

Molecular characterization of quinolate phosphoribosyltransferase (QPRTase) in *Nicotiana*

Steven J. Sinclair, Kristina J. Murphy, Carlie D. Birch and John D. Hamill*

Department of Biological Sciences, Monash University, Clayton Campus, Melbourne, Victoria 3168, Australia
(*author for correspondence; e-mail: john.hamill@sci.monash.edu.au)

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Abstract

Quinolate acid phosphoribosyltransferase (QPRTase), a key enzyme in nicotinamide adenine dinucleotide (NAD) biosynthesis, also plays an important role in ensuring nicotinic acid is available for the synthesis of defensive pyridine alkaloids in *Nicotiana* species. In this study, cDNAs for QPRTase were characterized from *N. rustica* and *N. tabacum*. Deduced proteins from both cDNAs are almost identical and contain a 24 amino acid N-terminal extension, not reported in other QPRTases, that has characteristics of a mitochondrial targeting sequence. In *N. tabacum* and *N. sylvestris*, both of which contain nicotine as the major pyridine alkaloid, QPRTase transcript was detected in roots, the site of nicotine synthesis, but not in leaves. QPRTase transcript levels increased markedly in roots of both species 12–24 h after damage to aerial tissues, with a concomitant rise in transcript levels of putrescine *N*-methyltransferase (PMT), another key enzyme in nicotine biosynthesis. In *N. glauca*, however, in which anabasine represents the major pyridine alkaloid, QPRTase transcript was detected in both leaf and root tissues. Moreover, wound induction of QPRTase but not PMT was observed in leaf tissues, and not in roots, 12–24 h after wounding. Southern analysis of genomic DNA from the *Nicotiana* species noted above, and also several others from within the genus, suggested that QPRTase is encoded by a small gene family in all the species investigated.

Abbreviations: LDC, lysine decarboxylase; ODC, ornithine decarboxylase; PMT, putrescine *N*-methyl transferase; QPRTase, quinolate phosphoribosyltransferase; SPDS, spermidine synthase

Introduction

The plant kingdom is a rich source of biologically active secondary metabolites with the largest and most diverse group being the alkaloids, many of which have therapeutic value (Kutchan, 1995). There is increasing evidence that such metabolites in plants have important ecological roles, particularly as defensive agents against herbivory (Wink, 1988; Baldwin and Preston, 1999). The production of secondary metabolites, particularly nitrogen-containing alkaloids, is metabolically expensive, and many plants have the capacity to

regulate alkaloid levels in response to environmental or developmental cues (Baldwin and Ohnmeiss, 1993; Wink, 1997; Baldwin and Preston, 1999).

The pyridine alkaloid nicotine exhibits toxicity to animals due to its ability to mimic acetylcholine, and high concentrations can result in paralysis and death (Karban and Baldwin, 1997). Studies involving commercial tobacco (*Nicotiana tabacum* L.) have shown that nicotine is synthesized in the roots and is translocated to aerial tissues (Dawson, 1941, 1942) where it is stored in vacuoles and acts as a defensive agent (Baldwin and Schmelz, 1994; Ohnmeiss *et al.*, 1997). The practice of removing the flowering shoot (topping) shortly before harvesting has long been practiced in the tobacco industry as a means of increasing the

The nucleotide sequences reported here have been deposited in the EMBL database under accession numbers AJ243436 (*N. rustica* QPRTase cDNA) and AJ243437 (*N. tabacum* QPRTase cDNA).

alkaloid content of leaves. One or two days after such damage to the aerial tissues of *N. tabacum*, a substantial increase in the activity of nicotine biosynthetic enzymes is observed in the roots (Mizusaki *et al.*, 1973; Saunders and Bush 1979), followed by an increase in nicotine levels for a period lasting several days (Saunders and Bush, 1979). Wounding of the aerial tissues of *N. sylvestris* Speg. and Comes, the maternal progenitor species of *N. tabacum*, produces a similar rise in nicotine levels (Baldwin, 1988; Baldwin and Schmelz, 1994). The jasmonate signalling system has been implicated as a key component of the mechanism(s) governing wound stimulation of nicotine biosynthesis in *Nicotiana* (Zhang and Baldwin, 1997; Baldwin *et al.*, 1997). Significantly, transcript levels of several genes important in nicotine biosynthesis were found to be up-regulated in cell suspension cultures of *N. tabacum* in response to treatment with methyl jasmonate, followed by a significant rise in the nicotine content of tissues (Imanishi *et al.*, 1998).

In *N. glauca* Grah., the tree tobacco, anabasine is the main pyridine alkaloid in leaf tissues (>85% of the alkaloid fraction), with a mixture of pyridine alkaloids present in root tissues (>50% anabasine, 35% nicotine, 10% anatabine) (Saitoh *et al.*, 1985; Sisson and Severson, 1990). This alkaloid is also toxic to herbivores and is regarded as being responsible for human fatalities and life-threatening paralysis following the consumption of tree tobacco foliage (Castorena *et al.*, 1987; Mellick *et al.*, 1999). Anabasine levels increase in leaf tissues of *N. glauca* in response to wounding and it is thought that this alkaloid may protect against excessive herbivory in a manner similar to that of nicotine in leaf tissue of *N. tabacum* and *N. sylvestris* (Baldwin and Ohnmeiss, 1993).

The production of both nicotine and anabasine involves the condensation of the primary metabolite nicotinic acid with another nitrogen containing metabolite. In the case of nicotine this is *N*-methyl pyrrolinium (Leete, 1979; Feth *et al.*, 1986; Wagner *et al.*, 1986a), and in the case of anabasine, nicotinic acid is condensed with Δ^1 -piperideinium (Leete, 1979; Walton and Belshaw, 1988). Each of these metabolites is derived from a separate area of primary metabolism (Figure 1).

Nicotine synthesis requires the concerted regulation of the metabolic pathways leading to both the production of nicotinic acid and to *N*-methyl pyrrolinium (Feth *et al.*, 1986; Wagner *et al.*, 1986b). The pathway leading from the primary metabolite putrescine to *N*-methyl pyrrolinium is primarily regu-

lated by the enzyme putrescine *N*-methyltransferase (PMT) (EC 2.1.1.53) (Feth *et al.*, 1986; Hibi *et al.*, 1992) and transcript levels of PMT have been shown to be strongly up-regulated in the roots of *N. tabacum* within 24 h of foliage damage (topping) (Hibi *et al.*, 1994; Riechers and Timko, 1999). PMT cDNAs from *N. tabacum* (Hibi *et al.*, 1994), *N. sylvestris* (Hashimoto *et al.*, 1998a) and also from the tropane alkaloid-synthesizing species *Atropa belladonna* (Suzuki *et al.*, 1999) show significant deduced amino acid sequence homology to spermidine synthase (SPDS) (EC 2.5.1.16), an enzyme of primary metabolism required for polyamine synthesis which also uses putrescine as a substrate. Such sequence homology strongly suggests that PMTs are derived from SPDSs (Hibi *et al.*, 1994; Hashimoto *et al.*, 1998a, b; Suzuki *et al.*, 1999) supporting the more general hypothesis that many secondary metabolic pathways are derived from primary metabolic pathways.

The regulation of Δ^1 -piperideinium production has also received attention in recent years with studies indicating that lysine decarboxylase (LDC) (EC 4.1.1.18) is an important rate-limiting step in anabasine synthesis in *Nicotiana* (Figure 1). Feeding of cadaverine to *N. rustica* L. root cultures led to the production of increased anabasine, at the expense of nicotine (Walton *et al.*, 1988). Over-expression of a bacterial gene encoding LDC in transformed roots of *N. tabacum* produced lines with elevated anabasine levels and altered anabasine: nicotine ratios (Fecker *et al.*, 1993; Herminghaus *et al.*, 1996). Together, these results highlight the close link between nicotine and anabasine synthesis, which apparently compete for nicotinic acid.

