MONASH UNIVERSITY
THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
ON..... 11 February 2004 .....
.....[redacted].....
Sec. Research Graduate School Committee

Response to PhD Examiners Report

This thesis was examined by Dr. Phillip Larkin (CSIRO, Plant Industry, Canberra) and Prof. Michael P. Timko (University of Virginia, USA). While both examiners recommended that the thesis be passed without amendment, Prof. Timko posed a number of questions to me, in lieu of a formal defence. These questions are repeated below, along with my responses, on the basis of which Prof. Timko recommended that the thesis be passed.

"During evolution, plants were presented with the struggle for survival against predation and pathogenesis. In* Nicotiana*, some but not all progenitor species appear to have developed sophisticated mechanisms for the control of inducible secondary metabolism (nicotine in* N. sylvestris*, anabasine in* N. glauca *are examples). Why didn't all develop some form, or is it a matter of degree of induction?

There are a number of scenarios which can explain the variation in ability of related *Nicotiana* species (and presumably some of their ancestors) to respond to predation with inducible chemical defences. For example:

Some, but not all, *Nicotiana* species may have independently evolved the ability to produce and/or induce defensive secondary metabolites. This suggestion seems extremely unlikely, unless the progenitors of the modern species carried a "predisposition" to the evolution of alkaloid production and/or induction in the form of an inherited mutation. For example, if a "proto-*Nicotiana*" plant carried a duplicated *SPDS/PMT* gene and a duplicated *QPRTase* gene, along with a transcription factor already responsive to foliage damage, the evolution of the inducible defence system presently observed might not require much genetic change. In this example, the relevant genes would be required to evolve sensitivity to the relevant transcription factor, possibly following alterations in their promoter sequence. The evolution of a nicotine synthase (NS) enzyme able to recruit nicotine precursors would also be a fundamental step. Recent studies suggest this may have occurred via genetic change in a duplicated isoflavone reductase-like gene, resulting in the formation of the likely NS-encoding '*A622*' (Shoji *et al.*, 2002). If this change occurred independently in different species, the observed variation in induction capacity could easily result. This is analogous to some inherited diseases in humans, where germ-line mutation causes the descendants to have an elevated probability of

developing a disease which would otherwise require an unlikely combination of multiple, independent mutations (eg. Evans, 1988; Sturgis and Wei, 2002).

The converse may also be argued. A proto-*Nicotiana* may have had the full capacity to produce and induce defensive alkaloids. Some of its descendants may have lost this ability. Such loss might be likely in species evolving in habitats where defoliation was a minor risk, or where it exerted relatively little selection pressure. In these species, the loss of a defence system may have been selectively neutral, or even beneficial due to the high metabolic (and possibly "genetic") cost of maintaining (elements of) such a system (eg. Baldwin *et al.*, 1998).

These two scenarios are opposite extremes, but they are not mutually exclusive. Components of the various production and induction mechanisms may be gained, lost or masked in isolation from each other. For example, it is likely that an ancestral proto-*Nicotiana* species was able to produce, say, nicotine, and was equipped with a signal transduction mechanism able to cause some increase in its production after wounding. Subsequent speciation may have been followed by different changes in different species, involving the loss or gain of different functions. *Nicotiana alata*, for example, may have lost the function of a transcription factor responsible for activating alkaloid genes, hence its low alkaloid profile. *Nicotiana glauca*, on the other hand, may have gained an ODC/LDC enzyme with the ability to produce enough cadaverine and Δ^1-piperideinium to out-compete *N*-methylpyrrolinium for the available nicotinic acid, thus causing an increase in anabasine content. A transcription factor responsible for regulating some of the alkaloid genes (say, *QPRTase*, *ODC/LDC*, '*A622*') may also have evolved an altered expression pattern in this species, being active in the leaves rather than the roots. These changes could have caused *N. glauca* to display the leaf inducible, anabasine-rich profile we presently observe. These noted changes are simply examples- many other permutations of loss, gain and alteration of function can be put forward to explain different alkaloid phenotypes. Many of these, particularly those involving alterations in the function of upstream transcription factors may require little genetic change, and are thus feasible in the ~25 million years the *Nicotiana* genus is thought to have existed (Meagher *et al.*, 1989; Ioerger *et al.*, 1990). Pyridine alkaloid profile is a variable trait, known to be influenced by many genes (~15 enzymes, some duplicated -five times in the case of PMT- probably numerous transcription factors, along with genes involving signal transduction, alkaloid transport and storage, a likely total of >30 genes). Pyridine alkaloid production also interacts with numerous other

physiological processes, and as such its alteration involves complex selection "trade-offs". Therefore, it is not surprising that it shows as much variation as other multi-factorial traits in plants. For example, leaf shape, indumentum, plant stature and flower colour are highly variable in many genera, and the genetic explanations for these differences are likely to be equally complex for pyridine alkaloid content in *Nicotiana*.

It is also possible that some of the reported variation in alkaloid content among *Nicotiana* species is an artefact of measurement, since only two studies have sampled large numbers of species (Saitoh *et al.*, 1985; Sisson and Severson, 1990).

Where does N. tomentosiformis stand with regard to inducible alkaloid production?

I am aware of no published experiments directly addressing this question, however some preliminary results from experiments by Karen Cane and Kristina Murphy in our laboratory have provided some insights. HPLC analysis of certain *N. tomentosiformis* accessions showed no evidence of nicotine induction after foliage damage. Similarly, Northern analysis suggests that alkaloid biosynthetic enzymes are not induced in the roots following wounding. These results accord well with the results of both Saitoh *et al.* (1985) and Sisson and Severson (1990) who found relatively low levels of nicotine and nor-nicotine in wounded plants of this species. These observations suggest that *N. tomentosiformis'* induction capabilities are limited or lacking. Thus, it seems likely that *N. tabacum* inherited crucial components of its induction capacity from *N. sylvestris*. This is borne out in my studies, where *N. sylvestris* is shown to be a strongly inducible species, apparently similar to *N. tabacum* in all relevant respects. Nonetheless, further studies would need to be undertaken in order to clarify this. These should include *N. otophora*, since *N. tabacum* may have inherited some of the genome of this species (Reichers and Timko, 1999; Ren and Timko, 2001; Murad *et al.*, 2002).

When hybridisation occurred leading to the formation of the tetraploid* N. tabacum *species, one might expect that the regulatory circuits (modes of gene regulation) in place in the diploid progenitors would be transferred to the tetraploids. How would you identify/determine what members of the* QPRTase/LDC-ODC *multigene families were derived from each progenitor, and can you devise experiments that directly test if members of these families have modes of regulation exactly like those found in* N. sylvestris *and* N. tomentosiformis*?

The determination of parentage for specific gene copies has been undertaken in the case of *PMT* in *N. tabacum*, where three gene copies have been shown to be derived from *N. sylvestris*, one from *N. tomentosiformis* (Hashimoto *et al.*, 1998) and another from *N. otophora* (Reichers and Timko, 1999). These studies exploited the differences in length between the gene copies, due to a variable 5' repeat region. Such length variation may be absent from *QPRTase* in *N. tabacum* (Sinclair, 2003), however parallel 'parentage studies' in *QPRTase* could be approached in a number of other ways.

Restriction digests followed by Southern blot analysis (ie. RFLP, or similar analysis) may be able to distinguish fragments containing individual gene copies. This was already successful in one case, using *Hind*III, where an individual RFLP band representing an *NtxQPT2*-like *QPRTase* gene copy was present in *N. tomentosiformis* and *N. tabacum* cv. *Xanthi* DNA, but was absent in *N. sylvestris* DNA. (Figure 2.2.2 in Sinclair, 2003. Band size ~2kb). If the question of individual gene-parentage became important, similar Southern analysis could be carried out using multiple enzymes, until an unambiguous pattern was found for all other gene copies.

Another approach is to sequence fragments of all individual gene copies in the library (using the same sorting and sequencing approach detailed in Sinclair, 2003), and design copy-specific primers. These primers could be used as probes or in PCR against genomic DNA from the parental species to locate the gene copies in their parent species. This technique may be limited in two ways: Firstly, practical copy-specific sequence segments may not exist, and secondly, the genes may have diverged sufficiently since the formation of *N. tabacum* that they are no longer identifiable between species using primers. Nonetheless, present indications suggest that these primer-based techniques are likely to be informative, and a number of such experiments are presently being carried out in our laboratory.

Analgous studies for *ODC/LDC* have commenced in our laboratory, as part of the PhD program of Kath DeBoer. So far this study has focussed on *N. glauca*. There are a number of *ODC/LDC* gene copies in this species, and the sequence data so far suggests that they may be very similar to one another (DeBoer, pers. comm. 1.8.03).

Experiments have already been completed which have examined the expression of individual *QPRTase* gene classes (which may include several gene copies) (Sinclair,

2003). These studies used short oligonucleotide probes that were able to distinguish the gene classes on the basis of distinct sequences in a 5' UTR region spanning ~35bp. These studies could be extended to include the parental species, providing these 35bp regions are sufficiently conserved. In addition to using oligonucleotide probes, these same regions could be exploited in RT-PCR studies for all species, as was discussed for *N. tabacum* in my thesis (Sinclair, 2003, Section 2.2.4). The continuing PCR studies presently being undertaken by other students in our laboratory will hopefully contribute to our understanding of how individual gene copies are expressed in both diploid and tetraploid species.

Another approach to this question would be to manipulate individual gene copies in transgenic plants. If, for example, a particular copy could be disrupted, then the ability of the other genes to be induced etc. could be assessed. This may possibly be done using an RNAi approach, exploiting the same variation noted above (RNAi is mediated by short ~25bp molecules, as discussed in Sinclair, 2003). In theory, individual gene copies may also be disrupted at the genomic DNA level, however homologous recombination technology to selectively mutate *N. tabacum* genes is not presently avaiiable.

What factors might lead to the maintenance or loss of regulatory circuits after hybridisation?

While the phenotype of a tetraploid may be the predictable "sum" of its parental phenotypes, previously impossible interactions between genes and proteins from different parents may also produce new effects. For example, if one parent contained a transcription factor which was induced by some stimulus, and the second parent contained a gene/promoter which was responsive to this factor, the resultant tetraploid may be capable of the transcriptional induction of this gene, of which neither parent was capable. Thus, tetraploid formation can lead to new regulatory circuits.

In addition to these factors, independent evolutionary change may occur in the diploid and tetraploid descendants, obscuring relationships between the modern tetraploid species and the modern counterparts of its diploid parents. This is particularly likely in the tetraploid offspring, since they contain duplicated genes which are somewhat free from selective constraints, and are able to accumulate or "withstand" mutations. The situation may be further complicated by larger-scale

genetic change, such as gene loss, which is known to occur commonly in tetraploids (Soltis and Soltis, 1995; Volkov *et al.*, 1999).

These three factors are the most obvious explanations for the difference (or loss) of regulatory circuits between diploid species and their tetraploid descendants. Obviously, the tetraploid may retain the phenotype of one or both of its parents. Maintenance would be anticipated in circumstances where the relevant trait provides a selective advantage.

How did your work directly shed light on this question?

Although the effect of tetraploidy on alkaloid regulation was not a stated aim of my PhD studies, my work has provided information that addresses aspects of this question, or will assist in further studies. For example,

- The basic alkaloid phenotype of one parent (*N. sylvestris*) was studied in detail.
- Molecular tools were partially established which may enable the expression of individual *QPRTase* gene copies to be measured in *N. tabacum*, *N. sylvestris* and *N. tomentosiformis*.
- Promoter studies set the foundation for future studies to discover transcription factors that interact with specific genes, enabling comparisons between the diploid and tetraploid species at a regulatory level.

Dr Larkin also noted the following errors:

p 51 para 2 line 1, "posses" should read "possess"

p 58 line 1, "intron 3" should read "intron 4"

p 85 Figure legend line 10, the word "each" is repeated.

p 87 para 2 line 10, "This is may" should read "This may"

ADDITIONAL REFERENCES CITED IN THIS RESPONSE:

Evans HJ (1988) Mutation as a cause of genetic disease. *Philosophical transactions of the Royal Society of London- B: Biological Sciences* **319:** 325-340.

Shoji T, Winz R, Iwase T, Nakajima K, Yamada Y and Hashimoto T (2002) Expression pattern of two tobacco isoflavone reductase-like genes and their possible roles in secondary metabolism in tobacco. *Plant Molecular Biology* **50:** 427-440.

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QPRTase: A wound-induced defence gene in *Nicotiana*

A thesis submitted for the degree of Doctor of Philosophy

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March, 2003

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PUBLICATION: Sinclair *et al.*, 2000.

ABSTRACT

QPRTase (Quinolinate phosphoribosyltransferase) is an essential enzyme in all organisms involved in the *de novo* synthesis of NAD (nicotinamide adenine dinucleotide). In some plants, including *Nicotiana* species, it plays an additional role in regulating the flow of nitrogen into wound-induced defensive alkaloids.

In this study, cDNAs isolated in the preceding honours project from *N. tabacum* and *N. rustica* were shown to encode functional QPRTase enzymes by the complementation of an *E. coli* mutant lacking endogenous QPRTase activity. The deduced *Nicotiana* QPRTases showed significant amino acid conservation with QPRTases from other plants, animals and micro-organisms. Unlike the non-plant sequences, however, QPRTases from plants all possessed N-terminal extensions, with the characteristics of mitochondrial and/or chloroplast targeting signals (Sinclair *et al.*, 2000). Future experiments to test the function of this N-terminal region are discussed.

Genomic Southern and sequence analysis indicated that *QPRTase* exists as a small gene family in *N. tabacum*, comprising genes in two classes, characterised by divergent sequences in their 5' non-coding regions (*NtxQPT1* and *NtxQPT2*). Northern analysis using copy-specific oligonucleotide probes from the 5' UTRs of each gene showed that *NtxQPT2*-like transcripts are strongly expressed in *N. tabacum* roots, and induced 24h after foliage damage, consistent with the hypothesis that this gene-class is regulated to facilitate the biosynthesis of defensive alkaloids. Although it appears able to encode QPRTase, *NtxQPT1* gene expression was not detected in the roots or leaves of *N. tabacum*, even after wounding. A number of studies to further assess the functionality of *NtxQPT1* and the regulation of *NtxQPT2* are outlined.

To determine whether the transcription of alkaloid-synthesis genes is correlated with the distinct alkaloid phenotypes of different *Nicotiana* species, alkaloid- and Northern analyses were carried out on a nicotine-rich species (*N. sylvestris*), a low-alkaloid species (*N. alata*) and an anabasine-rich species (*N. glauca*). In *N. sylvestris*, the key biosynthetic genes *QPRTase*, *PMT* (Putrescine *N*-methyltransferase) and *ODC* (Ornithine decarboxylase), and the putative alkaloid biosynthetic gene '*A622*' were all induced ~4-6 fold in the roots 24h after foliage damage, preceding a 2 fold increase in leaf nicotine-levels 1wk after wounding. This

is consistent with published studies showing that *N. sylvestris* increases nicotine production in its roots after foliage damage, before the alkaloid is relocated to the foliage. In contrast, *N. alata* showed no gene- nor alkaloid-induction after foliage damage, supporting the hypothesis that low gene transcription plays a role in determining the low-alkaloid profile of this species. In *N. glauca*, leaf anabasine levels were induced 2 fold 1wk after foliage damage. Unlike in *N. sylvestris*, however, no prior gene induction was observed in the roots. Rather, *QPRTase* was strongly induced in the wounded leaves 24h after damage, supporting the hypothesis that increased gene transcription in the foliage itself enables this species to induce anabasine synthesis directly in its leaves, rather than its roots. Although they have no known role in anabasine synthesis, *ODC* and '*A622*' were co-induced with *QPRTase* in wounded *N. glauca* foliage. On the basis of this data, together with published findings, a modified scheme for alkaloid synthesis, involving *ODC* and '*A622*' in both nicotine and anabasine synthesis is developed. Future experiments to begin testing this model are also discussed.

DECLARATION

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or institute. To the best of my knowledge this thesis contains no material previously published or written by another person, except where due reference is made in the text.

Steven J. Sinclair.

ACKNOWLEDGMENTS

I would like to thank all the people who assisted and supported me during the course of this project. In particular, I express appreciation to Prof. John Hamill, who has been an impressively thoughtful and supportive supervisor. Dr Alan Neale and Karen Cane have also provided continuing support. Prof. David Smyth, Assoc. Prof. Steve McKechnie and Dr. Martin Burd all provided constructive criticism after my seminars. The technical support of a number of workers in other institutions is also acknowledged: Dr Kelly Hughes (University of Washington, Seattle) provided the cell line TH265, Mr Bruce McGuinness (University of Melbourne) provided *Nicotiana* seeds, Dr Ed Newbigin (University of Melbourne) donated a sample of the *N. alata* genomic library, and my brother Robert Sinclair (University of Melbourne) contributed two accurate scale diagrams (Figures 2.2.6, 3.2.1). Many others have provided specific help, and great lab company. The list is long, but the thanks is always meant: Thanks Kath, Eug, Carlie, Robin, Elaine, Assunta, Penny, Yupynn, Tuan, Calida, Kris, Clare, Richard, and Rochelle. Christina Flann and Cecilia Davis are thanked for their critical reading of several drafts. Finally, my parents, my family, my friends Christina and James, and lovely Cecilia have been constantly fantastic.

FORMAT OF THE THESIS & LIST OF ABBREVIATIONS

The format of this thesis follows that of a number of journals in this field, including *Plant Cell* (American Society of Plant Physiologists), *The Plant Journal* (Blackwell Science, Society for Experimental Biology), and *Phytochemistry* (Elsevier, UK). The Harvard system of referencing is used. In line with current conventions, the authors of plant species are given, but not those of animal or bacterial species. All protein names are written in upright characters, while those of genes or transcripts are written in italics.

The abbreviations used are listed below. These are used as the original authors used them, even if this introduces minor inconsistencies when brought together in this thesis. Obvious abbreviations such as 'DNA', standard units, and the chemical element symbols are deliberately omitted from this list. Those abbreviations used only once, which are defined where they occur in the text (*eg*; the names of genes from other fields), are also omitted. The enzyme catalogue numbers follow the International Union of Biochemistry and Molecular Biology (IUBMB) (www.chem.qmu.ac.uk/iubmb/).

Enzymes/Proteins:

'A622'	An unidentified enzyme probably involved in pyridine alkaloid biosynthesis (Hibi *et al*., 1994).
ADC	Arginine decarboxylase (EC 4.1.1.19).
AP2/EREBP	A class of plant DNA transcription factors, named after the first known members AP2 (apetala2) and EREBP (ethylene responsive element binding protein) (reviewed in Meshi and Iwabuchi, 1995).
bZIP	A class of transcription factors characterised by a binding domain with a basic region followed by a leucine zipper motif (reviewed in Meshi and Iwabuchi, 1995).
DHS	Deoxyhypusine synthase (EC 2.5.1.46).
Dof	A class of transcription factors possessing a zinc-finger motif (Yanagisawa, 1995; Meshi and Iwabuchi, 1995).
GT-1	A class of DNA binding proteins possessing a characteristic α-helix binding domain (reviewed in Meshi and Iwabuchi, 1995).
HD	Homeodomain, referring to a class of transcription factors named after the first studied member (HD) binding the homeobox (reviewed in Meshi and Iwabuchi, 1995).
HSS	Homospermidine synthase (EC 2.5.1.44).

LDC	Lysine decarboxylase (EC 4.1.1.18).
MPO	*N*-methylputrescine oxidase (a diamine oxidase) (EC 1.4.3.6).
MYB	A class of transcription factors possessing a characteristic binding domain, named after the first studied member, the *myb* proto-oncogene (reviewed in Meshi and Iwabuchi, 1995).
nadC	The abbreviation for QPRTase (below) in bacterial systems.
NAD-ppase	NAD-pyrophosphatase (NAD diphosphatase) (EC 3.6.1.22).
NADs	NAD-synthetase (NAD synthase) (EC 6.3.1.5).
NAMN-ATase	NAMN-adenylyltransferase (Nicotinate nucleotide adenylyltransferase) (EC 2.7.7.18).
NAMN-Ghase	NAMN glycohydrolase (related to EC 2.4.2.11, or 3.2.2.14; Wagner *et al*., 1986a, 1986b).
N-ase	Nicotinamidase (EC 3.5.1.19).
NAPRTase	Nicotinic acid phosphoribosyltransferase (Nicotinate phosphoribosyltransferase) (EC 2.4.2.11).
NMN-ATase	NMN-adenylyltransferase (EC 2.7.7.1).
NMN-Ghase	NMN-glycohydrolase (NMN nucleosidase) (EC 3.2.2.14).
ODC	Ornithine decarboxylase (EC 4.1.1.17).
PMT	Putrescine *N*-methyltransferase (EC 2.1.1.53).
QPRTase	Quinolinate phosphoribosyltransferase (Quinolinic acid phosphoribosyltransferase) (Nicotinate-nucleotide pyrophosphorylase) (EC 2.4.2.19).
SPDS	Spermidine synthase (EC 2.5.1.16).
Str	Strictosidine synthase (EC 4.3.3.2).
TDC	Tryptophan decarboxylase (EC 4.1.1.28).
WRKY	A class of plant transcription factors possessing a binding domain with the conserved motif WRKYGQK (reviewed in Eulgem *et al*., 2000).

Chemicals, metabolites and hormones:

ABA	Abscisic acid.
Ade	Adenine.

Gln	Glutamine.
Glu	Glutamic acid.
IAA	Indole-3-acetic acid.
IPTG	Isopropyl-β-D-galactopyranoside.
N	Nicotinamide.
NA	Nicotinic acid.
NAD	Nicotinamide adenine dinucleotide (Previously referred to as diphosphopyridine nucleotide (DPN). Officially abbreviated as NAD by the IUBMB Commission on Enzymes in 1961 (nomenclature discussed in Chaykin, 1967)).
NADP	Nicotinamide adenine dinucleotide phosphate (Previously TPN, see note above).
NAMN	Nicotinic acid mononucleotide.
NMN	Nicotinamide mononucleotide.
PPi	Pyrophosphotase.
PRPP	5-phosphoribosyl 1-pyrophosphate.
Qa	Quinolinic acid.
R	Ribose.

Other terms:

DH5α	A strain of *E. coli* routinely used in transformations and cloning, wild type in respect to *nad*C (Trademark of Life Technologies).
dw	dry weight.
HPLC	High Performance Liquid Chromatography.
NtxQPT1	*Nicotiana tabacum* genomic *QPRTase* gene copy "class 1" (Results 2.2.3).
NtxQPT2	*Nicotiana tabacum* genomic *QPRTase* gene copy "class 2" (Results 2.2.3).
RQPT1	A *QPRTase* cDNA isolated from *N. rustica* (Sinclair *et al.*, 2000).
SAR	Scaffold attachment region, referring to a portion of DNA involved in binding the chromatin to the chromosomal scaffold.
TH265	A QPRTase deficient *E. coli* strain with the *nadC-aceF* region deleted (refer to section 2.1).
TQPT1	A *QPRTase* cDNA isolated from *N. tabacum* (Sinclair *et al.*, 2000).

1 INTRODUCTION

1.1 Plant secondary metabolites

1.1.1 Overview

Since all plants grow in the same manner... it may be asked how it comes to pass that their liquors... are of such different kinds. (N. Grew, 1682)

In the plant kingdom, many chemical compounds accumulate to high levels in some species, but not others. Their sporadic occurrence suggests that these compounds are superfluous to basic plant survival. This simple observation has long intrigued scientists (Grew, 1682; Dawson, 1962; Voelckel *et al.*, 2001). Despite considerable recent progress, many fundamental questions relating to the evolution, manufacture and function of phytochemicals remain unanswered.

Non-essential compounds with limited distribution among plant species are generally classed as *secondary* metabolites. These have been defined as those chemicals which have no known role in the "internal economy" (physiology) of the plant (Mothes, 1979[i]; Williams *et al.*, 1989; Luckner, 1990; Maplestone *et al.*, 1992). Secondary metabolites have been particularly well studied in the plant kingdom (Kinghorn, 2001), but are also known to occur in profusion in fungi, and to a lesser extent in animals and micro-organisms (Hartmann, 1991; Kinghorn, 2001).

The hypothesis that secondary metabolites were metabolically redundant, being 'ballast' from superseded cellular reactions, or the inert end products of primary metabolism, appears to have been commonly accepted until the 1960s (James, 1953; Dawson, 1962; reviewed in Mothes, 1969; Wink, 1998). This view, however, became increasingly untenable, as it became clear that secondary metabolites have characteristics incompatible with their supposed non-function. For example, it was noted that secondary compounds are numerous and structurally complex, requiring special biosynthetic pathways, many of which had seemingly arisen in relatively recent plant taxa. Furthermore, many contain nitrogen, an atom

[i] Albrecht Kossel first used the term "secondary" component in a lecture to the Physiological Society of Berlin in 1891. The relevant passage is provided in translation by Mothes (1979).

essential for primary metabolism, and often of limited availability to plants (Dawson, 1962; Robinson, 1974; Mothes, 1979; Wink, 1988, 1997; Seigler, 1998).

Current evidence suggests that many chemicals lacking roles in the basic physiology of a plant may have enormous bearing on the plant's performance in the external environment, enabling plants to survive under varying growth conditions, and the influences of other organisms (Bell, 1979; Wink, 1988; Williams *et al.*, 1989; Baldwin, 1999). For example, some secondary metabolites play a role in attracting pollinators (Luckner, 1990; Seigler, 1998). Anthocyanins are well known to accumulate in some species as flowers develop (Sablowski *et al.*, 1994), colouring them brightly, and making them visible to animals. The foul-smelling aliphatic monoamines which are given off by the flower of *Arum maculatum* L. (Araceae) have a similar function, attracting insects to effect pollination (Wink, 1997). Phenolics and terpenoids in numerous plants act to absorb damaging UV-radiation, and the accumulation of some, such as the flavonoids of birch trees (*Betula* spp., Betulaceae), are induced by UV-light (Lavola, 1998). Other metabolites play specific roles in plants that blur the line between primary and secondary metabolism, such as plant-specific growth regulators (*ie*; hormones), or energy reserves present in some seeds (Wink, 1997; Seigler, 1998).

Currently, the most commonly assigned function of secondary metabolites is as defences against herbivores and pathogens (Seigler and Price, 1976; Baldwin, 1993; Wink *et al.*, 1998). Our understanding of this role developed partially from the observations that many secondary metabolites are toxic, and their formation is common only in plants, fungi and those few animals which are vulnerable to attack by being immobile or lacking an immune response (Ehrlich and Raven, 1964; Bell, 1978; Maplestone *et al.*, 1992). Secondary metabolites with likely defensive roles include some amines (*eg*; histamine in nettles (*Urtica* spp., Urticaceae)), non-protein amino acids (*eg*; the neurotoxin β-cyanoalanine), cyanogenic glycosides (*eg*; dhurrin in many pasture species, which yields cyanide after cell damage) and numerous alkaloids, including the pyridine alkaloids discussed below (Baldwin, 1993; Wink, 1997).

1.1.2 Alkaloids

The term *alkaloid* is derived from the Arabic *al-qali*, meaning 'soda', referring to their ease of extraction under alkaline conditions (Kutchan, 1995). These metabolites have been defined differently by various authors, sometimes so broadly as to include all nitrogen-containing natural products that are not primary metabolites (Wink, 1997). All definitions, however, concur in the inclusion of low molecular weight cyclic metabolites containing nitrogen, which often display toxicity to animals when ingested (Cordell, 1981; De Luca and St Pierre, 2000). The alkaloids as a group are extraordinarily diverse, and include up to ~100,000 known members (Hashimoto and Yamada, 1994; Verpoorte, 1998; Wink *et al.*, 1998; De Luca and St Pierre, 2000; Pichersky and Gang, 2000). Alkaloids are currently estimated to occur in about 20% of higher plants (De Luca and St Pierre, 2000), although less than 15% of plants have been properly analysed in this regard (Kinghorn, 2001).

Given their diversity, there are numerous systems of alkaloid classification, however the most useful in the present study is that based on their biochemical origins (Southon and Buckingham, 1989; Dewick, 1998; Roberts and Wink, 1998). In general, the nitrogen in alkaloids is derived from an amino acid precursor. The most commonly incorporated amino acids are lysine, arginine, tyrosine, tryptophan, histidine, and the non-protein amino acids ornithine, nicotinic acid (niacin), and anthranilic acid (Southon and Buckingham, 1989; Dewick, 1998). A minority of alkaloids are synthesised from non-amino acid precursors such as the purines adenine and guanine, and some obtain their nitrogen atom via a transamination reaction relatively late in their biosynthesis (Dewick, 1998). The subjects of the present study are the pyridine alkaloids, which are derived from nicotinic acid. This class of alkaloids includes nicotine, anabasine, nornicotine and anatabine found in tobacco (*Nicotiana* spp., Solanaceae), ricinine from castor beans (*Ricinus communis* L. Euphorbiaceae), dioscorine from wild yam (*Dioscorea hispida* Dennst. Dioscoreaceae), arecoline from betel nut (*Areca catechu* L. Araceae), wilfordic acid from the Chinese medicinal plant 'lei gong teng' (*Tripterygium wilfordii* Hook. F. Celestraceae), and the sesquiterpenoid alkaloids of the Egyptian drug plant 'khat' (*Catha edulis* (Vahl.) Forsk. Celestraceae) (Waller and Nowacki, 1978; Leete, 1979; Schmidt, 1984; Evans, 1989). Pyridine alkaloids have been reviewed extensively and

repeatedly over the last half century, in regard to their distribution, synthesis, analysis, structure and pharmacology, although fewer studies have focussed on their molecular genetics (Henry, 1949; Bentley, 1957; Swan, 1967; Hill, 1970; Waller and Nowacki, 1978; Dalton, 1979; Cordell, 1981; Kutchan, 1998)

Society's interest in alkaloids has long been focussed on their usefulness to mankind. References to alkaloid-containing medicinal plants have been found on 4000 year old Assyrian clay tablets (Wink, 1998). Alkaloids have also been used as arrow poisons in most parts of the world, probably since well before c.2000 B.C (Neuwinger, 1998).

An interesting local case demonstrates the social importance which alkaloid-containing plants may hold. *Duboisia* (Solanaceae) species were used by the Australian aborigines as sources of hunting poisons, anaesthetics, recreational drugs, hunger suppressants, and in religious ceremonies (Johnston and Cleland, 1933; Watson, 1983; Watson *et al.*, 1983; Floyd, 1989). *Duboisia hopwoodii* R. Br. yielded the drug 'pituri', which was produced from plants in a particular West Queensland region by men of a designated social group, and traded over some 500,000 square kilometres of country (Watson, 1983; Watson *et al.*, 1983; Dobkin de Rios and Stachalek, 1999). Pituri contains the tropane alkaloid hyoscyamine, along with the pyridine alkaloids nicotine and nornicotine, which are also the predominant alkaloids in most tobacco species (Luanratana and Griffin, 1982). In Australia, many native *Nicotiana* species were also exploited (Johnston and Cleland, 1933).

Tobacco was also important in pre-Columbian American societies. Shamans and initiates consumed such quantities that near death experiences and permanent alterations to the sight were achieved. These effects may have corroborated the shaman's belief in the ability to see spirits, ancestors, the future, and hidden objects (Wilbert, 1987). Among the Maya and other groups, tobacco was also used as a cleanser, as medicine, and, as it is today, a recreational drug. It was smoked, chewed, snuffed, drunk, licked, and even administered in enemas (Wilbert, 1987; Robicsek, 1978). Despite being denounced as "a wicked poison from the devil" (Benzoni, 1541; in Robicsek, 1978), Europeans were quick to embrace tobacco use, and by 1612 there were 7000 London "smoking houses" (Robicsek, 1978). In 17th century Europe, it joined an already extensive traditional pharmacopoeia of medicines and stimulants (Hatfield, 1999).

Commercial Western medicine is indebted to traditional knowledge of plant medicines (Hamburger and Hostettmann, 1991), as was recognised by the Phytochemical Society of Europe's opening conference lecture in 1993 entitled "Shaman as Scientist" (Hostettmann *et al.*, 1994). Approximately 25% of commercial Western medicines are derived from plant material (Kutchan, 1995), and about 75% of these are directly related to traditional remedies (Schmeller and Wink, 1998; Kinghorn, 2001). Many of these chemicals are alkaloids (Schmeller and Wink, 1998). The exploitation of traditional knowledge for pharmaceutical manufacture has caused considerable debate recently over intellectual property rights (Cox, 2001; Pei, 2001) and the damage that is being caused by the extinction of both knowledge and biodiversity (Cox, 2000; O'Neil, 2000; Sharma, 2001).

Many useful alkaloids have also been identified by the systematic chemical screening of plant material. The anti-cancer agent paclitaxel (a diterpenoid taxane derivative often referred to as "Taxol"), for example, was developed after the analysis of bark extracts of the Pacific yew (*Taxus brevifolia* Nutt. (Taxaceae)). It inhibits the routine disassembly of micro-tubules, and can arrest cancer cells in the stages of division which are most sensitive to radio-therapy (De Smet, 1997). In 1999, paclitaxal was the eighteenth biggest selling drug in the US, with sales in excess of US$1 billion (Kingston, 2000). It is now produced in the laboratory via a semi-synthetic process that employs intermediates extracted from the bark of various yew species (Kinghorn, 2001). Other plant alkaloids which have become successful medicines are vinblastine and vincristine, isolated from *Catharanthus roseus* G. Don. (Apocynaceae), both of which act in a similar manner to paclitaxal, and are effective treatments against numerous cancers (*eg*; Hodgkins disease and other lymphomas, renal, testicular and neck cancer) (Hamburger and Hostettmann, 1991). In addition to the direct use of natural plant products, some synthetic pharmaceuticals are based on plant-derived analogues (Hamburger and Hostettmann, 1991; Kem, 1997; Kinghorn, 2001).

The abundance of pharmacologically active alkaloids is thought to be directly related to their evolution as defence chemicals, shaping their structures to interfere with biological targets (Schmeller and Wink, 1998). As described below, transgenic studies are now being undertaken with the dual aims of understanding how plants regulate the production of defence chemicals, and also how some of these chemicals

can be produced in larger quantities, both by intact plants and by cultured plant tissues.

1.1.3 Patterns of alkaloid biosynthesis

Although many current questions in plant biology are being successfully addressed by large studies in model species such as *Arabidopsis* (*eg*; The *Arabidopsis* Initiative, 2000), questions regarding specialised metabolites require basic research on the plant species which produce them. In 1978, Waller and Nowacki reviewed the genetic control of alkaloid biosynthesis in numerous plant taxa. The value of this study was, however, limited due to the lack of molecular data at that time. It was not until a decade later that alkaloid biology emerged as a molecular genetic discipline, with the first cloning of alkaloid biosynthetic genes. These encoded strictosidine synthase (Str; EC 4.3.3.2) and tryptophan decarboxylase (TDC; EC 4.1.1.28), involved in the production of medicinally important monoterpenoid indole alkaloids including vinblastine and vincristine in *C. roseus* (Kutchan *et al.*, 1988; De Luca *et al.*, 1989). Since then, significant advances have been made towards understanding the regulatory mechanisms governing gene activity in metabolic pathways leading to the production of many alkaloids (*eg*; vom Endt *et al.*, 2002). Although there are a large number of biochemical pathways involved in alkaloid synthesis, many features initially uncovered in studies of monoterpenoid indole alkaloid biosynthesis are likely to be valuable as common principles in the synthesis of other alkaloids.

In *C. roseus*, the amino acid tryptophan is decarboxylated by TDC, forming tryptamine, which is subsequently condensed with secologanin by Str to produce strictosidine (Pasquali *et al.*, 1999). Strictosidine is the basis for the synthesis of numerous monoterpenoid indole alkaloids (Hashimoto and Yamada, 1994). It is thought that these alkaloids act *in planta* to deter pathogens and herbivores (Ouwerkerk and Memelink, 1999). Their biosynthesis is responsive to physical damage and chemical signals associated with infection (elicitors) (Aerts *et al.*, 1994). Both *Str* and *TDC* are transcriptionally up-regulated by elicitors related to fungal infection via a signal cascade involving jasmonic acid or its ester, methyl jasmonate (Menke *et al.*, 1999a). Responsiveness to environmental stimuli is a common feature of alkaloid biology, as well as other secondary metabolites with defensive functions (Baldwin, 1993; Rhodes 1994), and jasmonate signal transduction pathways are

widespread in plants (Sembdner and Parthier, 1993; Wasternack and Parthier, 1997). It is therefore likely that an understanding of the regulation of *Str* and *TDC* transcription will be of value in understanding other systems (vom Endt *et al.*, 2002). It is, however, important to note that in some species multiple parallel signal transduction pathways exist, as demonstrated by the differential jasmonate induction of a reporter gene driven by the *C. roseus Str* promoter in *N. tabacum*, when compared to the induction of the endogenous genes chitinase and basic β-1,3-glucanase (Imanishi *et al.*, 1998; Pasquali *et al.*, 1999). This demonstrates that while key regulatory mechanisms may be shared between systems, some components differ, reinforcing the need for complementary studies in different species.

Transcription factors involved in *Str* induction have recently been isolated. In 1999, a novel Jasmonate and Elicitor Responsive Element (JERE) was identified in the *Str* promoter using promoter deletion analysis in transformed *C. roseus* cells (Menke *et al.*, 1999b). Using a yeast one-hybrid screen, two cDNAs encoding novel transcription factors binding JERE were isolated (octadecanoid-derivative response *Catharanthus* AP2-domain proteins- ORCA1 and ORCA2) (Menke *et al.*, 1999b), followed by the separate isolation of a third (ORCA3) (van der Fits and Memelink, 2000; 2001). The overexpression of ORCA2 in *C. roseus* facilitated an increase in the transcription of a reporter gene fused to the *Str* promoter (Menke *et al.*, 1999b), and overexpression of ORCA3 caused an increase in *Str* and *TDC* mRNA levels, as well as an increase in alkaloids in *C. roseus* (van der Fits and Memelink, 2000). ORCA2 and ORCA3 are themselves transcriptionally induced by jasmonate, raising the question as to what signal cascade regulates their expression. It is currently thought that pre-existing ORCA proteins are activated via a jasmonate signal, allowing them to increase their own subsequent transcription, and induce genes encoding biosynthetic enzymes involved in alkaloid synthesis (vom Endt *et al.*, 2002). Studies in other systems (*eg*; flavonoid synthesis) suggest that co-ordinated transcription, regulated by "master" transcription factors is a common mechanism of regulating secondary metabolic pathways (Rhodes, 1994; vom Endt *et al.*, 2002), although post-transcriptional mechanisms are also likely to contribute to the precise regulatory control of alkaloid metabolism (vom Endt *et al.*, 2002).

In addition to being influenced by environmental stimuli, alkaloid production may be regulated developmentally, by the spatial and temporal control of

intermediate steps. In *C. roseus*, for example, TDC acts in the cytoplasm, while Str is located in the vacuole (Pasquali *et al.*, 1999). *TDC* and *Str* mRNAs are also spatially regulated at the whole-plant scale, with both transcripts reaching high levels in the epidermis of aerial tissues, and developing root tips (St-Pierre *et al.*, 1999). Some later steps in monoterpenoid indole alkaloid synthesis also take place in different organelles and organs in *C. roseus*, suggesting that pathway intermediates are transported within and between cells. For example, in vindoline production, *N*-methyltransferase (NMT; EC 2.1.1) activity is located in the leaf chloroplast (De Luca and Cutler, 1987), while desacetoxyvindoline 4-hydroxylase (D4H; EC 1.14.11.11) and deacetylvindoline 4-O-acetyltransferase (DAT; EC 2.3.1.107) mRNAs are restricted to lacitifer and idioblast cells in leaves, stems and buds (St-Pierre *et al.*, 1999; De-Luca and St Pierre, 2000). TDC and Str activities are also regulated over developmental time, with young seedlings and young leaves possessing high activity, and correspondingly high levels of alkaloid accumulation (Aerts *et al.*, 1994; St-Pierre *et al.*, 1999).

Similarly complex spatial and temporal organisation is observed in other alkaloid pathways, allowing regulation by keeping some intermediates separated, and others in close physical proximity, thus favouring certain sequences of reactions (reviewed by Luckner, 1990; Hashimoto and Yamada, 1994; Kutchan, 1998; Verpoorte *et al.*, 1998). This "metabolic channelling" may also be facilitated by the physical association of enzymes into multi-enzyme complexes (Luckner, 1990). As such complexities have become clear, the initial hypothesis that alkaloids are non-functional waste products (Introduction 1.1.1) has become increasingly unlikely.

Further insights into the complexity of alkaloid biosynthesis have come from attempts to manipulate alkaloid accumulation *in vivo* (Robins *et al.*, 1994). One of the first successful transgenic manipulations of alkaloid content dealt with the tropane alkaloid hyoscyamine, and its medicinally valuable derivative scopolamine. In *Hyoscyamus niger* L. (Solanaceae), hyoscyamine is converted via a two-stage reaction into scopolamine, by hyoscyamine-6-β hydroxylase (H6H; EC 1.14.11.11) (Hashimoto *et al.*, 1993a; Hashimoto and Yamada, 1994). In *Atropa belladonna* L. (Solanaceae), however, hyoscyamine levels far exceed scopolamine levels (Yun *et al.*, 1992), suggesting that H6H activity limits this conversion. When transformed with a *H. niger H6H* gene under the transcriptional control of the CaMV35S

promoter, *A. belladonna* plants and cultured roots were able to accumulate raised scopolamine levels (Yun *et al.*, 1992; Hashimoto *et al.*, 1993b), demonstrating that the pathway could be manipulated by overcoming the limiting step. More recent experiments have achieved similar success in producing increased scopolamine levels at the expense of hyoscyamine via the introduction of *H6H* into the hyoscyamine-producing *H. muticus* L. (Jouhikainen *et al.*, 1999).

Many other attempts at manipulating alkaloid production have, however, been less successful and less predictable. For example, when *TDC* was overexpressed in *C. roseus*, an increase in tryptamine was observed, without any increase in monoterpenoid indole alkaloid levels, probably because the production of the key intermediate secologanin remained prohibitively low (Goddijn *et al.*, 1995). When *TDC* was overexpressed in a different species (*Peganum harmala* L. Zygophyllaceae), the tryptamine levels themselves were not elevated, probably because tryptamine was rapidly converted into another metabolite (serotonin) by a competing pathway (Berlin, *et al.*, 1993; Verpoorte *et al.*, 1998). It is notable that these examples attempted to manipulate an upstream step, which has been termed "pushing" the pathway, whereas the successful *A. belladonna* experiments "pulled" the pathway by manipulating a downstream limiting step (Nessler, 1994). These studies demonstrate the fact that alkaloid pathways often intersect or overlap with other metabolic pathways, and that limiting steps may occur at different points in the pathways. The prevalence of multiple, interacting enzymes may have contributed to the evolution of alkaloid diversity, with many new structures being produced by novel combinations of enzymes and substrates (Luckner, 1990). This phenomenon is conceptually mimicked by "combinatorial chemistry" aiming to produce novel structures (Hamburger and Hostettmann, 1991; Kinghorn, 2001).

Further insights into the patterns of alkaloid biosynthesis are now emerging from the study of the alkaloids of *Nicotiana* species. These alkaloids are proving to be a particularly valuable model system, because an emerging body of molecular data is able to complement an already impressive body of ecological and physiological knowledge.

1.2 Pyridine alkaloids in *Nicotiana* species

1.2.1 Ecological roles

To date, much of the direct evidence that alkaloids may be ecologically active as defence agents has come from studies involving tobacco species, most notably *N. sylvestris* Speg and Comes, and *N. tabacum* L. These species produce the pyridine alkaloid nicotine as their major alkaloid. Both nicotine and its derivative nornicotine are toxic to vertebrates and invertebrates (Watson *et al.*, 1983; Budavari *et al.*, 1989). It has been shown that insect larvae (*Manduca sexta*) forced to feed on tobacco plants gain less weight and feed less than control insects (Baldwin, 1988a). Other studies confirm that larvae may not develop to maturity (Parr and Thurston, 1972), or are killed outright (Baldwin and Preston, 1999) by a tobacco diet.

Alkaloids may constitute ~3% of the dry mass of *N. sylvestris* leaves (Saitoh *et al.*, 1985). Of this, 95- 99% is nicotine and nornicotine, while the remainder is made up of the minor pyridine alkaloids anabasine and anatabine (Saitoh *et al.*, 1985; Sisson and Severson, 1990). Considering that each of these alkaloids includes two nitrogen atoms in its structure (Figure 1.2), *N. sylvestris* plants may invest a significant proportion of their metabolic budget in chemical defences. In certain circumstances, it has been estimated that up to 8% of *N. sylvestris'* nitrogen may be invested in nicotine (Baldwin *et al.*, 1998; Baldwin and Hamilton, 2000). Furthermore, this investment in nitrogen may not be available for further primary metabolism. Tso and Jeffrey (1961) suggested, on the basis of feeding experiments, that in *N. rustica* L., nicotine is in a "dynamic state that is connected with primary metabolic pathways". More recent reports, however, have concluded that *de-novo* produced nicotine is not recycled into primary metabolism in *N. sylvestris*, even under nitrogen starved conditions, and have raised the possibility that fed and synthesised nicotine are treated differently *in planta* (Baldwin and Ohnmeiss, 1994; Baldwin *et al.*, 1994a; Baldwin *et al.*, 1998).

In *N. tabacum* and *N. sylvestris*, and possibly other *Nicotiana* species, nicotine production is up-regulated in response to foliage damage such as that caused by insect attack (see Figure 1.1). Total leaf nicotine content in *N. sylvestris* leaves may increase by up to ~400% following foliage damage (Baldwin, 1988a, 1988b; 1989). Damaged

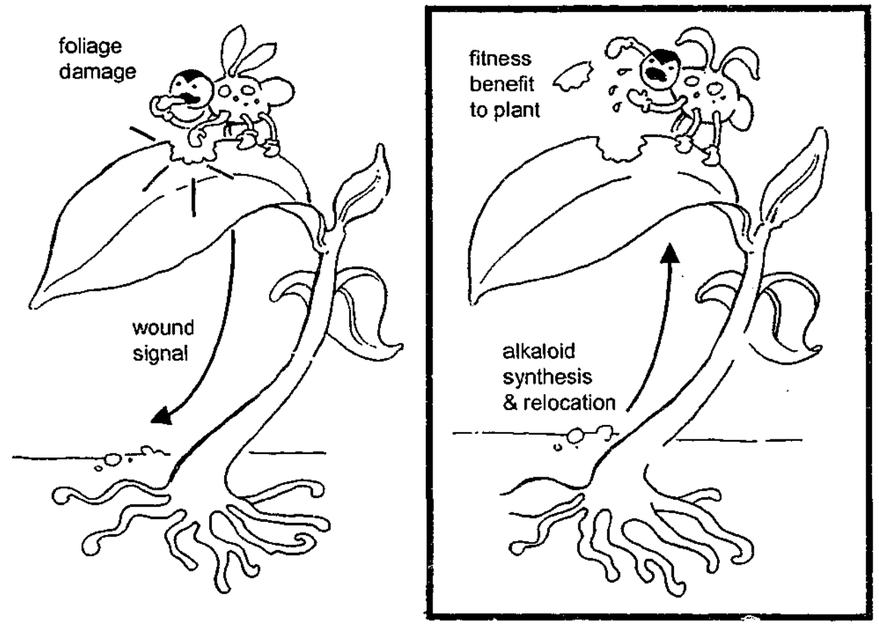


Figure 1.1 Representation of wound-induced alkaloid synthesis in tobacco.

