Effects of down-regulating ornithine decarboxylase upon putrescine-associated metabolism and growth in *Nicotiana tabacum* L.

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**Abstract**

Transgenic plants of *Nicotiana tabacum* L. homozygous for an RNAi construct designed to silence *ornithine decarboxylase* (*ODC*) had significantly lower concentrations of nicotine and nornicotine, but significantly higher concentrations of anatabine, compared with vector-only controls. Silencing of *ODC* also led to significantly reduced concentrations of polyamines (putrescine, spermidine and spermine), tyramine and phenolamides (caffeoylputrescine and dicaffeoylspermidine) with concomitant increases in concentrations of amino acids ornithine, arginine, aspartate, glutamate and glutamine. Root transcript levels of *S*-adenosyl methionine decarboxylase, *S*-adenosyl methionine synthase and spermidine synthase (polyamine synthesis enzymes) were reduced compared with vector controls, whilst transcript levels of arginine decarboxylase (putrescine synthesis), putrescine methyltransferase (nicotine production) and multi-drug and toxic compound extrusion (alkaloid transport) proteins were elevated. In contrast, expression of two other key proteins required for alkaloid synthesis, quinolinic acid phosphoribosyltransferase (nicotinic acid production) and a PIP-family oxidoreductase (nicotinic acid condensation reactions), were diminished in roots of *odc*-RNAi plants relative to vector-only controls. Transcriptional and biochemical differences associated with polyamine and alkaloid metabolism were exacerbated in *odc*-RNAi plants in response to different forms of shoot damage. In general, apex removal had a greater effect than leaf wounding alone, with a combination of these injury treatments producing synergistic responses in some cases. Reduced expression of *ODC* appeared to have negative effects upon plant growth and vigour with some leaves of *odc*-RNAi lines being brittle and bleached compared with vector-only controls. Together, results of this study demonstrate that ornithine decarboxylase has important roles in facilitating both primary and secondary metabolism in *Nicotiana*.

**Key words:** Alkaloid, gene expression, ODC, phenolamide, polyamine, putrescine, PMT, QPT, RNAi.
Introduction

Terrestrial plants have been subjected to herbivory since their emergence onto land ca. 450 million years ago and a wide array of physical and chemical defence systems have evolved to provide protection and facilitate their reproduction in native environments (Labandeira, 1998; Wellman and Gray, 2000). Alkaloids represent a diverse grouping of such chemical defences, with many thousands of chemical structures distributed widely across the plant kingdom (Aniszewski, 2015). Biosynthesis of alkaloids generally involves the diversion of amino acid precursors from primary into secondary metabolism via the action of decarboxylases and is often enhanced by exposure of plants to biotic and/or abiotic stress conditions (Shoji and Hashimoto, 2013a).

The genus Nicotiana (family Solanaceae) contains more than 75 species, native mainly to the Americas and mainland Australia, with representatives also on South Pacific islands and in southern Africa (Knapp et al., 2004). The genus is well known for its production of a range of pyridine alkaloids, particularly nicotine, nornicotine, anabasine and anatabine, which are found at various concentrations in all Nicotiana species (Saitoh et al., 1985). Acting as agonists on the nervous system of herbivores, both invertebrate and vertebrate, they discourage feeding and increase the rate of herbivore mortality and/or susceptibility to predatory attack (Steppuhn et al., 2004). Figure 1 provides an overview of alkaloid biosynthesis in N. tabacum and its relationship with other aspects of putrescine metabolism.

Synthesis of the alkaloid nicotine has been reported to be energy demanding (Bush et al., 1999) and diversion of nitrogen from primary metabolism, growth and reproduction into synthesis of this defence compound can also have fitness costs, as demonstrated in N. attenuata growing in native environments (Baldwin et al., 1990; Baldwin and Ohnmeiss, 1994; Ohnmeiss and Baldwin, 2000). Experiments with cultivated N. tabacum, and also native species N. sylvestris and N. attenuata, showed that damage to aerial tissues led to an increase in nicotine content of leaves within several days of wounding (Saunders and Bush, 1979; Baldwin, 1989; Baldwin and Ohnmeiss, 1993; Baldwin and Ohnmeiss, 1994; Sinclair et al., 2004). Studies in Nicotiana have attributed the transmission of wound signals resulting from leaf damage and apex removal (topping) from aerial to root tissues to increased JA and reduced auxin levels, respectively (Baldwin et al., 1994; Baldwin et al., 1996; Shi et al., 2006). Recent reports indicate that a convergence of both JA and auxin cross-signalling networks is likely to operate at the molecular level in vivo through shared components of these transduction pathways (Pauwels et al., 2010; Hentrich et al., 2013; He and Zhao, 2015).

