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Biosynthesis of alkaloids in Amaryllidaceae plants: a review

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Abstract Amaryllidaceae alkaloids are a group of specialized metabolites found predominantly in the Amaryllidaceae plant family. Approximately 600 naturally occurring Amaryllidaceae alkaloids have been identified, many of which possess a variety of potent biological and pharmacological properties. However, only the Amaryllidaceae alkaloid galanthamine, an acetylcholinesterase inhibitor used to treat symptoms of neurodegenerative disorders such as Alzheimer's disease, is currently on the market. Despite promising pharmacological potential, the clinical development of Amaryllidaceae alkaloids is hindered by limited commercial availability. In contrast to the large body of knowledge on the pharmacological and phytochemical aspects of Amaryllidaceae alkaloids, their molecular and physiological features are less explored. A better understanding of Amaryllidaceae alkaloid biosynthesis and metabolic regulation is crucial to take advantage of new metabolic engineering technologies for improving the efficiency and sustainability of plant or microbial Amaryllidaceae alkaloid production. Although there is still much to learn, over the past several years comparative transcriptomic and traditional biochemical approaches have led to significant advances in the identification of the molecular players involved in producing Amaryllidaceae alkaloids including enzymes from the shikimate and phenylpropanoid pathways. The identity of few Amaryllidaceae alkaloid biosynthetic enzymes and several genes encoding them has been reported. In this review, an overview of the current knowledge on the biosynthesis of Amaryllidaceae alkaloids is presented.

Keywords Alkaloid biosynthetic pathways · Phenylpropanoids · Plant specialized metabolism · Pathway gene discovery · Shikimate pathway · Metabolic engineering

Abbreviations

3,4-DHBA	3,4-dihydroxybenzaldehyde
4CL	4-hydroxycinnamic acid CoA ligase
ADH	Arogenate dehydrogenase
ADT	Arogenate dehydratase
AKR	Aldo-keto reductases
BIA	Benzylisoquinoline alkaloids
C4H	*Trans*-cinnamate hydroxylase (CYP73A1)
C3H	Coumaroyl shikimate hydroxylase (CYP98A3)
CCOMT	Caffeoyl CoA 3-O-methyltransferase
CES	Caffeoyl shikimate esterase
CM	Chorismate mutase
CYP96T	Cytochrome P450 monooxygenase 96T1

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GOGAT	Glutamine oxoglutarate aminotransferase
GS	Glutamine synthase
HBS	Hydroxybenzaldehyde synthase
HCT	Hydroxycinnamoyl CoA shikimate transferase
N4OMT	Norbelladine 4'-*O*-methlytransferase
NADPH	Nicotinamide adenine dinucleotide phosphate
NBS	Norbelladine synthase
NCS	Norcoclaurine synthase
NR	Noroxomaritidine reductase
OMT	*O*-methyltransferase
PAL	Phenylalanine ammonia lyase
PTAL	Phenylalanine/tyrosine ammonia lyase
PheH	Phenylalanine hydroxylase
PPA-AT	Prephenate aminotransferase
PPDiox	Phenylpropanoid 2,3-dioxygenase
SDR	Short-chain alcohol dehydrogenase/ reductase
TYDC	Tyrosine/DOPA decarboxylase
VpVAN	Vanillin synthase

Introduction

The Amaryllidaceae plant family consists of about 75 genera, whose 1600 species are naturally widely distributed in tropical and subtropical regions of the world (Christenhusz and Byng 2016). Amaryllidaceae plants have been used as traditional medicine for thousands of years and are among the top 20 most widely considered medicinal plant families to date (Jin and Yao 2019). For example, in Senegal, a mixture prepared from bulbs of *Crinum giganteum* has been used to treat and prevent infection of wounds, whereas in Nigeria, medicine produced from bulbs of *C. glaucum* or *C. jagus* has been traditionally recommended for various mental illness. A number of pharmacologically active compounds have been identified and characterized from this plant family.

A unique feature for plants of the Amaryllidaceae family is the production of a group of structurally diverse alkaloids. Alkaloids are a large and diverse class of plant specialized metabolites occurring in about 20% of all plant species (Facchini and De Luca 2008). About 20,000 alkaloids are known and mostly isolated from plants (Coqueiro and Verpoorte 2015). Amaryllidaceae alkaloids belong to the large group of isoquinoline alkaloids, one of the two largest groups of alkaloids along with the indole alkaloids (Coqueiro and Verpoorte 2015). Lycorine, was the first isolated Amaryllidaceae alkaloid in 1877 from *Narcissus pseudonarcissus* (Cook and Loudon 1952). Until now, up to 600 Amaryllidaceae alkaloids have been identified and this number is increasing every year (Jin 2007, 2009, 2011, 2013, 2016; Jin and Xu 2013; Jin and Yao 2019).

Interest in Amaryllidaceae alkaloid metabolism raised fundamental questions concerning the biological function of these molecules. As specialized metabolites, alkaloids do not appear essential for normal plant growth and development, but may play a key role in plant defense. Ironically, much more is known about the pharmacology of Amaryllidaceae alkaloids and relatively little study has been dedicated to determining their ecophysiological roles. Indeed, the pharmacological uses of Amaryllidaceae alkaloids are well defined including the potent inhibition of acetylcholinesterase (with potential application to Alzheimer's disease), cytotoxicity, antibacterial, antiviral, anti-inflammatory, antiparasitic, antihistaminic, antiproliferative, and anticancer [reviewed in (Cimmino et al. 2017; Hotchandani and Desgagne-Penix 2017; Kornienko and Evidente 2008; Nair and van Staden 2013; Ncube and Van Staden 2015)]. It has been proposed that Amaryllidaceae alkaloids are generally produced and used by plants for defense purposes to ward off pathogens, harmful insects and herbivores. For example, galanthamine serves as an insecticide by causing excess cholinergic stimulation in the insect (Houghton et al. 2006). Initially named galanthamine in 1947, because it was isolated and characterized from *Galanthus nivalis*, galantamine (the ''h'' was dropped in the 1990s when the international non-proprietary name was defined) generated more interest when its therapeutic usefulness was elucidated (Mucke 2015). Galanthamine inhibits the degradation of neurotransmitters acetylcholine (ACh) by binding to the enzyme, acetylcholinesterase (AChE) which is responsible for degrading ACh (McNulty et al. 2010). The resulting accumulation of ACh caused by galanthamine leads to increased neurotransmission causing continuous stimulation of the muscles, glands, and central nervous system, which can result in fatal convulsions. Another Amaryllidaceae alkaloid, lycorine, possesses antimicrobial and antiviral activities (Cao et al. 2013; Chen

et al. 2015) whereas narciclasine showed anti-cancer properties (Kornienko and Evidente 2008; Lefranc et al. 2009). Given these interesting and various biological properties, Amaryllidaceae alkaloids 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 Amaryllidaceae alkaloids, since many possess potent pharmacological properties (Bastida et al. 2011; Cimmino et al. 2017; Diamond and Desgagné-Penix 2015; Heinrich and Lee Teoh 2004; Hotchandani and Desgagne-Penix 2017; Kornienko and Evidente2008; Nair and van Staden 2013; Takos and Rook 2013).

Although several Amaryllidaceae alkaloids displayed interesting pharmacological applications, only one, galanthamine, has been used commercially to treat the symptoms of Alzheimer's disease (Heinrich and Lee Teoh 2004; Janssen 2014). The production and screening of Amaryllidaceae alkaloids in their native plant face several challenges. For example, many Amaryllidaceae alkaloids exist in trace amounts, and the methods for isolating and purifying them from plants are generally inefficient and environmentally unsustainable. Chemical synthesis can help alleviate the demands for limited Amaryllidaceae alkaloids, but the production of these complex molecules is often challenging and pricey. In addition, genetic engineering of plants or microorganisms to produce Amaryllidaceae alkaloids is unfortunately hindered due to the limited knowledge of the Amaryllidaceae alkaloid metabolic pathway. Few genes encoding biosynthetic enzymes are known, and the few known enzymes have often not been fully characterized. Therefore, the elucidation of the Amaryllidaceae alkaloid biosynthetic pathway, coupled with the ongoing pharmacological research on Amaryllidaceae alkaloids, will eventually help to better treat various human diseases. This review discusses recent discoveries involving the application of biochemical, molecular, cellular and genomic techniques for the elaboration of Amaryllidaceae alkaloid biosynthesis in plants.

Classification of Amaryllidaceae alkaloids

The large number of Amaryllidaceae alkaloid has been classified into different types according to their molecular skeletons and ring systems (Ding et al. 2017; He et al. 2015; Jin and Xu 2013; Jin and Yao 2019; Kornienko and Evidente 2008). For example, Kornienko and Evidente (2008), classified Amaryllidaceae alkaloids into 12 ring types, whereas He et al. (2015) grouped Amaryllidaceae alkaloids into 11 ring types and recently, Jin and Yao (2019) into 5 types (He et al. 2015; Jin and Yao 2019; Kornienko and Evidente 2008). For this review, a classification based on the Amaryllidaceae alkaloid biosynthetic origin and skeleton ring type proposes 9 types of Amaryllidaceae alkaloid including norbelladine, cherylline, galanthamine, lycorine, lycorenine, crinine, narciclasine, tazettine, and montanine (Table 1) of which their common biosynthetic origin, norbelladine, will be discussed later. A representative structure for each type is shown in Fig. 1. Other reported Amaryllidaceae alkaloid-types will not be discussed in this review because their *in planta* biosynthetic connection is not clear. For example, galanthindole contains a non-fused indole ring and might represent an artifact of homolycorine or tazettine-type derivatives (Unver et al. 2003). Another example, ismine is considered to be a catabolic product from the haemanthamine-type skeleton, thus not a specific type of Amaryllidaceae alkaloid (Bastida et al. 2011).

Biosynthesis of Amaryllidaceae alkaloids

In contrast to the considerable literature on the pharmacological effects of Amaryllidaceae alkaloids, information on their molecular genetics and biochemical pathways is still fragmentary. In general, the biosynthesis of alkaloids in plants often starts with the condensation of two amino acids derivatives to form a precursor scaffold as the entry point to a complex biosynthetic pathway. This is frequently followed by a series of reactions such as bond formations, rearrangements, breakages, functional group additions and modifications, yielding a vast array of diverse alkaloids. Amaryllidaceae alkaloid biosynthesis appears to follow this generic order of steps. Although the chemical structures of Amaryllidaceae alkaloids have great diversity, they are considered to share norbelladine as a common biosynthetic origin, which originally derived from the condensation of derivatives of the aromatic amino acids L-phenylalanine and L-tyrosine (Fig. 2).