The left panel shows an insect herbivore inflicting damage to the foliage of a tobacco plant. This damage is perceived by the plant, and a phloem-borne wound signal is sent from the foliage to the roots. The right panel depicts the induction of alkaloid synthesis in the roots in response to this signal. This induced alkaloid pool is then relocated from the roots to the foliage, via the xylem. The accumulation of alkaloids in the foliage acts as a feeding deterrent, providing the plant with protection against foliage damage, and hence a fitness benefit. This model has been deduced from experiments involving the model species *Nicotiana tabacum* and *N. sylvestris* (Dawson 1942; Baldwin 1988a, 1988b, Baldwin *et al.*, 1994b; 1997).

foliage releases a chemical signal, which is transported to the roots, via the phloem (Baldwin, 1989; Baldwin *et al.*, 1994b).

Jasmonic acid (or its ester methyl jasmonate) is known to be an important component of this signalling system (Baldwin *et al.*, 1994b; Baldwin *et al.*, 1997), however other plant hormones such as auxin and ethylene are known to influence nicotine production (Feth *et al.*, 1986; Shoji *et al.*, 2000b).

When the wound signal reaches the roots, gene expression is stimulated, enzyme activities increase, and increased rates of nicotine synthesis occur (Introduction 1.3). Nicotine is then transported in the xylem to the aerial tissues (Dawson, 1941; 1942; Wink and Roberts, 1998; Shoji *et al.*, 2000a), where it accumulates in cell vacuoles (Saunders, 1979; Baldwin and Schmelz, 1994; Ohnmeiss *et al.*, 1997). Nicotine has been shown to be produced only in growing root tips in *N. tabacum*, as demonstrated by the fact that pot-bound plants are unable to increase nicotine levels following wounding (Dawson, 1941, 1942; Baldwin, 1988b). Reciprocal grafting experiments have shown that nicotine is not produced in *N. tabacum* leaves or shoot in any great quantity (0.5-3% *de novo* synthesis in leaf; 0.5-8% in shoot), however nicotine is de-methylated in the leaves to form nornicotine (Dawson, 1945; Dawson and Solt, 1959; Wernsmann and Matzinger, 1968). Evidence also exists suggesting that foliage damage allows greater nicotine accumulation by facilitating a decrease in nicotine degradation (Yoshida, 1962), however this data has been questioned in later studies (Baldwin *et al.*, 1994a; Baldwin and Ohnmeiss, 1994; Baldwin and Hamilton, 2000).

The induction of nicotine in response to insect attack allows valuable nitrogen to be diverted from primary and into secondary metabolism when it is most required. Field studies have supported the suggestion that alkaloid production is so metabolically costly that it disadvantages the plant when it is induced at inappropriate times. For example, *Nicotiana attenuata* (Torrey ex S. Watson) plants treated with methyl jasmonate to increase their nicotine levels produced more seed than un-induced controls when herbivory levels were high. When, however, herbivory was reduced, the un-induced plants produced more seed (Baldwin, 1998). Other recent studies have also demonstrated that induced defences have associated costs (Karban and Baldwin, 1997; Baldwin *et al.*, 1998; Purrington, 2000).

In *N. sylvestris*, different degrees of herbivory or foliage damage induce different levels of alkaloid production (Baldwin and Ohnmeiss, 1993). Damage to the flowering stalk results in the largest alkaloid induction, and the puncturing of leaves results in a greater response than their surgical removal with a blade (Baldwin and Schmelz, 1994). It has been shown clearly in *N. sylvestris* that nicotine accumulation is related to leaf damage whether or not a plant is nitrogen-stressed, so that nicotine synthesis is not merely fuelled by overflow from primary metabolism, but is an active defence system (Ohnmeiss and Baldwin, 1994).

It is a matter of speculation as to what selective pressures enabled the *Nicotiana* species noted above to separate the site of nicotine synthesis from the site of foliar herbivory. One possibility is that this arrangement may prevent the site of production from being rapidly removed by the herbivore. Another possibility is that nicotine synthesis evolved in the roots, and was later co-opted to protect the foliage, which never evolved its own nicotine synthesis ability (Roddick, 1982). Nonetheless, many other defence chemicals are thought to be induced in the leaves of various plants species (Baldwin, 1988b; 1993; including the alkaloids of *C. roseus*), and it remains unclear what ecological impact results from the difference between leaf- and root-induced alkaloid defences.

1.2.2 Overview of the genus *Nicotiana*

The genus *Nicotiana* has been used as a model for genetic studies for over 200 years, since Kolreuter described a series of artificial crosses between various *N. tabacum* strains and *N. glutinosa* in 1761 (Darwin, 1859; Smith, 1979). It remains one of the primary model systems in biological research[ii]. Its interest to the biologist lies primarily in its amenability in the laboratory (Smith, 1979), its ease of genetic

[ii] When the literature database 'Bioabstracts' (1980-2002, Ovid) was searched using the names of dicot plants commonly used in research, *Nicotiana* (10753 hits) was second only to *Arabidopsis* (11209) in the number of papers mentioning its name. Other Solanaceous genera were also used extensively as models (*eg*; *Solanum* (9991) and *Lycopersicon* (8449)). Other model, agricultural or garden dicot plants have received far less attention in the publicly available literature (*eg*; *Eucalyptus* (6058), *Gossypium* (cotton, 5138), *Betula* (birch, 4087), *Lotus* (1938), *Petunia* (1666), *Catharanthus* (1277), *Rhododendron* (1197) and *Geranium* (927)). Some agriculturally important monocot species were, however, mentioned in many publications (*eg*; *Zea mays* (maize, 18830) and *Oryza* (rice, 10323)).

transformation, and the fact that aspects of many species' biochemistry- including the synthesis of their alkaloids from primary metabolic precursors- are well understood.

Nicotiana species are widely distributed on most of those landmasses that once made up the ancient Gondwanan continent. Most species are small, short lived shrubs or herbs (Goodspeed, 1954). The classic studies of Goodspeed (1945, 1954; Goodspeed and Thompson, 1959) have described in detail the morphology, distribution, taxonomy, evolution, genetics and chemistry of the genus. A number of species have since been described, including the only known African species (*N. africana* Merxm., Symon, 1991), and additional species from Australia (*eg*; *N. wutkeii*, Clarkson and Symon, 1991). Between 75 and 95 species have now been recognised (Symon, 1991; D'Arcy, 1991). The genus is relatively complex, being divided into three subgenera: *Rustica* (Don) Goodspeed and *Tabacum* (Don) Goodspeed, which are both distributed across South America, and *Petunioides* (Don) Goodspeed, which has members in North and South America, the Pacific Islands, Australasia and Africa. These Subgenera are further divided into sections (Goodspeed, 1954).

Various *Nicotiana* species have been used in a number of different research contexts. For example, *N. alata* Link and Otto has been a useful model for the study of self-incompatibility mechanisms (Newbigin *et al.*, 1993; Franklin-Tong and Franklin, 1993), and *N. benthamiana* Domin. has been useful in virological studies (Horvath, 1993). As already described, a number of species have also been the subjects of extensive research into the role and the synthesis of alkaloids, including the commercial tobaccos (*N. tabacum* and to a lesser extent *N. rustica*) and some wild tobaccos (mainly *N. sylvestris* and *N. attenuata*). The genus as a whole contains a great deal of variation in alkaloid content, and sixty-four *Nicotiana* species have been characterised in this respect (Smith and Smith, 1942; Smith and Abashian, 1963; Saitoh *et al.*, 1985; Parr and Hamill, 1987; Sisson and Severson, 1990). Essentially all species contain quantifiable amounts of nicotine, its derivative nornicotine, and the pyridine alkaloids anabasine and anatabine (Leete, 1983; Sisson and Severson, 1990). The structure and biosynthesis of these alkaloids are discussed below (Introduction 1.3). Over forty other alkaloids, mainly nicotine derivatives, have been reported in *Nicotiana* in minute quantities, and have been reviewed by Leete (1983).

The alkaloids of *Nicotiana* have also occasionally been reported in small quantities in other genera. Within the Solanaceae, nicotine has been reported in small

quantities in *Cyphanthera tasmanica* Miers (Bick *et al.*, 1974), *Cestrum* spp., *Datura* spp., *Duboisia* spp., *Petunia violacea* Lindl., *Lycopersicon esculentum* (tomato[iii]), *Solanum* spp. (including potato, *S. tuberosum* L.) and *Withania somnifera* (L.) Dunas., many of which also produce small quantities of nornicotine (Leete, 1983). Nicotine has also been reported (although not necessarily confirmed) in minute amounts in many other plants with widely disparate evolutionary origins, including *Acacia* spp. (Mimosaceae, wattles), *Cannabis sativa* L. (Cannabaceae, marijuana), *Equisetum* spp. (Equisetaceae, horsetails) and *Lycopodium* spp. (Lycopodiaceae, clubmosses). Anabasine has been reported in appreciable levels in *Anabasis aphylla* L. (Chenopodiaceae) after which it is named, and in the Solanaceous plants *Solanum carolinense* L. and *Duboisia* spp. (Leete, 1983) and the Australasian genera *Mackinlaya* (Araliaceae) and *Marsdenia* (Asclepiadaceae) (Fitzgerald *et al.*, 1966; Summons *et al.*, 1972). Interestingly, anabasine (and the structurally similar anabaseine), are also found in some invertebrate animals, including ants and worms (Leclercq, 2001), raising questions relating to the evolution of metabolic pathways.

Overall, however, the occurrence of *Nicotiana* alkaloids outside the genus is generally minimal, in striking contrast to the array of alkaloid profiles found within the genus. The intra-generic variation provides good opportunities for elucidating the mechanisms which control alkaloid accumulation, using both traditional and molecular techniques (Waller and Nowacki, 1978; Legg and Collins, 1971; Hibi *et al.*, 1994). For example, early work using inter-species grafts of *N. tabacum*, *N. glauca* and *N. glutinosa* first revealed that nornicotine was synthesised largely in *Nicotiana* leaves, at the expense of nicotine (Dawson, 1945). Similar variation in biosynthetic capacity is utilized in the present study, in order to investigate the role of differential gene expression in species with contrasting alkaloid profiles, each of which are reviewed below.

[iii] *Lycopersicon esculentum* was initially described as a member of *Solanum* by Linnaeus. Spooner and co-workers (1993) presented molecular evidence supporting its re-instatement within this genus as *Solanum lycopersicon* L., however this name has not been widely adopted.

1.2.3 *Nicotiana tabacum* (commercial tobacco) and *N. sylvestris* (wood tobacco)

Nicotiana tabacum is an allotetraploid (2n=4X=48) species which formed via the hybridisation of two diploid species (2n=4X=24), and subsequent chromosome doubling. The maternal parent is accepted as being a recent ancestor of *N. sylvestris* (2n=24), however the exact identity of the pollen parent has long been controversial (Goodspeed, 1954; Mann and Weybrew, 1958; Wernsman and Matzinger, 1968; Murad *et al.*, 2002). Some recent studies have supported the hypothesis that the plant was *N. tomentosiformis* Goodspeed (2n=24; subgenus *Tabacum*; section *Tomentosae*) (Lim *et al.*, 2000; Murad *et al.*, 2002), while others have suggested that the plant may have been an introgressed hybrid, carrying some of the genome of *N. otophora* Grisebach. (2n=24; section *Tomentosae*) (Riechers and Timko, 1999; Ren and Timko, 2001). Goodspeed (1954) implied that the hybridisation leading to the formation of *N tabacum* may have occurred under pre-Columbian agriculture, since no record of *N. tabacum* outside cultivation was then known. More recent molecular studies, however, suggest that this event may have taken place up to 6 million years ago (Okamuro and Goldberg, 1985; Fulnecek *et al.*, 2002). Interestingly, the other commercial tobacco, *N. rustica* (2n=48), is also an allotetraploid. An ancestor of *Nicotiana paniculata* L. (2n=24) is the likely maternal progenitor, and an ancestor of *N. undulata* Ruiz & Pavon (2n=24) is the likely paternal progenitor (Goodspeed, 1954; Cherep and Kormanitskii, 1991).

As already noted, the currently accepted model of alkaloid synthesis in *Nicotiana* was developed using *N. tabacum* and *N. sylvestris* as models, and the two species have qualitatively similar alkaloid profiles. Certain *N. tabacum* varieties have, however, been bred over many decades to possess desirable smoking traits (Valleau, 1949; Legg and Collins, 1971), including reduced alkaloid levels (*eg*; LAFC 53; 0.2% alkaloids, Chaplin, 1975; *cf.* Isogenic wild type strain NC95 with 3% alkaloid, Chaplin 1986).

1.2.4 *Nicotiana glauca* (tree tobacco)

Nicotiana glauca Grah. (2n=24) (Subgenus *Rustica*; section *Paniculatae*) stands apart from other *Nicotiana* species in a number of regards, most obviously as the only *Nicotiana* species which is a perennial tree. Goodspeed (1954) notes that its morphological and cytological distinctions from its counterparts point to its 'separate evolution', probably in isolation in Argentina. The species is, however, now very widespread, and is a common weed in many areas, including Australia. Here, it apparently forms hybrids with a number of native species (*eg*; *N. suaveolens* Lehm., *N. simulans* N. T. Burbidge and *N. goodspeedii* Wheeler; Horton, 1981; Walsh and Entwisle, 1999). It has occasionally been used as a drug by aboriginal people (Haegli *et al.*, 1982). *Nicotiana glauca* is, however, extremely toxic, and reports of stock poisoning (Haegli *et al.*, 1982) and even human fatalities following consumption of leaf material (Casterona *et al.*, 1987; Mellick *et al.*, 1999; Sims *et al.*, 1999) are more common in the literature than reports of controlled human use.

Nicotiana glauca is the only *Nicotiana* species which accumulates the toxic alkaloid anabasine as its major leaf alkaloid (~85-97% of 4-9 mg/g dw total) (Saitoh *et al.*, 1985; Sisson and Severson, 1990). Although studies extend over several decades, there is apparently still some confusion as to where this alkaloid is principally produced in the plant. Bagni and co-workers (1986) provided circumstantial evidence suggesting that lysine is decarboxylated in the roots to produce the intermediate cadaverine, which is transported to the foliage where most anabasine synthesis was purported to occur. Other workers, however, have assumed the opposite- that lysine is produced and decarboxylated in the leaf chloroplast, and an intermediate such as cadaverine is transported to the roots, where anabasine synthesis may occur (Herminghaus *et al.*, 1991; Fecker *et al.*, 1992). Perhaps the clearest experimental evidence, however, is the simple grafting experiments undertaken by Dawson (1945), who showed that *N. glauca* stocks and scions are both quite capable of anabasine synthesis when grafted onto non-anabasine producers. Furthermore, the enzyme responsible for the conversion of lysine (lysine decarboxylase; see Introduction 1.3.4) has been measured in both the leaves, where it is located in the chloroplast and possibly also the cytoplasm (Bagni *et al.*, 1986), and also in the roots of *N. glauca*, where it may be located in the leucoplast (Herminghaus

et al., 1996). Whatever the precise balance, it is clear that appreciable anabasine biosynthesis can take place in both the leaves and roots. This is in contrast to the production of nicotine, which essentially occurs only in the roots of all studied *Nicotiana* species, including *N. glauca* (Dawson, 1962; Baldwin 1988b).

Nicotiana glauca root cultures, like the roots of intact plants, contain both anabasine and nicotine. Dawson (1962) showed the kinetics of nicotine formation to be similar in cultured roots of *N. tabacum* and *N. glauca*, and suggested that nicotine is produced in actively growing root tips of both species. The kinetics of anabasine production in *N. glauca* root cultures was, however, different, suggesting that this alkaloid is produced in cells throughout the root.

Relatively few studies have examined the response of *N. glauca* to wounding. Baldwin and Ohnmeiss (1993) showed that leaf anabasine levels increased ~2 fold 7 days after foliage damage. This study did not determine whether increased leaf and/or root synthesis of anabasine was responsible for the induction. Preliminary observations in this laboratory have shown that one alkaloid biosynthesis gene (*QPRTase*; see introduction 1.3.6) is transcriptionally upregulated following wounding in the leaves themselves (Sinclair *et al.*, 2000), raising the possibility that *N. glauca* is able to increase synthesis of its main leaf alkaloid at the site of foliage damage, unlike other *Nicotiana* species studied so far.

1.2.5 *Nicotiana alata* (ornamental tobacco)

Nicotiana alata (2n=24) (Subgenus *Petunioides*; section *Alatae*) is a short-lived perennial, found naturally in rocky areas in Uruguay, Brazil, Argentina and Paraguay (Goodspeed, 1954). It has been cultivated since the early nineteenth century as an ornamental due to its large fragrant flowers (Goodspeed, 1954).

Nicotiana alata foliage is well known to contain only small amounts of alkaloids. It has been commented that were it not for its affinities with other *Nicotiana* species, it would probably not have been noticed that *N. alata* was capable of producing alkaloids (Waller and Nowacki, 1978). Saitoh and co-workers (1985) detected only 0.03 m/g dry weight in the foliage of wounded, flowering plants, all of which was nicotine. This is more than 1000 fold less than that found in *N. sylvestris*, and 300 fold less than in *N. glauca*. Sisson and Severson (1990) detected slightly

higher alkaloid levels (0.2 mg/g dw), with quantifiable levels of anabasine (21%) and nornicotine (10%) being identified along with the predominant nicotine fraction (69%) in the mature leaves of wounded, greenhouse-grown plants. A third report has produced yet another alkaloid spectrum. In young foliage, Friesen and Leete (1990) found 0.02 mg/g fresh weight of alkaloids, however 41% was anatabine, 6% anabasine, and only 53% nicotine. The old leaves yielded only 0.005 mg/g of alkaloid, in this case with more nornicotine (54%) than nicotine (28%). Although these results differ in detail, collectively they demonstrate that the foliage of *N. alata* contains substantially lower alkaloid levels than the leaves of *N. sylvestris* or *N. glauca*.

Although the leaves of *N. alata* accumulate extremely little alkaloid, an appreciable amount may be found in the roots. Saitoh and co-workers (1985) found 2.0 mg/g dw total alkaloids, of which 38% was nicotine, 46% nornicotine, and 16% anatabine. It is noteworthy that the roots of *N. sylvestris* plants grown under comparable conditions accumulated only a 4 fold greater alkaloid content than those of *N. alata*, in marked contrast to the 1000 fold difference found in the leaves (Saitoh *et al.*, 1985). The limited number of reports on *N. alata* root cultures suggest that they may be capable of producing ~4 fold higher levels of alkaloid than the roots of intact plants, although the alkaloid spectrum is similar (Friesen *et al.*, 1992; Botte *et al.*, 1997).

Interestingly, *N. alata* is very closely related to *N. sylvestris*, as shown by recent RAPD analysis (Yu and Lin, 1997). The vast difference in alkaloid phenotype between these two closely related species may be of value in determining what factors contribute to alkaloid accumulation in leaf tissue.

Although some of the plants used in past reports have been wounded (Sisson and Severson, 1990), the alkaloid response of *N. alata* to foliage damage remains unknown.

1.2.6 *Nicotiana hesperis* (a wild Australian tobacco)

During the 1950s, Nancy Burbidge collected extensively in arid Australia, and described a number of new *Nicotiana* species including *N. hesperis* (Burbidge, 1960) (2n=22; Subgenus *Petunioides*, section *Suaveolentes*) from a remote coastal area of Western Australia. In 1981, it was relegated to *N. occidentalis* subsp. *hesperis* (Horton, 1981; Haegli *et al.*, 1982), however, since then, it has been suggested that the species be restored, due to its distinctive seed morphology (Farooqui and Bahadur, 1983).

Despite the relative rarity and taxonomic obscurity of *N. hesperis*, it has attracted the most attention of the Australian *Nicotianas* in studies dealing with alkaloids. The distinction between *N. occidentalis* and *N. hesperis* seems sensible on chemical grounds, with Saitoh and co-workers (1985) showing that *N. occidentalis* accumulates large amounts of nicotine, nornicotine and anabasine in its roots (6.5 mg/g dw total; 42%, 15% and 40% respectively), but relatively little alkaloid in its leaves (0.52 mg/g dw). In contrast, *N. hesperis* has a relatively high leaf alkaloid content (4.1 mg/g dw), made up primarily of nicotine (52%) and anabasine (44%), but with a lower alkaloid content in the roots (1.9 mg/g dw), mostly anabasine (75%) and nicotine (22%). Sisson and Severson (1990) confirmed these data for *N. hesperis* leaves, without assaying roots.

The relatively high level of anabasine in *N. hesperis* makes it one of the most active anabasine producers in the genus, although it still produces far less anabasine than *N. glauca*. Parr and Hamill (1987) selected it as one of 8 *Nicotiana* species representative of alkaloid variation across the genus, and confirmed that of these species its root cultures were second only to those of *N. velutina* as anabasine producers (*N. glauca* was not included in this study). *Nicotiana hesperis* root cultures were found to contain similar anabasine levels to intact plant roots, but accumulated more nicotine and nornicotine (Walton and Belshaw, 1988).

No information has been published on the ability of *N. hesperis*, nor any other species in the largely Australian section *Suaveolentes*, to increase their alkaloid content following foliage damage.

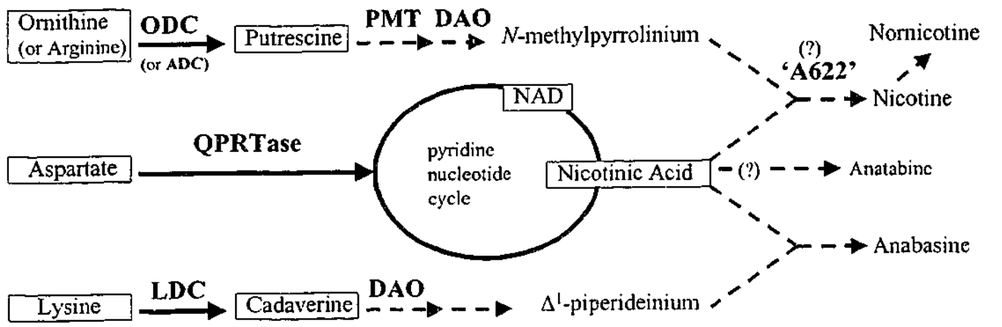
1.3 Pyridine Alkaloid Biosynthesis

1.3.1 Overview

The production of pyridine alkaloids involves the condensation of the pyridine nucleotide nicotinic acid (NA) with another metabolite (Dawson *et al.*, 1960; Leete, 1979; Evans, 1989). In most cases this other metabolite is unique to each particular pyridine alkaloid, and may be derived from apparently unrelated areas of metabolism. This general scheme, with NA as the central component of pyridine alkaloid formation, is shown in Figure 1.2. (Detailed discussion of these pathways is provided in the sections below, 1.3.2; 1.3.3; 1.3.4 and 1.3.5).

In the case of nicotine, NA is condensed with *N*-methylpyrrolinium salt (Leete, 1979; Feth *et al.*, 1986; Wagner *et al.*, 1986a; Verpoorte *et al*, 1998). For the synthesis of anabasine, NA reacts with Δ^1-piperideinium (Leete, 1979; Walton and Belshaw, 1988; Watson *et al.*, 1990; Verpoorte *et al.*, 1998). In anatabine production, both pyridine rings are probably derived from NA (Leete, 1979; Robins *et al.*, 1987). Nornicotine is derived directly from nicotine (Dawson, 1945).

Although the outline of these pathways is known, only some of the enzymes which are presumed to be involved have been characterised in detail. The condensation reaction which finally produces nicotine is presumed to be catalysed by nicotine synthase, an enzyme whose activity has been measured by some workers (Friesen and Leete, 1990), but which has evaded others, despite careful attempts to assay it (Hibi *et al.*, 1994). The nicotine synthase gene has not been characterised, however a cDNA designated '*A622*', isolated in a differential cDNA screen using high and low alkaloid varieties of *N. tabacum*, may represent this enzyme, or some component of a nicotine synthase complex (Hibi *et al.*, 1994). The '*A622*' cDNA sequence suggests that the corresponding gene has evolved from a sequence similar to isoflavone reductase, suggesting that the protein is an oxidoreductase of some sort, consistent with a role in nicotine synthesis. '*A622*' expression has been shown to be confined to the roots of *N. tabacum* and *N. sylvestris* plants, and it is induced by methyl jasmonate in *N. sylvestris*, consistent with the hypothesis that it represents an alkaloid biosynthetic gene (Hibi *et al.*, 1994; Shoji *et al.*, 2000b).



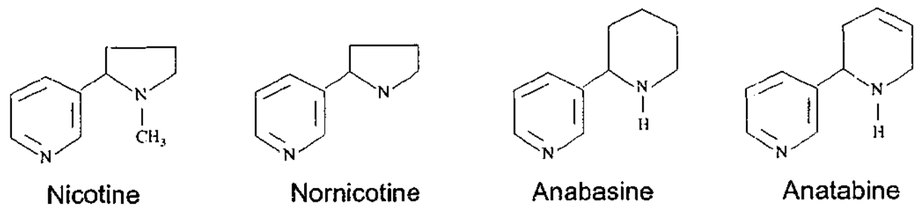


Figure 1.2 A simplified diagram of alkaloid synthesis in *Nicotiana*, as it is presently understood.

Primary metabolic reactions common to all/most organisms are shown with full arrows, whereas reactions specific to the production of secondary metabolites are shown with dotted arrows. Similarly, primary metabolites are boxed, while secondary metabolites are not boxed. Based on the schemes presented previously by Leete (1979), Feth and co-workers (1986), Wagner and co-workers (1986a, 1986b), and Walton and Belshaw (1988). A detailed explanation of the regulation of these converging pathways, and the enzymatic steps involved, is provided in the text (Introduction 1.3.2, 1.3.3, 1.3.4 and 1.3.5). The pathway from putrescine to *N*-methylpyrrolinium is shown in detail in Figure 1.3; and the pyridine nucleotide cycle is detailed in Figure 1.4. The molecular structures of nicotine, nornicotine, anatabine and anabasine are shown in the lower panel.

The final reactions which form anabasine and anatabine require further detailed analysis. Several experiments have shown that anabasine and nicotine synthesis compete for common precursors. Walton and co-workers (1988) found that feeding cadaverine to cultured roots of *N. rustica* caused an increase in anabasine levels, and a decrease in nicotine levels. These authors noted a number of hypotheses to explain this observation at the molecular level, and noted that a single enzymatic complex may be responsible for the final steps in the biosynthesis of both alkaloids. More recently, Herminghaus and co-workers (1996) were able to achieve the same result via the introduction of a bacterial gene encoding the cadaverine-producing enzyme lysine decarboxylase (LDC; EC 4.1.1.18) into *N. tabacum* cultured roots. Nicotine and anatabine biosynthesis reactions also compete or NA, and it is thus possible these alkaloids also share some enzymatic steps. This has been demonstrated recently via antisense suppression of the *N*-methylpyrrolinium-pathway enzyme putrescine *N*-methyltransferase (PMT; EC 2.1.1.53) in *N. tabacum* roots, which lead to reduced nicotine but elevated anatabine levels (Chintapakorn *et al.*, 1997; Chintapakorn, 2002). In addition, the feeding of NA and nicotinamide to cultured *N. rustica* roots, results in increased nicotine and anatabine levels. The proportionately higher stimulation in anatabine levels in these experiments is consistent with its synthesis from two NA molecules, without the need for other non-pyridine precursors (Robins *et al.*, 1987).

The upstream metabolic pathways which produce the intermediates *N*-methylpyrrolinium salt and Δ^1piperideinium are, however, much better characterised than the final stages of alkaloid synthesis.

1.3.2 The production of *N*-methylpyrrolinium salt for nicotine production

In plants, the primary metabolite putrescine is used for the manufacture of polyamines, and can also be used to produce pyridine or tropane alkaloids in some species (Hashimoto and Yamada, 1994) (Figure 1.3). Putrescine can be formed from the amino acids ornithine or arginine, via the actions of ornithine decarboxylase (ODC) (EC 4.1.1.17) or arginine decarboxylase (ADC) (EC 4.1.1.19) respectively

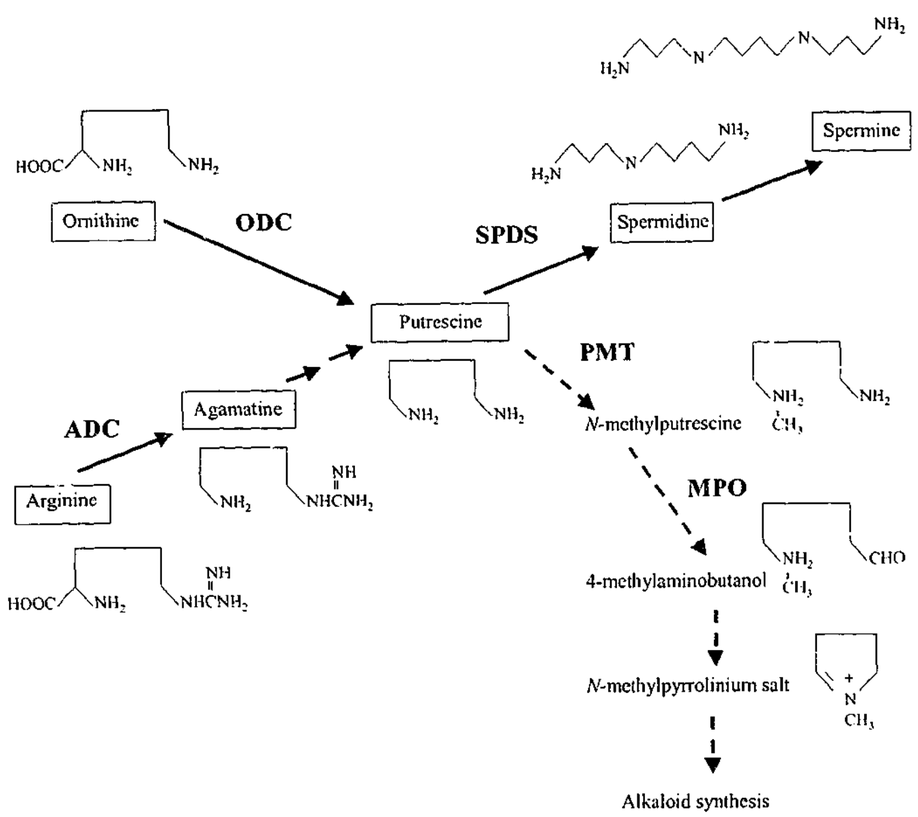


Figure 1.3 The production of *N*-methylpyrrolinium salt.

As in Figure 1.2, primary metabolic reactions which occur in all/most organisms are shown with full arrows, whereas secondary metabolic reactions committed to alkaloid production are represented by dotted arrows. Primary metabolites are boxed, secondary metabolites are not boxed. The full names, synonyms and EC catalogue numbers of all enzymes are given in the list of abbreviations. Based on schemes presented previously by Mizusaki and co-workers (1972), Hashimoto and Yamada (1994), and Imanishi and co-workers (1998). A detailed explanation, including the names of all relevant enzymes, is provided in the text (Introduction 1.3.2 and 1.3.3).

(Yoshida and Mitake, 1966; Hashimoto and Yamada, 1994; Michael *et al.*, 1996; Imanishi *et al.*, 1998).

The relative contribution of each of these routes to putrescine and nicotine production has long been debated, and remains unclear (Mizusaki *et al.*, 1971a; 1973; Leete, 1979, 1983; Tiburcio and Galston, 1986; Rastogi *et al.*, 1993; Garrido *et al.*, 1995). In one study, *ODC* but not *ADC* transcripts have been shown to increase under nicotine-producing conditions in *N. tabacum* (Imanishi *et al.*, 1998), however, both *ODC* and *ADC* cDNAs were recovered using a subtractive hybridisation screen seeking root transcripts induced by foliage damage in *N. tabacum* (Wang *et al.*, 2000). Despite the fact that questions remain, the majority of recent studies have focussed on ODC, and more molecular information on its transcriptional control is available (Michael *et al.*, 1996; Imanishi *et al.*, 1998; Shoji *et al.*, 2000a, 2000b).

ODC transcript levels increase in the roots, and to a lesser extent the leaves, of *N. tabacum* plants following wounding (Riechers and Timko, 1999; K. Cane pers. comm.). Over-expression of a yeast *ODC* gene in cultured *N. rustica* roots facilitated an increase in both putrescine and nicotine content (Hamill *et al.*, 1990). This increase in putrescine content may conceivably contribute not only to the production of alkaloids in the roots, but also to an increase in polyamines required for cellular growth/repair in the damaged leaves (Imanishi *et al.*, 1998). It has been shown, however, that when ODC is induced by methyl jasmonate in tobacco cell suspension cultures, putrescine and nicotine levels increase without an increase in polyamines such as spermidine (Imanishi *et al.*, 1998).

ODC has been cloned from animals, fungi (McConlogue *et al.*, 1984), several prokaryotes (Phillips *et al.*, 1987; Hanson *et al.*, 1992), the tropane alkaloid producing species *Datura stramonium* L. (Solanaceae) (Michael *et al.*, 1996), as well as from *N. tabacum* (Lidgett, 1997; Accession CAA71498; Imanishi *et al.*, 1998). *ADC* has been cloned from a number of organisms including tomato (Rastogi *et al.*, 1993) and *N. tabacum* (Wang *et al.*, 2000). A genomic clone of *ODC* has also been isolated from *N. tabacum* (Accessions: BAA83427; A8031066) and the available 1.4 kb promoter has been shown to direct methyl jasmonate induction in transgenic tobacco plants, when fused to a reporter gene (Imanishi *et al.*, 2000). No detailed molecular dissection of this promoter has been published to date.

1.3.3 Putrescine *N*-methyltransferase (PMT)

Putrescine is demethylated by putrescine *N*-methyltransferase (PMT; EC 2.1.1.53) (Mizusaki *et al.*, 1971b) to form *N*-methylputrescine in species which produce putrescine-derived alkaloids. PMT is probably present only in the roots of such species, and is thus the first enzyme in the pathway acting specifically for the synthesis of pyridine and tropane alkaloids (Mizusaki *et al.*, 1973; Saunders and Bush 1979; Hibi *et al.*, 1994; Riechers and Timko, 1999). *PMT* has been shown to be expressed in the pericycle of *Atropa belladonna* roots (Suzuki *et al.*, 1999), and the cortex, endodermis and xylem in *N. sylvestris* roots (Shoji *et al.*, 2000a).

As the first enzyme committed to alkaloid synthesis, PMT has been studied for its potential role in the regulation of alkaloid production. PMT activity has been shown to increase following foliage damage in *N. tabacum*, followed closely by an increase in alkaloid levels in the leaves (Mizusaki *et al.*, 1973; Saunders and Bush, 1979). PMT activity has also been shown to increase when nicotine accumulation is stimulated in *N. tabacum* callus following transfer to induction medium (Feth *et al.*, 1986). It has also been shown that *PMT* transcript levels in *N. sylvestris* plant roots increase following jasmonate treatment (Shoji *et al.*, 2000a, 2000b). Recent work shows that when *PMT* is down-regulated using antisense technology in *N. sylvestris*, nicotine levels are also lowered (Voelckel *et al.*, 2001; Chintapakorn and Hamill, in preparation).

Five *PMT* genes have been identified in *N. tabacum*, three of which are derived from *N. sylvestris*, one from *N. tomentosiformis* (Hashimoto *et al.*, 1998a), and the fifth is probably derived from *N. otophora* (Riechers and Timko, 1999). *PMT* genes have also been isolated from the tropane alkaloid producing species *A. belladonna* and *H. niger* (Suzuki *et al.*, 1999). In *N. tabacum*, the transcript levels of all 5 *PMT* genes increase following foliage damage (Riechers and Timko, 1999). The promoters of the 3 *N. sylvestris*-derived *PMT* genes in *N. tabacum* have been characterised (Shoji *et al.*, 2000a). They are all extremely similar for ~180bp upstream from the likely transcription start site. Further upstream, two of the promoter regions are conserved, while the other is widely divergent. All 3 are capable of up-regulating a reporter gene in *N. sylvestris* root cultures 2-4 fold following treatment with methyl jasmonate. As such, they probably contain one or

more unidentified jasmonate response elements in the ~180bp immediately upstream from the transcribed region (Shoji *et al.*, 2000a).

Close scrutiny of these genes has provided interesting insights into the evolution of *PMT*, and more broadly, into the evolution of alkaloid metabolism in plants. The deduced PMT protein is similar to the primary metabolic enzyme spermidine synthase (SPDS EC 2.5.1.16) (Hibi *et al.*, 1994). *PMT* is thought to have evolved from plant *SPDS* via gene duplication and subsequent modification, in the process opening a new route for putrescine metabolism, and the opportunity for pyridine and tropane alkaloid biosynthesis (Hashimoto *et al.*, 1998a; 1998b). Analogous examples have since been uncovered in other systems. For example in *Senecio vernalis* Waldst. & Kit (Asteraceae), homospermidine synthase, the first enzyme committed to pyrrolizidine alkaloid synthesis, shares significant amino acid homology to the primary metabolic enzyme deoxypusine synthase (Ober and Hartmann, 1999), from which it has apparently evolved via duplication and subsequent change of function (Ober and Hartmann 2000). It has been suggested that the diversification of duplicated genes, freed from the selective constraints of primary metabolism, is a common factor leading to secondary metabolic pathway evolution (Fabbri *et al.*, 2000; Ober and Hartmann, 2000; Pichersky and Gang, 2000; De Luca and Laflamme, 2001).

N-methylputrescine is oxidatively deaminated to form *N*-methylpyrrolinium salt. In tobacco, this reaction is catalysed by *N*-methylputrescine oxidase (MPO; a diamine oxidase (DAO; EC 1.4.3.6)) (Mizusaki *et al.*, 1972). This enzyme has been purified (McLauchlan *et al.*, 1993), but no cDNA has yet been isolated. The activity of MPO, like PMT, is correlated with the capacity for increased nicotine production in decapitated plants (Mizusaki *et al.*, 1973; Saunders and Bush; 1979) and in callus cultures (Feth *et al.*, 1986), and may be under common regulation with PMT. PMT is, however, considered to be the critical regulatory enzyme, being under more stringent control, and being the first enzyme committed to secondary metabolism, located at the branching point from the polyamine biosynthesis pathway (Feth *et al.*, 1986).

1.3.4 The production of Δ^1-piperideinium for anabasine synthesis

Anabasine is formed from NA and Δ^1-piperideinium, which is derived from the amino acid lysine. The *in planta* intermediates have long been a matter of debate (Leete, 1956; 1979; 1983; Leete and Chedekel, 1972; Leistner and Spenser, 1973; Watson *et al.*, 1990), however feeding and labelling experiments have shown that the diamine cadaverine is a precursor to anabasine (Walton *et al.*, 1988; Walton and Belshaw, 1988).

Lysine is first decarboxylated to form cadaverine by the action of lysine decarboxylase (LDC; EC 4.1.1.18) (Leete, 1979; Walton and Belshaw, 1988). This enzyme is thought to be the major control point in regulating Δ^1-piperideinium production for anabasine synthesis, however no *LDC* gene has thus far been isolated from any eukaryote. Transformed *N. glauca* and *N. tabacum* root cultures that over-expressed a lysine decarboxylase gene from the bacterium *Hafnia alvei* showed enhanced cadaverine and anabasine levels (Fecker *et al.*, 1992; 1993). When *N. tabacum* plants have been transformed to over-express *LDC*, substantially increased activity and cadaverine accumulation in the leaves has been observed only if the foreign gene is translated with a chloroplast transit peptide at its N-terminus. Thus, in leaves, the first stages of anabasine synthesis may take place inside this organelle (Herminghaus *et al.*, 1991). Similarly, if the same bacterial LDC is targeted to the root leucoplast, the LDC activity, cadaverine and anabasine accumulation are substantially elevated (Herminghaus *et al.*, 1996). These observations corroborate the work of Bagni and co-workers (1986), who demonstrated that LDC activity was present in the chloroplast.

Cadaverine is oxidised to form Δ^1-piperideinium by a diamine oxidase similar or identical to MPO (Walton and McLauchlan, 1990). This product competes with *N*-methylpyrrolinium salt for NA (Leete, 1983; Waller and Nowacki, 1978; Watson *et al.*, 1990). Thus, both anabasine and nicotine synthesis are analogous, in the flow from decarboxylated amino acid, requiring the action of a diamine oxidase, and the final condensation of the product with NA.

1.3.5 The role of quinolinate phosphoribosyltransferase (QPRTase) in producing nicotinic acid

Although it used to produce alkaloids in a small number of species, nicotinic acid (NA) is an important part of the pyridine nucleotide cycle (PN-cycle) in all organisms. This cycle consists of a series of primary metabolic reactions which produce and recycle the essential co-factor nicotinamide adenine dinucleotide (NAD) (Henderson, 1949; Preiss and Handler 1958a, 1958b; Gholson, 1966; Hillyard *et al.*, 1981; Wagner *et al.*, 1986a, 1986b) (see Figure 1.4). NAD plays an important role in accepting electrons in many oxidation reactions (*ie*; NAD^+ (oxidised form) is reduced to NADH), and is particularly prominent in reactions of energy metabolism, such as those which generate NADH during respiration in the mitochondria. Likewise, the NAD-derivative nicotinamide adenine dinucleotide phosphate (NADP) is particularly prominent as a co-factor in photosynthesis reactions (Wink, 1997).

In all organisms analysed to date, the entry point to the PN-cycle is catalysed by quinolinate phosphoribosyltransferase (QPRTase) (EC 2.4.2.19) (Figure 1.4). This enzyme catalyses the formation of nicotinic acid mononucleotide (NAMN) (along with CO_2 and pyrophosphate), from quinolinate and 5-phosphoribosyl 1-pyrophosphate (Henderson, 1949; Nishizuka and Hayaishi, 1963; Nakamura *et al.*, 1963; Iwai and Taguchi, 1973; Eads *et al.*, 1997; Cao *et al.*, 2002). NAMN is the first metabolite which is truly recycled in the operation of the PN-cycle, and is thus produced both *de novo* by QPRTase, and via the cycle itself.

The substrate of QPRTase, quinolinate, is derived from different sources in different organisms. In animals and most micro-organisms it is derived from tryptophan (Leete, 1983; Wink, 1997). In the plants *Nicotiana*, *Ricinus communis* and in some bacteria such as *Mycobacterium tuberculosis*, aspartic acid is the amino acid precursor (Mann and Byerrum, 1974a, 1974b; Leete, 1983; Luckner, 1990). Despite their different origins, both of these pathways proceed via quinolinate, and the PN-cycles in most organisms are largely equivalent beyond that point (Henderson, 1949; Chaykin, 1967; Hillyard *et al.*, 1981).

In the PN-cycle, NAMN is converted into nicotinic acid adenine dinucleotide (NAAD) by NAMN-adenylyltransferase (EC 2.7.7.18), and NAAD is subsequently converted into NAD by NAD-synthase (EC 6.3.1.5) (Wagner *et al.*, 1986a, 1986b).

NAD may then be recycled via nicotinamide mononucleotide (NMN), nicotinamide (N), and NA back into NAMN (Wagner *et al.*, 1986a, 1986b). These four recycling steps are catalysed by the enzymes NAD-pyrophosphatase (EC 3.6.1.22), NMN-glycohydrolase (EC 3.2.2.14), nicotinamidase (EC 3.5.1.19) and NA-phosphoribosyltransferase (EC 2.4.2.11), respectively.

Under nicotine-producing conditions in *N. tabacum* cell cultures, the PN-cycle is regulated to facilitate an accumulation of NA. This occurs in two ways. Firstly, the enzymes NAMN-adenyltransferase, NAD-pyrophosphatase and NMN-glycohydrolase are upregulated without the concomitant up-regulation of NA-phosphoribosyltransferase. Secondly, NAMN may be metabolised directly into NA, in a step that runs against the normal flow of the cycle, and is the reverse of the reaction catalysed by NA-phosphoribosyltransferase. The enzyme responsible for this, NAMN-glycohydrolase (related to EC 2.4.2.11 or 3.2.2.14), is upregulated under nicotine-inducing conditions in tobacco callus cultures. It has a relatively high K_m, such that it only functions effectively where its substrate NAMN is at relatively high concentrations (Wagner *et al.*, 1986a).

QPRTase, however, is thought to be the major limiting step in the production of both NAD, and NA for alkaloid synthesis (Wagner *et al.*, 1986a). QPRTase was first credited with a role in pyridine alkaloid synthesis in plants by Mann and Byerrum (1974a) in *R. communis*, which produces ricinine. In this species, QPRTase activity was shown to increase as etiolated seedlings developed, shortly before an increase in ricinine levels was observed (Mann and Byerrum, 1974a). These authors also predicted that QPRTase would be regulated to allow nicotine synthesis in *N. rustica* roots, as opposed to leaves, and showed that the enzyme activity was indeed higher in the roots than the foliage of this species. Saunders and Bush (1979) demonstrated that QPRTase, PMT and MPO enzyme activities all increased markedly in *N. tabacum* roots 24-48 hrs after plant decapitation, and before a marked increase in nicotine levels in the foliage. Interestingly, both the basal level and the degree of induction of these enzymes is less in low alkaloid mutants than in wild type *Nicotiana tabacum* (Saunders and Bush, 1979; Wagner *et al.*, 1986c). It has been speculated that these enzymes are under common molecular regulation (Hibi *et al.*, 1994), which may involve proteins encoded by the two nicotine-controlling loci long known to differ between high and low alkaloid strains of *N. tabacum*, designated *nic1* and *nic2* (Legg and Collins, 1971).