In contrast to *N*-methyl pyrrolinium and Δ^1 -piperideinium synthesis, little is known about the molecular controls governing the synthesis of nicotinic acid in *Nicotiana*, which is central to the production of all pyridine alkaloids. Analysis of the enzymes involved in nicotinic acid synthesis has shown that the activity of quinolinate phosphoribosyltransferase (QPRTase) (nicotinate-nucleotide pyrophosphorylase, EC 2.4.2.19) is of key importance in controlling the supply of nicotinic acid for pyridine alkaloid synthesis, whilst regulation of other enzymes in the pyridine nucleotide cycle may represent additional minor points of control (Wagner and Wagner, 1985; Wagner *et al.*, 1986c). In *N. tabacum*, the activity of QPRTase in roots, like that of PMT, has been shown to increase several-fold 24–48 h after wounding of aerial tissues (Mizusaki *et al.*, 1973; Saunders and Bush, 1979)

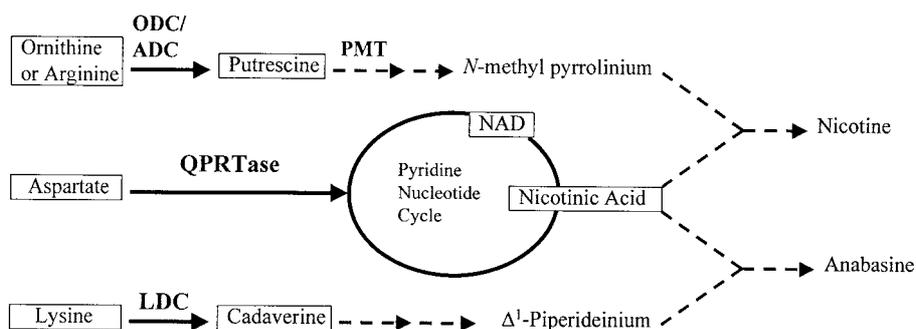


Figure 1. A simplified diagram of nicotine and anabasine synthesis in *Nicotiana*. Steps representing primary metabolism are shown with full arrows, whereas steps involved in the production of secondary metabolites are shown with dotted arrows. Similarly, primary metabolites are boxed, while secondary metabolites are not boxed. For further information, see Feth *et al.* (1986), Leete (1979), Wagner *et al.* (1986a, b) and Walton and Belshaw (1988).

and to be correlated with nicotine production both *in planta* and in callus tissue cultured *in vitro* (Wagner *et al.*, 1986a; Feth *et al.*, 1986).

Interestingly, QPRTase also plays another role as an essential enzyme of primary metabolism, catalysing the entry point step in the pyridine nucleotide cycle, which includes nicotinic acid as an intermediate (Figure 1). Thus, while PMT has apparently evolved from a gene involved in primary metabolism to have a specific function in secondary metabolism (Hibi *et al.*, 1994; Hashimoto *et al.*, 1998a), QPRTase activity is required for both primary and secondary metabolism in *Nicotiana*. It is in its role as a primary metabolic enzyme, essential for NAD synthesis, that QPRTase has mostly been studied. In human biology it may play a role in neurodegenerative disease (Fukuoka *et al.*, 1998), and it has recently been considered a potential target point for controlling infection by the pathogen *Mycobacterium tuberculosis* (Sharma *et al.*, 1998). Genes encoding QPRTase have been identified in *Salmonella typhimurium* (Hughes *et al.*, 1993), *Escherichia coli* (Bhatia and Calvo, 1995) and more than a dozen other microbes, including yeast (Eads *et al.*, 1997; Chang and Zylstra, 1999) and, recently, from man (Fukuoka *et al.*, 1998). Key regions of the enzyme are conserved amongst all these organisms. The crystal structure of QPRTase has been elucidated in *Salmonella* (Eads *et al.*, 1997) and in *M. tuberculosis* (Sharma *et al.*, 1998).

To date, molecular studies on QPRTase in plants have been limited. An *Arabidopsis* EST noted as a potential homologue of the *Salmonella nadC* (QPRTase) gene was submitted to the GenBank database in 1996 by Cook *et al.* (accession number F20096). A segment of expressed sequence from tobacco cells treated with

methyl jasmonate was subsequently reported, which also showed similarity to known QPRTases (accession number AB005979; Imanishi *et al.*, 1998). Recently, genomic sequence data from *Arabidopsis thaliana* chromosome II was made available (Lin *et al.*, 1999) which includes a possible complete coding sequence for QPRTase (accession number AC006200).

As has been noted, QPRTase is of particular interest in *Nicotiana* because it has important roles in both primary and secondary metabolism. This report details the molecular characterisation of QPRTase from *N. tabacum* and *N. rustica* L., allotetraploids derived from different progenitor species (Goodspeed and Thompson, 1959; Cherep and Komarnitskii, 1991; Riechers and Timko, 1999). Expression characteristics of QPRTase are reported from *N. tabacum* and *N. sylvestris* (mainly nicotine-producing species) and from *N. glauca* (mainly anabasine-producing) and compared with PMT expression in these species.

Materials and methods

Source of plant material and culture conditions

Seeds of high-alkaloid *N. tabacum* cultivar NC95 and the corresponding low-alkaloid cultivar LAFC53 (Chaplin, 1975) were kindly supplied by Dr V. Sisson, USDA. Seeds of *N. rustica* cv. V12 (Jinks *et al.* 1981) were kindly supplied originally by Professor J. Jinks, University of Birmingham, UK. Seeds of *N. sylvestris* were obtained from a commercial seed company (Fothergills, UK). Seeds of *N. glauca*, *N. tomentosiformis* and other species noted in the text were kindly supplied by Mr B. McGuinness, University of Melbourne, Australia. Several plants of each species

were initially grown in compost in a (PC2) greenhouse and seeds collected from a small number of founder plants for further analysis. Growth conditions for plants used for RNA analysis were as described previously (Lidgett *et al.*, 1995) except that growth was in rockwool fibre pellets (Growool Horticultural Systems, NSW, Australia) supported in open tubes with their roots submerged in ca. 2 cm of Hoagland's hydroponic medium (Mason, 1990). Plants were transferred to fresh medium 5–7 days before initiation of wounding experiments, which were undertaken at about midday using 6–8-week old plants. Root cultures of *N. tabacum*, used as a source of RNA for northern blotting and cDNA library construction, were grown as previously described (Hamill *et al.*, 1986) and harvested 11 days after subculture, in the early to mid stages of the growth cycle.

Screening cDNA libraries of *Nicotiana* to obtain cDNA clones for QPRTase

Libraries were synthesized in the vector UniZap (Stratagene) using mRNA isolated from alkaloid-producing transformed root cultures of *N. tabacum* cv. SC58 and *N. rustica* cv. V12 (Parr and Hamill, 1987), amplified once, stored at -70°C and tited as per the manufacturer's instructions (Stratagene). To obtain a probe for QPRTase, degenerate oligonucleotides were synthesized representing several conserved regions in the amino acid sequence of QPRTase from yeast, man and several bacteria. These were used to prime cDNA synthesis with mRNA from roots of wounded *N. tabacum* plants. Nested PCR was performed with oligonucleotides based on the putative QPRTase EST sequence AB005879 of *N. tabacum* (Imanishi *et al.*, 1998) to amplify a DNA fragment of ca. 350 bp which was purified from a 1% agarose gel (Gel Spin purification kit, Worthington Biochemica, ScimaR, Australia) and labelled for use as a probe with $\alpha^{[32]}\text{P}$ -dATP with a MultiPrime DNA labelling kit (Geneworks, Australia). Duplicate membranes (Hybond N⁺, Amersham) representing both cDNA libraries were fixed, hybridized to the probe and washed at high stringency according to the manufacturer's instructions, except that the hybridization solution contained $2\times$ SSPE, 7% SDS, 5 mg/ml skim milk powder, and 100 mg/ml PEG 20,000. Several purified plaques from each library were converted to plasmids with the *in vivo* excision capacity of the UniZap system. Those containing inserts of the expected size (ca. 1.3 kb deduced from preliminary northern blots) were identified

by PCR, using oligonucleotides representing T3 and T7 sequences in pBluescript and designated pTQPT (*N. tabacum*) and pRQPT (*N. rustica*).

