Biotechnology Journal

DOI 10.1002/biot.200900229

Biotechnol. J. 2009, 4, 1684–1703

Review

Medicinally important secondary metabolites in recombinant microorganisms or plants: Progress in alkaloid biosynthesis

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Plants produce a high diversity of natural products or secondary metabolites which are important for the communication of plants with other organisms. A prominent function is the protection against herbivores and/or microbial pathogens. Some natural products are also involved in defence against abiotic stress, *e.g.* UV-B exposure. Many of the secondary metabolites have interesting biological properties and quite a number are of medicinal importance. Because the production of the valuable natural products, such as the anticancer drugs paclitaxel, vinblastine or camptothecin in plants is a costly process, biotechnological alternatives to produce these alkaloids more economically become increasingly important. This review provides an overview of the state of art to produce alkaloids in recombinant microorganisms, such as bacteria or yeast. Some progress has been made in metabolic engineering usually employing a single recombinant alkaloid gene. More importantly, for benzylisoquinoline, monoterpene indole and diterpene alkaloids (taxanes) as well as some terpenoids and phenolics the proof of concept for production of complex alkaloids in recombinant *Escherichia coli* and yeast has already been achieved. In a long-term perspective, it will probably be possible to generate gene cassettes for complete pathways, which could then be used for production of valuable natural products in bioreactors or for metabolic engineering of crop plants. This will improve their resistance against herbivores and/or microbial pathogens.

Received 10 September 2009
Revised 28 October 2009
Accepted 2 November 2009

Keywords: Alkaloids · Biosynthesis · Metabolic engineering · *In-vitro* production · Terpenoids

1 Introduction

Plants produce a wide variety and high diversity of secondary metabolites (SM), which are not needed for primary or energy metabolism. They are not useless waste compounds, however, as previously assumed, but important for the ecological fitness of a plant producing them. Secondary metabolites have apparently evolved as a means for plants to protect themselves against insects, mammals and other herbivores, against bacteria, fungi, viruses and even other competing plants. Some plants use SM in addition to attract pollinating and seed dispersing animals or for UV protection [1–6].

Plants usually synthesize, transport and store SM in a specific and particular way [3, 4]. Even a single plant produces a complex mixture of SM, which often derives from different types of SM; *e.g.* most plants produce phenolics, such as flavonoids but concomitantly terpenoids, such as saponins. The types of SM produced are sometimes but not always typical for a certain systematic group of plants [3, 5, 6]. Among more than 100 000 structures of SM that have been identified so far, we can distinguish between nitrogen-containing and nitrogen-free SM. Among nitrogen-containing SM, alkaloids are the largest group with more than 20 000 structures, many of them with pronounced pharmacological and toxic properties [7–9]. Also impor-

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Abbreviations: BIA, benzylisoquinoline alkaloids; **MIA**, monoterpene indole alkaloid; **PA**, pyrrolizidine alkaloid; **SM**, secondary metabolite

tant are non-protein amino acids (700 structures), amines (100 structures), cyanogenic glucosides (60 structures), glucosinolates (100 structures), alkamides (150 structures), as well as lectins and other peptides (2000 structures). In the class of nitrogen-free SM, even more structures have been determined. The largest class is terpenoids with more than 20 000 known compounds, among them mono-, sesqui-, di-, and triterpenes with interesting bioactivities. Another bioactive group of SM, the polyphenols, is characterized by the presence of several phenolic hydroxyl groups, which can dissociate into O^- ions under physiological conditions. Members of polyphenols are flavonoids, anthocyanins, and tannins. In addition, phenylpropanoids, coumarins, lignans and anthraquinones often possess phenolic OH-groups [4–8].

Structures of most SM are not random but the consequence of millions of years of selection during evolution. Therefore, it is not surprising that pharmacologists have discovered that a number of SM exhibit significant biological properties and can interact with molecular targets in human cells or microorganisms [4–9]. Consequently, many of the drugs used in medicine today derive directly from plants or indirectly in that structures of bioactive SM were used as a lead for the chemical synthesis with improved activities. SM from plants either are used as isolated chemical entities or as complex extracts (typical for phytomedicine/phytotherapy) [7–9]. The latter approach has interesting aspects as the extracts apparently contain not only SM with additive but also synergistic properties [9]. Examples of isolated SM, which are being used in medicine include vinblastine, vincristine, paclitaxel (taxol), camptothecin, demecolcine, and podophyllotoxin (used in cancer therapy as probably the most important drugs), but also emetine, serpentine, ajmaline, reserpine, yohimbine, strychnine, ergobasine, ergotamine, quinine, quinidine, sparteine, ephedrine, lobeline, caffeine, berberine, sanguinarine, tubocurarine, papaverine, morphine, codeine, thebaine, noscapine, atropine, scopolamine, cardiac glycosides, artemisinin, anthraquinones and several others [7, 8]. As can be seen from this list, most of the interesting drugs are alkaloids; especially the anticancer drugs have a large market. So far, these drugs derive from plants, usually grown in plantations. Since their production in plants is usually low, the production costs are high and in consequence these drugs are costly.

Plant biotechnologists have explored possibilities to produce these valuable drugs using various *in vitro* systems, including bioreactors (Fig. 1). Callus cultures, suspension cell cultures, organ cultures (root and hairy root cultures) and even large-scale fermentation of suspended cells were successfully established over the last 40 years [10–21]. Thus, technically the *in-vitro* production should be feasible. However, the employment of callus and suspension cultures of medicinal plants often encountered the problem of very low or insufficient product yields. Apparently, the genes encoding the proteins necessary for biosynthesis, transport and storage of SM are not adequately expressed in most undifferentiated cell cultures [22]. There are a few notable exceptions with ginsenosides in *Panax ginseng*, shikonin in *Lithospermum erythrorhizon*, berberine in *Coptis japonica*, rosmarinic acid in *Coleus blumei*, anthraquinones in *Morinda citrofolia* or paclitaxel in *Taxus* sp. [21, 23]. More encouragingly, root and hairy root cultures, which are differentiated tissues, show excellent product yields for those SM that are produced in roots (which is unfortunately not the case for all SM) [21, 23, 24]. However, the large-scale fermentation of roots and hairy roots still is a challenge, although bioreactors have been developed for a sustainable *in vitro* cultivation [22–24].