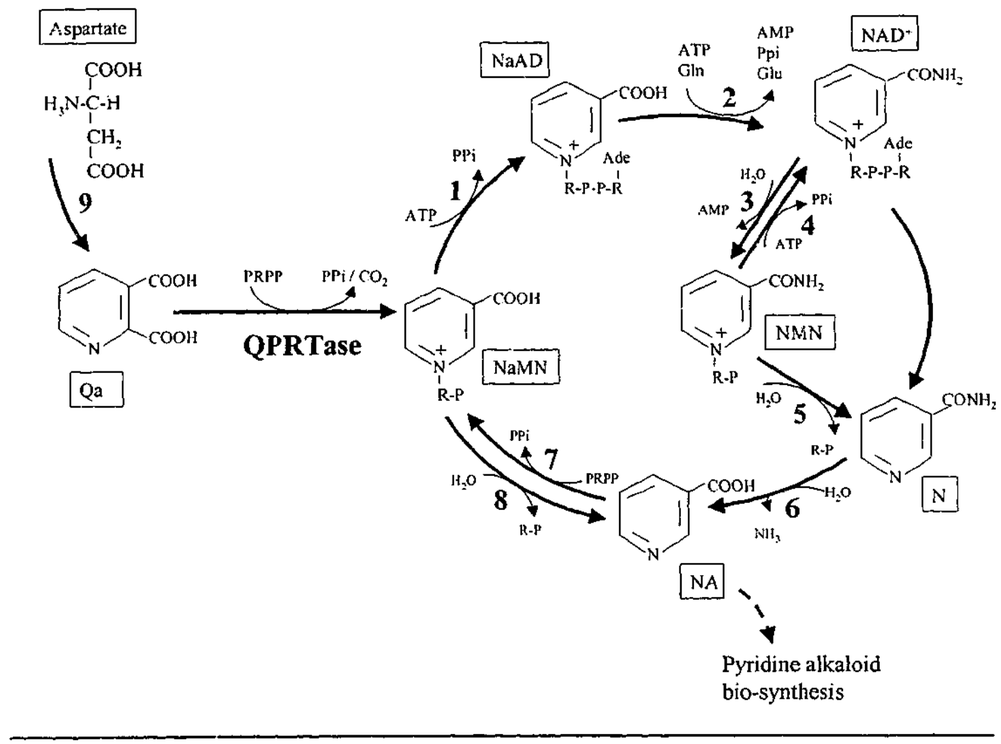


Figure 1.4 The pyridine nucleotide (PN-) cycle in *Nicotiana*.

As in Figures 1.2 and 1.3, primary metabolic reactions which occur in all/most organisms are shown with full arrows (with the exception of the path from aspartate to quinolinic acid, which originates from tryptophan in animals), whereas secondary metabolic reactions committed to alkaloid production are shown with dotted arrows. Similarly, the names of all primary metabolites are boxed. The reaction catalysed by the anaplerotic enzyme QPRTase is shown on the left of the cycle, while the other enzymes are numbered as follows:

1. NaMN-ATase
2. NADs
3. NAD-ppase
4. NMN-ATase
5. NMN-Ghase
6. N-ase
7. NaPRTase
8. NaMN-Ghase
9. L-aspartate oxidase

The full names, synonyms, and EC catalogue numbers of all enzymes are given in the list of abbreviations. The reaction linking NAD^+ directly to N has apparently not been confirmed in plants. The abbreviated names of the metabolites are given in both the text and the list of abbreviations. The scheme presented here is adapted from the published studies of Mann and Byerrum (1974a), Wagner and Wagner (1985) and Wagner and co-workers (1986b).

Unlike PMT, QPRTase activity must also be present in the leaves and stem of tobacco plants, albeit at low levels, to fulfil its primary metabolic role in NAD biosynthesis (Mann and Byerrum, 1974a). Consistent with this requirement, Wagner and Wagner (1984) showed that QPRTase activity was present in *N. tabacum* leaves, at a level ~20 fold less than that in roots. Thus, despite their co-regulation in nicotine-producing roots, these enzymes differ in the way they link secondary to primary metabolism in *Nicotiana*, with PMT catalysing a branch point committed to secondary metabolism, whilst QPRTase plays a dual role. In their early study of QPRTase, Wagner and Wagner (1984) acknowledged that it would be a "great challenge to explore how this enzyme is regulated, which is connected to both primary and secondary metabolism".

1.3.6 Molecular studies on QPRTase

QPRTase was initially described from rat liver (Nakamura *et al.*, 1963), and soon after from bovine liver (Gholson *et al.*, 1964). It was shown to have a molecular weight of 178 kDa (Packman and Jacoby, 1965a, 1965b, 1967), and has been shown to act as a homo-dimer in *Salmonella typhimurium* (Eads *et al.*, 1997).

Packman and Jacoby (1967) noted that its reaction kinetics suggested QPRTase reacted in a different manner to other phosphoribosyltransferases. Recent structural analysis in *S. typhimurium* and *Mycobacterium tuberculosis* confirmed that QPRTase possesses an unusual seven-stranded α/ß barrel structure containing its active site, resulting in a binding conformation unlike that of other phosphoribosyltransferases (Eads *et al.*, 1997; Sharma *et al.* 1998, Cao *et al.*, 2002). QPRTase binds first to PRPP, in the presence of magnesium, before it binds to QA (Bhatia and Calvo, 1995).

It is in its primary metabolic role that QPRTase has received the most attention in recent years. This interest has converged on QPRTase from a number of different quarters. Elevated levels of QA in the brain have been linked to neurodegenerative disorders such as epilepsy, Alzheimer's disease and Huntington's disease. The cDNA encoding human *QPRTase* has thus been isolated, and shown to have 30-40% amino acid identity to bacterial enzymes, along with the ability to

encode functional QPRTase in QPRTase deficient *E. coli* cells (Fukuoka *et al.*, 1998). The human *QPRTase* cDNA seems to possess a hydrophobic N-terminal signal sequence, and may be translocated into, and retained within, some vesicular compartment (Fukuoka *et al.*, 1998). In rat brains, QPRTase activity is concentrated in a fraction containing myelin, mitochondria, and synaptosomes, with the greatest activity in the latter component (Foster *et al.*, 1985). Antibodies have been used to detect the enzyme in the cytoplasm, and in small unidentified bodies possessing a single membrane (Koehler *et al.*, 1987). QPRTase has also been of interest as a target point in controlling the tuberculosis pathogen, *M. tuberculosis*. This organism is unusual in that it does not recycle NAD, but rather relies on *de novo* NAD synthesis via QPRTase (Sharma *et al.*, 1998). More recently, QPRTase has been studied in the bacterium *Burkholderia cepacia*, for its role in the degradation of phthalate, an environmental pollutant and toxin (Chang and Zylstra, 1999).

Molecular studies on plant *QPRTase* have until recently been limited by the lack of *QPRTase* sequence data. No plant sequences with homology to *QPRTase* existed on the database in 1998, with the exception of an EST fragment (Accession: AB005879) isolated among other jasmonate-inducible messages including *ODC* (Imanishi *et al.*, 1998; 2000) from *N. tabacum* var. Bright Yellow 2. This fragment was used to recover putative *QPRTase* cDNAs from *N. tabacum* and *N. rustica* in preliminary work preceding and facilitating the current study (Sinclair, 1998).

Since the commencement of this project, and the publication of *N. tabacum* and *N. rustica QPRTase* cDNAs during its early stages, new additions to the databases have included numerous plant sequences with similarity to *QPRTase:*

- Tobacco: After the submission of the putative *Nicotiana QPRTase* cDNAs during the course of this project, a full-length putative *QPRTase* cDNA sequence from suspended cells of *N. tabacum* var. Bright Yellow 2 (Accession: AB038494) was released by the same researchers who had submitted the partial cDNA noted above (Imanishi *et al.*, 1998).

- Arabidopsis: The *Arabidopsis* sequencing project has yielded a single putative *QPRTase* gene on chromosome 2. (Lin *et al.*, 1999; Accession AC006200; Ecotype Colombia). *Arabidopsis* (Columbia) EST fragments with homology to

QPRTase have since been released (Accessions: AY057685, AI998967, AV567706 and F20096). These sequences were similar (>90%) but not identical to the mRNA predicted from the locus on chromosome 2. It is unknown whether these represent gene copies which have evaded the sequencing project, or whether they are the result of polymorphisms or sequencing errors.

- Tomato: Five EST fragments with homology to *QPRTase* have recently been reported from *L. esculentum* (Accessions: AW934602, AW443537, AW735750 and AW934597), three derived from flower buds, and one after treating the plants with mixed elicitor. Three putative *QPRTase* ESTs have also been reported from the trichomes of *L. pennellii* (Accessions: AW160286; AW160287; AW398489).

- Rice: The rice (*Oryza sativa* L.(Poaceae)) genome database (http://www.tigr.org/-tdb/e2k1/osa1/) does not yet appear to contain putative *QPRTase* genes or ESTs (as of 8.3.2003). Only an un-annotated genomic fragment (Accession: AQ292009) shows any convincing homology to *QPRTase* (~80% identity over a region of ~95bp), and seems to include the rice equivalent of *Arabidopsis* putative exon 7.

- Other plants: EST fragments with homology to *QPRTase* have been reported from a number of other plants. Sequences with large segments of homology (>70% identity over >~75bp) include sequences from soybean (*Glycine max* (L.) Merr. (Fabaceae)) (Accessions: BE020114, AI938542, AI938548, AW568708, AW234858 and AI965771), sorghum (*Sorghum bicolor* (L.) Moench (Poaceae)) (Accessions: AW287300 and AW287359), cotton (*Gossypium arboreum* L. (Malvaceae)) (Accession: BE053586), and lotus (*Lotus japonicus* (Regel) Larsen (Fabaceae)) (Accession: AI967414).

This recent *QPRTase* sequence data heightens the amenability of studying pyridine alkaloid biosynthesis in plants. Together with the biochemical and ecological data reviewed above, the biosynthesis of pyridine alkaloids in *Nicotiana* provides a useful system for studying the molecular basis for these plants' ability to respond to their environments. The specific aims of the present study are presented below.

1.3.7 The AIMS and the scope of the present study

The aims of this project are broadly concerned with elucidating the molecular basis for the ways in which plants alter their resource allocation to defence chemicals, both as a short term response to their environment, and also over evolutionary time.

1 Characterisation of two QPRTase *cDNAs*

- To determine whether putative *QPRTase* cDNAs isolated previously from *N. tabacum* and *N. rustica* encode functional QPRTase, using the complementation of a bacterial mutant in conjunction with computational analysis.

2 QPRTase in the Nicotiana genome

- To isolate *QPRTase* gene copies, including their 5' regulatory regions, from *N. tabacum*, by screening a genomic library, in order to facilitate computational promoter sequence analysis and future experimental promoter delineation studies.
- To assess the origin of these genes in *N. tabacum*'s likely diploid progenitors, using Southern analysis.
- To assess the specific expression profiles of recognisable *QPRTase* genes, in order to identify gene copies which are likely to be regulated to facilitate alkaloid synthesis.
- To isolate *QPRTase* 5' regulatory DNA from the alkaloid-poor species *N. alata* for comparison with similar regions to those in *N. tabacum*.

3 *Differential patterns of gene expression in* Nicotiana *species with contrasting alkaloid profiles*

- To assess whether the alkaloid profiles of different *Nicotiana* species are correlated with the differential expression of the alkaloid biosynthetic genes *QPRTase*, *PMT*, *ODC* and the likely alkaloid biosynthesis gene'*A622*'.
- To determine whether the expression of these genes correlate with changes in pyridine alkaloid levels following foliage damage in the nicotine-rich species *N. sylvestris*, the anabasine-rich species *N. glauca*, and the alkaloid-poor species *N. alata*.
- To assess whether there is an alkaloidal or transcriptional wound-response in the lower, unwounded leaves on plants where a leaf response is recorded in the upper, wounded leaves.

2 RESULTS & DISCUSSION

2.1 Characterisation of two *QPRTase* cDNAs

2.1.1 Background

Two potential *QPRTase* cDNAs were isolated and sequenced prior to the commencement of the current project, one fro[illegible]*cotiana tabacum* and one from *N. rustica* (clones *TQPT1* and *RQPT1*) (Sinclair, 1998). Sequence analysis suggested that they represent QPRTase on the basis of deduced amino acid conservation with human, yeast and numerous prokaryotic QPRTase enzymes (Sinclair, 1998).

As full-length *QPRTase* cDNAs have not been previously isolated from any plant, it was deemed important to test whether these cDNAs did indeed encode functional QPRTase. To address this question, functional experiments involving the complementation of a bacterial mutant have been carried out, along with further sequence analysis using sequence data newly available on the database since 1998 (reviewed Introduction 1.3.6).

2.1.2 Complementation of a bacterial mutant to demonstrate that both cDNAs encode functional QPRTase

When expressed in mutant cells lacking QPRTase, cDNAs encoding functional QPRTase would be expected to complement the missing activity, restoring a normal phenotype. The mutant *Escherichia coli* strain TH265 was used here, since it lacks the *QPRTase* gene along with several other genes not essential for growth on minimal media (∇ *nad*C-*ace*F region; *E. coli* database: http://susi.bio.uni-giessen.de/ecdc). The same strain was previously used in the functional identification of a human brain *QPRTase* cDNA (Fukuoka *et al.*, 1998), and was kindly provided by Prof. Kelly T. Hughes, University of Washington, Seattle.

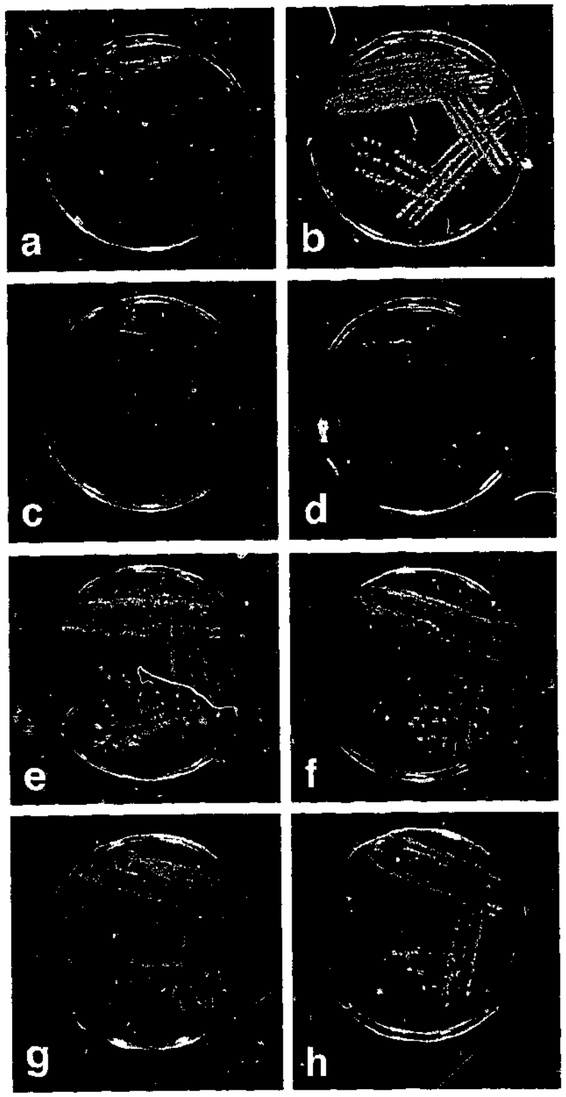


Figure 2.1.1 Complementation of QPRTase deficient (*nad C*$^-$) *E. coli* strain TH265 with *Nicotiana* cDNAs encoding QPRTase.

a TH265 cells cultured on minimal medium.
b TH265 cells cultured on minimal medium containing 0.2 µg/ml nicotinic acid.
c TH265 cells containing *pBluescript* cultured on minimal medium.
d TH265 cells containing *pBluescript* cultured on minimal medium containing 0.5mM 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 *pRQPT1* cultured on minimal medium containing IPTG.

Escherichia coli TH265 cells did not grow when streaked on minimal medium, but grew relatively slowly when cultured on either LB medium or minimal medium supplemented with nicotinic acid, thereby bypassing the need for QPRTase activity (Figure 2.1.1a, b).

These cells were transformed with the plasmid *pBluescript* (Stratagene), as a control, and also with *TQPT1* and *RQPT1* cloned into this vector. Transformation with *pBluescript* alone did not allow cells to grow on un-supplemented minimal medium (Figure 2.1.1c, d). When, however, cells were transformed with *pBluescript* containing *TQPT1* or *RQPT1* they were able to form colonies on this medium (Figure 2.1.1e, g). As in the study describing the isolation of human *QPRTase* (Fukuoka *et al.*, 1998), this result, in conjunction with sequence analysis, was accepted as providing firm evidence that both *TQPT1* and *RQPT1* encode QPRTase enzymes that are functional, even across wide phylogenetic boundaries (Sinclair *et al.*, 2000).

It was originally intended to use IPTG to induce the resident *lacZ* promoter in *pBluescript*, to express a fusion protein of lacZ-QPRTase. No IPTG was necessary, however, to induce colony growth, with both *pTQPT1*- and *pRQPT1*-transformed cells growing equally well with and without IPTG. Furthermore, *pRQPT1* was able to complement the *E. coli* mutant despite being out of frame, demonstrating that the anticipated re-cloning was not necessary in order to demonstrate its function. These observations suggest that 'leaky' transcription (and/or possibly translation) was able to produce a sufficient level of QPRTase to facilitate complementation (Sinclair *et al.*, 2000). This is consistent with the published results of Fukuoka and co-workers (1998) who also used *pBluescript* to express an out of frame human *QPRTase* in *E. coli* strain TH265, and noted that their system was 'leaky' with respect to IPTG induction.

2.1.3 Sequence analysis of the *QPRTase* cDNAs

The preliminary *TQPT1* sequence data presented previously contained some ambiguities (Sinclair, 1998), so the cDNAs were partially re-sequenced in the present study. The confirmed sequence data was submitted to the EMBL database (Accession numbers: AJ243347 (*N. tabacum* cDNA, *TQPT1*) and AJ243436 (*N.*

rustica cDNA, *RQPT1*)). These sequences are nearly identical (98%) (Sinclair *et al.*, 2000), and *TQPT* will be used in most sequence analysis presented here.

Comparison of Nicotiana tabacum QPRTase with QPRTases from other organisms:

A line-up of several QPRTase deduced amino acid sequences is presented in Figure 2.1.2. It displays the sequences of *TQPT1*, lined up alongside the *N. tabacum* 'Bright Yellow' putative QPRTase, a segment deduced from the 5' end of the soybean EST likely to represent QPRTase, and the *Arabidopsis* putative *QPRTase* (all described in Introduction 1.3.6). During analysis, the annotated database sequence from the *Arabidopsis* genome was found to contain a substantial error. The EST AY057685 revealed the presence of an un-annotated exon at the 5' end of the gene, beginning from an additional upstream ATG (*ie*; AUG) start codon. The *Arabidopsis* sequence included here has been amended such that it is different to that listed on the database, and from that referred to previously (in Sinclair *et al.*, 2000). Some non-plant sequences have also been included to provide a selection of QPRTases from other eukaryotic and a wide phylogenetic range of prokaryotic organisms (See Figure 2.1.2 legend for details including accession numbers). Figure 2.1.2b displays an alignment which includes the smaller sequence fragments deduced from the available 5' ends of putative *QPRTase* genes from *L. esculentum*, *N. alata* (Johnson, 2000) and *N. glauca* (DeBoer, 2001).

Plant QPRTases probably carry an N-terminal extension:

The data presented in Figure 2.1.2 show that deduced QPRTase proteins from a range of plants are apparently longer at their N-terminus than QPRTases from the prokaryotes, and other eukaryotes. Every plant QPRTase sequence characterised to date also contains a second in-frame ATG start codon downstream, as noted above for *Arabidopsis*. Thus the plant sequences could conceivably encode one or both of two proteins, with or without an N-terminal extension.

Generally, an mRNA is translated from only one AUG codon (Kozac, 1995). The currently accepted 'scanning' model of translation asserts that initiation occurs on the first AUG start codon present in the mRNA, unless other circumstances prevent this from occurring (Kozac, 1995; Joshi *et al.*, 1997). Analysis of the

available sequence data suggests that the first initiation codon in the *N. tabacum QPRTase* mRNA, as well as in the other plants, is indeed utilised, allowing a protein to be produced that carries an N-terminal extension:

- It is not closely followed by a 'stop' codon in any frame (Kozac, 1995).
- The 5' leaders to the upstream ATG are not exceptionally short (Kozac, 1995).
- The leader sequences are rather A-T rich (*eg*; 69% A-T in *N. tabacum*), and the formation of stable secondary structure does not seem likely (Kozac, 1995; Gallie, 1996). This is in contrast, for example, to mammalian *ODC*s whose 5'UTRs are G-C rich (*eg*; 34.5% A-T in the hamster) and are known to form secondary structures which inhibit/regulate translation (Grens and Scheffler, 1990; van Steeg *et al.*, 1991)
- The first initiation codon is apparently in a suitable sequence context in each case (Joshi *et al.*, 1997). In *N. tabacum* it scores a relatively high index of 0.7 when a translation initiation prediction program is used (Netgen2; http://www.cbs.dtu.dk) (Pedersen and Nielsen, 1997).
- There is no evidence to suggest that the cDNAs sequenced originated from 'abnormally' or 'alternatively' spliced mRNA. There are no 'donor sites' or 'branch sites' in the appropriate region that would flag an un-spliced intron (Simpson and Filipowicz, 1996).

It is not, however, possible to completely exclude the possibility of a shorter protein being produced, if for example, transcription produced an mRNA lacking the first 'AUG' codon. The sequence context of the second AUG is in fact similar to that of the first AUG (0.7; Netgen2, Pedersen and Nielsen, 1997).

It is also possible that both start codons are utilised *in vivo*. This has been demonstrated in a few plant genes (*eg*; Mireau *et al.*, 1996; Frappier *et al.*, 1998) and in other organisms (Muralidhar *et al.*, 1994; Kozac, 1995), and the isoforms produced may have distinct functions. For example, the *Arabidopsis* Alanyl-tRNA synthetase (*AlaRS*) mRNA encodes both a cytosolic enzyme and another enzyme with an N-terminal target directing it to the mitochondrion (Mireau *et al.*, 1996).

Figure 2.1.2 Alignment of the deduced *N. tabacum* QPRTase (TQPT1) sequence with other QPRTase sequences.

Constructed using PILEUP (via ANGIS; www.angis.org.au) (*NB*: the lineup in the N-terminal region has been altered "by hand"). Panel (a) includes full length QPRTase sequences from a range of species. Dark shading shows completely conserved residues, while lighter shading shows residues conserved in the majority of plant sequences. # denotes the active site, as determined by Eads *et al.*, 1997. Panel (b) includes additional partial QPRTase sequence from Solanaceous species, a number of which have been compiled from multiple EST entries, and may thus represent artificially composite proteins. The light shading in this panel represents residues which depart from the consensus sequence. The lower case characters at the N-terminal end of the *L. pennellii* sequence represent residues whose existence cannot be confirmed without further 5' DNA sequence including the upstream ATG codon. The sequences were deduced/sourced as follows:

N. tab (TQPT)	(*Nicotiana tabacum* cv. SC58 (*TQPT1*), Accession AJ243437, this study, Sinclair *et al.*, 2000)
N. tab (BY)	(*N. tabacum* cv. Bright Yellow 2 cDNA, Accession AB038494)
A. thaliana	(*Arabidopsis thaliana*, genomic & EST sequence, Accessions AC006200; AY057685)
G. max	(*Glycine max* (soybean), compiled from multiple ESTs, Accessions BE020114; AI938542; AI938548; AW568708; AW234858 and AW287359)
H. sapiens	(*Homo sapiens* (human), cDNA; Accession D78177, Fukuoka *et al.*, 1998)
S. cerevisae	(*Saccharomyces cerevisae* strain S288C/AB972 (yeast) genomic; Accession P43619)
E. coli	(*Escherichia coli* strain K12, genomic, Accession AAC73220)
R. rubrum	(*Rhodospirillum rubrum* strain UR1, genomic, Accession P77938)
M. leprae	(*Mycobacterium leprae* strain TN (leprosy), genomic, Accession P46714)
B. subtilis	(*Bacillus subtilis*, genomic, Accession CAB14746)
N.alata	(*N. alata*, genomic, Johnson, 2000)
N.glauca	(*N. glauca*, genomic, DeBoer, 2001)
L.esculentum	(*Lycopersicon esculentum* (tomato), compiled from 2 ESTs, Accessions 308467 and 353494)
L.pennellii	(*Lycopersicon pennellii*, compiled from 2 ESTs, Accessions 290144 and 290145)

Figure 2.1.2 Panel (a)

```
N. tab. (TQPT)  ------------------------MFRALPFTATVHPYAITAPRLVVKMSAIATKN--TR
N. tab. (BY)    ------------------------MFRAIPFTATVHPYAITAPRLVVKMSAIATKN--TR
A. thaliana     ------------------------MIGVSRFL-SPQFYAI--PRSFVKMSASATQT--AG
G. max          MAISCNKQEFLLRPVFHARESTTAFLPPLSLSLKLPPQSHSKVTRVVKMSATEVTSSTIS
H. sapiens      ----------------------------------------------------MDA---------
S. cerevisae    ------------------------------------------------------------
E. coli         ----------------------------------------------------MPPRRYN
R. rubrum       ------------------------------------------------------------
M. leprae       ------------------------------------------------------------
B. subtilis     ------------------------------------------------------------

N. tab. (TQPT)  VESLEVKPPAHPTYDLKGVMQLALSEDAGN---LGDVTCKATIPVDMESDAHFLAKEDGI
N. tab. (BY)    VESLEVKPPAHPTYDLKEVMKLALSEDAGN---LGDVTCKATIPLDMESDAHFLAKEDGI
A. thaliana     EVSMGIKPPSHPTYDLKAVIKLALAEDAGH---TGDVTCMATIPFDMEVEAYFLAKEDGI
G. max          YESFAIKPAEHPTYDLKGIIKLALEEDAGD---RGDVTCLATIPFDMEVEAYXLAKEDGI
H. sapiens      EGLALLLPP---VTLAA---LVDSWLREDCP------GLNYAALVSGAGPSQAALWAKSPGV
S. cerevisae    --MPVYEHLLPVNGAWRQDVTNWLSEDVP------SFDFGGYVVGSDLKEANLYCKQDGM
E. coli         PDTRRDELLERINLDIPGAVAQALREDLGGTVDANNDITAKLLPENSRSHATVITRENGV
R. rubrum       -MRTNHPVAALSPFAIDEAVRRALAEDLGR---AGDITSTATIPAATRAHARFVARQPGI
M. leprae       ------MLSDCEFDAARDTIRRALHEDLRY---GLDITTQATVPAGTVVTGSMVPREPGV
B. subtilis     ----------MNHLQLKKLLNHFFLEDIG----TGDLTSQSIFGEQS-CEAEIVAKSEGI
```



```
N. tab. (TQPT)  IAGIALAEMIFAEVDP--SLKVEWYVNDGDKVHKGLK------FGKVQGNAYNIVIAERV
N. tab. (BY)    IAGIALAEMIFAEVDP--SLKVEWYVNDGDKVHKGLK------FGKVQGNAYNIVIAERV
A. thaliana     VAGVALADMIFEHVDP--SLKVEWMRKDGDYVHKGLK------FGKVSGNAHKIVVAERV
G. max          IAGIALAEMICHEVDP--SLKVEWSKYDGDLVHTWLQ------FGR...→
H. sapiens      LAGQPFFDAIFTQLN----CQVSWFLPEGSKLVPVAR------VAEVRGPAHCLLLGERV
S. cerevisae    LCGVPFAQEVFNQCE----LQVEWLFKEGSFLEPSKNDSGKIVVAKITGPAKNILLAERT
E. coli         FCGKRWVEEVFIQLAG-DDVTIIWHVDDGDVINANQS------LFELEGPSRVLLTGERT
R. rubrum       LAGLGCARSAFALLD--DTVTFTTPLEDGAEIAAGQT------VAEVAGAARTILAAERT
M. leprae       IAGVDVALLVLDEVFGVDGYRVLYRVEDGARLQSGQP------LLTVQAAARGLLTAERT
B. subtilis     FAGAAIIKEGFSLLDEN--VQSILHKKDGDMLHKGEV------IAELHGPAAALLSGERV

                                                ###                  ##
N. tab. (TQPT)  VLNFMQRMSGIATLTKEMADAAHPA----YILETRKTAPGLRLVDKWAVLIGGGKNHRMG
N. tab. (BY)    VLNFMQRMSGIATLTKEMADAAHPA----YILETRKTAPGLRLVDKWAVLIGGGKNHRMG
A. thaliana     LLNFMQRMSGIATLTKLMADAASPA----CILETRKTAPGLRLVDKWAVLIGGGRNHRMG
H. sapiens      ALNTLARCSGIASAAAAAVEAARGAGWTGHVAGTRKTTPGFRLVEKYGLLVGGAASHRYD
S. cerevisae    ALNILSRSSGIATASHKIISLARSTGYKGTIAGTRKTTPGLRRLEKYSMLVGGCDTHRYD
E. coli         ALNFVQTLSGVASKVRHYVELLEGTN--TQLLDTRKTLPGLRSALKYAVLCGGGANHRLG
R. rubrum       ALNFLGHLSGIATRTRRFGDAIAHT--RARLTCTRKTTPGLRGLEKYAVRCGGGSNHRFG
M. leprae       MLNLVCHMSGIATVTVAWVDAVRGT--KAKIRDTRKTLPGLRALQKYAVRVGGGVNHRLG
B. subtilis     VLNLIQRLSGIATMTREAVRCLDDE--QIKICDTRKTTPGLRMLEKYAVRAGGGYNHRFG

                        #                       #
N. tab. (TQPT)  LFDMVMIKDNHISAAGGVGKALKSVDQYLEQNKLQIGVEVETRTIAEVREVLEYASQTKT
N. tab. (BY)    LFDMVMIKDNHISAAGGVGKALKSVDQYLEQNKLQIGVEVETRTIEEVREVLDYASQTKT
A. thaliana     LFDMVMIKDNHISAAGGIVNAVKSVDEYLKQKNLEMDVEVETRTLEEVKEVLEYASGSET
H. sapiens      LGGLVMLKDNHVVPPGGVEKAVRAARQAADFALK---VEVECSSLQEVVQAAEAG-----
S. cerevisae    LSSMVMLKDNHIWATGSITNAVKNARAVCGFAVK---IEVECLSEDEATEAIEAG-----
E. coli         LSDAFLIKENHIIASGSVRQAVEKASWLHPDAP----VEVEVENLEELDEALKAG-----
R. rubrum       LDDAVLIKDNHIAVAGGVSAALSRARAGVGHMVR---IEIEVDTLEQLAEVLAVGG-----
M. leprae       LGDTALIKDNHVAAVGSVVDALRAVRAAAPELP----CEVEVDSLEQLDAMLAEE-----
B. subtilis     LYDGIMIKDNHIAACGSILEACKKARQAAGHMVN---IEVEIETEEQLREAIAAG-----

                        #                       #
N. tab. (TQPT)  SLTRIMLDNMVVPLSNGDIDVSMLKEAVELINGRFDTEASGNVTLETVHKIGQTGVTYIS
N. tab. (BY)    SLTRIMLDNMVVPLSNGDIDVSMLKEAVELINGRFDTEASGNVTLETVHKIGQTGVTYIS
A. thaliana     RLTRIMLDNMVVPLENGDVDVTMLKDAVELINGRFETEASGNVTLETVHKIGQSGVTFIS
H. sapiens      -ADLVLLDNFKPEELHPT--ATALKAQFPS----VAVEASGGITLDNLPQFCGPHIDVIS
S. cerevisae    -ADVIMLDNFKGDGLKMCAQSLKNKWNGKKH---FLLECSGGLNLDNLEEYLCDDIDIYS
E. coli         -ADIIMLDNFETEQMREAVKRTNGKALL---------EVSGNVTDKTLREFAETGVDFIS
R. rubrum       -AEVVLLDNMDAPTLTRAVDMVAGR---------LVTEASGGVSLDTIAALAESGVDYIS
M. leprae       -PELILLDNFPVWQTQVAVQRRDIRAPT------VLLESSGGLSLENAAIYAGTGVDYLA
B. subtilis     -ADVIMFDNCPPDTVRHFAKLTPANIKT---------EASGGITLESLPAFKGTGVNYIS

N. tab. (TQPT)  SGALTQSVKALDISLKIDTELALEVGRRTKQA*
N. tab. (BY)    SGALTHSVKALDISLKIDTELALEDGRRTKRA*
A. thaliana     SGALTHSVKALDISLKIDTELALEVGRRTKRA*
H. sapiens      MGMLTQAVPALDFSLKLFAKEVAPVPKIH*---
S. cerevisae    TSSIHQGTPVIDFSLKLAH*------------
E. coli         VGALTKHVQALDLSMRFR*-------------
R. rubrum       VGALTHSVTTLDIGLDIVVAPPKAERA*-----
M. leprae       VGALTHSVRILDIGLDL*--------------
B. subtilis     LGFLTHSVKSLDI*------------------
```

Figure 2.1.2 (continued) panel (b)

```
N.tab (TQPT)      MFRALPFTATVHPYAITAPRLVVKMSAIATKNT--RVESLEVKPPAHPTYDLKGVMQLAL
N.alata           MFRAIPFTATVHPYAITAPRLVVKMSAIATKNT--RVESLEVKPPAHP - - - - - -
N.glauca          MFRAIPFTATVHPYAITAPRLVVKMSAIATKNT--RVESLEVKPPAHPTYDLKEVMQLAL
L.esculentum      MFRVLPFTTTVHPCAITAPRLVVKMSAMATKNAGRTVESLVVKPPAHPTYDLKGVIQLAL
L.pennelli        - - - - - vhpcaiaaprlvvkMSAMATKNAGRTVESLVVKPPAHPTYDLKGVIQLAL

N.tab (TQPT)      SEDAGNLGDVTCKATIPVDMESDAHFLAKEDGIIAGIALAEMIFAEVDPSLKVEWYVNDG
N.glauca          SEDAGNLGDVTCKATIPLDMESDAHFLAKEDGIVAGIALAEMIFAEVDPSLKVEWYVNDG
L.esculentum      SEDAGDLGDVSCKATIPVELESEAYFIAKEDGIVAGIALAEMIFAEVDPSLKVEWFIKDG
L.pennelli        SEDAGDLGDVSCKATIPVDLESEAYFIAKEDGIVAGIALAEMIFAEVDPSLKVEWFIKDG

N.tab (TQPT)      DKVHKGLKFGKVQGNAYNIVIAERVVLNFMQRMSGIATLTKEMADAAHPAYILETRKTAP
N.glauca          DKVHKGLKFGKVQGNAYNIVIAERVVLNFMQRMSGIATLTK- - - - - - - - - -
L.esculentum      DKVHKGLKFGKVQGKAHNIVIAERVVLNFMQRMSGIATLTKAMADAAHPAYILETRKTAP
L.pennelli        DKVHKGLKFGKVQGKAHNIVIAERVVLNFMQRMSGIATLTKAMADAAHPAYILETRKTAP
```

In some other cases, different isoforms are produced due to alternative transcription producing long and short mRNAs, each of which are only translated from their first start codon (*eg*; yeast Valyl-tRNA synthetase; Chatton *et al.*, 1988).

Cases of multiple codon usage, however, are exceptional (Kozac, 1995) and it is likely that most, if not all, plant QPRTases carry an N-terminal extension. The possible functional significance of this observation is discussed later (Discussion 3.1).

2.2 *QPRTase* in the *Nicotiana* genome

2.2.1 Background

The position of QPRTase at the interface between primary and secondary metabolism in pyridine alkaloid producing species means that its regulation is of fundamental interest. The mechanisms enabling *QPRTase* to perform its dual function are unknown, however preliminary studies show that transcriptional control plays an important role in regulating QPRTase in *Nicotiana* (Sinclair *et al.*, 2000). There is as yet no evidence for any post transcriptional regulation.

As a basis for future investigation into the molecular controls governing *QPRTase* expression, the first aim of the work presented below was to isolate *QPRTase* genes from *N. tabacum*, including substantial regions of 5' flanking sequence which are likely to contain important regulatory elements. As described in the Introduction (1.1.3), a substantial body of analogous research based on the *Str* genes of *C. roseus* has culminated in the isolation of important regulatory proteins which control the expression of many pathway genes.

Comparable work is underway in the cases of *ODC* and *PMT* from *Nicotiana*, which act together to produce the pyrrolidine ring necessary for nicotine synthesis. *ODC* and *PMT* promoter isolation and partial characterisations have been published, although no regulatory proteins have yet been described (Imanishi *et al.*, 1998; Shoji *et al.*, 2000a, 2000b). Although both *ODC* and *PMT* transcripts in *N. tabacum* roots are induced by jasmonates (Imanishi *et al.*, 1998), and their activities are co-elevated following foliage damage (Mizusaki *et al.*, 1973), the signal cascades leading to their transcription differ. While PMT jasmonate induction is inhibited by cyclohexamide, demonstrating that *de novo* protein synthesis is required for transcription, ODC transcriptional induction is unaffected by this inhibitor (Imanishi *et al.*, 1998). QPRTase transcript and activity levels are similarly induced by jasmonates and wounding, leading to the production of the pyridine ring for alkaloid synthesis in *Nicotiana* (Saunders and Bush, 1979; Imanishi *et al.*, 1998), however it is unknown whether its induction pathway is similar to those of ODC or PMT. It will thus be interesting, in future studies, to discover what mechanisms induce the wound and jasmonate responses of these genes, and whether they share any common upstream elements.

The isolation of genomic QPRTase sequences may also contribute to our understanding of how *QPRTase* has evolved in *Nicotiana*, in a manner analogous to the work of Hashimoto and co-workers (1998a) and of Riechers and Timko (1999), which traced the evolution of the *N. tabacum* PMT genes from their diploid ancestors. A further objective of the present study was to assess the specific expression profiles of recognisable *QPRTase* gene copies, in order to identify copies which may be wound induced, and hence appropriate for further promoter analysis seeking to understand the mechanism of this response.

2.2.2 Characterisation of genomic sequences encoding *QPRTase* from *N. tabacum*

Approximately 2 million plaques from an *N. tabacum* cv. Xanthi genomic library were screened at high stringency, using the *QPRTase* cDNA *RQPT1* as a probe, as described in Materials and Methods. Given that *N. tabacum* has a haploid genome of ~4,000 Mb, and the library contained average inserts of 16 kb, this screen therefore included ~8 haploid genome equivalents. Thirty one positive plaques were obtained, consistent with there being ~3 copies of *QPRTase* per haploid genome in *N. tabacum*.

Fourteen plaques were ultimately purified. This number was regarded as being sufficient for the objectives already noted, and is comparable to the 18 plaques used by Riechers and Timko (1999) to recover all five *N. tabacum PMT* copies. A number of approaches were used to determine which of these 14 plaques represented independent gene copies. Initially, a restriction digest method was employed. Due to difficulties encountered extracting DNA from numerous phage, this method was deemed impractical for categorising more than a few plaques (data not shown).

A PCR approach was then employed, using reverse primers (see Materials and Methods 4.3.?) designed to facilitate amplification of sequences upstream from the known coding sequence. The forward primers in these reactions were located in the phage arms (Materials and Methods 4.3.2). Because the orientation of the insert was unknown, a set of nested forward primers were designed in each phage arm (see Materials and Methods). This approach was expected to distinguish independent phage by both insert orientation and PCR fragment size. Using this method, only 4

phage templates produced clear PCR bands, however these were used as tools to characterise the remaining phage.

Those phage hybridising to the *QPRTase* probe which did not produce a PCR band using the primers noted above were spotted in an array onto agarose plates which had been spread with *E. coli*. Negative control plaques that did not contain any *QPRTase* DNA were included, selected at random from the same *N. tabacum* library as the positive plaques. These plates were blotted, and probed at high stringency with two of the PCR fragments of different size noted above, derived from the 5' flanking regions of different phage. (Figure 2.2.1). All 14 of the plaques hybridised to either one or the other PCR product, and none hybridised to both. Assuming that all of the *N. tabacum QPRTase* genes are represented in these 14 library plaques, this result is consistent with there being two classes of *QPRTase* in *N. tabacum* distinguishable by their 5' flanking sequences.

Two phage corresponding to each *QPRTase* gene class were selected, and their upstream regions were amplified, purified, and partially sequenced. In the case of the 'class 1' *QPRTase* phage, the two selected for sequencing displayed different PCR fragment lengths (2.4kb, 2.8kb), and were inserted in the phage in different orientations. The larger of the two inserts was, however, 100% identical to the smaller over the several hundred base pairs at each end which were sequenced, apart from a small region adjacent to the vector which may represent a cloning artefact. Thus, these two inserts were cloned independently during library construction, but are apparently identical. The phage without the likely cloning artefact was selected for further analysis and designated ***NtxQPT1***.

In the case of the 'class 2' *QPRTase* genes, DNA from both phage that was sequenced was 100% identical over ~1 kb. One of these phage was chosen for further analysis, and designated ***NtxQPT2***. When referring to the entire co-hybridisation class, not the specific gene copies selected here, the terms '*NtxQPT1*-like genes' and '*NtxQPT2*-like genes' will be used.

It is interesting to note that of the 14 purified *QPRTase* plaques, 9 hybridised to *NtxQPT1*, whereas only 5 hybridised to *NtxQPT2*. Assuming once more that the library is representative of the genome from which it was constructed, this is consistent with there being two copies of *NtxQPT1* and one copy of *NtxQPT2* in each *N. tabacum* haploid genome.

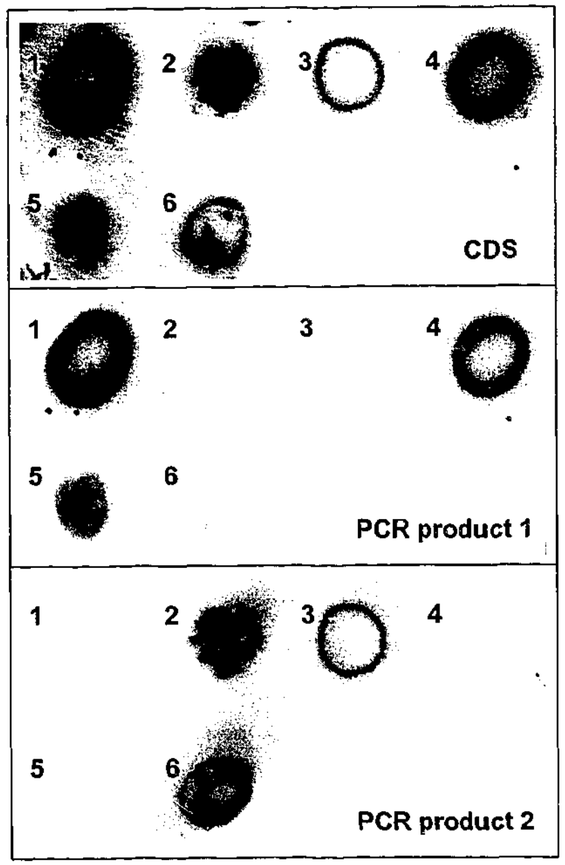


Figure 2.2.1 The *QPRTase* plaques fall into two mutually exclusive classes.

All three panels show the same membrane probed with three separate probes, after stripping between each probing. This is one of several blots of phage DNA, some of which contained negative control plaques.

CDS	The *QPRTase* coding sequence (*RQPT1* probe), which hybridises to all plaques carrying a *QPRTase* insert.
PCR product 1	The PCR product amplifying the 5' flanking region of the plaque designated *NtxQPT1*.
PCR product 2	The PCR product amplifying the 5' flanking region of the plaque designated *NtxQPT2*.

2.2.3 The origin of the two classes of *QPRTase* in the *N. tabacum* genome

Since *N. tabacum* is an allotetraploid, any given gene may be derived from either of its diploid progenitors. In the case of *QPRTase*, where there are at least two distinguishable gene classes making up a small gene family, there are a number of potential evolutionary routes which may have led to the current gene distribution. Alternative hypotheses explaining the evolutionary origin of *NtxQPT1*- and *NtxQPT2*-like genes are:

(i) That each class of *QPRTase* gene is derived from a different diploid progenitor species, such that each progenitor species contains only one class.

(ii) That each progenitor species contains both classes of *QPRTase* gene, such that *N. tabacum* has inherited *NtxQPT1*- and *NtxQPT2*-like gene(s) from both progenitor species.

Southern blotting was used to distinguish experimentally between these hypotheses. *NtxQPT1* and *NtxQPT2* 5' flanking regions were each used separately as probes against *Hind*III-digested genomic DNA isolated from the modern counterparts of the diploid progenitor species, *N. sylvestris* and *N. tomentosiformis*, along with *N. tabacum* cv. Xanthi (Genomic DNA kindly provided by Karen Cane). *Hind*III was chosen as the restriction enzyme because it produced a clear distinction between *N. sylvestris* and *N. tomentosiformis* on previous blots (Sinclair *et al.*, 2000). The results of this Southern analysis are shown in Figure 2.2.2. It is clear that both classes of *QPRTase* are present in all of the species tested. This result disproves hypothesis (i), and strongly suggests that (ii) is correct. The data are also consistent with there being more *NtxQPT1*- than *NtxQPT2*-like genes, and there being no other *QPRTase* gene classes in the genomes of the species tested.

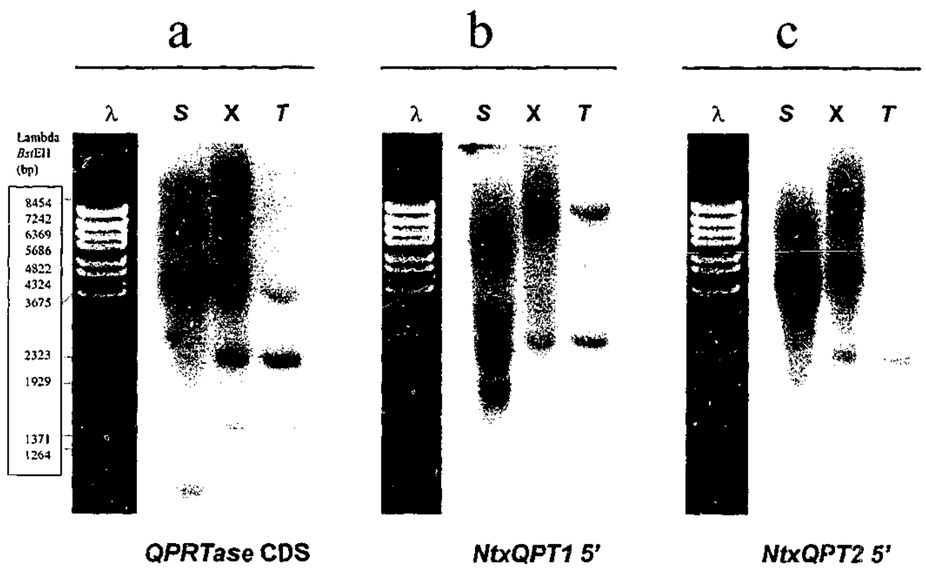


Figure 2.2.2 *NtxQPT1*- and *NtxQPT2*-like gene copies are both present in *N. tabacum* cv. Xanthi and the modern counterparts of both its diploid parents.

