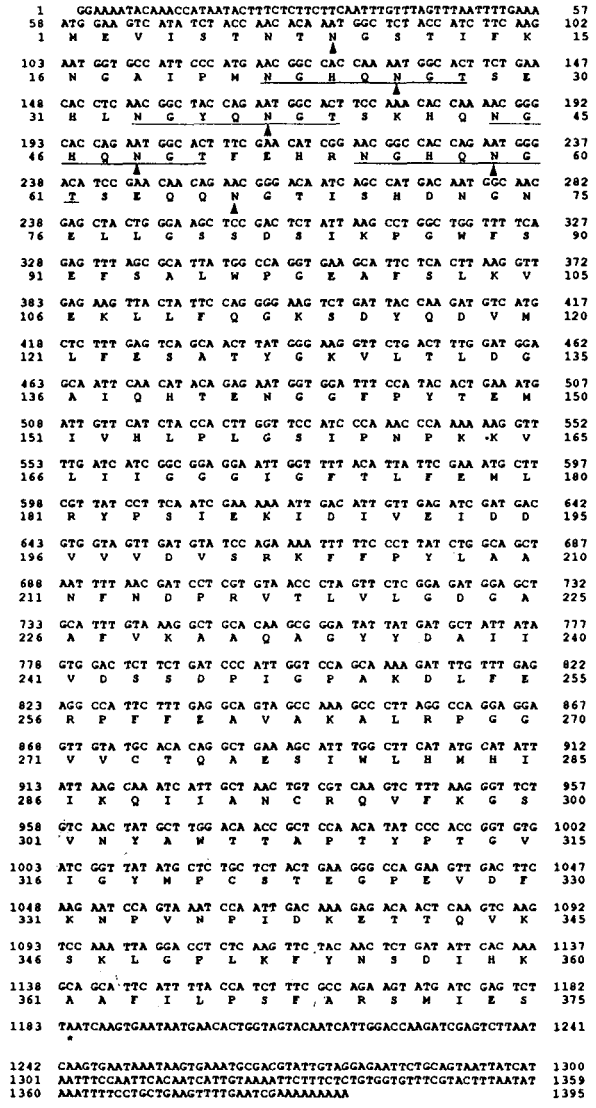

Figure 3. Nucleotide and Deduced Amino Acid Sequences of the *A411* cDNA Clone.

The nucleotide sequence is numbered from the 5′ end of the cDNA clone. The first methionine of the open reading frame is designated as the putative translational start site. Four repeats of the related heptapeptide are underlined, and putative N-glycosylation sites are marked by arrowheads. The DDBJ/EMBL/GenBank accession number of the *A411* cDNA sequence is D28506.

similar to mouse SPDS (DDBJ accession No. L19311), and 31 and 58% similar to *Escherichia coli* SPDS (Tabor and Tabor, 1987). Amino acid sequences similar to the proposed binding motifs for *S*-adenosylmethionine (SAM) (Bugos et al., 1991) were found in A411, as well as in SPDS (region I to V in Figure 4). It is interesting to note that the hydrophilic N-terminal extension is unique to A411 and is not present in any of the three SPDSs.

A411 Encodes PMT

SPDS transfers the aminopropyl moiety of decarboxylated SAM (dSAM) to putrescine, whereas PMT transfers the methyl moiety of SAM to putrescine (Figure 1). In addition, studies on substrate specificity and inhibition kinetics by amines have predicted that SPDS (Shirahata et al., 1991) and PMT (Hibi et al., 1992) may have similar active sites for enzyme catalysis. Therefore, we examined the possibility that *A411* may encode SPDS, PMT, or both (i.e., bifunctional enzyme). The *A411* cDNA was cloned into a pET-based vector to give pET411 so that an intact A411 protein would be expressed under the strong T7 promoter after induction by isopropyl β-D-thiogalactopyranoside. The enzyme activities of SPDS and PMT were measured in crude protein preparations from bacteria. As a host bacterial strain, we used an *E. coli* deletion mutant HT551 that lacks the *SPDS* gene (Tabor et al., 1986) and does not show either of the enzyme activities (Figure 5A). When the *E. coli SPDS* gene cloned in pSPD19 was expressed in HT551, we detected SPDS activity but not PMT activity (Figure 5B). However, when the *A411* cDNA was expressed, only PMT activity was detected (Figure 5C). Therefore, we concluded that *A411* encodes PMT, but not SPDS.

A622 Is Homologous to Isoflavone Reductase

A622 is 1179 bp long and contains one long open reading frame that encodes a polypeptide of 310 amino acids with a molecular weight of 34,648 (Figure 6). A search of the GenBank data base revealed that A622 is similar to isoflavone reductase (IFR) over the entire length (Figure 7). Identity and homology were 58 and 79% similar to alfalfa IFR (Paiva et al., 1991) and 56 and 80% similar to chick-pea IFR (Tiemann et al., 1991). The *A622* cDNA was expressed as an intact, soluble protein in *E. coli*, as with the expression of *A411*, and IFR activity was measured using 2′-hydroxyformononetin as a substrate. However, we could not detect any activity in cell extracts from the transformed bacteria; IFR activity was clearly detectable in the control extract from bacteria transformed with pIFRalf1, which encodes alfalfa IFR (Paiva et al., 1991) (data not shown). Thus, *A622* probably encodes a reductase, but not IFR.

