The evolution of chemical diversity in plants : pyrrolizidine alkaloids and cytochrome P450s in Jacobaea

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Chapter 5

Tests of cytochrome P450 candidates for the pyrrolizidine alkaloid pathway of *Jacobaea* species using an expression system in yeast

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Abstract

Pyrrolizidine alkaloids (PAs) are a typical class of secondary metabolites with high structural diversity. It is assumed that specific structural diversification of the backbone structure senecionine *N*-oxide to other PAs resulting in unique PA bouquets provides a powerful strategy for plants to cope with biotic and abiotic stresses. Senecionine *N*-oxide undergoes structural transformations including site-specific oxidative modifications and therefore, cytochrome P450s (CYPs) could be involved in PA biosynthesis. Here eight CYP candidates of *Jacobaea* species were tested for their functions by using heterologous expression in yeast and in vitro enzyme assays. The enzyme assays were performed using extracted microsomal membranes and the CYP geraniol 10-hydroxylase was used as a positive control. None of the eight CYP enzymes showed catalytic activities in structural conversion using senecionine/integerrimine, seneciphylline, jacobine, erucifoline or a PA mixture as well as the respective *N*-oxides as substrates. Although this might due to the fact that more optimal reaction conditions are required, most likely these CYPs are not enzymes invoved in the transformation of the tested substrates.

Keywords

yeast, pYeDP60u, microsome, senecionine, intergerrimine, seneciphylline, jacobine, erucifoline

Introduction

Pyrrolizidine alkaloids (PAs) are a characteristic group of plant secondary metabolites (SMs) with a high diversity of chemical structures. Of the senecionine type PAs, senecionine *N*-oxide (Fig. 1) has been identified as the primary product of the biosynthesis of the 12-membered macrocyclic senecionine-type PAs by tracer-feeding experiments (Hartmann and Toppel 1987; Hartmann *et al*., 1989; Hartmann and Dierich, 1998). In shoots of plants of the Senecioneae tribe, senecionine *N*-oxide was found to be diversified into species- or chemotype-specific bouquets of biosynthetically derived PAs including seneciphylline, jacobine and erucifoline (Hartmann and Dierich, 1998). These PAs have been extensively studied with regards to their ecological roles in defense (Macel *et al*., 2005; Nuringtyas *et al*., 2014; Liu *et al*., 2017; Leiss *et al*., 2009; Cheng *et al*., 2011; Wei *et al*., 2015). These studies demonstrated that PAs with different structures have different bioactivities on herbivores. It is assumed that specific structural conversion of senecionine *N*-oxide to other PAs resulting in individual PA bouquets provides a powerful strategy for plants to cope with a dynamic environment (Hartmann and Dierich, 1998). Still an open question is how the PA bouquet is brought about in individual plants. Thus, it is of interest to elucidate the underlying genes of the PA biosynthesis.

Transformation between most of the 12-membered senecionine type PAs can be explained by simple one- or two-step reactions from the primary PA senecionine *N*-oxide (Hartmann and Dierich, 1998; Pelser *et al*., 2005; Langel *et al*., 2011). Senecionine *N*-oxide (Fig. 1) undergoes position-specific and stereoselective structural transformations resulting in the rearrangement of the skeletal structure and in oxidative modifications (Hartmann and Dierich, 1998). The oxidative modifications include 18-hydroxylation, 13,19-dehydrogenation, 15,20-epoxidation, 12,13-epoxidation, 19-hydroxylation and 8-oxidation (see Fig. 1 and Fig. S1 of Chapter 4), which could be catalyzed by cytochrome P450s (CYPs) as CYPs catalyze the oxidative modifications of various substrates using oxygen and NAD(P)H in various SM biosynthesis pathways. Through metabolic and transcriptomic profiling techniques, initial CYP candidates for involvement in PA biosynthesis were selected from *Jacobaea* species (Chapter 4) and their functions remain to be tested.

In order to unequivocally characterize the enzymatic activities of CYPs, heterologous expression of CYP proteins is a critical step. All plant CYPs described so far are bound to membranes, usually to the endoplasmic reticulum (ER). To be active, CYPs need to be coupled with electron-donating proteins, CYP reductases or cytochrome b_5, which are also anchored to the surface of ER. Most commonly, via the NADPH-dependent CYP reductase (CPR) heme-bound O_2 is activated by the successive transfer of two electrons from NADPH (Bak *et al*., 2011), leading to regiospecific and stereospecific oxidative attack of a plethora of substrates. Therefore, yeast is the most often used system for heterologous expression of CYPs. For analysis of plant CYPs the yeast strain WAT11 (Pompon *et al*., 1996) which expresses an NADPH reductase from *Arabidopsis thaliana* instead of the yeast NADPH reductase and the

yeast expression vector pYeDP60 (Urban *et al*., 1990) has been traditionally used (Duan and Schuler, 2006; Miettinen *et al*., 2014; Liu *et al*., 2016; Liu *et al*., 2018). However, the vector pYeDP60 offers a limited selection of restriction sites which complicates the cloning of CYPs. The adaption of the vector to the uracil-excision based (USER^{TM}) cloning technique (Nour-Eldin *et al*., 2006) resulting in pYeDP60u was advocated as more optimal in high-throughput screening of CYPs (Hamann and Møller, 2007).

Here the functional tests of eight CYP candidates available in *Jacobaea vulgaris* were performed. After recombination of selected genes into the pYeDP60u vector, CYP genes were introduced in yeast. Subsequently, microsomes were extracted for *in vitro* enzyme assays using isolated PAs or a PA mixture as substrates, and reaction products were analyzed by LC-MS/MS. Geraniol 10-hydroxylase (G10H; CYP76B6) from *Catharanthus roseus* (Collu *et al*., 2001) was used as a positive control in this study.

Materials and Methods

Plant material and cDNA synthesis

Tissue culture plants of *J. vulgaris* were grown and treated with methyl jasmonate (MeJA) as reported in Chapter 4. The shoots of cultured plants with the MeJA treatment of eight days were ground into fine powder in liquid nitrogen, and were stored at -80 °C. Total RNA was extracted by phenol/chloroform extraction followed by overnight precipitation with ca. 3 M lithium chloride, washed with 70% ethanol, and resuspended in water. First strand cDNA was synthesized using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit.

