Cytochrome P450 Monooxygenase (CYP450) Analysis in *Lolium rigidum* Gaudin

Natalie Dillon

A DISSERTATION

Thesis submitted for the degree of Doctor of Philosophy,

Department of Applied and Molecular Ecology,

Faculty of Agricultural and Natural Resource Sciences,

The University of Adelaide, Waite Campus

July 2001

Table of Contents

2.   Materials and Methods

3.   Induction of Cytochrome P450 Monooxygenases in *L. rigidum*

4.   Inhibition of Cytochrome P450 Monooxygenases by Mechanism-based Inhibitors

Abstract

Cytochrome P450 monooxygenases have been implicated as a major detoxification system in *Lolium rigidum* for the metabolism of herbicides. Nevertheless, isolation of these genes has been impeded due to low levels of the enzyme within plants, interference of other pigments and difficulties in their extraction. In this study, whole plant studies demonstrated metabolism of diclofop-methyl occurred at a rate 1.3-fold faster in the herbicide-resistant *L. rigidum* biotype (SLR31) when compared to herbicide-susceptible biotype (VLR1). Subsequently, pre-treatment of seedlings for 24 h with either 2,4-dichlorophenoxyacetic acid (2,4-D) or clofibrate showed an increase in the *in vivo* levels of diclofop-methyl metabolism for both herbicide-resistant and herbicide-susceptible biotypes. These induction experiments indicated that 2,4-D exerted a greater effect in increased diclofop-methyl metabolism in the herbicide-susceptible (VLR1) *L. rigidum* biotype than in the herbicide-resistant biotype (SLR31).

Inhibition of diclofop-methyl metabolism was seen on the addition of 2.5 µM suicide substrate (compound 2). Both the herbicide-resistant (SLR31) and herbicide-susceptible (VLR1) biotypes showed up to 1.3-fold increases in the level of diclofop-methyl and diclofop acid remaining and a 50% decrease in metabolite production 24 h post-treatment.

Northern blot analysis indicated low level constitutive expression of LrCYP in 12 day old *L. rigidum* seedlings. Transcript expression was sharply enhanced in seedlings pre-treated with either 2,4-D or clofibrate. Similar findings were seen with CA4H (CYP73.301

isolated from *Triticum aestivum)* induction. Following the addition of 2,4-D to both herbicide-resistant and herbicide-susceptible *L. rigidum* seedlings a sharp increase in the transcript levels was seen as soon as 3 h post-treatment. This increase in transcript expression began to decrease slowly until 12 h post-treatment, after which levels returned to that of the control seedlings. On the addition of clofibrate to both herbicide-resistant and herbicide-susceptible *L. rigidum* seedlings a sharp increase in the transcript levels was also seen but not until 6 h post-treatment. Increased transcript levels again returned to basal levels by 60 h post-treatment. The increase in transcript expression was greater on the addition of 2,4-D than with the addition of clofibrate. The correlation between inducible diclofop-methyl metabolism and increased transcript expression of the LrCYP clone on the addition of either 2,4-D or clofibrate within *L. rigidum* requires further investigation.

A PCR-based approach enabled the isolation of a short, 3'-end fragment of a putative cytochrome P450 monooxygenase from *L. rigidum*. This fragment showed 63% sequence identity to the 3'-end of CYP71C2 (indolin-2-one hydroxylase), previously isolated from *Zea mays*. Alignment of sequences from the CYP71C sub-family enabled the design of a novel primer at a compatible region at the 5'-end of these cytochrome P450 monooxygenases enabling the amplification of a larger gene fragment (designated LrCYP). Due to primer design all clones lacked approximately 70 amino acids at the 5'-end and 30 amino acids at the 3'-end of the gene.

Despite numerous screenings of *L. rigidum* cDNA libraries (even low stringency) a full-length LrCYP clone could not be isolated. The encoded partial cDNA's isolated showed greater than 90% positional amino acid identity with other *L. rigidum* clones identified and only 56.5% amino acid identity with CYP71C2 from *Z. mays*. Divergence of the partial cDNA sequences of identified cytochrome P450 monooxygenases is sufficient to account for different substrate or reaction specificities within *L. rigidum* seedlings. Only isolation of full-length genes and expression in a suitable host expression vector will potentially identify the *in vivo* function of these cytochrome P450 monooxygenases within *L. rigidum* and identify their possible association in diclofop-methyl metabolism.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made.

I give my consent that this work may be photocopied or loaned from the university library.

Signed:

Natalie Dillon

Date: 25th October, 2002

Acknowledgments

Australia

I wish to thank my supervisors, Steve Powles, Chris Preston and Jill Karotam for their invaluable advice and assistance throughout the period of my PhD.

Thank you to Debbie Lorraine-Colwill for help with the HPLC, Rachael Burton and Uli Theopold for advice with molecular techniques, Neil Shirley for sequencing and technical advice, and Mary Rieger for reading my thesis.

Thank you to Kirsty Villis, Kelly Scott, Jan Fair, Rick Roush, Peter Ninnes, Jim Fortune, and Jo Speck, for all their help in the CRC for Weed Management Systems office. Thank you also to Otto Schmidt and Anke Johnson for their support in the Department of Applied and Molecular Ecology office, University of Adelaide.

To all the members of the CRC for Weed Management Systems, Kathy Evans, Gwen Mayo, Craig Clarke, Janine Lloyd, Stephen Hole, Chris Soar, Anuja Kumaratilake, and Midori Jones, thank you all for your support, help and friendship during the term of my PhD.

Gai McMichael, Scott Donner, and Andi Sebastian, I can't express how much your friendship and advice has supported me through my PhD.

I am extremely grateful for the financial support for the project supplied by the University of Adelaide (Australian Postgraduate Award) and the CRC for Weed Management Systems. Support was also provided by Centre National Recherché Scientific (CNRS) with PICS grant (No. 344) and the grant from the International Science and Technology Program provided by the Department of Education, Training and Employment.

France

Je dois également remercier Francis Durst, Jean-Pierre Salaün, Irene Benveniste, Danielle Werck-Reichhart, Franck Pinot, Agnes Lesot, Rachel Kahn, Miyuki Shimoji, Marc Morant, Guillaume, Nathalie Forthoffer, Svetlana Nedlikana, Marie-France Poulet et Monique Le Ret de m'accueillir dans leur laboratoire. Merci pour votre aide, amitié et pour rendre mon séjour à Strasbourg plus mémorable. J'évalue certainement l'aide que j'ai reçue de vous tout.

Finally I would like to thank my family who supported and encouraged me through the endless and seemingly eternal roller coaster ride throughout the course of my PhD.

List of Figures

List of Tables

List of Abbreviations

ABT	1-aminobenzotriazole
ACCase	acetyl coenzyme A carboxylase (E.C. 6.4.1.2)
AHAS	acetohydroxyacid synthase
ALS	acetolactate synthase (E.C. 4.1.3.18)
Amp	ampicillin
AOS	allene oxide synthase (E.C. 4.2.1.92)
APP	aryloxyphenoxypropionate
ARGT	annual ryegrass toxicity
ATP	adenosine 5'-triphosphate
BA	benzyladenine
BLAST	basic local alignment search tool
Borax	di-sodium tetraborate or sodium borate decahydrate
BSA	bovine serum albumin
bp	base pair(s)
BPB	bromophenol blue
°C	degrees centigrade
^{14}C	radiolabelled carbon 14
CA4H	*trans*-cinnamic 4-hydroxylase (E.C. 1.14.13.11)
cDNA	complimentary deoxyribonucleic acid
C3H	ρ-coumarate-3-hydroxylase
CHD	cyclohexanedione
cm	centimetre(s)
clofibrate	2-(ρ-chlorophenoxy)-2-methyl-propionic acid ethyl ester
CO	carbon monoxide
CsCl	caesium chloride