All panels (a, b, c) show *the same* blot, probed with three separate probes, after stripping between each probing. The blot contains *Hind*III digested genomic DNA extracted from the tetraploid *N. tabacum* cv. Xanthi (X), and from the modern relatives of its diploid progenitors *N. sylvestris* (maternal, S) and *N. tomentosiformis* (paternal, T). Panel (a) has been probed with the coding sequence of *QPRTase* (*RQPT1*). This probe yields a pattern similar to that shown previously (Murphy, 1999; Sinclair *et al.*, 2000, Figure 7; Karen Cane in preparation). Panel (b) shows the same blot re-probed with a fragment from the 5' flanking region of *NtxQPT1*. Panel (c) shows the same blot re-probed with a fragment from the 5' flanking region of *NtxQPT2*. The gel photograph is of *Bst*EII-digested lambda DNA, which is commonly used as a size standard. It is interesting that the *N. tabacum* cv. Xanthi genome does not appear to contain the sum total of *QPRTase* bands present in each of the modern counterparts of its diploid progenitors. The precise inheritance pattern of *QPRTase* is being studied in detail by another student in this laboratory (K. Cane, pers. comm.).

Given that *N. sylvestris* and *N. tomentosiformis* are in different subgenera, (*Petunioides* and *Tabacum* respectively; Goodspeed, 1954; Sisson and Severson, 1990), the data suggest that *QPRTase* probably underwent duplication before much of the speciation in the genus occurred, and may in fact be a basic feature of all *Nicotiana* genomes.

The fact that *Nicotiana* species posses different *QPRTase* gene copies with distinctive 5' flanking regions is of interest. As noted previously, it is thought that many new secondary metabolic pathways have arisen via gene duplication and subsequent specialisation. In this context, it was thought to be important to determine the individual expression profiles of *NtxQPT1*- and *NtxQPT2*-like genes in *N. tabacum*.

2.2.4 *NtxQPT1* and *NtxQPT2* are differentially expressed

It is known that *QPRTase* transcript levels are strongly induced in roots within 24h of foliage damage in both *N. tabacum* and *N. sylvestris* (Sinclair *et al.*, 2000). It is of interest to determine the relative contributions of *NtxQPT1*-like and *NtxQPT2*-like genes to this observed increase in *QPRTase* transcript. A number of techniques are available to address this question, however all of these have limitations. RT-PCR can be used to amplify specific gene copies which can be distinguished by differential primer specificity and/or fragment size. This technique has been successfully employed to examine the differential expression of *N. tabacum* PMT genes (Riechers and Timko, 1999), however accurate quantification is not a trivial task (Raval, 1994; Bustin, 2002; Wedemeyer, 2002). *NtxQPT1* and *–2* promoter sequences fused to an appropriate reporter gene could also be used to asses the transcriptional activity of these genes, however this technique requires numerous independent transformations to overcome variation in transcription caused by the effects of different integration positions (Allen *et al.*, 1993). Misleadingly low reporter gene expression could also result from the use of constructs which do not include full-length promoters, or, in some cases, from constructs lacking intronic sequences which may contain regulatory elements. In the current study, Northern analysis using 35bp oligonucleotide probes has been employed in an attempt to individually measure *NtxQPT1* and *NtxQPT2*

transcripts. This technique is attractive because it permits accurate, absolute quantification, and directly detects endogenous transcripts.

Northern blots are usually probed with relatively long probes which exhibit strong binding to the specific target RNA species (Trayhurn *et al.*, 1994). In the present case, however, the area of transcribed sequence differing between *NtxQPT1* and *NtxQPT2* was known to be relatively small, and so the opportunity for designing copy-specific probes was extremely limited. For this reason, 35mer ^{32}P end-labelled oligonucleotides, *oQPT1* and *oQPT2*, were used as molecular probes, being specific to *NtxQPT1* and *NtxQPT2*, respectively. These probes were designed to take advantage of a small, highly divergent region in the 5' untranslated regions of *NtxQPT1* and *NtxQPT2* (Their sequences are shown later in Figure 2.2.7 and 2.2.8). The same short probes were also used later against cDNA library plaques (Figure 2.2.4). The use of oligonucleotides against RNA blots is a technique which appears not to have been performed using plant tissue, and only to have been used relatively few times in non-plant systems (*eg*; Griffiths *et al.*, 1990; Beyer, 1991; Smith *et al.*, 1992; Trayhurn *et al.*, 1994).

In addition, few Northern studies have directly compared the levels of transcripts detected by different probes. Such comparisons are potentially problematic, since it cannot be assumed that both probes will be equally well radio-labelled, nor that the different probes will bind equally well to their respective targets. In order to surmount this difficulty, a series of standards were employed, so that molar quantities of RNA could be independently estimated for both transcripts, and then this data compared between transcripts. For use as standards, two PCR products- one amplified from *NtxQPT1* (350bp) and another from *NtxQPT2* (369bp)- were purified, concentrated in solution, and then quantified using spectrophotometry. These were serially diluted, rendered single stranded by boiling, and loaded into the gel in wells adjacent to the RNA samples. The signals obtained from these samples were used as standard curves to determine RNA amounts (see Appendix 1). Interestingly, a similar method has recently been independently employed by O'Hara and co-workers (2002), using conventional non-oligo probes and RNA standards, which enabled them to quantify mRNA levels of lipid biosynthetic genes in *Brassica napus* L. (Brassicaceae) in terms of transcript number per mass of total RNA.

Figure 2.2.3 shows the results of Northern analysis using *N. tabacum* cv. Xanthi tissue harvested from both control and wounded plants, 24h after wounding.

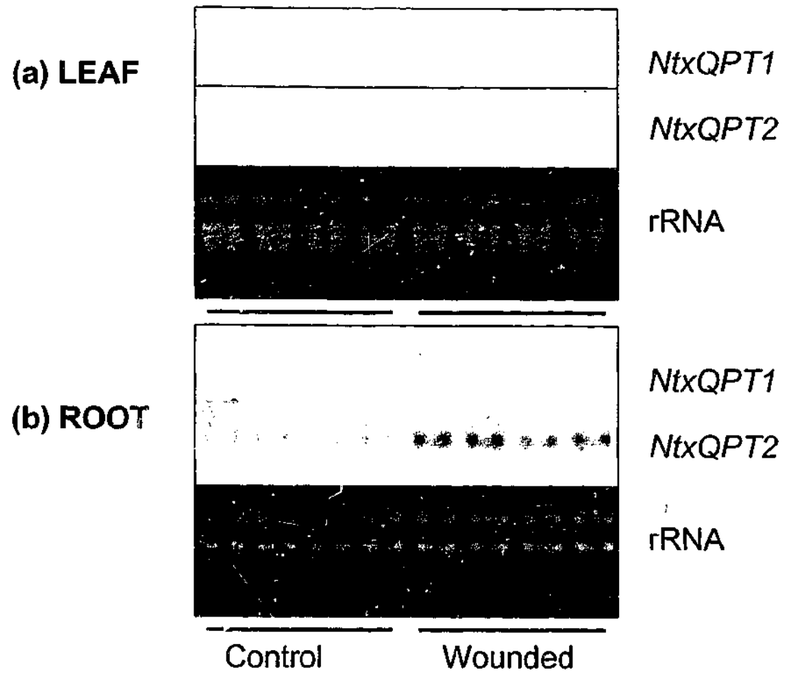


Figure 2.2.3 Differential expression of *NtxQPT1* and *NtxQPT2*.

Panels (a) and (b)

Northern blot analysis of wounded (w) and control (c) *N. tabacum* cv. Xanthi leaf (a) and root (b) tissues 24h after treatment, probed with *oQPT1* (*NtxQPT1*-specific oligonucleotide), then stripped and re-probed with *oQPT2* (*NtxQPT2*-specific oligonucleotide). Every blot included standard curves made up of *NtxQPT1* and *NtxQPT2* PCR products (Appendix 1). These standards confirm that each blot was successfully probed, and enable quantification. Gel photographs showing ribosomal RNA bands (rRNA) demonstrate comparable loading levels. Band intensities have not been normalised against an internal standard, since such standardisation is incompatible with the numerical quantification used here.

Panel (c)

NtxQPT2 transcript levels in root tissue in control (C) and wounded (W) plants, shown as number *NtxQPT2* transcripts per pg of total RNA. The bars represent the mean of 4 samples, ±SE. The dotted line represents the limit of detection for *NtxQPT1*, such that any undetectable *NtxQPT1* expression must fall below this level.

When probed with *oQPT1*, no signal was detected in any leaf or root sample, despite the fact that the standard containing only 50pg of *NtxQPT1* PCR product is detectable (this is equivalent to 25pg of sense strand). Since the formula weight of the sense strand of this standard is known[iv], the lowest standard signal detected can be calculated to represent 2.38 $x10^{-16}$mol of *oQPT1* DNA binding sites (This is numerically equivalent to 1.43 $x10^{8}$ target molecules, using Avogadro's number). Since DNA-RNA hybrids are generally more heat stable than DNA-DNA hybrids (Casey and Davidson, 1977; Anderson, 1999), an equivalent 2.38 $x10^{-16}$mol (1.43 $x10^{8}$ numerically) of *NtxQPT1* mRNAs would be expected to show a detectable signal. Given that no signal was detected in any 20µg total RNA sample probed with *oQPT1*, *NtxQPT1* transcripts must be present at levels less than 1.19 $x10^{-17}$mol/µg total RNA in both leaves and roots, even in wounded plants. This is equivalent to < 7.3 transcripts in every 1pg of total RNA.

The relatively low abundance of *NtxQPT1* transcripts may be seen in context when *NtxQPT2* is considered. Although *NtxQPT2*-like transcripts were not detected in leaf tissues when probed with *oQPT2*, they were readily detected in the roots of unwounded plants, and were increased ~2 fold in RNA from wounded plants. The average level of *NtxQPT2*-like gene expression in control *N. tabacum* roots was 2.5 $x10^{-17}$mol/µg total RNA, or 15.1 molecules/pg total RNA. After wounding, this level increased to 4.3 $x10^{-17}$mol/µg total RNA , or 25.9 molecules/pg total RNA. This data is presented graphically in Figure 2.2.3, with an indication of the *NtxQPT1* detection threshold.

The levels of detection achieved in this experiment are consistent with those found recently by O'Hara and co-workers (2002) in *B. napus*. Using a 1185bp DNA probe, these researchers were able to detect 10pg of RNA standard, which enabled them to deduce that their transcripts were present at levels equivalent to approximately ~2-5 molecules/pg RNA. Thus, the level of sensitivity in the experiments here, using short oligonucleotide probes was ~2-3 fold less sensitive than

[iv] The composition of the 350bp sense strand is: A(115); C(73); T(107); G(35). Using the formula weights of the bases (A: 326.21; C: 299.15; T: 317.19; G: 341.2), the formula weight of the strand can be calculated as 105233.4. The formula weight of the *NtxQPT2* PCR product's sense strand has been calculated in the same manner (118354.51).

analogous experiments using much longer probes. The published data of O'Hara and co-workers (2002) confirms the validity of the mRNA quantification presented here.

In order to gain more information on the expression profile of *NtxQPT1* in *N. tabacum*, a number of other techniques were also used. Twelve uncharacterised *QPRTase* cDNAs isolated from the *N. tabacum* SC58 root library screen completed in the lead-up to this project were available as semi-pure phage stocks (Sinclair, 1998). These were spotted in an array onto an agarose plate spread with *E. coli*, and screened using the oligonucleotides *oQPT1* and *oQPT2* as probes. As the sample blot shown in figure 2.2.4 demonstrates, all 12 *QPRTase*-positive plaques hybridised to *oQPT2*, but not *oQPT1*. Assuming that all cDNAs are present in the library at representative levels, this suggests that in *N. tabacum* wounded roots, *NtxQPT1* transcripts are present at a level ≤~12 fold less than *NtxQPT2* transcripts.

In addition, *NtxQPT1* transcripts were sought in RNA samples enriched for mRNA. From 375µg of total RNA available from wounded *N. sylvestris* roots (see Results 2.3), ~3µg of mRNA was purified. Figure 2.2.5 shows 2.5µg of this sample loaded alongside 20µg of the original sample before mRNA enrichment. When probed with *oQPT2*, the mRNA-enriched sample showed a signal ~5 fold greater than the signal obtained from the original RNA sample. When probed with *oQPT1*, however, no signal was visible. This result suggests that in *N. sylvestris*, like *N. tabacum*, *NtxQPT1* transcripts are not abundant in root tissue, even after foliage damage.

Another method which may be used to detect specific mRNAs according to their ability to hybridise to certain oligonucleotides is RT-PCR. This technique involves reverse-transcribing mRNA, and using the resultant cDNA as a PCR template. RT-PCR was used here in an attempt to identify *NtxQPT1* transcripts. While *NtxQPT2*-specific primers were able to produce a band, no *NtxQPT1* bands could be obtained from leaf or root tissue, and this technique was discontinued.

Together, these data demonstrate that *NtxQPT1*-like gene(s) are not strongly expressed in *N. tabacum* leaves or roots, nor in *N. sylvestris* roots. Due to the inherent limitations of experimentation, it is not possible to ascertain whether *NtxQPT1*-like genes are not expressed at all, expressed (and possibly induced) to a level below the detection limit of the experiments described above, and/or expressed in tissues not so far tested.

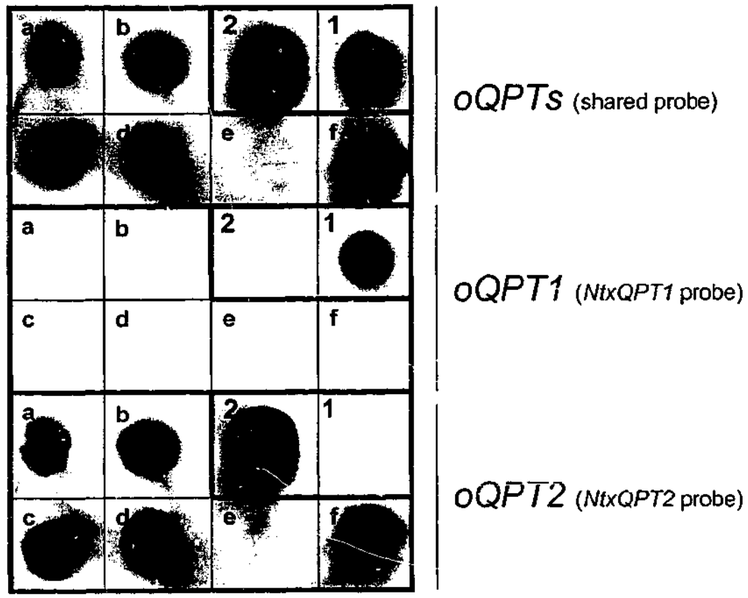


Figure 2.2.4 *NtxQPT2*-like mRNAs are more common in *N. tabacum* roots than *NtxQPT1*-like mRNAs.

All three panels show the same blot, probed with three different probes. The top panel is probed with *oQPTs* (conserved in the 5' UTR of all *QPRTase* sequences known in *N. tabacum*), the middle panel is probed with *oQPT1* (specific to *NtxQPT1*) and the bottom panel is probed with *oQPT2* (specific to *NtxQPT2*). Positions '1' and '2' represent *NtxQPT1* and *NtxQPT2* control phage, respectively. Positions a-f represent phage carrying different *QPRTase* cDNA inserts, which were previously purified from an *N. tabacum* root cDNA library (Sinclair *et al.*, 2000), but never characterised. cDNAs a,b,c,d and f are thus clearly derived from an *NtxQPT2*-like gene. cDNA e did not hybridise to any probe, it may be a false positive (*ie*; not *QPRTase*), or its 5' end may be truncated, such that none of the probes hybridise to it.

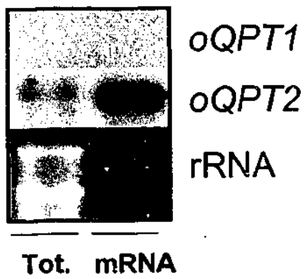


Figure 2.2.5

The left lane (Tot.) contains 20µg of total RNA, extracted from wounded *N. sylvestris* root tissue. The right lane (mRNA) contains 2.5µg of mRNA, derived from the same sample. This enrichment causes a ~5 fold increase in the *NtxQPT2* signal, detected with *oQPT2*, while NtxQPT1 remains undetectable.

It is worth noting that the *PMT* genes of *N. tabacum* are all wound inducible, but to considerably different levels (Riechers and Timko, 1999). One or more *NtxQPT2* genes, however, are clearly expressed in *N. tabacum* roots, and facilitate *QPRTase* induction following foliage damage. It is thus likely that *NtxQPT2*-like gene(s) are responsible for up-regulating QPRTase to facilitate a wound induced increase in alkaloid production.

2.2.5 The DNA sequence of *NtxQPT1*

Analysis of the *NtxQPT1* sequence is particularly important, since the integrity of its coding sequence may provide evidence as to whether the gene is functional or not. The insert in the *NtxQPT1* phage was completely sequenced using a series of amplified PCR products to 'walk' along its length. The insert was 7677bp in length, and it contained sequence similar to the complete *TQPT1* coding sequence noted above (Sinclair *et al.*, 2000), along with introns and a large stretch of 5' flanking DNA. The complete *NtxQPT1* sequence is presented in Figure 2.2.7, at the end of section 2.2.5. Its features are discussed below.

2.2.5.1 The introns and coding sequence of *NtxQPT1*

NtxQPT1 contains 10 regions of sequence highly homologous to the known *Nicotiana QPRTase* cDNAs (*ie*; exons), interrupted by 9 introns. One intron lies in the region of the N-terminal extension, between the two ATG codons noted previously. The 9 introns vary greatly in length, from 75 -1140bp, although all of them are within the common size range of plant introns (70-3000bp) (Simpson and Filipowicz, 1996).

In *NtxQPT1*, all of the intron-exon boundaries conform to the established consensus sequence, which is well defined in plants and other organisms (Simpson and Filipowicz, 1996) (refer to Figure 2.2.7b, later). The integrity of these splice sites may provide preliminary evidence that *NtxQPT1* is functional, since a non-functional gene may be expected to accumulate mutations over evolutionary time.

Interestingly, the positions of the *NtxQPT1* introns are extremely similar to those in the putative *Arabidopsis QPRTase* (*AtQPT*) with one exception being the

very short intron 3 in *NtxQPT1*, which is not found in *AtQPT*. Despite this conservation, however, the lengths of the introns, and their sequences are markedly different. Figure 2.2.6 shows the conservation of intron position, but not of intron length, between *NtxQPT1* and *AtQPT*. The introns in *NtxQPT1* are generally larger than those in *AtQPT*, and show more variation in size. It is difficult to determine whether this has been the result of intron elongation in *Nicotiana* or intron shortening in *Arabidopsis* (or both), however, the sequence of the first intron suggests that the former process has occurred in at least one case. In *NtxQPT1*, but not *AtQPT*, the first intron contains a 120bp region which is ~86% identical to a nearby 129bp region (indicated later on Figure 2.2.7a). It would seem that *NtxQPT1*, unlike *AtQPT*, has experienced the elongation of its first intron. Interestingly, this repeat is also absent in the corresponding introns from *N. alata* (Johnson, 2000) and *N. glauca* (DeBoer, 2001) genomic *QPRTase* sequence.

The phenomenon of conservation in intron position, together with variation in intron length, has been documented in other plant genes (Sahrawy *et al.*, 1996). There is evidence that intron length is generally larger in organisms with larger genomes (Vinogradov, 1999). For example, Vinogradov (1999) found that *Arabidopsis*, with a 1c genome of 0.15pg, had introns with an average length of ~113bp; whereas tomato, with a 1c genome of 1.00pg, had introns on average ~208bp long. The data obtained here, comparing the introns of an *N. tabacum* cv. Xanthi *QPRTase* (average intron size 419bp; 1c=~4.4pg; Bennett and Leitch, 1997) with an *Arabidopsis* counterpart (average *QPRTase* intron 104bp) conform to this pattern. The functional significance (if any) of this observation is unknown, however Vinogradov (1999) suggests that both intron size and genome size may relate to the level of DNA-protein interactions necessary to package the DNA in a given organism.

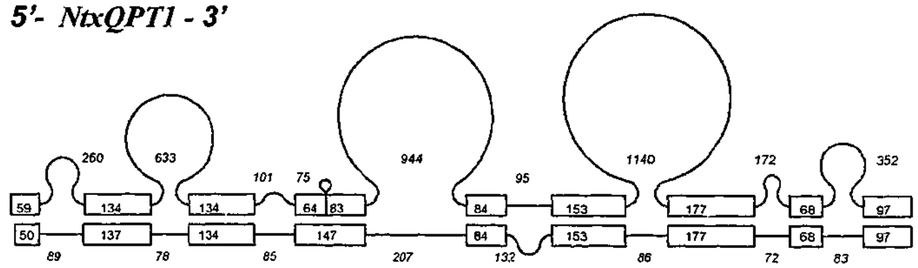


Figure 2.2.6 Scale diagram demonstrating the conservation of intron position, but not length, between *NtxQPT1* (top) and *Arabidopsis thaliana QPRTase* (bottom).

All of the exons in *NtxQPT1* have obvious counterparts in the *Arabidopsis QPRTase* gene, with each pair showing substantial sequence homology. The exons are represented in the diagram above by boxes, lined up beside their homologs. The introns are represented by intervening lines, such that the longer of the two introns at each position is forced to "bulge" out of alignment in order to permit the exons to remain paired. The lengths (bp) of all DNA segments are indicated. The lengths of all lines (including curves) and the (horizontal) lengths of all boxes are proportional to the length of the DNA segment they represent. The (vertical) width of the boxes is arbitrary.

Plant introns are generally AT(U) rich (Simpson and Filipowicz, 1996). *NtxQPT1* conforms to this trend, with an obvious anomaly in the case of intron 2. It contains a run of ~21 consecutive G residues, flanked by a few additional G and C residues on either side, forming a ~28bp island that contains only 2 A and no T residues (indicated later on Figure 2.2.7a). BLAST searches (using 'NR nucleic' database) show that there are G (or C) runs of comparable length (20-30bp) in the genomes of many organisms. Amongst the plant entries, the first 7 matches are all from rice. There were no matches from *Nicotiana* or *Arabidopsis*. Given that the *Arabidopsis* genome is essentially sequenced, the prevalence of matches from rice is unlikely to be due only to bias in the database. In rice, 6 of the 7 repeats were well apart from any annotated features, such as genes. The seventh was in the rather G-C rich fourth intron of a gene of unknown function (BAB16491). The only other G-repeat which was detected in the vicinity of a gene was the G_{17} run in the promoter of a barley chalcone synthase gene, which is followed directly by a series of repeated regions (Rohde *et al.*, 1991; Accession X58339). Without further data, it is not possible to draw conclusions regarding this G-C run, other than to note that such repeats are known, but uncommon, in plant introns. Shorter G-C repeats have, however, been studied in introns, and they may have functional roles. For example, the chicken β-tropomysoin pre-mRNA contains the repeated motif (A/U)GGG which enhances the splicing of an alternate mRNA version (Sirand-Pugnet *et al.*, 1995). Future reporter gene studies could perhaps examine whether the G-repeat in *NtxQPT1* plays any functional role.

Like the intron-exon boundaries, the coding sequence of *NtxQPT1* is apparently intact, consistent with the hypothesis that the gene is functional. The deduced amino acid sequence is similar to *TQPT1* (92% identity; see later, Figure 2.2.9) and contains all of the residues known to be important for enzymatic function (Eads *et al.*, 1997). The N-terminal extension noted previously is also present (79% identity until second ATG), as are the two in-frame ATG codons in the 5' region (Results 2.1.3).

2.2.5.2 The 5' flanking region of *NtxQPT1*

Overview:

The *NtxQPT1* insert includes ~2.7 kb of 5' flanking sequence. Given that *NtxQPT1* transcripts were undetectable in leaf or root tissues, it is unknown whether this region is capable of promoting transcription. Computational sequence analysis of the 5' flanking region is important, since it could provide further evidence on *NtxQPT1* expression or non-expression, and any putative DNA elements may assist in designing future promoter delineation experiments.

The 2.7 kb sequence available is, according to other studies, of appropriate size to form the basis of future promoter analysis. Most promoters contain response elements within 1-2 kb from the transcription start site (Klotz and Lagrimini, 1996; Imanishi *et al.*, 2000; Menke *et al.*, 1999b), although some studies have found functional response elements several kb upstream (Klotz and Lagrimini, 1996).

The *NtxQPT1* 5' flanking region does not contain any identifiable non-related features, such as the open reading frames of other genes. Database searches (BLAST) did not recover any known sequences displaying substantial homology to this region of *NtxQPT1* (data not shown). The sequence is A/T rich, particularly in the 1 kb region proximal to the coding sequence (68.8% A/T; in comparison to 57.4% A/T in the *NtxQPT1* coding sequence). This 1 kb region also includes numerous short A/T repeats, including 21 segments where an A or T is repeated 5 or more times in a row (The longest being 12 consecutive A residues), while the longest run of G or C is only 4bp. Although small repeats may be anticipated to accumulate in non-functional DNA, functional promoter sequences are also known to contain A and T repeats (*eg;* the promoter of the tyrosine/ dihydroxyphenylananine decarboxylase gene (*tydc7*) of *Papaver somniferum* L. (Park *et al.*, 1999)).

The core promoter- The TATA and CAAT boxes:

Most promoters share core elements such as the TATA- and CAAT-boxes, which allow the basic transcription machinery to operate. The TATA box is normally located 20-35bp upstream from the transcription start site in plants, and has the

consensus 5'–TATA(AA)-3' (Kuhlemeier, 1992; Guilfoyle, 1997). The *NtxQPT1* promoter has several possible TATA boxes, such as the motif TATATTT (2658-2664; 78bp upstream from the first ATG (the transcription start site has not been determined in *NtxQPT1*) noted in Figure 2.2.7a). The CAAT-box has the consensus 5'-GGCCAATCT-3', and may be found at many different positions in eukaryotic promoters, most often ~80bp upstream from the transcription start site (Kuhlemeier, 1992; Guilfoyle, 1997). The *NtxQPT1* promoter has a number of putative CAAT boxes, including the motif GGCCAAATTT (2582-2591; 152bp upstream from first ATG), noted in Figure 2.2.7a.

Promoter analysis using databases of known cis*-elements:*

There are three major databases suitable for locating putative *cis*-acting regulatory elements in plant promoters (Burks, 1999; Hehl and Wingender, 2001). Each has been used to analyse *NtxQPT1*:

- PlantCARE (http://sphinx.rug.ac.be:8080/PlantCARE/)
- PLACE (Higo *et al.*, 1999) (http://www.dna.affrc.go.jp/htdocs/PLACE/)
- TRANSFAC / TFSEARCH (http://www.gene-regulation.de/)

Due to their small size, sequences similar to any given *cis*-acting element will be relatively common in DNA sequence data. Thus, only a minority of the large number of putative elements will represent actual *cis*-elements, and some rationale must be used to attempt to distinguish these sequences. In the present case, sequences have been favoured which resemble the binding sites of characterised transcription factors. Although such sequences may not be functional in the context described in the literature, they may be indirectly relevant, because they may bind to un-characterised relatives of the known proteins. Plants may have large families of transcription factors, many of which bind to conserved DNA domains (Meshi and Iwabuchi, 1995) (*eg*; *Arabidopsis* is predicted to contain ~145 proteins in the DREB/ERF-family (Sakuma *et al.*, 2002)). Other putative response elements have only been identified as regulatory sequences in the analysis of a particular promoter. In the absence of any further information, such elements have been discounted, unless

their reported context has some relevance to *QPRTase* expression (*eg*; they are wound- or jasmonate-responsive).

The response elements discussed below have been considered regardless of their position or orientation on the DNA strands. Some elements may be functional when artificially placed in reverse (Matton *et al.*, 1993; Goldsborough *et al.*, 1993), or when shifted over 1 kb upstream of their normal position (Matton *et al.*, 1993; Goldsborough *et al.*, 1993; Elliot and Shirsat, 1998). Putative response elements in *NtxQPT1* are described below and indicated on Figure 2.2.7a, while those which were assessed in detail but are probably spurious are detailed in Appendix 2. These specific DNA motifs may be useful in designing future promoter delineation studies to identify regions which control transcription.

Potential binding domains for MYB transcription factors:

A number of putative MYB binding domains were identified in *NtxQPT1*, which are labelled on Figure 2.2.7a as described below. MYB-proteins are numerous in some plants (Meshi and Iwabuchi, 1995), with *Petunia hybrida* Vilm. (Solanaceae) containing at least 30 MYB-encoding genes (Avila *et al.*, 1993).

- Seven sequences containing the consensus MYB-binding domain (5'-CNGTT(A/G)-3') are all denoted *put*MYB (Meshi and Iwabuchi, 1995).
- *put*MYB1 resembles a binding site of MYB-Ph3, which is active in the petal epidermal cells of *P. hybrida* (Avila *et al.*, 1993; Solano *et al.*, 1995: 5'-aaaAaa**C**(C/G)**GTTA**-3'). Further upstream, the sequence labelled *put*MYB2 is similar to another binding site of the same transcription factor (5'-aaaAGT**Ta**G**TT**A-3'; Solano *et al.*, 1995).
- *Put*MYB3 resembles a putative binding site for MYBSt1 (5'-GGATA-3'), a transcription factor of unknown function, which is active in all potato tissues tested, but mostly in buds, flowers and roots (Baranowskij *et al.*, 1994).
- *Put*MYB4 resembles the "P-box" (5'-AGTTAGGTTC-3') from the promoter of a phenylalanine ammonia lyase promoter in *Phaseolus vulgaris* L. (Fabaceae) (Sablowski *et al.*, 1994). This binds the *N. tabacum* homolog of *Antirrhinum majus* L. (Scrophulariaceae) Myb305, which directs petal-specific expression.

- *Put*MYB5 resembles the binding site of the MYB encoded by the *P* gene of maize (*Zea mays* L. (Poaceae)), which regulates phenylpropanoid biosynthesis (5'-ACC(T/A)ACC-3') (Grotewold *et al.*, 1994).
- *Put*MYB6 resembles the non-consensus binding site of GAMyb (5'-TAACAAA-3'), which was isolated from barley, and is involved in gibberellin-regulated gene expression in barley (*Hordeum vulgare* L., Poaceae) (Gubler *et al.*, 1995).

Potential binding sites for bZIP transcription factors:

bZIP DNA binding proteins are widespread in eukaryotic organisms, including plants and may be involved in both developmentally and environmentally regulated gene expression (Meshi and Iwabuchi, 1995). Most plant bZIP proteins bind to DNA motifs containing a 5'-ACGT-3' core (Foster *et al.*, 1994). A number of potential bZIP binding sites are present in *NtxQPT1*, and are labelled on Figure 2.2.7a as described below.

- *put*bZIP1 resembles the binding site of the DPBF-1 and –2 transcription factors (5'-ACACNNG-3') which bind to the promoter of an embryo-specific gene (*Dc3*) in carrot (*Daucus carota* L., Apiaceae)) (Kim *et al.*, 1997).
- *put*bZIP2 marks the position of a G-box (5'-CACGTG-3'), which is present in numerous plant promoters and is bound by the *N. tabacum* DNA binding protein TAF-1 (Oeda *et al.*, 1991; Pasquali *et al.*, 1999).
- *put*bZIP3 resembles a number of characterised bZIP binding sites, including the HEX motif (5'-ACGTCA-3'), found upstream of many histone genes (Tabata *et al.*, 1989), the *as-1* element of the CaMV35S promoter (Lam *et al.*, 1989), and the B5 binding site of the Opaque-2 (O2) factor of maize (Lohmer *et al.*, 1991). The sequences in *NtxQPT1* labelled *put*bZIP3 also resemble motifs in the Lipoxygenase 1 promoter in barley, which is methyl jasmonate- and wound-induced (Rouster *et al.*, 1997). In *Lox*1, they occur as dispersed palindromes (CGTCA(N_{14})TGACG). In *NtxQPT1*, the *put*bZIP3 located at position 63 has the sequence CGTCA(N_{13})TGAAC, or CGTCA(N_{42})TGACG.

Potential binding sites for WRKY transcription factors:

The 5' flanking region of *NtxQPT1* contained 5 putative WRKY binding sites, which are labelled *put*WRKY on Figure 2.2.7a. WRKY transcription factors bind to W-box DNA motifs consisting of 5'-ttTGAC(C/T)-3' (Euglem *et al.*, 2000).

Potential binding sites for HD family transcription factors:

Three homeodomain (HD) protein binding sites were identified in the *NtxQPT1* 5' flanking region. These proteins are a family of transcription factors known in both plants and animals, which often regulate growth and development (Meshi and Iwabuchi, 1995).

- *put*HD2 is identical to a region in the soybean vegetative storage protein gene *Vsp*B, which is bound by a HD protein involved in phosphate-modulated gene expression (Tang *et al.*, 2001).
- *put*HD1 is identical to the binding site of the *Arabidopsis* HD protein Athb2 (Sessa *et al.*, 1993).
- *put*HD3 resembles the L1-box, which is present in the promoters of several *Arabidopsis* genes expressed in shoot primordia, which are regulated by the HD-protein ATML1 (Abe *et al.*, 2001).

Potential binding sites for GT-1 transcription factors:

GT-I factors bind the G-box, a *cis*-element present in many plant genes, which is particularly prominent among the promoters of light-regulated genes (Meshi and Iwabuchi, 1995). The G-box has the degenerate consensus 5'-G(G/A)(A/T)TT(A/T)-3', and is hence difficult to distinguish from similar sequences in raw sequence data (Zhou, 1999). Active GT-1 elements are, however, usually present as several closely spaced repeats (Zhou, 1999). *NtxQPT1* contains 32 potential GT-1 elements, however none of these are grouped as closely spaced repeats. Being so numerous, these regions are not shown on Figure 2.2.7a, although their positions are noted in the legend.

Potential binding sites for Dof family transcription factors:

Dof proteins comprise a plant-specific family of DNA binding proteins which are associated with many processes including hormone- and stress-responses (Yanagasiwa, 1995; Meshi and Iwabuchi, 1995). Dof proteins require a core 5'-(A/T)AAAG-3' motif for binding (Yanagisawa and Schmidt, 1999). Forty eight potential Dof core domains exist in the promoter of *NtxQPT1*. This large number is presumably due to the A-T richness of the *NtxQPT1* 5' flanking sequence (*NB*: only ~5 would be expected in a random sequence of similar length).

One region is of particular interest, as it contains a repeated region, where each repeat is made up of a Dof core domain palindrome. This region is marked with a dotted underline in Figure 2.2.7a, and the putative Dof binding domains within it are marked *put*D. Palindromes and repeats are known to be features of transcription factor binding sites (Meshi and Iwabuchi, 1995). The other putative Dof binding sites are noted in the legend to Figure 2.2.7a.

Comparisons with previously reported PMT and ODC promoters:

Although the evidence obtained here (Results 2.2.4) suggests that *NtxQPT2* is more strongly induced following wounding, *NtxQPT1* cannot be discounted as a possible alkaloid biosynthetic gene, and as such it may be co-regulated with other alkaloid genes. The *NtxQPT1* 5'flanking region does not, however, show substantial sustained homology with any of the 3 characterised *N. sylvestris PMT* promoters (Shoji *et al.*, 2000a), nor with the *N. tabacum ODC* promoter (Imanishi *et al.*, 2000) (data not shown). *Cis*-acting regulatory elements may, however, be very short (4-12bp) (Kuhlemeier, 1992; Guilfoyle, 1997), and the lack of obvious homology does not preclude the possibility that *NtxQPT1*, *PMT* and/or *ODC* share direct transcriptional regulation, as do other alkaloid biosynthetic genes (van der Fits and Memelink, 2000). The fact that response elements may be interrupted by non-specific nucleotides (Piechulla *et al.*, 1998) increases the difficulty in recognising them without experimental data.

The program 'COMPARE' (http://www.angis.org.au) was used to find small segments of identical DNA between any regions of the *NtxQPT1* 5' flanking region and the promoters of *N. tabacum ODC* and *N. sylvestris PMT*. The longest identical

stretch between *NtxQPT1* and the *PMT*s is only 10bp (5'-TAATGATTTT-3' in *NsPMT2*, and one nucleotide different in the other *PMT*s). This area is labelled NsP in Figure 2.2.7a. The largest segment of identity between *NtxQPT1* and *ODC* is the 12bp motif 5'-TCTTTCTTTATC-3' designated NtO in Figure 2.2.7a. These conserved blocks resemble a number of known *cis*-elements, however these have already been discounted in the survey just described.

Figure 2.2.7a The annotated DNA sequence of the *NtxQPT1* insert

Layout:

The left arm of EMBL3 abuts the 5' end of the insert sequence given below, and the right arm follows at the 3' end (after a short insert of 58bp (not shown)). The sequence is numbered from the 5' end of the insert, and the 5' flanking region is also numbered in reverse from the first in frame ATG codon.

Putative response elements:

The putative response elements identified in Results 2.2.5.2 are underlined, and annotated as described in the text. Putative TATA and CAAT boxes are highlighted and labelled. The putative Dof binding sites noted in Results 2.2.5.2, but not annotated below, occur on the positive strand at positions 42, 203, 739, 1051, 1099, 1237, 1272, 1336, 1405, 1459, 1576, 1595, 1724, 2014, 2308, 2355, 2423, 2438 and 2600; and on the negative strand at 185, 246, 252, 573, 812, 886, 1125, 1152, 1464, 1679, 1830, 1836, 1901, 1912, 1930, 1954, 2046, 2134, 2141, 2165, 2185, 2298, 2314, 2391 and 2461. The GT-1 sites noted in Results 2.2.4.2, but not annotated below, occur on the positive strand at positions 39, 356, 736, 850, 1286, 1333, 1401, 1572, 1584, 1734 and 1849; and on the negative strand at 247, 325, 460, 813, 887, 1029, 1126, 1185, 1217, 1518, 1647, 1831, 2063, 2129, 2155, 2207, 2301, 2464, 2488, 2194 and 2668.

Other features:

Exons are shown in bold, and numbered (E1, E2 etc.) in the left margin. The deduced amino acid sequence of *NtxQPT1* is shown below each exon, and numbered in bold at the right of the figure. The introns are also numbered (I1, I2 etc.) in the left margin. The repeats in I1 (noted in Results 2.2.4.1) are shown, one copy is underlined, the other highlighted. The G-repeat in I2 (noted in Results 2.2.4.1) is double underlined. The primer *oQPT1* which was used as a probe in Results 2.2.4 is indicated with a thick underline.

```
GGATCAAACAGGGTTTCATTGGGTCGGGTCACGTAACGGGTAAAGGAGATGGGACGAGGG 60
                                  putMYB                        (ATG-2683)
CCGTCAGATCAACCTGGTTTGAACGGCTGAGATGGGTTGACCTTGAAATGACGTAGTTTT 120
putbZIP3  putMYB5                                 putbZIP3      (ATG-2623)
GGTGTCAAACTACGTCGTTTGGTGGCCTGGGAGATGGGTCGCTTGGACCGGCTGCCTTGG 180
  PutWRKY      putMYB4                                          (ATG-2563)
GCTGCTTTTGGGCCTCAAATTTAAAGAAAACAGGCCCAATCCGATTTCTTAAAACAATTT 240
                                                               (ATG-2503)
GCACTCTTTTTCTTTTATTTTCTAATTTAAAACCAATACCTAATTAAAATGAAATTAAAC 300
                                                               (ATG-2443)
ACCCATTAATTAGCACTTAACACAATTATCACACACATATTAAAATATTTAAATAGGTAA 360
   putHD2                                                      (ATG-2383)
AATTACACCATGGCGACAAATAGAATCAAAGATGCATATTTGTGATTTTTTTTTAATAAC 420
                                                               (ATG-2323)
```

Figure 2.2.7a (continued)

```
CGAATTATGGTTTAATTACACACGACATATATTTTTTGTATTTTCGTTTGATTAGACTAA 480
putMYB1          putbZIP1                                      (ATG-2263)
ATAAGAATGGACAAAACCACAAATAACTACCAAAAATATCATGTAAATTCCAAAAATTGT 540
                                 putMYB5                       (ATG-2203)
ACAGCAAGACCATTTGTTATTATTTTTGATTTCTTTTTGGAGTGATTGTCGCGTAAACAA 600
              putMYB6                                          (ATG-2143)
AAATCACGTGCTCACACTAACCGCCATCCTGCTCGAACCTTTGCTGCCTGAGTTATATAG 660
   putbZIP2       putMYB                                      (ATG-2083)
CTGACTGCGAGAAGGAAGGTTCAAGCTATGGCTGCCCCATGGGATGCTCATTTGAAACCT 720
                                                              (ATG-2023)
TTGTCTGGTCAATGCGAAAAAGAGAGAAGTGATGTAAACATAAGAATGGAAGGTTTGCTT 780
      putWRKY                                                 (ATG-1963)
GTCAGCTGTCGAGCCAATGAGGCTCTTCCAGCTTTACCTACACGGCAAGGGAAAGATCAA 840
                                      putMYB5                 (ATG-1903)
AGGCGAGGTGGAAAATGCCTCGCACGAACTATCTGATCAAGTTTTCTTTTTCCTATCACA 900
                                                              (ATG-1843)
GGCAGCTGGCAAAGCCGTGTTTGATCGTCGCGTACATACTCCGTTACAGCTTCGTTCATG 960
                                          putMYB              (ATG-1783)
CGCCAATCAATTGATAGTGAAGCAGAGAAGGAAGAGTTTTGATCAACGTGTGGGTAGTCT 1020
                                                              (ATG-1723)
CTATTAGGATTTCCATTTCATGTGCACTAAAAAGGAAGGAAGACTGGTGCGACTATAGTA 1080
                                                              (ATG-1663)
ATATCAAGCATACGAGCAAAAGACTCTACCTACCCCTACTCTTTCTTTATCGGGTTATAA 1140
                                 putMYB5      NtO             (ATG-1603)
TGATTTTGTGACTTTTTTAGATTCTTTCTCTCGTCTTACTACTGTTTATCAACAAATATC 1200
 NsP                                                          (ATG-1543)
TAAGGATGGAGTTAGTTTTATCCTTCCTTTGTATCAAAAGAATGTTAAAATTCCATATAA 1260
      putMYB2  putMYB3                                        (ATG-1483)
TGCTTCTATAAAAAGACTTAAGTTGGATAATGGTAGAAAGTACATCTCTCATAAACTGCT 1320
                                 PutMYB3                      (ATG-1423)
GCAAGGGTATTTGAAAAAGCAAGGTATTGAGTCTCAAATGACATGCCCATACACCCCATA 1380
                                                              (ATG-1363)
GCAAAATGTTGTTGCAGAATGGAAAAAGAGACATTTATTGGAACTTATTAGGACACCTTC 1440
                                                              (ATG-1303)
CTTTGAGACGAATCTTCAAAAGTCTTTATGATCTGAACCATCATTATTCTTGGGAGGATC 1500
                                                              (ATG-1243)
AACAATGATTGGTCAACCCCCCTCCCCCACCTCCACCCAAATAATTTATAACTCGTCATA 1560
 putHD   putWRKY                                  putbZIP3     (ATG-1183)
TATGACTAACTGATAAAAGAAATGATAATTTAAAAAAGAAACGCCTAAGCTTATGTATTT 1620
      putMYB                                                  (ATG-1123)
ATCTGTTTGTTTTTATACATTTATTTATTATCTGTCAATCGAATATTGTATCTTGCTTCT 1680
                                 putWRKY                      (ATG-1063)
TTACAAATTTGATATTCTGAAAGGCATATACCAATGTTCCATAAAAGGGAAGTGAAAAAT 1740
                                                              (ATG-1003)
AAGCTATTGTGGTCACCCTGTTAAACCATATGGACCAAAACAACAACAAATTGACATAAT 1800
                    putMYB                putWRKY             (ATG-943)
TGGAGGACTATTTTGTTTTAGAAAACCTTCTTTTCCTTTTCAAGAATAGAAAAAATGACA 1860
                                                              (ATG-883)
AAAATGGTCCCGTATGTATGAGGATAGGTTCAAAGTTGTCCTTTAGGTATGCTTTAAGCA 1920
                      putMYB3                                 (ATG-823)
GTTATGGTTCTTTAAGTTTGTAAAAATTTAATACTTTTGATCTTTGTACAAAATTTACAA 1980
putMYB                                                        (ATG-763)
ATTATATCTGTTAATTTAATGAGAACTGTAAAAAAAGAGATTTAGTGGGAACTCATATGC 2040
      putMYB                                                  (ATG-703)
AGTAACTTTTGTAGTTTGTACTATTTTCCGTATATATTAGAGTTTGATGCAAACTTCTTT 2100
                                                              (ATG-643)
AGGTATGATTTTTGCTATTTTTAGTATATTTTTCTTTTGTCTTTAGTACAATTTTTTTTC 2160
                                                              (ATG-583)
TTCGCTTTTTAGGATTTGATAGACCTTTCCTAAGGTTAAATCTTTTTTTATCATAATACT 2220
     NsP                                                      (ATG-523)
CATCAAATCTAAGAGACACAGAGCTCATTAATAGCCCGTTTGGCCAAGCTGCAAAAATCA 2280
                                                              (ATG-463)
GCTTATTTTGATAAGTGCTTTTTTTCAAAAGTACTTTTGATGAGAAGCAGTTTGTGTTTG 2340
                                                              (ATG-403)
GCTAATTAGTTTAAAAAGCACTTCTGAGTAGCAATTAGTGTTTCGCCAAGCTTTAAAAAA 2400
                                                              (ATG-343)
CTGTTTCTAAGTGTATTTCTCAAAAGTGCTTCTCAAAAAGTAGTTTTGGAGAGAAGCTA 2460
putMYB1                                                       (ATG-283)
CTTTTTTTCTGCTTCTCCAAAATTATTTTTTTCCTTCCAGAAGCTTGGCCAAACCCCTCA 2520
                                                              (ATG-223)
ATTTTTGGCCAAAAGTACTTTTGGCAAAAAAAAAAAAGAACTTTTGGCCAAAAATAAGCT 2580
      putD   putD          putD   putD                        (ATG-163)
TGGCCAAATTTTAGGACCAAAAGTATTCAGAGTATACTTAAGGGACTACTTTGAACTTAT 2640
```