Genomic DNA Gel Blot Analysis

Genomic DNA from tobacco and *Nicotiana sylvestris*, one of the two progenitor species, was cleaved with BamHI, EcoRI, or KpnI, separated by agarose gel, and blotted onto nylon membranes. Blots were probed with ^{32}P-labeled *PMT* cDNA (Figure 8A) or *A622* cDNA (Figure 8B), both of which detected

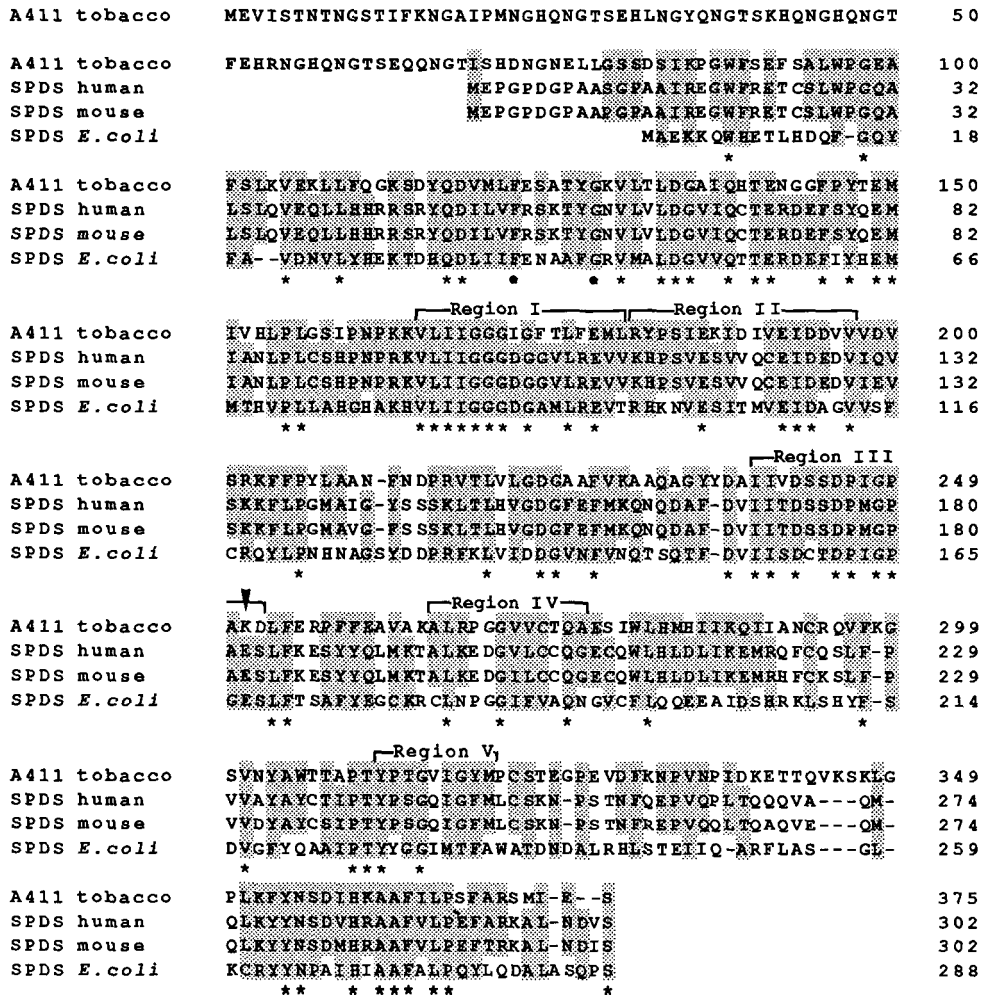

Figure 4. A411 Is Homologous to SPDS.

Identical amino acid residues are indicated by asterisks, and identical and similar amino acid residues are shaded. Structurally similar amino acids are grouped as follows: M, L, I, V, C, F; F, W, Y, H; A, G, S, T, P; D, E, N, Q; and R, K, H. Gaps introduced to optimize the alignment are shown by dashes. An arrowhead indicates a putative amino acid change that might be important in differentiating between the enzyme reactions of PMT and SPDS (see Discussion). Proposed binding regions for SAM and dSAM are also shown.

two to five strongly hybridized fragments in the two genomes. The *A622* probe also detected a few faintly hybridized DNA fragments only in the tobacco genome (arrowheads in Figure 8B). The tobacco genome contained a few extra fragments of the *PMT* gene in addition to those corresponding to the fragments in the *N. sylvestris* genome, whereas the tobacco fragments of the *A622* gene all had counterparts in the *N. sylvestris* genome, if we take into account one polymorphism detected with EcoRI digestion (asterisks). These results indicated that the *PMT* gene is represented by at least two more highly homologous DNA sequences in the tobacco genome and that the *A622* gene is probably a unique sequence in the tobacco genome derived from *N. sylvestris*. Thus far, we have been unable to detect any restriction fragment length polymorphisms for Burley 21 tobacco and the *nic1 nic2* mutant by using either cDNA probes, indicating that the *PMT* and *A622* genes in the mutant are not grossly different from those in the wild type (data not shown).

PMT and *A622* mRNA Levels Are Coordinately Regulated

Steady state levels of *PMT* and *A622* transcripts in cultured tobacco roots were analyzed (Figure 9). As a positive control, a probe for the β-ATPase gene (Boutry and Chua, 1985) was

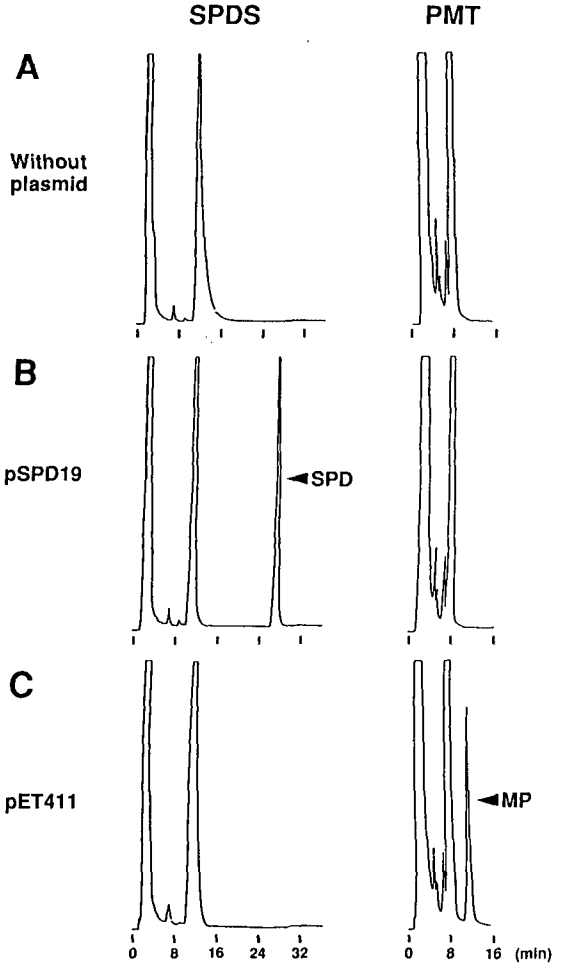

Figure 5. *A411* Encodes PMT, But Not SPDS.