An alternative to the production of valuable plant drugs in plant cells was discussed already more than 30 years ago but even more so these days [25–39]. Once the genes are known that encode the enzymes of a biosynthetic pathway, it should be possible to functionally express these genes in a microbial system, such as bacteria or yeasts, and let them do the production (Fig. 1). As a first step, it could be shown in 1989 that *Escherichia coli* transformed with the plant gene encoding phenylalanine ammonium lyase (PAL) would convert phenylalanine into cinnamic acid, an important intermediate in the biosynthesis of flavonoids and some phenylpropanoids [39]. As a prerequisite for the production of complex natural products we need to isolate and characterize all the genes involved in the synthesis and storage of SM and then find a way to co-express them concomitantly in a microbial system. The search for the genes of plant secondary metabolism turned out to be very difficult and slow, because the genes of a biosynthetic pathway are usually not clustered as in bacteria, but apparently well dispersed over the plant genome. Secondly, mutants to select the genes were also not available. Therefore, each individual enzyme of a pathway had to be isolated beforehand, sequenced and then the genes could be isolated by employing corresponding primers for PCR or cDNA synthesis. Once such a gene became known it was usually much easier to find homologous genes in other plants. In the last 20 years, an impressive number of genes of secondary metabolism has been isolat-

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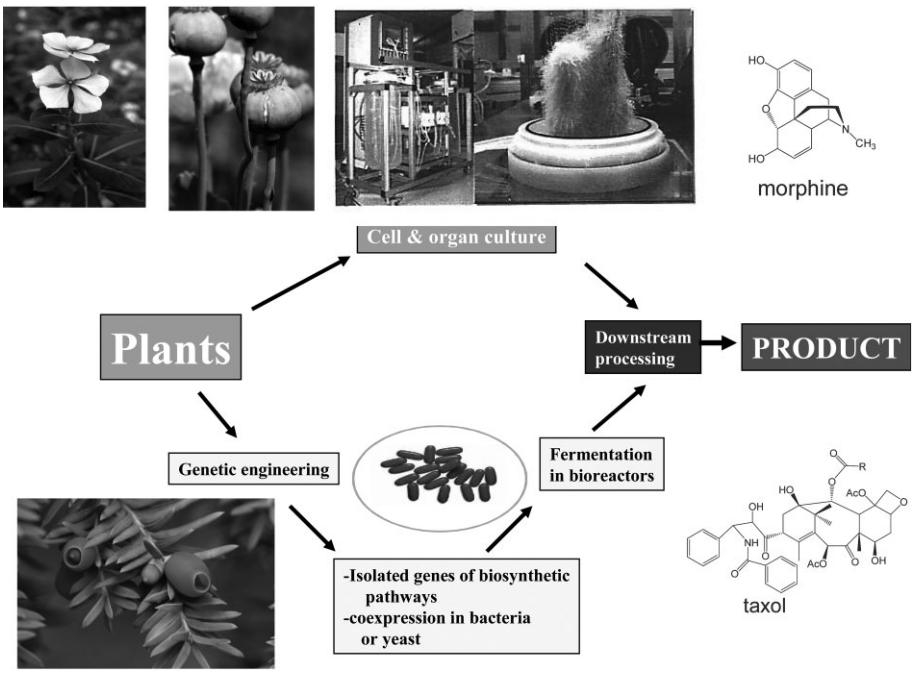


Figure 1. Some strategies for the production of secondary metabolites.

ed (see next Sections). Several of the genes could be expressed in recombinant microorganisms and plants (reviewed in [25–39]). Most excitingly, researchers were successful to functionally co-express two or even more genes of a pathway. Thus, through metabolic engineering it was possible to produce a few selected benzylisoquinoline alkaloids and key intermediates of artemisinin or taxane biosynthesis in recombinant *E. coli* or *Saccharomyces cerevisiae* [28, 33–35]. Although these processes are still incomplete and not yet commercial, they are important steps forward to the *in vitro* production of SM.

This review will mainly focus on the progress made in finding the genes involved in alkaloid biosynthesis and in expressing these genes in recombinant systems. Alkaloids were selected as this group contains many candidates of medical importance and are therefore especially interesting for biotechnology. The few success stories that have been reported so far on the *in-vitro* production of alkaloids will be discussed in more detail as well as an outlook for the future developments in this challenging field of biotechnology.

For the purpose of this review the weekly updated database Biosis Previews® (Thomson Scientific, Inc.) was searched systematically for relevant publications using the names of enzymes involved in alkaloid biosynthesis, of substrates or products as keywords. With the goal of giving a comprehensive summary of any progress made in metabolic engineering, literature was cited only, if a gene had been both cloned and functionally characterized, *e.g.* in a heterologous system.

2 Biosynthesis of alkaloids

The enzymes that catalyze the biosynthesis of SM are usually substrate-specific, whereas enzymes of breakdown or turnover (such as esterases, glycosidases) have a much broader substrate spectrum. The nitrogen atom in alkaloids derives from amino acids in most cases (except steroidal alkaloids) with phenylalanine, tyrosine, tryptophan, lysine, and ornithine being a precursor most often involved. In a first reaction, the amino acids are decarboxylated by decarboxylases with phenylethylamine, dopamine, tryptamine, cadaverine, and putrescine as the corresponding amines. Further reactions often involve the reaction of the amino group of the amine with an aldehyde function in the same or a second molecule. Aldehydes and amines are known to spontaneously form a Schiff's base under physiological conditions. Other reactions include ring closure, oxidation or reductions of double bonds, the addition of functional groups (hydroxyl-, methyl, methylene dioxy groups) and the further modifications of OH-groups (esterification, glycosylation, methylation) (reviewed in [40–46]). A scheme of the pathways leading to major groups of alkaloids is illustrated in Fig. 2.

