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par

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Identification and functional characterization of the first two aromatic prenyltransferases implicated in the biosynthesis of furanocoumarins and prenylated coumarins in two plant families: Rutaceae and Apiaceae

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Dedicated to my beloved mother

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List of abbreviations

4CL:	4-Hydroxycinnamoyl CoA ligase
4HB:	4-hydroxybenzoic acid
5-MOP:	Bergapten
8-MOP:	Xanthotoxin
AgPt:	Putative prenyltransferase of *A. graveolens*
AngPt:	Putative prenyltransferase of *A. gigas*
AS:	Angelicin synthase
C2'-H:	*p*-coumaroyl CoA 2'-hydroxylase
C3'-H:	p-Coumaroyl ester 3'-hydroxylase
C4H:	Cinnamate-4-hydroxylase
CCoAOMT:	Caffeoyl CoA *O*-methyltransferase
CliPt:	Putative prenyltransferase of *C. limon*
ClPt:	Putative prenyltransferase of *C. latifolia*
CoA:	Co-enzyme A
DMAPP:	Dimethylallylpyrophosphate
DMS:	Demethylsuberosin
DNA:	Deoxyribonucleic acid
DXP:	Deoxyxylulose 5-phosphate pathway
F6'H:	Feruloyl CoA 6-hydroxylase
FGPP:	Farnesylgeranyl diphosphate
FPP:	Farnesyl pyrophosphate
G4DT:	Pterocarpan 4-Dimethylallyltransferase
GGPP:	Geranyl-geranyl pyrophosphate
GPP:	Geranyl diphosphate
HCT:	Hydroxycinnamoyl-Coenzyme A shikimate
HGA:	Homogentisic acid
HGGT:	Homogentisate geranyl-geranyl transferase
HPLC:	High performance liquid chromatography
HPLC–MS:	High performance liquid chromatography–mass spectrometry
HQT:	Hydroxycinnamoyl-Coenzyme A quinate
IPPI:	Isoprenyl diphosphate isomerase
kan:	Kanamycin
*La*PT1:	Genistein 3-dimethylallyl prenyltransferase *L. albus*
LePGT:	Geranyl Diphosphate: 4-Hydroxybenzoate Geranyltransferase of *L. erythrorhizon*
M*and*S:	Murashige and Skoog
MEP:	Methylerythritol 4-phosphate (MEP) pathway
MS:	Mass spectrometry
MVA:	Cytosolic mevalonic pathway
OD:	optical density
PAL:	Phenylalanine ammonia lyase
PcPt:	Putative prenyltransferase of *P. crispum*
PS:	Psoralen synthase
PsPt:	Putative prenyltransferase of *P. sativa*
Pt:	Prenyltransferase
PTGS:	Post-transcriptional gene silencing
RgPt:	Putative prenyltransferase of *R. graveolens*
rif:	Rifampicin
RNA:	Ribonucleic acid
SfG6DT:	Genistein 6-dimethylallyl Prenyltransferase of *S. flavescens*

SfiLDT: Isoliquiritigenin dimethylallyltransferase of *S. flavescens*
SfN8DT-1: Naringenin 8-dimethylallyl transferase of *S. flavescens*
Umb: Umbelliferone

List of species cited in the manuscript

Plants

Allium ampeloprasum
Amaranthus hypochondriacus
Ammi majus
Angelica archangelica
Apium graveolens
Arabidopsis thaliana
Citrus bergamia
C. latifolia
C. limon
Coffea canephora
Cuphea avigera
Echinochloa crus-galli
Ficus carica
Glycine max
Glycyrrhiza glabra
Helianthus annuus
Hevea brasiliensis
Hierochloe odorata
Hordeum vulgare
Humulus lupulus
Humulus lupulus
Ipomoea batatas
Ipomoea purpurea
Lactuca sativa
Lavandula angustifolia
Lithospermum erythrorhizon
Lotus japonica
Lupinus albus
Melilolotus alba
Melilotus officinalis
Nicotiana benthamiana
Oryza sativa
Pastinaca sativa
Petroselinum hortense
P. crispum
Petunia hybrid
Psoralea corylifolia
Ruta graveolens
Sophora flavescens
Triticum aestivum
Zea mays

Fungal and bacterial strains

Alternaria carthami
Alternaria helianthi
Aspergillus niger
Candida albicans

Cryptococcus neoformans
Escherichia coli
Fusarium oxysporum
Helminthosporium carbonum
Mycobacterium phlei
Mycobacterium smegmatis
Mycobacterium fortuitum
Phytophthora megasperma (Pmg)
Phytophthora palmivora
Plasmodium falciparum
Pseudomaonas aeruginosa
Pythium aphanidermatum
Saccharomyces cerevisiae
Salmonella typhimurium

Introduction

Introduction

1 Phenylpropanoids

Phenylpropanoids constitute a large class of few thousand molecules that have been identified in plants. They form a subclass of a larger category of secondary metabolites commonly referred to as "phenolics". These phenolics are widely distributed in the plant kingdom, and until today more than 8000 structures have been identified in plants (Dai and Mumper, 2010). These molecules have been classified on the basis of the presence of a common phenol unit in their structures, completed by, at least one hydroxyl substituent (Figure 1) (Croteau *et al.,* 2000).

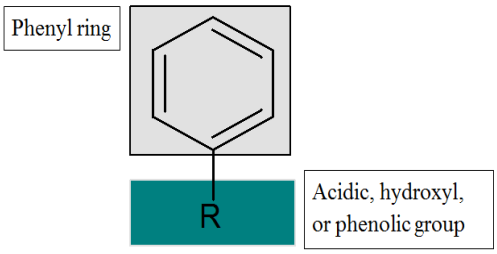


Figure 1. Basic structure of phenol ring

Phenylpropanoids play important roles in plant responses to environmental biotic and abiotic stresses. They help plants to resist to various environmental agressors like microbes (La Camera *et al.,* 2004; Mazid *et al.,* 2011), herbivores (Nuringtyas *et al.,* 2012) and help the plants in adapting to new environments (Bais *et al.,* 2003) through the mechanism of allelopathy and successful reproduction (Dudareva *et al.,* 2004). In addition they play a role as signaling molecules in plant-microbe interactions (La Camera *et al.,* 2004). Although secondary metabolites are generally described as molecules present in low concentration in plants, lignin are the phenylpropanoids that constitute the second most prevalent biopolymer of earth and are a basic structural unit of angiosperms and gymnosperms.

Beside their pivotal roles specific for plants, several phenylpropanoids have been described as molecules of medicinal importance. Indeed they can serve as antioxidants (Borchardt *et al.,* 2009), anticancer (Ferrazzano *et al.,* 2011), anti-inflammatory (Chang *et al.,* 2010), antibacterial , wound healing and UV screens (Korkina 2007).

1.1 Biosynthetic pathway of phenylpropanoids

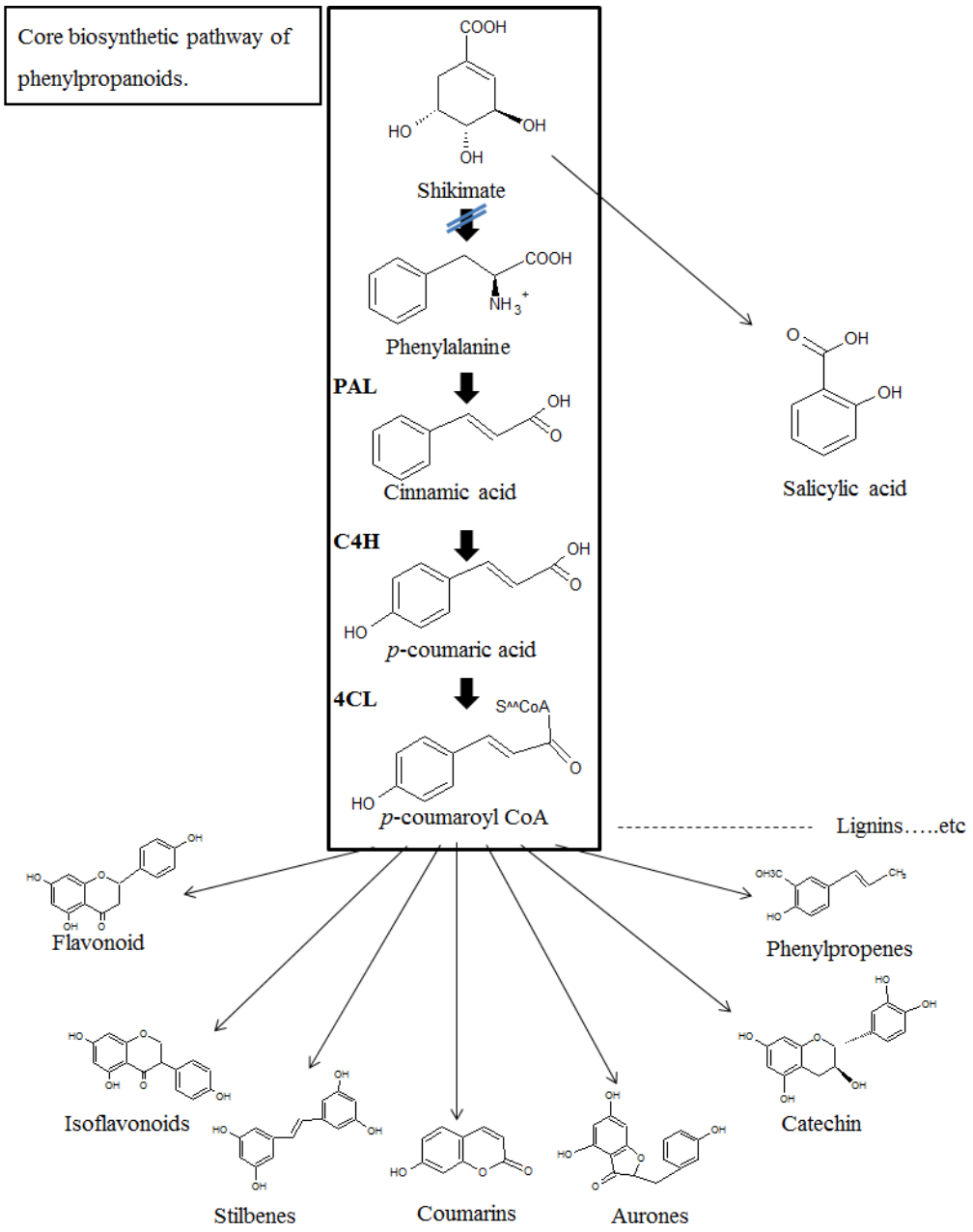


Figure 2. Simple schematic representation of the biosynthetic pathway of phenylpropanoids and derived products

PAL= Phenylalanine ammonia lyase, C4H= cinnamate 4- hydroxylase, 4CL= 4-Coumaroyl CoA ligase.

The biosynthesis pathway of phenylpropanoids is derived from the shikimate pathway of plants. This pathway starts with the step of deamination of phenylalanine into *trans*-cinnamic acid which is catalyzed by an enzyme, Phenylalanine Ammonia Lyase (PAL)

(Rosler *et al.,* 1997) (Figure 2). In all studied plants, it has been found that PAL enzymes belong to multi-gene families (Hamberger *et al.,* 2007), where the number of members varies from some as in case of the raspberry (Kumar *and* Ellis, 2001) to dozen as in potato (Hahlbrock *and* Joos, 1992). The second step consist of the hydroxylation of *trans*-cinnamic acid into 4-*para*-coumaric acid and is catalyzed by an enzyme known as cinnamate 4-hydroxylase (C4H) (Hotze *et al.,* 1995) (Figure 2). One hundred and seven different potential C4H have been isolated and classified in the CYP73A cytochrome P450 enzyme superfamily (http://drnelson.uthsc.edu/CytochromeP450.html). Several work done on the Arabidopsis C4H showed that inhibiting this enzyme resulted in drastic effects on growth and development of plants (Schilmiller *et al.,* 2009), highlighting the importance of this enzyme in this pathway. In the subsequent step catalyzed by a *p*-coumarate CoA ligase (4CL), *p*-coumaric acid is esterified by addition of a Coenzyme A (Costa *et al.,* 2005) (Figure 2). This step represents one of the most important branch points in the biosynthetic pathway of phenylpropanoids in plants. As represented in (Figure 2), a large number of plant natural products are deriving from this pathway like stilbene, catechine, aurones, lignin, flavonoids, chlorogenic acid, coumarins, and furanocoumarins.

1.2 Coumarins and furanocoumarins

1.2.1 Coumarins

Coumarins are simple phenolic molecules deriving from the phenylpropanoid pathway. They are denominated as coumarins because these molecules were isolated for the first time in 1820 from a plant tonka bean (*Diteryx odorata*), for which common name is "coumarou" (Bruneton, 1999). They are derived from 1,2-benzopyrones (Bourgaud *et al.,* 2006), and are made up of a benzene ring attached with a pyrone ring (Figure 3).

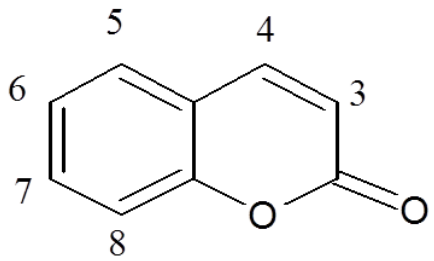


Figure 3. Basic structure of coumarins

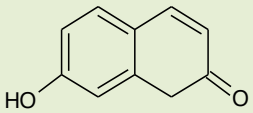
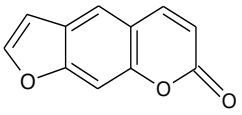
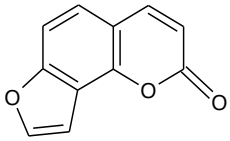
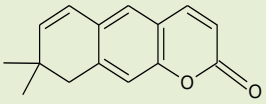
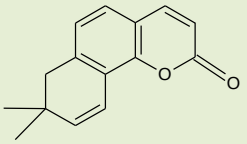
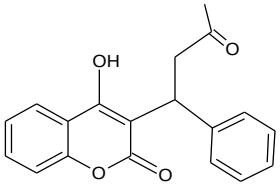
Table 1. Derived coumarins by various modifications (modified from Kostova, 2005)

	Compound	R3	R4	R5	R6	R7	R8
1	Coumarin	H	H	H	H	H	H
2	Umbelliferone	H	H	H	H	OH	H
3	Esculetin	H	H	H	OH	OH	H
4	4-hydroxycoumarin	H	OH	H	H	H	H
5	6-nitro-7-hydroxycoumarin	H	H	H	NO_2	OH	H
6	3,6,8-nitro-7-hydroxycoumarin	NO_2	H	H	NO_2	OH	NO_2
7	Daphnetin	H	H	H	H	OH	OH
8	Coumarin-3-caboxylic acid	COOH	H	H	H	H	H
9	Fraxetin	H	H	H	OMe	H	H
10	Esculin	H	H	H	OGl	OH	H
11	Auraptene	H	H	H	H	oGer	H
12	Scopoletin	H	H	H	OMe	OH	H
13	Scopolin	H	H	H	OMe	OGl	H
14	Herniarin	H	H	H	H	OMe	H
15	Scoparone	H	H	H	OMe	OMe	H
16	Isofraxidin	H	H	H	OMe	OH	OMe
17	Isoscopoletin	H	H	H	OH	OMe	H
18	Hydrangetin	H	H	H	H	OH	OMe
19	3-methylcoumarin	Me	H	H	H	H	H
20	Pervilleanine	Ph	H	H	H	H	H
21	Coumarin	H	Ph	OH	Me	OH	3-phenylpropinol

Modification of the basic structure of coumarins resulted in the production of a large number of new molecules (Figure 3 and Table 1). Coumarins (Bourgaud et al., 2006) are sub classified into the following groups as demonstrated in (Table 2):

- Simple coumarins (benzo-α-pyrones or 1,2-benzopyrone): these molecules possess the same architecture than the "parent molecule" but are hydroxylated, alkoxylated, alkylated and glycosylated;
- Furanocoumarins (furocoumarins or furocobenzo-α-pyrones): these derivatives are developed by the attachment of a C5 furan ring to the basic coumarin core molecule. This group is further divided into linear and angular furanocoumarins by modification of the position of the furan ring;
- Pyranocoumarins (benzodipyran-2-ones): these molecules are similar to furanocoumarins except that they contain a C6 ring in place of a furan ring;
- Phenylcoumarins (benzo-benzopyrones): derivatives that are substituted in the pyrone ring.

Table 2. The four coumarins subtypes. Main structural feature and an example of each subtype is represented in the table

Classification	Features	Examples
Simple coumarins	Hydroxylated, alkoxylated or alkylated at benzene ring	 7-hydroxycoumarin
Furanocoumarins	Furan ring attached to the benzene ring. Linear or Angular forms are described	 Psoralen  Angelicin
Pyranocoumarins	Pyran ring attached to the benzene ring. Linear or Angular forms are described	 Xanthyletin  Seselin
Phenylcoumarins	Substitution on the pyrone ring, often at position C3 or C4	 Warfarin

Coumarins are found in many dicotyledonous plant families, including Apiaceae, Fabaceae, Moraceae, Rutaceae, Solanaceae, Rubiaseae, Rosaceae or Asteraceae (Ojala 2001). They are distributed in upper parts of plants i.e., leaves and stems (De Castro *et al.,* 2006) and their abundance in plant organs varies with the age of plants, seasonal changes and environmental conditions. Many coumarins produced by plants serve as phytoalexin and play

important role in the defense system against phytopathogens (Afek *et al.,* 1995; Gutiérrez-Mellado *et al.,* 1996; Kai *et al.,* 2006; Sharan *et al.,* 1998; Stange *et al.,* 1999). In most cases, the attack leads to an enhanced production of these molecules. Insect attack and mechanical injury constitute factors which have been reported for induced production of coumarins in plants (Olson *and* Roseland, 1991). Coumarins showed diverse bioactivities such as, antimicrobial (Ojala *et al.,* 2000), anti-inflammatory (Cho *et al.,* 2012), anticoagulant (Mueller 2004) and antifungal (Bais *et al.,* 2000; Cowan 1999). For example, two fungal strains including non-pathogenic *Helminthosporium carbonum* and pathogenic *Alternaria helianthi* were reported to be capable to induce the biosynthesis of scopoletin and ayapin in *Helianthus annuus* L (David *and* Robeson, 1985; Tal *and* Robeson, 1986). While *Fusarium oxysporum* could develop the similar type of reaction (induction of biosynthesis of scopoletin, scopolin and ayapin) against fungal attack in *Ipomoea purpurea* (Shimizu *et al.,* 2005). Furthermore induced biosynthesis of scopoletin was reported in *Hevea brasiliensis* using *Phytophthora palmivora* as elicitor (Churngchow *and* Rattarasarn, 2001).

Finally, to complete the picture, coumarins have also been described to give a bitter taste to forage plants (Gorz *and* Haskins, 1962).

1.2.2 **Biosynthetic pathway of coumarins**

The coumarin biosynthetic pathway branches out the general phenylpropanoid pathway at the cinnamic acid derivatives level as presented in (Figure 4). A large family of acyltransferase enzymes (D'Auria 2006) catalyzes the conversion of the *p*-coumaroyl CoA esters into *p*-coumaroyl shikimic and quinic acid esters (Comino *et al.,* 2007, 2009; Hoffmann *et al.,* 2004; Niggeweg *et al.,* 2004). Silencing of these genes in *Nicotiana benthamiana* and *Arabidopsis thaliana* resulted in dwarf plants and changed the lignin composition (Hoffmann *et al.,* 2004). The subsequent hydroxylation step is catalyzed by another cytochrome P450 enzyme which belongs to the CYP98A subfamily. The first committed enzyme described in this subfamily is CYP98A3 (Schoch *et al.,* 2001) which has been described to metabolize these shikimic and quinic esters to produce caffeoyl shikimate and caffeoyl quinate (chlorogenic acid) respectively.

The hydroxylation of cinnamic acid, *p*-coumaric acid, ferulic acid and caffeic acid at the position 2 (or 2' if the molecules are esterified with a Coenzyme A, a shikimic acid or a quinic acid) was until recently, supposed to be catalyzed by a P450 enzyme. Indeed, in earlier studies based on biochemical experiments, it was shown that a chloroplast fraction from

Melilolotus alba (Gestetner, and Conn 1974) and *Petunia hybrida* (Ranjeva *et al.,* 1977) contains the enzyme that is capable of catalyzing the *ortho*-hydroxylation of cinnamic acid, involved in the biosynthesis of coumarins. However these experiments couldn't be repeated using the approach explained in these studies. Early tracer experiments explained the synthesis of coumarins in plants *via* two steps. *Trans*-cinnamic acid is first *ortho*-hydroxylated and the resulting *o*-coumaric acid undergoes a *trans* to *cis* isomerization to form coumarins (Kosuge and Conn 1959). Experiments carried out on *Hierochloe odorata* and *Melilotus officinalis* supported this assumption for the biosynthesis of umbelliferone (umb) (Brown *et al.,* 1960; Stoker 1962). In addition, analyses carried out for labeled molecules using *Lavandula angustifolia* demonstrated that the *para*-hydroxylation of cinnamic acid is an essential step in the biosynthesis of umb before the step of *ortho*-hydroxylation.

More recently different studies demonstrated that this *ortho* hydroxylation step of cinnamic acid derivatives is catalyzed by an oxoglutarate-dependent dioxygenase. Early evidences for the implication of this enzyme family were provided by Kai and coworkers in 2008. These authors made a comparative analysis of the metabolic profiles of KO mutant and wild type *A. thaliana* plants. They showed that knock-out mutants for the corresponding CYP98A3 gene plants were unable to produce functional *p*-coumaroyl ester 3'-hydroxylase (CYP98A3) or caffeoyl CoA *O*-methyltransferase 1 (CCoAOMT1) and accumulated reduced contents of scopoletin and scopolin (Kai *et al.,* 2008). These preliminary experiments led the authors to hypothesize that the 2'-hydroxylated feruloyl CoA is a precursor of the synthesis of scopoletin (Kai *et al.,* 2008). Further experiment provided evidences to conclude that the enzyme able to hydroxylate feruloyl CoA was a Fe(II)/α-oxoglutarate-dependent enzyme, and was named feruloyl CoA-6'-hydroxylase (F6'H) (Figure 4 A). Another study carried out by Vialart and coworkers (2012) described the isolation and characterization of a *p*-coumaroyl CoA 2'-hydroxylase, involved in the biosynthesis of umbelliferone (a coumarin) in *Ruta graveolens*. This 2-oxoglutarate-dependent dioxygenase has been characterized as a bifunctional enzyme capable to metabolize both the feruloyl CoA into scopoletin and *p*-coumaroyl CoA into umb with an equal activity (Figure 4 B). Similar results were demonstrated using *ortho*-hydroxylases isolated from sweet potato (*I. batatas* (L.) Lam.) (Matsumoto *et al.,* 2012).

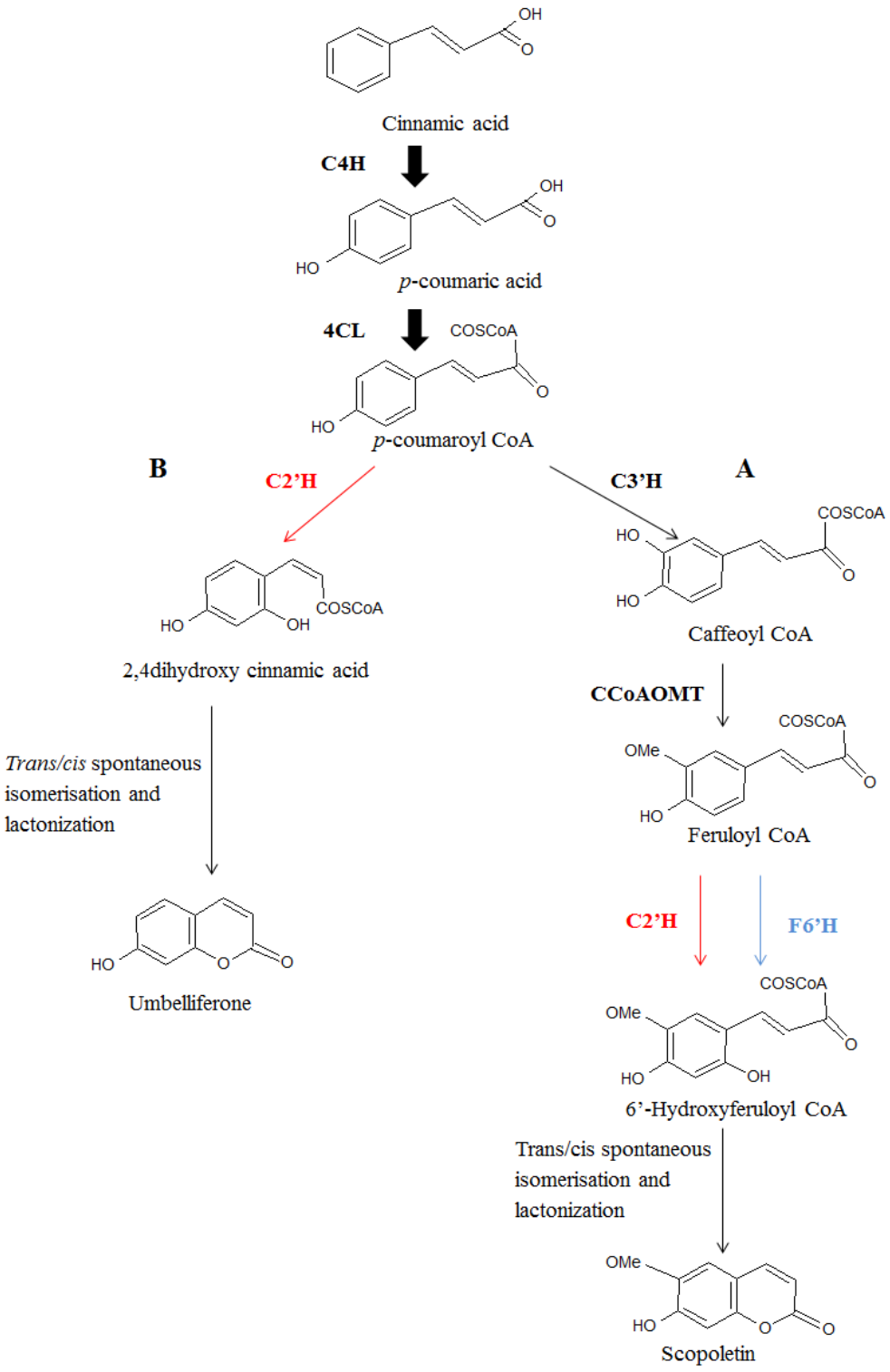
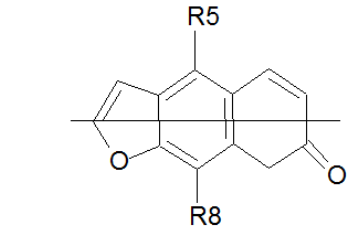


Figure 4. Biosynthetic pathway of coumarins in higher plants

(A); Biosynthetic pathway of scopoletin via F6'H is presented by blue arrow, B: biosynthetic pathway of umbelliferone and scopoletin via C2'H is presented by red arrows.PAL= Phenylalanine ammonia lyase, C4H= cinnamate 4-hydroxylase, 4CL= 4-Coumarate CoA ligase, C3'H= *p*-coumaroyl shikimate/quinate 3'hydroxylase, CCoAOMT1= Caffeoyl CoA *O*-methyltransferase, F6'H= Feruloyl CoA-6'-hydroxylase and C2'H= *p*-coumaroyl CoA 2'-hydroxylase.

1.3 Furanocoumarins

Furanocoumarins are obtained by the fusion of a coumarin moiety and a furan ring. Two different positions of the furan ring on the coumarin nucleus can be observed resulting in the production of either linear furanocoumarins or angular furanocoumarins (Table 2) (Figure 5). Further modifications of this basic heterocyclic molecule such as, hydroxylation, alkylation, methylation, prenylation or glycosylation resulted in the production of a very large family of molecules.

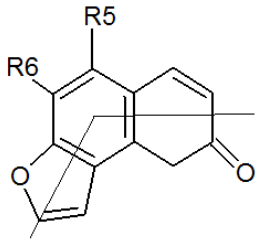


Psoralen = R5, R8: H

Bergapten = (5-MOP): OMe

Xathotoxin = (8-MOP): R8: OMe

Isopimpinellin = (5-8-MOP): R5, R8: OMe



Angelicin = R5, R6: H

Sphondin = R6: OMe

Pimpinellin = R5, R6: OMe

Figure 5. Simplified, basic chemical structure of linear and angular furanocoumarins

All these molecules can be detected within the same plant. Therefore different analytical method for isolation, separation and identification are used depending on the characteristic features of the molecules, supposed to be analyzed. Coumarins and furanocoumarins are more soluble in organic solvents such as, chloroform, diethyl ether, ethyl alcohol as well as in fats and fatty oils (Lozhkin *and* Sakanyan, 2006). These molecules have a characteristic absorbance spectrum at a wavelength of 210 nm to 320 nm due to presence of the aromatic ring (Lombaert *et al.,* 2001). Furanocoumarins can be separated efficiently using different technical approaches as thin layer chromatography (TLC), high performance liquid chromatography (HPLC), high performance liquid chromatography–mass spectrometry (HPLC–MS), high-speed countercurrent chromatography (HSCCC), gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS), capillary electrophoresis (CE) and pressurized capillary electrochromatography (pCEC) (Chen *et al.,* 2007; Wang *et al.,* 2007).

1.3.1 **Distribution of furanocoumarins in the plant Kingdom**

Furanocoumarins have been described in only 4 plant families: Apiaceae, Rutaceae, Fabaceae and Moraceae. Among these plant families, some contain only linear furanocoumarins such as Rutaceae (*R. graveolens, Citrus bergamia*…. etc), Moraceae (*Ficus carica*) and some species of Apiaceae (*Ammi majus*) (Damjanic *and* Akacic, 1974; Gattuso *et al.,* 2007; Pokrovskii *et al.,* 2009; Schimmer *and* Kühne, 1990). Other plant families have acquired the ability to synthesize both the linear and angular furanocoumarins such as, leguminoseae (genera Psoralea and Coronilla) and certain species of Apiaceae family (*Apium graveolens, Pastinaca sativa*) (Bourgaud *et al.,* 1994; Innocenti *et al.,* 1997; Sah *et al.,* 2006). However no plant is known till now, capable to synthesize only angular furanocoumarins.

In addition to these major four plant families, furanocoumarins have also been reported in low concentrations from other plant families also such as Asteraceae, Pittosporaceae, Rosaceae, Solanaceae and Thymelaeaceae (Bourgaud *et al.,* 2001; Murray *et al.,* 1982).

1.3.2 **Role of furanocoumarins**

Furanocoumarins, as coumarins, are produced by plants as phytoalexins (Beier *and* Oertli, 1983). Being considered as defense molecules they can be produced as a result of elicitation by biotic stresses (insects, microorganisms, and fungi) or abiotic stresses (UV radiation, environmental pollutants and mechanical breakage).

These molecules have been described as allelochemicals and can affect the growth and development of surrounding biological systems (Razavi 2011). For example, plant extracts containing furanocoumarins, prepared from Umbelliferae demonstrated potential inhibition of germination of anastatica, tomato and lettuce (Friedman *et al.,* 1982), while extracts prepared from Rutaceae family could inhibit the germination and growth of *Amaranthus hypochondriacus* (prince's feather) and *Echinochloa crus-galli* (Cockspur Grass)(Baskin *et al.,* 1967; Hale *et al.,* 2004; Luisa *et al.,* 2005).

Another work reported that the furanocoumarin production can be induced in plants as a response to pathogen attack (Solecka, 1997) and in addition, various studies demonstrated the presence of insecticidal properties in plant extracts and provided the evidence that these properties are correlated to furanocoumarins contents of extracts (Pavela *and* Vrchotová,

2013; Stevenson *et al.,* 2003). These molecules were described to be lethal for insects as they can act as anti-feeding molecules, growth inhibitors and chronic toxics (Guo *et al.,* 2012; Pavela and Vrchotová 2013). In addition, they display anti-feedant properties for herbivores (Berenbaum 1978). Taken together, all these elements make evidence that furanocoumarin are important in the plant defense system against phytopathogenic organisms.

As these molecules are involved in defense responses, their synthesis can be induced by various elicitors. Different environmental aggressors have been listed, which can induce the biosynthetic pathway of furanocoumarins e.g. pathogens and herbivores which are biotic aggressors (Kitamura *et al.,* 1998; Zangerl *et al.,* 2002). Abiotic stresses, as radiation (UV), abnormal concentrations of environmental pollutants or the nutrients were also described as potent inducers (Dercks *et al.,* 1990; Eckey-kaltenbach *et al.,* 1994; Zangerl *and* Berenbaum, 1987; Zobel *and* Brown 1993). For example, furanocoumarins content can be modified *in vitro* or *in vivo* in several plants by the addition of pathogen extracts either in the culture medium or by direct application on intact plants or cells in culture. Indeed treatment of cultured parsley cells (*Petroselinum hortense)* with different phytopathogenic fungi (*P. megasperma* (*Pmg)* and *A. carthami)* showed increased production of furanocoumarins (Tietjen *et al.,* 1983). Going in the same way, another study provided evidences that a similar increase in furanocoumarin concentration was observed when cultured cells of *A. majus* were treated with *P. megasperma* (Matern *and* Daria, 1988). The impact of abiotic stresses has been studied through longer exposure to UV light that can serve as an efficient elicitor for the induction of their synthesis in plants (Zobel and Brown 1993), which ultimately results in the production of thick epicuticular layer and decrease the amount of UV penetrating in leaves (Martin *et al.,* 1991). Similarly induced biosynthesis of furanocoumarins could be obtained more efficiently by controlling other factors along with the elicitors. For example, depletion of growth hormone (2,4-dichlorophenoxyacetic acid and benzylaminopurine) from media of *Angelica archangelica* cultured cells grown in dark in the presence of *Pythium aphanidermatum*, resulted in a 15 times increase of production of coumarins in comparison to the controlled plants (Siatka *and* Kašparová, 2009).

1.3.3 **Localization of furanocoumarins in plants**

Furanocoumarins are produced essentially in leaves, roots and fruits of plants (Page *et al.,* 2006; Pathak *et al.,* 1962; Zobel *et al.,* 1991; Zobel *and* Brown, 1990). To a lesser extent, their presence has also been reported in seeds and embryos of Leguminoseae, Umbelliferae

and Rutaceae (Milesi, 2001; Zobel *and* Brown, 1990). However, for *R. graveolens* a member of Rutaceace family, accumulation of furanocoumarins in roots was also reported (Milesi *et al.,* 2001).

Moreover, accumulation of furanocoumarins has also been observed on the leave surfaces and in seeds (Zobel and Brown 1991; Zobel *et al.,* 1991). This storage pattern of furanocoumarins on either the reproductive parts or the parts mostly exposed to predation is explained as an evolution mechanism by the optimal defense theory (Wittstock *and* Gershenzon, 2002; Zangerl *and* Rutledge, 1996) and supports the fact that they are involved in plant defense system against predation by aggressors (Milesi, 2001).

Depending on the plant system, synthesis of furanocoumarins can be constitutive or inducible. They are mostly produced in a constitutive manner in plant organs that are at highly exposed to attack, while induced production is predicted for other parts of the plants (Wittstock *and* Gershenzon, 2002). For example in wild parsnip (*P. sativa*) reproductive organs that are frequently attacked by herbivores showed high constitutive synthesis levels of xanthotoxin (8-MOP). In contrast, roots that are rarely attacked demonstrated low concentrations of this molecule even if it readily increases through wounding (Zangerl and Rutledge 1996).

1.3.4 **Storage of furanocoumarins in plant cell**

Furanocoumarins are toxic molecules (see below, paragraph 1.3.6) and can be lethal for the plant cells if present in too large amounts. To avoid cell death, various mechanisms for storing the excess of furanocoumarins have been developed by plants.

One of these mechanism, involves the glycosylation of furanocoumarins in the cytosol by specific enzymes which results in the production of less toxic glycosylated forms (Taguchi *et al.,* 2000). These molecules can easily be remobilized, when a pathogen attacks the plant, by releasing aglycon forms of furanocoumarins through the action of β-glucosidases (Berenbaum, 1991; Wittstock *and* Gershenzon, 2002).

The presence of psoralen and 8-MOP has also been found in xylem vessels through an approach using immuno-histolocalization (Massot *et al.,* 2000). This localization of furanocoumarins is probably related to a systemic release of the molecules through the plants.

1.3.5 **Biosynthetic pathway of furanocoumarins**

The branch point starting from the core part of the phenylpropanoid pathway leads to the synthesis of Umb (7-hydroxycoumarin) and has been associated to an oxoglutarate dependent dioxygenase in *R. graveolens* and in *I. batatas* (1.2.2).

Hence, the first specific step devoted to the furanocoumarin pathway is the prenylation step of Umb (Figure 6). Addition of isoprene unit occurs either on C6 position (linear furanocoumarins) or C8 position (angular furanocoumarins) to give rise to demethylsuberosin (DMS) (Figure 6 (6)) (linear furanocoumarin) or osthenol (Figure 6 (14)) (angular furanocoumarin) respectively. Early reports for the presence of an enzyme in plants capable to catalyze the addition of isoprene unit to Umbe were provided by the work of Ellis and Brown in 1974 (Ellis and Brown 1974). Their work supported the existence of a specific transferase that can transfer dimethylallylpyrophosphate (DMAPP) to the C6 position of Umb and results in the formation DMS. Another study conducted by Dhillon and Brown (1976) dedicated to the biochemical characterization of this enzyme in *R. graveolens* demonstrated that for an optimal activity of the enzyme, the presence of a divalent cation (Mg^{2+}) is required. This work demonstrated the existence of an enzyme that displays a high specificity for an isoprene unit DMAPP and for the position of the attack on the molecule. In this case, no prenylation at position C8 could be detected. These authors also provided evidence, by using tracer molecules, that the enzyme responsible of the DMAPP transfer is localized in chloroplasts (Dhillon and Brown 1976). Hamersky and collaborators showed that two prenyltransferases (Pts) with different activities were produced in *P. megasperma* treated *A. majus* cells (Hamerski *et al.,* 1990). Their biochemical characterization showed that one of these enzymes was able to mediate the prenylation at position C6 of Umb in the presence of Mg^{2+} resulting in the formation of DMS. The second enzyme was found to be an *O*-prenyltransferase that can catalyze an *O*-prenylation of Umb at position O7 resulting in the formation of *O*-prenyl Umb.

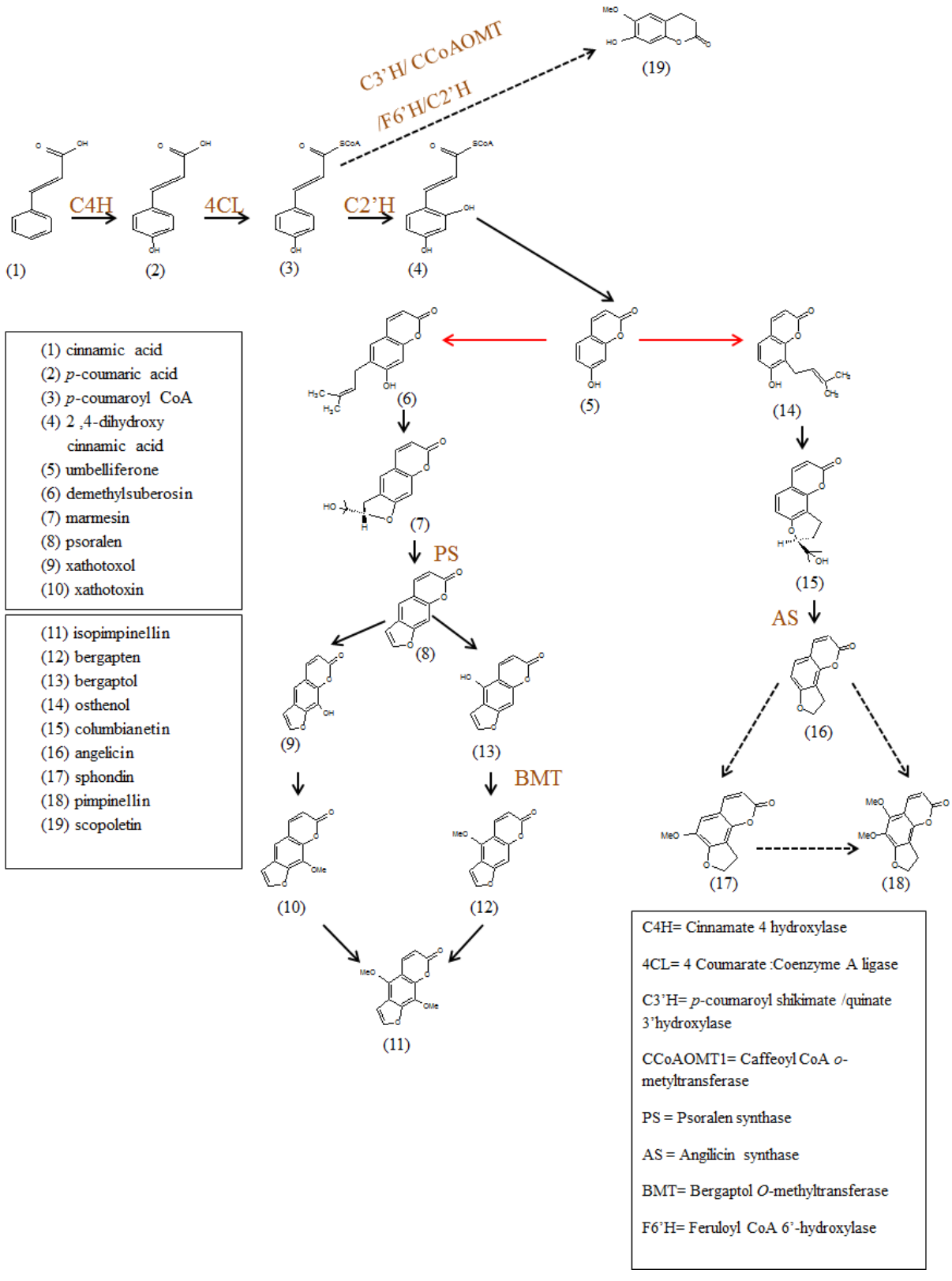


Figure 6. Hypothetic pathway of the furanocoumarin biosynthesis along with the recently explored biosynthetic pathway of scopoletin

Enzymes involved in the pathway which have been isolated and characterized are represented with their names. Branching point of the pathway for successive synthesis of linear and angular furanocoumarins mediated by Pts are showed by red arrows.

This study also reported the Pts as membrane bound enzymes, which were located in the endoplasmic reticulum. Concerning the isoprene moiety, experiments using labeled molecules in *A. graveolens* reported that DMAPP needed for the 6-prenylation of Umb is synthesized through the non-mevalonate 1-Deoxy-D-xylulose (DOX) pathway (Volker *et al.,* 1999). However, all these studies used biochemical approaches to explore these steps and did not provide any clue concerning the genes involved. These steps of biosynthetic pathway will be discussed in details latter under the heading (2.1.1.1).

The second specific step in the furanocoumarin biosynthetic pathway is accomplished by the conversion of DMS into marmesin (Figure 6 (7)) for linear furanocoumarins, and the conversion of osthenol into columbianetin (Figure 6 (15)) for angular furanocoumarins (Bourgaud *et al.,* 2006). This step always remained elusive at the molecular level although, Hamerski and Matern (Hamerski and Matern 1988) demonstrated using a biochemical approach that the mechanism of reaction is P450-dependent. The subsequent step leading to the synthesis of psoralen (Figure 6 (8)) and angelicin (Figure 6 (16)) has been precisely described. This reaction is catalyzed by two P450s of the CYP71AJ family in Apiaceae. These enzymes known as psoralen synthase (PS) and angelicin synthase (AS) cause the elimination of an acetone group of DMS and columbianetin (Larbat *et al.,* 2007; 2009). Both P450s enzymes are localized in the endoplasmic reticulum in *A. majus* (Hamerski and Matern 1988). DMS and columbianetin are further hydroxylated at either position C5 (Matern *and* Daria, 1988) or position C8 to form xanthotoxol and bergaptol respectively. These molecules are rapidly metabolized by *O*-methyltransferases to give the final products of this pathway (Figure 6 (11)) (Figure 6 (17)) (Figure 6 (18)).

1.3.6 **Biological properties of furanocoumarins**

Furanocoumarins are bioactive molecules that interact with a wide range of bio-macromolecules such as deoxyribonucleic acid (DNA), protein and lipid (Schoonderwoerd *et al.,* 1991) especially when they are subjected to ultraviolet radiation (UV-A, 320-400 nm) (Potapenko *et al.,* 2004). They generally develop adducts through the process of photobinding (Beijersbergen van Henegouwen *et al.,* 1989*)*.

1.3.6.1 Photolysis and photo-oxydation of furanocoumarins

Furanocoumarin undergo photolysis when UV radiation is applied to their aqueous solutions. This process results in the formation of many photoproducts depending upon the nature of the molecule and experimental conditions applied (Figure 7). These photoproducts have been grouped into the two classes on the basis of the mechanism of their production: developed through anoxic mechanism (products formed through cyclodimerization or solvent addition) or oxic mechanism (products arise by oxidative breakage of molecules or oxidative addition of solvent to molecules)(Caffieri, 2002).

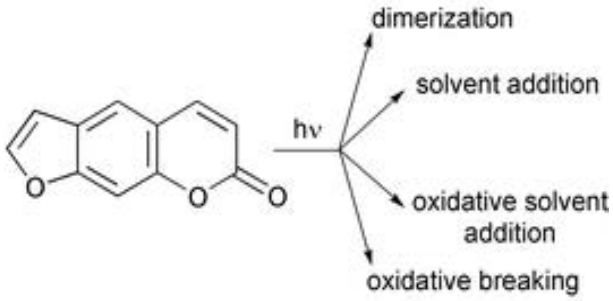


Figure 7. Schematic representation of photolysis and photoproducts of furanocoumarins (Caffieri, 2002)

3-carbethoxyangilicin is a synthetic furanocoumarin that is highly sensitive to UV radiation and rapidly undergoes photolysis. Only one photoproduct of this molecule has been identified that is a single oxygen and is capable to induce single stand breaks in DNA *in vivo* (Marzano *et al.,* 1997). UV treated furanocoumarins can also form active oxygen species (O^{-}_2, H_2O_2, and OH^{-}), which have already found to be involved in the process of apoptosis (Mignotte *and* Vayssiere, 1998).

1.3.6.2 Photocylcloaddition and photo-dimerization

Furanocoumarins and coumarins can develop dimers through cyclo-dimerization and results in the formation of various configurational isomers (Zdero *et al.,* 1990; Rojas-Lima *et al.,* 1999). This dimerization can occur at the C3=C4 double bond level and results in the formation of four isomers; *trans*-head-to-head (tHH), *trans*-head-to-tail (tHT), *cis*-head-to-head (cHT), and *cis*-head-to-tail (cHT) (Schönberg *et al.,* 1968) (Figure 8) in case of coumarins, and principally two isomers; *trans*-head-to-head (tHH), *trans*-head-to-tail (tHT)

for furanocoumarins (Krauch *et al.,* 1965; Zdero *et al.,* 1990). This process of dimerization can result in the production of molecules that are of great value for pharmaceutical industry (light-sensitiveintelligent drug dilvery systems and disease therapy fields) (Lin *et al.,* 2010).

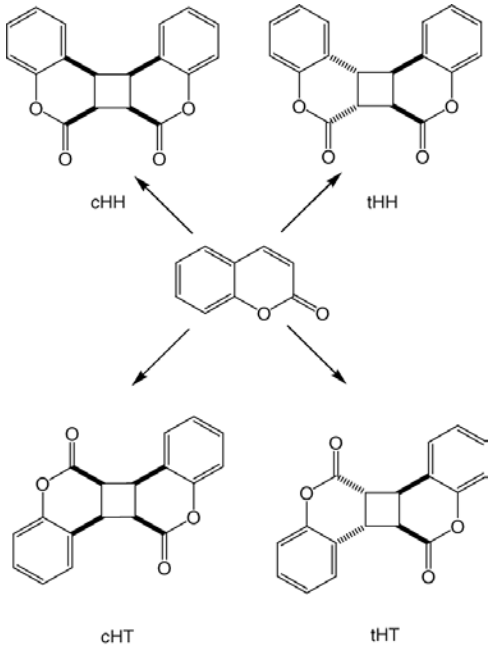


Figure 8. Photo cyclo-dimerization of coumarins (Kitamura *et al.,* 2005)

(*trans*-head-to-head (tHH), *trans*-head-to-tail (tHT), *cis*-head-to-head (cHT), and *cis*-head-to-tail (cHT))

Psoralen interaction with unsaturated molecules to form dimers is a very complex interaction (Figure 9). Mono adducts are formed when psoralen interacts with a molecule at the C3=C4 double bond level, the resultant cyclobutane can be *cis* and *trans* or *sys* and *anti,* depending upon the orientation of the X molecule (modified molecule due to addition of photoreactive molecule) with respect to the psoralen. One cyclobutane can give rise to four isomers thus psoralen can have eight isomers.

In addition to the C3=C4 double bond of psoralen, the C4'=C5' can undergo photoinduced cyclization and form double adducts with a double number of isomers (16).

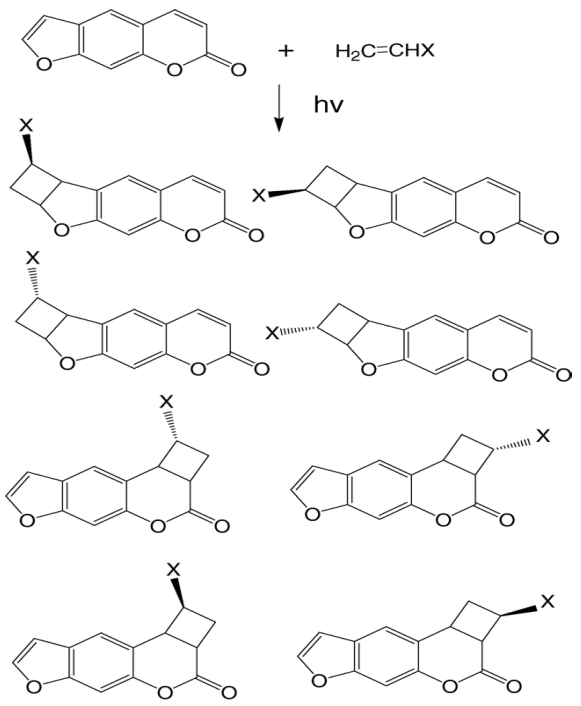


Figure 9. Photocyloaddition products of psoralen with unsaturated ethylene (Lin *et al.*, 2010)
(Eight isomers of mono adducts of psoralen with ethylene)

1.3.6.3 Reaction of furanocoumarins with nucleic acid

Photoaddition of furanocoumarins in nucleic acid is known since very long time (Kittler 1976; Musajo *et al.,* 1965). It has been found that the reaction of photosensitization of DNA in the presence of psoralen and its derivatives occurs in the nucleus (Cadet *et al.,* 1992; Sasaki *et al.,* 1988).

8-MOP, another furanocoumarin, can intercalate into DNA after activation by UV radiation. Once activated, the molecule reaches into the nucleus and integrates itself into DNA. This process occurs in three steps; (i) The 8-MOP enters into paired bases of DNA chain, (ii) it absorbs a photon of UV light and forms the bond with nucleotide present on one base. Finally, (iii) after absorbing another photon, it can establish a bond with the base present on the other strand (Figure 10) (Kitamura *et al.,* 2005). Besides these observations, it has been observed that psoralen, when subjected to light of a specific wavelength (320-410nm), interacts with DNA through a covalent bond by the same mechanism as explained above (Figure 11). This newly formed structure can block any DNA interaction with any transcriptase and polymerase and therefore inhibit the replication (Rocha *et al.,* 2004). Psoralen has been found to make photoadducts with pyrimidine bases, and preferentially with thymine bases rather than with adenine bases.

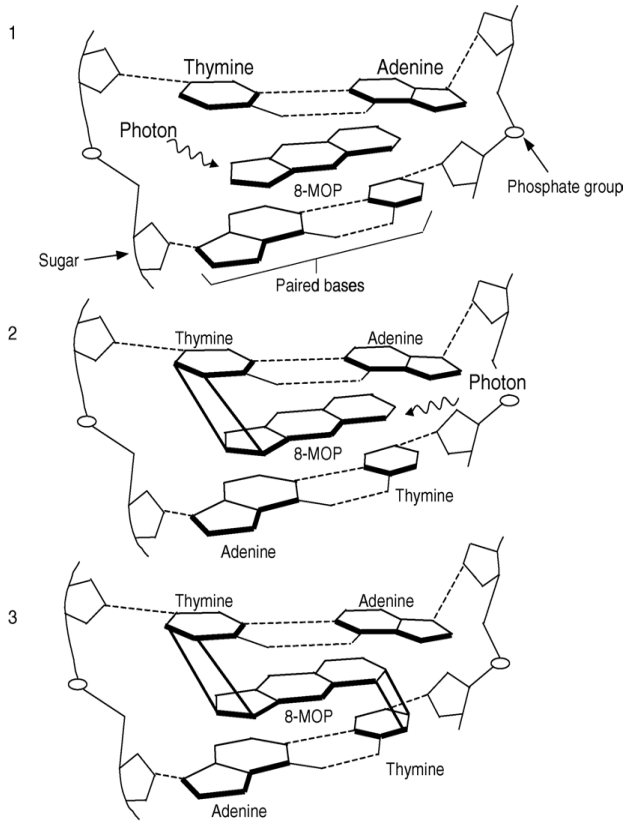


Figure 10. Schematic representation of the chemical bond formation between Xanthotoxin (8-MOP) and nucleotide bases of different DNA strands (Kitamura *et al.*, 2005)

DNA portion saturated with sequences d(TpA):d(TpT) are preferred for formation of bond by furanocoumarins (Friedberg *et al.,* 2005; Houten *et al.,* 1986).This mechanism explains the capability of furanocoumarins to be mutagenic agents (Bernd *et al.,* 1999; Kevekordes *et al.,* 1999).

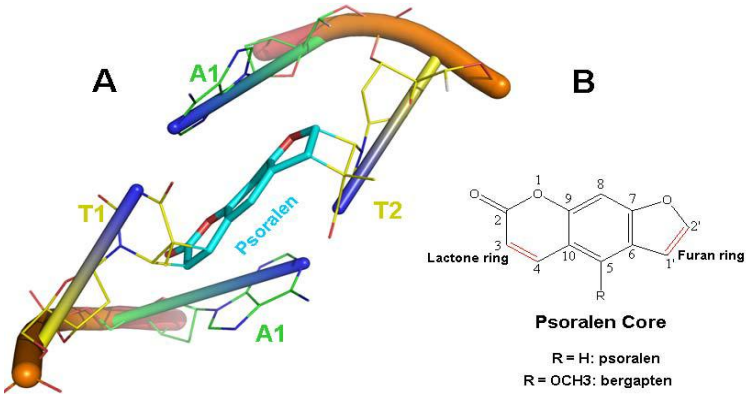


Figure 11. (A) Model prepared for demonstrating the cross linkage of psoralen between thymine bases of DNA (T1-T2)

Psoralen intercalated between adenine and thymine. (B) Psoralen core structure. Furan ring and lactone ring that form covalent bonds are shown in red color (da Silva *et al.,* 2009).

1.3.6.4 Reaction of furanocoumarins with lipids

Furanocoumarins subjected to UV light can damage the cellular membranes which are mainly made up of lipids. Two types of interaction between furanocoumarins and lipids membranes have been observed; (i) oxygen dependent reaction between furanocoumarins and lipids results in lipid peroxidation while (ii) oxygen independent interaction result in cycloaddition of furanocoumarins in unsaturated fatty acids (Dall'Acqua *and* Martelli, 1991). C3=C4 covalent bonds of pyran ring and C4=C5 covalent bond of furan ring to form cycloadducts, are established by furanocoumarins with lipids by the same mechanism as explained earlier for nucleic acid (Dall'Acqua and Martelli 1991; Frank *et al.,* 1998). This type of reaction can be used for psoralen ultraviolet-A treatment (Dos Santos *and* Eriksson, 2006).

Photoaddition of furanocoumarins to phospholipids occur through cyclobutane, from which photoadducts of fatty acids are released through the action of enzyme known as phospholipase (PAL_2). These furanocoumarin-fatty acid adducts are transferred from the membrane to the cytosol, where they can enhance the 1,2-dioctanoylglycerol (DAG) dependent activation of a protein kinase C (PKC) (Anthony *et al.,* 1997) and, hence, can induce melanogenesis in human cells through unknown pathway (Gordon *and* Gilchrest, 1989) (Figure12).

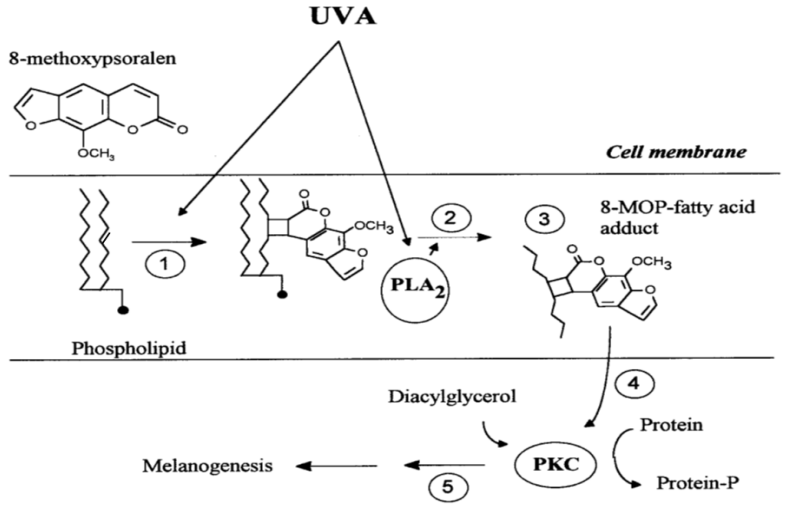


Figure12. Photoaddition of furanocoumarins to unsaturated fatty acids

Schematic representation of a proposed pathway of biosynthesis of melanogensis by photoaddition of furanocoumarin to unsaturated fatty acids (Zarebska *et al.,* 2000). Circled numbers indicated the different stages of reactions where, (1): UVA-excited 8-MOP undergoes cyclobutane-type attachment to phospholipids, (2): PLA_2 utilize 8-methoxypsoralen-phospholipid photoadductsas substrate to generate 8-methoxypsoralen-fatty acid adducts, (3): 8-MOP-fatty acid adducts are transferred to the cytosol, (4): protein kinase C is activated through DGA and (5): finally lead to the melanogenesis.

1.3.6.5 **Reaction of furanocoumarins with proteins**

Furanocoumarins interact not only with DNA and lipids but also with proteins. Epidermic cells of rats treated successively with 8-MOP and UVA, demonstrated that 17% of 8-MOP is bound to DNA and 26% to lipids while the rest is found to be linked with proteins (57%) (Bensasson *et al.,* 1978). Furanocoumarins could form photoadducts through photoaddition to a wide range of proteins that includes ribonucleases, bovine serum albumin, lysozyme, nucleic histone, glutamate dehydrogenases and *Escherichia coli* DNA polymerases (Schmitt *et al.,* 1994). In addition it has been found that the presence of oxygen is necessary to all these reaction except for the reaction mediating interaction of furanocoumarin with DNA polymerase I (Schmitt *et al.,* 1995). A photoadduct formed by bonding of psoralen with tyrosine was characterized by HPLC, UV-visible absorption, fluorescence and mass spectrometry (MS) (Sastry, 1997). Photoaddition of furanocoumarins to a wide range of proteins results in the production of prominent effects such as modification of intracellular signals, induction/repression of transcription factors and inactivation of enzymes, etc...).

Furthermore, furanocoumarins have been documented as potent inhibitors of P450s of insects and vertebrates (Fouin-Fortunet *et al.,* 1986; Koenigs *and* Trager, 1998; Mays *et al.,* 1989; Zumwalt *and* Neal, 1993). They are refereed as "suicide inhibitors" or mechanism-based inactivators (MBIs) because of their ability to bind irreversibly to enzymes, after their activation into reactive electrophilic intermediates by P450s enzymes (Mays *et al.,* 1989). However it is obvious that plants having high concentrations of furanocoumarins are surviving in nature also, these observation thus provide the clue that their P450s have some characters that make them different from the P450s exist in a non furanocoumarin producing plants. The verification of this hypothesis was done through a study carried out by Gravot and his collaborators, in which the researchers compared the inhibition of C4H of furanocoumarin producing plants (CYP73A32 isolated from *R. graveolens* and CYP73A10 isolated from *P. crispum*) with the C4H from a plant that doesn't produce furanocoumarins (CYP73A1 from *H. tuberosus)*. Both cytochromes CYP73A32 and CYP73A10 showed less sensitivity to furanocoumarins (Gravot *et al.,* 2004). More recently the functional characterization of a C2'H of *R. graveolens*, demonstrated that psoralen in high concentration can exert a feedback inhibition on this enzyme, might be involved in down regulation of coumarin/furanocoumarin biosynthetic pathway and therefore influence the amounts of furanocoumarins produced in plants (Vialart *et al.,* 2012).

1.3.7 **Furanocoumarins in the pharmaceutical industry**

Furanocoumarins have been used in traditional medicine for different skin diseases from a long time ago. Ancient Egyptians used the extracts of *A. majus* for the treatment of leukoderma (vitiligo). Nowadays as the physical properties are better characterized, furanocoumarins represent an interesting source of molecules for the treatment of many diseases.

1.3.7.1 **Photochemotherapy (PUVA)**

Phototherapy uses an artificial source of UV radiation for the treatment of skin diseases (Conforti *et al.,* 2009) while photochemotherapy consists to simultaneously use a photoabsorbing molecule along with the ultraviolet light to treat some of the diseases (Parrish *et al.,* 1974). PUVA therapy is a term used for photochemotherapy when psoralen is used in combination with UV-A (320-340nm). Methoxalen (i.e. methoxypsoralen), 5-methoxypsoralen and 4,5',8-trimethylpsoralen are commonly used for such treatment (Danno, 1999; Marzano *et al.,* 2002; Roelandts 1991). Three mechanisms of application of P-UVA have been used depending on the administration of the photoabsorbing molecule, that could be oral, topical or in bathes (Danno, 1999; Santana *et al.,* 2004) followed by UV exposure. Concerning the mechanisms involved, it has been demonstrated that furanocoumarins are not only able to form adducts with DNA and, hence, could be antipoliferative, but they can also induce immunosuppression and apoptosis in living cells (Godar, 1999; Moor *and* Gasparr, 1996). This protocol is used to treat a large number of diseases such as psoriasis, eczema, graft-versus-host disease, vitiligo, *mycosis fungoides*, large-plaque parapsoriasis and cutaneous T-cell lymphoma (Domínguez *et al.,* 2004; James *et al.,* 2006; Poiesz *et al.,* 1980).

For example, psoriasis is characterized by epidermal thickness due to a hyperproliferation, an abnormal differentiation and a decreased rate of cell apoptosis that leads to hyperkeratosis (Wrone-Smith *et al.,* 1997). Thus the ability of furanocoumarins to form photoadducts of DNA and ultimately block the replication helps to cure the disease (Calzavara-Pinton et al. 1997; Sasaki *et al.,* 1988). Vitiligo is another disease characterized by the depigmentation of skin due to a loss of normal functioning of melanocytes related to either autoimmune system or apoptosis (Conforti *et al.,* 2009).

Cutaneous T cell lymphoma (CTCL) is a type of cancer of the immune system, which is caused by mutations in T-cells (Poiesz *et al.,* 1980). The mutated cells are transferred to

skin and generate lesions. A new type of extracorporeal PUVA therapy or photopheresis has been developed, which is a cell-based immunomodulatory therapy and involves a collection of leukocytes from blood. These collected cells are then exposed to photosensitizing furanocoumarins and subjected to UV radiations. These cells are then re-infused into the body of the patient. This treatment results in the formation of photoadducts of DNA pyrimidine bases and can cause massive apoptosis of treated cells (Edelson *et al.,* 1987; Rook et al. 1989). In addition, this technique has been used for the treatment of graft-versus-host disease (Klassen, 2010).

1.3.7.2 Other implications of furanocoumarins as pharmaceutics

Depression is a disease that may arise due to abnormalities in catabolism of monoamines (MO), lipid metabolism (MDA) or hypothalamic–pituitary–adrenal (HPA) axis. Manic-depressive patients also display increased activities of superoxide dismutase (SOD) (Abdalla *et al.,* 1986; Bao *et al.,* 2005; Bilici *et al.,* 2001). Recently a study done by Chen and colleagues (Chen *et al*. 2007) showed that a total furanocoumarin contents (TFC) extract from seeds of *Psoralea corylifolia* can reverse the defects induced by chronic mild stress in mice. These results suggest that TFC are potent antidepressant that can regulate the functioning of MO, MDA, HPA axis and SOD (Chen *et al.,* 2007).

In addition furanocoumarins possess bacteriostatic properties. They are able to inhibit the formation of biofilms synthesized by bacteria such as *E. coli O157*, *Salmonella typhimurium* and *Pseudomaonas aeruginosa* for any protection purpose. They can also inhibit the activities of molecules necessary for intra and interspecific communication between gram positive and gram negative strains for the expression of pathogen genes (Girennavar *et al.,* 2008). Geranylated furanocoumarins isolated from rutaceae family showed antimycobacterial properties against *Mycobacterium fortuitum*, *M. smegmatis* and *M. phlei* (Adams *et al.,* 2006, 2007), and could therefore be used in treatment of infection caused by these microorganisms.

It has been reported that angular furanocoumarins (angelicin and its derivatives) possess antifungal properties against *Candida albicans, Cryptococcus neoformans, Saccharomyces cerevisiae* and *Aspergillus niger*. Furthermore these molecules were also found to be less toxic against KB cell line of human cells (Sardari *et al.,* 1999).

1.3.8 **Toxicity caused by furanocoumarins**

Beside the fact that furanocoumarins are used for the treatment of many diseases, they can also be at the origin of various ailments.

1.3.8.1 **Phytophotodermatitis**

Phytophotodermatitis is a skin disease that develops through the contact of skin with photosensitizing molecules (like furanocoumarins) of plants and successive exposure to UV light. It can be characterized by the appearance of either one or more symptoms, which includes erythematous plaques, edema, vesicles and blisters. These symptoms appear normally within 5 to 7 days after the exposure and are followed by hyperpigmentation (Moreira *et al.,* 2010). Earlier studies showed that a small number of plant families can cause this toxicity of skin notably Rutaceae, Apiaceae and Moraceae (Roland, 1997), but recent observation showed that plants belonging to the Chenopodiaceae family could also cause phototoxic dermatitis (Bilgili *et al.,* 2011) due to the presence of furanocoumarins.

1.3.8.2 **Melanoma**

Melanoma has been described as a cancer of melanocytes (cells producing melanin in human body). A study demonstrated that patients undergoing PUVA treatment display high risks to develop melanoma, especially those who received 250 or more treatments of PUVA (Stern *et al.,* 1997). Furanocoumarins are present at high concentration in citrus (belonging to the Rutaceae family). A recent study showed that a group of nurses preferentially taking lemon juice for a long time are at high risks to develop cutaneous melanoma (Feskanich *et al.,* 2003). It was also demonstrated that increased incidence of melanoma is directly correlated with P-UVA treatments and proportion of photocarcinogenic furanocoumarins in diet (Sayre *and* Dowdy, 2008).

1.3.8.3 **Interaction of furanocoumarins with drugs**

Cytochrome P450s play an important role in the metabolisation of drugs and it has been found that 60% of currently marketed drugs are metabolized by furanocoumarins, and hence are considered important in the process of drug interaction with human body. Many different furanocoumarins present in the juice of grapefruit can have inhibitory effects on the activity of CYP450s enzymes. It has been found that grape fruit juice can inhibit different families of cytochrome i.e.CYP3A4, CYP2C9, and CYP2D6. In addition 5 furanocoumarins (paradisin

A, dihydroxybergamottin, bergamottin, bergaptol and geranylcoumarin) display inhibitory effects against these three families of cytochromes P450s (Girennavar *et al.,* 2007).

2 Prenyltransferases

As it has been discussed under heading (1.3.5) of this manuscript, prenylation of umbelliferone is a key step in the biosynthesis of furanocoumarins. None of the enzymes has yet been described in the biosynthetic pathway of these molecules. However a number of Pts encoding genes have been characterized in other secondary metabolic pathways.

Prenylation consists to add a hydrophobic isoprenoid (Figure 13) side chain of different lengths (5 C atoms: DMAPP), 10 C atoms: geranyl diphosphate (GPP), 15 C atoms: farnesyl pyrophosphate (FPP), 20 C atoms geranyl-geranyl pyrophosphate (GGPP) and phytyl diphosphate (PDP) to an accepting molecule (Heide, 2009). This prenylation reaction plays a crucial role in the biosynthesis of chemically complex and structurally diverse molecules, performing important biological functions in phylogenetically diverse groups of organisms i.e. bacteria, fungi, plants and mammals. Further, it has been reported that these prenylated products are biologically more active than their parent molecules. Enzymes that are able to catalyze these prenylation reactions are known as Pts (Liang *et al.,* 2002).

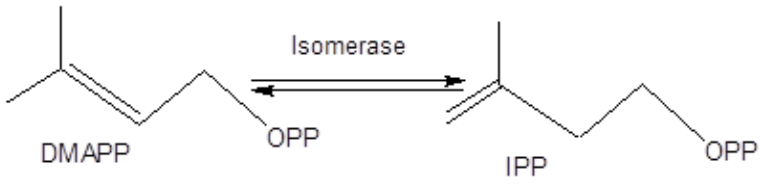


Figure 13. Basic unit of hydrophobic isoprenoid chain

2.1 Role of prenyltransferases in plants

Plant Pts are involved in the biosynthesis of numerous primary and secondary metabolites and prenylated proteins of plants.

2.1.1 Implication of prenyltransferases in the biosynthesis of primary metabolites

Pts play a pivotal role in the biosynthesis of many essential primary metabolites which are necessary for the survival of plants. Some important classes are described here.

2.1.1.1 Biosynthesis of isoprenoids

Isoprenoids, in plants are described both as primary and secondary metabolites. Here I will focus only on the products of this pathway involved in primary metabolism of plants while those involved in secondary metabolites will be discussed in paragraph (2.1.2) These molecules (which are also called terpenoids) constitute the most functionally and structurally diverse group of plant metabolites. They are synthesized by all organisms but are highly diverse and abundant in plants, with thousands of natural products described to date (Chappell, 1995, 2002; McGarvey *and* Croteau, 1995) and many enzymes involved in their biosynthesis have been documented in literature.

2.1.1.1.1 Isopentenyl diphosphate isomerase (IPPI)

This group of enzymes catalyzes the chain elongation of allylic pyrophosphate substrates through consecutive condensation reactions with isopentenyl pyrophosphate (IPP) (Figure 14). All these enzymes possess two conserved DDxxD motifs that coordinate with two or three Mg^{2+} ions in the reaction mixture, to serve as binding sites for both the allylic and homoallylic substrates (Guo *and* Yamazoe, 2004; Hosfield *et al.,* 2004; Tarshis *et al.,* 1996).

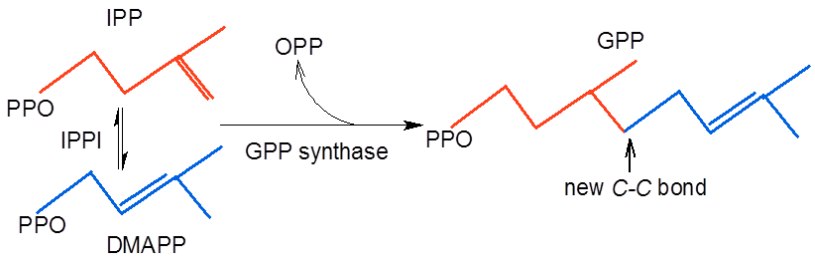


Figure 14. Formation of GPP

2.1.1.1.1.1 Reaction mechanism of GPP synthase

The reaction mechanism of these enzymes could be explained by three consecutive steps i.e. ionization-condensation-elimination. In the ionization process a chemical group is leaving, leading to the formation of a carbocation. For example, in (Figure 15), the PPO (pyrophosphate) group of DMAPP is leaving, resulting in the production of an allylic carbocation. During the condensation step (addition), the double C-C bond of IPP attacks the electrophilic carbon of DMAPP, leading to the formation of a new carbon-carbon bond and a

second carbocation intermediate. A proton is then released in the elimination phase, and a double bond is reestablished in the GPP product.

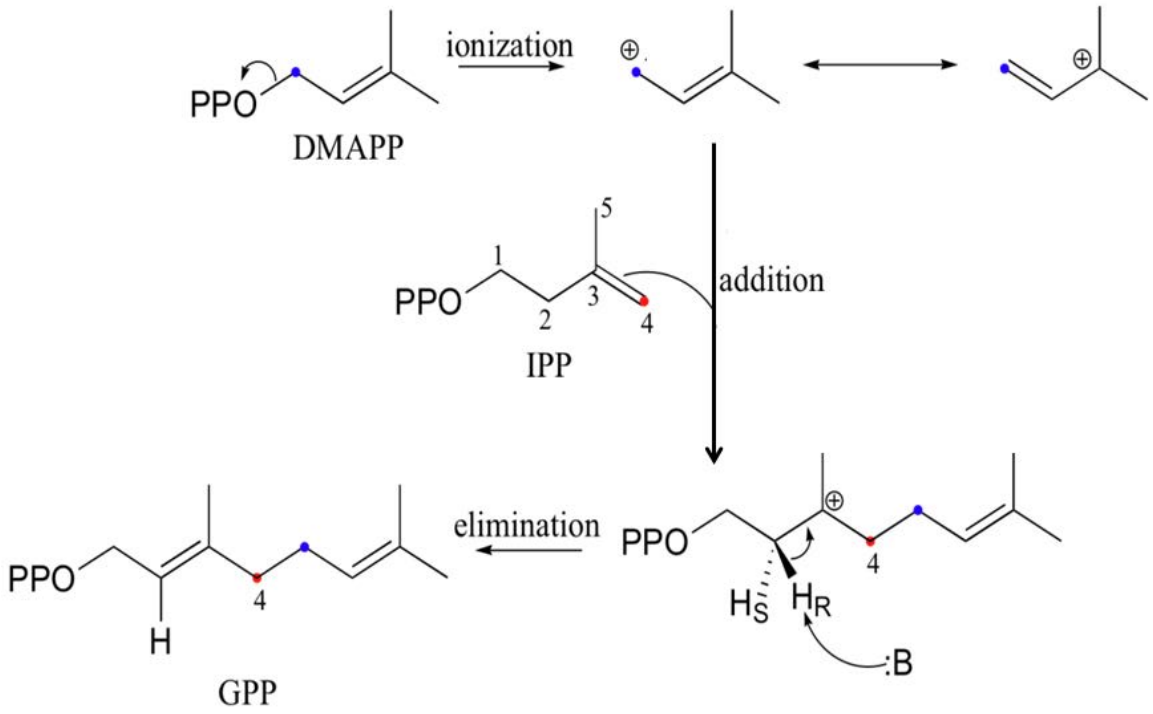


Figure 15. Mechanism of reaction for GPP synthase
(http://chemwiki.ucdavis.edu/).

This process of condensation and elimination keeps going and results in the formation of isoprenoid chains of various lengths e.g. GPP (C10), FPP (C15) GGPP (C20) and farnesylgeranyl diphosphate (FGPP) (C25) etc… (Figure 16).

