RNAi-mediated down-regulation of ornithine decarboxylase (ODC) leads to reduced nicotine and increased anatabine levels in transgenic *Nicotiana tabacum* L.

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ABSTRACT

In leaf and root tissues of *Nicotiana tabacum* L. (common tobacco), nicotine is by far the predominant pyridine alkaloid, with anatabine representing only a minor component of the total alkaloid fraction. The pyrrolidine ring of nicotine is derived from the diamine putrescine, which can be synthesized either directly from ornithine *via* the action of ODC, or from arginine *via* a three enzymatic step process, initiated by ADC. Previous studies in this laboratory have shown that antisense-mediated down-regulation of *ADC* transcript levels has only a minor effect upon the alkaloid profile of transgenic *N. tabacum*. In the present study, RNAi methodology was used to down-regulate *ODC* transcript levels in *N. tabacum*, using both the *Agrobacterium rhizogenes*-derived hairy root culture system, and also disarmed *Agrobacterium tumefaciens* to generate intact transgenic plants. We observed a marked effect upon the alkaloid profile of transgenic tissues, with *ODC* transcript down-regulation leading to reduced nicotine and increased anatabine levels in both cultured hairy roots and intact greenhouse-grown plants. Treatment of *ODC*-RNAi hairy roots with low levels of the wound-associated hormone methyl jasmonate, or wounding of transgenic plants by removal of apices – both treatments which normally stimulate nicotine synthesis in tobacco – did not restore capacity for normal nicotine synthesis in transgenic tissue but did lead to markedly increased levels of anatabine. We conclude that the ODC mediated route to putrescine plays an important role in determining the normal nicotine:anatabine profile in *N. tabacum* and is essential in allowing *N. tabacum* to increase nicotine levels in response to wound-associated stress.

1. Introduction

The plant kingdom produces a plethora of specialized metabolites, production of which involves complex regulation of both primary and secondary biosynthetic pathways. Increasingly, genetic approaches are being utilized to understand the relationship between primary and secondary metabolism and to manipulate flux between pathways. A useful model species in this regard is common tobacco – *Nicotiana tabacum* L., which accumulates moderate-high levels of the toxic pyridine alkaloid nicotine. A second alkaloid, anatabine, also constitutes a small but significant proportion of the alkaloid fraction in *N. tabacum* and in many other species in the genus *Nicotiana* (Saitoh et al., 1985; Sisson and Severson, 1990). Other alkaloids characteristic of the genus *Nicotiana*, such

as anabasine or nornicotine are not usually present at appreciable quantities in most varieties of *N. tabacum*. In the case of anabasine, this is due to low levels of lysine decarboxylase in root tissues so leading, in turn, to a low inherent capacity to produce cadaverine which is essential for anabasine synthesis (Fecker et al., 1993; Herminghaus et al., 1991, 1996). Nornicotine, considered undesirable in commercial tobacco, is produced *via* the action of nicotine demethylase which may be active in growing plants or in tissues post-harvest depending upon the genotype (Bush et al., 1999). It is now generally accepted that alkaloids in *Nicotiana* species are important specialized metabolites which have roles in defending plants against predators in their natural environments (Jackson et al., 2002; Steppuhn et al., 2004; Cane et al., 2005 and references therein). Nicotine itself is synthesized in the roots of *Nicotiana* species and is translocated to leaves *via* the xylem stream (Dawson, 1941, 1942; Baldwin, 1989), where conversion to nornicotine may occur if active nicotine demethylase enzyme, encoded by one or more *CPY82E* gene family members, is present (Siminszky et al., 2005; Gavilano et al., 2006, 2007; Chakrabarti et al., 2007; Gavilano and Siminszky, 2007; Xu et al., 2007; Lewis et al., 2010). Levels of nicotine in leaf tissues have been shown to increase in a number of *Nicotiana* species in response to aerial damage inflicted by herbivorous pests and/or simple mechanical damage. Indeed, it is com-