Figure 2.2.7a (continued)

```
   putCAAT                                          putMYB3  (ATG-103)
CCTCAAACATAAGGGACTATATTTGTCATTTTCTCGAAGTTCTCACAACCCAAGAAACCA 2700
                  putTATA                                    (ATG-43)
CTAAGCTCAGCAAAGCTATTTGCTCCAAAAATCAAAATTTCAATGTTTAAAGTTTTTCCT 2760
  OQPT1 probe                          M  F  K  V  F  P      6
E1 TTCACTGCAATAGTGCACCCTCATGCAATTACAGCACCAAGGTTTCTTTAGAACCCCTCA 2820
   F  T  A  I  V  H  P  H  A  I  T  A  P  R                 20
I1 ATTTAACCCAGAAGCAAAAAAAAAAAAAAAGGATTCTTGAAAATCATGTTTTTATTTTTGT 2880
   TATATATACTGATAAAGATTGTTCTTTTATGCAATTACAGCTCCCAATGTTTCTTTAAAA 2940
   CCACTCAATTCCACCCAGAAAGGAAAAAAAAAGATTCTTGAAAATCATGTTCTTATTGTT 3000
   TTGTACTGATAAAGATTATTCCTTTATGAGTTTCTGATTAAATTTTGTCTGTAAATTGCA 3060
E2 GGTTGGTTGTGAAAATGTCAGCAATAGCCACCAAAAATGCAGTGGAGTCATTAGTAGTGA 3120
     L  V  V  K  M  S  A  I  A  T  K  N  A  V  E  S  L  V  v  K 40
   AGCCACCAGCACACCCAACTTATGATTTAAAGGGTGTTATTCAACTTGCCCTCTCTGAAG 3180
     P  P  A  H  P  T  Y  D  L  K  G  V  I  Q  L  A  L  S  E  D 60
   ATGCTGGGGATTTAGGTTTGGTTTTAGCTCATATTATTTTAATCTTATTATATTGTCAAA 3240
     A  G  D  L  G                                           65
I2 TGAGGTGCACTCAGTTTCAATACTTTAGTAAATACTGTTAAGGGAAGCTAATGGTAGACT 3300
   TTTTAATTAATTGGGAGTAGCTTTATGAAGTTTAGCTATAGTCTTTTTTTGTTTTGTGTG 3360
   AACTCCAAGCTTCAATTTTTCTTGTGTAGCCTCAATTAATTTATAGCTAATTGTGTTCTT 3420
   TTTCCATAAGAACAGCTAATGTGTTCTTAATAAGGATTATCTTCAGTTTAACCGGGGGGG 3480
   GGGGGGGGGGGGGGAGAATAGTTCTGCCAAAAGTTGTTTTCCTTTCTTGTTTCCCAC 3540
   CTGGTGTCCGGTACCCG TTGTGGCCCAACTAATCCGGAGAACTCTTGTTAAGGATTGA 3600
   GGAGTATGTATCGCCCCACCGCAACCTTTGGTGTCAATAGTTGTTTTTTTAAGTTCAAGA 3660
   GCCCGAGGATATTGTCATGTACTGTCTGACTCTCTGCGTGATGGAAAAGTTTGTTTGAGT 3720
   AAATTTCTTGGTACTAGCAATATTTTCAGCACCTAGCTACCATTCTGAATTTGAGTACTC 3780
   AATTATAAAAAACCAATTTGTCCTGTGTATTATTTGTGTGAAATGCAGGAGATGTGACTT 3840
                                                  D  V  T  C 69
E3 GTAAGGCAACAATTCCTATTGACATGGAATCCGAAGCTCATTTTCTAGCAAAGGAAGACG 3900
     K  A  T  I  P  I  D  M  E  S  E  A  H  F  L  A  K  E  D  G 89
   GGATTGTAGCAGGAATTGCACTTGCTGAGATGATATTCGCAGAGGTTGATCCTTCACTAA 3960
     I  V  A  G  I  A  L  A  E  M  I  F  A  E  V  D  P  S  L  K 109
   AGGTATTGATTTTTTCAATTAGGTTGTTCTGGTAACCGGTATTTCTACTTTGAAGAAATG 4020
I3 TCTGAAAAAAGGTTTTTTAATTTCTTCATGTTCTCATTATCAGATGGAGTGGTCTATAAA 4080
                                          M  E  W  S  I  N  115
E4 TGATGGTGATAAAGTTCATAAAGGCTTGAAATTCGGCAAAGTACAAGGTAAACTATATGT 4140
     D  G  D  K  V  H  K  G  L  K  F  G  K  V  Q  G           131
I4 GAGAAATGATGAATTACTAATGATTTTTTGAATTAATAATAATCTTGCCTTGACGTCTGC 4200
E5 AGGAAAGGCTCACAGCATTGTTATACCTGAGAGAGTTGTTCTCAATTTCATGCAAAGAAT 4260
        K  A  H  S  I  V  I  A  E  R  V  V  L  N  F  M  Q  R  M 150
   GAGCGGAATAGCTACACTAACTAAGGTGTGTTTGTCCTCAATGTAAGTAGCACATCATTA 4320
     S  G  I  A  T  L  T  K                                   158
I5 ATAAAGATCTGGAAAGATAGCAATGAATGCAAATGGTTATGTCATTGATGAGTTGGTTTC 4380
   AGTATCCCAAAGTTAAGTCAAGCGTACCAGATCTGAGACTGTCAATTGTTCTATGCCCGA 4440
   AATTACTGGATTTTGTTGCAGAACATATGCATTGATCTAAATTTACACATTCCTGTATGA 4500
   TATATAAAATCAATGAAATATCCTTCAACGGTTCAAGTACAATGGTCAAGAGTTCAAGTC 4560
   TTAGAAACAGATTCCGCGTATTCTTTATAAGGCTGTGTACGTCCACTCTTCTGATACATT 4620
   AGATGGTGTGAGAGCCGCACACTGAAAACAATTCTGCAATCAGTCTATGACAAAGATTCC 4680
   TCAGTTTATTCCATCACAAACAAAAGGTGCAAATGCACATGCAAAGACTTAACTATACAC 4740
   ATGCCATTTTCTAAAACTCAGTAGACGAGAATAAATTAACTAATGAATGTTAACATAAAT 4800
   TATGAAGTACCAACAAAATTTGTAACTAATAAGAACGGTACAAATATTCACCCGGAATGA 4860
   AATAATGTGTGAAATCTAAACAGATATTGCTGTTAGAATTTGCAAACGGAGCTGTGAGTT 4920
   TTGACACTTTTGGTACAACTACTTGCGTTTACCTTTCAATGGAACCCCTTTGGTTCTCTA 4980
   AATCAGGTAATAAATATCAAATATCTGTCCACAAAAATTAATTCCATGTGTTCCTTTTTC 5040
   AGGATATGGACACTGCTGCAGGTGTCATGTTTTAAAAGAATTTGAAAGATTTCATTGAAC 5100
   CAGAACCAAACATCTTCATCTCTTT ATGAATAGACAAACTAGACATTTTGTACCTGATA 5160
   ATGCTATCTCAAGATGTCATGGAGTTCATTGTCTTGCCTCTGTAGTAATGATTAGAATTT 5220
   AGAAGAGAGTGAGTTCTTTAATATTTGCAGGCGATGGCAGATGCTGCACACCCTGCTACC 5280
                                        A  M  A  D  A  A  H  P  A  T  168
E6 ATCTTGGAGACTAGGAAAACTGCTCCAGGATTACGTTTGGTGGATAAATGGGCGGTAAAT 5340
     I  L  E  T  R  K  T  A  P  G  L  R  L  V  D  K  W  A      186
I6 ATTTACTCGCCAAAATCATCCATTGTGAGAGGCTATCACATGTATATATAGTTCATCTTG 5400
   GCTGACTTTCTACCTGGCTTTAAGTGAAGGTATTGATCGGTGGGGGGAAGAATCACAGAA 5460
                                        V  L  I  G  G  G  K  N  H  R  M 197
E7 TGGGCTTATTTGATATGGTAATGATAAAAGATAATCACATATCTGCTGCTGGAGGTGTCA 5520
     G  L  F  D  M  V  M  I  K  D  N  H  I  S  A  A  G  G  V  S 218
   GCAAAGCTCTAAATTCCGTGGATCACTATTTGGAACAAAATAAACTCCAGATGGGGGTTG 5580
     K  A  L  N  S  V  D  H  Y  L  E  Q  N  K  L  Q  M  G  V  E 238
   AGGTTGGCCCCATTTACCTTTGGATTAAATTGTGTGGTTATAGTTATGTTTATTTCGACC 5640
I7 TGACAGTTCAATTACCTATTCTGCTGGAATACGATTAATCATTTAATCTTAGCTTCTGCC 5700
   ATCAGTTGTGCAATACTCTTTTGTCAGTTTGTATTTGGTCAGATAAATATCTTTCTAGA 5760
   GCTATTTTCAATCAAAAACTATTTATAGTGTTGATGATATAAACTTTATAATTGATGCTA 5820
   TTTCTGATATCCTTTCATTGAAGAATTGAATTCTTATCTGTTGAAATCAAATGTTAGTCC 5880
```

Figure 2.2.7a (continued)

```
      AAGTTCAGAGATGTTTGCACTACACCTCGAGACTTTTATTTTACATTTTTGTATCTAGAT 5940
      ACACTTGTAGGCGGTTGAGAGCAACAACAACAAACCCAGTATAATCCCACAAGTGGGGTCT 6000
      GGTGAGGGTGGTGTATACGCAGACCTTGAAGCTAGAGAGGCTGTTTTCGAAAGACCCCCA 6060
      GCGTAGAGGTTGAGAGTAGTATCCCAGAATATATCATGATTTTTGATAACTGAAACAAGA 6120
      GTGGAAAATTGTGTTATTTGACCATGTTAAAGCAGTTAGCGAACAAAATAGTTTGTCAGG 6180
      TTACATTTTGGACTAGTTTTCAAGGTCATCTTTTTTGGATTTCGGCACACAACAATGTTC 6240
      ATGGTTCCACTCCCTTGGGTATTCTTTCTTGATAATGTTTTGCTTTTGTTACAAAAGTAA 6300
      AGGGAAAAGTAAAAATAACATTATATATTGCTGAAAGAGCTGGGAAAATTTCTTCTTCCA 6360
      GGAATTTTTCAATGTTATCATCCACCCTAATATAACCTAAGAGCCAAAAGGTTTGGGGGA 6420
      TATTGTATTTTAGGTCTGTCTTTGCATACTACTTATTGGTGTTGTCTTTCATTAAGTTGG 6480
      TAATTTGCACCTACTTTTGGATAGTTAAGAATACTAATAATGTAGATACTTTTTTTATAA 6540
      GGTAAGATCTTATAAATGTGTCAGCATCAAGAATATGCTGGTAGGAACCCAAATTGTCTG 6600
      ATTTAAGATACCCGAACTTACGTTATTGTATTTAGCAGTTTTGAGCATATGGTCCTTTCT 6660
      GAATCTGCTCAATTTCTGGTAATTATTGGCAGAGTTTTAAACCTTTTCTCCTATCCAACA 6720
      GGTTGAAACCAGGACAATTGCAGAAGTACGAGAGGTTCTGGACTATGCATCTCAAACAAA 6780
       V  E  T  R  T  I  A  E  V  R  E  V  L  D  Y  A  S  Q  T  K  258
E8    GACTTCGTTGACTAGGATAATGCTGGACAATATGGTTGTTCCATTATCTAACGGAGATAT 6840
       T  S  L  T  R  I  M  L  D  N  M  V  V  P  L  S  N  G  D  I  278
      TGATGTATCCATGCTTAAGGAGGCTGTAGAATTGATCAATGGGAGATTTGACACGGAGGT 6900
       D  V  S  M  L  K  E  A  V  E  L  I  N  G  R  F  D  T  E     297
I8    AAACCCTGGTTTCATTTTCATTAACTATTTCACACAAGTGTCATTGTGTCAGAATATAGA 6960
      AGACTTAATGTGATGCAATGCCTTGATTTTCATAAAAAAGAAGTACCGCATGGATTTCCT 7020
      GTAAACGTATATCTTTTGGTGACATGCTGTTGCTGTTGTAATGGTGGCAGGCTTCAGGAA 7080
                                                    A  S  G  N  300
E9    ATGTTACCCTTGAAACAGTACACAAGATTGGACAAACTGGAGTTACCTACATTTCTAGGT 7140
       V  T  L  E  T  V  H  K  I  G  Q  T  G  V  T  Y  I  S  R     320
I9    ATGCATTTCGCTCTTGCTATTTTTTTCAGAAGATAACCATACAAGACAGCAGAAGACGAC 7200
      ATATATAAGAGATTTCTCAGTGTTTGTTATTAGTATAGCTGGGACATCTTTCTGTTCCTT 7260
      TTCTTTTCCTTCTTATAATAAGATGGTTAGTATACTTGCCTTGTTCAGACAAACGAAAAA 7320
      GATGGTTAGGTTACTTGCACATGTTAGACCAAAAATGGTCTCAGTAGATAGTCTATGTTA 7380
      TGTAACAATTCTTTTGATAGGCAAAAGTGACAGAGGTATTTGTTTCCTTAAGTATTAGGC 7440
      GCGTGAAGCTTTGTTCTGATATTAAACTTATAGAGTTTAAATCTTTGCAGTGGTGCCCTG 7500
                                                    G  A  L     323
E10   ACGCATTCTGTGAAAGCACTTGACATTTCCCTGAAGATCGATACAGAGCTCGCCCTTGAA 7560
       T  H  S  V  K  A  L  D  I  S  L  K  I  D  T  E  L  A  L  E  343
      GTTGGAAGGCGTACAAAACGAGCATGAGCGCCATTACTTCTGCTGTAGGGTTGGAATAAA 7620
      V  G  R  R  T  K  R  A  *                                    351*
      AGCAGCTGAATAGCTGAAAGGTGCAAATAAGAATCATTTTACTAATGTCAAACAATA     7677
```

Figure 2.2.7b Summary of exon-intron boundaries, against the dicot consensus

```
Exon 1   CCAAG /  GTTTC---Intron 1---TGCAG /  GTTGG  Exon 2
Exon 2   TTTAG /  GTTTG---Intron 2---TGCAG /  GAGAT  Exon 3
Exon 3   TAAAG /  GTATT---Intron 3---ATCAG /  ATGGA  Exon 4
Exon 4   ACAAG /  GTAAA---Intron 4---TGCAG /  GAAAG  Exon 5
Exon 5   CTAAG /  GTGTG---Intron 5---TGCAG /  GCAAT  Exon 6
Exon 6   GGGCG /  GTAAA---Intron 6---TGAAG /  GTATT  Exon 7
Exon 7   TTGAG /  GTTGG---Intron 7---AACAG /  GTTGA  Exon 8
Exon 8   CGGAG /  GTAAA---Intron 8---GGCAG /  GCTTC  Exon 9
Exon 9   TCTAG /  GTATG---Intron 9---TGCAG /  TGGTG  Exon 10

         ---AG /  GTAag----------TgCAG /  gt---
```

The consensus sequence (bottom line) is taken from Simpson and Filipowicz (1996). Consensus nucleotides shown in lower case occurred at this position in 40-60% of dicot genes surveyed, those in capitals in 60-99%, and those in bold in 100%. *NtxQPT1* concurs well with this consensus, as shown by the bold nucleotides in the *NtxQPT1* sequence shown above. The most "unusual" base is the residue G, 4bp into intron 7, which occurs in this position in only 3% of dicots, but is nonetheless no barrier to splice site function.

2.2.6 The DNA sequence of *NtxQPT2*

The DNA sequence obtained from the *NtxQPT2* insert is included at the end of this section, as Figure 2.2.8, after all of its features have been discussed.

2.2.6.1 The 5' flanking region of *NtxQPT2*

Overview:

The *NtxQPT2* insert contained ~2.4 kb of 5' flanking sequence. Like that of *NtxQPT1*, the *NtxQPT2* 5' flanking region is A-T rich (71%), and contains numerous short A or T repeats (data not shown). The *NtxQPT1* and *NtxQPT2* 5' flanking regions do not, however, show any substantial homology to each other, as expected, given that these two fragments do not cross hybridise at moderate-high stringency (see Figure 2.2.1, above). The longest stretch of identity between the two ~2 kb sequences at any point is only 12bp (using COMPARE; data not shown). The *NtxQPT2* sequence does, however, show significant sequence homology to sequences already present in the databases.

Comparisons to sequences present on the database:

When the *NtxQPT2* 5' flanking region was used to retrieve similar sequences from DNA sequence databases, it was found to be essentially identical to the promoter sequence of *RD2* from *N. tabacum* cv. Wisconsin 38 (Conkling *et al.*, 1998; see below). The two sequences showed 99.6% identity (differing at only 9 nucleotides over 2005 bp). The *NtxQPT2* sequence is some 275 bp longer at the 5' upstream end. This strongly suggests that *RD2* is an *NtxQPT2*-like *QPRTase*.

RD2 was first isolated as a cDNA from a screen to identify root-specific transcripts in tobacco (Conkling *et al.*, 1990). It was determined to be a member of a small gene family, and shown to be substantially under transcriptional control, however no indications of its function, nor its sequence, have as yet been published in the peer-reviewed literature.

The *RD2* promoter subsequently became available (Accession: AR059247), after being isolated from a genomic library and patented as a root-specific promoter

in 1[illegible] without the gene's function being noted (cited as "Conkling *et al.*, 1998"; US 583,876 available at: http://www.uspto.gov). Examination of the patent description showed that a considerable amount of unpublished data had previously been gathered relating to *RD2*, including the PhD project of Song (1997). A copy of this thesis has recently been examined, and the identity of *RD2* as *QPRTase* is acknowledged therein. *RD2* will be hereafter referred to as *Ntw38QPT2*.

The transcription start site of *Ntw38QPT2* was determined using primer extension experiments (Conkling *et al.*, 1998). This site was 5 bp upstream from the start of the previously reported cDNA *TQPT1* (Sinclair *et al.*, 2000). A putative TATAA box was located at positions –23 to –29 (Figure 2.2.8).

Using GUS reporter gene constructs, the *Ntw38QPT2* promoter was shown by Song (1997) to direct expression almost exclusively to the roots of regenerated transgenic *N. tabacum* plants, at a level approximately four times higher than the *CaMV35S* promoter. The same construct also directed weaker root-preferential reporter gene expression in transgenic tomato plants (Song, 1997). Deletion analysis enabled a region (–545- –596) to be identified as being important for root-specific expression in tobacco. This region is completely conserved in the *NtxQPT2* sequence recovered here[v] (Figure 2.2.8).

The root-specific nature of expression directed by the promoter of *Ntw38QPT2* (Song, 1997) is consistent with this gene playing a role in alkaloid biosynthesis. This strengthens the hypothesis presented above that an *NtxQPT2*-like gene is likely to be involved in regulating alkaloid metabolism. The strong root-specific nature of the *Ntw38QPT2* promoter is the central concern of the work of Song (1997) and Conkling and co-workers (1998), and the potential of this promoter for wound-induction has not been investigated by these researchers.

In 2001 an "Application for the Determination of Non-Regulated Status" for a genetically engineered *Nicotiana tabacum* cv. Burley 21 LA line producing low nicotine was made to the United States Department of Agriculture (USDA) ("the application"; Petition Number 01-121-01p). This line was transformed with an antisense cassette containing the *Ntw38QPT2* cDNA, and as a result possesses a very low nicotine content. This application also notes that two *QPRTase* gene copies have been isolated from *N. tabacum* cv. Wisconsin 38 which share >80% promoter

[v] In *NtxQPT2* it is located between –546 and –560, due to a single nucleotide addition at position –276.

homology. The authors also mention other more weakly-hybridising bands which they believe may provide the low, constitutive QPRTase levels required for primary metabolism. Presumably, the two genomic clones mentioned in the patent application which share strong promoter homology represent two *NtxQPT2*-like genes, while the other bands hybridising to the coding sequence probe, which were not recovered, may correspond to *NtxQPT1*-like gene copies, like that characterised for the first time in the present study.

A portion of the *NtxQPT2* promoter also showed substantial sequence homology to another group of sequences present in the EMBL-Genbank databases. This region was not noted by any of the reports describing *Ntw38QPT2* that have been reviewed above. The *NtxQPT2* promoter possesses a region of ~830bp which is 81% identical to a portion of the promoter of the *T85* gene of *N. tabacum*, encoding an auxin-binding protein (Watanabe and Shimomura, 1998; Accession X70902) (Appendix 2.2.6). Abruptly on either side of this region, no homology is identifiable. This segment is in a similar relative location in each gene, 716bp upstream from the ATG codon in *NtxQPT2*, and 750bp upstream in *T85*. A region sharing 88% identity with 655bp of this same region also occurs in the promoter of an *N. rustica* (nuclear) gene encoding *PSI-E* subunit of photosystem 1 (Kubota *et al.*, 1995; Accession D42070). Small sections <100bp showing homology to this region also occur in the promoter of *cp31*, an *N. sylvestris* 31kD chloroplast ribonucleoprotein gene that is expressed in both leaves and roots (Li *et al.*, 1991; Accession X57079) (data not shown). These genes are apparently unrelated in function to *QPRTase*, and it seems unlikely that the region noted is involved in the co-regulation of their transcription. There is also no evidence that this region encodes part of a conserved exon from an unannotated gene.

The presence of a discrete, conserved DNA region in the promoters of several genes within the same genus is consistent with a number of hypotheses. Perhaps the most obvious explanation is that the DNA segment represents a piece of DNA which is commonly replicated and highly mobile. Plants contain numerous transposable elements, which may be as short as several hundred base pairs. No further insights could be gained by modelling the predicted RNA secondary structure for this region (using SQUIGGLES and FOLDRNA, http://www.angis.org.au), in comparison to known mobile DNA elements of similar length from Solanaceous species (*eg*;

Tomato macho viroid (Accession K00817); *Capsicum annum* L. Alien transposable element (Accessions X87903, X87869)) (data not shown).

A second possibility is that the conserved region represents a scaffold-attachment region (SAR). SARs are DNA domains which are thought to assist in DNA packaging, by binding to the protein scaffold within the chromosome (Gasser *et al.*, 1989; Avramova and Bennetzen, 1993; Kipp *et al.*, 2000). SARs are distributed unevenly throughout eukaryotic genomes (Gasser *et al.*, 1989). Their position is strongly associated with promoter regions *in vivo* (Hall *et al.*, 1991; Galliano *et al.*, 1995) and in plants they are able to enhance the expression of adjacent transgenes (Breyne *et al.*, 1992; Allen *et al.*, 1993, 1996). It remains unknown how SARs influence transcription, but the effect is probably related to the packing and un-packing of discrete DNA regions that lie between SARs, within which transcription is insulated from the packaging state of adjacent regions (Galliano *et al.*, 1995; Kipp *et al.*, 2000). SARs are characteristically several hundred base pairs long, markedly A-T rich (70-75%), and contain conserved motifs (T- and A-boxes, which resemble each other) (Gasser *et al.*, 1989; Dietz *et al.*, 1994). SARs also contain numerous putative binding sites for topoisomerase II (AAATAT), one of several known protein components of the scaffold (Gasser *et al.*, 1989; Kipp, *et al.*, 2000). The putative SAR in *NtxQPT2* displays all of these features. Its A/T-content is 73% in the region shared between *PS1-E* and *T85*. It possesses four regions resembling the A- and T-boxes, along with 5 completely conserved putative topoisomerase II binding sites (labelled *put*AT and *put*TOPII, respectively, on Figure 2.2.8), while only 1 would be expected to be present in random sequence of 2kb. Furthermore, the sequence in *NtxQPT2* shows homology to a number of known SAR regions in the *Drosophila* genome (*eg*; 68% identity over 92bp to the SAR at position 164957-165048 of sequence AE 003685).

It is notable that one of the first proven plant SARs (Hall *et al.*, 1991; Allen *et al.*, 1996) was originally isolated from the 3' flanking region of an *N. tabacum* gene (*RB7*) in the same screen for root-specific genes which originally yielded RD2 (Conkling *et al.*, 1990). *RB7* is known to encode a water channel protein (Yamamoto *et al.*, 1991; Song, 1997), however the relevant 3' sequence is not available on the public databases. It is unclear whether the presence of a putative SAR in both *NtxQPT2* and *RB7* has any functional significance. It is, however, possible that the strong root-specific nature of their transcripts in *N. tabacum* (Conkling *et al.*, 1990) is

influenced by the close proximity of a SAR, since SARs are known to enhance transcription. Furthermore, some proteinaceous components of the scaffold are known to be tissue specific (Kipp *et al.*, 2000), and it is thus conceivable that genes close to SARs would be strongly expressed in certain tissues.

Interestingly, some SARs may be derived from transposable elements, and thus the two hypotheses noted above relating to the identity of the *NtxQPT2* region are not mutually exclusive. In *Petunia hybrida*, a cross-hybridising domain was found in many places throughout the genome, including promoter regions, which was able to bind the nuclear scaffold (Galliano *et al.*, 1995). This fragment was related to *Copia*-like retrotransposons, and had probably dispersed throughout the genome, before losing its mobility (it contains a number of frameshift mutations) (Galliano *et al.*, 1995). The *NtxQPT2* region may have had a similar origin and a similar function. Further experimental work could determine whether this region is able to bind the nuclear scaffold, using plant-based assays that have been described in the literature (Hall *et al.*, 1991; Avramova and Bennetzen, 1993; Galliano *et al.*, 1995).

Promoter analysis using databases of known cis*-elements:*

Although *Ntw38QPT2* has been partially functionally characterised, no computational analysis of its promoter has been presented (Song, 1997; Conkling *et al.*, 1998). In the present study, the 5' flanking region of *NtxQPT2* was scanned for putative *cis*-acting regulatory elements, using the same techniques as for *NtxQPT1* (Results 2.2.4.2). As for *NtxQPT1*, the elements which were assessed, but deemed to be invalid or irrelevant based on experimental evidence in the literature are described in Appendix 2. *NtxQPT2* contained a number of putative response elements which have already been noted in *NtxQPT1*. The putative elements marked on Figure 2.2.8 as *put*MYB, *put*MYB1, *put*MYB3, *put*bZIP1, *put*bZIP3, *put*D and *put*WRKY have already been described (Results 2.2.5.2). Those elements which were found in *NtxQPT2*, but not in *NtxQPT1* are described below.

Potential binding domains for MYB transcription factors:

The sequence labelled *put*MYB7 is identical to the binding site of CCA1 (5'-AAAAATCT-3'), a MYB transcription factor which regulates photosystem II genes (*LhcbII*) in *Arabidopsis* (Wang *et al.*, 1997).

Potential binding domains for AP2/EREBP transcription factors:

AP2/EREBP proteins are a family of plant transcription factors (Meshi and Iwabuchi, 1995) which include the ORCA proteins which are important in controlling alkaloid synthesis in *C. roseus* (Menke *et al.*, 1999; van der Fits and Memelink 2000, 2001). The members DBF1 and DBF2 from maize are known to bind the sequence 5'-ACCGAC-3' in the promoter of the drought-induced *rab17* gene (Kizis and Pages, 2002). This sequence occurs in the 5' flanking region of *NtxQPT2*, and is labelled *put*AP2/E.

Figure 2.2.8 (facing page) The annotated DNA sequence of the *NtxQPT2* insert.

Layout:

The left arm of EMBL3 abuts the 5' end of the sequence given below, and the right arm the 3' end (after a 58bp insert (not shown)). The sequence is numbered from the 5' end of the insert. The 5' flanking region is also numbered in reverse from the transcription start site (*).

Putative response elements:

The region directing root-specific reporter gene expression is shown in bold (Conkling *et al.*, 1998). Putative response elements identified in Results 2.2.6.1 are underlined. Putative TATA and CAAT boxes are highlighted and labelled. Putative Dof binding sites noted in Results 2.2.6.1, but not annotated, occur on the positive strand at positions 41, 133, 432, 565, 683, 1012, 1082, 1096, 1158, 1180, 1271, 1648, 1893 and 2019; and on the negative strand at 185, 208, 218, 302, 323, 351, 369, 474, 845, 968 and 1401. The putative GT-1 sites noted in Results 2.2.6.1, but not annotated, occur on the positive strand at positions 442, 578, 710, 898, 1016, 1104, 1183, 1388, 1643, 1765, 1815 and 2235; and on the negative strand at 211, 292, 352, 808, 903, 1134, 1430, 1468, 1507, 1604 and 2062.

Other features:

Exons are shown in bold, and numbered (E1, E2 etc.) in the margin. The deduced amino acid sequence is shown below each exon, and numbered in bold at the right of the figure. Introns are also numbered (I1, I2 etc.) in the margin. The putative SAR noted in Results 2.2.6.1 is indicated with a dotted underline. Putative topoisomerase II consensus sites within that region are labelled *put*TOPII (Those not within this region occur at positions 119, 425, 488, 552, 580, 733, 1668, 2239). The oligonucleotide *oQPT2*, used as a probe in Results 2.2.4 is indicated by a thick underline.


```
GGATCCACATAAGAAGATATCAAGGCATTGCAATACTACAAAAGGTCAAGAATGAAGCTT 60
                                        PutWRKY/putbZIP3       (-2189)
GAAACTACAATTTACAATTATGGCTCATTTGCTCATTTTTGTGTTGATTTTAATTAGCAA 120
                                                               (-2129)
ATATAAATAGCTAAAGTGCAGGGTTTAGTTAGGAAGTTAGATTTTGATTAATGAGTTGAT 180
                                                               (-2069)
ACTCCTTTTGGTGTATTATGGTTATATCTTTTTATCTCTTTAATGAACCTAATTTGGACT 240
                                                               (-2009)
TCCCTTGTTACGGATCTAAATTGTGAGTTCAATCTCTTCCCTATTGGATTGATTATCCTT 300
                                                    putMYB3    (-1949)
TCTTTTCTTCCAATTTGTGTTTCTTTTTGCCTAATTTATTGTGTTATCCCCTTTATCCTA 360
                                          putMYB3  putMYB3     (-1889)
TTTTGTTTCTTTACTTATTTATTTGCTTCTATGTCTTTGTACAAAGATTTAAACTCTATG 420
                                                               (-1829)
GCACATATTTTAAAGTTGTTAGAAAATAAATTCTTTCAAGATTGATGAAAGAACTTTTTA 480
                                                               (-1769)
ATTGTAGATATTTCGTAGATTTTATTCTCTTACTACCAATATAACGCTTGAATTGACGAA 540
                                                putWRKY/putbZIP3 (-1709)
AATTTGTGTCCAAATATCTAGCAAAAAGGTATCCAATGAAAATATATCATATGTGATCTT 600
                                    putMYB3                    (-1649)
CAAATCTTGTGTCTTATGCAAGATTGATACTTTGTTCAATGGAAGAGATTGTGTGCATAT 660
                                                               (-1589)
TTTTAAAATTTTTATTAGTAATAAAGATTCTATATAGCTGTTATAGAGGGATAATTTTAC 720
                                          putMYB    putMYB3    (-1529)
AAAGAACACTATAAATATGATTGTTGTTGTTAGGGGTGTCAATGGTTCGGTTCGACTGGT 780
                                          PutWRKY              (-1469)
TATTTTATAAAATTTGTACCATACCATTTTTTCGGATATTCTATTTTGTATAACCAAAAT 840
                                    putMYB3                    (-1409)
TAGACTTTTCGAAATCGTCCCAATCATGTCGGTTTCACTTCGGTATCGGTACCGTTCGGT 900
                                       putAP2/E      putMYB    (-1349)
TAATTTTCATTTTTTTTTAAATGTCATTAAAATTCACTAGTAAAAATAGAATGCAATAAC 960
                                                               (-1289)
ATACGTTCTTTTATAGGACTTAGCAAAACTCTCTAGACATTTTTACTGTTTAAAGGATAA 1020
                                                    putMYB3    (-1229)
TGAATTAAAAAACATGAAAGATGGCTAGAGTATAGATACACAACTATTCGACAGCAACGT 1080
                                                               (-1169)
AAAAGAAACCAAGTAAAAGCAAAGAAAATATAAATCACACGAGTGGAAAGATATTAACCA 1140
                                 putTOPII/putAT putbZIP1       (-1109)
AGTTGGGATTCAAGAATAAAGTCTATATTAAATATTCAAAAAGATAAATTTAAATAATAT 1200
                                       putTOPII                (-1049)
GAAAGGAAACATATTCAATACATTGTAGTTTGCTACTCATAATCGCTAGAATACTTTGTG 1260
                                                               (-989)
CCTTGCTAATAAAGATACTTGAAATAGCTAAGTTTAAATATAAATAGCATAATAGATTTT 1320
                                          putTOPII/putAT       (-929)
AGGAATTAGTATTTTGAGTTTAATTACTTATTGACTTGTAACAGTTTTTATAATTCCAAG 1380
                                       putWRKY  putMYB         (-869)
GCCCAATGAAAAATTTAATGCTTTATTAGTTTTAAACTTACTATATAAATTTTTCATATG 1440
                                                               (-809)
TAAAATTTAATCGGTATAGTTCGATATTTTTTCAATTTATTTTTATAAAATAAAAAACTT 1500
                                    putTOPII   putAT   putAT   (-749)
ACCCTAATTATCGGTACAGTTATAGATTTATATTAAAAATCTACGGTTCTTCAGAAGAAAC 1560
                              putMYB      putMYB               (-689)
CTAAAAATCGGTTCGGTGCGGGACGGTTCGATCGGTTTAGTCGATTTTCAAATATTCATT 1620
                                                   putTOPII    (-629)
GACACTCCTAGTTGTTGTTATAGGTAAAAAGCAGTTACAGAGAGGTAAAATATAACTTAA 1680
putWRKY                              putMYB    putGT1         (-569)
AAAATCAGTTCTAAGGAAAAATTGACTTTTATAGTAAATGACTGTTATATAAGGATGTTG 1740
                 putGT1 putWRKY/putD          putMYB           (-509)
TTACAGAGAGGTATGAGTGTAGTTGGTAAATTATGTTCTTGACGGTGTATGTCACATATT 1800
                                       putWRKY/putbZIP3        (-449)
ATTTATTAAAACTAGAAAAAACAGCGTCAAAACTAGCAAAAATCCAACGGACAAAAAAAT 1860
                              putbZIP3/putWRKY      putMYB     (-389)
CGGCTGAATTTGATTTGGTTCCAACATTTAAAAAAGTTTCAGTGAGAAAGAATACGGTGA 1920
                                                               (-329)
CTGTTGATGATATAAACAAAGGGCACATTGGTCAATAACCATAAAAAATTATATGACAGC 1980
putMYB                               putWRKY putMYB1           (-269)
TACAGTTGGTAGCATGTGCTCAGCTATTGAACAAATCTAAAGAAGGTACATCTGTAACCG 2040
  putMYB                                              putMYB   (-209)
GAACAGCACTTAAATGACTAAATTACCCTCATCAGAAAGCAGATGGAGTGCTACAAATAA 2100
                                                               (-149)
```

Figure 2.2.8 (continued)

```
    CACACTATTCAACAACCATAAATAAAACGTGTTCAGCTACTAAAACAAATATAAATAAAT 2160
                                                                 (-89)
    CTATGTTTGTAAGCACTCCAGCCATGTTAATGGAGTGCTATTGCCTGTTAACTCTCACTT 2220
                                                  PutMYB         (-29)
    ATAAAATAGTAGTAGAAAAAATATGAACCAAAACACAACCAAAGAAAGCATTAAGCTCCC 2280
    putTATA                        *                           (+32)
    CAAAAACTATTTTCCACAAAATTCATTTCACAACCCCCCCAAAAAAAAACCATGTTTAGA_2340
E1                      oQPT2 probe                M F R   3
    GCTATTCCTTTCACTGCTACAGTGCATCCTTATGCAATTACAGCTCCAAGGTCTCTTTAA 2400
    A I P F T A T V H P Y A I T A P R                        20
I1  GAAGAAAAAAAGATTCTTGAAAATCATGTTTTTTTGGTTATATTCTGATAAAGATTGGGT 2460
     TTTTTCTGAGTTTGTGATTAAATTTTGTGTGTAAAATTGCAGGTTGGTGGTGAAAATGTC 2520
E2                                        L V V K M S   26
    AGCAATAGCCACCAAGAATACAAGAGTGGAGTCATTAGAGGTGAAACCACCAGCACACCC 2580
     A I A T K N T R V E S L E V K P P A H P                  46
    AACTTATGATTTAAAGGAAGTTATGAAACTTGCACTCTCTGAAGAT...           2626
     T Y D L K E V M K L A L S E D                            61
```

2.2.6.2 The coding sequence and introns of *NtxQPT2*

The coding region of *Ntw38QPT2* has been sequenced fully by the authors of the "Application for Deregulation" (referenced above). This DNA sequence data is not publicly available, however the amino acid sequence of *Ntw38QPT2* was given by Song (1997). Given the fact that *NtxQPT2* and *Ntw38QPT2* are likely to be almost identical (Given their 99.6% promoter identity), it was deemed unnecessary to fully sequence *NtxQPT2*. Rather, only the first two exons were sequenced (Figure 2.2.8), to provide enough DNA data to permit comparisons with other *QPRTase* sequences (Discussion 3.3).

The amino acid sequence of *Ntw38QPT2* (Song, 1997) is shown in Figure 2.2.9, alongside the deduced NtxQPT1 protein, deduced QPRTase sequence from *N. tabacum* cv. Bright Yellow (Imanishi *et al.*, 1998), and the deduced protein from the previously reported cDNAs from *N. tabacum* cv. SC58, and *N. rustica* cv. V12 (Sinclair *et al.*, 2000). Ntw38QPT2 is highly similar to all of these sequences except NtxQPT1, from which it differs to a larger degree. It is notable that NtxQPT1 has an amino acid missing in comparison to all other *Nicotiana* QPRTases (position 33), in a region encoded by exon 2 (also apparent as a 3bp difference when compared to Arabidopsis *QPRTase*, Figure 2.2.6). The fact that the *NtxQPT1* gene remains in frame despite this deletion may offer support to the hypothesis that it encodes a functional enzyme.

Figure 2.2.9 Comparison of the deduced amino acid sequences from various *N. tabacum* *QPRTase* genes.

The shading shows those residues which do not conform to the consensus sequence. The underlined residues are referred to in the text below (Results 2.2.6.2) The sequences were sourced as follows:

N.tab (RD2) (*ie*; *NtxQPT2*)	Song, 1997.
N.tab (SC58)	TQPT1, Sinclair *et al.*, 2000, Accession AJ243437
N.tab (BY)	Accession AB038494
N.tab (*NtxQPT1*)	This study
N. rustica	RQPT1, Sinclair *et al.*, 2000, Accession AJ 243436

```
N.tab (RD2) (NtxQPT2)   MFRAIPFTATVHPYAITAPRLVVKMSAIATKNTRVESLEVKPPAHPTYDLKEVMKLALSE
N.tab (SC58)            MFRALPFTATVHPYAITAPRLVVKMSAIATKNTRVESLEVKPPAHPTYDLKGVMQLALSE
N.tab (BY)              MFRAIPFTATVHPYAITAPRLVVKMSAIATKNTRVESLEVKPPAHPTYDLKEVMKLALSE
N.tab (NtxQPT1)         MFKVFPFTAIVHPHAITAPRLVVKMSAIATKN-AVESLVVKPPAHPTYDLKGVIQLALSE
N.rustica               MFRALPFTATVHPYAITAPRLVVKMSAIATKNTRVESLEVKPPAHPTYDLKEVMQLALSE

N.tab (RD2) (NtxQPT2)   DAGNLGDVTCKATIPLDMESDAHFLAKEDGIIAGIALAEMIFAEVDPSLKVEWYVNDGDK
N.tab (SC58)            DAGNLGDVTCKATIPVDMESDAHFLAKEDGIIAGIALAEMIFAEVDPSLKVEWYVNDGDK
N.tab (BY)              DAGNLGDVTCKATIPLDMESDAHFLAKEDGIIAGIALAEMIFAEVDPSLKVEWYVNDGDK
N.tab (NtxQPT1)         DAGDLGDVTCKATIPIDMESEAHFLAKEDGIVAGIALAEMIFAEVDPSLKVEWYVNDGDK
N.rustica               DAGNLGDVTCKATIPLDMESDAHFLAKEDGIIAGIALAEMIFAEVDPSLKVEWYVNDGDK

N.tab (RD2) (NtxQPT2)   VHKGLKFGKVQGNAYNIVIAERVVLNFMQRMSGIATLTKEMADAAHPAYILETRKTAPGL
N.tab (SC58)            VHKGLKFGKVQGNAYNIVIAERVVLNFMQRMSGIATLTKEMADAAHPAYILETRKTAPGL
N.tab (BY)              VHKGLKFGKVQGNAYNIVIAERVVLNFMQRMSGIATLTKEMADAAHPAYILETRKTAPGL
N.tab (NtxQPT1)         VHKGLKFGKVQGKAHSIVIAERVVLNFMQRMSGIATLTKAMADAAHPATILETRKTAPGL
N.rustica               VHKGLKFGKVQGNAYNIVIAERVVLNFMQRMSVIATLTKEMADAAHPAYILETRKTAPGL

N.tab (RD2) (NtxQPT2)   RLVDKWAVLIGGGKNHRMGLFDMVMIKDNHISAAGGVGKALKSVDQYLEQNKLQIGVEVE
N.tab (SC58)            RLVDKWAVLIGGGKNHRMGLFDMVMIKDNHISAAGGVGKALKSVDQYLEQNKLQIGVEVE
N.tab (BY)              RLVDKWAVLIGGGKNHRMGLFDMVMIKDNHISAAGGVGKALKSVDQYLEQNKLQIGVEVE
N.tab (NtxQPT1)         RLVDKWAVLIGGGKNHRMGLFDMVMIKDNHISAAGGVSKALNSVDHYLEQNKLQMGVEVE
N.rustica               RLVDKWAVLIGGGKNHRMGLFDMVMIKDNHISAAGGVGKALKSVDQYLEQNKLQIGVEVE

N.tab (RD2) (NtxQPT2)   TRTIEEVREVLDYASQTKTSLTRIMLDNMVVPLSNGDIDVSMLKEAVELINGRFDTEASG
N.tab (SC58)            TRTIAEVREVLEYASQTKTSLTRIMLDNMVVPLSNGDIDVSMLKEAVELINGRFDTEASG
N.tab (BY)              TRTIEEVREVLDYASQTKTSLTRIMLDNMVVPLSNGDIDVSMLKEAVELINGRFDTEASG
N.tab (NtxQPT1)         TRTIAEVREVLDYASQTKTSLTRIMLDNMVVPLSNGDIDVSMLKEAVELINGRFDTEASG
N.rustica               TRTIEEVREVLEYASQTKTSLTRIMLDNMVVPLSNGDIDVSMLKEAVELINGRFDTEASG

N.tab (RD2) (NtxQPT2)   NVTLETVHKIGQTGVTYISSGALTHSVKALDISLKIDTELALEVGRRTKRA
N.tab (SC58)            NVTLETVHKIGQTGVTYISSGALTQSVKALDISLKIDTELALEVGRRTKQA
N.tab (BY)              NVTLETVHKIGQTGVTYISSGALTHSVKALDISLKIDTELALEDGRRTKRA
N.tab (NtxQPT1)         NVTLETVHKIGQTGVTYISSGALTHSVKALDISLKIDTELALEVGRRTKRA
N.rustica               NVTLETVHEIGQTGVTYISSGALTHSVKALDISLKIDTELALEVGRRTKRA
```

Visual inspection of the QPRTase proteins in Figure 2.2.9, along with known ODC and PMT sequences (*eg*; Hibi *et al.*, 1994; Imanishi *et al.*, 1998) reveals that each carries a motif similar to "VLIIGGG" (underlined in figure 2.2.9). 'A622' (Hibi *et al.*, 1994) apparently lacks such a motif. A search of the SWISSPROT database using the sequence "SKMT**VLIIGGG**NHRTLFDTASAAVRSALEQHF" (a random composite of ODC, PMT and QPRTase sequence surrounding the VLIIGGG motif)

returns 11 ODC matches, 3 QPRTase matches and 2 PMT matches among the top 65 results, out of a total database of 846,659 sequences. The presence of a similar motif in unrelated proteins undertaking different reactions, but whose genes are co-regulated is potentially interesting, and may warrant further analysis.

It was noted above (Results 2.2.5.1) that the first intron of *NtxQPT1* displayed an obvious repeat region. The equivalent intron in *NtxQPT2* does not contain this repeat, and is thus more like the genes from *N. glauca*, *N. alata* and *Arabidopsis* in this regard.

2.2.7 A preliminary study to isolate an *N. alata* promoter

Nicotiana alata may be a useful model species in future studies, to elucidate the molecular differences between species with contrasting alkaloid profiles (Introduction 1.2.4), and hence the promoters of its alkaloid biosynthetic genes are of interest.

Nine plaques hybridising to *RQPT1* were isolated before the commencement of the present study (Sinclair, 1998) from an *N. alata* genomic library (prepared by Royo *et al.*, 1996). Johnson (2000) continued this work in this laboratory, showing that these plaques represented two independent gene copies, and attempting (unsuccessfully) to isolate their promoters. From one plaque only, *Hind*III-*Eco*RI insert fragments were successfully cloned into pBluescript, but promoter sequence was not isolated, since a *Hind*III site separated it from all sequence homologous to the probes then available (Johnson, 2000).

Once *NtxQPT1* and *NtxQPT2* were recovered in the present study (Results 2.2.2), 200 *Hind*III-*Eco*RI ligation colonies available from the *N. alata* genomic plaque (Johnson, 2000) were re-screened using a mixed probe consisting of both these promoter sequences. Three positives were recovered, all of which contained the same sized insert, of 589bp. Since all the colonies screened represented ligation products derived from a single library plaque, and hence a single *QPRTase* gene copy, it was expected that the 589bp promoter fragment would lie adjacent to the *QPRTase* fragment already isolated by Johnson (2000). To verify this, a PCR reaction was performed on the original, uncut phage DNA, across the *Hind*III boundary of the two fragments, and this fragment was sequenced to confirm that the sequences were contiguous.