Crude enzyme preparations from *E. coli* cells that harbored the indicated plasmid were analyzed for SPDS and PMT. The products from the enzyme reactions are shown by arrows on the HPLC chromatograms.

(A) Host *E. coli* strain HT551.

(B) *E. coli* cells transformed with pSPD19 expressing *E. coli SPDS*. SPD, spermidine.

(C) *E. coli* cells transformed with pET411 expressing *A411*. MP, *N*-methylputrescine.

also used for hybridization to the same blot. A *PMT* mRNA of ~1.4 kb was most abundant in the wild type and decreased in abundance in the order of *nic2* > *nic1* > *nic1 nic2*. Densitometric quantification indicated that the levels of *PMT* mRNA in *nic2*, *nic1*, and *nic1 nic2* were 73, 68, and 7%, respectively, of that in the wild type. Expression of the *A622* gene was affected similarly by the *nic* mutations: the levels of *A622* mRNA (~1.2 kb) in *nic2*, *nic1*, and *nic1 nic2* were estimated to be 82, 58, and 10%, respectively, of the wild type. These results showed that the *nic1* mutation has stronger effects on the expression levels of *PMT* and *A622* than the *nic2* mutation and that strong synergistic effects exist between the two mutant alleles.

Both the *PMT* and *A622* mRNAs were abundant in roots and were absent, or present at only very low levels, in stems and leaves of Burley 21 tobacco (Figure 10). In *nic1 nic2* tobacco, both transcripts were similarly detectable only in roots, but at levels much lower than those in wild-type roots.

PMT and *A622* mRNA Levels Are Down-Regulated by Auxin

Removal of the flower head and several young leaves causes a profound physiological adjustment in tobacco plants. This

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   1                                                AAAAATCCGATTTAATTCC    19
  20  TAGTTTCTAGCCCCTCCACCTTAACCCGAAGCTACTTTTTTTCTTCCCCTAGGAGTAAA    78
  79  ATG GTT GTA TCA GAG AAA AGC AAG ATC TTA ATA ATT GGA GGC ACA    123
   1   M  V  V  S  E  K  S  K  I  L  I  I  G  G  T                 15

 124  GGC TAC ATA GGA AAA TAC TTG GTG GAG ACA AGT GCA AAA TCT GGG    168
  16   G  Y  I  G  K  Y  L  V  E  T  S  A  K  S  G                 30

 169  CAT CCA ACT TTC GCT CTT ATC AGA GAA AGC ACA CTC AAA AAC CCC    213
  31   H  P  T  F  A  L  I  R  E  S  T  L  K  N  P                 45

 214  GAG AAA TCA AAA CTC ATC GAC ACA TTC AAG AGT TAT GGG GTT ACG    258
  46   E  K  S  K  L  I  D  T  F  K  S  Y  G  V  T                 60

 259  CTA CTT TTT GGA GAT ATA TCC AAT CAA GAG AGC TTA CTC AAG GCA    303
  61   L  L  F  G  D  I  S  N  Q  E  S  L  L  K  A                 75

 304  ATC AAG CAA GTT GAT GTG GTG ATT TCC ACT GTC GGA GGA CAG CAA    348
  76   I  K  Q  V  D  V  V  I  S  T  V  G  G  Q  Q                 90

 349  TTT ACT GAT CAA GTG AAC ATC ATC AAA GCA ATT AAA GAA GCT GGA    393
  91   F  T  D  Q  V  N  I  I  K  A  I  K  E  A  G                105

 394  AAT ATC AAG AGA TTT CTT CCT TCA GAA TTT GGA TTT GAT GTG GAT    438
 106   N  I  K  R  F  L  P  S  E  F  G  F  D  V  D                120

 439  CAT GCT CGT GCA ATT GAA CCA GCT GCA TCA CTC TTC GCT CTA AAG    483
 121   H  A  R  A  I  E  P  A  A  S  L  F  A  L  K                135

 484  GTA AGA ATC AGG AGG ATG ATA GAG GCA GAA GGA ATT CCA TAC ACA    528
 136   V  R  I  R  R  M  I  E  A  E  G  I  P  Y  T                150

 529  TAT GTA ATC TGC AAT TGG TTT GCA GAT TTC TTC TTG CCC AAC TTG    573
 151   Y  V  I  C  N  W  F  A  D  F  F  L  P  N  L                165

 574  GGG CAG TTA GAG GCC AAA ACC CCT CCT AGA GAC AAA GTT GTC ATT    618
 166   G  Q  L  E  A  K  T  P  P  R  D  K  V  V  I                180

 619  TTT GGC GAT GGA AAT CCC AAA GCA ATA TAT GTG AAG GAA GAA GAC    663
 181   F  G  D  G  N  P  K  A  I  Y  V  K  E  E  D                195

 664  ATA GCG ACA TAC ACT ATC GAA GCA GTA GAT GAT CCA CGG ACA TTG    708
 196   I  A  T  Y  T  I  E  A  V  D  D  P  R  T  L                210

 709  AAT AAG ACT CTT CAC ATG AGA CCA CCT GCC AAT ATT CTA TCC TTC    753
 211   N  K  T  L  H  M  R  P  P  A  N  I  L  S  F                225

 754  AAC GAG ATA GTG TCC TTG TGG GAG GAC AAA ATT GGG AAG ACC CTC    798
 226   N  E  I  V  S  L  W  E  D  K  I  G  K  T  L                240

 799  GAG AAG TTA TAT CTA TCA GAG GAA GAT ATT CTC CAG ATT GTA CAA    843
 241   E  K  L  Y  L  S  E  E  D  I  L  Q  I  V  Q                255

 844  GAG GGA CCT CTG CCA TTA AGG ACT AAT TTG GCC ATA TGC CAT TCA    888
 256   E  G  P  L  P  L  R  T  N  L  A  I  C  H  S                270