Transcription of key structural genes required for alkaloid biosynthesis is regulated via the action of several transcription factors, including MYC2 and APETAL2/ETHYLENE RESPONSE FACTOR (AP2/ERF) types, which are themselves regulated by these hormones (reviewed in Dewey and Xie, 2013; Shoji and Hashimoto, 2013a). Alkaloid transport from roots to the aerial tissues occurs via the xylem system, with loading/unloading into the xylem and storage in leaf vacuoles involving several MULTIDRUG AND TOXIC COMPOUND EXTRUSION (MATE)-type transporters, as well as NICOTINE UPTAKE PERMEASE (NUP1) proteins (Morita et al., 2009; Shitan et al., 2009; Shoji et al., 2009; Hildreth et al., 2011; Shitan et al., 2014a; Shitan et al., 2014b; Kato et al., 2014).

The diamine putrescine is an important intermediate precursor in the synthesis of higher amines, spermidine and spermine, which play important roles in metabolic, physiological and developmental processes in all living organisms (Fariduddin et al., 2013; Kusano and Suzuki, 2015). In most plant species, putrescine can be synthesized from either ornithine or arginine via the activity of the decarboxylating enzymes, ORNITHINE DECARBOXYLASE (ODC) or ARGinine DECARBOXYLASE (ADC), respectively (Shoji and Hashimoto, 2013a; Michael, 2015). Plant polyamines exist predominantly as conjugates with hydroxycinnamic acids in the Solanaceae family, collectively described as phenolamides (Fig. 1; Smith et al., 1983; Martin-Tanguy, 1985; Kaur et al., 2010; Onkokesung et al., 2012). Such conjugated polyamines have been reported to occur throughout the plant kingdom and appear to have roles in chemical defence as well as aspects of plant development (Kaur et al., 2010; Fellenberg et al., 2012; Onkokesung et al., 2012).

In many solanaceous genera, putrescine is also an important precursor for alkaloid synthesis, including nicotine and nornicotine (reviewed in Dewey and Xie, 2013; Shoji and Hashimoto, 2013a). Synthesis of nicotine involves the condensation of a nicotinic acid-derived pyridine ring, sourced from the pyridine nucleotide cycle, with a pyrrolidine ring derived from putrescine (Dewey and Xie, 2013; Shoji and Hashimoto, 2013a). A further step involving the N-demethylation of nicotine is the primary means of producing nornicotine (Simin szky et al., 2005; Lewis et al., 2008; Lewis et al., 2010). Anatabine, the other main alkaloid in N. tabacum, is derived entirely from two molecules of nicotinic acid (Leete and Slattery, 1976; Leete, 1992). Using antisense and RNAi methodology, our previous studies indicated that marked reductions in ODC, but not ADC, transcript levels had a marked effect on the capacity of transgenic N. tabacum to synthesise nicotine (Chintapakorn and Hamill, 2007; DeBoer et al., 2011a). In the current study, utilizing T2 generation plants of N. tabacum homozygous for an introduced odc-RNAi construct (DeBoer et al., 2011a), we undertook a detailed analysis of the effects of down-regulating ODC upon the production of amines and associated pools of amino acids, as well as the changes in defence chemistry and components of the associated root transcriptome.

Materials and methods

Plant material

Homozygous T2 plants were generated from transgenic N. tabacum (SC 58 variety, AABB genotype; Chaplin, 1966; Cane et al., 2005) lines At-Nt odc-RNAi-3 and At-Nt-odc-RNAi-4 plants, which were described fully in DeBoer et al. (2011a). Comparable T2 homozygous plants containing the T-DNA insert from an empty pART27 vector (vector-only control; VC) were used as a transformation
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control. These plants were identical in growth habit and morphology to those of non-transgenic parental line SC 58. Seeds of all lines were surface-sterilized and germinated in vitro on Murashige and Skoog (MS) agar plates containing 3% sucrose and 75 μg mL⁻¹ kanamycin sulphate according to Chintapakorn and Hamill (2003) and maintained in a 25 °C/16 h photoperiod and allowed to grow for ~4 weeks before transfer to 250 mL glass jars containing 50 mL of agar-solidified MS medium. Two weeks later seedlings (~4–6 leaf stage) were placed in rockwool blocks and transferred to communal hydroponic trays to acclimatize for a further 2 weeks before being placed in individual hydroponic containers each containing 200 mL of full strength Hoagland’s medium, formulated as described previously (Cane et al., 2005). Hydroponic chambers were randomly distributed on a communal bench at 20 cm intervals in an insect-proof (PC2) glasshouse at 25 ± 2 °C and plants grown under uniform supplemental fluorescent lighting and a 16/8 h photoperiod, with bi-weekly media changes and daily liquid replenishment to 200 mL with deionized water as described in previous work (DeBoer et al., 2009; DeBoer et al., 2011a; DeBoer et al., 2013). For further seed production, additional seedlings of each line (~4–6 leaf stage) were grown on a common damp mat in the same greenhouse at 25 ± 2 °C, under ambient lighting, in 250 mL pots of compost (3 parts seed raising mix : 1 part perlite) containing a single application of controlled release complete fertilizer (Osmocote®, 15g L⁻¹ of compost mix) as recommended by the manufacturer (Scotts Australia Pty Ltd).