Amplification and cloning of CYP genes

Eight CYP candidate genes, i.e. CYP71AT158, CYP71AV19, CYP76S36, CYP706C72, CYP706E12, CYPP81B113, CYP82D180 and CYP82Q5, from different CYP families/subfamilies were selected based on the association of PA abundances with differential gene expression of CYPs between different *Jacobaea* samples (Chapter 4). G10H (CYP76B6) from *C. roseus* was used as a positive control. The full-length coding sequences were amplified by PCR using Phusion® High-Fidelity DNA Polymerase (New England Biolabs) according to manufacturer's instruction (for primers see Table S1). Purified PCR fragments were ligated into the pJET1.2 vector using the Thermo Scientific CloneJET PCR Cloning Kit and introduced into *Escherichia coli* strain XL1-Blue. The resulting plasmids were sent for sequencing of both strands by BaseClear B.V. (The Netherlands).

Preparation of pYeDP60u vector for cloning

The yeast vector pYeDP60u (Hamann and Møller, 2007) was a kind gift from Nicolas Navrot from Strasbourg University. This vector was prepared for cloning according to Nour-Eldin *et al*. (2006) with minor modification. One μg pYeDP60u plasmid was digested with 10 U PacI (New England Biolabs) overnight in a total volume of 50 μL. An additional 5 U PacI was

added in together with 5 U Nt.BbvCI (New England Biolabs), and the digestion was incubated for 2 hours at 37 °C. The linearized vector was purified using the Thermo Scientific GeneJET Gel Extraction Kit.

Expression of CYP genes in yeast

CYP genes were PCR re-amplified using KlearTaq DNA polymerase with the pJET1.2 plasmid constructs as templates. Uracil-containing primers were designed according to Hamann and Møller (2007): forward 5'-GGATTAAU + A + sequence of coding strand of targeted DNA; reverse 5'-GGGTTAAU + optimized stop codon (TAA) + sequence complementary to coding strand of target DNA (Table S1). The PCR fragments were inserted into yeast/*E. coli* shuttle vector pYeDP60u using USER enzyme (New England Biolabs) and introduced in *E. coli* strain XL1-Blue. The genes inserted in the resulting plasmids were re-sequenced with the plasmid primers (forward: 5'-CACGCAAACACAAATACACACAC-3'; reverse: 5'- AAGCACCACCACCAGTAGAG-3'). Subsequently, the obtained constructs were transformed into the *Saccharomyces cerevisiae* WAT11 yeast strain which expresses the *ATR1* CPR from *Arabidopsis thaliana* (Pompon *et al*., 1996) by the improved lithium acetate procedure (Gietz *et al*., 1992) with minor modifications. The transformed cells were plated on SGI plates for autotrophic selection (Pompon *et al*., 1996). Yeast transformed with empty pYeDP60u plasmid and G10H was used as the negative control and the positive control, respectively.

Yeast microsome isolation

Preparation of microsomes from transformed yeasts was carried out according to Liu *et al*. (2016) with minor modifications. Colonies streaked onto SGI plates were pre-cultured in 10 mL liquid SGI medium (20 g/L glucose, 1 g/L bactocasamino acids, 7 g/L yeast nitrogen base without amino acid without ammonium sulfate, 5 g/L ammonium sulfate, 40 mg/L L-tryptophan) at 30 °C for 18 hours in a shaking incubator. Ten milliliters of pre-culture were used to inoculate 200 mL of YPGE medium (10 g/L yeast extract, 10 g/L bactopeptone, 5 g/L glucose, 3% ethanol by volume) and grown for 30 hours at 30 °C at 150 rpm. Induction of gene expression was started by adding 10 mL of 200 g/L galactose and further incubation at 20 °C for 16 hours.

Yeast cells were pelleted at 4,000 rpm for 10 min at 4 °C, washed with TEK buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM KCl), and resuspended in 2 mL of TES buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 600 mM sorbitol) supplemented with 2-mercaptoethanol and 10 g/L bovine serum albumin, farction V (extraction buffer). The cell resuspensions were homogenized with ca. 5mL of glass beads (450 - 600 μm) by shaking up and down by hand for 5 min in a cold room. Glass beads were washed twice with 10 mL of cold extraction buffer, and lysates were pooled. Cell debris and remaining glass beads were removed from the pooled lysates by centrifugation at 4,000 rpm for 10 min at 4 °C. Supernatants were filtered through Miracloth (Calbiochem®) and microsomes were pelleted by centrifugation at 100,000 g at 4

°C for 1 hour. Pelleted microsomal fractions were resuspended in 1 mL of TEG buffer (50 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 30% (v/v) glycerol) with a Potter-Elvehjem homogenizer. Protein concentrations of microsome preparations were measured by using Bradford reagent (BioRad) in cuvettes on a Nanodrop 2000c spectrophotometer (Thermo Scientific). Microsomal preparations were aliquoted and flash frozen in liquid nitrogen and stored at -80 ℃ until enzyme assays.

***In vitro* enzyme assays**

Enzyme assays were done in a 200 µL final volume of 20 mM potassium phosphate buffer (pH 7.5), containing 200 µg microsomal protein, 500 µM NADPH and 100 µM PA substrate. The mixture was incubated for 30 min at 28 °C and the reaction was terminated by addition of 10 µL of 50% acetic acid. Samples were diluted 10-fold with water and centrifuged at 14,000 rpm for 2 min and the supernatants were passed through a 0.2 µm regenerated cellulose membrane filter (Sartorius). PA substrates (Fig. 1) including a reduced PA extract of leaves of *J. vulgaris*, a mixture containing senecionine and integerrimine, seneciphylline, jacobine, erucifoline (isolated by ExPlant Technologies B.V., The Netherlands) and their corresponding *N*-oxides obtained by incubation with 300 µL of 30% H_2O_2 (Sigma) for 16 hours at 60°C in methanol at a concentration of 5 mg/mL were used for each CYP enzyme of *J. vulgaris* (Table 1). For the positive control G10H geraniol (approx. 98% purity; Sigma) was used as the substrate, and the reaction volume was enlarged to 1 mL final volume as liquid-liquid extraction was required in order to prepare samples for GC-MS which has a low sensitivity. After incubation, the aqueous reaction mixture was extracted three times with 1 mL of ethyl acetate. The ethyl acetate layers were combined and dried using a CentriVap concentrator (Labconco). The residue was redissolved in 100 µL of ethyl acetate before injection into GC-MS. The enzyme assay of the positive control was carried out in duplicate.