cyometrinil	(a-[(cyanomethoxy)imino]benzeneacetonitrile)
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytosine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
dTTP	deoxythymidine 5'-triphosphate
dNTP's	deoxynucleotide 5'-triphosphates
DTT	dithiothreitol
E.C.	enzyme commission number
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EREBP	ethylene-responsive element binding protein
EtBr	ethidium bromide
EtOH	ethanol
Fe[II]-CO	ferrous.carbon monoxide complex
F5H	ferulate 5-hydroxylase (no E.C. number assigned)
g	gram(s)
g	relative centrifugal force
G	guaiacyl unit
GST	glutathione S-transferase (E.C. 2.5.1.18)
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
h	hour(s)
IC-LAH	in-chain lauric acid hydroxylase
IPTG	isopropylthio-β-D-galactoside
jasmonic acid	3-oxo-2-(2'-pentenyl)-cyclopentaneacetic acid

kb	kilo base pair(s)
kDa	kilo Dalton(s)
kPa	kilo Pascal(s)
L	litre(s)
LAH	lauric acid hydroxylase
LB	Luria-Bertani medium
LiCl	lithium chloride
LSS	liquid scintillation spectroscopy
m	metre(s)
M	molar (moles per litre)
maltose	4-0-α-D-Glucopyranosyl-D-glucose
mCi	milli Curie(s)
MeOH	methanol
mfo	mixed function oxidase
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mM	millimolar
Mn-SOD	Mn-superoxide dismutase
MOPS	3-[*N*-Morpholino]propane-sulfonic acid
mRNA	messenger ribonucleic acid
MW	molecular weight
N	normal
NA	naphthalic acid anhydride
NAA	1-naphthaleneacetic acid
NaAc	sodium acetate
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide - reduced

NADPH	nicotinamide adenine dinucleotide phosphate - reduced
NCBI	National Centre for Biotechnology
ng	nanogram(s)
nm	nanometer(s)
nmol	nanomole(s)
NP-40	nonidet P-40
OD	optical density
OMT	O-methyltransferase
ORF	open reading frame
O_2	molecular oxygen
PAL	phenylalanine ammonia lyase (E.C. 4.3.1.5)
PB	phenobarbitol
PBO	piperonyl butoxide
pCMA	p-chloro-N-methylaniline
PCR	polymerase chain reaction
PEG_{8000}	polyethylene glycol$_{8000}$
pfu	plaque forming unit(s)
pmol	picomole(s)
PPARα	peroxisome proliferator-activated α receptor
PPRE	peroxisome proliferator response element
PSII	photosystem II
PVP$_{40}$	polyvinylpyrrolidone, Mr 40,000
P450$_{CAM}$	bacterial camphor-hydroxylating cytochrome P450
REB	RNA extraction buffer
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
rpp	rubber particle protein

RT	room temperature
RT-PCR	reverse transcription - polymerase chain reaction
RXRα	retinoid X receptor α
SDS	sodium dodecyl sulphate
s	second(s)
ss	single stranded
S	syringyl unit
sodium deoxycholate	deoxycholic acid (5β-Cholan-24-oic acid-3α,12α-diol) Sodium salt
SSC	sodium citrate buffer
TAE	tris-acetate/EDTA electrophoresis buffer
TAL	tyrosine ammonia lyase
TB	Terrific broth
TE	Tris/EDTA buffer
tetcyclasis	5-(4-chlorophenyl)-3,4,5,9,10-pentaazatetracyclo$[5,4,10^{2,6},O^{8,11}]$ dodeca-3,9-diene
TMV	tobacco mosaic virus
TNV	tobacco necrosis virus
Tris	tris(hydroxymethyl)aminomethane hydrochloride
Tween 20	polyoxyethylene sorbitan monolaurate
UDYS	undec-n-yne-1-sulfonic acid (sodium salt)
UV	ultraviolet light
V	volt(s)
v/v	volume/volume
w/v	weight/volume
X-gal	5-Bromo-4-chloro-3-indolyl-ß-D-galactoside
11-DDNA	11-dodecenoic acid
2,4-D	2,4-dichlorophenoxyacetic acid

4CL	4 coumarate:CoA ligase (E.C. 6.2.1.12)
λ	wavelength
μ	micro
μg	microgram(s)
μl	microlitre(s)
μM	micromolar
ρ	para
ω	omega
%	percent

Chapter 1

Literature Review

Chapter 1

Literature Review

1.1   Introduction

Lolium rigidum Gaudin (also known as annual ryegrass, Wimmera ryegrass, or rigid ryegrass) is well adapted to the Mediterranean regions of southern Australia. Due to its high productivity, especially for winter growth and regrowth after grazing, rapid spring growth, good forage quality producing a large bulk of palatable herbage, and prolific seed production, *L. rigidum* was integrated into the successful *Triticum aestivum* (L.) Beauv. (wheat)/sheep rotation of southern Australia as a valuable pasture species. Although widely cultivated as a pasture grass, it has become the major grass weed of winter field crops in the southern cropping belt (Terrell, 1968; Monaghan, 1980) where control of this species has posed serious problems for farmers. The major impact of *L. rigidum* is a reduction in crop yields, mainly through direct competition but other aspects of crop production are also affected. These weeds may reduce crop quality, increase energy consumption in their control, be a host to disease, such as annual ryegrass toxicity (ARGT) and *Rhizoctonia* bare patch, harbour detrimental insects, and contaminate produce.

For the last two decades, widespread herbicide use has been extensively adopted to minimise crop yield losses from the impact of *L. rigidum*. The development of the cereal-selective aryloxyphenoxypropionate (APP) and sulfonylurea herbicides allowed successful control of *L. rigidum* within the cropping phase. Nevertheless, intense selection pressure placed on this evasive weed from the reliance on herbicide use targeting specific

physiological or biochemical processes soon resulted in failure of control due to the onset of herbicide resistance. The first case of herbicide resistance in *L. rigidum* (biotype SLR5) to the post-emergent graminicide diclofop-methyl was reported in 1980 (Heap & Knight, 1982) only four years after the release of this herbicide. Further studies revealed that this biotype displayed cross-resistance to the unrelated herbicide chlorsulfuron (Heap & Knight, 1986). Since then, thousands of herbicide resistant populations of *L. rigidum* have appeared across the country (Powles & Matthews, 1991; Matthews, 1994; Gill, 1995; Preston *et al.*, 1999). A successful farming system aims to maximise crop yields while minimising the impact of weeds species by maintaining them at densities below tolerable economic loss levels (Powles *et al.*, 1997). The evolution of herbicide resistance in weedy plant species is a threat to the success of world crop production and the continued efficacy of herbicide use within these systems. Therefore, it is essential that we understand the mechanisms that underlie the onset of herbicide resistance in these species.

With many species displaying herbicide-resistance through a combination of mechanisms, a range of terms has been developed to describe the types of resistance mechanisms exhibited. For the purpose of this review the term 'resistance' will donate the inherited ability, due to the selection of traits, of a population to survive xenobiotic challenge at a level that would otherwise be detrimental. 'Tolerance' will refer to the naturally occurring ability of a species to survive xenobiotic challenge without prior exposure to that particular xenobiotic. 'Multiple-resistance' will designate a population that has developed more than one resistance mechanism to xenobiotics following exposure to each of the xenobiotics that resistance is exhibited. The term 'cross-resistance' will refer to the means by which a population exposed to one xenobiotic exhibits resistance to one or more different xenobiotics (of different chemical structure) to which it has never previously been exposed. Mechanisms endowing cross-resistance can be divided into two broad categories, target site cross-resistance and non-target site cross-resistance (Hall *et al.*, 1994). Target site

cross-resistance occurs when an alteration at the site of action of one herbicide also confers resistance to herbicides of a different chemistry that also inhibits the same target site. This does not necessarily confer resistance to all herbicides within one chemistry or with a similar mode of action. Non target site cross-resistance is conferred by mechanisms other than modification at the site of action. Mechanisms may include reduced herbicide uptake, translocation or enhanced metabolism of the herbicide (Hall *et al.*, 1994).

