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The regulation of defence chemistry in *Nicotiana*

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Abstract

Plants have to balance their allocation of resources to growth, reproduction, and defence to survive in complex environments. To defend against a diversity of herbivores, plants produce a variety of defensive metabolites and as such *Nicotiana* species have evolved a cocktail of jasmonate (JA)-regulated inducible defence molecules including pyridine alkaloids and phenylpropanoid-polyamine conjugates. In several species of *Nicotiana*, alkaloid and phenolamide levels increase following wound- and/or herbivore-associated stresses. Studies of plant-insect interactions can improve our understanding of the regulation of plant secondary metabolites and defensive protein production.

Possession of a molecular clock enables plants to control and synchronise internal rhythms to day/night cycles, thereby providing a mechanism to respond to daily stresses and modulate metabolism. The accumulation of plant defence signalling compounds, including JAs, salicylic acid (SA) and ethylene (ET), can alter under circadian control and activate biotic stress response pathways and cyclically accumulate under circadian control. The molecular systems that regulate plant metabolome dynamics and rechannel metabolic fluxes towards the production of a specific spectrum of defensive metabolites following herbivore-associated stresses remain poorly understood. Many genes associated with hormone-dependent defence response pathways are clock-regulated and a number of defence metabolites follow diurnal regulation. These include phenylpropanoids, diterpene glycosides and nicotine. Entrainment of plants by light controls JA, SA and ET rhythms as well as diurnal accumulation of defence metabolites. Clock control of hormone signalling is thought to underlie this wide spread phenomena enhancing plant resistance to herbivores amongst diverse crop species.

The research presented in this thesis used molecular and biochemical tools to examine the molecular controls that underlie circadian clock-hormone controls as well as integral genes involved in putrescine-associated metabolism, which drive nitrogen defence pathways in *Nicotiana* species.

In the present work, RNAi-mediated silencing of *TIMING OF CAB EXPRESSION 1* (*TOC1*) demonstrates that nicotine synthesis and accumulation in *N. attenuata* is strongly correlated with the repression and accumulation of genes involved in ET and nicotine biosynthesis, respectively. It is also demonstrated that the control over herbivore-induced

ET production by the *TOC1* evening component enables plants to control induction regimes and resource input into nicotine, a costly area of nitrogen metabolism. Further exploration was undertaken of down-stream effects of complex early herbivore-induced signalling events, connecting clock-signalling networks with JA-mediated responses to herbivory using rapid patterns of accumulation of defensive phenolamides as case studies. It was determined that the transcript levels of early herbivore-induced stress signalling components, nitrogen assimilation, JA-activated regulatory genes and defence biosynthetic genes in clock-JA response networks are mediated by *TOC1*. *TOC1*-specific alterations in allocation of nitrogen in *N. attenuata* defensive chemistry has a major effect on the insect fitness of a specialist herbivore, *Manduca sexta*, and an intermediate effect on a generalist, *Spodoptera littoralis*.

It was found that *TOC1*, acting with additional defence signals and molecular components, exerts independent regulatory control over branched areas of secondary metabolism. *TOC1* was shown to function as a positive regulator in phenylpropanoid, polyamine and diterpene metabolism and may additionally play a role in nitrogen uptake, prioritisation or investment of nitrogenous defence resources. These findings provide insight into how plants use the clock to coordinate various defence signals, enabling optimal accumulation of clock-associated nitrogen defences, which may underlie differential resistance against herbivores.

Research described in this thesis also examined *ORNITHINE DECARBOXYLASE* (*ODC*) and its role in putrescine-associated metabolism in *N. tabacum*. Down-regulation of *ODC* produced a marked effect upon the alkaloid profile, specifically reducing nicotine and concomitantly increasing anatabine levels. Additionally the role of *ODC* in the synthesis of anabasine in *N. glauca* was examined. Reduced expression of *ODC* in both hairy root cultures and transgenic plants of *N. glauca*, resulted in a diminished ability to elevate anabasine concentrations in response to wound-associated stress.

It was concluded that *ODC* plays an important role in determining the normal amino-acid, polyamine and polyamine-associated defence profile in *N. tabacum* and is essential in allowing plants to increase levels in response to wound-associated stress. Additionally, *ODC* plays an important role in enabling plants to elevate levels of anabasine in response to wound-associated stress.

General Declaration

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Declaration for thesis based or partially based on conjointly published work

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

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

This thesis includes 2 original papers published in peer reviewed journals and 3 unpublished manscripts intended for submission. The core theme of this thesis encompasses the regulation of defence chemistry in tobacco species. The ideas, development and writing up of all the manuscripts in this thesis were the principal responsibility of myself, the candidate, working within the Biological Sciences unit under the supervision of Cecilia Blomstedt, John Hamill, Alan Neale and Roslyn Gleadow.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of chapter's 2, 3, 4, 5 and 6 my contribution to the work involved the following:

Thesis Chapter	Publication title	Publication status	Nature and extent of candidate's contribution
2	Timing of CAB expression 1 (TOC1) mediates *Nicotiana attenuata* nitrogen-investment into nicotine biosynthesis during insect herbivory through modulations of hormonal signalling interactions	In preparation*	80%
3	Defending the clock: Silencing of timing of CAB expression 1 (TOC1) impedes herbivory-induced phenolamide accumulation dynamics and insect resistance in *Nicotiana attenuata*	In preparation*	80%

4	RNAi-mediated down-regulation of ornithine decarboxylase (ODC) leads to reduced nicotine and increased anatabine levels in transgenic *Nicotiana tabacum* L.	Published*	35%
5	RNAi-mediated down-regulation of ornithine decarboxylase (ODC) impedes wound-stress stimulation of anabasine synthesis in *Nicotiana glauca*	Published*	30%
6	Effects of down-regulating ornithine decarboxylase (ODC) upon putrescine associated metabolism and growth in *Nicotiana tabacum* L.	Accepted*	80%

* Research article

I have not re-numbered sections of published papers in order to generate a consistent presentation within the thesis.

Student signature: [redacted] **Date: 26/10/2015**

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student and co-authors' contributions to this work.

Main Supervisor signature: [redacted] **Date: 26/10/2015**

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Abbreviations

1°	primary (metabolism)
2°	secondary (metabolism); also known as specialised
ABA	abscisic acid
ABAR	abscisic acid receptor
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	1-aminocyclopropane-1-carboxylic acid oxidase
ACS	1-aminocyclopropane-1-carboxylic acid synthase
ADC	arginine decarboxylase
AOC	allene oxidase cyclase
AOS	allene oxide synthase
Arg	arginine
AT1	*N*-acyltransferase
AUX	auxin
BBL	berberine bridge-like
bHLH	basic helix-loop-helix
bp	base pair
cDNA	complementary deoxyribonucleic acid
CCA1	circadian clock-associated 1
CHLH	Magnesium - chelatase H subunit
COI1	coronatine-insensitive 1
CoP	*N*–coumaroylputrescine
CP	*N*–caffeoylputrescine
CTR1	constitutive triple response 1
CV86	*N*-acyltransferase
CYP82E	nicotine *N*-demethylase
DAO	Diamine oxidase
DCoS	*N*–dicoumaroylspermidine
DCS	*N*–dicaffeoylspermidine

DH29	*N*-acyltransferase
DNA	deoxyribonucleic acid
EIL	ethylene insensitive 3-like
EIN	ethylene insensitive
ERF	ethylene response factor
ET	ethylene
ETR1	ethylene resistant 1
EV	empty vector (vector-only control)
FAC	fatty acid-amino acid-conjugate
FP	feruloylputrescine
Glu	glutamic acid or glutamate
Gln	glutamine
GOX	glucose oxidase
GUN5	genomes uncoupled 5
h	hour
H_2O_2	hydrogen peroxide
HGL-DTG	17–hydroxygeranyllinalool diterpene glycoside
HPL	hydroperoxide lyase
HPLC	high pressure liquid chromatography
HPOT	hydroperoxy linolenic acid
Ile	isoleucine
ir	inverse repeat (RNAi)
JA	jasmonic acid or jasmonate
JAR	jasmonate resistant
JAs	jasmonates (comprises jasmonate and its derivatives and conjugates)
JAT1	jasmonate-inducible alkaloid transporter 1
JAZ	jasmonate ZIM domain
JMT	jasmonyl-O-methyltransferase
LDC	lysine decarboxylase

LHY	late elongated hypocotyl
LOX	lipoxygenase
MAPK	mitogen activated protein kinase
MATE	multidrug and toxic compound extrusion
MeJA	methyl jasmonate
MeSA	methyl salicylate
MPO	*N*–methylputrescine oxidase
MQ	Milli-Q $_{PLUS}$ ultrapure water, resistivity 18.2 MΩcm
MS	Murashige and Skoog medium
MYB	family of transcription factors containing tandem 50 amino acids repeats (R1, R2 and R3). Generally two and three repeats are found in plants and animals (R2R3-MYB class
MYC	transcription factor characterised by basic helix-loop-helix protein structural motif
n	number of replicates
N	nitrogen
NO_3^-	nitrate
NaAD	nicotinic acid adenine dinucleotide
nAChR	nicotinic acetyl choline receptors
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	the reduced form of NADP
NaMN	nicotinate mononucleotide
NaNG	nicotinic acid-β-*N*-glucoside
NaPRT	nicotinic acid phosphoribosyltransferase
NIC1/2	two non-linked loci controlling nicotine levels
NPR1	non-expressor of pathogenesis-related 1
NUP1	nicotine uptake permease 1
ODC	ornithine decarboxylase
ODT	optimal defence theory
OPDA	12-oxo-phytodienoic acid

OPR3	OPDA reductase 3
Orn	ornithine
OS	oral secretions
PAL	phenylalanine ammonia-lyase
PAO	polyamine oxidase
PAP1	production of anthocyanin pigment 1
PCR	polymerase chain reaction
PIP	pinoresinol–lariciresinol reductase, isoflavone reductase, and phenylcoumaran benzylic ether reductase family protein
PMT	putrescine *N*–methyltransferase
PPC	phenylpropanoid-polyamine conjugate
PR	pathogenesis-related protein
PRR	pseudo-response regulator
PUFA	polyunsaturated fatty acid
Put	putrescine
qPCR	quantitative (real-time) polymerase chain reaction
QA	quinolinic acid
QPT	quinolinate phophoribosyltransferase
QS	quinolinate synthase
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
SA	salicylic acid
SAM	*S*–adenosylmethionine
SAMDC	*S*–adenosylmethionine decarboxylase
SAMS	*S*–adenosylmethionine synthase
SAR	systemic acquired resistance
SCF	Skp1/Cullin/F-box
SE	standard error
SIPK	salicylic acid induced protein kinase
Spd	spermidine

SPDS	spermidine synthase
Spm	spermine
SPMS	spermine synthase
TF	transcription factor
TIC	time for coffee
TOC1	timing of CAB expression 1
TPI	trypsine protease inhibitors
VOC	volatile organic compound
W	(leaf) wounding treatment
WIPK	wound induced protein kinase
W+OS	leaf wounding plus OS treatment
w/v	weight per volume
ZTL	Zeitlupe

Chapter 1: Literature review and thesis introduction

1.1. Introduction

Plants produce a multitude of secondary metabolites as chemical barriers against herbivory. Thus, the study of chemically mediated plant-herbivore interactions has become an important area of research, which encompasses numerous disciplines to accurately describe the range of chemical and ecological processes that influence interactions of plants and herbivore. Although considerable advances have been made, several key questions remain, the most urgent of which is to understand the molecular mechanisms and ecological consequences of induced plant secondary metabolite and defensive protein production in response to herbivore-associated stresses.

In this chapter the literature will be reviewed to provide background information pertinent to the work presented in this thesis. The following literature review is divided into five broad areas. These areas encompass; plant defence responses and early signalling events at the plant-herbivore interface, the production of specialised nitrogen-containing metabolites focussing on biosynthesis of polyamines and their relationship with the production of alkaloids and phenolamides. The regulation of plant defence responses including the role of hormone signal transduction networks and stimulation by wound treatments, which have an important bearing upon the defence chemistry of plants, is also discussed. And finally the role of the circadian clock in regulating these metabolic pathways in tobacco species is examined.

1.2. Plant-herbivore interactions

1.2.1. Evolution of plant-herbivore interactions

In both their native environment and under cultivation in agricultural settings, plants are frequently exposed to various stresses. These include both abiotic (e.g. insufficient or high light, variable temperature, water deficit, salinity stress, nutrient limitations) and biotic (e.g. pathogen and herbivore attack). To deal with a multitude of potentially harmful environmental conditions, plants have developed complex strategies to first detect and then adapt to sub-optimal conditions to increase their chances of survival and reproduction.

Terrestrial plants have been consumed by a range of invertebrate herbivores since their emergence onto land ca. 450 million years ago (Labandeira, 1998; Wellman and Gray, 2000). Plant-herbivore interactions have been shaped through evolution in an antagonistic way and in recent years we have begun to understand the sophisticated defence systems that have evolved to deal with phytophagic insects, which use plants as food, mating and oviposition sites. Whilst plants have continually evolved mechanisms to tolerate herbivory, insects have co-evolved with plants and exhibit a wide range of physical and chemical adaptations to deal with such phytodefences (Ehrlich and Raven, 1964; Berenbaum and Zangerl, 1998). In some cases, certain insects (or more commonly their larvae) can inactivate or detoxify specific chemical defences to enable them to feed on plant tissues (reviewed in Cornell and Hawkins, 2003). For example, the cabbage white butterfly actively seeks out and lays eggs on glucosinolate containing leaves of *Brassicaea*. These eggs hatch into larvae capable of consuming leaf tissues containing levels of glucosinolates that are toxic to larvae of many other species (Huang and Renwick, 1994; Renwick and Lopez, 1999; Miles *et al*., 2005). In other cases, herbivores can utilise plant chemical defence traits for protection against their own predators, or as mating signals, in a phenomenon known as sequestration (Conner *et al*., 2000; Kuhn *et al*., 2007; Kumar *et al*., 2014). The ability to become more tolerant to plant defence metabolites by sequestration or detoxifying mechanisms can benefit insect fitness (Labeyrie and Dobler, 2004; Dobler *et al*., 2012; Kumar *et al*., 2014). This specialisation in defence mechanism provides a new ecological niche but narrowing down the range of possible host plants, sometimes to a single genus (Self *et al*., 1964; Dyer, 1995; Wink and Theile, 2002; Ali and Agrawal, 2012). More than 90% of all herbivorous insect species are estimated to feed on less than three different plant families (Bernays and Graham, 1988) and as such the diets of these insects become specialised, collectively referred to as specialist herbivores (reviewed in Ali and Agrawal, 2012). Alternatively, some insects can feed on a wide-range of plants and are typically branded as generalist herbivores including *Heliothis* and *Spodoptera* species (Futuyma and Gould, 1979; Meijden, 1996; Diezel *et al*., 2009; Jing *et al*., 2012).

Among plant populations, feeding behaviour of insects affects the level of damage inflicted on the plant. For example, chewing herbivores such as *Epitrix* species (flea beetles), *Spodoptera* species (armyworm) and larvae of *Manduca* species (hornworm), use their strong mandibles causing extensive plant tissue loss and cellular damage

(Karban and Baldwin, 1997). Conversely, piercing/sucking insects such as *Tupiocoris notatus* (mirid) and *Empoasca* species (leafhopper), use their mouthparts to penetrate and consume contents of plant cells or vasculature, inflicting minimal wounding to the plant but causing localised cell death (Walling, 2000).

1.2.2. Plant defence responses

Defence strategies utilised by plants to deter consumption by herbivores are very diverse and conventionally are classified according to whether they are direct or indirect in nature. Timing of deployment may also be important, with defences being constitutive, inducible or a combination of both, leading to increased rates of herbivore mortality directly and/or decreased rates of herbivore growth and reproduction. This in turn forms the basis for strong natural selection pressure and has spurred the evolution of new species of both plants and herbivores.

Direct versus indirect plant defence responses

Direct defence, traits that negatively affect host selection and overall herbivore fitness (growth, reproduction and/or fecundity), encompass mechanical barriers (physical structures such as thorns and trichomes) and anti-feedants (e.g. anti-nutritive proteins and anti-digestive proteins as well as a range of toxic or deterrent metabolites) (Schardl and Chen, 2001; Kessler and Baldwin, 2002; Chen, 2008). In general, any compound that leads to appetite suppression, paralysis, or death in herbivores is considered an anti-feedant (Duffey and Stout, 1996; Pichersky and Lewinsohn, 2011; Mithöfer and Boland, 2012). Anti-feedants such as anti-digestive proteins can interfere with insect protease and amylase digestive enzymes in the gut and digestive tract whilst anti-nutritive proteins (e.g. amino acid deaminases and polyphenol oxidases) decrease the nutritional value of plant tissues by depleting essential amino acids in the midgut of herbivores (Jongsma *et al*., 1995; Zavala *et al*., 2004b; Chen *et al*., 2005; Chen, 2008; Hartl *et al*., 2010). Toxic compounds (e.g., glucosinolates, alkaloids, cyanogenic glycosides, terpenoids, and phenolics) increase plant fitness by diminishing herbivore performance. These specialised chemicals, commonly referred to as secondary (2°) metabolites, can be present at low-moderate levels constitutively, with concentrations often increasing locally and in more distant parts of the plant directly in response to herbivory (Karban and Baldwin, 1997; Howe and Jander, 2008).

Indirect chemical defences do not affect herbivore performance *per se*, but rather attract, aid or reward natural enemies leading ultimately to reduced numbers of herbivores feeding on the plant and its neighbours (Kessler and Baldwin, 2002; Arimura *et al*., 2005; Wu and Baldwin, 2010; Kessler and Heil, 2011). Release of volatile organic compounds (VOCs) from plants tends to repel ovipositing herbivores (Kessler and Baldwin, 2001) or act as a guide for natural enemies by revealing the location of feeding herbivores (Paschold *et al*., 2006; Dicke, 2009; Allmann and Baldwin, 2010). In addition to VOCs, plants may also provide rewards such as extrafloral nectar to attract natural enemies of herbivores (Heil *et al*., 2004; Choh *et al*., 2006).

Constitutive versus induced defence responses

Constitutive deployment of defences to mitigate the consequences of a potential attack can be metabolically costly on plant carbon and nitrogen resources and may compromise plant growth and reproduction, ultimately reducing plant fitness (Baldwin and Preston, 1999; Zavala and Botto, 2002; Tian *et al*., 2003; Zavala *et al*., 2004a; 2004b). Therefore, plants often use sophisticated regulatory networks to maintain a balance between growth and herbivore defence. Plant defence responses can be categorised into two broad and not mutually exclusive categories according to the timing of manifestation: constitutive or induced upon attack. Constitutive defences, or phytoanticipins, are expressed constantly regardless of the presence of herbivores and may act to kill or deter attacking herbivores. An example of a phytoanticipin is cyanogenic glycosides which are produced constitutively and stored intracellularly in the plant. Cyanogenic glycosides are broken down in response to tissue damage to release HCN, which acts as a respiratory inhibitor to grazing herbivores (reviewed in Gleadow and Møller, 2014). On the other hand, inducible defences are synthesised or mobilised in response to plant tissue damage, a good example being the pyridine alkaloids of plants of the *Nicotiana* genus (reviewed in Dewey and Xie, 2013).

As the production of defence compounds may be metabolically expensive in the absence of herbivores (Baldwin, 1998), mounting a defence only when necessary, can save resources and provide metabolic flexibility, allowing plants to respond to other coexisting abiotic and biotic constraints. Most plants rely heavily on inducible defences and thus, the majority of defences are consitutively present only at low levels in plant tissues and amplified to high levels following herbivore pressure (Karban and Myers, 1989; Karban *et al*., 1997). Studies have shown that some metabolites are toxic to both herbivores and

the plants themselves, including iso-thio-cyanates and hydrogen cyanide. To avoid negative effects on themselves, such plants generally accumulate inactive forms of these compounds as glucosinolates and cyanogenic glycosides, respectively. Activation of catabolic pathways rapidly occur following herbivore attack, leading to toxic breakdown products. Inducible defence mechanisms provide a number of potential advantages over constitutive defences including metabolic flexibility and activation of stress responses, but one substantial disadvantage is time lapse between attacks and the activation or presence of defences (Karban, 2011). Many studies have suggested that plants can recall herbivory patterns and strengthen these 'protective' reactions through priming strategies to quickly respond to herbivores (Baldwin, 1998; Agrawal, 1999; Kessler and Baldwin, 2002; Cipollini *et al*., 2004; Kessler and Baldwin, 2004; Voelckel and Baldwin, 2004; Zavala *et al*., 2004a; Frost *et al*., 2008; Steppuhn and Baldwin, 2008; Mithöfer and Boland, 2012).

1.2.3. Plant perception of insect attack

When plants are attacked by insect herbivores, they can recognise insect feeding by the perception of multiple sequential wounding events (Mithöfer *et al*., 2005) or herbivore-associated elicitor components present in the oral secretions of the larvae (Alborn *et al*., 1997; Halitschke *et al*., 2003; Schmelz *et al*., 2009), or a combination of both. Fatty acid-amino acid-conjugates (FACs) belong to a widely distributed family of herbivore-associated elicitors present in the oral secretions of a large number of lepidopteran larvae. A wide variety of plant species including maize (*Zea mays*), soybean (*Glycine max*), eggplant (*Solanum melongena*), black nightshade (*S. nigrum*) and wild tobacco (*N. attenuata*) are able to trigger and tailor herbivore-specific defence responses against lepidopteran larval folivory after perception of elicitors (Alborn *et al*., 1997; Tumlinson and Lait, 2005; Schmelz *et al*., 2009; Bonaventure *et al*., 2011). Herbivore-associated elicitors are diverse in chemical structure and are usually specific to a particular plant species-insect association (Schmelz *et al*., 2009; Bonaventure *et al*., 2011). Herbivore-associated elicitor perception induces a plant-specific cocktail of direct and indirect defence responses (Schwachtje and Baldwin, 2008) against insect feeding. When perceived by the plants, herbivore-associated elicitors induce the production of phytohormones, ethylene (ET) and JA derivatives, which in turn activate herbivore-specific defence responses (Schmelz *et al*., 2009).

The first insect elicitor to be isolated was the FAC, volicitin (*N*-17- hydroxylinolenoyl-L-Glu; 17-OH-18:3-Gln), which was found in the oral secretions of beet armyworm (*Spodoptora exigua*) larvae feeding on maize plants (Alborn *et al*., 1997). Volicitin was shown to function through a ligand-receptor interaction, binding to a membrane-associated protein (Truitt *et al*., 2004). In contrast to the volatile terpenes induced by wounding alone, *S. exigua* herbivory induced a volicitin-specific blend of terpenes that attracted parasitic wasps (Turlings *et al*., 1993). In *S. litura*, the main FAC is *N*-linolenoyl-L-glutamine (18:3-Gln; Yoshinaga *et al*., 2010), while tobacco hornworm (*M. sexta*) oral secretions the main elicitors are FACs composed predominantly of linoleic acid (18:2) or α-linolenic acid (18:3) conjugated to glutamic acid (Glu) or glutamine (Gln; Halitschke *et al*., 2001; Diezel *et al*., 2009). Subsequently, many chemically diverse herbivore-associated elicitors were identified in the oral secretions of a number of other herbivorous insect species including enzymes, such as glucose oxidase (GOX; Musser *et al*., 2002), sulfated α-hydroxy fatty acids termed caeliferins (Alborn *et al*., 1997), fragments of cell walls (e.g., oligogalacturonides) and peptides released from digested plant proteins (e.g., the proteolytic fragments of chloroplastic ATP synthase γ-subunit, termed inceptins) (Schmelz *et al*., 2006). Inceptins, found in *S. frugiperda* oral secretions, had the capacity to induce the differential production of JA, SA and volatiles in cowpea (*Vigna unguiculata*) when larvae fed on these plants (Schmelz *et al*., 2006). While elicitors derived from plant pathogens have been well characterised, the role of FACs in insect physiology and mode of action is not fully understood. It was recently suggested that Glu-containing FACs are stored forms of glutamine used for the assimilation of nitrogen by the developing lepidopteran larvae (Yoshinaga *et al*., 2010).

1.2.4. Early signalling events in herbivore-related plant defence

Since the discovery of herbivore-associated elicitors, relatively little progress has been made in identifying and characterising the signal transduction mechanisms underlying plant perception and response to herbivore-associated elicitors. Within minutes of an insect feeding on a leaf, a cascade of signalling events is initiated rapidly, driven by the recognition and perception of wounding and/or herbivore-associated elicitors (Halitschke *et al*., 2003; Mithöfer *et al*., 2005; Schmelz *et al*., 2009). The FACs present in the oral secretions from *M. sexta*, have been shown to cause rapid activation in signalling pathways (Halitschke *et al*., 2003). When applied to wounded *N. attenuata* leaves, the major FAC components in *M. sexta* OS, rapidly increase mitogen-activated protein kinase

(MAPK) signalling (Liu and Zhang, 2004; Wu *et al*., 2007a) that modulate downstream transcriptional targets. In the hours following elicitation, leaves of *N. attenuata* plants significantly induce the differential production of defence signalling molecules ET, JA and SA (Kahl *et al*., 2000; Halitschke and Baldwin, 2003). Altered hormone signalling extensively reconfigures the transcriptome and proteome of the plant (Halitschke *et al*., 2003), causes release of plant volatiles (Gaquerel *et al*., 2009) and in doing so triggers most of the defence responses initiated by *M. sexta* larvae feeding (Halitschke *et al*., 2001). Compared with wounding alone, applying oral secretions to wounds amplifies and extends the kinase response. Transcript levels of two specific MAPKs, SA- and wound-induced protein kinase (SIPK and WIPK, respectively) are induced within 60 min following elicitation and subsequently decline within 120 min (Wu *et al*., 2007; Kallenbach *et al*., 2010). Activation and phosphorylation of SIPK and WIPK has been shown to control JA- and ET-mediated defence responses and facilitate plant resistance to herbivores (Zhang and Klessig, 2001; Kandoth *et al*., 2007; Wu *et al*., 2007a; Wu and Baldwin, 2009). When FACs are removed from *M. sexta* oral secretions, the remaining FAC-free fraction loses its capacity to elicit insect specific responses in *N. attenuata* (Halitschke *et al*., 2003; Giri *et al*., 2006; Gaquerel *et al*., 2009).

1.3. Specialised nitrogen-containing plant defence-related metabolites

1.3.1. Diversity of specialised plant-defence metabolites

The plant kingdom produces an extensive and diverse variety of biologically active specialised compounds generally referred to as secondary (2°) metabolites (Oksman-Caldentey and Inze, 2004; Oksman-Caldentey, 2007). Plant-derived 2° metabolites have been proved to be useful to mankind and are currently utilised in pharmaceuticals, fragrances, pesticides, flavourings, dyes and food additives. Once considered to be non-essential, waste products of primary (1°) metabolism, the term 2° metabolites was used to classify compounds for which no role had yet been found in growth, photosynthesis, reproduction and/or development (reviewed in Croteau *et al*., 2000; Hartmann, 2007). Many of these 2° metabolites are now recognised as essential components of plant defence responses. Plant 2° metabolites can confer adaptive characteristics and increase the fitness and capacity of the plant to grow, adapt and interact with its surrounding environment (Oksman-Caldentey and Inze, 2004; Sinclair *et al*., 2004; Wink, 2003). Plant 2° metabolites are usually formed through a complex chain of extensive, multi-step enzymatic reactions, which are tightly regulated by a highly-integrated signalling network

(Nugroho and Verpoorte, 2002). Based on their chemical structure and biosynthesis plant 2° metabolites can be classified into three major groups: 1) terpenes (e.g., mono-, sesqui-, di- and tri-terpenes, saponins), 2) phenolics (e.g., lignins, flavonoids, quinones), and 3) nitrogen-containing compounds (e.g., cyanogenic glycosides, glucosinolates, alkaloids; (reviewed in Croteau *et al*., 2000).

Until recently, little has been known regarding the molecular factors controlling the accumulation of these compounds *in vivo*. The study of plant biosynthetic pathways and factors that regulate them, using gene-based techniques (e.g. mutant analysis, differential gene expression, over-expression and gene silencing), has proven to be of value in understanding the relationship between 1° and 2° metabolism and how metabolism flux is manipulated in *planta*.

1.3.2. *Nicotiana* species as a model for studying plant-animal interactions

The widespread use by society of pleasurable and addictive pyridine alkaloids, particularly nicotine from common tobacco, *Nicotiana tabacum*, has been one of the factors driving investigations into the pathways that produce these compounds (reviewed in Dewey and Xie, 2013). These alkaloids are present in all *Nicotiana* species including *Nicotiana attenuata* (coyote tobacco), a wild summer annual tobacco species that has been well-studied in recent years. *N. attenuata* is native to south-western North America and colonises the immediate post-fire environment after exposure of surviving seed to smoke-related germination cues (Goodspeed, 1954). Seeds germinate from buried long-lived seed to form monocultures in the nitrogen-enriched soils (Baldwin and Morse, 1994; Preston and Baldwin, 1999). The synchronized post-fire germination behaviour of *N. attenuata* creates low interspecific competition and intense intra-specific competition within populations. These plants tend to allocate a large proportion of their resources to rapid growth and increased lifetime seed production, which resembles the life history of monocultural crops. Due to their geographical distribution in the Great Basin Desert, *N. attenuata* plants also experience high UV irradiance and extreme temperatures and thus have to utilise sophisticated strategies to balance and allocate their resources to growth, reproduction, and defence to adapt and survive in complex environments (Baldwin, 1998; 2001).

As a pioneer plant in a post-fire environment, *N. attenuata*, like many other species, is exposed to diverse insect communities. These include herbivores from different feeding

guilds such as piercing-sucking herbivores, such as mirids (*Tupiocoris notatus*) and chewing herbivores, such as grasshoppers (*Trimerotropis spp*), armyworm larvae (*Spodoptera spp*) and larvae of the specialist herbivores, the tomato hornworm (*M. quinquemaculata*) and the tobacco hornworm (*M. sexta*). The particular type of insect that will undertake the major assault cannot be predetermined. Tobacco species respond strongly and specifically to attack by herbivores from different feeding guilds (Baldwin, 1998; Voelckel and Baldwin, 2004; Diezel *et al*., 2009). To defend against this great diversity of herbivores, *N. attenuata* has evolved a cocktail of jasmonate (JA)-regulated inducible defence molecules including nicotine (Baldwin, 2001; Steppuhn *et al*., 2004), trypsine protease inhibitors (TPIs) (Ussuf *et al*., 2001; Steppuhn and Baldwin, 2007), 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) (Heiling *et al*., 2010) and phenylpropanoid-polyamine conjugates (PPCs) (Kaur *et al*., 2010). When *N. attenuata* was genetically engineered to produce reduced amounts of HGL-DTGs, nicotine, PPCs and TPIs, using RNA interference (RNAi) approaches, the plants become severely compromised in their capacity to survive *M. sexta* attack, and thus *M. sexta* larvae grow larger (Steppuhn *et al*., 2004; Zavala *et al*., 2004b; Jassbi *et al*., 2008; Kaur *et al*., 2010). It can be concluded that these highly specialised metabolites are required to mount a full defence response against herbivores such as *M. sexta*, which is a Solanaceous-feeding specialist and can tolerate high doses of nicotine (Wink and Theile, 2002). Feeding by larvae of *M. sexta*, frequently accounts for most of the leaf area removed in a given season resulting in environmental loss (Kessler and Baldwin, 2004) and thus *N. attenuata* has been meticulously studied with regard to how it responds to this Solanaceous-feeding specialist.

Several features make *N. attenuata* and *N. tabacum* useful model plants for the ecological and molecular studies of plant biochemical processes against tissue damage and/or herbivore attack These include a short generation time, well-known natural history including native plant-herbivore interactions, complexity of induced defences, self-compatibility and the availability of genetic transformation tools. Hence, these Solanaceous models are an asset for studying the regulation of metabolic systems in nature and results from such studies are likely to be agriculturally beneficial for Solanaceous crops.

1.3.3. Polyamine biosynthesis

Putrescine formation for primary and specialised metabolism

Polyamines are small, positively charged, aliphatic nitrogen containing compounds, ubiquitous in the 1° metabolism of prokaryotes, as well as plants and animals, and have been implicated in the control and regulation of a wide array of growth and developmental processes (Martin-Tanguy, 2001). Three polyamines; putrescine (Put), spermidine (Spd) and spermine (Spm), can be found in free and conjugated forms and are generally recognised as being essential for cell viability across all taxa (Evans and Malmberg, 1989). The triamine, Spd, and tetramine, Spm, are formed from the diamine, Put, by the sequential addition of aminopropyl groups to Put and Spd *via* the activity of the enzymes, Spd synthase (SPDS) and Spm synthase (SPMS), respectively (Figure 1; reviewed in Bagni and Tassoni, 2001). Polyamines are active as free molecules but are also conjugated to a wide range of specialised metabolites including antibiotics, alkaloids and phenylpropanoids. The use of specific inhibitors of polyamine biosynthesis, augmented by gene silencing based approaches, has allowed the manipulation of polyamine biosynthetic pathways, leading to an advance in our understanding of the important role of these organic polycations in plant developmental and other physiological processes (Bagni and Tassoni, 2001; Bitrián *et al*., 2012; Fariduddin *et al*., 2013).

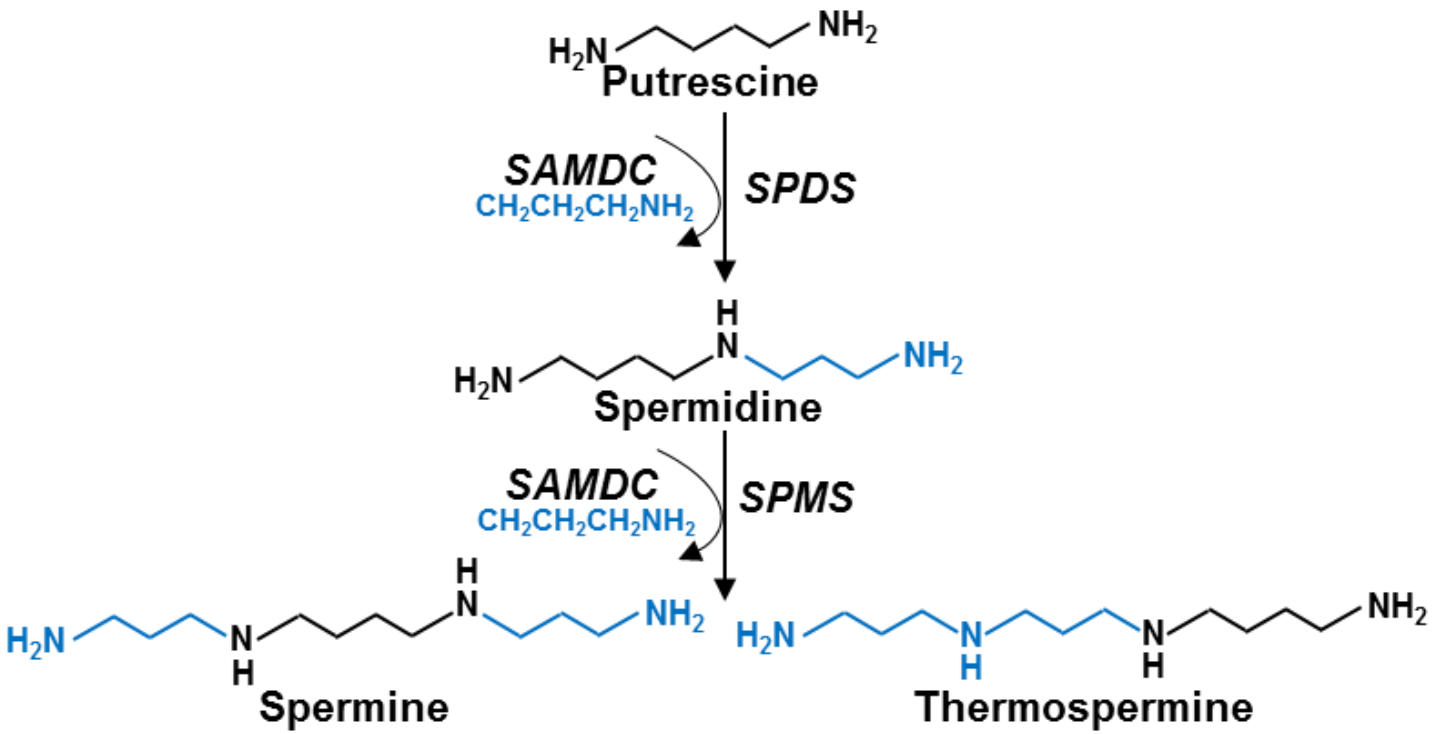

Figure 1. Chemical structure of the four main polyamines, putrescine, spermidine, spermine and thermospermine, found in plants (Bitrián *et al.*, 2012). Aminopropyl moieties (in blue), provided by SAMDC, are added to the four carbon chain skeleton of putrescine (in black) to synthesise tri- and tetra-amines, spermidine, spermine and thermospermine. Solid arrows indicate defined enzymatic steps.

In plants, enhanced synthesis of polyamines is associated with processes such as growth, cell division, DNA synthesis, cell proliferation and differentiation. Polyamines also have a role during the developmental processes of organogenesis, embryogenesis, senescence, floral development and fruit ripening and in the plant response to environmental stresses (Evans and Malmberg, 1989; Martin-Tanguy, 2001; Alcázar *et al*., 2010). It has also been suggested that they may act as secondary messengers in signalling, playing a role in controlling the flux between 1° and 2° metabolism (Evans and Malmberg, 1989; Martin-Tanguy, 2001).

Arginine decarboxylase (ADC), ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (SAMDC) and Spd/Spm *N*-acetyltransferases are well-studied enzymes that are known to regulate cellular levels of polyamines (Cohen *et al*., 1982; Bitrián *et al*., 2012; Fariduddin *et al*., 2013). The polyamine biosynthetic pathway is subject to divergent metabolic demands and is regulated at the transcriptional, post-transcriptional, translational and post-translational levels within the cell. Due to the fundamental biological functions of polyamines, it is unsurprising that intracellular levels of Put, Spd and Spm is tightly controlled within very narrow limits and that levels vary primarily during the transition of developmental stages or in response to stress conditions (Paschalidis and Roubelakis-Angelakis, 2005; Bitrián *et al*., 2012; Fellenberg *et al*., 2012; Fariduddin *et al*., 2013). Polyamine homeostasis is mainly achieved through their *de novo* biosynthesis, interconversion and catabolism or terminal degradation, which suggests that control of both areas of the polyamine pathway, is responsible for maintaining levels *in vivo* (Peremarti *et al*., 2010). Interconversion between Put, Spd and Spm maintains polyamine pools, where they are first acetylated by Spd/Spm *N*-acetyltransferases and then oxidised by diamine oxidase (DAO)/polyamine oxidase (PAO) back to Put. SAMDC is thought to be a critical point of regulation of polyamine homeostasis in all organisms (Kumar *et al*., 1996; Martin-Tanguy, 1997; Illingworth and Michael, 2011) and might be responsible for antagonism between synthesis of higher polyamines and ET. In addition to signalling, polyamines have other direct and indirect effects on plant development *via* mechanisms that also impact plant adaptation and defence. These include electrostatic binding to macromolecules such as DNA, RNA and protein (Serafini-Fracassini and Del Duca, 2008).

Terminal degradation or catabolism of polyamines involves the oxidation of primary terminal amino groups to produce polyamine derivatives that cannot be converted back

into polyamines. Polyamine degradation produces hydrogen peroxide (H_2O_2) as a bi-product, which contributes to polyamine bioactivity. H_2O_2 is a signalling molecule that can enter the stress signal transduction cascade promoting activation of an anti-oxidative defence response and providing another level of regulation of plant growth and development (Cona *et al*., 2006). H_2O_2 generated by oxidation of polyamines has been shown to be important for lignification and cross-linking of cell wall extension proteins in response to stress and wounding (Cona *et al*., 2006) and was suggested by Cane *et al.*, (2005) as a possible explanation for rapid increases in transcript levels of *ODC*, *ADC* and *SAMDC*, without a concomitant increase in polyamine content in leaves of tobacco after wounding. The body of knowledge surrounding polyamine biosynthesis is continually growing in the literature and there is a considerable amount of data to demonstrate that under many types of abiotic stresses including drought, salinity and osmotic stress, an accumulation of the three main polyamines, Put, Spd and Spm can occur. This is thought to aid in plant protection and tolerance under stressful conditions (Alcázar *et al*., 2006). Both abiotic and biotic stress can induce the export of Spd/Spm into the apoplast for DAO/PAO-mediated catabolism resulting in H_2O_2 production. Accumulation of H_2O_2 results either in the tolerance response or plant cell death, depending on the levels of intracellular polyamines (Moschou *et al*., 2008). Regulation of polyamine levels in plants is also controlled by the conjugation with hydroxycinnamic acids to form phenolamides such as coumaroylputrescine (CoP), caffeoylputrescine (CP), feruloylputrescine (FP), dicaffeoylspermidine (DCS) or dicoumaroylspermidine (DCoS; Martin-Tanguy, 1997). The complexity of this regulation indicates the importance of maintaining polyamine homeostasis, while still allowing rapid response of the pathway to abiotic and biotic factors (Reviewed in Bagni and Tassoni, 2001; Fariduddin *et al*., 2013).

Role of arginine and ornithine decarboxylases

In *Nicotiana* species, Put can be synthesised from $_L$-arginine (Arg) or $_L$-ornithine (Orn) *via* the activity of the decarboxylating enzymes ADC and ODC, respectively. Put can be metabolised to higher polyamines, serving as a precursor in the synthesis of Spd and Spm, which are known to play important roles in metabolic, physiological and developmental processes. Put and Spd are also present as conjugates to a number of phenylpropanoids (Figure 2; Nugroho and Verpoorte, 2002). Alternatively, Put can be converted into *N*-methylputrescine to provide the pyrrolidine moiety that is incorporated into some alkaloids, such as nicotine (Leete, 1958). Until recently, it was unclear whether the ADC

or ODC-mediated route, was preferentially utilised in directing polyamine and pyridine alkaloid production to synthesise nicotine (Katoh *et al*., 2005).

Unlike animals and fungi, many higher plants such as tobacco species, and some bacteria, have the capacity to synthesise the diamine Put, *via* two independent pathways. Put can be formed directly from $_{L}$-Orn, by the action of ODC, or indirectly from $_{L}$-Arg through the production of the intermediates agmatine and *N*-carbamoylputrescine, in reactions initiated by ADC (Figure 2; Hashimoto and Yamada, 1994; Hibi *et al*., 1994; Martin-Tanguy, 2001; Nugroho and Verpoorte, 2002). Plants possessing both functional pathways, such as those in the *Nicotiana* genus, may have evolved separate roles in 1° or 2° metabolism for the two decarboxylating enzymes that catalyse these reactions (Tiburcio *et al*., 1993; Alcázar *et al*., 2010; Fuell *et al*., 2010). In *Arabidopsis* however, the annotated genome contains no predicted ODC gene sequence (Hanfrey *et al*., 2001). Regulation of ODC activity appears to have evolved differently in plants compared to mammals. For example, ubiquitin-independent proteasome degradation of ODC by antizyme, an important regulatory mechanism preventing overproduction of putrescine in mammals, has not been identified in plants (Murakami *et al*., 1992; Kahana, 2007).

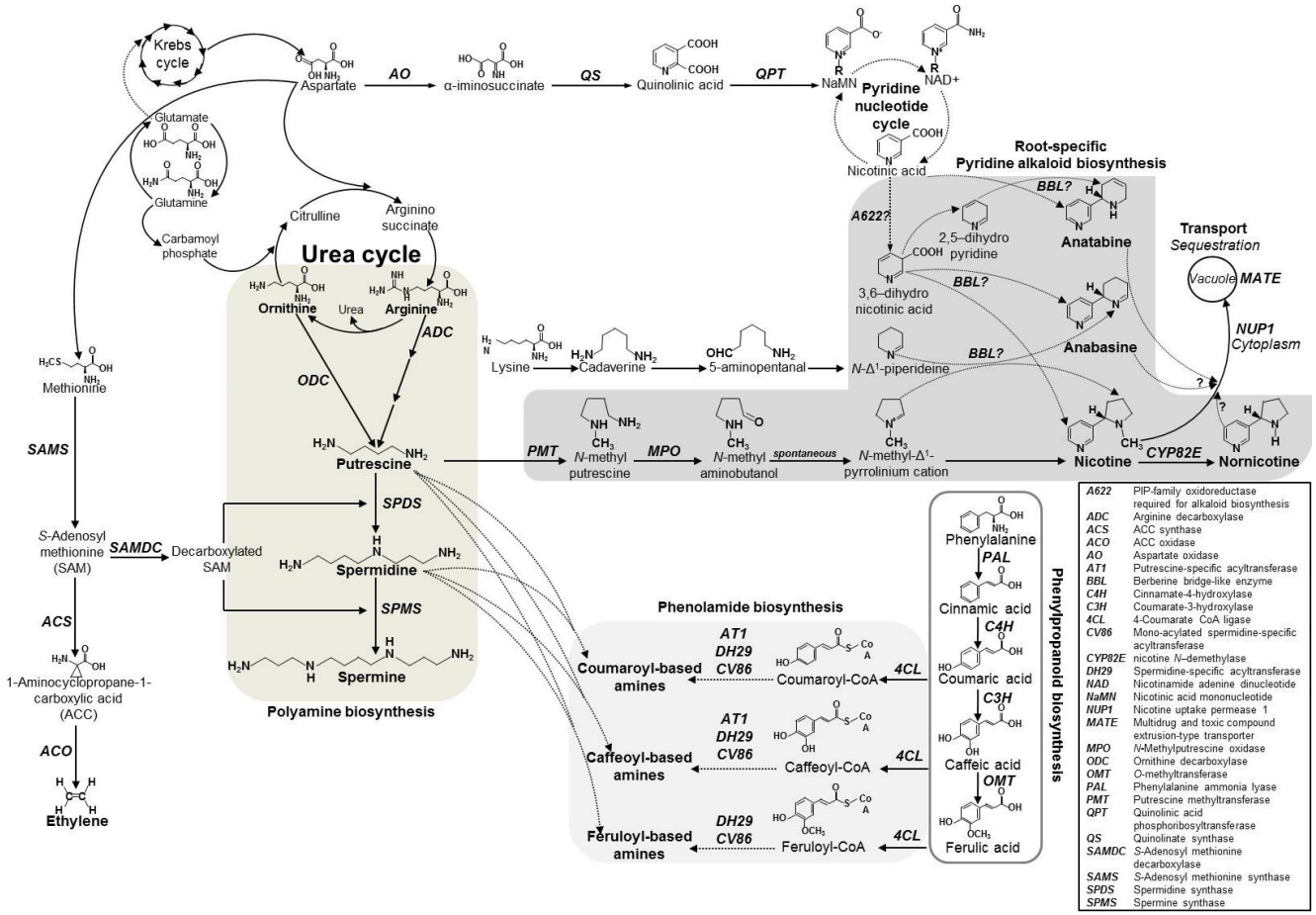

Figure 2. Schematic diagram of biosynthesis of polyamines and connections with other metabolic pathways including pyridine alkaloid, phenolamide and phenylpropanoid pathways in tobacco (Cane *et al.*, 2005; Onkokesung *et al.*, 2012; Shoji and Hashimoto, 2012). Solid lines indicate defined steps, while broken lines indicate undefined steps or steps including multiple reactions. Both pyridine alkaloids and polyamines/phenolamides metabolites share early biosynthetic steps in common, starting from arginine and ornithine to produce putrescine. The biosynthesis of alkaloids in tobacco is exclusively made in the 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*-methylpyrrolinium cation is not yet clear, however it is suggested that A622, a protein bearing significant similarities to a PIP-family isoflavone reductase, is involved in the late step of synthesis of the pyridine moiety. Berberine bridge-like (BBL) enzymes are thought to be involved in the final conjugations steps, while MATE and NUP1 transporters are involved in the uptake of nicotine into vacuoles and the cytoplasm, respectively, in tobacco roots.

Conversion of arginine and ornithine in the urea cycle

The possibility of biochemical conversion between $_{L}$-Arg and $_{L}$-Orn *via* the urea cycle, has contributed to a degree of confusion surrounding the primary source of Put in plant metabolism. Arg metabolism is complex and highly regulated and is involved in a number of metabolic processes that play diverse roles across taxa. Arg acts as a precursor to a wide range of compounds, including nitric oxide, creatine, urea, Orn, agmatine, proline, Glu and polyamines (Morris, 2009). In higher plants, Arg is utilised in a functionally diverse range of processes. In addition to its role in protein synthesis, it is thought to function as nitrogen storage and transport compound in *planta* due to its high nitrogen: carbon ratio (4:6). Classified as a semi-essential amino acid, Arg is required at adequate levels to meet the metabolic demand under conditions of maximal growth, severe stress and injury (Flynn *et al*., 2002; Tong and Barbul, 2004; Morris, 2009). The hydrolysis of Arg by arginase produces urea and Orn, the latter serving as a precursor for synthesis of polyamines, proline or Glu. Arg may thus play a role in plants as another source of Orn, which in turn can serve as a precursor to support the biosynthesis of polyamines and nicotine/nornicotine in *Nicotiana* species (Flynn *et al*., 2002; Tong and Barbul, 2004; Lea *et al*., 2007; Kalamaki *et al*., 2009a; 2009b; Morris, 2009).

Cellular localisation of ADC and ODC enzymes

Lack of knowledge of the cellular and subcellular localisation of polyamine biosynthetic enzymes has been one of the obstacles in our understanding of the biological role of the ODC/ADC polyamine system. Whilst both pathways appear to be functional and active in different tissues in the majority of higher plants, their relative contribution to the biosynthesis of Put, during development and in response to abiotic and biotic stress remains relatively unclear (Tiburcio *et al*., 1993). The enzymes of ODC and ADC have been suggested to play distinct physiological roles in the supply of Put and are regulated separately in various phases of growth, development and stress response of plants and are physically separated within the plant cell (Imanishi *et al*., 1998).

Studies have shown that polyamines are present in cell wall fractions, vacuoles, mitochondria and chloroplasts (Torrigiani *et al*., 1986; Slocum, 1991; Tiburcio *et al*., 1997). Subcellular localisation of the polyamine biosynthetic enzymes, SAMDC and SPDS located them predominantly in the cytoplasm, while PAO was reported in the cell wall of plant tissues (Kaur-Sawhney *et al*., 1981). The first ODC gene from plants, isolated from *Datura stramonium* and homologous to other eukaryotic ODCs and ADCs,

was shown to have higher transcript levels in root tissue compared to stems or leaves (Michael *et al*., 1996). ODC activity was reported to be localised in the cytoplasm of plants (Borrell *et al*., 1996; Tiburcio *et al*., 1997). Conversely, subcellular compartment localisation of ADC was demonstrated in the chloroplast thylakoid membrane of photosynthetic tissues and nuclei of non-photosynthetic tissues (Borrell *et al*., 1995; Borrell *et al*., 1996; Tiburcio *et al*., 1997; Bortolotti *et al*., 2004). Differences in the compartmentalisation of ADC and ODC may be associated with distinct functions of these proteins in different cell types. The ODC-mediated route to Put is generally suggested to be important for providing sufficient levels of polyamines for normal cellular division, differentiation and development in actively growing tissues (e.g. roots), while the ADC-mediated route is reported to be necessary for cell expansion and also response to stress factors such as drought or nutritional stress (Cohen *et al*., 1982; Evans and Malmberg, 1989; Malmberg *et al*., 1998; Martin-Tanguy, 2001; Tiburcio and Galston, 1986). Bouchereau *et al*. (1999) and Soyka and Heyer (1999) demonstrated that increases in ADC activity were induced by treatment of plants with JA and abscisic acid (ABA), as well as exposure to osmotic stress and wounding. The activity of the ADC enzyme in the chloroplast of photosynthetic tissues has been suggested to be related to regulation of polyamines induced by osmotic stress. These polyamines may be involved in maintaining photosynthetic activity in senescing leaves (Borrell *et al*., 1995; Bortolotti *et al*., 2004). While *ODC* transcript levels are present in roots, the site of alkaloid synthesis, it is unclear why transcript levels should be elevated in non-alkaloid producing tissues such as sepals, stamens and carpels, and in leaf tissues following wounding (Wang *et al*., 2000; Cane *et al*., 2005). Few studies have examined the over-lap and potential cross-talk in regulation of *ODC* and *ADC* during polyamine and alkaloid biosynthesis.

Contribution of ADC and ODC to putrescine formation

Many plants analysed to date possess ODC as well as ADC activity and there has been debate in the plant literature regarding the relative contributions and importance of ODC and ADC in facilitating the supply of Put destined for 1° (polyamine) and 2° (alkaloid) metabolism (Tiburcio and Galston, 1986; Robins *et al*., 1990; Walton *et al*., 1990; Tiburcio *et al*., 1993; Walden *et al*., 1997).

It has been suggested that the Arg/agmatine/N-carbamoylputrescine/Put route involving ADC is more important for nicotine biosynthesis in tobacco than the Orn/Put route *via* ODC. This conclusion was based on biochemical experiments involving callus cell

cultures of tobacco using specific enzyme inhibitors. Nicotine production was effectively inhibited by the specific ADC suicide inhibitor, DFMA, rather than by treatment with the ODC inhibitor, DFMO (Tiburcio and Galston, 1986; Robins *et al*., 1990). These authors also showed that incorporation of ^{14}C into nicotine was more efficient from labelled Arg than that from labelled Orn. These observations concurred with previous studies by Tiburcio *et al*. (1985) who showed increased levels of ADC activity, but not ODC activity, were correlated with increased accumulation of nicotine in *N. tabacum* callus. Observations by Feth *et al*. (1986) supported this hypothesis where no change in ODC enzymatic activity in *N. tabacum* callus that was grown under conditions promoting nicotine synthesis were reported, despite subsequent increases in the activities of other enzymes involved in nicotine biosynthesis. However, these studies assume absolute inhibition of ODC and ADC activity with DMFO and DFMA inhibitor binding, respectively. In fact there can be a wide range of effects with inhibitor studies on enzyme specificity and inhibition *in vivo*. This was demonstrated in studies by Slocum and Galston (1985a; b) and Slocum *et al*. (1988), which showed that DFMA treatment in flowers of *N. tabacum* reduced the activity of ODC by arginase-mediated conversion of DFMA to DFMO.

Using hairy roots and regenerated transgenic plants as an experimental system rather than callus tissues, Chintapakorn and Hamill (2007) reported that antisense down-regulation of *ADC* transcript in transgenic *N. tabacum* had little effect upon capacity of *N. tabacum* to synthesise nicotine. No measurements were made of the effects of these manipulations on polyamine metabolism in this study. In a study undertaken by Nölke *et al*. (2005), which used immuno-modulation to inhibit ODC enzymatic activity in *N. tabacum*, a decrease in polyamine levels was correlated with a reduction in ODC enzyme activity and associated with reduced rates of growth resulting in stunted plants. Interestingly, ADC enzyme activity was seen to increase in lines exhibiting reduced ODC activity, suggesting a possible compensatory regulatory mechanism employed by the plant in order to maintain Put concentrations in *Nicotiana* plants (Nölke *et al*., 2005). Effects upon pyridine alkaloid metabolism were not examined in ODC reduced plants produced in this study. Recently, and of direct relevance to the current investigation, DeBoer *et al*., (2011) showed that RNAi-mediated down regulation of ODC effectively reduced ODC transcript and enzyme levels in transgenic hairy roots and plants of *N. tabacum* leading to reduced nicotine and elevated anatabine levels compared to vector-only controls. The elevated anatabine levels

were suggested by Chintapakorn and Hamill (2003) to be indicative of reduced putrescine supply *in vivo* for alkaloid production in tobacco. No measurements of the effects of reducing ODC levels on polyamine metabolism were undertaken by DeBoer *et al*., (2011) and no obvious effects on growth of hairy roots and transgenic plants were observed either. In agreement with the report of Nölke *et al*. (2005), elevated levels of ADC transcript and activity were reported in hairy roots and transgenic plants in which ODC transcript and enzyme levels were markedly reduced (DeBoer *et al*., 2011). The biochemical conversion of Orn into Arg and vice versa *via* the urea cycle makes the interpretation of some studies, like this, unclear.

1.3.4. Alkaloids

Alkaloids represent one of the largest and best studied classes of nitrogen containing specialised 2° metabolites and are detected in over 20% of all plant species (Kutchan, 1995). Many alkaloids have complex and unique structures and as a group they represent a diverse collection of ecologically active, low molecular weight organic compounds. To date, more than 20,000 different alkaloids have been described and classified into several subclasses according to their chemical structure and the amino acids from which they are derived (Wink, 2003; Oksman-Caldentey, 2007; Yang and Stöckigt, 2010). As well as being widely distributed throughout the plant kingdom, alkaloids are also found in insects, birds, marine invertebrates and fungi (Memelink *et al*., 2001; Wink, 2003; Oksman-Caldentey, 2007). Due to their potent biological activity, many groups of alkaloids including benzylisoquinine, pyridine, pyrrolizidine and terpenoid indole have long been exploited in a range of medical and social applications as useful therapeutics (e.g. vincristine and vinblastine), analgesics (e.g. morphine and codeine), sedatives (e.g. scopolamine), antimicrobial agents (e.g. sanguinarine and berberine), stimulants (e.g. caffeine and nicotine), narcotics (e.g. opium and cocaine) and also as powerful poisons, insecticides and fumigants (e.g. anabasine, atropine and nicotine) (Wink, 1998b; Kutchan, 1995; Oksman-Caldentey, 2007; O'Connor, 2010; Yang and Stöckigt, 2010).

Alkaloid biosynthesis in plants involves the diversion of amino acid precursors from 1° (vital) metabolism into 2° (specialised) metabolism – most being derived from a limited group of amino acid precursors (arg, histidine, lysine, orn, tryptophan and tyrosine) by the action of decarboxylases (Kutchan, 1995). Biosynthesis and accumulation patterns of alkaloids may be tissue specific and levels vary greatly within plants throughout growth, development and in response to environmental signals (Hashimoto and Yamada, 2003).

Production is often enhanced both locally and systemically in response to plant tissue damage, both mechanical and caused by herbivory; and by exposure of plants to abiotic stress conditions and nutrient limitations (reviewed in Facchini, 2001).

Alkaloids may be acutely toxic, due to their ability to interfere with neuro-receptors and ion channels in many microbes, insects and herbivores. It is now generally accepted that alkaloids have important ecological roles with many acting as plant defensive compounds to provide protection from excessive herbivory and predation (Wink, 1988; Kutchan, 1995; Baldwin and Preston, 1999; Ohnmeiss and Baldwin, 2000; Wink, 2000; Jackson *et al*., 2002; Shoji *et al*., 2002; Hashimoto and Yamada, 2003; Sinclair *et al*., 2004; Steppuhn *et al*., 2004; Cane *et al*., 2005; Katoh *et al*., 2005). The great diversity in structure of plant alkaloids may result from evolutionary pressure to counteract the adaptation of animals to the toxicity of these plant defence compounds (Wink, 1998a; 1998b). Alkaloids may also display anti-microbial and anti-fungal activity, whilst some act as allelochemicals, displaying properties that inhibit the growth of competing plants in close proximity (Krug and Proksch, 1993; Wink, 1998b).

While alkaloids as a group are prevalent across the plant kingdom, the production of specific alkaloids is often restricted to certain plant families (Wink, 2003). For example, several different classes of alkaloids are synthesised within the Solanaceae family, the most prominent being the tropane, pyridine and steroid alkaloids (Wink, 1998a; 1998b). *Nicotiana* is a large genus within the Solanaceae family containing more than 75 naturally occurring species, including diploid (n=24) species *N. attenuata* (coyote tobacco) and *N. glauca* (tree tobacco), as well as a number of natural and manmade hybrid species such as *N. tabacum* (commercial tobacco; Knapp *et al*., 2004). *N. attenuata* is one of many species commonly known as wild tobacco. It is an annual herb that is native to western North America and propagates in many types of habitat. *N. glauca*, a noctifloroid and sister species to the *Suaveolentes* section, is a branched tree-like shrub naturalised in many warm-temperate regions around the world and grows vigorously in open and disturbed habitats such as roadside verges and lakeshores. Interspecies hybridisation, genome doubling and aneuploidy reductions have played an integral role in the speciation of *Nicotiana* and thus, accounted for the variation in chromosome number throughout the genus (Goodspeed, 1954). Segregation analysis of artificial hybrids supported *N. sylvestris*–type species as the maternal S genome donor and *N. tormentosiformis* as the paternal T genome donor of *N. tabacum*, a cultivated allotetraploid (2n=48) species

(Goodspeed, 1954; Knapp *et al*., 2004. *N. attenuata*, *N. glauca* and *N. tabacum* were the subject of experimental investigation in the current study.

The social and economic benefits arising from the exploitation of 2° metabolites have prompted in depth studies into the regulation of pathways that synthesise some alkaloids. The emerging ability to investigate the molecular controls governing alkaloid biosynthetic pathways is increasing our understanding of how and why the plant produces particular alkaloids. However, restricted knowledge thus far relating to the fundamental mechanisms involved in the regulation of alkaloid metabolism places limitations on our ability to harness the biotechnological potential of these important 2° metabolites (Facchini, 2006). Hence, there is increasing interest in using genetic approaches to address fundamental questions relating to the genetic and biochemical controls that regulate 1° and 2° metabolism.

1.3.5. Pyridine alkaloids of the *Nicotiana* genus

The accumulation of pyridine alkaloids in leaf and root tissues is a characteristic feature of all species of the genus *Nicotiana*. Pyridine alkaloids, so called because of the characteristic pyridine ring in their structures, are derived from nicotinic acid *via* the pyridine nucleotide cycle (Dawson, 1962). The four most abundant pyridine alkaloids found within the genus *Nicotiana* are nicotine, nornicotine, anabasine and anatabine (Figure 3). In addition to the common pyridine ring, the major alkaloids each contain a second heterocyclic ring. Nicotine and nornicotine both contain a pyrrolidine ring derived from Put (Bush *et al*., 1999). Anabasine contains a piperidine ring derived from lysine, while the second ring of anatabine is formed from the condensation of two nicotinic acid derived molecules (Bush *et al*., 1999).

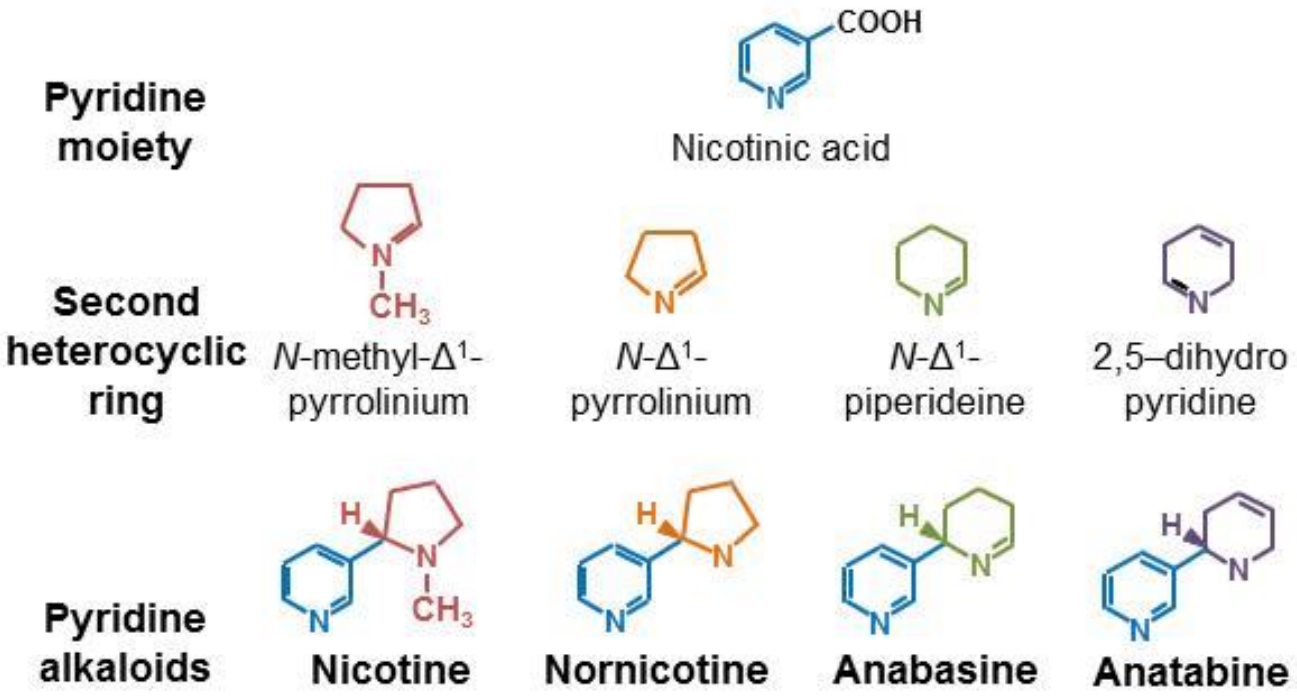

Figure 3. Chemical structure of the four main pyridine alkaloids found in *Nicotiana* species (Cordell, 2013). A nicotinic acid-derived molecule (in blue) provides the pyridine moiety that is combined with a second heterocyclic ring (in red, orange, green and purple) to form nicotine, nornicotine, anabasine and anatabine.

While pyridine alkaloids have been detected in all known *Nicotiana* species, the type and abundance of alkaloids varies substantially within the genus, with the majority of species containing predominately one or two main alkaloids (Saitoh *et al*., 1985; Sisson and Severson, 1990). Separate studies conducted by Saitoh *et al*. (1985) and Sisson and Severson (1990) found that most species, including the well-studied tobacco species *N. tabacum, N. sylvestris* and *N. attenuata*, contain either nicotine, or its demethylated derivative nornicotine, as their main alkaloid, with the remaining alkaloids forming a minor constituent of the alkaloid sector. The leaves of *N. tabacum* contain high levels of nicotine, which usually accounts for more than 90% of the total alkaloid fraction (Sachan and Falcone, 2002; Saitoh *et al*., 1985; Sisson and Severson, 1990). On the other hand, a limited number of species such as *N. glauca, N. noctiflora, N. petunioides* and *N. debneyi*, contain anabasine as their major alkaloid component (Saitoh *et al*., 1985; Sisson and Severson, 1990; Shoji *et al*., 2002). A small number of species, including the widely grown ornamental tobacco *N. alata* have been found to contain alkaloids primarily in their roots, with their leaf tissues remaining virtually alkaloid-free (Saitoh *et al*., 1985; Pakdeechanuan *et al*., 2012).

Synthesis of pyridine alkaloids

Overview of the pyridine alkaloid biosynthetic pathway

Alkaloid synthesis in *Nicotiana* species occurs when certain amino acid based precursors are diverted from 1° to 2° metabolism. Pertinent steps of the complex biosynthetic pathway and the main enzymes that drive the metabolism of pyridine alkaloid precursor compounds leading to the synthesis of nicotine, nornicotine, anabasine and anatabine and their connections with other closely linked metabolic pathways is shown in Figure 2. Nicotine represents the prominent pyridine alkaloid present in tissues of tobacco species such as *N. tabacum* and *N. attenuata*, with anatabine comprising only a small portion of the alkaloid fraction. Anabasine and nornicotine, two less common pyridine alkaloids present in the *Nicotiana* genus, are typically present at low quantities in these species.

The production of nicotine involves the condensation reaction combining the Put-derived *N*-methyl-Δ^1-pyrrolinium cation with the pyridine ring from nicotinic acid. Both of these rings are derived from separate areas of 1° metabolism (Feth *et al*., 1986; Wagner *et al*., 1986a). Nicotinic acid can also be conjugated to a lysine-derived *N*-Δ^1-piperideinum to produce anabasine (Leete, 1979; Walton and Belshaw, 1988). It was reported that the

nicotinic acid-derived pyridine ring of nicotine and anabasine may be formed from a nicotinic acid derivative, 3,6–dihydronicotinic acid (Dawson, 1962; Leete, 1992). The structure of anatabine contains two pyridine rings that are derived solely from a condensation reaction with two nicotinic acid-derived molecules (Leete and Slattery, 1976; Leete, 1979; Häkkinen *et al*., 2004). It is thought that anatabine is formed from the condensation of nicotinic acid with a decarboxylated version of the nicotinic acid-derivative, 3,6–dihydronicotinic acid (Leete and Slattery, 1976; Leete, 1992). A further step involving the *N*-demethylation of nicotine is the primary mode of producing nornicotine (Siminszky *et al*., 2005).

Source of the pyridine moiety for pyridine alkaloid biosynthesis

As noted, the pyridine ring common to all *Nicotiana* alkaloids is derived from nicotinic acid. This was demonstrated by Dawson *et al*. (1958; 1960a; 1960b) and Solt *et al*. (1960) who independently showed through feeding experiments that radioactively labelled nicotinic acid is incorporated into the pyridine ring of nicotine, anatabine and anabasine in *N. tabacum* and *N. glauca*, respectively.

The NAD biosynthetic pathway

The *de novo* biosynthesis of nicotinamide adenine dinucleotide (NAD) is conserved among all living organisms and stems from the conversion of aspartate and/or tryptophan to produce quinolinic acid (QA) from quinolinate synthase (QS). QA acts as the initial precursor in the NAD cycle to replenish nicotinic acid mononucleotide (NaMN) by the action of quinolinate phosphoribosyltransferase (QPT) and is subsequently channelled into nicotinic acid adenine dinucleotide (NaAD) and NAD (Figure 4; Wagner and Wagner, 1985; Sharma *et al*., 1998; Rongvaux *et al*., 2003; Chappie *et al*., 2005; Noctor *et al*., 2006). However, there has been little evidence to date that suggests the existence of functional enzymes encoding the kynurenine (tryptophan route) pathway in dicotyledonous plants, nor the incorporation of labelled tryptophan into nicotine (Leete, 1957). It has therefore been suggested that QA, in dicotyledonous plants, is synthesised solely from the aspartate pathway (Katoh *et al*., 2006), as opposed to monocotyledonous species, where it can be produced *via* either pathway (Katoh and Hashimoto, 2004). A recent study by Schippers *et al*. (2008), involving QS mutants of *Arabidopsis*, reported that pyridine nucleotide and salvage pathway genes may be co-regulated together as part of a homeostatic feedback mechanism to maintain steady NAD production for plant metabolism.

