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Cytochrome P450s in Plants

Kirsten Annette Nielsen and Birger Lindberg Møller

1. Introduction

Plants are sessile organisms that cannot avoid exposure to adverse climatic conditions or attack from herbivores and pests by escaping. To survive and protect themselves, they are dependent on the ability to (a) redirect their overall metabolism to meet environmental constraints, (b) construct physical barriers that are difficult to penetrate, (c) produce chemicals that make the plant toxic to pests and herbivores, and (d) communicate with the environment, for example, to attract pollinators. Cytochrome P450 enzymes (P450s) play a key role in enabling plants to achieve these main goals.

1.1. Natural Products

Plants are the best organic chemists in nature as evidenced by their ability to synthesize all necessary carbon compounds with carbon dioxide as the sole carbon source and by their ability to synthesize a vast number of natural products. Currently, structures for more than 100,000 different natural products isolated from plants are known[1–3], and with time this number will increase into millions. Natural products are classified as phytoanticipins, phytoalexins, and/or attractants. In the last decade, the majority of the biosynthetic pathways responsible for natural product synthesis have been shown to include P450s as key enzymes. Such pathways include the biosynthetic pathways for cyanogenic glucosides, glucosinolates, isoflavonoids, and alkaloids. In addition, a number of plant P450s have been shown to catalyze detoxification of harmful agents including herbicides[4].

1.2. Chemical Warfare

The chemical warfare between plants and herbivores and pests is complex and takes place at many trophic levels. The plant *Apium graveolens* (celery) is known to combat *Helicoverpa zea* (corn earworm) by producing allelochemicals including furanocoumarins in a P450-dependent series of reactions[5]. The herbivore, however, is able to detoxify the furanocoumarins. The detoxification pathway is induced by jasmonate, a wound-induced plant signal compound. Jasmonate activates the transcription at least of four herbivore P450 genes[6]. The continuous chemical warfare between plants on one side and herbivores and pests on the other may enforce plants to constantly evolve new natural products and insects to find means to detoxify these. This requires recruitment of enzymes with altered biological functions probably mediated by modifications and duplications of existing genes. P450s are key players in securing recruitment of these new functions as exemplified by the recruitment of an allele specific P450 in Drosophila to acquire resistance to chemical insecticides[7].

1.3. Chemical Communication

Plants are dependent on intense communication with their surroundings via biochemical

Kirsten Annette Nielsen and Birger Lindberg Møller • Plant Biochemistry Laboratory, Royal Veterinary and Agricultural University, 40, Thorvaldsensvej, DK-1871 Frederiksberg C, Copenhagen, Denmark.

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signalling, for example, to mediate pollination and seed spreading[1]. In cases with insect-mediated pollination, plants synthesize insect attractants. A special group of secondary metabolites, glucosinolates, is produced within the taxonomic order Capparales. Glucosinolates have dual roles acting both as attractants for specialized insects and as deterrents for generalist herbivores. P450s are key enzymes in the biosynthesis of glucosinolates[8, 9]. *Nicotiana tabacum* (tobacco) from the taxonomic order Solanes produces cembranoid-type terpenes as insect attractants. The cembranoid terpene gland exudates contain α- and β-epimers of cembra-2,7,11-triene-4,6-diol and these attract different pollinating insects. Unfortunately, they also enhance oviposition of the unwanted insect *Myzus nicotiana* (red aphid)[10].

1.4. Medicinal Agents

Humans take advantage of plant natural products as drugs or lead compounds in medicine, for example, as anesthetics and anticarcinogens. Alkaloid-containing plants have been used in human medicine for thousands of years. One very large and structurally diverse group of alkaloids are the tetrahydrobenzylisoquinoline alkaloids. *Papaver somniferum* (opium poppy) from the taxonomic order Ranunculales produces more than 100 such L-tyrosine derived alkaloids including the potent anesthetic morphine[11, 12]. The biosynthesis of tetrahydrobenzylisoquinoline alkaloids involves P450s with unique catalytic properties[13].

The nutraceuticals daidzein and genistein belong to the isoflavonoids and are phytoestrogens preventing breast and prostate cancers[14]. The dietary compounds are synthesized especially in Leguminosae belonging to the Fabales order. Isoflavonoid biosynthesis also requires a unique P450 enzyme that catalyzes aryl group migration[15]. Alkaloids and isoflavonoids play important roles in plant defense and their biosynthesis are tightly regulated and inducible processes[2, 3].

The purpose of this review is to highlight recent key findings on plant P450s. The genome sequencing programs have identified the P450s as the largest superfamily in plants. The catalytic properties of most of these P450s remain elusive. We focus on P450s involved in the synthesis of natural products belonging to the groups of cyanogenic glucosides, glucosinolates, alkaloids, and isoflavonoids. Special emphasis is on the plant model *Arabidopsis thaliana* for which the complete genome sequence is available[16] and which is easily amenable to genetical modifications[17] and provides an excellent model plant for metabolic engineering. Exploitation of P450s to reach increased production levels for desired natural compounds and transfer of entire biosynthetic pathways into other plant species will be discussed. The first part is a short presentation of different tools used to achieve gene—to function relationship, the bottleneck in P450 functional genomics[18].

2. The P450 Superfamily in Plants

So far genomic and expressed sequence tag (EST) sequencing projects have revealed a total of 1,059 plant P450 sequences[19]. Phylogenetic analyses based on translated raw DNA sequence data have spaced the P450s into 10 clans that include 59 families and an extensive number of subfamilies[18, 20]. In the *A. thaliana* genome alone, a superfamily of 272 cytochrome P450 genes including 26 pseudo genes were annotated and named[16, 21]. These genes represent members of 45 out of the 59 currently assigned plant P450 families[20, 22]. P450s constitute the largest and continuously expanding superfamily in plants. From the *Oryza sativa* cvs *japonica* and *indica* (rice cultivars)[23, 24] genome sequence projects, as many as 458 predicted P450 genes were annotated by the end of September 2002[25].

2.1. Nomenclature

The large number of P450 enzymes found in the Plant Kingdom are named and categorized based on protein sequence identity and phylogenetic relationships[26]. P450s assigned to the same family share more than 40% sequence identity at the amino acid level. Correspondingly, P450s assigned to the same subfamily share more than 55% sequence identity.

In plants, the identity rule has some exceptions due to gene duplications and shuffling as pointed out in Werck-Reichart *et al.* (2002)[22]. The plant P450s are categorized into the following

families: CYP51, CYP71-99, CYP701-727, and CYP736[20]. The CYP51 family is unique because the sequence identity of the P450s belonging to this family is well-enough conserved across phyla to contain plant, fungal, bacterial as well as animal sequences[27]. The precise structure of the sterols that serve as substrates for CYP51s varies among different eucaryotes. This variability has been suggested to represent adaptation to the availability of different sterol precursors in different Kingdoms.

Plant P450s are membrane-bound proteins. They are classified into the A-type and the non-A-type P450s[28, 29]. It has been proposed that the plant-specific A-type P450s originate from a single ancestral P450[28]. A-type P450s share a simple gene organization with a single phase 0 intron with a highly conversed position[29]. Typically, the P450 genes are found to cluster with close relatives on short stretches of all five *A. thaliana* chromosomes indicating recent duplication events[29]. P450s involved in the biosynthesis of plant natural products belong to the A-type. In this review, we focus on A-type P450s belonging to the CYP71, CYP79, CYP80, CYP83, and CYP93 families and their respective involvement in the biosynthesis of cyanogenic glucosides, glucosinolates, alkaloids, and isoflavonoids. A short description of the biological function of non-A-type plant P450s is confined to members of the CYP85 and CYP90 families that are involved in the production of polyhydroxylated steroidal molecules.

3. Tools Available to Identify Biological Functions

Only very few of the 246 predicted P450 enzymes present in *A. thaliana* have had a biological function assigned. Functional assignments of the *A. thaliana* P450s are restricted to 23 enzymes belonging to 14 of the 45 plant families represented in the *A. thaliana* genome. These identified enzymatic activities are: *CYP51*, obtusifoliol 14α-demethylase[30]; *CYP72B1*, brassinolide 26-hydroxylase[31]; *CYP73A5*, cinnamate-4-hydroxylase[32]; *CYP74A1*, allene oxide synthase[33, 34]; *CYP74B2*, hydroperoxide lyase[35]; *CYP75B1*, flavonoid 3′-hydroxylase[36]; *CYP79A2*, phenylalanine *N*-hydroxylase[37]; *CYP79B2* and *CYP79B3*, tryptophan *N*-hydroxylases[38]; *CYP79F1* and *CYP79F2*, methionine *N*-hydroxylases[39–41]; *CYP83A1* and *CYP83B1* enzymes converting indole-3-acetaldoxime, *p*-hydroxyphenylacetaldoxime, and phenylacetaldoxime into the corresponding *S*-alkyl-thiohydroximates[42–44]; *CYP84A1*, ferulic acid hydroxylase[45]; *CYP85A1*, steroid C-6-hydroxylase[46]; *CYP86A1* and *CYP86A8*, fatty acid ϖ-hydroxylases[47, 48]; *CYP88A3* and *CYP88A4* enzymes converting *ent*-kaurenoic acid to GA_{12}[49]; *CYP90A1*, steroid C-23 hydroxylase[50]; *CYP90B1*, steroid C-22 hydroxylase[51]; *CYP98A3*, 3′-hydroxylase of phenolic esters[52]; and *CYP701A3*, *ent*-kaurene oxidase[53].

3.1. Phylogenetic Relationships

The categorization of P450s from different plant species into families and subfamilies based on sequence identity and phylogenetic relationships as discussed above typically does not concomitantly lead to an assignment of biological and/or enzymatic function. In some families, the P450s all appear to catalyze the same enzymatic reaction. In other families, members of the same family clearly catalyze very different enzymatic reactions. These differences are illustrated with the following examples. The CYP73 family is composed of one subfamily, CYP73A with 37 members. CYP73As from *Helianthus tuberus* (artichoke)[54], *Phaseolus aureus* (mung bean)[55], *Medicago sativa* (alfalfa)[56], *Petroselium crispum* (parsley)[57], *Popupus tremuloides* (querken aspen)[58, 59], *A. thaliana*[32], *Triticum aestivum* (wheat)[60], *Cicer arietinum* (chickpea)[61], have all been demonstrated to be cinnamate 4-hydroxylases. The rest of the members of the CYP73A subfamily are therefore with great confidence assigned as cinnamate 4-hydroxylases solely based on their amino acid sequence identity. Members of the CYP74A subfamily have been characterized as allene oxide synthases in *A. thaliana*[33, 34], *Linum usitatissium* (flaxseed)[62], *Hordeum vulgare* (barley)[63], and *Lycopersicon esculentum* (tomato)[64]. However, members of the closely related CYP74B subfamily possess fatty acid hydroperoxide lyase activity as demonstrated in *A. thaliana* and *L. esculentum* (tomato)[35, 64]. Members of a single subfamily may also catalyze different and consecutive steps in a biosynthetic pathway as reported for members of CYP90A and CYP90B (see Section 4.1). The different steps of

entire biosynthetic pathways may be mediated by P450s belonging to the same subfamily as exemplified by the CYP71C subfamily (see Section 5.3.2). In contrast to the latter examples, members of five different subfamilies of the CYP79 family are all *N*-hydroxylases (see Section 5.2). A final example on the existence of numerous subfamilies with widely different biological functions is the 18 subfamilies CYP71A to CYP71R in the CYP71 family[20]. Enzymatic activities have solely been demonstrated for members of subfamilies CYP71C, CYP71D, and CYP71E as described in detail in Section 5.1.1. One member of a fourth subfamily, the CYP71A10 was shown to possess enhanced detoxifying properties against phenylurea-derived herbicides, an activity unlikely to be the major biological function of the enzyme[65]. Of the 110 known members of the CYP71 family, 97 belong to the subfamilies CYP71A to CYP71D. No catalytic function has been assigned to any of the 37 members of the CYP71B subfamily.