The ability to exhibit both multiple and cross-resistance to herbicides of unrelated chemistries and modes of action is prevalent within *L. rigidum*. This is possibly a consequence of genetic plasticity of this species also evidenced by its adaptability and variable morphology. Wide genetic variation due to large differences at the isoenzyme level is evident in this species (Matthews, 1994). Therefore, given sufficient time and selection pressure, evolutionary responses altering resistance traits are inevitable. Target site resistance has been identified in *L. rigidum* to acetyl coenzyme A carboxylase (ACCase; E.C. 6.4.1.2)- and acetolactate synthase (ALS; E.C. 4.1.3.18)-inhibiting herbicides (Christopher *et al.*, 1992; Tardif *et al.*, 1993; Tardif & Powles, 1994). Evidence for enhanced metabolism of herbicides has also been demonstrated in populations of this species and is believed to involve the ubiquitous superfamily of enzymes, cytochrome P450 monooxygenases (Christopher *et al.*, 1991; Holtum *et al.*, 1991; Burnet *et al.*, 1993a; 1993b). This will be discussed further in Section 1.5.

Literature pertaining to the field of cytochrome P450 monooxygenases and their functions is broad and by no means is this review able to embrace the comprehensive diversity of this family of enzymes. Therefore, this review will cover the background literature on some of the more extensively studied plant cytochrome P450 monooxygenases, their identified endogenous functions within secondary plant biosynthesis and their possible involvement in herbicide metabolism and detoxification.

1.2 Cytochrome P450 Monooxygenases

Cytochrome P450 monooxygenases are a superfamily of stereospecific monooxygenases, widely distributed in organisms from bacteria, yeast and fungi to insects, mammals and higher plants (Guengerich, 1991; Porter & Coon, 1991; Nelson *et al.*, 1993). In higher plants, these membrane-bound cytochrome P450 hemeproteins catalyse the oxidation of a wide range of physiological substrates during normal cellular biosynthesis. These include phenylpropanoids (lignins, pigments, ultraviolet protectants, defence molecules), terpenes (sterols, hormones, aroma, defence molecules) and fatty acids (cutin and suberin precursors, defence molecules), and are also involved in the metabolism of foreign compounds which they may encounter in the environment, including agricultural pesticides and herbicides (i.e. xenobiotics). Cytochrome P450 metabolism has been implicated as being largely responsible for detoxification of herbicides in a variety of crop species (selective toxicity).

Until recently, the study of plant cytochrome P450 enzymes has been limited, compared to those of microbial or animal origin, due to factors such as low concentrations of enzyme components in plant tissue, difficulties in obtaining large quantities of material, the presence of often large amounts of interfering plant pigments and instability on purification.

1.2.1 Early Studies of Plant Cytochrome P450 Monooxygenases

Observations of cytochromes in microsomal fractions of plant tissues were first reported in the mid 1950s (Bendall & Hill, 1956; Martin & Morton, 1957), but it was not until 1967 that evidence for carbon monoxide (CO)-binding pigments, with maximal wavelength absorptions at 450nm and 420nm (characteristic of cytochrome P450 monooxygenases) in microsomes of *Pisum sativum* L. (pea) cotyledons, was gathered (Moore, 1967, cited in

West, 1980). The first reports of cytochrome P450 in membrane fractions of higher plants appeared in the late 1960s (Frear *et al.*, 1969; Murphy & West, 1969) and all plant cytochrome P450 monooxygenases reported so far have been associated with microsomal fractions (defined as those cellular membranes sedimenting between 10,000 and 100,000g; Benveniste *et al.*, 1977; 1978; Rich & Lamb, 1977; Salaün *et al.*, 1978; Durst, 1991). These enzymes are believed to be linked to the endoplasmic reticulum, although association with the plasma membrane (Kjellbom *et al.*, 1985) and provacuolar fractions (Madyastha *et al.*, 1977) have also been reported. Cytochrome P450 activity has been detected in all plant tissues except in dry seed, however, their activity can be detected in imbibed seeds (Young & Beevers, 1976).

The first plant cytochrome P450 gene, CYP71A1, was cloned in 1990 from *Persea americana* Mill. (avocado; Bozak *et al.*, 1990). This enzyme catalyses chloro-*N*-methylamine demethylase activity and is believed to be involved in fruit ripening, however, no function *in vivo* has yet been assigned (O'Keefe & Leto, 1989). The first plant cytochrome P450 gene with a known physiological role, CYP73A1, was cloned in 1993 (Teutsch *et al.*, 1993). CYP73A1 is the cinnamate 4-hydroxylase (CA4H), which produces p-coumaric acid, the common precursor for all lignin monomers, flavonoids, pigments and a range of phytoalexins (Werck-Reichhart, 1995b). Since 1993, more than 500 cytochrome P450 genes have been cloned from higher plants although the enzymatic function of many of these genes is not known (Nelson, 1999).

1.2.2 General Structure of Cytochrome P450 Monooxygenases

Current evidence indicates that plant monooxygenases have many similarities to the microsomal cytochrome P450 enzymes in animals. Cytochrome P450 proteins generally have a molecular mass of between 45 – 62 kDa, a highly conserved **FXXGXRXCXG** heme-binding motif in their catalytic site (near the C-terminus) and conserved amino acids

in their oxygen-binding pocket and activation groove near the centre of helix I, approximately 150 residues upstream on the distal side of the heme (Nelson *et al.*, 1993). Apart from these two areas of the protein, cytochrome P450 sequences are highly diverse. Membrane-bound cytochromes P450 also have a hydrophobic *N*-terminal transmembrane anchor, unlike the soluble bacterial cytochromes P450. Sequence identity of greater than 40% is required to classify a sequence as a potential member of the cytochrome P450 gene superfamily (Nelson *et al.*, 1993; Durst & Nelson, 1995). Cytochrome P450 genes are further classified into 150 distinct families, 43 of which exist in animals, 33 in bacteria and archaebacteria, 25 in lower eukaryotes and 49 in plants (see URL:http://drnelson.utmem.edu/CytochromeP450.html) although an accurate count is difficult to make (Table 1.1). Members of each subfamily must share at least 68% sequence similarity to other genes within that same subfamily and amino acid sequence of a protein in one subfamily must be 40 to 65% similar to that in any other of the P450 subfamilies (Nebert & Gonzalez, 1987). Those sequences >97% identical are related at the allelic variant level. Most of the differences between these enzymes are usually observed around the substrate binding pocket.

Three distinct cytochrome groups (a, b and c) can be distinguished by their maximal absorption wavelength in the visible range in their reduced state (Soret band). Cytochrome P450 monooxygenases are type b cytochromes and the letter "P" (for pigment) recognises their colouration in the visible range due to a Soret band around 420 nm (Omura & Sato, 1964a; 1964b). The name "cytochrome P450" was derived from the unusual spectral properties exhibited by the reduced cytochrome P450 ferrous.carbon monoxide complex (Fe[II]-CO; Omura & Sato, 1962; Mason, 1965; Dawson & Eble, 1986). The most characteristic feature of cytochrome P450 is the red-shifted Soret peak of this CO-reduced enzyme complex which occurs at a maximal wavelength of 450 nm (447 nm to 452 nm),

Table 1.1 List of sequenced plant cytochrome P450 monooxygenases and their proposed enzymatic functions.