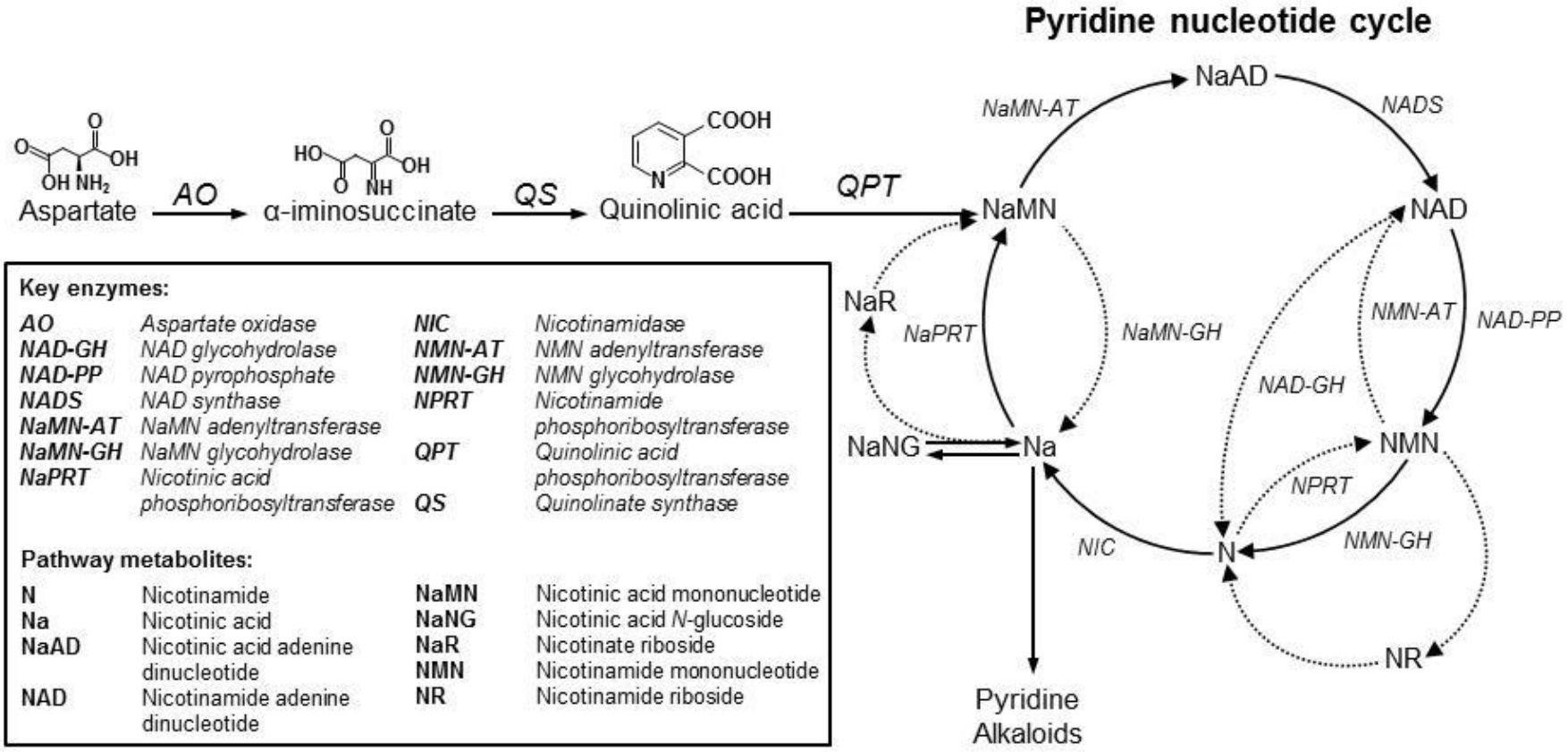

Figure 4. Schematic diagram of the biosynthesis of nicotinic acid *via* the pyridine nucleotide cycle. Solid lines indicate defined steps in the *de novo* synthesis of NAD *via* the aspartate pathway, while broken lines indicate additional routes for NAD degradation and consumption. It is suggested that NaNG accumulates as a detoxification mechanism of nicotinic acid when A622 activity is diminished (Kajikawa *et al*., 2009).

The pyridine nucleotide cycle

The final steps in the *de novo* biosynthesis of NAD or its recycling following consumption or break down and resynthesis are often collectively referred to as the pyridine nucleotide cycle (Rongvaux *et al*., 2003; Hunt *et al*., 2004; Katoh and Hashimoto, 2004; Noctor *et al*., 2006; Wang and Pichersky, 2007). Normal cellular function in organisms including signalling and gene regulation of development and stress processes rely heavily on NADP and its reduced derivative, NADPH, which mediate redox reactions. Furthermore in plants, it is also essential for electron transfer in photosynthesis and respiration processes (Noctor *et al*., 2006).

NAD salvage pathway

Recovery pathways that act to produce NAD by the salvaging of pyridine nucleotides are described as either Preiss-Handler independent or dependent pathways, named after the person who described the pathways (Preiss Handler, 1958a; 1958b). The independent pathway is formed by resynthesising NAD, while the dependent pathway re-introduces NAD by converting nicotinamide, a degradation product, to nicotinic acid and subsequently NAMN *via* nicotinic acid phosphoribosyltransferase (NaPRT). All evidence to date suggests that degradation of pyridine nucleotides is the only source of nicotinamide in plants, formed from the hydrolysis of the ribose-nicotinamide bond by NAD enzymes (Kaplan, 1961).

Role of quinolinate phosphoribosyltransferase (QPT)

The pyridine moiety of pyridine alkaloid has been demonstrated to be produced as a component of the pyridine nucleotide cycle (or NAD cycle), which is derived from aspartic acid and converted to QA by the enzyme QPT (Katoh *et al*., 2006). Therefore, QPT has known functions in 1° and 2° metabolism and is essential for supplying QA precursors for the *de novo* synthesis of NAD (Katoh *et al*., 2006). Mann and Byerrum (1974a; 1974b) demonstrated the strong association between QPT activity, consumption of nicotinic acid, production of the pyridine moiety and increased supply of the alkaloids ricinine and nicotine in castor beans and *Nicotiana*, respectively. In *N. tabacum* expression of *QPT*, along with other pertinent alkaloid biosynthetic genes such as *PMT*, *A622* and *BBL*, is predominantly observed in root tissues and is amplified by wound-associated stress known to stimulate pyridine alkaloid biosynthesis (Conkling *et al*., 1990;

Shoji *et al*., 2000; Sinclair *et al*., 2000; Cane *et al*., 2005; DeBoer *et al*., 2009; Kajikawa *et al*., 2009; 2011).

Source of the pyrrolidine ring for pyridine alkaloid biosynthesis

The pyrrolidine ring common to a number of methylputrescine-derived alkaloids such as pyrollidines, tropanes and some pyridine alkaloids, is derived from Put. It was demonstrated through feeding experiments that radioactively labelled Put and methylputrescine are incorporated into the pyrrolidine moiety of nicotine in *Nicotiana* species (Leete, 1955; Dewey *et al*., 1955; Leete *et al*., 1964; Mizusaki *et al*., 1968).

Role of putrescine methyltransferase (PMT)

The first enzymic step committing Put to the synthesis of alkaloids such as the pyridines (nicotine and nornicotine), tropanes (hyoscyamine, scopolamine, tropinone and tropine), pseudotropanes and calystegines, is the *N*–methylation of Put into *N*-methylputrescine (Hashimoto and Yamada, 1994; Biastoff *et al*., 2009a; 2009b). This step is catalysed by the key regulatory enzyme putrescine methyltransferase (PMT), which directs the flow of 1° nitrogen metabolites destined for alkaloid (2°) biosynthesis, away from polyamine (1°) metabolism (Feth *et al*., 1986; Wagner *et al*., 1986a; 1986b; Hibi *et al.,* 1992; Shoji *et al*., 2000b; Sato *et al*., 2001; Sachan and Falcone, 2002). The root-specific transcript expression and enzymic activity of PMT in *Nicotiana* has been well correlated with the level of nicotine formation (Mizusaki *et al*., 1971; 1973; Saunders and Bush, 1979; Hibi *et al*., 1994; Hashimoto *et al*., 1998; Riechers and Timko, 1999; Winz and Baldwin, 2001; Liu *et al.,* 2008). This has also been shown to be true for other *N*-methylputrescine-derived alkaloids including tropanes in a number of other Solacaneous plants, including *Datura* species (Hashimoto *et al*., 1989a; 1989b; Hibi *et al*., 1992). Over-expression of PMT increased the nicotine content in *N. sylvestris*, whereas suppression of endogenous PMT activity severely decreased the nicotine content and was also associated with induced abnormal morphologies (Sato *et al*., 2001). Enhanced expression of PMT in root tissues and increased leaf nicotine is largely associated with wound-associated stress, including decapitation, known to heavily stimulate pyridine alkaloid biosynthesis (Mizusaki *et al*., 1973; Saunders and Bush, 1979). Experiments involving antisense- or RNAi-mediated gene silencing with transgenic *Nicotiana* hairy roots and plants have shown that a decrease in *PMT* expression levels can lead to low nicotine content (Sato *et al*., 2001; Voelckel *et al*., 2001a; Chintapakorn and Hamill, 2003; Wang *et al*., 2009). Chintapakorn and Hamill (2003) also found that anti-sense mediated down-regulation of

PMT expression in transformed roots of *N. tabacum* resulted in reduced nicotine levels and, in addition was associated with significantly increased anatabine levels. The latter observation was deemed to be due to an effective over-supply of nicotinic acid *in vivo*, thereby remobilising the pyridine moiety precursors to provide more substrate for anatabine synthesis (Chintapakorn and Hamill, 2003). This finding was subsequently also reported by Steppuhn *et al*. (2004) and Wang *et al*. (2009).

Methylputrescine oxidase (MPO)

The second step involving commitment of Put to alkaloid formation involves the oxidative deamination of *N*-methylputrescine by the action of methylputrescine oxidase (MPO). MPO, a subclass of copper diamine oxidases, specifically recognises methylated substrates, to form 1-methylaminobutanal, an unstable intermediate that spontaneously cyclises to form the pyrrolinium cation (Mizusaki *et al*., 1968; 1971; 1972; Heim *et al*., 2007; Katoh *et al*., 2007). Comparable with PMT, root-specific activity of MPO is associated with *N*-methylputrescine-derived alkaloids, including nicotine (Mizusaki *et al*., 1971; 1973; Wagner *et al*., 1986b). Transcript expression of *MPO* was also amplified by wound-associated stress, removal of shoot apices and methyl jasmonate (MeJA) application (Saunders and Bush, 1979; Heim *et al*., 2007; Katoh *et al*., 2007). Experiments by Shoji and Hashimoto (2008) using RNAi-mediated gene silencing techniques to suppress MPO activity in hairy root cultures of *N. tabacum* demonstrated a strong correlation between decreased expression and activity of MPO and reduced production of the pyrrolidine ring-derived pyridine alkaloids, nicotine and nornicotine. In line with previous reports involving silenced PMT transgenic plants, silenced MPO hairy root cultures displayed elevated levels of anatabine. On top of this, this study also showed that silenced MPO was correlated with elevated levels of anabasine, anatalline (normally a minor alkaloid in tobacco containing 3 pyridine-derived rings) and also free and conjugated forms of Put and methylputrescine.

Final stages of pyridine alkaloid biosynthesis

Some questions remain regarding the final stages of pyridine alkaloid formation in *Nicotiana.* Experimental evidence points to the co-involvement of a protein bearing significant similarities to the PIP (pinoresinol–lariciresinol reductase, isoflavone reductase, and phenylcoumaran benzylic ether reductase) family of enzymes and is likely to play an important role in the condensation of the pyridine ring with a pyrrolidine, piperidine or another pyridine ring to form nicotine, anabasine and anatabine structures,

respectively (Hibi *et al*., 1994; Shoji *et al*., 2002; DeBoer *et al*., 2009; Kajikawa *et al*., 2009). A berberine bridge-like (BBL) enzyme is also suggested to be involved in the concluding steps of alkaloid biosynthesis in *Nicotiana* (Kajikawa *et al*., 2011).

Role of an isoflavone reductase

The PIP family NADPH-dependent reductase protein encoded by the *A622* gene was identified in the same study that isolated the cDNA encoding PMT, following molecular comparisons of root cDNA from low alkaloid mutants and wild type *N. tabacum* (Hibi *et al*., 1994). The A622 gene was shown to be upregulated at the site of synthesis of a number of related pyridine alkaloids including nicotine, anatabine and anabasine in *N. tabacum*, *N. sylvestris* and *N. glauca* following leaf wounding, removal of flowering heads and several young leaves, known as 'topping' and MeJA application (Shoji *et al*., 2002; Sinclair *et al*., 2004; Cane *et al*., 2005; DeBoer *et al*., 2009; Kajikawa *et al*., 2009; Shoji *et al*., 2010). Subsequent studies by DeBoer *et al*. (2009) and Kajikawa *et al*. (2009) using RNAi-mediated suppression of A622 in *N. glauca* and *N. tabacum*, respectively, showed that the entire pyridine alkaloid profile, which differs between the two species, was severely diminished in both plant species. Collectively, these results provide evidence for A622 acting, either in the usage of nicotinic acid, formation of the nicotinic acid derivative or the condensation of rings, to form pyridine alkaloids. A build-up of *N*-methylpyrrolinium cation and nicotinic acid-β-N-glucoside (NaNG) intermediates was reported in A622 silenced cell and hairy root cultures of *N. tabacum* and transgenic *N. glauca* plants that were fed with excess nicotinic acid (DeBoer *et al*., 2009; Kajikawa *et al*., 2009). The elevated levels of intermediates suggests A622 is involved in the formation of the NA derivative or the final pyridine synthesising reactions.

Role of berberine bridge-like (BBL) proteins

A gene isolation study by Kajikawa *et al*. (2011) led to the characterisation of a new berberine bridge-like enzyme, BBL, required for formation of pyridine alkaloids in *Nicotiana*. Through its suppression in cell cultures, hairy roots and transgenic plants of *N. tabacum*, BBL was shown to be implicated in an oxidation reaction, which may be the final phase of pyridine alkaloid construction, succeeding condensation of pyridine rings.

Synthesis of nornicotine

Nornicotine is both an effective anticholinergic agent and a powerful carcinogen and considerable efforts have been expended by breeders to ensure minimal levels accumulate

in commercial tobacco varieties (reviewed in Dewey and Xie, 2013). This alkaloid can be synthesised directly from nicotine by demethylation or alternatively, from a non-methylated pyrrolinium cation, produced by the action of DAO/MPO upon putrescine itself (Dawson, 1945; Tso and Jeffrey, 1956; Saunders and Bush, 1979; Siminszky *et al*., 2005; Gavilano and Siminszky, 2007). While both routes are thought to exist in *Nicotiana*, it is generally surmised that most nornicotine is normally produced *via* the action of a subclass of cytochrome P450 enzymes, CYP82E, which specifically demethylate nicotine. The secondary route to nornicotine is likely to be utilised to compensate and achieve desired nornicotine levels when plants are subjected to certain stress events. This was demonstrated in studies by Lewis *et al*. (2010), which showed that silencing CYP82E in *N. tabacum* plants did not completely eliminate nornicotine levels. Comparable to previous reports on nornicotine biosynthesis using CYP82E mutants, experiments by Kajikawa *et al*. (2011) using silenced BBL transgenic *N. tabacum* plants, which displayed severely reduced nicotine levels, had no effect on the levels of nornicotine.

Site of alkaloid synthesis

Classical grafting experiments between *N. tabacum* and tomato found that the main site of nicotine synthesis occurred in the roots, and particularly in the growing root tips, of *N. tabacum* plants (Dawson, 1942; Solt, 1957; Saedler and Baldwin, 2004). Subsequent research showed that Put-derived pyridine alkaloids are generally produced in the roots of *Nicotiana* plants and then transported through the xylem to the shoots and leaves, where they are stored in vacuoles of mesophyll cells (Saunders and Bush, 1979; Baldwin, 1989; Hashimoto and Yamada, 1994; Nugroho and Verpoorte, 2002). Nakajima and Hashimoto (1999) showed that PMT was subcellularly localised to the pericycle, the outermost layer of the vascular tissue in the root. The close proximity of PMT to the vascular tissue, where amino acids are also transported, means Arg and Orn, are readily accessible. Localisation studies have since revealed that many alkaloid biosynthetic enzymes, precursors, intermediates and the resulting metabolites are not exclusively present in vacuoles, but undergo dynamic intracellular trafficking and can also localise in smaller amounts in diverse subcellular organelles including the cytosol, tonoplast membrane, endoplasmic reticulum, chloroplast stroma and thylakoid membranes (reviewed in De Luca and St Pierre, 2000; Facchini, 2001; Shitan *et al*., 2014). While the formation of nicotine is thought to occur in the vacuolar lumen, the process of incorporation of

alkaloids into other subcellular organelles remains unclear (Kajikawa *et al*., 2011; Shitan *et al*., 2014).

Spatially separating substrates, enzymes and intermediates in compartments for synthesis or storage purposes ensures local concentrations of products remain high for biosynthesis, whilst preventing feedback inhibition of their synthesis (Facchini, 2001). Sequestering of alkaloids in subcellular compartments other than the cytosol can also provide an additional level of metabolic regulation to minimise cytotoxicity. Compartmentalising precursors, intermediates and associated enzymes of alkaloid biosynthetic pathways can provide a mechanism to limit or impede these reactions within space and/or time (Stafford, 1981; Facchini, 2001).

Following earlier demonstrations in *N. tabacum* (Saunders and Bush, 1979) and *N. sylvestris* (Baldwin, 1989), Baldwin and Ohnmeiss (1993) showed that the level of nicotine within the leaves of *N. attenuata* doubled in response to leaf damage and quadrupled after excision of the shoot apex, demonstrating the alkaloid response is related to the amount and type of damage incurred by the plant. Sequestration of nicotine biosynthesis capacity in the root was suggested to be an evolutionary adaptation to heavy predation as it ensures that the plant is still able to up-regulate the synthesis of this metabolic deterrent even if the major portion of the leaf tissue has been grazed away (Karban and Baldwin, 1997; Karban, 2011).

Transport of pyridine alkaloids

Most of our knowledge about transport of pyridine alkaloids stems from studies investigating the conveyance of nicotine within the plant. Above ground, nicotine allocation follows the predictions of the optimal defence theory (ODT) whereby seed capsules and young leaves receive proportionally greater allocations of nicotine than do older leaves (Baldwin *et al*., 1990). Therefore, to be an effective defence strategy, the plant relies substantially on transporters mediating the efflux of nicotine, and related alkaloids, and intake through plasma and vacuolar membranes. Recent studies have identified multidrug and toxic compound extrusion (MATE) transporters as important proteins enabling vacuolar sequestration of nicotine (Morita *et al*., 2009; Shoji *et al*., 2009), whilst nicotine uptake permease 1 (NUP1), acts as a plasma membrane transporter to direct apoplastic nicotine into the cytoplasm of root tip cells (Hildreth *et al*., 2011).

Multidrug and toxic compound extrusion (MATE) transporter

Together with genes directly involved in nicotine synthesis such as *QPT, PMT, MPO, A622* and *BBL*, transcript levels of MATE (and NUP1) genes are induced following wound- and MeJA stress (Shoji *et al*., 2009; Hildreth *et al*., 2011). Cytosolic nicotine is shuttled out of cells *via* a proton exchange across the plasma membrane by MATE transporters. This was concluded using protein localisation, feeding experiments and a series of exogenous treatment studies applied to suppressed and over-expressing MATE cell lines of *N. tabacum*. In a similar fashion, jasmonate-inducible alkaloid transporter 1 (JAT1), a different vacuole-localised multisubstrate proton antiporter isolated from tobacco, was presented as a separate aerially expressed nicotine shuttling service, mediating mobilisation of stem and leaf nicotine to the vacuoles for storage (Shitan *et al*., 2009; Shitan *et al*., 2014). MATE transporters may also have the capacity to facilitate transport of other alkaloids including other pyridine alkaloids and also tropanes (Morita *et al*., 2009; Shoji *et al*., 2009; Shitan *et al*., 2014).

Nicotine uptake permease 1 (NUP1)

NUP1, a plasma membrane-localised transporter, displaying a greater specificity for uptake of nicotine than other alkaloids, accumulates in higher quantities in root tissues than leaves and was recently shown to mediate apoplastic nicotine translocation to the cytoplasm in roots of *Nicotiana* (Hildreth *et al*., 2011; Kato *et al*., 2014). NUP1 positively influences the expression of *ETHYLENE RESPONSE FACTOR 189* (ERF189), which in turn positively regulates structural genes that function in nicotine biosynthesis and transport. This was demonstrated in studies by Kato *et al*. (2014), which showed that down-regulation of NUP1 led to severely reduced nicotine in both root and foliar tissues, whereas levels of nicotine in the roots of NUP1 over-expression plants quadrupled whilst the aerial tissues contained significantly reduced nicotine levels. These experiments demonstrated that NUP1 positively regulates nicotine biosynthesis and accumulation by controlling the mobilisation of nicotine from the roots to the shoots, as well as ERF189 expression. Kato *et al*. (2015) noted that in addition to tobacco alkaloids, NUP1 displays substrate specificity for pyridine ring-containing compounds and can also transport vitamin B6 (pyridoxine and its derivatives). This was concluded following the use of transport assays and exogenous treatment studies applied to over-expressing NUP1 cell lines of *N. tabacum.*

Toxicity of pyridine alkaloids

Insecticidal action

Many alkaloids are extremely toxic to insects and mammals and are synthesised by plants to provide protection from predation by acting on the nervous system of herbivores (Wink, 1998b; Baldwin and Preston, 1999; Katoh *et al*., 2005; Kutchan, 1995; Ohnmeiss and Baldwin, 2000; Steppuhn *et al*., 2004). Thus it is not surprising that nicotine was one of the first insecticides used to control pests in agriculture (Schmeltz, 1971). Its acute toxicity can be attributed to its ability to function as a neurotransmitter agonist at high concentrations (Soeda and Yamamoto, 1968), mimicking the conformation and electronic makeup of acetylcholine (Beers and Reich, 1970). Free nicotine penetrates into the insect body, where the ionic form binds agonistically to nicotinic acetylcholine receptors (nAChR) in the central nervous system (Yamamoto *et al*., 1962; Schmeltz, 1971), subsequently decreasing ingestion and increasing the mortality of insects (Wink, 1998b).

In *N. sylvestris* the nicotine content in leaves increases several fold within days after leaf damage by insects or wounding, suggesting a protective role following herbivory (Baldwin, 1989). Low alkaloid mutants of *N. tabacum* were shown to be more prone to attack by *Heliothis virescens* (Jackson *et al*., 2002). Studies by Steppuhn *et al*. (2004) showed increased levels of herbivory on leaves of transgenic *N. attenuata* plants that had a reduced ability to synthesise nicotine as a result of *PMT* down-regulation. However, not all species in the *Nicotiana* genus are characterised by high levels of nicotine in their foliage. As has been noted, foliage of *N. glauca* contains predominantly anabasine (Saitoh *et al*., 1985; Sisson and Severson, 1990), which like nicotine, is highly toxic to both insects and mammals. Like nicotine, anabasine accumulates in foliage of *N. glauca* in response to decapitation and it has been proposed to also protect plants against excessive herbivory (Baldwin and Ohnmeiss, 1993; Sinclair *et al*., 2004). While nicotine is produced mainly in the roots, there is strong evidence that increased anabasine in wounded *N. glauca* is due to upregulation of genes required for anabasine synthesis, including ODC, in upper leaf tissues themselves. It is thought that ODC may possess a dual role as lysine decarboxylase (LDC) to provide cadaverine required for synthesis of the piperidine ring of anabasine in *N. glauca* (Sinclair *et al*., 2004; DeBoer *et al*., 2009; 2013).

1.3.6. Phenylpropanoid biosynthesis

One of the most widespread 2° metabolic pathways, assisting plants to survive stressful conditions (Ferrer *et al*., 2008), involves the biosynthesis of phenylpropanoid derivatives. These specialised metabolites are quite different from alkaloids in that they lack nitrogen, are found ubiquitously across the plant kingdom and contain at least one aromatic ring with one or more hydroxyl groups attached (Vogt, 2010). Phenylpropanoid metabolism stems from phenylalanine and comprises a complex series of branching biochemical pathways that provide plants with a wide range of hydroxycinnamoyl acids and esters (Figure 5). These metabolites are widely used as: major structural cell components (lignin, suberin and other cell wall-associated phenolics), pigments (flavonoids and anthocyanins), defence signals (SA) and toxins (coumarins, furanocoumarins and phenolamides) (Dixon *et al*., 2002; Vogt, 2010). These phenolic compounds have essential functions in development as well as regulatory molecules in signal transduction and communication with other organisms, plant defence against biotic challenges and UV light protection (Ferrer *et al*., 2008). The phenylpropanoid pathway and species-specific transcriptional regulators are positively regulated by JA and its derivate MeJA, which has been shown to induce the accumulation of phenylalanine ammonia-lyase (PAL; Kazan and Manners, 2008).

The need to improve crop resistance and decrease the annual yield loss caused by drought and pest outbreaks motivates the research into manipulating the phenylpropanoid pathway to produce plants resistant to insects. Enhanced resistance can be achieved by elaborate breeding programs, but advances in molecular biology may allow for the potential use of transgenic crops to obtain resistant plants.

Synthesis of phenylpropanoids

The phenylpropanoid pathway begins with PAL catalysing the deamination of phenylalanine to cinnamate (Figure 5). Production of coumaric acid follows by the introduction of a hydroxyl group in the phenyl ring of cinnamic acid, a process catalysed by cinnamate-4-hydroxylase. Simple hydroxycinnamoyl acids and esters are formed as part of the core shikimate pathway, following modifications such as oxidation, reduction, methylation, polymerisation or addition of various small molecules (Vogt, 2010). Phenolic compounds including hydroxycinnamic acid derivatives and flavonoids have been reported to be located mainly in the vacuoles of the tissues in which they accumulate (Grob and Matile, 1979; Moskowitz and Hrazdina, 1981; Sharma and Strack, 1985).

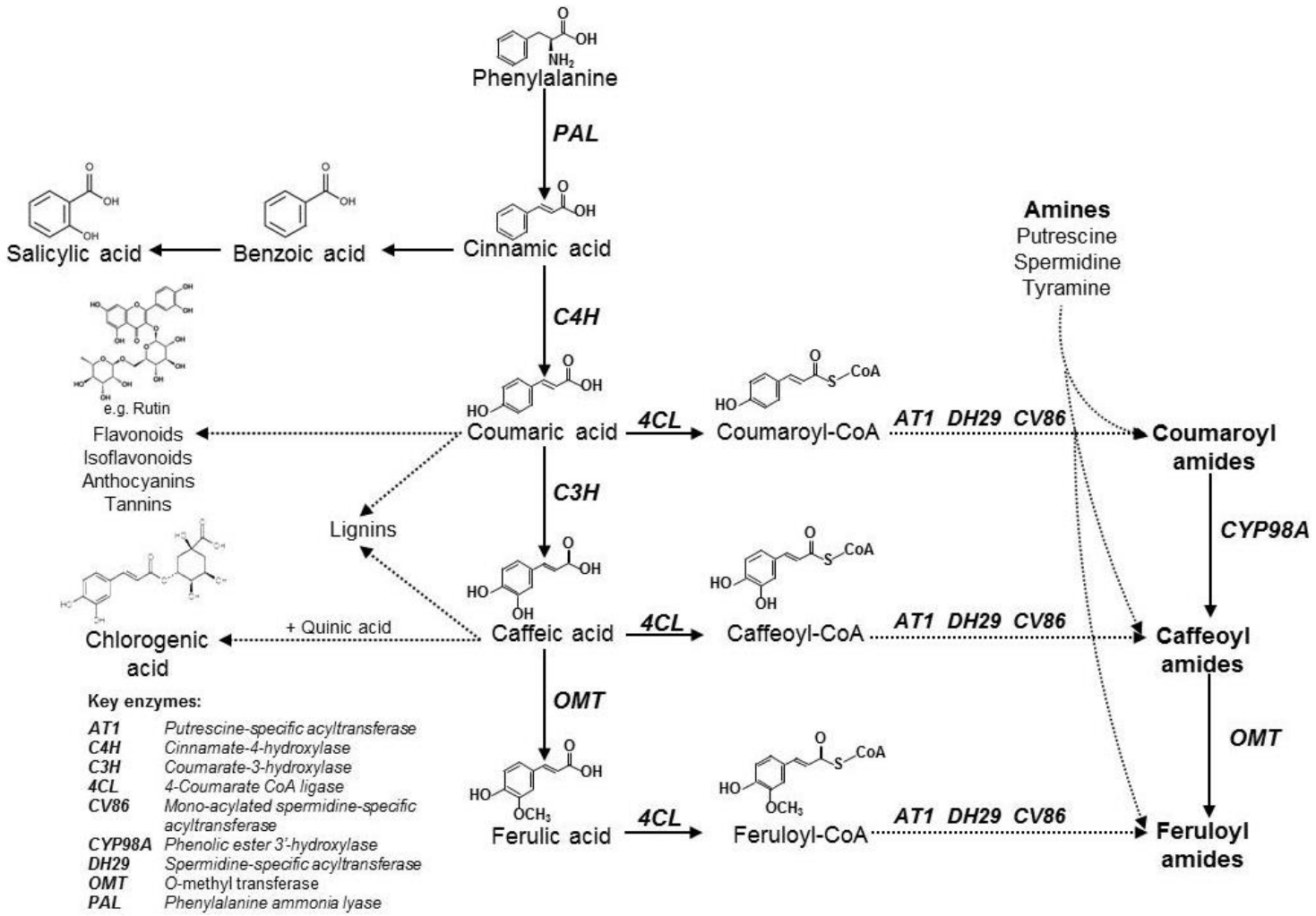

Figure 5. C**onnection of phenolamides to phenolic metabolism (adapted from Dixon and Paiva, 1995; Bassard *et al.*, 2010; Gaquerel *et al.*, 2013).** Defined enzymatic steps are shown by solid lines, while broken lines indicate undefined steps or steps including multiple reactions. Both phenolic (e.g. flavonoids, isoflavonoids, anthocyanins, lignins and tannins) and phenolamide metabolites share early biosynthetic steps in common, starting from phenylalanine to produce cinnamic acid.

Phenolamide biosynthesis

Phenolamides are abundant in plants and constitute a large, diverse class of 2° metabolites, which result from the conjugation of a phenolic moiety with polyamines or with deaminated aromatic amino acids. The key entry point into phenolamide biosynthesis involves the conjugation of phenolic acids with amine moieties, catalysed by *N*-acyltransferases. Three *N*-acyltransferase enzymes, AT1, DH29 and CV86, have recently been identified in *N. attenuata*, which are proposed to mediate Put acylation, the initial acylation step of Spd conjugation and the second acylation step of mono-acylated Spd conjugates, respectively (Onkokesung *et al*., 2012). These conjugated metabolites, sometimes referred to as PPCs, phenolic amines, phenylamides, hydroxycinnamic acid amides or more accurately as *N*-hydroxycinnamoyl-amine conjugates, have been reported throughout the plant kingdom, usually as the main phenolic constituents of reproductive organs and seeds. They were generally regarded as either a product of polyamine catabolism or as storage forms of polyamine and/or phenolic compounds. However, the intermediates and final products of these pathways seem to have specific functions in plant development and defence (Morant *et al*., 2007; Fellenberg *et al*., 2008; 2009; Matsuno *et al*., 2009; Kaur *et al*., 2010; Fellenberg *et al*., 2012).

Conjugation of phenolic and polyamine metabolites

Hydroxycinnamic acids such as coumaric, caffeic and ferulic acids in combination with either aryl monoamines (such as tyramine, tryptamine, octopamine or anthranilate) or polyamines (Put and Spd) are the main building blocks of phenolamide molecules. A large proportion of the Spd found in plants is mono-, di- or tri-substituted with amides of hydroxycinnamic acids. This provides a large array of structures that may be additionally modified after conjugation (Fellenberg *et al*., 2008; 2009; 2012; Matsuno *et al*., 2009), resulting in a large range of structures with varying physicochemical properties and biological functions. In the Solanaceae and many other plant families, polyamines occur as conjugates to amides of hydroxycinnamic acids (Smith *et al*., 1983). However, mono-acylated Put conjugates and mono- or di-acylated Spd's in coumaric, caffeic or ferulic acid combinations are the main phenolamides formed in tobacco species (Figure 6). The relative proportions of free and conjugated polyamines vary among different plant species, with approximately 90% of the polyamine pool in tobacco species present in the conjugated form (Torrigiani *et al*., 1987).

Conjugation of polyamines with phenolics significantly reduces their polarity and hydrophilicity. This may favour their translocation, stability and compartmentalisation. Conjugation can be a means to regulate the pools of both parent compounds and to store phenolics and bioactive polyamines. While conjugates were often regarded as final and accumulated products, turnover and translocation of conjugates was described by Martin-Tanguy (1985; 1997) and Havelange *et al*. (1996) and, more recently, interconversion between free and conjugated precursors in *Arabidopsis* seed has been reported (Luo *et al*., 2009). Studies conducted by Morant *et al*. (2007) and Matsuno *et al*. (2009) suggest that polyamine conjugates may also act as signals in cross-talk between nitrogen and phenolic metabolism.

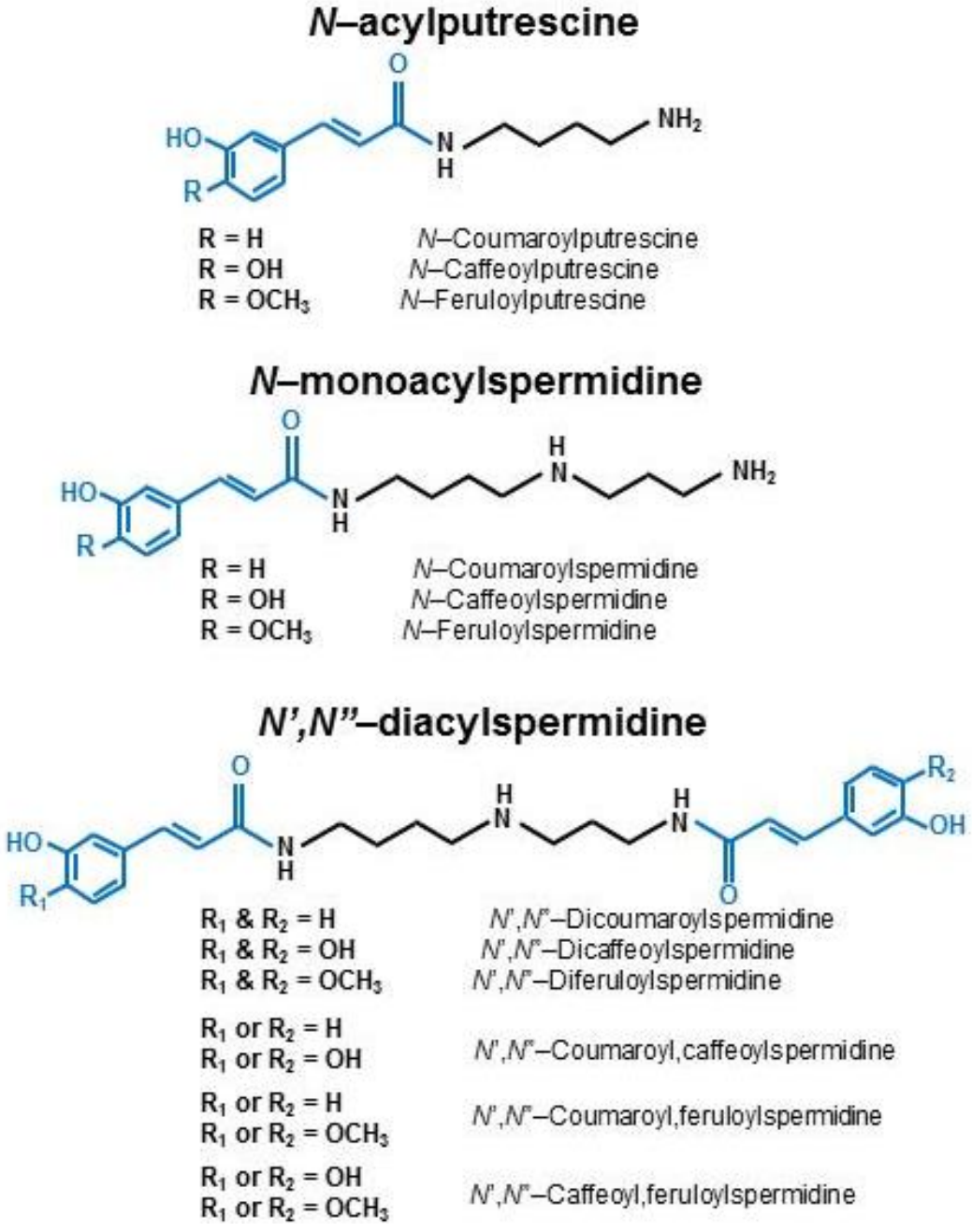

Figure 6. Schematic of the main putrescine and spermidine phenolamide structures found in leaves of *Nicotiana* species (adapted from Gaquerel *et al*., 2014). Representative structures are shown of the most commonly encountered coumaroyl-, caffeoyl- and feruloyl-containing mono-acylated putrescine molecules and mono- or di-acylated spermidines. A coumaric, caffeic or ferulic-derived molecule provides the hydroxycinnamic acid moiety (in blue) that is conjugated to either the four or seven carbon chain skeleton of putrescine or spermidine (in black) as monomers or dimers.

Role of phenolamides

Abundance, diversity and distribution of phenolamides are well documented (Martin-Tanguy *et al*., 1978; Martin-Tanguy, 1985; Bienz *et al*., 2005; Rogoza *et al*., 2005). Surprisingly little is known about their biological functions in plants. However, phenolamides may have roles in plant development, in responses to abiotic stress and in defence (reviewed in Facchini *et al*., 2002; Edreva *et al*., 2007). Phenolamides have been found as major pollen constituents. They are present in a number of vegetative and floral organs, and more recently have suggested roles in floral development, cell-wall cross-linking and adaptation to abiotic stress and plant defences against microorganisms and insects (reviewed in Bassard *et al*., 2010).

Role in plant defence

Phenolamides have been described as bioactive compounds with anti-viral, anti-bacterial anti-fungal or insecticidal activities and have attracted attention for their hypotensive effects and anti-trypanosomal activity (Funayama *et al*., 1980; 1995; Ponasik *et al*., 1995). Put-, Spd- or tyramine-derivatives appear to occur ubiquitously in higher plants. Specific diverse assortments of phenolamides have been shown to accumulate upon pathogen attack or herbivory, suggesting they may act as phytoanticipins or phytoalexins. JA was shown to promote a strong increase in the local and systemic concentrations of Put- and Spd-based phenolamides in barley leaves. This increase in phenolamide concentration was correlated with a reduction in powdery mildew infection (Walters *et al*., 2002). However, their defensive function has only been deduced from a correlation between metabolite accumulation and resistance to pathogens or herbivores.

Basic and neutral polyamine conjugates have been detected in a range of plant species such as maize, rice and tobacco and a number of different plant tissues and it is likely that they have differing roles in plant development and defence (Martin-Tanguy, 1985; Bonneau *et al*., 1994; Kaur *et al*., 2010). It has been shown that the structures of these plant-based phenolamides is very closely related to polyamine conjugates found in the venoms of predaceous spiders and wasps and it was surmised that they may have potential insecticidal or anti-feedant qualities (Fixon-Owoo *et al*., 2003; Pereira *et al*., 2007). Their defensive roles were recently demonstrated in studies by Tebayashi *et al*. (2007), which showed that the ability of *Capsicum annuum* (sweet pepper) to accumulate

caffeoylputrescine (CP) with age or following JA treatment was correlated with ovipositional deterrence of leaf miners (*Liriomyza trifolii*).

Furthermore, exogenous treatment of *C. annuum* with synthetic Put conjugates, such as coumaroylputrescine (CoP), was also seen to heavily influence oviposition behaviour. Transgenic *N. attenuata* plants genetically engineered to produce reduced amounts of these conjugated compounds, *via* RNAi-mediated silencing of biosynthesis enzymes or down-regulation of related transcription factors (TFs), has recently provided tools to investigate the role of these conjugates in plant defence (Kaur *et al*., 2010; Onkokesung *et al*., 2012; Gaquerel *et al*., 2013). Plants silenced for MYB8, a JA-activated TF, significantly reduced the amounts of phenolamide compounds present in the plants, which were shown to become severely compromised in their capacity to survive attack by both specialist (*M. sexta)* and generalist (*S. littoralis*) insect predators (Kaur *et al*., 2010). The marked induction of these metabolites in response to insect herbivory (Gaquerel *et al*., 2010; Kaur *et al*., 2010; Onkokesung *et al*., 2012) suggests they could act as indicators of herbivore-induced changes, which includes large alterations in defence signals, inter-connected metabolic pathways (Gaquerel *et al*., 2013) and increased resistance to insect herbivores (Kaur *et al*., 2010).

Additionally, two phenolamides in particular, CP and dicaffeoylspermidine (DCS), are known to play important roles in *N. attenuata's* defence response against herbivores such as *M. sexta* (Kaur *et al*., 2010). The toxicity of phenolamide compounds was determined in this study by the exogenous application of synthetic CP to leaves, which severely impaired growth of *M. sexta* caterpillars. Similar findings were reported in studies utilising *A. thaliana* and *N. tabacum* plants that constitutively over-express the PAP1 (Production of Anthocyanin Pigment 1)/AtMYB75 or AtMYB12 regulatory proteins of biosynthesis. These plants showed significant increases in the concentration of glycosylated anthocyanins, flavonols, and cell wall-esterified phenolamides when compared to wild-type plants and exhibited enhanced resistance to larvae of several important lepidopteran agricultural pests such as *S. frugiperda, S. litura* and *Helicoverpa armigera* (Borevitz *et al*., 2000; Johnson and Dowd, 2004; Tohge *et al*., 2005; Malone *et al*., 2009; Misra *et al*., 2010).

1.4. Regulation of defence responses

1.4.1. Hormone signalling

Phytohormones are important signal compounds that integrate information perceived from external and internal cues into the regulatory processes of plants to regulate growth and development. Plant-environment interactions, specifically the activation of wound-, herbivore- and pathogen-induced defence responses, involve a network of plant signalling cascades that are tightly regulated by plant phytohormones such as SA, ET, ABA, auxin (AUX) and JA (Rojo *et al*., 2003; Fujita *et al*., 2006). In the hours following wound-elicitation in leaves of *N. attenuata*, plants produce a JA and/or SA burst (Stork *et al*., 2009) and also release ET (Kahl *et al*., 2000), which is intensified by FACs elicitors (Halitschke *et al*., 2001).

1.4.2. Oxylipins – signalling molecules in response to stress

Oxylipins are a large family of oxygenated fatty acids derived from the peroxidation of membrane-derived lipids and are prevalent in a diverse range of organisms including mosses, algae, higher plants, bacteria, fungi and mammals (Funk, 2001; Andreou *et al*., 2009; Brodhun and Feussner, 2011). Oxylipins play essential roles as signalling molecules during plant responses to environmental stresses. Their production from polyunsaturated fatty acids (PUFAs) is induced in response to diverse stresses including wounding, insect and pathogen attacks (Farmer, 1994; Creelman and Mullet, 1995; Turner *et al*., 2002; Farmer *et al*., 2003; Mueller, 2004; Taki *et al*., 2005; Matsui, 2006; Howe and Jander, 2008; Browse, 2009). The enzymatic biosynthesis of oxylipins in plants is initiated by lipoxygenases (9- and 13-LOXs), which catalyse di-oxygenation of PUFAs, such as linoleic acid and α-linolenic acid, to yield 9- and 13-hydroperoxy linolenic acid (HPOT), respectively (Sanz *et al*., 1998; Hamberg *et al*., 1999; Feussner and Wasternack, 2002; Howe and Schilmiller, 2002). These hydroperoxides can be metabolized by allene oxide synthase (AOS) to initiate the biosynthesis of JA or by hydroperoxide lyase (HPL) to initiate the biosynthesis of C6 aldehydes and C12 ω-oxo-acids (Mosblech *et al*., 2009). Most research into plant oxylipins has focused predominantly on the phytohormone JA and its derivatives, collectively referred to as jasmonates (JAs), which play a well-documented role in plant responses to wounding (Farmer and Ryan, 1992; Glauser *et al*., 2008), herbivore attack (Kessler and Baldwin, 2002; Zavala and Baldwin, 2006; Browse and Howe, 2008) and pathogen infection (Glazebrook, 2005).

1.4.3. Jasmonate – important mediator in the wound and herbivory response

JAs are important signal molecules known to play a role in plant growth and development. Plant processes influenced by JA signalling include; root growth (Staswick *et al*., 1992), trichome initiation (Li *et al*., 2004; Qi *et al*., 2011), fruit ripening (Pérez *et al*., 1997; Fan *et al*., 1998), anthocyanin accumulation (Shan *et al*., 2009), senescence (Parthier, 1990; He *et al*., 2002; Shan *et al*., 2011), pollen maturation and flower development (McConn and Browse, 1996; Stintzi and Browse, 2000; Li *et al*., 2004; Mandaokar *et al*., 2006). JAs also function as essential mediators of plant defence responses following wounding (Glauser *et al*., 2008), herbivore attack (Halitschke and Baldwin, 2003; Glazebrook, 2005; Zavala and Baldwin, 2006; Browse and Howe, 2008) and pathogen infection (Vijayan *et al*., 1998). Hence, JAs are crucial natural integrators of plant defence and development (reviewed in Wasternack, 2007; Balbi and Devoto, 2008; Howe and Jander, 2008). To date, JA synthesis- or JA perception-deficient plants have been used to conduct many advanced functional studies of JAs, revealing their ubiquitous function in plants' defence and development.

In *N. attenuata,* defence responses are governed by a strong, transient release of JA within one hour of herbivore attack which is further amplified by herbivore-associated elicitors (Halitschke *et al*., 2001; Howe and Jander, 2008; Schmelz *et al*., 2009; Stork *et al*., 2009). In un-elicited mature leaves, JA is maintained at very low levels, however, upon specific wound- and/or insect- stimulations JA biosynthesis is induced within a few minutes (Halitschke and Baldwin, 2003; Glauser *et al*., 2008). This rapid response results from the activation of constitutively expressed JA biosynthetic enzymes, either directly by substrate availability and/or following activation by posttranslational modifications (Glauser *et al*., 2008). Field studies have demonstrated the essential role of the activation of JA biosynthesis and the induction of JA-regulated defences in *N. attenuata* in response to insect herbivore attack (Kessler *et al*., 2004; Paschold *et al*., 2007; Stitz *et al*., 2011a). Further evidence for this was demonstrated with *N. attenuata* plants rendered deficient in JA biosynthesis by anti-sense-mediated silencing of JA pathway components including LOX3 (Halitschke and Baldwin, 2003), AOS (Halitschke *et al*., 2004), jasmonate-resistant 4 (JAR4), JAR6 and JAR4/6 (Wang *et al*., 2007; 2008), which resulted in diminished JA and defences. These plants also suffered increased damage by herbivores (Kessler *et al*., 2004).

JA is synthesised from membrane-derived fatty acids *via* the well-characterized octadecanoid pathway in chloroplasts and peroxisomes (Figure 7; reviewed in Schaller and Stintzi, 2009). The biosynthesis of JA is initiated with the production of linolenic acid, which serves as a substrate for 13-hydroperoxy linolenic acid (13-HPOT) by 13-LOX enzymes in plastids. 13-HPOT is converted into a highly unstable allene oxide intermediate by the action of AOS, which is processed by an allene oxidase cyclase (AOC) to generate 12-oxo-phytodienoic acid (OPDA; Stenzel *et al*., 2003; Kallenbach *et al*., 2012). OPDA is then transported from the plastid into the peroxisome where it is reduced by the action of OPDA reductase 3 (OPR3) and submitted to cycles of β-oxidation, which ultimately leads to the production of JA. JA can be modified and subsequently metabolised to several derivatives, such as methyl-JA (MeJA; Seo *et al*., 2001; Stitz *et al*., 2011a; 2011b) by jasmonyl-O-methyltransferase (JMT) or it can be conjugated to amino acids (e.g. JA-Ile, JA-Leucine, JA-Valine) by JAR enzymes. However, JA is active at the molecular level only when conjugated to isoleucine (JA-Ile; Staswick and Tiryaki, 2004). JA-Ile activates the Skp1/Cullin/F-box (SCF)-COI1-JAZ complex (Fonseca *et al*., 2009), interacting with coronatine-insensitive 1 (COI1), releasing its targets from jasmonate ZIM domain (JAZ) protein repression, and thus triggering transcriptional activation of genes involved in the biosynthesis of defence molecules and other JA-mediated responses (Xie *et al*., 1998; Chini *et al*., 2007; Paschold *et al*., 2007; Oh *et al*., 2012). Consistent with studies demonstrating that JAs are involved in stimulating production of defence metabolites in numerous *Nicotiana* species, the induction of key defence biosynthetic and regulatory genes and the concomitant surge in production of defence metabolites is strongly associated with prior application of MeJA in all *Nicotiana* species studied so far (Shoji *et al*., 2000b; Winz and Baldwin, 2001; Sinclair *et al*., 2004; Kazan and Manners, 2008; DeBoer *et al*., 2009; 2011; 2013; Shoji *et al*., 2009; Heiling *et al*., 2010; Shoji *et al*., 2010; Shoji and Hashimoto, 2011b; Shoji and Hashimoto, 2015).

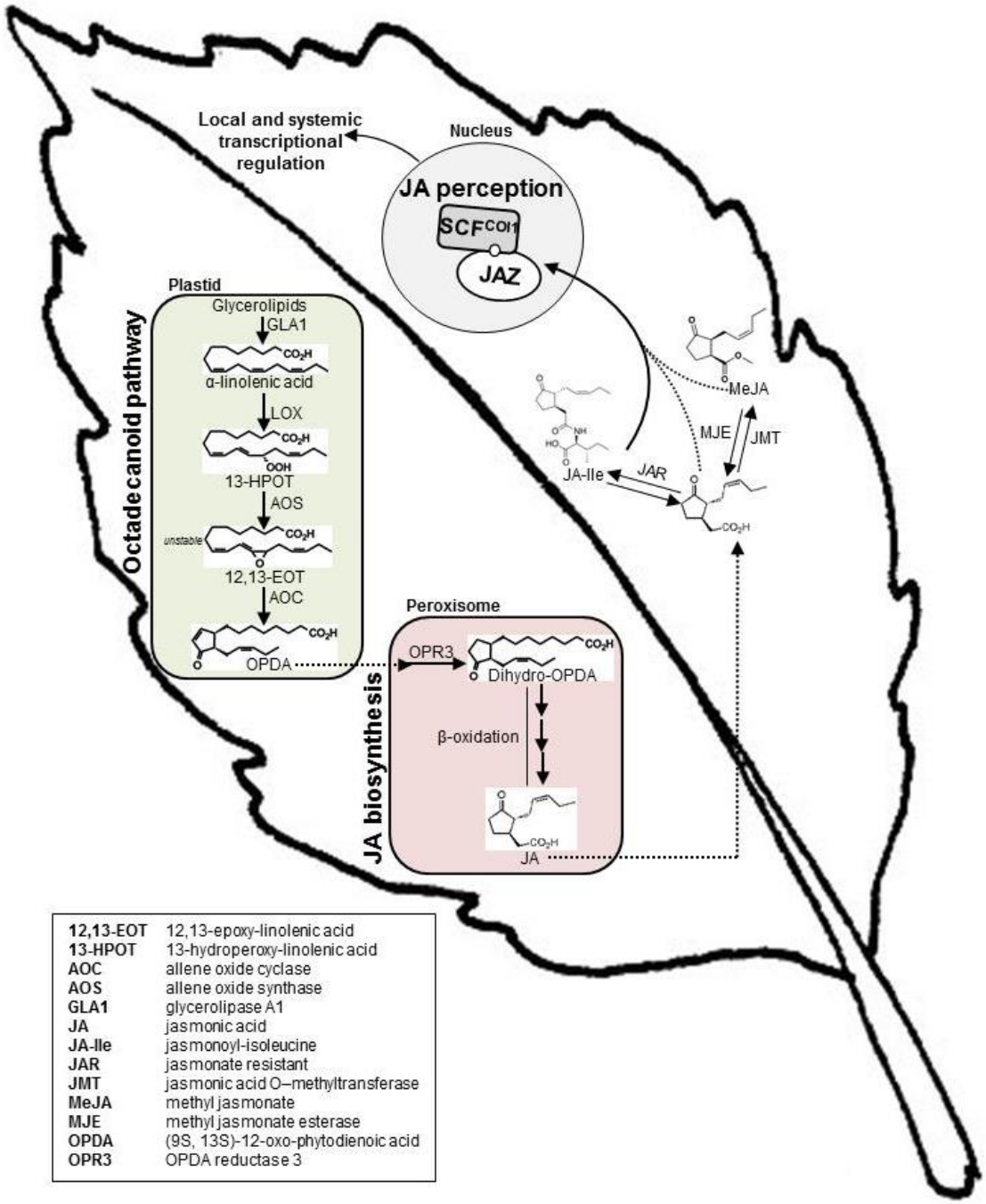

Figure 7. Schematic representation of the biosynthesis of JA in plants (adapted from Zhao *et al*., 2005). Solid lines indicate defined steps, while broken lines indicate mobilisation steps or not favoured reactions.The octadecanoid pathway for JA biosynthesis is initiated when lipases release free linolenic acid (C18:3) from plastidial membranes. Plastidic enzymes lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) enzymes convert linolenic acid to 13-hydroperoxy-linolenic acid (13-HPOT), a highly instable 12,13-epoxy-linolenic acid (12-13-EOT) and oxo-phytodienoic acid (OPDA), respectively. OPDA is then transported from the chloroplast to the peroxisome where it is then converted to JA by the peroxisomal enzymes OPDA reductase 3 (OPR3) and three cycles of β-oxidation. JA can be modified to form a number of derivatives including methyl Jasmonate (MeJA) and jasmonoyl-isoleucine (JA-Ile) by jasmonic acid methyltransferase (JMT) and jasmonate resistant (JAR) ligases, respectively. The formation of the JA-Ile conjugate has been shown to mediate the final step in JA activation, which requires COI1, an F-box protein that targets JAZ regulators of the signalling pathway for ubiquitin-dependent proteasome degradation.

Activation of the signalling cascade

In contrast to the mechanisms acting upstream of JA biosynthesis, the mechanisms mediating downstream JA defence responses are better characterised and understood (Kazan and Manners, 2008; Shoji *et al*., 2008; Browse, 2009; Pauwels *et al*., 2010; Shoji *et al*., 2011b). The current understanding of the signalling cascade and JA-mediate regulation of other defence pathways in tobacco is shown in Figure 8. Knowledge surrounding JA-mediated plant responses has dramatically expanding since the discovery of core components of the signalling cascade, COI1, JAZ and JA-Ile. COI1, an F-box protein that acts as a receptor of JAs (Feys *et al*., 1994; Xie, 1998; Devoto *et al*., 2005; Katsir *et al*., 2008; Paschold *et al*., 2008; Yan *et al*., 2009; VanDoorn *et al*., 2011; Ye *et al*., 2012) and participates in JA perception and regulation of gene expression through the interaction with JAZ repressors (Chini *et al*., 2007; Thines *et al*., 2007). JAZ proteins are characterised by two highly conserved motifs, essential for suppression of JA signalling (Shikata *et al*., 2004; Shoji *et al*., 2008; Ye *et al*., 2009; Vanholme *et al*., 2007; Yan *et al*., 2007; Melotto *et al*., 2008). In the absence of JA, JAZ acts as repressor protein that binds to TFs such as the basic helix-loop-helix (bHLH) protein MYC2 *via* a Jas domain to stop activation of JA responsive genes and other downstream TFs (Lorenzo *et al*., 2004; Boter *et al*., 2004; Dombrecht *et al*., 2007; Chini *et al*., 2007). On the other hand, in the presence of the active hormone JA-Ile, JAZ proteins are targeted for ubiquitination and degraded by the action of the SCFCOI1-E3 ubiquitin ligase complex and 26S proteasome respectively, releasing JA-responsive TFs (e.g. MYC2/3/4) from repression, and triggering expression of JA-dependent genes (Thines *et al*., 2007; Chico *et al*., 2008; Wasternack and Dombrink, 2010). In *A. thaliana* and *N. tabacum*, many JAZ proteins are both induced by wounding, MeJA and herbivory and also more rapidly degraded following JA treatment (Chini *et al*., 2007; Thines *et al*., 2007; Chico *et al*., 2008; Chung *et al*., 2008; Shoji *et al*., 2008). Consistent with the proposed mechanism surrounding JA signalling, JAZ repressors are also known to be rapidly degraded in roots of *N. tabacum* in the presence of JAs or following wound-associated stress (Shoji *et al*., 2008). These observations further support the model whereby JAZ degradation is necessary to instigate JA-associated induction of pyridine alkaloids (Shoji *et al*., 2008; Pauwels *et al*., 2010; Fernández-Calvo *et al*., 2011; Zhang *et al*., 2011; 2012).

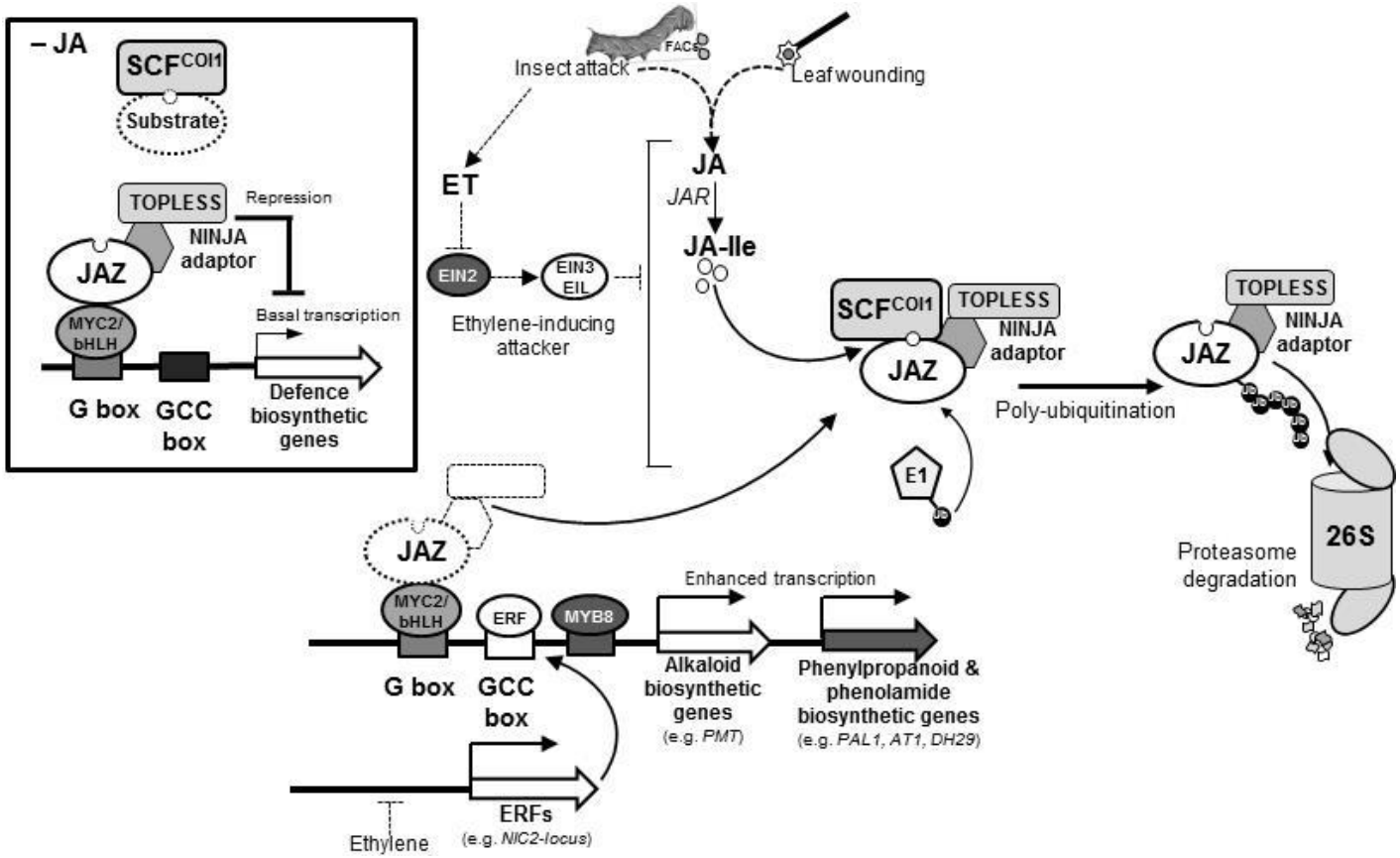

Figure 8. Schematic diagram depicting the jasmonate (JA) signalling cascade and JA-mediated regulation of alkaloid and phenylpropanoid biosynthesis in tobacco (adapted from Shoji and Hashimoto, 2011b; DeBoer *et al*., 2011b; Zhang *et al*., 2012). Solid lines indicate defined steps, while broken lines indicate undefined steps or steps including multiple reactions. In the absence of JA, MYC2 is bound by JAZ, which inhibits the ability of MYC2 to activate transcription of defence biosynthetic genes. JAZ repressors also recruit TOPLESS, a co-repressor, which suppresses MYC2-targeted genes, through an adaptor protein, NINJA. Leaf wounding and/or herbivore attack induces JA and JA-Ile bursts. The accumulation of JA-Ile mediates the COI1-JAZ interaction resulting in the ubiquitination of JAZ repressors by the SCF-COI1 complex and degraded by the 26S proteasome. The removal of JAZ, releases the downstream TF, MYC2, which is proposed to activate the transcription of defence-related genes, either directly by binding to the G-box motif or indirectly by activating secondary TFs such as NIC2-locus ERFs and MYB8 (in cooperation with MYC2). MYC2 up regulates the transcription of ERF TFs, enabling further stimulation of alkaloid-related metabolic and transport genes by the binding of the ERF proteins to the GCC-box. Ethylene signalling has also been shown to negatively regulate JA-mediated regulation of nicotine biosynthesis, through suppression of the NIC2-locus ERF and/or JA-signalling cascade by EIN and EIL TFs.

Co-regulators of JAZ proteins

The exact mechanism(s) by which JAZ proteins repress transcription of target TFs is/are still unclear. However, recently it was shown that JAZ proteins are co-regulated by a number of novel interactors, including inositol pentakisphosphate (InsP5), Groucho/Tup1-type co-repressor TOPLESS, novel interactor of JAZ (NINJA) and DELLA proteins. Using *Arabidopsis* mutants, InsP5 was shown to interact with amino acid residues of COI1 and act as a co-receptor of COI1-JAZ, regulating the expression of the wound- and JA-induced genes AtWRKY70 and AtAOS, altering root growth inhibition and performance of herbivores (Stevenson-Paulik *et al*., 2005; Sheard *et al*., 2010; Mosblech *et al*., 2011). JAZ proteins have also been found to recruit a co-repressor protein, TOPLESS, with and without NINJA assistance, to repress JA-associated genes targeted by MYC2 (Kazan, 2006; Szemenyei *et al*., 2008; Shyu *et al*., 2012). NINJA can act as an adaptor protein to the co-repressor TOPLESS *via* an N-terminal ERF-associated amphiphilic repression (EAR) motif interaction found in most AUX/IAA proteins, which has be shown to be required for the regulation of JA-responsive genes (Chung and Howe, 2009; Grunewald *et al*., 2009; Pauwels *et al*., 2010). RNAi-mediated silencing of NINJA demonstrated that NINJA, like JAZ, is a negative regulator of JA signalling, which is required for suppression of JA-responses (Pauwels *et al*. 2010). Lastly, DELLA proteins, a subfamily of the GRAS family of putative transcriptional regulators (Pysh *et al*., 1999; Richards *et al*., 2000), have been demonstrated to be essential in order to fully elicit JA-mediated responses by acting to directly repress the interaction of JAZ and MYC2, aswell as the antagonist relationship of JAZ/MYC2 with the TOPLESS-NINJA complex (Hou *et al*., 2010; Wild *et al*., 2012).

1.4.4. Transcription factors associated with JA defence responses

MYC

MYC TFs are characterised by the presence of a bHLH protein structural motif and are known to regulate many essential biological processes, including the biosynthesis of secondary metabolites after wounding and herbivore attack in *Nicotiana* (Rushton *et al*., 2008; Shoji and Hashimoto, 2011b; Todd *et al*., 2010; DeBoer *et al*., 2011b), *S. lycopersicum* (Boter *et al*., 2004), *A. thaliana* (Boter *et al*., 2004; Lorenzo *et al*., 2004; Chung *et al*., 2009; Fernández-Calvo *et al*., 2011) and *Catharanthus roseus* (Zhang *et al*., 2011; 2012). MYC TFs have been associated with the regulation of glucosinolate production by *Arabidopsis* plants in response to insect attack (Schweizer *et al*., 2013).

This was demonstrated in studies by Schweizer *et al*. (2013), which showed that MYC2 binds to the promoter of 14 glucosinolate biosynthesis related genes. Triple MYC mutants lacking MYC2, MYC3 and MYC4 (*myc234*) of *Arabidopsis* were shown to have severely diminished concentrations of glucosinolates (Schweizer *et al*., 2013). Myc234 mutants were more prone to attack by the generalist herbivore *S. littoralis*, while the specialist herbivore, *Pieris brassicae*, preferred feeding on wild type plants (Schweizer *et al*., 2013). MYC2 was shown to be the first TF identified to be transcriptionally supressed by JAZ proteins (Chini *et al*., 2007; Thines *et al*., 2007). Furthermore, MYC2-like genes have been associated with regulating a number of stress responses including: nicotine related NIC2-loci ERF transcription factors (TFs) and nicotine biosynthesis in *Nicotiana* (Todd *et al*., 2010; Shoji and Hashimoto, 2011a; 2011b; Woldemariam *et al*., 2013) and tolerance to drought stress in rice (Seo *et al*., 2011).

R2R3-MYB

The MYB family of proteins is one of the largest and most highly diverse gene families represented in all eukaryotes, with most functioning as TFs. TFs of the MYB family can be classified into four different classes depending on the number of tandem 50–53 amino acids repeats (1R, 2R, 3R and 4R; Stracke *et al*., 2001). The highly conserved DNA-binding domain consists of up to four repeats (R1, R2, R3 and R4) that each form a helix-turn-helix structure and can bind the major groove of DNA (Stracke *et al*., 2001; Dubos *et al*., 2010). Generally the MYB DNA-binding domain of plant proteins consists of two imperfect repeats (R2R3-MYB class; Rosinski and Atchley, 1998; Stracke *et al*., 2001).

Recently, the R2R3-MYB (2R-MYB) subfamily of TFs was shown to be associated with JA-mediated responses and identified as a target of JAZ proteins (Qi *et al*., 2011; Song *et al*., 2011). Numerous functional studies involving a diverse range of plant species have shown many R2R3-MYB TFs play roles in diverse biological processes including: (1) 1° and 2° metabolism: biosynthesis of glucosinolates, flavonoids, anthocyanins, tannins and phenylpropanoids, and cell wall construction (lignin biosynthesis, lignin, cellulose, and xylan deposition); (2) morphogenesis: determination of cell fate and identity such as trichome and root hair initiation, extension, and patterning, control of petal shape, and cell differentiation; (3) development: anther and pollen development, axillary meristem formation, side shoot formation, and lateral organ separation; and (4) stress: response to abiotic and biotic stresses such as hypersensitive cell death program against pathogen attack, systemic resistance against fungi and bacteria, regulation of stomata movements in

response to drought stress and disease resistance, cold tolerance, and phosphate starvation (Martin and Paz Ares, 1997; Schwinn *et al*., 2006; Baumann *et al*., 2007; Dubos *et al*., 2010; Schwinn *et al*., 2014).

MYC TFs associated with JA-responsive expression of several glucosinolate biosynthesis genes have been shown to coordinately interact with related MYB TFs to positively control glucosinolate production (Schweizer *et al*., 2013; Frerigmann *et al*., 2014). Recently, a specific member of the MYB family in tobacco, MYB8, was shown to function as a major defence regulator in tobacco plants, controlling phenylpropanoid production in local and systemic leaves following herbivore attack (Kaur *et al*., 2010; Onkokesung *et al*., 2012; Ullmann-Zeunert *et al*., 2013).

1.4.5. Salicylic acid – an important mediator of plant responses to pathogens

SA has been implicated in a number of growth and developmental processes such as seed germination, photosynthesis, thermogenesis and flowering as well as responses to biotic and abiotic stresses (Rivas-San Vicente and Plasencia, 2011). JA-SA cross-signalling networks are primarily activated to tailor pathogen-related plant defence responses. Plants have the capacity to synthesise SA *via* two dependent pathways indirectly from cinnamic acid; either by the decarboxylation followed by hydroxylation of cinnamic acid and benzoic acid, respectively, or *via* the hydroxylation of cinnamic acid to form o-coumaric acid followed by subsequent decarboxylation to form SA (Lee *et al*., 1995).

A key signal for induction of anti-microbial defences, SA has also been shown to be necessary for establishing systemic acquired resistance (SAR) and thus plays a crucial role in the elicitation of defence responses against biotrophic pathogens, including the production of pathogenesis-related (PR) proteins (Ryals *et al*., 1996). An antagonistic relationship exists between SA- and JA-mediated resistance pathways to pathogens and herbivores, respectively. The development of SAR to subsequent pathogen attack is achieved by pre-inoculation of plants with a necrosis-causing pathogen (Ryals *et al*., 1994). Silencing PAL, an enzyme synthesizing precursors to SA, was shown to reduce SAR and also to enhance herbivory resistance against insects in both *Arabidopsis* and tobacco (Pallas *et al*., 1996; Felton *et al*., 1999; Stotz *et al*., 2002). On the other hand, over-expression of PAL enhanced SAR and increased resistance against microbial pathogens (Kim and Hwang, 2014) but reduced the JA-mediated wound response after insect herbivory (Felten *et al*., 1999). Additionally, in *Arabidopsis*, plants pre-exposed to

Pieris rapae caterpillars were shown to be more sensitive to pathogen-induced SA and primed for enhanced SA-mediated resistance following herbivore-associated ET emissions (De Vos *et al*., 2006). Collectively, these results demonstrate phenylpropanoid-mediated cross-talk between pathogen and insect herbivore resistance mechanisms, which modifies acquired systemic resistance.

