UNIVERSITÉ DU QUÉBEC

MÉMOIRE PRÉSENTÉ À
L'UNIVERSITÉ DU QUÉBEC À TROIS-RIVIÈRES

COMME EXIGENCE PARTIELLE
DE LA MAÎTRISE EN BIOLOGIE CELLULAIRE ET MOLÉCULAIRE

PAR
TARUN HOTCHANDANI

ÉTUDE DU TRANSCRIPTOME ET DU MÉTABOLOME CIBLÉE AUX ALCALOÏDES DE *NARCISSUS PAPYRACEUS* À DIVERS STADES DE DÉVELOPPEMENT

AOÛT 2018

Université du Québec à Trois-Rivières

Service de la bibliothèque

Avertissement

L'auteur de ce mémoire ou de cette thèse a autorisé l'Université du Québec à Trois-Rivières à diffuser, à des fins non lucratives, une copie de son mémoire ou de sa thèse.

Cette diffusion n'entraîne pas une renonciation de la part de l'auteur à ses droits de propriété intellectuelle, incluant le droit d'auteur, sur ce mémoire ou cette thèse. Notamment, la reproduction ou la publication de la totalité ou d'une partie importante de ce mémoire ou de cette thèse requiert son autorisation.

REMERCIEMENTS

Je tiens à exprimer ma sincère gratitude à ma directrice de recherche, Prof. Isabel Desgagné-Penix, pour m'avoir accueilli au sein de son groupe de recherche et de m'avoir guidé tout au long de ma maîtrise. Je la remercie aussi pour sa patience et sa gentillesse.

Je tiens aussi à remercier chaleureusement Prof. Hugo Germain pour sa générosité et son aide au cours de ma recherche.

Un grand merci à Justine de Villers qui a travaillé sérieusement sur mon projet de recherche durant son stage.

Je remercie également tous les membres de notre laboratoire et de ceux de Prof. Germain qui m'ont aidé à poursuivre ma recherche.

Merci aussi à Prof. Céline Van Themsche pour m'avoir donné l'accès à ses instruments de laboratoire.

Merci également à Mme Catarina Leote Franco Pio pour son aide dans la mise en forme de ce mémoire.

RÉSUMÉ

Les plantes de la famille des Amaryllidacées produisent des alcaloïdes ayant de puissants effets biologiques (neurologiques, cytotoxiques, cytostatiques, etc.). Ces effets rendent les alcaloïdes d'Amaryllidacée (AA) appropriés pour l'utilisation potentielle comme médicaments. Malheureusement, étant donné qu'ils sont synthétisés en quantités infimes dans les plantes, l'utilisation des AA est limitée. L'ingénierie métabolique aiderait grandement à atténuer ce problème. Pour cela, cependant, une connaissance complète de la voie de biosynthèse des AA est nécessaire. Afin d'obtenir plus d'informations sur les gènes impliqués dans la biosynthèse des AA, des études transcriptomiques (profilage de l'expression des gènes) et métabolomiques (profilage des alcaloïdes) ont été réalisées. L'espèce d'Amaryllidacée *Narcissus papyraceus* a été choisie à cause de sa facilité à croître à l'intérieur, comparé à d'autres espèces d'Amaryllidacées, permettant de faire pousser les plantes dans des conditions contrôlées. Jusqu'à cinq parties à six stades de développement de la plante ont été analysées. Le séquençage de l'ARN ainsi que l'assemblage *de novo* et l'annotation du transcriptome ont permis d'identifier tous les gènes biosynthétiques proposés des AA dans le transcriptome de *N. papyraceus*. La chromatographie en phase liquide à haute performance a été utilisée pour séparer et identifier les AA extraits des différents échantillons de plantes. Les résultats suggèrent que la lycorine était présente dans tous les échantillons de plantes, tandis que la galanthamine et la narciclasine n'étaient pas détectées. De plus, il a été remarqué que les plantes ont toujours tendance à accumuler préférentiellement les alcaloïdes dans les parties aériennes telles que les feuilles et les fleurs. En revanche, il n'y avait pas de tendance particulière concernant l'expression des gènes. Autrement dit, l'expression des gènes variait considérablement parmi les différentes parties de la plante au courant de sa croissance et développement. L'hypothèse du projet de recherche était qu'il y aurait une corrélation positive entre les niveaux d'expression génique et la quantité d'AA accumulés dans la plante. Cependant, en raison d'un transport probable d'enzymes et d'alcaloïdes dans la plante, une corrélation négative a été observée dans certains cas.

Mots-clés : alcaloïde d'Amaryllidacée, pharmacologique, gène biosynthétique, stade de développement, *N. papyraceus*, transcriptome, expression.

TABLE DES MATIÈRES

REMERCIEMENTS ii

RÉSUMÉ iii

LISTE DES FIGURES ET TABLEAU vii

LISTE DES SIGLES ET ACRONYMES viii

CHAPITRE I
INTRODUCTION **1**

1.1 Les métabolites 1

1.2 Les alcaloïdes 2

1.3 La famille des Amaryllidacées 3

1.4 Les alcaloïdes d'Amaryllidacée 4

1.5 Les propriétés pharmacologiques des alcaloïdes d'Amaryllidacée 5

1.5.1 Effets neurologiques 5

1.5.2 Propriétés anticancéreuses 6

1.5.3 Propriétés antimicrobiennes 7

1.6 Problématique 7

1.7 Voie biosynthétique proposée des AA 8

1.8 Objectif 11

1.9 Hypothèse 12

1.10 Méthodologie 12

1.10.1 Échantillons de plante 12

1.10.2 Étude transcriptomique 14

1.10.3 Étude métabolomique 16

CHAPITRE II
DEVELOPMENTAL REGULATION OF THE EXPRESSION OF AMARYLLIDACEAE ALKALOID BIOSYNTHETIC GENES IN *NARCISSUS PAPYRACEUS* **18**

Résumé de l'article 18

Abstract 19

Introduction 20

Materials and methods 25

RNA extraction, next-generation Illumina sequencing and *de novo* transcriptome assembly 25

Chemicals 26

Plant material 27

cDNA synthesis 27

Primer design 28

Reverse transcription PCR for primer testing 28

Gel electrophoresis 29

Sequencing of amplicons 29

Reverse transcription quantitative real-time PCR 29

Alkaloid extraction 30

HPLC 30

Accession numbers 31

Results and discussion 32

RNA sequencing of *Narcissus papyraceus* bulbs 32

Confirmation of candidate genes 34

Plant growth 35

Gene expression analysis with RT-qPCR 35

Alkaloid profiling 41

Comparison of gene expression and compound content profiles 43

Acknowledgements 44

References 45

Tables 52

Figure legends 55

Figures 57

Supplementary data 63

CHAPITRE III
CONCLUSION 69

3.1 Étude du transcriptome 70

3.2 AA détectés 71

3.3 Expression des gènes et comparaison avec les profils alcaloïdiques 71

3.4 Sommaire des applications pharmacologiques des AA 73

3.5 Génie génétique et ingénierie métabolique 77

RÉFÉRENCES BIBLIOGRAPHIQUES 80

ANNEXE A
ARTICLE DE REVUE 87

LISTE DES FIGURES ET TABLEAU

Figure **Page**

1.1 Espèces ornementales d'Amaryllidacées 3

1.2 Structures chimiques de certains AA représentatifs 4

1.3 Voie de biosynthèse proposée des AA 9

1.4 Échantillons de plante de *N. papyraceus* 14

1.5 Schématisation des méthodes utilisées 15

3.1 Structures chimiques des AA décrits au tableau 3.1 76

3.2 Étapes nécessaires pour l'ingénierie métabolique de microorganismes 79

Tableau

3.1 Activités pharmacologiques de plusieurs AA 74

LISTE DES SIGLES ET ACRONYMES

AA	Alcaloïde(s) d'Amaryllidacée
ADN	Acide désoxyribonucléique
ARN	Acide ribonucléique
3,4-DHBA	3,4-dihydroxybenzaldéhyde
PAL	*Phenylalanine ammonia lyase*
C4H	*Cinnamate 4-hydroxylase*
C3H	*Coumarate 3-hydroxylase*
4CL	*4-Hydroxycinnamoyl CoA ligase*
HCT	*Hydroxycinnamoyl transferase*
TYDC	L-tyrosine/L-dopa décarboxylase
N4OMT	Norbelladine 4'-*O*-méthyltransférase
CYP96T1	Cytochrome P450 monooxygénase 96T1
NorRed	Noroxomaritidine réductase
PCR	Réaction en chaîne par polymérase
RT-PCR	PCR à transcription inverse
RT-qPCR	RT-PCR en temps réel de façon quantitative
HPLC	Chromatographie en phase liquide à haute performance
AChE	Acétylcholinestérase
nAChR	Récepteurs neuronaux nicotiniques de l'acétylcholine
SAM	*S*-adénosyl méthionine

CHAPITRE I

INTRODUCTION

1.1 Les métabolites

Les métabolites sont de petits composés organiques ayant une masse de moins de 1,5 kDa produits naturellement par les réactions biochimiques dans une cellule [1, 2]. Les métabolites ont diverses fonctions : agissent comme source d'énergie, participent dans la signalisation, ont des effets stimulants et inhibiteurs sur les enzymes, et jouent des rôles dans la défense et les interactions avec d'autres organismes. Chez les plantes, par exemple, les métabolites peuvent être divisés en deux catégories : primaires et spécialisés. Un métabolite primaire est directement impliqué dans la croissance, le développement et la reproduction de l'organisme. Certains acides aminés, l'acide lactique et l'éthanol sont des exemples de métabolites primaires. Un métabolite spécialisé (aussi appelé secondaire) n'est pas directement impliqué dans ces processus, mais joue un rôle important dans les interactions avec d'autres organismes (par exemple en lien avec la défense et la compétition). Les phénols, les terpènes et les alcaloïdes sont des classes de métabolites spécialisés.

Les phénols sont des hydrocarbures aromatiques qui possèdent un groupement hydroxyle [3]. Plus de 8000 phénols ont été identifiés dans les plantes. Certaines classes de phénols sont les coumarines, les flavonoïdes/anthocyanes, les stilbènes, les hydroxycinnamates, les lignanes et les lignines [4, 5]. Les phénols exercent de nombreuses fonctions physiologiques. Par exemple, la lignine fournit un support mécanique et un environnement hydrophobe permettant le transport d'eau et de nutriments [6, 7]. En outre, de nombreux phénols solubles protègent les plantes contre les stress biotiques et abiotiques, agissent comme des pigments attirant les pollinisateurs, et servent comme molécules de signalisation responsables pour les interactions entre les plantes et microbes [8].

Les terpènes sont des hydrocarbures produits par diverses plantes, en particulier les conifères, et par certains insectes [9, 10]. De nombreuses plantes produisent des terpènes volatils afin d'attirer des insectes spécifiques pour la pollinisation ou pour dissuader les herbivores. Des terpènes moins volatils qui sont fortement amers ou toxiques agissent comme biopesticide. De plus, les terpènes jouent un rôle important en tant que molécules de signalisation et phytohormones [10].

1.2 Les alcaloïdes

Les alcaloïdes sont des molécules organiques hétérocycliques azotées [11]. La position de l'atome d'azote dans le cycle carboné varie avec différents alcaloïdes. La plupart des alcaloïdes sont des solides cristallins incolores au goût amer. Ils possèdent de faibles poids moléculaires, ont un point de fusion défini et sont optiquement actifs. Les alcaloïdes sont en majorité dérivés de précurseurs d'acides aminés simples comme la tyrosine, la phénylalanine, le tryptophane, l'ornithine, l'arginine ou la lysine. Le terme *alcaloïde* a été introduit par le chimiste allemand W. Meissner en 1819 et s'appliquait à tous les composés organiques basiques obtenus à partir de matériaux animaux ou végétaux [12]. Environ 12 000 alcaloïdes différents ont été identifiés dans les plantes et sont répartis dans plusieurs classes distinctes basées sur leurs structures chimiques, leurs origines biosynthétiques, et leurs activités biologiques et écologiques [13]. Les alcaloïdes possèdent des propriétés toxiques, notamment des propriétés cytotoxiques, cytostatiques et antimicrobiennes [14-16]. Il est donc supposé qu'ils jouent un rôle important pour les plantes dans la défense contre les herbivores et les pathogènes [11]. Par exemple, l'alcaloïde galanthamine se lie à l'enzyme acétylcholinestérase qui est responsable de la dégradation du neurotransmetteur acétylcholine, entraînant ainsi une augmentation de l'acétylcholine [17]. L'accumulation d'acétylcholine résulte en une augmentation de la neurotransmission ce qui cause une stimulation continue des muscles, des glandes et du système nerveux central pouvant conduire à la paralysie. À de fortes doses, il peut produire des convulsions fatales. En conséquence, il est proposé que la galanthamine sert d'insecticide à la plante en provoquant une stimulation cholinergique excessive dans un insecte nuisible [18]. Les propriétés toxiques des alcaloïdes les rendent utilisables,

à faibles doses, comme médicaments. Par exemple, la galanthamine est utilisée dans le traitement des symptômes de la maladie d'Alzheimer (voir sous-section 1.5.1 Effets neurologiques) [19, 20]. Un autre alcaloïde, la vinblastine, est utilisé comme agent anticancéreux [21], tandis que la morphine et la codéine sont deux alcaloïdes analgésiques bien connus [22].

1.3 La famille des Amaryllidacées

Une famille de plantes connue pour sa production d'une grande variété d'alcaloïdes avec des potentiels médicinaux prometteurs est celle des Amaryllidacées [23]. Les plantes appartenant à cette famille sont des plantes vivaces herbacées qui poussent à partir de bulbes. Cette famille comprend une soixantaine de genres dont les 1100 espèces sont largement réparties dans plusieurs pays du monde. Ils sont également cultivés comme plantes ornementales pour leurs belles fleurs et pour la production d'huile volatile. Certains genres et espèces ornementaux communs des Amaryllidacées sont l'*Amaryllis*, les narcisses (*Narcissus* spp.) et les perce-neiges (*Galanthus* spp.) (Figure 1.1).

Figure 1.1 Espèces ornementales d'Amaryllidacées.
Affichées de gauche à droite, elles sont l'*Amaryllis*, les narcisses (*Narcissus* spp.) et les perce-neiges (*Galanthus* spp.) [24].

Les Amaryllidacées sont utilisées depuis plusieurs siècles déjà pour leurs propriétés médicinales [25]. Des extraits d'Amaryllidacées étaient utilisés à l'époque de la Grèce ancienne dans le traitement du cancer. Dans d'autres régions, comme l'Afrique, l'Asie et la Polynésie, ces plantes étaient plutôt utilisées pour les soins de santé primaires.

Les Amaryllidacées font d'ailleurs partie des 20 plantes les plus utilisées, que ce soit pour leur propriétés ornementales ou pharmacologiques [26]. Les effets pharmacologiques des Amaryllidacées sont fréquemment associés à plusieurs alcaloïdes typiques qu'elles synthétisent.

1.4 Les alcaloïdes d'Amaryllidacée

L'étude des alcaloïdes d'Amaryllidacée (AA) a commencé en 1877 avec l'extraction de l'AA lycorine (Figure 1.2) de *Narcissus pseudonarcissus* [25]. De nos jours, plus de 500 alcaloïdes d'Amaryllidacée ont été identifiés [27]. Les AA sont classés selon leur structure squelettique du carbone et de leur origine biogénique. Ainsi, il existe 8 types structurels chimiques distincts d'AA dans la famille des Amaryllidacées : les AA de type lycorine, homolycorine, galanthamine, hémanthamine, montanine, narciclasine, tazettine et crinine. L'AA représentatif de chaque type structurel chimique est présenté à la figure 1.2. Plusieurs de ces alcaloïdes possèdent des propriétés biologiques importantes.

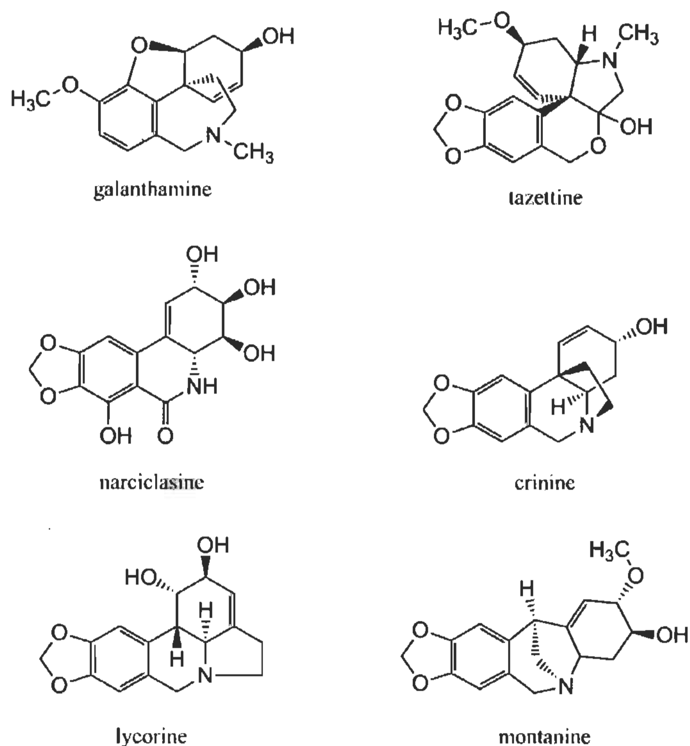


Figure 1.2 Structures chimiques de certains AA représentatifs [23].

1.5 Les propriétés pharmacologiques des alcaloïdes d'Amaryllidacée

Les AA ont des effets neurologiques, cytotoxiques, cytostatiques, etc. Ils perturbent l'intégrité de la biomembrane, inhibent efficacement les canaux ioniques et altèrent la fonction des microtubules ou des microfilaments. De plus, ils peuvent se lier à l'ADN et peuvent aussi agir sur la polymérase de l'ADN et de l'ARN [11].

Compte tenu de ces propriétés biologiques intéressantes, les AA sont des candidats appropriés pour traiter les troubles neurologiques, inhiber la croissance des tumeurs ou tuer les micro-organismes. C'est pourquoi de nombreuses recherches sont en cours sur les applications médicales potentielles des AA [19, 25]. Une brève description est fournie ci-après.

1.5.1 Effets neurologiques

Le seul AA actuellement utilisé en médecine est la galanthamine. Elle peut traverser la barrière hémato-encéphalique qui est une caractéristique essentielle pour traiter les problèmes neurologiques. Cette caractéristique est souvent manquante dans de nombreux autres médicaments [23]. Commercialisé sous les noms de Reminyl® et Razadyne® par Janssen Inc., une filiale de Johnson & Johnson Inc., elle est utilisée pour traiter les symptômes de la maladie d'Alzheimer. La galanthamine est un inhibiteur sélectif, réversible, compétitif et à action prolongée de l'enzyme acétylcholinestérase [19, 28]. Avec l'administration de la galanthamine, le niveau du neurotransmetteur acétylcholine est augmenté, aidant ainsi à contrer la sensibilité réduite à l'acétylcholine présente chez les patients souffrant de la maladie d'Alzheimer. Reminyl® a été approuvé par la Food and Drug Administration des États-Unis en 2001 et plusieurs versions génériques du médicament ont maintenant été approuvées [18, 23].

En plus d'être un inhibiteur de l'acétylcholinestérase, la galanthamine est un modulateur allostérique des récepteurs neuronaux nicotiniques de l'acétylcholine auxquels l'acétylcholine se lie [18, 23]. En conséquence, la galanthamine est étudiée pour des applications visant à traiter d'autres affections neurologiques. Un autre AA ayant des effets

bénéfiques sur le système nerveux est la montanine. Selon da Silva *et al.*, elle possède des activités psycho-pharmacologiques incluant des effets anxiolytiques, anti-dépressifs et anticonvulsivants [29].

1.5.2 Propriétés anticancéreuses

Plusieurs AA sont actuellement étudiés pour servir comme agents chimio-thérapeutiques potentiels. La lycorine, l'un des alcaloïdes les plus fréquents chez les Amaryllidacées, est un inhibiteur puissant du cycle cellulaire à l'étape de l'interphase, comme en témoignent ses effets cytostatiques *in vitro* sur plusieurs lignées de cellules cancéreuses humaines [30]. La pseudolycorine, un dérivé de la lycorine, possède des propriétés cytotoxiques et fait l'objet d'études pour le traitement de cellules cancéreuses métastatiques résistantes à de nombreux médicaments anticancéreux actuellement disponibles. D'autres alcaloïdes de type lycorine, tels que la caranine, la galanthine et la 2-*O*-acétylpseudolycorine, sont également actifs contre diverses cellules tumorales.

Les alcaloïdes de type hémanthamine, tels que l'hémanthamine, l'hémanthidine et la vittatine, ont également des propriétés anticancéreuses prometteuses. Par exemple, l'hémanthamine s'est révélée être un inhibiteur puissant de la croissance cellulaire et un inducteur de l'apoptose dans les cellules tumorales à des concentrations micromolaires [17, 31].

Un autre agent anticancéreux efficace est la narciclasine. Elle a été trouvée à avoir une activité puissante contre les tumeurs humaines de glioblastome multiforme [32]. Ce type de tumeur cérébrale primaire la plus maligne se propage de façon agressive dans les tissus cérébraux normaux et est résistante aux traitements conventionnels qui déclenchent l'apoptose.

Une activité cytotoxique modérée a été rapportée pour la tazettine et la prétazettine. Ces composés ont montré une cytotoxicité remarquable contre un certain nombre de

lignées cellulaires tumorales, étant efficaces de façon thérapeutique contre plusieurs types de leucémies et de carcinomes [30].

1.5.3 Propriétés antimicrobiennes

Les AA isolées d'*Amaryllis belladonna* L. (également appelée *Hippeastrum equestre*), incluant la pancracine, la vittatine, la 11-hydroxyvittatine et l'amarbellisine, ont toutes une activité antibactérienne contre l'*Escherichia coli*. De plus, six AA isolés d'*A. belladonna* L., à savoir lycorine, pancracine, vittatine, 11-hydroxyvittatine, amarbellisine et hippeastrine, ont tous démontré une activité cytotoxique contre *Candida albicans* [33].

L'activité antivirale est encore une autre propriété thérapeutique présentée par certains alcaloïdes de type lycorine. La lycorine et ses dérivés, tel que la pseudolycorine, bloquent la réplication de l'ARN viral et suppriment la synthèse des protéines virales, inhibant un certain nombre de virus à ARN et à ADN [34].

1.6 Problématique

Compte tenu de l'utilisation médicale prometteuse des alcaloïdes d'Amaryllidacée, beaucoup de recherche est concentré à établir ou à améliorer leur production pour que celle-ci soit commercialement viable. Actuellement, la production commerciale de galanthamine consiste à l'extraire à partir de plantes *Narcissus pseudonarcissus* cultivar Carlton en raison de sa grande disponibilité et sa grande résistance aux stresses environnementaux [35]. Dans certains pays, *Leucojum aestivum* et *Lycoris radiata* sont également cultivés et utilisés pour la production de la galanthamine. Pour ce qui est des AA lycorine et narciclasine, de petites quantités sont généralement extraites de diverses espèces de *Narcissus* et de *Lycoris*. Cependant, la production et le dépistage des AA dans les plantes font face à plusieurs défis. Par exemple, beaucoup d'AA n'existent qu'en quantités infimes dans les plantes, et les méthodes pour les isoler et les purifier des plantes sont généralement inefficaces et non viables sur le plan environnemental. La synthèse

chimique totale peut aider à alléger les demandes des AA, mais la synthèse de ces composés, si elle a été élucidée, est souvent ardue et coûteuse en raison de leur stéréochimie complexe. En revanche, les cultures de cellules ou de tissus peuvent être un système de production durable mais les coûts associés à ces méthodes sont élevés pour un rendement assez faible. Le meilleur exemple de ceci est probablement la production commerciale à base de cellules végétales de paclitaxel, mieux connue sous le nom de taxol [36]. Des cultures ont également été développées pour la production des AA galanthamine et lycorine en utilisant les espèces d'Amaryllidacées *Leucojum aestivum* et *Pancratium maritimum* [37]. Par exemple, Pavlov *et al.* ont créé une lignée de culture de pousses de *L. aestivum* qui produisaient des quantités stables de galanthamine [38]. Plus tard, Georgiev *et al.* ont pu augmenter la production de galanthamine en modifiant la concentration de divers nutriments (nitrate, ammonium, ions phosphates et saccharose) dans des cultures de pousses de *L. aestivum* [39].

Alors, deux méthodes plus économiques et écologiques sont : (1) le développement de nouveaux cultivars capables de produire de plus grandes quantités d'alcaloïdes, ou (2) l'ingénierie métabolique permettant la production des AA dans des systèmes hétérologues tels que les microbes ou d'autres espèces végétales. Cela, par contre, nécessite une compréhension complète des réactions biochimiques et de la génétique moléculaire de la biosynthèse des AA qui est présentement manquante. Bref, pour produire plus d'AA d'intérêt médicinal, une meilleure compréhension de la biosynthèse des AA est requise.

1.7 Voie biosynthétique proposée des AA

Il est connu que des structures similaires sont fort probablement synthétisées par des voies métaboliques impliquant des gènes biosynthétiques similaires [40]. Puisque des composés similaires aux composés précurseurs et intermédiaires des AA sont présents dans des plantes appartenant à d'autres familles de plantes produisant des alcaloïdes, comme les familles des Papaveracées, Brassicacées, Orchidacées et Solanacées, où les gènes biosynthétiques sont connus, il a été proposé que des gènes homologues aux gènes

biosynthétiques connus puissent être impliqués dans la voie de biosynthèse des AA (Figure 1.3).

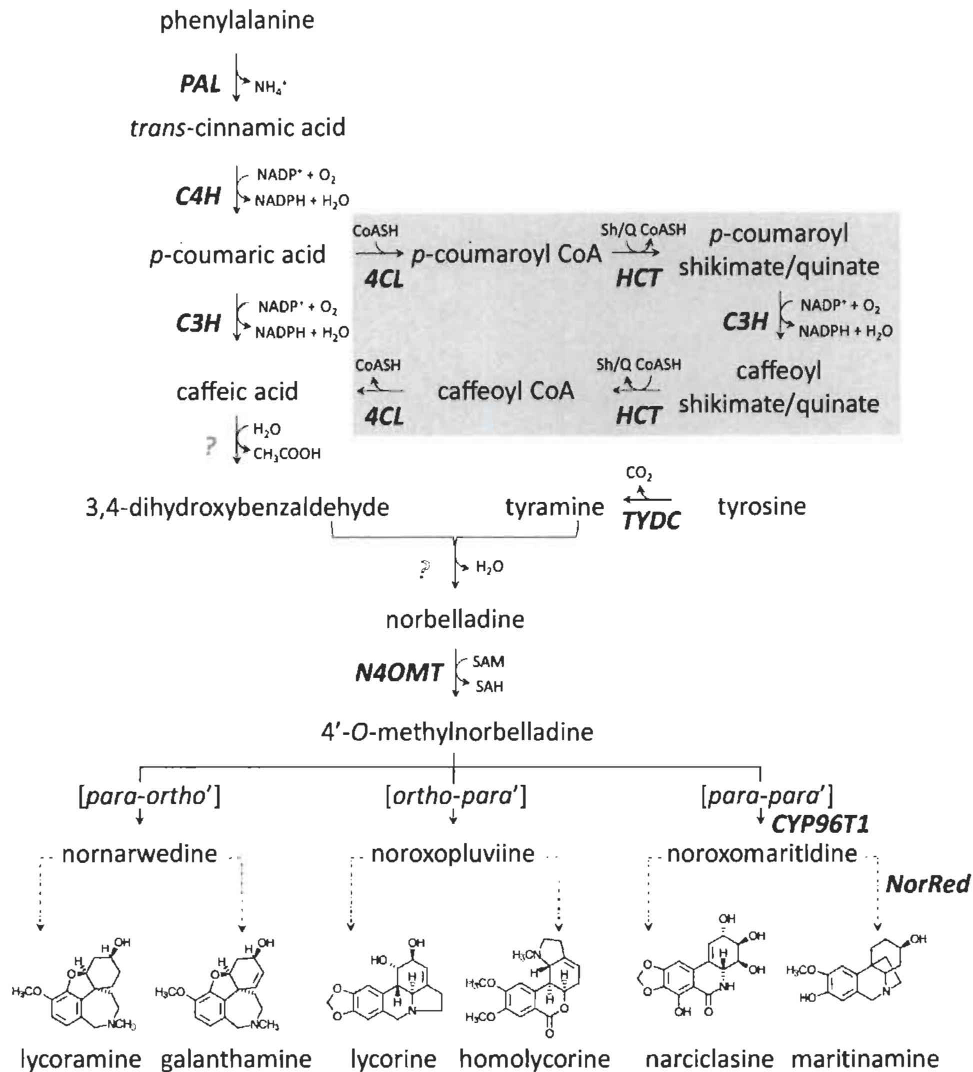


Figure 1.3 **Voie de biosynthèse proposée des AA.** Les gènes qui ont été isolés à partir de plantes d'Amaryllidacée sont indiqués à côté des flèches en caractères gras-italiques. Les réactions dans la zone grisée représentent une voie alternative à la synthèse des précurseurs. Les flèches pointillées représentent plus d'une réaction biochimique. Les noms complets des gènes sont écrits à la page viii.

Des expériences consistant à introduire des composés précurseurs radiomarqués dans des plantes d'Amaryllidacée, suggèrent que, malgré leur grande diversité structurale, tous les AA sont dérivés du composé central norbelladine et des analogies avec des voies

biosynthétiques d'autres alcaloïdes végétaux suggèrent que la norbelladine est formée à partir de la condensation des composés précurseurs 3,4-dihydroxybenzaldéhyde (3,4-DHBA) et tyramine [12, 15]. De telles réactions de condensation ont déjà été identifiées dans la biosynthèse des alcaloïdes benzylisoquinoline, par exemple, où l'intermédiaire central norcoclaurine est issu de la condensation de la 4-hydroxyphénylacétaldéhyde et la dopamine [41].

La synthèse du précurseur 3,4-DHBA implique une série de réactions similaires à la voie de biosynthèse des phénylpropanoïdes. En comparaison avec cette voie, il est considéré que la synthèse de 3,4-DHBA commence par la désamination de l'acide aminé phénylalanine par l'enzyme *phenylalanine ammonia lyase* (PAL). Les réactions d'hydroxylations ultérieures par les enzymes *cinnamate 4-hydroxylase* (C4H) et *coumarate 3-hydroxylase* (C3H), et un clivage par une enzyme actuellement inconnue aboutissent à la formation de 3,4-DHBA. Les enzymes C4H et C3H appartiennent à la superfamille des cytochromes P450. Les cytochromes P450 végétaux constituent l'une des plus grandes superfamilles génétiques des génomes des végétaux [42]. Elles sont impliquées dans diverses voies biochimiques pour produire des métabolites primaires et spécialisés comprenant les phénylpropanoïdes, les alcaloïdes, les terpénoïdes, les lipides, et les hormones végétales [43, 44]. Dans le métabolisme spécialisé, les cytochromes P450 catalysent une grande variété de réactions de monooxygénation et d'hydroxylation, ainsi que la formation de ponts méthylènedioxy, les réactions de couplage phénoliques, le réarrangement oxydatif des squelettes carbonés et le clivage oxydatif des liaisons C-C [42].

Concernant la transformation de l'acide *p*-coumarique en acide caféique par la C3H, une voie alternative, affichée dans la zone grisée de la figure 1.3, impliquant les enzymes additionnels *4-hydroxycinnamoyl CoA ligase* (4CL) et *hydroxycinnamoyl transferase* (HCT), a aussi été proposée. Le précurseur tyramine est, de son côté, obtenu à partir de la décarboxylation de l'acide aminé tyrosine supposément par l'enzyme L-tyrosine/L-dopa décarboxylase (TYDC) qui est une enzyme de régulation clé dans la formation des alcaloïdes benzylisoquinoline tels que la morphine et la codéine dans le pavot somnifère [41].

Le premier composé intermédiaire dans la voie de biosynthèse des AA, la norbelladine, est converti en 4'-*O*-méthylnorbelladine par la norbelladine 4'-*O*-méthyltransférase (N4OMT) [45]. Ensuite, une étape cruciale dans la biosynthèse des AA est la cyclisation de la 4'-*O*-méthylnorbelladine par trois voies différentes de couplage phénolique oxydatif intramoléculaire nommé *ortho-para'*, *para-ortho'* et *para-para'*, générant diverses structures de squelette. Ces squelettes sont à la source de la diversité des AA [30]. De là, un réseau complexe de réactions enzymatiques existe pour produire un spectre de composés qui diffère entre les espèces, les cultivars et les variétés d'Amaryllidacée. Ces modifications biochimiques sont réalisées par une multitude d'enzymes catalysant divers types de réactions, telles que les oxydoréductions, les hydroxylations, les formations de liaisons C-C et C-O, les acétylations, les déméthylations et les *O*- et *N*-méthylations. Ces réactions conduisent à la formation des plusieurs centaines d'AA tous structurellement apparentés [26]. Par exemple, l'AA anti-tumorale lycorine est dérivé de la réaction de couplage phénolique *ortho-para'*, alors que la galanthamine, utilisée pour le traitement des symptômes de la maladie d'Alzheimer, provient du couplage *para-ortho'* (Figure 1.3) [23, 30].

1.8 Objectif

La plupart des gènes biosynthétiques codant pour les enzymes responsables de la production des différents AA ne sont pas connus avec certitude. L'analyse transcriptomique, qui permet l'étude de l'expression des gènes, couplée à une analyse métabolomique, axée sur la quantité d'alcaloïdes présents, est devenue un outil populaire pour la découverte de nouveaux gènes codant pour les enzymes impliqués dans la biosynthèse des alcaloïdes [46]. Une telle approche a grandement contribué à l'élucidation de plusieurs voies métaboliques spécialisées de diverses plantes médicinales et nutritionnelles [47-50]. Un exemple est celui de *Cathanranthus roseus* dans lequel des transcrits de gènes et des métabolites associés aux alcaloïdes de type terpénoïdes-indoles ont été identifiés [51]. De la même manière, une meilleure compréhension a été obtenue de la voie de biosynthèse conduisant à la production des alcaloïdes anticancéreux camptothécine et anthraquinones [52].

