Introduction to the Different Classes of Biosynthetic Enzymes

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Abstract Plant natural products are intimately associated with traits such as quality, yield, disease resistance, stress tolerance, color, and fragrance, in addition to being important dietary components and phytomedicines. In spite of the apparent complexity of natural product biosynthesis, much of the rich chemical diversity of the plant kingdom arises from a limited number of chemical scaffold types, modified by specific chemical substitutions such as hydroxylation, glycosylation, acylation, prenylation, and *O*-methylation. The molecular genetic basis underlying plant natural product chemistry has recently been the subject of concerted genomic and genetic approaches, facilitated by the fact that many of the key enzymatic steps in scaffold formation and substitution are catalyzed by proteins originating from recognizable gene families (e.g. polyketide synthase, glucosyltransferase) that have undergone significant expansion throughout plant evolution. This overview summarizes the types of enzymatic reactions involved in plant secondary metabolism from a pathway organization perspective that highlights the entry points from primary metabolism, general scaffold formation and scaffold modification (Box 1).

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The Primary–Secondary Metabolism Interface

Phenylpropanoids

The aromatic amino acid L-phenylalanine (primary metabolite) is directed into the phenylpropanoid pathway leading to hydroxycinnamic acids, lignin and flavonoids by the activity of L-phenylalanine ammonia-lyase (PAL), which brings about its nonoxidative deamination yielding ammonia and *trans*-cinnamic acid (Fig. 1). PAL is one of the most studied plant enzymes, and its crystal structure has recently been solved [2]. PAL is related to the histidine and tyrosine ammonia-lyases of amino acid catabolism. A class of bifunctional PALs found in monocotyledonous plants and yeast can also deaminate tyrosine [3]. A single His residue is responsible for this switch in substrate preference [3, 4]. All three enzymes share a unique MIO (4-methylidene-imidazole-5-one) prosthetic group at the active site. This is formed autocatalytically from the tripeptide Ala-Ser-Gly by cyclization and dehydration during a late

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Box 1 A functional classification of natural product biosynthetic enzymes.

The immense variety of plant natural products is generated by an equally large and at first sight confusing array of enzymes. However, these enzymes catalyze a relatively limited number of reaction types [1]. For the purpose of this survey, the enzymes will be divided into three major groups based on their positions in the overall scheme of secondary metabolite biosynthesis:

- Enzymes at the interface between primary and secondary metabolism.
  - A small number of key enzymes catalyze the first committed steps which direct compounds from primary into secondary metabolism (Fig. 1). They control flux into natural products without depleting pools of primary metabolites, and must therefore act quickly and efficiently in response to developmental or environmental cues. For this reason they are often regulated both transcriptionally and post-transcriptionally.
- Enzymes forming plant secondary metabolite scaffolds
  - This group of enzymes directs flux into the major classes of plant natural products: e.g. polyketides (including flavonoids), alkaloids and terpenes. The initially formed scaffold molecules then enter different branch pathways as precursors for further downstream modifications.
- Enzymes for modification of scaffold structures
  - Modification reactions create the enormous diversity of plant natural products, providing new molecules with different biological activities from the basic scaffolds outlined above. The plant kingdom contains a large number of enzymes that catalyze hydroxylation, epoxidation, aryl migration, glycosylation, methylation, sulfation, acylation, prenylation, and reduction of secondary metabolite skeletons. Figure 6 shows how a single molecule (the isoflavone genistein) can be converted to a range of different products by such enzymes.

stage of chain folding [5]. Two reaction mechanisms have been proposed for the elimination of ammonia, with a Friedel-Crafts-like acylation mechanism being most favored [3].

Many plants utilize different PAL isoforms for stress responses or for biosynthesis of structural components, and these different PALs exhibit differential expression in distinct tissues. Metabolic channeling may help control the flux of phenylalanine through PAL into the different phenylpropanoid branch pathways [6, 7].

Polyketides

Many polyketide-derived plant natural products originate in part from acetyl CoA via malonyl CoA (Fig. 1). For example, the key reaction in flavonoid biosynthesis, catalyzed by chalcone synthase (CHS) (Fig. 2), combines a phenylpropanoid-derived moiety, 4-coumaroyl CoA, with three molecules of malonyl CoA. Although acetyl CoA carboxylase, the enzyme forming malonyl CoA, is essentially an enzyme of primary metabolism (Fig. 1), it is often co-regulated with the enzymes of plant polyketide biosynthesis [8].

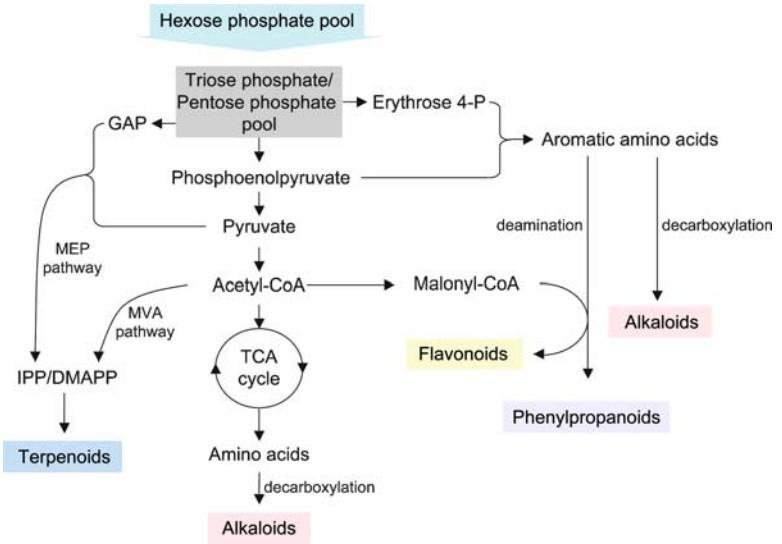

Fig. 1 The interface between primary and secondary metabolism in plants. GAP, glyceraldehyde 3-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; MEP, methylerythritol phosphate; MVA, mevalonic acid; TCA, tricarboxylic

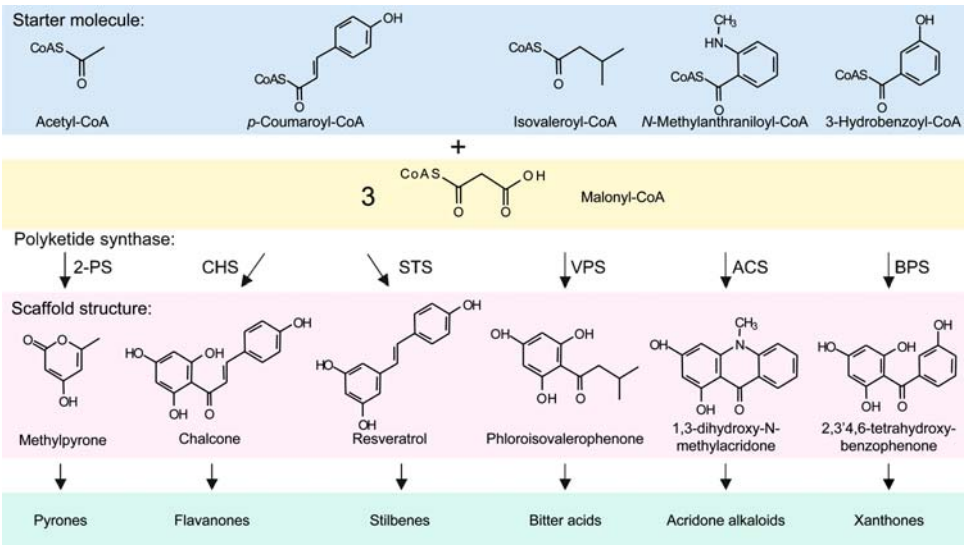

Fig. 2 Reaction types catalyzed by plant type III polyketide synthases. PS, pyrone synthase; CHS, chalcone synthase; STS, stilbene synthase; VPS, valerophenone synthase: ACS, acridone synthase; BPS, benzophenone synthase