1.4.6. Ethylene – a modulator of JA responses

ET has been shown to play a central role in physiological and developmental processes including seed germination; root, shoot and flower development; fruit ripening; leaf senescence and stress responses (Wang *et al*., 2002; von Dahl and Baldwin, 2007; Yang *et al*., 2008). The use of inhibitors and chemical treatments has been used to study the role of ET in plant-herbivore interactions and defence responses by impairing the production, accumulation and perception of ET (reviewed in von Dahl and Baldwin, 2007). In these interactions, ET acts as a modulator of the responses triggered by JA (von Dahl and Baldwin, 2007).

Ethylene (ET) biosynthesis

The biosynthesis of ET occurs in the cytoplasm where *S*-adenosyl-methionine (SAM) is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by the action of 1-aminocyclopropane-1-carboxylic acid synthase (ACS). It is then oxidized by ACC oxidase (ACO) to produce ET (Wang *et al*., 2002).

1.4.7. Cross-signalling between JA, SA and ET in plant defence responses

Although JAs play the prominent role in the induction of defence responses against necrotrophic pathogens and chewing insects in plants, SA and ET can either positively or negatively modulate JA-induced defence responses (Doherty *et al*., 1988; Doares *et al*., 1995; O'Donnell *et al*., 1996; Reymond and Farmer, 1998; Stotz *et al*., 2002; Zarate *et al*., 2007; Diezel *et al*., 2009; Pieterse *et al*., 2009). Alternatively, JAs can also positively or negatively affect responses moderated by SA and ET.

In the hours following wounding in leaves of *N. attenuata*, plants produce a JA burst (Stork *et al*., 2009) and emit ET (Kahl *et al*., 2000), which is intensified by *M. sexta* herbivory, oral secretions and FACs elicitors (Halitschke *et al*., 2001). Synergistic and antagonistic cross-talks between JA and ET mediators are known to regulate the expression of a number of wound- and herbivore-responsive 2° metabolite biosynthetic

genes (Rojo *et al*., 1999; Mikkelsen *et al*., 2003; Lorenzo *et al*., 2004) and in addition tailors the majority of plant defence responses to herbivory (Bari and Jones, 2009). MYC2, EIN3 and EIL-1/2 transcription regulators interact bi-directionally to modulate this antagonism and control differential defence pathway activation (Shin *et al*., 2012; Song *et al*., 2014). Previous studies have shown that after *M. sexta* herbivory, *N. attenuata* plants accumulate 2° metabolites such as nicotine, phenolics and TPIs, the accumulation of which depends on JA production and is modulated by ET (von Dahl and Baldwin, 2007). Defence biosynthetic genes and metabolites, including nicotine, are upregulated by wound- and herbivore-induced JA, while endogenous ET emissions amplified by *M. sexta* herbivory (Kahl *et al*., 2000; Halitschke *et al*., 2001) negates the normal "wound" induction of nicotine (Baldwin, 1989; Baldwin *et al*., 1994a) and as such restricts or inhibits stimulated inductions in nicotine through its antagonism with JA (Baldwin *et al*., 1994a; von Dahl *et al*., 2007; Voelckel *et al*., 2001b). Increased nicotine production has also been correlated with enhanced activity of the regulatory enzyme, *PMT*, and the PIP protein A622, which are directly inhibited by ET production, despite induction of endogenous JA (Kahl *et al*., 2000; Schittko *et al*., 2000; Shoji *et al*., 2000a; Voelckel *et al*., 2001b; Winz and Baldwin, 2001; Shoji *et al*., 2002).

ET and JAs are also known to synergistically regulate plant development and defence responses (Robert-Seilaniantz *et al*., 2011). Recently, Zhu *et al*. (2011) demonstrated in *Arabidopsis* that JAZ directly interacts with EIN3/EIL1 TFs, which are positive regulators of JA/ET-responsive defence-related genes and JA-induced root hair development, but not fertility or pigment metabolism. This study also showed that AtJAZ directly interacts with EIN3/EIL1 TFs and inhibits their transcriptional activity (Zhu *et al*., 2011).

The relative production of defence signals (JAs, SA and ET) is largely herbivore-specific and thus both plants and insects attempt to adjust or prevent, respectively, the induction of defence responses (Cipollini *et al*., 2004; Heidel and Baldwin, 2004; Diezel *et al*., 2009). *N. attenuata* defence responses were attenuated after *S. exigua* elicitation when compared to those after *M. sexta* elicitation. In *N. attenuata,* when leaves are elicited with oral secretions from S. *exigua*, a generalised herbivore, it stimulates the accumulation of SA to levels higher than those induced by *M. sexta* a specialist herbivore (Diezel *et al*., 2009). In contrast, *M. sexta* induces the accumulation of higher levels of JA and ET and consequently lower SA, a consequence of its antagonism with ET (Diezel *et al*., 2009). In

N. sylvestris, JA- or wound-induction of nicotine accumulation is prevented by applying methyl salicylate (MeSA) to wounds (Baldwin *et al*., 1997). A classic example of JAs suppressing SA is the production of the phytotoxin coronatine by *P. psyringae*, which mimics JA-Ile and suppress SA-dependent plant defence responses, thus allowing bacteria to overcome plant defence responses. In tomato (*Lycopersicon esculentum*), it has been shown that the SA and acetyl-SA inhibits the accumulation of JA- and systemin-induced TPIs (Doares *et al*., 1995). In contrast, ET and JA are required for the wound induction of TPI genes in tomato; and this positive regulation of TPI genes is suppressed by exogenous application of SA (O'Donnell *et al*., 1996). In *Arabidopsis*, the non-expressor of PR1 (NPR1) is an important regulator of SA-JA cross-talk, though the final defence response depends on ET (Leon- Reyes *et al*., 2009). Infestation of plants by thrips, which do not induce ET, requires NPR1 for the SA-mediated suppression of JA responses, whereas *Alternaria brassicicola*, a necrotrophic pathogen, which induces JA and ET, bypasses the NPR1 requirement (Leon-Reyes *et al*., 2009). Additionally, in *Arabidopsis*, the TFs WRKY70 and WRKY62 regulate the SA-mediated suppression of JA responses (Li *et al*., 2004). Interestingly, in *N. attenuata*, NPR1 plays a different role by negatively regulating SA production, independently of ET emission, allowing unfettered elicitation of JA-mediated defences against herbivores (Rayapuram and Baldwin, 2007).

1.5. Stimulation of defence metabolism

The activation of defence metabolism can be metabolically costly and therefore the levels are regulated in response to various developmental, environmental, chemical and stress signals (Facchini, 2001). Inducibility of synthesis of such compounds is one strategy employed by plants to avoid wasting resources in the absence of herbivores (McKey, 1979; Karban *et al*., 1999; Wink, 1999; Zangerl, 2003), especially during conditions of limited nitrogen and other nutrient availability (Baldwin and Ohnmeiss, 1994; Baldwin *et al*., 1993). For example, after wounding, topping and leaf damage through herbivory, *N. tabacum* plants stimulate nicotine biosynthesis in roots, manifested by elevated transcript and enhanced activity of a suite of key enzymes required for nicotine biosynthesis, such as ODC, ADC, PMT, QPT, A622 and BBL within 24–48 hours of wounding, which is followed by elevated levels of nicotine concentration in both damaged and intact leaf tissues several days later (Baldwin *et al*., 1997; Green and Ryan, 1972; Mizusaki *et al*., 1971; 1973; Saunders and Bush, 1979; Sinclair *et al*., 2000; Shoji *et al*., 2002; Cane *et al*.,

2005; Kajikawa *et al*., 2009; 2011). A number of specialised inducible chemicals, including nicotine and phenylpropanoid-polyamine conjugates, have been shown to be protective in the wake of wound- and herbivore- associated stress in *Nicotiana* species (Voelckel *et al*., 2001a; 2001b; Steppuhn *et al*., 2004; Kaur *et al*., 2010). Maintaining balance in 1° and 2° resources is critical for optimum plant growth, reproduction and protection from herbivores or pathogens. This ongoing battle for the plant thus requires a constant trade-off between the demand for protection and the expense of defence metabolites (Whittaker and Feeny, 1971; Herms and Mattson, 1992; Baldwin, 1999; Agrawal, 2000; Strauss *et al*., 2002).

1.5.1. Apex removal

Apex removal or topping 1–2 weeks prior to harvest is a common practice in the cultivation of commercial tobacco, increasing the nicotine content of leaves, as well as the size and mass of leaves (Bush *et al*., 1999). This action causes a temporary loss of apical dominance, drastically reducing the principle source of auxin within the plant, which is known to suppress nicotine synthesis (Takahashi and Yamada, 1973). The stimulation of nicotine synthesis can also be mimicked in intact plants by application of an auxin inhibitor to the stem, below the main inflorescence (Shi *et al*., 2006). Similarly, application of auxin in a lanolin paste immediately to leaf wounds (Baldwin, 1989; Baldwin *et al*., 1997) or to the cut surface of the severed stem (Shi *et al*., 2006) largely eradicates the wound response of increased alkaloids that would normally occur. Complicated cross-signalling networks are likely at play; however, promotion of auxin signalling through its application may supersede the JA leaf-root wound response. Taken together, this suggests that auxin represses pyridine alkaloid biosynthesis and its removal is primarily associated with topping practices.

1.5.2. Leaf wounding or damage

JA is an important component of the leaf-root signalling system that facilitates the wound response, functioning as a signalling molecule mediating changes in 2° metabolism (Baldwin *et al*., 1997; Hashimoto and Yamada, 1994; Hibi *et al*., 1994; Kutchan, 1995; Shoji *et al*., 2000b; Paschold *et al*., 2007; 2008; Shoji *et al*., 2008; Pauwels *et al*., 2010; Stitz *et al*., 2011a). Leaf wounding or leaf damage, typically through leaf puncture methods, is mediated by a signal transduction cascade, regulated by JA signalling, which has been found to regulate production of a number of defence related 2° compounds (Baldwin *et al*., 1994a; 1994b; 1996; Baldwin, 1989; Shi *et al*., 2006). Rapid, transient

expression of JA is produced initially in wounded leaves *via* the octadecanoid pathway, and rapidly transmitted from damaged tissues to the root within two hours (Baldwin *et al*., 1994a; 1996; 1997; Baldwin, 1996; Ohnmeiss *et al*., 1997; Shi *et al*., 2006). Experiments that damaged the stem of *N. sylvestris* plants by steam-girdling indicated that the wound signal flows *via* the phloem (Baldwin, 1989) and treatment with JA inhibitors further highlighted this through inhibition of nicotine synthesis in the roots (Baldwin *et al*., 1996).

1.6. The circadian clock

1.6.1. Overview of the plant endogenous 'clock'

Understanding mechanisms by which organisms anticipate and adapt to changing environments is of fundamental importance in biology. Circadian systems, entrained by 24-hour rotations of the earth, enables organisms to coordinate and time their physiological, metabolic and developmental changes to predictable day/night cycles of light and temperature in their environment (Dunlap, 1996; Wenden *et al*., 2011; Pokhilko *et al*., 2012). The circadian clock requires external signals for entrainment and allows plants to adapt to different photoperiods and seasons (Harmer, 2009). The clock and its endogenous mechanisms, which drive day-night synchronised regimes, are known to influence plant processes including germination, hypocotyl elongation, cotyledon movement, leaf movement, photosynthesis, bolting floral transition, flower opening, senescence, volatile emission and stress resistance (Barak *et al*., 2000; Harmer *et al*., 2000; Kolosova *et al*., 2001; Yakir *et al*., 2007; Sanchez *et al*., 2011; Yon, 2014).

Plant circadian rhythmicity has been extensively studied using the genetic model plant *Arabidopsis thaliana.* A three-loop model has been postulated consisting of interlocking transcriptional and post-translational feedback loops to generate entrained rhythmic regimes (Nagel and Kay, 2012). The core network of the clock has been termed the central oscillation loop and is responsible for resetting or fine-tuning the clock (Figure 9). It is comprised of two partially redundant morning-expressed MYB TFs, CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) and an evening component, TIMING OF CAB EXPRESSION 1 (TOC1) (Wang and Tobin, 1998). During the day, CCA1 and LHY bind directly to the *TOC1* promoter forming the first 'loop' and repressing transcriptional expression of *TOC1*. The second loop is established through negative feedback between *PSEUDO-RESPONSE REGULATOR* (*PRR*) genes *PRR7/PRR9* and CCA1/LHY. Over the course of the day, levels of CCA1

and LHY drop, allowing *TOC1* expression to increase resulting in suppression of the transcription of *CCA1* and *LHY* at dusk by TOC1 protein (Gendron *et al*., 2012; Pokhilko *et al*., 2012), establishing the final negative feedback loop (Alabadí *et al*., 2001). In conjunction with these feedback loops, TOC1 and PRR5 proteins are post-translationally regulated at night by an F-box protein ZEITLUPE (ZTL). Just as TOC1 executes degradation of its targets (Harmer, 2009), ZTL directly binds to TOC1 and PRR5 proteins, resulting in their ubiquitination and proteasome-mediated degradation by the sequential action of E3 ubiquitin ligase SCF complex and the 26S proteasome (Mas *et al*., 2003; Kiba *et al*., 2007; Kim *et al*., 2007).

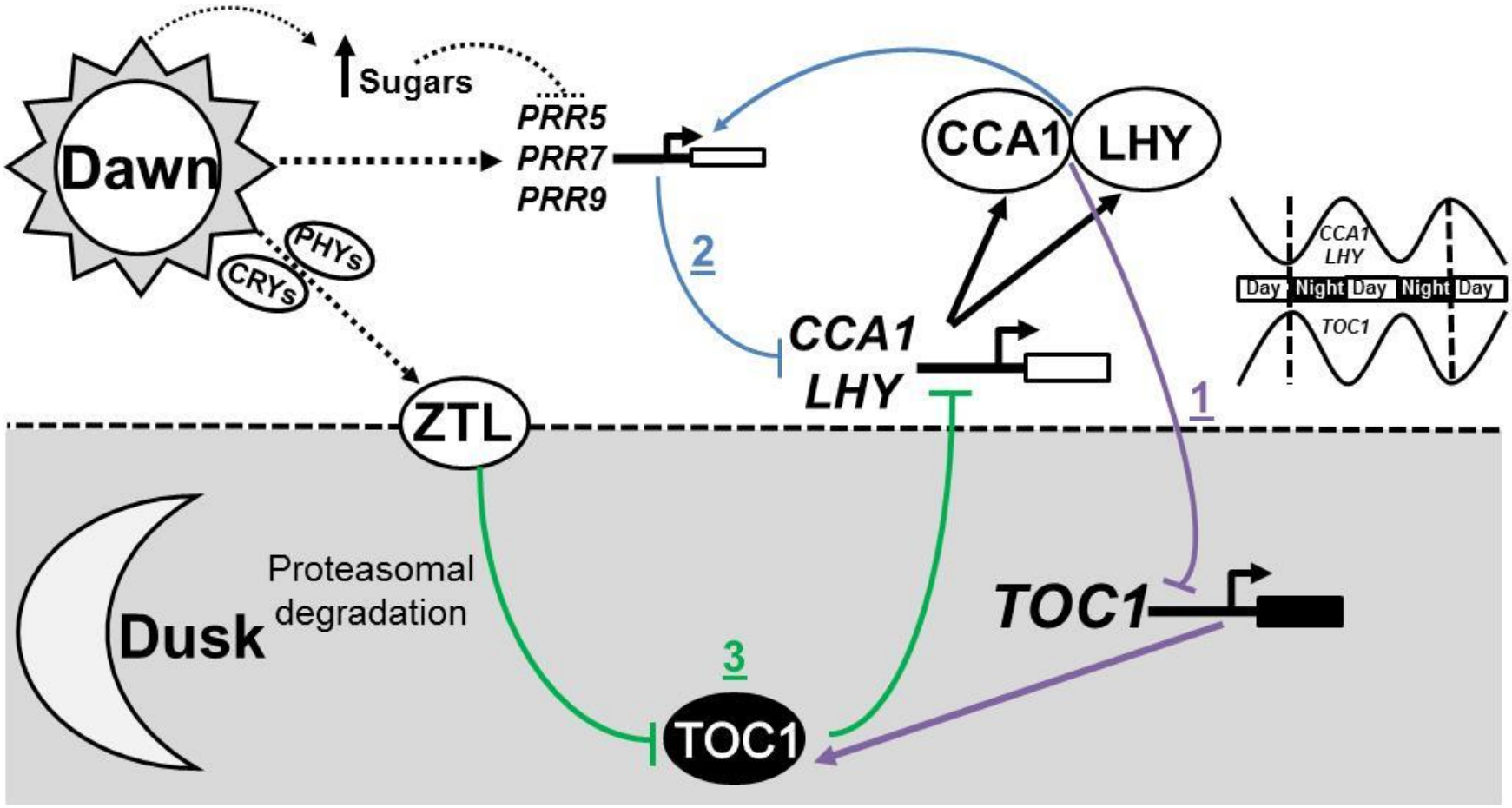

Figure 9. Schematic representation of the three-loop core oscillation clock model (adapted from McClung, 2006; Nagel and Kay, 2012). Solid lines indicate defined steps and interactions, while broken lines indicate undefined steps or steps including multiple reactions. Diagram shows core morning and evening circadian clock elements, CCA1/LHY and TOC1, and clock regulators PRR's and ZTL in *Nicotiana.* CCA1, circadian clock associated 1; CRYs, cryptocrome's; LHY, late elongated hypocotyl; PHYs, phytochrome's; PRR, pseudo-response regulators; TOC1, timing of CAB expression; ZTL, zeitlupe.

Circadian clock components in Nicotiana

In addition to the model plant *Arabidopsis*, circadian clock genes have been characterised from various plant species including rice, tobacco, soybean, maize and poplar (Murakami *et al*., 2007; Liu *et al*., 2009; Takata *et al*., 2009; Wang *et al*., 2011b; Kim *et al*., 2011), demonstrating a high degree of conservation of this system across the plant kingdom. While it should be noted that no gene or protein sharing significant similarities to the CCA1 oscillator described for *Arabidopsis* has been identified in the genome of *N. attenuata*, the general architecture of circadian networks is expected to be broadly conserved across plants (Song *et al*., 2010). A number of clock components have since been identified in *N. attenuata*, including *LHY*, *TOC1*, *ZTL* and *PRR5*, which share significant homology with *Arabidopsis* clock genes. In *N. attenuata*, clock entrainment is involved in controlling flower aperture, movement and benzyl acetone emission (Yon *et al*., 2012; Yon, 2014; Yon *et al*., 2015). Despite the clear importance of an entrained clock in influencing various biological processes in *Arabidopsis*, the connection to hormone signalling and defence networks in *N. attenuata* has not been investigated in great detail. However, it is reasonable to hypothesise that regulation of flux between 1° and 2° metabolism, orchestrated through the circadian clock, would help *N. attenuata* plants adapt to fluctuating environments and tailor an effective chemical defence response.

1.6.2. Circadian regulation of plant metabolism

Signalling networks coordinating metabolism and growth provide plants with the ability to cope with environmental variations and selective pressures (Thain *et al*., 2000; Kragler and Hulskamp, 2012). The core clock system controls a large fraction (~36%) of the *A. thaliana* transcriptome, affecting genes involved in many aspects of plant growth and development (Covington *et al*., 2008) as well as genes involved in plant defence (Goodspeed *et al*., 2013a). This was demonstrated in studies by Bläsing *et al*. (2005), which showed that daily changes in carbohydrate concentrations was associated with the regulation of a large portion of the *Arabidopsis* transcriptome, including a number of known circadian-regulated genes. In the past two decades a number of vital plant processes have been specifically linked to the clock, including photosynthesis and starch metabolism (Harmer *et al*., 2000; Dodd *et al*., 2005; Graf *et al*., 2010; Noordally *et al*., 2013). In the case of sugar metabolism, they have been shown to influence the circadian oscillator in *Arabidopsis* plants, particularly in response to their exongenous application, which shortened the circadian period when grown under constant light (Knight *et al*.,

2008; Haydon *et al*., 2013) and depending on the time of application advanced or delayed the circadian phase (Haydon *et al*., 2013). It is clear that the production and allocation of resources needed for growth and reproduction is strictly regulated by the circadian clock.

Role in adjusting hormone metabolism

In the past decade, oscillation of hormone metabolism, catabolism, perception and signalling related genes have also been shown, in part, to be controlled by core clock components and diurnal cues (Thain *et al*., 2004; Nováková *et al*., 2005; Salomé *et al*., 2006; Covington and Harmer, 2007; Yin *et al*., 2007; Covington *et al*., 2008; Michael *et al*., 2008; Mizuno and Yamashino, 2008; Robertson *et al*., 2009; Goodspeed *et al*., 2012). This has resulted in the elucidation of an array of hormone-signalling systems that function as key output pathways that are gated by the clock, including JA (Covington *et al*., 2008; Goodspeed *et al*., 2012; Shin *et al*., 2012), SA (Bhardwaj *et al*., 2011; Zhou *et al*., 2015), ABA (Nováková *et al*., 2005; Legnaioli *et al*., 2009; Seung *et al*., 2012; Portolés and Zhang, 2014), ET (Thain *et al*., 2004), AUX (Covington and Harmer, 2007) and gibberelins (Arana *et al*., 2011). Together, hormone-regulated developmental processes and stress signalling are clear targets of clock outputs, revealing the interaction of hormone-clock cross-talk systems. The high degree of interconnection of hormone signalling systems with the circadian clock is further exemplified by evidence of hormones, such as AUX, ABA and cytokinin acting as clock input factors. These in turn modulate the core oscillator, altering circadian amplitude, period, and phase, respectively (Hanano *et al*., 2006). Although evidence of cross-regulation between the circadian clock and hormone pathways has started to emerge, the mechanistic basis for such relationships awaits further elucidation.

Recently, a number of core circadian oscillators, have been associated with a number of hormone signalling networks. Basal JA levels in leaves follow sharp diurnal rhythms, peaking in the middle of the day, which follows the peak expression of many JA-regulated genes at dusk (Covington *et al*., 2008; Goodspeed *et al*., 2012). TIME FOR COFFEE (TIC) has been shown to be a necessary regulator of the circadian-clock, maintaining circadian period and accumulation of JA (Hall *et al*., 2003; Ding *et al*., 2007). TIC activity prevents accumulation of MYC2, a TF that regulates JA-responsive genes (Shin *et al*., 2012). Both CCA1 and TOC1 have been shown to regulate ET production through clock control of ACS, an intermediate step in ET biosynthesis (Thain *et al*., 2004). Interestingly ET displayed no effect on rhythmicity of the clock or its outputs

however and so questions around the role of rhythmic ET in hormone-clock systems remains (Thain *et al*., 2004). Legnaioli *et al*. (2009) showed that the dusk-peaking element, TOC1, controls ABA signalling and its responses by repressing transcription of the proposed ABA receptor ABAR/CHLH/GUN5 through direct binding to its promoter. They furthered indicated the daytime specific regulation of *TOC1* through exogenous application of ABA. Despite the increasing knowledge of the molecular networks underpinning the circadian oscillator, our understanding of TOC1's biochemical and molecular role in controlling clock outputs and regulating other areas of plant metabolism in addition to ABA and ET signalling, is still largely unknown.

Role in adjusting primary and defence metabolism

Several defence metabolites including, phenylpropanoid polyamine conjugates, diterpene glycosides and nicotine, and the biosynthetic genes that regulate them, were recently shown to follow daily oscillation in *N. attenuata* (Kim *et al*., 2011; Gulati *et al*., 2014). The mechanism(s) by which plants experiencing predation by grazing insect larvae redirect metabolites towards the production of a spectrum of chemical defences is largely unknown. However, a number of core circadian oscillators have recently been implicated in defence networks through their relationship with hormone metabolism. Goodspeed *et al*. (2012) showed that the accumulation of wound-responsive genes, JAs and defences in *A. thaliana* was timed with the synchronised feeding of an atypical generalist herbivore, *Tricoplusia ni*. Additionally, CCA1 has been shown to control fluctuation in diurnal patterns of plant defence responses to elicitation by the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Bhardwaj *et al*., 2011). These experiments demonstrated that a higher plant defence response to pathogen attack was achieved when plants were exposed to attack in the morning, as opposed to inductions conducted in the evening, a response that depended on the morning-phased TF, CCA1. Wang *et al*. (2011) also highlighted a number of key genes involved in pathogen resistance that show rhythmic expression patterns and contain clock-related elements within their promoters. Interestingly, it was uncovered through integrated network models that *CCA1* may monitor glutamic acid levels and affect N-utilisation by regulating transcription of key genes associated with N-assimilation (Roenneberg, 1994; Green *et al*., 2002; Dodd *et al*., 2005; Gutiérrez *et al*., 2008).

Recent research has highlighted in *Arabidopsis* important roles for circadian entrainment of plant 1° metabolism (Thain *et al*., 2004; Dodd *et al*., 2005; Covington *et al*., 2008;

Arana *et al*., 2011; Seung *et al*., 2012), as well as evidence of the plant circadian clock affecting 2° metabolism and herbivore feeding (Goodspeed *et al*., 2012; 2013; Zhou *et al*., 2015). While there is extensive transcriptional regulation of 2° metabolism in *Nicotiana* (reviewed in Meldau *et al*., 2012; Dewey and Xie, 2013; Shoji and Hashimoto, 2013), we currently know little about the circadian-driven mechanisms behind rhythmic fluxes of these specialised metabolites in *N. attenuata* (Kim *et al*., 2011). JA-ET and JA-SA cross-signalling networks are known to tailor herbivore- and pathogen-related plant defence responses. Hence, their synergistic and antagonistic interactions may also be dictated by the circadian clock.

1.7. Thesis aims and objectives

1.7.1. Aims

The overarching aim of the research presented in this thesis was to investigate the regulation of defence chemistry in two species in the *Nicotiana* genus, relating to the control of nitrogen-based defence metabolites in response to abiotic and biotic stress factors. As has been noted above, many plants have adapted defence strategies in response to various stress stimuli to produce a plethora of biologically active compounds with toxic, anti-digestive and therapeutic potential. A greater understanding of the mechanisms by which plant metabolic systems are regulated, using a combination of molecular, genetic and biochemical approaches, may have long term value in the quest to advance health and global food security simultaneously with ecosystem sustainability.

The thesis itself is divided into five sections, grouped in two broad and inter-related parts that either have formed, or will form, the basis of published journal articles. The thesis investigates pertinent aspects of genes within the plant circadian clock (TOC1) and urea cycle (ODC) and their subsequent roles in regulating or facilitating synthesis of polyamines (1° metabolism), pyridine alkaloids and phenolamides (2° metabolism) that act as defensive agents against herbivores in native environments.

1.7.2. Objectives and structure of thesis

Part A – Circadian clock

Defence systems in the context of circadian clock components and hormone signalling pathways are discussed. Molecular and biochemical tools were used to elucidate the mechanisms that underlie TIMING OF CAB EXPRESSION 1 (TOC1*)* control of nicotine

and phenolpropanoid defence patterns in *Nicotiana attenuata*, a tobacco species native to the Great Basin Desert, Utah.

A.1. Investigation into the role of *TOC1* in regulating hormone signalling and nicotine biosynthesis in *N. attenuata*

Transcriptional and metabolic changes following wounding, MeJA application and herbivore-associated stress were explored in *toc1*-RNAi plants.

A.2. Investigation into the role of *TOC1* in regulating phenylpropanoid conjugates in *N. attenuata*

The herbivore-response was explored in *TOC1* suppressed plants; particularly the role this clock component plays in regulating early stress signalling, nitrogen assimilation, JA-regulated components, herbivory-induced phenolamide accumulation dynamics and insect resistance.

Part B – Ornithine decarboxylase

Plants in the *Nicotiana* genus, such as *N. tabacum* and *N. glauca*, provide an interesting eukaryotic system for investigating the role of ODC and polyamines because both a direct route from ornithine and an additional indirect route to Put from arginine is functional.

B.1. Investigation into the role of *ODC* in regulating pyridine alkaloid biosynthesis in *N. tabacum*

Transcriptional and metabolic responses associated particularly with nicotine biosynthesis after wound-associated stress was under investigation.

B.2. Investigation into the role of *ODC* in anabasine biosynthesis in *N. glauca*

The role of *ODC* in regulating anabasine synthesis after wound-stress was examined.

B.3. Investigation into the wider effects of manipulating *ODC* on putrescine-associated metabolism in *N. tabacum*

Exploring selected *odc*-RNAi down-regulated homozygous lines of *N. tabacum* in more detail. The question of conversion of arginine and ornithine substrates and whether transcriptional activity of *ADC* may be employed as a contingency to supply Put under particular conditions were of particular interest here.

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Monash University

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
- Planned, performed, analysed and interpreted all experiments - Main author of the manuscript	**80%**

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Prof Ian Baldwin	- Contribution to planning of experiments - Provided supervisory support	N/A
Dr Emmanuel Gaquerel *	- Contribution to planning of experiments - Provided supervisory support and was involved with drafting of the manuscript	N/A

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** **26/10/2015**
Main Supervisor's Signature	[redacted]	**Date** **26/10/2015**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Chapter 2: Timing of CAB expression 1 (TOC1) mediates *Nicotiana attenuata* nitrogen-investment into nicotine biosynthesis during insect herbivory through modulations of hormonal signalling interactions

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Abstract

Biological processes controlled by hormones largely overlap with those regulated by the clock, which suggests an interaction between the circadian clock and jasmonate (JA), ethylene (ET) and salicylic acid (SA) in defence signal networks is possible. Silencing *TIMING OF CAB EXPRESSION 1* (*TOC1*) in *Nicotiana attenuata* led to higher basal and wound-induced levels of nicotine compared to empty vector (EV) plants. This difference was further exacerbated by the application of *Manduca sexta* oral secretions to leaf wounds to simulate herbivory. We found that JA and ET emissions were reduced in silenced *TOC1* (ir-*toc1*) plants following herbivory simulations, whilst SA levels were markedly higher. Moreover, transcript levels of *ACO* (ET biosynthesis) were diminished in leaves of ir-*toc1* plants following simulated herbivory. This was accompanied by higher root transcript levels of the key nicotine biosynthetic gene, *PMT*, in down-regulated *TOC1* plants. Pre-treatment of plants with an inhibitor of ET perception (1-MCP) prior to herbivory simulations was sufficient to alleviate the differences in herbivory induced nicotine in leaves of ir-*toc1* plants compared to EV plants. Allocation of labelled nitrogen into shoots and root nicotine pools were also substantially elevated in silenced *TOC1* transgenics. Together, results of this study sheds light on clock mechanisms utilised by plants to modulate defence pathways, demonstrating that *TOC1* has important roles in regulating herbivory-induced levels of ET, JA and SA (defence-related phytohormones) and nicotine.

Keywords:

Circadian clock, defence, ethylene, herbivory, hormone, Timing of CAB expression 1, *Nicotiana attenuata*, nicotine, secondary metabolism

2.1. Introduction

How organisms anticipate and adapt to their ever-changing environment remains a fundamental and challenging question in biology. Circadian systems, entrained by 24-hour rotations of the earth, enables organisms to coordinate and time their physiological, metabolic and developmental changes to predictable day-night cycles of light and temperature in their environment (Dunlap, 1996; Wenden *et al*., 2011; Pokhilko *et al*., 2012). The genetic model plant, *Arabidopsis thaliana,* has been extensively studied in recent decades with respect to the molecular genetics of the core clock network, which is responsible for resetting or fine-tuning the clock system and generating entrained rhythmic regimes (reviewed by de Montaigu *et al.*, 2010). The clock based on interlocking feedback loops underpinning a time-keeping system, enables plants to respond to anticipated daily stresses (Wang and Tobin, 1998; Dunlap, 1996; Alabadí *et al*., 2001; Mas *et al*., 2003; Kiba *et al*., 2007; Gendron *et al*., 2012; Pokhilko *et al*., 2012).

A three-loop model has been postulated comprising two partially redundant morning-expressed MYB transcription factors (TF's), CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) and an evening component, TIMING OF CAB EXPRESSION 1 (TOC1) (Figure 1A; Wang and Tobin, 1998; Nagel and Kay, 2012). During the day, CCA1 and LHY bind directly to the *TOC1* promoter repressing transcriptional expression of *TOC1*. Over the course of the day, levels of CCA1 and LHY are negatively regulated by PSEUDO-RESPONSE REGULATOR (PRR) genes PRR7/PRR9. This allows *TOC1* expression to increase, resulting in suppression of the transcription of *CCA1* and *LHY* at dusk by TOC1 protein (Alabadí *et al*., 2001; Gendron *et al*., 2012; Pokhilko *et al*., 2012). In conjunction with these feedback loops, TOC1 and PRR5 proteins are post-translationally regulated at night by ZEITLUPE (ZTL), an F-box protein that directly binds to these proteins, resulting in their proteasome-mediated degradation in the E3 ubiquitin ligase SCF complex (Mas *et al*., 2003; Kiba *et al*., 2007; Kim *et al*., 2007).

The importance of circadian networks in promoting plant fitness is becoming increasingly clear, with a substantial portion (~36%) of the *A. thaliana* transcriptome controlled by this system (Covington *et al*., 2008; Michael and McClung, 2003). The core genes of this circuit affect genes controlling many plant processes, such as germination, hypocotyl elongation, photosynthesis, floral transition, flower opening, volatile emission and more recently with recognised roles in stress resistance and plant defence (Barak *et*

al., 2000; Harmer *et al.*, 2000; Kolosova *et al*., 2001; Yakir *et al*., 2007; Mizuno and Yamashino, 2008; Sanchez *et al*., 2011; Goodspeed *et al.*, 2012; Yon *et al*., 2015). It is clear that the daily production and allocation of resources needed for metabolic and physiological processes is strictly regulated by the circadian clock (Rensin and Ruoff, 2002; Müller *et al*., 2014; Grundy *et al*., 2015; Haydon *et al*., 2015). In the past decade, oscillation of hormone metabolism, catabolism, perception and signalling related genes have also been shown, in part, to be controlled by core clock components and diurnal cues (Covington *et al*., 2008; Hanano *et al*., 2006; Michael *et al*., 2008; Mizuno and Yamashino, 2008; Robertson *et al*., 2009). This has resulted in the elucidation of an array of hormone-signalling systems that function as key output pathways that are gated by the clock, including jasmonic acid (JA; Covington *et al*., 2008; Goodspeed *et al*., 2012; Shin *et al*., 2012); salicylic acid (SA; Bhardwaj *et al*., 2011; Liu *et al*., 2011; Zhou *et al*., 2015); ethylene (ET; Thain *et al*., 2004); abscisic acid (ABA; Nováková *et al*., 2005; Legnaioli *et al.*, 2009; Seung *et al*., 2012; Portolés and Zhang, 2014); auxin (Covington and Harmer, 2007) and gibberelins (Arana *et al*., 2011). Hormone-clock signalling networks are highly interconnected with evidence of hormones, such as ABA, auxin and cytokinin, acting as clock input factors to modulate circadian amplitude, period, and phase (Hanano *et al*., 2006). Although studies of cross-regulation between the circadian clock and hormone pathways have started to emerge, the mechanistic basis for such relationships awaits further elucidation.

The clock clearly targets hormone signalling and developmental processes; however, to date there is only limited evidence for the role of hormones in delivering information from the clock to output stress signalling processes (Legnaioli *et al*., 2009; Bhardwaj *et al*., 2011; Goodspeed *et al*., 2012; Zhou *et al*., 2015). Basal JA levels in leaves follow sharp diurnal rhythms, peaking in the middle of the day following the peak expression of many JA-regulated genes at dusk (Covington *et al*., 2008; Goodspeed *et al*., 2012). *TIME FOR COFFEE* (*TIC*) has been shown to be a circadian-clock regulator necessary for maintaining circadian period and amplitude of JA (Hall *et al*., 2003; Ding *et al*., 2007), by repressing the accumulation of MYC2, an important TF that regulates JA-responsive genes (Shin *et al*., 2012). Goodspeed *et al*. (2012) showed that the accumulation of wound-responsive genes, JAs and defences in *A. thaliana* were timed with the synchronised feeding of a non-native herbivore, *Tricoplusia ni*. Rhythmic emission of ET is controlled by CCA1 and TOC1 genes, specifically by regulating the

expression of *1-AMINO-CYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE* (*ACS*; Thain *et al*., 2004). ET displayed no effect on rhythmicity of the clock or its outputs and so questions around the role of rhythmic ET in hormone-clock systems remain, particularly following herbivore-associated stress. Additionally, CCA1 was shown to control fluctuation in diurnal patterns of plant defence responses to elicitation by the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Bhardwaj *et al*., 2011). Interestingly, a higher plant defence response to pathogen attack was achieved when plants were induced in the morning, as opposed to inductions in the evening, a response that depended on the morning-phased TF, CCA1. Wang *et al*. (2011) also highlighted a number of key genes involved in pathogen resistance that show rhythmic expression patterns and contain clock-related elements within their promoters. It was uncovered through integrated network models that *CCA1* may play a role in nutrient availability, regulating key genes associated with N-assimilation through regulation by glutamic acid (Gutiérrez *et al*., 2008). Legnaioli *et al*. (2009) showed that the dusk-peaking element, TOC1, controls ABA signalling and its responses by repressing transcription of the proposed ABA receptor (ABAR) through direct binding to its promoter. They further indicated the daytime specific regulation of *TOC1* gene activity through exogenous application of ABA.

JA and its oxylipin derivatives regulate many aspects of defence chemistry in plants (Kessler and Baldwin, 2002; Halitschke and Baldwin, 2003; Paschold *et al*., 2007). JA-ET and JA-SA cross-signalling networks are known to tailor herbivore- and pathogen-related plant defence responses, respectively. Hence, their synergistic and antagonistic interactions may also be dictated by the circadian clock. In recent decades numerous studies have explored the cost of defence in *Nicotiana attenuata*, revealing that plants maximise resistance by coordinating their defences against biotic stress to limit fitness trade-offs imposed by activation of such costly defence networks (Baldwin and Preston, 1999; Voelckel *et al*., 2001; Kessler and Baldwin, 2004; Zavala *et al*., 2004; Ullmann-Zeunert *et al*., 2013). Recent observations found a number of defence metabolites including, phenylpropanoid polyamine conjugates, diterpene glycosides and nicotine, and the biosynthetic genes that regulate them, follow daily oscillation patterns in *N. attenuata* (Kim *et al*., 2011; Gulati *et al*., 2014). A number of clock components, *LHY*, *TOC1*, *ZTL* and *PRR5*, which share significant homology with *Arabidopsis* clock genes, have since

been identified in *N. attenuata*, revealing the clock entrainment of flower aperture, movement and benzyl acetone emission (Yon *et al*., 2012; 2015).

N. attenuata has been meticulously studied with regard to how it responds to its major insect herbivore, the tobacco hornworm (*Manduca sexta*), which can tolerate comparatively high levels of nicotine (Halitschke *et al*., 2001; 2003; Schittko *et al*., 2001; Winz and Baldwin, 2001; Wink and Theile, 2002; Steppuhn and Baldwin, 2007; Gaquerel *et al*., 2009). Nicotine, a potent alkaloid toxin, forms an essential component of *N. attenuata's* direct defence strategy by acting as an agonist on the nervous system of herbivores, inhibiting ingestion and increasing the rate of insect mortality (Wink, 1988; Wink, 1998a; 1998b; Baldwin and Preston, 1999; Kutchan, 1995; Ohnmeiss and Baldwin, 2000; Shoji *et al.*, 2002; Hashimoto and Yamada, 2003; Steppuhn *et al.*, 2004; Katoh *et al.*, 2005). Within minutes of the initiation of insect feeding, a cascade of signalling events is activated upon recognition and perception of wound- and/or herbivore- specific elicitors (Halitschke *et al*., 2003; Mithöfer *et al*., 2005; Schmelz *et al*., 2009). This response has been accredited to fatty acid-amino acid conjugates (FACs) present in the oral secretions (OS) from *M. sexta*, which rapidly modulate downstream transcriptional changes and induce the differential production of signalling molecules and large-scale transcriptomic and proteomic reconfigurations (Kahl *et al*., 2000; Halitschke and Baldwin, 2003; Halitschke *et al*., 2003; Liu and Zhang, 2004; Wu *et al*., 2007). In the hours following wound elicitation in leaves of *N. attenuata*, plants rapidly produce a JA burst (Stork *et al*. 2009) and emit ET (Kahl *et al*., 2000), which is intensified by FACs elicitors (Halitschke *et al*., 2001). With their synergistic and antagonistic actions, JA-ET cross-talk regulates the majority of defence chemicals to leaf wounding and herbivory (Mikkelsen *et al*., 2003; Bari and Jones, 2009). Specifically, nicotine biosynthesis is upregulated by OS-induced JA, whilst ET inhibits the synthesis of nicotine (Baldwin *et al*., 1994; 1996; Voelckel *et al*., 2001; Halitschke and Baldwin, 2003; von Dahl *et al*., 2007). These studies suggest a possible role for circadian regulation of nicotine accumulation, particularly in herbivore-challenged plants, where JA-ET cross-talk is well known to shape plant defence responses. Coordinating the flux between primary and secondary metabolism through clock-hormone networks would help plants adapt to fluctuating environments and may provide a mechanism to direct resources and execute highly specific stress responses.

In the current study, we undertook a detailed analysis of the effects of RNAi-mediated down-regulation of *TOC1* upon the production of defence-related hormones and nicotine in *N. attenuata*. Here we provide insight into how the plant clock integrates various synergistic and antagonistic phytohormone signals, connecting *TOC1* with nitrogen incorporation and nicotine accumulation in *N. attenuata*. Through the use of an ethylene inhibitor, 1-methylcyclopropene (1-MCP), to block ethylene perception, we demonstrate the importance of ET-clock cross-talk in nicotine accumulation, particularly following herbivore-associated stress.

2.2. Experimental Procedures

Plant material, growth and harvesting conditions

Transgenic plants of *N. attenuata* homozygous for an introduced invert repeat (ir) construct (Gase *et al*., 2011) were utilised that specifically silenced different clock elements (LHY, PRR5, TOC1 and ZTL; Yon *et al*., 2012; 2015). Homozygous T_3 generation plants were generated from lines ir-*lhy*-406, ir-*toc1*-205, ir-*prr5*-458 and ir-*ztl*-314, which displayed >90% silencing efficiency and were selected following characterisation of several independent clock-silenced transgenic lines in Yon *et al*. (2012; 2015). Plants containing an empty vector (EV) generated from line EV-266 that was comparable in growth and morphology to other independent EV transgenics, as well as those of the non-transgenic parental line Torr. Ex. Wats (30^{th} inbred generation), was used as a transformation control. Seeds were sterilised and germinated on agar plates containing Gamborg's B5 medium (Duchefa; http://www.duchefa.com) as described previously by Krügel *et al*. (2002) and maintained in a 26 °C/16-h, 155 µmol m^{-2} s^{-1} light: 24 °C/8-h dark cycle (Percival) for 10 days. Young seedlings were planted individually in TEKU plastic pots (Pöppelmann) with Klasmann plug soil (Klasmann-Deilmann) and ten days later, early rosette plants were transferred to 1-L individual pots with sand to facilitate sampling of root tissue. Plants were grown in a glasshouse at 26 °C-28 °C for 16 hour under supplemental light from Master Sun-T PIA Agro 400- or 600-W high pressure sodium lamps (Phillips) and an 8 hour dark cycle (22 °C-24 °C). *TOC1* silenced plants were previously observed to be delayed developmentally compared to EV plants (Yon *et al*., 2012), remaining on average 7–10 days longer in the rosette stage before bolting, which resulted in delayed flowering. Unless otherwise stated, experiments utilised silenced *TOC1* plants that were developmentally synchronised with EV plants by germinating ir-*toc1* seeds ~10 days prior (Yon *et al*., 2012).

Insect rearing and collection of oral secretions

Tobacco hornworm (*Manduca sexta*) eggs, obtained from Carolina Biological Supply (Burlington, USA) and bred in an in-house colony, were cultivated in climate chambers until hatching. Oral secretions and regurgitants (OS) were collected on ice from third to fifth instar larvae reared on wild type *N. attenuata* plants as described in Roda *et al*. (2004) and stored under Argon at -20 °C until use.

Plant treatments

After ~four weeks growth, early elongating plants were randomly divided and assigned into treatment groups of five independent plants per genotype (n=5), unless otherwise stated. Transgenic *N. attenuata* plants remained either untreated as a control (C) or where stated were mechanically wounded at 13:00 in one of two ways. (1) – Designated 'W" for leaf wounded; the lamina of three experimental leaves per plant were mechanically damaged using a serrated pattern wheel to puncture three rows of holes on both sides of the midvein and 20 µL of water then applied directly to the wound and gently dispersed on the leaf lamina. (2) – Designated 'W+OS' for wound plus oral secretions; 20µL of eliciting solution of 1:5 (v/v) water-diluted OS from specialist *M. sexta* were applied immediately to fresh leaf wounds and gently dispersed across the leaf surface with a gloved finger to simulate insect feeding and evaluate non-destructively the response of transformed plants. Equivalent phyllotactic positions from untreated and treated local leaves, along with their systemic leaves and complete root systems were collected from plants at the same time point and immediately flash frozen in liquid nitrogen. Roots were briefly washed in water and blotted dry with paper towel to remove sand prior to freezing.

1-Methylcyclopropene (1-MCP) treatment

Endogenous ethylene (ET) production is induced by OS treatment, which in turn negates the "wound" induction of nicotine and as such causes a reduction in nicotine. To inhibit ET perception and uncouple the negative effects on induced nicotine production by OS-induced ET, plants were exposed to 1-MCP, a gas which binds irreversibly to ET receptors and inhibits ET-induced responses (Sisler *et al*. 1996). Plants were pre-exposed overnight (22:00 till 06:00) to 1-MCP before W+OS treatments as described above. To selectively expose plants to this gaseous ET-receptor antagonist, 10 plants along with the activated solution of 1-MCP were placed within growth chambers fitted with Plexiglas lids and a 14.5 cm fan for air circulation. Following Kahl *et al*. (2000) 500 mg of Ethylblock (0.43% 1-MCP [van der Sprong, Postbus, Netherlands]) was dissolved in a

vial containing 50 mL of alkaline solution (0.75% KOH + NaOH in a 1:1 ratio) to release the active substance, 1-MCP and immediately placed within the chamber. To control for any potential stress responses associated with confinement in chambers and exposure to 1-MCP, control plants were kept in identical chambers supplied with an equivalent amount of alkaline solution.

^{15}N pulse labelling

For ^{15}N-labeling, three days prior to W+OS elicitation as described above plants were pulse labelled with 5.1 mg of labelled nitrogen which was administered as $K^{15}NO_3$. Incorporation of ^{15}N into nitrogen-containing metabolites was determined as described by Ullmann-Zeunert *et al*. (2012).

Analysis of leaf and root metabolites

One hundred milligrams of liquid nitrogen-ground leaf or root powder was pre-weighed and aliquoted into 1.5 mL Eppendorf tubes containing a steel ball to aid extraction for subsequent metabolite analyses. Nicotine was extracted from local leaf, systemic leaf and root samples using an optimised 40% methanol extraction for *N. attenuata* as described by Gaquerel *et al*. (2010). Approximately 1 mL of extraction buffer per 100 mg of aliquoted tissue [50 mM acetate buffer, pH 4.8, containing 40% methanol] was added and samples were homogenised using a Genogrinder 2000 (SPEX CertiPrep) for 1 min at 250 strokes min^{-1}. After centrifugation (13.2 rpm, 20 min, 4 °C), the supernatant was transferred to a clean 1.5 mL Eppendorf tube and centrifuge again and 100 µL of supernatant was transferred to a HPLC vial. Two microliters of extract was typically separated using a Dionex rapid separation liquid chromatography (RSLC) system equipped with an Acclaim 150 mm x 2 mm i.d., 2.2 µm, 120-A, RP-18 column and 2 mm x 4 mm i.d guard column of the same material (Dionex, Sunnyvale, USA), applying either a short (10 min) or long (40 min) separation binary gradient according to Onkokesung *et al*. (2012) or Heiling *et al*. (2013), respectively. The flow rate was 200 µL min^{-1} and eluted compounds were detected by a MicroTOF quadrupole time-of-flight mass spectrometer (qTOF-MS; Bruker Daltonics) operated in electrospray positive mode. Typical instrument settings were as follows: capillary voltage, 4500 V; capillary exit, 130 V; dry gas temperature, 200 °C; dry gas flow, 8 L min^{-1}. Ions were detected from *m/z* 50 to 1400 at a repetition rate of 1 Hz. Mass calibration was performed using sodium formate clusters (10 mM solution of NaOH in 50/50% [v/v] isopropanol:water containing 0.2% formic acid). Raw data files were converted to the netCDF format using the export

function of the Data Analysis v4.0 software and processed using the Quant Analysis software package (Bruker Daltonik, Bremen, Germany). Concentration of nicotine was quantified relative to known concentrations of standards and is graphically presented per mg fresh weight of tissue which was extracted.

Phytohormone and ethylene analysis

Phytohormones (JA, JA-Ile, SA and ABA) were extracted from locally treated leaves and their control counterparts as described by Wu *et al*. (2007). Briefly, 1 mL of ethyl acetate [containing 200 ng mL^{-1} D_2-JA and 40 ng mL^{-1} of D_6-ABA; D_4-SA and JA-$^{13}C_6$-Ile internal standards] per 100 mg of aliquoted tissue powder was added and samples were homogenised using a Genogrinder (SPEX CertiPrep) at a frequency of 1200 strokes min^{-1} for 1 min. After centrifugation (13.2 rpm, 20 min, 4 °C), the supernatant was transferred to a clean 1.5 mL Eppendorf tube and pellets were re-extracted in 0.5 mL ethyl acetate and supernatants combined. The supernatant was evaporated using a vacuum concentrator (Eppendorf) and resuspended in 0.5 mL 70% methanol. Re-suspended samples were centrifuged again and 10 µL of supernatant analysed on Varian 1200L Triple-Quadrupole-LC-MS tandem mass spectrometer system (Varian; http://www.varianinc.com) using a ProntoSIL column (C_{18}; 5 µm; 50 x 2 mm) with a guard column (C_{18}; 4 x 2 mm, Phenomenex). ET emissions were measured following 5 hours of incubation in sealed glass vials with a photoacoustic laser spectrometer (INVIVO; http://www.invivo-gmbh.de) as described in von Dahl *et al*. (2007). Leaves were excised immediately after treatment and transferred to sealed 1 mL vials where the headspace was allowed to accumulate over a 5 hour time period. During measurements, vials were flushed with a constant flow of purified air at 40 mL min^{-1}, which had previously passed through a liquid N_2 cooling trap to remove CO_2 and water. The ET concentration was quantified by comparing ET peaks with peak areas generated by a standard ET gas as described previously by von Dahl *et al*. (2007).

Quantitative Real Time – PCR

Total RNA was isolated from snap frozen leaf and root tissues using the Trizol method (Invitrogen).DNA-free RNA samples were reverse transcribed using oligo$(dT)_{18}$ primer and cDNA was synthesised from 2 µg of RNA with Superscript II Reverse Transcriptase (Invitrogen) and a poly-T primer following the manufacturer's recommendations. Quantitative RT-PCR (qRT-PCR) was performed with approximately 150 ng of cDNA using qPCR core kit for SYBR Green I (Eurogentec) and a MX3005P Multiplex qPCR

instrument (Stratagene) following the manufacturer's recommendations. Gene-specific primers used for SYBR Green-based analyses were previously described (von Dahl *et al*., 2007; Fragoso *et al*., 2014) and produced an amplicon of ~100 bp representing all known gene family members (Table S1). Results were obtained from five independent samples per genotype and treatment, each containing two technical replicates. Data was analysed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and is presented as the fold change in gene expression, normalised to the elongation factor 1 alpha (EF1α, Acc. No. D63396) gene from tobacco and relative to the corresponding untreated EV at time-zero.

2.3. Results

Wound- and herbivory-induced nicotine accumulation is altered in ir-toc1 plants

To determine the influence of the circadian clock and confirm the involvement of specific clock components (LHY and TOC1) and their regulators (PRR5 and ZTL) in defence metabolism, particularly nicotine accumulation, we first examined the responses of transgenic clock-silenced *N. attenuata* lines (ir-*ztl*; ir-*toc1*; ir-*prr5*; ir-*lhy*) to mechanical damage using a pattern wheel to inflict 'wound' stress (Figure 1A). Silencing of *TOC1* resulted in an increased capacity (p-value <0.05) of plants to stimulate nicotine synthesis in response to leaf wounding (relative to basal genotype levels) than empty vector (EV) plants (Figure 1B). Induction of nicotine in ir-*ztl*, ir-*prr5* and ir-*lhy* plants was found to be similar to that of EV plants (p-value >0.05). To examine whether the contribution of *TOC1* to nicotine defence responses differs under various wound stress regimes, we measured the levels of nicotine in locally treated and systemic leaves following leaf wounding and simulated herbivory (wound plus oral secretion; W+OS) after 48 h. Basal levels of nicotine in local and systemic tissues were 5–10% higher (p-value <0.01) in ir-*toc1* compared to EV plants (Figure 1C and 1S). Whilst leaf wounding generally had a stimulatory effect on both local (Figure 1C) and systemic (Figure 1S) levels of leaf nicotine, ir-*toc1* plants displayed marginally higher concentrations of nicotine (5–10%; p-value <0.05) in leaf tissues compared to EV. W+OS elicitation caused ir-*toc1* plants to accumulate markedly higher (~30%; p-value <0.001) levels of nicotine than their EV counterparts, albeit lower in concentration compared to leaf damage. Insect herbivory by *M. sexta* or application of its OS to leaf wounds amplifies JA and ET emissions (Kahl *et al*., 2000; Halitschke *et al*., 2001), the latter specifically inhibits the synthesis of nicotine (Voelckel *et al*., 2001; von Dahl *et al*., 2007) compared with wounding alone (Baldwin, 1989; Baldwin *et al*., 1994).

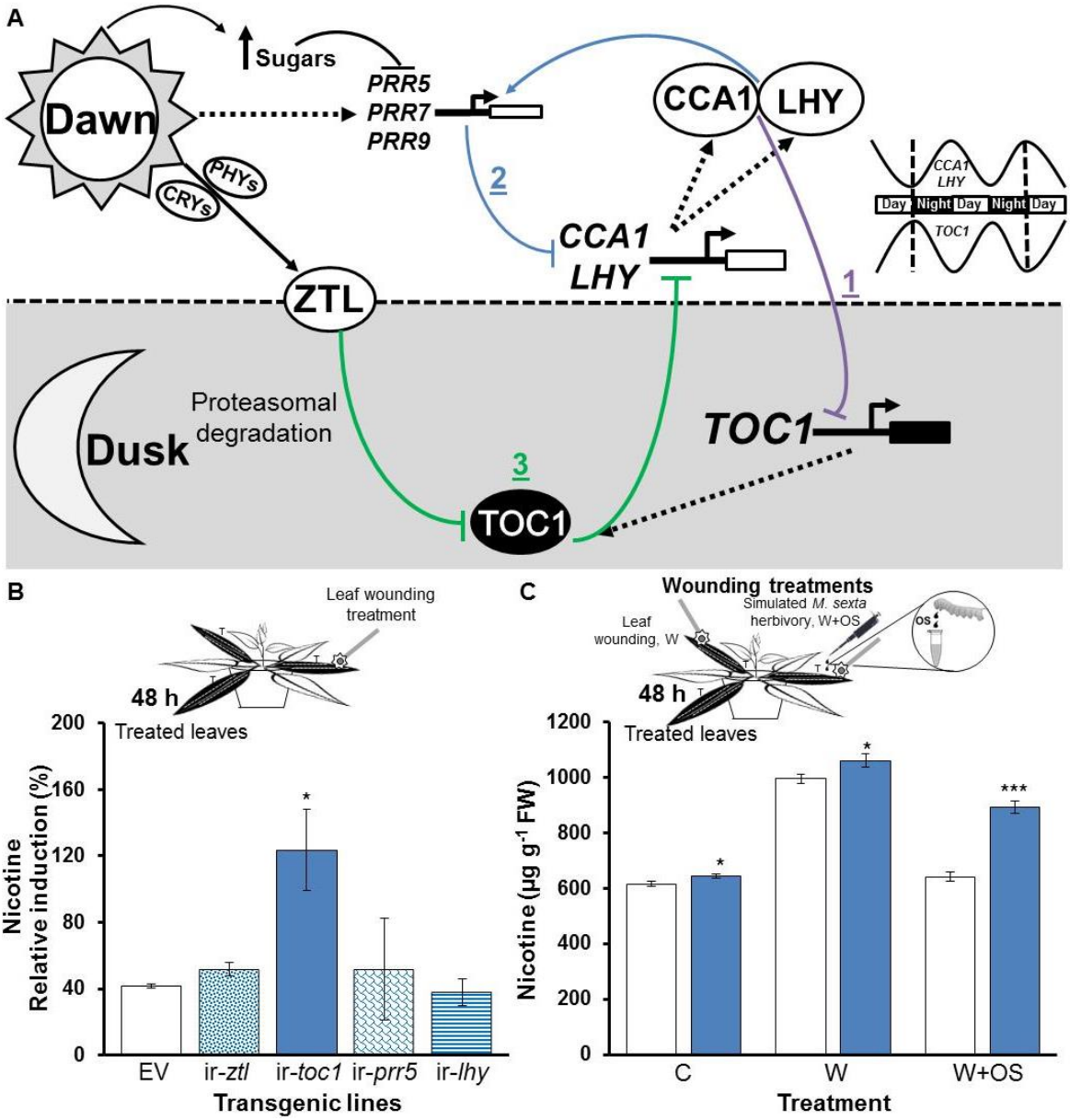

Figure 1. *TOC1* influences nicotine levels during wounding and simulated herbivory. (A) Schematic representation of the three-loop core oscillation clock model (adapted from McClung, 2006; Nagel and Kay, 2012). Diagram shows relationship between core morning and evening plant circadian clock components, CCA1/LHY and TOC1, and clock regulators PRR's and ZTL in *N. attenuata*. At 13:00, ten days after transfer to 1L pots, rosette leaves of five independent (n=5) developmentally synchronised empty vector (EV) and ir-*toc1* plants either remained untreated (C), were wounded (W) with a fabric pattern wheel, or 20 µL of 1:5 diluted *M. sexta* oral secretion (OS) was directly applied to the freshly wounded leaf lamina (W+OS) to simulate *M. sexta* herbivory (C). **(B)** Mean (±SE) induction of nicotine (relative to time 0 controls) in EV and *ir-toc1* locally treated leaves. **(C)** Mean (±SE) concentrations of nicotine in locally treated leaves 48h after W and W+OS treatments in EV and *ir-toc1* plants. Significant differences in nicotine concentration between EV and ir-*toc1* plants were determined by Student's *t* test at P<0.05 (*); P<0.01 (**) and P<0.001 (***). CCA1, circadian clock associated 1; CRYs, cryptochrome's; h, hours; LHY, late elongated hypocotyl; PHYs, phytochrome's; PRR, pseudo-response regulators; TOC1, timing of cab expression; W+OS, wound and OS application; ZTL, zeitlupe.

Silencing TOC1 alters the herbivory-induced hormonal profile

Given that the synergistic and antagonistic actions of hormones play a significant role in regulating nicotine induction, we examined in this study, the role of *TOC1* in specifically modulating the W+OS-induced production of phytohormones such as JA, JA-Ile, SA and ABA in locally elicited leaves (Figure 2). The mean concentration of JA in untreated *TOC1* silenced plants was ~10% higher (p-value <0.01) than basal JA content of EV plants. Similarly, ir-*toc1* plants contained ~20% higher (p-value <0.05) basal levels of JA-Ile, the bioactive form of JA, compared to average JA-Ile levels of EV plants. Consistent with previous JA metabolite analyses (Halitschke *et al*., 2001), W+OS elicitation in EV plants resulted in substantial increases in both JA and JA-Ile, peaking 1 hour after treatment. In both cases, levels plummeted almost 10-fold thereafter, with JA and JA-Ile levels returning to almost pre-elicited basal amounts after 5 hours in EV plants. Thirty minutes after W+OS elicitation, ir-*toc1* plants displayed slightly higher JA (~10%; p-value <0.05) and JA-Ile (~20%; p-value <0.01) levels than comparable EV plants (Figure 2). This was followed by a sharp reduction in JA production in silenced *TOC1* plants, with observably lower levels 1 (~50%; p-value <0.001) and 5 hours (~45%; p-value <0.05) after elicitation than in comparable EV plants. Interestingly, peak JA-Ile levels in ir-*toc1* plants 30 min after W+OS application were 15% lower than the corresponding peak amounts at 1 hour in EV plants (p-value <0.01). JA-Ile levels in ir-*toc1* plants rapidly declined thereafter, contrary to rising levels in EV plants, resulting in silenced plants containing ~35–50% less JA-Ile at 1 hour (p-value <0.001) and 5 hours (p-value <0.05) after elicitation compared to EV plants. It was concluded that silencing *TOC1* led to accelerated or shifted peaking of JA-Ile, and concomitantly lower JA and JA-Ile accumulation thereafter. Interestingly, nicotine levels of ir-*toc1* plants were restored to EV levels following JA supplementation with methyl jasmonate (MeJA) application in both local and systemic leaves (Figure S2).

Simulated herbivory of EV plants produced a modest increase in levels of SA and ABA (Figure 2). Basal levels of SA were similar between ir-*toc1* and corresponding EV plants. However, following W+OS elicitation, we observed higher levels of SA (~30-35%) in silenced *TOC1* plants compared to EV plants at 30 min (p-value <0.01), 1 hour (p-value <0.01) and 5 hours (p-value <0.001) after treatment. Basal ABA levels were ~10% higher in ir-*toc1* compared to EV plants (p-value <0.01). Following simulated herbivory, ABA accumulation patterns of EV plants peaked at 1 hour and rapidly declining 5 hours

post-treatment (Figure 2). On the contrary, ir-*toc1* plants exhibited a logistic asymptote pattern of accumulation, where levels of ABA were lower than EV plants at 30 min (~35%; p-value <0.001) and 1 hour (~15%; p-value <0.01) after elicitation. Interestingly, 5 hours after treatment ABA levels were observably higher (~25%) in ir-*toc1* plants compared to EV plants (p-value <0.001). The changes in ABA accumulation patterns over 5 hours following simulated herbivory between genotypes may be a result of manifest herbivore-associated phytohormonal responses in ir-*toc1* plants.

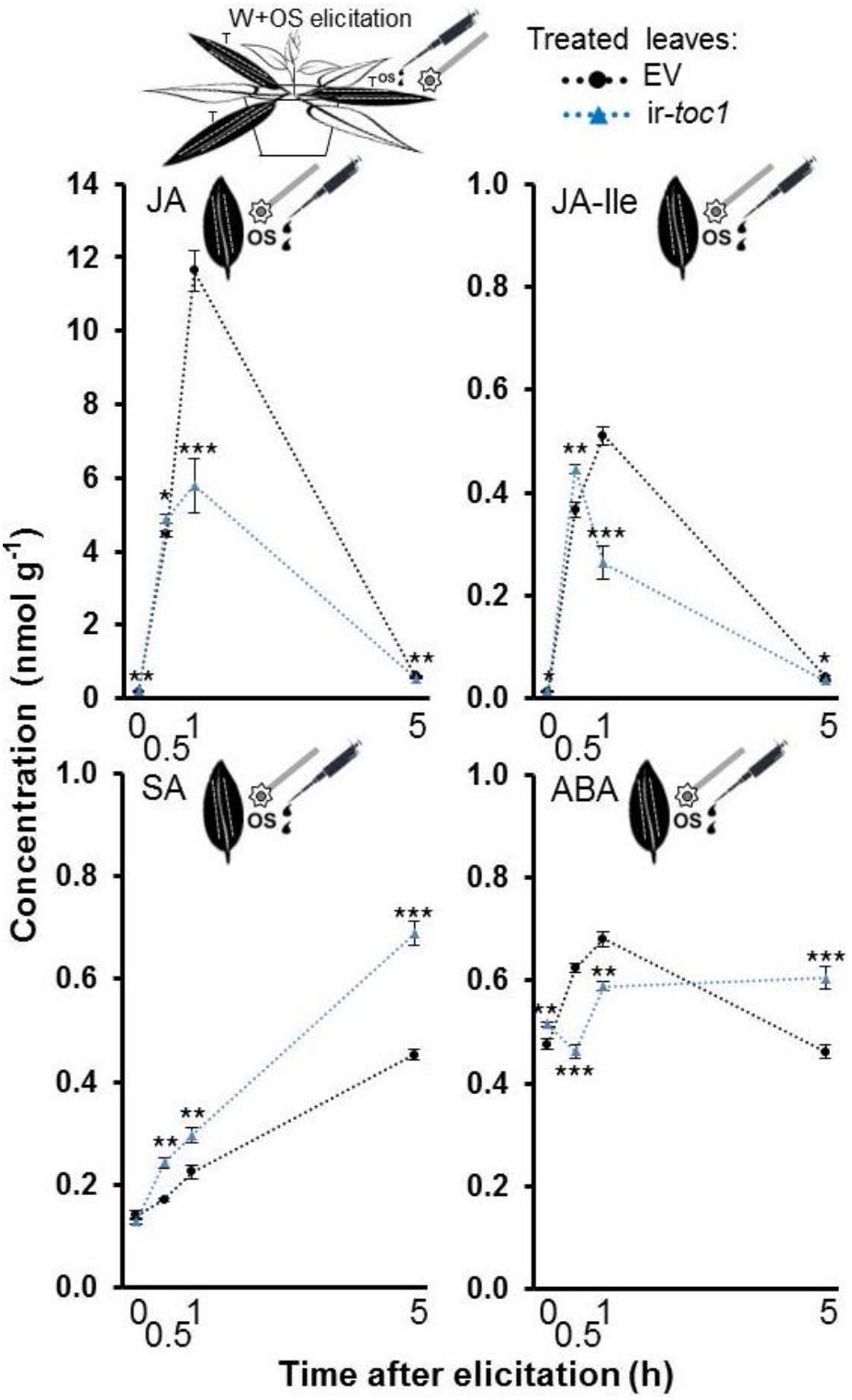

Figure 2. *TOC1* influences W+OS induced levels of defence-related phytohormones. Developmentally synchronised EV and ir-*toc1* plants were W+OS treated as described in Figure 1 and mean (±SE) levels of ABA, JA, JA-Ile and SA levels quantified over 5 hours from locally treated leaves of five independent plants (n=5) per genotype. Asterisks represent significantly different levels of phytohormones between EV and *ir-toc1* counterparts at specific time points by Student's *t* test at P<0.05 (*); P<0.01 (**) and P<0.001 (***).

Silencing TOC1 alters the OS-specific ethylene suppression of nicotine biosynthesis

Nicotine levels and its biosynthetic genes decrease following herbivory or W+OS treatment in *N. attenuata*, which is known to be tailored specifically by OS-induced ET production (von Dahl *et al*., 2007). We therefore examined the expression of two genes, *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID OXIDASE* (*ACO)* and *PUTRESCINE METHYLTRANSFERASE* (*PMT*), which are transcriptional markers for ET and nicotine biosynthesis, respectively (Figure 3A and B). These enzymes catalyse the final committed step to ET and the first committed step from polyamine metabolism into nicotine biosynthesis, respectively, (Nakatsuka *et al*., 1998). Consistent with previous studies on ET signalling (von Dahl *et al*., 2007), EV plants displayed a significant increase (~35-fold) in transcript levels of *ACO* 1 hour after W+OS-elicitation relative to basal levels (Figure 3A). Expression of *ACO* progressively declined thereafter in EV plants 5, 9 and 21 hours after elicitation. Silenced *TOC1* plants displayed ~30% lower basal *ACO* expression (p-value <0.05). Supporting a role for *TOC1* in herbivore-induced ET signalling, silenced *TOC1* plants displayed a 35–40% reduction in expression of *ACO* transcript compared to EV plants at 1 hour (p-value <0.01), 5 hours (p-value <0.01) and 21 hours (p-value <0.01) after W+OS elicitation. In addition, silenced *TOC1* plants displayed 40% lower basal *PMT* expression than EV plants (p-value <0.01; Figure 3B). Root transcript levels of *PMT* was significantly induced (~1.8-fold; p-value <0.001) 5 hours after W+OS elicitation in EV plants. *PMT* expression in ir-*toc1* plants increased 3-fold, ~15% higher than that of EV plants (p-value <0.001). This was followed by a marked reduction in *PMT* gene transcripts in EV plants 9 hours post-treatment, and to a lesser extent ir-*toc1* plants, which exhibited higher (~1.8-fold; p-value <0.001) levels of *PMT* than EV plants. Levels of *PMT* transcripts in both EV and ir-*toc1* plants returned almost to pre-elicited levels 21 hours after elicitation, with ir-*toc1* plants displaying ~15% lower expression of *PMT* compared to EV (p-value <0.001).

As expected, silencing *TOC1* in plants significantly diminished the normal W+OS-induced ET emissions of EV plants by ~25% (p-value <0.001; Figure 3C). Plants perceive ethylene through a family of 5 receptors (ETR1, ETR2, ERS1, ERS2 and EIN4) (Bleecker, 1999; Chang and Shockey, 1999). To test whether *TOC1* affects ET perception and confirm its involvement in the regulation of nicotine biosynthesis we inhibited the plant response to endogenous ET production. Prior to W+OS treatment, plants were exposed overnight to 1-MCP, a competitive inhibitor of ET perception, which renders

plants insensitive to ET, through its binding to ETR receptors (Figure 3D; Sisler *et al*., 1996). Both treated and systemic leaves (Figure 3E) and root (Figure S3) tissues of W+OS elicited ir-*toc1* plants pre-treated with mock solution (–1-MCP) show significantly higher nicotine than elicited EV plants (p-value <0.01). Pre-treatment with 1-MCP was sufficient to alleviate W+OS-induced differences in nicotine levels in both local and systemic leaf tissues of ir-*toc1* plants. It was concluded that *TOC1* regulates nicotine metabolism through alteration in ET signalling, upstream of receptors of ET. Pre-treatment with 1-MCP had no effect on restoring differences seen in root nicotine concentrations of W+OS treated ir-*toc1* plants compared to EV (Figure S3).