The Amaryllidaceae alkaloid biosynthetic pathway can be conveniently divided into five stages:

Table 1 Ring types and representative Amaryllidaceae alkaloids

	Amaryllidaceae alkaloid-type	Ring structure	Phenol coupling	Representative Amaryllidaceae alkaloids
1	Norbelladine	N-(3,4-Dioxybenzyl)-4-oxyphenethylamine		Norbelladine, Rystilline
2	Cherylline	Tetrahydroisoquinoline		Cherylline, Gigantelline
3	Galanthamine	6H-Benzofuro[3a,3,2-e,f]-2-benzazepine	*para-ortho'*	Narwedine, Galanthamine
4	Lycorine	Pyrrolo[d,e]phenanthridine	*ortho-para'*	Lycorine, Galanthine
5	Lycorenine	2-Benzopyrano-[3,4-g]indole	*ortho-para'*	Homolycorine, Hippeastrine
6	Crinine	5,10b-Ethanophenanthridine	*para-para'*	Crinine, Haemanthamine
7	Narciclasine	Lycoricidine	*para-para'*	Narciclasine, Pancratistatin
8	Tazettine	2-Benzopyrano[3,4-c]indole	*para-para'*	6a-Deoxytazettine, Pretazettine
9	Montanine	5,11-Methanomorphanthridine	*para-para'*	Pancracine, Montanine

1. The shikimate pathway, which provides the aromatic amino acid subunits L-phenylalanine and L-tyrosine, and is considered as primary metabolism.
2. The phenylpropanoid pathway that leads to the formation of 3,4-dihydroxybenzaldehyde (3,4-DHBA) from phenylalanine, which provides the aldehyde moiety of Amaryllidaceae alkaloid precursor.
3. The core pathway which involves the biosynthesis of tyramine (the amine moiety of Amaryllidaceae alkaloid precursor) from tyrosine and its condensation with 3,4-DHBA to form the central precursor norbelladine, as well as its subsequent *O*-methylation.
4. The intermediate pathway where phenol coupling of the 4-*O*-methylnorbelladine followed by a reduction step will gives rise to a series of unstable intermediates.
5. The late pathway(s) for the biosynthesis of the different types of Amaryllidaceae alkaloids found in different plant species and whose pathways remain largely uncharacterized.

The shikimate pathway

The substrates and enzymes necessary for the first reactions in plant specialized metabolism are often recruited from primary metabolic pathways (Bruneton 2016; Chu et al. 2011). Indeed, substrates for the initial reactions of alkaloids biosynthesis are derived from primary metabolism, especially the aromatic amino acids L-phenylalanine and L-tyrosine.

The amino acids in plants are not only essential components of protein synthesis, but also serve as precursors for a wide range of primary and specialized metabolites (Wink 2010). The aromatic amino acids (L-phenylalanine, L-tyrosine, and L-tryptophan) are synthesized via the shikimate pathway, which is a seven step chloroplastic metabolic route in plants (Fig. 3). Regulation of the entire pathway is accomplished at multiples levels (e.g. transcriptional, enzyme activity regulation, and post-transcriptional), thereby explaining the multiple factors contributing to the expression of these pathway genes (Tzin and Galili 2010; Vogt 2010). For example, the transcriptional control of 3-deoxy-D-arabinose-heptulosonate synthase (DAHPS) was reported, whereas feedback inhibition of arogenate and prephenate dehydratase by phenylalanine was demonstrated (Vogt 2010). Also, the response of individual genes of the shikimate pathway to variations in light or nutrient content is tightly regulated and very complex but highly efficient in controlling the carbon flux in cells (Maeda and Dudareva 2012; Vogt 2010). For example, microalga *Euglena gracilis* has two genes encoding DAHPS enzyme that are differentially expression by light and are located in different cell compartments (Tohge et al. 2013). PAL variants can also be regulated by light at the transcriptional or activity levels (Zhang et al. 2016; Zhang and Liu 2015). In addition to their role as the basic structural units of proteins, aromatic amino acids

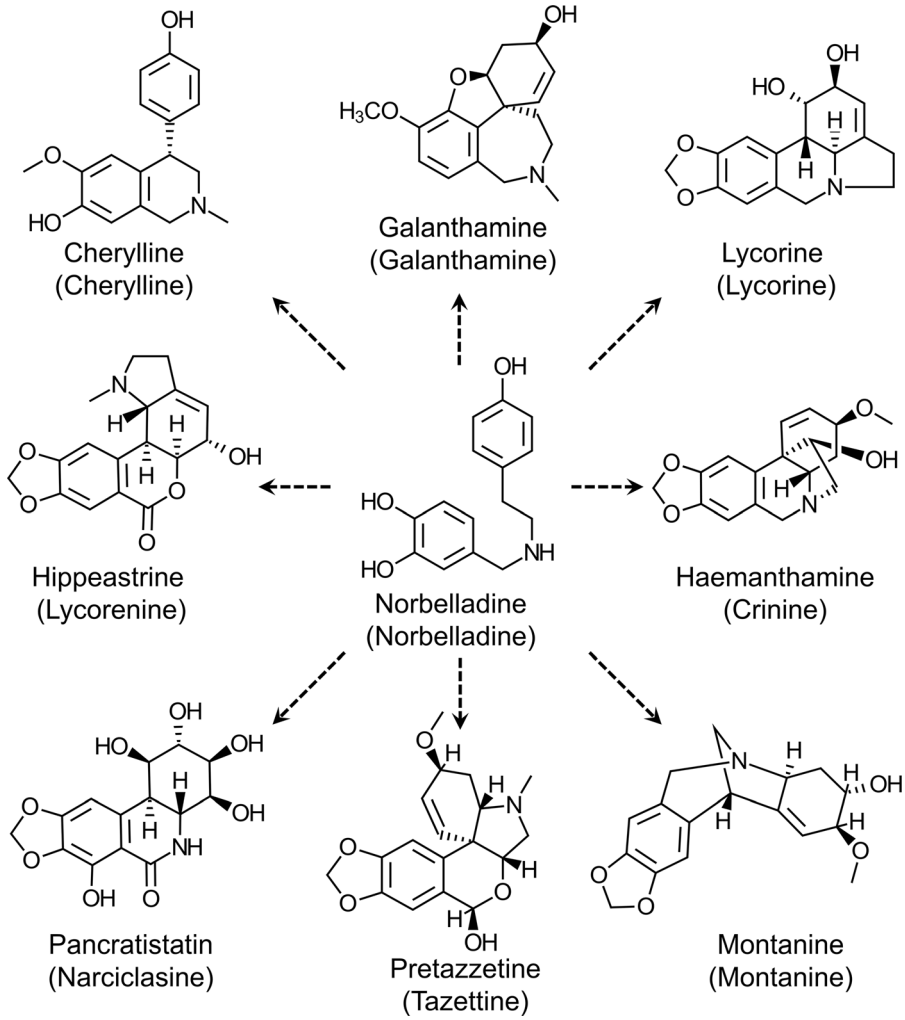

Fig. 1 Amaryllidaceae alkaloid structural scaffolds, represented by a signature metabolite of each type, derived from the norbelladine backbone

also serve as biosynthetic precursor for important specialized metabolites such as phenylpropanoids, flavonoids, lignins, coumarins, and alkaloids.

Chorismate, the final product of the shikimate pathway, serves as the precursor to all aromatic amino acids produced within the plastids. First, chorismate is converted into prephenate by the chorismate mutase (CM) followed by arogenate by an aminotransferase (PPA-AT or GOGAT) (Fig. 3). From arogenate, the arogenate dehydratase (ADT) yields phenylalanine whereas the arogenate dehydrogenase (ADH) forms tyrosine (Fig. 3). The amino acids synthesized in the plastids are exported to the cytosol where they can be used for protein synthesis, specialized metabolism, or enters other pathways. Although most studies showed that enzymes from the shikimate pathway in higher plants are generally synthesized as precursors containing a plastid transit peptide that directs them to the plastid, few studies have reported shikimate enzymes in the cytosol (Fig. 3). As far as the importance in the framework of Amaryllidaceae alkaloids biosynthesis, it suggests that intermediates and/or end-products of the shikimate pathway are transported out of the plastid to the cytosol where the 'cytosolic' enzymes, acting at the border between primary and specialized metabolism (e.g. PAL, TYDC), can catalyze these important reactions leading to precursors entering alkaloid pathway. Such transporters are therefore