### Plant treatments

After 4 weeks growth in hydroponics, transgenic *N. tabacum* plants (~10–12 leaf stage) either remained non-wounded (C) or were mechanically injured in one of three ways as follows. (1) Designated ‘W’ for wounded; a fabric pattern wheel was drawn across the lamina twice on each side of the mid-vein of the two uppermost (>50%) expanded leaves to simulate insect attack (Ohnmeiss and Baldwin, 1994). (2) Designated ‘A’ for apex removal; a sharp scalpel blade was used to remove the shoot apex and young leaves (less than 50% expanded) to simulate ‘topping’ (Saunders and Bush, 1979; Bush et al., 1999). (3) Designated ‘W+A’; a combination of both damage treatments was used. Unless otherwise stated, chemical analysis was performed using wounded leaves or the two leaves located immediately below the apex removal point. Tissues were harvested 24 h or 7 days after treatment for RNA or metabolite analysis, respectively.

**Fig. 1.** Schematic diagram of the biosynthesis of polyamines and connections with other metabolic pathways, including pyridine alkaloid, phenolamide and phenylpropanoid metabolism, in *N. tabacum* (adapted from Cane et al., 2005; Onkokesung et al., 2012; Shoji and Hashimoto, 2012; Winter et al., 2015). Solid lines indicate defined steps, while dotted lines indicate undefined steps or steps including multiple reactions. Both pyridine alkaloid and polyamine/phenolamide metabolites share early biosynthetic steps, starting from arginine and ornithine to produce putrescine. The biosynthesis of alkaloids in tobacco occurs mainly in roots, while the phenolamide and phenylpropanoid pathways are present predominantly in above-ground (leaf) tissues. The final condensation reactions with nicotinic acid derivatives and N-methyl pyrrolinium cation are not yet clear; however, it is suggested that a PIP-family soffavone reductase-like protein, A622, is involved in the late step of synthesis of the pyridine moiety prior to synthesis steps involving enzymes from a Berberine Bridge Like gene family. Several toxin transport proteins including MATEs and NUP1 are involved in vacuolar storage and transport of alkaloids to aerial tissues (Shoji et al., 2009; Monka et al., 2009; Hildreth et al., 2011; Shtian et al., 2014a, b).
in line with previous work from this laboratory (Cane et al., 2005; DeBoer et al., 2009; DeBoer et al., 2011a; DeBoer et al., 2013). In non-damaged control plants, cotton thread was tied loosely around the petiole of phyllo-tactically equivalent leaves on day 0, with these leaves being harvested for analysis at the same time point as in damaged plants.

Targeted analysis of leaf and root primary and secondary metabolites
Concentrations of amino acids, amines, alkaloids and phenolamides were determined using portions of homogeneous powdered tissue that had previously been freeze-dried for a minimum of 48 h. One hundred milligrams of freeze-dried ground leaf or root powder was pre-weighed and aliquoted into 1.5 mL Eppendorf tubes containing a sterile stainless steel ball to aid extraction for subsequent metabolite analyses. Alkaloids and phenolamides were extracted from leaf and root samples using an optimized 40% methanol extraction method described by Gaquerel et al. (2010). Amino acids were analysed and quantified by LC-MS/MS. Samples were prepared as reported above for alkaloid analysis and aliquots of the supernatant were diluted and analysed as described by Jander et al. (2004). Amines (putrescine, spermidine, spermine and tyramine) were extracted using an optimized hydrochloric and boric acid extraction and supernatant aliquots were analysed as ortho-phthalaldehydehydruethanethiolfluorenylmethoxycarbonyl derivatives as described by Fellenberg et al. (2012). Concentrations of amino acids, amines, alkaloids and phenolamides were quantified relative to known concentrations of standards and are graphically presented per milligram dry weight of tissue sample that was extracted.

Quantitative real time PCR
Total RNA was isolated from leaf and root tissues snap-frozen in liquid N\textsubscript{2} using a hot phenol method adapted from Verwoerd et al. (1989) and previously found to be suitable for extraction of high quality RNA from both leave and root tissue of Nicotiana species (Cane et al., 2005; DeBoer et al., 2011a). DNase treated RNA was reverse transcribed with Superscript III reverse transcriptase (Invitrogen) using oligo (dT)\textsubscript{18} following the manufacturer’s recommendations. Quantitative RT-PCR (qRT-PCR) was performed with approximately 150 ng of cDNA on a Lightcycler 480 real-time instrument (Roche) using SensiMix\textsuperscript{TM} SYBR no-ROX (Bioline) following the manufacturer’s recommendations. Previously published gene-specific primers (Shoji et al., 2008; Shoji et al., 2010; Schmidt and Delaney, 2010; Shoji and Hashimoto, 2011) were used, with slight modifications where stated, so that each primer pair combination produced an amplicon of ~100 bp representing all known respective gene family members (see Supplementary Table S1 at JXB online). Results were obtained from analysis of three independent samples per treatment, each containing three technical replicates. Data were analysed using the 2\textsuperscript{-ΔΔCT} method (Livak and Schmittgen, 2001) and are presented as the fold change in gene expression for that particular gene family, each normalized to elongation factor 1α (EF1α) and relative to the corresponding non-wounded VC at time-zero.