The difficulties in assigning function to a P450 solely based on its amino acid sequence will be partly alleviated as more catalytic functions become known and diagnostic sequence elements identified. The matter is particularly complicated for the A-type P450s involved in natural product synthesis. Plants are known to produce more than 100,000 different natural products with P450s involved in most pathways and sometimes being multifunctional[66–68]. To illustrate the preponderance of A-type P450s, they account for 153 out of the predicted 246 P450 genes in the *A. thaliana* genome.[20] In general, an A-type P450 is thought to possess high substrate specificity and its function to be limited to a single or a few parallel biosynthetic pathway(s).

The wide diversity in amino acid sequences found among the P450s is evident by the fact that in *A. thaliana*, P450s[16, 20] belong to 45 of the 59 plant P450 families.

3.2. Mutant Collections in *A. thaliana*

The biological function of some *A. thaliana* P450s have been elucidated *in planta* by taking advantage of the availability of knockout mutants in this model plant. Mutant collections have been generated by either T-DNA insertion[69–71], ethyl methanesulphonate (EMS) mutagenesis[72, 73], or ionizing radiation[74]. Special attention in screening programmes has been paid toward phenotypic mutants showing aberrant growth characteristics. This way, non-A-type P450s was shown to affect, for example, dwarfism[50, 51] (Section 4) and A-type P450s to affect excessive lateral root formation[43] (Section 7). Genetic analysis of phenotypes recognized by a lack of blue-green autofluorescence caused by absence of sinapoyl malate identified additional members of A-type P450s. Sinapoyl malate is a phenylpropanoid that serves as a biochemical sunscreen[75] (Section 7).

Methodologies to provide gain-of-function in mutants in existing knockout collections use activation tagging in weak-mutant-allelic backgrounds[76]. This facilitates identification of dominant suppressor genes, which will show enhanced expression after incorporation of multimeric, positive *cis*-acting elements close to suppressor genes. Using activation tagging, it was found that expression levels of a gene encoding a non-A-type P450 proved to influence regulation of light responsiveness and accumulation of steroid phytohormones[31] (Section 4.1).

3.3. Reverse Genetics

Reverse genetics provides a tool to identify the mutant genotype causing specific phenotypic characteristics. Using T-DNA tagged phenotypic mutants (Section 3.2), genomic DNA sequences flanking the T-DNA integration site are identified. Subsequently, the wild-type allele is identified and cloned and inserted into the mutant to revert its phenotype into wild type. Catalytic properties of the P450 are thereafter studied by heterologous expression of the plant cDNA in microorganisms.

3.4. Heterologous Expression in Microorganisms

Heterologous expression of individual cDNAs in *Escherichia coli* followed by enzyme assays in the presence of putative substrates have been used extensively for characterization of plant P450s[77], for example, for the CYP79s involved in

cyanogenic glucoside and glucosinolate synthesis[9, 78]. Recombinant P450 protein can be subjected to classical protein characterization including CO difference spectroscopy[79] and recording of substrate-binding spectra[80] and finally assayed for desired catalytic properties. The first plant P450 cDNA was isolated from ripening fruits of *Persea americana* (avocado)[81]. It was designated CYP71A1. Expression of the cDNA in *Saccharomyces cerevisia*[82] yielded high amounts of recombinant protein, but the predicted catalytic property of CYP71A1 was not identified[83].

Cinnamic acid 4-hydroxylase from *Helianthus tuberosus* (Jerusalem artichoke) was the first plant P450 to be functionally characterized[54]. CYP73A1 was designated as the first member of the CYP73 family. This cDNA was isolated from an expression library using antibodies raised against the isolated P450 protein (Section 3.5). Cinnamic acid 4-hydroxylase catalyzes an essential step in the phenylpropanoid pathway and it is considered to be ubiquitous in plants (see Section 3.1).

3.5. Isolation of Enzymes

Cinnamate 4-hydroxylases catalyze the hydroxylation of *trans*-cinnamic acid into *trans*-*p*-coumaric acid. The ability to monitor this enzyme activity in Jerusalem artichoke allowed isolation of the P450 enzyme CYP73A1 using conventional chromatography and generation of specific antibodies[84, 85].

A general isolation procedure based on dye affinity chromatography has been developed and has been used to isolate CYP79A1 that converts L-tyrosine into *p*-hydroxyphenylacetaldoxime[86]. This *N*-hydroxylase catalyzes the first committed step in the production of the cyanogenic glucoside dhurrin in *Sorghum bicolor*. Isolated CYP79A1 was catalytically active as demonstrated by its ability to convert tyrosine into *p*-hydroxyphenylacetaldoxime when reconstituted in artificial liposomes in the presence of NADPH-cytochrome P450 oxidoreductase, NADPH, and molecular oxygen[87]. Based on partial amino acid sequencing, the corresponding cDNA sequence was cloned from expression libraries of sorghum seedlings and subsequently used to produce recombinant protein[88, 89] (see Section 5.1.2).

3.6. Homology-Based Cloning

The CYP79A1 cDNA sequence[88] has been used to design degenerate DNA oligonucleotide primer sequences for identification of homologous genes in other cyanogenic crops like *Manihot esculenta* (cassava) using polymerase chain reactions (PCR). Cassava was found to express two P450 isoforms belonging to the CYP79 family. They showed 53% and 54% amino acid sequence identity, respectively, to CYP79A1[90]. Because the sequence identity to the CYP79A1 is below 55%, the two cassava homologues established a new subfamily and were named CYP79D1 and CYP79D2. The two isoforms exhibit 85% sequence identity and the recombinant proteins catalyze the same biochemical reaction (Sections 5.2). A similar PCR strategy served to identify additional CYP79 homologues from *Triglochin maritima* (seaside arrowgrass)[91].

Based on known cinnamate 4-hydroxylase sequences from Jerusalem artichoke and mung bean[54, 55], a homology search in an EST library identified an EST clone with 84–86% sequence identity, which was then used as a probe to isolate the *CYP73A5* from a genomic library[32].

To identify and clone cDNAs encoding inducible P450s involved in the biosynthesis of tetrahydrobenzylisoquinoline alkaloids, a PCR strategy based on the conserved sequence elements in the haem-binding domain of A-type P450s was applied[11, 92]. Based on mRNA isolated from induced, tetrahydrobenzylisoquinoline alkaloid producing plant tissue, 17 different P450 sequences were found. The sequences were compared with existing sequence data and heterologous expression assays based on predicted enzymatic activities that identified two alleles of (*S*)-*N*-methylcoclaurine 3′-hydroxylase[11] (Section 6).

4. Non-A-Type P450s Mediating Steroid Biosynthesis

Like vertebrates and fungi, plants produce polyhydroxylated steroidal hormones to regulate and control tissue morphology. In plants these types of hormones are designated brassinosteroids[93–96] and they are built on a campestanol carbon skeleton (Figure 12.1). The brassinosteroids

are nonessential phytohormones with impact on morphological characteristics, for example, leaf shape and dwarfism[50, 51]. Biological functions of brassinosteroids are controlled by specific receptors and suppressors (see Figure 12.2). These mediate signal transduction and control regulation of target genes including those for brassinosteroid biosynthesis[97, 98]. Brassinosteroids may potentiate plant fitness and defense in response to pathogen attack, since brassinosteroids induce systemic defense responses in tobacco and rice[99].

The biosynthetic pathway for brassinosteroids has not yet been fully elucidated. Models as presented in figure 12.3 for two parallel pathways assigned as "the early C-6 oxidation" and "the late C-6-oxidation" pathways have been suggested[100-102]. Non-A-type plant P450s participate in the production of the plant sterols that are brassinosteroid precursors. A key enzyme is obtusifoliol 14α-demethylase. This belongs to the CYP51 family and gene sequences encoding this ubiquitous plant enzyme that has been obtained from *S. bicolor* (sorghum)[103, 104] and *T. aestivum* (wheat)[105, 106]. Recently, an orthologue of obtusifoliol 14α-demethylase was identified in *A. thaliana* based on its ability to complement a lanosterol 14α-demethylase mutant of yeast[29]. The expression level of another gene *CYP72B1*, also assigned as *BAS1* belonging to the A-type family and encoding a brassinolide 26-hydroxylase, has been shown to regulate light perception and control accumulation of brassinosteroids[31]. Two non-A-type P450 families with known enzymatic activities, CYP90 and CYP85, participating in brassinosteroid biosynthesis are selected as representatives for detailed description (Sections 4.1 and 4.2).

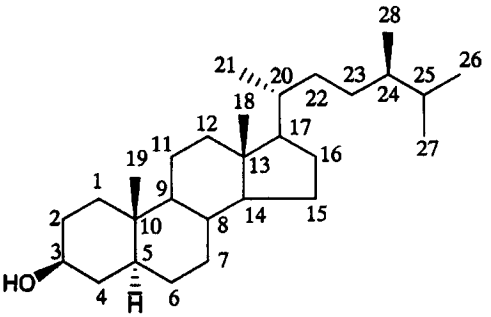

Figure 12.1. Polyhydroxylated steroids in plants indicating the carbon numbers of brassinosteroids as reprinted with permission from Bishop and Yokota (2001)[94].

4.1. CYP90s

CYP90A1, also assigned as *constitutive photomorphogenesis and dwarfism* (cpd) from *A. thaliana* encodes an enzyme in steroid biosynthesis that catalyzes hydroxylation (Figure 12.3) of cathasterone to testarone and of 6-deoxycathasterone to 6-deoxytestarone[50]. CYP90A1 was the first plant P450 identified by reverse genetics using a morphological screen for aberrant growth

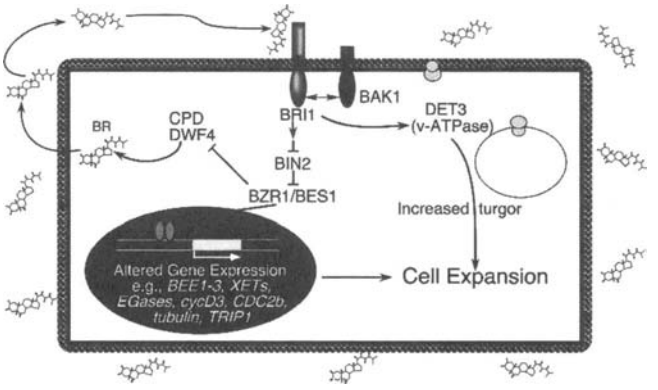

Figure 12.2. A model of the regulatory machinery for brassinosteroid sensing and biosynthesis. Upon perception of brassinosteroids, the receptor BRII signals via a phosphorylation cascade to regulate gene expression and cell expansion. Reprinted with permission from Thummel and Chory (2002)[97].