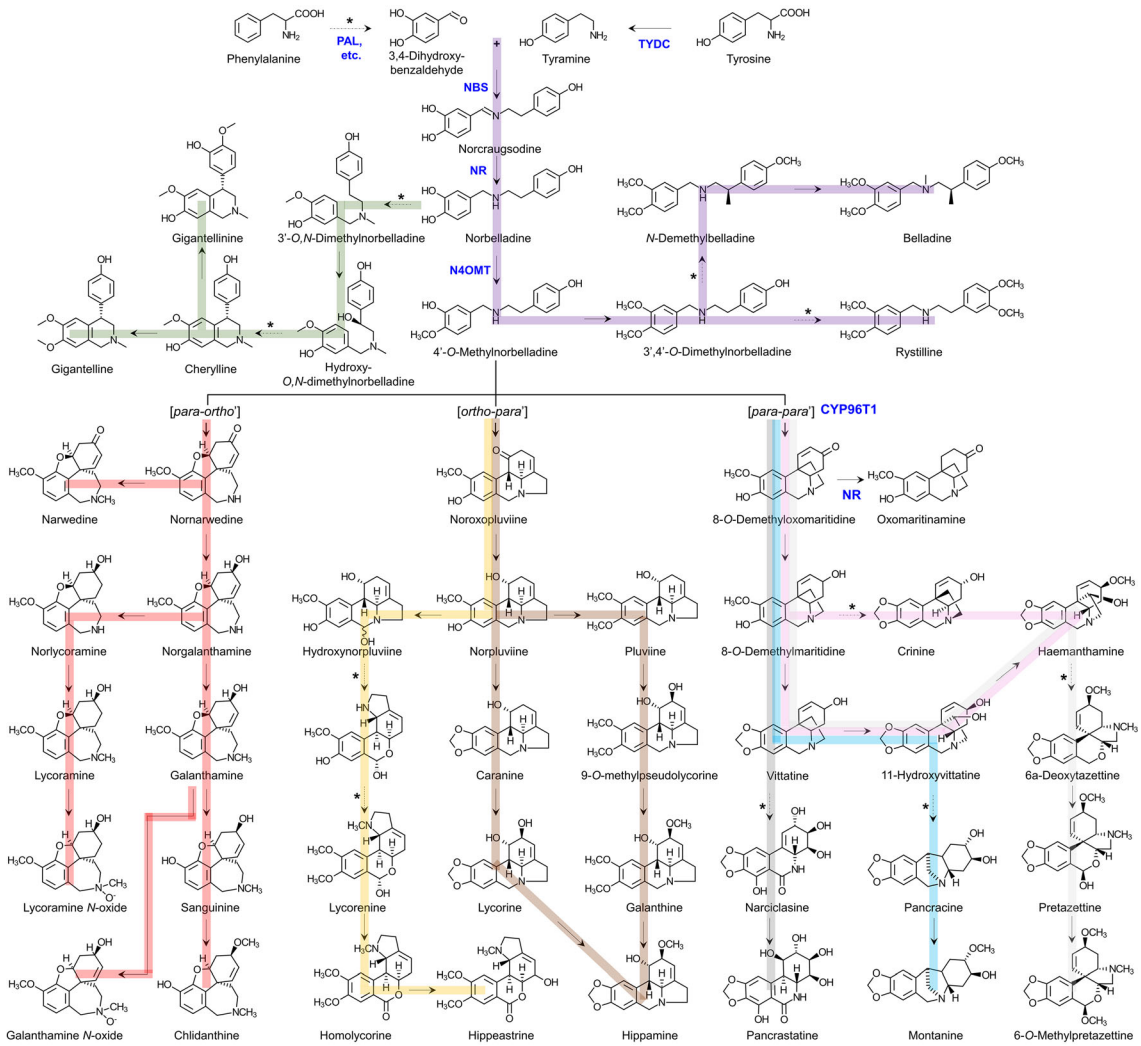

Fig. 2 Major proposed Amaryllidaceae alkaloid biosynthetic pathways leading to norbelladine (purple), cherylline (green), galanthamine (red), lycorenine (yellow), lycorine (brown), crinine/haemanthamie (pink), pancrastatin (dark gray), montanine (blue), and tazettine (light gray). All enzymes shown in bold blue have been identified from Amaryllidaceae. A solid arrow represents one enzymatic reaction whereas a dashed arrow with an asterisk mark represents multiple enzymatic steps. Abbreviations are PAL, phenylalanine ammonia lyase; TYDC, tyrosine decarboxylase; NBS, norbelladine synthase; N4OMT, norbelladine 4'-O-methyltransferase; CYP96T1, cytochrome P450 monooxygenase 96T1; and NR, noroxomaritidine reductase. (Color figure online)

potentially involved in the regulation of alkaloids biosynthesis by limiting the amount of precursors available.

Feedback activation and inhibition occurs to fine tuned and regulate the level of L-tyrosine and L-phenylalanine within the cells (Schenck and Maeda 2018) which can also participate to availability of these precursors to alkaloid production. As such, CM, ADT and ADH are allosterically regulated enzymes (Tzin and Galili 2010). For example, two of the three Arabidopsis CM isoforms are feedback-inhibited by phenylalanine and tyrosine whereas AtCM2 is insensitive to this allosteric regulation (Mobley et al. 1999). Interestingly, ADT activity was positively regulated by tyrosine and negatively regulated by phenylalanine in tobacco, spinach, and sorghum bicolor (Tzin and Galili 2010). None of these enzymes activity and regulation mechanism have been investigated in Amaryllidaceae plants.

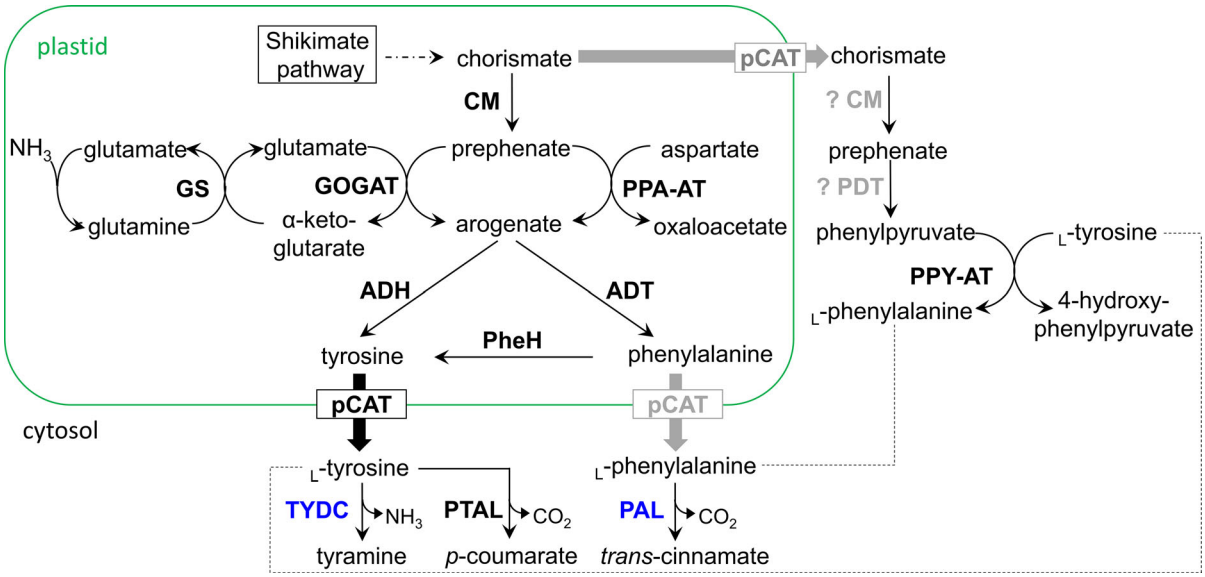

Fig. 3 Chloroplastic biosynthetic pathways leading to aromatic amino acids, L-tyrosine and L-phenylalanine, in plants. Enzymes and transporters (boxes) for which corresponding genes have been isolated from Amaryllidaceae plants are shown in blue whereas those for which corresponding genes have been isolated in other plants are shown in black. Uncharacterized enzymes and transporters are written in gray or with question marks. Dashed arrow represents more than one reaction. Abbreviations: CM, chorismate mutase; PPA-AT, prephenate aminotransferase; GOGAT, glutamine oxoglutarate aminotransferase; GS, glutamine synthase; PDT, prephenate dehydratase; PPY-AT, phenylpyruvate aminotransferase; ADH, arogenate dehydrogenase; ADT, arogenate dehydratase; PheH, Phenylalanine hydroxylase; pCAT, plastidial cationic amino acid transporter; TYDC, tyrosine decarboxylase; PTAL, phenylalanine/tyrosine ammonia lyase and PAL, phenylalanine ammonia lyase. (Color figure online)

The enzymes responsible for the commitment of aromatic amino acids to specialized metabolic pathways typically have key regulatory functions since they act at the interface between primary and specialized metabolism (Maeda 2019). For Amaryllidaceae alkaloid biosynthesis, the enzymes proposed to catalyze these firsts committed steps include L-tyrosine/L-DOPA decarboxylase (TYDC) and phenylalanine ammonia lyase (PAL). Thus, the formation of the Amaryllidaceae alkaloid requires the involvement of the shikimate pathway followed by two major routes, namely the tyramine and the phenylpropanoid pathways (Fig. 3). Furthermore, the digital expression profile of *TYDC* and *PAL* variants from two different *Narcissus* transcriptome (Table 2) were comparable suggesting that they may be coordinately regulated since both these genes encode enzymes catalyzing the first reactions in the Amaryllidaceae alkaloid biosynthetic pathway (Fig. 2).

The TYDC and PAL reactions link specialized metabolism to primary metabolism leading to the production of a variety of phenolic compounds including flavonoids and cell wall structural polymer lignin (Zhang and Liu 2015). This suggest that they both control carbon flux into the Amaryllidaceae alkaloid pathway. Thus, Amaryllidaceae alkaloid biosynthesis starts with the modifications of aromatic amino acids phenylalanine and tyrosine, both produced from the shikimate pathway. The response of individual genes of the shikimate pathway to environmental stressors appears tightly regulated and more complex than the known transcriptional responses of the entry genes of the phenylpropanoid and tyramine biosynthetic pathways, encoded by PAL and TYDC respectively (Desgagné-Penix and Facchini 2011; Facchini et al. 2000; Hagel and Facchini 2005; Vogt 2010).

The subsequent condensation of an amine and an aldehyde precursors forms the norbelladine backbone. Fundamentally, Amaryllidaceae alkaloid biosynthesis begins with the formation of the two amino acids derivatives precursors; (1) the amine tyramine derived from L-tyrosine and (2) the aldehyde 3,4-DHBA obtained from modifications of L-phenylalanine.

Table 2 Digital expression of *TYDC* and *PAL* gene transcripts in bulbs of *N. pseudonarcissus* 'king Alfred 'and *N. papyraceus*. Data are expressed in fragments per kilobase million (FPKM) and extracted from (Hotchandani et al. 2019; Singh and Desgagne-Penix 2017)

Name	*N. pseudonarcissus* 'king Alfred' (FPKM)	*N. papyraceus* (FPKM)
TYDC1	1881	593
TYDC2	1111	1044
PAL1	1911	367
PAL2	1790	125

These initial precursors are synthesized from different pathways: the tyramine and the phenylpropanoid pathways.

The tyramine pathway

The reaction leading to tyramine (the amine precursor) formation is well known and involves the decarboxylation of L-tyrosine by TYDC (Figs. 2, 3). The TYDC reaction represents one of the initial reaction in the biosynthesis of a wide variety of metabolites including the phenylethanoid glycosides (*e.g.* verbascoside), protoalkaloids (*e.g.* *N*-methyltyramine and hordenine), and benzylisoquinoline alkaloids (*e.g.* morphine and codeine) (Alipieva et al. 2014; Facchini et al. 2000; Pellati and Benvenuti 2007; Wheaton and Stewart 1970). Since TYDC enzymes operate at the interface between primary and specialized metabolism, they may have key regulatory roles in the control of Amaryllidaceae alkaloid end-product biosynthesis. TYDCs have been isolated and purified from a variety of plants species and shown to exhibit developmental, tissue-specific, and inducible expression. TYDC is encoded by a single-copy gene in Arabidopsis, five putative genes in lotus and about 15 genes in opium poppy that are differentially regulated (Deng et al. 2018; Desgagné-Penix and Facchini 2011; Facchini et al. 2000). Native TYDCs were shown to be homodimeric enzymes capable of decarboxylating L-tyrosine and L-DOPA, but inactive towards L-phenylalanine and L-tryptophan. Isolated *TYDC* genes have been used in plant genetic engineering studies which contributed to our understanding in the regulation of tyrosine-derived specialized metabolites (Hagel and Facchini 2005).

