Molecular investigations of plant cytochrome P450

by

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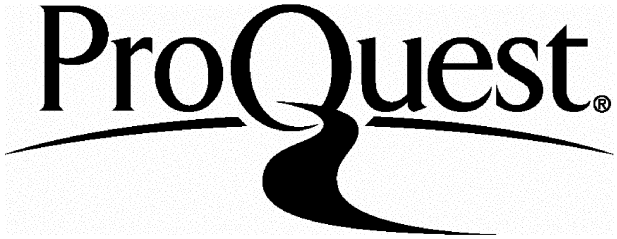

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Abstract

Purification of plant cyt P450s has been difficult, due to their general low abundance in plant tissues and instability during purification. This has meant that only a few have been directly purified from plant tissue to date. Thus, conventional methods for studying their catalytic activity, such as cloning procedures involving the use of antibodies, or the generation of oligonucleotide probes from amino acid sequence, have proven of limited success. An alternative molecular approach was therefore adopted to study cyt P450s from *Zea mays* and *Arabidopsis*. For *Z. mays*, degenerate PCR primers directed to two conserved regions of sequence were used to amplify a cyt P450 fragment with greatest homology to the CYP71 family of plant cyt P450s. Despite extensive screening of phage λ *Z. mays* leaf, safener-treated root and seedling cDNA libraries, a full-length clone was not isolated using this fragment as a probe. Genomic southern analysis confirmed that a gene corresponding to the cyt P450 fragment was part of the *Z. mays* genome. Northern analysis and reverse transcriptase-polymerase chain reaction indicated that it represented a transcript of low abundance, was present in shoot and root tissues and was constitutive with respect to age and treatments known to induce cyt P450s capable of herbicide metabolism.

For *Arabidopsis*, an Expressed Sequence Tag (EST) from the Michigan State University EST programme, previously identified as a potential full-length cyt P450, was obtained for further analysis in an effort to assign function. Detailed sequence analysis confirmed it was a full-length cyt P450 of the CYP71 family, and it was designated CYP71B7. Northern analysis revealed it was expressed most strongly in rosette leaves and was also present in roots, leaves, siliques and flowers. Southern analysis indicated that it represented a single copy gene in the *Arabidopsis* genome. CYP71B7 was successfully expressed in *Saccharomyces cerevisiae* and biochemical analysis revealed it was active in ethoxycoumarin *O*-deethylation. 7-ethoxycoumarin is a general artificial substrate of cyt P450s. Several natural compounds were identified which inhibited this activity, and thus are candidate physiological substrates of CYP71B7.

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To Mum and Dad

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Abbreviations

λ	lambda
µCi	microcurie
µg	microgram
µl	microlitre
µM	micron or micromolar
abs.	absolute
AMPS	ammonium persulphate
AOS	allene oxide synthase
ATP	adenosine triphosphate
bp	basepair
C	carbon
C4H	cinnamate 4-hydroxylase
ca.	*circa*
cDNA	complementary DNA
CH	cumene hydroperoxide
chlorsulfuron	2-chloro-N-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)aminocarbonyl]benzenesulfonamide
Ci	curie
CO	carbon monoxide
CoA	coenzyme A
CPR	cytochrome P450 reductase
cyt P450	cytochrome P450
cyt b_5	cytochrome b_5
ddH_2O	double distilled water
DEPC	diethylpyrocarbonate
dH_2O	distilled water
DIMBOA	2,4-di-hydroxy-7-methoxy-1,4-benzoxazin-3-one
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECOD	ethoxycoumarin *O*-deethylase
epr	electron paramagnetic resonance
EROD	ethoxyresorufin *O*-deethylase
EST	Expressed sequence tag
F5H	ferulate 5-hydroxylase
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GAL	galactose promoter
GCG	Genetics Computer Group
gDNA	genomic DNA
GST	glutathione-s-transferase
h	hours
IPTG	isopropyl-thiogalactoside
kb	kilobase
kDa	kilodaltons

l	litre
LB	Luria - Bertani
M	molar
mA	milliamps
MES	2-[N-morpholino]ethane sulfonic acid
mg	milligram
min	minutes
mM	millimolar
MOPS	3-(N-morpholino) propane sulphonic acid
mRNA	messenger RNA
NA	naphthalic anyhdride
Na_2HPO_4	disodium hydrogen phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
ng	nanogram
nm	nanometres
OD	optical density
PAGE	polyacrylamide gel electrophoresis
*p*CMA	*p*-chloro-*N*-methylaniline
PCR	polymerase chain reaction
PEG	polyethyleneglycol
pfu	plaque forming units
PGK	phosphoglycerate kinase promoter
pmol	picomole
PMSF	phenylmethylsulphonylfluoride
psi	pounds per square inch
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
rubisco	ribulose 5-bisphosphatase carboxylase
s	second
SC	synthetic complete
SD	synthetic dextrose
SM	Suspension Medium
TAE	Tris acetate electrophoresis buffer
TBE	Tris borate electrophoresis buffer
TE	Tris-EDTA
TEMED	N,N,N'N'-tetramethylethyldiamine
Tris	Tris[hydroxymethyl]aminomethane
U	unit
UV	ultraviolet
v/v	volume by volume
V	volts
vols	volumes
w/v	weight by volume
XGAL	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
YNB-AA/AS	yeast nitrogen base without amino acids/ammonium sulphate
YPD	yeast extract peptone dextrose

Chapter 1

Introduction

1.1 General background

Cytochrome P450s (cyt P450) represent a large superfamily of heme-containing proteins, which act as monooxygenases, typically inserting a single oxygen atom into a variety of different substrates. These enzymes are ubiquitous and cyt P450-dependent reactions have been identified in biological sources as diverse as mammals, insects, microorganisms and plants (Porter and Coon 1991; Guengerich 1992; Halkier 1996). In prokaryotes, cyt P450s are soluble proteins, whereas in eukaryotic organisms they are membrane-bound to either the endoplasmic reticulum (Sato, Sakaguchi, *et. al.* 1990) or, in certain cases, to the mitochondrial membrane (Guengerich 1992; Halkier 1996; Schuler 1996).

The name 'cytochrome P450' was assigned since all these enzymes show a characteristic absorption maximum at 450 nanometres (nm) when carbon monoxide (CO) binds to a reduced heme moiety in the CO difference spectrum (Omura and Sato 1964). Binding of CO is inhibitory to activity of the enzyme and reversible since exposure to light at 450 nm causes CO to be displaced from the heme (Estabrook, Cooper, *et. al.* 1963). This inhibition and light reversal is one of the classic methods of establishing the involvement of cyt P450 in a reaction (West 1980). However, the term 'cytochrome P450' is in fact a holdover from the time when the protein was given its provisional name and these proteins are not, in fact, cytochromes in the true sense of the word, i.e iron-containing protein pigments which act as electron carriers. For this reason the term 'heme-thiolate protein' is the preferred term of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (Nelson, Koymans, *et. al.*

1996). Other physiological characteristics of cyt P450s are discussed in Section 1.3.

Cyt P450 enzymes have been the subject of intense research over the last three decades following their discovery in the late 1950s with over 20,000 scientific papers published on the topic (Boobis, Edwards, *et. al.* 1996). As a result a great deal is known about their role *in vivo*, particularly in mammalian systems. This enormous research interest is a clear reflection on their importance in many areas of medical research, since the animal enzymes are known to play a major role in the metabolism of many important chemicals. Cyt P450s in animals play a pivotal role in processing the daily burden of many exogenous compounds including drugs, carcinogens, steroids, pesticides, hydrocarbons and natural products (Guengerich 1992), detoxifying them, in many instances, to more benign substances. The role of cyt P450 in the metabolism of many physiologically important endogenous compounds has also been identified, with substrates including steroids, eicosanoids, fat-soluble vitamins, fatty acids and the mammalian alkaloids (Guengerich 1992). In general, cyt P450s involved in metabolism of xenobiotics exhibit unusually broad and overlapping substrate specificities, whereas those involved in biosynthesis of endogenous compounds, such as steroids, appear to have narrow substrate specificities (Porter and Coon 1991).

1.2 Discovery, research history and distribution of cyt P450

The carbon monoxide binding pigment was independently discovered by Klingenberg and Garfinkel in 1958 (Garfinkel 1958; Klingenberg 1958) during research into the metabolism of drugs and chemicals by mammalian liver microsomes. The CO binding pigment was confirmed to be a hemoprotein (Omura and Sato 1962; Omura and Sato 1964), and the physiological function of cyt P450 as the oxygen-activating terminal oxidase of the

monooxygenase system was determined (Estabrook, Cooper, *et. al.* 1963).

The assignment of a physiological role gave real impetus to research at that time. Estabrook and co-workers demonstrated the role of cyt P450 in steroid C-21 hydroxylation in adrenal microsomes (Estabrook, Cooper, *et. al.* 1963). The principal role of cyt P450 in many other mixed-function oxidase reactions including hydroxylations and oxidative dealkylations of various foreign compounds by liver microsomes was later demonstrated (Cooper, Levin, *et. al.* 1965).

Despite the fact that early biochemical studies had suggested the presence of more than one monooxygenase system in liver microsomes, (Axelrod 1955; Posner, Mitoma, *et. al.* 1961), only a single form of cyt P450 could initially be distinguished spectroscopically. However, the first report of the partial purification of a form of cyt P450 from rabbit liver microsomes (Lu and Coon 1968) was followed by an explosion of activity in cyt P450 purification over the next two decades. These efforts resulted in definitive evidence that multiple forms of cyt P450 existed (Haugen, van der Hoeven, *et. al.* 1975). Subsequent research spanning many biological and biophysical disciplines provided great insights into the multiplicity, substrate specificity, catalytic mechanism and regulatory pathways of this important group of enzymes.

It was initially thought that cyt P450s existed only in mammalian systems, where they were found in all tissues examined (Porter and Coon 1991). However, the 1960s brought reports of cyt P450s in other organisms. Cyt P450 was discovered in 1964 in the yeast *Saccharomyces cerevisiae* (Lindenmayer and Smith 1964) and 1967 saw the first report of a bacterial cyt P450, the camphor-hydroxylating cyt P450 of *Pseudomonas putida* (Appleby 1967). The soluble nature of the latter cyt P450 made it easier to purify than membrane-bound cyt P450, and its crystallisation (Poulos, Perez, *et. al.* 1982; Poulos,

Finzel, *et. al.* 1987) provided an abundance of structural information. The structure of this enzyme, along with that of cyt P450BM-3, a bacterial fatty acid monooxygenase from *Bacillus megaterium* (Kurumbail, Ravichandran, *et. al.* 1993), has served as a model for understanding detailed structure-function relationships of cyt P450s, a subject which will be described in further detail later in this chapter. Cyt P450-dependent reactions are now known to be very widespread and have been detected in such diverse groups as insects (Agosin 1982), an elasmobranch (Bend, Pohl, *et. al.* 1977), yeasts (Käpelli 1986) and other fungi (Ghosh, Dutta, *et. al.* 1983; Shoun, Sudo, *et. al.* 1983) and plants (Bolwell, Bozak, *et. al.* 1994; Durst and O'Keefe 1995; Halkier 1996; Schuler 1996). Genes encoding cyt P450 have now been described in 85 eukaryotic and 20 prokaryotic species and a total of over 480 cyt P450 genes and 22 pseudogenes have been isolated to date (Nelson, Koymans, *et. al.* 1996), a fact which demonstrates their great abundance throughout living species.

1.3 Physiological characteristics of cyt P450 and reaction mechanism

As mentioned above, Cyt P450s are hemoproteins in which the heme is available for oxygen or CO binding. In the CO difference spectrum, binding of CO shifts the absorption maximum to 450 nm (Figure 1.1) and blocks further redox activity (Donaldson and Luster 1991). The 450 nm absorption peak is often accompanied by a peak at 420 nm and solubilisation with detergents and/or prolonged exposure to oxygen may increase the 420 nm peak at the expense of the 450 nm peak (Omura and Sato 1964). For this reason, the CO-hemoprotein adduct with the 420 nm peak is often thought to be a degradation product of cyt P450.

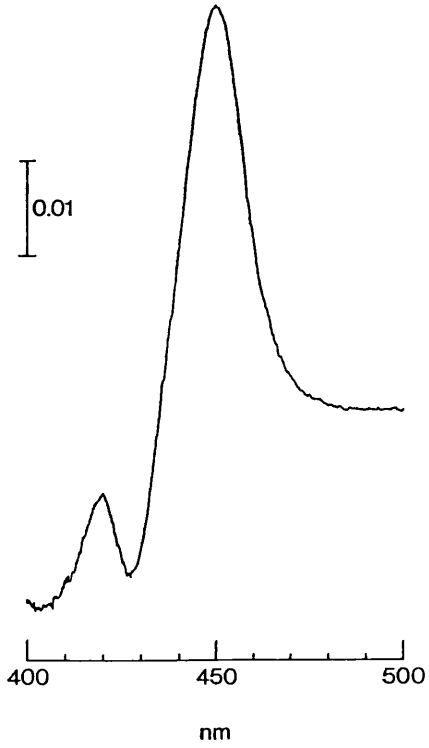


Figure 1.1 ***A typical cyt P450 carbon monoxide difference spectrum showing the classic absorption maximum at 450 nm*** *(CO spectrum is from pumpkin seed endosperm microsomes, Hallahan, unpublished).*

The type of reactions Cyt P450 enzymes are known to carry out include hydroxylations, epoxidations, peroxygenations, desulphurations, dehalogenations and reductions, and most substrates are lipophilic (Porter and Coon 1991).

Cyt P450s range in molecular mass from 45 to 62 kilodaltons (kDa) with an average size of 55 kDa (Schuler 1996). The enzymes are b-type hemoproteins which contain iron protoporphyrin IX at their active site, held in place partly by hydrophobic forces (Porter and Coon 1991). Four ligands of the heme iron can be attributed to the porphyrin ring. The fifth (axial) ligand is a thiolate anion provided by a conserved cysteine residue (Porter and Coon 1991) located towards the carboxy terminus of the protein (Bolwell, Bozak, *et. al.* 1994). The sixth (axial) ligand is probably a hydroxyl group from an amino acid residue, or a water molecule (Goeptar, Scheerens, *et. al.* 1995).

Cyt P450s require molecular oxygen and reducing equivalents for activity. Reducing equivalents from NADPH or NADH are supplied *via* a flavin-containing reductase in microsomal systems or a non-heme iron protein in mitochondrial and bacterial systems (Porter and Coon 1991). Figure 1.2 shows the catalytic cycle of cyt P450s.

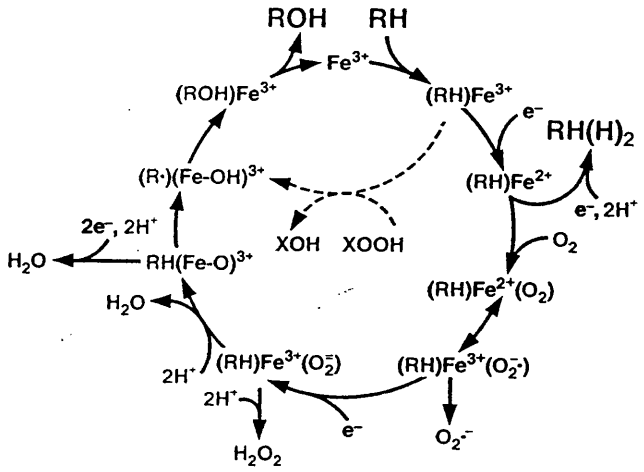


Figure 1.2 ***Scheme for mechanism of action of cyt P450***
Fe represents the heme iron at the active site, RH the substrate, $RH(H)_2$ a reduction product, ROH a monooxygenation product and XOOH a peroxy compound that can serve as an alternative oxygen donor. Reproduced from (Porter and Coon 1991).

Step 1 in the catalytic cycle is binding of the substrate (RH). As water is displaced, the iron spin state alters from low to high spin as iron shifts from a hexa-coordinated to a penta-coordinated spin state (White and Coon 1980; Ortiz de Montellano 1986). Certain cyt P450s exist in the high spin state without the presence of substrate (Guengerich 1990). In the next step, the heme iron is reduced by an electron from the ferric (Fe^{3+}) to the ferrous (Fe^{2+}) state. This ferrous, substrate-bound high spin cyt P450 readily binds molecular oxygen (White and Coon 1980), the next step in the catalytic cycle. The ferrous dioxygen complex is then reduced by a second electron *via* NADPH cyt P450

reductase or cyt b_5 (Bonfils, Balny, *et. al.* 1981). Depending upon the substrate and cyt P450 species, the role of cyt b_5 in supplying the second electron can vary (Halkier 1996) and there has been only one case demonstrated of an obligatory requirement for cyt b_5 to date (Sugimaya, Uiki, *et. al.* 1979).

The final step in the catalytic cycle involves the splitting of dioxygen and the generation of an "activated oxygen" (Porter and Coon 1991) $[FeO]^{3+}$ and the release of water. One oxygen atom is inserted into the substrate, a process believed to involve hydrogen abstraction and recombination of the resulting carbon and iron-bound hydroxyl radicals to produce the product (Porter and Coon 1991). The hydroxylated molecule is believed to possess a lower affinity for the active site, a situation which favours its release and the subsequent binding of new substrate, thus perpetuating the cycle (White and Coon 1980).

The entire reaction can be summarised thus,

$$\mathbf{RH + O_2 + NADPH + H^+ \longrightarrow ROH + H_2O + NADP^+}$$

where RH is the substrate and ROH is the product. The reaction stoichiometry, that is one mole of NADPH, oxygen and substrate utilised for each mole of product formed, is another characteristic of cyt P450 reactions (West 1980).

In addition to the characteristic absorption maximum at 450 nm caused by binding of CO to the heme, characteristic spectral features are caused by binding of substrates to cyt P450, due to the shift in the Soret visible absorption band caused by an alteration in the cyt P450 heme iron spin state. These changes can be easily followed by optical difference binding spectra (Jefcoate 1978). Three main types of difference spectra have been described, type I, reverse-type I and type II, these are shown in Figure 1.3. Type I spectra are characterised by a peak at about 385 nm and a trough at around 420 nm.

Reverse type I, the mirror image of type I spectra, show a peak at 420 nm, and a trough at around 385 nm. Type II spectra are characterised by a broad trough between 390 nm and 410 nm, and a peak varying between 425 and 435 nm. Type I spectra are believed to reflect the formation of a true enzyme-substrate complex, reflecting an increase in high spin character upon binding the ligand, whereas a reverse type I spectral change reflects the opposite change (Jefcoate 1978). Compounds which effect type II difference spectrum are usually poor substrates and generally act as cyt P450 inhibitors. The technique of optical difference spectroscopy is widely used to identify and characterise cyt P450 substrates and inhibitors and can be correlated with electron paramagnetic resonance (epr) spectra for semi-quantitative measurement of spin states (Jefcoate 1978).

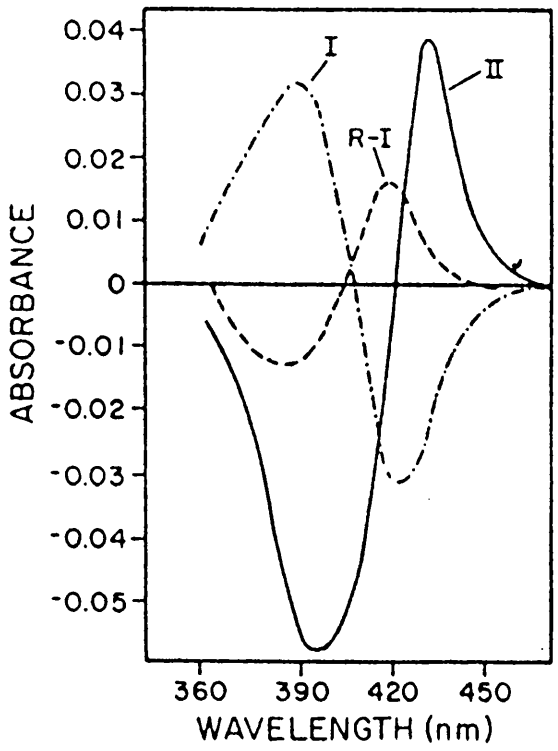


Figure 1.3 ***Examples of types of difference spectra produced in liver microsomes*** *I. Type I is characterised by* λ_{max} *~ 385 nm,* λ_{min} *~ 420 nm;* ***R-I****. reverse type I is characterised by* λ_{max} *~ 420 nm,* λ_{min} *~ 385 nm;* ***II****. type II is characterised by a broad trough between 390 and 410 nm, and a peak varying between 425 and 435 nm. Reproduced from (Jefcoate 1978).*

1.4 Structure and membrane topology of cyt P450

Cyt P450s have been divided into classes depending on the nature of their redox partner. Class I cyt P450s are found in mitochondrial membranes of eukaryotes and in bacteria and require a flavin adenine dinucleotide (FAD)-containing reductase and an iron-sulphur protein as their redox partner. Class II cyt P450s are bound to the endoplasmic reticulum and interact directly with NADPH cyt P450 reductase which contains both FAD and flavin mononucleotide (FMN) (Kurumbail, Ravichandran, *et. al.* 1993). Recently a third class of cyt P450s has been identified which do not require a redox partner. These catalyse rearrangements of endoperoxides and hydroperoxides (Peterson and Graham-Lorence 1995).

Studies on the correlation between eukaryotic cyt P450 structure and function have been hampered by their membrane-bound nature which makes these proteins difficult to purify and solve the nature of their tertiary or three-dimensional structure. To date no tertiary structure for a eukaryotic cyt P450 has been elucidated. Structure determination has had to rely largely on computer modelling of structures and comparison of models with the tertiary structure of the soluble bacterial cyt P450s. Much of what is known about the tertiary structure of cyt P450s is based on the tertiary structure of four soluble bacterial cyt P450 enzymes, P450cam (Poulos, Finzel, *et. al.* 1987), the heme domain of P450BM-3 (Kurumbail, Ravichandran, *et. al.* 1993), P450terp (Hasemann, Ravichandran, *et. al.* 1994) and P450eryF (Cupp-Vickery and Poulos 1995). Strong structural similarity is evident between soluble bacterial cyt P450s and their microsomal counterparts, despite large differences in primary sequence (Kalb and Loper 1988; Nelson and Strobel 1988; Tretiakov, Degtyarenko, *et. al.* 1989; Ouzounis and Melvin 1991; Kurumbail, Ravichandran, *et. al.* 1993). Thus, the bacterial cyt P450 structures can serve as models

for structure/function correlation at least until the three-dimensional structure for a microsomal cyt P450 is solved. P450cam, shown in Figure 1.4, a soluble 5-exo-hydroxylase of camphor, was the first cyt P450 whose tertiary structure was solved, and as such served as an archetypal model for the non-membranous portion of eukaryotic cyt P450 structure. However, it is a cyt P450 belonging to Class I and shares only 10 - 20 % sequence identity with microsomal cyt P450s. P450BM-3 is a soluble fatty acid hydroxylase from *Bacillus megaterium* consisting of an N-terminal cyt P450 domain and a C-terminal flavoprotein reductase (Narhi and Fulco 1987). Cyt P450BM-3 shows functional and structural similarity to the eukaryotic class II cyt P450 with about 25 - 30 % identity between its cyt P450 domain and the microsomal fatty acid ω-hydroxylase and *n*-alkane-inducible microsomal cyt P450s (Ruettinger, Wen, *et. al.* 1989). As such it is considered a better prototype for microsomal cyt P450s (Poulos 1991).

Those cyt P450 structures solved exhibit a significant degree of secondary and tertiary homology with the overall shape resembling a triangular prism (Kurumbail, Ravichandran, *et. al.* 1993). Secondary structure elements are found in similar locations in all four structures, with approximately 50 % of the amino acids found in α-helices and approximately 15 % in β-sheets (Halkier 1996). The tertiary structure is composed of an asymmetric arrangement of helices on one side of the protein and a β-sheet structure on the other (Porter and Coon 1991; Kurumbail, Ravichandran, *et. al.* 1993). One of the main structural characteristics of cyt P450 enzymes is the heme-binding core structure which is highly conserved containing helices I and L and the substrate binding pocket. The carboxy-terminal portion of the I helix, the L helix and the loop containing a conserved cysteine residue are structurally similar and all cyt P450s exhibit amino acid sequence similarity in this region. The substrate binding pocket is adjacent to the heme

and is accessible through a long hydrophobic channel. It appears that the access route to the binding pocket can vary between cyt P450s (Poulos 1995). Most differences between the enzymes are observed around the substrate binding pocket and regions suggested to be important for binding of the redox partner (Hasemann, Ravichandran, *et. al.* 1994). Differences around the substrate binding pocket are not entirely unexpected due the variety of substrates metabolised by various cyt P450s.

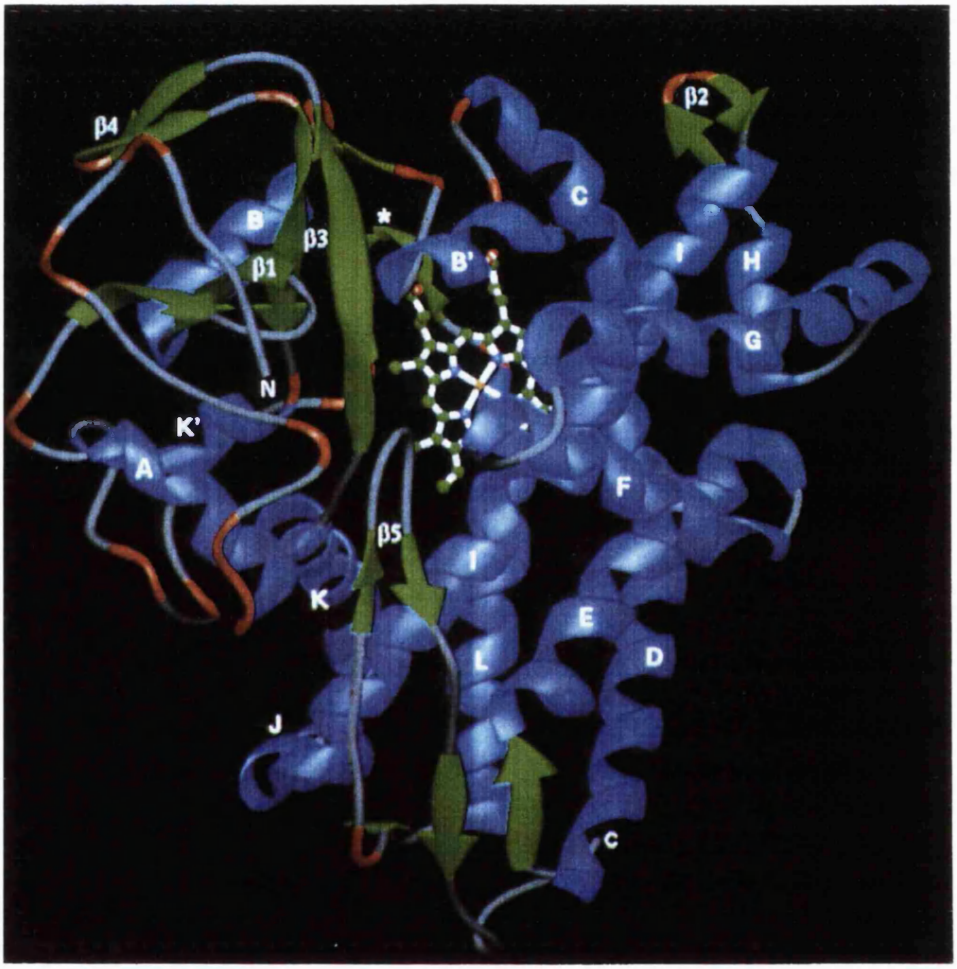


Figure 1.4 ***Three- dimensional structure of cyt P450cam***
α-helices are indicated as blue spirals, β-strands are depicted as green arrows, β-turns are shown in red and random coil is represented in grey. The 'cys-binding loop/β-bulge' is indicated by an asterisk. Reproduced from Black 1993.

The microsomal membrane-bound cyt P450s are known to contain four discrete regions of sequence similarity (Kalb and Loper 1988) known as domain A, B, C and D. Domain D contains the highly-conserved heme-binding region, the cysteine residue of which provides the thiolate ligand to the heme (Black and Coon 1986). Residues found in Domain A correspond to a segment in P450cam that was demonstrated to span the heme-distal surface and to contain residues which contact the substrate camphor. Microsomal cyt P450s are believed to be bound to the membrane by a hydrophobic region at the amino terminus consisting of 20 to 25 amino acids (Nelson and Strobel 1988) which functions as an insertion signal and a stop-transfer sequence (Sakaguchi, Mihara, *et. al.* 1984). Unlike mitochondrial cyt P450s where the transfer peptide is proteolytically cleaved from the molecule following its insertion into the membrane, microsomal cyt P450 retain their signal peptide (Schuler 1996). The heme lies parallel to the membrane in bacterial cyt P450s (Hasemann, Ravichandran, *et. al.* 1994) and EPR spectroscopy has demonstrated that in membrane-bound cyt P450s the heme plane is nearly parallel to the membrane surface (Kamin, Batie, *et. al.* 1985). The membrane-bound cyt P450s are thought to lie flat on the membrane surface with the distal end of the active site and the substrate channel facing the membrane (Nelson and Strobel 1988). The N-terminus contains a proline-rich region within which is the PPGP tetrapeptide. The proline-rich region has been postulated to be of critical importance in orienting the cyt P450 enzyme, and this region is discussed in greater detail in Chapter 4. A great deal of controversy exists regarding the orientation of the N-terminal signal anchor region in the membrane and as a result numerous studies have been carried out on this subject. There are two main models, as indicated in Figure 1.5, A, a bitopic N_{exo}, C_{cyt} topology with a single transmembrane spanning region, and B, a polytopic two anchor hairpin loop structure (Black 1992).

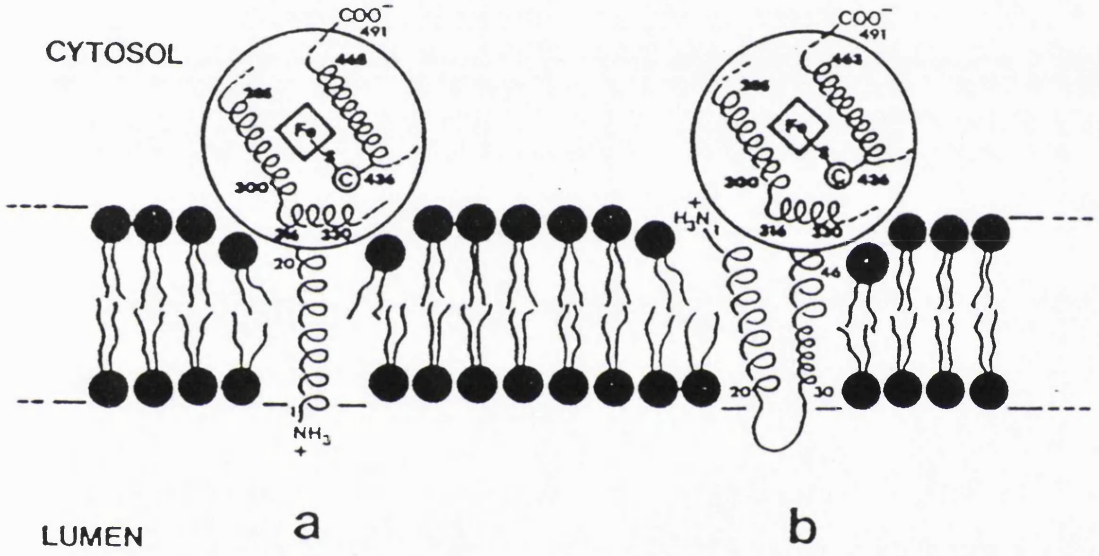


Figure 1.5 ***Membrane topology of cyt P450 on the endoplasmic reticulum***
Model A is a bitopic N_{exo} C_{cyt} topology; Model B is a polytopic, two-anchored hairpin model. Reproduced from Black, 1992.

1.5 Evolution of cyt P450 and nomenclature

As of October 1995, 481 genes and 22 putative pseudogenes of the cyt P450 superfamily have been characterised, each one almost always encoding a single protein (Nelson, Koymans, *et. al.* 1996). The number of cyt P450 genes characterised continues to increase at a significant rate each year and to date 21 plant cyt P450 gene families have been identified representing a total of over 80 separate cDNA or gene sequences. The first plant cyt P450 was isolated relatively recently, a ripening-induced transcript from *P. americana* mesocarp (Bozak, Yu, *et. al.* 1990).

The cyt P450 superfamily is very ancient, the ancestral gene having existed for over 3.5 billion years, based on proposals that any gene occurring in eubacteria and eukaryotes arose from a single ancestral gene (Loomis 1988). Analysis of phylogenetic data shows an 'explosion' of new genes during the past 400 million years, most if not all of which are believed to have arisen from animal-plant 'warfare'. As animals and plants diverged approximately 1200 million years ago, animals began to eat plants and plants countered by producing defence compounds to make them less palatable and/or digestible. This was followed by the emergence of new cyt P450 genes in animals capable of detoxifying these compounds, a process which probably intensified as animals advanced on land and encountered new plant species (Gonzalez and Nebert 1990). The vast array of cyt P450s seen in many different organisms today probably arose through gene divergence in response to these pressures.

Cyt P450 genes are classified according to a nomenclature system based on divergence evolution of the superfamily, a system originally devised in 1987 (Nebert, Adesnik, *et. al.* 1987). The gene is denoted by the italicised root symbol *CYP*, representing cytochrome P450 (*Cyp* for mouse). This is followed by an Arabic number

designating the cyt P450 family, a letter indicating the subfamily (when two or more exist) and an Arabic numeral representing the individual gene. The same nomenclature is used for the corresponding gene product (enzyme) and cDNA, but without the use of italics. Thus CYP71A1, the first plant cyt P450 to be isolated, represents a cyt P450 of family 71 subfamily A, gene 1. The criteria for placing cyt P450s into a particular family is that they must exhibit at least 40 % sequence identity at the amino acid level. Mammalian sequences within the same subfamily are always more than 55 % identical. If a new cyt P450 sequence is less than 40 % identical to all other cyt P450 sequences it constitutes the first member of a new family. Such a system for classification has proved extremely useful allowing related cyt P450s to be readily recognised regardless of their source or catalytic activity.

Considering the plant cyt P450s, the subject of this thesis, it has been shown that plant cyt P450s form two distinct groups (Durst and Nelson 1995), group A comprising families 71, 73, 75, 76, 77, 78, 79, 80, 81, 83 and 89 which all appear to branch off a phylogenetic tree at the same node and may derive from a common ancestor. The second group are denoted non-A cyt P450s and do not form a group in the phylogenetic sense and comprise families 72, 74, 86, and 87. These sequences cluster near to animal, fungal and microbial families and apparently diverged from group A cyt P450s before the divergence into animals and plants (Figure 1.6).

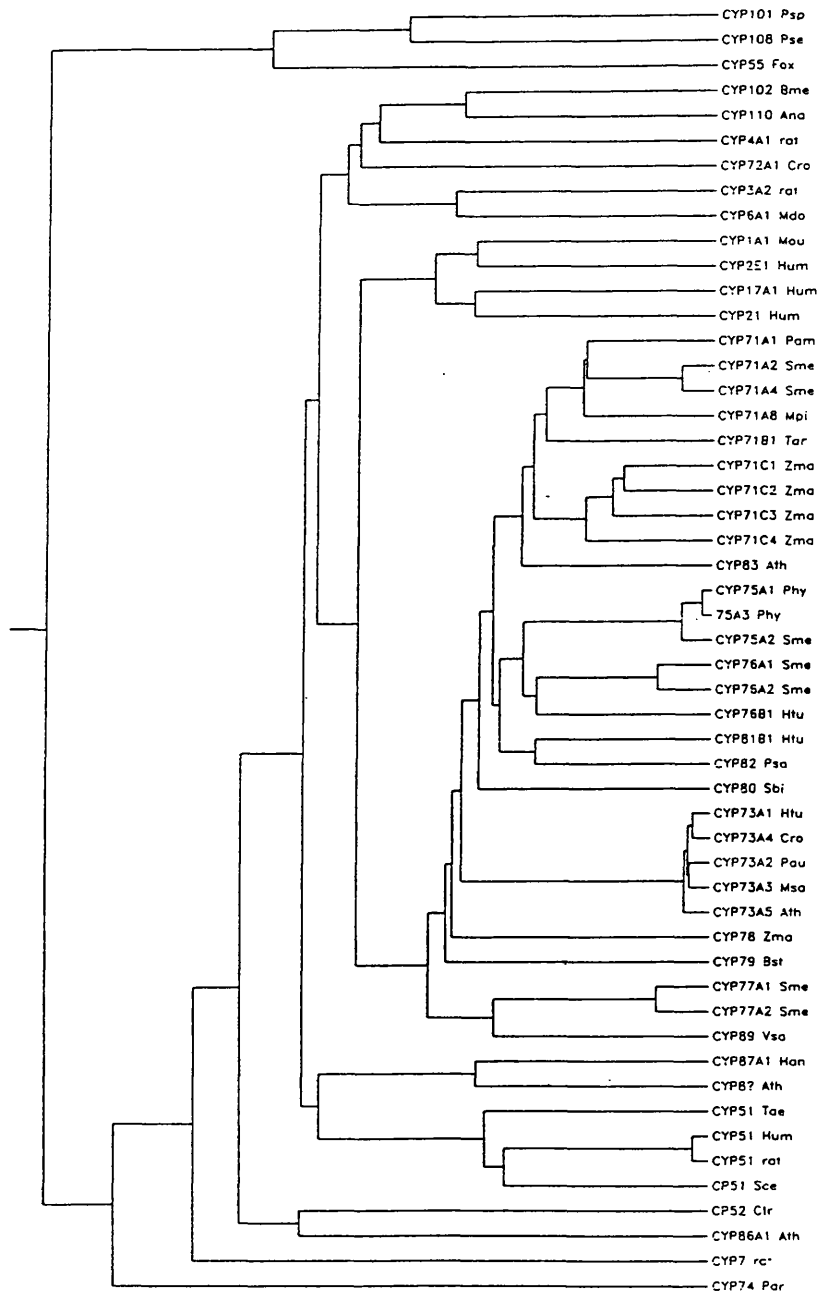


Figure 1.6 ***Phylogeny of cyt P450 from plants and other organisms***
Cyt P450s are named according to the official nomenclature
Reproduced from Durst and Nelson, 1995.

1.6 Function of plant cyt P450s

The first evidence for cyt P450 in plants came in 1967 when Moore observed a CO binding pigment with absorption maxima at 450 nm and 420 nm in pea microsomes (Moore 1967), almost a decade after the same phenomena was observed in mammalian liver microsomes. Research into the plant cyt P450s has lagged well behind that of mammalian and bacterial cyt P450s due in part to difficulties in their purification. This has been largely caused by the presence of proteases and interfering compounds such as phenolics, as well as the relatively low amounts of cyt P450 in plant tissues (5 - 50 pmol per mg (Durst and O'Keefe 1995)). For these reasons, only a handful of cyt P450s have been directly purified from plants to date. These include cinnamate 4-hydroxylase from *Helianthus tuberosus* (Jerusalem artichoke) tubers (Gabriac, Werck-Reichhart, *et. al.* 1991), *Phaseolus vulgaris* (bean) cell cultures (Rodgers, Zimmerlin, *et. al.* 1993) and mung bean seedlings (Mizutani, Ohta, *et. al.* 1993); 3,9 dihydroxypterocarpan 6a-hydroxylase from *Glycine max* (soybean) cell cultures (Kochs and Grisebach 1989; Kochs, Werck-Reichhart, *et. al.* 1992); digitoxin 12β-hydroxylase from *Digitalis lanata* (foxglove) cell cultures (Petersen and Setz 1988); a *p*-chloro-*N*-methylaniline demethylase from *Persea americana* (avocado) mesocarp (O'Keefe and Leto 1989); an N-hydroxylase ($P450_{TYR}$) from *Sorghum bicolor* (sorghum) seedlings (Sibbesen, Koch, *et. al.* 1995); berbamunine synthase from *Berberis stolonifera* (barberry) cell cultures (Stadler and Zenk 1993) geraniol 10-hydroxylase from *Catharanthus roseus* (periwinkle) cell cultures (Meijer, DeWaal, *et. al.* 1993) and a 7-ethoxycoumarin *O*-deethylase from *H. tuberosus* tubers (Batard, Zimmerlin, *et. al.* 1995). Studies have indicated that cyt P450 enzymes in higher plants bear close physiological and structural similarities to their mammalian counterparts, with the minimal active system composed of cyt P450 and cyt P450 reductase (West

1980). Plant cyt P450s are known to catalyse the same types of reactions as their animal counterparts including aliphatic and aromatic hydroxylation, epoxidation, N- and S-oxidation, C-, N- and O-dealkylation (Batard, Zimmerlin, *et. al.* 1995).

The difficulty in purification of plant cyt P450s has meant that much research has tended to focus on molecular biology techniques, using cDNA and gene cloning strategies to isolate full length clones encoding plant P450s. These approaches have led to the isolation of over 69 plant P450s genes or cDNAs spanning 21 discrete families and this number is increasing rapidly. Various different techniques, described in Section 1.7 below, have been used to clone full or partial plant cyt P450 cDNA sequences and genes.

The metabolism of well over 50 different endogenous chemical substrates have been attributed to plant cyt P450s (Bolwell, Bozak, *et. al.* 1994; Durst and O'Keefe 1995; Schuler 1996). Plant cyt P450s play key roles in the biosynthesis of a wide variety of secondary metabolites including lignin intermediates, sterols, plant hormones, terpenes, flavonoids and furanocoumarins (Schuler 1996). These enzymes have also been shown to be involved in metabolism of a range of foreign chemicals (xenobiotics). As is the case with their mammalian counterparts, cyt P450(s) metabolise xenobiotics, including herbicides (O'Keefe, Romesser, *et. al.* 1987; Jones and Caseley 1989), to compounds, in most cases, with reduced phytotoxicity (Hallahan, Cheriton, *et. al.* 1993). The reactions of plant cyt P450s that have been identified to date are described below.

A) Phenylpropanoid biosynthesis

Phenylpropanoids are a large class of plant secondary metabolites which derive from the C6-C3 structure formed by deamination of phenylalanine to *trans*-cinnamic acid. Cinnamic acid is then 4-hydroxylated by a cyt P450, cinnamate 4-hydroxylase (C4H), a reaction which is responsible for the distribution of the hydroxycinnamate skeleton into

many phenylpropanoid pathways (Durst and Benveniste 1993). These pathways lead to the production of many important plant constituents including cell wall components (lignins), pigments (flavonoids), compounds involved in protection from ultraviolet light (coumarins, flavonoids, furanocoumarins) and plant defence compounds (isoflavonoids, coumarins, furanocoumarins) (Werck-Reichhart 1995; Schuler 1996). Because of its central role with respect to all of these pathways, C4H activity exists in all plants at a relatively high level compared to many other cyt P450s and for this reason was one of the first plant cyt P450 activities characterised and the only activity identified in a wide range of plant tissues and cell types (Schuler 1996). The enzyme has been purified from *H. tuberosus* (Gabriac, Werck-Reichhart, *et. al.* 1991), french bean (Rodgers, Zimmerlin, *et. al.* 1993) and mung bean (Mizutani, Ohta, *et. al.* 1993).

Lignin biosynthesis

In the lignin biosynthetic pathway, the 5-hydroxylation of ferulate, a lignin precursor, is known to be catalysed by cyt P450 ferulate 5-hydroxylase (F5H) (Grand 1984). This is a key enzyme determining the relative abundance of different monomer types of lignin (Schuler 1996) and has recently been cloned from *Arabidopsis thaliana* (Meyer, Cusumano, *et. al.* 1996).

Flavonoid and coumarin biosynthesis

Modified flavonoids are important constituents of the pathway leading to production of the anthocyanins which are important flower pigments. The types of anthocyanins synthesised are controlled by the cyt P450 enzymes flavonoid 3' hydroxylase and flavonoid 3'5' hydroxylase, cDNAs for which have been cloned from *Petunia hybrida* (Holton, Brugliera, *et. al.* 1993) and *S. melongena* (eggplant) (Toguri, Azuma, *et. al.* 1993). These cyt P450s belong to the family CYP75 and expression of the CYP75 gene

is tissue specific and strictly dependent on UV/blue light (Werck-Reichhart 1995). Interest in these enzymes stems from continuous efforts to create novel flower varieties by manipulation of flower colour. Developments in molecular biology have enabled the alteration of characteristics such as flower colour by the introduction of new genes, an alternative approach to classical plant breeding experiments.

Modified flavonoids (and coumarins) are also significant as plant defence compounds (phytoalexins) and many of the modifications are cyt P450-dependent. Isoflavone synthesis and hydroxylation have been demonstrated to be cyt P450-dependent as is the synthesis of the coumarins, marmesin (Wendorff and Matern 1986), psoralen (Hamerski and Matern 1988) and bergaptol (Hamerski and Matern 1988). Cyt P450 reactions involved in phenylpropanoid biosynthesis and flavonoid and coumarin modification are listed in Table 1.1 with the appropriate references and cyt P450 family where reported.

Table 1.1 ***Cyt P450s involved in phenylpropanoid metabolism and flavonoid metabolism***

Enzyme	Reference(s)
Cinnamate 4-hydroxylase (CYP73)	(Gabriac, Werck-Reichhart, *et. al.* 1991) (Mizutani, Ohta, *et. al.* 1993) (Rodgers, Zimmerlin, *et. al.* 1993) (Teutsch, Hasenfratz, *et. al.* 1993) (Fahrendorf and Dixon 1993)
Ferulate 5-hydroxylase (*CYP*84)	(Grand 1984) (Meyer, Cusumano, *et. al.* 1996)
Flavonoid 3' hydroxylase (CYP75) Flavonoid 3 '5'-hydroxylase (CYP75)	(Larson and Bussard 1986) (Holton, Brugliera, *et. al.* 1993)
Isoflavone synthase	(Hakamatsuka, Hashim, *et. al.* 1990) (Kochs and Grisebach 1986)
Isoflavone 2'-hydroxylase Isoflavone 3'-hydroxylase	(Clemens, Hinderer, *et. al.* 1993)
Marmesin synthase	(Wendorff and Matern 1986)
Psoralen synthase	(Hamerski and Matern 1988)
Psoralen 5-monoxygenase (bergaptol synthase)	(Hamerski and Matern 1988)

The table lists cyt P450 enzymes with the appropriate references and cyt P450 family where reported.

B) Fatty acid biosynthesis

Cyt P450s have been shown to catalyse hydroxylation and/or epoxidation of medium and long chain (C10 to C18) fatty acids in plants (Salaün and Helvig 1995). These reactions are essential to the biosynthesis of cutins and suberins (essentially biopolymers of hydroxylated C10 and C18 fatty acids) the role of which is to protect the aerial (cutins) and underground (suberins) plant tissues from water loss, chemical penetration and microbial attack (Salaün and Helvig 1995).

Fatty acid hydroxylation

Capric (C10:0), lauric (C12:0) and myristic (C14:0) acids have been shown to be hydroxylated at the terminal position (ω-hydroxylation) in *Vicia sativa* (Salaün, Simon, *et. al.* 1986). A lauric acid ω-hydroxylase has also been characterised in *Pisum sativum* (pea) (Benveniste, Salaün, *et. al.* 1982). The fatty acid hydroxylases exhibit varying regioselectivity and as well as ω-hydroxylation also catalyse in-chain hydroxylation of fatty acids. An example is the in-chain hydroxylation at positions 10, 9 and 8 (predominantly at position 9) of lauric acid in *H. tuberosus* (Salaün, Benveniste, *et. al.* 1981; Salaün, Benveniste, *et. al.* 1982). The ω and in-chain lauric acid hydroxylases appear to be extremely regio-specific for the position of hydroxylation relative to the terminal methyl group and also species exclusive (i.e no two lauric acid hydroxylase activities co-exist in the same plant) (Zimmerlin, Salaün, *et. al.* 1992). More recently a third type of hydroxylation has been identified in wheat where the fatty acid is hydroxylated at the subterminal position (Zimmerlin, Salaün, *et. al.* 1992).

The hydroxylation of the long chain fatty acids was historically the first to be studied (Soliday and Kolattukudy 1978) and this research showed that palmitic acid (C16:0) is ω-hydroxylated in *V. sativa* microsomes. More recently, omega hydroxylation

of oleic (C18:1), 9,10-epoxystearic, 9,10-dihydroepoxystearic and linoleic acids (C18:2) has been shown to occur in *V.sativa* (Pinot, Salaün, *et. al.* 1992; Pinot, Bosch, *et. al.* 1993) and also the terminal and subterminal hydroxylation of oleic acid has been demonstrated in wheat (Pinot, Alayrac, *et. al.* 1994).

Epoxidation

The first epoxidation reaction described was the conversion of 18-hydroxylinoleic acid into 18-hydroxy-*cis*-9,10-epoxystearic acid in spinach (Croteau and Kolattukudy 1975). This is an unusual cyt P450 which requires ATP and coenzyme A (CoA). More recently, unsaturated analogues of lauric acid have been shown to be epoxidised by a cyt P450 from *H. tuberosus* (Salaün, Weissbart, *et. al.* 1989; Salaün, Weissbart, *et. al.* 1993). These reactions appear to be catalysed by the same cyt P450 responsible for hydroxylation of lauric acid. Similarly, 11-dodecenoic acid (the terminally unsaturated lauric acid analogue) is expoxidised in *V.sativa* by the lauric acid ω-hydroxylase in this material (Salaün, Weissbart, *et. al.* 1989). Thus, single cyt P450 isoforms are capable of catalysing both epoxidation or hydroxylation of a single fatty acid. To date no cyt P450s involved in fatty acid biosynthesis have been purified or cloned.

Allene oxide synthase

A particularly unusual cyt P450, allene oxide synthase (AOS), has been purified from *Linus usitatissimum* (flaxseed) (Song and Brash 1991) which converts the 13-hydroperoxide of linoleic acid to its corresponding allene oxide *via* a dehydration reaction. This allene oxide is a metabolic precursor of jasmonic acid, an important plant growth regulator (Meyer, Miersch, *et. al.* 1984). The reaction is unusual in that unlike other cyt P450 reactions it does not require molecular oxygen or NADPH. In addition, the reaction proceeds at a turnover rate of 2000 - 6000 per second, much higher than most other cyt

P450 activities measured (Lau, Harder, *et. al.* 1993). In addition to its purification the enzyme has been cloned from *L. usitatissimum* (Song, Funk, *et. al.* 1993), *A. thaliana* (Laudert, Pfannschmidt, *et. al.* 1996) and *Parthenium argentatum* (guayule) rubber particles (Pan, Durst, *et. al.* 1995). It has been suggested that AOS is a particularly abundant cyt P450 particularly in monocotyledons (Lau, Harder, *et. al.* 1993). Table 1.2 details cyt P450s involved in fatty acid biosynthesis with the appropriate reference(s) and cyt P450 family where reported.

