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Biosynthesis of coumarins in plants: a major pathway still to be unravelled for cytochrome P450 enzymes

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Abstract Coumarins (1,2-benzopyrones) are ubiquitously found in higher plants where they originate from the phenylpropanoid pathway. They contribute essentially to the persistence of plants being involved in processes such as defense against phytopathogens, response to abiotic stresses, regulation of oxidative stress, and probably hormonal regulation. Despite their importance, major details of their biosynthesis are still largely unknown and many P450-dependent enzymatic steps have remained unresolved. *Ortho*-hydroxylation of hydroxycinnamic acids is a pivotal step that has received insufficient attention in the literature. This hypothetical P450 reaction is critical for the course for the biosynthesis of simple coumarin, umbelliferone and other hydroxylated coumarins in plants. Multiple P450 enzymes are also involved in furanocoumarin synthesis, a major class of phytoalexins derived from umbelliferone. Several of them have been characterized at the biochemical level but no monooxygenase gene of the furanocoumarin pathway has been identified yet. This review highlights the major steps of the coumarin pathway with emphasis on the cytochrome P450 enzymes involved. Recent progress and the outcomes of novel strategies developed to uncover coumarin-committed CYPs are discussed.

Keywords Cytochrome P450 · Monooxygenase · *Ortho*-hydroxylase · Coumarin · Hydroxycinnamic acid · Furanocoumarin · Biosynthesis

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Introduction

Coumarins are derived from 1,2-benzopyrones. These molecules are found in higher plants where they originate from the general phenylpropanoid pathway (Harborne 1999) and are subject to numerous modifications. Coumarins continue to receive attention for their diverse bioactivities. Some natural coumarins have been used as human therapeutics, while 4-hydroxycoumarins are prominent examples of microbial modification which gave rise to the first generation molecules developed along with aspirin and heparin as anticoagulants (Mueller 2004). Other applications appear possible in the course of new developments in various therapeutic fields, like

symptomatic treatment of multiple sclerosis (Wulff et al. 1998), photochemotherapy of T cell lymphoma (Plumas et al. 2003), chemotherapy of multidrug resistant tumors (Kawase et al. 2005), organ transplants (Damjanovich et al. 2004), or treatment of smokers for nicotine addiction (Malaiyandi et al. 2005).

Despite the importance of coumarins for plant life and human uses, major details of their biosynthesis have remained unresolved. This review will give an update of coumarin biogenesis in plants with emphasis on the cytochrome P450 enzymes involved.

Occurrence and functions of coumarins in plants

Coumarins may be subclassified as simple coumarins (benzo-α-pyrones syn 1,2-benzopyrone), 7-oxygenated coumarins (furanocoumarins syn. furobenzo-α-pyrones or furocoumarins), pyranocoumarins (benzodipyran-2-ones), and phenylcoumarins (benzo-benzopyrones) (Estévez-Braun and González 1997; Murray 1991; Murray et al. 1982). Simple coumarins, furanocoumarins and pyranocoumarins derive from the same pathway, whereas the most common phenylcoumarins (i.e., coumestans) originate from isoflavone metabolism and will not be considered in this review.

Simple coumarins

These compounds are widespread in plants and more than 700 structures have already been described (Harborne 1999).

Coumarin

Coumarin (Fig. 1) is a natural product well-known for its pleasant vanilla-like odor. It was reported from many plants of a variety of families, including Fabaceae i.e., Tonka bean (*Coumarouna odorata*) (Ehlers et al. 1995) or sweetclover (*Melilotus alba*) (Akeson et al. 1963), Lamiaceae i.e., lavender (*Lavandula officinalis*) (Brown 1962), and Lauraceae i.e., cinnamon (*Cinnamonum verum*) (Miller et al. 1996). More recent studies have revealed the presence of *o*-coumaric acid in *Arabidopsis thaliana* root exudates (Walker et al. 2003). As *cis*-*o*-coumaric acid is unstable under acidic or neutral conditions and lactonizes spontaneously to coumarin it is conceivable that coumarin is formed in *Arabidopsis thaliana*.

There have been many reports on the effect of coumarin in plants, at the organ, tissue and cellular levels (Brown 1981). These observations tend to demonstrate that coumarin acts as a plant hormone. However, until now neither solid evidence for a physiological function nor the molecular mode of action of coumarin has been provided in plant tissues.

Hydroxylated and methoxylated coumarins

Prevalent hydroxylated coumarins are umbelliferone, herniarin and scoparone (2 methoxylated derivatives of umbelliferone), esculetin, fraxetin, isofraxidin, isoscopoletin, daphnetin and their corresponding glucosides (Fig. 1). As for scopoletin, these molecules are involved in plant responses to stressors like salicylic acid (Pastirova et al. 2004; Repcak et al. 2001). Herniarin was demonstrated to be demethylated to umbelliferone by C4H from *Helianthus tuberosus* (CYP73A1) heterologously expressed in yeast (Pierrel et al. 1994); However, the *K*m was so high compared to cinnamate substrate that the implication of C4H for herniarin demethylation remains questionable.

