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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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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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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-

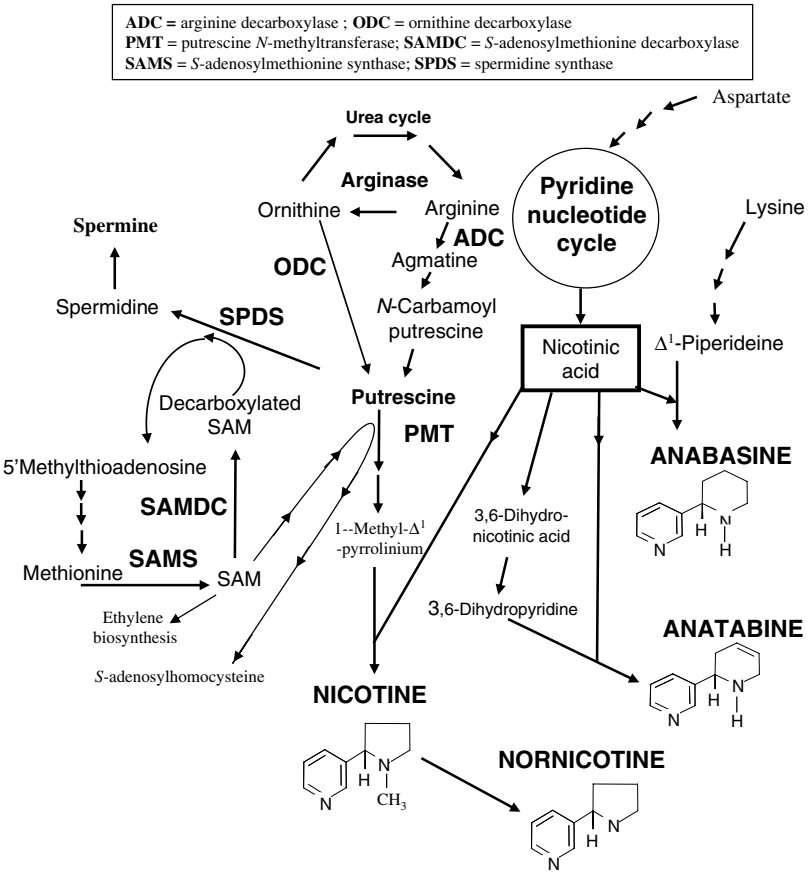

Fig. 1. Schematic diagram showing main steps in the synthesis of the 4 main alkaloids in *Nicotiana* species (adapted from Luckner, 1990; Imanishi et al., 1998b; Cane et al., 2005). In roots of *N. tabacum* varieties of *AABB* genotype (includes NC 95 used in the current study), nicotine comprises ~80–85% 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.