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Phytochemistry 68 (2007) 2465–2479
PHYTOCHEMISTRY
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Antisense-mediated reduction in ADC activity causes minor alterations in the alkaloid profile of cultured hairy roots and regenerated transgenic plants of *Nicotiana tabacum*

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Received 6 June 2006; received in revised form 11 May 2007
Available online 5 July 2007

Abstract

In species of the genus *Nicotiana*, as in most plants, the important polyamine precursor putrescine can be derived from the amino acids ornithine and/or arginine *via* the activity of ornithine decarboxylase (ODC) and/or arginine decarboxylase (ADC), respectively. *Nicotiana* species also utilize putrescine to provide the pyrollidine ring of the defensive alkaloid nicotine and its derivatives. Previous biochemical studies, involving callus tissues cultured *in vitro*, suggested that the ADC-mediated route to putrescine is used preferentially to provide the putrescine that is utilized for nicotine synthesis in *N. tabacum*. To ascertain if this is the case in *N. tabacum* plants, where nicotine synthesis takes place exclusively in roots, we used an antisense approach to diminish ADC activity in transformed roots which were cultured *in vitro*. Several independent lines were recovered possessing markedly reduced levels of ADC transcript and ADC activity compared to controls. Transcript levels of other genes in this general area of metabolism, including *ODC*, were not altered as a result of the antisense-mediated downregulation of ADC. Concentrations of nicotine were comparable in antisense-ADC and control hairy root lines throughout most of their respective culture cycles, except at the latter stages of growth when the nicotine content of antisense-ADC hairy root lines was observed to be ~20% lower than in controls. Levels of anatabine, the second most abundant alkaloid typically found in *N. tabacum*, which is not derived from putrescine, were slightly elevated in two antisense-ADC hairy root lines at the latter stages of their culture cycles compared to controls. Comparison of alkaloid levels in leaves of transgenic plants that were regenerated from separate antisense-ADC and control transformed root lines indicated that the former possessed slightly elevated levels of anatabine but did not contain average levels of leaf nicotine that were different from that of controls. Our overall conclusion is that the ADC mediated route to putrescine plays a role, but is not of prime importance, in providing the pyrollidine ring which is used for nicotine synthesis in cultured hairy roots of *N. tabacum* and in roots of healthy greenhouse-grown plants.

Keywords: Alkaloid; Arginine decarboxylase; Anatabine; Antisense; Hairy roots; Metabolism; *Nicotiana*; Nicotine; Solanaceae; Tobacco

1. Introduction

The plant kingdom contains tens of thousands of secondary (natural) metabolites, most of which are likely to be important in the eco-physiology of host plants and a considerable number of which are also utilized by mankind as medicines, toxins, flavorings and fragrances (Luckner, 1990; Croteau et al., 2000; Wink, 2003; Barnes and Prasain, 2005). There is increasing interest in using gene-based strategies to address fundamental questions relating to the genetic and biochemical controls that regulate secondary

Abbreviations: ADC, arginine decarboxylase; DFMA, DL-α-difluoromethylarginine; DFMO, DL-α-difluoromethylornithine; GUS, β-glucuronidase; HPLC, high performance liquid chromatography; ODC, ornithine decarboxylase; PMT, putrescine *N*-methyltransferase; QPT, quinolinate phosphoribosyltransferase; SAMDC, *S*-adenosylmethionine decarboxylase; SAMS, *S*-adenosylmethionine synthase; SPDS, spermidine synthase.

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doi:10.1016/j.phytochem.2007.05.025

metabolism and to also explore the capacity for metabolic engineering of plant biosynthetic pathways for commercial utilization (Memelink, 2005; Dixon, 2005).

Alkaloids represent one of the largest and most diverse groups of natural products in the plant kingdom with more than 12,000 structures having been described (Kutchan, 1995, 1998; Southon and Buckingham, 1989; De Luca and St Pierre, 2000). The bitter taste and/or physiological activity of many alkaloids render them effective as chemical defense agents against potential insect and vertebrate predators. Some alkaloids also have been shown to exhibit allelochemical activities, being inhibitory to the growth of competitors that are in the vicinity of plants which produce them (Lovett and Hoult, 1998; Wink, 1998b, 2003). Most alkaloids contain one or more heterocyclic nitrogen atoms derived from a limited number of amino acids (Roberts and Wink, 1998; Wink, 1998a, 2003; Croteau et al., 2000). With a capacity to constitute several % of the dry weight of plant tissues in some species, production of alkaloids can also represent a significant allocation of nitrogenous resources that otherwise might be utilized for primary metabolic purposes to maximize growth and reproduction (Baldwin and Ohnmeiss, 1994). It is becoming clear that the relationships between primary and secondary metabolism in alkaloid-producing tissues are influenced by a complex interplay of processes operating at the genetic and biochemical/cellular level with strong influences also from a range of developmental, physiological and environmental factors (Hughes and Shanks, 2002; Kutchan and Dixon, 2005; Kutchan, 2005).

The diamine putrescine, and its polyamine derivatives spermidine and spermine are small, aliphatic and positively charged amines that are important components of primary metabolism in bacteria, fungi, plants and animals (Tiburcio et al., 1997). In most plants, (though apparently not in the model plant *Arabidopsis thaliana*, (Hanfrey et al., 2001)), putrescine is synthesized by ODC-mediated decarboxylation of ornithine and via an alternative route, which involves ADC-mediated decarboxylation of arginine as the first step (Malmberg et al., 1998; Kakkar and Sawhney, 2002) (Fig. 1). Although, biochemical investigations have produced some conflicting results, collectively they have suggested that the ODC-mediated route to putrescine is particularly important in providing polyamines for normal cellular division, differentiation and development in most plants whilst putrescine which is synthesized via the ADC-route is necessary for cell expansion and environmental stress responses (Malmberg et al., 1998; Martin-

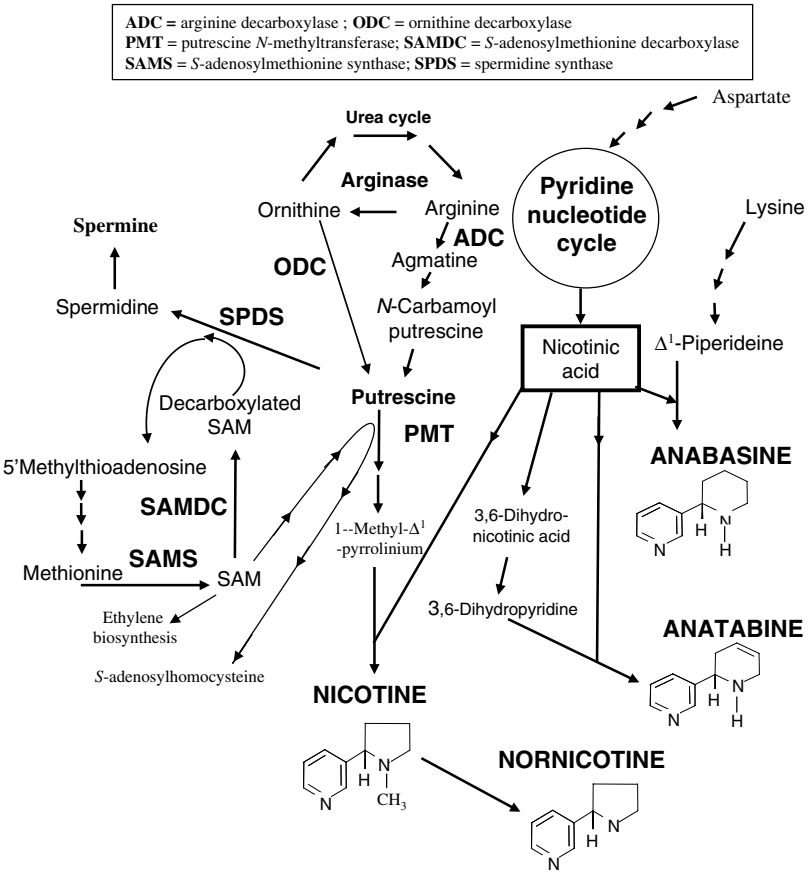

Fig. 1. Schematic diagram showing main steps in the synthesis of the 4 main alkaloids in *Nicotiana* species (adapted from Luckner, 1990; Imanishi et al., 1998b; Cane et al., 2005). In roots of *N. tabacum* varieties of *AABB* genotype (includes NC 95 used in the current study), nicotine comprises ~80–85% of the alkaloid fraction with anatabine comprising most of the remainder. Enzymic steps for which molecular probes were used for northern analysis in the present study are shown in bold and listed in the insert box.

Tanguy, 2001; Kakkar and Sawhney, 2002; Acosta et al., 2005).

Notwithstanding its important role in the primary metabolism of plants, putrescine is also an important intermediate in the synthesis of a range of secondary metabolites. These include pyridine and tropane alkaloids, which are commonly found in many genera within the Solanaceae, and pyrrolizidine alkaloids which are found in genera belonging to a range of families including the Compositae, Leguminoseae and Boraginaceae (Luckner, 1990; Kutchan, 1995, 1998; Griffin and Lin, 2000; Wink, 2003). The relative importance of ODC and ADC in facilitating the supply of putrescine that is destined for primary vs. secondary metabolism in these species has been the topic of some debate in the literature (reviewed in Tiburcio et al., 1997; Martin-Tanguy, 2001). In part, this may be due to the diverse array of plant species, tissue types and experimental systems that have been the subject of these studies. The possibility of biochemical interconversion between arginine and ornithine has also been a contributory factor in this debate and questions have been asked regarding the *in vivo* specificity of DFMO and DFMA which are effective enzyme-activated biochemical inhibitors of ODC and ADC activity respectively (Bey et al., 1987). For example, in developing ovary tissues of tobacco flowers after fertilization, despite the fact that ODC represented >95% of detectable decarboxylase activity, treatment with DFMA was inhibitory to ODC activity *in vivo* and tissue growth. This was attributed to arginase-mediated conversion of DFMA to DFMO in these tissues (Slocum and Galston, 1985a,b; Slocum et al., 1988). DFMA was observed to be more effective, on an equimolar basis, than DFMO in causing inhibition of tropane alkaloid production when added to the growth medium of *Datura stramonium* hairy roots (Walton et al., 1990) Interestingly, Walton et al. (1990) detected high levels of arginase in extracts of these root tissues, albeit using likely non-physiological *in vitro* assay conditions of pH 9.0 and high concentrations of Mn^{2+}. In addition, the same research group also observed that concentrations of DFMA as low as 1–2.5 mM reduced ODC activity to 40% of levels that were observed in untreated controls whilst addition of DFMO at concentrations up to 10 mM had no inhibitory effect upon ODC activity (Robins et al., 1991).