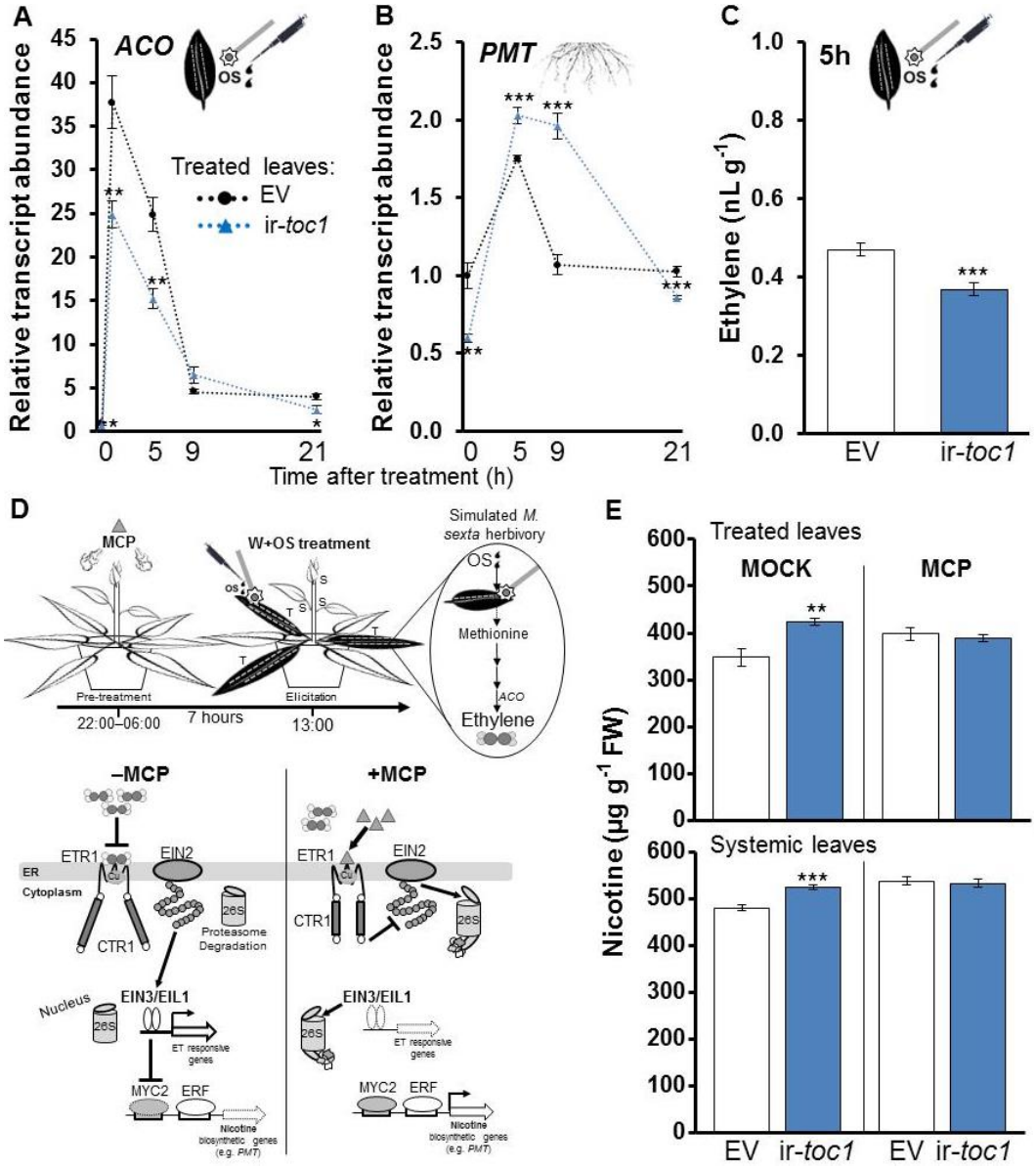

Figure 3. *TOC1* influences W+OS-specific elicitation of nicotine in an ET–dependent manner. Rosette leaves of developmentally synchronised empty vector (EV) and ir-*toc1* plants were W+OS-treated as described in Figure 1. Transcript levels of ethylene (A) and nicotine (B) biosynthetic marker genes were measured over 21 hours, whileethylene (C) production was quantified over 5 hours from five independent plants per genotype (n=5). Mean (±SE) transcript abundance (relative to Na*EF-1α*) of W+OS elicited expression of *ACO* (A) and *PMT* (B) were determined from locally treated leaves and root tissue, respectively. (D) Schematic representation demonstrating the MCP pre-treatment and elicitation strategy as well as the antagonistic relationship between ethylene and nicotine biosynthesis. To inhibit ethylene perception and uncouple the negative effects on induced nicotine production by OS-induced ethylene, plants were pre-exposed overnight (22:00 till 06:00) to MCP, a gas which binds irreversibly to ETR receptors and inhibits ethylene-induced responses (Sisler *et al.* 1996). Five plants per genotype were placed within growth chambers fitted along with either alkaline (mock; -MCP) solution or activated solution of MCP (+MCP) as described by Kahl *et al.* (2000). Seven hours after exposure was complete, plants were W+OS treated as described previously. (E) Mean (±SE) concentrations of W+OS-induced nicotine levels in EV and *ir-toc1* plants 21 hours after W+OS-elicitation in plants that had been pre-treated with either a mock or activated MCP solution. Significant differences between EV and *ir-toc1* plants were determined by Student's *t* test and are indicated by asterisks at P<0.05 (*); P<0.01 (**) and P<0.001 (***). ACO, 1-aminocyclopropane-1-carboxylic acid oxidase; CTR1, constitutive triple response 1; EIL1, ethylene insensitive3-like1; EIN2/3, ethylene insensitive 2/3; ER, endoplasmic reticulum; ERF, ethylene response factor; ETR1, ethylene resistant 1; MCP, 1-methylcyclopropene; Mock, alkaline solution; OS, oral secretion; PMT, putrescine methyltransferase.

TOC1 effects are independent of developmental differences

To confirm that the effects of *TOC1* on nicotine defences are independent of known developmental differences, we examined both the total shoot and root biomass over a period of 17 days (from day 30–47 after germination) of ir-*toc1* plants, which were germinated 7 days earlier than EV, and delayed by approximately 2–3 days in bolting and flowering (Figure 5A). The corresponding shoots and root biomass (Figure 5B and C) and pools of nicotine (Figure 5D and E) were measured to estimate the impact of *TOC1* on the synthesis of nicotine in *N. attenuata* throughout growth and development. Herbivory places extreme demand on many metabolic aspects of plant fitness and as such is known to alter allocation of resources within plants (Bazzaz *et al*., 1987; Gomez *et al*., 2010). To estimate the impact of *TOC1* on the synthesis of nicotine in *N. attenuata* under metabolic constraint, we also compared the total shoot and root biomass and pools of nicotine in ir-*toc1* and EV plants after simulated herbivory (W+OS). W+OS treatment did not dramatically alter the shoot or root biomass compared to non-wounded plants within a genotype. In general, we observed that the total shoot biomass of EV plants was markedly larger than ir-*toc1* plants (p-value <0.05; Figure 5B). This finding was observed under both basal (~15% reduction) and W+OS (~20% reduction) conditions. On the contrary, EV and ir-*toc1* plants did not differ in their root biomass under basal or W+OS-elicited conditions (Figure 5C). We observed basal shoot (Figure 5D) and root (Figure 5E) nicotine pools were significantly higher (~30%) in ir-*toc1* plants across all harvest points compared to EV plants (p-value <0.001). Similarly, following W+OS elicitation ir-*toc1* plants displayed ~25% higher root and shoot levels of nicotine across all harvest times (p-value <0.001). Whilst basal and elicited shoot nicotine pools of ir-*toc1* plants largely followed the same accumulation patterns as observed in EV, absolute levels of nicotine was elevated in silenced transgenics (Figure D and E). Likewise, basal root nicotine pools followed similar nicotine patterning. Interestingly, W+OS treatment led to lower levels of nicotine in roots of ir-*toc1* plants compared to respective non-wounded control plants. This is in stark contrast to EV, where W+OS elicited plants in general contained marginally higher nicotine pools compared to their non-wounded controls (Figure E).

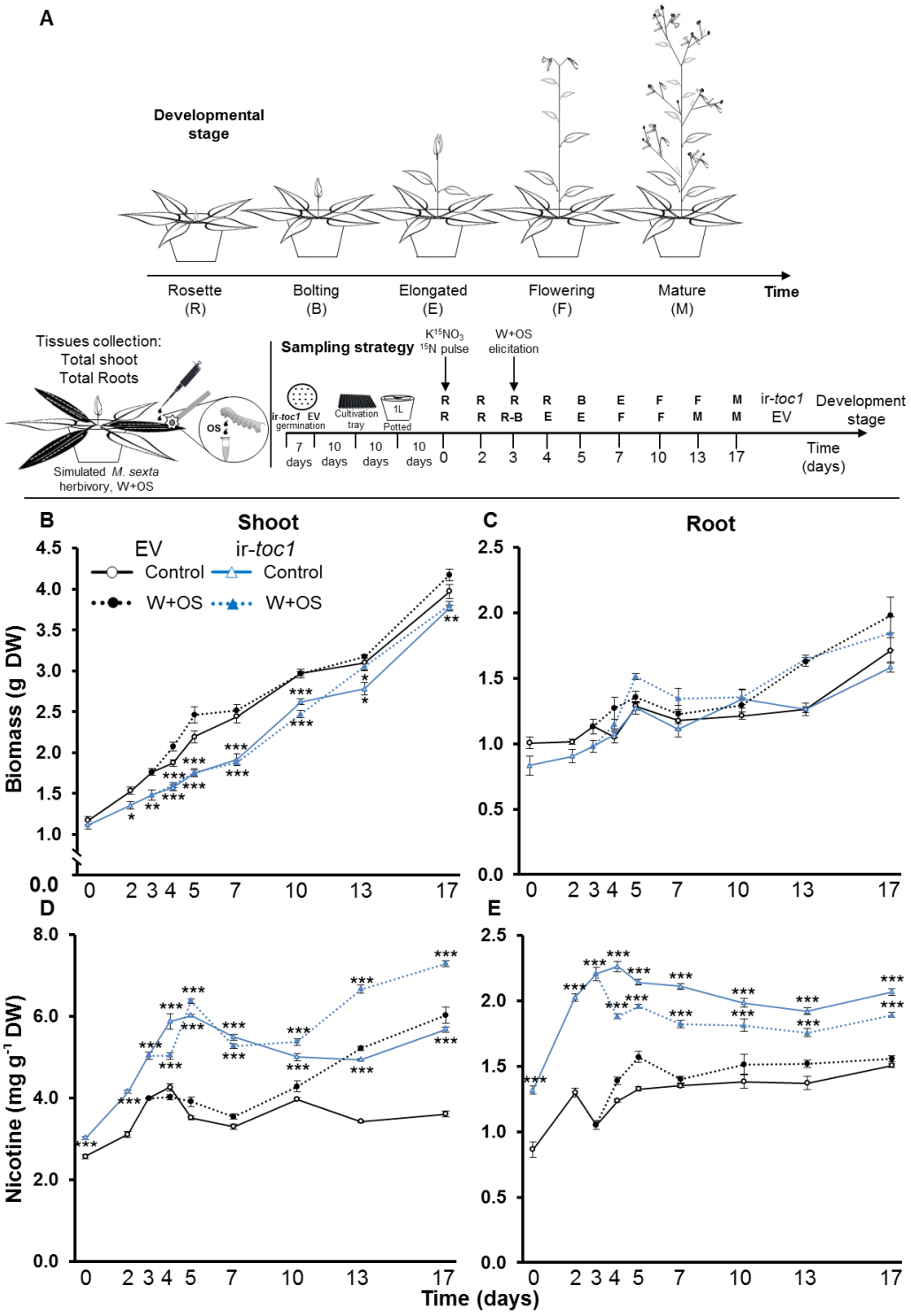

Figure 5. *TOC1* influences the incorporation of ^{15}N into whole plant nicotine pools in control and herbivory simulated (W+OS) plants. (A) Schematic representation demonstrating the elicitation and collection strategy used to study the *TOC1*-controlled dynamics of nicotine accumulation throughout growth and development. Ten days after transfer to individual 1L pots, five independent plants plants per genotype and treatment (n=5) were pulse labelled with 5.1 mg nitrogen delivered as $K^{15}NO_3$. Three days later, the first three fully expanded rosette leaves of *N. attenuata* plants (randomised; n=5 biological replicates per treatment and genotype) were W+OS-elicited as described in Figure 1. Mean (±SE) shoot (B) and root (C) biomass (g) and nicotine concentration (mg g^{-1} DW) in total shoot (D) and root (E) tissue of EV and ir-*toc1* plants in control and W+OS treated plants were measured over 17 days Asterisks represent significantly different incorporations or concentrations of nicotine between *ir-toc1* and corresponding EV plant determined by Student's *t* test at P<0.05 (*); P<0.01 (**) and P<0.001 (***).

TOC1 influences the incorporation of ^{15}N into herbivory-induced de novo nicotine synthesis

Consistent with previous nitrogen flux studies (Ullmann-Zeunert *et al*., 2013), we were able to calculate tissue-specific and pooled nitrogen investment into nicotine before and after simulated herbivory. This was done to explore the role of *TOC1* on nitrogen incorporation at the leaf-scale into local and systemic leaves, as well as into total shoot and root pools of nicotine (Figure 6). We used ^{15}N pulse labelling experiments to investigate and follow the investment of a defined pool of nitrogen into local and systemic nicotine defence responses over 4 days, as well as total plant nicotine pools over a longer time-frame. Labelled nitrogen (^{15}N) was administered as a pulse to plants in order to determine whether *TOC1* affected the incorporation of ^{15}N into nicotine biosynthesis, particularly in herbivory-stressed plants. Leaf and root tissues of ir-*toc1* plants had in general 10–15% higher basal and W+OS-elicited levels of nicotine compared to EV plants (p-value <0.05; Figure 6A, B and C). This was accompanied by 10–15% higher (p-value <0.05) incorporation of ^{15}N into nicotine in local and systemic leaves and root tissues in both control and W+OS elicited ir-*toc1* plants compared to EV (Figure 6D, E and F). We also observed that the incorporation of ^{15}N into nicotine was significantly higher in basal, as well as *de novo* incorporation into total root (~45%; p-value <0.001) and shoot (~35%; p-value <0.001) pools of ir-*toc1* plants compared to EV following W+OS induction (Figure 6G and H). While we observed similar accumulation patterns and incorporation of ^{15}N into nicotine in EV and ir-*toc1* plants, silencing *TOC1* was shown to positively influence the investment of nitrogen into nicotine and its overall accumulation in the plant.

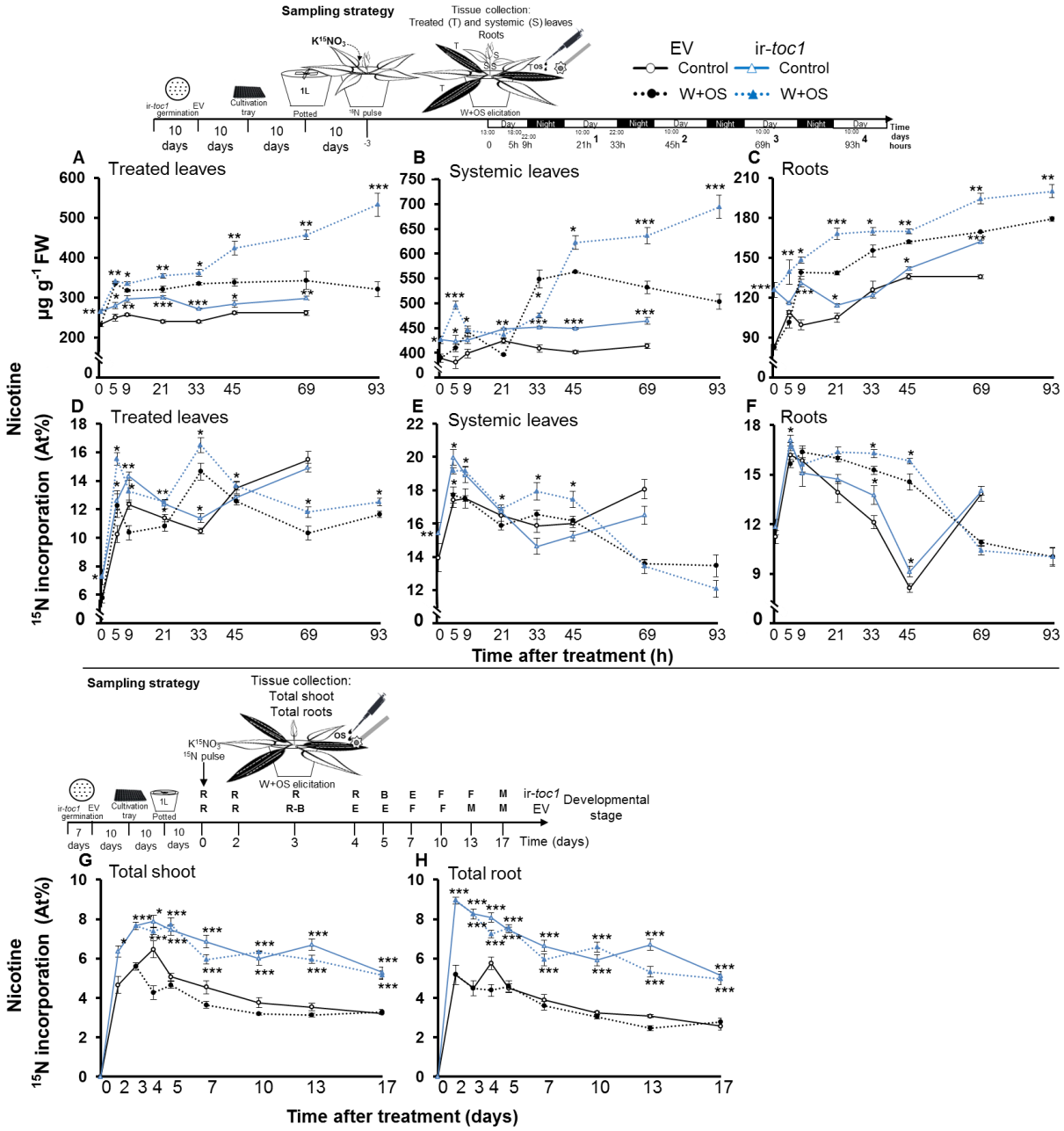

Figure 6. ***TOC1*** **influences the channelling and incorporation of ^{15}N into nicotine, and its accumulation, in control and treated (W+OS) local leaves, systemic leaves, root tissues and total shoot and root pools.** After transfer to individual 1L pots, five independent plants per genotype and treatment (n=5) were pulse labelled with ^{15}N and rosette leaves W+OS treated as described in Figure 5. Mean (±SE) concentrations of nicotine and incorporation of ^{15}N into nicotine were measured and quantified in locally treated leaves (A, D), systemic leaves (B, E) and root tissue (C, F) from synchronised empty vector (EV) and *ir-toc1* plants. Mean (±SE) incorporation of ^{15}N into nicotine in total shoot (G) and root (H) pools from EV and *ir-toc1* plants following W+OS treatment throughout development were measured. Asterisks represent significantly different incorporations or concentrations of nicotine by Student's *t* test in *ir-toc1* compared to corresponding EV plants at specific time points at P<0.05 (*); P<0.01 (**) and P<0.001 (***). At%, atomic percentage; B, bolting; E, elongated; F, flowering; FW, fresh weight; M, mature; R, rosette; W+OS, wound plus oral secretion.

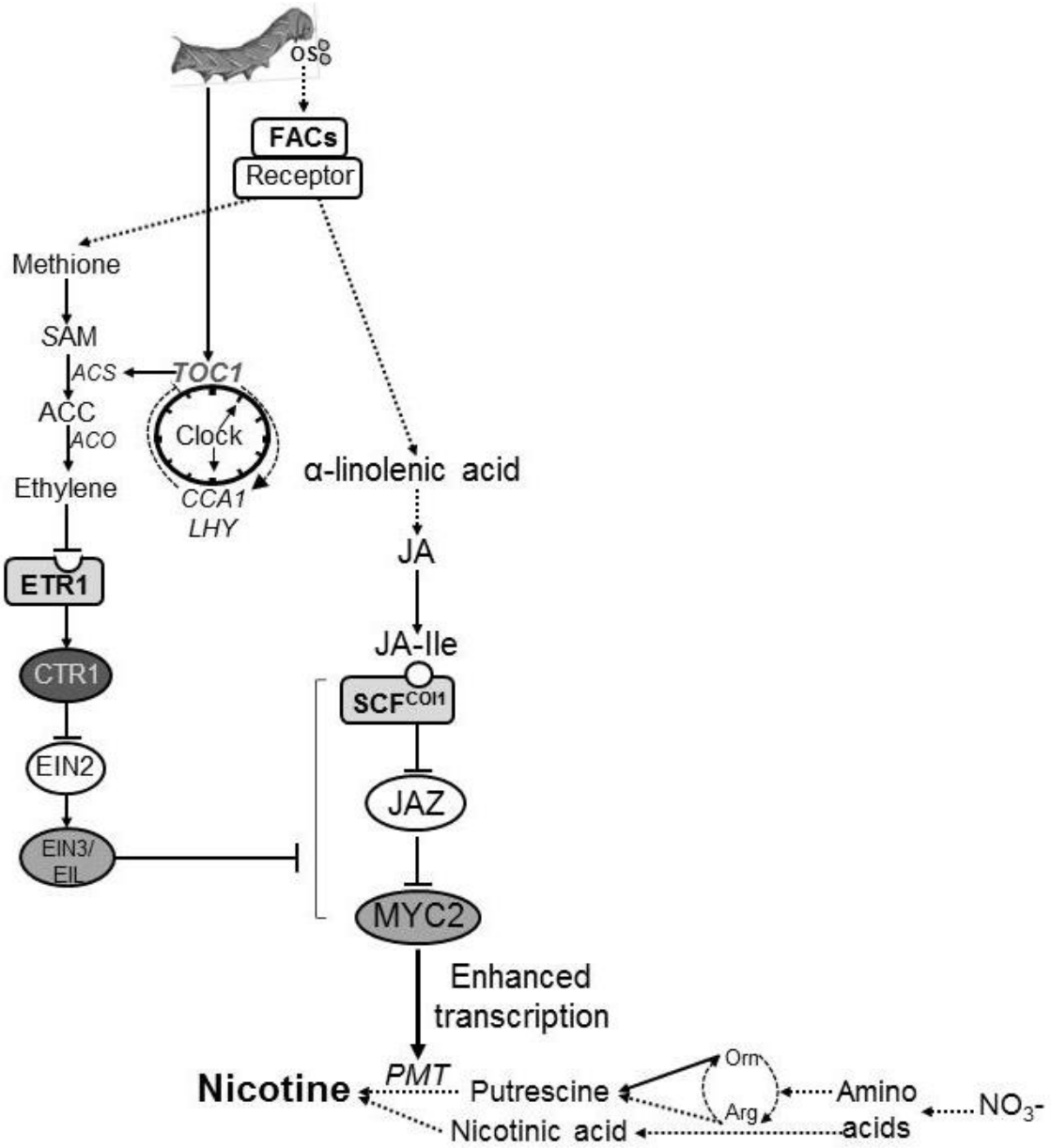

Figure 7. A simplified schematic representation of the proposed involvement of *TOC1* in hormone-clock signalling of nicotine defence responses to *M. sexta* attack in *N. attenuata*. Herbivore attack by *M. sexta*, composed of wound signal and release of insect elicitors in the OS to the wounds, induces a strong burst of defence hormones: JA and ET. Both JA and ET are required to trigger accumulation of a number of defence chemicals, including nicotine, via the actions of JA- and ET-specific transcriptional regulators. The involvement of *TOC1* in ET hormonal homeostasis, as a means of tuning the defence response in plants is hypothesised. ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC oxidase; ACS, ACC synthase; Arg, arginine; COI1, coronatine-insensitive 1; CTR1, constitutive triple response 1; EIL1, ethylene insensitive3-like1; EIN2/3, ethylene insensitive 2/3; ETR1, ethylene resistant 1; EV, empty vector; FACs, fatty acid-amino acid conjugates; Ile, isoleucine; JA, jasmonic acid; JAZ, jasmonate ZIM domain; NO_3^-, nitrate; Orn, ornithine; OS, oral secretion; PMT, putrescine methyltransferase; SAM, *S*-adenosylmethionine, SCF, Skp1/Cullin/F-box.

2.4. Discussion

Most evidence to date has shown that the clock, primarily CCA1, is important for regulating primary metabolites or JA- and SA-associated defences in *Arabidopsis* (Covington *et al*., 2008; Bhardwaj *et al*., 2011; Goodspeed *et al*., 2012; Shin *et al*., 2012). Few studies have investigated the importance of TOC1 and hormonal cross-talk in controlling abundant defence compounds such as nicotine. Previous research has shown that synthesis of nicotine, as well as expression of pertinent genes in its biosynthesis, is under diurnal control (Kim *et al*., 2011; Gulati *et al*., 2014). In this study, we identified cross-talk mechanisms between the circadian system and ET signalling, which was mediated by *TOC1*. We found that *TOC1* influenced nicotine accumulation through regulatory control over nitrogen investment and deregulation in hormone signalling. *TOC1* acts as a negative regulator of basal and herbivore-stress nicotine biosynthesis in an ET-dependent manner, highlighting a possible role for ET-TOC1 cross-talk in modulating nitrogen defences in plants.

Application of *M. sexta* OS to wounded leaves dramatically alters the normal wound-induced response of JA, ET and SA hormone signals. Nicotine biosynthesis is both upregulated by JA and down-regulated by OS-induced ET (Baldwin *et al*., 1994; Voelckel *et al*., 2001; von Dahl *et al*., 2007). Interestingly, the mitigation of induced nicotine following W+OS is not to the same extent in silenced *TOC1* plants as it is in EV plants. The large differences in nicotine accumulation and induction capacity are exacerbated following simulated herbivory compared to wounding alone, which is likely to solely result from the difference of W+OS-induced ET signalling. The *M. sexta* specific elicitation of ET emissions is clearly distinguishable from the wound-induced ET response in *N. attenuata* plants (Kahl *et al*., 2000; von Dahl *et al*., 2007) and as such attenuates the induction of nicotine in W+OS-treated plants through its antagonism with JA (Kahl *et al*., 2000). The production of defensive compounds is metabolically costly. Their inducibility allows plants to minimise metabolic constraint and fitness consequences associated with their production and is thought to give plants the ability to time their production to demand (Givnish, 1986; Purrington, 2000). We concluded that *TOC1* alters nicotine biosynthesis through negative signalling, which is amplified under inducible situations such as herbivory simulations.

We found that *TOC1* influences the overall amplitude or concentration of whole-plant and tissue-specific pools of nicotine. Overall, silencing *TOC1* resulted in higher

concentrations of shoot- and root-pools of nicotine, suggesting that *TOC1* may influence the nitrogen demand for basal and W+OS-stimulated nicotine biosynthesis. With regards to tissue-specific changes in nicotine, we found that basal and W+OS elicited levels of nicotine were significantly higher in local and systemic leaves of ir-*toc1* plants, suggesting *TOC1* may regulate tissue-specific partitioning of nitrogenous resources. Interestingly, simulated herbivory led to lower total root pools of nicotine in ir-*toc1* plants compared to non-wounded ir-*toc1* plants. This is in stark contrast to EV, where W+OS treated plants in general contained marginally higher nicotine pools compared to their controls. This could be due to higher turnover of nicotine from root to shoot in ir-*toc1* plants following W+OS treatment. Although nicotine accumulation and incorporation of ^{15}N into nicotine in silenced *TOC1* plants was robust, and the effect on total pools clear, tissue-specific investments were generally much less pronounced. This could be due to vigorous trade-offs within and between local and systemic tissues, resulting in smaller, albeit still significant, differences. Whether *TOC1* governs the basal rhythm of nicotine by close root-shoot signalling still remains an essentially unresolved question. It would be of interest to explore whether *TOC1* aids in root-to-shoot communication of whole-plant circadian rhythms. This could be addressed by the use of micro-grafting techniques to test the signal and function of *TOC1* in roots.

Previous ^{15}N labelling experiments in *N. attenuata* have found no evidence that herbivore stress changes the assimilation rate of nitrogen (Baldwin and Ohnmeiss, 1994; Lynds and Baldwin, 1998). The clock has hypothesised roles in adjusting root nitrogen metabolism (Gutiérrez *et al*., 2008) through regulation of assimilatory enzymes, which may also influence the uptake of nitrogen for nicotine biosynthesis, which exclusively takes place in the roots. We found that silencing *TOC1* was sufficient to increase the capacity for plants to synthesis nicotine. This was supported by labelling experiments which demonstrated higher incorporation of ^{15}N into basal- and *de novo* synthesised nicotine following W+OS elicitation at the leaf scale and in whole-shoot and root pools of nicotine compared to EV plants. This suggests that *TOC1* may regulate nitrogen uptake, storage and/or usage, which clearly changed the prioritisation of nitrogen into nicotine in ir-*toc1* plants. Future experiments could examine root nitrogen pools or its storage forms. This may be in the form of primary metabolites that may be preferentially allocated to other areas of metabolism and could also influence the rate of nitrogen uptake in order to compensate for nicotine demand.

Silencing of *TOC1* in *Arabidopsis* is shown to cause arrhythmia under constant darkness and red light, while in constant darkness and red and blue light conditions its over-expression abolishes clock rhythmicity (Más *et al*., 2003). However, silencing *TOC1* in *N. attenuata* does not disrupt the overall running of the clock or produce an arrhythmic/dis-rhythmic plant, but rather disrupts the engagement of *TOC1* in developmental (Yon *et al*., 2012) and metabolic processes, as shown here. In *N. attenuata, TOC1* silenced plants were shown to be developmentally delayed compared to EV (Yon *et al*., 2012), remaining in the rosette stage longer before bolting and thus being delayed in flowering, similar to *toc1* mutants in *Arabidopsis* (Somers *et al*., 1998). To confirm that the effects of *TOC1* on nicotine defences are independent of developmental differences, we examined the total plant biomass, whole- shoot and root pools of nicotine and the incorporation of ^{15}N into these respective pools. The ability to perceive seasonal changes by the circadian clock is required for the successful transition from vegetative to reproductive stages, while basal and induced levels of nicotine depend largely on the developmental stage of the plant (Ohnmeiss and Baldwin, 2000). It was shown that smaller biomass and slight developmental retardation at the transition from rosette to bolting stage in ir-*toc1* plants was uncoupled from major differences in the production of nicotine. Consistently higher concentrations of nicotine throughout development confirm nicotine is unrelated to allometric differences.

Given the strong connection of clock components to hormone signalling and plant resistance and also the large role hormonal cross-talk plays in regulating *N. attenuata* defence responses to *M. sexta*, we examined the involvement of *TOC1* in specifically modulating the W+OS-induced production of phytohormones. The data shows that *TOC1* influences distinct herbivore-associated phytohormonal responses. Silencing *TOC1* resulted in severe deregulation of herbivory-induced phytohormones, including diminished emission of ET and peak concentrations of JA and Ja-Ile. Silenced *TOC1* plants displayed a weakened JA burst, which hastened the timing or peaking of JA-Ile compared to EV-elicited plants. Given the central role of JA in regulating wound- and herbivore- induced accumulation of nicotine in the leaf tissue (Baldwin *et al*., 1994; 1996; Baldwin, 1989) we examined whether the addition of MeJA, the volatile methyl ester of JA, known to further stimulate JA signalling, would further exacerbate *TOC1*-control of nicotine biosynthesis. Interestingly, the responsiveness of EV and ir-*toc1* plants to JA supplementation in both local and systemic leaves was able to restore differences in

nicotine levels to that of EV plants, indicating TOC1-mediated JA synthesis dynamics is not essential for influencing nicotine accumulation. While manipulating JA signalling alone was able to complement these differences, questions still remain around the involvement of *TOC1* in JA-regulation of nicotine defence. Our data suggests that it may be upstream of JA, perhaps at the core of transcriptional regulation.

TOC1 additionally affected the accumulation of SA and ABA in plants. These two hormones are known to display antagonistic interactions with both JA and ET signalling (Leslie and Romani, 1988; Niki *et al*., 1997; Anderson *et al*., 2004; Cipollini *et al*., 2004; Takahashi *et al*., 2004; Adie *et al*., 2007). W+OS-elicited ir-*toc1* plants showed rapidly escalating levels of SA compared to EV plants. A feedback mechanism between ABA and TOC1 exists, particularly in plant responses to drought where Legnaioli *et al*. (2009) showed that TOC1 was important for sensitised ABA activity. Basal ABA levels were observably higher in ir-*toc1* compared to EV plants; however, alterations in ABA after W+OS elicitation were more complex. Initially, elicitation caused a reduction in ABA in ir-*toc1* plants compared to EV plants, up to 1 hour following herbivory simulation. This was interestingly followed by higher ABA levels in ir-*toc1* plants than in EV plants, 5 hours after elicitation. Reciprocal regulation between ABA and TOC1 may be important for distinct herbivore-related hormone responses. However, large reductions in W+OS-induced ET emissions in ir-*toc1* plants could also account for the differential alterations in the induction pattern of JA, JA-Ile, SA and ABA. ET is known to supress both ABA- and SA-mediated signalling, the latter of which also inhibits JA (Cipollini *et al*., 2004; Leon-Reyes *et al*., 2009). Exogenous application of SA to leaf wounds was shown to reduce levels of nicotine (Baldwin *et al*., 1997). However, induced levels of SA do not appear to negatively affect the W+OS-induced nicotine observed in ir-*toc1* plants, which is consistent with studies using exogenous JA or MeJA in conjunction with SA (Baldwin *et al*., 1996; 1997). Kahl *et al*. (2000) demonstrated that nicotine accumulation following *M. sexta* herbivory, OS application and MeJA induction in *N. attenuata* is effectively attenuated by ET and moreover that excess SA in plant systems does not inhibit the release of ET following simulated insect herbivory. In *Arabidopsis*, plants pre-exposed to herbivores were shown to be more sensitive to pathogen-induced SA and primed for enhanced SA-mediated resistance following herbivore-associated ET emissions (De Vos *et al*., 2006). Additionally, Diezel *et al*. (2009) demonstrated that *N. attenuata* plants impaired in the biosynthesis or perception of ET accumulate higher levels of herbivory-

induced SA. Collectively, these studies could suggest that increased SA levels in ir-*toc1* may result from abolished ET signalling. However, perturbation of SA status, along with NPR1 (non-expressor of pathogenesis-related gene 1), has recently been shown to gate plant immune responses directly through expression of morning and evening clock genes, principally by boosting *TOC1* expression (Zhou *et al*., 2015). The differential responses in defence-related hormones observed in ir-*toc1* plants are clearly complex. Here we show that *TOC1* stringently affects ABA, ET, JA and SA responses to herbivore pressure, which support the notion that clock-dependent gating of defence-related phytohormones is presumably important for metabolic homeostasis under stress environments. Hormone-clock feedback mechanisms are known to tailor expression of clock genes and regulate plant stress and immune responses to drought, herbivore attack and pathogen invasion (Nováková *et al*., 2005; Legnaioli *et al*., 2009; Goodspeed *et al*., 2012; 2013a; Zhou *et al*., 2015), which may be a reflection of distinct defence- and stress-related perturbations of phytohormones in silenced *TOC1* plants.

Increased nicotine production is correlated with enhanced activity of the regulatory enzyme, *PMT*, which is directly inhibited by ET production (Winz and Baldwin, 2001) and directs the flow of nitrogen from the diamine precursor, putrescine, into nicotine biosynthesis (Shoji *et al.* 2002). Silencing *TOC1* was shown to effect transcriptional markers for both ET and nicotine biosynthesis, with ir-*toc1* plants displaying reduced *ACO* and induced *PMT* transcript levels following herbivory simulations. Circadian rhythm of ET production in unstressed seedlings is tightly controlled by TOC1-driven ACS expression (Thain *et al*., 2000; Thain *et al*., 2004). Collectively, these results support the theory that impaired ET signalling in ir-*toc1* plants has a diminished antagonistic effect on nicotine synthesis (Figure 7). The use of inhibitors and chemical treatments has long been used to study the role of ET in plant-herbivore interactions by impairing the production, accumulation and perception of ET (reviewed in von Dahl and Baldwin, 2007). To further corroborate the involvement of *TOC1* in the regulation of herbivore-elicited nicotine biosynthesis, mediated specifically through ET signalling, we inhibited the plant response to endogenous ET production using exogenous 1-MCP.

Consistent with previous findings (Kahl *et al*., 2000), treatment with 1-MCP was shown to abolish the inhibitory effect of ET on W+OS-induced nicotine in EV plants and was sufficient to eradicate differences in nicotine accumulation observed between

induced EV and ir-*toc1* plants. These results demonstrate that *TOC1* affects the ability to produce ET following herbivory, but not the perception or sensitivity to ET and thus mediates W+OS-specific nicotine production in an ET-dependent manner. Interestingly, pre-treatment with 1-MCP had no effect on restoring the differences seen in nicotine concentrations in the root tissue of W+OS treated ir-*toc1* plants compared to EV plants. How to interpret this difference in induced response of nicotine in the roots of ir-*toc1* plants with and without 1-MCP treatment remains an open question. JA, together with some of its precursors and derivatives, are important signal components of the leaf-root signalling system that facilitates the plants' wound, anti-herbivore and anti-pathogen responses, produced initially in wounded leaves via the octodecanoid pathway, and rapidly transmitted from damaged tissues to the root (Baldwin *et al.*, 1997; Hashimoto and Yamada, 1994; Hibi *et al.*, 1994; Kutchan, 1995). *TOC1* may additionally play a role in leaf-root signalling, perhaps through regulation of microRNAs (Bozorov *et al*., 2012), coordinating the expression of target genes involved in responses to herbivore attack, consequently up-regulating nicotine in roots independent of ET signalling in the leaves.

Here we provide evidence for a mechanism of circadian clock gating of plant defence responses, connecting *TOC1* with hormonal responses to herbivory. Our data indicates that *TOC1*-ET regulation has a significant effect on the major defensive metabolite, nicotine, produced by *N. attenuata*. Based on our data, we speculate that clock control of hormone cross-talk systems may provide a mechanism plants can utilise to reconfigure their metabolome to fluctuating environments, particularly for optimal defence responses against herbivores. Questions still remain about whether *TOC1* specifically affects the ability of the plant to take up nitrogen and therefore promote higher turnover into nitrogen metabolites or whether it affects the partitioning or trade-off of nitrogen resources into different aspects of metabolism during herbivory. Recent studies have suggested that the function of the clock is not on 'anticipation' but on the timing of response to herbivore attack (Goodspeed *et al*., 2012). While we controlled for timing of attack through set wounding times, data from the current study may suggest that *TOC1*, and thus the clock, acts with defence-related hormone signals, to fine-tune resource expenditure and optimise investments into defence, particularly during times of stress. Previous reports suggested that the burst of ET in *N. attenuata*, induced by *M. sexta* herbivory, may be a tool to prolong the feeding of caterpillars on host plants (Voelckel *et al*., 2011). Plants may have evolved coordinated clock-hormonal signalling

regimes to compensate for this and direct their valuable resources into specific spectrums of metabolites. Events like herbivory place extreme metabolic demand on plants, especially in the production of defences at the expense of primary metabolism. In the case of attack from caterpillars such as *M. sexta*, which are able to detoxify doses of nicotine (Wink and Theile, 2002; Kumar *et al*., 2014), we postulate that *TOC1* alleviates the allocation of nitrogen into nicotine defence through the use of sophisticated hormone signalling networks and may direct nitrogen into alternate defence pathways. It would be beneficial to investigate whether *TOC1* exerts dependent regulatory control over branched areas of nitrogen-defence in *N. attenuata*.

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2.6. Supporting Information

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

Gene	Forward primer (5'–3')	Reverse primer	Reference
EF-1a	CCACACTTCCCACATTGCTGTCA	CGCATGTCCCTCACAGCAAAAC	Fragoso *et al*. (2014)
ACO1	CTATTGAATCTGATGTCAAGCTG	TATGTAGTAGGGACACACGCTT	von Dahl *et al*. (2007)
PMT1/2	TCATTGGACCAAGATCGAG	TGGAAATTATGATAATTACTGCA	Fragoso *et al*. (2014)

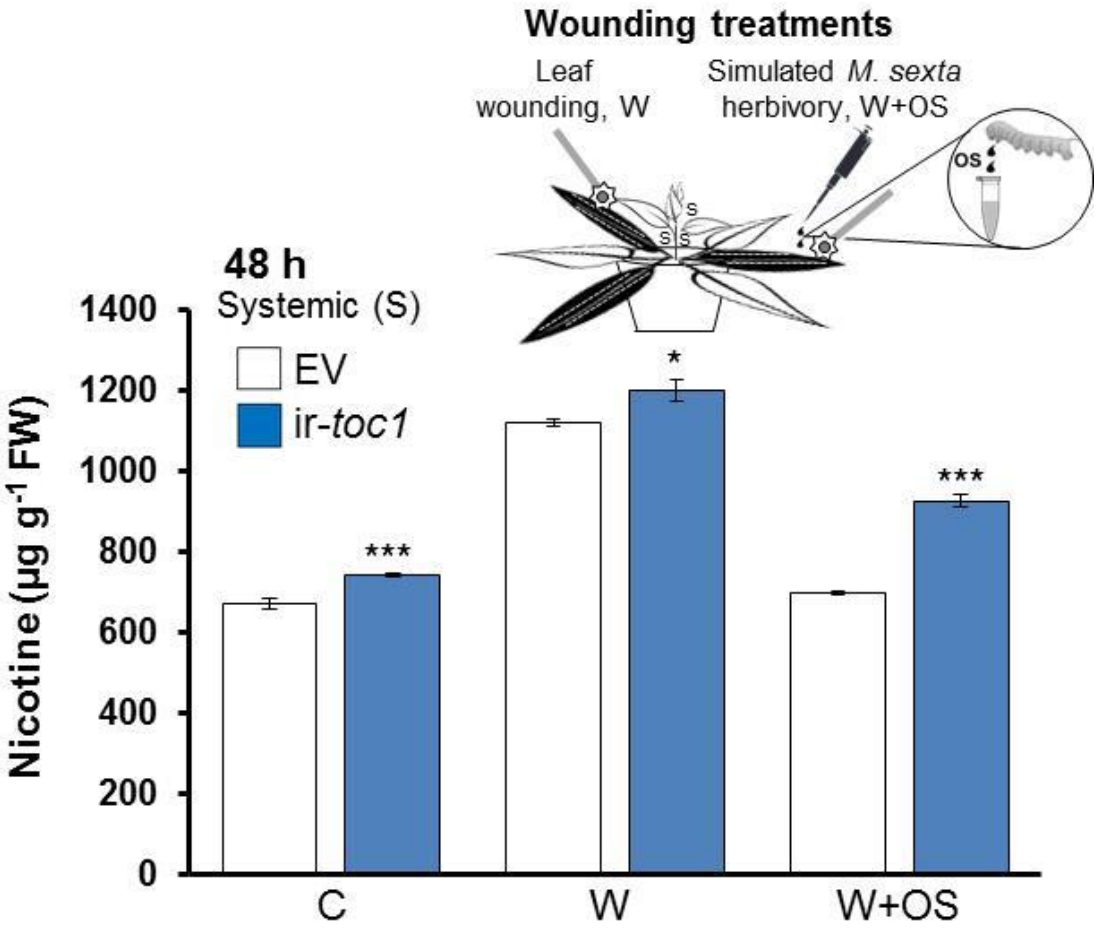

Figure S1. ***TOC1*** **influences systemic levels of nicotine.** A schematic representation of the leaf wounding (W) and simulated herbivory (W+OS) treatments is shown. Rosette leaves of developmentally synchronised empty vector (EV) and ir-*toc1* plants were treated as described in Figure 1. Mean (±SE) concentration of nicotine in systemic (S) tissues 48 hours (h) after W- and W+OS-elicitation treatments in EV and *ir-toc1* plants. Significant differences in nicotine concentration between EV and ir-*toc1* plants were determined by Student's *t* test at P<0.05 (*); P<0.01 (**) and P<0.001 (***; n=5).

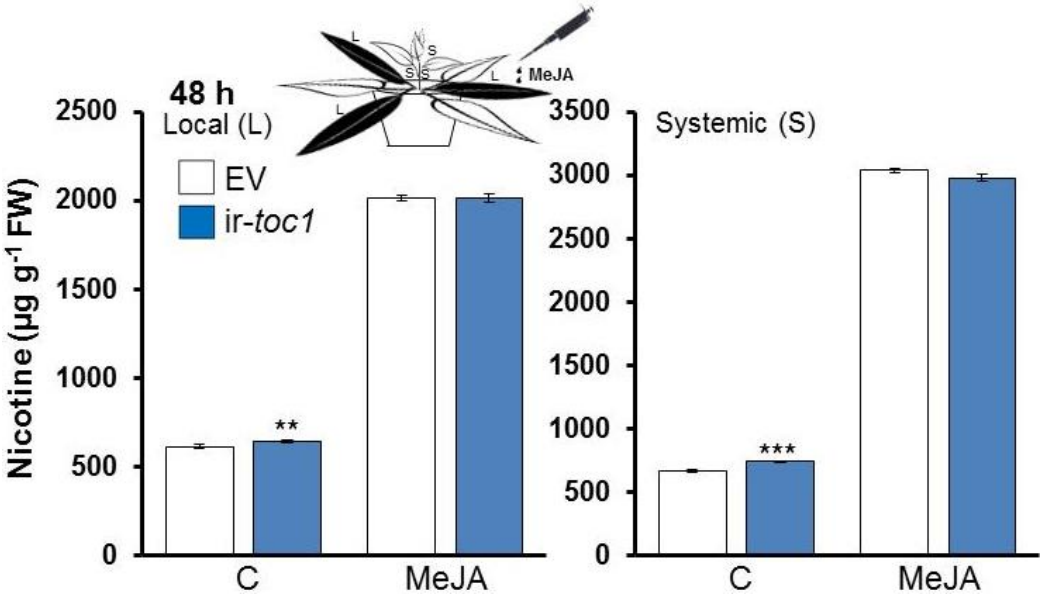

Figure S2. Differences in levels of nicotine between EV and ir-*toc1* plants are restored with JA supplementation. Rosette leaves of developmentally synchronised empty vector (EV) and ir-*toc1* plants were elicited with 20 µL of lanolin paste containing 150 µg methyjasmonate (MeJA) to supplement JA production or with 20 µL of lanolin to control (C) for solvent effects and plants were harvested 48 hours (h) later. Figure shows mean (±SE) concentration of nicotine in locally treated (L) and systemic (S) leaves 48 h after MeJA-elicitation. Asterisks indicate significant differences determined by Student's *t* test from the EV at P<0.05 (*); P<0.01 (**) and P<0.001 (***; n=5).

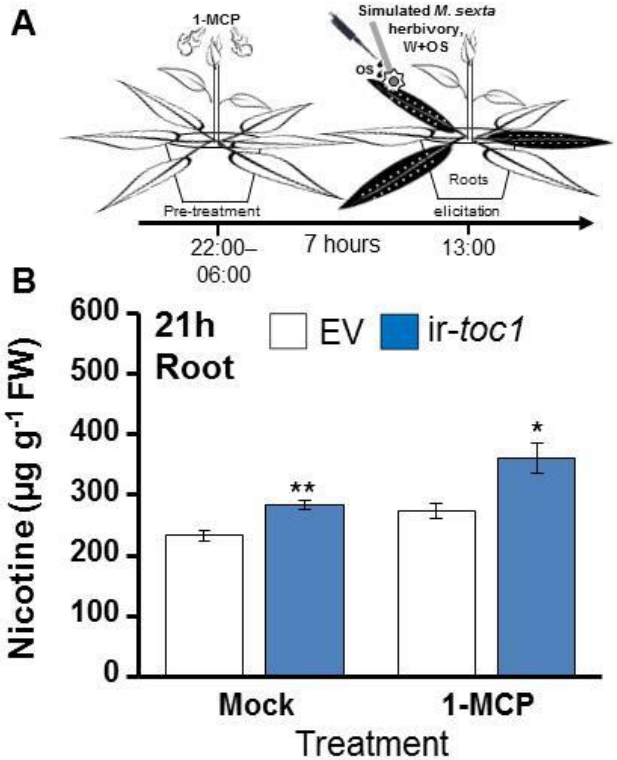

Figure S3. Differences in levels of root-based nicotine between EV and ir-*toc1* plants are not restored with MCP treatment. (A) Schematic representation of the MCP pre-treatment and W+OS elicitation strategy is shown and is further described in Figure 3 along with the antagonistic relationship between ethylene and nicotine biosynthesis. (B) Mean (±SE) concentration of nicotine in roots of EV and *ir-toc1* plants 21 hours after W+OS-elicitation in plants that had been pre-treated with either a mock or activated MCP solution. Significant differences from EV were determined by Student's *t* test and are indicated by asterisks at P<0.05 (*); P<0.01 (**) and P<0.001 (***; n=5). MCP, 1-methylcyclopropene; Mock, alkaline solution; W+OS, wound plus application of oral secretions.

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Monash University

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
- Planned, performed, analysed and interpreted all experiments - Main author of the manuscript	**80%**

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Prof Ian Baldwin	- Provided supervisory support - Contribution to experiment planning	N/A
Dr Emmanuel Gaquerel *	- Provided supervisory support - Contribution to experiment planning and editing of the manuscript	N/A

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** **26/10/2015**
Main Supervisor's Signature	[redacted]	**Date** **26/10/2015**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Chapter 3: Silencing of timing of CAB expression 1 (TOC1) impedes herbivory-induced phenolamide accumulation dynamics and insect resistance in *Nicotiana attenuata*

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Abstract

Plants possess a molecular clock which enables them to control and synchronise internal rhythms to predictable/recurring day-night cycles, thereby providing a robust mechanism to respond to anticipated daily stresses. In the present study involving *Nicotiana attenuata*, we found that silencing of the circadian clock component *TIMING OF CAB EXPRESSION 1* (*TOC1*) had a depressive effect upon transcript levels of early herbivore-induced stress signalling components (*SIPK*), nitrogen assimilation (*GS*), jasmonic acid (JA)-activated transcriptional regulators (*MYC2* and *MYB8*) and phenylpropanoid biosynthetic genes (*PAL1*) following simulated herbivory. This was accompanied by attenuated accumulation of putrescine and plant defence metabolites, including diterpene glycosides, chlorogenic acid, caffeoylputrescine and dicaffeoylspermidine in silenced *TOC1* plants. Interestingly, incorporation of labelled nitrogen into phenolamide metabolites was also found to be diminished in silenced *TOC1* transgenics compared to empty vector controls. Additionally we found that down-regulation of *TOC1* led to increases in concentrations of upstream precursors including tyramine and spermidine, as well as amino acids following herbivore-associated stress. These alterations in primary and secondary metabolite levels in silenced *TOC1* plants had a major effect on the insect fitness of the specialist herbivore, *Manduca sexta*, and an intermediate effect on the generalist, *Spodoptera littoralis*. Together, these results support important roles for *TOC1* in coordinating signalling processes and rechanneling metabolic fluxes towards the production of a specific spectrum of defensive metabolites following herbivore-associated stresses.

Keywords: circadian clock; defence metabolism; herbivory; hormone; phenylpropanoid-polyamine conjugate; phenolamide; Timing of CAB expression 1

3.1. Introduction

Organisms have developed robust molecular circuits to acclimatise to their ever-changing environment and coordinate their processes to specific times of the day. Plant circadian rhythms, generally equating to 24 hour cycles, are generated by an endogenous circadian clock and entrained by environmental cues, such as light and temperature, and function to reset and fine-tune a plant's overall metabolism to recurring environmental day-night cycles (Dunlap, 1996; Alabadí *et al.,* 2001; Wenden *et al.,* 2011; Gendron *et al.,* 2012; Pokhilko *et al.,* 2012). The central clock oscillator is composed of three interlocking feedback loops, with both positive and negative transcriptional regulators to generate entrained rhythms (Harmer, 2009; Nagel and Kay, 2012). These core oscillation circuits, responsible for tweaking rhythmic gene expression cycles, are comprised of two partially redundant morning-phased MYB transcription factors (TFs), CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) and an evening-phased component, TIMING OF CAB EXPRESSION 1/PSEUDO-RESPONSE REGULATOR 1 (TOC1/PRR1) (Wang and Tobin, 1998). Post-translational regulation plays an important role in clock systems, establishing and maintaining regular feedback loops. ZEITLUPE (ZTL), an F-box protein directly targets TOC1 and PRR5 proteins, regulating their levels through proteasome-mediated degradation in the E3 ubiquitin ligase SCF-type E3-ubiquitin ligase complex (Mas *et al.,* 2003; Kiba *et al.,* 2007; Kim *et al.,* 2007).

Most molecular studies to date have been undertaken in the model plant, *Arabidopsis thaliana*, extensively expanding our knowledge of plant circadian rhythmicity (reviewed by Harmer, 2009; de Montaigu *et al.,* 2010). These studies have revealed that the core oscillator controls about one third of all plant genes, affecting genes participating in plant growth, development, carbon metabolism and water uptake, as well as numerous genes with known functions in plant defence (Harmer *et al.,* 2000; Michael and McClung, 2003; Covington *et al.,* 2008; Mizuno and Yamashino, 2008; Goodspeed *et al.,* 2012). The identification of key plant processes, such as photosynthesis, stomatal opening, starch turnover and plant synchronisation to pathogen infection and insect behaviour as clock-controlled outputs, highlights the importance of molecular clocks in regulating resource acquisition and allocation (Bhardwaj *et al.,* 2011; Graf *et al.,* 2011; Chen *et al.,* 2012; Goodspeed *et al.,* 2012). It still remains poorly understood however, what systems are in place for rechannelling metabolic fluxes towards the production of a

specific spectrum of metabolites, particularly in response to expected daily biotic and abiotic stresses.

The discovery of a collection of key growth, developmental and defence related hormone signals, including abscisic acid (ABA; Nováková *et al*., 2005; Legnaioli *et al.,* 2009; Seung *et al*., 2012; Portolés and Zhang, 2014); auxin (Covington and Harmer, 2007; Yin *et al.,* 2007); cytokinins (CKs; Salomé *et al.,* 2006); ethylene (ET; Thain *et al.,* 2004); gibberelins (Arana *et al.,* 2011); jasmonic acid (JA; Covington *et al.,* 2008; Goodspeed *et al.,* 2012; Shin *et al.,* 2012) and salicylic acid (SA; Bhardwaj *et al.,* 2011) that act as clear clock output pathways, suggests development, defence and stress related responses that are largely controlled by hormone-signal networks largely overlap with biological processes also regulated by the clock. This extends also to their biosynthesis, perception and breakdown being under rhythmic control (Thain *et al.,* 2000; Nováková *et al.,* 2005; Salomé *et al.,* 2006; Covington and Harmer, 2007; Covington *et al.,* 2008; Michael *et al.,* 2008; Mizuno and Yamashino, 2008; Robertson *et al.,* 2009; Goodspeed *et al.,* 2012). Not only is hormonal homeostasis an output coordinated by clock components, such as *CCA1* and *TOC1*, but the phase, amplitude and period of the clock can also be modulated by hormones (such as ABA, auxins and CKs), demonstrating bidirectional regulation between clock and hormone networks in plants (Hanano *et al.,* 2006).

Recent studies on the circadian control of plant defence metabolism have revealed rhythmical accumulation of plant primary and secondary metabolites (Kim *et al.,* 2011), and that the feedback of these pathways directly influence the clock system (Kerwin *et al.,* 2011). Goodspeed *et al*. (2012) demonstrated that herbivore-induced defences were also diurnally regulated with rhythmic insect behaviour. It was shown using *cca1* mutants that JAs, JA-responsive genes and JA-mediated defences are synchronised with daily herbivore activity. MYC2, a JA-activated TF, has been indirectly linked with core clock components, which gate JA responses, interacting with a regulator of the clock, TIME FOR COFFEE (TIC) that was shown to inhibit MYC2 protein production (Shin *et al.,* 2012). These results highlight the complex interaction of the metabolic rhythms of plants and herbivores, suggesting that it may be advantageous to synchronise metabolite accumulation and clock controlled resource allocation to maximise and time the accumulation of plant defence products to deter insect herbivory. Through analysis of integrated network models it has been predicted that the morning-phased clock component, *CCA1*, may play a role in nutrient availability, adjusting key genes associated

with nitrogen assimilation through regulation of glutamic acid (Gutiérrez *et al.,* 2008). The plant circadian clock has additionally been linked to pathogen infection and immune responses through SA signalling (Bhardwaj *et al.,* 2011; Wang *et al.,* 2011; Zhou *et al.,* 2015). This was linked to *CCA1* and directly mediated oscillations in patterns of plant antimicrobial defence responses to pathogen elicitation. The transcriptional repressors/regulators, TOC1 and CCA1 clock components, target an expanded network of genes, primarily through binding interactions in the promoter region, involved in multiple processes associated with growth, development and stress responses (Harmer *et al.,* 2000; Covington *et al.,* 2008; Legnaioli *et al.,* 2009; Nagel and Kay, 2012). In *Arabidopsis* Nagel *et al*. (2015) has recently uncovered that a subset of clock output pathways do not cycle with a circadian rhythm. Furthermore, CCA1 targeted promoters of both morning- and evening-phased genes, some of which were enriched with multiple circadian-related motifs, suggesting that gene networks may respond largely to or be targeted by multiple clock regulatory inputs.

Based on homology to genes in *Arabidopsis*, core clock components, *LHY*, *TOC1*, and regulators, *ZTL* and *PRR5*, have recently been identified and silenced using RNAi-mediated approaches in *Nicotiana attenuata* (Yon *et al.,* 2012; 2015) to ascertain the function of these genes in plant growth, development and defence. A number of clock entrained behaviours, such as flower movement, flower aperture and benzyl acetone emission (Kim *et al.,* 2011; Yon *et al.,* 2012; 2015) have recently been discovered and explored in *N. attenuata*. Many land plants, including *N. attenuata*, an annual wild tobacco plant, have evolved highly effective, inducible defence systems, utilising hormone cascades to modulate growth, development and to produce a wide spectrum of biologically active toxic compounds in response to leaf wounding, herbivore attack and pathogen invasion (Gatehouse, 2002; Oksman-Caldentey and Inze, 2004; Schwachtje and Baldwin, 2008; Bari and Jones, 2009). Herbivore-induced plant defence responses are known to be specifically shaped by hormone-signalling systems. It is plausible then, that the downstream regulatory interactions and signalling networks that these hormones activate to elicit plant defence reactions may also be controlled by the circadian clock. JA mediators regulate the expression of a number of wound- and herbivore-receptive defence biosynthetic genes (Rojo *et al.,* 1999; Lorenzo *et al.,* 2004; Fernández-Calvo *et al.,* 2011), to mediate the majority of direct defence responses in plants (Bari and Jones, 2009). When an insect feeds on a leaf this rapidly activates a cascade of signalling events, driven

by the recognition and perception of wound- and/or fatty acid-amino acid conjugates (FACs) present in the oral secretions (OS) of many herbivores, including the tobacco hornworm *Manduca sexta* (Halitschke *et al.,* 2001; 2003; Mithöfer *et al.,* 2005; Schmelz *et al.,* 2009). Following detection of the FAC elicitors, *N. attenuata* plants rapidly increase mitogen activated protein kinase (MAPK) signalling (Liu and Zhang, 2004; Wu *et al.,* 2007), downstream transcriptional targets, hormone signalling molecules, JA, SA and ET (Kahl *et al.,* 2000; Halitschke and Baldwin, 2003), and extensively reconfigure the transcriptome and proteome of the plant (Halitschke *et al.,* 2003). Activation and phosphorylation of specific MAPKs, including SA-induced protein kinase (SIPK), has been shown to control JA- and ET-mediated defence responses and facilitate plant resistance to herbivores (Zhang and Klessig, 2001; Kandoth *et al.,* 2007; Wu *et al.,* 2007; Wu and Baldwin, 2009).

The circadian clock has emerged as an additional mechanism controlling ability of plants to direct resources into areas of growth and defence for optimal plant fitness (Goodspeed *et al*., 2012; 2013a; 2013b; Chapter 2). Several genes and herbivory-induced defence metabolites and their precursors, which are metabolically costly to produce, have been shown to follow diurnal regulation in *N. attenuata*, including phenylpropanoid precursors, phenolamides (also frequently referred to as phenylpropanoid-polyamine conjugates, hydroxycinnamic acid amides and phenolic amines) and diterpene glycosides (DTGs) (Kim *et al.,* 2011). Biosynthesis of phenylpropanoid derivatives comprises a complex series of branching biochemical pathways that provide plants with a wide range of hydroxycinnamoyl acids and esters that are widely used as major structural cell components (e.g. lignin), pigments (flavonoids and anthocyanins), immunity signals (SA) and toxins (e.g. phenolamides) (Dixon *et al.,* 2002; Vogt, 2010). Their metabolic intermediates and final products have essential functions in plant development as well as defence against biotic and abiotic challenges (Ferrer *et al.,* 2008; Kaur *et al.,* 2010). Phenylpropanoid metabolism and species-specific transcriptional regulators in many Solanaceous plants are positively regulated by JA and its derivate MeJA, which has been shown to induce the accumulation of *PHENYLALANINE AMMONIA-LYASE 1* (*PAL1*; Tebayashi *et al.,* 2000; Paschold *et al*., 2007; Kazan and Manners, 2008; Onkokesung *et al.,* 2012; Ullmann-Zeunert *et al.,* 2013). Phenolamides, abundant in plant tissues, are assembled from the conjugation of a phenolic moiety with polyamines (putrescine and spermidine) or with aryl monoamines (e.g. tyramine and tryptamine) building blocks. The

entry point into their biosynthesis is at the interface between carbon (phenylpropanoid) and nitrogen (polyamine) metabolism. In many plants families, in particular in the Solanaceous family (Smith *et al.*, 1983), aryl mono- and poly-amines are present as amide conjugates to hydroxycinnamic acids (such as coumaric, caffeic and ferulic acids). However, mono-acylated putrescine conjugates and mono- or di-acylated spermidines in coumaric, caffeic or ferulic acid combinations exist as the main phenolamides formed in tobacco species.

Using RNAi methodology, a recent study indicated that down-regulation of *TOC1* had a significant inhibitory effect on ET signalling and enhanced nicotine defence responses in transgenic *N. attenuata* (Chapter 2). The control over herbivore-induced ET production by *TOC1* was shown to enable plants to control induction regimes and input of nitrogen into nicotine. Relatively little is known about the involvement of clock-associated regulatory networks to coordinate the flux of resources into closely linked defence pathways, particularly in herbivore-challenged plants. It is clear harmonising specific defence responses to herbivore and pathogen activity requires clock-control of cycling JA and SA defence signals, particularly CCA1 in *Arabidopsis* (Covington *et al.*, 2008; Bhardwaj *et al.*, 2011; Goodspeed *et al.*, 2012; Shin *et al.*, 2012). However, the involvement of other oscillators (such as TOC1) in regulating JA-responsive metabolites has been largely overlooked. *TOC1* has been shown to shape aspects of plant metabolism including ABA- and ET- related stress responses (Thain *et al.*, 2004; Legnaioli *et al.*, 2009; Chapter 2). It is likely regulation of complex gene networks for optimal defence in herbivore-induced plant systems responds to or is targeted by multiple oscillator and clock-output genes. We hypothesise that *TOC1* may also have roles in regulating primary and secondary metabolite fluxes, which could help plants maintain a healthy balance between growth and defence. The work presented here explores down-stream effects of complex early herbivore-induced signalling events, connecting *TOC1*-signalling networks with JA-mediated responses to herbivory using rapid accumulation patterns of defensive phenolamides as a case study. We provide insight into how plants integrate JA defence signals and closely coordinate activation of a number of different branched defence pathways and contribute to an optimal direct defence in *N. attenuata*.

3.2. Materials and Methods

Plant material, growth and harvesting conditions

Homozygous T_3 plants containing an invert-repeat (ir) construct targeted for the silencing of TOC1 (ir-*toc1*), a circadian clock component, were generated from line ir-*toc1*-205 which was described fully in Yon *et al*. (2012) and selected following characterisation of several independent transgenic lines in which *TOC1* transcript levels were reduced by >90%. Plants containing an empty vector (EV) were used as a transformation control (EV-266), which were identical in growth and morphology to other independent EV transgenics as well as those of the non-transgenic parental line Torr. Ex. Wats (30^{th} inbred generation). Seeds were sterilised and germinated on agar plates containing Gamborg's B5 medium (Duchefa; http://www.duchefa.com) as described previously by Krügel *et al*. (2002) and maintained in a 26°C/16-h, 155 µmol m^{-2} s^{-1} light: 24°C/8-h dark cycle (Percival) for 10 days. Young seedlings were planted individually in TEKU plastic pots (Pöppelmann) with Klasmann plug soil (Klasmann-Deilmann) and approximately ten days later, early rosette plants were transferred to 1-L individual pots with sand to facilitate sampling of root tissue. Plants were grown in the glasshouse at 26 °C-28 °C for 16 h under supplemental light from Master Sun-T PIA Agro 400- or 600-W high pressure sodium lamps (Phillips) and an 8 h dark cycle (22°C-24°C). *TOC1* silenced plants were previously observed to be delayed developmentally compared to EV plants (Yon *et al*., 2012), remaining on average 7–10 days longer in the rosette stage before bolting, which resulted in delayed flowering. Unless otherwise stated, silenced *TOC1* plants were developmentally synchronised by germinating ir-*toc1* seeds ~10 days prior to EV plants (Yon *et al*., 2012).

Insect rearing and collection of oral secretions

Tobacco hornworm (*Manduca sexta*) eggs, obtained from Carolina Biological Supply (Burlington, USA) and bred in an in-house colony, were cultivated in climate chambers until hatching. Egyptian cotton leafworm (*Spodoptera littoralis)* were obtained from the Entomology department at the Max Planck Institute for Chemical Ecology. Oral secretions and regurgitants (OS) were collected on ice from third to fifth instar larvae reared on wild type *N. attenuata* plants as described in Roda *et al*. (2004) and stored under Argon at -20 °C until use.

Plant treatments

Early elongating plants (~four weeks) were randomly divided and unless otherwise stated five biological replicates per genotype were assigned to treatment groups (n=5). Plants were either left untreated as a control (C) or to simulate insect feeding (W+OS) the lamina of three experimental rosette leaves per plant were mechanically wounded at 13:00 using a serrated fabric pattern wheel to puncture three rows of holes on both sides of the midvein and fresh wounds were immediately treated with 20µL of eliciting solution of 1:5 (v/v) water-diluted OS from *M. sexta* (W+OS). Equivalent phyllotactic positions from untreated and treated local leaves, along with their systemic leaves and complete root systems were collected from plants at designated time points and immediately flash frozen in liquid nitrogen. Roots were briefly washed in water and blotted dry with paper towel to remove sand prior to freezing. To evaluate the growth performance of native specialist (*M. sexta*) and generalist (*S. littoralis*) herbivores of *N. attenuata* on transformed plants, one freshly hatched *M. sexta* neonate and one six-day old *S. littoralis* caterpillar was placed on two separate stem leaves of ten independent EV and *ir-toc1* replicate plants (n=10) and allowed to feed. *S. littoralis* neonates were first reared on artificial diet for 4 days due to the high sensitivity of the unadapted generalist to *N. attenuata* leaf defences and then placed on EV- or *ir-toc1* leaves for 2 days to get rid of any artificial diet present in their guts. Caterpillars were subsequently weighed and similar weight caterpillars were then placed directly on EV/*ir-toc1* stem leaves. Caterpillars were removed and their mass measured every 2 days and placed thereafter on undamaged stem leaves. Similarly positioned systemic leaf tissues were collected for metabolite analysis on day 12 from all treatment groups.

1-Methylcyclopropene (1-MCP) treatment

Endogenous ET production is induced by OS treatment, which in turn acts synergistically and antagonistically with OS-induced JA signals to regulate a number of tobacco defences. To inhibit ET perception and uncouple any negative effects on induced phenolamide production by OS-induced ET, plants were exposed to 1-MCP, as described by (Sisler *et al*. 1996).

^{15}N pulse labelling

For ^{15}N-labelling, plants were pulse labelled with 5.1 mg nitrogen administered as $K^{15}NO_3$ and ^{15}N-incorporation into nitrogen-containing metabolites was determined

according to Ullmann-Zeunert *et al*. (2012; 2013). Experimental strategy (treatment and sampling) is depicted in Figure 3A.

Analysis of leaf and root metabolites

One hundred milligrams of liquid nitrogen-ground leaf or root powder was pre-weighed and aliquoted into 1.5 mL Eppendorf tubes containing a steel ball to aid extraction for subsequent metabolite analyses. Secondary metabolites were extracted from local leaf, systemic leaf and root samples using an optimised 40% methanol extraction for the analysis of a wide range of metabolites (alkaloids; 17-hydroxygeranyllinalool diterpene glycosides [HGL-DTGs]; phenolamides; chlorogenic acid and rutin) in *N. attenuata* (Gaquerel *et al*., 2010). High performance liquid chromatography coupled to photodiode array detection (HPLC-PDA) was used for the quantification of secondary metabolites using known concentrations of dicaffeoylspermidine (DCS), caffeoylputrescine (CP), chlorogenic acid (CGA), crypto-chlorogenic acid (crypto-CGA) and rutin standards as described previously (Kaur *et al*., 2010) standards. Data is graphically presented per mg fresh weight of tissue extracted.

Polyamine analysis

Putrescine, spermidine, spermine and tyramine were extracted from locally treated leaves and their control counterparts and quantified by HPLC with fluorescence detection (FLD-HPLC) as described by Fellenberg *et al*. (2012). Briefly, 1 mL of 0.1M HCl extraction buffer per 50 mg of aliquoted tissue powder was added and samples were homogenised using a Genogrinder (SPEX CertiPrep) at a frequency of 1250 strokes min^{-1} for 1 min. After centrifugation (13.2 rpm, 20 min, 4 °C), 400 µL of supernatant and 300 µL of 0.5 M borate buffer was transferred to a new tube, mixed and incubated at room temperature for approximately 15 minutes before centrifuging again for 5 min. Aliquots of 50 µL supernatant was transferred to a HPLC vial for derivatisation and subsequent analysis. Samples were derivatised firstly with 50 µL OPA for 1 min and a further 2 min with 2 µL FMOC. Typically 30 µL of double derivatised sample was separated and analysed with Agilent-HPLC 1100 series (http://www.chem.agilent.com) according to Fellenberg *et al*. (2012).