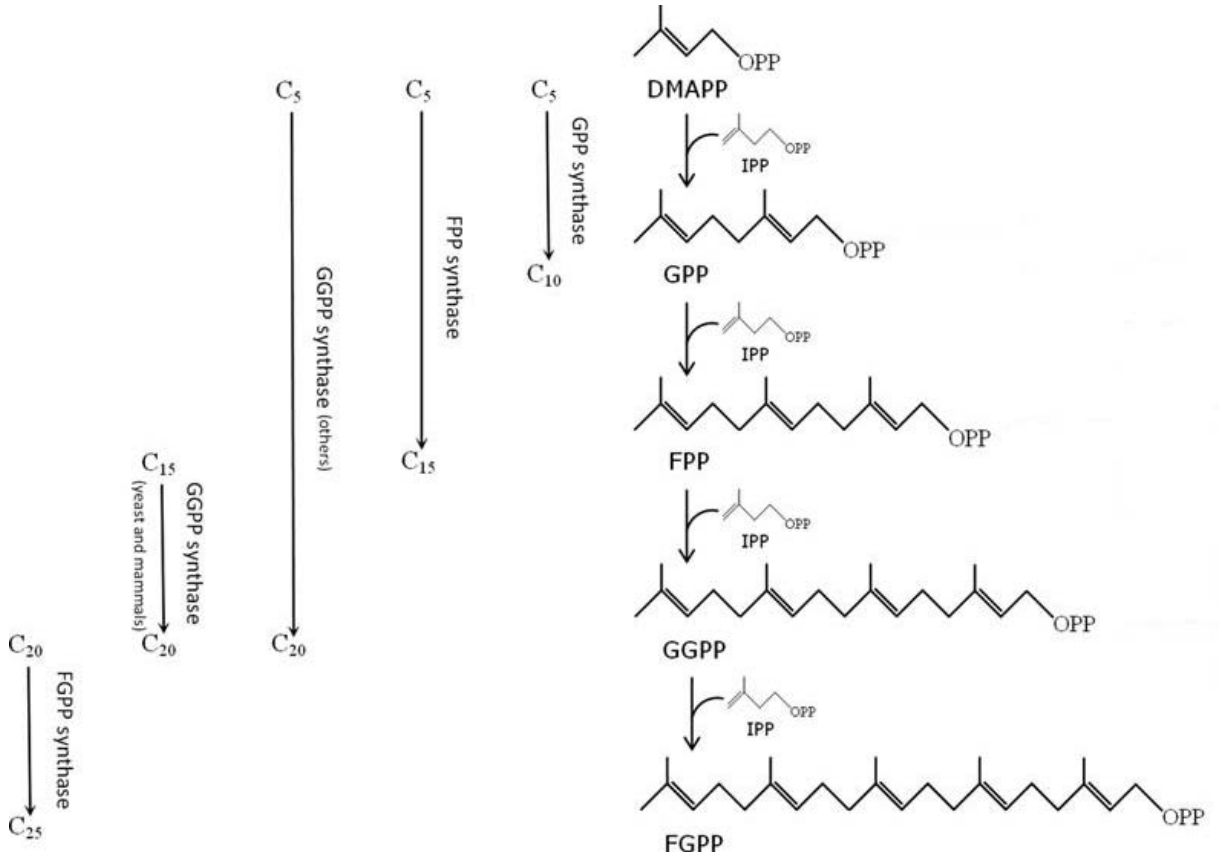


Figure 16. Elongation reaction catalyzed by short chain Pts

Taken and modified from (Vandermoten *et al.,* 2009).

2.1.1.1.2 Subcellular Localization of the biosynthetic pathway of isoprenoids

In 1950, a cytosolic mevalonic pathway (MVA) producing IPP from acetyl-CoA which, then, isomerize to DMAPP (precursor molecule of the isoprenoid biosynthetic pathway) was described as the only pathway in yeast and animal (Chappell, 1995; McGarvey *and* Croteau, 1995). More recent experiments carried out to understand the biosynthetic pathway of isoprenoids in plants, didn't support these previous results (Eisenreich *et al.,* 1998 2001; Lichtenthaler *et al.,* 1997). Thus, experimental approaches using labeled molecules, reported the presence of an alternative MVA-independent pathway for the biosynthesis of isoprenoids not only in bacteria (Broers 1994; Rohmer *et al.,* 1993) but also in plants (Lichtenthaler *et al.,* 1997; McCaskill *and* Croteau, 1999). This pathway is known by various names selected and modified with course of time including non-mevalonate pathway, Rohmer pathway, pyruvate/glyceraldehayde 3-phosphate [G3P] pathway or deoxyxylulose 5-phosphate pathway (DXP). Finally this pathway is now designed as the "methylerythritol 4-phosphate (MEP) pathway" in reference to its first committed precursor and therefore using the same rule as it was used to name the MVA pathway.

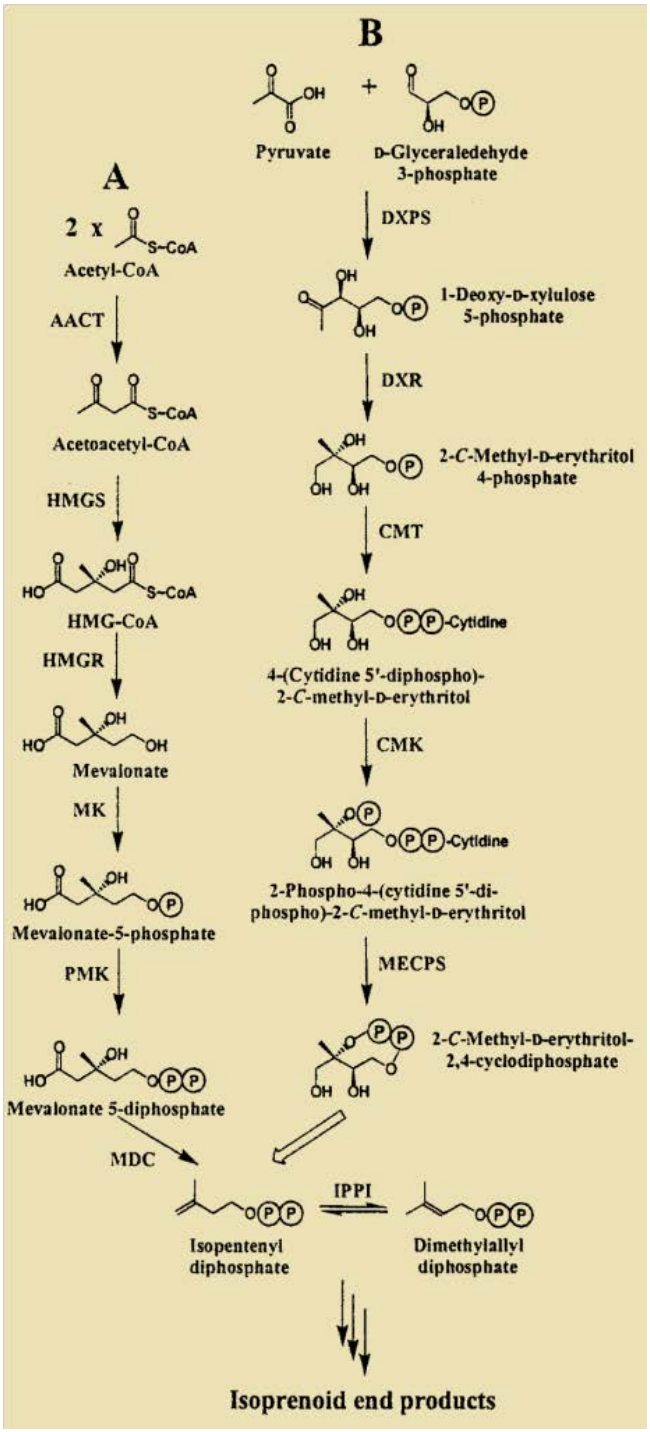


Figure 17. Biosynthesis of IPP via two different pathways

IPP is synthesized via the mevalonate pathway (MVA) (A) and via the methylerythritol-4-phosphate pathway (MEP). The circled Ps represents the phosphate moieties. Large opened arrows indicate several unidentified steps. Pts (IPPI) are also shown (Lange *et al.,* 2000)**.**

Both pathways for the synthesis of isoprenoids do not exist in all the living organisms. Thus, the MEP pathway is the only one present in eubacteria and malarial parasite (*Plasmodium falciparum*), while archaebacteria, fungi and animals synthesize their isoprenoids exclusively

through the MVA pathway (Rodrıguez-Concepcion *and* Boronat, 2002). In plants, isoprenoids are derived from both the MEP pathway and MVA pathway. These pathways are localized in two different subcellular compartments (Eisenreich *et al.,* 1998, 2001; Lichtenthaler *et al.,* 1997); plastidic for the MEP pathway and cytosolic for the MVA pathway (Figure 17). Recently, it has been demonstrated on the basis of bioinformatics approaches that additional enzymes of this pathway may be localized to peroxisomes, suggesting that part of the MVA pathway is compartmentalized in the peroxisomes (Sapir-Mir *et al.,* 2008).

In addition, it has been demonstrated that the MVA pathway provides IPPs for the synthesis of isoprenoids in mitochondria (Lichtenthaler 1999). Downstream little exchanges of IPPs or presence of intermediates have also been reported between plastid and cytoplasm (Eisenreich *et al.,* 1998, 2001; Lichtenthale *et al.,* 1997; Lichtenthaler 1999). This exchange could explain the reason for the late discovery of the MEP pathway, because labeled precursor could be incorporated (although in very low concentration) into the most plastidic isoprenoids.

2.1.1.1.3 Diversity of molecules derived from the isoprenoid biosynthetic pathway

Isoprenoid chains deriving from isoprenoid biosynthetic pathway are further utilized by plants by two different mechanisms.

- They undergo various condensation and isomerization process in the presence of specific enzymes (for example, squalene synthases) and results in the production of large molecules e.g. plant sterols also known as phytosterols, photosynthetic pigments and plant hormones. Phytosterols are integral components of the membrane lipid bilayer and regulate membrane fluidity and permeability (Gül *and* Amar, 2006; Hac-Wydro *et al.,* 2007; Schuler *et al.,* 1991). Furthermore, carotenoids constitute photosynthetic pigments while plant hormones include gibberellins, brassinosteroids, abscisic acid, retinoic acid, juvenile hormone (Vandermoten *et al.,* 2009);
- These isoprenoid chains can also be used for the prenylation process of a variety of acceptor molecules which will be discussed below.

2.1.1.2 **Biosynthesis of isoprenoid quinones**

In plants these molecules, also known as prenylquinones (Lichtenthaler 1978), function in electron transport chains, as membrane-bound and mobile hydrogen carriers. Some of these molecules are permanently attached to proteins and help in electron transport within protein complexes (Nowicka *and* Kruk, 2010). It has also been reported that they participate in the regulation of gene expression and signal transduction within the cells (Kawamukai, 2002). Other studies have described their important role in photosynthesis (phylloquinine and plastoquinone) and aerobic respiration (ubiquinone).

Pts involved in the biosynthesis of these molecules are able to catalyze the addition of an isoprenoid moiety to an aromatic substrate and result in the production of molecules essential for survival of plants. These Pts contain an aspartate rich motif (e.g. NDxxDxxxD) and their activity is absolutely dependent on the presence of Mg^{2+} or similar cations like Mn^{2+}, Co^{2+} or Ni^{2+}. They have been described as membrane bound proteins (Heide, 2009).

2.1.1.2.1 DHNA phytyltransferases

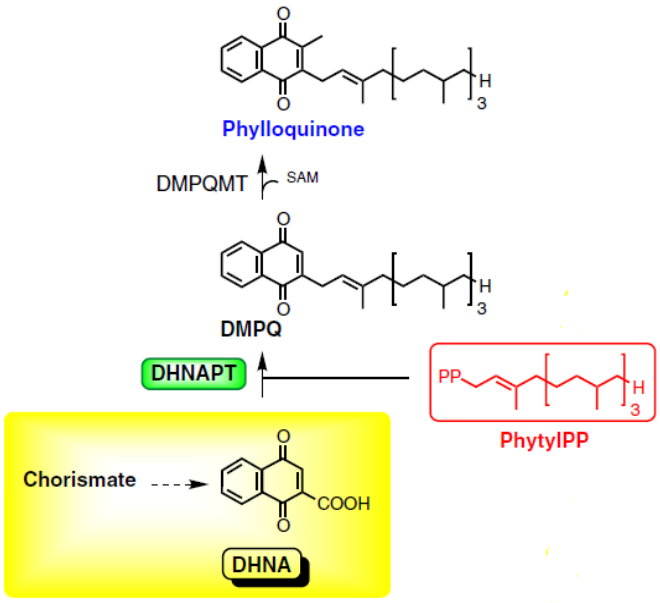


Figure 18. Biosynthesis of phylloquinone

Substrates abbreviations: DHNA (1,4-dihydroxy-12- naphtoate) and DMPQ (demethylphylloquinone).Enzymes abbreviation: DHNAPT (1,4-dihydroxy-2-naphtoate phytyltransferase) and DMPQMT (demethylphylloquinone methyltransferase). Modified and taken from Bouvier *et al*. (2005).

Phylloquinone (also known as vitamin K1) exists in photosynthetic membranes and functions as an electron bound cofactor in the photosystem I (PSI)(Bouvier *et al.,* 2005). A Pt is involved in the synthesis of these molecules. This enzyme catalyzes the fusion of 1,4-dihydroxy-12-naphtoate (DHNA) and PhytylIPP to give rise to demethylphylloquinone (DMPQ) and is known as 1,4-dihydroxy-2-naphtoate phytyltransferase or DHNA-phytyltransferase (DHNAPT) (Figure 18). This reaction occurs in plastids. The *A. thaliana abc4* mutants showed a phenotype characterized by pale-green young leaves and white old leaves. A metabolic analysis showed that these plants are devoid of phylloquinone. Cloning and subsequent characterization of the *ABC4* gene demonstrated that it encodes for a DHNAPT (Shimada *et al.,* 2005).

2.1.1.2.2 HGA Prenyltransferases

Plastoquinone is another plant quinone that functions as an electron bound cofactor but in the photosystem II (PSII). Solanesyl diphosphate (SPP), a C45 isoprenoid derivative, is covalently attached to a homogentisic acid (HGA) by the action of a HGA solanesyl transferase (HST) (Figure 19). A HGA prenyltransferase designed as AtHST has been isolated and characterized from *A. thaliana* (Sadre *et al.,* 2006).

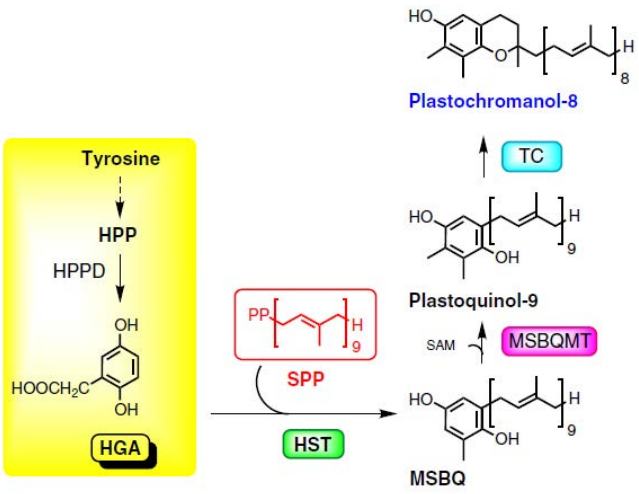


Figure 19. Biosynthesis of plastoquinone

Substrate abbreviations: HPP (*p*-hydroxyphenylpyruvate), HGA (homogentisic acid), SPP (solanesyl diphosphate) and MSBQ (2-methyl-6-solanesyl-1, 1,4-benzoquinol). Enzymes abbreviations: HST (3,Homogentisate solanesyl transferase), MSBQMT (2-methyl-6 solanesyl- 1,4 –benzoquinol methyltransferase) and TC (Tochopherol cyclase). Taken and modified from Bouvier *et al.* (2005).

Tocopherol and tocotrinol (vitamin E) are other essential compounds described in plants which are derived from HGA acid by prenylation. The biosynthetic pathway of both molecules could be differentiated depending on the origin of the phenol ring, the methyl groups and the formation of their isoprenoid chain (Figure 20). Prenylation of HGA acid is catalyzed by two different Pts. A homogentisate phytyl transferase (HPT) initiates the tocopherol branch while a homogentisate geranyl-geranyl transferase (HGGT) catalyzes a step for biosynthesis of tocotrienol. Both of these genes have been cloned from various plants and the effects of their overexpression have been studied (Hunter *and* Cahoon, 2007). As previously described, these enzymes have also been localized in plastids.

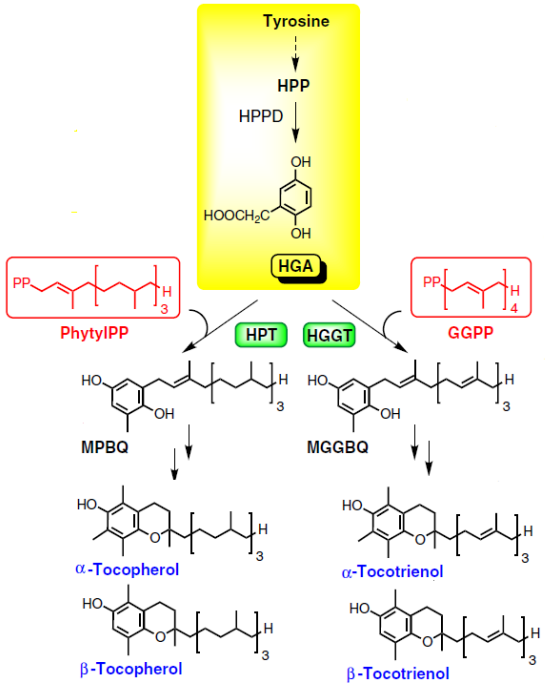


Figure 20. Biosynthetic pathway of vitamin E (tocopherol and tocotrienol)

Abbreviation used for enzymes: HPPD (*p*-Hydroxyphenylpyruvate dioxygenase), HPT (Homogentisate phytyl transferase) and HGGT (Homogentisate geranyl-geranyl transferase). Abbreviations used for substrates; GGPP (Geranyl-geranyl diphosphate), PhytyIPP (Phytyl diphosphate), MGGBQ (2-methyl-6-geranylgeranyl benzoquinol) and MPBQ (2-methyl-6-phytyl-1,4-benzoquinol); While others have been explained in text. Double arrows show the multiple steps. Taken from Bouvier *et al.* (2005).

2.1.1.2.3 *p*-hydroxybenzoic acid prenyltransferase

Ubiquinone serves as an essential component of the electron transport chain of respiration in all living organisms. *p*-hydroxybenzoic acid polyprenyl diphosphate transferases (PHBPT) (Figure 21) have been reported and characterized in *A. thaliana* (Okada *et al.,* 2004) and *Oryza sativa* (Ohara *et al.,* 2006). These studies reported

mitochondria as the subcellular localization of both enzymes. In addition, they demonstrated that PHBPT is capable to catalyze the reaction with a broad range of aromatic substrates.

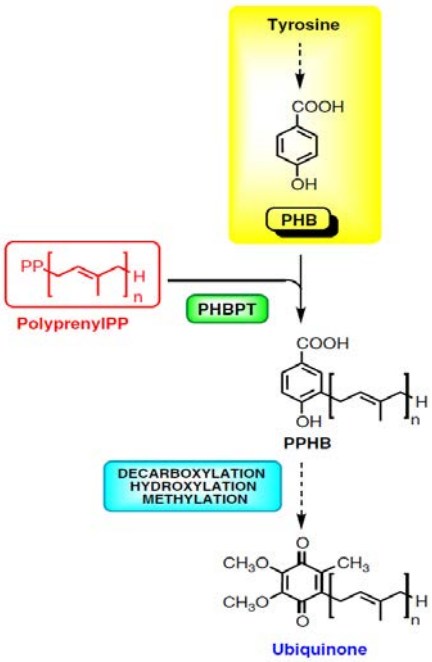


Figure 21. Biosynthetic pathway of ubiquinone

Abbreviations used for substrates: PolyprenylIPP (Polyprenyl diphosphate), PHB (*p*-hydroxybenzoic acid) and PPHB (Polyprenyl *p*-hydroxybenzoic acid).Abbreviations used for enzymes PHBPT (*p*-hydroxybenzoic acid polyprenyl diphosphate transferases). Shaded arrow shows the presence of multiple reactions. Taken from Bouvier *et al.* (2005).

2.1.1.3 Prenyltransferases involved in the biosynthesis of other plant essential products

2.1.1.3.1 Cytokinins (Zeatin)

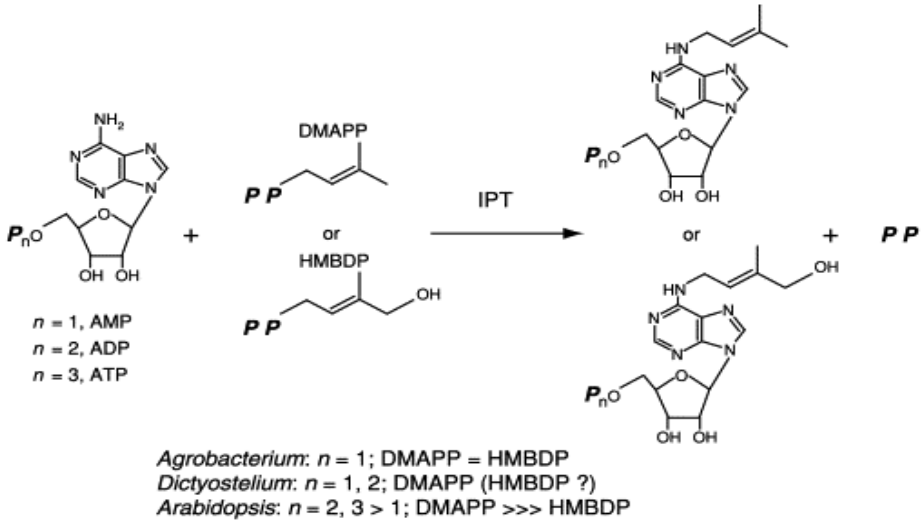


Figure 22. Primary reaction of cytokinin biosynthesis in bacteria, slime molds, and higher plants

Substrate specificity of IPT in each organism is noted in the bottom. **PP**, (diphospholic acid) HMBDP (hydroxymethylbutenyl diphosphate) (Sakakibara, 2005).

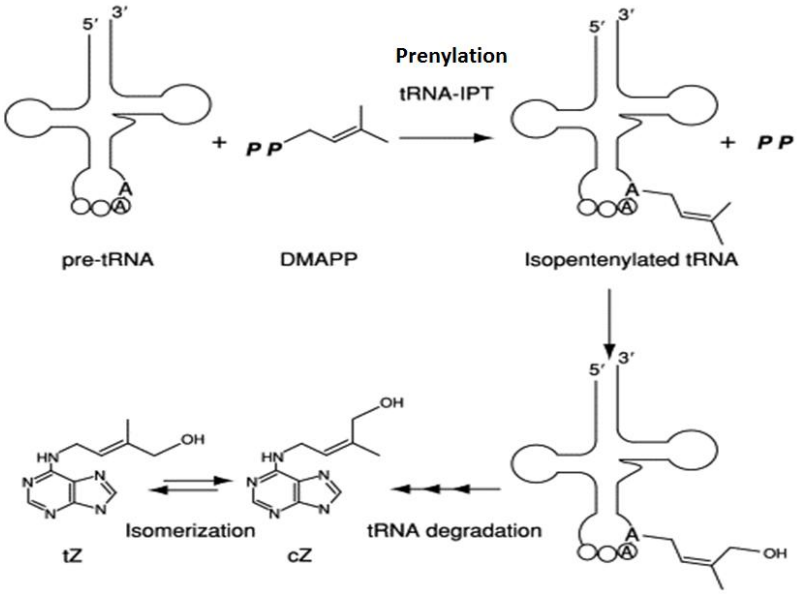


Figure 23. Hypothesized pathway for tRNA-mediated cis-Zeatin biosynthesis

PP (diphospholic acid), *cis*-Zeatin can be reversibly converted to *trans*-Zeatin by CK *cis–trans* isomerases (Bassil *et al.,* 1993).

Cytokinins are plant hormones that regulate cell division and differentiation in plants. They are adenine derivatives carrying an isopentenyl side chain that may be hydroxylated. It has been reported that adenosine phosphate isopentenyl transferase (Figure 22) and tRNA isopentenyl transferase (Figure 23) are involved in biosynthesis of *trans*-Zeatin and *cis*-Zeatin in *A. thaliana* (Miyawaki *et al.,* 2006) respectively by two completely separated pathways.

GFP fused protein studies in *A. thaliana* demonstrated the presence of four adenosine phosphate-isopentenyltransferases (AtIPT1, AtIPT3, AtIPT5, and AtIPT8) in the plastid. This study supported the fact that the prenyl moiety of *trans*- Zeatin is produced through the MEP pathway. In contrast, AtIPT2, a tRNA isopentenyltransferase, was detected in the cytosol and therefore allows concluding that the prenyl moiety of tRNA is of MVA origin.

2.1.1.3.2 Biosynthesis of photosynthetic pigments

Involvement of Pts in the biosynthesis of chlorophyll has been reported by Lopez and coworkers (Lopez *et al.,*1996) and other authors demonstrated that a chlorophyll synthase adds a prenyl group to a chlorophyllide (Kropat *et al.,* 1997; Oster *et al.,* 1997).

2.1.1.4 Post translational modification of proteins

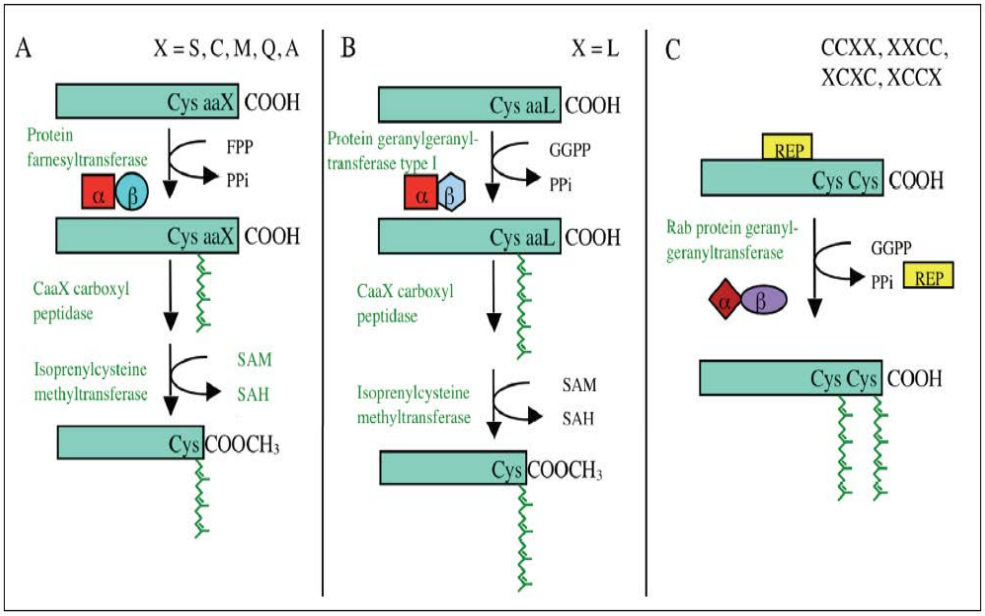


Figure 24. Protein prenylation

(A) Protein prenylation catalyzed by farnesyltransferaes at CaaX motif. (B) Protein prenylation catalyzed by geranyl-geranyl transferase at CaaX motif. (C) Prenylation of RAB protein by RAB GGT.

Modification of protein through posttranslational prenylation has been reported as an important step for their normal functioning in plants. Protein Pts involve the covalent attachment either of FPP or geranyl-geranyl diphosphate GGPP to a carboxyl terminal CaaX motif (C=Cys; a=aliphatic; X=Ser, Cys, Met, Gln, Ala) in the protein. A third enzyme known as a protein geranylgeranyl type II (PGGT II), also called RAB geranyl-geranyl transferase (RAB GGT), catalyzes the covalent attachment of GGPP to RAB proteins which are important proteins involved in vesicular membrane traffic (Andrews *et al.,* 2010) (Figure 24). All these three enzymes have been reported in pea(Qian *et al.,* 1996; Yang *et al.,* 1993), tomato (Loraine *et al.,* 1996; Motif *et al.,* 1996) and arabidopsis (Caldelari *et al.,* 2001).

Prenylated proteins have been reported to play pivotal roles in signaling, vesicle transport and cell cycle regulation that suggest a link between cellular growth control and cytoplasmic isoprenoid biosynthesis (Yalovsky et al. 1999; Zhang *and* Casey, 1996). Abnormal phenotypes observed for mutant plants that lack functional Pts, include enlargement of shoot apical meristem, retarded growth delayed flowering and hypersensitivity to abscissic acid (Allen *et al.,* 2002; Bonetta *et al.,* 2000; Cutler *et al.,* 1996; Johnson *et al.,* 2005; Pei *et al.,* 1998; Running *et al.,* 2004; Yalovsky *et al.,* 2000; Ziegelhoffer *et al.,* 2000).

2.1.2 **Prenyltransferases in biosynthesis of secondary metabolites**

2.1.2.1 **General introduction**

Pts give rise to a huge diversity of secondary metabolites of plants e.g. carbon prenylated molecules (C-prenyl), oxyprenylated molecules (O-prenyl) and azoprenylated molecules (N-prenyl). The above cited groups have been differentiated on the basis of linkage formed between different atoms of the prenyl moiety and its acceptor molecules (a terpenoid, an aromatic group etc…). These groups are described below.

- Oxyprenylated secondary metabolites

This group of plant natural products has been described as interesting and important biologically active phytochemicals. Almost 300 oxyprenylated molecules have been isolated from plants. Most of them belong to the Rutaceae and Compositae families. No oxyprenylated terpenoid pathway has been documented till now.

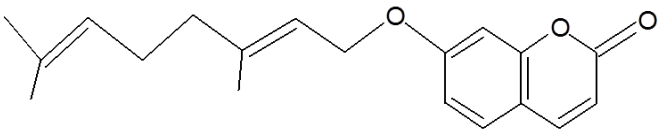
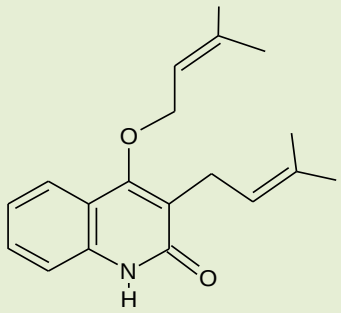
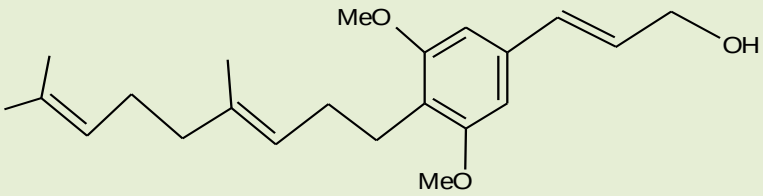
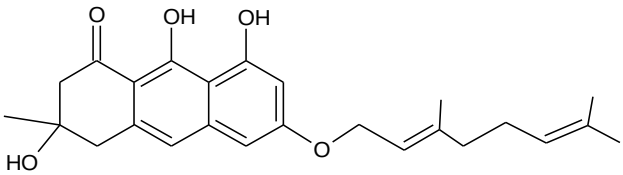


Figure 25. First documented prenyloxy secondary metabolite (auraptene)

Table 3. Some of the examples of oxyprenylated secondary metabolites of plants

Oxyprenylated polyphenols		
Type of prenylated molecule	**Biological activities**	**Reference**
Prenyloxyalkaloids		
 **3-isopentenyl-4-isopentenyloxyquinolin-2-one**	Anti HIV	(McCormick *et al.,* 1996)
Oxyprenylated phenylpropanoids		
 **3,5-dimethoxy-4-O-geranylcinnamyl alcohol**	Anti-cancer	(Zhao *et al.,* 2006)
 **vismione D**	Anti-protozoal	(Mbwambo *et al.,* 2004)

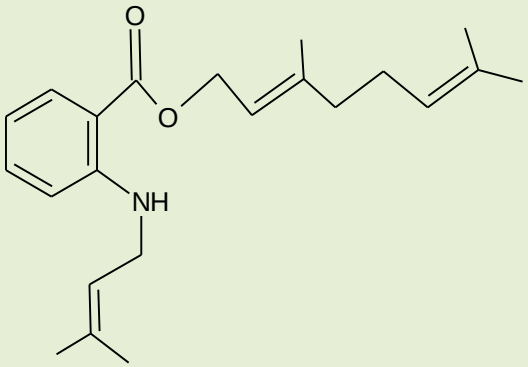
The oxyprenylated polyphenols are formed in plants by the prenylation of an alkaloid or phenylpropanoids core using prenyl diphosphate as alkalyting agent (Kuzuyama *et al.,* 2005). Three types of prenyloxy chains have been identified, that were incorporated in

oxyprenylated molecules. Some of the oxyprenylated molecules along with their biological activities are listed in the following table (Table 3).

- Azoprenylated secondary metabolites

N-prenyl secondary metabolites are an extremely rare class of natural products of plants. This class has been recently exploited and till date only one alkaloid prenylated by a linkage between N atom of acceptor molecule and C atom of isoprenyl moiety have been documented. This azoprenylated alkaloid has been isolated from *Esenbeckia yaaxhokob* Lundell (syn. *E. berlandieri* Baillon ex Hemsley) belonging to the Rutaceae family and named as Geranyl N-dimethylallylanthranilate (Table 4). Structural characterization of this product demonstrated that it contained both N-prenyl and O-prenyl moieties in the same structure. This is the first example of a secondary metabolite having two different terpenyl side chains attached to different heteroatoms (Genovese *et al.,* 2009).

Table 4. Example of azoprenylated isolated from plant

Azoprenylated polyphenols		
Type of prenylated molecule	**Biological activities**	**Reference**
Azoprenylated alkaloids		
 **Geranyl N-dimethylallylanthranilate**	Anti-bacterial	(Aguilar-Guadarrama *and* Rios, 2004)

In this document, I will focus only on prenylated secondary metabolites formed through the development of a linkage between carbon-carbon atom of isoprenyl moiety and acceptor molecule.

2.1.2.2 **Biosynthesis of plant prenylated polyphenols**

2.1.2.2.1 Polyphenols

Plants produce thousands of phenolic and polyphenolic compounds as secondary metabolites that are widely distributed in all plant species. Plant polyphenols are aromatic compounds and have been divided into various classes depending upon the type of phenolic rings e.g., phenolic acids (benzoic acid and cinnamic acid derivatives), flavonoids, coumarins, stilbenes, lignans and polyphenolic amides (Pereira *et al.,* 2009). These aromatic compounds are synthesized by two different pathways either the shikimate pathway or the acetate/malonate pathway. Most of the plant polyphenols are derived from the shikimate pathway (Figure 26). The acetate/malonate pathway is of less significant in plants but is important for the biosynthesis of phenolics of fungi and bacteria (Taiz *and* Eduardo, 2002; Tzin *and* Galili, 2010).

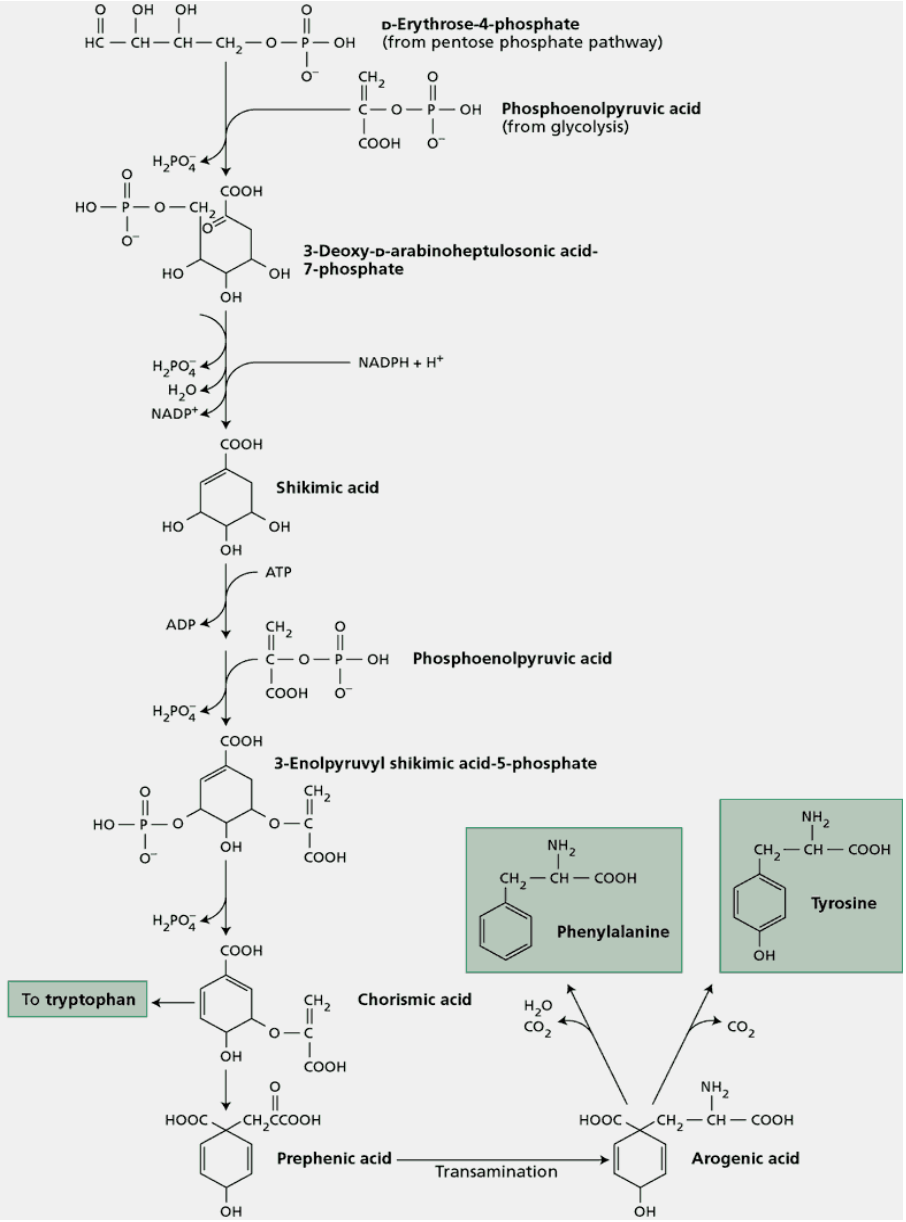


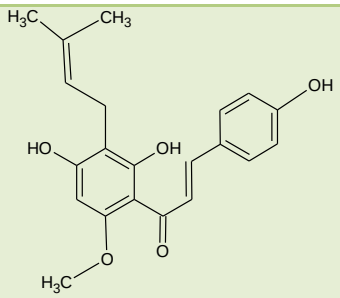
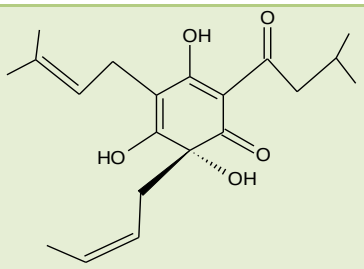
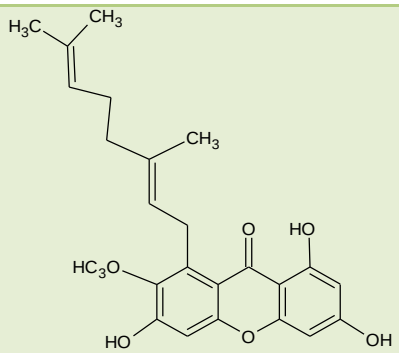
Figure 26. Shikimate pathway for the biosynthesis of aromatic amino acid (precursor of all plant poly phenols)

2.1.2.2.2 Biological activities of prenylated polyphenols

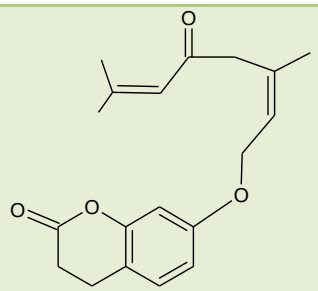
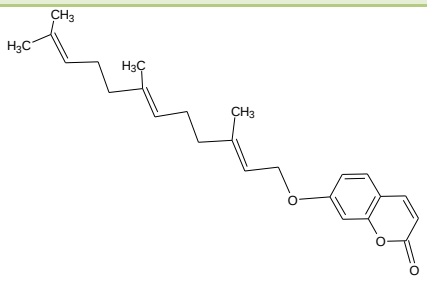
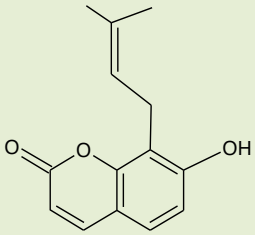
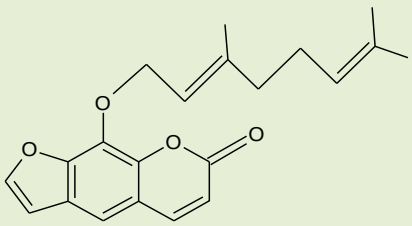
Polyphenols or more precisely aromatic compounds are also described as substrates for Pts. The difference of the prenylation position on the aromatic ring, the various lengths of the prenyl chains and also further decoration of the prenyl moieties (e.g. hydroxylation and cyclization) leads to a wide diversity of secondary metabolites displaying a large panel of biological activities (Botta *et al.,* 2005). For example, prenylated polyphenols serve as phytoalexins and are involved in plant defense mechanism (Morandi 1996; Tahara *and*

Ibrahim, 1995). They also have been reported as potential medicines (Miranda *et al.,* 2000; Wang *et al.,* 1997) (Table 5).

Table 5. Structures and biological activities of some prenylated polyphenols

Prenylated polyphenols		
Type of prenylated molecule	Biological activities	Reference
Prenylated flavonoid		
 **Xanthohumol**	Esterogenic Antioxidant Anti-tumor	(Erkoc *et al.,* 2002)
Prenylated Phloroglucinol		
 **Humulone**	Inhibition of angiogenesis Induction of apoptosis Inhibition of tumor promotion Suppression of cyclooxygenase-2 gene transcription	(Siegel *et al.,* 2008)
Prenylated Xanthone		
 **Rubraxanthone**	Antithrombotic Anti-allergic Anti-inflammatory	(Jantan *et al.,* 2002)
Prenylated Coumarins		

Following part of table 5 is on next page.

 **Diversinin**	Anti-fungal Anti-bacterial	(Kurdelas *et al.,* 2010)
 **Umbelliprenin**	Antioxidant Anti-inflammatory Lipoxygenase inhibitory activities	(Iranshahi *et al.,* 2009)
 **Osthenol**	Anti-bacterial	(Souza *et al.,* 2005)
Prenylated Furanocoumarin		
 **8-Geranyloxypsoralen**	Inhibitor of Cytochrome P450 (CYP3A4)	(Row *et al.,* 2006)

2.1.2.2.3 Biochemical studies of polyphenol prenyltransferases of plants

Keeping in view all the literature cited above, prenylation of aromatic compounds (polyphenols) could be described as an important step of coupling two major biosynthetic pathways of plants i.e. biosynthetic pathway of aromatic compounds for example shikimate or malonate pathways and the biosynthetic pathway of isoprenoid moiety for example MVA or MEP pathway (Figure 27). Various biochemical studies were carried out to explore this key

step of biosynthesis of prenylated polyphenols that reported the presence of both the soluble type and membrane bound type Pts in plants.

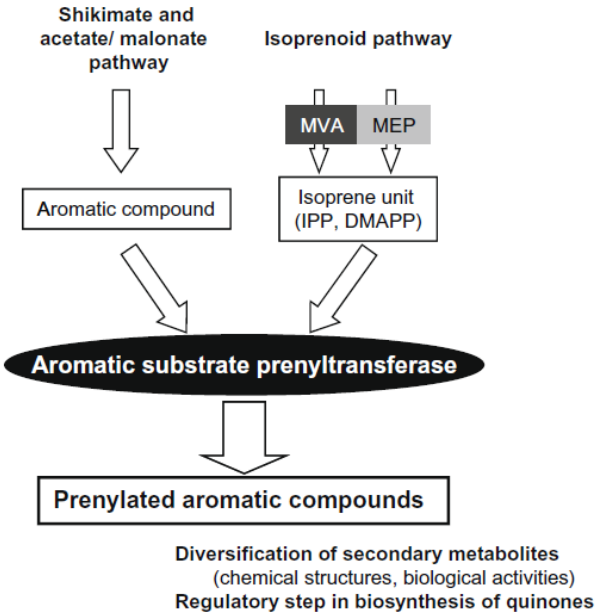


Figure 27. Schematic representation of the prenylation of aromatic compound (Yazaki *et al.*, 2009)

IPP (isopentyl diphosphate), DMAPP (dimethylallyl diphosphate), MVA (mevalonic pathway) and MEP (methylerythritol 4-phosphate).

2.1.2.2.3.1 Biochemical studies for soluble-type aromatic prenyltransferases

Soluble type Pts were reported to be involved in the biosynthesis of terpenophenolics (cannabinoids) in hemp plant. This enzyme was found to be able to catalyze the condensation reaction of olivetolic acid with GPP, and its activity was found in a soluble fraction (Fellermeier *and* Zenk, 1998). Another soluble Pt was reported, from *Humulus lupulus* involved in the biosynthesis of humolone (Zuurbier *et al.,* 1999). In this case, authors demonstrated that the prenyl moiety was derived from the MEP pathway.

2.1.2.2.3.2 Biochemical studies for membrane bound aromatic prenyltransferases

From the 1970s, many studies have been conducted to understand the prenylation of polyphenols. Enzymatic activity of Pts involved in the biosynthesis of prenylated coumarins was reported from two different plants of Rutaceae family (Dhillon *and* Brown, 1976; Ellis *and* Brown, 1974). These enzymes catalyze the addition of an isoprenoid moiety, DMAPP to the aromatic acceptor molecules, and were reported to be localized in plastids. In another

work done using soybean (*Glycine max*), a prenylation step in the synthesis of glycinol has been studied. Glycinol is a well-known phytoalexin, derived from pterocarpan. In this synthesis a dimethylallyl moiety is added to the aromatic ring. This activity was reported by Zahringer and his collaborators in the late 1970s (Zähringer *et al.,* 1979). A third study demonstrated that the microsomal fraction prepared from cell cultures of *Phaseolus vulgaris* can catalyze the prenylation of an isoflavonoid, 3,9-dihydroxypeterocarpan and results in phaseollidin, that can serve as a precursor molecule for biosynthesis of phaseollin (a phytoalexin) (Biggs *et al.,* 1987). Similarly, it was demonstrated that microsomal fraction prepared from cell cultures of *Lithospermum erythrorhizon* could catalyze the prenylation of 4HB with two different prenyl moiety, hexaprenyl diphosphate and GPP resulting in the production of ubiquinone and shikonin respectively (Boehm *et al.,* 1997). By another study, researchers showed that the dimethylallyl moiety used for the prenylation of glabrol in *Glycyrrhiza glabra* was derived from the MEP pathway, which also suggest that the corresponding Pt is localized in plastids (Asada *et al.,* 2000). Microsomal fractions prepared from cell suspension culture of *Sophora flavescens* can prenylate various flavanones including naringenin as major substrate while liquiritigenin, hesperetin and taxifolin were reported as minor substrates. In addition, it was reported that the presence of an hydroxyl group at position 2' could increase the amounts of products produced (Yamamoto *et al.,* 2000). More recently, both *O* and *C*-Pt activities were detected in microsomal fractions prepared from lemon peel. These microsomal fractions showed broad range specificity for coumarins substrate but were demonstrated to be highly specific for the prenyl moiety. The prenylation can only take place with GPP (Munakata *et al.,* 2012). In general, when it has been investigated using the various biochemical approches, all these studies showed that the prenyl moieties of polyphenols are derived from the MEP pathway.

The biochemical studies demonstrated that various plant aromatic Pts are involved in the synthesis of secondary metabolites. However identification of coding sequences and purification of the enzymes to homogeneity of any of the member of this family of enzymes remained a question to be solved for researchers for long time.

2.1.2.2.3.3 Isolation of aromatic prenyltransferases of plant polyphenols

2.1.2.2.3.3.1 Shikonin biosynthesis

Shikonin, a plant napthoquinone, is a secondary metabolite that specifically occurs in a Boraginaceous plant. It is an active medicinal component and has anti-inflammatory, anti-tumor and antibacterial properties (Lu *et al.,* 2011; Yang *et al.,* 2009; Yazaki *et al.,* 2002). The biosynthetic pathway of this molecule involves a Pt which has been investigated from the biochemical point of view as described above. It is in this biosynthetic pathway that the first gene encoding for a plant Pt has been described by Yazaki and his coworkers.

- Geranyl Diphosphate:4-Hydroxybenzoate Geranyltransferase

This group cloned two different Geranyl Diphosphate: 4-Hydroxybenzoate Geranyltransferase (*Le*PGT1 and *Le*PGT2) both involved in the shikonin biosynthesis in *L. erythrorhizon.* These proteins are closely related to the PPT of ubiquinone biosynthesis (Yazaki *et al.,* 2002) (Figure 28). Each of these Pts shares six transmembrane domains in their predicted aminoacid sequences. They display an aspartate-rich motif (and both prenylation activates are Mg^{2+}-dependent). Another conserved amino acid sequence (GX(K/Y)STAL) is present and has been reported to be a putative 4HB binding site (Melzer *and* Heide, 1994). However, in contrast to the ubiquinone Pt, both of these genes possess a transit peptide for targeting the endoplasmic reticulum and showed a strict substrate specificity for prenyl substrate and catalyzed the reaction only with GPP (Yazaki *et al.,* 2002).

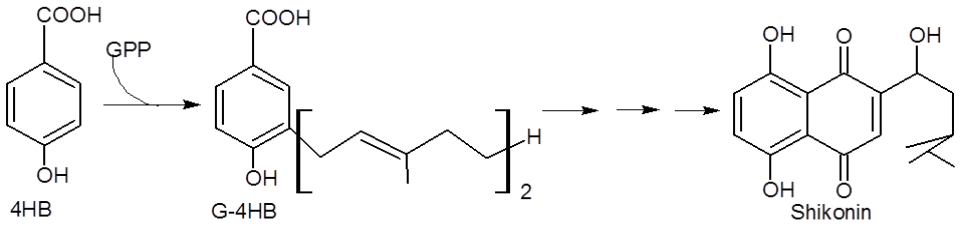


Figure 28. Enzymatic reactions of *Le*PGT1 and *Le*PGT2 in biosynthesis of shikonin

2.1.2.2.3.3.2 Flavonoid and isoflavonoid biosynthesis

Prenylated flavonoids are described as strong antibacterial and anti-fungal compounds in plants (Sohn *et al.,* 2004). In addition many prenylated flavonoids have shown important medicinal properties including anti-cancer, anti-androgen, anti-leishmania and anti-nitric oxide production (Ahmed-Belkacem *et al.,* 2005; De Naeyer *et al.,* 2004; Han *et al.,* 2006).

- Naringenin 8-Pt *(SfN8DT-1)*

Yazaki and his coworkers (Sasaki *et al.,* 2008) used three basic criteria for the identification of candidate genes encoding for flavonoid Pts;

- Presence of conserved sequences that are characteristic of Mg^{2+}-dependent polyprenyl transferases i.e. an aspartate rich motifs (NDxxDxxxD,NQxxDxxxD, NQxxExxxD, DDxxDxxxD, DQxxDxxxD, or DQxxExxxD) that is used for binding of prenyl diphosphate *via* Mg^{2+} ion;
- Presence of a transit peptide (plastid targeting peptide) at N-terminus of the protein.
- Presence of at least one trans-membrane α-helix;
- Based on these criteria, they performed PCR screening for interesting sequences in an EST data bank prepared from a methyl jasmonate induced cell cultures of *S. flavescens*.

This search led to the identification of a naringenin 8-dimethylallyl transferase encoding an open reading frame (*Sf*N8DT-1) (Figure 29). This enzyme was very close to the HGA Pt involved in the biosynthesis of vitamin E. It contained 9 transmembrane α-helices as well as an aspartate-rich motif for substrate binding. The corresponding protein was expressed in a yeast heterologous expression system. The enzyme is Mg^{2+}-dependent, specific for flavonones and DMAPP as substrates and is localized in plastids.

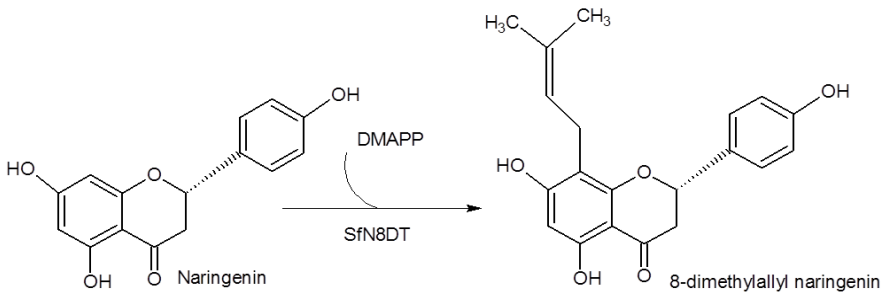


Figure 29. Enzymatic reactions of *Sf*N8DT-1 in the biosynthesis of 8- dimethylallyl naringenin

An additional work done by Sasaki showed that when the expression of this enzyme is realized in *A. thaliana* (used as an expression system), a large number of flavonones including apigenin, quercetin, kaempferol and 8-prenylnaringenin could be prenylated (Sasaki *et al.,* 2008).

- Pterocarpan 4-Dimethylallyltransferase (*G4DT)*

In order to identify genes encoding for Pts, Akashi and co-workers explored the publically available EST databases using HGA phytyltransferase of *A. thaliana* as a probe. They succeeded to identify an aromatic Pt involved in the biosynthesis of glyceollins. Glyceollins are the principle phytoalexins having a pterocarpan skeleton, which contain a decoration originated from a C_5 isoprenyl moiety. The ORF of the enzyme was isolated from young seedlings. These enzymes contain a transit peptide targeting to plastid and display high sequence homologies to the sequence of *Sf*N8DT-1. The corresponding protein was expressed in a yeast expression system in order to determine its catalytic constants. It was found that this enzyme could catalyze the prenylation of a pterocarpan skeleton with a dimethylallyl moiety (Figure 30). A study involving tracer molecules showed that the dimethylallyl moiety of pterocarpan glycinol is derived from the MEP pathway (Akashi *et al.,* 2009).

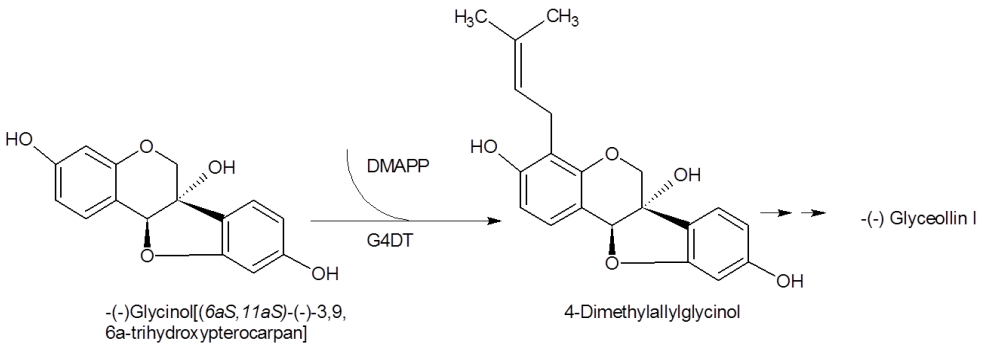


Figure 30. Enzymatic reactions of *Sf*G4DT in the biosynthesis of Glyceollin

- Genistein 6-dimethylallyl Pt (*SfG6DT*) and isoliquiritigenin dimethylallyltransferase (*SfiLDT*)

Both genes were isolated from *S. flavescens*, using a PCR approach and specific primers directed against *SfN8DT-1*. The corresponding proteins were membrane-bound enzymes, involved in the prenylation of either isoflavones (*Sf*G6DT) (Figure 31) or chalcone (*Sf*iLDT) (Figure 32). *Sf*iLDT was found to be specific for isoliquiritgenin as the aromatic substrate and for DMAPP as the prenyl moiety. While *Sf*G6DT preferred genistein as the aromatic substrate but could utilize biochanin also. Similarly for the prenyl moiety, DMAPP was the preferred substrate over GPP. In addition, flavonoid specificity of enzymes was checked by preparing a serie of chimeric enzymes between *Sf*N8DT-1 (flavone specific) and *Sf*G6DT (isoflavone-specific). This analysis showed that domain made up of 33 amino acids

present in the neighbouring of the fifth transmembrane α-helix determines the substrate specificity (Sasaki *et al.,* 2011).

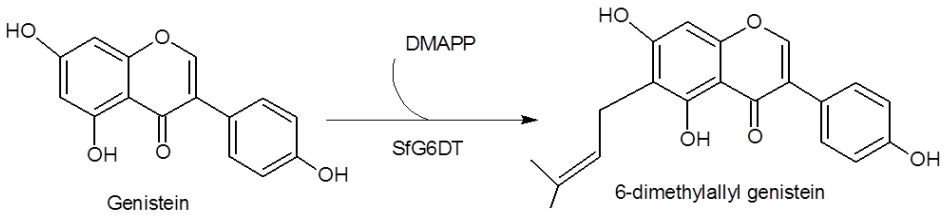


Figure 31. Enzymatic reactions of *Sf*G6DT in the biosynthesis of prenylated isoflavone (6-dimethylallyl genistein)

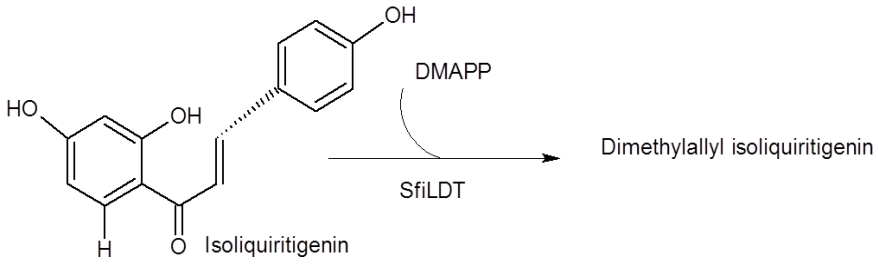


Figure 32. Enzymatic reactions of *Sfi*LDT in the biosynthesis of prenylated chalcone (dimethylallyl isoliquiritigenin)

- Genistein 3-dimethylallyl prenyltransferase (*La*PT1)

The isolation and characterization of an isoflavonoid Pt *(La*PT1*)* from *Lupinus albus* has been done. The sequence isolated from this plant shows clear similarity with other isoflavonoid Pts and is localized in plastids. This enzyme is highly specific for a DMAPP moiety as substrate and among different flavonoids it is specific for only genistein and 2'-hydroxygenistein. It catalyzes the introduction of the prenyl moiety at position 3' of molecule as shown in (Figure 33). Surprisingly, a truncated protein prepared by removing the transit peptide has more activity then the original one (Sasaki *et al.,* 2011).

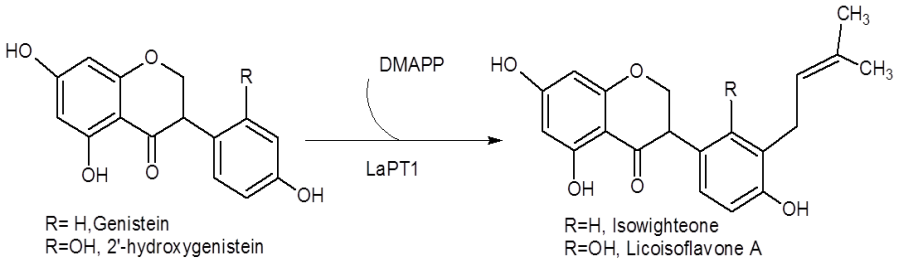


Figure 33. Enzymatic reactions of *La*PT1 in the biosynthesis of prenylated isoflavonoids

- Biosynthesis of other prenylated polyphenols in hop (*Humulus lupulus* L.)

Hop is a perennial and dioecious plant for which the female plants are cultivated for the flowers (hop cones) that are used in beer brewing process. Hop cones possess lupulin glands, in which a variety of aromatic compounds are biosynthesized and accumulated e.g., prenylated phloroglucinols also known as bitter acid (lupulone (β-acid) and humulone (α-acid)) and prenylated chalcone (xanthohumol). However no aromatic Pt was reported from a non-leguminous plant such as hop.

For the identification and isolation of an aromatic Pt from hop, a cDNA library was prepared and EST data were analyzed using HG phytyltransferase of *A. thaliana* as a query. This search allowed the identification of a putative aromatic Pt (*HP*T-1), which has all the characteristics of aromatic Pt i.e., two conserved motifs: NQxxDxxxD (motif 1) and KD(I/L)xDx(E/D)GD (motif 2), nine transmembrane α-helices and a transit peptide at the N-terminus. This enzyme was demonstrated to be localized in plastids (Tsurumaru *et al.,* 2010).

In contrast to other aromatic Pts, HPT-1 was reported to have unique characteristics. For example, this enzyme could catalyze the addition of a dimethylallyl moiety to a broad range of aromatic substrates i.e., various phloroglucinol derivatives (Figure 34) (PIVP, phloroisovalerophenone (n-humulone and lupulone type); PIBP, phloroisobutyrophenone (co-humulone and lupulone type); PMBP, phloromethylbutanophenone (ad-humulone and lupulone type) that are intermediate products of the biosynthesis of humulone and lupulone respectively) and flavonoid (Figure 35) (naringenin chalcone). In addition, this enzyme was found to be exclusively Mg^{2+}-dependent and its optimum activity was observed at a pH 7.0 (Tsurumaru *et al.,* 2012).

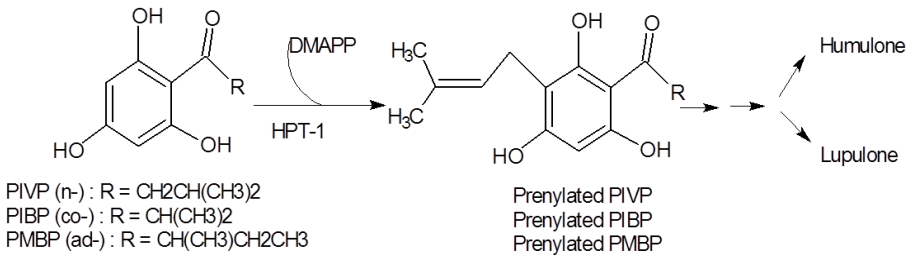


Figure 34. Enzymatic reactions of HPT-1 in the biosynthesis of prenylated phloroglucinols

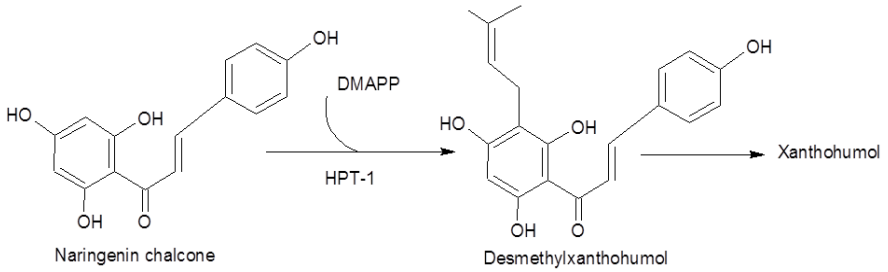


Figure 35. Enzymatic reactions of HPT-1 in the biosynthesis of prenylated flavonoid

2.1.2.2.3.4 Structure activity relationship of Prenyltransferases

2.1.2.2.3.4.1 Three dimensional modeling of aromatic prenyltransferase

Aromatic Pts are membrane bond enzymes, so that purification and crystallization of these proteins is rather difficult. Only few data are available for three dimensional structures and modeling of these enzymes. However, the membrane bound 4HB oligoPt (UbiA) from *E. coli* has been studied in detail for its structure. This enzyme consists of 290 amino acids residues. Early studies about UbiA model showed the amino acids, necessary for enzymatic catalysis, and reported that they are distributed in two different putative active sites (Bräuer *et al.,* 2004). Later on, another study was carried out to build new X-ray structure of the enzyme. Five point mutations (D71A, D75A, D191A, R137A and D195A) of amino acids present in both active sites (one represented byAsp71 and Asp75, and another by Asp191 and Asp195) were generated (Bräuer *et al.,* 2008). Alignment of various enzymes showed that these five residues are well conserved in all enzymes. A loss of activity to form geranylated 4HB was reported for each single mutant. These observations suggested that either these sites are interrelated or that there is only one site. Based on the experimental results and using 5-epi-aristolochene synthase from *N. tabacum* (pdb-code 5eau) (Starks *et al.,* 1997) as a template, a new X-ray structure of UbiA was presented. Threading (a homology modeling software available in Schrodinger's modeling software tools) then provided a 3D-model of UbiA, which showed that the enzyme consisted of ten α-helical structural elements likely located in the membrane (Figure 36 A and B).

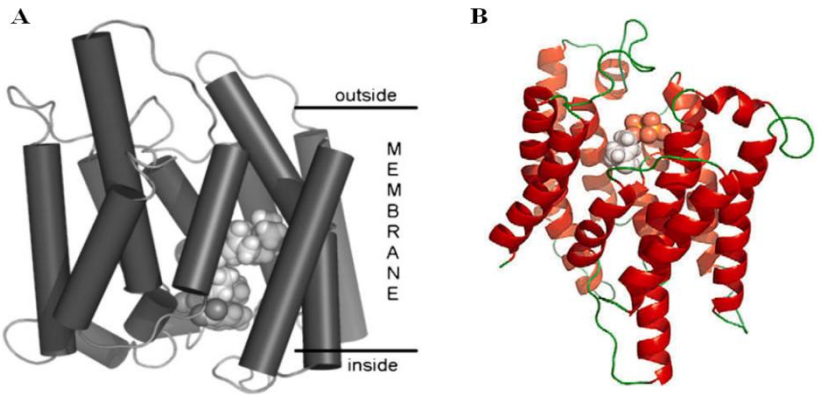


Figure 36. A) UbiA secondary and tertiary structure side view with putative location of the membrane

Substrates 4-hydroxybenzoate (PHB) and geranyl pyrophosphate are located in the center of the membrane bundle (Bräuer *et al.,* 2008). (B) Model of the all-a-helical tertiary structure of UbiA with bound substrates 4-hydroxy benzoic acid (PHB) in front, and hidden geranyldiphosphate behind PHB (Brandt *et al.,* 2009).

UbiA fold allows hypothesizing that this enzyme has an evolutionary relationship to terpene synthase (cyclases). In addition, the model indicates that Asp71 and Asp75 are responsible for the fixation of one magnesium ion and through this fixation they can activate the diphosphate. Asp191 can directly attach to 4-hydroxybenzoate to stabilize the intermediate phenolate (Figure 37). Asp195 is indirectly involved in the stabilization of the side chain of Arg 137, which can form a bridge with the diphosphate group. Through this it could play a role in positioning correctly prenyl substrate relative to 4HB. In addition the side chain of Trp152 is located in a co-planer orientation to the first prenyl moiety of the bound geranyl diphosphate (Bräuer *et al.,* 2008).

Interaction of the prenyl residue with its aromatic amino acid residues could form an intermediate prenyl cation, which yields energy: the *ab initio* calculations showed almost 85 kj/mol. In addition, co-planar orientation of 4HB resulted in intrinsic stabilization along with a considerable energy gain. This energy supported not only the cleavage of the diphosphate-geranyl bond but it could also overcome the transition state energy required for the formation of an intermediate prenyl cation complex. Furthermore substrate activation is completed by deprotonated Asp191 (Brandt *et al.,* 2009).

More recently, on the basis of the UbiA model, the structure of geranyl diphosphate:4HB geranyltransferase (isolated from *L. erythrorhizon*)(Yazaki *et al.,* 2002) which represented 33% sequence identity with UbiA, was created. As described above, unlike

UbiA, this enzyme is specific for prenyl moiety (geranyl diphosphate). The calculated structure of the active site of an aromatic plant Pt was presented for the first time (Figure 38).

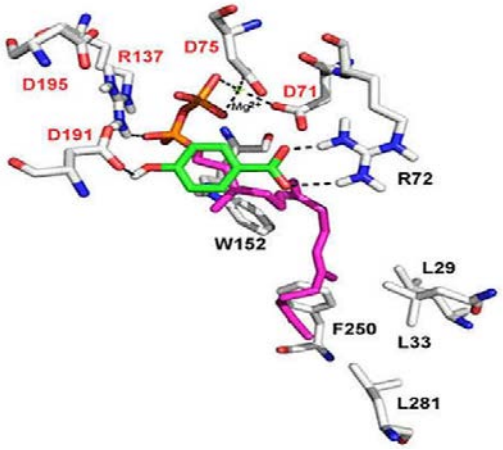


Figure 37. Model of the active site of UbiA with bound 4-hydroxy benzoic acid (green), and geranyl-geranyl diphosphate (magenta)

Red labeled amino acids were mutated to alanine and caused almost complete loss of enzymatic activity (Brandt *et al.,* 2009).

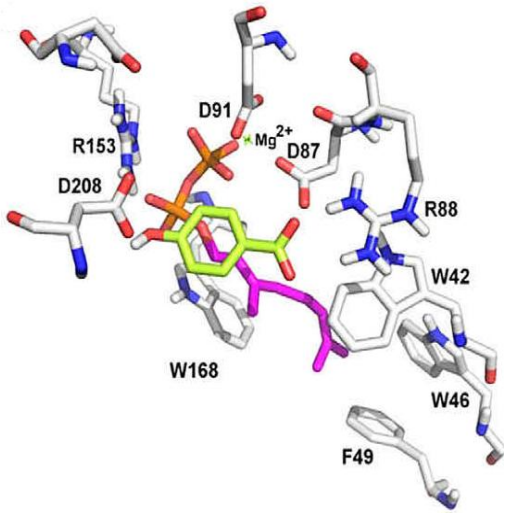


Figure 38. Model of the active site of the aromatic Pt from *Lithospermum erythrorhizon* with bound 4HB (green) and geranyl diphosphate (magenta)

In comparison to the UbiA-enzyme, the prenyl recognition cleft is restricted by tryptophans 42 and 46 (Brandt *et al.,* 2009).

The reorganization site of diphosphate group was reported to be identical to the *E. coli* UbiA site. The main differences were localized at the interaction positions of the tail isoprene moiety, which was recognized by two tryptophans and one phenylalanine. These residues

were supposed to control the length of the binding site in this area which could explain the enzyme specificity for the prenyl moiety (Brandt *et al.,* 2009).

2.1.2.2.3.4.2 Reaction mechanism of aromatic prenyltransferases of plant polyphenols

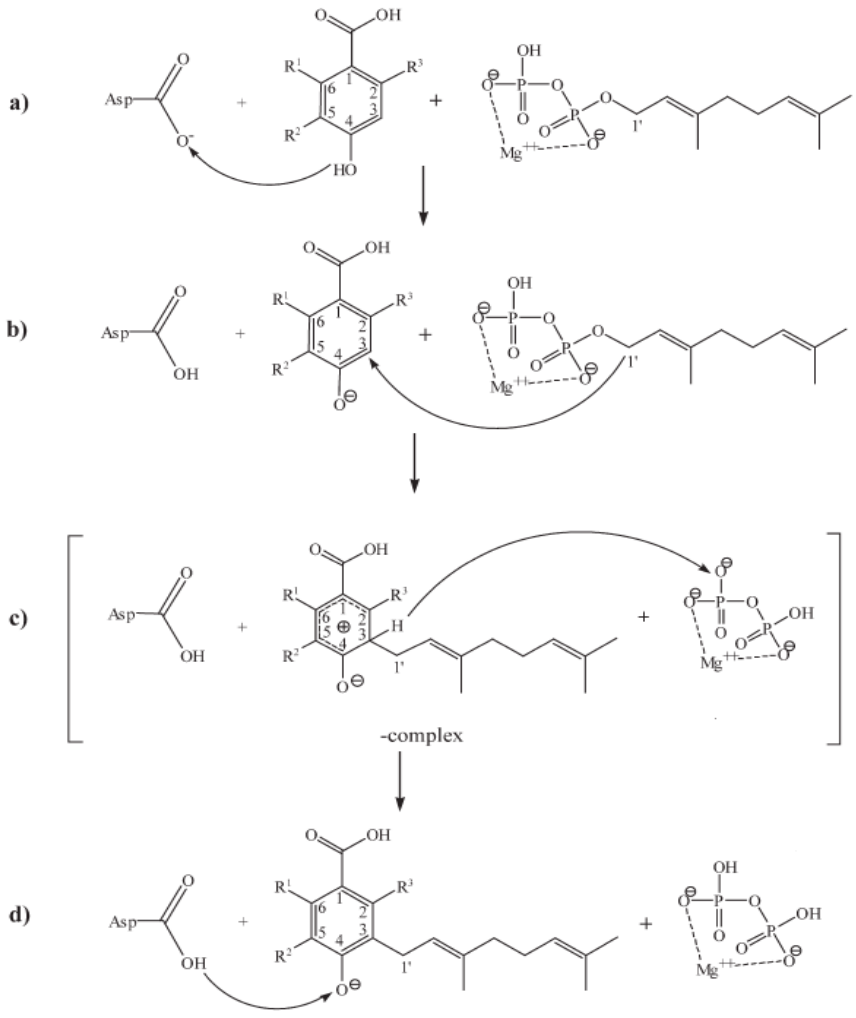


Figure 39. Simplified representation of complete formal mechanism of prenylation reaction, including the intermediates (amino acid that don't take part directly are not shown) (Bräuer *et al.,* 2004)

The reaction is initiated with the deprotonation (activation) of the phenolic compound. This step consists in the recognition and the nucleophilic activation of the phenolic hydroxyl group by an aspartate, which results in the abstraction of a phenolic proton by the negatively charged side chain of aspartate. This step leads to the formation of a phenolate anion, causing the negative charge at the *ortho* position (Figure 39 (a)). The appearance of a charge makes

the *ortho* and *para* position of phenol compound susceptible to the attack of a prenyl cation. However, only the former orientation has been found effective for binding. GPP is activated by a Mg^{2+} complex for leaving the diphosphate group and attacked by a phenolate *ortho* carbon at position 1 (Figure 39 (b)). σ-complex is formed between oppositely charged molecules and a proton from the C3 position of phenolate is transferred to the oxygen of the diphosphate group of prenyl cation, which subsequently results in the cleavage of the GPP (C-O) bond (Figure 39 (c)). Products of the reaction are produced and aspartate is recovered after protonation (Figure 39 (d)). In addition, aromatic prenylation occurs preferentially at a position close to phenolic OH-group or another donor substituent that could be activated by strong base (Brandt *et al.,* 2009).

2.1.2.2.3.5 Evolution of aromatic prenyltransferases of plants

Aromatic Pts of plants have been divided into two groups (1) 4-HB Pts and (2) HGA Pts. Members of both groups of enzyme catalyze similar type of reaction i.e., proton substitution with a prenyl chain either on 4-HB or HGA molecules. Different characteristics that were used as basis for the identification of new membrane-bound aromatic Pts of plants could also be used to understand the evolution pattern of aromatic Pts. It appears likely that the 4-HB geranyltransferase involved in the biosynthesis of shikonin has evolved from a 4-HB Pt involved in the biosynthesis of ubiquinone, only adopting a single peptide targeting to endoplasmic reticulum in spite of mitochondria. On the other hand, aromatic Pts of plants localized in plastids and involved in secondary metabolism seems to have evolved from HGA Pts also located in plastids (Yazaki *et al.,* 2009).

2.1.2.2.3.6 Heterologous expression systems for the characterization of plants membrane-bound aromatic prenyltransferases

Plant aromatic Pts have been characterized using various heterologous expression systems, The transient expression system which has been the most described, is the yeast expression system (Sasaki *et al.,* 2008, 2011; Yazaki *et al.,* 2002). However, it has also been reported that baculovirus-infected insect cells system can be used successfully (Tsurumaru *et al.,* 2012).

Beside the transient expression system, stable expression of various aromatic Pts have been described in different hosts. For example, *Sf*N8DT-1 was ectopically expressed in *A. thaliana* (a plant that do not produce prenylated polyphenols) under the control of the

constitutive 35S promoter. Analysis of transformed plants provided unexpected results. Actually, 8-prenylated kaempferol was detected as the only product (Sasaki *et al.,* 2008). However, feeding of these transformed plants by an exogenous supply of naringenin resulted in the production of a large variety of products including 8-dimethylnaringenin, similar level of 8-prenylated kaempferol, prenylated apigenin and quercetin. These molecules were not substrates in *in vitro* assays. These results allow to make the following hypotheses (Yazaki *et al.,* 2009):

- Naringenin, an *in vitro* substrate for SfN8DT-1, can also serve as initial substrate for the enzyme when overexpressed in transgenic *A. thaliana*. However the prenylated naringenin is not necessarily the final product in the transformed plants. Thus prenylated naringenin may be metabolized by any endogenous enzyme and is further modified leading to the production of unexpected molecules.
- The storage mechanisms of secondary metabolites could also explain the low concentration of prenylated naringenin. Analysis of transformed *A. thaliana*, demonstrated that 8-prenylated kaempferol was mainly stored in leaves, which could be due to a plant-specific pattern of accumulation for any excess of product. Perhaps prenylated naringenin is not the preferred form of storage and hence is converted to kaempferol before storage.
- A lot of factors can influence the expression of a gene when it is introduced in a host plant for the stable expression. These factors may influence the substrate specificity of respective gene, which could explain the above described situation.

Another study demonstrated that ectopic expression of *Sf*N8DT-1 and *SfG6DT* in *Lotus japonica* without exogenous supply of substrate, does not lead to the formation of any prenylated product. However, when these plants were fed with naringenin or genistein, *Sf*N8DT transformants produced 8-dimethylallylnaringenin while *Sf*G6DT transformants produced 6-dimethylallyl genistein. However, no accumulation of 8-dimethylallyl kaempferol was reported, which is in contrast to the previously demonstrated results. In addition, mutant plants overexpressing *Sf*N8DT and *Sf*G6DT, displayed metabolic profiles with significant differences among the quantities of various prenylated polyphenols. This study also suggests that properties of Pts modify in transgenic plants in a host specific manner (Sugiyama *et al.,* 2011).