The majority of *Nicotiana* species contain moderate-high levels of the pyridine alkaloid nicotine or its demethylated derivitive nornicotine (Saitoh et al., 1985; Sisson and Severson, 1990). The effects of adding biochemical inhibitors of ADC and/or ODC to plants of *Nicotiana* species have not been well documented with respects to their effects upon alkaloid productivity. However, treatment of disorganised callus cultures of *N. tabacum* L. cv 'Burley 21' with either 1 mM DFMA or 1 mM DFMO did show the former to be more effective at inhibiting nicotine production without negatively affecting growth (Tiburcio and Galston, 1986). In this study, no arginase activity was detectable in callus extracts and as higher incorporation rates of ^{14}C-labelled arginine into nicotine were observed compared to rates of ^{14}C-ornithine incorporation, it was concluded that the ADC route is preferentially utilized to provide putrescine which is used in nicotine synthesis in tobacco (Tiburcio and Galston, 1986). However, whilst this may be the case for tobacco callus cells when cultured in vitro, it is unlikely to be the case for all cultural conditions. For example, Imanishi et al. (1998b) showed that a marked increase in ODC but not ADC transcripts occurred within hours of addition of methyljasmonate to tobacco BY-2 cells cultured in liquid medium, followed by increased levels of nicotine but not polyamines during subsequent days. In contrast, cDNA-AFLP based profiling of the transcriptome of jasmonate-treated BY-2 tobacco cells provided evidence that ADC is transcriptionally up-regulated shortly after treatment with jasmonate together with ODC and a number of other genes encoding enzymes required for pyridine alkaloid biosynthesis (Goosens et al., 2003).

In tobacco plants, nicotine is synthesized in roots before transport to aerial tissues (Dawson, 1941; Dawson and Solt, 1959; Baldwin, 1989; Ruiz et al., 2005). Increased rates of nicotine synthesis occur in roots following removal of the shoot apex and previous studies suggested that ODC is particularly importance in providing putrescine for increased rates of nicotine synthesis in such wounded tobacco plants (Mizusaki et al., 1973; Saunders and Bush, 1979; Yang et al., 1984). More recently, up-regulation of both ADC and ODC transcripts in root tissues has been demonstrated to occur within a few hours of damage to aerial tissues of tobacco and preceding an increase in leaf nicotine concentrations during subsequent days (Wang et al., 2000; Cane et al., 2005). Rapid increases in ADC and ODC transcript can also be simulated in cultured tobacco roots by either reducing the auxin levels in media of non-transformed tissues (Reed and Jelesko, 2004) or by adding low levels of methyljasmonate to culture media of transformed hairy roots which typically are grown in auxin-free media (Shoji et al., 2000; Cane et al., 2005).

These observations suggest that transcriptional up-regulation of both ODC and ADC is important for ensuring adequate supplies of putrescine are available to enable increased rates of nicotine synthesis in *Nicotiana* roots in response to chemical signals emanating from damaged shoots (Hibi et al., 1994; Baldwin et al., 1994, 1997; Zhang and Baldwin, 1997). Interestingly however, although early biochemical studies did indicate that the ADC mediated route to putrescine is utilized during nicotine synthesis in non-wounded plants (Yoshida and Mitake, 1966), it remains unclear whether the ADC-mediated route to putrescine is preferentially utilized for nicotine production in tobacco roots when plants are grown or roots are cultured under non-stressful conditions. To address this question, we employed an antisense approach to reduce ADC transcript and associated enzymic activity in cultured transformed roots of tobacco with the specific aim of assessing the downstream effects upon alkaloid productivity in non-stressed roots at various stages during a culture cycle

in vitro. To provide an insight into what also occurs in intact non-stressed plants, we assessed the alkaloid profiles in leaf tissues from plants that were regenerated from selected ADC-antisense transformed root lines and grown in compost in a temperature-controlled insect-proof greenhouse with access to ample supplies of water, nutrients and light.

2. Results

2.1. Northern analysis

After establishment of *N. tabacum* root clones from single transformed root tips, RNA was extracted from four control root lines and nine antisense-ADC lines that were harvested midway through a typical culture cycle of ~3.5–4 weeks. Parallel northern blots were prepared and probed separately with ^{32}P-labeled strand-specific ADC probes. Several antisense-ADC hairy root lines were identified with relatively high signals representing the ~1.2 kb antisense-ADC transcript which also had markedly reduced signals representing the ~2.3 kb endogenous sense-ADC transcript compared to controls (e.g. antisense-ADC lines II-4, II-26, II-32, II, 34 and II-39) (Fig. 2a and b). After stripping of hybridizing signals, blots were sequentially re-probed to detect transcripts of *ODC*, *SAMDC*, *SAMS*, *SPDS*, *PMT* genes to determine whether there were any obvious differences between antisense-manipulated *vs* control lines. Results indicated that the introduction of the ADC-antisense construct had very little, if any, effect upon relative transcript levels of other genes encoding enzymes that are associated with putrescine metabolism in cultured hairy roots of *N. tabacum* (Fig. 3).

2.2. ADC activity

The mean ADC activity of three control hairy root lines (lines III-12, IV-3, and IV-9) was determined to be 1.61 pkat/mg protein (±S.D. 0.23) at day 14 of the culture cycle. This is comparable to the ADC levels that were observed

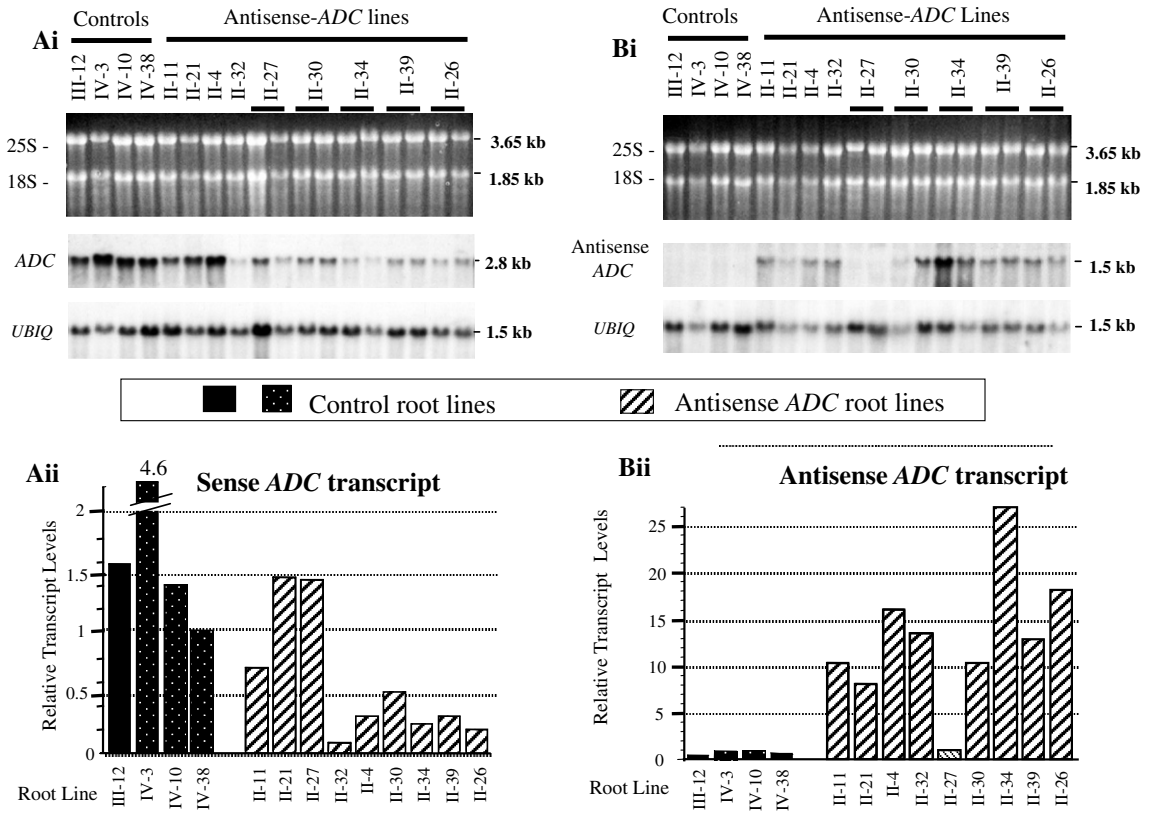

Fig. 2. Northern blot analysis to detect sense-ADC (panel A) and antisense-ADC (panel B) transcripts in control and antisense-ADC clonal transformed root lines harvested at day 12 of the culture cycle. Sufficient high quality RNA was available from duplicate cultures of lines II-26, II-27, II-30, II-34, II-39 to enable separate tracks to be loaded and allow mean relative transcript levels for these samples to be calculated. For other samples, RNA from single cultures was used to determine relative levels of sense ADC and antisense ADC transcripts. The upper gel image in each panel shows the 28S and 18S ribosomal RNA signals in the formaldehyde agarose gel prior to blotting onto membranes indicating that RNA was of high quality in all samples and that efforts to load equal quantities of RNA (20 μg) in each track were largely successful. The upper northern image in each panel shows results from parallel northern blots probed with either ^{32}P-labelled antisense-ADC DNA to detect endogenous ADC transcript of ~2.8 kb (Ai) or labelled sense-ADC DNA to detect antisense-ADC transcript of ~1.5 kb (Bi). The lower northern image in both panels shows each respective filter re-probed with ^{32}P-labelled ubiquitin DNA to enable relative transcript levels to be determined as shown in Aii and Bii after quantification of signals using a phosphoimager. The lowest relative level of transcripts detected amongst control lines (line IV-38) was arbitrarily assigned a value of 1.0. The antisense-ADC transcript levels depicted in control lines (Bii) effectively represent background readings in each track after quantification of signals.