Sequence analysis of clones

Two putative full-length cDNAs (pTQPT1 from *N. tabacum* and pRQPT1 from *N. rustica*) were fully sequenced with pBluescript T7 and T3 primers, together with several oligonucleotides specific to the cDNA inserts. Sequencing was performed using an ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer-Applied Biosystems), and an Applied Biosystems 373 A DNA sequencer. DNA sequence analysis was done via the Australian National Genomic Information Service (ANGIS) (<http://www.mel1.angis.org.au>).

Functional complementation in *E. coli*

QPRTase-deficient (*nadC*⁻) *E. coli* cells (TH265) were kindly provided by Prof. K. Hughes (University of Washington) and maintained on LB medium as described by Fukuoka *et al.* (1998). The inability of these cells to grow on minimal medium (Yates and Pardee, 1956) was confirmed, as was their ability to grow on this medium when supplemented with nicotinic acid (0.2 $\mu\text{g}/\text{ml}$). Plasmids pTQPT1 and pRQPT1 were transformed into these cells by the method described by Chung and Miller (1988). Control TH265 cells were transformed with pBluescript only. Transformants were selected and maintained on LB-ampicillin plates.

In pTQPT1, the coding sequence is in frame with the 5' region of the *lac-Z* coding sequence present in pBluescript. Thus a fusion protein consisting of the N-terminal portion of the Lac-Z protein as well as the *N. tabacum* QPRTase protein is expected upon induction with IPTG. In the case of pRQPT1, the *N. rustica* QPRTase coding sequence is not in frame with the *Lac-Z* gene in pBluescript. Here, expression of QPRTase would be expected to rely on the occasional translation of a functional protein using a downstream AUG codon. This approach was successfully employed by Fukuoka *et al.* (1998) to demonstrate the functional cloning of the human QPRTase cDNA, also with *E. coli* strain TH265.

Analysis of transcript levels in *Nicotiana*

Aerial tissues of 6–8-week old plants grown in hydroponic medium were wounded with a fabric pattern

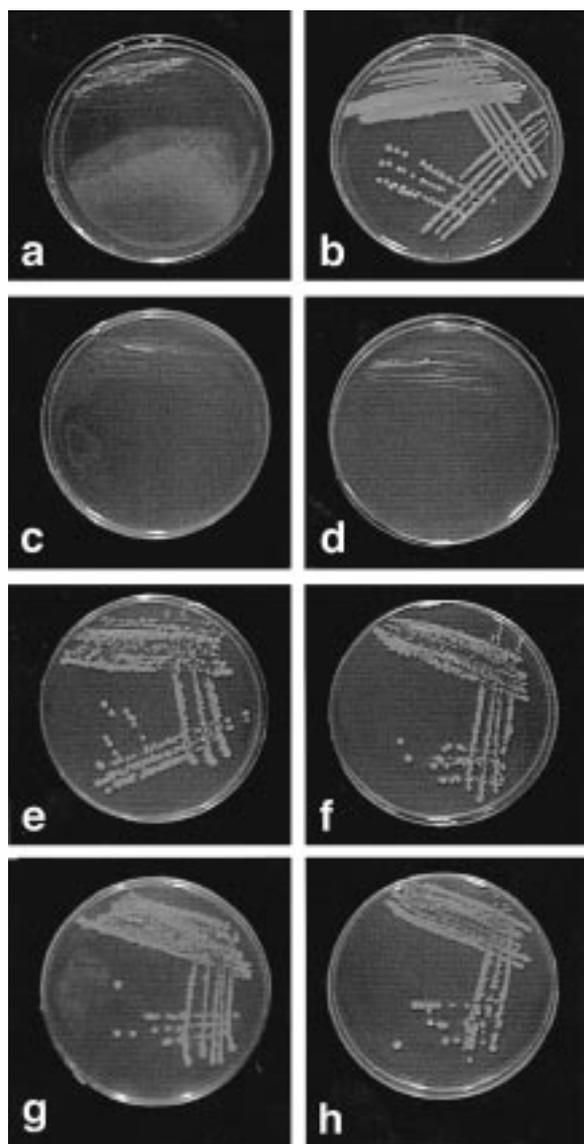


Figure 2. Complementation of QPRTase-deficient (*nad C*⁻) *E. coli* strain TH265 with *Nicotiana* cDNAs encoding QPRTase. a. TH265 cells cultured on minimal medium. b. TH265 cells cultured on minimal medium containing 0.2 $\mu\text{g/ml}$ nicotinic acid. c. TH265 cells containing pBluescript cultured on minimal medium containing 0.5 mM IPTG. d. TH265 cells containing pBluescript cultured on minimal medium containing 0.5 mM IPTG. e. TH265 cells containing pTQPT1 cultured on minimal medium. f. TH265 cells containing pTQPT1 cultured on minimal medium containing IPTG. g. TH265 cells containing pRQPT1 cultured on minimal medium. h. TH265 cells containing pBluescript cultured on minimal medium containing IPTG.

wheel (Baldwin *et al.*, 1994) to trace four lines of holes on the two upper-most fully developed leaves, together with the removal of the apical growing tip, to simulate decapitation. Root and leaf tissue was harvested, rinsed in cold water, blotted dry, snap-frozen in liquid nitrogen and stored at -70°C until required. RNA extraction, electrophoresis in a formaldehyde gel and transfer to positively charged nylon membranes (Amersham N⁺), together with northern hybridization and washing was undertaken according to Hamill and Lidgett (1997) with ExpressHyb (Clontech) hybridization solution in accordance with the manufacturer's instructions. An RNA ladder (Gibco-BRL, 0.24–9.5 kb) was used as a standard to determine transcript sizes. The insert in pTQPT1 was excised as an *EcoRI/XbaI* fragment of 1.3 kb and used as a probe for QPRTase. To generate a probe for PMT, the coding sequence of PMT (Hibi *et al.*, 1994) was excised from PET-PMT as a *BamHI/EcoRI* fragment. The plasmid PET-PMT was kindly supplied by Prof. T. Hashimoto, Nara Institute of Technology, Japan.

Southern analysis of QPRTase in Nicotiana

DNA was extracted from young leaf material from healthy plants on a CsCl_2 gradient as described in Hamill and Lidgett (1997) and 10 μg DNA per sample was digested to completion with appropriate restriction enzymes in a large volume as detailed in Sambrook *et al.* (1989). DNA was transferred to positively charged nylon membranes by capillary action (Amersham) after electrophoresis through a 1% agarose TBE gel containing ethidium bromide. Membranes were hybridized in ExpressHyb hybridization solution (Clontech) and washed at high stringency in accordance with the manufacturer's instructions.

Results

Identification and characterization of cDNA clones

When screened with a PCR product representing the putative QPRTase EST sequence reported by Imanishi *et al.* (1998), the root cDNA libraries of both *Nicotiana* species yielded a large number of positive plaques (ca. 1/600 plaques from the *N. rustica* library, and 1/2500 plaques from the *N. tabacum* library). Sequencing of *N. tabacum* cDNA clone pTQPT1 (accession number AJ243437) and *N. rustica* clone pRQPT1 (AJ243436) showed that both are capable of encoding almost identical proteins, each consisting of 351

amino acids. The deduced proteins differ from each other at only seven amino acid residues. Correspondingly, the homology at the DNA level between both cDNAs is extremely high in the coding region (98.2% identity). The untranslated (UTR) regions are also very similar, the 67 bp 5'-UTRs sharing 82% identity. The *N. tabacum* cDNA, however, contains a 66 bp region at the extreme 3' end of its 3'-UTR which is not found in the *N. rustica* cDNA. In the regions that are shared, however, the 3'-UTRs show 94% identity. The 3'-UTRs are both AT(U)-rich, and contain several possible polyadenylation sites (Rothnie, 1996).