L'objectif de mon projet de recherche était d'augmenter les connaissances sur l'implication des gènes biosynthétiques proposés dans la production des AA en comparant les niveaux d'expression de ces gènes avec la quantité d'AA présents dans des plantes d'une espèce d'Amaryllidacée. De plus, cette étude a été faite pour différentes parties à divers stades de développement de la plante permettant d'observer les différences dans l'expression des gènes et l'accumulation des AA parmi les différentes parties aux divers stades. Cela a aussi permis d'étudier les effets de la croissance et du développement de la plante sur la production des AA dans les différentes parties.

1.9 Hypothèse

En regardant la voie de biosynthèse des AA (Figure 1.3), il semble que toute augmentation de l'expression des gènes biosynthétiques indiqués conduira, dans la plupart des cas, à une plus grande production des enzymes correspondantes. Cela, en revanche, conduira très probablement à des quantités élevées des composés précurseurs et intermédiaires qui devraient produire une plus grande quantité d'AA. Donc, l'hypothèse était qu'il y aura des corrélations positives entre l'expression des gènes et la quantité d'alcaloïdes présente. Autrement dit, plus l'expression des gènes sera élevée, plus la quantité d'alcaloïdes présente sera grande aussi.

1.10 Méthodologie

1.10.1 Échantillons de plante

Pour mener ce projet de recherche, l'espèce d'Amaryllidacée *Narcissus papyraceus* a été choisie à cause de sa facilité à croître à l'intérieur, comparé à d'autres espèces d'Amaryllidacées, permettant d'étudier, dans un environnement contrôlé, les effets de la croissance et du développement sur la production et l'accumulation des AA.

Avec six stades de développement identifiés et jusqu'à cinq parties (bulbe, racines, feuilles, tige et fleurs) présentes à plusieurs de ces stades, 24 échantillons de

N. papyraceus ont été obtenus (Figure 1.4). Chaque échantillon se consiste d'un triplicata. Plus précisément, parmi 24 bulbes de *N. papyraceus*, 3 ont été utilisés pour le 1er stade et parmi les 21 bulbes plantés, 3-4 plantes ont été sélectionnées pour chaque stade. L'analyse de différentes parties à divers stades a fourni de l'information qui pourra être utile par rapport à l'extraction de la plus grande quantité d'alcaloïdes des plantes en sachant à quel moment de la vie de la plante et dans quelle partie de la plante les AA s'accumulent le plus.

Il a déjà été rapporté pour trois espèces différentes de *Narcissus* que certains échantillons des plantes accumulent plus d'alcaloïdes que d'autres. Par exemple, Viladomat *et al.* ont trouvé que le bulbe de *Narcissus assoanus* accumulait la plus grande quantité d'alcaloïdes [53], alors que Kreh a observé que la concentration de galanthamine était relativement élevée dans les bulbes de *N. pseudonarcissus* L. cultivar Carlton [54]. Plus récemment, Shawky *et al.* ont étudié différentes parties de *N. papyraceus* aux stades de pré-floraison, la floraison et post-floraison. Les alcaloïdes se sont révélés plus abondants dans les échantillons de bulbes aux stades de pré- et post-floraison, de racines et tiges au stade de la floraison, et d'écaille au stade de pré-floraison [55]. Des différences dans l'expression de certains gènes de biosynthèse des AA parmi divers échantillons d'une espèce ont également été rapportées. Par exemple, dans une étude menée sur *PAL* dans une autre espèce d'Amaryllidacée, la *Lycoris radiata*, Jiang *et al.* ont identifié deux isoformes de *PAL* qui différaient dans leur expression dans différentes parties de la fleur au stade de la floraison; l'expression de *LrPAL2* était toujours beaucoup plus élevée que celle de *LrPAL1* [56]. Singh et Desgagné-Penix ont aussi noté, dans leur étude récente de *N. pseudonarcissus* cultivar King Alfred, différents profils d'expression de deux isoformes de *PAL* dans cinq parties de la plante au stade de la floraison [57]. De telles différences dans l'expression des gènes peuvent, probablement, expliquer les différents profils d'accumulation d'alcaloïdes observés dans une plante à différents stades.

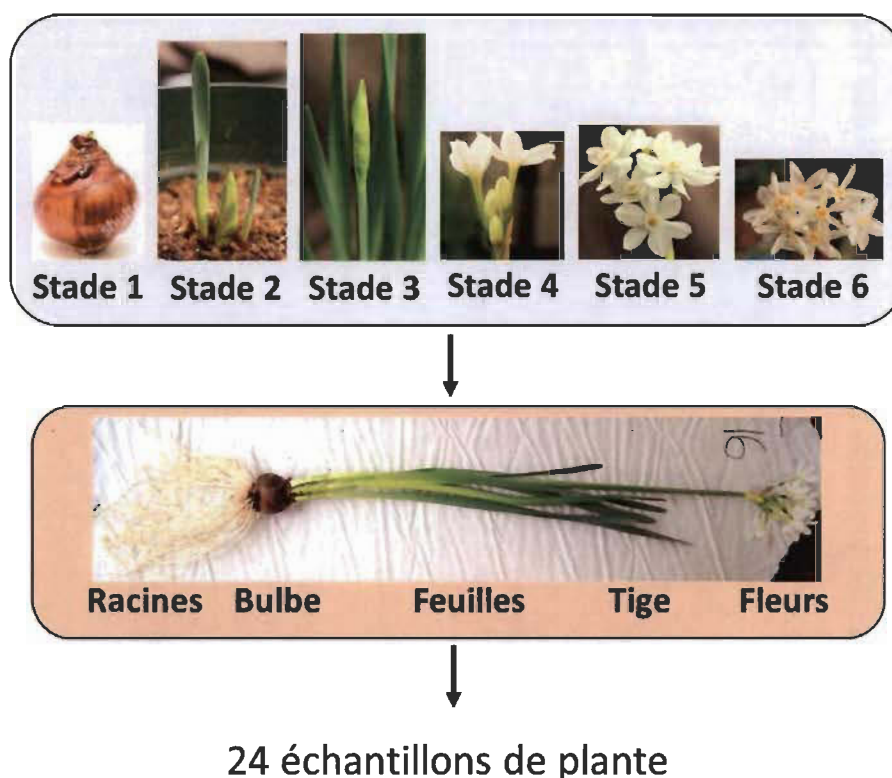


Figure 1.4 Échantillons de plante de *N. papyraceus*.
Le stade 1 consiste du bulbe non-planté. Le stade 2, pour sa part, est caractérisé par l'apparence des premières feuilles, tandis que la présence d'une tige avec un bourgeon caractérise le stade 3. Les stades 4-6 ont été déterminés par rapport à la maturation des fleurs. Les feuilles sont basales, alors la tige ne porte que les fleurs. Vingt-quatre échantillons de *N. papyraceus* ont été obtenus à partir des six stades auxquels il y avait jusqu'à cinq parties présentes.

1.10.2 Étude transcriptomique

Le transcriptome est l'ensemble de toutes les molécules d'ARN dans une cellule ou une population de cellules [58]. Il peut se référer à tous les ARN, ou seulement aux ARN messagers, selon l'expérience effectuée. Puisque le génome de *N. papyraceus* n'était pas disponible dans les bases de données publiques, il a fallu procéder à l'assemblage *de novo* du transcriptome, c'est-à-dire créer un transcriptome sans l'aide d'un génome de référence.

Pour ce faire, les ARN messagers extraits du bulbe au stade 1 de *N. papyraceus* ont, d'abord, été séquencés à l'aide de la méthode de séquençage à haut débit Illumina

HiSeq 2000. Par la suite, l'assemblage *de novo* du transcriptome a été réalisé à l'aide du logiciel informatique Trinity. Éventuellement, avec l'utilisation d'outils bio-informatiques, tels que Trinotate et BLAST, les séquences correspondant à tous les gènes biosynthétiques proposés des AA, dans le transcriptome assemblé, étaient identifiées.

Ces séquences des gènes ont permis, entre autres, de concevoir des amorces spécifiques à ces gènes pour permettre d'étudier leur expression dans les différents échantillons de *N. papyraceus* par une méthode de la réaction en chaîne par polymérase (PCR) (Figure 1.5).

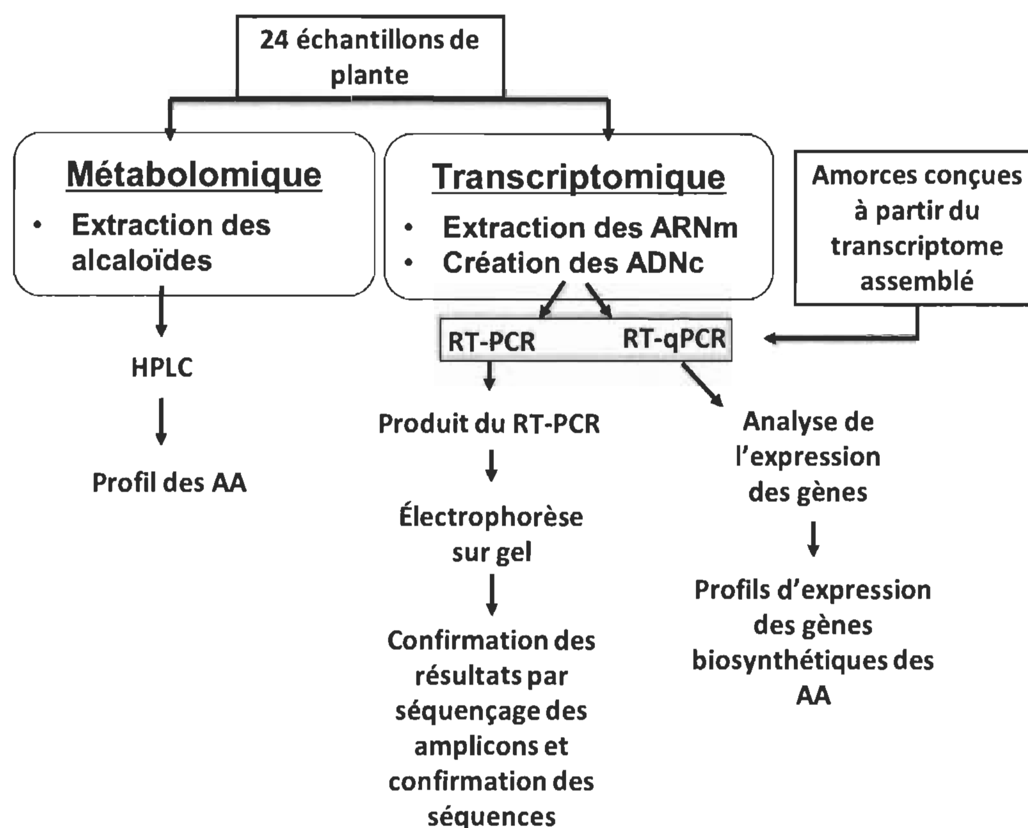


Figure 1.5 Schématisation des méthodes utilisées.

La technique de la PCR permet d'amplifier, plus précisément d'augmenter, le nombre de copies d'une séquence d'ADN connue de plusieurs ordres de grandeur générant des millions de copies de la séquence d'ADN. Puisque l'ARN n'est pas une

molécule stable, elle doit être convertie en ADN avant de pouvoir l'amplifier par la PCR. Donc, il faut procéder à la transcription inverse de l'ARN pour générer l'ADN complémentaire. Le processus d'amplification de l'ADN complémentaire par la PCR est appelé la PCR à transcription inverse (RT-PCR). La technique de la RT-PCR en temps réel de façon quantitative (RT-qPCR), quant à elle, permet de quantifier l'ADN complémentaire préalablement rétrotranscrit. Ainsi, les quantités d'ARN messagers (converti en ADN complémentaire) d'un gène présent dans les différents échantillons de plante peuvent être comparées entre eux, relativement à un ou des gène(s) de référence, en utilisant la formule mathématique $2^{-\Delta\Delta C_T}$ [59]. La quantité d'ARN messager d'un gène indique le niveau d'expression de ce gène.

1.10.3 Étude métabolomique

Pour l'étude du métabolome spécifique aux AA, ces alcaloïdes ont été extraits des divers échantillons de *N. papyraceus* par une méthode d'extraction acido-basique optimisée par notre laboratoire. Les extraits ont été analysés par la chromatographie en phase liquide à haute performance (HPLC) et un détecteur de barrette de photodiode afin d'obtenir de l'information sur les alcaloïdes présents dans les échantillons de *N. papyraceus* (Figure 1.5). La technique de la HPLC permet de séparer divers composés présents dans un échantillon selon leur taille et leur polarité. Un détecteur de barrette de photodiode fournit des informations spectrophotométriques sur les composés détectés et permet de calculer leur concentration.

La technique de la HPLC repose sur des pompes pour faire passer un échantillon liquide et un solvant liquide, sous haute pression, à travers une colonne remplie de matériau adsorbant solide [60, 61]. Le matériau adsorbant est appelé phase stationnaire et est constitué de particules solides, telle que la silice, dont la taille varie de 2 à 50 μm. Lorsque l'échantillon est injecté dans la colonne du système de la HPLC, les différents composés de l'échantillon adhèrent à divers degrés à la phase stationnaire parce que chaque composé de l'échantillon interagit légèrement différemment avec la phase stationnaire. Le solvant liquide est appelé phase mobile. Il est passé à travers la colonne

afin d'éluer les composés adsorbés à la phase stationnaire. Les composés qui sont relativement faiblement liés à la phase stationnaire sont facilement élués par la phase mobile et sont les premiers composés à être détectés par le détecteur. Plus un composé est fortement lié à la phase stationnaire, plus il faut de temps pour qu'il soit élué et détecté par le détecteur.

La méthode de la HPLC la plus couramment utilisée est la HPLC en phase inverse [60, 61]. Dans cette méthode, la phase stationnaire est non polaire, et la phase mobile est modérément polaire se constituant d'eau et d'un solvant organique (généralement de l'acétonitrile ou du méthanol). Dans la présente étude de l'analyse des AA par HPLC, la phase mobile consistait d'un mélange d'une solution aqueuse d'acétate d'ammonium à 1 % et d'acétonitrile. La composition de la phase mobile est souvent modifiée au cours du processus d'élution en ce que le pourcentage d'eau dans la phase mobile diminue avec le temps, tandis que le pourcentage du solvant moins polaire augmente simultanément. Cela signifie que la phase mobile devient progressivement plus non-polaire. Ceci est appelé élution par gradient. Son avantage, par opposition à l'élution isocratique dans laquelle la composition de la phase mobile reste constante, est que les composés qui normalement seront élués plus tardivement (c'est-à-dire les composés plus non polaires) s'élueront plus rapidement, donnant des pics plus étroits et plus haut sur le chromatogramme pour la plupart de ces composés. En d'autres termes, les pics seront plus aigus. Dans le cas présent, le rapport de la solution d'acétate d'ammonium à l'acétonitrile variait de 90:10 pendant 11 minutes, à 69:31 pendant 4 minutes, à 30:70 pendant 1 minute et finalement à 10:90 pendant 5 minutes. C'était un protocole optimisé pour permettre une séparation efficace des alcaloïdes dans les différents échantillons de plantes en fonction de leur polarité. Le rapport initial de 90:10 a permis d'éluer les composés polaires. Ensuite, les ratios de 69:31 et 30:70 ont permis de bien séparer les composés légèrement non-polaires incluant les AA. Finalement, les composés non-polaires sont sortis grâce au ratio de 10:90 de la phase mobile.

CHAPITRE II

DEVELOPMENTAL REGULATION OF THE EXPRESSION OF AMARYLLIDACEAE ALKALOID BIOSYNTHETIC GENES IN *NARCISSUS PAPYRACEUS*

Tarun Hotchandani, Justine de Villers and Isabel Desgagné-Penix*

Manuscrit en préparation pour soumission dans *Journal of Experimental Botany*.

Résumé de l'article

Les alcaloïdes synthétisés par les plantes d'Amaryllidacée ont des effets biologiques multiples, ce qui explique leur utilisation dans les industries pharmaceutiques et agricoles. Malheureusement, la production à grande échelle des alcaloïdes d'Amaryllidacée (AA) est difficile en raison du manque d'information sur leur biosynthèse. Ainsi, nous nous sommes concentrés à augmenter nos connaissances sur l'implication des gènes biosynthétiques des AA proposés et confirmés dans la production des AA, et sur l'observation des différences spatiales et temporelles concernant la biosynthèse des AA dans la plante. Pour ce faire, d'abord, le transcriptome de *Narcissus papyraceus* a été généré par le séquençage de l'ARN. La présence de tous les gènes biosynthétiques des AA a été confirmée dans le transcriptome de *N. papyraceus*. Une banque d'ADN complémentaire a ensuite été créée à partir de différentes parties à différents stades de développement de *N. papyraceus*. L'expression des gènes biosynthétiques des AA a été analysée dans chaque échantillon de *N. papyraceus* par RT-qPCR. Pour les gènes biosynthétiques proposés des AA, les amorces ont été conçues à partir de séquences de transcrits de gènes homologues aux gènes proposés. Pour la plupart des gènes biosynthétiques des AA, les niveaux d'expression variaient considérablement entre les différentes parties de la plante aux différents stades de développement. Pour étudier la teneur en alcaloïdes, chaque

échantillon de *N. papyraceus* a été analysé par la HPLC. Les feuilles et les fleurs avaient la plus grande abondance de composés hétérocycliques, alors que les bulbes, les plus bas. En outre, la lycorine était l'AA prédominant. Les résultats de l'expression des gènes ont été comparés avec les profils des composés hétérocycliques obtenus par la HPLC pour chaque échantillon de la plante. Une corrélation positive a généralement été observée entre les niveaux d'expression génique et la quantité d'AA accumulés dans la plante. Cependant, en raison d'un transport probable d'enzymes et d'alcaloïdes dans la plante, une corrélation négative a été observée dans certains cas.

Abstract

The alkaloids synthesised by Amaryllidaceae plants have multiple biological effects, which explains their use in the pharmaceutical and agricultural industries. Unfortunately, large-scale production of Amaryllidaceae alkaloids (AAs) is difficult due to the lack of information on their biosynthesis. Thus, we focused on gaining more knowledge on the involvement of the proposed and confirmed AA biosynthetic genes in AA production, and on observing spatial and temporal differences regarding AA biosynthesis in the plant. To do so, first, the transcriptome of *Narcissus papyraceus* was generated by RNA-Seq. The presence of all the AA biosynthetic genes was confirmed in the *N. papyraceus* transcriptome. A cDNA library was then created from different parts at various developmental stages of *N. papyraceus*. The expression of the AA biosynthetic genes was analysed in each *N. papyraceus* sample by RT-qPCR. For the proposed AA biosynthetic genes, primers were designed from transcript sequences of genes homologous to the proposed genes. For most genes, expression levels varied considerably among the *N. papyraceus* samples. To study the alkaloid content, each sample was analysed by HPLC. Leaves and flowers were found to have the highest abundance of heterocyclic compounds, whereas the bulbs, the lowest. Also, lycorine was the predominant AA. The gene expression results were compared with the heterocyclic compound profiles obtained by HPLC for each sample. A positive correlation was generally observed between the gene expression levels and the amount of

AAs accumulated. However, due to a probable transport of enzymes and alkaloids in the plant, a negative correlation was also observed.

Keywords

alkaloids, Amaryllidaceae, biosynthetic genes, developmental stage, expression, *N. papyraceus*, HPLC, RT-qPCR, transcriptome.

Abbreviations

λ_{max}: absorption maximum

3,4-DHBA: 3,4-dihydroxybenzaldehyde

4CL: 4-hydroxycinnamoyl CoA ligase

AA: Amaryllidaceae alkaloid

C3H: coumarate 3-hydroxylase

C4H: cinnamate 4-hydroxylase

CYP96T1: cytochrome P450 monooxygenase 96T1

HCT: hydroxycinnamoyl transferase

HIS: histone

N4OMT: norbelladine 4'-*O*-methyltransferase

NorRed: noroxomaritidine reductase

PAL: phenylalanine ammonia lyase

PDA: photodiode array

Rt: retention time

RT-PCR: reverse transcription PCR

RT-qPCR: quantitative real-time RT-PCR

TYDC: L-tyrosine/L-dopa decarboxylase

Introduction

Plants produce specialised (secondary) metabolites having a wide range of biological activities. One class of specialised metabolites is that of the alkaloids.

These compounds are mainly characterised by the occurrence of at least one nitrogen atom in a heterocyclic ring. Since alkaloids are toxic, they are believed to play an important role in the defense against herbivores and pathogens (Mahajan et al., 2010). The toxic properties (cytotoxic, cytostatic, anti-microbial, etc.) of alkaloids makes them suitable for use, at low doses, as medical drugs. For example, the alkaloid vinblastine is used as an anticancer drug (Evidente and Kornienko, 2009), whereas morphine and codeine are two well-known analgesic alkaloids (Mascavage et al., 2006).

One family of plants known to produce a wide variety of alkaloids with promising medicinal potential is the Amaryllidaceae family (Hotchandani and Desgagné-Penix, 2016). It includes ornamental species such as daffodils, narcissuses, jonquils and snowdrops. More than 500 alkaloids synthesised by the Amaryllidaceae family have been identified so far (Jin and Xu, 2013). However, only one Amaryllidaceae alkaloid (AA), galanthamine, is currently available as a commercial drug, marketed under the names Reminyl® and Razadyne® by Janssen Pharmaceuticals (Takos and Rook, 2013). Owing to its acetylcholinesterase inhibitory property, it is used to treat the symptoms of Alzheimer's disease (Heinrich and Teoh, 2004, Keller et al., 2011).

Many other AAs are being studied for eventual clinical use. For example, lycorine and narciclasine exhibit anticancer effects. Lycorine is a powerful inhibitor of cell growth and cell division (Lamoral-Theys et al., 2009, Hayden et al., 2010), whereas narciclasine has been shown to be effective against human glioblastoma multiform tumours in preclinical animal models (Lefranc et al., 2009). Another AA, pancracine, has anti-bacterial activity against the Gram-negative bacterium *Escherichia coli* and cytotoxic activity against the yeast *Candida albicans* (Evidente et al., 2004). The AAs haemanthamine and haemanthidine possess activity against the malarial protozoan parasite *Plasmodium falciparum* (Şener et al., 2003, Kaya et al., 2011, Cedrón et al., 2012).

Unfortunately, large-scale production of AAs is difficult and expensive. Firstly, many AAs exist only in trace amounts in their native plants, and growing a large

number of Amaryllidaceae plants in order to obtain reasonable amounts of AAs is environmentally unsustainable. Secondly, total chemical synthesis can help alleviate the demands for a limited number of AAs, but since these compounds have complex structures, their synthesis is quite challenging. Therefore, genetic engineering of plants or microorganisms to produce high concentrations of AAs would be a more environmentally sustainable and cheaper approach. This, however, requires a better understanding of the AA biosynthetic pathway.

Early feeding experiments using radiolabelled precursors led to the biochemical elucidation of the initial steps in AA biosynthesis (Barton and Cohen, 1957, Barton and Kirby, 1962, Barton et al., 1963, Bhandarkar and Kirby, 1970, Eichhorn et al., 1998, Tahchy et al., 2010, El Tahchy et al., 2011, Saliba et al., 2015). These studies suggest that, despite their large structural diversity, all AAs are derived from norbelladine (Fig. 1). Furthermore, experimental studies coupled with analogies with other plant alkaloid pathways suggest that the central compound norbelladine is formed from the condensation of 3,4-dihydroxybenzaldehyde (3,4-DHBA) and tyramine (Fig. 1) (Bastida et al., 2011, Takos and Rook, 2013, Singh and Desgagné-Penix, 2014, Diamond and Desgagné-Penix, 2015).

The biosynthesis of the AAs begins with the two amino acids phenylalanine and tyrosine. A series of biochemical reactions eventually leads to the production of the more than 600 AAs known to date (Singh and Desgagné-Penix, 2017). In the pathway, all the compounds synthesized from these two amino acids, leading to the formation of norbelladine, are referred to as the AA precursor compounds. On the other hand, the compounds produced from norbelladine, leading to the formation of all the AAs, are referred to as the AA intermediate compounds.

At present, the AA precursors such as *trans*-cinnamic acid, *p*-coumaric acid, caffeic acid, 3,4-DHBA, tyramine, etc. and a few of the initial intermediates (4'-*O*-methylnorbelladine, nornarwedine, noroxomaritidine, etc.) are known, however most of the biosynthetic genes involved in their production are not known. A central

dogma of medicinal chemistry and chemical biology is that compounds with similar structures have similar activities (Bohlin et al., 2010). Hence, similar structures suggest similar metabolic pathways involving similar biosynthetic genes. Since compounds similar to the AA precursors and intermediates are present in plants belonging to other alkaloid-producing plant families, such as Papaveraceae, Brassicaceae, Orchidaceae and Solanaceae, where the biosynthetic genes are known, it has been proposed that genes homologous to the known biosynthetic genes may be involved in AA biosynthesis. Therefore, the biosynthetic genes *PAL* (coding for phenylalanine ammonia lyase), *C4H* (coding for cinnamate 4-hydroxylase (CYP73A1)) and *C3H* (coding for coumarate 3-hydroxylase (CYP98A3)) have been proposed, so far, as being responsible for the production of 3,4-DHBA from phenylalanine (Hotchandani and Desgagné-Penix, 2016). Regarding caffeic acid, it is uncertain whether it is produced directly from *p*-coumaric acid involving only *C3H* or whether it is produced via the sequence of reactions, shown in the grey shaded area in the pathway (Fig. 1), involving the additional proposed AA biosynthetic genes *4CL* (coding for 4-hydroxycinnamoyl CoA ligase) and *HCT* (coding for hydroxycinnamoyl transferase). Concerning tyramine, it is assumed *TYDC* (coding for L-tyrosine/L-dopa tyrosine decarboxylase) is responsible for its production from tyrosine (Facchini et al., 2000). It should be noted that no biosynthetic gene has yet been proposed for the production of norbelladine from the condensation of 3,4-DHBA and tyramine. Also, it is only recently that the biosynthetic genes *N4OMT* (coding for norbelladine 4'-*O*-methyltransferase), *CYP96T1* (coding for cytochrome P450 monooxygenase 96T1) and *NorRed* (coding for noroxomaritidine reductase), all involved in the production of AA intermediates, were confirmed (Kilgore et al., 2014, Kilgore et al., 2016a, Kilgore et al., 2016b). Therefore, there are 6 proposed (*PAL*, *C4H*, *C3H*, *4CL*, *HCT* and *TYDC*) and 3 confirmed (*N4OMT*, *CYP96T1* and *NorRed*) AA biosynthetic genes in the pathway at the moment. The reason behind the great diversity of AAs is due mainly to the 3 different types of oxidative phenol coupling reactions (*para-ortho'*, *ortho-para'* and *para-para'*) which convert 4'-*O*-methylnorbelladine (produced by N4OMT) into a myriad of different alkaloids (Fig. 1).

In the present work, we attempt to gain a better understanding of the involvement of the AA biosynthetic genes in the production of AAs, and to study spatial and temporal differences regarding AA biosynthesis in the plant. To do this, we analysed different parts of the Amaryllidaceae species *Narcissus papyraceus* at various developmental stages. This species was selected since, among Amaryllidaceae species, it can be easily grown indoors in a controlled environment. Also, differences in AA accumulation and biosynthetic genes' expression among different parts and developmental stages of a plant have been reported for *Narcissus* and other Amaryllidaceae species (Viladomat et al., 1986, Kreh, 2002, Jiang et al., 2013, Shawky et al., 2015, Singh and Desgagné-Penix, 2017).

Initially, the *N. papyraceus* bulb transcriptome was sequenced, using RNA-Seq, to investigate the AA biosynthetic genes. Recently, next-generation high-throughput sequencing methods, such as RNA-Seq, have emerged as cost-effective approaches that are well adapted to the analysis of the transcriptomes of both model and non-model species (Singh and Desgagné-Penix, 2017). Not only can RNA-Seq help identify a large number of expressed sequences, but it can also allow the discovery of novel genes via deep sequencing, effectively revealing the expression of many rare transcripts. This report describes an RNA-Seq analysis of *N. papyraceus* bulbs and discovers a large number of candidate transcripts that have significant sequence similarities to proposed genes encoding AA biosynthetic enzymes.

Next, the levels of expression of the AA biosynthetic genes and AA content in each sample of *N. papyraceus* were examined. A total of 24 samples of *N. papyraceus* were obtained from up to 5 parts at 6 developmental stages of the plant (Fig. 2). RNA was extracted from each plant sample and reverse transcribed to cDNA. Primers specific to the AA biosynthetic genes were used in reverse transcription PCR (RT-PCR) experiments. Subsequent sequencing of the amplicons and their alignment with the *N. papyraceus* transcripts allowed us to confirm the presence of the genes in the *N. papyraceus* transcriptome. The levels of expression of the genes in each plant sample were studied by reverse transcription quantitative real-time PCR (RT-qPCR). To gain information on the

AA content in the plant samples, AAs were extracted from each sample and analysed by HPLC. The gene expression profiles were compared with the AA profiles to get a better picture of which genes are supposedly involved in the production of the AAs and to what extent.

All genes and isoforms analysed were confirmed to be present in the *N. papyraceus* un-planted bulb transcriptome. For most genes, their expression levels varied considerably among the *N. papyraceus* samples. Regarding the content of AAs in the *N. papyraceus* samples, the detected compounds tended to preferentially accumulate in specific parts of the plant, namely roots, flowers and, especially, leaves. When comparing the gene expression levels with the amount of AAs accumulated, a negative correlation was observed in certain cases perhaps due to a probable transport of enzymes and/or alkaloids in the plant.

Materials and methods

RNA extraction, next-generation Illumina sequencing and *de novo* transcriptome assembly

Two grams of triplicate bulbs, stems, roots, leaves or flowers of *N. papyraceus* were crushed using a pestle and a mortar with liquid nitrogen and transferred to pre-chilled 50 ml tubes to proceed with the CTAB (cetrimonium bromide) method for total RNA extraction as described in (Singh and Desgagné-Penix, 2017). After RNA extraction, bulb RNA was selected for Illumina sequencing, and stem, root, leaf and flower RNA were used for RT-qPCR analysis. For transcriptome analysis, integrity of the bulb RNA was checked on a nanodrop and bioanalyzer. Nanodrop quantification yielded 1820.42 ng/µl of total RNA with the absorbance ratios 260 nm/230 nm of 2.50 and 260 nm/280 nm of 2.04. Bioanalysis of RNA gave a RNA integrity number (RIN) of 8.1 with 28S/18S of 1.54 confirming the integrity and quality of the RNA. The pipeline used for trimming and *de novo* assembly is described in "*De novo* transcript sequence reconstruction from RNA-Seq using the Trinity platform for reference generation and analysis" (Haas et al., 2013) and mostly based on the Trinity assembly software suite (Grabherr et al., 2011).

Briefly, mRNA were converted into cDNA library and sequenced through Illumina HiSeq 2000, PE 100 paired ends, at McGill University and Genome Quebec Innovation Centre (Montreal, Canada). Raw paired reads were trimmed from 3'-end to have a phred score of at least 30. Illumina sequencing adapters were removed, maintaining 50 bp of minimum read length to obtain surviving paired reads. Trimming and clipping was done using Trimmomatic (http://www.usadellab.org/cms/index.php?page=trimmomatic) (Bolger et al., 2014) for quality filtering to have clean reads.

Once surviving pair data were generated, Trinity normalization was performed to eliminate redundant reads in datasets without affecting its *Kmer* content (Brown et al., 2012). *De novo* assembly of cleaned and normalized reads was done using Trinity assembler (https://github.com/trinityrnaseq/trinityrnaseq/wiki) (Grabherr et al., 2011). Final obtained unigenes were functionally annotated using Trinotate (http://trinotate.github.io/). To quantify the gene transcript abundance, the raw RNA-Seq reads were mapped to assembled transcripts applying Bowtie (Langmead et al., 2009) using default parameters. The gene transcript abundance was calculated as *fragments per million mapped reads per kilobase* (FPKM) using the RSEM package (Li and Dewey, 2011).

Chemicals

Anhydrous ethyl alcohol was purchased from Commercial Alcohols (Brampton, Ontario, Canada). Ammonium acetate (anhydrous, ACS reagent grade) was from MP Biomedicals (Solon, Ohio, USA). Ammonia (ca. 7N solution in methanol) was obtained from ACROS Organics™ (www.acros.com). Agarose (protein electrophoresis grade, high gelling temperature); Tris base (for molecular biology); ethylenediamine tetraacetic acid (EDTA), disodium salt dihydrate; sulfuric acid; acetic acid, glacial (certified ACS); methanol (HPLC grade); chloroform (HPLC grade); and acetonitrile (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). The alkaloids narciclasine and galanthamine were purchased from Tocris Bioscience

(Bristol, U.K.), whereas lycorine and papaverine were obtained from Sigma-Aldrich (Ontario, Canada).

Plant material

Twenty-four *N. papyraceus* bulbs were purchased from Veseys (York, Prince Edward Island, Canada). Three bulbs were kept aside and 21 were planted. AGRO MIX® G6 potting soil (Fafard, Saint-Bonaventure, Quebec, Canada) was autoclaved for 40 minutes. The plants were kept at room temperature with exposure to tube lighting for 16 hours daily until being harvested. The plants were watered when necessary to keep the soil moist. No other nutrients, such as fertilizer or plant food, were added. The plants were harvested at each developmental stage (Fig. 2) over an 85-day period.

The three unplanted bulbs represent the first stage of development. These bulbs were cut open, and a total of 10 g of material from the center of the three bulbs was extracted and stored in Ziploc bags at -80 °C. Among the planted bulbs (for the five subsequent developmental stages), 3-4 plants were collected for each stage and the same amount (10 g) was extracted from different parts of the plant (bulbs, roots, leaves, and/or stems and flowers) and stored in Ziploc bags at -80 °C. Up to five parts being studied at six developmental stages resulted in a total of 24 plant samples.

cDNA synthesis

A mass of 0.1 g of each plant sample was frozen with liquid nitrogen and ground to a fine powder with a mortar and pestle. RNA was extracted from each ground-up plant sample using GENEzol™ TriRNA Pure Kit (Geneaid, www.geneaid.com). The RNA sample obtained was diluted with autoclaved water to a concentration of 100 ng μl^{-1}. It was then immediately used for reverse transcription or stored at -80 °C. Just prior to reverse transcription, the purity of the RNA was verified using the NanoVue spectrophotometer (GE Healthcare Life Sciences, www.gelifesciences.com). According to Gallagher and Desjardins, a ratio of the absorbance at 260 and 280 nm ($A_{260/280}$) greater

than 1.8, and the ratio $A_{260/230}$ greater than 2.0 are considered to be suitable for gene expression measurements (Gallagher and Desjardins, 2007). cDNA synthesis was carried out using Omniscript® Reverse Transcription Kit (QIAGEN, www.qiagen.com) with the aid of oligo-dT. The cDNA created was stored at -20 °C.