3 Objective of the project

Identification and functional characterization of genes involved in the biosynthesis of furanocoumarins in higher plants

Furanocoumarins constitute a class of secondary metabolites of plants that has emerged as potent molecules of plant defense system. Furthermore their properties made them as molecule of great interest for the pharmaceutical industry. Despite the interest of these molecules less is known about the molecular aspects of their synthesis. Indeed, various biochemical approaches have been implemented since the 1960's to explore this pathway but the first elements concerning the genes involved in this pathway were only published in 2004 (identification of the bergaptol o-methyltransferase).

The "Furanocoumarin" project started in 1988 in the "Laboratoire d'Agronomie et Environnement". The first studies used agronomic approaches in order to understand and increase the production of furanocoumarins in different plant families (Massot *et al.,* 2000; Milesi *et al.,* 2001). In 2001, these studies switched to molecular approaches in order to characterize the genes involved in the pathway but also to understand how this pathway appeared in only four different plant species. First, the scientists of this group focused on genes encoding P450s because most of the steps in the pathway were catalyzed by these enzymes. This work led us to identify a C4H of *R. graveolens,* which is indirectly implicated in the biosynthesis of furanocoumarins (Gravot *et al.,* 2004). Important results for the understanding of this biosynthetic pathway were presented by Romain Larbat with the identification and characterization of two P450s (psoralen synthase and AS), which are directly involved in the biosynthesis of furanocoumarins (Larbat *et al.,* 2007, 2009). Even if not all the P450-mediated steps are identified, we recently discovered another enzymatic family involved in the synthesis of these molecules. Indeed, a 2-oxoglutarate dependent dioxygenase was demonstrated to catalyze the *ortho* hydroxylation of *p*-coumaroyl CoA in order to generate umbelliferone (Vialart *et al.,* 2012).

The objective of my thesis project was to go on with the molecular characterization of the genes involved the synthesis of these molecules. In this work we focused on the prenylation step of umbelliferone which is the real entry into the furanocoumarin pathway. The result part of this manuscript is divided into 4 parts;

The first part explains how we isolated new candidate genes. This part has been started just before the beginning of my thesis and also involves Japanese collaborators;

The second part explains how we managed to express the Pts using the heterologous expression system of *N. benthamiana*-based. To adapt this system to Pts, I used different proteins such as GFP, a P450 and an already described Pt. Therefore, in this part I report the original results I obtained for the functional characterization of CYP98A22 which has been published in 2012 (Karamat *et al.,* 2012);

The third part concerns the molecular and functional characterization of Pt/Pts involved in the synthesis of osthenol and demethylsuberosine. This chapter constitutes the main part of my PhD work;

In the last part, I describe the preliminary results obtained for two other Pts and their involvement in the synthesis of various prenylated coumarins and furanocoumarin

Materials and Methods

Material and Methods

1 Material

1.1 Plant material

1.1.1 *P. crispum*

Plant used for these studies were grown from seeds of *P. crispum* variety *crispum* provided by Clause Semences (Bretigny Sur Orge, France).

P. crispum is a biennial crop that belongs to the Apiaceae plant family. Plant grows normally from 0.3 m up to 0.6 m. The plant is of Central and South European (probably East Mediterranean) origin and was naturalized in Britain. Flowering of this plant occurs from June to August and seeds ripen from July to August. The flowers are yellow and hermaphrodite. Apart from the botanical classification, Parsley has been divided into two main cultivated types, i.e. Leaf parsley and Root parsley, which depends on the nature of their uses. Leaf parsley (Figure 40) can have curly (used for garnishing) or flat leaves (preferred for its strong flavor). On the other hand, Root parsley produces much thicker roots that are used for soups and stews.

Figure 40. ***Petroselinum crispum***

1.1.2 ***N. benthamiana***

N. benthamiana (Figure 41A) seeds were provided by Dr Etienne Herrbach (INRA Colmar, France). This plant is widely used for scientific studies and especially as a system of transient expression of proteins using an agro-infiltration approach.

N. benthamiana is an herbaceous plant that belongs to the Solanaceae plant family, and is widely distributed on the rocks present on hills and cliffs in northern areas of Australia. An enormous size variation has been described for these plants depending on their height (0.2to1.5 m) and habit. The flowers produced by these plants are white in color (Figure 41B).

Figure 41. *N. benthamiana*

1.1.3 ***R. graveolens***

R. graveolens were obtained from Conrad Appel, Samen and Pfanzen GmbH (BismarckstraBe 59, 64 293 Darmstadt, Germany).

Figure 42. *R. graveolens*

(A) presence of pentamerous and tetramerous flower of *R. graveolens* (B)

R. graveolens (Figure 42 A), known through many different names such as common rue, garden rue and herby grass, is a traditional medicinal plant and belongs to the Rutaceae plant family. This plant has first been described in Eastern and Southeastern Europe but is now widely distributed all over the temperate and tropical regions of the world. It is a small evergreen subshrub, semiwoody and perennial, varying in size from 2 to 3 m height and almost in width. Leaves are 7 to 13 cm long and are dissected pinnately into oblong or spoon shaped segments. The peniculate clusters of small yellow flowers appear in mid-summer. Rue displays both pentamerous (5-parted) and tetramerous (4-parted) flowers on the same plant (Figure 42 B), with the pentamerous flower usually located in the center of the inflorescence (cyme). This rare event could be explained as "terminal flower effect". Fruits are five lobed capsules, containing many seeds.

1.2 Bacterial strains

1.2.1 *E. coli* Top 10

This bacterial strain was used for the replication of different plasmids. It is provided by Invitrogen.

Genotype: [F- mcrA _(mrr-hsdRMS-mcrBC) Φ80lacZ_M15 _lacX74 recA1 araD139_(araleu) 7697 galU galK rpsL (StrR) endA1 nupG]

1.2.2 *E. coli* GeneHogs® (Invitrogen)

This bacterial strain derives from *E. coli* DH10^{TM}. This strain is described as being highly efficient for the amplification of large sized plasmids.

Genotype: [F- mcrA D(mrr-hsdRMS-mrcBC) f80lacZDM15 DlacX74 deoR recA1 endA1 araD139 D(ara-leu)7697 galU galK l rpsL nupG].

1.2.3 *E. coli* M15

This bacterial strain is derived from *E. coli* K12. It harbors a low copy plasmid (pREP4) which confers kanamycin resistance (Kan^{r}) and constitutively expresses the *lac* repressor protein encoded by the *lac* I gene (Farabaugh *et al.,* 1978).

Genotype: [*nal*s *str*s *rif*s *thi- lac- ara+ gal+ mtl- f rec*A+ *uvr*+ *lon*+]

1.2.4 ***E. coli* BL21 (DE3) (Novagen)**

This strain is used for the expression of recombinant proteins. It has the lysogen DE3, containing the gene coding for the T7 RNA polymerase under the control of the lacUV5 promoter. The expression of the T7 RNA polymerase is induced by Isopropyl β-D-1-thiogalactopyranoside (IPTG).

Genotype: F– ompT hsdSB(rB–, mB–) gal dcm (DE3)

1.2.5 ***E. coli* ccdb survival**

This bacterial strain is used for the amplification of plasmids containing the *ccdB* gene. The product of this gene is lethal for "normal" *E. coli.*

Genotype: F- mcrA (mrr-hsdRMS-mcrBC) Φ80lacZ_M15 _lacX74 recA1 ara_139_(araleu)7697 galU galK rpsL (StrR) endA1 nupG tonA::Ptrc-ccdA.

1.2.6 ***E. coli* HB101 (pRK2013)**

This bacterial strain contains a helper plasmid pRK2013. pRK2013 contains a gene *mob* encoding for a protein known as mob (mobilizing) and another gene known as *tra* (transfer) that helps in transfering binary plasmid from donor strain to recipient agrobacteria strain.

Genotype: supE44, hsdS20 (rB- mB-), recA13, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5,mtl-1.

1.2.7 ***E. coli* MC1061**

This strain is used in triparental conjugation as a donor strain. These bacteria are transformed with binary vectors containing the required inserts. Thus plasmid pBIN-X is transferred in the presence of *E. coli* HB101 (pRK2013) helper strain to *Agrobacterium tumefaciens* C58C1Rif^{R}, through the mechanism of triparental conjugation.

Genotype: hsdR2, hsdM+, hsdS+, araD139 _ (ara-leu)7697, _(lac)X74, galE15, galK16, rpsL (StrR) mcrA, mcrB1.

1.3 Agrobacterial strains

1.3.1 *A. tumefaciens* C58C1Rif^R (pGV2260)

This bacterial strain contains a Ti plasmid pGV2260 that contains the genes of virulence, necessary for the infection of plants. This plasmid also contains a gene of resistance against carbenicillin. This strain displays a resistance against rifampicin (rif) and is used as a vector for genetic transformation of plants. This strain was provided by Dr. Brigitte Thomasset, UMR 6022 CNRS Génie Enzymatique et Cellulaire, Université de Technologie de Compiègne, France.

1.3.2 *A. tumefaciens* LBA4404 (pAL4404)

A. tumefaciens strain LBA4404 and the binary vector method was generated by Dr. P.J.J Hooykaas at Leiden University in the Netherlands. This strain has a disarmed Ti plasmid pAL4404 plasmid, which only contains the T-DNA vir region (genes responsible for vir gene induction and T-DNA transfer), and is a widely used for plant transformation. This strain is resistant against rifampicin while plasmid pAL4404 provides resistance against streptomycin.

1.4 Yeast strains

1.4.1 *Saccharomyces cerevisiae* WAT11 and WAT21

S. cerevisiae WAT11 and WAT21 were generated by Dr. Denis Pompon (Laboratoire d'Ingénierie des Protéines Membranaires (LIPM), Gif-sur-Yvette, France). They are derived from the W303 Strain. In this strains, the endogenous cytochrome P450 reductase gene (CPR) was replaced by the *Arabidopsis thaliana* reductases AtR1 or AtR2 (for WAT11 and WAT21 respectively) under the control of the GAL10-CYC1 promoter. This promoter is repressed by glucose and induced by galactose. These strains are ADE2 and therefore are unable to grow on a medium without adenine.

1.5 Recombinant plasmids and vectors

1.5.1 pYeDP60

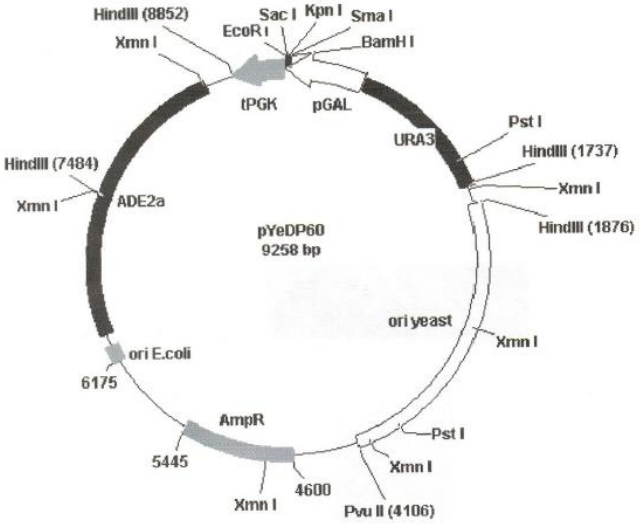


Figure 43. Map of plasmid; pYeDP60

This plasmid has been generated by Dr. D. Pompon (Laboratoire d'Ingénierie des Protéines Membranaires (LIPM), Gif-sur-Yvette, France). It is a shuttle vector and can therefore replicate in yeast and *E. coli*. In yeast, this vector contains an origin of replication of yeast (2μ) and provides auxotrophy for adenine and uracile (ADE2 and URA3). In bacteria, the plasmid contains an *E. coli* origin of replication (ColE1) and confers resistance to ampicillin (amp).

A Multiple Cloning Site (*Bam* HI, *Sma* I, *Kpn* I, *Sac* I, *Eco* RI) allows cloning of open reading frames downstream the GAL10/CYC1 promoter and upstream the PGK terminator. The GAL10-CYC1 promoter is induced by galactose and repressed by glucose (Figure 43).

1.5.2 pQE30 (Qiagen)

This plasmid possesses a ColE1 origin of replication and confers the resistance to amp (*bla* gene); this plasmid is devoted to protein overexpression. It contains a strong T5 promoter which controls the transcription of open reading frame inserted in a multiple cloning site, along with a double operator lacO (Figure 44). IPTG is used as an inducer for the overexpression of the corresponding proteins. In the absence of IPTG, tetramers of LacI

repressor interact with the *LacO* operator and repress the expression of the genes of interest. The MCS allows the addition of a poly-histidine tag at the N-terminus of the protein of interest. The plasmid contains a transcription terminal region *rrn*B T1.

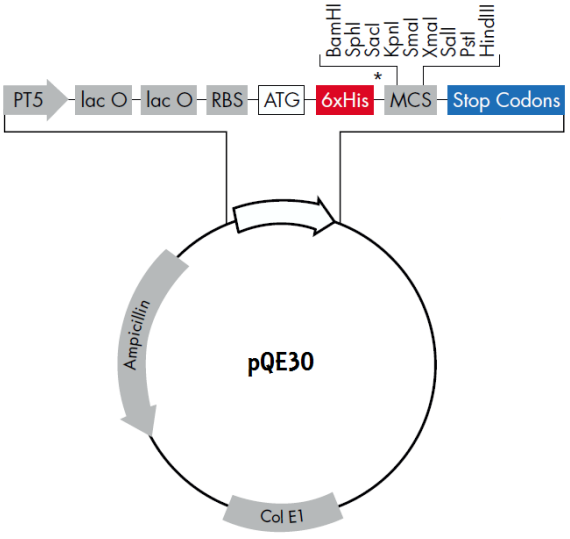


Figure 44. Map of plasmid; pQE30 (Qiagen)

1.5.3 pGEX-KG (Amersham Biosciences)

This plasmid derives from pBR322 and contains the gene providing resistance to amp. It contains a promoter tac that can be induced by IPTG for the expression of glutathione *S*-transferase (GST)-tagged recombinant proteins.

1.5.4 pCR®8/GW/TOPO® (Invitrogen)

pCR®8/GW/TOPO® (Invitrogen) is a kit developed by Invitrogen in order to allow efficient cloning of PCR product. This plasmid can serve as an entry vector for the Gateway® technology. This vector is provided linearized with single 3'-thymidine (T) overhangs coupled with a Topoisomerase. *Taq* DNA polymerases are enzymes that are known to add a single deoxyadenosine (A) to 3' end of their PCR products. When these PCR products are mixed to the pCR®8/GW/TOPO®, the topoisomerase allows a quick and efficient insertion of the PCR product to generate a recombinant plasmid (Figure 45).

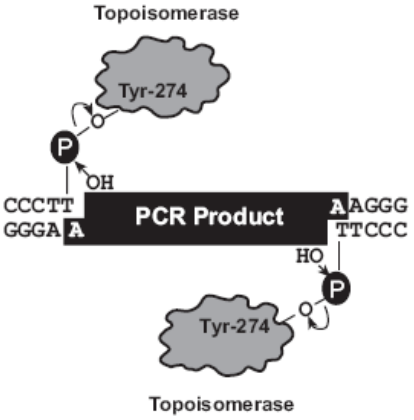


Figure 45. Mechanism of ligation of insert into pCR®8/GW/TOPO® (Invitrogen)

Furthermore, pCR®8/GW/TOPO® vector is equipped with two specific recombinantion sites, *att*L1 and *att*L2, that allows the transfer of the insert into a destination vector by the "LR recombination" process. The vector contains a gene providing resistance to spectinomycin. The origin of replication derives from a pUC plasmid.

1.5.5 Binary vector systems

A binary system is an efficient system to perform transformation of Agrobacteria strains. This system requires two types of plasmids, a binary plasmid and a helper plasmid. In such a system the T-DNA (containing the gene of interest) and *vir* genes (necessary to enable the transfer of the T-DNA in the genome of plants) are present on separate replicons. The binary vectors contain modified T-DNA region. These plasmids contain an origin of replication that is able to be active both in *E. coli* and *A. tumefaciens.* The *vir* genes, known as *vir* helpers, are localized on an independent plasmid.

1.5.5.1 pBin-GW

pBIN-GW is a binary vector that was constructed in our laboratory by Sébastien Doerper during his PhD. It derives from the plasmid pBIN m-gfp-5ER provided by Prof. Jim Haselhoff, Division of Cell Biology, MRC Laboratory of Molecular Biology, Addenbrookes Hospital, Hills Road, Cambridge, CB2 2QH, UK. It is a low copy Gateway® destination vector. The *npt*II gene confers resistance against kan (Kan^R) in transformed plant cells and a cassette of recombination (RfB) flanked by two site *att*R1 and *att*R2, are present between the

right and left borders of the T-DNA region. The *npt*II gene is under the control of the Nopaline synthase promoter and terminator. The RfB recombination cassette includes a gene providing resistance against chloramphenicol and a *ccdB* survival gene (Figure 46). Borders of this cassette are made up by the CaMV 35S-promoter upstream and by the 3'nos-terminator downstream. A specialized *E. coli* strain resistant to the product of the *ccdb* gene is necessarily used for the replication of this plasmid. A second gene that provides resistance against kan is present outside T-DNA region of plasmid and used for selection of recombinant *E. coli* bacteria.

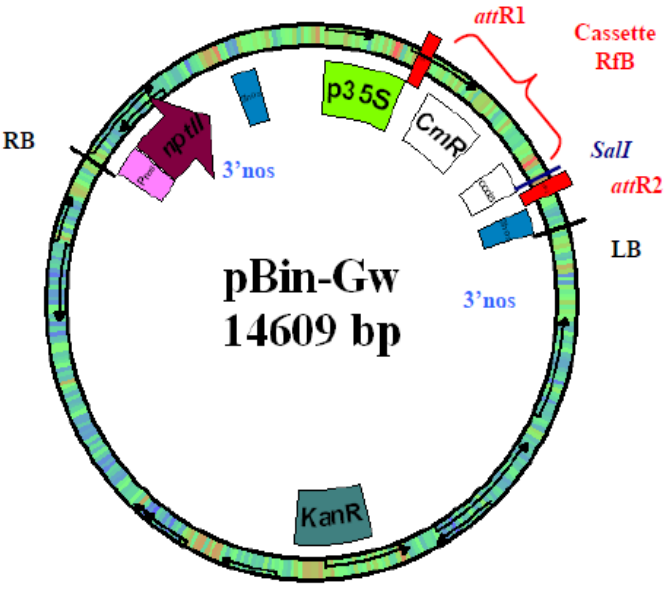


Figure 46. Map of plasmid; pBIN-GW

1.5.5.2 **pGWB2**

The plasmid has been provided by Dr Tsuyoshi NAKAGAWA (Center for Integrated Research in Science Shimane University, Matsue 690-8504, Japan). This high copy binary vector has been constructed to permit the overexpression of genes under the control of the CaMV 35S promoter. As pBIN-GW, this plasmid contains a GATEWAY® cassette allowing efficient recombination with a DONOR plasmid (like pCR8®). Moreover, the T-DNA region contains two gene dedicated to selection *nptII* and *hpt* which provides resistance to kan and hygromycin respectively for the transformed plant cells (Figure 47). Expression of *npt*II is controlled by the Nopaline Synthase promoter and terminator, while the CaMV *35S* promoter

controls the expression of *hpt.* The Gateway® Recombination Cassette lies between two sites *att*R1 and *att*R2 and contains both a gene providing resistance against chloramphenicol and a *ccdb* gene.

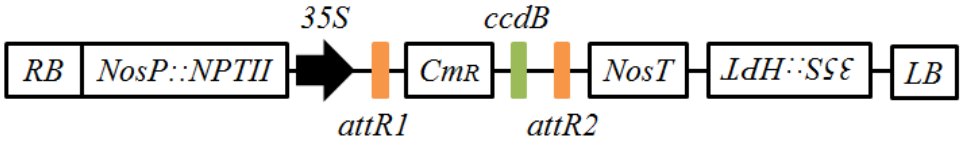


Figure 47. T-DNA region of plasmid pGWB2

1.6 Culture media

1.6.1 For bacteria

1.6.1.1 Liquid LB

This LB medium (Luria-Bertani) is used for the growth of all the *E. coli* strains. This media was prepared by dissolving 25 g of LB (Sigma Aldrich) powdered medium in 1000 ml of water. This solution was autoclaved (20 min, 1 bar) and stored at room temperature. The composition of the medium is presented in table (Table 6).

Table 6. Composition of LB medium

Composition	Tryptone	Yeast extract	NaCl	H_2O
Concentration (g/L)	10	5	5	1 L

1.6.1.2 Solid LB

To prepare this medium 15g/L agar (sigma, Aldrich) was added to the LB liquid medium before autoclaving. This medium is then solidified by cooling in Petri plates. These plates are Stored at 4°C.

1.6.1.3 YEB

This liquid medium is used for the growth of *A. tumefaciens*. Its composition (Table 7) is presented below.

Table 7. Composition of YEB medium

Composition	Beef extract	Yeast extract	peptone	Sucrose	H_2O
Concentration (g/L)	5	1	5	5	1 L

Solid YEB medium is obtained by adding 15g/L agar to YEB liquid medium. YEB Mg^{2+} solid medium is obtained by adding 15g/L agar and 0.5 g/L $MgSO_4$ to liquid YEB before autoclaving the medium.

1.6.2 Yeast medium

Table 8. Composition of media used for yeast

	YPGA	SGI	YPGE	YPL
Yeast extacts (g/L)	10		10	10
Bactopeptone (g/L)	10		10	10
Bactocasamino acid (g/L)		1		
Yeast Nitrogen base without amino acids (g/ L)		6.7		
Tryptophan (mg/L)		40		
Adenine (mg/L)	30			
Glucose (g/L)	20	20	20	
Galactose (200 mg/L)				20ml

1.6.2.1 **YPGA**

This is a complete medium supplemented with adenine, and used for the growth of wild type yeast strains WAT11 and WAT21 (Table 8).

1.6.2.2 **SGI**

SGI is a minimal selective medium deficient for adenine that allows the selection of transformed yeast. This yeast deficiency for adenine is complemented by introducing the pYeDP60 plasmid in the cells. Thus, only the transformed yeast can grow on this medium (Table 8).

1.6.2.3 **YPGE**

YPGE is used for growth of transformed yeast. The presence of glucose in the medium inhibits the expression of the *A. thaliana* a CPR as well as the protein of interest incorporated in pYeDP60. YPGE is a complete medium and therefore generates no selection pressure (Table 8).

1.6.2.4 **YPL**

YPL is a complete medium used to induce the expression of the CPR and the protein of interest, cloned into pYeDP60. This medium contains galactose that activates the *GAL10-CYC1* promoter, which controls the synthesis of these two proteins in parallel. As YPGE, YPL is a complete medium and therefore generates no selection pressure (Table 8).

Solid media for yeast were prepared by adding 15 g/L of agar to liquid medium before autoclaving.

1.6.3 **Culture media and conditions used for plants culture**

1.6.3.1 **Murashige and Skoog (M*and*S)**

In vitro culture of *R. graveolens* was done on a solid and sterile Murashige and Skoog (M*and*S) (Duchefa Biochemie, UK) medium, prepared by addition of 8 g/L of agar and 20 g/L of saccharose to the remaining components. Basic composition of the M*and*S medium is presented in (Table 9).

Table 9. Composition of M*and*S

Macroelements mg/L	
NH_4NO_3	1650
KNO_3	1900
$CaCL_2$, 2 H_2O	440
$MgSO_4$, $7H_2O$	370
KH_2PO_4	170

Vitamins mg/L	
Inositol	100
Nicotinic Acid	0.5
Pyridoxin HCL	0.
Thiamin HCL	0.1
Glycine	2

Microelements mg/L	
KI	0.83
H_3BO_3	6.2
$MnSO_4$, H_2O	16.9
$ZnSO_4$, 7 H_2O	8.6
$NaMoO_4$, 2 H_2O	0.25
$CuSO_4$, 5 H_2O	0.025
$CoCL_2$, 6 H_2O	0.025

Fer mg/L	
$FeSO_4$, 7 H_2O	27.8
Na_2EDTA, 2 H_20	37.8
pH	8.0

The basic M*and*S medium is modified by different ways to be used on the various steps of the development of transgenic plants of *R. graveolens* (the plant transformation protocol is described in Lièvre *et al.,* 2005). These modifications are as following:

- Liquid M*and*S medium supplemented with benzylaminopurine (BAP, 0.1 mg/L) is used for the preparation of agrobacteria inoculum for stable transformation of plants.
- Solid M*and*S media supplemented with BAP (0.1 mg/L) and acetosyringone (50 mg/L) is used for the co-culture of explants with agrobacteria.
- Solid M*and*S medium supplemented with BAP (0.1 mg/L) and Cefotaxime (300 mg/L) is used as a medium of regeneration for *R. graveolens* plantlets of after the co-culture step of explants with Agrobacteria.

Solid M*and*S medium supplemented with BAP (0.1 mg/L), Cefotaxime (300 mg/L) and kan (75 mg/L) is used as a selection medium for transformed *R. graveolens* cells.

1.6.3.2 ***In vitro* culture of *R. graveolens***

In vitro culture of *R. graveolens* was carried out in growth chamber with specific conditions. i.e., 23 °C during the day and 20 °C during the night, 40% relative humidity, and 160 µmol/m^2/s light intensity with a photoperiod of 16 hours (h). Multiplication by micropropagation of *R. graveolens* plants is carried out in glass jars of 200 ml. All steps of *in vitro* culture (seeds or plants) were carried out in a vertical laminar flow hood (Thermo Electron Corporation, Germany). Forceps and scalpels used for experimentations were pastor sterilized in an oven at 180 °C for 24 h before any use. Any other material used was autoclaved before use.

1.6.4 **Growth of plants in soil**

Plants in soil were grown in individual pots in greenhouse at a temperature of 22°C and were watered once a week.

1.7 Antibiotics

Table 10. Final concentrations of antibiotics

Antibiotics	Concentration of stock solution (mg/ml)	Solvent used to make solution	Final concentrations
Ampicillin	100	Water (H_2O)	50 µg/ml
Carbenicillin	100	Water (H_2O)	100 µg/ml
Chloramphenicol	100	Ethanol (100%)	30 µg/ml
Kanamycine	50	Water (H_2O)	50 µg/ml and 75mg/L
Rifampicin	100	DMSO (Dimethyl Sulfoxide)	100 µg/ml
Spectinomycin	100	Water (H_2O)	50 µg/ml
Cefotaxime	100	Water (H_2O)	300mg/L

Antibiotics are added to the media for selection of transformed bacteria or plants. Solutions of these antibiotics were prepared using respective solvent (Table 10) and then these solutions were sterilized using filters of 0.2 µm sizes. The antibiotics are added to the media after autoclave and by cooling down the media up to 55 °C.

2 Molecular biology

2.1 DNA Extraction from plant

DNA Extraction was performed from different plants such as, *R. graveolens*, *P. sativa* and *P. crispum.* Organs were grounded in liquid nitrogen with pistil and mortar. The powder obtained was then used for extraction of genomic DNA following the protocol provided with the kit “DNeasy Plant Mini Kit” (Qiagen). Qquality of extracted DNA was checked thanks to gel electrophoresis. And concentrations were measured before storage at -20 °C.

2.2 RNAs Extraction from plant material

Extraction of RNAs from plants is delicate due to the fact that a large quantity of RNase is present in the working environement. In addition RNAs are very sensitive to temperature and degrades very quickly at room temperature. Therefore special care is taken in account to solve these problems. A RNAse neutralizing solution (RNaseZap® - Ambion) was applied to the working bench and the user wear gloves for the whole extraction.

Extraction was performed using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's recommendations. Immediately after the extraction, samples were used for measurement of concentrations, aliquoted and freezed at -80 °C. The quality of samples was tested by migrating samples on agarose gel and quantified.

2.3 Amplification of DNA fragments by a Polymerase chain reaction (PCR) approach

2.3.1 Classic Polymerase chain reaction

PCR is a method for amplifying DNA *in vitro*. The PCR starts with a heat denaturation step at 95°C which leads to the separation of the two DNA strands. The second step allows the annealing of oligonucleotidic primers to the DNA matrix (generally 50 to 65 °C depending o3n the primer sequence). The melting temperature of the primers is based on the length and quantity of purine basis and is calculated as follow:

Tm= 69.3+0.41 x (%GC) – 650/N, where N is the number of nucleotides present in the primer.

The annealing temperature must be fixed close to the melting temperature, it is generally fixed as Tm= -5 °C.

The third step is performed at 72 °C which is generally the optimal working temperature of the *Taq* DNA polymerase. This step allows the synthesis of the complementary strand of the DNA Matrix starting from the primer. The enzyme needs free dNTPs present in reaction mixture to synthesize this strand. Time duration for this elongation step depends on

the length of the sequence to be amplified (generally 1 min/1000 base pairs). These three main cycles are repeated for specific numbers that permit to amplify specific fragments of DNA.

Table 11. Different steps of a classic PCR

Steps	Duration of each step	Temperature	cycle
Initial denaturation	10 min	95 °C	1
Step of denaturation	30 to 60 sec	95 °C	25 to 35 cycles
Step of Hybridization	30to 90 sec	50 °C to 65 °C	
Step of Elongation	30 to 120 sec	72 °C	
Step of Elongation	10 min	72 °C	1

All PCRs were carried out using two different machines: either an Icycler (Bio Rad) or or Gradient Cycler PTC-200 (MJ Research). Reaction mixtures prepared for the PCR contains 0.4 µM of each sense and antisense primer, 0.4 mM of dNTP, 10X PCR buffer, 2 to 5 U of *Taq* DNA polymerase and 10 to 100 ng of DNA.

2.3.2 High Fidelity PCR

High fidelity PCR were done with Platinum® *Pfx* DNA polymerase (Invitrogen). Unlike *Taq* DNA polymerase, this enzyme got a proof reading activity which increases the fidelity of the PCR and also generate amplified fragment without adding an additional A at their end (cloning in the pCR8 TA system is therefore not possible without an additional step in the presence of a classical polymerase, see the paragraph 2.3.1 for details). For PCR using this enzyme, the reaction mix contains 0.8 µM of each primer, 2 U of Platinum® *Pfx* DNA polymerase, and 0.3 mM each of dNTPs, 10 pg-200 ng of DNA, 1 mM $MgSO_4$ and 10 X *Pfx* amplification buffer for a 50 µL of total reaction volume. The PCR starts with an initial denaturation step at 94 °C for 5 min, and then 35 cycles of amplification (30 sec at 94 °C, 30 °C at 55 to 60 °C, and then 1-1:30 min at 68 °C). A final elongation step was carried at 68 °C for 10 min. To add an adenine at the 3' ends of the amplified products 2 U of *Taq* DNA polymerase (Invitrogen) was added to the reaction mixture and an additional incubation at 72 °C for 10 min is performed.

2.3.3 Reverse transcription

Reverse Transcription leads to the synthesis of cDNA (Complementary deoxyribonucleic acid) using a reverse transcriptase. These cDNAs can be subsequently use in a PCR reaction to amplify and therefore check the expression of a specific gene. Such a RT PCR can be performed either in one step or in a two-step process. In a two-step RT-PCR, cDNA is first synthesized by a RNA dependent DNA polymerase, which uses deoxyribonucleotides to generate cDNA. This reaction is done following the protocol provided with the «high capacity RNA -to-cDNA master mix» kit by Applied Biosystem (Annex).

For the single step RT-PCR the reverse transcription and the PCR amplification is done sequentially in the same tube. For this approach, we followed the protocol provided with the «SuperScript™ One-Step RT-PCR with Platinum® *Taq*» kit. Once this step is done, the RT is inactivated and the Platinum® *Taq* DNA polymerase activated at 95 °C followed by classical PCR conditions (see above). Single step RT-PCR reduces the risks of contamination, mixing of sample and RNA degradations.

2.3.4 Verification of the stably transformed plants using the Phire plant direct PCR kit

Phire plant direct PCR kit is a kit developed specifically with purpose of performing the PCR directly from plant material (leaves and seed) skipping the process of DNA purification.

2.3.4.1 Preparation of samples

One leaf of transformed plant of *R. graveolens* was harvested. Samples of consistent sizes were obtained using a convenient sampling tool (Harris Uni-Core and Cutting Mat.) provided by the Thermo scientific "Phire Plant Direct PCR Kit", and ejected into 20µl of dilution buffer. These samples prepared were then directly used for PCR following the instructions of provider.

2.3.4.2 Polymerase chain reaction for Phire plant direct PCR kit

This PCR differs from a classical reaction only for the primers annealing temperature, which were measured using the Tm calculator and instructions available at www.thermoscientific.com/pcrwebtools. Thermo Scientific Phire Hot Start II DNA Polymerase has an engineered DNA-binding domain that enhances the polymerase activity. Additionaly, it exhibits extremely high resistance to many PCR inhibitors found in plants. Reaction mix for PCR contained, 0.5 µM of each primer, 0.4 µl of Phire Hot Start II DNA Polymerase, 10 µl of 2x Phire Plant PCR Buffer and 0.5 µl of Plant tissue (dilution protocol) for a 20 µL of total reaction volume.

The PCR starts with an initial denaturation step at 98°C for 5 min, and then 40 cycles of amplification (5 sec at 98°C, 5 sec at 62 to 78°C, and then 20-40 sec at 72°C). A final elongation step was carried at 72°C for 1 min. Two different primer pairs (*npt*II dir and *npt*II rev (i) and 35S dir and x-transgene rev (ii)) were utilized for checking the presence of two genes (*npt*II (i) and transgene along with the part of 35S promoter: 35S:: transgene (ii)) in different potentially transformed palnts of *R. graveolens.* Primer pair utilized for the amplification of the *npt*II gene along with the temperature of hybridization is listed in atable (Table 12), while the second pair of primers will be discussed latterely in the respective part.

Table 12. Sequences of the primers used for amplifying *nptII*

	Direct	Annealing temp.
***nptII* Dir**	ATTGAACAAGATGGATTGCAG	62 °C
***nptII* Rev**	TCGTCAAGAAGGCGATAGAA	

2.4 Analysis of DNA and RNA by agarose gel electrophoresis

Agarose gel electrophoresis is a technic that is used to separate DNA or RNA on the basis of their sizes. Concentration of the gel can be adjusted depending on the type of molecule to be analyzed. A gel with a concentration of 1% will be used for analysis of a wide range of molecules. This gel is prepared by dissolving 1g agarose in 100 ml TAE 1X buffer (Tris acetate 40 mM, EDTA 1 mM). Samples are supplemented with 6X loading buffer (Tris

50 mM pH 7.5; EDTA 100 mM pH 7.5; 10% glycerol; 0.05% bromophenol blue) and loaded on the gel. Separation of the sample is realized at 100 V (10 V/cm) for 20 to 45 min depending on type of sample. In addition to samples, a colored marker of size (Fermentas massruler ready -to-use # SM0403) is loaded on the gel to help to determine the approximate sizes of the bands appearing on the gel after staining with Ethidium Bromide (EtBr) or Sybr Safe. EtBr, is an intercalating agent and commonly used as fluorescent tag in molecular biology. Once intercalated into DNA it fluoresces when exposed to Ultraviolet light (λ=254 nm). EtBr is highly toxic. Syber® Safe DNA Gel stain (Invitrogen) is an alternative to EtBr. It has been classified as less toxic than EtBr for users as well as for environment. It is also an intercalating agent and is available in solution 10.000 time concentrated (Invitrogen) in Dimethyl sulfoxide (DMSO). This solution is finally diluted in a ratio of 1:10000. DNA stained with Syber® Safe DNA Gel stain can be visualized by blue light transilluminator or a standard UV transilluminator. However it is advised to avoid the exposure of DNA stained with Syber® Safe to UV if it is going to be used in cloning process.

2.5 Extraction of DNA from agarose gel

Once migration and visualization of samples on agarose gel is finished, DNA can be extracted from the gel. For this purpose, the band is cut out from the gel using a clean scalpel and is transferred in a 2 ml microtube. The DNA is than extracted from the gel using the QIAquick Gel Extraction Kit (Annex). Once extracted, the DNA can be used for subsequent experiments such as cloning, sequencing or re-amplification by PCR.

2.6 Digestion of DNA fragment by restriction enzymes

All the restrictions have been done with the FastDigest® Restriction Enzymes commercialized by Fermentas (Thermo scientific). These enzymes allow a short time incubation (reducing the time of incubation by a factor of 10 as compared to classical enzymes) and provide to do most of the digestion in a single and common buffer. Digestion reaction is generally carried out in a final volume of 20 µl containing 2 µl of 10X buffer, 1 µl of restriction enzyme and 1 µg of DNA. Reaction mixture is incubated at 37 °C for 5 min to 1 h.

2.7 Ligation

2.7.1 pCR8®/GW/ TOPO®

Ligation of inserts of interest into the pCR8 vector is done using the pCR8®/GW/TOPO® TA Cloning kit as described by the supplier (Invitrogen). To be efficient, the PCR product needs to have an additional A at its 3' end (see the paragraph 1.5.4 for details).

Reaction is carried out in a total volume of 6 µl with 10 ng of linearized pCR8 vector, 1.2 µmol of NaCl, 0.06 µmol of $MgCl_2$ and 50 to 100 ng/µl of PCR product. Reaction mixture is incubated for 30 min at room temperature to ensure 95% of ligation of PCR product into vector.

2.7.2 Ligation in other vectors

Ligation in standard vectors is done using the T4 DNA ligase provided by Invitrogen. This system allows the ligation of DNA fragments either blunt or sticky ended into linearized plasmids. Reaction is done in a total volume of 20 µl containing 4 µl of Ligase buffer 5X, 0.1U of T4DNA ligase, 3 to 30 fmol of vector and 9 to 90 fmol of insert. Reactions are done at 16 °C for night.

2.8 Construction of recombinant binary plasmid for genetic transformation of plant cells using the Gateway® Technology

Gateway® is a universal cloning technology developed by Invitrogen, and based on site-specific recombination properties of bacteriophage lambda. The system provides a rapid and highly efficient system to transfer DNA into the pBIN-GW or the pGWB2 plasmids.

2.8.1 Recombination reaction *via* Gateway® technology

Recombination reaction is carried out using the Gateway® LR Clonase enzyme mix provided by Invitrogen. This enzyme mix includes a bacteriophage lambda recombination protein Integrase and Excisionase and the Integration Host Factor (IHF) as an *E. coli* encoded protein (Invitrogen).

On a general point of view, the recombination reaction is carried out using an entry clone of Gateway technology and a destination vector of Gateway technology. In our experiments, the entry plasmid is any recombinant pCR8 plasmid (see heading 1.5.4) and the destination plasmid is either pBIN-GW or pGWB2. The DNA of interest which is present between the *att*L1 and *att*L2 sites of plasmid PCR8-GW-X is recombined with the sequence present between two sites *attR1and att*R2 of destination vector (Figure 48)

Figure 48. schematic representation of LR recombination reaction (Invitrogen)

This reaction is done in a total volume of 10 µl containing 100 ng of pCR8-GW-X, 200 ng of destination plasmid pBIN-GW (or pGWB2) and 2 µl of LR Clonase® (Invitrogen) and was incubated at 25 °C for 15h. Reaction is then stopped by adding 1µl of proteinase K (Invitrogen) to the reaction mixture and incubated at 37 °C for 10 min. The reaction mixture is then used to transform chemically competent *E. coli* TOP10. Transformed bacteria were spread on selective LB media and incubated at 37 °C for whole night.

2.9 Preparation of competent *E. coli* bacteria

2.9.1 Preparation of electro-competent bacteria

A preculture of bacteria was prepared by inoculating 10 ml of LB medium with one colony of *E. coli*. After one night incubation under agitation at 37 °C, 1 ml of this preculture is used to inoculate 100 ml of LB medium. This new culture is incubated at 37 °C until an OD_{600} of 0.7 is reached. Culture is then centrifuged at 5000 g at 4 °C for 15 min. The resulting bacterial is smoothly re-suspended and washed successively, two times with cold sterile water and two times with cold glycerol 10%. Finally the washed and ultra-concentrated competent bacteria are suspended in 1ml of glycerol 10%, aliquoted (40 µl in each tube) and freezed in liquid nitrogen before storage at -80 °C for maximum 6 months.

2.9.2 Preparation of chemically competent bacteria

A preculture was prepared by inoculating 10 ml of LB medium with one colony of *E. coli* in the presence of selective antibiotic, if necessary. This preculture is incubated during 15h at 37 °C. One ml of this preculture is used to inoculate 50 ml of LB containing the same antibiotic. This new culture is incubated at 37 °C until an OD_{600} of 0.7 is reached. The culture is then centrifuged at 4000 g for 10 min. The pellet is suspended in 10 ml of sterile $CaCl_2$ (100 mM). Bacteria are again collected by centrifugation at 4000 g for 10 min and finally in 1 ml of $CaCl_2$. These bacteria were incubated on ice for at least one hour before being used for transformation by heat shock.

2.10 Transformation of competent *E. coli* bacteria

2.10.1 Electroporation

The application of a short electrical shock of high voltage destabilizes the membrane of the bacterial cells and makes it porous and permeable for a plasmid. An aliquot of 40 µl of electro competent cells is mixed with 1 to 10 ng of plasmid or ligation product. The bacterial suspension is introduced into an electroporation cuvette (2 mm of distance between two electrodes), and placed in an electroporator (BioRad micropulser^{TM}). An electric shock is applied to the samples (V=2.5 kV; R=200 Ω; C= 25 µF) immediately followed by addition of 500 µl of LB medium and an incubation at 37 °C for one hour. This time of recovery allows the transformed bacteria to develop the resistance against the antibiotic provided by the recombinant plasmid. The bacterial suspension is spread on selective plates and transformed colonies appear after one night incubation at 37 °C.

2.10.2 Heat shock

1 µl of plasmid is added to 100 µl of chemically competent bacteria and suspension is stored on ice for 30 min. A thermal shock is applied to the mixture by incubating the sample for 45 sec in a water bath at 42 °C immediately followed by addition of 500 µl of ice cold LB medium. The transformed bacteria are, than, incubated at 37 °C for 1h. Finally the bacterial suspension is spread on selective media plates and incubated at 37 °C for one night to get transformed colonies.

2.11 Transformation of *A. tumefaciens*

A. tumefaciens is used as a tool in the process of production of genetically transformed plant cells. The binary plasmids are introduced into *A. tumefaciens* thanks to two different methods: heat shock or triparental mating.

2.11.1 Heat shock

2.11.1.1 Preparation of chemically-competent Agrobacteria

This technique is used for the transfection of *A. tumefaciens* LBA 4404 strain. A preculture is prepared by inoculating 2 ml of YEB medium with a single colony of agrobacteria in the presence of selective antibiotic (rif 100 µg/ml and streptomycin 100 µg/ml). This culture is incubated at 28 °C for 15 h. 2 ml of the preculture is used to inoculate 50 ml of YEB medium and is incubated at 28 °C for 4 h under gentle agitation. A centrifugation carried out at 3000 g for 20 min and 4 °C allows harvesting the bacteria. The pellet is washed twice in 5 ml cold TE (Tris10 mM, pH=8 with HCl; EDTA 1 mM). Finally the pellet is suspended in 2 ml of YEB and 500 µl aliquots were prepared.

2.11.1.2 Heat shock transformation of Agrobacteria

500 µl of competent cells are mixed with 0.5 to 1 µg of plasmid in a 2 ml microtube. The suspension is incubated on ice for 5 min, freezed for 5 min in liquid nitrogen before being transferred to a water bath at 37 °C for 5 min. Finally, 1ml of YEB medium is added and the transformed bacteria are incubated at 30 °C for 4 h under gentle agitation. 200 µl are spread on solid selective media plates. The remaining suspension is centrifuged at 3000 g for 2 min, the supernatant is removed and the pellet suspended in 200 µl of YEB medium which are spread on a solid selective media too. Transformed colonies were obtained by incubating the plates at 30 °C for almost 60 h.

2.11.1.3 Transformation of agrobacteria using the triparental mating method

The triparental mating method is a technique developed to transfer recombinant plasmids into agrobacteria using two *E. coli* strains known as helper and donor strain. The

helper strain is *E. coli* HB101 containing a self-transmissible plasmid pRK2013 and the donor strain is a recombinant MC1061transformed with any recombinant pBIN-GW or pGWB2 plasmid. With the help of these two *E. coli* strains, recombinant plasmid pBIN-GW or pGWB2 can be introduced into *A. tumefaciens* C58C1Rif® (pGV 2260).

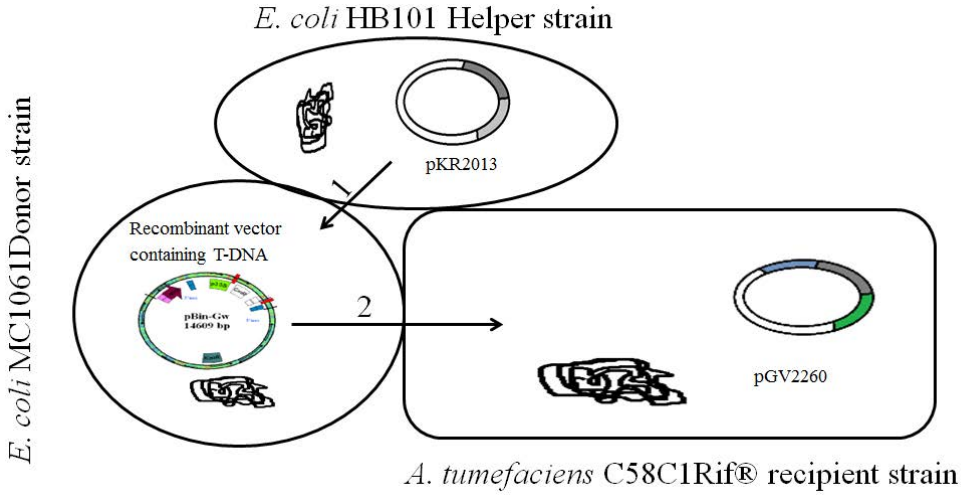


Figure 49. Schematic representation of triparental mating

Cultures of the three strains are mixed together. In a first step the *E. coli* helper strain HB101 transfers the self-transmissible plasmid pRK2013 to the MC1061 *E. coli* donor strain. This recombinant plasmid is mobilizable but not self-transmissible. As soon as the *E. coli* donor strain acquires the transfer ability due to presence of pRK2013, it transfers the recombinant plasmid to the *A. tumefaciens* C58C1Rif recipient strain (Figure 49).

2.11.1.3.1 Technical approach

E. coli MC1061 is transformed with the recombinant plasmid pBIN-GW (carrying the gene of interest) by heat shock as described before. 2 ml of LB medium containing kanamycine (25 µg/ml) are inoculated with one colony of this strain and incubated at 37 °C for 15h. In parallel, 2 ml of LB medium containing kanamycine (25 µg/ml) is inoculated with one colony of *E. coli* HB101 and incubated at 37 °C for 15h. And finally one colony of *A. tumefaciens C58C1Rif* is inoculated 2 ml of YEB medium containing carbenicillin and kanamycine and incubated at 28 °C for 15h. 100 µl of each culture is mixed, spread on solid YEB plates supplemented with Mg^{2+} and incubated at 28 °C for 15h so that triparental mating

could occur among the different strains. The transformed bacteria are selected by using appropriate antibiotics. Only transformed colonies of *A. tumefaciens* having both pGV2260 and recombinant pBIN-GW-X, are able to grow on selective media. Incubation is done during 48 h at 28°C and the resulting colonies are sub-cultured on a new media.

2.12 Extraction of plasmidic DNA

To amplify a plasmid, transformed single *E. coli* colonies are inoculated in LB medium in the presence of an appropriate selective antibiotic. After 15 h culture at 37 °C, bacteria are collected by a centrifugation of 10 000 g for 1 min. The supernatant is removed and the bacterial pellet used to extract the plasmid using the Genelute™ plasmid minikit provided by Sigma-Aldrich (Annex). The plasmids are eluted from the column with 50 µl of elution buffer and are stored at -20 °C.

2.13 Sequencing

The sequence of each plasmid is verified. Sequencing is done by Eurofins MWG Operon.

2.14 Induction of the expression of genes by UV

2-3 months old parsley plants are subjected to UV radiation for 24h using a monochromatic lamp (312 nm). The plants are 25 cm distant from the UV source. After 24 h, the leaves are harvested and used for subsequent analysis (coumarins, DNA or RNA extraction).

3 Heterologous expression system

These systems are used as tools to find out whether transcription and translation of a particular gene produces mRNA and/ or protein and whether the protein produced is functional or defective. In the frame of this work, several expression systems have been used based on prokaryotes (bacteria) or eukaryotes (yeast, plants).

3.1 Prokaryote expression system

The system used in this work is a two-step inducible system. In this system, the first step consists in production of bacterial biomass without any expression of the protein of interest. The second culturing step is dedicated to production of recombinant proteins. The partition of this system into two steps makes the system specialized to protect the recombinant organism from the toxic effects raises due to the accumulation of heterologous recombinant protein.

3.1.1 Expression

A colony of recombinant *E. coli* (M15 [pRP4] or BL21-DE3) is used to inoculate 5 ml of LB medium in the presence of appropriate antibiotics and incubated at 37 °C for 15 h. 1 ml of this preculture is used to inoculate 50 ml of LB medium in the presence of appropriate antibiotics and incubated at 37 °C until they reach an OD_{600} comprised between 0.4 and 0.6. 1 mM sterile IPTG is added to the culture and incubation is continued during 22 h at room temperature for M15 [pRP4]-pQE30/4CL1 and for 20 h at 18 °C for BL21-DE3-pGEX/HCT. The bacteria are harvested by a 5000 g centrifugation for 15 min at 4 °C. The bacterial pellet is washed 3 times in 5 ml of cold PBS (8.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, pH 7, 4) and finally ressuspended in 1 ml PBS. The cells are hydrolyzed by 3 sonications (Bandelin Sonoplus HD 2070 MS73 probe with an intensity of 200W/cm2), and samples were placed for 30 sec on ice between two successive sonication. After a final centrifugation at 16,000 g for 10 min at 4 °C, the supernatant and pellets are separated and stored at -20 °C.

3.1.2 Purification of protein

In some cases, a His-Tag has been added at the N or C-terminus of recombinant proteins. This tag allows the purification of the corresponding protein by using a Ni-NTA purification system kit provided by Qiagen. The supernatant is loaded on column and, after 3 washes; the protein is released by 300µl of solution containing 500 mM imidazole, 300 mM NaCl and 50 mM NaH_2PO_4, pH 8.0. The protein is then directly used either to perform an enzymatic test activity or is stored at -80 ° C.

3.2 Eukaryotes expression system

Two eukaryote expression systems were used during this work: a yeast expression system and plant expression systems.

3.2.1 Yeast Expression system

3.2.1.1 Preparation of competent yeast

A colony of WAT11 yeast strain is inoculated to 10 ml YPGA and incubated at 30°C for 15h. The preculture is diluted in YPGA to obtain an OD_{600} near 0.2. This fresh culture is then incubated until OD_{600} reaches 0.9. Yeast is harvested by a centrifugation at 4000 g for 10 min. The resulting pellet is first washed in 1 ml sterile ultrapure water and second in 1 ml of 100 mM lithium acetate. Finally after a final centrifugation the pellet is suspended in 200 µl of lithium acetate 100 mM.

3.2.1.2 Transformation of yeast

50 µl of competent yeast cells are mixed to 100 ng of plasmid, 10 µl of denatured salmon sperm DNA (DNA (10 mg/ml) is denatured by heating at 100 °C for 20 min) and 500 µl of a PEG solution (100 mM lithium acetate/ TE 1X diluted in PEG 4000 (50%)). This suspension is sequentially incubated for 30 min at 30 °C, 15 min at 42 °C and stored on ice for one minute. Yeasts are harvested by centrifugation at 3000 g for 2 min and the resulting pellet re-suspended in 500 µl YPGA before of an incubation of 2 h at 30 °C. Finally the transformed yeast cells are spread on a solid selective SGI media and incubated at 28 °C for 3 nights until colonies appear.

3.2.1.3 Protein expression in yeast

3.2.1.3.1 Conditions of culture

The heterologous expression system of yeast proposed by Pompon and Diesperger (Diesperger *et al.,* 1974; Pompon *et al.,* 1996) is widely used for the expression and functional analysis of Cytochrome P450.

3.2.1.3.2 Preparation of yeast culture

The process for the expression of recombinant proteins is splitted in 3 steps: 1) yeast culture in selective medium, 2) production of biomass, 3) induction of the synthesis of the recombinant proteins.

A preculture is prepared by inoculating of 10 ml of SGI with one colony of yeast and incubated at 28 °C for 24 h. This preculture is used to inoculate 200 ml of YPGE medium which is incubated at 28 °C for 24 h with orbital shaking. The culture is centrifuged for 5 min at 3000 g and the pellet is re-suspended in 200 ml YPL (induction phase). This culture is incubated at 18 °C for 16 h. Many different modification in this system were adopted which will be discussed later.

3.2.1.3.3 Microsomes preparation

P450 proteins are very sensitive to temperature i.e., they degrade very quickly at ambient temperature. Thus it is necessary to do all the experimentations for P450s at 4 °C so that the recombinant proteins can be recovered without affecting their functionality.

The YPL culture is centrifuged at 5000 g for 7 min at 4 °C. The pellet is washed with 20 ml of TEK buffer (Table 13). Samples are centrifuged again at 5000 g for 7 min and the pellet is re-suspended in 2 ml cold extraction buffer. The cells are broken by mechanical agitation thanks to glass beads (Glass BEADS 400-625 µm, Sigma) (5 shaking periods of 1 min separated by 1 min on ice). Finally the glass beads are washed 4 times with 5 ml of cold extraction buffer. The collected suspension is then centrifuged for 15 min at 10 000 g in order to remove all the debris. Pelleting of microsomes is done by ultracentrifugation (Rotor Surespin 360 - Thermo Scientific T-1250) for one hour at 100 000 g at 4 °C (Thermo Scientific Sorvall WXultra 80°). Finally the microsomal pellet is re-suspended in 2 ml of TEG buffer using a potter and aliquoted before storage at -80 °C.

Table 13. Composition of buffers used for preparation of microsomes of yeast

TEK	Tris HCL pH 7.5	50 mM
	EDTA	1 mM
	KCL	100 mM
TEG	Tris HCL pH 7.5	50 mM
	EDTA	1 mM
	Glycérol	30 % (v/v)
Extraction buffer	Tris HCL pH 7.5	50 mM
	EDTA	1 mM
	Sorbitol	0.6 M
	BSA*	1 % (p/v)
	β-mercapto éthanol*	20 mM
	*these products were added at the time of utilization.	

3.3 Heterologous Expression in plants

3.3.1 Transient expression in *N. benthamiana*

This transient expression system is fast, flexible and is not affected by chromosomal position of the T-DNA. This system works on the principle of expressing the gene of interest in the nucleus of plant cells, without its insertion into the genome of the plant cell. As the recombinant gene is not inserted into a genetic background the expression of proteins is not long lasted.

3.3.2 Inoculum Preparation

A colony of transformed agrobacteria containing pBIN-GW-X (pGWB2) and a colony of agrobacteria containing plasmid pBIN-GW-P19 (provided by Prof. David Baulcombe, University of Cambridge) were used to inoculate 10 ml of YEB medium in the presence of appropriate antibiotics separately in 2 tubes. Cultures were incubated at 30 °C for 24 h. The bacteria are centrifuged at 4000 g for 10 min. Three washings are performed: the first one with YEB medium and then two successive washes with sterile ultrapure water to remove the excess of antibiotics that could be lethal for plant cells after infiltration. The bacterial pellet is suspended in 2 ml sterile water. To optimize the expression, an infiltration mixture was

prepared in a manner to obtain final OD_{600} of 0.2 for agrobacteria containing the recombinant gene and an OD_{600} of 0.4 for agrobacteria containing *p19* gene. This mixture was then used to infiltrate the leaves of tobacco using a syringe (Figure 50).

3.3.3 Agro-infiltration of leaves

Three to four healthy leaves (second to fourth leaf from the apical meristem) of a 1 to 2 month old tobacco plants are selected for agro-infiltration. Regions to be inoculated are selected on the underside of the leaves and are punctured with a small needle. This preliminary treatment allows infiltrating the leaves with less pressure and reduces chances of damaging the cells. A 1 ml syringe (without needle) carrying the agrobacteria suspension is placed on the underside of leaf on the mark of needle. The suspension is gently injected in the leaf (Figure 50). After infiltration, the plants are placed for 3-4 days in a growth chamber in specific boxes in order to prevent any contamination of other plants with agrobacteria. Leaves inoculated with agrobacteria containing pBIN-GW-PT can be used for a secondary inoculation with potential substrates (see Results).

Figure 50. Infiltration of lower epidermis of leaves of *N. benthamiana*

3.4 Stable transformation of *R. graveolens*

To generate genetically transformed plants of *R. graveolens*, a protocol has been developed by Karine Lièvre during her PhD (Lièvre *et al.,* 2005)

3.4.1 Germination of *R. graveolens* seeds

R. graveolens seeds have very tough seed coat, thus they are scarified before they are sown in culture media. This treatment is done by dipping the seeds in 95% sulfuric acid. After

this preliminary step, the seeds are sterilized by dipping them, in a solution of calcium hypochlorite 7% (m/v) for 5 min and washed three times with ultrapure sterilized water before putting them on a solid MS media in the sterile conditions (Figure 51).

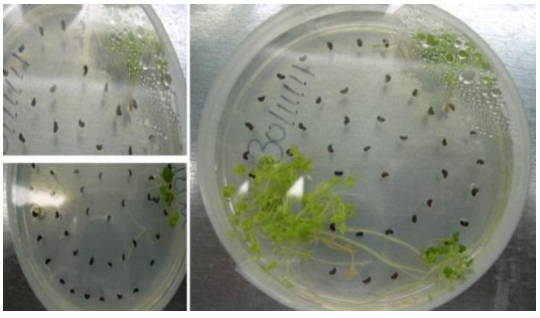


Figure 51. Germination of seed of *R. graveolens*

3.4.2 Preparation of bacterial inoculum

LBA4404 and C58C1Rif^R Agrobacteria were cultured separately on solid media plates supplemented with appropriate plates for 3 days at 28 °C. A colony of each type of bacteria is used to inoculate 10 ml of YEB in two separate tubes and cultured at 28 °C for16 h under a shaking of 200 g. When the OD_{600} approximately reached 0.5, acetosyringone with the final concentration of 125 µM is added to the culture. This molecule can activate the virulence gene present on the T-DNA of the binary vector. The culture is than incubated at 28°C until OD_{600} is 0.8 to 1.1. Agrobacteria are then harvested by centrifugation at 5000 g for 3 min. The resulting pellet is suspended in 10 ml of YEB liquid medium, re-centrifuged and finally suspended again in 10 ml M*and*S liquid medium. These two washings allow the removal of antibiotics that could interrupt the process of genetic transformation. Finally bacterial suspension is prepared with 35 ml of liquid M*and*S (30) BAP (0.1 mg/ml) medium.

3.4.3 Transformation of hypocotyls of *R. graveolens*

Hypocotyls of Ruta plantlets obtained 2 to 3 weeks after sowing of seeds on M*and*S medium were used for genetic transformation (Figure 52). The hypocotyls are co-cultured with *A. tumefaciens* in order to transfer T-DNA to plant cells and then transferred on selective medium in order to select genetically transformed explants.

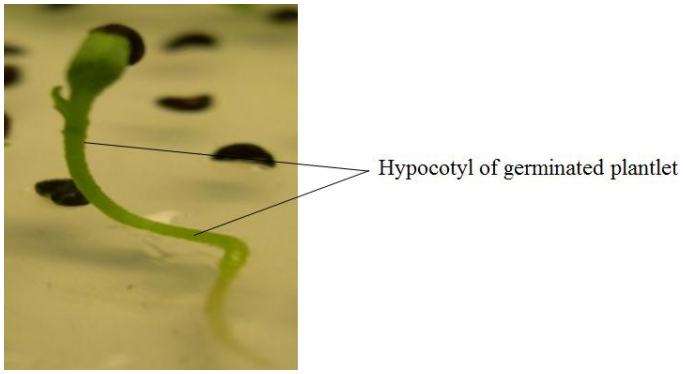


Figure 52. Plantlet of *R.graveolens* of the age of 2-3 weeks

Explants are seperated from the remaining plantlet under sterile conditions and dipped in the solution of Agrobacteria inoculum for 30 min. Excess of solution is removed from explants by squeezing on filter paper. Finally they are transfered to plates of solid media of co-culture [M*and*S(30) BAP(0.1 mg/l) acetosyringone (50 mg/L)].

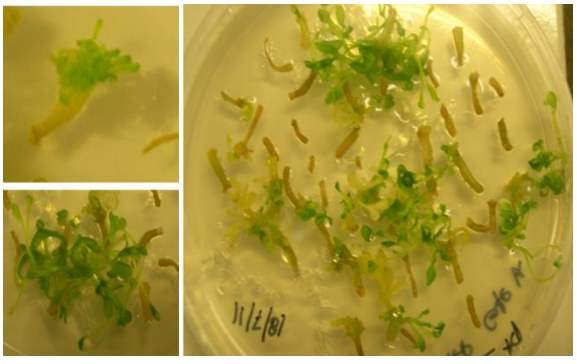


Figure 53. Regeneration of leaf buds from explants

Explants are co-cultured with agrobacteria for 3 days and then agrobacteria were eliminated. To this purpose, explants were dipped for 15 min in ultrapure sterile water and then for 15 min in a liquid medium containing M*and*S(30), BAP(0.1 mg/l) and cefotaxime (500 mg/L). Excess of solution is removed with the help of filter paper and then explants are transfered on a medium known as regeneration medium [M*and*S(30), BAP(0.1 mg/l) cefotaxime (300 mg/l)]. After 8 days, explants are transferred on a selection media [M*and*S(30), BAP(0.1 mg/l), kan (75 mg/L) and cefotaxime (300 mg/L)]. First leaf buds appear within one month (Figure 53).

The regeretaed plants are transferred into pots of 200 ml containing medium M*and*S (30), BAP (0.1) and Kan (75 mg/L), which maintain a selection pressure (Figure 54).

Figure 54. Development of genetically transformed plants on selective medium

4 Methods of biochemical analysis

4.1 Quantification of proteins

Concentration of purified protein overexpressed in *E. coli* is measured by fluorometry using the Qubit fluorimeter TM (Invitrogen). This technic is based on the capability of fluorofores to fluoresce when they bind to proteins. This technic is 1000 times more sensitive than the Bradford quantification technic. Diluted sample (1 µl in 20 µl) is added to 200 µl of reaction solutions. Concentrations are measured after an incubation of 15 min at room temperature.

4.2 Quantification of P450 by CO spectrum

This technique is used to detect the presence of functional protein of P450 in solution (Sato, 1964a)(Sato, 1964b). Microsomes are diluted 5 times in TEG buffer, supplemented with Sodium dithionite and divided into two spectrophotometer cuvette. The microsomal suspension of cuvette is saturated with CO by bubbling for 30 sec. The measurement of absorbance is done between 400 and 500 nm using a spectrophotometer. The CO reacts with iron of the heme of P450, causing a change in the spinning state of iron and the appearance of a specific absorption peak at 450 nm, which shows to the presence of active P450 in the microsomal preparation.

4.3 Measurement of enzymatic parameters

4.3.1 For Cytochrome P450

4.3.1.1 Determination of kinetic constants

To determine the catalytic parameters of P450, enzymatic assays were made as described by (Larbat *et al.,* 2007). Microsomes prepared for CYP98A22 were incubated in a total volume of 200 µl of NaPi buffer (0.1 M pH=7), containing 0.2 mM NADPH and 100 µM of substrate (*p*-coumaroyl shikimate, *p*-coumaroyl quinate, *p*-coumaroyl tyramine or tricoumaroyl spermidine). Reactions are incubated at 28 °C for 30 min and stopped by adding 75 µl HCl (0.1 M). The mix is centrifuged for 30 min at 10 000 g to separate supernatant from pellet. 50 µl of the supernatant is used to be analyzed by reverse phase HPLC. All experiments were repeated three times and quantities of total extracted proteins and time of incubation were adjusted so that quantity of substrates used was not more than 50% of the reaction volume. The kinetic parameters were calculated using the SIGMAPLOT software program (Systat Software Inc., http://www.sigmaplot.com/).

4.3.2 For prenyltransferases

4.3.2.1 Determination of enzyme kinetics

Enzymatic essays for measuring catalytic parameters of Pt is carried out in a total volume of 200 µl containing 50 mM Tris /HCl pH 8, 1 mM $MgCl_2$, 25 µl of total microsomal proteins extracted from inoculated tobacco leaves and substrates with increasing concentration, from 0.2 to 1 mM for DMAPP and from 2 µm to 750 µM for Umb. Reactions are incubated at 25°C for 2 h and stopped by adding 1 µl of trifluroacetic acid. An additional vortex (MS1 minishaker IKA) of 1 min for each sample is done to make sure the loss of activity of the enzyme. Reaction mixtures are centrifuged at 16000 g for 10 min to separate the supernatant that is analyzed by HPLC. All experiments were carried out in triplicate and quantities of total extracted proteins and time of incubation were adjusted so that quantity of substrates used was not more than 50% of the reaction volume. The kinetic parameters were calculated using the SIGMAPLOT software program (Systat Software Inc., http://www.sigmaplot.com/).

4.3.2.2 Measurement of the inhibition of the Pt activity by furanocoumarins and determination of the inhibition constant

To carry out the tests of inhibition of Pts by furanocoumarins, enzymatic assays are done in the same conditions and with the same components as described above except that to the reaction we added different fuanocoumarins. Different concentrations of psoralen are tested (sigma Aldrich) ranging from 10 to 60 µM in the presence of different concentrations of Umb (10 to 80 µM). Each series of incubations is repeated 3 times and the corresponding data are fitted into graphical representation of Michaelis-Menten model and, *K*i was determined using a graphical representation of Lineweaver-Burk.

4.4 HPLC Analysis

4.4.1 Metabolisation of substrate

Samples were prepared for analysis on HPLC as explained earlier in this chapter. Molecules present in each sample are separated using reverse phase HPLC (LiChrospher 250-4 5µm BP18e). This separation is obtained by using two solvents: solvent A (H_2O, formic acid 0.1%) and solvent B (methanol, formic acid 0.1%). This method uses linear gradient thus the concentration of solvent B increases from 10 to 100% in 34 min with a flow rate of 0.7 ml/min. The presence of hydroxylated product is verified at 320 nm while an absorbance at 333 nm is checked for the prenylated products.

4.4.2 Analysis of extracts of phenylpropanoids

Samples are separated using a reverse phase HPLC (LiChospher 250-4 5µm BP18e). This separation is obtained by using two solvents: solvent A (H_2O, formic acid 0.1%) and solvent B (methanol, formic acid 0.1%). This method uses a program of 90 min with a linear gradient where concentration of solvent B increases from10- 60% in 45 min and reached up to 100% till 76 min with a flow rate 0.7 ml/minute. Identification of different molecules is done at 300 nm.

4.5 Analysis by mass spectrometry (MS)

MS is a technique of analysis that allows identification and detection very precisely the molecules of interest by measuring their masses. Basic principle of this technique is the separation of charged molecules in gaseous phases, on the basis of ratios between their masses and charges (m/z).

4.5.1 Preparation of samples for analysis for quantification

Samples of phenylpropanoids contents of different plants were prepared finally with 200 µl of 70% methanol as it has been explained earlier. After that some modification were carried to complete the analysis on HPLC/LCMS. For this purpose a molecule as control was added to sample (always at the same final concentration) that is of the same type as the molecules to be analyzed, but it is not natural metabolite of plant and not present in sample naturally. Here coumarine serves as control molecule. In addition two series of dilutions (10 and 30 folds dilution) for all sample were prepared to be sure that molecules present in very high or very low concentrations can be quantified precisely. Once the samples are prepared they were analyzed by HPLC/LCMS along with the series of different concentrations of standard molecules.

4.5.1.1 Detection and quantification of hydroxylated products

The HPLC-MS system consisted of a binary solvent delivery pump and a linear ion trap mass spectrometer (LTQ-MS, Thermo Scientific, San Jose, CA, USA). The LTQ was equipped with an atmospheric pressure ionization interface operating in the ESI negative ion mode. The data were processed using Xcalibur software (version 2.1). The operational parameters of the mass spectrometer are given below. The spray voltage was 4.5kV and the temperature of the heated capillary was set at 300°C. The flow rates of sheath, auxiliary and sweep gases were set (in arbitrary units min-1) to 40, 10, and 10, respectively. The capillary voltage was set at -36V, the split lens was set at 44 V and the front lens was set at 3.25 V. All the parameters were optimized by infusing a standard solution of isopimpinelline (0.1 g. L^{-1}) in mobile phase [water + acetic acid 0.1% / methanol + acetic acid 0.1% (90/10)] at a flow rate of 5 µl min^{-1}]. The caffeoylshikimic acid isomers were monitored through MS_2 (335)-specific

and full (100-400 m/z) scans. Standard solution of caffeoyl quinate and caffeoyl shikimate were used for quantification.

4.5.1.2 **Detection and quantification of prenylated products**

Quantitation of a pool of coumarins and furanocoumarines compounds was performed using a HPLC-MS system (ThermoFisher Scientific, San Jose, CA, USA) equipped with an LTQ ion trap as mass analyzer (Linear Trap Quadripole). Data were processed using Xcalibur software (version 2.1). Chromatographic separation was performed on a C18 Alltima reverse phase column (150*2.1mm, 5µm porosity – Grace/Alltech, Darmstadt, Germany) equipped with a C18 Alltima pre-column (7.5*2.1mm, 5µm porosity – Grace/Alltech, Darmstadt, Germany) at 25°C and mobile phases consisted in water modified with formic acid (0.1%) for A and methanol modified with formic acid (0.1%) for B. Compounds were eluted using a mobile phase composition gradient (A:B; v/v) : 90:10 at 0 min, 80:20 at 5 min, 53:47 between 15 and 30 min, 0:100 from 35 to 40 min, and finally 90:10 from 40.10 to 46 min, the analyze was performed at a flowrate of 0.2 $mL.min^{-1}$. A double detection photodiode array and mass was performed during all the time of the run. The mass spectrometric conditions were as follows: positive ionization mode ElectroSpray Ionization (ESI) was used source gases were set (in arbitrary units min^{-1}) for sheath gas, auxilliary gas and sweep gas at 40, 10 and 10, respectively; capillary temperature was set at 300°C; capillary voltage was set at 36V; tube lens, split lens and front lens voltages were set at 80 V, -44V and -3.25V, respectively. The ion optics parameters were optimized by automatic tuning using a standard solution of isopimpinellin at 0.1 gL^{-1} infused in mobile phase (A/B: 50/50) at a flow rate of $5\mu Lmin^{-1}$. Highly sensible and specific detection of compounds was realized through monitoring of daughter ions obtained from MS^2 fragmentation of pseudo-molecular $[M+H]^+$ ions, as follows: MS^2 (163) for umbelliferone, MS^2 (187) for psoralen and angelicin, MS^2 (217) for bergapten (5-MOP), 8MOP, sphondin and isobergapten, MS^2 (231) for osthenol and DMS, MS^2 (247) for marmesin, colombianetin, isopimpinellin and pimpinellin. Coumarin (M=146) was used as internal mass spectrometric standard (5 µM) and monitored thanks to MS^2 (147) operation. It allowed us to plot five points internal calibration curves for target compounds from 0.5 to 20 µM with a very good linear correlation in all cases ($r>0.99$). Using chromatographic conditions offer a very good selectivity especially for the four considered pairs of isomers, as

regards the retention times of the different species: umbelliferon (18.08 min), psoralen (27.79 min), angelicin (30.04 min), marmesin (25.12 min), colombianetin (27.27 min), isopimpinellin (35.47 min), pimpinellin (37.23 min), 8-MOP (28.62 min), 5-MOP (36.50 min) sphondin (30.72 min), isobergapten (30.02 min), osthenol (37.85 min) DMS (38.86 min) and internal standard coumarin (20.33min).

4.6 Synthesis of substrats

Potential substrates for CYP98A22 are not available commercially. Thus they were prepared enzymatically in our laboratory. These substrates were prepared in two steps; in a first step different acidic molecules are esterified with a co-enzyme A (CoA), and in a second step ester of these molecules are synthesized containing shikimic or quinic acid.

4.6.1 Synthesis of CoA esters

4CL is an enzyme that catalyzes the addition of a group of coenzyme A, at the different derivative molecules of cinnamic acid. 4CL enzymes is produced and purified according to protocol already explained above (see paragraph under heading 3.1).

4.6.1.1 Production of CoA esters

Stock solutions of commercially available substrates are prepared in 100% of ethanol (10 mM). These substrates are coumaric acid and caffeic acid. Required amounts of these substrates are transferred to the reaction tubes and dried (ethanol is evaporated through a stream of nitrogen). The synthesis of different CoA esters is carried out in a final volume of 1 ml containing 0.2 mM of respective acid, 0.2 mM Coenzyme A (Sigma), 2.5 mM ATP(Sigma), 1 mM DTT, 2.5 mM $MgCl_2$, purified 4CL enzyme (20 mg protein/ml) and NaPi buffer (0.1 M and pH=7). Reaction mixture is incubated at room temperature in dark with agitation for 30 min

4.6.1.1.1 Kinetics of chemical reactions

The efficiency of the enzymatic synthesis of CoA esters is verified by spectrophotometry by measuring the absorption at 333 nm for *p*-coumaroyl CoA, and at 346

nm for feruloyl CoA and Caffeoyl CoA. This absorption is measured during 20 min (Figure 55).

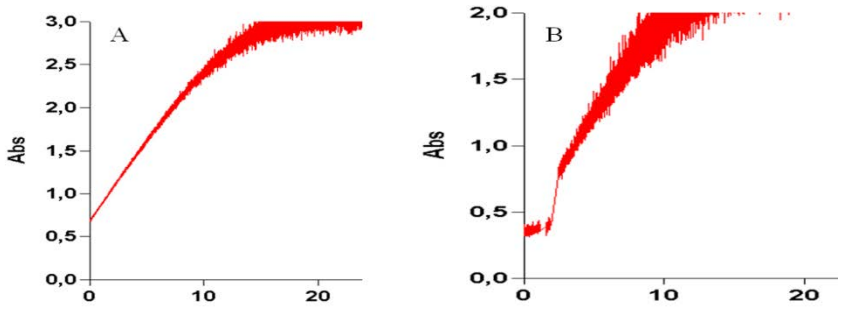


Figure 55. Measurement of absorption during the synthesis of Caffeoyl CoA (A) and Coumaroyl CoA (B) in the presence of 4CL

4.6.1.2 Synthesis of shikimic and quinic acid esters

Hydroxycinnamoyl-Coenzyme A shikimate (HCT) and Hydroxycinnamoyl-Coenzyme A quinate (HQT) are two enzymes that can catalyze the conversion of CoA esters into shikimic acid and quinic acid esters. Syntheses of HCT and HQT enzymes have been above (see paragraph under heading 3.1).

4.6.1.2.1 Synthesis of shikimic acid and quinic acid derivatives

For this second reaction, products of first step described above are used as substrates. Thus the reaction mixture is prepared by adding 0.2 mM of CoA ester, 100 mM of either shikimic acid or quinic acid and 0.02 mg protein/ml of HCT when substrate is shikimic acid, or of HQT when substrate is quinic acid. The reactions were performed in dark for 1 h at 30 °C.

The reactions are stopped by addition of 4 N HCL in a proportion of V/15 of total volume. Finally the products are extracted 2 times in the presence of 2 volumes of ethyl acetate. A centrifugation at 1000 g for 10 min is done between each extraction to separate the two phases of the solution. Finally the solvent is dried by using a speed vacuum concentrator (Jouan) and analyzed by HPLC (Figure 56).

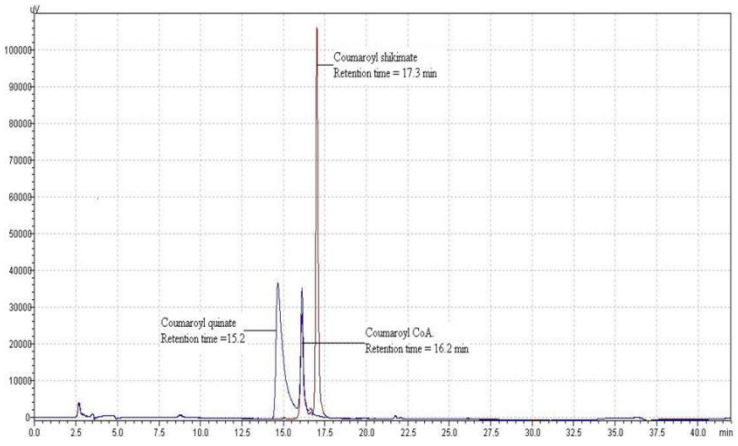


Figure 56. HPLC chromatogram for three different mixtures of reaction

(peak 1) *p*- Coumaroyl CoA without enzyme, (peak 2) with HCT and peak depicts presence of *p*-coumaroyl shikimate and (peak 3) with HQT and peak shows the metabolisation of substrate into *p*-coumaroyl quinate.