 889  GTT TTT GTT AAT GGA GAT TCT GCA AAC TTT GAG GTT CAG CCT CCT    933
 271   V  F  V  N  G  D  S  A  N  F  E  V  Q  P  P                285

 934  ACA GGT GTC GAA GCC ACT GAG CTA TAT CCA AAA GTG AAA TAC ACA    978
 286   T  G  V  E  A  T  E  L  Y  P  K  V  K  Y  T                300

 979  ACC GTC GAC GAG TTC TAC AAC AAA TTT GTC TAGTTTGTCGATATCAATC   1027
 301   T  V  D  E  F  Y  N  K  F  V  *                            310

1028  TGCGGTGACTCTATCAAACTTGTTGTTTCTATGAATCTATTGAGTGTAATTGCAATAAT   1086
1087  TTTCGCTTCAGTGCTTTTGCAACTGAAATGTACTAGCTAGTTGAACGCTAGCTAAATTC   1145
1146  TTTACTGTTGTTTTCTATTTTTCGTCTTATTCCA..                          1179
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Figure 6. Nucleotide and Deduced Amino Acid Sequences of *A622* cDNA Clone.

The nucleotide sequence is numbered from the 5′ end of the cDNA clone. The first methionine of the open reading frame is designated as the putative translational start site. The DDBJ/EMBL/GenBank accession number of the *A622* cDNA sequence is D28505.

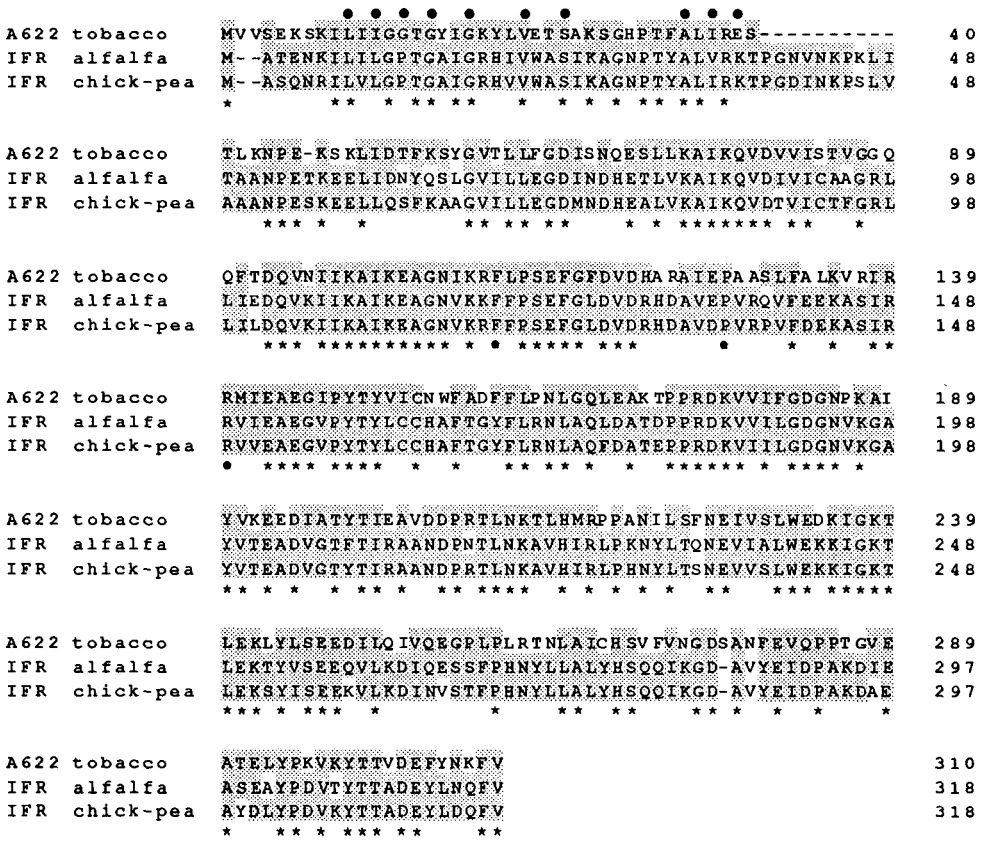

Figure 7. A622 Is Homologous to IFR.

Homology was compared as indicated in Figure 4. Amino acid residues that are proposed to interact with NAD(H) or NADP(H) are shown by closed circles.

practice is common among tobacco growers and eliminates apical dominance as well as increasing root growth and nicotine content (Akehurst, 1981). It has been previously shown that PMT enzyme activity and nicotine content in the root increase severalfold 1 day after decapitation (Mizusaki et al., 1973). Figure 11 shows that the level of *PMT* mRNA increased within 30 min after decapitation, peaked after 1 hr, and declined thereafter. Similar changes in the level of *A622* mRNA were also observed after decapitation. The maximum induction levels were approximately twofold for *PMT* and threefold for *A622* over the nondecapitated control at 1 hr.

Because auxin synthesized in young expanding leaves is transported down the stem (Phillips, 1975), removal of the tip region may cause a reduced auxin supply from the aerial parts into the root, which may result in activation or derepression of structural genes involved in nicotine biosynthesis. Therefore, we examined whether auxin in the culture medium affects expression of the *PMT* and *A622* genes in wild-type cultured tobacco roots (Figure 12). When cultured roots that had been grown in a medium containing 3 μM indolebutyric acid (IBA) were transferred into an auxin-free medium, both the *PMT* and *A622* mRNA levels increased in less than 30 min, peaked at 1 hr, and slowly increased thereafter: the addition of IBA (3 μM) to the culture after 20 hr caused a rapid decrease in both transcripts. These results indicated that the *PMT* and *A622* genes are down-regulated by auxin.