Primer design

To design primer pairs specific to a region of a particular gene, 3-4 partial or complete homologous mRNA sequences from closely related species were selected from the Nucleotide database of the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov). The similarity of the selected sequences was verified by performing a multiple sequence alignment using CLUSTALW (Kyoto University Bioinformatics Center, www.genome.jp/tools-bin/clustalw). From the aligned sequences, the longest one having the least number of gaps was selected. This sequence was queried against the *N. papyraceus* transcriptome using NCBI's megablast BLASTN program. Among the transcripts selected by BLASTN, the one with the highest value of *Query cover* and *Ident* was used in the designing of primers. They were designed using the online PrimerQuest® tool (Integrated DNA Technologies, www.idtdna.com/PrimerQuest). Primer pairs producing short amplicons of 100-300 bp (a requirement for quantitative real-time PCR experiments) were chosen. Primers were also designed for the gene encoding histone (*HIS*) which was used as reference gene in the RT-qPCR experiments. The primers are listed in Supplementary Table S1.

Reverse transcription PCR for primer testing

Using the gene-specific primers on the prepared cDNA, reverse transcription PCR (RT-PCR) was performed to amplify the targeted region of each gene. Reaction mixtures (50 μl each) were prepared with the TopTaq DNA Polymerase kit (QIAGEN) following the provided PCR Using TopTaq DNA Polymerase protocol. For all genes, the same cDNA sample of the 4th stage bulb was used. The reaction was carried out using T100™ Thermal Cycler (Bio-Rad, www.bio-rad.com).

Gel electrophoresis

The RT-PCR products were analysed by gel electrophoresis using FisherBiotech™ Horizontal Electrophoresis Systems (Fisher Scientific). The gel consisted of 2% agarose in 1x Tris-acetate-EDTA buffer and was stained with Invitrogen™ SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, Carlsbad, California, USA). Also, the 1 Kb Plus DNA Ladder (Thermo Fisher Scientific) was used. One fifth of the volume of the RT-PCR product of each gene (i.e. 10 µl), mixed with 2 µl of GelPilot® DNA Loading Dye 5x (QIAGEN), was loaded in separate wells of the gel. Electrophoresis was carried out for 1 h at 100 V. The bands on the gel were observed using ChemiDoc™ Touch Imaging System (Bio-Rad).

Sequencing of amplicons

The remaining RT-PCR product (40 µl) of each gene was sent to Génome Québec for Sanger sequencing. Sanger sequencing was performed on both the forward and reverse strands to increase the chances of obtaining an accurate sequenced amplicon. The amplicon sequence (forward and reverse strands) obtained for each gene was aligned with the sequence (forward and reverse strands) of the corresponding candidate transcript in the *N. papyraceus* transcriptome using CLUSTALW. A value indicating the homology of the aligned sequences was obtained.

Reverse transcription quantitative real-time PCR

Reverse transcription quantitative real-time PCR (RT-qPCR) was performed on triple technical replicates of each plant sample using CFX Connect™ Real-Time System (Bio-Rad). The reaction mixtures were prepared using SensiFAST™ SYBR® Lo-ROX Kit (Bioline, www.bioline.com/sensifast). The included protocol was followed using 1 µl of template cDNA (100 ng/µl). For each gene, the primers used and the temperature at the annealing/extension step of the reaction were the same as those for the RT-PCR experiments (Supplementary Table S1). The reaction conditions were 95 °C for 3 min, followed by 40 cycles of denaturation (95 °C for 10 s) and annealing/extension for 30 s.

The last steps of the reaction were 95 °C for 10 s, 65 °C for 5 s and 95 °C for 5 s. The threshold cycle (C_T) value of each gene was normalised against the C_T value of the gene encoding histone, which served as reference gene. Mean C_T values were calculated from the technical triplicates and used for relative quantitative gene expression analysis involving the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). The statistical error was calculated using the method stated in the CFX Connect™ Real-Time System manual (2013).

Alkaloid extraction

Amaryllidaceae alkaloids were extracted from the 24 *N. papyraceus* samples with methanol and purified following an optimised acid-base extraction protocol designed by us. For a given sample, 2 g of plant material were ground to a fine powder in liquid nitrogen with a mortar and pestle. The powder was transferred to a 15 ml centrifuge tube. Ten microlitres of papaverine (3 $\mu g\ ml^{-1}$ in water), used as internal standard for data quantification, and 5 ml of methanol were added to the powder, which was then placed at 50 °C for 2 h. The tube was vortexed briefly and then centrifuged at 7000 *g* for 2 min. The supernatant (crude extract of alkaloids) was collected in a new 15 ml centrifuge tube and allowed to evaporate until approximately 1.5 ml remained. The tube was once again vortexed and centrifuged briefly. The supernatant was transferred to a 1.7 ml microcentrifuge tube and allowed to evaporate to dryness. The dry crude extract of alkaloids was re-suspended in 300 µl of methanol and subjected to acid-base extraction. First, the sample was acidified with H_2SO_4 (2% v/v in water). Organic impurities were removed by washing twice with chloroform. Next, alkalisation was achieved using ammonia. The purified alkaloid extract obtained was dried under N_2 gas and finally solubilised in 300 µl of methanol.

HPLC

The alkaloid extract samples were analyzed by reversed-phase HPLC with photodiode array (PDA) detector using the Prominence-i LC-2030C system (Shimadzu,

www.ssi.shimadzu.com). Separations were performed at a flow rate of 0.5 ml min^{-1} using a Kinetex® C18 column (150 × 4.6 mm, 5 µm particle size; Phenomenex, www.phenomenex.com). Gradient elution was carried out using a 1% aqueous ammonium acetate solution with pH 5 and 100% acetonitrile. The ratio of the ammonium acetate solution to acetonitrile was: 90:10 for 11 min, 69:31 for 4 min, 30:70 for 1 min, 10:90 for 5 min and 90:10 for 2 min. Compounds were monitored at 280 nm.

The average retention time (Rt) and average absorption maxima (λ_{max}) were determined for the AAs lycorine, galanthamine and narciclasine serving as standards, and for the alkaloid papaverine serving as internal standard for the relative quantification of the concentration of detected compounds (Supplementary Table S2).

Hundred microlitres of the alkaloid extract of each plant sample was injected. The absorbance values of all compounds detected in each sample were normalised to the absorbance value of the internal standard papaverine. After having set an arbitrary minimum relative absorbance cut-off value of 0.20, compounds presenting interesting trends regarding their detection in the various plant samples were selected (Supplementary Table S3). The absorbance of a compound represents its concentration.

The relative concentration of lycorine was determined for each plant sample by dividing its relative absorbance (compound 4.22 in Supplementary Table S3) by the total relative absorbance of all compounds in that sample (row B in Supplementary Table S3). The relative concentrations of lycorine are indicated in row C of Supplementary Table S3.

Accession numbers

The sequences described in this paper have been deposited in the National Center for Biotechnology Information Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra/) under the accession number SRR6041662. Gene transcript sequences were deposited in Genbank with the following accession numbers for nucleotide sequences: tyrosine decarboxylase 1 (MF979854), tyrosine decarboxylase 2

(MF979855), phenylalanine ammonia lyase 1 (MF979856), phenylalanine ammonia lyase 2 (MF979857), phenylalanine ammonia lyase 3 (MF979858), cinnamate 4-hydroxylase 1 (MF979859), cinnamate 4-hydroxylase 2 (MF979860), coumarate 3-hydroxylase (MF979861), 4-coumarate-CoA ligase 1 (MF979862), 4-coumarate-CoA ligase 2 (MF979863), 4-coumarate-CoA ligase 3 (MF979864), 4-coumarate-CoA ligase 4 (MF979865), hydroxycinnamoyltransferase 1 (MF979866), hydroxycinnamoyltransferase 2 (MF979867), hydroxycinnamoyltransferase 3 (MF979868), norbelladine 4'-*O*-methyltransferase (MF979869), noroxomaritidine synthase 1 (MF979770), noroxomaritidine synthase 2 (MF979871), noroxomaritidine/norcraugsodine reductase 1 (MF979872), noroxomaritidine/norcraugsodine reductase 2 (MF979873), noroxomaritidine/norcraugsodine reductase 3 (MF979874) and histone (MF979875).

Results and discussion

RNA sequencing of *Narcissus papyraceus* bulbs

To gain a better understanding of the AA biosynthetic pathway and to enable the discovery of genes involved in their formation, we performed RNA sequencing of *N. papyraceus*. Bulbs were chosen based primarily on their alkaloid accumulation profiles reported in the literature. For example, Viladomat *et al.* found that the bulb of *Narcissus assoanus* accumulated the greatest amount of alkaloids (Viladomat et al., 1986), whereas Kreh reported that the concentration of galanthamine was relatively high in the bulbs of *N. pseudonarcissus* L. cultivar Carlton (Kreh, 2002). In a more recent study by Shawky *et al.*, in which nine different samples (different parts at different developmental stages) of *N. papyraceus* were analysed, alkaloids were found to be most abundant in bulbs compared to other parts (Shawky et al., 2015). Thus, RNA from *N. papyraceus* bulbs was extracted and screened for sufficient quality and quantity prior to cDNA library creation and deep sequencing using Illumina. A total of 70,409,091 raw paired reads were obtained, which were trimmed to give 64,038,268 surviving paired reads that corresponds to 91% of the initial raw reads (Table 1). For non-model plants such as Amaryllidaceae,

for which genomic information is lacking, a *de novo* assembly is necessary. Detailed overview of the sequencing output, trimming, assembly, and transcript length distribution is provided in Supplementary Table S4. A total of 8,945,044 paired reads were obtained after the normalization step corresponding to 14% of the initial raw reads. Normalised reads were used to assemble the transcriptome generating 148,563 transcripts (or unigenes) (Table 1). For identification, the transcripts were aligned against the uniprot_sprot.trinotate_v2.0.pep protein database. BLAST annotation yielded 8,866 transcripts of an average length of 1,695 bp (Supplementary Table S4). Thus, the remaining 139,697 transcripts represented a large number of transcripts showing no similarity to known genes. They appeared to represent transcripts of uncharacterised genes or sequences specific to *N. papyraceus*. Altogether, we concluded that a good quality transcriptome was developed with a high number of surviving paired reads and full-length assembled transcripts.

To identify specific transcripts encoding enzymes involved in AA biosynthesis, we performed local BLASTx (translated nucleotide → protein) analyses (Table 2). Several transcript isoforms of orthologous genes were identified. For example, *TYDC* genes have been identified from various plant species including *N. pseudonarcissus* where two isoforms, *TYDC1* and *TYDC2*, were reported (Singh and Desgagné-Penix, 2017). Together, *NpapyTYDC1* and *NpapyTYDC2* share 57% homology of the amino acid sequence; *NpapyTYDC1* is 90% homologous to *N. pseudonarcissus TYDC1* and *NpapyTYDC2* shares 94% with *N. pseudonarcissus TYDC2* (Singh and Desgagné-Penix, 2017). BLASTx analysis reported that *NpapyTYDC1* shared 70% homology with opium poppy *TYDC2*, which is involved in the synthesis of benzylisoquinoline alkaloids, whereas *NpapyTYDC2* was 74% homologous to the rice *TYDC1* (Table 2).

PAL genes have also been reported from various plant species including the Amaryllidaceae *Lycoris radiata* and *N. pseudonarcissus*, where two isoforms were identified in each species (Jiang et al., 2011, Jiang et al., 2013, Singh and Desgagné-Penix, 2017). BLASTx searches for *PAL* genes in the transcriptome led to the identification of three full-length isoforms (Table 2). Similarly, several transcript isoforms were also

identified for *C4H*, *4CL*, and *HCT* (Table 2). For gene transcripts "specific" to AA biosynthesis, we identified one isoform of *N4OMT*, two of *CYP96T* and three of *NorRed* (Table 2). Altogether, we were able to identify full-length transcripts and several isoforms with Expect values of zero, or very close to zero, corresponding to genes encoding biosynthetic enzymes involved in AA production.

Comparative fragments per kilobase million (FPKM) digital expression of the transcriptome of the *N. papyraceus* bulbs indicated that the "AA-specific" gene transcripts such as those of *N4OMT*, *CYP96T* and *NorRed*, were more abundantly expressed than transcripts encoding enzymes involved in the "AA-precursor" biochemical reactions including those of *TYDC*, *PAL* and others (Table 2). Similar levels of expression were observed for *TYDC* and *PAL* genes (Table 2). Since these genes encode enzymes involved in steps at the entry point of other pathways, it may suggest coordinated regulation for the formation of AA precursors (Fig. 1).

Confirmation of candidate genes

In order to confirm the presence and expression of candidate genes in the bulbs of *N. papyraceus*, specific primer pairs were designed (Supplementary Table S1) and tested using RT-PCR. Gel electrophoresis of the PCR products revealed bands of the expected size between the 100 and 300 bp markers (Supplementary Fig. S1). This means that the targeted region of each AA biosynthetic gene was properly amplified by RT-PCR since the gene-specific primers were designed to create amplicons ranging between 100-300 bp in length (Supplementary Table S1). Regarding *HIS*, the two bands observed between the 100 and 300 bp markers suggest that there are two *HIS* transcripts. These are either splicing variants of the same *HIS* gene or products of two *HIS* gene isoforms. Each PCR product was purified and sequenced to verify accuracy compared to the corresponding sequence from the bulb transcriptome. We concluded that all AA biosynthetic genes are present in the *N. papyraceus* transcriptome. The primers were later used for expression profile analysis by RT-qPCR.

Plant growth

The complete life cycle of *N. papyraceus*, from bulb to senescence, lasted between 10-12 weeks and different parts (bulbs, roots, leaves, stems, and flowers) were collected at each stage (Fig. 2). A total of six stages of development were selected for the study, from non-germinated bulbs (stage 1) to mature plants with senescing flowers (stage 6). The second stage of development, at around 44.5 ± 5 days old, is characterised by the appearance of the first leaves, whereas the third stage (circa 58.5 ± 5 days old) involved the presence of a stem with flower buds (Table 3). The last three stages, i.e. stages 4-6, were determined by the maturation of the flowers (Fig. 2). Plants reached flowering stages after 70 days with 5-12 leaves and ranging in length from 36-48 cm (Table 3). The differences in age, leaf count and leaf length between stages is due to differences between the growth rates of individual plants. A total of 24 samples was collected including bulbs at each of the six stages (6 samples), roots and leaves for stages 2 to 6 (10 samples), and stems and flowers for the last four stages (8 samples). Triplicates for each stage representing these differences were harvested, pooled and used for the subsequent study.

Gene expression analysis with RT-qPCR

In order to profile AA biosynthetic gene expression in *N. papyraceus* parts in response to development, total RNA was isolated from the various parts at the six developmental stages of the plant (Fig. 2). Quantitative expression analysis was performed on twelve genes which included nine genes encoding enzymes involved in AA-precursors' biosynthesis such as *TYDC* (*1* & *2*), *PAL* (*1* & *2*), *C4H*, *C3H*, *4CL* and *HCT* (*1* & *2*) and three genes encoding enzymes involved in AA-intermediates' biosynthesis such as *N4OMT*, *CYP96T* and *NorRed* (Fig. 1). The expression for each gene was normalised against the reference gene coding for histone to obtain the relative gene expression. The results (Fig. 3) indicate that the transcripts exhibited different expression kinetics that can be described as early (stage 1), mid (stages 2-4) and late (stages 5-6) responses. The fold-expression varied from relatively low (< 2 fold) to high (> 4000 fold), compared to each other.

As mentioned earlier, Amaryllidaceae alkaloids are derived from norbelladine (Bastida et al., 2011), which itself is considered to be generated from the condensation of 3,4-DHBA and tyramine (Fig. 1) (Bastida et al., 2011, Singh and Desgagné-Penix, 2014, Diamond and Desgagné-Penix, 2015). Tyramine is produced from tyrosine, while 3,4-DHBA is a product of the phenylpropanoid pathway (Singh and Desgagné-Penix, 2017). The biosynthetic genes *PAL*, *C4H*, *4CL*, *HCT* and *C3H* are involved in phenylpropanoid biosynthesis. The phenylpropanoid biosynthetic pathway is an important specialised metabolic pathway responsible for the production of a large number of metabolites including coumarins, flavonoids, anthocyanins, stilbenes, hydroxycinnamates, lignans, lignin and benzoic acids (Vogt, 2010, Fraser and Chapple, 2011). These compounds play essential roles in plant growth and development, and plant–environment interactions (Zhang and Liu, 2015). Being the first biosynthetic gene of the pathway, *PAL* is responsible for converting the amino acid phenylalanine, a product of primary metabolism, to *trans*-cinnamic acid (Fig. 1). Thus, *PAL* resides at a metabolically important position, linking primary metabolism to specialised metabolism (Singh et al., 2009). The same is true for the biosynthetic gene *TYDC* which transforms the amino acid tyrosine (primary metabolite) to tyramine. In addition to AAs, *TYDC* is involved in the biosynthesis of several other types of specialised metabolites (Facchini et al., 2000) including benzylisoquinoline alkaloids (Desgagné-Penix and Facchini, 2012) and hydroxycinnamic acid amides of tyramine (HCAATs) (Hagel and Facchini, 2005). Morphine and codeine are the best known examples of benzylisoquinoline alkaloids produced by the opium poppy. The production of HCAATs has been associated with plant stress responses. Following oxidative polymerization, HCAATs are believed to reinforce cell walls rendering them less susceptible to penetration by pathogens. Since *PAL* and *TYDC* are the first biosynthetic genes involved in the production of many types of specialised metabolites, they have attracted considerable attention.

Several isoforms of *PAL* exist since *PAL* is encoded by a multi-gene family in plants (Chang et al., 2008). For example, there are four isoforms of *PAL* in *Arabidopsis*, five in poplar, nine in rice (Hamberger et al., 2007) and more than a dozen copies in potato and tomato (Chang et al., 2008). We analysed the expression of two isoforms of *PAL* in our

study of *N. papyraceus* (Fig. 3). *PAL1*, in general, was expressed at similar levels in all parts at each developmental stage. *PAL2*, for its part, was expressed the highest in bulbs at almost all stages, followed by stems, indicating it is more specifically involved in the synthesis of phenylpropanoids in bulbs and stems. This profile of *PAL2* expression was also reported by Singh and Desgagné-Penix for *N. pseudonarcissus* 'King Alfred' *PAL2* (Singh and Desgagné-Penix, 2017). The two isoforms being expressed at different levels in the various *N. papyraceus* plant samples suggests they are involved to different degrees in the phenylpropanoid pathway for the production of various specialised metabolites in the plant.

Such differential spatial and/or temporal expression of isoforms of *PAL*, associated with specific metabolic activities, has also been reported for other plants species. For example, in a study conducted on *PAL* in another Amaryllidaceae species, *Lycoris radiata*, Jiang *et al.* identified two isoforms of *PAL* which differed in their expression in different parts of the flower at the blooming stage of the plant; the expression of *LrPAL2* was always much higher than that of *LrPAL1* (Jiang et al., 2013). Singh and Desgagné-Penix also noted, in their study of *N. pseudonarcissus* 'King Alfred', different expression profiles for two *PAL* isoforms in five parts of the plant at the flowering stage (Singh and Desgagné-Penix, 2017). In both studies, the authors suggest that the *PAL* isoforms may have distinct functions in different branches of the phenylpropanoid pathway.

Differential expression of two isoforms of *PAL* having specific functions was also observed by Kao *et al.* for quaking aspen (*Populus tremuloides* Michx.) (Kao et al., 2002). They found that *PtPAL1* was expressed in the cells of non-lignifying parts such as stems, leaves and roots. However, *PtPAL2* was expressed in both the heavily lignified structural cells of shoots and in the non-lignifying cells of root tips. It was suggested that *PtPAL1* is implicated in the biosynthesis of non-lignin metabolites while *PtPAL2* is involved in lignin formation.

In *Arabidopsis thaliana*, among the four isoforms of *PAL*, *AtPAL1*, *-2* and *-4* were observed to be highly expressed in inflorescence stem, a part rich in lignifying cells (Raes et al., 2003, Rohde et al., 2004), and have been shown to play an important role in lignin biosynthesis (Oh et al., 2003, Rohde et al., 2004, Huang et al., 2010).

Regarding the biosynthetic gene *TYDC*, similar to *PAL*, it is encoded by multiple genes. For example, eight copies of the *TYDC* gene have been detected in the opium poppy (Facchini and De Luca, 1994), whereas four similar genes encode TYDC in parsley (Kawalleck et al., 1993). In our study of *N. papyraceus*, when comparing the expression profiles of *TYDC1* and *TYDC2*, the most interesting feature is that they are practically the same in the first three developmental stages (Fig. 3). This may be a sign that these two isoforms are regulated in a similar manner early in the life of the plant. However, differences in gene expression levels between *TYDC1* and *TYDC2* appear in the later stages 4-6 (Fig. 3). At stage 4, *TYDC1* is expressed at about the same level in all parts of the plant, whereas *TYDC2* is highly expressed in the leaves. At stages 5 and 6, *TYDC1* is expressed more in certain parts than others: bulbs and roots at stage 5, whereas flowers at stage 6. On the other hand, expression of *TYDC2* is not particularly pronounced in any part of the plant during the last two stages.

Different expression profiles for different *TYDC* isoforms have also been observed by Facchini and De Luca who studied their expression in the mature opium poppy (Facchini and De Luca, 1995). They noted that *TYDC1*-like genes were predominantly expressed in roots and, to a much lower extent, in stems. The opposite was true for *TYDC2*-like genes. In the opium poppy, the *TYDC* gene is involved in the synthesis of benzylisoquinoline alkaloids of which morphine and codeine are well-known examples (Desgagné-Penix and Facchini, 2012). Two benzylisoquinoline alkaloids which Facchini and De Luca detected and analysed were sanguinarine and morphine, each of which is synthesised by different branch pathways further downstream in the benzylisoquinoline alkaloid biosynthetic pathway (Desgagné-Penix and Facchini, 2012). The presence of high quantities of sanguinarine in the roots, where *TYDC1*-like genes were highly expressed, and the lack of detectable quantities of sanguinarine in aerial parts,

where *TYDC1*-like genes were lowly expressed, led the authors to believe that the *TYDC1*-like genes were particularly involved in the production of sanguinarine and other alkaloids synthesised in the same branch pathway as sanguinarine. On the other hand, the abundance of morphine in aerial parts and its presence in roots, together with the high levels of *TYDC2*-like transcripts in stems and roots, led the authors to suggest that *TYDC2*-like genes may be implicated in the synthesis of morphine and other closely related alkaloids. Based on these observations, the authors propose that the *TYDC1*-like genes may be coordinately regulated with genes present in the branch pathway involving sanguinarine biosynthesis, whereas *TYDC2*-like genes may be coordinately regulated with genes present in the branch pathway involving morphine biosynthesis.

HCT is another gene for which we analysed the expression levels of two isoforms (Fig. 3). Like for *PAL* and *TYDC*, there are considerable differences between the expression level profiles of the *HCT* isoforms. We assume that, like for *PAL*, the different isoforms of *HCT* are involved to varying extents in the phenylpropanoid pathway. *HCT* has been reported to be quite active in lignin formation (Hoffmann et al., 2004, Lepelley et al., 2007). Lignins are phenylpropanoid-derived compounds involved in cell wall formation. Therefore, maybe the relatively higher expression of *HCT1* during stages 3-6, for example, could be due to a possibly greater production of lignins during these stages and that *HCT1* in *N. papyraceus* is perhaps more involved in lignin formation than *HCT2*.

C4H and *C3H* are two biosynthetic genes in the phenylpropanoid pathway which code for cytochrome P450 enzymes. Curiously, both genes display the same expression pattern for stages 2-6 (Fig. 3). It, thus, seems that these two genes are being coordinately regulated as the plant is growing and developing. Regarding *C4H*, Fock-Bastide *et al.* reported that its expression level in *Vanilla planifolia* pods was highest during the earlier stages of its maturation, but declined during the later stages (Fock-Bastide et al., 2014). This expression profile resembles that observed by us for *N. papyraceus* in that *C4H* expression is high during the 1st and 2nd developmental stages (Fig. 3). Phimchan *et al.*, in their study of capsaicinoid biosynthesis, noted that C4H activity remained constant in

various cultivars of *Capsicum* regardless of fluctuations in capsaicinoid accumulation in these cultivars (Phimchan et al., 2014). Similarly, the level of expression of *C4H* remains constant during stages 3-5 (Fig. 3) even though the *N. papyraceus* plant continues to grow and develop during these stages.

Unlike the genes discussed so far, *N4OMT* is specific to AA biosynthesis. It is responsible for the methylation of norbelladine, the compound from which all AAs are derived (Fig. 1) (Bastida et al., 2011). The expression profile we obtained for *N4OMT* is not in concordance with that obtained by Kilgore *et al.* (Kilgore et al., 2014) and Singh and Desgagné-Penix (Singh and Desgagné-Penix, 2017). They analysed the expression of *N4OMT* in *Narcissus* species at the flowering stage in different parts: bulbs, leaves, flowers, and/or roots and stems. Kilgore *et al.* noted the highest expression was in bulbs, followed by flowers in close second, while it was barely expressed in leaves. Singh and Desgagné-Penix's results demonstrated that highest expression was once again in bulbs, but very low in all other parts. In our results of *N4OMT*'s expression in *N. papyraceus* at developmental stage 4 (flowering stage) (Fig. 3), we observed that the expression in leaves was slightly higher than that in bulbs, and the lowest expression was in flowers. The only similarity with our results is that Singh and Desgagné-Penix also observed the flowers to be among the plant parts where *N4OMT* was expressed the lowest. One reason for the discrepancies in the results of *N4OMT* expression between our and the other two studies may be because the same *Narcissus* species was not analysed in each study.

Looking at the expression levels of any gene in all the 24 samples of *N. papyraceus* (Fig. 3), it is obvious its expression differs among the various parts and usually changes as the plant grows and develops. The physiological conditions of an organism as well as environmental stimuli have long been known to affect the transcriptional regulation of *PAL* (Zhang and Liu, 2015), for example. The same must, undoubtedly, be true for all the other AA biosynthetic genes, which can explain the observed spatial and temporal differences in gene expression. Obviously, as time goes on, a plant adapts to its changing needs, and responds to changes in its environment to varying extents in the various parts of the plant.

Alkaloid profiling

In order to establish the alkaloid profile of each part at each developmental stage, HPLC was performed on all 24 samples of *N. papyraceus* following an optimised acid-base extraction method for alkaloids. First, the average retention times (Rt) and absorption maxima (λ_{max}) were determined for the AAs lycorine, galanthamine and narciclasine, which served as standards, as well as for the alkaloid papaverine (internal standard) which was used for relative quantification (Supplementary Table S2). Among the various compounds detected in all the plant samples, 28 were of interest. Some of these were detected in all or almost all the samples, while others were present in particular parts throughout the life of the plant. The presence of the 28 compounds in the 24 plant samples, listed by their average Rt, is presented in Supplementary Table S3.

By comparing these results with those of the AA standards (Supplementary Table S2), the following observations can be made. The compound with the average Rt of 4.22 min (average λ_{max} of 221 and 285 nm), seems to be lycorine since these values are quite close to those of lycorine (4.13 min; 237 and 285 nm). Contrary to lycorine, galanthamine does not seem to be present in any of the samples as no compound was detected having an average Rt similar to that of galanthamine (4.77 min). Narciclasine also does not seem to be present even though there is a compound detected in almost all of the samples with an average Rt of 7.69 min, which is very close to that obtained for narciclasine (7.70 min). However, the mean λ_{max} values (221 and 283 nm) of this compound differ considerably from those of narciclasine (251 and 304 nm).

Regarding their detection in the 24 plant samples, many compounds share common trends with each other. The compounds, henceforth, will be referred to by their average Rt value. For example, compounds 4.22 (supposedly lycorine), 9.19 and 9.52 were present in all 24 plant samples (Supplementary Table S3). On the other hand, compounds 10.03, 10.66 and 13.66 were present in particular parts throughout the life of the plant: 10.03 and 10.66 in the leaves and flowers, while 13.66 in the roots. This suggests specificity of these three compounds for their respective parts regardless of the developmental stage. Interestingly, compounds 11.66, 12.17, 12.89 and 14.03 were found in only one sample:

stage 6 flowers. These four compounds may be involved in a physiological process exclusive to the stage 6 flowers. What is peculiar, however, is that the six compounds with Rt ranging from 6.43 to 7.20 were present at extremely high concentrations in the leaves of the 2nd developmental stage. The first 3 of these 6 compounds (6.43, 6.60 and 6.73) were also present at high concentrations in the leaves of the 5th developmental stage.

Compound 4.22, supposedly lycorine, has been detected in all plant samples and has a high relative concentration in most samples (Supplementary Table S3). This is in accordance with the findings reported by Shawky *et al.* who found that lycorine was the predominant alkaloid in *N. papyraceus* (Shawky et al., 2015), and with the fact that lycorine is one of the most abundant AAs in the *Narcissus* genus (Torras-Claveria et al., 2014). A reason for this is that lycorine is possibly, phylogenetically, among the oldest AAs in *Narcissus* (Berkov et al., 2014). Furthermore, lycorine is present in higher concentration in the leaf samples compared to the bulb samples (Supplementary Table S3). This is in agreement with the results obtained by Torras-Claveria *et al.* regarding the GC-MS analysis of six ornamental species of *Narcissus* (Torras-Claveria et al., 2014).

The relative concentration of lycorine was determined for each plant sample and is shown in Fig. 5A. It is interesting to note that the fraction of the concentration of lycorine out of the total concentration of heterocyclic compounds in each plant sample remains more or less constant as the plant ages. This suggests a constant production of lycorine, in each part, throughout the growth and development of the plant.

Altogether, the results show different compound profiles for the different parts of *N. papyraceus* with aerial parts such as leaves and flowers having the highest abundance of compounds, whereas the bulbs, the lowest. A similar finding was reported in a recent study of AA biosynthesis in *N. pseudonarcissus* 'King Alfred' in that leaves had the highest concentration of AAs (Singh and Desgagné-Penix, 2017).

Comparison of gene expression and compound content profiles

To better understand the involvement of the proposed AA biosynthetic genes in the production of the AAs at each developmental stage of *N. papyraceus*, we compared the expression profiles (Fig. 3) of the ten AA biosynthetic genes and isoforms common to all Amaryllidaceae alkaloids (i.e. down to and including *N4OMT* (Fig. 1)) with the compound content profiles (Figs. 5B and 5C). Some of the noteworthy observations are described below.

At the 2nd stage, relative to the other stages, *PAL2*, *C4H*, *C3H*, *TYDC1*, *TYDC2* and *N4OMT* all exhibit very high expression in bulbs, considerably lower in roots, and low in leaves. Therefore, at this stage, during which the plant begins its growth from the 1st stage bulb, the expression of most of the genes remains high in the bulbs compared to the roots and leaves. According to this, a great amount of alkaloids would be expected to be produced in the bulbs and least in the leaves. However, the opposite is observed in Figs. 5B and 5C where the bulbs have the lowest amount of compounds and the leaves have the highest. A plausible explanation for this may be that the biosynthetic enzymes and/or alkaloids produced in the bulb are being transported to other parts of the plant, leading to a decrease in the amount of alkaloids in the bulb and higher amounts in the roots and leaves. Such transport has been previously described in the opium poppy (Bird et al., 2003, Ziegler and Facchini, 2008, Onoyovwe et al., 2013).

At the 3rd developmental stage, the greater amount of compounds being accumulated in the leaves and flowers is in accordance with the high or very high expression, in these parts, of several genes such as *PAL1*, *4CL*, *HCT1* and *N4OMT*. The surprisingly high content of compounds in the stems, where almost all genes are expressed at very low levels, points, once again, to a possible transport of enzymes and/or alkaloids from other parts of the plant to the stem.

The analysis of the levels of expression of various genes at stage 4 shows that *PAL2* is highest in the flowers, *C4H* is high in the flowers, *4CL* is higher in all aerial parts, *HCT1* is highest in the leaves and stems, and *TYDC2* is highest in the leaves. This is consistent

with the high accumulation of compounds in the aerial parts. It should further be noted that at this stage, with the advent of the blossoming of flowers, compounds begin to increase in the underground extremity, i.e. roots, of the plant. The roots at this stage have the highest concentration of compounds (Fig. 5C) specifically due to compound 4.22, assumed to be lycorine (Supplementary Table S3). The high concentration of lycorine in the stage 4 roots may be due to high expression levels of many other, yet unknown, genes implicated further downstream in the pathway, more precisely, in the *ortho-para'* phenol coupling subgroup which is specific to lycorine synthesis (Fig. 1).

To summarise, as the plant grows, it has a tendency to store the compounds in certain parts rather than others regardless of the level of gene expression. This is probably due, as mentioned for the 2nd and 3rd stages, to a transport of enzymes and AAs within the plant. When the plant is young, i.e. at stages 2 and 3, it is clear there is a greater accumulation of compounds in all aerial parts (leaves, stems and un-blossomed flowers) (Figs. 5B and 5C). At stage 4, the leaves and flowers continue to accumulate large amounts of compounds but a decrease occurs in the stems, whereas in the roots the amount of compounds begins to increase. The presence of a high amount of compounds in the aerial parts is in line with the theory that alkaloids act as defense molecules for a plant (Mahajan et al., 2010) as the aerial parts, besides being vulnerable to various pathogens, are also visible and easily accessible to herbivores. The reason the roots also possess a high amount of compounds is that they can always be attacked by pathogens present in the soil (Facchini and De Luca, 1995). However, the decrease of compounds in the stems at stage 4, and the much lesser content of compounds in the roots during stages 2-3, is rather puzzling.