5 Methods of analytical analysis

5.1 Preparation of phenylpropanoids extracts

The same extraction procedure of phenylpropanoids is used for different plants i.e., *R. graveolens*, *P. crispum* and *N. benthamiana*.

100 mg of fresh tissue were harvested and freezed in liquid nitrogen. This material is crushed into powder in liquid nitrogen with the help of pestle and mortar. 2 ml of 95% ethanol is added along with 20 µl of an internal standard taxifoline (2 mg/ml). This internal standard helps to remove the error that could occur at two different steps, firstly during the preparation of sample and secondly during the injection of samples on HPLC. Samples are then homogenized using an ultraturax (Poly-Tron, PT2100) for one minute and furthermore are mixed by shaking them at a speed of 1400 rpm at 4 °C using a thermomixer (Eppendorf). A final centrifugation was done at 4 °C for 15 min at 10 000 g to separate supernatant and pellet. All the supernatant was collected and solvent was evaporated using a speed vaccum concentrator to concentrate the samples (Jouan). The resulting pellets were suspendend in 200 µl of 70% methanol before the analysis on HPLC.

5.2 Quantification of expression of gene by real time PCR

Expression levels of different genes were assessed by real time PCR. In order to get reproducible results, the data was compared to reference housekeeping genes (18S or 28S). Relative quantification is measured by respective efficiencies of target and reference gene, and Ct values obtained for sample and calibrator are measured by the method of ΔΔCt.

The results are expressed as a ratio of target gene / reference for each sample, normalized by the ratio of target / reference of a sample called calibrator (18S). The results are calculated using the software known as "StepOne software V2.2.2."

5.2.1 Preparation of material

Total RNA is extracted from the plant samples, according to the protocol provided with kit « Plant RNeasy mini kit » provided by Qiagen (Annex). High-capacity RNA-to-cDNA master mix (Applied Biosystems, http://www.appliedbiosystems.com/) (Annex) is used for the first-strand cDNA synthesis with a Poly $(T)_{18}$ as the primer. The resulting cDNAs samples are diluted 10-fold before being used for real-time PCR.

5.2.2 Preparation of the reaction mix

In order to do relative quantification, specific primers of the reference gene (18S) of *R. graveolens* (forward primer 5'-CATTCGGCCCGTCTTGAA-3' and reverse primer 5'-CCGTTGACTCGCACACATGT-3') and of the reference gene (28S) of *P. crispum* (forward primer, 5'-GCTACCGTGCGCTGGATT-3' reverse primer, 5'-GCTTCTAGCCCGGATTCTGA-3') were ordered. Specific primers of target genes (Table 14) of Pt of parsley and lemon were designed using the Primer Express 3.0 Software program (Applied Biosystems, http://www.appliedbiosystems.com). To measure the efficiency of each pair of primer were necessary for the validation of the primers, so that 5 series of 1:5 fold diluted samples was realized with *R. graveolens* and *P. crispum* gDNA and the efficiency of the primers was checked to be around 95-105%. Results showed that primers of target and reference gene are efficient for successive experiments. Experimentally this result was represented by an absolute value obtained from the slope for each sample ΔCt = f (successive dilutions of sample) is less than 0.1.

Reaction mixture contains 12.5 µl of Power Syber Green PCR Master Mix (Applied Biosytems) 2X, 0.5 µl of each couple of primers at a final concentration of 0.1 µM, 5 µl of 20 times diluted cDNA and 6.5 µl of ultrapure sterile water. Thermocycler machine utilized was Real-Time PCR StepOneplus of Applied Biosystems.

Table 14. Primers that were used for quantitative real time PCR

Names of genes	Direct	Reverse
PT of *P. crispum*	TCA-GAC-GGC-GAG-GCT-ATT-G	ATT-CTG-CCA-TGT-GAC-TTC-GAA-A
28S of *P. crispum*	GCT-ACC-GTG-CGC-TGG-ATT	GCT-TCT-AGC-CCG-GAT-TCT-GA
PT of *Citrus limonum*	CGTCCCTTCGCTATGATTGG	TGCAGCGGCAGAATAGCA
18 S of *R. graveolens*	CATTCGGCCCGTCTTGAA	CCGTTGACTCGCACACATGT

The PCR conditions are as following:

95 °C for 10 min necessary to activate the polymerase, then 40 cycles starting first step of denaturation at 95 °C for 30 sec followed by annealing/amplification at 60 °C for 15 sec, and a final step at 72 °C for 15 sec. At each cycle the hybridization temperature of 60 °C initially increases by 0.3 °C

6 Statistical analysis of data

Data was analyzed statistically by T-teast using Microsoft Excel.

Results and discussion

Results and discussion

Chapter I: New putative aromatic prenyltransferases

1 Identification of candidate genes encoding for enzymes belonging to the aromatic prenyltransferases family

In order to identify new candidate genes, we adopted two different strategies. The first strategy was consisted of using known sequences of characterized Pts in order to screen *in silico* libraries and to find out orthologous ORF. The second approach was based on the presence of conserved domains among different Pts subfamilies. Degenerated primers were designed against these particular sequences and used to try to amplify new undisclosed genes by using the PCR approach.

1.1 Identification and isolation of candidate genes

1.1.1 *In silico* data mining

Flavonoids, coumarins and furanocoumarins constitute subclasses of phenylpropanoids and prenylated forms of some of these molecules have been documented in literature (Figure 57 A). Moreover recently, *Sf*N8DT-1 has been described as a Pt involved in the biosynthesis of prenylated flavonoids (Figure 57 B). The reaction mechanism of this enzyme seemed to be close to the prenylation reaction mechanism of umbelliferone (Figure 57 C) which is the first step of the furanocoumarin biosynthesis pathway. This evidence led us assume that the prenylation of umbelliferone could be catalyzed by one or two enzymes sharing high sequence homologies with *Sf*N8DT-1. Therefore, the first strategy to identify putative Pts involved in this pathway consisted to use the peptidic sequence of the *S. flavescens* enzyme (*Sf*N8DT-1) (NCBI ID AB325579) to screen public or private *in silico* databases.

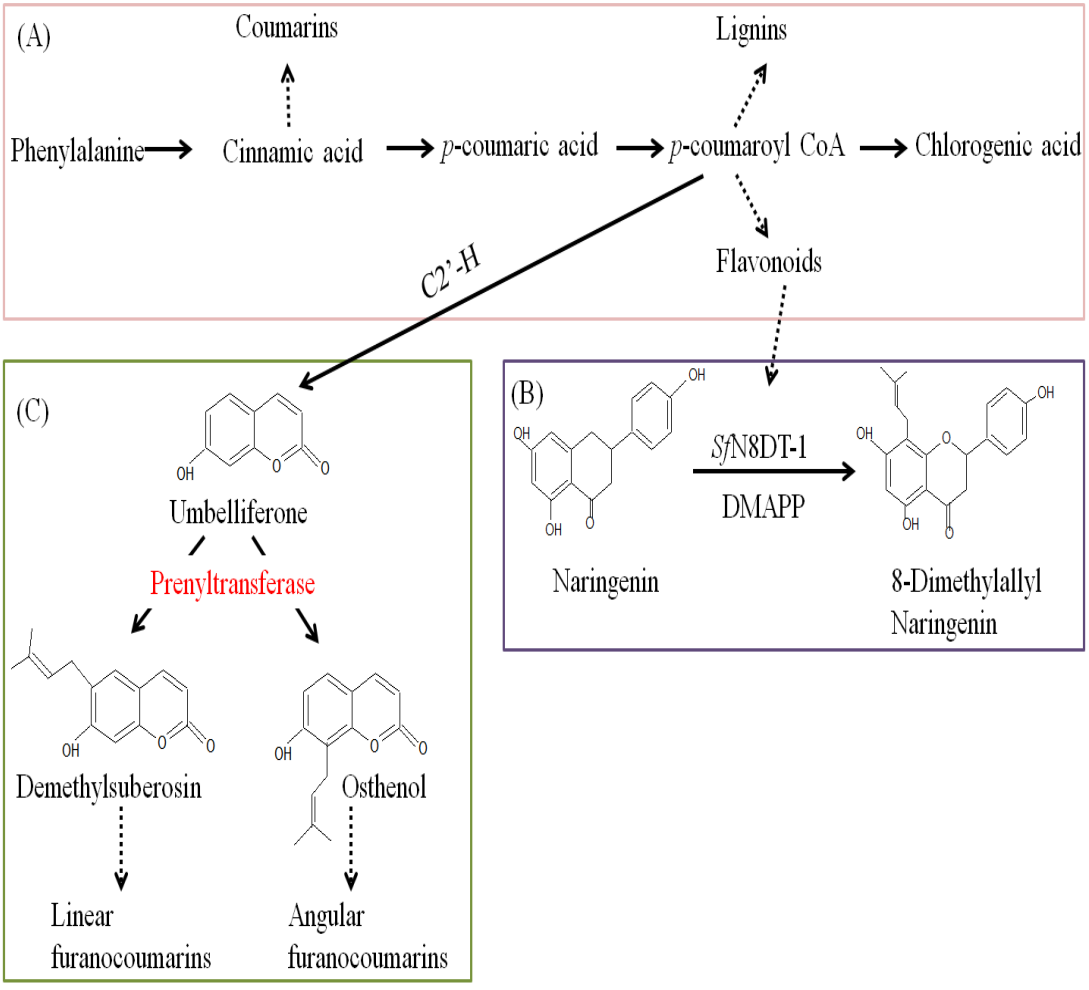


Figure 57. Simplified phenylpropanoid pathway
(A). Comparison of the prenylation of naringenin (B) and umbelliferone (C).

1.1.1.1 *A. gigas* homogentisic acid phytyltransferases

```
Query  4     MGSMLLASFPGASSITTGGSCLRSKQYAKNYDASSYVTTSWYKKRKIQKEHCAAIFSKHN  183

             M S+L++SFP A S                 D SS  +T   +  + Q  +    +S  N
Sbjct  1     MESLLISSFPKACSFPVSA------------DFSSSHSTD-LRVLRCQAWNTIESYSATN  47

Query  184   LKQHYKVNE-GGSTSNTS--KECEKKYVVNAISEQSFEYEPQTRDPESIWDSVNDALDIF  354

              +QH  +   GG T+N S  +  + K++VNA SEQ  E EP +  P+S+  S+ +AL+ F
Sbjct  48    CRQHLVMQHAGGFTNNLSLYRRGDTKFLVNAASEQPLESEPLSYQPKSLQGSLQNALNAF  107

Query  355   YKFCRPYAMFTIVLGATFKSLVAVEKLSDLSLAFFIGWLQVVVAVICIHIFGVGLNQLCD  534

             YKF RP+ +    L     SL+AVEKLSD S  FF G L+ +VA + ++I+ VGLNQL D
Sbjct  108   YKFSRPHTVIGTALSIISVSLLAVEKLSDFSPIFFTGVLEAIVAALFMNIYIVGLNQLSD  167

Query  535   IEIDKINKPDLPLASGKLSFRNVVIITASSLILGLGFAWIVDSWPLFWTVFISCMVASAY  714

             IEIDK+NKP LPLASG+ S    VI+ AS  I+     W V S PL   +FIS ++ +AY
Sbjct  168   IEIDKVNKPYLPLASGEYSVATGVILVASFAIMSFCLGWSVGSQPLLLALFISFILGTAY  227

Query  715   NVDLPLLRWKKYPVLTAINFIADVAVTRSLGFFLHMQTCVFKRPTTFPRPLIFCTAIVSI  894

             ++++P LRWK+Y V+ A+  +A  AV   + F+LH+QT V+ RP  FP+P+IF TA +S
Sbjct  228   SINIPFLRWKRYAVVAAMCILAVRAVIVQIAFYLHVQTHVYGRPAIFPKPVIFATAFMSF  287

Query  895   YAIVIALFKDIPDMEGDEKFGIQSLSLRLGPKRVFWICVSLLEMTYGVTILVGATSPILW  1074

             +++VIALFKDIPD+ GD+ +GI+S ++RLG KRVFWIC++LL+M Y   I+VGA+S   W
Sbjct  288   FSVVIALFKDIPDIVGDQIYGIRSFTVRLGQKRVFWICIALLQMAYATAIIVGASSSTPW  347

Query  1075  SKIITVLGHAVLASVLWYHAKSVDLTSNVVLHSFYMFIWKLHTAEYFLIPLFR  1233
             SK+ITVLGH +L+S+LW  AKSVDL S V + +FYMFIWKL  AEY LIPL R
Sbjct  348   SKLITVLGHMMLSSILWIRAKSVDLDSKVAITTFYMFIWKLFYAEYLLIPLVR  400
```

Figure 58. Identification of a putative Pt protein available in the NCBI genomic database
Subject: *A. gigas*, Query: *Sf*N8DT-1.

```
LOCUS       EU407262               1370 bp    mRNA    linear   PLN 01-OCT-2008
DEFINITION Angelica gigas homogentisic acid phytyltransferase mRNA, complete cds.
ACCESSION   EU407262
VERSION     EU407262.1  GI:190611657
KEYWORDS.
SOURCE      Angelica gigas
  ORGANISM Angelica gigas
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
            asterids; campanulids; Apiales; Apiaceae; Apioideae; apioid
            superclade; Selineae; Angelica.
REFERENCE   1  (bases 1 to 1370)
  AUTHORS   Sung,M.-J., Chun,J.-A. and Chung,C.-H.
  TITLE     Expression of homogentisic acid phytyltransferase in transgenic
            plants
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1370)
  AUTHORS   Sung,M.-J., Chun,J.-A. and Chung,C.-H.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-JAN-2008) Biotechnology, Don-A University, 840,
            Ha-Dan-Dong, Sa-Ha-Gu, Busan, Busan 604-714, South Korea
FEATURES             Location/Qualifiers
                     source          1..1370
                     /organism="Angelica gigas"
                     /mol_type="mRNA"
                     /db_xref="taxon:85712"
                     CDS             16..1218
                     /codon_start=1
                     /product="homogentisic acid phytyltransferase"
                     /protein_id="ACE80263.1"
                     /db_xref="GI:190611658"
                     /translation="MESLLISSFPKACSFPVSADFSSSHSTDLRVLRCQAWNTIESYS
                     ATNCRQHLVMQHAGGFTNNLSLYRRGDTKFLVNAASEQPLESEPLSYQPKSLQGSLQN
                     ALNAFYKFSRPHTVIGTALSIISVSLLAVEKLSDFSPIFFTGVLEAIVAALFMNIYIV
                     GLNQLSDIEIDKVNKPYLPLASGEYSVATGVILVASFAIMSFCLGWSVGSQPLLLALF
                     ISFILGTAYSINIPFLRWKRYAVVAAMCILAVRAVIVQIAFYLHVQTHVYGRPAIFPK
                     PVIFATAFMSFFSVVIALFKDIPDIVGDQIYGIRSFTVRLGQKRVFWICIALLQMAYA
                     TAIIVGASSSTPWSKLITVLGHMMLSSILWIRAKSVDLDSKVAITTFYMFIWKLFYAE
                     YLLIPLVR"
ORIGIN
        1 atcaaatcaa atcaaatgga gtctctgctt ataagctcct ttcccaaagcttgttcattc
       61 ccagtttctg ctgatttttc atcttcacac tctactgatc tacgagttct gaggtgccaa
      121 gcatggaaca ctatagaaag ttactctgcc acgaattgtc ggcaacattt ggtgatgcaa
      181 catgctggag gctttaccaa caatttgtca ctgtaccgga gaggggatac aaagtttctg
      241 gtgaatgcag cttctgaaca gcccctcgaa tctgagcctc tctcttatca acccaagagt
      301 ctccagggat cactacaaaa tgcattaaat gctttctaca agtttttctag gcctcacaca
      361 gtgataggaa cagcattgag cattatttca gtttctctcc ttgcagttga gaaactatca
      421 gatttttctc caatttttt cactggggta ttagaggcaa ttgttgctgc tcttttcatg
      481 aatatctata tagtcggtct taatcagttg tccgatatag aaatagacaa ggttaataag
      541 ccatatcttc ctttggcatc aggggagtat tctgtcgcca ccggagtcat cctagttgca
      601 tcttttgcta ttatgagttt ttgccttgga tggtctgttg gttcacaacc gttactgttg
      661 gccctgttca ttagttttat acttgggact gcatattcaa tcaatatacc ttttttgaga
      721 tggaagagat atgctgtagt tgcagccatg tgcatcctgg ctgtacgagc agtgatcgtt
      781 caaattgcat tttatctgca tgtgcagact catgtttatg gcagaccagc tatcttcccg
      841 aagccggtga tctttgcaac cgcattcatg agtttttct ctgttgtcat agcactattt
      901 aaggacatac ctgatattgt cggggaccag atatatggca ttcggtcatt tactgtccgg
      961 ttaggtcaaa agcgggtatt ctggatttgt atagcactac ttcaaatggc ttatgcgact
     1021 gctataatcg taggggcatc ctcttccacc ccatggagca aattaataac ggtgttgggc
     1081 catatgatgc tgtcatcgat actttggatc cgtgccaaat cagttgatct ggatagcaaa
     1141 gtagcaataa ctacatttta catgtttata tggaagctat tttatgcaga gtacctgctc
     1201 ataccactgg tgagatgaat gtttagaagt agacagtaga aagacgcatc ttttccccgc
     1261 tgctgctttt gatcatttag ttaatctatt catgaaactg ttgatgtatc taagaatcca
     1321 gagagcaaat ttcagtcccc ccccccccc tcctttccga acgatttat
```

Figure 59. NCBI ID of a putative Phytyltransferase of *A. gigas*

To perform our investigation we first used the Basic Local Alignment Search Tool available on the NCBI website. A first BLASTx research was realized with the *Sf*N8DT-1 nucleotidic sequence on the whole protein database, which demonstrated the presence of hundreds of sequences potentially displaying a Pt activity. As the pathway we would like to focus on (i.e. the furanocoumarins biosynthetic pathway) was present in only few plant species, we decided to restrict our investigations to the plants that had been described as furanocoumarin producing plants.

Angelica is well-known for its ability to produce angelicin and has a strong reputation of medicinal plant (Kyline, 2010). By making a limited screening on this plant family we were able to identify a sequence (Figure 58) which displayed 47% homology with *Sf*N8DT-1. The full length sequence corresponding to this gene was available (Figure 59, NCBI ID EU407262) and was annotated as a homogentisic acid phytyltransferase. However a bibliographic research showed that no experimental details for the functional characterization of this gene were available.

Based on this nucleotide sequence we designed specific primers with additional restriction sites (*Bam*HI and *Nde*I) in order to amplify the ORF and subsequently clone it into an expression vector (Table 15).

Table 15. Sequence of PCR primers used to amplify Pt encoding genes

Name	Sequences
Prenyl-Ang-*Nde*I For	GGCCCAT ATGCCTCAAGCTAACAAACA
Prenyl-Ang-*Bam*HI Rev	GGCGG GTTGCTTATCCCAACGAACAAAAGGAATTAG

Total RNAs were extracted from *A. gigas* leaves cultivated in green house and used to carry out a RT-PCR. The resulting amplification product was cloned into a pCR8 vector and sequenced. The sequence we amplified corresponded to another Angelica putative transferase (homogentisate geranylgeranyl transferase) referred as EU402478.1. A comparison of this peptidic sequence with the one available in the database displayed 98% identity. Even if this sequence seems to be a Pt, it only shares 43% identity with the targeted *A. gigas* protein (EU407262) and only 33% identity with *Sf*N8DT-1 (Figure 60). Moreover, an alignment of the 3 different sequences highlighted that this protein was lacking a part of the N-terminus.

As this first experiment didn't allow us to clone the gene we were looking for, we repeated the experiment several times. As we were unsuccessful we concluded that this gene was not expressed in our plants.

Therefore, as we were seeking for new enzyme activities, we finally decided to try to characterize the activity of the only gene we isolated (i.e. EU402478.1) which will be named AngPt in this manuscript.

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
EU402478        ---------- ---------- ---------- ---------- ----------
EU407262        MESLLISSFP KACSFPVSAD FSSS------ -------HST DLRVLRCQAW
SfN8DT-1        MGSMLLASFP GASSITTGGS CLRSKQYAKN YDASSYVTTS WYKKRKIQKE

                ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
EU402478        ---------- ------MPQA N--------- ---------- ----KQEVSP
EU407262        NTIESYSATN CRQHLVMQHA GGFTNNLSLY RRGDTKFLVN AASEQPLESE
SfN8DT-1        HCAAIFSKHN LKQHYKVNEG GSTSN---TS KECEKKYVVN AISEQSFEYE

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
EU402478        PLSQN----- --LWRKVDAF YRFTRPHTII GSIVGITSVS LLPLVSFEDL
EU407262        PLSYQPKSLQ GSLQNALNAF YKFSRPHTVI GTALSIISVS LLAVEKLSDF
SfN8DT-1        PQTRDPESIW DSVNDALDIF YKFCRPYAMF TIVLGATFKS LVAVEKLSDL

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        160        170        180        190        200
EU402478        SPAFFVGLLK VMIPIVCVNI YVVGLNQLYD VEIDKVNKPN LPIASGEYSM
EU407262        SPIFFTGVLE AIVAALFMNI YIVGLNQLSD IEIDKVNKPY LPLASGEYSV
SfN8DT-1        SLAFFIGWLQ VVVAVICIHI FGVGLNQLCD IEIDKINKPD LPLASGKLSF

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        210        220        230        240        250
EU402478        ETGKAIVSAF GLMSIIMGIM FQSPPVLYCL LVCFFFGTAY SIDVPLFRWK
EU407262        ATGVILVASF AIMSFCLGWS VGSQPLLLAL FISFILGTAY SINIPFLRWK
SfN8DT-1        RNVVIITASS LILGLGFAWI VDSWPLFWTV FISCMVASAY NVDLPLLRWK

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        260        270        280        290        300
EU402478        KNAFLAAMCI VIVRAITVQL TVFYHIQQYV LGRPVLFSRS LAFAIICMTL
EU407262        RYAVVAAMCI LAVRAVIVQI AFYLHVQTHV YGRPAIFPKP VIFATAFMSF
SfN8DT-1        KYPVLTAINF IADVAVTRSL GFFLHMQTCV FKRPTTFPRP LIFCTAIVSI

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350
EU402478        FVTVIALFKD IPDVDGDRDF GIQTITVTLG KKRVFWLCIT ILLIAYGSAV
EU407262        FSVVIALFKD IPDIVGDQIY GIRSFTVRLG QKRVFWICIA LLQMAYATAI
SfN8DT-1        YAIVIALFKD IPDMEGDEKF GIQSLSLRLG PKRVFWICVS LLEMTYGVTI

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        360        370        380        390        400
EU402478        VIGSSSSLLL SKLVTVTGHC ILASILWSRA ISVDLESNKS ITSFYMFIWK
EU407262        IVGASSSTPW SKLITVLGHM MLSSILWIRA KSVDLDSKVA ITTFYMFIWK
SfN8DT-1        LVGATSPILW SKIITVLGHA VLASVLWYHA KSVDLTSNVV LHSFYMFIWK

                ....|....| ...
                        410
EU402478        LFYAEYLLIP FVR
EU407262        LFYAEYLLIP LVR
SfN8DT-1        LHTAEYFLIP LFR
```

Figure 60. Alignement of the peptidic sequence of EU402478, EU407262 and *Sf*N8DT-1

1.1.1.2 Identification of a gene encoding for a putative Pt of *P. sativa*

P. sativa is a plant producing both angular and linear furanocoumarins. This plant has been successfully used for the molecular characterization of two genes: the first is involved in the biosynthesis of linear furanocoumarins and the second is specific for the angular furanocoumarins. These genes belong to the cytochrome P450 enzyme family and encode for a PS (CYP71AJ3) and AS (CYP71AJ4) (Larbat *et al.,* 2007, 2009) respectively. Hence, as this plant is a good model plant to identify the whole furanocoumarin pathway, we decided to realize a *de novo* transcriptome sequencing of the total RNA extracted from *P. sativa* plantlets by using the GS FLX technology (Eurofins MWG Operon). To identify any putative Pt sequence, Celia Krieger (Ph.D Student in the lab) realized a tBlastn to screen this library using a Pt sequence. This analysis highlighted a sequence sharing 42% identity with the *Sf*N8DT-1 sequence. This parsnip Pt was identified as the contig633 in the database and was named as PsPt (Figure 61).

In order to characterize the function of this new protein, we synthesized the corresponding gene (GenScript). The codon bias of the gene sequence was optimized for its expression in *N. benthamiana*. This synthesized sequence shares 76% nucleotidic identity and 100% peptidic sequence identity with the putative PsPt identified in *P. sativa* database. This synthesized gene was cloned by the manufacturer in pCR®8/GW/TOPO®, which was utilized then for our further experiments.

A) Nucleotidic sequence of PsPt

```
ATGGCTCAAACAATTATGCATTCACGATTATCGTCCGGTTTCTTACATCTTCAACGAGAC
AAAGGCTTTCGTACACTTCCAACGCAAAGAAGACATGCTAAAGTAGTGAACGGAGACCAA
GAATTTGCTTTCAGGGTGGTTTCATGCGATAAAAATTTAGATTCAACAAAGAATTTTAGC
GGCAGTTGTGAAAAGCCAATTAGAACACACACAAACAAACTTTTACAAACAATTAGTGCC
ACATCAGACAGAGAGGCTATTATACAGCCCAAAGATGATTACGAAGCACCATGGCAGAAT
ACTTTACGGAGAAAATGGGATGCATTTTGCACGTTTGGTCGTCCATACTCTGCCATCTGC
ACAATTATTGGAATAAGTTCGGTTTCTCTGCTGCCCTTAACTTCCGTTAAAGATTTCTCG
GCTCCATATTTTGTGGGATTACTACAGGCATTGATCCCATTTCTTTGTGCCAACATCTAT
ACCTCGGGGATAAATCAACTTGTTGATGTGGACATAGACAAAATAAACAAGCCTTACCTG
CCACTGGTTTCGGGAGAATTTTCCTTGGGCGAAGGGAGAGCAATTGTCTCAGCACTGGCT
TTTATGTGCCTGGCCGTGGGAATCCTATCTCATTCTACACCATTGTTTGTCGGGGTTCTT
GTCTATTTTTTAATTGGAACCGCATATTCTGTTGAGCTACCTCTACTTAGATGGAAAACA
AAACCGGCCATGGCTGCATTTTCTATGGCCGGCCTAATGGGACTCACGATCCAGCCTGCT
GTCTTCTATCACATCCAGAATGCACTCGGTAAACCAATGGTATTTTCAAAGACAGTGGCC
TTTGCTACCATTTTCTTCAGTGTCTTTGCAGCTGTTCTTGGAGCGATCAAGGACGTACCT
GATGTTGAAGGAGACACAGCGTTCGGCAACCGAACATTCAGTGTGAGATATGGCCAAGAG
AAAGTCTTTTCTGTCTGTCTAAATATCCTGCTGCTTGCCTACGGGTTTGCTGTGGTGGTA
GGAGCTTCATCCTCGTTCCTATTATGCAAGATTGTATCTGTGATGGGTCACACTACACTT
GCCTCCCTACTTTTGCTTCGAGCAAAATCTACTAATCCGAAAGATCCTGAATCAACGCAG
TCCTTTTACATGTTTTTATTTAAGCTATTGTACGCGGAGTATGTGCTAATTCATTTCATG
CGCTGA
```

B) Peptidic sequence of PsPt