Statistical analysis
All statistical tests were performed using R 3.1.2 (http://www.r-project.org/) and R-Studio (v.0.98.976, http://www.rstudio.com/).

Results
Down-regulation of ODC reduces concentrations of amines in leaves and roots
Concentrations of polyamines in both leaves and roots, and tyramine in roots, were significantly lower in non-wounded odc-RNAi transgenic plants compared with corresponding tissues of non-wounded VC plants (Fig. 2A–D). Concentrations of each amine in VC plants increased following wounding treatments, being particularly evident for tyramine, which showed significant increases of ~250–500% in leaf tissues of plants damaged by leaf wounding and apex removal, respectively (Fig. 2A–D). In general, odc-RNAi transgenics showed a reduced capacity to increase concentrations of these amines in leaves and roots in response to damage of aerial tissues, and no cases were observed where concentrations were elevated significantly above levels present in corresponding tissues of non-wounded VC plants (Fig 2A–D).

Analysis of pyridine alkaloids and phenolamides in odc-RNAi plants
As anticipated (Hamill et al., 1986; Parr and Hamill, 1987), pyridine alkaloid analysis of upper leaves and roots of non-wounded N.\textit{tobacum} VC plants revealed mainly nicotine, with lower concentrations of anatabine and nornicotine also being present (Fig. 3A–C). The two uppermost expanded leaves of non-wounded VC plants contained ~1.3 mg nicotine g\textsuperscript{-1} dwt, with similar concentrations of nicotine observed in the roots (Fig. 3A). Nornicotine levels were 100-fold lower than nicotine in these leaves (~12 µg g\textsuperscript{-1} dwt) and 25-fold lower in roots (50 µg g\textsuperscript{-1} dwt; Fig. 3B). Anatabine levels were also low in non-wounded VC plants, being ~50 µg g\textsuperscript{-1} dwt in leaves and at trace levels in roots (Fig. 3C). Consistent with previous observations of T\textsubscript{1} odc-RNAi transgenics (DeBoer et al., 2011a), analysis of alkaloid concentrations in non-wounded T\textsubscript{2} odc-RNAi plants revealed markedly different profiles from correspondingly non-wounded VC plants. Nicotine concentrations were significantly reduced in roots and uppermost expanded leaves of non-wounded odc-RNAi plants (Fig. 3A). Nornicotine concentrations in non-wounded odc-RNAi plants were also significantly reduced, dropping to ~8 µg g\textsuperscript{-1} dwt in leaves and ~35 µg g\textsuperscript{-1} dwt in roots (Fig. 3B). In marked contrast, anatabine concentrations were significantly elevated in leaves of non-wounded odc-RNAi plants, rising to ~600 µg g\textsuperscript{-1} dwt, which was >10-fold higher than in non-wounded VC plants. Unlike non-wounded VC plants, anatabine was also readily detectable in roots of non-wounded odc-RNAi plants (~200 µg g\textsuperscript{-1} dwt; Fig. 3C).

Wounding of VC plants had a stimulatory effect on the concentrations of all alkaloids in leaves and roots, with combined leaf wounding and apex removal causing the greatest increase in both leaves and roots of plants. In the latter treatment group, there was a ~3.5-fold increase in nicotine content of leaves (rising to ~4 mg g\textsuperscript{-1} dwt) and a ~2-fold increase in nicotine content of roots (rising to ~2.4 mg g\textsuperscript{-1} dwt); a ~2-fold increase in nornicotine concentrations (rising to ~20 µg g\textsuperscript{-1} dwt in leaves and ~80 µg g\textsuperscript{-1} dwt in roots); and a ~10-fold increase in anatabine concentrations (rising to ~1 mg g\textsuperscript{-1} in leaves and ~250 µg g\textsuperscript{-1} dwt in roots; Fig. 3A–C). Alkaloid analysis of odc-RNAi plants that had been subjected to either the apex removal or combined leaf-wounding and apex-removal treatment showed some capacity to increase concentrations of nicotine and nornicotine, but levels in both leaves and
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roots were never significantly higher than in comparable tissues of non-wounded VC plants (Fig 3A–B). On the other hand, anatabine concentrations were significantly elevated across all wounding treatments in odc-RNAi plants, representing an increase of 50–70% over that of similarly damaged VC plants. Thus, anatabine concentrations reached a maximum of ~2.5 mg g\(^{-1}\) dwt in leaves and ~750 μg g\(^{-1}\) dwt in roots of odc-RNAi plants that experienced the combined apex removal and leaf wounding treatment, compared with ~1.2 mg g\(^{-1}\) dwt in leaves and ~250 μg g\(^{-1}\) dwt in roots of comparable VC plants (Fig. 3C).