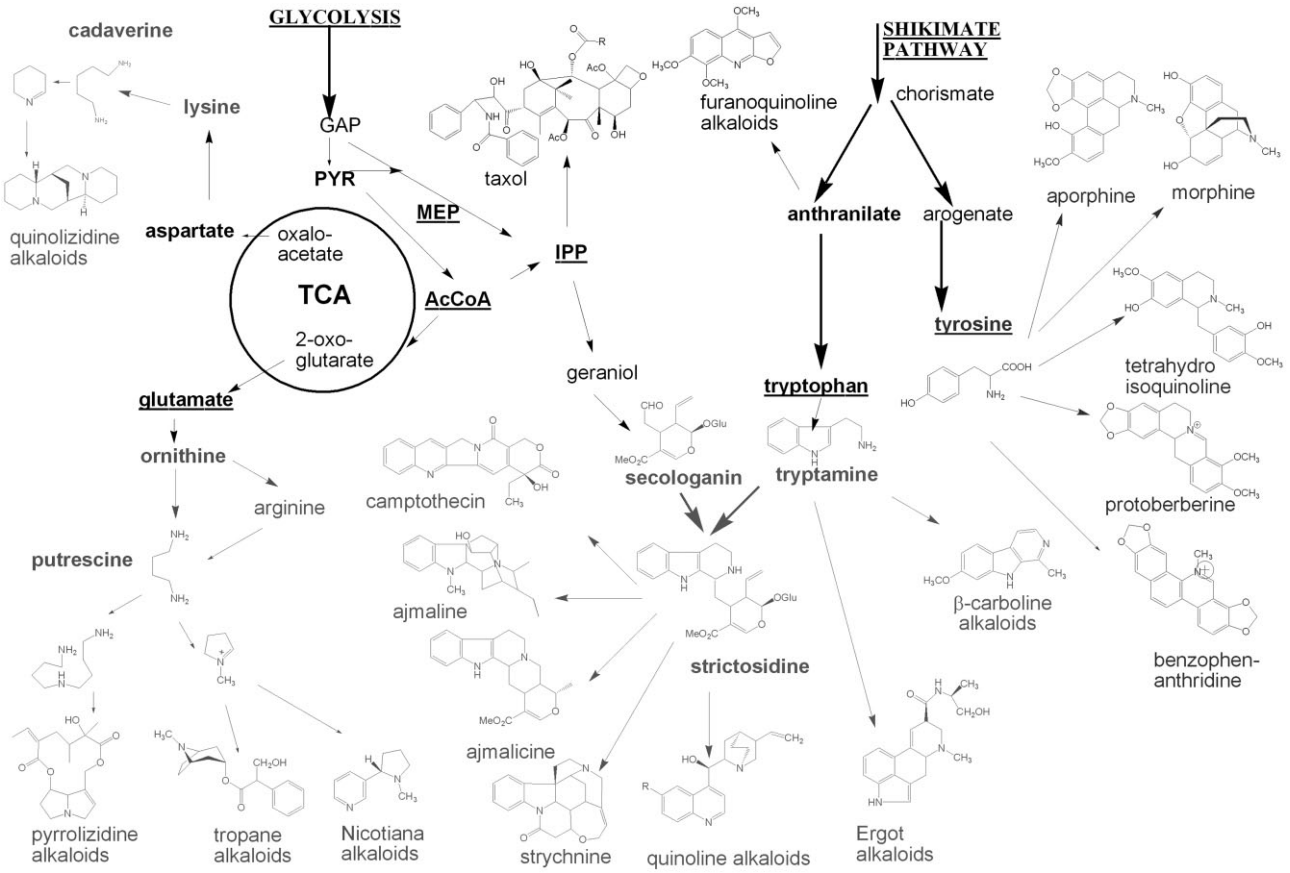


Figure 2. Overview of biosynthetic pathways of major groups of alkaloids.

The search for the enzymes involved in the biosynthesis of alkaloids is an ongoing process and so far has been successful (at least partially) for a number of alkaloidal groups, such as morphinane-, protoberberine-, monoterpene indole-, Taxus-, ergot-, purine-, tropane-, Nicotiana-, pyrrolizidine and furanoquinoline alkaloids (see reviews [40–55]). However, considering the high structural diversity of alkaloids and other SM, much work still needs to be done until the picture is complete. Pathways have usually been explored in a few plants that produce a certain type of SM and it is silently assumed that pathways are identical in all plants that produce such compounds. This assumption still needs to be tested, but it is likely that different organisms evolved different solutions for the same task.

3 Genes of alkaloid biosynthesis

Once the enzymes had been isolated and purified, they could be sequenced and using the genetic code, primers for PCR and cDNA synthesis could be deduced. With some luck full-length cDNA clones could be generated encoding specific enzymes in a particular biosynthetic pathway. The corresponding genes could then be characterized and expressed in recombinant systems. This topic will be explored in more detail for major alkaloid groups and the relevant newer literature is summarized in the following. Earlier and recent work has been summarized in [40–55].

3.1 Benzylisoquinoline alkaloids

Benzylisoquinoline alkaloids form one of the major groups with several drugs of medical importance, such as morphine, thebaine, codeine, papaverine, berberine or sanguinarine. The biosynthesis of benzylisoquinoline alkaloids, which include structural types such as tetrahydroisoquinoline, morphinan, protoberberine, benzophenanthridine and aporphine alkaloids (Fig. 3) has been reviewed recently [47, 48, 51, 55–57]. The pathway leading to tetrahydroisoquinoline, morphinane, and protoberberine alkaloids has been elucidated almost completely by now and most of the responsible genes have been cloned and characterized (Table

Table 1. Cloned genes and characterized enzymes involved in the biosynthesis of benzylisoquinoline alkaloids (see also [42–46, 48, 50, 54] for earlier publications)

Enzyme	Plant	Reference
Tyrosine decarboxylase (TYDC)	*Papaver somniferum, Oryza sativa*, Thalictrum flavum, Arabidopsis thaliana**	[61–64]
Norcoclaurine synthase (NCS)	*Thalictrum flavum, Coptis japonica*	[65, 66]
Norcoclaurine 6-*O*-methyltransferase (6OMT)	*Thalictrum tuberosum, Thalictrum flavum, Coptis japonica, Papaver somniferum,*	[63, 67–70]
Coclaurine *N*-methyltransferase (CNMT)	*Thalictrum flavum, Papaver somniferum, Coptis japonica*	[63,69,71]
Berbamunine synthase (Cyp80A1)	*Berberis stolonifera*	[72]
N-methylcoclaurine 3'-hydroxylase (Cyp80B)	*Thalictrum flavum, Eschscholzia californica*	[63, 73–75]
3-Hydroxy-*N*-methylcoclaurine 4-*O*-Methyltransferase (4'OMT)	*Thalictrum flavum, Coptis japonica, Papaver somniferum*	[63, 68, 69]
Reticuline 7-*O*-methyltransferase (7OMT)	*Papaver somniferum*	[70]
Berberine bridge enzyme (BBE)	*Thalictrum flavum, Eschscholzia californica, Papaver somniferum*	[63, 76,77]
Cheilanthifoline synthase (Cyp719A5)	*Eschscholzia californica*	[78]
Stylopine synthase (Cyp719A2, 3)	*Eschscholzia californica*	[79]
Tetrahydroprotoberberine *N*-methyltransferase (TNMT)	*Papaver somniferum*	[56]
Cyp719A1	*Thalictrum flavum, Coptis japonica*	[63, 80]
Scoulerine 9-*O*-methyltransferase (SOMT)	*Thalictrum flavum, Coptis japonica*	[63, 81]
Columbamine *O*-methyltransferase (CoOMT)	*Coptis japonica*	[82]
Salutaridine reductase (SalR)	*Papaver somniferum*	[83]
Salutaridinol 7-*O*-acetyltransferase (SalAT)	*Papaver somniferum*	[84]
Codeinone reductase (COR)	*Papaver somniferum*	[85]
Cyp80G2	*Coptis japonica*	[86]

* These plants do not produce benzylisoquinoline alkaloids.

1). For a few enzymes X-ray data are available, such as berberine bridge enzyme (BBE) [58, 59] and norcoclaurine synthase [60].

3.2 Monoterpene indole alkaloids

Monoterpene indole alkaloids are especially abundant in Apocynaceae and contain several drugs of medicinal importance, such as the dimeric vinblastine and vincristine from *Catharanthus roseus* (important anticancer drugs), reserpine, ajmaline, ajmalicine, strychnine and yohimbine. Reviews of the biosynthesis of monoterpene indole alkaloids, which combine tryptamine and a monoterpene (secologanin) in their skeleton, have recently been published [47, 52, 55, 87, 88]. Strictosidine synthase (STR) is the key enzyme of this pathway and has been studied by many groups. It was the first alkaloid gene to be cloned in 1988 by T. Kutchan and M. Zenk [89]. The pathway leading to a few complex monoterpene indole-alkaloids (ajmalicine, serpentine, ajmaline, tabersonine, vindoline, catharanthine, and dimeric vinblastine) has been elucidated partially by now (Fig. 4) and several of the responsible genes have been cloned and characterized (Table 2). For a few enzymes X-ray data are available, such as STR [90], strictosidine beta-D-glucosidase (SGD) [91, 92] and vinorine synthase [110, 122] from *Rauvolfia serpentina*.