Table 1.2 ***Cyt P450s involved in fatty acid metabolism***

Enzyme	Reference(s)
Hydroxylases	
Capric and myristic acid	(Salaün, Simon, *et. al.* 1986)
Lauric acid	(Salaün, Simon, *et. al.* 1986) (Salaün, Benveniste, *et. al.* 1981) (Salaün, Benveniste, *et. al.* 1982) (Salaün, Weissbart, *et. al.* 1993) (Zimmerlin, Salaün, *et. al.* 1992)
Oleic acid	(Soliday and Kolattukudy 1978) (Pinot, Salaün, *et. al.* 1992) (Pinot, Bosch, *et. al.* 1993)
Epoxidases	
Lauric acid analogues	(Salaün, Weissbart, *et. al.* 1993) (Salaün, Weissbart, *et. al.* 1989)
18-hydroxylinoleic acid	(Croteau and Kolattukudy 1975)
Linoleic acid hydroperoxide	(Croteau and Kolattukudy 1975) (Song and Brash 1991) (Song, Funk, *et. al.* 1993) (Laudert, Pfannschmidt, *et. al.* 1996) (Pan, Durst, *et. al.* 1995)

C) Terpenoid biosynthesis

Terpenoids are a large class of plant secondary metabolites which include plant hormones (gibberellin, abscisic acid), plant defence compounds, photosynthetic pigments (carotenoids), structural membrane components and electron carriers (Schuler 1996). Monoterpenes (C_{10}), diterpenes (C_{20}), sesquiterpenes (C_{15}) and triterpenes (C_{30}) have all been shown to be substrates of cyt P450 enzymes. Monoterpenes, widely distributed in the plant kingdom, are the predominant components of plant essential oils and are commercially used as flavourings, decongestants, external analgesics and antiseptics (Templeton 1969; Klocke, Darlington, *et. al.* 1987). Plant monoterpenes also possess insect attractant and repellant, feeding deterrent and ovipositional stimulant activities towards various insect species (Sangwan, Verma, *et. al.* 1990). The C10-hydroxylation of the monoterpene geraniol (and its *cis* isomer nerol) was also one of the first cyt P450 activities characterised in plants (Meehan and Coscia 1973) and this enzyme was one of the first from plants to be partially purified (Madyastha, Meehan, *et. al.* 1976; Madyastha and Coscia 1979). More recently it has been purified to homogeneity (Meijer, DeWaal, *et. al.* 1993). Geraniol, nerol and citronellol hydroxylation have been demonstrated in *Nepeta racemosa* (catmint) (Hallahan, Dawson, *et. al.* 1992; Hallahan, Lau, *et. al.* 1994; Hallahan and West 1995) and *C. roseus* (Meijer, DeWaal, *et. al.* 1993). In *P. americana*, epoxidation, rather than a hydroxylation, of these compounds appears to be catalysed by CYP71A1 (Hallahan, Lau, *et. al.* 1994). The CYP71 family is further discussed in Chapter 4.

Hydroxylations of other monoterpenes catalysed by cyt P450s include those of limonene (Karp, Mihaliak, *et. al.* 1990), sabinene (Karp, Harris, *et. al.* 1987), pinene (Karp and Croteau 1992), camphor (Karp and Croteau 1992) and terpineol (Lee, Hirata, *et. al.*

1983). Limonene hydroxylase has been purified and is extremely specific for its substrate but exhibits regioselectivity in different plant species such as *Mentha* and *Perilla* (Karp, Mihaliak, *et. al.* 1990).

Reactions involving cyt P450s in the metabolism of diterpenes, sesquiterpenes and triterpenes include the hydroxylation of kaurene (in the pathway leading to synthesis of the gibberellins) (Murphy and West 1969; Coolbaugh and Moore 1971), the hydroxylation of ipomeamarone (to form the sweet potato phytoalexin, ipomeamaronol) (Fujita, Oba, *et. al.* 1982) and the 14α - demethylation of the sterol obtusifoliol (Rahier and Taton 1986) respectively. Table 1.3 below shows examples of cyt P450 involved in terpenoid biosynthesis, with the appropriate reference(s).

Table 1.3 Cyt P450s involved in terpenoid metabolism

Enzyme	Reference(s)
Monoterpenes	
Geraniol/nerol hydroxylase	(Meehan and Coscia 1973) (Madyastha and Coscia 1979) (Madyastha, Meehan, *et. al.* 1976) (Meijer, DeWaal, *et. al.* 1993) (Hallahan, Dawson, *et. al.* 1992) (Hallahan, Nugent, *et. al.* 1992) (Hallahan, Lau, *et. al.* 1994)
Limonene hydroxylase	(Karp, Mihaliak, *et. al.* 1990)
Sabinene hydroxylase	(Karp, Harris, *et. al.* 1987)
Pinene hydroxylase	(Karp and Croteau 1992)
Camphor hydroxylase	(Karp and Croteau 1992)
Terpineol hydroxylase	(Lee, Hirata, *et. al.* 1983)
Diterpenes	
Kaurene oxidase	(Coolbaugh and Moore 1971) (Murphy and West 1969)
Abscisic acid 6-hydroxylase	(Gillard and Walton 1976)
Sesquiterpenes	
Ipomeamarone 15-hydroxylase	(Fujita, Oba, *et. al.* 1982)
Triterpenes	
Obtusifoliol 14α - demethylase	(Rahier and Taton 1986)

D) Cyanogenic glucoside biosynthesis

Cyt P450s have been shown to be involved in the biosynthesis of plant defence compounds the cyanogenic glucosides, which are found in over 2,500 plant species (Halkier, Sibbesen, *et. al.* 1995). Upon tissue disruption caused by wounding, feeding and infection, these compounds become available to enzymes which lead to the release of hydrogen cyanide. Two cyt P450 enzymes involved in cyanogenic glucoside biosynthesis have been characterised in *S. bicolor* (Halkier and Møller 1991; Halkier, Sibbesen, *et. al.* 1995), and *Manihot esculenta* (cassava) (Koch, Neilsen, *et. al.* 1992). The *S. bicolor* enzymes are both multi-functional and catalyse two steps in the biosynthesis of dhurrin, the N-hydroxylation of tyrosine to yield *N,N*-dihydroxytyrosine (cyt $P450_{TYR}$) and the conversion of the *p*-hydroxyphenylacetaldehyde oxime to *p*-hydroxymandelonitrile (cyt $P450_{OX}$) (Schuler 1996). Cyt $P450_{TYR}$ has been cloned and is assigned to the family CYP79 (Koch, Sibbesen, *et. al.* 1995). Cyt $P450_{TYR}$ represents the first amino acid sequence of a functionally characterised cyt P450 from a monocotyledous plant and the first multi-functional N-hydroxylase cyt P450 reported in a living organism (Halkier, Sibbesen, *et. al.* 1995).

E) Alkaloid biosynthesis

Two major alkaloid groups, the benzylisoquinolines and the monoterpenoid indoles contain steps in their biosynthetic pathway known to be cyt P450 catalysed (Bolwell, Bozak, *et. al.* 1994). In the case of the isoquinoline alkaloids, salutaridine biosynthesis from reticuline is cyt P450-catalysed in *Papaver somniferum* (opium poppy) (Zenk, Gerardy, *et. al.* 1989; Gerardy and Zenk 1993). An unusual cyt P450 involved in the biosynthesis of the isoquinoline alkaloid berbamunine has been characterised, purified (Stadler and Zenk 1993) and cloned (Kraus and Kutchan 1995). This enzyme, of the cyt

P450 family CYP80, is unusual in that it catalyses the regio- and stereo-selective formation of a C-O phenol couple without concomitant incorporation of oxygen into the substrate as is the case for most cyt P450s.

Other examples of cyt P450 involved in alkaloid biosynthesis are reviewed in Bolwell, Bozak, *et. al.* 1994.

F) Xenobiotic metabolism

The first reaction attributed to a cyt P450 in plants was the N-demethylation of monuron, a substituted phenylurea herbicide, in cotton microsomes (Frear, Swanson, *et. al.* 1969). The first purified plant cyt P450 to exhibit xenobiotic metabolism was the *P. americana* CYP71A1, which was shown to catalyse demethylation of the model cyt P450 substrate *p*-chloro-*N*-methylaniline (O'Keefe and Leto 1989). Ethoxycoumarin and ethoxyresorufin, two model cyt P450 substrates used in the study of animal cyt P450s involved in detoxification, have also been shown to be metabolised (undergoing deethylation) by a variety of plant cyt P450s (Werck-Reichhart, Gabriac, *et. al.* 1990). Cyt P450 involvement in xenobiotic metabolism including herbicides and insecticides is well documented. The interest in herbicide metabolising cyt P450 is not surprising considering the importance of herbicides in modern crop production. The metabolism of herbicides by cyt P450 is discussed in detail in Chapter 3.

Plant cyt P450s, therefore, play a significant role in the detoxification of xenobiotics, much as their mammalian counterparts do. It is unclear whether the same cyt P450 enzymes responsible for biosynthetic metabolism also catalyse detoxification of xenobiotics or whether distinct cyt P450 isoforms have evolved in response to increasing xenobiotic challenge. Evidence does exist for the former suggestion however, with evidence that lauric acid hydroxylase and a cyt P450 responsible for detoxification of the

herbicide diclofop are in fact the same enzyme (Zimmerlin and Durst 1992). One thing that is certain is the continued interest and financial investment in research into the xenobiotic metabolising plant cyt P450s, particularly those involved in herbicide metabolism. This is due largely to the huge potential in using molecular biological techniques to engineer transgenic crops with novel and multiple pesticide resistance.

1.7 Cloning of plant cyt P450

The first plant cyt P450 to be cloned was the cDNA encoding CYP71A1 from *P. americana* (Bozak, Yu, *et. al.* 1990). The mesocarp of *P. americana* is a rich source of cyt P450 (O'Keefe and Leto 1989). This facilitated the purification to homogeneity of a ripening-related cyt P450 from this tissue, and the sequence of 40 amino acid residues at the N-terminal end of the protein were determined (O'Keefe and Leto 1989). A ripening-related *P. americana* cyt P450 cDNA was later isolated, using differential colony hybridisation with cDNA from ripe and unripe fruit, which was assigned to a new cyt P450 family, CYP71 (Bozak, Yu, *et. al.* 1990). The N-terminal amino acid sequence of the purified protein described above was shown to be identical to the predicted N-terminal amino acid sequence of the isolated cDNA sequence. Thus, the *P. americana* CYP71A1 cyt P450 was the first plant cyt P450 to be characterised at both the molecular and biochemical level. The precise function of this cyt P450 and others within the same family in plants has yet to be established. The availability of a plant cyt P450 sequence, however, had a dramatic impact on the field of cyt P450 research, as, for the first time, sequence similarities between plant and other eukaryotic cyt P450s could be observed. This meant that molecular biological techniques could be exploited to facilitate isolation of further plant cyt P450 clones. Knowledge of the sequence of CYP71A1 allowed the

technique of polymerase chain reaction (PCR) to be used to isolate a cyt P450 from *Thlaspi arvense* (Udvardi, Metzger, *et. al.* 1994). The degenerate PCR primers used were based on two of the four main regions of conserved sequence in the CYP71A1, around the heme co-ordinating cysteine and around the Helix I region conserved threonine discussed earlier in this chapter. A PCR strategy was used to isolate a number of cyt P450 fragments from *C. roseus* by a research group interested in the cyt P450 geraniol 10-hydroxylase (Meijer, De Waal, *et. al.* 1993). The group had set out to purify the enzyme directly from *C. roseus* cell suspension cultures. In view of difficulties anticipated in purification however, they also decided to adopt a molecular approach in an attempt to clone geraniol 10-hydroxylase, thus perhaps circumventing the need to purify the enzyme. A degenerate PCR primer was designed, corresponding to the cyt P450 consensus of the haem binding domain of CYP71A1 and mammalian cyt P450s (Bozak, Yu, *et. al.* 1990), and an unpublished petunia cyt P450 cDNA. PCR was carried out on a cDNA library using this degenerate primer and primers specific for cDNA sequence poly-A tails and a portion of the vector sequence close to the poly A tail of the sequences within the library. As a result, 16 cyt P450 fragments corresponding to the C-terminal end of the sequence were isolated, one of which was of the CYP71 family (CYP71A7). Others belonged to the families CYP72 and CYP73, and the remainder were not assigned to specific families. None of the cloned fragments could be identified as representing part of a geraniol 10-hydroxylase sequence at that point, but the group did, however, succeed in purifying this enzyme directly from *C. roseus* (Meijer, DeWaal, *et. al.* 1993).

A different strategy for cloning geraniol 10-hydroxylase from *C. roseus* was adopted by another research group, carried out prior to the work described above. This involved screening a cDNA library prepared from cell cultures of a *C. roseus* line selected

for high indole alkaloid production and induced for indole alkaloid biosynthesis by altering the growth medium composition (Vetter, Mangold, *et. al.* 1992). The library was screened in two stages to obtain clones corresponding to induced mRNAs of the size expected for cyt P450 proteins (1.3 - 1.9 kb). Differential hybridisation was used to obtain induced mRNAs by screening with cDNAs prepared from non-induced and induced cultures. Selection of a second subset of cDNAs which were potentially cyt P450s was achieved by isolating only those clones which, on northern analysis, hybridised to ~~indole-alkaloid~~-induced mRNA of the expected size for cyt P450 transcripts. This lead to the isolation of two closely related (98 % identity at the amino acid level) cyt P450 cDNAs, assigned to family CYP72. The cDNAs were heterologously expressed in *E. coli* and comparison of the induction kinetics of these cyt P450 genes to those of geraniol 10-hydroxylase were similar, thus these cyt P450s were tentatively assigned as geraniol 10-hydroxylases. However, no geraniol 10-hydroxylase activity could be detected when the cyt P450 clones were heterologously expressed in yeast (Vetter, Mangold, *et. al.* 1992) or plants (Mangold, Eichel, *et. al.* 1994) and, therefore, definitive function as a geraniol 10-hydroxylase was not assigned.

The fourth and fifth cyt P450 cDNAs to be isolated were the first with clear physiological activity, the cinnamate 4-hydroxylases (C4H) from mung bean (Mizutani, Ward, *et. al.* 1993) and Jerusalem artichoke (Teutsch, Hasenfratz, *et. al.* 1993). The N-terminal portion of the previously purified protein from mung bean (Mizutani, Ohta, *et. al.* 1993) was sequenced and this amino acid sequence used to design degenerate oligonucleotide primers for PCR. A 1.3 kb cyt P450 cDNA fragment was amplified using PCR on mung bean cDNA. Comparison of stretches of the deduced amino acid sequence to that of the purified protein revealed it to represent C4H. This probe was used to isolate

two near full-length sequences from a mung bean cDNA library.

A similar approach was used to clone the allene oxide synthase (AOS) cyt P450 from flaxseed (Song, Funk, *et. al.* 1993). Regions of amino acid sequence of the previously purified protein (Song and Brash 1991) were used to prepare degenerate primers and reverse-transcriptase used to amplify a short cDNA sequence. Nested-PCR was used to obtain the remaining C-terminal end of the cDNA and this was then used to screen a library prepared from flaxseed at a developmental stage previously determined to be optimal for AOS activity. Another full-length AOS cDNA, from *Arabidopsis thaliana* (Laudert, Pfannschmidt, *et. al.* 1996), and a putative AOS from guayule rubber particles (Pan, Durst, *et. al.* 1995) have been cloned recently using a very similar approach. This strategy, using protein sequencing, followed by degenerate PCR and library screening, was also used to isolate a full length limonene-6-hydroxylase from *Mentha spicata* (spearmint) (Lupien, Karp, *et. al.* 1995) and a C-O phenol coupling cyt P450 from *Berberis stolonifera* (Kraus and Kutchan 1995).

Other strategies have involved using an understanding of the physiological processes of the pathways the particular cyt P450s are involved in as was the case with the cloning of the flavonoid 3'-, 5'-, hydroxylases from *Petunia hybrida* (Holton, Brugliera, *et. al.* 1993) and *S. melongena* (eggplant) (Toguri, Azuma, *et. al.* 1993; Toguri, Umemoto, *et. al.* 1993) and ferulate 5-hydroxylase (F5H) from *Arabidopsis* (Meyer, Cusumano, *et. al.* 1996).

Generation of antibodies raised to a particular purified cyt P450 in order to screen for the corresponding cDNA from a library has also been used and a C4H from Jerusalem artichoke (Teutsch, Hasenfratz, *et. al.* 1993) and P450TYR from *Sorghum bicolor* (Koch, Sibbesen, *et. al.* 1995) were isolated using this technique.

Plant cyt P450 genes, as well as cDNA sequences, have been isolated. Four *Z. mays* cyt P450 genes were isolated using differential screening of a seedling cDNA library prepared from *Z. mays* lines with a high level of a cyt P450 catalysing a step in the biosynthesis of 2,4-di-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), a secondary metabolite associated with defence against insect herbivory in monocotyledonous plants (Frey M., Kliem, *et. al.* 1995). Also a gene from *C. roseus* corresponding to *CYP72A1* has been isolated (Mangold, Eichel, *et. al.* 1994).

As can be seen, the strategies used to isolate cyt P450 clones from plants are numerous and the number of plant cyt P450 cDNAs isolated continues to rise steadily as a result of such efforts. Genes encoding the majority of known plant cyt P450 enzymes have not been identified to date, but many 'orphan' cDNAs have been isolated for example from the *Arabidopsis* EST programme, discussed in more detail in Chapter 4. The development and refinement of suitable heterologous expression systems for plant cyt P450s will continue to facilitate the assignment of function to particular genes. Heterologous expression of cyt P450s is discussed in greater detail in Chapter 5.

1.8 Aims

The difficulties in purification of plant cyt P450s, for reasons described in Section 1.2 above, have resulted in a situation where only a few have been isolated directly from plant tissue to date. This has meant that conventional methods for studying their catalytic activity, such as cloning procedures involving the use of antibodies, or the generation of oligonucleotide probes from amino acid sequence, have proven of limited success. This project aimed to circumvent these problems by adopting an alternative molecular approach using degenerate polymerase chain reaction (PCR) techniques to isolate full length cyt

P450 clones. The ultimate aim of the project was to characterise isolated plant cyt P450 clones with respect to their expression patterns in the host plant and to assign function to the corresponding cyt P450 enzyme by biochemical assay of the enzyme expressed in a suitable heterologous system such as yeast.

Chapter 2

Materials & Methods

2.1 DNA vector host strains

The *Escherichia coli* (*E.coli*) strains used were XL1-Blue (Bullock, Fernandez, *et. al.* 1987), DH5α (Hanahan 1983; BRL 1986), DH10B^{TM} (Lorow and Jessee 1990) and Y1090r^{-} (Huynh 1985).

The *Saccharomyces cerevisiae* yeast strains R3 (*leu*2-3,*leu*2-112,*his4,ura3*) (Fang, Venkateswarlu, *et. al.* 1994) and DBY746 (American Type Culture Collection (ATCC) 44773; (*mat-α,his3-Δ1,leu2-3,leu2-112,ura3-52,trp1-289*) (Falco, Li, *et. al.* 1982) used were a kind gift from Dr. S. L. Kelly, University of Sheffield. The R3 strain was selected as a suitable host for heterologous expression of cyt P450 due to its low endogenous cyt P450 levels.

2.2 DNA vectors

Plasmids

Plasmids used were pBluescript® KS (Stratagene), pZL1 (Life Technologies$_{TM}$), pCR-Script^{TM} SK$^{(+)}$ (Stratagene), pCR^{TM} II (Invitrogen), pGEM® (Promega), pYES2 (Invitrogen) and pGY. The latter was constructed at Rothamsted by Dr. S. Crank, by replacing the inducible galactose promoter of the *(GAL)* yeast-*E.coli* shuttle vector pYES2 with the constitutive promoter for phosphoglycerate kinase (PGK).

Bacteriophage

Bacteriophage lambda (λ) libraries used were constructed in λZAP® II (Short, Fernandez, *et. al.* 1988), (Stratagene) and λgt11 (Young and Davis 1983) (Clontech).

2.3 *Growth and storage media*

Autoclave conditions for all media were 121^0C, 15 pounds per square inch (p.s.i) pressure, 20 minutes (min) on liquid cycle. Agar plates were prepared by adding Bacto-agar (Difco) to liquid medium at a concentration of 1.5% (w/v) and 2% (w/v) for bacterial and yeast plates respectively. Following autoclaving, the agar was allowed to cool to approximately 50^0C prior to pouring into sterile disposable petri dishes in a laminar flow hood. Antibiotics, when required, were prepared as previously described (Sambrook, Fritsh, *et. al.* 1989) and added aseptically to cooled media prior to pouring (to prevent denaturation of the antibiotic at high temperature). Plates were left in the air flow for approximately 30 min to dry off excess moisture and stored at 4^0C if not used immediately.

E.coli strains were routinely cultured in Luria-Bertani (LB) medium (Sambrook, Fritsh, *et. al.* 1989). Selection for transformed and recombinant *E.coli* was achieved by addition of the appropriate antibiotics to either liquid or solid medium.

Non-transformed yeast were routinely grown in yeast extract peptone dextrose (YPD) medium (1% (w/v) Bacto-yeast extract (Difco), 2% (w/v) Bacto-peptone (Difco), 2% (w/v) dextrose). Dextrose was added as a 20% (w/v) filter-sterilised stock solution to avoid caramelisation through autoclaving. Transformed and recombinant yeast were grown in either synthetic dextrose (SD) medium (0.17 % (w/v) yeast nitrogen base without amino acids or ammonium sulphate (YNB-AA/AS; Difco), 0.5 % (w/v) ammonium sulphate, 2% (w/v) dextrose) or synthetic complete medium (SC). SD medium was supplemented only with amino acids that the yeast strains ATCC 44773 and R3 cannot synthesise, namely 20 mg/l histidine, 40 mg/l tryptophan and 60 mg/l leucine. SC medium was SD medium plus all the amino acids. Amino acids were prepared and

added as previously described (Ausubel 1989). Uracil was omitted from both media when transformed and recombinant yeast were grown since this is the selectable marker provided by the yeast expression plasmids used in this work. Yeast cells transformed with vectors carrying the *PGY* constitutive promoter, were routinely grown in SD (minus uracil) for 24 h at 30^0C. Cells transformed with vectors carrying the inducible promoter *GAL* were grown in SD (mins uracil) with the addition of 2.5 % glycerol, 0.1 % dextrose for 24 h at 30^0C. Galactose was then added to a final concentration of 2 % to initiate induction of the vector promoter and cells were grown for a further 10-12 h prior to harvesting.

Storage and dilution of bacteriophage λ was carried out in Suspension Medium (SM) (Sambrook, Fritsh, *et. al.* 1989). Chloroform (0.3 % (v/v)) was added to prevent bacterial contamination.

Chromogenic substrates and inducers

Blue/white selection for recombinants was possible for vectors where the cloning site is located within the β-Galactosidase (*lacZ*) gene, (including pBluescript®, pGEM® and pCR-Script™). This was achieved by addition of the chromogenic substrate of β-galactosidase, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), to solid medium in the presence of the gene inducer isopropyl-thiogalactoside (IPTG). This results in blue staining of non-recombinant colonies. X-Gal and IPTG stock solutions were prepared and added to solid media as previously described (Sambrook, Fritsh, *et. al.* 1989).

2.4 *Growth and stock maintenance of bacteria, yeast and bacteriophage*

Bacteria

Bacteria taken from a single colony were streaked onto LB agar plates with the appropriate antibiotic selection where possible, and grown overnight at 37^0C. Plated bacteria were stored for up to one month at 4^0C before being restreaked to fresh LB plates. For liquid culture, cells were grown in LB medium overnight at 37^0C with shaking (250 rpm).

Yeast

Yeast cells taken from a single colony were streaked onto the appropriate agar plates as described above, and grown for up to 48 h at 30^0C. Plated yeasts were stored for up to 2 months at 4^0C before restreaking to fresh plates. For liquid culture, cells were grown in the appropriate medium (Section 2.3) overnight at 30^0C with shaking (200 rpm).

For long-term storage, bacteria or yeast were placed in the appropriate medium containing 15% (v/v) glycerol in screw capped, air-tight tubes. Tubes were initially quick-frozen in liquid nitrogen and subsequently stored at -70^0C. Bacteria or yeast glycerol stocks were revived by scraping the surface of the suspension with a cooled sterile loop and streaking the cells onto the appropriate agar plates. Cells were grown as specified above. Glycerol stocks were replaced quickly to -70^0C storage before full thawing occurred.

Bacteriophage

Host bacteria for bacteriophage plating were prepared as previously described (Sambrook, Fritsh, *et. al.* 1989). Phage plating to isolate pure plaques and titration of phage stocks was also carried out as previously described (Sambrook, Fritsh, *et. al.* 1989). Isolated plaques were used to generate high titre stocks by large-scale plate lysis as described below. All bacteriophage stocks were stored at 4^0C in suspension medium (SM)

(Sambrook, Fritsh, *et. al.* 1989) containing 0.3 % (v/v) chloroform.

Large-scale isolation of bacteriophage was carried out using either a plate or liquid lysate procedure. The method of choice was found to be plate lysis since it yielded more phage and was easier to carry out. Plate lysis was carried out essentially as previously described (Sambrook, Fritsh, *et. al.* 1989), with the following modifications. Up to 25 large (150 mm diameter) plates of bacteriophage were prepared and grown to confluency overnight at 37^0C. The top agarose was scraped off each plate using a sterile scalpel blade and pooled into a sterile 250 ml polypropylene centrifuge bottle. SM (5 ml) was added to each plate and the plates left on a rotating platform for approximately 5 min. This solution, containing the remaining bacteriophage particles, was recovered from each plate using a 5 ml micropipetter and added to the top agarose from the original scraping. Residual SM was recovered by tilting the plates allowing it to drain to the bottom of the plate. The agarose/phage suspension was vortexed vigorously for several minutes until the agarose was completely dispersed. Chloroform (2.5 ml) was added and the suspension shaken (250 rpm) for 15 min at 37^0C to facilitate lysis of any bacteria present. The suspension was then centrifuged at 4,000 x g for 10 min at 4^0C. The supernatant was carefully recovered and filtered through sterile Miracloth (Calbiochem) to remove residual agarose. The amplified phage were now ready for titration (Sambrook, Fritsh, *et. al.* 1989) and further manipulations such as purification of bacteriophage DNA (Section 2.7).

For liquid lysis, host plating bacteria (routinely 1 litre) were grown at 37^0C, with shaking (250 rpm) in LB medium supplemented with 10 mM $MgSO_4$ and 0.2% (w/v) maltose to an optical density at 600 nm (OD_{600}) of 0.6. A volume of bacteriophage equivalent to 3 x 10^{10} plaque forming units (pfu) was added to the bacteria and the culture replaced at 37^0C with shaking for a further 6 - 10 h until lysis occurred. When

lysis has occurred the culture appears clear with bacterial debris present. Foaming also gives a good indication of lysis. Following lysis, 10 ml of chloroform was added and the culture shaken for a further 15 min at 37^0C. The culture was centrifuged at 7,200 x g for 10 min at 4^0C in polypropylene centrifuge bottles and the supernatant containing bacteriophage particles recovered. Chloroform (0.3 % v/v) was added and the suspension stored at 4^0C.

For long-term storage, bacteriophage isolated from plaques were placed in SM containing 7 % (v/v) dimethylsulphoxide (DMSO) and small aliquots stored at -70^0C following quick-freezing in liquid nitrogen. Single aliquots of frozen bacteriophage were thawed and stored at 4^0C as a working stock without re-freezing since repeated freeze-thaw cycles can detrimentally affect the titre of a bacteriophage stock. Alternatively, if absolutely necessary, bacteriophage were recovered by scraping the surface of the frozen liquid stock with a sterile 18-gauge needle. The needle was then streaked over the surface of a plate containing the appropriate plating bacteria. Frozen stocks were replaced immediately at -70^0C to prevent complete thawing. Plates were grown at 37^0C for 12 h to obtain isolated plaques as previously described (Sambrook, Fritsh, *et. al.* 1989).

2.5 Standard solutions and chemicals

Chemicals (molecular biology grade) were purchased from BDH, Sigma and Aldrich, unless otherwise stated. All solutions used routinely for molecular biology techniques were prepared and stored as previously described (Sambrook, Fritsh, *et. al.* 1989). Wherever possible, water used to prepare solutions had been purified by reverse osmosis (Elgastat® UHQ II). Type III Loading buffer was used for all DNA agarose gels (Sambrook, Fritsh, *et. al.* 1989). Phenol was obtained already equilibrated with

Tris[hydroxymethyl]amino methane (Tris), pH 8.0 (Biorad) for use with RNA and DNA techniques. Acrylamide (19:1 acrylamide:bisacrylamide for manual sequencing gels; and 37.5:1 acrylamide:bisacrylamide for polyacrylamide gels) was obtained as a pre-weighed powder from Sigma.

2.6 Preparation and sterilisation of equipment and solutions

All equipment used for molecular biology techniques was handled with gloves before and after sterilisation to prevent possible contamination with nucleases present on the skin. Centrifuge tubes, microfuge tubes, disposable pipette tips and solutions were sterilised by autoclaving as described in Section 2.3. Solutions which could not be autoclaved such as dextrose and 2-[N-morpholino]ethane-sulfonic acid (MES) buffer were sterilised by passing through a 0.2 μm filter (Schleicher and Schuell) into a sterile container.

Glassware was wrapped in aluminium foil and baked at 180 ^{0}C for at least 2 h to sterilise and to destroy contaminating ribonucleases (RNases) when required for working with RNA.

Solutions used for RNA extraction and manipulation were treated to inactivate contaminating RNases wherever possible using 0.1 % (v/v) diethylpyrocarbonate (DEPC) as previously described (Sambrook, Fritsh, *et. al.* 1989). Certain solutions, including Tris buffers, which could not be treated with DEPC, were prepared using RNase-free sterile distilled water (dH_2O) and baked glassware.

When required for running RNA gels, electrophoresis equipment was treated to destroy RNases by soaking the tank, gel former and comb for 10 min in 3 % (v/v) hydrogen peroxide followed by rinsing in RNase-free water. The gel pouring platform was soaked in 0.1 M NaOH for at least 10 min and rinsed with RNase-free sterile dH_2O.

Contaminating RNases were eliminated from the pH electrode prior to pH adjustment of RNase-free solutions by soaking the bulb in 0.1 M NaOH for 10 min followed by soaking in RNase-free sterile dH_2O to completely rinse off the strong alkali.

2.7 DNA and RNA isolation techniques

Plasmid DNA isolation

DNA was routinely isolated from bacterial cultures using Wizard^{TM} Mini and Maxi-Prep kits (Promega) or Plasmid Mini and Maxi preparations (QIAGEN) according to the manufacturer's instructions.

Plant genomic DNA isolation

Plant genomic DNA (gDNA) was isolated by using a scaled-up, modified version of the plant DNA minipreparation version II previously described (Dellaporta, Wood, *et. al.* 1983). Plant tissue was harvested and immediately frozen in liquid nitrogen. Tissue was stored at - 70^0C if gDNA was not extracted immediately. The method was scaled up 10-fold, and as a result, modification to the procedure included using a sterile 5 inch pestle and mortar and, following grinding to a powder, the tissue was transferred to 250 ml sterile centrifuge bottles to which the required quantity of extraction buffer and sodium dodecylsulphate (SDS) was added. The remainder of the procedure was carried out as described in the original protocol, scaling up volumes as required. Centrifugations were carried out in sterile 50 ml Oakridge tubes. Following addition of Tris-EDTA (TE) (Sambrook, Fritsh, *et. al.* 1989)) to the washed DNA pellet it was placed at 4^0C overnight to ensure complete re-hydration, thus avoiding the risk of shearing by the vortexing specified in the original protocol.

Bacteriophage λ DNA isolation

A) λ DNA isolation using QIAGEN columns

Bacteriophage λ DNA was isolated from plate lysates using Lambda Mini or Maxi kits (depending upon the volume of plate lysate) according to the manufacturer's instructions (Qiagen).

B) λ DNA isolation using polyethylene glycol (PEG) precipitation and caesium chloride step gradients

Liquid lysate of bacteriophage λ was prepared as described in Section 2.4. DNA was prepared from the lysate as described in the λgt11 library (Clontech) handbook supplied with a commercial bacteriophage library (details of which can be found in Chapter 3, Section 3.2). The DNA was purified using caesium chloride step gradients as previously described (Sambrook, Fritsh, *et. al.* 1989).

Quick plasmid DNA preparation from yeast cells

Quick analysis of transformants from yeast was carried out as previously described (Strathern and Higgins 1991). A 10 ml culture of transformed yeast was grown at 30^0C overnight with shaking (250 rpm) in SD (minus uracil) medium. A 1.5 ml volume of cells was pelleted using a microfuge by centrifugation at 14,000 x g for 20 s. The cell pellet was resuspended in 200 µl breaking buffer (100 mM NaCl, 1 mM EDTA, 0.5 % (w/v) SDS, 10 mM Tris.Cl, pH 8.0) and a volume equivalent to 200 µl sterile acid-washed glass beads was added. The cells were vortexed with the beads for 1 min and then phenol/chloroform extracted and ethanol precipitated to purify as described below. The DNA pellet was resuspended in 50 µl TE and 5 µl used to transform *E.coli* as described in Section 2.8. Transformants were analysed by either colony hybridisation (Section 2.11) using the original insert DNA as a probe, or by minipreparation of DNA and analysis by

gel electrophoresis (Section 2.9) of restriction fragments.

Plant RNA isolation

Plant RNA was isolated using a modification of the method of Logemann, Schell, *et. al.* 1987. The guanidine extraction buffer was prepared in a baked beaker using guanidine hydrochloride (molecular biology grade, Sigma) and sterile RNase-free stock solutions. Following pH adjustment (See Section 2.6), the solution was filter sterilised (MES cannot be autoclaved) into a baked bottle. β-Mercaptoethanol was added to a final concentration of 50 mM immediately prior to use. Tissue (frozen in liquid nitrogen) was ground to a fine powder using a baked pestle and mortar. Centrifugations were carried out in baked Corex® (Corning Glass) tubes.

Yeast RNA isolation

RNA was extracted from transformed yeast for northern analysis as previously described (Treco 1989). Yeast were cultured in SD (minus uracil) overnight at 30^0C with shaking (250 rpm) to an OD_{600} of 0.5 - 1.0. The cell density was determined using a haemocytometer and a volume containing 2 x 10^8 cells (for cells in mid-log phase, 1 - 2 x 10^7 cells/ml has an OD_{600} of 0.5 - 1.0) was centrifuged at 1,200 x g for 10 min at 4^0C. The supernatant was discarded, cells resuspended in 1 ml Yeast RNA Buffer (0.5 M NaCl, 10 mM EDTA, 0.2 M Tris.Cl, pH 7.5), and re-centrifuged at 14,000 x g in a microfuge for 5 s at 4^0C. The supernatant was discarded and the cells resuspended in 300 µl Yeast RNA Buffer. Yeast cells were disrupted by adding an equivalent of 200 µl of sterile acid-washed glass beads (0.4 - 0.55 mm diameter, Braun Melsungen, ref 85417011B) and 300 µl phenol/chloroform/isoamyl alcohol, 25:24:1 (previously equilibriated with Yeast RNA Buffer). The yeast cells were mixed thoroughly by inversion and vortexed vigorously with the beads for 2 min at 4^0C. Phenol/chloroform

extraction of the aqueous layer was repeated (as described below) until no white precipitate could be seen at the organic/aqueous interface and the RNA precipitated with 2.5 volumes of ice-cold absolute ethanol as described below.

Determination of DNA and RNA concentration and purity

DNA and RNA concentration and purity was measured over an absorption range of 230 - 300 nm using a Cary 3 spectrophotometer. Calculations of concentration were based on the fact that DNA and RNA at 50 μg/ml and 40 μg/ml respectively have an absorbance at 260 nm (A_{260}) of 1.0 (Sambrook, Fritsh, *et. al.* 1989). Purity was determined by calculating the ratio of the A_{260}/A_{280}. DNA and RNA relatively free of protein and carbohydrate impurities should have a ratio of 1.8 - 2.0.

In cases where only small amounts of DNA were available which cannot be easily estimated accurately by spectrophotometric methods without significant loss of sample, DNA concentration was estimated by comparison with a known concentration of λ *Eco*RI/*Hind*III molecular weight markers by agarose gel electrophoresis (See Section 2.9). When the DNA and markers were sufficiently resolved, the gel was visualised using ultraviolet trans-illumination and the intensity of the fluorescence of the ethidium bromide stained target DNA compared to the intensity of each marker band. Since the amount of DNA in each marker band could be calculated, the concentration of target DNA was estimated to be approximately equal to the marker band of equivalent intensity.

DNA and RNA purification and storage procedures

DNA and RNA were purified using phenol/chloroform/isomamyl alcohol extraction, chloroform extraction and ethanol precipitation as previously described (Sambrook, Fritsh, *et. al.* 1989). DNA for routine use was stored in TE buffer or dH_2O at -20^0C, or precipitated under 2 volumes of absolute ethanol for long term storage. RNA was stored

at -70^{0}C in RNase-free dH_2O or precipitated under 2.5 volumes of absolute ethanol containing 0.3 M sodium acetate for long-term storage.

2.8 DNA Manipulations

Restriction digestion

Restriction endonucleases were obtained from Boehringer Mannheim, Life Technologies$_{TM}$ and Stratagene. Enzymes were used with buffers supplied according to the manufacturer's instructions. For multiple enzyme digests, the buffer which supported enzyme activity most efficiently was used as determined by manufacturer's specifications. Routinely, 1 - 5 units (U) enzyme per μg DNA was used. In most cases digestion was carried out for 2 h, but reactions containing gDNA or large quantities (i.e. > 20 μg) of plasmid DNA were allowed to proceed overnight.

Ligation

In cases where restriction digestion of DNA created 5'- or 3'- single-stranded overhangs of vector and target insert DNA, ligation was carried out in a total reaction volume of 20 μl containing a 1:1 molar ratio of insert to vector DNA (routinely 50 - 100 ng of each DNA component dependent on size), 3 Weiss units of T4 DNA Ligase (Promega) and 1 x ligase buffer (10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.5 mM rATP, 30 mM Tris.Cl, pH 7.8; Promega). Ligation was carried out overnight at 12^{0}C and terminated by heating at 75^{0}C for 10 min.

Blunt-end ligation was carried out where restriction digestion generated no single-stranded DNA overhangs or following 'filling in' (see below) of non-compatible overhangs to generate blunt ends. The dynamics of the blunt end ligation reaction required that more insert than vector DNA be present to drive the ligation reaction towards recombination

of vector with insert rather than self-ligation of vector. Blunt-end reactions were therefore carried out using a 3:1 molar ratio of insert DNA to vector DNA (routinely 100 - 300 ng of each DNA component dependent on size). Reactions were carried out overnight using the same enzymes and buffers as used for ligation of cohesive ends at room temperature and terminated as described above. The amount of insert DNA to be included in a ligation experiment was calculated according to the following equation.

$$\frac{\text{ng vector DNA} \times \text{size insert DNA (kb)}}{\text{size vector (kb)}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} \quad \text{i.e :-} \quad \frac{3}{1}$$

In all ligations a positive control, consisting of restriction-digested vector DNA and a negative control, consisting of restriction-digested, dephosphorylated (to prevent re-ligation, see below) vector DNA were included.

Filling in of 5'- single-stranded DNA overhangs

The Klenow fragment of DNA Polymerase I (Ultra pure sequencing grade, Stratagene) was used to fill in 5'- single-stranded overhangs to generate blunt ends for ligation from non-compatible ends. The reaction conditions were 4 U Klenow per μg DNA, 20 μM each dNTP (Life Technologies$_{TM}$), 1 mM DTT, 7 mM $MgCl_2$, 50 mM Tris.Cl, pH 7.5 (Stratagene's T4 DNA ligase buffer at a concentration of 1 x was used since it was compatible with the identical reaction conditions required). Reactions were incubated at room temperature for 15 min and terminated by heating at 75^0C for 15 min.

Dephosphorylation

Removal of 5'- phosphate residues from DNA was catalysed by shrimp alkaline phosphatase (United States Biochemical) according to the manufacturer's instructions for dephosphorylation of blunt and 5'- overhanging DNA ends. Reactions were carried out

in the supplied buffer, (10 mM $MgCl_2$, 20 mM Tris.HCl, pH 8). A 12.5 to 25 - fold excess of the recommended amount of enzyme was used (0.04 U and 0.08 U per μg for 5'- overhangs and blunt-ended DNA respectively) and the reaction time increased from 1 to 4 h at 37^0C to ensure complete dephosphorylation had taken place. The reaction was terminated by heating at 65^0C for 15 min.

Polymerase chain reaction (PCR)

PCR of DNA and bacteriophage λ

PCR primers were custom-synthesised by a number of commercial suppliers (Genosys, Pharmacia, or Cruachem). 50 pmol was used per reaction for routine DNA amplifications with up to 1 μg of template DNA. PCR reactions were catalysed by *Thermus aquaticus* DNA Polymerase (AmpliTaq^R, Perkin Elmer). Reactions were carried out using the GeneAmp^R PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 10 mM Tris.HCl, pH 8.3), according to the manufacturer's instructions (Perkin Elmer). Amplification was carried out in an Omnigene thermal cycler (Hybaid), with cycle lengths, number of cycles and temperatures as indicated for specific experiments (Chapter 3).

Reverse transcriptase PCR (RT-PCR) was carried out on total RNA using the GeneAmp^R RNA PCR kit according to the manufacturer's instruction's (Perkin Elmer), using 0.5 - 1 μg total RNA per reaction. Initial reverse transcription of RNA was primed using the oligo $d(T)_{16}$ primer supplied. Cycle lengths, number of cycles and temperatures used for each individual experiment were as indicated for specific experiments (Chapter 3).

PCR reactions in which oil had been used were purified by chloroform extraction as described in Section 2.7. The use of a heated-lid facility on the PCR machine when available obviated the need for oil and hence a chloroform extraction step prior to loading

on an agarose gel for analysis.

DNA sequencing

Sequencing of double-stranded DNA templates was based on the dideoxy chain termination method (Sanger, Nicklen, *et. al.* 1977). Sequence data for publication was obtained by sequencing both strands of DNA at least 3 times in both directions using custom-synthesised (Pharmacia, Cruachem) vector-specific and sequence-specific oligonucleotides as primers. The vector- specific primers M13 Forward (5'-TGTAAAACGACGGCCAGT), T7 (5'- AATACGACTCACTATAG), T3 (5'-ATTAACCCTCACTAAAG), λgt11 Forward (5'-TGGTGGCGTCGACTCCTCGAGCCCGT) and λgt11 Reverse (5'-TTTGACTCGAGACCAACTAGTAATGG) and sequence-specific primers were used as indicated for specific experiments in Chapters 3 and 4. Sequence details on sequence-specific primers can also be found in these chapters.

For manual sequencing, double-stranded plasmid DNA template was prepared using Wizard^{TM} Mini or Maxiprep kits according to the manufacturer's instructions (Promega). Sequencing reactions were performed using the Sequenase® (Version 2) kit according to the manufacturer's instructions (United States Biochemical). 3 - 5 μg DNA and 5 μC*i* [α-^{35}S]dATP was used per reaction. Sequencing reactions were electrophoresed on a 6 % acrylamide gel and gels were dried under vacuum using a gel dryer according to the manufacturer's instructions (Biorad). Autoradiography of gels by exposure to X-ray film for visualisation of reactions was carried out as described in Section 2.11.

For automated sequencing, plasmid DNA was prepared as for manual sequencing described above. Sequencing reactions were carried out using the Prism^{TM} Ready

Reaction DyeDeoxy^{TM} Terminator Cycle Sequencing kit according to the manufacturer's instructions (Perkin Elmer) and analysed on an ABI 373 Stretch Sequencer (Applied Biosystems).

Sequence data were analysed and suitable sequencing primers selected using the Staden and University of Wisconsin Genetics Computer Group (GCG) software packages. Putative sequence-specific primers were checked for potential mismatches to the sequence itself and to vector DNA using the GCG programs WORDSEARCH and SEGMENTS.

Labelling and purification of DNA probes

DNA was radiolabelled for use as probes in southern and northern analysis using the random-priming method (Feinberg and Vogelstein 1983). Routinely, 25 ng of DNA were labelled using 50 μC*i* [α-^{32}P]dATP or dCTP (3000 C*i*/mMol) in a 50 μl reaction volume. The amount of DNA labelled was increased to 100 ng when the probe was required for genomic southern analysis or northern analysis of rare transcripts. The reaction was catalysed with the Klenow fragment of DNA Polymerase I and allowed to proceed for at least 4 h. After radiolabelling, DNA was purified from unincorporated radionucleotides using NucTrap^{R} Probe purification columns with the Pushcolumn Beta Shielding device according to the manufacturer's instructions (Stratagene).

Transformation of E.coli

A 10 ml starter culture of *E.coli* cells was grown at 37^{0}C overnight with shaking (250 rpm) in LB medium (with the appropriate antibiotic where selection was possible) until a high cell density (saturation) was reached. The next day, 1 ml of the culture was removed and added to 100 ml of LB and grown as before until the OD_{550} of the culture was 0.5. The culture was chilled on ice for 10 min and then centrifuged in sterile 50 ml polypropylene tubes (Apex, Alpha Laboratories) at 4,000 x g for 5 min at 4^{0}C. The

of the pellet
supernatant was removed and resuspended in 50 ml ice-cold sterile Cell Resuspension (CR) buffer (50 mM $CaCl_2$, 10 mM Tris.Cl, pH 8.0). The cells were placed on ice for 15 min and then re-centrifuged as before. The cell pellet was resuspended very gently on ice in 7 ml CR buffer and 200 µl aliquots dispensed into pre-chilled sterile microfuge tubes using pre-chilled pipette tips. Cells were left on ice for at least 1 h before being used for transformation. If not required for transformation the same day, cells were quick-frozen in liquid nitrogen and stored at - 70^0C.

Approximately 25 ng plasmid DNA from DNA ligation experiments or intact plasmid for transformation controls was mixed with each competent cell aliquot using a pipette tip. Cells and DNA were placed on ice for 30 min and then heat-shocked at 42^0C for exactly 2 min. 1 ml of LB medium was added and the tubes left at 37^0C for 1 h without agitation. This step allows the bacteria to express the antibiotic resistance carried by the introduced vector DNA prior to plating. Small aliquots, i.e. 50 µl, 100 µl and 200 µl were plated onto LB agar plates with the appropriate antibiotic for selection. The remainder of the transformed cells were stored at 4^0C for a maximum of 2 days in case more transformants were subsequently required to be plated. Where vectors used allowed blue/white selection of recombinants, plates were previously treated with X-Gal and IPTG (See Section 2.3). Plates were left for approximately 10 min to allow the liquid cell suspension to sink into the agar, then inverted and grown at 37^0C overnight.

Transformation of yeast

Yeast were transformed as previously described (Schiestl, Manivasakam, *et. al.* 1993).

2.9 *Electrophoresis*

Agarose gel electrophoresis of DNA

DNA was routinely separated by horizontal slab agarose gel electrophoresis (Southern 1979) using Mini, Wide-Mini, and Maxi gel apparatus (Biorad) depending upon the length of gel run required and the number of samples to be separated. Agarose (Ultrapure grade, Life Technologies$_{TM}$) was melted in a glass flask in the required amount of 1 x TAE or 1 x TBE gel running buffer (Sambrook, Fritsh, *et. al.* 1989) in a microwave on high setting. The molten agarose was allowed to cool to approximately 50^0C before adding ethidium bromide to a final concentration of 0.5 μg/ml and swirling gently to mix. Gels were poured on a level platform and left at room temperature until completely set. Partially set gels were transferred to the fridge to speed up the setting process if required. Type III gel loading buffer (used at 1 x concentration) was added to aqueous DNA samples prior to loading and gels were run in the presence of 1 x TAE or TBE using a powerpack to provide a constant voltage supply across the electrodes.

When separation of very small amounts (less than 50 ng) of DNA was required, electrophoresis using gels prepared on glass microscope slides was performed. Agarose was prepared in the same way as for larger gels and a mini-gel comb was suspended over the short axis of the glass slide to create 4 - 5 wells. The gel was poured by carefully dispensing molten agarose onto the glass slide using a 5 ml micropipetter until the whole surface was covered and a maximum surface tension was reached. The gel was left to set and then electrophoresis carried out in the normal way. Small volumes of DNA (up to 5 μl including loading buffer) could be separated in this way.

Polyacrylamide gel electrophoresis (PAGE) of DNA

PAGE was used in cases where high resolution of DNA fragments was required e.g. for

detection of low concentrations of PCR products. Acrylamide gels (8 % w/v) were prepared using 37.5:1 acrylamide:bis-acrylamide, 1 x TBE, 0.08 % (w/v) ammonium persulphate and 30 µl N,N,N'N'-Tetramethylethylenediamine). The wells were washed out in 1 x TBE using a syringe (Hamilton) and up to 100 µl of DNA sample was loaded per well. The gel was electrophoresed at 30 mA in 1 x TBE in a protein gel electrophoresis system (Protean^{TM} II, Biorad) connected to a cooling water supply. Gels were stained in 250 ml 1 x TBE containing 10 µg/ml ethidium bromide for 30 min, destained in 1 x TBE for 10 min and visualised using UV transillumination.

Agarose gel electrophoresis of RNA

Electrophoresis apparatus was treated for elimination of RNases as described in Section 2.6. RNA agarose gels contained 1.2 % (w/v) agarose, 1 x MOPS/EDTA buffer (20 mM 3-(N-morpholino) propane sulfonic acid (MOPS), 1 mM EDTA, 5 mM sodium acetate, pH 7.0). Each RNA sample contained up to 30 µg RNA (suspended in RNase-free H_2O), 6 % (v/v) formaldehyde, 1 x MOPS/EDTA buffer and 50 % (v/v) formamide. Ethidium bromide was added to a final concentration of 400 µg/ml and the samples heated at 65^0C for 10 min. Following a pulse centrifugation to collect any condensate, 1 µl of RNA gel loading buffer (50 % (v/v) glycerol, 0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol, 1 mM EDTA, pH 8.0) was added to each sample. The sample wells were washed out with 1 x MOPS/EDTA buffer prior to loading the samples, and the gel was electrophoresed at 3 V/cm in the same buffer with constant re-circulation using a peristaltic pump.

Molecular weight markers for gel electrophoresis

Up to 1 µg per well of the 123 bp ladder (Life Technologies$_{TM}$) or Lambda *Eco*R1/*Hind* III markers (Promega) were used for DNA gel electrophoresis. Up to 5 µg per well of

the 0.24 - 9.5 kb RNA Ladder, (Life Technologies$_{TM}$) was used for RNA gel electrophoresis.

Visualisation and photography

DNA and RNA gels were visualised on a UV transilluminator (Stratagene). Permanent records of gels were made on black and white film using a Polaroid camera. Additionally, and where gel pictures would not be required for publication, gels were visualised and permanent records made using an EagleEye^{TM} II image analyser (Stratagene).

2.10 Isolation of DNA from agarose gels

Several different methods were used to isolate DNA from agarose gels (Section 2.9), depending upon the amount of DNA present and the availability of equipment and materials. In all cases DNA was excised in a minimal volume of agarose under UV transillumination using a sterile scalpel blade. The methods used are described in detail below.