The *N. alata* 5' flanking sequence is 95% identical to *NtxQPT2* for about 380bp from the ATG codon, suggesting that this *N. alata* gene copy is an *NtxQPT2*-like gene. Upstream from this region, however, the homology ends abruptly, and the 209bp sequence further upstream shares only 40% identity with *NtxQPT2* (Figure 2.2.10). As would be predicted, the *N. alata* gene isolated showed no homology to *NtxQPT1* outside of the coding region.

Figure 2.2.10 Comparison between the 5' flanking regions of *NtxQPT2* (top) and an *N. alata NtxQPT2*-like *QPRTase* gene (bottom). *NtxQPT2* is numbered as in Figure 2.2.8, the first exon is shown in bold in both sequences.

```
ACTTAAAAAATCAGTTCTAAGGAAAAATTGACTTTTATAGTAAATGACTGTTATATAAGG (1095)
           | ||||        |||||  ||   ||   ||       | |||||
...........GAATTCTCTTCCCTTATTGAGTTAGCTAAATAA.....AATCTATAATT 44

ATGTTGTTACAGAGAGGTATGAGTGTAGTTGGTAAATTATGTTCTTGACGGTGTATGTCA (1155)
||  | ||| | |   |||    |    |   |    | | |   | |      || |
ATCATTTTAAAAACTTGTACTTCTTCTTTAACTTTTCTTTTTAACTCAAACATAATTTTT 104

CATATTATTTATTAAAACTAGAAAAAACAGCGTCAAAACTAGCAAAAATCCAACGGACAA (1215)
 | || || | |  | | |  |||||      ||||  ||  |   ||  |    |
AAGATAATATCTATATAATTTTAAAAA.......AAAAATATTATGTATTTAGATAAGGT 157

AAAAATCGGCTGAATTTGATTTGGTTCCAACATTTAAAAAAGTTTCAGTGAGAAAGAATA (1275)
| |         |   |    |  | |  ||||||||||| || || ||||||||||
ACACGCGCAAAGCGCGTACCCTAAGACTAGTATTTAAAAAAGGTTGAGCGAGAAAGAATA 217

CGGTGACTGTTGATGATATAAACAAAGGGCACATTGGTCAATAACCATAAAAAATTATAT (1335)
 ||||||| || ||||||| ||||||||||||||||||||||||||||||||||||||||
TGGTGACTATTTATGATATCAACAAAGGGCACATTGGTCAATAACCATAAAAAATTATAT 277

GACAGCTACAGTTGGTAGCATGTGCTCAGCTATTGAACAAATCTAAAGAAGGTACATCTG (1395)
|||||||||||||||||||||||||||||||| |||||||||||||||||||||||||
GACAGCTACAGTTGGTAGCATGTGCTCAGCTATTAAACAAATCTAAAGAAGGTACATCTG 337

T.AACCGGAACAGCACTTAAATGACTAAATTACCCTCATCAG.AAAGCAGATGGAGTGCT (1454)
| || | ||||||||||||||||||||||||||||||||| | ||||||||||||||||||
TAAATCAGAACAGCACTTAAATGACTAAATTACCCTCATCGGCAAAGCAGATGGAGTGCT 397

ACAAATAACACACTATTCAACAACCATAAATAAAACGTGTTCAGCTACTAAAACAAATAT (1514)
|||||||| ||| |  | ||||||||||||||||||||||||||||||||||||||||||
ACAAATAATACATTGATAAACAACCATAAATAAAACGTGTTCAGCTACTAAAACAAATAT 457

AAATAAATCTATGTTTGTAAGCACTCCAGCCATGTTAATGGAGTGCTATTGCCTGTTAAC (1574)
|||||||||||| |||||||||||||||||| ||||||||||||||||||||| ||||||
AAATAAATCTATATTTGTAAGCACTCCAGCCCTGTTAATGGAGTGCTATTGCCAGTTAAC 517

TCTCACTTATAAAATAGT......AGTAGAAAAAATATGAACCAAAACACAACCAAAGAA (1628)
||||||||||||||||      ||||||||||||||||||||||||||||||||||||
TCTCACTTATAAAATAGTAGTAGAAGTAGAAAAAATATGAACCAAAACACAACCAAAGAA 577

AGCATTAAGCTCCCCAAAAACTATTTTCCACAAAATTCATTTCACAACCCCCCCAAAAAA (1688)
| |||||||||| | ||||||||||||||||||||| |||||||| |||||  |||
ACCATTAAGCTTCTCAAAAACTATTTTCCACAAAAATCATTTCAC.ACCCCCAAAAATTT 636

AAACCATGTTTAGAGCTATTCCTTTCACTGCTACAGTGCA    NtxQPT2    (1728)
|||  ||||||||||||  ||||||||||||||  ||||||||
AAATCATGTTTAGAGCAATTCCTTTCACTGCAACAGTGCA    N. alata   676
```

This abrupt end in homology is of unknown significance, but is of possible interest since it may indicate a past deletion or insertion event. Future experiments could investigate whether this promoter difference influences *QPRTase* gene expression in each of these species.

2.3 Differential patterns of gene expression in *Nicotiana* species with contrasting alkaloid profiles

2.3.1 Background

In the previous section (Results 2.2), the arrangement and expression of *QPRTase* genes within *N. tabacum* was investigated. The present section takes a broader view, in assessing the expression of a set of genes in different *Nicotiana* species with contrasting alkaloid profiles.

The genus *Nicotiana* is well known to contain species with a range of alkaloid phenotypes (Smith and Abashian, 1963; Saitoh *et al.*, 1985; Sisson and Severson, 1990), however there is a paucity of data relating to the underlying molecular differences which influence these profiles. One of the aims of the work presented below was to assess whether the differential expression of a shared set of biosynthesis genes was correlated to the alkaloid phenotype of different *Nicotiana* species.

The alkaloid content, along with *QPRTase*, *PMT*, *ODC* and '*A622*' gene expression, has been measured in the roots and leaves of wounded and control *Nicotiana sylvestris*, *N. glauca*, and *N. alata* plants, using Northern and HPLC analysis. The results of a preliminary study on *N. Hesperis* are also presented. For each species, leaf and root tissue has been harvested from hydroponically grown plants, and the same individual samples have been used for all analyses.

If transcriptional regulation appears to be a determinant of different alkaloid contents in different *Nicotiana* species, as it is in other genera (Rhodes, 1994; vom Endt *et al.*, 2002), future studies may aim to elucidate the molecular basis for these transcriptional differences. Such information will contribute to our knowledge of how different species have evolved different defence strategies using common biosynthetic pathways.

2.3.2 Patterns of gene expression and alkaloid accumulation following wounding in *N. sylvestris*

All of the data presented below from *N. sylvestris* are consistent or easily reconcilable with published reports, and confirm that the chosen experimental conditions enable *N. sylvestris* to be used a "reference point" when assessing the characteristics of the other species examined.

Gene expression in N. sylvestris *leaf tissue:*

In both control and wounded *N. sylvestris* leaves, Northern analysis showed that *PMT* and'*A622*' transcript levels were very low or un-detectable (Figure 2.3.1), consistent with previous studies which have shown these transcripts to be root-specific in *N. sylvestris* and *N. tabacum* (Hibi *et al.*, 1994; Shoji, 2000b). *ODC* and *QPRTase* transcripts were detectable only at low levels in all samples, but were not wound-induced at the timepoints examined, consistent with their known role in the primary metabolism of the leaf.

Gene expression in N. sylvestris *root tissue:*

In contrast to the leaves, *QPRTase*, *PMT*, *ODC*, and '*A622*' transcripts were readily detectable in RNA samples extracted from root tissues of all plants, and all were up-regulated (4.1, 4.8, 3.8 and 6.6 fold respectively) in wounded plants 24h after foliage damage (Figure 2.3.2). The levels of all transcripts had returned almost to the levels seen in roots of unwounded plants by ~72h after wounding.

N. sylvestris leaf tissue:

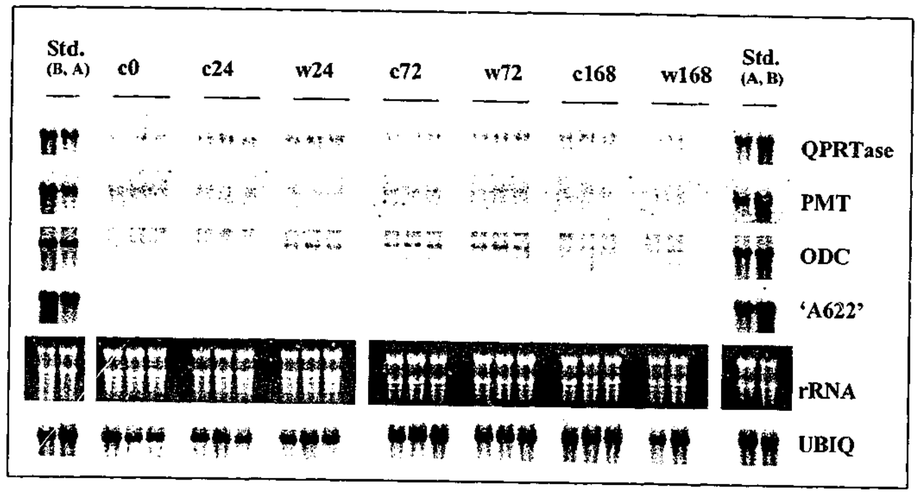
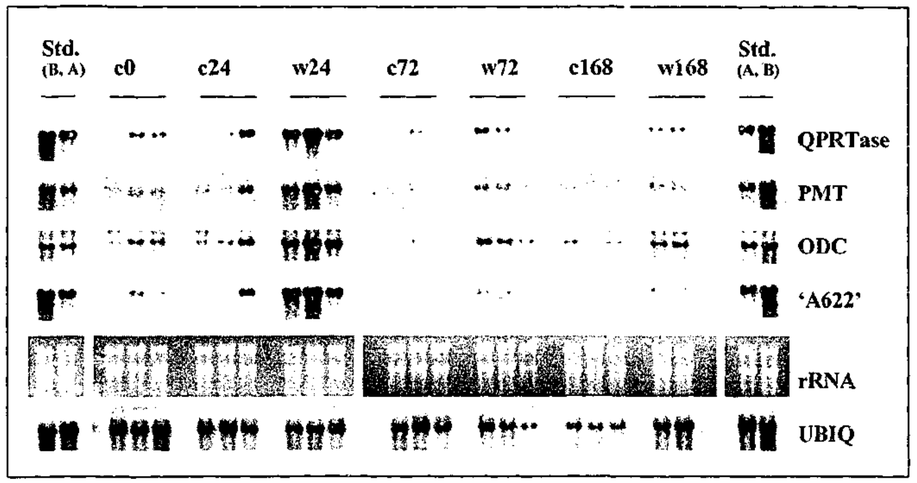


Figure 2.3.1: Alkaloid biosynthetic gene expression in *N. sylvestris* leaf tissue.

Northern blot analysis showing *QPRTase*, *PMT*, *ODC* and '*A622*' transcript levels in the upper leaves of un-wounded control (c) and foliage-wounded (w) *N. sylvestris* plants 0h, 24h, 72h and 168h (1 week) after wounding. The gel photograph (rRNA) is included to demonstrate that the lanes were evenly loaded and the RNA was not degraded. Loading levels have also been standardised against the signal obtained from a ubiquitin (UBIQ) probe, which hybridises to a band that is not influenced by wounding (Lidgett *et al.*, 1995). The analysis was carried out on two gels (the first including 0h and 24h samples), each of which includes standard RNA samples (Std.) taken from the roots of unwounded (A) and wounded (B) *N. sylvestris* plants. These lanes demonstrate that each blot has been successfully probed, and facilitate comparisons between blots for each each gene, as described in Materials and Methods. Relative transcript levels for each gene are shown on the histograms, which show the average of three samples (±1SE). Their values are standardised against ubiquitin and expressed relative to the transcript level in standard A (value 1; shown with a dotted line).

N. sylvestris root tissue:



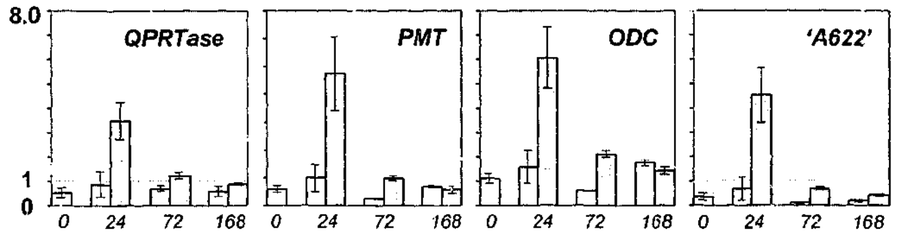


Figure 2.3.2: Alkaloid biosynthetic gene expression in *N. sylvestris* root tissue.

Northern blot analysis showing *QPRTase*, *PMT*, *ODC* and *'A622'* transcript levels in the roots of un-wounded control (c) and foliage-wounded (w) *N. sylvestris* plants 0h, 24h, 72h and 168h (1 week) after wounding. For further information, see the legend to Figure 2.3.1.

Alkaloid levels in N. sylvestris *leaf tissue:*

In all *N. sylvestris* leaf samples, regardless of treatment, nicotine accounted for 96-99.5% of the total alkaloid content detected using HPLC analysis. No nornicotine was detected in any leaf tissue, the remainder being composed of low levels of anabasine (<0.01mg/g dry weight (dw)) and anatabine (<~0.005mg/g dw). Nicotine levels were substantially increased in the wounded leaves of damaged *N. sylvestris* plants one week after wounding, with an average of 1.9 mg/g dw being observed in the wounded leaves, in contrast to the 0.8 mg/g dw average found in the un-wounded leaves (Figure 2.3.3).

The relative content of different alkaloids reported here differs from that reported in the literature for *N. sylvestris*, where nornicotine accounts for a substantial proportion of total leaf alkaloid (Smith and Abashian, 1963; Saitoh *et al.*, 1985; Sisson and Severson, 1990). These discrepancies are probably due to the fact that previous studies used leaf tissue from flowering plants, unlike the leaves from young plants which were used here. *Nicotiana sylvestris* leaves are known to convert nicotine into nornicotine at late developmental or senescence stages (Wernsmann and Matzinger, 1968). Similarly, the total quantity of nicotine in leaf tissue was less than that reported in other studies, which have detected nicotine levels up to 14 fold higher than those reported here (Saitoh *et al.*, 1985; Baldwin, 1989). This may also be related to the age of the plants and/or the growth conditions (Yoshida, 1962; Waller and Nowacki, 1978).

The published data relating to the alkaloid-induction capabilities of *N. sylvestris* are variable (1.5-5 fold), and the results from the present study fall well within the range previously reported (Baldwin, 1988a, 1988b, 1989; Baldwin and Ohnmeiss, 1994; Baldwin and Schmelz, 1994).

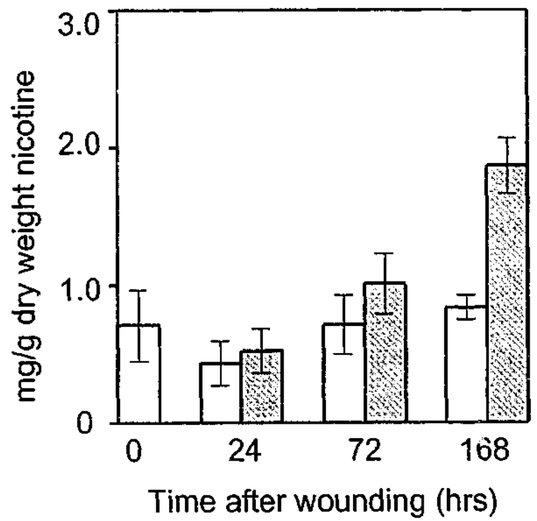


Figure 2.3.3 Nicotine accumulation is responsive to wounding in *N. sylvestris* leaves.

Nicotine levels in the upper leaves of wounded and non-wounded control *N. sylvestris* plants at 0h, 24h, 72h and 168h (1 week) after wounding, determined using HPLC analysis. The bars represent means (±1SE) of three samples, taken from the same plants as those analysed in figures 2.3.1 and 2.3.2, with the exception of the 168h samples, where only two plants were available for testing.

Alkaloid accumulation in N. sylvestris *root tissue:*

HPLC analysis showed that the roots of *N. sylvestris* contained on average ~1.2 mg/g dw of alkaloids, of which ~85% was nicotine, ~10% nornicotine, ~5% anabasine, and only a trace of anatabine. This profile is extremely similar to the only other analysis of *N. sylvestris* root tissue available in the literature (Saitoh *et al.*, 1985). No increase in nicotine levels in the roots was observed in response to foliage damage, however it is noteworthy that nicotine levels in roots did fluctuate up to ~3 fold between individual plants, which may have masked a very small wound induction. There was no obvious relationship between nicotine levels in the leaf and root of individual plants, regardless of whether wounding was considered (data not shown).

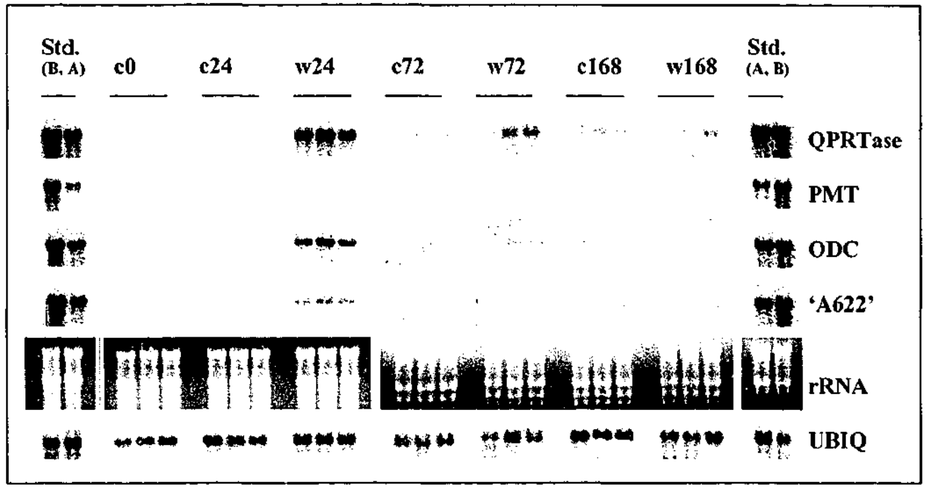
2.3.3 Patterns of gene expression and alkaloid accumulation following wounding in *N. glauca*

Gene expression in N. glauca *leaf tissue:*

As noted above, preliminary studies suggested that *QPRTase* was up-regulated in wounded leaves of *N. glauca* (Sinclair *et al.*, 2000). The present experiments investigate the magnitude of this change, and whether it was accompanied by induction in any of the other key biosynthetic genes involved in alkaloid synthesis. In addition, undamaged leaves were analysed from all plants, in order to determine whether the *QPRTase* transcriptional wound-response was foliage wide, or confined to the wounded tissue itself.

In the upper leaves of *N. glauca*, *QPRTase*, *ODC* and '*A622*' transcripts were present in low abundance in all control samples, but were markedly up-regulated in wounded leaves harvested 24h after foliage damage (7.5, 12.1 and 5.9 fold respectively) (Figure 2.3.4). Transcript levels had receded to the control levels by 72 hours after treatment. These results differ from those obtained from *N. sylvestris*, where none of the genes showed evidence of wound-induction. In *N. glauca* leaves, *PMT* transcripts were not detectable in any samples, consistent with previous preliminary observations (Sinclair *et al.*, 2000).

N. glauca upper leaf tissue:



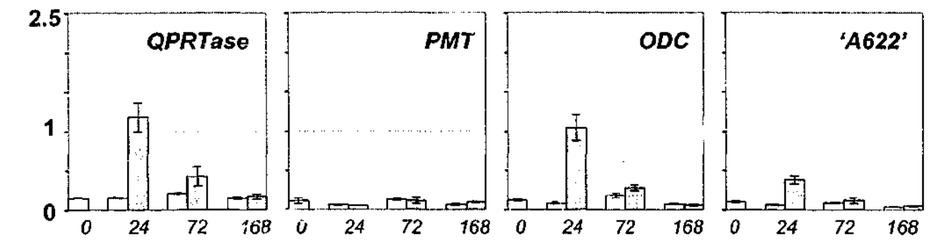


Figure 2.3.4: Alkaloid biosynthetic gene expression in *N. glauca* upper leaf tissue.

Northern blot analysis showing *QPRTase*, *PMT*, *ODC* and *'A622'* transcript levels in the upper leaves of un-wounded control (c) and upper-foliage-wounded (w) *N. glauca* plants 0h, 24h, 72h and 168h (1 week) after wounding. For further information, see the legend to Figure 2.3.1. The scale on the histrograms presented here has been expanded in comparison to the histograms presented in Figures 2.3.1 and 2.3.2. Thus, although *QPRTase*, *ODC* and *'A622'* transcripts are strongly induced by wounding, the absolute levels of all transcripts remain low in comparison to the *N. sylvestris* samples (value 1 represents unwounded *N. sylvestris* Std A signal).

In the lower unwounded leaves, *PMT*, *ODC* and *'A622'* transcript levels were barely detectable using Northern analysis, and were not induced by the wounding of upper leaves. *QPRTase* transcript levels were detectable in the lower, unwounded leaves, and showed a very slight (~2 fold) induction 24h after upper foliage damage (Figure 2.3.5).

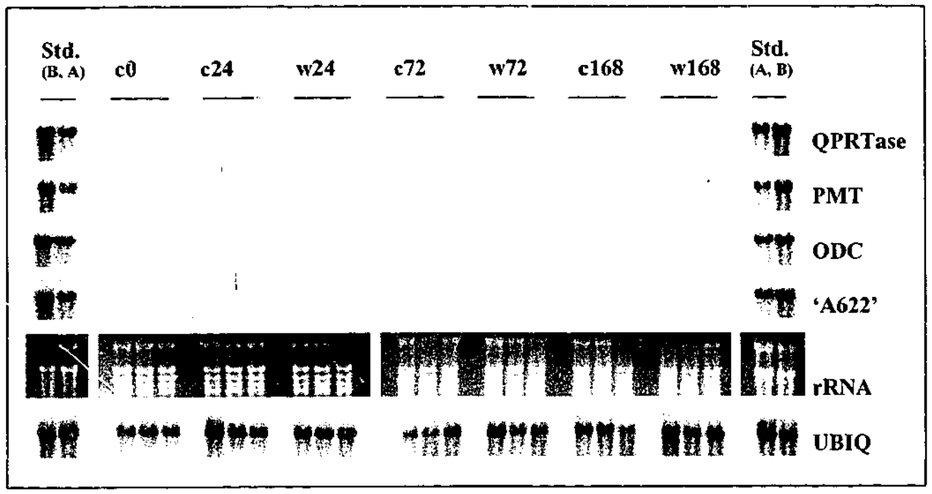
Gene expression in N. glauca *root tissue:*

In the roots of *N. glauca*, transcripts of *QPRTase*, *PMT*, *ODC* and *'A622'* were present at low levels, and again in contrast to *N. sylvestris*, none of the genes were induced by foliage damage (Figure 2.3.5). The levels of all these transcripts were slightly higher in the 0h controls than in any other tissue. This could suggest that the wounded plants had released a signal, and that all of the plants, both wounded and un-wounded, subsequently downregulated their genes to a similar level. This seems highly unlikely, and it is more sensible to suggest that an external factor altered the gene expression in all plants, independent of wounding. This may have been related to the light-, temperature-, or nutrient-environment of the plants. The latter appears particularly plausible, since the addition of new nutrient solution was stopped just prior to wounding, so that plants were not given a "burst" of fertiliser during the experiment. Nutrient levels are known to influence alkaloid content (Baldwin and Ohnmeiss, 1994; Ryan, 2001), and it is possible that the fall in nutrient availability over the course of the experiment caused the observed decrease in transcript levels for all the genes analysed.

Alkaloid accumulation in N. glauca *leaf tissue:*

The upper leaves of *N. glauca* contained predominantly anabasine. Anatabine and nornicotine were present as traces in most samples, whilst no trace of nicotine was observed in any leaf sample. The resting level of anabasine, in the control plants, was approximately 4 mg/g dw. One week after wounding, the wounded leaves contained on average 8.4 mg/g dw- 2 fold more than in the controls (Figure 2.3.6).

N. glauca lower leaf tissue:



N. glauca root tissue:

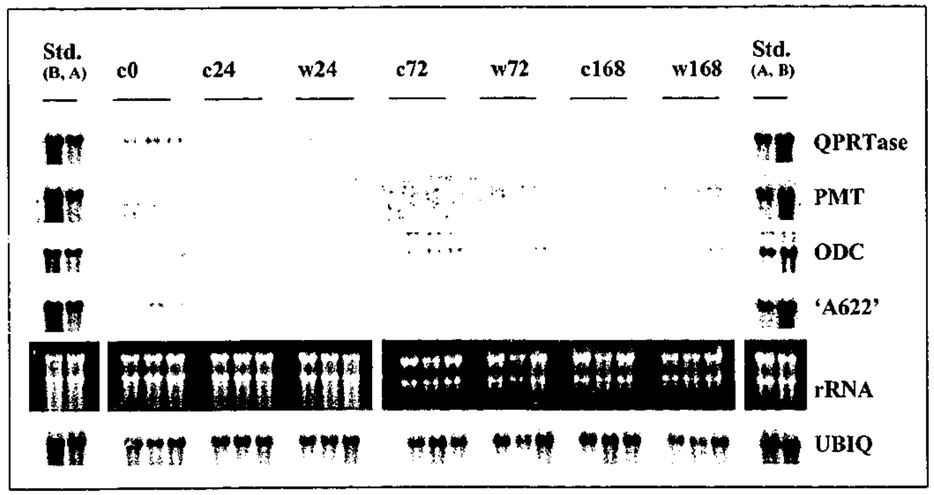


Figure 2.3.5: Alkaloid biosynthetic gene expression in *N. glauca* lower leaf and root tissue.

Northern blot analysis showing *QPRTase*, *PMT*, *ODC* and '*A622*' transcript levels in the roots and lower leaves of un-wounded control (c) and upper-foliage-wounded (w) *N. glauca* plants 0h, 24h, 72h and 168h (1 week) after wounding. Although histograms were presented in Figures 2.3.1, 2.3.2 and 2.3.4 to show relative transcript levels, they have not been included in the present case, since neither root nor lower leaf tissue showed appreciable transcript levels. For further information, see the legend to Figure 2.3.1.

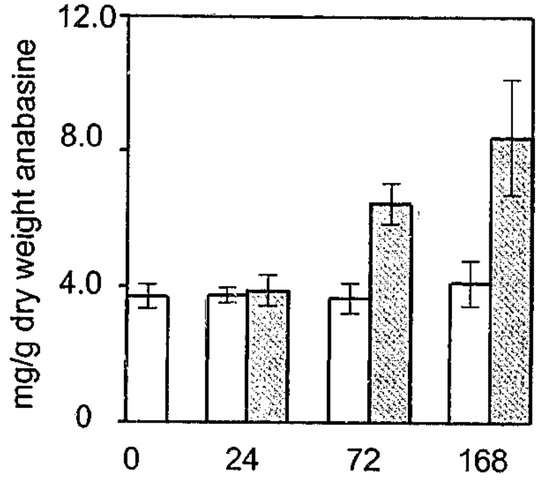


Figure 2.3.6 Anabasine accumulation is responsive to wounding in *N. glauca* upper leaves.

Anabasine levels in the upper leaves of wounded and non-wounded control *N. glauca* plants at 0h, 24h, 72h and 168h (1 week) after wounding, determined using HPLC analysis. The bars represent means (±1SE) of three samples, taken from the same plants as those analysed in figures 2.3.4 and 2.3.5.

The lower unwounded leaves also contained predominantly anabasine, although generally at lower levels than the upper leaves (1-3 mg/g dw). No increase in anabasine levels was observed in these lower leaves following the wounding of the upper leaves. This result, taken together with the results of Northern analysis on the lower leaves, is consistent with the hypothesis that in *N. glauca*, there is no movement of a wound signal, nor of anabasine, from wounded foliage, to foliage further down the stem.

The results presented here accord well with previously published accounts of *N. glauca* leaves, both in terms of alkaloid levels and alkaloid ratios (Saitoh *et al.*, 1985; Sisson and Severson, 1990). A number of unidentified peaks in the HPLC trace were present in *N. glauca* foliage, with one peak with an average retention time of 5.70 minutes (±0.028SE) being prominent. These peaks were larger in the upper leaves than the lower, but were not correlated to anabasine levels nor responsive to wounding, and were not investigated further.

Alkaloid accumulation in N. glauca *root tissue:*

The roots of *N. glauca* assayed here were found to have a very low alkaloid content. Nornicotine was always absent, anatabine was occasionally present as a trace, while anabasine and nicotine were both present at levels less than 0.05 mg/g dw. No stimulation was evident for any alkaloid in *N. glauca* roots following the wounding of aerial tissues. These results differ markedly from results published by Saitoh and co-workers (1985) who found that the roots of soil-grown *N. glauca* plants produced larger amounts of alkaloids (5.25 mg/g dw). This difference is likely to be due to differences in culture conditions or the age of the plants harvested.

The data presented above are informative when considered together with previous data showing that unwounded leaves above wounded leaves on the stem of *N. glauca* plants accumulated increased anabasine levels after wounding (Baldwin and Ohnmeiss, 1993). Together, the data would seem to suggest that in *N. glauca*, wound-induced anabasine is not relocated from the roots to the foliage in large quantity, in contrast to the relocation of nicotine in *N. sylvestris*. Instead, the data are consistent with the hypothesis that the wound response is largely facilitated by the foliage. It would seem that this induced anabasine is not transported to leaves below the site of wounding. It remains unknown whether the upper leaves which

accumulate increased anabasine levels after wounding (Baldwin and Ohnmiess, 1993) receive the alkaloid after biosynthesis, or whether a signal transduction cascade permits these upper leaves to increase their own anabasine biosynthesis.

2.3.4 Patterns of gene expression and alkaloid accumulation following wounding in *N. alata*

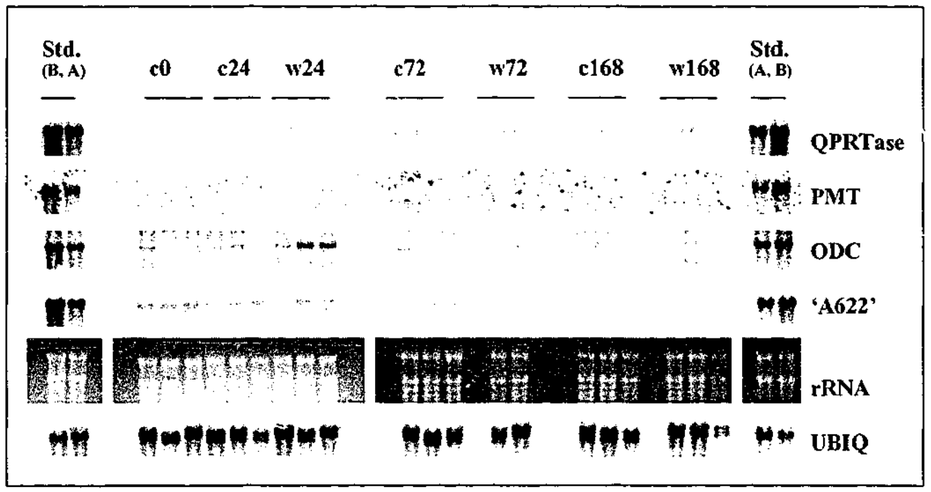
Gene expression in N. alata *leaf tissue:*

In the unwounded leaves of *N. alata*, *QPRTase*, *ODC* and *'A622'* transcripts were detected at very low levels, whilst *PMT* transcripts were un-detectable (Figure 2.3.7). Wounding did not lead to elevated transcript levels of *QPRTase* or '*A622*' in leaf tissue, however *ODC* transcripts were slightly (less than 2 fold) more abundant in wounded than control leaves 24h after treatment. In terms of alkaloid biosynthetic gene expression and wound-response, therefore, the leaves of *N. alata* were found to be similar to *N. sylvestris* leaves but in marked contrast to those of *N. glauca*.

Gene expression in N. alata *root tissue:*

Unlike leaves, the analysis of *N. alata* roots yielded data more similar to those obtained from the roots of *N. glauca*, than to that obtained from *N. sylvestris* (Figure 2.3.7). Transcripts for *PMT* and '*A622*' were barely detectable. *QPRTase* and *ODC* transcripts were detectable at relatively low levels. In contrast to *N. sylvestris*, none of the genes tested showed evidence of transcriptional up-regulation in roots following foliage damage. Although none of the genes were induced by wounding, their resting levels in some plants displayed higher transcript levels than others. For example, the middle sample in the w72h lanes on Figure 2.3.7 displays relatively high *QPRTase* and '*A622*' transcript levels, but low *ODC* and *PMT* levels, raising the possibility that *QPRTase* and '*A622*' are co-regulated in *N. alata*, in a manner which does not involve the other genes.

N. alata leaf tissue:



N. alata root tissue:

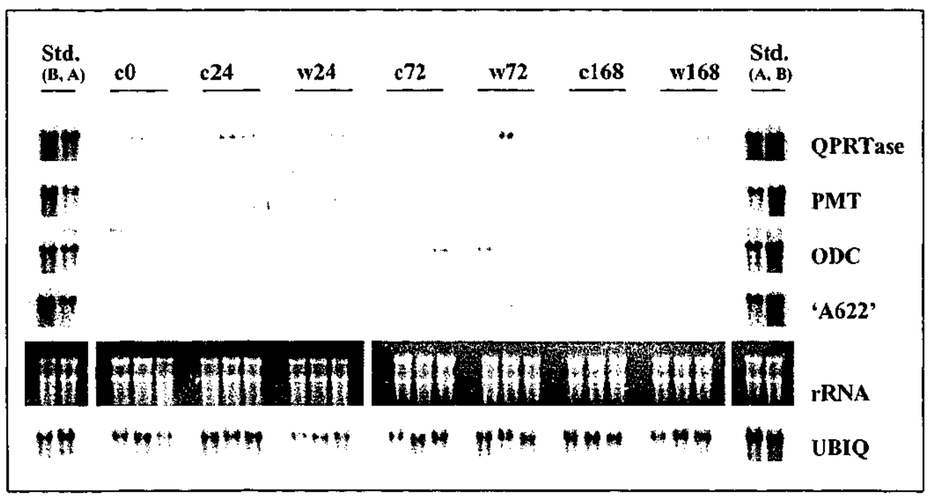


Figure 2.3.7: Alkaloid biosynthetic gene expression in *N. alata* leaf and root tissue.

Northern blot analysis showing *QPRTase*, *PMT*, *ODC* and *'A622'* transcript levels in the roots and leaves of un-wounded control (c) and foliage-wounded (w) *N. alata* plants 0h, 24h, 72h and 168h (1 week) after wounding. Although histograms were presented in Figures 2.3.1, 2.3.2, and 2.3.4 to show relative transcript levels, they have not been included in the present case, since neither root nor leaf tissue showed substantial wound-induced changes in the levels of any transcript measured. The only exception was ODC, which was induced by a relatively small 1.9 fold in wounded leaf tissue, from a level 0.29 relative to standard A, to a level of 0.56. For further information, see the legend to Figure 2.3.1.

Alkaloid accumulation in N. alata *leaf tissue:*

Consistent with the molecular data showing no induction of biosynthetic gene expression in roots or leaves, wounding had no detectable effect on alkaloid levels in the leaves of *N. alata*, even up to 7 days following wounding. Very low alkaloid levels were detected in the leaves of *N. alata*. Nicotine, nornicotine and anatabine were either totally absent, or present at levels less than 0.02mg/g dw.

Anabasine was present in all samples, but rarely exceeded 0.05 mg/g dw. These data are consistent with previous reports which also recorded low leaf alkaloid levels in this species (Waller and Nowacki, 1978; Saitoh *et al.*, 1985; Sisson and Severson, 1990), although the data presented here show, for the first time, that these low levels are not increased after wounding.

Alkaloid accumulation in N. alata *root tissue:*

In the roots of un-wounded *N. alata* plants, higher alkaloid levels were found than in leaves, in general agreement with other reports (Saitoh *et al.*, 1985; Friesen *et al.*, 1992). Nornicotine was present at 1-1.5 mg/g dw, and anatabine at levels up to ~0.4 mg/g dw. Consistent with a lack of gene induction, levels of these alkaloids did not increase in the roots of *N. alata* plants when tissues were analysed at 24, 72 or 168h after foliar damage.

N. hesperis tissue:

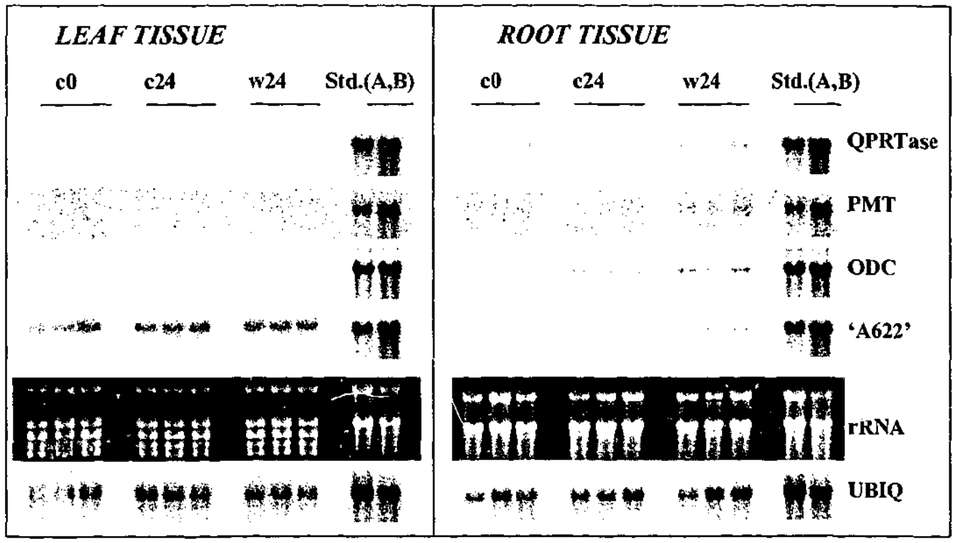


Figure 2.3.8: Alkaloid biosynthetic gene expression in *N. hesperis* roots and leaves.

Northern blot analysis showing *QPRTase*, *PMT*, *ODC* and '*A622*' transcript levels in the leaves and roots of un-wounded control (c) and foliage-wounded (w) *N. hesperis* plants 0h and 24h after wounding. For further information, see the legend to figure 2.3.1.

2.3.5 Preliminary study into gene expression and alkaloid accumulation in *N. hesperis*

As shown in Figure 2.3.8, wounding had no effect on any of the transcripts measured in *N. hesperis* leaves, 24h after treatment. *PMT*, *QPRTase* and *ODC* transcripts were barely detectable.

Interestingly, '*A622*' transcripts were found to be present at relatively high constitutive levels in *N. hesperis* leaf tissue. The inclusion of the *N. sylvestris* standards in this experiment permits a comparison of '*A622*' levels between species. The transcript levels in the leaves of all *N. hesperis* plants were found to equal to or higher than the resting level of '*A622*' in the roots of *N. sylvestris* (when standardised against the ubiquitin signal). Consequently, they were higher than in the leaves of any other species, even if wounded. The induced levels of '*A622*' in *N glauca* leaves, for example, were only ~ 30% of the resting levels found in *N. hesperis* leaves. In fact, the only tissue possessing higher '*A622*' transcript levels was *N. sylvestris* root tissue 24 hours after wounding. The possible significance of this finding is discussed later (Discussion 3.4).

In the roots of *N. hesperis*, none of the transcripts representing *QPRTase*, *PMT*, *ODC* nor '*A622*' were induced 24h after foliage damage, with each being present at levels lower than the *N. sylvestris* standards. '*A622*' transcripts were present in *N. hesperis* roots at a level ~3.5 fold lower than in the leaves.

This preliminary investigation into *N. hesperis* did not include a detailed alkaloid analysis of wounded and non-wounded plants, as it did in the species described above. Instead, a small number of unwounded plants were assayed using HPLC, in order to compare their alkaloid contents with the published data. Only unquantifiable traces of nicotine, and probably of anabasine and nornicotine were found in the sample leaves. These results differ from the analyses of *N. hesperis* available in the literature, which have detected 3-4 mg/g dw alkaloids in leaf tissue (Saitoh *et al.*, 1985; Sisson and Severson, 1990). It is unknown whether this difference was due to the line of plants used, or their growth/ harvest conditions. The roots of these plants were not tested.

3 CONCLUSIONS & FUTURE DIRECTIONS

3.1 Molecular characterisation of *Nicotiana QPRTase* cDNAs

In Results 2.1.3, the cDNAs *TQPT1* and *RQPT1* were shown to encode functional QPRTase. The deduced proteins were highly similar to putative QPRTases from other plants, all of which carry an N-terminal extension which apparently has no counterpart in other organisms. It is of interest to speculate as to the function of this region.

The N-terminal region of the shorter human QPRTase has been identified as a putative cleavable target sequence (Residues 1-16), which may direct the protein to subcellular vesicles *in vivo* (Fukuoka *et al.*, 1998). Although the N-terminal regions of the deduced human and plant QPRTase proteins are not related, beyond their generally hydrophobic nature (Figure 2.1.2a, Results 2.1.3), it is possible that the plant N-terminal regions also act as signals to target the proteins to specific sub-cellular locations.

Computer analysis supports the suggestion that plant QPRTases are targeted to organelles. Initial analysis of the *Nicotiana* sequence by "P-Sort" (Nakai and Kanehisa, 1992), suggested that *Nicotiana* QPRTase was targeted to the mitochondria, as reported previously (Sinclair *et al.*, 2000). When, however, the same data were analysed using the updated "iP-Sort" (2002), a plastid targeting sequence was predicted. The QPRTase sequences deduced from the recently available plant DNA sequences (reviewed Introduction 1.3.6) were also predicted to have either a mitochondrial or a plastid targeting sequence (data not shown). When the yeast and prokaryotic sequences were analysed as if they were plant sequences, they returned negative results for targeting to the mitochondria or the plastid. When the program was informed of the real origin of these sequences, the prokaryotic sequences are predicted to be cytosolic or possibly membrane bound. The human sequence is assessed as possessing a targeting sequence, consistent with the speculation of Fukuoka and coworkers (1998), noted above.

The inability of P-sort/iPsort to distinguish clearly between a plastid and a mitochondrial target highlights the current difficulty in making sound functional predictions from many types of plant sequence data, where the database of proven

cases often remains small. This difficulty is exacerbated by the well-recognised similarity of signal sequences for plant chloroplasts and mitochondria (von Heijne *et al.*, 1989; Small *et al.*, 1998; Bannai *et al.*, 2002). The difference between these signals is indeed subtle. It is even possible for an "ambiguous" peptide to act in both capacities *in planta* or in isolated organelles (Creissen *et al.*, 1995; Small *et al.*, 1998; Cleary *et al.*, 2002). Both plastid and mitochondrial target peptides are N-terminal and are cleaved from the protein. Neither contains any characteristic areas of conserved sequence, and both share generally similar amino acid compositions (von Heijne *et al.*, 1989). Mitochondrial targeting pre-sequences (mTPs) are known to contain many S, R, A, L and T residues, and few C, H, E, Y and D residues (von Heijne *et al.*, 1989; Moore *et al.*, 1994; Sjoeling and Glaser, 1998). The most N-terminal part of the signal is likely to form an amphiphilic α-helix (Duby *et al.*, 2001; Cleary *et al.*, 2002). Similarly, chloroplast (plastid) targeting pre-sequences (cTPs) are known to contain many S and T residues, and few E, Y or D residues. They probably consist of three sections, with an uncharged amino section, a central region lacking acidic residues, and a likely amphiphilic β-strand (von Heijne *et al.*, 1989; Cleary *et al.*, 2002). Those proteins destined for specific destinations *within* an organelle, such as the mitochondrial inter-membrane space, have yet more complex signals, sometimes consisting of a general pre-sequence to deliver the protein to an organelle, with a second target peptide directing it to a location within the organelle (von Heijne *et al.*, 1989). Von Heijne and co-workers (1989) suggested that the most recognisable difference between cTPs and mTPs was their S content (19% for cTPs, and 11% for mTPs), and the fact that cTPs almost always posses an A immediately after the initial M, while this position is rarely (3% of cases then known) occupied by an A in mTPs. On this basis, visual scrutiny of the plant QPRTase sequences would suggest that the *N. tabacum* sequences are marginally more likely to be mitochondrial targeted, whilst the *G. max* sequence may be plastid targeted. This determination is, however, limited, since percent composition cannot be determined without precise knowledge of the cleavage site

Published research provides little information which could be used to refine any such prediction. The only system where the location of QPRTase has been speculated is in animals. In rat neurons and glial cells, where QPRTase has been studied for its possible role in mammalian brain dysfunction, the enzyme has been

located by immunocytochemistry primarily in an unidentified compartment 0.1-0.8 nm in diameter bound by a single membrane (Koehler *et al.*, 1987). As noted above, Fukuoka and co-workers (1998) suggested, on the available evidence, that human QPRTase was translocated into the secretory pathway, and then retained within this ill-defined vesicular compartment. Clearly, these systems are not directly comparable to plant cells.

In addition to there being little relevant information on the location of QPRTase, a paucity of general information is available on the location of other interacting biochemical reactions. Despite its fundamental importance, the physical sub-cellular location(s) of the PN-cycle is not well known in any organism. Early cell fractionation studies investigated the location of some PN-cycle enzymes in mammalian cells. NMN-ATase, for example, was active in the nuclei of mouse liver cells (Hogeboom and Schneider, 1952; Branster and Morton, 1956). NAPRTase was found to be active in the cytoplasm of beef liver cells, the subsequent enzymatic step catalysed by NAMN-ATase was measured in the nuclear fraction, while the following step catalysed by NADs was located in the cytoplasmic fraction (Preiss and Handler, 1958b; Imsande and Handler 1961). Together these results suggest complex patterns of intermediate transport, however subsequent publications (Grunicke *et al.*, 1975; Behr *et al.*, 1981; Pearson and Wilson, 1997) have been unable to further elucidate any such processes. Observations that isolated rat liver mitochondria are apparently capable of NAD synthesis from radiolabelled N (but not from radiolabelled Qa or NA) (Grunicke *et al.*, 1975) have proven particularly difficult to interpret (Behr *et al.*, 1981), and this area of study has apparently not been recently investigated. Studies in plants have been even more limited. Pearson and Wilson (1997) showed that isolated potato mitochondria are capable of the rapid turnover of radiolabelled NAD, however none of the enzymes of the pyridine nucleotide cycle have been localised to the plant mitochondria. In 2001, Agius and co-workers stated clearly that it remains unknown whether NAD breakdown and/or synthesis occurs primarily in the plant mitochondria *in vivo*. Thus, published information on the PN-cycle is of little use in predicting where QPRTase may be located. Any such prediction is made even more difficult since NAD and its derivatives (*eg*; NADP) are used in so many cellular reactions, including many reactions which are plant-specific (Wink, 1998). Some secondary metabolic reactions may also take place in certain subcellular compartments (Hashimoto and Yamada, 1994; Wink, 1997; Facchini,

2001). Together, this circumstantial evidence cannot be used to suggest a likely location for QPRTase, however the mitochondria certainly cannot be excluded.