Scopoletin and scopolin (7-*β*-D-glucoside of scopoletin, Fig.1) were reported from many plants, e.g., rubber tree (Giesemann et al. 1986; Silva et al. 2002) and cassava (Gomez-Vasquez et al. 2004) or carrot (Coxon et al. 1973) and cotton (Zeringue 1984), but have been mainly studied in tobacco (Fraissinet-Tachet et al. 1998; Maier et al. 2000) and sunflower (Cabello-Hurtado et al. 1998; Gutierrez et al. 1995). Scopoletin is a typical phytoalexin (Kùc 1982). Its synthesis is post-infectionally activated in plants (Sharan et al. 1998), but can also be triggered by various abiotic stresses (Gutierrez et al. 1995). Scopoletin also displays radical scavenging properties toward reactive oxygen species and may be involved in

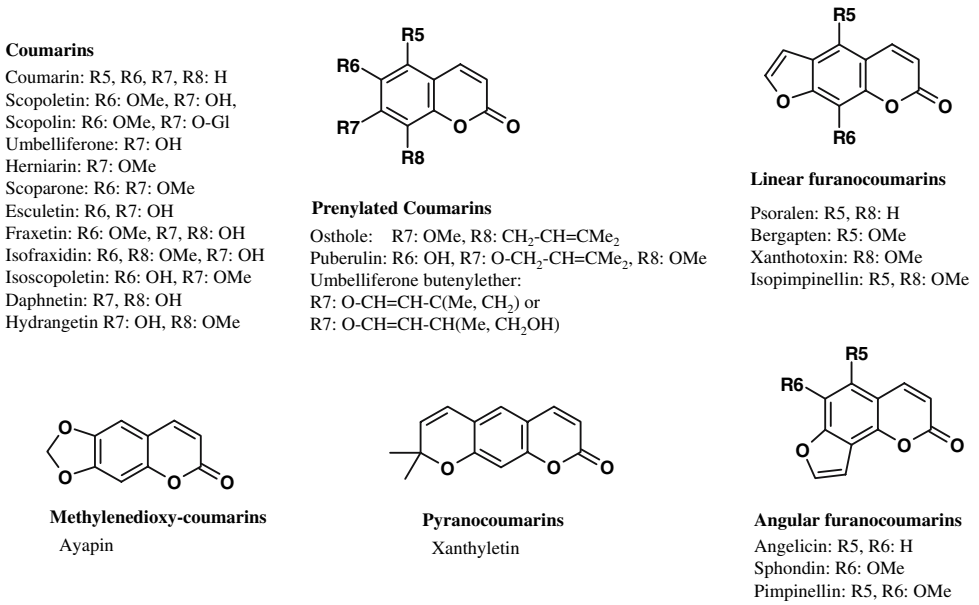

Fig. 1 Types of coumarins found in higher plants

the reduction of oxidative stress in plant cells .(Chong et al. 2002). Until recently, there was no report of hydroxylated coumarins in *Arabidopsis*, however, recent metabolic studies have revealed that this plant can accumulate scopolin in stems (Rohde et al. 2004) and roots (Bednarek et al. 2005). These findings demonstrate that stress-induced hydroxylated coumarins are more common in higher plant species than previously assumed. As frequently described for other secondary metabolites (Harborne 1999), scopoletin is glucosylated to scopolin (Fig. 1) in the cytosol and then transferred to the vacuole (Taguchi et al. 2000).

Derivatives of daphnetin have attracted most attention recently. Cold acclimated rye expresses an *O*-methyltransferase with attenuated specificity for position 8. The product, 7-hydroxy-8-methoxycoumarin (hydrangetin) (Fig. 1), had been reported as a protein kinase inhibitor (Yang et al. 1999), and the modulating effect on protein kinases was proposed to function during exposure of rye to high photosystem II excitation pressure and cold acclimation (Ndong et al. 2003). This might be the first example of a coumarin involved in hormone-like signaling.

Polyhydroxylated coumarins, like 6,7,8-trihydroxycoumarin, have been described from *Pelargonium sinoides* (Kayser and Kolodziej 1995; Latte et al. 2000), which demonstrates that plants are capable of multiple-step hydroxylations leading to more complex coumarin patterns.

Minor coumarins

There have been reports on many other minor coumarins in the phytochemical literature, which are beyond the scope of this review. Amongst this vast chemical diversity, methylenedioxy-substituted coumarins, i.e., ayapin (Fig. 1), and prenylated coumarins, like osthole and puberulin (Fig. 1), deserve mentioning. Ayapin has been described from Asteraceae only (Cabello-Hurtado et al. 1998; Scio et al. 2003) and was characterized as a phytoalexin (Gutierrez et al. 1995). Methylenedioxy bridge-formation commonly occurs through cyclization of an *ortho*-methoxyphenol and is catalyzed by cytochrome P450-dependent activities (Clemens and Barz 1996; Ikezawa et al. 2003). Such compounds are difficult to detoxify by phytopathogenic fungi (George and VanEtten 2001), and it is noteworthy that the methylenedioxy moiety is known as a potent P450 inhibitor group requiring bioactivation (Murray and Redy 1990). Osthole and puberulin have been frequently reported from

Rutaceae (Brophy et al. 2002; Brown et al. 1984) and Apiaceae (Tosun et al. 2005). *O*-Prenylated coumarins may be desaturated further to the corresponding butenylethers (Fig. 1) as shown in *Ammi majus* (Hamerski et al. 1990a), and these reactions are likely also catalyzed through P450 enzymes. The butenylethers are labile and release a potentially toxic aldehyde moiety, which contributes to their role as phytoalexins. Thus, the aliphatic substitution of umbelliferone may provide new substrates for further cytochrome P450 modifications, but neither of these enzymes has so far been identified. As in case of ayapin in Asteraceae, the P450 monooxygenases must be considered as essential ecological factors (see below).