DISCUSSION

PMT Has Evolved from Spermidine Synthase

The amino acid sequence of PMT, except for its unique N-terminal extension, is highly homologous to the sequences of SPDS from mammals and *E. coli*. Although plant *SPDS* genes have not been reported, we found that PMT is even more homologous to the peptide fragment (73% identity and 88% homology in 66 amino acid residues) encoded by a partial cDNA sequence of rice (GenBank accession No. D15401) that

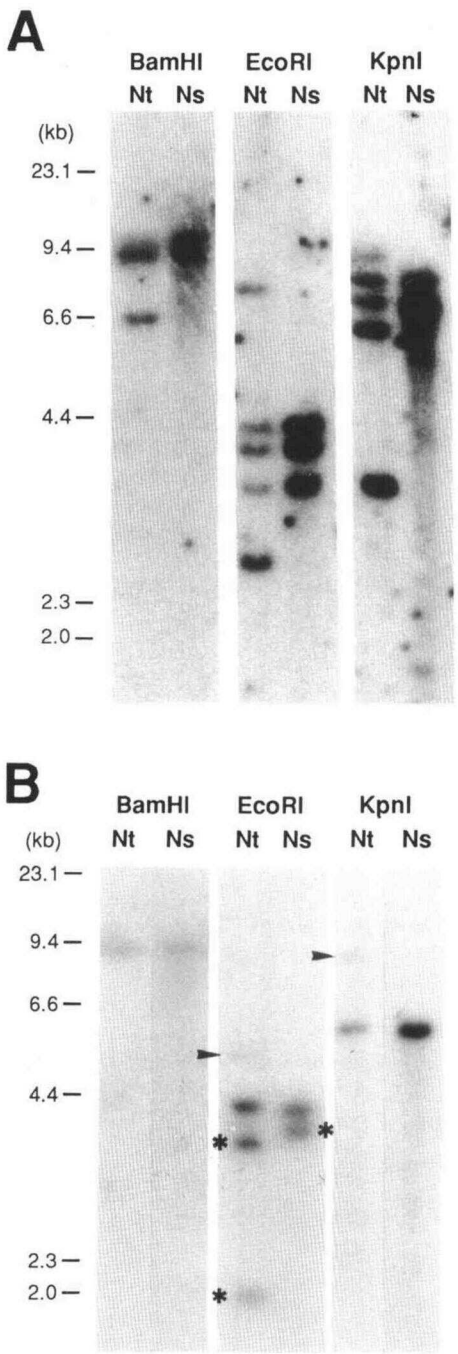

Figure 8. DNA Gel Blot Analysis.

Genomic DNA (20 μg) from tobacco (Nt) and *N. sylvestris* (Ns) was digested with either BamHI, EcoRI, or KpnI, electrophoresed on 0.8% agarose gels, and transferred to nylon membranes. Molecular length markers are given at left in kilobases.

(A) The blot was probed with the *PMT* cDNA.

(B) The blot was probed with the *A622* cDNA. Faintly hybridized DNA fragments are shown by arrowheads. Asterisks indicate DNA fragments that differ between the two genomes.

may represent part of the rice *SPDS* gene. In contrast, PMT is much less homologous to various *N*- and *O*-methyltransferases from plants and other organisms. A phylogenetic tree constructed from a matrix of sequence similarities (Nei, 1987) indicates that mammalian SPDS is more closely related to PMT than to *E. coli* SPDS and that PMT and SPDS form one particular subfamily, distinct from other methyltransferases (Figure 13). Thus, it is likely that *PMT* has evolved from *SPDS* in tobacco during the diversification of the Solanaceae.

Despite similarities in their primary structures, PMT and SPDS catalyze different reactions with high specificities; PMT does not have SPDS activity and vice versa. Transformation from SPDS to PMT would require two major changes at the enzyme's active site. First, SAM would have to become preferentially utilized in place of dSAM, which is bound by SPDS more strongly than SAM (Hibasami et al., 1980). One possibility here is that a positively charged amino acid is introduced to the binding motifs to effectively interact with the carboxyl group of SAM by ion pairing. One such candidate (a change from Glu to Lys) can be found at motif III of the proposed SAM binding site (arrowhead in Figure 4). Second, when dSAM is bound to SPDS, its aminopropyl moiety would have to be situated close to the deprotonated amino group of putrescine, and the methyl group of the cofactor should be away from the amino group, whereas SAM bound in PMT should have its methyl group close to the amino group of putrescine. The simplest

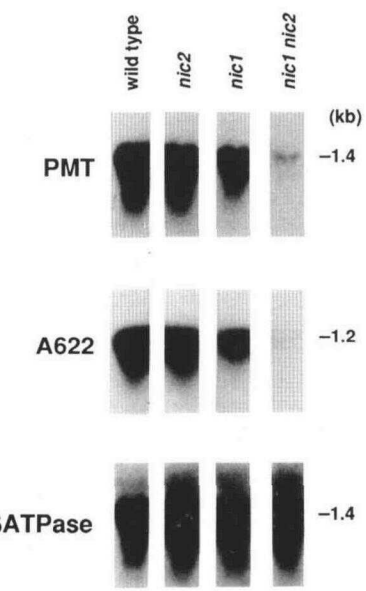

Figure 9. Transcript Levels of *PMT* and *A622* Are Down-Regulated in *nic* Mutants.

RNA gel blots were made from total RNA (20 μg per lane) from cultured roots of four tobacco genotypes and were successively probed with *PMT*, *A622*, and β-ATPase cDNAs. Molecular lengths of the respective transcripts are given on the right in kilobases.

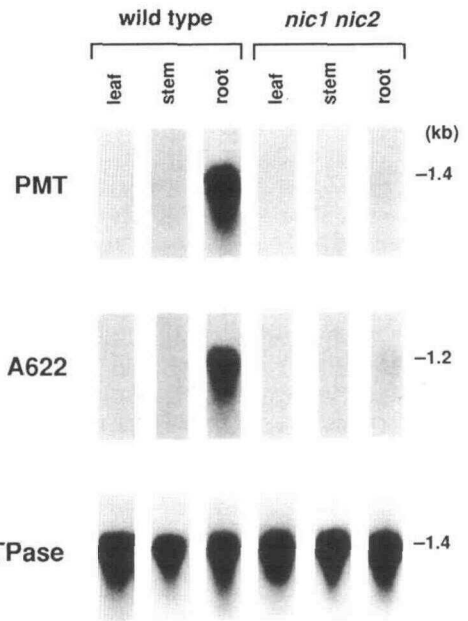

Figure 10. *PMT* and *A622* Are Expressed in the Root.