```
MAQTIMHSRLSSGFLHLQRDKGFRTLPTQRRHAKVVNGDQEFAFRVVSCDKNLDSTKNFS
GSCEKPIRTHTNKLLQTISATSDREAIIQPKDDYEAPWQNTLRRKWDAFCTFGRPYSAIC
TIIGISSVSLLPLTSVKDFSAPYFVGLLQALIPFLCANIYTSGINQLVDVDIDKINKPYL
PLVSGEFSLGEGRAIVSALAFMCLAVGILSHSTPLFVGVLVYFLIGTAYSVELPLLRWKT
KPAMAAFSMAGLMGLTIQPAVFYHIQNALGKPMVFSKTVAFATIFFSVFAAVLGAIKDVP
DVEGDTAFGNRTFSVRYGQEKVFSVCLNILLLAYGFAVVVGASSSFLLCKIVSVMGHTTL
ASLLLLRAKSTNPKDPESTQSFYMFLFKLLYAEYVLIHFMR
```

Figure 61. Nucleotidic and peptidic sequence of a Pt identified in the *P. sativa* cDNA library

1.1.2 PCR approach using degenerated primers

This part of the work has been realized by Dr Takao Koeduka in Prof. Yazaki's group (Kyoto University, Japan)

Single-strand cDNAs were synthesized from total RNA isolated from 4-week-old plant leaves with Oligo-dT primer and Superscript III reverse transcriptase (Invitrogen). Degenerated primers corresponding to conserved amino acid sequences of known aromatic Pts were designed. A first RT-PCR was carried out with the specific primer sets. The resulting PCR products were cloned and sequenced. 5'- and 3'-RACE transcripts were performed using the SMART RACE cDNA Amplification Kit (Clontech) following the manufacturer's instructions to obtain the complete coding sequence of the different genes

This approach led to isolate 5 different putative Pts genes from different furanocoumarin producing plants, which were *P. crispum*, *Citrus limon*, *C. latifolia*, *A. graveolens* and *R. graveolens* (Figure 62).

Putative prenyltransferase of *P. crispum* (PcPt)

MSQTLMHSRFSSGFLHHQPEKGFLTLQTQRRHAKTLKGEKEFPSRVVSCHKNVDSSKNFS
SSCEKSKTQEKILAQTLGATSDGEAIVQPNNDFEVTWQNTLRRKWDAFSIFSRPYSAICT
IIGISSVSLLPLTSVADFSPAYFVGLLQALIPFLCANIYTSAINQLVDVDIDKINKPYLP
LVSGEFSMGEGRAIVSALTFTCFAMAIMSHSVPLFVGVLVYFLIGTAYSVEHPLLRWKTK
PAMAAFSMAGLMGLTIQPTVFYHIQNVLGKPMVFSRSVAFATMFFSIFAACLGAIKDIPD
VEGDREFGNLTFSVRYGQEKVFSFCLNVLLLAYGSAVVVGASSSSLLCKTVSVIGHTVLA
SLLVLRAKSTNPKDPESTQSFYMFLFKLLYAEYVLIHFMR*

Putative prenyltransferase of *C. latifolia* (ClPt)

MLQMHSSSSFSPKYYYPLQHAGCVKTLQLPLTKVHGGSNRSESKNYAIKCTQSDSVYSTN
KIRNNENASRRNCKPFNKNRVAVTLQQEGWASNNEDDINSTSFRDVLLKKLHALYVFTRP
FAMIGTIVGITSIAILPLQSFADLTPKYFMEFLKALLSAVLMNNYVGTVNQVADVEIDKV
NKPGLPLASGDLSMGTGLAITLILSLTSLAIALSLQSPPLIFGLIVWFLLGTAYSVDLPF
LRWKTNPFLAGMSMVIVFGLVYQFSFFIHFQKYVLGRPVVITRPLIFAAAIISTISAVMS
LLKDIPDEDGDKQFGYQSISSMLGKEKVLRLCVYALFFAYGVAVIVGASSSFQLGKLVSI
IGHSTLAFLLWLRAQTVDLSNNASAYSFYMFVWKLFYAEYLLIHFLR*

Putative prenyltransferase of *R. graveolens* (RgPt)

MPLQMNLCSSVSVKSHLLQDHHKFLLQRAEGLPRLHRHGAAVRQTPGLVSRSDQGKIDNK
LTAKCSLSSFNLTTKFWNNEDTTTTTSKSQQSCASSSSNEHYYDDSTSSGGTLAKKLTAL
WNFLRPFAIIGSIVSLTSVSFLPLESLSDLTPQYFLGFLKALVPFLLMNIYVGTLNQLSD
IEIDKVNKPYLPLASGELSVATGTVVALISPLLSLAMAIGLKSPPLVVGLGAWFLLGTAY
SVHLPLLRWKATTFLAGLCVVIPFGFIHTFSFYMHMQQHVLGRPLLFNRPILFVSAFMCL
FSTAVSILKDIPDEKGDEKFGIQSMSSRLGKEKVLSISVYVLLLAYGGALVAGASASSLL
GRVVSVIGHSVLASVLWHRSQNTDLSSPDSTFSFYMFIWQLFYAEYLLAHFLR*

Putative prenyltransferase of *C. limon* (CliPt)

MLQMHSNSSFSPKCYYPLQHAGCVKTLQLPLTKVHGGLNRSESKNYAIKCTQSDSFYSTN
KIRNNENSSSRNCKPFNKYRVAVTLQQQDCASNNEDDINSTSFRDVLLKKLHALYVFTRP
FAMIGTIVGITSIAILPLQSFADLTPKYFMEFLKALLSAVLMNNYVGTVNQVADVEIDKV
NKPGLPLASGDLSVGTGLAITLILSLTSLAIALSLQSPPLIFGLIVWFLLGTAYSVDLPF
LRWKTNPFLAGMCMVIVFGLVYQFSFFIHFQKYVLGRPVVITRPLIFAAAIISTISAVMS
LLKDIPDEDGDKQFGYQSISSKLGKENVLRLCVYALFFAYGVAVIVGASSSFQLGKLVSI
IGHSTLAFLLWLRAQTVDLSNNASTYSFYMFIWKLFYAEYLLIHFLR*

Putative prenyltransferase of *A. graveolens* (AgPt)

MTQTFIHPRFSSGFLHHQPEKGFLTSRTQRRHARIWNGDKEFPFRVVSCHQNLDSAKNFS
SSHEKPITTHKKKLVQTLGATSDGELLIQPSDNVQVTWQNTLRRKWDAFCIFSRPYSAIC
TIIGISSVSLLPLTSVGDFSSAYFVGLLQALIPFLCANIYTSAINQLVDVDIDKVNKPYL
PLVSGEFSMGEGSAIVSALTFTCLAMAIMSHSAPLLVGVLVYFLIGTAYSVELPFLRWKT
KPAMAAFSMAGLMGLTIQPAVFYHIQNALGKPMVFSRSVGFATIFFSIFAAVLGAVKDIP
DVEGDREFGNLTFSVRYGQERVFSFCLNVLLMAYGAAVVVGASSSFLLCKIVSVIGHTTL
ASLLMLRAKSTNPTDSESTQSFYMFLFKLLYAEYVLIHFMR*

Figure 62. Peptidic sequences of the putative prenyltransferases isolated and provided by Professor Yazaki in the frame of collaboration with our group.

1.1 *In silico* analysis of the putative prenyltransferase sequences

1.1.1 Consensus sequences

The presence of conserved domains in Pts has already been discussed and is considered as one of the most important criteria for their grouping in various classes. Two important consensus sequences (NQxxDxxxD and KDxxDxE/DGD) were described to be necessary for the activity of plant aromatic Pts (Ohara et al. 2009; Yazaki *et al.,* 2009). An analysis done one the putative Pts described in the frame of this project, highlighted the presence of both conserved aspartate rich motifs (NQxxDxxxD) (Figure 63, outlined in red color) and (KDxPDxxGD) (Figure 63, outlined in green).

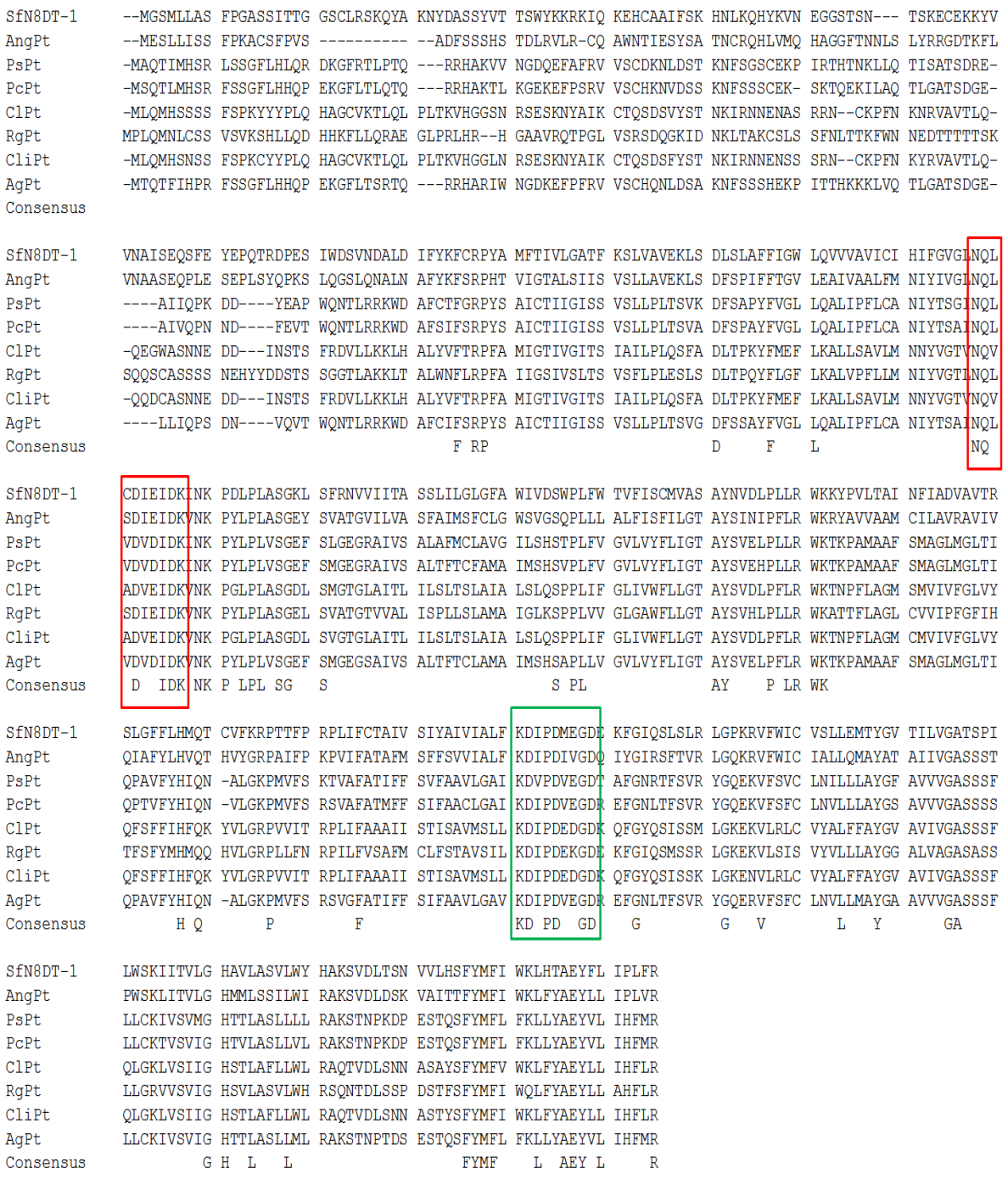


Figure 63. Alignment of peptidic sequences of SfN8DT-1 and of putative prenyltransferases isolated from different plants using the various approaches

Two Conserved regions (1: NQxxDxxxD) and (2: KDxxDxE/DGD) found among the SfN8DT-1 and the putative Pts are framed in Red and Green boxes respectively and has been reported as important sequences for reaction of prenylation (Ohara *et al.,* 2009). Where *Sf*N8DT-1 represents; 8-dimethylallyl naringenin of *S. flavescens*, AngPt; putative Pt of *A. gigas*, PsPt; putative Pt of *P. sativa*, PcPt; putative Pt of *P. crispum*, ClPt; putative Pt of *C. latifolia*, RgPt; putative Pt of *R. graveolens*, CliPt; putative Pt of *C. limon* and AgPt; putative Pt of *A. graveolens*.

1.1.2 **Protein typology**

The study done by Sazaki on *Sf*N8DT-1 showed that this Pt contains nine putative transmembrane α-helices (Sasaki *et al.,* 2008). In order to study the structure of the putative Pt isolated from the different plants described earlier, we performed a typology analysis using the TMHMM server of the center of biological sequence analysis of the technical university of Denmark (www.cbs.dtu.dk). This analysis showed that, in contrast to *Sf*N8DT-1, PcPt contained only 8 transmembrane helices (TMHs) (Figure 64).

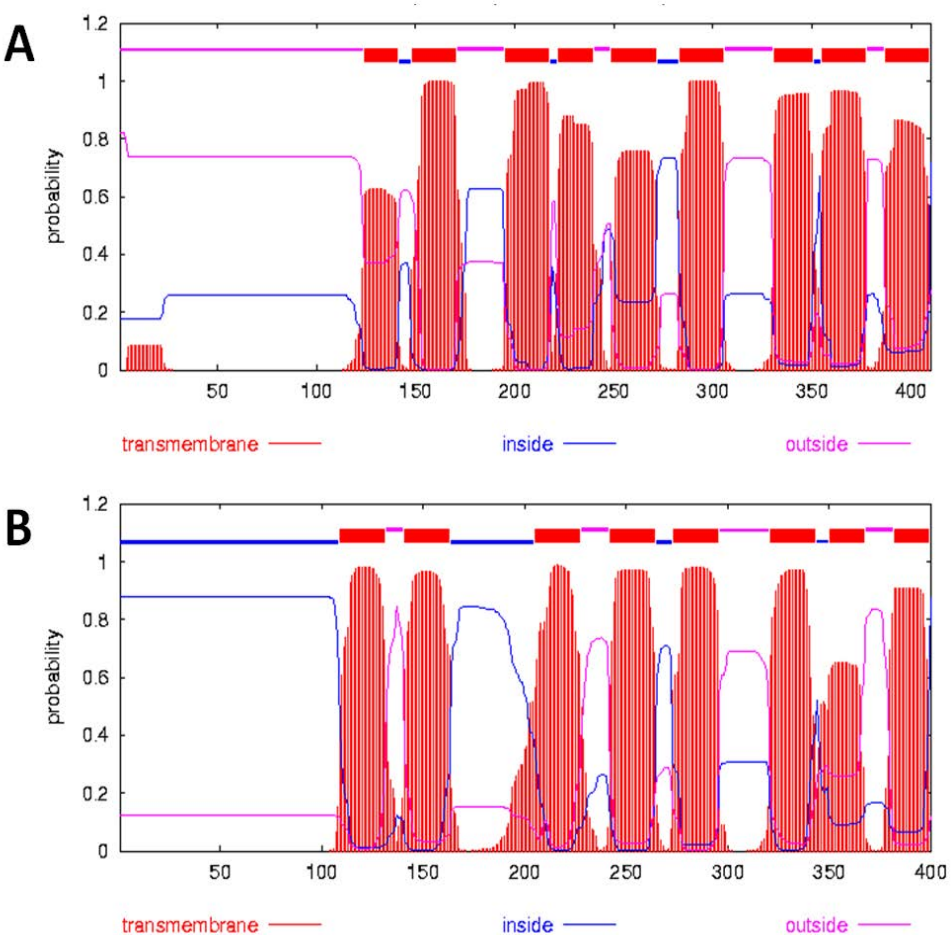


Figure 64. Results obtained for predicting the number of putative transmembrane domains present in protein structure of *Sf*N8DT-1 (A) and PcPt (B) using the software TMHMM

The same approach was used to analyze the protein structure of all the other sequences identified above. These analyses showed that RgPt, AgPt, ClPt and CliPt have the same protein topology as PcPt. In contrast to these putative Pts, analysis for protein structure of

AngPt and PsPt demonstrated the presence of only 7 and 6 putative transmembranes respectively (Figure 65). The results obtained for AngPt is consistent with the analysis done previously and is probably related to the absence of part of the N-terminus sequence.

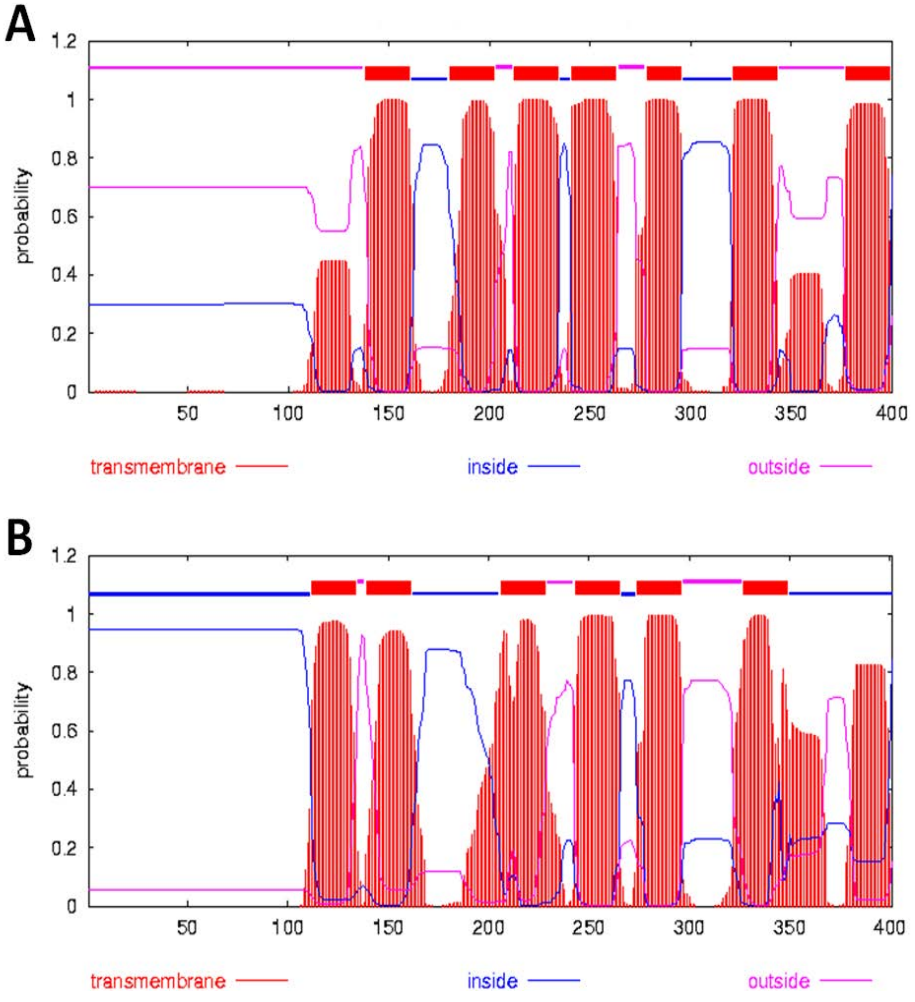


Figure 65. Results obtained for predicting the number of putative transmembranes present in protein structure of AngPt (A) and PsPt (B) using the software TMHMM

Analysis for the protein topology to determine the number of the putative transmembranes present in AngPt and PsPt was completed and it has been found that PsPT contained 6 putative transmembranes while AngPt possessed 7 putative transmembranes. Where, AngPt represents putative Pt of *A. gigas* and PcPt; putative Pt of *P. sativa*.

1.1.3 Subcellular localization

A bibliographic analysis demonstrated that the various plant aromatic Pt described till now are mostly membranous enzymes (Akashi *et al.,* 2009; Biggs *et al.,* 1990; Sasaki *et al.,*

2008, 2011). However, different kind of membranes can be targeted. To check the possible subcellular localizations of our putative Pts, we analyzed these peptidic sequences using TargetIP available on the CBS website (www.cbs.dtu.dk). This software checks the presence of signal peptides specific for various subcellular compartments that include chloroplast (cTP), mitochondria (mTP), secretory pathway (SP) or others (o). The relationship between the scores for each location predication can serve as an indication of how accurate the prediction is. There are 5 reliability classes (RC). 1 indicates the strongest prediction. Results obtained for the various putative Pts are summarized in (Table 16). According to these *in silico* analysis PcPt, AgPt and AngPt are most likely localized in chloroplast, while RgPt and PsPt were found to have mitochondrial targeting-peptides. On other hand, no possible localization could be detected for ClPt, and CliPt.

Table 16. Results obtained for the subcellular localization of various putative Pt using the software TargetIP

Name of sequence	Len of sequence	cTP	mTP	SP	o	Loc	RC	Tp Len
PcPt	400	0.480	0.257	0.010	0.144	C	4	48
RgPt	413	0.093	0.378	0.02	0.208	M	5	39
AgPt	401	0.556	0.262	0.009	0.172	C	4	48
AngPt	400	0.52	0.038	0.158	0.201	C	4	18
PsPt	401	0.427	0.559	0.019	0.059	M	5	116
ClPt	407	0.122	0.133	0.039	0.277	-	5	-
CliPt	407	0.147	0.121	0.044	0.299	-	5	-

Various putative proteins were analyzed using the software TargetIP for their subcellular localization. Where cTP demonstrates; chloroplast targeting-peptide, mTP; mitochondria targeting-peptide, SP; signal peptide, Len of sequence; length of sequence, Loc; localization and Tp Len; length of targeting peptide. Where, AngPt represents putative Pt of *A. gigas*, PsPt; putative Pt of *P. sativa*, PcPt; putative Pt of *P. crispum*, RgPt; putative Pt of *R. graveolens* and AgPt; putative Pt of *A. graveolens*.

1.1.4 A new group of prenyltransferases on the basis of phylogenetic analysis

In order to highlight their evolution and the phylogenetic relationship (if ever there is one) between the putative Pts, we gathered various sequences of plant aromatic Pts available in databases omitting the limitation of plant's ability to produce or not the furanocoumarins. Using this approach we collected almost 22 different either putative or characterized sequences of plant aromatic Pts, which included homogentisate phytyltransferase VTE2-1

[*Allium ampeloprasum*] (ABB70124), homogentisate phytyltransferases-1 [*A. thaliana*] (NP_849984), polyprenyltransferase-1 [*A. thaliana*] (NP_567688), homogentisate solanesyltransferase [*A. thaliana*](NP_001078138), homogentisate phytyltransferase VTE2-1 [*Cuphea avigera* var. pulcherrima] (ABB70125), homogentisate phytyltransferase-1, chloroplastic-like [*G. max*] (NP_001241496), homogentisic acid geranylgeranyl transferase [*Hordeum vulgare*] (AAP43911), 4-hydroxybenzoate geranyltransferase 1 (Q8W405), homogentisic acid geranylgeranyl transferase [*Oryza sativa* Japonica Group] (AAP43913), Os08g0322600 [*O. sativa* Japonica Group] (NP_001061539), isoliquiritigenin dimethylallyltransferase [*S. flavescens*] (BAK52290), homogentisic acid geranylgeranyl transferase [*Triticum aestivum*](AAP43912), homogentisate phytyltransferase VTE2-1 [*Triticum aestivum*](ABB70123), homogentisate phytyltransferase VTE2-1 [*Zea mays*](NP_001105877), homogentisate phytyltransferase VTE2-2 [*G. max*] (NP_001237900), glycinol 4-dimethylallyltransferase [*G. max*] (NP_001235990), genistein 6-dimethylallyltransferase [*S. flavescens*](BAK52291), genistein 3'-dimethylallyltransferase [*L. albus*](AER35706), 4-hydroxybenzoate geranyltransferase 2 (Q8W404), naringenin 8-dimethylallyltransferase [*S. flavescens*](BAG12673), 8-dimethylallyltransferase [*S. flavescens*](BAK52289), Naringenin 8-dimethylallyltransferase 1 Chloroplastic; Short=SfN8DT-1; Flags: Precursor (B1B3P3) and completed the phylogentic analysis.

The different sequences collected from databases were aligned using the MEGA 5.1 software and used to construct a phylogenetic tree (Figure 66). Through this approach we observed a clearly separated clustering of different classes of aromatic Pts that include homogentisate Pts, 4HB Pts and flavonoid Pts. Furthermore, in this clustering pattern it was very interesting to note that among the 7 putative Pts studied in this project, one (AngPt) appeared in the cluster of homogentisate Pts while all the 6 other formed a new clad of their own.

Hence, these putative aromatic Pts could be involved in the biosynthesis of molecules that have never been reported at the molecular level in the literature, such as furanocoumarins. In addition through this analysis, it was observed that the putative Pts were distinct from flavonoid and homogentisate Pts but were close to these classes on the phylogenetic basis. Thus, we can assume that our putative Pt were derived from homogentistae Pt as it has been

cited for flavonoid Pt in literature (Yazaki *et al.,* 2009), and perhaps they acquired the capability to utilize other aromatic substrates.

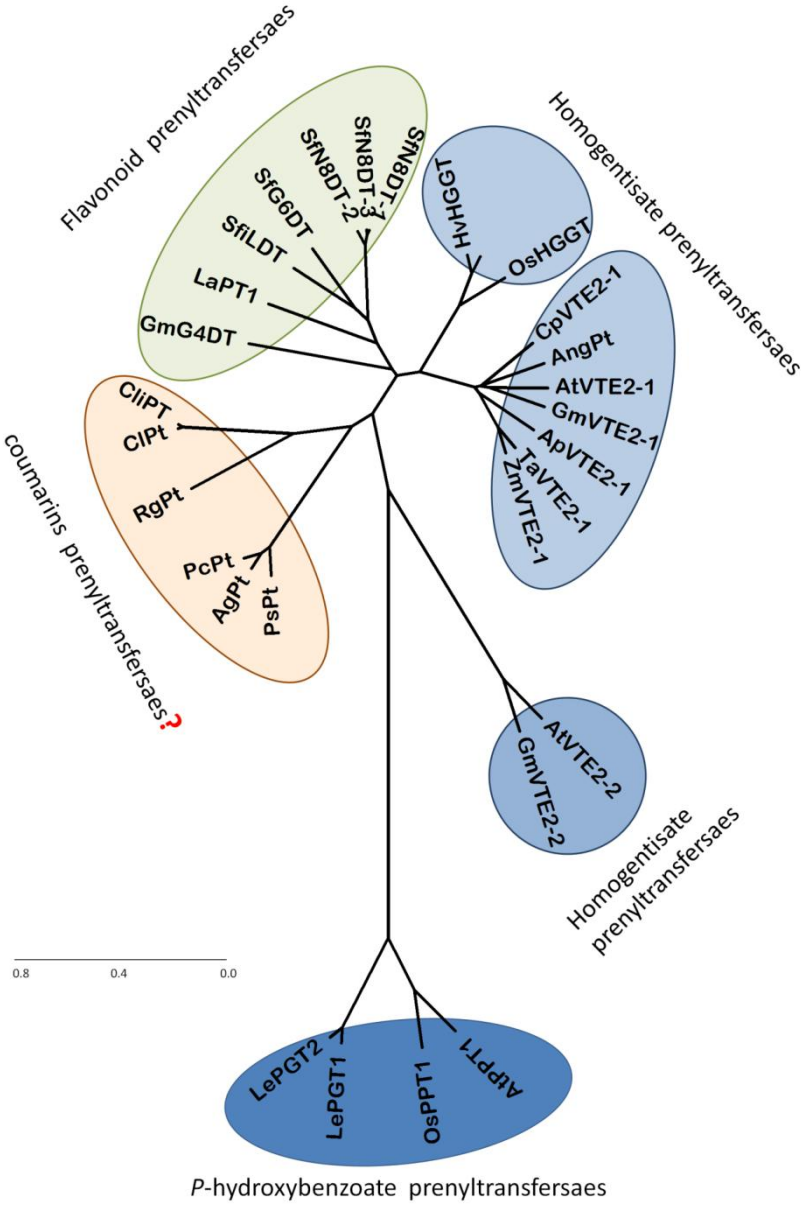


Figure 66. Phylogenetic analysis of 22 different sequences of either putative or characterized aromatic Pts of plants found in database along with the seven potential candidate genes of Pts discussed in this chapter was accomplished

Alignment has been done with MEGA5.1. NCBI ID of the 22 sequences are: ABB70124, NP_849984, NP_567688, NP_001078138, NP_001241496, AAP43911, Q8W405, AAP43913, NP_001061539, BAK52290, AAP43912, ABB70123, NP_001105877, NP_001237900, NP_001235990, BAK52291, AER35706, Q8W404, BAG12673, BAK52289, B1B3P3 and ABB70125. Ap; Allium ampeloprasum, At; Arabidopsis thaliana, Cp; Cuphea avigera, Gm; Glycine max, Hv; Hordeum vulgare, Os; Oryza sativa, Sf; S. flavescens, Ta; Triticum aestivum, Zm; Zea mays, La; Lupinus albus and Le; Lithospermum erythrorhizon.

1.2 Conclusion

At the beginning of the thesis project, little information was available concerning the plant aromatic Pts. In order to overcome this problem we used various approaches to find new putative Pts. For this purpose, we started collaboration with professor Yazaki (University of Kyoto) who provided 5 sequences isolated from furanocoumarin producing plants. In addition we isolated 2 other genes from the same group of plants. Analysis of these putative Pts by various bioinformatic approaches demonstrated that they all shared similarities with already described Pts such as the presence of two consensus sequences (NQxxDxxxD and KDxxDxE/DGD) or the presence of transmembrane domains. An additional subcellular localization analysis supported their use for studies of functional characterization of coumarins and furanocoumarins specific Pts. Finally, a phylogenetic analysis clearly showed the emergence of a new separate group of enzymes potentially involved in the prenylation of furanocoumarins or coumarins.

Chapter II: Development of a heterologous transient expression system for membranous proteins using *Nicotiana benthamiana*

2 Development of heterologous expression system of *N. benthamiana*

2.1 Introduction

Most of the enzymes involved in the furanocoumarin biosynthesis pathway are membranous. For example, till today 3 different enzymes psoralen synthase, AS (Larbat *et al.,* 2007, 2009) and C2'-H (Vialart *et al.,* 2012) directly involved in biosynthetic pathway of furanocoumarins have been characterized and out of these 3, PS and AS are membrane bound proteins. Beside these major enzymes other steps of this pathway could be catalyzed by membrane bound enzymes. Recently the group of Yazaki (Kyoto University, japan) showed that several plant aromatic Pts involved in the biosynthesis of prenylated flavonoids were membrane-associated enzymes (Akashi *et al.,* 2009; Sasaki *et al.,* 2008, 2011). A similar prenylation step has been described in the synthesis of furanocoumarins (Bourgaud *et al.,* 2006) and the analysis done on the basis of bioinformatics approaches described in previous chapter (under heading 1.1.3) provided us the clue that our putative Pts were also membrane-associated enzymes. The functional characterization of these kinds of proteins has always remained challenging for scientists. The bottleneck for the study of these proteins is often related to not only their membranous nature but also to their low abundance, their cell specific expression patterns or the availability of cofactors (for example for P450s/redox partner) (Dixon, 1999; Morant *et al.,* 2003; Schuler *and* Werck-Reichhart, 2003). As stated previously, in introduction (under heading 3), the goal of this research work is to functionally characterize several candidate **prenyltransferases** and to highlight their possible involvement in the synthesis of furanocoumarins. To achieve this goal, we needed to develop an accurate and reproducible heterologous expression system for the expression of membrane-bound proteins.

Since more than 10 years, several enzymes that belong to the P450 family have been successfully expressed in our laboratory using a yeast heterologous expression system (Gravot *et al.,* 2004; Larbat *et al.,* 2007, 2009). Preliminary experiments done by Yazaki and collaborators made evidence that this system was not the best suited for the expression of Pts

(K. Yazaki, personal communication). Therefore, in order to carry out, not only the functional characterization of such membrane-bound proteins but also to understand their physiological role we chose to focus on a plant expression system. For this purpose, we selected two different strategies to work with. The first strategy was to develop stably transformed *R. graveolens* plants that could over express the genes of interest. The stable genetic transformation of *R. graveolens* has already been established during the PhD of Karine Lièvre (Lièvre *et al.,* 2005) in our laboratory and will be discussed later in this manuscript. As stable transformation of Ruta plants takes a very long time (at least one year to have enough plant material to make biochemical analyses) we decided to develop an alternative and faster strategy by realizing plant transient expression of our candidate genes. Such transient gene expression in plants is a suitable strategy because it allows to produce the proteins in large amounts within a short time period (Gleba *et al.,* 2005; Lindbo, 2007). Numerous techniques have been described that focused on different methods for introducing DNA into plant cell like electroporation (To *et al.,* 1996), particle bombardment (Seki *et al.,* 1999), plant viral vectors (McCormick *et al.,* 2008; Wagner *et al.,* 2004) and *Agrobacterium*-mediated T-DNA transfer (Wroblewski *et al.,* 2005; Wydro *et al.,* 2006). Among all these different approaches available, we focused on an *Agrobacterium*-mediated method for the transfer of T-DNA into plant cell because of the advantage that this technique could be utilized both for transient and stable transformation of plants.

2.2 Validation of a transient expression system in *N. benthamiana* using the Green Fluorescent Protein (GFP)

Transient expression systems using *N. benthamiana* as host have been used for research purposes since many years and various protocols developed using this technique are available in literature. To use this method for heterologous expression and functional characterization of membranous enzymes we needed, first, to verify that we have enough expertise to develop a functional system in our laboratory. This validation step was completed using a soluble reporter protein, GFP.

2.2.1 Recombinant plasmid: pBIN-m-gfp5-ER

The recombinant pBIN m-gfp5-ER plasmid used in this preliminary experiment was provided by Prof. Jim Haselhoff, Division of Cell Biology, MRC Laboratory of Molecular Biology, Addenbrookes Hospital, Hills Road, Cambridge, CB2 2QH, UK.

2.2.2 Preparation of *Agrobacterium* inoculum and inoculation of *N. benthamiana* leaves

The recombinant pBIN m-gfp5-ER plasmid was introduced into the LBA4404 *A. tumefaciens* strain using the heat shock method as described in the "Material and Methods" chapter. As the agrobacteria are resistant to rif and as the pBIN plasmid confers resistance to kan, the transformed agrobacteria were selected on a selective media that contained both antibiotics. Colonies obtained were used to inoculate a liquid medium and were incubated at 28 °C for 16 h. These cultures were finally used to infiltrate leaves of young tobacco plants. To perform our experiments, we strongly inspired from a recently published work by Sparkes and his collaborators (Sparkes *et al.,* 2006) who described infiltration experiments of tobacco leaves. However, in order to adapt the system for our research work we had to make some improvements. Different steps of the modified protocol are as follows. We used 10 ml of LB medium which were inoculated with a colony of freshly spread transformed agrobacteria in the presence of kan and rif. This culture was incubated overnight (16 h) at 28 °C with a strong shaking (200 rpm). After one night, the culture was centrifuged at 5000 g for 10 min (room temperature) in order to pellet the bacteria. After removing the supernatant, the pellet was re-suspended in 2 ml sterile water. This step was repeated 3 times to remove the excess of antibiotics that could be harmful for the plant.

The overexpression of any protein can be lethal to plant tissues. Therefore it is necessary to control the amount of protein produced and, hence respectively to know the amount of bacteria which is injected in the leaves. For this purpose, we measured the optimal optical density (OD_{600nm}) of the bacterial culture. We tested 3 different concentrations of GFP transformed agrobacteria (OD_{600nm} 0.2, 0.4, and 0.6) for the inoculation of *N. benthamiana* leaves. The bacteria were prepared in a 2 ml microtube and stored at room temperature until the inoculation.

Concerning plants, we used 4-6 weeks old *N. benthamiana* plantlets as described in the "Material and Methods" chapter (Figure 67 (1)). The selected plants were removed from the growth chamber and were left in day light for almost 1 h before the infiltration to facilitate the opening of stomata. Leaves onwards to the 4th leaf on the plant were used for infiltration. Regions to be infiltrated were selected and prepared by puncturing the leaf with a small needle (Figure 67 (2)) that facilitate the infiltration and reduce the risk of damaging leaves.

To do the infiltration, the diluted *Agrobacterium* samples were taken in a 1ml syringe (without needle). The syringe was then placed on the underside of the leaf lamina on the puncture made by the needle and the plunger of syringe was pushed slowly while supporting the same point on upper side of lamina with the fingertip (Figure 67 (4)). This process was continued until the liquid had diffused in a large part of the lamina by filling the mesophyll air spaces. Once the infiltration was done, the plants were put back in the growth chamber (under optimal growth conditions) for various time periods, in separate boxes to prevent the contamination of other plants.

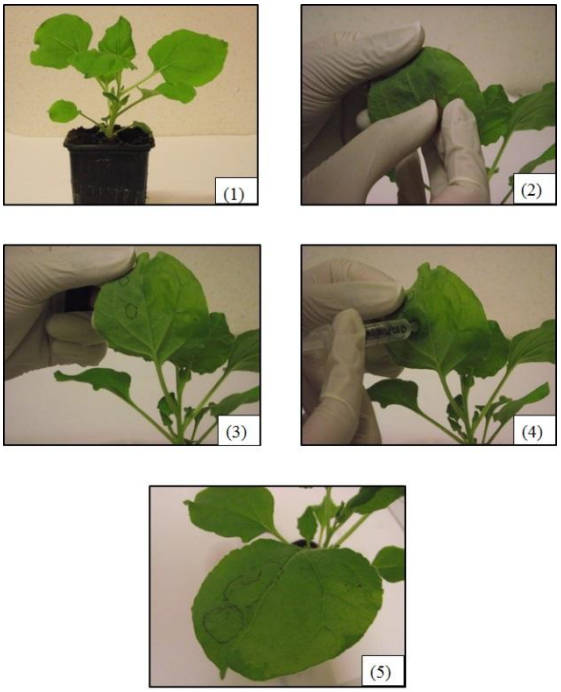


Figure 67. Different steps for the infiltration of *N. benthamiana* leaves
(1) Selection of leaves on healthy plant suitable for infiltration, (2) puncturing of the underside of leaf lamina, (3-4) infiltration of leaf with *Agrobacterium* inoculum using the marked punctured zones and (5) marking of infiltrated zones so they can be used to prepare sample for examining the expression of GFP 4 days post inoculation (IN).

Analysis was done from a 1cm diameter round shaped segment of the infiltrated zone of leaf. This piece of leaf was mounted on a microscope slide (in water) and covered with a coverslip. These samples were then examined for the expression of GFP using a binoculars light compound microscope or confocal microscope.

These preliminary experiments showed that 4 days was the most suitable time for the expression of GFP (data not shown).

2.2.3 Determination of the best concentration of agrobacteria to be used for infiltrating *N. benthamiana* leaves

In order to determine the optimal concentration of agrobacteria to get the best expression of GFP, tobacco leaves were infiltrated using the protocol described above with three different OD_{600nm}: 0.2, 0.4 and 0.6.

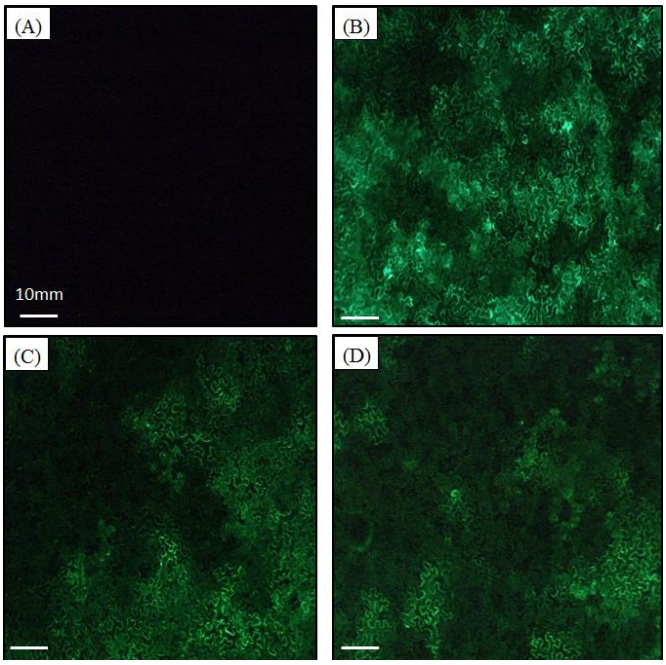


Figure 68. Examining the expression of GFP in samples infiltrated with *Agrobacterium* inoculums of various optical densities (OD)

(A) Leaf infiltration with sterilized water. Leaf infiltration with a recombinant *Agrobacterium* strain containing pBIN-m-gfp5-ER (B) OD_{600nm} of 0.2. (C) OD_{600nm} of 0.4 and (D) OD_{600nm} of 0.6. Analyses of the GFP expression were done by using a binoculars compound light microscope. While the scale bar utilized is equal to10 mm.

A negative control was prepared by infiltrating only sterilized water. Four days post inoculation; the samples were examined for their expression of GFP using a binoculars compound light microscope. Results showed that the GFP is expressed in all infiltrated leaves (Figure 68 B, C and D). However, these preliminary results clearly demonstrated that the expression level was the best with 0.2 OD_{600nm} (Figure 68 A).

2.2.4 Improvement of the expression of GFP

Numerous modifications and improvements have been suggested for the transient expression system of tobacco that could enhance the expression of proteins (Lindbo, 2007; Wydro *et al.,* 2006). One of the limiting factor for a good expression of the gene of interest is the establishment of a post-transcriptional gene silencing (PTGS) (Johansen *and* Carrington, 2001). To overcome this problem, Voinnet and collaborators propose to co-express, along with any protein of interest, a viral encoded suppressor of gene silencing, the p19 protein of Tomato Busy Stunt Virus (TBSV) (Voinnet *et al.,* 2003).

2.2.4.1 Co-expression of GFP with p19

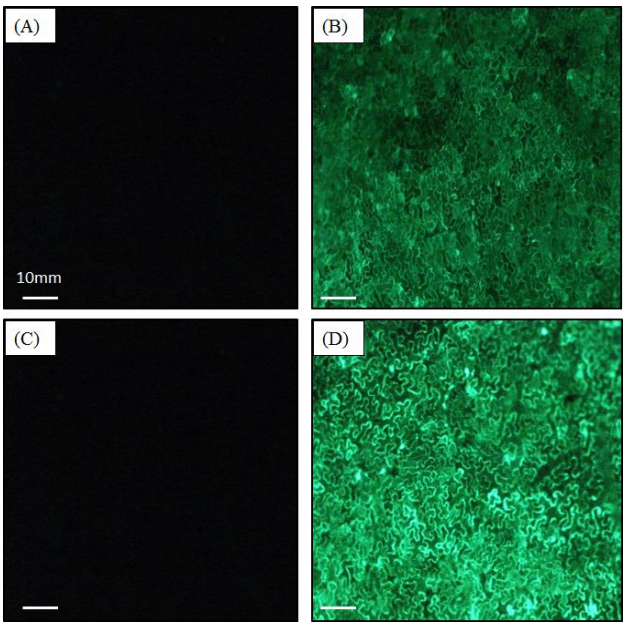


Figure 69. Co-infiltration of *N. benthamiana* leaves with *Agrobacterium* strains containing pBIN-mgfp5-ER and pBIN61-p19

(A) Leaf infiltration with sterilized water, (B) Leaf infiltration with recombinant *Agrobacterium* containing pBIN-mgfp5-ER, (C) Leaf infiltration of *Agrobacterium* containing pBIN61-P19 and (D) sample prepared by co infiltration of *Agrobacteria* containing pBIN-mgfp5-ER and *Agrobacterium* containing pBIN61-P19. Analyses of the GFP expression were done by using binoculars. While the scale bar utilized is equal to10 mm.

In order to increase the expression level of GFP, we considered the co-expression of p19. For this purpose we contacted Dr David Baulcombe who provided us an agrobacteria strain containing a recombinant plasmid having the gene, leading to the expression of the TBSV p19 protein.

The protocol used for the preparation of *Agrobacterium* inoculum containing the pBIN61-p19 plasmid was similar as described above. Two different inoculums were prepared. The first contained pBIN-m-gfp5-ER and the second pBIN61-p19. Both bacterial suspensions were mixed, using as a ratio, a final OD_{600nm} of 0.2 for pBIN-m-gfp5-ER and 0.4 for pBIN61-p19 and infiltrated into leaves. Control samples were prepared by infiltrating leaves only with *Agrobacteria* transformed with pBIN61-p19 (OD_{600nm} of 0.2) or with pBIN-m-gfp5-ER (OD_{600nm} of 0.4). Four days post inoculation, the infiltrated leaves were observed with a binoculars compound light microscope (Figure 69) or with a confocal microscope (Figure 70). The results obtained made evidence that the co-expression of p19 clearly increased the fluorescence observed in infiltrated leaves (Figure 69-D)(Figure 70-3 c) in comparison with a single infiltration of Agrobacteria transformed with the pBIN-m-gfp5-ER (Figure 69-B)(Figure 70-2 c).

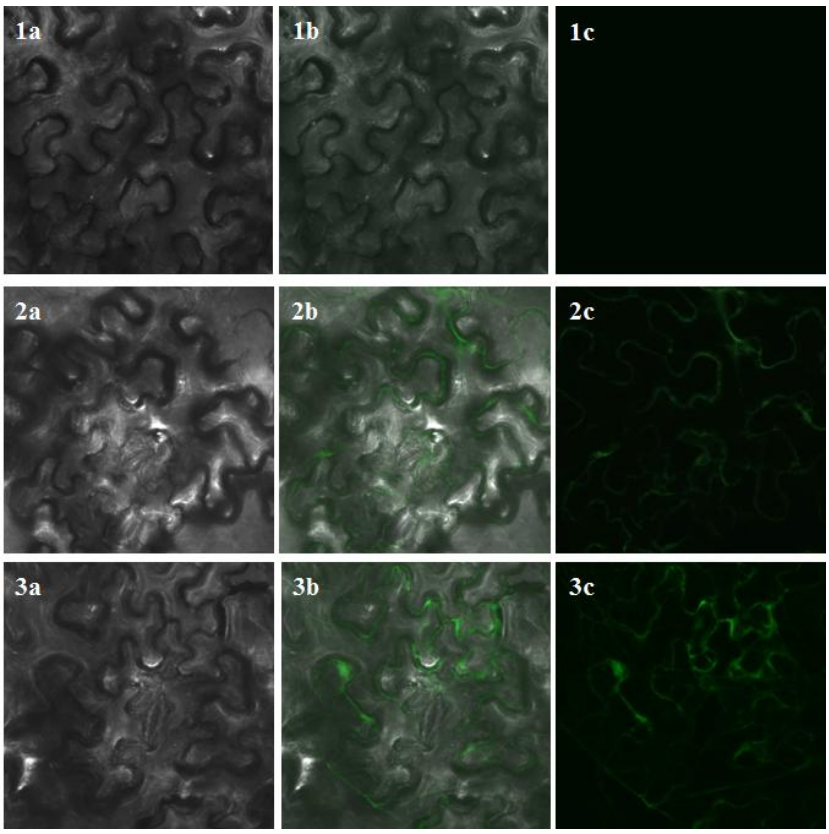


Figure 70. Analysis of GFP expression by confocal microscopy

(1a,1b and 1c) leaves infiltrated with a recombinant *Agrobacterium* inoculum carrying the pBIN61-p19 plasmid, (1b, 2b, and 2c) leaves infiltrated with *Agrobacterium* inoculum carrying the pBIN-mgfp5-ER plasmid and (1c, 2c, and 3c) leaves sample co-infiltrated with a mixture of *Agrobacterium* containing both pBIN61-p19 and pBIN-mgfp5-ER plasmids.

2.3 Development of a *N. benthamiana* -based transient expression system for the expression and the functional characterization of a membranous enzyme (CYP98A22)

These preliminary results led me to direct my research work to the functional characterization of a membranous enzyme. Before working with a Pt, we focused on a cytochrome P450 for which several information were available in literature. Recently a new candidate gene encoding for a *p*-Coumaroyl ester 3'-hydroxylase (C3'-H) was isolated from *R. graveolens* using the CODEHOP approach in our laboratory. This protein was submitted to the international committee of nomenclature of P450, which named this it as CYP98A22 (Hehn, 2007).

In order to characterize its function, we expressed the protein in 3 different expression systems. We tested the efficiency and the substrates specificity of the enzyme in the yeast expression system which was classically in use in the laboratory. Beside this first approach, during his PhD, S. Doerper constructed transgenic Ruta plants overexpressing this protein. We analyzed the metabolic profile of these transgenic plants. And finally I used the *N. benthamiana* transient expression system described above.

The results we obtained have been published in BMC Plant Biology as Karamat F, Olry A, Doerper S, Vialart G, Ullmann P., Werck-Reichhart D., Bourgaud F, Hehn A. (2012) CYP98A22, a phenolic ester hydroxylase specialized in the synthesis of chlorogenic acid, as a new tool for enhancing the furanocoumarin concentration in *Ruta graveolens BMC Plant Biology. Volume 12, p152*

Karamat et al. BMC Plant Biology 2012, 12:152
http://www.biomedcentral.com/1471-2229/12/152

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CYP98A22, a phenolic ester 3′-hydroxylase specialized in the synthesis of chlorogenic acid, as a new tool for enhancing the furanocoumarin concentration in *Ruta graveolens*

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Abstract

Background: Furanocoumarins are molecules with proven therapeutic properties and are produced in only a small number of medicinal plant species such as *Ruta graveolens*. *In vivo*, these molecules play a protective role against phytophageous insect attack. Furanocoumarins are members of the phenylpropanoids family, and their biosynthetic pathway is initiated from *p*-coumaroyl coA. The enzymes belonging to the CYP98A cytochrome P450 family have been widely described as being aromatic *meta*-hydroxylases of various substrates, such as *p*-coumaroyl ester derivatives, and are involved in the synthesis of coumarins such as scopoletin. In furanocoumarin-producing plants, these enzymes catalyze the step directly downstream of the junction with the furanocoumarin biosynthetic pathway and might indirectly impact their synthesis.

Results: In this work, we describe the cloning and functional characterization of the first CYP98A encoding gene isolated from *R. graveolens*. Using *Nicotiana benthamiana* as a heterologous expression system, we have demonstrated that this enzyme adds a 3-OH to *p*-coumaroyl ester derivatives but is more efficient to convert *p*-coumaroyl quinate into chlorogenic acid than to metabolize *p*-coumaroyl shikimate. Plants exposed to UV-B stress showed an enhanced expression level of the corresponding gene. The *R. graveolens cyp98a22* open reading frame and the orthologous *Arabidopsis thaliana cyp98a3* open reading frame were overexpressed in stable transgenic Ruta plants. Both plant series were analyzed for their production of scopoletin and furanocoumarin. A detailed analysis indicates that both genes enhance the production of furanocoumarins but that CYP98A22, unlike CYP98A3, doesn't affect the synthesis of scopoletin.

Conclusions: The overexpression of CYP98A22 positively impacts the concentration of furanocoumarins in *R. graveolens*. This gene is therefore a valuable tool to engineer plants with improved therapeutical values that might also be more resistant to phytophageous insects.

Background

The adaptation of plants to their environment and their survival under stressing conditions, e.g., pathogenic attacks, requires secondary metabolites, such as polyphenols. These molecules are broadly distributed in the plant kingdom with more than 8,000 phenolic structures currently known, ranging from simple molecules, such as phenolic acids, to highly polymerized substances, such as tannins [1]. The furanocoumarins constitute one of these classes of polyphenols. Despite their importance in plant life, their biosynthesis remains relatively poorly documented at the molecular level. These molecules exist mainly in 4 plant families: Rutaceae, Apiaceae, Fabaceae and Moraceae where they play diverse functions in plant adaptation to the environment as phytoalexins in defense systems [2] or in plant-insect interactions [3]. These molecules also display remarkable physicochemical properties, making them potentially toxics. They can interfere in enzymatic

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reactions through the inhibition of cytochrome P450 (P450) enzymatic activities [4,5]. They also interact with nucleic acids through the photocycloaddition of pyrimidic bases [6]. These characteristics make furanocoumarins attractive candidates for therapeutic use. For example, furanocoumarin derivatives have been used for decades as treatments for skin diseases, such as psoriasis and vitiligo [7]. In addition, there are other applications for furanocoumarins in various therapeutic fields, such as the symptomatic treatment of multiple sclerosis [8], photochemotherapy of T cell lymphoma [9], or chemotherapy of multidrug-resistant tumors [10]. Thus, it would be beneficial to increase the production of furanocoumarins in plants to match pharmaceutical demand. To reach this goal, it is essential to understand the biosynthetic pathway of furanocoumarins, and to determine how the production of these molecules could be enhanced.

Furanocoumarin-producing plants are not model plants for the scientific community. Therefore, little is known about their genomes and the genes that encode the enzymes involved in their biosynthetic pathways. Only four genes have been functionnaly characterized so far. Two P450s, psoralen synthase and angelicin synthase, have been described and are specifically involved in the synthesis of these molecules. These synthases catalyze the transformation of marmesin and colombianetin in psoralen and angelicin, respectively [11,12]. Another study reported the identification and the characterization of a *o*-methyl transferase in *Ammi majus* that catalyzes the transformation of bergaptol into bergapten [13]. Finally, a Fe^{2+}/α-ketoglutarate-dependent dioxygenase was recently identified in *Ruta graveolens*, which is able to metabolize *p*-coumaroyl coA leading to the synthesis of umbelliferone, the immediate precursor of furanocoumarins [14].

CYP98A (C3'H) are enzymes belonging to the cytochrome P450 family that catalyze the *meta*-hydroxylation of *p*-coumarate derivatives, an important step in the phenylpropanoid pathway (Figure 1). This *meta*-hydroxylation is not operating on free *p*-coumaric acid, but on *p*-coumarate esterified with shikimic, quinic, tyramine or phenyllactic acids [15]. In most of the studies,

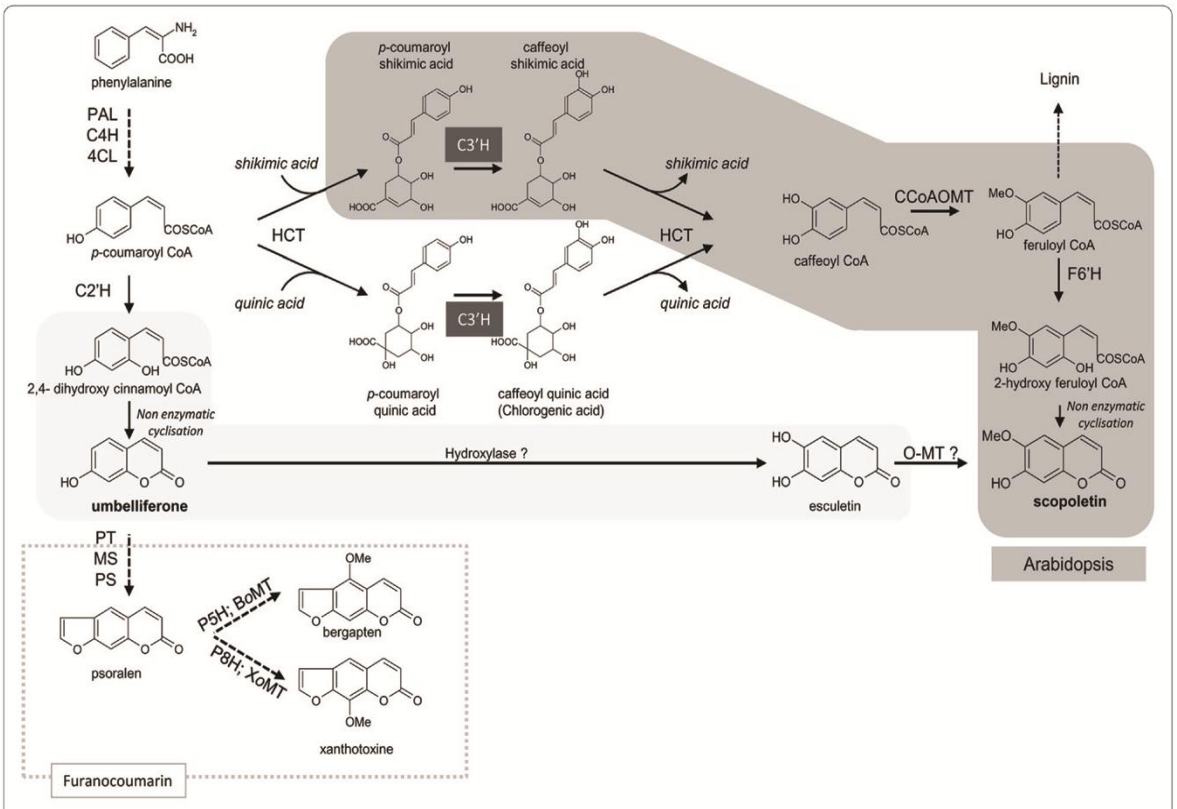

Figure 1 Simplified phenylpropanoid biosynthesis pathway (PAL: phenyl ammonia lyase, C4H: cinnamate 4-hydroxylase, 4CL: 4-coumaroyl CoA ligase, HCT: hydroxycinnamoyl transferase, C3'H: cinnamoyl ester 3'-hydroxylase), C2'H: *p*-coumaroyl CoA 2'-hydroxylase, PT: prenyltransferase, MS: marmesin synthase, PS: psoralen synthase, P5H: psoralen 5-hydroxylase, BoMT: bergaptol *o*-methyltransferase, P8H: psoralen 8-hydroxylase, XoMT: xanthotoxol *o*-methyltransferase, F6'H: feruloyl CoA hydroxylase, CCoAOMT: caffeoyl CoA O-methyltransferase Black dashed arrows: multistep reaction.

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these enzymes showed preferential affinity for the 5-*O*-shikimate and 5-*O*-D-quinate esters of *trans*-*p*-coumaric acid [16-18], however 4-coumaroyl-3', 4'-dihydroxyphenyl-lactate and *p*-coumaroyl tyramine are also precursor molecules that form rosmarinic acid and caffeoyltyramine, respectively [18-20]. Recently, another study demonstrated a complex process of evolution of the CYP98A family to acquire new functions implicated in the mechanism of pollen development in plants [21]. Other studies have assigned roles for these enzymes in the response of the plants to various molecules, such as salicylic acid derivatives and isonicotinic acids [22], and the expression level of the corresponding genes is highly increased after UV-C treatment [23]. Therefore, the CYP98A subfamily plays a major role in the phenylpropanoid pathway, and its action is not limited to the direct synthesis of caffeoyl ester derivatives but has a larger impact on plant metabolism.

In the present work, we describe the identification and *in vitro*/*in vivo* enzymatic characterization of a new gene encoding CYP98A22, a *p*-coumaroyl ester 3'-hydroxylase from *R. graveolens,* which constitutes the first CYP98 characterized from a furanocoumarin-producing plant. The biochemical characterization was assayed in 3 different systems: i) heterologous expression in yeast using the galactose-inducible strain pYeDP60/WAT11, ii) heterologous transient expression in the leaves of *N. benthamiana* together with the TBSV P19-silencing suppressor, and iii) stable expression in *R. graveolens* plants. Our results clearly show that CYP98A22 preferentially hydroxylates *p*-coumaroyl quinate to a greater extent than *p*-coumaroyl shikimate and, therefore seems to be involved in chlorogenic acid metabolism. Leaf exposure to UV-B light and further analyses of the expression level of *CYP98A22* revealed an increased *cyp98a22* mRNA accumulation. Finally, the analyses of the coumarin and furanocoumarin extracted from transgenic *R. graveolens* overexpressing *cyp98a22* or *cyp98a3* clearly showed an increase in the concentration of furanocoumarins in both cases whereas the accumulation scopoletin could only be observed for the CYP98A3 plants. Therefore, the work described here demonstrates that CYP98A22 can be used as a tool to modulate the furanocoumarins content in *R. graveolens.*

Results

Identification of a cyp98a orthologous gene in *R. graveolens*

To identify *cyp98a* genes present in furanocoumarin-producing plants, we used a PCR-based approach and the CODEHOP strategy described by Morant et al. [24]. First, we focused on the identification of genes belonging to the CYP98A subfamily. To achieve this, we performed an alignment of 9 sequences of CYP98A available in databases which allowed us to identify two peptidic consensus domains (EWAMAEL and PFGAGRR) and define degenerated primers. The PCR reactions were performed on genomic DNA extracted from young *R. graveolens* seedlings. A DNA fragment of 389 nucleotides corresponding to the internal sequence of a gene encoding a cytochrome P450 was amplified and subsequently cloned. A Genbank homology search using the Blast tool showed 89% identity at the amino acid level with a C3'H isolated from *Ocinum basilicum* (AAL99200.1). The corresponding full length open reading frame was isolated by using PCR conducted on a SMART cDNA library produced from RNA extracted from the leaves of young *R. graveolens* seedlings [14] as described in material and methods. The resulting sequence (GenBank JF799117, Additional file 1) was 1527 bp long and encoded for a 508 amino acid protein, which displayed 81% identity with the Arabidopsis CYP98A3.

In vitro biochemical characterization of CYP98A22

To characterize the activity of CYP98A22, the open reading frame was cloned into the pYeDP60 plasmid and expressed in the *Saccharomyces cerevisae* WAT11 strain as described by Larbat and collaborators [11]. The P450 functional expression was determined at 450 nm using a CO differential spectrum. Unfortunately, for the yeast expressing CYP98A22, no peak could be detected with microsomes prepared from cells grown in conventional or modified culture conditions (increase of the induction length from 12 to 24 h and a decrease in the induction temperature from 28 to 18°C). Despite undetectable levels of expression, incubations were performed using the substrates previously described for the enzymes of this P450 family. We were unable to detect any significant activity using HPLC-DAD measurements with *p*-coumaroyl quinate, *p*-coumaroyl shikimate, *p*-coumaroyl tyramine and *p*-tricoumaroyl spermidine as substrates. In parallel, microsomes prepared from yeast expressing CYP98A3 were perfectly functional and consistent with the results described elsewhere, which showed that the preparation of microsomes was efficient.

The absence of CYP98A22 activity in the yeast expression system led us to produce the protein in another heterologous expression system, *i.e. Nicotiana benthamiana*, using a modified protocol of Voinnet *et al.* [25]. In an initial attempt to monitor the expression of the corresponding proteins, the YFP protein was fused to the C terminus of the gene encoding CYP98A22. The recombinant coding sequence was cloned into the binary plasmid pBIN-GW [14] and introduced into a LBA4404 *Agrobacterium tumefaciens* strain [14]. Young leaves of *N. benthamiana* were infiltrated with *A. tumefaciens* LBA4404 which was transformed with either pBIN-CYP98A22-YFP or the empty pBIN plasmid. The coinfiltration was performed

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along with Agrobacterium transformed with pBIN61-P19. A strong fluorescence signal was observed at the endoplasmic reticulum level in the case of the coexpression of P19 and CYP98A22-YFP (Figure 2D), whereas no signal could be detected with the control empty plasmid (Figure 2C). These experiments demonstrated that *N. benthamiana* was an effective system for the expression of CYP98A22.

To assess the function of CYP98A22, a second set of experiments was conducted using a recombinant pBIN plasmid containing *cyp98a22* as a single sequence (without the presence of YFP). The agrobacteria transformed with this plasmid were coinfiltrated into *N. benthamiana* leaves along with recombinant *A. tumefaciens* containing pBIN61-P19. In parallel, as two independent controls, the inoculation of separate plants was performed with *A. tumefaciens* transformed with pBIN-CYP98A3 or with the pBIN-GW void plasmid. Four days post-inoculation, microsomes were prepared from the infiltrated *N. benthamiana* leaves as described in the material and methods section. These microsomes were tested for *in vitro* enzymatic activity with 100μM of various potential substrates, i.e., *p*-coumaroyl shikimate, *p*-coumaroyl quinate, *p-tri*coumaroyl spermidine or *p*-coumaroyl tyramine. For both proteins (CYP98A22 and CYP98A3), no metabolisation could be detected in the presence of *p-tri*coumaroyl spermidine or *p*-coumaroyl tyramine but *p*-coumaroyl shikimate and *p*-coumaroyl quinate, were transformed in two products that shared similar retention time and UV spectrum than caffeoyl shikimate and caffeoyl quinate standards, respectively. The identity of the caffeoyl products was confirmed using LC/MS analyses (see Additional files 2 and 3). No metabolization of any substrate was observed when the infiltration was performed with the void plasmid showing that the expression level of the endogenous CYP98A from *N. benthamiana* was not high enough to be taken into account. The V_i/K_m and apparent K_m kinetic parameters were determined for CYP98A22 by titration of the product formed using mass spectrometry. Results showed a higher apparent affinity (K_m 3.4 times lower) as well as a higher catalytic efficiency (V_i/K_m 9 times higher) for *p*-coumaroyl quinate than for *p*-coumaroyl shikimate (Table 1, Additional file 4).

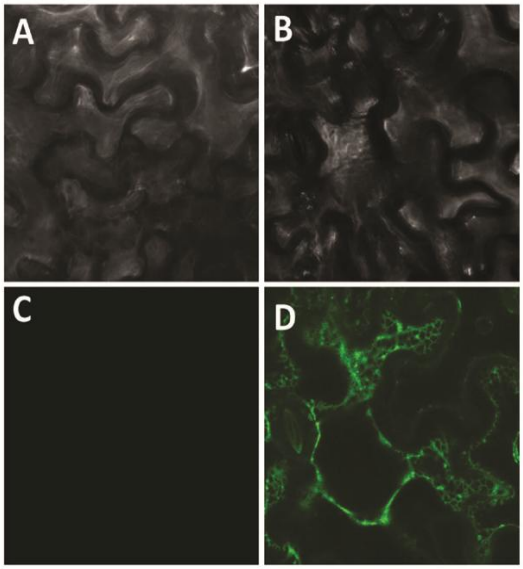

Figure 2 Confocal microscopy images showing subcellular CYP98A22-YFP localization in *Nicotiana benthamiana* leaves coinfiltrated with *Agrobacterium tumefaciens* containing pBIN61-P19 and A and C) the empty pBIN-GW plasmid or B and D) pBIN-CYP98A22-YFP. The images were recorded at 3 days post-infiltration. **A** and **B**: visible light, **C** and **D**: 488nm excitation.

In planta biochemical characterization of CYP98A22

A first series of phytochemical investigations (LC/MS) were achieved on wild type leaves of *N. benthamiana* and revealed that these tissues contain a significant pool of *p*-coumaroyl shikimate and quinate. Thus, we assumed that *in planta* functional characterization of CYP98A22 could be examined following transient expression in epidermal cells, because the potential substrates of this enzyme were present. This type of approach has 2 main advantages: the accuracy of the *in vitro* results can be demonstrated and the risk of the loss of enzymatic activity during the protein purification process is reduced. To this end, we infiltrated *N. benthamiana* leaves with recombinant *A. tumefaciens* containing pBIN35S::CYP98A22, pBIN35S::CYP98A3 or an empty pBIN-GW vector. All infiltrations were analyzed along with Agrobacterium containing pBIN61-P19. The LC/MS analyses of the extracts prepared four days post-infiltration showed an increase of chlorogenic acid and caffeoyl shikimate derivatives when any *cyp98a* was transiently overexpressed (Figure 3A and 3B). When comparing the accumulation of each molecule in relation with the overexpression of *cyp98a3* or *cyp98a*22, we observed that CYP98A22 is more efficient for the production of chlorogenic acid (Figure 3A) whereas CYP98A3 seems to be more dedicated to the synthesis of caffeoyl shikimate (Figure 3B) These results are consistent with those obtained for the *in vitro* characterization in the plant microsomes and shows that CYP98A22 is more specific for *p*-coumaroyl quinate (the precursor of chlorogenic acid).

Over-expression of cyp98a22 in *R. graveolens*

To establish a link between CYP98A22 and/or CYP98A3 and the synthesis of coumarins or furanocoumarins, transgenic *R. graveolens* were generated which overexpressed the corresponding gene. The same recombinant *A. tumefaciens* strains, used for transient expression in

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Table 1 Kinetic parameters of CYP98A22 determined with *p*-coumaroyl quinate and *p*-coumaroyl shikimate as substrate

Substrate	Product	V_{max} (pmol.min^{-1}.g^{-1} FW)	K_m (μM)	V_i / K_m (μM.pmol^{-1}.min^{-1}.g^{-1} FW)
p-coumaroyl quinate	caffeoyl quinate	0.49 +/- 0.16	7 +/- 5	0.07 +/- 0.02
p-coumaroyl shikimate	caffeoyl shikimate	0.19 +/- 0.03	24 +/- 7	0.008 +/- 0.002

Reactions were carried out at 28°C during 30 min in a KPi buffer 20Mm pH 7.0 as described in material and methods. Reactions were stopped with addition of 0.1% trifluoroacetic acid. Data obtained were fitted with the Michaelis-Menten equation.

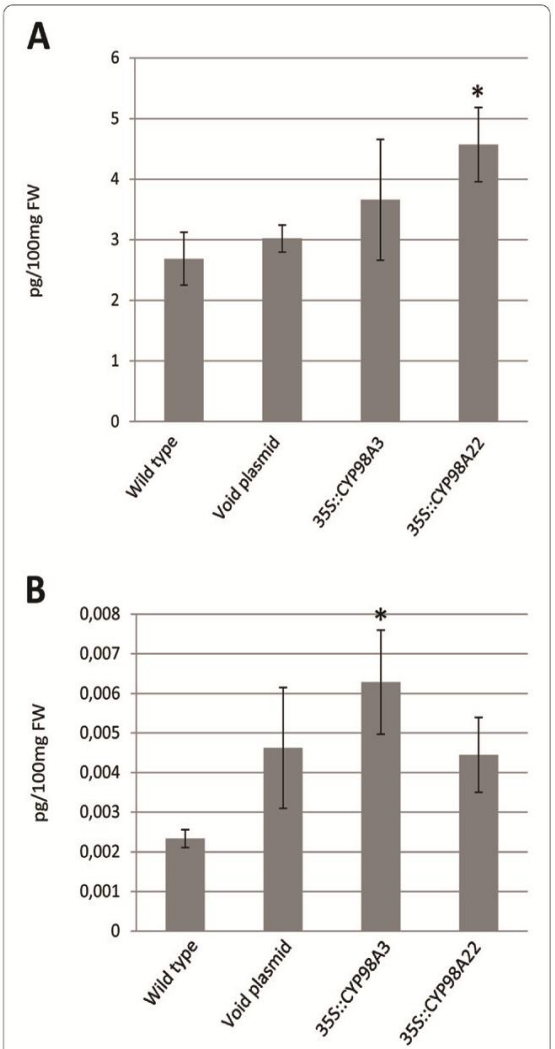

Figure 3 Chlorogenic acid (A) and caffeoyl shikimic (B) content in *N. benthamiana* infiltred leaves. A) Difference between 35S::98A22 and the wild type is statistically significant (*: Student test T statiscally significant, $p < 0.05$). Difference between 35S::98A3 and the wild type is statistically not significant but shows a tendency ($0.1 > p > 0.05$). **B**) Difference between 35S::98A3 and the wild type is statistically significant (*: Student test T statiscally significant, $p < 0.05$). Difference between 35S::98A22 and the wild type is statistically not significant ($p > 0.05$). Analyses have been done on 6 independently infiltrated leaves.

N. benthamiana, were used for stable transformation (either pBIN-CYP98A22 or CYP98A3). The transformation protocol was performed as described by Lièvre *et al.* [26]. Four 35S:CYP98A22 and nine 35S:CYP98A3 plants were generated and characterized at the molecular level using PCR directed against the *35S- cyp98a22* (the endogenous *cyp98a22* gene was not amplified) and the *cyp98a3* sequences. For each plant, the samples were divided into two halves. The first half was used to perform an RNA extraction to confirm the expression level of the transgenes using real-time PCR. The second half was used to extract phenolic molecules, which were further analyzed using HPLC. These analyses were compared with a set of extractions from wild-type plants. The content of scopoletin, umbelliferone and the three major furanocoumarins (psoralen, bergapten and xanthotoxin) present in *R. graveolens* was analyzed. For the 35S:CYP98A3 plants (Figure 4A), an increase in the concentration of scopoletin and umbelliferone could be observed (2-fold and 3.6-fold respectively; $p < 0.05$). For the 35S::CYP98A22 (Figure 4B) plants, no impact was observed for the production of scopoletin whereas an important decrease of umbelliferone (3.2 times, $p < 0.05$) could be highlighted. Moreover, in both cases, the results showed a statistically significant increase in the furanocoumarin content (3.2 times increase for the 35S:CYP98A3 plants ($p < 0.05$), and 2.9 times increase for the 35S:CYP98A22 plants ($p < 0.05$)) (Figure 4A and B).

Tissue-specific expression pattern of cyp98a22 in *R. graveolens*

Total RNA was extracted from various organs of 3-month-old *R. graveolens plants* (leaves, seeds, petals, pistils, roots, and stems) and was used to establish the relative expression pattern of *cyp98a22* using a real-time PCR approach. Two sets of primers were selected for the quantification of *cyp98a22* mRNA, and the two probes gave similar results. The results showed a broad expression pattern of the mRNA (Figure 5A). Indeed, mRNA was detected in all the tissues tested and was not restricted to lignified tissues. The results showed that the expression level is highest in the roots and petals and is also significantly high in the petioles and pistil. For the stems, leaves and seeds, the expression was much lower.

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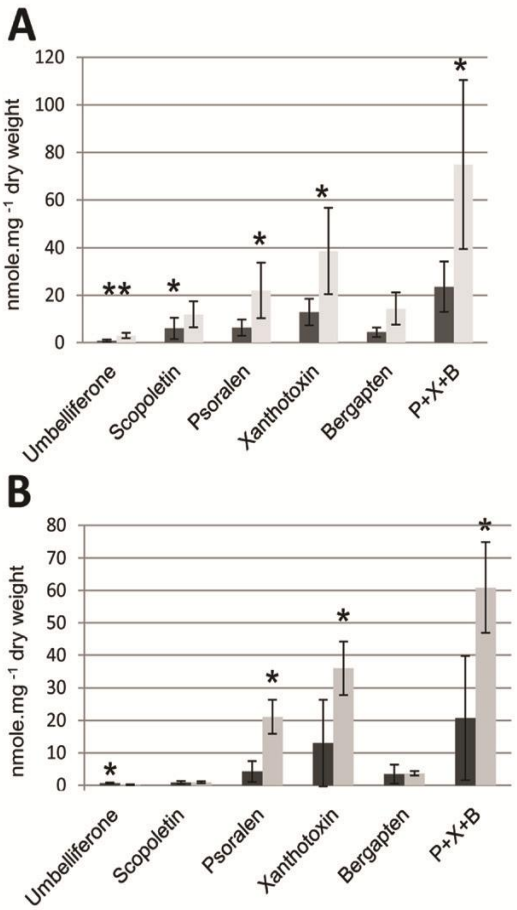

Figure 4 Concentration level of umbelliferone, scopoletin, psoralen, bergaptol and xanthotoxin in *35S: cyp98a3*, *35S: cyp98a22* transgenic *Ruta graveolens* and wild type plants. Black bars are corresponding to wild type plants, grey bars are corresponding to transgenic plants. The results corresponds to the mean value of analyses performed on **A**) 6 wild type plants and 9 35S::CYP98A3 transgenic plants and **B**) 4 wild type plants and 4 35S:: CYP98A22 transgenic plants. *: Student test T statiscally significant ($p < 0.05$), **: Student test T statiscally highly significant ($p < 0.01$).

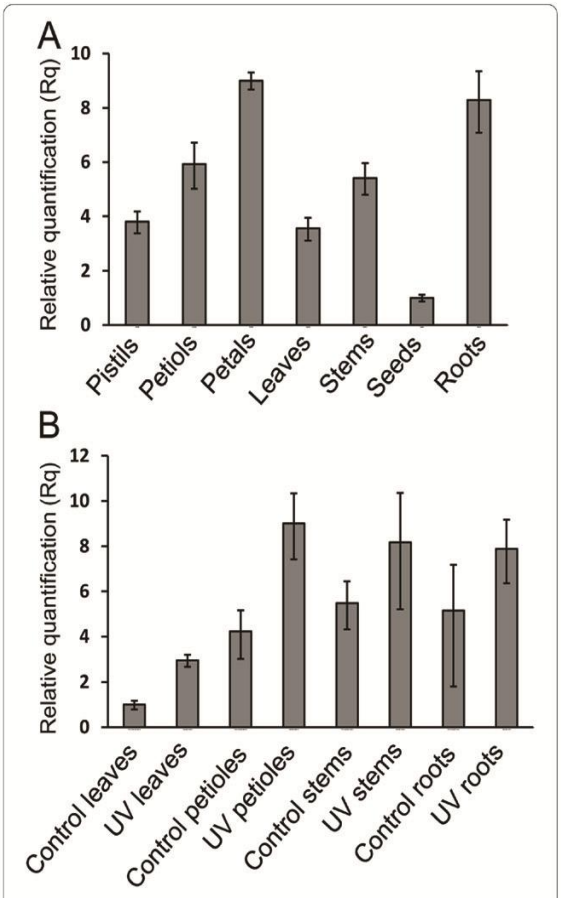

Figure 5 Expression level of *cyp98a22* in *Ruta graveolens*. Vertical bar chart showing the results of real-time RT-PCR experiments **A**) in different organs of the plants **B**) with or without UV-B stress conditions. UV indicates UV-B induced. Control indicates UV-B non induced. (The data $2^{-\Delta\Delta Ct}$ were deduced using the Ct method; the error bars represent the standard errors of 3 replicates).

UV-B treatment of *R. graveolens*

As described by different authors [14,27], the biosynthesis of furanocoumarins in *R. graveolens* plants can be enhanced through exposure to UV-B radiations. After a 24-hour exposure to UV-B light (312 nm), the leaves, stems, petioles and roots of *R. graveolens* plants were collected and both total RNA and furanocoumarins were extracted. As a control, the same extractions were performed on non-UV-treated plants. The LC-MS quantification was conducted for bergapten, xanthotoxin, psoralen, marmesin and umbelliferone in the treated and nontreated plants. The results showed a 3.5-fold increase of umbelliferone content in the UV-treated plants (1.4 _ 0.1 mg g^{-1} FW) compared to the control plants (0.4 _ 0.1 mg g^{-1} FW) as described in Vialart *et al.* [14]. Concerning other molecules, no statistically difference could be demonstrated. Real-time PCR experiments were performed on the same set of material as published in Vialart et al. The expression of *cyp98a22* was significantly stimulated in the UV-treated leaves (3-fold increase), whereas no statistically significant change was observed in the petioles, stems or roots (Figure 5B).

Discussion

The function of the P450 CYP98A gene subfamily has been extensively discussed in the literature for over 10 years. More than 60 different genes have been sequenced and referenced in the international P450 database (http://drnelson.uthsc.edu/cytochromep450.html) as belonging to this subfamily. Various functions have been attributed to these proteins. Their activity is classically described as

a C3'H catalyzing hydroxylation in the 3' position of *p*-coumaroyl shikimate and *p*-coumaroyl quinate [15,23,28]. However, other substrates have also been described, such as 4-*p*-coumaroyl-3' 4'-dihydroxyphenyllactate [19], *p*-coumaroyl tyramine [18] or spermidine esters [21]. Among the enzymes that metabolize *p*-coumaroyl shikimate, some were demonstrated to hydroxylate *p*-coumaroyl quinate at a lower rate and efficiency [15], while others were simply unable to metabolize both substrates [28]. In some cases, these enzymes have been described as having a larger effect on metabolism. For instance, Schoch and collaborators [17] indicated that the presence of C3'H is necessary for the biosynthesis of many divergent compounds such as, lignins, UV-absorbing pigments, antioxidants, flavors, fragrances and coumarins. Together, all these data show that this enzyme plays a central (Figure 1) and pivotal role in the phenylpropanoid pathway and that it has an indirect impact on the synthesis of several molecules. In this work, we hypothesized that an orthologous gene isolated from a furanocoumarin-producing plant might have impact the synthesis of some of these molecules and play an important role in the answer to various stresses.

Little is known concerning the genome of *R. graveolens*, a furanocoumarin-producing plant. Only a few accessions (less than 80) are available in the nucleic acid or protein databases, and no information concerning a putative gene corresponding to a *cyp98a* orthologous gene was found. However, each P450 subfamily shares several conserved consensus domains which makes it possible to use sequence-based PCR approaches for cloning and identifying plant cytochrome P450 genes. Accordingly, we used a PCR approach with degenerated primers [24] to isolate a gene from the *R. graveolens* genome that potentially encodes a C3'H. The translational product of the resulting open reading frame (ORF), *cyp98a22* (Genbank JF799117), was compared with 25 peptidic sequences available in the databases. The results showed that the protein is clustering with enzymes described as classical C3'H involved in the synthesis of lignin precursor, among which the first C3'H CYP98A3 ever functionally described and not with divergent CYP98 as the *Arabidopsis thaliana* CYP98A8 and CYP98A9 which are involved in the synthesis of N^{10}-monosinapoyl-N^{1},N^{5}-dihydroxyferuloyl spermidine, an important constituent of pollen [21]. This first set of data seemed to indicate that this enzyme might be a conventional C3'H that potentially metabolized *p*-coumaroyl quinate and *p*-coumaroyl shikimate.

The biochemical characterization of plant cytochrome P450s using a yeast expression system [29] is often difficult due to the low abundance and instability of these membrane-bound proteins. Thus, several different heterologous systems for the expression of the P450s, including yeast, *E. coli* and baculovirus, have been described in the literature. To perform the enzymatic characterization of CYP98A22, we selected the yeast expression system described by Pompon and collaborators [29] which is extensively used for the heterologous expression and characterization of enzymatic activities of proteins belonging to the P450 family. This system is efficient for the production of certain enzymes [30] but was limited for the production of other plant enzymes. Various improvements have been proposed in the literature, including changes in the media and culture conditions [31], the modification or exchange of nucleotide sequences (partial or complete recoding of genes) [32,33] or the replacement of the membrane anchor [32] [12]. A carbon monoxide differential spectrum is generally performed to assess the expression level of P450 in such a system. To achieve the expression of *cyp98a22*, several of these strategies were employed. Although no peak at 450 nm could be observed with a CO spectrum for any strategy tested, we used the prepared microsomes to conduct metabolisation tests using the following substrates: *p*-coumaroyl quinate, *p*-coumaroyl shikimate, *p*-coumaroyl tyramine, and *p*-tricoumaroyl spermidine. No transformation of any substrate could be detected, whereas the metabolisation of *p*-coumaroyl quinate and shikimate was observed in the presence of CYP98A3 yeast microsomes. This lack of expression could not been explained so far but led us to move to another protein heterologous expression system.

To determine the activity of CYP98A22, we used *N. benthamiana* as an alternative heterologous expression system. The transient transformation of the plant petals with cytochrome P450s using particle bombardment as a technique for transferring a T-DNA has been described in the literature [34,35]. The use of this system has also been reported for the functional characterization of a P450 belonging to the CYP71A subfamily involved in the biosynthetic pathway of alkaloid phytoalexins [36].

To test the efficiency of this plant heterologous expression system to allow the expression of CYP98A22, we first constructed a fusion protein comprising CYP98A22 and the yellow fluorescent protein (YFP). This recombinant ORF was placed under the control of the CaMV 35S promoter and agroinfiltrated in the epidermal cells of *N. benthamiana*. Because no fluorescence could be observed, a second set of infiltrations was performed in the presence of a plasmid allowing the expression of the Tomato Bushy Stunt Virus silencing suppressor protein P19 as described by Voinnet *et al.* [25]. In this case, the analyses of the infiltrated leaves revealed a strong fluorescence signal localized in the endoplasmic reticulum. This result is consistent with the work of Ro and collaborators who demonstrated that the cinnamate 4-hydroxylase (C4H), a cytochrome P450 enzyme of the phenylpropanoid pathway, is bound in the ER membrane [37]. Our results show that this plant system is efficient for the functional expression of CYP98A22 in the presence of the gene silencing suppressor protein.

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To realize the functional characterization of the enzyme, a new set of plasmids were constructed containing the ORF of *cyp98aA22* and *cyp98a3* under the control of the 35S promoter in the pBIN vector prior to agroinfiltration to *N. benthamiana* in the presence of TBSV P19. As a first attempt, we prepared microsomes from the leaves at 4 days post-infiltration, and the enzymatic tests showed that both *p*-coumaroyl quinate and *p*-coumaroyl shikimate were hydroxylated to their caffeic counterparts by CYP98A22 and CYP98A3. However, CYP98A22 showed a much higher efficiency with *p*-coumaroyl quinate as a substrate than with *p*-coumaroyl shikimate, whereas CYP98A3 seems to preferentially metabolize *p*-coumaroyl shikimate. This first experiment revealed that the *N. benthamiana* plant system is a more efficient tool for the expression and the *in vitro* characterization of CYP98A22 than the yeast system. The use of this plant heterologous system for the expression of CYP98A22 also provides a rapid method to test the *in vivo* activity of the concerned protein. In addition to a pool of *p*-coumaroyl esters of quinate and shikimate, the dominant phenylpropanoids of tobacco is chlorogenic acid [38]. The presence of these molecules in plants provides an interesting tool to determine the activity of the overexpressed CYP98A enzymes and to highlight the natural function of these enzymes. The analysis of the metabolic profile and the quantification of chlorogenic and caffeoyl shikimic acid were performed on leaves infiltrated with recombinant agrobacteria containing pBIN-CYP98A22 and on non-infiltrated leaves. The results showed a statistically significant increase of the chlorogenic acid content.

To complete this study, we examined the tissue-specific expression pattern using real-time PCR. Indeed, as discussed above, most of the members of the CYP98A P450 subfamily catalyze efficient 3'-hydroxylation of *p*-coumaroyl shikimate and much slower synthesis of caffeoyl quinate [15,39]. Because the hydroxylation of *p*-coumaroyl-shikimate is a key step in the formation of the monolignol lignin precursor, this observed activity, along with the relatively high level of expression of most of the described C3'H in stem and vascular bundles, indicates that these genes play an important role in lignification [15,16,39]. The results we obtained in our investigation concerning the expression pattern of *cyp98a22* were somewhat different. Unlike other C3'H, *cyp98a22* displays a broad expression pattern. Strong expression was observed in the petals and roots, whereas a relatively weak expression was observed in the petioles, stems and pistils.

Together, these results show that even if CYP98A22 is phylogenetically related to CYP98A3, it might play a more complex or, at least, a different role *in planta*. Indeed, caffeoyl shikimate plays an important role in lignification, while the caffeoyl quinate derivatives, such as chlorogenic acid, are described as growth regulators, disease resistance factors, antioxidants and compounds affecting the organoleptic quality of fruits [40,41]. The ability of CYP98A22 to be more dedicated to the synthesis of chlorogenic acid constitutes an evidence of its involvement in the stress response.

To explore this hypothesis, plantlets were exposed to UV-B light for 24 hours as described by Vialart *et al.* [14]. Altough the analysis of the furanocoumarin content showed no increase in comparison with the non-treated plants, the UV-B elicitation stimulated the synthesis of umbelliferone, which is a precursor of this pathway. Moreover, these analyses demonstrated that the expression level of *cyp98a22* gene was strongly increased (3-fold) in UV-treated leaves as compared with non-treated leaves. Recently, the elicitation experiments were utilized in *Cynara cardunculus* to induce the expression of *cyp98a49* using UV-C light [23]. Although this does not provide irrefutable evidence, these elements are consistent with the hypothesis that CYP98A22 is involved in responses against several stresses in *R. graveolens*. These preliminary results must now be explored in depth.

Scopoletin is an important defense compound ubiquitously found in higher plants for which two biosynthetic routes have been described (Figure 1). A first route, operating through feruloyl-CoA was demonstrated by Kai and collaborators [42] in Arabidopsis, which implies the conversion of *p*-coumaroyl-shikimic acid into caffeoyl-shikimic acid by CYP98A3, upstream to feruloyl-CoA [43]. Scopoletin content was dramatically decreased in knock-out *cyp98a3* mutants, confirming the role of the Arabidopsis C3'H in the synthesis of this compound. An alternate route to scopoletin was proposed in plants such as *Daphne mezereum* L [43] and *Agathosma puberula* [44] where umbelliferone and esculetin are the direct precursors of scopoletin. It is unestablished in *R. graveolens* if the two pathways are coexisting or if only one of the two is prevalently operating. To assess the involvement of CYP98A enzymes in the synthesis of scopoletin in *R. graveolens*, transgenic Ruta plants overexpressing either *cyp98a3* or *cyp98a22* where generated. The analysis done on the *in vitro* plants highlighted a statistically significant increase of the scopoletin content in 35S::CYP98A3 plants whereas plants overexpressing *cyp98a22* did not show any modification in scopoletin content when compared to control plants. The results obtained on CYP98A3 are consistent with the findings of Kay *et al.* [45] who described the key-role of C3'H in the synthesis of scopoletin in Arabidopsis. In parrallel, the results obtained on CYP98A22 fit well with our *in vitro* investigations obtained with *N. benthamiana*, which demonstrate the preferential role of this enzyme in the synthesis of chlorogenic acid *vs* caffeoyl-shikimate, the precursor of scopoletin in the first

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biosynthetic route described above. The analyses were extended to other coumarin which led us to show that the overexpression of both CYP98A impact significantly the concentration of furanocoumarins in transgenic plants. This constitute an unexpected result because C3'H is not directly involved into the synthesis of furanocoumarins (Figure 1). The exact reason for this increase in furanocoumarin content remains unknowned and will require further metabolomic investigations to be elucidated.

Conclusions

The results described in this work showed that members of the CYP98A family, even if sharing high sequence homologies, don't have the same function in all the plants. Indeed, CYP98A22 seems to be more dedicated to the synthesis of chlorogenic acid in *R. graveolens*. As mentioned previously, furanocoumarins are molecules of pharmaceutical interest for humans. *In planta*, these molecules have been described in plant defense against phytophageous insect attacks. The selection of plants synthesizing higher amounts of these molecules would be useful to facilitate their production. According to our results, CYP98A22 is a good candidate for this purpose: it has an impact on the synthesis of furanocoumarins in *R. graveolens*. These properties can be used in a metabolic engineering strategy to enhance the production of molecules of therapeutic interest. This work also confirms that the study of metabolic networks opens a wide field of investigation for the production of plants of interest for pharmaceutical applications.

Methods

cDNA library

The total RNA was extracted from the *Ruta graveolens* plantlets using the Plant Rneasy kit (Qiagen). The cDNA library was prepared as described by Vialart et al. [14].

Amplification and cloning of CYP98A from *R. graveolens*

Design of CODEHOP primers to amplify CYP98A