Electroelution

A 4 - 5 inch portion of 1 inch wide dialysis tubing was cut using a sterile scalpel blade and washed thoroughly in sterile double-distilled water (ddH_2O) using a 5 ml micropipetter. One end of the tubing was folded twice and clipped securely with a mediclip ensuring a complete seal. The agarose gel slice containing the DNA was placed in the open end of the tubing and 1 ml of 1 x TAE added. After expelling any bubbles present by gently squeezing the tubing, the open end of the tubing was folded over and secured with another mediclip. The tubing was positioned in a mini-gel electrophoresis tank so that the slice was at the end of the dialysis tube furthest away from the positive

electrode. The tank was filled with 1 x TAE so that the tubing was just covered with buffer and the lid of the apparatus replaced. A power pack was connected and a constant 45 V applied across the electrodes for 2 h. This allowed the DNA to move out of the gel slice in to the buffer within the dialysis tubing. The electrodes were then reversed for exactly 15 s to ensure no DNA was sticking to the dialysis tubing. The tubing was removed from the gel tank and the DNA purified from the buffer as described in Section 2.7. In some cases, particularly when multiple elutions were required, DNA was purified using dedicated electroelution apparatus according to the manufacturer's instructions (Model 422 Electro-Eluter, Biorad).

Glassmilk extraction

DNA was purified using the 'Geneclean' glassmilk extraction kit according to the manufacturer's instructions (BIO101).

DE81 paper

DNA was separated on a agarose gel until the band of interest was clearly resolved from any other DNA bands. The gel was removed from the tank and placed on a UV transilluminator to visualise the bands. Two cuts were made with a sterile scalpel blade, one just above and one just below the band of interest right through the gel in the region sliced. DE81 paper (Whatman) rectangles were cut 5 mm greater than the width and 5 mm greater than its depth and inserted into the cuts in the gel with gloved hands. Electrophoresis was continued for approximately 15 min, or until the band could be seen to have migrated onto the paper below it when viewed under UV illumination. The paper below the well with the DNA of interest adhered to it was then removed and placed into a microfuge tube. 400 µl of Extraction Buffer (10 mM arginine, 1 M NaCl), was then added to the paper and the tube incubated at 65^0C for 1 h. The paper was then

transferred to a 0.5 ml microfuge tube with a small hole pierced in its base. This tube was placed within a larger 1.5 ml microfuge tube and centrifuged at 14,000 x g for 5 min in a microfuge. The paper pellet was discarded and the small volume of buffer which had been spun from the paper added to the buffer in the original incubation tube. The DNA was purified from the Extraction Buffer and any remaining paper using phenol/chloroform extraction and ethanol precipitation as described in Section 2.7.

Low melt agarose

DNA was separated on a gel prepared using low-melt agarose (Life Technologies$_{TM}$) and the gel slice containing the DNA placed in a microfuge tube with 5 vols of 1 mM EDTA, 20 mM Tris.Cl, pH 8.0. The gel was incubated at 65^0C for 5 min until the agarose had melted completely. The DNA was purified from the agarose at room temperature using phenol/chloroform extraction and ethanol precipitation as described in Section 2.7

2.11 Southern blotting analysis of DNA

Two different methods were adopted to transfer DNA from agarose gels onto nylon membrane (Hybond-N, (Amersham) or Zeta-Probe®GT (Genomic Tested), (Biorad) were used), both based on the capillary transfer method of (Southern 1975). The more recently published alkaline transfer method (method B, detailed below) was the method of choice since it was found to produce less background hybridisation and was faster.

A) Transfer in the presence of 20 x SSC

Following electrophoresis, the gel was photographed with a ruler to allow subsequent size estimation of hybridisation signals. The gel was then soaked for 30 min in denaturing solution (1.5 M NaCl, 0.5 M NaOH) in a sterile dish on a rotating platform to ensure even

coverage of solution. The denaturing solution was replaced with neutralisation solution (1.5 M NaCl, 1 M Tris.Cl, pH 8.0) and the gel soaked for a further 30 min. The DNA was then blotted overnight onto Hybond-N or Zeta-Probe® GT membrane in the presence of 20 x SSC (Sambrook, Fritsh, *et. al.* 1989) by capillary transfer assembled as previously described (Sambrook, Fritsh, *et. al.* 1989). The next day, the capillary blot was disassembled and the position of the wells marked onto the upper side of the membrane (the side not containing the DNA) with a soft pencil. The membrane was carefully removed from the gel, rinsed gently in 2 x SSC and left to air dry for 2 min. The DNA was then irreversibly bound to the membrane using a UV cross-linking device on 'Autocrosslink' setting according to the manufacturer's instructions (Stratalinker; Stratagene). Filters not used immediately for hybridisation were stored at room temperature between 2 pieces of filter paper.

B) Alkaline transfer method

DNA was separated by electrophoresis and a capillary transfer blot set up as described above. DNA was transferred overnight in the presence of 0.4 M NaOH (Reed and Mann 1988) and the DNA bound to the membrane by UV cross-linking as described above.

Hybridisation of southern blots

Detection of specific sequences of DNA bound to nylon membranes was carried out by hybridisation of the appropriate radiolabelled probes (See Section 2.8) to the membrane. All hybridisation was carried out using a minimal volume of fluid, (sufficient to cover the membrane at all times) in glass hybridisation tubes (Schott) in a hybridisation oven equipped with a tube rotation facility (Techne). The purified radiolabelled probe was boiled for 10 min before addition to the prehybridisation solution and hybridisation carried out (depending upon which nylon membrane was used) as described below.

A) Hybridisation of Hybond-N membranes

When Hybond-N was used, the filter was prehybridised for a minimum of 4 h at 42^0C in a solution containing 5 x SSC, 0.1 % (w/v) SDS, 50 % (v/v) formamide, 100 µg/ml salmon sperm DNA (denatured before addition by boiling for 10 min) and 5 x Denharts solution (Sambrook, Fritsh, *et. al.* 1989). Filters were washed initially in 1 x SSC, 0.1 % (w/v) SDS at room temperature for 30 min to remove excess unbound probe. Filters were then washed further to remove background and non-specific signals with increasing stringency by increasing the temperature and length of each wash and decreasing the concentration of SSC and SDS used. Filters were monitored between washes with a hand-held geiger counter to determine the level of stringency of each wash required for each particular experiment.

B) Hybridisation of Zeta-Probe membranes

When Zeta-Probe®GT (Genomic Tested) membrane was used, the formamide hybridisation protocol was followed according to the manufacturer's instructions (Biorad). Briefly, the filter was prehybridised for 5 min at 43^0C in a solution containing 50 % (v/v) formamide, 0.25 M NaCl, 7 % (w/v) SDS, 0.12 M Na_2HPO_4, pH 7.2. Following prehybridisation radiolabelled probe was added at a concentration of 10 ng/ml and hybridisation carried out for approximately 18 h. Membranes were washed in the same way as Hybond-N membranes as described above.

Polyethyleneglycol (PEG) hybridisation of genomic DNA

In certain cases where extreme hybridisation sensitivity was required, for example for genomic southern analysis of maize DNA, a PEG hybridisation protocol was used. gDNA was transferred to Hybond-N nylon membrane from an agarose gel in the presence of 20 x SSC as described above. The filter was prehybridised in 10 % (w/v) PEG (MW 8,000,

Sigma), 5 mM EDTA, 0.6 x SSC, 100 μg/ml salmon sperm DNA (pre-boiled for 10 min), 7 % (w/v) SDS, 10 mM PO_4^{3-} for a minimum of 4 h at 60^0C. Hybridisation was carried out in a minimal volume of fluid using 100 ng probe for approximately 18 h at 60^0C. Filters were washed in 0.25 x SSC, 0.2 % (w/v) SDS for 2 x 5 min at room temperature followed by 1 h at 60^0C in 0.25 x SSC, 0.2 % (w/v) SDS. Autoradiography of the filter (as described below) was carried out to assess the amount of further washing required.

Screening of bacteriophage λ libraries by plaque hybridisation

Bacteriophage libraries (details of which can be found in Chapter 3) were screened for full length DNA sequences identical or homologous to the probe DNA using plaque hybridisation. Bacteriophage were plated at high density (up to 30,000 pfu per 150 mm diameter plate, but ensuring individual plaques could still be discerned) as described in Section 2.4 and plates cooled for several hours at 4^0C. Phage DNA was transferred onto circular nylon membranes (Hybond-N, 0.45 μm, Amersham) and plaques of interest located by hybridisation of the probe to the membrane.

For DNA transfer, each filter was labelled with a soft lead pencil and carefully lowered onto the surface of the plate using sterile forceps avoiding air bubbles between the plate and filter. Sets of asymmetrical orientation marks were made through the membrane and agarose/agar layers using an 18-gauge needle. The plate was held up to the light and marks made in the same position on the underside of the plate with a permanent pen. After 30 - 60 s of contact with the plate surface the filter was removed and placed 'DNA side-up' on filter paper soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 1 - 5 min. The filter was then transferred to neutralisation solution (1.5 M NaCl, 0.5 M Tris.Cl, pH 7.2) for 5 min. Meanwhile, a second duplicate filter was placed on the agarose surface and orientation marks made using a needle through the filter and

agarose/agar layers corresponding exactly to the position of the pen marks made on the underside of the plate. This duplicate filter was left on the agarose surface for up to 5 min. The second filter was denatured and neutralised in the same way as the first. After neutralisation, filters were rinsed briefly and gently in 2 x SSC, air dried and the DNA fixed to the membrane by UV cross-linking as described above. Hybridisation and washing of membranes was carried out as described above for northern and southern analysis.

Bacterial colony hybridisation

Detection of transformants among large numbers of bacterial colonies following transformation experiments was carried out using colony hybridisation as previously described (Sambrook, Fritsh, *et. al.* 1989). DNA transfer from bacterial colonies to circular nylon membranes, hybridisation and washing of membranes was carried out in the same way as for bacteriophage plaques as described above.

Autoradiography

Autoradiography of filters probed with radiolabelled DNA was carried out using X-ray film as previously described (Sambrook, Fritsh, *et. al.* 1989). All films were exposed at -70^0C with intensifying screens except when sequencing gels using ^{35}S were used when films were exposed at room temperature and no intensifying screens were used. Films were developed using an X-OGraph Compact X2 X-ray film developer (Kodak).

2.12 Northern blotting analysis of RNA

Transfer of RNA to nylon membrane

RNA was transferred to nylon membrane using the 20 x SSC transfer method, or by a modification of the alkaline transfer method using 5 x SSC, 10 mM NaOH as previously

described (Low and Rausch 1994). When the latter method was used, the transfer time was increased to 2 h and RNA bound to the membrane in the same way as for DNA using a UV cross-linking device.

Hybridisation of northern blots

Hybridisation, subsequent washing and autoradiography of membranes was carried out in the same way as for DNA described in Section 2.11 above depending on whether Hybond-N or Zeta-Probe membrane was used.

Staining of RNA markers

RNA markers which had been immobilised on membrane were stained by soaking the membrane in 5 % (v/v) acetic acid for 15 min then soaking in 0.04 % methylene blue, 0.5 M sodium acetate, pH 5.2 for 5 - 10 min. The dye solution was rinsed off in dH_2O for 5 - 10 min until the RNA markers bands (now stained blue) became visible against the background of the membrane. The stained membrane was dried, wrapped in clingfilm and photocopied for a permanent record.

2.13 Plant growth and treatments

Growth of* Zea mays *(maize) plants for genomic DNA extraction

Maize seed were sown in plastic seed trays containing coconut peat and grown under ambient (standard seasonal day/night) conditions in a greenhouse. Shoots were harvested at 14 days post-germination into liquid nitrogen and used immediately or stored at - 70^0C.

Growth of* Z. mays *plants for RNA extraction

Maize plants used for RNA extraction (Section 2.7) were grown in seed trays containing vermiculite and watered only with deionised dH_2O (except when treated for cyt P450 induction as described below). Conditions for growth were either the same as those used

for gDNA extraction or under controlled conditions of 16 h day (25^0C), 8 h night (20^0C) where indicated in the results. Plants grown for subsequent herbicide treatment as described below were grown separately in 4 inch pots (1 plant per pot) in Rothamsted Prescription Mix soil (Petersfield Products).

Growth of* Arabidopsis thaliana *plants for RNA extraction

Arabidopsis thaliana (Var. Landsberg) plants for RNA extraction purposes were grown in seed trays in Rothamsted Prescription Mix soil under glasshouse conditions. Plant tissue, including leaf, stem, root, siliques, rosette leaf and flowers was harvested 38 days after germination (when plants were in full flower) into liquid nitrogen and stored at -70^0C until required for RNA extraction.

Application of herbicides, safeners and other cyt P450 inducers to* Z. mays *plants

Maize plants for herbicide and/or naphthalic anhydride treatment were grown under controlled conditions (16 h day (25^0C), 8 h night (20^0C)) for 15 days prior to treatment with herbicide. Naphthalic anhydride was ~~used~~ ~~was~~ applied as a pre-germination treatment by shaking the seeds with the powder at a concentration of 10 g/kg seed for approximately 10 min prior to sowing. The herbicide Chlorsulfuron (2-chloro-N-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)aminocaronyl]benzenesulfonamide) was applied at a concentration of 30 g active ingredient per hectare using a laboratory sprayer delivering circa 165 litres of water per hectare at 210 kilopascals through a single 'Lurmark' 01/F110 flat fan nozzle. The sprayer was used at a speed of 0.4 metres per second and a pressure of 30 p.s.i and the spray nozzle set at a height of 50 cm from the tip of the plants. Following herbicide application, plants were returned to the glasshouse until leaf tissue was harvested at various post-application time intervals as indicated in the results. Tissue was harvested into liquid nitrogen and stored at -70^0C.

Application of ethanol to Z. mays plants

Maize tissues were examined for cyt P450 induction by first germinating the seeds in vermiculite, watering for 5 days with dH_2O, followed by 5 days post-germination watering with 10 % (v/v) ethanol prior to harvesting. After RNA extraction, northern blotting was carried out as described in Section 2.12.

2.14 Microsome preparation and assay

Preparation of avocado microsomes

Avocado (*Persea americana* cv. Hass) fruits were purchased locally and ripened at room temperature. Microsomes were prepared from 500 g batches of mesocarp tissue as previously described (O'Keefe and Leto 1989).

Preparation of yeast microsomes

Yeast cells were cultured for microsome preparation at 30^0C in SD medium (minus uracil) either in flasks with shaking at 250 rpm, or, when a large concentration of microsomes was required, 5 litre cultures in SD (minus uracil), 0.1 % antifoam A (Sigma) were prepared using a LH Series 210 fermenter (Inceltech). Growth of cells was also monitored over time by counting of cells in a haemocytometer under a microscope. Cells were sonicated (0.5 inch probe, 140 W; Soniprep 150, MSE) for approximately 60 s prior to counting since yeast cells often clump, a factor which can give misleading OD_{600} readings. The correlation between OD_{600} and the number of cells/ml was calculated in order to accurately monitor cell growth stage each time a large scale culture was prepared to consistently achieve optimal cell lysis. If not used immediately for microsome preparation, cells were isolated by centrifugation at 4,500 x g for 10 min at $4\ ^0C$, washed with sterile dH_2O, re-centrifuged and stored in 15 % (v/v) glycerol at -70^0C. Two

different methods used to prepare yeast microsomes are described below.

A) Lytic enzyme method

The method of (Guengerich, Brian, *et. al.* 1991) was used to prepare yeast microsomes. Yeast lytic enzyme from *Arthrobacter luteus* was initially obtained from ICN Biomedicals Inc (Cat. No. 152270) In later experiments, a different yeast lytic enzyme (ICN, Cat. No. 360942) was used since it was found to be of equal quality and performance to the former which is habitually used by researchers but is much more expensive. Yeast cells grown for approximately 24 h to an OD_{600} of 1.6 - 1.8 which is optimal for cell lysis. This stage appeared to correlate with the late-log growth phase (5×10^7 - 2×10^8 cells/ml). Cells were centrifuged at 4,500 x g for 10 min and resuspended in a solution containing 2 M sorbitol, 0.1 mM DTT, 0.1 mM EDTA, 0.4 mM phenylmethylsulphonylfluoride (PMSF), 10 mM Tris.Cl, pH 7.5 (10 ml resuspension buffer per litre original culture) in sterile 50 ml polypropylene tubes. Yeast lytic enzyme was added to a final concentration of 5 mg/ml and the cells incubated at 30^0C with gentle shaking for 1 h. Cells were centrifuged at 120 x g for 10 min at 4^0C to pellet the spheroplasts, resuspended in ice-cold 0.65 M sorbitol, 0.1 mM DTT, 0.1 mM EDTA, 0.4 mM PMSF, 10 mM Tris.Cl, pH 7.5, (10 ml per litre original culture) and re-centrifuged as before. Washed spheroplasts were resuspended in ice-cold 0.65 M sorbitol, 0.1 mM DTT, 0.1 mM EDTA, 0.4 mM PMSF, 10 mM Tris.Cl, pH 7.5 (30 ml per litre original culture) and lysed by sonication on ice in 50 ml polypropylene tubes with 6 x 15 s pulses using a 0.5 inch probe at 140 watts. Intervals of 15 s were left between pulses to prevent overheating. Lysis of cells was monitored by placing a drop of sonicated cell solution on a glass slide and viewing under a microscope at 100 x magnification under oil-immersion. When over 95 % of cells were lysed, the spheroplasts were pelleted by centrifugation at 10,000 x g for 30 min

in sterile 30 ml Corex® tubes. The supernatant was recovered and microsomes pelleted at 100,000 x g for 1 h at 4^0C. The microsomal pellet was resuspended very carefully on ice in a minimal volume (routinely 0.5 ml per litre original culture) in 20 % (v/v) glycerol, 1 mM EDTA, 10 mM Tris.Cl, pH 7.5 and stored in liquid nitrogen.

B) Glass bead method

A 50 ml starter culture of yeast in SD (minus uracil) medium was grown at 30^0C for 24 h with shaking (250 rpm). This was used to inoculate 250 ml of SD (minus uracil) medium and cells were grown under the same conditions for a further 24 h until a high cell density was reached. Cells were harvested by centrifugation at 4,500 x g for 10 min at 4 ^{0}C. The cell pellet was resuspended in 40 ml Extraction Buffer (1 mM EDTA, 0.6 M sorbitol, 50 mM Tris.Cl, pH 7.5) and centrifuged in 50 ml polypropylene tubes as before. Cells were resuspended in a further 40 ml of Extraction Buffer containing 10 mM β-mercaptoethanol and left to stand for exactly 10 min at room temperature. Cells were centrifuged under the same conditions and the cell pellet resuspended in an equivalent volume of Extraction buffer to yield a thick mixture. Glass beads were added until only the meniscus of the liquid was visible and cells and beads shaken manually for 5 - 10 min, placing on ice occasionally to prevent over-heating. Cell breakage was verified under a microscope and once breakage was optimal, cells were filtered through Miracloth into a 50 ml polypropylene centrifuge tube on ice. The beads were washed in three times in 10 ml Extraction Buffer and washes combined in the centrifuge tube. Cells were centrifuged at 20,000 x g for 20 min at 4^0C and the resulting supernatant was centrifuged at 100,000 x g for 1 h at 4^0C. The microsomal pellet was resuspended and stored as described above.

Cyt P450 difference spectroscopy

Microsomal cyt P450 concentration was estimated from the carbon monoxide (CO) absorption difference spectrum (Omura and Sato 1964) using a Cary 3 spectrophotometer. Microsomes (approximately 2 mg protein) were suspended in 20 % (v/v) glycerol, 1 mM EDTA, 50 mM potassium phosphate, pH 7.0, to give a total volume of 1 ml and a few grains of sodium dithionite added and mixed by inversion. The microsomal suspension was split between two matched 0.5 ml quartz cuvettes and a baseline recorded between 400 and 500 nm. The sample cuvette was removed and CO bubbled through the solution for 60 s at a rate of approximately 1 bubble per s (to reduce frothing) using a syringe needle. The spectrum was recorded between 400 and 500 nm and cyt P450 concentration calculated using an extinction coefficient of 91 $mM^{-1}cm^{-1}$ (Omura and Sato 1964).

Estimation of microsomal protein content

Microsomal protein concentration was estimated using a modified Lowry procedure (Markwell, Haas, *et. al.* 1978).

Western blotting

Denaturing PAGE was carried out using 7.5 to 15 % (w/v) acrylamide gradient gels in the presence of SDS as previously described (Laemmli 1970). Microsomal protein (50 μg) was loaded into each lane against pre-stained protein molecular weight markers (Sigma). Western blotting of separated proteins onto Immobilon PVDF (Millipore) was performed as previously described (Towbin, Staehelin, *et. al.* 1979) using the Trans-Blot^{TM} Cell blotting apparatus (Biorad). Antisera, (the polyclonal Anti-ARP 1 (O'Keefe and Leto 1989) and a monoclonal raised to rat liver cyt P-448, (Form C) (Boobis, McQuade, *et. al.* 1985)) were used at a dilution of 1:4000. Visualisation of antigen was achieved using biotinylated secondary antibody (Sigma) in combination and ECL (Amersham). The

polyclonal antibody was a kind gift from Dr D.P.O'Keefe, Dupont, USA and the monoclonal a kind gift from Dr. A. Dismore, Zeneca Pharmaceuticals.

Assay for NADPH cyt P450 reductase (CPR) activity

NADPH - Ferricytochrome P450 reductase was assayed according to previously published methods (Masters 1976; Fujita and Asahi 1985).

Substrate screening using optical difference spectroscopy

Binding of potential substrates to cyt P450 expressed in yeast microsomal preparations was monitored by difference spectroscopy (Jefcoate 1978) using a Cary 3 spectrophotometer. Microsomes (approximately 10.5 mg protein) were suspended in 20 % (v/v) glycerol, 1 mM EDTA, 50 mM potassium phosphate, pH 7.0, to give a total volume of 2 ml and divided between 2 matched 1 ml quartz cuvettes. A baseline was recorded between 400 and 500 nm and substrate (dissolved in dimethylsulphoxide (DMSO)) was then added to the sample cuvette and an equal volume of DMSO was added to the reference cuvette. The spectrum was recorded again between 400 and 500 nm to assess the effect of adding the compound to the microsomes.

Measurement of ethoxycoumarin O-deethylase (ECOD) activity

NADPH-dependent ECOD activity of yeast microsomes was assayed in the presence of 250 μM of the substrate 7-ethoxycoumarin (7-EC) as previously described (Werck-Reichhart, Gabriac, *et. al.* 1990). A Perkin Elmer LS-5B luminescence spectrometer was used to directly record the change in fluorescence at 460 nm as a function of time, using an excitation wavelength of 380 nm. Cumene hydroperoxide-dependent ECOD activity was measured in the same way, except 1μl 5.4 M cumene hydroperoxide (CH) (Sigma, 80 % in cumene) was added to start the reaction and NADPH, glucose 6-phosphate (G6-P) and glucose 6-phosphate dehydrogenase (G6-PDH;

(Boehringer grade II) were omitted. The assay was calibrated using known amounts (0 - 50 pmol) of product 7-hydroxycoumarin (umbelliferone) in the assay mixture. ECOD activity was recorded using a chart recorder.

Measurement of ethoxyresorufin O-deethylase (EROD) activity

NADPH-dependent EROD activity was assayed as previously described (Werck-Reichhart, Gabriac, *et. al.* 1990). The fluorescence change at 585 nm was recorded as a function of time, using an excitation wavelength of 530 nm. Cumene hydroperoxide-dependent EROD activity was measured in the same way as cumene-dependent ECOD activity described above. The assay was calibrated using known amounts (0 - 50 pmol) of the product resorufin.

Measurement of p-chloro-N-methylaniline (pCMA) demethylase activity

*p*CMA demethylase activity was assayed as previously described (Rifkind and Petchke 1981). *p*CMA was obtained from Sigma and recrystallised to the HCl salt before use. Microsomes (containing approximately 100 pmol cyt P450 as measured by CO difference spectroscopy) were added to a 1 ml assay mixture containing 10 µM G-6P, 0.15 U G6-PDH, 500 µM NADPH, 1 µM DTT, 100 µM magnesium acetate, 0.5 µM *p*CMA 0.1 M potassium phosphate, pH 7.5. The reaction was incubated at 30^0C for 1 h and terminated with 0.2 ml 30 % TCA. dH_2O (0.4 ml) was added and tubes placed on ice. A 'zero activity' control was included in the assay where 0.2 ml 30 % TCA was included prior to incubation of the reaction at 30^0C for 1 h. The reaction was centrifuged for 5 min at 14,000 x g to pellet the protein and 0.8 ml of the supernatant transferred to a fresh microfuge tube. 0.2 ml 1 M sodium acetate (pH 4.0) and 50 µl fluorescamine were added to this portion of the deproteinised solution. After 15 min at room temperature, the fluorescence of 0.5 ml of reaction mixture in 2.5 ml dH_2O was measured at 500 nm (with

an excitation wavelength of 410 nm) after adding it to 2.5 ml dH_2O. The assay was calibrated by adding known amounts of pCA (0 - 30 nmol) in a total volume of 750 µl dH_2O to 200 µl 1 M sodium acetate (pH 4.0) and 50 µl fluorescamine. After incubation at room temperature for 15 min the fluorescence of 0.5 ml (in a total volume of 3 ml) was assayed as described above.

Substrate screening by ECOD inhibition

In order to test whether compounds were possible substrates of a cyt P450 expressed in yeast microsomes, the ability to inhibit cumene hydroperoxide-dependent ECOD activity was investigated. An ECOD assay was set up as described above (omitting NADPH, G6P and G6PDH) and the reaction initiated by addition of 1 µl cumene hydroperoxide (5.4 M) as described above. Reactions were carried out at 30^0C in cuvettes (with stirring) and 2 µl of each potential substrate (dissolved in DMSO) was added by direct injection through the hole above the cuvette using a syringe (Hamilton) to give a final concentration of 250 µM. The sensitivity of ECOD activity to the addition of each compound was noted and recorded using a chart recorder as described above.

Chapter 3

Molecular Analysis of cyt P450 in Zea mays

3.1 Introduction

As discussed in Chapter 1, in addition to their involvement in the biosynthetic pathways of a wide variety of endogenous compounds, plant cyt P450s are also known to be involved in the detoxification of xenobiotics including herbicides (O'Keefe, Romesser, *et. al.* 1987). The first reaction attributed to a cyt P450 in plants was the N-demethylation of monuron, a substituted phenylurea herbicide, in cotton microsomes (Frear, Swanson, *et. al.* 1969).

The use of herbicides remains an important tool in modern crop production. These compounds are the most heavily used class of pesticides world wide and account for 46 % of the pesticides used, representing 11.7 billion dollars in 1993 (Barrett 1995). It is their selectivity, the ability to kill weeds whilst leaving the crop undamaged, which has made them invaluable in the war against weeds. There are various mechanisms of herbicide selectivity. These include reduced absorption, a less sensitive target site, or more efficient metabolism to an inactive compound in the crop compared to that in the weed plant (Devine, Duke, *et. al.* 1993). Cyt P450s are involved in the latter mechanism, that is they actively metabolise the herbicide (Barrett 1995), to a form with reduced phytotoxicity towards the crop, or to a form which can undergo subsequent hydroxylations and conjugations ~~with~~ leading to the ultimate detoxification of the herbicide (Devine, Duke, *et. al.* 1993). In most cases the target weed is unable to do this and therefore dies. There is a downside to this particular type of herbicide selectivity, in that certain weed biotypes have been identified which have acquired resistance to multiple herbicides, one

of the mechanisms for which is due to increased cyt P450 activity. This is the case with certain biotypes of *Lolium rigidum* (rigid ryegrass) (Burnet, Loveys, *et. al.* 1993) and *Alopecurus myosuroides* (blackgrass) (Gonneau, Pasquette, *et. al.* 1988). A number of crop plants have been shown to demonstrate herbicide metabolism attributed to cyt P450s. Reactions carried out by cyt P450 on herbicides include ring hydroxylations, alkyl hydroxylations, and N- and O- demethylations (Barrett 1995). Since the demonstration of cyt P450-mediated metabolism of bentazon (McFadden, Gronwald, *et. al.* 1990), an additional 13 herbicides, from six classes of herbicide chemistry, have been shown to be metabolised by cyt P450 in *Zea mays* (corn). These include the ring hydroxylation of the phenylurea, chlortoluron (Fonné-Pfister and Kreuz 1990), the sulfonylurea herbicides, chlorsulfuron (Moreland, Corbin, *et. al.* 1993) and primisulfuron (Fonné-Pfister, Gaudin, *et. al.* 1990), and the sulfonamide, flumetsulam (Frear, Swanson, *et. al.* 1993). In wheat, 12 herbicides, covering seven classes of herbicide chemistry, have been shown to be metabolised by cyt P450. These include the aryl hydroxylation of the arylphenoxypropanoate herbicide, diclofop (McFadden, Frear, *et. al.* 1989; Zimmerlin and Durst 1992) and the alkylhydroxylation and N-demethylation of chlortoluron (Mougin, Cabanne, *et. al.* 1990). It is now known that representatives from every major new group of herbicides introduced for weed management in *Z. mays* and wheat are metabolised by cyt P450 (Barrett 1995; Frear 1995). It is highly likely that the number of cyt P450s capable of herbicide metabolism in these crops is much higher since the numbers shown are limited by the number of analogues tested and only commercially useful herbicides are represented.

An important aspect of cyt P450-dependent herbicide metabolism is that it is very often inducible in the crop plant, i.e. levels of cyt P450 activity can be elevated in

response to certain chemical treatments. Classic treatments known to induce cyt P450s involved in herbicide metabolism include ethanol (Potter, Moreland, *et. al.* 1995), CGA 154281 (4-[dichloroacetyl]-3,4-dihydro-3-methyl-2H-1,4-benzoxazine), and 1,8- naphthalic anhydride (NA). The latter two compounds are herbicide 'safeners', which protect the crop by elevating levels of cyt P450 which metabolise a herbicide before its application and thus can form an important aspect of crop protection (Barrett 1995). Examples of cyt P450-mediated metabolism of herbicides induced by such safeners include the induction of triasulfuron (Persans and Schuler 1995) and imazethapyr (Barrett and Maxson 1991) metabolism in *Z. mays* by NA. Both CGA 155281 and NA also induce other enzymes involved in herbicide detoxification, the glutathione S-transferases (GSTs), as well as cyt P450s (Dean, Gronwald, *et. al.* 1990; Dean, Gronwald, *et. al.* 1991). GSTs acts by conjugating the parent herbicide, or a hydroxylated or demethylated intermediate, to glutathione (Devine, Duke, *et. al.* 1993). The metabolism of all of the herbicides mentioned above in wheat and *Z. mays* was shown to be higher in treated tissue. Although constitutive cyt P450 activity can be demonstrated for many of these herbicides in *Z. mays* for instance, the level of activity is generally too low to permit detailed experimentation.

It is possible that a few cyt P450s are capable of metabolising a wide range of different herbicide substrates. However, there is evidence to suggest that separate cyt P450 enzymes are responsible for detoxification of a single herbicide in non-induced and induced tissue, as is the case with detoxification of bentazon in *Z. mays* (Barrett 1995). There is some evidence to suggest that the plant cyt P450s which detoxify herbicides actually have a role in endogenous metabolism within the plant. An example of this is shown in wheat where hydroxylation of diclofop and lauric acid are thought to be

metabolised by the same cyt P450 isozyme (Zimmerlin and Durst 1992). This situation has not been seen in *Z. mays* however, where lauric acid hydroxylase, and also cinnamic acid hydroxylase, are separate cyt P450 isoforms not involved in herbicide metabolism in the crop plant (Barrett 1995). Despite, therefore, the wealth of knowledge about cyt P450 involvement in herbicide metabolism, conclusively ascribing a role in herbicide metabolism to a particular plant cyt P450 has proved difficult. This is due in part to the difficulties mentioned in Chapter 1, regarding cyt P450 purification. Molecular approaches are increasingly being adopted to attempt to isolate the cyt P450 genes responsible and produce the cyt P450 protein potentially involved in herbicide metabolism using gene expression techniques. Once such genes are isolated, strategies to engineer crop plants with increased herbicide resistance, one of the major goals in this area of molecular research, can be initiated and ultimately put in place in the field, with obvious commercial significance. Also, a greater understanding of cyt P450s which metabolise herbicides might lead to better strategies for the avoidance of herbicide resistance in weeds. As described above, certain methods can be employed and exploited to try and induce and ultimately isolate cyt P450s capable of herbicide metabolism. One particular example is the construction of cDNA libraries from plant tissue previously treated with cyt P450 inducers in an attempt to enrich with cDNAs for these particular cyt P450s which are presumably present in lower amounts in non-induced tissue. This chapter details an attempt to isolate and characterise cyt P450s from *Z. mays,* with emphasis on those responsible for herbicide metabolism in this important crop plant.

3.2 Results

3.2.1 Isolation of a cyt P450 fragment from Zea mays for cDNA library screening

Polymerase chain reaction (PCR)

When this work was initiated, the only plant cyt P450 cloned was CYP71A1 from *P. americana* (Bozak, Yu, *et. al.* 1990). Degenerate oligonucleotide primers were designed, designated primer A and primer C, encoding two of four conserved cyt P450 domains of CYP71A1. Primers A and C were based on the heme co-ordinating cysteine domain and the Helix I region (containing the conserved threonine) respectively, shown in Figure 3.1. The nucleotide and amino acid sequence of the two primers is shown below.

Primer A:

```
CGGGATCCCCTCCTACCTGCTCCAAA
          G  GC  A  A  G
             G
             T
```

Amino acid sequence: **FGAGRRG**

Primer C:

```
CCATCGATGTTCTCTGGTGGAACTGATAC
           TC    A  T  A  C
                    C
```

Amino acid sequence : **MFSGGTDT**

gDNA and root cDNA were a kind gift from Dr. Ian Jepson, Zeneca Agrochemicals. PCR was carried out on both gDNA and cDNA as described in Section 2.8. The root cDNA was prepared from five-day old *Z.mays* root tissue (variety SD10) which had been treated with the herbicide safener dichloromid for 24 h prior to harvesting. PCR reaction conditions were 35 cycles of 1 min at 94^0C, 1 min at 55^0C, and 2 min at 72^0C. A major

band of the expected size of *ca.* 450 bp was produced from PCR on gDNA. No bands were seen following PCR using the cDNA. Controls employed indicated that the product from PCR on gDNA was not an artefact since no bands were seen in the absence of gDNA using both primers. The PCR product was isolated using low melt agarose gel electrophoresis of the reaction products (Section 2.10) and ligated (Section 2.8) into pBluescript (Section 2.2) using a TA cloning strategy according to the manufacturer's instructions (Invitrogen). The resulting plasmid, designated pBSMZERG.450, was transformed (Section 2.8) into *E. coli* XL1Blue (Section 2.1). Manual sequencing (Section 2.8) of Wizard^{TM} minipreparation of plasmid DNA (Section 2.7) and sequence analysis using GCG software revealed that the product was a cyt P450 fragment, 424 bp in size (Figure 3.2). The deduced amino sequence had homology to other plant cyt P450s (Figure 3.3), including the CYP71A1 cyt P450 from *P.americana* (Bozak, Yu, *et. al.* 1990) (56.4 % identity, 124 amino acid overlap), CYP76A2 from *S. melongena* (Toguri, Kobayashi, *et. al.* 1993) (46.8 % identity, 124 amino acid overlap, CYP71A8 from *Mentha piperata* (Kang and Choi, unpublished) (47.2 % identity, 123 amino acid overlap) and CYP76A1 from *S. melongena* (Toguri, Kobayashi, *et. al.* 1993) (48.4 % identity, 124 amino acid overlap).

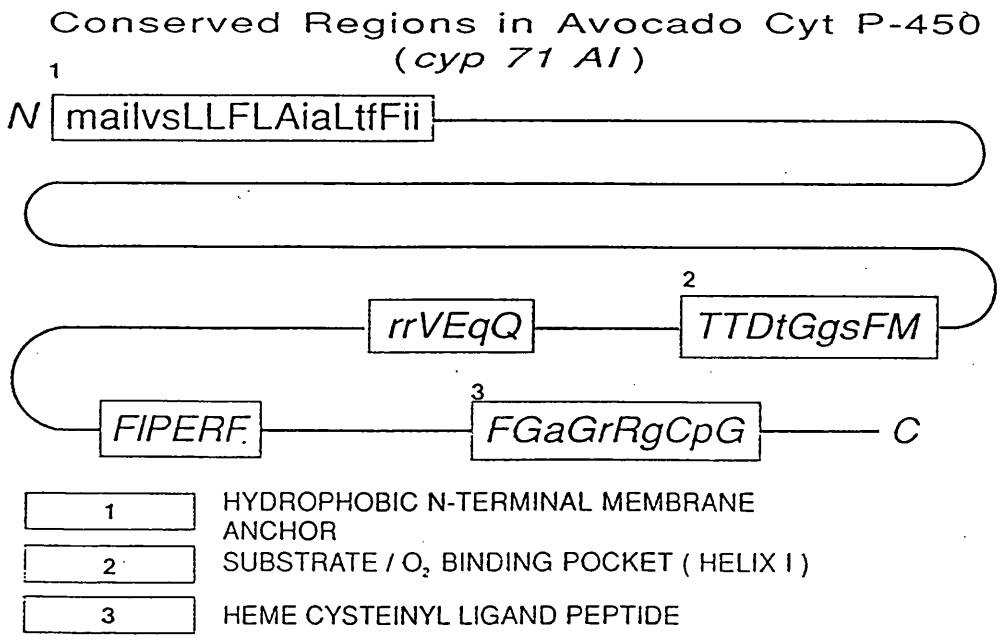


Figure 3.1 ***Schematic diagram showing conserved regions of the P. americana mesocarp CYP71A1 cyt P450***

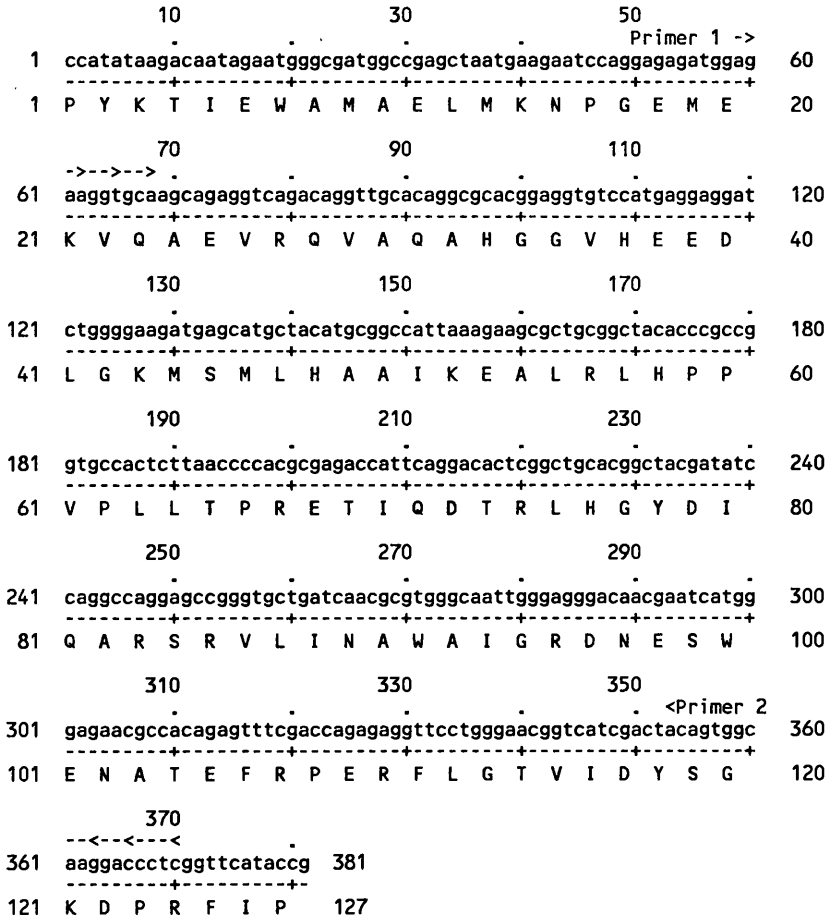


Figure 3.2 Nucleotide and deduced amino acid sequence of MZERG.450

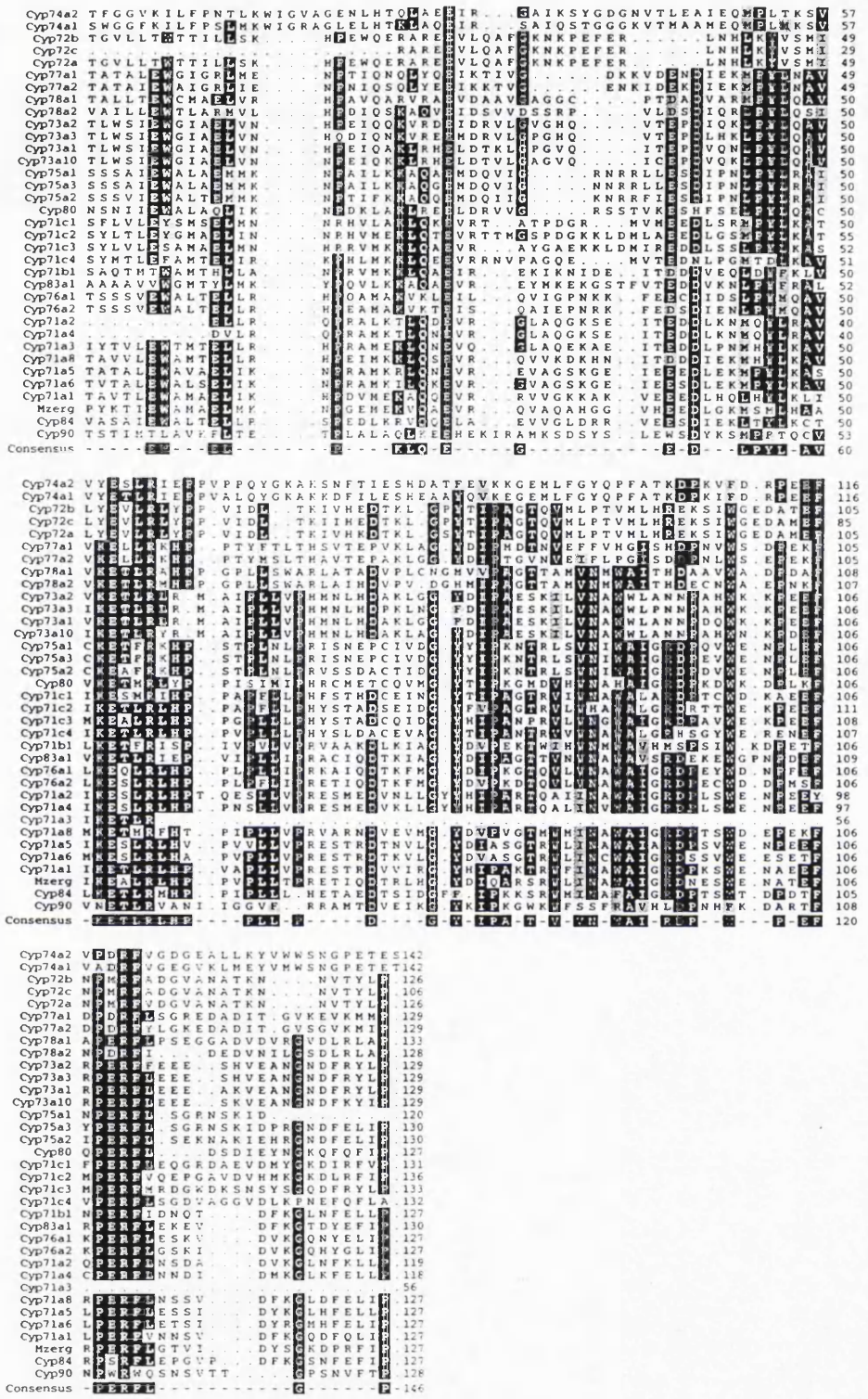


Figure 3.3 ***A comparison of the amino acid sequence of MZERG.450 to other plant cyt P450 sequences.***

Black shaded amino acids indicate identical amino acids between sequences, grey shaded areas indicate similar amino acids between sequences

3.2.2 Screening of Z. mays *cDNA libraries using MZERG.450*

The MZERG.450 insert was gel-purified (Section 2.10) from pBSMZERG.450 for use as a probe for library screening carried out as described in Section 2.11. The MZERG.450 probe was used to screen three different *Z. mays* cDNA libraries to isolate the full-length cDNA for MZERG.450 and also possible *Z. mays* cyt P450 homologues to the MZERG.450 sequence.

The first library was prepared in the vector λZAP II (Section 2.2) according to the manufacturer's instructions (Stratagene) by Dr. I. Jepson at Zeneca using the original dichloromid-treated five-day old root cDNA described above. A preliminary primary screen of *ca.* 300,000 plaques by I. Jepson had produced eleven putative positives for cyt P450 sequences. The plaques were purified and passed to the author for secondary screening. Between *ca.* 300 and 2000 plaques were screened per putative positive depending on the titre of the original plaque isolate. This resulted in the identification of four putative secondary positives from the original eleven primary positives identified. A total of five plaques were isolated per master plate and the pBluescript phagemids carrying cDNA inserts were purified from the λZAP II vector using '*in vivo* excision' according to the manufacturer's instructions (Stratagene). Restriction digestion (Section 2.8) was carried out using the restriction enzyme *Eco*RI to release the cDNA inserts. These inserts were separated using gel electrophoresis and southern analysis was carried out (Section 2.11) using MZERG.450 as a probe. A positive control using 2 μg of pBSMZERG.450 previously digested using *Bam*H1 and *Eco*R1 to release the MZERG.450 insert and a negative control of *Eco*RI digested pBluescript vector DNA were also included. The putative cyt P450 pBluescript clones did not produce any hybridisation signals using MZERG.450 as a probe and hybridisation was only seen with the positive

control. This confirmed that the four secondary putative positive clones did not represent *Z.mays* cyt P450 cDNA sequences.

The original eleven primary positives were then plated for plaque amplification (Section 2.4) prior to a repeat round of secondary screening as before to further confirm whether these were in fact representative of cyt P450 clones. All eleven plaques failed to grow when plating was carried out at University College London (previous plating had been carried out at IACR Rothamsted). Rigorous investigation by testing batches of all the components of the top and bottom agar and agarose plating media, including the dH_2O, maltose, tryptone, NaCl, yeast extract, agar and agarose conclusively showed that the problem was due to the top agarose used. The top agarose for the original plating had contained a Gibco agarose product (Ultra pure range Cat No. 540-551OUA), whereas at UCL, Sigma agarose (Molecular Biology Reagent Cat. No A9539) was used in the top agarose layer. This Sigma agarose was found to completely inhibit phage growth. This was surprising considering the supposed high purity of the reagent and the general hardiness of phage. All subsequent plating was carried out using the Gibco agarose and no further problems of this nature occurred. The repeat secondary screening of the original putative positives clones did not produce any positive signals, further confirming that the original eleven putative positives were indeed not representative of phage recombinant for cyt P450.

The reason that the eleven phage isolates may have initially resulted in positive signals in the first of the secondary screens may have been due to non-specific binding of MZERG.450 resulting in false positive signals. In addition, the original primary screen had been carried out using single rather than duplicate filters, thus reducing the confidence that any signal from the master plate in fact represented a cyt P450 clone. All subsequent

screening was carried out using duplicate filters.

To further check that the original eleven putatives and the total root cDNA library did not contain cyt P450 clones, PCR was carried out on the eleven putative positives (representing *ca.* 1000 pfu) and aliquots of the total library containing 10^5 pfu. The degenerate primers A and C (500 pmol per reaction) were used in conjunction with the vector specific primers T3 or T7 (50 pmol per reaction). Prior to PCR, the library aliquots were heated to 80^0C for 10 min and phenol chloroform extracted. Reaction conditions were 1 cycle of 4 min at 93^0C, 35 cycles of 1 min at 93^0C, 2 min at 45 ^{0}C, 3 min at 72^0C, 1 cycle of 10 min at 72^0C. In addition, sense and antisense versions of a third degenerate primer, designated primer N, were designed based on a proline-rich region of the plant cyt P450 sequences available at that time corresponding to amino acid sequence LPPGP (Figure 3.4). It has been hypothesised that this region may be of critical importance in the correct positioning of the orientation of the cyt P450 protein with respect to the active site and the membrane (Szczesna-Skorupa, Straub, *et. al.* 1993). PCR was carried out on the eleven putative positives and the total library using primer N in conjunction with the vector primers T3 and T7 (Section 2.8) as described above. PCR on the library samples using these degenerate and vector-specific primers failed to produce any products of the expected size. It was concluded therefore that the putative positives were definitely not cyt P450 clones and in addition it was unlikely that further conventional screening of the library would yield a full-length cyt P450 clone since PCR on the total library had failed to amplify any cyt P450 fragments from the cDNA pool. The fact that both conventional screening of the root cDNA library and PCR on aliquots of the library did not produce any cyt P450 clones was not entirely unexpected. The original PCR on the same cDNA which was also used to prepare the library did not

produce a product, suggesting that this particular population of cDNAs did not contain any cyt P450s with conserved domains highly similar to primers A and C described above.

Species	*Amino acids*	*Nucleotide sequence*
P. americana	LPPSP	CTACCCCCTTCTCCT
Mung bean	LPPGP	CTCCCGCCGGGCCCA
Helianthus	LPPGP	CTCCCACCTGGCCCA
Petunia	LPPGP	CTACCGCCGGGGCCA
Alfalfa	LPPGP	CTCCCACCAGGTCCA

LPPGP CTACCICCIGGCCCA Sense
C G T
T

AGGGCCIGGIGGGAG
Antisense T C T
A

5' ==========> 3'

Figure 3.4 ***Design of a degenerate primer based on the proline rich region of several plant cyt P450s***

The nucleotide sequence of the proline rich region of five plant cyt P450s is shown with the corresponding amino acid sequence. The consensus was used to design the degenerate primer N in the sense and antisense format. I represents inosine.

Based on the root cDNA library screening and PCR results it was decided to proceed with screening a second *Z. mays* library provided by Zeneca. This had been prepared in λ ZAP II (Section 2.2) according to the manufacturer's instructions (Stratagene) by I. Jepson, using cDNA prepared from five-week old leaf tissue (*Z. mays* variety UE95). Despite screening *ca.* 220,000 plaques no positive signals were seen using MZERG.450 as a probe. Since mRNA transcripts coding for cyt P450s often are present in low abundance in plant tissues, it is likely that in a population of cDNAs in a library only a very small number of phage would be recombinant for cyt P450 cDNAs. It would therefore have been beneficial to have been able to screen a larger numbers of plaques from the leaf cDNA library to isolate a full-length cyt P450. However, in this case primary screening of more plaques was not possible due to the volume of library available and the extremely low titre of the library (10^5 pfu/ml) requiring large volumes (200 µl per plate to obtain 20,000 plaques per plate) to be plated each time. In addition, since the library had already been amplified once at Zeneca, further amplification to increase the numbers of phage for each clone would have been likely to cause selective or unequal amplification of the phage population resulting in a decreased likelihood of the library containing low abundance clones such as cyt P450s.

In addition, PCR carried out on aliquots of the library using both the degenerate primers A and C and N and vector specific primers as described above did not produce any products of the expected size. It was concluded therefore that neither the root or leaf cDNA libraries used contained clones identical or related to MZERG.450.