Total RNA was extracted from leaf, stem, and root of wild-type and *nic1 nic2* tobacco and was used as described in the legend to Figure 9.

way to change the orientation of the methyl group would be to rotate the methyl group around the sulfur atom of the cofactor toward the amino group of putrescine. This might be accomplished by the above amino acid mutation that was introduced to bind the carboxyl group of SAM. The N-terminal hydrophilic extension unique to PMT might also be important in determining the reaction specificity of PMT. Alternatively, this N-terminal extension might be used to target PMT protein to a specific subcellular organelle, such as the alkaloid vesicle involved in biosynthesis of isoquinoline alkaloids (Amann et al., 1986), although the structure of the N terminus of PMT does not resemble that of any known signal peptide, and alkaloid vesicles have not been found that are specific for nicotine biosynthesis. Cloning of *SPDS* genes from tobacco and *PMT* genes from other alkaloid-producing species, in combination with protein engineering, should give further insight into the molecular evolution of *PMT.*

Because PMT is the first enzyme committed to the nicotine pathway, it is reasonable to assume that its expression is closely correlated with nicotine formation in tobacco. Reciprocal grafting experiments between tobacco and non-nicotine-producing species have suggested that nicotine is synthesized in the root and translocated, probably through xylem, to the aerial parts (reviewed in Tso, 1972). Accordingly, PMT enzyme activity is detected only in the root of tobacco plants (Mizusaki et al., 1971). Root-specific expression of the *PMT* gene (Figure 10) further confirms the site of nicotine synthesis in tobacco. Down-regulation of the *PMT* gene by auxin also reflects the inhibitory effects of auxin on nicotine formation in tobacco callus (Ohta et al., 1978; Feth et al., 1986).

A622 Is Homologous to IFR

A622 is highly homologous to IFR over its entire length, but did not show IFR enzyme activity when expressed in *E. coli.* Because IFR is specifically involved in the biosynthesis of isoflavonoid phytoalexins in the Leguminosae and because tobacco apparently does not use isoflavonoid phytoalexins in its response to fungal attack (Dixon et al., 1983), tobacco is not expected to have an *IFR* gene. The very high similarity between the *A622* gene and the *IFR* gene indicates that these genes diverged relatively recently and that the *A622* gene codes for an oxidoreductase that utilizes NADP(H) or NAD(H) as a cofactor. Possibly, the substrate for A622 may be structurally related to isoflavonoids. Similar to the *PMT* gene, the *A622* gene is specifically expressed in the root, controlled by the *Nic* genes, and down-regulated by auxin, which suggests that it may be a structural gene for the biosynthetic pathway of nicotine or related alkaloids. The enzyme activity of QPT, the first enzyme in the biosynthesis of nicotinic acid, has been reported to be low in the root of the *nic1 nic2* mutant (Saunders and Bush, 1979). However, because QPT requires 5-phosphoribose-1-pyrophosphate but not nicotinamide as a cofactor, it is an unlikely candidate for A622.

One step that involves reduction of a double bond in the nicotine pathway is condensation between the 1-methyl-Δ^1-pyrrolinium cation and nicotinic acid, which forms nicotine. This is based on a feeding experiment using tobacco plants in which hydrogen from C-6 of nicotinic acid is lost during its incorporation into nicotine (Leete and Liu, 1973). Although detection of weak "nicotine synthase" activity in tobacco root extract was

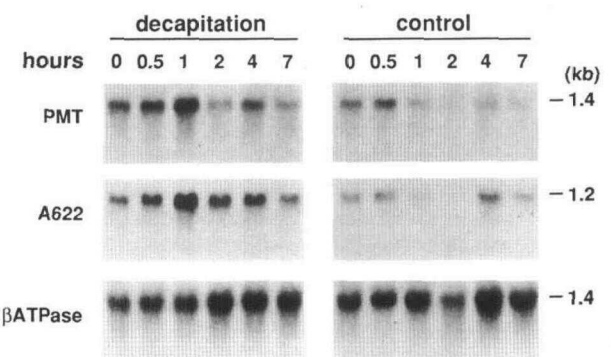

Figure 11. Decapitation Transiently Increases Transcript Levels of *PMT* and *A622.*

Total RNA was extracted from roots of three wild-type plants at the indicated time after decapitation and was used after pooling as described in the legend to Figure 9. Control plants were not decapitated.

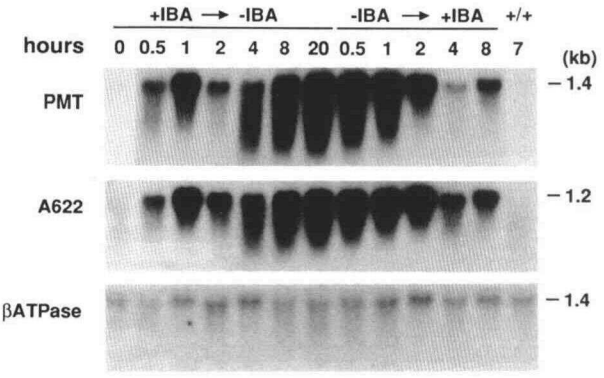

Figure 12. Auxin Down-Regulates Expression of *PMT* and *A622*.

Wild-type cultured roots that had been grown in the presence of 3 μM IBA were transferred to an auxin-free culture medium (+IBA → −IBA) or to the same IBA-containing medium (+/+). After 20 hr, IBA was added to the auxin-free medium to a final concentration of 3 μM (−IBA → +IBA). Total RNA was extracted from cultured roots at the indicated time after the treatments and used as described in the legend to Figure 9.

reported and the enzyme was proposed to require NADPH (Friesen and Leete, 1990), we have been unable to measure this elusive enzyme activity in enzyme preparations from either cultured roots or recombinant bacteria that expressed the *A622* gene (T. Hashimoto and M. Fukuyama, unpublished results).

The apparent lack of a DNA sequence that is highly homologous to *A622* in the genome of *N. tomentosiformis* may imply that A622 is involved in secondary metabolism that is present in *N. sylvestris* but absent in *N. tomentosiformis.* Both species produce nicotine, nornicotine, anabasine, and anatabine at somewhat different ratios, but no putrescine-derived alkaloids specific to either species have been found (Saitoh et al., 1985). It is difficult to suggest a probable function for A622 with the currently available phytochemical and biochemical information in *Nicotiana.* Antisense expression, as well as overexpression, of the *A622* gene in transgenic *N. sylvestris* should tell us whether it is a component of "nicotine synthase" or if it is involved in alkaloid synthesis at all.