Acknowledgements

We thank Professor Hugo Germain at Université du Québec à Trois-Rivières (UQTR) for his suggestions and access to his laboratory equipment. We also thank Professor Céline Van Themsche (UQTR) for access to her laboratory equipment. Doctoral students Aparna Singh and Geneviève Laperrière are thanked for their help in the experiments.

References

Barton, D., Cohen, T. 1957. Some biogenetic aspects of phenol oxidation. *Festschrift Arthur Stoll*, 117-143.

Barton, D., Kirby, G. 1962. 153. Phenol oxidation and biosynthesis. Part V. The synthesis of galanthamine. *Journal of the Chemical Society (Resumed)*, 806-817.

Barton, D., Kirby, G., Taylor, J., Thomas, G. 1963. 866. Phenol oxidation and biosynthesis. Part VI. The biogenesis of amaryllidaceae alkaloids. *Journal of the Chemical Society (Resumed)*, 4545-4558.

Bastida, J., Berkov, S., Torras, L., Pigni, N. B., de Andrade, J. P., Martínez, V., Codina, C., Viladomat, F. 2011. Chemical and biological aspects of Amaryllidaceae alkaloids. *Recent Advances in Pharmaceutical Sciences,* 2011, 65-100.

Berkov, S., Martínez-Francés, V., Bastida, J., Codina, C., Ríos, S. 2014. Evolution of alkaloid biosynthesis in the genus Narcissus. *Phytochemistry,* 99, 95-106.

Bhandarkar, J., Kirby, G. 1970. Structure and biosynthesis of chlidanthine. *Journal of the Chemical Society C: Organic*, 1224-1227.

Bird, D. A., Franceschi, V. R., Facchini, P. J. 2003. A tale of three cell types: alkaloid biosynthesis is localized to sieve elements in opium poppy. *The Plant Cell,* 15, 2626-2635.

Bohlin, L., Göransson, U., Alsmark, C., Wedén, C., Backlund, A. 2010. Natural products in modern life science. *Phytochemistry Reviews,* 9, 279-301.

Bolger, A. M., Lohse, M., Usadel, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics,* 30, 2114-2120.

Brown, C. T., Howe, A., Zhang, Q., Pyrkosz, A. B., Brom, T. H. 2012. A reference-free algorithm for computational normalization of shotgun sequencing data. *arXiv preprint arXiv:1203.4802*.

Cedrón, J. C., Gutiérrez, D., Flores, N., Ravelo, Á. G., Estévez-Braun, A. 2012. Synthesis and antimalarial activity of new haemanthamine-type derivatives. *Bioorganic & medicinal chemistry,* 20, 5464-5472.

Chang, A., Lim, M.-H., Lee, S.-W., Robb, E. J., Nazar, R. N. 2008. Tomato phenylalanine ammonia-lyase gene family, highly redundant but strongly underutilized. *Journal of biological chemistry,* 283, 33591-33601.

Desgagné-Penix, I., Facchini, P. J. 2012. Systematic silencing of benzylisoquinoline alkaloid biosynthetic genes reveals the major route to papaverine in opium poppy. *The Plant Journal,* 72, 331-344.

Diamond, A., Desgagné-Penix, I. 2015. Metabolic engineering for the production of plant isoquinoline alkaloids. *Plant biotechnology journal.*

Eichhorn, J., Takada, T., Kita, Y., Zenk, M. H. 1998. Biosynthesis of the Amaryllidaceae alkaloid galanthamine. *Phytochemistry,* 49, 1037-1047.

El Tahchy, A., Ptak, A., Boisbrun, M., Barre, E., Guillou, C., Dupire, F. o., Chrétien, F. o., Henry, M., Chapleur, Y., Laurain-Mattar, D. 2011. Kinetic study of the rearrangement of deuterium-labeled 4′-O-methylnorbelladine in Leucojum aestivum shoot cultures by mass spectrometry. Influence of precursor feeding on Amaryllidaceae alkaloid accumulation. *Journal of natural products,* 74, 2356-2361.

Evidente, A., Andolfi, A., Abou-Donia, A. H., Touema, S. M., Hammoda, H. M., Shawky, E., Motta, A. 2004. (-)-Amarbellisine, a lycorine-type alkaloid from Amaryllis belladonna L. growing in Egypt. *Phytochemistry,* 65, 2113-8.

Evidente, A., Kornienko, A. 2009. Anticancer evaluation of structurally diverse Amaryllidaceae alkaloids and their synthetic derivatives. *Phytochemistry Reviews,* 8, 449-459.

Facchini, P. J., De Luca, V. 1994. Differential and tissue-specific expression of a gene family for tyrosine/dopa decarboxylase in opium poppy. *Journal of Biological Chemistry,* 269, 26684-26690.

Facchini, P. J., De Luca, V. 1995. Phloem-specific expression of tyrosine/dopa decarboxylase genes and the biosynthesis of isoquinoline alkaloids in opium poppy. *The Plant Cell,* 7, 1811-1821.

Facchini, P. J., Huber-Allanach, K. L., Tari, L. W. 2000. Plant aromatic L-amino acid decarboxylases: evolution, biochemistry, regulation, and metabolic engineering applications. *Phytochemistry,* 54, 121-38.

Fock-Bastide, I., Palama, T. L., Bory, S., Lécolier, A., Noirot, M., Joët, T. 2014. Expression profiles of key phenylpropanoid genes during Vanilla planifolia pod development reveal a positive correlation between PAL gene expression and vanillin biosynthesis. *Plant physiology and biochemistry,* 74, 304-314.

Fraser, C. M., Chapple, C. 2011. The phenylpropanoid pathway in Arabidopsis. *The Arabidopsis Book*, e0152.

Gallagher, S. R., Desjardins, P. R. 2007. Quantitation of DNA and RNA with absorption and fluorescence spectroscopy. *Current Protocols in Human Genetics*, A. 3D. 1-A. 3D. 21.

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature biotechnology*, 29, 644.

Haas, B. J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P. D., Bowden, J., Couger, M. B., Eccles, D., Li, B., Lieber, M. 2013. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nature protocols,* 8, 1494.

Hagel, J. M., Facchini, P. J. 2005. Elevated tyrosine decarboxylase and tyramine hydroxycinnamoyltransferase levels increase wound-induced tyramine-derived hydroxycinnamic acid amide accumulation in transgenic tobacco leaves. *Planta,* 221, 904-914.

Hamberger, B., Ellis, M., Friedmann, M., de Azevedo Souza, C., Barbazuk, B., Douglas, C. J. 2007. Genome-wide analyses of phenylpropanoid-related genes in Populus trichocarpa, Arabidopsis thaliana, and Oryza sativa: the Populus lignin toolbox and conservation and diversification of angiosperm gene families. *Botany,* 85, 1182-1201.

Hayden, R. E., Pratt, G., Drayson, M. T., Bunce, C. M. 2010. Lycorine sensitizes CD40 ligand-protected chronic lymphocytic leukemia cells to bezafibrate-and medroxyprogesterone acetate-induced apoptosis but dasatanib does not overcome reported CD40-mediated drug resistance. *haematologica,* 95, 1889-1896.

Heinrich, M., Teoh, H. L. 2004. Galanthamine from snowdrop-the development of a modern drug against Alzheimer's disease from local Caucasian knowledge. *Journal of ethnopharmacology,* 92, 147-162.

Hoffmann, L., Besseau, S., Geoffroy, P., Ritzenthaler, C., Meyer, D., Lapierre, C., Pollet, B., Legrand, M. 2004. Silencing of hydroxycinnamoyl-coenzyme A shikimate/quinate hydroxycinnamoyltransferase affects phenylpropanoid biosynthesis. *The Plant Cell,* 16, 1446-1465.

Hotchandani, T., Desgagné-Penix, I. 2016. Heterocyclic Amaryllidaceae Alkaloids: Biosynthesis and Pharmacological Applications. *Curr Top Med Chem.*

Huang, J., Gu, M., Lai, Z., Fan, B., Shi, K., Zhou, Y.-H., Yu, J.-Q., Chen, Z. 2010. Functional analysis of the Arabidopsis PAL gene family in plant growth, development, and response to environmental stress. *Plant Physiology,* 153, 1526-1538.

Jiang, Y., Xia, B., Liang, L., Li, X., Xu, S., Peng, F., Wang, R. 2013. Molecular and analysis of a phenylalanine ammonia-lyase gene (LrPAL2) from Lycoris radiata. *Molecular biology reports,* 40, 2293-2300.

Jiang, Y., Xia, N., Li, X., Shen, W., Liang, L., Wang, C., Wang, R., Peng, F., Xia, B. 2011. Molecular cloning and characterization of a phenylalanine ammonia-lyase gene (LrPAL) from Lycoris radiata. *Molecular biology reports,* 38, 1935-1940.

Jin, Z., Xu, X.-H. 2013. Amaryllidaceae Alkaloids. *In:* Ramawat, K. G. & Mérillon, J.-M. (eds.) *Natural Products: Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes.* Berlin, Heidelberg: Springer Berlin Heidelberg.

Kao, Y.-Y., Harding, S. A., Tsai, C.-J. 2002. Differential expression of two distinct phenylalanine ammonia-lyase genes in condensed tannin-accumulating and lignifying cells of quaking aspen. *Plant physiology,* 130, 796-807.

Kawalleck, P., Keller, H., Hahlbrock, K., Scheel, D., Somssich, I. 1993. A pathogen-responsive gene of parsley encodes tyrosine decarboxylase. *Journal of Biological Chemistry,* 268, 2189-2194.

Kaya, G. I., Sarıkaya, B., Onur, M. A., Somer, N. U., Viladomat, F., Codina, C., Bastida, J., Lauinger, I. L., Kaiser, M., Tasdemir, D. 2011. Antiprotozoal alkaloids from Galanthus trojanus. *Phytochemistry Letters,* 4, 301-305.

Keller, C., Kadir, A., Forsberg, A., Porras, O., Nordberg, A. 2011. Long-term effects of galantamine treatment on brain functional activities as measured by PET in Alzheimer's disease patients. *Journal of Alzheimer's Disease,* 24, 109-123.

Kilgore, M. B., Augustin, M. M., May, G. D., Crow, J. A., Kutchan, T. M. 2016a. CYP96T1 of Narcissus sp. aff. pseudonarcissus Catalyzes Formation of the Para-Para'CC Phenol Couple in the Amaryllidaceae Alkaloids. *Frontiers in plant science,* 7.

Kilgore, M. B., Augustin, M. M., Starks, C. M., O'Neil-Johnson, M., May, G. D., Crow, J. A., Kutchan, T. M. 2014. Cloning and characterization of a norbelladine 4'-O-methyltransferase involved in the biosynthesis of the Alzheimer's drug galanthamine in Narcissus sp. aff. pseudonarcissus. *PloS one,* 9, e103223.

Kilgore, M. B., Holland, C. K., Jez, J. M., Kutchan, T. M. 2016b. Identification of a Noroxomaritidine Reductase with Amaryllidaceae Alkaloid Biosynthesis Related Activities. *Journal of Biological Chemistry*, jbc. M116. 717827.

Kreh, M. 2002. *Studies on galanthamine extraction from Narcissus and other Amaryllidaceae*, Taylor & Francis: London, UK.

Lamoral-Theys, D., Andolfi, A., Van Goietsenoven, G., Cimmino, A., Le Calvé, B., Wauthoz, N., Mégalizzi, V., Gras, T., Bruyère, C., Dubois, J. 2009. Lycorine, the main phenanthridine Amaryllidaceae alkaloid, exhibits significant antitumor activity in cancer cells that display resistance to proapoptotic stimuli: an investigation of structure-activity relationship and mechanistic insight. *Journal of medicinal chemistry,* 52, 6244-6256.

Langmead, B., Trapnell, C., Pop, M., Salzberg, S. L. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome biology,* 10, R25.

Lefranc, F., Sauvage, S., Van Goietsenoven, G., Mégalizzi, V., Lamoral-Theys, D., Debeir, O., Spiegl-Kreinecker, S., Berger, W., Mathieu, V., Decaestecker, C. 2009. Narciclasine, a plant growth modulator, activates Rho and stress fibers in glioblastoma cells. *Molecular cancer therapeutics,* 8, 1739-1750.

Lepelley, M., Cheminade, G., Tremillon, N., Simkin, A., Caillet, V., McCarthy, J. 2007. Chlorogenic acid synthesis in coffee: An analysis of CGA content and real-time RT-PCR expression of HCT, HQT, C3H1, and CCoAOMT1 genes during grain development in C. canephora. *Plant Science,* 172, 978-996.

Li, B., Dewey, C. N. 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC bioinformatics,* 12, 323.

Livak, K. J., Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *methods,* 25, 402-408.

Mahajan, M., Kumar, V., Yadav, S. K. 2010. Alkaloids: Properties, Application and Pharmacological Effects. *In:* CASSIANO, N. M. (ed.) *Alkaloids: Properties, Applications and Pharmacological Effects.* Nova Science Publishers, Inc.

Mascavage, L., Wilson, M., Dalton, D. 2006. Syntheses of Morphine and Codeine (1992-2002): Templates for Exploration of Synthetic Tools. *Current Organic Synthesis,* 3, 99-120.

Oh, S., Park, S., Han, K.-H. 2003. Transcriptional regulation of secondary growth in Arabidopsis thaliana. *Journal of Experimental Botany,* 54, 2709-2722.

Onoyovwe, A., Hagel, J. M., Chen, X., Khan, M. F., Schriemer, D. C., Facchini, P. J. 2013. Morphine biosynthesis in opium poppy involves two cell types: sieve elements and laticifers. *The Plant Cell,* 25, 4110-4122.

Phimchan, P., Chanthai, S., Bosland, P. W., Techawongstien, S. 2014. Enzymatic changes in phenylalanine ammonia-lyase, cinnamic-4-hydroxylase, capsaicin synthase, and peroxidase activities in Capsicum under drought stress. *Journal of agricultural and food chemistry,* 62, 7057-7062.

Raes, J., Rohde, A., Christensen, J. H., Van de Peer, Y., Boerjan, W. 2003. Genome-wide characterization of the lignification toolbox in Arabidopsis. *Plant Physiology,* 133, 1051-1071.

Rohde, A., Morreel, K., Ralph, J., Goeminne, G., Hostyn, V., De Rycke, R., Kushnir, S., Van Doorsselaere, J., Joseleau, J.-P., Vuylsteke, M. 2004. Molecular phenotyping of the pal1 and pal2 mutants of Arabidopsis thaliana reveals far-reaching consequences on phenylpropanoid, amino acid, and carbohydrate metabolism. *The Plant Cell,* 16, 2749-2771.

Saliba, S., Ptak, A., Laurain- Mattar, D. 2015. 4'- O- Methylnorbelladine feeding enhances galanthamine and lycorine production by Leucojum aestivum L. shoot cultures. *Engineering in Life Sciences,* 15, 640-645.

Şener, B., Orhan, I., Satayavivad, J. 2003. Antimalarial activity screening of some alkaloids and the plant extracts from Amaryllidaceae. *Phytotherapy Research,* 17, 1220-1223.

Shawky, E., Abou- Donia, A. H., Darwish, F. A., Toaima, S. M., Takla, S. S., Pigni, N. B., Bastida, J. 2015. HPTLC and GC/MS Study of Amaryllidaceae Alkaloids of Two Narcissus Species. *Chemistry & biodiversity,* 12, 1184-1199.

Singh, A., Desgagné-Penix, I. 2014. Biosynthesis of the Amaryllidaceae alkaloids. *Plant Science Today,* 1, 114-120.

Singh, A., Desgagné-Penix, I. 2017. Transcriptome and metabolome profiling of Narcissus pseudonarcissus 'King Alfred'reveal components of Amaryllidaceae alkaloid metabolism. *Scientific reports,* 7, 17356.

Singh, K., Kumar, S., Rani, A., Gulati, A., Ahuja, P. S. 2009. Phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H) and catechins (flavan-3-ols) accumulation in tea. *Functional & integrative genomics,* 9, 125.

Tahchy, A. E., Boisbrun, M., Ptak, A., Dupire, F., Chrétien, F., Henry, M., Chapleur, Y., Laurain-Mattar, D. 2010. New method for the study of Amaryllidaceae alkaloid biosynthesis using biotransformation of deuterium-labeled precursor in tissue cultures. *Acta Biochimica Polonica,* 57, 75.

Takos, A., Rook, F. 2013. Towards a Molecular Understanding of the Biosynthesis of Amaryllidaceae Alkaloids in Support of Their Expanding Medical Use. *International Journal of Molecular Sciences,* 14, 11713.

Torras-Claveria, L., Berkov, S., Codina, C., Viladomat, F., Bastida, J. 2014. Metabolomic analysis of bioactive Amaryllidaceae alkaloids of ornamental varieties of Narcissus by GC–MS combined with k-means cluster analysis. *Industrial Crops and Products,* 56, 211-222.

Viladomat, F., Llabrés, J. M., Bastida, J., Cusidó, R. M., Codina, C. 1986. Ontogenic variations in the alkaloids of Narcissus assoanus. *Physiologia Plantarum,* 68, 657-661.

Vogt, T. 2010. Phenylpropanoid biosynthesis. *Molecular plant,* 3, 2-20.

Zhang, X., Liu, C.-J. 2015. Multifaceted regulations of gateway enzyme phenylalanine ammonia-lyase in the biosynthesis of phenylpropanoids. *Molecular plant,* 8, 17-27.

Ziegler, J., Facchini, P. J. 2008. Alkaloid biosynthesis: metabolism and trafficking. *Annu. Rev. Plant Biol.,* 59, 735-769.

Tables

Table 1. Summary of the transcriptome database generated from one lane of Illumina sequencing of *N. papyraceus* stage 1 bulbs.

	N. papyraceus
Total number of raw paired reads[a]	70 409 091
Total number of surviving paired reads trimmed[b]	64 038 268
Total number of surviving paired reads normalized[c]	8 945 044
Number of transcripts[d]	148 563
Number of components[e]	86 994
Number of annotated transcripts[f]	8 866

[a] Number of Paired Reads obtained from the sequencer.
[b] Number of Remaining Paired Reads after the trimming step.
[c] Number of Remaining Paired Reads after the normalization step.
[d] Number of transcripts: Trinity has created a list of transcripts representing the transcriptome isoforms.
[e] The transcripts are grouped in components loosely representing genes. Transcript names are prefixed by the component/gene name e.g. transcripts c115_g5_i1 and c115_g5_i2 are derived from the same isolated de Bruijn graph and therefore share the same component/gene number c115_g5.
[f] Each transcript has been aligned against the *uniprot_sprot.trinotate_v2.0.pep* protein database using the blastx program from the NCBI BLAST family.

Table 2. Summary of the biosynthetic genes identified from the *N. papyraceus* stage 1 bulb transcriptome known to be involved in Amaryllidaceae alkaloid biosynthesis.

Name	Reads (FPKM)	Length (bp)	ORF (bp)	Top annotation	Species	Expect value	Accession number
TYDC1	1881	1641	1536	tyrosine/DOPA decarboxylase 2	*Papaver somniferum*	0	P54769.1
TYDC2	1111	2004	1356	tyrosine decarboxylase 1	*Oryza sativa*	0	Q7XHL3.1
PAL1	1911	2277	2130	phenylalanine ammonia-lyase 3	*Petroselinum crispum*	0	P45729.1
PAL2	1790	2395	2136	phenylalanine ammonia-lyase 1	*Prunus avium*	0	O64963.1
PAL3	1790	2395	2136	phenylalanine ammonia-lyase 1	*Prunus avium*	0	O64963.1
C4H1	3309	1792	1518	cinnamic acid 4-hydroxylase	*Zinnia violacea*	0	Q43240.1
C4H2	3309	1743	1518	cinnamic acid 4-hydroxylase	*Zinnia violacea*	0	Q43240.1
C3H	2018	1830	1530	*p*-coumarate 3-hydroxylase	*Arabidopsis thaliana*	0	O22203.1
4CL1	1645	1846	1710	4-coumarate:CoA ligase 2	*Oryza sativa*	0	Q42982.2
4CL2	1173	1994	1683	4-coumarate:CoA ligase-like 7	*Oryza sativa*	0	Q69RG7.1
4CL3	1173	1834	1614	4-coumarate:CoA ligase-like 7	*Arabidopsis thaliana*	0	Q9M0X9.1
4CL4	1173	2023	1653	4-coumarate:CoA ligase-like 4	*Oryza sativa*	0	Q10S72.1
HCT1	2572	1633	1311	hydroxycinnamoyltransferase 1	*Oryza sativa*	0	Q0JBZ8.1
HCT2	2572	1807	1416	hydroxycinnamoyltransferase 2	*Nicotiana tabacum*	2e-96	Q8GSM7.1
HCT3	2572	1612	1308	hydroxycinnamoyltransferase 3	*Oryza sativa*	1e-105	Q5SMM8.1
N4OMT	21722	1062	720	norbelladine 4'-*O*-methyltransferase	*Narcissus aff. pseudonarcissus MK-2014*	2e-171	A0A077EWA5.1
CYP96T1	16956	1866	1605	noroxomaritidine synthase (CYP96T1)	*Narcissus aff. pseudonarcissus MK-2014*	0	A0A140IL90.1
CYP96T2	16956	1839	1605	noroxomaritidine synthase (CYP96T1)	*Narcissus aff. pseudonarcissus MK-2014*	0	A0A140IL90.1
NorRed1	6876	1049	810	noroxomaritidine/norcraugsodine reductase	*Narcissus aff. pseudonarcissus MK-2014*	2e-169	AOP04255.1
NorRed2	6876	1049	780	noroxomaritidine/norcraugsodine reductase	*Narcissus aff. pseudonarcissus MK-2014*	1e-164	AOP04255.1
NorRed3	6876	1049	768	noroxomaritidine/norcraugsodine reductase	*Narcissus aff. pseudonarcissus MK-2014*	3e-173	AOP04255.1
HIS	9658	437	410	histone H3.3	*Arabidopsis thaliana*	3e-95	P59169.2

Table 3. Growth details of *N. papyraceus* plants measured by the number and length of their leaves.

	Developmental stage					
	1	**2**	**3**	**4**	**5**	**6**
Days	0	44.5 ± 5.0	58.5 ± 5.0	72.5 ± 3.5	74.5 ± 6.4	79.0 ± 8.5
Leaf count	-	7.5 ± 2.6	8.6 ± 4.9	6.0 ± 1.6	8.3 ± 1.0	8.5 ± 3.8
Shortest leaf (cm)	-	4.1 ± 1.0	10.7 ± 2.8	21.7 ± 8.5	12.4 ± 7.9	26.5 ± 7.7
Longest leaf (cm)	-	14.3 ± 5.7	40.5 ± 4.0	44.6 ± 2.4	39.2 ± 2.9	42.4 ± 6.5

Figure legends

Fig. 1. Proposed AA biosynthetic pathway. Abbreviations of enzymes for which corresponding genes have been isolated from Amaryllidaceae plants are written in bold-black font, whereas from those isolated from other plants are written in bold-grey font. Reactions in the grey-shaded area represent an alternative route for caffeic acid synthesis. Broken arrows represent more than one biochemical reaction. Abbreviations: PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase (CYP73A1); 4CL, 4-hydroxycinnamoyl CoA ligase; HCT, hydroxycinnamoyl transferase; C3H, coumarate 3-hydroxylase (CYP98A3); TYDC, tyramine decarboxylase; N4OMT, norbelladine 4'-*O*-methyltransferase; NR, noroxomaritidine reductase; Sh/Q, shikimate/quinate.

Fig. 2. Photos showing the developmental stages of *Narcissus papyraceus* used in this study. (a) Stage 1: unplanted bulb; (b-c) stage 2: emergence with presence of young leaves; (d-e) stage 3: presence of stems with unopened flower buds; (f-g) stage 4: blossoming with emergence of young flowers; (h-i) stage 5: flowering with mature flowers; and (j-k) stage 6: senescence with wilting flowers.

Fig. 3. Expression profiles of genes involved in Amaryllidaceae alkaloid biosynthesis obtained by quantitative real-time PCR analysis in different parts (bulbs, roots, leaves, stems and flowers) at six different developmental stages of *Narcissus papyraceus*. The graphs are plotted using normalized $\Delta\Delta C_T$ values scaled to the lowest. The gene for histone was used as reference gene. The gene expression fold change was calculated using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). The error bars represent the mean standard deviation of three independent replicates and were calculated using the method stated in the CFX Connect™ Real-Time System (Bio-Rad, www.bio-rad.com) manual (2013). Abbreviations: *TYDC*, *tyramine decarboxylase*; *PAL*, *phenylalanine ammonia lyase*; *C4H*, *cinnamate 4-hydroxylase*; *C3H*, *p-coumarate 3-hydroxylase*; *4CL*, *4-hydroxycinnamoyl CoA ligase*; *HCT*, *hydroxycinnamoyl transferase*; *N4OMT*, *norbelladine 4'*-O-*methyltransferase*; *CYP96T*, *cytochrome P450 monooxygenase 96T1* and *NorRed*, *noroxomaritidine reductase*. Developmental stages are detailed in Fig. 2.

Fig. 4. Representative HPLC chromatograms. A) Alkaloid standards lycorine (LYC), galanthamine (GAL), narciclasine (NAR) and papaverine (PAP). B-C) Compounds extracted from *N. papyraceus* stage 1 bulbs and stage 5 leaves, respectively.

Fig. 5. Accumulation profiles of compounds extracted from different parts (bulbs, roots, leaves, stems and flowers) at six different developmental stages of *Narcissus papyraceus* obtained by HPLC-PDA analysis. A) Relative concentration of lycorine representing the concentration of lycorine out of the total concentration of compounds in each plant sample. B) The number of compounds detected in each plant sample. C) The relative concentration of compounds detected in each plant sample. In panel C, the bars representing samples 2-Leaves and 5-Leaves, which actually reach the very high values of 731 and 85, respectively, are cut-off at the value of 50 making it easier to view and compare the bars representing all the other samples. The histogram in panel C was plotted using absorbance values normalized to the absorbance of papaverine (internal standard).