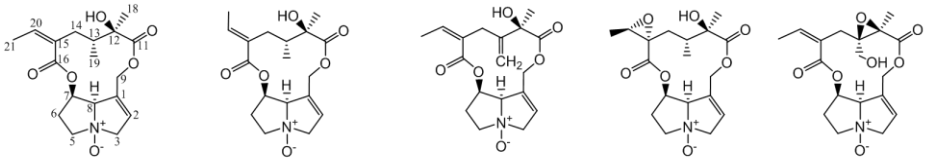

Figure 1. Structures of isolated PAs in *N*-oxide form used as substrates in enzyme assays.

LC-MS/MS analysis of PA conversion

Analysis of reaction mixtures was performed on an LC-MS/MS system consisting of a Waters Acquity UPLC coupled to a Xevo TQ-S tandem mass spectrometer (Waters, Milford, MA, USA), run in positive electrospray mode. Before analysis aliquots (50 µL) of the filtered supernatants were diluted an additional 20 times with water before injection into LC-MS/MS.

Chromatographic separation was achieved on an Acquity BEH C18 analytical column, 150 × 2.1 mm, 1.7 μm particle size (Waters, Milford, MA, USA). Eluent A consisted of water containing 10 mM ammonium carbonate pH 9.0 and acetonitrile was used as eluent B. A gradient elution was performed as follows: 0.0 min 100% A/0% B, 0.1 min 95% A/5% B, 3.0 min 90% A/10% B, 7.0 min 76% A/24% B, 9.0 min 70% A/30% B, 12.0 min 30% A/70% B, 12.1-15.0 min 100% A/0% B. The column was kept at 50ºC and a flow rate of 400 μL/min was applied; 2 μL of the sample extracts was injected.

For each analyte at least two selected precursor to product ion MRM transitions were measured. Cone energy was 40V and collision energy settings were optimized for the individual compounds. Quantification was performed against a range of mixed standard solutions (0-5-10-25-50-100-200 μg/L) of the PAs in a diluted extract of *Tanacetum vulgare* (tansy). The extract of *T. vulgare* material was used to mimic a PA-free plant extract. The range of mixed standard solutions was injected at the beginning of the series and at the end. The mixed standard solution of 50 μg/L in *T. vulgare* extract was injected every 30-40 samples, to monitor the performance of the system (drift in retention times, changes in detector sensitivity) during the analysis. For each PA the averaged response of two MRM transitions was used for quantification. Data processing was conducted with MassLynx 4.1 software (Waters Corporation, Milford, MA, USA).

GC-MS analysis of geraniol conversion

The ethyl acetate solutions were analyzed with a 7890A gas chromatograph equipped with a 7693 automatic sampler and coupled to a 5975C mass single-quadrupole detector (Agilent, Folsom, CA, USA). Samples were separated using a DB-5 GC column (30m×0.25 mm, 0.25 μm film, J&W Science, Folsom, CA, USA) and He (99.9% purity) as a carrier gas at a flow rate of 1 mL/min. The oven temperature was programmed starting at 60 °C and increased to 150 °C at 5 °C/min, then to 240 °C at 15 °C/min. The injector was set at 250 °C and 1 μL of sample was injected in splitless mode. The interface temperature was 280 °C, and the ion source and quadrupole temperature of the mass detector were 230 °C and 150 °C, respectively. Ionization energy in EI mode was 70 eV and peaks were identified by comparison of their ion spectra with the NIST library (version 2008), and by comparison of their retention time and spectra with the standards geraniol and 10-hydroxygeraniol (synthesized by Chiralix B.V., Nijmegen, The Netherlands).

Results

The purpose of this study is to check whether the selected CYP candidates are involved in PA biosynthetic pathway. Aiming to characterize the enzymatic activities of CYPs, heterologous expression of CYP proteins in the yeast expression system was conducted. *In vitro* enzyme

assays were performed using extracted microsomal membranes and different PA substrates, and reaction products were analyzed by LC-MS/MS.

CYP sequences

The initial step of expression of CYPs was to amplify selected CYP sequences from cDNA of the shoots of cultured plants with the MeJA treatment of eight days. The sequence information of CYPs used for amplification were obtained from the *de novo* assembled transcriptome of *J. vulgaris* (Chapter 3; Chapter 4). Six of the eight CYPs showed identical amino acid sequences between Sanger sequencing and RNA-Seq (three CYPs showed identical nucleotide sequences, while the other three had 1-2 different nucleotides). The remaining two CYPs showed two and three different amino acids between two different sequencing approaches.

Geraniol conversion

In order to make sure that the heterologous expression of plant CYPs in yeast and *in vitro* enzyme assays were conducted properly, G10H of *C. roseus* was used as the positive control in our study. In the enzyme assay, geraniol incubated with microsomes expressing CYP76B6 was successfully converted to 10-hydroxygeraniol (Fig. 2A). Most of the geraniol was evaporated during vacuum drying of ethyl acetate extracts (Fig. 2A-B). Metabolic background of enzyme assays (Fig. 2B) as well as background noise of the GC-MS instrument (Fig. 2E) were observed.

Enzyme assays of CYPs from *Jacobaea vulgaris*

None of the eight CYP enzymes selected as candidates for PA biosynthesis showed catalytic activities in structural transformation of PAs using a PA extract, a mixture of senecionine and integerrimine, seneciphylline, jacobine, erucifoline or the respective PAs in *N*-oxide form as substrates (Table 1).

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Discussion

In the current study, eight CYP candidates (CYP71AT158, CYP71AV19, CYP76S36, CYP706C72, CYP706E12, CYPP81B113, CYP82D180 and CYP82Q5) were chosen from the candidate list obtained in Chapter 4. All amplified CYPs in this study showed identical amino acid sequences to *de novo* assembled sequences of RNA-seq analysis except two (CYP71AT158 and CYP706C72) which showed two and three different amino acids to their virtual sequences. These results provided an evidence for the good quality of *de novo* assembled transcriptome of *J. vulgaris*.