3.3 Ergot alkaloids

Claviceps purpureus and other fungi produce indole alkaloids of the ergot alkaloid type with pronounced activities in neuronal signal transduction (such as found in LSD, ergotamine, ergometrine). Ergot alkaloids have also been detected in a few plants of the family Convolvulaceae. It could be shown recently, that the ergot alkaloid production in *Ipomoea* is due to an endophytic fungus that lives in symbiosis with its host plant [113]. The biosynthesis of the lysergic acid skeleton starts from

tryptamine to which a unit of active isoprene is added. The corresponding 4-dimethylallyltryptophan synthase or 7-dimethylallyltryptophan synthase have been cloned, characterized (Table 3) and heterogeneously expressed in *E. coli* [114, 115].

3.4 Purine alkaloids

Purine alkaloids, such as caffeine, theobromine and theophylline mediate the known stimulating bioactivities of coffee and tea (inhibition of phosphodiesterase, adenosine receptor antagonist) [4, 7]. The biosynthesis of purine alkaloids, such as caffeine, starts with xanthosine as a precursor. The pathway to xanthosine is assumed to follow the general pathway to purines, which is required for the purine bases adenine and guanine of DNA. The modifications of xanthosine are relatively simple and involve consecutive *N*-methylations with SAM as a methyl donor [40, 41, 123–125]. The responsible genes have been cloned and characterized (Table 4).

3.5 Paclitaxel (Taxol®)

Paclitaxel is a complex diterpene alkaloid, which has been introduced as a powerful anticancer drug (Taxol®) during the last 20 years [130]. Originally isolated from the bark of the Pacific yew tree (*Taxus brevifolia*), the production of this important drug was not sustainable at first. Later it was found that leaves from other *Taxus* species could be used to isolate taxanes in a more sustainable way: The taxanes can be converted into paclitaxel in a semisynthetic process [40, 41, 130]. Nevertheless, researchers have started programs for the chemical

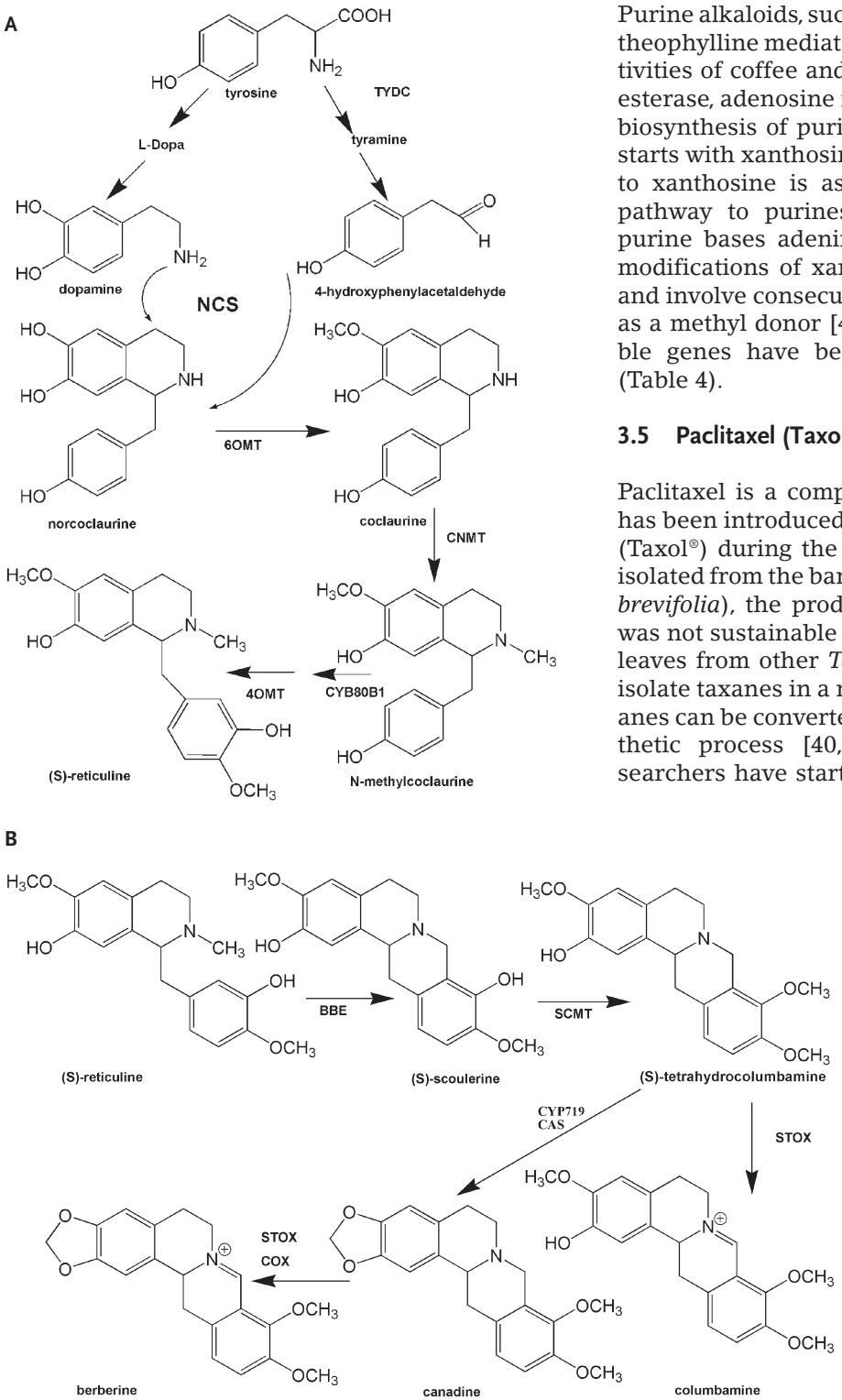
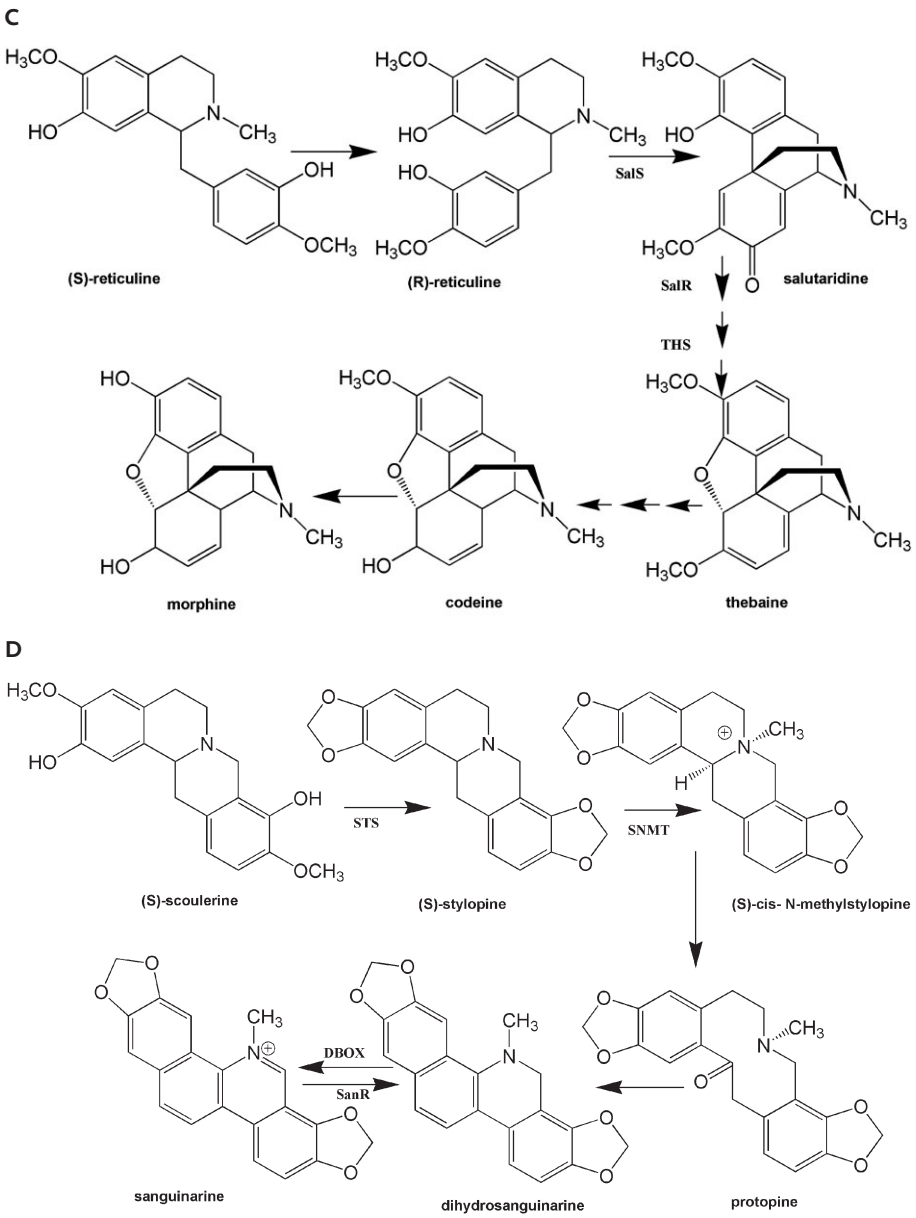