A tobacco *TP7* gene (Drews et al., 1992) has been reported to encode a protein with high homology (56% identity and 88% similarity) to the alfalfa IFR. Although we could not directly compare the DNA sequences of *TP7* and *A622* because the *TP7* sequence was unavailable, these homologous tobacco genes appear to be different. The *TP7* and *A622* genes digested with EcoRI give totally different fragment patterns on genomic DNA gel blots of tobacco and *N. sylvestris* (Drews et al., 1992; Figure 8). In addition, *TP7* mRNA is detected predominantly in the limb region of the corolla, with some expression in the stem and very low expression in the root (Drews et al., 1992). In contrast, *A622* mRNA was highly abundant in the root, but not detectable in the stem (Figure 10) or the corolla (N. Hibi, unpublished results). In the tobacco genome, there seem to be several DNA sequences that are homologous to *IFR* but do not necessarily encode proteins with the same function.

Nic1 and *Nic2* May Be Regulatory Genes for Nicotine Biosynthesis

Enzyme activities of PMT and QPT (Saunders and Bush, 1979) and transcript levels of *PMT* and *A622* are simultaneously reduced by either of the two mutations. Both mutations caused a decrease in PMT activity and a concomitant increase in polyamine contents, indicating that putrescine formation from arginine and ornithine is much less affected than nicotine synthesis by the *Nic* genes. These results indicate that multiple biosynthetic enzymes in the nicotine pathway are affected in each *nic* mutant and that the *Nic* genes may be regulatory genes that act specifically, or primarily, on nicotine biosynthesis. The semidominant phenotype of *nic1* and *nic2* (Legg and Collins, 1971) further suggests that Nic1 and Nic2 limit the regulation of the nicotine pathway.

Strong synergistic effects were observed between the *nic1* allele and the *nic2* allele, judging from their effects on the transcript levels of *PMT* and *A622.* The synergy between the two

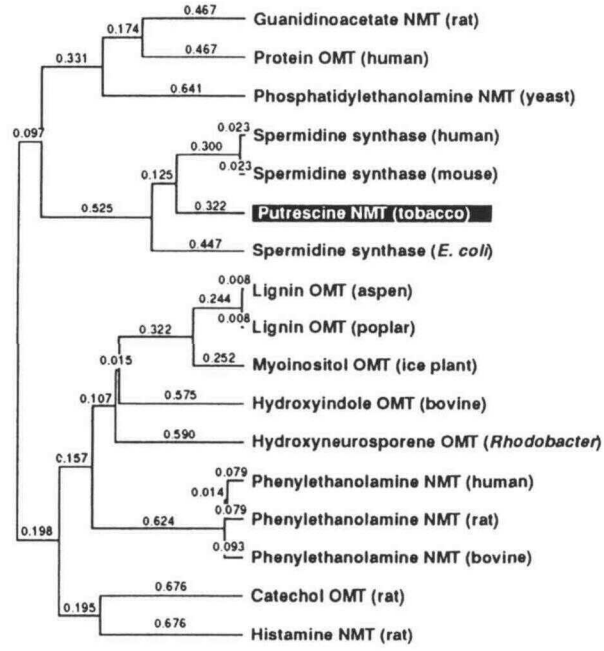

Figure 13. PMT and SPDS Form a Distinct Subgroup of Methyltransferases.

A phylogenetic tree showing the evolutionary relationship among methyltransferases and SPDS was constructed by the UPGMA Tree program in GeneWorks (IntelliGenetics, Inc., Mountain View, CA). The length of the horizontal lines indicates the evolutionary distance between one protein and another. NMT and OMT, respectively, represent *N*-methyltransferase and *O*-methyltransferase.

mutant alleles may be explained by postulating that Nic1 and Nic2 perform the same function in regulating target genes (i.e., *PMT*, *A622*, and probably several other genes). The most probable case is that *Nic1* and *Nic2* are duplicate genes, each originating from one of the two tobacco progenitors. The stronger allele *nic1* might be a null mutation, whereas the weaker *nic2* allele might be a non-null mutation with residual Nic function. In this case, a mutation at one of the two *Nic* genes would reduce Nic function to no less than half of the wild type, whereas a double mutation would severely affect Nic function. Further molecular characterization, including cloning of the *Nic* loci, is necessary to elucidate their functions.

METHODS

Plant Materials

Seeds of four Burley 21 tobacco lines (germ plasms HPB21, HIB21, LIB21, and LAB21) were obtained from the U.S. Department of Agriculture (Beltsville, MD). These lines were isogenic, except for the two low-nicotine loci, and had genotypes of *Nic1/Nic1 Nic2/Nic2*, *Nic1/Nic1 nic2/nic2*, *nic1/nic1 Nic2/Nic2*, and *nic1/nic1 nic2/nic2*, which had been previously referred to as AABB, AAbb, aaBB, and aabb, respectively (Legg and Collins, 1971).

Sterile seedlings of the four genotypes were grown in light on half-strength auxin-free B5 medium (Gamborg et al., 1968) supplemented with 1.5% (w/v) sucrose and 1% (w/v) agar. Seedling roots were excised and transferred to HF medium (Mano et al., 1989), supplemented with 3% (w/v) sucrose and 3 μM indolebutyric acid (IBA), and cultured in the dark on a rotary shaker at 90 rpm. Two weeks after subculture, the cultured roots were harvested, immediately frozen with liquid nitrogen, and kept at −80°C until use. In one experiment, the cultured roots were rinsed well with HF medium without auxin and transferred to the auxin-free medium. Twenty hours after the transfer, filter-sterilized IBA solution was added to the culture medium to a final concentration of 3 μM.

Tobacco plants were grown from seed in soil for a month and transferred to pots containing a nutrient solution described by Takahashi and Yoshida (1957). Six plants were hydroponically grown in aerated nutrient solution in a greenhouse for 3 months, during which they developed ~10 flowers or flower buds. Flower heads and several young leaves were then removed from three plants, leaving 16 leaves on each plant. A portion of the roots were harvested from each plant after indicated intervals and pooled for RNA extraction. Three control plants were not decapitated.