Aiming at characterizing the functions of CYP candidates from *Jacobaea* species to see whether they are involved in PA biosynthesis, we used heterologous expression in yeast and *in vitro* enzyme assays. The successful conversion of geraniol to 10-hydroxygeraniol demonstrated that the yeast expression system followed by *in vitro* enzyme assays can be used

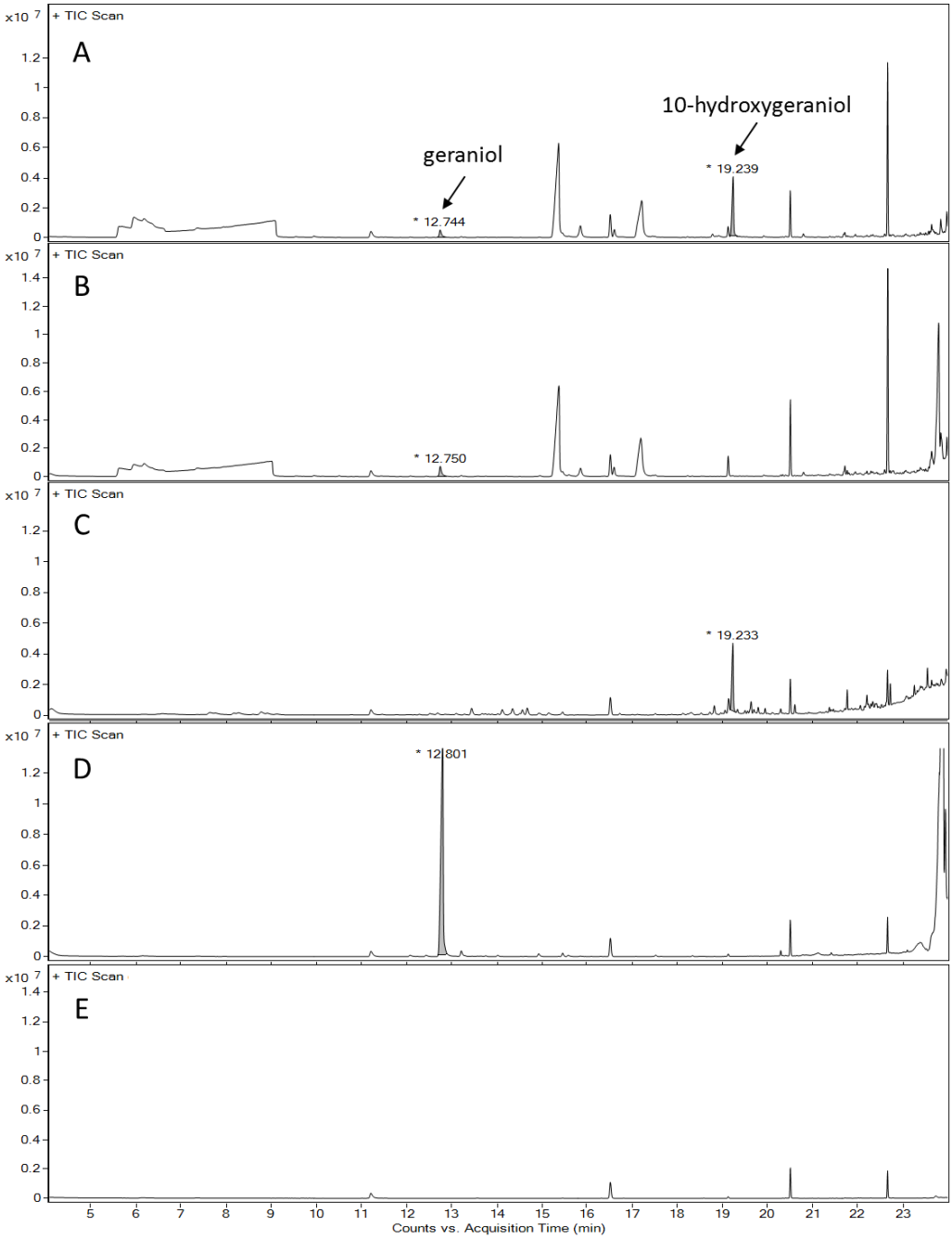

Figure 2. GC-MS analyses of geraniol conversion in enzyme assays with yeast microsomes. (A) Microsomes from yeast expressing G10H; (B) microsomes from yeast expressing the empty pYeDP60u vector; (C) 10-hydroxygeraniol standard; (D) geraniol standard; (E) blank solvent ethyl acetate.

Table 1. Tests of cytochrome P450s (CYPs) for pyrrolizidine alkaloid (PA) conversions by *in vitro* enzyme assays with different PA substrates.