Figure 3. Biosynthesis of benzylisoquinoline alkaloids. (A) Pathway from tyrosine to S-reticuline; (B) pathway from reticuline to protoberberine alkaloids; (C) pathway from reticuline to morphinan alkaloids, (D) pathway from scoulerine to benzophenanthridine alkaloids.

and biotechnological production of paclitaxel or its precursors. Several steps in the biosynthesis of taxanes have been elucidated [40, 41, 46] (Fig. 5) and some of the corresponding genes have been isolated and characterized (Table 5).

3.6 Tropane alkaloids and nicotine

Hyoscyamine and scopolamine are tropane alkaloids of medical importance since they are antagonist of the muscarinic acetylcholine receptor [4, 7, 40, 41]. Nicotine is a major alkaloid of tobacco; it is an agonist at the nicotinic acetylcholine receptor [4, 7, 40, 41] and had been used as a natural insecticide for many years [151]. The biosynthetic pathway leading to tropane alkaloids (including the polyhydroxyalkaloids of the calystegine type) and nicotine has been intensely studied and the initial steps have been elucidated (Fig. 2 and 6), but the corresponding synthase genes are still enigmatic. In nicotine biosynthesis two pathways can lead to the intermediary putrescine: (i) via ornithine decarboxylation and (ii) from ornithine to arginine and after decarboxylation (ADC) via agmatine. Several genes have been isolated, expressed and characterized (Table 6).



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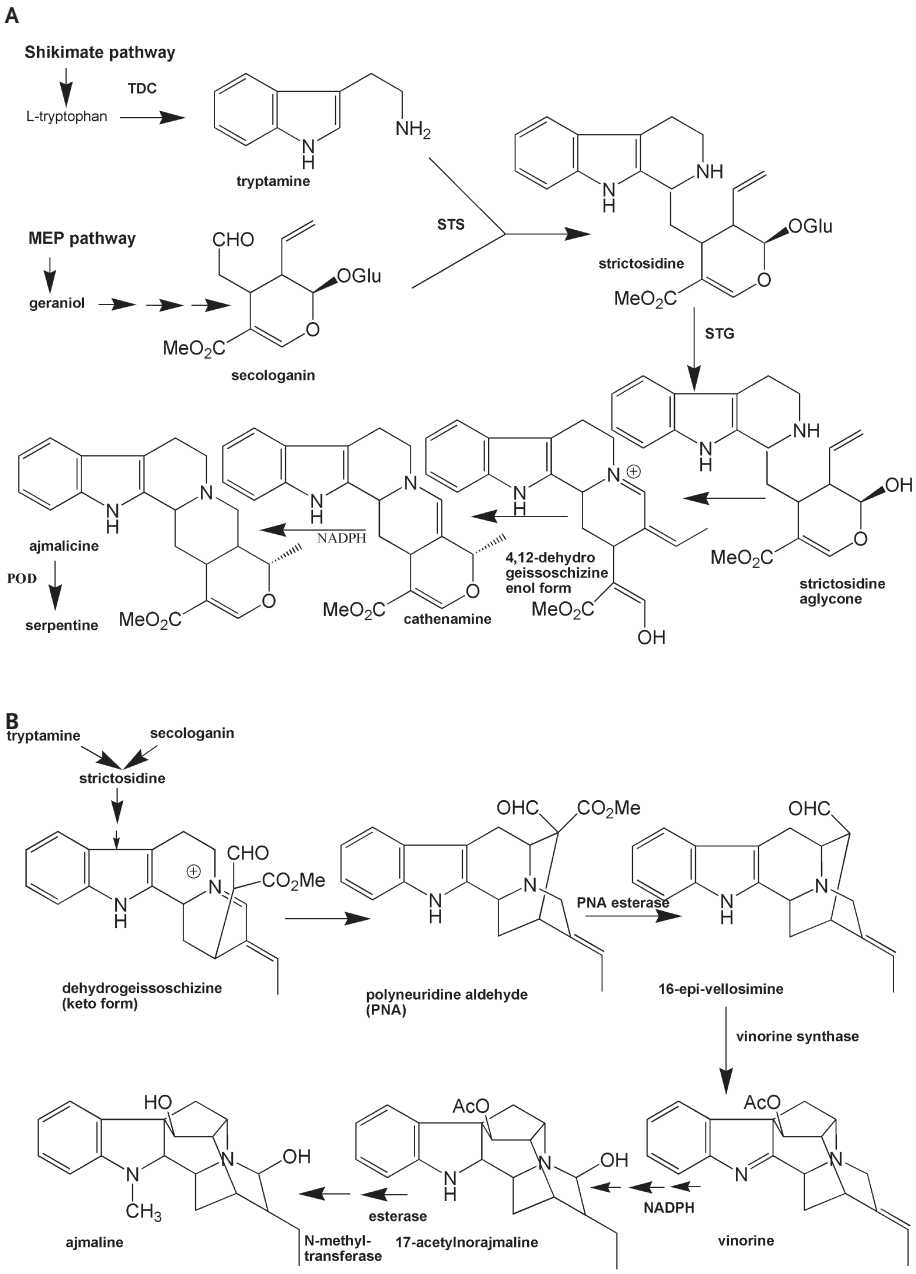

Figure 4. Biosynthesis of monoterpene indole alkaloids. (A) Pathway from tryptophan to ajmalicine; (B) pathway from strictosidine to ajmalin; (C) pathway from strictosidine to vinblastine.