Similarly, it would not be surprising to find that QPRTase was targeted to the plastid *in vivo*. Photosynthesis requires NADP as a co-factor, and this could provide a clue as to why plants may target QPRTase differently from animals. If QPRTase was active in plant plastids, this organelle may have been exploited by some plants to produce NA-derived alkaloids. Interesting but highly circumstantial evidence exists that some steps in alkaloid pathways related to pyridine alkaloid biosynthesis occur in the plastid. In oat (*Avena sativa* L., Poaceae), for example, ADC activity has been localised to the plastid (Borrell *et al.*, 1995). LDC activity has also been localised to the plastid in some species (*eg*; *Lupinus polyphyllus* Lindl., Fabaceae; Wink and Hartmann, 1982), and its substrate lysine is manufactured in plant chloroplasts (Shaul and Galili, 1992). Furthermore, the inclusion of an N-terminal chloroplast signal can enhance the activity of a bacterial LDC protein as well as the accumulation of anabasine in transformed *N. tabacum* plants and root cultures. (Herminghaus *et al.*, 1991, 1996). Circumstantial evidence cannot, however, be used to infer that other components of nicotine or anabasine synthesis such as QPRTase are also located in the plastid, since the transport of pathway intermediates between compartments is a common feature of alkaloid biosynthesis (Facchini, 2001).

On balance, then, it is difficult to justify the assignment of either a mitochondrial or a plastid location for QPRTase in *Nicotiana* at the present time. It is, moreover, possible that QPRTase is located in multiple compartments. This is possible through 'ambiguous' targeting (Small *et al.*, 1998), or by the presence of different genes or differentially translated mRNA copies encoding differentially targeted proteins. Taken together, the current data and the presently available literature cannot resolve the sub-cellular location of QPRTase in plants, and as such an experimental program designed specifically to answer this question is required.

Future work:

The subcellular location of plant QPRTase could be determined using two parallel lines of research. The first is to carefully measure QPRTase activity in sub-cellular fractions. This method has long been used to determine the location of enzyme activities, sometimes in combination with antibody detection techniques (*eg*; Borrell

et al., 1995). The second approach, taking advantage of information gained in the current study, would be to assemble two reporter gene constructs, one including the coding sequence encoding the N-terminal region of QPRTase fused to the reporter gene, the second construct lacking this region. Other studies have successfully detected GUS and GFP proteins targeted to specific subcellular compartments in plant cells (*eg*; Small *et al.*, 1998). If the N-terminal region was involved in directing QPRTase to a subcellular location *in vivo*, it would be predicted that the reporter gene product would be directed to this same location by the construct able to encode the N-terminus of QPRTase. Preliminary steps towards these experiments have already commenced in this laboratory (Brimblecombe, 2002). Such constructs could also be used in non-alkaloid producing plants, in order to determine whether the subcellular location of QPRTase in *Nicotiana* is related to alkaloid synthesis. Once data from animals are available, it will be of interest to determine whether the location of the fundamental PN-cycle differs between photosynthetic and non-photosynthetic organisms.

3.2 *QPRTase* gene transcription

One of the primary aims of isolating *QPRTase* 5' flanking sequences was to facilitate future experiments investigating the regulation of *QPRTase* gene expression. The work presented here will enable the spatial and temporal patterns of *QPRTase* expression to be further elucidated through reporter gene studies using *QPRTase* promoter sequences. It will also permit the isolation of upstream *cis*- and *trans*-regulatory components through promoter deletion analysis and yeast one-hybrid studies, as has been done in *C. roseus* leading to the isolation of ORCA transcription factors (vom Endt *et al.*, 2002).

The work presented in 2.2.4 showed that *NtxQPT2* transcripts are strongly expressed in *N. tabacum* roots and are induced after wounding. This, along with the fact that it is almost identical to a jasmonate-induced *QPRTase* mRNA (Discussion 3.3; Imanishi *et al.*, 1998) increases the likelihood that *NtxQPT2* is involved in alkaloid metabolism. Conversely, *NtxQPT1* expression could not be detected in the roots or leaves of *N. tabacum* even after wounding, and it remains unknown whether this gene copy is transcriptionally active. Although no *QPRTase* transcripts have been detected in *N. tabacum* leaf tissue, here or in other studies (*eg*; Hamill *et al.*,

2000; Sinclair *et al.*, 2000; Cane and Hamill, in preparation.), and QPRTase transcripts have only been weakly detected in *N. sylvestris* leaf tissue (Results 2.3.2), low levels of QPRTase activity have been measured in tobacco leaves and stems (Wagner and Wagner, 1984). Together these observations make it likely that *QPRTase* transcripts are indeed present in *N. tabacum* leaves, but that they are present at levels below the detection limit of Northern analysis. Whether they are *NtxQPT1*- and/or *NtxQPT2*-like transcripts remains to be determined.

If *NtxQPT1*-like genes are not expressed at all, then *NtxQPT2* must be under a regulatory regime able to facilitate both QPRTase's contribution to the pyridine nucleotide cycle, and to alkaloid synthesis in *N. tabacum*. Conversely, if an *NtxQPT1*–like gene is expressed, it is quite likely regulated in some way alongside *NtxQPT2* to achieve *QPRTase*'s dual role. One such possibility is that *NtxQPT1* is regulated in concert with *NtxQPT2*, but at a level which has so far not been detectable. This situation would be somewhat similar to that of *PMT* expression in *N. tabacum*, where the five *PMT* genes are all root-specific and wound induced to varying degrees (Riechers and Timko, 1999; Shoji *et al.*, 2000a). It is also possible that *NtxQPT1*- and *NtxQPT2*-like genes are differentially regulated, possibly to facilitate primary and secondary metabolic reactions respectively. This situation would be analogous to other situations where genes have been duplicated, and the duplicate copy has subsequently mutated to take on a new role (*eg*; *PMT* and *SPDS*; Introduction 1.3.3). In the latter case, *NtxQPT1* and *NtxQPT2* would be a novel example of duplicated alkaloid biosynthesis genes which have evolved divergent induction capabilities without significantly changing their enzymatic function.

If the *QPRTase* gene classes are differentially regulated in *N. tabacum*, it would be important to discover where and when *NtxQPT1*-like genes were expressed. If *NtxQPT-1* and *-2* expression does ever coincide in the same cell, it would be interesting to discover whether they encode proteins which are physically partitioned in order to carry out their separate roles (*ie*; a form of metabolic channelling, Introduction 1.1.3; Luckner, 1990), or whether the enzymes produced by *NtxQPT-1* and *-2* may simply enter a pool of QPRTase enzyme, regulated as a whole.

Future work:

The questions raised above may be somewhat clarified if more evidence could be found for the functionality or non-functionality of *NtxQPT1*. A number of approaches could be used. In the current study, Northern analysis employing short oligonucleotide probes was used, as a direct way to seek evidence for *NtxQPT1* transcription. These experiments demonstrated clearly that *NtxQPT1* transcript levels were much lower than those of *NtxQPT2*, but they were unable to answer the more elusive question as to whether *NtxQPT1* was expressed at all. Proof of non-expression is, by its nature, difficult to obtain, although a number of additional strategies could be used in future to gather more evidence.

Initially, a cDNA library could be screened with the aim of detecting *NtxQPT1*-like mRNAs, using a probe which distinguishes between *NtxQPT2* and *NtxQPT1* gene copies. This would be conceptually identical to the screen shown in Figure 2.2.4, although it would necessarily involve a larger number of phage cDNAs. Further Northern, or RT-PCR analysis using copy-specific probes could also be performed using mRNA purified from other tissues not so-far examined (*eg*; anthers, petals, stem sections), allowing the oligonuceotide probe method refined in Results 2.2.4 to be further explored. In addition, reporter gene analysis similar to that described above could also be performed, using the *NtxQPT1* promoter to direct expression of the reporter. Such a strategy may elucidate the expression pattern of *NtxQPT1*, however it may prove difficult to distinguish between low level expression directed by the *NtxQPT1* promoter, and "promoter leakage" caused by surrounding pieces of chromosomal DNA, such as enhancers or SARs (Allen *et al.*, 1993).

An alternative way to test whether *NtxQPT1* is functional would be to assess the effects of disabling it. Recent studies suggest that it may be feasible to suppress *NtxQPT1* without suppressing *NtxQPT2* (or *vice versa*) with the use of RNA silencing techniques (Baulcombe, 2002; Hutvágner and Zamore, 2002). Plant genes have been routinely suppressed at the transcriptional level using antisense technology, whereby a construct expresses a specific antisense transcript, which acts to suppress its endogenous counterpart (*eg*; Voelckel *et al.*, 2001). The mechanism of this silencing is currently being elucidated. It appears that double stranded RNA (such as that produced by the hybridisation of a sense and antisense transcript) is recognised by the cell, and "diced" into 21-25bp fragments of both orientations (siRNAs)

(Zamore *et al.*, 2000; Vance and Vaucheret, 2001; Baulcombe, 2002). These siRNAs are then incorporated into a multimeric RNase complex, that is able to recognise homologous messages and destroy them (Baulcombe, 2002). These insights have accompanied advances in RNA silencing techniques, and it is now known that the deliberate creation of double stranded RNA from sense-antisense constructs able to form stable hairpins is a more reliable and potent method of silencing specific transcripts than standard antisense suppression (Vance and Vaucheret, 2001). A number of 'RNA interference' (RNAi) vectors have recently become available (*eg*; pHANNIBAL; Wesley *et al.*, 2001). Given that the silencing is effected by 21-25mers, it is likely that *NtxQPT1* or *NtxQPT2* transcripts could be specifically silenced by siRNAs homologous to the oligonucleotides already used as probes in this study (Results 2.2.4).

Although the work presented above showed, for the first time, that an *NtxQPT2*-like gene was up-regulated in the roots of *N. tabacum* following foliage damage, nothing is yet known about the DNA-protein interactions which enable this to take place. In order to delineate the *cis*-acting elements responsible, a series of promoter deletion fragments could be constructed, each one directing the expression of a reporter gene. Substantial changes in wound-induced reporter gene expression between different constructs would indicate that the region differing between them contains a DNA motif involved in wound-induction. The important bases in the binding site can be identified in a number of ways, including DNase footprinting and gel mobility shift assays (*eg*; Ishiguro and Nakamura, 1992; Nagao *et al.*, 1993; Ulmasov *et al.*, 1999; Plesch *et al.*, 2001). The transcription factors binding to such elements may be isolated as cDNAs using the yeast one hybrid system (Wang and Reed, 1993; Menke *et al.*, 1999b).

3.3 The origin and arrangement of *QPRTase* genes in the *Nicotiana* genus

Southern analysis was used in Results 2.2.3 to demonstrate that the differentiation of *QPRTase* into recognisable *NtxQPT1*- and *NtxQPT2*-like gene classes preceded the formation of *N. tabacum*, an event thought to have occurred some ~5-6 million years ago (Okamuro and Goldberg, 1985; Fulnecek *et al.*, 2002). Further insights into the

origin and arrangement of *QPRTase* genes in *Nicotiana* species can be gained from sequence analysis.

Figure 3.3.1 shows three dendrograms representing the genetic distance between some plant *QPRTase* DNA and amino acid sequences. Although these separate analyses are based on different portions of sequence data (*ie*; limited to what is currently available, Introduction 1.3.6), all three dendrograms show similar trends, suggesting that the analysis is robust.

The dendrograms show that *NtxQPT1* is relatively dissimilar to the other *Nicotiana QPRTase* sequences included, all of which clearly represent *NtxQPT2*-like genes. It is significant that sequences from *N. alata*, *N. glauca* and *N. rustica* cluster **within** the sequences which encompasses *N. tabacum*, suggesting that *QPRTase* duplication preceded not only the formation of *N. tabacum*, but much/all of the speciation within the genus, as suggested in Results 2.2.3.

Furthermore, *NtxQPT1* is more similar to the tomato *QPRTase* than it is to the other *Nicotiana* sequences. This clustering may reflect very ancient divergence of *NtxQPT1* from *NtxQPT2*, before the reproductive isolation of the *Nicotiana* and *Lycopersicon (Solanum)* genera, some 25-40 million years ago (Meagher *et al.*, 1989; Ioerger *et al.*, 1990). This possible antiquity of the *QPRTase* duplication is particularly interesting when considered in conjunction with the question of *NtxQPT1*'s functionality. Although it is divergent from the other *Nicotiana QPRTases*, *NtxQPT1* displays conserved intron-exon boundaries, all of the residues required for the active site are present, and the only deletion/insertion (3bp) does not cause a frameshift (Results 2.2.5) (Eads *et al.*, 1997; Sinclair *et al.*, 2000). A redundant gene may be expected to accumulate deleterious mutations over evolutionary time. Taken together, these data are consistent with the suggestion that *NtxQPT1* is required in addition to *NtxQPT2* for some basic function. For example, *NtxQPT1* may be required for NAD or alkaloid production in an isolated cell or tissue type which has not been detected by the broad Northern analysis applied so far.

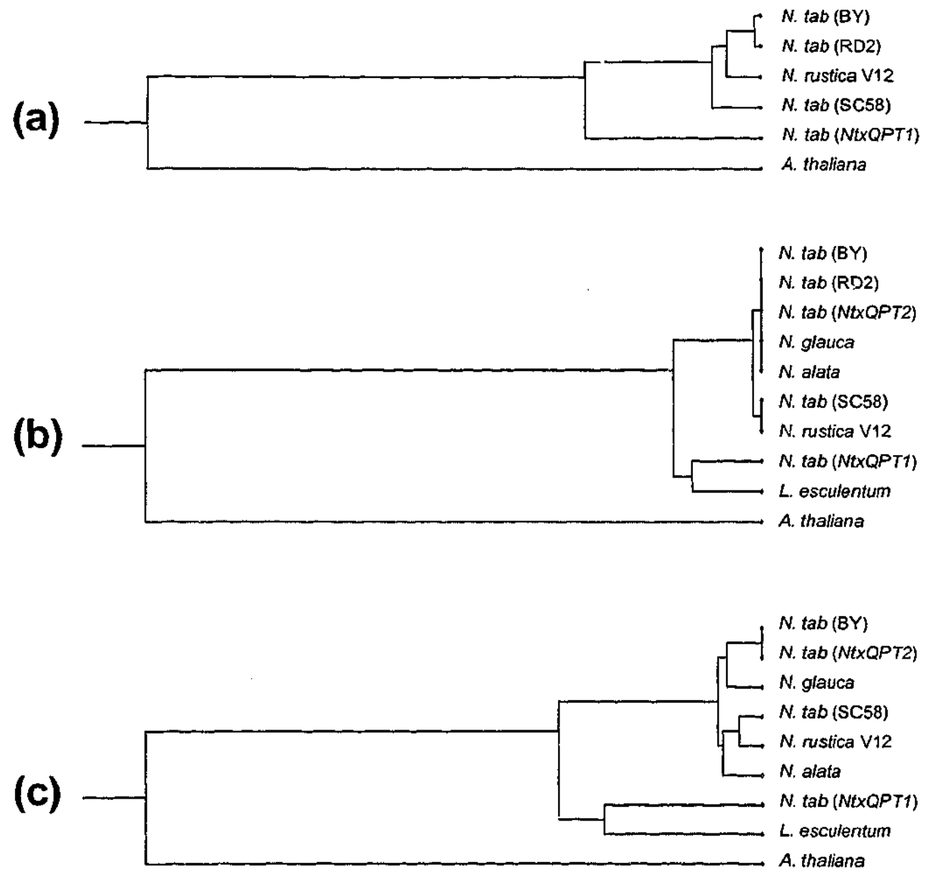


Figure 3.3.1 Dendrograms showing the genetic distances between various plant QPRTase sequences.

The sources of the sequence data have already been described in the legends to Figures 2.1.2 and 2.2.9. Panel (a) has been generated from full length deduced amino acid sequences. Panel (b) has been generated from partial amino acid sequences, representing the first 44-49 N-terminal residues in the various sequences, up to and including the sequence PP(X)HP which is conserved in all sequences. This analysis has been limited by the availability of *N. glauca* and *N. alata* sequence data. Panel (c) has been generated from DNA sequence including the 5' UTR, exon 1, and part of exon 2. The first intron has been removed from the analysis to permit the inclusion of cDNA sequences. The dendrograms were created via the Australian National Genomic Information Service (ANGIS) (www.angis.org.au) using ENEIGHBOR (UPGMA method), based on sequence alignments generated by ECLUSTLW.

It is, however, important to acknowledge that the validity of the dendrogram presented in Figure 3.3.1 is itself **dependant** on *NtxQPT1* being functional. The integrity of non-functional DNA is not maintained by selective pressure, and it will thus appear to evolve faster than functional DNA, distorting or invalidating dendrogram analysis (Page and Holmes, 1998). If *NtxQPT1* is non-functional, then Figure 3.3.1 cannot be used to argue for an ancient divergence between *NtxQPT1* and *NtxQPT2*.

Future work:

Further experimental data may distinguish between the two possible interpretations of the dendrograms noted above and to help clarify when *NtxQPT-1* and *–2* may have diverged. It is important to determine whether *NtxQPT1* is functional in order to do this, possibly using the techniques noted above (Discussion 3.2). Identification of the relative time of *QPRTase* gene duplication may enable the determination of whether this duplication is independent of pyridine alkaloid production, or an important factor permitting its evolution. It may be, for example, that other Solanaceous genera which produce nicotine (*eg*; *Duboisia*; Leete, 1983), also inherited a duplicate *QPRTase* gene which predisposed them towards evolving alkaloid synthesis. The anaylsis of genomic *QPRTase* clones from other Solanaceous species may strengthen any estimate of when *QPRTase* genes diverged.

3.4 Alkaloid and gene expression patterns in different *Nicotiana* species

Differential gene transcription and alkaloid content in different species:

One of the aims of the experiments described in Results 3.3 was to document the responses of *Nicotiana sylvestris*, *N. glauca* and *N. alata* to foliage damage, in order to further our understanding of how different species have evolved variations on a defence strategy. The new data obtained in the present study for *N. glauca* and *N. alata* support the hypothesis, noted previously (Sinclair *et al.* 2000), that differential gene transcription is a major factor contributing to the different alkaloid patterns found in different *Nicotiana* species. This hypothesis does not, of course, preclude

the possibility that post-transcriptional regulation, alkaloid degradation or intermediate transport may also play roles, and further experiments would be needed in future to explore these issues.

Taken together, the results presented here for *N. sylvestris* are consistent with the generally accepted model of alkaloid biosynthesis, confirming that the hydroponic growth conditions used here enable appropriate comparisons to be made with past studies. *QPRTase*, *PMT*, *ODC* and '*A622*' transcripts are all up-regulated in the roots of *N. sylvestris* plants 24 hours after foliage damage. This is followed several days later by a measurable increase in nicotine content in the leaves of the plant, which can be interpreted as a result of nicotine redistribution from the roots to the leaves (Dawson, 1945; Baldwin and Preston, 1999).

In *N. alata* plants, however, there was no increase in the transcript levels of any of the four genes measured, nor in alkaloid content, in root or leaf tissue after foliage damage. Thus, it appears that *N. alata* possesses a wound-response that differs from *N. sylvestris* in both biosynthetic gene transcription, and the subsequent alkaloid response. The *N. alata* promoter fragment isolated here (Results 2.2.7) may enable further studies to elucidate the molecular basis for this difference. One simple hypothesis to explain the lack of an *N. alata* wound response is that the relevant *N. alata QPRTase* promoter lacks the requisite *cis*-elements. This is consistent with the *Ntw38QPT2* promoter delineation undertaken by Song (1997) and Conkling and co-workers (1998), which found important root specific elements to be located upstream of the region conserved between the *N. alata* promoter and *NtxQPT2/Ntw38QPT2*. This hypothesis does not, however, explain the low level expression of the other alkaloid biosynthetic genes *PMT*, *ODC* and '*A622*', and thus some other factor must also differ between *N. alata* and *N. sylvestris*.

Although previous studies indicated that *N. glauca* differs from all other *Nicotiana* species in accumulating anabasine as its major alkaloid (Saitoh *et al.*, 1985; Sisson and Severson, 1990; Baldwin and Ohnmeiss, 1993), these reports made no suggestion that the basic pattern of foliage-damage followed by root alkaloid-synthesis may also differ. The data presented here (Results 2.3.3), showing wound-induced *QPRTase* gene expression in *N. glauca* leaves but not roots, suggest that the anabasine-response in this species is fundamentally different from the nicotine-response in *N. sylvestris*. These data raise the possibility that *Nicotiana glauca* relies on an elevated capacity for anabasine biosynthesis in the wounded foliage, apparently

without partitioning induced alkaloid synthesis to the roots. This finding advances the early discoveries of Dawson (1945) who showed, using inter-species grafts, that isolated *N. glauca* foliage could produce anabasine.

Furthermore, the data presented here suggest that wound-induced anabasine synthesis does not occur foliage wide, since the lower unwounded leaves display no *QPRTase* gene induction, nor any anabasine increase following wounding. Thus, there is no evidence that wounded *N. glauca* leaves send a basipetal (*ie*; downward) signal to the lower leaves or roots in order to induce alkaloid synthesis, unlike *N. sylvestris* and *N. tabacum*.

Baldwin and Ohnmeiss (1993) have, however, demonstrated that unwounded apical leaves do accumulate higher anabasine levels when the lower leaves have been wounded. This response may involve an acropetal (*ie*; upward) signal which induces anabasine synthesis in the upper leaves, or on the other hand, restricted anabasine synthesis within the damaged tissue, and subsequent acropetal anabasine transport. Further experiments to uncover the nature of this response may involve further Northern- and alkaloid-analysis, using upper leaves from plants wounded differently to those here (*ie*; not decapitated above the wounded leaf).

It is conceivable that this foliar-defence strategy has evolved in *N. glauca* because it is more effective in a perennial tree, where long distance transport of alkaloids from the roots to the leaves may be inefficient. If this is the case, it is interesting to note that the foliage-based wound response operates not only in mature trees, but also in young plants such as those used here, which would, presumably, be physically able to relocate alkaloids in the same manner as similar sized young *N. sylvestris* plants.

Potential wound related roles of ODC in Nicotiana *leaves:*

Some of the other Northern data obtained from *N. glauca* were somewhat unexpected. Whereas it might be predicted that the expression of *ODC*, *PMT* and '*A622*' would be unlikely to increase in a tissue accumulating elevated levels of anabasine, but not nicotine (refer to Figure 1.2), *ODC* and '*A622*' transcript levels were found to be substantially elevated in *N. glauca* leaves 24 hours after wounding, 12.1 and 5.9 fold respectively. It is interesting to consider the potential wound-

related roles of ODC and 'A622' in the leaves of *N. glauca*, since this tissue does not produce significant quantities of nicotine.

Interpreting the putative wound-related role of ODC is complicated by the fact that it is involved in the production of polyamines as well as alkaloids. Thus, like QPRTase, it is involved in both primary and secondary metabolism. The precise functions of polyamines are not well defined, but these metabolites are generally thought to play roles in cell division (Kumar *et al.*, 1997; Watson *et al.*, 1998), and are known to be associated in plants with numerous stresses including drought, salt and cold (Erdei *et al.*, 1990; Kramer and Wang, 1989; Espartero *et al.*, 1994). Given this, it is conceivable that ODC is up-regulated following wounding to allow the increased production of polyamines to facilitate some aspect of tissue repair and/or wound recovery. However, if this hypothesis were correct, then the striking difference between the ODC response in *N. glauca* and both *N. sylvestris* and *N. alata* would suggest that these species have a fundamentally different wound-repair response, not only a different alkaloid response. There is no evidence to support such a conclusion.

An intriguing alternative interpretation, consistent with the current literature and the new data presented here, is that ODC may be involved not only in the production of nicotine, but also in the production of anabasine. Specifically, ODC may contribute to the LDC activity required for the biosynthesis of cadaverine, which is the source of the piperideine ring in anabasine (Walton *et al.*, 1988). Such a suggestion would certainly account for the particularly large induction of *ODC* transcript levels in the leaves of *N. glauca* following foliage damage, and before anabasine accumulation.

Some recent independent work has given weight to this suggestion. Takatsuka and co-workers (1999; 2000) characterised an enzyme from the bacterium *Selemonas ruminatum* that was able to decarboxylate both ornithine and lysine. The sequence of the gene demonstrated that it was related to eukaryotic *ODC*s, but not to any previously characterised bacterial *LDC*s. These researchers were able to alter the enzyme's preference for ornithine or lysine by altering only a few amino acids (Takatsuka *et al.*, 2000), demonstrating that the molecular difference between ODC and LDC is small, and raising the possibility that an enzyme with LDC activity could evolve from ODC. Plant secondary metabolic enzymes with different substrate specificities are indeed known to differ by as little as a single amino acid (*eg*; O-

methyltransferases from *Thalictrum tuberosum* L., Ranunculaceae; Frick and Kutchan, 1999).

A second line of evidence that supports this hypothesis comes from the recent work of Lee and Cho (2001), who demonstrated that *N. glutinosa* L. ODC was capable of decarboxylating lysine. It was shown that the same polypeptide can decarboxylate both lysine and ornithine, at the same catalytic site, with the amino acid preference being pH dependant. Under lower pH conditions, the enzyme behaved as an LDC (optimum 6.8), while ODC activity was favoured in more basic conditions (optimum pH=8.0). *Nicotiana glutinosa* does not produce large amounts of anabasine, with only 0.3-1.4% having been recorded in the leaves, and 3% in the roots (Saitoh *et al.*, 1985; Sisson and Severson, 1990), and Lee and Cho (2001) made no reference to alkaloids in their work.

The data presented here are consistent with the hypothesis that in *N. glauca*, and possibly in other anabasine producers, this LDC activity linked to *ODC*(-like) gene expression has been exploited for defensive alkaloid production. This could have occurred via a number of evolutionary routes.

For example, *ODC* duplication and modification via mutation may have yielded an (*ODC*-like) *LDC* gene, such that the mRNAs hybridising to the *ODC* probe are in fact representative of two functionally independent genes. It is notable that *N. glauca* LDC apparently has an optimum pH of 8.4 (Bagni *et al.*, 1986), far higher than that noted in *N. glutinosa* ODC/LDC (6.8) (Lee and Cho, 2001). This difference in pH optimum may result from an evolutionary modification of ODC/LDC in *N. glauca*, however it is not possible to exclude the possibility that the assayed LDC in *N. glauca* (Bagni *et al.*, 1986) was encoded by a non-ODC-like gene.

Alternatively, the ODC and LDC activities in *N. glauca* foliage may be derived from the same gene. pH conditions could conceivably alter the balance between the ODC and LDC activity of a single peptide *in vivo*. This could be achieved, for example, if the protein was situated in a compartment where the pH was non-constant, such as the chloroplast. When photosynthetically active, the chloroplast stroma becomes increasingly alkaline, while the thylakoid space becomes acidified (pH=7.5-8 and pH=5-6 respectively, Robinson, 1985; Laasch *et al.*, 1993; Oja *et al.*, 1999). Some enzymes are known to be regulated by such changes, although most are directly involved in photosynthesis (Hager, 1980; Laasch *et al.*, 1993). This hypothesis is given credence by the fact that LDC activity is thought to

be located in the plastid in at least some plants (Wink and Hartmann, 1982; Shaul and Galili, 1992; Herminghaus *et al.*, 1991, 1996). Nonetheless, the hypothesis is limited by the fact that the observed transcriptional induction would be partially nullified during times when the pH conditions favoured ODC rather than LDC. Furthermore, no information is yet available as to whether alkaloid synthesis fluctuates between day and night conditions in *N. glauca*.

A number of experiments would need to be carried out before these hypotheses can be verified or extended. The subcellular location, amino acid composition and pH preference of *N. glauca* ODC(s) must be compared to the ODCs of other species (*ie*; *N. glutinosa*, *N. tabacum*) as a starting point. Work is currently underway in this laboratory (K. DeBoer, pers. comm.) with the aim of characterising the genomic fragments in *N. glauca* which hybridise to the same ODC probe used here.

Potential wound-related roles of 'A622' in Nicotiana leaves:

The observation that '*A622*', like *QPRTase* and *ODC*, is also upregulated by wounding in *N. glauca* leaves, prompts a reinterpretation of 'A622''s possible role in alkaloid synthesis. At present, the basic function of 'A622' is unknown, although it is thought to be involved in nicotine production, since its expression has until now been closely linked with that of *PMT* in *N. sylvestris* (Hibi *et al.*, 1994; Shoji *et al.*, 2000a, 2000b). Analysis of '*A622*' sequence data by Hibi and co-workers (1994) indicated that it probably encodes a reductase. It is notable that the condensation between the 1-methyl-Δ^1-pyrrolinium cation and nicotinic acid involves the reduction of a double bond, a reaction thought to involve the enzyme 'nicotine synthase' (NS) (Friesen and Leete, 1990). Hibi and co-workers (1994) speculated that '*A622*' may encode this enzyme (or a component of it), however NS activity has proven difficult to measure, and it has not been demonstrated that '*A622*' encodes this enzyme.

The data presented here are consistent with the hypothesis that '*A622*' encodes an enzyme which may be involved not only in the synthesis of nicotine, but also other pyridine alkaloids such as anabasine. This hypothesis is also consistent with previously reported data showing that the nicotine- and anabasine-biosynthesis pathways compete for nicotinic acid. For example, anabasine levels were increased at

the expense of nicotine by feeding cadaverine to cultured *N. rustica* roots (Walton *et al.*, 1988). Similarly, anabasine levels have been increased at the expense of nicotine in *N. tabacum* via the over-expression of a bacterial *LDC* gene (Herminghaus *et al.*, 1996). Taken together with the observation that '*A622*' is up-regulated in a tissue accumulating anabasine but not nicotine, it is possible that '*A622*' encodes not only NS, but also "anabasine synthase".

This hypothesis is also compatible with the preliminary data presented here involving *N. hesperis* (Results 2.3.5). The high constitutive level of '*A622*' expression observed in the leaves, without correspondingly high levels of *QPRTase*, *PMT* or *ODC*, would be expected to result in the production of very small amounts of nicotine, limited by the availability of NA and *N*-methylpyrrolinium salt. If 'A622' was also implicated in anabasine synthesis, then it would be expected to draw on existing intermediates to produce a similar (small) amount of anabasine. Alkaloids were almost absent from the *N. hesperis* foliage analysed here, possibly due to low levels of *QPRTase*, *PMT* and *ODC* transcription. Previous studies have, however, recorded similar relative amounts of both nicotine and anabasine (~2mg/g dw each[vi]; Saitoh *et al.*, 1985), consistent with a pathway which can be "pulled" (Nessler, 1994) by relatively high levels of an 'A622' enzyme able to utilise both the pyridine and piperideine intermediates of nicotine and anabasine.

A simplified model for pyridine alkaloid biosynthesis in Nicotiana*:*

Although the hypotheses noted above have yet to be tested experimentally, they merit investigation because they enable a simpler model of pyridine alkaloid biosynthesis in *Nicotiana* to be generated than that which is currently accepted (*ie*; Figure 1.2). If ODC and 'A622' are indeed able to perform steps involved in both nicotine and anabasine synthesis, then genes specific to anabasine synthesis may not be required. Taking up this possibility, Figure 3.4.1 presents a hypothetical model of alkaloid synthesis, where anabasine and nicotine are both products of the same non-specific metabolic pathway.

[vi] This similar weight also represents similar stoichiometric amounts, since the formula weights of anabasine (162.24) and nicotine (162.23) are almost identical.

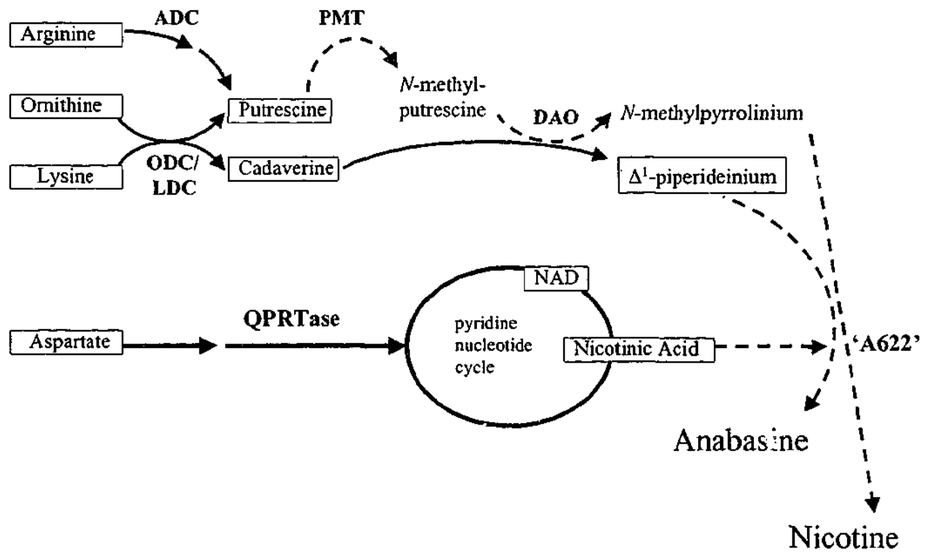


Figure 3.4.1 A modified scheme representing the hypothetical production of pyridine alkaloids in *Nicotiana*

The arrangement of biochemical pathways presented above has been modified from the scheme presented in Figure 1.2, to better accommodate the new data obtained in the present study. In Figure 1.1, the production of *N*-methyl pyrrolinium and Δ^1-piperideinium were depicted as entirely separate pathways. In the hypothetical scheme presented here, they both share steps catalysed by common enzymes (DAO and ODC/LDC). Furthermore, the final synthesis of both anabasine and nicotine are hypothesised to be catalysed by 'A622'. A detailed justification of these modifications is given in the text (Discussion 3.4). As in Figure 1.1, primary metabolic reactions common to all/most organisms are shown with full arrows, whereas reactions specific to the production of secondary metabolites are shown with dotted arrows. Similarly, primary metabolites are boxed, secondary metabolites are not boxed.

In this model, the only qualitative enzymatic difference between the routes leading to anabasine and nicotine is the presence of PMT in the latter pathway.

In this modified pathway, there is still sufficient scope for the independent regulation of nicotine and anabasine synthesis to account for the variation in alkaloid content which is observed in different species within the genus *Nicotiana*. The proportion of nicotine could be increased, for example, via the up-regulation of PMT. The nicotine : anabasine ratio could also be altered if the ratio of ODC : LDC activity was altered. These or similar changes could readily facilitate changes in alkaloid profile, both in response to wounding, or over evolutionary time in different species.

The scheme presented in Figure 3.4.1 is particularly attractive from an evolutionary perspective. Assigning multiple functions to identical or clearly co-derived enzymes suggests new ways of interpreting the evolutionary development of the different defensive alkaloid profiles found in nature, without the need for the evolution and subsequent co-regulation of additional genes.

Future work:

Continuing research may address both the molecular differences between *Nicotiana* species, in terms of the wound-response mechanisms, along with specifically addressing the predicted wound-related roles of ODC and 'A622'.

One specific question of interest is to find out what molecular difference(s) allows *N. glauca* to transcriptionally up-regulate *QPRTase*, *ODC* and *PMT* in the leaf after foliage damage, rather than the root as in *N. sylvestris* and *N. tabacum*. The introduction of a construct containing a reporter gene driven by the *NtxQPT2* promoter into *N. glauca* may be informative. If this construct directed strong, wound-inducible expression in the leaf, the data would suggest that *N. glauca* utilises a transcription factor to regulate its alkaloid response which is similar or identical to that used in the roots of *N. tabacum* and *N. sylvestris*. This outcome is not unlikely, since the alteration of a transcription factor's activity is a simple way, in evolutionary terms, of envisaging how the wound responses of *N. glauca* and *N. tabacum/N. sylvestris* differ. Contrastingly, if the construct directed root-specific wound-induced expression, as it does in its native *N. tabacum*, this would suggest that *N. glauca* possesses a *QPRTase* gene with a differentially responsive promoter to that of *NtxQPT2*, interacting with a different transcription factor, possibly related to one of

those already known to be wound-induced in the leaves of Solanaceous species (*eg*; WIZZ, a WRKY induced within minutes of wounding in *N. tabacum*, Hara *et al.*, 2000). Numerous cross-species reporter experiments can be designed, which test similar questions.

It is also possible to address relevant questions with the use of inter-species grafts, similar to those which underpinned some of the classic early work in alkaloid synthesis (Dawson, 1945). Although no evidence was found to suggest that *N. glauca* roots were able to *respond* to any basipetal signal from the wounded leaves, it would be interesting to discover whether *N. glauca*, like *N. sylvestris*, possesses the ability to *send* such a signal to its roots, by determining whether wounded *N. glauca* scions can induce gene transcription in *N. sylvestris* roots. Alternatively, the reciprocal graft would determine whether *N. glauca* roots were able to respond to a signal from *N. sylvestris* foliage, which may be lacking *in vivo* in *N. glauca*. Analogous experiments involving reciprocal grafts between *N. sylvestris* and *N. alata* may help identify whether *N. alata*'s inability to respond to foliage damage by upregulating alkaloid biosynthesis is influenced by an inability to perceive or send such a wound signal.

A number of experiments could be used to investigate the function of the wound-induced transcripts that hybridised to the *ODC* probe in RNA extracted from *N. glauca* foliage. Given the availability of detailed data on the sequence and active site of ODC(-LDC) in plants (Michael *et al.*, 1996; Takatsuka *et al.*, 1999; 2000; Lee and Cho, 2001), it is possible that an increased affinity for lysine would be evident from careful sequence analysis of *N. glauca ODC* cDNA or genomic clones. Functional confirmation could be achieved by expressing any such cDNA(s) in a microbial system and assaying ODC and LDC activity (Lee and Cho, 2001). Antisense suppression experiments may also provide evidence for any involvement of *ODC*-like transcripts in anabasine synthesis, if the suppression of *ODC* led to a decrease in anabasine levels in transformed *N. glauca* plants.

Appropriate parallel work would directly address the possibility that LDC is not related to ODC, as is the case in bacterial systems (Takatsuka *et al.*, 1999; 2000). Such work could pursue a non-ODC-like LDC using bacterial sequences as molecular probes, or to design primers. This work is underway already in this laboratory, with the identification of 5 genes in the *Arabidopsis* genome which somewhat resemble

bacterial LDC genes (K. DeBoer, pers. comm.). DeBoer is currently attempting to assay LDC activity in *E. coli* cells expressing these putative LDC genes.

Specific experiments could also be designed to further characterise the role of 'A622' in alkaloid biosynthesis. Hibi and co-workers (1994) already suggested both antisense and over-expression studies in *N. sylvestris*. The data presented here suggest that such studies would also be valuable in *N. glauca* and *N. hesperis*. If, for example, RNA interference/suppression of 'A622' in these species lead to a decrease in both nicotine and anabasine levels, this would provide strong evidence that 'A622' was able to participate in anabasine synthesis.

3.5 Summary

The experimental data, and the interpretations discussed above, have made a number of contributions to our understanding of pyridine alkaloid synthesis in *Nicotiana* species:

1: Characterisation of two QPRTase *cDNAs:*

- cDNAs from *N. tabacum* and *N. rustica* have been shown to encode functional QPRTase enzymes by functional complementation of a bacterial QPRTase mutant.
- These QPRTase enzymes show significant sequence homology to other QPRTase enzymes, particularly those deduced from plant DNA sequences.
- Plant QPRTase enzymes are predicted to carry an N-terminal extension not found on their animal, yeast or prokaryotic counterparts. This extension has the characteristics of a chloroplast and/or mitochondrial targeting signal.

Key future directions:

- Reporter gene studies to determine the subcellular location of QPRTase in *Nicotiana* would prove valuable. Such studies may enable future comparisons with other organisms, to assess whether the location of QPRTase differs according to pyridine alkaloid biosynthetic capacity, or between plants and animals.

2: QPRTase in the Nicotiana *genome:*

- An *N. tabacum* cv. Xanthi genomic library was shown to contain two distinct classes of *QPRTase* gene (exemplified by *NtxQPT1* and *NtxQPT2*), distinguishable by their different 5' flanking regions.
- *NtxQPT1*-like genes are likely to be more numerous than *NtxQPT2* genes in the *N. tabacum* genome.

- Both classes of *QPRTase* are present in the genomes of each of the diploid progenitors of *N. tabacum*. Thus, *QPRTase* genes must have duplicated before the genus *Nicotiana* had fully speciated.
- At least one *NtxQPT2*-like gene is strongly expressed in *N. tabacum* root (but not leaf) tissue. This gene is transcriptionally up-regulated in roots, but not leaves following foliage damage.
- All *NtxQPT1* genes are either non-expressed, or expressed at very low levels in roots and leaves of *N. tabacum*.
- The 5' flanking region of *NtxQPT1* shows no substantial homology with other available DNA sequences. The 5' flanking region of *NtxQPT2*, on the other hand, is essentially identical to a promoter previously patented, but not published in the public literature, which directs root specific reporter gene expression, consistent with the expression profile of *NtxQPT2* noted above.
- The promoter of *NtxQPT2* contains a ~830bp region which is similar to sequences in several other *N. tabacum* gene promoters, and which may represent a scaffold attachment region.
- *NtxQPT1* possesses 9 introns, each of which is located in the same relative position as the introns of the single putative *Arabidopsis QPRTase* gene, however, 8 of the *NtxQPT1* introns are longer than their *Arabidopsis* counterparts. One *NtxQPT1* intron contained a direct repeat, possibly due to a relatively recent duplication event. Another intron contained an unusual run of 23 consecutive G residues, of unknown significance.

Key future directions:

- Studies to determine whether *NtxQPT1*-like genes are transcribed would help clarify whether this gene copy is required for primary metabolism, or whether *NtxQPT*-like genes are able to perform both primary and secondary metabolic functions. Valuable experiments would involve screening a cDNA library with *NtxQPT1*-specific probes to try to identify transcripts. RNA interference techniques may also be able to address the effects of silencing *NtxQPT1*. Another valuable approach would be to determine whether the *NtxQPT1* 5' flanking region

was able to induce the transcription of a reporter gene in transformed *N. tabacum* cells.
- The *NtxQPT2* promoter may be dissected, in order to isolate DNA regions capable of directing root specific and wound-induced transcription. A knowledge of such *cis*-elements may provide insights into the function of the *NtxQPT2*-like *N. alata* promoter which was isolated here. These regions may be used in further studies to isolate DNA binding proteins which regulate *QPRTase* gene expression.

3: Differential patterns of gene expression in Nicotiana *species with contrasting alkaloid profiles:*

- The co-ordinated 3-7 fold up-regulation of *QPRTase*, *PMT*, *ODC* and '*A622*' in the roots of *N. sylvestris* 24h after foliage damage precedes a two-fold increase in nicotine levels in the leaves ~5 days later.
- Transcript levels of *QPRTase*, *PMT*, *ODC* and '*A622*' were very low in the leaves and roots of *N. alata*, in comparison to *N. sylvestris*. *Nicotiana alata* accumulates almost undetectable amounts of any alkaloid in its leaves, even after wounding, and a moderate level of nornicotine in its roots. This is consistent with the hypothesis that alkaloid levels are heavily influenced by the transcriptional activity of these genes.
- *QPRTase*, *ODC* and '*A622*' (but not *PMT*) transcripts were up-regulated in the wounded leaves of the anabasine producer, *N. glauca*, 24h after foliage damage. This gene expression was followed by a four-fold increase in anabasine accumulation in these same leaves ~5 days later.
- The lower, unwounded leaves, and the roots of the same wounded *N. glauca* plants showed no increase in gene expression or alkaloid accumulation. These results are consistent with the hypothesis that *N. glauca* does not rely on the acropetal transport of anabasine to facilitate its leaf anabasine response, but rather increases leaf anabasine levels via local synthesis.
- Transcripts detected by an *N. tabacum ODC* probe were up-regulated 24 hrs after foliage damage in the leaves of *N. glauca* - a tissue where nicotine was apparently

not produced. It is suggested here that this ODC-like transcript is involved in anabasine production, possibly by encoding LDC activity.

- Transcripts hybridising to an *N. tabacum* '*A622*' probe were also up-regulated following wounding in the leaves of *N. glauca*, without the concomitant production of nicotine. The protein encoded by such transcripts is also suggested to play a role in anabasine production, possibly encoding an enzyme able to synthesise both nicotine and anabasine.
- If correct, these proposals for the roles of *ODC*- and '*A622*'-like transcripts permit a simplification of the current model of pyridine alkaloid biosynthesis, where no specific enzymes are required for anabasine synthesis.

Key future directions:

- The work noted above will serve as the basis for investigations to uncover the molecular basis for the different gene expression and alkaloid profiles found in *N. sylvestris*, *N. glauca* and *N. alata*. The long-distance signal transduction capacity of these species could be examined using inter-species grafts, or inter-species reporter gene experiments.
- The role of *ODC*-like transcripts in *N. glauca* foliage could be investigated using RNA interference experiments, or by assaying the activity of the enzyme encoded by them in a bacterial system.
- The role of '*A622*'-like transcripts in *N. glauca* foliage could be investigated in a similar manner, using enzyme assays and RNA interference studies.

4 MATERIALS AND METHODS

4.1 Demonstrating that the cDNAs *pTQPT1* and *pRQPT1* encode *QPRTase*

4.1.1 The complementation of a bacterial mutant

nadC deficient *E. coli* cells (TH265) were obtained from Prof. Kelly T. Hughes (Dept. Microbiology, University of Washington, Seattle, USA). These cells were derived from the wild type *E. coli* strain K-12, and have the *ace-nadC* region deleted (K-12 *E. coli* chromosome map is available at: http://susi.bio.uni-giessen.de/ecdc/ecmap0.htm). TH265 cells were maintained using LB media (Tryptone 10g/L; NaCl 10g/L; Yeast extract 5g/L; pH = 7.5) (on plates, 15g agar/L) without any antibiotics or supplements. Their *nadC*$^-$ genotype was confirmed by their inability to grow on minimal medium (Yates and Pardee, 1956; Fukuoka *et al.*, 1993) unless supplemented with filter sterile nicotinic acid ($C_6H_5NO_2$), kept as a 0.25M stock (pH = ~6.5), and diluted to 0.025mM for use (pH = ~7). TH265 cells, and *E. coli* DH5α control cells, were made competent and transformed using the heat shock method described by Chung and Miller (1988).