Prior to obtaining and screening a third cDNA library, genomic southern analysis, northern analysis and RT-PCR was carried out to gain more information regarding expression characteristics of MZERG.450 and its presence in the genome of *Z. mays*.

3.2.3 Genomic southern analysis

gDNA was isolated (Section 2.7) from 14-day old *Z. mays* plants grown as described in Section 2.13 and southern analysis was carried out (Section 2.11) to confirm the presence of MZERG.450 in the *Z. mays* genome. Initial genomic southern analysis using Hybond-N membrane hybridisation did not detect the presence of MZERG.450 in the genome. However, it was calculated that based on the large size of the *Z. mays* genome (1.5×10^9 bp), and the size of the cyt P450 target fragment (424 bp), the fraction of the genome being targeted for detection represented only 3 picograms of gDNA per 10 μg of restriction digested gDNA per lane. It was therefore decided that more sensitive hybridisation techniques were required and PEG southern analysis was carried out (Section 2.11). This method did reveal two distinct signals with *Eco*RI and *Hin*dIII digested gDNA (Figure 3.5), which represented between 2 and 10 picograms of the target DNA sample as compared to the positive control lanes (not shown). Unfortunately, size markers included could not be detected and the size of the fragment of gDNA which produced the positive signal could not be calculated. Despite extensive repetition using the existing gDNA and samples of freshly prepared gDNA this result could not be repeated. However, without a full-length version of MZERG.450 and the accompanying restriction map information, it would not be possible to gain an accurate estimate of the number of copies of the gene representing the full-length of the MZERG.450 fragment or the size of the stretch of gDNA the gene is located. This is because the presence of additional restriction sites for *Eco*RI and *Hin*dIII within the gene corresponding to MZERG.450 cannot be discounted, rendering any possible calculation of size invalid.

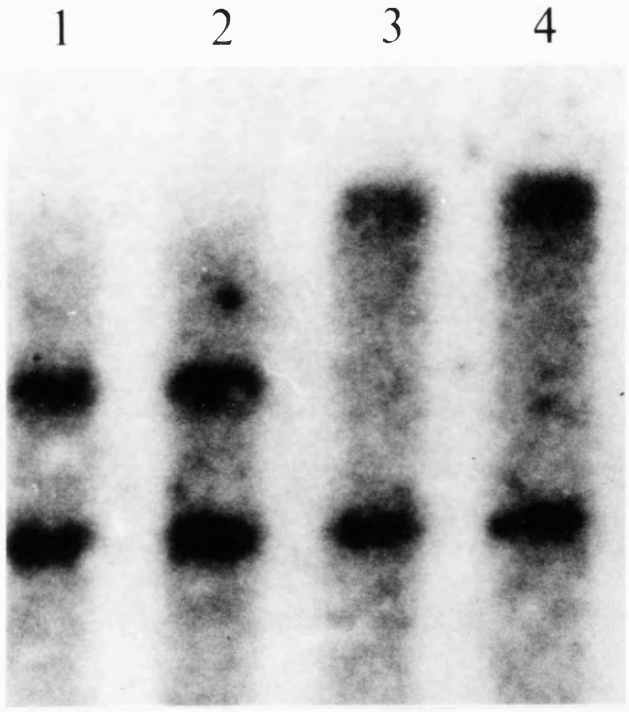


Figure 3.5 ***Southern analysis of* Z.mays *gDNA***

Total genomic DNA from Z. mays *shoot tissue was digested with restriction enzymes* EcoR*I (lanes 1 and 2) and* Hind*III (lanes 3 and 4) prior to 0.8 % agarose gel electrophoresis (10 µg/lane). The DNA was then transferred to a nylon membrane and hybridised to [α-^{32}P]dCTP labelled MZERG.450. Final wash conditions were 0.25 x SSC, 0.2 % SDS, 60°C, 1 hr.*

To further investigate the presence of MZERG.450 in the *Z. mays* genome, due to the fact that genomic southern analysis had proved difficult, PCR was repeated on new samples of gDNA prepared as described in Section 2.7. PCR was carried out using the degenerate primers A and C (100 pmol per reaction) and reaction conditions were 1 cycle of 2.5 min at 94 ^{0}C, 35 cycles of 1 min at 94 ^{0}C, 1 min at 55 ^{0}C, 2 min at 72^{0}C, 1 cycle of 10 min at 72^{0}C. The annealing temperature was increased to 55^{0}C compared to 45^{0}C used in the original PCR to increase the stringency of the reaction thus avoiding amplification of non-specific products to increase the likelihood that any reaction products represented fragments of the genome. This PCR yielded products of the same size as those amplified using the positive control of MZERG.450 insert only (Figure 3.6). This confirmed the presence of a similar or identical gene to MZERG.450 in the genome and further indicated that the original PCR product was not merely a PCR artefact.

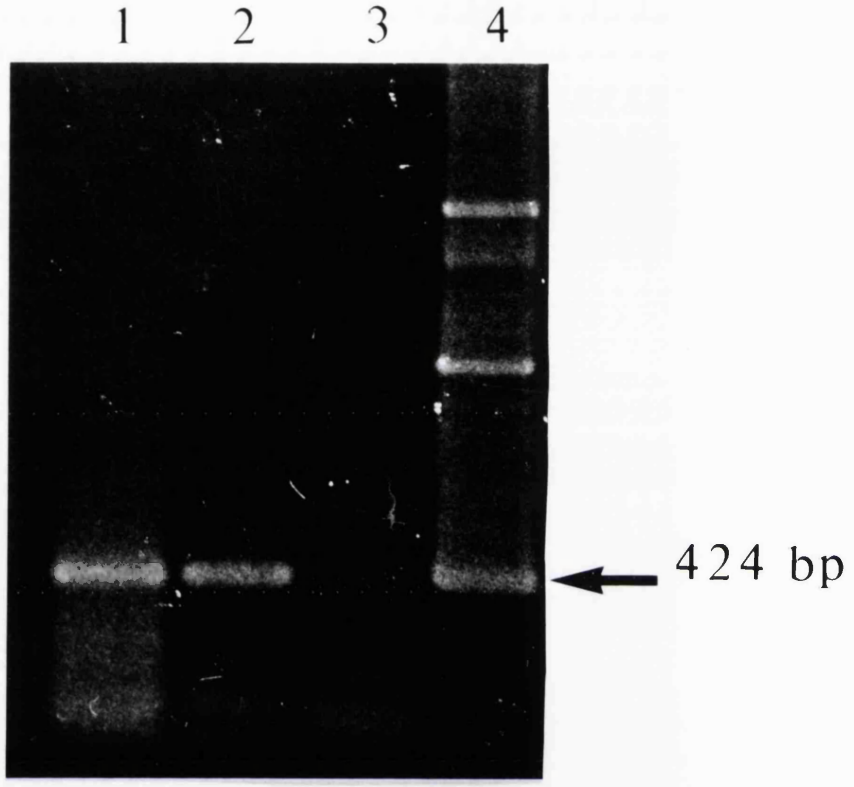


Figure 3.6 ***PCR amplification of* Z. mays *gDNA***

PCR was carried out on Z. mays *gDNA (1 µg) using primers A and C (100 pmol). PCR products were separated by electrophoresis (1 % agarose) and visualised by ethidium bromide staining. Lanes 1 and 2: gDNA; lane 3: no gDNA; lane 4: MZERG.450 (1 µg).*

3.2.4 Investigation of expression patterns of MZERG.450 using northern analysis and RT-PCR

A number of different RNA samples were prepared from *Z. mays* tissues in order to investigate the expression characteristics of MZERG.450 with respect to tissue type, tissue age, *Z. mays* variety and induction due to treatments known to induce cyt P450s involved in herbicide detoxification. Details regarding the RNA samples prepared are shown in Table 3.1 below and details regarding growth of plants, harvesting and treatment application can be found in Section 2.13.

Initial northern analysis using untreated 7-day old root and shoot RNA of *Z. mays* variety SD10 (samples 1 and 2, Table 3.1) did not produce any definite signal with MZERG.450 as a probe. However, northern analysis using RNA prepared from untreated UE95 tissue and RNA prepared from UE95 tissue treated with ethanol, naphthalic anhydride and ethanol and naphthalic anhydride in combination (samples 3 to 6 respectively) produced a signal of the expected size at *ca.* 1.6 kb (Figure 3.7). The intensity of each signal indicated that MZERG.450 was not an inducible cyt P450 with respect to the treatments with naphthalic anhydride and ethanol. A smaller band of *ca.* 1.2 kb remained present despite high stringency washing and it was unclear what this band might represent. Since subsequent attempts to repeat this northern analysis result proved unsuccessful, and because of this fact together with the difficulties experienced screening libraries with MZERG.450, it was decided to investigate expression of MZERG.450 using the more sensitive technique of RT-PCR (Section 2.8).

Table 3.1 *Details of RNA samples prepared from Z. mays tissue*

Tissue	Variety	Age	Inducer Treatment
1. Root	SD10	7	Untreated
2. Shoot	SD10	7	Untreated
3. Shoot	UE95	9	Untreated
4. Shoot	UE95	9	10 % Ethanol
5. Shoot	UE95	9	Naphthalic anhydride
6. Shoot	UE95	9	Naphthalic anhydride/ethanol
7. Shoot	UE95	2	Untreated
8. Shoot	UE95	5	Untreated
9. Shoot	UE95	8	Untreated
10. Shoot	B73	8	Untreated
11. Shoot	UE95	18	Untreated 3 day post spray control
12. Shoot	UE95	20	Untreated 5 day post spray control
13. Shoot	UE95	18	Chlorsulfuron 3 day post spray
14. Shoot	UE95	20	Chlorsulfuron 5 day post spray
15. Shoot	UE95	18	Chlorsulfuron/NA 3 day post spray
16. Shoot	UE95	20	Chlorsulfuron/NA 5 day post spray
17. Root	UE95	20	Chlorsulfuron/NA 5 day post spray

(NA = Naphthalic anhydride)

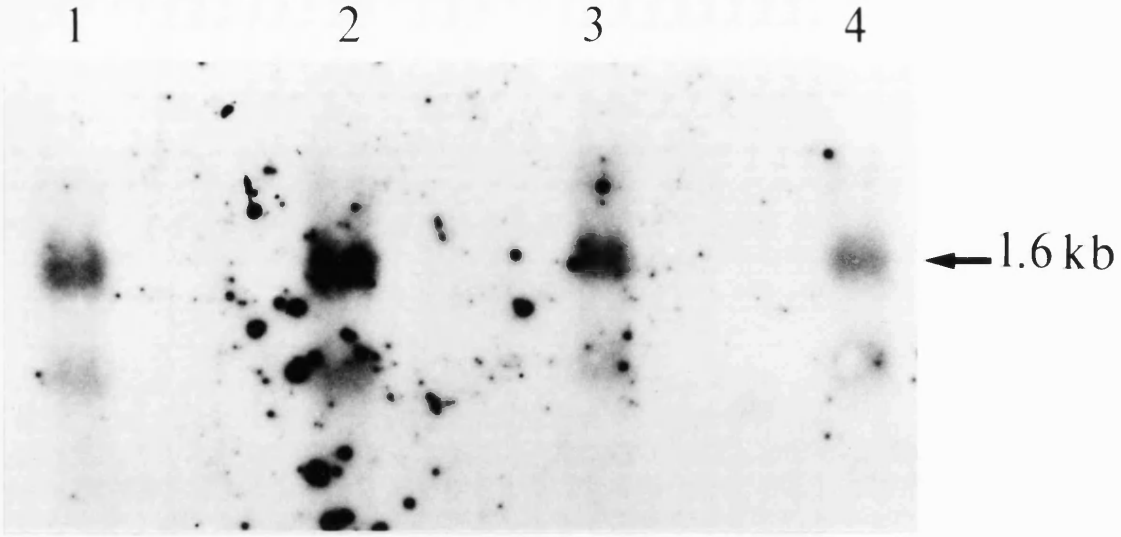


Figure 3.7 ***Northern analysis of*** Z.mays ***RNA***

RNA prepared from 9-day old UE95 Z.mays *shoot tissue was separated by electrophoresis (30 µg/lane), transferred to nylon membrane and hybridised to [α-^{32}P]dCTP labelled MZERG.450. The RNA samples were isolated from untreated tissue (lane 1), ethanol treated tissue (lane 2), naphthalic anhydride treated tissue (lane 4) and ethanol/naphthalic anhydride treated tissue. Final wash conditions were 0.1 x SSC, 0.1 % SDS,55^{0}C, 1 hr.*

RT-PCR was carried out using RNA prepared from untreated 9-day old UE95 tissue using degenerate primers A, C and N (100 pmol per reaction) and oligo dT (50 pmol per reaction), specific for the poly A tails of messenger RNA (mRNA). PCR reaction conditions were 1 cycle of 4 min at 94 ^{0}C, 35 cycles of 1 min at 94^{0}C, 1 min at 55^{0}C, 2 min at 72^{0}C and 1 cycle of 10 min at 72^{0}C. This yielded several amplified bands up to *ca.* 1 kb in size. No bands were seen in the negative control where only one primer, oligo dT, was used. A band of the expected size of 308 bp was seen using the positive control RNA and primers supplied with the RT-PCR kit (Figure 3.8 A). Southern analysis of PCR reactions using MZERG.450 as a probe indicated that the products amplified using primers A and C were identical or highly homologous to MZERG.450 (Figure 3.8 B) indicating that this cyt P450 was present in the untreated shoot tissue of *Z.mays* of variety UE95. None of the amplified fragments resulting from RT-PCR using primer A and oligo dT in combination hybridised to MZERG.450 in the southern analysis experiment. This would be expected since oligo dT and primer A are both antisense primers and therefore would not have been expected to amplify cyt P450 fragments homologous to MZERG.450.

Due to the presence of multiple bands in the RT-PCR using degenerate primers, more specific sense and antisense primers to MZERG.450 were designed :- 5'-GAGATGGAGAAGGTGCAAGC and 5'- GAACCGAGGGTCCTTGCCACTG (as shown previously in Figure 3.2). RT-PCR was carried out on RNA prepared from untreated 7-day old SD10 root and shoot tissue (*ca.* 4 μg RNA per reaction) using the MZERG.450-specific primers. This produced a major band of the expected size of 324 bp (Figure 3.9 A) which produced a strong signal on southern analysis (Figure 3.9 B). This confirmed that the cyt P450 represented by the MZERG.450 fragment, or a close

homologue, was present in 9-day old untreated shoot and root tissue of the variety SD10. The intensity of the hybridisation signal with the root RT-PCR product was lower than that seen with shoot RT-PCR product, despite the fact that on the original gel, there appeared to be a higher concentration of amplified shoot cDNA. This may suggest that MZERG.450 is more highly expressed in shoot tissue than in root tissue, however more detailed investigation, perhaps using quantitative PCR methods would be required to confirm this. RT-PCR conditions for this experiment were identical to those used with SD10 RNA except the annealing temperature for the primers was increased to 57 ^{0}C.

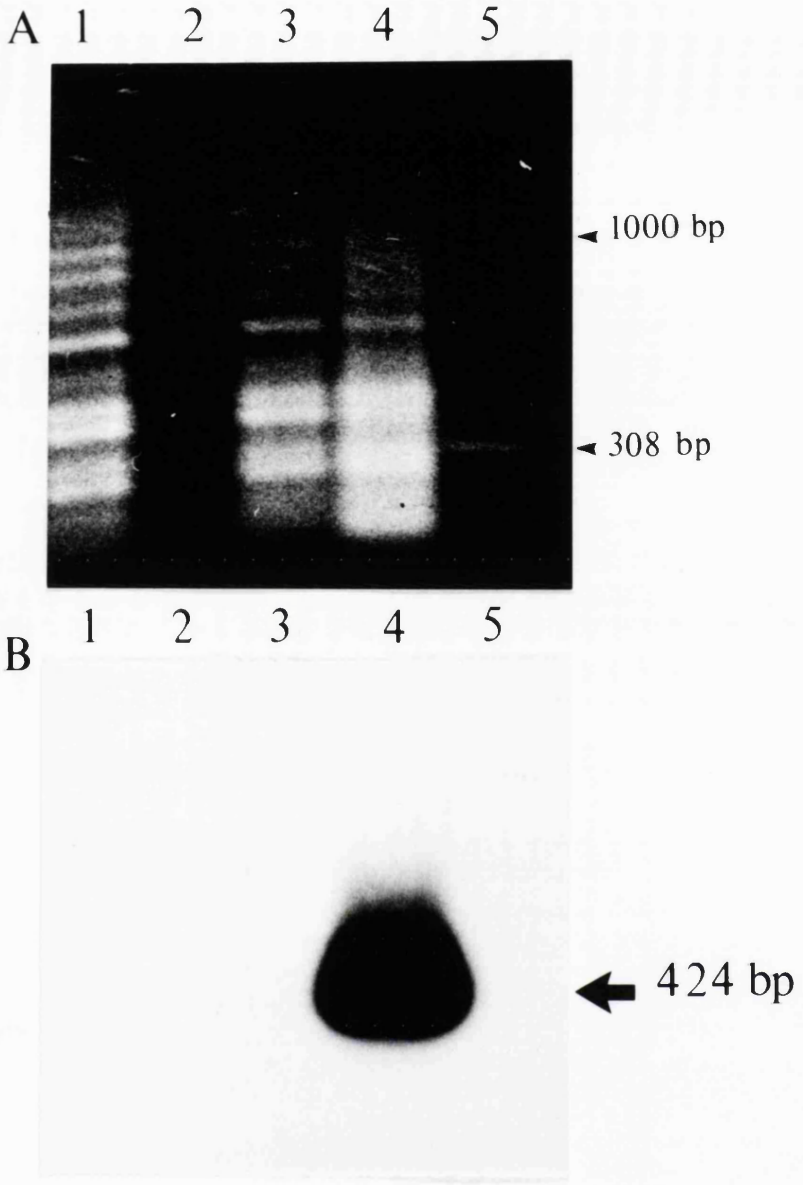


Figure 3.8 ***Amplification of a cyt P450 fragment from UE95 Z.mays using RT-PCR***

A) RT-PCR was carried out on RNA (1 µg) prepared from 9-day old untreated shoot tissue. RT-PCR products were separated by electrophoresis (1 % agarose) and visualised by ethidium bromide.
B) RT-PCR products were transferred to nylon membrane and hybridised to [α-^{32}P]dCTP labelled MZERG.450.

The primers used were A/oligo dT (lane 1); oligo dT alone (lane 2); N/A (lane 3); A/C (lane 4); RT-PCR kit control primers and RNA (lane 5). 100 pmol of degenerate primers and 50 pmol oligo dT primer were used per reaction. Southern analysis final wash conditions were 0.1 x SSC, 0.1 % SDS, 42^{0}C, 1 hr.

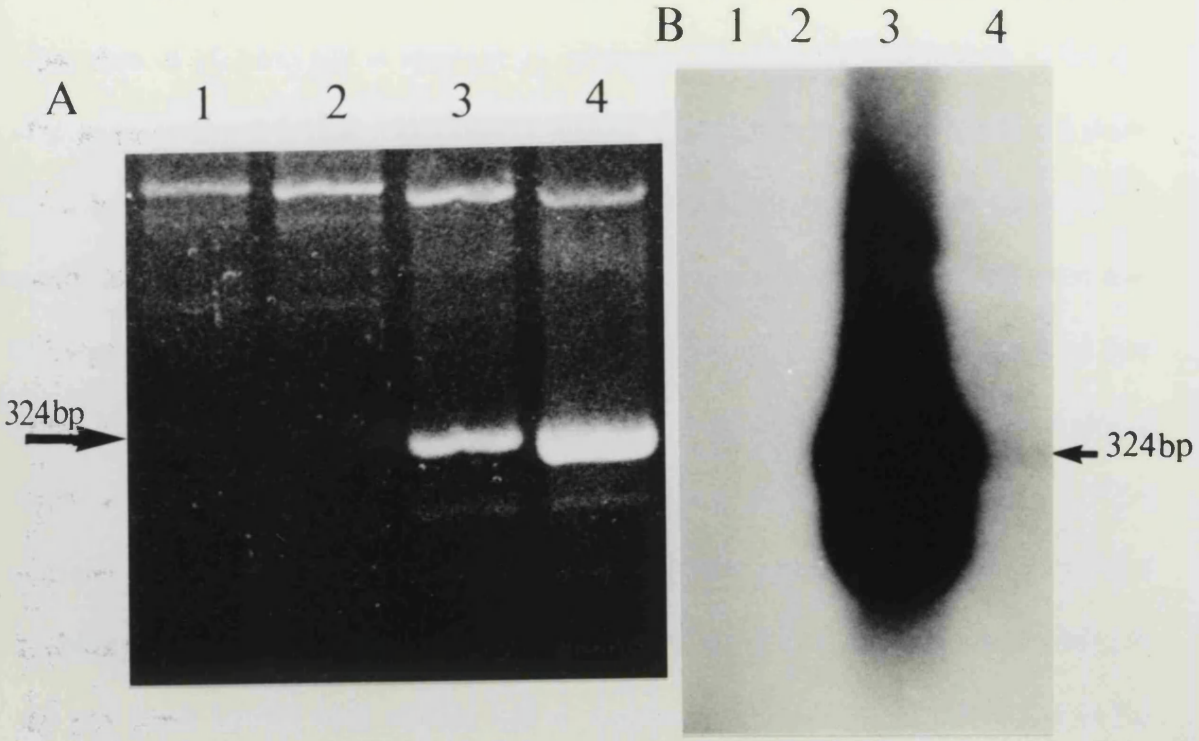


Figure 3.9 ***Amplification of a cyt P450 fragment from SD10 Z.mays using RT-PCR***

A) RT-PCR was carried out on RNA (5 µg) prepared from 7-day old untreated shoot and root tissue. RT-PCR products were separated by electrophoresis (1 % agarose) and visualised by ethidium bromide.
B) RT-PCR products were transferred to nylon membrane and hybridised to [α-^{32}P]dCTP labelled MZERG.450.

MZERG.450 specific primers were used (15 pmol per reaction) with root RNA - 1 primer control (lane 1); shoot RNA - 1 primer control (lane 2); root RNA (lane 3) shoot RNA (lane 4). Southern analysis final wash conditions were 0.1 x SSC, 0.1 % SDS, 42^{0}C, 1 hr.

The RT-PCR using the specific primers detailed above was extended to examine expression of MZERG.450 in response to inducer treatments and with respect to the age of *Z. mays* tissue. RT-PCR was therefore carried out on RNA prepared from UE95 shoot tissue treated with ethanol, naphthalic anhydride and ethanol and naphthalic anhydride in combination, RNA prepared from untreated 5-day old and 8-day old UE95 shoots and also RNA prepared from 8-day old B73 untreated shoot tissue. A band of the expected size of *ca.* 324 bp was seen with all samples (Figure 3.10 A), all of which produced a strong signal on southern analysis using MZERG.450 as a probe (Figure 3.10 B). This confirmed that a cyt P450 represented by MZERG.450 or a close homologue was present in shoot tissue of 2, 5 and 9-day old untreated UE95 and B73 *Z. mays* tissue and also 9-day old tissue treated with ethanol and/or naphthalic anhydride. No difference in the intensity of the signal was seen between untreated and treated tissue indicating, as suggested by the northern analysis using these RNA samples, that these treatments classically used to induce herbicide detoxifying cyt P450 had no induction effect on MZERG.450.

To confirm that the original PCR product could be isolated from cDNA, a separate RT-PCR experiment was carried out as described above using RNA prepared from untreated 2- and 9-day old UE95 shoot tissue and degenerate primers A and C (100 pmol per reaction). This resulted in products of the expected size of *ca.* 424 bp which were subsequently isolated from a PAGE gel (Section 2.9) and cloned into pCR-Script^{TM} and also pCR^{TM} II according to the manufacturer's instructions (Stratagene and Invitrogen respectively). Preliminary sequencing showed that these clones were identical to the original clone MZERG.450 which had been produced by PCR of gDNA on a separate occasion. This indicated that despite the fact that the original PCR on cDNA using primers A and C had not produced a product, MZERG.450 was expressed in *Z. mays* shoot tissue.

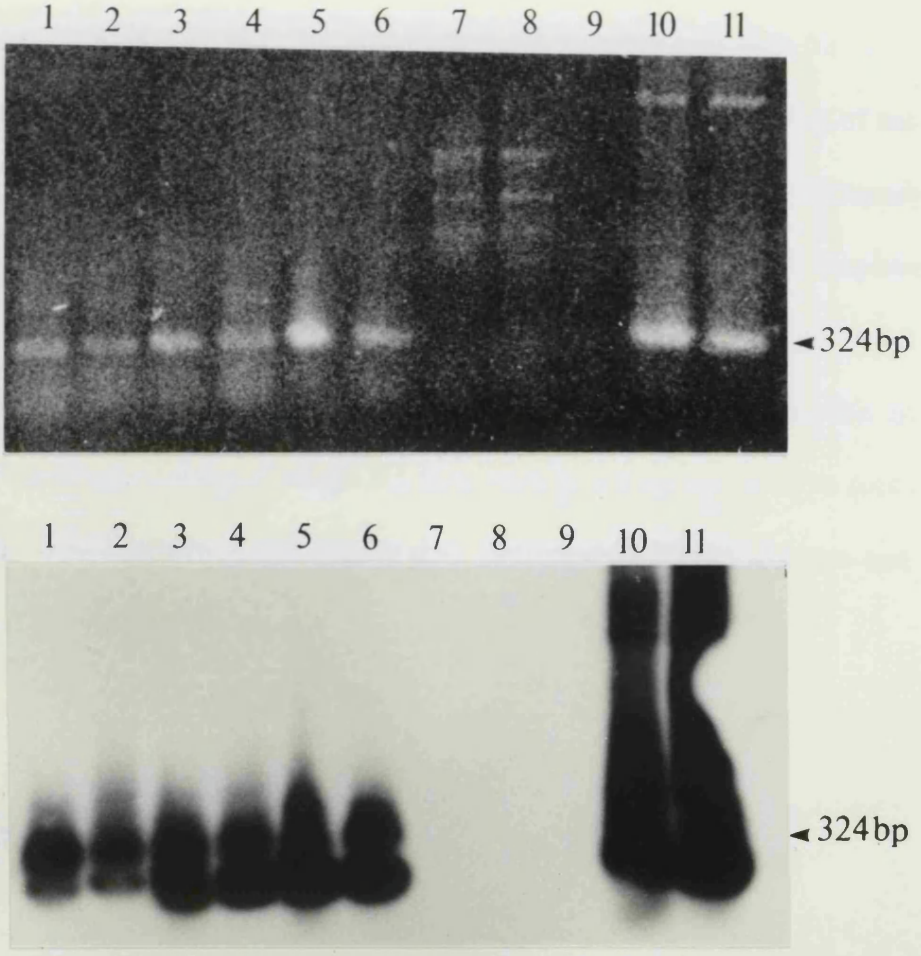


***Figure 3.10** Amplification of a cyt P450 fragment from* **Z.mays** *using RT-PCR*

A) RT-PCR was carried out on RNA (1 µg) prepared from a range of* Z.mays *shoot tissues. RT-PCR products were separated by electrophoresis (1 % agarose) and visualised by ethidium bromide.
B) RT-PCR products were transferred to nylon membrane and hybridised to [α-^{32}P]dCTP labelled MZERG.450.

RT-PCR was carried out using MZERG.450 specific primers (15 pmol per reaction) on RNA (1 µg) prepared from UE95 shoot tissue treated with ethanol (lane 1), naphthalic anhydride (NA) (lane 2) or ethanol/NA (lane 3); 8-day old (lane 4) and 5-day old (lane 5) untreated UE95 tissue; 8-day old B73 tissue (lane 6); 8-day old B73 tissue - 1 primer control (lane 7); 8-day old untreated UE95 tissue - 1 primer control (lane 8). RT-PCR was also carried out using kit control RNA and primers (lane 9) and MZERG.450 DNA and MZERG.450-specific primers (lanes 10 and 11). Southern analysis final wash conditions were 0.1 x SSC, 0.1 % SDS, 55^{0}C, 30 min.

Northern analysis carried out using all the samples shown in Table 3.1, using MZERG.450 as a probe did not produce a hybridisation signal was seen for any of the RNA samples. In order to test the integrity of the RNA used, and the loading of samples, the northern blot was stripped according to the manufacturer's instructions (Zetaprobe GT membrane, Biorad) and reprobed using the large subunit of ribulose 5-bisphosphate carboxylase (rubisco) as a positive control. All RNA samples except those prepared from root tissue produced a strong signal with rubisco as would be expected since this enzyme is involved in photosynthesis and would not be present in non-photosynthetic root tissue (Figure 3.11). The intensity of the signals also confirmed that RNA samples had been loaded equally throughout the gel.

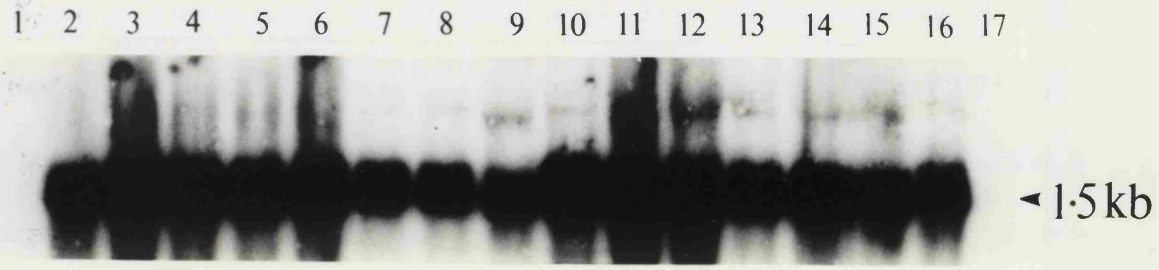


Figure 3.11 ***Northern analysis of* Z.mays *shoot and root RNA***

RNA prepared from a range of Z.mays *tissues was separated by electrophoresis (30 µg/lane), transferred to nylon membrane and hybridised to [α-^{32}P]dCTP labelled large subunit of rubisco. The RNA samples were isolated from 7-day old untreated SD10 root (lane 1) and shoot (lane 2) tissue; 9-day old UE95 untreated (lane 3), ethanol treated (lane 4), naphthalic anhydride (NA) treated (lane 5) and ethanol/NA treated (lane 6) shoot tissue; UE95 untreated 2-day old (lane 7), 5-day old (lane 8) and 8-day old (lane 9) shoot tissue; 8-day old untreated B73 shoot tissue (lane 10); untreated UE95 shoot tissue, 3-day (lane 11) and 5-day (lane 12) post spray control; chlorsulfuron treated UE95 shoot tissue, 3-day (lane 13) and 5-day (lane 14) post spray; chlorsulfuron/NA treated UE95 shoot tissue, 3-day (lane 15) and 5-day (lane 16) post spray; chlorsulfuron/NA treated UE95 root RNA, 5-day post spray (lane 17); Final wash conditions 0.1 x SSC, 0.1 % SDS, 60^{0}C, 1hr*

3.2.5 Screening of a commercial B73 seedling library using MZERG.450

A third library was obtained for screening for a full-length cyt P450. This was a commercially available cDNA library prepared from 5-day old seedling tissue of *Z. mays* variety B73 (Clontech). The presence of MZERG.450 or a homologue in B73 shoot tissue had already been investigated using RT-PCR to determine whether screening of such a commercial 'off-the-shelf' library was a feasible approach. Since RT-PCR on B73 shoot RNA had produced a product with MZERG.450-specific primers, it was decided that it was reasonable to assume that a cDNA library prepared from B73 seedlings may contain a cDNA encoding the full-length version of MZERG.450.

Approximately 360,000 plaques from this library were screened, and three putative cyt P450 clones identified through primary, secondary and tertiary rounds of screening. Phage recombinant for the first clone were isolated using a liquid lysis procedure (Section 2.4) and phage DNA isolated according to the manufacturer's instructions (Clontech) and purified using caesium chloride step gradients (Section 2.7). The clone was determined by restriction digestion and gel electrophoresis to be 1500 bp in size. The liquid lysis and caesium chloride procedure gave low DNA yields and the phage DNA was re-purified for manual sequencing using plate-lysis (Section 2.4) and the QIAGEN column purification procedure (Section 2.7). This method for phage DNA purification was used in subsequent experiments. Manual sequencing using the λgt11 vector forward and reverse primers (Section 2.8) was carried out and sequence analysis using GCG software revealed that the putative cyt P450 clone isolated had 97.4 % identity (114 bp overlap) at the nucleotide level with exon 1 of a photolyase gene from *E.coli* (Genbank accession no. X57399). Additionally the two areas of sequence flanking the vector cloning site bore no resemblance to those specified by the manufacturer.

The remaining two putative cyt P450 clones, since they were only *ca.* 500 bp and therefore not full-length cyt P450 clones, were isolated using PCR with the λgt11 forward and reverse primers (Section 2.8) and subcloned into the pCR^{TM}II and pGEM® plasmids according to the manufacturer's instructions (Invitrogen and Promega respectively). Manual sequencing revealed that these putatives were in fact stretches of λgt11 DNA which had been cloned into the multiple cloning site when the library had been produced by Clontech.

It was surprising and disappointing that a commercially prepared library was contaminated with *E.coli* DNA and that certain clones contained only λgt11 DNA within the cloning site. The library was obviously of extremely poor quality and it remained unclear as to why these particular non-cyt P450 clones remained positive up to tertiary screening levels. Negative screening controls had been employed by including plates of phage plaques which had not produced a primary or secondary positive signal on screening, and these remained negative on tertiary screening, thus the signal with these putative clones was not due to poor experimental technique. It was concluded that due to the quality problems of the library and the time-consuming nature of screening the library and isolating phage DNA for sequencing, it would not be productive to continue with this approach.

3.3 Discussion

A gDNA fragment representing part of a novel *Z.mays* cyt P450 was successfully amplified using the PCR technique. This fragment showed homology to other plant cyt P450 cDNAs (Figure 3.3). Genomic southern analysis confirmed the presence of a gene for this cyt P450 in the *Z. mays* genome.

Difficulties experienced both with library screening and northern analysis using MZERG.450 indicated that the particular cyt P450 mRNA represented by this fragment was likely to be present at very low concentrations in *Z. mays* tissue. Nevertheless, use of the more sensitive technique of RT-PCR for examining expression characteristics of the cyt P450 clone confirmed that this cyt P450 was present in root and shoot tissue of various ages and in different *Z.mays* varieties (SD10, UE95 and B73). The reason that the original PCR on root cDNA did not produce a product may have been due to the fact that this particular cyt P450 was not present in this cDNA pool or at such low quantities that PCR failed to detect it. The use of treatments known to induce herbicide metabolising cyt P450s including ethanol, the herbicide safener naphthalic anhydride and the herbicide chlorsulfuron, appeared to have no effect on the levels of MZERG.450 mRNA in root and shoot tissue, indicating that this cyt P450 was not inducible by these treatments.

Despite the fact that the original aim (to isolate cyt P450 involved in herbicide metabolism by screening a root library prepared from safener treated root tissue) was not achieved, a *Z. mays* cyt P450 fragment was successfully isolated and information gained regarding its expression. This fragment may prove useful for future work and perhaps could be used with different *Z. mays* cDNA or gDNA libraries of higher quality for isolating the corresponding full-length cDNA from this plant. In addition, the greater

number of plant cyt P450 sequences now available would enable better primers to be designed to be used to attempt to isolate probes for library screening. These primers could be designed so that cyt P450 sequences such as cinnamate hydroxylase and allene oxide synthase which have highly conserved sequences are not amplified when PCR is employed. Since allene oxide synthase is known to be the predominant cyt P450 in many plant tissues, particularly monocotyledonous tissue such as *Z. mays* (Lau, Harder, *et. al.* 1993), design of primers with bias away from this enzyme may help in the isolation of other less common cyt P450s. Three years after the isolation of MZERG.450, four full-length cyt P450s were isolated from *Z. mays* (Frey M., Kliem, *et. al.* 1995). Examination of the conserved regions of these four cyt P450s, corresponding to the regions spanned by primers A and C used in this project, showed that the helix I region of all four cyt P450s differed markedly to the amino acid sequence of primer C. This was probably the reason that these particular cyt P450s were not isolated at the same time as MZERG.450 when PCR was carried out using primers A and C.

Despite the fact that the treatments used in this study did not result in induction of MZERG.450, it is still possible that this particular cyt P450 could be induced by other agents, chemical, developmental or environmental. When considering the problem of isolating a cyt P450 involved in herbicide detoxification, it would be of value to more thoroughly investigate induction characteristics using a wider range of herbicides and safeners including detailed time course experiments to investigate levels of induction at different times. There is evidence that the effect of certain xenobiotics on cyt P450 induction is restricted to a precise developmental stage such as is the case with the induction of cyt P450 mediated triasulfuron metabolism in *Z.mays* (Persans and Schuler 1995). Such an approach, paying closer attention to induction profiles from the outset of

the study, could allow the optimal time for expression of a particular inducible cyt P450 to be determined, hence the best time to isolate mRNA for library preparation could be identified. This might increase the likelihood of obtaining a cyt P450 involved in herbicide metabolism. Specific herbicide-detoxifying cyt P450s could be targeted by examining the metabolism of herbicides in microsomal membrane fractions prepared from plants previously treated with inducing agents and using the tissue with optimal herbicide detoxification activity to prepare cDNA libraries for screening for the cyt P450 responsible for this metabolism.

Due to the fact that it had not been possible to isolate a full-length cDNA corresponding to MZERG.450 for use to investigate its corresponding enzyme activity, it was decided not to pursue the *Z. mays* work further. In order to pursue the goal of analysing the activity of an uncharacterised cyt P450, a full-length plant cyt P450 cDNA was obtained from the *Arabidopsis* Expressed Sequence Tag programme for heterologous expression to attempt to assign function to this particular plant cyt P450 by biochemical assay. This work is detailed in Chapters 4 and 5.

Chapter 4

Molecular characterisation of an Arabidopsis thaliana *Expressed Sequence Tag (EST) coding for cyt P450*

4.1 Introduction

The large-scale functional identification of genes through the use of genetic map location and mutational analysis data, a strategy known as 'Chromosome Walking' has severe limitations for most organisms. Limited resources and relative genome sizes has meant the technique is technically unfeasible in most species. The sequencing of the human (Martin-Gallardo, McCombie, *et. al.* 1992; McCombie, Martin-Gallardo, *et. al.* 1992), yeast (*Saccharomyces cerevisiae)* (Oliver, van der Aart, *et. al.* 1992) and nematode (*Caenorhabditis elegans*) (Sulston, Du, *et. al.* 1992) genomesis well underway and the only plant genome being targeted for complete sequencing by the end of the decade is that of *Arabidopsis thaliana,* a small flowering plant of the family Cruciferae (Brassicaceae) (Foundation 1990). *A. thaliana* was chosen because of its short life cycle, small size, large seed output, relatively small genome (100 Mb) and the presence of relatively few repetitive sequences.

Although genomic sequencing data are informative for gene identification, faster, more cost-effective approaches involving the analysis of cDNAs have become of increasing importance. The advent of high turn-over automated sequencing has also contributed to the feasibility of such approaches. Such strategies have had particular impact on the study of plant cyt P450s in recent years since, for reasons discussed earlier in Chapter 1, direct purification of these enzymes from plants for biochemical analysis remains difficult. The use of degenerate PCR techniques and low stringency screening

of cDNA libraries has resulted in the isolation of a rapidly growing number of plant cyt P450s and over 60 full-length plant cyt P450 cDNAs have been isolated to date spanning 20 discrete cyt P450 families. Most of these however are 'orphan' clones, that is they have yet to be assigned physiological function. In certain cases the sequence of a particular cyt P450 alone can provide strong evidence as to its physiological function. This is true of the cinnamate hydroxylases (CYP73) and allene oxide synthases (CYP74). The genes from both these families show high homology between members enabling the function to be 'guessed' from the sequence data. However, such 'assignment by analogy' is not possible for those cyt P450s which do not fall into families with known activity, and, indeed, it is known that even a single amino acid change can dramatically alter the substrate specificity of a particular cyt P450 (Lindberg and Negishi 1989). In mammalian cyt P450 research, cyt P450s with very similar sequences have been shown to have significantly different substrate specificities (Ryan, Thomas, *et. al.* 1982; Aoyama, Yamano, *et. al.* 1989). Clearly, other approaches are required to allow accurate identification of cyt P450 function. One method of investigating physiological function of a cyt P450 is to express the protein encoded by the isolated cDNA in a suitable heterologous system such as yeast, a technique which is discussed in more detail in the next chapter.

One example of the success of cDNA analysis has been the assignment of putative function to a large number of maize cDNAs by comparing the sequence with those available in public sequence databases and coupling the data with analysis of map location and gene expression (Shen, Carneiro, *et. al.* 1994). Recently a faster strategy has been developed, also based upon the analysis of expressed sequences, to identify genes. In this method, the putative identity of a significant proportion of large numbers of anonymous

cDNAs (Expressed Sequence Tags) has been assigned based solely on single-pass automated sequencing and comparison of sequences with those on the public databases without the use of mapping or mutational information. Since its initial use with human cDNAs (Adams, Kelley, *et. al.* 1991; Adams, Dubnick, *et. al.* 1992), the strategy has been used with nematode (McCombie, Adams, *et. al.* 1992; Waterston, Martin, *et. al.* 1992) and plant cDNAs including *Brassica napus* (Park, Kwak, *et. al.* 1993), *Oryza sativa* L (rice) (Uchimiya, Kidou, *et. al.* 1992), *Zea mays* (Keith, Hoang, *et. al.* 1993) and *A. thaliana* (Hofte, Desprez, *et. al.* 1993; Newman, de Bruijn, *et. al.* 1994). To date the technique has resulted in over 37,000 plant Expressed Sequence tags (ESTs) being available with tens of thousands more expected over the next few years.

A number of cyt P450s have been identified in various species in this way. Since the ESTs are publicly available, detailed identification of function can be pursued by independent research groups with specific interests, thus the approach has served to facilitate progress in many areas of plant biology. The programme at Michigan State University alone (Newman, de Bruijn, *et. al.* 1994) has made available over 5000 ESTs from *A. thaliana*. Of 1500 ESTs analysed, 32% were putatively identified and at least 165 had significant deduced amino acid homology to proteins or gene products not previously characterised from higher plants. This project revealed several sequences resembling cyt P450s one of which, designated clone pZL15G6, appeared from preliminary sequence analysis to encode a full length cyt P450. This clone was obtained for further analysis in an effort to assign function using a combination of detailed sequence analysis and comparison, expression pattern analysis, genomic southern analysis and, ultimately, biochemical characterisation of the gene product by heterologous expression. This chapter details the sequence analysis, tissue expression analysis and genomic southern analysis

carried out on this clone.

4.2 *Results*

4.2.1 Sequencing and analysis

Plasmid DNA of clone pZL15G6 was prepared from the host bacteria DH10B (Section 2.1) for automated sequencing as described in Section 2.7. Initial sequencing of both ends of the cDNA was carried out using the vector primers, -21 M13 (Forward) and T7 (Section 2.8). Subsequent sequencing was carried out using pZL15G6 specific primers selected from analysis of sequencing data obtained. The primers selected for sequencing from the N-terminus were designated Ant1 (5'-GCAGCCGACCAGAGACTGTAGC), Ant2 (5'-TCGCGTTCGGGGTGAATATCC) and Ant3 (5'-GTGCAAGACGAGATTAGG). Primers selected for sequencing the C-terminus were designated Act1 (5'-CGGTGGTGATTCCCAGC), Act2 (5'-GCTTGAAGTAGTGAACTTGG) and Act3 (5'-GCCTACACACGATACTCGCG). Clone pZL15G6 was sequenced at least 3 times in both directions until firm sequence data were obtained. The resulting full nucleotide and deduced amino acid sequence is shown in Figure 4.1. The cDNA was 1684 bp in length and contained an open reading frame of 504 amino acids with a start codon at nucleotide 53 and the first stop codon located at nucleotide 1565.

The deduced amino acid sequence was compared with the sequences available in the GenEmbl database using the GCG software program TFASTA. This showed that pZL15G6 was most closely related to a cDNA from the related species *Thlaspi arvense* (Udvardi, Metzger, *et. al.* 1994), exhibiting 42.7 % identity with the deduced amino sequence of this clone (501 amino acid overlap). The next most closely related sequences were those of *Persea americana* (avocado) CYP71A1 (43.3 % identity, 473 amino acid overlap); (Bozak, Yu, *et. al.* 1990), *Petunia hybrida* pCGP147; (37.6 % identity, 502 amino acid overlap, Patent No. EPO522880, 1993, International Flower Developments

Ltd.), *Solanum melongena* (eggplant) CYP71A2 (39.1 % identity, 501 amino acid overlap);(Umemoto, Kobayashi, *et. al.*1993).

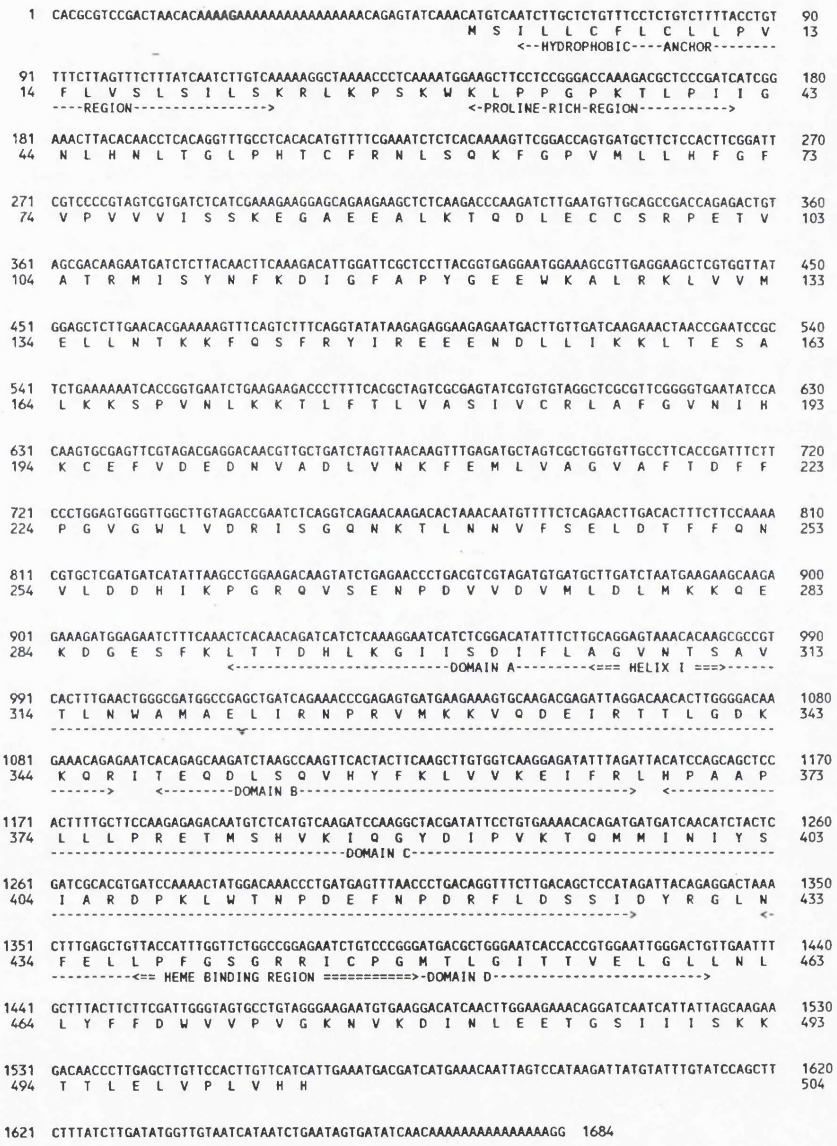


Figure 4.1 ***Nucleotide and deduced amino acid sequence of* A. thaliana *clone pZL15G6 (CYP71B7)***
Conserved domains A, B, C and D and the N-terminal membrane spanning regions are indicated as dashed lines below the amino acid sequence

and *Z. mays* CYP71C4 (36.2 % identity, 475 amino acid overlap); (Frey M., Kliem, *et. al.* 1995). This indicated that pZL15G6 was likely to be a member of the family CYP71.

Figure 4.2 shows a comparison of clone pZL15G6 with CYP71 cyt P450s generated using the GCG software. The comparison shows the high degree of similarity the *A. thaliana* sequence has with members of this family. Sequence comparison of pZL15G6 with known plant cyt P450s using the GCG software revealed the presence of a number of domains characteristic of cyt P450s indicated in Figure 4.1. These included an N-terminal hydrophobic membrane anchor region (Nelson and Strobel 1988) spanning amino acids 3 to 22. A hydrophobicity plot (Figure 4.3) prepared using GCG program PEPPLOT also indicated the presence of a hydrophobic membrane spanning region at the far N-terminus of the deduced amino acid sequence. The N-terminus of clone pZL5G6 also contained a proline-rich region characteristic of many cyt P450s, between amino acids 31 and 41. This region contains the PPGP tetrapeptide. The proline rich region has been postulated to be of critical importance in orienting the cyt P450 and this region, including PPGP, serves to provide a β-turn in the polypeptide chain following the membrane insertion region. Alteration of this region in another cyt P450, through deletion of the tetrapeptide, has been shown to result in loss of activity and stability of the enzyme, perhaps due to conformational changes at the active site or the entire orientation of the enzyme, without altering its insertion into the endoplasmic reticulum membrane (Szczesna-Skorupa, Straub, *et. al.* 1993).

Four discrete domains A, B, C and D, (Kalb and Loper 1988) characteristic of the cyt P450 group of enzymes, were also present in the pZL15G6 sequence. These included Domain D, spanning amino acids 433 to 460 which contains the highly-conserved heme-binding domain between amino acids 437 and 448. The heme binding domain is

represented by a highly conserved peptide FxxGxxxCxG, (Nebert and Gonzalez 1987), the cysteine residue of which provides the thiolate ligand to the heme in all cyt P450s (Black and Coon 1986).

Plant P450s have been shown to form two distinct family groups: Group A comprises families 71, 73, 75, 76, 77, 78, 79, 80, 81, 83 and 89. These are believed to derive from a common ancestor. The second group (non-A) comprises families 72, 74, 86 and 87 and do not form a discrete phylogenetic group (Durst and Nelson 1995). pZL15G6 can therefore be classed as a group A P450. This group has a unique consensus for Domain D described above, P F G A/S/V G R R X C X P/A/V G and a recent alignment by David Nelson of the P450 Nomenclature Committee detected this motif only in plant P450s of group A. PZL15G6 would therefore be expected to contain the unique consensus for this domain and it is indeed found in this P450 sequence.

Figure 4.4 shows the phylogenetic tree prepared for plant cyt P450s including the pZL15G6 clone using the PHYLIP software. The tree clearly shows the close relationship clone pZL15G6 has with cyt P450s of the family CYP71, serving to further confirm that this clone is a member of this particular cyt P450 family.