3.7 Pyrrolizidine alkaloids

Pyrrolizidine alkaloids (PA), which occur in most taxa of the Boraginaceae and several Asteraceae, are of general toxicological importance, since most PA have mutagenic and even carcinogenic properties [40, 41]. Only one enzyme (homospermidine synthase) of PA biosynthesis and the corresponding gene have been isolated and characterized so far (Fig. 2 and Table 7) [177, 178]. When overexpressed in tobacco cells, an overproduction of homospermidine was observed [179].

3.8 Acridone alkaloids

Acridone alkaloids are common in Rutaceae and use anthranilate as a key intermediate (Fig. 2). So far, two enzymes plus corresponding genes have been characterized (Table 8). The key enzyme acridone synthase has high sequence similarity to chalcone synthase (a key enzyme in the formation of flavonoids); only three amino acids differ between both enzymes [180].

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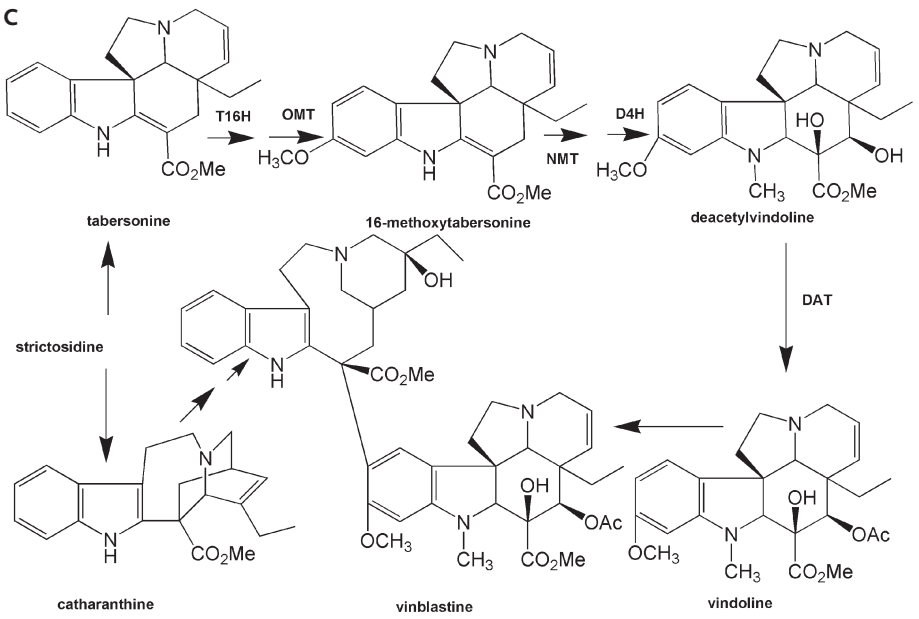

Figure 4. Continued

Table 2. Cloned genes and characterized enzymes involved in the biosynthesis of monoterpene indole alkaloids

Enzyme	Plant	Reference
Tryptophan decarboxylase (TDC)	*Oryza sativa*, Catharanthus roseus, Camptotheca acuminata, Ophiorrhiza pumila*	[62, 93–95]
Geraniol 10-hydroxylase (G10H or Cyp76B6)	*Catharanthus roseus*	[96]
Cyp72A1	*Catharanthus roseus*	[97]
Loganic acid *O*-methyltransferase (LAMT)	*Catharanthus roseus*	[98]
Strictosidine synthase (STR)	*Rauvolfia serpentina, Ophiorrhiza pumila, Catharanthus roseus, Rauvolfia mannii, Rauvolfia verticillata*	[89, 95, 99–101]
Strictosidine beta-*D*-glucosidase (SGD)	*Catharanthus roseus, Rauvolfia serpentina*	[102, 103]
Tabersonine 16-hydroxylase (T16H or Cyp71D12)	*Catharanthus roseus*	[104]
16-Hydroxytabersonine-16-*O*-methyltransferase (16OMT)	*Catharanthus roseus*	[105]
Desacetoxyvindoline-4-hydroxylase (D4H)	*Catharanthus roseus*	[106]
Deacetylvindoline 4-*O*-acetyltransferase (DAT)	*Catharanthus roseus*	[107]
Minovincinine-19-*O*-acetyltransferase (MAT)	*Catharanthus roseus*	[108]
Polyneuridine aldehyde esterase (PNAE)	*Rauvolfia serpentina*	[109]
Vinorine synthase (VS)	*Rauvolfia serpentina*	[110, 122]
Acetylajmalan esterase (AAE)	*Rauvolfia serpentina*	[87]
Raucaffricine-*O*-beta-*D*-glucosidase	*Rauvolfia serpentina*	[111]
Perakine reductase (PR)	*Rauvolfia serpentina*	[112]

* Not an alkaloidal plant.

3.9 Betalains

Betalains are nitrogen-containing red-orange or yellow colored SM (and therefore alkaloids), which replace anthocyanins in some taxonomic groups (*e.g.* Caryophyllales) as flower pigments. They also occur in some fungi. A few steps of their biosynthesis have been elucidated in detail (Table 9).

Table 3. Cloned and characterized enzymes involved in the biosynthesis of ergot alkaloids

Enzyme	Fungus	Reference
4-Dimethylallyltryptophan synthase	*Claviceps purpurea, Neotyphodium sp., Aspergillus fumigatus, Malbranchea aurantiaca*	[116–119]
7-Dimethylallyltryptophan synthase	*Aspergillus fumigatus*	[120]
4-Dimethylallyltryptophan *N*-methyltransferase	*Aspergillus fumigatus*	[121]
Brevianamide F prenyltransferase	*Aspergillus fumigatus*	[113]

Table 4. Cloned and characterized enzymes involved in the biosynthesis of purine alkaloids

Enzyme	Plant	Reference
Xanthosine 7-*N*-methyltransferase (XMT) or 7-methylxanthosine synthase (XRS)	*Coffea arabica*	[126, 127]
Caffeine synthase (CS)	*Camellia sinensis, Coffea arabica*	[124, 126, 128]
7-Methylxanthine methyltransferase (MXMT)	*Coffea arabica*	[127, 129]
Dimethylxanthosine methyltransferase (DXMT)	*Coffea arabica*	[127]

4 Metabolic engineering and alkaloid production in recombinant systems

The enzymes mentioned in Section 3 were characterized either as proteins purified from plant sources, or after the corresponding genes had been expressed in recombinant bacteria or yeasts in order to have enough material. Usually the focus of such work was not the functional expression of these genes in terms of metabolic engineering. A few experiments have been reported in which a single key enzyme was cloned in a heterologous system, usually another plant species, such as tobacco, and a change in the profile of primary and secondary metabolites was recorded. Sometimes the recombinant plants were fed with the appropriate precursor and it was analyzed if a biotransformation took place.