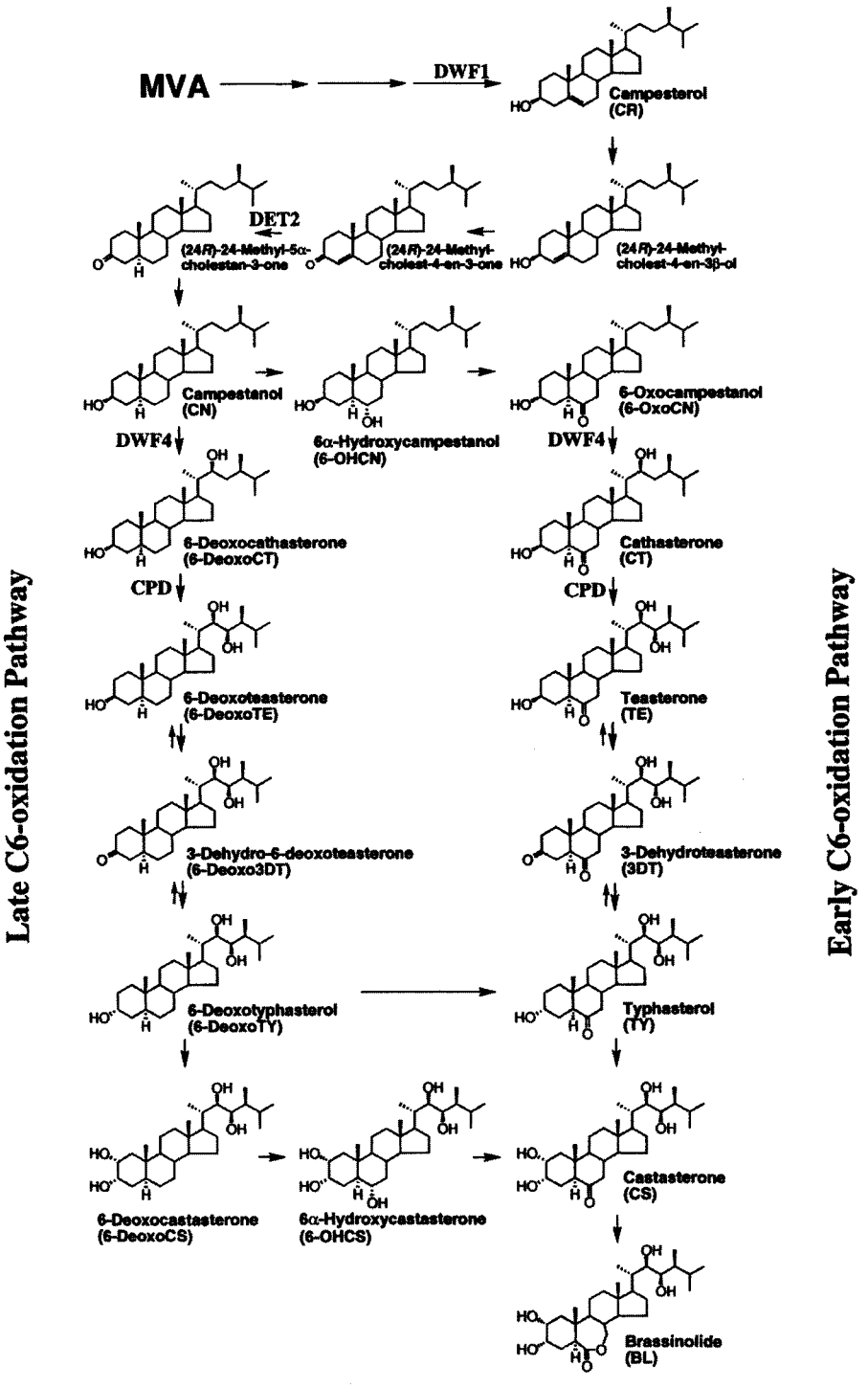

Figure 12.3. The proposed late and the early C-6 oxidation pathways for biosynthesis of brassinolides as outlined and reprinted with permission from Nogushi *et al.* (2000)[102].

characteristics among a collection of T-DNA tagged mutants[69]. CYP90A1 shared 24% amino acid sequence identity to the rat testosterone-16α-hydroxylase, CYP2B1[107]. Transcription of *CYP90A1* is negatively controlled by brassinosteroids[95, 108], most likely as part of a regulatory mechanism to ensure optimal physiological levels of endogenous brassinosteroids during growth (see Figure 12.2). The ability of CYP90A1 to hydroxylate the steroid side chain of both cathasterone and 6-deoxycathasterone illustrates that steps in "the early C-6 oxidation" and "the late C-6-oxidation" pathways may be mediated by the same enzyme. Homologues categorized into the CYP90A family have subsequently been identified in *Saccharum* sp. (sugar cane) and in *Vigna radiata* (mung bean)[20]. CYP90A encoding genes are expected to be ubiquitous in the Plant Kingdom.

A second dwarfed phenotype, *dwarf 4 (dwf4)*, in *A. thaliana* was also characterized using reverse genetics[51]. The mutation affects a 22-α-hydroxylase, assigned as CYP90B1, that hydroxylates the brassinosteroid side chain (Figure 12.3). The enzymatic activity of CYP90B1 provides the substrate of CYP90A1. CYP90B1 shares 40% amino acid sequence identity with CYP90A1 and, like CYP90A1, functions in "the early C-6 oxidation" as well and in "the late C-6-oxidation" pathways[46]. Although the *A. thaliana* CYP90B1 is currently the only member of this subfamily, it is thought to be ubiquitous in plants. Two additional subfamilies CYP90C and CYP90D have been established each containing one gene from *A. thaliana*[20]. The enzymatic activities of these P450s remain to be elucidated.

4.2. CYP85s

The CYP85 family is also involved in brassinosteroid biosynthesis. cDNA sequences encoding enzymes belonging to this non-A-type family has been obtained from *A. thaliana* and *Solanum lycopersicon* (potato). Members of the CYP85 family share approximately 35% identity to those of CYP90[95]. Recombinant versions of the two CYP85 plant genes were expressed in yeast and both enzymes were shown to catalyze multiple steps from 6-deoxoteasterone to teasterone, from 3-dehydro-6-deoxoteasterone to 3-dehydroteasterone, from 6-deoxotyphasterol to typhasterol, and from 6-deoxocastasterone to castasterone[46, 109]. The enzymatic activity of CYP85 enables crosstalk between "the early C-6 oxidation" and "the late C-6-oxidation" pathways for brassinosteroid formation and transforms the two pathways into a metabolic grid (Figure 12.3). Transcriptional activity of the gene is negatively regulated by brassinosteroids[95].

5. A-Type P450s Mediating Plant Protection

Plants need to defend and protect themselves against attack from herbivores and microorganisms. Toward this goal, plants produce a vast array of natural products some of which mediate broad resistance toward herbivores and pests and some of which are highly specific. Accordingly, the ability of plants to produce natural products enhances plant fitness by efficiently counteracting otherwise damaging biotic and abiotic stresses.

5.1. Broad Defense: Cyanogenic Glucosides

Cyanogenesis is the ability of plants to release hydrogen cyanide upon tissue damage. Cyanogenesis is an old trait widely distributed in the Plant Kingdom[78, 110–112] and currently documented in more than 2,650 plant species[113]. Cyanogenesis is mediated by cleavage of cyanogenic glucosides into the corresponding cyanohydrin and glucose by the action of β-glucosidase. Subsequent cleavage of the cyanohydrin into a ketone or aldehyde and hydrogen cyanide proceeds catalyzed by an α-hydroxy-nitrilase or non-enzymatically. Cyanogenic glucosides belong to the class of natural products known as phytoanticipins. They are also present in healthy plant tissues anticipating and ready to combat pathogen attack. Cyanogenic glucosides are present in many important crop plants like barley, sorghum, and cassava[113].

The release of poisonous hydrogen cyanide upon tissue disruption may render the presence of cyanogenic glucosides in a crop plant, a nutritional problem. This is of special concern in

cassava where use of this crop as a staple food requires careful processing to remove the cyanogenic glucosides or their degradation products before consumption[114]. In barley, major focus has been on cyanide potential in malt (5-day-old seedlings) and breeding programs have established genotypes assigned as low, medium, and high producers[115]. Despite domestication and controlled breeding, null-mutants have neither been identified in cassava nor in barley. It has been hypothesized that the ability of humans to remove cyanide by food processing explains why humans have continued to select and use cyanogenic crops as important components in the diet. In the early phases of plant breeding, selection of cyanogenic crops may have afforded protection from herbivore damage and may have helped to prevent theft of the crop[116].

Cyanogenic glucosides are derived from the amino acids L-valine, L-isoleucine, L-leucine, L-phenylalanine, and L-tyrosine and from the nonprotein amino acid cyclopentenyl glycine[78, 110]. Typically, a cyanogenic plant contains only one or two different cyanogenic glucosides. The biosynthetic pathway for cyanogenic glucosides has been elucidated using dhurrin production in *S. bicolor* as a model system. A general scheme for biosynthesis of cyanogenic glucosides involving two membrane-bound P450s and a soluble UDPG-glucosyltransferase was established as described below (see Figure 12.4).