P450 Family/ Subfamily	Enzyme Name	Species	P450 Family/ Subfamily	Enzyme Name	Species
CYP51A1	Obtusifoliol 14α-demethylase	Sorghum bicolor	CYP73A1	Cinnamate-4-hydroxylase	Helianthus tuberosus
CYP51A2	Obtusifoliol 14α-demethylase	Triticum aestivum	CYP73A2	Cinnamate-4-hydroxylase	Phaseolus aureus
			CYP73A3	Cinnamate-4-hydroxylase	Medicago sativa
CYP71A1	Unknown	Persea americana	CYP73A4	Cinnamate-4-hydroxylase	Catharanthus roseus
CYP71A2	Unknown	Solanum melongena	CYP73A5	Cinnamate-4-hydroxylase	Arabidopsis thaliana
CYP71A3	Unknown	Solanum melongena	CYP73A9	Cinnamate-4-hydroxylase	Pisum sativum
CYP71A4	Unknown	Solanum melongena	CYP73A10	Cinnamate-4-hydroxylase	Petroselinum crispum
CYP71A5	Unknown	Nepeta racemosa	CYP73A11	Cinnamate-4-hydroxylase	Glycine max
CYP71A6	Unknown	Nepeta racemosa	CYP73A12	Cinnamate-4-hydroxylase	Zinnia elegans
CYP71A7	Unknown	Catharanthus roseus	CYP73A13	Cinnamate-4-hydroxylase	Populus tremuloides
CYP71A9	Unknown	Glycine max	CYP73A14	Cinnamate 4-hydroxylase	Glycyrrhiza echinata
CYP71A10	Capable of phenylurea herbicide metabolism	Glycine max	CYP73A16	Cinnamate-4-hydroxylase	Populus kitakamiensis
			CYP73A19	Cinnamate-4-hydroxylase	Cicer arietinum
			CYP73A20	Cinnamate-4-hydroxylase	Pinus taeda
CYP71B1	Unknown	Thlaspi arvense			
CYP71B2	Unknown	Arabidopsis thaliana	CYP74A1	Allene oxide synthase	Linum usitatissimum
CYP71B3	Unknown	Arabidopsis thaliana	CYP74A2	Rubber particle protein	Parthenium argentatum
CYP71B4	Unknown	Arabidopsis thaliana	CYP74A3	Allene oxide synthase	Arabidopsis thaliana
CYP71B5	Unknown	Arabidopsis thaliana			
CYP71B6	Unknown	Arabidopsis thaliana	CYP74B2	Fatty acid hydroperoxide lyase	Arabidopsis thaliana
CYP71C1	HBOA synthase	Zea mays	CYP75A1	Flavonoid-3',5'-hydroxylase	Petunia hybrida
CYP71C2	Indolin-2-one hydroxylase	Zea mays	CYP75A2	Flavonoid-3',5'-hydroxylase	Solanum melongena
CYP71C3	HBOA-N-hydroxylase	Zea mays	CYP75A3	Flavonoid-3',5'-hydroxylase	Petunia hybrida
CYP71C4	Indolin-2-one synthase	Zea mays	CYP75A4	Flavonoid-3',5'-hydroxylase	Gentiana triflora
			CYP75A8	Flavonoid-3',5'-hydroxylase	Catharanthus roseus
CYP71D6	Unknown	Solanum chacoense			
CYP71D7	Unknown	Solanum chacoense	CYP75B2	Flavonoid-3'-hydroxylase	Petunia hybrida
CYP71D8	Unknown	Glycine max			
CYP71D9	Unknown	Glycine max	CYP76A1	Unknown	Solanum melongena
CYP71D11	Unknown	Lotus japonicus	CYP76A2	Unknown	Solanum melongena
CYP71D12	Tabersonine 16-hydroxylase	Catharanthus roseus			
CYP71D18	Limonene-hydroxylase	Mentha spicata	CYP76C1	Unknown	Arabidopsis thaliana
CYP71D19	Unknown	Capsicum annuum			
			CYP76F2	Unknown	Vitis vinifera
CYP71E1	Unknown	Sorghum bicolor			
			CYP77A1	Unknown	Solanum melongena
CYP72A1	Unknown	Catharanthus roseus	CYP77A2	Unknown	Solanum melongena

P450 Family/ Subfamily	Enzyme Name	Species	P450 Family/ Subfamily	Enzyme Name	Species
CYP78A1	Unknown	*Zea mays*	CYP85	Unknown	*Lycopersicon esculentum*
CYP78A2	Unknown	*Phalaenopsis* sp.			
			CYP86	Unknown	*Arabidopsis thaliana*
CYP79A1	Tyrosine-*N*-hydroxylase	*Sorghum bicolor*			
			CYP88A1	Unknown	*Zea mays*
CYP79B1	Unknown	*Sinapis alba*			
CYP79B2	Unknown	*Arabidopsis thaliana*	CYP89A2	Unknown	*Arabidopsis thaliana*
CYP80A1	Berbamunine synthase	*Berberis stolonifera*	CYP90A1	Cathasterone-23-hydroxylase	*Arabidopsis thaliana*
CYP80B1	(S)-*N*-methylcoclaurine 3'-hydroxylase	*Eschscholzia californica*	CYP90C1	Unknown	*Arabidopsis thaliana*
CYP80B3	(S)-*N*-methylcoclaurine 3'-hydroxylase	*Papaver somniferum*	CYP92A2	Unknown	*Nicotiana tabacum*
			CYP92A3	Unknown	*Nicotiana tabacum*
CYP81B1	IC-fatty acid hydroxylase	*Helianthus tuberosus*	CYP93A1	Unknown	*Glycine max*
			CYP93A3	Unknown	*Glycine max*
CYP81D1	Unknown	*Arabidopsis thaliana*	CYP93A?	Unknown	*Glycyrrhiza echinata*
CYP81E1	Isoflavone 2'-hydroxylase	*Glycyrrhiza echinata*	CYP93B1	(2S)-flavanone 2-hydroxylase	*Glycyrrhiza echinata*
			CYP93B3	flavanone synthase	*Antirrhinum majus*
CYP81F1	Unknown	*Arabidopsis thaliana*	CYP93B4	flavanone synthase	*Torenia hybrid*
CYP82A2	Unknown	*Glycine max*	CYP93C2	isoflavanone synthase	*Glycyrrhiza echinata*
CYP82A3	Unknown	*Glycine max*	CYP93C5	isoflavanone synthase	*Glycine max*
CYP82A4	Unknown	*Glycine max*	CYP93C6	isoflavanone synthase	*Vigna radiata*
			CYP93C7	isoflavanone synthase	*Medicago sativa*
CYP83A1	Unknown	*Arabidopsis thaliana*	CYP94A1	Unknown	*Vicia sativa*
CYP83A2	Unknown	*Arabidopsis thaliana*	CYP94?	Unknown	*Pisum sativum*
CYP84A1	Ferulate-5-hydroxylase	*Arabidopsis thaliana*			
CYP84A2	Ferulate-5-hydroxylase	*Solanum lycopersicum*	CYP98A1	Unknown	*Sorghum bicolor*
CYP84A3	Coniferyl aldehyde-5-hydroxylase	*Liquidamber stryaciflua*	CYP99A1	Unknown	*Sorghum bicolor*
CYP84A4	Ferulate-5-hydroxylase	*Populus trichocarpa*			
CYP84A?	Unknown	*Glycyrrhiza echinata*	CYP701A3	ent-kaurene oxidase	*Arabidopsis thaliana*

30 nm longer than the usual CO-heme complex. The reduced P450.CO enzyme complex has been shown to be photodissociable (i.e. exposure to light will cause the CO to be displaced from the heme) therefore, cytochrome P450 enzymes exhibit photoreversible inhibition by CO. When this occurs a second Soret peak can be detected at a maximal wavelength of 420 nm. Solublization with detergents and/or prolonged exposure to O_2 may increase the 420 nm peak at the expense of the 450 nm peak (Omura & Sato, 1962; 1963; 1964a; 1964b). The protein with the Soret peak of 420 nm (P420) is inactive and, therefore, regarded as a degradation product of cytochrome P450.

Cytochrome P450 proteins contain a heme (in the form of iron protoporphyrin IX) as the prosthetic group bound to the central metal ion (Figure 1.1). The iron is always penta- or hexa-coordinate with four of the ligands being contributed by the planar tetradentate porphyrin ring. The fifth coordination axial heme ligand is a cysteine sulfhydryl (Mason, 1965; Murakami & Mason, 1967), which covalently links the protoporphyrin IX heme group to a hydrophobic apoprotein, while the sixth coordination site of the iron is occupied by an easily exchangeable ligand, presumed to be water in the native, substrate-free, ferric state of P450 (Kumaki *et al.*, 1978; Dawson *et al.*, 1982; White & Coon, 1982; Dawson & Eble, 1986). Upon reduction of the iron, the sixth position becomes the site of dioxygen binding and activation. Other small diatomic ligands, such as carbon monoxide (CO), nitric oxide and cyanide may alternatively occupy this sixth coordination position under appropriate conditions (Omura & Sato, 1962).

