MINI REVIEW

Biosynthesis of the Amaryllidaceae alkaloids

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Abstract

Amaryllidaceae alkaloids (AAs) are a structurally diverse group of plant specialized metabolites with powerful biological activities. The medicinal properties of many AAs have been identified including the antitumor agent narciclasine and galanthamine, used for Alzheimer's disease. Tracer studies have led to proposed pathways but AA biosynthesis remains molecularly uncharacterized. The use of systems biology-based approaches could lead to the unraveling of AA metabolic pathways. The elucidation of AA biosynthesis will provide necessary tools required to enhance AA production in plants as well as the development of microbial production platforms as an alternative to plants as a commercial source of valuable AAs.

Keywords: amaryllidaceae alkaloid; plant secondary metabolism; galanthamine biosynthesis; natural products; systems biology.

Abbreviations: AA, Amaryllidaceae alkaloid; 3,4-DHBA, 3,4-dihydroxybenzaldehyde; PAL, phenylalanine ammonia lyase; Ca4H, cinnamate-4-hydroxylase; Ca3H, coumarate-3-hydroxylase; TYDC, tyrosine decarboxylase; CYP450, cytochromes P450.

1. Introduction

For centuries, a large number of plants have been used as essential resource for therapeutic agents against various human illnesses. Among these traditional sources

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of medicines, the bulbous monocot Amaryllidaceae family, comprising over 1100 species, are among the top 20 of the most widely applied plant families (Jin, 2013). In addition to their pharmacological interests, the well-known horticultural ornamental value of Amaryllidaceae plants (Amaryllis, Narcissus and Galanthus), has long been recognized and exploited.

The medicinal properties of the Amaryllidaceae are owed to the presence of specialized metabolites, the Amaryllidaceae alkaloids (AAs), which are specific to this plant family. To date, more than 500 structurally diverse AAs have been isolated (Jin, 2013) and modern phytochemical studies have shown the various pharmacological activities of numerous AAs. These include analgesic, anticancer, and antimicrobial activities (Bastida *et al.*, 2011; Heinrich & Lee Teoh, 2004; Kornienko & Evidente, 2008; Nair & van Staden, 2013). For example, narciclasine displays potent antitumor activity (Kornienko & Evidente, 2008) whereas galanthamine, an acetylcholinesterase inhibitor, is used to treat symptoms of Alzheimer's disease (Heinrich & Lee Teoh, 2004). As it is the case for many valuable plant natural products, the commercial use of most AAs is restricted by their limited availabilities due to their low concentrations in plants. For example, the yield of galanthamine from leaves of *Leucojum aestivum* is less than 0.2% DW and varies between 0.059-0.166% DW from plants of different geographical origin (Berkov, Bastida, Viladomat, & Codina, 2011). Narciclasine levels are higher in *Narcissus tazetta* (65mg/kg) compared with other Amaryllidaceaea species such as *L. aestivum* (30mg/kg) and *Galanthus nivalis* (10mg/kg) however this yield is still too low for lucrative applications (Kornienko & Evidente, 2008). Currently, only galanthamine is used clinically and almost entirely produced from *Leucojum* and *Narcissus* cultivated plant species (Takos & Rook, 2013). Most AAs available commercially are obtained from their natural sources and only a few are produced synthetically. Although several chemical syntheses for galanthamine, narciclasine and others AAs have been described (Kornienko & Evidente, 2008; Takos & Rook, 2013), chemists are still struggling to

devise a reasonably short synthetic pathway that can be used to produce these compounds on an economically viable commercial scale. Since most AAs display promising biological activities [but are produced in small quantities relative to the potential clinical and commercial demand], there are obvious interests in engineering AA production in plants or in heterologous expression systems. However, the lack of information on AA biosynthetic pathways, regulation and transport makes this task very challenging. To date, several AA pathways remain hypothetical and enzymes and metabolic intermediates await discovery. Recently, systems biology-based approaches have facilitated the discovery of biosynthetic genes involved in natural products metabolic pathways in other plant families through the integration of multiple 'omics' (genomic, transcriptomic, proteomic and metabolomic) datasets. This strategy appears to be a promising avenue for the elucidation of AA biosynthesis in order to identify and characterize biosynthetic enzymes. In the scope of this review, we are focusing on the molecular basis of AA biosynthesis and we will propose putative gene families involved in AA biosynthetic pathways.