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Terpenoids

The interface between primary and secondary metabolism is less easy to define in the case of terpene (isoprenoid-derived) metabolites, because terpene units are also found in many compounds associated with primary metabolism, such as hormones and vitamins. The precursors of all isoprenoids, isopentenyl pyrophosphate (IPP) and its allylic isomer dimethylallyl pyrophosphate (DMAPP), are synthesized in higher plants by two independent pathways (Fig. 1). The mevalonic acid (MVA) pathway is localized in the cytosol and starts with the condensation of three molecules of acetyl-CoA. In plastids, IPP is formed from pyruvate and glyceraldehyde-3-phosphate via the methylerythritol phosphate (MEP) pathway [9]. MEP pathway enzymes are nuclear-encoded and imported into plastids [10]. In flowers of snapdragon (*Antirrhinum majus*), plant volatiles are assembled from IPP units originating from the MEP pathway, and flux through this pathway in controlled by a circadian clock following a diurnal rhythm [11]. Archaebacteria, fungi and animals synthesize isoprenoids exclusively through the MVA pathway, whereas plants employ both pathways [9].

The cytosolic MVA pathway provides precursors for sterols and the side chain of ubiquinone, whereas synthesis of monoterpenes, certain sesquiterpenes, diterpenes, carotenoids, and the side chains of chlorophylls and plastoquinone is carried out in plastids [12]. Cross-talk occurs between the MVA and MEP pathways, and appears to be mainly unidirectional from plastids to cytosol, although limited import of intermediates into the plastid has been observed [13].

The initial reactions of terpene biosynthesis are catalyzed by short-chain prenyltransferases belonging to the class of *trans*- or *cis*- isoprenyl pyrophosphate synthases that catalyze chain elongation of allylic pyrophosphate substrates with IPP to generate linear polymers with defined chain length. Geranyl pyrophosphate synthase (GPPS), farnesyl pyrophosphate synthase (FPPS) and geranylgeranyl pyrophosphate synthase (GGPPS) catalyze formation of the linear precursors of monoterpenes (C10), sesquiterpenes (C15) and diterpenes (C20), respectively. GPPS is a plastidic, homo- or hetero-dimeric enzyme that catalyzes the head-to-tail condensation of one IPP molecule and one DMAPP molecule to form *trans*-GPP (C10) [14]. *Trans*-GGPP is produced in plastids by GGPPS, which sequentially adds three IPP molecules to a DMAPP molecule [15]. The biosynthesis of *trans*-FPP occurs in the cytosol, where FPPS combines two IPP molecules with DMAPP.

Alkaloids

Most alkaloids are derived from amino acids (Fig. 3) and the first reaction in the otherwise independent pathways is the decarboxylation of the respective amino acid by an amino acid decarboxylase (AADC) (Figs. 1 and 3); this step is often under complex regulation. Plant and animal AADCs share high amino acid identity, with significant similarities in subunit structure and kinetic characteristics. In contrast to their mammalian and insect counterparts, plant AADCs exhibit high specificity for their respective substrates. The reaction is pyridoxal-5'-phosphate (PLP)-dependent.

Plant aromatic L-amino acid decarboxylases (AADCs) catalyze the initial reactions in the formation of terpenoid indole alkaloids (TIAs) such as quinine and strychnine, and benzylisoquinoline alkaloids (BIAs) such as morphine and codeine (Fig. 3). L-tryptophan decarboxylase (TDC) initiates TIA synthesis with the formation of tryptamine. TDC is encoded by two genes in *Cola accuminata*; *TDC1* is expressed as part of a developmentally regulated chemical defense system, whereas *TDC2* is induced after elicitation with yeast extract or methyl jasmonate (MJ).

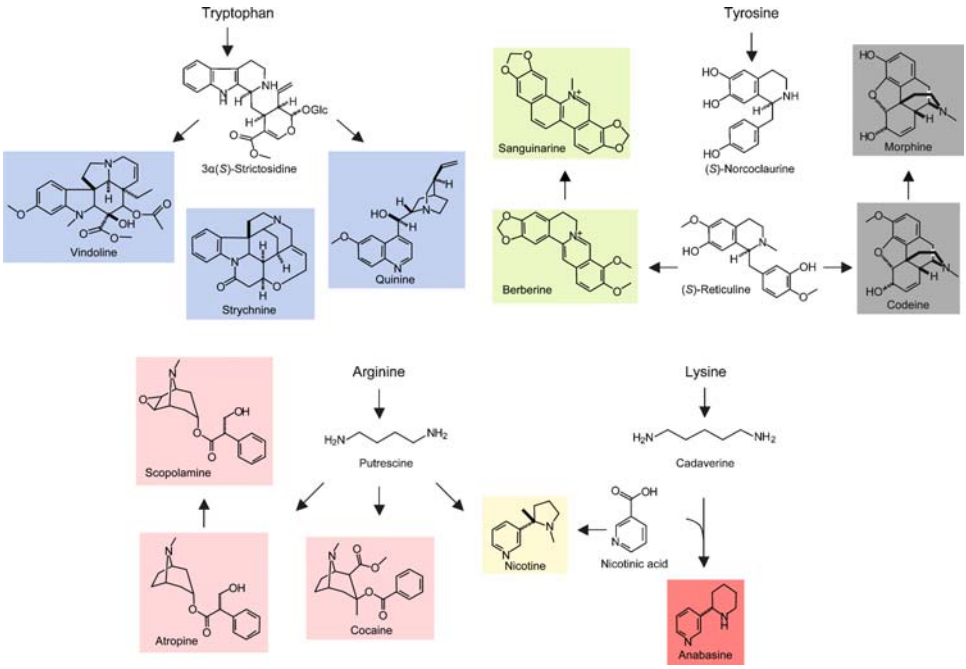

Fig. 3 Primary precursor–end product relationships in alkaloid biosynthesis. Color codes: blue, terpene indole alkaloids; green, benzophenanthridine alkaloids; gray, benzylisoquinoline alkaloids; red, quinolizidine alkaloids; yellow, pyridine alkaloids; pink, tropane alkaloids

L-tyrosine decarboxylase (TYDC) converts tyrosine and L-DOPA to tyramine and dopamine, respectively, the precursors for BIA biosynthesis [16]. TYDC is encoded by a single-copy gene in *Arabidopsis* (which does not produce BIAs), but a *TYDC* gene family of about 15 members divided into two subgroups is present in opium poppy (*Papaver somniferum*) [16]. The Arabidopsis *TYDC* gene contains 12 introns, whereas all other plant *AADC* genes described to date lack introns. TYDC is induced during plant defense responses, where it is involved in the synthesis of cell wall-bound hydroxycinnamic amides that provide a physical barrier against pathogens.