Sections of sequence in both pTQPT1 and pRQPT1 are almost identical to the methyl jasmonate-induced EST (AB005879) reported previously by Imanishi *et al.* (1998) and suggested by these authors to be a fragment of the *N. tabacum* QPRTase gene. Furthermore, both cDNAs are capable of encoding proteins with residues characteristic of conserved regions in previously reported QPRTases (Eads *et al.*, 1997) (see also Figure 3 below). Together, these observations suggest that pRQPT1 and pTQPT1 encode QPRTase. Recently, a full-length cDNA identified as QPRTase was reported from *N. tabacum* strain Bright Yellow (accession number AB0038494). This sequence has over 99% identity with the pTQPT1 sequence noted here.

Functional complementation of QPRTase-deficient E. coli cells

In order to confirm that these cDNAs encode QPRTase, complementation tests were undertaken with the QPRTase-deficient (*nadC*⁻) *E. coli* strain TH265, which normally requires a supplement of nicotinic acid to grow on minimal medium (Fukuoka *et al.*, 1998) (Figure 2a, b). Transformation of these cells with pBluescript alone did not allow TH265 cells to form colonies in the absence of a nicotinic acid supplement (Figure 2c, d). When transformed with pRQPT1 or pTQPT1, however, TH265 cells were able to form colonies on minimal medium lacking a nicotinic acid supplement, without the need for induction by IPTG (Figure 2e–h). These results provide compelling evidence that both pRQPT1 and pTQPT1 encode QPRTase. The observation that TH265 cells containing pTQPT1 and pRQPT1 grew equally well on minimal media, with or without the presence of IPTG, is consistent with the results of Fukuoka *et al.* (1998) who used pBluescript to express human QPRTase in *E. coli* strain TH265, and found this system to be

somewhat leaky with respect to IPTG induction. In their study, the human QPRTase cDNA was inserted out of frame with respect to the *lacZ* gene in pBluescript and thus relied on 'leaky scanning' to express a non-fusion QPRTase protein (Fukuoka *et al.*, 1998). The same interpretation can presumably account for complementation of TH265 cells with pRQPT1. In pTQPT1, however, the insert was cloned in frame with the *lacZ* gene. At the present time, it remains unclear whether a functional fusion protein is produced, or if expression from pTQPT1 also relies on leaky scanning.

Detailed comparison of Nicotiana QPRTase deduced protein sequences with QPRTase sequences from other organisms

The deduced proteins from *N. rustica* and *N. tabacum* show significant amino acid similarity to previously published QPRTase sequences from man, yeast, several prokaryotes, and also the presumed QPRTase sequence identified in *Arabidopsis* (Figure 3). The active site in QPRTase, as determined by Eads *et al.* (1997), is highly conserved between QPRTase from all these species. The level of sequence homology between the *Nicotiana* deduced amino acid sequences and the *Arabidopsis* sequence (79.8% amino acid identity) is particularly striking. The deduced *Nicotiana* QPRTase proteins are, however, longer at the N-terminus than any other QPRTases characterised to date. In fact, the predicted *Nicotiana* translation initiation codon is over fifty nucleotides upstream from the approximately equivalent location in other species, most obviously the presumed QPRTase from *Arabidopsis* (Figure 3). Thus, *Nicotiana* QPRTase apparently carries an N-terminal extension not found on other QPRTase enzymes.

QPRTase is expressed preferentially in roots of N. tabacum and is up-regulated after foliage damage

Previous work had established that increased QPRTase enzyme activity and increased PMT activity were found in roots of *N. tabacum* after damage to the foliage (Mizusaki *et al.*, 1973; Saunders and Bush, 1979). Saunders and Bush (1979) also demonstrated higher basal levels of QPRTase and PMT activity in the roots of a high-alkaloid variety of *N. tabacum* relative to those observed in a low-alkaloid variety. The high-alkaloid variety also had a much greater capacity to increase the activities of both enzymes in response

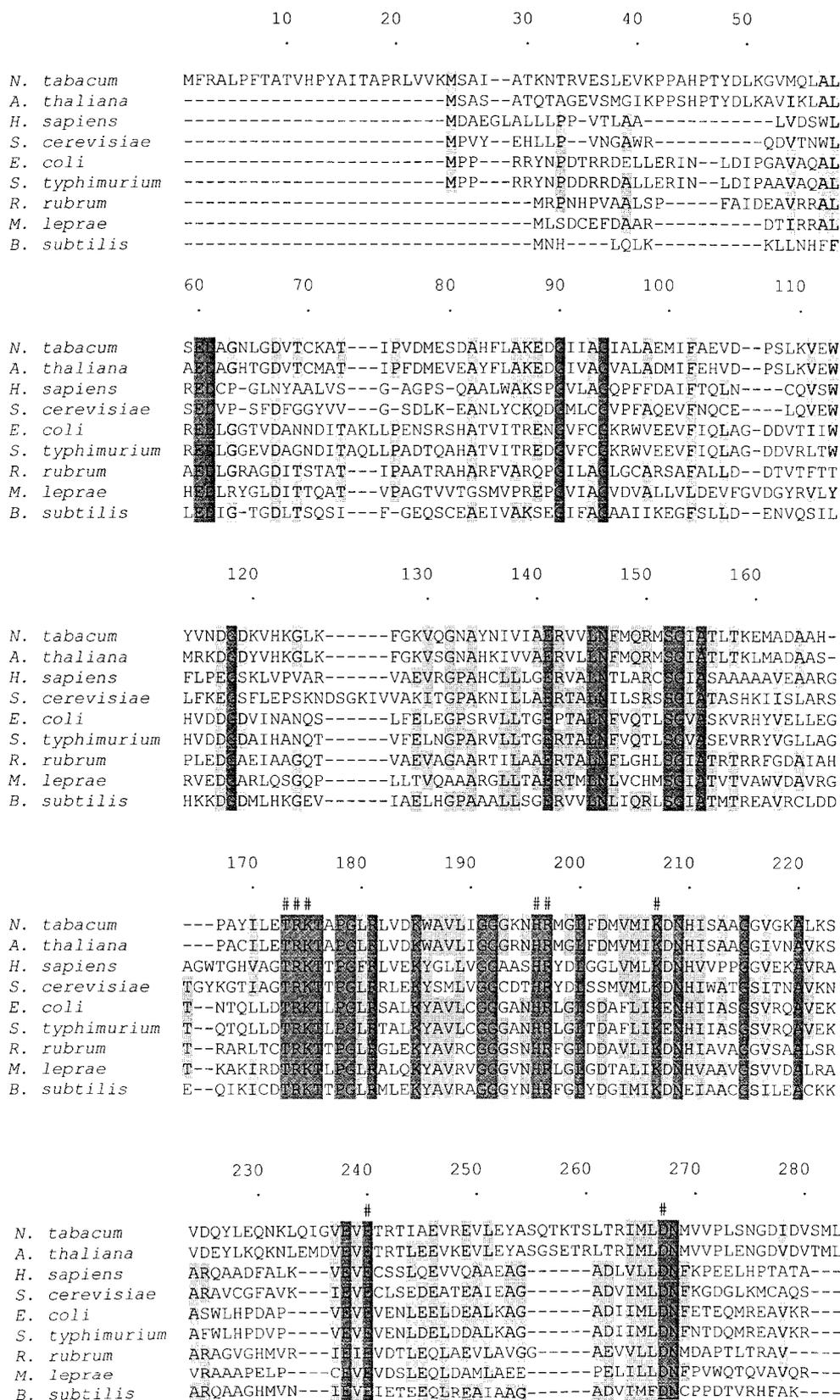


Figure 3. Alignment of the deduced amino acid sequence of *N. tabacum* QPRTase (TQPT1, 351 amino acids) with other QPRTase sequences. *Arabidopsis thaliana* (AC006200), *Homo sapiens* (D78177), *Saccharomyces cerevisiae* (P43619), *Escherichia coli* (S05571), *Salmonella typhimurium* (L07292), *Rhodospirillum rubrum* (U20508), *Mycobacterium leprae* (P46714), and *Bacillus subtilis* (D4071). Totally conserved residues are shaded dark grey, while areas of 'similar' residues determined by 'PRETTYBOX' are shaded lighter grey. Conserved residues found at the active site of *S. typhimurium* QPRTase (Eads *et al.*, 1997) are denoted #.