CYP Candidates	PA substrates	Sum FB	Sum Nox	Sn (336)	Sn-ox (352)	Ir (336)	Ir-ox (352)	Sv (336)	Sv-ox (352)	Rt (352)	Rt-ox (368)	Us (352)	Us-ox (368)	Sp (334)
CYP706E12		99.2	0.8	58.6	0.1	34.6	0.2	2.1	0.2	[b]	-	-	-	3.7
CYP81B113		99.5	0.5	59.3	0.1	34.0	0.0	2.3	0.2	-	-	-	-	3.7
CYP71AV19		99.3	0.7	59.3	0.1	33.9	0.2	2.3	0.2	-	-	-	-	3.6
CYP82D180		99.2	0.8	59.2	0.1	34.0	0.2	2.1	0.2	-	-	-	-	3.7
CYP76S36	Sn + Ir	99.2	0.8	59.2	0.1	33.9	0.2	2.2	0.2	-	-	-	-	3.8
CYP71AT158		99.2	0.8	59.4	0.1	33.8	0.2	2.0	0.2	-	-	-	-	3.7
CYP706C72		99.3	0.7	59.3	0.1	34.1	0.2	2.1	0.2	-	-	-	-	3.6
CYP82Q5		99.2	0.8	59.2	0.1	33.7	0.2	2.5	0.2	-	-	-	-	3.6
NC[a]		99.3	0.7	59.4	0.1	34.0	0.2	2.1	0.2	-	-	-	-	3.6
CYP706E12		0.2	99.8	0.2	59.9	0.1	33.8	-	-	-	-	-	-	-
CYP81B113		0.5	99.5	0.3	58.6	0.1	33.8	-	-	-	-	-	-	-
CYP71AV19		0.1	99.9	0.1	59.6	0.0	33.9	-	-	-	-	-	-	-
CYP82D180		0.6	99.4	0.4	59.2	0.2	33.3	-	-	-	-	-	-	-
CYP76S36	Sn-ox + Ir-ox	0.2	99.8	0.2	59.0	0.1	33.8	-	-	-	-	-	-	-
CYP71AT158		0.2	99.8	0.2	59.4	0.1	34.0	-	-	-	-	-	-	-
CYP706C72		0.2	99.8	0.1	59.5	0.1	33.4	-	-	-	-	-	-	-
CYP82Q5		0.3	99.7	0.1	60.1	0.0	33.5	-	-	-	-	-	-	-
NC		0.3	99.7	0.2	59.5	0.1	33.7	-	-	-	-	-	-	-
CYP706E12		99.9	0.1	1.5	-	0.2	-	-	-	-	-	-	-	98.0
CYP81B113		99.9	0.1	1.6	-	0.2	-	-	-	-	-	-	-	98.0
CYP71AV19		99.9	0.1	1.6	-	0.2	-	-	-	-	-	-	-	98.0
CYP82D180		99.9	0.1	1.6	-	0.2	-	-	-	-	-	-	-	98.0
CYP76S36	Sp	99.9	0.1	1.6	-	0.2	-	-	-	-	-	-	-	98.0
CYP71AT158		99.9	0.1	1.6	-	0.2	-	-	-	-	-	-	-	98.0
CYP706C72		99.9	0.1	1.6	-	0.2	-	-	-	-	-	-	-	98.0
CYP82Q5		99.9	0.1	1.6	-	0.2	-	-	-	-	-	-	-	98.0
NC		99.9	0.1	1.6	-	0.2	-	-	-	-	-	-	-	98.0
CYP706E12		0.3	99.7	-	1.8	-	0.2	-	-	-	-	-	-	0.3
CYP81B113		0.6	99.4	-	1.8	-	0.2	-	-	-	-	-	-	0.6
CYP71AV19		0.2	99.8	-	1.8	-	0.2	-	-	-	-	-	-	0.2
CYP82D180		0.8	99.2	-	1.8	-	0.2	-	-	-	-	-	-	0.8
CYP76S36	Spox	0.2	99.8	-	1.7	-	0.2	-	-	-	-	-	-	0.2
CYP71AT158		0.2	99.8	-	1.8	-	0.3	-	-	-	-	-	-	0.2
CYP706C72		0.4	99.6	-	1.8	-	0.2	-	-	-	-	-	-	0.4
CYP82Q5		0.2	99.8	-	1.8	-	0.3	-	-	-	-	-	-	0.2
NC		0.4	99.6	-	1.8	-	0.2	-	-	-	-	-	-	0.4
CYP706E12		99.9	0.1	-	-	-	-	-	-	-	-	-	-	0.1
CYP81B113		99.9	0.1	-	-	-	-	-	-	-	-	-	-	0.1
CYP71AV19		99.9	0.1	-	-	-	-	-	-	-	-	-	-	0.1
CYP82D180		99.9	0.1	-	-	-	-	-	-	-	-	-	-	0.1
CYP76S36	Jb	99.9	0.1	-	-	-	-	-	-	-	-	-	-	0.1
CYP71AT158		99.9	0.1	-	-	-	-	-	-	-	-	-	-	0.1
CYP706C72		99.9	0.1	-	-	-	-	-	-	-	-	-	-	0.1
CYP82Q5		99.9	0.1	-	-	-	-	-	-	-	-	-	-	0.1
NC		99.9	0.1	-	-	-	-	-	-	-	-	-	-	0.1

Sp-ox (350)	St (334)	St-ox (350)	Rd (350)	Rd-ox (366)	Jb (352)	Jb-ox (368)	Jl (370)	Jl-ox (386)	Jn (388)	Jn-ox (404)	Jz (350)	Jz-ox (366)	Er (350)	Er-ox (366)	Un-ox (366)	Ot (382)
-	-	-	-	-	0.1	-	-	-	-	-	-	-	0.1	-	0.2	-
-	-	-	-	-	0.0	-	-	-	-	-	-	-	0.1	-	0.2	-
-	-	-	-	-	0.1	-	-	-	-	-	-	-	0.1	-	0.2	-
-	-	-	-	-	0.1	-	-	-	-	-	-	-	0.1	-	0.2	-
-	-	-	-	-	0.0	-	-	-	-	-	-	-	0.1	-	0.2	-
-	-	-	-	-	0.0	-	-	-	-	-	-	-	0.1	-	0.2	-
-	-	-	-	-	0.0	-	-	-	-	-	-	-	0.1	-	0.2	-
-	-	-	-	-	0.0	-	-	-	-	-	-	-	0.1	-	0.2	-
-	-	-	-	-	0.0	-	-	-	-	-	-	-	0.1	-	0.2	-
2.7	-	-	-	-	-	0.1	-	0.1	-	-	-	-	0.0	0.0	3.2	-
2.7	-	-	-	-	-	0.1	-	0.1	-	-	-	-	0.0	0.9	3.3	-
2.7	-	-	-	-	-	0.1	-	0.1	-	-	-	-	0.0	0.0	3.3	-
2.6	-	-	-	-	-	0.1	-	0.1	-	-	-	-	0.0	0.9	3.2	-
2.7	-	-	-	-	-	0.1	-	0.1	-	-	-	-	0.0	0.8	3.3	-
2.7	-	-	-	-	-	0.1	-	0.1	-	-	-	-	0.0	0.0	3.3	-
2.6	-	-	-	-	-	0.1	-	0.1	-	-	-	-	0.0	0.8	3.2	-
2.6	-	-	-	-	-	0.1	-	0.1	-	-	-	-	0.1	0.0	3.2	-
2.6	-	-	-	-	-	0.1	-	0.1	-	-	-	-	0.0	0.1	3.2	-
0.0	-	0.0	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-
0.0	-	0.0	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-
0.0	-	0.0	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-
0.1	-	0.1	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-
0.1	-	0.0	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-
0.1	-	0.0	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-
0.0	-	0.0	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-
0.0	-	0.0	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-
0.0	-	0.0	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-
95.7	-	0.0	-	1.4	-	0.1	-	-	-	-	0.0	0.1	-	0.1	0.3	-
95.5	-	0.0	-	1.5	-	0.1	-	-	-	-	0.0	0.0	-	0.0	0.3	-
95.9	-	0.0	-	1.4	-	0.1	-	-	-	-	0.0	0.0	-	0.0	0.3	-
95.4	-	0.0	-	1.3	-	0.1	-	-	-	-	0.0	0.1	-	0.0	0.3	-
95.9	-	0.0	-	1.4	-	0.1	-	-	-	-	0.0	0.1	-	0.0	0.3	-
95.8	-	0.0	-	1.4	-	0.1	-	-	-	-	0.0	0.1	-	0.0	0.3	-
95.7	-	0.0	-	1.4	-	0.1	-	-	-	-	0.0	0.1	-	0.0	0.3	-
95.8	-	0.0	-	1.4	-	0.1	-	-	-	-	0.0	0.1	-	0.0	0.3	-
95.7	-	0.0	-	1.3	-	0.1	-	-	-	-	0.0	0.1	-	0.0	0.3	-
-	-	-	-	-	96.3	0.1	0.3	-	0.2	-	2.7	-	0.2	-	-	-
-	-	-	-	-	96.3	0.1	0.3	-	0.2	-	2.7	-	0.2	-	-	-
-	-	-	-	-	96.2	0.1	0.3	-	0.2	-	2.8	-	0.2	-	-	-
-	-	-	-	-	96.1	0.1	0.4	-	0.2	-	2.9	-	0.2	-	-	-
-	-	-	-	-	96.2	0.1	0.3	-	0.2	-	2.8	-	0.2	-	-	-
-	-	-	-	-	96.1	0.1	0.4	-	0.2	-	2.8	-	0.4	-	-	-
-	-	-	-	-	96.2	0.1	0.4	-	0.2	-	2.7	-	0.2	-	-	-
-	-	-	-	-	96.2	0.1	0.4	-	0.2	-	2.8	-	0.2	-	-	-
-	-	-	-	-	96.2	0.1	0.4	-	0.2	-	2.8	-	0.2	-	-	-