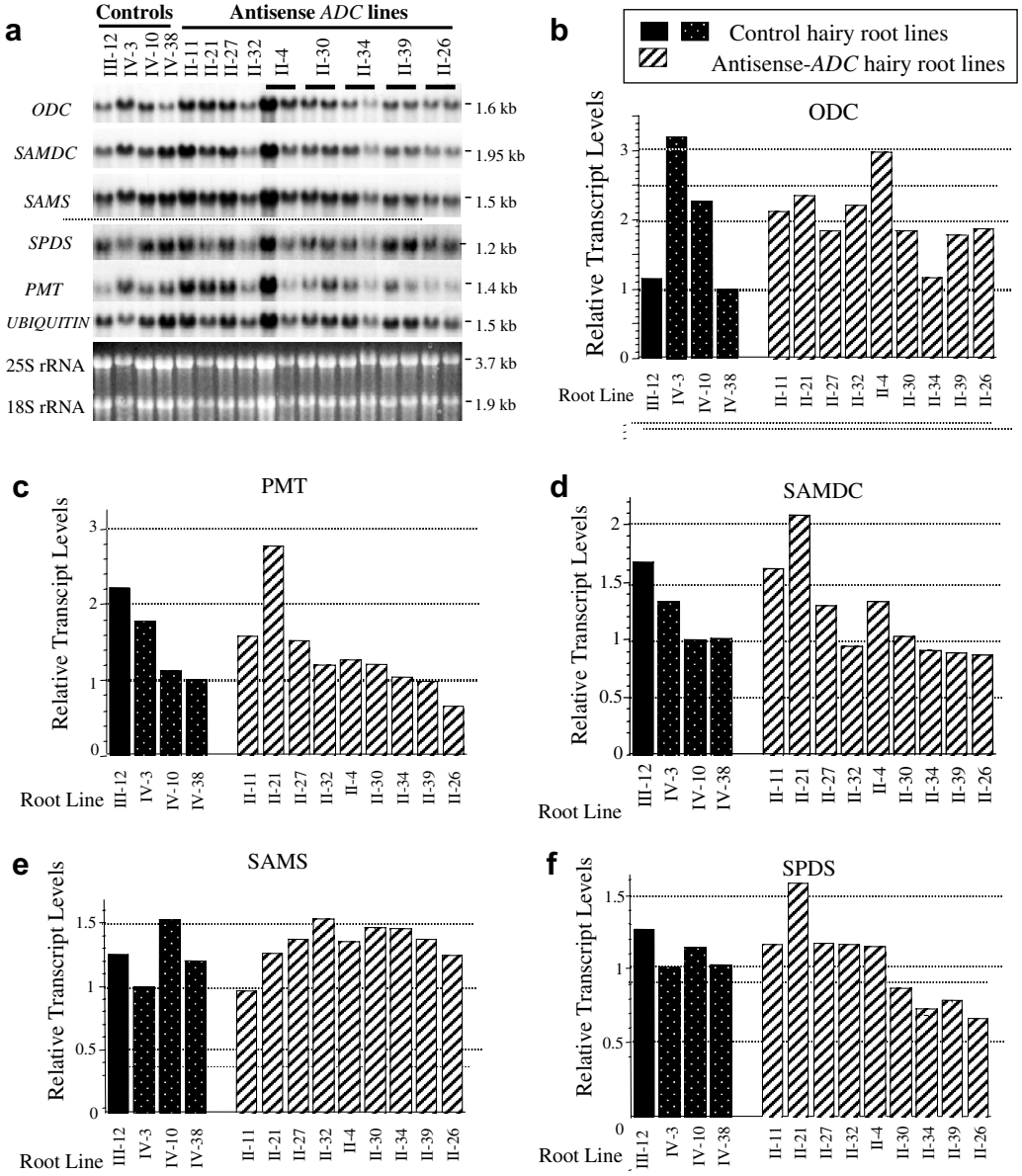

Fig. 3. Northern blot analysis to determine relative transcript levels of ODC, PMT, SAMDC, SAMS and SPDS genes in antisense-ADC root lines vs controls. (a) Shows northern blot images suggesting that transcript levels are similar for each respective gene in all root lines. (b–f) Shows relative transcript levels of each gene relative to the levels of *UBIQUITIN* transcript in each lane with the lowest relative level of each respective transcript on the blot being assigned a value of 1.0. As noted in Fig. 2, histograms relating to lines II-4, II-30, II-34, II-39 and II-26 represent the mean relative transcript level of duplicate root culture samples from each transformed hairy root line with the remaining histograms representing data from single cultures of each hairy root line harvested at day 12 of their culture cycle.

previously in hairy roots of the related species *N. rustica* when they too were analysed at the mid-point point of their culture cycle (Hamill et al., 1990). ADC activity was also determined at the same time point in six antisense-ADC lines of *N. tabacum* (noted above) which possessed low levels of endogenous ADC transcript compared to controls (lines II-4, II-26, II-30, II-32, II-34, and II-39) and also in two additional antisense-*ADC* lines that had not previously been analysed for ADC transcript levels (lines II-7 and II-35). Results are shown in Fig. 4.

Following a one way ANOVA analysis which suggested a difference between mean ADC activity in the control population and antisense population, a Dunnett's statistical test indicated that reduced levels of ADC activity in lines II-7, II-26, II-32, II-34 and II-39 were likely to be significantly different ($P \leqslant 0.05$) from the average ADC activity of control lines as a group. Four of these antisense-ADC lines each had an ADC activity that was less than 30% of the mean activity that was found in control lines (Fig. 4).

2.3. Nicotine analysis

Our previous studies have shown that alkaloid concentration in transformed roots of *N. tabacum* can vary several-fold during a culture cycle with levels generally increasing to maximum levels during the latter half of a

culture cycle of ~3.5–4 weeks as one or more nutrients become limiting and growth rates become slower (Chintapakorn and Hamill, 2003). Accordingly, preliminary analysis of the nicotine content of five control hairy root lines and eleven antisense-ADC hairy root lines was determined at the 20 day stage of the culture cycle to assess whether nicotine levels were reduced in antisense-ADC root lines in accordance with the results of the northern and enzymic analysis noted above. Initial evaluation of results suggested that average levels of this alkaloid were broadly comparable in both groups of cultures, ranging from ~2800–3750 μg nicotine g^{-1} fwt. It was noticed however that there was a tendency for control lines to possess levels at the higher end of this range and for antisense-ADC lines to possess levels at the lower end of this range (Fig. 5). This observation was supported by the results of a statistical analysis whereby an analysis of variance (one way ANOVA) suggested a small but likely significant ($P = 0.018$) difference between the mean nicotine content of con-

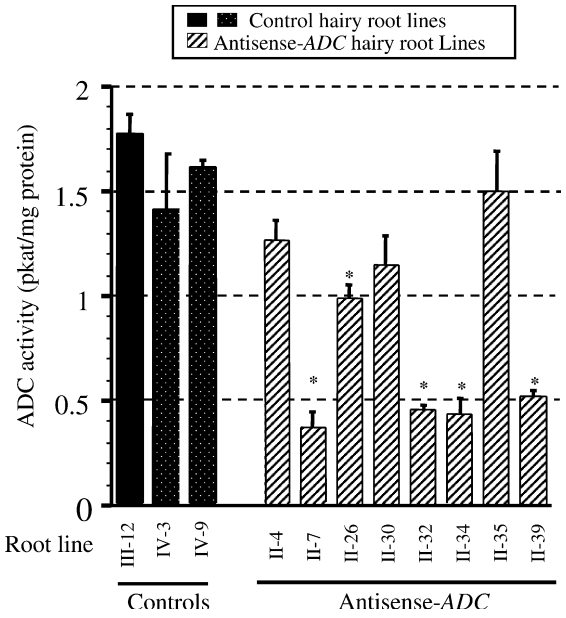

Fig. 4. Comparison of ADC activity in control root lines vs antisense-ADC hairy root lines harvested at day 14 of the culture cycle. Each histogram represents the mean activity (±SE) of roots harvested from two culture flasks. Individual lines showing a difference from the mean of controls, as determined by a Dunnett's statistical test at $P \leqslant 0.05$, are indicated by *.

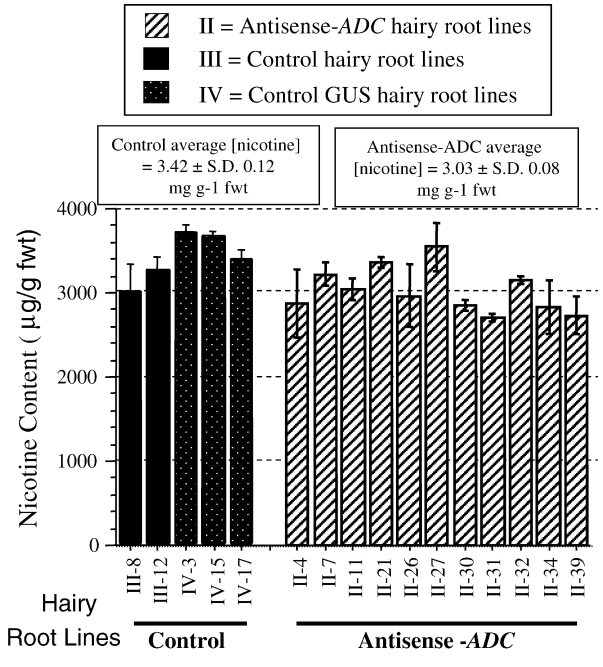

Fig. 5. Nicotine concentration in separate control vs antisense-*ADC* transformed root lines of *N. tabacum* var. NC95 at day-20 of the culture cycle. Control lines from group III resulted from infection of tobacco with *A. rhizogenes* strain LBA 9402 whilst control lines from group IV resulted from infection of tobacco leaves with *A. rhizogenes* LBA 9402 + pBI 121. Each histogram represents the mean (±SE) nicotine content of duplicate samples.

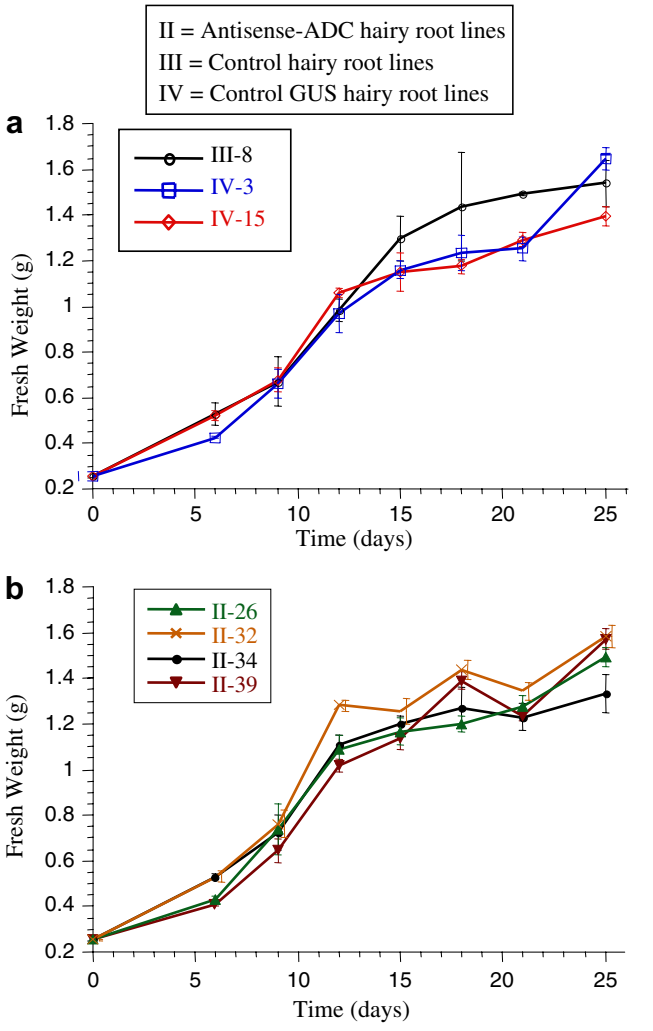

Fig. 6. Growth curves of 3 control root lines (a) and 4 antisense-ADC root lines with reduced ADC activity (b). For most lines, each data point represents the mean (±SE) fresh weight (g) of root tissue from four separate culture vessels harvested at days 6, 9, 12, 15, 18, 21 and 25 of the culture cycle. For control line III-8, the contents of two vessels were harvested at each time point.

trol lines as a group compared to the mean nicotine content of the antisense-ADC lines as a group. These results encouraged us to undertake a more detailed analysis of alkaloid levels in several selected antisense-ADC hairy root lines compared to control hairy root lines at various points throughout their respective culture cycles.