Figures

Fig. 1

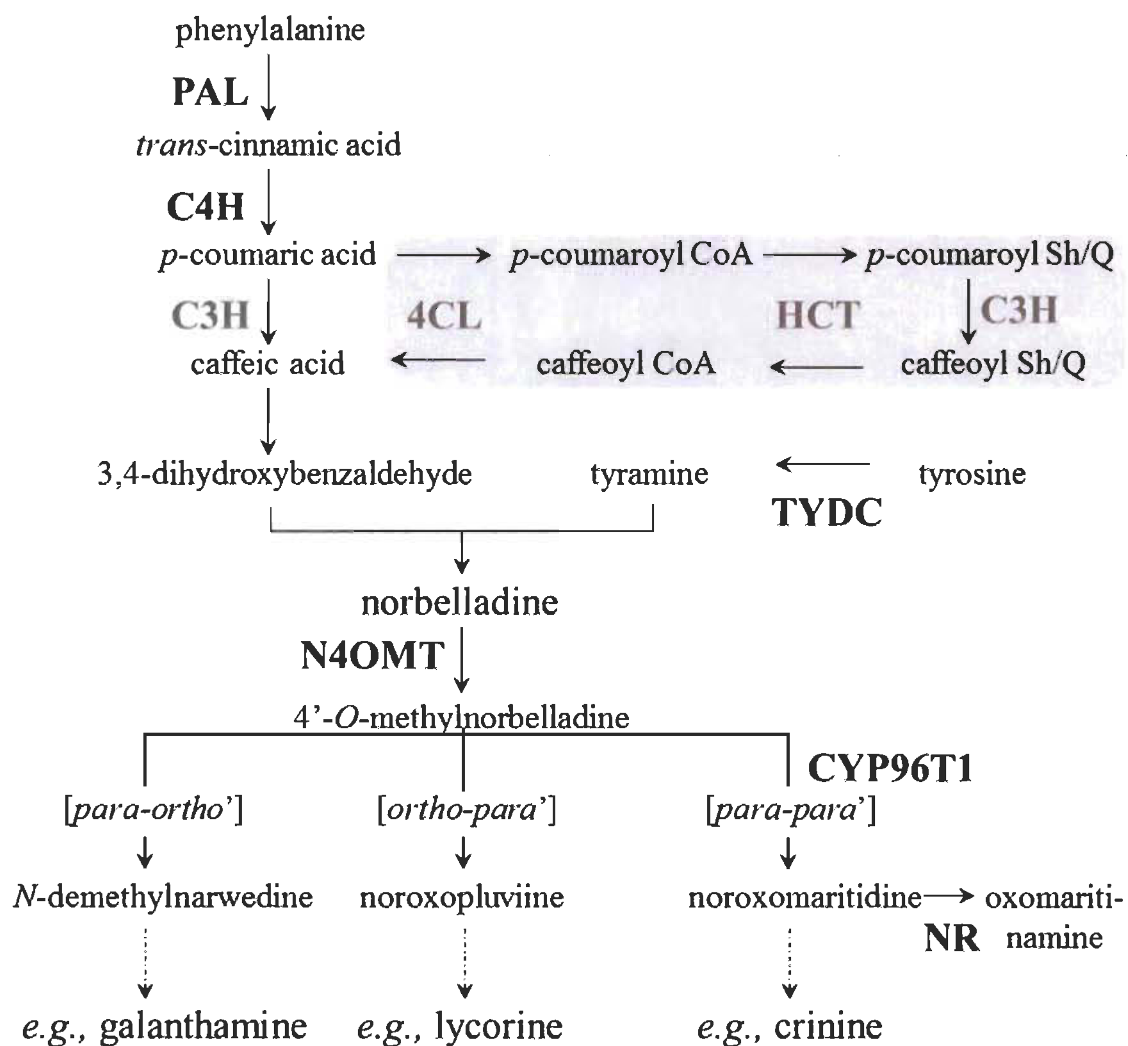


Fig. 2

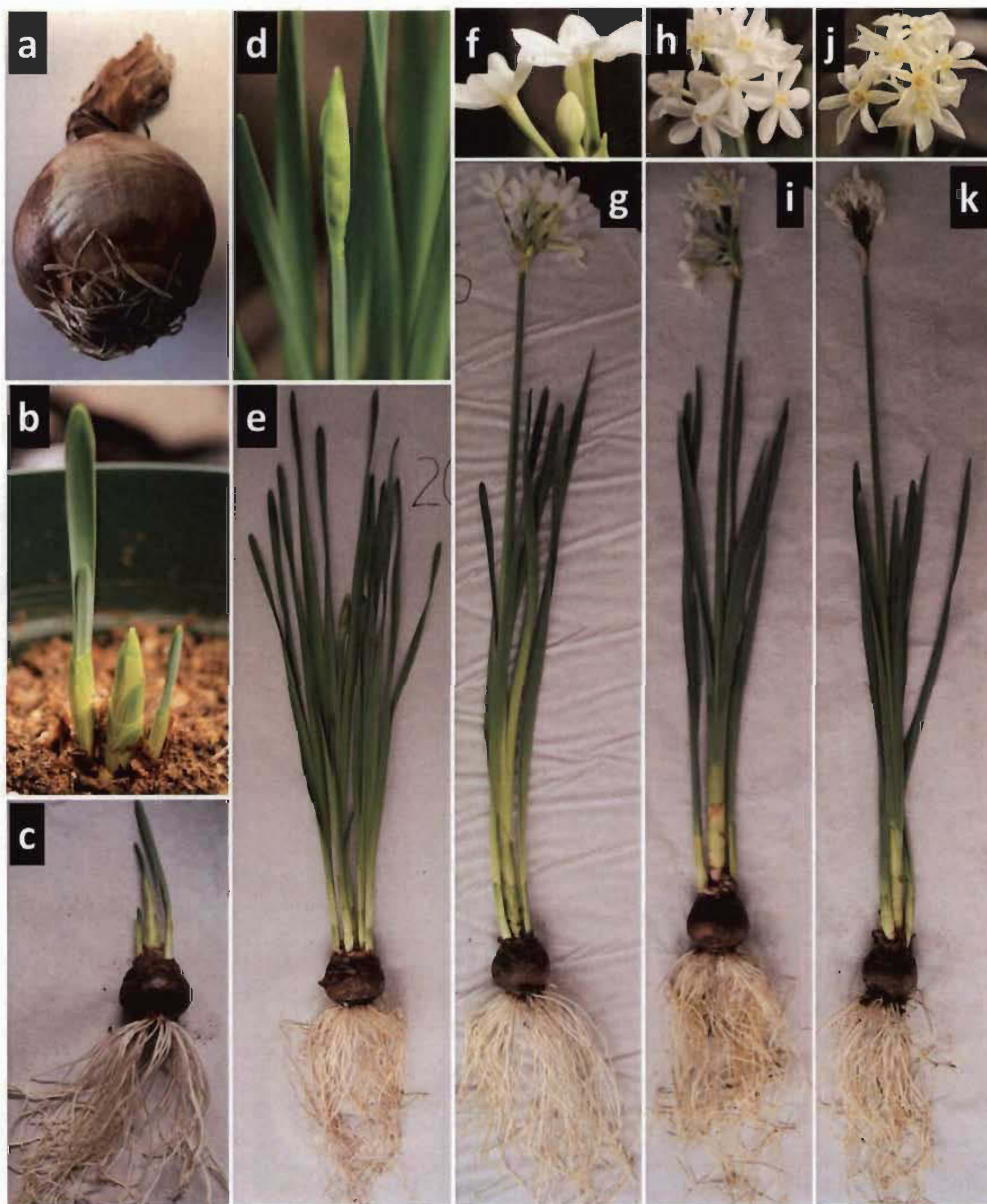


Fig. 3

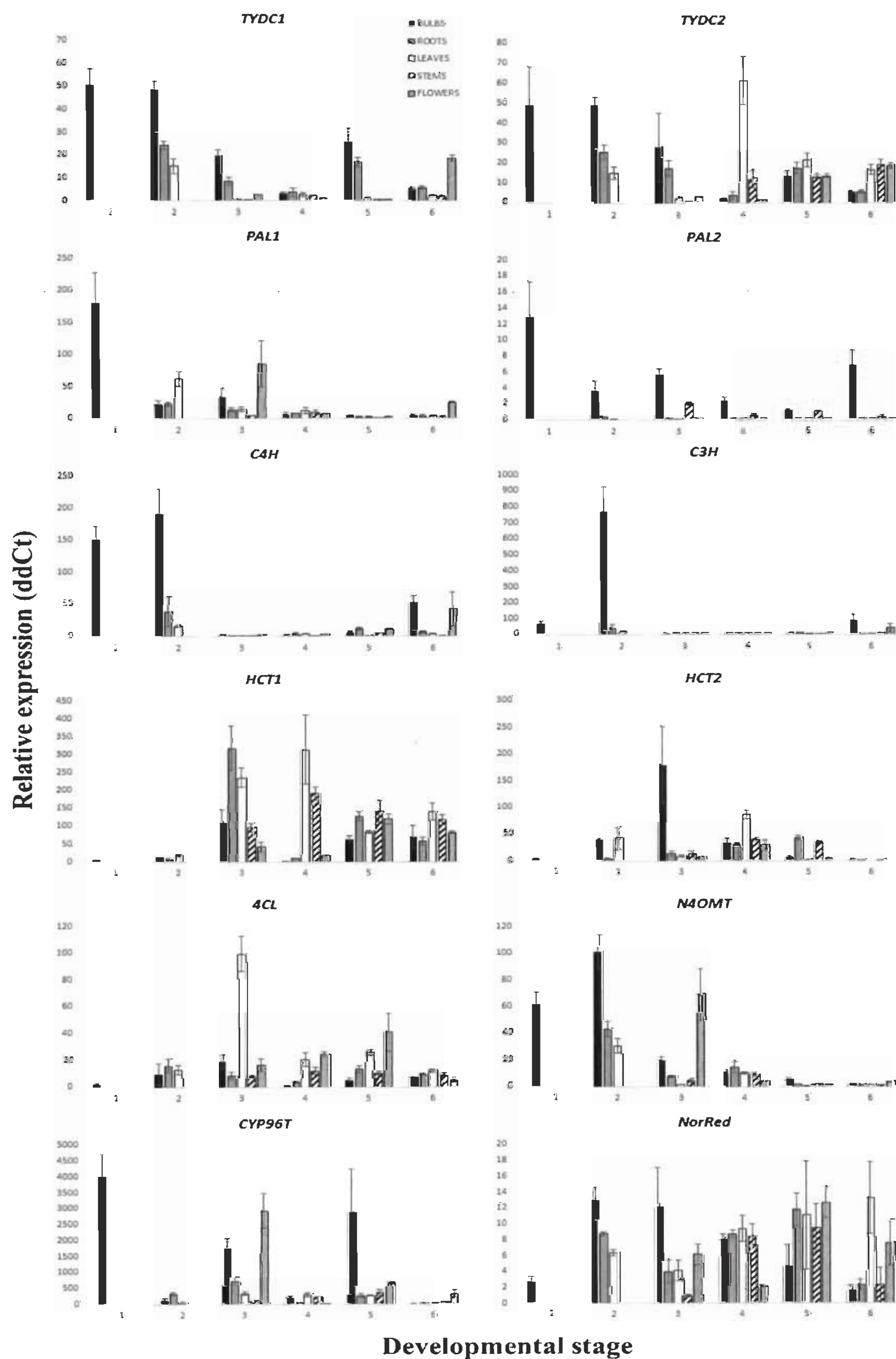


Fig. 4

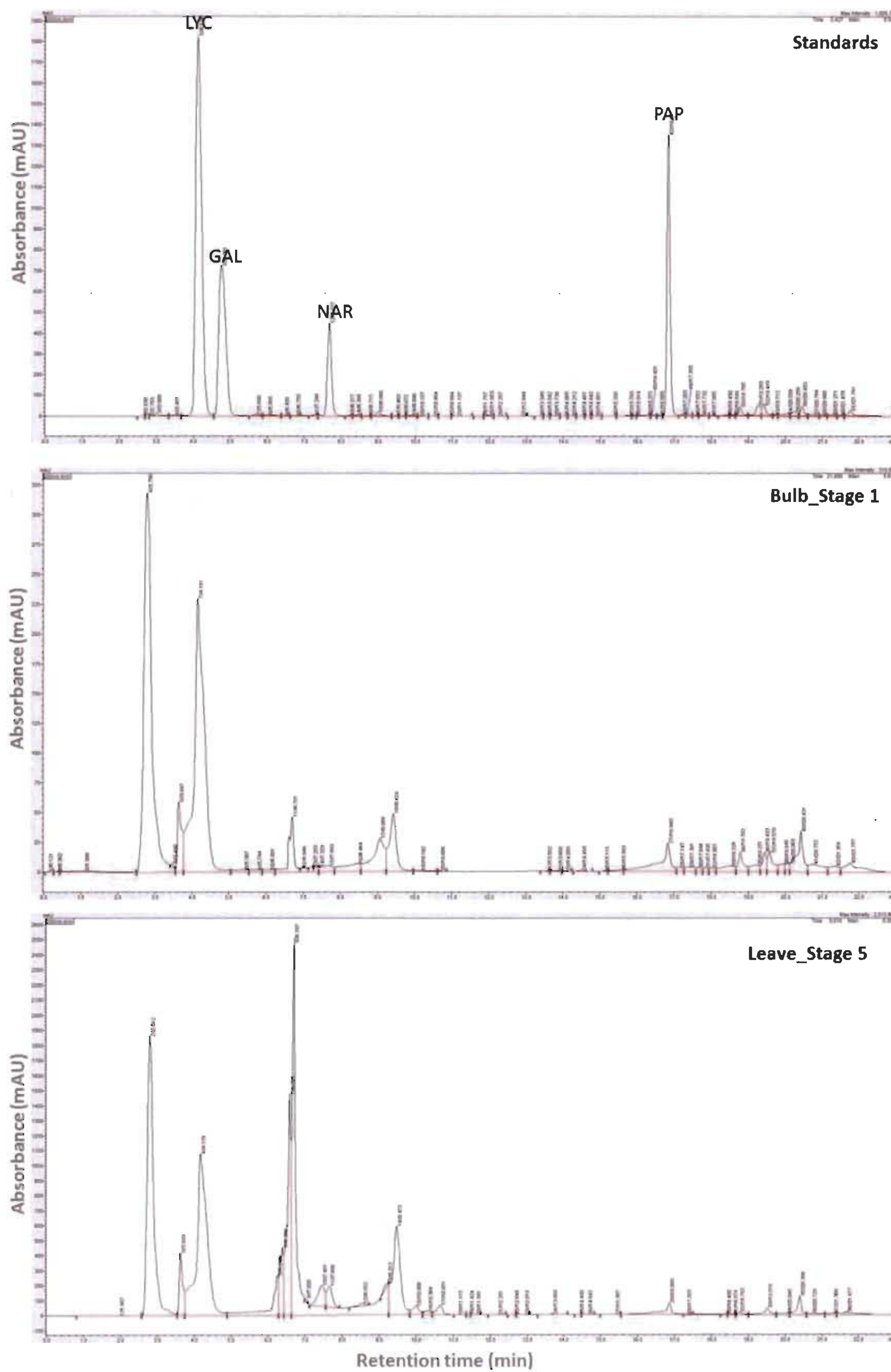


Fig. 5

A

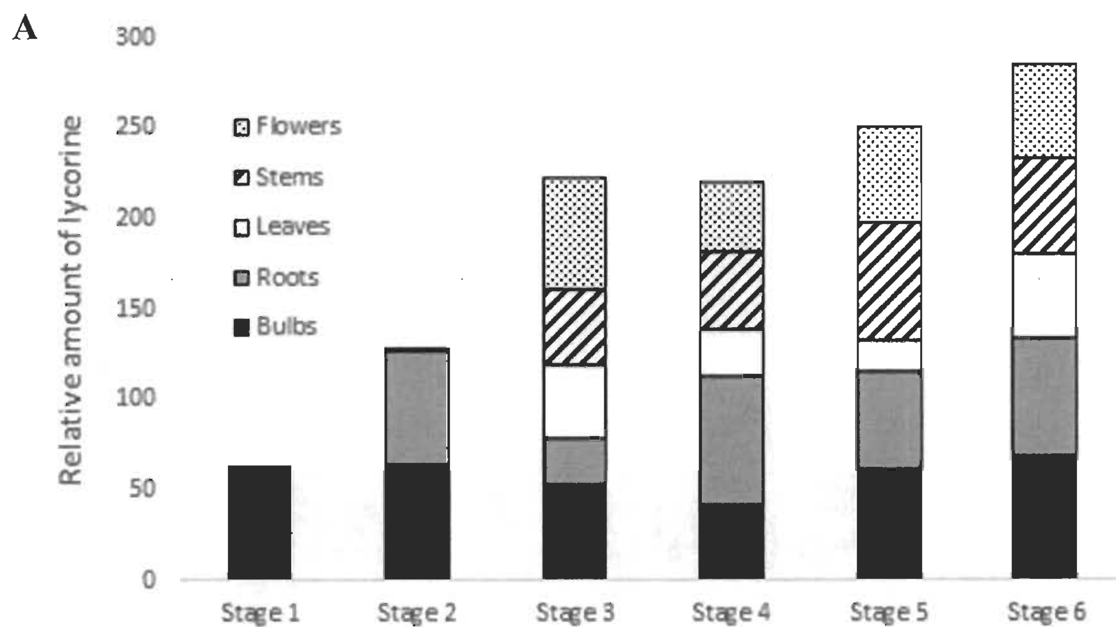
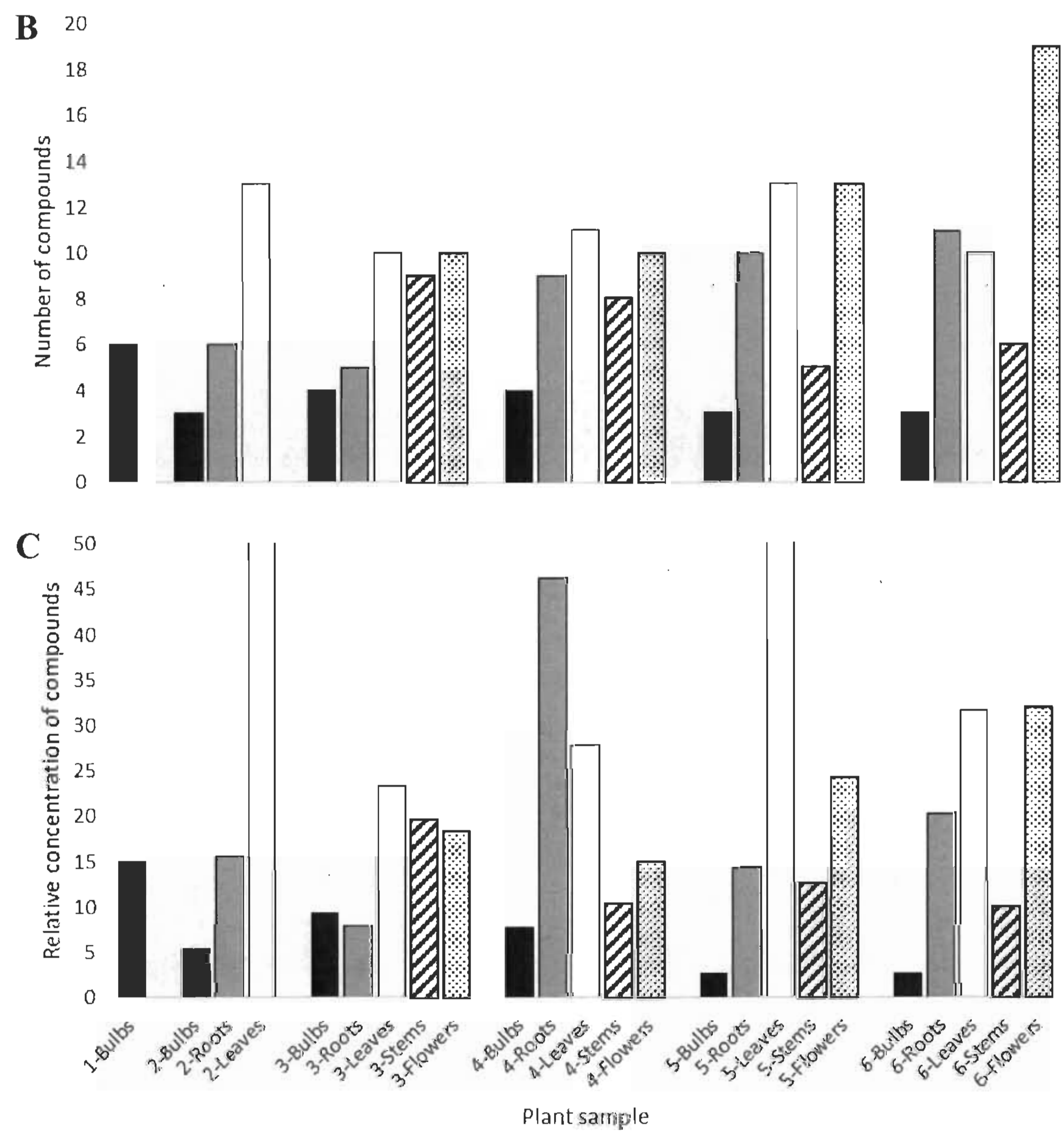


Fig. 5 (continued)



Supplementary data

Supplementary Table S1. List of primers used in, and amplicon lengths pertaining to, the RT-qPCR experiments.

Gene	Forward primers (5′-3′)	Reverse primers (5′-3′)	Amplicon size (bp)
TYDC1	TTGATGTCGGCGGATGAG	TGCTTCTCCTCGGTGAGG	245
TYDC2	GTGCCAGGCGTAACCCATTGG	CTGCCCAGAAGACAGGAATTGG	219
PAL1	GAGGAGAATCTGAAGAGC	CATTAAAGGGTAAGTGC	186
PAL2	GTACCCGTTGATGCAGAAGC	TCCTATTGGCGATTGGTGC	197
C4H	GTATTTGAAGATTTGCAAGC	GCTGCAACATTGATGTTCTC	210
C3H	CAGGTGCTTCGCCGAGTGG	CCTCACCTTCACGTAGTGGG	226
4CL	GGCTATTTGAATGACCCTGAGG	CCGAGCTTTTCGACGGAACTAC	277
HCT	CGACGAGGAGTATGTGAGG	CTCCAACGAAACCAGAACC	197
N4OMT	GTGAAGCTCGTCAGGATTGG	CAGGTTTCTGAAAGAGAGCC	127
CYP96T	TGCTATGGCGAGGATGAAGG	ACATGTCCCTTCACCATCTG	228
NorRed	TCCGGGAGCCATAAGAACGC	GGTGATGTAGGAAGCAGATGG	160
HIS	GTCTGCCCCAACAACTGGAGG	GCTTCCTAATCAGTAGCTCG	113

Supplementary Table S2. Average retention time and absorption maxima for each alkaloid standard obtained by HPLC-PDA analysis.

Standard	Average retention time (min)	λ_{max} (nm)
Lycorine	4.13	237, 285
Galanthamine	4.77	234, 286
Narciclasine	7.70	251, 304
Papaverine	16.85	233, 278, 314, 325

Supplementary Table S3. Relative absorbance values of 28 compounds of interest obtained by HPLC-PDA analysis of 24 *N. papyraceus* alkaloid extract samples. The compounds are listed by their retention time (min) values. For each sample, all absorbance values (mAU) were normalised to that of the internal standard papaverine (compound 16.87) to obtain the relative absorbance values shown. Cells containing values below 0.20 are shaded and were not considered. An empty cell indicates that the compound was not detected in that plant sample. Row A indicates the total number of compounds in each sample. Row B indicates the total concentration of compounds in each sample. Row C indicates the relative concentration of lycorine, i.e. the fraction of the concentration of compound 4.22 (assumed to be lycorine) out of the total concentration of compounds in each sample.

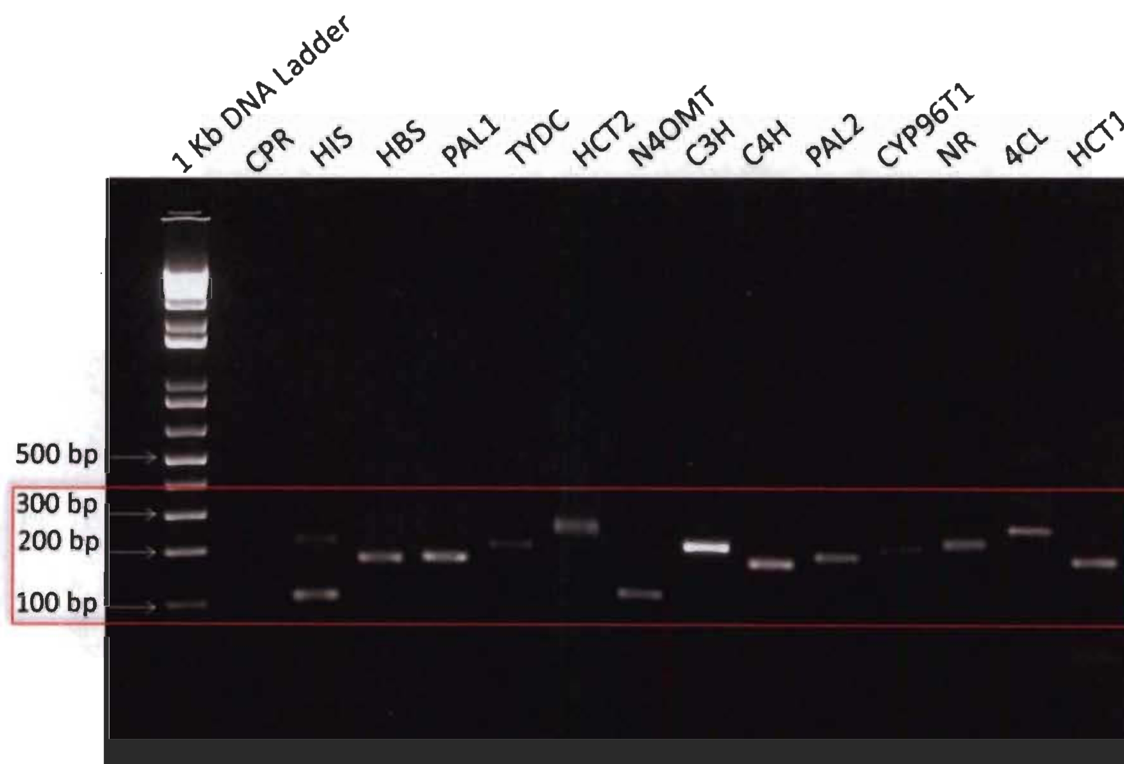
Compound	*N. papyraceus* samples																							
	1-Bulbs	2-Bulbs	2-Roots	2-Leaves	3-Bulbs	3-Roots	3-Leaves	3-Stems	3-Flowers	4-Bulbs	4-Roots	4-Leaves	4-Stems	4-Flowers	5-Bulbs	5-Roots	5-Leaves	5-Stems	5-Flowers	6-Bulbs	6-Roots	6-Leaves	6-Stems	6-Flowers
4.22	9.54645	3.62571	10.0262	15.1074	5.12088	2.24088	9.7118	8.22177	11.748	3.33005	33.6834	7.34482	4.43735	6.03968	1.86321	8.4682	13.8236	8.84955	13.243	1.88465	13.7389	14.8462	5.55926	16.293
5.86	0.08896		0.14438	0.13448		0.05503			0.13502		0.33502			0.27491	0.04677	0.15862		0.16203	0.40908	0.00722	0.22525			0.47064
6.43				140.473					0.00983								5.83154							
6.60				151.408					0.03454								18.9491	0.17433						
6.73	1.9098	0.02429		70.0722			0.56094				0.36662	0.13669		0.38243			32.2128				0.22301	0.685	0.02186	
6.88				123.866											0.04712									
7.01	0.0162			57.8245					0.00507								0.21241							
7.20	0.02681			161.256		0.17588																		
7.47	0.01715	0.07696	0.26465		0.12191		1.99316	0.64168	0.63745	0.09343		1.50761	0.31519	1.10151			1.8114					2.3957	0.39644	
7.69	0.20937		0.29744	0.89358	0.15355		1.72348	0.62491	0.61855		0.57446	1.77883	0.4209	1.52125	0.10941	0.25399	1.82675	0.91303	1.62284	0.0462	0.37974	2.46448	0.61424	1.6749
8.54	0.277	0.15311		0.15287			0.78579	0.58487	0.09577	0.07277		1.14177	0.28423	0.64362	0.09222	0.31115	0.2022	0.26415		0.06339		1.04112	0.20242	
9.19	1.15472	0.61868	1.78641	2.16029	0.97608	1.75099	1.92364	2.56502	0.78203	0.97981	3.92351	3.69688	1.31471	1.62643	0.25392	1.19156	0.25309	0.80934	4.01744	0.25049	1.54593	2.41645	0.79665	5.14181
9.52	2.01815	1.08042	2.5003	4.9581	2.61471	3.2873	4.94	5.01951	1.77862	2.60047	4.83805	8.4884	2.36962	1.76742	0.51383	1.97515	7.59339	1.71632	2.03108	0.44006	2.21338	6.46592	2.3196	2.94835
10.03	0.02896		0.01407	0.25086			0.65184	0.22416	0.25522		0.06308	0.5707	0.09652	0.68123			0.77002	0.14207	0.59338			0.5392	0.06288	0.5073
10.30			0.01044	0.51466			0.28987	0.14956				0.19398	0.06701				0.33284	0.06908				0.24256	0.01875	
10.66	0.00891		0.04176	2.21219	0.12191		0.68696	0.89904	0.29666		0.10683	2.12074	0.56797	0.79805	0.00786	0.16474	0.84778	0.16541	0.66691	0.0058	0.01878	0.45855	0.10256	0.41459
10.88						0.01551					0.07649					0.24811		0.01208	0.16654		0.23031		0.01715	0.28443
11.18						0.00443			0.05925		0.02288					0.15247			0.22786		0.14819	0.01018		0.353
11.66		0.01049	0.00598		0.04913	0.01702	0.06677		0.02005	0.07559	0.01606			0.01943	0.01681	0.13869	0.00494	0.00382	0.11441	0.01737				0.26177
12.17			0.00739			0.0266	0.06003		0.02272					0.02105	0.00546	0.15692	0.01028	0.01008	0.11831			0.01053		0.22277
12.89		0.01049	0.01214	0.01052		0.04312	0.06952	0.11054	0.02301		0.05373			0.02854	0.01013	0.15123	0.01857	0.04428	0.11979	0.00841	0.17242	0.02879	0.06666	0.2452
13.66	0.00912	0.03856	0.6532	0.02988		0.32192	0.11031	0.08282	0.10624	0.10939	1.52048		0.08563	0.1145	0.04555	0.92256	0.03073	0.09733	0.22795	0.02273	0.79693	0.04203	0.10113	0.47525
14.03	0.01081		0.08038											0.04509					0.16612		0.13781	0.01134		0.27097
14.64	0.03037	0.02199	0.07672	0.00544		0.06352	0.07383		0.16895		0.07838			0.05753	0.01942	0.17649	0.02711	0.05452	0.20401	0.01235	0.17444	0.02676	0.04136	0.33636
15.11	0.00613		0.09088			0.19481			0.27764		0.10126			0.1196		0.18938			0.2034		0.19821		0.03716	0.29936
15.37				0.01593		0.15684			0.94153	0.0615	0.14349			0.15015		0.2039	0.02981		0.3745		0.21104			0.56604
15.69	0.04873		0.1763						0.88275		0.30493	0.23024				0.24933			0.36584		0.27562			0.58706
16.46				0.09069	0.62346	0.32138		0.73956		0.76995	0.6335	0.70957	0.62313			0.44313					0.43692			0.50028
16.87	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	1-Bulbs	2-Bulbs	2-Roots	2-Leaves	3-Bulbs	3-Roots	3-Leaves	3-Stems	3-Flowers	4-Bulbs	4-Roots	4-Leaves	4-Stems	4-Flowers	5-Bulbs	5-Roots	5-Leaves	5-Stems	5-Flowers	6-Bulbs	6-Roots	6-Leaves	6-Stems	6-Flowers
A	6	3	6	13	4	5	10	9	10	4	9	10	8	10	3	10	13	5	13	3	11	10	6	19
B	15.1155	5.32481	15.5282	730.997	9.33513	7.92248	23.2675	19.5205	18.2185	7.68028	46.18	27.7835	10.3331	14.8365	2.63096	14.2671	84.667	12.5524	24.1873	2.5752	20.277	31.5552	9.88862	31.8531
C	0.63157	0.68091	0.64568	0.02067	0.54856	0.28285	0.4174	0.42119	0.64484	0.43358	0.72939	0.26436	0.42943	0.40708	0.70818	0.59355	0.16327	0.70501	0.54752	0.73185	0.67756	0.47048	0.56219	0.5115

Supplementary Table S4. Summary of the output of Illumina sequencing of the *N. papyraceus* stage 1 bulb.

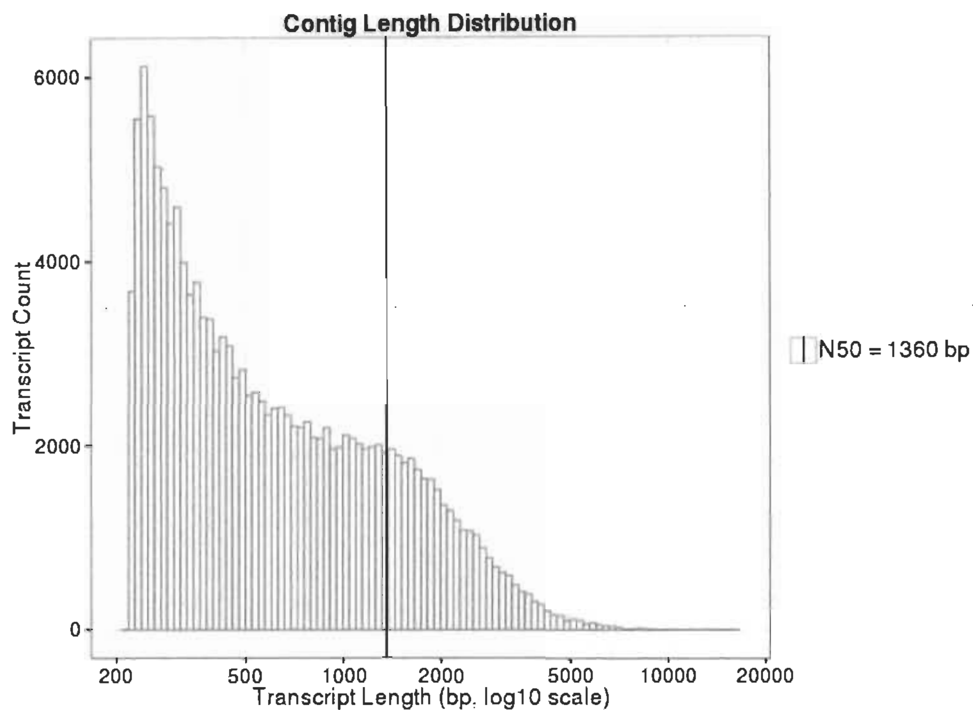
Read Trimming and Clipping of Adapters	
Raw Paired Reads[a]	70 409 091
Surviving Paired Reads[b]	64 038 268
Surviving Paired Reads[c] (%)	91
Normalization	
Surviving Paired Reads after normalization[d]	8 945 044
Surviving Paired Reads after normalization[e] (%)	14
Trinity *de novo* Assembly	
Number of Transcripts[f]	148 563
Number of Components[f]	86 994
Total Transcripts Length (bp)	126 994 523
Max. Transcript Length (bp)	15 491
Min. Transcript Length (bp)	224
Median Transcript Length (bp)	514
Mean Transcript Length (bp)	854
N50 (bp)[g]	1 360
BLAST annotation and filtered annotated components	
Number of Transcripts	8 866
Number of Components	3 802
Total Transcripts Length (bp)	15 030 163
Max. Transcript Length (bp)	8 887
Min. Transcript Length (bp)	297
Median Transcript Length (bp)	1 504
Mean Transcript Length (bp)	1 695
N50 (bp)[h]	2 043

[a] Number of Paired Reads obtained from the sequencer.
[b] Number of Remaining Paired Reads after the trimming step.
[c] Percentage of Surviving Paired Reads / Raw Paired Reads.
[d] Number of remaining Paired Reads after the normalization step.
[e] Percentage of Surviving Paired Reads after normalization / Surviving Paired Reads after trimming.
[f] Trinity has created a list of transcripts (contigs) representing the transcriptome isoforms. The transcripts are grouped in components loosely representing genes. Transcript names are prefixed by the component/gene name e.g. transcripts c115_g5_i1 and c115_g5_i2 are derived from the same isolated de Bruijn graph and therefore share the same component/gene number c115_g5.
[g] Corresponding contig length distribution figure N50 = 1360 bp below.
[h] Corresponding contig length distribution figure N50 = 2043 bp below.

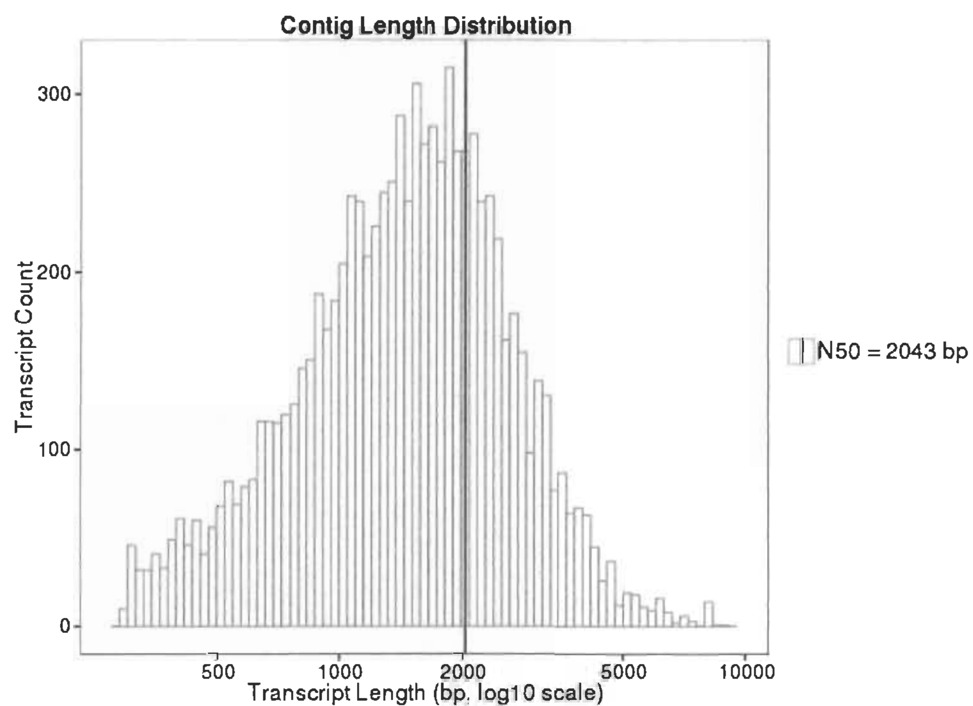
Supplementary Fig. S1. Representative electrophoresis gel image. The RT-PCR product of each AA biosynthetic gene is located between the 100 and 300 bp markers (highlighted by the red contour).



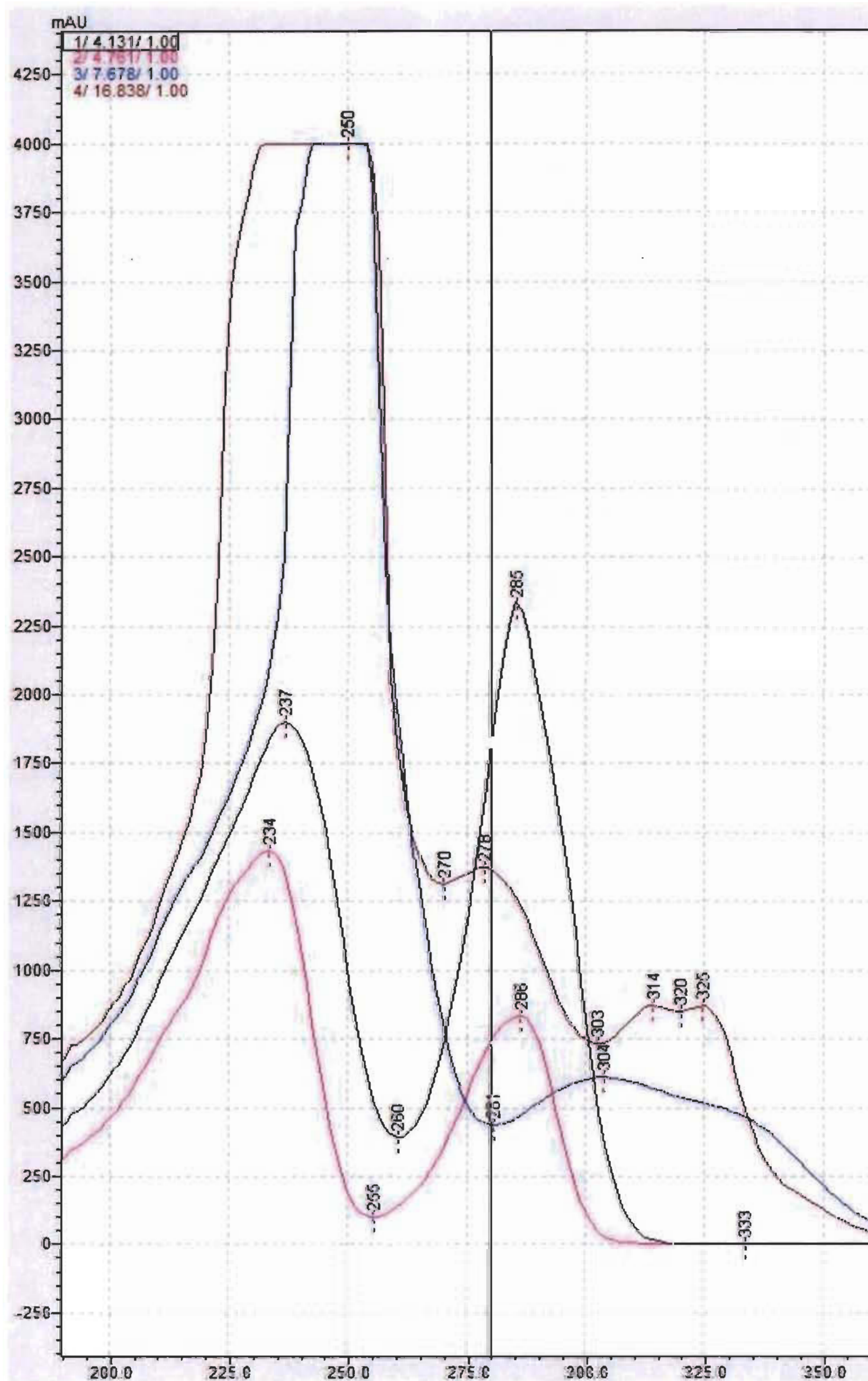
Supplementary Fig. S2. Transcript length distribution after Trinity *de novo* assembly of the *N. papyraceus* stage 1 bulb transcriptome.