Lysine decarboxylase (LDC) catalyzes the formation of cadaverine by decarboxylation of lysine (Fig. 3). As an enzyme participating in polyamine biosynthesis, LDC links primary metabolism with biosynthesis of quinolizidine alkaloids like lupinine, which occurs in mesophyll chloroplasts of legumes. LDC is assumed to be the rate-limiting step in the biosynthesis of anabasine (Fig. 3), a pyridine alkaloid produced by tobacco species [17].

Ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) are the first enzymes involved in the formation of tropane alkaloids (TPAs) such as atropine and cocaine (Fig. 3). Decarboxylation of ornithine yields putrescine, whereas arginine is converted to agmatine, which is metabolized to putrescine via a second route. ADC is assumed to play the primary role in TPA synthesis [18].

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Scaffold Formation

Polyketides

Type III polyketide synthases (PKS) form the primary scaffolds for the synthesis of a range of secondary metabolites including flavonoids, stilbenes, bibenzyls, xanthones and pyrones [19] (Fig. 2). They catalyze a reaction similar to their fatty acid synthase (FAS) ancestors by facilitating the sequential head-to-tail addition of two-carbon acetate units to a growing polyketide chain. Type III PKSs differ from their type I and type II relatives by a simpler structure and the use of a CoA thioester substrate instead of an acyl carrier protein (ACP)-linked substrate [19].

CHS (Fig. 2), the most studied member of the type III PKS family, is a ubiquitous enzyme in plants that catalyzes the first committed step in flavonoid biosynthesis, the elongation of the starter molecule 4-coumaroyl-CoA by addition of three acetate units derived from three molecules of malonyl-CoA [19]. After binding of the 4-coumaroyl moiety to the active site Cys164, sequential polyketide chain elongation is initiated by the decarboxylation of malonyl-CoA to form an acetyl-CoA carbanion, followed by an intramolecular Claisen condensation step and subsequent cyclization and aromatization, yielding chalcone [20].

Other CHS-like PKSs accept different starter molecules, vary the length of the polyketide chain, or achieve molecular diversity through alteration of cyclization regiospecificity (Fig. 2). For example, stilbene synthase (STS) catalyzes a reaction similar to that of CHS in the initial stages, but the tetraketide intermediate undergoes a different cyclization reaction involving an intramolecular aldol condensation, hydrolysis from Cys164 and an additional decarboxylation step during formation of resveratrol. The structural bases for the differences in starter molecule, control of chain length, and overall cyclization mechanism (i.e. CHS- vs STS-type reactions) are now understood, and product formation has been altered rationally by point mutation to convert chalcone synthase to either stilbene synthase or pyrone synthase (Fig. 2) [21–23].

Phlorisovalerophenone synthase (VPS) from flower cones of hop (*Humulus lupulus* L.) utilizes isovaleryl-CoA or isobutyryl-CoA as starter molecules [24] (Fig. 2). Three molecules of malonyl-CoA are added to these starters to form phlorisovalerophenone or phorisobutyrophenone, respectively, precursors for the biosynthesis of hop bitter acids. The first committed step of cannabinoid biosynthesis in glandular trichomes of *Cannabis sativa* is catalyzed by a stilbene synthase carboxylate-like (STCSL) polyketide synthase using n-hexanoyl-CoA as starter molecule, yielding olivetolic acid [25].

Terpenoids

One group of terpene synthases (TPSs) use GPP, FPP or GGPP as substrates to form monoterpene (C10), sesquiterpene (C15) or diterpene (C20) scaffold molecules, respectively, which then undergo a variety of secondary modifications. This family of synthases is structurally distinct from triterpene (C30) or tetraterpene (C40) synthases [26]. The scaffolds produced are themselves highly divergent due to different folding patterns prior to cyclization (Fig. 4). TPSs fall into two major groups with regard to their modes of cyclization. Type A cyclization begins with the ionization of the polyprenyl pyrophosphate molecule, whereas type B cyclizations start with protonation at the terminal double bond. Both types of reactions are followed by cyclization and rearrangement ending with deprotonation of the final carbocation [27].

The sequence of reactions catalyzed by the type A limonene synthase (monoterpene cyclase) (Fig. 4) is initiated by ionization-isomerization

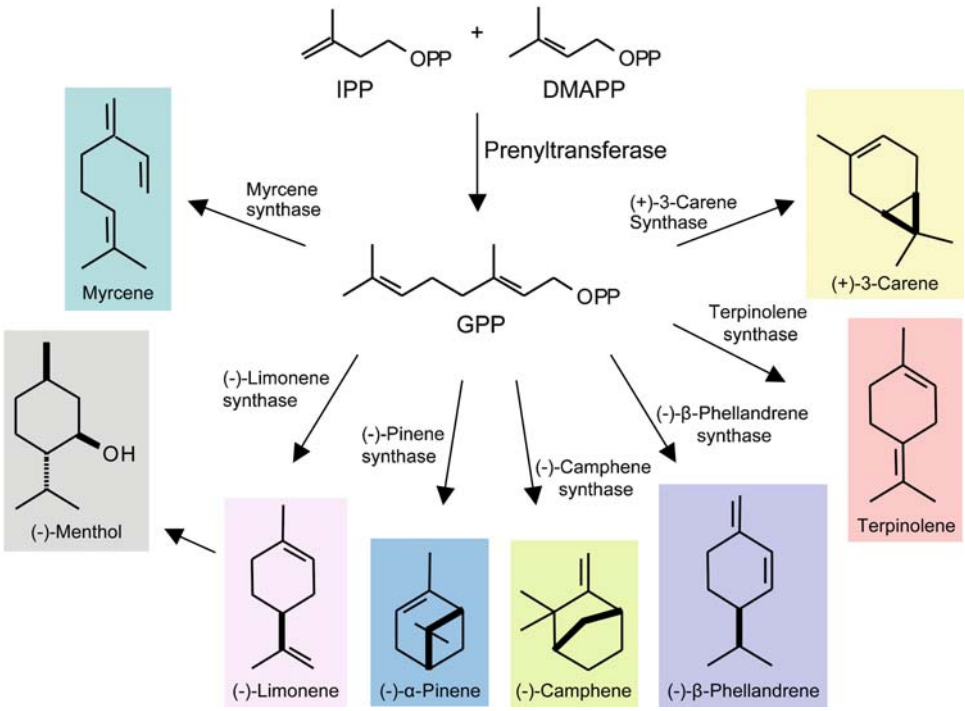

Fig. 4 Selected reactions catalyzed by monoterpene cyclases, illustrating the diversity of products that can be formed from a single precursor molecule (GPP, geranyl pyrophosphate)

of GPP to form the intermediate linalyl pyrophosphate in order to overcome the *trans*-geometry of the C2-C3 double bond, which prevents direct cyclization. This intermediate then undergoes an ionization-cyclization step resulting in a (*4R*)- or (*4S*)-α-terpinyl carbocation • pyrophosphate anion pair and subsequent termination reactions [28]. Many sesquiterpene and diterpene synthases employ type A cyclization [26].

5-*epi*-Aristolochene synthase (EAS) is one of the most studied sesquiterpene synthases. It catalyzes the conversion of FPP to 5-*epi*-aristolochene, an intermediate in the formation of sesquiterpene phytoalexins in tobacco, having (+) germacrene A and eudesmyl carbocation as intermediates [29]. EAS was the first terpene synthase for which a reaction mechanism could be confirmed by determination of the enzyme's crystal structure [30].