The minimal system for expressing oxygenase activity comprises: a hemeprotein (cytochrome P450) and an electron transport system, either the flavoprotein NADPH-cytochrome c reductase (E.C. 1.6.2.4), or alternatively, the NADH-cytochrome b5 reductase system, consisting of the flavoprotein NADH-cytochrome b5 reductase
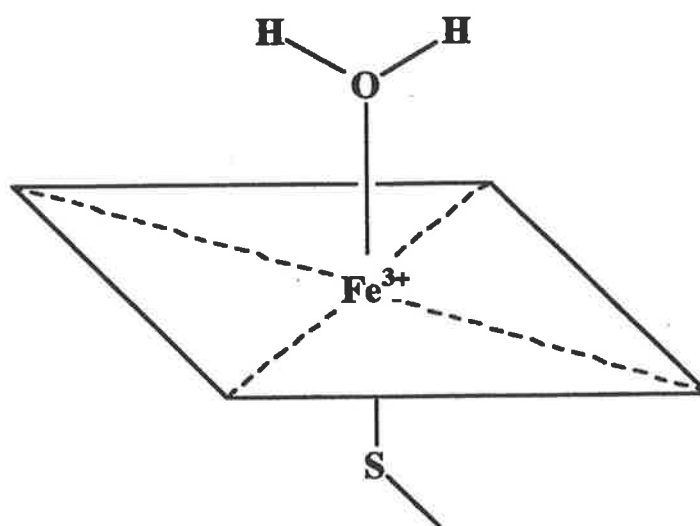
Figure 1.1 Proposed model of the cytochrome P450 enzyme complex with heme (in the form of iron protoporphyrin IX) bound to the central metal ion. The fifth axial heme ligand is a cysteine sulfhydryl and the sixth coordination site is presumed to be water.

(E.C. 1.6.2.2) and cytochrome b5 (Benveniste *et al.*, 1986; 1991). Separation of these two protein components results in complete loss of oxygenase activity. NADPH-cytochrome c reductase serves as the means for transfer of electrons from the external reductant NADPH to the catalytic cycle reactions occurring with cytochrome P450.

1.2.3 General Mechanisms of Cytochrome P450 Metabolism

Molecular oxygen (O_2) plays an essential role in metabolism and generation of energy in all aerobic organisms. Independently, both Hayaishi and co-workers (1955) and Mason and co-workers (1955) were able to utilise the stable isotope $^{18}O_2$ to provide the first evidence for the direct incorporation of oxygen atoms from atmospheric O_2 into organic substrates. In 1956 the name "oxygenase" was proposed for those enzymes that catalyse the enzymatic incorporation of oxygen, from molecular oxygen, into the carbon chains of organic substrates (Hayaishi *et al.*, 1956). It is now established that oxygenation reactions are catalysed by a number of these enzymes which are further classified as monooxygenases (E.C. 1.14.13.- to 1.14.18.-) and dioxygenases (E.C. 1.14.11.- to 1.14.12.-) depending on whether one or both oxygen atoms are inserted into the organic substrate. The cytochrome P450 enzymes are monooxygenases belonging to the subset known as hydroxylases. In this case the covalent bond of dioxygen is broken with one oxygen atom being incorporated into an organic substrate in the form of a hydroxyl group, while the second oxygen atom is reduced to water (Hayaishi, 1974). Cytochromes P450 are also classified as mixed function oxidases (mfos; Mason, 1957) as the oxidant, molecular oxygen, receives four electrons from two different reducing agents, the substrate (RH) to be oxygenated and a cofactor which is usually NADPH (Omura, 1993), simultaneously catalysing an oxygenation and an oxidase reaction (the reduction of oxygen to water).

The general equation of the cytochrome P450 catalysed reaction is:

$$RH + O_2 + (2H^+ + 2e^-) \longrightarrow ROH + H_2O$$

$$NADPH, H^+ \longrightarrow NADP^+$$
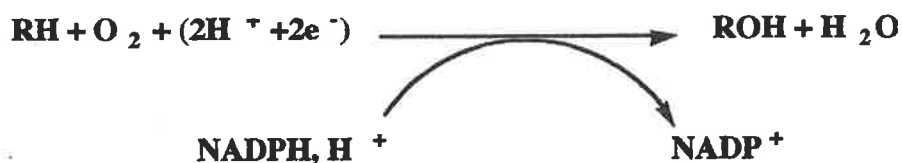
where **RH** represents the organic substrate and **ROH** represents the oxidised product.

The catalytic cycle (Figure 1.2) proposed for plant cytochromes P450 is based partly on the mammalian liver microsomal system (Estabrook *et al.*, 1971), but primarily on the soluble camphor-oxidising cytochrome P450 (P450$_{CAM}$) from the bacterium *Pseudomonas putida* (Gunsalus *et al.*, 1974).

In the resting state, the iron of cytochrome P450 has two axial ligands, the cysteine sulfur and an oxygen donor, which may be water (Dawson & Eble, 1986). At this stage, the enzyme has heme in the low-spin, ferric (Fe[III]) state (Figure 1.2 1). The organic substrate for cytochrome P450 must bind first (2), and does so in a precise orientation within the active site of the enzyme (Dawson & Eble, 1986). Therefore, when the reactive state is formed, the organic substrate is already in place. The substrate does not bind directly to the iron, but rather promotes formation of a state with only the cysteine as axial ligand. Physical studies indicate that the organic substrate displaces the oxygen donor (Dawson & Eble, 1986) causing changes in both the conformation and spectral properties of the protein. Substrate binding is also accompanied by a shift of the ferric complex from low spin to high spin state (as the iron shifts from a hexa- to a penta-coordinated state), as seen in other heme proteins (Tsai *et al.*, 1970; Peterson *et al.*, 1971). One electron is then supplied (3), from an external electron transport chain with NADPH (or NADH in the case
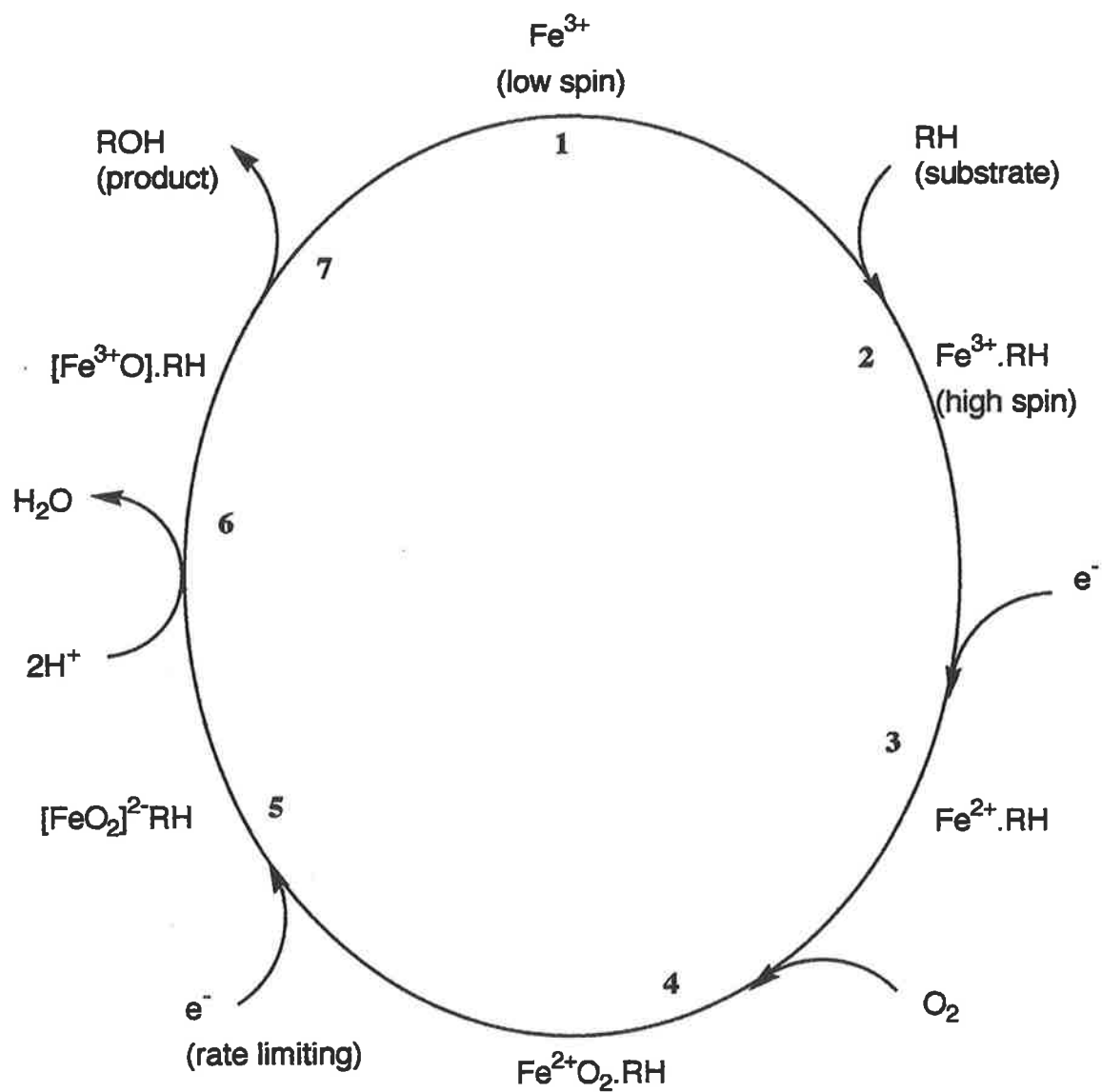
Figure 1.2 The catalytic cycle of cytochrome P450 monoxygenases (Porter & Coon, 1991).