Supplementary Fig. S3. Transcript length distribution after BLAST annotation and filtered annotated components of the *N. papyraceus* stage 1 bulb transcriptome.



Supplementary Fig. S4. Absorption maxima for each alkaloid standard obtained by HPLC-PDA analysis.



CHAPITRE III

CONCLUSION

Les plantes sont utilisées depuis des milliers d'années pour prévenir et traiter les maladies de toutes sortes. Les caractéristiques thérapeutiques des plantes sont dues à la variété de métabolites spécialisés qu'elles produisent. Une famille de plantes connue pour ses valeurs médicinales bénéfiques est la famille des Amaryllidacées. Ses propriétés bénéfiques peuvent principalement être attribuées à la présence d'une myriade d'une classe particulière de métabolites spécialisés, les alcaloïdes. Plusieurs alcaloïdes d'Amaryllidacée (AA) présentent des activités pharmaceutiques telles que anticancéreuses, antimicrobiennes, neurologiques, etc. Malgré les plus de 500 AA identifiés à ce jour, un seul, la galanthamine, est actuellement utilisé comme médicament par la médecine moderne pour le traitement des symptômes de la maladie d'Alzheimer.

Les méthodes de production à usage commercial de la galanthamine reposent aujourd'hui, principalement, sur la culture d'un certain nombre d'espèces d'Amaryllidacée afin d'extraire les alcaloïdes accumulés dans les plantes. Les inconvénients de cette méthode, cependant, sont que les AA n'existent qu'en quantités infimes dans les plantes, et que leur extraction et leur purification sont généralement inefficaces et coûteuses. Ainsi, des méthodes de production à grande échelle à faible coût et écologiquement durables sont indispensables pour la poursuite de la production viable de la galanthamine, ainsi que pour la production éventuelle d'autres AA ayant des propriétés pharmacologiques actuellement à l'étude.

Une méthode de choix est celle de l'ingénierie génétique pour la production des AA dans des systèmes hétérologues tels que les microbes ou d'autres espèces végétales. Cela, par contre, nécessite une compréhension plus complète de la voie de biosynthèse des AA. Le manque de connaissances sur cette voie a été la source d'inspiration de mon projet de recherche.

L'objectif de la recherche présentée dans ce mémoire était de faire la lumière, en particulier, sur les gènes qui ont été proposés pour être impliqués dans les diverses réactions biochimiques constituant la voie de biosynthèse proposée des AA. L'approche utilisée consistait à combiner une analyse du transcriptome avec une analyse du métabolome de l'espèce d'Amaryllidacée *N. papyraceus*. Vingt-quatre échantillons de *N. papyraceus*, obtenus à partir de différentes parties de la plante à plusieurs stades de développement, ont été étudiés. L'analyse transcriptomique a impliqué l'étude de la présence des gènes biosynthétiques proposés et récemment confirmés des AA dans le transcriptome du bulbe non-planté du stade 1, suivie du profilage de l'expression de ces gènes dans les 24 échantillons de plantes. L'analyse métabolomique, d'autre part, impliquait le profilage de la teneur en AA dans les 24 échantillons de plantes. La comparaison des profils d'expression géniques avec les profils alcaloïdiques a permis d'approfondir nos connaissances en ce qui concerne l'implication des gènes biosynthétiques des AA dans la production des AA.

3.1 Étude du transcriptome

Pour les plantes non-modèles telles que les Amaryllidacées, pour lesquelles il y a une absence d'information génomique, un assemblage *de novo* du transcriptome était nécessaire. L'ARN a été extrait des bulbes non-plantés de *N. papyraceus* et éventuellement a été séquencé en utilisant la méthode d'Illumina. Après le taillage et la normalisation d'environ 70 millions *raw paired reads*, environ 8 millions *paired reads* étaient obtenus. Ces *paired reads* ont été utilisées pour assembler le transcriptome qui a généré environ 150 000 transcrits. Après l'annotation du transcriptome, l'annotation BLAST a donné environ 9000 transcrits d'une longueur moyenne de 1695 paires de bases. Avec ces résultats, il a été conclu qu'un transcriptome de bonne qualité a été développé.

Parmi les environ 9000 transcrits annotés, des transcrits correspondant à tous les gènes biosynthétiques des AA ont été identifiés. Plusieurs isoformes de transcription de gènes orthologues ont été identifiées. Dans le cas de *PAL* et de *TYDC*, deux isoformes ou plus ont été identifiées. D'autres études ont aussi rapporté l'identification de

deux isoformes de chacun des deux gènes [56, 57, 62]. Plusieurs isoformes de transcription ont également été identifiées pour les autres gènes biosynthétiques des AA. Bref, des transcrits de pleine longueur et plusieurs isoformes de transcription avec des valeurs de Expect à zéro, ou très près de zéro, correspondant aux gènes biosynthétiques des AA ont été identifiés.

3.2 AA détectés

L'analyse par la HPLC des différentes parties de la plante à différents stades de développement a montré que seule la lycorine est très probablement présente dans tous les échantillons de *N. papyraceus*. Ceci est en lien avec le fait que la lycorine semble être phylogénétiquement le plus ancien AA [63], et donc le plus abondant [64].

3.3 Expression des gènes et comparaison avec les profils alcaloïdiques

Les niveaux relatifs d'expression des neuf gènes biosynthétiques des AA, et de leurs isoformes, communs à tous les alcaloïdes d'Amaryllidacée ont été analysés en termes de leur expression à chaque stade de développement de *N. papyraceus*. Les résultats montrent qu'au stade 1 (bulbe non-planté), il y a une expression élevée de *PAL1*, *TYDC* et *N4OMT*, alors que *C4H* et *C3H* ont été exprimés à de faibles niveaux, relativement aux autres stades, dans ces bulbes. Le *4CL* et les deux isoformes de *HCT* ont été exprimées à des niveaux très bas, signifiant une absence possible de l'implication de la voie alternative (zone grisée de la figure 1.3) de transformation de l'acide *p*-coumarique en acide caféique. Les niveaux d'expression de *C4H*, *C3H*, *TYDC* et *N4OMT*, qui vont de faibles à élevés, explique la quantité faible d'AA dans les bulbes non-plantés du stade 1.

Au deuxième stade, l'expression de *4CL* et des deux isoformes de *HCT* est faible, relativement aux autres stades. Ces observations suggèrent que l'acide caféique est principalement produit directement par la C3H dans le bulbe et les racines, alors que dans les feuilles, la synthèse de l'acide caféique se produit probablement par les deux voies. D'autres part à ce stade, au cours duquel la plante commence sa croissance à partir du

bulbe du premier stade, de nombreux gènes, y compris *C4H*, *C3H*, *TYDC* et *N4OMT*, montrent la tendance suivante : expression la plus élevée dans les bulbes, niveau moyen dans les racines et faible niveau dans les feuilles. Au contraire, la quantité d'alcaloïdes dans les bulbes est faible et celle dans les feuilles est élevée même si l'expression des gènes est beaucoup plus élevée dans les bulbes. Une explication plausible à ceci peut être que les enzymes et/ou les alcaloïdes produits dans les bulbes sont transportés vers d'autres parties de la plante, conduisant à une diminution de la quantité d'alcaloïdes dans les bulbes et à des quantités plus élevées dans les racines et les feuilles. Le transport d'enzymes et d'alcaloïdes d'une partie vers d'autres de la plante a déjà été décrit dans la littérature [65-67]. Concernant les alcaloïdes benzylisoquinoline dans le pavot à opium, la morphine et d'autres alcaloïdes apparentés se trouvent dans le latex du pavot à opium. Le latex se trouve dans de grandes vésicules membranaires appelées laticifères. Cela a conduit à la croyance depuis longtemps que les alcaloïdes sont synthétisés dans les laticifères. Cependant, plusieurs enzymes clés impliquées dans la biosynthèse de la morphine n'étaient pas trouvées dans le latex. Les transcrits des gènes biosynthétiques ont été trouvés dans les cellules compagnes (*companion cells*) alors que les enzymes ont été trouvés dans les éléments de tamis. Cette découverte a montré que les alcaloïdes accumulent dans les laticifères, mais sont synthétisés ailleurs dans la plante.

Au 3e stade, puisque l'expression de *C3H* est extrêmement faible, il semble peu probable que la conversion de l'acide *p*-coumarique en acide caféique se fait uniquement par la C3H. D'un autre côté, les niveaux d'expression de *4CL*, *HCT1* et *HCT2* vont de faibles à élevés, relativement aux autres stades. Ainsi, on peut supposer que la voie alternative est la voie prédominante de production d'acide caféique à partir de l'acide *p*-coumarique. L'expression élevée ou très élevée des gènes *PAL1*, *PAL2*, *4CL*, *HCT1* et *N4OMT* dans les feuilles ou les fleurs est en accord avec une plus grande quantité d'AA présents dans ces parties de la plante. La teneur étonnamment élevée des AA dans les tiges, où presque tous les gènes sont exprimés à des niveaux très bas, suggère, encore une fois, un transport probable d'une certaine quantité d'enzymes et/ou d'alcaloïdes des autres parties de la plante vers la tige.

4CL, *HCT1* et *HCT2* sont activement transcrits dans toutes les parties de la plante au stade 4 comparé à *C3H* qui a une expression extrêmement faible au cours de ce stade. Il semble donc que la principale voie de production de l'acide caféique continue d'être par la voie alternative plutôt que par la C3H seule. En outre, *PAL2*, *C4H*, *4CL*, *HCT1* et *TYDC* ont montré une forte expression dans diverses parties aériennes donnant lieu à la forte accumulation d'AA dans les parties aériennes.

Au cours des stades finaux, 5 et 6, la caractéristique la plus frappante est la diminution prononcée des niveaux d'expression de plusieurs gènes. Cela pourrait être un signe du vieillissement de la plante.

3.4 Sommaire des applications pharmacologiques des AA

La galanthamine est présentement le seul alcaloïde utilisé de manière commerciale comme médicament. Cependant, de nombreux autres AA sont actuellement en train d'être évalués pour leurs activités pharmacologiques qui sont résumées dans le tableau 3.1. Les structures chimiques des alcaloïdes décrits au tableau 3.1 sont illustrées à la figure 3.1. Tout comme la galanthamine, on espère que ces AA seront également utilisés comme médicaments dans un avenir pas si lointain.

Tableau 3.1

Activités pharmacologiques de plusieurs AA.
Les sigles apparaissant dans ce tableau sont définis à la page viii.

Propriété thérapeutique	**Activités biologiques**	**AA**
Neurologique	Inhibiteur d'AChE Modulateur des nAChR	Galanthamine (**7**)
	Anxiolytique Antidépressive Anticonvulsive	Montanine (**10**)
Anti-cancer	Cytostatique	Lycorine (**5**) Hémanthamine (**8**) Bulbispermine (**13**)
	Cytotoxique	Pseudolycorine (**14**) Hémanthamine (**8**) Hémanthidine (**15**) Vittatine (**16**) Narciclasine (**11**) Pancrastatine (**17**) Tazettine (**12**) Prétazettine (**18**)
Anti-bactérienne	Cytotoxique	Ungeremine (**19**) Vittatine (**16**) 11-hydroxyvittatine (**20**) Amarbellisine (**21**) Pancracine (**22**) Clivatine (**23**) Nobilisine (**24**) Nobilisitine A et B (+)-8-déméthylmaritidine (**27**)
Anti-fongique	Cytotoxique	Lycorine (**5**) Amarbellisine (**21**) Vittatine (**16**) 11-hydroxyvittatine (**20**) Hippeastrine (**25**) Pancracine (**22**) Clivatine (**23**) Clivimine (**26**) Nobilisine (**24**) Nobilisitine A et B (+)-8-déméthylmaritidine (**27**)

Propriété thérapeutique	Activités biologiques	AA
Anti-parasitique	Cytotoxique	Hémanthamine (**8**) Hémanthidine (**15**) Lycorine (**5**)
Anti-virale	Inhibiteur de la réplication	Lycorine (**5**) Pseudolycorine (**14**)
		Pancratistatine (**17**)
Diverses	Anti-inflammatoire	Lycorine (**5**) Hémanthidine (**15**)
	Antioxidante	Hémanthamine (**8**)
	Hypotensive	Homolycorine (**6**) 8-*O*-déméthylhomolycorine
	Analgésique	Hémanthidine (**15**) Lycorine (**5**)

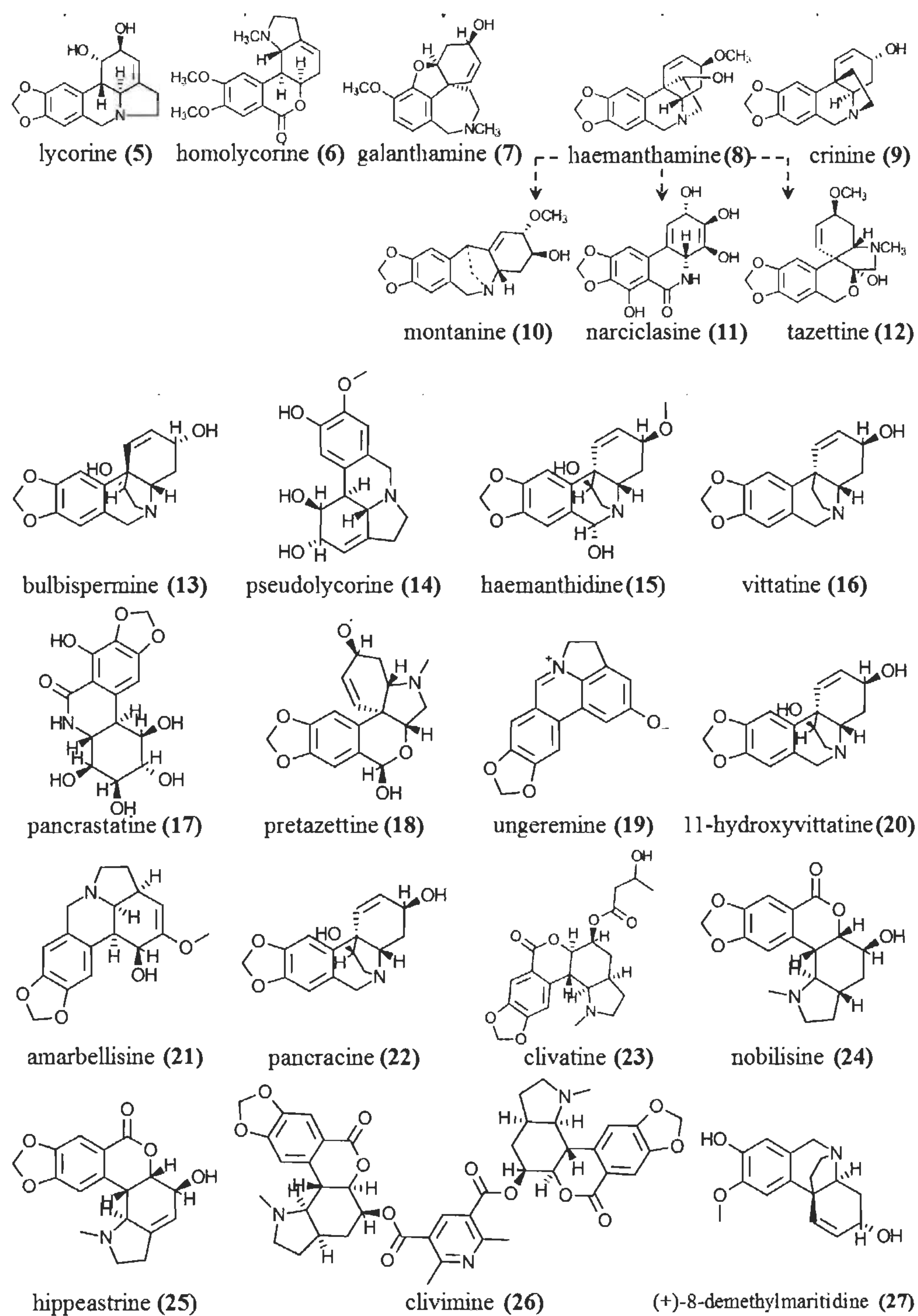


Figure 3.1 Structures chimiques des AA [68] décrits au tableau 3.1.

3.5 Génie génétique et ingénierie métabolique

Parmi les différents gènes proposés et confirmés dans la voie de biosynthèse des AA, il serait intéressant de connaître quels gènes sont cruciaux pour la biosynthèse des AA. Cela peut être étudié par l'utilisation d'un éliciteur. Par exemple, dans des cultures cellulaires de pavot à opium traitées avec un éliciteur fongique [69], et dans des cultures de *Catharanthus roseus* et de tabac traitées au jasmonate de méthyle [46, 51], une augmentation était observée de l'expression des gènes impliqués dans la biosynthèse des alcaloïdes. De plus, l'expression de gènes contrôlant le recyclage de SAM (*S*-adénosyl méthionine) a augmenté dans toutes les trois cultures, ce qui implique l'importance des gènes impliqués dans le recyclage des SAM dans la biosynthèse des alcaloïdes. En tant que donneur de méthyle [70], SAM joue un rôle de cofacteur dans les réactions enzymatiques responsables de la méthylation de divers composés. Dans le cas de la biosynthèse des AA, SAM est impliqué dans la méthylation de la norbelladine par N4OMT (Figure 1.3).

Concernant les plantes produisant des alcaloïdes, le génie génétique vise à augmenter la synthèse des alcaloïdes par la surexpression de certains gènes. Cela a été démontré par Yun *et al.* qui ont observé que l'introduction du gène *H6H*, exprimé constitutivement, augmentait la synthèse de scopolamine, un alcaloïde d'Amaryllidacée, chez les plantes *Amaryllis belladonna* [71]. La surexpression d'un gène biosynthétique d'alcaloïde pourrait même éventuellement entraîner l'induction transcriptionnelle coordonnée des gènes dans d'autres voies [72]. Il faut, par contre, considérer les conséquences négatives sur la physiologie de la plante qui pourraient survenir suite à la surexpression d'un gène biosynthétique et d'une quantité élevée d'alcaloïdes. La surexpression génique pourrait avoir un coût métabolique sur la plante et il faudrait évaluer les effets néfastes que pourraient causer une quantité élevée de composés toxiques tels les alcaloïdes.

Le concept de la surexpression des gènes pourrait être appliqué à la voie de biosynthèse des AA, soit à tous les gènes ou à certains gènes clés (Figure 1.3). L'ingénierie métabolique consiste à altérer les processus génétiques et biochimiques, entre autres,

d'une cellule pour que celle-ci puisse synthétiser des composés désirés [73]. L'ingénierie métabolique pourrait, donc, permettre l'introduction de gènes biosynthétiques d'AA surexprimés dans un hôte microbien, par exemple. Des millions de ces hôtes microbiens fonctionneraient comme une usine cellulaire produisant des AA en grande quantité. Cette méthode de production à grande échelle des AA nécessite la sélection d'un hôte qui conviendrait à la production des AA.

Un microorganisme qui pourrait éventuellement servir à synthétiser les AA à grande échelle est la microalgue *Chlamydomonas reinhardtii* [74, 75]. Des avantages d'utiliser une microalgue sont qu'elle utilise le dioxyde de carbone, qui est facilement disponible, comme source de carbone. Elle peut même pousser dans des milieux de culture contenant une source de carbone faible. En outre, elle exprime des gènes codant pour des enzymes appartenant à la superfamille des cytochromes P450. De nombreuses réactions dans la voie de biosynthèse des AA sont supposément effectuées par des enzymes cytochromes P450 (voir section 1.7 Voie biosynthétique proposée des AA). Un avantage de l'espèce *Chlamydomonas reinhardtii* est qu'elle s'agit d'un organisme modèle faisant en sorte qu'elle est particulièrement bien étudiée.

L'ingénierie métabolique des hôtes microbiens pour produire des composés à grande échelle implique généralement les étapes décrites dans la figure 3.2.

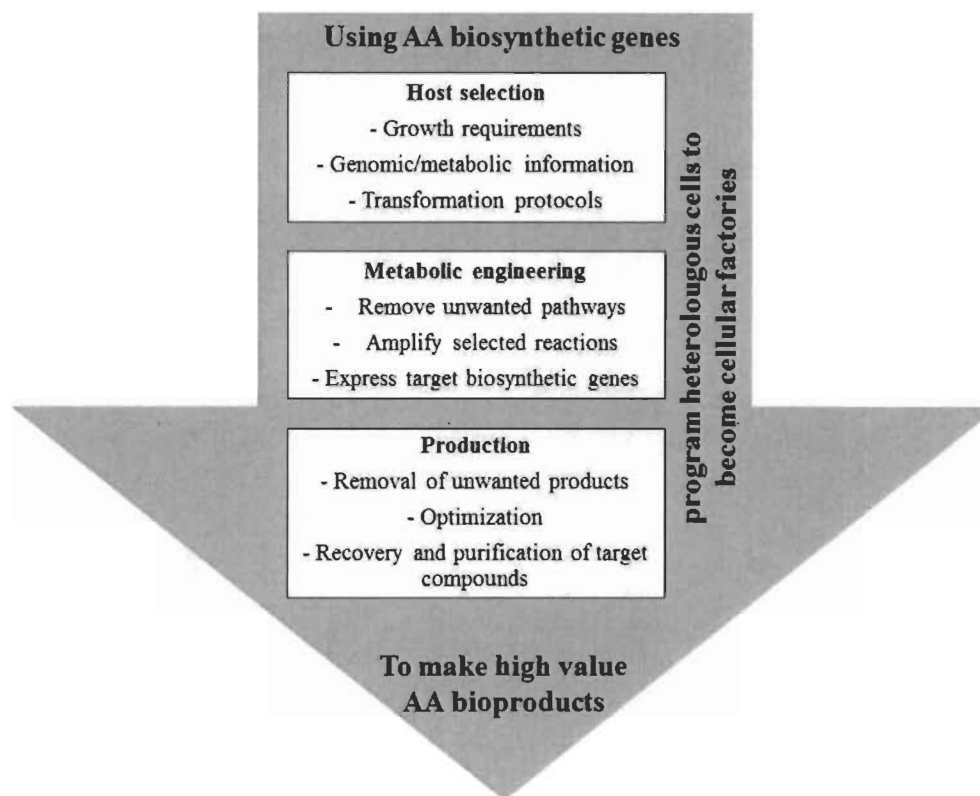


Figure 3.2 Étapes nécessaires pour l'ingénierie métabolique de microorganismes [27].

Les plantes d'Amaryllidacées sont utilisées depuis longtemps par la médecine traditionnelle. Cependant, jusqu'à maintenant, beaucoup de recherche n'a pas été menée sur la variété des effets médicaux bénéfiques possibles que les alcaloïdes produits par ces plantes peuvent avoir. Avec les recherches que j'ai mené sur *N. papyraceus*, nous avons maintenant plus d'information sur quelles parties durant quels stades de développement accumulent plus d'AA. Cela peut être utile pour la récolte des plantes de *N. papyraceus*, ou peut-être même d'autres plantes d'Amaryllidacées, pour pouvoir extraite une grande quantité d'alcaloïdes. De plus, les analyses transcriptomiques et d'expression génique ont contribué à approfondir nos connaissances sur les gènes biosynthétiques des AA. Éventuellement, une compréhension complète de la biosynthèse des AA au niveau génique permettra l'utilisation de méthodes moins coûteuses et plus durables, telles que l'ingénierie métabolique, pour la production des AA à grande échelle.

RÉFÉRENCES BIBLIOGRAPHIQUES

1. Oliver, S.G., et al., *Systematic functional analysis of the yeast genome.* Trends in biotechnology, 1998. **16**(9): p. 373-378.

2. Harris, E.D., *Professor Biochemistry and Biophysics and Faculty of Nutrition Texas A&M University.*

3. Khoddami, A., M.A. Wilkes, and T.H. Roberts, *Techniques for analysis of plant phenolic compounds.* Molecules, 2013. **18**(2): p. 2328-2375.

4. Vogt, T., *Phenylpropanoid biosynthesis.* Molecular plant, 2010. **3**(1): p. 2-20.

5. Fraser, C.M. and C. Chapple, *The phenylpropanoid pathway in Arabidopsis.* The Arabidopsis Book, 2011: p. e0152.

6. Whetten, R. and R. Sederoff, *Lignin biosynthesis.* The plant cell, 1995. **7**(7): p. 1001.

7. Vanholme, R., et al., *Lignin biosynthesis and structure.* Plant physiology, 2010. **153**(3): p. 895-905.

8. Dixon, R.A. and N.L. Paiva, *Stress-induced phenylpropanoid metabolism.* The plant cell, 1995. **7**(7): p. 1085.

9. Davis, E.M. and R. Croteau, *Cyclization enzymes in the biosynthesis of monoterpenes, sesquiterpenes, and diterpenes*, in *Biosynthesis*. 2000, Springer. p. 53-95.

10. Breitmaier, E., *Terpenes: flavors, fragrances, pharmaca, pheromones*. 2006: John Wiley & Sons.

11. Mahajan, M., V. Kumar, and S.K. Yadav, *Alkaloids: Properties, Application and Pharmacological Effects*, in *Alkaloids: Properties, Applications and Pharmacological Effects*, N.M. Cassiano, Editor. 2010, Nova Science Publishers, Inc. p. 1-36.

12. Hesse, M., *Alkaloids: nature's curse or blessing?* 2002: John Wiley & Sons.

13. Wink, M., *Introduction: biochemistry, physiology and ecological functions of secondary metabolites.* Annual Plant Reviews Volume 40: Biochemistry of Plant Secondary Metabolism, Second Edition, 2010: p. 1-19.

14. Schrader, K.K., et al., *A survey of phytotoxic microbial and plant metabolites as potential natural products for pest management.* Chemistry & biodiversity, 2010. **7**(9): p. 2261-2280.

15. Van Goietsenoven, G., et al., *Amaryllidaceae Alkaloids Belonging to Different Structural Subgroups Display Activity against Apoptosis-Resistant Cancer Cells†.* Journal of natural products, 2010. **73**(7): p. 1223-1227.

16. Wang, P., et al., *Novel lycorine derivatives as anticancer agents: synthesis and in vitro biological evaluation.* Molecules, 2014. **19**(2): p. 2469-2480.

17. McNulty, J., et al., *Structure-activity studies on the lycorine pharmacophore: A potent inducer of apoptosis in human leukemia cells.* Phytochemistry, 2009. **70**(7): p. 913-919.

18. Houghton, P.J., Y. Ren, and M.-J. Howes, *Acetylcholinesterase inhibitors from plants and fungi.* Natural Product Reports, 2006. **23**(2): p. 181-199.

19. Heinrich, M. and H.L. Teoh, *Galanthamine from snowdrop-the development of a modern drug against Alzheimer's disease from local Caucasian knowledge.* Journal of ethnopharmacology, 2004. **92**(2): p. 147-162.

20. Keller, C., et al., *Long-term effects of galantamine treatment on brain functional activities as measured by PET in Alzheimer's disease patients.* Journal of Alzheimer's Disease, 2011. **24**(1): p. 109-123.

21. Evidente, A., et al., *Biological evaluation of structurally diverse Amaryllidaceae alkaloids and their synthetic derivatives: discovery of novel leads for anticancer drug design.* Planta medica, 2009. **75**(5): p. 501.

22. Mascavage, L., M. Wilson, and D. Dalton, *Syntheses of Morphine and Codeine (1992-2002): Templates for Exploration of Synthetic Tools.* Current Organic Synthesis, 2006. **3**(1): p. 99-120.

23. Takos, A. and F. Rook, *Towards a Molecular Understanding of the Biosynthesis of Amaryllidaceae Alkaloids in Support of Their Expanding Medical Use.* International Journal of Molecular Sciences, 2013. **14**(6): p. 11713.

24. Contributors. *Amaryllidaceae*. Wikipedia, The Free Encyclopedia. 29 April 2018 09:19 UTC [cited 2016; Available from: https://en.wikipedia.org/w/index.php?title=Amaryllidaceae&oldid=838789556

25. Kornienko, A. and A. Evidente, *Chemistry, Biology and Medicinal Potential of Narciclasine and its Congeners.* Chemical reviews, 2008. **108**(6): p. 1982-2014.

26. Jin, Z. and X.-H. Xu, *Amaryllidaceae Alkaloids*, in *Natural Products: Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes*, K.G. Ramawat and J.-M. Mérillon, Editors. 2013, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 479-522.

27. Singh, A. and I. Desgagné-Penix, *Biosynthesis of Amaryllidaceae Alkaloids: A Biochemical Outlook*, in *Alkaloids: Biosynthesis, Biological Roles and Health Benefits*, E. Sobarzo-Sánchez, Editor. 2015, Nova Science Publishers, Inc. p. 53-76.

28. Hilmas, C.J., et al., *Galantamine is a novel post-exposure therapeutic against lethal VX challenge.* Toxicology and applied pharmacology, 2009. **240**(2): p. 166-173.

29. da Silva, A.F.S., et al., *Anxiolytic-, antidepressant-and anticonvulsant-like effects of the alkaloid montanine isolated from Hippeastrum vittatum.* Pharmacology Biochemistry and Behavior, 2006. **85**(1): p. 148-154.

30. Bastida, J., et al., *Chemical and biological aspects of Amaryllidaceae alkaloids.* Recent Advances in Pharmaceutical Sciences, 2011. **2011**: p. 65-100.

31. Hohmann, J., et al., *Antiproliferative amaryllidaceae alkaloids isolated from the bulbs of Sprekelia formosissima and Hymenocallis x festalis.* Planta medica, 2002. **68**(5): p. 454-457.

32. Lefranc, F., et al., *Narciclasine, a plant growth modulator, activates Rho and stress fibers in glioblastoma cells.* Molecular cancer therapeutics, 2009. **8**(7): p. 1739-1750.

33. Evidente, A., et al., *(-)-Amarbellisine, a lycorine-type alkaloid from Amaryllis belladonna L. growing in Egypt.* Phytochemistry, 2004. **65**(14): p. 2113-8.

34. Chen, D., et al., *Lycorine-derived phenanthridine downregulators of host Hsc70 as potential hepatitis C virus inhibitors.* Future, 2015. **7**(5): p. 561-570.

35. Lubbe, A., et al., *Analysis of metabolic variation and galanthamine content in Narcissus bulbs by 1H NMR.* Phytochemical analysis, 2010. **21**(1): p. 66-72.

36. Nosov, A., *Application of cell technologies for production of plant-derived bioactive substances of plant origin.* Applied biochemistry and microbiology, 2012. **48**(7): p. 609-624.

37. Diamond, A. and I. Desgagné-Penix, *Metabolic engineering for the production of plant isoquinoline alkaloids.* Plant biotechnology journal, 2015.

38. Pavlov, A., et al., *Galanthamine production by Leucojum aestivum in vitro systems.* Process Biochemistry, 2007. **42**(4): p. 734-739.

39. Georgiev, V., et al., *Optimized nutrient medium for galanthamine production in Leucojum aestivum L. in vitro shoot system.* Zeitschrift für Naturforschung C, 2009. **64**(3-4): p. 219-224.

40. Bohlin, L., et al., *Natural products in modern life science.* Phytochemistry Reviews, 2010. **9**(2): p. 279-301.

41. Desgagné-Penix, I. and P.J. Facchini, *Systematic silencing of benzylisoquinoline alkaloid biosynthetic genes reveals the major route to papaverine in opium poppy.* The Plant Journal, 2012. **72**(2): p. 331-344.

42. Mizutani, M. and F. Sato, *Unusual P450 reactions in plant secondary metabolism.* Archives of Biochemistry and Biophysics, 2011. **507**(1): p. 194-203.

43. Mizutani, M. and D. Ohta, *Diversification of P450 genes during land plant evolution.* Annual review of plant biology, 2010. **61**: p. 291-315.

44. Nelson, D.R., et al., *Comparison of cytochrome P450 genes from six plant genomes.* Tropical Plant Biology, 2008. **1**(3-4): p. 216-235.

45. Kilgore, M.B., et al., *Cloning and Characterization of a Norbelladine 4'-O-Methyltransferase Involved in the Biosynthesis of the Alzheimer's Drug Galanthamine in Narcissus sp. aff. pseudonarcissus.* 2014.

46. Goossens, A., et al., *A functional genomics approach toward the understanding of secondary metabolism in plant cells.* Proceedings of the National Academy of Sciences, 2003. **100**(14): p. 8595-8600.

47. Gahlan, P., et al., *De novo sequencing and characterization of Picrorhiza kurrooa transcriptome at two temperatures showed major transcriptome adjustments.* BMC genomics, 2012. **13**(1): p. 126.

48. Ge, G., et al., *The first insight into the tissue specific taxus transcriptome via Illumina second generation sequencing.* PLoS one, 2011. **6**(6): p. e21220.

49. Hao, D., et al., *De novo characterization of the root transcriptome of a traditional Chinese medicinal plant Polygonum cuspidatum.* Science China Life Sciences, 2012. **55**(5): p. 452-466.

50. Hyun, T.K., et al., *De novo transcriptome sequencing of Momordica cochinchinensis to identify genes involved in the carotenoid biosynthesis.* Plant molecular biology, 2012. **79**(4-5): p. 413-427.

51. Rischer, H., et al., *Gene-to-metabolite networks for terpenoid indole alkaloid biosynthesis in Catharanthus roseus cells.* Proceedings of the National Academy of Sciences, 2006. **103**(14): p. 5614-5619.

52. Yamazaki, M., et al., *Coupling deep transcriptome analysis with untargeted metabolic profiling in Ophiorrhiza pumila to further the understanding of the biosynthesis of the anti-cancer alkaloid camptothecin and anthraquinones.* Plant and cell physiology, 2013. **54**(5): p. 686-696.

53. Viladomat, F., et al., *Ontogenic variations in the alkaloids of Narcissus assoanus.* Physiologia Plantarum, 1986. **68**(4): p. 657-661.