Clustal W was used to perform a peptidic alignment between *cyp98a*1 (AF029856), *cyp98a*2 (AF022458), *cyp98a*3 (AC002409), *cyp98a*6 (AB017418), *cyp98a*8 (AC011765), *cyp98a*9 (AC011765), *cyp98a*13 (AAL99200), *cyp98a*19 (AY064170), and *cyp98a*20 (AY065995). The specific primers were directed against the EWAMAEL CYP98A sequence and the large PFGAGRR P450 consensus domain to generate 98-11DIR: 5'-GAGTGGGC TATGGCTGARHTNRT-3' and 98-1R: 5'-CCTCCTGCC NGCNCCRAANGG-3'. The 5' ends of the primers were not degenerate, and the codon usage was based on *Citrus sinensis,* which also belongs to the *Rutaceae* family. The 3' end was completely degenerate. The PCR conditions were set as described in a previous study [24], and the starting hybridization temperature was 70°C and decreased by 1°C at each step.

5' and 3' end amplification

The CDS III and SMART IV primers (Smart cDNA Library Construction Kit, Clontech) designed against λTriplEx were used in association with primers designed to the internal sequence of the partial *cyp98a22* gene amplified using the CODEHOP approach. *cyp98a22* was amplified using PCR (98FLREV: 5'-GGGGTACCTTACA AATCAGCAGCAACACGTTT-3', 98FLDIR: 5'-GGGG TACCATGGGTCTCCCACTCATCCC-3'), and a *Kpn*I restriction site was inserted at the 5' and 3' ends. The gene encoding *cyp98a*3 was amplified from the pYeDP69-CYP98A3 plasmid [15], using the primers 98A3DIRBam: 5'-GGGATCCATGTCGTGGTTTCTAAT AGCG-3' and 98A3REVEco: 5'-GGAATTCTTACAT ATCGTAAGGCACGCG. The PCR was conducted using the following conditions: 94°C for 5 min, 30 cycles of 94°C for 15 sec, 50°C for 15 sec, and 72°C for 90 sec followed by a final extension step at 72°C for 10 min. The PCR fragments were introduced into the pCR8-GW-TOPO-TA vector according to the manufacturer's recommendations.

Construction of pYeDP60 recombinant plasmids

The recombinant pCR8 plasmids containing the *cyp98a22* sequence were inserted into the pYeDP60 yeast expression plasmid [29]. The empty pYeDP60 and pCR8 plasmids containing *cyp98a22* were digested with *Kpn* I and were purified following agarose gel electrophoresis using the MinElute Gel Extraction Kit (Qiagen). The *cyp98a22* inserts and the pYeDP60 vector were mixed and ligated using the T4 DNA ligase from Invitrogen. The resulting clones obtained in *E. coli* cells were examined using colony PCR to determine the insertion and orientation of the coding sequence.

Construction of the YFP fusion proteins and cloning of the cyp98a22 coding sequences in plant vectors

The design of the *cyp98a22* C-terminal fusion in frame with a fluorescent reporter was a two-step procedure. The first set of the PCR reactions was designed to yield *cyp98a22* with a 18-nucleotide 3' end overlapping the first 18 nucleotides of *YFP* (forward primer 98FLDIR, reverse primer 98A22YFPREV: 5'-CCTTGCTCACCATGTGGCGACCGGTACCCCCC AAATCAGCAGCAAC-3') and the *YFP* sequence with an additional sequence at the 5' end corresponding to the last 18 nucleotides from *cyp98a22* (forward primer 98A22YFPDIR: 5'- GTTGCTGCTGATTTGGG GGGTACCGGTCGCCACATGGTGAGCAAGG-3' and reverse primer YFPREV: 5'-TTACTTGTACAGCTCGT CCATGCC-3'). In a second set of PCR reactions, the

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products from the previous PCR were mixed, and the amplification was performed using the CYP98A22 direct primer and the YFP reverse primer to generate the chimeric *cyp98a22-YFP* fusion gene. The recombinant sequence was inserted into the pCR8 vector (Invitrogen) according to the manufacturer's recommendations.

Subcloning in the pBIN-GW plasmid

The genes inserted in the pCR8 plasmid were transferred to the pBIN-GW plasmid using LR recombination as recommended by Invitrogen. The pBIN recombinant plasmids were introduced into the LBA4404 *A. tumefaciens* strain ([46]), and the resulting clones were used for the transient expression of *N. benthamiana* leaves or the stable transformation of *R. graveolens.*

Yeast expression

The *Saccharomyces cerevisiae* WAT11 strain were transformed as described by Pompon [29] using the different pYeDP60 recombinant plasmids. The expression of the corresponding proteins and preparation of microsomes were performed as described by Larbat et al. [11].

Transient expression in *N. benthamiana*

For *N. benthamiana* transient protein expression, 4- to 6-week-old plants were transiently transfected with *A. tumefaciens* containing pBIN 35S:98A3, pBIN 35S:98A22, pBIN 35S:98A22-YFP and pBIN61-P19 in accordance with a previously published protocol [47]. A volume of 5 mL of overnight *A. tumefaciens* culture was pelleted, washed three times with water and resuspended in water. The leaves were coinfiltrated with Agrobacterium (at a final OD_{600} of 0.2) containing one of the pBIN-98 constructs and *Agrobacterium* (at a final OD_{600} of 0.4) containing pBIN61-P19. The infiltration was performed in the lower epidermis using a 1-ml syringe (without a needle) and gentle pressure.

Fluorescence analysis in infiltrated *N. benthamiana* leaves

Fluorescence (in the form of a fusion protein) was observed 96 hours postinoculation. For the observation of the fluorescent proteins using confocal microscopy, the leaf disks were excised, mounted between slides and coverslips, and vacuum infiltrated using water. The leaf discs were observed using a laser scanning confocal microscope (Fluoview, FV10i, Olympus). The excitation and emission wavelengths were 488 nm and 505 to 545 nm for YFP, respectively. The images presented are single focal sections. The image processing was performed using ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij/) and Photoshop 6.0 (final image assembly; Adobe Systems, San Jose, CA).

Plant microsomes preparation and enzymatic activity test

Approximately 4 g of fresh leaves were harvested at 96 hours postinoculation, frozen in liquid nitrogen and ground in a mortar. The cell debris was resuspended in 0.1 M of KPi buffer (pH 7.0) containing a cocktail of protease inhibitors (Roche) and centrifuged for 30 min at 10,000 g. The supernatant was filtered through miracloth and centrifuged for 1 h at 100,000 g using a SORVALL WX80 ultracentrifuge. The resulting supernatant was carefully removed, and the pellet was resuspended in 200 µl of 0.1 M KPi buffer (pH 7.0). All the microsomal preparations were prepared at 4°C.

The enzymatic syntheses of *p*-coumaroyl quinate and *p*-coumaroyl shikimate were performed as described by Morant *et al.* [18]. The enzymatic assays were conducted as described by Larbat and collaborators [11]. Microsomes overexpressing CYP98A22 were incubated with 0.2 mM NADPH in 20 mM sodium phosphate buffer (pH 7.0) containing 100 µM *p*-coumaroyl shikimate or *p*-coumaroyl quinate and *p*-coumaroyl tyramine or tricoumaroylspermidine. The reaction was performed for 30 min at 28°C and 0.1 M HCl were added to terminate the reaction. The range of substrate used for the determination of kinetic parameters was 4µM, 5, 7.5, 10, 20, 30, 50µM for *p*-coumaroyl shikimate and 1µM, 2, 4, 5, 7.5, 10, 20, 30µM for *p*-coumaroyl quinate. The reaction mix was centrifuged for 30 min at 10,000 g, and 50 µl of the supernatant was analyzed using reverse phase HPLC (LiChrospher 100 RP-18 column) coupled with mass spectrometry. The HPLC program was set as follows: buffer A (H_2O, 0.1% formic acid) and buffer B (100% MeOH, 0.1% formic acid); 5 min with 10% isocratic in buffer B followed by a linear gradient from 10 to 80% buffer B for 15 min and final a washing step. The absorbances of the hydroxylated products were measured at 330 nm with a diode array detector. The identity of the metabolized products caffeoyl shikimate and quinate were analyzed using an electrospray mass spectrometer in the negative ion mode as described by Matsuno et al. [21]. Apparent K_m values were measured by fitting the data obtained for both substrates to the Michaelis-Menten equation using the software SigmaPLOT 12.0 (Systat Software Inc).

Chlorogenic acids and caffeoyl shikimic derivatives extracted from *N. benthamiana* and quantification

Four days postinfiltration, 6 independently infiltrated leaves were collected and crushed separately in liquid nitrogen using a mortar. A total of 0.1 g of each preparation was collected, and the quinic derivative molecules were extracted in 1 ml of 80% MeOH, vortexed vigorously for 1 min and centrifuged at 13,000 g for 20 min. The resulting supernatant was removed and evaporated to dryness. The solid extract was then resuspended in 100% MeOH prior

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to HPLC analysis. The chlorogenic acids were followed and identified as described by Hoffmann et al. [48]. The quantification of the esters was performed in comparison with taxifolin, an external standard, which was added at the beginning of the extraction procedure.

LC/MS analyses of the caffeoyl derivatives

The HPLC-MS system consisted of a binary solvent delivery pump and a linear ion trap mass spectrometer (LTQ-MS, Thermo Scientific, San Jose, CA, USA). The LTQ was equipped with an atmospheric pressure ionization interface operating in the ESI negative ion mode. The data were processed using Xcalibur software (version 2.1). The operational parameters of the mass spectrometer are given below. The spray voltage was 4.5 kV and the temperature of the heated capillary was set at 300°C. The flow rates of sheath, auxiliary and sweep gases were set (in arbitrary units min-1) to 40, 10, and 10, respectively. The capillary voltage was set at -36 V, the split lens was set at 44 V and the front lens was set at 3.25 V. All the parameters were optimized by infusing a standard solution of isopimpinelline (0.1 g L^{-1}) in mobile phase [water + acetic acid 0.1% / methanol + acetic acid 0.1% (90/10)] at a flow rate of 5 μl min^{-1}. The caffeoylshikimic acid isomers were monitored through MS^2 (335)-specific and full (100-400 m/z) scans. Standard solution of caffeoyl quinate and caffeoyl shikimate were used for quantification.

Plant Material and UV-B treatments

The *R. graveolens* seeds were germinated on soil. The experimental setup under glasshouse conditions was similar to that described by Vialart and collaborators [14].

Real-Time PCR

To examine tissue-specific gene expression, the total mRNA was extracted from 100 mg of fresh tissues from the leaves, roots, stems, petioles, petals, seeds, and flowers of *R. graveolens* plants using the RNeasy plant-mini-kit (Qiagen). A high-capacity RNA-to-cDNA master mix (Applied Biosystems) was used for the first-strand cDNA synthesis. A total of 1 μg of RNA from each organ was reverse-transcribed using oligo$(dT)_{17}$ as a primer. The obtained cDNA samples were diluted 1:100 before use. Real-time PCR was performed using the following procedure. Specific 16S ribosomal RNA primers from *R. graveolens* were used as an internal control for data normalization (Rg16SDIR: 5'-CATTCGGCCCGTCTTGAA-3' forward and Rg16SRev: 5'-CCGTTGACTCGCACACATGT-3'). Two pairs of primers were designed for the quantification of *cyp98a22*. The following sequences were used for the first set of primers: 98RTPCRDIR1: 5'-CACGGAGTTG GCGAAGGA-3', 98RTPCRREV1 5'-GGTGCCTGTCAGC CAATTG-3'. The following sequences were used for the second set of primers: 98RTPCRDIR2: 5'-ACAGCAGA GTGGGCAATGG-3', 98RTPCRREV2 5'-CCTGTGCTT TGTGTTGCACTCTA-3'. The three sets of probes were designed using the Primer Express software version 3.0 (Applied Biosystems), and their efficiency was determined using the standard linear curve method. The relative transcript level of the same gene in various organs was expressed as the log2 ratio of the 16S normalized transcript levels in a given organ in comparison with the average expression of the gene in all the organs tested.

Plant stable transformation

The transformation of *R. graveolens* was performed as described previously [26] The regenerated transgenic plants were characterized at the molecular level using a PCR approach. The genomic DNA was extracted using the Plant DNeasy Kit (Qiagen). The PCR amplification was performed using specific primers. The forward primer was designed against the 35S promoter. This primer was the same for all the tested plants (35SDIR100: 5'-CACTATCCTTCGCAAGACCCTTCCTCTATATAAGG AA-3'). To determine the presence of transgenic *cyp98a22* and *cyp98a3*, we used the same reverse primers that were used to perform the cloning steps described above.

Phylogenetic analysis

25 sequences of previously described CYP98A gene were aligned using the Clustal X software. An additional sequence of a divergent CYP was added to the alignment as an outgroup sequence. A molecular phylogenetic tree was generated from this alignment using the neighbor-joining method. The tree was visualized using the TreeView software.

Additional files

Additional file 1: A) Alignment done on 9 CYP98A (CYP98A1 (AF029856) ; CYP98A2 (AF022458); CYP98A3 (AC002409); CYP98A6 (AB017418); CYP98A8 (AC011765); CYP98A9(AC011765); CYP98A13 (AAL99200); CYP98A19 (AY064170); CYP98A20 (AY065995). Only the C-terminal ends of the proteins are represented. The conserved sequences used to design the CODEHOP primers are highlighted in yellow. B) Nucleotidic and peptidic sequence of CYP98A22 (Genbank JF799117).

Additional file 2: Identification of the metabolization product of p-coumaroyl quinate. A) MS analysis of the metabolization product: B) MS analysis of caffeoyl quinate; C) MS^2 analysis of the metabolization product: D) MS^2 analysis of caffeoyl quinate.

Additional file 3: Identification of the metabolization product of *p*-coumaroyl shikimate. A) MS analysis of the metabolization product: B) MS analysis of caffeoyl shikimate; C) MS^2 analysis of the metabolization product: D) MS^2 analysis of caffeoyl shikimate.

Additional file 4: Measurement of apparent K_m of CYP98A22. Reaction rates *versus* different concentrations of *p*-coumaroyl shikimate (4μM-50μM) (A) and of *p*-coumaroyl quinate(1μM-30 μM) (B). Apparent

K_m values were measured by fitting the data to the Michaelis-Menten equation using the software SigmaPLOT 12.0.

Competing interest
There are no competing interests either financial or not financial concerning the work described in the present manuscript.

Authors' contributions
KF performed the yeast and plant transient expression and the enzymatic characterization. OA conducted the metabolomic and transcriptomic analysis and the YFP fusion construction. DS constructed the transgenic *Ruta graveolens* and conducted the corresponding analysis. VG treated the *R. graveolens* with UV and performed the corresponding analysis. PU prepared and synthesized the cinnamoyl ester derivatives. DW participated to the design of the work with the yeast expression system (WAT11-CYP98A3) HA conducted the plylogenetic analysis and the isolation and cloning of the gene. HA and BF conceived the study, participated in its design. AH and BF coordinated and finalized the written manuscript. All authors read and approved the final manuscript.

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2.4 Development of a *N. benthamiana* heterologous expression system in order to do the functional characterization of aromatic prenyltransferases

As the first step of the work consisting in developing an expression system for membrane proteins was completed, I could then start to develop a system for the study of Pt/Pts involved in the synthesis of furanocoumarins. To achieve this goal we had to work on two key elements. The first element was the availability of substrate for the enzyme that we wanted to study. In our case, the substrate of the Pt(s) should be umb, but until now no evidences for the presence of these potential substrates in tobacco were available in literature, may be, because they are not detectable. The second point concerns the expression of respective proteins of putative Pt. The tobacco system had never been used for the expression of Pts. To check if this system was suitable for the expression of this enzyme family, we chose to perform an experiment with a Pt whose function has already been described in the literature.

2.4.1 Presence and stability of potential substrates for Prenyltransferases involved in the biosynthesis of furanocoumarins

The very first step was to determine whether umb exists naturally in tobacco plants or not. To answer this question I infiltrated tobacco leaves with *Agrobacterium* transformed with the pBIN61-p19 plasmid as described above. Four days post inoculation, the total phenylpropanoids content was extracted from the 100 mg leaves. The same extraction was done on non-inoculated plants. In order to make the experiments more accurate and reproducible, 0.4 µg of taxifoline (a flavonoid used as an external standard) was added to samples prior to extraction. The phenylpropanoids content of the different sample of non-inoculated leaves and Agro-pBIN-p19 inoculated leaves were extracted and analyzed using HPLC as described in the "Materials and Methods" chapter. The results we obtained highlighted the absence of umbelliferone in all the samples (data not shown). To resolve this problem of the absence of potential substrate, we had to find a way to provide the substrate to the plants if we wanted to use them to realize an *in vivo* characterization of the enzyme.

To achieve this objective I infiltrated the *N. benthamiana* leaves with a 200 µM umb solution. Some inoculated leaves were harvest and frozen immediately after infiltration, while others were collected 24 h later. The HPLC analyses of the phenylpropanoids content of all samples showed the presence of a high concentration of umb only in the samples that were inoculated and harvested immediately (Figure 71B). Negligible amounts of umb were

detected in samples that were left for 2 h attached to the plants (Figure 71 C). Given these experimental results, we assume that excessive Umb inoculated in leaves of plants is transferred to other parts of plants probably through diffusion.

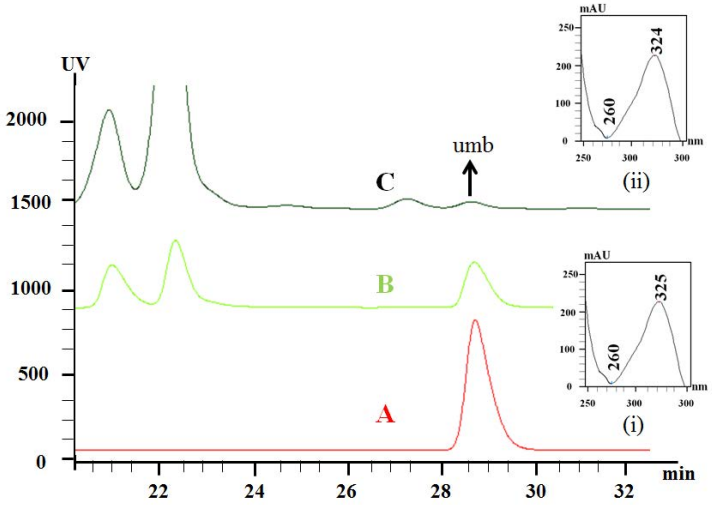


Figure 71. Detection of umb in leaves of *N. benthamiana* using the HPLC

Chromatograms obtained on HPLC by analyzing the samples prepared for leaves inoculated with 200 µM of umb, (A in red) standard umb, (B in green) samples prepared from leaves inoculated and detached from plant instantaneously and (C in black) represents the samples prepared from leaves inoculated with umbelliferone and left for 2 hours on plant. The spectrum of absorbance (i) present on the right side represents the absorbance spectrum for standard Umb and (ii) represents the absorbance spectrum of the peak obtained in samples (B), however no absorbance spectrum was detected for peak present in sample (C black line). Where umb stands for umbelliferone.

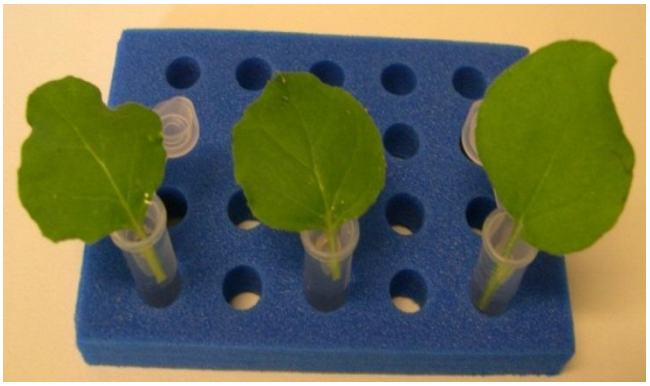


Figure 72. Inoculation of *N .benthamiana* leaves with potential substrate

Leaves were inoculated with Umb (200 µM) and dipped into the solution of Umb (200 µM) for 2 to 24 h.

This observation was a real drawback in our experimental protocol since we found above that for having a good expression of any heterologously expressed protein, we needed to perform the experiment of the expression for 4 days. Furthermore, additional time is required so that protein produced by the expression of respective gene, could metabolize the substrate into product. Therefore we had to find out a way to maintain the concentration of the

substrate in the infiltrated cells so that the enzyme could metabolize it. To overcome this problem, leaves were infiltrated with Umb, were immediately cut off the plants and their petioles were dipped in a 200 µM solution of Umb (Figure 72). These samples were collected after 2 h and 24 h and their phenylpropanoids content was extracted.

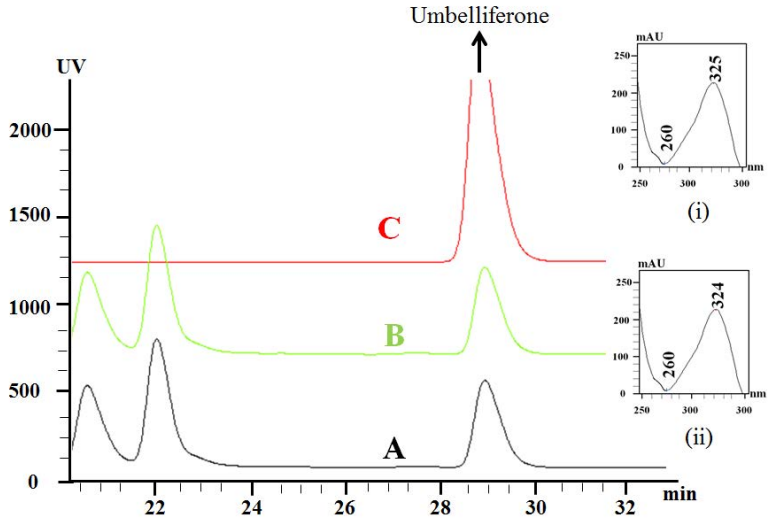


Figure 73. Detection of Umb in infiltrated leaves of *N. benthamiana* using the HPLC

Chromatograms obtained on HPLC by analyzing the samples prepared for leaves inoculated with 200 µM of Umb, (C in red) standard Umb, (B in green) samples prepared from leaves inoculated and detached from plant instantaneously and dipped in a solution of Umb of same concentration for 2 h, (A in black) represents the samples prepared from leaves inoculated with Umb, detached instantaneously and dipped in a solution of Umb of same concentration for 24h. The spectrum of absorbance present on right side represents the absorbance spectrum for standard Umb (i) and of the peaks obtained in samples (ii). Where Umb stands for umbelliferone.

HPLC analyses demonstrated the presence of a high concentration of a molecule in all the samples (Figure 73 B and C), molecule which corresponds to Umb as shown by the UV absorbance spectrum, identical to one obtained for the standard Umb. (Figure 73 (i) and (ii)).

By this way we could manage to make available any potential substrates for an appropriate time period and hence make the transient expression system of *N. benthamiana* possible for an enzyme *in vivo* functional characterization.

2.4.2 Validation of the *N. benthamiana* transient expression system for the expression of *Sf*N8DT-1, a flavonoid prenyltransferase isolated from *S. flavescens*

The last step in developing the setup of the expression system was to check if it was suitable for the study of our enzymes, and hence to perform a test with a Pt for which the

function has already been characterized. For this purpose, we selected a Pt described by Sasaki and collaborators which has been described as a Naringenin 8-Dimethylallytransferase (Sasaki *et al.,* 2008).

2.4.2.1 Recombinant plasmid: pGWB5-SfN8DT-1

This recombinant plasmid pGWB5-*Sf*N8DT-1 was provided by Prof. Yazaki. The gene was cloned in a modified pGWB5 and its expression was controlled by a strong CaMV 35S promoter as explained in (Sasaki *et al.,* 2008). This plasmid was used to transform agrobacterium strain LBA4404 (pAL4404) following the protocol described in the "Material and Methods" chapter.

2.4.2.2 Preparation of inoculum and infiltration of plants

Two different *Agrobacterium* inoculums were prepared. The first inoculum contained pBIN61- p19 and the second had pGWB5-*Sf*N8DT-1. Both bacterial suspensions were mixed, so that the final OD_{600nm} was 0.2 for pGWB5-*Sf*N8DT-1 and 0.4 for pBIN61-p19. This mix was infiltrated to leaves as set up previously. Control samples were prepared by infiltrating leaves independently with only Agrobacteria transformed with pBIN61-p19 (OD_{600nm} of 0.4). Four days post inoculation; the infiltrated leaves were collected and used for the preparation of microsomes.

2.4.2.3 Development of *N. benthamiana* transient expression system for *in vitro* functional characterization

2.4.2.3.1 Preparation of plant microsomes and test for the enzyme activity

Pts are membrane-bound enzymes and therefore the isolation of membranes is necessary to assess their activity *in vitro*. Many methods have been described in the literature. Several of them have been tested in our laboratory with more or less success. The one which worked the best in our hands was as follows;

Infiltrated leaves were cut off from plants 96 h post inoculation. 4 g of these leaves were divided into small pieces using a razor blade and immediately mixed to 1 ml KPi buffer (0.1 M, pH 7.0) containing a cocktail of protease inhibitors (Complete Mini, EDTA free from Roche), 10 mM of DTT (Dithiothreitol) (Sigma) and 0.1 g PolyVinylPolyPyrrolidone (PVPP) (Sigma Aldrich) per gram of fresh weight plant tissue. The plant material was further shredded using an ultraturax (Poly-Tron, PT2100) by realizing three cycles of one minute

shredding/one minute storage on ice. The mixture was centrifuged for 30 min at 10 000 g, prior to the filtration through a Whatman filter. The suspension was then centrifuged for 1h at 100 000 g using a SOR-VALL WX80 ultracentrifuge and finally the resulting pellet resuspended in 500 µl of Tris HCl (0.5 M, pH 8.0). The whole microsomes preparation procedure needs to be done at 4°C.

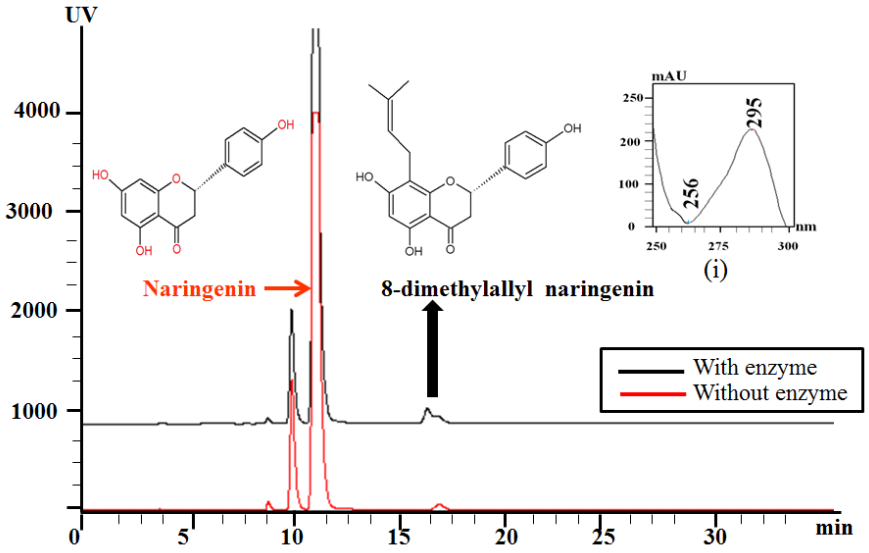


Figure 74. Enzymatic characterization of *Sf*N8DT-1 by HPLC

HPLC analysis showed that naringenin could be metabolized into 8-dimethylallyl naringenin by the microsomes prepared for *N. benthamiana* leaves expressing *Sf*N8DT-1.

Once the microsomes preparation was completed, the crucial step was to test the functionality of the *Sf*N8DT-1 Pt. For this purpose, we utilized a protocol based on experiments carried out by Yamamoto and collaborators who tested this activity with crude extracts of leaf cells in the presence of naringenin and DMAPP as substrates (Yamamoto *et al.,* 2000).

The reaction product were analyzed by HPLC as it has been described in the "Material and Methods" chapter and, as shown in (Figure 74), a small peak could detected in the presence of the enzyme. In this case, we didn't have the standard molecule and hence the comparison of UV spectrum (Figure 74) was done with the data provided in (Sasaki *et al.,* 2008), which led us to conclude that the new peak obtained in the presence of the recombinant *Sf*N8DT-1 was 8-dimethylallyl naringenin. This result was further confirmed by MS as described in (Figure 75).

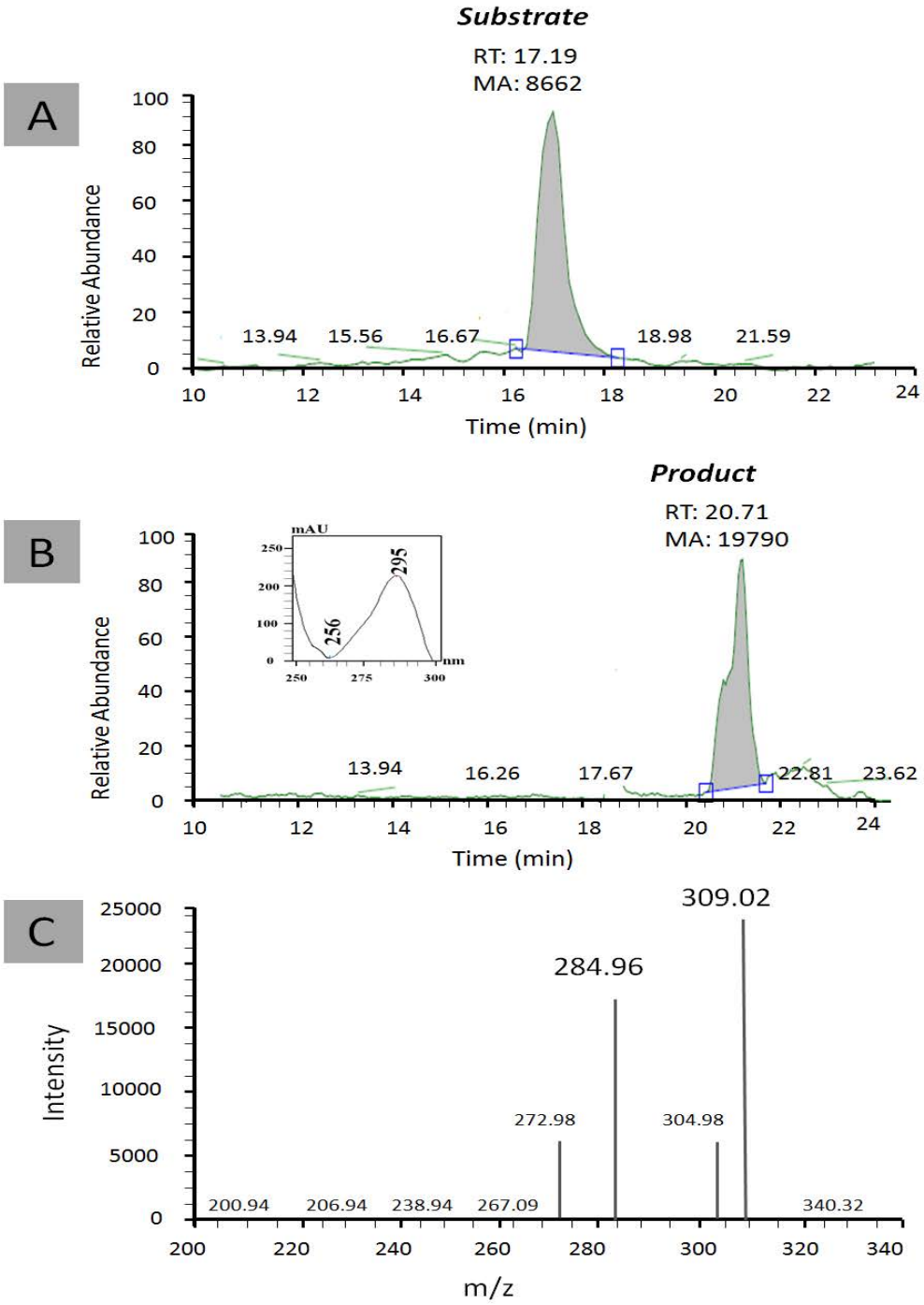


Figure 75. Identification of prenylated naringenin using mass spectrometry in *N. benthamiana* expressing the *Sf*N8DT-1

Analysis of molecules present in *N. benthamiana* leaves expressing *Sf*N8DT-1 by transient expression; analysis has done using mass spectrometry. Presence of the substrate (naringenin) in samples with and without enzyme *Sf*N8DT-1 was verified by the HPLC/LCMS (A), the presence of a new product was detected only in the presence of enzyme *Sf*N8DT-1 (B). Further confirmation of the presence of prenylated molecule was obtained by mass spectrometry, where m/z chromatogram demonstrated the mass positiove mode of ionization of naringenin (272.98) after a loss of molecular weight of 56 Da that corresponds to the mass of positiove mode of ionization (M-H)+) of DMAPP(C).

2.4.2.4 *N. benthamiana* transient expression system for *in vivo* characterization of *Sf*N8DT-1

In order to determine if the activity of the enzymes was the same *in vitro* and *in vivo*, we wanted to express the enzyme in leaves and to supply the substrate through a second infiltration of the leaves. To test the feasibility of such an approach, we used again the SfN8DT-1 as a positive control. As described above (under heading 2.4.2.2), we inoculated *N. benthamiana* leaves with a mix of agrobacteria transformed with pBIN61-p19 and pGWB5-GW-*Sf*N8DT. As a control, we infiltrated plants only with agrobacteria containing pBIN61-p19. Since aromatic Pts are described as very slow enzymes and as the highest expression of GFP was observed 96h post-inoculation, we decided to do the substrat infiltration 3 days after the agro-infiltration and to let the freshly added substrat for 24 h more in the plant before final extraction. Therefore, 72 h post inoculation, a second infiltration was carried out with 200µM naringenin solution. Naringenin is supposed to be metabolized into 8-dimethyllyl naringenin. Thus, the infiltrated leaves were cut off from the plants and their petioles were dipped in a solution containing naringenin as described (under heading 2.4.1). After 24 h, the samples were collected and their phenylpropanoids content were extracted. These extracts were analyzed by LCMS and the presence of prenylated naringenin derivatives could be highlighted only in plants infiltrated with *Sf*N8DT-1 (Figure 75 B and C) whereas such products couldn't be detected in plants transiently expressing only p19 with pBin-GW (Figure 75 A).

2.4.3 Discussion and conclusions

Selection of a heterologous expression system to realize the functional characterization of proteins is a basic and very important element to perform the study of any type of enzymes. Different important factors must be considered for the selection of any heterologous expression system: cost and simplicity of use, codon preference, post translation modification, mechanism of attachment of the proteins to the membrane, compatibility with the host cell such as coupling efficiency with endogenous redox partners (if any) and purification (Gonzalez *and* Korzekwa, 1995; Schuler *and* Werck-Reichhart, 2003). A simplified figure is presented in order to explain the host and protein interaction in terms of various factors (Figure 76). Plant aromatic Pts are enzymes which have recently been discovered and were reported as membrane-bound enzymes. Like other membrane bound enzymes, they are

difficult to express in yeast. In this work we showed that the use of a plant expression system could be an attractive alternative as most of the criteria listed above are fulfilled.

The Laboratoire Agronomie et Environnement is working with cytochromes P450s since a fairly long time and has acquired a good expertise for this class of enzymes. P450s are most relevant enzymes, for the development of such a plant expression system since most of them (and especially those which belong to plants) are membrane-bound enzymes. Among the 7000 accession available in databases, we selected the CYP98A P450 subfamily to work with. This subfamily has already been widely studied and demonstrated to be involved in the synthesis of molecules of the phenylpropanoids pathway and, more precisely, described C3'-H (Mahesh *et al.,* 2007; Schoch *et al.,* 2001). We focused on a CYP98A which has been previously isolated from *R. graveolens* and named as CYP98A22 (see the article added under heading 2.3).

The first step of this research work was the production of a functional protein of CYP98A22. The system commonly employed in the laboratory for the expression of P450, is the yeast expression system. The use of this heterologous expression system was first reported in 1985 (Oeda *et al.,* 1985). In 1996, a well-known and efficient protocol of expression and preparation of microsomes was documented by Pompon and collaborators (Pompon *et al.,* 1996). To start the functional characterization I, therefore, started to work with the yeast approach, but the results I obtained were not very fruitful. Various factors affect the efficiency of this heterologous expression system. These factors include low abundance of proteins and cell specific expression patterns, availability of specific endogenous redox partner (Dixon, 1999; Morant *et al.,* 2003; M. a Schuler *and* Werck-Reichhart, 2003). To overcome these problems various methods have been proposed. For example the protocol developed by Pompon was modified by Morgan. This author compared the expression levels of C4H and ferulate 5-hydroxylase (F5H) in modified media and by using a different protocol to perform the yeast culture (Jiang *and* Morgan, 2004). Keeping in view these protocols and modifications I made the changes not only to improve the expression of CYP98A22 but also to improve the efficiency of preparation of functional microsomes.

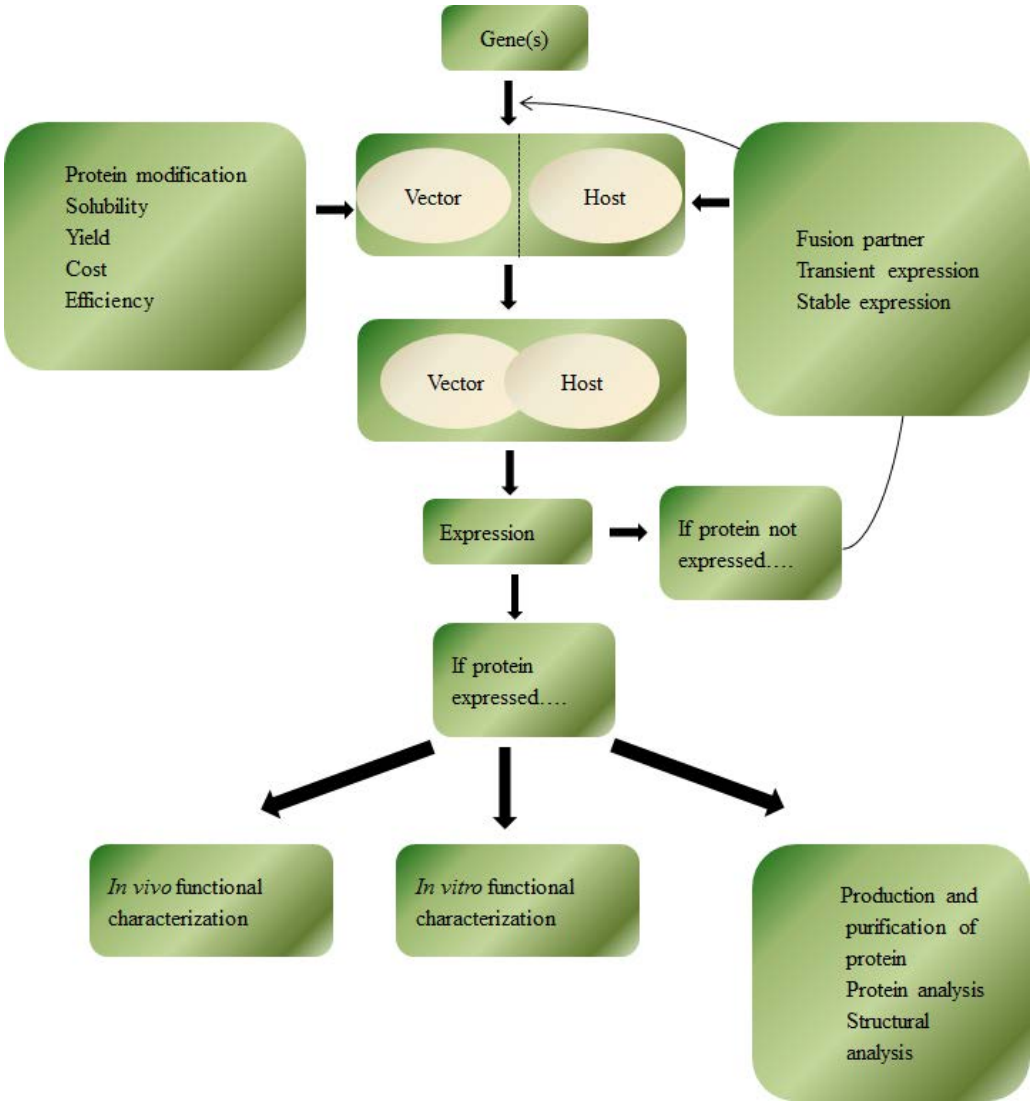


Figure 76. Simplified flow chart of heterologous expression system

Proteins are complex in nature and are, in general, difficult to express in large amounts, they are sensitive to temperature and can be cytotoxic for the organism in which they are produced. Thus, to modulate the quantity of protein produced within the host organism, the time of induction could be reduced or increased to overcome the problem of less quantity or high doses of protein. I used different time periods of induction (12 to 72 h) and found out that 48 h was the most suitable length for the expression of CYP98A22. The sensitivity of the enzyme to the extraction protocol is also a limiting step. To avoid an early degradation of the proteins, I added a cocktail of protease inhibitors (Complete Mini, EDTA free from Roche) to the extraction buffer. Furthermore Pompon suggested the use of beads that are added to liquid solution to grind cells by manual agitation. In order to make the grinding step more reproducible, I used an automatic machine (Vibro Broyeur Retsch MM40) for

mechanic grinding. This grinding procedure was done at a frequency of 1/30 and repeated three times for 1 min. All these modifications were not very efficient to produce functional proteins in yeast heterologous expression system, but efficient enough to get a metabolization of *p*-coumaroyl-shikimate into *p*-caffeoyl shikimate, which is one of the substrate generally described in literature. However this result was not easily reproducible.

These preliminary results we obtained for CYP98A22 seemed to be partial since we could detect only the metabolization of one substrate which is inconsistent to general observation reported for the members of this family. However, these results provided us the evidences that we had a functional protein and, by the way, that the concerned nucleotidic sequence was correct. Keeping in view these results we decided to switch to a plant expression system. Plant cells offer many advantages over prokaryotic and other eukaryotic expression systems like high biomass, ease of producing protein in high concentrations, correct folding of protein through post translational modification, low risks of contamination by pathogens and, finally, production is inexpensive (Ma *et al.,* 2003; Twyman *et al,.* 2003). Among all the systems available, we focused on a transient gene expression because, according to literature, it allows the production of protein in large amounts within a short time period (Gleba *et al.,* 2005; Lindbo 2007). *Agrobacterium*-mediated transient expression of protein was documented by different studies (Fraley *et al.,* 1984; Horsch *and* Klee, 1986).

The first step to appropriate this system was the selection of a plant well adapted for transient expression, which is easy to handle, grows quickly and can easily be infiltrated. *N. benthamiana* has been used for most of the studies of *Agro*-infiltration transient expression even if this system has also been optimized for other plants like *Lactuca sativa* (lettuce), *L. serriola* (wild lettuce), *Solanum lycopersicum* (tomato) and some cultivars of *A. thaliana* (Wroblewski *et al.,* 2005). In our case, we focused on *N. benthamiana.*

Different ways for introducing the agrobacteria in the leaves have been used. Kapila and collaborators, made expression of β-glucuronidase in intact leaves of *Phaseolus vulgaris* using a vacuum infiltration approach (Kapila *et al.,* 1997). More recently another work described the utilization of the agro-infiltration of *N. benthamiana* leaves to study GFP expression (Sheludko, 2008; Wydro *et al.,* 2006).

The second step of my research work was to learn to use the system with a model protein prior to start working with “more difficult” proteins. On the basis of all the collected information, I started working with transient expression system of *N. benthamiana* using the

GFP as a marker. My first priority was to get a strong and persistent expression of GFP. This goal was achieved by co-expression of a post transcriptional gene silencing suppressor protein (p19 described for tombusviruses) together with GFP. We determined a suitable OD value of inoculums of agrobacteria i.e., 0.2 for GFP and 0.4 for p19 protein (Figure 68). In addition it has been found that the maximum expression of GFP could be observed 4 days post inoculation.

The third step was to use the method for the expression of a model membrane-bound protein and therefore we switched to CYP98A22. The use of this protein led us to develop a protocol for the preparation of microsomes which finally allowed us to functionally characterize CYP98A22 *in vitro*. Comparison of the results obtained for CYP98A22 with that of CYP98A3, demonstrated that CYP98A22 preferred *p*-coumaroyl quinate as substrate rather than *p*-coumaroyl shikimate. Similar type of results were reported in a non-published research work for the C3'-H enzyme of *A. majus* (Million-rousseau, 2006). In addition, another study carried out in 2007 (Mahesh *et al.,* 2007) demonstrated the ability of the *Coffea canephora* CYP98A cytochrome P450 subfamily to acquire new substrate preferences through the evolution process. This study explained that among two different enzymes, CYP98A36 could metabolize only the hydroxylation of *p*-coumaroyl shikimate while CYP98A35 has the ability to use both substrates with the same efficiency.

Beside the *in vitro* experiments, I checked the activity of CYP98A22 *in vivo* using the *N. benthamiana* system. It was easy to use this system due to the fact that potential substrates of this enzyme were available in *N. benthamiana* plant. These analyses supported the *in vitro* results since I found a higher content of chlorogenic acids in samples overexpressing the CYP98A22 while the samples overexpressing CYP98A3 demonstrated the presence of higher amounts of caffeoyl shikimate. Therefore, this study showed that CYP98A22 is more dedicated to the synthesis of chlorogenic acid, a molecule known as being involved in defense reactions of plants. These results also supported the hypothesis of the implication of this gene in plant defense system. This expression system also offers new insights for the heterologous expression of P450s. These enzymes are well-known to be hardly expressed and the system that we have developed in this work could be an interesting solution to the functional study of many orphan enzymes for which the function has not yet been identified. Furthermore, *in vivo* charaterization of CYP98A22 carried out in our laboratory by S. Doerper, using the technique of developing the stably transformed plants of *R. graveolens*. The analyses of metabolic profiles of these plants demonstrated that although this gene is not directly involved in the

biosynthesis of furanocoumarins however, its overexpression resulted in the 3 folds increase in furanocouamrins contents of the transformed plants as compared to the wild plants. These results also supported the hypothesis of the implication of this gene in plant defense system.

As stated in the introduction under headings (3) of this chapter, the general aim of my PhD work was to identify and characterize the function of coumarins specific aromatic Pts. For this purpose, a preliminary study was carried out to analyze the presence of potential substrate for these enzymes in *N. benthamiana* leaves. Previous studies demonstrated that tobacco plant display very low concentration of scopoletin and scopoline (glycosylated form of scopoletin) but no presence of Umb was reported (Chong *et al.,* 2002; Costet *et al.,* 2002). Another study identified chlorogenic acid and rutin as the major polyphenols of tobacco plant (Wang *et al.,* 2008). Since Umb is the precursor of furanocoumarins (Bourgaud *et al.,* 2006), we performed analyses in order to check its presence in tobacco leaves. The results we obtained clearly demonstrated the absence of this molecule in wild type *N. benthamiana* (data not shown). To overcome this problem, an exogenous supply of Umb was provided through a second infiltration that was carried out 3 days after the agro-inoculation. In addition, in order to prevent the spread of the molecule in the whole plant after injection, and to ensure its presence in sufficiently high concentration to be detected, the petiole of the inoculated leaves was dipped into the substrate solution. These approaches permitted us to have enough quantity retained in infiltrated leaves for specific time periods (Figure 73).

To test the system for the expression of an aromatic Pt, I selected *Sf*N8DT-1, a Pt recently characterized and involved in the biosynthesis of flavonoids. As a first attempt, we prepared microsomes 4 days post-infiltration from agrobacteria inoculated leaves. The enzymatic tests done with naringenin showed that the microsomal fraction was able to generate 8-dimethylallyl naringenin (Figure 74). After the verification of *in vitro* activity of *Sf*N8DT-1 using the *N. benthamiana* system, I switched towards the use of the same system for *in vivo* characterization of the enzyme. For this purpose, *N. benthamiana* leaves were agro-infiltrated. Three days post inoculation, a second infiltration was done with the potential aromatic substrates (naringenin) of enzyme. Analyses done 24h latter highlighted the presence of prenylated products (Figure 75) demonstrating, by the way, that the *in vivo* approach was fully functional. These results made evidence that the *N. benthamiana* plant system was definitely an efficient tool for the expression and the functional characterization of aromatic Pts.

The functional characterization of orphan proteins is a process which is done in two successive steps. The first step consists in expressing the putative enzymes and the second step is their metabolic screening. Doing the second without being sure that the protein is properly expressed makes no sense. In this chapter I described the development of the setup of an expression system dedicated for the expression of membranous proteins.

During the methodological development process, we investigated the role of CYP98A22, a putative C3'H. The work that we have done has shown that this enzyme, isolated from *R. graveolens*, probably does not have the same role as in *A. thaliana*. It appears that the enzyme is more involved in defense reactions as it seems to favor the synthesis of chlorogenic acid.

Finally, to confirm that the system is well adapted for the expression of Pts, we successfully tested the expression of an enzyme involved in the prenylation of a flavonoid.

This system will now be used to carry on the study of several putative Pts. We will first express the different genes available prior to the doing a metabolic screening for all of them.

Chapter III: Functional characterization of the first aromatic prenyltransferase which catalyzes the prenylation of umbelliferone to produce demethylsuberosin and osthenol

3 Functional characterization of the umbelliferone prenyltransferase

3.1 Introduction

Among seven different potential candidate genes encoding putative aromatic Pts, I first focused on a gene isolated from parsley; gene which has been named *PcPt* for ***P****etroselinum* ***c****rispum* **p**renyl**t**ransferase. To realize the functional characterization of this gene, in addition to the transient expression system developed in chapter 1, I used *R. graveolens* as a stable expression system to observe the effects of the gene overexpression on the metabolic profile of transformed plants. Beside these heterologous expression systems, I also studied the behavior of this enzyme in *P. crispum*, in order to highlight the relationship between the gene expression and the furanocoumarin content in plants. Finally, using a dioxygenase proven to be involved in the synthesis of Umb (Vialart *et al*, 2012), I reconstructed the first part of the furanocoumarin pathway from *p*-coumaroyl CoA up to DMS and osthenol in tobacco.

3.2 Cloning of the PcPt coding sequence into the pBIN-GW plasmid

The PcPT coding sequence, provided by Prof Yazaki has to be introduced into the pBIN-GW plasmid which contains a strong constitutive promoter (35S promoter of the Cauliflower mosaic virus (CaMV)) giving us good chances to have a strong expression of the corresponding protein. This was done in two steps. In a first step the gene was amplified by PCR using the pENTR1A recombinant plasmid that contained the gene of PcPT provided by Prof. Yazaki as a template. The reaction was done as explained in paragraph (under heading

2.3.1) of the "Material and Methods" part, except for the annealing temperature which has been adapted to the primers described in (Table 17).

Table 17. Sequences of specific primers used to amplify *PcPt* by PCR

Sequences of primers utilized		
PcPtRev	TCAGCGCATGAAATGAATTAGCACATACTCTGCATACAG	Annealing Temp. = 59°C
PcPtDir	ATGTCTCAAACACTTATGCATTCACGATTCTCCTCCG	

Amplified PCR product was ligated into pCR8/GW/TOPO according to the instructions of the manufacturer described in "Materials and Methods" part. After the verification of the presence of right insert in the suitable orientation in pCR8/GW/TOPO, this recombinanat plasmid was utilized for LR recombination reaction to transfer the insert into plant expression vector (pBIN-GW) following the protocol described in "Material and Methods". The presence of the transgene was assessed in the pBIN-GW vector using two approaches: 1) a PCR using a forward primer directed against the 35S promoter and a reverse primer specific of the PcPt gene (Table 18) a restriction digestion using *PvuII* which cuts the plasmid at five different points (Figure 77).

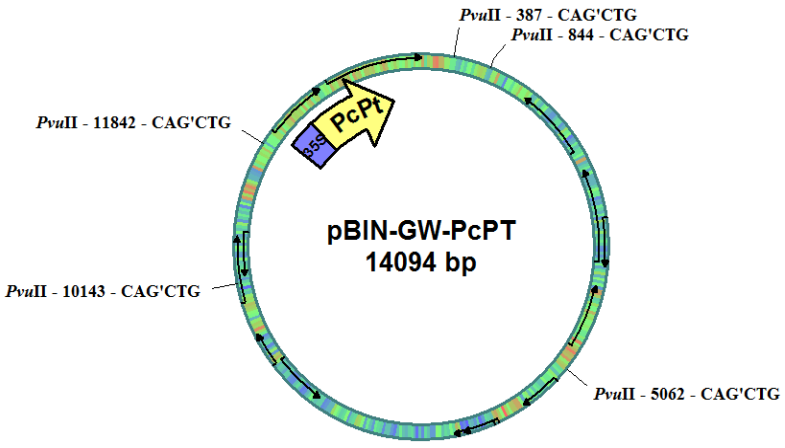


Figure 77. Map of the pBIN-PcPT plasmid

First, the PCR was done using the primers described in (Table 18) at an annealing temperature of 56°C. The expected size of the PCR product was almost 2000 bp which has been obtained as reported in (Figure 78 A). Further verification of the presence of the insert

into the plasmid was done through a restriction analysis using *Pvu*II as enzyme. The results reported in (Figure 78 B) are consistent with the map represented in (Figure 78 B) (we expected 5 bands: 5081, 4218, 2639, 1699, 457). Note that the shortest fragment is not visible on the gel because of a too poor staining. As both tests are complementary, they allow me to conclude without any doubt that the recombinant plasmid contained the gene encoding for the PcPt.

Table 18. Sequences of primers used for the PCR amplification of 35S-PcPt using pBIN-GW-PcPt plasmid as the template

Sequences of primers utilized		
PcPtRev	TCAGCGCATGAAATGAATTAGCACATACTCTGCATACAG	Annealing Temp. = 56°C
35SDir	TGCATGCCTGCAGGTCCCCAGATTG	

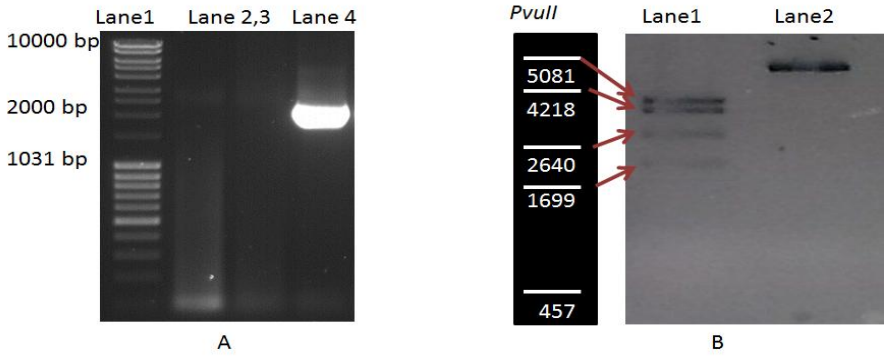


Figure 78. Construction of pBIN-GW-PcPt

(A); PCR product corresponding to 35S-PcPt. Lane 1: MM (molecular marker), Lane 2 and 3: negative control (-ve C) (genomic DNA template and a void pBIN-GW). Lane 4 : PCR has done on bacterial colony. Size of respective molecular marker is noted along to the gel.

(B); Restriction digestion of the recombinant plasmid extracted form the above described colony of bacteria. Lane 1: pBIN-GW-PcPt digested with *Pvu*II, Lane 2: pBIN-GW-PcPt not digested.

3.3 Transient expression of PcPT in *N. benthamiana*

In order to perform transient expression, the pBIN-GW-PcPt plasmid was introduced into the LBA4404 agrobacterium strain and the recombinant LBA4404::pBIN-PcPt were cultured in the same condition that were described for the LBA4404::pBIN-*Sf*N8DT-1. When I started this work, the function of the PcPt was, of course, unknown. Therefore the absence of any activity could be related to either a real lack of activity or to a poor expression level of the protein. To test the quality of the infiltration, and to make sure that the tobacco plants

were in good physiological conditions to achieve the expression of any recombinant protein, I always made a control infiltration with LBA4404::pBIN-*Sf*N8DT1 agrobacteria. Thus, the presence of a metabolization of naringenin for *Sf*N8DT-1 became the positive control for my experiments.

3.3.1 *In vivo* characterization of PcPt

To investigate the nature of the reaction catalyzed by the PcPt, I initiated a broad functional screening by testing a large number of different molecules**.**

Table 19. List of substrates used for the inoculation of *N. benthamiana* leaves 72 hours after a first inoculation

Mixes of substrates	
Mix 1: coumarins and furanocoumarins	Mix 2: flavonoids
Xanthotoxol	Naringenin
Bergaptol	7,8-dihydroxyflavanone
Umbelliferone	7-hydroxyflavanone
6-methoxy coumarin	6-hydroxyflavanone
6,7-methoxy coumarin	5-hydroxyflavanone
7-ethoxy coumarin	Taxifolin
Coumarin	Rutin
	Liquiritigenin

To carry out the first experiments, different inoculums of agrobacteria were prepared by mixing the different agrobacteria strains cultured independently. On one hand, we realized a mix of Agrobacteria transformed with pBin61-p19 and pBIN-GW-PcPt and, on the other hand, control plants were infiltrated with inoculum prepared by agrobacteria containing independently pBin61-P19 or pBIN-GW. The concentrations of bacteria were the same as used above to prepare the final mixture of inoculum. These inoculums were infiltrated in two leaves/plant and 6 different plants were used. 72 h post-inoculation, the leaves were infiltrated a second time with various substrates. In order to avoid too many infiltrations we chose to do this secondary infiltration using a mix of different substrates (Table 19). In a first mix, we used coumarins and furanocoumarins, whereas in the second mix we used flavonoids. For each mix, the final concentration of each molecule was adjusted to 200 µM. Each mix was

infiltrated in 3 plants. As described in the previous chapter, these "re-infiltrated" leaves were cut off from the plant and their petioles dipped in solutions containing the different molecules present in the mix. Finally, 24 h later, the phenylpropanoids contents were extracted from the leaves as described in the "Material and Methods" chapter and analyzed by LCMS.

The LC-MS method (described in the "Material and Methods" chapter) is very sensitive and precise and can detect very little amounts of any product present in the sample. As the mass spectrometric screening is done regarding the molecular mass of the products, the samples were analyzed for any possible prenylated product corresponding to the molecules present in each mix (MW of the molecule +68 for dimethylallyl moiety, MW of the molecule +136 for geranyl moiety). The analysis done on the control plants made evidence that no prenylated products corresponding to the molecules present in the mix could be detected. However, in plants co-expressing p19 and PcPt, the results I obtained demonstrated that Umb was metabolized in two distinct molecules P1 and P2 (Figure 79 A) sharing the same molecular weight (Umb +68) and the same MS spectrum (Figure 79 B). In order to identify unequivocally each molecule I analyzed independently (and also as a mix) osthenol and DMS which were the expected products. The analysis of the mix with both molecules, generate the same chromatogram as presented in (Figure 79 A). The analysis of each standard molecule allowed us to identify the P1 product as DMS and the P2 product as Osthenol.

Among all the molecules tested in both mixes, no other metabolization could be highlighted. This first experiment using a mix of 7 or 8 different molecules allowed us to identify C-prenylated forms of Umb. In a second step, we wanted to confirm these results by infiltrating only the molecule that has given us a positive result. Therefore, new *N. benthamiana* leaves were inoculated with the same bacteria mix described previously for PcPt. The secondary infiltration was done 72 h post inoculation only with a 200 µM solution of Umb. After 24 additional hours, the phenylpropanoids were extracted and analyzed by LC-MS. The results obtained were exactly the same to the one discussed above, making evidence that the reaction is reproducible and that there was no interaction between the different molecules present in the coumarins/furanocoumarin mix.

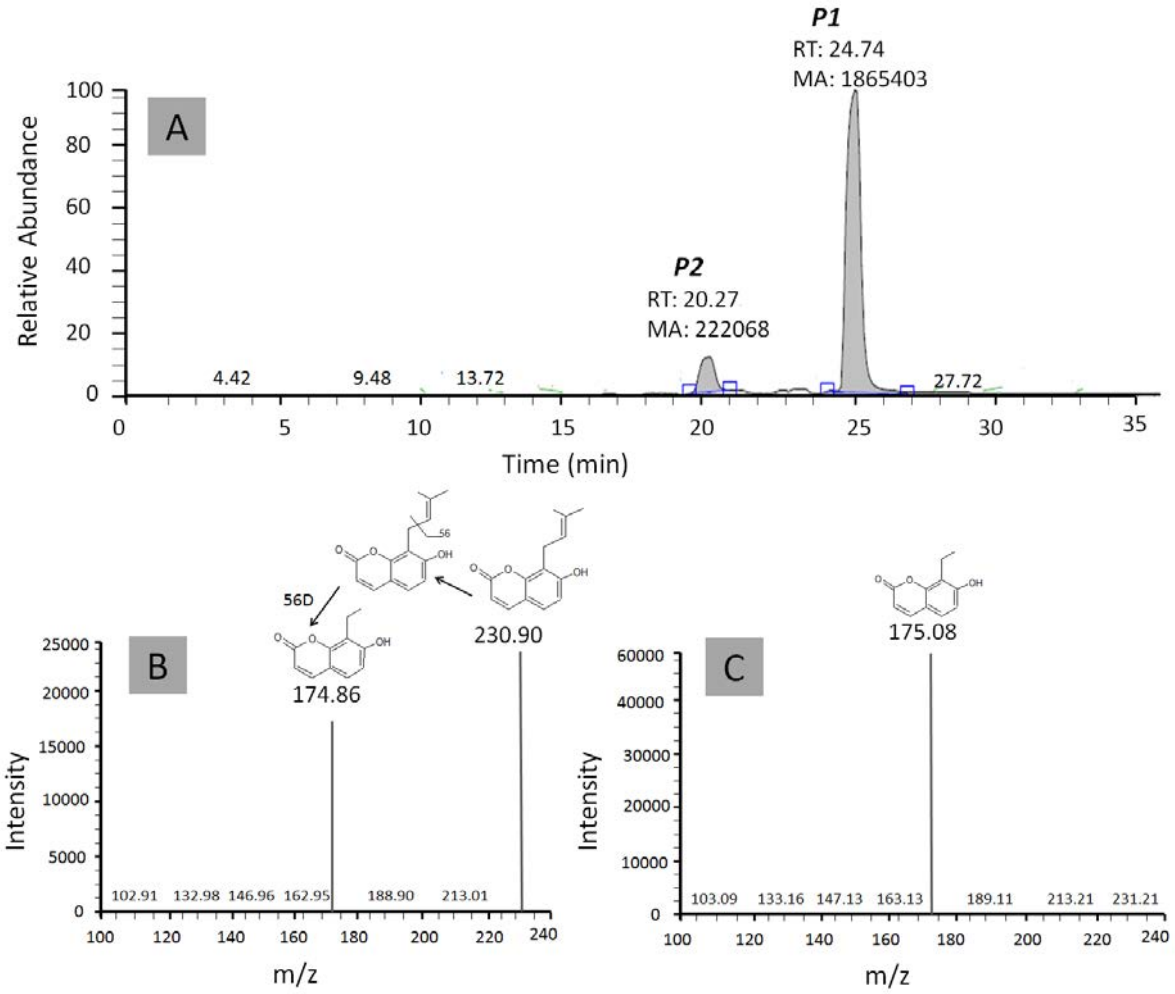


Figure 79. Identification of prenylated Umb using mass spectrometry in *N. benthamiana* expressing the PcPT

Analysis of molecules present in *N. benthamiana* leaves expressing PcPT by transient expression; analysis done using mass spectrometry demonstrated the presence of two prenylated products (A). Both of these products demonstrated same m/z chromatogram corresponding to the mass of Umb after a loss of molecular weight of 56 Da that corresponds to the mass of positiove mode of ionization (M-H)+) of DMAPP (B). While (C) represents the m/z specific both for the standard DMS and osthenol.

These results showed for the first time an enzyme of the Pt family able of metabolizing a coumarin-like molecule. In addition, this enzyme displays a double activity since it is able to synthesize two different products; DMS and osthenol from Umb as a starting molecule. This enzyme is therefore a key enzyme in the biosynthetic pathway of furanocoumarins. This main result will be discussed in details later.

3.3.2 Biochemical *(in vitro)* characterization of PcPt

In vivo expression of PcPT provided the first results of the capability of enzyme of prenylation of Umb. However, it is quite evident that the conditions available for *in vivo* reaction are different from controlled *in vitro* conditions. Other studies have shown that sometimes, the expression of gene under *in vivo* conditions could modify the substrate specificity of enzymes (Sugiyama *et al.,* 2011). In addition to it, availability of prenyl moieties DMAPP, FPP or GPP could be unequal within the various compartments of plants cells, and hence it is difficult to be sure that these moieties are available for enzymatic activity. To answer these questions, I started the *in vitro* characterization of PcPt using the same system of *N. benthamiana* as it has been described for *Sf*N8DT-1 previously. Thus utilization of this technique permitted me to test other aromatic substrates along with three different prenyl moieties (DMAPP, GPP and FPP) on one hand, while, on other hand provided me the chance to measure the various kinetic parameters. With the help of these kinetic parameters (Km_{app} and $Vmax_{app}$), it is possible to detect whether DMS or osthenol is preferentially synthesized.

To achieve this characterization, inoculum of agrobacteria containing independently transformed bacteria with recombinant plasmid pBin61-p19 and pBin-GW-PcPt was prepared and used for inoculating *N. benthamiana* leaves using the same protocol as described previously. Instead of doing a secondary infiltration with a mix of molecules, the leaves were harvested 96 h post infiltration and used for extraction of microsomal proteins following the protocol that had been described for *Sf*N8DT in the previous Chapter (under heading 2.4.2.3.1) of results.

3.3.2.1 Enzymatic characterization

Extracts of total membrane-bound proteins, prepared from infiltrated leaves of tobacco were used as enzyme solution for performing the Pt assays. The reaction mix was prepared in 500 mM Tris HCl (pH 8.0) that contained 200 µM of "coumarinic" substrate (17 different molecules, see Table 20), 10 mM of the prenyl moiety (DMAPP, FPP or GPP), 1mM of $MgCl_2$, 50 µl of enzyme solution and the final volume was made up to 200 µl with buffer. This reaction mix was incubated at 25°C for 3 h. The reaction was stopped by addition of 1µl of TFA and vortexed for one minute. A 20 min final centrifugation at 16000 g was carried out to remove all cellular debris before analyzing the supernatant samples by HPLC. This

incubation protocol was used for microsomes extracted from test and control plants (Agro-(pBin61-p19 + pBin-GW-PcPt and pBin61-p19 + pBin-GW)).

3.3.2.1.1 Screening for other potential substrates

Aromatic prenylation is a reaction of addition of a prenyl moiety on an aromatic molecule in the presence of a suitable enzyme. This reaction can result in the production of diverse products depending upon the nature, the size and the attachment position of the prenyl moiety (either at O or C atom of aromatic substrate). To verify the *in vivo* activity of PcPt and in order to screen other possible substrates (either aromatic moiety or prenyl moiety) a number of coumarins and furanocoumarins were tested in the presence of the three most abundant prenyl moieties described (DMAPP, GPP and FPP).

Table 20. List of substrates used for *in vitro* screening of possible substrates of PcPt

Coumarin and furanocoumarin moiety/ prenyl moiety	DMAPP	GPP	FPP
Xanthotoxol	-	-	-
Bergaptol	-	-	-
Umbelliferone	+	-	-
6- methoxy coumarin	-	-	-
7- methoxycoumarin	-	-	-
6,7-methoxycoumarin	-	-	-
Coumarin	-	-	-
7-ethoxycoumarin	-	-	-
5-methoxypsoralen	-	-	-
8-methoxypsoralen	-	-	-
Psoralen	-	-	-
8-OH Xanthotoxin	-	-	-
5-OH Bergapten	-	-	-
5,8-di OH Psoralen	-	-	-
5,6-di OH Angelicin	-	-	-
Isobergaptol	-	-	-
Sophondinol	-	-	-

Different aromatic molecules were used in the presence of three different prenyl moieties as possible substrate of PcPt. A cross in the table indicates a metabolized with its corresponding prenyl moiety. It was observed that PcPt is highly specific for Umb as aromatic substrate and for DMAPP as prenyl moiety.

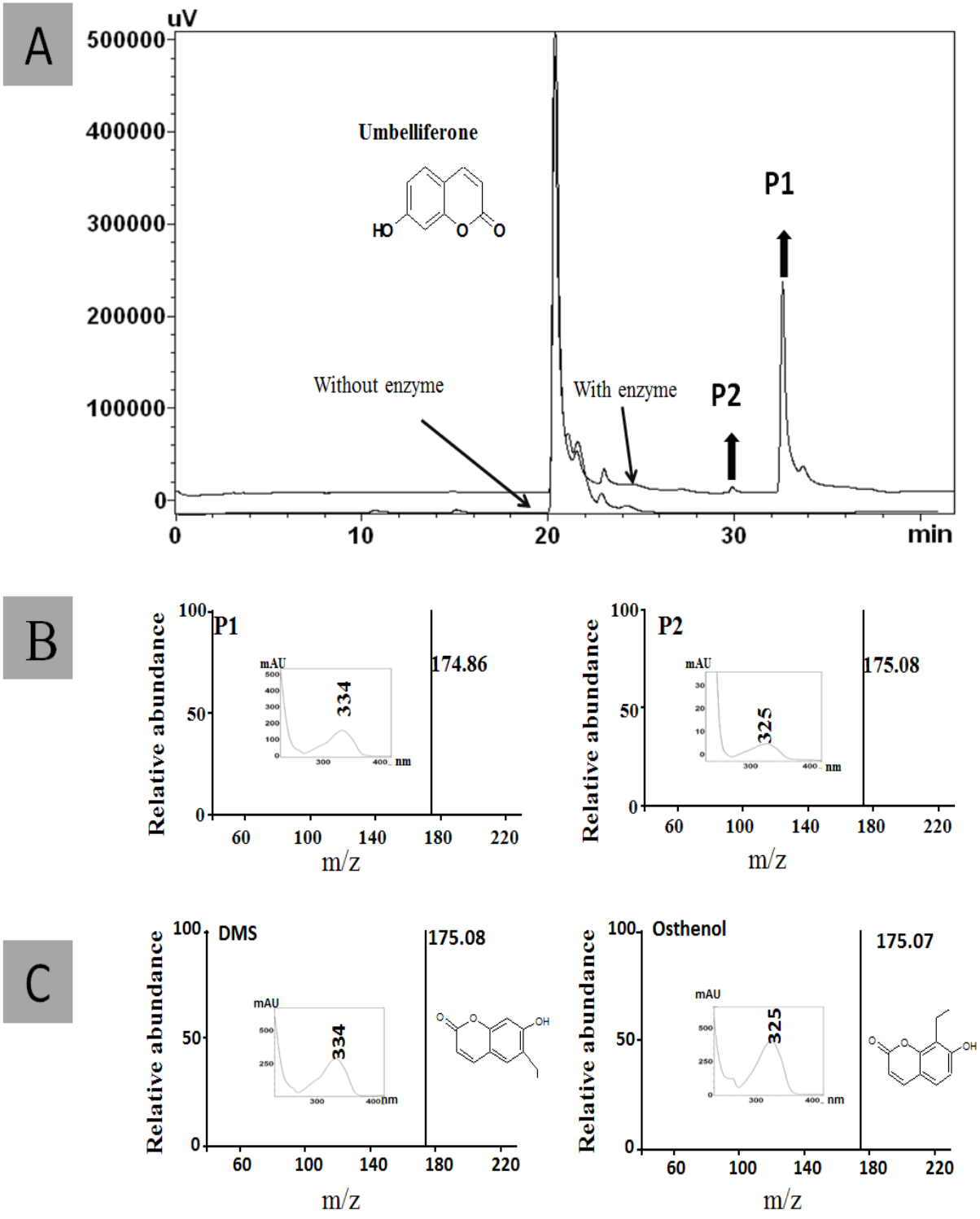


Figure 80. Functional characterization of the parsley Pt

HPLC-DAD analysis showed that Umb was metabolized into two products P1 and P2 in the presence of enzyme PcPt (A). Absorbance spectra of UV and mass spectra of product P1 and P2 obtained by analysis on HPLC and LC-MS are shown (B) and correspond exactly to the absorbance spectra and mass spectra of standard DMS and Osthenol commercially available (C).

All of these substrates are listed above in the (Table 20). All the samples were analyzed by LC-MS and the results, we obtained clearly highlighted only the metabolization of Umb in the presence of DMAPP (Table 20). As shown *in vivo*, two different peaks were obtained (P1 and P2 product, Figure 80 A). In order to check the accuracy of the results obtained *in vivo*, I compared again the products I obtained to commercial standards corresponding to DMS and Osthenol. The retention time, the UV spectrum and also the mass spectrum, with out any doubt provided the evidences that P1 corresponded to DMS whereas P2 was identified as Osthenol.

The *in vivo* and *in vitro* results both demonstrate that, among all the substrate tested, PcPt is specific to Umb and DMAPP and that this enzyme catalyze the synthesis of two different molecule by the addition of DMAPP either on the Carbon C6 or C8 of Umb forming the DMS and osthenol respectively.

3.3.2.1.2 Biochemical and kinetic characterization of PcPt

Before starting the measurement of the enzymatic kinetic parameters, it is necessary to optimize the reaction conditions. The different parameters I focused on were the incubation time, the pH and the optimal concentration of DMAPP.

3.3.2.1.2.1 Optimal incubation time

The preliminary experiments described above were realized with a long incubation time. In order to determine a relationship between the incubation time and the concentration of product, reaction mixes were prepared as described in the "Material and Methods" chapter. Different incubation periods were tested (15 min, 30 min, 45 min, 60 min, 90 min, 120 min, 150 min and 180 min). At the end of each time interval, the reaction was stopped by addition of 1 µl TFA followed by a 20 min centrifugation (protocol described well in paragraph 3.3.2.1). The results obtained are described in (Figure 81). A linear relationship between the time of incubation and concentration of the product could be observed. Relying on these results, I chose **90-120 min** as a suitable time period to have enough concentration of product that is easily detectable and analyzable on HPLC and perhaps could be used for further analysis.

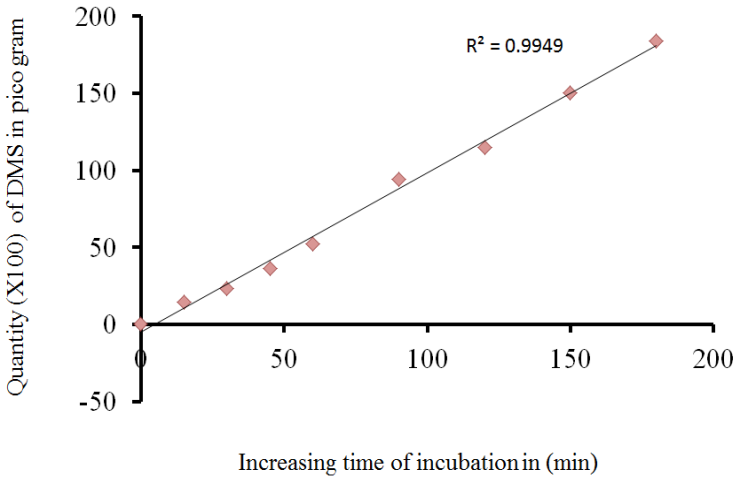


Figure 81. Determination of a relationship between time of incubation and concentration of DMS

3.3.2.1.2.2 Optimal pH

For measuring the optimal pH, reaction mixes were prepared as described in the "Material and Method" chapter except for the pH of the reaction buffer. Buffer with a 5 to 9.5 range of pH was prepared (5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, and 9.5). Keeping all other factors constant except pH, these incubations were made following the protocol already described. The analysis of the results was made using the Gaussian distribution method, based on the equation (Equation 1) and found a maximum of the activity of enzyme at **pH of 8.2** (Figure 82).

$$f(x) = \frac{1}{\sigma\sqrt{2x}} e - \frac{(x-\mu)^2}{2\sigma^2}$$

Equation 1. Formula for Gaussian distribution

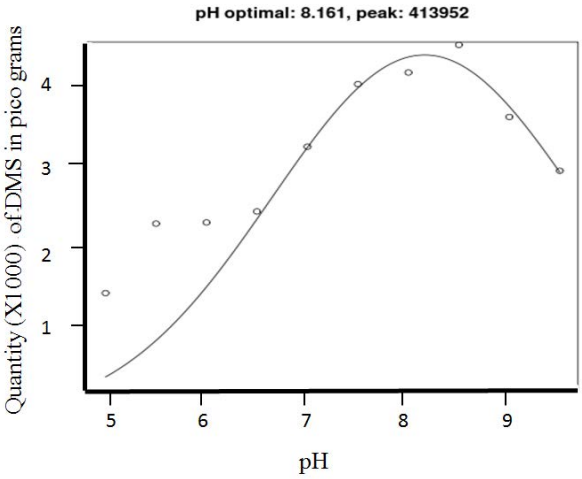


Figure 82. Measurement of optimal pH for maximum activity of PcPt

Optimal pH for maximum activity of enzyme was measure using the Gaussian distribution method.

3.3.2.1.2.3 Saturated concentration of DMAPP

DMAPP is the second substrate (or cofactor) necessary for the enzymatic reaction catalyzed by PcPT. Before starting the measurement of kinetic parameters it was necessary to know the saturated concentration of DMAPP. For this purpose, the reaction mixes were prepared and incubated using the protocol described in the "Material and Methods" chapter. The only factor that varied was the concentration of DMAPP. Different concentrations of DMAPP (0.2 mM, 0.5 mM, 0.75 mM and 1 mM) were tested. The analysis of the results of these incubation obtained by HPLC (Figure 83) showed that **0.75 mM** is the most suitable concentration for a maximum activity of the PcPT enzyme.

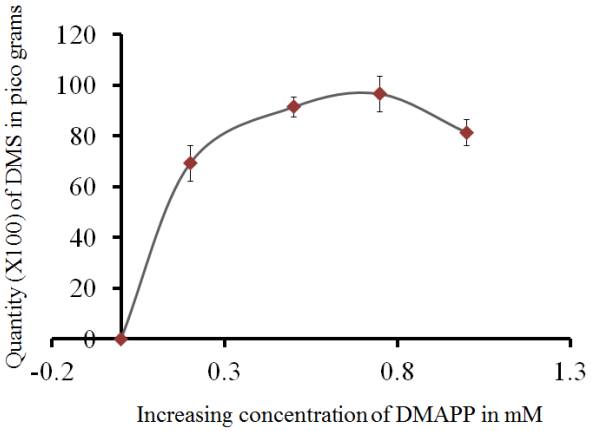


Figure 83. Measurement of saturated concentrations of DMAPP for enzymatic activity of PcPt

Each point on the graph represents the average quantity of DMS in pmoles, where n = 2 ("n" is the number of replicates).

3.3.2.1.2.4 Measurement of the kinetic constants of PcPt

To determine the kinetic parameters of the enzyme, the reactions were performed in optimal conditions (pH, Temperature, DMAPP concentration determined above) and in the presence of Umb concentration ranging from 2 µM to 1 mM. The reaction products were analyzed by HPLC. The results [Substrate] / [Product] obtained were distributed as a hyperbolic curve, which was the characteristic of Michaelis-Menten type enzymes and hence kinetic constants of the PcPt could be measured using a Michaelis-Menten kinetic model. To measure the kinetic parameters, the hyperbolic equation was converted into a linear equation, i.e. y = mx+b following the Lineweaver-Burke representation. And hence, $1/Vmax_{app}$ was plotted against 1/[S] both for DMS and osthenol independently (Figure 84 and Figure 85) and used to calculate the $Vmax_{app}$ and Km_{app}. In addition, kinetic constants were also measured using the Sigma Plot software [Scientific computing]. The results summarized in (Table 21) show that the Km_{app} is nearly the same for the synthesis of DMS (Km_{app} = 21 ± 3, V_i / Km_{app} = 0.83 ± 0.08) and osthenol (Km_{app} = 25 ± 3, V_i / Km_{app} = 0.017 ± 0.002). On the other hand, reaction of the synthesis of DMS is almost 40 times faster than that of osthenol.

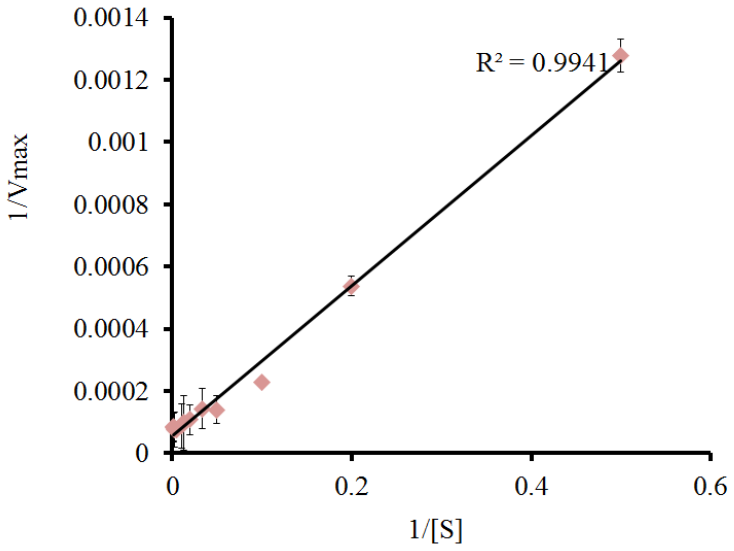


Figure 84. Lineweaver-Burke representation for the appearance of DMS when the PcPt is incubated with Umb

A relation between concentrations of substrate Umb (2 µM to 1 mM) and respective quantity of DMS is represented. Each point on the graph represents an average value of 3 replicates.

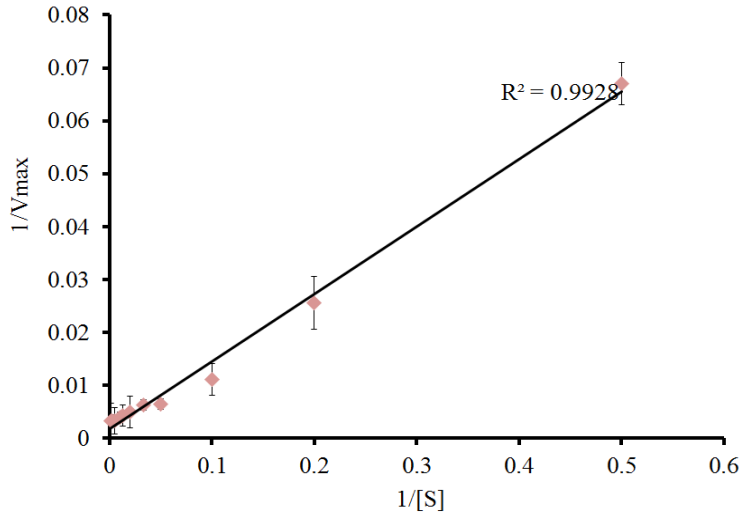


Figure 85. Lineweaver-Burke Representation for the appearance of osthenol when the PcPt is incubated with Umb

A relation between concentrations of substrate Umb (2 µM to 1 mM) and respective quantity of DMS is represented. Each point on the graph represents an average value of 3 replicates.

Table 21. Kinetic constants measured for PcPt using the Sigma Plot software [Scientific Computing]

Substrate	Product	V_i (pmol.min.g FW)	$Km_{app\ umb}$ (µM)	$V_i / Km_{app\ umb}$ (μM^{-1}.pmol.min.g FW)
Umbelliferone	Demethylsuberosin	17.5 +/- 0.8	21 +/- 3	0.83 +/- 0.08
	Osthenol	0.41 +/- 0.01	25 +/- 3	0.017 +/- 0.002

3.3.2.1.2.5 Measurement of uncompetitive inhibition

Coumarins and furanocoumarins have frequently been described as potential enzymatic inhibitors. Moreover, due to their physical properties, some of them are toxic for the cell in which they are synthesized. Therefore, it is not impossible that a control system has been set up in the cell in order to limit their synthesis, system that could actually result from the inhibition of the enzymes involved in the biosynthesis pathway by these same molecules as a retro-control. In order to find out if the activity of PcPt is affected by these molecules I did a number of incubations in the presence of several coumarins and furanocoumarins (Xanthotoxol, Bergaptol, Psoralen, 5-MOP, 8-MOP, Isopimpinellin, 6-methoxycoumarin, 7-methoxycoumarin, Coumarin, and 7-ethoxycoumarin). As a preliminary experiment, incubations were performed with 200 µM of each molecule prior adding 200µM Umb in the reaction mix. The first set of data obtained (Figure 86) showed that, among all the tested

molecules, only psoralen had a significant effect on the enzyme activity and led to a 50% reduced production of DMS.

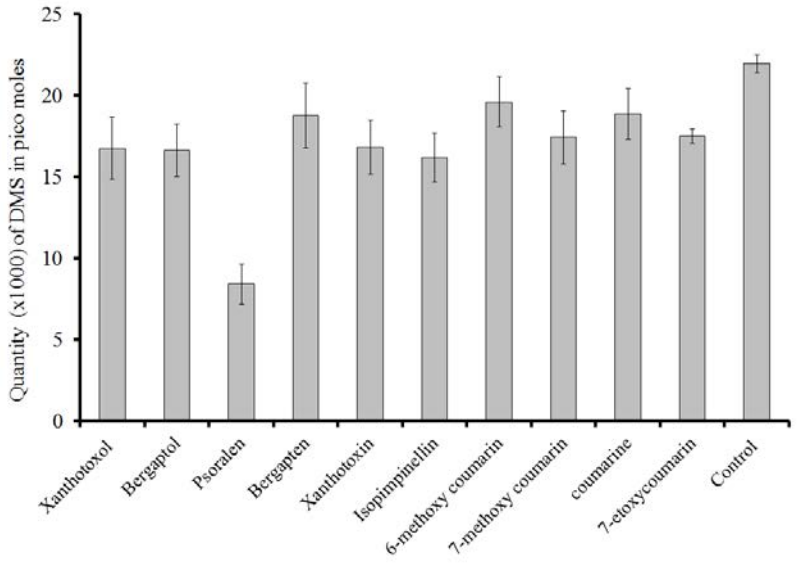


Figure 86. Effect of the presence of different coumarins and furanocoumarins on the activity of PcPt enzymatic activity

Each bar represents the average quantity of DMS in pmoles, where n = 2 ("n" is the number of replicates).

In order to determine the inhibition constants related to the presence of psoralen in the reaction mix, new sets of incubation have been done with various concentration of psoralen. For a first rough experiment, the reaction mix was realized with 200 µM Umb and 0 µM, 25 µM, 50 µM or 100 µM of psoralen. After 2 hours, the reaction was stopped and analyzed by HPLC. This experiment helped me to select the range of concentrations of psoralen suitable for measuring the kinetic of inhibition for the PcPt by psoralen. The analysis of these incubations represented in (Figure 87), showed that the presence of even a small concentration of psoralen (25 µM) can inhibit the enzymatic activity.

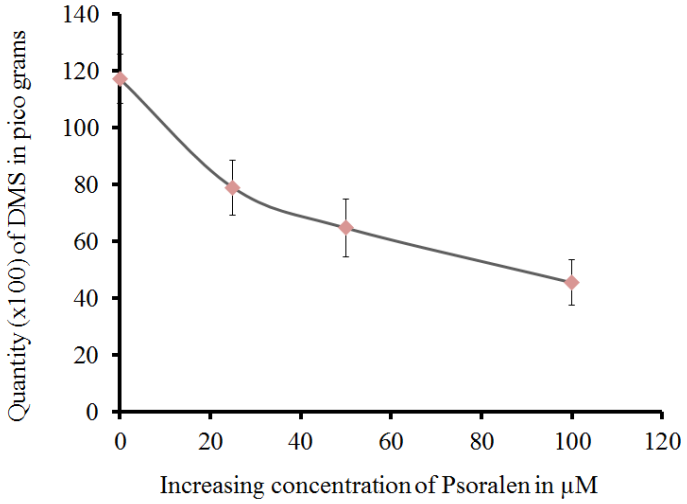


Figure 87. Measurement of inhibition effects of PcPT in the presence of various concentration of Psoralen

Each point on the graph represents the average quantity of DMS in pmoles/µl, where n = 2 ("n" is the number of replicates).

Based on these results I selected three concentration of psoralen (10 µM, 20 µM, and 40 µM) for the measurement of the inhibition constant (*Ki*). To carry out these experiments 6 different concentrations of Umb (10 µM, 15 µM, 20 µM, 40 µM, 60 µM and 80 µM) were incubated in all possible combinations with the different concentrations of psoralen. These incubations were made following the protocol already described in the "Material and Methods" chapter, except for the concentrations of Umb and psoralen. Reactions were stopped and analyzed by HPLC to observe the relevant quantity of product formed.