	290	300	310	320	330	340
			#			
<i>N. tabacum</i>	KEAVELING--RFDT	ASGNVTLETVHKIGQ	TGVTYI	SSGALTQSVKAL	DISL	KIDTELA
<i>A. thaliana</i>	KDAVELING--RFET	ASGNVTLETVHKIGQ	SGVTFI	SSGALTHSVKAL	DISL	KIDTELA
<i>H. sapiens</i>	--LKAQFPS---VAV	ASGGITLDNLPQFC	GHIDVISM	GMLTQAVPAL	DFSL	KLFAKEV
<i>S. cerevisiae</i>	--LKNKWN	GKKHFLLECGGL	NLDNLEEYLC	DDIDIYST	SSIHQ	GTTPVIFSLKL----
<i>E. coli</i>	-----TNG--KALL	EVGNVTDKTLRE	FAETGVDFI	SVGALTKHVQAL	QLSM	-----
<i>S. typhimurium</i>	-----VNG--QARL	EVGNVTAETLRE	FAETGVDFI	SVGALTKHVRAL	QLSM	-----
<i>R. rubrum</i>	----DMVAG--RLVT	ASGGVSLDTIAA	LAESGV	DYISVGAL	THSVTTL	IGL--DIVVA
<i>M. leprae</i>	---RDIRAP--TVLL	ESGGLENAAI	YAGTGV	DYLAVGAL	THSVRIL	IGL--DL*--
<i>B. subtilis</i>	-----LTPA--NIKT	ASGGITLES	LPAFKGT	GVNYISL	GFLTHSVKSL	DI*-----
	350					
<i>N. tabacum</i>	LEVGRRTKQA*					
<i>A. thaliana</i>	LEVGRRTKRA*					
<i>H. sapiens</i>	APVPKIH*---					
<i>S. cerevisiae</i>	-----AH*---					
<i>E. coli</i>	RFR*-----					
<i>S. typhimurium</i>	RFC*-----					
<i>R. rubrum</i>	PPKAERA*---					
<i>M. leprae</i>	-----					
<i>B. subtilis</i>	-----					

Figure 3. Continued.

to wounding. Furthermore, Hibi *et al.* (1994) demonstrated that PMT transcript is up-regulated in roots, but not leaves, of a high-alkaloid variety upon removal of the flowering shoot (topping). Consistent with each of these observations, we observed that QPRTase transcript levels, together with those of PMT, are higher in roots of unwounded high-alkaloid *N. tabacum* than in similar tissues of low-alkaloid *N. tabacum*, and that transcript levels of both QPRTase and PMT increased in roots of the high-alkaloid variety but not in roots of the low-alkaloid variety 24 h after foliage damage (Figure 4a). Additional experiments have shown that cultured roots of high-alkaloid *N. tabacum* contain substantially higher levels of both QPRTase and PMT transcripts, when compared to the levels found in cultured roots of the low-alkaloid variety (Chintapakorn *et al.*, in preparation; and demonstrated as a component of Figures 5 and 6). Furthermore, northern analysis showed that both QPRTase and PMT transcript levels were not readily detectable in the foliage of both unwounded or wounded plants of high-alkaloid *N. tabacum*, unlike the situation in roots (Figure 4b).

Expression of QPRTase in *N. sylvestris* and *N. glauca*

The expression characteristics of QPRTase were assessed in *N. sylvestris*, a progenitor species of *N. tabacum* with a similar alkaloid profile (Saitoh *et al.*, 1985; Sisson and Severson, 1990) and also in *N. glauca*, a species that accumulates primarily

anabasine in leaf tissue (Saitoh *et al.*, 1985; Sisson and Severson, 1990). Northern analysis of RNA extracted from roots and leaves revealed substantial differences in patterns of basal QPRTase expression in these species and in their capacity to increase transcript levels of QPRTase upon wounding of aerial tissues.

In *N. sylvestris*, transcript levels of QPRTase and PMT in roots increased markedly 12–24 h after wounding of aerial tissues, with levels of QPRTase remaining substantially elevated relative to controls for at least 72 h after wounding (Figure 5a). As was also the case in *N. tabacum*, levels of both QPRTase and PMT transcript were not detected in both unwounded and wounded leaf tissues of *N. sylvestris* harvested 12–72 h after wounding (Figure 5b).

In *N. glauca*, however, the pattern of QPRTase expression was quite different. In this species, relatively high levels of QPRTase transcript were detected in roots of unwounded plants, but these levels did not increase substantially over basal levels within 72 h of wounding aerial tissues (Figure 6a). PMT transcript levels were relatively low in the roots of *N. glauca*, and did not increase significantly over a period of 12–72 h after wounding (Figure 6a). Also in contrast to the situation in *N. tabacum* and *N. sylvestris*, QPRTase transcript was detected in unwounded leaf tissue, being approximately equivalent to levels observed in cultured roots of *N. tabacum* cv. LAFC53 (Figure 6b).

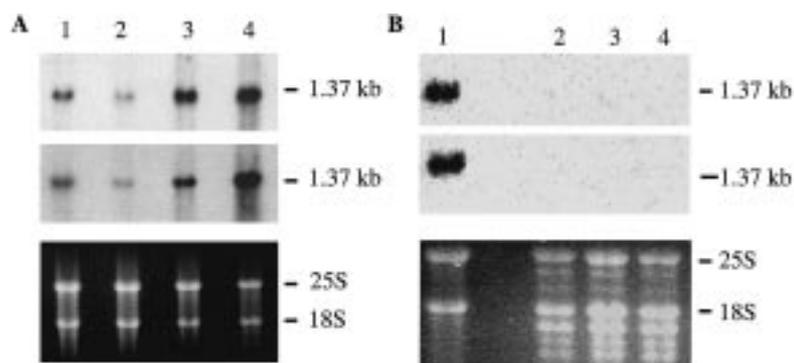


Figure 4. Northern/RNA gel blot analysis of QPRTase and PMT transcript levels in *N. tabacum*. Aerial tissues were wounded as described in Materials and methods and RNA was extracted from roots and leaves 24 h after wounding. The upper blot in each panel shows the signal obtained by probing the membrane with insert from pTQPT1 (QPRTase). The lower blot in each panel shows the signal on the same membrane after stripping and re-probing with PMT coding sequence. Panel A contains RNA isolated from roots of low- and high-alkaloid *N. tabacum*. Lanes: 1, unwounded control plants, low-alkaloid variety; 2, wounded plants, low-alkaloid variety; 3, unwounded control plants, high-alkaloid variety; 4, wounded plants, high-alkaloid variety. The lower gel photograph in panel A shows that RNA levels were comparable in each track, except for track 4 which is slightly under-loaded relative to the other tracks. Panel B contains RNA from high-alkaloid *N. tabacum* as follows. Lanes: 1, RNA from roots of wounded plants as noted above; 2, RNA from unwounded leaves; 3 and 4, RNA from wounded leaf tissues.

Transcript levels in leaf tissue of *N. glauca* increased markedly 12–24 h after wounding before returning to basal levels by 48 h after wounding (Figure 6b). As in *N. tabacum* and *N. sylvestris*, PMT transcript was not detectable in leaf tissues in either unwounded or wounded *N. glauca* plants.