of P450$_{CAM}$) as the reductant, and the heme is reduced to the ferrous (Fe[II]) state (Ullrich *et al.*, 1968; Ishimura *et al.*, 1971). The reduced Fe(II) state is readily able to bind molecular O_2 to form a ternary complex (4; Estabrook *et al.*, 1971; Ishimura *et al.*, 1971) and a second electron is supplied forming a peroxoiron [III] complex (5). This second reduction results in a short-lived active state, whereby the oxygen-oxygen bond is split by heterolytic cleavage resulting in the concurrent formation of water (6) and the oxidised (hydrolysed) substrate (7). This final step, completing the cycle, regenerates the low-spin ferric state of oxidised cytochrome P450, which is then ready to undergo a second catalytic cycle.

1.3   Reactions Catalysed by Cytochrome P450 Monooxygenases

Plant cytochrome P450 monooxygenases catalyse an abundance of reactions including hydroxylation, epoxidation, isomerisation, oxidation, hydrolysis, reduction, *N*-dealkylation, *O*-dealkylation, *S*-dealkylation, desulfuration, dehalogenation, dehydrohalogenation, dehydrogenation and conjugation reactions (Figure 1.3; Casida & Lykken, 1969). The actual number of reactions that could be catalysed by these enzymes may be enormous and the diversity of reactions catalysed by cytochromes P450 make them important, not only for the biosynthesis of endogenous compounds but also for the metabolism of toxic substances (xenobiotics) they may encounter in the environment.

All the substrates noted for cytochromes P450 are essentially lipophilic, rather inert, poorly soluble compounds containing certain functional groups that may undergo a biochemical reaction. These enzymes are the only mechanism existing in nature that are able to convert aliphatic or aromatic compounds to other functional derivatives and are, therefore, crucial

$$R - CH_3 \rightarrow R- CH_2OH$$

Aliphatic Hydroxylation

$$R-\langle\bigcirc\rangle \longrightarrow \left[R-\langle\bigcirc\rangle^O \right] \longrightarrow R-\langle\bigcirc\rangle-OH$$

Aromatic Hydroxylation

$$R - NH - CH_3 \rightarrow [R - NH - CH_2OH] \rightarrow R - NH_2 + HCHO$$

N-Dealkylation

$$R - O - CH_3 \rightarrow [R - O - CH_2OH] \rightarrow R - OH + HCHO$$

O-Dealkylation

$$R - S - CH_3 \rightarrow [R - S - CH_2OH] \rightarrow R - SH + HCHO$$

S-Dealkylation

$$R-\underset{NH_2}{CHCH_3} \longrightarrow \left[R-\underset{NH_2}{\overset{OH}{C}}-CH_3 \right] \longrightarrow R-\overset{O}{\overset{\|}{C}}-CH_3 + NH_3$$

Oxidative Deamination

$$R_1-S-R_2 \xrightarrow{H^+} \left[R_1-\overset{OH}{\underset{}{C}}-R_2 \right]^+ \longrightarrow R_1-\overset{O}{\overset{\|}{S}}-R_2 + H^+$$

Sulfoxide Formation

$$(CH_3)_3N \xrightarrow{H^+} \left[(CH_3)_3N-OH\right]^+ \longrightarrow (CH_3)_3N^+-O^- + H^+$$

N-Oxidation

$$R_1-NHR_2 \longrightarrow R_1-\overset{OH}{\underset{}{N}}-R_2$$

N-Hydroxylation

$$R_1-\underset{R_2}{CHX} \longrightarrow \left[R_1-\underset{R_2}{\overset{OH}{CHX}} \right] \longrightarrow R_1-\underset{R_2}{C}=O + HX$$

Oxidative Dehalogenation

Figure 1.3 Examples of monooxygenase activities mediated by different cytochrome P450 enzymes (Nebert & Gonzalez, 1987; Schuler, 1996).

in metabolism. The resulting metabolic products either fulfil functions in cell metabolism, regulation, or may be conjugated with sulphate, glucuronic acid or glutathione. Generally, the products of these monooxygenases are more polar and less toxic than the parent compound, however, as in the case of proherbicides, the resulting metabolite can be highly toxic to the organism compared to the original starting compound (Cole, 1994). Each substrate has a characteristic quantitative pattern of metabolites that can be detected for each plant species and potentially could be used for species classification.

It is difficult to classify cytochrome P450 enzymes solely according to function due to the diversity of reactions catalysed and the many substrates utilised. Difficulty in isolation of monooxygenase systems from plants, due to their multicomponent nature and membrane-bound structure, makes it hard to assign a particular enzyme activity to a particular protein. Multiple forms (or isozymes) of cytochromes P450 have been shown to exist, based on several lines of evidence. Firstly, in a single plant species different chemicals may induce different cytochrome P450-dependent activities; secondly, certain inhibitor substrates (including suicide substrates and triazoles) will selectively inhibit certain cytochrome P450 isoforms; and thirdly, the same substrate may be oxidised with different regioselectivity and stereoselectivity in different plant species. It is assumed that each form of cytochrome P450 reacts with different substrates at different rates. Those enzymes isolated from mammalian liver appear to have broad, overlapping substrate specificities (Coon & Koop, 1983) whereas, in contrast, $P450_{CAM}$, from bacteria, is highly substrate specific utilising only camphor.

1.3.1 Fatty Acid Oxygenation

The fatty acid biosynthetic pathway is a primary metabolic pathway, essential for the growth and function of plant cells. Hydroxylation and/or epoxidation of fatty acids (from 10 to 18 carbons in length) has been shown to be mediated by cytochromes P450 (Durst *et*

al., 1992).   These reactions are essential in the biosynthesis of insoluble, high polymer membrane materials, cutins and suberins, which form part of the protective waxy barriers of plants that aid in the protection against infection by pathogenic microorganisms, limit water loss and pesticide penetration (Kolattukudy, 1981).

Cutin is the structural component of the cuticle of all the aerial parts of the plant (except the periderms), and some internal tissues such as inner seed coats and the juice sacs of citrus (Espelie *et al.*, 1980).   Two families of monomers (hydroxy- and epoxy- fatty acids) are the main components of cutin: a C16 and a C18 family. The most predominant component of the C16 family is 10,16-dihydroxypalmitic acid and/or its position isomers, in which the mid-chain hydroxyl group is at C-9, C-8 or C-7.   Palmitic acid and 16-hydroxypalmitic acid are also included in this family but are found in smaller quantities. Major components of the C18 family of monomers are 18-hydroxyoleic acid, 18-hydroxy-9,10-epoxystearic acid and *threo*-9,10,18-trihydroxystearic acid, all of which contain an additional double bond at C-12 (Salaün & Benveniste, 1998).   The monomeric composition of cuticles, however, seems to be dependent on plant species, stage of development, the part of the plant, and environmental conditions during cuticle deposition (Matzke & Riederer, 1991).