4.1.2 Sequence analysis

Sequencing reactions were performed using 6µl of "BigDye" sequencing premix (supplied by Microbiology Department, Monash University, Australia), 20ng primer, and ~0.1-1µg template DNA in a reaction volume of 15µl. The following temperature regime in the PCR machine was used to carry out the reaction:

96°C for 30 seconds,
50°C for 15 seconds,
60°C for 4 minutes, for 25 cycles, then storage at 4°C.

The whole reaction volume was then precipitated in 50µl 95% Ethanol, and 2µl 3M Sodium Acetate (pH=4.5) at room temperature for 15 minutes. The DNA was pelleted, washed twice in 70% ethanol and dried. The sequencing gels were run and read using the ABI prism system (Perkin Elmer), by the DNA Sequencing Facility, Microbiology Department, Monash University.

Most sequence manipulation and analysis was performed via the Australian National Genome Information Service (ANGIS) (http://www.angis.org.au) The programs MAP, TRANSLATE, REVERSE, GAP, COMPARE, PILEUP, CLUSTL, E-NEIGHBOR, SQUIGGLES, FASTA and BLAST were used. Details on the use of each program can be found at the above web address. Sequencing primers were designed by hand, or with the help of PRIMER-3 (Whitehead Institute/MIT Center for Genome Research; www.genome.wi.mit.edu) and Net Primer (Premier biosoft; www.PremierBiosoft.com). Some additional sequence analysis was done making use of various free web-based databases, the details of which are given in the text.

4.2 Investigating the Genomic arrangement of *QPRTase* genes in *Nicotiana*

4.2.1 Genomic library screen

A genomic library constructed from *N. tabacum* cv. Xanthi in the phage EMBL3 was purchased commercially (Clontech, USA, Cat# FL107d). It had been amplified once, so that the resultant plaques were not all independent. The library was plated using *E. coli* K803 cells in accordance with the manufacturer's instructions, except that the agarose concentration was reduced to 4g/L, and the volume of host cells was reduced ~2 fold, in order to obtain sufficiently large plaques. (Clontech's generic library manual is apparently unsuited to EMBL3, which is an "enfeebled" plaque former with its red⁻gam⁻ genotype (Sambrook *et al.*, 1989)).

Phage DNA was transferred by contact (~2min) to Hybond N+ (Amersham) nylon membranes, in duplicate, and fixed by immersing the membranes in denaturer (1.5M NaCl; 0.5M NaOH; pH ≥ 12) for 2min; neutraliser (1.5M NaCl; 1M Tris-HCl; pH = 8) for 5min, 2XSSC for ~30sec, then air drying and exposing the membranes to UV light for ~2min.

The membranes were prehybridised in 20XSSPE; 7%SDS; 0.5% normal milk powder; 1%PEG 20,000; 0.5mg/mL freshly boiled herring sperm DNA, for 1 hour at 65°C. The *N. rustica* cDNA sequence (Sinclair *et al.*, 2000; Accession: AJ243436) was radiolabelled for use as a probe using the 'Giga-prime kit' (Bresatec (now Geneworks), Australia), and the unincorporated radio-nucleotides removed using a sephadex G-50 column. The purified probe was added directly to the hybridisation chamber. The membranes were hybridised overnight at 65°C, with continual shaking, then washed at high stringency (2XSSC/0.1%SDS for 15min at room temperature; 0.5XSSC/0.1%DSD for 15 minutes at 65°C).

After detection on X-ray film (Fuji, Japan), any positive plaques were picked into SM buffer (0.1M NaCl; $MgSO_4$ 0.01M; 0.05M Tris-HCl), a drop of chloroform was added, and this stock was used to infect the cells in the next round of titering/screening. Three rounds of screening were necessary to purify most plaques, and a fourth was taken to confirm the purity of the stock.

4.2.2 Characterising the genomic library inserts

In order to screen and begin sequencing the inserts, portions of DNA were amplified using PCR between each end of the *QPRTase* coding sequence and the phage arms. The nested reverse primers used to amplify between the 5' end of the coding region and the phage arms were *xqpt*R1 (5'-cttggtggctattgctgaca-3') and *xqpt*R2 (5'-gcactgttgcagtgaaagga-3'), designed using the *N. tabacum QPRTase* cDNA AJ243437. The primers in the phage arms were designed using the EMBL3 database sequences U02425 and U02435. In the left arm, the nested forward primers *embl3*L (5'-gcaactcgtgaaaggtaggc-3') and *em*L2 (5'-atgcccgagaagatgttgag-3') were used, while in the right arm, *embl3*R (5'-aagcagaagtccaacccaga-3') and *em*R2 (5'-aaacatgccacacatgagga-3') were used.

To determine which inserts were independent, and which were equivalent, the phage were cross hybridised with 2 non-conserved 5' flanking regions obtained using the PCR technique just described. In order to do this, an efficient method of screening numerous plaques on the same membrane was devised. An LB agar plate was covered with LB top agarose (0.4%) mixed with the *E. coli* host strain K803. This was allowed to set for ~20mins, and then 5µl of the various un-diluted *QPRTase* phage stocks were added as discrete drops to the surface of the plate. These were allowed to dry. The plate was then incubated overnight at 37°C, allowing small circles of confluent plaque growth to form, each corresponding to a different *QPRTase* phage. Interestingly, many of these appeared as hollow rings of plaques, rather than solid circles (See Figure 2.2.1). It is unknown whether this was caused by physical (*eg*; gravity pulling surface-borne phage to the periphery of the drop) or biological factors (*eg*; aspects of the growth kinetics of phage).

These plates were then blotted, as described above (4.3.1) and the membranes were probed using the non-conserved 5' flanking regions as DNA probes. These were purified from agarose gels. The DNA band was first excised from the gel with a scalpel, and an equal volume of phenol was added. This was mixed using a bench 'vortex' for ~10min. The homogenised samples were frozen at ~70°C for ~1h, and then melted at ~60°C. The aqueous layer was separated by centrifugation at 13,000rpm for 30min, and removed to a new tube. This was extracted twice with chloroform, and the DNA precipitated from the final aqueous phase by adding 2vols

ethanol and 0.1vol sodium acetate. The resultant DNA was radio-labelled as described above (4.3.1).

The inserts contained in selected plaques were sequenced using a series of PCR products as templates. These were amplified using various PCR temperature programs based on the following:

92°C 30 seconds
~52°C 1 minute
70°C 1 minute (+ 1 additional minute for each kb of expected fragment length)
for 30 cycles.

The PCR fragments were purified (as described above) and sequenced (Materials and Methods 4.2.2).

4.2.3 Genomic Southern blot analysis

In order to detect the presence of the *QPRTase* genes in the genomes of *Nicotiana* species, Southern blot analysis was undertaken. Genomic DNA from *N. tabacum* cv. Xanthi, *N. tabacum* cv. NC95, *N. sylvestris* and *N. tomentosiformis*, was kindly provided by fellow graduate student Karen Cane. 15µg of DNA was digested overnight at 37°C using 50 units of *Hind*III (Promega, USA) in a total volume of 400µl, containing 0.25mM spermidine, 20µg BSA (Promega), and 1X the buffer supplied by Promega (*ie*; Promega buffer 'E' in the case of *Hind*III). The digested DNA was precipitated from the reaction using 100% ethanol, pelleted, and resuspended in 20µl of water, before being electophoresed.

After electrophoresis, the DNA was blotted onto Hybond N+ (Amersham) nylon membranes using the capillary transfer method, described elsewhere (Sambrook *et al.*, 1989; Hamill and Lidgett, 1997). DNA probes were radio-labelled and purified as described above (4.3.1). Pre-hybridisation and hybridisation were carried out using ExpressHyb solution (Clontech, USA), as described by the manufacturer. Signals were detected using X-ray film (Fuji, Japan) and a Molecular Dynamics (USA) Storm phosphor imager and the associated IMAGEQUANT software.

4.2.4 Northern analysis with oligonucleotide probes

In order to determine the gene-specific expression profiles of the two *QPRTase* gene classes, extracted RNA was probed with short oligonucleotide probes (*oQPT1* and *oQPT2*) which were able to distinguish *NtxQPT1* transcripts from *NtxQPT2* transcripts, respectively. Their sequences are complementary to the regions noted in Figures 2.2.7 and 2.2.8. These oligonucleotides were designed carefully, to have similar TMs (61 and 62°C respectively), and to avoid any uneven cross hybridisation.

Nicotiana tabacum cv. Xanthi plants were grown hydroponically as described below (Materials and Methods 4.4.1). When the wounding experiment commenced, they were ~50 days old. Four plants were wounded as described below (Materials and Methods 4.4.1), and four left undamaged. Twenty four hours later, their leaves and roots were harvested, as described below (Materials and Methods 4.4.1).

RNA extraction and Northern blotting was carried out exactly as described below (4.3.2). Oligonucleotides to be used as probes were end-labelled using terminal deoxynucleotidyl tranferase (TdT) (Promega, USA). 50 units TdT were added to ~150ng of oligonucleotide, 2mg BSA, 8µl 5XTdT buffer (Promega) and 10µl of ($^{\alpha 32}$P)dATP in a total volume of 40µl. This reaction was incubated at 37°C for ~1h, and then the labelled oligonucleotides were separated from un-incorporated nucleotides using a sephadex G-50 column. Trials confirmed that sephadex G-50 was suitable for separating ~30mers from the un-incorporated radio-nucleotides. This labelling technique is modified from the report of Henderson and co-workers (1991). Pre-hybridisation and hybridisation were carried out using ExpressHyb solution (Clontech, USA), as described in the manufacturer's specific instructions for probing using oligonucleotides. The washing conditions were established by trial and error, based on the report of Henderson and co-workers (1991). Membranes were washed in the hybridisation bottle once, as it cooled to room temperature, in 2XSSC/0.05%SDS, then in the same solution at room temperature with shaking for 30 min, and then again for 10 min. Finally, the membranes were washed at 37°C for 25 min in 0.1XSSC/0.1%SDS. The signals were detected and quantified using a Molecular Dynamics (USA) Storm phosphor imager and the associated IMAGEQUANT software. Because actual numerical estimates of transcript

abundance were to be calculated, no standardisation to account for loading errors was undertaken. Standardisation was included for all other Northern blots in this study, where relative levels only were required. Thus, the variation introduced by loading error must be assessed independently, with reference to a UV-illuminated gel photograph.

Two other methods were also used to assess the relative transcript levels of *NtxQPT1* and *NtxQPT2*. Because *NtxQPT1* could not be detected in roots or leaves using the method just described, it was hoped that it could be detected in RNA samples enriched for mRNA. Most samples did not contain enough total RNA to warrant mRNA purification (mRNA makes up <5% of total RNA in most cells; Sambrook *et al.*, 1989; Leary *et al.*, 1998; Wilkinson, 2000), with the exception of the *N. sylvestris* standards, which were extracted from large volumes of control and wounded root tissues (described below Materials and Methods 4.4.2). mRNA was purified from the wounded standard (Std. B) using oligo $(dT)_{25}$ tagged magnetic beads (Dynabeads, Dynal, Norway), according to the manufacturers instructions. It was found that from 375µg of total RNA, ~3µg mRNA were obtained. mRNA was electrophoresed, blotted and probed as described immediately above. *NtxQPT1* transcripts were also sought among 12 *N. tabacum QPRTase* cDNAs which were previously isolated, but never characterised (Sinclair, 1998; Sinclair *et al.*, 2000). These were cultured and screened using *oQPT1* and *oQPT2* as probes, in the same manner as the genomic library plaques, described in Materials and Methods 4.3.2.

4.3 Measuring biosynthetic gene expression and alkaloid accumulation

4.3.1 The cultivation of plants

The seeds of *N. sylvestris* were purchased commercially (Fothergills, UK). *N. hesperis* seeds were originally obtained from the United States Department of Agriculture (Parr and Hamill, 1987). *Nicotiana tabacum* cv. Xanthi seeds were obtained from CSIRO Plant Industries, Canberra. *Nicotiana alata* and *N. glauca* seeds were kindly supplied by Mr B. McGuiness, University of Melbourne, Australia. They were germinated in punnets of seed raising mixture (Debco) with a small amount of perlite (Chillagoe), and a few pellets of "Osmocote" slow-release fertiliser

(The Scotts Company). Glasshouse conditions have been described previously (Lidgett *et al.*, 1995). When they were ~2cm high, the seedlings were supported in rockwool fibre pellets (Growool Horticultural Systems, Australia) in plastic tubs 11.5cm diameter at the rim, 8.5cm diameter at the base, and 4.5cm deep, with ~2cm of Hoagland's hydroponic medium covering the roots (Mason, 1990). A ventilated cover on the tub minimised light penetration and prevented algal growth. Plants were randomly assigned to treatments and bench positions. Plants were ~6 weeks old and had begun to bolt when experiments commenced, after which time no further nutrients were supplied to the plants, only deionised water.

Other researchers in this laboratory (Murphy, 1999) have grown their plants in communal trays of nutrient solution. Anecdotal observations suggest that this method facilitates faster plant growth. The individual-pot method employed here, however, was developed to allow the plants to be randomised in their bench positions, and hence for them to be treated as independent replicates, and also to prevent plants from communicating a wound signal through the liquid medium.

Three control plants were harvested at 0h, immediately followed by the wounding of 'wounded' plants, at about midday. Plants were wounded by running a fabric pattern wheel three times down each side of the lamina of the upper 3 fully-expanded leaves, puncturing numerous holes in the leaf, imitating insect attack. This method has also been employed by other laboratories (Baldwin *et al.*, 1994b). In addition, the apices were pinched out using gloved fingers, to imitate the procedure of "topping" which is also used to induce alkaloid accumulation (Baldwin 1988b).

Three unwounded control and three wounded plants were harvested at 24h, 72h, and 168h; times chosen based on previous studies (*eg*; Mitzusaki *et al.*, 1973; Saunders and Bush, 1979; Baldwin, 1989). During harvesting, the midribs were removed and discarded. The flat green laminas were then each diced into ~6-8 small slices, before being mixed together haphazardly and frozen in ~0.5-~0.75g lots; one sample for RNA analysis, one for alkaloid analysis. The root system was similarly blotted dry and divided for alkaloid and RNA analysis, ensuring (somewhat subjectively) that each sample contained an equal portion of root tips and older roots. The precise portions of tissue types included within each sample are likely to be important. Root tips, for example, are known to produce more nicotine than fully formed roots (Dawson, 1962; Baldwin, 1988b), and the leaf edges are known to accumulate more alkaloids than the inner areas of the lamina (Burton *et al.*, 1992). It

is, however, difficult to snap freeze all samples at their correct time of harvest, and homogenise them so that each sample is representative of its tissue type. The 'dicing' and 'splitting' method described here is a practical, albeit imperfect solution to this problem.

The leaves harvested from wounded plants were those individual leaves which were damaged. Since *QPRTase* was found, in preliminary studies to be active in wounded *N. glauca* leaves (Murphy, 1999; Sinclair *et al.*, 2000), it was decided to also harvest lower, always unwounded, leaves in this species (leaves 6-9 from apex) to test for leaf-leaf signalling or transport.

4.3.2 Northern analysis

RNA extraction and Northern analysis were undertaken as described elsewhere (Hamill and Lidgett, 1997) with only one modification to this procedure. RNA samples were found to be stable for months in ethanol at –20°C. This allowed the samples to be ground over a period of days, then stored in ethanol, and precipitated together. This allowed all RNA samples to be quantified by spectrophotometry, diluted and electrophoresed on the same day, removing the need for re-freezing and re-thawing of the RNA samples, and overcoming any error existing between different quantification runs. Hybridisation was undertaken using ExpressHyb (Clontech), according to the manufacturer's instructions. Band intensities on Northern blots were quantified using a Molecular Dynamics (USA) Storm phosphor imager and the associated IMAGEQUANT software.

The *QPRTase* probe was the cDNA reported from *N. rustica* (Sinclair *et al.*, 2000; Accession: AJ243436). The *PMT* probe (Hibi *et al.*, 1994) was kindly supplied by Prof. T. Hashimoto, Nara Institute of Technology, Japan. The *ODC* probe was from a *N. tabacum* cDNA cloned in this laboratory (Lidgett 1997; Accession: CAA71498). The '*A622*' probe was a PCR product amplified from *N. tabacum* root cDNA (nucleotides 71 –1015 in Hibi *et al.*, 1994; confirmed by sequencing). Band intensities were normalised using the signal from an *Antirrhinum majus* L. ubiquitin probe, which hybridises to a transcript not affected by wounding in tobacco species (Lidgett *et al.*, 1995).

Comparisons were made between blots by loading aliquots of a shared sample onto each blot, providing a common internal 'reference point'. RNA samples from roots of mature *N. sylvestris* plants (both control (Std. A) and wounded (Std. B)) were used. In the histograms presented in Results 2.3, the relative transcript levels of each gene are expressed numerically, in relation to the un-wounded *N. sylvestris* root standard (Std. A) in terms of relative transcript number per total mass of RNA. The numerical value for transcript level was determined in two steps. First, every raw transcript measurement was corrected for uneven loading by dividing its value by the signal obtained for ubiquitin in that same lane. At this point, the standards are treated no differently than the test samples. Next, all values are divided by the standard's value, such that the standard is expressed as '1.0'. These values permit direct comparisons to be made between experiments for a given transcript, but do not permit direct comparisons to be made between different transcripts which have been detected by different probes.

This analysis is based on the assumption that sequence divergence does not cause the under-estimation of levels of more distantly related transcripts. This assumption is likely to be essentially valid, since the known *QPRTase* sequence from *N. tabacum*, *N. rustica*, *N. alata* and *N. glauca* are all >94% identical (Sinclair *et al.*, 2000; Murphy, 1999; Johnson, 2000; DeBoer, 2001).

4.3.3 Alkaloid analysis

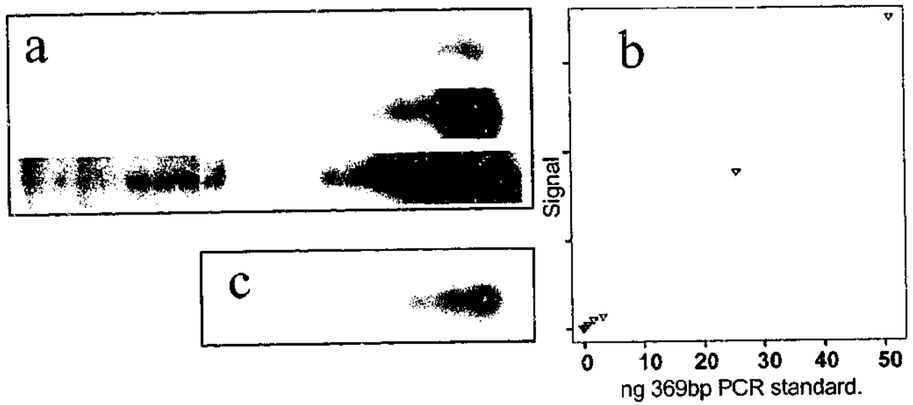
The Plants used for alkaloid analysis were the same plants as those used in RNA analysis (Materials and Methods 4.4.2). Alkaloids were analysed according to Saunders and Blume (1981). Approximately 0.2g of freeze-dried tissue was homogenised at room temperature using a 'Polytron' (Kinematica AG, Switzerland), in ~5ml of extraction buffer. Samples were analysed using a Waters (USA) 600E HPLC with a Waters 486 absorbance detector (260nm).

Standard curves were constructed using authentic samples of nicotine (Sigma), nornicotine (Sigma), anabasine (Sigma) and anatabine (kindly supplied by Dr Patrick Lippiello, R. J. Reynolds Ltd, USA). Identities of sample peaks were confirmed by spiking with authentic alkaloid standards.

Peaks representing less than ~0.01 mg alkaloid/g dry weight are referred to as traces. This limit of detection corresponds with similar studies (Sisson and Severson, 1990).

Appendix 1

Standards for the quantification of *NtxQPT1* and *NtxQPT2* transcripts



Examples of standard curves used to calculate molar amounts (and hence numbers of molecules) in RNA samples, as presented in Figure 2.2.3. Panel (a) shows a blot probed with *oQPT2*. On the right hand side, the standard curve is visible. The same blot is presented 3 times, adjusted to different display levels using the Molecular Dynamics software (ImageQuant), in order to show the large range of DNA concentrations detected. The left of the panel shows the actual RNA samples, at the same signal intensities as the standard curves. Panel (b) shows the signal intensities measured by phosphorimaging plotted against the mass of DNA in the same standard curve shown in panel (a). It is important to note that the standard curve is linear throughout its range, despite a 250 fold variation in DNA quantity. Panel (c) shows an example of a standard curve probed with *oQPT1*, used to determine the limit below which any *NtxQPT1* mRNA levels must fall. An oligonucleotide probe (*oQPTs*; 5'-gctgtaattgcatRaggRtgcactRtWgca-3') which hybridised to a region conserved between exon 1 of *NtxQPT1* and *NtxQPT2* was used to verify that the *NtxQPT1* and *NtxQPT2* standard curves were comparable in the amount of DNA added.

Appendix 2

Putative response elements not discussed in Results 2.2.5 or 2.2.6

The databases used to search the 5' flanking sequences for putative response elements returned several hundred results, few of which will be of relevance *in vivo* in *NtxQPT1*. Those results which were initially considered in detail (because they were related to a wound- or jasmonate-response etc.), but thought unworthy of inclusion on Figures 2.2.7 and 2.2.8 as putative response elements are detailed below.

Putative elements identified in NtxQPT1:

- *Wound-, elicitor- and jasmonate-response elements:*

Both fungal elicitors and wounding may be detected via a jasmonate signal cascade (Menke *et al.*, 1999a; Baldwin *et al.*, 1997). *Nicotiana* pyridine alkaloids are known to be induced by wounding and to applications of jasmonates. Given their close association, the putative elements thought to be related to wound-, elicitor- or jasmonate-induction are discussed here together.

A sequence similar to an elicitor-responsive element (EIRE), in the promoter of the pathogenesis related protein (PR-2d) gene of *N. tabacum* (Shah and Klessing, 1996) was noted in *NtxQPT1*. The sequence in *NtxQPT1* lacks the CC di-nucleotide and an adjacent GGGG-motif known to be involved in binding nuclear extract (Shah and Klessing, 1996), and has not been considered further here.

NtxQPT1 contains a sequence similar to a putative element (WAR) in the *extensin A* promoter of canola (*Brassica napus* L. Brassicaceae) (Elliot and Shirsat, 1998), which is expressed in response to tensile stress, wounding, jasmonic acid and elicitors, in roots, stems, leaves and carpels. The *NtxQPT1* sequence has not been considered further because it is not palindromic, unlike WAR.

The wound and/or elicitor response elements denoted "WUN", "WUN-1" and "ELI-box" by PlantCARE are only supported by a secondary reference (Pastuglia *et al.*, 1997). This work does not include any experimental promoter delineation, and provides misleading references to the elements mentioned, none of which define the

elements as implied. These putative elements were not considered further due to the lack of any supporting experimental data.

- *Auxin responsive elements:*

It is known that auxins suppress nicotine production, both *in planta* and when added to cell cultures, however the mechanism is not understood. Auxins are known to decrease jasmonic acid levels as well as alkaloid synthesis, and can effectively stop the wound response (Baldwin, 1989). Auxins can also suppress nicotine production and *QPRTase* activity in cell cultures, possibly via an indirect effect on cell growth and differentiation (Feth *et al.*, 1986). It is unclear whether auxins have any effect on *QPRTase* transcription, other than through interaction with other hormones. The databases identified 6 classes of putative auxin response elements. All are known to induce, rather than repress, gene expression in the presence of auxins, and each one of them has been shown to be a component of a larger sequence motif, which *NtxQPT1* does not possess (Lam *et al.*, 1989; Liu *et al.*, 1994; Sakai *et al.*, 1996; Xu *et al.*, 1997). None of these elements are thought likely to be relevant to *NtxQPT1* expression.

- *Solanaceae-active response elements:*

Some elements from *Solanum tuberosum* L. (potato) were noted as being similar to segments in the *NtxQPT1* promoter. These elements were portions of complex light responsive elements, which consist of modular parts (Arguello-Astroga and Herrera-Estrella, 1996). Although nicotine levels fluctuate diurnally, peaking at midnight (Wink, 1997), this fluctuation is not known to be related to gene transcription. The light-responsive elements have not been considered in detail.

Nine regions similar to previously noted TC-rich repeats (consensus ATTTTCTT(C/A)(C/A) Klotz and Lagrimini, 1996) were identified by PlantCARE. Similar elements have been noted in the *N. tabacum* anionic peroxidase promoter, which is stress-induced, and may be related to wound repair. These TC-rich repeats were, however, not characterised functionally, and were themselves only highlighted because they showed homology to a 10bp motif (consensus TCATCTTCTT) found in

many plant stress-related promoters. The *NtxQPT1* sequences hardly resemble this characterised element, and are not regarded further here.

Both PLACE and PlantCARE highlighted the motif 5'-CAAAAATATG-3' occurring in *NtxQPT1* as being identical to a putative circadian clock motif 5'-CAA$(N)_4$ATG-3' (Piechulla *et al.*, 1998). This motif has not been functionally defined, but is present in 13 different promoter segments which direct circadian expression in *N. tabacum*. Given that the original element has not been functionally characterised, and that *QPRTase* transcription is not known to show a circadian rhythm (Murphy, 1999; Sinclair *et al.*, 2000), this element has not been considered further.

Another putative element located by PLACE was represented by 3 5'-ATAGAA-3' motifs. These have not been considered further here because they are plastid derived sequences (Kapoor and Sugiura, 1999), while previous Southern blot evidence in the tetraploid *N. tabacum* is consistent with *QPRTase* being a nuclear encoded gene (Sinclair *et al.*, 2000; Karen Cane, in preparation).

PLACE also identified a series of sequences in the *NtxQPT1* promoter which correspond to 5'-(A/T)AAG-3' motifs noted previously in the promoter of the guard cell specific *KST1* gene of *S. tuberosum* (Plesch *et al.*, 2001). This element has not been considered further since *NtxQPT1* expression is not thought to be restricted to guard cells.

The AGAAA-element identified by PLACE was originally defined in the promoter of the *lat52* gene of tomato, which directs pollen specific reporter gene expression in tomato and tobacco (Bate and Twell, 1998). Another putative element was noted as being similar to the GTGA-motif from the *g10* gene of *N. tabacum*, which also directs pollen specific gene expression (Rogers *et al.*, 2001). These elements have not been investigated further due to their pollen-specificity. The AGAAA-element can further be discounted because *in situ* it merely enhances the strength of an adjacent element (Bate and Twell, 1998).

PLACE highlighted a region in *NtxQPT1* identical to the SP8b sequence (5'-TACTATT-3') in the promoters of genes encoding B-type sporamin and a subunit of β-amylase from sweet potato (*Ipomea batatas* L.; Convolvulaceae) (Ishiguro and Nakamura, 1992). SP8b is bound by the uncharacterised transcription factor(s) SP8BF (Ishiguro and Nakamura, 1992). SP8BF is found in sweet potato tuberous

roots and petioles, and in the stems of *Nicotiana tabacum* (Ishiguro and Nakamura, 1992).

In the *NtxQPT1* promoter, the sequence 5'-AAGAATAGAAAAA-3' is identical to the SURE-1 sequence known to be involved in sucrose induction of the largely tuber-specific *patatin* gene of potato *Solanum tuberosum*. SURE-1 forms part of an assemblage of repeated elements, which confer tuber specificity and sucrose-inducible expression (Grierson *et al.*, 1994). The SURE-1 element is not a likely regulatory element in *NtxQPT1* because the surrounding DNA elements are not present, and *QPRTase* expression is not known to be influenced by sucrose.

- *Response elements in* NtxQPT1 *showing high similarity to known elements:*

When searching the TRANSFAC database, it is possible to set a threshold such that only sequences with substantial sequence homology to known elements are noted. The motif CATTTATTG in *NtxQPT1* closely resembles the binding sites of the *Arabidopsis* homeodomain (HD) and a leucine zipper (Zip) domain transcription factors Athb-1 and –2 (5'-CAAT(A/T)ATTG-3' and 5'-CAAT(G/C)ATTG-3' respectively) (Sessa *et al.*, 1993). The *NtxQPT1* motif is unlikely to be related to either of the above binding sites, since Sessa and co-workers (1993) tested many potential binding domains, and found that the central residues were essentially invariant.

Putative elements identified in NtxQPT2*:*

- *Wound-, elicitor- and jasmonate-response elements:*

Two sequences found in *NtxQPT2* resemble an elicitor-responsive element repeated in the promoter of the chalcone synthase 1 gene in pea (*Pisum sativum* L. Fabaceae) (Seki *et al.*, 1996). In pea, the sequence 5'-TAAAATACT-3' appears twice, 16bp apart, and deletion analysis shows that at least the promoter-distal copy is required for elicitor induction. The sequences in *NtxQPT2* are slightly different, (5'-TAAAAAAACT-3' and 5'-TAAAATAGT-3') and are ~731bp apart.

- *Auxin responsive elements:*

The *NtxQPT2* promoter contains a sequence resembling one component of the auxin responsive promoter of the *parC* gene of *N. tabacum* (a gene resembling *parB*, which encodes glutathione-*S*-transferase). This putative element in *NtxQPT2*, however, is unlikely to be relevant, given that it lacks a nearby *as-1*-like sequence known to be essential for auxin responsiveness, and also possess a slightly different sequence from the defined element (Sakai *et al.*, 1996).

- *Solanaceae-active response elements:*

Several regions within *NtxQPT2* resemble the 5'-CTCAYTYY-3' motif found overlapping the transcription start site in some *N. tabacum* light responsive genes which lack a TATA box (Nakamura *et al.*, 2002). The sections of *NtxQPT2* resembling this motif are far upstream from the transcribed region.

Many putative response elements noted above from *NtxQPT1* were also noted in *NtxQPT2*. The following elements were discounted as irrelevant in *NtxQPT2* for the same reasons as they were in *NtxQPT1*:

- (A/T)AAG element of *KST1* (Plesch *et al.*, 2001)
- SURE-1 element of *patatin* (Grierson *et al.*, 1994)
- SP8b element (Ishiguro and Nakamura, 1992)
- AGAAA element of *lat52* (Bate and Twell, 1998)
- GTGA element of *g10* (Rogers *et al.*, 2001)
- ATAGAA element (Kapoor and Sugiura, 1999)
- Circadian motif CAA(N)$_4$ATG (Piechulla *et al.*, 1998)
- WAR element (Elliot and Shirsat, 1998)
- Light responsive element (described by Sakai *et al.*, 1996)

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Plant Molecular Biology **44:** 603–617, 2000.

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

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Received 3 February 2000; accepted in revised form 19 July 2000

Key words: gene expression, nicotinic acid, pyridine alkaloids, secondary metabolism, polyploidy, wound-induced

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; QRPTase, quinolinate 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 regulated 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)

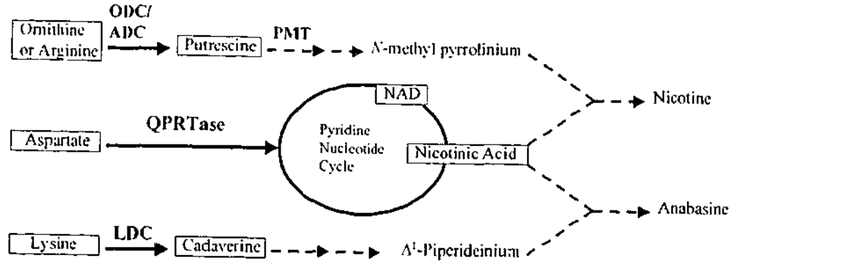

Figure 1. A simplified diagram of nicotine and anabasine synthesis in *Nicotiana*. Steps representing primary metabolism are shown with full arrows, whereas steps involved in the production of secondary metabolites are shown with dotted arrows. Similarly, primary metabolites are boxed, while secondary 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 titred 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 $\alpha[^{32}]$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× 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 μg/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 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 *Eco*RI/*Xba*I 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 *Bam*HI/*Eco*RI 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.

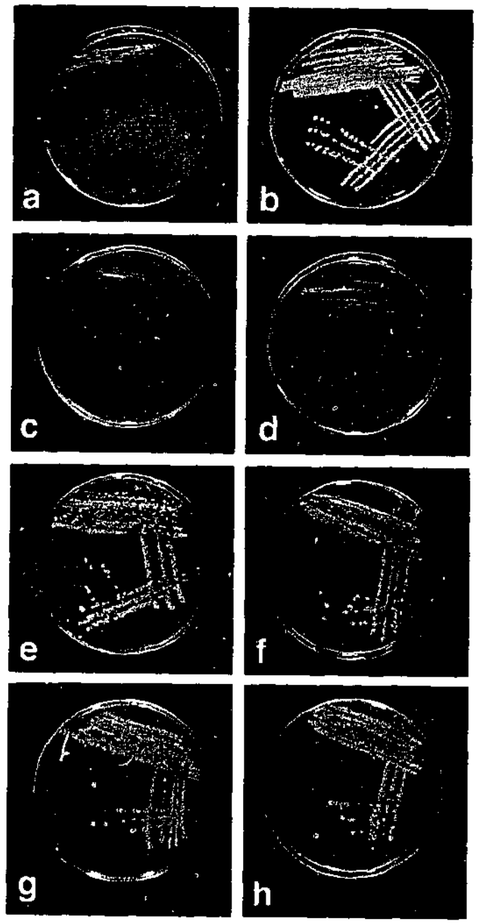

Figure 2. Complementation of QPRTase-deficient (*nad C*$^-$) *E. coli* strain TH265 with *Nicotiana* cDNAs encoding QPRTase. a. TH265 cells cultured on minimal medium. b. TH265 cells cultured on minimal medium containing 0.2 μg/ml nicotinic acid. c. TH265 cells containing pBluescript cultured on minimal medium. 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.

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 cell's 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

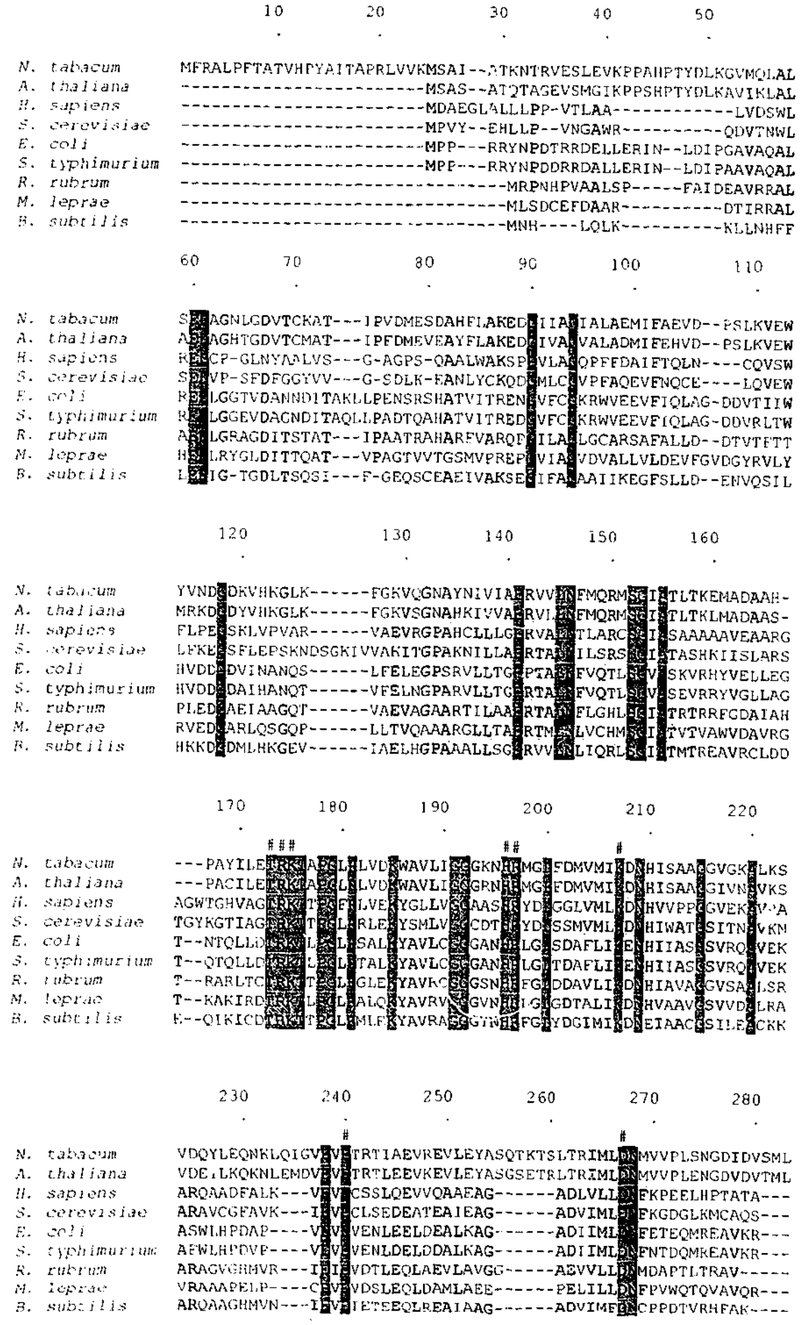

Figure 3. Alignment of the deduced amino acid sequence of *N. tabacum* QPRTase (TQPT1, 351 amino acids) with other QPRTase sequences. *Arabidopsis thaliana* (AC006200), *Homo sapiens* (D78177), *Saccharomyces cerevisiae* (P43619), *Escherichia coli* (S05571), *Salmonella typhimurium* (L07292), *Rhodospirillum rubrum* (U20508), *Mycobacterium leprae* (P46714), and *Bacillus subtilis* (D43071). 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 #.

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                    290         300         310         320         330         340
                     .           .           .           .           .           .
                                 #
N. tabacum      KEAVELING--RFDTGASGNVTLETVHKIGQTGVTYISSGALTQSVKALGISLKIDTELA
A. thaliana     KDAVELING--RFETGASGNVTLETVHKIGQSGVTFISSGALTHSVKALGISLKIDTELA
H. sapiens      --LKAQFPS---VAVGASGGITLDNLPQFCGPHIDVISMGMLTQAVPALGFSLKLFAKEV
S. cerevisiae   --LKNKWNGKKHFLLGCAGGLNLDNLEEYLCDDIDIYSTSSIHQGTPVIGFSLKL-----
E. coli         ------TNG--KALLGVAGNVTDKTLREFAETGVDFISVGALTKHVQALGLSM-------
S. typhimurium  ------VNG--QARLGVAGNVTAETLREFAETGVDFISVGALTKHVRALGLSM-------
R. rubrum       ----DMVAG--RLVTGASGGVSLDTIAALAESGVDYISVGALTHSVTTLGIGL--DIVVA
M. leprae       ---RDIRAP--TVLLGSAGGLSLENAAIYAGTGVDYLAVGALTHSVRILGIGL--DL*--
B. subtilis     -----LTPA--NIKTGASGGITLESLPAFKGTGVNYISLGFLTHSVKSLGI*--------

                    350
                     .
N. tabacum      LEVGRRTKQA*
A. thaliana     LEVGRRTKRA*
H. sapiens      APVPKIH*---
S. cerevisiae   -----AH*---
E. coli         RFR*-------
S. typhimurium  RFC*-------
R. rubrum       PPKAERA*---
M. leprae       ----------
B. subtilis     ----------
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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).

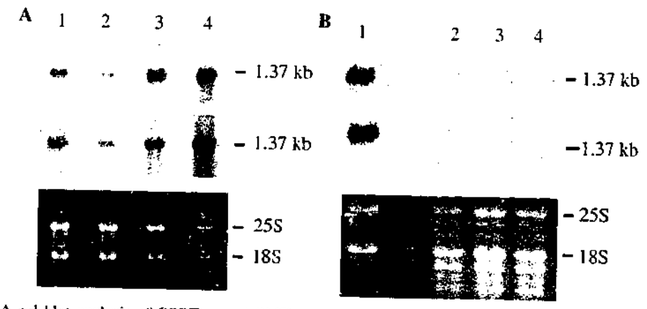

Figure 4. Northern/RNA gel blot analysis of QPRTase and PMT transcript levels in *N. tabacum*. Aerial tissues were wounded as described in Materials and methods and RNA was extracted from roots and leaves 24 h after wounding. The upper blot in each panel shows the signal obtained by probing the membrane 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′

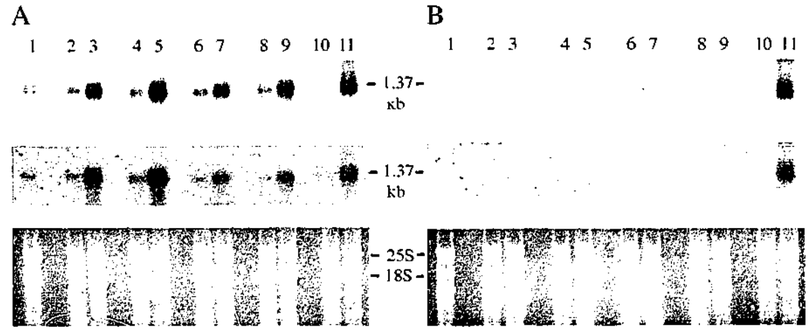

Figure 5. Northern/RNA gel blot analysis of QPRTase and PMT transcript levels in control (unwounded) and wounded plants of *N. sylvestris*. Aerial tissues were wounded as described in Materials and methods and RNA was extracted from roots (panel A) and leaves (panel B). The upper blot in each panel shows 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.)

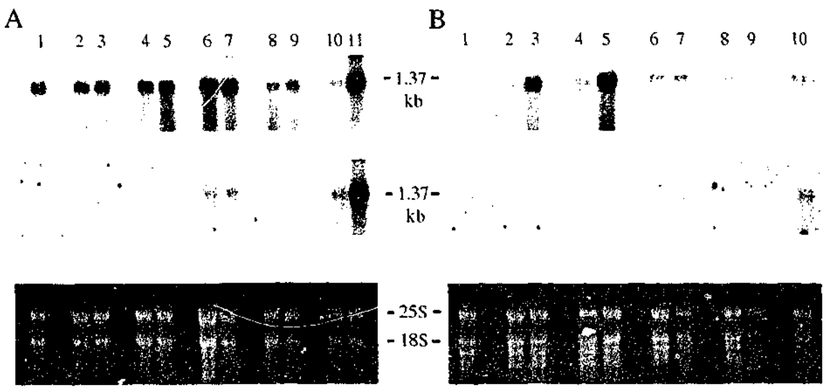

Figure 6. Northern/RNA gel blot analysis of QPRTase and PMT transcript levels in control (unwounded) and wounded plants of *N. glauca*. Aerial tissues were wounded as described in Materials and methods and RNA was extracted from roots (panel A) and leaves (panel B). The upper blot in each panel shows 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*.)

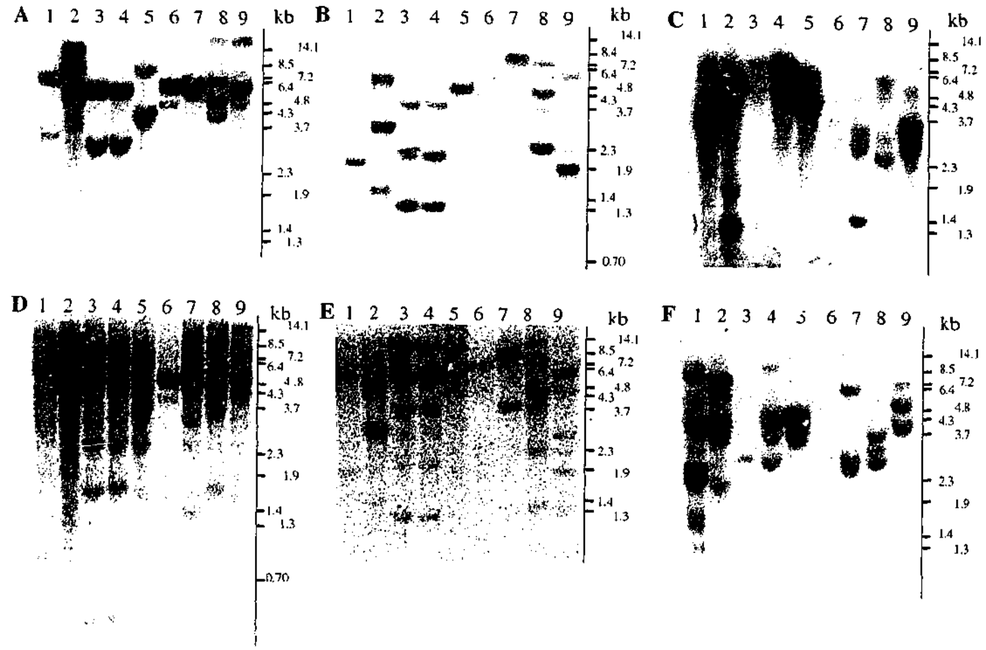

Figure 7. Southern analysis of QPRTase in a range of *Nicotiana* species. Genomic DNA from a number of *Nicotiana* species was digested with a range of restriction enzymes and agarose gels were loaded as follows. Lanes: 1, *N. glauca*; 2, *N. rustica*; 3, *N. tomentosiformis*; 4, *N. tabacum* cv. NC95; 5, *N. sylvestris*; 6, *N. langsdorfii*; 7, *N. longiflora*; 8, *N. alata*; 9, *N. hesperis*. Membranes containing DNA digested with *Xba*I (A), *Hin*dIII (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, *Hin*dIII). 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 an 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 profile 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 lev[illegible] 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]).

Acknowledgements

We are grateful to Prof. Hughes for *E. coli* strain TH265; Prof. Hashimoto for plasmid PET-PMT; Dr

A. Lidgett for construction of the *N. tabacum* root cDNA library; Dr V. Krishnapillai, Dr A. Neale and Prof. K. Nakamura for discussions; Y. Chintapakorn and K. Cane for practical assistance and advice. The receipt of ARC grant A19701779 is also gratefully acknowledged.

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