The conserved region within Domain A specified by amino acids A/G G X D/E T T/S, within Helix I could also be seen (amino acids 306 to 311). This region is believed to play a role in forming of the oxygen binding pocket (Poulos, Finzel, *et. al.* 1987) although three-dimensional modelling studies of P450BM-3 have cast some doubt on this theory since dioxygen bound to iron was not seen to protrude into this region (Kurumbail, Ravichandran, *et. al.* 1993). Notably, in the sequence of pZL15G6 some deviation has occurred from the consensus of this domain, with an asparagine (N) residue replacing aspartate/glutamate (D/E) at the fourth position. This is not entirely unexpected

since despite their relatedness, group A P450s show considerable variation in this domain. For example, P450 from families CYP71C, CYP73 and CYP79 have an alanine and CYP80 has a proline in place of A/G in the first position, and CYP79 has an asparagine in place of the conserved threonine at position 5. The asparagine residue at postion 4 in the Helix I region of pZL15G6 is a charged residue, whereas the residues normally found at this position in the domain A consensus, aspartate or glutamate, are uncharged. It may be, therefore, that this change confers a conformational change related to the substrate specificity of this particular CYP71 cyt P450. In fact, it has been speculated that deviations from the consensus relate to the unusual reactions catalysed by these enzymes, for example CYP80 cyt P450 catalyses the inter-molecular formation of a C-O bond, a reaction which proceeds without the transfer of oxygen. However, some cyt P450s which catalyse conventional reactions, such as the cinnamate hydroxylases, also deviate from the consensus and so perhaps these differences are significant more from a phylogenetic point of view than a catalytic one (Durst and Nelson 1995). The sequence of pZL15G6 was submitted to Dr. D Nelson (University of Memphis, Tennessee, USA) for assignment of the CYP family name (Nelson, Kamataki, *et. al.* 1993) and was confirmed to be a member the CYP71 family as the sequence and phylogeny analysis had indicated. It was designated CYP71B7 and submitted to the GENEMBL sequence database, and has the accession number X97864.

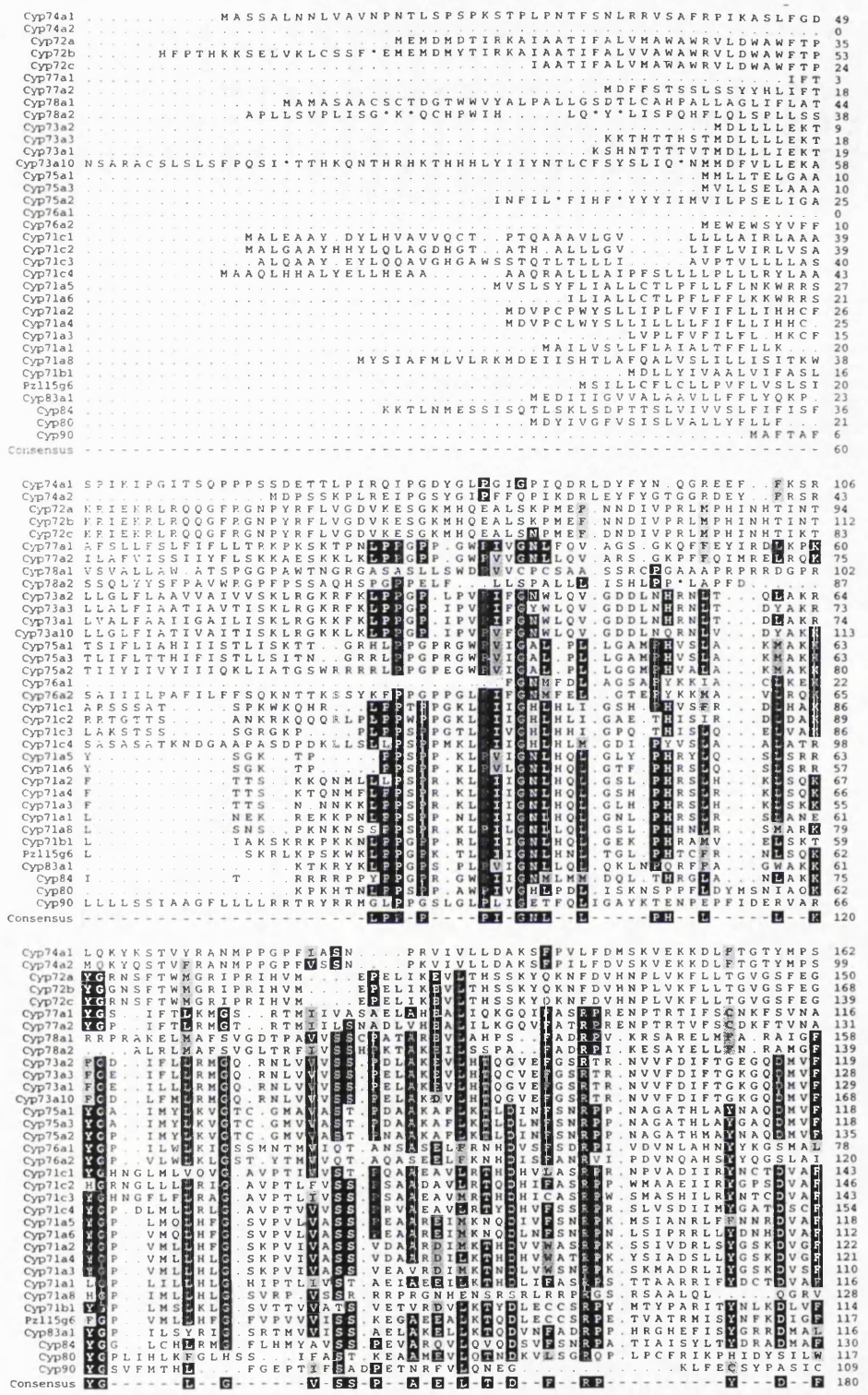
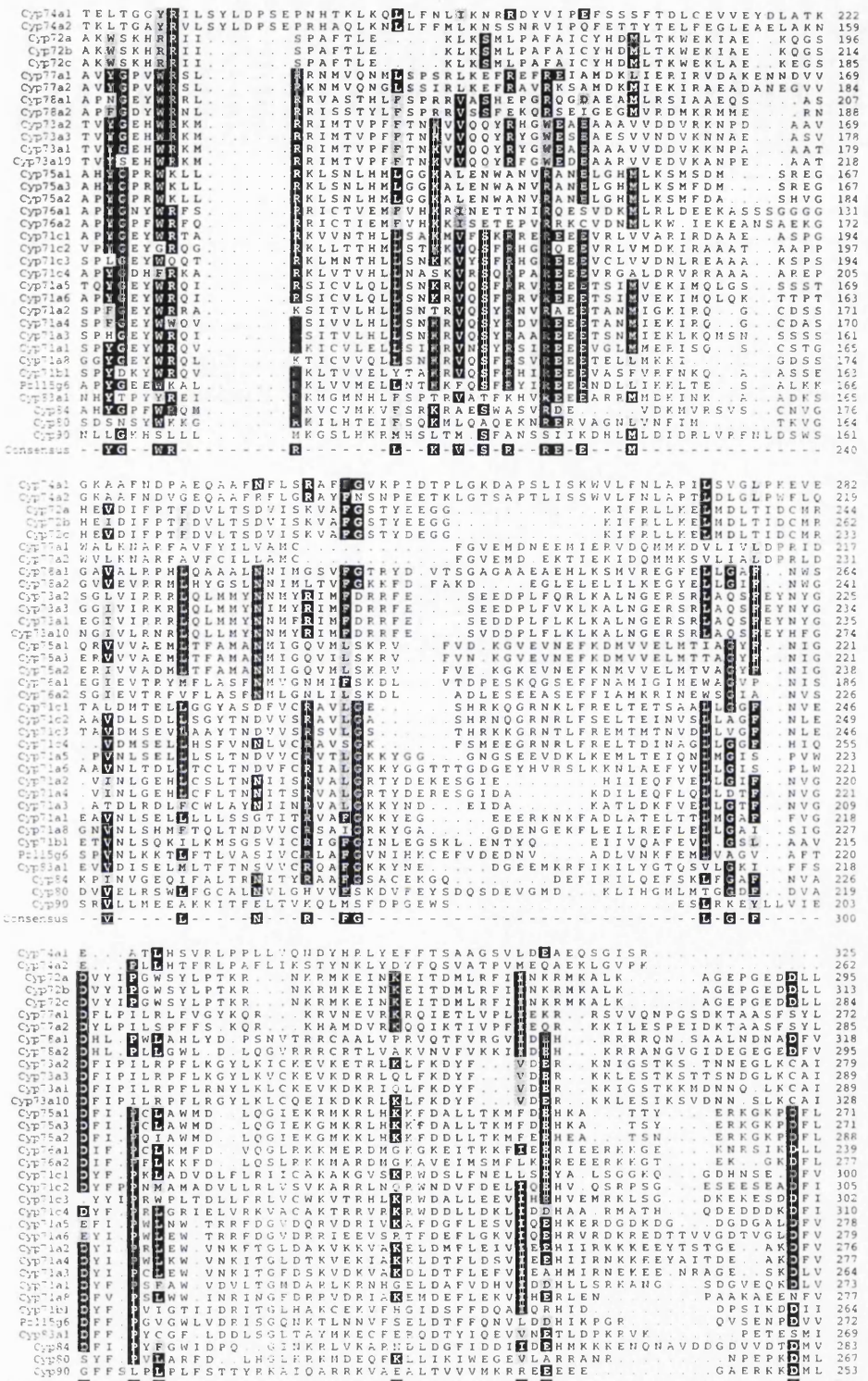
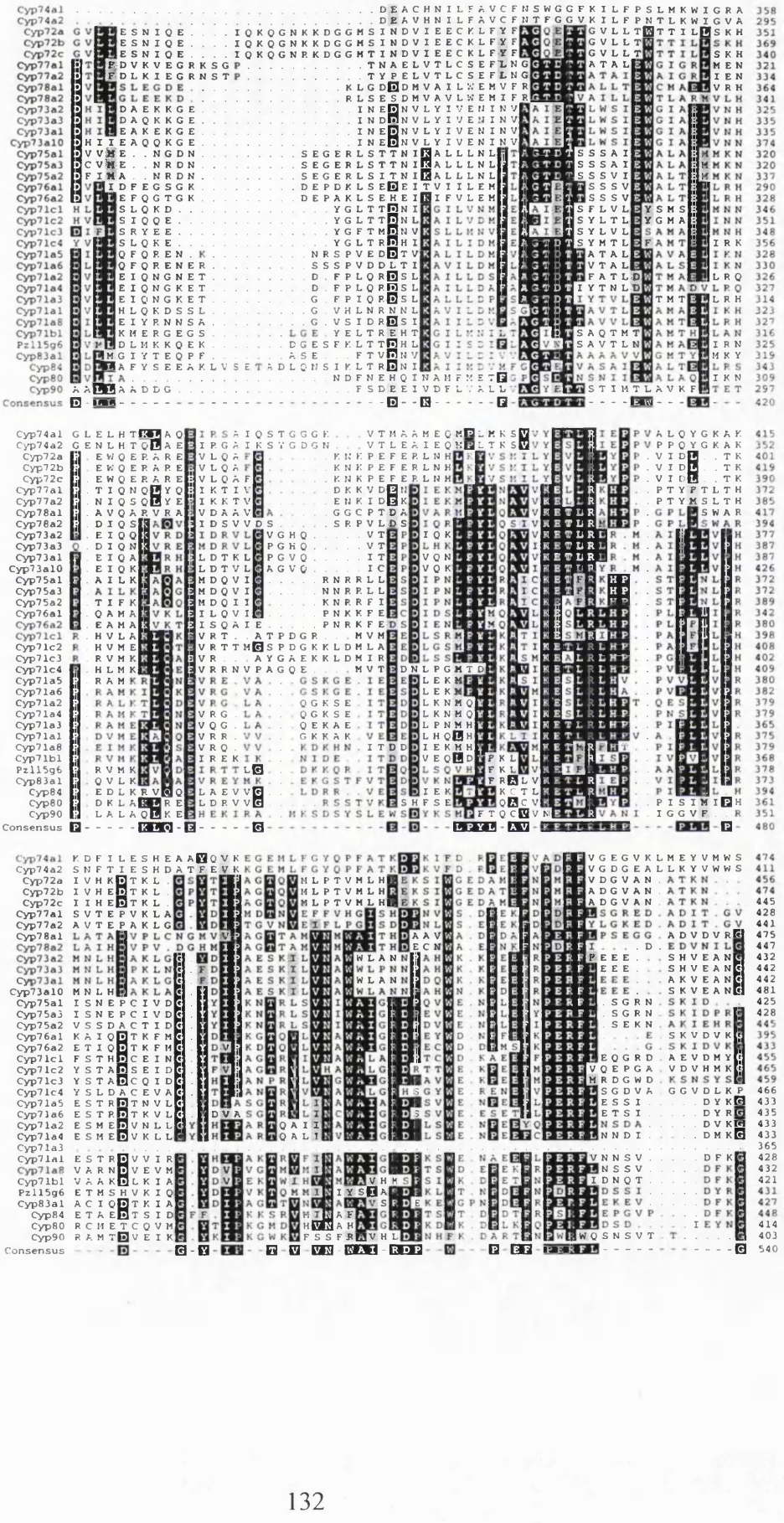
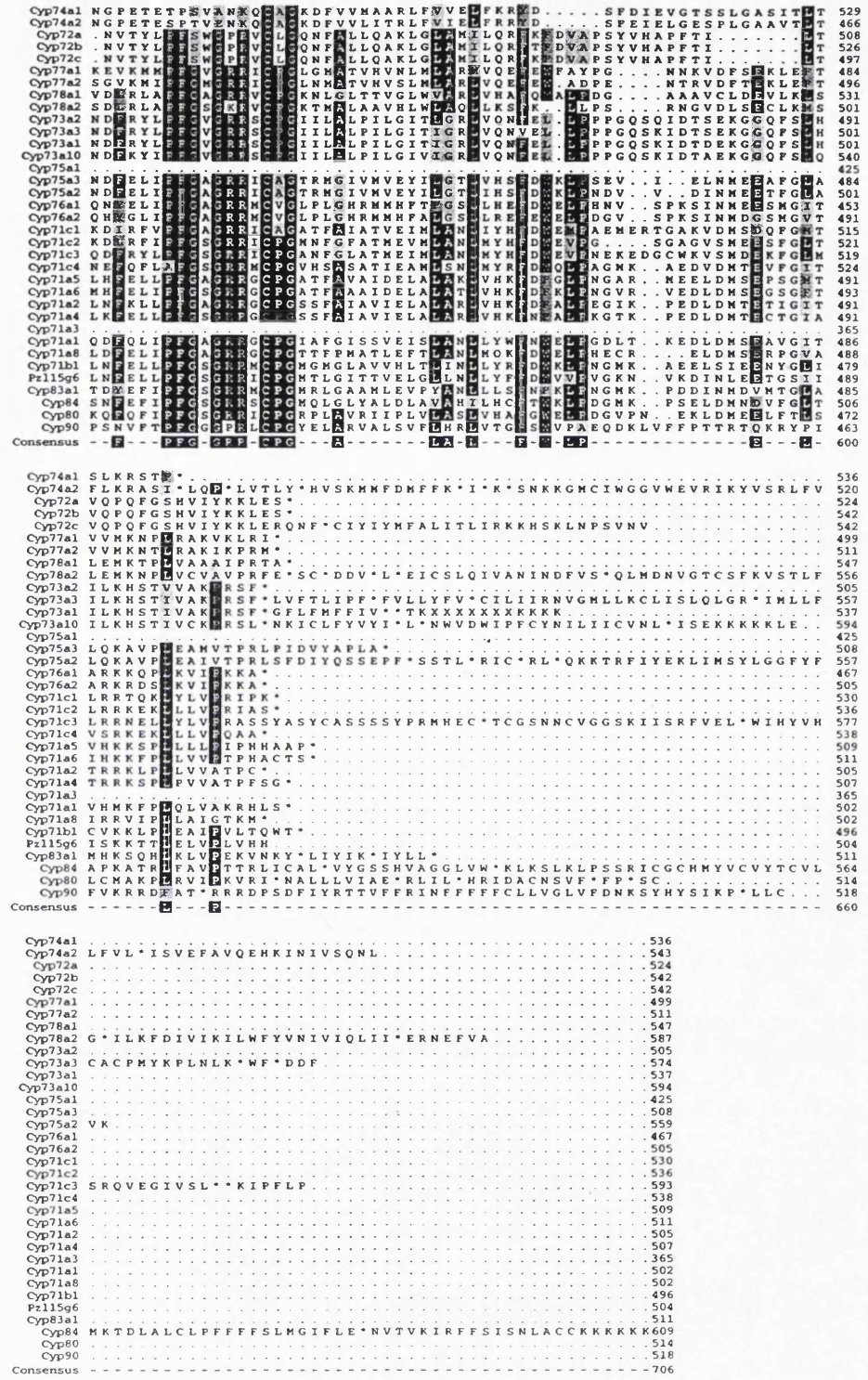


Figure 4.2 ***A comparison of the deduced amino acid sequence of* A. thaliana *pZL15G6 (CYP71B7) with related plant cyt P450s***

Black shaded areas indicate completely conserved residues and grey shaded areas indicate similar residues. The sequence comparison was prepared using the GCG programs PILEUP and PRETTYBOX. Cyt P450s are named according to the official nomenclature (Nelson, Koymans et. al. 1996).






Cyp74a1
Cyp74a2
Cyp72a
Cyp72b
Cyp72c
Cyp77a1
Cyp77a2
Cyp78a1
Cyp78a2
Cyp73a2
Cyp73a3
Cyp73a1
Cyp73a10
Cyp75a1
Cyp75a3
Cyp75a2
Cyp76a1
Cyp76a2
Cyp71c1
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Cyp71c4
Cyp71a5
Cyp71a6
Cyp71a2
Cyp71a4
Cyp71a3
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Cyp71a8
Cyp71b1
Pz115g6
Cyp83a1
Cyp84
Cyp80
Cyp90
Consensus

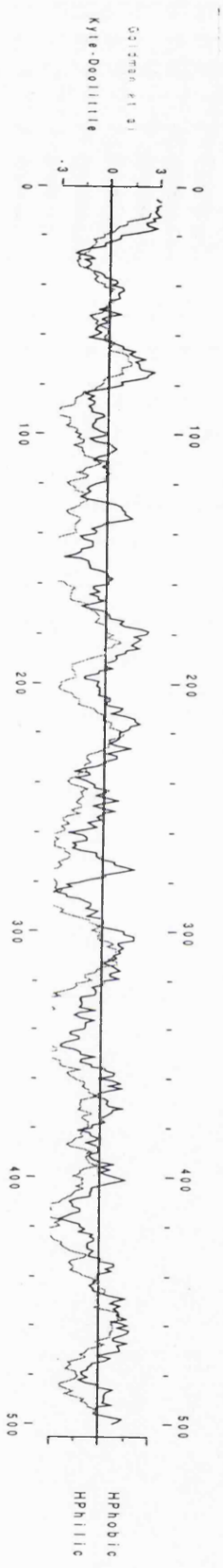

Figure 4.3 ***Hydrophobicity plot of* A. thaliana *pZL15G6 (CYP71B7)***
The plot was prepared using the GCG program PEPLOT. The solid line represents a Kyte-Doolittle plot and the broken line a Goldman plot.

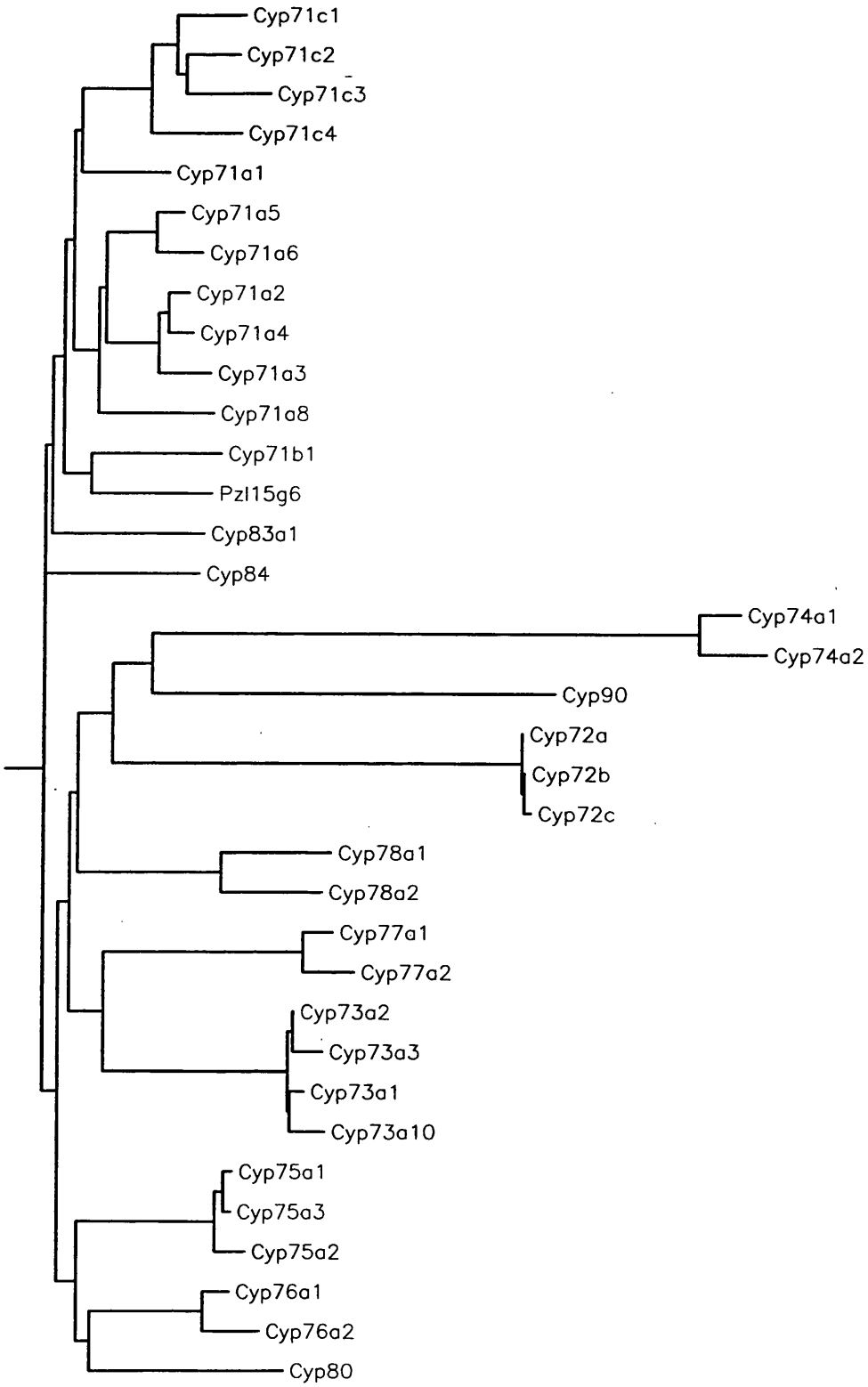

Figure 4.4 ***Phylogeny of plant cytochromes P450***
The phylogeny tree was prepared using the PHYLIP software program NEIGHBOUR. Reproduced from (Durst and O'Keefe, 1995). Cyt P450s are named according to the official nomenclature (Nelson, Koymans et. al. 1996).

4.2.2 Northern analysis

The expression of CYP71B7 in specific *A. thaliana* tissues (grown as described in Section 2.13) was investigated by northern analysis carried out using the rapid alkaline blotting method and Zeta Probe® (GT) membrane as described in Section 2.12. Figure 4.5 shows that CYP71B7 cDNA hybridised to a *ca.* 1.7 kb band which was detected primarily in RNA from rosette leaves. Hybridising bands were also seen in RNA from roots, leaves and siliques with a very faint band present in flower RNA. No hybridisation signal was detected with RNA isolated from stem tissue. The band observed in silique RNA was noticeably larger in size than those present in RNA from the other tissues examined. This may represent hybridisation to a related P450 isoform, as the Michigan University EST programme identified a number of clones with some sequence identity to pZL15G6. However, even the most closely related *A. thaliana* cyt P450 was only 35.2 % related (494 amino acid overlap) and this was assigned to a different family (CYP83) so it is unlikely that under the high stringency washing conditions used CYP71 was cross hybridising to any other cyt P450 isoform. Approaches which could have been used to confirm this could be to probe with a shorter section of the CYP71B7 cDNA spanning a non-conserved region such as the N-terminus and/or the use of a quantitative reverse-transcriptase PCR technique using CYP71B7-specific oligonucleotide primers. The latter technique has been known to distinguish between targets that differ by only a single nucleotide (Foley, Leonard, *et. al.* 1993) and may have been suitable to preclude quantification of different but related isoforms of the *A. thaliana* CYP71 P450. Unfortunately time constraints within the project did not allow this to be pursued further.

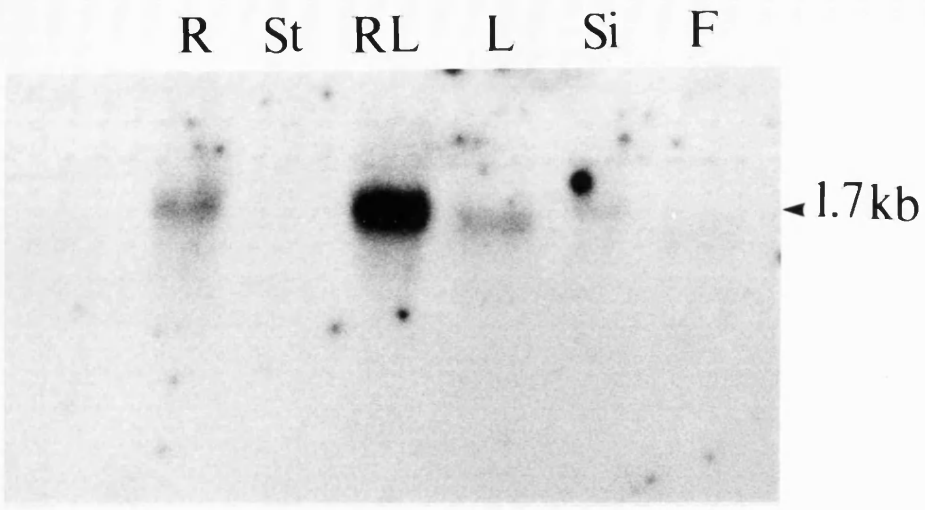

Figure 4.5 ***Northern analysis of* A. thaliana *tissue***
RNA prepared from a range of A. thaliana *tissues was separated by electrophoresis (20 µg/lane), transferred to nylon membrane and hybridised to [α-^{32}P]dCTP labelled CYP71B7. RNA was isolated from root (R); stem (St); rosette leaf (RL); leaf (L); silique (Si) and flower (F) tissue. Final wash conditions were 0.1x SSC, 0.1 % SDS, 55^{0}C for 60 min.*

4.2.3 Genomic southern analysis

For southern analysis, restriction digested *A. thaliana* genomic DNA blotted onto Hybond-N was provided by Dr. H. Zhang, Biochemistry & Physiology Department, Rothamsted Experimental Station, and hybridised to CYP71B7 as described in Section 2.11. Figure 4.6 shows the results of this genomic southern analysis using CYP71B7 as a probe. Figure 4.7 shows the restriction map of CYP71B7 cDNA digested with these 5 enzymes to allow analysis of the results.

The number of bands observed following digestion with *Bgl*II or *Hin*dIII was consistent with the presence of two restriction sites for these enzymes within the cDNA sequence. From the sequence of the CYP71B7 cDNA, the smallest fragment expected from digestion with *Bgl*II is 774 bp and with *Hin*dIII 984 bp. The bands observed, which are likely to derive from digestion within the coding sequence, are slightly larger than those predicted from the sequence, at *ca.* 1.0 kb for *Bgl*II and *ca.* 1.23 kb for *Hin*dIII. This may indicate the presence of a short intron of up to 250 bp, located between the sites for these enzymes within the gene. If so, such an intron is far shorter than that observed with the gene from the same CYP71 family, CYP71A1 from *P. americana*, which is approximately 5.0 kb in size (Christoffersen, Percival, *et. al.* 1995). However, a *Z. mays* (CYPZm1) cyt P450 gene also of the CYP71 family, was demonstrated to have one intron of only 70 bp located close to the dioxygen binding site (Frey M., Kliem, *et. al.* 1995). Bands observed with *Eco*RV are consistent with a single restriction site for this enzyme within the sequence, and those with *Eco*RI and *Bam*HI consistent with the absence of sites for these enzymes within the cDNA sequence. Digestion with *Eco*RI yielded a single band *ca.* 3.0 kb implying that the gene is located within a fragment of DNA of this size in the *A. thaliana* genome. The high stringency wash conditions used would tend to

preclude the cross-hybridisation of related isoforms (as with the northern analysis), which if it were occurring would make interpretation of the southern analysis results more difficult. Overall, southern analysis indicated that the gene for CYP71B7 is present as a single copy within the *A. thaliana* genome.

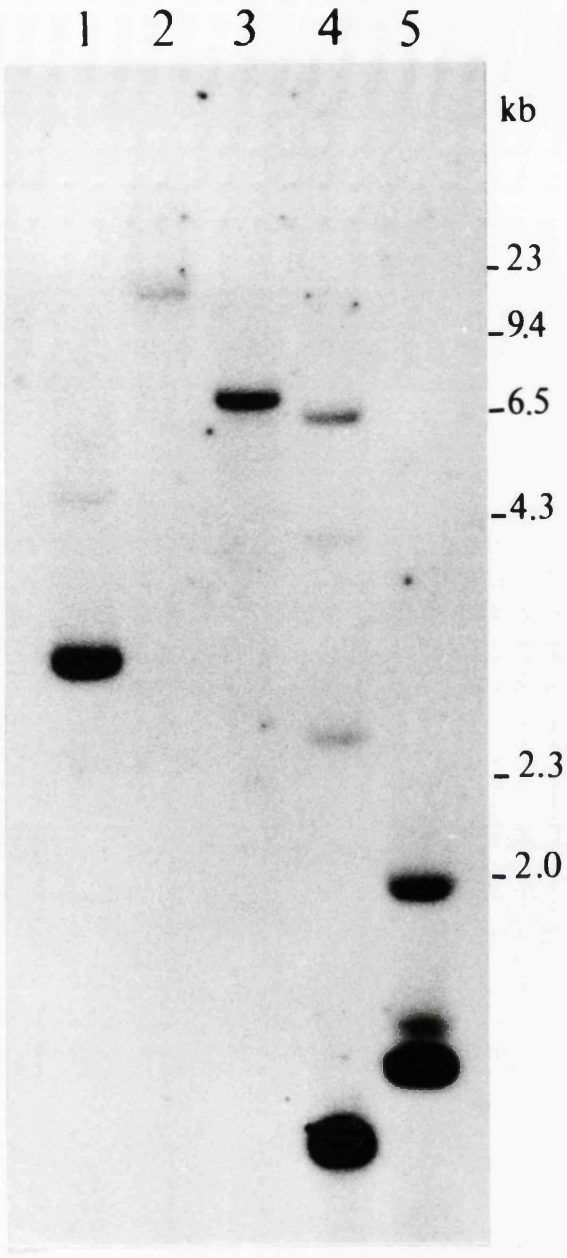

Figure 4.6 ***Southern analysis of* A. thaliana *gDNA***

Total genomic DNA from A. thaliana *tissue was digested with restriction enzymes* Eco*RI (lane 1),* Bam*HI (lane 2),* Eco*RV (lane 3)* Bg*lII (lane 4) and* Hin*dIII (lane 5) prior to 0.8 % agarose gel electrophoresis (10 µg/lane). The DNA was then transferred to a nylon membrane and hybridised to [α-^{32}P]dCTP labelled CYP71B7. Final wash conditions were 0.1 x SSC, 0.1 % SDS, 65 °C, 30 min.*

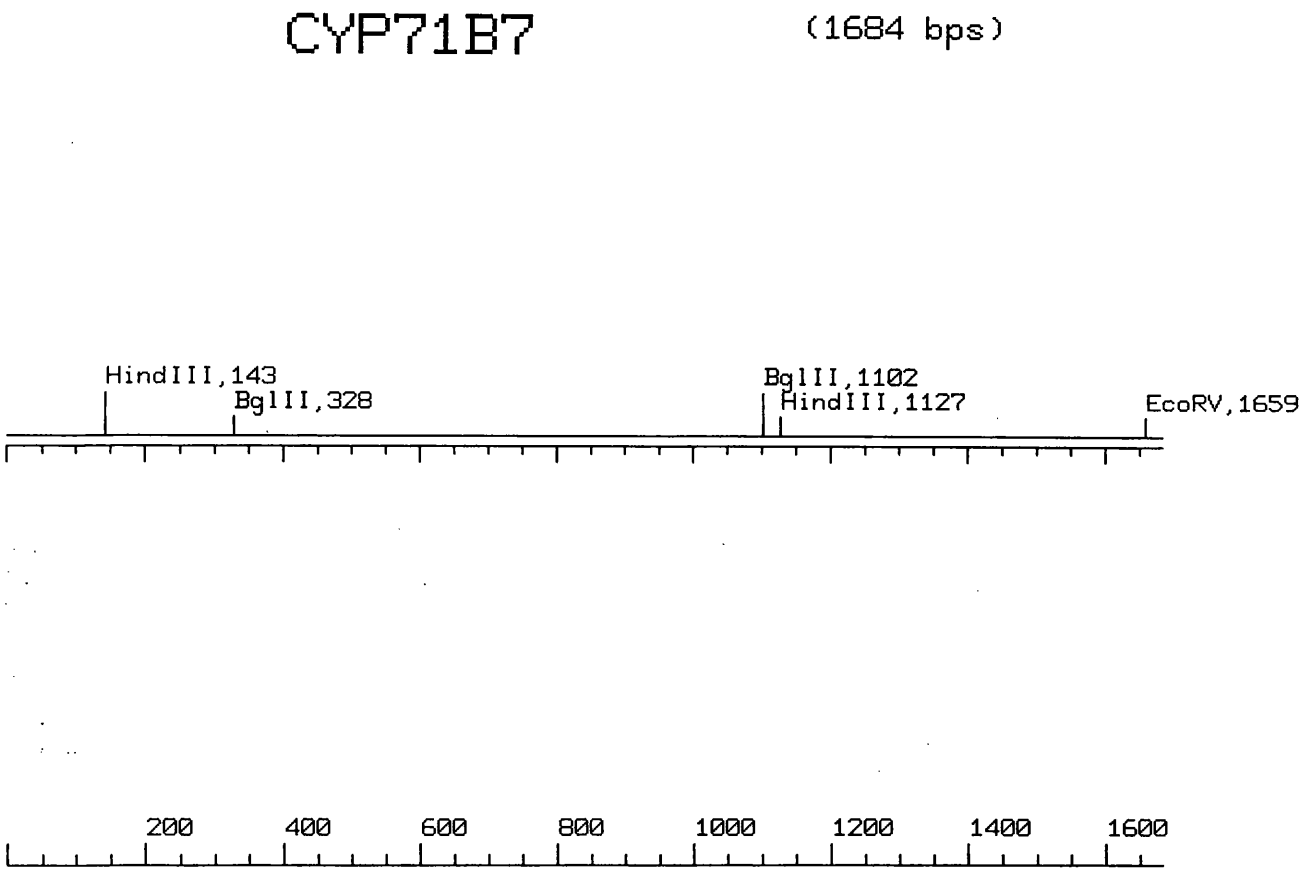

Figure 4.7 ***Restriction map of pZL15G6 (CYP71B7) cDNA***

Restriction sites corresponding to Bgl*II,* Hin*dIII,* Eco*RI,* Eco*RV, and* Bam*HI are indicated. Numbers refer to the position on the nucleotide sequence.*

4.3 *Discussion*

Sequence analysis of the *A. thaliana* EST clone pZL15G6 indicated that it encoded a cyt P450 within the CYP71 family, subfamily B and was designated CYP71B7. The CYP71 family is likely to be very diverse, with known examples isolated from *S. melongena* (three members of the CYP71 family to date) (Umemoto, Kobayashi, *et. al.* 1993), *Thlaspi arvense* (Udvardi, Metzger, *et. al.* 1994), *Z. mays* (four CYP71s) (Frey M., Kliem, *et. al.* 1995), *Nepeta racemosa* (catmint) (two CYP71s) (I. Clark, D.Hallahan, unpublished) and *P. americana* (Bozak, Yu, *et. al.* 1990). Up to ten genes are believed to encode CYP71s in *P. americana* alone (Christoffersen, Percival, *et. al.* 1995) and it would appear therefore that this gene family is widely dispersed within the plant kingdom and has diverged to a greater extent than other cyt P450 families such as CYP73 (coding for t-cinnamate hydroxylases) and CYP75 (coding for flavonoid 3',5'-hydroxylases (Christoffersen, Percival, *et. al.* 1995).

Although as yet no physiological role has been identified for members of the CYP71 family, the evidence to date indicates an involvement in terpenoid metabolism (Hallahan, Lau, *et. al.* 1994). The diversity of CYP71 isoforms is consistent with the diversity of terpenoids and their metabolites produced by plants, as well as with the number of reactions in terpenoid metabolism known to involve cyt P450s (Mihaliak, Karp, *et. al.* 1993). The only activities which have been conclusively ascribed to a member of the CYP71 family have been the epoxidation of the monoterpenoids nerol and geraniol (Hallahan, Lau, *et. al.* 1994), demethylation of the model substrate *p*-chloro-N-methylaniline (pCMA) (O'Keefe and Leto 1989; Bozak, O'Keefe, *et. al.* 1992) and deethylation of the model substrate 7-ethoxycoumarin (Hallahan, Nugent, *et. al.* 1992). Little or no data on terpenoid biosynthesis in *A. thaliana* are available in the literature, and

this species is not known to accumulate terpenoids. It may be however, that *A. thaliana* CYP71B7 does play a role as yet undiscovered in metabolism of monoterpenoids in this plant.

A further possibility when considering the potential function of CYP71B7 may lie in the close similarity it has with the CYP71 cDNA from the related species *Thlaspi arvense* (Udvardi, Metzger, *et. al.* 1994). Both *Thlaspi* and *Arabidopsis* are members of the family Cruciferae (Figure 4.8). The characteristic odours and flavours of crucifers are primarily due to glucosinolate hydrolysis products and both plants show evidence of common glucosinolates e.g 2-propenyl (allylglucosinolate or sinigrin) in the seed (Daxenbichler, Spencer, *et. al.* 1991). There is evidence to suggest that cyt P450s are involved in biosynthesis pathway of certain aromatic glucosinolates (Bennett, Kiddle, *et. al.* 1996), and also in the hydroxylation of certain alkenyl glucosinolates (Rossiter, James, *et. al.* 1990). *A. thaliana* has been shown to contain 23 different glucosinolates arising from three amino acid precursors (Haughn, Davin, *et. al.* 1991). Given the abundance of glucosinolates in *A. thaliana*, and also their presence in many species of *Thlaspi* including *T.arvense*, the possibility that CYP71B7 and perhaps the related *T. arvense* cyt P450 play a role in the biosynthesis and/or hydroxylation of common or even distinct glucosinolates in these plants cannot be ruled out. Northern analysis indicated that CYP71B7 was not represented in stem tissue but was most highly represented in the leaves of the rosette. This fact also may point towards a possible role in glucosinolate biosynthesis or modification since glucosinolates are found to be most abundant in leaves (Bennett, Kiddle, *et. al.* 1996). However, whether the enzyme is involved in terpenoid or glucosinolate biosynthesis, or a completely unrelated cyt P450 function, the catalytic activity associated with the protein corresponding to the CYP71B7 cDNA can only be

conclusively ascribed by analysis of the heterologously expressed enzyme. The next chapter details heterologous expression of and biochemical analysis of CYP71B7 in an attempt to elucidate its role *in vivo*.

Figure 4.8 ***The morphology of*** **Thlaspi arvense *and* A. thaliana**

Mature plants of Thlaspi arvense *(A) and* Arabidopsis thaliana *(B).*

Chapter 5

Heterologous expression and biochemical characterisation of A. thaliana *cyt P450 (CYP71B7)*

5.1 Introduction

The definitive assignment of function to specific amino acid sequences requires that the catalytic activities of the corresponding proteins are conclusively delineated. A variety of systems for the heterologous expression of foreign proteins have been developed for this purpose including those based on bacterial, mammalian, insect, fungal and yeast cells.

The success of any heterologous expression system often hinges upon the choice of system used and in many cases is dictated by the nature of the protein to be expressed. This is certainly the case with expression of eukaryotic cyt P450s (Gonzalez and Korzekwa 1995) due to the fact that they are membrane-associated and require reconstitution with NADPH-cyt P450 reductase for activity. Proteolysis of the expressed protein can pose a serious problem in prokaryotic bacterial systems (Porter, Wilson, *et. al.* 1987) unless specific constructions are used to direct the protein either to the cytoplasmic membrane, outer membrane, or into the periplasmic space using secretion vectors (Ghrayeb, Kimura, *et. al.* 1984; Shen, Porter, *et. al.* 1989). Even then, bacterial systems are devoid of NADPH-cyt P450 reductase and the expressed protein must be completely or partially purified and reconstituted for catalytic analysis (Guengerich, Brian, *et. al.* 1991).

Mammalian expression systems offer an alternative for expression of eukaryotic cyt P450s, however the expression levels tend to be low precluding catalytic analysis without the use of highly sensitive methods. Expression of foreign proteins in mammalian

cells can often be transient (Guengerich, Brian, *et. al.* 1991). Additionally, such systems are expensive to use and not suitable for large-scale preparation of expressed cyt P450s. As such, mammalian cell expression is often unfeasible in many instances.

Expression systems based on *Saccharomyces cerevisiae* have been the method of choice for expression of cyt P450s since they offer several distinct advantages over other systems. On a general level, *S. cerevisiae* can be manipulated biochemically and genetically as easily as bacteria and is relatively cheap to use. In addition, with particular relevance to cyt P450 expression, *S. cerevisiae* contain an endoplasmic reticulum membrane, does not appear to have problems associated with proteolysis (Guengerich, Brian, *et. al.* 1991) and contain NADPH cyt-P450 reductase and cyt b_5. In addition, endogenous yeast cyt P450s are devoted primarily to sterol oxidation (Käpelli 1986), thus newly introduced enzyme activities can usually be distinguished from background activity of endogenous cyt P450s. Many different eukaryotic cyt P450s have been successfully expressed in yeast including those from human (Yasumori, Murayama, *et. al.* 1989; Renaud, Cullin, *et. al.* 1990), rabbit (Imai 1988; Pompon 1988), rat (Oeda, Sakaki, *et. al.* 1985) and plants (Bozak, O'Keefe, *et. al.* 1992; Vetter, Mangold, *et. al.* 1992; Holton, Brugliera, *et. al.* 1993; Pierrel, Batard, *et. al.* 1994; Urban, Werck-Reichhart, *et. al.* 1994; Kraus and Kutchan 1995).

As discussed in Section 4.1, the sequence of a particular plant cyt P450 alone can provide strong evidence as to its physiological function if the function of homologues has already been identified, as with the CYP73 and CYP74 cyt P450s. This takes much of the guesswork out of deciding upon a strategy for biochemical analysis, allowing informed decisions to be made from the outset. Also, the family a particular plant P450 is assigned to can provide some clues as to its possible physiological role albeit not as firmly as is

the case with the CYP73 and CYP74 cyt P450s. For example, as discussed in Section 4.3, members of the CYP71 family are believed to be involved in terpenoid biosynthesis. It would therefore be prudent to include a detailed investigation with these substrates if sequence analysis reveals a cyt P450 belongs to this family, as does the *A. thaliana* cyt P450 CYP71B7. However, in most situations, sequence data alone do not provide any indications as to physiological role, as is the case with the majority of plant cyt P450 cDNAs isolated to date. In these cases, pinpointing catalytic activity is more difficult and often relies on extensive screening of substrates and exhaustive biochemical assays.

Heterologous expression provides a platform to investigate function by allowing the production of the sort of quantities of the corresponding cyt P450 protein which could not usually be obtained directly from the plant. However, the approach is not without its shortcomings, since, with no knowledge of the physiological function, it may be difficult to distinguish between a poor substrate and an inactive or poorly expressed enzyme. For this reason almost all the plant cyt P450s clearly identified to date have been as a result of direct protein purification from the source tissue. Exceptions are the flavonoid 3'- 5'-hydroxylase (CYP75) (Holton, Brugliera, *et. al.* 1993) and the ferulic acid hydroxylase (CYP84) (Meyer, Cusumano, *et. al.* 1996) which were recently cloned using genetic techniques and an understanding of the physiological processes of the plant. Nevertheless, in the absence of techniques to easily purify plant cyt P450 enzymes directly, heterologous yeast expression remains the method of choice for studying the catalytic activity of cloned plant cyt P450s with a view to identifying their natural substrate. As more efficient heterologous systems are designed it is likely that the method will help to identify the physiological function of many more unidentified plant cyt P450s in years to come.

To date only one other cyt P450 originating from an *Arabidopsis* EST program

(Morris, Guerrier, *et. al.* 1993) has been successfully expressed in *S. cerevisiae*, a CYP86 (clone accession number X90458) (Benveniste and Durst 1995). This chapter details the heterologous expression of a second EST from an *A. thaliana* EST programme (Newman, de Bruijn, *et. al.* 1994), CYP71B7, and its biochemical characterisation in an attempt to assign it a physiological function.

5.2 *Results*

5.2.1 S. cerevisiae *expression of CYP71B7*

Wizard^{TM} maxiprep pZL1CYP71B7 plasmid DNA from the original DH10B host strain (Section 2.1) was prepared as described (Section 2.7), the CYP71B7 cDNA insert excised by *Eco*RI/*Not*I restriction digestion (Section 2.8) and gel purified using 'Geneclean' (Section 2.10). The insert was then ligated (Section 2.8) into *Eco*RI/*Not*I digested yeast expression shuttle vectors pYES2 and pGY (Section 2.2). The resulting plasmids, denoted pYES2CYP71B7 and pGYCYP71B7, were verified by gel electrophoresis (Section 2.9) which showed the ligations had been successful.

Following verification, plasmids pYES2CYP71B7 and pGYCYP71B7 were initially transformed (Section 2.8) into *E.coli* strain DH5α (Section 2.1) to allow amplification of the plasmids for ease of subsequent manipulation. Transformants were verified by *Eco*RI/*Not*I digestion of Wizard^{TM} miniprep plasmid DNA. Plasmid DNA from colonies which had been successfully transformed with pYES2CYP71B7 and pGYCYP71B7 was then used to transform (Section 2.8) two *S. cerevisiae* strains, R3 and ATTC44773 (Section 2.1). Transformants were selected on SD medium minus uracil. Negative controls for CYP71B7 P450 yeast expression were included by transformation of both *S. cerevisiae* strains with pGY and pYES2 vectors without CYP71B7 inserts. Restriction analysis of plasmid DNA prepared as described in Section 2.7 showed that pGYCYP71B7 and pYES2CYP71B7 had been successfully introduced into the *S. cerevisiae* strains R3 and ATTC44773, together with their respective negative controls, pYES2 and pGY.

In order to ascertain whether the transformed yeast expressed the CYP71B7 cyt P450, CO difference spectroscopy (Section 2.14) was carried out on whole yeast cells grown in liquid culture as described in Section 2.3. Whole yeast cells prepared from

ATTC44773 cells transformed with pGYCYP71B7 exhibited a faint CO difference spectrum characteristic of cyt P450 (data not shown). No such cyt P450 CO spectrum was seen with the corresponding negative control. Spectroscopic detection of cyt P450 in intact cells was difficult, with the amount of cyt P450 detected typically low. All subsequent spectroscopic analyses were therefore performed on yeast microsomal membrane fractions prepared using the methods described in Section 2.14.

Analysis of microsomal membranes allowed more definitive cyt P450 CO difference spectra to be obtained. For initial analyses, the glass bead method (Section 2.14) was used to prepare microsomal membranes. Figures 5.1 and 5.2 show the CO difference spectra exhibited by microsomes prepared from R3 and ATTC44773 cells transformed with pGYCYP71B7, and also with the pGY control. A characteristic cyt P450 CO spectrum, with an absorbance peak at 450 nm, was seen with pGYCYP71B7 in both *S. cerevisiae* strains, which was considerably lower or absent in the vector controls. CO difference spectroscopy on microsomes prepared from *S. cerevisiae* transformed with pYES2CYP71B7 showed weak P450 spectra (data not shown), but results were inconclusive and inconsistent with this vector. Galactose induction was more time consuming than growth of *S. cerevisiae* with the constitutive vector and the R3 *S. cerevisiae* strain was found to grow better than the ATTC44773 cells. Thus, only the pGYCYP71B7 and pGY R3 *S. cerevisiae* transformants were selected for use in fermentation scale-up procedures (Section 2.14) for microsomal analyses. These are referred to as 'expressing' (microsomes prepared from pGYCYP71B7 transformed *S. cerevisiae*) and 'control' (microsomes prepared from pGY transformed *S. cerevisiae*) microsomes in subsequent text.

Prior to analyses of large-scale cultures, some studies were performed to optimise

membrane and therefore cyt P450 recovery. It was found that microsomes prepared using the lytic enzyme method (Section 2.14) generally gave improved cyt P450 CO spectra and higher levels of cyt P450 than those prepared with the glass bead method (the method used to prepare the microsomes used in Figures 5.1 and 5.2). The lytic enzyme method was therefore adopted for all subsequent microsome preparations. The reason for this difference may have been due to the fact that with the glass bead method, cells are grown to a high cell density prior to harvesting. It may be that the peak for maximal activity for CYP71B7 lies at an earlier growth stage, such as that used in the lytic enzyme method. This was the case for mouse P450IA1 expressed in yeast also grown in minimal medium (Urban, Cullin, *et. al.* 1990). The optimal yeast cell density for efficient P450IA1 expression, measured in terms of P450IA1 EROD activity, was found to lie in a narrow growth stage around 2 - 3 x 10^7 cells ml^{-1}. It would be useful therefore to investigate CYP71B7 expression levels with respect to growth stage of the host cells for future optimisation of expression systems with the CYP71B7 cyt P450.

Analysis of CO difference spectra showed there was considerable variation in the amount of cyt P450 present in microsomes prepared from different batches of transformed R3 cells. On average, cells contained 17.46 +/- 4.6 pmol cyt P450 mg^{-1} microsomal protein (+/- standard error of the mean (SEM), n = 6), with a maximum of 60 pmol cyt P450 mg^{-1} microsomal protein in one preparation. Cyt P450 could also be detected in control microsomes prepared from R3 cells transformed with pGY, but again the level varied between batches. Cyt P450 levels were consistently lower in control microsomes than in expressing microsomes, averaging 8.0 +/- 3.3 pmol cyt P450 mg^{-1} microsomal protein (SEM, n = 5), with a maximum of 32 pmol mg^{-1} microsomal protein in one preparation. In certain batches of control microsomes, cyt P450 was barely detectable or completely absent.