mon practice in commercial tobacco cultivation to remove the floral apex (topping of plants) several days prior to harvest to ensure higher levels of nicotine in leaf tissues (Bush et al., 1999). Previous studies demonstrated a significant increase in the activity of several nicotine biosynthetic enzymes in the roots of *N. tabacum* plants when they were analysed 24–48 h after removal of the apex. This increase, which included ornithine decarboxylase (ODC), putrescine methyltransferase (PMT) and quinolinate phosphoribosyltransferase (QPT), preceded a marked rise in nicotine content of remaining leaf tissues during the following week (Mizusaki et al., 1973; Saunders and Bush, 1979). More recent molecular studies have reiterated these more classical findings and have indicated the presence of sophisticated signaling cascades and transcription factors involved in the up-regulation of key genes encoding enzymes responsible for providing both the pyridine and the pyrrolidine ring of nicotine (Hibi et al., 1994; Imanishi et al., 1998; Sinclair et al., 2000, 2004; Cane et al., 2005; Goossens et al., 2003; De Sutter et al., 2005; Shoji et al., 2008, 2010; Todd et al., 2010).

An important precursor to the formation of the pyrrolidine ring of nicotine is putrescine, a ubiquitous diamine that is utilized in the synthesis of the polyamines spermidine and spermine. These organic cations are found in cells of all organisms. In plants, spermidine is generally regarded as being essential for viability whilst spermine is not essential for life *per se* but plays an important role in enhancing tolerance to a number of abiotic environmental stresses. Thermospermine, an isomer of spermine, has also been identified as being important for growth of the flowering stem. These and other aspects of polyamine metabolism in plants have been thoroughly reviewed recently (Kusano et al., 2008; Takahashi and Kakehi, 2009; Alcázar et al., 2010; Fuell et al., 2010).

In most plants, including *Nicotiana* species, putrescine is derived directly from the amino acid ornithine, *via* the activity of ornithine decarboxylase (ODC), or indirectly from the amino acid arginine, *via* a three enzymatic step process which is initiated by arginine decarboxylase (ADC) (Alcázar et al., 2010; Fuell et al., 2010). The pyrrolidine ring of nicotine is derived from putrescine, *via* activity of the key regulatory enzyme putrescine methyltransferase (PMT) leading to the production of the methylpyrrolinium cation, which is condensed with a nicotinic acid derivative to produce nicotine. Recent reports confirm the earlier suggestion of Hibi et al. (1994) that a PIP family protein, encoded by the *A622* gene, is crucial for the synthesis of nicotine and other related pyridine alkaloids such as anatabine and anabasine (DeBoer et al., 2009; Kajikawa et al., 2009). A simplified scheme illustrating pertinent steps in alkaloid synthesis in *Nicotiana* is shown in Fig. 1.

For many years, there was some debate in the scientific literature as to whether the ODC or the ADC route to putrescine is the more important in enabling nicotine production in *Nicotiana* (Tiburcio and Galston, 1986; Walden et al., 1997; also see Chintapakorn and Hamill, 2007 and references therein for a fuller discussion). Previous work in this laboratory used an antisense approach to down-regulate *ADC* transcript levels in both hairy roots cultured *in vitro*, and in regenerated transgenic plants grown in soil. Despite a substantial decrease in both *ADC* transcript levels and enzyme activity, only minor effects upon the capacity of *N. tabacum* to synthesize nicotine were observed (Chintapakorn and Hamill, 2007). Together with previous observations that showed *ODC* transcript levels and enzymatic activity were strongly up-regulated in plants in response to wounding, and also in cell and root cultures treated with methyl jasmonate (Mizusaki et al., 1973; Saunders and Bush, 1979; Imanishi et al., 1998; Wang et al., 2000; Xu et al., 2004; Cane et al., 2005), these results suggested that the presence of ODC in *N. tabacum* is essential for nicotine production, particularly in response to wound-associated stress. To test this suggestion experimentally, we have undertaken experiments to diminish *ODC* transcript levels *in vivo* and assess effects

on alkaloid metabolism in tissues ± wound-associated stress. In addition to observing a significant reduction in the ability of transgenic tissues to produce nicotine, we also observed an increased capacity for *ODC*-RNAi transgenic hairy roots and plants to accumulate anatabine. As noted in previous work, a substantial increase in levels of this alkaloid is indicative of an imbalance in the supply of putrescine-derived intermediates required for nicotine synthesis in transgenic tissues (Chintapakorn and Hamill, 2003; Wang et al., 2009).