To observe what type of inhibition relationship exist between psoralen and the reaction catalyzed by PcPt, the apparent reaction velocity ($Vmax_{app}$) in terms of quantity of DMS or osthenol were plotted against the concentration of Umb. The resultant curves I obtained were exploited according to the Michaelis-Menten kinetic model and were further rearranged according to the Lineweaver-Burke plot both for DMS (Figure 88) and osthenol (Figure 89). This arrangement of the data resulted in the appearance of parallel lines when the values of $1/Vmax_{app}$ were plotted against the reciprocals of substrates concentrations (1/[S]). These results demonstrated the presence of an **uncompetitive type inhibition** on the basis of Lineweaver-Burke plot method. Wile the uncompetitive inhibition is a type of inhibition, where inhibtior binds exclusively to the enzyme-substrate complex yielding an inactive enzyme-substrate-inhibitor complex.

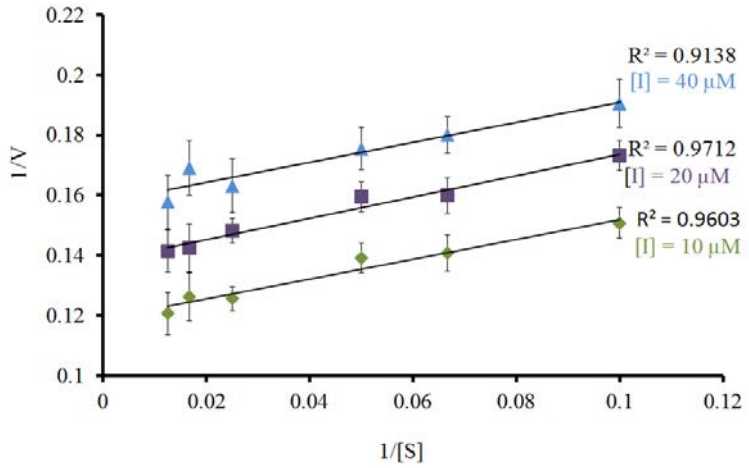


Figure 88. Graphical representation of an uncompetitive inhibition of PcPt for DMS following the Lineweaver-Burke plot

Each point on graph represents the average of reciprocals of quantity of DMS in nmoles in the presence of specific concentration of psoralen (n = 3, "n" is the number of replicates).

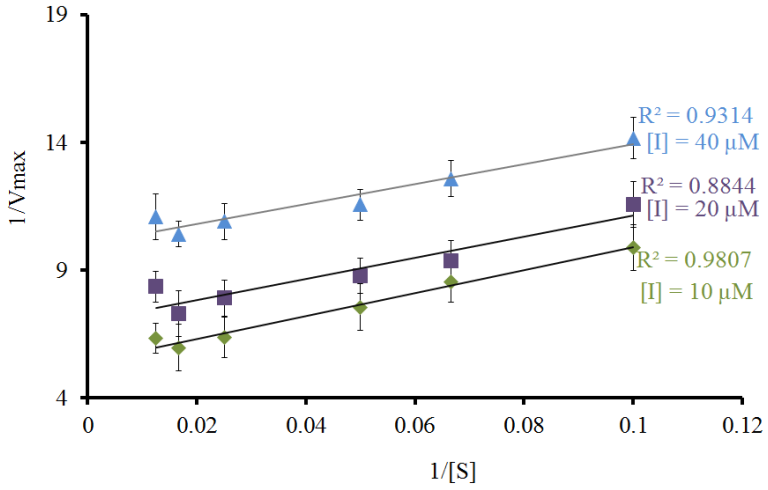


Figure 89. Graphical representation of an uncompetitive inhibition of PcPt for Osthenol following the Lineweaver-Burke plot

Each point on graph (B) represents the average of reciprocals of quantity of DMS in nmoles in the presence of specific concentration of psoralen (n = 3, "n" is the number of replicates).

Finally, using the Lineweaver-Burke plot method, I could determine the Km_{app} and the $Vmax_{app}$ for each of the data set obtained for a specific concentration of psoralen both for DMS and osthenol. These results showed that both, the apparent Km_{app} and $Vmax_{app}$ decreased with an increased concentration of inhibitors (Table 22), while the ratios of $Km_{app}/Vmax_{app}$ remained unchanged (Table 22). This observation is also characteristic of an **uncompetitive inhibition**.

Table 22. Measurement of *Vmax* app, *Km* app and *Km* app /*Vmax* app for various concentrations of psoralen using Lineweaver-Burke plot method

Apparent Km_{app} and $Vmax_{app}$ measured for DMS using Lineweaver-Burke plot method			
Concentration of psoralen in µM	Km_{app} (µM)	$Vmax_{app}$ (pmol.min^{-1}.g^{-1} FW)	Km_{app} /$Vmax_{app}$ (µM pmol^{-1}.min^{-1}.g^{-1} FW)
10	2.95	8.3	0.35
20	2.59	7.25	0.36
40	2.12	6.01	0.35
Apparent K_m and V_{max} measured for osthenol using Lineweaver-Burke plot method			
Concentration of psoralen in µM	$K_{m\ app}$ (µM)	$V_{max\ app}$ (pmol.min^{-1}.g^{-1} FW)	$K_{m\ app}$ /$V_{max\ app}$ (µM pmol^{-1}.min^{-1}.g^{-1} FW)
10	8.22	0.184	44.6
20	6.35	0.14	45.3
40	4.5	0.10	45.0

According to this theory, in case of uncompetitive inhibition both the Km_{app} and $Vmax_{app}$ decreases with increasing concentrations of inhibitors while Km_{app} /$Vmax_{app}$ remains unchanged. Results obtained in our experiment supports this theory.

In order to measure the inhibition constants, a second type of graphs was constructed. These graphs were based on the $1/Vmax_{app}$ and $1/Km_{app}$ values that were calculated for three concentrations of psoralen in three independent experiments for both DMS and osthenol. These $1/Vmax_{app}$ (Figure 90 A and Figure 91 A) and $1/K_{m\ app}$ (Figure 90 B and Figure 91 B) value were plotted against the concentration of psoralen in order to measure the *K*i. Values of *K*i were measured by the intercept on the horizontal axis, where it is equal to $-1/K_m$. This whole procedure permitted me to calculate the values of *K*i, which were **31µM and 34µM for DMS and osthenol respectively**.

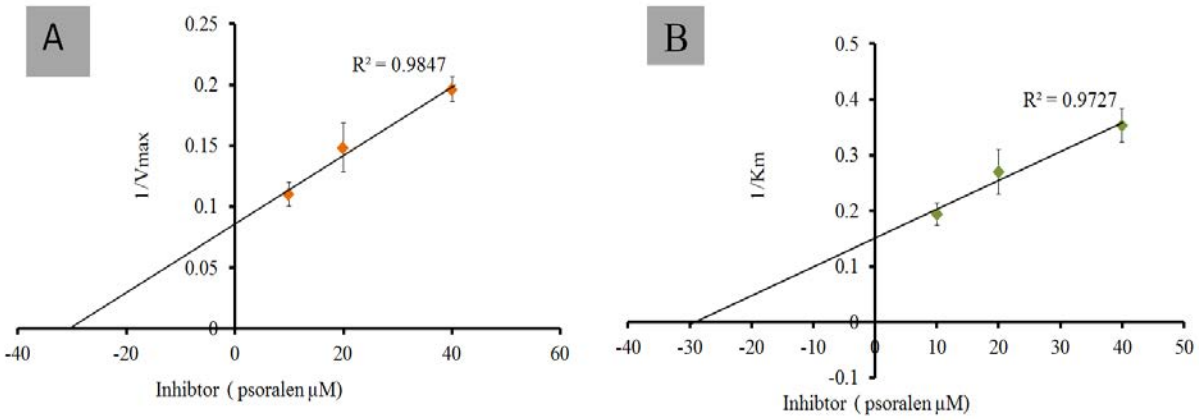


Figure 90. Measurement of the inhibtion constant (*Ki*) of PcPt for DMS, either via 1/*Vmax*$_{app}$(A) or 1/*Km*$_{app}$ (B) following the Lineweaver-Burke plot method

Each point on the graph represents the average reciprocal value of either *Vmax*$_{app}$ or *Km*$_{app}$.(where n = 3, "n" is the number of replicates).

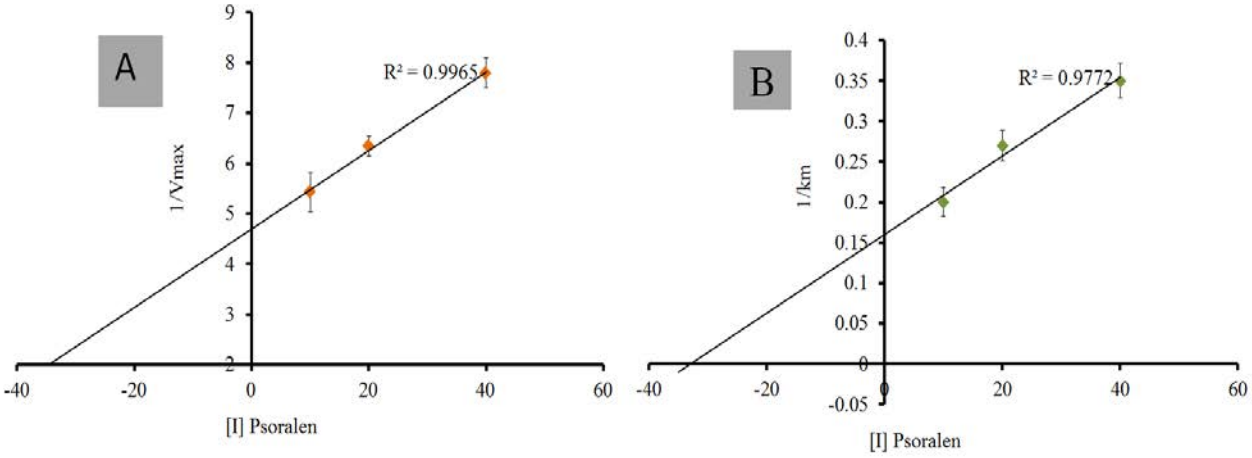


Figure 91. Measurement of the inhibtion constant (*Ki*) of PcPt for osthenol, either via 1/V$_{max\ app}$(A) or 1/*K*$_{m\ app}$ (B) following the Lineweaver-Burke plot method

Each point on the graph represents the average reciprocal value of either *Vmax*$_{app}$ or *Km*$_{app}$. (where n = 3, "n" is the number of replicates).

3.4 Stable expression of PcPt

Up to now all our experimental approach was focused on the characterization of the PcPT using the tobacco transient expression system. Another aspect had to be explored. As tobacco doesn't produce furanocoumarins, we wanted to investigate the effect of the overexpression of PcPt in a furanocoumarin producing plant in order to check if there is a modification of the metabolic profile or not.

3.4.1 Construction of transgenic *R. graveolens* plants using PcPt as a transgene

Transformation of *R. graveolens* plants was carried out using hypocotyls as "explants" through the technique of genetic transformation developed by Karine Lièvre during her PhD (Lièvre *et al.,* 2005). A simple schematic representation of this technique is presented in (Figure 92).

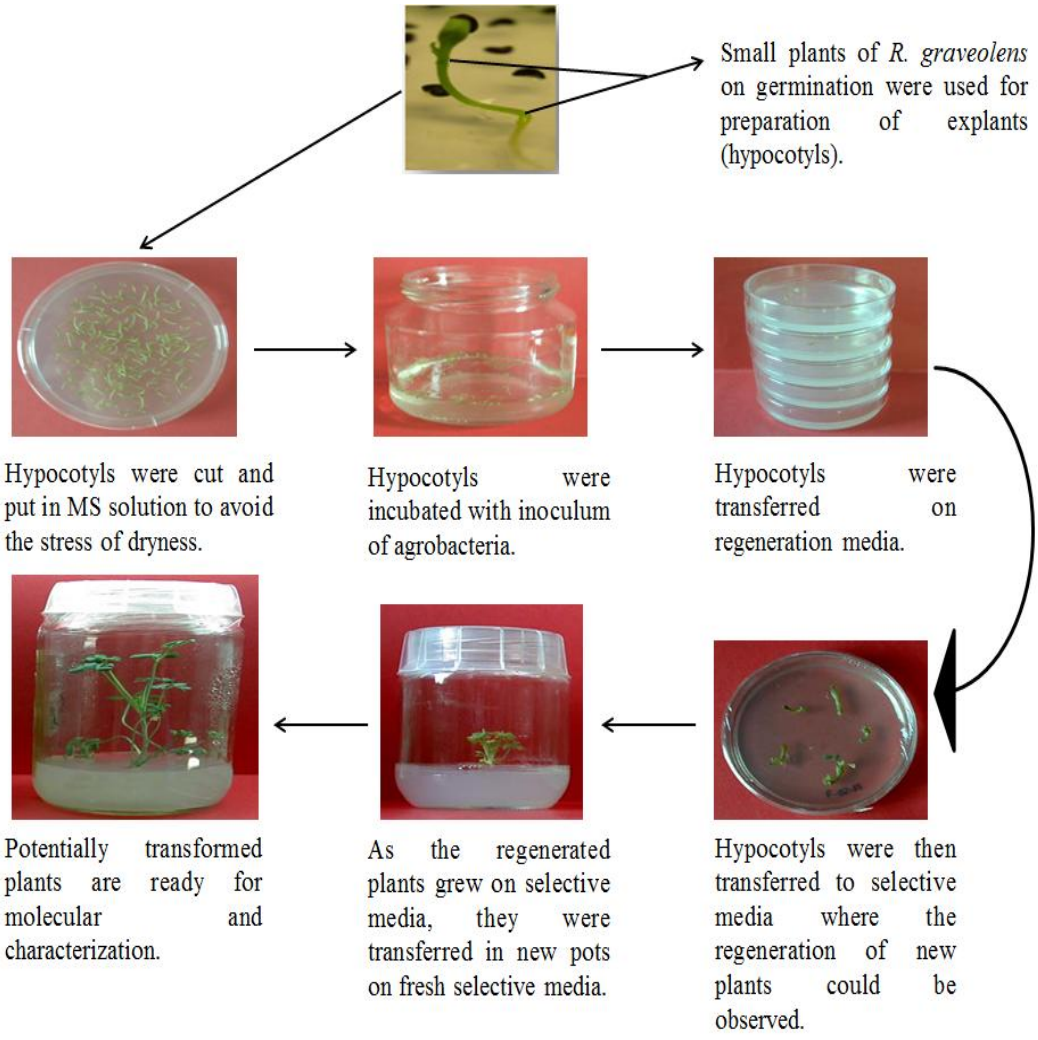


Figure 92. Simple representation of the protocol used to develop the PcPt genetically transformed *R. graveolens* plants (Modified from the Ph D manuscript of Karine Lièvre (Lièvre, 2004))

For this procedure an inoculum of Agrobacteria containing pBIN-GW-PcPt or pBIN-GW (= control plants) was prepared and the transformation performed as described in the "Material and Methods" chapter. After washing them to remove the bacteria, hypocotyls were incubated on a regeneration media before being transferred to a selective media containing kana. When the transformed hypocotyls developed new leafy stems of almost 1 cm, they were

transferred to 200 ml culture pots as described in the "Material and Methods" part (Figure 93). No clear different phenotype could be observed between pBIN-GW-PcPt and pBIN-GW plants except that pBIN-GW-PcPt transgenic plants display a very slow growth rate, especially for roots.

All these plants, transferred on the new selective media are resistant to kana. Therefore they were potentially transformed and contained either the PcPt gene under the control of the constitutive 35S CaMV promoter and (or) the T-DNA containing the *nptII* gene (for the plants transformed with void pBIN-GW plasmid). Six different individually transformed plants were propagated on a selective medium and were used for further molecular and biochemical characterization.

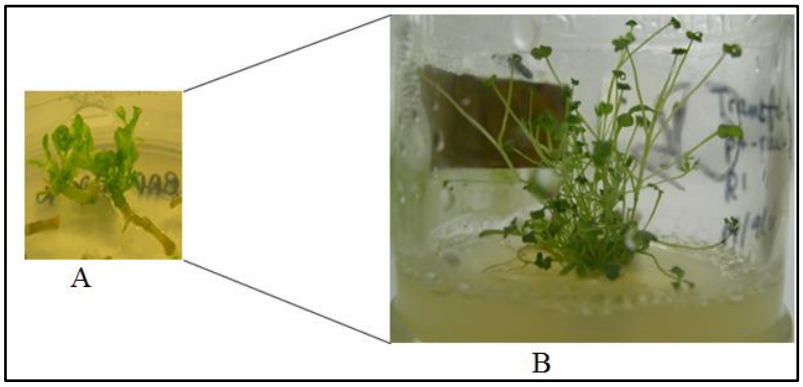


Figure 93. New leafy stems grown from potentially transformed hypocotyls before (A) and after (B) transfer on the new selective media

3.4.2 Molecular characterization of transgenic plants

Molecular characterization is the verification process of the presence of the transgene in the genome of the putative transformed plants. To perform this molecular characterization I used a PCR approach in order to amplify and therefore to highlight the presence of the transgene in the genome of the plant. Since the PcPt plants are growing very slowly, and a small quantity of plant material was available. Extraction of genomic DNA using a "classical way" was therefore not possible. To overcome this problem I used the « Phire plant direct PCR kit », which had been developed to perform PCR directly on very small amounts of plant material without doing a DNA extraction. The use of this kit has been reported in the "Material and Methods" chapter.

To realize the molecular characterization of transformed PcPt plants, we performed a PCR directed against the two different genes present on the T-DNA: the *nptII* gene and the 35S-PcPt DNA fragment. DNA of non-transformed agrobacteria was utilized as negative control to verify the absence of any type of amplification for above described primer pairs. For the pBIN-GW plants, this PCR was only directed against the *nptII* gene. The PCR conditions used were as described previously for *nptII* in the "Material and Methods" part. While, the *35S-PcPt* transgene was amplified using specific primers displaying 77 °C as annealing temperature (Table 18). As described in (Figure 94 and Figure 95 A) all the control plants tested, got the *nptII* gene listed in (Table 23), however I couldn't amplify the PcPt gene on all the plants supposed to be transgenic for this gene (Figure 95 B). The Results obtained for the molecular characterization of transgenic plants with PcPt are summarized in (Table 24).

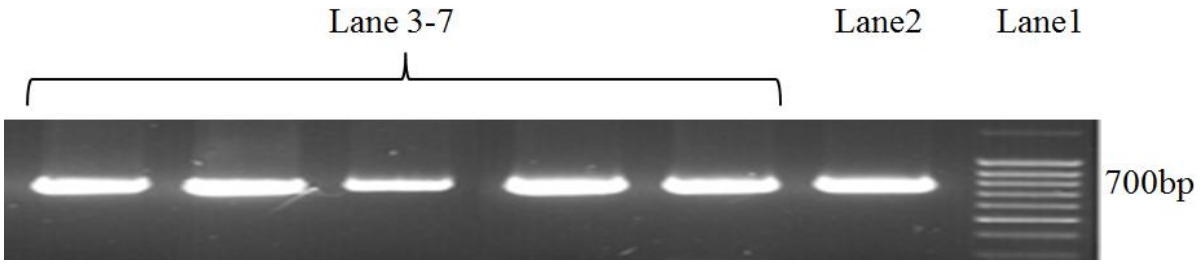


Figure 94. Agarose gel analysis of PCR-amplified *nptII* genes from pBIN-GW transformed *R. graveolens*

PCR was performed using primers *nptII*Dir and *nptII*Rev. The observed bands are of expected size (800 bp). Lane 1: (MM is the molecular marker), Lane 2: (+ve C is the positive control using the original plasmid as template) and Lane 3-7: (five different transformed plants of *R. graveolens* for the construction pBIN-GW (*nptII*)).

Table 23. Summary of the results of amplification of gene *nptII*

Transgenic *R. graveolens*	*neptII*
T1-Pbin void-1	+
T1-Pbin void -2	+
T1-Pbin void -3	+
T1-Pbin void -4	+
T1-Pbin void -5	+
T1-Pbin void -6	+

DNA samples for PCR were prepared through Phire plant direct PCR kit from leaves of potentially transformed plants of *R. graveolens*

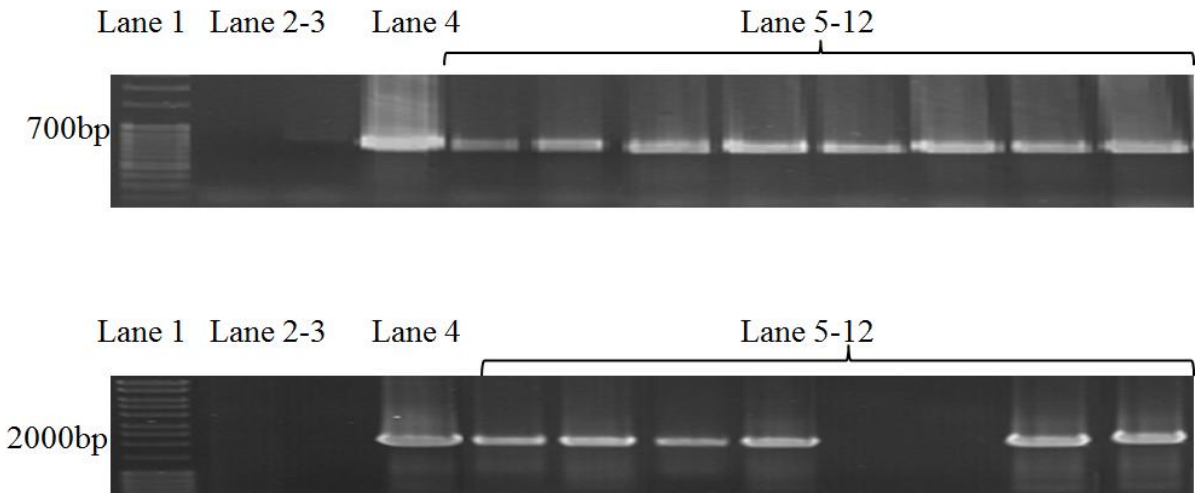


Figure 95. Agarose gel analysis of PCR-amplified *nptII* and 35S-PcPt genes in pBIN-GW-PcPt transformed *R. graveolens*

(A) PCR done on *nptII*. Expected size: 700 bp. Lane 1: (MM is the molecular marker), Lane 2 and 3: negative control (reaction mixture without DNA template and wild type plant), Lane 4: positive control (recombinant plasmid), Lane 5-12: (eight different transformed plants of *R. graveolens* for the construction pBIN-GW (*nptII*)). (B) PCR done on the 35S-PcPt gene. Expected size (2000 bp) Lane 1: (MM is the molecular marker), Lane 2 and 3: negative control (reaction mixture without DNA template and wild type plant), Lane 4: positive control (recombinant plasmid), Lane 5-12: (eight different transformed plants of *R. graveolens* for the construction pBIN-GW (35S-PcPt)).

Table 24. Summary of the amplification results for *nptII* and 35S-PcPt

Transgenic *R. graveolens*	*neptII*	35S-PcPt	Transgenic *R. graveolens*	*neptII*	35S-PcPt
T1-pt-per-1	+	+	T1-pt-per-6	+	+
T1-pt-per-3	+	+	T1-pt-per-11	+	+
T1-pt-per-7	+	+	T1-pt-per-13	+	-
T1-pt-per-9	+	+	T1-pt-per-16	+	-

PCR was done using the Phire plant direct PCR kit on potentially transformed leaves of *R. graveolens* plants.

The results we obtained made evidence that among the 8 tested plants, only 6 contained the whole T-DNA integrated in their genome, while 2 had only partially integrated T-DNA (only the *nptII* could be detected).

3.4.3 Relative Expression level of PcPt assessed by real time PCR

The second step of the characterization of the transgenic plants consisted to check if the gene is really expressed. For this purpose I started to analyze the presence of the corresponding mRNAs through Real Time quantitative PCR.

Each transformed plant was collected and the material divided into two parts. The first part served for the quantification of coumarins/furanocoumarins (see next 3.4.4) while the second part was used for the mRNA quantification.

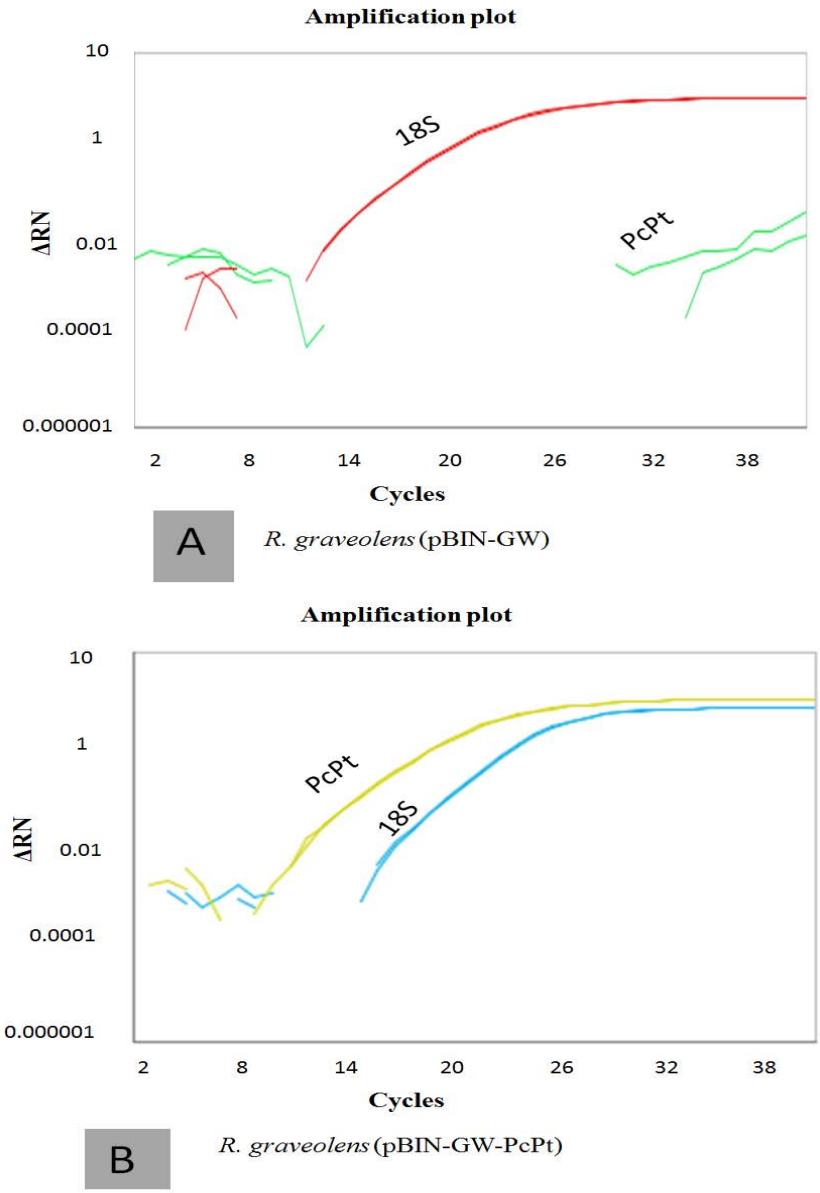


Figure 96. Real time PCR observed for the expression pattern of PcPt together with the 18S housekeeping gene in A) control plantes (void pBIN-GW) and B) PcPt transgenic plants (pBIN-PcPt)

Total RNA were extracted from *R. graveolens* (pBIN-PcPt and void pBIN-GW) as described previously. Specific primers directed toward the PcPt ORF and toward a housekeeping gene (18S) were designed using the Primer Express 3.0 software. Sequences of these primers along with the real time PCR conditions are described in the "Material and Methods" chapter. The results obtained are consistent with what was expected and provided the evidence for the expression of RNA of PcPt only in positive transgenic plant samples (Agro-pBIN-PcPt) whereas it couldn't be detected in control plants (Agro-pBIN-GW) (Figure 96). While in controleed plant only some amplication was observed that perhaps due to the non-specific hybridization of primers (Figure 96 A). The expression level is nearly the same in all the plants which were tested for the presence of transgene (Agro-pBIN-PcPt).

3.4.4 Quantification of coumarins and furanocoumarins

To study the effects of the expression of PcPt on the metabolic profiles of coumarins and furanocoumarins in transformed plants, a quantitative analysis was carried out for the concentration 14 different molecules (Table 25).

Table 25. List of the different molecules which were quantified in PcPt transgenic plants by using a LCMS /MS approach

Coumarins and Linear Furanocoumarins	Angular furanocoumarins
1. Umbelliferone	2. Osthenol
3. Demethylsuberosin	4. Columbianetin
5. Marmesin	6. Angelicin
7. Psoralen	8. Sphondin
9. Xanthotoxol	10. Pimpinellin
11. Xanthotoxin	
12. Bergaptol	
13. Bergapten	
14. Isopimpinellin	

Different techniques and strategies could be used to perform these analyses. The easiest one consists to do a simple HPLC analysis. In our case, the different molecules are present in various concentrations ranging from low to very high, which make this approach less efficient. Thus, in order to quantify the different molecules in these extracts, another technique consisted to use an LCMS/MS approach. This technique allows the unambiguous identification of a molecule, as well as its subsequent quantification, even if the concerned molecule is present in very small concentration. To adapt this approach to our expectation,

preliminary analyses were performed using non-transformed plants. These analyses confirmed that a lot of molecules were present in the plant extracts at various concentrations. Some molecules such as psoralen were highly concentrated, while other molecules such as Umb could be detected in very small amounts. This makes sense since several molecules are intermediates and are readily converted to other forms. In order to realize accurate measures, I prepared two different series of samples prepared from a master preparation. In the first series, the master sample was diluted 10 times to precisely quantify the molecules present in smaller quantity and for the second series, the dilution was 30 times to measure the precise amount of molecules present at higher concentration (protocol described in the "Material and Methods" chapter.

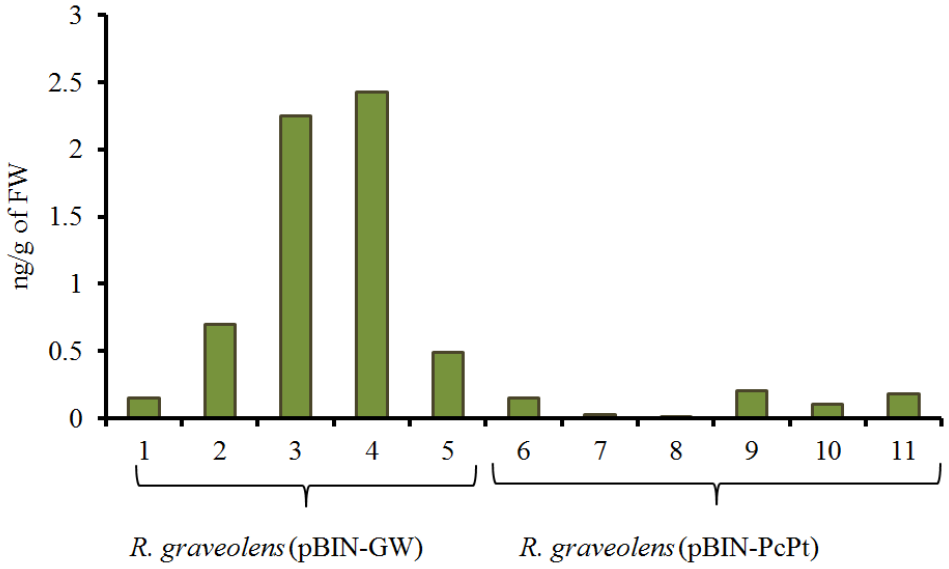


Figure 97. Umb content in plants transformed with the void pBIN-GW (1 to 5) and with the pBIN-PcPt (6 to 11) plasmids

In a first attempt, I focused on the concentration of Umb which has been demonstrated to be a putative substrate. The results we obtained reported in (Figure 97) are consistent with the experiments done by transient expression in *N. benthamiana*. The difference between all the plants can be related to the insertion site of the T-DNA but also to the fact that the different plants result from individual transformation events on plants which are not homozygous. The mean value shows a concentration of 1.20 ± 0.879 $ng.g^{-1}$ FW Umb in the control plants and this concentration dropped to 0.114 ± 0.079 $ng.g^{-1}$ FW in the case of PcPt plants. This was expected since Umb should be transformed into DMS and osthenol when the enzyme is overproduced. In order to check if these differences are statistically significant, the

collected values for all these different plants were further used to run a statistical analysis. Thus, a T-test demonstrated that the Umb content in transgenic PcPt plants were significantly different ($p < 0.05$) from that of control plants.

Once we had demonstrated that the concentration of Umb decreased, I had to investigate in which molecule it could be metabolized. Therefore, other molecules were quantified using the same approach than above. The results I obtained are summarized in (Table 26). All the molecules sought could be detected except xanthotoxol and bergaptol. Osthenol was detected at very low concentration in both kinds of plants. This was the only detected molecule from the "angular furanocoumarin" pathway which is consistent with our expectations. We carried out a statistical analysis for all the plants in order to compare the results obtained for PcPT plants and for void pBIN-GW plants. This analysis made evidence that there was only a slight difference for the concentration of osthenol (tendency: $0.05 < P < 0.1$). No difference was observed for all the other molecules investigated.

Table 26. Selected coumarins/furanocoumarins investigated in control (pBIN-GW) and PcPT transgenic plants

Molecules	Control plants ($ng.g^{-1}$ FW)	PcPT Plants ($ng.g^{-1}$ FW)	T-Test
Demethylsuberosin	0.834 ± 0.536	0.411 ± 0.1811	$P > 0.1$
Osthenol	0.275 ± 0.157	0.337 ± 0.186	$0.05 < P < 0.1$
Marmesin	1.590 ± 0.876	1.153 ± 0.527	$P > 0.1$
Psoralen	6.434 ± 4.341	7.883 ± 3.584	$P > 0.1$
Xanthotoxin	11.702 ± 5.276	9.636 ± 3.509	$P > 0.1$
Bergapten	33.482 ± 15.142	27.299 ± 7.707	$P > 0.1$
Isopimpinellin	2.349 ± 0.883	2.112 ± 0.812	$P > 0.1$
Xanthotoxol	ND	ND	
Bergaptol	ND	ND	

Where ND means not-detcted.

These results were somewhat surprising. The absence of difference between the PcPT and the control plants for the concentration of most of the molecules described in furanocoumarin pathway is intriguing and led us to do some additional experiments. We first thought that these molecules might be stored in a glycosylated form. I realized a new series of analyses taking care of these other kind of molecules. These new analyses, however, were

unsuccessful since no difference between both kinds of plants could be observed. Our second hypothesis was that these molecules could be further prenylated and may be excreted in the growth media. As stated before, no other prenylated molecule was detected in the plants. Therefore we performed an analysis on 100 mg of media. I extracted the phenylpropanoids content of the gel following the protocol described in the "Materials and Methods" part. I collected the media from five control plants and 6 PcPT plants. The analyses done couldn't detect any difference among all the media tested (data not shown).

In this part we could make evidence that the PcPT is efficiently expressed in transgenic plants. This overexpression is accompanied by a decrease of the umbeliferone content which is the natural substrate of the enzyme. This latest result is consistent with transient expression experiments done *in vivo* and *in vitro* in *N. benthamiana*.

3.4.4.1 Is there a relationship between a tissue specific expression pattern of PcPt and the coumarin/furanocoumarin content in Parsley (*P. crispum*)?

All the experiments done so far were performed in a heterologous expression system: transient expression in *N. benthamiana* and stable expression in *R. graveolens*. Furthermore, these expressions were realized thanks to a strong and constitutive promoter. These conditions are rather far away from the physiological conditions. Therefore, to complete my work I started to analyze the expression pattern of the PcPt gene in the plant of its origin, ie Parsley. These analyses were focused on two different aspects. The first question I wanted to answer was: is it possible to induce the expression level of the gene along with the synthesis of coumarins? The second question was: is the gene expression pattern/coumarin production different regard to the plant organs? Recently, Vialart and collaborators demonstrated that UV light could induce the expression of the *p*-coumaroyl CoA 2'-hydroxylase (C2'-H) in *R. graveolens*, an enzyme involved in the furanocoumarin pathway and catalyzing the synthesis of Umb (Vialart *et al.,* 2012). Thus, I chose the same approach to try to induce the expression of *PcPt* and enhance the production of any coumarin/furanocoumarin.

P. crispum plants were cultured for 1-2 months in growth chamber using controlled conditions (see "Material and Methods"). 18 different plants were selected and separated in 6 different pools. Three pools were exposed to UV light for 7 hours before being stored in normal conditions for 8 additional hours to allow the plants to produce coumarins and

furanocoumarins. Finally, 15h after the beginning of the experiment, the plant material was harvested and separated, taking care to separate leaves, roots and stems. The 3 pools of non-treated plants were collected in the same way and at the same time. Each organ sample was further divided into two parts in order to perform either a phenylpropanoid or a RNA extraction.

3.4.4.1.1 Analysis of the coumarin and furanocoumarin composition in *P. crispum* plants

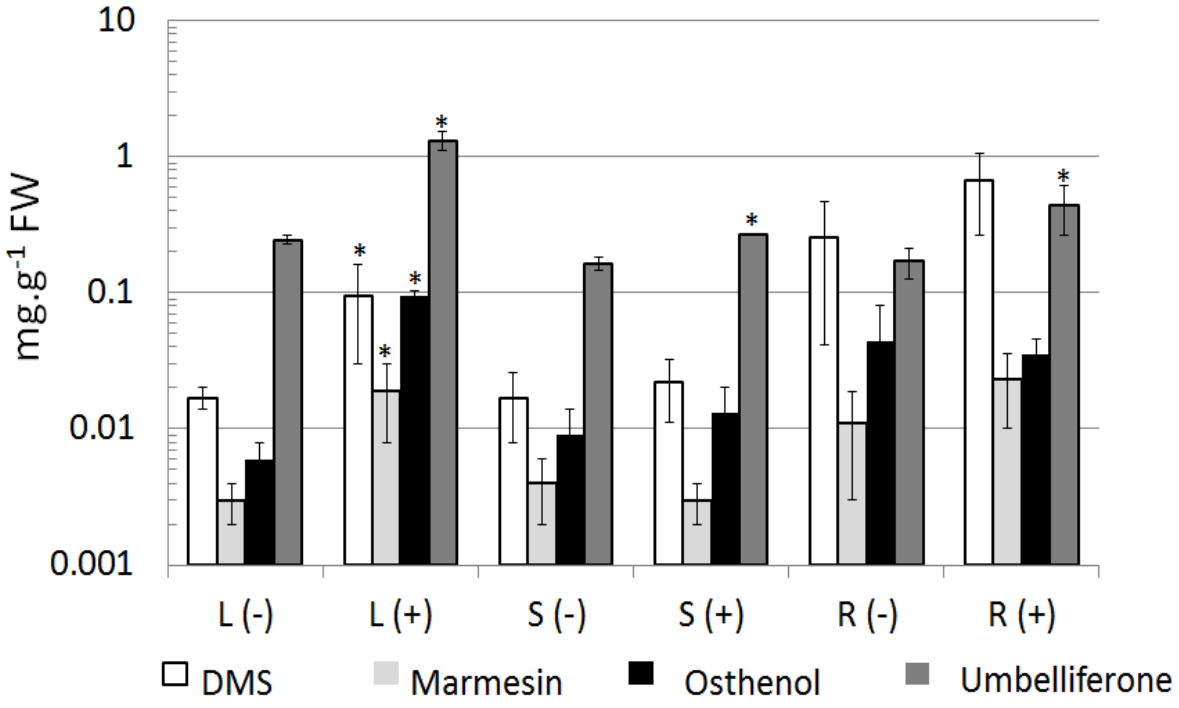


Figure 98. Quantitative analysis of coumarins (Umb, DMS, osthenol and marmesin) in leaves, roots and stem of *P. crispum* with and without UV stress

Quantitative analysis of coumarin content was carried out in the leaves (L), stems (S) and roots (R) of 6 independent pools of plants of *P. crispum*. These analyses were realized on UV treated plants (+) and compared to control plants (-). A T-Test was performed to compare each (+) / (-) result. * means that $p < 0.05$.

Total coumarins and furanocoumarins were extracted from the 18 pools following the protocol described in the "Material and Methods" chapter. For each extraction, I analyzed 2 different dilutions. A non-diluted series was dedicated for the analyses of molecules present in low concentration whereas the second one (10 time dilution) was used for the quantification of molecules present in higher concentration. The molecules were separated and analyzed by LC-MS. For this study, we focused on the 14 different molecules already analyzed previously

(Table 26) which were quantified with the help of standard curves which were obtained by analyzing a range of various concentrations of each molecule (commercially available) as described in the "Material and Methods" chapter.

The results I obtained for this experiment are summarized in (Figure 98 and Figure 99).

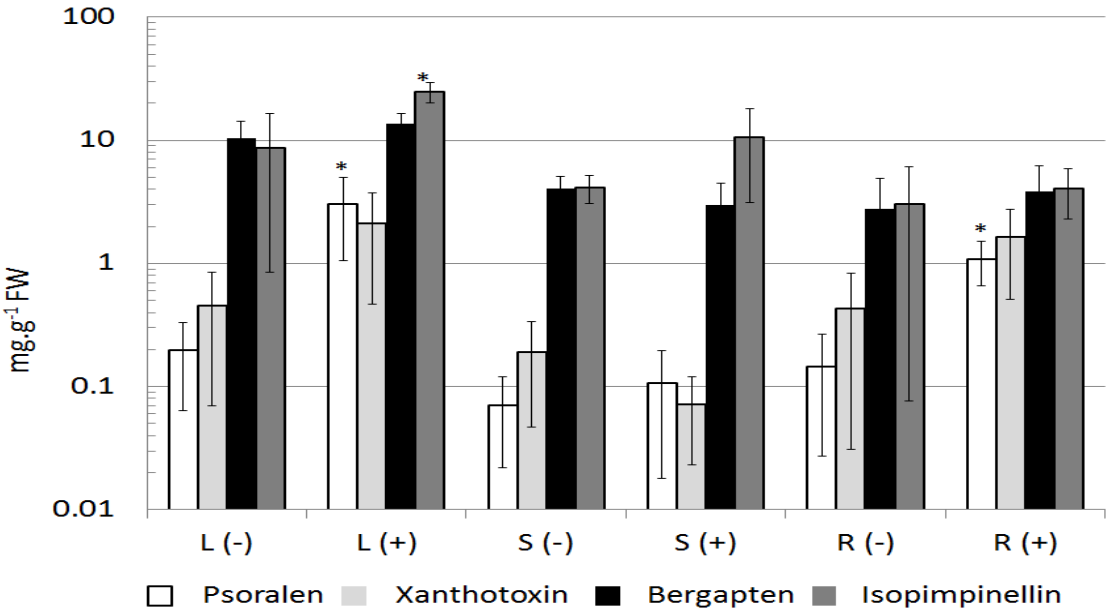


Figure 99. Quantitative analysis of furanocoumarin (psoralen, 8-MOP, 5-MOP and isopimpinellin) content in leaves, roots and stem of *P. crispum* with and without UV stress

Quantitative analysis of furanocoumarin content was carried out in the leaves (L), stems (S) and roots (R) of 6 independent pools of plants of *P. crispum*. These analyses were realized on UV treated plants (+) and compared to control plants (-). A T-Test was performed to compare each (+) / (-) result. * means that $p < 0.05$.

Among the various linear and angular furanocoumarins that were investigated and quantified in the different extracts, xanthotoxol and bergaptol could not be detected. Angular furanocoumarins have never been reported so far in parsley. In this study we could highlight the presence of osthenol (Figure 98) which could be consistent with the analysis done previously in the heterologous expression experiments of *R. graveolens* for an overexpression of the PcPt. Osthenol and DMS, were shown to be more concentrated in roots than in leaves and stems. In addition, osthenol was less distributed than DMS in all the observed organs (Figure 98).

Apart from these results regarding DMS and osthenol, the study of other furanocoumarins has highlighted some interesting features. 5-MOP and Isopimpinellin, and to

a lesser extent Umb and 8-MOP, are the most prominent furanocoumarins observed. The distribution of all the tested molecules in plants is not the same. For example, Marmesin was among those furanocoumarins that were found to be more concentrated in roots than in stems and leaves (Figure 98). For their part, Umb (Figure 98) and psoralen (Figure 99), under controled conditions, were more concentrated in leaves, than roots and were found to be lowly concentrated in stems. Concerning the three other linear furanocoumarins 8-MOP, 5-MOP and isopimpinellin, they were mainly distributed in leaves rather than in stems and roots (Figure 99).

In addition to the distribution of coumarins and furanocoumarins in various parts of plants, a comparison was made between the UV-treated and non-treated plants using a statistical analysis. These analyses demonstrated that UV exposure resulted in a significant increase in Umb content in all the three tested parts of plants i.e. leaves, roots and stem (Figure 98). Similar analyses were made for the contents of prenylated molecules (DMS and osthenol). These comparisons provided the evidences that the UV exposure resulted in a significant increase ($p < 0.05$) in DMS contents only in leaves of *P. crispum*. No significant effect on the DMS contents of the other two parts (roots and leaves) was observed. Similar results were observed for osthenol contents where the significant increase was observed only for the leaves ($p < 0.05$) (Figure 98). In addition it was observed that UV stress caused an almost similar increase in DMS and osthenol contents of leaves. Surprisingly, an increase of DMS (but not osthenol) could also be observed in roots when the leaves were exposed to UV light even if this increase is not statistically significant.

Concerning the furanocoumarins that were quantified in the samples, I observed that the concentration of marmesin and isopimpinellin was significantly increased ($p < 0.05$) in the UV treated leaves (Figure 99). For its part, psoralen concentration sharply increased in UV treated leaves but also in roots whereas no effect was observed in stems (Figure 99). Finally, we couldn't detect an impact of the UV treatment on the concentration of 8-MOP or 5-MOP (Figure 99).

3.4.4.1.2 Quantification of the PcPt expression within various organs of *P. crispum*

To find out if there was a correlation between the expression of PcPt and the content of coumarin/furanocoumarin in *P. crispum*, I had to analyze the expression level of this gene. For this purpose, I used a real time PCR approach.

Total RNAs were extracted from the vegetative material of the 18 samples prepared previously (Leaves, stems and roots collected from 3 plants exposed to UV light and 3 control plants). The resulting RNAs were used to synthesize cDNA which were finally subjected to real time PCR according to the conditions and primers that had presented in "Material and Methods". The results described in (Figure 100) were processed with the StepOne software.

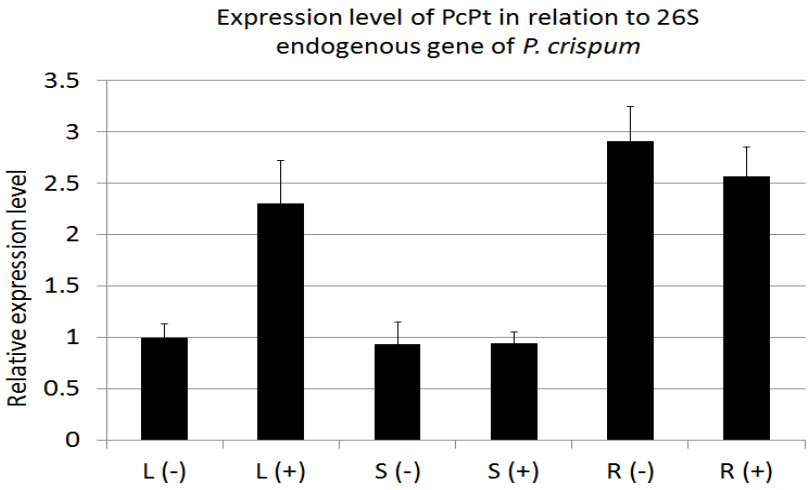


Figure 100. Relative quantification of the expression level of PcPt mRNA performed using leaves, stems and roots of *P. crispum* UV treated or not

Real time PCR realized on RNA extracted from leaves (L), stems (S) and roots (R) of 6 independent pools of plants of *P. crispum*. These analyses were realized on UV treated plants (+) and compared to control plants (-).

These results made evidence that the PcPt was expressed in all organs tested but its expression level was highest in roots. The comparison of the results obtained on UV-treated and control plants, demonstrated that a 7 h exposure of plants to UV light induced a 2 folds enhancement of the expression level of PcPt in the leaves of the treated plants of *P. crispum* (Figure 100) whereas no modification was noticed in stems or root.

When we compare these results with the metabolic analysis, it becomes clear that there might be a correlation between the PcPt expression level and the demethylsuberosin but also osthenol contents in *P. crispum*.

In vivo experiments carried out on *P. crispum* demonstrated that the prenylated coumarins (DMS and osthenol) are most abundant in roots, which is correlated to the expression level of *PcPt*. Furthermore, an exposure of 7 h of plants to UV light almost doubled the expression level of PcPt in leaves and is consistent with the increased contents of prenylated molecules in treated leaves

3.4.5 Metabolic engineering of the biosynthetic pathway of furanocoumarin in *N. benthamiana*

The establishment of a biosynthetic pathway is a matter of cost for plants but also a matter of organization of metabolic fluxes. To investigate this costs and fluxes, we considered to reconstruct a complete biosynthetic pathway in a plant that does not contain it before and to compare the transiently transformed plants to wild type plants. To achieve this goal, the first step is to collect all the genes involved in the considered pathway.

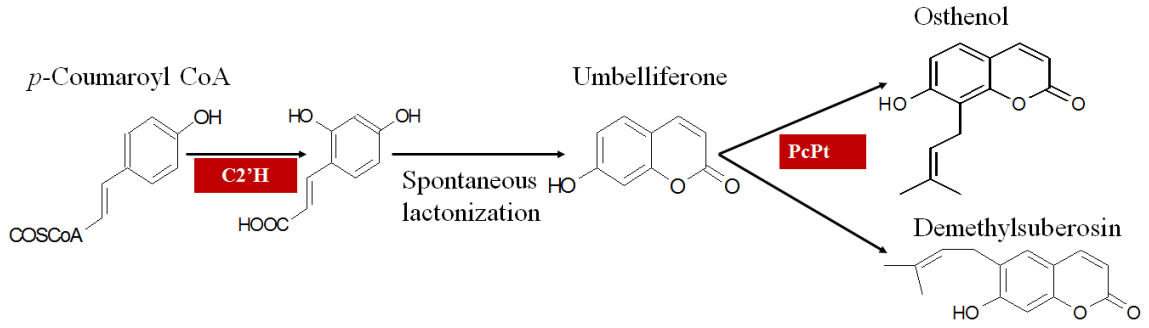


Figure 101. Hypothesized metabolic engineering of biosynthetic pathway of furanocoumarins in *N. benthamiana*

In our research group we are working with the furanocoumarin biosynthetic pathway since a long time, and hence, have characterized some of the genes involved in this biosynthetic pathway. Recently a *p*-coumaroyl CoA 2'-hydroxylase (C2'-H), was isolated and characterized from *R. graveolens* (Vialart *et al.,* 2012) and was shown to be involved in the biosynthesis of Umb. The same authors demonstrated that the *in vivo* transient expression of C2'-H in *N. benthamiana* system worked very well to realize the functional characterization. This led us to hypothesize that the co-expression of both C2'-H and PcPt in the presence of

p19 could result in the production of DMS and osthenol without the exogenous supplementation of substrates necessary for the PcPt activity (Figure 101) which would be the very first step for the reconstruction of the whole furanocoumarin metabolic pathway.

3.4.5.1 Co-infiltration of tobacco leaves with Agro-pBIN-p19, Agro-pBIN-C2'H and Agro-pBIN-PcPt

These experiments were carried out following the protocol already described in chapter 2 of the "Results" part (under heading 2.4.2). Recombinant agrobacteria strains containing the 3 different genes p19, C2'H and PcPt were prepared. They were mixed together with a final OD_{600nm} value 0.2 for Agro-pBIN-void, Agro-pBIN-C2'H and Agro-pBIN-PcPt, while 0.4 for Agro-pBIN61-p19. A mix of Agro-pBIN-P19, Agro-pBIN-C2'H and Agro-pBIN-PcPt were infiltrated to leaves. Three negative control samples were prepared by mixing different agrobacteria i.e., 1) Agro-pBIN61-p19 + Agro-pBIN-void, 2) Agro-pBIN-P19 + Agro-pBIN-C2'H and 3) Agro-pBIN-P19 + Agro-pBIN-PcPt. All samples were collected 96 h post infiltration.

3.4.5.2 Analyses of samples by mass spectrometry

After extraction, the different samples were analyzed by LCMS. We first checked the presence or absence of four different molecules: Umb, scopoletin, DMS and osthenol. In a second step we quantify them using the same protocol and procedure explained for quantification of phenylpropanoids contents in plant extracts in "Materials and Methods" part. The results we obtained were surprising and are presented in (Figure 102). We couldn't detect any of the above listed molecules in the samples prepared from Agro-pBIN61-p19 + Agro-pBIN-void except some traces of Umb. We could clearly observe a significant amount of Umb and scopoletin in samples where the p19 and the C2'H were expressed together, which agreed with the results presented in Vialart's work (Vialart *et al.,* 2012). Quantification of phenylpropanoid content of these samples demonstrated the presence of 10.18 ± 3.42 $ng.g^{-1}$ FW of Umb and 2.32 ± 1.08 $ng.g^{-1}$ FW of scopoletin respectively.

The analyses of samples infiltrated with Agro-pBIN-P19 + Agro-pBIN-PcPt, didn't allow us to detect Umb, scopoletin, DMS and osthenol. These results supported the fact that the potential substrate (Umb) of the PcPt was not available in plants. However, when the C2'H is produced along with the PcPt and the p19 we could highlight the presence of Umb and osthenol but also observe quantifiable amounts of DMS. Quantification of these

molecules in the samples was completed using LCMS/MS and we found 3.00 ± 0.74 $ng.g^{-1}$ FW of Umb, 0.93 ± 0.33 $.g^{-1}$ FW of scopoletin and 3.93 ± 1.95 $ng.g^{-1}$ FW of DMS respectively. Osthenol was also detected in these samples but only in trace amounts.

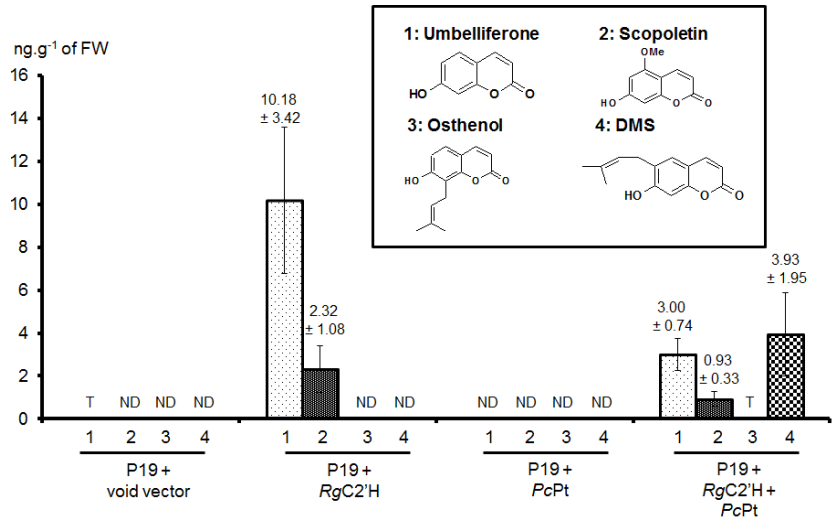


Figure 102. Metabolic pathway reconstruction using transient expression of proteins in *N. benthamiana* leaves

Void vector: pBIN-GW, *Rg*C2'H: *p*-Coumaroyl CoA 2'-Hydroxylase from *Ruta graveolens*, PcPt: Pt from *Petroselinum crispum*. ND: Not Detected, T: Trace amounts, concentration below the limit of quantification. Each value is the result of 4 independent experiments.

3.4.6 Conclusion and Discussion

Furanocoumarins are important molecules for the interaction of plants with their environment. But also have pharmaceutical importance and could therefore be used for the development of new medicines. Due to these facts, to develop the better understanding of biosynthetic pathways of these molecules in plants is of higher interest for researchers. Aromatic prenylation, "addition of a hydrophobic prenyl moiety to an aromatic substrate", is an important step in this pathway that results not only in the addition of DMAPP to Umb but also constitutes the entry step into biosynthetic pathway of furanocoumarins. Different studies based on the biochemical characterization of enzyme reported the presence of the enzymatic activity of prenylation in the extracts prepared from *R. graveolens* and *A. majus* (Dhillon *and* Brown, 1976; Ellis *and* Brown, 1974; Hamerski *et al.,* 1990; Munakata *et al.,* 2012), possibly located in chloroplast (Dhillon *and* Brown, 1976). However, none of these genes involved in the synthesis has neither been identified nor characterized till today. Recently a study published by Sasaki and his collaborators (Sasaki *et al.,* 2008) has contributed to identify and characterize the first aromatic Pt of plant implicated in the biosynthesis of prenylated

flavonoids. These authors demonstrated that a Pt, naringenin 8-dimethylallyltransferase (*Sf*N8DT-1), is involved in the biosynthesis of sophoraflavanone G (a flavonoid) in *S. flavescens*. Since this pioneer study, a number of other flavonoid Pts have been reported in literature (Akashi *et al.,* 2009; Sasaki *et al.,* 2011). But in contrast, no gene playing a role in the biosynthesis of prenylated coumarin had been identified on the molecular level so far.

Based on the idea that the enzymatic activity of *Sf* N8DT-1 Pt is close to the Umb Pt activity, we used this gene/protein as a probe to identify different candidate genes from a number of furanocoumarin producing plants. Based on a bioinformatic analyses, all of these candidate genes seem to belong to the same separate class of Pts which we assume to be aromatic Pts. In this chapter, I functionally characterize one of these candidate genes which was isolated from *P. crispum* and named as PcPt. To realize the functional characterization of this gene we first decided to use the *N. benthamiana* heterologous expression system which was developed in the previous chapter (under heading 2).

The recombinant agrobacterium strains where infiltrated in leaves. In order to investigate the function of the enzyme, we performed a secondary infiltration of a mix of different kind of substrates. Two mixtures containing two different subgroups of phenylpropanoids were prepared. The first one was made up of coumarins and furanocoumarins and the second one with flavonoids (Table 19). By this way, a number of different molecules could be tested simultaneously. The analyses of these samples were oriented toward the identification of prenylated products. These results demonstrated the synthesis of DMS and osthenol (Figure 79) starting from Umb.

To verify the *in vivo* results, *in vitro* characterization of PcPt was carried out using the same expression system but after extraction of the total proteins and subsequent *in vitro* incubation in the presence of different kind of substrates. These *in vitro* experiments provided more information concerning the enzymatic characteristics of PcPt. The first point was the specificity of the enzyme for its substrates. Unlike *Sf*N8DT-1 that could add a prenyl moiety to three different aromatic substrates, this enzyme presented a tight specificity for Umb as aromatic substrate even if, as for *Sf*N8DT-1, DMAPP was the only prenyl moiety introduced in the product (Table 20) (Figure 80). These results agree with the results of biochemical studies carried out for coumarins Pt of *R. graveolens* by Ellis and Brown thirty years ago (Ellis *and* Brown, 1974). The second important feature was that, this enzyme is able to add the prenyl moiety both on C-6 and C-8 position of Umb leading to the production of both

DMS as major product (serves as precursor of linear furanocoumarins) and osthenol as minor product (serves as the precursor of angular furanocoumarins). Such a prenylation on two different positions of a molecule has, so far, never been observed for plant Pts. This is also in contrast to the results provided by Dhillon and Brown (Dhillon *and* Brown, 1976) for the Umb Pt of *R. graveolens,* which was demonstrated to be specific to C-6 prenylation only, leading to the sole formation of DMS.

In addition to the substrate specificity, other catalytic parameters and optimum conditions of reaction were measured using the protein extracts prepared through the same heterologous expression system. These results demonstrated that the preferred reaction conditions of PcPt for maximum enzymatic activity (optimum pH and presence of metal ion) were almost identical to other aromatic Pts (Dhillon *and* Brown, 1976). Furthermore, calculated apparent catalytic parameters, Km_{app} and $Vmax_{app}$ (Table 21) were found to be very close to that reported for other aromatic Pts of flavonoids (Akashi *et al.,* 2009; Sasaki *et al.,* 2008; Vitali *et al.,* 2004; Yazaki *et al.,* 2002).

Psoralen was identified as a molecule that could drastically reduce the activity of PcPt enzyme (Figure 87). The study of the inhibition mechanism demonstrated that it was an un-competitive inhibition type (Figure 88 and Figure 89). Linear furanocoumarins are allelochemicals and are known for their phototoxic properties. Their toxicity could be explained due to their ability to form covalent adducts with double stranded DNA, and therefore block the replication of DNA (Ojala *et al.,* 2000; Wber *and* Kittler, 1977). This molecule has also been described to inhibit cytochrome P450s (Guo and Yamazoe 2004) (Gravot *et al.,* 2004). Thus it is obvious that these molecules are toxic to all organisms especially for the plants that produce them in higher concentrations. The experimentally determined *Ki* values concerning to the inhibition of PcPt by psoralen for the DMS and Osthenol were higher than the measured values of apparent Km_{app}. These results demonstrated that the affinity of PcPt is less important for psoralen compared to its substrate. However it is conceivable for psoralen to control the activity of PcPt by a negative feedback inhibition mechanism when it is present in an excessive concentration that could be harmful for the plant cell. This negative feedback mechanism could regulate the biosynthetic pathway of furanocoumarins and adjusts the amounts of plant furanocoumarins. *P. crispum* has been reported as a plant that constitutively synthesizes furanocoumarins in all plant parts (Chaudhary *et al.,* 1986; Eckey-kaltenbach *et al.,* 1994) and hence the presence of a negative feedback mechanism is strongly supported for plant health. These results are supported by

studies (Tietjen *et al.,* 1983) which reported that *P. crispum* generally accumulated psoralen as major furanocoumarins and hence could regulate the production of furanocoaumarins in plants by negative feed back mechanism. In addition, these agree with results presented by vialart for the inhibition of another enzyme of this biosynthetic pathway (C2'-H) by psoralen (Vialart *et al.,* 2012)

Different types of enzymes are involved in the biosynthetic pathway of furanocoumarins. Most of these enzymes belong to the cytochrome P450s enzymatic family. However, other classes of enzymes were also found to play a role in this pathway including dioxygenases, Pts and methyltransferases. Two different cytochrome P450s of this pathway (marmesine synthase and psoralen synthase) have been localized in the endoplasmic reticulum (Hamerski and Matern 1988). Sazaki and collaborators demonstrated that the Sophora Pt was localized close to the plastids (Sasaki *et al.,* 2008). Our Japanese collaborators (Ryousuke Munakata and Prof. Kazufumi Yazaki) used the same system to investigate the subcellular localization of the PcPt. For this purpose, they fused the transit peptide (Tp) of PcPt to the GFP, and the gene was placed under the control of the 35S promoter. This construction was introduced in onion cells by particle bombardment. The analysis of the expression pattern demonstrated that the fluorescence of PcPt-GFP was localized in dot shaped organelles, whose sizes and shapes were identical to that of isoprene synthase, a typical plastid protein, used as a positive control (Figure 103). These results suggested that the PcPt was localized to plastids. Similar results were presented by other studies for aromatic Pt of other plants (Biggs *et al.,* 1990; Dhillon *and* Brown, 1976).

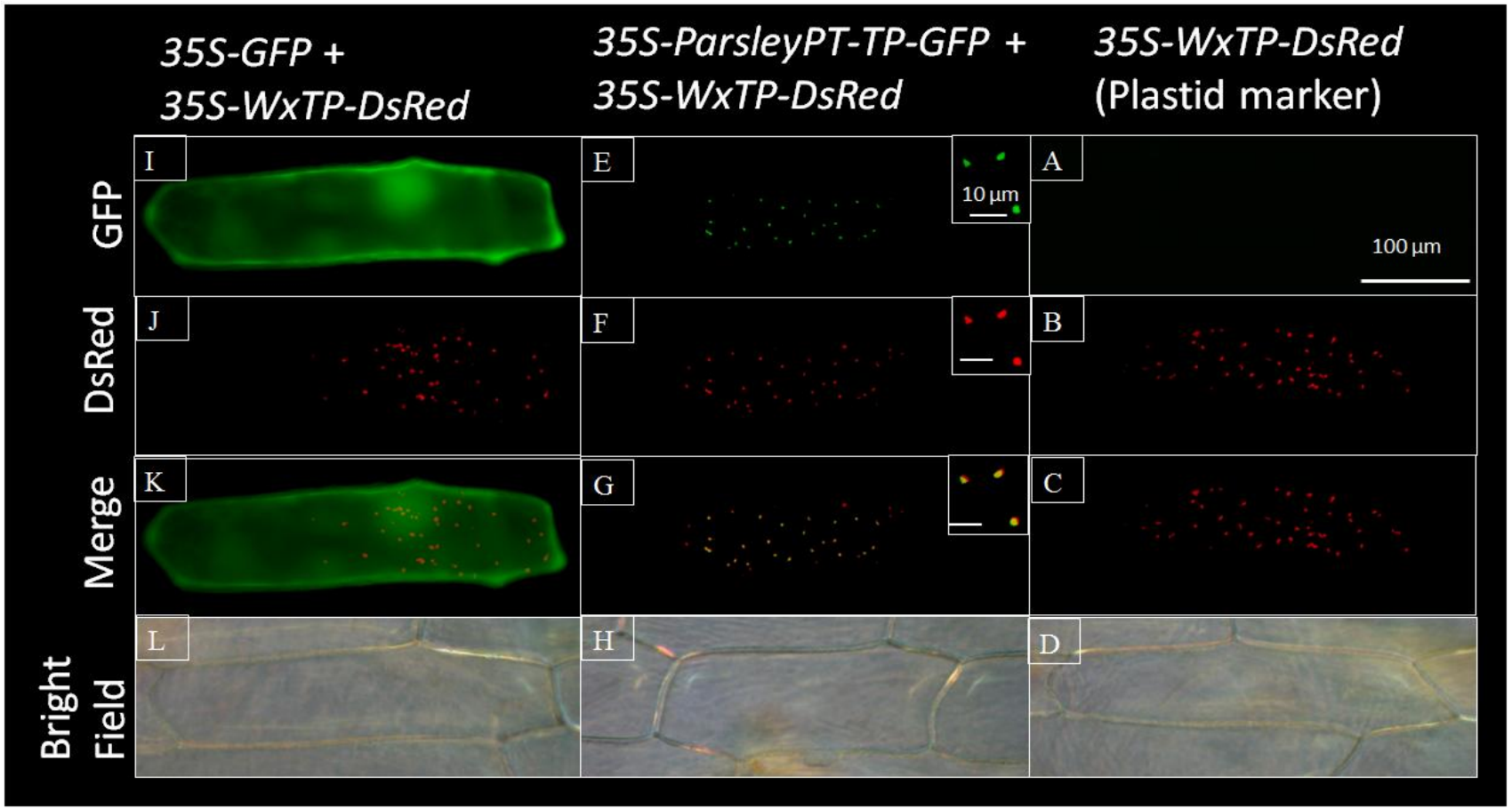


Figure 103 : Subcellular localization of PcPt-GFP fused protein by transient expression in onion cells

Plasmid containing the PcPt (TP)-GFP was introduced into onion cells (E-H) by particle bombardment. Scale bars utilized are 100µm and 10µm as shown in the figure. (A-D); way gene trans peptide (WxTP) fused with RsRed (Red flouresecent protein (DsRed)) as the plastid targeted control, (I-L); expression of non-fused GFP and plastid targeted marker (WxTP-DsREd) and (I-L) main combination and expression of TP-PcPt-GFP +WxTP-DsRed.

Furanocoumarins are molecules of pharmaceutical importance i.e. they are not only used in dermatology to treat various diseases (psoriasis and vitiligo) (Conforti *et al.,* 2009; Llano *et al.,* 2003) but also in the treatment of T-cell lymphoma (Van Iperen *and* Beijersbergen van Henegouwen, 1997), in the treatment of depression (Chen *et al.,* 2007) and β-thalassemia (Fimiani *et al.,* 1994; Viola *et al.,* 2008). To fulfill these requirements of the pharmaceutical industry these molecules could be either chemically synthesized or extracted from furanocoumarin producing plants by using a large amount of biomass of respective plants. In parallel, it has been observed that chemical synthesis of these compounds is a very expensive process and extraction from plant not very efficient since these molecules are produced in small quantities in plants under natural conditions. Generally furanocoumarins were extracted from bergamot oil (*C. bergamia*) cultivated in Italy (Calabria) and Ivory Coast. However, rapidly decreasing cultivation of bergamot trees provided the idea of development of new efficient plant production system. Milesi and collaborators reported the *Ruta* plant as an efficient candidate for furanocoumarins bio-production due to its higher furanocoumarins contents and best biomass production (Milesi *et al.,* 2001) as compared to other plant families producing the similar type of molecules (Apiacae, Moraceae and Fabaceae). Furthermore, a protocol for developing genetically transformed plant of *R. graveolens* was optimized by Karine Lièvre and used by Sébastien Doerper in their PhD (Doerper, 2008; Lièvre *et al.,* 2005). The overexpression of the PcPT in *R. graveolens* could help not only to understand the physiological function of the gene in the plant but also provide a chance to observe the effects of the overexpression of the gene on the metabolic profiles of transformed plants. I transformed the *R. graveolens* plants with two construction separately: control (Agro-pBIN-GW) and positive (Agro-pBIN-PcPt). Potentially transformed plants were obtained by growing the treated plants on a selective media (containing kan 75mg/L). I started the verification of the presence of the transgene when they reached a specific size (Figure 93). Plants growing in *in vitro* conditions normally pose different problems e.g. contamination and death. In addition, in the case of PcPt, I observed that growth of potentially transformed plant was slow as compared to control plants and that they produce fewer roots. Similar results were observed by Yazaki and collaborators when they tried to construct transformed plants with flavonoid aromatic Pt (Prof.Yazaki, personal communication). However, to overcome the problem of slow growth I selected a system of verification of the presence of transgene in the potentially transformed plants that allows skipping the normal procedure of DNA extraction. I used only a dot size piece of leaf of size (0.50 mm)(Phire Plant Direct PCR kit,

Thermo scientific) to extract the DNA and to perform the PCR. Using this approach I tested five different plants transformed with construction (Agro-pBIN-GW) and found that all of these plants showed a positive amplification for *nptII* gene (Figure 94) and hence justified the growth of these plants on the selective media. On the other hands, in the case of PcPt, eight different plants were tested. Once again all of these plants showed a positive amplification for the *nptII* gene but only six out of eight showed the presence of the PcPt transgene (Figure 95). Further verification of the expression of PcPt gene was done using real time PCR. These results supported the simple PCR results and it was observed that the expression of the transgene was found only in plants which were positive for the previous verification steps (Figure 96). Phenylpropanoids were extracted from the positive plants as it has been done by Sébastien Doerper during his thesis (Doerper, 2008). Quantification of the furanocoumarin content of transformed plant was done using a highly precise and sophisticated technique of LCMS/MS. I could observe a significantly lower amount of Umb in all the plants overexpressing the PcPT gene (Figure 97) in comparison to control plants, which led us to hypothesize that the substrate was metabolized by the enzyme. However, no increase could be observed neither in the quantity of DMS nor in any of the subsequent intermediate or final molecule of the furanocoumarin biosynthetic pathway. Regulation of the production and storage of secondary metabolites is a complex system. As the excess of secondary metabolites could be dangerous for plant, they are often glycosylated and stored in plant cells or tissues (Jones *and* Vogt, 2001; Wang *and* Hou 2008). Keeping in view this fact, I made an analysis for glycosylated forms of all the molecules that were quantified in the previous experiments. Unfortunately, the quantification results demonstrated that there was no difference related to these downstream molecules between control and transgenic plants. Another possibility was that molecules were prenylated and further excreted in the culture media. This was investigated but no differences of quantities were found in the media of the different plants. So far, no real explanation could be found for the non-modification of the concentration of the downstream furanocoumarins in plants. But it was reported that stable expression of an aromatic Pt in a host plant could be affected by various factors and specific properties of respective enzyme could be modified in host specific manner (Sugiyama *et al.,* 2011). In addition to all other explanations, a negative feedback mechanism could be possible to maintain a non-toxic level of furanocoumarins content even after the overexpression of PcPt.

We demonstrated that the PcPt was implicated in the synthesis of angular furanocoumarins (osthenol). In contrast to linear furanocoumarins, angular furanocoumarins

are less widely distributed and are mainly confined to some Fabaceae and Apiaceae plants. Presence of both types of furanocoumarins has been found to be more toxic for plant insects than the presence of linear furanocoumarins alone which provides the evidences of evolutionary adaptation of plants to the new herbivores that have acquired the ability to detoxify psoralen (Wen *et al.,* 2003). Both kinds of molecules are detected in parsnip, whereas only linear furanocoumarins (except osthenol, the intermediate precursor) are present in Parsley. It seems that the step of prenylation is not the limiting step that differentiates the plants having the capacity to produce only linear furanocoumarins from other that could produce both types of furanocoumarins. If the first step of the synthesis of both kind molecules is available in parsley, it seems that all the other steps are lacking but might be present in other apiaceous plants as it is the case for parsnip.

In planta functioning of PcPt was further explored by quantifying the coumarins and furanocoumarins contents in UV-treated and non-treated *P. crispum* plants. These analyses demonstrated the presence of only linear furanocoumarins in plant extracts with the exception of osthenol as only "angular furanocoumarin". Similar results for the presence of linear furanocoumarins were reported for parsley by other studies (Beier *et al.,* 1994; Chaudhary *et al.,* 1986). Distribution of furanocoumarins is unequal in plants. For example for prenylated furanocoumarins, I observed that they were most abundant in roots in comparison to the aerial parts which is correlated to the expression level of PcPt (Figure 98 and Figure 100). Similar results were presented for a flavonoid Pt *Sf*N8DT-1 of *S. flavescens* (Sasaki *et al.,* 2008). In contrast to prenylated molecules and marmesin (Figure 98) that were abundantly present in roots, other furanocoumarins (psoralen, 8- MOP, 5-MOP and isopimpinellin) were found mostly in leaves of *P. crispum* (Figure 99). A study of several species of apiaceous and rutaceous plants families carried out by Zobel and Brown (Zobel *et al.,* 1991) reported that a large proportion of each furanocoumarins was located on the leaf surface in most of plant models used for this study.

UV has been reported as an elicitor that could increase the furanocoumarin contents of plants. A research work carried out by Larbat showed that small increases in furanocoumarin contents could be achieved using UV light (Larbat, 2006). And more recently, it has been demonstrated that UV could induce the expression of C2'-H, a gene of the biosynthetic pathway of furanocoumarins in *R. graveolens*. Similarly in this study I found that the expression level of PcPt in *P. crispum* was positively correlated with the accumulation of

DMS and osthenol in leaves treated with UV light (Figure 98 and Figure 100). These results also provide the evidence of the bi-functionality of PcPt in parsley where it controls the production of both DMS and osthenol. Furthermore, significant increase in the contents of Umb in all three organs of the plants permitted to make the hypothesis that the Umb is perhaps involved in more than one biosynthetic pathway and hence has variable functions. In addition, this could be due to the induced expression of more than one gene that are involved in the synthesis of Umb as it was hypothesized by Vialart *et al.* (2012). Significant increase in marmesin, psoralen and isopimpinellin contents in leaves was also observed (Figure 99), which demonstrated that UV stress could enhance the production of furanocoumarins. A study already proposed that the presence of coumarins and furanocoumarins on the leave surfaces could play an important role for the communication between the plant and its environment (Zobel *et al.,* 1991).

For the metabolic engineering of the biosynthetic pathway of furanocoumarins, I not only used furanocoumarins producing plant but I worked also with a plant that does not produce furanocoumarins naturally. I selected the *N. benthamiana* plant system as a model system for the construction of a partial biosynthetic pathway of furanocoumarins using metabolic engineering approach. I co-expressed two successive genes of this pathway: the former C2'H of *R. graveolens* (recently characterized (Vialart *et al.,* 2012)) which is implicated in the biosynthesis of Umb and the precedent one, PcPt (identified as the Umb Pt) and quatified the amonuts of four molecules Umb, scopoletin, DMS and osthenol. These analyses demonstrated that transient expression of C2'H could result in the appearance of Umb and scopoletin that agreed with the results presented by Vialart (Vialart *et al.,* 2012). Furthermore co-expression of both C2'H and PcPt resulted in the production of prenylated Umb (DMS and osthenol). Among the two prenylated products, DMS was found to be quantifiable while osthenol was detected in trace amount which is consistent with the results obtained by *in vitro* functional characterization of PcPt (reaction was 50 times faster for the production of DMS than for osthenol). These results allowed us to assume that this step is not the limiting step for the synthesis of furanocoumarins but probably the absence of the downstream genes.

To conclude, although furanocoumarins have been reported as important molecules in the plant defense system, very little is known at the molecular level for the biosynthetic pathway of linear and angular furanocoumarins. Results that have been presented in this chapter describe the functional characterization of an enzyme implicated in the biosynthesis of furanocoumarins. Heterologous expression system of *N. benthamiana* was used both for *in vitro* and *in vivo* functional characterization. This system was selected as it has been found to be the best fitted system for the expression of membrane-bound enzymes. These experiments permitted me to characterize the first coumarin Pt identified and isolated from *P. crispum*. This enzyme (PcPt) was found to be able to catalyze the addition of a prenyl moiety (DMAPP) both to the carbon 6 and 8 of Umb to produce DMS and osthenol. I further demonstrated that the activity of this enzyme was inhibited by psoralen through the mechanism of uncompetitive inhibition. Thus psoralen, a furanocoumarin which is highly toxic could control the biosynthetic pathway of furanocoumarins by a negative feedback mechanism in Parsley. In addition to the *in vivo* expression of PcPt, genetically transformed plants of *R. graveolens* were developed which demonstrated that overexpression of PcPT could modify the metabolic profile of plants and supported the results of functional characterization. A positive correlation was found between the expression level of PcPt and the content of prenylated Umb in roots of *P. crispum*. Furthermore, analyses of UV induced plants demonstrated the increased expression level of PcPt in leaves that was positively correlated to the significant increase in contents of prenylated molecules (DMS and osthenol) in leaves. In addition to it, stress caused by UV exposure resulted in increase in contents of other linear furanocoumarins also, which demonstrated the direct implication of PcPt in the biosynthetic pathway of furanocoumarins. Construction of a partial biosynthetic pathway of furanocoumarins using *N. benthamiana* as a heterologous expression system provided a plant model system that could be used to produce higher amounts of furanocoumarins but also to investigate the energetic cost necessary for a plant to develop a biosynthetic pathway.

Chapter IV: Functional characterization of other putative aromatic prenyltransferases

4 Functional characterization of other putative aromatic prenyltransferases

In the first Chapter, I described 7 different candidate genes encoding putative Pts. In chapter 3 I focused on one of these genes "PcPt", and demonstrated its involvement in the biosynthesis of DMS and osthenol. In this chapter I will describe the results obtained for the functional characterization of the remaining 6 putative Pts.

4.1 Cloning of the six aromatic prenyltransferase coding sequence into a plant expression vector (either pBIN-GW or pGWB2)

As described in the first part of the manuscript, these genes were obtained either by a combination of *in silico* and molecular approaches in our laboratory or through a collaboration with Japanese colleagues. They were subcloned into a plant expression vector (pBIN-GW for CliPt, AgPt, RgPt, AngPt, ClPt and pGWB2 for PsPt) using a LR recombination reaction as described previously. The presence of the transgene in the expression vector was assessed is the same way than before (data not shown). Finally the recombinant plasmids containing each Pt were introduced into the LBA4404 agrobacteria strain in order to transiently or stably transform *N. benthamiana* or *R. graveolens.*

4.2 Functional characterization of PsPt

4.2.1 General presentation

The phylogenetic analysis in chapter 1 made evidence that the candidate Pt isolated from parsnip was closed to the one of parsley and shares more than 82% homology. This observation is not so surprising since parsnip and parsley belong to the same apiaceae plant family. However, different work demonstrated that their respective metabolic profiles regard to coumarins and furanocoumarins were clearly different. Whereas only linear furanocoumarins (psoralen, 8-MOP, 5-MOP and isopimpinellin) were described in parsley (Beier *et al.,* 1994; Chaudhary *et al.,* 1986), the analysis of parsnip plants demonstrated the

presence of angular furanocoumarins also (such as angelicin, sphondin and pimpinellin) (Diawara *et al.,* 1995; Ivie *et al.,* 1981; Lombaert *et al.,* 2001).

We demonstrated in chapter 3 that PcPt could catalyze the synthesis of DMS and osthenol. Since prenylation of Umb constitutes the entry step into the biosynthetic pathway either of linear or angular furanocoumarins, we tried to understand if this step is responsible for the difference existing between parsley and parsnip. For this purpose we focused on PsPt and performed its functional characterization.

4.2.2 **Umb, a substrate for PsPt ?**

The recombinant LBA4404::pGWB2-PsPt were grown using the same condition as for the LBA4404::pBIN-PcPT strain. Three sets of *N. benthamiana* plants were selected. One set of 3 plants was agroinfiltrated with LBA4404::pGWB2-PsPt, the second set was inoculated with the LBA4404::pBIN-PcPt strain (positive control) while the last one was infiltrated with LBA4404::void pGWB2 (negative control). In each case, the agrobacteria strains were co-infiltrated along with the pBin61-p19 strain as done in the previous experiments described in chapter 2 and 3 of this manuscript. The leaves were harvested 96 h post infiltration and used for extraction of microsomal proteins following the protocol that had been described earlier in the chapter 2 of "Results" (under heading 2.4.2.3.1).

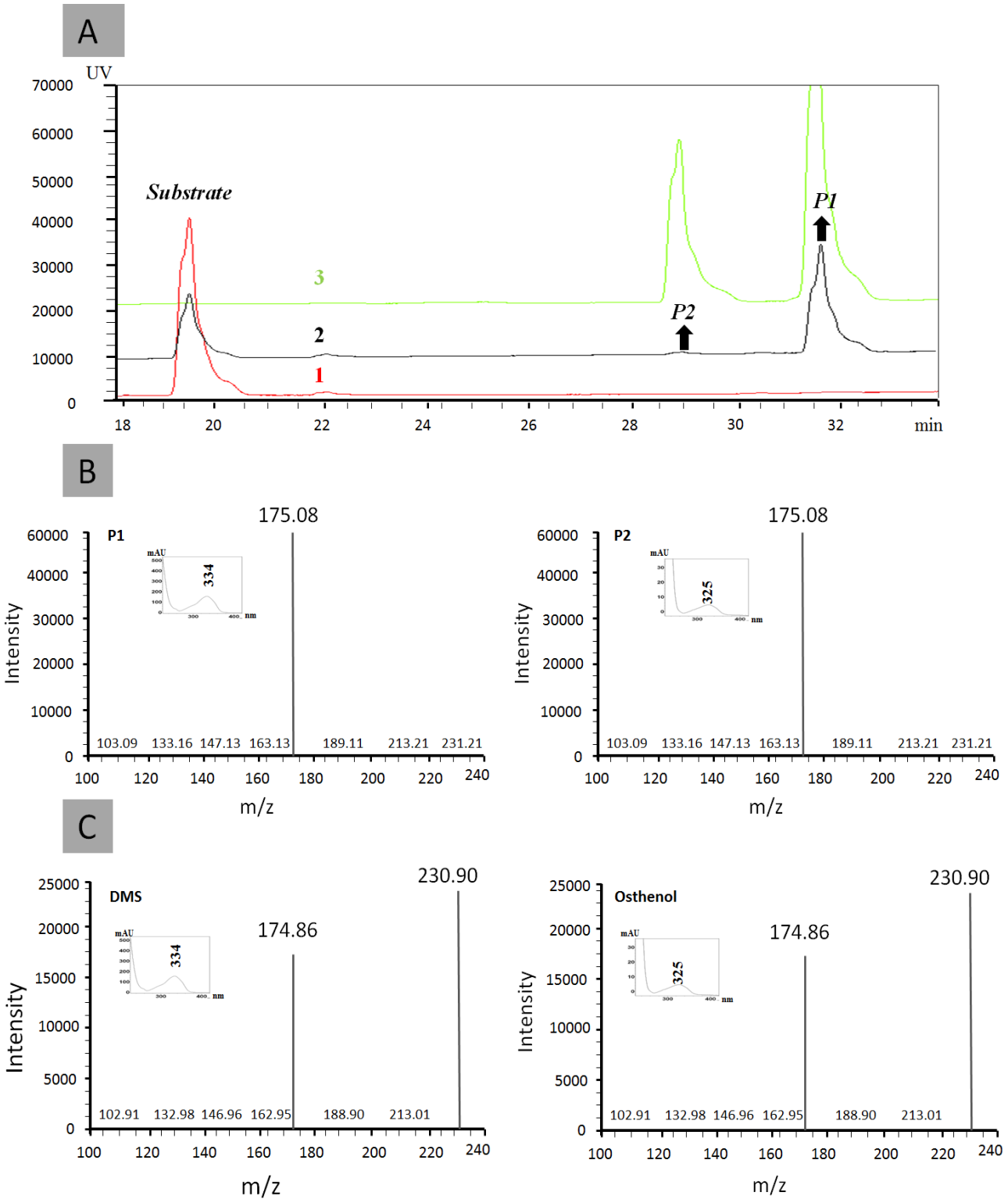


Figure 104. Metabolization of Umb by PsPt

A) HPLC-DAD analysis of a negative control (1), the incubation mix (2), DMS and ostheno (3). B) UV spectrum and mass analysis of P1 and P2 product. C) UV spectrum and mass analysis of commercial DMS and Osthenol.

In contrast to PcPt, I directly focused on Umb (as the aromatic substrate) and DMAPP (as the prenyl moiety). The reaction mixture was prepared as described for PcPt in chapter 3 (3.3.2.1) and the incubation conditions were similar to the one used for the characterization of PcPt. In order to have comparable results, the same incubations were made using the microsomes prepared from plants inoculated with all the recombinant agrobacteria mixes described above.

The reaction mixes were analyzed by HPLC-MS and the results clearly showed a metabolization of Umb in the presence of DMAPP for PcPt and PsPt. In both samples two different peaks were obtained (P1 and P2 product) (Figure 104 A). These molecules have been compared to commercial standards corresponding to DMS and Osthenol (Figure 104 C). The retention time, the UV absorbance and also the mass fragmentation spectrum (Figure 104 B) provided evidences that P1 corresponded to DMS whereas P2 was osthenol as it has already been demonstrated in the case of PcPt. In addition, the chromatographic profiles of each reaction catalyzed either by PcPt or PsPt were remarkably similar (compare Figure 104 to Figure 80). In both cases, DMS was the major product of the reaction while osthenol was the minor product. Hence apparently no difference between PcPt and PsPt was observed.

These preliminary experiments showed that PsPt displays the same activity as it was observed for PcPt. This enzyme is able to metabolize Umb in the presence of DMAPP into DMS (as major product of reaction) and osthenol (as minor product of reaction).

This study must now be completed in order to check if the enzymatic characteristics of both Pts are the same and should also be extended to other molecules in order to test if the substrate specificity is restricted to a single molecule as for PcPtor not.

4.3 Functional characterization of CliPt

A candidate gene among all the available candidate genes of aromatic Pts was isolated from *C. limon* and provided by our Japanese collaborators. *C. limon* is a plant that belongs to the Rutaceae family, and is well known for producing linear furanocoumarins (Waksmundzka-Hajnos *et al.,* 2008).

4.3.1 Transient expression of prenyltransferase of CliPt in *N. benthamiana*

4.3.1.1 *In vivo* characterization of CliPt

As it was described for PcPt, we performed a first functional screening for the CliPt using the *N. benthamiana* as a transient expression system. An agrobacteria inoculum containing both strains pBIN61-p19 and pBIN-CliPt was prepared and used to infiltrate leaves of *N. benthamiana* following the protocol that has been described previously. 72 hours after infiltration, the inoculated leaves were re-infiltrated independently with the molecule mixes that were used for the characterization of PcPt (see Table 19 of this chapter). After 24 additional hours, the infiltrated leaves were used to extract the total phenylpropanoids and analyzed by LCMS/MS. We tried to detect the presence of any prenylated molecules corresponding to the molecules that were present in the initial infiltration mix (3.3.1). Unfortunately, this first attempt was unsuccessful and we couldn't detect any expected prenylated products. In order to verify the accuracy of these results and also to check the involvement of another type of co-factor in the reaction (another prenyl moiety for example), we decided to use an *in vitro* approach to investigate the function of this enzyme.

4.3.1.2 *In vitro* characterization of CliPt

These transient expression experiments were realized in Nancy by Alexandre Olry and Ryosuke Munakata. The recombinant LBA4404::pBIN-CliPt were grown as described earlier and an inoculum of agrobacteria that contained the recombinant plasmid pBin61-p19 and pBIN-CliPt was prepared to infiltrate the *N. benthamiana* leaves. As done previously, a control inoculum was used by mixing recombinant agrobacteria containing the pBin61-p19 plasmid and the void pBin-pGWB2. These different inoculums were used to infiltrate three independent sets of plants, the corresponding leaves were harvested 96 h post infiltration and used to extract microsomal proteins as already described in chapter 2 (under heading 2.4.2.3.1). The microsomal proteins (CliPt and control samples) were used to perform different incubations by using a large number of aromatic substrates (listed in Table 27). The reaction mixes were analyzed by HPLC/LC-MS and the results showed a clear metabolization of only two different aromatic molecules: Umb and esculetin. However, these reactions could only be observed when the prenyl moiety was GPP.

The product we obtained has the same retention time than a standard geranylated Umb (Figure 105 A). The analysis of the UV spectra and the mass spectra (Figure 105 B and C)

provided strong evidences that the molecule detected in the reaction mixture in the presence of CliPt is 8-geranyl-Umb.

Table 27. Aromatic molecules and prenyl moieties used for *in vitro* screening too find out the possible substrates of CliPt

Coumarin and furanocoumarin moiety/ prenyl moiety	DMAPP	GPP
Xanthotoxol	-	-
Bergaptol	-	-
Umbelliferone	-	+
6- methoxy coumarin	-	-
7- methoxycoumarin	-	-
6,7-methoxycoumarin	-	-
Coumarin	-	-
7-ethoxycoumarin	-	-
5-methoxypsoralen	-	-
8-methoxypsoralen	-	-
Psoralen	-	-
8-OH Xanthotoxin	-	-
5-OH Bergapten	-	-
5,8-di OH Psoralen	-	-
5,6-di OH Angelicin	-	-
Isobergaptol	-	-
Sophondinol	-	-
Esculetin	-	+
Scopoletin	-	-

Esculetin was the second molecule which was metabolized by CliPt (Figure 106 A). The metabolisation rate seems to be less important than for Umb. This molecule was therefore more difficult to identify especially because we didn't have any relative standard molecule. However, a fragmentation profile obtained through a LCMS/MS analyses highlighted the presence of GPP, as a loss of 314 D could be observed (Figure 106 C).

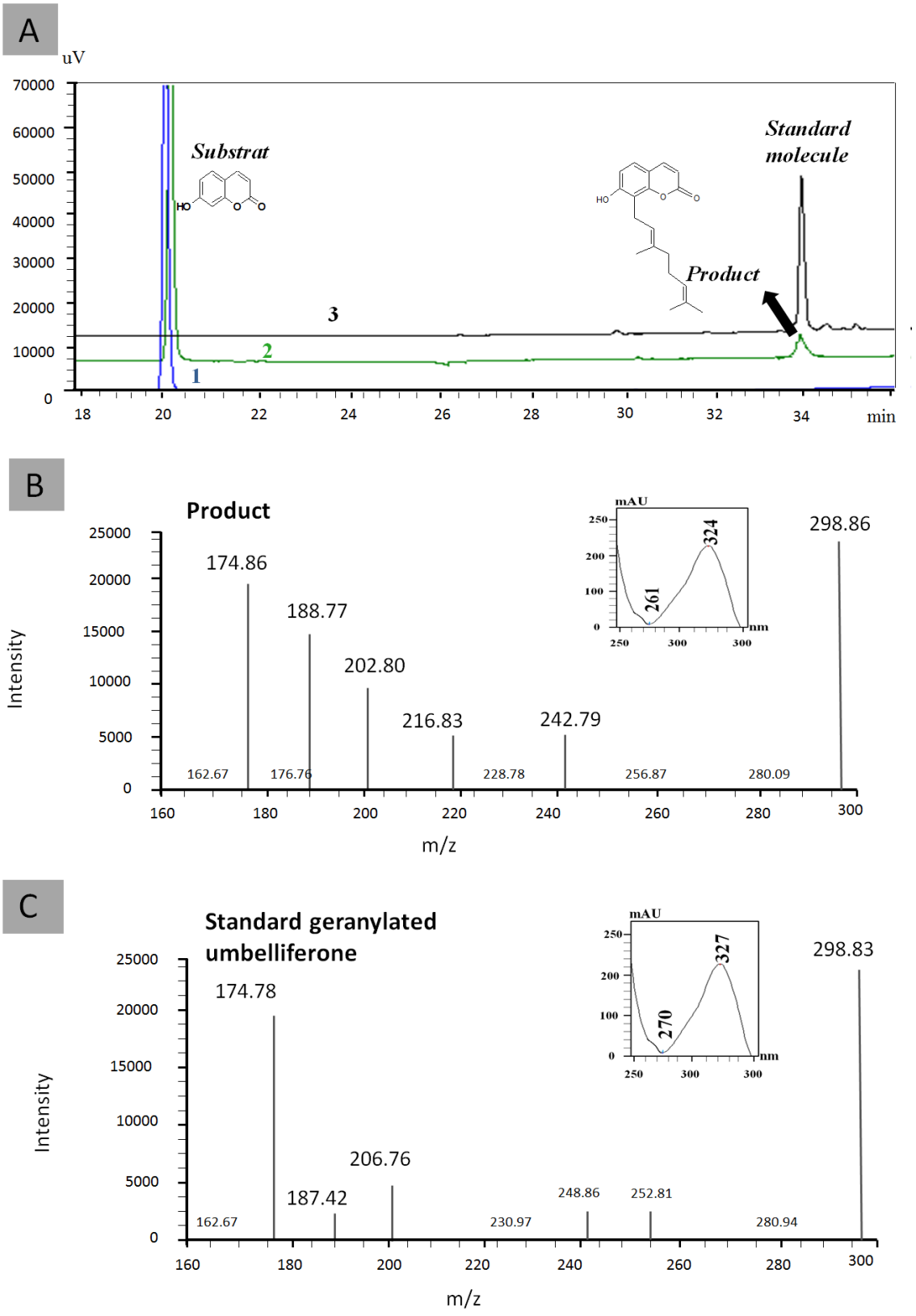


Figure 105. Metabolization of Umb by CliPt

A) Incubation samples were analyzed by HPLC-DAD. 1) Negative control (plant infiltrated with the void plasmid), 2) Metabolization of Umb, 3) Standard molecule. B) UV spectrum and mass spectrum of the product, C) UV spectrum and mass spectrum of standard 6-geranyl- Umb synthesized in the laboratory by our collaborators.

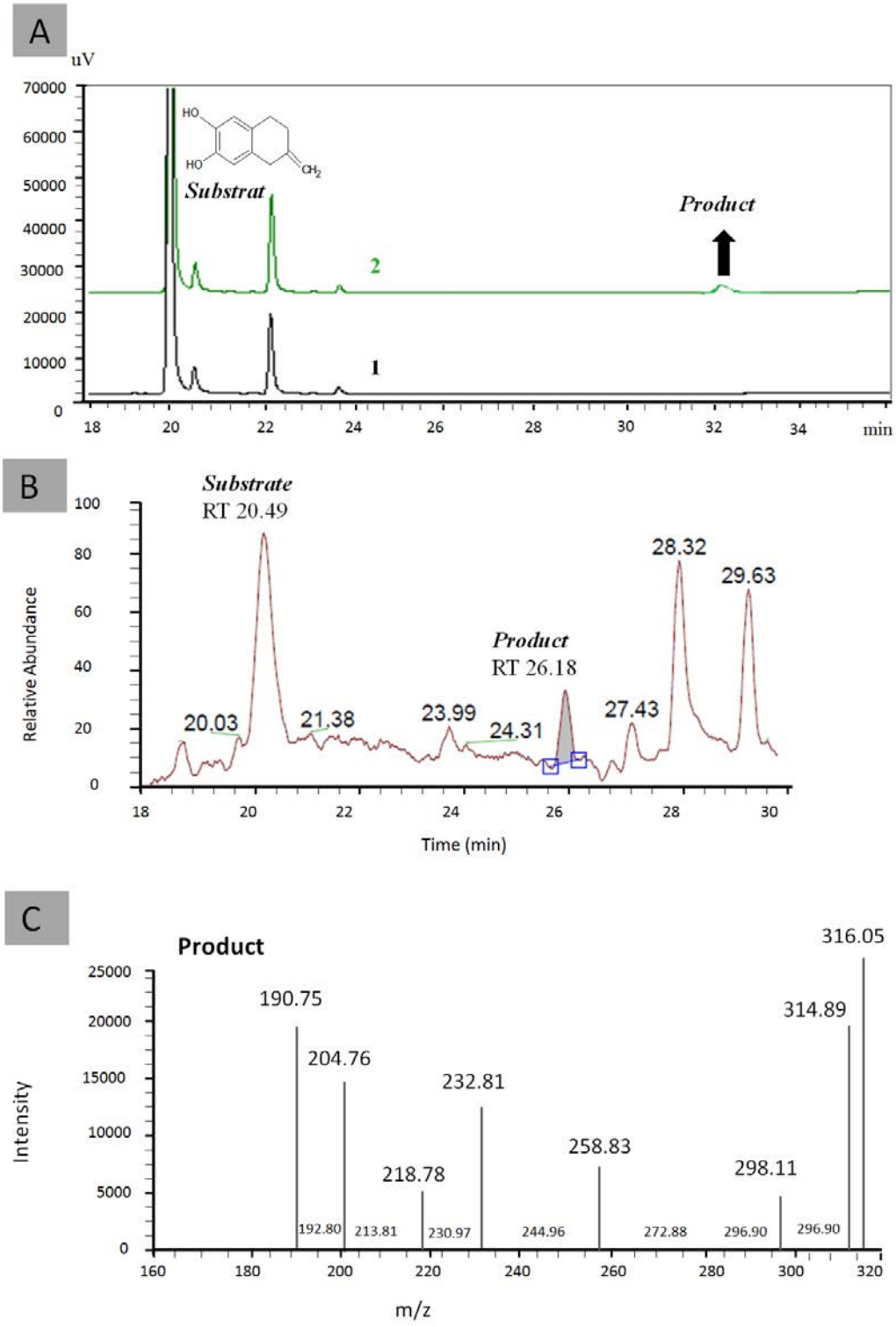


Figure 106. Metabolisation of esculetin by CliPt

A) HPLC-DAD analysis 1) Incubation done with the void plasmid, 2) Incubation done with CliPt. B) MS spectra obtained for the esculetin and geranyl-esculetin. C) MS^2 Mass spectra of the synthesized product. A 314 D ion is generated.

4.3.2 Construction of transgenic *R. graveolens* plants overexpressing CliPt

Transformation of *R. graveolens* plants was carried out using the genetic transformation protocol already described in the "Materials and Methods" section. To achieve this transformation, an inoculum of LBA4404 agrobacteria containing pBIN::CliPt was prepared. In order to make comparative analyses, I also generated transgenic plants transformed with the void pBIN-GW as discussed for PcPt in chapter 3. Treated hypocotyls were incubated on a regeneration media before being transferred to a selective media containing Kanamycin as it has already been described. When the transformed hypocotyls, developed new leafy stems of almost 1 cm they were transferred to 200 ml pots (Figure 107). No clear different phenotype was highlighted between CliPt plants and void plasmid control plants except that CliPt plants showed a slow growth rate as it has been already observed for transgenic plants developed for PcPt plants.