Wounding of VC plants produced significant increases in caffeoylputrescine and dicaffeoylspermidine concentrations compared with non-wounded counterparts (Fig. 3D–E). The stimulatory effects on phenolamides concentrations varied in magnitude in relation to the damage inflicted, with leaf-only wounded < apex removal < combined leaf wounding and apex removal treatment (Fig. 3D–E). Concentrations of caffeoylputrescine and dicaffeoylspermidine were reduced significantly in leaves of non-wounded odc-RNAi plants relative to non-wounded VC plants (Fig. 3D–E). Unlike VC plants, wounding produced no stimulatory effect upon caffeoylputrescine concentrations in the odc-RNAi lines (Fig. 3D). Dicaffeoylspermidine concentrations were elevated 3- to 4-fold in odc-RNAi plants that experienced apex removal, but total levels remained significantly lower compared with similarly damaged VC plants (Fig. 3E).

Amino acid analysis of odc-RNAi versus vector control plants

Silencing of ODC resulted in a significant increase (2- to 3-fold) in baseline levels of ornithine in leaf and root tissues of non-wounded odc-RNAi transgensics compared with VC plants (Fig. 4). Ornithine concentrations in leaf tissues were not increased further in response to wounding in either VC or odc-RNAi plants. Roots of VC plants also did not show an increase in ornithine concentrations as a result of any of the wounding treatments. However, in the roots of odc-RNAi transgensics, ornithine concentrations were significantly enhanced (~3-fold) by leaf wounding alone and by apex removal (5- to 6-fold) relative to similarly treated VC plants (Fig. 4). Interestingly, odc-RNAi plants also showed significant increases in baseline concentrations of arginine in leaf (20–25%) and root (30–40%) tissues compared with VC plants. Wounding did not significantly alter arginine concentrations in leaves of VC plants but there was a significant, albeit <2-fold, increase in arginine concentration of roots in response to the combined leaf wounding and apex removal treatment (Fig 4). Wounding increased arginine concentrations further in root, but not leaf, tissues of odc-RNAi plants relative to comparable VC controls (Fig. 4).

Baseline leaf and root glutamate concentrations were increased by 20–25% in odc-RNAi transgensics compared with VC counterparts (Fig. 4). Similarly, silencing of ODC

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**Fig. 2.** Down-regulation of ODC influences levels of amines in leaf and root tissues of non-wounded and wounded N. tabacum plants. Four weeks after transfer to hydroponics, VC and odc-RNAi transgenic plants were left non-wounded (C), mechanically damaged with a pattern wheel applied to the two uppermost expanded leaves (W), wounded by removing (`topping`) the apical region containing leaves that were less than 50% expanded (A), or subjected to a combination of both wounding treatments (W+A). Phyllotactic equivalent leaves from untreated plants that were not damaged were analysed as a wound control comparison. Mean (±SE) putrescine (A), spermidine (B), spermine (C), and tyramine (D) content (nmol) per gram dry weight (wt) in the two uppermost expanded leaves (top panel) or root tissues (bottom panel) harvested from odc-RNAi transgenic vs. VC plants 7 d post-wounding and quantified by UHPLC. Significantly different concentrations of amines among the genotypes and treatment groups at P<0.05 were determined by two-way analysis of variance (ANOVA) followed by Tukey’s honest significant difference (HSD) test and are indicated by different letters (n=4).
resulted in higher baseline concentrations of glutamine relative to non-wounded vector plants. In both odc-RNAi and VC plants, glutamine levels did not increase in response to leaf wounding alone, but interestingly did increase ~2-fold in leaf tissues as a result of apex removal. Glutamine levels were 20–30% higher in these apex removed odc-RNAi plants than in corresponding VC plants (Fig. 4). Aspartate levels were generally 20–35% higher in non-wounded and wounded odc-RNAi plants, relative to correspondingly treated VC plants. In all genotypes, there was a 20–30% increase in aspartate levels of leaf, but not root, tissues of plants wounded by apex removal or combined with leaf damage relative to comparable non-wounded plants (Fig. 4).

Analysis of key polyamine and alkaloid biosynthetic gene activity in roots of odc-RNAi versus vector control plants

As alkaloid synthesis occurs predominantly in roots of N. tabacum (Dewey and Xie, 2013 and references therein), we undertook a detailed comparative analysis of transcript abundance relating to genes of alkaloid and polyamine metabolism in roots of odc-RNAi transgenics vs. VC plants. Consistent with previous studies involving wounded N. tabacum (Cane et al., 2005; Shoji and Hashimoto, 2011 and references therein), analysis of VC plants 1 day post-treatment showed that leaf wounding only, apex removal only, and both leaf wounding and apex removal in combination generally caused progressively larger increases in transcript levels of genes involved in putrescine and spermidine synthesis (ODC, ADC and SPDS) and also alkaloid production and mobilization (A622, PMT, QPT and MATE) (Fig. 5). In contrast, transcript levels of other genes involved in polyamine synthesis either remained relatively constant (SAMDC) or were reduced (SAMS) in roots of wounded vs. non-wounded VC control plants (Fig 5).