Quantitative Real Time – PCR

Total RNA was isolated from snap frozen leaf and root tissues using the Trizol method (Invitrogen). DNA-free RNA samples were reverse transcribed using oligo$(dT)_{18}$ primer

and cDNA was synthesised from 2 µg of RNA with Superscript II Reverse Transcriptase (Invitrogen) and a poly-T primer following the manufacturer's recommendations. Quantitative RT-PCR (qRT-PCR) of the genes of interest was performed with approximately 150 ng of cDNA using qPCR core kit for SYBR Green I (Eurogentec) and a MX3005P Multiplex qPCR instrument (Stratagene) as recommended by the manufacturer's recommendations. All gene-specific primers used for SYBR Green-based analyses of genes were previously described (Table S1; Kang *et al*., 2006; Meldau *et al*., 2009; Onkokesung *et al*., 2012; Woldemariam *et al*., 2013). Results were obtained from five independent samples per genotype and treatment, each containing two technical replicates. Data was analysed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and is presented as the fold change in gene expression, normalised to the elongation factor 1 alpha (EF1α, Acc. No. D63396) gene from tobacco and relative to the corresponding untreated EV at time-zero.

3.3. Results

Untreated and W+OS-induced accumulation patterns of secondary metabolites are altered in ir-toc1 plants

In previous studies of *N. attenuata*, we observed diurnal changes in secondary metabolite profiles and a host of important related genes (Kim *et al*., 2011), begging the question as to whether these patterns are regulated by the circadian clock. To address this question, we measured the production of abundant secondary metabolites, including 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) and three frequently identified phenylpropanoid derivatives, chlorogenic acids (CGAs), caffeoylputrescine (CP), and dicaffeoylspermidine (DCS), in untreated and W+OS-elicited empty vector (EV) control and ir-*toc1* plants. Levels of DCS were broadly similar between local (Figure 1) than systemic (Figure S1) leaves of untreated EV plants whilst CP, CGAs and HGL-DTGs were consistently lower in local leaves than their systemic counterparts. In line with previous observations (Gális *et al*., 2010; Kaur *et al*., 2010; Ullmann-Zeunert *et al*., 2013; Gaquerel *et al*., 2014), combined leaf wounding and OS application had a marked stimulatory effect on levels of CP and DCS in locally treated leaves of EV plants (Figure 1), which was further exacerbated in systemic leaves (Figure S1). However, W+OS elicitation did not overtly increase levels of CGAs above those produced in untreated EV plants (Figure 1 and S1). While levels of HGL-DTGs were not substantially increased following W+OS elicitation in locally treated leaves of EV plants relative to their

untreated EV controls (Figure 1), systemic leaves on the other hand were increased ~2-fold (Figure S1). Silencing *TOC1* led to significantly reduced baseline levels of CP (55–60%; p-value <0.01), DCS (30–40%; p-value <0.01) and HGL-DTGs (35–40%; p-value <0.01) in local (Figure 1) and systemic (Figure S1) leaves of untreated ir-*toc1* plants compared to EV. Whilst levels of these metabolites in ir-*toc1* plants were generally stimulated in response to W+OS-elicitation and followed the same trends as observed in EV controls, the amplitude or overall production of these compounds were significantly attenuated (35–45%; p-value <0.01) compared to EV plants. Silenced *TOC1* plants also displayed 15–20% lower basal (p-value <0.01) and W+OS-elicited (p-value <0.01) levels of CGAs in local (Figure 1) and systemic (Figure S1) leaves compared to EV plants. From these results, we concluded that *TOC1* plays a major role in tuning constitutive as well as W+OS-induced levels of three major defensive phenylpropanoid conjugates, as well as total HGL-DTGs.

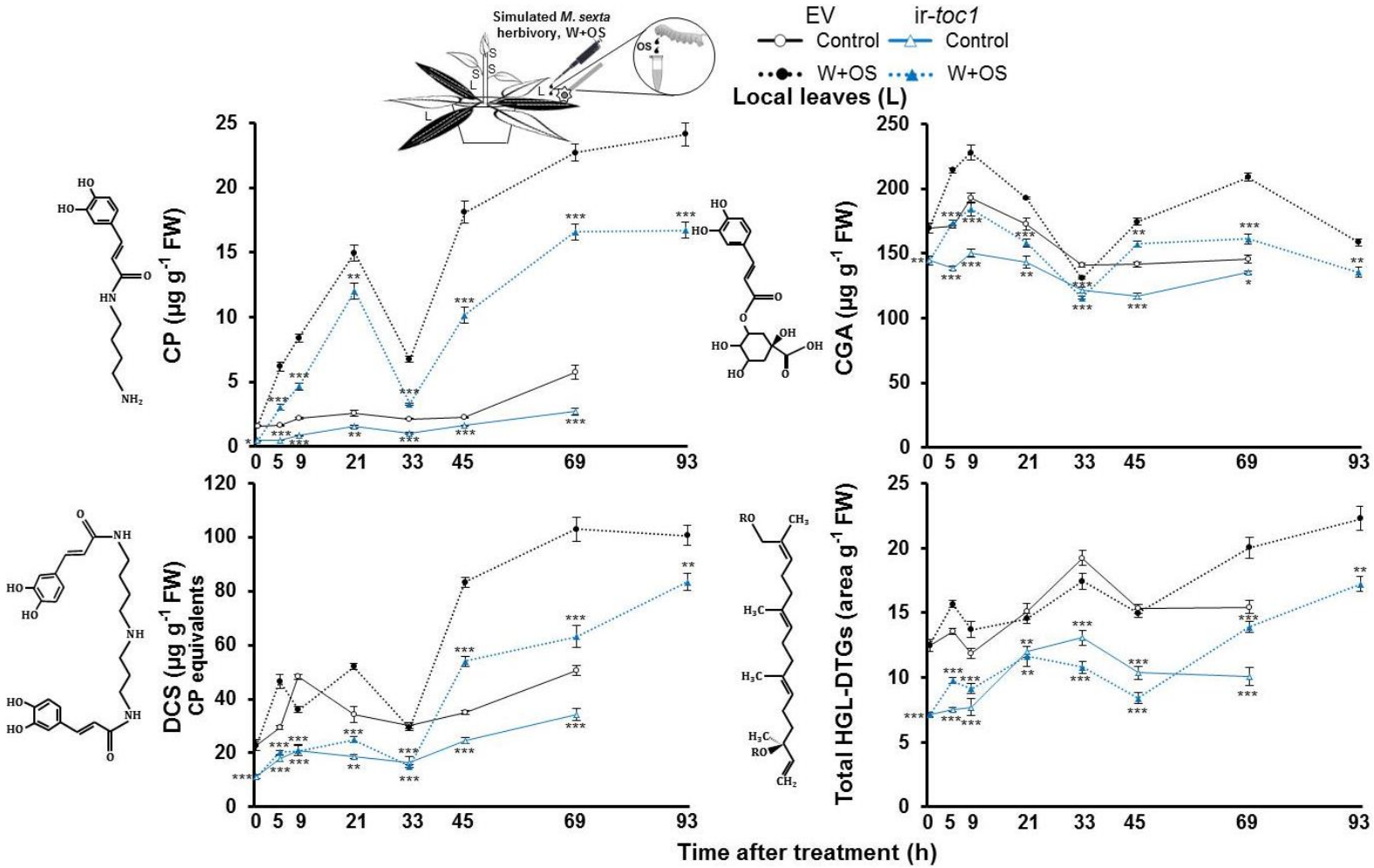

Figure 1. *TOC1* regulates the accumulation of CP, DCS, CGA and HGL-DTGs in untreated and W+OS elicited leaves *N. attenuata* plants. Ten days after transfer to 1L pots, rosette leaves of five independent (n=5) empty vector (EV) and ir-*toc1* plants were wounded (W) with a fabric pattern wheel, and 20 µL of 1:5 diluted *M. sexta* oral secretion (+OS) was directly applied to the leaf lamina at 13:00. Mean (±SE) concentrations of total caffeoylputrescine (CP); dicaffeoylspermidine (DCS), chlorogenic acid (CGA) and 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) following wound and OS application (W+OS) in locally treated leaves of EV and ir-*toc1* plants were quantified by UHPLC-UV. Concentration values along the y-axis of metabolites are not all to the same scale. Asterisks represent significantly differences between EV and comparably treated ir-*toc1* plant tissues at specific time points determined by Student's *t* test at P<0.05 (*); P<0.01 (**) and P<0.001 (***).

TOC1 regulates the accumulation of phenylpropanoid intermediates and amine building blocks for biosynthesis of phenolamides

Central clock genes have hypothesised roles in regulating nitrogen assimilation that directly influences nitrate nutritional status (Gutiérrez *et al.*, 2008). This begs the question as to whether the largest metabolic changes we observed in phenolamides (aforementioned CP and DCS) of ir-*toc1* plants, which are directly linked with both carbon (phenylpropanoid) and nitrogen (polyamine) metabolism, could be translated from alterations in upstream precursors. We therefore examined the accumulation of nitrogen-containing aromatic amino acid precursors of phenylpropanoid derivatives and amine building blocks for the biosynthesis of the phenolic and amide moieties integrated to form phenolamides (Figure 2). Wound and OS treatment of EV plants led to sharp increases in tyramine (~80%; p-value <0.001) and phenylalanine (~55%; p-value <0.001) compared to phyllotactic equivalent tissues of non-wounded plants. The polyamine pathway is under tight control in plants (reviewed in Kusano and Suzuki, 2015) and thus smaller differences in accumulation of putrescine and spermidine were observed between untreated and W+OS-treated EV plants. Whilst accumulation patterns of these phenolamide precursors in ir-*toc1* plants generally followed the same trends as observed in EV plants, levels of phenylalanine (p-value <0.05) and tyramine (p-value <0.01) were overall 15–25% higher in untreated and W+OS-elicited ir-*toc1* plants. Differences between EV and ir-*toc1* plants were greatest at 9 hours with silenced *TOC1* plants having ~50% more tyramine and ~70% more phenylalanine than comparative EV plants. Levels of spermidine were also generally higher in both untreated (~10%; p-value <0.05) and W+OS-elicited (~15%; p-value <0.05) silenced *TOC1* plants compared to comparative EV control plants, with differences greatest 1 hour following W+OS-elicitation (~30% increase; p-value <0.001). Interestingly, putrescine levels were reduced in ir-*toc1* plants, with untreated and elicited plants having 10–15% lower (p-value <0.05) levels than in corresponding EV plants (Figure 2).

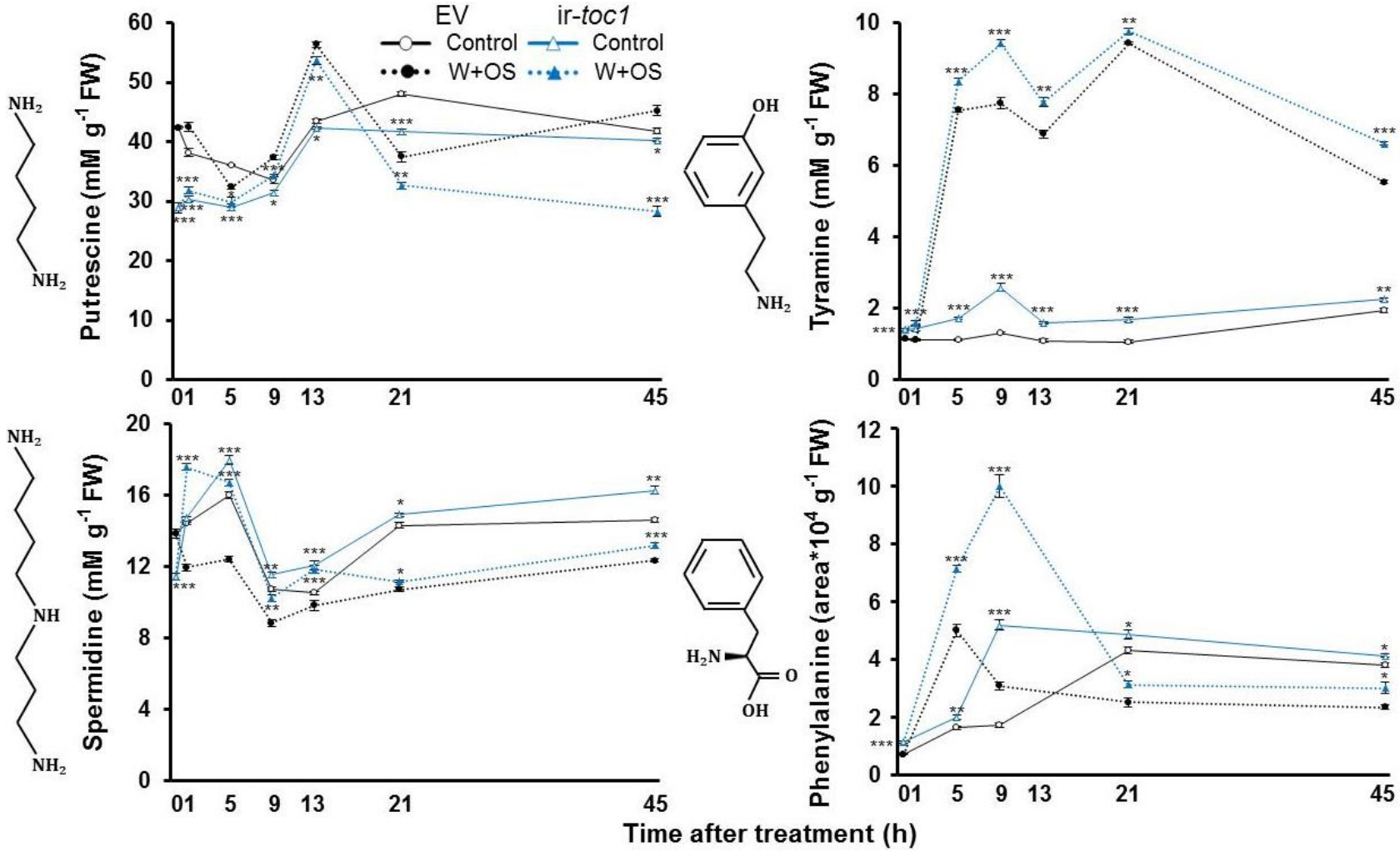

Figure 2. Na*TOC1* regulates the accumulation of a phenylpropanoid intermediate and amine 'building blocks' of phenolamides, in untreated and W+OS elicited leaves of *ir-toc1* plants. Rosette leaves of five independent (n=5) empty vector (EV) and ir-*toc1* plants were W+OS-treated as described in Figure 1. Mean (±SE) concentrations of tyramine, putrescine, spermidine and phenylalanine, following wound and OS application (W+OS) in locally treated leaves (L) of EV and ir-*toc1* plants, quantified by HPLC-/fluorescence. Asterisks represent significantly different concentrations of metabolites determined by Student's *t* test in EV compared to comparably treated ir-*toc1* plant tissues at specific time points at P<0.05 (*); P<0.01 (**) and P<0.001 (***).

TOC1 modulates the incorporation of ^{15}N into leaf phenolamides

By calculating the percentage of ^{15}N-investment into phenolamide defences before and after simulated herbivory, we were able to explore *TOC1* control of nitrogen allocation into phenylpropanoid biosynthesis. We used labelled nitrogen (^{15}N) pulse labelling to follow the investment of a defined N-pool into phenolamide leaf pools over time (Figure 3A). All regio-isomers of known tobacco phenolamides were screened for in local and systemic leaves (Figure S2 and S3), and only those which were consistently detected are shown (Figure 3B). Whilst analysis of silenced *TOC1* plants showed similar incorporation patterns to EV plants; incorporation of ^{15}N into inducible mono-acylputrescine and mono- and di-acylspermidine conjugates was generally 20–30% lower in ir-*toc1* plants (Figure 3B). We identified a number of specific isomers of CP, DCS, caffeoylferuloylspermidine (CFS) and diferuloylspermidine (DFS) (Figure S3 and S3), which were also present prior to W+OS treatment and *TOC1* was shown to influence the incorporation of ^{15}N into these metabolites in untreated conditions, as well as during their de novo synthesis following simulated herbivory (Figure 3B). We found that incorporation of ^{15}N into total coumaric-, caffeic- or ferulic-based phenolamide budgets was significantly reduced in ir-*toc1* plants, revealing similar reductions regardless of the hydroxycinnamic conjugated, which was regulated by *TOC1*. We conclude that *TOC1* regulates the overall extent or amount of nitrogen (^{15}N) integrated, and not the patterning or period of incorporation of ^{15}N into both untreated and elicited phenolamides.

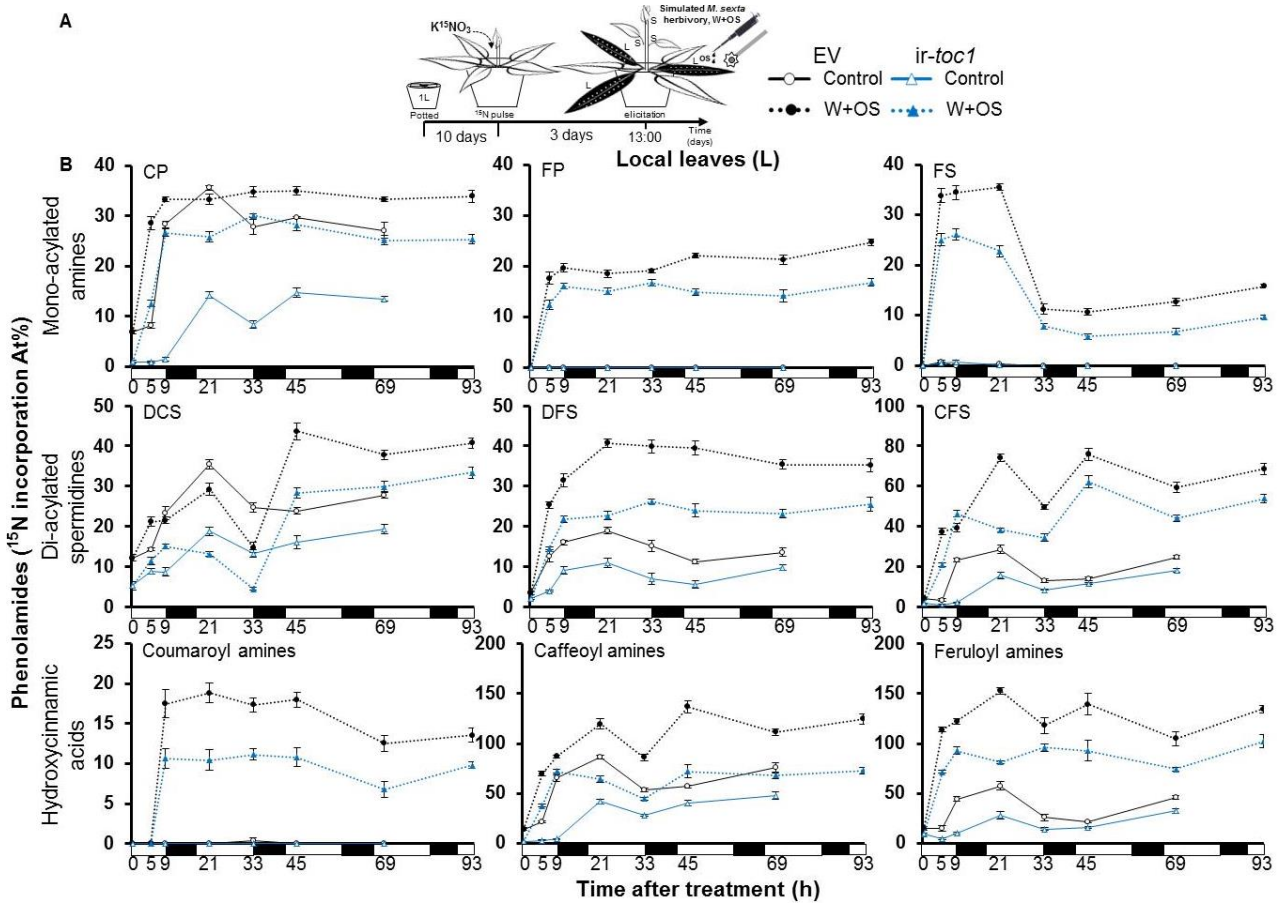

Figure 3. The dynamics of ^{15}N-incorporation into major phenolamides and total courmaroyl-, caffeoyl- and feruloyl-amine conjugates in EV versus ir-*toc1* plants. (A) Schematic representation demonstrating the elicitation and collection strategy used to study the *TOC1*-controlled dynamics of ^{15}N-incorporation into major phenolamides. Ten days after transfer to individual 1L pots, plants were pulse labelled with 5.1 mg nitrogen delivered as $K^{15}NO_3$. Three days later, the first three fully expanded rosette leaves of *N. attenuata* plants (randomised; n=5) biological replicates per treatment and genotype were W+OS-elicited as described in Figure 1. ^{15}N-incorporation was determined as described by Ullmann-Zeunert *et al*. (2012). **(B)** Mean (±SE) of ^{15}N incorporation into the main mono-acylated amines (top row), di-acylated amines (middle row) and total phenolic acid conjugated forms (bottom row) in locally treated leaves from five independent (n=5) empty vector (EV) and *ir-toc1* plants. *TOC1* influences the channelling and incorporation of ^{15}N into phenolamides over time in untreated and herbivory stressed (W+OS) plants. Significant differences between EV and comparable *ir-toc1* plants at specific time points were determined by Student's *t* test and are indicated by asterisks at P<0.05 (*); P<0.01 (**) and P<0.001 (***). At%, atomic percentage.

Key defence regulatory genes are regulated by TOC1

In EV plants, and consistent with previous studies in *N. attenuata* (Kang *et al*., 2006; Kaur *et al*., 2010; Onkokesung *et al*., 2012; Woldemariam *et al*., 2013), W+OS treatment resulted in substantial increases in transcript levels of key genes involved in MAPK stress signalling (*SALICYLATE-INDUCED PROTEIN KINASE; SIPK*), defence metabolism (*PAL1* and *THREONINE DEAMINASE; TD*), JA-associated TFs (*MYC2* and *MYB8*) in leaves of EV plants, as well as root expression of *GLUTAMINE SYNTHETASE* (*GS*), a key enzyme marker that acts in assimilation and flux of nitrogen (Figure 4; Miflin and Habash, 2002).

While we generally observed a rapid increase in gene expression in EV plants between 1 and 5 hours after W+OS-elicitation, this was strikingly attenuated in ir-*toc1* plants. A marked reduction (~30%; p-value <0.01) in gene transcript levels of *SIPK* was observed in the first 5 hours after W+OS treatment of ir-*toc1* compared to EV plants (Figure 4B). This difference was further exacerbated in ir-*toc1* plants at 9 hours, with silenced transgenics being ~70% lower than comparative EV plants (p-value <0.001). Silenced *TOC1* plants also exhibited substantially lower transcript levels of *MYC2* (~70%; p-value <0.001) and *MYB8* (~75%; p-value <0.001) 1 hour after W+OS treatment (Figure 4B). Interestingly, gene expression of *MYC2* in locally treated leaves of ir-*toc1* plants was ~20% higher at 5 (p-value <0.05) and 21 hours (p-value <0.01) after W+OS-elicitation than in comparative EV plants. Similarly, *MYB8* gene expression was also increased (~30%; p-value <0.05) in ir-*toc1* plants 9 hours following W+OS treatment, which was reduced thereafter (~30%; p-value; <0.05) in silenced transgenics compared to EV plants (Figure 4B). Expression levels of *PAL1* and *TD* were also clearly altered in locally treated leaves of silenced *TOC1* plants (Figure 4B and S4). Whilst their expression was significantly lower at 1 (15–20%; p-value <0.01), 9 (40–60%; p-value <0.001) and 21 hours (40–45%; p-value <0.01) after W+OS-elicitation, levels were 30–40% higher (p-value <0.001) than comparative EV plants 5 hours after treatment. We additionally found that expression of GS was modestly down-regulated (20–40%) in W+OS-elicited ir-*toc1* plants.

JA-ET cross-talk is known to specifically tailor *M. sexta* associated defence responses in *N. attenuata*. We thus inhibited the plant perception to endogenous ET using 1-MCP and observed that *TOC1*-regulation of induced phenylpropanoids is uncoupled from OS-induced ET production (Figure S5 and S6).

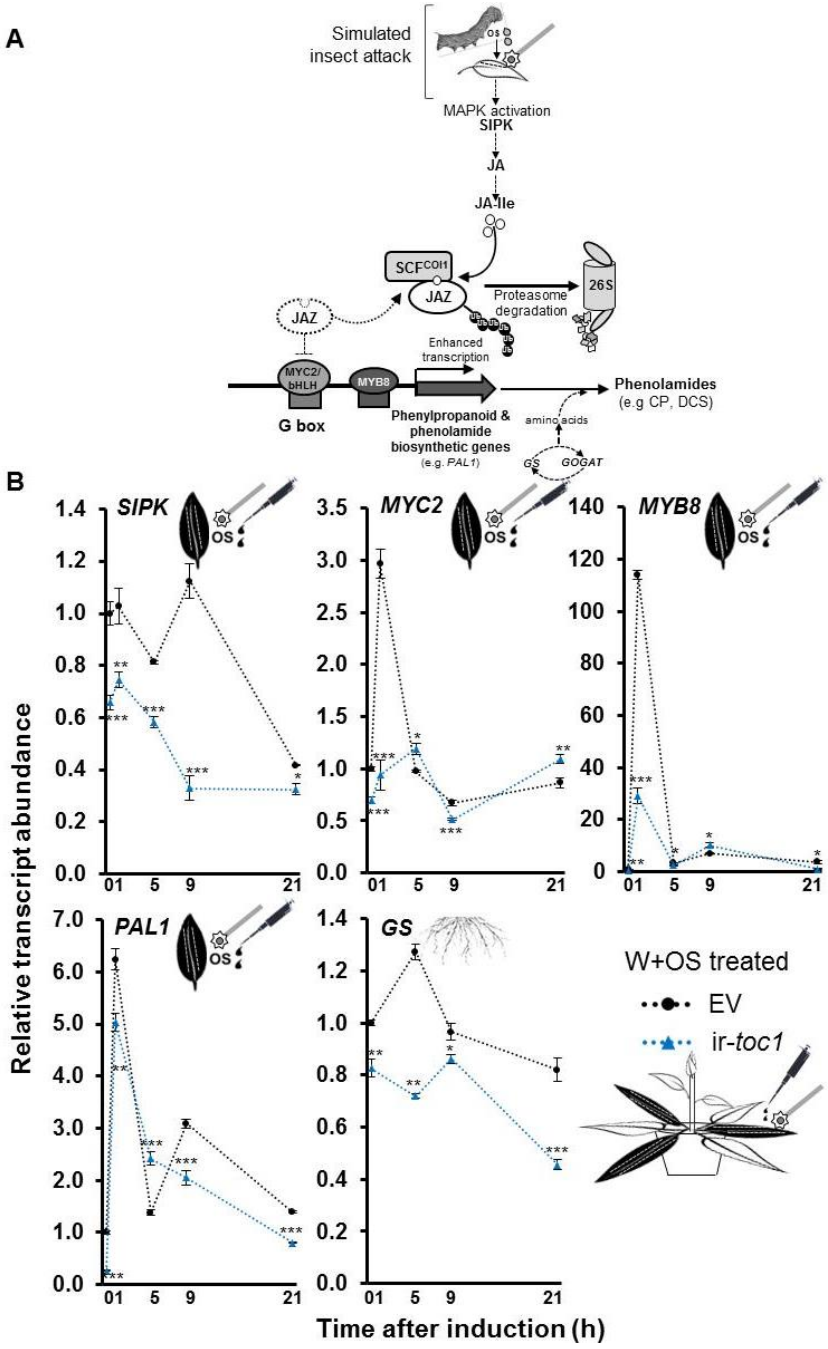

Figure 4. Na*TOC1* influences transcript levels of key marker genes involved in herbivore perception (*SIPK*), nitrogen assimilation (*GS*), jasmonate (JA) regulatory networks (*MYC2* and *MYB8*) and phenylpropanoid biosynthetic genes (*PAL1*) in *N. attenuata*. (A) Schematic diagram depicting a simplified version of the JA signalling cascade and JA-mediated regulation of phenylpropanoid biosynthesis in tobacco (adapted from Shoji and Hashimoto, 2013; Zhang *et al.*, 2012). Leaf wounding and/or herbivore attack induces JA and JA-Ile bursts. The accumulation of JA-Ile mediates the COI1-JAZ interaction resulting in the ubiquitination of JAZ repressors by the SCF-COI1 complex and degraded by the 26S proteasome. The removal of JAZ, releases the downstream TF, MYC2, which is proposed to activate the transcription of defence-related genes, either directly by binding to the G-box motif or indirectly by activating secondary TFs such as MYB8 (in cooperation with MYC2). **(B)** Mean (±SE) transcript abundance (relative to Na*EF-1α*) of salicylate-induced protein kinase (SIPK), glutamine synthetase (GS), MYC2 and MYB8 (JA-responsive) transcription factors, and phenylalanine ammonia lyase 1 (PAL1) in leaf lamina (from locally treated leaves) from five independent (n=5) samples per genptype, each containing two technical replicates. Asterisks represent significantly different levels of transcripts between empty vector (EV) and ir-*toc1* at specific time points determined by Student's *t* test at P<0.05 (*); P<0.01 (**) and P<0.001 (***).

TOC1 regulates the production of induced amino acids and phenolamides in response to herbivore attack

In order to examine the wider targets of *TOC1* on nitrogen-containing primary metabolites in *N. attenuata,* we determined the production of herbivore-induced amino acids in EV and ir-*toc1* plants that were fed on by *M. sexta* or *S. littoralis* caterpillars for 12 days (Figure 5). We found in general that the concentration of all amino acids increased in ir-*toc1* plants compared to EV plants, regardless of whether the plants were fed on by *M. sexta* or *S. littoralis* caterpillars. Silencing of *TOC1* led to markedly higher (15–30%) levels of alanine, asparagine, glycine, glutamine, histidine, isoleucine, leucine, lysine, ornithine, proline, tryptophan, tyrosine and valine when fed on by *M. sexta* caterpillars compared to EV plants (p-value <0.05; Figure 5). Levels of arginine, aspartic acid, cysteine, glutamic acid, methionine, phenylalanine, serine and threonine were also substantially elevated (40–60%; p-value <0.01) in *M. sexta* fed ir-*toc1* plants. It is noteworthy that in most cases, accumulation of amino acids in ir-*toc1* plants attacked by either *S. littoralis* or *M. sexta* were increased to the same extent in comparison to EV plants, except for alanine, histidine, leucine, methionine, ornithine, threonine, which were found to accumulate even higher in silenced transgenics fed on by *S. littoralis*.

To elucidate the involvement of *TOC1* in elicitation of phenylpropanoid biosynthesis and disentangle this from upstream herbivore-specific modulations, we directly measured the production of phenylpropanoid metabolites in systemic leaves of EV and ir-*toc1* plants that were fed on by *M. sexta* or *S. littoralis* caterpillars for 12 days. Analysis by UPLC-Time-of-Flight mass spectrometry (UPLC-ToF-MS) revealed clear differences in specific phenylpropanoids between EV and ir-*toc1* plants (Figure 6). Levels of phenylalanine following feeding from either *M. sexta* or *S. littoralis* were ~2-fold higher in ir-*toc1* plants compared to EV (p-value <0.001). Silencing *TOC1* led to lower levels of mono-acyl conjugates such as CP (2–3-fold; p-value <0.001), feruloylputrescine (~20%, p-value <0.05) and feruloylspermidine (~2-fold; p-value <0.01) following caterpillar herbivory. Similarly, di-acylspermidine conjugates (such as CFS, DCS, DCoS, DFS) and quinate conjugates such as CGA, were reduced by ~2-fold (p-value <0.01) in ir-*toc1* plants on which caterpillars had fed, demonstrating ir-*toc1* plants had significantly diminished phenylpropanoid defences (Figure 6). Interestingly, the effect of the two different herbivores on phenylpropanoids was not substantially different in ir-*toc1* plants compared to EV.

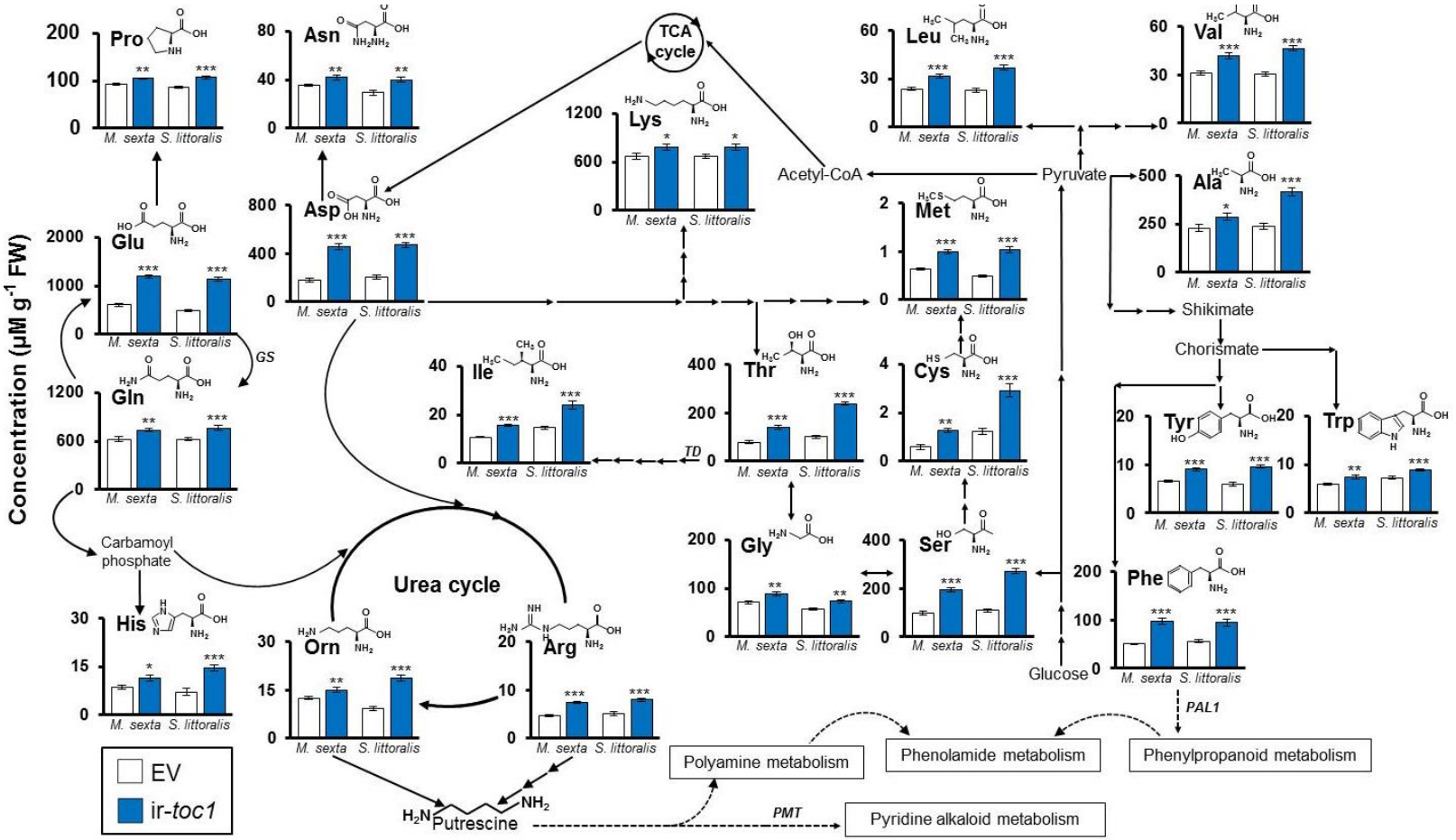

Figure 5. Na*TOC1* regulates the accumulation of amino acids in *M. sexta*- and *S. littoralis*- attacked plants. Caterpillars were allowed to feed on *N. attenuata* empty vector (EV) and ir-*toc1* plants for 12 days before harvesting systemic leaves for analysis. Mean (±SE) concentrations of amino acids in systemic leaves of sixteen independent (n=16) empty vector (EV) and ir-*toc1* plants following feeding from either specialist (*M. sexta)* or generalist (*S. littoralis*) caterpillars. Asterisks represent significant differences between comparably treated EV and *ir-toc1* genotypes determined by Student's *t* test at P<0.05 (*); P<0.01 (**) and P<0.001 (***). Ala, alanine; Asn, asparagine; Asp, aspartic acid; Arg, arginine; Cys, cysteine; FW, fresh weight; Glu, glutamic acid; Gln, glutamine; GS, glutamine synthetase; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Orn, ornithine; PAL1, phenylalanine ammonia lyase; Phe, phenylalanine; PMT, putrescine methyltransferase; Pro, proline; Ser, serine; TCA, The citric acid/Kreb's cycle; TD, Threonine deaminase; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine.

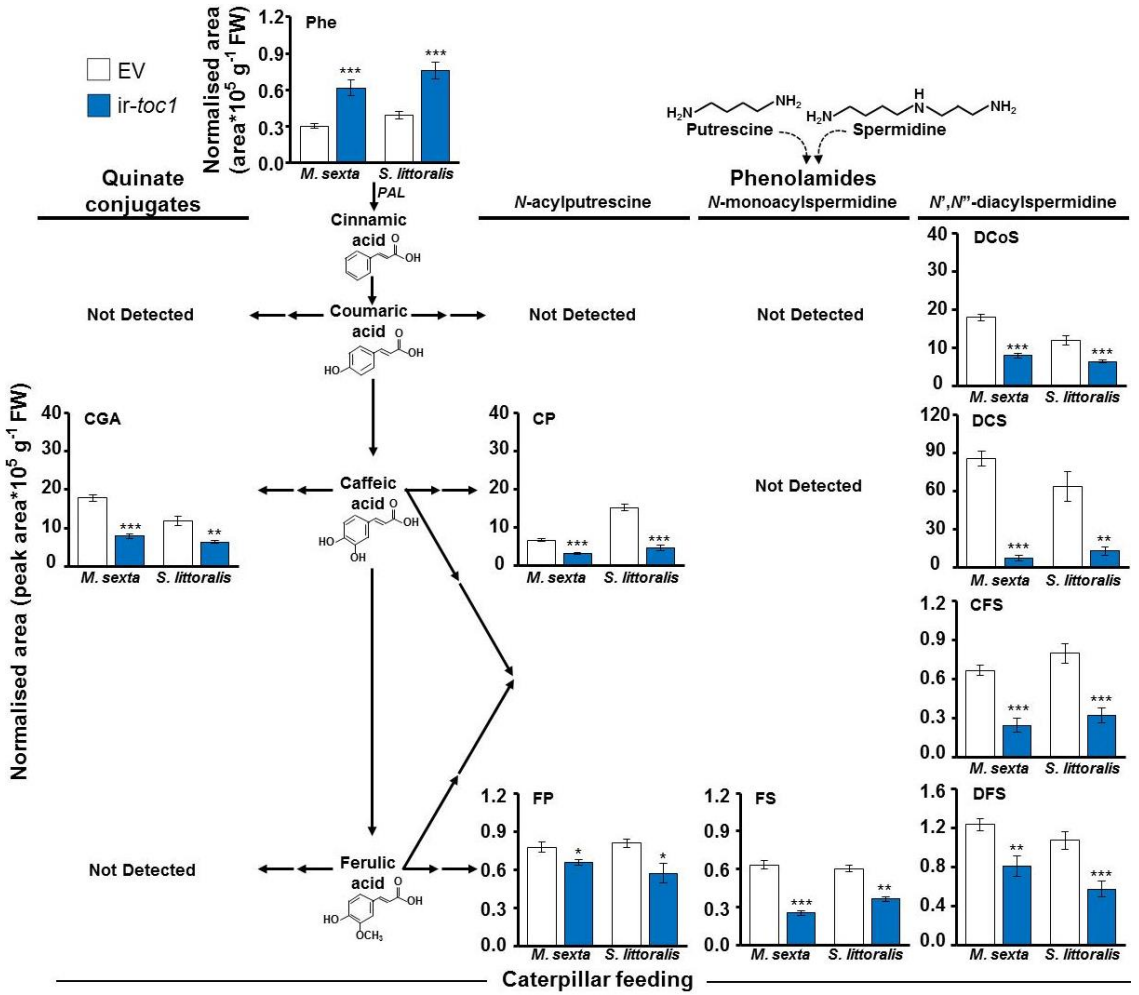

Figure 6. Silencing *TOC1* in *N. attenuata* affects the production of phenylalanine (Phe) intermediates, mono- acylputrescine, mono- and di- acylspermidine and quinate conjugates in caterpillar-fed plants, in a herbivore unspecific manner. Mean (±SE) concentrations of systemic leaf phenylpropanoids detected in sixteen independent (n=16) empty vector (EV) and ir-*toc1* plants following feeding from either specialist (*M. sexta)* or generalist (*S. littoralis*) caterpillars are shown. Significant differences among comparable EV and ir-*toc1* treated plants are represented by asterisks determined by Student's *t* test at P<0.05 (*); P<0.01 (**) and P<0.001 (***). CP, caffeoylputrescine; CFS, caffeoylferuloylspermidine; CGA, chlorogenic acid; DCoS, dicoumaroylspermidine; DCS, dicaffeoylspermidine; DFS, diferulyolspermidine; FP, feruloylputrescine; FS, feruloylspermidine; FW, fresh weight; Phe, phenylalanine.

Silencing TOC1 renders N. attenuata more vulnerable to attack by two native herbivores

In order to further clarify the mechanisms of *TOC1*-control on defence metabolism and resistance to herbivore attack, we examined the caterpillar performance of the day-active specialist, *M. sexta* and the nocturnal generalist, *S. littoralis* on EV and ir-*toc1* plants (Figure 7). We found that silencing *TOC1* positively influenced *M. sexta* larval mass gain. *S. littoralis* caterpillars were larger when fed on ir-*toc1* plants between days 7 and 9 only. We conclude that *TOC1* influences the vulnerability of plants to insect attack in a herbivore specific manner. A working model illustrating the role of *TOC1* in modulating JA-mediated phenolamide defences is shown in Figure 8.

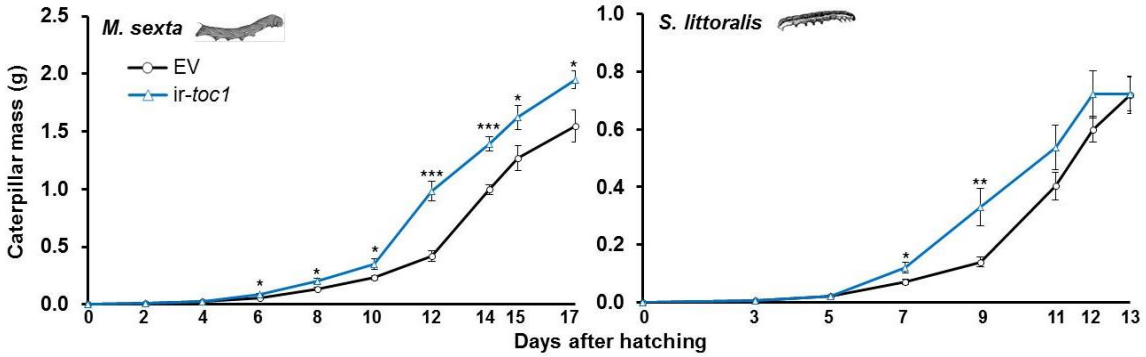

Figure 7. Silencing *TOC1* makes *N. attenuata* plants vulnerable to insect attack in a herbivore specific manner. Mean (±SE) mass gained by *N. attenuata* generalist herbivore (*S. littoralis*) and adapted herbivore (*M. sexta*) larvae that fed on stem leaves ofempty vector (EV) and *ir-toc1* plants. Two freshly hatched *M. sexta* neonates and six-day old *S. littoralis* larvae were placed on separate transition leaves of ten independent rosette-stage EV and *ir-toc1* plants (2 caterpillars per plant; n=20) and enclosed individually in well-aerated clip cages. After weighing, the caterpillars were placed on a new previously undamaged leaf of the same plant. Asterisks represent significantly different growth responses of herbivores that fed on EV and *ir-toc1* plants determined by Student's *t* test at specific time points at P<0.05 (*); P<0.01 (**) and P<0.001 (***).

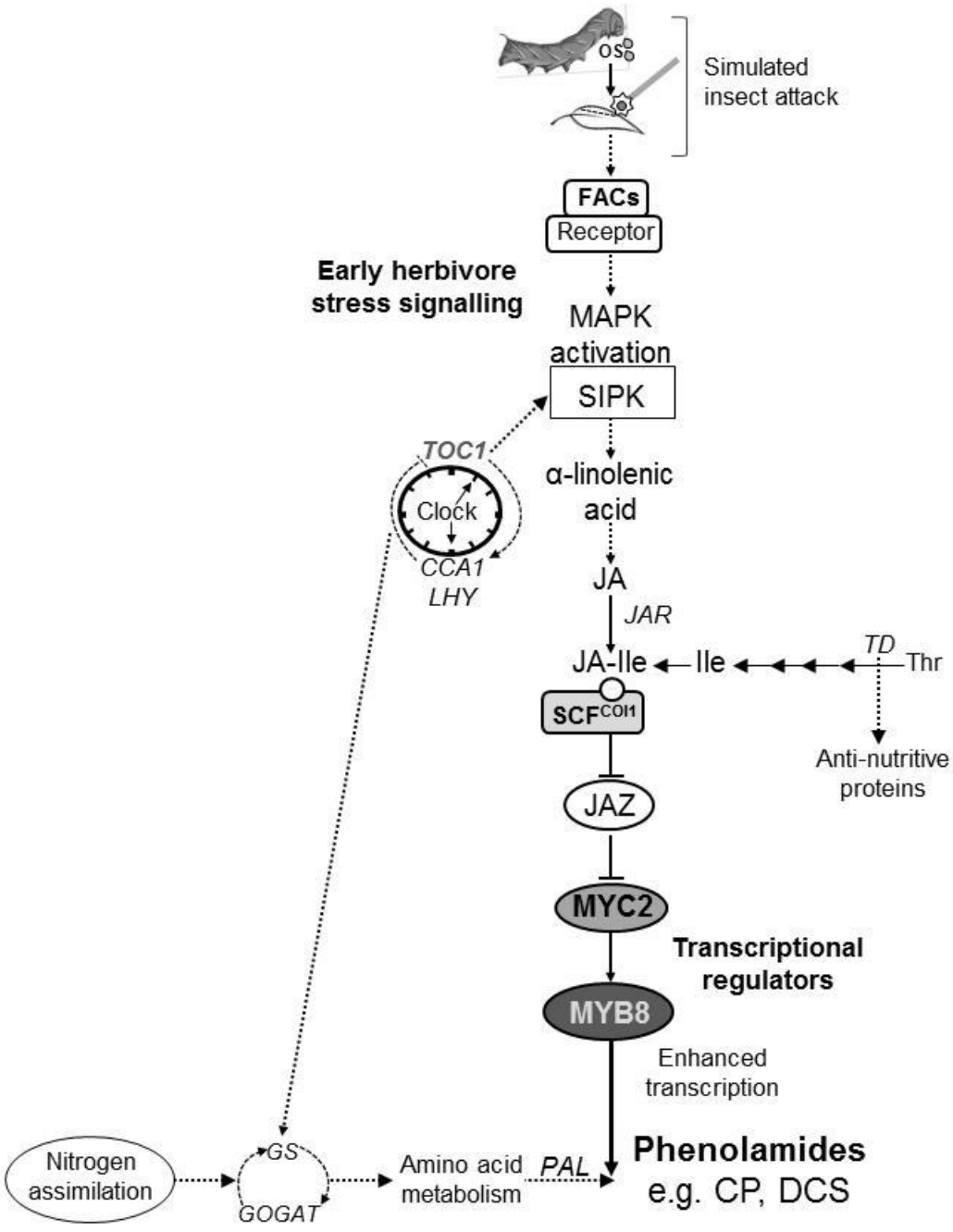

Figure 8. A working model of the *TOC1*–regulation of plant defence responses in *N. attenuata via* interaction of major defence hormone signals and transcriptional regulators. Herbivore attack, composed of wound signal and release of insect elicitors to the wounds, induces a strong burst of the defence hormone JA. JA is required to trigger accumulation of a number of defence chemicals, including phenolamides. The involvement of TOC1 in hormonal and nitrogen homeostasis, as a means of tuning the defence response in plants is hypothesised.

3.4. Discussion

The significance of tightly disciplined molecular networks in driving rhythms in hormone signalling systems has become increasingly clear over recent years (reviewed in Grundy *et al*., 2015). Hormones affect most of the known circadian-controlled processes in plants as well as their synthesis and turnover being under rhythmic control (Thain *et al.,* 2004; Nováková *et al.,* 2005; Hanano *et al.,* 2006; Covington and Harmer, 2007; Covington *et al.,* 2008; Michael *et al.,* 2008; Mizuno and Yamashino, 2008; Legnaioli *et al.,* 2009; Robertson *et al.,* 2009; Arana *et al.,* 2011; Shin *et al.,* 2012; Goodspeed *et al.,* 2013a). It is likely, at least in some cases that the clock operates through changes in hormone levels or hormone perception. These advances have led to the exploration of the connection between hormone signalling and defence networks, which are gated by one or more core circadian oscillators. This has revealed that hormone cascades, as well as downstream TFs (Shin *et al.,* 2014), that drive the transcriptional activation (Bhardwaj *et al.,* 2011; Goodspeed *et al.,* 2012) or repression (Chapter 2) of key biosynthetic genes and defence metabolites are also mitigated by the clock. In the present study, we examined the wider metabolic consequences of down-regulating *TOC1* in *N. attenuata,* utilising a characterised transgenic line derived from the study of Yon *et al*. (2012). In addition to determining the effects of *TOC1* down-regulation upon phenylpropanoid and diterpene glycoside metabolism in plants, we also studied effects upon amine and amino acid levels in leaves of herbivore-attacked plants. The results presented here provides insights into how plants utilise the clock to integrate and coordinate phytohormone signals to accomplish resistance responses, particularly the combination of JA and ET signals in herbivore-challenged plants, to orchestrate and regulate the flux of nitrogen precursors into closely linked nitrogen defence pathways.

Given the large role *TOC1* and JA-ET cross-talk plays in regulating nicotine biosynthesis in *N. attenuata* (Chapter 2) as a defence against a Solanaceous specialist, *M. sexta*, we examined the involvement of *TOC1* in modulating other abundant *N. attenuata* defences including HGL-DTGs, phenylpropanoid derivatives and phenolamides under basal and W+OS-induced conditions. The production of these metabolites are known to be upregulated by both wounding, application of caterpillar OS and herbivory (Jassbi *et al*., 2008; Kaur *et al.,* 2010; Heiling *et al.,* 2010; Kim *et al.,* 2011). In this study we found that *TOC1* acts as a positive regulator in shaping basal and herbivory stimulated increases in HGL-DTG, phenylpropanoid and phenolamide biosynthesis (Figure 1), which is in

strong contrast to its action on nicotine biosynthesis (Chapter 2). In general, silencing *TOC1* severely attenuated the local (Figure 1) and systemic (Figure S1) accumulation of HGL-DTGs, CP, DCS and CGAs in untreated and W+OS-elicited ir-*toc1* plants compared to their phyllotactic equivalent tissues in EV plants. Many of these metabolites have been previously shown to display daily cycling patterns (Kim *et al.*, 2011), which supports the influence of *TOC1* expression on their constitutive and de novo synthesis. The mechanism underlying the effects observed on these pathways in silenced *TOC1* plants is not well understood; however, their accumulation is known to be strongly regulated by JA and associated TFs (Kaur *et al.*, 2010; Heiling *et al.*, 2010; Kim *et al.*, 2011; Woldemariam *et al*., 2013). Metabolism of phenylpropanoids and HGL-DTGs are not directly relying on metabolism of similar products. However, their pathways appear to be conjointly regulated by the clock, perhaps through similar co-opted transcriptional regulatory systems. Inducibility of defence metabolites allows plants to minimise metabolic constraint and fitness consequences associated with the production of costly defence resources to demand. In order to efficiently articulate such costly metabolic responses, data presented here suggests that close coordination of *TOC1* in plants drives hormone-specific defence responses, shown by silenced *TOC1* plants that displayed a reduced ability to mount a defence response.

When plants are challenged by different herbivores, a substantial increase in JA synthesis generally occurs through transcriptional induction of key biosynthetic genes, as well as downstream transcription factors (TFs) MYC2 and MYB8 (Halitschke and Baldwin, 2003; Kandoth *et al*., 2007; Paschold *et al*., 2007; Kazan and Manners, 2008; Kaur *et al.*, 2010; Woldemariam *et al.*, 2013). However, mechanisms by which herbivore-signals trigger defence signals in local tissues, and the regulatory controls that are involved, are not fully understood. In the current study we examined transcript levels of relevant genes associated with early herbivore-induced stress signalling, nitrogen assimilation and JA-mediated transcriptional responses, which mediate specific defence reactions including phenolamide metabolism. In line with previous studies (Kang *et al*., 2006; Kandoth *et al*., 2007; Paschold *et al*., 2007; Wu *et al*., 2007; Kaur *et al.*, 2010; Onkokesung *et al.*, 2012; Ullmann-Zeunert *et al.*, 2013; Woldemariam *et al.*, 2013) in *N. attenuata* we showed that transcript levels of SIPK, MYC2, MYB8, PAL1, GS and TD genes are rapidly upregulated in leaves of EV plants in response to simulated herbivory (Figure 4). In the initial hours following simulated herbivory plants harbouring an ir-*toc1*

construct in general displayed reduced capacity for stimulation of relevant gene transcripts.

We observed marked reductions in levels of *SIPK* transcript in leaves of ir-*toc1* plants following simulated herbivory compared to EV control plants. Activation of SIPK, a key kinase in *N. attenuata*, regulates TFs and phytohormone biosynthetic enzymes that modulate JA- and ET-mediated defence responses involved in facilitating plants' resistance to herbivores (Zhang and Klessig, 2001; Liu and Zhang, 2004; Kandoth *et al.*, 2007; Wu *et al.*, 2007). Comparable with silencing *SIPK* activity in *N. attenuata*, which abolished half of the W+OS-elicited ET produced (Wu *et al*., 2007), reductions in expression of *SIPK* (Figure 4B) may account for diminished production of both OS-induced ET and JA observed in ir-*toc1* plants (Chapter 2). The speed at which *SIPK* levels decrease in ir-*toc1* plants following simulated herbivory suggests that positive regulation of *SIPK* to herbivore attack may to some degree, be dictated by the clock. The possibility that the clock has a direct bearing upon regulation of SIPK signalling needed for triggering a metabolic elicitation response is intriguing and deserves further attention. Such a suggestion is compatible with results presented by Thain *et al*. (2004), who reported that *TOC1* regulates rhythmic ET production through clock control of 1-amino-cyclopropane-1-carboxylic acid synthases (ACS). SIPK was shown to directly phosphorylate a number of ACSs involved in ET biosynthesis in *Arabidopsis*, dramatically increasing their stability (Liu and Zhang, 2004), as well the expression of a number of genes involved in JA biosynthesis in *Nicotiana* (Kandoth *et al*., 2007; Wu *et al*., 2007). Reduced 'perception' of herbivory, seen by lower transcripts of *SIPK* in ir-*toc1* plants, we speculate results in a weakened ability or sensitivity to initiate the plethora of herbivory-induced signal cascades and mount an efficient defence response. We predict that *TOC1* tightly regulates a number of hormone signals under both basal and stressed conditions. This may enable plants to coordinate antagonistic and synergistic cross-talk signals that differentially regulate and gate hormone-mediated plant responses towards the production of specific spectrums of specialised metabolites. Coordinated clock regulation of plant responses in both antagonistic and synergistic manners would help plants adapt to fluctuating environments. It may be advantageous for the clock in *N. attenuata*, and perhaps most plants, to act *via* components of the MAPK signalling network or co-opted JA- and/or ET-dependent TFs to coordinate the necessary transcriptomic and/or proteomic reconfigurations that occur in response to herbivory and

possibly a multitude of other stresses. This is supported by dramatic and abrupt changes in herbivore-induced phytohormone responses and differential effects on defence metabolism in plants lacking *TOC1* (Chapter 2). Specifically, synthesis of nicotine was elevated while levels of JA, ET, phenolamides, phenylpropanoid quinate conjugates and HGL-DTGs were reduced; demonstrating that the differential regulation of carbon- and nitrogen-based defences in plants by the circadian clock is more complex than first assumed.

With regards to downstream TFs associated with JA and defence, we found that gene transcript levels of *MYC2* and *MYB8* were significantly down-regulated in leaves of ir-*toc1* plants compared to EV plants, particularly within the initial hours following simulated herbivory (Figure 4B). It has been suggested that there is a strong clock-controlled correlation between primary and secondary metabolism and the expression of genes involved in nitrogen uptake and remobilisation (Harmer *et al.,* 2000; Gutiérrez *et al.,* 2008). We found that expression of *GS*, a primary nitrogen assimilatory enzyme that acts at the centre of nitrogen flux to influence nitrate and ammonia nutritional status and nitrogen use efficiency (reviewed in Miflin and Habash, 2002), was diminished in ir-*toc1* plants in response to herbivory simulation. This suggests that the uptake, assimilation or efficient use of nitrogen may be, in part, controlled by *TOC1*. Members of the family of R2R3-MYB TFs in plants also have important roles in controlling expression of genes involved in nitrogen assimilation (Imamura *et al.,* 2009; Miyake *et al.,* 2003) and thus *TOC1*, *via* action of *MYB8*, may mediate changes in growth-defence resource allocations by playing a role in assimilation and apportionment of nitrogen fluxes in plants.

Previous ^{15}N experiments in *N. attenuata* using ir-*toc1* plants demonstrated that whole-plant and tissue-specific investment of ^{15}N into nicotine was negatively regulated by the clock (Chapter 2). In stark contrast, we demonstrated that *TOC1* positively affected incorporation of ^{15}N into a large number of phenolamides (Figure 3B). Silenced *TOC1* plants displayed lower incorporation of ^{15}N into both monoacyl putrescine and di- and mono-acyl spermidine conjugates. *TOC1* regulation was not restricted to particular hydroxycinnamic acids and thus it was shown that *TOC1* positively influenced the incorporation of ^{15}N into all present combinations of coumaric-, caffeic- and ferulic-based phenolamides similarly. Comparison of ^{15}N-investment into nicotine and phenolamide defence compounds revealed that ir-*toc1* plants allocated proportionally higher percentage of ^{15}N into nicotine (Chapter 2) and less into phenolamide metabolites

compared to EV (Figure 2). This suggests that *TOC1* may mediate changes in N-assimilation and/or N-investment into defence compounds. Herbivore-induced differential partitioning of newly fixed carbon and nitrogen into separate secondary metabolite pathways with the aid of *TOC1* may lead to additional reallocation of resources. Further experiments with detailed analysis of different nitrogen and carbon pools are required to fully understand the role the circadian clock plays in carbon and nitrogen partitioning important for growth and fitness of plants with and without hormone perturbations.

It is unclear whether these transcriptional changes are downstream effects of altered SIPK or MYB8 and thus, it may be particularly insightful to ascertain the mechanistic basis accounting for these alterations. Similar to its role in ABA signalling (Legnaioli *et al*., 2009), *TOC1* may regulate a large subset of JA-mediated responses, directly through interaction with associated TFs. Our data suggests a possibility that *TOC1* interacts with other clock components or regulators such as CCA1 and TIME FOR COFFEE (TIC), which have demonstrated roles in regulating JA responses and associated TFs (Covington *et al*., 2008; Shin *et al*., 2012; Goodspeed *et al*., 2012; 2013a; Nagel *et al*., 2015). TIC gene activity specifically, is required for maintaining circadian period and amplitude of JA (Hall *et al.*, 2003; Ding *et al.*, 2007), which is achieved by TIC-directed repression of MYC2 (Shin *et al.*, 2012) and could account for some of the differences we observed. Whether regulation of JA-defences, is attained only through control of MAPK signalling components, or whether it achieves these reactions *via* interactions with other clock-regulators or TFs are possibilities. Previous reports employing biochemical analysis of *MYB8* silenced *N. attenuata* plants (Onkokesung *et al*., 2012) suggested a hypothesis for wound and OS-elicited ET in mediating stabilised and increased expression of *MYB8* in *N. attenuata*. While reductions in both ET (Chapter 2) and *MYB8* gene expression following W+OS-elicitation in *TOC1* silenced plants may support this theory, we demonstrated through the exogenous application of 1-MCP that *TOC1* regulation of W+OS-induced phenolamide metabolism is uncoupled from OS-induced ET production (Figure S5–S6).

As noted previously, synthesis of phenolamides is directly at the junction of nitrogen and carbon metabolism and as such relies on both nitrogenous-amine elements, as well as carbon-based hydroxycinnamoyl chains derived from the phenylpropanoid pathway (Matsuno *et al.*, 2009; Fellenberg *et al.*, 2012; Ullmann-Zeunert *et al.*, 2013). We examined levels of phenylalanine and amines that form the phenolic and amide

components of phenolamides and found that the accumulation profile of these components was regulated by *TOC1* (Figure 2). Accumulation patterns of phenylalanine and tyramine have previously been shown to display rhythmic cycles that are highly inducible following wounding or herbivory (Kim *et al.*, 2011). Their regulation, predicted to be mitigated by the clock, was confirmed in silenced *TOC1* plants, which displayed elevated levels of phenylalanine and tyramine in both untreated and W+OS-elicited plants. These observations suggest that phenylalanine and tyramine may be more prone to clock-dependent alterations translating from changes in hormone signalling, particularly following herbivory. It is noteworthy that leaves of untreated and W+OS-elicited ir-*toc1* plants had significantly reduced levels of putrescine compared to comparative EV plants while levels of spermidine in leaves of silenced *TOC1* plants were surprisingly elevated. This is of particular interest as, in addition to its role in the biosynthesis of higher derived polyamines, putrescine serves as an intermediate in the synthesis of the pyrrolidine ring of nicotine. These observations may accord with previous studies (Chapter 2) that demonstrated elevated levels of nicotine in transgenic *N. attenuata* with down-regulated *TOC1* and may be attributed to its preferential use as a substrate for nicotine and spermidine biosynthesis. Given their pivotal role in growth and development (Kusano and Susuki, 2015), homeostasis of free polyamine levels is under tighter constraints across all *planta* and, compared to phenylalanine and tyramine, is likely controlled by multiple transcriptional stimuli. These results highlight that the constraints on putrescine utilisation destined for nicotine or phenolamide metabolism may be larger than that of spermidine. We concluded that *TOC1* aids in regulating both untreated- and simulated herbivory-levels of major JA-regulated defence metabolites, primarily in branched areas of nitrogen metabolism leading to phenolic and polyamine conjugates.

Experiments investigating primary nitrogen resources in ir-*toc1* plants revealed that *TOC1* exerts dependent regulatory control over inter-related areas of amino acid biosynthesis in *N. attenuata*. This was observed in systemic leaves of ir-*toc1* plants that were previously fed on by two native herbivores. Interestingly, ir-*toc1* plants had not only increased levels of related precursors including ornithine, arginine and phenylalanine, but displayed elevated levels of almost all amino acids, when compared to EV plants. The mechanism underlying the effects observed on primary metabolism as a result of reducing *TOC1* is not well understood; however, their accumulation may result from an effective over-supply of substrates, such as amines, for areas of defence metabolism, which

resulted in remobilisation of excess precursors into areas of amino acid metabolism. It is also possible that large increases in branched areas of primary metabolism could result from perturbations in phytohormones such as JA and SA, contributing to the strong increases in amino acids that were observed (Figure 5). Such a suggestion may agree with the observations of Yun *et al*. (2015), who demonstrated that the accumulation of amino acids is correlated with rising levels of SA, which has been shown to be important in disease resistance in sweat-treated Satsuma mandarin fruit. TOC1 has additionally been linked to SA and the immune regulator, non-expressor of pathogenesis-related gene 1 (NPR1), which in their presence assess the redox state of a plant and regulate the expression of morning and evening clock genes to tune immune responses to the morning (Zhou *et al*., 2015). Together, these findings shed new light on an intriguing relationship between *TOC1* and branched areas of primary and specialised metabolism and reconfirms complexity of the clock-hormone mechanisms that act on these metabolic networks. It emphasises the importance of being able to differentially regulate disparate defence pathways following herbivore pressure and that *TOC1* may act in inducing these defences for efficient utilisation. Interestingly, 5h following W+OS *TOC1* silenced plants exhibited higher transcript levels of *TD*, a gene with suggested roles in defence as an anti-nutritive protein (Kang *et al*., 2006).

We showed that clock-gated defence responses into a spectrum of JA-regulated metabolites following simulated herbivory in the afternoon (Figure 1) or when fed on continuously by *M. sexta* or *S. littoralis* herbivores (Figure 5 and 6) is severely compromised in *TOC1* silenced plants. Activation of herbivore-defence responses by *M. sexta* and *S. littoralis* has been previously reported to be tailored by JA-ET and JA-SA cross-talk, respectively (reviewed in Diezel *et al.*, 2009; Smith *et al.*, 2009; Onkokesung *et al.*, 2010). This may suggest a condition-specific role for *TOC1* regulation of JA, ET and SA hormone targets.

The current study also addressed whether herbivore performance was altered on plants with silenced *TOC* gene activity. We found that *M. sexta* performed better on ir-*toc1* plants than EV plants, while *S. littoralis*, gained more mass on ir-*toc1* only between days 7 and 9 of feeding. Although the effects on phenolamide accumulation is similar in ir-*toc1* plants fed on by either herbivore, the difference in hormone responses induced by feeding by these two herbivores (Onkokesung *et al.*, 2010) highlight possible interactions between the signalling systems that regulate these responses and even other clock

components. For example, JA-mediated responses are regulated by *TOC1* in our analyses but CCA1 has also been identified as a regulator of JA and SA defence responses to herbivore and pathogen attack, respectively. The potential for gene networks to respond largely to or for combinatorial targeting by multiple clock regulatory oscillators and inputs appears to exist. Nagel *et al*. (2015) confirmed that in fact multiple clock regulators, such as *TOC1*, *CCA1*, *PRR5* and *PRR7*, have overlapping interactions with promoter regions of clock-targeted genes. It was shown previously that ir-*toc1* plants contain extremely high levels of nicotine (Chapter 2) and may account for the variation in severity of herbivore resistance between *M. sexta* and *S. littoralis* caterpillars, which differ in their tolerance to nicotine. It would be beneficial to establish the direct cause of increased performance. While our data suggests that the major effects on insect fitness of a specialist herbivore, *M. sexta*, and intermediate effects on a generalist, *S. littoralis* could be due to large reductions in defence chemistry (HGL-DTGs, phenylpropanoid and phenolamide defences), increased accumulation of amino acids and amines cannot be discounted.

The importance of tight diurnal regulation of a plant's defence arsenal is rapidly being recognised. The advances outlined here in understanding the use of sophisticated hormonal networks communicated through the core clock, to coordinate resource acquisition and allocation into dynamic defence metabolites offers new possibilities for manipulating these central metabolite pools. In this study we show that the circadian clock may be involved in resource allocation or prioritisation and thus, primary (amino acids and amines) metabolites were elevated while their defence responses to herbivory were compromised in *TOC1*-silenced plants. This finding suggests that plants are equipped with the transcriptional and metabolic tools to adapt to fluctuating environments. In order to conserve particularly 'expensive' chemical resources for when they are required, clock regulation of defence pathways may provide a mechanism to direct resources and execute highly specific stress responses. These findings may correlate with the necessity for reducing costs of nicotine and increasing the production of other toxins such as phenolamides and HGL-DTG's. The clock may provide a mechanism to change allocation into specific defence spectra, which may be the key to reducing the chances of one herbivore becoming resistant to a number of toxins produced by plants. The relatively large spectrum of regulators that are affected by *TOC1*, that could promote accumulation of various specialised metabolites, suggests that *TOC1* may mediate a broad range of

resistances to fungi, necrotrophic pathogens and herbivores. Our data suggests, at least, during the day, when attacked by two different herbivores, that responses are quite similar. However, this still does not answer the question about plant metabolic priorities or sensitivity during day-time versus night-time responses and whether they prioritise their resources into the production of chemical defences following attack. It would be of interest to explore whether plant clocks have varying sensitivity or can differentiate between different attackers during the day and night. This could be addressed by examining the diurnal metabolic response at specific times to both day active (*M. sexta*) and nocturnal (*S. littoralis*) herbivores to ascertain whether the clock controls nitrogen investment and activation of induced-defences in response to specific attackers at precise times of the day.