5.1.1. Biosynthesis

Initial studies on the biosynthetic pathway for the L-tyrosine-derived cyanogenic glucoside dhurrin demonstrated that the covalent bond linking the α-and β-carbon atoms in L-tyrosine was preserved throughout dhurrin synthesis[117]. Subsequently, it was shown that the C–N bond in the parent amino acid is preserved during the biosynthetic process[118]. These studies were carried out by administration of double-labeled tyrosine to excised, biosynthetically active sorghum seedlings. Based on these observations, a biosynthetic pathway including an aldoxime, a nitrile, and an α-hydroxynitrile as intermediates was proposed, although no such compounds were detectable[119, 120]. A major breakthrough in the elucidation of the dhurrin pathway was based on the

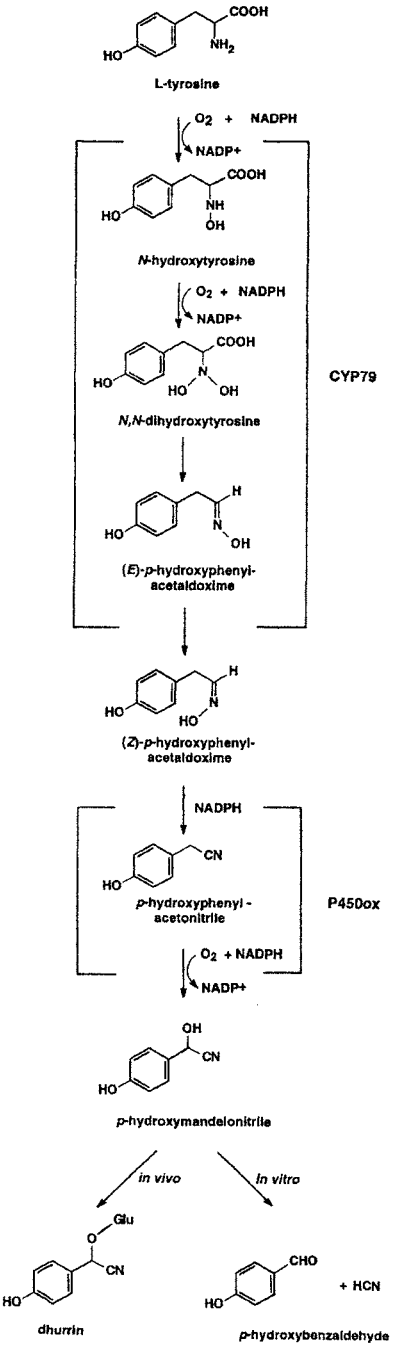

Figure 12.4. The biosynthetic pathway for the cyanogenic glucoside dhurrin is catalyzed by two multifunctional cytochrome P450s, CYP79A1, and CYP71E1 (P450ox) and by a glucosyltransferase, UGT85B1.

isolation of a biosynthetically active microsomal system. Upon administration of NADPH, molecular oxygen, and radiolabeled L-tyrosine to this experimental system in the presence of a large surplus of unlabeled putative intermediates, it was possible to trap radiolabeled *N*-hydroxytyrosine, (*E*)-*p*-hydroxyphenylacetaldoxime, (*Z*)-*p*-hydroxyphenylacetaldoxime, and *p*-hydroxymandelonitrile[121, 122].

The enzymes responsible for cyanogenic glucoside synthesis have been characterized using the sorghum microsomal system as biological starting material[86, 123]. The conversion of the parent amino acid L-tyrosine into the corresponding (*Z*)-aldoxime is catalyzed by CYP79A1 (Figure 12.4). This multifunctional P450 monooxygenase constitutes the first identified member of the CYP79 family and catalyzes two consecutive *N*-hydroxylations, a decarboxylation and a dehydration reaction. Isolated CYP79A1 was successfully reconstituted into artificial liposomes also containing isolated NADPH cytochrome P450 oxidoreductase[87]. Administration of radiolabeled tyrosine to this reconstituted enzyme system in the presence of putative intermediates as unlabeled compounds permitted identification of *N*-hydroxytyrosine, *N,N*-dihydrotyrosine, and (*E*)-*p*-hydroxyphenylacetaldoxime as intermediates in the conversion of tyrosine to the (*Z*)-aldoxime. From stoichiometric analyses, it was shown that two molecules of oxygen are consumed in this conversion. Enzyme assays carried out in an $^{18}O_2$ atmosphere using either tyrosine or *N*-hydroxytyrosine as substrates demonstrated that the two oxygen atoms introduced in the *N*-hydroxylation steps are enzymatically distinguishable as demonstrated by specific loss of the oxygen atom introduced by the first *N*-hydroxylation reaction in the subsequent conversion of *N,N*-dihydroxytyrosine into the (*Z*)-aldoxime[78, 112, 124]. This demonstrates that the intermediate *N,N*-dihydroxytyrosine is bound to the active site of CYP79A1 in a manner that prevents free rotation around the C–N single bond.

The further conversion of the (*Z*)-aldoxime into the cyanohydrin was demonstrated to also be mediated by a multifunctional P450 using the microsomal system isolated from sorghum as the biological starting material. This P450 was assigned CYP71E1 as the first member of the CYP71E subfamily. CYP71E1 catalyzes an unusual dehydration of an oxime to the corresponding nitrile, which subsequently is *C*-hydroxylated to the cyanohydrin (Figure 12.4)[123]. The nitrile intermediate in the CYP71E1 catalyzed reaction was demonstrated using trapping experiments[123, 125]. A single oxygen molecule is consumed in the CYP71E1 catalyzed reaction sequence[126, 127].

The last step in cyanogenic glucoside synthesis involves conversion of a cyanohydrin into the corresponding cyanogenic glucoside. Using dye-column affinity chromatography, a soluble UDP-glucose:*p*-hydroxymandelonitrile-*O*-glucosyltransferase, designated UGT85B1[128], was isolated from etiolated sorghum seedlings and shown to glucosylate the cyanohydrin function of *p*-hydroxymandelonitrile to produce dhurrin (Figure 12.4). Reconstitution of CYP79A1 and CYP71E1 into artificial liposomes in the presence of UGT85B1 resulted in the formation of dhurrin, that is, in reconstitution of the entire pathway for dhurrin production from its parent amino acid tyrosine[128] (Figure 12.5).

cDNA sequences encoding CYP79A1, CYP71E1, and UGT85B1 have been isolated[88, 125, 128] and functionally active proteins were obtained by heterologous expression of each of the cDNA clones in *E. coli*. The entire pathway for dhurrin synthesis has been transferred to *A. thaliana*[129], a plant species that in nature does not possess the ability to produce cyanogenic glucosides. Sequential introduction of each of the three enzymes into *A. thaliana* demonstrated that dhurrin is produced only after coordinated expression of all three sorghum genes[129]. Importantly, expression of UGT85B1 proved obligatory despite the availability in the *A. thaliana* genome of 120 family 1 glycosyl transferase genes[21, 130]. In transgenic plants co-expressing CYP79A1 and CYP71E1[131], *p*-hydroxymandelonitrile is the final product produced by the enzymes introduced. In such transgenic plants, *p*-hydroxymandelonitrile is metabolized by endogenous enzymes into a large number of different products. This is in sharp contrast to the results obtained when CYP79A1 and CYP71E1 are expressed together with UGT85B1, in which case only dhurrin formation is observed[129]. The transgenic dhurrin-producing *A. thaliana* plants showed improved resistance against the flea beetle *Phyllotreta nemorum*, which is a crucifer specialist[129].

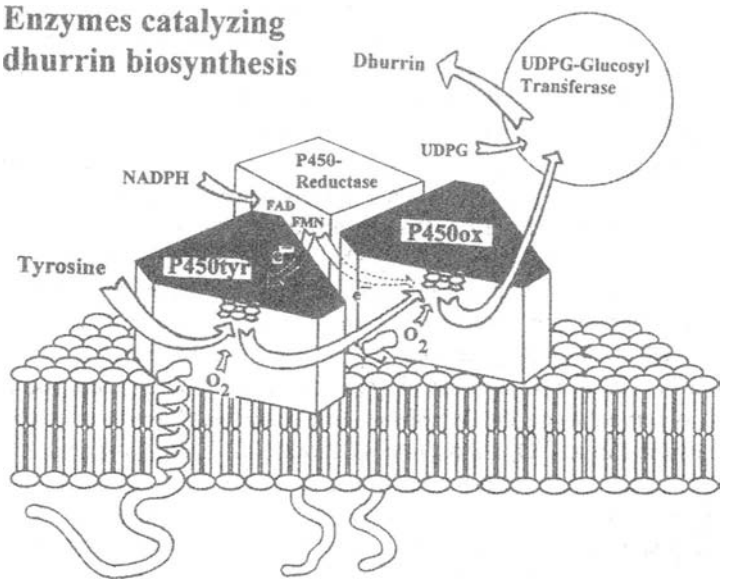

Figure 12.5. A model for metabolon formation of the three biosynthetic enzymes CYP79A1 (P450Tyr), CYP71E1 (P450ox), and UGT85B1 (glucosyltransferase) at the cytosolic surface of endoplasmic reticulum. Modified after Nelson and Strobel (1988).

5.1.2. Substrate Channeling and Metabolon Formation

Administration of radiolabeled tyrosine to etiolated sorghum seedlings resulted in a 49% incorporation into dhurrin, but surprisingly no radio labeled intermediates involved in this conversion were detectable[119, 120]. Biosynthetic studies using highly active microsomal enzyme preparations demonstrated efficient channeling of the intermediates in the pathway and provided an explanation as to why no intermediates accumulate[122] (Section 5.1.1). Likewise, biosynthetic studies with recombinant CYP79A1 and CYP71E1 reconstituted with NADPH cytochrome P450 oxidoreductase (ATR2) in artificial liposomes demonstrated efficient flux through the pathway with barely detectable levels of intermediates accumulating. Upon inclusion of cytosolic sorghum extracts or heterologously expressed UGT85B1 in the assays, almost complete stereospecific glycosylation of *p*-hydroxymandelonitrile into dhurrin was observed[123, 128]. These different sets of data suggest that the combined presence of CYP79A1, CYP71E1, and UGT85B1 results in the formation of an active metabolon (Figure 12.5).

The possible organization of the enzymes catalyzing a specific biosynthetic pathway into multi-enzyme complexes, also denoted metabolons, has for many years been a point of discussion in plant biology. The existence of metabolons in plants becomes increasingly apparent[132], for example, in the biosynthesis of cyanogenic glucosides[122], phenylpropanoid, and flavonoid pathways[133–135]. Metabolon formation may serve to overcome kinetic constraints, for example, by mediating a considerable local increase in substrate availability and concentration and secure that labile and/or toxic intermediates are swiftly converted into more stable and less toxic constituents. Evolution of a metabolon for dhurrin synthesis would appear essential to ensure rapid conversion of the toxic *p*-hydroxymandelonitrile intermediate by UGT85B1 to prevent its dissociation into hydrogen cyanide and aldehyde at the same time as gaining efficacy in dhurrin production. To demonstrate metabolon formation and to identify the subcellular compartment into which the metabolon accumulates, expression plasmids harboring DNA sequences encoding fusion proteins between the biosynthetic enzymes and spectral variants of green fluorescent protein (GFP)[136, 137]

were designed. Fusion proteins in which each of the three enzymes, CYP79A1, CYP71E1, and UGT85B1, were *C*-terminally linked to either cyano fluorescent protein (CFP) or yellow fluorescent protein (YFP) were functionally active when heterologously expressed in *E. coli* or *A. thaliana*. Dhurrin-producing *A. thaliana* plants were obtained by simultaneous expression of CYP79A1, CYP71E1-CFP, and UGT85B1-YFP, but not by simultaneous expression of CYP79A1-YFP, CYP71E1-CFP, and UGT85B1. This indicates prevention of proper interaction between CYP79A1 and CYP71E1 when both are fused to fluorescent protein in spite of a retained functionality of each separate P450 fusion. Examination of the transgenic plants by confocal laser scanning microscopy (CLSM) demonstrated that a metabolon visualized by UGT85B1-YFP is indeed formed after coordinated expression of the three biosynthetic genes. The metabolon located in distinct domains at the cytosolic surface of the endoplasmic reticulum appressed against the plasma membrane at the periphery of biosynthetically active cells (Figure 12.6A, B, see color insert). When UGT85B1-YFP was expressed alone, it showed an even cytosolic distribution (Figure 12.6C, see color insert).