Southern analysis of QPRTase in *Nicotiana* species

Genomic DNA from *N. tabacum* cv. NC95, its progenitor species *N. sylvestris* and *N. tomentosiformis* and several other *Nicotiana* species was digested with *Xba*I, *Hind*III and *Eco*RI. Southern/gel blot analysis was undertaken with a fragment of QPRTase from the 5' region of pTQPT1 as a probe, which lacks these restriction sites. This analysis revealed a relatively simple hybridization pattern in all species analysed, with 2–3 bands strongly hybridizing to the probe in most cases (Figure 7a–c). This is suggestive of a small gene family encoding QPRTase in these species. Interestingly, and rather unexpectedly however, the QPRTase hybridization pattern obtained for *N. tomentosiformis* was identical to that obtained for *N. tabacum* cv. NC95 and was quite different from that of *N. sylvestris* (tracks 3, 4 and 5 in Figure 7a–c). This suggested that some or all of the *N. sylvestris* genetic information encoding QPRTase was not present in the genome of *N. tabacum* cv. NC95. To check whether a truncated region of *N. sylvestris* QPRTase is present in *N. tabacum* cv. NC95, blots were stripped of hybridizing signal and re-probed with the entire coding sequence from pTQPT1. This analysis revealed additional bands

present in *N. tabacum* cv. NC 95 that are also present in *N. tomentosiformis*. However, no bands could be identified as being characteristic of *N. sylvestris* (e.g. Figure 7d, e). To eliminate the possibility that samples had been inadvertently mixed during DNA extraction, filters were stripped of hybridizing signal and re-probed with the coding sequence of the PMT gene. For each restriction enzyme, characteristic hybridizing bands from the *N. sylvestris* genome were observed in the *N. tabacum* sample, in addition to bands from the *N. tomentosiformis* genome. This is demonstrated clearly in Figure 7f where 5 bands are seen in *Eco*RI-digested genomic DNA from *N. tabacum* cv. NC95 (track 4) with 3 of these bands being present in DNA extracted from *N. sylvestris* and one being present in *N. tomentosiformis* DNA. This is in agreement with results of Hashimoto *et al.* (1998a) and also recent results from Riechers and Timko (1999). Thus it appears that all genetic information from *N. sylvestris* which encodes QPRTase has been eliminated from the genome of *N. tabacum* cv. NC95.

Discussion

Analysis of the QPRTase gene in *Nicotiana*

In this study we have characterized almost identical cDNAs encoding QPRTase from *Nicotiana tabacum* and *N. rustica*, each displaying sequence homology to QPRTase from a range of organisms. Both cDNAs contain two ATG codons, in frame, close to their 5'

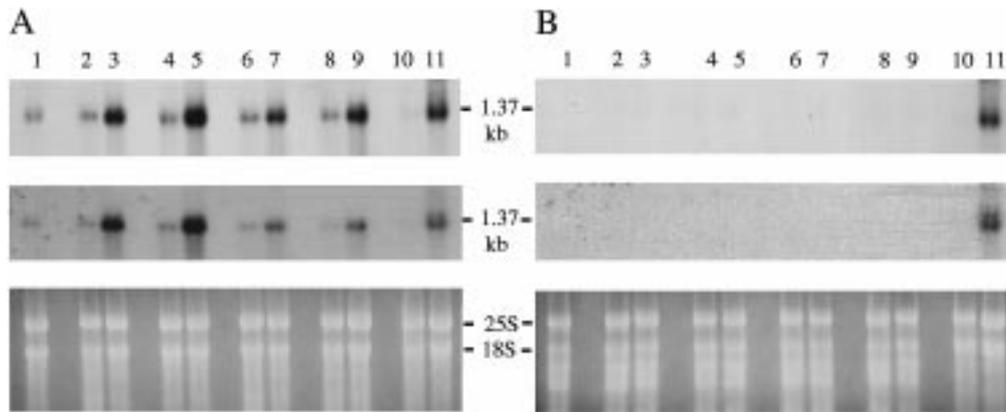


Figure 5. Northern/RNA gel blot analysis of QPRTase and PMT transcript levels in control (unwounded) and wounded plants of *N. sylvestris*. Aerial tissues were wounded as described in Materials and methods and RNA was extracted from roots (panel A) and leaves (panel B). The upper blot in each panel shows the signal obtained by probing the membrane with insert from pTQPT1 (QPRTase). The lower blot in each panel shows the signal on the same membrane after stripping and re-probing with PMT coding sequence. The lower gel photograph in each panel shows that RNA levels were comparable in all tracks. Designation of samples in each lane: 1, control (unwounded) time zero; 2, control (unwounded) and 3, wounded, 12 h after wounding; 4, control (unwounded) and 5, wounded, 24 h after wounding; 6, control (unwounded) and 7, wounded, 48 h after wounding; 8, control (unwounded) and 9, wounded, 72 h after wounding; 10 and 11, RNA extracted from actively growing hairy root cultures of low-alkaloid *N. tabacum* (lane 10) and high-alkaloid *N. tabacum* (lane 11). (These samples were included primarily to serve as internal standards to allow comparison between strength of signal on different blots but also show that both QPRTase and PMT are expressed at much lower levels in cultured roots of low-alkaloid *N. tabacum* cv. LAFC53 than in cultured roots of high-alkaloid *N. tabacum* cv. NC95.)

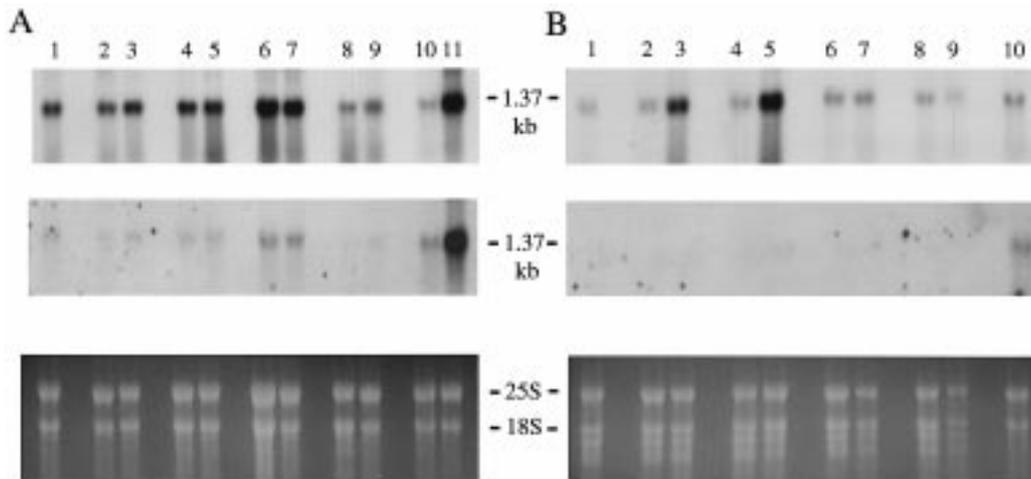


Figure 6. Northern/RNA gel blot analysis of QPRTase and PMT transcript levels in control (unwounded) and wounded plants of *N. glauca*. Aerial tissues were wounded as described in Materials and methods and RNA was extracted from roots (panel A) and leaves (panel B). The upper blot in each panel shows the signal obtained by probing the membrane with insert from pTQPT1 (QPRTase). The lower blot in each panel shows the signal on the same membrane after stripping and re-probing with PMT coding sequence. The lower gel photograph in each panel shows that RNA levels were comparable in all tracks except lanes 6 and 7 of panel A which were slightly over-loaded relative to others. Designation of samples: 1, control (unwounded) time zero; 2, control (unwounded) and 3, wounded, 12 h after wounding; 4, control (unwounded) and 5, wounded, 24 h after wounding; 6, control (unwounded) and 7, wounded, 48 h after wounding; 8, control (unwounded) and 9, wounded, 72 h after wounding; 10 and 11, RNA extracted from root cultures of low-alkaloid *N. tabacum* (lane 10) and high-alkaloid *N. tabacum* (lane 11). (Note that the same batch of *N. tabacum* root culture RNA was used as in Figure 5 to enable comparison between blots with reference to signal strength in lanes 10 and 11. Insufficient RNA was available to enable track 11 to be loaded in panel B. However, reference to the signals observed in track 10 of panels A and B allows a direct comparison of signal strengths for QPRTase and PMT transcripts in roots and leaves of *N. glauca*.)