2. Biosynthesis

In contrast to the extensive literature on the pharmaceutical effects of AAs, information on their molecular genetics and biochemical pathways is incomplete. Previous studies using radiolabeled precursors led to the biochemical elucidation of the first steps in AA biosynthesis (Barton & Cohen, 1957; Barton, Kirby, Taylor, & Thomas, 1963; Battersby, Fales, & Wildman, 1961; Eichhorn, Takada, Kita, & Zenk, 1998). Despite the vast structural diversity, AAs share a common central intermediate, norbelladine, which is formed through the condensation of aromatic amino acids derivatives; 3,4-dihydroxybenzaldehyde (3,4-DHBA also named protocatechuic aldehyde) and tyramine (Fig. 1). Interestingly, similarities to several plant specialized biosynthetic pathways can be observed. For example, the formation of 3,4-DHBA features the initial steps of the phenylpropanoid biosynthesis whereas the formation of the tyramine precursor is analogous to isoquinoline alkaloid biosynthesis. The resulting tetrahydroisoquinoline core of norbelladine is central to the biosynthesis of many structural types of AAs.

2.1 Initial biosynthetic reactions

The substrates and enzymes necessary for the first committed steps in plant specialized metabolism often appear to have been recruited from primary metabolic pathways (Chu, Wegel, & Osbourn, 2011). Substrates for the initial reactions of alkaloids biosynthesis are derived from primary metabolism, especially the aromatic amino acids L-phenylalanine and L-tyrosine. Additionally, since gene duplication is one of the most important components

for the evolution of specialized metabolism, there are several "later" enzymes in these pathways that have ancestors in primary metabolism. For example, the enzyme homospermidine synthase, which catalyzes the first committed step to pyrrolizidine alkaloids, has most likely been developed by gene duplication from deoxyhypusin synthase, an enzyme from the polyamine biosynthetic pathway involved in cell division and growth (Moll *et al*., 2002).

In the initial stages of AA biosynthesis, the enzyme phenylalanine ammonia lyase (PAL) catalyzes the elimination of ammonia to generate trans-cinnamic acid (Fig. 1). Two hydroxylation reactions catalyzed by cytochrome P450s, cinnamate-4-hydroxylase (Ca4H) and coumarate-3-hydroxylase (Ca3H) followed by the loss of two carbon atoms leads to the formation of the C_6C_1 precursor, 3,4-DHBA (Bastida *et al*., 2011; Eichhorn *et al*., 1998; Grisebach, 1973). These reactions are also part of phenylpropanoid metabolism, so that AA biosynthesis nicely shows the connections between two plant specialized pathways, alkaloid and phenylpropanoid. On the other hand, tyrosine is decarboxylated to tyramine by tyrosine decarboxylase (TYDC) (Bastida *et al*., 2011; Eichhorn *et al*., 1998; Grisebach, 1973; Takos & Rook, 2013). TYDC is a unique and key regulatory enzyme in many alkaloid-producing plants. In addition to controlling the transition from primary to specialized metabolism, TYDC ensures an adequate supply of tyramine for the synthesis of various isoquinoline alkaloids including the well-known narcotic analgesics morphine and codeine (Desgagné-Penix & Facchini, 2011; Facchini, 2001; Facchini, Huber-Allanach, & Tari, 2000; Hagel & Facchini, 2013; Ziegler & Facchini, 2008). Although the enzymatic reactions leading to the formation of AA precursors still need confirmation, feeding experiments in *N. pseudonarcissus* (Eichhorn *et al*., 1998) and recent transcriptome analyses of *Lycoris aurea* (Wang *et al*., 2013) have proven the presence of metabolite intermediates and transcripts for PAL, C4H, C3H and TYDC. These findings support the current proposed pathways. However no gene sequences or enzymes have been isolated and characterized to date.