2.4. Alkaloid analysis of selected antisense-ADC root lines

Detailed analysis of alkaloid profiles at regular time intervals throughout a growth cycle of 25 days was undertaken involving four antisense-ADC hairy root lines, each with reduced ADC activities. Results obtained were compared with comparable results from the analysis of three control transformed root lines. Overall, all lines had the normal characteristics of healthy, transformed root cultures of tobacco (Hamill et al., 1986; Hamill and Lidgett, 1997) indicating that the antisense-ADC manipulation had no obvious deleterious effect upon root morphology and growth *in vitro* (Fig. 6a and b). As we observed previously (Chintapakorn and Hamill, 2003), nicotine levels in control transformed root lines were typically in the low-middling range when they were harvested early in their culture cycle (days 6–9; ~1.5–2 mg nicotine g^{-1} fwt). Nicotine concentrations rose to comparatively high levels when root tissues were harvested in the latter stages the culture cycle (days 21–25; ~5–5.5 mg nicotine g^{-1} fwt) (Fig. 7). Interestingly, nicotine levels in each of the four selected antisense-ADC root lines were quite comparable to those of controls when they were harvested at the early-mid stages of their culture cycles (Fig. 7). However, the final levels of nicotine that were detected in each antisense-ADC root line at days 21 and 25 of the culture cycle were ~20% lower than those of control lines when roots harvested during the latter phase of their culture cycles (Fig. 7). A Dunnett's statistical test, following a one way ANOVA, indicated that the difference in concentration between control and antisense lines was statistically significant ($P \leqslant 0.05$) in tissues harvested at this late stage in the growth cycle. This suggested that the antisense-ADC manipulation had caused a real, but relatively modest reduction in the capacity of transformed root cultures of *N. tabacum* to produce nicotine and that this reduction in alkaloid content only became apparent during the latter stages of the culture cycle when one or more nutrients would likely to be have become growth limiting.

To explore this suggestion from another perspective, we also calculated levels of the minor alkaloid anatabine in

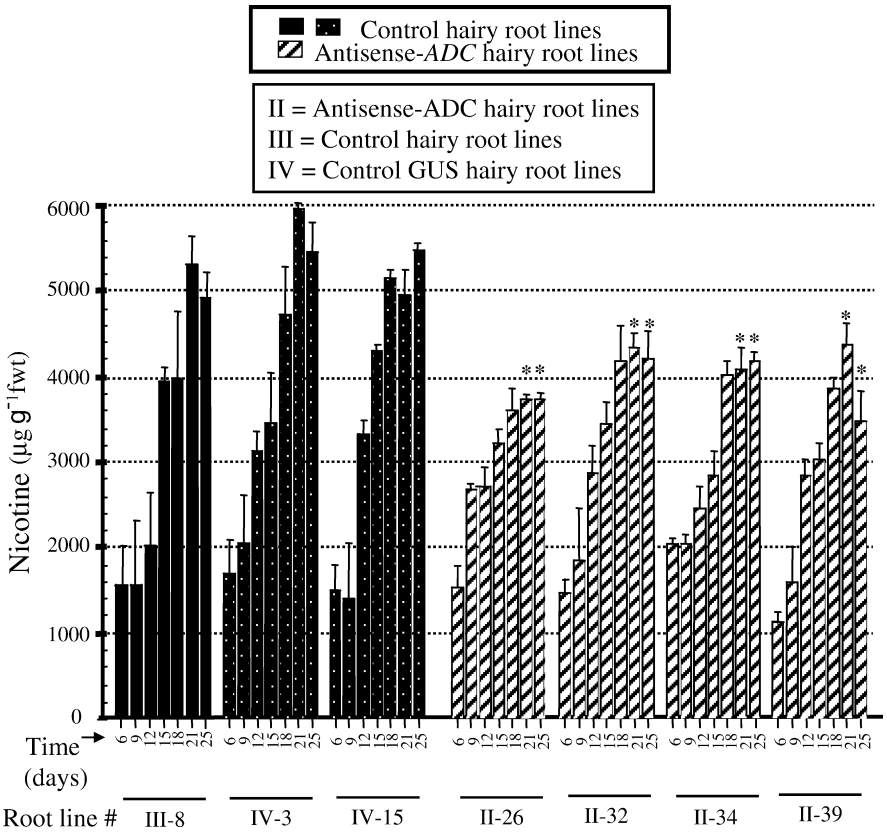

Fig. 7. Nicotine profiles of control and antisense ADC root lines harvested at days 6, 12, 15, 18, 21 and 25 of their culture cycle. Each histogram represents the mean (±SE) nicotine content (µg/g^{-1} fwt) of duplicate samples, except for lines IV-3, II-26 and II-34 from day 12 to 25, and lines IV-15, II-32 and II-39 from day 15 to 25, where data points represent the mean nicotine content (±SE) of triplicate samples. One way ANOVA indicated likely significant differences between nicotine concentrations of control and antisense-ADC groups at day 21 ($P = 0.001$) and day 25 ($P < 0.001$). Individual lines showing a difference from the mean of controls at that time point, as determined by a Dunnett's statistical test, are indicated by *.

tissue extracts from each control and antisense-ADC line noted above. Normally this alkaloid, which is derived entirely from nicotinic acid, comprises ~15% of the total pyridine alkaloid fraction of *N. tabacum* roots (Saitoh et al., 1985; Hamill et al., 1986; Parr and Hamill, 1987). Previous studies involving *Nicotiana* species have indicated that reduced ability to produce nicotine due to reduced supply of intermediates derived from putrescine is associated with increased capacity of cultured roots and soil-grown transgenic plants to produce anatabine (Chintapakorn and Hamill, 2003; Steppuhn et al., 2004).

In the present study, analysis of anatabine in control *N. tabacum* hairy root lines showed that levels ranged from a low of ~300–350 μg anatabine g^{-1} fwt, when tissues were harvested at the early stages (days 6–9) of their culture cycle to a high of ~600–700 μg anatabine g^{-1} when tissues were harvested during the latter stages (days 21–25) of their culture cycle (Fig. 8). Anatabine levels in each antisense-ADC line were quite similar to those of control values, when tissues were harvested in the early and also the middle stages of their growth cycle (Fig. 8). This was expected given that nicotine levels in antisense-ADC and control lines were also very comparable when roots were harvested at these points in the culture cycle (Fig. 7). Slightly elevated levels of anatabine were observed in two antisense-ADC lines (lines II-34 and II-39) when tissues were harvested at the latter stages of their culture cycle (Fig. 8). Whilst these increases in anatabine content were very modest, a Dunnett's statistical test following a one way ANOVA, indicated that the difference in anatabine concentration between control and antisense lines was statistically significant ($P \leqslant 0.05$) in line II-34 roots were when harvested at days 21 and 25 of their culture cycle and in line II-39 when roots were harvested at days 12, 15, 18 and 21 of their culture cycle (Fig. 8).

2.5. Alkaloid analysis of plants regenerated from antisense-ADC hairy root lines

Plants were successfully regenerated from three antisense-ADC hairy root lines noted above (lines II-26, II-32 and II-39) and were grown under comparable conditions in soil, in an insect-free PC2 greenhouse, alongside plants that were regenerated from three control root lines (lines III-8, IV-3 and IV-15) at the same time. Plants that regenerated from antisense-ADC hairy roots were observed to

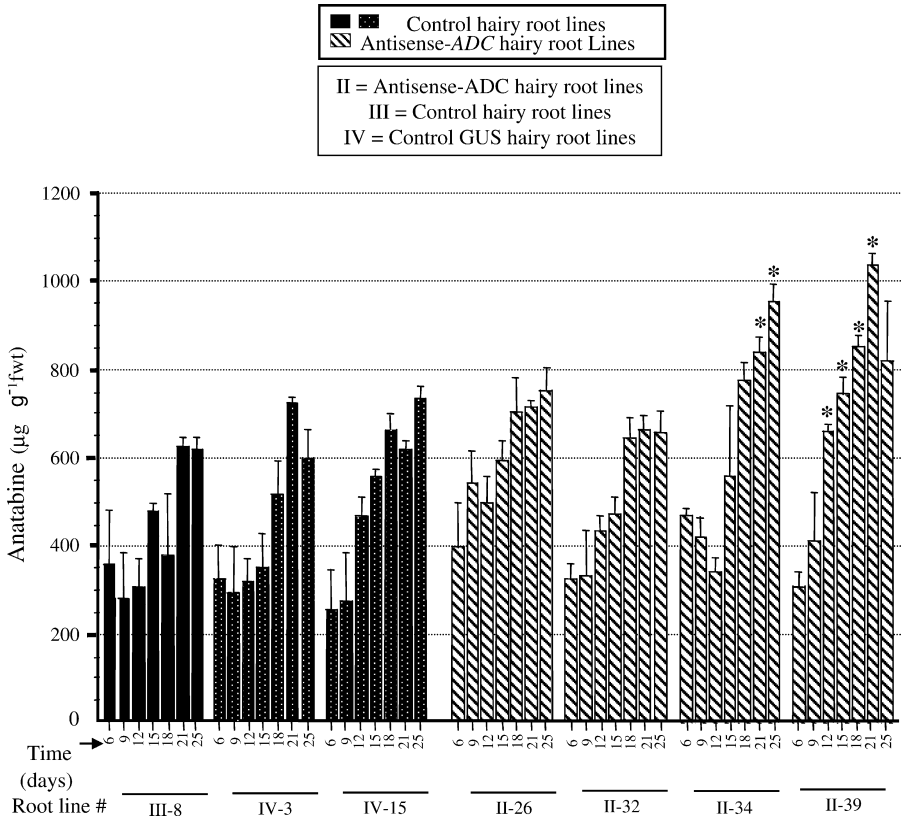

Fig. 8. Anatabine profiles of control and 1.2 kb antisense ADC root lines at days 6, 12, 15, 18, 21 and 25 of their growth cycle. Each histogram represents the mean (±SE) anatabine content (μg g^{-1} fwt) of duplicate samples, except lines IV-3, II-26 and II-34 from day 12 to 25, and lines IV-15, II-32 and II-39 from day 15 to 25, which represents the mean anatabine content (±SE) of triplicate samples. One way ANOVA indicated likely significant differences between anatabine concentrations of control and antisense-ADC groups at day 12 ($P = 0.006$); day 15 ($P = 0.069$); day 18 ($P = 0.017$); day 21 ($P < 0.001$) and day 25 ($P = 0.016$). Individual lines showing a difference from the mean of controls at that time point, as determined by a Dunnett's statistical test, are indicated by *.

be similar in overall appearance to plants that regenerated from control hairy roots. All plants grew vigorously and possessed, as anticipated and as reported previously for these transgenic control plants (Chintapakorn and Hamill, 2003), a mild T-phenotype consisting of slightly shorter internodes and slight leaf wrinkling (Fig. 9a). The latter