Since tracer studies suggested that tyramine is one of the precursors of norbelladine (Battersby et al. 1961), analogies with elucidated biosynthetic pathways proposed that TYDC is involved (Fig. 2) (Bastida et al. 2011; Kilgore and Kutchan 2015; Singh and Desgagne-Penix 2014; Singh and Desgagné-Penix 2015; Takos and Rook 2013). Plant TYDCs share extensive amino acid identity and display remarkable similarities (*e.g.* structure, molecular mass and kinetic properties). Indeed, BLAST searches to identify the sequence of TYDC in the transcriptome of *N. pseudonarcissus*'king Alfred' led to the identification of two *TYDC* transcript variants, *Nps*TYDC1 and *Nps*TYDC2 sharing 57% identity of the amino acid sequence (Singh and Desgagne-Penix 2017).

Similarly, two TYDC variants, *NpaTYDC1* and *NpaTYDC2*, were identified within the *N. papyraceus* transcriptome, in addition to *LraTYDC2* and *LauTYDC1* identified from *L. radiata* and from *L. aureus,* respectively (Hotchandani et al. 2019; Park et al. 2019; Wang et al. 2019). In all case, the expression of each of these Amaryllidaceae *TYDCs* was higher in the underground parts (*i.e.* roots and bulbs) compared to the aerial parts (Hotchandani et al. 2019; Park et al. 2019; Singh and Desgagne-Penix 2017; Wang et al. 2019) fitting the models for galanthamine accumulation *in planta*.

Phylogenetic analysis of TYDCs was carried out and the Amaryllidaceae TYDCs divided into two main clusters (Fig. 4). Notably, the hereby named ''TYDC1'' cluster including *Lau*TYDC1, *Nps*TYDC1, *NpsMK2014*TYDC1, and *Npa*TYDC1 shares higher similarity with proteins from the alkaloid-producing plants TYDCs such as *Pso*TYDC1 and *Tfl*TYDC (Fig. 4, purple). The second cluster, the ''TYDC2'' cluster composed of *Nps*TYDC2, *Npa*TYDC2 and *Lra*TYDC, shares higher similarity with asparagus TYDC (*Aof*TYDC) (Fig. 4, green). This suggests that the Amaryllidaceae 'TYDC1' cluster

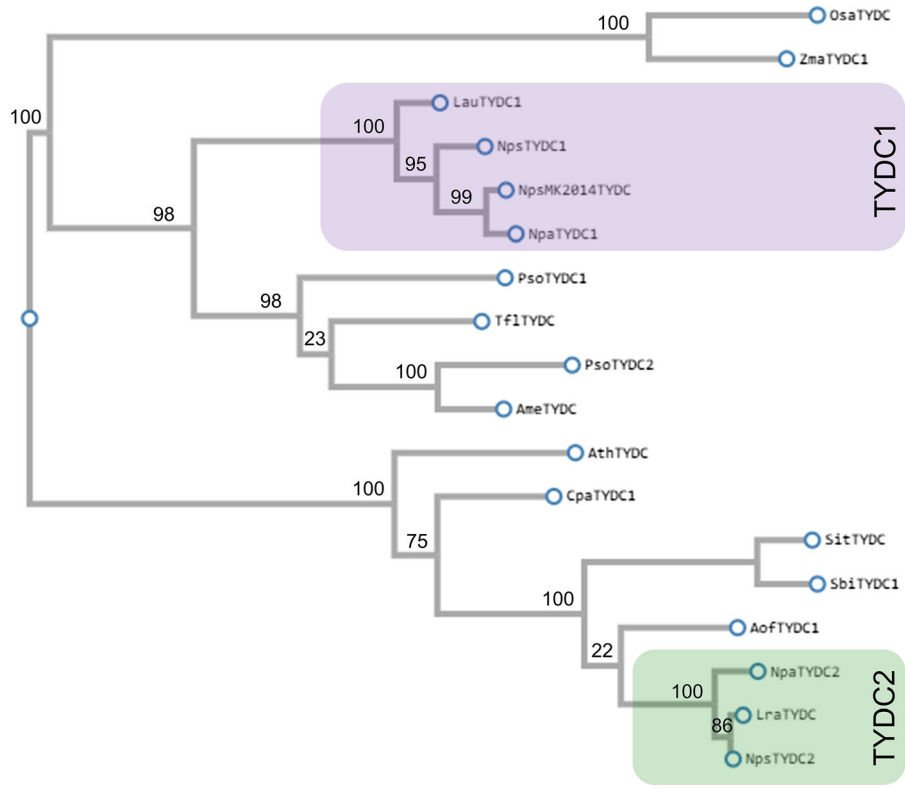

Fig. 4 Phylogenetic tree analysis of TYDC proteins from different species. Alignment and phylogenetic reconstructions were performed using the function "build" of ETE3 v3.1.1 (Huerta-Cepas et al. 2016) as implanted on the GenomeNet (https://www.genome.jp/tools/ete/). The tree was constructed using fasttree with slow NNI and MLACC = 3 (to make the maximum-likelihood NNIs more exhaustive) (Price et al. 2009) and branch supports were computed out of 100 bootstrapped trees with amino acid sequences of plant *Osa*TYDC (*Oryza sativa japonica*, Q94EE9.1); *Zma*TYDC1 (*Zea mays*, XP_008651749.1); *Lau*TYDC1 (*Lycoris aurea*, MG932082.1); *Lra*TYDC (*Lycoris radiata*, QFQ50504.1); *Nps*TYDC1 (*Narcissus pseudonarcissus*, AUG71932.1); *Nps*TYDC2 (*Narcissus pseudonarcissus*, AUG71933.1); *Npa*TYDC1 (*Narcissus papyraceus*, AXU39890.1); *Npa*TYDC2 (*Narcissus papyraceus*, AXU39891.1); *Nps*MK2014TYDC (*Narcissus pseudonarcissus MK 2014*, ALM26449.1); *Pso*TYDC1 (*Papaver somniferum*, P54768.1); *Pso*TYDC2 (*Papaver somniferum*, XP_026405553.1); *Tfl*TYDC (*Thalictrum flavum*, AAG60665.1); *Ame*TYDC (*Argemone mexicana*, ACJ76782.1); *Ath*TYDC (*Arabidopsis thaliana*, CAB56038.1); *Cpa*TYDC (*Carica papaya*, XP_021893770.1); *Sit*TYDC (*Setaria italica*, XP_004956078.1); *Sbi*TYDC (*Sorghum bicolor*, XP_002459778.2); *Aof*TYDC (*Asparagus officinalis*, XP_020265332.1)

may be involved in the synthesis of alkaloids, whereas the 'TYDC2' cluster, more similar to a TYDC from a non-alkaloid producing plant, may operate in other primary metabolic pathways. Recently, the enzyme activity of *Lau*TYDC1 was functionally characterized using heterologous expression in *E. coli*. *Lau*TYDC1 efficiently catalyzed the conversion of tyrosine to tyramine, localized to the cytoplasm, and was regulated by methyl jasmonate (MeJA) treatment (Wang et al. 2019).

It is known that despite variable functions, plant aromatic amino acid decarboxylases (AAAD) such as TYDC and tryptophan decarboxylase (TDC) have high sequence identity, but do not have easily recognizable motifs in primary sequences. However, an investigation on the structural aspects of TYDC and TDC through extensive sequence comparison, site-directed mutagenesis and enzyme assay, led to the identification of a single active site amino acid residue responsible for substrate specificity (Torrens-Spence et al. 2014). Results indicated that the phenolic and indolic substrates in plant AAADs could be primarily dictated by this single active site amino acid. The stringent conservation for serine 372 in TYDC and glycine 372 in TDC, support this consideration (Torrens-Spence et al. 2014). Indeed, *Papaver*

somniferum TYDC was mutated from serine 372 to glycine, which enables the *P. somniferum* TYDC to use 5-hydroxytryptophan as a substrate, and reduces the enzyme activity toward DOPA. Additionally, the reverse mutation in *Catharanthus roseus* TDC enables the mutant enzyme to utilize tyrosine and DOPA as substrates with a reduced affinity towards tryptophan (Torrens-Spence et al. 2014). Sequence alignment of Amaryllidaceae TYDC sequences revealed that all members of the ''TYDC1'' cluster had a serine residue at position 372 (Fig. 4, purple) whereas the ''TYDC2'' cluster (Fig. 4, green) displayed a glycine 372. This suggest that the members of the Amaryllidaceae 'TYDC1' cluster may be involved in the synthesis of alkaloids using the phenolic substrates, whereas members of the 'TYDC2' cluster, more similar to a TDC, may operate towards indolic substrates in metabolic pathways other than the Amaryllidaceae alkaloids pathway.

The phenylpropanoid pathway

The sequence of reactions leading to the conversion of L-phenylalanine into the aldehyde precursor 3,4-DHBA are not known. However, studies suggested that 3,4-DHBA originates from the phenylpropanoid pathway (Barton et al. 1963) as in the formation of phenolic compounds like the fragrant molecule, vanillin, in *Vanilla planifolia* plants (Havkin-Frenkel et al. 1996; Negishi et al. 2009; Walton et al. 2003; Zenk, 1965). Interestingly, vanillin metabolites have been reported in Amaryllidaceae plant (Benedec et al. 2018; Nikolova and Gevrenova, 2005) and they are also known to be involved in the biosynthesis of alkaloids such as the capsaicinoids (benzylamine alkaloids) responsible of the spicy flavor in chili peppers (Aza-Gonzalez et al. 2011). This suggests that vanillin reactions may be present in Amaryllidaceae to participate in 3,4-DHBA synthesis. The pathway from phenylalanine to 3,4-DHBA was determined by feeding radiolabeled precursors to *N. pseudonarcissus* and monitoring production of haemanthamine (Suhadolnik et al. 1963). The study led to the conclusion that the pathway to 3,4-DHBA is: phenylalanine, *trans*-cinnamic acid, 4-hydroxycinnamic acid, 3,4-dihydroxycinnamic acid or 4-hydroxybenzaldehyde, and 3,4-DHBA (Suhadolnik et al. 1962, 1963). In contrast to alkaloids, data on phenolic metabolites in Amaryllidaceae plants are limited. Only a few studies have been published reporting the presence of phenolic acids such as protocatechuic, 4-hydroxybenzoic, vanillic, caffeic, syringic, *p*-coumaric, and ferulic acids in *N. pseudonarcissus, N. poeticus and G. nivalis* (Benedec et al. 2018; Ferdausi et al. 2020; Nikolova and Gevrenova 2005). In addition, 3,4-DHBA was detected in plants outside the Amaryllidaceae family (Prachayasittikul et al. 2008). Altogether, this suggest that the initial reactions and biosynthetic enzymes of the phenylpropanoid pathway participate in the synthesis of the aldehyde precursor 3,4-DHBA.