2.2 Formation of the norbelladine skeleton

All AAs are viewed as derivatives of the common skeleton structure of norbelladine (Fig. 1). The combination of 3,4-DHBA and tyramine results in the formation of a Schiff base intermediate which following reduction yields norbelladine (Dewick, 2009; Ghosal, Shanthy, & Singh, 1988). Several similar condensation reactions have been described in other plant alkaloid biosynthetic pathways. For example, the first committed step in the formation of benzylisoquinoline alkaloids (BIA) is the combination of two L-tyrosine derivatives (4-hydroxyphehylacetaldehyde and dopamine) by a

Pictet-Spengler condensation catalyzed by norcoclaurine synthase (NCS) to produce the trihydroxytetrahydroisoquinoline alkaloid norcoclaurine (Luk, Bunn, Liscombe, Facchini, & Tanner, 2007). NCS catalyzes a two-step reaction mechanism; a condensation reaction followed by an intramolecular cyclization (Luk et

al., 2007). The electron-donating oxygen of the hydroxyl group of dopamine (missing in tyramine) is essential for the reaction to proceed and may provide a mechanistic explanation for the absence of cyclization in the condensation step of AA biosynthesis. Two different protein families (pathogenesis-related PR10-Betv1 and
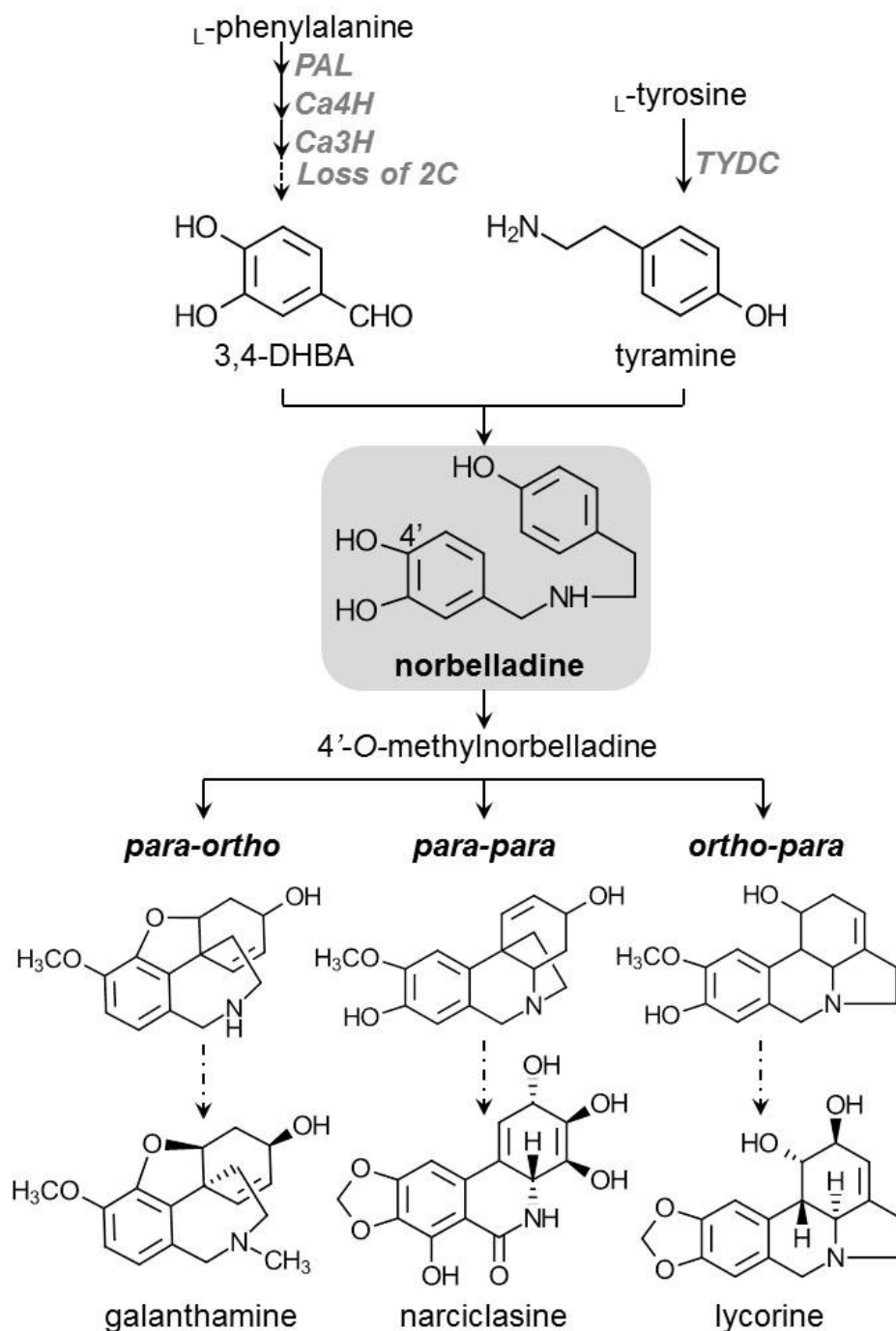