Furanocoumarins

Furanocoumarins can be grouped into the linear type, where the (dihydro)furan ring is attached at C(6) and C(7), and the angular type, carrying the substitution at C(7) and C(8). Linear furocoumarins (syn. psoralens) are principally distributed in four angiosperm families: Apiaceae, Moraceae, Rutaceae and Leguminosae (restricted to Psoralea and Coronilla generae). The angular (dihydro)furanocoumarins are less widely distributed and primarily confined to the Apiaceae and Leguminosae (Bourgaud et al. 1989). The most abundant linear furanocoumarins are psoralen, xanthotoxin, bergapten and isopimpinellin, whereas the angular type is mostly represented by angelicin, sphondin, and pimpinellin (Fig 1). As was mentioned for the simple coumarins, numerous minor furocoumarins have been described in the literature, like bergamottin (5-geranoxy-psoralen) (Stanley and Vannier 1967) which has received attention recently as a major grapefruit component interfering with drug metabolism by intestinal CYP3A4 (Paine et al. 2005; Wen et al. 2002).

Furanocoumarins are recognized as potent phytoalexins (Beier and Oertli 1983) and allelochemical compounds (Baskin et al. 1967; Beier 1990). An outstanding feature of linear furanocoumarins is their ability to intercalate into dsDNA and create covalent cross-links primarily with thymidine residues (Dall'Acqua et al. 1978). Crosslinking proceeds readily under photoactivation and potentially blocks DNA replication and transcription. Accordingly, psoralens exhibit strong genotoxicity toward all living organisms, whereas the angular furanocoumarins are just capable of forming mono-adducts with DNA creating much less damage (Wamer et al. 1995). Another remarkable property of furanocoumarins is their reactivity to inactivate P450 enzymes as mentioned above for bergamottin. This kind of enzyme inhibition has been demonstrated for P450s from vertebrate (Koenigs and Trager 1998), insect (Zumwalt and Neal 1993) and plant sources (Gravot et al. 2004). Psoralens inactivate by a mechanism-based inhibition (also referred as suicide inhibition) which requires their conversion to reactive intermediates by the enzyme itself. These intermediates form covalent links to the apoprotein and permanently inactivate the enzyme (Fouin-Fortunet et al. 1986; Mays et al. 1989).

The reactivity of furanocoumarins bears considerable ecotoxicological consequences, i.e., attributing these compounds an important role as allelochemicals during plant-insect interactions (Schuler and Berenbaum 2003). Only herbivores able to tolerate furanocoumarins can feed on psoralen-rich plants, and xanthotoxin-insensitive P450 forms have been described from *Papilio polyxenes*, a papilionid butterfly adapted to furanocoumarin-accumulating host plants. This insensitivity was supposed to be the result of coevolution of insect detoxifying enzymes and the particular phytochemical defense since *Papilio glaucus*—whose host-plants do not contain furanocoumarins—exhibits sensitive P450s (Zumwalt and Neal 1993). The race of coevolution of butterflies on Apiaceae host plants has been studied in detail. The capacity of *Papilio polyxenes* to detoxify furanocoumarins through CYP6B1 follows the order xanthotoxin > psoralen > angelicin (Wen et al. 2003), but a synergistic effect has been described between angular furanocoumarins and psoralen or xanthotoxin in response to insect attack (Berenbaum and Zangerl 1993). Considering the minor direct toxicity of angular furanocoumarins, the synergism is conceivably based on the inhibition of psoralen-detoxifying CYP by angelicin. Furthermore, the

accumulation of angular furanocoumarins is confined to a few taxons only. It was hypothesized, therefore, that the capacity for angular furanocoumarin biosynthesis has evolved later and presumably as a consequence to compensate for the success of herbivores in the detoxification of psoralens. (Berenbaum and Zangerl 1998). It remains to be established, whether the enzymes for angular furanocoumarin biosynthesis have evolved from the biosynthesis of linear furanocoumarins.

Most plants accumulating furanocoumarins possess a highly inducible biosynthetic pathway. which can be triggered by various biotic (Hagemeier et al. 1999; Hamerski and Matern 1988b) and abiotic stresses (Eckey-Kaltenbach et al. 1994; Katz et al. 1998). *Ruta graveolens*, and possibly other Rutaceae, are exceptional because they do not respond to stressors and synthesize constitutively furanocoumarins in all tissues (Eilert 1989). However, the elicitation is still possible in *Ruta graveolens* dedifferentiated cells (Bohlmann et al. 1995).

The tissue-specific distribution of furanocoumarins has been studied in Apiaceae (Nitao and Zangerl 1987) and Rutaceae (Milesi et al. 2001). Obviously, these compounds accumulate in cells as well on the surface of plants. The pronounced accumulation on seeds and reproductive organs matches the optimal defense theory which predicts that defense compounds are principally allocated to the organs that play a key-role in plant fitness (McKey 1979). The subcellular localization of furanocoumarins is still unknown, but glucosylated forms have been frequently reported (Nguyen et al. 1997; Zobel and Brown 1988), suggesting a probable vacuolar compartmentation.

Pyranocoumarins

Pyranocoumarins, like xanthyletin (Fig. 1), have been mainly described from Rutaceae (Anaya et al. 2005; Sarker et al. 2002) and Apiaceae (Zgórka et al. 1998). As for furanocoumarins, linear and angular forms can be distinguished. To our knowledge, there is no proposal on their functions in plants, however, due to the structural relationship with furanocoumarins, a role as phytoalexins may be assumed. The biosynthesis of pyranocoumarins has not yet been investigated.

Biosynthesis of coumarins in plants

Main enzymes and genes implicated in coumarins biosynthesis and that have been sufficiently documented are presented in Table 1.