Alkaloid and Polyamine Analyses

Nicotine and nornicotine were analyzed by gas liquid chromatography as described previously (Hibi et al., 1992), except that the column temperature was held at 120°C for 8 min and then increased from 120 to 250°C at a rate of 8°C/min.

Polyamines were first divided into water-soluble, trichloroacetic acid–soluble, and trichloroacetic acid–insoluble forms, and each form was quantified separately by HPLC after converting it to dansyl derivatives (Hibi et al., 1992).

RNA Isolation and cDNA Synthesis

Total RNA was extracted from plant tissues as described by Mohnen et al. (1985). Poly(A)$^+$ RNA (5 μg) was purified from total RNA of cultured roots of wild-type and *nic1 nic2* mutant tobacco lines using an mRNA purification kit (Pharmacia), and the purified RNA was used to construct double-stranded cDNA for the subtracted cDNA probes, using cDNA Synthesis System Plus (Amersham). A phagemid cDNA library was constructed from the wild-type poly(A)$^+$ RNA using a Librarian II kit (Invitrogen, San Diego, CA).

Differential Screening

Subtractive hybridization was performed essentially as described by Wang and Brown (1991). Briefly, double-stranded cDNAs made from wild-type and mutant roots were fragmented and ligated to linkers for polymerase chain reaction amplification. Excess mutant cDNA was biotinylated and hybridized with wild-type cDNA, after which DNA fragments common to both cDNA pools were removed by binding to streptavidin and extraction with organic solvent. One subtraction cycle consisted of long (20 hr) and short (2 hr) hybridization steps. After one cycle, the subtracted cDNA pool was amplified by polymerase chain reaction, and the subtraction cycle was repeated two more times to yield the subtracted cDNA probe. A more detailed description of this procedure is provided elsewhere (Hashimoto et al., 1993).

Duplicate filters made from the wild-type cDNA library were hybridized separately with the subtracted probe and the nonsubtracted cDNA probe from the mutant root in 50% formamide, 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 20 mM sodium phosphate, 5 mM EDTA, 0.1% SDS, and 100 μg/mL sonicated salmon sperm DNA at 42°C for 16 hr and then washed twice with 0.2 × SSC, 0.1% SDS at 42°C for 15 min and twice with the same buffer at 65°C for 30 min. DNA probes were radiolabeled by random hexamer priming. Clones that hybridized strongly to the subtracted probe and weakly, or not at all, to the mutant probe were chosen and further screened by dot blot hybridization. Eleven clones were confirmed to be positive and were classified into two groups based on cross-hybridization between them.

Nucleotide sequences of the cDNA inserts of the representative clones were determined on both strands using an ALF I DNA sequencer (Pharmacia) and a DNA polymerase sequencing kit (Pharmacia).

RNA and DNA Gel Blot Analyses

For RNA gel blot analysis, 20 μg of total RNA was electrophoresed on 1% formaldehyde agarose gels, blotted onto GeneScreen Plus membranes (Du Pont), and hybridized with the indicated radiolabeled probes at 42°C for 20 hr as described for differential screening. Whole cDNA inserts were used for the putrescine *N*-methyltransferase (*PMT*), *A622*, and β-ATPase probes. The levels of hybridization signals were quantified by the Radioanalytic Imaging System (AMBIS Systems, San Diego, CA). For normalizing the sample loading on each lane, the levels of *PMT* and *A622* mRNAs were divided by the level of β-ATPase mRNA.

Total genomic DNA was isolated from tobacco leaves (variety Burley 21) as described by Murray and Thompson (1980), and 20 μg of genomic

DNA was digested with restriction enzymes, followed by electrophoresis on 0.8% agarose gels. The gels were subsequently denatured and neutralized by standard procedures (Meinkoth and Wahl, 1984). Subsequent procedures were performed as described above.

Expression in *Escherichia coli*

An NcoI site was introduced at the first ATG of the *A411* and *A622* cDNA inserts in pcDNA II using a Transformer site-directed mutagenesis kit (Clontech, Palo Alto, CA). NcoI-BamHI fragments containing *A411* and *A622* coding sequences were excised and cloned into pET-3d (Novagen, Madison, WI) to give pET411 and pET622, respectively. pET411 was introduced into *E. coli* HT551, which has deletions at the *speE* and *speD* loci (Tabor et al., 1986), and the recombinant bacteria were cultured in M9 medium containing 10 μg/mL calcium pantothenate, 1 μg/mL thiamine, 0.1% casein HCl hydrolysate, and 50 μg/mL ampicillin at 37°C until the OD_{600} of the culture reached 0.5, when isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 mM. After 1 hr, M13/T7 phage (8×10^{10} plaque-forming units) was added to the culture and incubation continued at 16°C overnight. Bacterial cells were then recovered by centrifugation, suspended in 100 mM potassium phosphate buffer, pH 7.5, 0.5% sodium ascorbate, 5 mM EDTA, and 5 mM DTT, and sonicated for 1 min. After centrifugation of the homogenate at 22,000*g* for 15 min, the supernatant was passed through a PD-10 column (Pharmacia), from which the desalted crude enzyme was eluted with 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM DTT. *E. coli* HT573, which contains pSPD19 (Tabor and Tabor, 1987), was used as a source of spermidine synthase (SPDS).

pET622 was introduced to *E. coli* BL21(DE3). The recombinant bacteria were cultured, and the recombinant A622 protein was expressed according to the method of Studier et al. (1990). Crude enzyme solution was prepared as described for A411.

Enzyme Assays

PMT activity was measured by HPLC as described previously (Hibi et al., 1992). For the assay of SPDS, the *S*-adenosylmethionine (SAM) component in the PMT assay mixture was replaced with decarboxylated SAM (dSAM). The reaction product spermidine was converted to the dansyl derivative and quantified by HPLC, as described previously (Hibi et al., 1992). Enzyme reactions were carried out at 30°C for 60 min using 66 μg of crude protein. Isoflavone reductase (IFR) activity was measured according to Paiva et al. (1991).

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