Fig. 1. Proposed AA biosynthetic pathway. Top: Early biosynthetic reactions leading to the norbelladine precursor (shaded gray). Middle: representation of the three backbone structures derived from the alternative phenol coupling reactions. Bottom: examples of chemical structures of AAs in each subgroups. Abbreviations defined in text.

2-oxoglutarate-dependent dioxygenase) have been reported to catalyze this reaction in vitro. However the PR10/Betv1-NCS was shown to be the major one involved in BIA biosynthesis in *Papaver somniferum* and *Thalictrum flavum* plants (Lee & Facchini, 2010; Minami, Dubouzet, Iwasa, & Sato, 2007; Samanani, Liscombe, & Facchini, 2004). Another example of a Pictet-Spengler condensation is the combination of tryptamine and secologanin resulting in strictosidine, the general precursor of monoterpenoid indole alkaloids. In *Rauvolfia serpentina*, this reaction is catalyzed by strictosidine synthase, a member of the six-bladed four-stranded β-propeller fold protein family (Stockigt, Barleben, Panjikar, & Loris, 2008).

The examples above show that the initial condensation reactions in alkaloid biosynthesis can be catalyzed by members of very different protein families. This suggests that the type of enzyme recruited for the non-cyclizing condensation reaction in AA biosynthesis may belong to one of these families. However, additional classes of enzymes should not be ruled out.

It has been proposed that O-methylation of norbelladine precedes the oxidative phenol coupling, which yields the structurally diverse AAs. Plant O-methylation reactions are common transformation in the biosynthesis of alkaloids and are most often catalyzed by S-adenosyl-L-methionine (SAM)-dependent methyltransferases (MTs) (Liscombe, Louie, & Noel, 2012). Thus, it is assumed that norbelladine must be 4'-O-methylated to form 4'-O-methylnorbelladine. This compound then serves as the central intermediate from which multiple biosynthetic pathways lead to various structural types of AAs (Fig. 1).