Cinnamic acid to coumarin

The pathway of coumarin biosynthesis has been largely outlined during the ‘60s and ‘70s, with the help of tracer feeding experiments (Brown 1981). Radiolabeled cinnamic acid was incorporated into coumarin and 7-hydroxycoumarins (Brown et al. 1960). Other tracer experiments conducted with *Lavandula officinalis*, a plant that produces coumarin as well as 7-hydroxylated coumarins, revealed that in the latter instance *para*-hydroxylation preceded the *ortho*-hydroxylation required for lactonization (Brown 1962). This indicated that umbelliferone (Fig. 1) is derived from *cis-p*-coumaric acid, whereas coumarin originates from *cis*-cinnamic acid (Fig. 2), and may imply different enzymes for the *ortho*-hydroxylation/lactonization of coumarin versus umbelliferone.

The *ortho*-hydroxylation is a key step of coumarin biosynthesis, that has received insufficient attention. In initial experiments, double-labeled (*ortho*-^{3}H, *ring*-1-^{14}C) cinnamic acid was fed to *Melilotus alba* shoots or *Gaultheria procumbens* leaves, and the retention of label was monitored upon conversion to *o*-coumaric acid (Ellis and Amrhein 1971). An NIH shift was proposed because of insignificant decrease of the ^{3}H:^{14}C ratio, which is an indication of a cytochrome P450 monooxygenase reaction mechanism. A following report addressed the formation of coumarin with extracts from *Melilotus alba*, a plant that produces high levels of coumarin. This study allocated the *ortho*-hydroxylation of cinnamic acid to the chloroplast and again suggested a P450-dependent hydroxylation mechanism (Gestetner and Conn 1974). Unfortunately, the in vitro results could not be reproduced, and the class of the enzyme involved as well as its

Table 1 Enzymes of coumarin and furanocoumarin biosynthesis characterized in vitro or by precursor feeding studies. Cinnamate 4-hydroxylase, not mentioned here, is described by Ehlting et al. in this issue. N.D. means not determined, *Pmg: Phytophthora megasperma*

Activity	Substrate	Kinetic constants	Plant	Comments	Authors
Benzoic acid 2—hydroxylase	Benzoic acid	N.D.	*Nicotiana tabacum*	P450, Soluble enzyme	Leon et al. 1995
Coumarate/Cinnamate/ Ferulate 2-hydroxylase	*p*-Coumaric acid; Ferulic acid; Cinnamic acid	N.D.	*Hydrangea macrophylla*	Chloroplastic activity	Kindl 1971
Esculetin synthase	Umbelliferone	N.D.	*Cichorium intybus*	Precursor feeding studies only	Brown 1985
Daphnetin synthase	Umbelliferone	N.D.	*Daphne mezereum*	Precursor feeding studies only	Brown 1986
Umbelliferone 7-*O*-prenyltransferase	Umbelliferone	K_m Umbelliferone = 6.5μM K_m DMAPP = 30μM	*Ammi majus*	*Pmg* elicited cell cultures	Hamerski et al. 1990
Umbelliferone 6-prenyltransferase	Umbelliferone	N.D.	*Ammi majus*	*Pmg* elicited cell cultures	Hamerski et al. 1990
Umbelliferone 6-prenyltransferase	Umbelliferone	N.D.	*Ruta graveolens*	Plastidic enzyme	Dhillon and Brown 1976
Marmesin synthase	Demethylsuberosin	K_m DMS = 10.3μM K_m NADPH = 19.6μM	*Ammi majus*	P450, *Pmg* elicited cell cultures optimal pH = 7.5/optimal temp. = 20°C	Hamerski and Matern 1988
Marmesin 5-hydroxylase	(+)-Marmesin	N.D.	*Ruta graveolens* *Ficus carica*	Precursor feeding studies only	Caporale et al. 1981
Psoralen synthase	(+)-Marmesin	K_m NADPH = 52μM	*Petroselinum crispum*	P450, optimal pH = 7–7.25	Wendorf and Matern 1986
Angelicin synthase	(+)-Columbianetin	N.D.	*Heracleum mantegazzianum*	Precursor feeding studies only	Stanjek et al. 1998
Psoralen 5-monooxygenase	Psoralen	K_m Psoralen = 12μM K_m NADPH = 200μM	*Ammi majus*	P450, *Pmg* elicited cell cultures	Hamerski and Matern 1988
Bergaptol *O*-methyltransferase	Bergaptol	K_m Bergaptol = 2.8μM K_m SAM = 6.5μM	*Ammi majus*	Genbank accession AY443006, *Pmg* elicited cell cultures, optimal pH = 8/temp = 42°C	Hehmann et al. 2004
Bergaptol *O*-methyltransferase	Bergaptol/5-Hydroxyxanthotoxin	K_m Bergaptol = 4μM V_{max} = 980μkat/kg K_m SAM = 3.1μM V_{max} = 1160μkat/kg K_m 5-H-Xanthotoxin = 1μM V_{max}=1250μkat/kg	*Petroselinum crispum*	Pmg elicited cell cultures, purified enzyme optimal pH = 8–8.5	Hauffe et al. 1986