Suberin is found tightly attached to the cell walls of the periderms (mainly the underground part of plants, ie: roots and tubers) and is also produced in response to wounding of any part of the plant.   The most common aliphatic components of suberin include fatty acids, fatty alcohols, ω-hydroxyfatty acids and dicarboxylic acids, usually containing more than 18 carbon atoms (Kolattukudy, 1980).
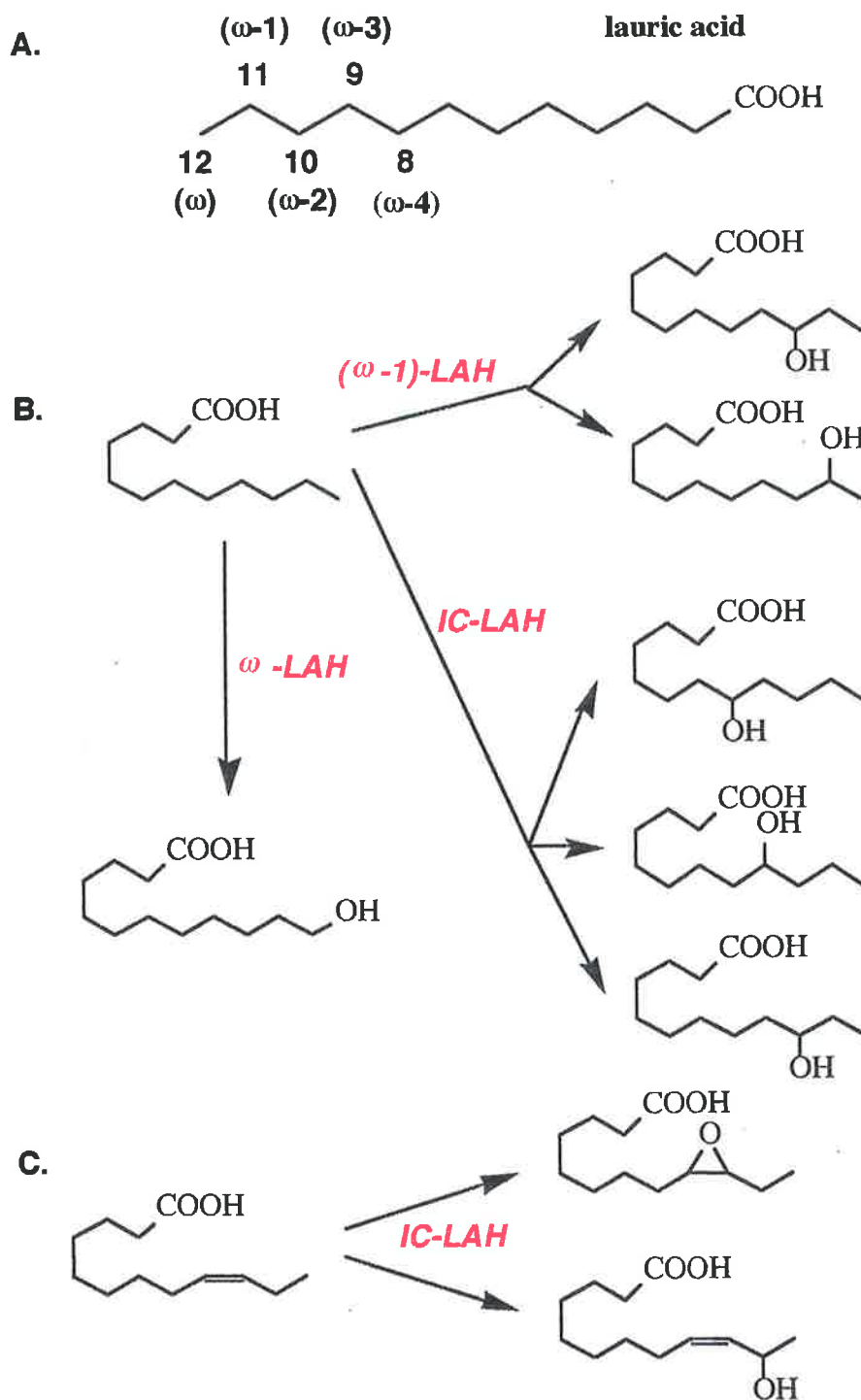
Figure 1.4 Lauric acid hydroxylases (LAH). (A) Chemical structure of lauric acid and position of carbons. (B) The regiospecificities of *Vicia sativa* ω-LAH, *Triticum aestivum* (ω-1)-LAH and *Helianthus tuberosus* IC-LAH. (C) The epoxidation and hydroxylation reactions catalysed by IC-LAH on unsaturated lauric acid analogues (Schuler, 1996).

The fatty acids capric acid (C10:0), lauric acid (C12:0) and myristic acid (C14:0) are all hydroxylated at the terminal position (ω-hydroxylation) by a cytochrome P450 in *Vicia sativa* (vetch; Salaün *et al.*, 1978; 1981; 1986). A single cytochrome P450 (ω-LAH) is believed to be involved in the ω-hydroxylation of these medium-chain fatty acids based on evidence from induction and inhibition studies (Weissbart *et al.*, 1992). In *P. sativum* microsomes, lauric acid is also exclusively hydroxylated at the methyl terminus or ω-position (Figure 1.4A; Benveniste *et al.*, 1982). ω-LAH appears specific for short and medium chain fatty acids as ω-LAH is not inhibited by oleic acid (C18:1, a long chain fatty acid) at a concentration ten times higher than laurate (Pinot *et al.*, 1993). However, in *Helianthus tuberosus* L. (Jerusalem artichoke) tubers, *Tulipa gesneriana* L. (tulip) bulbs and *Zea mays* L. (maize) seedlings these same lipids are hydroxylated at different carbons, (ω-2), (ω-3) and (ω-4) (carbons 10, 9 and 8, with position 9 being dominant; Figure 1.4B). The enzymes involved are termed in-chain lauric acid hydroxylases (IC-LAH; Salaün *et al.*, 1978; 1981; 1982). *T. aestivum* microsomes show hydroxylation of lauric, caprate and myristic acids predominantly at the subterminal or ω-1 position (carbon 11; Figure 1.4B & 1.4C; Zimmerlin *et al.*, 1992). This (ω-1)-LAH from *T. aestivum* is postulated to catalyse the hydroxylation of the herbicide diclofop in addition to the fatty acid substrates (Zimmerlin & Durst, 1992; Zimmerlin *et al.*, 1992). Depending on the plant, both short and medium length fatty acids can be hydroxylated at any carbon position from 8 to 14. However, the two activities, hydroxylation at the methyl terminus or in-chain hydroxylation of lauric acid, are yet to be found to coexist in a single plant species (Durst, 1991). It appears that a single plant species will contain only one lauric acid hydroxylation mechanism (Durst *et al.*, 1992). Unlike the mammalian (Ichihara *et al.*, 1979) and microbial (Miura & Fulco, 1975) fatty acid hydroxylases, the lauric acid hydroxylase in higher plants shows very strict substrate specificity and regiospecificity (Salaün *et al.*, 1981).

Hydroxylation of long chain fatty acids also occurs in plants. Palmitic acid (C16:0) is ω-hydroxylated while ω-hydroxypalmitate is in-chain hydroxylated in seedling microsomes of *Vicia faba* L. (Walton & Kolattukudy, 1972; Soliday & Kolattukudy, 1977; 1978). Omega (ω-) hydroxylation of oleic acid (C18:1), 9,10-epoxystearic acid, 9,10-dihydroxystearic acid and linoleic acid (C18:2) by a cytochrome P450 (ω-OAH) has been demonstrated in microsomes from etiolated *Vicia sativa* L. seedlings (Pinot *et al.*, 1992; 1993; Figure 1.4B). In *T. aestivum* microsomes, ω-1 hydroxylation of oleic acid is the predominant reaction catalysed by cytochromes P450 (Pinot *et al.*, 1994). The two cytochrome P450 hydroxylases involved in ω-hydroxylation and σ-hydroxylation, are distinct from each other, require NADPH and O_2 as cofactors, and have stringent substrate specificities (Pinot *et al.*, 1993).