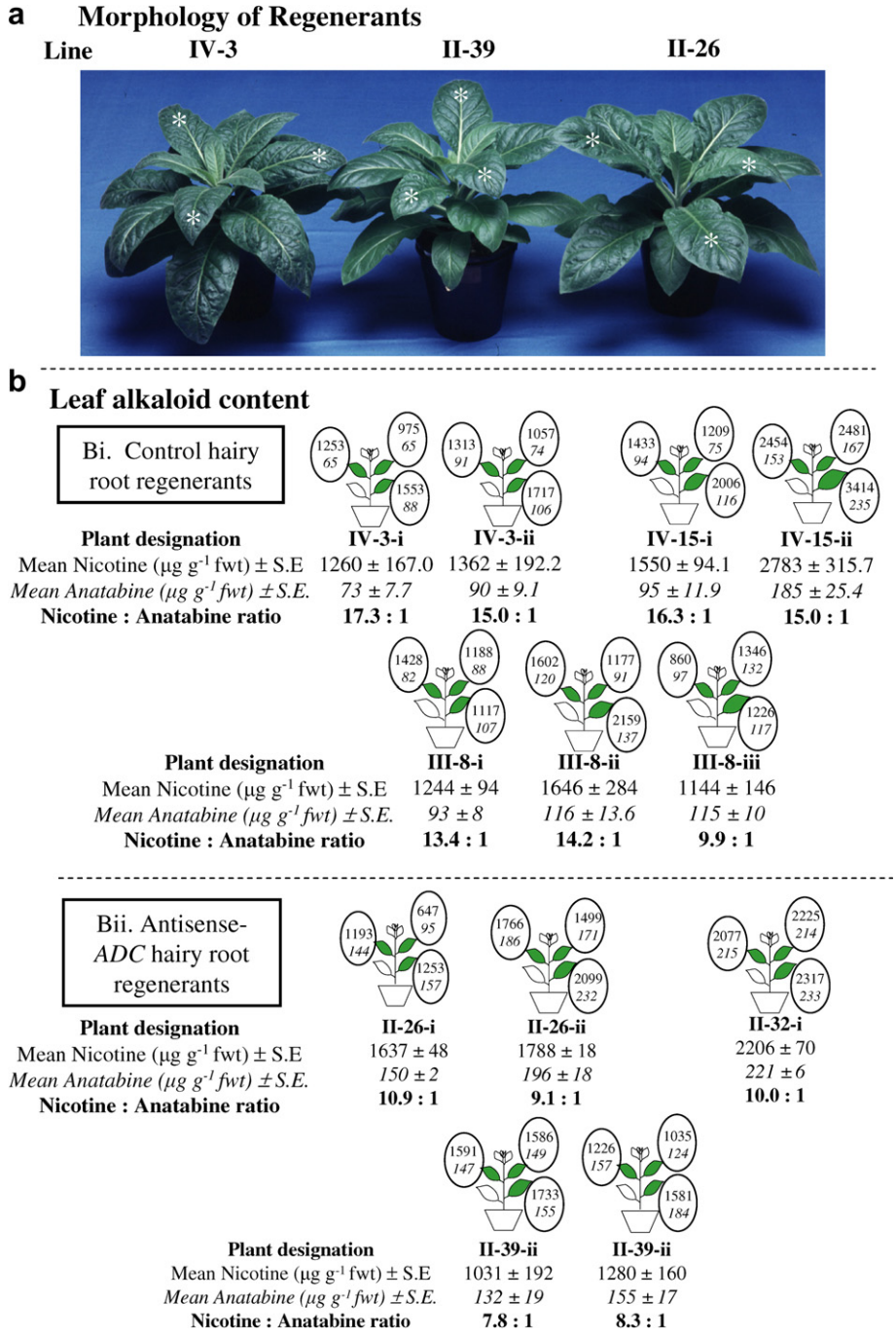

Fig. 9. Leaf alkaloid analysis of transgenic plants regenerated from three control hairy roots (lines III-8, IV-3 and IV-15) and three antisense-ADC hairy root lines (II-2, II-32, and II-39) which, relative to controls, showed evidence of diminished ADC activity at day 12 and also altered alkaloid levels at days 21 and 25 of their culture cycles. (a) Example of soil grown plants regenerated from control hairy root line IV-3 and antisense-ADC hairy root lines II-26 and II-39 approximately 8 weeks after transfer of shoots from *in vitro* conditions to compost and growth in the greenhouse. Apart from a slight wrinkling of leaves and shortened internodes due to the presence of Ri T-DNA in all regenerants, no phenotypic alterations were evident in antisense-ADC transgenic plants compared to control transgenic plants Three upper leaves from each plant (asterisked) were individually analysed for their alkaloid content as noted below. (b) Schematic representation of plants regenerated from control hairy roots and antisense-ADC hairy roots showing alkaloid titres in 3 leaves analysed from each plant (leaf numbers 6, 7 and 8 from the apex – shaded on schematic diagram). Upper value in each schematic leaf = μg nicotine g^{-1} fwt and lower value in each schematic leaf (*italicised*) = μg anatabine g^{-1} fwt of that leaf. The mean nicotine and anatabine levels (μg g^{-1} fwt) ±SE per plant are shown below each schematic plant which enabled the average nicotine: anatabine ratio for that plant to be determined.

phenotype is attributable to the presence of Ri T-DNA from *A. rhizogenes* in the genome of the regenerated plants (Tepfer, 1984).

Previous analysis in this laboratory has indicated that the alkaloid content and nicotine: anatabine ratios of leaf extracts from *N. tabacum* var. NC-95 regenerants containing Ri T-DNA (± the CaMV35S-*uidA* construct) are very similar to those of leaf extracts from non transgenic seed-derived plants of the same variety when plants were grown under the same greenhouse conditions and leaf tissues harvested at a similar (pre-flowering) stage of development (Chintapakorn, 2003). In the present study, HPLC analysis of leaf extracts from pre-flowering plants indicated that nicotine was the dominant alkaloid in all leaf samples with, as anticipated, anatabine being the only other pyridine alkaloid that was detectable at quantifiable levels. Nicotine levels varied from ~1 to 3 mg g^{-1} fwt in leaf tissues of controls and also antisense ADC transgenics, with a mean of ~1.5 mg nicotine g^{-1} fwt in leaves from both groups of plants (Fig. 9b). Average levels of anatabine were somewhat different between leaves from control- and antisense-ADC transgenic plants, with ~110 μg^{-1} fwt being the mean for control plants compared to ~180 $\mu g\ g^{-1}$ fwt as the mean value for antisense-ADC transgenic plants (Fig. 9b). By arbitrarily setting the relative anatabine level of each leaf sample noted above as 1.0, and calculating the relative nicotine level in precisely the same leaf extract, we observed a slight increase in the anatabine component as a proportion of the total leaf alkaloid fraction in antisense-ADC transgenic plants compared to their controls. Thus, together, leaves of control plants had an overall nicotine:anatabine ratio of ~14:1 whereas, together, leaves of antisense-ADC transgenic plants had an overall nicotine:anatabine ratio of ~9:1 (Fig. 9b). These results are generally consistent with conclusions from hairy root analysis noted above and suggest that the effect of antisense-mediated reduction of ADC activity in roots of non-stressed tobacco plants only slightly perturbed the normal supply of metabolic intermediates that are available for nicotine synthesis. At the stage of development that was chosen for leaf alkaloid analysis in the current study, this perturbance appears to have been minimal with the only evidence for it being a very small increase in the anatabine content of leaves with a concomitant modest decrease in the nicotine: anatabine ratio of antisense ADC-transgenic plants compared to that of control transgenic plants.

3. Discussion

Numerous previous studies have shown that alkaloid levels in *Nicotiana* species and tissues cultured *in vitro* can vary markedly in response to a range of intrinsic genetic factors and environmental factors (e.g. Legg et al., 1969; Legg and Collins, 1971; Saunders and Bush, 1979; Baldwin, 1989; Baldwin and Ohnmeiss, 1994; Baldwin et al., 1997a,1997b; Jackson et al., 2002; Chintapakorn and Hamill, 2003; Sinclair et al., 2004; Cane et al., 2005). Thus, is it important to utilize a robust experimental system when assessing effects of gene manipulation upon alkaloid metabolism, particularly if resultant changes are relatively small. Axenic root culture procedures have been used successfully for more than 60 years to study alkaloid production by roots of *Nicotiana* species (Dawson, 1942). However, careful attention must be paid to phytohormone levels in the medium of cultured roots, particularly auxin, to prevent possible repression of alkaloid biosynthesis due to reduced levels of key genes and key enzymes that are essential for alkaloid biosynthesis (Rhodes et al., 1989; Hibi et al., 1994; Reed and Jelesko, 2004). Transformed (hairy) roots of several *Nicotiana* species, as well as species of many other genera, have been utilized for more than two decades for secondary metabolism studies due to their ability to synthesis bioproducts characteristically produced by roots of the plant in question and also their general capacity for relatively rapid growth in phytohormone-free media (Hamill et al., 1987b; Flores et al., 1987; Hamill and Lidgett, 1997; Guillon et al., 2006; Georgiev et al., 2007). The stimulus for hairy root growth results from the transformation of wounded plant cells by Ri T-DNA from a large plasmid resident in *A. rhizogenes*. The Ri T-DNA contains several *rol* genes that, together with other genes, collectively alter endogenous levels and/or sensitivity of tissues to auxin and cytokinin (Slightom et al., 1986; Schmülling et al., 1988; Maurel et al., 1994 – reviewed in Gelvin, 2000; Tzfira et al., 2004). Recent work has demonstrated that the protein encoded by the *rol*B gene, of central importance in triggering hairy root formation, interacts with 14:3:3 proteins (Moriuchi et al., 2004). This class of proteins includes an auxin-inducible H^+-ATPase which may explain previous observations that levels of auxin that are required to induce a transmembrane potential in cultured protoplasts are much lower for leaf mesophyll protoplasts isolated from *rol* B-transformed plants compared to mesophyll protoplasts from non-transformed control plants (Maurel et al., 1994). Whether there are any direct effects of Ri T-DNA gene expression upon alkaloid biosynthesis and its underlying metabolism in roots of any species is uncertain. Studies undertaken to date, that have noted differences in alkaloid and associated polyamine metabolism in transgenic *Nicotiana* plants containing *A. rhizogenes* DNA, have generally not discriminated the effects of individual and combinatorial *rol* gene expression upon root growth from effects upon alkaloid biosynthesis *per se* (Altabella et al., 1995; Palazón et al., 1997, 1998). From a practical viewpoint, if reasonable care is taken to ensure to minimise the proliferation of disorganised callus, e.g. by avoiding excessive wounding during subculture, *Nicotiana* hairy root cultures typically produce pyridine alkaloids that are characteristic of the species from which they are derived (Hamill et al., 1986; Parr and Hamill, 1987). They also provide a simple and robust experimental system to study alkaloid metabolism and to assess the effects of binary vector-mediated alterations in

expression of genes which may be important in alkaloid biosynthesis (Hamill et al., 1987b, 1997; Chintapakorn and Hamill, 2003, Cane et al., 2005). A further benefit in using such cultures is that healthy plants can be often be regenerated from individual clonal root lines. Although these plants may have slightly altered phenotypes due to the presence of Ri T-DNA from *A. rhizogenes* (Tepfer, 1984; Hamill and Rhodes, 1988), their capacity for vigorous growth in soil allows the effects of gene manipulation upon alkaloid levels in transgenic plants to be assessed and compared to that of the cultured root from which they are derived (Chintapakorn and Hamill, 2003).