5.1.3. Substrate Specificities

The type of cyanogenic glucoside present in a given plant species is defined by the substrate specificity of the enzyme catalyzing the first committed step in the pathway. This conclusion was reached from investigations of the amino acid specificity of active microsomal systems from sorghum that is specific to L-tyrosine, the precursor of dhurrin[86], seaside arrowgrass showing specificity to L-tyrosine, the precursor of taxiphyllin[138, 139], cassava, flax, and white clover, which are all specific to L-valine and L-isoleucine, the precursors of linamarin and lotaustralin[140–145], and barley with specificity to L-leucine, the precursor of epiheterodendrin[146]. These same specificities are also observed in *in vitro* assays using recombinant protein from sorghum, cassava, and seaside arrowgrass[90, 91, 123].

The enzymes catalyzing the subsequent steps in cyanogenic glucoside synthesis, that is, the conversion of oximes into cyanohydrins are not nearly as substrate specific. Again this knowledge was obtained from studies of microsomal preparations. The broadest substrate specificity is observed with the cassava microsomal preparation that is able to metabolize oximes derived from L-valine, L-isoleucine, L-phenylalalnine, L-tyrosine as well as from cyclopentenylglycine[142]. Sorghum microsomal preparations are able to metabolize oximes derived from L-tyrosine and L-phenylalanine[127]. Barley contains five different L-leucine-derived cyanoglucosides of which only one is cyanogenic. These are thought to be formed by the action of a single P450 that is able to hydroxylate all individual carbon atoms of the nitrile intermediate and to facilitate multiple hydroxylations as well as dehydrations (Figure 12.7)[146]. So far, the only P450 known to catalyze this set of reactions is CYP71E1 isolated from sorghum.

5.2. Functional Uniformity within the CYP79 Family

To date the CYP79 family consists of six subfamilies denoted CYP79A, -B, -C, -D, -E, and -F[20]. Currently, the CYP79A subfamily has eight members covering four plant species of which sorghum, *T. aestivum* (wheat) and *H. vulgare* (barley) belong to the *Poacea*[20]. The fourth plant species is Arabidopsis that does not contain cyanogenic glucosides. Instead, Arabidopsis is able to synthesize glucosinolates, a closely related group of natural products[9, 147]. The amino acid sequence identity between CYP79A1 from sorghum and CYP79A2 from Arabidopsis is 53%, slightly below the 55%[18, 20, 22, 26] criterion usually required to assign P450s to the same subfamily. Whereas the precise catalytic properties of the CYP79C subfamily remain to be established, all other members of the CYP79 family have been shown to catalyze the conversion of an amino acid to the corresponding oxime. Subfamilies CYP79A, -D, and -Es are involved in cyanogenic glucoside synthesis whereas the subfamilies CYP79A, -B, and -F are involved in glucosinolate synthesis[9]. Introduction of the sorghum CYP79A1 gene into *A. thaliana* by genetic engineering resulted in the production of large amounts of the tyrosine-derived glucosinolate *p*-hydroxyglucosinolate[148]. This illustrates that the oxime produced by the "cyanogenic" CYP79A1 serves as an efficient substrate for the endogenous *A. thaliana* downstream biosynthetic enzymes mediating

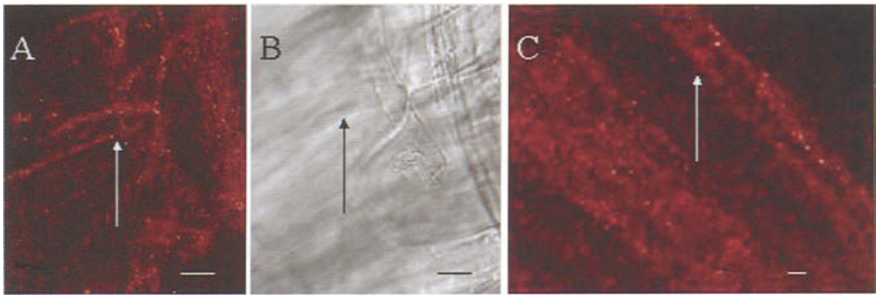

Figure 12.6. Confocal laser scanning microscopy of *A. thaliana* roots. (A) YFP fluorescence monitored using a color code gradient ranging from black over red to orange to illustrate increased fluorescence intensities. The arrow indicates the confined fluorescence at the periphery of cells co-expressing CYP79, CYP71, UGT85B1-YFP. (B) Transmitted light image to visualize the cell shape. Arrow as in (A). (C) YFP fluorescence in cells expressing UGT85B1-YFP shows even cytosolic distribution and high accumulation in and around the nucleus (arrow). Bar = 5 μm. According to Tattersall *et al.*, unpublished.

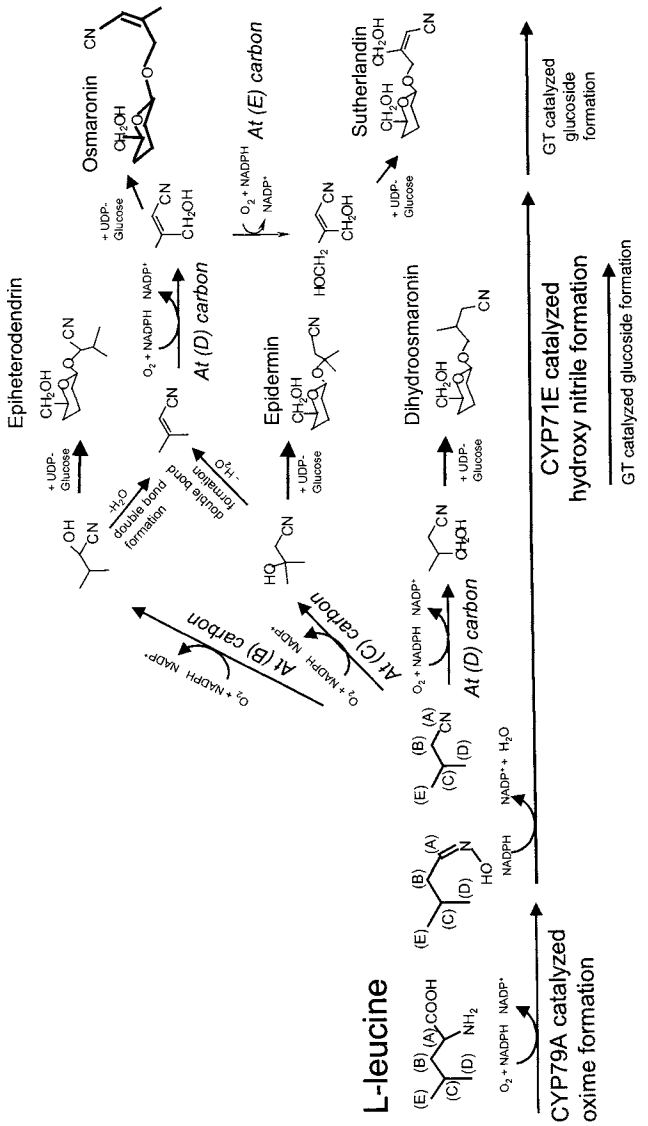

Figure 12.7. Proposed biosynthetic pathway for the different Leu-derived cyanoglucosides in barley. Reprinted with permission from Nielsen *et al.* (2002)[146].

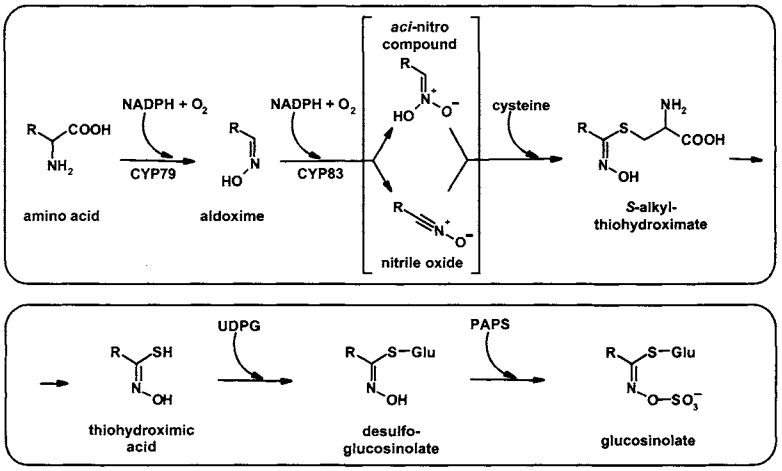

Figure 12.8. The biosynthetic pathway for glucosinolate production. Reprinted with permission from Wittstock and Halkier (2002)[9].

glucosinolate formation. Most likely, this involves formation of a metabolon as demonstrated in sorghum (Section 5.1.2). An Arabidopsis double mutant knocked out in both CY79B2 and CYP79B3 completely lack indole-derived glucosinolates, but show subtle morphological mutant phenotype. The subsequent conversion of oximes to glucosinolates is catalyzed by members of the CYP83 family (Section 7; Figure 12.8).

5.3. Functional Diversity among CYP71s

In contrast to the CYP79 family, the CYP71 family is functionally diverse and constitutes the largest A-type plant P450 family with a total of 110 members divided into 18 subfamilies.

5.3.1. CYP71A and CYP71B Subfamilies

The CYP71A subfamily contains 28 members including 17 annotations from the *A. thaliana* genome. The first member of this subfamily was derived from avocado[81]. No specific enzymatic activity has been demonstrated for the members of the CYP71A subfamily. CYP71A10 from *Glycine max* (soybean) catalyzes conversion of the phenylurea herbicides, fluometuron, linuron, chlortoluron, and diuron into more polar compounds[65]. This is unlikely to be the *in planta* biological function of the enzyme and surely does not explain the apparent evolutionary need for maintenance of 17 isoforms in the *A. thaliana* genome[21]. The CYP71B family is very large and composed of 36 members all annotated from the *A. thaliana* genome. The subfamily was first established based on a sequence with unknown biological function from *Thlaspi arvense* (field penny-cress), which like *A. thaliana* belongs to the *Brassicacea*[92].