54. Kreh, M., *Studies on galanthamine extraction from Narcissus and other Amaryllidaceae.* 2002: Taylor & Francis: London, UK.

55. Shawky, E., et al., *HPTLC and GC/MS Study of Amaryllidaceae Alkaloids of Two Narcissus Species.* Chemistry & biodiversity, 2015. **12**(8): p. 1184-1199.

56. Jiang, Y., et al., *Molecular and analysis of a phenylalanine ammonia-lyase gene (LrPAL2) from Lycoris radiata.* Molecular biology reports, 2013. **40**(3): p. 2293-2300.

57. Singh, A. and I. Desgagné-Penix, *Transcriptome and metabolome profiling of Narcissus pseudonarcissus 'King Alfred'reveal components of Amaryllidaceae alkaloid metabolism.* Scientific reports, 2017. **7**(1): p. 17356.

58. Martin, J.A. and Z. Wang, *Next-generation transcriptome assembly.* Nature Reviews Genetics, 2011. **12**(10): p. 671.

59. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method.* methods, 2001. **25**(4): p. 402-408.

60. Petruczynik, A., *Analysis of alkaloids from different chemical groups by different liquid chromatography methods.* Open Chemistry, 2012. **10**(3): p. 802-835.

61. Cohrt, K.O.H. *Running You Through HPLC: How Does it Work?* [cited 2018; Available from: https://bitesizebio.com/20419/running-you-through-hplc-how-does-it-work/

62. Jiang, Y., et al., *Molecular cloning and characterization of a phenylalanine ammonia-lyase gene (LrPAL) from Lycoris radiata.* Molecular biology reports, 2011. **38**(3): p. 1935-1940.

63. Berkov, S., et al., *Evolution of alkaloid biosynthesis in the genus Narcissus.* Phytochemistry, 2014. **99**(0): p. 95-106.

64. Torras-Claveria, L., et al., *Metabolomic analysis of bioactive Amaryllidaceae alkaloids of ornamental varieties of Narcissus by GC-MS combined with k-means cluster analysis.* Industrial Crops and Products, 2014. **56**: p. 211-222.

65. Bird, D.A., V.R. Franceschi, and P.J. Facchini, *A tale of three cell types: alkaloid biosynthesis is localized to sieve elements in opium poppy.* The Plant Cell, 2003. **15**(11): p. 2626-2635.

66. Onoyovwe, A., et al., *Morphine biosynthesis in opium poppy involves two cell types: sieve elements and laticifers.* The Plant Cell, 2013. **25**(10): p. 4110-4122.

67. Ziegler, J. and P.J. Facchini, *Alkaloid biosynthesis: metabolism and trafficking.* Annu. Rev. Plant Biol., 2008. **59**: p. 735-769.

68. Hotchandani, T. and I. Desgagné-Penix, *Heterocyclic Amaryllidaceae Alkaloids: Biosynthesis and Pharmacological Applications.* Curr Top Med Chem, 2016.

69. Zulak, K.G., et al., *Gene transcript and metabolite profiling of elicitor-induced opium poppy cell cultures reveals the coordinate regulation of primary and secondary metabolism.* Planta, 2007. **225**(5): p. 1085-1106.

70. Ding, W., et al., *s-Adenosylmethionine Levels Govern Innate Immunity through Distinct Methylation-Dependent Pathways.* Cell Metab, 2015. **22**(4): p. 633-45.

71. Yun, D.-J., T. Hashimoto, and Y. Yamada, *Metabolic engineering of medicinal plants: transgenic Atropa belladonna with an improved alkaloid composition.* Proceedings of the National Academy of Sciences, 1992. **89**(24): p. 11799-11803.

72. Larkin, P.J., et al., *Increasing morphinan alkaloid production by over- expressing codeinone reductase in transgenic Papaver somniferum.* Plant biotechnology journal, 2007. **5**(1): p. 26-37.

73. Kulkarni, R., *Biological Art of Producing Useful Chemicals.* Resonance, 2016.

74. Mathieu-Rivet, E., P. Lerouge, and M. Bardor, *Chlamydomonas reinhardtii: Protein Glycosylation and Production of Biopharmaceuticals*, in *Chlamydomonas: Biotechnology and Biomedicine.* 2017, Springer. p. 45-72.

75. Rochaix, J.-D., *Chlamydomonas reinhardtii as the photosynthetic yeast.* Annual review of genetics, 1995. **29**(1): p. 209-230.

ANNEXE A

ARTICLE DE REVUE

Send Orders for Reprints to reprints@benthamscience.ae

Current Topics in Medicinal Chemistry, 2017, *17*, 1-10

REVIEW ARTICLE

Heterocyclic Amaryllidaceae Alkaloids: Biosynthesis and Pharmacological Applications

Tarun Hotchandani and Isabel Desgagné-Penix*

Department of Chemistry, Biochemistry and Physics, Université du Québec à Trois-Rivières, Trois-Rivières, Québec, Canada

ARTICLE HISTORY

Received: July 07, 2015
Revised: October 21, 2015
Accepted: April 18, 2016

DOI: 10.2174/1568026616666160824104052

Abstract: Amaryllidaceae alkaloids (AAs), which are natural heterocyclic compounds, are isolated from Amaryllidaceae plants such as narcissus, snowdrop and spider lily. AAs have been extensively studied due to their multiple pharmacological properties. Nevertheless, knowledge of AA synthesis in plants is lacking and most genes encoding enzymes involved in their production remain unknown. AAs are structurally complex compounds which are challenging for total chemical synthesis that is economically viable. Therefore the understanding of AA biosynthesis could allow for the development of biotechnologies for the production of natural AAs or analogues, maintaining or improving their pharmacological properties. In this review, we describe the progress regarding the biosynthesis and pharmacological properties of AAs. The most recent developments in neurological, anti-cancer and anti-microbial bioactivities of heterocyclic AAs are covered.

Isabel Desgagné-Penix

Keywords: Alkaloids, Amaryllidaceae, biosynthetic pathways, plant secondary metabolism, galanthamine.

1. INTRODUCTION

Alkaloids represent a diverse group of specialized metabolites, mainly characterized by the occurrence of at least one nitrogen atom in a heterocyclic ring. The position of the nitrogen atom in the carbon ring varies with different alkaloids [1]. Most alkaloids are colorless crystalline solids with a bitter taste. They possess low molecular weights, have definite melting point and are optically active. The term *alkaloids* was first introduced by German chemist W. Meissner in 1819 and applied to all basic, organic compounds obtained from animal or plant materials [2, 3]. The basicity of alkaloids depends on the lone pair of electrons on the nitrogen atom. For example, if the functional groups adjacent to the nitrogen are electron-releasing (*e.g.* an alkyl group), the compound is more basic due to the increased availability of the nitrogen electrons. The basicity of the alkaloids renders them extremely susceptible to decomposition, particularly by heat or light in the presence of oxygen [1]. Alkaloids can form salts with acids making them more water soluble and preventing their decomposition which is why they are available commercially in their salt form [1].

Amaryllidaceae alkaloids (AAs) are a diverse group of alkaloids that includes approximately 500 known structures [4]. Their chemical structure feature a ring system composed of a C_6-C_1 unit (**1**) derived from phenylalanine and an N-C_2-C_6 unit (**2**) derived from tyrosine (Fig. **1**). These alkaloids are only produced by plants of the Amaryllidaceae family, known for its horticultural and ornamental appeal as well as its medicinal value (*e.g.* daffodils (*Narcissus* spp.), snowdrops (*Galanthus* spp.), spider lily (*Lycoris radiata*) and summer snowflakes (*Leucojum aestivum*)). AAs, in general, are used by plants for defense purposes to ward off pathogens, harmful insects and herbivores. For example, the AA galanthamine (**7**) serves as an insecticide by causing excess cholinergic stimulation in the insect [5]. Galanthamine (**7**) inhibits the degradation of neurotransmitters acetylcholine (ACh) by binding to the enzyme acetylcholinesterase (AChE) which is responsible for degrading ACh (Fig. **2**) [6]. The resulting accumulation of acetylcholine, caused by galanthamine (**7**), leads to increased neurotransmission causing continuous stimulation of the muscles, glands, and central nervous system. At high doses, it can result in fatal convulsions. Another AA, lycorine (**5**), is an anti-microbial and anti-viral agent [7, 8], whereas narciclasine (**11**) possesses anti-cancer properties [9, 10]. Thus, AAs exhibit effects on the nervous system, as well as cytotoxic and cytostatic effects, just to name a few. Given these interesting biological properties, AAs are suitable candidates to treat neurological disorders, inhibit growth of tumours or kill microorganisms. This is why there is much ongoing research on the potential medical applications of AAs, since many possess potent pharmacological properties [9, 11-15].

*Address correspondence to this author at the Department of Chemistry, Biochemistry and Physics, Université du Québec à Trois-Rivières, 3351 boulevard des Forges, Trois-Rivières, QC, G9A 5H7, Canada; Tel: 1-819-376-5011; E-mail: Isabel.Desgagne-Penix@uqtr.ca

1568-0266/17 $58.00+.00 © 2017 Bentham Science Publishers

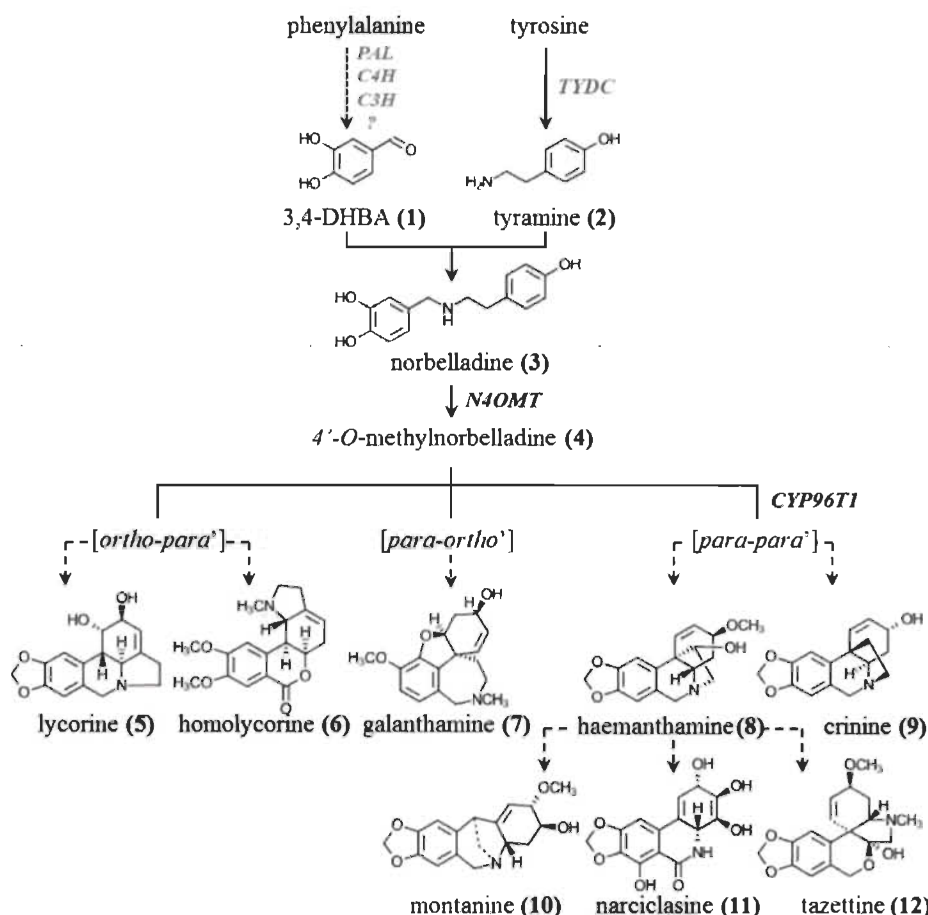


Fig. (1). Proposed AA biosynthetic pathways. Three major subgroups of AAs (*ortho-para'*, *para-ortho'*, *para-para'*) are represented along with the nine proposed AA structural types: norbelladine-, lycorine-, homolycorine-, galanthamine-, haemanthamine-, montanine-, narciclasine-, tazettine- and crinine-type. Broken arrows indicate more than one enzymatic step. The genes encoding enzymes involved in the biosynthesis are indicated next to the arrows in bold-italics. Genes that have been cloned, characterized and identified are labeled in black italics whereas putative enzymes are labeled in grey italics. Arrows without labeling reflect chemical reactions that have not been enzymatically characterized. Abbreviations: *PAL*, phenylalanine ammonia lyase; *C4H*, cinnamate 4-hydroxylase; *C3H*, coumarate 3-hydroxylase; *TYDC*, L-tyrosine/L-dopa decarboxylase; *N4OMT*, norbelladine-4'-*O*-methyltransferase; and *CYP96T1*, cytochrome P450 monooxygenase 96T1.

Once an AA is approved for medical use, however, the problem of producing it in large quantities at a reasonable cost arises. Currently, large numbers of Amaryllidaceae plants must be grown in order to obtain reasonable amounts of AAs (because they are produced at low concentrations in plants). Galanthamine (7) is currently isolated from *Narcissus pseudonarcissus* cv. Carlton because of its wide commercial availability and high resistance to environmental stresses [14, 16]. In some countries, *Leucojum aestivum* and *Lycoris radiata* are also grown and used for the commercial production of galanthamine (7) [17]. Small amounts of lycorine (5) and narciclasine (11) are generally extracted from various *Narcissus* and *Lycoris* species [16, 18-21]. However, the production and screening of AAs in their native plant face several challenges. For example, many AAs exist only in trace amounts, and the methods for isolating and purifying them from plants are generally inefficient and environmentally unsustainable. Total chemical synthesis can help alleviate the demands for limited AAs, but the production of these complex molecules is often challenging and costly. In addition, genetic engineering of plants or microorganisms to produce high concentrations of AAs is unfortunately limited due to the lack of knowledge of the AA biosynthetic pathway.

The biosynthetic enzymes responsible for the production of the various AAs are not known with certainty. The genes encoding these enzymes have also not been fully characterized. Therefore, the elucidation of the AA biosynthetic pathway, coupled with the ongoing pharmacological research on AAs, will eventually help to better treat various human diseases. In this short review, we examine the proposed AA biosynthetic pathway by analyzing the various steps and the enzymes presumed to be involved. We then report the therapeutic properties of certain important AAs.

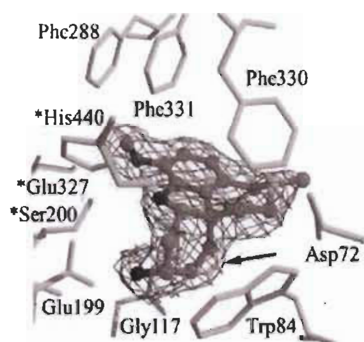


Fig. (2). Representation of the complex galanthamine-AChE in the active site of the enzyme. Galanthamine is drawn as ball and stick model with contouring shown in thin gray lines. Selected amino acid residues of AChE are rendered in stick form and labelled appropriately. The arrow indicates the double bond of the cyclohexene ring in galanthamine. *Amino acids part of the catalytic triad of AChE. Figure adapted from Greenblatt *et al.* (1999) [40].

2. BIOSYNTHESIS

A central dogma of medicinal chemistry and chemical biology is that compounds with similar structures have similar activities. But there are numerous examples for or against this [22]. Correspondingly, similar structures suggest similar metabolic pathways with similar enzymes being involved. Information on the biochemical pathways and molecular genetics of AAs is complex and still fragmentary. Early feeding experiments using radiolabelled precursors have led to the biochemical elucidation of the initial steps in AA biosynthesis [23-30]. These studies suggested that, despite their large structural diversity, all AAs are derived from the core, norbelladine (**3**) (Fig. **1**). Experimental studies coupled with analogies with other plant alkaloid pathways suggest that norbelladine (**3**) is formed from the condensation of two amino acid derivatives: 3,4-dihydroxybenzaldehyde (3,4-DHBA) (**1**) and tyramine (**2**) (Fig. **1**) [12, 15, 31].

AA biosynthesis starts with the formation of the two precursors (3,4-DHBA (**1**) and tyramine (**2**)). On the one hand, phenylalanine is metabolized by various steps: a deamination by phenylalanine ammonia lyase (PAL), a hydroxylation by cinnamate 4-hydroxylase (C4H), another hydroxylation by coumarate 3-hydroxylase (C3H) and a cleavage by a currently unknown enzyme to yield the precursor 3,4-DHBA (**1**) (Fig. **1**). On the other hand, tyrosine is decarboxylated by L-tyrosine/L-dopa decarboxylase (TYDC) to form the precursor tyramine (**2**). In animals, TYDCs participate in the formation of monoamine neurotransmitters (*e.g.* serotonin, dopamine, epinephrine, and norepinephrine), whereas in plants, they are involved in the biosynthesis of several types of specialized metabolites [32]. The condensation of 3,4-DHBA (**1**) and tyramine (**2**) forms the first committed intermediate, norbelladine (**3**) [12, 14, 15, 31]. All AAs share norbelladine (**3**) as a common biosynthetic origin (Fig. **1**).

Until recently, none of the genes involved in AA biosynthesis had been characterized nor confirmed. However, molecular systematics approaches combined with investigations of crude AA extractions from *Narcissus* spp. led to the identification of the first AA biosynthetic gene, *norbelladine 4'-O-methyltransferase* (*N4OMT*). Heterologous expression and characterization of the enzyme encoded by *N4OMT* showed that it is involved in the formation of 4'-*O*-methylnorbelladine (**4**) from norbelladine (**3**) (Fig. **1**) [33].

A crucial step in AA biosynthesis is the cyclization of 4'-*O*-methylnorbelladine (**4**) by three different ways of intramolecular oxidative phenol coupling named *ortho-para'*, *para-ortho'* and *para-para'*, generating various backbone structures. These core skeletons form the basis of further alkaloid diversity [12, 15, 31]. From there, a complex network of enzymatic reactions exists to produce a spectrum of compounds that differs between Amaryllidaceae plant species, cultivars and varieties. These biochemical modifications are carried out by a multitude of decorating enzymes catalyzing diverse types of reactions, such as oxidoreductions, hydroxylations, C-C and C-O bond formations, acetylations, demethylations, and *O*- and *N*-methylations. These reactions lead to the formation of the several hundreds of structurally related AAs known to date [4]. For instance, the anti-tumour AA lycorine (**5**) is derived from the *ortho-para'* phenol coupling step, whereas galanthamine (**7**) originates from the *para-ortho'* coupling (Fig. **1**) [12, 14, 15, 31]. Recently, a cytochrome P450 monooxygenase (*CYP96T1*) was identified and is capable of *para-para' C-C* phenol coupling to yield haemanthamine (**8**) precursors from 4'-*O*-methylnorbelladine (**4**) (Fig. **1**) [34]. Based on the proposed biosynthetic pathway, we discern nine distinct structural types of alkaloids in the Amaryllidaceae family: norbelladine-, lycorine-, homolycorine-, galanthamine-, haemanthamine-, montanine-, narciclasine-, tazettine-, and crinine-type. The representative AA of each structural type is shown in Fig. (**1**).

The presence or absence of certain AA structural types among the genera of Amaryllidaceae plants suggests that some biosynthetic pathways are genera-specific (Fig. **1**). For example, all but the crinine-type AAs, have been isolated from the genus *Narcissus* [21]. This suggests that specific biosynthetic enzyme(s) involved in crinine-type AA production are absent, or not expressed, in *Narcissus* plants. Furthermore, this information also supports the presence of two independent pathways (haemanthamine- and crinine-type) for the *para-para'* AAs (Fig. **1**).

3. PHARMACOLOGICAL APPLICATIONS

A number of studies have confirmed the biological properties of AAs including neurological, anti-cancer, antimicrobial, *etc.* A brief summary is presented in Table **1** and corresponding chemical structures are presented in Fig. (**3**).

3.1. Neurological Activities

The only AA being used medicinally is galanthamine (**7**). It can cross the blood-brain barrier, a characteristic necessary to treat neurological problems, which is often lacking in many drugs [35]. Marketed under the name Reminyl® by Janssen Pharmaceuticals, part of Johnson & Johnson, it is used to treat the symptoms of Alzheimer's disease [11, 35, 36]. With the administration of galanthamine (**7**), the levels of ACh are increased, helping to compensate for the reduced sensitivity to ACh in Alzheimer's disease [37].

Table 1. Therapeutic and biological effects of various AAs.

Therapeutic properties	Biological activities	AAs	AA types	References
Neurological	AChE inhibitor/ nAChRs modulator	galanthamine (7)	galanthamine	[11, 14, 37-39, 42-47]
	anxiolytic, antidepressive, anticonvulsive	montanine (10)	montanine	[48]
Anti-cancer	cytostatic	lycorine (5)	lycorine	[7, 12, 49-53]
		haemanthamine (8)	haemanthamine	[54, 55]
		bulbispermine (13)	crinine	[56]
	cytotoxic	pseudolycorine (14)	lycorine	[57]
		haemanthamine (8)	haemanthamine	[54, 55, 57-62]
		haemanthidine (15)	haemanthamine	[55, 57]
		vittatine (16)	haemanthamine	[63]
		narciclasine(11) / pancrastatine(17)	narciclasine	[10, 64-66]
		tazettine (12) / pretazettine (18)	tazettine	[67, 68]
Anti-bacterial	cytotoxic	ungeremine (19)	lycorine	[69, 70]
		vittatine (16) / 11-hydroxyvittatine (20)	haemanthamine	[71]
		amarbellisine (21)	lycorine	[71]
		pancracine (22)	montanine	[71]
		clivatine(23) /nobilisine (24) / nobilisitine A & B	homolycorine	[72]
		(+)-8-demethylmaritidine (27)	crinine	[72]
Anti-fungal	cytotoxic	lycorine (5) / amarbellisine (21)	lycorine	[69, 70]
		vittatine (16) / 11-hydroxyvittatine (20)	haemanthamine	[71]
		hippeastrine (25)	homolycorine	[71]
		pancracine (22)	montanine	[71]
		clivatine(23) / clivimine (26) / nobilisine(24) / nobilisitine A and B	homolycorine	[72]
		(+)-8-demethylmaritidine (27)	crinine	[72]
Anti-parasitic	cytotoxic	haemanthamine (8) / haemanthidine (15)	haemanthamine	[73-75]
		lycorine (5)	lycorine	[76, 77]
Anti-viral	inhibitor of replication	lycorine (5) / pseudolycorine (14)	lycorine	[8, 78-81]
		pancratistatine (17)	narciclasine	[82, 83]
Miscellaneous	anti-inflammatory	lycorine (5)	lycorine	[7, 84, 85]
		haemanthidine (15)	haemanthamine	[85]
	antioxidant	haemanthamine (8)	haemanthamine	[86]
	hypotensive	homolycorine (6) / 8-*O*-demethylhomolycorine	homolycorine	[87]
	analgesic	haemanthidine (15)	haemanthamine	[88, 89]
		lycorine (5)	lycorine	[89]

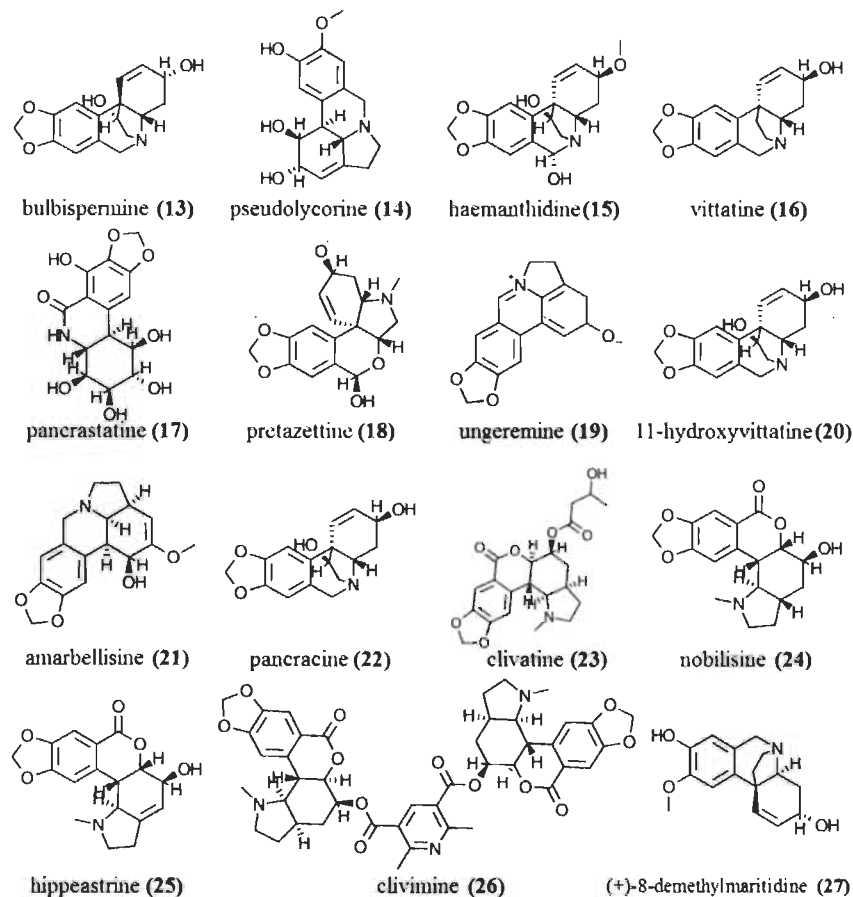


Fig. (3). Chemical structures of diverse Amaryllidaceae alkaloids.

In 2001, Reminyl® was approved by the U.S. Food and Drug Administration (FDA) and several generic versions of the drug have now been approved [5, 35].

Galanthamine (7) is a long-acting, selective, reversible and competitive inhibitor of the enzyme acetylcholinesterase (AChE) [38, 39]. It binds at the base of the active site of AChE interacting with both, an acyl-binding pocket and the indole ring of tryptophan 84 (Trp84) (Fig. 2). The binding with Trp84 is not achieved by the tertiary amino group, but by the double bond in the cyclohexene ring by means of a π-π interaction (Fig. 2) [6, 40]. Trp84 is part of a subsite located near the catalytic triad (Ser200, Glu327, His440) of AChE and has been identified as the binding site for ACh [41].

In addition to being an AChE inhibitor, galanthamine (7) is an allosteric modulator of the neuronal nicotinic acetylcholine receptors (nAChRs) to which ACh binds [38, 46, 47]. As a result, galanthamine (7) is being studied for applications as a drug to treat other neurological conditions [42]. For example, it has exhibited neuroprotective effects. This was shown on brain tissues subjected to oxidative stress, or oxygen and glucose deprivation in both *in vitro* and *in vivo* models [43]. The neuroprotective effect of galanthamine (7) was again demonstrated in *in vivo* models when administered within three hours following an episode of reduced blood flow to the brain (cerebral ischemia), aiding in memory recovery [44]. Thus, galanthamine (7) may have therapeutic potential for preventing neuronal death following a stroke, as well as for patients with mixed or vascular dementia (resulting from vascular disease). Galanthamine (7) could also be used as an effective antidote against poisoning with organophosphorus compounds (OPs) such as pesticides and nerve agents (*e.g.,* soman and VX), counteracting both the acute toxicity as well as OP-induced neurodegeneration [45, 46].

Another AA found to have beneficial effects on the nervous system is montanine (10). According to da Silva *et al.* (2006), it has psycho-pharmacological activities including anxiolytic, anti-depressive and anticonvulsive effects [48].

3.2. Anti-cancer

Several AAs are being studied as potential chemotherapeutic agents. Lycorine (5), one of the most frequently occurring alkaloids in Amaryllidaceae plants, is a powerful inhibitor of the cell cycle during interphase as demonstrated

by its *in vitro* cytostatic effects against several human cancer cell lines [7, 12, 49-53]. Pseudolycorine (**14**), a derivative of lycorine (**5**), has cytotoxic properties and is being investigated for the treatment against metastatic cancer cells that are resistant to many currently available anti-cancer drugs [57]. Other lycorine-type alkaloids, such as caranine, galanthine and 2-*O*-acetylpseudolycorine, are also active against a variety of tumour cells [35, 53].

Haemanthamine-type alkaloids, namely haemanthamine (**8**), haemanthidine (**15**) and vittatine (**16**), also have promising anti-cancer properties. For instance, haemanthamine (**8**) has been shown to be a potent cell growth inhibitor and apoptosis inducer in tumour cells at micromolar concentrations [54]. Furthermore, it was shown to have pronounced cytotoxicity against a number of different cancer cell types [57, 58]. An assay determining interactions with DNA and RNA revealed a strong growth inhibitory effect of haemanthamine (**8**) on L5178 mouse lymphoma cells resulting from its complex formation with RNA [55]. Recently, the first reports indicating apoptosis-mediated cytotoxic activity on cancer cell lines following haemanthamine (**8**) exposure have been published [54, 61, 62, 65]. Haemanthidine (**15**), obtained from haemanthamine (**8**) *via* an irreversible enzymatic reaction occurring in Amaryllidaceae plants, has shown cytotoxicity against different types of mouse lymphoma cells [55, 57]. The AA vittatine (**16**) has shown cytotoxic activity against HT29 colon adenocarcinoma, lung carcinoma and RXF393 renal cell carcinoma [63]. Despite the growing evidence of anti-tumour activities of haemanthamine (**8**), haemanthidine (**15**) and vittatine (**16**), the mechanisms of their action at the cellular level are still not fully understood.

Another effective anti-cancer agent is the AA narciclasine (**11**). It has been found to have potent activity against human glioblastoma multiforme (GBM) tumours [10]. This most malignant type of primary brain tumour is characterised by aggressive invasive behaviour into normal brain tissues and resistance to conventional therapies that trigger apoptosis [35]. To this end, narciclasine (**11**) arrested the growth of GBM tumours and significantly extended the survival time of immunodeficient mice with xenografts of GBM tumours into their brain [10, 64, 65]. These experiments also demonstrated the ability of narciclasine (**11**) to cross the blood-brain barrier. Thus, narciclasine (**11**) is a promising candidate for anti-cancer therapy due both to its remarkable apoptosis-mediated cytotoxic effects on human cancer cells and its high selectivity by not affecting normal fibroblasts [64]. Pancratistatine (**17**), another narciclasine-type AA, displays antineoplastic activity at low concentrations against several cancer cell lines through activation of apoptosis [66].

Moderate cytotoxic activity has been reported for tazettine (**12**) and pretazettine (**18**) present in plants [67, 68, 90]. These compounds have shown remarkable cytotoxicity against a number of tumour cell lines, being therapeutically effective against several types of leukemias and carcinomas [12]. More recently, cytostatic activity against a panel of apoptosis resistant brain cancer cell lines was reported for bulbispermine (**13**), a crinine-type AA [56].

3.3. Anti-microbial

The anti-bacterial, anti-fungal, anti-parasitic and anti-viral properties of AAs are often due to their powerful cytotoxic effects. Table **1** and the text below list some examples.

Anti-bacterial

Studies, involving two isolates of *Flavobacterium columnare* (ALM-00-173 and BioMed), demonstrated that the AA ungeremine (**19**) and its hemi-synthesized analogues possess strong anti-bacterial activities [69, 70]. Ungeremine (**19**) is a lycorine-type AA primarily obtained from *Pancratium maritimum* L. [91]. AAs isolated from *Amaryllis belladonna* L. (also named *Hippeastrum equestre*), including pancracine (**22**), vittatine (**16**), 11-hydroxyvittatine (**20**) and amarbellisine (**21**), all have anti-bacterial activity against Gram-negative *Escherichia coli*, while pancracine (**22**) also showed activity against *Pseudomonas aeruginosae* [71]. AAs extracted from *Clivia nobilis*, including clivatine (**23**), nobilisine (**24**), (+)-8-demethylmaritidine (**27**) and nobilisitine A and B, all exhibited anti-bacterial activity against Gram-positive *Staphylococcus aureus* [72, 92, 93]. The anti-bacterial activity of *Crinum angustum* extracts was evaluated against several strains of bacteria, including drug-resistant clinical isolates *S. aureus* (methicillin-resistant) and *Klebsiella pneumonia* (carbapenemase-resistant) [94]. Several extracts containing AAs exhibited more pronounced anti-bacterial activity on Gram-positive bacteria than on Gram-negative ones and IC_{50} values ranged from 156 to 625 µg/mL [94]. However, the exact AAs responsible for this action remain unknown.

Anti-fungal

Six AAs isolated from *A. belladonna* L., namely lycorine (**5**), pancracine (**22**), vittatine (**16**), 11-hydroxyvittatine (**20**), amarbellisine (**21**) and hippeastrine (**25**), all demonstrated cytotoxic activity against *Candida albicans* [71]. The anti-fungal activity was higher with lycorine (**5**), amarbellisine (**21**) and hippeastrine (**25**) [71]. *C. nobilis* AAs clivatine (**23**), clivimine (**26**), nobilisine (**24**), (+)-8-demethylmaritidine (**27**), as well as nobilisitine A and B, all showed anti-fungal activity against *C. albicans* [72, 92, 93]. *C. angustum* extracts displayed mycostatic activity against *C. albicans* with an IC_{50} of 78 µg/mL [94].

Anti-parasitic

Since several AAs, due to their cytotoxic effects, are used by amaryllidaceous plants to fend off pests, they can also serve to kill human parasites. This is the case with lycorine (**5**), haemanthamine (**8**) and haemanthidine (**15**) which possess activity against the malarial protozoan parasite *Plasmodium falciparum* [73-75]. Lycorine (**5**) is also effective against other protozoan parasites such as *Trichomonas vaginalis*, the cause of the sexually transmitted disease trichomonosis [76, 77, 95].

Anti-viral

Anti-viral activity is yet another therapeutic property exhibited by certain lycorine-type alkaloids. Lycorine (**5**) and its derivatives such as pseudolycorine (**14**) block viral RNA replication and suppress viral protein synthesis, inhibiting a number of RNA and DNA viruses [8, 78-81]. Pancratistatine

(**17**), a narciclasine-type AA, also possesses anti-viral activities [82, 83].