In the present study, we report on the use of an antisense approach to reduce ADC activity in *N. tabacum* with the aim of assessing the capacity of transgenic lines to produce nicotine, the main alkaloid that is characteristically present in roots and leaves of this species (Saitoh et al., 1985; Sisson and Severson, 1990). We reasoned that if the ADC mediated-pathway is indeed the preferential route to putrescine which is destined for alkaloid synthesis in roots of non-stressed *N. tabacum*, as has been reported to be case for disorganised callus cultures of tobacco (Tiburcio and Galston, 1986), we might have expected that such substantial reductions in ADC activity in our antisense-ADC hairy root lines would have had a markedly depressive effect upon nicotine levels – possibly akin to what was observed previously in *Nicotiana* hairy roots expressing an antisense-PMT gene construct (Chintapakorn and Hamill, 2003). Additionally, we may have expected a substantial increase in concentrations of the (normally) minor tobacco alkaloid anatabine due to, presumably, concomitant relative increases in levels of pyridine nucleotide intermediates *in vivo* (Chintapakorn and Hamill, 2003; Steppuhn et al., 2004). We identified several antisense-ADC hairy root lines with markedly reduced levels of sense-ADC transcript and which also possessed only ~25–30% residual ADC activity of controls. As the results demonstrate, very slight differences in alkaloid content and profile were observed between control and antisense-ADC hairy root lines. Reductions in nicotine levels, together with concomitantly slight increases in anatabine content of some antisense-ADC hairy root lines, were observed but only when hairy root tissues were harvested at the latter stages of their culture cycle. There was no evidence of a depressive or stimulatory effect upon transcript levels of a number other genes encoding important enzymes in this general area of alkaloid and associated polyamine metabolism, including ODC which catalyses the alternative route to putrescine *in vivo*. This is in general agreement with our previous observations following the down-regulation of PMT activity in hairy roots of *N. tabacum* (Chintapakorn and Hamill, 2003). These results are also in accordance with observations of Trung-Nghia et al. (2003) who reported that expression of an antisense oat-ADC construct in transgenic rice produced significantly lower levels of ADC activity but had no effects upon transcript levels of ODC or other enzymes important in polyamine metabolism such as SAMDC and spermidine synthase. In the current study, small increases in anatabine content were observed in leaf tissues of plants that were regenerated from antisense-ADC lines, compared to controls, but there was no convincing evidence of alterations in the leaf nicotine content of both classes of plant. In this respect, regenerated anti-ADC transgenic plants were quite different from corresponding regenerated anti-PMT transgenic plants where marked reductions in nicotine content were accompanied by substantial increases in anatabine concentration in leaves (Chintapakorn and Hamill, 2003).

The results of the current study suggest that the ADC mediated route to putrescine does play a role, but is not of prime importance, in providing the pyrollidine ring that is required for nicotine synthesis in cultured transformed roots of *N. tabacum* and also in roots of healthy greenhouse-grown plants. They do not of course indicate whether down-regulation of ADC activity is likely to affect nicotine synthesis in cultured root tissues that are exposed to methyljasmonate which is known to increase alkaloid levels (Cane et al., 2005). Similarly, whilst effects of down-regulating ADC upon alkaloid levels are minimal in well-nourished, healthy plants that are cultivated in insect-free glasshouses, the effects upon plants that are subjected to mechanical/insect-mediated damage to aerial tissues or when grown under a range of nutritional regimes are as yet unknown. The availability of antisense-ADC transgenic root lines and plants of *N. tabacum*, produced as a result of the current study, may enable further insights to be obtained regarding the importance of ADC in providing putrescine for nicotine biosynthesis in roots of *Nicotiana* species when plants or cultured hairy roots are subjected to stress conditions.

4. Experimental

General procedures for growth of *AABB* genotype *N. tabacum* variety NC-95 (Moore et al., 1962); production and maintenance of transformed root cultures *in vitro* and regenerated transgenic plants in soil; extraction of alkaloids and quantification using HPLC; details of DNA probes for *ODC*, *SAMDC*, *SAMS*, *SPDS* and *PMT* and determination of relative transcript levels in *ADC*-transgenics vs. controls by northern hybridisation and standardization against signals obtained by re-probing with a ^{32}P-labelled ubiquitin gene from *Antirhinum* were undertaken as described previously (Chintapakorn and Hamill, 2003; Sinclair et al., 2004; Cane et al., 2005) Whilst the latter approach may be somewhat more time-consuming and labour intensive for determining relative transcript abundance, compared to many currently used procedures involving quantitative PCR, we have consistently observed that it is a reproducible and reliable technique which is useful when one is considering a limited number of co-expressed genes such as those involved in alkaloid synthesis in tobacco. In addition, the procedure allows the operator

to be assured that quantification of relative transcript abundance involves consideration of only full length transcripts of the genes in question.

4.1. Production of transformed roots expressing an antisense-ADC gene sequence

Conserved sections of deduced ADC protein from ADC cDNA sequences of tomato (Genbank accession no. L16582; Rastogi et al., 1993) and pea (Genbank accession no. Z37540; Perez-Amador et al., 1995) were used to design forward oligonucleotide 5′CTTGTTGTCGCCTTCCTGAT 3′ (designated YC1F) and reverse oligonucleotide 5′ TGGATTGGAACAATAGGAAA 3′ (designated EY1R) which, respectively, represent nucleotide transcript positions 31–50 and 1255–1275 (amino acids 2–7 and 411–417 in deduced protein) in the ADC gene sequence that was reported by Rastogi et al. (1993). Each oligo was synthesized (Geneworks Ltd., South Australia) with the addition of an XbaI recognition site at its 5′ end to facilitate further manipulation. Using YC1F and EY1R in a standard PCR reaction involving genomic DNA (e.g. Hamill et al., 1991), from *N. tabacum* var. NC-95, a 1.2 kb PCR product was recovered which was ligated into a high copy plasmid pGEM-T (Promega). Sequencing of ~250 bp from both the 5′ and 3′ ends (ABI automated DNA sequencer and ABI PRISM Big Dye Terminator Cycle Sequencing – Perkin Elmer, USA), and database analysis (via Australian National Genomic Information Service [ANGIS] Australia) indicated a high level of similarity (~90%) at the deduced protein level with the tomato ADC sequence noted previously (Rastogi et al., 1993). Comparison with sequences of two ADC cDNAs from *N. tabacum* var. Xanthi that were subsequently reported in the Genbank data base revealed that the partial ADC sequence recovered here from *N. tabacum* var. NC-95 was ~99% identical to ADC2 (Genbank accession no. AF127241) and ~97% identical to ADC1 (Genbank accession no. AF127240). The cloned 1.2 kb ADC DNA fragment from var. NC-95 was ligated into the XbaI site of binary expression vector pFIH10, a modified version of pBin 19 (Bevan, 1984) containing a CaMV35S promoter with duplicated upstream enhancer sequences and a CaMV35S terminator sequence (Hamill et al., 1987a). Previous experiments using these binary vectors in conjunction with the agropine strain of *Agrobacterium rhizogenes* LBA9402, showed high transfer rates of both Ri T-DNA and vector T-DNA transfer to wounded *Nicotiana* leaf tissues resulting in efficient production of kanamycin-resistant transformed root cultures possessing high levels of heterologous gene expression (Hamill et al., 1990; Chintapakorn and Hamill, 2003). A similar approach was undertaken here to produce a number of independent, single root tip-derived, kanamycin-resistant transformed root cultures of *N. tabacum* var. NC-95 containing the antisense-ADC construct noted above. As controls, hairy roots were generated using *A. rhizogenes* LBA 9402 only (series III) or using *A. rhizogenes* LBA 9402 containing pBI 121 (Jefferson et al., 1987) (series IV). As for antisense-ADC lines, roots of control series IV were grown in the presence of 25 μg ml^{-1} kanamycin to select for the presence of binary vector T-DNA. All kanamycin-resistant roots of series IV showed strong indigo-blue staining ~1 h after immersion in 1 mL of X-gluc staining solution (1 mM X-gluc, 50 mM sodium phosphate buffer [pH 7.0]; 37 °C) confirming the presence of high levels of GUS activity in each separately transformed root line.

4.2. Estimation of relative of sense and antisense-ADC transcript levels in kanamycin-resistant transformed root lines

An asymmetric PCR procedure, based on the procedure described by Taylor (1991), was used to synthesise single-strand DNA probes for detection of mainly either sense- or antisense-ADC transcripts in selected transformed root lines by northern hybridization. Briefly, double-strand ADC DNA was first synthesized using oligonucleotides YC1F and EY1R and employing standard PCR methodology. Probes, capable of hybridising predominantly to either sense- or antisense- RNA strands, were subsequently produced by taking a 1 μl aliquot of the previous PCR reaction mix without purification and repeating the amplification process using ^{32}P-dATP rather than cold dATP and also adding normal concentrations of only one oligonucleotide primer (i.e. either YC1F or EY1R) to the PCR reaction mix. This process diluted the second primer to less than 2% of normal levels and allowed a small amount of double-stranded DNA synthesis during the subsequent amplification process which then served as a template for synthesis of predominantly single-strand DNA for use as sense or antisense probes in subsequent northern hybridization experiments. Radiolabelled DNA fragments were separated from unincorporated nucleotides by passage through a short glass column (Pasteur pipette) packed with Sephadex G-50 (medium grade; Amersham UK) using TE buffer (pH 8.0).

4.3. Quantification of ADC enzyme activity in transformed root lines

ADC enzyme assay procedures were based on those described in Robins et al. (1990). Briefly, root tissues were harvested at appropriate time points during the culture cycle; rinsed with several volumes of cold sterile milliQ water, blotted dry on absorbant paper until no further external moisture was evident, weighed, securely wrapped in aluminium foil, snap frozen in liquid nitrogen and stored at −70°C until required. To undertake the ADC assay, approximately 0.5 g of frozen tissue was ground to a fine powder under liquid N_2, using a mortar and pestle and in the presence of 50 mg insoluble polyvinylpyrrolidine (PVP, Sigma). Immediately upon evaporation of liquid N_2, the frozen powder was added to 1.5 mL volumes of cold buffer A (100 mM Tris–HCl [pH 8.0], 20 mM EDTA

[pH 8.0], 10 mM dithiothreitol [DTT] and 2 mM pyridoxal phosphate). After vortexing for approximately 20 s, each sample was stored on ice before all tubes were centrifuged together at 15,000*g* for 20 min at 4 °C to remove insoluble debris. Supernatants were transferred to a clean centrifuge tube and volumes adjusted to 3.5 mL with cold buffer A. To remove low molecular-weight contaminants, a pre-packed PD-10 desalting column (Pharmacia) was used in conjunction with cold 10% buffer A to first equilibrate the column and then elute soluble proteins from the column in accordance with manufacturer's instructions. Desalted plant extracts were kept on ice until required. Protein concentrations were determined by the protein-dye binding method using Bradford reagent (Biorad) according to the manufacturer's instructions (Bradford, 1976) with bovine serum albumin (BSA Fraction V; Promega) used as a standard.