CYP706E12		0.5	99.5	-	-	-	-	-	-	-	-	-	-	-
CYP81B113		1.5	98.5	-	-	-	-	-	-	-	-	-	-	-
CYP71AV19		0.3	99.7	-	-	-	-	-	-	-	-	-	-	-
CYP82D180		1.9	98.1	-	-	-	-	-	-	-	-	-	-	-
CYP76S36	Jb-ox	0.4	99.6	-	-	-	-	-	-	-	-	-	-	-
CYP71AT158		0.4	99.6	-	-	-	-	-	-	-	-	-	-	-
CYP706C72		0.5	99.5	-	-	-	-	-	-	-	-	-	-	-
CYP82Q5		0.3	99.7	-	-	-	-	-	-	-	-	-	-	-
NC		1.1	98.9	-	-	-	-	-	-	-	-	-	-	-
CYP706E12		99.8	0.2	-	-	-	-	-	-	0.0	-	0.0	-	-
CYP81B113		99.8	0.2	-	-	-	-	-	-	0.0	-	0.0	-	-
CYP71AV19		99.8	0.2	-	-	-	-	-	-	0.0	-	0.0	-	-
CYP82D180		99.8	0.2	-	-	-	-	-	-	0.0	-	0.0	-	-
CYP76S36	Er	99.8	0.2	-	-	-	-	-	-	0.0	-	0.0	-	-
CYP71AT158		99.8	0.2	-	-	-	-	-	-	0.0	-	0.0	-	-
CYP706C72		99.8	0.2	-	-	-	-	-	-	0.0	-	0.0	-	-
CYP82Q5		99.8	0.2	-	-	-	-	-	-	0.0	-	0.1	-	-
NC		99.8	0.2	-	-	-	-	-	-	0.1	-	0.0	-	-
CYP706E12		0.5	99.5	-	-	-	-	-	-	-	0.2	-	0.0	-
CYP81B113		1.8	98.2	-	-	-	-	-	-	-	0.2	-	0.0	-
CYP71AV19		0.4	99.6	-	-	-	-	-	-	-	0.1	-	0.3	-
CYP82D180		2.3	97.7	-	-	-	-	-	-	-	0.2	-	0.3	-
CYP76S36	Er-ox	0.7	99.3	-	-	-	-	-	-	-	0.1	-	0.3	-
CYP71AT158		0.6	99.4	-	-	-	-	-	-	-	0.2	-	0.3	-
CYP706C72		0.6	99.4	-	-	-	-	-	-	-	0.2	-	0.3	-
CYP82Q5		0.6	99.4	-	-	-	-	-	-	-	0.2	-	0.3	-
NC		1.3	98.7	-	-	-	-	-	-	-	0.2	-	0.3	-
CYP706E12		99.8	0.2	6.4	0.0	2.6	0.0	0.2	-	3.0	-	1.8	-	22.5
CYP81B113		99.8	0.2	6.3	0.0	2.6	0.0	0.2	-	2.9	-	1.7	-	22.4
CYP71AV19		98.9	1.1	6.1	0.0	2.6	0.9	0.2	-	2.9	-	1.8	-	22.5
CYP82D180	A mixture of	99.1	0.9	6.2	0.8	2.6	0.0	0.2	-	2.9	-	1.8	-	22.2
CYP76S36	PA extract	98.2	1.8	6.0	0.8	2.5	0.9	0.2	-	2.9	-	1.8	-	22.1
CYP71AT158		98.1	1.9	6.0	0.8	2.6	0.9	0.2	-	2.8	-	1.7	-	22.5
CYP706C72		98.2	1.8	6.2	0.8	2.7	0.9	0.1	-	2.9	-	1.8	-	22.3
CYP82Q5		98.1	1.9	6.2	0.8	2.6	0.9	0.2	-	2.9	-	1.8	-	21.9
NC		99.0	1.0	6.2	0.8	2.6	0.0	0.2	-	2.9	-	1.8	-	22.5
CYP706E12		0.4	99.6	0.0	9.4	-	4.0	-	-	0.0	3.7	-	3.4	0.0
CYP81B113		1.1	98.9	0.1	9.3	-	3.9	-	-	0.0	3.7	-	3.2	0.2
CYP71AV19		0.3	99.7	0.0	9.6	-	3.9	-	-	0.0	3.7	-	3.3	0.0
CYP82D180	A mixture of	1.3	98.7	0.1	9.4	-	4.0	-	-	0.1	3.7	-	3.3	0.2
CYP76S36	PA extract	0.5	99.5	0.0	9.3	-	4.0	-	-	0.0	3.7	-	3.5	0.1
CYP71AT158	in *N*-oxide form	0.5	99.5	0.0	9.3	-	4.0	-	-	0.0	3.7	-	3.5	0.1
CYP706C72		0.6	99.4	0.0	9.3	-	4.1	-	-	0.0	3.7	-	3.5	0.1
CYP82Q5		0.3	99.7	0.0	9.3	-	3.9	-	-	0.0	3.7	-	3.4	0.0
NC		0.9	99.1	0.1	9.4	-	3.9	-	-	0.0	3.7	-	3.3	0.1

The contents of PAs are shown in their relative abundances (%). Sum FB: the sum of all PA free bases. Sum Nox: the sum of all PA *N*-oxides. Sn: senecionine. Ir: integerrimine. Sv: senecivernine. Rt: retrorsine. Us: usuramine. Sp: seneciphylline. St: spartioidine. Rd: riddelliine. Jb: jacobine. Jl: jacoline. Jn: jaconine. Jz: jacozine. Er: erucifoline. Un: unkown. Ot: otosenine.