The first reaction of the phenylpropanoid pathway is catalyzed by the phenylalanine ammonia-lyase (PAL), a key regulatory enzyme promoting the deamination of phenylalanine to yield *trans*-cinnamic acid (Figs. 2 and 3). PAL directs the carbon flow from the shikimate pathway to the various branches of the phenylpropanoid metabolism. Thus, PAL reaction not only controls carbon flux into this pathway but also links primary to specialized metabolism leading to the production of a variety of phenolic compounds. This enzyme is among the most studied in plant specialized metabolism and is encoded by a multi-gene family, ranging from four in Arabidopsis to a dozen or more copies in some higher plants (Vogt 2010). For example, 20 putative *PAL* genes were identified in tomato but only one single transcript was strongly expressed in all tissues while all other appears silenced (Chang et al. 2008).

Several *PAL* transcripts have been identified and characterized from the Amaryllidaceae species (Hotchandani et al. 2019; Jiang et al. 2011, 2013; Li et al. 2018a; Park et al. 2019; Singh and Desgagne-Penix 2017). The *LraPAL1* and *LraPAL2* genes from *L. radiata*, sharing 83% amino acid identity, were isolated and characterized (Jiang et al. 2011, 2013). Expression analysis of *LraPAL* transcripts suggested differential regulation mechanisms. *LraPAL2* was detected in all tissues, with higher abundance in aerials parts (*e.g.* petal, stamen, stem and ovary), whereas *LraPAL1* was mostly expressed in underground tissues such as bulbs and roots correlating with the content of galanthamine (Jiang et al. 2011, 2013). These results suggest that *LraPAL2* might play distinct roles in different branches of the phenylpropanoid pathway.

Phylogenetic analysis of PAL proteins shown that Amaryllidaceae PALs divided into 2 main clusters notably, the hereby named ''PAL1'' and the ''PAL2''

clusters (Fig. 5). The ''PAL1'' cluster, includes *Lra*PAL_AWW24969.1 (corresponding to *LraPAL1* (Jiang et al. 2011) and *Lra*PAL_ACM61988.1 (reported as *Lra*PAL3 (Li et al. 2018a)) which shared 98% amino acid identity (Fig. 5, yellow). The ''PAL1'' cluster also included *Nps*PAL1 with *Npa*PAL1 and *Nta*PAL sharing 94% and 95% amino acid identity, respectively (Fig. 5, yellow). The ''PAL2'' cluster is composed of *Lra*PAL_QFQ50498.1 [reported as *LraPAL2* by (Park et al. 2019)] which is 92%, 95% and 90% identical to *Nps*PAL2, *Npa*PAL2 and *Npa*PAL3, respectively (Fig. 5, red). Between the two clusters, there is between 80 and 83% amino acid identities. The difference in phylogeny, transcript expression and regulation of the Amaryllidaceae PALs (''PAL1'' vs ''PAL2'' clusters) suggest different functional role for PAL1 versus PAL2.

A recent study on the expression of transcript variants of PAL in *N. papyraceus* revealed that at each developmental stage (*e.g.* dormance, germination, elongation, flowering, senescence), *NpaPAL1* was ubiquitously expressed [similar levels in all parts (e.g. root, bulb, stem, leaf, flower)], whereas *NpaPAL2* expression was the highest in the bulb, at almost all stages (Hotchandani et al. 2019). A similar profile of expression was reported for *NpsPAL2* (Singh and Desgagne-Penix 2017). This indicates that *Narcissus PAL2* variants encode enzymes that may have distinct

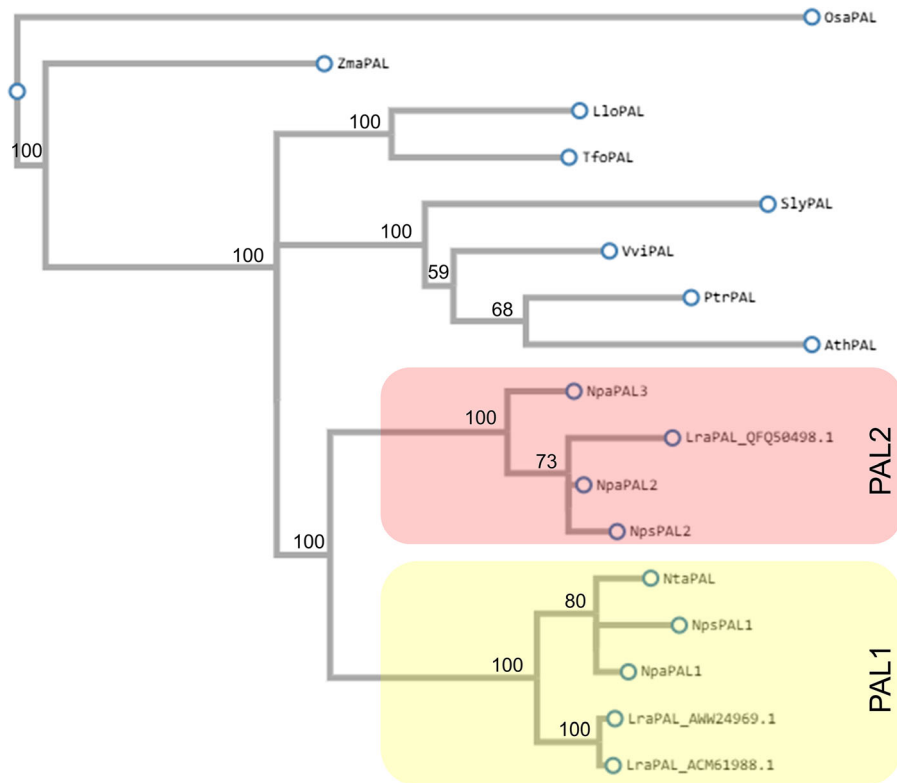

Fig. 5 Phylogenetic tree analysis of PAL proteins from different species. Alignment and phylogenetic reconstructions were performed using the function "build" of ETE3 v3.1.1 (Huerta-Cepas et al. 2016) as implanted on the GenomeNet (https://www.genome.jp/tools/ete/). The tree was constructed using fasttree with slow NNI and MLACC = 3 (to make the maximum-likelihood NNIs more exhaustive) (Price et al. 2009) and branch supports were computed out of 100 bootstrapped trees with amino acid sequences of plant *Osa*PAL (*Oryza sativa*, S06475); *Zma*PAL (*Zea mays*, NP_001147433.2); *Llo*PAL (*Lilium longiferum*, ACZ44833.1); *Tfo*PAL (*Tulipa fosteriana*, AHY20029.1); *Sly*TYDC (*Solanum lycopersicum*, AAA34179.1); *Vvi*PAL (*Vitis vinifera*, AEX32790.1); *Ptr*PAL (*Populus trichocarpa*, XP_002322884.2); *Ath*PAL (*Arabidopsis thaliana*, AAC18870.1); *Lra*PAL (*Lycoris radiata*, QFQ50498.1); *Lra*PAL (*Lycoris radiata*, AWW24969.1); *Lra*PAL (*Lycoris radiata*, ACM61988.1); *Nps*PAL1 (*Narcissus pseudonarcissus*, AUG71934.1); *Nps*PAL2 (*Narcissus pseudonarcissus*, AUG71935.1); *Npa*PAL1 (*Narcissus papyraceus*, AXU39892.1); *Npa*PAL2 (*Narcissus papyraceus*, AXU39893.1); *Npa*PAL3 (*Narcissus papyraceus*, AXU39894.1); and *Nta*PAL (*Narcissus tazetta*, ADD82537.1)

functions in different branches of the phenylpropanoid pathway. An analysis of three *PAL*-genes in poplar with organ specific expression shown that each may have different function. Specifically, of the two poplar *PAL*-genes expressed in lignifying tissues, only one was involved in lignin formation whereas the third *PAL*-gene was associated with flowering (Hamberger et al. 2007).

Recently, it was shown that monocot grasses of the Poaceae family use a pathway leading to phenylpropanoids more efficiently than dicots (Barros and Dixon 2020; Barros et al. 2016). This pathway involves the bifunctional phenylalanine/tyrosine ammonia-lyase (PTAL) generating *p*-coumarate directly from tyrosine (Figs. 3, 6). Since Amaryllidaceae are monocots phylogenetically relatively close to the Poaceae family, it is possible that such pathway is present in these plants. If so, this efficient 'monocot' pathway could potentially contribute to Amaryllidaceae alkaloid biosynthesis, but none of the genes or enzymes involved in this pathway have as been reported to date.

Following the PAL reaction, the 4-hydroxylation of the benzyl ring of *trans*-cinnamic acid is catalyzed by the cytochrome P450-dependent monooxygenase (CYP) cinnamate-4-hydroxylase (C4H) (Russell 1971; Russell and Conn 1967), which belongs to the CYP73 subfamily, and results in the formation of *p*-coumaric acid (Fig. 6) (Ehlting et al. 2006). *C4H* was one of the first phenylpropanoid pathway genes to be cloned in 1993, concurrently from mung bean, Jerusalem artichoke, and alfalfa (Fahrendorf and Dixon 1993; Mizutani et al. 1993; Teutsch et al. 1993). Surprisingly, among over 300 genes encoding cytochrome P450s in Arabidopsis, *C4H* is present in one single copy.