Ultimately, it will be necessary to transform plants or microbes with a series of genes or complete pathways and co-express them in order to produce a given compound in a recombinant system (Fig. 1). For the synthesis of antibiotics, such an approach has been shown to be feasible [190, 191]. However, here the situation is somehow easier, as the genes come in a cassette already and contain all the appropriate signal elements. In this Section, the

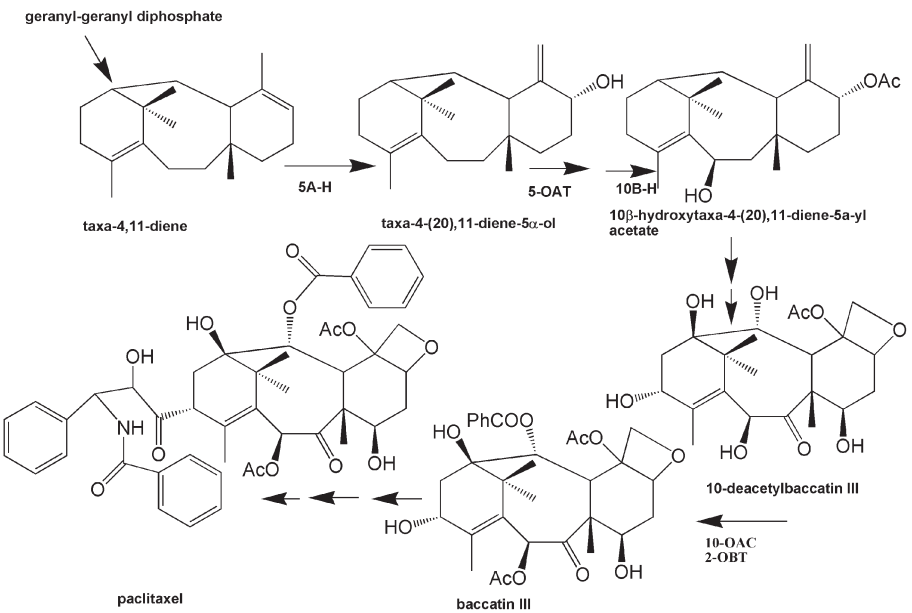

Figure 5. Biosynthesis of paclitaxel.

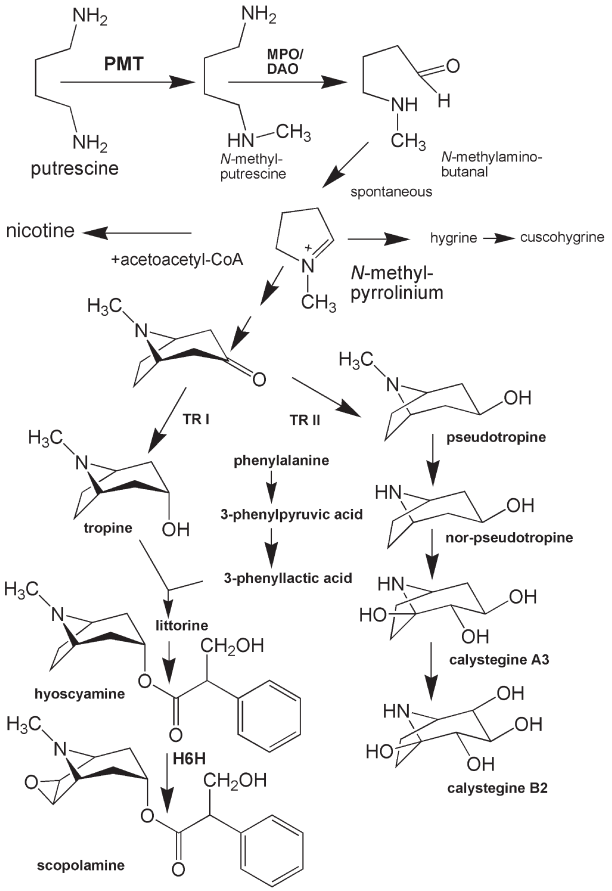

Figure 6. Biosynthetic pathway from ornithine to tropane alkaloids.

progress in the line of metabolic engineering of natural products from plants will be discussed.

4.1 Benzylisoquinoline alkaloids

In terms of metabolic engineering two alkaloid genes from *Coptis japonica* (6OMT and 4'OMT) were overexpressed in another alkaloid plant, *Eschscholzia californica*. Consequently, the alkaloid content was elevated 7.5 times [192]. When berberine bridge enzyme was knocked down by RNAi in *Eschscholzia californica,* an enhanced reticuline accumulation was observed [193]. In another approach salutaridinol 7-O-acetyltransferase in opium poppy was overexpressed or suppressed by RNAi [194].

When multiple genes in the BIA pathway were functionally co-expressed in yeast and *E. coli* the production of simple benzyliosquinoline alkaloids such as reticuline and related alkaloids [195], or reticuline, magnoflorine, and scoulerine was demonstrated [33]. These findings are especially encouraging because they are a proof of concept that the production of complex alkaloids is possible by using recombinant microorganisms. However, this approach is not optimal yet, since the production was not carried out in a single recombinant system with some steps done in *E. coli*, some *in vitro* and others through transformations in yeast.

Table 5. Cloned and characterized enzymes involved in the biosynthesis of paclitaxel

Enzyme	Plant	Reference
Taxa-4(5), 11(12)-diene synthase	*Taxus brevifolia, T. chinensis, T. media*	[131–133]
Geranylgeranyl diphosphate synthase	*Taxus canadensis*	[134]
Taxane 2α-O-benzoyltransferase	*Taxus cuspidata*	[135]
10-Deacetylbaccatin III-10-O-acetyltransferase	*Taxus cuspidata, T. media*	[136, 137]
Taxa-4(20),11(12)-dien-5alpha-ol-*O*-acetyl transferase	*Taxus sp.*	[138]
Taxane 13α-hydroxylase	*Taxus sp.*	[139]
Taxane 10β-hydroxylase	*Taxus sp., T. media*	[140, 141]
Taxoid 14β-hydroxylase	*Taxus sp.*	[142]
Taxoid 2α-hydroxylase	*Taxus sp.*	[143]
Taxoid 7β-hydroxylase	*Taxus sp.*	[144]
Taxadiene 5α-hydroxylase	*Taxus sp.*	[145]
C-13 phenylpropanoid side chain-CoA acetyl transferase	*Taxus sp.*	[146]
C13-side-chain *N*-benzoyl transferase	*Taxus sp.*	[147]
Phenylalanine ammonium mutase	*Taxus cuspidata, T. chinensis*	[148, 149]
Taxadiene 5α-ol-*O*-acetyltransferase	*Taxus sp.*	[150]