Consistent with a previous study involving T1 odc-RNAi plants (DeBoer et al., 2011a), ODC transcript levels were reduced by >95% in T2 odc-RNAi plants compared with VC plants, and did not increase significantly even after the combined wounding treatments (Fig. 5). The ODC wound response was enhanced in odc-RNAi plants where we observed significantly higher (~2- to 3-fold) levels of ADC transcript in roots of wounded odc-RNAi plants compared with VC counterparts, increasing in magnitude with plants damaged by leaf wounding < apex removal < combined leaf wounding and apex removal treatments (Fig. 5). Silenced ODC plants also displayed ~1.5- to 2-fold higher basal and wound-elicited levels of PMT transcript compared with VC plants. Basal transcript levels of MATE were similar in non-wounded VC and odc-RNAi plants. However, following wounding, we observed significantly higher levels of MATE transcripts in roots of odc-RNAi plants than in corresponding VC plants (Fig. 5). Interestingly, and unexpectedly, basal QPT transcript levels in roots of non-wounded odc-RNAi
Effects of silencing ODC upon the spatial distribution of polyamine, alkaloid and phenolamide metabolites in decapitated N. tabacum plants

Experiments were performed to assess the effects of removing plant apices upon spatial distribution of polyamines, pyridine alkaloids and phenolamides throughout the plant (Figs 6–7). One week after apex removal, older leaves located progressively lower on the stem of plants, together with the stem tissue and roots of each plant, were analysed and compared
with levels of these metabolites in phyllotactic equivalent tissues of non-wounded plants. In concurrence with previous experiments noted above, apical bud tissues, leaves, stem and root tissues from non-wounded odc-RNAi transgenic plants contained significantly lower concentrations of polyamines, tyramine, nicotine/nornicotine and phenolamides, compared with equivalent tissues in VC plants (Figs 6–7). The capacity to increase polyamines, tyramine, nicotine/nornicotine and phenolamide concentrations in response to apex removal was also significantly compromised in odc-RNAi plants compared with VC plants with the largest differences between both groups of plants being detected in upper (younger) leaves, as well as in the stem and roots. Tyramine concentrations were also lower in leaves and roots of wounded odc-RNAi lines than in VC plants but, interestingly, the converse was true in stem tissues where levels were ~2-fold higher in wounded odc-RNAi lines compared with similarly wounded VC plants (Fig. 6). Consistent with our previous study involving T1 odc-RNAi transgenic plants (DeBoer et al., 2011a), the present study found that anatabine concentrations were significantly elevated in roots and leaves of T2 odc-RNAi plants, both non-wounded and wounded, compared with corresponding tissues of VC plants (Fig. 7).

Effects of silencing ODC upon growth and flowering in N. tabacum

In a separate study, Nölke et al. (2005) reported that the use of immuno-modulation to inhibit ODC enzymatic activity in transgenic N. tabacum led to a decrease in levels of all three polyamines. Morphological changes were also observed, including stunted plants with elongated leaves that produced smaller and fewer flowers. In our previous experiments involving T1 offspring of transgenic plants, we did not observe an obvious negative effect upon phenotype in hydroponically grown plants containing the odc-RNAi construct (DeBoer et al., 2011a). However, in the present study, using T2 offspring homozygous for the introduced empty pART27 vector and odc-RNAi constructs, careful observation did reveal a number of negative effects upon leaf morphology, growth and reproductive parameters in hydroponically grown odc-RNAi transgenics compared with VC plants (Fig. 8). These alterations became progressively more obvious with age and, although much less pronounced, were reminiscent of the effects observed by Nölke et al. (2005). Thus, at ~11 weeks old, hydroponically grown odc-RNAi plants had produced on average, one fewer leaf than their equivalently aged, similarly cultivated VC counterparts (Fig. 8A). This was
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accompanied by reductions in stem length (Fig. 8B), internode length (Fig. 8C), root biomass (Fig. 8D), and rate of axillary bud emergence and outgrowth following decapitation of plants to 10 cm in height (Fig. 8E). We also noticed that leaves of hydroponically grown odc-RNAi plants displayed tendencies for sporadic bleaching and occasional chlorosis (Fig. 8F) of entire leaves, which were slightly epinastic and brittle compared with leaves of hydroponically grown VC plants (Fig 8F). Although these alterations in leaf morphology bore some resemblance to classic symptoms of mineral deficiencies, separate growth experiments showed they were not prevented by more frequent replenishment of the Hoagland’s nutrient medium in each container (three times per week) or by separately altering the concentrations of nitrate, iron or manganese over a range from one-quarter strength to double strength that of normal Hoagland’s, with twice weekly changes in medium (data not shown). In contrast to hydroponically grown plants, seedlings transplanted to compost, with the aim of generating sufficient seed for further studies, showed odc-RNAi lines to be markedly slower growing and delayed in their time of flowering compared with VC plants (Fig 8G).