One single *C4H* transcript was identified in the transcriptome of *N. pseudonarcissus* and *N. papyraceus* respectively (Hotchandani et al. 2019; Singh and Desgagne-Penix 2017). *NpaC4H* expression was highest in bulbs of the dormant and early germination stages (Hotchandani et al. 2019). In flowering plants, *NpsC4H* expression was high in stems, roots and bulbs of *N. pseudonarcissus* 'king Alfred' (Singh and Desgagne-Penix 2017), but highest in flowers of *N. papyraceus* and *L. radiata* (Hotchandani et al. 2019; Li et al. 2018a). The presence of single copy of *C4H* in Amaryllidaceae suggest that any carbon flux through phenylpropanoid metabolism is mediated by the activity of the protein encoded by this single gene.

Fock-Bastide et al. (2014) reported that *C4H* expression level in *V. 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 for *N. papyraceus* in that *NpaC4H* expression is high during the first two developmental stages to drop and remained constant during stages 3-5 (elongation to

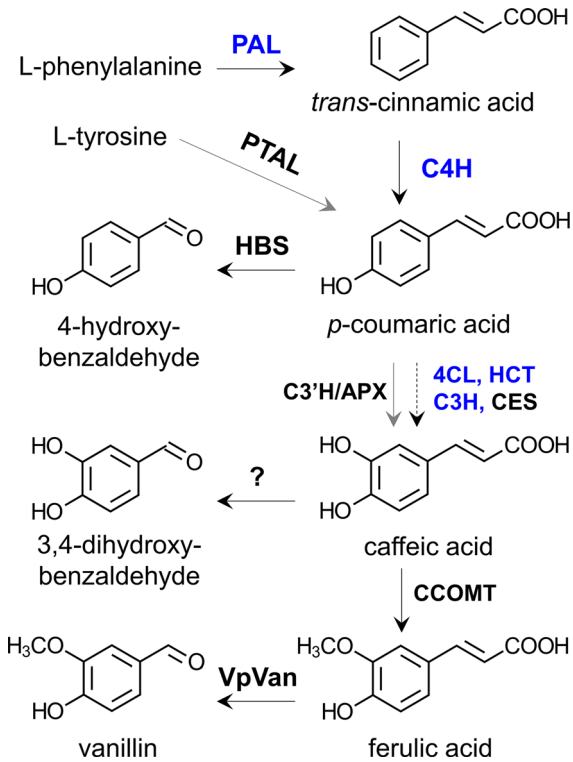

Fig. 6 Phenylpropanoid biosynthetic pathway leading to Amaryllidaceae alkaloid precursors. Broken arrow represents more than one biochemical reaction. Enzymes for which corresponding genes have been isolated from Amaryllidaceae plants are shown in blue, whereas those for which corresponding genes have been isolated in other plants are shown in black. An alternative 'direct' pathway from L-tyrosine to *p*-coumaric acid to caffeic acid is shown with a gray arrow. Abbreviations: PAL, phenylalanine ammonia lyase; PTAL, phenylalanine/tyrosine ammonia lyase C4H, *trans*-cinnamate hydroxylase (CYP73A1); 4CL, 4-hydroxycinnamic acid CoA ligase; HCT, hydroxycinnamoyl CoA shikimate transferase; C3H, coumaroyl shikimate hydroxylase (CYP98A3); CES, caffeoyl shikimate esterase; C3'H/APX, bifunctional coumarate 3-hydroxylase/ascorbate peroxidase; CCOMT, caffeoyl CoA 3-O-methyltransferase; HBS, hydroxybenzaldehyde synthase; VpVan, vanillin synthase. (Color figue online)

flowering) (Hotchandani et al. 2019). Similarly, C4H activity remained constant in various cultivars of Capsicum regardless of fluctuations in capsaicinoid accumulation in these cultivars (Phimchan et al. 2014).

Recently, *LraC4H* was characterized and results showed the selective 4-hydroxylation conversion of *trans*-cinnamic acid into *p*-coumaric acid (Li et al. 2018a). Also, *LauC4H* from *L. aurea* was functionally characterized results showed its subcellular localization at the endoplasmic reticulum membrane in protoplasts of *A. thaliana* (Li et al. 2018b). Since, *C4H* has been cloned and characterized from many plant species and has been used in synthetic biology for the production of specialized metabolites of interest. For example, *LauC4H* together with *AthPAL1* were assembled in *E. coli* for the production of *p*-coumaric acid. With glucose as the sole carbon source and via the phenylalanine route, recombinant *E. coli* cells achieved a titer of 156 μM of *p*-coumaric acid in shake flasks (Li et al. 2018b).

The *p*-coumaric acid is activated to its corresponding CoA thioester by the 4-coumarate CoA ligase (4CL) (Fig. 6). From there, a series of reactions involving the hydroxycinnamoyl CoA shikimate transferase (HCT), coumaroyl shikimate hydroxylase (C3H) and caffeoyl shikimate esterase (CES) lead to the formation of caffeic acid (Fig. 6). Several transcripts encoding these enzymes were identified in the transcriptome of *Narcissus* species (Hotchandani et al. 2019; Singh and Desgagne-Penix, 2017). For example, four transcript variants of *4CL* and three of *HCT* were identified in the *N. papyraceus* transcriptome (Hotchandani et al. 2019). The expression of *NpaHCT1* was highest in aerial parts (*e.g.* stem, leaf, flower) whereas *NpaHCT2* was highest in the underground parts (*e.g.* root and bulb) suggesting their potential role in phenylpropanoid metabolism in these parts. Recently, a non-membrane bound ascorbate peroxidase C3H (C3'H/APX) enzyme catalyzing the direct 3-hydroxylation of *p*-coumaric acid to caffeic acid was characterized from the model plants *Brachypodium distachyon* and *A. thaliana* (Barros et al. 2019). This path (Fig. 6, gray arrow) was proposed by Zenk in 1965 to be involved in vanillin biosynthesis (Zenk 1965) but not such genes or enzymes have been reported in Amaryllidaceae so far.

Interestingly, a single copy of CYP450 *C3H* was found in each Amaryllidaceae transcriptome and *NpaC3H* expression was highest in bulbs at dormant and early germination developmental stages, whereas *NpsC3H* was highest in stems of flowering plants compared to all others tissues (Hotchandani et al. 2019; Singh and Desgagne-Penix 2017). The enzymatic activity of CYP450 C3H from *L. radiata* was confirmed to participate in caffeic acid biosynthesis (Li et al. 2009). The subsequent efficient *O*-methylation of caffeic acid to ferulic acid was demonstrated in grasses (Barros et al. 2019) thereby revising the currently accepted models of lignin biosynthesis, suggesting new gene targets to improve forage and bioenergy crops but also for other specialized metabolites.

The chain shortening (loss of two carbon atoms) reaction from caffeic acid to form 3,4-DHBA is not known, but appears very similar to reactions reported in the biosynthesis of vanillin from *V. planifolia*. Specifically, the last controversial step involving the conversion of ferulic acid to vanillin by the vanillin synthase (*Vp*Van) (Fig. 6) (Gallage et al. 2014, 2018). Interestingly, there is a debate regarding the substrate specificity of this unusual cysteine protease. Indeed, a re-evaluation of the final step of vanillin biosynthesis demonstrated that the recombinant *Vp*VAN, whether expressed in an *in vitro* transcription-translation system, *E. coli*, yeast, or plants, is unable to convert ferulic acid to vanillin (Yang et al. 2017). Rather, *Vp*VAN is a component of an enzyme complex that preferentially converts 4-coumaric acid to 4-hydroxybenzaldehyde (initially named hydroxybenzaldehyde synthase; HBS; Fig. 6), as demonstrated by the purification of this complex and peptide sequencing (Podstolski et al. 2002; Yang et al. 2017). Recently, a second enzyme named phenylpropanoid 2,3-dioxygenase (PPDiox) was shown to be involved in the cleavage of the ferulic acid side chain to form vanillin and glyoxylic acid in *V. planifolia* (Negishi and Negishi 2017). Because of substrate and reaction similarity, it is possible that the formation of 3,4-DHBA in Amaryllidaceae alkaloid biosynthesis is catalyzed by an enzyme related to *Vp*VAN or PPDiox. Remarkably, two proteins in two different protein families can catalyze the same reaction. Nevertheless, the relative participation of each enzyme in Amaryllidaceae alkaloid biosynthesis must still be determined. Further study are necessary to define the roles of 3,4-DHBA-forming enzymes in Amaryllidaceae alkaloid metabolism.

The intermediate pathway

A key feature of plant specialized metabolism is the formation of key scaffold molecules through the use of a small repertoire of chemical reaction using substrates derived from primary metabolism as building blocks. In alkaloids biosynthesis, the starting materials for these scaffold-generating reactions are typically only a few metabolic steps from amino acids. For example, initial reactions in the synthesis of benzylisoquinoline alkaloids (BIAs) start with the formation of (*S*)-norcoclaurine, the scaffold precursor to all BIAs (Beaudoin and Facchini 2014; Hagel and Facchini 2013). Similarly, first reactions in the synthesis of monoterpenoid indole alkaloids (MIAs) and monoterpenoid isoquinoline (MIQs; ipecac) alkaloids start with the formation of the strictosidine precursor (De Luca 2011; De Luca et al. 2014; Kutchan 1993; Nomura and Kutchan 2010; O'Connor and Maresh 2006). The majority of the research done on Amaryllidaceae alkaloid biosynthesis is supported by early feeding experiments and retro-biosynthetic studies using labeled precursors. This led to the biochemical elucidation of the initial steps (Barton and Cohen 1957; Barton and Kirby 1962; Barton et al. 1963; Bhandarkar and Kirby 1970; Eichhorn et al. 1998; El Tahchy et al. 2010, 2011; Saliba et al. 2015). Same as for the case of BIA, MIA and MIQ alkaloids, these studies suggested that despite their large structural diversity, all Amaryllidaceae alkaloids are derived from an isoquinoline core, the scaffold molecule norbelladine (Fig. 2, purple).

Norbelladine is formed from the condensation of tyramine (amine precursor) and 3,4-DHBA (aldehyde precursor) (Fig. 2). Similar condensation reactions, involving an amine and an aldehyde precursor, have been reported. For example, the condensation of tryptamine and secologanin by strictosidine synthase, a key Pictet-Spengler reaction, to yield strictosidine, the general precursor of MIAs and MIQ alkaloids (Kutchan 1993; Treimer and Zenk 1979). Another example is the reaction of norcoclaurine synthase (NCS) which catalyzes the condensation of dopamine and 4-hydroxyphenylacetaldehyde as the first step in BIA biosynthesis (Samanani et al. 2004). NCS catalyzes a Pictet-Spengler condensation by a two-step reaction mechanism that involves a condensation step followed by an intramolecular cyclization (Luk et al. 2007). The fact that the electron-donating oxygen of the hydroxyl group at C-2 of dopamine, not present in tyramine, is essential for this reaction to proceed provides a mechanistic explanation for the absence of cyclization in the condensation step of Amaryllidaceae alkaloid biosynthesis (Singh and Desgagné-Penix 2015; Takos and Rook 2013).