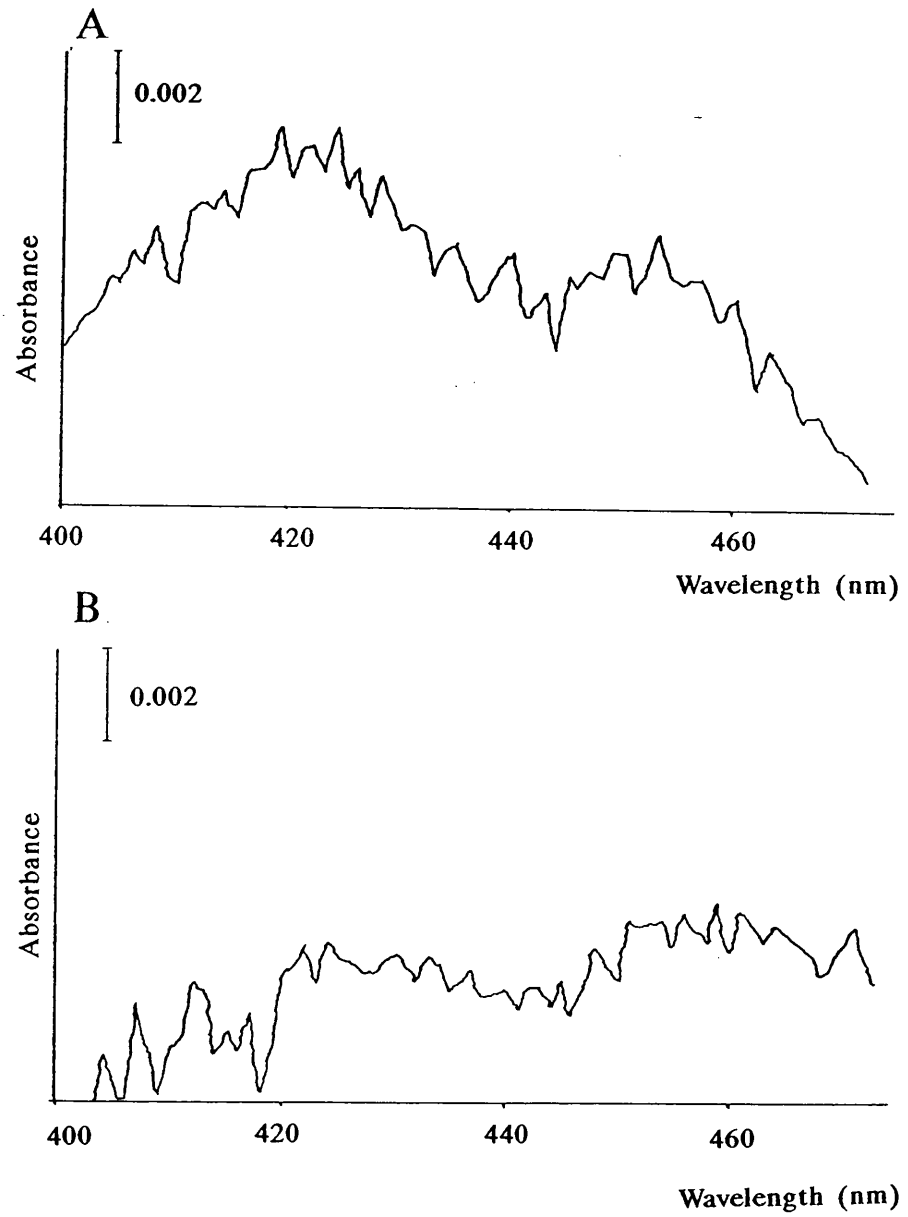

Figure 5.1 ***Cyt P450 CO difference spectrum of microsome from* S. cerevisiae *(R3) transformed with pGYCYP71B7 and pGY***

Microsomes (ca. 2 mg microsomal protein) prepared from pGYCYP71B7 (A) and pGY (B) transformed R3 cells were suspended in 20 % (v/v) glycerol, 1 mM EDTA, 10 mM Tris.Cl (pH 7.5) to 1 ml and a few grains of sodium dithionite added. The suspension was split between two 0.5 ml matched quartz cuvettes and a baseline recorded between 400 and 500 nm. CO was bubbled through the front sample cuvette for 60 s and the spectrum between 400 and 500 nm recorded. Membranes were isolated using the glass bead method.

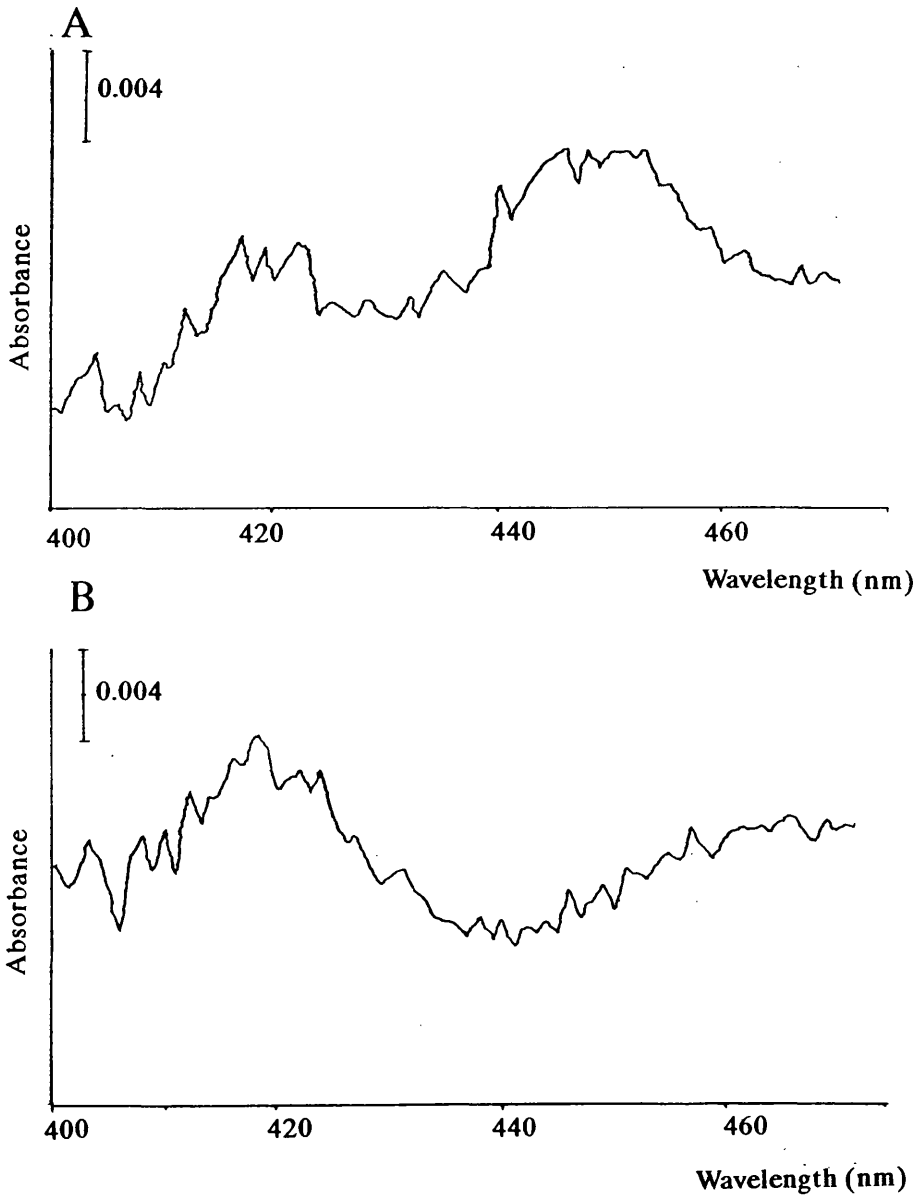

Figure 5.2 ***Cyt P450 CO difference spectrum of microsomes from*** **S.cerevisiae** ***(ATTC44773) transformed with pGYCYP71B7 and pGY***

Microsomes (ca. 2 mg microsomal protein) prepared from pGYCYP71B7 (A) and pGY (B) transformed ATTC44773 cells were suspended in 20 % (v/v) glycerol, 1 mM EDTA, 10 mM Tris.Cl (pH 7.5) to 1 ml and a few grains of sodium dithionite added. The suspension was split between two 0.5 ml matched quartz cuvettes and a baseline recorded between 400 and 500 nm.CO was bubbled through the front sample cuvette for 60 s and the spectrum between 400 and 500 nm recorded. Membranes were isolated using the glass bead method.

An analysis of variance, for a randomised block design with each preparation being considered as a block, showed that these means for control and expressing microsomes were significantly different ($P<0.05$). Figure 5.3 shows the CO difference spectra obtained for microsomes (prepared using the lytic enzyme method) from R3 cells transformed with pGYCYP71B7 and pGY. In CYP71B7 expressing microsomes the maximum absorbance occurred at 448 nm, whereas the endogenous cyt P450 isoform exhibited maximum absorbance at 452 nm. Examination of the spectrum obtained for expressing microsomes (Figure 5.3 A) shows that the absorbance peak centred at 448 nm is broad by comparison with that of control microsomes (Figure 5.3 B), with a discernable shoulder at higher wavelength. This feature implies that CYP71B7 and the endogenous yeast cyt P450 are most likely co-expressed. In previous studies with the R3 yeast strain, the spectral maximum of cyt P450 was 448 nm (Fang, Venkateswarlu, *et. al.* 1994), thus it was surprising therefore to observe a maximum of 452 nm in microsomes prepared from yeast transformed only with the pGY vector. However, this was observed consistently in all control microsome preparations. Overall the CO difference spectra clearly demonstrated the enhanced levels of cyt P450 seen with cells transformed with CYP71B7 in comparison to the control microsomes, (the spectra of which represent endogenous *S. cerevisiae* cyt P450(s)) confirming that a novel cyt P450 isoform was expressed in yeast transformed with pGYCYP71B7 at a level at least equivalent to that of endogenous cyt P450 isoforms.

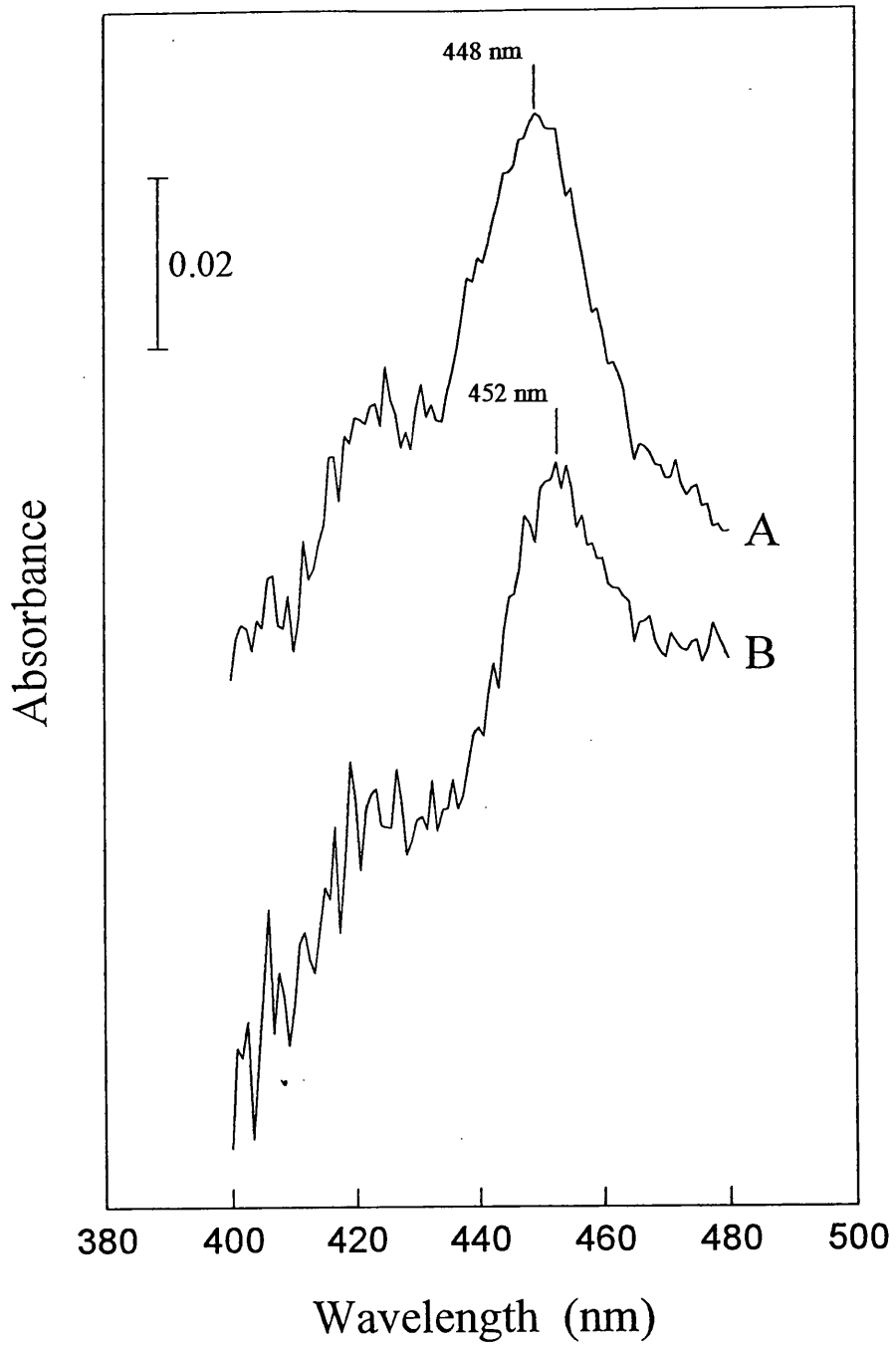

Figure 5.3 ***A comparison of the cyt P450 CO difference spectrum in microsomes from R3* S. cerevisiae *transformed with pGYCYP71B7 and pGY***

Microsomes (ca. 10 mg microsomal protein) prepared from pGYCYP71B7 (A) and pGY (B) transformed R3 cells were suspended in 20 % (v/v) glycerol, 1 mM EDTA, 10 mM Tris.Cl (pH 7.5) to 1 ml and a few grains of sodium dithionite added. The suspension was split between two 0.5 ml matched quartz cuvettes and a baseline recorded between 400 and 500 nm. CO was bubbled through the front sample cuvette for 60 s and the spectrum between 400 and 500 nm recorded. Membranes were isolated using the lytic enzyme method.

Expression of CYP71B7 in R3 cells was also verified by northern analysis (Section 2.12). *S. cerevisiae* RNA was prepared as described in Section 2.7, and hybridised to CYP71B7 cDNA. Figure 5.4 shows that CYP71B7 hybridised to a *ca.* 1.7 kb band which is the expected size for CYP71B7 transcript. RNA from cells transformed with pGY vector alone showed no such band. This result indicated that CYP71B7 cDNA was being successfully transcribed under the direction of the phosphoglycerate kinase *(PGK)* promoter in R3 *S. cerevisiae* cells.

Denaturing SDS-PAGE gel electrophoresis and western blot analysis (Section 2.14) was carried out in an attempt to detect the presence of CYP71B7 protein in microsomes from R3 yeast. SDS-PAGE analysis showed no extra or enhanced bands of the expected size in microsomes expressing CYP71B7, as compared to control microsomes (Figure 5.5). It is likely that the proportion of CYP71B7 in the microsomal protein loaded (approx 50 μg pGYCYP71B7 microsomal protein from transformed yeast contained 2 pmol or 100 picograms cyt P450) onto the gel was simply too low to detect using this approach.

Western blot analysis was performed using the antibody ARP-1, which was raised against another CYP71, from *P. americana* (O'Keefe and Leto 1989). The antibody bound to a band of *ca.* 60 kDa in the lane containing CYP71B7 R3 microsomes. No band was seen in the PGY R3 control lane (Figure 5.6). Since the expected size of CYP71B7, calculated using GCG software, is 57.2 kDa (predicted isoelectric point 8.24), the signal seen in the western may have been due to the presence of CYP71B7. The *P. americana* positive control gave a signal at *ca.* 45 kDa and the expected size for this protein is 48 kDa (O'Keefe and Leto 1989; Bozak, O'Keefe, *et. al.* 1992), serving to indicate the accuracy of the molecular weight markers and provide more confidence in this result.

Western analysis using a monoclonal antibody (Boobis, McQuade, *et. al.* 1985), designed to recognise many common epitopes of cyt P450s, did not produce clear results. Although there was a band present in the CYP71B7 lane which was not present in the PGY R3 control lane, the sizes of the bands detected in the *P.americana* control lane did not appear to correspond that expected of CYP71A1. Hence it seemed this antibody was unlikely to be binding to CYP71B7. Since ARP-1 was raised against a CYP71 cyt P450 it could be more likely to bind to CYP71B7 than the monoclonal antibody and indeed the results were clearer with the ARP-1 antibody.

Overall, CO difference spectroscopy, northern and western analysis clearly showed that CYP71B7 was being successfully expressed in R3 *S. cerevisiae*.

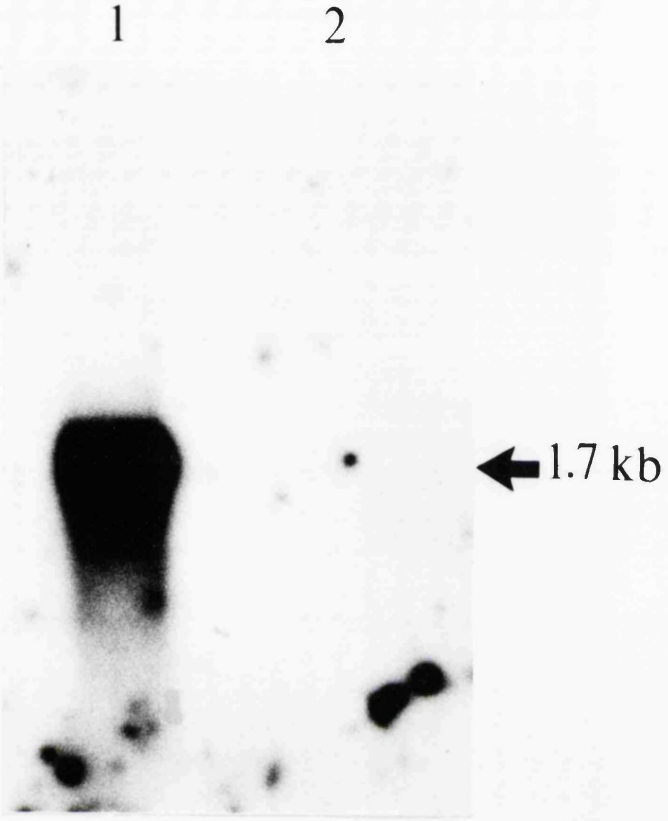

Figure 5.4 ***Northern analysis of* S. cerevisiae *transformed with CYP71B7***

RNA prepared from R3 S. cerevisiae *cells transformed with pGYCYP71B7 (lane 1) and pGY (lane 2) was separated by electrophoresis (5 µg/lane), transferred to nylon membrane and hybridised to [α-^{32}P]dCTP labelled CYP71B7. Final wash conditions were 0.1 x SSC, 0.1 % SDS, 55^{0}C for 60 min.*

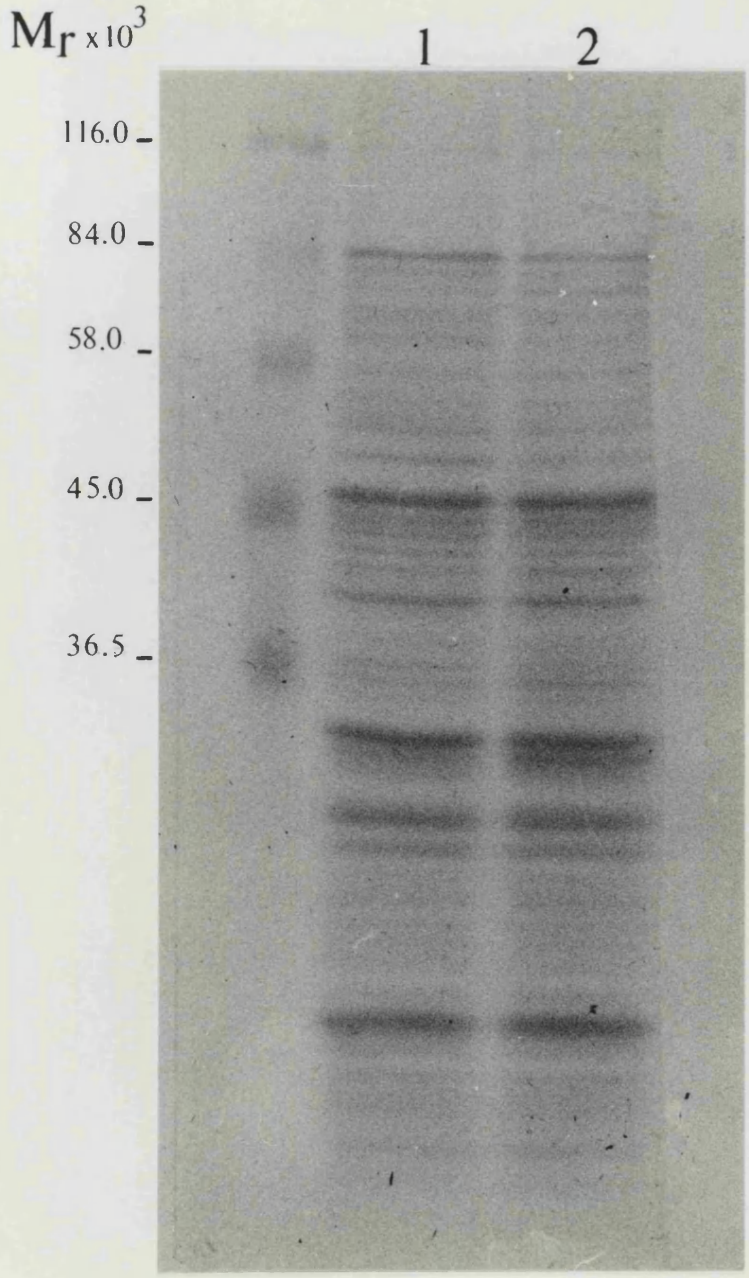

Figure 5.5 SDS-PAGE electrophoresis of control and expressing microsomes

Microsomal protein (50 µg/lane) prepared from pGYCYP71B7 transformed R3 S. cerevisiae *(lane 1) and pGY transformed* R3 S. cerevisiae *(lane 2) was separated by SDS-PAGE. Following electrophoresis the gel was coomassie-stained for visualisation of protein.*

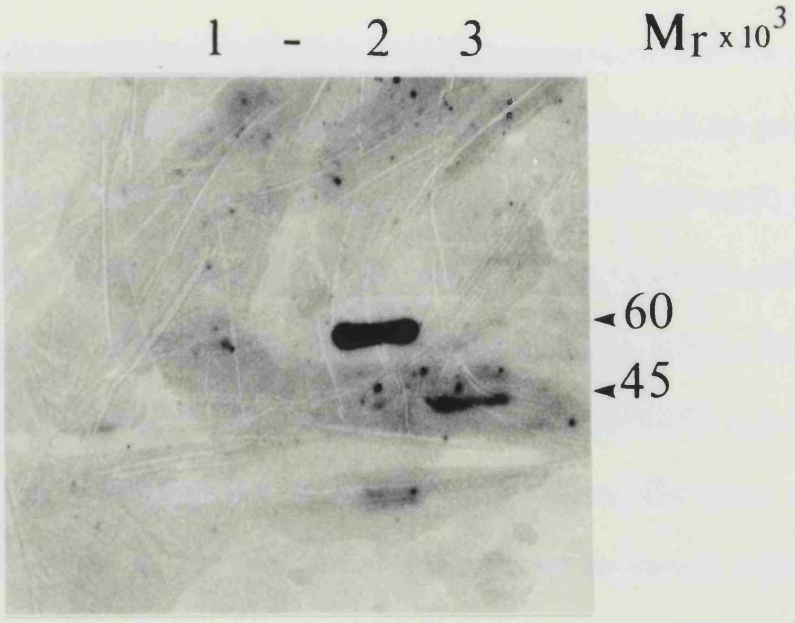

Figure 5.6 ***Western blot analysis of control and expressing microsomes***

Microsomal protein (50 µg/lane) prepared from pGY transformed R3 S. cerevisiae *(lane 1), pGYCYP71B7 transformed R3* S. cerevisiae *(lane 2) and* P. americana *mesocarp (lane 3), was separated by SDS-PAGE, transferred to nylon membrane and probed with the polyclonal Anti-ARP1. Bound antibody was visualised using enhanced chemiluminescence (Amersham).*

5.2.2 Characterisation of yeast-expressed CYP71B7

Control and expressing microsomes were assayed for activity towards the synthetic model cyt P450 substrate 7-ethoxycoumarin (7-EC). No NADPH-dependent *0*-deethylation of 7-EC (ECOD activity) could be observed in microsome preparations from both control and expressing yeasts. Control and expressing microsomes were assayed for endogenous *S. cerevisiae* cytochrome P450 reductase (CPR) and had activities of 0.13 and 0.15 μmol cyt c min^{-1} mg^{-1} respectively. These results indicated that the *S. cerevisiae* cells contained active NADPH cyt P450 reductase, therefore were potentially capable of supporting CYP71B7 catalytic activity. It was possible that the lack of NADPH-supported ECOD activity was due to inactivation of CPR through loss of flavin. The effect of including FAD and FMN (2.5 μM per assay) in the ECOD assay mixture, as well as incubation of microsomes for 10 min at room temperature in the presence of FMN and FAD was therefore investigated. Neither of these modifications to the standard ECOD assay resulted in NADPH-supported ECOD activity in CYP71B7 expressing microsomes. It appeared therefore that CYP71B7 is incapable of accepting electrons from the endogenous yeast CPR proteins(s) present in R3 yeast.

However, addition of cumene hydroperoxide (CH) to the ECOD assay mixture resulted in significant enhancement of the ECOD activity of microsomes from cells expressing CYP71B7 (Figure 5.7). Cumene hydroperoxide, like a number of organic hydroperoxides, is known to support cyt P450 catalysis by donation of active oxygen to the cyt P450 heme (Porter and Coon 1991), thus bypassing the need for electrons to be transferred from NADPH via CPR. In the presence of CH, an average rate of 5.41 +/- 1.32 pmol min^{-1} mg^{-1} (SEM, n = 11) was observed in expressing microsomes which was fairly consistent between experiments. No such enhancement was seen with the control

(Figure 5.7), or with expressing microsomes which had been boiled for 10 min prior to assaying ECOD activity. The CH-supported ECOD activity was unaffected by addition of cyt c, which might be expected to inhibit activity involving electron donation from the flavoprotein CPR.

The ECOD activity exhibited by expressing microsomes upon addition of CH was unlikely to be due merely to the presence of more endogenous cyt P450 in those microsomes since in eight separate preparations of both control and expressing microsomes, cyt P450 concentration in expressing microsomes was consistently higher (up to 4 fold) than in the control batches (see CO difference spectroscopy results above). It would appear that CYP71B7 is catalytically competent as expressed in *S. cerevisiae* as a 7-EC deethylase when the reaction is supported by cumene hydroperoxide, but not by NADPH. The rate of CH-supported ECOD activity (5.41 +/- 1.32 pmol min^{-1} mg^{-1}) observed in CYP71B7 expressing microsomes is comparable to that observed in certain other non-induced plant tissues. ECOD rates observed in wheat, wild mint, watercress and tulip for instance, were 9, 6, 5.4 and 3 pmol min^{-1} mg^{-1} respectively (Werck-Reichhart, Gabriac, *et. al.* 1990). These rates, however, are much lower than those observed in microsomes prepared from plant tissues treated with chemical agents. The rate of ECOD activity in wheat microsomes, for instance, increases to 94 pmol min^{-1} mg^{-1} when the wheat seedlings are pre-treated for 48 hrs with 5 mM phenobarbital.

The CH-supported ECOD activity supported by CYP71B7 was very stable with the reaction proceeding for up to 30 mins. This slowing of the cyt P450 catalysed ECOD activity may have been due to in part the presence of CH as this compound has been shown to inhibit the cyt P450 enzyme activity (Werck-Reichhart, Gabriac, *et. al.* 1990). However, the ECOD activity of CYP71B7 appeared to be more resilient to CH inhibition

than that of the ECOD activity of Jerulsalem artichoke microsomes where, using 125 μM CH (a concentration of over 14-fold lower than that used with CYP71B7), the reaction was linear for only 1 min. Reducing the CH concentration to 25 μM increased this linearity to 5 min. (Werck-Reichhart, Gabriac, *et. al.* 1990). However, the ability to assay ECOD inhibition with NADPH-supported activity would have been more ideal. It was concluded therefore that the rate seen for CH-dependent ECOD activity in expressing microsomes was due to the CYP71B7 cyt P450.

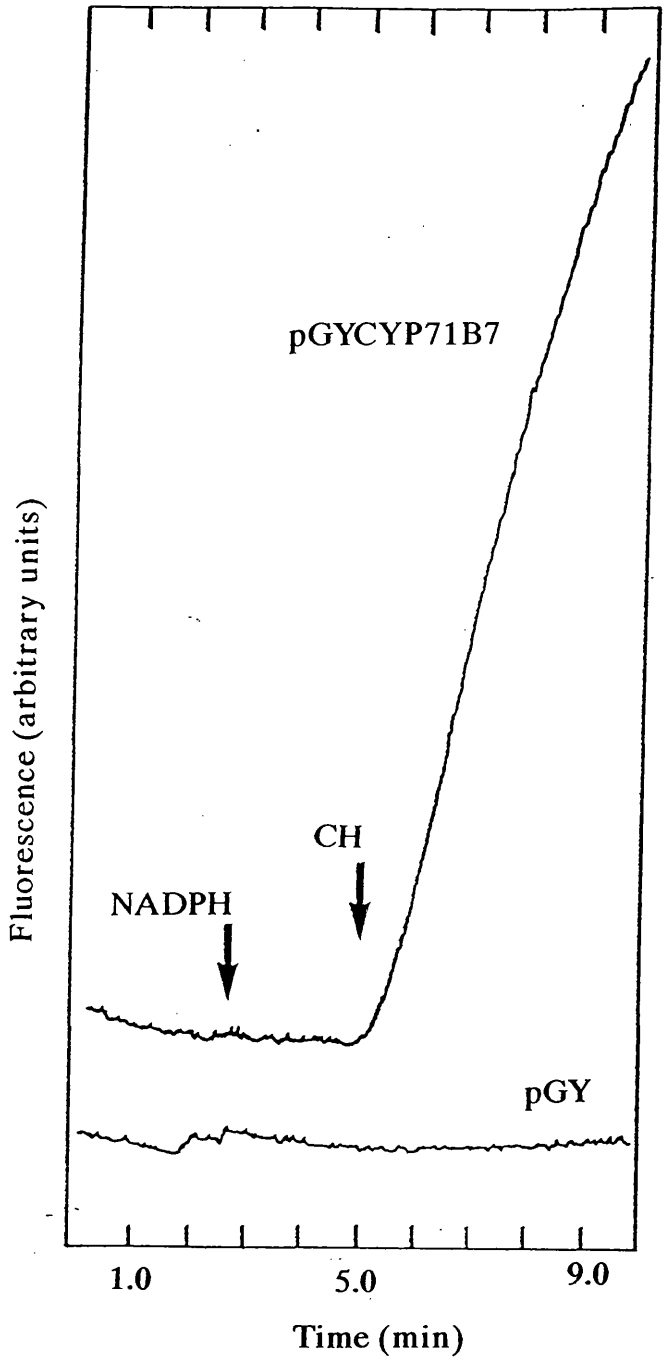

Figure 5.7 ***NADPH and cumene hydroperoxide-supported ECOD activity of CYP71B7 expressing and control microsomes.***
The assay contained 250 µM 7-ethoxycoumarin, microsomes (ca. 1 mg protein) in 50 mM Tris.Cl pH 8.6. 50 µM NADPH was added to the reaction, followed by 1 µl CH (5.4 M). The assay was carried out at 30 ^{0}C.

Microsomes prepared from pGYCYP71B7 and pGY transformed *S. cerevisiae* R3 cells were analysed for activity towards the synthetic model cyt P450 substrate 7-ethoxyresorufin (7-ER). No NADPH *0*-deethylation of 7-ER (EROD activity) was seen in each case and no enhancement of the rate was seen with the addition of cumene hydroperoxide for either control or expressing microsomes.

The artificial substrate *p*-chloro-N-methylaniline (*p*CMA) is also used as a model substrate, for studies of N-demethylation by a variety of cyt P450s from mammals, insects and some plants (Dohn and Krieger 1984). Control and expressing microsomes were assayed for *p*CMA activity. In addition, microsomes prepared from *P. americana* (var. Hass) mesocarp were also assayed. Considering the *P. americana* positive control, NADPH-supported *p*CMA activity was 57 nmol *p*CA [nmol cyt P450]$^{-1}$ mg^{-1} microsomal protein or 1.733 nmol *p*CA [nmol cyt P450]$^{-1}$ min^{-1}. When supported by CH, *p*CMA demethylation activity increased to 106 nmol *p*CA min $^{-1}$ mg^{-1} or 5 nmol *p*CA [nmol cyt P450]$^{-1}$ min^{-1}. These results compare to those observed by other researchers where an enhancement of *p*CMA activity of *P. americana* microsomes due to CH was also observed (O'Keefe and Leto 1989). Thus it appeared that the *P. americana* microsomes preparation was suitable as a positive control for *p*CMA demethylation.

Results showed that *p*CMA activity varied significantly between individual yeast microsome preparations with NADPH-supported activity of control microsomes proceeding at a rate of 0.8 pmol hr^{-1}mg^{-1} in one batch and 4.15 pmol hr^{-1}mg^{-1} in a second batch prepared on a separate occasion. NADPH-supported activity of expressing microsomes also varied between microsome preparations and was measured to be 1.28 and 6.03 pmol hr^{-1}mg^{-1} in two separate batches of expressing microsomes. There were no significant differences in NADPH-dependent *p*CMA demethylase activity between batches

of control and expressing microsomes prepared at the same time. Thus, the endogenous *S. cerevisiae* cyt P450 appeared capable of *p*CMA activity and may account for all the activity observed. When expressed as nmol *p*CA min^{-1} mg^{-1}, the values for pCMA activity are comparable to those obtained by Bozak *et al* with control microsomes prepared from *S. cerevisiae* (strain BJ5465). They reported a *p*CMA activity of 10 pmol min^{-1} mg^{-1} for the yeast transformed with pYES2 vector (Bozak, O'Keefe, *et. al.* 1992). Control and expressing microsomes of the first preparation showed an average rate of 13 and 20 pmol *p*CA min^{-1} mg^{-1} respectively, which are quite similar to the result of Bozak *et al* with control microsomes. In the case of the first microsome preparations, the control microsomes contained 49 pmol endogenous cyt P450 per assay and the expressing microsomes contained 100 pmol cyt P450 in total. If CYP71B7 were capable of *p*CMA activity it would be expected that microsomes expressing this enzyme would show at least twice the *p*CMA activity than that which was exhibited by the control microsomes. In the case of the second microsome preparation, the control microsomes contained 17 pmol cyt P450 and the expressing microsomes 100 pmol cyt P450. Despite having five times more cyt P450 than their control counterpart, the *p*CMA activity of expressing microsomes was not significantly greater, and control microsomes showed at least 70 % of the activity seen in expressing microsomes. This again indicated that CYP71B7 is incapable of *p*CMA activity. The slightly higher activity seen with expressing microsomes as compared to the control in both microsome preparations may have been due to a higher level of endogenous cyt P450, since it is not possible to separate the proportions of endogenous cyt P450 and CYP71B7 cyt P450 responsible for *p*CMA demethylation in the 100 pmol used in the assay with expressing microsomes.

The effect of adding cumene hydroperoxide (CH) into the *p*CMA demethylase

assay was measured. If CYP71B7 expressing microsomes did support *p*CMA demethylation as they do ECOD activity, it would be expected that CH would produce a similar enhancement effect as seen within the ECOD assay carried out on expressing microsomes. The addition of CH to the assay with expressing and control microsomes had no significant effect on *pCMA* activity with CH-supported activity proceeding at a rate of 6.47 and 6.56 pmol $hr^{-1}mg^{-1}$ for control and expressing microsomes respectively. The results indicated that CYP71B7 is incapable of *p*CMA demethylation.

5.2.3 Substrate screening using optical difference binding spectra

As discussed in Chapter 1, the binding of compounds to cyt P450 can be monitored using difference spectroscopy. On binding of a substrate, a type I difference spectrum is typically obtained with a peak at 390 nm and a trough at 420 nm (Jefcoate 1978). A variety of potential cyt P450 substrates, including the terpenes, kaurene, cineole, nerol and S-limonene, the fatty acids oleic and lauric acid and the phenylpropanoids ferulic, benzoic and cinnamic acid were tested using optical difference spectroscopy carried out as described in Section 2.14. In addition, the cyt P450 inhibitors clotrimazole (1-[chloro-α,α-diphenylbenzyl]imidazole) and paclobutrazol were tested.

No type I binding spectra were seen for any of the natural substrates of cyt P450 tested. These included cineole, nerol and S-limonene which are monoterpenoids, potential substrates for the CYP71 cyt P450s (Hallahan, Dawson, *et. al.* 1992; Hallahan, Nugent, *et. al.* 1992). However, it may be that these particular terpenoids are not substrates of CYP71B7 and there are many other terpenoids which may be possible substrates. On the other hand, it may have been the amount of cyt P450 which was used in this particular type of assay was limiting. In these assays, 5.25 mg microsomal protein, corresponding to 80 pmol cyt P450 per cuvette, was used. This is the same amount which showed a

clearly detectable CO difference spectrum with these microsomes and it would be reasonable to assume that it would also yield detectable type I binding spectra for natural substrates. However, with *P. americana* microsomes, containing CYP71A1, 385 pmol cyt P450 per cuvette were used to generate clear type I binding spectra with the terpenoids nerol and geraniol (Hallahan, Nugent, *et. al.* 1992). It may well be that 80 pmol of cyt P450 in expressing microsomes, some of which must also represent endogenous cyt P450, was not sufficient to detect substrate binding in this way. Since only a certain amount of protein can be included in the assay, due to the turbidity of the sample, it appeared that it was not feasible to exploit difference spectroscopy to investigate substrate binding to CYP71B7 expressed in yeast. Another drawback of this technique was the fact that some light-absorbing compounds, such as the flavonoid naringenin, cannot be assayed for binding due to spectral interference. For these reasons, the more sensitive technique of investigating substrate binding by inhibition of the ECOD activity of CYP71B7 was used as described below.

The expressing microsomes did appear to exhibit type II binding spectra however, characterised by a broad trough at 390 nm to 410 nm and a peak between 425 nm and 435 nm, with the cyt P450 specific inhibitor clotrimazole. This spectrum was also seen with the control microsomes (Figure 5.8). The difference in absorbance between peak to trough ($\Delta A_{425-390}$) is proportional to inhibitor-complexed cyt P450. This was the same for both control and expressing microsomes when 1.0 μM clotrimazole was used (($\Delta A_{425-390}$ = 0.007). There was a slight increase in the value for $\Delta A_{425-390}$ with expressing microsomes as compared to the control when 10 μM ($\Delta A_{425-390}$ = 0.007 and 0.009 for control and expressing microsomes respectively) and 20 μM clotrimazole ($\Delta A_{425-390}$ = 0.008, and 0.0122) was used. Since $\Delta A_{425-390}$ did not increase with

increasing clotrimazole concentration for control microsomes, it indicated that the endogenous cyt P450 (lanosterol demethylase) was probably saturated by 1.0 μM clotrimazole. The increase in $\Delta A_{425-390}$ seen with the expressing microsomes at higher concentrations indicated that this cyt P450 inhibitor might be binding to CYP71B7. Since each sample studied contained an equivalent amount of cyt P450 (80 pmol per 0.25 ml cuvette, 0.32 μM), this also indicated that a cyt P450 other than endogenous *S. cerevisiae* cyt P450 was binding to clotrimazole. This cyt P450 was likely to have been CYP71B7, but any absorbance changes seen with the addition of clotrimazole were small and may not therefore have been significant. Thus, it was not possible to determine whether CYP71B7 was capable of binding this inhibitor from these results. Clotrimazole is a substituted imidazole which has previously been shown to strongly inhibit limonene hydroxylase from *Mentha spicata* (spearmint), *Mentha piperita* (peppermint), and to a much lesser extent inhibited a limonene hydroxylase from a different plant species, *Perilla frutescens* (perilla), thus allowing the different regiospecific forms of limonene hydroxylase systems to be distinguished from each other (Karp, Mihaliak, *et. al.* 1990).

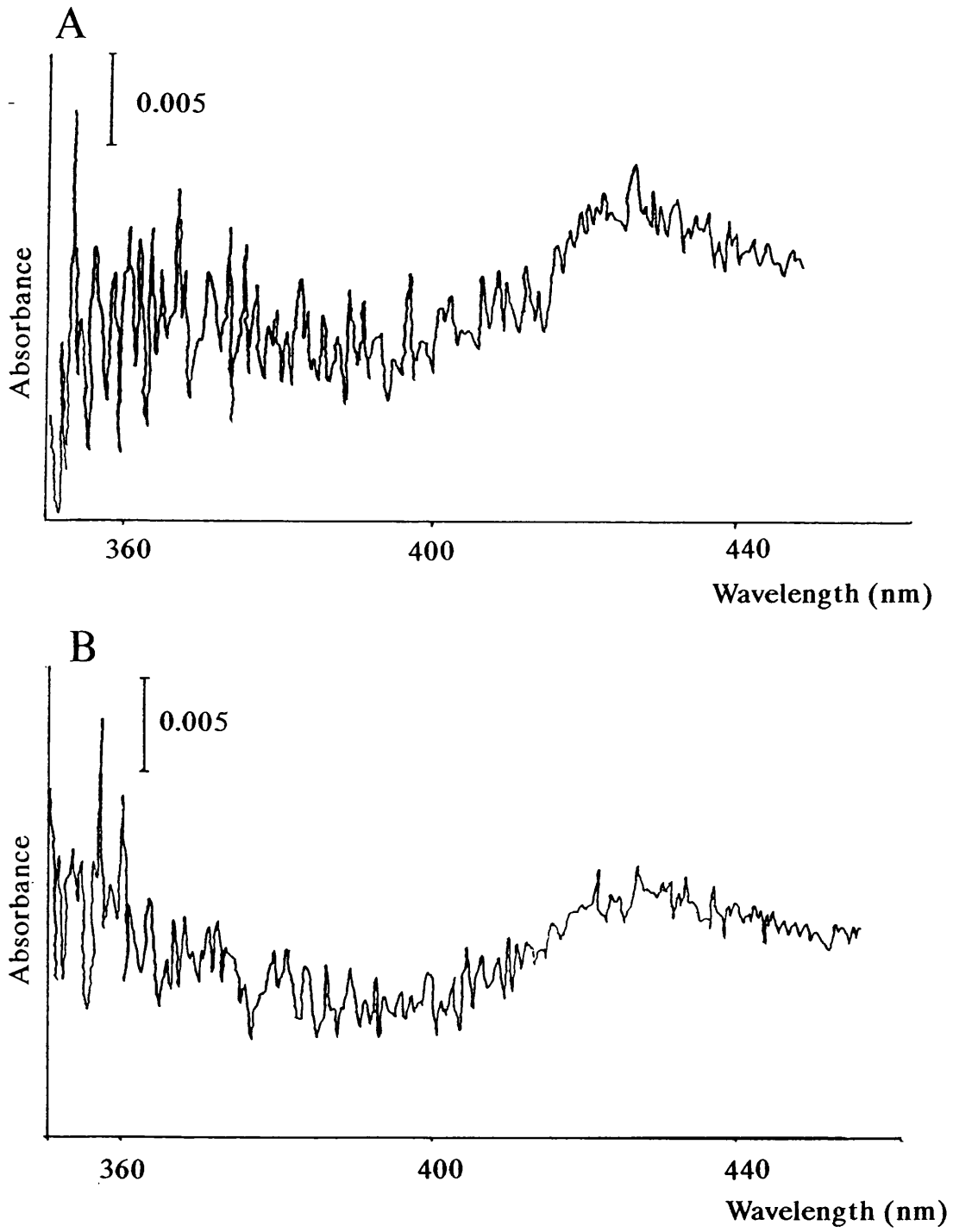

Figure 5.8 ***Clotrimazole-induced Type II difference spectra***

Microsomes (ca. 1 mg microsomal protein) prepared from CYP71B7 expressing (A) or pGY control (B) R3 cells were suspended in 20 % (v/v) glycerol, 1 mM EDTA, 10 mM Tris.Cl (pH 7.5) to 1 ml. The suspension was split between two 0.5 ml matched quartz cuvettes and a baseline recorded between 350 and 500 nm. Clotrimazole (1 μM) was added to the front sample cuvette and the spectrum re-recorded.

The binding to cyt P450 in control and expressing microsomes of two different enantiomers of paclobutrazol, one a plant growth retardant (PAC-PGR) which specifically inhibits the plant cyt P450 kaurene oxidase involved in gibberellin biosynthesis, the other a fungal cyt P450 lanosterol 14α-demethylase inhibitor (PAC-Fungal) (Burden, Carter, *et. al.* 1987), was investigated.

A type II binding spectrum was seen with expressing microsomes incubated with PAC-PGR. This was also seen with the control microsomes (Figure 5.9), but the spectrum was not as clear as that with expressing microsomes at all concentrations (1.0, 10 and 90 μM) of inhibitor tested. The difference in absorbance between peak to trough ($\Delta A_{435-390}$) was the same for control and expressing microsomes respectively using 1.0 μM PAC-PGR, ($\Delta A_{435-390}$ = 0.003). There was a slight increase in the value for $\Delta A_{435-390}$ with expressing microsomes as compared to the control when 10 μM PAC-PGR was used, ($\Delta A_{435-390}$ = 0.005) which was not seen with control microsomes with the same concentration of inhibitor. However, this increase was minor and may not have been significant. There was no further increase seen for expressing microsomes when 90 μM PAC-PGR was used ($\Delta A_{435-390}$ = 0.003.) and since results were generally inconsistent with this inhibitor it was not possible to determine whether CYP71B7 was capable of binding to PAC-PGR on the basis of these results.

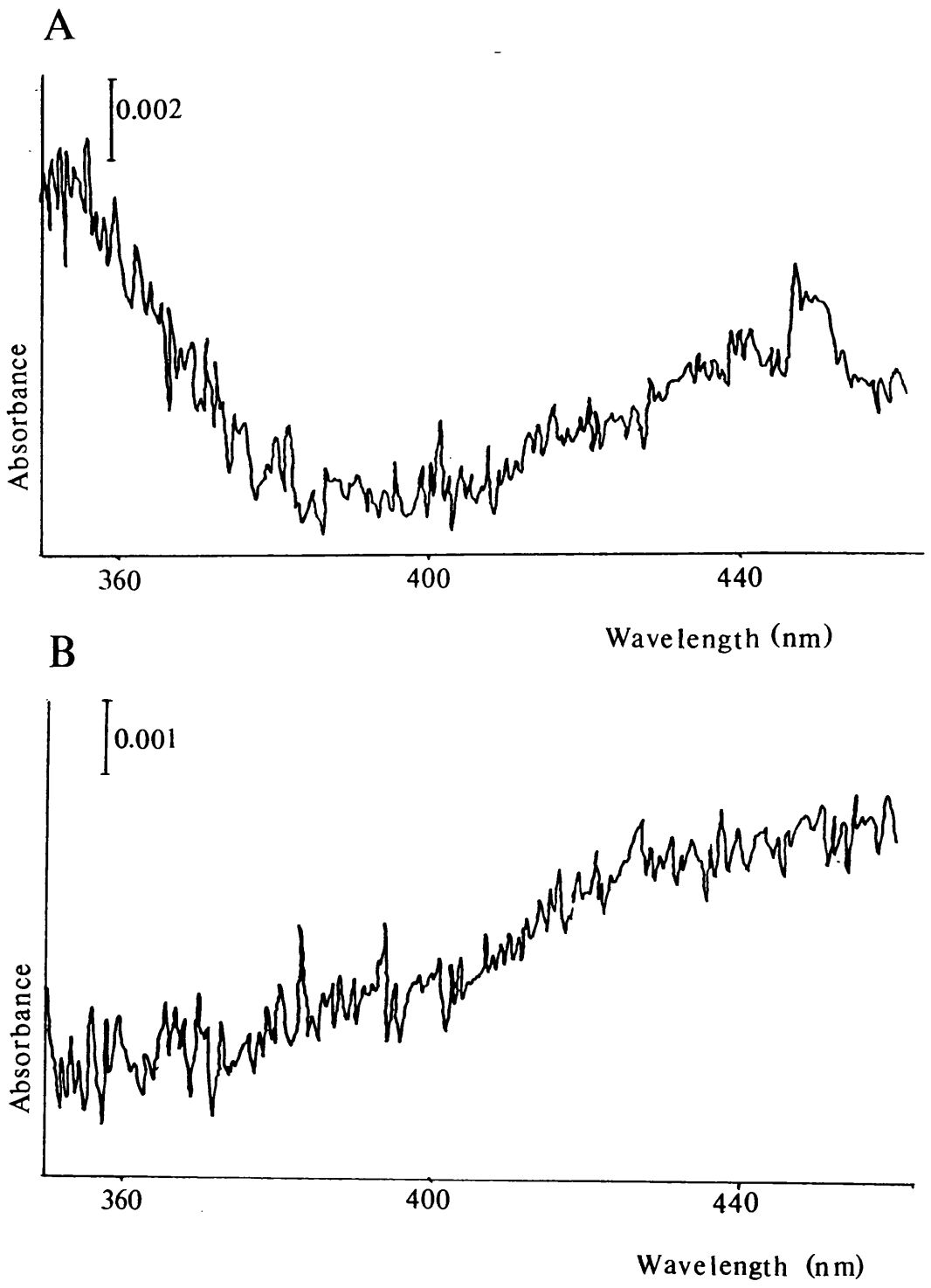

Figure 5.9 PAC-PGR-induced Type II difference spectra

Microsomes (ca. 1 mg microsomal protein) prepared from CYP71B7 expressing (A) or pGY control (B) R3 cells were suspended in 20 % (v/v) glycerol, 1 mM EDTA, 10 mM Tris.Cl (pH 7.5) to 1 ml. The suspension was split between two 0.5 ml matched quartz cuvettes and a baseline recorded between 350 and 500 nm. PAC-PGR (90 μM) was added to the front sample cuvette and the spectrum re-recorded.

A type II binding spectrum was seen for both control and expressing microsomes with 10 μM of the lanosterol 14α-demethylase specific form of paclobutrazol (Figure 5.10). The difference in absorbance from peak to trough at this concentration was similar for control and expressing microsomes ($\Delta A_{435-390}$= 0.002 and 0.003 respectively). This would be expected since both sets of microsomes would contain a similar amount of endogenous cyt P450 represented by lanosterol 14α-demethylase in yeast. No real type II spectrum was observed at the lower concentration of 1.0 μM PAC-Fungal. It was not possible to determine whether the CYP71B7 also bound PAC-Fungal since no further concentrations were tested. Lanosterol 14α-demethylation is a key step in the synthesis of ergosterol, the principal sterol in yeasts. The cyt P450 responsible, $P450_{DM,}$ is only known to exhibit a type I binding spectrum with lanosterol, indicating it has a very a narrow substrate specificity (Yoshida and Aoyama 1984). It has also been shown that azole antifungal agents, such as paclobutrazol, have a extremely high affinity to this enzyme. Indeed, ketoconazole, another azole antifungal compound has an apparent Kd (representing the binding affinity) of 0.01 μM (Yoshida and Aoyama 1987). Since this reaction is not known to occur in plants, PAC-Fungal was highly likely to be only binding to *S. cerevisiae* lanosterol 14α-demethylase present in the microsomes.