3.4. Miscellaneous Properties

Certain other effects of AAs have been observed. For example, lycorine (**5**) possesses anti-inflammatory characteristics since it can block lipopolysaccharide (LPS)-induced production of pro-inflammatory mediators and decreases LPS-induced mortality in mice [7, 84, 85]. Homolycorine (**6**) and 8-*O*-demethylhomolycorine have a hypotensive effect on normotensive rats [87]. Haemanthamine (**8**) has anti-oxidant activity quantified as scavenging action on the pre-formed free radicals determined by 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazinyl radical (DPPH) assay [86]. In addition, haemanthidine (**15**) has been reported to be an anti-inflammatory agent in mice by the carrageenan-induced oedema test [85] and an analgesic with higher activity than acetylsalicylic acid [88].

4. GALANTHAMINE

Since galanthamine (**7**) is the only AA currently used in medicine, we briefly report, the clinical studies conducted, as well as the pharmacokinetic data and adverse effects of the drug.

4.1. Clinical Studies

Four randomized, double-blind, placebo-controlled clinical trials of galanthamine (**7**) were conducted in patients with Alzheimer's disease [96]. The doses studied ranged from 8 to 32 mg/day given as twice daily doses. In 3 of the 4 studies, patients were started on a low dose then titrated weekly. In the fourth study, dose escalation of 8 mg/day occurred over 4 week intervals. The mean age of patients participating in the four trials was 75 years with a range of 41 to 100 years. The patients sex distribution was 62% women and 38% men, and the racial distribution was 94% white, 3% black and 3% others. In all studies, the improvement observed in the groups treated with galanthamine (**7**) was statistically significantly higher than in the placebo group [36, 96].

4.2. Pharmacokinetics

Rapid absorption was observed with a single dose of 8 mg of galanthamine (**7**) taken orally by 12 healthy males. After 1.2 hours (T_{max}), the peak plasma concentration (C_{max}) reached 43 ± 13 ng/mL. With that dose, the maximum inhibition of AChE activity, after about 1 hour was ~40%. The absolute oral bioavailability of galanthamine (**7**) is 88.5% [36]. One-hour intravenous infusion of the same dose in 12 healthy males, showed blood distribution of galanthamine (**7**) as follows: 52.7% to blood cells, 39.0% to plasma water and only 8.4% bound to plasma proteins [36].

Galanthamine (**7**) is metabolised by hepatic enzymes, such as the cytochrome P450 monooxygenases and the glucuronosylases or is excreted unchanged in the urine. *In vitro* studies indicate that CYP2D6 and CYP3A4 are the major cytochrome P450 isoenzymes involved in the metabolism of galanthamine (**7**) [97]. Galanthamine (**7**) elimination is biphasic, with a terminal half-life in the order of 7–10 hours. Difference between total plasma clearance after oral administration was observed between patients with Alzheimer's disease and healthy subjects (13.2 L/h versus 19.4 L/h). Thus, the plasma concentrations of galanthamine (**7**) in elderly patients (median age 75) with Alzheimer's disease are 30–40% higher than in young subjects (median age 28) [36].

4.3. Adverse Effects

The most common adverse effects are nausea and vomiting which tend to gradually disappear as the body adapts to the treatment. Other possible side effects, among many, include: abdominal pain, diarrhea, indigestion, loss of appetite, dehydration, headache, depression and weight loss [36]. Some serious side effects observed in a few cases include difficulty urinating and pain while urinating, seizures, and fainting [98].

CONCLUSION

The exact function of most alkaloids in plants remains mysterious. Hypotheses have included views such as metabolic waste, nitrogen storage molecules, defense compounds or ecological adaptors [99, 100]. Regardless of the validity of any of these hypotheses, the large structural variation and wide range of biological activities associated with alkaloids make them highly interesting compounds for medicinal chemists and molecular biochemists. To date, over 500 structurally diverse AAs have been isolated and identified from plants. However, given the large number of uncharacterized plant species in this family, we can expect that many new AAs will be isolated from Amaryllidaceae plants in the future.

Research is often limited to a very small number of compounds such as galanthamine (**7**), the only AA pharmaceutically and commercially utilized. This might be due to the restricted availability of AAs due to their low concentrations in plants and the absence of economically viable chemical synthesis protocols. In view of the significantly biological activities exhibited by AAs, development of new medicines derived from these natural compounds of great importance is expected. For example, narciclasine (**11**), pancratistatine (**17**) and their hemisynthetic derivatives are in preclinical development as very promising anti-tumour agents. Consequently, chemical synthetic approaches to AAs and related analogues represent an important focus for future investigations.

In addition, biotechnological approaches for the production of AAs provides an inexpensive, efficient and practical alternative [15]. However, a better understanding of the AA biosynthetic pathway at the molecular and biochemical levels is needed to provide tools, and create the opportunity for the metabolic engineering of plants and reconstitution of the AA pathway in heterologous systems as a potential alternative commercial source for pharmacologically active AAs.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

This work was financially supported by the discovery grant program from the Natural Sciences and Engineering Research Council of Canada (RGPIN-2014-05294).

REFERENCES

[1] Mahajan, M.; Kumar, V.; Yadav, S.K. Alkaloids : properties, applications and pharmacological effects. **2010**: Nova Science Publishers 185.

[2] Hesse, M. Alkaloids: Nature's Curse Or Blessing? **2002**: Verlag Helvetica Chimica Acta.

[3] Martin, S.F. *The amaryllidaceae alkaloids.*, in *The alkaloids*. **1988**, Academic Press. p. 251.

[4] Jin, Z.; Xu, X.-H. *Amaryllidaceae Alkaloids*, in *Natural Products*, K.G. Ramawat and J.-M. Mérillon, Editors. **2013**, Springer Berlin Heidelberg. p. 479-522.

[5] Houghton, P.J.; Ren, Y.; Howes, M.-J. Acetylcholinesterase inhibitors from plants and fungi. *Natural Product Reports*, **2006**. *23*(2): p. 181-199.

[6] McNulty, J.; Nair, J.J.; Little, J.R.; Brennan, J.D.; Bastida, J. Structure-activity studies on acetylcholinesterase inhibition in the lycorine series of Amaryllidaceae alkaloids. *Bioorg Med Chem Lett*, **2010**. *20*(17): p. 5290-4.

[7] Cao, Z.; Yang, P.; Zhou, Q. Multiple biological functions and pharmacological effects of lycorine. *Science China Chemistry*, **2013**. *56*(10): p. 1382-1391.

[8] Chen, D.; Cai, J.; Yin, J.; Jiang, J.; Jing, C.; Zhu, Y.; Cheng, J.; Di, Y.; Zhang, Y.; Cao, M.; Li, S.; Peng, Z.; Hao, X. Lycorine-derived phenanthridine downregulators of host Hsc70 as potential hepatitis C virus inhibitors. *Future Med Chem*, **2015**. 7(5): p. 561-70.

[9] Kornienko, A.; Evidente, A. Chemistry, biology, and medicinal potential of narciclasine and its congeners. *Chem Rev*, **2008**. *108*(6): p. 1982-2014.

[10] Lefranc, F.; Sauvage, S.; Van Goietsenoven, G.; Megalizzi, V.; Lamoral-Theys, D.; Debeir, O.; Spiegl-Kreinecker, S.; Berger, W.; Mathieu, V.; Decaestecker, C.; Kiss, R. Narciclasine, a plant growth modulator, activates Rho and stress fibers in glioblastoma cells. *Mol Cancer Ther*, **2009**. *8*(7): p. 1739-50.

[11] Heinrich, M.; Lee Teoh, H. Galanthamine from snowdrop--the development of a modern drug against Alzheimer's disease from local Caucasian knowledge. *J Ethnopharmacol*, **2004**. *92*(2-3): p. 147-62.

[12] Bastida, J.; Berkov, S.; Torras, L.; Pigni, N.B.; de Andrade, J.P.; Vanessa Martinez, V.; Codina, C.; Viladomat, F. Chapter 3: Chemical and biological aspects of Amaryllidaceae alkaloids., E.D. Muñoz-Torrero, T.R. Network, F.P.O. 37/661 (2), T.-. 023, and I. Kerala, Editors. **2011**. Recent Advances in Pharmaceutical Sciences.

[13] Nair, J.J.; van Staden, J. Pharmacological and toxicological insights to the South African Amaryllidaceae. *Food Chem Toxicol*, **2013**. *62C*: p. 262-275.

[14] Takos, A.M.; Rook, F. Towards a molecular understanding of the biosynthesis of amaryllidaceae alkaloids in support of their expanding medical use. *Int J Mol Sci*, **2013**. *14*(6): p. 11713-41.

[15] Diamond, A.; Desgagné-Penix, I. Metabolic engineering for the production of plant isoquinoline alkaloids. *Plant Biotechnology Journal* **2015**. *13*.

[16] Lubbe, A.; Pomahacova, B.; Choi, Y.H.; Verpoorte, R. Analysis of metabolic variation and galanthamine content in Narcissus bulbs by 1H NMR. *Phytochem Anal*, **2010**. *21*(1): p. 66-72.

[17] Berkov, S.; Georgieva, L.; Kondakova, V.; Atanassov, A.; Viladomat, F.; Bastida, J.; Codina, C. Plant Sources of Galanthamine: Phytochemical and Biotechnological Aspects. *Biotechnology & Biotechnological Equipment*, **2009**. *23*(2): p. 1170-1176.

[18] Wang, Y.; Zhu, G.; Li, X.; Hao, Z. Simultaneous determination of galanthamine and lycorine in Lycoris radiata by a capillary electrophoresis with an electrochemiluminescence method. *J Sep Sci*, **2014**. *37*(20): p. 3007-12.

[19] Berkov, S.; Martinez-Frances, V.; Bastida, J.; Codina, C.; Rios, S. Evolution of alkaloid biosynthesis in the genus Narcissus. *Phytochemistry*, **2014**. *99*: p. 95-106.

[20] Berkov, S.; Bastida, J.; Viladomat, F.; Codina, C. Development and validation of a GC-MS method for rapid determination of galanthamine in Leucojum aestivum and Narcissus ssp.: a metabolomic approach. *Talanta*, **2011**. *83*(5): p. 1455-65.

[21] Torras-Claveria, L.; Berkov, S.; Codina, C.; Viladomat, F.; Bastida, J. Metabolomic analysis of bioactive Amaryllidaceae alkaloids of ornamental varieties of Narcissus by GC–MS combined with k-means cluster analysis. *Industrial Crops and Products*, **2014**. *56*(0): p. 211-222.

[22] Bohlin, L.; Göransson, U.; Alsmark, C.; Wedén, C.; Backlund, A. Natural products in modern life science. *Phytochemistry Reviews*, **2010**. *9*(2): p. 279-301.

[23] Barton, D.H.R.; Cohen, T. Some biogenetic aspects of phenol oxidation. *Basel: Festschrift Arthur Stoll. Birkhäuser*, **1957**. .

[24] Barton, D.H.R.; Kirby, G.W. Phenol oxidation and biosynthesis. Part V. Synthesis of galanthamine. *J. Chem. Soc.*, **1962**: p. 806-817.

[25] Barton, D.H.R.; Kirby, G.W.; Taylor, G.B.; Thomas, G.M. Phenol oxidation and biosynthesis. Part VI. The biogenesis of Amaryllidaceae alkaloids. *J. Chem. Soc.*, **1963**: p. 4545-4558.

[26] Bhandarkar, J.G.; Kirby, G.W. Structure and biosynthesis of chlidanthine. *Journal of the Chemical Society C: Organic*, **1970**(9): p. 1224-1227.

[27] Eichhorn, J.; Takada, T.; Kita, Y.; Zenk, M.H. Biosynthesis of the Amaryllidaceae alkaloid galanthamine. *Phytochemistry*, **1998**. *49*(4): p. 1037-1047.

[28] El Tahchy, A.; Boisbrun, M.; Ptak, A.; Dupire, F.; Chretien, F.; Henry, M.; Chapleur, Y.; Laurain-Mattar, D. New method for the study of Amaryllidaceae alkaloid biosynthesis using biotransformation of deuterium-labeled precursor in tissue cultures. *Acta Biochim Pol*, **2010**. *57*(1): p. 75-82.

[29] El Tahchy, A.; Ptak, A.; Boisbrun, M.; Barre, E.; Guillou, C.; Dupire, F.; Chretien, F.; Henry, M.; Chapleur, Y.; Laurain-Mattar, D. Kinetic study of the rearrangement of deuterium-labeled 4'-O-methylnorbelladine in Leucojum aestivum shoot cultures by mass spectrometry. Influence of precursor feeding on amaryllidaceae alkaloid accumulation. *J Nat Prod*, **2011**. *74*(11): p. 2356-61.

[30] Saliba, S.; Ptak, A.; Laurain-Mattar, D. 4'-O-Methylnorbelladine feeding enhances galanthamine and lycorine production by Leucojum aestivum L. shoot cultures. *Engineering in Life Sciences*, **2015**: p. n/a-n/a.

[31] Singh, A.; Desgagne-Penix, I. Biosynthesis of the Amaryllidaceae alkaloids. *Plant Science Today*, **2014**. *1*(2): p. 114-120.

[32] Facchini, P.J.; Huber-Allanach, K.L.; Tari, L.W. Plant aromatic L-amino acid decarboxylases: evolution, biochemistry, regulation, and metabolic engineering applications. *Phytochemistry*, **2000**. *54*(2): p. 121-38.

[33] Kilgore, M.B.; Augustin, M.M.; Starks, C.M.; O'Neil-Johnson, M.; May, G.D.; Crow, J.A.; Kutchan, T.M. Cloning and Characterization of a Norbelladine 4'-O-Methyltransferase Involved in the Biosynthesis of the Alzheimer's Drug Galanthamine in Narcissus sp. aff. pseudonarcissus. *PLoS One*, **2014**. *9*(7): p. e103223.

[34] Kilgore, M.; Augustin, M.M.; May, G.D.; Crow, J.A.; Kutchan, T.M. CYP96T1 of Narcissus sp. aff. pseudonarcissus Catalyzes Formation of the Para-Para' C-C Phenol Couple in the Amaryllidaceae Alkaloids. *Frontiers in Plant Science*, **2016**. *7*.

[35] Takos, A.; Rook, F. Towards a Molecular Understanding of the Biosynthesis of Amaryllidaceae Alkaloids in Support of Their Expanding Medical Use. *International Journal of Molecular Sciences*, **2013**. *14*(6): p. 11713.

[36] Janssen REMINYL® ER -galantamine hydrobromide extended release capsules 8 mg, 16 mg, 24 mg galantamine base. **2014**.

[37] Barik, J.; Dajas-Bailador, F.; Wonnacott, S. Cellular responses to nicotinic receptor activation are decreased after prolonged exposure to galantamine in human neuroblastoma cells. *British journal of pharmacology*, **2005**. *145*(8): p. 1084-1092.

[38] Sweeney, J.E.; Puttfarcken, P.S.; Coyle, J.T. Galanthamine, an acetylcholinesterase inhibitor: a time course of the effects on performance and neurochemical parameters in mice. *Pharmacol Biochem Behav*, **1989**. *34*(1): p. 129-37.

[39] Thomsen, T.; Kewitz, H. Selective inhibition of human acetylcholinesterase by galanthamine *in vitro* and *in vivo*. *Life Sci*, **1990**. *46*(21): p. 1553-8.

[40] Greenblatt, H.M.; Kryger, G.; Lewis, T.; Silman, I.; Sussman, J.L. Structure of acetylcholinesterase complexed with (-)-galanthamine at 2.3 A resolution. *FEBS Lett*, **1999**. *463*(3): p. 321-6.

[41] Sussman, J.L.; Harel, M.; Frolow, F.; Oefner, C.; Goldman, A.; Toker, L.; Silman, I. Atomic structure of acetylcholinesterase from Torpedo californica: a prototypic acetylcholine-binding protein. *Science*, **1991**. *253*(5022): p. 872-9.

[42] Santos, M.D.; Alkondon, M.; Pereira, E.F.; Aracava, Y.; Eisenberg, H.M.; Maelicke, A.; Albuquerque, E.X. The nicotinic allosteric potentiating ligand galantamine facilitates synaptic transmission in the mammalian central nervous system. *Molecular pharmacology*, **2002**. *61*(5): p. 1222-1234.

[43] Egea, J.; Martin-de-Saavedra, M.; Parada, E.; Romero, A.; Del Barrio, L.; Rosa, A.; Garcia, A.; Lopez, M. Galantamine elicits neuroprotection by inhibiting iNOS, NADPH oxidase and ROS in hippocampal slices stressed with anoxia/reoxygenation. *Neuropharmacology*, **2012**. *62*(2): p. 1082-1090.

[44] Lorrio, S.; Sobrado, M.; Arias, E.; Roda, J.M.; García, A.G.; Lopez, M.G. Galantamine postischemia provides neuroprotection and memory recovery against transient global cerebral ischemia in gerbils. *Journal of Pharmacology and Experimental Therapeutics*, **2007**. *322*(2): p. 591-599.

[45] Hilmas, C.J.; Poole, M.J.; Finneran, K.; Clark, M.G.; Williams, P.T. Galantamine is a novel post-exposure therapeutic against lethal VX challenge. *Toxicology and applied pharmacology*, **2009**. *240*(2): p. 166-173.

[46] Pereira, E.F.; Aracava, Y.; Alkondon, M.; Akkerman, M.; Merchenthaler, I.; Albuquerque, E.X. Molecular and cellular actions of galantamine: clinical implications for treatment of organophosphorus poisoning. *Journal of molecular neuroscience*, **2010**. *40*(1-2): p. 196-203.

[47] Oikawa, S.; Mano, A.; Iketani, M.; Kakinuma, Y. Nicotinic receptor-dependent and -independent effects of galantamine, an acetylcholinesterase inhibitor, on the non-neuronal acetylcholine system in C2C12 cells. *Int Immunopharmacol*, **2015**.

[48] da Silva, A.F.S.; de Andrade, J.P.; Bevilaqua, L.R.; de Souza, M.M.; Izquierdo, I.; Henriques, A.T.; Zuanazzi, J.Â.S. Anxiolytic-, antidepressant-and anticonvulsant-like effects of the alkaloid montanine isolated from Hippeastrum vittatum. *Pharmacology Biochemistry and Behavior*, **2006**. *85*(1): p. 148-154.

[49] Hayden, R.E.; Pratt, G.; Drayson, M.T.; Bunce, C.M. Lycorine sensitizes CD40 ligand-protected chronic lymphocytic leukemia cells to bezafibrate- and medroxyprogesterone acetate-induced apoptosis but dasatanib does not overcome reported CD40-mediated drug resistance. *Haematologica*, **2010**. *95*(11): p. 1889-96.

[50] Lamoral-Theys, D.; Decaestecker, C.; Mathieu, V.; Dubois, J.; Kornienko, A.; Kiss, R.; Evidente, A.; Pottier, L. Lycorine and its derivatives for anticancer drug design. *Mini Rev Med Chem*, **2010**. *10*(1): p. 41-50.

[51] Li, Y.; Liu, J.; Tang, L.J.; Shi, Y.W.; Ren, W.; Hu, W.X. Apoptosis induced by lycorine in KM3 cells is associated with the G0/G1 cell cycle arrest. *Oncol Rep*, **2007**. *17*(2): p. 377-84.

[52] Liu, X.S.; Jiang, J.; Jiao, X.Y.; Wu, Y.E.; Lin, J.H.; Cai, Y.M. Lycorine induces apoptosis and down-regulation of Mcl-1 in human leukemia cells. *Cancer Lett*, **2009**. *274*(1): p. 16-24.

[53] Wang, P.; Yuan, H.H.; Zhang, X.; Li, Y.P.; Shang, L.Q.; Yin, Z. Novel lycorine derivatives as anticancer agents: synthesis and *in vitro* biological evaluation. *Molecules*, **2014**. *19*(2): p. 2469-80.

[54] McNulty, J.; Nair, J.J.; Bastida, J.; Pandey, S.; Griffin, C. Structure-activity studies on the lycorine pharmacophore: A potent inducer of apoptosis in human leukemia cells. *Phytochemistry*, **2009**. *70*(7): p. 913-919.

[55] Hohmann, J.; Forgo, P.; Molnár, J.; Wolfard, K.; Molnár, A.; Thalhammer, T.; Máthé, I.; Sharples, D. Antiproliferative amaryllidaceae alkaloids isolated from the bulbs of Sprekelia formosissima and Hymenocallis x festalis. *Planta medica*, **2002**. *68*(5): p. 454-457.

[56] Luchetti, G.; Johnston, R.; Mathieu, V.; Lefranc, F.; Hayden, K.; Andolfi, A.; Lamoral-Theys, D.; Reisenauer, M.R.; Champion, C.; Pelly, S.C. Bulbispermine: A Crinine-Type Amaryllidaceae Alkaloid Exhibiting Cytostatic Activity toward Apoptosis-Resistant Glioma Cells. *ChemMedChem*, **2012**. *7*(5): p. 815-822.

[57] Van Goietsenoven, G.; Andolfi, A.; Lallemand, B.; Cimmino, A.; Lamoral-Theys, D.; Gras, T.; Abou-Donia, A.; Dubois, J.; Lefranc, F.; Mathieu, V. Amaryllidaceae Alkaloids Belonging to Different Structural Subgroups Display Activity against Apoptosis-Resistant Cancer Cells†. *Journal of natural products*, **2010**. *73*(7): p. 1223-1227.

[58] Nair, J.J.; Rárová, L.; Strnad, M.; Bastida, J.; van Staden, J. Apoptosis-inducing effects of distichamine and narciprimine, rare alkaloids of the plant family Amaryllidaceae. *Bioorganic & medicinal chemistry letters*, **2012**. *22*(19): p. 6195-6199.

[59] Evidente, A.; Kireev, A.S.; Jenkins, A.R.; Romero, A.E.; Steelant, W.F. Biological evaluation of structurally diverse Amaryllidaceae alkaloids and their synthetic derivatives: discovery of novel leads for anticancer drug design. *Planta medica*, **2009**. *75*(5): p. 501.

[60] Evidente, A.; Kornienko, A. Anticancer evaluation of structurally diverse Amaryllidaceae alkaloids and their synthetic derivatives. *Phytochemistry Reviews*, **2009**. *8*(2): p. 449-459.

[61] Evidente, A.; Kireev, A.S.; Jenkins, A.R.; Romero, A.E.; Steelant, W.F.; Van Slambrouck, S.; Kornienko, A. Biological evaluation of structurally diverse amaryllidaceae alkaloids and their synthetic derivatives: discovery of novel leads for anticancer drug design. *Planta Med*, **2009**. *75*(5): p. 501-7.

[62] Havelek, R.; Seifrtova, M.; Kralovec, K.; Bruckova, L.; Cahlikova, L.; Dalecka, M.; Vavrova, J.; Rezacova, M.; Opletal, L.; Bilkova, Z. The effect of Amaryllidaceae alkaloids haemanthamine and haemanthidine on cell cycle progression and apoptosis in p53-negative human leukemic Jurkat cells. *Phytomedicine*, **2014**. *21*(4): p. 479-90.

[63] Silva, A.F.; de Andrade, J.P.; Machado, K.R.; Rocha, A.B.; Apel, M.A.; Sobral, M.E.; Henriques, A.T.; Zuanazzi, J.A. Screening for cytotoxic activity of extracts and isolated alkaloids from bulbs of Hippeastrum vittatum. *Phytomedicine*, **2008**. *15*(10): p. 882-5.

[64] Dumont, P.; Ingrassia, L.; Rouzeau, S.; Ribaucour, F.; Thomas, S.; Roland, I.; Darro, F.; Lefranc, F.; Kiss, R. The Amaryllidaceae isocarbostyril narciclasine induces apoptosis by activation of the death receptor and/or mitochondrial pathways in cancer cells but not in normal fibroblasts. *Neoplasia*, **2007**. *9*(9): p. 766-76.

[65] Van Goietsenoven, G.; Hutton, J.; Becker, J.P.; Lallemand, B.; Robert, F.; Lefranc, F.; Pirker, C.; Vandenbussche, G.; Van Antwerpen, P.; Evidente, A.; Berger, W.; Prevost, M.; Pelletier, J.; Kiss, R.; Kinzy, T.G.; Kornienko, A.; Mathieu, V. Targeting of eEF1A with Amaryllidaceae isocarbostyrils as a strategy to combat melanomas. *FASEB J*, **2010**. *24*(11): p. 4575-84.

[66] McLachlan, A.; Kekre, N.; McNulty, J.; Pandey, S. Pancratistatin: a natural anti-cancer compound that targets mitochondria specifically in cancer cells to induce apoptosis. *Apoptosis*, **2005**. *10*(3): p. 619-30.

[67] Zupkó, I.; Réthy, B.; Hohmann, J.; Molnar, J.; Ocsovszki, I.; Falkay, G. Antitumor Activity of Alkaloids Derived from Amaryllidaceae Species. *in vivo*, **2009**. *23*: p. 41-48.

[68] Weniger, B.; Italiano, L.; Beck, J.P.; Bastida, J.; Bergonon, S.; Codina, C.; Lobstein, A.; Anton, R. Cytotoxic activity of Amaryllidaceae alkaloids. *Planta Med*, **1995**. *61*(1): p. 77-9.

[69] Schrader, K.K.; Andolfi, A.; Cantrell, C.L.; Cimmino, A.; Duke, S.O.; Osbrink, W.; Wedge, D.E.; Evidente, A. A survey of phytotoxic microbial and plant metabolites as potential natural products for pest management. *Chemistry & biodiversity*, **2010**. *7*(9): p. 2261-2280.

[70] Schrader, K.K.; Avolio, F.; Andolfi, A.; Cimmino, A.; Evidente, A. Ungeremine and Its hemisynthesized analogues as bactericides against Flavobacterium columnare. *J Agric Food Chem*, **2013**. *61*(6): p. 1179-83.

[71] Evidente, A.; Andolfi, A.; Abou-Donia, A.H.; Touema, S.M.; Hammoda, H.M.; Shawky, E.; Motta, A. (-)-Amarbellisine, a lycorine-type alkaloid from Amaryllis belladonna L. growing in Egypt. *Phytochemistry*, **2004**. *65*(14): p. 2113-8.

[72] Evidente, A.; Andolfi, A.; Abou-Donia, A.H.; Darwish, F.A.; Hammoda, H.A.M.; Motta, A. Minor alkaloids from Clivia nobilis Regel. *Alex J Pharm Sci.*, **2005**. *19*: p. 49-53.

[73] Kaya, G.I.; Sarıkaya, B.; Onur, M.A.; Somer, N.U.; Viladomat, F.; Codina, C.; Bastida, J.; Lauinger, I.L.; Kaiser, M.; Tasdemir, D. Antiprotozoal alkaloids from Galanthus trojanus. *Phytochemistry Letters*, **2011**. *4*(3): p. 301-305.

[74] Şener, B.; Orhan, I.; Satayavivad, J. Antimalarial activity screening of some alkaloids and the plant extracts from Amaryllidaceae. *Phytotherapy Research*, **2003**. *17*(10): p. 1220-1223.

[75] Cedrón, J.C.; Gutiérrez, D.; Flores, N.; Ravelo, Á.G.; Estévez-Braun, A. Synthesis and antimalarial activity of new

haemanthamine-type derivatives. *Bioorganic & Medicinal Chemistry*, **2012**. *20*(18): p. 5464-5472.

[76] Giordani, R.B.; de Brum Vieira, P.; Weizenmann, M.; Rosemberg, D.B.; Souza, A.P.; Bonorino, C.; De Carli, G.A.; Bogo, M.R.; Zuanazzi, J.A.; Tasca, T. Lycorine induces cell death in the amitochondriate parasite, Trichomonas vaginalis, *via* an alternative non-apoptotic death pathway. *Phytochemistry*, **2011**. *72*(7): p. 645-650.

[77] Giordani, R.B.; Junior, C.O.; de Andrade, J.P.; Bastida, J.; Zuanazzi, J.A.; Tasca, T.; de Almeida, M.V. Lycorine derivatives against Trichomonas vaginalis. *Chemical biology & drug design*, **2012**. *80*(1): p. 129-133.

[78] He, J.; Qi, W.B.; Wang, L.; Tian, J.; Jiao, P.R.; Liu, G.Q.; Ye, W.C.; Liao, M. Amaryllidaceae alkaloids inhibit nuclear-to-cytoplasmic export of ribonucleoprotein (RNP) complex of highly pathogenic avian influenza virus H5N1. *Influenza Other Respir Viruses*, **2013**. *7*(6): p. 922-31.

[79] Li, S.Y.; Chen, C.; Zhang, H.Q.; Guo, H.Y.; Wang, H.; Wang, L.; Zhang, X.; Hua, S.N.; Yu, J.; Xiao, P.G.; Li, R.S.; Tan, X. Identification of natural compounds with antiviral activities against SARS-associated coronavirus. *Antiviral Res*, **2005**. *67*(1): p. 18-23.

[80] Szlavik, L.; Gyuris, A.; Minarovits, J.; Forgo, P.; Molnar, J.; Hohmann, J. Alkaloids from Leucojum vernum and antiretroviral activity of Amaryllidaceae alkaloids. *Planta Med*, **2004**. *70*(9): p. 871-3.

[81] Hwang, Y.C.; Chu, J.J.; Yang, P.L.; Chen, W.; Yates, M.V. Rapid identification of inhibitors that interfere with poliovirus replication using a cell-based assay. *Antiviral Res*, **2008**. *77*(3): p. 232-6.

[82] Havelek, R.; Seifrtova, M.; Kralovec, K.; Bruckova, L.; Cahlikova, L.; Dalecka, M.; Vavrova, J.; Rezacova, M.; Opletal, L.; Bilkova, Z. The effect of Amaryllidaceae alkaloids haemanthamine and haemanthidine on cell cycle progression and apoptosis in p53-negative human leukemic Jurkat cells. *Phytomedicine*, **2014**. *21*(4): p. 479-490.

[83] Liu, J.; Hu, W.-X.; He, L.-F.; Ye, M.; Li, Y. Effects of lycorine on HL-60 cells *via* arresting cell cycle and inducing apoptosis. *FEBS letters*, **2004**. *578*(3): p. 245-250.

[84] Kang, J.; Zhang, Y.; Cao, X.; Fan, J.; Li, G.; Wang, Q.; Diao, Y.; Zhao, Z.; Luo, L.; Yin, Z. Lycorine inhibits lipopolysaccharide-induced iNOS and COX-2 up-regulation in RAW264. 7 cells through suppressing P38 and STATs activation and increases the survival rate of mice after LPS challenge. *International immunopharmacology*, **2012**. *12*(1): p. 249-256.

[85] Çitoğlu, G.; Tanker, M.; Gümüşel, B. Antiinflammatory effects of lycorine and haemanthidine. *Phytotherapy Research*, **1998**. *12*(3): p. 205-206.

[86] Oloyede, G.; Oke, M.; Raji, Y.; Olugbade, A. Antioxidant and anticonvulsant alkaloids in Crinum ornatum bulb extract. *World journal of chemistry*, **2010**. *5*(1): p. 26-31.

[87] Schmeda-Hirschmann, G.; RodrÍGuez, J.A.; Loyola, J.I.; Astudillo, L.; Bastida, J.; Viladomat, F.; Codina, C. Activity of Amaryllidaceae Alkaloids on the Blood Pressure of Normotensive Rats. *Pharmacy and Pharmacology Communications*, **2000**. *6*(7): p. 309-312.

[88] Tanker, M.; Çitoglu, G.; Gümüşel, B.; Hener, B. Alkaloids of Sternbergia clusiana and their analgesic effects. *Pharmaceutical Biology*, **1996**. *34*(3): p. 194-197.

[89] Citoglu, G.S.; Acikara, O.B.; Yilmaz, B.S.; Ozbek, H. Evaluation of analgesic, anti-inflammatory and hepatoprotective effects of lycorine from Sternbergia fisheriana (Herbert) Rupr. *Fitoterapia*, **2012**. *83*(1): p. 81-7.

[90] de Andrade, J.P.; Pigni, N.B.; Torras-Claveria, L.; Berkov, S.; Codina, C.; Viladomat, F.; Bastida, J. Bioactive alkaloid extracts from Narcissus broussonetii: mass spectral studies. *J Pharm Biomed Anal*, **2012**. *70*: p. 13-25.

[91] Abou-Donia, A.H.; Abib, A.-A.; El Din, A.S.; Evidente, A.; Gaber, M.; Scopa, A. Two betaine-type alkaloids from Egyptian Pancratium maritimum. *Phytochemistry*, **1992**. *31*(6): p. 2139-2141.

[92] Evidente, A.; Abou-Donia, A.H.; Darwish, F.A.; Amer, M.E.; Kassem, F.F.; Hammoda, H.A.; Motta, A. Nobilisitine A and B, two masanane-type alkaloids from Clivia nobilis. *Phytochemistry*, **1999**. *51*(8): p. 1151-1155.

[93] Jeffs, P.W.; Mueller, L.; Abou-Donia, A.H.; Seif el-Din, A.A.; Campau, D. Nobilisine, a New Alkaloid from Cli*via* nobilis. *Journal of Natural Products*, **1988**. *51*(3): p. 549-554.

[94] Iannello, C.; Bastida, J.; Bonvicini, F.; Antognoni, F.; Gentilomi, G.A.; Poli, F. Chemical composition, and *in vitro* antibacterial and antifungal activity of an alkaloid extract from Crinum angustum Steud. *Nat Prod Res*, **2014**. *28*(10): p. 704-10.

[95] Garreau de Loubresse, N.; Prokhorova, I.; Holtkamp, W.; Rodnina, M.V.; Yusupova, G.; Yusupov, M. Structural basis for the inhibition of the eukaryotic ribosome. *Nature*, **2014**. *513*(7519): p. 517-522.

[96] https://clinicaltrials.gov/ct2/show/NCT00253214. **2005**.

[97] Noetzli, M.; Guidi, M.; Ebbing, K.; Eyer, S.; Zumbach, S.; Giannakopoulos, P.; von Gunten, A.; Csajka, C.; Eap, C.B. Relationship of CYP2D6, CYP3A, POR, and ABCB1 genotypes with galantamine plasma concentrations. *Ther Drug Monit*, **2013**. *35*(2): p. 270-5.

[98] https://www.nlm.nih.gov/medlineplus/druginfo/meds/a699058.html#side-effects. **2015**.

[99] Robinson, T. Metabolism and function of alkaloids in plants. *Science*, **1974**. *184*(4135): p. 430-5.

[100] Mithofer, A.; Boland, W. Plant defense against herbivores: chemical aspects. *Annu Rev Plant Biol*, **2012**. *63*: p. 431-50.