Recently, a transcriptome-mining search for *NCS* orthologs in *N. pseudonarcissus* database led to the identification of a transcript sequence named norbelladine synthase (*NBS*). *NpsNBS* encodes for a small protein of 19 kDa which from a unique clade of PR10/Bet v1 proteins and shared 41% amino acid identity to opium poppy NCS1 (Singh et al. 2018). Expression of *NpNBS* in *E. coli* produced a recombinant enzyme able to condense tyramine and 3,4-DHBA into norbelladine (Fig. 2) (Singh et al. 2018). Although the level of norbelladine was low, no norcraugsodine was detected in the assay. It should be noted that a background reaction was observed in the control/non-induced protein assay and in the boiled *Nps*NBS assay (*i.e.* when the two substrates were in contact without or with boiled *Nps*NBS) suggesting a low level of non-enzymatic condensation. Considering the basic chemistry required for the formation of the imine intermediate norcraugsodine, it was proposed that the condensation reaction was either non-enzymatic (spontaneous) or in the active site of an enzyme reducing norcraugsodine to norbelladine (Kilgore et al. 2016b). For example, tetrahydroalstonine synthase in *Catharanthus roseus* is an imine reductase, from the short-chain alcohol dehydrogenase/reductases (SDR) superfamily, involved in the conversion of strictosidine to tetrahydroalstonine in the MIA pathway (Stavrinides et al. 2015). A *N. pseudonarcissus* SDR gene was identified and characterized to reduce the carbon–carbon double bond of noroxomaritidine to oxomaritinamine (Kilgore et al. 2016b). Interestingly, this enzyme, named noroxomaritidine reductase (NR), could also produce low amount of norbelladine from tyramine and 3,4-DHBA, but 400-fold lower than noroxomaritidine conversion (Kilgore et al. 2016b).

The imine reduction of the presumated norcraugsodine intermediate by *Nps*NBS is confusing. It is possible low amount of remaining bacterial components such as reducing cofactors (Wetzl et al. 2015), present in the assay media, would participate in the reduction since norbelladine was also detected in the control assay (with proteins from non-induced bacteria

or boiled *Nps*NBS). Furthermore, norcraugsodine reduction by NR was also at low yield although SDRs are able to catalyze this reaction (Roth et al. 2018). It is possible that enzymes such as NBS and NR work together in a metabolon to rapidly and effectively channel the substrates for their condensation into norcraugsodine followed by an immediate reduction into norbelladine (Fig. 2). This would improve catalytic activity, avoid degradation of unstable norcraugsodine by decreasing its transit time while coordinate and prevent feedback regulation of norbelladine production. One thing is sure, further studies are clearly required to confirm the role of NBS, NR or another enzyme in norbelladine formation.

The late pathways

Norbelladine-type Amaryllidaceae alkaloids biosynthesis

Norbelladine is methylated to 4'-*O*-methylnorbelladine which has been shown to be incorporated several Amaryllidaceae alkaloid skeletons (*e.g.* galanthamine, lycorine, and crinine) (Fig. 2) (Barton and Kirby 1962; Eichhorn et al. 1998; Fuganti 1973). The reaction is catalyzed by the norbelladine 4'-*O*-methlytransferase (N4OMT), a class I methyltransferase which was the first Amaryllidaceae alkaloid biosynthetic gene identified (Kilgore et al. 2014). *N4OMT* was isolated from the transcriptome of daffodil *N.* sp. *aff. pseudonarcissus* using BLAST searches for sequences of plant OMTs coupled to the program HAYSTACK used to identify MTs that fit a model for galanthamine biosynthesis (Kilgore et al. 2014). *NpN4OMT* was shown to be expressed highly in bulbs of *N.* sp. *aff. pseudonarcissus* (Kilgore et al. 2014). Heterologous expression, purification and characterization of N4OMT enzyme showed the specific 4'-*O*-methylation of norbelladine and *N*-methylnorbelladine to similar Km (Kilgore et al. 2014).

Since, several *N4OMT* sequences and transcript variants have been identified and characterized from several Amaryllidaceae species including *N. pseudonarcissus, N. papyraceus, L. radiata, L. aurea* and *Rhodophiala bifida* (Hotchandani et al. 2019; Li et al. 2019; Park et al. 2019; Reis et al. 2019; Singh and Desgagne-Penix 2017; Sun et al. 2018). Similarly to *NpN4OMT*, transcripts for *NpsN4OMT, NpaN4OMT, LraOMT1* and *RbiN4OMT* were highest in bulbs (Hotchandani et al. 2019; Li et al. 2019; Park et al. 2019; Reis et al. 2019; Singh and Desgagne-Penix 2017) whereas and *LauOMT1* expression was highest in aerial parts such as flower stalk and ovary (Sun et al. 2018). Differently to *Np*N4OMT which activity was specific to the 4'-*O* position, the biochemical analysis of recombinant *Lycoris* OMTs, *Lau*OMT1 (highly identical to *Np*N4OMT) displayed both, *meta* (3') and *para* (4'), *O*-methylation activities with norbelladine, caffeic acid and 3,4-DHBA, but showed a strong preference for the *meta* position (Sun et al. 2018). Similarly, enzymatic promiscuity of *Lra*OMT was also demonstrated with the 3'- and 4'-*O*-methlyation of caffeic acid, 3,4-DHBA, and norbelladine with a preference for the 4'-*O*-methylation of norbelladine (Li et al. 2019). Subcellular localization showed that *Lau*OMT1 is mainly localized in cytoplasm and the endosome (Sun et al. 2018). Altogether, these studies showed that N4OMT preferentially catalyzes the 4'-*O*-methylation of norbelladine.

In addition to norbelladine and its 4'-*O*-methylated form, few norbelladine-type Amaryllidaceae alkaloid have been identified in Amaryllidaceae plants but the biosynthetic reactions leading to their synthesis are not know (Fig. 2, purple). For example, *N*-demethylbelladine and its *N*-methylated form belladine have been detected in plants proposing the presence of a *N*-methyltransferase enzyme able to catalyze this reaction. Also, rystilline detected in Amaryllidaceae plants must involve a series of enzymes capable of *O*-methylations and hydroxylation reactions (Fig. 2).

Cherylline-type Amaryllidaceae alkaloids biosynthesis

The cherylline skeleton is thought to originate from the hydroxylation at the 11-position of the norbelladine scaffold followed by the subsequent cyclization with the dioxygenated phenol group (Chan 1973). Although the order of reactions is not determined, norbelladine may be 3'-*O*- and *N*- methylated to form *O,N*-dimethylnorbelladine (Fig. 2, green), which would be hydroxylated at the C-2 position to give 2-hydroxy-*O,N*-dimethylnorbelladine by dehydratation into a quinone methide structure followed by cyclization and tautomerization to cherylline (Ding et al. 2017). Recently, derivatives of cherylline including gigantellinine and gigantelline (Fig. 2, green) were identified in *C. jagus* supporting further

enzymatic modifications of the cherylline scaffold within this species (Ka et al. 2020). No biosynthetic genes or enzymes have been identified to be involved in cherylline formation (Fig. 2, green), but it was demonstrated that various recombinant N4OMT could perform 3'-*O*-methylation activities on norbelladine (Li et al. 2019; Sun et al. 2018).

The late–late pathways

Phenol coupling and reduction reactions

An important step in the biosynthesis of Amaryllidaceae alkaloids, downstream of *O*-methylnorbelladine, is the coupling of phenolic rings to yield crucial intermediate structures. The reaction involves hydrogen abstraction from a phenol followed by the delocalization of the unpaired electron via resonance forms in which the free electron is dispersed to positions *ortho* and *para* to the original oxygen. These phenol-derived radicals are quenched by coupling with other radicals and the coupling of two of these resonance structures, in various combinations, gives a range of dimeric systems. Hence, C–C bonds involving positions *ortho* or *para* to the original phenols may be formed. Thus, the cyclization of the 4'-*O*-methylnorbelladine, by three alternative ways of C–C phenol coupling, leads to Amaryllidaceae alkaloid with different core skeletons (Fig. 2). These three common divisions at 4'-*O*-methylnorbelladine are the *para-ortho'* coupling leading to galanthamine-type Amaryllidaceae alkaloids, the *ortho-para'* phenol coupling elaborating lycorine- and lycorenine-type, and the *para-para'* phenol coupling leading to Amaryllidaceae alkaloids of the crinine-, narciclasine-, tazettine-, and montanine-type (Fig. 2).

The three common phenol-phenol' coupling reactions require the same basic biochemistry to function and are likely catalyzed by cytochrome P450 enzymes, laccases, or peroxidases (Kilgore and Kutchan 2015; Kilgore 2016; Singh and Desgagne-Penix 2014; Singh and Desgagné-Penix 2015; Takos and Rook 2013). Recently, a candidate cytochrome P450 sequence was identified through comparative transcriptomics of *N.* sp. *aff. pseudonarcissus*, *Galanthus* sp., and *G. elwesii*. Through heterologous expression, purification and in vitro characterization, *Np*CYP96T1 showed to be capable of forming the products (10b*R*,4a*S*)-noroxomaritidine and (10b*S*,4a*R*)-noroxomaritidine from 4'-*O*-methylnorbelladine supporting its involvement as a *para-para' C–C* phenol coupling cytochrome P450 (Kilgore et al. 2016a). Furthermore, *Np*CYP96T1 was also shown to catalyzed, at very low level, the formation of the *para-ortho'* phenol coupled, *N*-demethylnarwedine (Kilgore et al. 2016a). Until now, few orthologous sequences and transcript variants of CYP96T were found including two variants in the transcriptome of *N. pseudonarcissus* 'king Alfred' and *N. papyraceus* (Hotchandani et al. 2019; Singh and Desgagne-Penix, 2017). The expression of *NpsCYP96T1, NpaCYP96T1* and *LraCYP96T* was highest in bulbs (Hotchandani et al. 2019; Park et al. 2019; Singh and Desgagne-Penix 2017). In various *Narcissus* transcriptomes, *CYP96T1* co-expressed with *N4OMT* and correlated with lycorine and/or galantamine accumulation (Hotchandani et al. 2019; Kilgore et al. 2016a; Singh and Desgagne-Penix 2017). Interestingly, CYP96T1 is the first phenol-coupling enzyme characterized from a monocot.