Table 1 continued

Activity	Substrate	Kinetic constants	Plant	Comments	Authors
Bergaptol *O*-methyltransferase	Bergaptol/5-Hydroxyxanthotoxin	N.D.	*Ruta graveolens*	Separated from xanthotoxol OMT purified enzyme	Sharma et al. 1979
Xanthotoxol *O*-methyltransferase	Xantothoxol	K_m Xanthotoxol = 9.8μM V_{max} = 5750μkat/kg K_m SAM = 4.4μM V_{max} = 7480μkat/kg	*Petroselinum crispum*	*Pmg* Elicited cell cultures, purified enzyme	Hauffe et al. 1986
Xanthotoxol *O*-methyltransferase	Xanthotoxol, 8-Hydroxybergapten	N.D.	*Ruta graveolens*	Separated from bergaptol OMT purified enzyme	Sharma et al. 1979
Isopimpinellin synthase	Xanthotoxin, Bergapten	N.D.	*Heracleum lanatum*	Precursor feeding studies only	Brown and Sampathkumar 1977
Isopimpinellin synthase	5,8-Hydroxypsoralen	N.D.	*Ruta graveolens*	Precursor feeding studies only	Innocenti et al. 1983

subcellular site remain to be established. As revealed later, the early experiments may have suffered from fundamental analytical problems, since the chromatography and recrystallization techniques employed were likely insufficient to separate the various cinnamic acids. Nevertheless, the proposed conversion of cinnamic to *o*-coumaric acid received some support by precursor feeding studies done with *Petunia* chloroplasts, which ascribed cinnamate 2-hydroxylase, including the formation of coumarin, and lack of cinnamate 4-hydroxylase to these organelles (Conn 1984; Ranjeva et al. 1977). In light of the studies done since with *Ammi majus* microsomes (Hamerski and Matern 1988a, b; Stanjek et al. 1999a) on the biosynthesis of furanocoumarins it appears possible that the 'ortho-hydroxylase' is an exceptionally labile CYP enzyme, in contrast to the CYPs hydroxylating cinnamic acids in *para*- or *meta*-position (Fig. 2). Overall, the *ortho*-hydroxylation of cinnamic (or 4-coumaric) acid, being of pivotal importance for all coumarins, remains a missing link in the network of phenylpropanoid biosynthesis.

Cinnamic acid to umbelliferone and other hydroxylated coumarins

The formation of umbelliferone proceeds from 4-coumaric acid or its ester derivatives (Fig. 2). The conversion of cinnamic acid to 4-coumaric acid is catalyzed by cinnamate 4-hydroxylase, a cytochrome P450 monooxygenase from the CYP73A family (Teutsch et al. 1993). This enzyme constitutes the P450 enzyme most studied to date and sets the stage for several branch pathways, such as the lignification (Anterola and Lewis 2002) or flavonoid biosynthesis (Harborne 1999) (see Ehlting et al. 2006 in this issue for a review).

Following the pertaining literature, 4-coumaric acid is *ortho*-hydroxylated to 2,4-dihydroxycinnamic acid. The respective enzyme activity was reported exclusively from *Hydrangea macrophylla* and assigned to the chloroplasts (Kindl 1971). This enzyme fraction was demonstrated to slowly convert cinnamic acid to *o*-coumaric acid but was more active to transform *p*-coumaric acid and ferulic acid respectively to umbelliferone and