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3.5. Supplemental Material

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

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Reference
EF-1a	CCACACTTCCCACATTGCTGTCA	CGCATGTCCCTCACAGCAAAAC	Chapter 2
MYB8	AACCTCAAGAAACTCAGGACATACAA	GATGAATGTGTGACCAAATTTTCC	Onkokesung *et al*. (2012)
MYC2	CAACAAGGGATCAAACTATACCG	TTTCCATTTACTGTATTTCTCTTCA	Woldemariam *et al*. (2013)
PAL1	TGCATACGCTGATGAC	TGGAAGATAGAGCTGTTCGC	Woldemariam *et al*. (2013)
SIPK	GTTGACGAATTTTCCAAAACAAAGT	CCGGAATATTATCCATACCGGCC	Meldau *et al*. (2009)
TD	TAAGGCATTTGATGGGAGGC	TAAGGCATTTGATGGGAGGC	Kang *et al*. (2006)

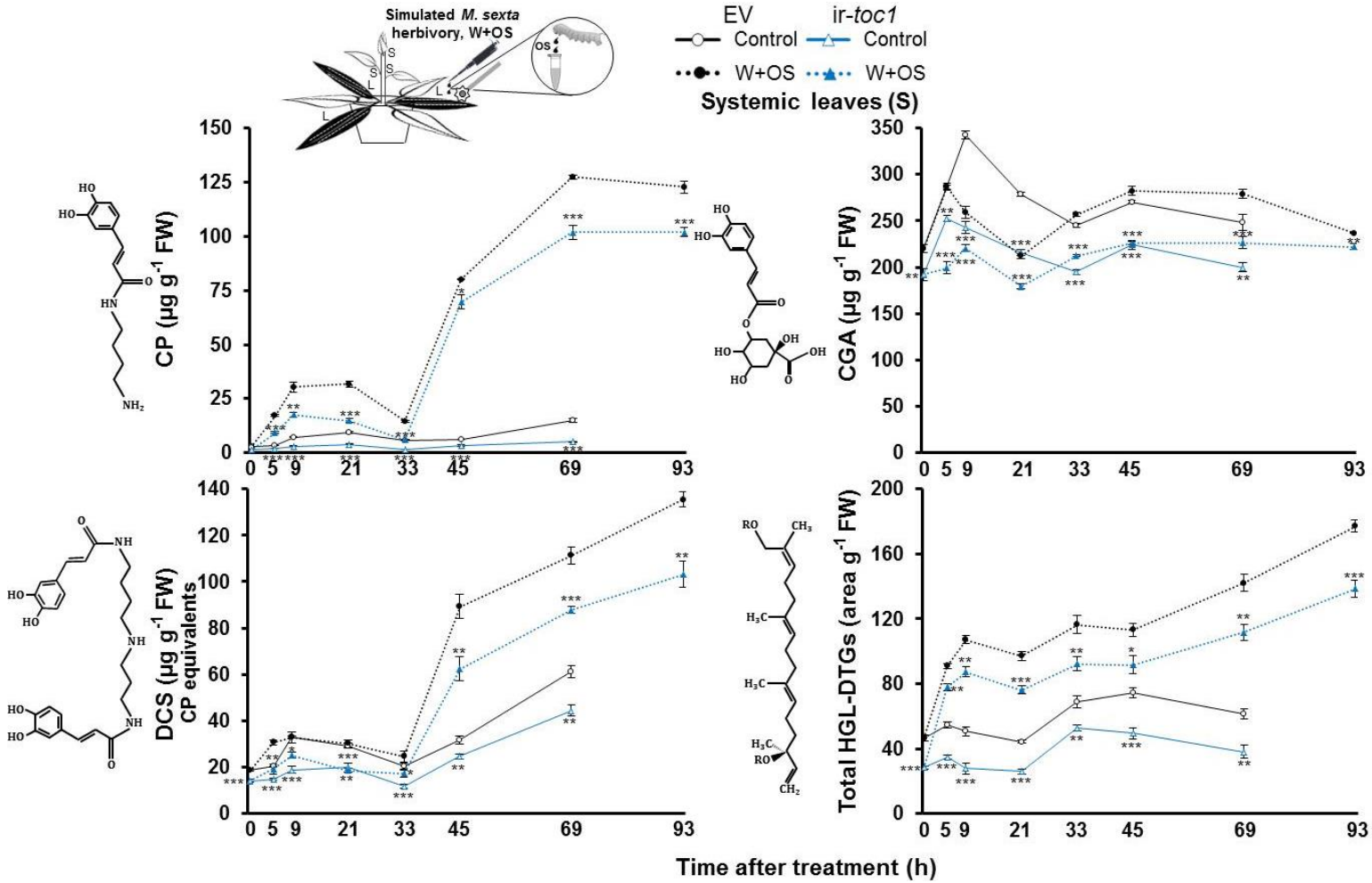

Figure S1. *TOC1* regulates the accumulation of CP, DCS, CGA and HGL-DTGs in untreated and W+OS systemic leaves of *N. attenuata* plants. Rosette leaves of empty vector (EV) and ir-*toc1* plants were wounded as described in Figure 1. Mean (±SE) concentrations of total caffeoylputrescine (CP); dicaffeoylspermidine (DCS), chlorogenic acid (CGA) and 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) following wound and OS application (W+OS) in systemic leaves (S) of five independent (n=5) EV and ir-*toc1* plants were quantified by UHPLC-UV. Concentration along the y-axis of secondary metabolites are not all to the same scale. Asterisks represent significantly differences between EV and comparably treated ir-*toc1* plant tissues at specific time points determined by Student's *t* test at P<0.05 (*); P<0.01 (**) and P<0.001 (***).

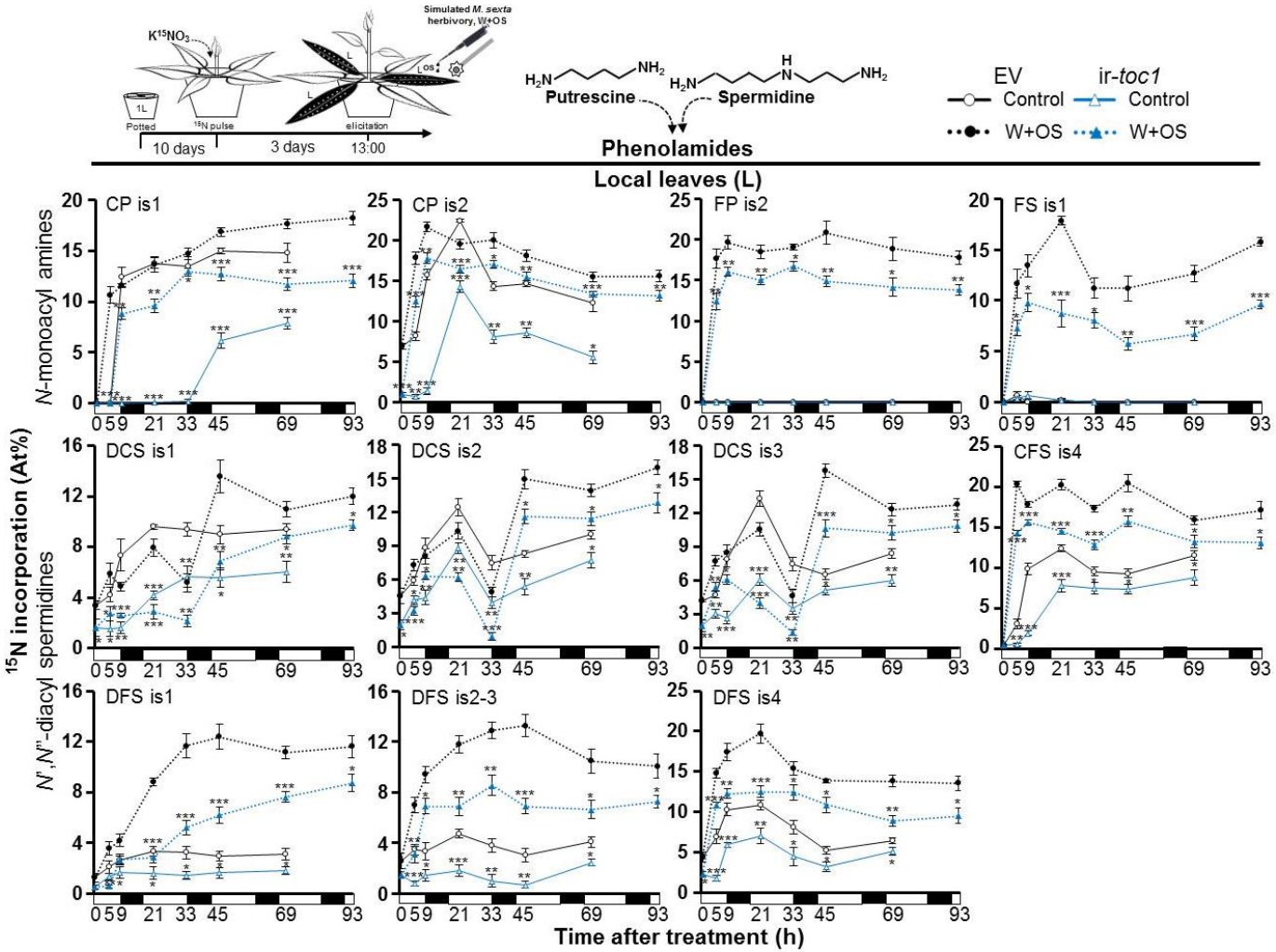

Figure S2. Silencing *TOC1* shows large-scale alterations in incorporation of ^{15}N into regioisomers of known *N. attenuata* phenolamides over time. Treatment of ^{15}N, elicitation and collection strategy were as described in Figure 3. Mean (±SE) incorporation of ^{15}N into the main mono- and di-acylated amines in locally treated leaves from five independent (n=5) empty vector (EV) and *ir-toc1* plants. All regioisomers of known tobacco phenolamides were screened for in and only those which were consistently detected are shown. Significant differences between EV and comparable *ir-toc1* plants at specific time points were determined by Student's *t* test and are indicated by asterisks at P<0.05 (*); P<0.01 (**) and P<0.001 (***). At%, atomic percentage.

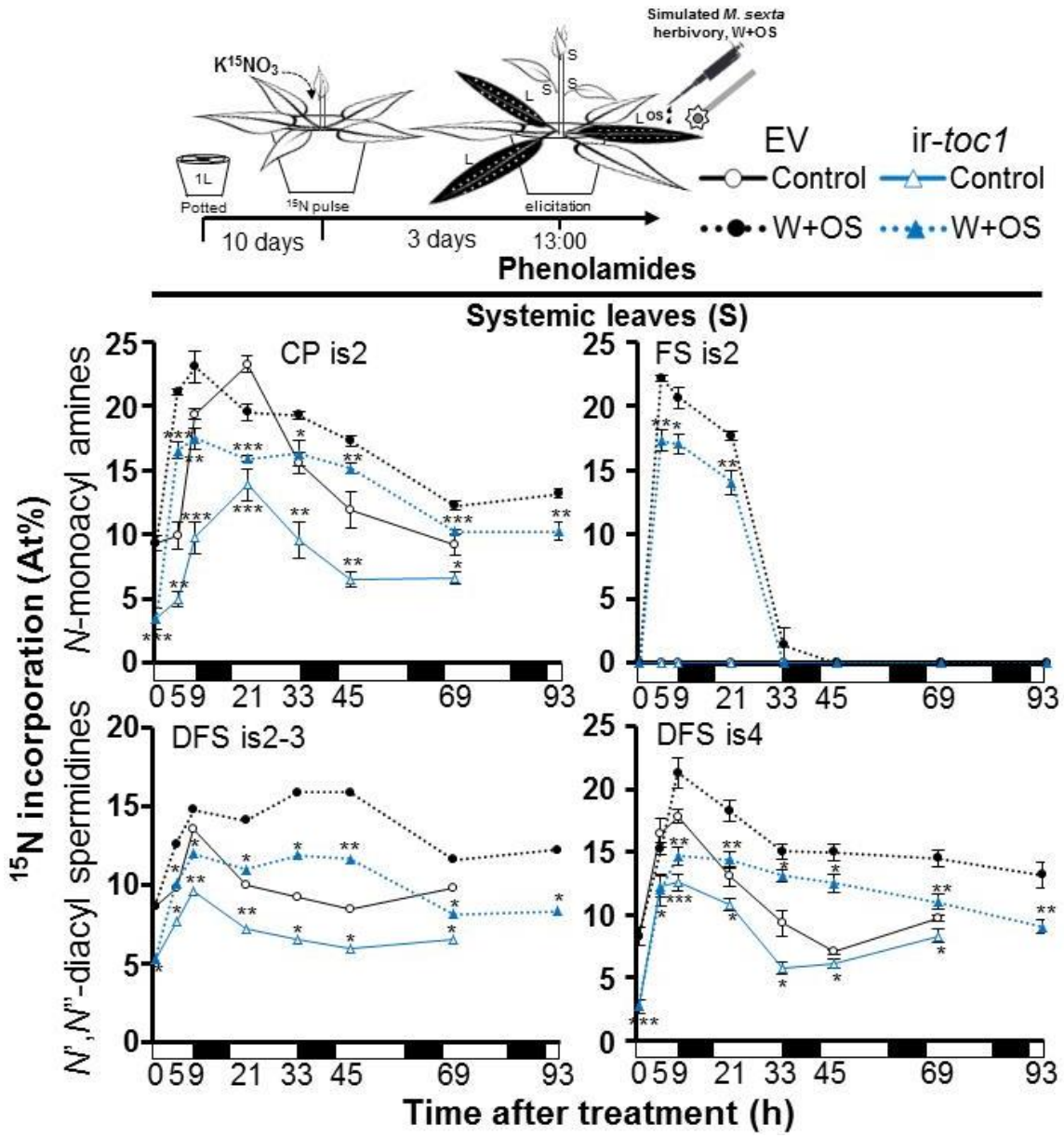

Figure S3. *TOC1* affects the dynamics of ^{15}N incorporation into regioisomers of known *N. attenuata* phenolamides in systemic leaves. Treatment of ^{15}N, elicitation and collection strategy were as described in Figure 3. Mean (±SE) incorporation of ^{15}N into the main mono- and di-acylated amines in systemic (S) leaves from five independent (n=5) empty vector (EV) and *ir-toc1* plants. All regioisomers of known tobacco phenolamides were screened for in and only those which were consistently detected are shown. Significant differences between EV and comparable *ir-toc1* plants at specific time points were determined by Student's *t* test and are indicated by asterisks at P<0.05 (*); P<0.01 (**) and P<0.001 (***). At%, atomic percentage.

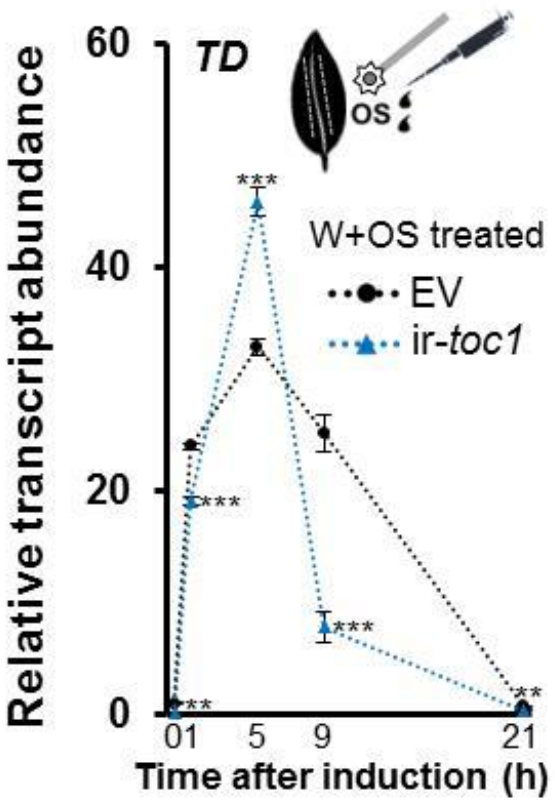

Figure S4. Na*TOC1* influences transcript levels of key marker genes involved in defence metabolism in *N. attenuata*. Mean (±SE) transcript abundance (relative to Na*EF-1α*) of threonine deaminase (TD) in leaf lamina (from locally treated leaves) from W+OS elicited plants. Asterisks represent significantly different levels of transcripts between five independent (n=5) empty vector (EV) and ir-*toc1* plants at specific time points determined by Student's *t* test at P<0.05 (*); P<0.01 (**) and P<0.001 (***;).

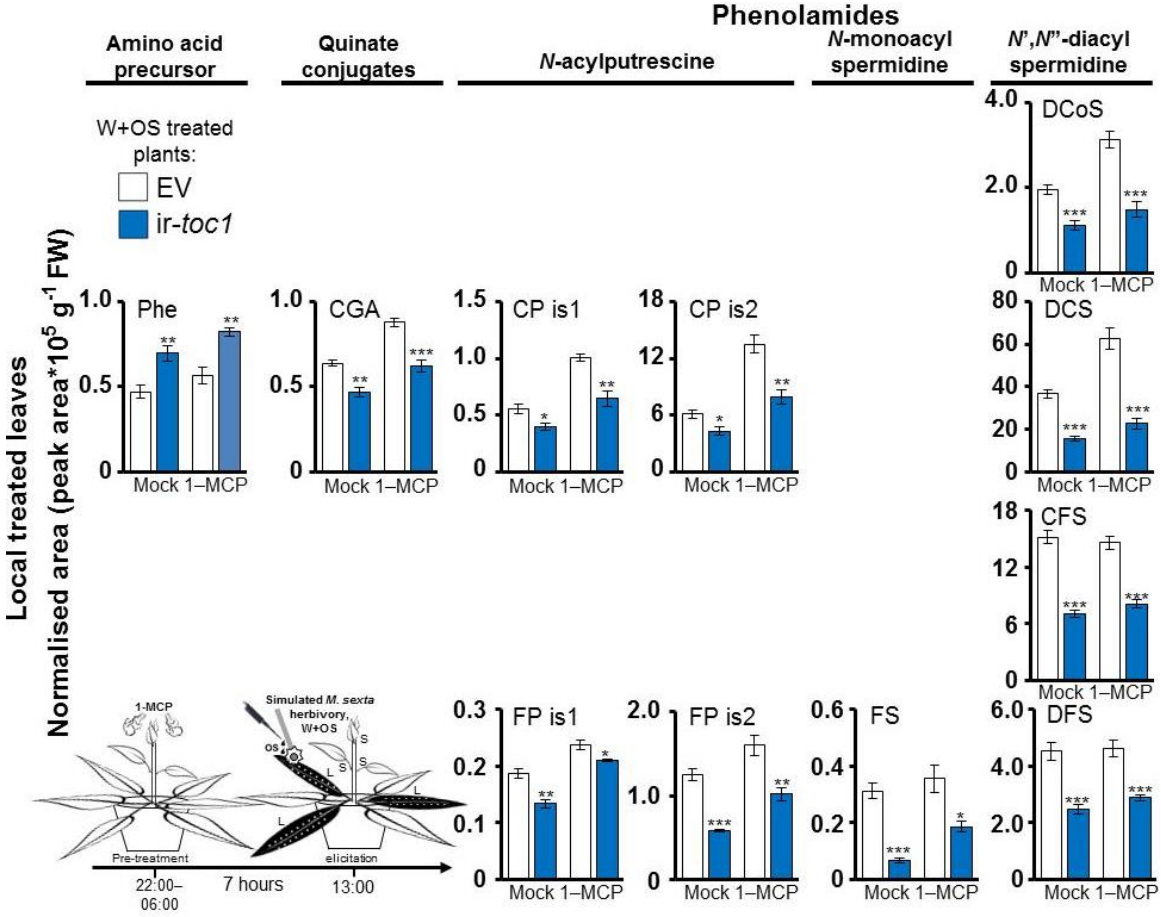

Figure S5. *TOC1* influences W+OS-specific accumulation of phenolamides in local leaves in an ET-independent manner. Developmentally synchronised plants were pre-exposed overnight (22:00 till 06:00) to 1-MCP and rosette leaves were W+OS treated at 13:00 the following day as per previous experiments. To selectively expose plants, 5 plants per genotype were placed within growth chambers fitted along with either alkaline (mock) solution or activated solution of 1-MCP as described by Kahl *et al.* (2000). Mean (±SE) concentrations of W+OS-induced levels of phenylalanine (Phe), chlorogenic acid (CGA) and known phenolamides in empty vector (EV) and *ir-toc1* plants 21 hours after W+OS-elicitation in plants that had been pre-treated with either a mock or activated 1-MCP solution. Significant differences between *ir-toc1* and corresponding EV plants were determined by Student's *t* test and are indicated by asterisks at P<0.05 (*); P<0.01 (**) and P<0.001 (***; n=5). 1–MCP, 1-methylcyclopropene; CP, caffeoylputrescine; CFS, caffeoylferuloylspermidine; DCoS, dicoumaroylspermidine; DCS, dicaffeoylspermidine; DFS, diferuloylspermidine; FP, feruloylputrescine; is, isomer; L, local; S, systemic.

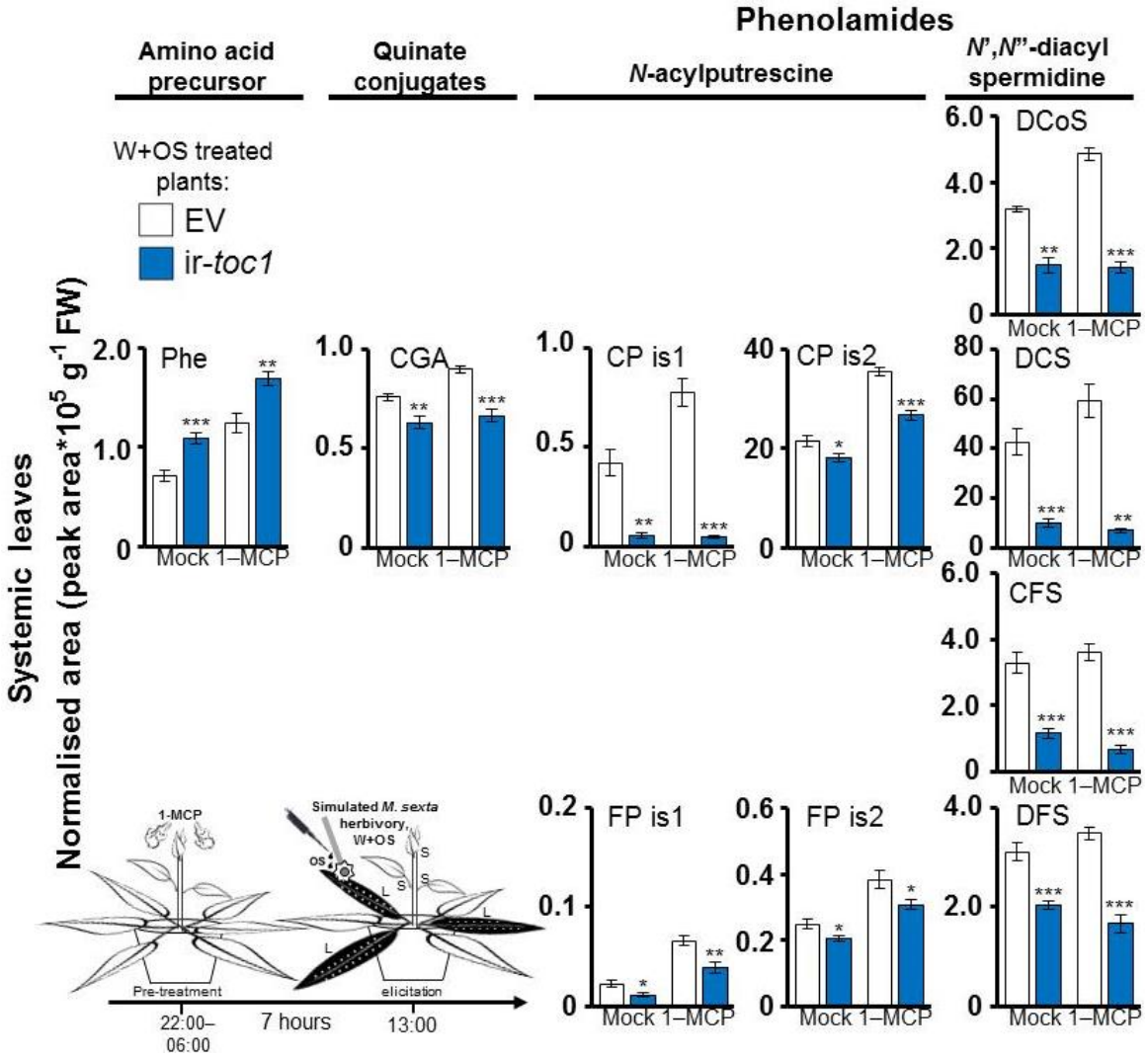

Figure S6. *TOC1* influences W+OS-specific accumulation of phenolamides in systemic leaves in an ET-independent manner. Developmentally synchronised plants were pre-exposed to 1-MCP and W+OS treated as described in Figure S7. Mean (±SE) concentrations of W+OS-induced levels of phenylalanine (Phe), chlorogenic acid (CGA) and known phenolamides in systemic leaves of empty vector (EV) and *ir-toc1* plants 21 hours after W+OS-elicitation in plants that had been pre-treated with either a mock or activated 1-MCP solution. Significant differences between *ir-toc1* and corresponding EV plants were determined by Student's *t* test and are indicated by asterisks at P<0.05 (*); P<0.01 (**) and P<0.001 (***; n=5). For abbreviations see Figure S6.

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Monash University

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
- Planned, performed and analysed all experiments associated with the production of figures: 6 and 7 - Main author of the following sections: Introduction, 2.3. Plant growth conditions and wounding experiments, 3.5. Alkaloid levels in *ODC*-RNAi transgenic plants - Heavily involved in drafting and editing of the manuscript	**35%**

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Kathleen DeBoer	- Created and produced all transgenic plants - Planned, performed and analysed experiments associated with the production of figures: 2, 3, 4 and 5 - Main author of the manuscript and heavily involved in drafting and editing of the manuscript	**50%**
Felicity J Edward	- Performed preliminary experiments during Honours - Assisted with enzyme assays	**5%**
Prof John Hamill *	- Contribution to planning of experiments - Provided supervisory support and was heavily involved with drafting and editing of the manuscript	N/A

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date 26/10/2015**
Main Supervisor's Signature	[redacted]	**Date 26/10/2015**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Chapter 4: 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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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-

Abbreviations: ADC, arginine decarboxylase; HPLC, high performance liquid chromatography; MeJa, methyl jasmonate; ODC, ornithine decarboxylase; PIP proteins, a family of NADPH-dependent reductases (Kajikawa et al., 2009); PMT, putrescine *N*-methyltransferase; QPT, quinolinate phosphoribosyltransferase.

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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 down-regulation 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′ ATCGATATGCCAA GGGCTAAAACGTA 3′) (primer set two: Forward 5′ CTCGAGGCC ATTCTTCAGTCCACAAT 3′; Reverse 5′ GGTACCATGCCAAGGGCT 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

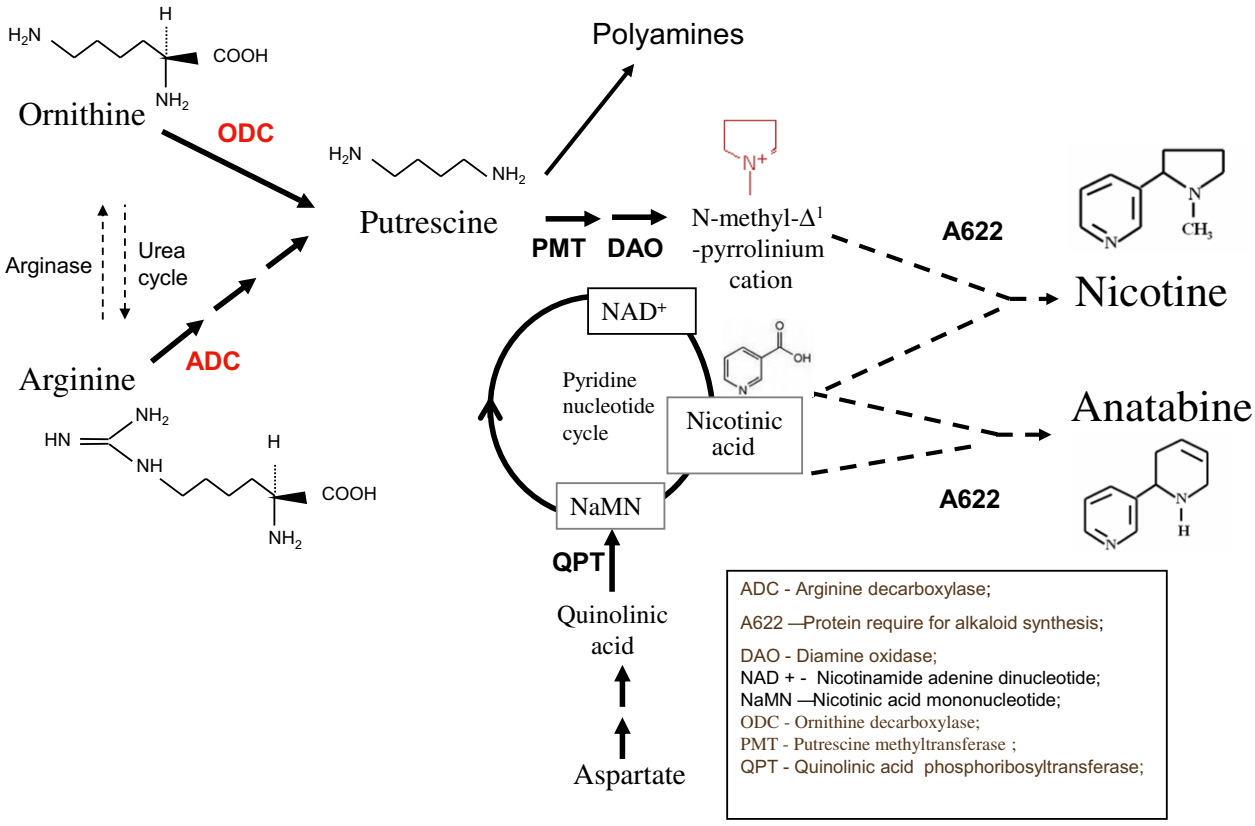

Fig. 1. Alkaloid synthesis pathway in *Nicotiana tabacum* L. Simplified schematic shows the relative positions of ornithine decarboxylase (ODC) and arginine decarboxylase(ADC), in addition to several other important enzymatic steps required for synthesis of the main alkaloids nicotine and anatabine (adapted from Cane et al., 2005; Chintapakorn and Hamill, 2007).

Australia Pty. Ltd.) containing MeJa at appropriate concentrations was added to each culture to achieve 2.5 mM or 25 mM final concentrations. Control cultures were treated with 20 µl of AnalaR absolute ethanol. These concentrations of MeJa have previously been shown to lead to a rapid increase in transcript levels of nicotine-associated enzymes and 2–3-fold increase in nicotine content of cultures within 48 h of treatment (Cane et al., 2005).

2.3. Plant growth conditions and wounding experiments

Seeds of *N. tabacum* variety SC 58, a high alkaloid (*AABB* genotype) variety of flue cured tobacco (Chaplin, 1966; Legg et al., 1969) were initially obtained from Dr. George White, USDA, Agricultural Research Service, Beltsville, MD, USA, and maintained by selfing (Sinclair et al., 2004; Cane et al., 2005). Seeds were germinated in punnets containing Debco seed raising mix and vermiculite (2:1 ratio) supplemented with a slow release fertilizer (Osmocote, 100 g per 6 L of soil mix). Plants were grown under a 16 h photoperiod (Gro-Lux fluorescent lights (photon flux density: 150 µmol m^{-2} s^{-1}) (Sylvania, Germany) (25 ± 2 °C)), in an insect free (PC2) greenhouse. When seedlings were ~2 cm in height (4–6 leaf stage) they were transferred to individual pots containing the same soil mix, or to individual rock wool blocks for growth in hydroponics. Plants in the rock wool blocks were placed initially in communal trays (~8 plants per tray) containing 50% modified Hoagland's nutrient solution (Cane et al., 2005). After 10–14 days, healthy growing plants of similar size and developmental stage were transferred to individual containers containing 200 ml of 50% modified Hoagland's solution. These plants were placed in randomized positions ~0.5 m below the light source. Nutrient solution changes were undertaken twice weekly, with distilled H_2O used daily to replenish water lost due to transpiration. Experiments were performed on pre-flowering *N. tabacum* plants when they were ~10 cm in height (~8–9 leaves). For wounding, the plant apex, containing the apical meristem and young unexpanded leaves, was removed, with analysis performed on the two upper leaves immediately below the apex removal point. Tissues were harvested 24 h and 7 days after wounding treatment for northern and alkaloid analysis, respectively, as described in Sinclair et al. (2004) and DeBoer et al. (2009).

2.4. Molecular and biochemical analysis of tissues

RNA extractions, northern blots and DNA-probe hybridization procedures were performed as described previously (Hamill and Lidgett, 1997; Chintapakorn and Hamill, 2003, 2007; Cane et al., 2005). Following hybridization, membranes were exposed to a phosphor screen (Molecular Dynamics) overnight and signal intensities determined using a Typhoon Trio scanner (Amersham Biosciences). Quantification was undertaken relative to levels of *ubiquitin* transcript levels by using ImageQuant (version 5) software (Molecular Dynamics). DNA fragments which were α^{32}P-dATP labelled for northern analysis were as follows.

ODC: a DNA fragment of ~360 bp which was PCR-amplified from *N. tabacum* genomic DNA using Forward oligonucleotide: 5′ CCGTCGCCGTTAAATCAG 3′ and Reverse oligonucleotide 5′ TTAGGAAAAACCAGCCAATCA 3′ (95 °C for 2 min [1 cycle]; 95 °C for 45 s, 50 °C for 90 s, 72 °C for 90 s [30 cycles]; 72 s for 10 min [1 cycle]). The PCR product recovered represented the 3′ region of *ODC* coding sequence (position 924–1302 in *N. tabacum* cDNA; Accession number AF321138; Lidgett, 1997) and did not overlap with the section of *N. glauca* cDNA which was used to create the

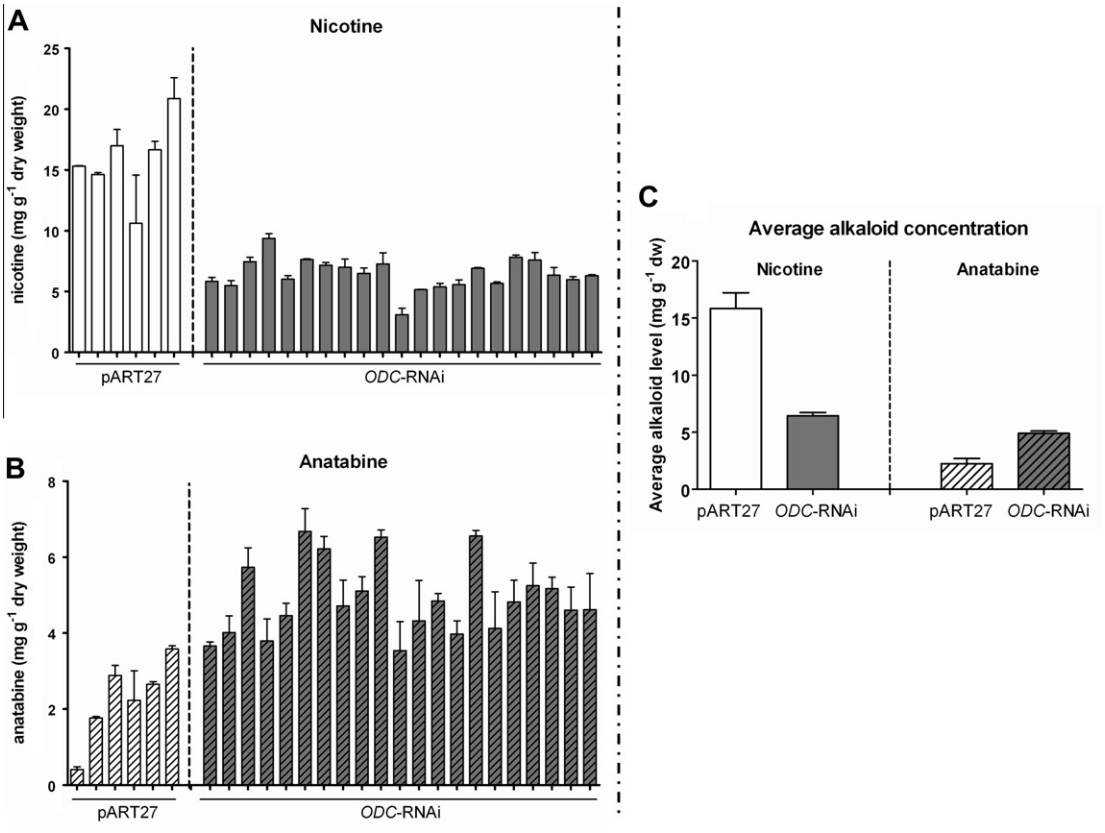

Fig. 2. Preliminary analysis of alkaloid levels in separate clonal hairy root lines of *N. tabacum* containing either empty vector (pART27) or CaMV35S *ODC*-RNAi construct. (A and B) Show the mean levels of nicotine and anatabine, respectively, in duplicate cultures (±SE) harvested in the latter part (day 21) of a typical growth cycle. (C) Shows a summary of average (±SE) nicotine (left panel) and anatabine (right panel) concentrations in pART27 control hairy root lines *vs.* CaMV35S *ODC*-RNAi hairy root lines at day 21 of the growth cycle.

CaMV35S *ODC*-RNAi construct. This was deemed to be important as preliminary experiments involving *N. tabacum ODC* sequences from the 5′ region of the cDNA led to the detection of the primary, non-processed RNAi-construct in RNA from transgenic lines on northern filters (data not shown). *ADC:* 1.2 kb *XbaI* fragment representing part of the *N. tabacum ADC* cDNA (Accession number AF127241; details noted in Chintapakorn and Hamill, 2007). *Ubiquitin:* 1.8 kb *EcoRI* cDNA fragment representing the ubiquitin gene from *Antirrhinum majus* (courtesy of Prof. C. Martin, Norwich UK; details noted in Chintapakorn and Hamill, 2003).

Pyridine alkaloids were extracted from freeze-dried tissue (approximately 0.05 g per sample) in glass tubes containing 4 ml acidic methanol (40% v/v HPLC grade methanol; 0.1% v/v 1 M HCl) and homogenizing for 1.5 min at 2500 rpm using a Kinematica AG polytron (PT 1200). Samples were centrifuged at 3000 rpm for 3 min and the supernatant filtered through a 0.45 µm filter (PALL Life Sciences). Analysis was undertaken using a Waters HPLC system (Waters 600 Controller; Waters In line degasser AF; Waters 717plus Autosampler; Waters 296 Photodiode Array detector) with a C-18 reverse phase column (GraceSmart RP18 Column; 250 mm × 4.6 mm, 5u 120A) and Empower2 software (Waters). The mobile phase (40% v/v HPLC grade methanol; 0.2% phosphoric acid; made up to volume with H_2O and buffered to pH 7.25 with triethylamine) was run at a flow rate of 1 ml min^{-1}. Pyridine alkaloids were identified on the basis of their diode array spectrum and quantified at 260 nm using known amounts of authentic standards (Sigma). ODC and ADC enzyme assays were performed as outlined previously (Hamill et al., 1990; Cane et al., 2005; Chintapakorn and Hamill, 2007).

3. Results

3.1. Alkaloid levels in N. tabacum ODC-RNAi hairy root cultures

Alkaloid levels were examined in tissue harvested from 21 independent *ODC*-RNAi hairy root lines, and six independent vector-only control lines, harvested in the latter part of a typical growth cycle (Fig. 2). In line with other observations within our laboratory involving hairy roots of wild type *N. tabacum* (Chintapakorn and Hamill, 2003, 2007; Cane et al., 2005), nicotine comprised more than 85% of the total alkaloid fraction, with nicotine concentrations in vector-only control lines being, on average, 15.84 mg g^{-1} d wt (±1.37 SE). Levels of the second most abundant alkaloid, anatabine, were on average, 2.25 mg g^{-1} d wt (±0.45 SE) in these vector-only control lines. In contrast, *ODC*-RNAi hairy root lines had an average nicotine content of 6.45 mg^{-1} d wt (±0.28 mg SE) and an average anatabine content of 4.89 mg g^{-1} d wt (±0.21 SE) (Fig. 2). Thus, the relative proportion of nicotine:anatabine in *ODC*-RNAi lines was altered from a typical ratio of ~7:1 in the vector-only control hairy root lines, to ~2:1 in *ODC*-RNAi hairy root lines.

3.2. Transcript analysis in ODC-RNAi hairy root lines

Previous studies involving hairy root cultures of *Nicotiana* and other Solanaceous species have indicated that it is appropriate to assay ODC and ADC transcript levels, and also enzyme activity, during the early-mid-stages of a culture growth cycle (Hamill et al., 1990; Michael et al., 1996; Chintapakorn and Hamill, 2007). Northern analysis, using healthy tissue of separate hairy root lines harvested at the mid-point of a typical growth cycle,

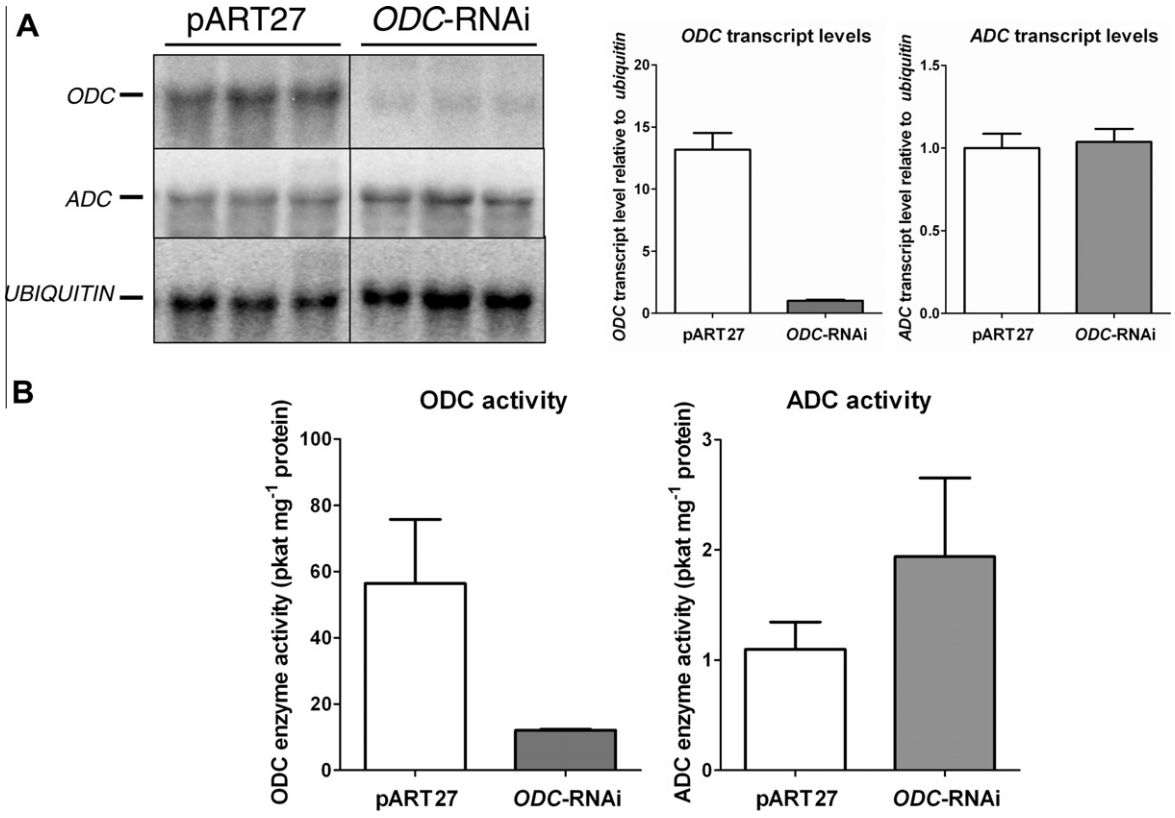

Fig. 3. Analysis of *ODC* and *ADC* transcript and enzymatic activity in three pART27 vector control hairy root lines *vs*. three CaMV35S *ODC*-RNAi hairy root lines harvested in the mid-stage (day 12) of a typical growth cycle. (A) Shows a Northern blot (left panel) of total RNA from each line probed sequentially with *ODC*, *ADC* and *ubiquitin* coding sequences. Relative mean transcript levels of each class of hairy root line (±SE) are also shown (right panel) after standardizing against the *ubiquitin* transcript signal within each lane. (B) Shows mean (±SE) ODC and ADC enzyme activity of the same three pART27 and three *ODC*-RNAi hairy root lines noted in A.

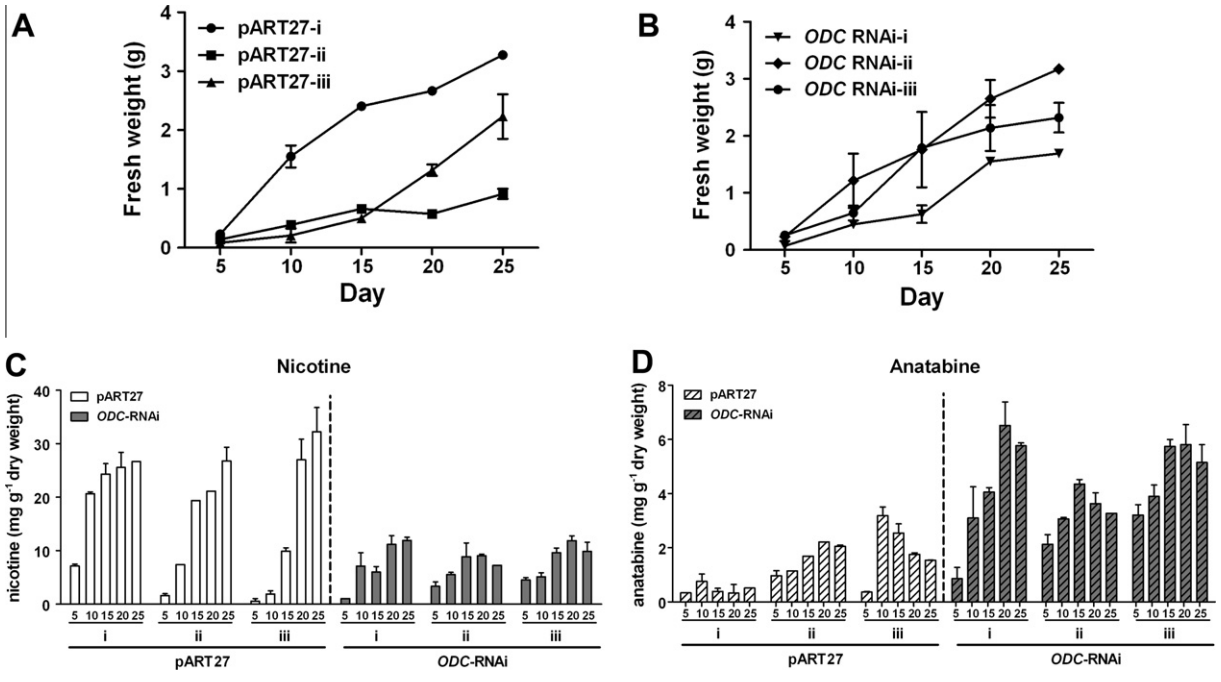

Fig. 4. Alkaloid levels in hairy roots at various stages of the growth cycle. Growth curves (A and B) and alkaloid content (C and D) were undertaken using three separate pART27 vector control root lines and three separate CaMV35S *ODC*-RNAi root lines with reduced *ODC* transcript levels. In most cases, each data points represent the mean (±SE) fresh weight (g) of root tissue from two separate culture vessels harvested at days 5, 10, 15, 15, 20 and 25 of the culture cycles.

indicated that levels of *ODC* transcript were markedly reduced in *ODC*-RNAi lines compared to vector-only controls (Fig. 3A). In contrast, *ADC* transcript levels were comparable between vector-only controls lines and *ODC*-RNAi hairy root lines (Fig. 3A). In line with

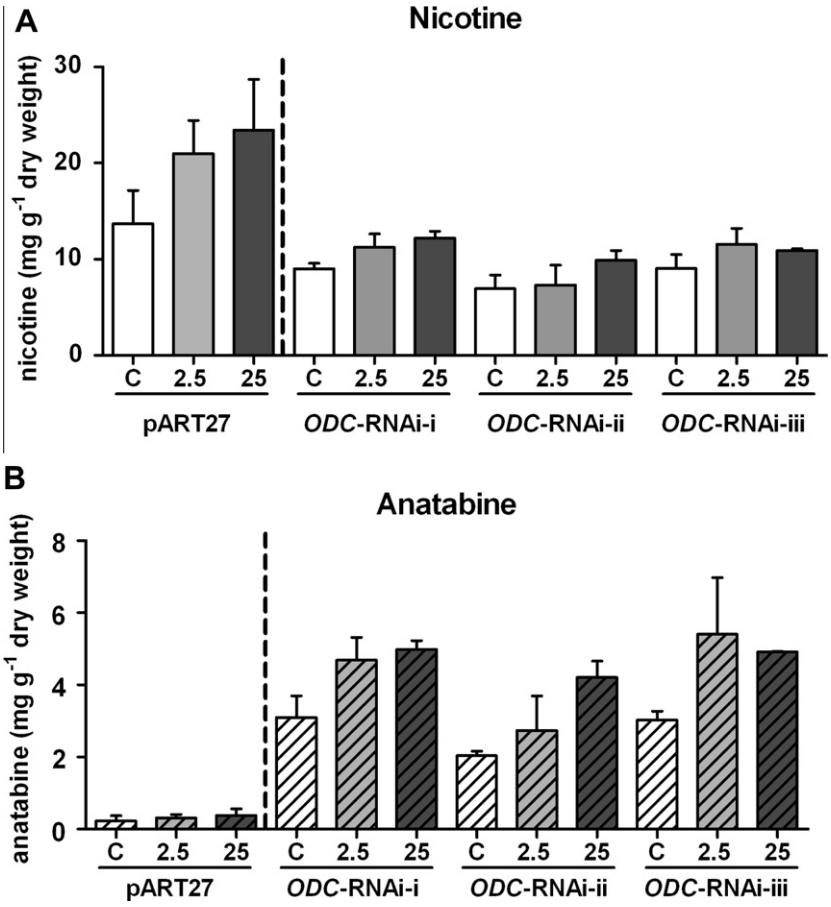

Fig. 5. Effect of adding methyl jasmonate (MeJa) to vector control *vs*. *ODC*-RNAi hairy root lines. At day 11 of the growth cycle, non-treated control (C) tissues received 20 μL ethanol/50 ml culture medium whilst jasmonate-treated cultures received 20 μL ethanol/50 ml containing sufficient MeJa to bring the final concentration to either 2.5 or 25 μM which leads to a 2–3-fold increase in nicotine levels in hairy roots of several *Nicotiana* species (Sinclair et al., 2004; Cane et al., 2005). Each histogram represents mean alkaloid levels (±SE) in roots from two to three separate culture vessels which were harvested 48 h after treatment.

this northern data, *ODC* enzyme activity in *ODC*-RNAi lines was also greatly reduced compared to vector-only control root lines (Fig. 3B). ADC activity in these *ODC*-RNAi root extracts on the other hand, was approximately 2-fold higher than the average level observed in the vector-only control lines (Fig. 3B).

3.3. Alkaloid levels throughout the growth cycle of hairy roots

Previous studies involving antisense-mediated down-regulation of *PMT* transcript levels, and to a much lesser extent *ADC* transcript levels, showed that changes in the alkaloid concentration of hairy roots were less obvious during the early stages of growth but became progressively more evident during mid-latter stages of the growth cycle (Chintapakorn and Hamill, 2003, 2007). To determine whether this was also the case for hairy root lines containing the *ODC*-RNAi construct, a detailed analysis was undertaken using comparatively cultured tissues harvested from three separate *ODC*-RNAi root lines and three separate pART27 vector-only control root lines. Replicate cultures were harvested at 5-day intervals throughout a typical growth cycle of ~3 weeks and their alkaloid content at each time point was determined (Fig. 4). Although some variation in growth rate was observed between separate clonal hairy root lines which were chosen for this analysis, similar growth trends were observed in the group of vector-only hairy root cultures as in the group of *ODC*-RNAi hairy root cultures. Such clone-clone differences in growth are commonly observed and are likely due to position effects following insertion of Ri T-DNA from *Agrobacterium rhizogenes* at random into host chromosomes during the transformation process. The observation that similar ranges of growth rates occurred in both the vector-control group and the *ODC*-RNAi group of hairy roots suggests that RNAi-mediated down-regulation of *ODC* transcript levels has little, if any negative effect upon growth of *N. tabacum* hairy roots *in vitro*.

In the early stages of the growth cycle, nicotine concentrations were comparable between the *ODC*-RNAi and the pART27 control lines (Fig. 4C). Nicotine levels increased substantially in pART27 lines as the growth cycle progressed (day 15–20), which is consistent with previous observations in wild-type hairy root lines of *N. tabacum* (Chintapakorn and Hamill, 2003; Cane et al., 2005). In contrast, *ODC*-RNAi lines harvested at comparable stages of the growth cycle exhibited little increase in nicotine content. At day 20, pART27 lines contained nicotine at levels between 21 and 27 mg g^{-1} d wt, whereas *ODC*-RNAi root lines contained nicotine at levels between 9 and 12 mg g^{-1} d wt (Fig. 4C). Although alkaloid concentrations in media of all hairy roots were low compared to root tissues, in general, nicotine concentrations were reduced in media of *ODC*-RNAi lines compared to that of vector control hairy roots. For example, nicotine concentrations ranged from 36 to 57 μg ml^{-1} in media of pART27 control lines harvested at day 25 of the growth cycle, compared to concentrations between 13 and 35 μg ml^{-1} in the media of *ODC*-RNAi root lines harvested at the same stage of growth.

As with nicotine, differences in anatabine levels of *ODC*-RNAi and pART27 hairy roots were most pronounced in tissues that were harvested during the latter half of the growth cycle (Fig. 4D). In pART27 vector control lines, anatabine concentrations were

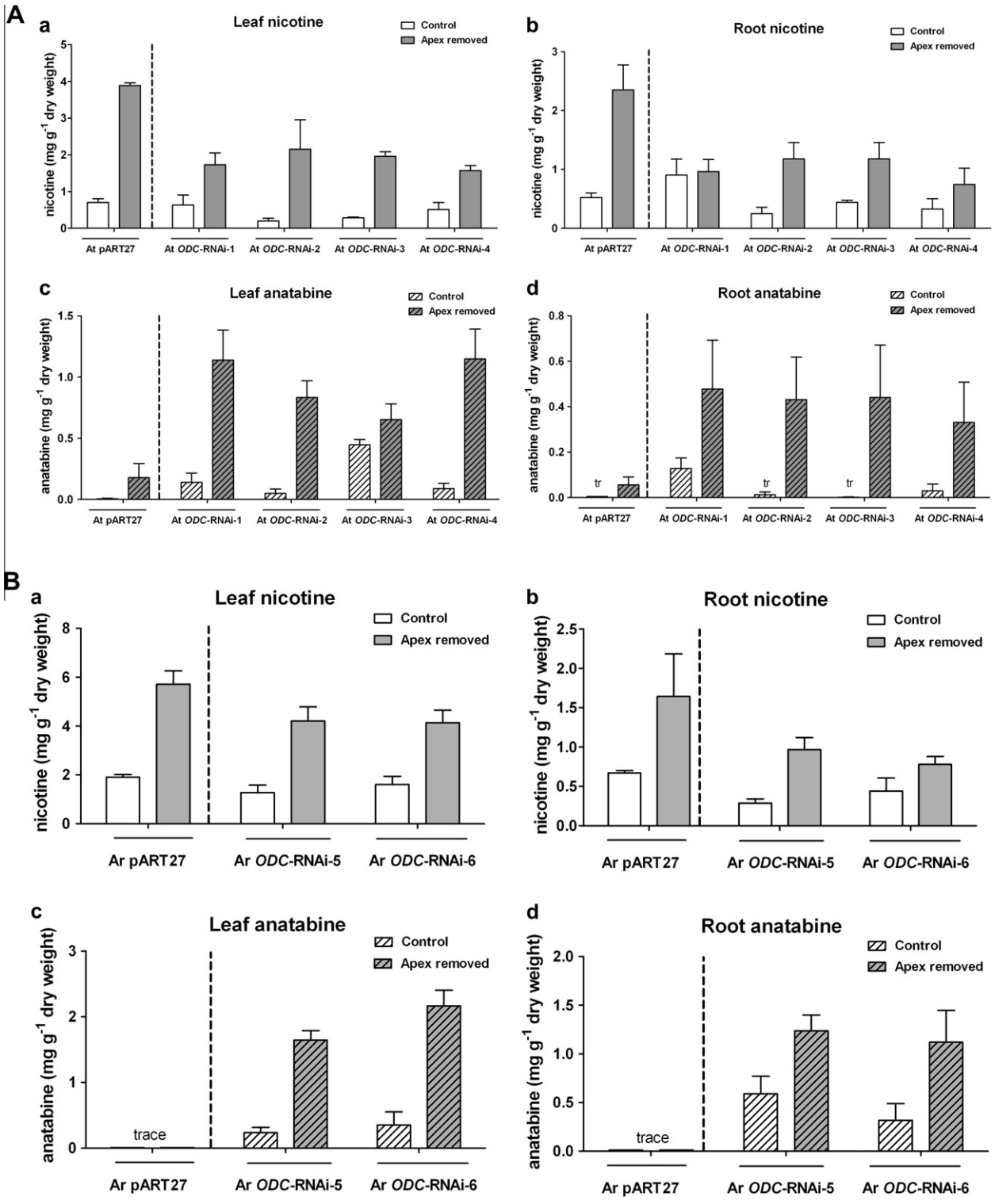

Fig. 6. Effects of wounding upon alkaloid levels in *ODC*-RNAi transgenic plants *vs.* pART27 vector controls. (A) Represents kanamycin resistant T1 offspring of plants transformed with disarmed *A. tumefaciens* whilst (B) represents T1 offspring of plants regenerated from hairy roots produced by transformation with *A. rhizogenes*. Plants were either unwounded (control) or wounded (Apex removed) by severing the apical region containing leaves which were less than 50% expanded. Panels a and b shows nicotine levels in uppermost fully expanded leaf and root tissues, respectively, harvested 7 days post-wounding. Panels c and d shows anatabine levels in uppermost fully expanded leaf and root tissues, respectively, harvested 7 days post-wounding. Data represents mean alkaloid content per g dry weight (±SE) of three separate plants for each line and treatment.

between 0.3 and 0.9 (average 0.55 ± 0.21 SE mg g^{-1} d wt) at day 5 of the of the growth cycle, comprising approximately 50% of the total alkaloid fraction. Levels of anatabine in the pART27 vector control lines increased to 2–3 mg g^{-1} d wt during the mid-latter stages of the growth cycle, before falling somewhat to an average of 1.4 ± 0.45 SE mg mg g^{-1} d wt at the end of the experiment (day 25). Anatabine comprised only about 5% of the total alkaloid fraction at this stage of the growth cycle of the vector-control hairy

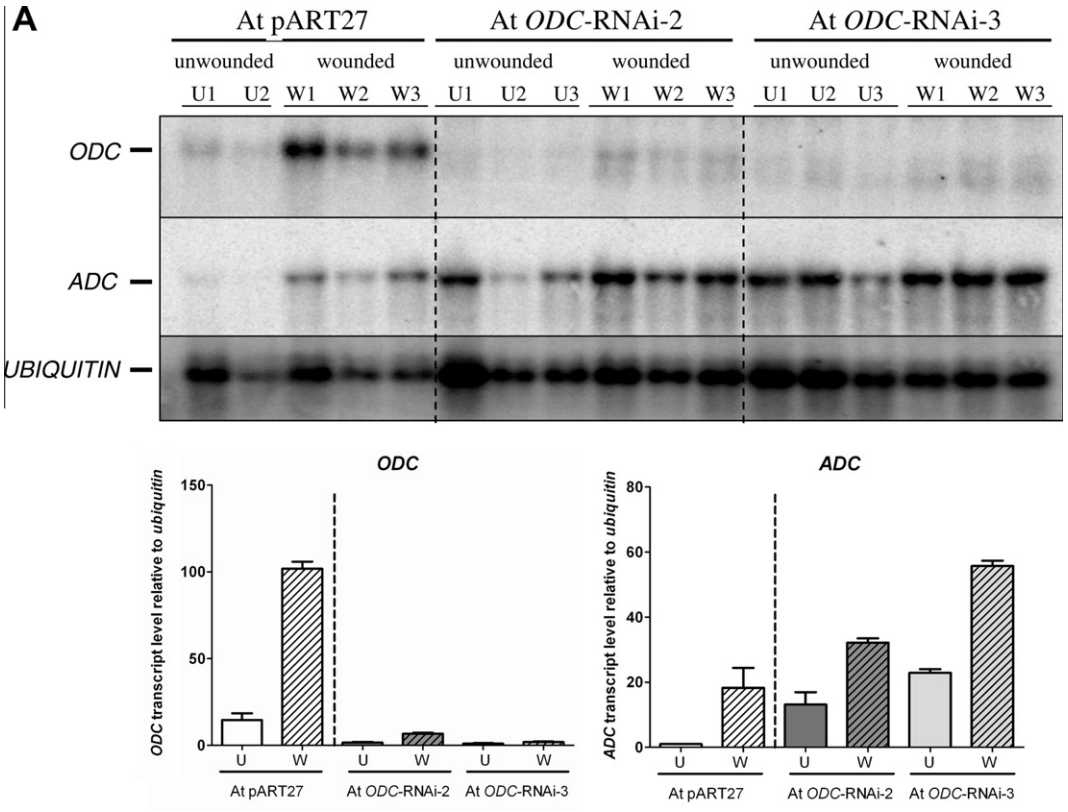

Fig. 7. Effects of wounding upon transcript levels in separate *ODC*-RNAi transgenic plants *vs.* vector controls. Root tissues from a subset of plants noted in Fig. 6 were sampled for RNA extraction 24 h after apex removal (W1–W3), with unwounded plants (U1–U3) serving as controls. Probing of RNA blots, and quantification of *ODC* or *ADC* transcript levels relative to *ubiquitin* transcript levels in each sample was undertaken as described in Fig 3. Data shown in A represent kanamycin-resistant T1 offspring of designated transgenics produced using disarmed *A. tumefaciens* whilst those in B represent kanamycin-resistant offspring of designated transgenics following regeneration from *A. rhizogenes*-derived hairy roots.

root lines. At day 5 of the growth cycle, anatabine concentrations in *ODC*-RNAi lines, were between 0.8 and 3 (average 2.1 ± 0.68 SE) mg g^{-1} d wt, and as in the pART27 controls, comprised approximately 50% of the total alkaloid fraction at this early stage in the growth cycle. By the mid-stages of the growth cycle however, anatabine levels in all *ODC*-RNAi lines were already higher than at any stage of growth in any of the pART27 vector control lines. At the end of the experiment (day 25), anatabine levels averaged 4.7 ± 0.75 mg g^{-1} d wt in *ODC*-RNAi root lines, typically representing more than 30% of the total alkaloid pool in tissues. The media of *ODC*-RNAi hairy root lines also contained higher levels of anatabine than comparable tissues of vector-only controls. For example, anatabine levels in media of hairy roots harvested at days 20–25 were in the range 9–23 μg ml^{-1}, compared to 3–9 μg ml^{-1} anatabine in media of comparable pART27 vector-only controls.

3.4. Effects of methyl jasmonate treatment on alkaloid levels and profiles

Treatment of actively growing *N. tabacum* hairy roots with low levels of MeJa has previously been shown to be associated with a rapid stimulation of transcript levels of key alkaloid synthesis genes, including *ODC*, followed by 2–3-fold stimulation in alkaloid levels, when tissues are analysed within 48 h of treatment (Cane et al., 2005). To determine the ability of *ODC*-RNAi hairy root cultures to increase nicotine biosynthesis in response to MeJa, compared to that of vector-only control hairy roots, cultures were treated with 2.5 μM and 25 μM MeJa on day 11 of the growth cycle. Alkaloid levels were examined in treated *vs.* control tissues, 48 h after treatment, as described previously (Cane et al., 2005). Nicotine levels were observed to be in the range of 15–20 mg g^{-1} d wt in non-MeJa treated pART27 cultures, with this level rising approximately 2-fold in response to MeJa treatment. In contrast, nicotine levels observed in non-MeJa treated *ODC*-RNAi lines were ~7–10 mg g^{-1} d wt, and were found to increase only slightly in response to MeJa treatment (Fig. 5A). Levels of anatabine were ~5-fold higher in non-MeJa treated *ODC*-RNAi hairy root lines (~2–3 mg g^{-1} d wt) than in corresponding control pART27 root lines (~0.5 mg g^{-1} d wt). The addition of MeJa caused a further increase in anatabine levels in *ODC*-RNAi lines, with levels rising to 4–5 mg g^{-1} d wt. In contrast, anatabine levels in pART27 control lines remained at low levels following treatment with MeJa (Fig. 5B).

3.5. Alkaloid levels in ODC-RNAi transgenic plants

3.5.1. Preliminary analysis of T_0 transgenic plants

Plants were regenerated from several hairy root lines, and grown in hydroponics as described previously (Sinclair et al., 2004; Cane et al., 2005) to facilitate preliminary nicotine analysis in both the roots and the leaves of pre-flowering plants. The average nicotine content (±SE) of plants regenerated from three separate pART27 vector hairy root controls, analysed 7 days after apex removal, was 3.93 ± 0.33 mg g^{-1} g d wt in leaves and 1.27 ± 0.39 mg g^{-1} g d wt in roots. This compared with average nicotine content (±SE) of similarly treated plants regenerated from 5 separate hairy root lines containing the *ODC*-RNAi construct, which was 3.61 ± 1.25 mg g^{-1} g d wt in leaves and 1.12 ± 0.45 mg g^{-1} g d wt in roots – i.e. only a small difference overall between the alkaloid levels of the *ODC*-RNAi hairy root-derived T_0 plants and their

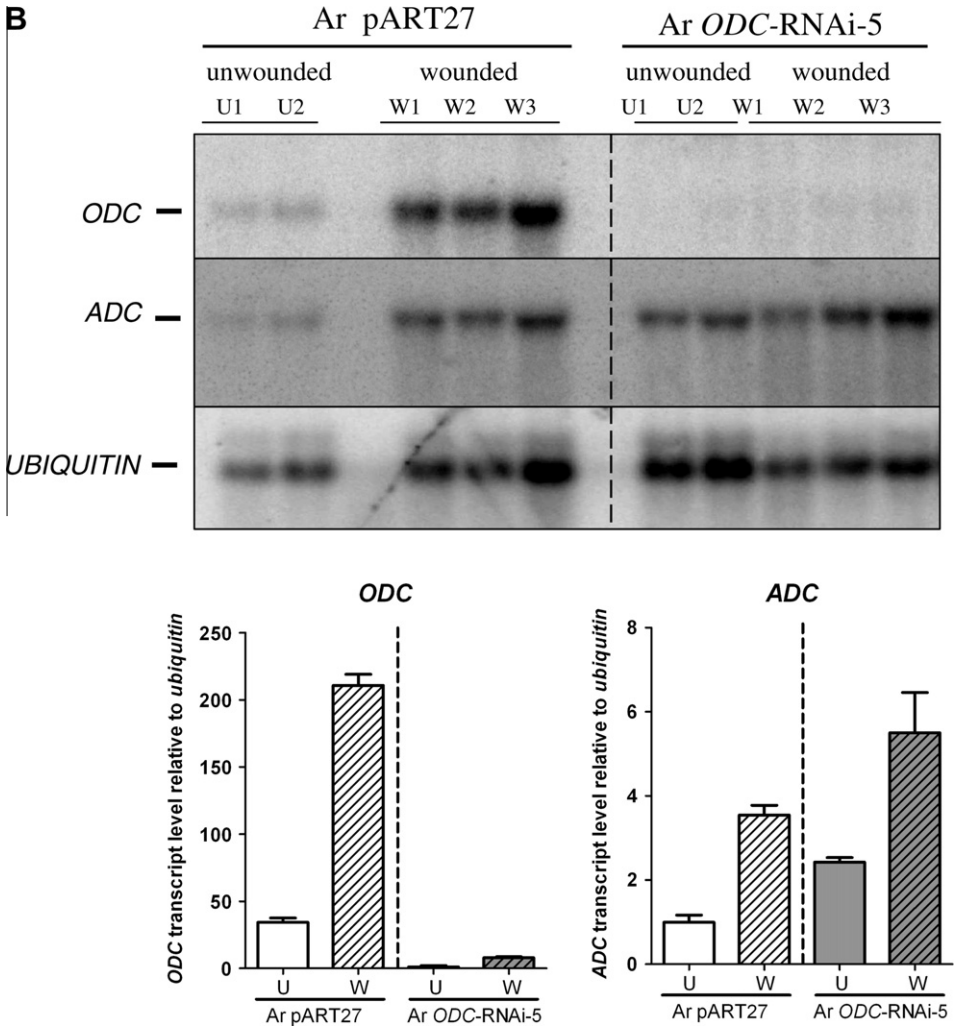

Fig. 7 (*continued*)

vector-only controls. This contrasted somewhat with the analysis of a separate set of primary transgenic plants in which the *ODC*-RNAi binary vector and the pART27 vector control, were separately introduced into *N. tabacum* using disarmed *A. tumefaciens* strain LBA 4404 to infect leaf pieces, with transgenic plants recovered by selection on regeneration medium containing high levels of kanamycin. In this case, alkaloid content of plants, analysed 7 days after apex removal, detected 6.3 ± 0.7 mg g^{-1} d wt nicotine in leaves of *ODC*-RNAi transgenics *vs.* 10 ± 0.8 mg g^{-1} d wt for vector-only controls. Anatabine was also observed in the leaves of these *ODC*-RNAi transgenics (average 1.1 ± 0.4 mg g^{-1} d wt) whilst only trace levels of this alkaloid (<0.1 mg g^{-1} d wt) were observed in leaves of the vector-only controls. This observation was suggestive of an imbalance in supply of putrescine-derived intermediates required for normal patterns of alkaloid synthesis in *ODC*-RNAi plants of *N. tabacum* (Chintapakorn and Hamill, 2003; Steppuhn et al., 2004; Wang et al., 2008, 2009) and indicated that more detailed experiments involving the offspring of transgenic plants were warranted (see below).

With respect to the overall morphology of T_0 transgenic plants, those which were regenerated from hairy roots containing either the pART27 vector or the *ODC*-RNAi construct varied slightly from wild type *N. tabacum* var. SC58 in that they possessed somewhat shorter internodes and slightly crinkled leaves compared to wild-type *N. tabacum*. This can be attributed to the presence of Ri T-DNA from *A. rhizogenes* (Tepfer, 1984). No obvious differences in morphology were noted between *ODC*-RNAi transgenics and their vector-only controls however. In the case of the *A. tumefaciens*-derived plants, no phenotypic differences were evident between *ODC*-RNAi transgenics and vector-only controls and all appeared morphologically similar to normal seed-derived *N. tabacum* var. SC58 plants. All T_0 plants typically flowered 10–14 weeks after transfer to soil (~400 ml compost per pot) and, once again, no obvious differences in inflorescences or floral structures were apparent between *ODC*-RNAi transgenics and their respective vector-only controls.