Table 6. Cloned and characterized enzymes involved in the biosynthesis of tropane alkaloids and nicotine

Enzyme	Plant	Reference
Ornithine decarboxylase (ODC)	*Datura stramonium, Nicotiana glutinosa, N. tabacum, Capsicum annuum*	[152–155]
Putrescine *N*-methyltransferase (PMT)	*Nicotiana tabacum, Atropa belladonna, Hyoscyamus niger, Anisodus tanguticus, Solanum spec, Calystegia sepium, Datura spec., Physalis divaricarpa, Anisodus acutangulus*	[156–161]
N-methylputrescine oxidase (MPO)	*Nicotiana tabacum*	[162, 163]
Tropinone reductase I (TRI)	*Hyoscyamus niger, Datura stramonium, Solanum tuberosum*	[164–166]
Tropinone reductase II (TRII)	*Hyoscyamus niger, Datura stramonium, Solanum tuberosum*	[164, 165, 167]
Cyp80F1	*Hyoscyamus niger*	[168]
Hyoscyamine 6β-hydroxylase (H6H)	*Hyoscyamus niger, Atropa belladonna, Anisodus tanguticus, A. acutangulus, Brugmansia candida, Atropa beatica*	[169–174]
Nicotine *N*-demethylase (NND)	*Nicotiana tabacum*	[175]
Arginine decarboxylase (ADC)	*Nicotiana tabacum*	[176]

Table 7. Cloned and characterized enzymes involved in the biosynthesis of pyrrolizidine alkaloids

Enzyme	Plant	Reference
Homospermidine synthase (HSS)	Boraginaceae (*Heliotropium, Cynoglossum, Symphytum*), Asteraceae (*Eupatorium, Senecio, Petasites*), Convolvulaceae (*Ipomoea hederifolia*), Solanaceae (*Nicotiana tabacum*), Fabaceae (*Crotalaria*), Orchidaceae (*Phalaenopsis*)	[178]

4.2 Monoterpene indole alkaloids (MIA)

Metabolic engineering with genes of the MIA pathway in homologous and heterologous cell culture systems has been carried out with tryptophan decarboxylase and strictosidine synthase. The different experiments and results have been summarized in Table 10. The production of MIA in recombinant microorganisms is more complicated than that of BIA since the biosynthesis of the terpenoid precursor, secologanin is complex and the corresponding biosynthesis genes are not available.

4.3 Purine alkaloids

When tobacco plants were transformed with the genes of caffeine biosynthesis, such as XMT, MXMT and DXMT a caffeine production was recorded; it has been argued that such a transformation could be interesting for crop plants, because caffeine apparently functions as a natural pest repellent [205]. The down-regulation of the genes of caffeine biosynthesis produced coffee plants without caffeine; this approach is interesting for the production of decaffeinated coffee [206].

Table 8. Cloned and characterized enzymes involved in the biosynthesis of acridone alkaloids

Enzyme	Plant	Reference
Acridone synthase	*Ruta graveolens, Huperzia serrata*	[181, 182]
Anthranilate *N*-methyltransferase	*Ruta graveolens*	[183]

4.4 Paclitaxel

Because of the importance of paclitaxel as an anticancer drug, research with regard of metabolic engineering of the taxane pathway was quite active during the last 10 years [29, 30]. The key enzyme taxadiene synthase, which converts geranyl-ger-

Table 9. Cloned and characterized enzymes involved in the biosynthesis of betalains

Enzyme	Plant	Reference
DOPA 4,5-dioxygenase	*Amanita muscaria, Portulacca grandiflora, P. americana,*	[184–187]
Polyphenol oxidase	*Phytolacca americana*	[188]
Betanidin 5-*O*-glucosyl-transferase	*Dorotheanthus bellidiformis*	[189]

Table 10. Expression of tryptophan decarboxylase and strictosidine synthase; metabolic engineering of the monoterpene indole alkaloid biosynthesis

Enzyme	Source	Transgenic plant	Goal/remark	Ref.
Tryptophan decarboxylase	*Catharanthus roseus*	*Brassica napus*	Reduction of tryptophan derived glucosinolates	[196]
	Catharanthus roseus	*Nicotina tabacum*	Investigation of influence of subcellular localization on enzyme activity and tryptamine accumulation	[197]
	Camptotheca acuminata	*Populus sp.*	Study of tryptamine accumulation and effect on insect pests	[198]
	Catharanthus roseus	*Catharanthus roseus*	Stimulation of tryptamine production but no change of MIA content	[199]
Tryptophan decarboxylase + strictosodine synthase	*Catharanthus roseus*	*Nicotina tabacum*	Plasmid construct with both genes	[200]
	Catharanthus roseus	*Cinchona officinalis*	Functional expression of TDC and STR; enhanced levels of strictosidine and Cinchona alkaloids in hairy root cultures	[201]
	Catharanthus roseus	*Catharanthus roseus*	Over-expression of TDC and STR with stimulation of MIA synthesis	[202, 203]
	Catharanthus roseus	*Nicotina tabacum*	Functional expression of TDC and STR in tobacco cell cultures and strictosidine production after feeding od secologanin	[204]

anyl diphosphate into the diterpene skeleton, could be functionally expressed and taxadiene was detected in *Arabidopsis thaliana* [207], in transgenic tomato with carotenoid deficiency [208] and in the moss *Physcomitrella patens* [209].

A few groups were already successful in co-expressing several genes of paclitaxel biosynthesis in recombinant microbes: The expression of isopentenyl diphosphate isomerase, geranylgeranyl diphosphate synthase and taxadiane synthase in *E. coli* made it possible to synthesize taxadiene from isopentenyl diphosphate both in cell-free extracts and in recombinant bacteria [28, 31]. The co-expression of reductase and oxygenase enhances the formation of hydroxylated taxanes in recombinant yeast cells [210]. A significant step in the same direction was reported from the Croteau lab [28], which functionally expressed eight genes of taxane biosynthesis in yeast and could demonstrate a formation of taxadiene-5α-acetoxy-10β-ol from precursors of primary metabolism. However, a cytochrome p450 hydroxylation step seems to be a bottleneck in the present process [28].

5 Conclusions and outlook

Research towards the production of valuable alkaloids has seen much progress during the last two decades [38, 211, 212], but we are still not at a stage where these results can be used directly in the biotech industry. The proofs of concept obtained with isoquinoline alkaloids and taxanes are very encouraging but also show the problems that have to be overcome. It should be mentioned that similar approaches have been followed with pathways leading to mono- and sesquiterpenes (several terpene synthases have been isolated and functionally expressed) [25, 26, 34, 35, 38, 211–213]. Research in the biosynthesis of flavonoids and related polyphenols has the longest tradition so far and many papers show the impact of metabolic engineering and practical applications [26, 27, 32, 37, 38, 211, 212].

In the long run, once the expression cassettes for different pathways have been established, these systems are likely to be used for the production of valuable drugs in fermented microorganisms, for the biotransformation of critical steps in a chemical synthesis or when transferred into crop plants for the enhancement of resistance against microbes and/or herbivores.

The authors have declared no conflict of interest.

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