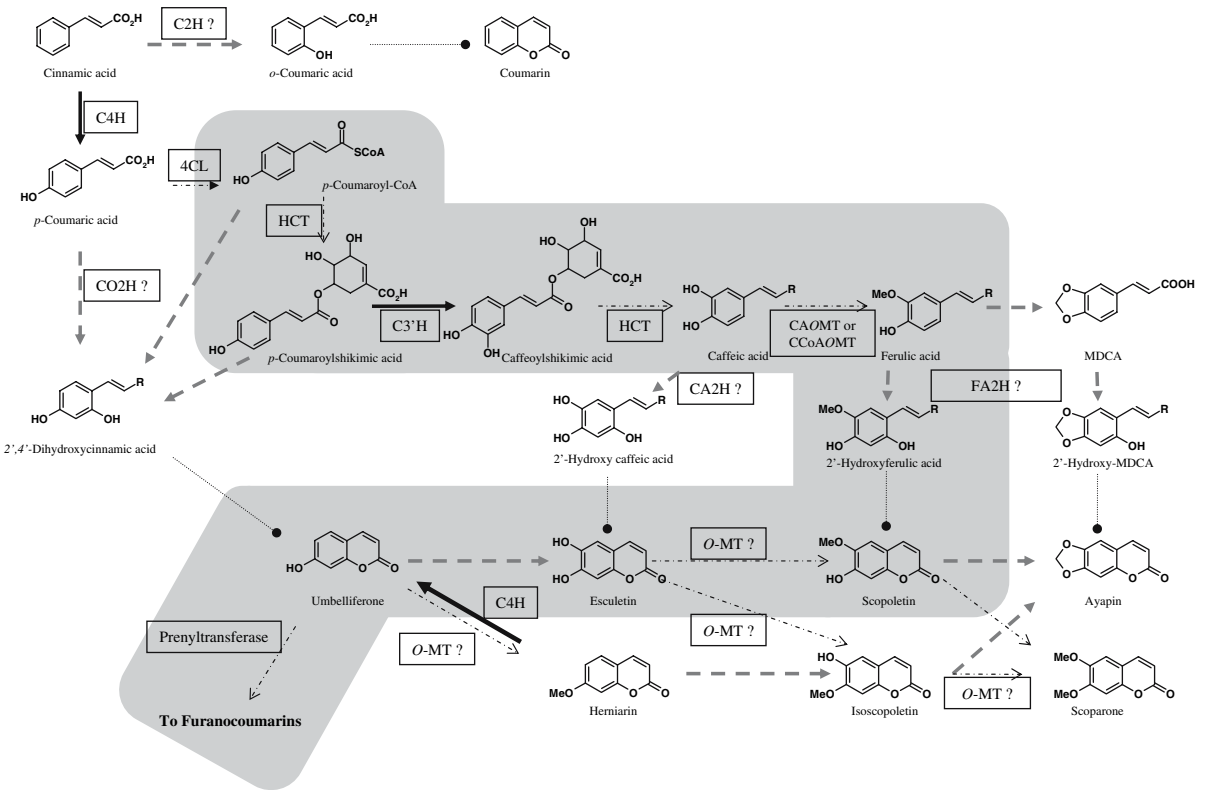

Fig. 2 Phenylpropanoid pathway leading to coumarins Pathways in gray have been unequivocally established. Glycosylated compounds are not shown for clarity. Enzymes assigned by a question mark are hypothetical. R = CO_2H or CO-SCoA C2H, cinnamic acid 2-hydroxylase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CO2H, 4-coumaric acid 2-hydroxylase; HCT, hydroxycinnamoyl-transferase; CA*O*MT, caffeic acid *O*-methyltransferase; CCoA*O*MT, caffeoyl CoA *O*-methyltransferase, CA2H, caffeic acid 2-hydroxylase; FA2H, ferulic acid 2-hydroxylase; MDCA2H, methylenedioxycinnamic acid 2-hydroxylase; *O*-MT, *O*-methyltransferase

scopoletin. Although this report is unique in describing the *ortho*-hydroxylation of a hydroxylated coumarin in vitro and suggesting one plastidic fraction for the *o*-hydroxylation of both *p*-coumaric and ferulic acid as well as benzoic acid, the conversion of ferulic acid to scopoletin had been postulated before from precursor feeding studies in tobacco tissue cultures (Fritig et al. 1970). This biosynthetic course of scopoletin/scopolin has been recently established in *Arabidopsis thaliana* (Kai et al. 2006). T-DNA insertion mutants within the gene encoding CYP98A3, which catalyzes 3'-hydroxylation of *p*-coumarate, revealed a dramatic decrease in both scopoletin and scopolin contents, confirming the origin from ferulic acid in *Arabidopsis*. This is in contrast to the results obtained for puberulin (Fig. 1) biosynthesis in *Agathosma puberula* (Brown et al. 1988). Here, as well as in *Daphne mezereum*, ferulic acid was not readily incorporated as opposed to umbelliferone, therefore making esculetin (Fig. 1) a likely precursor for the synthesis of scopoletin (Brown 1986). The details were discussed before (Murray et al. 1982), and different pathways may operate in different plants.

The formation of esculetin (Fig. 1; 6,7-dihydroxy coumarin) was examined in *Cichorium intybus* (Brown 1985). These studies revealed that umbelliferone was an efficient precursor but not

caffeic acid, suggesting 6-hydroxylation of umbelliferone, probably by the action of a P450 monooxygenase. This deserves mentioning, because the conversion of caffeic acid to esculetin is readily accomplished in vitro with various plant extracts containing phenoloxidase activity (Kneusel 1987; Sato 1967), but has not been confirmed *in planta*. Similar to esculetin, daphnetin (Fig. 1, 7,8-dihydroxycoumarin) in *Daphne mezereum*, was shown to be derived from umbelliferone rather than caffeic acid.

The *ortho*-hydroxylation: a common route with salicylic acid

Analogous to C2H, another major *ortho*-hydroxylation step in phenolic metabolism is still controversial. Salicylic acid is a pivotal signal molecule in plant defense mechanisms (Shah 2003) but the biosynthesis pathway is still matter of debate. Two routes have been proposed. A pathway already shown to occur in bacteria has been proposed in tobacco through chorismate and isochorismate, via the general shikimic acid metabolism (Wildermuth et al. 2001). Another route has been documented in tobacco (Coquoz et al. 1998; Yalpani et al. 1993) and rice (Silverman et al. 1995), via decarboxylation of *trans*-cinnamic acid to benzoic acid and subsequent 2-hydroxylation. This benzoic acid 2-hydroxylase was characterized as a P450 enzyme but important biochemical characteristics are atypical for an eucaryotic P450 as it appears to be soluble and it exhibits an unusually high molecular weight (Leon et al. 1995). The corresponding P450 gene has not been reported so far. This benzoic acid 2-hydroxylase is unable to transform cinnamic acid into *o*-coumaric acid (Yalpani et al. 1993) and consequently is unlikely to interfere with the coumarin pathway.

Biosynthesis of furanocoumarins in plants

While coumarin biosynthesis remains a black box, several enzymes of the furanocoumarin pathway have been isolated and characterized (Fig. 3).

Entry of umbelliferone into the furanocoumarin pathway

Umbelliferone rather than coumarin is the parent compound of furanocoumarins, as was reported a long time ago (Floss and Mothes 1964). It is first prenylated in 6- (for linear furanocoumarins) or 8-position (for angular furanocoumarins) to yield demethylsuberosin and osthenol, respectively (Fig. 3). Dimethylallyl diphosphate required for the 6-prenylation at least is provided in celery (*Apium graveolens*) by the deoxy-D-xylulose pathway and not through the mevalonate-dependent pathway (Stanjek et al. 1999b). This is conceivably also the case in other plants, because the prenyltransferase has been identified in *Ruta graveolens* as a plastidic enzyme (Dhillon and Brown 1976; Ellis and Brown 1974), and the activity was also documented in *Ammi majus* (Hamerski et al. 1990b) The homologous enzyme for the angular furanocoumarins has not been isolated so far.