Following the formation of the three C–C phenol coupled scaffolds: nornarwedine (*para-ortho'*), noroxolpluviine (*ortho-para'*) and noroxomaritidine (*para-para'*), these are further reduced into metabolite intermediates that will be further modified to yield numerous Amaryllidaceae alkaloid end-products (Fig. 2). In alkaloid metabolism, reductases mostly come from two different superfamilies: the SDRs and the aldo–keto reductases (AKRs). In BIA metabolism members of both family are involved with salutaridine reductase, sanguinarine reductase, and noscapine synthase from the SDR superfamily, whereas 1,2-dehydroreticuline reductase and codeinone reductase are members of the AKR superfamily (Dastmalchi et al. 2018). Although SDRs and AKRs are derived from distinct ancestral sources, their catalytic mechanisms and conformations display features indicative of convergent evolution. The SDR discussed earlier, capable of reducing norcraugsodine to norbelladine at very low level was initially identified as a noroxomaritidine reductase (NR) catalyzing the formation of oxomaritinamine from noroxomaritidine (*para-para'*) through a C–C double bond reduction (Kilgore et al. 2016b). Since, several orthologous sequences and transcript variants of NR were found including three variants in the transcriptome of *N. papyraceus* (Hotchandani et al. 2019). The expression of *NpsNR, NpaNR* and *LraNR* was ubiquitous in different tissues of Amaryllidaceae plants (*e.g.* bulb, root, stem, leaf,

flower) (Hotchandani et al. 2019; Park et al. 2019; Singh and Desgagne-Penix 2017).

The three core skeletons obtained from the phenol coupling and reduction steps form the basis of further alkaloid diversity. A complex network of enzymatic reactions exists to produce a spectrum of compounds that differs between species, varieties and cultivars and even between the different tissues and vegetative phases of the same plant. These biochemical modifications are achieved by a multitude of enzymes catalyzing various types of reactions, such as C–C and C–O bond formations, O- and *N*-methylations, demethylations, hydroxylations, oxidations and reductions. The various products obtained from these reactions yields the several hundred of structurally related Amaryllidaceae alkaloids known to date (Table 1, Figs. 1 and 2) (Bastida et al. 2011; Jin 2013; Jin and Yao 2019; Kilgore and Kutchan 2015; Kilgore 2016; Kornienko and Evidente 2008; Nair and van Staden 2013; Singh and Desgagne-Penix 2014; Singh and Desgagné-Penix 2015; Takos and Rook 2013). In addition, substrate promiscuity, which is characteristic for several of these enzyme families, add to the variation and diversity of alkaloids. Also, cellular and subcellular localization of these enzymes may directly influence the alkaloid profile obtained from different parts of the Amaryllidaceae plant as cellular relocalization primes specialized metabolic diversification (Schenck and Last 2019).

The extensive work studying the synthesis of the derivatives generated from phenol–phenol' coupling products has been reviewed in details and is beyond the scope of this review (Bastida et al. 2011; Ding et al. 2017; He et al. 2015; Jin 2013, 2016; Jin and Yao 2019; Kornienko and Evidente 2008; Singh and Desgagné-Penix 2015). It is however briefly summarized below with an emphasis on biosynthetic steps.

The para-ortho' phenol coupled: galanthamine-type Amaryllidaceae alkaloids

Downstream of nornarwedine, there are no genes or enzymes that have been reported so far. A proposed pathway is presented in Fig. 2, based on radiolabeled studies (Eichhorn et al. 1998; El Tahchy 2010; El Tahchy et al. 2010, 2011). Briefly, the stereospecific reduction of nornawedine yields norgalanthamine, which is *N*-methylated to galanthamine (Fig. 2, red). Alternatively, nornarwedine is *N*-methylated to narwedine (Fig. 2, red). Norgalanthamine may also be reduced to norlycoramine and *N*-methylated to lycoramine. All these *N*-methylation reactions could be catalyzed by similar *N*-methyltransferases putatively involved these steps (Fig. 2, red). Further modifications of galanthamine or lycoramine lead to various galanthamine-type alkaloids (*e.g.* sanguine and chlidanthine).

The ortho-para' phenol coupled: lycorine- and lycorenine-type Amaryllidaceae alkaloids

Downstream of noroxopluviine, there are no genes or enzymes that have been identified so far in the formation of lycorine- or lycorenine-type alkaloids. Their biosynthesis starts with the reduction of noroxopluviine to yield norpluviine (Fig. 2). From there the pathway separates towards lycorine- or lycorenine-type Amaryllidaceae alkaloids. Briefly, the path to lycorine-type involves a methyledioxy bridge formation of norpluviine to form caranine followed by its hydroxylation to lycorine (Fig. 2, brown). The route to lycorenine-type Amaryllidaceae alkaloids starts with the hydroxylation of norpluviine followed by a series of modifications including methylation, oxidation, reduction, etc. (Figure 2, yellow).

The para-para' phenol coupled: crinine-, narciclasine-, tazettine-, and montanine-type Amaryllidaceae alkaloids

Aside from CYP96T1 and NR, there are no other steps (genes or enzymes) that have been identified in the formation of *para-para'* Amaryllidaceae alkaloid-types. The crinine-type belong to the largest class of the Amaryllidaceae alkaloid, a significant taxonomic feature (Ding et al. 2017; Jin and Yao 2019). Their biosynthesis starts with the reduction of noroxomaritidine to normaritidine (Fig. 2). Form there, the pathway separates and several reactions are involved. For example, the formation of narciclasine-type involves the rearrangements of the vittatine skeleton with several hydroxylation and reduction reactions to yield narciclasine, which can be hydroxylated to pancrastatine (Fig. 2, dark gray). Another example is the modification and reorganization of 11-hydroxyvittatine to pancracine which can be *O*-methylated to yield montanine (Fig. 2, blue). 11-hydroxyvittatine can be *O*-methylated to yield haemanthamine (Fig. 2,

pink), which can be modified into tazettine-type Amaryllidaceae alkaloids (Fig. 2, light gray). Further studies are necessary to determine the exact sequence of reactions to form these Amaryllidaceae alkaloids-types.

Biotechnological production of Amaryllidaceae alkaloids

The pharmaceutical potential of Amaryllidaceae alkaloids has been acknowledged through the commercialization of galanthamine as an Alzheimer's drug due to its potent and selective inhibitory activity against the acetylcholinesterase enzyme. Other Amaryllidaceae alkaloids are of commercial interest, but are only in low amounts in their native plants. Every Amaryllidaceae species studied until now display specific alkaloid composition (types and quantity), often with a few dominant ones and a larger number at low abundant alkaloids (Berkov et al. 2014; He et al. 2015; Jin and Yao 2019). Although the molecular origin of this biochemical diversity has not yet been elucidated, these Amaryllidaceae alkaloid compositions likely result from differences in the expression level and substrate specificity of the various biosynthetic enzymes involved. Large-scale production of Amaryllidaceae alkaloids is difficult and costly and growing large number of Amaryllidaceae plants in order to obtain reasonable amounts of Amaryllidaceae alkaloids is not always environmentally sustainable. Thus, it is important to develop sustainable methods to produce valuable Amaryllidaceae alkaloids for pharmaceutical applications. Plant in vitro technologies such as cell/tissue culture has become increasingly attractive as cost-effective alternative for the sustainable mass production of plant-derived metabolites. Several Amaryllidaceae in vitro cultures have been studied and the level of some interesting alkaloid was regulated (El Tahchy 2010; El Tahchy et al. 2010, 2011; Ferdausi 2017; Ivanov et al. 2011, 2013; Laurain-Mattar and Ptak 2018; Pavlov et al. 2007; Ptak et al. 2010; Resetár et al. 2017; Schumann et al. 2012, 2013; Tarakemeh et al. 2019; Trujillo-Chacon et al. 2019). For example, (El Tahchy et al. 2011) have reported that auxin analogs changed alkaloids composition in Amaryllidaceae cell cultures. Specifically, adding 2,4-D enhanced the biosynthesis of galanthamine, lycorine, crinine and demethylmaritidine in *L. aestivum* shoot cultures whereas adding naphthalene-1-acetic acid led to more diversity in alkaloids in *G. elwesii* shoots cultures (El Tahchy et al. 2011). The level of alkaloids in Amaryllidaceae in vitro cultures still need to be investigated to become a commercially attractive opportunity. The genetic engineering of plants or microorganisms offers promising potential alternative sources for the production of high concentrations of alkaloids and would be environmentally sustainable and cost-effective. This, however, requires a better understanding of the Amaryllidaceae alkaloids biosynthetic pathway.

In summary, a precise knowledge of Amaryllidaceae alkaloids biosynthetic networks is mandatory to the emerging field of metabolic engineering and synthetic biology, which aims to reconstruct plant pathways in microorganisms with the ultimate goal of creating alternative systems for the production of valuable bioproducts. To date, several strategies have been developed to engineered microbial hosts for the production of alkaloids and theirs precursors (Diamond and Desgagne-Penix 2016; Facchini et al. 2012; Fossati et al. 2014, 2015; Hawkins and Smolke 2008; Matsumura et al. 2018; Minami et al. 2008; Narcross et al. 2016; Pyne et al. 2019; Slattery et al. 2018).

Conclusion

The commercial demand to increase certain Amaryllidaceae alkaloids in plants is very difficult to achieve by conventional breeding techniques but current technologies may help us to reach these goals. Until recently, non-model plants such as Amaryllidaceae were recalcitrant to modern molecular biology approaches for gene and pathway discovery due in part to the lack of transformation protocols. However, the advent of quick and low-cost sequencing coupled with rapid progress in the development of integrative approaches such as genomics, transcriptomics, and metabolomics, has provided essential information for the understanding of several complex biosynthetic pathways. Correlations between plant transcriptome and metabolome have been successfully used for the identification of novel genes involved in Amaryllidaceae alkaloid biosynthesis (Hotchandani et al. 2019; Kilgore et al. 2014, 2016a, b; Park et al. 2019; Singh and Desgagne-Penix 2017; Singh et al. 2018; Wang et al. 2013). This has opened the door to facilitate the

elucidation of the Amaryllidaceae alkaloids biosynthetic pathways.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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