The diterpene synthase copalyl diphosphate synthase (CDP) is one of the most studied type B synthases. It forms the bicyclic intermediate (-)-copalyl pyrophosphate ((-)-CPP) or its diastereomer (+)-copalyl pyrophosphate ((+)-CPP) from GGPP. Several CDPs are expressed in rice; OsCyc1 forms (+)-CPP, while OsCyc2 and OsCPS1 form (-)-CPPs. OsCPS1 is believed to be involved in gibberellin biosynthesis and OsCyc2 in diterpene phytoalexin biosynthesis [31].

The abietadiene synthase from grand fir (*Abies grandis*) catalyzes two cyclization reactions at separate but interdependent active sites. The enzyme first converts GGPP in a type-B cyclization at one active site to (+)-CPP, which then undergoes a type A cyclization and additional reactions at the second active site to form a mixture of abietadiene isomers [26, 32].

Abietadiene is the main diterpenoid resin acid of oleoresin, which is secreted in response to wounding and herbivore attack.

The diterpene taxol from Pacific yew is one of the most powerful anticancer agents in therapeutic use today. In a series of seminal discoveries, the group of Croteau has dissected the pathway to this complex molecule at the enzymatic and molecular genetic levels, leading to recent success in partial reconstruction of the pathway in yeast [33]. The diterpene synthase taxadiene synthase catalyzes the cyclization of GGPP as the first committed step in taxol formation [34].

Several terpene synthases appear to be multi-functional. For example, a sesquiterpene synthase from *Zea mays* produces a complex mixture of terpene volatiles. The closely related TPS4 and TPS5 from different maize varieties each synthesize the same complements of sesquiterpenes from FPP, but in different proportions as a result of the different ratios of (*S*)- versus (*R*)-bisabolyl cation formation. This difference in stereoselectivity is determined by four amino acid residues in the active site [35].

Oxidosqualene cyclases (OSCs) or triterpene synthases convert oxidosqualene to one or more cyclic triterpene alcohols which are the precursors of sterols, steroids and saponins [36]. The reaction catalyzed is mechanistically similar to those of monoterpene, sesquiterpene and diterpene synthases, but the enzymes are phylogenetically distinct [37].

Alkaloids

A key reaction in assembly of scaffolds for several classes of alkaloid is the coupling of the amine derived by decarboxylation of an amino acid with a second molecule, the product of which then serves as the precursor for secondary modifications. Strictosidine synthase (STR) catalyzes the formation of strictosidine, the precursor for monoterpenoid indole alkaloids such as quinine (Fig. 3) [38]. The reaction involves the condensation of tryptamine and the monoterpenoid secologanin, a unique reaction called a Pictet-Spengler-type reaction. Formation of a Schiff base between the aldehyde group of secologanin and the primary amine group of tryptamine is followed by electrophilic cyclization between the iminium ion and carbon 2 of tryptamine. The crystal structure of STR1 from *Rauvolfia serpentina* has been elucidated and consists of a six-bladed four-stranded β-propeller fold [38]. The protein has a signal peptide that directs it to the vacuole. STR activity has been detected in several members of the Apocynaceae and Rubiaceae [39]. Interestingly, *Arabidopsis thaliana* contains a number of *STR*-like genes, but none has been ascribed a function in alkaloid biosynthesis to date [40].

Norcoclaurine synthase (NCS) catalyzes the condensation of dopamine and 4-hydroxyphenylacetaldehyde (4-HPAA), the first committed step in BIA biosynthesis (Fig. 3). The reaction mechanism is an asymmetric Pictet-Spengler reaction, similar to that of STR although no sequence homology exists between the two proteins. Deacetylipecoside synthase is the third enzyme known to utilize this reaction type, facilitating the condensation of dopamine and secologanin. It has been purified from *Alangium lamarchii* [41]. The homodimeric NCS from meadow rue (*Thalictrum flavum*) exhibits positive cooperativity towards dopamine but not 4-HPAA [42]. NCS belongs to the PR10 (pathogenesis-related) and Betv1 protein family [43]. *Coptis japonica* contains both a PR10-like NCS activity and an additional enzyme, with amino acid sequence similarity to 2-oxoglutarate-dependent dioxygenases (see below) (but lacking the 2-oxoglutarate binding domain), that likewise catalyzes formation of norcoclaurine from dopamine and 4-HPAA [44].

(*S*)-reticuline is a central branch-point metabolite in BIA biosynthesis (Fig. 3). It serves as a precursor for sanguinarine after conversion to (*S*)-scoulerine by the berberine bridge enzyme (BBE), originally purified from *Berberis beaniana*

[45]. The unique reaction catalyzed by BBE comprises the conversion of the *N*-methyl moiety of (*S*)-reticuline into the berberine bridge carbon of (*S*)-scoulerine through oxidative cyclization with a methylene iminium ion as reaction intermediate [45]. The enzyme uses FAD as cofactor, bi-covalently bound to the protein via a histidine and a cysteine residue. An N-terminal signal peptide targets the protein to the ER, and an adjacent vacuolar sorting determinant then directs BBE into the vacuole, where sanguinarine (Fig. 3) accumulates after elicitation with fungal elicitor in opium poppy. The low vacuolar pH suggests that alkaloid synthesis is completed before the ER-derived vesicles fuse with the vacuole [46].

Modifications of Secondary Metabolite Scaffolds

Modification reactions create the enormous diversity of plant natural products, providing new molecules with different biological activities from the basic scaffolds outlined above. The plant kingdom contains a large number of enzymes that catalyze hydroxylation, epoxidation, aryl migration, glycosylation, methylation, sulfation, acylation, prenylation, oxidation and reduction of secondary metabolite skeletons, examples of which are reviewed below, and illustrated, for phenylpropanoid and flavonoid biosynthesis, in Fig. 5. Figure 6