Linear furanocoumarins

Demethylsuberosin is transformed to marmesin and further to psoralen by two separate cytochrome P450 enzymes (Hamerski and Matern 1988b; Wendorff and Matern 1986). The enzymes were biochemically characterized, and evidence for their P450 nature was obtained from characteristic blue-light-reversible inhibition of the activities by carbon monoxide, and the use of specific inhibitors. The two enzymes formally catalyze very different reactions, the first forming the dihydrofuran-ring from the *ortho*-prenylated phenol (marmesin synthase) and the second catalyzing the oxidative carbon-carbon chain cleavage reaction (psoralen synthase). The mechanism of marmesin synthase has not been solved yet, but it might be speculated that some analogy exists to menthofuran synthase from *Mentha piperita* which belongs to the CYP71 family (Bertea et al. 2001; Croteau et al. 2005). Psoralen synthase was found to operates by *syn*-elimination of acetone and one hydrogen from position 3' (Fig. 3) (Stanjek et al. 1999a). This release of acetone is unique in plants. Psoralen synthase is

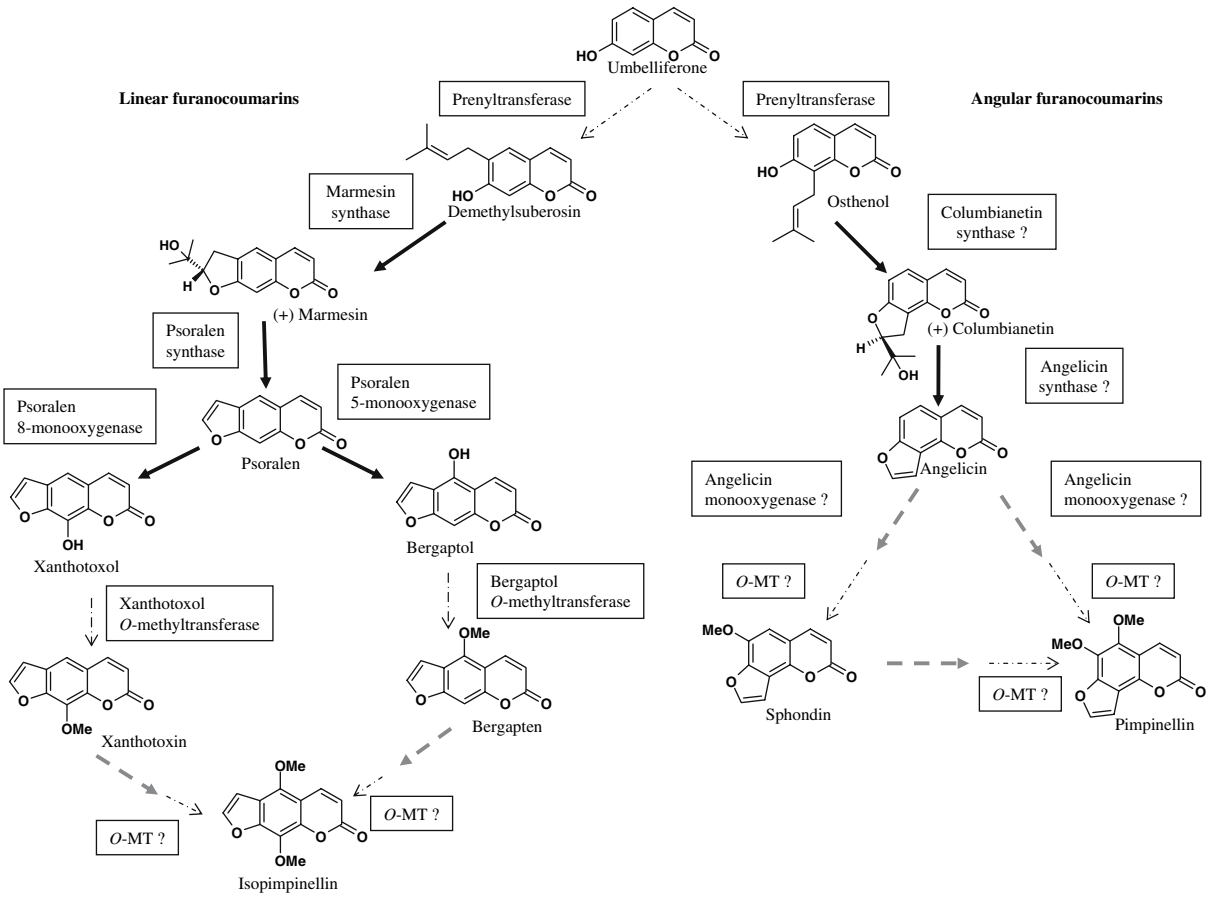

Fig. 3 Furanocoumarin pathway Glycosylated forms are not represented for clarity. Enzymes designated with a question mark are hypothetical. *O*-MT, *O*-methyltransferase

P450

putative P450

non-P450 enzyme

very specific for (+)-marmesin and does not accept the (–)-stereoisomer (nodakenetin) as a substrate. Neither of the two P450s has been characterized at the gene level.

Psoralen 5-monooxygenase catalyzes the subsequent hydroxylation of psoralen to bergaptol and was also characterized as a cytochrome P450 enzyme (Hamerski and Matern 1988a) from *Ammi majus* cell suspensions. Nevertheless, there is still the possibility that bergaptol could be formed from 5-hydroxymarmesin. This possibility was strongly favored by the work of Caporale and coll. (Caporale et al. 1981). Different plants might thus have developed a slightly different sequences. Bergaptol is then *O*-methylated to bergapten. The cDNA encoding the *O*-methyltransferase catalyzing this reaction was recently cloned and functionally characterized from *Ammi majus* (Hehmann et al. 2004). The enzyme was shown to be highly specific for bergaptol and does not accept xanthotoxol, the C(8) corresponding phenol. This corroborates previous reports on the separation of bergaptol and xanthotoxol methyltransferases from *Ruta graveolens* (Sharma et al. 1979) or *Petroselinum crispum* (Hauffe et al. 1986).