5.3.2. CYP71C Subfamily: Grass-Specific Defense Compounds

The CYP71C subfamily is comprised of a total of 23 members with 11 from *Zea mays* (corn), 11 from *Trititum aestivum* (wheat), and a single member from *H. vulgare* (barley), all belonging to *Poacea*. The CYP71C subfamily possesses some very special enzymatic features related to the fact that together different members of this subfamily are able to mediate the synthesis of the grass-specific phytoalexin 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA)[149]. Each of the 23 members catalyzes one of four consecutive enzyme reactions

in the DIBOA pathway (Figure 12.9). Thus, coordinated enzymatic activities of CYP71C1, CYP71C2, CYP71C3v1, and CYP71C4 from maize mediate the production of DIBOA that is further metabolized to yield the cyclic hydroxamic acid 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA)[150].

Biosynthetic experiments using maize seedling and radiolabeled [3-^{13}C]-indole as precursor demonstrated that CYP71C4, CYP71C2, CYP71C1, and CYP71C3 catalyze the consecutive conversions into [3-^{13}C]-indolin-2-one, [3-^{13}C]-hydroxyindonin-2-one, 2-hydroxy-1, 4-benzoxazin-3-one (HBOA), and DIBOA, respectively (Figure 12.9). An additional hydroxylation at the C-7 position followed by C-7 specific methylation gave rise to the formation of DIMBOA. The C-7 hydroxylating enzyme was obtained by screening a maize EST collection in combination with a reverse genetics approach that revealed C-7 hydroxylation of DIBOA forming 2,4,7-trihydroxy-2H-1,4-benzoxazin-3(4H)-one (TRIBOA) by a 2-oxoglutarate-dependent dioxygenase[151] (Figure 12.10). The high sequence identity among CYP71C4, CYP71C2, CYP71C1, and CYP71C3 does not compromise substrate specificity as demonstrated by determining the catalytic activities of the recombinant proteins expressed in yeast[152].

From an evolutionary perspective, it is interesting that the phylogenetically closely related genes Bx2 (encoding CYP71C4), Bx3 (encoding CYP71C2), Bx4 (encoding CYP71C1), and Bx5 (encoding CYP71C3) co-locate to the short arm on chromosome 4 in the maize genome and to chromosome 5 on wheat genomes[153]. A fifth gene,

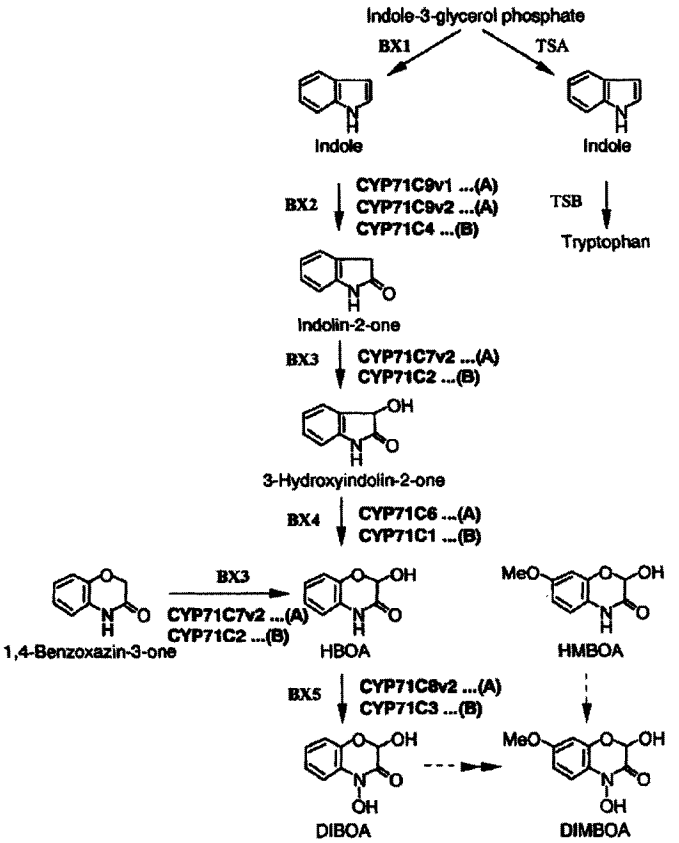

Figure 12.9. The biosynthetic pathway for DIMBOA. Bx1–Bx5 are gene names encoding the corresponding CYP71Cs as indicated in (A) wheat and (B) maize. Reprinted with permission from Nomura *et al.* (2002)[153].

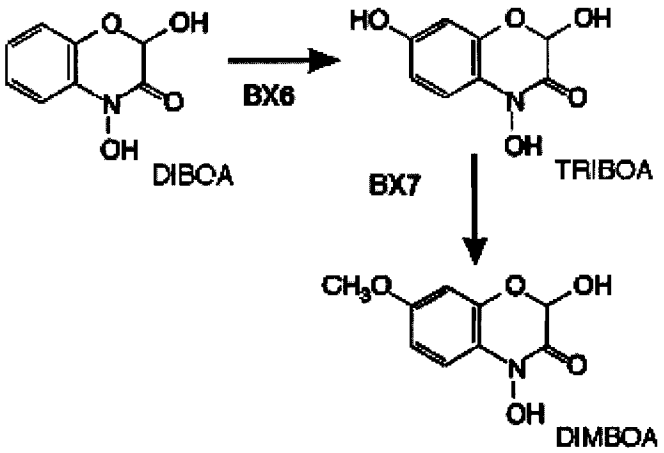

Figure 12.10. The 2-oxoglutarate-dependent dioxygenase Bx6 catalyzes hydroxylation of DIMBOA to produce TRIBOA. Reprinted with permission from Frey *et al.* (2003)[151].

Bx6, encoding the oxoglutarate-dependent dioxygenase clusters with the CYP71Cs at the short arm of chromosome 4. In maize, DIMBOA confers resistance to herbivores like *Ostrinia nubilalis* (European corn borer) and *Rhophalosiphum maydis* (maize plant aphid) and to the fungal pathogen *Helminthosporium turcicum* (Northern corn blight). The DIMBOA pathway may exemplify an evolutionary recent recruitment of new biological activities of P450s. The substrate for DIMBOA synthesis, indole or indole-3-glycerol phosphate is suggested to derive from a branch point in L-tryptophan synthesis. A sixth gene *Bx1* encoding a tryptophan synthase homologue is situated together with the cluster of DIMBOA genes on chromosome 4 in maize and was shown to be essential for DIMBOA production[149]. A homologue of this gene was activated by a herbivore elicitor, thus strengthening the suggestion of an introduction of a branch point in L-tryptophan biosynthesis for DIMBOA production in response to herbivore attack[154]. Transcription of the maize genes encoding CYP71C1 (Bx4) and CYP71C3 (Bx5) are induced in response to the maize bacterial pathogen *Acidovorax avenae* and in response to wounding[155]. No CYP71C homologues are identified in the Arabidopsis genome. However, the structure of DIMBOA is sufficiently close to the indole-derived phytoalexin camalexin that is produced by *A. thaliana* to allow speculations on a tight functional relationship between CYP71Cs and Arabidopsis P450 candidates[154]. In support of this working hypothesis, Zhou *et al.* (1999)[156] have published that a *pad3 A. thaliana* mutant unable to accumulate camalexin is defective in a putative P450 monooxygenase gene, annotated as CYP71B15[18, 20].

5.3.3. CYP71D, -F, and -R Subfamilies

CYP71D subfamily is also large and currently comprises a total of 22 members from 10 different plant species. At present, the catalytic properties of five CYP71D enzymes have been determined and the enzymes assigned to specific steps in indole alkaloid, sequiterpenoid, cyclic terpenoid, and flavonoid synthesis. Accordingly, enzymes belonging to the CYP71D subfamily do not necessarily share similar functional characteristics.

The first member to be functionally characterized was CYP71D12 from *Catharanthus rosea* (Madagascar periwinkle). CYP71D12 was identified as the tabersonine 16-hydoxylase enzyme involved in the biosynthetic pathway for the two medically important bisindole alkaloids vinblastine and vincristine[157] (Figure 12.11). Microsomal preparations from etiolated seedlings of Madagascar periwinkle were shown to be low in tabersonine 16-hydroxylase activity in comparison to light grown seedling. Interestingly, the light regulation was retained in suspension cultures of Madagascar periwinkle. A cDNA clone encoding tabersonine

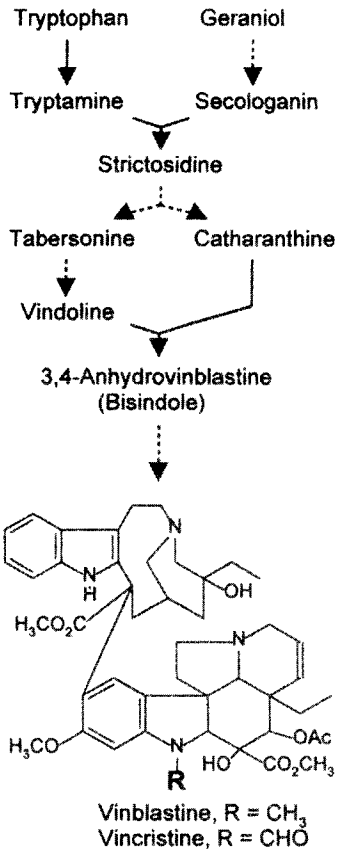

Figure 12.11. The biosynthetic pathway for the bisindole alkaloids vinblastine and vincristine. Reprinted with permission from Schroeder *et al.* (1999)[157].

16-hydroxylase was isolated from a cDNA library prepared from light-induced cells using degenerate oligodeoxynucleotide primers and verified by heterologous expression in *E. coli*. The other enzymes involved in the conversion of tabersonine to vindoline may also be light induced and this may provide a route for their isolation and cloning and for the production of vinblastine and vincristine by expression of the entire pathway from the precursors tryptamine and secologanin in cell cultures. A transcriptional regulator *O*ctadecanoid-derivative *r*esponsive *Catharanthus A*P2-domain protein (ORCA3) activates the expression of genes mediating L-tryptophan and tryptamine production as well of several genes in the synthesis of vindoline from tryptamine and secologanin[158]. A cytochrome P450, CYP72A1, was shown to convert loganin into serologanin (Figure 12.11)[159]. Activation of the ORCA3 gene is regulated by methyl jasmonate. This plant hormone is produced in response to stress and wounding[160] thereby enabling synthesis of the bisindole alkaloids as response to herbivore attack.

A second functionally identified member of the CYP71D subfamily is CYP71D20[161]. This enzyme from tobacco mediates production of the sesquiterpene capsidiol, an antimicrobial compound. The enzyme catalyzes hydroxylations of 5-epi-aristone as well as of 1-deoxy-capsidiol to capsidiol[161]. The functional and mechanistic features of CYP71D20 were determined in a coupled assay using substrate production by sesquiterpene synthases and a microsomal system[162]. CYP71D20 was found to catalyze unique stereo- and regiospecific hydroxylations first at carbon atom-1 followed by rotation of the molecule in the active site and a second hydroxylation at carbon atom-3 of the bicyclic sesquiterpene hydrocarbon skeleton. The CYP71D20 gene is induced in response to fungal elicitors like paraciticein[163].