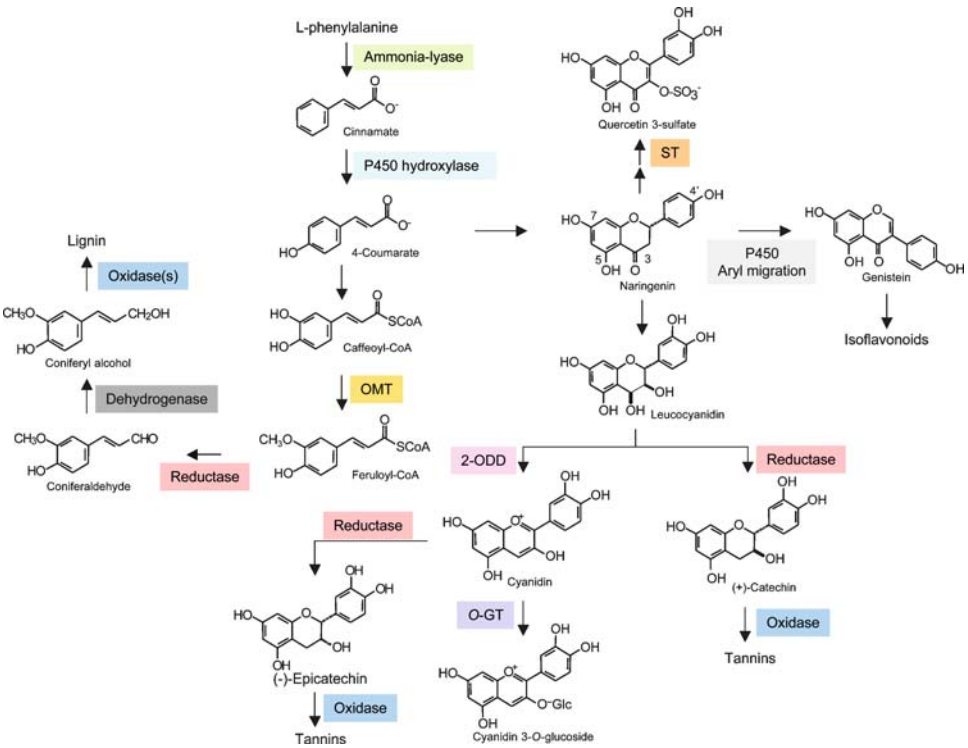

Fig. 5 Schematic representation of enzymatic reactions responsible for the modification of plant-derived secondary metabolite scaffolds, using phenylpropanoids/flavonoids as an example. Glc, glucose residue; 2-ODD, 2-oxoglutarate-dependent dioxygenase; *O*-GT, *O*-glycosyltransferase; OMT, *O*-methyltransferase; P450, cytochrome P450 monooxygenase; ST, sulfotransferase

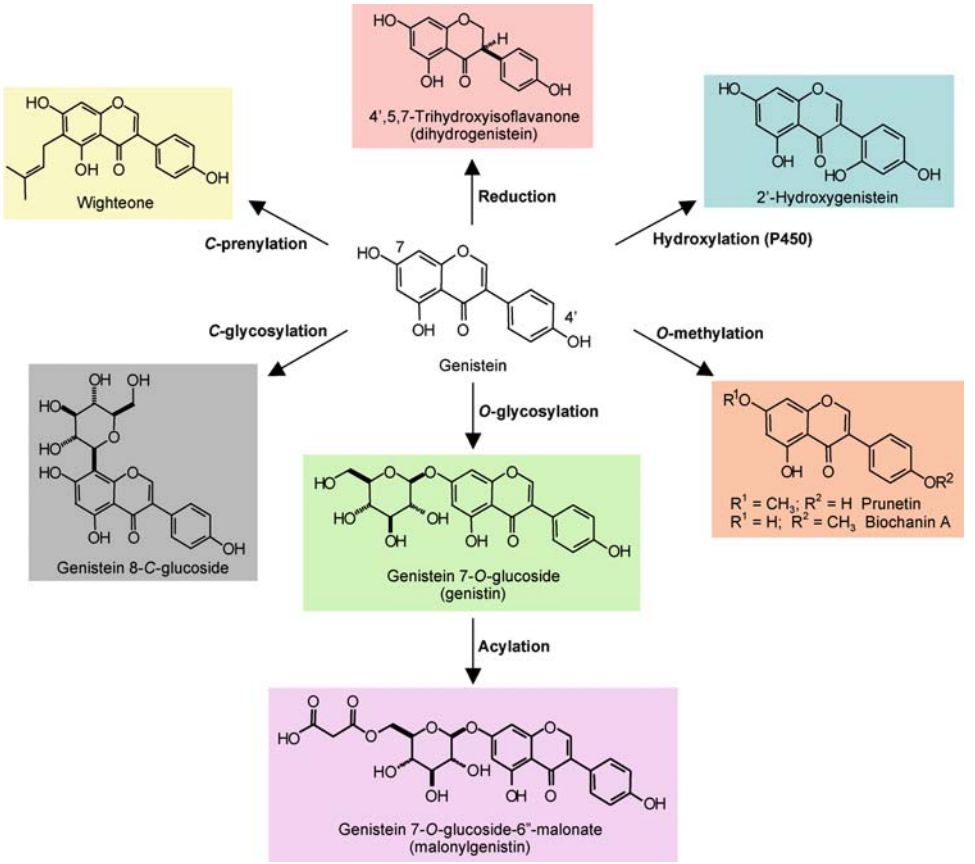

Fig. 6 Enzymatic modifications of the isoflavone genistein

shows how a single molecule (in this case the isoflavone genistein) can be converted to a range of different products.

Oxygenation Reactions Catalyzed by Cytochrome P450s

Cytochromes P450 (CYPs) are versatile biocatalysts. Their name derives from the fact that they contain a cytochrome (heme-protein) pigment that exhibits maximum absorption at 450 nm upon binding of CO. These enzymes form the largest family of plant proteins (http://drnelson.utmem.edu/CytochromeP450.html). NADPH-dependent regio- and stereo-specific oxygenations of lipids, phenolics, terpenoids and alkaloids catalyzed by P450 enzymes include simple hydroxylation or epoxidation, dealkylation, isomerization and aryl migration [47].

Hydroxylation reactions are very common in the biosynthesis of phenylpropanoid/polyketide, alkaloid and terpenoid secondary metabolites. The most abundant plant cytochrome P450 is the cinnamate 4-hydroxylase (CYP73A family) that catalyzes the second committed step of the central phenylpropanoid pathway (Fig. 5) leading to hydroxycinnamic acids, flavonoids and lignin [48]. CYP84A1 is responsible for the 5-hydroxylation of coniferaldehyde during lignin

biosynthesis, and the *Arabidopsis thaliana* EMS mutant *fah I* defective in CYP84A1 expression has altered lignin composition and suppressed sinapoyl malate accumulation [49]. CYP75A1 is a flavonoid 3',5'-hydroxylase involved in the biosynthesis of anthocyanin pigments [50] (Fig. 5). Two mutant alleles of this gene in Petunia (*Hf1* and *Hf2*) exhibit altered flower color (from blue to pink) as a result of loss of delphinidin and residual pigmentation from cyanidin or pelargonidin [50].

Geraniol 10-hydroxylase (CYP76B6) from *Catharanthus roseus* is a key regulatory enzyme in the synthesis of the terpene indole alkaloid vindoline [51]. Alkaloids such as vincristine and vinblastine (derived from vindoline) are used in modern medicine as anti-neoplastic agents (Fig. 3).

In recent years, a range of cytochrome P450 enzymes involved in cyclic terpene hydroxylation has been characterized at the molecular level. These include monoterpene hydroxylases involved in the biosynthesis of essential oils in commercial mint [52], a sesquiterpene hydroxylase performing successive hydroxylations of 5-*epi*-aristolochene in the biosynthesis of the tobacco phytoalexin capsidiol [53], diterpene hydroxylases involved in the formation of taxol [54, 55], and triterpene hydroxylases involved in the biosynthesis of saponins [56] and brassinosteroids [57].

Lutein, the most abundant carotenoid in photosynthetic tissues, is produced via hydroxylation of the ε-ring of zeionoxanthin catalyzed by CYP97C1 [58]. CYP707A1 and CYP707A2 are critical for the control of seed dormancy and germination in Arabidopsis by hydroxylating abscisic acid at the C-8' position for catabolism of this carotenoid-related phytohormone [59].

DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) is a cyclic hydroxamic acid produced by members of the Gramineae as a defense against herbivores or microbial pathogens [60]. Maize CYP71C1 catalyzes the hydroxylation of indolin-2-one at the 3-position to form the precursor molecule for DIMBOA biosynthesis [61]. Transposon-tagged mutations (*Bx3::Mu*) in *CYP71C1* suppress the production of DIMBOA and make maize plants more susceptible to pathogens [61].