3.5.2. Alkaloid and molecular analysis of T_1 transgenic plants

Following self-fertilization of plants described above, seeds were sown *in vitro* on MS agar containing 75 μg ml^{-1} kanamycin sulphate, to select for transgenic T_1 offspring, and also to identify those T_0 lines which segregated in a 3:1 Mendelian fashion, thereby indicating the likelihood of one intact transgenic locus in the corresponding T_0 plants. A subset of these T_1 plants, were then grown in hydroponics to determine whether their *ODC* transcript levels were reduced, and also to assess their capacity for alkaloid synthesis. The data from these experiments are summarized in Figs. 6 and 7.

With respect to alkaloid analysis of transgenic plants (Fig. 6A and B), the nicotine content in both leaf and root tissue of non-wounded plants was broadly similar in both ODC-RNAi transgenics and their respective vector-only controls. When analysed 7 days

after removal of plant apices, the leaves and roots of both vector-only controls and ODC-RNAi transgenics showed a several-fold increase in nicotine content, compared to non-wounded respective control plants. It was noteworthy however that the absolute levels of nicotine in tissues of the wounded ODC-RNAi transgenics was usually substantially lower than in the corresponding tissues of the wounded vector only control plants (Fig. 6A and B).

On the other hand, analysis of the anatabine content of plants revealed a more dramatic difference between ODC-RNAi transgenics and corresponding vector-only controls. Levels were notably higher in both leaf and root tissues of the non-wounded ODC-RNAi transgenic plants than in non-wounded pART27 vector control plants, where only trace levels were observed. Moreover, anatabine levels were observed to have increased several-fold in both leaves and roots of ODC-RNAi transgenic plants which had been wounded by apex removal. This contrasts with leaf and root tissues harvested from pART27 wounded plants where, again, only trace levels of anatabine were detected (Fig. 6A and B). The ratio of nicotine:anatabine in leaves and roots of ODC-RNAi T_1 transgenic plants, both wounded and non-wounded, was approximately 2:1. This was true for plants regenerated from hairy roots, where transformation was effected by *A. rhizogenes*, and also for plants derived from regenerating callus where transformation was effected by disarmed *A. tumefaciens*. These results are in stark contrast to vector-only controls where the nicotine:anatabine ratio of all tissues was greater than 20:1.

The most noticeable feature of the molecular analysis of transgenic plants, as depicted in Fig. 7A and B, was the very low level of detectable ODC transcript in the roots of both hairy root derived- and disarmed *A. tumefaciens*-derived ODC-RNAi transgenic plants – even when analysed 24 h after removal of plant apices. This contrasts with the pART27 vector-only control plants, in which ODC transcript was detectable in RNA isolated from non-wounded plants, with ODC transcript levels increasing several-fold in response to removal of plant apices, as expected from previous studies involving wild type *N. tabacum* (Cane et al., 2005). An additional aspect of this analysis is that ADC transcript levels were elevated in the root RNA from ODC-RNAi transgenics, when compared to the pART27 vector only controls, in both the unwounded and wounded plants.

4. Discussion

The synthesis of nicotine occurs in roots of *N. tabacum* and increases, both in intact plants and in roots cultured axenically *in vitro*, in response to wound-associated stresses such as removal of the plant apex (a major source of auxin) or jasmonate treatment of tissues (Saunders and Bush, 1979; Baldwin, 1989; Baldwin et al., 1994; Cane et al., 2005; Shi et al., 2006). The increase in nicotine-synthesising capacity of plants following damage to aerial tissues is thought to provide protection in their natural environment (Steppuhn et al., 2004). Typically, it is preceded by a marked rise in root transcript levels and associated enzyme activity of key genes required for alkaloid synthesis, including *ODC*, within hours of damage to aerial tissues (Mizusaki et al., 1973; Saunders and Bush, 1979; Wang et al., 2000; Cane et al., 2005). Associated studies have also indicated that transcript levels of many biosynthetic genes, including *ODC*, are rapidly up-regulated in tissues grown *in vitro*, such as disorganized cell cultures and hairy root cultures, following treatment with low levels of methyl jasmonate (Imanishi et al., 1998; Goossens et al., 2003; Xu et al., 2004; Cane et al., 2005). Similar results have also been observed in experiments involving auxin-dependent non-transformed roots following transfer to media lacking this phytohormone (Reed and Jelesko, 2004). Together these studies strongly suggest an important role for ODC in enabling roots of *N. tabacum* plants to increase nicotine levels in response to such wound-associated stresses. To date however, there has been rather a lack of experimental evidence for this assumption. In the current study, we addressed this question by using an RNAi-mediated approach to reduce *ODC* expression in both cultured hairy roots and also intact transgenic plants of *N. tabacum*, and assessed their capacity for alkaloid synthesis following either methyl jasmonate treatment of hairy roots or removal of plant apices. We observed a significant decrease in nicotine content of cultured hairy roots, selected for the RNAi construct, without apparent negative effects on growth rates. Moreover, unlike vector-only controls, *ODC*-RNAi hairy roots had greatly diminished capacity to increase nicotine content in response to treatment with low levels of methyl jasmonate. In addition, decreased capacity for nicotine production in *ODC*-RNAi-lines was accompanied by increased capacity to produce anatabine. This alkaloid, normally a minor component in hairy roots of *N. tabacum* and related species (Hamill et al., 1986; Parr and Hamill 1987), is derived entirely from nicotinic acid (Leete, 1975; Leete and Slattery, 1976). Previous studies involving down-regulation of *PMT* transcript levels in both transgenic *N. tabacum* plants and cultured roots, have indicated that a substantial increase in the anatabine:nicotine ratio results from an imbalance in supply of putrescine derivatives from polyamine metabolism, so effectively causing an oversupply *in vivo* of derivatives from the pyridine-nucleotide cycle (Chintapakorn and Hamill, 2003; Steppuhn et al., 2004; Wang et al., 2009).

The demonstration from the current study that ODC is indeed important in supplying putrescine for normal nicotine synthesis capacity in *N. tabacum*, particularly when plants are wounded by removal of apices or roots are treated with methyl jasmonate, is not surprising given that previous experiments have shown that ODC and alkaloid synthesis are stimulated in tobacco roots by either reduction in supply of apically-derived auxin in decapitated plants (Shi et al., 2006) or direct treatment of cultured cells or hairy roots with jasmonate (Imanishi et al., 1998; Cane et al., 2005). These observations do contrast somewhat however with previous biochemical studies involving *in vitro* cultured callus tissues of *N. tabacum* which suggested that ADC, not ODC, is mainly responsible for the production of putrescine that is channeled into the synthesis of nicotine (Tiburcio and Galston, 1986). However, it is likely that the results obtained in the current study, are reflective of what actually occurs *in planta* particularly when we consider that antisense-mediated reductions in ADC activity in *N. tabacum* had very little effect upon the nicotine content or the nicotine:anatabine ratio in cultured hairy roots or regenerated transgenic plants (Chintapakorn and Hamill, 2007). It is interesting that we did not recover *ODC*-RNAi transgenics with nicotine levels that were below a threshold of several mg g^{-1} d wt, either in cultured hairy roots or intact transgenic plants. This may be because the ADC route to putrescine functions in these transgenic lines to ensure sufficient putrescine is produced for baseline nicotine synthesis, as well as allowing for normal growth in the absence of normal ODC activity. Indeed, there was some evidence from the current study that both *ADC* transcript and enzyme levels were elevated in *ODC*-RNAi lines compared to vector only controls. In this regard it is noteworthy that studies by Nölke et al. (2005) observed increases in ADC enzymatic activity in *N. tabacum* transgenic lines in which ODC enzymatic activity had been largely inhibited by expression of a gene encoding an ODC antibody. Unfortunately, effects of the immunomodulation of ODC upon nicotine levels were not reported and thus direct comparison of the immunomodulation approach of Nölke et al. (2005) *vs.* the RNAi approach as used in the current study to down-regulate *ODC* transcript levels is not possible. It is also noteworthy that in the immunomodulation study of Nölke et al. (2005), severe phenotypic effects were observed in transgenic plants with reduced ODC enzymatic activity, with these plants

being twisted and dwarfed. This is unlike our experience involving the use of an RNAi approach to down-regulate *ODC* transcript levels in *N. tabacum*, where obvious negative effects on phenotype of transgenic plants were not observed. Further studies to compare effects of *ODC*-RNAi transcript down-regulation *vs.* immunomodulation of ODC may be informative with respect to the effects upon alkaloid and polyamine metabolism specifically and other areas of the tobacco metabolome in general.

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Declaration for Thesis Chapter 5

Declaration by candidate

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
- Completed *ODC* and *ADC* probing for figure 2A - Planned, performed and analysed all experiments associated with the production of Figures: 5 - Main author of the following sections: 2.3. Plant growth conditions and wounding regimes, 3.4. Analysis of anabasine levels in *ODC*-RNAi *N. glauca* transgenic plants - Heavily involved in drafting and editing of the manuscript	**30%**

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Kathleen DeBoer	- Created and produced all transgenic plants - Planned, performed and analysed experiments associated with the production of Figures: 2B, 3 and 4 - Main author of the manuscript and heavily involved in drafting and editing of the manuscript	**50%**
Felicity J Edward	- Performed preliminary experiments during Honours	**5%**
Suzanne M Ryan	- Completed *UBIQUITIN* probing for Figure 2A	**5%**
Prof John Hamill *	- Contribution to planning of experiments - Provided supervisory support and was heavily involved with drafting and editing of the manuscript	N/A

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date 26/10/2015**
Main Supervisor's Signature	[redacted]	**Date 26/10/2015**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Chapter 5: RNAi-mediated down-regulation of ornithine decarboxylase (ODC) impedes wound-stress stimulation of anabasine synthesis in *Nicotiana glauca*

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RNAi-mediated down-regulation of ornithine decarboxylase (ODC) impedes wound-stress stimulation of anabasine synthesis in *Nicotiana glauca*

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Solanaceae

ABSTRACT

Unlike most *Nicotiana* species, leaf tissues of the globally significant weed *Nicotiana glauca* Grah. (Argentinian tree tobacco) contains anabasine as the main component of its alkaloid pool, with concentrations typically increasing several fold in response to wounding of plants. The Δ^1-piperidinium ring of anabasine is synthesised from cadaverine, *via* the decarboxylation of lysine, however the identity of the protein catalysing this reaction remains unknown. Recent studies indicate that ornithine decarboxylase (ODC), an enzyme involved in the synthesis of the diamine putrescine, may also possess LDC activity. Previously we found that *ODC* transcript is markedly up-regulated in leaves of *N. glauca* in response to wounding. In order to examine the role of ODC in the synthesis of anabasine in *N. glauca*, transcript levels were constitutively down-regulated in hairy root cultures and transgenic plants *via* the introduction of a CaMV35S driven *ODC*-RNAi construct. In addition to the anticipated marked reduction in nicotine concentrations, demonstrating that the *ODC*-RNAi construct was functioning *in vivo*, we observed that *N. glauca ODC*-RNAi hairy root cultures had a significantly diminished capacity to elevate anabasine synthesis in response to treatment with the wound-associated hormone methyl jasmonate, when compared to vector-only controls. We observed also that *ODC*-RNAi transgenic plants had significantly reduced ability to increase anabasine concentrations following removal of the plant apex. We conclude that ODC does have an important role in enabling *N. glauca* to elevate levels of anabasine in response to wound-associated stress.

1. Introduction

The presence of pyridine alkaloids is a characteristic trait of species in the genus *Nicotiana* and provides an effective chemical defence against herbivory (Baldwin and Ohnmeiss, 1993; Steppuhn et al., 2004). Most *Nicotiana* species accumulate mainly nicotine or its derivative nornicotine as the principal component of their alkaloid pool, combined with relatively low levels of anatabine and/or anabasine (Saitoh et al., 1985; Sisson and Severson, 1990). The diamine putrescine is a key intermediate in the production of nicotine, and is a precursor of the essential polyamines, spermidine and spermine. In *Nicotiana*, as in many plants, putrescine can be synthesised indirectly from arginine *via* the sequential action of arginine decarboxylase (ADC), agmatine iminohydrolase (agmatine deiminase) and *N*-carbamoylputrescine amidohydrolase, or alternatively, directly from ornithine, *via* the action of ornithine decarboxylase (ODC) (Fuell et al., 2010; Fig. 1). Recent *in vivo* transcript down-regulation experiments indicate that ODC is more crucial than ADC in ensuring adequate levels of putrescine are available for nicotine synthesis in roots of *Nicotiana tabacum* (Chintapakorn and Hamill, 2007; DeBoer et al., 2011a). The latter stages of nicotine production involve the conversion of putrescine to *N*-methylpyrrolinium, *via* the sequential action of putrescine *N*-methyltransferase (PMT) and *N*-methylputrescine oxidase (MPO), which is condensed with a derivative of nicotinic acid to produce nicotine. A PIP family-like protein (A622) and a berberine bridge enzyme-like protein (BBL) are essential for the final stages of nicotine synthesis (DeBoer et al., 2009; Kajikawa et al., 2009, 2011). Nornicotine is formed in *Nicotiana* species by demethylation of nicotine by a small family of closely related cytochrome P450 enzymes collectively known as nicotine *N*-demethylase and encoded by *CYP82E* genes (Siminszky et al., 2005; Pakdeechanuan et al., 2012 and references therein).

A few *Nicotiana* species accumulate relatively high proportions of anabasine in leaf and/or root tissues. The most extreme example

Abbreviations: ADC, arginine decarboxylase; HPLC, high performance liquid chromatography; LDC, lysine decarboxylase; MeJa, methyl jasmonate; ODC, ornithine decarboxylase; PMT, putrescine *N*-methyltransferase; QPT, quinolinate phosphoribosyltransferase.

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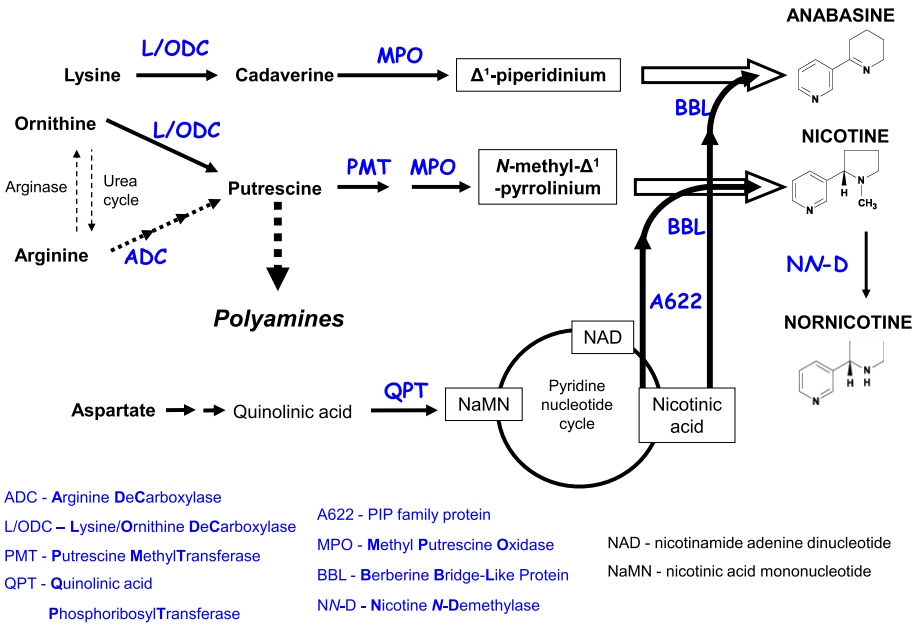

Fig. 1. Diagrammatic representation of pyridine alkaloid synthesis in *Nicotiana*, illustrating the close association of putrescine-nicotine/nornicotine synthesis and cadaverine-anabasine synthesis.

is *Nicotiana glauca* (tree tobacco), an invasive, drought tolerant, weedy shrub of global significance (Florentine and Westbrooke, 2005), where the root alkaloid fraction is comprised of nicotine and anabasine in roughly equal proportions whilst that of leaves is almost totally dominated by anabasine (Saitoh et al., 1985; Sisson and Severson, 1990). The high levels of anabasine in *N. glauca* is responsible for congenital deformities in grazing stock (Keeler, 1979; Keeler and Crowe, 1983; Panter et al., 1990, 2000; Green et al., 2010, 2012) and respiratory illness and death in humans who have mistakenly used its leaves as a food source (Castorena et al., 1987; Steenkamp et al., 2002; Furer et al., 2011). Experiments using radio-labelled precursors undertaken more than half a century ago, demonstrated that the Δ^1-piperidinium ring of anabasine is derived from cadaverine (1,5-diaminopentane) which is itself synthesised *via* the decarboxylation of lysine (Leete, 1956, 1958; Solt et al., 1960; Fig. 1). Subsequent experimental studies involving hairy roots of the predominately nicotine-producing species *Nicotiana rustica* supported this suggestion, with markedly increased levels of anabasine observed in cultures that were exposed to millimolar concentrations of cadaverine in their culture media (Walton et al., 1988). High levels of anabasine were also found in *N. tabacum* hairy roots engineered to over-express a bacterial lysine decarboxylase (*LDC*) gene, particularly when the protein was targeted to the plastid (Fecker et al., 1993; Herminghaus et al., 1996). Despite these observations, the molecular nature of the *Nicotiana* LDC protein has yet to be resolved. The subsequent biochemical reactions involved in the synthesis of anabasine are less clear, but likely involve the oxidation of cadaverine to 5-aminopentanal, before spontaneous cyclization, and finally, condensation with a nicotinic acid derivative to produce anabasine. A622 and BBL are also likely to be involved in the final stages of anabasine synthesis (DeBoer et al., 2009; Kajikawa et al., 2009, 2011).

In *N. glauca*, anabasine levels of leaves increase several fold above baseline concentrations following wounding of plants (Baldwin and Ohnmeiss, 1993; Sinclair et al., 2004; DeBoer et al., 2009; DeBoer, 2010). A rapid increase in the transcript levels of key genes involved in anabasine synthesis, such as *QPT* and *A622*, occurs in the upper leaves of *N. glauca* 12–24 h after shoot damage. This is followed by a more gradual rise in anabasine levels in the upper leaves and developing lateral side shoots over a period of approximately 1 week (Sinclair et al., 2004; DeBoer et al., 2009; DeBoer, 2010). Interestingly, *ODC* transcript levels also increase markedly in the upper leaves of *N. glauca* plants, in concert with *QPT* and *A622*, in response to apex removal despite only trace levels of nicotine being detected in these tissues when analysed 3–10 days post-wounding (Sinclair et al., 2004; DeBoer et al., 2009; DeBoer, 2010). Sinclair et al. (2004) suggested that the elevated protein derived from this *ODC* transcript facilitates the increase of anabasine in *N. glauca* following such wounding. This working hypothesis was based on the observation that the protein encoded by the *ODC* gene from the closely related species *Nicotiana glutinosa*, efficiently decarboxylates both L-ornithine and L-lysine, albeit functioning maximally under different pH optima (LDC maximum activity pH 6.8; ODC maximum activity pH 8.0) (Lee and Cho, 2001). If correct, it might be expected that disruption of *ODC* transcript levels in *N. glauca*, by the introduction of an *ODC*-RNAi construct, would reduce or possibly prevent elevations in anabasine levels in response to wound-associated stress. In the current study, we explore the validity of this hypothesis by undertaking targeted RNAi-mediated down-regulation of the authentic *N. glauca ODC* gene in hairy root cultures and regenerated transgenic plants of *N. glauca* to assess ability of these transgenic tissues to produce alkaloids in response to wound-associated stress.

2. Materials and methods

2.1. Screening of N. glauca genomic library for ODC sequences

Approximately 1.3 million plaques (average insert size 15 kb; ~3–4 haploid genome equivalents) of a custom-produced *N. glauca* genomic library were screened under high stringency conditions using a α-^{32}P dATP labelled *N. tabacum* ~800 bp *ODC* cDNA XbaI fragment (GenBank accession number Y10472) as outlined in DeBoer et al. (2009). Nine independent phage clones exhibiting strong hybridisation to the *N. tabacum ODC* probe were purified to the tertiary stage and inserts classified strictly into two separate groups (*NgODC1* or *NgODC2*) based upon Southern hybridisation restriction fragment length (RFLP) patterns and also partial sequence analysis (DeBoer, 2010). Approximately 4 kb of DNA

sequence was obtained from a purified phage of *NgODC1* type and approximately 4.3 kb of DNA sequence was obtained from a purified phage of *NgODC2* type (DeBoer, 2010). These sequences were separately sub-cloned into pBluescript and sequenced (GenBank accession number FR691073 [*NgODC1*] and FR691072 [*NgODC2*] respectively).

2.2. Generation of hairy root cultures and transgenic plants

A *CaMV35S-ODC*-RNAi expression cassette, containing a 347 bp *NgODC2* cDNA fragment cloned in both the sense and anti-sense direction to generate a double stranded hairpin RNA structure when expressed *in planta*, was cloned into pART27 binary vector, as described in DeBoer et al. (2011a). The *ODC*-RNAi vector and the empty pART27 binary control vector were transformed separately into *Agrobacterium rhizogenes*, and kanamycin-resistant clonal *N. glauca* hairy root cultures were generated and maintained with a ~3 week sub-culture regime essentially as described previously (Hamill and Lidgett, 1997; DeBoer et al., 2009, 2011a,b). Healthy root cultures, 11 days into their culture cycle, were treated with 2.5 μM MeJa to induce alkaloid synthesis (Cane et al., 2005; DeBoer et al., 2011a,b) and transgenic plants were regenerated from hairy root cultures of *N. glauca* and cultivated in an insect-free greenhouse essentially as described previously for *N. tabacum* (DeBoer et al., 2011a). The phenotype of most plants was similar to wild-type plants with very little evidence of the leaf wrinkling and reduced stature that has often been observed in primary regenerants of *N. tabacum* containing *A. rhizogenes* Ri T-DNA (Tepfer, 1984; Chintapakorn and Hamill, 2003, 2007). Prior to the onset of flowering, inflorescences of transgenic *N. glauca* plants were covered with paper bags to prevent cross fertilisation and seeds harvested from plants approximately 6 weeks after first opening of flowers.

2.3. Plant growth conditions and wounding regimes

Nicotiana glauca pART27 and *ODC*-RNAi seedlings were germinated *in vitro* on Murashige & Skoog (MS) agar plates containing 3% sucrose and also 75 mg l^{-1} kanamycin sulphate, to select seedlings containing binary vector T-DNA. Kanamycin-resistant plants, ~3–4 cm in height, were transferred to an insect-proof greenhouse (25 ± 2 °C; 16 h photoperiod) and cultured in individual hydroponic containers containing 200 mL of 50% modified Hoagland's nutrient solution as described previously (Sinclair et al., 2004; Cane et al., 2005; DeBoer et al., 2011a). One day prior to the commencement of experiments, when plants were ~20 cm in height, nutrient medium was replaced with 200 mL of 75% modified Hoagland's solution. Liquid lost due to transpiration or evaporation was replenished daily with de-ionised water for the duration of the experiment. Plants were wounded by surgical removal of the apex and young leaves less than 1 cm in length using a scalpel blade. The two upper leaves immediately below the excision point were harvested 24 h and 7 days following treatment for transcript and alkaloid analysis respectively. Phyllotactic equivalent leaves were harvested from untreated control plants at the same time points throughout the duration of the experiment. Alkaloid levels were determined by HPLC analysis (Cane et al., 2005; DeBoer et al., 2009, 2011a). For northern analysis, *N. tabacum ODC* and *ADC* cDNA probes were used, with signal levels quantified relative to the *Ubiquitin* signal detected using a cDNA probe from *Antirrhinum majus*, as described previously (Lidgett et al., 1995; Cane et al., 2005; DeBoer et al., 2009, 2011a). One-way ANOVA analysis in combination with a Tukey post hoc test was performed, unless otherwise stated, to determine whether transcript and alkaloid levels were significantly different between treatment groups.

3. Results

3.1. Nicotiana glauca ODC gene sequences

Analysis of phage DNA inserts in plaques isolated from the *N. glauca* genomic library revealed two closely related but distinct classes of DNA sequences, each possessing >90% nucleotide identity to other *Nicotiana ODC* sequences in genetic databases. We sequenced a representative of both classes and designated these sequences *NgODC1* and *NgODC2* (GenBank accession number FR691073 and FR691072 respectively). The *NgODC1* sequence possesses a high level of nucleotide identity with the 5′ end of a *N. tabacum ODC* cDNA sequence (accession number AB031066), with ~92% overall identity beginning at the putative transcription initiation site (as determined for *N. tabacum ODC* sequences; Xu et al., 2004) but ending ~400 bp downstream from the putative translational start site. The remainder of the *NgODC1* phage insert possesses low overall similarity with the remaining 3′ region of *N. tabacum ODC* cDNA, indicating that *NgODC1* most likely encodes a truncated *ODC* pseudo-gene sequence. Using a cDNA probe specific to the 3′ region of the *NgODC1* gene sequence, we were unable to detect expression in either leaf or root tissue of *N. glauca* by northern analysis (data not shown) indicating that *NgODC2* is likely to encode ODC in *N. glauca*. A similarly truncated *ODC* pseudo-gene sequence has also been reported in *N. tabacum* (*NtODC4*) (accession number AF233850; Xu et al., 2004).

NgODC2 exhibits a very high level of similarity to *N. tabacum ODC* cDNA sequences in current genetic databases, translating into a putative full length protein of 433 amino acids possessing >98% identity to the deduced *N. glutinosa* ODC (accession number: AAG45222), *N. tabacum* ODC1 (accession number: BAA83427) and *N. tabacum* ODC2 (accession number: AAK13622) proteins. Typical of other plant *ODC* gene sequences (Jimenez-Bremont et al., 2004), *NgODC2* does not contain introns. Recently the *Lupinus angustifolius* L/ODC protein was predicted to be localised in the chloroplast using the program WoLF PSORT (http://wolfpsort.org/), with a probability score of chloroplast: 10.0; nucleus; 2.0; cytosol; 2.0 (Bunsupa et al., 2012). Additional experimental analysis revealed that the L/ODC protein was indeed localised to the chloroplast in *L. angustifolius* (Bunsupa et al., 2012). Interestingly, WoLF PSORT analysis of NgODC2 also predicts a plastidic subcellular location (chloroplast 10.0, cytosol 2.0, mitochondria 1.0) for this deduced protein.

3.2. Alkaloid and transcript levels in N. glauca ODC-RNAi hairy root cultures

Although only trace amounts of pyridine alkaloids are detected in the roots of young hydroponically grown *N. glauca* plants (Sinclair et al., 2004; DeBoer, 2010), previous studies have indicated that axenic *N. glauca* root cultures, both non-transformed (Solt et al., 1960) and hairy roots transformed by *A. rhizogenes* T-DNA (DeBoer et al., 2009), contain substantial levels of both nicotine and anabasine. In order to examine the involvement of ODC in the synthesis of these alkaloids, *ODC* transcript levels were down-regulated in *N. glauca* hairy root cultures using a comparable RNAi approach to the one reported to be effective in *N. tabacum* (DeBoer et al., 2011a). Eight independent, healthy *N. glauca ODC*-RNAi-containing clonal hairy root lines were generated, along with three independent *N. glauca* clonal hairy root lines containing the empty pART27 binary vector as experimental controls. Transcript levels of *ODC* and *ADC* were examined by northern analysis in all clonal root cultures harvested at an actively growing stage (day 12) of their growth cycles (Fig. 2A). Average relative *ODC* transcript levels in the *ODC*-RNAi hairy root cultures were less than 30% of

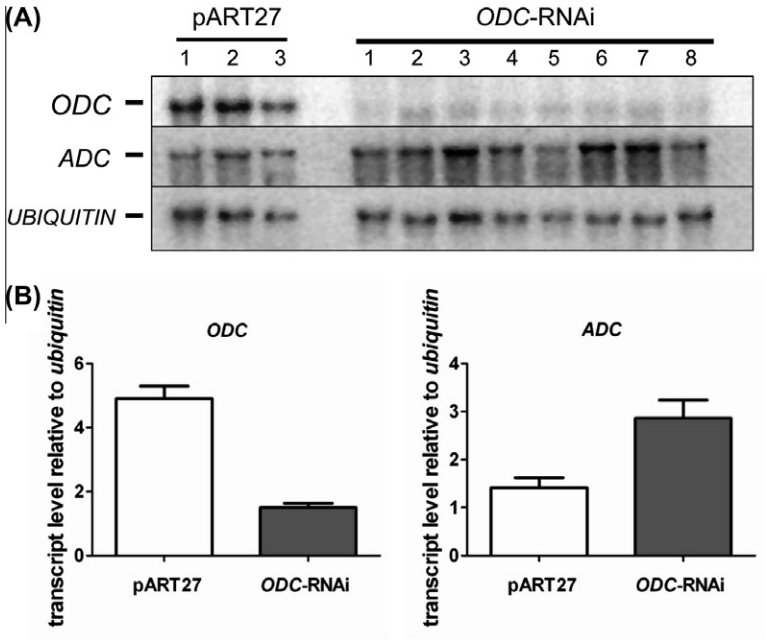

Fig. 2. (A) Northern analysis of *ODC*, *ADC* and *Ubiquitin* transcript levels in pART27 (empty vector control) vs. *CaMV35S ODC*-RNAi hairy root lines, harvested at day 12 of their growth cycle. (B) *ODC* and *ADC* transcript levels were normalised relative to *Ubiquitin* transcript levels. Bars represent the average transcript level in *ODC*-RNAi ($n = 8$) and pART27 ($n = 3$) hairy root cultures (±SE).

those typically observed in otherwise equivalent pART27 control hairy root lines. Interestingly, and in concordance with previous results involving the down-regulation of *ODC* transcript levels in transgenic plants of *N. tabacum* (DeBoer et al., 2011a), mean relative *ADC* transcript levels were observed to be ~2-fold higher in *N. glauca ODC*-RNAi hairy root cultures compared to pART27 vector controls (Fig. 2B).

Alkaloid concentrations were examined in each clonal hairy root culture noted above, using tissues that were harvested during the latter part (day 21) of a typical growth cycle. Analysis revealed a significant ($p = 0.03$ students *t*-test) reduction in mean baseline nicotine levels of *ODC*-RNAi *N. glauca* hairy root lines (average 1.8 mg g^{-1} dry wt.; SE ± 0.4) compared to nicotine levels of comparable pART27 control hairy root lines (average 3.8 mg g^{-1} dry wt.; SE ± 0.9). Baseline levels of anabasine, however, were comparable ($p > 0.05$) between *ODC*-RNAi (average 3.5 mg g^{-1} dry wt.; SE ± 0.7) and pART27 (3.2 mg g^{-1} dry wt.; SE ± 1.0) hairy root lines.

Previous studies involving *N. tabacum* hairy root cultures have noted that the effect of down-regulating *PMT*, *ADC* and *ODC* transcript levels on alkaloid levels can vary depending on the stage of the culture cycle at which tissues were harvested (Chintapakorn and Hamill, 2003, 2007; DeBoer et al., 2011a). Thus, a detailed analysis of hairy roots of two representative *N. glauca ODC*-RNAi lines and two pART27 control root lines were undertaken, at various stages during the course of a 25 day growth cycle (Fig. 3). In general, hairy roots of pART27 controls produced more biomass at each time point than comparable *ODC*-RNAi hairy root cultures, particularly when harvested during the latter stages of the culture growth cycle (Fig. 3A). Such diminished vigour of *ODC*-RNAi hairy root cultures may be due to a reduced capacity for putrescine/polyamine biosynthesis, as also noted in comparable experiments where *ADC* or *ODC* transcript levels were down-regulated in *N. tabacum* hairy root cultures (Chintapakorn and Hamill, 2007; DeBoer et al., 2011a). In terms of effects upon alkaloid levels, the main focus of the current study, it is clear that *ODC*-RNAi lines produced significantly decreased baseline levels of nicotine ($p < 0.05$) at all stages of the growth cycle when compared to the average of the pART27 control lines harvested at comparable time points (Fig. 3C). The effect of *ODC*-RNAi transcript down-regulation on baseline anabasine concentrations was less pronounced than the effect upon nicotine levels however. Though levels of anabasine in *ODC-RNAi* roots were, in general reduced compared to levels of anabasine in vector control roots harvested at comparable time points during the growth cycle, the magnitude of these differences were found not to be significant at $p = 0.05$. Anabasine and nicotine were detected at trace levels only, or not at all, in the culture media of both *N. glauca ODC*-RNAi and pART27 hairy root cultures sampled at all stages of their respective growth cycles.

3.3. MeJa elicitation of *N. glauca ODC-RNAi* hairy root cultures

Direct wounding of hairy root tissues is generally not undertaken as this often results in prolific callus formation, particularly in *Nicotiana* species, which is undesirable as loss of root integrity is typically associated with down-regulation of alkaloid synthesis (Rhodes et al., 1994). Thus in order to examine the effect of *ODC* transcript down-regulation on the capacity of *N. glauca* to increase pyridine alkaloid concentrations in response to wound-associated stress, hairy root cultures were treated with micromolar levels of the wound-associated hormone MeJa which has previously been shown to induce synthesis of alkaloids in *Nicotiana* hairy roots (Cane et al., 2005; DeBoer et al., 2009, 2011a). In line with previous observations involving *N. glauca* hairy roots (DeBoer et al., 2009), treatment of pART27 control cultures with 2.5 μM MeJa, produced a significant increase ($p < 0.05$) in both nicotine and anabasine concentrations when tissues were analysed 48 h following treatment (Fig. 4). In agreement with data presented in Fig. 3, nicotine levels were significantly lower in untreated *ODC*-RNAi lines when compared to vector controls ($p < 0.05$) and MeJa-treatment did not cause any significant increase in nicotine levels (Fig. 4A). No significant difference in average anabasine concentration was found in untreated *ODC*-RNAi hairy roots compared to untreated experimental pART27 control cultures. Interestingly however, anabasine levels in *ODC*-RNAi hairy roots treated with MeJa (~7 mg g^{-1} dry

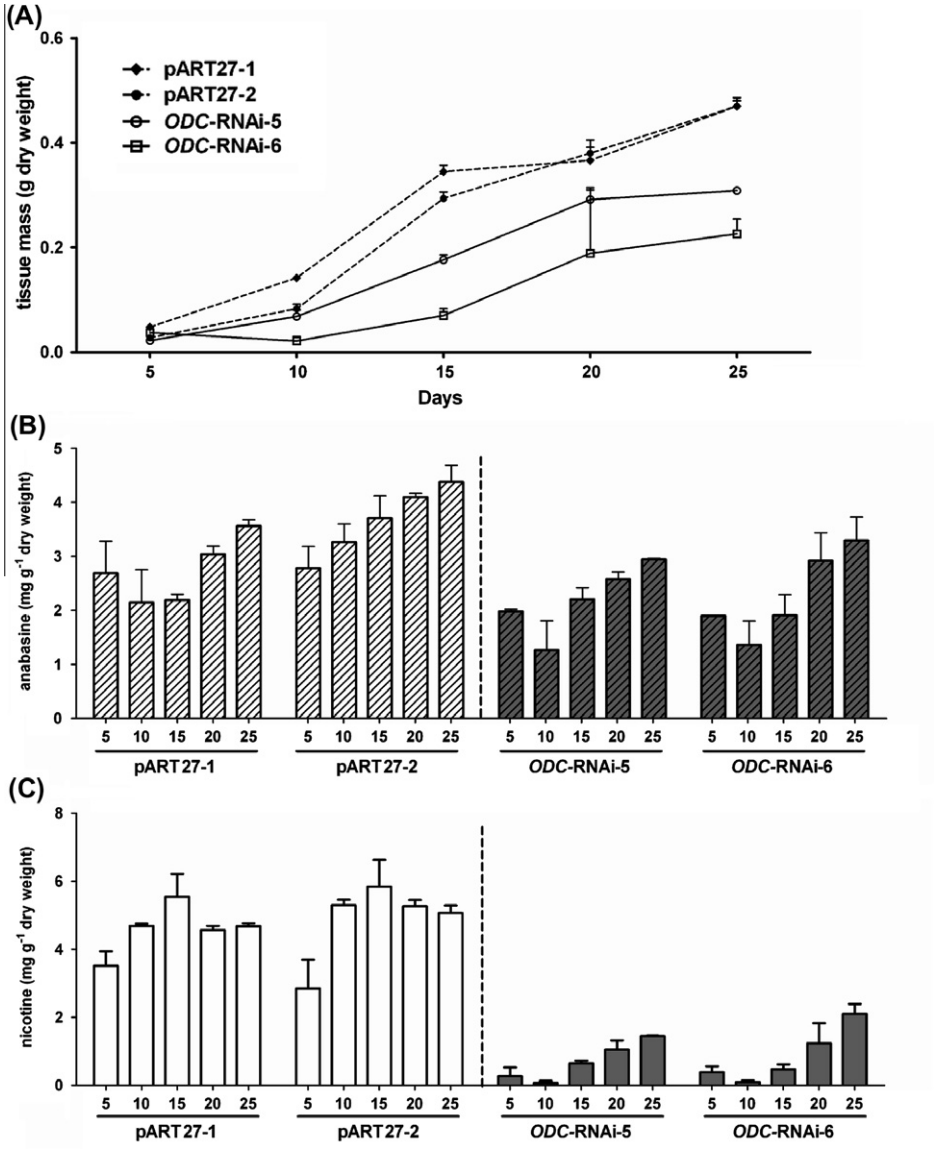

Fig. 3. The tissue mass of two independent *CaMV35S-ODC*-RNAi lines and two pART27 control lines (empty vector) was examined in tissues harvested at various stages during the course of a typical growth cycle. Panel A represents the average dry weight (±SE) of two separate vessels harvested at 5, 10, 15, 20 and 25 days of the culture cycle. Panels B and C respectively represent the average anabasine and nicotine concentrations (mg g^{-1} dry weight ± SE), in root tissue lines throughout the growth cycle.

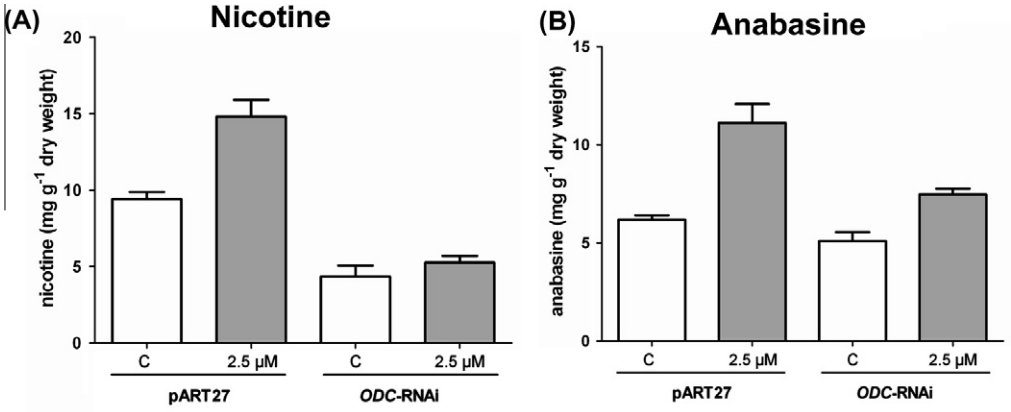

Fig. 4. Nicotine (Panel A) and anabasine (Panel B) concentrations (mg g^{-1} dry weight ± SE) were examined in pART27 and also *CaMV35S-ODC*-RNAi hairy root cultures in response to treatment with MeJa. Bars represent the average of three independent vessels (±SE) treated with ethanol only (C = control) or MeJa (2.5 µM final concentration). Treatment was undertaken at day 11 of the culture cycle. Final concentration of ethanol in all cultures was 0.04% v/v which is below toxic threshold level for *Nicotiana* hairy roots (Cane et al., 2005).

wt.) were significantly lower ($p < 0.05$) than in similarly MeJa treated vector control hairy roots (~12 mg g^{-1} dry wt.) (Fig. 4B).

3.4. Analysis of anabasine levels in ODC-RNAi N. glauca transgenic plants

Whole plants were regenerated from hairy root cultures in order to ascertain the effect of *ODC* transcript down-regulation on alkaloid synthesis *in planta*. Kanamycin-resistant offspring of T_0 transgenic plant lines that segregated in a 3:1 Mendelian fashion (likely indicating a single locus T-DNA insertion) were selected for detailed alkaloid and molecular analysis. All kanamycin-resistant *ODC*-RNAi T_1 plants were slightly smaller in stature than pART27 controls, but appeared healthy with no obvious leaf wrinkling or other developmental abnormalities. Northern analysis of RNA extracted 24 h after apex removal, demonstrated that leaves of T_1 *ODC*-RNAi *N. glauca* plants did not noticeably up-regulate *ODC* transcript levels, unlike similarly wounded pART27 vector controls (Fig. 5A). Anabasine was the only pyridine alkaloid detected in leaf tissues of all *N. glauca* plants analysed 1 week after wounding, in agreement with previous observations involving hydroponically grown *N. glauca* (Sinclair et al., 2004; DeBoer et al., 2009). Differences in baseline anabasine levels of leaf tissues of non-wounded *ODC*-RNAi transgenic plants and pART27 vector controls were observed (Fig. 5B), but were statistically non-significant ($p > 0.05$). However, levels of anabasine in upper leaves of wounded *ODC*-RNAi transgenic plants (~10 mg g^{-1} dry wt.) were found to be significantly lower ($p < 0.05$) than levels of anabasine found in upper leaves of similarly wounded pART27 vector control plants (~25 mg g^{-1} dry wt.) (Fig. 5B). Together with results presented in Fig. 4B, these results strongly suggest that ODC does indeed play an important role in enabling *N. glauca* to synthesise anabasine, particularly following exposure to wound-associated stress.

4. Discussion

In many higher plants, the diamine putrescine can be synthesised directly *via* the decarboxylation of ornithine by ODC, or indirectly from arginine *via* the initial action of ADC (Fuell et al., 2010; Fig. 1). In addition to being a precursor for polyamine synthesis, putrescine is also used to provide the pyrrolidine ring of nicotine and nornicotine in *Nicotiana* species (Leete, 1958; Solt et al., 1960). Recently, we reported that ODC plays a key role in providing putrescine to enable normal levels of nicotine synthesis in cultured hairy roots and also in whole plants of *N. tabacum*, particularly following exposure to wound-associated stress (DeBoer et al., 2011a). Similarly, results presented here show that RNAi-mediated reduction of *ODC* transcript markedly reduces nicotine concentrations in *N. glauca* hairy root cultures and largely removes capacity for stimulation of nicotine synthesis following treatment of such cultures with methyl jasmonate. Results from both of these studies contrast with the observations of Tiburcio and Galston (1986), whose analysis of *in vitro* cultured *N. tabacum* callus tissues suggested the predominance of the ADC-putrescine pathway in providing the pyrrolidine ring of nicotine.

Cadaverine, a diamine chemically similar to putrescine, is derived from lysine *via* the action of LDC, and is used to provide the Δ^1-piperidinium ring of anabasine in *Nicotiana* and a large number of pyrrolizidine and quinolizidine alkaloids in a wide range of species, including many legumes (Schoofs et al., 1983; Bunsupa et al., 2012 and references therein). Transcript levels of *ODC*, in addition to that of genes encoding other key enzymes such as *PMT*, *QPT2* and *A622*, are rapidly up-regulated in nicotine-producing roots of *Nicotiana sylvestris* and *N. tabacum* following exposure to wound-associated stress (Sinclair et al., 2000, 2004; Cane et al., 2005; DeBoer et al., 2011a; Ryan et al., 2012). Transcript levels of *ODC*, *QPT2* and *A622*, but not *PMT*, are also up-regulated in the upper leaves of *N. glauca* in response to wounding (Sinclair

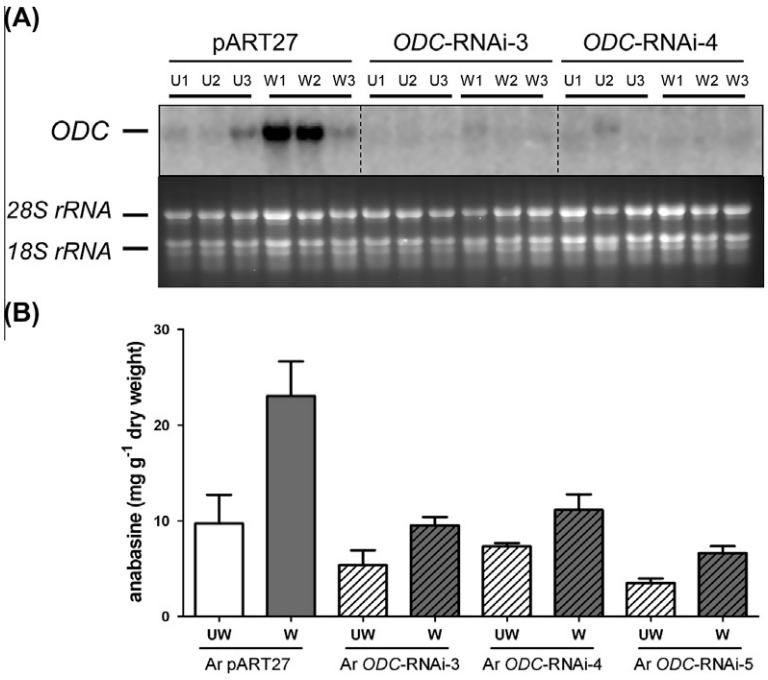

Fig. 5. The capacity for wound induced alkaloid synthesis was assessed in T_1 kanamycin-resistant offspring of *CaMV35S-ODC*-RNAi and pART27 – hairy root-derived regenerated transgenic plants. Transcript levels of *ODC* were examined by northern analysis in the uppermost expanded leaves, 24 h following removal of the plant apical region (W). Transcript levels in the phyllotactically equivalent leaves of intact control plants were also examined (U) (Panel A). Anabasine concentrations were examined in the leaves of both unwounded (UW) and wounded (W) plants 7 days following removal of the apical region (Panel B). Bars represent the average (mg g^{-1} dry wt. ± SE) alkaloid levels of 3–6 plants analysed per transgenic line.

et al., 2000, 2004; DeBoer et al., 2009; Ryan et al., 2012). Whilst it is clear that QPT and A622 are essential for anabasine synthesis (DeBoer et al., 2009; Kajikawa et al., 2009), the question of a role for ODC in facilitating elevated anabasine levels in wounded *N. glauca* has, until now, been uncertain. A working hypothesis, proposed by Sinclair et al. (2004), suggested that the marked increase in *ODC* transcript levels which is observed in upper leaves of *N. glauca* plants within 24 h of wounding, plays a key role in facilitating increased synthesis of anabasine by these tissues over several days, by virtue of the fact that *Nicotiana* ODC can decarboxylate lysine, under appropriate pH conditions (Lee and Cho, 2001). In the present study, whilst levels of anabasine in non-stressed hairy roots and non-wounded plants were not significantly reduced relative to appropriate control tissues, possibly due to normal functioning of the pyridine nucleotide cycle acting in concert with residual L/ODC enzymatic activity, we clearly did observe that RNAi-mediated down-regulation of *ODC* transcript levels in hairy roots of *N. glauca* does significantly impede any increase in anabasine levels following methyl jasmonate-treatment, unlike vector controls. Also, in separate experiments, we observed significantly lower levels of anabasine in leaves of *ODC*-RNAi transgenic plants of *N. glauca*, wounded by removal of their apices, compared to similarly treated vector controls. Together, these results strongly support the L/ODC hypothesis of Sinclair et al. (2004) with regards to *N. glauca* tissues experiencing wound-stress.

Recent studies indicate the presence of a bi-functional L/ODC protein in the legume *L. angustifolius*. The enzyme encoded by the *L. angustifolius* L/ODC gene is capable of catalysing the decarboxylation of both ornithine and lysine at almost equal efficiency, an evolutionary adaption that seems to have occurred specifically within quinolizidine alkaloid-synthesising plants within the Leguminosae family (Bunsupa et al., 2012). Key to acceptance of lysine as a substrate is a mutation which alters a His residue at position 344 in authentic ODC proteins to become a Phe residue at position 344 in the L/ODC protein of *L. angustifolius*. This mutation is postulated to affect the active site of the enzyme by altering the length of the 310-helix and thus the cavity size of the substrate pocket (Bunsupa et al., 2012). With respect to the present study, the protein encoded by the *NgODC2* gene sequence utilised to produce the *ODC*-RNAi construct, as used here, contains the amino acid Histidine at residue 344. The question therefore arises as to how ODC may function as LDC intracellularly in *N. glauca* to facilitate increased anabasine synthesis in response to wound-stress. We cannot answer this question definitively at present but it may be noteworthy that *N. glauca*, and indeed other *Nicotiana* ODC proteins, are predicted to be localised in the chloroplast. It is interesting to note also that lysine synthesis in plants is mainly associated with chloroplast fractions (Bunsupa et al., 2012 and references therein) and that previous experiments have demonstrated targeting of bacterial LDC to the plastid of *N. tabacum* hairy roots led to significantly increased capacity to produce cadaverine and anabasine (Fecker et al., 1993; Herminghaus et al., 1996). As has been noted, the *N. glauca* ODC2 deduced protein has >98% identity with *N. glutinosa* ODC, which was shown to have optimal ODC activity at pH 8.0 and optimal LDC activity at pH 6.8 (Lee and Cho, 2001). It is conceivable that alterations in sub-cellular pH levels, such as those which occur in the chloroplast stroma during alternate periods of darkness and light (Werden et al., 1975), might provide the appropriate microenvironment to allow ODC to function efficiently as LDC in *N. glauca* and thus facilitate anabasine synthesis. Clearly further detailed investigative experimentation here is warranted.

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Declaration for Thesis Chapter 6

Declaration by candidate

In the case of Chapter 6, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
- Planned, performed, analysed and interpreted all experiments - Main author of all sections of the manuscript	**80%**

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) (student co-authors only)
Dr Cecilia Blomstedt	- Provided supervisory support - Contribution to manuscript editing	N/A
Dr. Alan Neale	- Provided advisory and general editorial support	N/A
A/Prof Roslyn Gleadow	- Provided general editorial support	N/A
Dr Kathleen DeBoer	- Provided transgenic plants and general editorial support	N/A
Prof John Hamill *	- Provided laboratory and supervisory support - Contribution to experiment planning - Involved in field-specific editing of the manuscript	N/A

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** **26/10/2015**
Main Supervisor's Signature	[redacted]	**Date** **26/10/2015**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Chapter 6: Effects of down-regulating ornithine decarboxylase (ODC) upon putrescine-associated metabolism and growth in *Nicotiana tabacum* L.

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Running title: Effects of *ODC* down-regulation in tobacco.

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Highlight

RNAi-mediated reduction of ornithine decarboxylase gene activity in tobacco has negative effects on plant growth and leads to widespread alterations in primary and secondary metabolism, particularly in wounded plants.

Abstract

T_2 transgenic lines 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 to 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 ornithine, arginine, aspartate, glutamate and glutamine in spatially coincident tissues. Root transcript levels of *SAMDC*, *SAMS* and *SPDS* (polyamine synthesis enzymes) were reduced compared to vector controls, whilst transcript levels of *ADC* (putrescine synthesis), *PMT* (nicotine production) and *MATE* (alkaloid transport) genes were elevated. In contrast, expression of *QPT* (nicotinic acid production) and *A622* (nicotinic acid condensation reactions) were diminished in *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 affect 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 plants being brittle and bleached compared to vector-only controls. Together, results of this study demonstrate that ODC has important roles in facilitating both primary and secondary metabolism in *Nicotiana*.

Keywords

Alkaloid, gene expression, ODC, phenolamide, polyamine, putrescine, PMT, RNAi, QPT

Abbreviations

A622, PIP-family oxidoreductase; ADC, arginine decarboxylase; JA, jasmonate; MATE, Multi-drug and toxic compound extrusion; ODC, ornithine decarboxylase; PMT, putrescine methyltransferase; QPT, quinolinic acid phosphoribosyltransferase; RNAi, RNA interference; SAMDC, *S*–adenosyl methionine decarboxylase; SAMS, *S*–adenosyl methionine synthase; SPDS, spermidine synthase; VC, vector-only control.

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 (Aniszewshi, 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. attentata* 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, 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, 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 are regulated *via* the action of several transcription factors, including MYC2 and APETALA2/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, 2014; Shoji *et al*., 2009; Hildreth *et al*., 2011; 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 synthesised 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 (Fellenberg *et al*., 2012; Kaur *et al*., 2010; 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 (Siminszky *et al*., 2005; Lewis *et al*., 2008; 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 down-regulation of *ODC*, but not *ADC*, 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, utilising T_2 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 T_2 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 T_2 homozygous plants containing the T-DNA insert from an empty pART27 vector (vector-only control; VC), were used as a transformation 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 sterilised 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 ~four weeks before transfer to 250 mL capacity 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 acclimatise for a further two 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°C (±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 deionised water as described in previous work (DeBoer *et al*., 2009, 2011a, 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 dose of controlled release complete fertiliser (Osmocote) as recommended by the manufacturer (Scotts, Australia).

Plant treatments

After four 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 hours or 7 days after treatment for RNA or metabolite analysis, respectively, in line with previous work from this laboratory (Cane *et al*., 2005; DeBoer *et al*., 2009, 2011a, 2013). In non-damaged control plants, cotton thread was tied loosely around the petiole of phyllotactic 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 homogenous 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 optimised 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 optimised hydrochloric and boric acid extraction and supernatant aliquots were analysed as *ortho*-phthaldialdehyde/ethanethiol/fluorenylmethoxycarbonyl 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 mg dry weight of tissue which was extracted.

Quantitative Real Time PCR

Total RNA was isolated from snap frozen leaf and root tissues using the hot phenol method adapted from Verwoerd *et al*. (1989) and previously found to be suitable for extraction of high quality RNA from both leaf 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)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 SensiMixTM SYBR no-ROX (Bioline) following the manufacturer's recommendations. Previously published gene-specific primers (Shoji *et al*., 2008, 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 (Table S1). Results were obtained from analysis of three independent samples per treatment, each containing three technical replicates. Data was analysed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and is presented as the fold change in gene expression for that particular gene family, each normalised to 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 to 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 whereby 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. tabacum* 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^{-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^{-1} dwt) and 25-fold lower in roots (50 µg g^{-1} dwt; Fig. 3B). Anatabine levels were also low in non-wounded VC plants, being ~50 µg g^{-1} dwt in leaves and at trace levels in roots (Fig. 3C). Consistent with previous observations of T_1 *odc*-RNAi transgenics (DeBoer *et al*., 2011a), analysis of alkaloid concentrations in non-wounded T_2 *odc*-RNAi plants revealed markedly different profiles than 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^{-1} dwt in leaves and ~35 µg g^{-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^{-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^{-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^{-1} dwt) and a ~2-fold increase in nicotine content of roots (rising to ~2.4 mg g^{-1} dwt); a ~2-fold increase in nornicotine concentrations (rising to ~20 μg^{-1} dwt in leaves and ~80 μg^{-1} dwt in roots) and a ~10-fold increase anatabine concentrations (rising to ~1 mg g^{-1} in leaves and ~250 µg $^{-1}$ 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 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 to ~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 to non-wounded counterparts (Fig. 3D–E). The stimulatory effects upon phenolamide 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 3–4-fold in *odc*-RNAi plants that experienced apex removal, however total levels remained significantly lower compared to similarly damaged VC plants (Fig. 3E).

Amino acid analysis of odc-RNAi versus vector control plants

Silencing of *ODC* resulted in a significant increase (2–3-fold) in baseline levels of ornithine in leaf and root tissues of non-wounded *odc*-RNAi transgenics compared to 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 transgenics, ornithine concentrations were significantly enhanced (~3-fold) by leaf wounding alone and by apex removal (5–6-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 to 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 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 transgenics compared to VC counterparts (Fig. 4). Similarly, silencing of *ODC* 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 one 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 mobilisation (*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 T_1 *odc*-RNAi plants (DeBoer *et al*., 2011a), *ODC* transcript levels were reduced by >95% in T_2 *odc*-RNAi plants compared to VC plants, and did not increase significantly even after the combined wounding treatments (Fig. 5). The *ADC* wound-response was enhanced in *odc*-RNAi where we observed significantly higher (~2–3-fold) levels of *ADC* transcript in roots of wounded *odc*-RNAi plants compared to VC counterparts, increasing in magnitude in plants damaged by leaf wounding < apex removal < combined leaf wounding and apex removal treatments (Fig. 5). Silenced *ODC* plants also displayed ~1.5–2-fold higher basal and wound-elicited levels of *PMT* transcript compared to 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 plants were found to be significantly lower (~3-fold) than in comparable VC plants. We observed even greater relative differences in roots of wounded *odc*-RNAi plants, where *QPT* transcript levels were 5–6-fold lower than in similarly wounded VC plants. Even in plants that experienced the most severe combined leaf wounding and apex removal treatment, *QPT* transcript levels of *odc*-RNAi plants were not significantly elevated above levels observed in non-wounded VC plants (Fig 5). Transcript levels of *A622* in roots of wounded *odc*-RNAi plants were also significantly lower than in VC counterparts, albeit the magnitude of reduction was much less pronounced than was observed for *QPT* (Fig. 5).

Transcript levels of genes encoding polyamine-related enzymes were also clearly altered in *odc*-RNAi plants compared to VC plants. *SPDS* was significantly up-regulated in roots in response to leaf wounding compared to VC plants (~5-fold) and/or apex removal (6–7-fold) in VC plants compared to non-wounded counterparts. A marked reduction (~50%) in relative baseline *SPDS* transcript levels was observed in non-wounded *odc*-RNAi plants compared to VC plants. This difference was further exacerbated in wounded *odc*-RNAi plants, with *SPDS* transcript levels being only 75–80% less than that of similarly wounded VC plants. Silencing of *ODC* also resulted in ~1.5–2-fold reductions in relative *SAMS* and *SAMDC* transcript levels in both non-wounded and wounded *odc*-RNAi-plants, compared to appropriate VC plants (Fig. 5).

Effects of silencing ODC upon the spatial distribution of polyamine, alkaloid and phenolamide metabolites in decapitated N. tabacum plants

We examined the response of *odc*-RNAi plants to apex removal with respect to putrescine and polyamine-derived defence profiles throughout the plant. Additional experiments were undertaken to assess the effects of removing plant apices upon spatial distribution of polyamines (Fig. 6) as well as pyridine alkaloids and phenolamides (Fig. 7). One week after apex removal, older leaves located progressively lower on the stem of plants, as well 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 to equivalent tissues in VC plants (Figs 6–7). The capacity to increase polyamines, tyramine, nicotine/nornicotine and phenolamides concentrations in response to apex removal was also significantly compromised in *odc*-RNAi plants compared to 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 to similarly wounded VC plants (Fig. 6). Consistent with our previous study involving T_1 *odc*-RNAi transgenic plants, (DeBoer *et al*., 2011a), the present study found that anatabine concentrations were observed to be significantly elevated in roots and leaves of T_2 *odc*-RNAi plants, both non-wounded and wounded, compared to 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 T_1 offspring of transgenic plants, we did not observe an obvious negative effect upon phenotype in plants containing the *odc*-RNAi

construct (DeBoer *et al*., 2011a). However, in the present study, using T_2 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 *odc*-RNAi transgenics compared to 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 less leaf than their equivalently aged, similarly cultivated VC counterparts (Fig. 8A). This was 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 (e.g. Fig. 8F) of entire leaves, which were slightly epinastic and brittle compared to leaves of hydroponically grown VC plants (e.g. 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 Hoaglands nutrient medium in each container (3 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 Hoaglands, with twice weekly changes in medium (data not shown). In addition, seedlings grown in compost for seed production showed *odc*-RNAi lines to be markedly slower growing and delayed in their time of flowering compared to VC plants (e.g. 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, (reviewed in Dewey and Xie, 2013), 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 the *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 levels in *odc*-RNAi transgenics,

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). However, the observation that levels of arginine, glutamate, glutamine and aspartate were also elevated in leaves and roots of *odc*-RNAi transgenics, relative to VC plants, may not have been anticipated. As discussed recently, arginine is derived from ornithine whilst ornithine is derived ultimately from glutamate (Winter *et al*., 2015). The possibility of biochemical conversion *in vivo* between arginine and ornithine, *via* the urea cycle, has also been a discussion point in the literature concerning the relative importance of the ODC- versus the ADC-route to putrescine (Shargool *et al*., 1988; Robins *et al*., 1991; Winter *et al*., 2015). Further work is required to determine the precise reasons for elevations in concentrations of arginine, glutamate and other amino acids in *odc*-RNAi transgenic plants, relative to VC plants.

Analysis of nicotine and anatabine alkaloid concentrations in non-wounded and wounded plants were consistent with other studies involving 'wild type' *N. tabacum* (e.g. Saunders and Bush, 1979; Shi *et al*., 2006) and those conducted in our laboratory involving 'wild type', VC and *odc*-RNAi lines (e.g. Sinclair *et al*., 2000; Cane *et al*., 2005; DeBoer *et al*., 2011a). In the present study we also observed that *odc*-RNAi plants had significantly lower levels of nornicotine in leaves and roots of both non-wounded and wounded plants, compared to VC counterparts. Although a minor component of the alkaloid profile in most commercial *N. tabacum* varieties, nornicotine is considered to be particularly undesirable due to its potential for biochemical conversion into strongly carcinogenic nitrosamines (Lewis *et al*., 2008, 2010).

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). The current study indicates that ODC plays a particularly important role in enabling production of a range of defensive metabolites in such tissues of *N. tabacum*, particularly in response to aerial damage. Thus, the largest disparities between alkaloid levels of wounded VC and *odc*-RNAi plants were observed in leaves positioned in the upper half of the plant. Marked reduced concentrations of phenolamides were also particularly evident in the upper leaves *odc*-RNAi plants compared to phyllotactic equivalent tissues of VC

plants. These putrescine derivatives have also 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* (Guillet and De Luca, 2005; Kim *et al*., 2011). Though not directly associated with putrescine or polyamine 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* to resist herbivory in an external environment, though we might expect such plants to be more susceptible to insect attack than normal. Although they had enhanced ability to produce anatabine in response to wounding, compared to VC plants, it is unlikely that this would be sufficient to protect these *odc*-RNAi plants from insect larval attack, in the absence of normal capacity to produce nicotine (e.g. Steppuhn *et al*., 2004) or other likely defensive metabolites as noted above. Further experimentation here is warranted.

In addition to changes in the metabolic profile of *ODC*-silenced plants, and unlike our earlier observations (DeBoer *et al*., 2011a), we noted negative effects upon plant growth and morphology in hydroponically grown T_2 lines that were homozygous for the introduced *odc*-RNAi construct, compared to 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 hydoponically grown *odc*-RNAi transgenics such as shorter internode expansion, slower rates of development and release of dormant axillary buds following decapitation, and also reduced vigour and delayed onset of flowering in *odc*-RNAi plants grown in soil, all relative to VC plants, 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 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).

In the current study we also undertook a more detailed analysis of the effects of down-regulating *ODC* on relevant components of the associated polyamine and alkaloid root transcriptome. Comparatively elevated levels of *ADC* transcript in roots of *odc*-RNAi lines, relative to VC plants, was 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). Such a compensatory increase may have enabled partial restorative production of putrescine-derived metabolites for primary and specialised metabolism requirements and is consistent with suggestions that putrescine supply is not solely under transcriptional control, but is also influenced biochemically by the metabolic flux of associated metabolites (Page *et al*., 2012). In contrast to *ADC*, transcript levels of *SPDS*, *SAMDC* and *SAMS* were significantly diminished in roots of *odc*-RNAi plants compared to VC counterparts, possibly indicating a mechanism whereby availability of putrescine affects the regulation of downstream genes essential for polyamine synthesis. With regards to genes more closely associated with alkaloid production, our observations that *PMT* and *MATE* gene transcripts were up-regulated to a greater extent in roots of wounded *odc*-RNAi plants than in similarly treated VC plants is in contrast to markedly reduced transcript abundance of *A622* and *QPT*. The latter observation is of particular interest and was not anticipated as several molecular systems that regulate wound- and JA-induction of alkaloid biosynthesis in *Nicotiana* including AP2/ERF- and MYC2-type transcription factors, are known to impact directly upon the capacity for up-regulated expression of key genes such as *PMT*, *QPT, A622* and *MATE* in wound-stressed tissues of *Nicotiana* (Shoji *et al*., 2009; DeBoer *et al*., 2011b; Shoji and Hashimoto, 2013b). The possibility of an important role for putrescine or its derivatives in the regulation of *QPT* transcript abundance in *Nicotiana*, as suggested by the results of the current study, is intriguing and may in fact help explain previous observations whereby over-expression of a yeast *ODC* gene was reported to cause both elevated putrescine levels and increased concentrations of nicotine in hairy roots of *N. rustica* (Hamill *et al*., 1990). Given the ubiquitous importance of QPT as the anaplerotic enzyme of the pyridine nucleotide cycle, in addition to providing nicotinic acid for pyridine alkaloid synthesis in *Nicotiana* and other genera (Wagner *et al*., 1986; Katoh *et al*., 2006; Noctor *et al*., 2006), it would not be surprising if additional regulatory controls have evolved to regulate this vital area of metabolism at the interface between primary and secondary metabolism in *Nicotiana*. In this

respect it may be pertinent to note the findings recently reported by Li *et al*. (2015) who demonstrated that a miRNA decoy accumulates in roots of *N. tabacum,* in response to topping, leading to sequestration of a *QPT*-associated miRNA repressor and thereby enabling increased levels of *QPT* transcript levels in wounded plants.

Competing interests

The authors declare no competing or financial interests.

Author contributions

HLD designed, collected, performed data analysis and prepared figures. JDH assisted in the design of experiments. HLD, CKB, and JDH prepared the manuscript. ADN, RG and KDD provided editorial support.

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Figure Legends

Figure 1. Schematic diagram of the biosynthesis of polyamines and connections with other metabolic pathways, including pyridine alkaloid, phenolamide and phenylpropanoid metabolism, in *Nicotiana* (adapted from Cane *et al*., 2005, Onkokesung *et al*., 2012, Shoji and Hashimoto, 2012, Winter *et al*., 2015). Solid lines indicate defined steps, while broken 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 isoflavone reductase, 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 NUP1 and MATEs are involved in vacuolar storage and transport of alkaloids to aerial tissues.

Figure 2. *ODC* influences non-wounded and wound-induced levels of amines in leaf and root tissues of *N. tabacum* plants. Four weeks after transfer to hydroponics, VC and *odc*-RNAi transgenic plants were either left non-wounded (C); mechanically damaged with a pattern wheel to the two uppermost (<50%) expanded leaves (W); wounded by removing the apical region containing leaves which were less than 50% expanded (A) or a combination of both wounding treatments (W+A). Phyllotactic equivalent leaves from untreated plants that were not damaged were analysed as a wounding control comparison. Mean (±SE) putrescine (A), spermidine (B), spermine (C) and tyramine (D) content (nmol) per g dry weight (wt) in the two uppermost expanded leaves (top panel) or root tissues (bottom panel) were harvested from *odc*-RNAi transgenic *vs*. VC plants 7 days 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 the Tukey-HSD and are indicated by different letters (n=4).

Figure 3. *ODC* affects the accumulation of pyridine alkaloids (A–C) and phenolamides (D and E) in both non-wounded and wounded plants. Leaves of VC and *odc*-RNAi transgenic plants were treated and harvested 7 days post-treatment as described in Fig. 2. Mean (±SE) concentrations of nicotine (A), nornicotine (B), anatabine (C) in the two uppermost expanded leaves (top panel) and root (bottom panel) tissue from *odc*-RNAi *vs*. VC plants is shown. The mean (±SE) concentration of caffeoylputrescine (D) and dicaffeoylspermidine (E) in the two uppermost expanded leaves are shown. Roots were analysed for phenolamides but were present at trace levels and not quantifiable in all cases. Significant differences ($P<0.05$) between the lines and treatment groups were determined by two-way ANOVA followed by the Tukey-HSD test and are indicated by letters (n=4). **A**, apex removed; **C**, control; **W**, leaf wounded; **W + A**, wound plus apex removal.

Figure 4. Silencing *ODC* affects accumulation of inter-related amino acids. VC and *odc*-RNAi transgenic plants were treated and harvested 7 days post-treatment as described in Fig. 2. A schematic diagram depicting a simplified version of the metabolic connections between various amino acids, pyridine nucleotide cycle components, polyamines (putrescine and spermidine) and pyridine alkaloid (nicotine) metabolic pathways is shown**.** Solid lines indicate single defined steps, while broken lines indicate steps comprised of multiple reactions. Mean (±SE) arginine, aspartate, glutamate, glutamine and ornithine content (nmol) per g dry weight (wt) in the two uppermost expanded leaves (top panel) and roots (bottom panel) from vector-only *vs*. *odc*-RNAi transgenic plants. Significantly different levels of amino acids among the lines at $P<0.05$ were determined by two-way ANOVA followed by the Tukey-HSD test and are indicated by different letters (n=4).

Figure 5. The effect of wounding on root transcript levels of key genes involved in polyamine and pyridine alkaloid synthesis in vector-only control and *odc*-RNAi plants. RNA was extracted from roots harvested 24 hours after wounding treatments as described in Fig. 2. Data shown in the schematic diagram represent the average (±SE) transcript abundance of designated alkaloid- and polyamine-associated genes normalised to the endogenous reference gene (EF1α) and relative to the non-wounded VC control at time-zero which was arbitrarily designated as "1". Significant differences ($P<0.05$) in levels of transcripts among VC and *odc*-RNAi

plants were determined by two-way ANOVA followed by the Tukey-HSD test and are indicated by letters (n=4). For description of abbreviations see Fig. 1.

Figure 6. The spatial distribution of amines in non-wounded and wounded vector-only control and *odc*-RNAi plants. Four weeks after transfer to hydroponics, pre-flowering VC and *odc*-RNAi transgenic plants were either not wounded (C) or wounded by removing the apex, along with leaves less than 50% expanded (A). Different tissues were harvested and quantified for amines 7 days post-treatment. Mean (±SE) putrescine, spermidine, spermine and tyramine content (µmol) per g dry weight (wt) in apical buds, leaf (unexpanded through to leaf 7), stem and root tissue from *odc*-RNAi transgenic plants *vs*. VC plants. Significant differences ($P<0.05$) among plant lines were determined by two-way ANOVA followed by the Tukey-HSD test and are indicated by letters (n=4). Unexpanded, leaves less than 50% expanded.