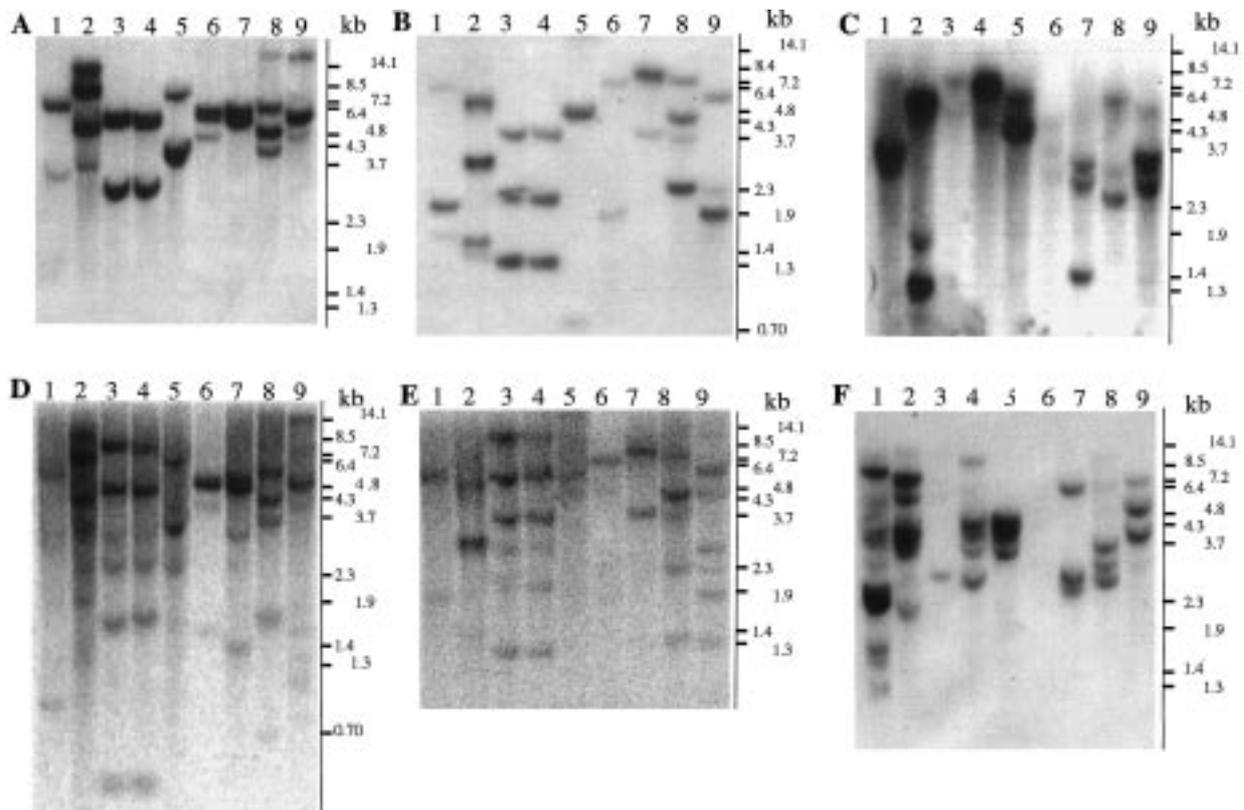


Figure 7. Southern analysis of QPRTase in a range of *Nicotiana* species. Genomic DNA from a number of *Nicotiana* species was digested with a range of restriction enzymes and agarose gels were loaded as follows. Lanes: 1, *N. glauca*; 2, *N. rustica*; 3, *N. tomentosiformis*; 4, *N. tabacum* cv. NC95; 5, *N. sylvestris*; 6, *N. langsdorfii*; 7, *N. longiflora*; 8, *N. alata*; 9, *N. hesperis*. Membranes containing DNA digested with *Xba*I (A), *Hind*III (B) or *Eco*RI (C) were probed with a 32 P-labelled 350 bp PCR fragment from the 5' region of pTQPT1 lacking these restriction sites. To test whether truncated copies of QPRTase are present in the genome of *N. tabacum* cv. NC95 which could be identified as having been derived from *N. sylvestris*, membranes were re-probed with the entire coding DNA of pTQPT1 (D, *Xba*I; E, *Hind*III). To confirm that DNA in lane 4 was from *N. tabacum*, each filter was stripped of all hybridizing sequences and probed with 32 P-labelled PMT-coding sequence. F (*Eco*RI digest) shows the presence of a characteristic single band in *N. tomentosiformis* of ca. 2.5 kb, 5 bands ranging from ca. 2.5 kb to ca. 9 kb in *N. tabacum* and 3 bands ranging from ca. 3.5 kb to ca. 5 kb in *N. sylvestris* (Hashimoto *et al.*, 1998a; Riechers and Timko, 1999).

ends, which are also present in the QPRTase sequence reported recently from *N. tabacum* strain Bright Yellow (AB038494). Considering the currently accepted 'scanning model' of translation in plants (Kozac, 1986, 1995), it seems likely that the first methionine codon represents the initiating AUG in the mRNA as it is not followed by a stop codon in any frame and nor is the 5'-UTR particularly short (>65 bp) (Kozac, 1995). The leader sequences are also AT-rich in both cDNAs (62% in *N. rustica*, 69% in *N. tabacum*), and thus the formation of stable secondary structures does not seem particularly likely (Gallie, 1996). Furthermore, the first AUG is apparently in a suitable sequence context (Joshi *et al.*, 1997) and scores a relatively high index of 0.7 when a translation initiation prediction algorithm is applied (Netgen2) (Pedersen and

Nielsen, 1997). Thus it seems likely that *Nicotiana* QPRTase has a 24 amino acid N-terminal extension not found in previously characterized QPRTases. It is of interest to speculate cautiously as to the function of this predicted region as a putative cleavable N-terminal signal sequence has also been identified on the shorter human QPRTase (residues 1–16, Figure 3) which may direct the protein to subcellular vesicles *in vivo* (Fukuoka *et al.*, 1998). Although the N-terminal regions of the deduced human and *Nicotiana* QPRTase proteins are not related by sequence homology, beyond their generally hydrophobic nature, it is possible that the *Nicotiana* N-terminal region also acts as a sorting signal to target the protein to a particular subcellular location. Analysis of the *Nicotiana* sequences by the P-Sort program, designed to identify

potential sorting signals in *Arabidopsis*, shows that while indices designed to predict the presence or absence of a signal sequence ('McG' and 'GvH' scores; Nakai and Kanehisa, 1992) suggested that none was present, the algorithm used to predict mitochondrial signal peptides returned a favourable result. The sequence 'TRVESL', just downstream from the second methionine in *Nicotiana* QPRTase, was identified as a putative cleavage site. The amino acid composition of the N-terminal region is also consistent with that of a mitochondrial signal peptide as amino acids S, R, A, L and T are known, empirically, to be prevalent in mitochondrial signalling peptides, while the residues W, C, H, E, Y and D are relatively uncommon (Sjoeling and Glaser, 1998).