2.3 Phenol coupling reaction and formation of diverse AA subgroup backbones

A crucial step in AA biosynthesis is the cyclization of 4'-O-methylnorbelladine by oxidative C-C phenol coupling, which can occur in ortho-para, para-ortho and para-para positions. These alternative phenol coupling reactions generate three backbone structures. Accordingly, the different AA subgroups are referred to as ortho-para, para-ortho and para-para (Fig. 1). For example, antitumor AAs of the narciclasine-type are derived by a para-para phenol coupling step (Bastida *et al*., 2011; Kornienko & Evidente, 2008; Takos & Rook, 2013).

Recently, several enzymes have been discovered in various alkaloids pathways, which catalyze oxidative C-C phenol coupling reactions. All these enzymes were found to be members of the plant cytochromes P450 (CYP450s) (Mizutani & Sato, 2011). CYP450s catalyze a wide variety of monooxygenation/hydroxylation reaction in specialized metabolism and some of them are involved in unusual reactions such as methylenedioxy-bridge formation, oxidative C-C bond cleavage, oxidative rearrangement of carbon skeletons and phenol coupling reactions. For example, the intramolecular C-C phenol coupling catalyzed by CYP80G2 participates in the formation of aporphine-type alkaloid in Coptis japonica (Ikezawa, Iwasa, & Sato, 2008). Substrate specificity studies of CYP80G2 revealed the promiscuity of this enzyme as it reacted with several derivatives of reticuline such as (R,S)-orientaline, (R,S)-codamine, (S)-N-methylcoclaurine, and (S)-coclaurine informing that it could accept different functional group on the C-ring. However, CYP80G2 did not react with derivatives whose functional group were different from reticuline on the A-ring suggesting that the A-ring plays an important role in substrate recognition (Ikezawa *et al*., 2008). Another example is salutaridine synthase, a member of the CYP719B1, which catalyzes the para-ortho coupling of reticuline in the biosynthesis of morphinan alkaloids in *P. somniferum*. CYP719B1 is highly stereospecific as it catalyzes the C-C phenol coupling of only (R)-reticuline resulting in formation of salutaridine (Gesell *et al*., 2009). These examples demonstrate that specific CYP450s are able to catalyze intramolecular C-C phenol coupling reactions in alkaloid biosynthesis and that members of two CYP450 families, CYP80 and CYP719, have been identified. A recent comparative analysis of plant CYP450 sequences has shown that only the CYP80 family occurs in monocot plant although no Amaryllidaceae species were represented (Nelson & Werck-Reichhart, 2011). The CYP80 family has been associated with phenolic coupling reactions in several species whereas CYP719s are restricted to Ranunculales plants including *P. somniferum* and *C. japonica*. In addition, the latter steps of AA biosynthesis involve multiple C-C and C-O bond creation including methyledioxy-bridge formation. In other alkaloid pathways such as the BIA, these reactions are catalyzed by CYP719s and CYP80s (Diaz Chavez, Rolf, Gesell, & Kutchan, 2011; Gesell *et al*., 2009; Ikezawa *et al*., 2008). Altogether, these studies suggest that the phenol coupling and the methyledioxy-bridge formation steps in AA biosynthesis are likely to involve CYP450 of the CYP80 family. However, additional families of enzymes should not be ruled out. Interestingly, AAs from all three subgroups of phenol coupling can co-occur in a single plant, whereas some species and cultivars contains only one subgroup of AA (Berkov, Martinez-Frances, Bastida, Codina, & Rios, 2014; Kornienko & Evidente, 2008), This further suggests the existence of several CYP450 genes with different substrate, and product specificity with respect to the positions of intramolecular oxidative C-C phenol coupling.