2. Materials and methods

2.1. Construction of the ODC-RNAi vector

Basic transformation and molecular analytical procedures were performed as described in Sambrook et al. (1989) and Hamill and Lidgett (1997). An *ODC*-RNAi vector was created as part of a parallel study examining *ODC* gene structure and transcript downregulation in *Nicotiana glauca* (DeBoer, 2010). Briefly, a 347 bp *ODC* PCR DNA fragment from the *N. glauca ODC* gene (Genbank accession number FR691072.1; ∼98% identity to the *N. tabacum ODC1* and *ODC2* genes (Accession numbers AB031066 and AF233849)) was cloned in both the sense and anti-sense direction in the vector pKANNIBAL (Genbank accession number: AJ311873; Wesley et al., 2001) through the use of restriction enzyme sites in the 5′ region of oligonucleotides (primer set one: Forward 5′ GGATTCCGCCATTCTTCAGTCCACAAT 3′; Reverse 5′ ATCGATATGC-CAA GGGCTAAAACGTA 3′) (primer set two: Forward 5′ CTCGAGGCC ATTCTTCAGTCCACAAT 3′; Reverse 5′ GGTACCATGC-CAAGGGCT AAAACGTA 3′) [for each reaction: 92 °C for 2 min (1 cycle); 92 °C for 30 s, 53 °C for 1 min 72° for 1 min (30 cycles); 72 °C for 10 min (1 cycle)]. The *ODC*-RNAi gene expression cassette was purified from the pKANNIBAL vector by digesting with *NotI* restriction enzyme, and ligated into the binary vector pART27 (Genbank accession number AJ311874; Gleave, 1992).

2.2. Creation of ODC-RNAi hairy root cultures and transgenic plants

Both the CaMV35S *ODC*-RNAi construct and the empty-control pART27 binary vector were transformed into *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens* using standard electroporation procedures. Transgenic hairy root cultures containing either the pART27 or the *ODC*-RNAi binary vector were created by *A. rhizogenes* transformation techniques as described previously (Hamill et al., 1987; Hamill and Lidgett, 1997; DeBoer et al., 2009). Cultures were established from single root tips derived from separate transformation events and sub-cultured every 3 weeks by placing approximately 0.2 g (fresh weight) of healthy white root tips into vessels containing 50 ml of B5 medium (B5 salts [Phytotechnology, Australia], 3% sucrose, pH 6.0 before autoclaving) supplemented with 200 mg L⁻¹ filter-sterilized ampicillin (Phytotechnology, Australia) and 25 mg L⁻¹ filter-sterilized kanamycin sulphate (Phytotechnology, Australia) to select for transgenics (Hamill et al., 1987). Selected hairy root cultures were regenerated into plants *via* methods outlined in Chintapakorn and Hamill (2003), except that 75 mg L⁻¹ kanamycin sulphate was also included in the regeneration medium. Intact, callus-derived transgenic plants were created separately *via A. tumefaciens* (strain LBA 4404) transformation techniques using the basic method of Horsch et al. (1985) and as outlined in DeBoer et al. (2009).

Hairy root cultures were treated with methyl jasmonate (MeJa) (Serva) based upon methods described in Cane et al. (2005). Experimental root cultures were initiated by placing 0.2 g of root tissue into 50 ml B5 medium and, after 11 days when cultures were beginning to grow rapidly, 20 µl of AnalaR absolute ethanol (BDH