The subcellular relationship between alkaloid production and the pyridine nucleotide cycle remains unclear, and thus the possibility that *Nicotiana* QPRTase possesses a sorting signal is intriguing. Several enzymatic steps involved in the synthesis of other alkaloids have been reported to be associated with compartments such as vesicles, vacuoles and the chloroplast (Hashimoto and Yamada, 1994; Wink, 1997). Parenthetically, it may be noted that *Nicotiana* PMT also has an N-terminal extension not found in the otherwise similar SPDS protein of primary metabolism from which the PMT gene is derived (Hibi *et al.*, 1994; Hashimoto *et al.*, 1998a, b; Suzuki *et al.*, 1999). The relationship between this sequence and the 24 amino acid N-terminal extension on the QPRTase deduced protein and their function, if any, is unknown at present. No significant sequence or structural homology is readily identifiable.

It is important to remain cautious, however, when making predictions from sequence data in plants and further experiments are required to investigate both translation initiation and the subcellular localization of QPRTase in *Nicotiana*. For example, both cDNAs reported here contain a second in-frame ATG codon close to their 5' ends which is in a very similar context to that of the first, also scoring a positive 0.7 index from 'Netgen2' (Pedersen and Nielsen, 1997). It is thus conceivable that a shorter QPRTase protein, without an N-terminal extension, is also produced in *Nicotiana*, which would probably be functional, given the position of the active site in the enzyme (Eads *et al.*, 1997). A shorter QPRTase protein could have a separate role *in vivo* in *Nicotiana*, for example in primary metabolism concerned with NAD production. Alternatively, both ATG codons may play a role in ensuring sufficient nicotinic acid is produced for nicotine

synthesis by providing an additional translation initiation point which may allow rapid and assured production of protein after wound-induced transcription of the QPRTase gene.

Expression of QPRTase in N. tabacum, N. sylvestris and N. glauca

The pattern of QPRTase expression we observed in different tissues and different strains of *N. tabacum* is consistent with previous studies relating to QPRTase and PMT enzymic activity and nicotine synthesis in *N. tabacum* plants and callus tissues cultured *in vitro*. Results obtained here are compatible with the hypothesis that regulatory genes that are mutated in low-alkaloid varieties, such as LAFC53 (Chaplin, 1975), are responsible for ensuring co-ordinated expression of QPRTase and PMT genes to enable nicotine production in the roots of *N. tabacum*. These regulatory genes were identified in genetic studies conducted by Collins and colleagues in the 1960s (Legg and Collins, 1971) and were subsequently designated *nic1* and *nic2* by Hibi *et al.* (1994). Additional experiments undertaken in this laboratory (Chintapakorn *et al.*, in preparation) and in other laboratories (e.g. unpublished observations noted by Riechers and Timko, 1999) have also indicated that other genes involved in alkaloid synthesis in tobacco, such as ornithine decarboxylase, are differentially expressed in roots of low- and high-alkaloid varieties, and respond to wounding of aerial tissues in a manner similar to QPRTase and PMT. This suggests that *nic1* and *nic2* may regulate the expression of numerous genes involved in the biosynthesis of nicotine in *Nicotiana* species.

Analysis of different species of the genus *Nicotiana*, with respect to QPRTase transcript abundance and induction in response to wounding, were also instructive. Rates of *de novo* synthesis of nicotine in roots of *N. sylvestris* have been reported to increase within 10–15 h after aerial tissues are wounded, with levels of nicotine continuing to rise steadily for several days after wounding (Ohnmeiss *et al.*, 1997). Our observations that QPRTase transcript levels in roots of *N. sylvestris* are markedly elevated 12–24 h after wounding of aerial tissues, remaining elevated for at least 72 h after wounding, suggest that elevated expression of QPRTase in roots facilitates an increase in the nicotine content of the foliage.

The expression profile of QPRTase in *N. glauca* is quite different from that observed in *N. sylvestris* and *N. tabacum*, but is consistent with the alkaloid pro-

file of the former species in which anabasine is the principal alkaloid in both roots and leaves (Saitoh *et al.*, 1985; Sisson and Severson, 1990). In contrast to *N. tabacum* and *N. sylvestris*, QPRTase transcript is readily detected in unwounded leaf tissue of *N. glauca* and levels are elevated markedly in these tissues 12–24 h after wounding. Classical experiments involving grafts between species have established that while nicotine is produced in the roots of *N. glauca*, as in *N. tabacum* and *N. sylvestris*, anabasine is produced in both the leaves and roots of *N. glauca* (Dawson, 1945, 1962). Increased anabasine concentrations in leaf tissue of *N. glauca* have been documented as a result of wounding (Baldwin and Ohnmeiss, 1993). Our results are thus consistent with the suggestion that up-regulation of QPRTase expression in leaf tissue of *N. glauca*, in response to wounding, facilitates increased synthesis of anabasine in these tissues. QPRTase expression in the roots of *N. glauca* may facilitate both nicotine and anabasine production. The absence of a strong wound response in roots upon damage to aerial tissues, with respect to QPRTase and PMT transcript levels, may be related to the fact that anabasine rather than nicotine is the main alkaloid component of *N. glauca* leaf tissue (Saitoh *et al.*, 1985; Sisson and Severson, 1990). The relatively high level of QPRTase transcript that we observed in roots of unwounded *N. glauca* plants, compared to PMT transcript levels in the same tissues, is also in contrast to observations made on comparable tissues of *N. tabacum* and *N. sylvestris* where transcript levels of both genes were broadly equivalent to each other within a particular tissue sample. This may be explained by reference to classical work undertaken with root cultures of *N. glauca* and *N. tabacum* which suggested that anabasine is produced in maturing root tissue while nicotine is produced in growing root tips (Dawson, 1962). We extracted RNA from the entire root system of hydroponic plants, which included a relatively high proportion of maturing root tissues and a low proportion of root tips.

Taken together, the contrasting patterns of QPRTase expression in *N. tabacum*, *N. sylvestris* and *N. glauca* appear to reflect the differences in alkaloid content in these species. Experiments are underway currently to improve our understanding of the mechanism(s) whereby separate species of *Nicotiana* are capable of differentially regulating QPRTase expression to provide nicotinic acid for synthesis of different types of pyridine alkaloids.

Southern analysis of QPRTase in a range of Nicotiana species

Analysis of genomic DNA extracted from *N. tabacum* and several other *Nicotiana* species suggests that QPRTase is present as a small gene family in these species. This is similar to the situation for the PMT gene where 3 copies of the gene have been identified in the genome of *N. sylvestris*, whilst in *N. tomentosiformis* and *N. otophora* one copy of the PMT gene is present (Hashimoto *et al.*, 1998a; Riechers and Timko, 1999). However, considering that *N. tabacum* contains five copies of the PMT gene, in agreement with the hypothesis that it resulted from a natural hybridisation between *N. sylvestris* and an introgressed hybrid of *N. tomentosiformis* and *N. otophora* several million years ago (Riechers and Timko, 1999), it was rather unexpected to find that genetic information encoding QPRTase from *N. sylvestris* was missing from the genome of *N. tabacum* cv. NC95. Although this may seem strange at first sight, given that this gene is important in nicotine synthesis, it is worth noting that the genomes of allotetraploids may be prone to rearrangement and loss of genetic information from one or both parental species (Jamet *et al.*, 1987; Song *et al.*, 1995; Soltis and Soltis 1995; Volkov *et al.*, 1999). Preliminary observations suggest that QPRTase gene sequences characteristic of the *N. sylvestris* genome are present in the genomes of some other *N. tabacum* varieties (Cane and Hamill, in preparation) raising the possibility that the loss of *N. sylvestris* QPRTase from the genome of *N. tabacum* cv. NC95 was associated with breeding programmes which led to the development of this variety (Chaplin, 1986). It would be interesting, therefore, to assess whether other genetic information characteristic of the *N. sylvestris* genome is missing from *N. tabacum* cv. NC95.

Note added in proof

Recent EST database entries suggest that QPRTase from tomato, also a member of the Solanaceae, may possess an N-terminal extension similar to that of tobacco unlike QPRTase from soybean (accession numbers AW443537 [tomato] and AW757286 [soybean]).

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