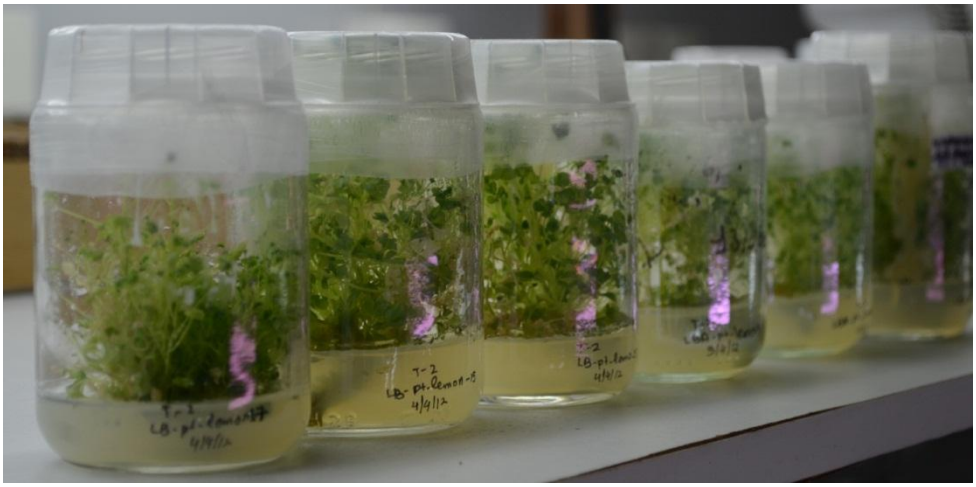


Figure 107. *In vitro* transgenic CliPt *R. graveolens*

These plants, transferred on the selective media were resistant to kan and therefore were potentially transformed.14 different *R. graveolens* plants were propagated on the selective medium and were used for subsequent molecular characterization.

4.3.2.1 Molecular characterization of CliPt transgenic plants

To characterize the CliPt plants, I used the same approach then for the PcPt. The presence of the transgene was assessed with the PCR kit « Phire plant direct PCR kit ». Using this PCR approach I checked the presence of the 2 genes present on the T-DNA: *nptII* and 35S::CliPt. The primers and annealing temperature for *nptII* were described in (Table 12) of this "Material and Methods". To detect the presence of the CliPt gene, we used a primer

targeting the 35S and a reverse primer specific to the CliPt gene and the annealing temperature of this second PCR was 73°C (Table 28). After amplification, all the samples were analyzed on 1% agarose gel (Figure 108). All the 14 different plants that were tested showed positive results for the amplification of *nptII* gene (Figure 108), but, unfortunately, no amplification for the 35S-CliPt transgene could be observed. Results obtained for molecular characterization of transgenic plants developed for construction Agro-pBIN-Pt-lemon have been summarized in (Table 29).

Table 28. Sequences of primers utilized for amplification of 35S-CliPt using the DNA of transgenic plants as the template

Sequences of primers utilized		
CliPt-Rev	TCATCGTAAGAAGTGAATAAGGAGATATTCAGC	Annealing temp. = 73°C
35SDir	TGCATGCCTGCAGGTCCCCAGATTG	

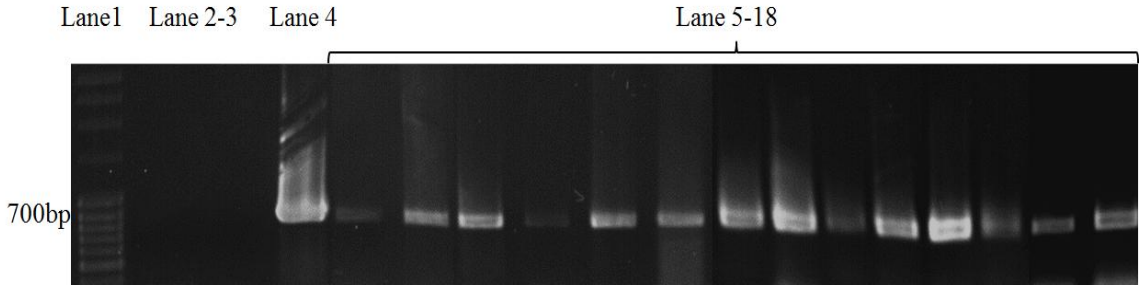


Figure 108. Agarose gel analysis of PCR-amplified *nptII* genes in transformed pBIN::CliPt *R. graveolens* plants

PCR was performed using two primers pairs; *nptII* (dir and rev). Fourteen, different plants potentially transformed with the pBIN::CliPt were checked. All the plants showed positive amplification for *nptII* (dir and rev) and bands are of expected size (700 bp) were observed. Lane 1: MM (molecular marker), Lane 2 and 3: Negative control -ve C (wild type plant and water), Lane 4: positive control +ve C (plasmid), Lane 5-19: fourteen plants issued from individual transformants events.

These results demonstrated that among the 14 tested plants, all of them had partially integrated the T-DNA (only the gene of *nptII* was present) however, no amplification of the transgene was observed. There were two main possible reasons for this observation: either all of them have only partially integrated T-DNA or the amplification conditions used for the transgene were not optimized. In order to go further in the molecular characterization of these plants I decided to use a more sensitive and specific approach.

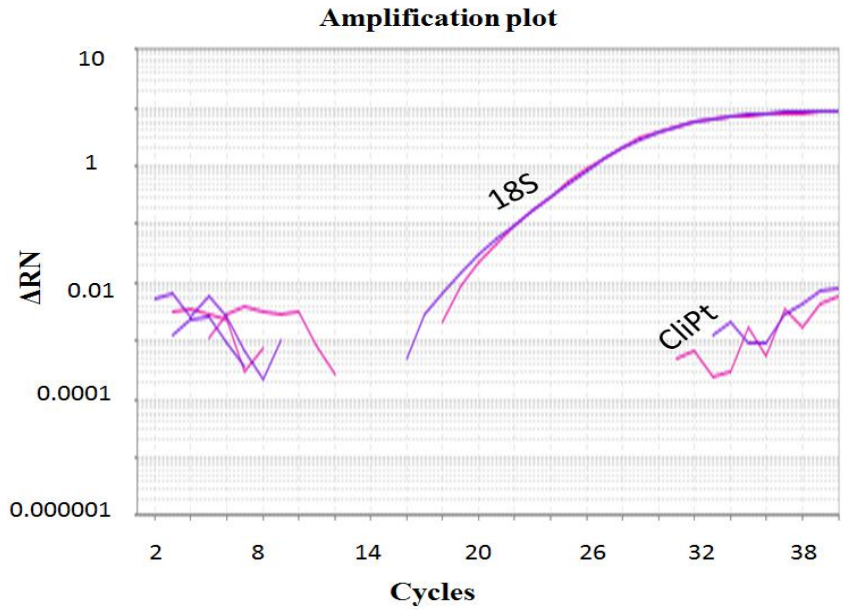
Table 29. Summary of the amplification results of *nptII* and 35S-CliPt using samples prepared with the Phire plant direct PCR kit from leaves of potentially transformed plants of *R. graveolens*

Plants of *R. graveolens*	*neptII*	35S-CliPt	Plants of *R. graveolens*	*neptII*	35S-CliPt
T1- ClPT -1	+	-	T1- ClPT -16	+	-
T1- ClPT -2	+	-	T1- ClPT -23	+	-
T1- ClPT -6	+	-	T1- ClPT -32	+	-
T1- ClPT -7	+	-	T1- ClPT -37	+	-
T1- ClPT -12	+	-	T1- ClPT -38	+	-
T1- ClPT -14	+	-	T1- ClPT -40	+	-
T1- ClPT -15	+	-	T1 ClPT -47	+	-

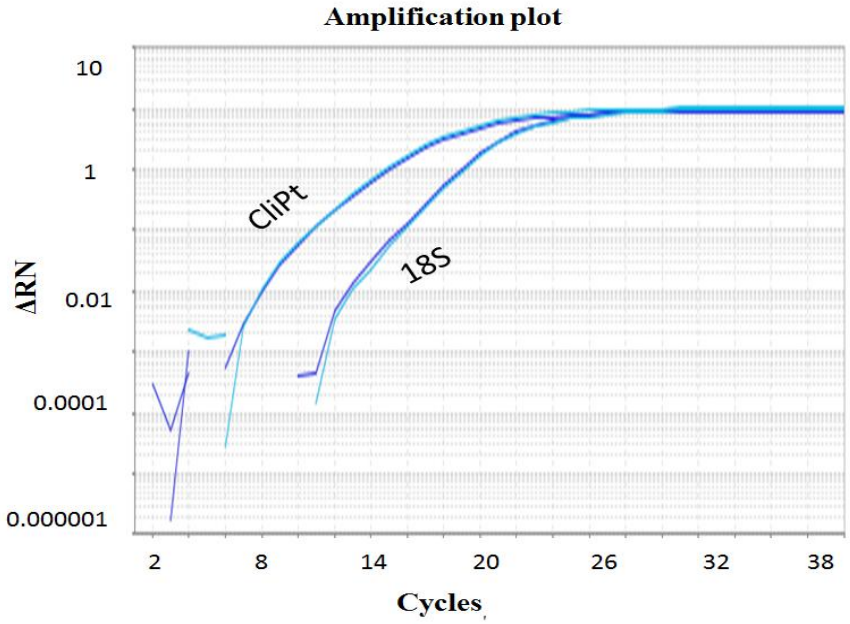
(+) demonstrates that positive amplification of required size was observed, while (-) indicates that no amplification of respective genes could be obtained.

4.3.2.2 Overexpression of CliPt: detection through RT-qPCR.

Total RNAs were extracted from fourteen whole plantlets of *R. graveolens* transformed with pBIN-GW-CliPt and five plantlets transformed with pBIN-GW (or control). Specific primers for CliPt and for the 16S endogenous housekeeping gene were designed using the Primer Express 3.0 software. Sequences of these primers as well as RT-qPCR conditions were described in the "Material and Methods" chapter. The results I obtained, demonstrated that the expression of the CliPt was detected only in five out of fourteen tested samples of transgenic plants developed for the construction (pBIN-CliPt) (Figure 109 B). Furthermore, as expected, this expression was absent in the five control plant samples (pBIN-GW) (Figure 109 A) and only a little amplification was observed due to some non-specific hybridization of primers. The results for Real time PCR are listed in (Table 30).



A *R. graveolens* (pBIN-GW)



B *R. graveolens* (pBIN-GW-CliPt)

Figure 109. Results of real time PCR

Results observed for the expression pattern of CliPt together with an endogenous gene (18S) in both the controlled (Agro-pBIN-GW)(A) and positive (Agro-pBIN-GW-CliPt) (B) transgenic plants of R. *graveolens*.

Table 30. Summary of the results of Real time PCR for the expression of endogenous gene (18S) and transgene (CliPt) in transformed plants of *R. graveolens* developed either for (pBIN-GW) or for (pBIN-GW-CliPt)

Control plants (transformed with a void pBIN-GW)	Housekeeping gene (18S)	Overexpression of CliPt	CliPt Plants	Housekeeping gene (18S)	Overexpression of CliPt
T1-void pBIN-1	+	-	T1-CliPt-6	+	+
T1-void pBIN-2	+	-	T1-CliPt-14	+	+
T1-void pBIN-3	+	-	T1-CliPt-15	+	+
T1-void pBIN-4	+	-	T1-CliPt-16	+	+
T1-void pBIN-5	+	-	T1-CliPt-23	+	+

(+) demonstrates that positive amplification was observed, while (-) indicates that no amplification of respective genes could be obtained.

These results demonstrated the presence of the transgene CliPt in at least five plants out the 14 tested plants of *R. graveolens*. In order to check whether the results obtained by *in vitro* technique for CliPt could be observed by *in vivo* expression, these plants were analyzed for their metabolic profiles.

4.3.2.3 Metabolic profiles of transformed *R. graveolens* plants: Quantification of geranylated Umb and geranylated esculetin

We detected the expression of CliPt in 5 different plants of *R. graveolens* using the Real time PCR technique. To analyze the possible effects of the overexpression of transgene, we realized the analyses of metabolic profiles of these plants. Based on the results obtained for *in vitro* functional characterization, we decided to quantify the geranylated molecules in the transformed and control plants. For these experiments, the total phenylpropanoids contents were extracted from the 5 different plants carrying the transgene of CliPt and five different plants transformed with empty plasmid (pBIN-GW) of *R. graveolens*. These samples were then used firstly for the detection of geranylated Umb, and secondly for the quantification of

Umb and geranylted Umb. Quantification of Umb content present in these plants was carried out as described for PcPt in this chapter (unde heading 3.4.4.1.1).

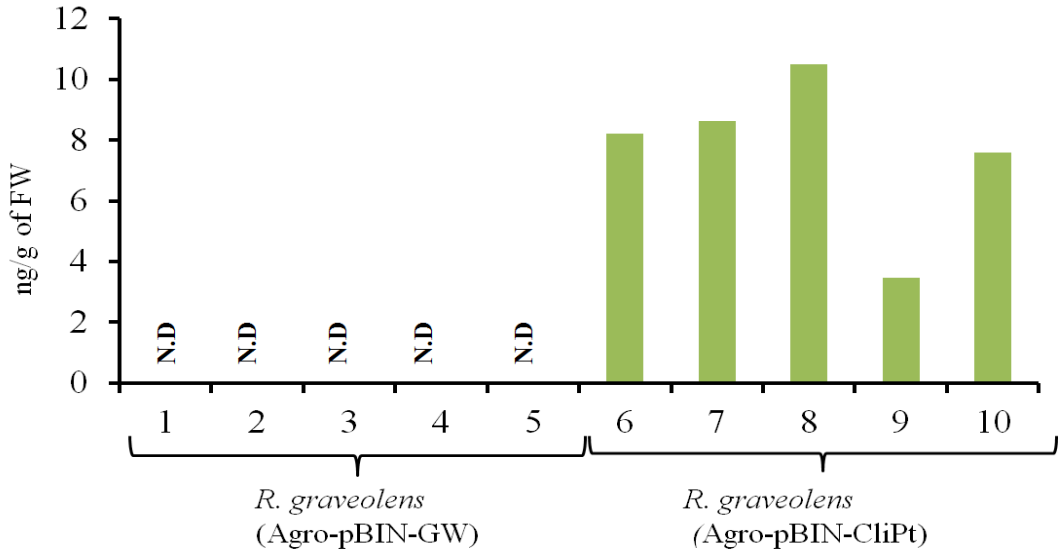


Figure 110. Quantification of geranylated Umb present in transgenic plants.

Where ND means; not detected.

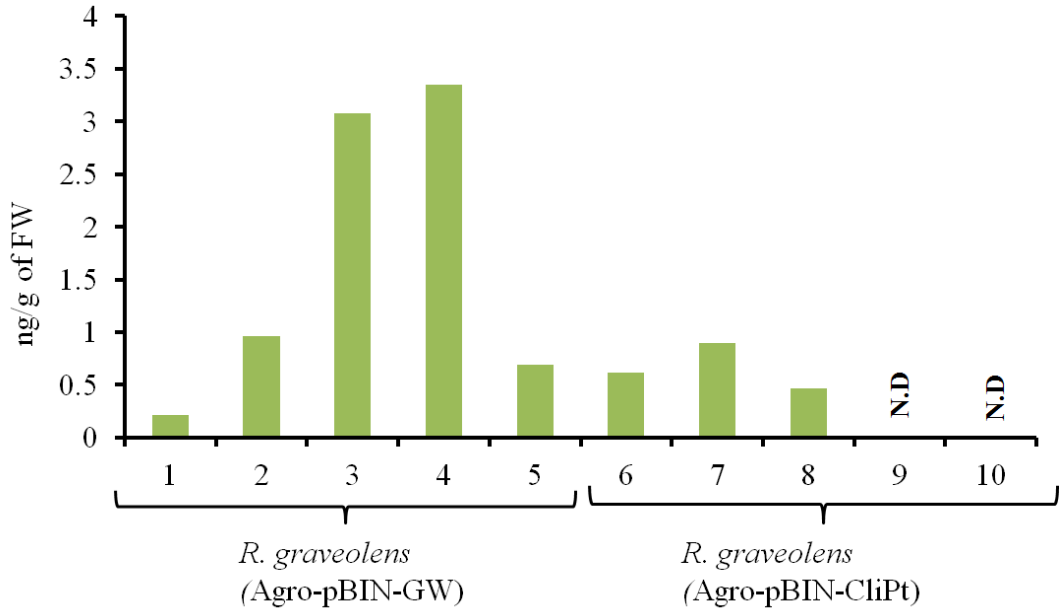


Figure 111. Quantification of Umb present in transgenic plants developed for pBIN-GW-CliPt and control plants

Where ND represents Not detected.

Preliminary quantification experiments of all the transgenic plants (control pBIN-GW and pBIN-GW-CliPt) highlighted the presence of geranylated Umb only in 5 out of the 14

stably transformed plants for the CliPt transgene (Figure 110) that agreed with the results obtained by the Real time PCR technique. Furthermore, a clear difference in Umb contents was observed. Umb, which was found as one of the aromatic molecule that could be used by CliPt in transient expression experiments, is detected in lower concentrations in transgenic CliPt plants than in control plants (pBIN-GW) (Figure 111).

The values of geranylted Umb of the 5 transgenic *R. graveolens* plants carrying the construction (pBIN-GW-CliPt) were plotted against the values of the control (pBIN-GW) plant and further used for a statistical analysis. This analysis showed that the Umb contents of transgenic plants overexpressing the CliPt were significantly reduced (0.396 ± 0.293 ng/g FW) in comparison to the transgenic void pBIN-GW plants which contain the *nptII* gene (1.659 ± 1.047 ng/g FW).

As esculetin has also been proven to be metabolized in *in vitro* assays, we also tried to detect the geranylated esculetin in transformed *R. graveolens* plants. However all our investigations concerning this molecule were unsuccessful and we couldn't detect this molecule neither in positive samples (containing transgene CliPt) nor in control samples (containing *nptII* gene) perhaps due to the lack of esculetin in rue plant tissues.

Except for esculetin, the results obtained in transgenic plants agree exactly with the *in vitro* results obtained *in vitro* expression of CliPt. Moreover, disappearance of the substrates and appearance of the product in these transformed plants were found to be positively correlated with the overexpression of transgene (only 5 plants expressing the gene accumulate the molecule).

4.3.3 Attempt for functional characterization for other aromatic prenyltransferases

4.3.3.1 Transient expression system of *N. benthamiana*

In addition to PcPt, PsPt and CliPt, I also tried to functionally characterize the 4 other available candidate genes (ClPt, AngPt, AgPt, and RgPt) that have been isolated in chapter 1 using the *N. benthamiana* transient expression system. As it has been already described for other genes, agrobacteria strains were infiltrated in tobacco leaves along with pBin61-p19 transformed bacteria. The infiltrated leaves were either used to undergo a secondary infiltration with different molecules or they were harvested 96 h post inoculation, used to prepare microsomes and to perform *in vitro* enzymatic reactions.

For both approaches (*in vivo* and *in vitro*), the same substrates that have been listed in Table 27 were tested. The results we obtained showed that the screening for prenylated products within these samples was unsuccessful for any of the tested substrate and any of the candidate genes.

4.3.3.2 Stable expression in *R. graveolens*

All the genes were stably introduced into the *R. graveolens* genome using the hypocotyl transformation method.

To realize the molecular characterization of the putatively transformed plants, DNA samples were prepared using the same kit as explained previoulsy (under heading 3.4.2). The PCRs done were dedicated to amplify a part of the "35S-transgene" fragment and, except for the angelica plants, to amplify the *nptII* gene present on the T-DNA. The primers used to perform these tests are summarized in table (Table 32). After amplification all the samples were analyzed on a 1% agarose gel and the results obtained are summarized in (Table 31).

Table 31. Molecular characterization of transgenic ruta plants

Plant	Name	*nptII*	35S-transgene	% of plants transformed with transgene
35S-RgPt	T0-Pt-rue-3	+	+	67
	T0-Pt-rue-5	+	+	
	T0-Pt-rue-7	+	+	
	T1-Pt-rue-1	+	-	
	T1-Pt-rue-2	+	+	
	T1-Pt-rue-4	+	+	
	T1-Pt-rue-5	+	-	
	T1-Pt-rue-6	+	-	
	T1-Pt-rue-8	+	+	
35S-AngPt	T0-Pt-ang-1	NT	+	89
	T0-Pt-ang-4	NT	+	
	T0-Pt-ang-5	NT	+	
	T0-Pt-ang-6	NT	-	
	T0-Pt-ang-7	NT	+	
	T0-Pt-ang-10	NT	+	
	T1-Pt-ang-3	NT	-	
	T1-Pt-ang-4	NT	+	
	T1-Pt-ang-7	NT	+	
35S-AgPt	T1-Pt-cel-1	+	-	53
	T1-Pt-cel-2	+	-	
	T1-Pt-cel-3	+	-	
	T1-Pt-cel-4	+	+	
	T1-Pt-cel-5	+	+	
	T1-Pt-cel-6	+	-	
	T1-Pt-cel-7	+	+	
	T1-Pt-cel-8	+	+	
	T1-Pt-cel-9	+	-	
	T1-Pt-cel-11	+	+	
	T1-Pt-cel-13	+	+	
	T1-Pt-cel-14	+	-	
	T1-Pt-cel-15	+	-	
	T1-Pt-cel-16	+	+	
	T1-Pt-cel-18	+	-	
	T1-Pt-cel-19	+	-	
	T1-Pt-cel-21	+	-	
	T1-Pt-cel-24	+	+	
	T1-Pt-cel-25	+	+	
35S-ClPt	T1-Pt-lim-1	+	-	0
	T1-Pt-lim-5	+	-	
	T1-Pt-lim-6	+	-	
	T1-Pt-lim-7	+	-	
	T1-Pt-lim-11	+	-	
	T1-Pt-lim-13	+	-	
	T1-Pt-lim-14	+	-	
	T1-Pt-lim-19	+	-	
	T1-Pt-lim-21	+	-	

Plants were transformed for RgPT, ClPt, AngPt and AgPt. (+) means that the gene is present, (-) means that the gene could not be detected, NT means not tested. % means transformation rate.

Table 32. Sequences of primers utilized for the characterization of various transgenic plants

Name	Sequence	Annealing temp.
35SDir	TGCATGCCTGCAGGTCCCCAGATTG	
AngPt- Rev	CTCGAGACGAACAAAAGGAATTAGTAGATATTCTGC	75°C
RgPt- Rev	TGAGTATCTCCTTGCACACTTCCTACGGTGA	77°C
AgPt-Rev	TCAGCGCATGAAATGAATTAGCACATACTCCGCATAC	79°C
ClPt-Rev	TCATCGTAAGAAGTGAATAAGGAGATATTCAGCAT	75°C

All the plants described were analyzed for the various linear and angular prenylated furanocoumarins that have been listed in (Table 25) of this chapter. These experiments didn't show any difference in the amounts of any analyzed molecules among the control plants (void pBIN-GW) or any Pt transformed plants. It seems that the expression of the corresponding gene has no impact on the plant metabolism.

4.4 Discussion and conclusions

In this chapter I made preliminary experiments in order to characterize the function of the 6 remaining enzymes described in the chapter 1. Among all the genes available, the one isolated from parsnip shares the highest homology with the parsley Pt. Parsley and parsnip are two plants belonging to the same plant species but are different since only parsnip can produce both linear and angular furanocoumarins (Diawara *et al.,* 1995; Ivie *et al.,* 1981; Lombaert *et al.,* 2001). In the conclusion of chapter 3, I assumed that the prenylation is not the limiting step for the synthesis of linear and angular furanocoumarins. In order to support this assumption, we performed the functional characterization of the PsPt using the *N. benthamiana* transient expression system. As our main concern was to understand the role of prenylation in the biosynthesis of linear and angular furanocoumarin, I directly used Umb and DMAPP as potential substrates. The analysis of these experiments showed similar results as those previously obtained for PcPt, and we detected the prenylated Umb in form of DMS and osthenol. A similar induced expression of Umb Pt activity was correlated to DMS production in induced cells of *A. majus* (another member of plant family Apiacae) (Hamerski *et al.,* 1990). Another interesting feature highlighted was that, like for PcPt, DMS was found as a major reaction product while osthenol was detected as a minor product. In addition to the enzymatic characterization of PcPt and PsPt, we investigated the occurence of various furanocoumarins present in leaves of parsley and parsnip. I performed these experiments for parsley leavesfor Umb, DMS, marmesin, psoralen, 8-MOP, 5-MOP, isopimpinellin and

osthenol as the main furanocoumarins. Such linear furanocoumarins were already described as the major furanocoumarins in parsley plant (Beier *et al.,* 1994; Chaudhary *et al.,* 1986). In parallel to my study, another work was carried out in our laboratory by Dr. Sandro Roselli with the objectif to quantify the furanocoumarin content of parsnip using a LCMS/MS approach. This study demonstrated the presence of Umb, DMS, marmesin, psoralen, 8-MOP, 5-MOP, isopimpinellin, angelicin, colambianetin, sphondin and pimpinellin. These results highlight two important results. The first one was that we could detect angular furanocoumarin only in parsnip plants. This confirmed results described in previous reports. The second important result is that osthenol couldn't be detected in parsnip leaves while it was detected in small amounts in parsley leaves. These observations make sense since osthenol, which is present in small amount in parsley, could be stored due to the absence of further enzymes to go on the synthesis of furanocoumarins. In contrast in parsnip, we couldn't detect osthenol because it is readily used by the downstream enzymes to convert it into angelicin and hence continue the pathway to angular furanocoumarins. All these observation supported the conclusion made in the last chapter which was that prenylation is not the limiting step in the biosynthesis of furanocoumarins.

In addition to the 2 Pts involved in the synthesis of DMS and osthenol, 5 additional genes were available for characterization. The phylogenetic analysis done in chapter 1 showed that the 6 proteins clustered in a separate group from the already described Pts. They are clearly different from flavonoid Pts. The results obtained for PcPt and PsPt strengthens the assumption that this new group is specialized for the prenylation of coumarin-like molecules. To check this assumption, I investigated the 6 remaining candidate genes that include CliPt, ClPt, RgPt, AngPt and AgPt. All of them were expressed either transiently in *N. benthamiana* or stably in *R. graveolens*. Since no metabolization was observed *in vivo* with two different mixes of substrates, we included the other prenyl moieties as potential substrates in our *in vitro* experiments. With the help of this second approach, we observed that among all the tested candidate genes, the Pt isolated from lemon (CliPt) was able to catalyze the addition of GPP to Umb (Figure 105 A). This kind of activity has recently been described by a biochemical study done by Munakata and coworkers with microsomes prepared from *C. limon* peel (Munakata *et al.,* 2012). These authors demonstrated that the microsomal fraction, was able to catalyze the addition of GPP to different aromatic substrates which includes xanthotoxol, bergaptol, 5-hydroxy-7-methoxycoumarin, 5, 7-dihydroxycoumarin and Umb. Keeping in view these observations, we checked other aromatic molecules as potential

substrates and found that this gene was also able to geranylate esculetin. The reaction rate was very low and hence, the product was difficult to be analyzed precisely by HPLC (Figure 106 A) and needed an LCMS/MS approach (Figure 106 B) to be detected. So, even if both enzymes were highly specific for the GPP prenyl moiety, in comparison to the microsomal fraction described above which was capable to prenylate different aromatic substrates, the CliPt activity was restricted to only 2 molecules, Umb and esculetin. Umb (Figure 112 A) has a very similar chemical structure to that of esculetin which has only one additional hydroxyl group (Figure 112 B).

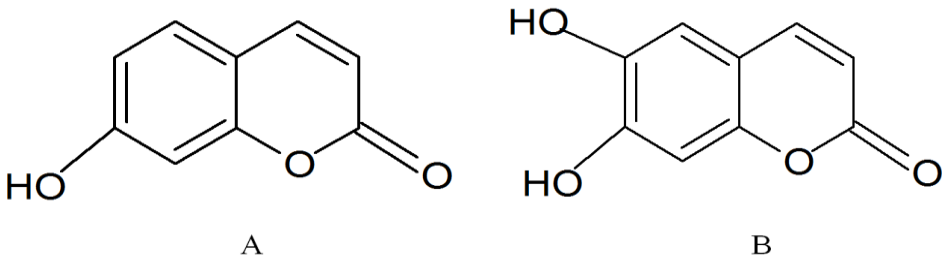


Figure 112. Chemical structures of Umb and esculetin

All these observations cumulatively allows us to assume that the microsomal fraction prepared from the lemon peel contained more than one enzyme that were able to catalyze the addition of GPP to various aromatic substrates. The CliPt used in our study could be one of these enzymes. These studies allowed us to identify the first Umb geranyltransferase at the molecular level.

Our results demonstrated that CliPt activity was observed only when the microsomal fraction was isolated from infiltrated leaves of *N. benthamiana* and incubated with potential substrates under *in vitro* conditions. No activity could be highlighted *in vivo*. One possible reason for the failure of this approach may rely on the fact that one of both substrates is not present in the cell in sufficient concentration. This hypothesis is more valid for the availability of the prenyl moiety since we exogenously supplied the aromatic molecules. The geranyl pyrophosphate synthase has been reported as a plastid localized protein and only small amounts of GPP have been detected in cytoplasm (Aharoni *et al.,* 2003, 2006; Aharoni 2005). Plant aromatic Pts have also been reported as plastid localized proteins (Akashi *et al.,* 2009; Sasaki *et al.,* 2008). Thus the only valid reason for the unavailability of GPP as a substrate is the involvement of this molecule as a precursor for the synthesis of a large number of products belonging to the biosynthetic pathway of monoterpenes (Nagegowda *et al.,* 2008)

Similar experiments were carried out to identify the function of the five other candidate genes available in our lab. Unfortunately, no metabolization of any of the aromatic substrates that were used could be detected for any of these candidate genes. To solve this problem it is necessary to find out new possible aromatic substrates to test with these enzymes. For this purpose, we transformed *R. graveolens* with each of these genes. As this plant is a high producer of coumarins and furanocoumarins, we expected to find out new metabolized molecules. The plants were constructed, characterized at the molecular level and subsequently investigated for their phenylpropanoids content. The CliPt plants were found to produce geranylated Umb which has never been reported to be present in *R. graveolens*. Furthermore, the LCMS/MS analyses done on 5 different CliPt plants showed the presence of significant quantity of geranylated Umb (Figure 110). In addition to these experiments, we also demonstrated that the concentration of Umb in these plants was significantly lower than the wild type plants (Figure 111), which make sense this molecule is transformed in its geranylated counterpart. On the other hand we didn't succeed to detect the geranylated esculetin in the transformed *R. graveolens* plants. This result is consistent with the *in vitro* results where the activity of CliPt was demonstrated to be not so efficient.

The absence of any modification of metabolic profiles of any transformed plants, for the 5 remaining enzymes should be further investigated. The absence of any new molecule can be explained in different ways. The first possibility is that we didn't search for the right molecules. We have to find out other screening parameters. One way would be to improve our extraction method and separation method in order to be able to compare more precisely the two different chromatographic profiles. This problem could be solved by searching a specific molecule on the basis of its mass. As each molecule has its own specific mass thus by utilization of this technique there is no need to compare two chromatographic profiles and absence or the presence of a specific molecule could be detected on the basis of mass. A research through the molecular mass of the molecule requires knowing precisely this mass. Hence, we should extend this technique of detection to other prenyl moieties or other coumarins and furanocouamrins. The molecules resulting from the prenylation could also undergo other modifications (hydroxylation, cyclisation…) which were not investigated in our study. Finally, it is possible that our putative Pts had a mutation in the sequence. Actually, we have no guarantee that the sequences we have are the correct sequences as no genomic information exist for the plants with which we are working. Therefore it would be interesting to do again the cloning of putative ORFs from their original plants. For example, concerning

the RgPt we could find an allelic variant in our database which is very close to the one we tested in this study. The modification of only a few amino acids can change the function of the enzyme. This second Pt will be tested for the respective enzymatic activity in our in future in the laboratory.

Two enzymes have been characterized in this study. Both are involved in the metabolization of coumarins: Umb dimethylallyl transferase isolated from parsnip and Umb geranyltransferase from lemon tree. These observations support the assumption that the different candidate genes identified in the first part of this manuscript belong to a same new group of Pt whose substrates are related to coumarins/furanocoumarins. This point has been recently reinforced by the discovery of another activity for a new Pt isolated from grapefruit, clustering with the 8 candidate genes and involved in the synthesis of bergamottine (results from our group, data not shown). It is likely that the other proteins are also involved in this kind of reaction but it has to be proven in the future.

General conclusion and perspectives

General conclusion and perspectives

5 General conclusion

The main objective of this project was to develop our knowledge for furanocoumarins biosynthesis at the molecular level. Till today, only four genes involved in this pathway were characterized in different plants: a psoralen synthase and an AS that were isolated from Parsnip, *A. majus* and celery; a bergaptol *o*-methyltransferase described in *A. majus* and a *p*-coumaroyl CoA *ortho*-hydroxylase (C2'-H) isolated from *R. graveolens* and *I. batatas*. Although all these enzymes are important in the synthesis of furanocoumarins, however the important, "Pt" which is the enzyme catalysing the synthesis of either linear or angular furnaocoumarins, remained elusive. On these bases, my thesis project consisted of two steps: first to identify new potential candidate genes that could catalyze this prenylation of Umb, and second, to perform their functional characterization.

Identification of new candidate genes belonging to the plant aromatic prenyltransferase family

The furanocoumarin biosynthetic pathway begins with the synthesis of Umb which is further prenylated (addition of DMAPP) either on the 6^{th} or 8^{th} carbon to produce linear or angular furanocoumarins respectively. Biochemical studies done in the seventies, reported the presence of enzymes that can catalyze the addition of a prenyl moiety on 6-C of Umb to produce DMS in *R. graveolens* (Dhillon *and* Brown, 1976; Ellis *and* Brown, 1974). Another work done by (Hamerski *et al.,*1990) highlighted the presence of two Pts in induced *A. majus* cell cultures. These two enzymes displayed two different activities. They could catalyze the 6-C-prenylation and 7-O-prenylation of Umb to give rise to relevant intermediates of biosynthetic pathway of dihydrofuranocoumarins and butenyl ethers respectively. More recently, Sazaki and coworkers identified an enzyme that could catalyze the addition of DMAPP to naringenin (a flavonoid) at position 8 (Sasaki *et al.,* 2008). The reaction mechanism that is necessary for the synthesis of naringenine 8-dimethylallyl seems to be close to the one involved in the synthesis of DMS and/or osthenol. Finally in a review article, Heide classified the plant aromatic Pts, to which our enzyme of interest might belong, into a separate group on the basis of the presence of specific conserved sequences and membrane

integration typology (Heide, 2009). Based on all these studies, we used a combination of bioinformatics and experimental approaches to isolate 7 different putative Pts (AngPt, PsPt, PcPt, RgPt, ClPt, CliPt and AgPt,) from various furanocoumarin producing plants that include *A. gigas*, *P. sativa*, *P. sativum*, *C. latifolia*, *C. limon*, *R. graveolens* and *A. graveolens*. This work was partly realized in collaboration with the group of professor Yazaki from the Kyoto University (Japan).

An *in silico* analysis performed on these different sequences confirmed the presence of essential domains necessary for the prenylation reaction (Ohara *et al.,* 2009). A TMHMM analysis also showed that all these proteins were embedded in the membrane as it was described for other Pts. But the most promising information was that a phylogenetic analysis of these proteins along with other Pts clearly highlighted a new cluster of enzymes, separated from all the other Pts characterized so far. This new group of enzymes might therefore display different substrate specificity and catalyze different kind of reaction. This assumption was investigated in the second part of the work.

Set up of an adapted heterologous expression system

Preliminary experiments: establishing an efficient expression system for membrane bound proteins using CYP98A22 as a model

The selection of a suitable heterologous expression system was a key step in the functional characterization of these membrane-bound enzymes. As less information was described concerning the characterization of Pt, and in order to set up an efficient system, we started to work with a P450 from the CYP98A subfamily for which the activity has already been widely described as C3'-H. To do the characterization of this enzyme, we focused on *N. benthamania* as a heterologous system. This first protein model led us to develop an efficient system for the production of functional P450s which are membrane proteins. Moreover, we also demonstrated that this enzyme catalyzed the hydroxylation of *p*-coumaroyl ester derivatives as expected but is more efficient to convert *p*-coumaroyl quinate into chlorogenic acid than *p*-coumaroyl shikimate. Since this result was rather original, we completed this study by transforming *R. gravoelens* plants, and made evidence that this enzyme could enhance the production of scopoletin and furanocoumarin.

Adaptation of the system for the utilization of a Pt: SfN8DT-1 as a control

Once the *N. benthamiana* system was established for a P450 enzyme, we wanted to assess its efficiency for an aromatic Pt. For this purpose, we selected *Sf*N8DT-1 which was characterized in 2008, and used it both for *in vitro* and *in vivo* functional characterization. As the substrate of this enzyme was not present in tobacco, we supplied it through a secondary infiltration in leaves. With this approach we could observe the prenylation of naringenin as described by Sazaki. Beside this *in vivo* approach, we also successfully checked the activity of the enzyme *in vitro* after total protein extraction. All these preliminary experiments clearly demonstrated that the *N. benthamiana* expression system was adapted for the functional characterization of membrane bound enzymes and, more precisely, of Pts.

Functional characterization of new Pt

Functional characterization of an Umb Prenyltransferase (Umb Pt) isolated from P. crispum: PcPt

As we didn't have any information concerning a potential substrate but we expected to find an involvement of the enzyme in the synthesis of coumarins or furanocoumarins, we chose first to express a putative Pt isolated from *P. crispum* in *N. benthamiana* and, second, perform a secondary infiltration using a mix of different molecules. This approach provided a prominent result which is that PcPt could catalyze the addition of DMAPP both onto the 6th and 8th Carbon of Umb to produce the DMS and osthenol respectively. *In vitro* approaches confirmed our results and kinetic parameters of enzymes were measured. The results we obtained highlighted the necessity of the presence of metallic ion (Mg^{2+}) for optimum enzymatic activity. Morever we showed that the optimal pH was 8.2 which is consistent with the results already described for Umb Pt in literature (Dhillon *and* Brown, 1976). The kinetic parameters measured for PcPt also demonstrated that the reaction for the synthesis of DMS is almost 50 times faster than the synthesis of osthenol. Furthermore, we could demonstrate that psoralen acts as an uncompetitive inhibitor of the prenylation reaction. Both the Km_{app} and $Vmax_{app}$ decreased when increasing the inhibitor concentration. This latest observation led us to assume that psoralen could act as feedback inhibitor for PcPt activity and hence could control the production of furanocoumarins when its concentration becomes excessive and could be lethal for plants.

The functionality of the enzyme was also tested through co-expression of PcPt and *p*-coumaroyl CoA 2-hydroxylase C2'-H (an enzyme catalyzing the step upstream in the furanocoumarin biosynthetic pathway) in *N. benthamiana.* This coexpression led to the synthesis of DMS and osthenol without an exogenous supply of Umb.

To assess the involvement of this enzyme in the synthesis of furanocoumarins in natural conditions, we investigated its function in parsley. We demonstrated a positive correlation between the expression level of PcPt (by using real time PCR approaches) and the relative contents of prenylated products (DMS and osthenol).

Finally, we overexpressed this gene in *R. graveolens*, a plant producing furanocoumarins. The furanocoumarin content was analyzed in these transgenic plants and we demonstrated a significant decrease of Umb compared to control plants.

Taken together, all these results led us to conclude that PcPt is an Umb Pt that is involved in the biosynthesis of DMS and osthenol in *P. crispum*.

Umb Pt of P. sativa

We showed that PcPt could catalyze the synthesis of linear and angular furanocoumarins. But intriguingly, parsley doesn't produce angular molecules. Therefore, we investigated the synthesis of these molecules in parsnip which has been widely described to produce both kinds of molecules. In our preliminary experiments we could isolate a Pt from *P. sativa* (PsPT) which shares high homologies with the parsley Pt. The expression and the characterization of this enzyme in the *N. benthamiana* expression system made evidence that these enzymes could catalyze the same reaction. When PsPt was incubated in the presence of Umb and DMAPP, we observed the appearance of DMS as a major product while osthenol was synthesized as a minor product. These results led us conclude that the Umb Pt is not the rate limiting step in the biosynthesis of angular furanocoumarins. It is therefore more likely that parsley lacks the downstream enzymes allowing the synthesis of angular furanocoumarins which is consistent with the study done by Dr. Larbat (personal communication).

Umb geranyl transferase (Umb gPt) of C. limon.

A similar approach was used to characterize the function of the candidate gene isolated from *C. limon*. As for both enzymes discussed above, this enzyme is clustering in a different group than the already characterized enzymes. In order to find out its function, we expressed it in tobacco and extracted the whole proteins to perform *in vitro* characterization. This way we could demonstrate that this enzyme could catalyze the addition of GPP to Umb and esculetin. These results were confirmed by stable expression in *R. graveolens*. Indeed, a significant decrease of Umb and the appearance of the geranylated Umb were detected in these transgenic plants. This result is important. It first shows that we discovered a new function of Pt which was never described before. Second, it confirms that the separate cluster of enzymes described in the phylogenetic analysis displays a new substrate specificity probably related to coumarin like molecules.

6 Perspectives

The *N. benthamiana* heterologous expression system has been successfully used here for both the *in vitro* and *in vivo* functional characterization of two enzymes that belong to two different families: P450 and Pts. The simplicity and rapidity of this system to perform the functional characterization of soluble and membrane bound proteins made it an efficient tool to decipher the function of many orphan genes. Additionally, this system could also be used as reactor to produce high amounts of any secondary metabolite if the genes involved in their biosynthesis are known as we demonstrated it through the synthesis of DMS when the plants were transformed with two successive genes (C2'H, Pt).

In this document, we used this system to successfully characterize the function of three out of 7 putative Pts isolated from two different plant families Rutaceae and Apiaceae. The next step will be to widen our range of potential substrates in order to find out the function of the 4 remaining putative Pts.

Development of new and more efficient sequencing methods will offer new databases. For example, new genomic sequencing programs (like phytozome) provide information concerning non-model plants. Through the analysis of these databases we could highlight 5 sequences of Pts isolated from *C. sinensis* and *C. clementina*. Preliminary experiments done on these genes made it possible to identify a gene involved in the synthesis of

pharmaceutically important prenylated molecules e.g. bergamottin. The characterization of this latest enzyme but also the identification of other activities will be the main priority in the next future.

Metabolic engineering techniques that are applied to medicinal plants open new horizons of research. Our results demonstrated that the constitutive expression of gene that is not directly involved in the biosynthesis of furanocoumarins could enhance the production of furanocoumarins. Overexpression of two C3'-H; one isolated from non furanocoumarin producing plant *A. thaliana* (CYP98A3), and second isolated from furanocoumarin producing plant *R. graveolens* (CYP98A22) demonstrated an increase in total furanocoumarins contents of *R. graveolens*. When we used the same technique for two Pts we could generate the synthesis of geranylated Umb. These observations provided the clue that this technique could be used not only to enhance the production of the pharmaceutically important molecules in plants, but also to produce molecules that don't exist in plant under normal conditions. However the use of this approach has its own limit since we observed a disappearance of Umb without any effect on the concentration of any other furanocoumarins, when PcPt is overproduced. Thus the production of an excess of furanocoumarin can stop their production. This is consistent with their potent inhibitor properties described for P450s and of other enzymes. This system could therefore be used as an interesting model to to study metabolic fluxes.

To conclude, with the development of knowledge of other enzymes supposed to be involved in the biosynthesis of furanocoumarins, we will certainly be able to rebuild the whole biosynthetic pathways of furanocoumarin in model plants such as *N. benthamiana* or *A. thaliana*. Moreover, since prenylated molecules are molecules with a high therapeutical potential, the study of these reaction has a major interest. These metabolic engineering could be used to fulfill requirements of various pharmaceutical applications (large scale production of furocoumarins), agronomic (phytoallexines) or health (phototoxicity of furocoumarins).

Résumé en français

7 Résumé en français pour validation par le conseil scientifique

Le métabolisme secondaire joue un rôle essentiel dans les processus d'adaptation des plantes à leur environnement. Les molécules qui composent ce métabolisme sont d'une très grande diversité et sont classées dans trois grandes familles : les alcaloïdes, les terpènes et les phénylpropanoïdes. Si ces molécules ont un rôle essentiel dans la survie des plantes dans un environnement souvent hostile, certaines d'entre elles disposent également de propriétés pharmaceutiques ou cosmétiques intéressantes et sont largement exploitées par l'Homme.

Un des projets de recherche développé au Laboratoire Agronomie et Environnement concerne l'étude de la voie de biosynthèse des furocoumarines chez les végétaux supérieurs.

Les furocoumarines sont des molécules appartenant à la famille des polyphénols qui ne sont représentées que chez quelques familles de végétaux : les fabacées, les rutacées, les apiacées et les moracées (Bourgaud *et al.,* 2006). Les propriétés décrites pour ces molécules sont nombreuses. Il a été démontré qu'elles sont impliquées dans des processus d'allélopathie et que ce sont des phytoaléxines qui confèrent à la plante une protection contre les herbivores et les micro-organismes pathogènes (McCloud *et al.,* 1992; Ojala *et al.,* 2000). D'autres travaux indiquent que ces molécules ont la capacité d'inhiber la germination et la croissance de plantes concurrentes et contribuent à la compétition interspécifique (Baskin *et al.,* 1967; Junttila, 1976). Enfin, certains travaux ont montré que ces molécules pouvaient être utilisées à des fins thérapeutiques pour traiter des maladies de la peau et plus récemment pour soigner la sclérose en plaque ou certains cancers (Conforti *et al.,* 2009; Lacy *and* O'Kennedy, 2004). Il existe 2 types de furocoumarines qui définissent deux voies de biosynthèse différentes: les furocoumarines linéaires d'une part et les furocoumarines angulaires d'autre part. Ces dernières sont probablement apparues suite à des phénomènes de co-évolution entre les plantes et les insectes, conférant aux plantes qui les synthétisent un avantage par leur capacité à limiter la prédation par les insectes (Li *et al.,* 2003).

Comme la lignine, l'acide chlorogénique, les coumarines ou encore les flavonoïdes, les furocoumarines dérivent de la voie de biosynthèse des phénylpropanoïdes (Figure 113) qui est présente chez tous les végétaux supérieurs. Si la compréhension de la synthèse de

ces molécules présente un intérêt tant fondamental qu'appliqué, le déterminisme moléculaire est encore mal connu à ce jour. De nombreuses études réalisées dans les années 70 ont permis de mettre en évidence, par des approches biochimiques, que presque 8 étapes de synthèse de ces molécules étaient catalysées par des enzymes de la famille des cytochromes P450. Pour l'instant, seules 3 de ces enzymes P450 ont fait l'objet de publications scientifiques. Ainsi, la cinnamate 4-hydroxylase (Gravot *et al.,* 2004; Hübner *et al.,* 2003), la psoralène synthase et l'angélicine synthase (Larbat *et al.,* 2007, 2009) ont été décrites chez diverses familles de plantes. Parallèlement, d'autres familles d'enzymes non-P450 appartenant à cette voie de biosynthèse ont été mises en évidence. Hehman et ses collaborateurs ont caractérisé la bergaptol *O*-méthyltransférase (Hehmann *et al.,* 2004) catalysant une des dernières étape de la voie de biosynthèse. Plus récemment, Vialart et ses collaborateurs ont démontré le rôle d'une dioxygénase α-cétoglutarate-dépendante dans l'*ortho*-hydroxylation du *p*-coumaroyl-CoA pour donner de l'Umb, précurseur direct des furocoumarines. Malgré ces nombreux travaux, il reste de nombreuses étapes qui sont encore peu documentées. Parmi ces étapes, la prénylation de l'umbelliférone (umb) (Figure 113 C) est particulièrement importante puisqu'il s'agit de l'étape qui contrôle la synthèse de la déméthylsubérosine (DMS) ou de l'osthénol qui sont respectivement les précurseurs des furocoumarines linéaires ou angulaires. Cette réaction consiste en l'ajout d'une molécule de dimethylallyl pyrophosphate (DMAPP) sur le carbone 6 ou le carbone 8 de l'Umb et est catalysée par une enzyme de la famille des prényltransférases (Pts).

Les Pts à substrats aromatiques sont relativement peu décrits chez les plantes. Des travaux réalisés par Brown et ses collaborateurs ont mis en évidence que des enzymes pouvaient catalyser l'addition d'un groupement prényl sur le carbone 6 de l'Umb, permettant ainsi la synthèse de DMS chez *R. graveolens* (Ellis *and* Brown, 1974) et que la localisation subcellulaire de cette activité était restreinte aux plastes (Dhillon *and* Brown, 1976). Ces travaux ont été complétés par Hamerski et coll. (Hamerski *et al.,*1990) qui ont montré que deux Pts différentes, exprimées dans des cultures cellulaires induites d'*A. majus,* étaient capables de catalyser la 6-C-prénylation et 7-O-prénylation de l'Umb, générant ainsi des intermédiaires de la voie de biosynthèse de buténynl-éthers et dihydrofurocoumarines. Ce n'est qu'en 2008 que Sasaki et ses collaborateurs ont apporté les premiers éléments moléculaires concernant les Pts aromatiques avec l'identification et la caractérisation fonctionnelle d'une enzyme capable de réaliser la prénylation d'un flavonoïde (Sasaki *et al.,* 2008). Dans cette étude, ces auteurs montrent qu'un gène isolé à

partir de *Sophora flavescens* était en mesure de transférer une molécule de DMAPP sur la naringénine. Cette enzyme a été nommée Naringenin 8-dimethylallyltransferase (*SfN8DT-1*).

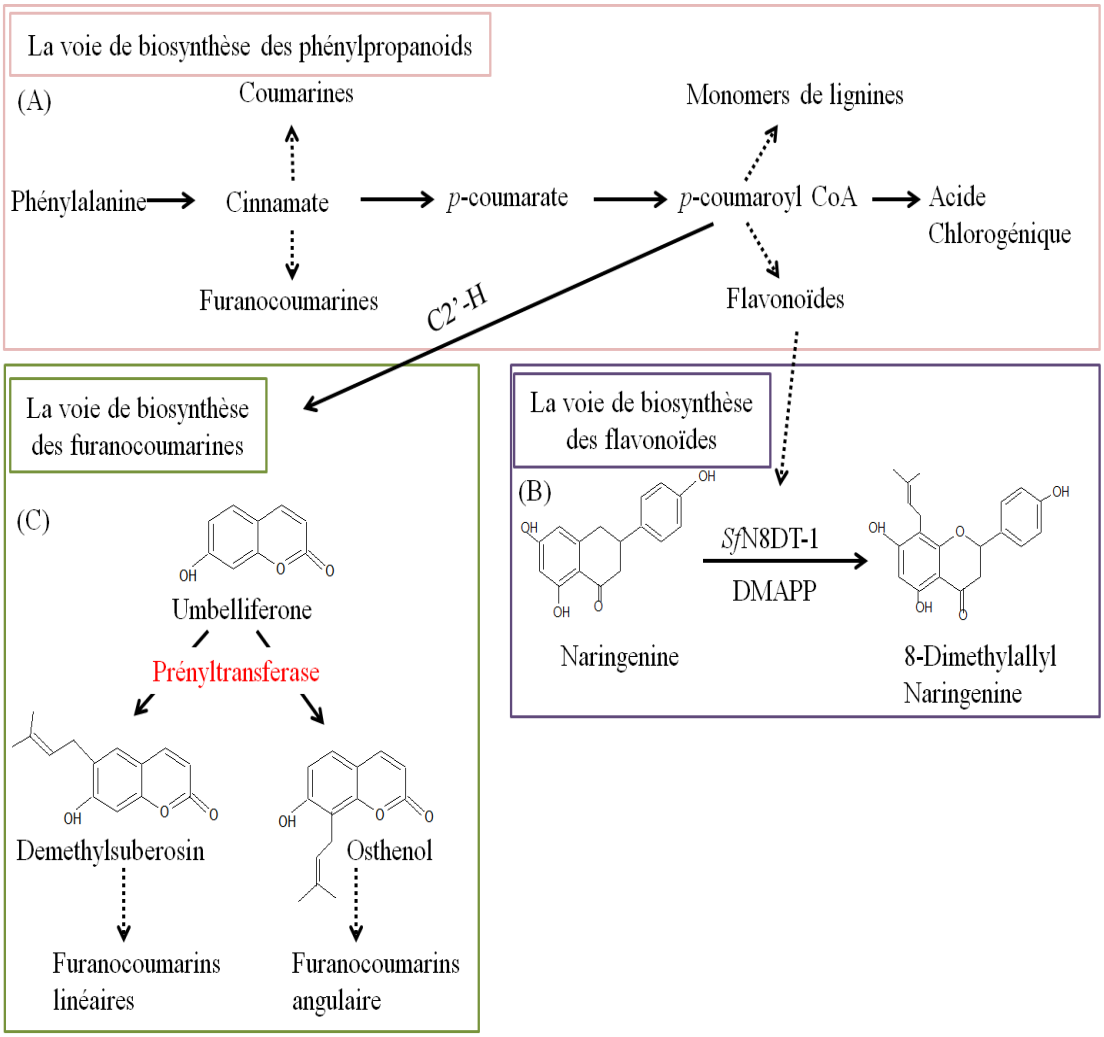


Figure 113. Représentation schématique de la voie de biosynthèse des phénylpropanoïdes

Afin de lever le voile sur cette étape essentielle de la voie de biosynthèse des furocoumarines, nous nous sommes fixés comme objectif, dans le cadre de cette thèse, d'identifier et de caractériser fonctionnellement de nouvelles Pts impliquées dans la synthèse de ces molécules. Ce travail a été effectué en trois étapes. La première étape a consisté à identifier de nouveaux gènes présentant des caractéristiques communes avec des Pts déjà décrites. Dans la seconde étape il a fallu développer un système d'expression hétérologue adapté à l'expression de ces protéines particulières. Enfin, la caractérisation fonctionnelle des enzymes a été effectuée dans la troisième étape de ce travail.

7.1 Chapitre 1 : Identification et clonage de nouveaux gènes codants pour des Prenyltransferases

Afin de trouver de nouveaux gènes appartenant à la famille des Pts et impliqués dans la synthèse de furocoumarines, nous avons utilisé deux approches complémentaires. La première approche a consisté à utiliser la séquence de *Sf*N8DT-1 pour cribler des bases de données informatiques (publiques et celles disponibles au laboratoire) et rechercher des gènes présentant des homologies de séquences importantes chez des plantes productrices de furocoumarines. Cette approche nous a permis d'identifier 2 gènes, le premier chez *A. gigas* et le second chez *P. sativa*. La seconde méthode d'identification de nouveaux gènes a été utilisée par des collègues japonais avec qui le LAE a établi une collaboration en 2009. Les Pts renferment des séquences consensus conservées. En se basant sur ces séquences, les collègues japonais ont fait synthétiser des amorces dégénérées qui ont été utilisées dans des approches de PCR pour cloner 5 gènes chez différentes plantes productrices de furocoumarines : le persil, *Ruta graveolens*, la Lime, le Citron et le Céleri. Une analyse bioinformatique nous a permis de confirmer la présence des séquences conservées essentielles à la prénylation (Ohara *et al.,* 2009) mais également de montrer que ces protéines adoptaient une structure proche de celle décrite pour SfN8DT-1, de *Sophora flavescens*, avec 6 à 9 domaines transmembranaires. Enfin, une analyse phylogénétique comparative de ces Pts candidates avec d'autres Pts dont les séquences étaient disponibles dans les bases de données a permis de montrer que 6 des 7 séquences étaient regroupées dans un groupe distinct mais proche de celui de Pts impliquées dans la prénylation des flavonoïdes. Ces observations nous ont donc amené à émettre l'hypothèse que ce groupe d'enzyme pourrait être impliqué dans la prénylation de molécules de la famille des coumarines et de leurs dérivés.

7.2 Chapitre 2 : Mise au point d'un système d'expression adapté pour l'expression de protéines membranaires

Le choix d'un système d'expression hétérologue approprié est une étape clé dans la caractérisation fonctionnelle de ces enzymes liées aux membranes. Le laboratoire dispose d'une expérience importante dans l'expression de protéines membranaires de la famille des cytochromes P450s. Nous avons donc choisi de démarrer la mise en point du système

d'expression par l'utilisation d'une enzyme de la sous-famille CYP98A, dont l'activité a déjà été largement décrite comme étant une 3'-hydroxylase d'ester de *p*-coumaroyl-CoA (C3'-H). CYP98A22, qui est le gène orthologue isolé à partir de *R. graveolens* a été exprimé transitoirement dans *N. benthamiana*. Pour cela, le gène codant pour CYP98A22 a été introduit dans des agrobactéries qui ont ensuite été infiltrées dans les feuilles en même temps que des agrobactéries permettant l'expression d'une protéine suppresseur de « gene silencing » (p19). Grâce à cette approche nous avons pu montrer que cette enzyme présentait de nombreuses similarités avec CYP98A3 d'*A. thaliana* et notamment sa gamme de substrats. Cependant nous avons également pu mettre en évidence des différences et notamment nous avons montré que cette enzyme métabolisait préférentiellement le *p*-coumaroyl quinate et favorisait la synthèse d'acide chlorogénique, molécule réputée pour son rôle dans les réactions de défense des plantes. Ces travaux ont été complétés par la surexpression stable de cette enzyme dans *R. graveolens*. Au travers de cette étude, nous avons observé que la surexpression de CYP98A22 entrainait une augmentation de la synthèse de scopolétine et de furocoumarines dans les plantes transgéniques en comparaison avec des plantes contrôle. Ces résultats ont fait l'objet d'une publication dans BMC plant biology.

Dans une seconde étape, nous avons choisi de valider le système d'expression en surexprimant *Sf*N8DT-1 dans *N. benthamiana*. Le substrat de cette enzyme n'étant pas présent dans le tabac, il a été nécessaire de le fournir en réalisant une infiltration secondaire, 72 h après l'inoculation par les agrobactéries. Cette approche nous a permis de confirmer les résultats obtenus par Sazaki, à savoir la prénylation de la naringénine (Sasaki *et al.,* 2008) mais également de confirmer la fonctionnalité du système d'expression pour une protéine similaire à celle que nous voulions étudier. Parallèlement à cette approche « *in vivo* », nous avons également confirmé la fonctionnalité de l'enzyme « *in vitro* »après l'extraction des protéines totales et leur utilisation dans des tests enzymatiques.

7.3 **Chapitre 3: Caractérisation fonctionnelle de nouvelles prenyltransferases impliquées dans la synthèse de furocoumarines.**

Dans un premier temps, nous nous sommes focalisés sur la Pt isolée à partir de Persil (*P. crispum*, PsPt). N'ayant initialement pas d'idée arrêtée et précise du substrat de l'enzyme, nous avons dans un premier temps effectué un criblage fonctionnel assez large. Pour cela, nous avons infiltré des agrobactéries permettant l'expression de la PcPt et

de la protéine P19, puis, après 72 h, nous avons effectué une infiltration secondaire avec un mélange de molécules constitué soit de coumarines et furocoumarines, soit un mélange de flavonoïdes. Cette approche nous a permis de montrer que l'Umb était métabolisé *in vivo* pour générer de la DMS et de l'osthenol. Ce résultat démontre que la protéine PcPt est bien l'enzyme que nous recherchions et qui permet la synthèse de la DMS, inermédiaire essentiel à la synthèse des furocoumarines linéaires. En revanche, la synthèse par cette même enzyme de l'osthenol est étonnante. En effet, l'osthenol est le précurseur de la voie de biosynthèse des furocoumarines angulaires, or ces molécules n'ont jamais été décrites chez le persil. Afin de vérifier cette activité, nous avons réitéré ces expérimentations mais en faisant l'extraction des protéines totales de feuilles de tabac agroinfiltrés et en réalisant les incubations enzymatique *in vitro* en présence d'une large gamme de molécule comprenant diverses coumarines mais également divers dérivés prénylés (DMAPP, GPP ou FPP). Pour effectuer ces réactions, différentes mises au point préliminaires ont été nécessaires. Nous avons montré que la présence d'ions métalliques (Mg^{2+}) ainsi qu'un pH 8,2 était nécessaire pour avoir une activité optimale de l'enzyme. Ces incubations *in vitro* ont confirmé la spécificité de substrat de l'enzyme pour l'Umb et le DMAPP ainsi que la synthèse des deux molécules : DMS et Osthenol. Cependant, les paramètres cinétiques mesurés pour PcPt ont mis en évidence que la synthèse de DMS était 50 fois plus rapide que la synthèse d'osthenol. Enfin, nous avons montré que cette réaction était inhibée lorsqu'on rajoute du psoralène dans le mix réactionnel. Cette inhibition est de type non-compétitif et les valeurs du Km_{app} et de la $Vmax_{app}$ diminuent en augmentant la concentration de l'inhibiteur. Cette dernière observation nous a conduits à émettre l'hypothèse que le psoralène pourrait effectuer un rétrocontrôle négatif sur la synthèse de furocoumarines lorsque la concentration en psoralène devient excessive et nocive pour la plante. Ce rétrocontrôle négatif permettrait de réguler la voie de biosynthèse des furocoumarines et d'ajuster les teneurs en furocoumarines chez la plante.

Pour compléter cette étude, nous avons testé la fonctionnalité de l'enzyme lorsque celle-ci est co-exprimée avec la *p*-coumaroylCoA 2-hydroxylase C2'-H (une enzyme qui catalyse l'étape en amont dans la voie de biosynthèse des furanocoumarine) chez *N. benthamiana*. Cette expérimentation a permis de mettre en évidence la présence de DMS et d'osthenol dans les tabac agroinfiltrés qui ne disposent initialement que de très peu d'Umbelliferone. Ceci démontre qu'il est possible de reconstruire cette voie métabolique dans une plante qui n'est pas capable de synthétiser ces molécules à la base.

L'ensemble de cette étude ayant été effectuée dans le tabac, qui n'est initialement pas producteur de furocoumarines, nous avons souhaité voir ce qui se passait lorsque ce gène était surexprimé dans *R. graveolens*. Pour cela nous avons construit des plantes transgéniques dans lesquelles le gène codant pour la PcPt était placée sous le contrôle du promoteur 35S du CaMV. L'analyse de la teneur en furocoumarines a mis en exergue une diminution significative de la concentration en Umb par rapport à des plantes témoins bien qu'une augmentation de la concentration en DMS et en osthenol n'ait pas été démontrée.
Enfin, une étude dans la plante d'origine, le persil, a permis de mettre en évidence une corrélation positive entre la variation de la concentration de DMS et d'osthenol dans les feuilles de *P. crispum* et le niveau d'expression de la PcPt.

Ce travail nous a donc permis de montrer que PcPt pouvait initier la synthèse de furocoumarines linéaires et angulaires bien que ces molécules soient absentes chez le persil. Pour compléter ce travail nous avons choisi, dans un second temps de nous focaliser sur une Pt que nous avons cloné à partir du panais (*P. sativa*, PsPt), une plante qui produit les deux types de furocoumarines. L'analyse informatique nous a permis de montrer que la PsPt est identique à 82% de la PcPt. L'expression et la caractérisation fonctionnelle de cette enzyme dans le système *N. benthamiana* nous a fourni la preuve que ces enzymes pouvaient catalyser exactement la même réaction. En effet, lorsque la PsPt a été incubée en présence Umb et de DMAPP, nous avons constaté l'apparition de DMS comme un produit majeur et l'osthenol comme un produit mineur. Ces résultats nous amènent à conclure que cette seconde étape de la voie de biosynthèse des furocoumarines n'est pas une l'étape qui fait défaut chez le persil, pour la synthèse des furocoumarines angulaires, puisqu'elle est présente à la fois chez le persil (producteur de furocoumarines linéaires) et le panais (producteur de furocoumarines linéaires et angulaires). L'absence de furocoumarines angulaires chez le persil serait donc plutôt liée à l'absence des enzymes en aval de cette étape.

La même approche a été utilisée pour caractériser la fonction des 5 autres gènes codant pour des Pts potentielles décrites dans le premier chapitre de ce travail. Le gène candidat isolé à partir de *C. limon* (CliPt) a été exprimé transitoirement dans des feuilles de *N. benthamiana* et de manière stable dans *R. graveolens*. L'approche de criblage *in vivo* par expression transitoire utilisée dans une première étape pour la caractérisation fonctionnelle de CliPt n'ayant pas été fructueuse, nous avons été amenés à faire les incubations *in vitro*.

De cette manière nous avons pu démontrer que la CliPt catalysait l'addition de GPP sur de l'Umb et sur l'esculétine. Ces résultats sont été confirmés chez des *R. graveolens* transgéniques surexprimant ce gène par l'observation d'une diminution significative d'Umb et par l'apparition d'Umb geranylée.

En ce qui concerne les 4 derniers gènes disponibles, aucune activité n'a pu être mise en évidence à ce jour.

Les résultats obtenus dans le cadre de ce travail de thèse ont donc permis de faire progresser de manière significative la compréhension du déterminisme moléculaire de la voie de biosynthèse des furocoumarines. Nous avons identifié trois enzymes impliquées directement dans la synthèse de dérivé prénylés en liens avec les furocoumarines (Figure 114). La mise en évidence de ces activités et des gènes correspondant renforce l'hypothèse d'un groupe d'enzymes impliquées dans la prénylation de molécules apparentées aux coumarines. Cette hypothèse devra maintenant être renforcée par la caractérisation fonctionnelle des 4 gènes codant pour des Pts potentielles restantes mais également pour d'autres gènes qui ont été isolés depuis le début de ce travail. Pour cela, il faudra probablement élargir la gamme de molécules à tester.

Le système d'expression hétérologue de *N. benthamiana* a été utilisé avec succès pour réaliser la caractérisation fonctionnelle « *in vitro* » et « *in vivo* »de quatre enzymes appartenant à deux familles enzymatique différentes: les P450 et les Pts.

Au vu de ces résultats, ce système pourrait être utilisé comme réacteur pour produire de grandes quantités de toutes sortes de métabolites secondaires avec cependant comme limite que les gènes impliqués dans leur voie de biosynthèse soient connus. Nous avons démontré la faisabilité de cette approche pour la synthèse de DMS lorsque les plantes expriment deux enzymes successives dans la synthèse des furocoumarines (C2'H, Pt). Ces approches de génie métabolique pourraient ainsi servir pour la production de molécules d'intérêt et satisfaire aux exigences de diverses industries pour la production de molécules ayant des applications pharmaceutiques (production à grande échelle de furocoumarines), agronomiques (phytoaléxines) ou pour la santé (phototoxicité des furocoumarines).

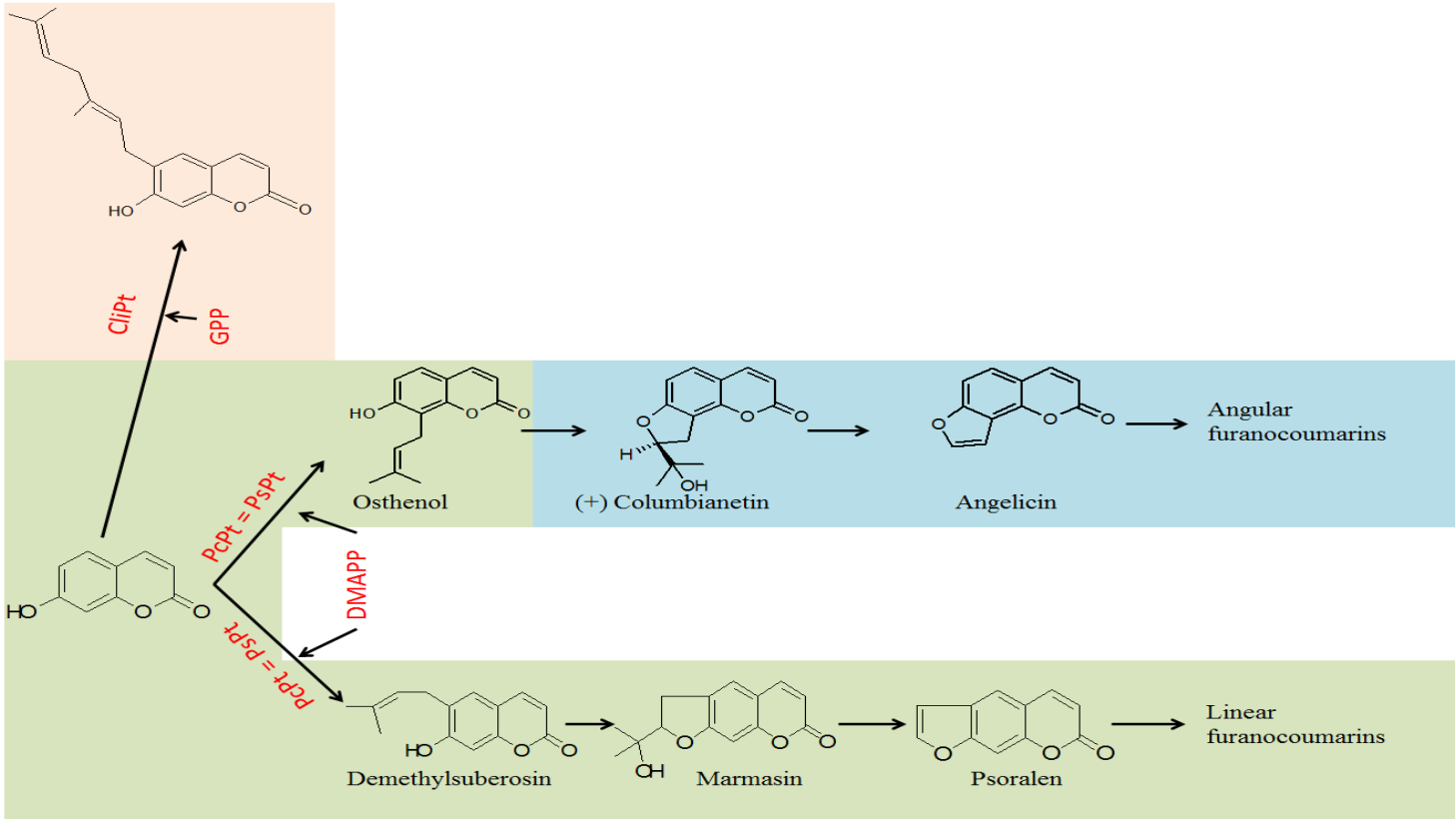


Figure 114. Représentation simplifiée de la voie de biosynthèse des furanocoumarines présentant l'implication des trois prényltransferases caractérisées dans le cadre de ce travail de thèse.

PcPt (prenyltransfersae de *P.crispum*), PsPt (prényltransférase de *P. sativa*), CliPt (prényltransférase de *C. limon*), DMAPP (diméthylallyl pyrophohphate) et GPP (géranyl pyrophosphate).Voie de biosynthèse des furanocoumarins est trouvés dans *P. crispum* sont représentées par de l'area vert, tandis que *P. sativa* est représentée par l'ombre verte et bleue ensemble et la couleur rose représente molécule trouvé chez *C. limon*.

Résumé en français

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List of scientific communications

List of scientific communications

Karamat F., Hehn A., Olry A., Yazaki K., Bourgaud F. Poster Presentation.

Identification moléculaire et caractérisation fonctionnelle des prényltransferases impliquées dans la voie de biosynthèse des furanocoumarins. 8ième Séminaire de l'Ecole Doctorale RP2E, 20 janvier 2011, Nancy, France.

Karamat F., Oral Presentation.

Functional characterization of the first prenyltransferase involved in the biosynthesis of coumarins, important molecules in the defense system of higher plants. 9ième Séminaire de l'Ecole Doctorale RP2E, 19 janvier 2012, Nancy, France.

Karamat F., Poster and Oral Presentation.

How C3'H (CYP98A22) can be used as an alternative tool for enhancing the furanocoumarin concentration in *Ruta graveolens.* 11th International Symposium on Cytochrome P450 Biodiversity *and* Biotechnology, 22-26 of June, 2012, Torino, Italy.

Karamat F., Olry A., Doerper S., Vialart G., Ullmann P., Werck-Reichhart D., Bourgaud F., Hehn A.
CYP98A22, a phenolic ester 3'-hydroxylase specialized in the synthesis of chlorogenic acid, as a new tool for enhancing the furanocoumarin concentration in Ruta graveolens. BMC Plant Biol. 2012 Aug 29;12:152.

Karamat F., Olry A., Munakata R., Koeduka T., Sugiyama A., Hehn A., Bourgaud F., Yazaki K. A prenyltransferase isolated from parsley (*Petroselinum crispum*) catalyzes the synthesis of demethylsuberosine and osthenol, the entry point in the furanocoumarin biosynthesis pathway. *Soumission prevue en avril 2013 dans PNAS*

UNIVERSITÉ DE LORRAINE

A Nancy, le 06 mai 2013

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Madame,

Par décision en date du 06 mai 2013, vous avez été autorisée à présenter en soutenance vos travaux en vue de l'obtention du diplôme :

Doctorat Sciences Agronomiques

La soutenance aura lieu le 21 mai 2013 à 14h00 à l'adresse suivante :

Salle Gallé - Ex Présidence de l'INPL - 2, Avenue de la Forêt de Haye 54500 VANDOEUVRE les NANCY

La soutenance sera publique.

Je vous prie d'agréer, Madame, l'expression de mes salutations distinguées.

Le Président

Pierre MUTZENHARDT

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Identification and functional characterization of the first two aromatic prenyltransferases implicated in the biosynthesis of furanocoumarins and prenylated coumarins in two plant families: Rutaceae and Apiaceae

Furanocoumarins constitute one of the classes of secondary metabolites deriving from the phenylpropanoid biosynthetic pathway. These molecules are described as phytoalexins in plants but are also used by humans for their pharmaceutical properties. A large number of biochemical studies were carried out to understand their biosynthetic pathway but little information was available concerning the genes involved in the pathway. In this study, we focused on the characterization of genes encoding for aromatic prenyltransferases which were described to be involved in this pathway.

Prenlyltransferases catalyze the entry step to the linear or angular furanocoumarin pathway. Hence they catalyze the addition of a dimethylallyl pyrophosphate (DMAPP) prenyl moiety to umbelliferone. Using a recently characterized aromatic prenyltransferase (*Sf*N8DT-1) as a probe, we isolated 7 different candidate genes from two plant families (Rutaceae and Apiaceae). As these enzymes were described as membrane bound proteins, we adapted a heterologous expression system made up of *Nicotiana benthamian a*and we validated its efficiency by using two membrane-associated enzymes: a cytochrome P450 (CYP98A22) and the already described prenyltransferase *Sf*N8DT-1. Subsequently, this system was used to perform the functional characterization of the 7 newly identified proteins. This way we succeeded to characterize the first umbelliferone prenyltransferase of *Petroselinum crispum* that was able to catalyze both the 6-C and 8-C prenylation of umbelliferone with DMAPP producing demethylsuberosin and osthenol respectively. We made evidence that these reactions occurred both *in vitro* and *in vivo*. In addition, *in planta* studies performed in *P.crispum* showed a positive relationship between the gene expression level and the content of prenylated umbelliferone. The overexpression of this gene was investigated in *Ruta graveolens* and we could provide evidences of a link between the enzymatic activity and the disappearance of umbelliferone. We also reported a similar activity for a prenyltransferase isolated from *Pastinaca sativa*, which makes us assume that the prenylation step is not a rate limiting step in the biosynthetic pathway of angular furanocoumarins since parsley is producing only linear furanocoumarins whereas parsnip is producing both linear and angular furanocoumarins. In addition, using the same *N. benthamiana* and *R. graveolens* heterologous expression systems, we identified a second aromatic prenyltransferase able to catalyze the addition of geranyl pyrophosphate (GPP) both to umbelliferone and esculetin.

Keywords: Prenyltransferase, Umbelliferone, Demethylsuberosin, Osthenol, Furanocoumarins, Heterologous expression system, *Nicotiana benthamiana*, *Ruta graveolens*

L'identification et caractérisation fonctionnelle des deux premiers prényltransférases aromatiques impliqués dans la biosynthèse de furanocoumarines et des coumarines prénylés chez deux familles de plantes: Rutaceae et Apiaceae

Les furocoumarines constituent l'une des classes demétabolites secondaires dérivant de la voie de biosynthèse des phénylpropanoïdes. Elles ont été décrites comme étant des phytoaléxines mais sont également très largement utilisées par l'Homme pour leurs propriétés thérapeutiques. Un certain nombre d'études biochimiques ont été réalisées afin d'en comprendrela biosynthèse mais peu de choses sont connues concernant leur déterminisme moléculaire. Dans cetteétude, nous nous sommes concentrés sur la caractérisation fonctionnelle de gènes appartenant aux prényltransférases aromatiques potentiellement impliquées dans cette voie de biosynthèse.

Les prényltransférases catalysent la première étape de la voie de biosynthèse des furocoumarines linéaires ou angulaires. Elles permettent l'addition d'un groupement dimethylally pyrophosphate (DMAPP) sur l'umbelliférone. En utilisant *Sf*N8DT-1, une prényltransférase aromatique récemment caractérisée, comme sonde, nous avons identifié 7 gènes candidats chez deux familles de plantes (Rutaceae et Apiaceae). Dans la mesure où il a été décrit que ces enzymes étaient des protéines membranaires, nous avons adapté un système d'expression hétérologue basé sur l'utilisation de *N. benthamiana*. Ce système a été validé par l'expression de deux protéines membranaires : un cytochrome P450 CYP98A22 et une prényltransférase déjà caractérisée *Sf*N8DT-1.Nous avons ensuite utilisé ce système d'expression pour réaliser l'étude des 7 gènes nouvellement isolés. Ces travaux nous ont permis de caractériser la première umbelliferone prenyltransferase de *Petroselinum crispum* capable de catalyser *in vitro* et *in vivo* la prénylation du carbone 6 ou 8 de l'umbelliférone en présence de DMAPP permettant ainsi la synthèse respectivement de demethylsuberosin et d'osthenol. Par ailleurs, une étude réalisée *in planta* chez le persil a permis de mettre en évidence une relation positive entre le niveau d'expression du gène et la teneur en umbelliférone prénylée. L'étude de la surexpression du gène chez *Ruta graveolens* a permis de mettre en évidence un lien entre l'expression du gène et la disparition de l'umbelliférone. Enfin nous avons identifié la même activité pour une prényltransférase de *Pastinaca sativa,* ce qui nous amène à émettre l'hypothèse que l'étape de prénylation n'est pas une étape limitante dans la biosynthèse des furocoumarines angulaires, étant donné que le persil ne produit que des furocoumarines linéaires, tandis que le panais produit des furocoumarines linéaires et angulaires. L'utilisation de ces mêmes systèmes d'expression hétérologue de *N. benthamiana et R. graveolens* nous a également permis d'identifier une seconde prényltransférase aromatique capable de catalyse rl'addition de géranylpyrophosphate (GPP) sur l'umbelliférone et sur l'esculétine.

Mots clés : Prényltransférase, Umbelliférone, Demethylsuberosin, Osthenol, Furocoumarines, système d'expression hétérologue, *Nicotiana benthamiana*, *Ruta graveolen*