Figure 7. Levels of pyridine alkaloids and phenolamides in tissues of non-wounded and wounded vector-only control and *odc*-RNAi plants. Plants were either not wounded (C) or had their apex removed (A) and harvested 7 days post-treatment as described in Fig. 5. Data represents mean (±SE) concentrations (mg) per g dry weight (wt) of nicotine, anatabine and nornicotine (pyridine alkaloids) and caffeoylputrescine and dicaffeoylspermidine (phenolamides) in apical buds, leaf (unexpanded through to leaf 7), stem and root tissue from VC *vs*. *odc*-RNAi transgenic plants. Letters represent significantly different ($P<0.05$) concentrations of metabolites determined by two-way ANOVA and followed by the Tukey-HSD test among *odc*-RNAi and VC plants (n=4). Unexpanded, leaves less than 50% expanded.

Figure 8. Growth and morphological traits in vector-only control and *odc*-RNAi plants. Seedlings cultured on MS medium *in vitro* for six weeks (~4–6 leaf stage) were transferred to either hydroponics (A–F) with continual nutrient replenishment or soil (G) with an initial dose of Osmocote® controlled release complete fertiliser. Five weeks after plants were transferred to hydroponics, leaf number (A), stem length (B), internode length (C), root biomass blotted to remove extra liquid (D) and leaf morphology (F) were recorded from non-wounded VC and *odc*-RNAi transgenic plants. The presence of chlorotic sections on leaves of *odc*-RNAi plants, unlike VC plants can be noted (F). Plants were then decapitated to a height of 10 cm, where all

leaves were removed and the rate of axillary bud growth and emergence following decapitation was measured over the following 8 days (E). Significant differences ($P<0.05$) between lines, represented by letters, was determined by one-way or two-way ANOVA and followed by the Tukey-HSD test (n=4). VC plants that were transferred to compost at the ~4–6 leaf stage with the aim of collecting seeds after self-fertilisation were ~60–80 cm high with inflorescence formation or were in flower ~12 weeks after transfer whilst *odc*-RNAi plants were much shorter in height (10–25 cm) with no signs of inflorescence development at this time point (G; lower senescent leaves removed prior to photography). The *odc*-RNAi plants shown here did flower however, after a further 8–12 weeks of greenhouse cultivation, with heights of plants at flowering being approximately half that of the VC controls. Floral morphology and self-fertility was not noticeably altered in these *odc*-RNAi transgenics compared to VC plants (data not shown). Fr. wt, fresh weight.

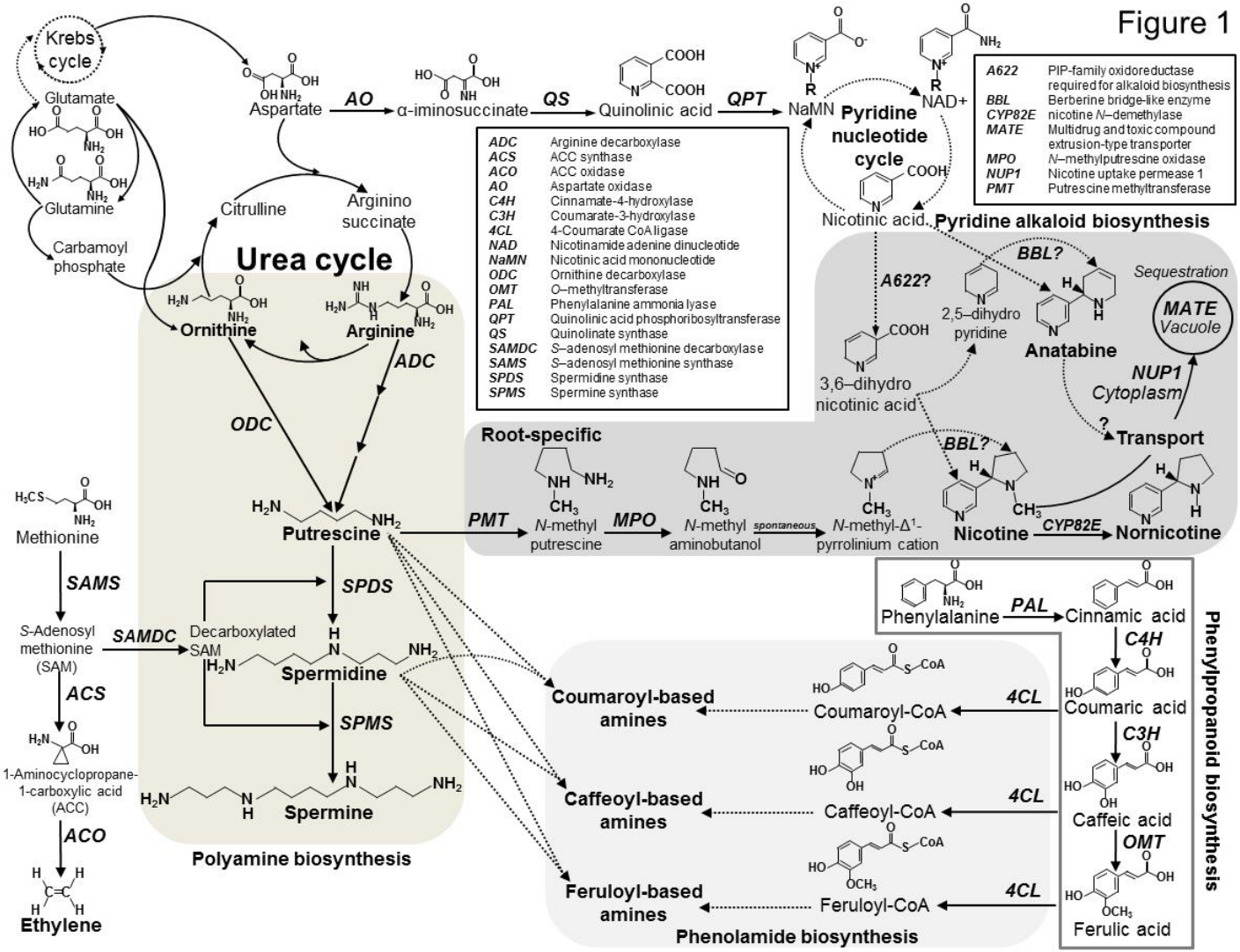


Figure 1
Krebs cycle
Glutamate
Glutamine
Carbamoyl phosphate
Aspartate
AO
α-iminosuccinate
QS
Quinolinic acid
QPT
NaMN
NAD+
Pyridine nucleotide cycle
Nicotinic acid
Pyridine alkaloid biosynthesis
Urea cycle
Citrulline
Arginino succinate
Ornithine
Arginine
ADC
ODC
Putrescine
Methionine
SAMS
S-Adenosyl methionine (SAM)
SAMDC
Decarboxylated SAM
Spermidine
SPDS
SPMS
Spermine
ACS
1-Aminocyclopropane-1-carboxylic acid (ACC)
ACO
Ethylene
Polyamine biosynthesis
Root-specific
PMT
N-methyl putrescine
MPO
N-methyl aminobutanol
spontaneous
N-methyl-Δ1-pyrrolinium cation
Nicotine
CYP82E
Nornicotine
A622?
2,5–dihydro pyridine
3,6–dihydro nicotinic acid
BBL?
Anatabine
Sequestration
MATE Vacuole
NUP1 Cytoplasm
Transport
ADC Arginine decarboxylase
ACS ACC synthase
ACO ACC oxidase
AO Aspartate oxidase
C4H Cinnamate-4-hydroxylase
C3H Coumarate-3-hydroxylase
4CL 4-Coumarate CoA ligase
NAD Nicotinamide adenine dinucleotide
NaMN Nicotinic acid mononucleotide
ODC Ornithine decarboxylase
OMT O–methyltransferase
PAL Phenylalanine ammonia lyase
QPT Quinolinic acid phosphoribosyltransferase
QS Quinolinate synthase
SAMDC S–adenosyl methionine decarboxylase
SAMS S–adenosyl methionine synthase
SPDS Spermidine synthase
SPMS Spermine synthase
A622 PIP-family oxidoreductase required for alkaloid biosynthesis
BBL Berberine bridge-like enzyme
CYP82E nicotine N–demethylase
MATE Multidrug and toxic compound extrusion-type transporter
MPO N–methylputrescine oxidase
NUP1 Nicotine uptake permease 1
PMT Putrescine methyltransferase
Phenylalanine
PAL
Cinnamic acid
C4H
Coumaric acid
C3H
Caffeic acid
OMT
Ferulic acid
4CL
Coumaroyl-CoA
Caffeoyl-CoA
Feruloyl-CoA
Coumaroyl-based amines
Caffeoyl-based amines
Feruloyl-based amines
Phenolamide biosynthesis
Phenylpropanoid biosynthesis

Figure 2

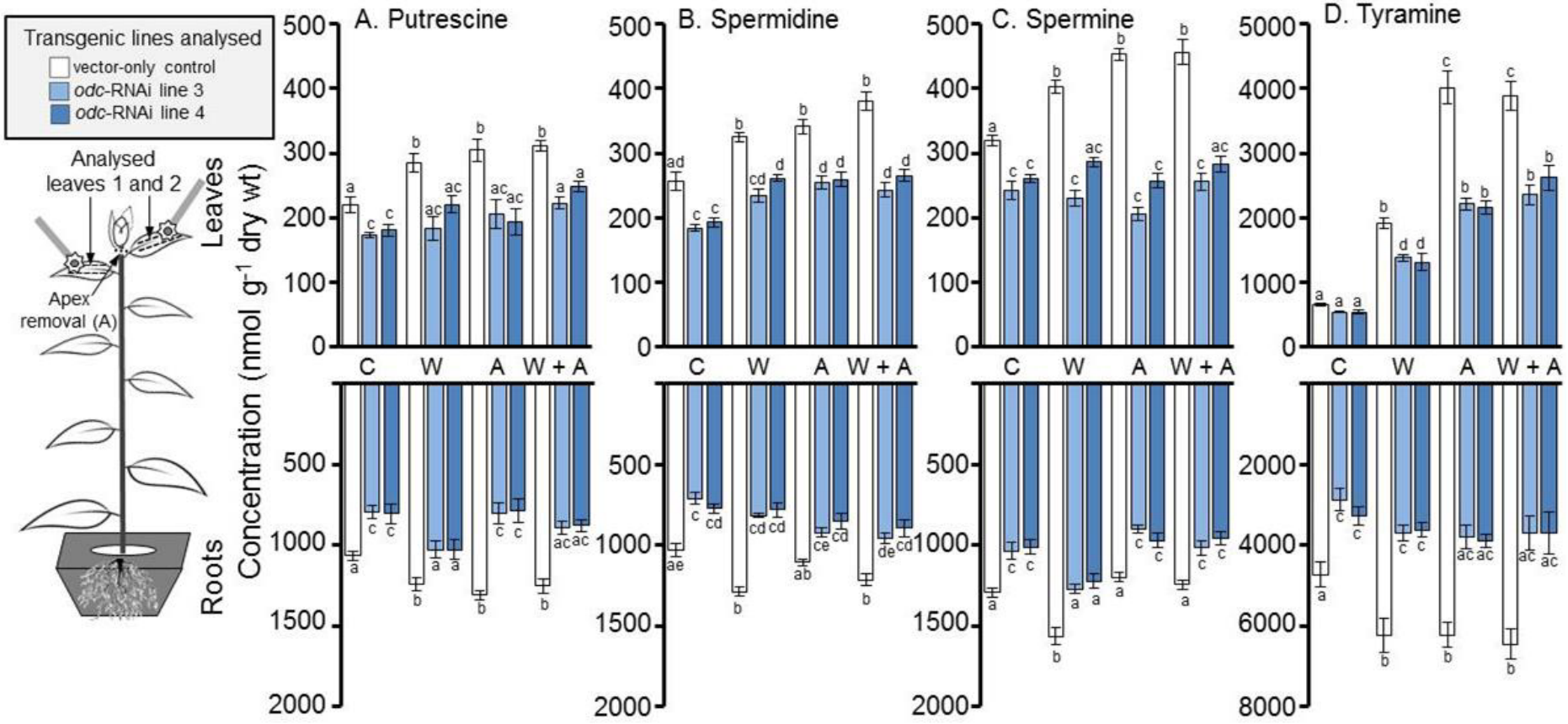

Transgenic lines analysed
vector-only control
odc-RNAi line 3
odc-RNAi line 4
Analysed leaves 1 and 2
Apex removal (A)
Leaves
Roots
Concentration (nmol g-1 dry wt)
A. Putrescine
B. Spermidine
C. Spermine
D. Tyramine
C
W
A
W + A

Figure 3

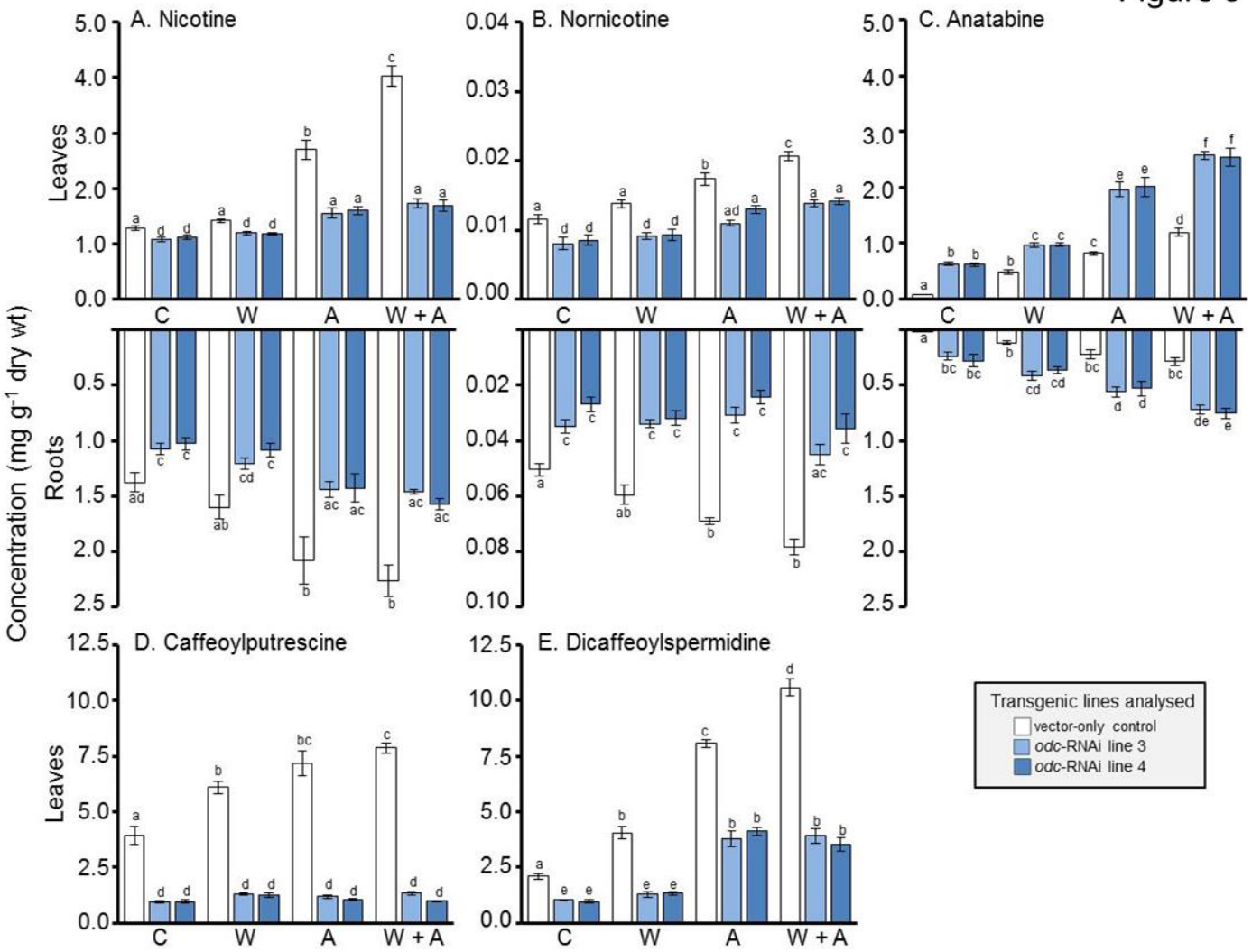

A. Nicotine
B. Nornicotine
C. Anatabine
D. Caffeoylputrescine
E. Dicaffeoylspermidine
Leaves
Roots
Concentration (mg g-1 dry wt)
C
W
A
W + A
Transgenic lines analysed
vector-only control
odc-RNAi line 3
odc-RNAi line 4

Figure 4

Concentration (nmol g^{-1} dry wt) of associated urea cycle amino acids

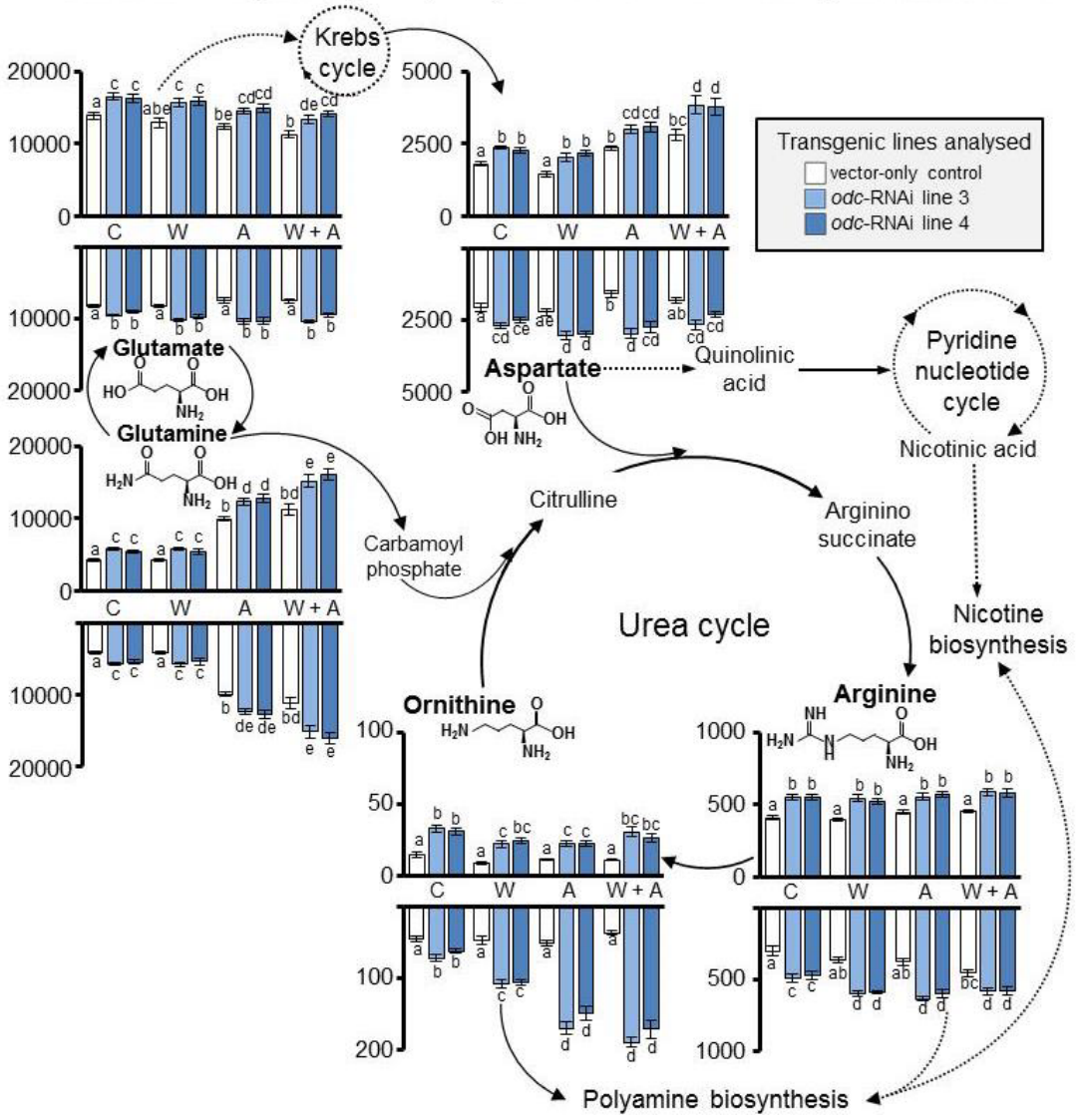
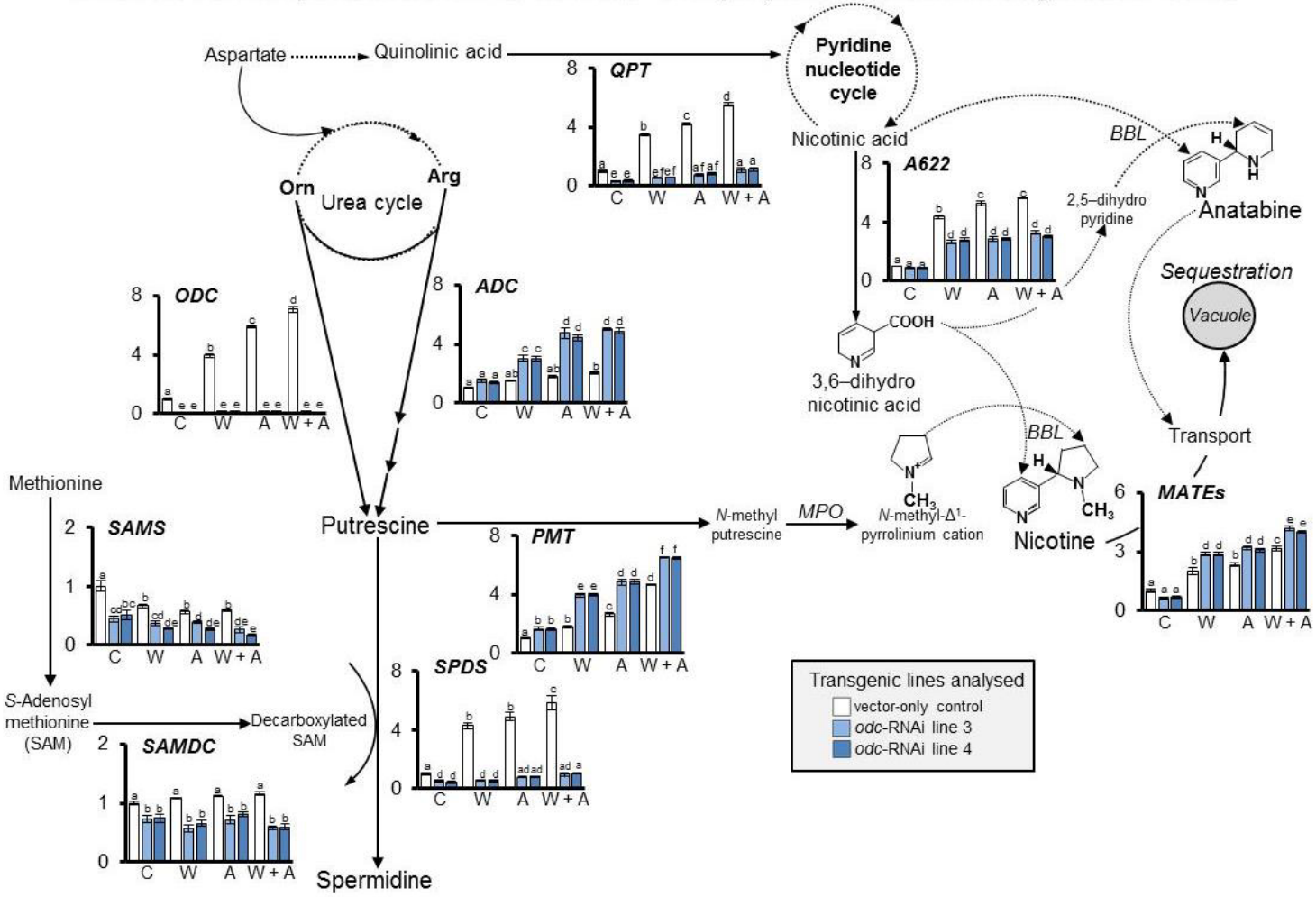


Figure 5

Relative transcript abundance of alkaloid- and polyamine-associated genes in roots



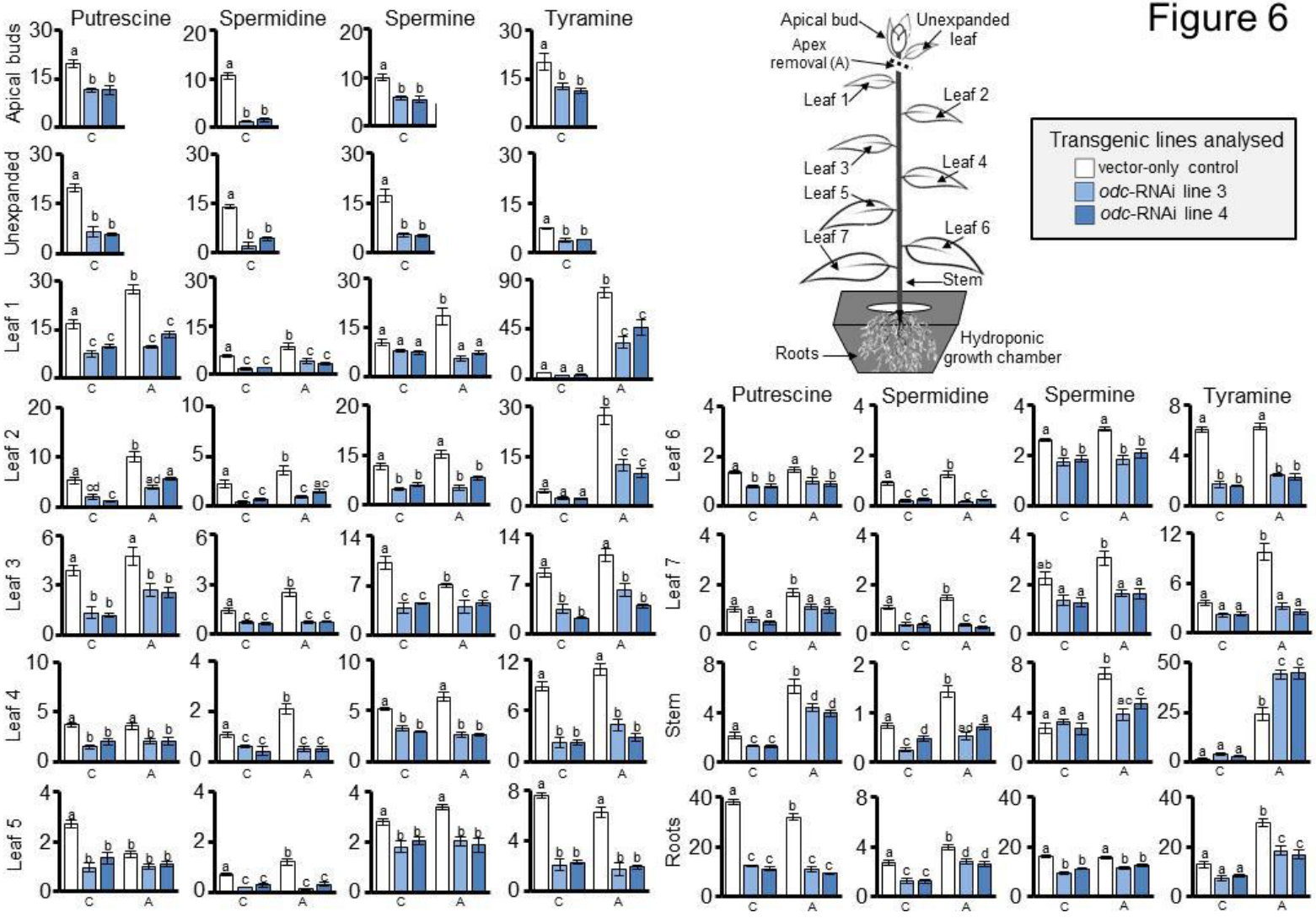


Figure 6
Putrescine
Spermidine
Spermine
Tyramine
Apical buds
Unexpanded
Leaf 1
Leaf 2
Leaf 3
Leaf 4
Leaf 5
Leaf 6
Leaf 7
Stem
Roots
Apical bud
Unexpanded leaf
Apex removal (A)
Hydroponic growth chamber
Transgenic lines analysed
vector-only control
odc-RNAi line 3
odc-RNAi line 4
C
A

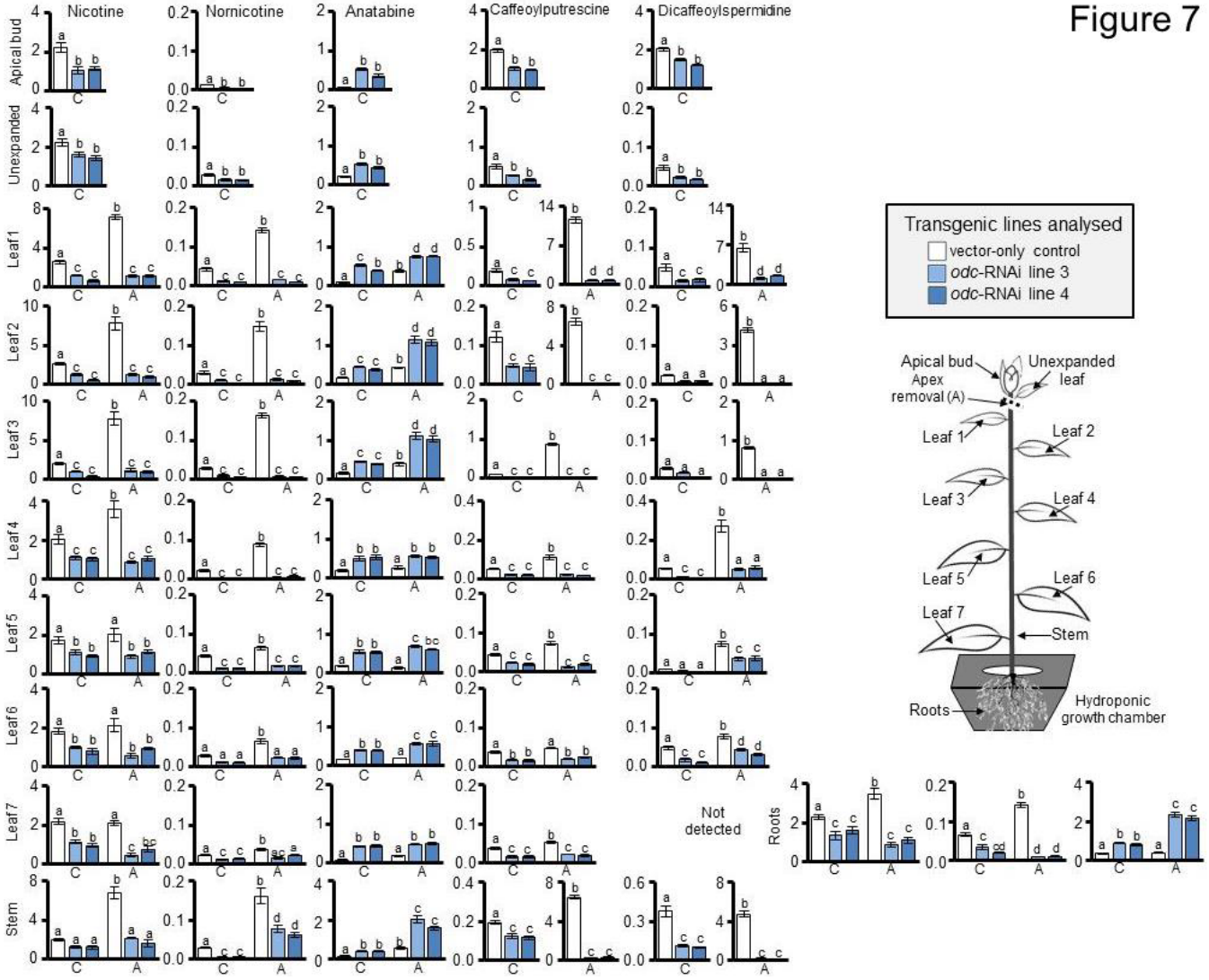
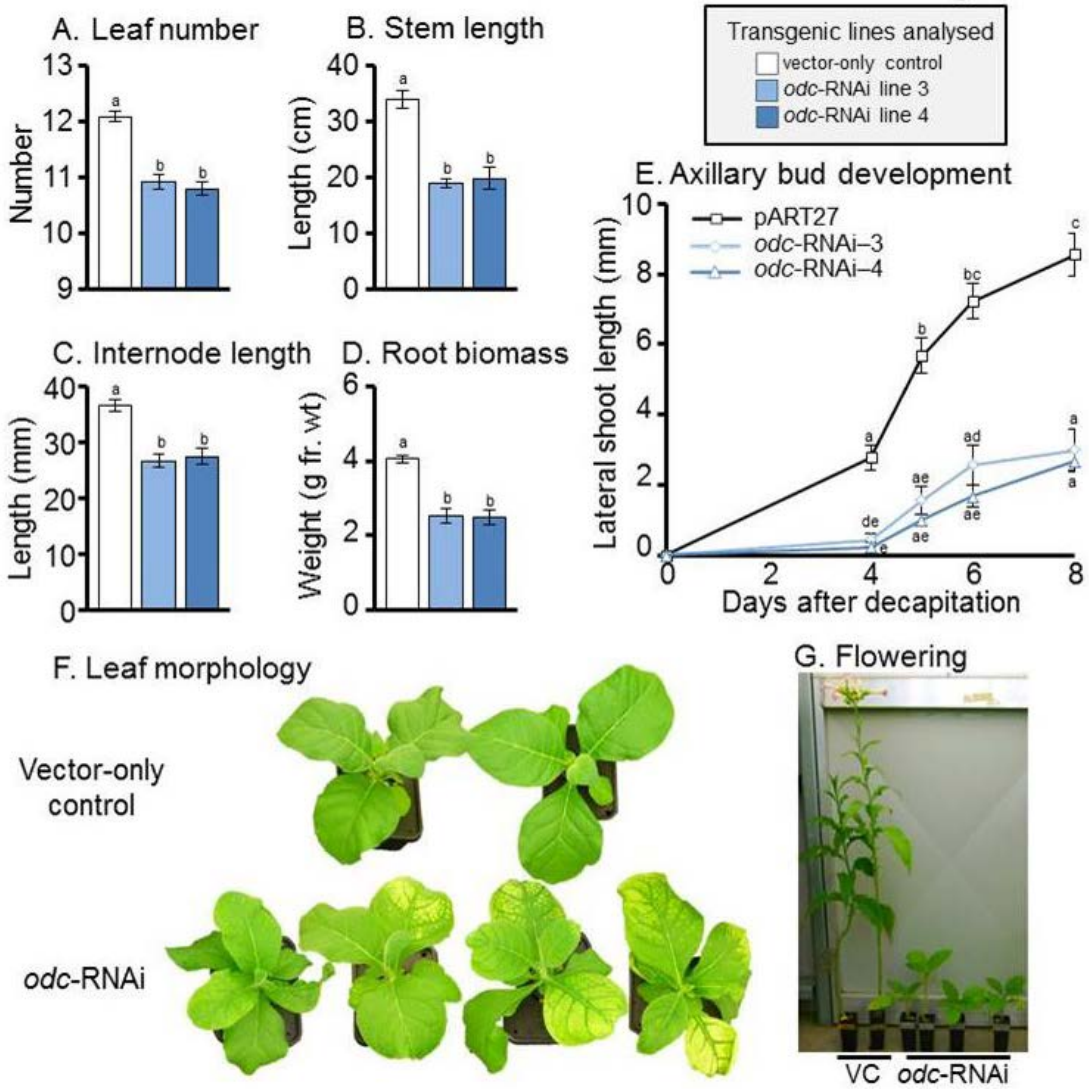

Nicotine
Nornicotine
Anatabine
Caffeoylputrescine
Dicaffeoylspermidine
Apical bud
Unexpanded
Leaf 1
Leaf 2
Leaf 3
Leaf 4
Leaf 5
Leaf 6
Leaf 7
Stem
Roots
Not detected
C
A
Transgenic lines analysed
vector-only control
odc-RNAi line 3
odc-RNAi line 4
Apical bud
Apex removal (A)
Unexpanded leaf
Leaf 1
Leaf 2
Leaf 3
Leaf 4
Leaf 5
Leaf 6
Leaf 7
Stem
Roots
Hydroponic growth chamber

Figure 7

Figure 8


A. Leaf number
Number
B. Stem length
Length (cm)
C. Internode length
Length (mm)
D. Root biomass
Weight (g fr. wt)
Transgenic lines analysed
vector-only control
odc-RNAi line 3
odc-RNAi line 4
E. Axillary bud development
Lateral shoot length (mm)
pART27
odc-RNAi–3
odc-RNAi–4
Days after decapitation
F. Leaf morphology
Vector-only control
odc-RNAi
G. Flowering
VC
odc-RNAi

Supplementary Table S1. Sequences of gene-specific primers used for qRT-PCR. Previously published primers were utilised or modified in order to amplify a template of ~100 bp as close to the 3' end of the respective gene as possible.

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Reference
EF-1a	AAGCCCATGGT TGTTGAGAC	CGTTCTTGATAA CACCAACAGC	Shoji *et al*., (2010)
α – tubulin	GCTACCATCAA GACTAAGCG	CTCCAGGAACA ACAGTTGG	Shoji *et al*., (2008)
UBC 2	TGCAAAATCAG TGGAGTCC	TCTGAATTAGCT GGCGAG	Modified from Schmidt and Delaney (2010)
A622	GGAAGACCCTC GAGAAGTTA	GAATGGCATAT GGCCAAAT	Modified from Shoji *et al*., (2010)
ADC	GCGGAGGAATT CTTGGAACA	CCACAAGGTAA GGCATGTTATG	Modified from Shoji *et al*., (2010)
MATE1/2	TCTAAACAAGG AATGAAGGTGG	GACTTCTTTCCC CTTGCATA	Modified from Shoji *et al*., (2010)
ODC	GTTAGCTGTTCT GTCGAATCG	AACAGTATCAA GAGCATCACA	Modified from Shoji *et al*., (2010)
PMT	GCAGCATTCAT TTTGCCATC	GACTCGATCTT GGTCCAATG	Modified from Shoji *et al*., (2010)
QPT1/2	CTTCGTTGACTA GGATAATGC	CCCATTGATCA ATTCTACAG	Shoji and Hashimoto (2011)
SAMDC	CAGTGTCCGTG TCTGTCTCTG	ACAAATCCGAA CGACACAGC	Shoji *et al*., (2010)
SAMS	CAAGGTGGACA GGAGTGGTG	TGCATAAGAAA CCTGGACAATG	Shoji *et al*., (2010)
SPDS	CAGATGTAGCT GTAGGATACG	CAGCATCGTAA GTTCCTGC	Shoji *et al*., (2010)

Chapter 7: General discussion

Plants have evolved sophisticated signalling networks in order to regulate and coordinate gene expression, metabolism and growth at the tissue and organ level. Such signalling networks also offer plants the ability to respond to environmental stimuli such as induced chemical defence against herbivory (Thain *et al*., 2000; Kragler and Hülskamp, 2012). Plants use a range of complex strategies to maintain the balance between growth and defence when attacked by herbivores. The costs *vs* benefits of these allocations and the effects on plant fitness are important factors affecting the capacity of the plant to survive and reproduce in variable environments (Baldwin and Preston, 1999; Zavala and Botto, 2002; Tian *et al*., 2003; Zavala *et al*., 2004a; 2004b; Chen, 2008). As nutrients, particularly nitrogen, is often limiting, investment in defence metabolites that contain elemental nitrogen may come at the expense of other aspects of plant metabolism. The optimal defence theory is often considered to be an important predictor of resource allocation and encompasses considerations of (i) the magnitude of the attack risk; (ii) the value of the tissues to be defended; and (iii) the size of the budget for investing in defence (McKey, 1974; Coley *et al*., 1985; Ohmeiss and Baldwin, 2000). When limited resources are available, the optimal defence theory predicts that plants will preferentially direct their defences to tissues that both have a higher probability of being attacked and are ultimately more 'valuable' than others, either in terms of reproduction or capacity for carbon assimilation. Different tissues have different growth/defence budgets depending on ontogeny and in general, plants tend to allocate more resources to reproductive, younger and developing tissues that may be considered more significant in terms of reproductive success according to this optimal allocation model (Baldwin *et al*., 1990; Ohnmeiss and Baldwin, 2000). These tissues, which are generally more nutritious to herbivores, are also usually more responsive to stress and typically have faster and stronger responses to ensure they receive greater allocation of defensive metabolites. The value of specific tissues to the plant also changes with ontogeny, such that different developmental stages of plants respond differently to the same type and strength of stress (reviewed in Meldau *et al*., 2012). Studies by Diezel *et al*. (2011) involving *N. attenuata*, showed that the magnitude of the stress response is greater in the vegetative stages than after plants start flowering. In the experimental studies presented in this thesis, experiments were thus conducted on pre-flowering plants of both *N. attenuata* and *N.*

tabacum to maximise chances of plants responding strongly to stress. With recent observations of diurnal fluctuations in defence regimes (Kim *et al*., 2011; Goodspeed *et al*., 2013b), the paradigm surrounding optimised cost-benefit relationship for maximised plant fitness appears more complex than first thought, with the circadian clock emerging as an additional mechanism controlling the ability of plants to direct resources into areas of growth and defence to optimise fitness.

7.1. The role of hormones in regulating circadian output

The significance of tightly disciplined molecular networks in driving rhythms in hormone response systems has become increasingly clear in recent years (reviewed in Grundy *et al*., 2015). Hormones affect most of the known circadian-controlled processes in plants, but the synthesis and turnover of the hormones themselves are also under rhythmic control (Thain *et al*., 2004; Covington and Harmer, 2007; Legnaioli *et al*., 2009; Arana *et al*., 2011; Bhardwaj *et al*., 2011; Goodspeed *et al*., 2012; Seung *et al*., 2012). It is likely, at least in some cases that the clock operates through changes in hormone levels or hormone perception. The clock clearly targets hormone signalling systems, but to date there is only limited evidence for the role of hormones in delivering information from the clock to output stress signalling processes (Legnaioli *et al*., 2009; Bhardwaj *et al*., 2011; Goodspeed *et al*., 2012; Zhou *et al*., 2015).

As information regarding the influence of the clock on defence and immune responses to herbivores and pathogens begins to emerge, it has become clear that key repressor/regulator clock components, such as *TIMING OF CAB EXPRESSION 1* (*TOC1*) and *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), target an extensive network of genes, primarily through binding interactions in the promoter region (Harmer *et al.*, 2000; Covington *et al.*, 2008; Legnaioli *et al.*, 2009; Nagel and Kay, 2012). Most studies to date have been undertaken in *Arabidopsis*, investigating aspects such as the influence of *CCA1* on jasmonic acid (JA)-regulated and salicylic acid (SA)-regulated responses to herbivory and pathogenesis, respectively (Dodd *et al*., 2005; Gutiérrez *et al*., 2008; Bhardwaj *et al*., 2011; Wang *et al*., 2011; Goodspeed *et al*., 2012; 2013a). Zhou *et al*. (2015) demonstrated that morning (*CCA1*) and evening (*TOC1*) clock genes were sensitive to changes in levels of NON-EXPRESSOR OF PATHOGENESIS-RELATED GENE 1 (NPR1) and SA signals. Responsiveness of clock genes to NPR1 and SA was enhanced in the absence and presence of pathogens and subsequently tuned their resistance to pathogens towards the morning. The role of *TOC1* in JA- and SA-associated stress

processes has been largely overlooked, albeit its importance is beginning to emerge (Chapter 2; Chapter 3; Zhou *et al*., 2015). However, the ability of TOC1 to control signalling and drought responses during abiotic stress is well established (Legnaioli *et al*., 2009). TOC1 mechanistically operates by repressing transcription of the proposed ABA receptor gene through direct binding to its promoter (Legnaioli *et al*., 2009). This modulates both basal cycles of abscisic acid (ABA) as well as the sensitivity of the clock to resetting following drought conditions and changes in ABA cues (reviewed in Seung *et al*., 2012; Portolés and Zhang, 2014).

7.1.1. The role of *TOC1* in plant defence against herbivory

TOC1 regulates diurnal emission of ethylene (ET) from unstressed plants, by driving the expression of *1-AMINO-CYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE* (*ACS*) genes (Thain *et al*., 2004). However, the significance of rhythmic production of ET is still somewhat elusive since ET itself displays no feedback effects on rhythmicity, sensitivity or output processes of the clock. This is unlike almost all phytohormones (e.g. ABA, auxin, cytokinin, gibberellin, JA and SA) tested up till now (Covington and Harmer, 2007; Legnaioli *et al*., 2009; Arana *et al*., 2011; Bhardwaj *et al*., 2011; Goodspeed *et al*., 2012). The attenuating role of ET on the wound-associated stimulation of nicotine has been well demonstrated (Kahl *et al*., 2000; Shoji *et al*., 2000; von Dahl *et al*., 2007). However, it is not known whether *TOC1* influences ET emission in response to herbivory. As demonstrated in Chapter 2 of this thesis, the emission of ET was compromised following simulated herbivory in *TOC1* down-regulated plants (ir-*toc1*). It was also shown that herbivore-induced phytohormonal responses of plants, particularly JA, JA-Ile, SA and ABA, were all rapidly altered in ir-*toc1* plants compared to equivalent vector-only control plants. Elevated levels of nicotine in ir-*toc1* plants compared to vector controls, in response to simulated herbivory was a direct result of impaired ET signalling. This was confirmed by treatment with an inhibitor of ET perception, 1-methylcyclopropene (1-MCP), which negated the differences in nicotine between ir-*toc1* and vector control plants.

The plant hormone JA is generally considered the most important signal for defence against herbivores (Halitschke and Baldwin, 2003; Zavala and Baldwin, 2006; Browse and Howe, 2008; Schmelz *et al*., 2009). When plants, such as tobacco and *Arabidopsis*, are challenged by different herbivores, an increase in JA synthesis generally occurs through transcriptional induction of key biosynthetic genes, as well as downstream transcription factors (TFs), such as *MYC2* and *MYB8*. However, the mechanism(s) by

which herbivory triggers defence signals in local tissues and the regulatory mechanisms behind these inductions are not fully understood. Recently, the clock has been implicated in the regulatory responses to herbivory, particularly those associated with *CCA1* and JA-regulated defences (Goodspeed *et al.*, 2012; 2013a). As demonstrated in Chapter 3, activation of early stress signalling, JA-dependent transcriptional regulators controlling HGL-DTG (17–hydroxygeranyllinalool diterpene glycoside), phenylpropanoid and phenolamide defences are regulated by *TOC1*. In marked contrast to the action on nicotine biosynthesis, *TOC1* here acts as a positive regulator in shaping basal and herbivore-induced production of phenylpropanoid derivatives and phenolamides. Thus, the ability of plants to synthesise phenylpropanoids and phenolamides were substantially decreased in ir-*toc1* plants. It was also shown that their accumulation was independent of *TOC1* regulation of herbivory-induced ET and may be modulated specifically through *TOC1* regulation of JA-associated transcriptional mediators. Expression of key defence-related TFs, *MYC2* and *MYB8*, were found to be diminished in ir-*toc1* plants, particularly within 5 hours following herbivory. It was additionally demonstrated that *TOC1* adjusts transcript levels of key genes associated with the assimilation of nitrogen, through regulation of *GLUTAMINE SYNTHETASE* (*GS*) and thus affected the incorporation of ^{15}N into N-containing compounds including phenolamides. Members of the R2R3-MYB TF family have roles in controlling nitrogen assimilation (Imamura *et al*., 2009) and thus *TOC1*, *via* the action of MYB8, may regulate changes in growth-defence allocations by playing a role in assimilation and distribution of nitrogen in plants.

The relationship of *TOC1* and hormone signalling has been recognised, specifically its role in driving ET and ABA biosynthesis (Thain *et al*., 2004; Legnaioli *et al*., 2009) and in response to SA production (Zhou *et al*., 2015). However, the results detailed in chapters 2 and 3 are the first reports of the effect of *TOC1* on defence metabolism. Rhythmic synthesis of JA and SA, as well as herbivore- and pathogen-related resistance, respectively, is generally thought to be dependent on *CCA1* (Covington *et al*., 2008; Bhardwaj *et al*., 2011; Wang *et al*., 2011; Goodspeed *et al.*, 2012). The possibility that SA levels are influenced by *TOC1 in vivo* and having a direct bearing upon regulation of plant metabolism, is intriguing and warrants further attention. It would be particularly interesting to ascertain whether *TOC1* similarly influences plant transcriptomic and metabolic responses to pathogen infection. Such a suggestion is compatible with the results presented by Zhou *et al*. (2015), who reported that feedback

of NPR1 and SA accumulation regulates transcriptional responses of *CCA1* and *TOC1* in *Arabidopsis*.

In the present study, it is shown that *TOC1* influenced the transcription levels of *SALICYLATE-INDUCED PROTEIN KINASE* (*SIPK*), a wound- and herbivory-responsive member of the MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) family. This finding is interesting because it adds another dimension to the current model surrounding clock-mediated control of the plant metabolome, particularly in the area of defence metabolism. SIPK activity has previously been observed to increase in response to insect elicitors and induce both ET and JA defence signals, facilitating plant resistance to herbivores (Zhang and Klessig, 2001; Liu and Zhang, 2004; Kandoth *et al*., 2007; Wu *et al*., 2007; Wu and Baldwin, 2009). Together, these hormones act synergistically and antagonistically on different areas of defence metabolism. Specifically, ET negates the JA-induction of nicotine, but acts synergistically with JA to promote synthesis of phenylpropanoids and phenolamides (reviewed in von Dahl and Baldwin, 2007). This may explain, at least in part, how the clock coordinates pathway specific responses against herbivores. It may be advantageous for the clock in *N. attenuata*, and perhaps most plants, to act *via* components of the MAPK signalling network to coordinate the necessary transcriptomic and/or proteomic reconfigurations that occur in response to herbivory and possibly a multitude of other stresses.

The interactions or mechanisms governing *TOC1* regulation of the leaf transcriptome in response to herbivory still remain to be elucidated. It is unclear whether the observed transcriptional changes on hormone, alkaloid and phenylpropanoid metabolism, as well as related TFs, are associated with down-regulation of *TOC1* or may be a downstream effect of perturbed *SIPK* gene activity. It may thus be particularly insightful to ascertain the mechanistic basis accounting for these alterations in plants with reduced expression of *TOC1*. While *TOC1* has been shown to influence *ACS* expression (Thain *et al.*, 2004), it is also known that after phosphorylation by SIPK, *ACS* genes gain higher stability and enhance ET biosynthesis (Liu and Zhang, 2004; Wu *et al*., 2007). Since the targets of SIPK are widespread, it would make sense that *TOC1* directly controls SIPK activity to transduce reactions into downstream molecular and/or metabolic responses. Reduced perception of herbivore elicitation, seen by lower transcripts of *SIPK* in ir-*toc1* plants, perhaps results in a weakened ability to initiate clock-controlled herbivory-induced signal cascades and mount an efficient defence response. Data

presented in chapter 3 may indicate that TOC1 controls expression of clock-regulators, such as TIME FOR COFFEE (TIC), in order to regulate JA-associated TFs. TIC activity has previously been shown to interact with MYC2 protein and inhibit its production (Shin *et al.*, 2012). It is possible that as a result of reducing *TOC1*, a decrease in *TIC* expression resulted in down-regulation of *MYC2*. It is unclear whether regulation of JA-defences (Chapter 3) is attained only through control of MAPK signalling components or whether it achieves these reactions *via* interactions with other clock-regulators. It was hypothesised by Onkokesung *et al*. (2012) that herbivory-elicited ET could mediate stabilised and increased expression of *MYB8* in *N. attenuata*. While reductions in both ET (Chapter 2) and *MYB8* gene expression (Chapter 3) in *TOC1* silenced plants following simulated herbivory (wounding plus addition of *Manduca sexta* oral secretions) support this theory, *TOC1* regulation of phenolamide metabolism was found to be independent of the perception of herbivore-induced ET.

As demonstrated in Chapter 3, down-regulated *TOC1* plants display a reduced ability to mount a defence response. The accumulation of phenylpropanoid derivatives, phenolamides and 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) was significantly reduced in ir-*toc1* plants compared to vector control plants, particularly following simulated herbivory. This was also supported in systemic leaves of ir-*toc1* plants that had been previously fed on by two native herbivores. Interestingly, down-regulated *TOC1* plants showed elevated levels of amino acids when compared to similarly treated vector control plants. The mechanism underlying the effects observed on these pathways in ir-*toc1* plants is not well understood, however, the accumulation of amino acids is strongly regulated by *TOC1*. The biosynthesis of polyamines, phenylpropanoids and HGL-DTGs do not rely on a common product to be metabolised. However, their pathways appear to be conjointly regulated, perhaps through similar co-opted transcriptional regulatory systems. It is not known to what extent *TOC1* and/or the affected transcriptional 'outputs' caused the biochemical changes in related areas of primary and secondary metabolism. It is possible that as a result of reducing *TOC1* and perturbing hormone metabolism, an effective over-supply of substrate precursors for areas of defence metabolism resulted in remobilisation of excess amino acid substrates precursors into other areas of amino acid metabolism.

The current study also found that herbivores performed better on ir-*toc1* plants than plants with normal capacity to express *TOC1*. While data from the current study

suggests that the major effects on insect fitness of a specialist herbivore, *M. sexta*, and intermediate effects on a generalist, *Spodoptera littoralis* could be due to the compromised defence chemistry (lower HGL-DTG, phenylpropanoid and phenolamide defences), increased accumulation of amino acids cannot be discounted. It would be beneficial to establish the direct cause of increased performance. The question remains as to whether plants prioritise their resources and/or responses into production of chemical defences following attack. Also questions relating to the importance of the time of elicitation, the attacker(s), and/or engagement of specific clock-hormone signalling networks, still remain essentially unresolved. It would be of interest to explore whether plants are metabolically equipped to perceive and differentiate between different attackers during the day and night. This could be addressed by examining the diurnal metabolic response at specific times to both day active (*M. sexta*) and nocturnal (*S. littoralis*) herbivores to ascertain whether the clock controls nitrogen investment and activation of induced-defences in response to specific attackers at precise times of day. Data presented here suggests that responses of plants attacked by two different herbivores are quite similar, at least during daytime. However, this still does not answer the question about day-time versus night-time responses. Nor does it answer sensitivity to attack throughout the day or through the night, as elicitation experiments were all controlled for time of day.

It is intriguing that *TOC1* differentially altered related defence pathways in tobacco. While there is no evidence in the literature for the direct involvement of the clock in herbivore perception, *TOC1* is rapidly repressed following herbivory (Emmanuel Gaquerel, unpublished) and this may suggest that *TOC1* acts as a repressor of defence metabolism under basal conditions. Rising levels of *TOC1* throughout the day, peaking at dusk, may suggest an important role for *TOC1* in disabling a response to herbivory during the night. If this is the case, perhaps *TOC1* expression is particularly useful for increasing resistance to pathogens at night. Together, this suggests the existence of a molecular response network that may act in parallel with the current 'anticipation' model previously put forward for clock-hormone regulation of defence metabolism (Goodspeed *et al*., 2012). While the precursors of many of these defence pathways do oscillate in daily rhythms (reviewed in Grundy *et al*., 2015), possibly in an anticipation of potential stress events, it may simply be an effective method to reduce the lag-time in production of particular metabolites, such as induced chemical defences.

It has been previously suggested that ET emissions may be a method exploited by *M. sexta* to allow continuous feeding on their host plants (Voelckel *et al.*, 2001). However, data presented here suggests that ET may also enable plants to fine-tune resource allocation. Data from the current study may indicate that this could be mediated through the plants' endogenous clock as a compensatory mechanism to optimise and partition defence investments against specialist and generalist herbivores, as well as pathogens, which are known to differentially regulate plant defence signals (e.g. ET, JA and SA) upon attack. These findings may correlate with the necessity to reduce nicotine production and increase the production of other toxins such as phenolamides and HGL-DTG's. In this instance, larger investments of recently assimilated nitrogen into phenolamides, such as CP and DCS, may be the 'toxic' force against depredation from a day-active tobacco specialist that can rigorously damage *Nicotiana* plants (Baldwin, 2001; Kessler and Baldwin, 2004; Steppuhn and Baldwin, 2007). Larvae of *M. sexta* are able to tolerate high doses of nicotine by detoxification, excretion or co-opting it for cytochrome P450-mediated exhalation through their spiracles (Self *et al*., 1964; Kumar *et al*., 2014). On the other hand, *M. sexta* is negatively affected by phenolamides (Kaur *et al*., 2010). *TOC1* expression peaks at dusk, declining throughout the night when nocturnal herbivores like *S. littoralis* are known to feed. Alleviating the strong pressure towards phenylpropanoid metabolism at night when herbivores are perhaps more susceptible to an arrangement of toxins including nicotine, may be a mechanism to adjust and conserve resources for day-time pressures. Conversely, the clock gene components may provide a mechanism to change allocation into specific defence spectra, which may be the key to reduce the chances of one herbivore becoming resistant to a number of toxins produced by plants.

In summary, inducibility of toxic compounds allows *N. attenuata* plants to minimise metabolic constraint and fitness consequences associated with the production of costly defence metabolites. In order to conserve particularly 'expensive' chemical resources for when they are most effective, clock regulation of defence pathways may provide a mechanism to direct resources and execute highly specific stress responses. Data presented here may suggest that close coordination of *TOC1* in plants drives hormone-specific defence responses following herbivore-associated stress. The mechanistic relationship associated with silencing of individual clock genes and production of chemical defences has not been previously reported. While some of the

molecular systems that regulate plant metabolome dynamics have been uncovered, the mechanistic basis behind *TOC1* and JA-mediated defence accumulation still awaits further elucidation. This study extends the current knowledge surrounding clock dependent regulation of hormone signalling pathways to include a number of inter-connected and independent defence pathways, including nicotine, phenylpropanoid derivatives and conjugates and HGL-DTGs. Results obtained here highlight the complex interaction of the metabolomic rhythms of plants and herbivores. Together, the current research activity involving circadian rhythm components suggests it is advantageous for *N. attenuata*, and perhaps most plants, to synchronously coordinate resource allocation and metabolite accumulation to deter insect herbivory. The clock may provide a means for rechannelling metabolic fluxes towards the production of specific spectrums of defence metabolites. Coordinated regulation of plant responses in both antagonistic and synergistic manners may help plants adapt to fluctuating environments and unpredictable stresses. This work provides insights into how plants utilise the clock to integrate and coordinate various phytohormone signals to accomplish differential resistance responses, particularly following herbivory.

7.2. Regulation of putrescine metabolism and relationship with chemical defence in *Nicotiana*

Polyamines may be considered as a potential 'metabolic hub', which connects plant growth with various biotic and abiotic environmental factors (Kusano *et al*., 2007). In the current study, evidence was presented that suggest that multiple levels of regulation exist to maintain homeostasis in polyamine and pyridine alkaloid production, but also the levels of associated genes and metabolites in inter-related areas of metabolism are affected by altering the capacity for polyamine production. While the growth-related synthesis of polyamines have been well addressed (reviewed in Kusano and Suzuki, 2015), the current study suggests their biosynthesis is in part regulated by the clock, which has not previously been reported (Chapter 3). It was also shown that down-regulation of *ornithine decarboxylase* (*ODC*) in *N. tabacum*, causes alterations not only in polyamine metabolism and in the capacity to produce pyridine alkaloids (Chapter 4 and 5; DeBoer *et al*., 2011a; 2013), but also on the levels of amino acids, aryl-amides and phenolamides in leaves and roots of plants (Chapter 6).

7.2.1. Functions of *ODC* in polyamine and pyridine alkaloid biosynthesis and connections with amino acid and phenolamide pathways

Putrescine acts as an important intermediate precursor in the synthesis of higher amines, spermidine and spermine, that are essential in all living organisms (reviewed by Kusano *et al*., 2007; Kusano and Suzuki, 2015). In Solanaceous plants, polyamines exist predominantly as conjugates with hydroxycinnamic acids and in several genera, the diamine, putrescine, can also be utilised in the metabolism of a number of tropane and pyridine alkaloids (Hashimoto *et al*., 1990; Dräger, 2004).

In Chapter 4 (DeBoer *et al*., 2011a), it was shown that *ODC* regulates the normal and wound-induced alkaloid profile in *Nicotiana tabacum*. Down-regulation of *ODC* (*odc*-RNAi) produced a marked effect upon the alkaloid profile, specifically reducing nicotine levels and concomitantly increasing anatabine production. Further analysis also found that reduced expression of *ODC* led to significantly diminished levels of nornicotine in both shoots and roots of non-wounded and wounded plants, compared to vector-only controls (Chapter 6). We also examined the role of *ODC* in the synthesis of anabasine in transgenic *N. glauca* also expressing the *odc*-RNAi construct (Chapter 5; DeBoer *et al*., 2013). It was concluded that *ODC* additionally plays a direct role in enabling hairy root cultures and transgenic plants of *N. glauca* to elevate anabasine synthesis following wound-associated stress. As we noted in DeBoer *et al*. (2013), this may indicate that the *Nicotiana* ODC enzyme also has a capacity to decarboxylate lysine *in vivo*, as was observed in enzymic studies conducted *in vitro* wherein the pH of buffer was raised from 6.8 to 8.2 (Lee and Cho, 2001). However, in the light of detailed subsequent analysis as described in Chapter 6 and discussed in more detail below, it is possible that the down-regulation of *ODC* in *N. glauca* plants noted in Chapter 5 (DeBoer *et al*., 2013) also had reduced capacity to upregulate *QPT* and *A622* in response to wound-associated stress. However, expression of these genes was not assessed in the work reported in Chapter 5 (DeBoer *et al*., 2013). If indeed reduced capacity to upregulate *QPT* and *A622* is a feature of *odc*-RNAi transgenics in *N. glauca*, this could conceivably reduce their capacity to produce anabasine as both enzymes play a key role in the production of this alkaloid in *N. glauca* (Sinclair *et al*., 2004; DeBoer *et al*., 2009). This is an important question to be addressed in future studies.

Experiments conducted in Chapter 6 explored the wider manipulations of down-regulating the expression of *ODC* in *N. tabacum*, which not only affected the levels of pyridine alkaloids, but also the production of amines, associated pools of amino acids,

polyamine-derived phenolamides and particular components of the root transcriptome associated with putrescine and alkaloid metabolism. It was found that reduced expression of *ODC* compromised the ability of plants to synthesise putrescine, spermidine, spermine, tyramine, caffeoylputrescine and dicaffeoylspermidine. This was accompanied by enhanced production of the amino acids ornithine, arginine, aspartate, glutamate and glutamine in *ODC* down-regulated plants. This may be a consequence of perturbed putrescine metabolism, resulting in utilisation of surplus ornithine and arginine substrate into connected areas of amino acid metabolism, at the expense of polyamine and defence metabolism.

Down-regulation of *ODC* also revealed differential effects on genes in downstream pathways of *ODC*, particularly nicotine production and transport, spermidine biosynthesis and the pyridine nucleotide cycle. It was shown that expression of genes involved in putrescine biosynthesis (*ADC*) as well as nicotine (*PMT*) production and mobilisation (*MATE*) were elevated in *odc*-RNAi plants, particularly after wound-associated stress. In contrast, capacity for wound stimulation of genes involved in spermidine biosynthesis (*SPDS*), nicotinic acid production (*QPT*) and synthesis of the pyridine moiety of pyridine alkaloids (*A622*) was substantially reduced in *odc*-RNAi plants. Results obtained here suggest that the wound stimulation of genes involved in NAD(P)(H), spermidine and alkaloid biosynthesis pathways may operate under different mechanisms. While data presented here may suggest genes and/or metabolites essential for polyamine and alkaloid metabolism are influenced by *ODC* activity or putrescine demand, the presence of a feedback mechanism associated with reduced availability of putrescine cannot be discounted. Marked reduction of *QPT* and *A622*, observed in *odc*-RNAi *N. tabacum* lines, may also account for the observed reductions in anabasine in *ODC*-silenced plants of *N. glauca*, rather than a direct consequence of ODC suppression as first suggested (Chapter 5). However, if this was the reason for such result, it may be surmised that anatabine concentrations also be substantially reduced, which was not the case (Chapter 4 and 6). Further transcriptional analysis of the effects of down-regulating *ODC* in *N. glauca* upon components of the associated alkaloid root transcriptome is warranted.

This study raises additional questions regarding the details of the wound-induced mechanisms that regulate components of the associated polyamine, pyridine nucleotide cycle and the alkaloid root transcriptome in *Nicotiana*. Advances have been made in

recent years in understanding the importance of APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) and MYC2 type TFs, as well as miRNA, in regulating expression of key genes essential for alkaloid production in tobacco, particularly in relation to wound and jasmonate induction (De Sutter *et al*., 2005; Shoji *et al*., 2010; Todd *et al*., 2010; DeBoer *et al*., 2011b; Fernández-Calvo *et al*., 2011; Shoji and Hashimoto 2011; 2012; 2013; 2015; Zhang *et al*., 2011; 2012; Sears *et al*., 2014; Li *et al*., 2015). In addition to its role in providing nicotinic acid for pyridine alkaloid synthesis in *Nicotiana* and many other genera, QPT is regarded as a vital component of primary metabolism enabling NAD(P)(H) supply in all organisms (Mann and Byerrum, 1974a; 1974b; Wagner *et al*., 1986; Katoh *et al*., 2006; Noctor *et al*., 2006). Recently, involvement of endogenous target mimicry (eTM) and its target microRNA have been reported to regulate expression of *QPT2* after topping in *N. tabacum* (Li *et al*., 2015), adding yet another dimension to the regulatory framework controlling this area of metabolism.

The possibility that *ODC* or feedback of putrescine availability *in vivo* differentially influence transcription of key genes required for primary and alkaloid metabolism, such as *QPT, SPDS, PMT,* and *A622*, is intriguing and warrants further attention. It would be particularly interesting to ascertain whether down-regulation of *ODC* similarly influences expression of alkaloid associated TFs and miRNA, particularly those involved in specifically regulating expression of *QPT*. Data presented here suggests that *QPT* is carefully regulated *in vivo*, likely at the level of transcription, translation and post-translation. Such a suggestion may be compatible with the results presented by Li *et al*. (2015), who recently reported that the accumulation of a miRNA decoy positively regulates transcriptional expression of *QPT* under wounding conditions in *N. tabacum* by sequestering a miRNA that specifically represses *QPT*. The fact that levels of anatabine are elevated in *odc*-RNAi lines suggests supply of nicotinic acid may also be increased, even if transcript expression of *QPT* and *A622* are suppressed. It is important to note that in the current study, enzyme levels and pyridine nucleotide cycle intermediates were not measured. Therefore, activity of related enzymes in the pyridine cycle (e.g. QPT) and also downstream enzymes involved in alkaloid production (e.g. A622) may still operate at sufficiently high levels to ensure that nicotinic acid is being produced at levels more than adequate for primary metabolism. Additional nicotinic acid may then be channelled into anatabine synthesis in the absence of an adequate supply of putrescine-derived intermediate metabolites, which produce the pyrrolidine ring of nicotine. Further detailed

analysis of metabolism, at the transcriptional and post-transcriptional level, as well as detailed analysis of all relevant intermediate metabolites, including nicotinic acid, would be of interest here. Together, the current research involving putrescine metabolism highlights the complexity of mechanisms that exist to regulate the plant transcriptome and metabolome. Our results suggest additional mechanisms exist controlling the ability of plants to direct resources into connected areas of primary (e.g. polyamine, NAD cycle) and secondary (e.g. alkaloid and phenolamide) metabolism.

7.3. Conclusions and future prospects in research of plant defence

This thesis sheds new light on metabolic regulations in *Nicotiana* plants. It underscores the complexity of these metabolic networks, particularly the regulation of nitrogen metabolites with primary or specialised functions. The circadian system clearly plays an extremely important role in the life of these plants, with proper clock function not only enhancing plant fitness and survival (Green *et al*., 2002; Michael *et al*., 2003; Dodd *et al*., 2005) but also providing advantages leading to improved growth vigour (Ni *et al*., 2009) and biomass (Chapter 2), as well as enhanced plant resistance (Chapter 3). In this sense, the circadian clock has been found to interact with other pathways, including those of hormone signalling and stress response (Chapter 2; Covington *et al*., 2008). The knowledge gained from research of the 'biological clock' *via* the action of phytohormone signalling could be utilised for future crop improvement. Functions such as redirecting primary resources including nitrogen flow (Chapter 2, 3), drought resistance (reviewed in Seung *et al*., 2012; Portolés and Zhang, 2014), growth (Ni *et al*., 2009; Michael *et al*., 2003), flowering (Yon *et al*., 2015), plant defence against pathogens (Bhardwaj *et al*., 2011; Wang *et al*., 2011; Zhou *et al*., 2015) and insect herbivores (Chapter 3; Goodspeed *et al*., 2012; 2013a; 2013b) could be of agricultural interest. Targeting clock-hormone pathways, crops might profit from suppressing or promoting herbivory induced responses to help reduce predation from pests or prevent changes in the resource allocation and perhaps other yield defining processes. By increasing our understanding of the mechanisms of the clock in plant defence and resource allocation, we can be better prepared to produce improved crops. Potential targets may include MAPK signalling components such as SIPK or JA-regulated TFs, such as MYC2 and MYB8, which are associated with specific JA-defence functions including stimulation of nicotine and phenolamide biosynthesis, respectively.

In spite of the considerable advances in our understanding of how the oscillator and input pathways function, there is still much that is not understood about how the circadian system is able to accurately regulate so many output processes. Information regarding the interactions between the signalling and regulatory elements involved in clock-hormone processes is still elusive. The mechanistic understanding of clock-regulation of hormone signalling is constantly expanding as many new signalling components and target TFs are reported. Research needs to be targeted towards identifying the remaining co-regulators and target proteins of TOC1, as well as other clock genes that moderate pleiotropic downstream responses. Deciphering the mechanisms by which these output processes are controlled may allow us to modify specific pathways that are regulated by the circadian system. It will also give us a better understanding of this fundamental aspect of plant growth and development.

The polyamine pathway could also be of interest for researchers working on ecological interactions and biodiversity research, as well as for crop improvement. Abiotic stresses such as drought, salinity and nutritional limitations are common to plant species adapted to a wide range of habitats and may affect production of polyamines. Much remains to be understood, however, regarding their role in conferring tolerance to environmental stresses, and their relationship with other areas of metabolism, both primary and specialised. As shown in the current study, production and characterisation of transgenic plants with stably down-regulated enzymes of importance in polyamine metabolism, such as ODC, offer a valuable resource to facilitate further experiments in this area.

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