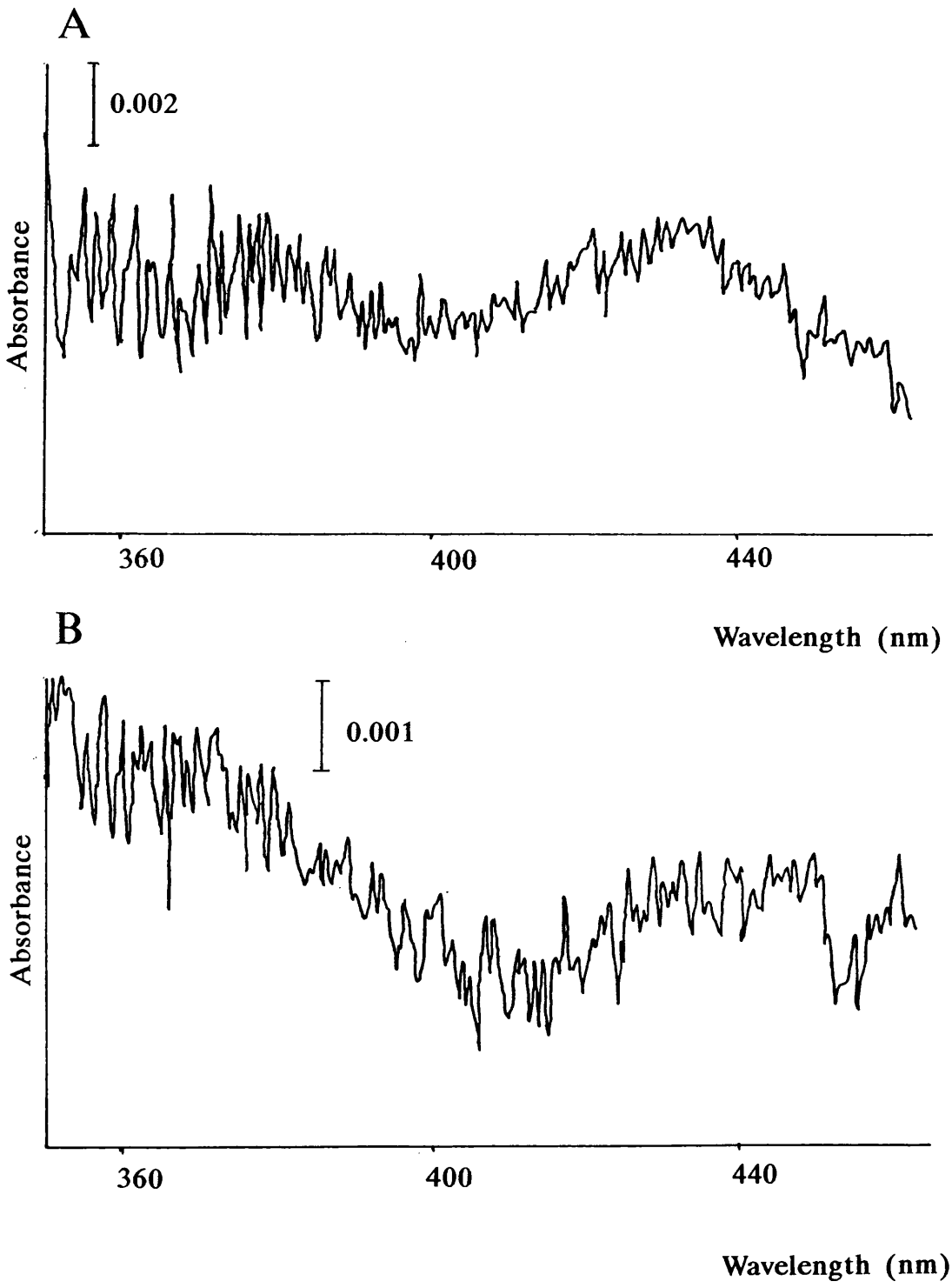

***Figure 5.10** PAC-Fungal-induced Type II difference spectra*

Microsomes (ca. 1 mg microsomal protein) prepared from CYP71B7 expressing (A) or pGY control (B) R3 cells were suspended in 20 % (v/v) glycerol, 1 mM EDTA, 10 mM Tris.Cl (pH 7.5) to 1 ml. The suspension was split between two 0.5 ml matched quartz cuvettes and a baseline recorded between 350 and 500 nm. PAC-Fungal (10 μM) was added to the front sample cuvette and the spectrum re-recorded.

5.2.4 Substrate screening by ECOD inhibition assay

A variety of potential substrates of CYP71B7 cyt P450, (detailed in Table 5.1), were screened by assaying for their ability to inhibit of CH-supported ECOD activity (at a concentration of 250 μM) as described in Section 2.14. These included physiological substrates for plant cyt P450s known to be involved in terpenoid hydroxylation, phenylpropanoid metabolism, flavonoid biosynthesis, fatty acid hydroxylation and xenobiotic metabolism. In addition, amino acid precursors of glucosinolate biosynthesis and two glucosinolates were included since there is evidence to suggest cyt P450 involvement in the biosynthesis and hydroxylation of these compounds in plants as discussed in the previous chapter, Section 4.3. Compounds capable of binding to the active site of CYP71B7, inhibiting CH-supported ECOD activity, could be potential natural CYP71B7 substrates.

The results of the ECOD inhibition substrate screening showed that out of the ten terpenoids tested, six clearly inhibited CH-dependent ECOD activity of CYP71B7 (Figure 5.11). These were S-limonene, terpinolene, p-menth-1-ene, citronellol, geraniol and nerol. A seventh monoterpene, linalool, slightly inhibited ECOD activity at the concentration tested. Figure 5.11 also shows the lack of inhibition of ECOD by the herbicide chlortoluron (3-(3-chloro-4-methylphenyl)-1,1-dimethylurea). One of the monoterpenes which demonstrated ECOD inhibition, S-limonene, had been included in the optical binding spectra studies (See Section 5.2.3 above) and had not shown a type I binding spectrum with expressing microsomes. This again indicated that the optical binding spectra may not have been sensitive enough to identify compounds which bind to CYP71B7 cyt P450, and therefore was not a technique suitable for substrate identification in this particular case. Alternatively, it is possible that the concentration of limonene used

induced compensating type I and reverse type I binding spectra, essentially leading to no spectral response (Jefcoate 1978). For this reason, compounds should ideally be tested over a wide range of concentrations since generally type I and reverse type I cyt P450 binding exhibit different spectral dissociation constant (Ks) (Jefcoate 1978).

Only two non-terpenoids inhibited ECOD activity of CYP71B7, ferulic acid and *p*-coumaric (4-hydroxycinnamic) acid (Figure 5.12), substrates of cyt P450s involved in phenylpropanoid metabolism. Another cyt P450 substrate involved in phenylpropanoid biosynthesis, cinnamic acid with a very similar structure had no effect as was the case with the structurally similar L-phenylalanine.

***Table 5.1** Potential CYP71B7 substrates tested by assay of ECOD inhibition*

Compound	Inhibition	Compound	Inhibition
S-Limonene	++	Ferulic acid	++
Terpinolene	++	*p*-coumaric acid	++
p-menth-1-ene	++	Cinnamic acid	-
Citronellol	++	Benzoic acid	-
Nerol	++	Salicylic acid	-
Geraniol	++	Kaempferol	-
Linalool	+	Lauric acid	-
Cineole	-	Chlortoluron	-
Kaurene	-	L-tryptophan	-
Abscisic acid	-	L-phenylalanine	-
Homo-methionine	-	L-methionine	-
Tri-homo-methionine	-	Phenylethyl glucosinolate	-
Tetra-homo-methionine	-	3-butenyl glucosinolate	-

++ indicates strong inhibition of ECOD activity observed, + indicates weak inhibition of ECOD activity observed and - indicates no ECOD inhibition observed.

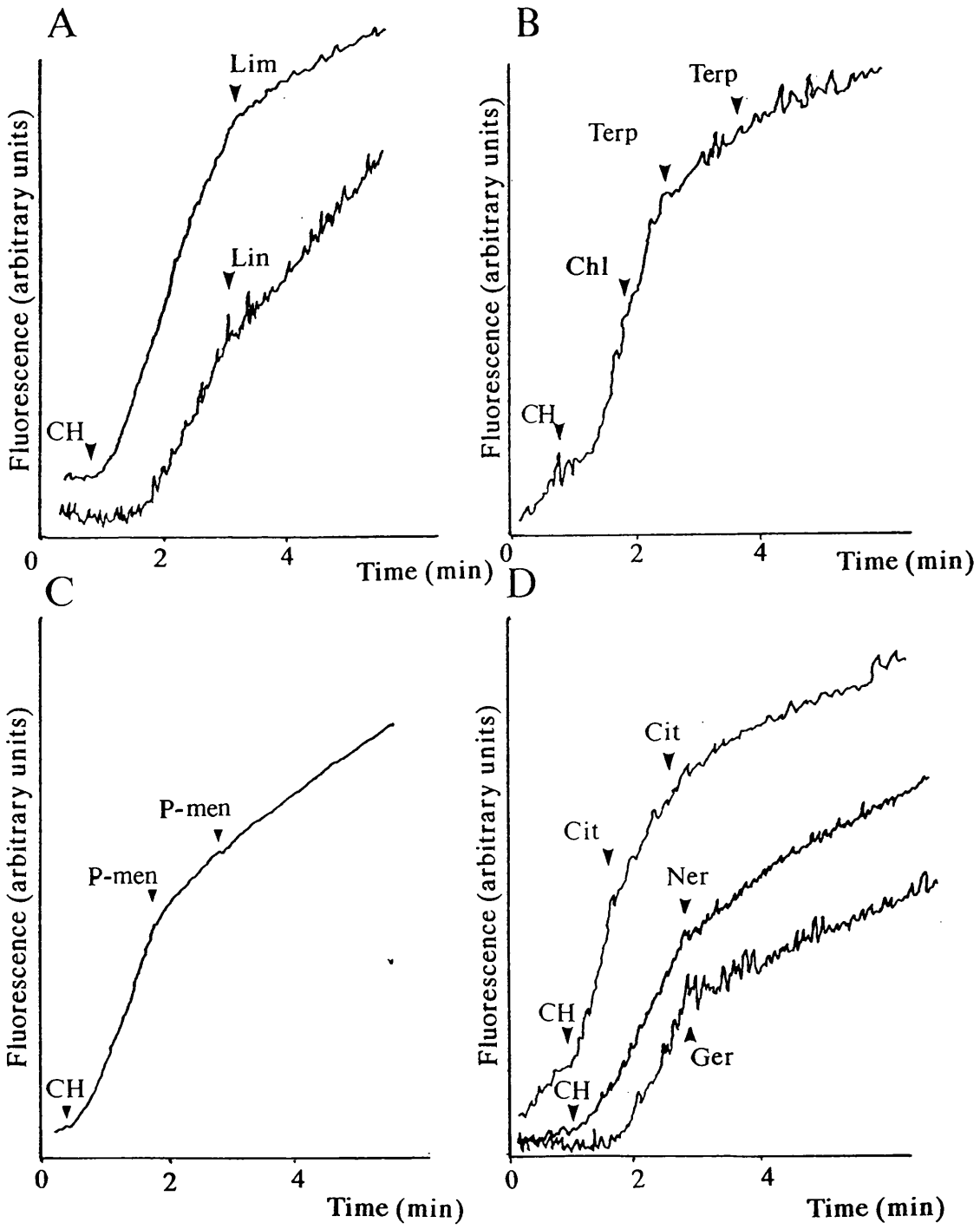

Figure 5.11 Inhibition of ECOD activity of yeast expressed CYP71B7 by monoterpenoids

The assay contained 250 µM 7-ethoxycoumarin, microsomes (ca. 1.0 mg protein) in 50 mM Tris.Cl pH 8.6. 1 µl CH (5.4 M) was added to start the reaction. The cyclic monoterpenoids linalool (Lin), limonene (Lim) (A), terpinolene (Terp) (B) and p-menth-1-ene (P-men) (C), and the acyclic monoterpenoids geraniol (Ger), nerol (Ner) and citronellol (Cit) (D) were added into the reaction at a final concentration of 250 µM. The assay was carried out at 30^{0}C. Graph B also shows the lack of inhibition by the herbicide chlortoluron (Chl).

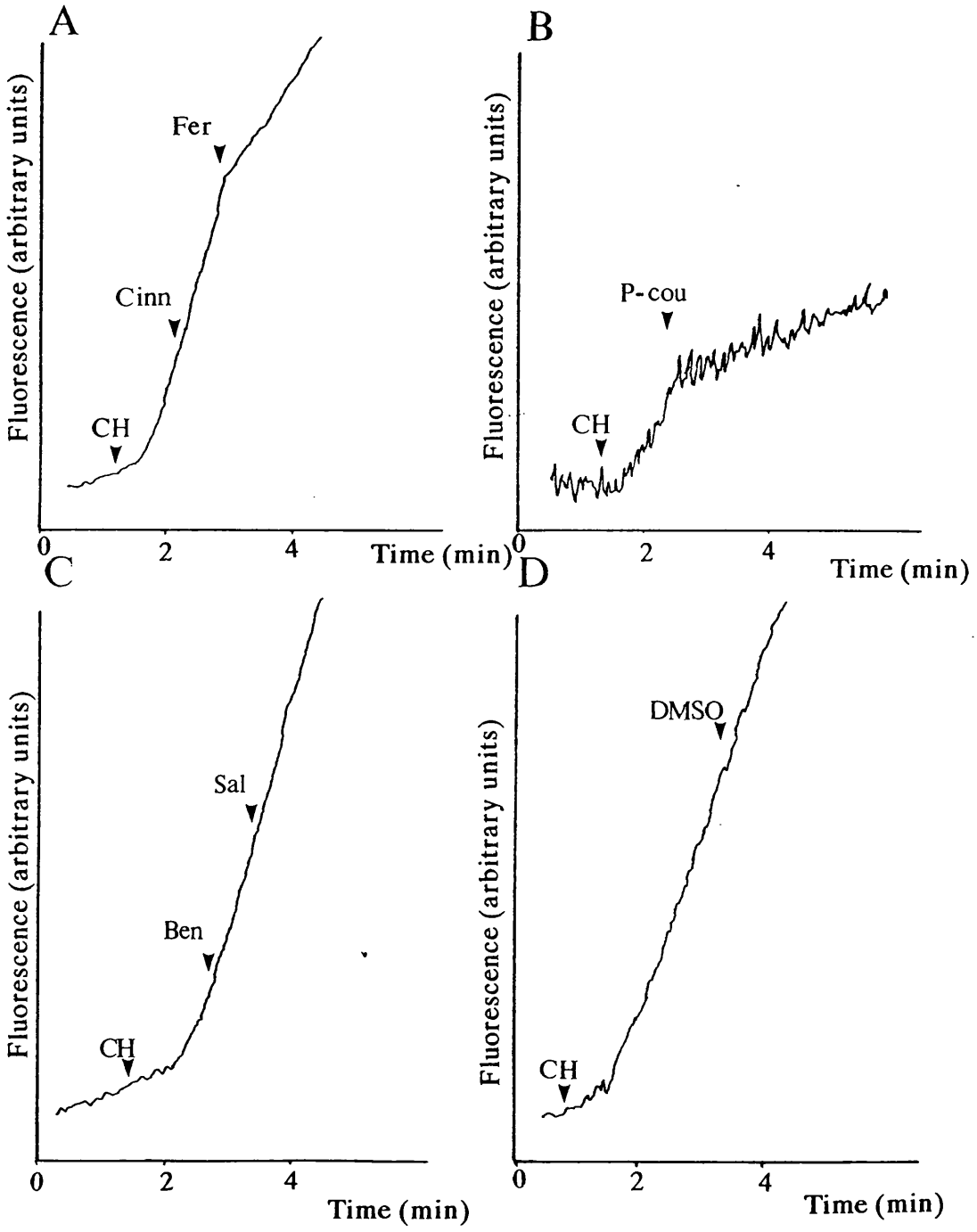

Figure 5.12 Inhibition of ECOD activity of yeast expressed CYP71B7 by phenylpropanoids

The assay contained 250 µM 7-ethoxycoumarin, microsomes (ca. 1 mg protein) in 50 mM Tris.Cl pH 8.6. 1 µl CH (5.4 M) was added to start the reaction. Ferulic (Fer) and cinnamic (Cinn) acid (A), p-*coumaric acid (P-cou) (B), benzoic (Ben) and salicylic (Sal) acid (C) and DMSO (D) were added into the reaction mixture after ca. 1 to 2 mins. The assay was carried out at* 30^0*C.*

5.3 *Discussion*

Successful yeast expression of the Arabidopsis EST clone 5G6 belonging to the cyt P450 family CYP71B7 was achieved in two different yeast strains under the direction of either a constitutive or inducible promoter. The highest and most consistent expression was obtained using the vector pGY with the constitutive phosphoglycerate kinase (*PGK*) promoter system in the yeast strain R3.

Cyt P450 levels of up to 60 pmol mg^{-1} microsomal protein were obtained using this vector and host system in microsomes prepared from cells expressing CYP71B7. Levels in these microsomes were routinely twice the level of cyt P450 in the pGY controls and in one preparation was four times the amount. These results are comparable to those obtained for plant cyt P450 expression in yeast by other researchers including expression of a *C. roseus* cyt P450 (80 +/- 8 pmol mg^{-1} microsomal protein) (Vetter, Mangold, *et. al.* 1992), *P. americana* CYP71A1 (50 pmol mg^{-1} microsomal protein) (Bozak, O'Keefe, *et. al.* 1992) and a *Helianthus tuberosus* cinnamate 4-hydroxylase (80 +/- 20 pmol mg^{-1} microsomal protein) (Urban, Werck-Reichhart, *et. al.* 1994).

Expression levels in yeast obtained with mammalian and human P450s are also comparable with these results, with levels ranging from 90 - 200 pmol cyt P450 mg^{-1} microsomal protein under similar conditions (Yasumori, Murayama, *et. al.* 1989; Renaud, Cullin, *et. al.* 1990). However, direct comparison of P450 levels is not always possible since different researchers utilise different promoter systems and host strains and in many of the cases cited above the inducible galactose promoter system rather than the constitutive *PGK* promoter system was used to direct expression.

The wide variation in the levels reported may be due to the fact that a range of different factors can influence expression levels. On a general level, the amount of

foreign cyt P450 expressed can be a function of the growth conditions used for the host yeast. Of primary importance in recombinant yeast production (and thus the level of expressed protein obtained) is the oxygen transfer across the medium which in turn is affected by agitation, vessel geometry, antifoam addition (in the case of fermentation scale up-experiments), culture viscosity, cell density, metabolic rate and temperature (Guengerich, Brian, *et. al.* 1991). In addition, the method used to prepare microsomes may be an important factor in the amount of product obtained, for example when lytic enzyme methods are used to lyse yeast cells, it is important the OD_{600} at cell harvest does not exceed 1.8 since older cells are difficult to lyse by this method (Guengerich, Brian, *et. al.* 1991). Use of mechanical breakage procedures can allow cells to be harvested at a later stage, but even then multiple generations of cells tend to encourage plasmid loss or genetic drift (Guengerich, Brian, *et. al.* 1991), and the host cell growth stage where the cyt P450 has maximal specific activity may have been missed.

Other factors which can influence the amount of foreign protein produced include inoculum age and amount, growth rate, pH, carbon source, nitrogen source and concentration (Guengerich, Brian, *et. al.* 1991). Clearly many of the factors discussed above may vary significantly between experiments, and, indeed, research laboratories, explaining perhaps why consistency in cyt P450 expression levels is not always obtained.

An additional factor which can influence the amount of cyt P450 produced in yeast is the promoter system used and again seems to depend on the particular cyt P450 being expressed and results vary widely. In the case of expression of CYP71B7, the *PGK* promoter gave higher expression levels than the inducible *GAL* promoter system, as was the case for the expression of certain mammalian P450s in yeast (Pompon 1988; Pompon and Cullin 1989). This is, however, not always the case and these promoters have been

found to direct cDNA transcription at similar efficiencies (Cullin and Pompon 1988). Thus, optimisation of expression may involve testing a number of different promoter systems.

The amount of non-coding flanking sequence can also affect cyt P450 expression and deletion of the 5'- and 3'- non-coding regions originally present in the heterologous cDNA before its insertion into a yeast transcription unit has been shown to enhance expression levels (Pompon 1988). However, the CYP71B7 cDNA used had 52 bp of 5' flanking region, which did not prevent expression. The complete or partial removal of this region might enhance cyt P450 expression levels in yeast, and this may well be worthy of future investigation. It may also be worth investigating alternative systems to yeast expression such as those based on mammalian cells or baculovirus infection of insect cells. However, the levels of expression obtained were comparable to other examples reported and sufficient to allow biochemical analysis of the plant cyt P450 enzyme it represented.

As discussed in the previous chapter, other CYP71 cyt P450s have been demonstrated to catalyse demethylation of *p*CMA and deethylation of 7-EC. CYP71B7 did exhibit ECOD activity but only in the presence of cumene hydroperoxide. Neither NADPH or CH-supported EROD activity was seen with CYP71B7. De-ethylation of alkoxycoumarins such as 7-EC and 7-ER were previously attributed to two distinct plant cytochrome P450 isoforms on the basis of their differing distribution between species and tissues, induction patterns and inhibitor susceptibility (Werck-Reichhart, Gabriac, *et. al.* 1990). In that study it was found that de-ethylation of 7-ER could not be supported by CH, but was only supported by NADPH. Given that activity of the *A. thaliana* cyt P450 cannot be supported by NADPH when expressed in yeast microsomes, the possibility that

this isoform is capable of alkophenoxazome metabolism cannot be ruled out.

Demethylation of *p*CMA by the CYP71B7 enzyme was not clearly demonstrated, but the results indicated that the endogenous yeast cyt P450 could catalyse this reaction, with lower activity than *P. americana* mesocarp microsomes.

The fact that, despite the presence of catalytically competent endogenous CPR, only CH-dependent deethylation of 7-EC was seen indicated that the CYP71B7 was incapable of accepting electrons from NADPH via the endogenous yeast CPR. Although at first sight yeast appears to be an ideal host for the reconstitution of heterologously expressed cyt P450, the data indicate that for certain cyt P450 isoforms the redox environment is inappropriate. Poor interaction between yeast expressed cyt P450 and endogenous yeast redox enzymes has previously been demonstrated. For example, the efficiency of coupling between yeast reductase and the mammalian cyt P450 IA1 was shown to be strongly dependant on the cyt P450 expression level. This resulted in a poor specific activity when expressed at a high level in yeast, indicating that the level of endogenous yeast reductase was limiting (Urban, Cullin, *et. al.* 1990). Another example, with the expression of the plant cyt P450 cinnamate hydroxylase, showed that the enzyme activity was affected by the levels of endogenous CPR (Urban, Werck-Reichhart, *et. al.* 1994). Since the *S. cerevisiae* host strain R3 had low endogenous levels of cyt P450, it is likely that the levels of CPR are perhaps the cause of problems with regard to coupling with the foreign CYP71B7 cyt P450. This type of situation was also suspected with the yeast expression of *P. americana* CYP71A1 (Bozak, O'Keefe, *et. al.* 1992). The specific activity of the R3 yeast reductase at *ca.* 0.14 μM cyt c reduced min^{-1} mg^{-1} seems low in comparison to that measured with purified plant CPRs with specific activities reported to be 50, 17, 29 and 33 μM cyt c reduced min^{-1} mg^{-1} for mung bean (Shet, Sathasivan,

et. al. 1993), *C. roseus*, sweet potato and Jerusalem artichoke respectively (Durst 1991). Thus it may well be that the level of CPR in R3 yeast was simply not high enough to sustain CYP71B7 activity. If this is the case it would be useful to attempt to increase CYP71B7 activity by transforming yeast strains engineered to have increased levels of endogenous reductase (Urban, Werck-Reichhart, *et. al.* 1994; Pompon, Louerat, *et. al.* 1995). These methods use vectors which allow integration of the particular reductase used into the yeast genome. The copy number is therefore stable independently of culture conditions and expression relies only upon promoter efficiency, thus overcoming problems of genetic instability.

Alternatively, the low specific activity of expressed CYP71B7 may be due to the inability of the yeast CPR to interact with the plant cyt P450. Indeed, unlike the situation in mammals, where a single reductase, encoded by a single gene delivers electrons to the different mammalian cyt P450s (Durst and Nelson 1995), at least two NADPH-cytochrome P450 reductase isoforms exist in plants, including *A. thaliana* (Mignotte, Kazmaier, *et. al.* 1992), and *H. tuberosus* (Lesot, Hasenfratz, *et. al.* 1995). The lack of interaction with yeast CPR by CYP71B7 may indicate that different cyt P450 isoforms in plants interact more specifically with particular CPRs than has previously been assumed. Two yeast strains have recently been developed containing the two different *A.thaliana* CPRs integrated into the genome (Pompon, Louerat, *et. al.* 1995). It would be of interest therefore to transform these strains with CYP71B7 to attempt to overcome the lack of NADPH-supported ECOD activity. Also, addition of purified plant reductase into the ECOD reaction using CY71B7-expressing R3 yeast may shed some light on this and it would be useful to obtain purified *A. thaliana* reductase to investigate this. In addition, it would be of interest to see whether in *A. thaliana* plant microsomes, ECOD activity can

be NADPH-supported. Alternatively, lack of NADPH-supported ECOD activity of the expressed plant enzyme may be due to targeting to membrane lacking CPR in yeast.

Several different approaches have been adopted by various researchers to try and overcome such difficulties including use of artificial gene fusions encoding chimeric proteins fused in frame to yeast, rat or human CPR (Murakami, Yabusaki, *et. al.* 1987; Shibata, Sakaki, *et. al.* 1990) or co-expression systems involving multiple expression cassettes on a single plasmid (Eugster, Bärtsch, *et. al.* 1992). Other approaches involved modification of the N-terminal region of the foreign reductase by addition of a sequence encoding the N-terminal region of the endogenous yeast CPR and co-expression of it with a human or rat cyt P450. This increased the stability of the foreign reductase expressed in the yeast and increased the specific activity of the expressed human or rat cyt P450 (Bligh, Wolf, *et. al.* 1992).

Despite the problems encountered in this work with CYP71B7, in many cases of cyt P450 expression NADPH-supported enzyme activity has been seen, such as with the expression of *P. americana CYP*71A1 (Bozak, O'Keefe, *et. al.* 1992). It is unclear as to why the problem occurs in certain cases and not others. Unravelling the specific reasons for low or absent cyt P450 activity in heterologous expression systems and overcoming them may not be an easy or straightforward task in certain situations. A greater understanding about the relationships between the cyt P450 and redox enzymes in these systems is required to facilitate progress.

Nevertheless, even without NADPH-supported activity of CYP71B7, many experiments can be carried out in the future to further investigate its physiological role. Such approaches could include spectral analysis such as electron paramagnetic resonance (EPR) to further investigate substrate binding and spin state as was done with *P.*

americana cyt P450 (Hallahan, Nugent, *et. al.* 1992). Analysis of metabolites generated could be carried out with the substrates so far identified to attempt to identify reaction products and characterise reactions. The transgenic R3 *S. cerevisiae* cells could also be used as a source from which to attempt to directly purify the CYP71B7 protein, thus facilitating biochemical characterisation.

The results of the substrate screening by assaying CH-dependent ECOD inhibition suggested that CYP71B7 may be involved in hydroxylation of terpenes since inhibition of ECOD activity was seen with the monoterpenes limonene, *p*-menth-1-ene, terpinolene, citronellol, geraniol, nerol and linalool. Interestingly, two other compounds which were not terpenes also inhibited ECOD activity.

Figure 5.13 shows the chemical structure of the substrates which inhibited ECOD activity as well as that of certain compounds which had no effect to allow structural comparisons when considering the sort of molecules which might have an affinity for the active site of CYP71B7. It can be seen that all the compounds which inhibited ECOD are of a similar size. The only monoterpene tested which did not inhibit ECOD activity, cineole, is unlike the other monoterpenes in that it possesses an oxygen bridge. Other terpenes, kaurene and abscisic acid may not be of the right size or shape for the active site of CYP71B7 and do differ considerably in structure to the monoterpenoids which inhibited ECOD activity.

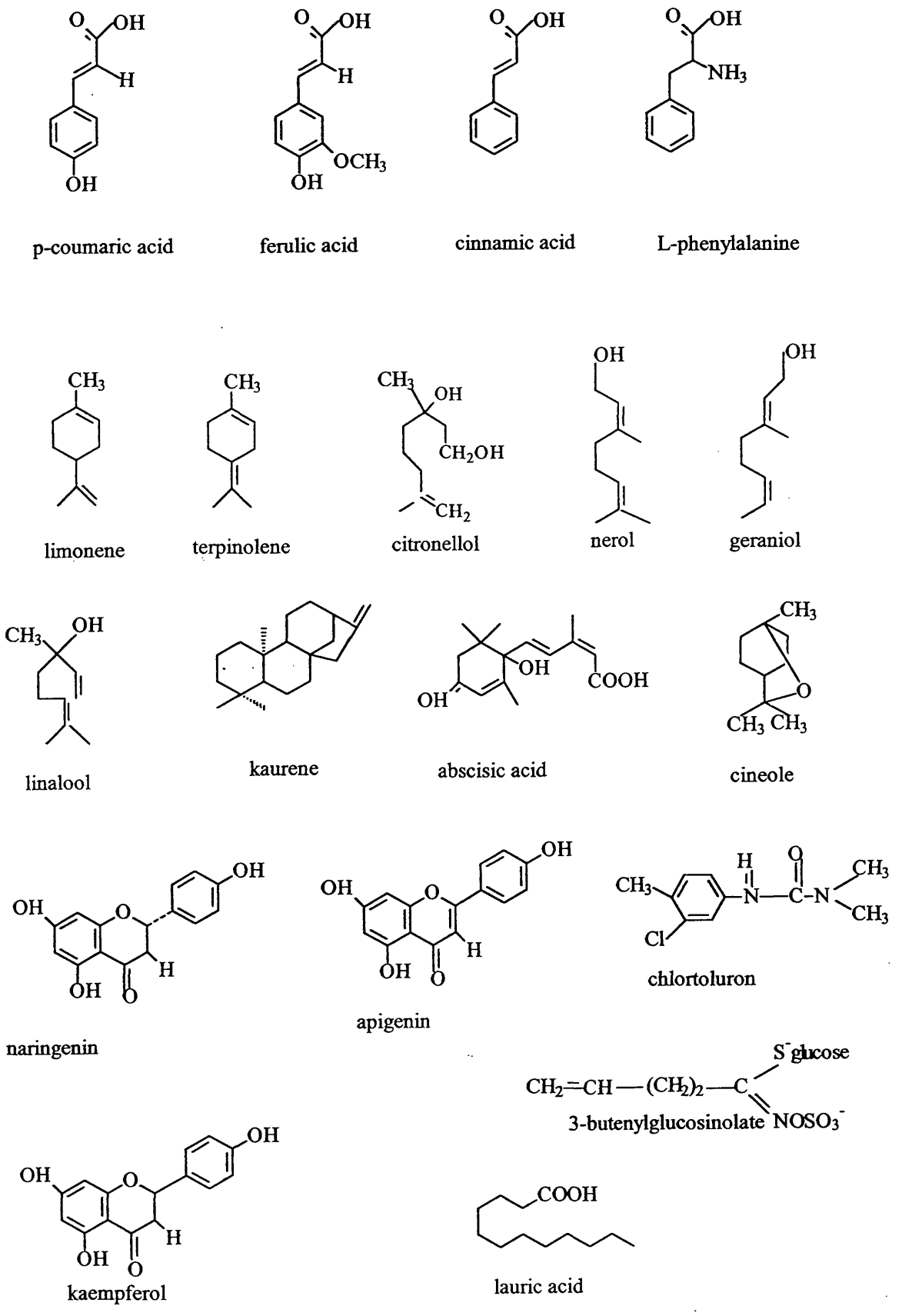

Figure 5.13 *Chemical structures of some compounds tested for their ability to inhibit CH-supported ECOD activity of CYP71B7*

Despite the fact that CYP71B7 was capable of binding two distinct classes of compounds, terpenoids and phenylpropanoids, it does not necessarily mean that it is catalytically competent with them. Some indication as to the possibility that the compounds shown to inhibit ECOD activity are natural substrates would be shown by calculation of the Km (Michaelis menten constant) for each substrate in turn. The lower the value for Km, the more likely that the compound is a natural substrate of CYP71B7. However, enzyme assays with the substrates using reconstitution with NADPH-cyt P450 reductase, and NADPH as the electron source, as in the natural system, is required to assess which, if any, of the compounds which bind to CYP71B7 are in fact natural substrates of this cyt P450 enzyme in *A. thaliana.* The fact that geraniol, citronellol and nerol inhibited CYP71B7 ECOD activity was particularly interesting, since in *C. roseus*, geraniol 10-hydroxylase is involved in synthesis of medically important indole alkaloids (Vetter, Mangold, *et. al.* 1992). If CYP71B7 turns out to be a geraniol 10-hydroxylase it may aid in isolation of the gene coding for the same enzyme in *C. roseus*, providing the potential for large-scale production of these alkaloids.

As discussed in the previous chapter, Section 4.3, the similarity between the CYP71B7 and a cyt P450 gene isolated from *T. arvense,* a plant also from the glucosinolate-producing crucifer family, suggested the possibility that CYP71B7 might be involved in glucosinolate biosynthesis or hydroxylation in *A. thaliana.* A cyt P450 from *Sorghum bicolor* has been isolated which acts as a N-hydroxylase converting the amino acid tyrosine to *p*-hydroxyphenylacetaldehyde oxime in the biosynthesis of the cyanogenic glucoside dhurrin (Koch, Sibbesen, *et. al.* 1995). When compared to other plant cyt P450s, this sequence was most like the flavonoid hydroxylase from petunia, a CYP75A1 (Holton, Brugliera, *et. al.* 1993), the next most similar being the *P. americana* CYP71A1

(Bozak, Yu, *et. al.* 1990). The N-hydroxylase was assigned to a new family however since its amino acid sequence identity to these sequences was less than 40 % (Koch, Sibbesen, *et. al.* 1995). Glucosinolates are anionic thioglucosides and both glucosinolates and cyanogenic glucosides share common features in their biosynthetic pathway. The initial steps in the biosynthesis of these compounds are the conversions of amino acid precursors to hydroxyamino acids then to aldoximes (Haughn, Davin, *et. al.* 1991). The first step for the conversion of amino acids to hydroxyamino acids is believed to be catalysed by a cyt P450 in *S. bicolor* for the ultimate synthesis of dhurrin (Sibbesen, Koch, *et. al.* 1995). It is possible therefore that a cyt P450 also catalyses this step in *A. thaliana* in the synthesis of glucosinolates. However, some controversy exists as to whether this step is cyt P450-catalysed in all plant species which synthesise cyanogenic glucosides and glucosinolates (Bennett and Wallsgrove 1994). Evidence also exists that implies that the initial step in the pathway to the glucosinolates in *Brassica napus* (oil seed rape) is in fact catalysed by a flavoprotein and not a cyt P450 (Bennett and Wallsgrove 1994; Bennett, Kiddle, *et. al.* 1996). Until more data is available with these species, and more cyt P450 sequences are characterised it is likely that this controversy will remain. However, as can be seen in Table 5.1, none of the amino acid precursors of glucosinolates in *Arabidopsis* or a glucosinolate thought to be possible cyt P450 substrate had an effect on ECOD activity. It appears therefore that CYP71B7 does not play a role in the biosynthetic pathway or subsequent hydroxylation of glucosinolates in *A. thaliana.*

In summary, it has been shown that the *A.thaliana* EST clone 5G6 encodes a functional cyt P450 monooxygenase following its successful heterologous expression in yeast. This was only the second cyt P450 originating from an *A.thaliana* EST sequencing programme to be expressed in a heterologous system (the other being a CYP86

(Benveniste and Durst 1995)). The expressed clone 5G6 was characterised as a 7-ethoxycoumarin *O*-deethylase. Several potential substrates for this cyt P450 were identified by assaying for their inhibition of ECOD activity, including the cyclic monoterpenoids terpenoids S-limonene, terpinolene, p-menth-1-ene, the acyclic monoterpenoids citronellol, geraniol, nerol and linalool and the phenylpropanoids ferulic and *p*-coumaric acid. The finding that terpenoids inhibited this activity of CYP71B7 was consistent with the evidence for the involvement of the CYP71 family of cyt P450s in hydroxylation of these compounds. In addition, clotrimazole, a cyt P450 inhibitor which binds specifically to a terpene hydroxylase, limonene hydroxylase in plants, appeared to bind to CYP71B7 exhibiting a type II binding spectra. Several approaches to further characterise and ultimately identify the natural substrate of CYP71B7 have been also suggested.

Chapter 6

Final Discussion and conclusions

The aim of this project was to isolate and characterise a cyt P450 from a higher plant using molecular and biochemical techniques. A novel cyt P450 genomic DNA fragment was isolated from *Zea mays* which, when compared to other plant cyt P450s sequences, was most like the CYP71A1 from *P. americana.* Thus the cyt P450 this clone represented was likely to belong to the CYP71 family of plant cyt P450s. Genomic southern analysis and PCR on gDNA confirmed the presence of a gene for this cyt P450 in the *Z. mays* genome. The *Z. mays* cyt P450 that the gDNA fragment represented appeared to represent a low abundance transcript within the plant, was present in both root and shoot tissue and also in three different varieties of *Z. mays* tested. It was also constitutive within *Z. mays* tissue with respect to age and was not inducible by treatments known to induce herbicide-detoxifying cyt P450s, namely ethanol, naphthalic anhydride and the herbicide chlorsulfuron. Extensive screening of three different *Z. mays* cDNA libraries using this cyt P450 fragment did not result in the isolation of a full length *Z. mays* cDNA. However, several details regarding the expression characteristics of this cyt P450 were elucidated and some strategies for future work highlighted. These included the construction of alternative *Z. mays* cDNA libraries for screening following detailed investigation into the conditions (including induction profiles) which are optimal for expression of this cyt P450 (and homologues) and exploitation of the increasing number of plant cyt P450 sequences now available to design superior oligonucleotide primers for use in the PCR. These could be used to isolate further *Z. mays* cyt P450 probes for library screening.

To allow the biochemical characterisation of a novel plant cyt p450, a different clone was obtained from the *Arabidopsis* Expressed Sequence Tag programme. This was fully sequenced, confirming that it represented a full length cyt P450. It was assigned to the family CYP71B7 and its expression characteristics in various *Arabidopsis* tissues was determined. The cyt P450 was successfully expressed in two different yeast strains under two different types of promoter. It was shown that the *A.thaliana* EST clone (5G6) encodes a functional cyt P450 monooxygenase following its successful heterologous expression in yeast. This was only the second EST originating from an *A.thaliana* EST sequencing programme to be expressed in a heterologous system. Biochemical analysis revealed CYP71B7 to be a 7-ethoxycoumarin *O*-deethylase. A number of potential substrates were identified as judged by the ability of the compound to inhibit the ECOD activity of CYP71B7. These included a number of monoterpenoids and two intermediates of phenylpropanoid biosynthesis. The finding that terpenoids inhibited this activity of CYP71B7 was consistent with the evidence for the involvement of the CYP71 family of cyt P450s in hydroxylation of these compounds. A number of future strategies for this work have been highlighted. These included the use of EPR to further investigate substrate binding, analysis of metabolites to attempt to identify reaction products, and exploitation of the transgenic R3 *S. cerevisiae* cells as a source from which to attempt to directly purify the CYP71B7 protein, thus facilitating biochemical characterisation. In addition, the cDNA could be used in an anti-sense or sense expression strategy with *A.thaliana* plants in an further attempt to determine the role of CYP71B7 *in vivo*.

In summary, cyt P450s from both a monocotyledonous and a dicotyledonous plant have been examined and characterised in different ways, yielding information which has furthered the knowledge of cyt P450 in plants.

References

Adams, M. D., M. Dubnick, A. R. Kerlavage, R. Moreno, J. M. Kelley, T. R. Utterback, J. W. Nagle, C. Fields and J. C. Venter (1992). Sequence identification of 2375 human brain genes. *Nature* **355**: 632-634.

Adams, M. D., J. M. Kelley, J. D. Gocayne, M. Dubnick, M. H. Ploymeropoulos, H. Xiao, C. R. Merril, A. Wu, B. Olde, R. F. Moreno, A. R. Kerlavage, C. R. McCombie and C. Venter (1991). Complementary DNA sequencing : expressed sequence tags and human genome project. *Science* **252**: 1651-1656.

Agosin, M. (1982). Multiple forms of insect cytochrome P-450:role in insecticide resistance. *Cytochrome P450; biochemistry, biophysics and environmental implications.* E. Hietanen. Amsterdam, Elselvier: 661-669.

Aoyama, T., S. Yamano, D. J. Waxman, D. P. Lapenson, U. A. Meyer, V. Ficher, R. Tyndale, T. Inaba, W. Kalow, H. V. Gelboin and F. J. Gonzale (1989). Cytochrome P-450hPCN3, a novel cytochrome P450 IIIA gene product that is differentially expressed in adult human liver. *J. Biol. Chem.* **264**: 10388-10395.

Appleby, C. A. (1967). A soluble hemoprotein from nitrogen-fixing Rhizopium bacteroids. *Biochem. Biophys. Acta* **147**: 399-402.

Ausubel, F. M., Ed. (1989). *Saccharomyces cerevisiae*. Current Protocols in Molecular Biology : Supplement 5. New York, John Wiley & Sons, Inc.

Axelrod, J. (1955). The enzymatic demethylation of ephedrine. *J. Pharmacol.Exp.Ther.* **114**: 430-438.

Barrett, M. (1995). Metabolism of herbicides by cytochrome P450 in corn. *Drug Metab. Drug Interact.* **12**(3-4): 299-315.

Barrett, M. and J. Maxson (1991). Naphthalic anhydride induces imazethapyr metabolism

and cytochrome P-450 activity in maize. *Journal of Biosciences* **46**(9-10): 897-900.

Batard, Y., A. Zimmerlin, M. LeRet, F. Durst and D. Werck-Reichhart (1995). Multiple xenobiotic-inducible P450s are involved in alkoxycoumarin and alkoxyresorufin metabolism in higher plants. *Plant Cell Environment* **18**: 523-533.

Bend, J. R., R. J. Pohl, E. Arinç and R. M. Philpot (1977). Hepatic microsomal and solubilized mixed-function oxidase systems from the little skate, *Raja erinacea*, a marine elasmobranch. *Microsomes and Drug Oxidations*. V. Ulrich, I. Roots, A. Hildebrandt, R. W. Estabrook and A. H. Conney. Oxford, Pergamon: 160-169.

Bennett, R. N., G. Kiddle, A. J. Hick, G. W. Dawson and R. M. Wallsgrove (1996). Distribution and activity of microsomal NADPH-dependent monooxygenases and amino acid decarboxylases in cruciferous and non-cruciferous plants, and their relationship to foliar glucosinolate content. *Plant, Cell and Environment* **19**: 801-812.

Bennett, R. N. and R. M. Wallsgrove (1994). Tansley Review No. 72 Secondary metabolites in plant defence. *New Phytol.* **127**: 617-633.

Benveniste, I. and F. Durst (1995). Cloning, sequencing and expression of CYP86, a new cytochrome P450 from *Arabidopsis thaliana. Plant Physiol.* **In press**.

Benveniste, I., J.-P. Salaün, A. Simon, D. Reichhart and F. Durst (1982). Cytochrome P-450 dependent ω-hydroxylation of lauric acid by microsomes from pea seedlings. *Plant Physiol.* **70**: 122-126.

Black, S. D. (1992). Membrane topology of mammalian P450 cytochromes. *FASEB* **6**: 680-685.

Black, S. D. (1993). Cytochrome P450 structure and function. *Cytochrome P450*. J. B. Schenkman and H. Greim. Berlin, Springer-Verlag. **105:** 155-168.

Black, S. D. and M. J. Coon (1986). Comparative structures of P-450 cytochromes.

Cytochrome P-450. O. de. Montellano. New York, Plenum: 161-216.

Bligh, H. F. J., C. R. Wolf, G. Smith and J. D. Beggs (1992). Production of cytochrome P450 reductase yeast-rat hybrid proteins in *Saccharomyces cerevisiae*. *Gene* **110**: 33-39.

Bolwell, G. P., K. Bozak and A. Zimmerlin (1994). Plant Cytochrome P450. *Phytochemistry*. **37**(6): 1491-1506.

Bonfils, C., C. Balny and P. Maurel (1981). Direct evidence for electron-transfer from ferrous cytochrome b_5 to the oxyferrous intermediate of liver microsomal cyt-P450 LM2. *J. Biol. Chem.* **256**: 9457-9465.

Boobis, A. R., R. J. Edwards, D. A. Adams and D. S. Davies (1996). Dissecting the function of cytochrome P450. *British Journal of Clinical Pharmacology* **42**: 81-89.

Boobis, A. R., J. McQuade, D. Sesardic, R. T. Robson, C. Hayward, E. A. Lock, C. R. Elcombe, M. S. Rose and D. S. Davies (1985). A monoclonal antibody raised to rat liver Cytochrome P-448 (Form C) which recognises an epitope common to many other forms of Cytochrome P450. *Biochem. Pharmacol.* **34**(10): 1671-1681.

Bozak, K. R., D. P. O'Keefe and R. E. Christoffersen (1992). Expression of a ripening-related avocado (*Persea americana*) cytochrome P450 in yeast. *Plant Physiol.* **100**: 1976-1981.

Bozak, K. R., H. Yu, R. Siervag and R. E. Christoffersen (1990). Sequence analysis of ripening-related cytochrome P-450 cDNAs from avocado fruit. *Pro. Natl. Acad. Sci. USA* **87**: 3904-3908.

BRL (1986). BRL pUC host: *E. coli* DH5α competent cells. *BRL Focus* **8**(2): 9.

Bullock, W. O., J. M. Fernandez and J. M. Short (1987). XL1-Blue: A high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. *BioTechniques* **5**(4): 376-378.

Burden, R. S., G. A. Carter, T. Clark, D. T. Cooke, S. J. Croker, A. H. B. Deas, P. Hedden, C. S. James and J. R. Lenton (1987). Comparative activity of the enantiomers of triadimenol and paclobutrazol as inhibitors of fungal growth and plant sterol and gibberellin biosynthesis. *Pestic. Sci* **21**: 253-267.

Burnet, M. W. M., B. R. Loveys, J. A. M. Holtum and S. Powles (1993). A mechanism of chlortoluron resistance in *Lolium rigidum*. *Planta* **190**: 182-189.

Christoffersen, R. E., F. W. Percival and K. R. Bozak (1995). Functional and DNA sequence divergence of the CYP71 gene family in higher plants. *Drug Metab. Drug Interact.* **12**(3-4): 207-217.

Clemens, S., W. Hinderer, U. Witkampf and W. Barz (1993). Characterisation of cytochrome P450-dependent isoflavone hydroxylases from chickpea. *Phytochemistry* **32**(3): 653-657.

Coolbaugh, R. C. and T. C. Moore (1971). Metabolism of kaurene in cell-free extracts of immature pea seeds. *Phytochemistry* **10**: 2401-2412.

Cooper, D. Y., S. Levin, S. Narasimhulu, O. Rosenthal and R. W. Estabrook (1965). Photochemical action spectrum of the terminal oxidase of mixed function oxidase systems. *Science* **147**: 400-402.

Croteau, R. and P. E. Kolattukudy (1975). Biosynthesis of hydroxyfatty acid polymers: enzymatic epoxidation of 18-hydroxyolieic acid to 18-hydroxy-cis-9,10-epoxystearic acid by a particulate preparation from spinach. *Arch. Biochem, Biophys.***170**: 61-72.

Cullin, C. and D. Pompon (1988). Synthesis of functional mouse cytochromes P-450 P1 and chimeric P-450 P3-1 in the yeast *Saccharomyces cerevisiae*. *Gene* **65**: 203-217.

Cupp-Vickery, J. R. and T. L. Poulos (1995). Structure of cytochrome P450ERYF involved in erythromycin biosynthesis. *Nature Struct. Biol.* **2**: 144-153.

Daxenbichler, M. E., G. F. Spencer, D. G. Carlson, G. B. Rose, A. M. Brinker and R. G. Powell (1991). Glucosinolate composition of seeds from 297 species of wild plants. *Phytochemistry* **30**(8): 2623-2638.

Dean, J. V., J. W. Gronwald and M. P. Anderson (1991). Glutathione S-transferase activity in nontreated and CGA-154281-treated maize shoots. *Z Naturforsch* **46c**: 850-855.

Dean, J. V., J. W. Gronwald and C. V. Eberlein (1990). Induction of glutathione S-transferase isozymes in sorghum by herbicide antidotes. *Plant Physiol.* **92**: 467-473.

Dellaporta, S. L., J. Wood and J. B. Hicks (1983). A plant DNA minipreparation: version II. *Plant Molecular Biology Reporter* **1**(4): 19-21.

Devine, M. D., S. O. Duke and C. Fedtke (1993). Herbicide metabolism. *Physiology of Herbicide Action.* Englewood Cliffs NJ, PTR Prentice Hall: 95-112.

Devine, M. D., S. O. Duke and C. Fedtke (1993). Herbicide Selectivity. *Physiology of Herbicide Action.* Englewood Cliffs NJ, PTR Prentice Hall: 5-6.

Dohn, D. R. and R. I. Krieger (1984). *N*-demethylation of *p*-chloro-*N*-methylaniline catalyzed by subcellular fractions from the avocado pear (*Persea americana*). *Arch. Biochem. Biophys.* **231**(2): 416-423.

Donaldson, R. P. and D. G. Luster (1991). Multiple forms of plant cytochromes P-450. *Plant Physiol.* **96**: 669-674.

Durst, F. (1991). Biochemistry and physiology of plant cytochrome P-450. *Microbial and Plant Cytochromes P-450: Biochemical Characteristics, Genetic Engineering and Practical Implications*. K. Ruckpaul and H. Rein. London, Taylor and Francis: 191-232.

Durst, F. and I. Benveniste (1993). Cytochrome P450 in plants. *Cytochrome P450.* J. B. Schenkman and H. Greim. Berlin, Springer-Verlag: 293-310.

Durst, F. and D. R. Nelson (1995). Diversity and evolution of plant P450 and

P450-reductases. *Drug Metab. Drug Interact.* **12**(3-4): 189-206.

Durst, F. and D. P. O'Keefe (1995). Plant cytochromes P450: an overview. *Drug Metab. Drug Interact.* **12**(3-4): 171-187.

Estabrook, R. W., D. Y. Cooper and O. Rosenthal (1963). The light reversible carbon monoxide inhibition of the steroid C21-hydroxylase system of the adrenal cortex. *Biochem. Z.* **338**: 741-755.