Activity of ADC was determined by measuring the amount of $^{14}CO_2$ released from L-(U-^{14}C)-arginine (Amersham; specific activity 12.7 GBq/mol) diluted with non-radioactive L-arginine-HCl (Sigma) to produce a 2.5 mM stock with a specific activity of 103 MBq/mmol. The final incubation mixture contained 200 µl of enzyme extract and 50 µl of arginine stock in a de-capped microcentrifuge tube placed within a 20 mL scintillation vial containing 100 µl of 20% (w/v) KOH. Vials were sealed with silicone rubber subaseals before incubation at 37 °C in a shaking water bath. At designated time points, reactions were terminated by the injection of 200 µl of 5% (v/v) perchloric acid through the subaseal cap into the microcentrifuge tubes. Vials were incubated at 37 °C for a further 50 min to ensure that all ^{14}C-labelled CO_2 was absorbed by the KOH. Microcentrifuge tubes were then removed and their outsides rinsed into the vials with 0.9 ml of sterile water. After addition of 9 ml of scintillant (Ready Value, Beckman) vials were capped and shaken vigorously until the solution was clear, indicating a homogenous mixture. The radioactivity of the sample was then determined using a scintillation counter.

Acknowledgements

We are very grateful to Dr. Nick. J. Walton (BBSRC Institute Food Research, Norwich, UK) for advice pertaining to the measurement of ADC activity in root extracts and also to Dr. Aidan Sudbury (School of Mathematics, Monash University, Australia) for assistance with statistical analysis of data. In addition, we thank previous members of this laboratory, particularly Karen Cane, Elaine Chow, Eugene Fredericks, Angela Lidgett, Assunta Pelosi and Steve Sinclair for advice and general assistance. Y.C. gratefully acknowledges receipt of a Royal Thai Postgraduate scholarship to undertake higher studies in Australia and also the Phyllis Hillgrove fund for additional scholarship assistance. This work was supported by Australian Research Council Grant A19701779 to J.D.H.

References

Altabella, T., Angel, E., Biondi, S., Palazón, J., Bagni, N., Piñol, M.T., 1995. Effect of the rol genes from *Agrobacterium rhizogenes* on polyamine metabolism in tobacco roots. Physiologia Plantarum 95, 479–485.

Acosta, C., Pérez-Amador, D., Carbonell, J., Granwell, A., 2005. The two ways to produce putrescine in tomato are cell-specific during normal development. Plant Science 168, 1053–1057.

Baldwin, I.T., 1989. Mechanism of damage-induced alkaloid production in wild tobacco. Journal of Chemical Ecology 15, 1661–1680.

Baldwin, I.T., Ohnmeiss, T.E., 1994. Swords into plowshares – *Nicotiana sylvestris* does not use nicotine as a nitrogen-source under nitrogen-limited growth. Oecologia 98, 385–392.

Baldwin, I.T., Schmelz, E.A., Ohnmeiss, T.E., 1994. Wound-induced changes in root and shoot jasmonic acid pools correlate with induced nicotine synthesis in *Nicotiana sylvestris* Spegazzini and Comes. Journal of Chemical Ecology 20, 2139–2157.

Baldwin, I.T., Zhang, Z., Diab, N., Ohnmeiss, T.E., McCloud, E.S., Lynds, G.Y., Schmelz, E.A., 1997. Quantification, correlations and manipulations of wound-induced changes in jasmonic acid and nicotine in *Nicotiana sylvestris*. Planta 201, 397–404.

Barnes, S., Prasain, J., 2005. Current progress in the use of traditional medicines and nutraceuticals. Current Opinion in Plant Biology 8, 324–328.

Bevan, M.W., 1984. Binary Agrobacterium vectors for plant transformation. Nucleic Acids Research 12, 8711–8723.

Bey, P., Danzin, C., Jung, M., 1987. Inhibition of basic amino acid decarboxylases involved in polyamine biosynthesis. In: McCann, P.P., Pegg, A.E., Sjoerdsma, A. (Eds.), Inhibition of Polyamine Metabolism. Orlando Academic Press, pp. 1–32.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Cane, K.A., Mayer, M., Lidgett, A.J., Michael, A.J., Hamill, J.D., 2005. Molecular analysis of alkaloid metabolism in AABB vs. aabb genotype *Nicotiana tabacum* in response to wounding of aerial tissues and methyljasmonate treatment of cultured roots. Functional Plant Biology 32, 305–320.

Chintapakorn, Y., 2003. An antisense approach to study the role of ADC and PMT in alkaloid metabolism in *Nicotiana tabacum* L. PhD thesis. School of Biological Sciences, Monash University, Melbourne, Australia.

Chintapakorn, Y., Hamill, J.D., 2003. Antisense-mediated down-regulation of putrescine *N*-methyltransferase activity in transgenic *Nicotiana tabacum* L. can lead to elevated levels of anatabine at the expense of nicotine. Plant Molecular Biology 53, 87–105.

Croteau, R., Kutchan, T.M., Lewis, N.G., 2000. Natural products (secondary metabolites). In: Buchanan, B., Gruissern, W., Jones, R. (Eds.), Biochemistry and Molecular Biology of Plants, American Society of Plant Physiologists.

Dawson, R.F., 1941. The localisation of the nicotine synthetic mechanism in the tobacco plant. Science 94, 396–397.

Dawson, R.F., 1942. Nicotine synthesis in excised tobacco roots. American Journal of Botany 29, 813–815.

Dawson, R.F., Solt, M.L., 1959. Estimated contributions of root and shoot to the nicotine content of the tobacco plant. Plant Physiology 34, 656–661.

De Luca, V., St Pierre, B., 2000. The cell and developmental biology of alkaloid biosynthesis. Trends in Plant Science 5, 168–173.

Dixon, R.A., 2005. Engineering of plant natural product pathways. Current Opinion in Plant Biology 8, 329–336.

Flores, H.E., Hoy, M.H., Pickard, J.J., 1987. Secondary metabolites from root cultures. Trends in Biotechnology 5, 64–69.

Gelvin, S.B., 2000. Agrobacterium and plant genes involved in T-DNA transfer and integration. Annual Review Plant Physiology and Plant Molecular Biology 51, 223–256.

Georgiev, M.I., Pavlov, A.I., Bley, T., 2007. Hairy root type plant in vitro systems as sources of bioactive substances. Applied Microbiology and Biotechnology 74, 1175–1185.

Goosens, A., Häkkinen, S.T., Laakso, I., Seppänen-Laakso, T., Biondi, S., De Sutter, V., Lammertyn, F., Nuutila, A.M., Söderlund, H., Zabeau, M., Inzé, D., Oksman-Caldentey, K.M., 2003. A functional genomics approach toward the understanding of secondary metabolism in plant cells. Proceedings of the National Academy of Science USA 100, 8595–8600.

Griffin, W.J., Lin, G.D., 2000. Chemotaxonomy and geographical distribution of tropane alkaloids. Phytochemistry 53, 623–637.

Guillon, S., Tremouillaux-Guiller, J., pati, P.K., Rideau, M., Gantet, P., 2006. Harnessing the potential of hairy roots:dawn of a new era. Trends in Biotechnology 24, 403–409.

Hamill, J.D., Lidgett, A.J., 1997. Hairy root cultures – opportunities and key protocols for studies in metabolic engineering. In: Doran, P.M. (Ed.), Hairy Roots: Culture and Application. Overseas Publishers Association, Amsterdam, pp. 1–29.

Hamill, J.D., Parr, A.J., Robins, R.J., Rhodes, M.J.C., 1986. Secondary product formation by cultures of *Beta vulgaris* and *Nicotiana rustica* transformed with *Agrobacterium rhizogenes*. Plant Cell Reports 5, 111–114.

Hamill, J.D., Evans, D.M., Robins, R.J., Rhodes, M.J.C., Martin, C., Prescott, A., 1987a. Foreign gene insertion into transformed roots with binary vectors and *Agrobacterium rhizogenes* – potential for genetic manipulation of plant secondary metabolism. In: Robins, R.J., Rhodes, M.J.C. (Eds.), Manipulating Secondary Metabolism in Culture. Cambridge University Press, Cambridge, pp. 145–153.

Hamill, J.D., Prescott, A., Martin, C., 1987b. Assessment of the efficiency of co-transformation by the T-DNA of disarmed binary vectors derived from *Agrobacterium tumefaciens* and the T-DNA from *A. rhizogenes*. Plant Molecular Biology 9, 573–584.

Hamill, J.D., Rhodes, M.J.C., 1988. A spontaneous, light independent and prolif plant regeneration response from hairy roots of Nicotiana hesperis transformed by *Agrobacterium rhizogenes*. Journal Plant Physiology 133, 506–509.

Hamill, J.D., Robins, R.J., Parr, A.J., Evans, D.M., Furze, J.M., Rhodes, M.J.C., 1990. Over-expressing a yeast ornithine decarboxylase gene in transgenic roots of *Nicotiana rustica* can lead to enhanced nicotine accumulation. Plant Molecular Biology 15, 27–38.

Hamill, J.D., Rounsley, S., Spencer, A., Todd, G., Rhodes, M.J.C., 1991. The polymerase chain reaction in plant transformation studies. Plant Cell Reports 10, 221–224.

Hamill, J.D., Lidgett, A.J., 1997. Hairy root cultures – opportunities and key protocols for studies in metabolic engineering. In: Doran, P.M. (Ed.), Hairy Roots. Gordon and Breach/Harwood Academic, UK, pp. 1–30.

Hanfrey, C., Sommer, S., Mayer, M.J., Burtin, D., Michael, A.J., 2001. Arabidopsis polyamine biosynthesis: absence of ornithine decarboxylase and the mechanism of arginine decarboxylase activity. The Plant Journal 27, 551–560.

Hibi, N., Higashiguchi, S., Hashimoto, T., Yamada, Y., 1994. Gene expression in tobacco low-nicotine mutants. The Plant Cell 6, 723–735.

Hughes, E.H., Shanks, J.V., 2002. Metabolic engineering of plants for alkaloid production. Metabolic Engineering 4, 41–48.

Imanishi, S., Hashizume, K., Nakakita, M., Kojima, H., Matsubayashi, Y., Hashimoto, T., Sakagami, Y., Yamada, Y., Nakamura, K., 1998b. Differential induction by methyl jasmonate of genes encoding ornithine decarboxylase and other enzymes involved in nicotine biosynthesis in tobacco cell cultures. Plant Molecular Biology 38, 1101–1111.

Jackson, D.M., Johnson, A.W., Stephenson, M.G., 2002. Survival and development of *Heliothis virescens* (Lepidoptera:Noctuidae) larvae on isogenic tobacco lines with different levels of alkaloids. Journal of Economic Entomology 95, 1294–1302.

Jefferson, R.A., Kavanagh, T.A., Bevan, M.W., 1987. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO Journal 6, 3901–3907.

Kakkar, R.K., Sawhney, V.K., 2002. Polyamine research in plants – changing perspective. Physiologia Plantarum 116, 281–292.

Kutchan, T.M., 1995. Alkaloid biosynthesis – the basis for metabolic engineering of medicinal plants. Plant Cell 7, 1059–1070.

Kutchan, T.M., 1998. Molecular genetics of plant alkaloid biosynthesis. In: Cordell, G.A. (Ed.), The Alkaloids, Chemistry and Pharmacology, vol. 50. Academic Press, San Diego, pp. 257–316.

Kutchan, T.M., 2005. A role for intra- and intercellular translocation in natural product biosynthesis. Current Opinion in Plant Biology 8, 292–300.