The third functionally characterized member of the CYP71D subfamily is CYP71D9. This enzyme has been identified in soybean as a flavonoid 6-hydroxylase. It was demonstrated that hydroxylation of carbon atom-6 of the A-ring precedes 1,2-aryl migration to produce isoflavonoids as described in Section 5.4[164].

Regiospecific hydroxylation of the monoterpene (−)-4*S*-limonene at the C-3 or C-6-allylic positions to yield (−)-menthol (peppermint) or (−)-carvone (spearmint), respectively, is accomplished by the last two functionally characterized CYP71Ds, the CYP71D13 and CYP79D18 found in commercial mint species (*Mentha* sp.)[165–167].

5.4. Specialized Defense—Isoflavonoids in Legumes

Plant isoflavonoids possess a wide range of biological activities. They are efficient antimicrobial agents, inducers of the nodulation genes of symbiotic *Rhizobium* bacteria and phytoestrogens that work through the human estrogen receptor causing alterations in serum lipids and bone metabolism[2, 168]. Isoflavonoids are produced almost exclusively in the Leguminosae in the order Fabales. Isoflavonoids are produced from L-phenylalanine that condenses with 4-coumaroyl

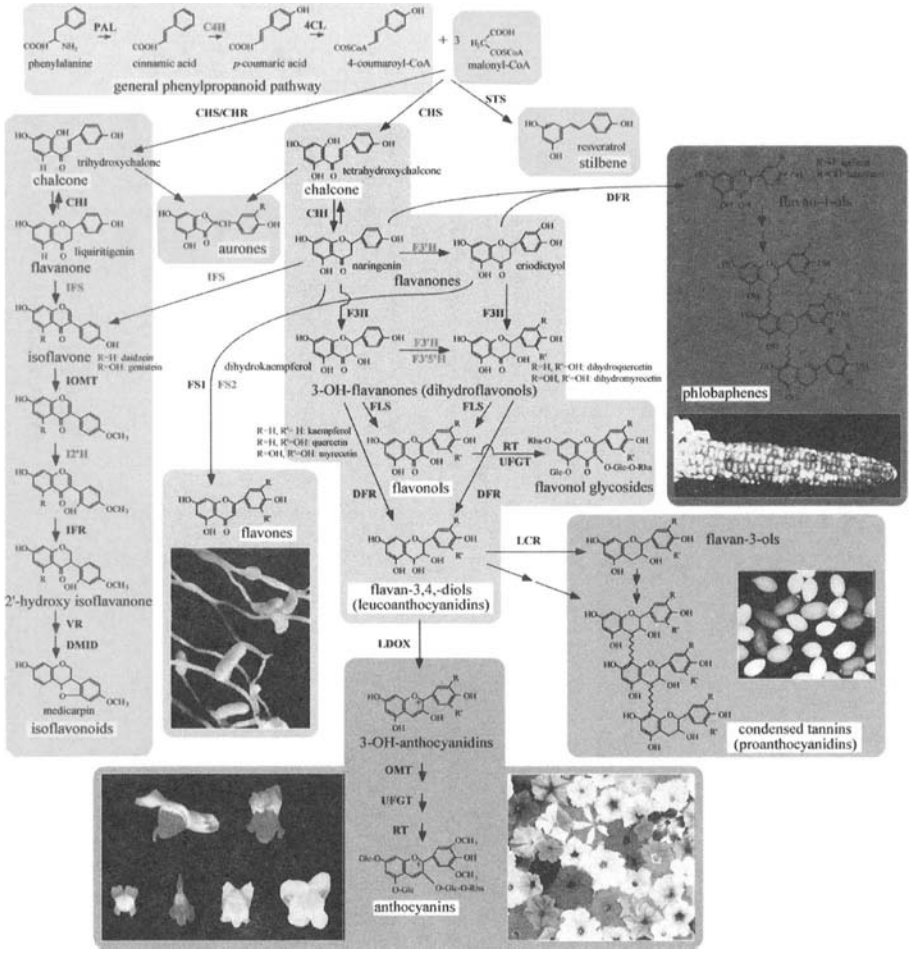

Figure 12.12. Biosynthetic pathways of flavonoid compounds from phenylalanine into isoflavonoids, flavones, flavonols, anthocyanidins, leucoanthocyanidins, proanthocyanidins, and phlobaphenes. The figure was kindly provided by Winkel-Shirley, B. (2003) as modified from Winkel-Shirley (2001). Reprinted with permission from Winkel-Shirley B., Grotewold E., Martins C., Hirsch A. M., and Quattrocchio F.

CoA and three molecules of malonyl CoA to produce chalcone and subsequently the flavanones naringenin and liquiritigenin (Figure 12.12). The synthesis of isoflavonoids from these flavanones is mediated by a CYP93C that catalyzes the migration of the B-ring to the 3-position followed by hydroxylation at the 2-position. The CYP93Cs are therefore termed 2-hydroxy-isoflavone synthases[15, 169]. CYP93C genes have been cloned from *G. max* (soybean; CYP93C1v2)[15], *Glycyrrhiza echinata* (licorice; CYP93C2)[170], and several other legumes: *Trifolium pratense* (red clover), *Trifolium repens* (white clover), mung bean, *M. sativa* (alfalfa), *Lens culinaris* Medik. (lentil), *Pisum sativum* L (snow pea), *Vicia villosa* (hairy vetch), and *Lupinus* spp. Lupin[171]. CYP93C enzymes catalyze the first committed step in the isoflavonoid pathway. Insertion of CYP93Cs into *A. thaliana* by genetic engineering enabled production of low levels of genistein in this non-leguminous plant[169, 171, 172]. Increased expression of CYP93Cv2 did not add to production[169]. When CYP93Cv2 was expressed in the tt3, tt6 double-mutant[173–175] that is blocked with respect to flavonol synthesis (see Figure 12.12), the genistein content was increased 3-fold[169]. Accordingly, competition for common substrates is an important parameter to consider in optimizing the production of desired natural products[169].

The production of the two isoflavonoids daidzein and genistein is highly induced by pathogen attack. Elicitation by crude polysaccharide preparations from yeast cell wall was used to facilitate biosynthetic studies in alfalfa cell suspension cultures[176]. A signal pathway dependent on endogenously generated nitric oxide is also responsible for the induction of daidzein and genistein synthesis[177]. Nitric oxide is generated from L-arginine by nitric oxide synthases (NOS). Although NOS belongs to the class of heme-thiolate proteins, the crystal structure of NOS clearly demonstrates that they belong to a different class of heme proteins as the P450 superfamily[178] enzymes. In alfalfa, improved protection against fungal pathogens is achieved by 4′-*O*-methylation of daidzein into formononetin followed by a number of unidentified hydroxylation steps to yield the highly antifungal phytoalexin, medicarpin (Figure 12.12)[176]. The 4′-*O*-methylation reaction has been studied in detail. Intricate physical interaction between the CYP93C isoflavonoid synthase and an isoflavone-*O*-methyltransferase, designated IOMT8 was suggested to guide 4′-*O*-methylation and to prevent 7′-*O*-methylation in spite of the fact that *in vitro* IOMT8 was found to catalyze 7′-*O*-methylation[176]. However, this intricate reaction mechanism has recently been challenged by the cloning and functional characterization of 2,7,4′-trihydroxyisoflavanone 4′-*O*-methyltransferases from *G. echinata* (licorice) and *Lotus japonicus* (Bird's foot trefoil) that exhibit high affinity for 4-*O*-methylation of daidzein[179].

6. P450 Mediated Production of Alkaloids with Medicinal Importance

In previous parts of this review, natural products with interesting medicinal uses have been mentioned like the bisindoles and isoflavonoids[14, 157]. In this context, a number of other alkaloids are important. The tetrahydrobenzylisoquinoline alkaloid berberine constitutes the first complex alkaloid for which the enzymes catalyzing the entire biosynthetic pathway from the primary precursor L-tyrosine have been identified. Biosynthesis of tetrahydrobenzylisoquinoline alkaloids involves a number of P450s with high substrate specificity and catalysing stereo- and regiospecific oxidations[180]. (*S*)-*N*-methylcoclaurine 3′-hydroxylase assigned as CYP80B1[11] catalyzes the conversion of (*S*)-*N*-methylcoclaurine to (*S*)-3′-hydroxy-*N*-methylcoclaurine, which by methylation is transformed into (*S*)-reticuline, which represents the branch point for formation of a vast number of different tetrahydroisoquinoline alkaloids including the berberine-, phenan-threne- and benzo[*c*]phenanthridine-type alkaloids (Figure 12.13).

The conversion of (*S*)-reticuline into berberine includes two remarkable P450 enzymes. The first is the unique berberine bridge enzyme (BBE) that catalyzes the introduction of a new C–C bond in its product, (*S*)-scoulerine[13, 180] (Figure 12.13). The second enzyme has been designated as canadine synthase and introduces a methylene dioxy-bridge[181]. The enzymatic mechanism for methy-lene dioxy-bridge formation[180] is outlined in Figure 12.14. Synthesis of the phenanthrine-type alkaloid morphine from (*S*)-reticuline via (*R*)-reticuline demands the involvement of three NADPH-dependent reductases