Epoxidation Reactions of P450 Monooxygenases

Oxylipins are biologically active signaling compounds of structural diversity generated by the coordinated action of lipases, lipoxygenases and P450s specialized in the metabolism of hydroperoxy fatty acids. CYP74A, CYP74B, and CYP74C catalyze epoxidation of the 9- and/or 13-hydroperoxides of linoleic and linolenic acid [62, 63]. CYP74C displays specificity toward 9-hydroperoxy fatty acid derivatives [62] while CYP74A and CYP74B are more selective for 13-hydroperoxy derivatives leading to the synthesis of the wound signal jasmonic acid (JA) [63]. Knock-out mutation in the *CYP74A* gene of Arabidopsis causes male sterility and impaired wound signal transduction due to suppression of JA production [64].

Hairy root cultures of *Catharanthus roseus* can synthesize tabersonine, a precursor of vindoline [65]. Methyl jasmonate induces the tabersonine 6,7-epoxidase P450 that converts tabersonine into lochnericine [65].

CYP714D1 (a catabolic enzyme) catalyzes 16α,17-epoxidation of non-13-hydroxylated gibberellins, and over-expression of this gene in *Oryza sativa* leads to a dwarf phenotype due to gibberellin deficiency [66].

Non-oxygenation Reactions Catalyzed by P450s

In isoquinoline alkaloid biosyntheses, several unique P450 reactions have been reported, such as methylenedioxy bridge formation, intramolecular C–C phenol-coupling and intermolecular C–O phenol-coupling reactions. Salutaridine

synthase, involved in the conversion of (*R*)-reticuline to salutaridine during the formation of morphine (Fig. 3), is a cytochrome P450 that catalyzes an intramolecular phenol-coupling reaction that does not involve incorporation of oxygen into the substrate [67]. Recently, heterologous expression in yeast has indicated that CYP80G2 from *Coptis japonica* likewise exhibits intramolecular C–C phenol-coupling activity to produce (*S*)-corytuberine (aporphine-type alkaloid) from (*S*)-reticuline (benzylisoquinoline type) [68]. Methylenedioxy-bridge forming cytochrome P450s have been described in alkaloid [69] and isoflavonoid [70] biosynthesis.

Hydroxylation/Aryl Migration Catalyzed by P450 Monooxygenases

Isoflavonoids are widely produced by legumes and function in defense against pathogen attack and attraction of symbiotic microbes. An unusual aryl migration reaction constitutes the first committed step in their biosynthesis [71]. Isoflavone synthase (IFS, more correctly known as 2-hydroxyisoflavanone synthase) is a member of the CYP93C family that catalyzes migration of the B-ring of the flavanones liquiritigenin and naringenin from C-2 to C-3 leading to the isoflavones daidzein and genistein (Fig. 5) respectively [71, 72]. The reaction proceeds by abstraction of a hydrogen at C-3 followed by B-ring migration and subsequent hydroxylation of the resulting C-2 radical; dehydration with loss of the C-2 hydroxyl to yield isoflavone occurs enzymatically *in vivo* and non-enzymatically *in vitro*. The enzyme is stereo-selective and recognizes only 2*S*-flavanones as substrates. Down-regulation of IFS in soybean hairy roots compromises defense against *Fusarium solani* f. sp. *glycinea* by suppressing accumulation of the glyceollins, daidzein-derived phytoalexins [73].

Isoflavones are not limited to the Leguminosae. For example, sugarbeet (Chenopodiaceae) produces isoflavones in response to pathogen attack. Two IFS cDNAs from sugarbeet share higher than 95% similarity to IFS1 from soybean [74].

Phenylphenalenones are polycyclic natural products of the Haemodoraceae, Musaceae and Strelitziaceae. The biosynthetic pathway for 8-phenylphenalenone production in the water hyacinth *Eichhornia crassipes* may occur via the formation of diarylheptanoid and 9-phenylphenalenone as intermediates, and includes a 1,2-aryl migration reaction that may follow a mechanism similar to that of IFS [75]. However, no P450 enzyme able to catalyze this reaction has been reported to date.

2-Oxoglutarate-Dependent Dioxygenases (2-ODDs)

Plant 2-oxoglutarate-dependent dioxygenases (2-ODDs) are cytosolic, non-heme iron-containing enzymes that utilize an oxoacid to oxidize a target metabolite. Ascorbate is required *in vitro,* probably to maintain the iron moiety in the reduced form (Fe^{2+}). 2-ODD enzymes are involved in the biosynthesis of some amino acids, hormones, signaling molecules, and a large number of secondary metabolites [76].

Flavanone 3β-hydroxylase (F3H) converts the basic flavonoid skeleton flavanone to dihydroflavonol through hydroxylation at the 3-position [77], a critical early step in anthocyanin flower pigment biosynthesis. Flavonol synthase (FLS) is a 2-ODD that catalyzes the formation of a double bond between C-2 and C-3 in dihydroflavonols [78]. Flavonol 6-hydroxylase (F6H) is also a 2-ODD [79].

Anthocyanidin synthase (ANS), the key enzyme in the biosynthesis of anthocyanins, catalyzes oxidation of leucoanthocyanidin (flavan-3,4-diol) to a 2-flaven-3,4-diol that spontaneously isomerizes to 3-flaven-2,3-diol (anthocyanidin) (Fig. 5). This is subsequently glycosylated at C-3, transported to the vacuole, and finally converted to the colored flavilium cation at the acidic

pH of the vacuole [77]. The first biochemical evidence for ANS being a 2-ODD came from studies with the recombinant enzyme from *Perilla* [80]. A catalytic mechanism has been proposed in which ANS promotes hydroxylation at C-3 as the initial step in anthocyanidin biosynthesis, and this is supported by structural data for ANS from Arabidopsis [81].

Hyoscyamine 6β-hydroxylase (H6βH) is a 2-ODD enzyme that catalyzes a critical step in the biosynthesis of the tropane alkaloid scopolamine (Fig. 3) in members of the Solanaceae [82].

Reductases

Several NADPH-dependent reductase enzymes play key roles in the biosynthesis of flavonoids. Isoflavone reductase (IFR), which is a member of the Reductase-Epimerase-Dehydrogenase (RED) family of proteins, catalyzes the stereospecific reduction of isoflavone to the corresponding (3*R*)-isoflavanone (Fig. 6), a key reaction in the biosynthesis of antimicrobial pterocarpan phytoalexins such as medicarpin in legumes. IFR from alfalfa catalyzes formation of (3*R*)-vestitone from 2'-hydroxyformononetin for the biosynthesis of medicarpin [83]. The IFR from soybean has activity toward 2'-hydroxydaidzein, 2'-hydroxyformononetin, and 2'-hydroxygenistein [84] while the IFRs from pea and chickpea recognize 7,2'-dihydroxy-4',5'-methylenedioxyisoflavone and 2'-hydroxyformononetin, respectively [85, 86].

IFRs belong to a large protein family that includes IFR-like proteins from non-legume plants. IFR-like proteins have high sequence identity to legume IFRs, but the functions of many are still unclear. Some clearly catalyze reduction reactions in pathways other than isoflavonoid biosynthesis. For example, pinoresinol-lariciresinol reductase and phenylcoumaran benzylic ether reductase are IFR-like proteins involved in the formation of lignans in *Forsythia intermedia* and *Pinus taeda*, respectively [87, 88], and it is thought that pinoresinol reductases may represent the progenitors of the IFRs.