2.4 Core backbone structure modifications and decorations

The three backbone structures obtained from the phenol coupling steps form the scaffolds of further alkaloid diversity leading to more than 500 AAs known to date (Jin, 2013). For example, in BIA biosynthesis, the reticuline

skeleton is a basic building block for several types of isoquinoline alkaloids divided in several categories including aporphines, benzophenanthridines, protoberberines and morphinans (Desgagné-Penix & Facchini, 2011). A complex network of enzymatic reactions will produce a spectrum of compounds that accumulate species-, cultivar-, tissue-, and development-specific. These chemical modifications are achieved by a multitude of enzymes catalyzing various types of reactions, such as O- and N-methylations (OMTs, NMTs), C-C and C-O bond formations, oxidations and reductions, demethylations, and hydroxylations resulting in a variety of different structures. Fig. 1 shows an example for each phenol coupling subgroup of AA. The numerous AA pathways for which the complete set of reactions and biosynthetic genes have not yet been compiled and remain a significant challenge. However, phylogenetic analyses with candidates of analogous alkaloid pathways, such as the isoquinoline alkaloids, appear to be a logical and promising strategy.

3. Systems Biology and future directions

Until recently, non-model organisms such as medicinal plants were recalcitrant to modern molecular biology approaches for gene and pathway discovery due to the lack of genomic information and experimental protocols. However, the recent advances in 'omics' platforms and systems biology have revolutionized our understanding of natural products metabolism in non-model species (Schilmiller, Pichersky, & Last, 2012; Xiao *et al*., 2013) and correlations between plant transcriptome, proteome and metabolome have been successfully used for the identification of novel genes involved in alkaloid biosynthesis (Desgagne-Penix & Facchini, 2012; Desgagne-Penix, Farrow, Cram, Nowak, & Facchini, 2012; Desgagne-Penix *et al*., 2010; Liscombe, Ziegler, Schmidt, Ammer, & Facchini, 2009; Ziegler, Diaz-Chavez, Kramell, Ammer, & Kutchan, 2005; Ziegler *et al*., 2006). Each Amaryllidaceae species studied to date displays a specific AA profile, often with a few dominant compounds and a larger number of compounds at lower concentrations (Bastida *et al*., 2011; Berkov *et al*., 2014; Kornienko & Evidente, 2008). Although the molecular origin of this chemical diversity has not yet been clarified, these profiles likely result from differences in the expression level and substrate specificity of the various biosynthetic enzymes. The development and subsequent integration of 'omics' databases for Amaryllidaceae plants displaying different AA profiles would allow for the identification of candidate genes involved in AA biosynthesis. For example, searches by sequence similarity to Arabidopsis, orthologous genes involved in synthesis of the precursors 3,4-DHBA and tyramine, e.g. PAL, Ca_4H, Ca_3H and TYDC, can be identified. Those could be targeted for silencing or over-expression in plants to assess their role(s) in AA biosynthesis. Recently, the transcriptome of the Amaryllidaceae *Lycoris aurea* was sequenced and assembled and putative genes involved in

AAs biosynthesis (PAL, TYDC, OMT, NMT, P450) were identified based on sequence analysis (Wang *et al*., 2013) however none of the biosynthetic enzymes involved in these pathways have been isolated or functionally characterized.

4. Conclusions

Over the past several years, extensive phytochemical and pharmacological analyses have reported the numerous biological activities of Amaryllidaceae alkaloids and numerous AAs display interesting and valuable pharmacological capabilities. Earlier biochemical tracer studies have led to the current AA biosynthetic proposed pathways. However the molecular identity of the biosynthetic enzymes remains unknown. The technological advances brought as part of the post-genomics era have revolutionized the study of alkaloid metabolism and discoveries made over the past years have relied largely on 'omics' tools including transcriptome libraries, proteomic analyses and metabolomics methods. Integration of these 'omics' resources such as comparative analyses of metabolite and transcript data from Amaryllidaceae species will lead to the identification and isolation of numerous enzymes involved in AA biosynthesis. A deeper understanding of the molecular mechanisms involved in AA biosynthesis will support breeding efforts to produced cultivars of Amaryllidaceae species with enhanced AA production. In addition, it will pave the way for the successful metabolic engineering of microbial systems for the production of valuable AAs.

Acknowledgments

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