Eugster, H.-P., S. Bärtsch, F. Würgler and C. Sengstag (1992). Functional co-expression of human oxidoreductase and cytochrome P450 1A1 in *Saccharomyces cerevisiae* results in increased EROD activity. *Biochem. Biophys. Res. Comm.* **185**(2): 641-647.

Fahrendorf, T. and R. Dixon (1993). Stress responses in Alfalfa (*Medicago sativa* L.) XVII: Molecular cloning and expression of the elicitor-inducible cinnamic acid 4-hydroxylase cytochrome P450. *Arch. Biochem. Biophys.* **305**: 509-515.

Falco, S. C., Y. Li, J. R. Broach and D. Botstein (1982). Genetic properties of chromosomally integrated 2 μm plasmid DNA in yeast. *Cell* **29**: 573-584.

Fang, G., K. Venkateswarlu, D. E. Kelly and S. L. Kelly (1994). Studies on yeast host strains for heterologous P450 expression. *Biotechnology Letters* **16**(12): 1253-1258.

Feinberg, A. P. and B. Vogelstein (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **132**: 6-13.

Foley, K. P., M. W. Leonard and J. D. Engel (1993). Quantitation of RNA using the polymerase chain reaction. *Trends in Genetics* **9**(11): 380-385.

Fonné-Pfister, R., J. Gaudin, K. Kreuz, K. Ramsteiner and E. Ebert (1990). Hydroxylation of primsulfuron by an inducible cytochrome P450-dependent monooxygenase system from maize. *Pesticide Biochemistry and Physiology* **37**: 165-173.

Fonné-Pfister, R. and K. Kreuz (1990). Ring-methyl hydroxylation of chlortoluron by an

inducible cytochrome P450-dependent enzyme from maize. *Phytochemistry* **29**(9): 2793-2796.

Foundation, N. S. (1990). A long-range plan for the multinational coordinated *Arabidopsis thaliana* genome research project (NSF 90-80). Washington, National Science Foundation.

Frear, D. S. (1995). Wheat microsomal cytochrome P450 monooxygenases:characterization and importance in the metabolic detoxification and selectivity of wheat herbicides. *Drug Metab. Drug Interact.* **12**(3-4): 329-357.

Frear, D. S., H. R. Swanson and F. S. Tanaka (1993). Metabolism of flumetsulam (DE-498) in wheat, corn and barley. *Pesticide Biochemistry and Physiology* **45**(3): 178-192.

Frear, D. S., H. R. Swanson and F. S. Tanaka, (1969). *N*-demethylation of substituted 3-(phenyl)-1-methylureas: isolation and characterization of a microsomal mixed function oxidase from cotton. *Phytochemistry* **8**: 2157-2169.

Frey M., R. Kliem, H. Saedler and A. Gierl (1995). Expression of a cytochrome P-450 gene family in maize. *Mol. Gen. Genetics* **246**: 100-109.

Fujita, M. and T. Asahi (1985). Purification and properties of sweet potato NADPH-Cytochrome *c* (P-450) reductase. *Plant Cell Physiology* **26**(3): 397-405.

Fujita, M., K. Oba and I. Uritani (1982). Properties of mixed function oxygenase catalyzing ipomeamarone 15-hydroxylation in microsomes from cut-injured and *Ceratocystis fimbriata*-infected sweet potato root tissues. *Plant Physiol.* **70**: 573-578.

Gabriac, B., D. Werck-Reichhart, H. Teutsch and F. Durst (1991). Purification and immuno-characterisation of a plant cytochrome P450: the cinnamic acid 4-hydroxylase. *Arch. Biochem. Biophys.* **288**: 302-309.

Garfinkel, D. (1958). Studies on pig liver micrsomes. I. Enzymic and pigment composition

of different microsomal fractions. *Arch. Biochem. Biophys.* **77**: 493-509.

Gerardy, R. and M. Zenk (1993). Formation of salutardine from (R)-reticuline by a membane-bound cytochrome-P-450 enzyme from Papaver-somniferum. *Phytochemistry* **32**: 79-86.

Ghosh, D. K., D. Dutta, T. B. Samanta and A. K. Mishra (1983). Microsomal benzo[*a*]pyrene hydroxylase in Aspergillus ochraceus TS: assay and characterization of the enzyme system. *Biochem. Biophys, Res. Comm.* **113**: 497-505.

Ghrayeb, J., H. Kimura, M. Takahara, H. Hsuing, Y. Masui and M. Inouye (1984). Secretion cloning vectors in *Escherichia coli*. *EMBO J.* **3**(10): 2437-2442.

Gillard, D. F. and D. C. Walton (1976). Abscisic acid metabolism by a cell-free preparation from *Echinocystis lobata* liquid endosperm. *Plant Physiol.* **58**: 790-795.

Goeptar, A. R., H. Scheerens and N. P. E. Vermeulen (1995). Oxygen and xenobiotic reductase activities of cytochrome P450. *Critical Reviews in Toxicology* **25**(1): 25-65.

Gonneau, M., B. Pasquette, F. Cabanne and R. Scalla (1988). Metabolism of chlortoluron in tolerant weed species: possible role of cytochrome P-450 mono-oxygenases. *Weeds Res.* **28**: 19-25.

Gonzalez, F. J. and K. R. Korzekwa (1995). Cytochromes P450 expression systems. *Annu. Rev. Pharmacol. Toxicol.* **35**: 369-390.

Gonzalez, F. J. and D. W. Nebert (1990). Evolution of the P450 gene superfamily: animal-plant 'warfare', molecular drive and human genetic differences in drug oxidation. *Trends in Genetics* **6**(6): 182-186.

Grand, C. (1984). Ferulic acid 5-hydroxylase: A new cytochrome P-450-dependent enzyme from higher plant microsomes involved in lignin biosynthesis. *FEBS Letters* **169**: 7-11.

Guengerich, F. P. (1990). Enzymatic oxidation of xenobiotic chemicals. *Critical Reviews in Biochemistry and Molecular Biology* **25**: 97-153.

Guengerich, F. P. (1992). Cytochrome P450: advances and prospects. *FASEB* **6**: 667-668.

Guengerich, F. P., W. R. Brian, M.-A. Sari and J. T. Ross (1991). Expression of mammalian cytochrome P450 enzymes using yeast-based vectors. *Methods Enzymol.* **206**: 130-145.

Hakamatsuka, T., M. F. Hashim, Y. Ebizuka and U. Sanwaka (1990). P-450-dependent oxidative rearrangement in isoflavone biosynthesis - reconstitution of P-450 and NADPH-P-450 reductase. *Tetrahedron* **47**: 5969-5978.

Halkier, B. A. (1996). Catalytic reactivities and structure/function relationships of cytochrome P450 enzymes. *Phytochemistry* **43**(1): 1-21.

Halkier, B. A. and B. L. Møller (1991). Involvemant of cytochrome-P-450 in the biosynthesis of dhurrin in Sorghum-bicolor (L) moench. *Plant Physiol.* **96**: 10-17.

Halkier, B. A., O. Sibbesen, B. Koch and B. L. Møller (1995). Characterisation of cytochrome $P450_{TYR}$, a multifunctional haem-thiolate N-hydroxylase involved in the biosynthesis of the cyanogenic glucoside dhurrin. *Drug Metab. Drug Interact.* **12**(3-4): 285-297.

Hallahan, D. L., G. W. Dawson, J. West and R. M. Wallsgrove (1992). Cytochrome P450 catalysed monoterpene hydroxylation in *Nepeta mussinii*. *Plant Physiol. and Biochemistry* **30**(4): 435-443.

Hallahan, D. L., S.-M. Lau, P. A. Harder, D. W. M. Smiley, G. W. Dawson, J. A. Pickett, R. E. Christoffersen and D. P. O'Keefe (1994). Cytochrome P-450 catalyzed monoterpenoid oxidation in catmint (*Nepeta racemosa*) and avocado (*Persea americana*) - evidence for related enzymes with different activities. *Biochem. Biophys. Acta* **1201**:

94-100.

Hallahan, D. L., J. H. A. Nugent, B. J. Hallahan, G. W. Dawson, D. W. M. Smiley, J. M. West and R. W. Wallsgrove (1992). Interactions of avocado (*Persea americana*) cytochrome P-450 with monoterpenoids. *Plant Physiol.* **98**: 1290-1297.

Hallahan, D. L. and J. M. West (1995). Cytochrome P-450 in plant/insect interactions:geraniol 10-hydroxylase and the biosynthesis of iridoid monoterpenoids. *Drug Metab. Drug Interact* **12**(3-4): 369-382.

Hallahan, D. L. H., A. K. Cheriton, R. Hyde, I. Clark and B. Forde (1993). Plant cytochrome P-450 and agricultural biotechnology. *Biochem. Soc. Trans.* **21**: 1068-1073.

Hamerski, D. and U. Matern (1988). Biosynthesis of psoralens - psoralen 5-monooxygenase activity from elicitor-treated ammi majus cells. *FEBS Letters* **239**: 263-265.

Hamerski, D. and U. Matern (1988). Elicitor-induced biosynthesis of psoralens in ammi-majus L suspension-cultures - microsomal conversion of demethyl suberosin into (+) marmesin and psoralen. *Eur. J. Biochem.* **171**: 369-375.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**: 557-580.

Hasemann, C. A., K. G. Ravichandran, J. A. Peterson and J. Deisenhofer (1994). Crystal-structure and refinement of P450(terp) at 2-center-dot-3 angstrom resolution. *J. Mol. Biol.* **239**: 1169-1185.

Haugen, D. A., T. A. van der Hoeven and M. J. Coon (1975). Purified liver microsomal cytochrome P-450: separation and characterization of multiple forms. *J. Biol. Chem.* **250**: 3567-3570.

Haughn, G. W., L. Davin, M. Giblin and E. W. Underhill (1991). Biochemical genetics

of plant secondary metabolites in *Arabidopsis thaliana. Plant Physiol.* **97**: 217-226.

Hofte, H., T. Desprez, J. Amselem, H. Chiapello, M. Caboche, A. Moisan, M.-F. Jourjon, L. Charpenteau, P. Berthomieu, D. Guerrier, J. Giraudat, F. Quigley, F. Thomas, D.-Y. Yu, R. Mache, M. Raynal, R. Cooke, F. Grellet, M. Delsney, Y. Parmentier, G. Marcillac, C. Gigot, J. Fleck, G. Philipps, M. Axelos, C. Bardet, D. Tremousaygue and B. Lescure (1993). An inventory of 1152 expressed sequence tags obtained by partial sequencing of cDNAs from *Arabidopsis thaliana. The Plant Journal* **4**(6): 1051-1061.

Holton, T. A., F. Brugliera, D. R. Lester, Y. Tanaka, C. Hyland, J. G. T. Menting, C.-Y. Lu, E. Farcy, T. W. Stevenson and E. C. Cornish (1993). Cloning and expression of cytochrome P450 genes controlling flower colour. *Nature* **366**: 276-279.

Huynh, T. V. (1985). Constructing and screening cDNA libraries in λgt10 and λgt11. *DNA Cloning*. Glover. Oxford, IRL Press. **1:** 49-78.

Imai, Y. (1988). Characterization of rabbit liver cytochrome P-450 (laurate ω - 1 hydroxylase) synthesised in transformed yeast cells. *J. Biochem.* **103**: 143-148.

Jefcoate, C. R. (1978). Measurement of substrate and inhibitor binding to microsomal P450 by optical difference spectroscopy. *Methods Enzymol.* **52**: 258-279.

Jones, O. T. G. and J. C. Caseley (1989). *Role of Cytochrome P450 in herbicide metabolism*. Brighton Crop Protection Conference, Brighton.

Kalb, V. F. and J. C. Loper (1988). Proteins from eight eukaryotic cytochrome P-450 families share a segmented region of sequence similarity. *Pro. Natl. Acad. Sci. USA* **85**: 7221-7225.

Kamin, H., C. Batie, J. D. Lambeth, J. Lancaster, L. Graham and J. C. Salerno (1985). Paramagnetic probes of multicomponent electron-transfer systems. *Biochem. Soc. Trans.* **13**: 615-618.

Käpelli, O. (1986). Cytochromes P-450 of yeasts. *Microbiol. Rev.* **50**: 244-258.

Karp, F. and R. Croteau (1992). Hydroxylation of (-)-β-pinene and (-)-α-pinene by a cytochrome P-450 system from hyssop (*Hyssopus officianalis*). *Secondary Metabolite Biosynthesis and Metabolism*. R. J. Petroski and S. P. McCormick. New York, Plenum Press: 253-260.

Karp, F., J. L. Harris and R. Croteau (1987). Metabolism of monoterpenes:demonstration of the hydroxylation of (+) sabinene to (+)-*cis*-sabinol by an enzyme preparation from sage (*Salvia officinalis*) leaves. *Arch. Biochem. Biophys.* **256**(1): 179-193.

Karp, F., C. A. Mihaliak, J. L. Harris and R. Croteau (1990). Monoterpene biosynthesis: specificity of the hydroxylations of (-) - limonene by enzyme preparations from peppermint (*Mentha piperita*), spearmint (*Mentha spicata*) and perilla. *Arch. Biochem. Biophys.* **276**(1): 219-226.

Keith, C. S., D. O. Hoang, B. M. Barrett, B. Feigelman, M. C. Nelson, H. Thai and C. Baysdorfer (1993). Partial sequence analysis of 130 randomly selected maize cDNA clones. *Plant Physiol.* **101**: 329-332.

Klingenberg, M. (1958). Pigments of rat liver microsomes. *Arch. Biochem. Biophys.* **75**: 376-386.

Klocke, J. A., M. V. Darlington and M. F. Balandrin (1987). Biologically active constituents of North-american plants .3. 1,8-cineole (eucalyptol), a mosquito feeding and ovipositional repellent from volatile oil of *Hemizonia fitchii* (asteraceae). *J. Chem. Ecol.* **13**: 2131-2141.

Koch, B., V. S. Neilsen, B. A. Halkier, C. E. Olsen and B. L. Møller (1992). The biosynthesis of cyanogenic glucosides in seedlings of cassava (manihot-esculenta crantz). *Arch. Biochem. Biophys.* **292**: 141-150.

Koch, B., O. Sibbesen, B. A. Halkier, I. Svendsen and B. L. Møller (1995). The primary sequence of cytochrome $P450_{TYR}$, the multifunctional *N*-hydroxylase catalyzing the conversion of L-tyrosine to *p*-hydroxyphenylacetaldehyde oxime in the biosynthesis of the cyanogenic glucoside dhurrin. *Arch. Biochem. Biophys.* **323**: 177-186.

Kochs, G. and H. Grisebach (1986). Enzymic-synthesis of isoflavones. *Eur. J. Biochem.* **155**(2): 311-318.

Kochs, G. and H. Grisebach (1989). Phytoalexin synthesis in soybean: purification and reconstitution of cytochrome P450 3,9-dihydropterocarpan 6a-hydroxylase and separation from cytochrome P450 cinnamate 4-hydroxylase. *Arch. Biochem. Biophys.* **273**: 543-553.

Kochs, G., D. Werck-Reichhart and H. Grisebach (1992). Further characterization of cytochrome P450 involved in phytoalexin synthesis in soybean:cytochrome P450 cinnamate 4-hydroxylase and 3,9-dihydropterocarpan 6a-hydroxylase. *Arch. Biochem. Biophys.* **293**: 187-194.

Kraus, P. F. X. and T. M. Kutchan (1995). Molecular cloning and heterologous expression of a cDNA encoding berbamunine synthase, a C-O phenol coupling cytochrome P450 from the higher plant *Berberis stolonifera. Pro. Natl. Acad. Sci. USA* **92**: 2071-2075.

Kurumbail, G., K. G. Ravichandran, S. Sekhar, S. S. Boddupalli, C. A. Hasermann, J. A. Peterson and J. Deisenhofer (1993). Crystal structure of hemoprotein domain of P450BM-3, a prototype for microsomal P450's. *Science* **261**: 731-736.

Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Larson, R. L. and J. B. Bussard (1986). Microsomal flavonoid 3'-monoxygenase from maize seedlings. *Plant Physiol.* **80**: 483-486.

Lau, S.-Z., P. Harder and D. O'Keefe (1993). Low carbon monoxide affinity allene oxide

synthase is the predominant cytochrome P450 in many plant tissues. *Biochemistry* **32**: 1945-1950.

Laudert, D., U. Pfannschmidt, F. Lottspeich, H. Holländer-Czytko and E. Weiler (1996). Cloning, molecular and functional characterisation of *Arabidopsis thaliana* allene oxide synthase (CYP 74), the first enzyme of the octadecanoid pathway to jasmonates. *Plant Mol. Biol.* **31**: 323-335.

Lee, Y. S., T. Hirata and T. Suga (1983). Biotransformation of 1-acetoxy-*p*-menth-4(8)-ene with a suspension of cultured cells of *Nicotiana tabacum*. *J. Chem. Soc. Perkin. Trans.* **1**: 2475.

Lesot, A., M.-P. Hasenfratz, Y. Batard, F. Durst and I. Benveniste (1995). Two messenger RNAs are encoding for NADPH-cytochrome P450 reductases in *Helianthus tuberosus* tuber tissues. *Plant Physiol. Biochem.* **33**(6): 751-757.

Lindberg, R. L. P. and M. Negishi (1989). Alteration of mouse cytochrome $P450_{coh}$ substrate specificity by mutation of a single amino-acid residue. *Nature* **339**(22): 632-634.

Lindenmayer, A. and L. Smith (1964). Cytochromes and other pigments of baker's yeast grown aerobically and anaerobically. *Biochem. Biophys.* **5**: 167-173.

Logemann, J., J. Schell and L. Willmitzer (1987). Improved method for the isolation of RNA from plant tissues. *Analytical Biochemistry* **163**: 16-20.

Loomis, W. F. (1988). *Four Billion Years: An Essay on the Evolution of Genes and Organisms*. Sunderlans, Massachusetts, Sinauer Associates Inc.

Lorow, D. and J. Jessee (1990). MAX efficiency DH10B^{TM}: a new host for cloning methylated DNA. *BRL Focus* **12**: 19-20.

Low, R. and T. Rausch (1994). Sensitive, non-radioactive northern blots using alkaline transfer of total RNA and PCR-amplified biotinylated probes. *BioTechniques* **17**:

1026-1030.

Lu, A. Y. H. and M. J. Coon (1968). Role of hemoprotein P450 in fatty acid ω - hydroxylation in a soluble enzyme system from liver microsomes. *J. Biol. Chem.* **243**: 1331-1332.

Lupien, S., F. Karp, K. Ponnamoeruma, M. Wildung and R. Croteau (1995). Cytochrome P450 limonene hydroxylases of *Mentha* species. *Drug. Metab. Drug. Interact.* **12**(3-4): 245-260.

Madyastha, K. M. and C. J. Coscia (1979). Detergent-solubilized NADPH-cytochrome *c* (P-450) reductase from the higher plant, *Catharanthus roseus*. *J. Biol. Chem.* **254**(7): 2419-2427.

Madyastha, K. M., T. D. Meehan and C. J. Coscia (1976). Characterization of a cytochrome P-450 dependent monoterpene hydroxylase from the higher plant *Vinca rosea*. *Biochemistry* **15**: 1097-1102.

Mangold, U., J. Eichel, A. Batschauer, T. Lanz, T. Kaizer, G. Spanberg, D. Werck-Reichhart and J. Schruder (1994). Gene and cDNA for plant cytochrome-P450 proteins CYP72 family from *Catharanthus roseus* and transgenic expression of the gene and a cDNA in tobacco and *Arabidopsis*. *Plant Science* **96**(1-2): 129-136.

Markwell, M. A., S. M. Haas, L. L. Bieber and N. E. Tolbert (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipid samples. *Analytical Biochemistry* **87**: 206-210.

Martin-Gallardo, A., W. R. McCombie, J. D. Gocayne, M. G. FitzGerald, S. Wallace, B. M. B. Lee, J. Lamerdin, S. Trapp, J. M. Kelley, L. I. Liu, M. Dubnick, L. A. Johnston-Dow, A. R. Kerlavage, P. de Jong, A. Carrano, C. Fields and J. C. Venter (1992). Automated DNA sequencing and analysis of 106 kilobases from human

chromosome 19q13.3. *Nature Genet.* **1**: 34-39.

Masters, B. S. S., C. H. Williams and H. Kamin (1976). The preparation and properties of microsomal TPNH-cytochrome C reductase from pig liver. *Methods Enzymol.* **10**: 565-573.

McCombie W.R., M. D. Adams, J. M. Kelley, M. G. FitzGerald, T. R. Utterback, M. Khan, M. Dubnick, A. R. Kerlavage, J. C. Venter and C. Fields (1992). *Caenorhabditis elegans* expressed sequence tags identify gene families and potential gene homologues. *Nature Genet.* **1**: 124-131.

McCombie, W. R., A. Martin-Gallardo, J. D. Gocayne, M. FitzGerald, M. Dubnick, J. M. Kelley, L. Castilla, L. I. Liu, S. Wallace, S. Trapp, D. Tagle, W. L. Whaley, S. Cheng, J. Gusella, A. M. Frischauf, A. Poustka, H. Lehrach, F. S. Collins, A. R. Kerlavage, C. Fields and J. C. Venter (1992). Expressed genes, *Alu* repeats and polymorphisms in cosmids sequenced from chromosome 4p16.3. *Nature Genet.* **1**: 348-353.

McFadden, J. J., D. S. Frear and E. R. Mansager (1989). Aryl hydroxylation of diclofop by a cytochrome P450 dependent monooxygenase from wheat. *Pesticide Biochemistry and Physiology* **34**: 92-100.

McFadden, J. J., J. W. Gronwald and C. V. Eberlein (1990). In vitro hydroxylation of bentazon by microsomes from naphthalic-anhydride treated corn shoots. *Biochem. Biophys. Res. Comm.* **168**: 206-216.

Meehan, T. D. and C. J. Coscia (1973). Hydroxylation of geraniol and nerol by a monooxygenase. *Biochem. Biophys. Res. Comm.* **53**(4): 1043-1048.

Meijer, A., A. De Waal and R. Verpoote (1993). Isolation of cytochrome P-450 cDNA clones from the higher plant *Catharanthus roseus* by a PCR strategy. *Plant Molecular Biology* **22**: 379-383.

Meijer, A. H., A. DeWaal and R. Verpoorte (1993). Purification of the cytochrome P-450 enzyme geraniol 10-hydroxylase from cell cultures of *Catharanthus roseus*. *Journal of Chromatography* **635**: 237-249.

Meyer, A., O. Miersch, C. Büttner, W. Dathe and G. Sembdner (1984). Occurence of the plant growth regulator jasmonic acid in plants. *J. Plant Growth Regul.* **3**: 1-8.

Meyer, K., J. C. Cusumano, C. Somerville and C. Chapple (1996). Ferulate-5-hydroxylase from *Arabidopsis thaliana* defines a new family of cytochrome P450-dependent monoxygenases. *Pro. Natl. Acad. Sci. USA* **93**: 6869-6874.

Mignotte, C., M. Kazmaier, F. Lacroute, M. Minet and D. Pompon (1992). Procede de clonage d'une sequence d'ADN codant pour une NADPH - cytochrome P450 reductase et sequences d'ADN codant pour une NADPH - cytochrome P450 reductase, notamment de Plants. *French Patent No. D13969/233445.*

Mihaliak, C., A., F. Karp and Croteau R. (1993). Cytochrome P-450 terpene hydroxylases. *Meths. Plant. Biochem.* **9**: 261-279.

Mizutani, M., D. Ohta and R. Sato (1993). Purification and characterization of a cytochrome P450 (*trans*-cinnamic acid 4-hydroxylase) from etiolated mung bean seedlings. *Plant Cell Physiology* **34**(3): 481-488.

Mizutani, M., E. Ward, J. DiMaio, D. Ohta, J. Ryals and R. Sato (1993). Molecular cloning and sequencing of a cDNA encoding mung bean cytochrome P450 ($P450_{C4H}$) possessing cinnamate 4-hydroxylase activity. *Biochem. Biophys. Res. Comms.* **190**(3): 875-880.

Moore, C. W. D. (1967). PhD Thesis, University of Cambridge.

Moreland, D. E., F. T. Corbin and McFarland J.E. (1993). Oxidation of multiple substrates by corn microsomes. *Pesticide Biochemistry and Physiology* **47**: 206-214.

Morris, P. C., D. Guerrier, J. C. Barbet and J. Giraudat (1993). The Arabidopsis thaliana transcribed genome: the GDR program. *Unpublished.*

Mougin, C., F. Cabanne, M.-C. Canivenc and R. Scalla (1990). Hydroxylation and N-demethylation of chlortoluron by wheat microsomal enzymes. *Plant Science* **66**: 195-203.

Murakami, H., Y. Yabusaki, T. Sakaki, M. Shibata and H. Ohwaka (1987). A genetically engineered P450 monoxygenase : construction of the functional fused enzyme between rat cytochrome P450c and NADPH-cytochrome P450 reductase. *DNA (NY)* **6**: 189-197.

Murphy, P. J. and C. A. West (1969). The role of mixed function oxidases in kaurene metabolism in *Echinocystis macrocarpa* green endosperm. *Arch. Biochem. Biophys.* **133**: 395-407.

Narhi, L. O. and A. J. Fulco (1987). Identification and characterization of 2 functional domains in cytochrome-P-450BM-3, a catalytically self-sufficient monooxygenase induced by barbiturates in *Bacillus megaterium*. *J. Biol. Chem.* **262**: 6683-6690.

Nebert, D. W., M. Adesnik, M. J. Coon, R. W. Estabrook, F. J. Gonzalez, F. P. Guengerich, I. C. Gunsalus, E. F. Johnson, B. Kemper, W. Levin, I. R. Phillips, R. Sato and M. R. Waterman (1987). The P450 gene superfamily: recommended nomenclature. *DNA* **6**: 1-11.

Nebert, D. W. and F. J. Gonzalez (1987). P450 genes:structure, evolution and regulation. *Annu. Review. Biochem.* **56**: 945-993.

Nelson, D. R., T. Kamataki, D. J. Waxman, F. P. Guengerich, R. W. Estabrook, R. Feyereisen, F. J. Gonzalez, M. J. Coon, I. C. Gunsalus, O. Gotoh, K. Okuda and D. W. Nebert (1993). The P450 Superfamily - update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biology*

12: 1-51.

Nelson, D. R., L. Koymans, T. Kamataki, J. J. Stegeman, R. Feyereisen, D. J. Waxman, M. R. Waterman, O. Gotoh, M. J. Coon, R. W. Estabrook, I. C. Gunsalus and D. W. Nebert (1996). P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **6**: 1-42.

Nelson, D. R. and H. W. Strobel (1988). On the membrane topology of vertebrate cytochrome P-450 proteins. *J. Biol. Chem.* **263**(13): 6038-6050.

Nelson, D. R. and H. W. Strobel (1988). Secondary structure prediction of 52 membrane-bound cytochromes P450 show a strong structural similarity to $P450_{cam}$. *Biochemistry* **28**: 656-660.

Newman, T., F. J. de Bruijn, P. Green, K. Keegstra, H. Kende, L. McIntosh, J. Ohlrogge, N. Raikhel, S. Somerville, M. Thomashow, E. Retzel and C. Somerville (1994). Genes galore:a summary of methods for accessing results from large-scale partial sequencing of anonymous *Arabidopsis* cDNA clones. *Plant Physiol.* **106**: 1241-1255.

Oeda, K., T. Sakaki and H. Ohkawa (1985). Expression of rat liver cytochrome P-450MC cDNA in *Saccharomyces cerevisiae*. *DNA* **4**(3): 203-210.

O'Keefe, D. P. and K. J. Leto (1989). Cytochrome P-450 from the mesocarp of avocado (*Persea americana*). *Plant Physiol.* **89**: 1141-1149.

O'Keefe, D. P., J. A. Romesser and K. J. Leto (1987). Plant and bacterial cytochromes P-450: involvement in herbicide metabolism. *Recent Advances in Phytochemistry*. J. A. Saunders, L. Kosak-Channing and E. E. Conn. New York, Plenum Press. **21:** 151-173.

Oliver, S. G., Q. J. M. van der Aart, M. L. Agostoni-Carbone, M. Aigle, L. Albergghina, D. Alexandraki, G. Antoine, R. Anwar, J. P. G. Ballesta, P. Benit, G. Berben, E. Bergantino, N. Biteau, P. A. Bolle, M. Bolotin-Fukuhara, A. Brown, A. J. P. Brown, J.

M. Buhler, C. Carcarno, G. Carignani, H. Cederberg, R. Chanet, R. Contreras, M. Crouzet, B. Daignan-Fornier, E. Defoor, M. Delgado, J. Demolder, C. Diora, E. Dubois, B. Dujon, A. Dusterhoft, D. Erdmann, M. Esteban, F. Fabre, C. Fairhead, G. Faye, H. Feldmann, W. Fiers, M. C. Francingues-Gaillard, L. Franco, L. Frontali, H. Fukuhara, L. J. Fuller, P. Galland, M. E. Gent, D. Gigot, V. Gilliquet, N. Glansdorff, A. Goffeau, M. Grenson, P. Grisanti, L. A. Grivell, M. de Haan, M. Haasemann, D. Hata, J. Hoenicka, J. Hegemann, C. J. Herbert, F. Hilger, S. Hohmann, C. P. Hollenberg, K. Huse, F. Iborra, K. J. Indge, K. Isono, C. Jacq, M. Jacquet, C. M. James, J. C. Jauniaux, Y. Jia, A. Jimenez, A. Kelly, U. Kleinhans, P. Kreisl, G. Lanfranchi, C. Lewis, C. G. van der Linden, G. Lucchini, K. Lutzenkirchen, M. J. Maat, L. Mallet, G. Mannhaupt, E. Martegani, A. Mathieu, C. T. C. Maurer, D. McConnell, R. A. McKee, F. Messenguy, H. W. Mewes, F. Molemans, M. A. Montague, M. Muzi Falconi, L. Navas, C. S. Newlon, D. Noone, C. Pallier, L. Panzeri, B. M. Pearson, J. Perea, *et. al.* (1992). The complete DNA sequence of yeast chromosome III. *Nature* **357**: 38-46.

Omura, T. and R. Sato (1962). A new cytochrome in liver microsomes. *J. Biol. Chem.* **237**: 1375-1376.

Omura, T. and R. Sato (1964). The carbon monoxide-binding pigment of liver microsomes. *J. Biochem* **239**: 2370-2378.

Ortiz de Montellano, P. R. (1986). Oxygen activation and transfer. *Cytochrome P450. Structure Mechanism and Biochemistry*. P. R. Ortiz de Montellano. New York, Plenum Press: 217-271.

Ouzounis, C. A. and W. T. Melvin (1991). Primary and secondary structural patterns in eukaryotic cytochrome P-450 families correspond to structures of helix-rich domain of *Pseudomonas putida* cytochrome $P\text{-}450_{cam}$. Indications for a similar overall topology.

Eur. J. Biochem **198**: 307-315.

Pan, Z. Q., F. Durst, D. Werck-Reichhart, H. W. Gardner, B. Camara, K. Cornish and R. A. Backhaus (1995). The major protein of guayale rubber particles is a cytochrome P-450 - characterisation based on cDNA cloning and spectroscopic analysis of the solubilised enzyme and its reaction products. *J. Biol. Chem.* **270**: 8487-8494.

Park, Y. S., J. M. Kwak, Y. S. Kim, D. S. Lee, M. J. Cho, H. Lee and H. G. Nam (1993). Generation of expressed sequence tags of random root cDNA clones of *Brassica napus* by single-run partial sequencing. *Plant Physiol.* **103**: 359-370.

Persans, M. and M. A. Schuler (1995). Differential induction of cytochrome P450-mediated triasulfuron metabolism by naphthalic anhydride and triasulfuron. *Plant Physiol.* **109**: 1483-1490.

Petersen, M. and H. U. Setz (1988). Reconstitution of cytochrome P450-dependent digitoxin 12β-hydroxylase from cell cultures of foxglove (*Digitalis lanata* EHRH.). *Biochemical Journal* **252**: 537-543.

Peterson, J. A. and S. E. Graham-Lorence (1995). Bacterial cytochrome P450s: structural similarities and functional differences. *Cytochrome P450: Structure, Mechanism and Biochemistry*. O. d. Montellano. New York, Plenum Press: 151-180.

Pierrel, M. A., Y. Batard, M. Kazmaier, C. Mignotte-Vieux, F. Durst and D. Werck-Reichhart (1994). Catalytic properties of the plant cytochrome *P*450 CYP73 expressed in yeast. *Eur. J. Biochem.* **224**: 835-844.

Pinot, F., C. Alayrac, C. Mioskowski, F. Durst and J.-P. Salaün (1994). New cytochrome P450-dependent reactions from wheat - terminal and subterminal hydroxylation of oleic acid by microsomes from naphthalic anhydride and phenobarbital induced wheat seedlings. *Biochem. Biophys. Res. Comm.* **198**: 795-803.

Pinot, F., H. Bosch, C. Alyrac, C. Mioskowski, A. Vendais, F. Durst and J.-P. Salaün (1993). ω-hydroxylation of oleic acid in *Vicia sativa* microsomes. Inhibition by substrates analogs and inactivation by terminal acetylenes. *Plant Physiol.* **102**: 1313-1318.

Pinot, F., J.-P. Salaün, H. Bosch, A. Lesot, C. Mioskowski and F. Durst (1992). ω-hydroxylation of Z 9-octadecanoic, Z 9,10 epoxystearic and 9,10 dihydroepoxystearic acids by microsomal cyt P450 systems from *Vicia sativa*. *Biochem. Biophys. Res. Comm.* **184**: 183-193.

Pompon, D. (1988). cDNA cloning and functional expression in yeast *Saccharomyces cerevisiae* of *B*-naphthoflavone-induced rabbit liver P-450 LM4 and LM6. *Eur. J. Biochem.* **177**: 285-293.

Pompon, D. and C. Cullin (1989). Functional Expression in yeast of chimeric and authentic P-450 isoenzymes. *Cytochrome P-450:Biochemistry and Biophysics*. I. Schuster. London.NewYork.Philadelphia, Taylor and Francis: 515-518.

Pompon, D., B. Louerat, A. Bronine and P. Urban (1995). Yeast expression of animal and plant P450s in optimised redox environments. *Meths. Enzymol.* **272**: 51-64.

Porter, T. D. and M. J. Coon (1991). Cytochrome P450: multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms. *J. Biol. Chem.* **266**: 13469-13472.

Porter, T. D., T. E. Wilson and C. B. Kasper (1987). Expression of functional 78,000 dalton mammalian flavoprotein, NADPH-cytochrome-P-450 oxidoreductase in *Escherichia-coli*. *Arch. Biochem. Biophys.* **254**: 353-367.

Posner, H. S., C. Mitoma and S. Udenfriend (1961). Enzymatic hydroxylation of aromatic compounds: II Further studies of the properties of the microsomal hydroxylating system. *Arch. Biochem. Biophys.* **94**: 269-279.

Potter, S., D. E. Moreland, K. Kreuz and E. Ward (1995). Induction of cytochrome P450

genes by ethanol in maize. *Drug Metabolism and Drug Interaction* **12**(3-4): 317-327.

Poulos, T. L. (1991). Modelling of mammalian P450s on basis of P450cam X-ray structure. *Methods Enzymol.* **206**: 11-30.

Poulos, T. L. (1995). Cytochrome P450. *Current Opinion in Structural Biology* **5**: 767-774.

Poulos, T. L., B. C. Finzel and A. J. Howard (1987). High-resolution crystal structure of cytochrome P450cam. *J. Mol. Biol.* **195**: 687-700.

Poulos, T. L., M. Perez and G. Wagner (1982). Preliminary crystallographic data on cytochrome P-450$_{CAM}$. *J. Biol. Chem.* **257**: 10427-10429.

Rahier, A. and M. Taton (1986). The 14α-demethylation of obtusifoliol by a cytochrome P-450 monooxygenase from higher plant microsomes. *Biochem. Biophys. Res. Comm.* **140**: 1064-1072.

Reed, K. C. and D. A. Mann (1988). Rapid transfer of DNA from agarose gels to nylon membranes. *Nucleic Acids Research* **13**: 7207-7221.

Renaud, J.-P., C. Cullin, D. Pompon, P. Beaune and D. Mansuy (1990). Expression of human liver cytochrome P450 IIIA4 in yeast.

A functional model for the hepatic enzyme. *Eur. J. Biochem.* **194**: 889-896.

Rifkind, A. B. and T. Petchke (1981). *p*-Chloro-*N*-methylaniline demethylase activity in liver and extrahepatic tissues of the human fetus and in chick embryo liver. *Journal of Pharmacology and Experimental Therapeutics* **217**(2): 572-578.

Rodgers, M. W., A. Zimmerlin, D. Werck-Reichhart and G. P. Bolwell (1993). Microsomally associated heme proteins from French bean:characterization of the cytochrome P450 cinnamate-4-hydroxylase and two peroxidases. *Arch. Biochem. Biophys.* **304**: 74-80.

Rossiter, J. T., D. C. James and N. Atkins (1990). Biosynthesis of 2-hydroxy-3-butenylglucosinolate and 3-butenylglucosinolate in *Brassica napus*. *Phytochemistry* **29**: 2509-2512.

Ruettinger, R. T., L. Wen and A. J. Fulco (1989). Coding, nucleotide 5' regulatory, and deduced amino-acid sequences of P450BM3, a single peptide cytochrome P-450-NADPH-P450 reductase from *Bacillus megaterium*. *J. Biol. Chem.* **264**: 10987-10995.

Ryan, D. E., P. E. Thomas and W. Levin (1982). Purification and characterisation of a minor form of hepatic microsomal cytochrome P-450 from rats treated with polychlorinated biphenyls. *Arch. Biochem. Biophys.* **216**: 272-288.

Sakaguchi, M., K. Mihara and R. Sato (1984). Signal recognition particle is required for co-translational insertion of cytochrome P-450 into microsomal membranes. *Pro. Natl. Acad. Sci. USA* **81**: 3361-3364.

Salaün, J.-P., I. Benveniste, R. Fonne, B. Gabriac, D. Reichhart, A. Simon and F. Durst (1982). Hydroxylations microsomales de l'acide laurique catalysées par le cytochrome P450 chez les plantes supérieures. *Physiologie Végétale* **20**: 613-621.

Salaün, J.-P., I. Benveniste, D. Reichhart and F. Durst (1981). Induction and specificity of a (cytochrome P-450)-dependent laurate in-chain hydroxylase from higher plant microsomes. *Eur. J. Biochem.* **119**: 651-655.

Salaün, J.-P. and C. Helvig (1995). Cytochrome P450-dependent oxidation of fatty acids. *Drug Metab. Drug Interact.* **12**(3-4): 261-283.

Salaün, J.-P., A. Simon and F. Durst (1986). Specific induction of lauric acid ω-hydroxylase by clofibrate, diethylhexyl-phthalate and 2,4-dichlorophenoxyacetic acid in higher plants. *Lipids* **21**: 776-779.

Salaün, J.-P., D. Weissbart, F. Durst, P. Pflieger and C. Mioskowski (1989). Epoxidation of *cis* and *trans* delta-9-unsaturated lauric acids by a cyt P-450 from higher plant microsomes. *FEBS Letters* **246**: 120-126.

Salaün, J.-P., D. Weissbart, C. Helvig, F. Durst and C. Mioskowski (1993). Regioselective hydroxylation and epoxidation of lauric acid and unsaturated analogs by cytochrome P450 in Jerusalem artichoke microsomes. *Plant Physiol. Biochem.* **31**: 285-293.

Sambrook, J., E. F. Fritsh and T. Maniatis (1989). *Molecular Cloning:A Laboratory Manual*. Cold Spring Harbour, New York, Cold Spring Harbour Press.

Sanger, F., S. Nicklen and A. R. Coulson (1977). DNA sequencing with chain terminating inhibitors. *Pro. Natl. Acad. Sci. USA* **74**: 5463-5467.

Sangwan, N. K., B. S. Verma, K. K. Verma and K. S. Dhindsa (1990). Nematicidal activity of some essential plant oils. *Pesticide Science* **28**: 331-335.

Sato, T., M. Sakaguchi, K. Mihara and T. Omura (1990). The amino-terminal structures that determine topological orientation of cytochrome P-450 in microsomal membrane. *EMBO J.* **9**: 2391-2397.

Schiestl, R. H., P. Manivasakam, R. A. Woods and R. D. Gietz (1993). Introducing DNA into yeast by transformation. *METHODS: A Companion to Methods Enzymol.* **5**: 79-85.

Schuler, M. A. (1996). Plant cytochrome P450 monoxygenases. *Critical Reviews in Plant Sciences* **15**(3): 235-284.

Shen, A. L., T. D. Porter, T. E. Wilson and C. B. Kasper (1989). Structural analysis of the FMN binding domain of NADPH-cytochrome P-450 oxidoreductase by site directed mutagenesis. *J. Biol. Chem.* **264**: 7584-7589.

Shen, B., N. Carneiro, I. Torres-Jerez, B. Stevenson, T. McCreery, T. Helentjaris, C. Batsdorfer, E. Almira, R. Ferl, J. E. Habben and B. Larkins (1994). Partial sequencing and

mapping of clones from two maize cDNA libraries. *Plant Molecular Biology* **26**: 1085-1101.

Shet, M. S., K. Sathasivan, M. A. Arlotto and M. C. Mehdy (1993). Purification, characterisation, and cDNA cloning of an NADPH-cytochrome P450 reductase from mung bean. *Proc. Nat. Acad. Sci.* **90**: 2890-2894.

Shibata, M., T. Sakaki, Y. Yabusaki, H. Murakami and H. Ohkawa (1990). Genetically engineered P450 monoxygenases: construction of bovine P450c17/yeast reductase fused enzymes. *DNA and Cell Biology* **9**(1): 27-36.

Short, J. M., J. M. Fernandez, J. A. Sorge and W. Huse (1988). λ ZAP: A bacteriophage λ expression vector with *in-vivo* excision properties. *Nucleic Acids Research* **16**(15): 7583-7600.

Shoun, H., Y. Sudo, Y. Seto and Beppu T. (1983). Purification and properties of a cytochrome P-450 of a fungus, Fusarium oxysorium. *J. Biol. Chem.* **94**: 1219-1229.

Sibbesen, O., B. Koch, B. A. Halkier and B. L. Møller (1995). Isolation of the heme-thiolate enzyme cytochrome $P450_{TYR}$, which catalyzes the committed step in the biosynthesis of the cyanogenic glucoside dhurrin in *Sorghum bicolor* (L.) Moench. *Pro. Natl. Acad. Sci. USA* **91**: 9740-9744.

Soliday, C. L. and P. E. Kolattukudy (1978). Midchain hydroxylation of 16-hydroxypalmitic acid by the endoplasmic reticulum fraction from germinating *Vicia faba*. *Arch. Biochem. Biophys.* **188**: 338-347.

Song, W.-C. and A. R. Brash (1991). Purification of an allene oxide synthase and identification of the enzyme as a cytochrome P450. *Science* **253**: 781-784.

Song, W.-C., C. D. Funk and A. R. Brash (1993). Molecular cloning of an allene oxide synthase: a cytochrome P450 specialized for the metabolism of fatty acid hydroperoxides.

Pro. Natl. Acad. Sci. USA **90**: 8519-8523.

Southern, E. (1979). Gel electrophoresis of restriction fragments. *Methods Enzymol.* **68**: 152-176.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**: 503-517.

Stadler, R. and M. Zenk (1993). The purification and characterization of a unique cytochrome P450 enzyme from *Berberis stolonifera* plant cell cultures. *J. Biol. Chem.* **286**(2): 823-831.

Strathern, J. N. and D. R. Higgins (1991). Recovery of plasmids from yeast into *Escherichia-coli*-shuttle vectors. *Methods Enzymol.* **194**: 319-329.

Sugimaya, T., N. Uiki and T. Yamano (1979). The obligatory requirement of cytochrome b_5 in the p-nitoanisole O-demethylation reaction catalyzed by cytochrome P-450 with a high affinity for cytochrome b_5. *Biochem. Biophys. Res. Comm.* **90**: 715-720.

Sulston, J., Z. Du, K. Thomas, R. Wilson, L. Hillier, Staden R., N. Halloran, P. Green, J. Thierry-Mieg, L. Qui, S. Dear, A. Coulson, M. Craxton, R. Durbin, M. Berks, M. Metzstein, T. Hawkins, R. Ainscough and R. Waterston (1992). The *C. elegans* genome sequencing project: a beginning. *Nature* **356**: 37-41.

Szczesna-Skorupa, E., P. Straub and B. Kemper (1993). Deletion of a conserved tetrapeptide, PPGP in P450 2C2 results in loss of enzymatic activity without a change in its cellular location. *Arch. Biochem. Biophys.* **304**(1): 170-175.

Templeton, W. (1969). *An Introduction to the Chemistry of Terpenoids and Steroids.* London, Butterworths and Co. Ltd.

Teutsch, H. G., M. P. Hasenfratz, A. Lesot, C. Stoltz, J.-M. Garnier, J.-M. Jeltsch, F. Durst and D. Werck-Reichhart (1993). Isolation and sequence of a cDNA encoding the

Jerusalem artichoke cinnamate 4-hydroxylase, a major plant cytochrome P450 in the general phenylpropanoid pathway. *Pro. Natl. Acad. Sci. USA* **90**: 4102-4106.

Toguri, T., M. Azuma and T. Ohtani (1993). The cloning and characterisation of a cDNA encoding a cytochrome P450 from the flowers of *Petunia hybrida. Plant Science* **94**: 119-126.

Toguri, T., O. Kobayashi and N. Umemoto (1993). The cloning of egg plant cDNAs encoding proteins from a novel cytochrome P-450 family (CYP76) *Biochem. Biophys. Acta* **1216**: 165-169.

Toguri, T., N. Umemoto, O. Kobayashi and T. Ohtani (1993). Activation of anthocyanin synthesis genes by white light in eggplant hypocotyl tissues, and identification of an inducible P-450 cDNA. *Plant Molecular Biology* **23**: 933-946.

Towbin, H., T. Staehelin and J. Gordon (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci . USA* **76**: 4350-4354.

Treco, D. A. (1989). Preparation of yeast RNA. *Current Protocols* **Supplement 5**: 13.12.11-13.12.13.

Tretiakov, V. A., K. N. Degtyarenko, V. Y. Uvarov and A. I. Archakov (1989). Secondary structure and membrane topology of cytochrome P450s. *Arch. Biochem. Biophys.* **275**(2): 429-439.

Uchimiya, H., S. Kidou, T. Shimazaki, S. Takamatsu, H. Hasimoto, R. Nishi, S. Aotsuka, Y. Matsubayashi, N. Kidou, M. Umeda and A. Kato (1992). Random sequencing of cDNA libraries reveals a variety of expressed genes in cultured cells of rice (*Oryza sativa* L). *Plant J.* **2**: 1005-1009.

Udvardi, K. M., J. D. Metzger, V. Krishnapillai, W. J. Peacock and E. S. Dennis (1994).

Cloning and sequencing of a full-length cDNA from *Thlaspi arvense* L. that encodes a cytochrome P450. *Plant Physiol.* **105**: 755-756.

Umemoto, N., O. Kobayashi, O. Ishizaki-Nishizawa and T. Toguri (1993). cDNA sequences encoding cytochrome P450 (CYP71 family) from eggplant seedlings. *FEBS Letters* **330**: 169-173.

Urban, P., C. Cullin and D. Pompon (1990). Maximising the expression of mammalian cytochrome P-450 monoxygenase activities in yeast cells. *Biochemie* **72**: 463-472.

Urban, P., D. Werck-Reichhart, H. G. Teutsch, F. Durst and S. Regnier (1994). Characterisation of recombinant plant cinnamate 4-hydroxylase produced in yeast. *Eur. J. Biochem.* **222**: 843-850.

Vetter, H.-P., U. Mangold, G. Schröder, F.-J. Marner, D. Werck-Reichhart and J. Schröder (1992). Molecular analysis and heterologous expression of an inducible cytochrome P-450 protein from periwinkle (*Catharanthus roseus* L.). *Plant Physiol.* **100**: 998-1007.

Waterston, R., C. Martin, M. Craxton, A. Coulson, L. Hillier, R. Durbin, P. Green, R. Shownkeen, N. Halloran, M. Metzstein, T. Hawkins, R. Wilson, M. Berks, Z. Du, K. Thomas, J. Thierry-Mieg and J. Sulston (1992). A survey of expressed genes in *Caenorhabditis elegans*. *Nature Genet.* **1**: 114-123.

Wendorff, H. and U. Matern (1986). Differential response of cultured parsley cells to elicitors from 2 non-pathogenic strains of fungi-microsomal conversion of (&)marmesin into psoralen. *Eur. J. Biochem.* **161**: 391-398.

Werck-Reichhart, D. (1995). Cytochromes P450 in phenylpropanoid metabolism. *Drug. Metab. Drug. Interact.* **12**(3-4): 221-243.

Werck-Reichhart, D., B. Gabriac, H. Teutsch and F. Durst (1990). Two cytochrome P450 isoforms catalysing *O*-de-ethylation of ethoxycoumarin and ethoxyresorufin in higher

plants. *Biochem. J.* **270**: 729-735.

West, C. A. (1980). Hydroxylases, monoxygenases and cytochrome P-450. *The Biochemistry of Plants*, Academic Press, Inc. **2:** 317-363.

White, R. E. and M. J. Coon (1980). Oxygen activation by cytochrome P-450. *Annu. Rev. Biochem.* **49**: 315-356.

Yasumori, T., N. Murayama, Y. Yamazoe, A. Abe, Y. Nogi, T. Fukasawa and R. Kato (1989). Expression of a human P-450IIC gene in yeast cells using galactose-inducible expression system. *Molecular Pharmacology* **35**: 443-439.

Yoshida, Y. and Y. Aoyama (1984). Yeast cytochrome-P-450 catalysing lanosterol 14-alpha-demethylation .2. lanosterol metabolism by purified P-45014DM and by intact microsomes. *J. Biol. Chem.* **259**: 1661-1666.

Yoshida, Y. and Y. Aoyama (1987). Interaction of azole antifungal agents with cytochrome-P-45014DM purified from *Saccharomyces cerevisiae* microsomes. *Biochem. Pharmacol.* **36**(2): 229-235.

Young, R. A. and R. W. Davis (1983). Efficient isolation of genes using antibody probes. *Pro. Natl. Acad. Sci. USA* **80**: 1194-1198.

Zenk, M., R. Gerardy and R. Stadler (1989). Phenol oxidative coupling of benzylisoquinoline alkaloids is catalysed by regio-selective and stereo-selective cytochrome P-450 linked plant enzymes. *Chem. Soc. Chem. Commun.* **22**: 1725-1727.

Zimmerlin, A. and F. Durst (1992). Aryl hydroxylation of the herbicide diclofop by a wheat cytochrome P-450 monoxygenase. *Plant Physiol.* **100**: 874-881.

Zimmerlin, A., J.-P. Salaün, F. Durst and C. Mioskowski (1992). Cytochrome P-450-dependent hydroxylation of lauric acid at the subterminal position and oxidation of unsaturated analogs in wheat microsomes. *Plant Physiol.* **100**: 868-873.