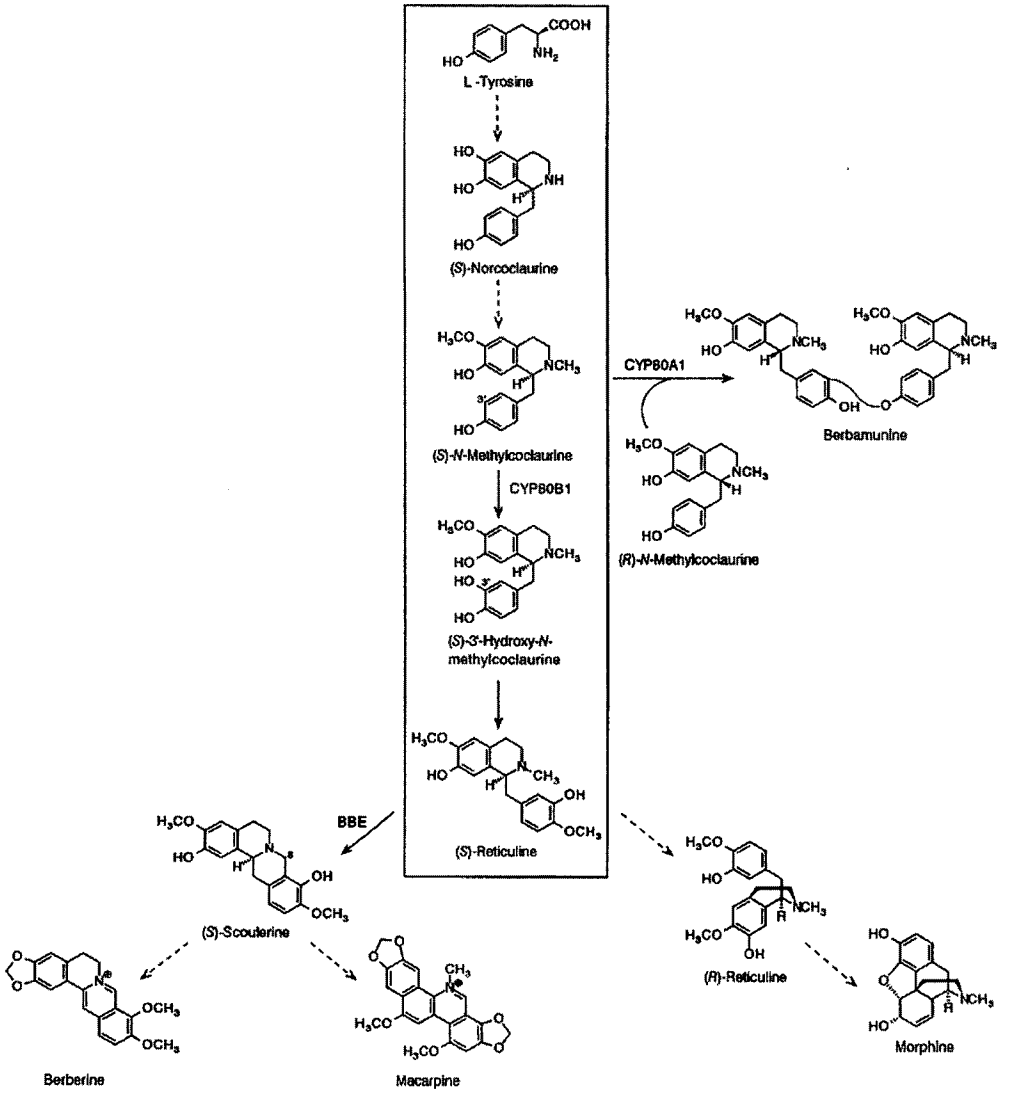

Figure 12.13. The biosynthetic pathway for L-tyrosine derived tetrahydrobenzylisoquinoline alkaloids. The core production of (*S*)-reticuline and the branch points for berberine, macarpine, and morphine production. Reprinted with permission from Chou and Kutchan (1998)[180].

and the P450 enzyme salutaridine synthase[180] that like the BBE introduces a new C–C bond in the product (Figure 12.15). So far the corresponding gene of only one of the reductases has been cloned[12]. Synthesis of the antimicrobial benzo[*c*]phenanthridine-type alkaloid macarpine (Figure 12.13) from (*S*)-reticuline involves the action of six P450s that have been studied in plant cell cultures[182–184]. New C–C bond formation and methylene dioxy-bridge formations are key catalytic features of the conversion[180].

The CYP80 family involved in the production of (*S*)-reticuline in the opium poppy is not at all represented in the Arabidopsis genome. This reflects the fact that the production of a specific alkaloid typically is restricted to a particular plant species or to a limited number of species within a family. Accordingly, it is normally not possible to

study such pathways in genetically well-characterized model plants. This greatly complicates elucidation of the biosynthetic pathways involved in alkaloid formation. Furthermore, alkaloids may accumulate very slowly over a period of months to years and in a highly tissue-specific manner. The establishment of cell cultures have helped to overcome some of these experimental difficulties[185]. Availability of native alkaloid producing plants as sources for isolation of important medicinal drugs remain of high importance because controlled production in, for example, transgenic *A. thaliana* is dependent on the availability of the genes encoding the entire pathway and hampered by technical problems in the co-expression of a multitude of heterologous genes.[186]

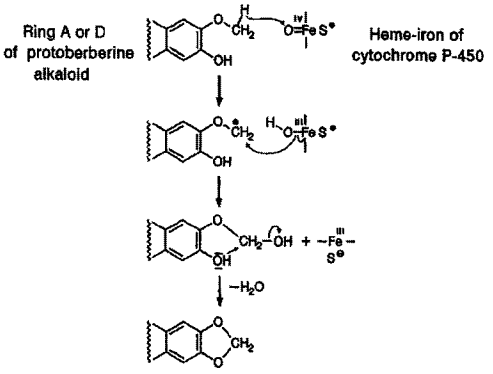

Figure 12.14. A proposed mechanism of methylene dioxy-bridge formation. Reprinted with permission from Chou and Kutchan (1998)[180].

7. Future Prospects: Crosstalk and Metabolic Engineering

The multigene family of plant P450s represents a very rich source for metabolic engineering. The A-type P450s involved in the synthesis of low molecular mass natural products is a key target because many of these compounds are of high value either as fine chemicals or as plant constituents that provide desired agronomical traits such as insect or fungal resistance. In all cases, the P450 enzymes catalyzing the first committed step in the different pathways leading to the production of natural products appear to exert a very high degree of substrate specificity. The successful transfer of the entire pathway for dhurrin formation from sorghum to *A. thaliana*[129] demonstrates that metabolon formation may be achieved also after heterologous expression of a biosynthetic pathway in a plant species that would not in nature produce the same type of natural products. Insertion of an incomplete pathway was shown to favor crosstalk with other metabolic pathways and the formation of side products[129]. When separately introduced into *A. thaliana*, sorghum CYP79A1 was able to establish highly efficient

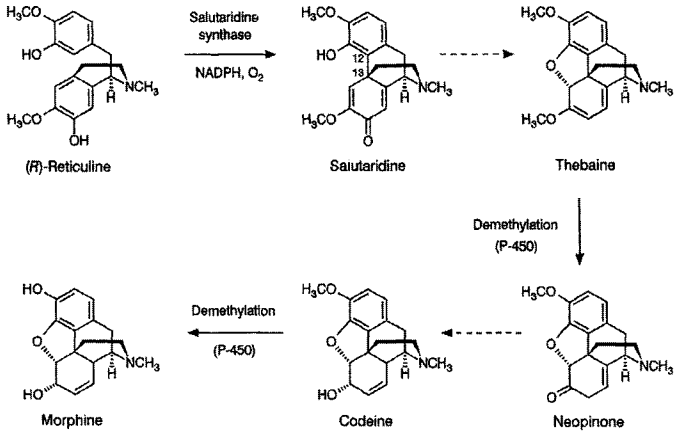

Figure 12.15. Biosynthesis of morphine from (*R*)-reticuline. Reprinted with permission from Chou and Kutchan (1998)[180].

interaction with downstream glucosinolate-producing enzymes to create a new metabolon that resulted in the accumulation of large amounts of *p*-hydroxybenzylglucosinolate in *A. thaliana*[148] and thereby changing the overall glucosinolate profile of *A. thaliana*[187].

The possibility to redirect L-tyrosine into the glucosinolate or cyanogenic glucoside pathways without loss of plant fitness[8, 129] demonstrates the existence of immanent routes for transport and storage of new classes of natural products introduced into plants by genetic engineering, and an inherent ability to redirect and optimize the flux of intermediates to counteract inbalances in primary and secondary metabolism[43]. The availability of a metabolic grid with numerous metabolic crosspoints to accommodate the synthesis of natural products upon demand is well documented. To enable the production of physiologically active amounts of DIMBOA in grasses without depleting the indole-3-glycerol phosphate pool for tryptophan synthesis, gene duplication has provided two modified genes each encoding enzymes that catalyze the same reaction but are directed toward different biochemical routes[154]. In periwinkle, a transcription factor ORCA3 upregulates the synthesis of L-tryptophan to provide efficient synthesis of the inducible bisindole alkaloids. Bisindole alkaloid synthesis is also dependent on the availability of secologanin and the rate-limiting step in its synthesis appears unaffected by ORCA3. The opposite situation where L-tryptophan accumulates due to blockage of natural product synthesis is also possible as observed in the double knockout mutant in Arabidopsis lacking the tryptophan metabolizing CYP79B2 and CYP79B3 enzymes[188]. Such plants completely lack indole-derived glucosinolates but only exhibit temperature-dependent phenotypic difference. So accumulation of free L-tryptophan does not appear to severely compromise wild-type growth characteristics, for example, by the formation of excess amounts of the tryptophan-derived indole acetic acid.

The ability to accommodate altered levels of intermediates depends on the type of compounds involved. In *A. thaliana*, tryptophan-derived oximes are key intermediates in the formation of the phytohormone indole acetic acid as well as in the synthesis of glucosinolates. CYP83A1 and CYP83B1 are the enzymes responsible for converting oximes into glucosinolates. Overexpression and knockout of these two enzyme activities result in altered phenotypes and pleiotrophic effects. Increased formation of lateral roots was associated with altered levels of indole acetic acid and provided evidence that fluxes of intermediates directed toward natural product formation may serve an important function to balance primary metabolism[43, 44, 189]. Surprisingly, disturbance of oxime metabolism affects phenylpropanoid metabolism and the monomer composition of lignin[75]. The link between these different phenomena is not yet understood.

In the synthesis of natural products, increased diversity is often achieved by a final set of modifications including hydroxylations, glucosylations, methylations, and acylations. As a result, the flavonol quercitin may be transformed into 300 different glucosides[190]. Berries of *Vitis vinifera* (grape wine) accumulate over 200 different aglycones that each may be decorated differently[191, 192]. Most likely, the synthesis of the basic structures of natural products is facilitated by metabolon formation. Dependent on cell type, developmental stage and elicitation as a result of abiotic or biotic stresses, additional enzyme activities may be bound to the basic metabolons to secure that desired specific modifications are obtained. The broad *in vitro* substrate specificity observed for *O*-methyltransferases[176, 193] and UDPG-glucosyltransferases[128, 194] may reflect that *in vivo* these will be associated to metabolons that prevent general access to their active sites. In this manner, the cell is able to maintain the potential to specifically decorate a large array of natural products without having to produce a separate enzyme for each reaction. As an added benefit, metabolon formation may prevent undesired reactions, for example, random glucolylation of plant hormones.

Based on the understanding of the basic principles for metabolon formation, in a foreseeable future it may be possible to transfer the entire pathways for synthesis of desired alkaloids into more convenient production plants from which these compounds can be isolated in high amounts. A main obstacle to reach these goals is knowledge of the proper P450, UDPG-glucosyltransferases, methyltransferases, and acyltransferases. Typically, these genes are not present in genetically well-defined model plants like *A. thaliana* and rice. They have to be traced often from exotic

plants for which no genome program and not even cDNA libraries are available. System biology technologies like metabolite profiling, proteomics, and transcriptomics may help to identify the proper enzymes and genes by unraveling coincidences of enhanced expression, protein appearance, and accumulation of specific metabolites. A genomics approach to elucidate the biosynthesis of the triterpene saponin in *Medicago truncatula* based on data mining of EST resources[195] and saponin metabolite profiles[196] resulted in the identification of three putative pathway enzymes[197]. In such approaches, metabolon formation may render it difficult to detect the true intermediates of a pathway. Reconstitution of a biosynthetic pathway by heterologous expression in a model plant is important to avoid wrong conclusions. In spite of the experimental limitations described above, progress on P450s and natural product synthesis moves quickly ahead thanks to hard work and the original approaches taken by many scientists involved in this research area.

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