Kutchan, T.M., Dixon, R.A., 2005. Physiology and metabolism. Secondary metabolism: nature's chemical reservoir under deconvolution. Current Opinion in Plant Biology 8, 227–229.

Legg, P.D., Collins, G.B., 1971. Inheritance of percent total alkaloids in *Nicotiana tabacum* L. II. Genetic effects of two loci in Burley 21 × LA Burley 21 populations. Canadian Journal of Genetics and Cytology 13, 287–291.

Legg, P.D., Chaplin, J.F., Collins, G.B., 1969. Inheritance of percent total alkaloids in *Nicotiana tabacum* L. Populations derived from crosses of low alkaloid lines with burley and flue-cured varieties. Journal of Heredity 60, 213–217.

Lovett, J.V., Hoult, A.H.C., 1998. Allelopathy in plants. In: Roberts, M.F., Wink, M. (Eds.), Alkaloids: Biochemistry, Ecology, and Medicinal Applications. Plenum Press, New York, pp. 337–347.

Luckner, M., 1990. Secondary Metabolism in Microorganisms, Plants, and Animals, third ed. Springer-Verlag, Berlin.

Malmberg, R.L., Watson, M.B., Galloway, G.L., Yu, W., 1998. Molecular genetic analyses of plant polyamines. Critical Reviews in Plant Sciences 17, 199–224.

Martin-Tanguy, J., 2001. Metabolism and function of polyamines in plants: recent developments (new approaches). Plant Growth Regulation 34, 135–148.

Maurel, C., Leblanc, N., Barbier-Brigoo, H., Perrot-Rochermann, C., Bouvier-Durand, M., Guern, J., 1994. Alteration of auxin perception in *rol*B-transformed protoplasts. Plant Physiology 105, 1209–1215.

Memelink, J., 2005. The use of genetics to dissect plant secondary pathways. Current Opinion in Plant Biology 8, 230–235.

Mizusaki, S., Tanabe, Y., Noguchi, M., Tamaki, E., 1973. Changes in the activities of ornithine decarboxylase, putrescine *N*-methyltransferase and *N*-methylputrescine oxidase in tobacco roots in relation to nicotine biosynthesis. Plant and Cell Physiology 14, 103–110.

Moore, E.L., Powell, N.T., Jones, G.L., Gwynn, G.R., 1962. Flue-cured tobacco variety NC-95; Resistant to root-knot, black shank and the wilt diseases. In: Agricultural Experiment Station, Bulletin 419, North Carolina State College, North Carolina, Raleigh, Raleigh USA, pp. 3–18.

Moriuchi, H., Okamoto, C., Nishihma, R., Yamashita, I., Machida, Y., Tanaka, N., 2004. Nuclear localization and interaction of RolB with plant 14-3-3 proteins correlates with induction of adventitious rots by the oncogene *rol*B. Plant Journal 38, 260–275.

Palazón, J., Cusidó, R.M., Roig, C., Piñol, M.T., 1997. Effect of rol genes from *Agrobacterium rhizogenes* TL-DNA on nicotine production in tobacco root cultures. Plant Physiology and Biochemistry 35, 155–162.

Palazón, J., Cusidó, R.M., Roig, C., Piñol, M.T., 1998. Expression of the rolC gene and nicotine production in transgenic roots and their regenerated plants. Plant Cell Reports 17, 384–390.

Parr, A.J., Hamill, J.D., 1987. Relationship between *Agrobacterium rhizogenes* transformed hairy roots and intact, uninfected *Nicotiana* plants. Phytochemistry 26, 3241–3245.

Perez-Amador, M.A., Carbonell, J., Granell, A., 1995. Expression of arginine decarboxylase is induced during early fruit development and in young tissues of *Pisum sativum* (L). Plant Molecular Biology 28, 997–1009.

Rastogi, R., Dulson, J., Rothstein, S.J., 1993. Cloning of tomato (*Lycopersicon esculentum* Mill) arginine decarboxylase gene and its expression during fruit ripening. Plant Physiology 103, 829–834.

Reed, D.G., Jelesko, J.G., 2004. The *A* and *B* loci of *Nicotiana tabacum* have non-equivalent effects on the mRNA levels of four alkaloid biosynthetic genes. Plant Science 167, 1123–1130.

Rhodes, M.J.C., Robins, R.J., Aird, E.L.H., Payne, A.J., Parr, A.J., Walton, N.J., 1989. Regulation of secondary metabolism in transformed root cultures. In: Kurz, W.G.W. (Ed.), Primary and Secondary Metabolism of Plant Cell Culture. Springer-Verlag, Berlin, pp. 58–72.

Roberts, M.F., Wink, M., 1998. Introduction. In: Roberts, M.F., Wink, M. (Eds.), Alkaloids: Biochemistry, Ecology, and Medicinal Applications. Plenum Press, New York, pp. 1–7.

Robins, R.J., Parr, A.J., Payne, J., Walton, N.J., Rhodes, M.J.C., 1990. Factors regulating tropane-alkaloid production in a transformed root culture of a *Datura candida* × *Datura aurea* hybrid. Planta 181, 414–422.

Robins, R.J., Parr, A.J., Walton, N.J., 1991. Studies on the biosynthesis of tropane alkaloids in *Datura stramonium* L. transformed root cultures 2. On the relative contributions of L-arginine and L-ornithine to the formation of the tropane ring. Planta 183, 196–201.

Ruiz, J.M., Blasco, B., Rivero, R.M., Romero, L., 2005. Nicotine-free and salt tolerant tobacco plants obtained by grafting to salinity-resistant rootstocks of tomato. Physiologia Plantarum 124, 465–475.

Saitoh, F., Noma, M., Kawashima, N., 1985. The alkaloid contents of sixty *Nicotiana* species. Phytochemistry 24, 477–480.

Saunders, J.W., Bush, L.P., 1979. Nicotine biosynthetic enzyme activities in *Nicotiana tabacum* L. genotypes with different alkaloid levels. Plant Physiology 64, 236–240.

Schmülling, T., Schell, J., Spena, A., 1988. Single genes from Agrobacterium rhizogenes influence plant development. EMBO Journal 7, 2621–2629.

Shoji, T., Nakajima, K., Hashimoto, T., 2000. Ethylene suppresses jasmonate-induced gene expression in nicotine biosynthesis. Plant and Cell Physiology 41, 1072–1076.

Sinclair, S.J., Johnson, R., Hamill, J.D., 2004. Analysis of wound-induced gene expression in *Nicotiana* species with contrasting alkaloid profiles. Functional Plant Biology 31, 1–9.

Sisson, V.A., Severson, R.F., 1990. Alkaloid composition of the *Nicotiana* species. Beiträge zur Tabakforschung International 14, 327–339.

Slightom, J.L., Durand-Tardif, M., Jouanin, L., Tepfer, D., 1986. Nucleotide sequence analysis of TL-DNA of *Agrobacterium rhizogenes* agropin-type plasmid: identification of openreading frames. Journal of Biological Chemistry 261, 108–121.

Slocum, R.D., Galston, A.W., 1985a. In vivo inhibition of polyamine biosynthesis and growth in tobacco ovary tissues. Plant & Cell Physiology 26, 1519–1526.

Slocum, R.D., Galston, A.W., 1985b. Arginase-mediated hydrolysis of DFMA to DFMO *in vivo*. Plant Physiology 77 (Suppl.), S45.

Slocum, R.D., Bitonti, A.J., McCann, P.P., Feirer, R.P., 1988. DL-α-difluoromethyl [3,4-^{3}H] arginine metabolism in tobacco and mammalian cells; inhibition of ornithine decarboxylase activity after arginase-mediated hydrolysis of DL-α-difluoromethylarginine to DL-α-difluoromethylornithine. Biochemical Journal 255, 197–202.

Southon, I.W., Buckingham, J. (Eds.), 1989. Dictionary of Alkaloids. Chapman and Hall, London.

Steppuhn, A., Gase, K., Krock, B., Halitschke, R., Baldwin, I.T., 2004. Nicotine's defensive function in nature. PLoS Biology 2, 1074–1080.

Taylor, G.R., 1991. Polymerase chain reaction: basic principles and automation. In: McPherson, M.J., Quirke, P., Taylor, G.R. (Eds.), PCR: A Practical Approach. Oxford University Press, pp. 1–14.

Tepfer, D., 1984. Transformation of several species of higher plants by *Agrobacterium rhizogenes*: sexual transmission of the transformed genotype and phenotype. Cell 37, 959–968.

Tiburcio, A.F., Galston, A.W., 1986. Arginine decarboxylase as the source of putrescine for tobacco alkaloids. Phytochemistry 25, 107–110.

Tiburcio, A.F., Altabella, T., Borrell, A., Masgrau, C., 1997. Polyamine metabolism and its regulation. Physiologia Plantarum 100, 664–674.

Trung-Nghia, P., Bassie, L., Safwat, G., Thu-Hang, P., Lepn, O., Rocha, P., Christous, P., Capell, T., 2003. Reduction of the endogenous arginine decarboxylase transcript levels in rice leads to depletion of the putrescine and spermidine pools with no concomitant changes in the expression of downstream genes in the polyamine biosynthesis pathway. Planta 218, 125–134.

Tzfira, T., Li, J., Lacroix, B., Citovsky, V., 2004. Agrobacterium–T-DNA integration: models and molecules. Trends in Genetics 20, 375–383.

Walton, N.J., Robins, R.J., Peerless, A.C.J., 1990. Enzymes of *N*-methylputrescine biosynthesis in relation to hyoscyamine formation in transformed root cultures of *Datura stramonium* and *Atropa belladonna*. Planta 182, 136–141.

Wang, J.M., Sheehan, M., Brookman, H., Timko, M.P., 2000. Characterization of cDNAs differentially expressed in roots of tobacco (*Nicotiana tabacum* cv. Burley 21) during the early stages of alkaloid biosynthesis. Plant Science 158, 19–32.

Wink, M., 1998a. A short history of alkaloids. In: Roberts, M.F., Wink, M. (Eds.), Alkaloids: Biochemistry, Ecology, and Medicinal Applications. Plenum Press, New York, pp. 11–44.

Wink, M., 1998b. Chemical ecology of alkaloids. In: Roberts, M.F., Wink, M. (Eds.), Alkaloids: Biochemistry, Ecology, and Medicinal Applications. Plenum Press, New York, pp. 265–300.

Wink, M., 2003. Evolution of secondary metabolites from an ecological and molecular perspective. Phytochemistry 64, 3–19.

Yang, YP., Lin, P.P.C., Bush, L.P., 1984. Induction of putrescine biosynthetic enzymes and its relation to polyamine and nicotine synthesis in roots of tobacco. Plant Physiology, 118–673.

Yoshida, D., Mitake, T., 1966. Agmatine and *N*-carbamylputrescine as intermediates in the formation of nicotine by tobacco plants. Plant and Cell Physiology 7, 301–305.

Zhang, Z.P., Baldwin, I.T., 1997. Transport of [2-^{14}C] jasmonic acid from leaves to roots mimics wound-induced changes in endogenous jasmonic acid pools in *Nicotiana sylvestris*. Planta 203, 436–441.