[a]NC: negative control using empty vector without CYP insertion.

[b]- percentages of PAs lower than 0.05%.

0.1	-	-	-	0.6	0.5	93.7	-	1.4	-	0.4	-	2.1	-	0.7	0.5	-
0.1	-	-	-	0.6	1.4	93.0	-	1.3	-	0.4	-	2.0	-	0.6	0.5	-
0.1	-	-	-	0.6	0.3	94.1	-	1.4	-	0.4	-	2.0	-	0.7	0.5	-
0.1	-	-	-	0.6	1.9	92.4	-	1.4	-	0.4	-	2.1	-	0.7	0.5	-
0.1	-	-	-	0.6	0.4	93.9	-	1.4	-	0.4	-	2.0	-	0.7	0.5	-
0.1	-	-	-	0.6	0.4	93.9	-	1.4	-	0.4	-	1.9	-	0.7	0.5	-
0.1	-	-	-	0.6	0.5	93.8	-	1.5	-	0.4	-	2.0	-	0.6	0.5	-
0.2	-	-	-	0.6	0.3	93.9	-	1.4	-	0.4	-	2.0	-	0.7	0.5	-
0.1	-	-	-	0.6	1.0	93.3	-	1.4	-	0.4	-	2.0	-	0.6	0.5	-
-	-	-	-	-	2.3	-	-	-	-	-	10.1	-	87.2	0.2	-	-
-	-	-	-	-	2.3	-	-	-	-	-	9.8	-	87.6	0.2	-	-
-	-	-	-	-	2.3	-	-	-	-	-	9.7	-	87.6	0.2	-	-
-	-	-	-	-	2.3	-	-	-	-	-	9.7	-	87.6	0.2	-	-
-	-	-	-	-	2.4	-	-	-	-	-	10.4	-	86.9	0.2	-	-
-	-	-	-	-	2.3	-	-	-	-	-	9.8	-	87.5	0.1	-	-
-	-	-	-	-	2.4	-	-	-	-	-	9.9	-	87.4	0.2	-	-
-	-	-	-	-	2.4	-	-	-	-	-	10.2	-	87.0	0.2	-	-
-	-	-	-	-	2.4	-	-	-	-	-	9.9	-	87.4	0.1	-	-
-	-	-	-	0.1	0.0	5.2	-	0.9	-	0.1	0.0	15.5	0.5	77.2	0.3	-
-	-	-	-	0.0	0.1	6.7	-	0.9	-	0.1	0.3	15.2	1.5	74.7	0.4	-
-	-	-	-	0.1	0.0	5.7	-	0.9	-	0.1	0.1	15.8	0.3	76.3	0.3	-
-	-	-	-	0.1	0.1	5.2	-	0.8	-	0.1	0.3	15.4	1.8	75.3	0.3	-
-	-	-	-	0.0	0.0	5.2	-	0.9	-	0.1	0.1	15.2	0.6	77.1	0.4	-
-	-	-	-	0.0	0.1	5.3	-	1.0	-	0.1	0.1	15.9	0.5	76.2	0.3	-
-	-	-	-	0.1	0.0	5.2	-	1.0	-	0.1	0.1	15.3	0.5	76.9	0.3	-
-	-	-	-	0.0	0.0	5.4	-	0.9	-	0.1	0.2	15.0	0.4	77.2	0.3	-
-	-	-	-	0.1	0.0	5.3	-	1.0	-	0.1	0.2	15.3	1.1	76.1	0.3	-
-	0.6	-	2.9	-	17.4	-	6.8	-	0.2	-	3.0	-	32.2	0.1	-	0.2
-	0.6	-	2.9	-	17.3	-	6.9	-	0.2	-	3.0	-	32.5	0.1	-	0.3
-	0.6	-	2.8	-	17.1	-	6.9	-	0.2	-	2.9	-	32.1	0.1	-	0.2
-	0.6	-	2.9	-	17.4	-	6.9	-	0.2	-	2.8	-	32.0	0.1	-	0.2
-	0.6	-	2.9	-	17.1	-	6.9	-	0.2	-	2.9	-	31.8	0.1	-	0.2
-	0.7	-	2.9	-	17.2	-	6.9	-	0.2	-	2.8	-	31.4	0.1	-	0.2
-	0.6	-	2.8	-	17.2	-	6.9	-	0.2	-	2.8	-	31.4	0.1	-	0.2
-	0.6	-	2.8	-	17.4	-	6.8	-	0.2	-	2.9	-	31.6	0.1	-	0.2
-	0.6	-	2.8	-	17.4	-	7.0	-	0.2	-	3.0	-	31.7	0.1	-	0.2
26.1	-	0.0	-	3.5	0.1	18.7	0.0	8.2	-	0.7	-	2.2	0.1	19.0	0.6	0.2
26.4	-	0.0	-	3.7	0.2	18.0	0.1	8.3	-	0.7	-	2.3	0.3	18.9	0.6	0.2
26.5	-	0.0	-	3.7	0.0	19.2	0.0	8.2	-	0.7	-	1.2	0.0	19.0	0.6	0.2
26.1	-	0.0	-	3.5	0.3	18.3	0.1	8.4	-	0.7	-	2.1	0.3	18.7	0.6	0.2
26.2	-	0.0	-	3.6	0.1	18.7	0.0	8.3	-	0.7	-	2.2	0.1	18.7	0.6	0.2
25.9	-	0.0	-	3.6	0.1	18.6	0.0	8.3	-	0.8	-	2.2	0.1	18.9	0.5	0.2
25.9	-	0.0	-	3.6	0.1	18.5	0.0	8.4	-	0.7	-	2.2	0.1	18.9	0.5	0.2
26.1	-	0.0	-	3.6	0.1	18.5	0.0	8.2	-	0.8	-	2.2	0.1	19.3	0.6	0.2
25.6	-	0.0	-	3.6	0.2	18.6	0.1	8.5	-	0.7	-	2.2	0.2	19.0	0.6	0.2