Discussion

Although N. tabacum has been used for many years as a model system to study wound-associated alterations in alkaloid biosynthesis and transport from roots to aerial tissues, (for recent reviews see Dewey and Xie, 2013; Wang and Bennetzen 2015), the relationship between ‘primary’ and ‘secondary’ metabolism in vivo remains unclear, particularly in wounded plants. In the present study, we examined the broader metabolic consequences of down-regulating ODC transcript levels in N. tabacum, comparing non-wounded and wounded lines homozygous for an odc-RNAi construct with a control line homozygous for VC T-DNA, all derived from the transgenic plants described in the study of DeBoer et al. (2011a). Higher ornithine concentrations in odc-RNAi transgensics, relative to that of VC plants, was not unexpected given their reduced capacity to produce ODC transcript which, as shown previously, results in diminished ODC activity (DeBoer et al., 2011a). As illustrated in Fig. 1,
discussed recently (Majumdar et al., 2013; Winter et al., 2015; Majumdar et al., 2016), there is a close relationship between ornithine metabolism, catabolism of glutamate and the synthesis of arginine in plants. It is possible therefore that such elevated concentrations of ornithine in odc-RNAi transgenics may have led directly to increased rates of arginine synthesis as well as a build-up of glutamate in tissues that, in turn, led to higher concentrations of glutamine. It is possible too that increased concentrations of aspartate in odc-RNAi transgenics, relative to VC plants, is directly linked to the diminished levels of QPT transcript in these plants, discussed in more detail below. Further work is thus warranted to examine both gene transcript levels and enzyme activities of the proteins responsible for conversion of glutamate–glutamine and glutamate–ornithine–arginine (Page et al., 2012) and also aspartate–quinolinic acid and additional key enzymes associated with NAD synthesis (Katoh et al., 2006; Noctor et al., 2006).

Co-ordinated up-regulation of many genes encoding enzymes involved in alkaloid production, some also with important roles in primary metabolism such as ODC and QPT (Sinclair et al., 2000; DeBoer et al., 2011a; Ryan et al., 2012), has been reported in several Nicotiana species (e.g. Goossens et al., 2003; Sinclair et al., 2004; Cane et al., 2005; Shoji et al., 2008) and involves concerted action of multiple JAZ-, MYC2-, and AP2/ERF-type regulatory proteins (Shoji et al., 2008; Shoji et al., 2010; DeBoer et al., 2011b; Shoji and Hashimoto, 2011; Zhang et al., 2012; Shoji and Hashimoto, 2012; Sears et al., 2014; Shoji and Hashimoto, 2013b; Shoji and Hashimoto, 2015; Yang et al., 2015). In previous experiments in which PMT and ADC genes were strongly down-regulated in N. tabacum hairy roots using an antisense approach, transcript levels of non-targeted alkaloid biosynthesis genes were generally similar in antisense lines compared with vector controls (Chintapakorn and Hamill, 2003; Chintapakorn and Hamill, 2007). Thus, our observations in the current work that PMT and MATE transcripts were significantly higher in roots of wounded odc-RNAi plants compared with similarly treated VC plants, whilst A622 and QPT transcripts were significantly lower, were not anticipated. These observations may indicate the existence of one or more hitherto undefined
additional regulatory control mechanism(s) controlling the synthesis of defensive alkaloids in *N. tabacum*, particularly in response to wounding. One or more mechanism(s) linking ornithine metabolism directly with pyridine alkaloid biosynthetic capacity in *Nicotiana* may also help explain previous observations whereby over-expression of a yeast *ODC* gene led to both elevated putrescine levels and increased concentrations of nicotine in hairy roots of *N. rustica* (Hamill et al., 1990). Conversely, reduced transcript levels of *QPT*, *A622* and possibly other genes encoding key enzymes required for production and incorporation of the pyridine ring of anabasine may have contributed to the reduced ability of *odc*-RNAi transgenic plants of *N. glauca* to produce this alkaloid, compared with vector controls, in response to the removal of plant apices (DeBoer et al., 2013). The biochemical nature of such regulatory mechanism(s) that may link primary and alkaloid specialized metabolism in *Nicotiana* remains speculative at the current time but it may be pertinent to note that
in addition to being an important intermediate for various catabolic reactions, ornithine has been proposed to be an important signalling molecule that plays a key role in controlling synthesis of associated amino acids and polyamines (Majumdar et al., 2013; Majumdar et al., 2016). Also of relevance here may be the recent discovery of an miRNA decoy that accumulates in roots of *N. tabacum* in response to apex removal, leading to sequestration of an miRNA that targets *QPT*, thereby enabling increased levels of *QPT* transcript levels in roots of wounded plants (Li et al., 2015). The use of RNAi and other approaches to alter gene expression will be valuable in exploring further the links between ornithine and alkaloid metabolism in a range of *Nicotiana* species and possibly also other plants that produce specialized metabolites derived from nicotinic acid (reviewed in Ashihara et al., 2015).