Dihydroflavonol 4-reductase (DFR) is involved in the biosynthesis of anthocyanins and proanthocyanidins (PAs). DFRs catalyze the stereospecific reduction of (2*R*,3*R*)-dihydroflavonols to (2*R*,3*R*,4*S*)-leucoanthocyanidins [77] (Fig. 5). Petunia possesses three different *DFR* genes (*dfrA-C*), but only *dfrA* is transcribed in floral tissues. DFR-A does not accept dihydrokaempferol, the precursor for the synthesis of pelar-gonidin-type anthocyanins. Consequently, no orange-colored petunia flowers are found in nature [89]. Dihydroquercetin and dihydromyricetin are also substrates for DFRs and provide leucocyanidin and leucodelphinidin, respectively.

Leucoanthocyanidin reductase (LAR), which is related to the isoflavone reductase group of plant enzymes, catalyzes the reduction of leucoanthocyanidins to (+)-afzelechin, (+)-catechin, and (+)-gallocatechin, building blocks for PA biosynthesis [77] (Fig. 5). The above catechin series of flavan-3-ols possess 2,3-*trans* stereochemistry. The corresponding (*epi*)-catechin series with 2,3-*cis* stereochemistry is formed by a different mechanism involving an unrelated reductase (ANR) that acts at the level of anthocyanidin [90] (Fig. 5).

Reduction of a coenzyme a ester to the corresponding aldehyde, catalyzed by cinnamoyl CoA reductases (CCRs), is an important reaction in lignin biosynthesis (Fig. 5). Plants contain small *CCR* gene families [91] encoding enzymes with differences in overall specificity for monolignol precursors with different aromatic ring substitution patterns.

Glycosyltransferases

Among the reactions for the modification of secondary metabolite scaffolds, glycosylation plays a particularly important role in plants, contributing to the biosynthesis and storage of

secondary metabolites, regulation of hormone homeostasis, detoxification of xenobiotics, enhancement of a molecules' solubility, and plant defense [92]. Glycosyltransferases of small molecules (UGTs) catalyze the transfer of sugar residues from uridine diphosphate sugars to an acceptor. The sugar moiety can be transferred to oxygen, nitrogen, or sulfur atoms of different classes of natural products [92]. UGTs comprise a superfamily of enzymes ubiquitous in living organisms (http://afmb.cnrsmrs.fr/CAZY/fam/acc_GT.html), the number of which in the plant kingdom is likely comparable to that of the cytochrome P450 enzymes.

The most common reaction catalyzed by UGTs is the transfer of sugar residues to oxygen atoms. Several plant UGT crystal structures have been reported in the literature, all for *O*-glucosyltransferases [93–95]. Glucose is the commonest sugar attached to plant secondary metabolites, although galactose, glucuronic acid and other "common" monosaccharides are also found; this contrasts to the situation in prokaryotes, where more "exotic" sugars are often found attached to secondary metabolites (e.g. in antibiotics).

Eight recombinant *Medicago truncatula* UGTs display *O*-glycosyltransferase activity toward (iso)flavonoids at different positions [96]. Recombinant UGT85H2 prefers flavonols whereas the substrate specificity of UGT78G1 is higher for isoflavones [95, 96]. UGT78G1 is regioselective, catalyzing *O*-glycosylation at C-7 unless the flavonoid has a hydroxyl group at the C-3 position (see labeling of naringenin in Fig. 5), in which case this position is preferred. UGT78G1 is also able to operate in the reverse direction, deglycosylating (iso)flavonoid glucoderivatives in the presence of uridine diphosphate [96]. Similar to grapevine *Vv*GT1 [94], UGT78G1 recognizes anthocyanidins as substrates [96]. Snapdragon 4'CGT converts chalcone to its 4'-*O*-glucoside derivative, an intermediate in the synthesis of yellow aurone flower pigments [97].

Three glucoyltransferases involved in the biosynthesis of the sweet diterpene glucosides of *Stevia rebaudiana* [98] and two involved in formation of triterpene saponins in *Medicago truncatula* [99], have been identified through genomic approaches. Whereas glycosylation often inactivates or targets plant natural products for storage, it is important for biological activity in the case of the triterpene saponins.

Arabidopsis UGT84B1 catalyzes the *O*-glycosylation of indole-3-acetic acid (IAA), and over-expression of *UGT84B1* leads to altered root phenotypes as a consequence of free IAA depletion [100]. UGT73C5 from Arabidopsis catalyzes the 23-*O*-glucosylation of brassinolide and castasterone, two plant steroid hormones. Brassinosteroid accumulation is dramatically reduced in transgenic plants over-expressing UGT73C5, and the phenotype is consistent with the deprivation of free steroid hormones [101]. UGT76C1 and UGT76C2 from *Arabidopsis thaliana* both exhibit activity toward cytokinins [102]. Plants over-expressing *UGT76C1* show increased accumulation of *trans*-zeatin 7-*N*-glucoside when supplemented with *trans*-zeatin [102]. Structural studies have recently revealed mechanisms controlling *N*- as compared to *O*-glucosylation in UGT72B1, a bifunctional N-/O-glucosyltransferase from Arabidopsis active in xenobiotic detoxification [103].

An *S*-GT from *Brassica napus* was the first thiohydroximate *S*-glycosyltransferase of the glucosinolate pathway to be partially characterized *in vitro* [104]. T-DNA insertions in the corresponding gene in Arabidopsis (UGT74B1) cause low levels of glucosinolates, leaf vein chlorosis, and impaired auxin metabolism [105].

Despite interest in the anti-microbial and anti-insect activities of flavonoid *C*-glycosides (e.g. from maize), and the potential therapeutic value of isoflavone *C*-glycosides such as genistein 8-*C*-glucoside (Fig. 6) or puerarin from *Pueraria lobata*, no plant gene encoding a *C*-glycosyltransferase has yet been cloned.

Methyltransferases

O-Methyltransferases (OMTs) catalyze the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to oxygen atoms of hydroxyl groups on an acceptor molecule to yield the methyl ether derivative (Figs. 5 and 6). OMTs are regio- and stereo-selective and can collectively mono- or poly-methylate a great number of plant natural products [106]. Methylation of flavonoids alters their solubility and intracellular compartmentalization, and can increase their antimicrobial activity [107].

Small molecule OMT enzymes from plants are classified in two distinct groups. Group I OMTs have molecular weights in the range of 38–43 KDa and target many acceptors such as flavonoids, phenylpropanoids, alkaloids, and coumarins. Group II OMTs are of lower molecular weight (23–27 KDa) and are dependent on Mg^{2+} for activity [108].

O-Methylation at C-3 of the isoflavonoid derivative 6a-hydroxymaackiain by PsHMM (group I) is the final step in the production of the phytoalexin pisatin in *Pisum sativum*; the non-methylated precursor lacks antimicrobial activity [109]. Caffeic acid 3-*O*-methyltransferase (group I) and caffeoyl CoA 3-*O*-methyltransferase (group II) (Fig. 5) play important roles in lignin biosynthesis [110]. Group II OMTs from cell suspension cultures of meadow rue are involved in the biosynthesis of the isoquinoline alkaloid beberine (Fig. 3). The recombinant enzymes OMT II 1.1, OMT II 2.2, OMT II 3.3, and OMT II 4.4 also recognize a range of phenylpropanoids and catechols with different specificities [111]. OMT II 1.1, but not OMT II 4.4 is active toward the isoquinoline alkaloid (*R,S*)-norcoclaurine, and these enzymes only differ from each other at amino acid residue 21 (Tyr in the former and Cys in the latter) [111].