as the system to test functions of CYP candidates. None of the eight CYPs were found to possess catalytic activities on PA modifications with the selected PA substrates. Most likely the negative results are due to the fact that the CYPs tested in this study were not the enzymes involved in PA biosynthesis. The study finding candidate genes involved in PA biosynthesis (Chapter 4) should be interpreted with caution as long as CYPs responsible for PA diversification have not been identified. As how PA modification is related to CYPs is unknown, defining candidate genes by setting a threshold for the *P*-value and fold change of differential gene expression can be ambiguous. As such, statistical studies do not show causation and are not always sensitive enough to clarify the contribution of CYP abundances on PA concentrations. More candidate genes need to be tested before we can conclude that the candidate approach described in Chapter 4 is not working. Before biochemical characterization, *in silico* enzyme-substrate interaction modelling analysis of binding site features and substrate selectivity might be a potential approach (Raunio *et al*., 2015; Liu *et al*., 2018) to refine the CYP candidate list in later studies. There is a possibility that the genes underlying PA biosynthesis are not CYP genes but other oxidative enzymes such as flavin-dependent monooxygenases and peroxidases (Burton, 2003), which can also be retrieved from transcriptomes described in Chapter 4.

The negative results might be also explained by the lack of efficient expression of CYP enzymes required for catalyzing PA conversions or by suboptimal reaction conditions of enzyme assays for PA conversion. In addition, the PA substrates isolated (Table 1) may contained impurities which might inhibit activities of CYPs. PA conversions might happen but in such low amounts that derived PAs only account for marginal percentages of the total PA concentration and thus is treated as technical variability in LC-MS detection.

Overall, we tested eight CYPs (CYP706E12, CYP81B113, CYP82D180, CYP71AT158, CYP71AV19, CYP706C72, CYP82Q5, CYP76S36) of *J. vulgaris* for their catalytic activities on isolated PAs and PA extracts but found no PA conversion in all combinations. Based on the current results, most likely is that these CYPs are not enzymes involved in PA biosynthesis, at least not with the tested substrates. More CYP candidates are suggested to be tested in further studies.

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Table S1. Primers used for amplification of CYP genes

	Length of coding region in nt	Normal primer sets	Uracil-containing primer sets
CYP706E12	1551	F: 5'-ATGATATCAAAGCTCAACCATAAGTTTC-3'	F: 5'-**GGATTAAUA**ATGATATCAAAGCTCAACCATAAGTTTCTTG-3'
		R: 5'-TCACATTCATAGAGGGTTGCATC-3'	R: 5'-**GGGTTAAU**TTAACATTCATAGAGGGTTGCATCAG-3'
CYP81B113	1524	F: 5'-ATGGAAACTTTTTATGTGATTCTCTCCATC-3'	F: 5'-**GGATTAAUA**ATGGAAACTTTTTATGTGATTCTCTCCATC-3'
		R: 5'-TCACAACTGAGATAACAATTCCATCATC-3'	R: 5'-**GGGTTAAU**TTACAACTGAGATAACAATTCCATCATC-3'
CYP71AT158	1506	F: 5'-ATGACATTTACATTTTTACTTCTTCTTCTCTCTTTC-3'	F: 5'-**GGATTAAUA**ATGACATTTACATTTTTACTTCTTCTTCTCTCTTTC-3'
		R: 5'-TCAGTACAAATAAGCTAAAAGGCAAAGCTC-3'	R: 5'-**GGGTTAAU**TTAGTACAAATAAGCTAAAAGGCAAAGCTC-3'
CYP82D180	1602	F: 5'-ATGAATATCCAAAGAAAAATTGATCTTC-3'	F: 5'-**GGATTAAUA**ATGAATATCCAAAGAAAAATTGATCTTCTAATGG-3'
		R: 5'-TCACATCGTAGTAGAAACCATTCG-3'	R: 5'-**GGGTTAAU**TTACATCGTAGTAGAAACCATTCGCG-3'
CYP71AV19	1503	F: 5'-ATGGAGCTTCAATCTCCTTTTTCTCTC-3'	F: 5'-**GGATTAAUA**ATGGAGCTTCAATCTCCTTTTTCTCTC-3'
		R: 5'-TTATTGTTGAGGCTTGTAATGGCTTGG-3'	R: 5'-**GGGTTAAU**TTATTGTTGAGGCTTGTAATGGCTTGG-3'
CYP706C72	1575	F: 5'-ATGTCGAACCTAGTCTTCACAGCC-3'	F: 5'-**GGATTAAUA**ATGTCGAACCTAGTCTTCACAGCC-3'
		R: 5'-TCACTCAAACAACGTTGCGTGT-3'	R: 5'-**GGGTTAAU**TTACTCAAACAACGTTGCGTGTGTC-3'
CYP82Q5	1584	F: 5'-ATGAATTTCTATCTCTCATCTTCAACATTTG-3'	F: 5'-**GGATTAAUA**ATGATGAATTTCTATCTCTCATCTTCAACATTTG-3'
		R: 5'-TCAAGCAACATGATACATATTAGAGGATAAAC-3'	R: 5'-**GGGTTAAU**TTAAGCAACATGATACATATTAGAGGATAAACG-3'
CYP76S36	1494	F: 5'-ATGGATAACACCACTTTCCTTCTCTTAT-3'	F: 5'-G**GATTAAUA**ATGGATAACACCACTTTCCTTCTCTTATTTTCG-3'
		R: 5'-TCAAAGACGGATTGGAATAGCCTT-3'	R: 5'-**GGGTTAAU**TTAAAGACGGATTGGAATAGCCTTGAG-3'
CYP76B6	1482	F: 5'-ATGGATTACCTTACCATAATATTAACTTTACT-3'	F: 5'-**GGATTAAUA**ATGGATTACCTTACCATAATATTAACTTTACT-3'
		R: 5'-TTAAAGGGTGCTTGGTACAGC-3'	R: 5'-**GGGTTAAU**TTAAAGGGTGCTTGGTACAGC-3'

The USER compatible linkers are marked in bold. All linkers at the 5' end of forward primers are identical, so are all linkers of reverse primers.