Deployment of chemical defences is often allocated with a higher preference for reproductively significant tissues or younger tissues that are potentially more vulnerable to attack by herbivores (Baldwin et al., 1990). Accordingly, in the current study the largest differences between alkaloid levels of wounded VC- and *odc*-RNAi transgenic plants were observed in leaves positioned in the upper half of plants. Markedly reduced concentrations of phenolamides were also found in the upper leaves of *odc*-RNAi plants compared with phylo-tactic equivalent tissues of VC plants. These putrescine deriva-tives have recently been shown to be important defence agents against insect herbivores (Gaquerel et al., 2010; Kaur et al., 2010; Onkokesung et al., 2012). Tyramine is also stimulated by wounding in *Nicotiana* (Guillett and De Luca, 2005; Kim et al., 2011). Though not directly associated with putrescine or poly-amine metabolism, it is noteworthy that *odc*-RNAi transgenics were also significantly less capable of increasing tyramine levels of leaves and roots following apex removal. At the present time, we can only speculate as to the capacity of *odc*-RNAi transgenic *N. tabacum* plants to resist herbivory in an external environment. However, it is likely that they would be much more susceptible to insect attack than normal, as was reported previ-ously for transgenic plants of *N. attenuata* in which overall levels of pyrindle alkaloids were reduced as a result of down-regulation of the *PMT* gene (Steppuhn et al., 2004).

With regards to effects of *ODC* gene manipulation upon poly-amine metabolism, previous experiments have demonstrated a several-fold increase in putrescine content, but little change in overall levels of spermidine and spermine, in transgenic *Nicotiana* tissues engineered to over-express *ODC* from a variety of organisms including yeast (Hamill et al., 1990), mouse (DeScenzo and Minocha, 1993) and the closely related solanaceous species *Datura stramonium* (Mayer and Michael, 2003). Experiments with cultured poplar cells also engineered to over-express mouse *ODC* reported similar results, with analysis of plant *ODC, ADC, SPDS,* and *SAMDC* transcript levels indicating that only the latter was significantly altered (reduced) in cells over-expressing the mouse *ODC* gene compared with vector controls (Page et al., 2007). In the current study employing a contrasting approach in which native *ODC* was down-regulated in tobacco using an RNAi approach, diminished transcript levels of the polyamine synthesis genes *SAMDC, SAMS,* and *SPDS* in roots of *odc*-RNAi plants was observed compared with vector-only controls. Such reductions in transcript abundance in *odc*-RNAi transgenics may be directly linked with reduced putrescine supply and likely underpin the lower spermidine and spermine levels that we observed in these plants compared with their VC counterparts. On the other hand, an increase in the arginine concentration of these plants, together with elevated *ADC* transcript, is in line with previous observations suggesting compensatory increases in ADC activity in plants with lowered ODC activity (Nölke et al., 2005; DeBoer et al., 2011a). This may have facilitated maintenance of adequate baseline concentrations of putrescine and higher polyamines for essential primary and also specialized metabolism requirements and is consistent with suggestions that putrescine supply in plants is influenced biochemically by the metabolic flux of associated metabolites (Page et al., 2012; Majumdar et al., 2016).

In addition to changes in the metabolic profile of *ODC*-silenced plants, and unlike our earlier observations with plants of the T1 generation (DeBoer et al., 2011a), we observed some negative effects upon plant growth and morphology in hydroponically grown T1 lines that were homozygous for the introduced *odc*-RNAi construct, compared with VC controls. Anomalies such as sporadic periodic production of chlorotic, bleached and brittle leaves may be linked to changes in photosynthetic machinery or nutritional deficiencies associated with reduced putrescine supply (Sfichi et al., 2004; Ioannidis et al., 2012). Other differences in these hydroponically grown *odc*-RNAi transgenics compared with vector-only controls, such as shorter internodes, reduced root biomass, slower rates of release of dormant axillary buds following decapitation, and also markedly reduced vigour and delayed onset of flowering in *odc*-RNAi plants when grown in soil, are broadly in line with morphological alterations that have been reported previously in polyamine mutants of tobacco (Malmberg and McIndoo, 1983); in *Nicotiana* plants treated with the ODC biochemical inhibitor difluoromethylornithine (DFMO; Burtin et al., 1991); and in transgenics with immuno-modulated ODC (Nölke et al., 2005). Such abnormalities may be indicative of spermine depletion rather than attenuation of putrescine or spermidine levels per se (Hanzawa et al., 2000; Imai et al., 2004; Nölke et al., 2005). It will be of interest to determine whether introduction of a more distantly related ODC gene less likely to be down-regulated by the *Nicotiana* *odc*-RNAi construct used here, for example the *ODC* gene from yeast that was previously expressed in *N. rustica* (Hamill et al., 1990), will lead to restoration of normal polyamine metabolism and patterns of growth in *odc*-RNAi transgenic lines of *N. tabacum*.

**Supplementary data**

Supplementary data are available at *JXB* online.

**Table S1.** Sequences of gene-specific primers used for qRT-PCR

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