The path for isopimpinellin formation (5, 8-dimethoxypsoralen) is uncertain. It was studied in *Heracleum lanatum*. In this plant xanthotoxin was the most efficient precursor (Brown and Sampathkumar 1977). However, bergapten was

found to be converted into isopimpinellin, although at a lower rate. Both 5- and 8-hydroxylation pathways can thus lead to final product, but 5,8-dihydroxypsoralen was also demonstrated to be a possible precursor in *Ruta graveolens* (Innocenti et al. 1983). Enzymatic turnover of the pathways could simply explain the prevalence of one of the three routes in a given plant.

Angular furanocoumarins

The transformation of columbianetin to angelicin is very similar from a mechanistic and stereochemical point of view to the conversion of marmesin to psoralen (Stanjek and Boland 1998). As demonstrated by feeding studies using fluor- or deuterium-labeled columbianetin with plants or leaf tissues. It is, thus, conceivable that the enzymes for angular furanocoumarin biosynthesis may have emerged by evolutionary adaptation from the linear pathway. This would be consistent with the fact that angular furanocoumarins are less abundant in plants than the linear type and that angular furanocoumarins are always found concomitantly with linear furanocoumarins. This hypothesis will be investigated once the genes for marmesin synthase and psoralen synthase, as well as those for umbelliferone 6- and 8-prenyltransferases, will be identified (Fig. 3). Unfortunately, no information is available yet at the genetic level.

Implication of P450s in furanocoumarin synthesis

Cytochrome P450 enzymes are pivotal enzymes of furanocoumarin biosynthesis, i.e., the formation of xanthotoxin relies, at least, on four sequential P450 reactions catalyzed by C4H, marmesin synthase, psoralen synthase and psoralen 8-monooxygenase. This was at a first glance puzzling because of the intrinsic capacity of furanocoumarins to inhibit very different cytochrome P450 enzymes, irrespective of the species, through a mechanism-based inactivation process (Fouin-Fortunet et al. 1986).

To understand how plants cope with this problem Gravot and co-workers compared inactivation by furanocoumarins (Gravot et al. 2004) of three different C4H: one from a plant that does not contain furanocoumarins (*Helianthus tuberosus*, CYP73 A1) and two from plants that synthesize furanocoumarins (*Ruta graveolens*, CYP73A32; *Petroselinum crispum* CYP73A10). They showed that K_{inact} and 1/*K*i in presence of psoralen were lower for CYP73A32 and CYP73A10 compared to CYP73A1, and, accordingly, the cinnamate hydroxylation activity of CYP73A32 and CYP73A10 appears more resistant to mechanism-based inactivation than that of CYP73A1 (respectively *k*inact/*K*i 7, 4.4, and 45 $min^{-1}mM^{-1}$). This would suggest that plants producing furanocoumarins have adapted their P450 enzyme repertoire to the need for reduced inactivation while retaining the high catalytic efficiency. It is reasonable to expect a similar adaptation of all the P450 enzymes in the same pathway.

The evolution toward furanocoumarin accumulation must have occurred under strong selection pressure, since the biocidal and enzyme inactivation properties of furanocoumarins appear to be lethal to plants unless quick adaptation can be accomplished. This pressure might have built up by the exposure to herbivores and the need for efficient antifeedant metabolites. This would be fully compatible with the scheme of furanocoumarins as allelochemicals in the warfare with insects only adapted to hatch on furanocoumarin producing plants (Schuler and Berenbaum 2003). It will be interesting to compare the cytochrome P450 families recruited for the synthesis of furanocoumarins in the plant and their detoxification in insects.

Perspectives

Although no monooxygenase of the furanocoumarin pathway *stricto sensu* has been characterized at the gene level, techniques such as differential display and RT-PCR strategies have been developed for P450s (Schoendorf et al. 2001; Schopfer and Ebel 1998) which should be readily applicable to furanocoumarin pathway. Such techniques already led to the characterization of the C4H and C3'H in the relevant plants. Inducible systems are needed to

differentiate and correlate the individual transcript abundances with product accumulation Elicitor-treated *Ammi majus* cultures appear to qualify for this purpose.

Numerous recent studies focused on the role of furanocoumarins as key allelochemicals, but the physiological relevance of coumarins reaches far beyond in the producing plants. This includes the potential role of simple coumarins as hormones and signaling molecules, which were shown in the past decade to be much more widespread in plant kingdom than previously assumed. More functional insight should be obtained once the mechanism, regulation of their biosynthesis and their subcellular localization will be known. Biosynthesis of L-phenylalanine proceeds in plastids while phenylalanine ammonia-lyase and C4H activities reside in the cytosol and endoplasmic reticulum. Subcellular localization of the pivotal *ortho*-hydroxylation of cinnamic or 4-coumaric acid, so far, remains unresolved. Investigation in *Ruta graveolens* assigned the subsequent 6-prenylation of umbelliferone to plastidial membranes (Dhillon and Brown 1976). Clarification of the localization of the 2-hydroxylation will be the further step to understand the physiological role of coumarins.

It is probable that different routes to coumarins will be discovered to operate in plants, some of them might be confined to a taxonomic group. The formation of scopoletin is an example and derives either from esculetin or ferulic acid according to the plant species considered. It is currently unknown, whether the P450s involved in the furocoumarin pathway belong to a single family, as is the case with CYP71s in benzoxazine synthesis, or to multiple P450 families as shown for biosynthesis of cyanogenic glucosides (CYP71E1 and CYP79A1). In either case, the discovery of genes involved in coumarin synthesis will add another stage of complexity to the phenylpropanoid pathway.

The recent detection of coumarin and hydroxylated coumarins in *Arabidopsis thaliana* have opened the way for new approaches. Metabolomics in conjunction with screening of mutant libraries is likely to reveal new players in the coumarin pathway.

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