Putrescine *N*-methyltransferase (PMT) catalyzes the first committed step in TPA and nicotine biosynthesis, the SAM-dependent methylation of putrescine (Fig. 3). The enzyme has a high similarity to mammalian and plant spermidine synthase (SPDS), from which it is assumed to have evolved. SPDS uses a slightly different co-substrate, decarboxylated SAM (dcSAM), but can also accept putrescine as substrate [18]. PMT is expressed primarily in roots, but has also been detected in young potato tuber sprouts and wounded leaves of tobacco [112]. SAM-dependent ***N*-**methyltransferases play a key role in caffeine biosynthesis, where three steps of methylation of nitrogen atoms take place.

Sulfo- and Aromatic Prenyl-Transferases

Sulfate transfer to flavonoids and glucosinolate precursors is catalyzed by a small family of soluble sulfotransferases (STs) that use 3'-phosphoadenosine 5'-phosphosulfate (PAPS) as sulfate donor (Fig. 5). Sulfated flavonols may play a role in the transport of auxins [113]. Four position-specific flavonol STs are found in plants of the genus *Flaveria,* with preferences for the 3-position of the flavonoid aglycone, the 3' and 4'-positions of 3-sulfate derivatives, and the 7-position of 3,3'- or 3,4'-disulfate derivatives [113] (Fig. 5).

Aromatic prenyltransferases (PTs) utilize allyl diphosphate for the prenylation of aromatic compounds in a process dependent on divalent cations (Mg^{2+} or Mn^{2+}). The UbiA family includes PTs that catalyze the prenylation of 4-hydroxybenzoate as a key step in the formation of ubiquinone (UQ), an electron carrier in the respiratory chain [114]. UbiA PTs exhibit a broad substrate specificity accepting prenyl pyrophosphates of different chain lengths originating from UQ6 in *Saccharomyces cerevisae* to UQ10 in tobacco. The *AtPP1* gene from Arabidopsis encodes a 4-hydroxybenzoate polyprenyl diphosphate transferase (4-HPT) and its expression in a yeast mutant lacking 4-HPT activity restores ubiquinone

synthesis as well as the cells' respiratory ability [114]. Disruption of the *AtPP1* gene through T-DNA insertion compromises the early stage of Arabidopsis embryo development [114]. PTs from soil bacteria also target small aromatic molecules catalyzing the production of antibiotic compounds. However, these bacterial PTs lack the prenyl diphosphate binding motif (N/D)DXXD characteristic of aromatic PTs from the UBiA family [115].

Prenylated (iso)flavonoids are relatively common antimicrobial compounds in plants (e.g. in fruits of Osage orange [*Maclura pomifera*] and roots of white lupin) (see wighteone in Fig. 6). The first characterization of a plant flavonoid PT at the molecular level was recently reported; naringenin 8-prenyltransferase from *Sophora flavescens* is related to the homogentisate PTs involved in the formation of tocopherols and tocotrienols [116].

Acyltransferases

Acylation of oxygen or nitrogen atoms to generate esters and amides, respectively, is a common reaction for natural product scaffold modification. Members of the large BAHD family of acyltransferases utilize CoA thioesters as a source of the acyl group. The term BAHD comes from the initials of the first four plant acyltransferases biochemically characterized (BEAT, AHCT, HCBT, and DAT) [117]. Benzylalcohol *O*-acetyltransferase (BEAT) is responsible for the production of the floral volatile benzylacetate in *Clarkia breweri*, whereas deacetylvindoline 4-*O*-acetyltransferase (DAT) participates in the last step in the biosynthesis of vindoline in *Catharanthus roseus*. *N*-hydroxycinnamoyl/benzoyltransferase (HCBT) acts in the production of anthramide phytoalexins in *Dianthus caryophyllus*, and anthocyanin *O*-hydroxycinnamoyltransferase (AHCT) is responsible for the 5-*O*-acylation of anthocyanins in *Gentiana triflora* [117]. Other BAHD acyltransferases include CmAAT4 from *Charentais melon* that catalyzes the formation of volatile medium-chain aliphatic esters, HMT/HLT, a tigloyltransferase crucial for the biosynthesis of quinolizidine alkaloids in *Lupinus albus* [117], and MtMat1–3, which catalyze the malonylation of the sugar residue on isoflavone 7-*O*-glucosides in *Medicago truncatula* [118] (Fig. 6).

Plants also possess various serine carboxypeptidase-like (SCPL) enzymes that function as acyltransferases [119]. In contrast to BAHD family acyltransferases, SCPL enzymes use 1-*O*-β-acyl acetals (most frequently the 1-*O*-β-ester of glucose) as the acyl donor. True serine carboxypeptidases are exclusively hydrolytic, and the discovery of serine carboxypeptidase-like enzymes with acyltransferase features brought a new perspective to gene annotation in plant secondary metabolism [120, 121]. Isolation of a cDNA encoding an SCPL protein responsible for the synthesis of glucose polyesters has been reported [120]. These compounds are produced in trichomes of *Lycopersicon pennellii* and *Solanum berthaultii* as a defense against insect attack. An SCPL enzyme responsible for the formation of UV-protecting sinapoyl malate in leaves of the Brassicaceae has also been described [121].

The Challenge of Predicting Enzyme Function in Plant Secondary Metabolism

The functional annotation of members of gene families involved in modification of secondary metabolite scaffolds is often challenging, and amino acid sequence identity by itself may be misleading for prediction of enzyme function. For example, although *Medicago truncatula* UGT71G1 clusters phylogenetically with UGT71C1 or UGT71C4 from *Arabidopsis thaliana* (enzymes known to glycosylate benzoic acid derivatives), recombinant UGT71G1

does not display activity toward benzoic acid [96]. Despite the high activity of recombinant UGT71G1 against quercetin, with all hydroxyl groups on the molecule being glycosylated, and much lower *in vitro* activity toward triterpenes, UGT71C1 is believed to catalyze the glycosylation of triterpenes *in vivo* based on correlated transcript and metabolite induction patterns, which seem to rule out an *in vivo* role in quercetin glycosylation [96, 99]. Difficulties associated with gene annotation have been widely discussed for methyltransferases, and the overlapping substrate specificities that are co-expressed in the same cell types becomes an additional issue [111, 122]. The existence of serine carboxypeptidases with acyl transfer but not hydrolytic properties, isoflavone reductase-like proteins in plants that do not synthesize isoflavonoids, and strictisodine synthase-like genes in Arabidopsis, clearly demonstrate that similarities in amino acid sequence *per se* are not enough for determination of protein function. Studies integrating spatially and temporally resolved metabolome and transcriptome analysis, together with loss of function genetic analysis using insertion/deletion mutants or transgenic plants (antisense or RNAi lines), will be crucial for elucidating the individual roles of these enzymes *in vivo*. Gain of function analyses alone may be confusing for enzymes with promiscuous *in vitro* substrate preferences.

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