An unusual cytochrome P450 (family CYP74), allene oxide synthase (AOS; E.C. 4.2.1.92), first characterised (Zimmerman & Vick, 1970) and subsequently identified (Song & Brash, 1991) in *Linum usitatissimum* L. (flaxseed) microsomes is the first enzyme involved in the octadecanoid metabolic pathway leading to the production of jasmonic acid (3-oxo-2-(2'-pentenyl)-cyclopentaneacetic acid). Jasmonic acid, a plant growth regulator, is derived from unsaturated fatty acids, predominantly linoleic and linolenic acids present in membrane lipids (Zimmerman & Vick, 1970; Vick & Zimmerman, 1984). Jasmonic acid and it's derivatives are known to act as chemical messengers during plant stress and insect, herbivore and pathogen defence (Farmer *et al.*, 1992; Gundlach *et al.*, 1992), and also modulate aspects of fruit ripening, production of viable pollen, root growth and tendril coiling (Creelman & Mullet, 1997). AOS catalyses the first committed step in jasmonic acid biosynthesis, the dehydration of 13-(S)-hydroperoxylinoleic acid (18:3ω3) to its unstable allene oxide (12,13-epoxy-9(Z),11(E)-octadecadienoic acid) without the involvement of molecular oxygen (Zimmerman & Vick, 1970; Song and Brash, 1991) or

Linolenic Acid, C18.3 ω3

13-Lipoxygenase

13-Hydroperoxy - linolenic acid

H_2O

Allene Oxide Synthase
CYP74

Allene Oxide (unstable)

Cyclase
Reductase
β-oxidation
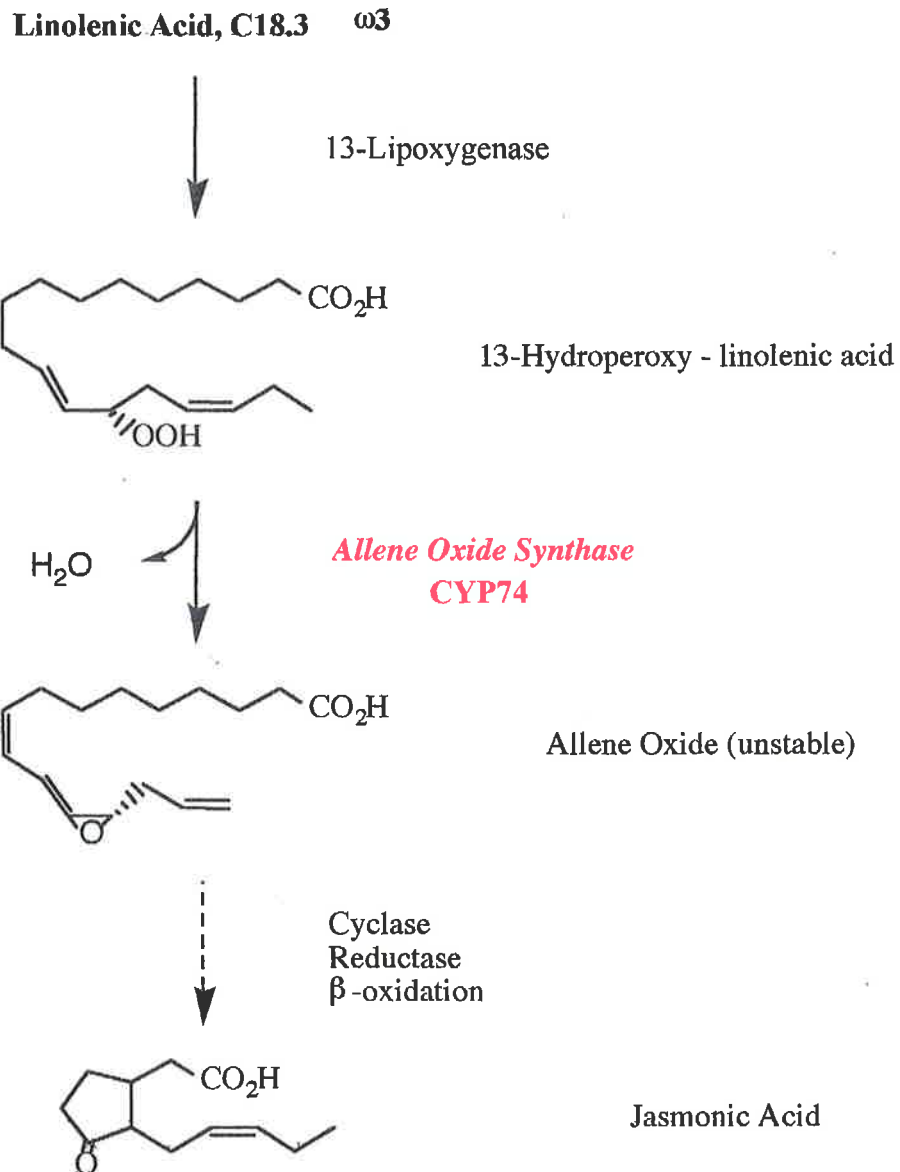
Jasmonic Acid

Figure 1.5 General biosynthetic pathway for jasmonic acid, catalysed by the unusual cytochrome P450 monooxygenase, allene oxide synthase (CYP74; Song *et al.*, 1993).

interaction with a NADPH-dependent cytochrome P450 reductase of the usual cytochrome P450 system. This indicates that the enzyme does not require electrons for activity (Lau *et al.*, 1993). The general reaction catalysed by AOS is shown in Figure 1.5. The gene encoding AOS does not show the typical hydrophobic membrane domain (anchor sequence) found in all the other plant P450 sequences, rather the *N*-terminus resembles that of a chloroplast targeting leader sequence. The bulb of *T. gesneriana* (Lau *et al.*, 1993), and leaves of both *Spinacia oleracea* L. (spinach; Higashi *et al.*, 1985; Blée & Joyard, 1996; Vick & Zimmerman, 1987) and *Arabidopsis thaliana* (L.) Heynh. (Laudert *et al.*, 1996) have also been shown to contain a similar AOS to that identified in flaxseed. However, the AOS-homologous cytochrome P450, rubber particle protein (RPP), purified from the membrane surrounding rubber particles in latex of the desert shrub, *Parthenium argentatum* Gray (guayle), shows an unusual *N*-terminus, lacking both the amino terminal membrane anchor and organelle targeting sequences (Pan *et al.*, 1995). RPP constitutes 50% of the protein in rubber particles and is the first eukaryotic cytochrome P450 to be identified outside the endoplasmic reticulum, mitochondria or plastids (Pan *et al.*, 1995).

1.3.2 Phenylpropanoid Biosynthesis

The shikimate pathway, only found in microorganisms, fungi and plants, is the biosynthetic route to the aromatic amino acids phenylalanine, tyrosine and tryptophan. Higher plants use these amino acids not only as protein building blocks but as precursors for a large number of secondary metabolites. A plant-specific branch of the shikimate pathway is the phenylpropanoid pathway, responsible for the biosynthesis of the simple phenylpropanoids, coumarins, flavonoids, suberins, lignans and lignins (Herrmann, 1995a; 1995b). Phenylpropanoid compounds are so named because of the basic structure of a three-carbon side chain on an aromatic ring derived from L-phenylalanine (Whetten & Sederoff, 1995). Sixteen cytochromes P450 have been characterised in the phenylpropanoid pathway, involved in hydroxylations, dealkylations, cyclisations or ring

migration (Werck-Reichhart, 1995b) with the resulting metabolites involved in many plant defence responses (Nicholson & Hammerschmidt, 1992).

The first step in this pathway involves the deamination of phenylalanine by phenylalanine ammonia-lyase (PAL; E.C. 4.3.1.5) to produce *trans*-cinnamic acid, a key intermediate in the general phenylpropanoid pathway found in all plants (Figure 1.6). The first cytochrome P450-mediated step in this pathway is the well-characterised hydroxylation of *trans*-cinnamic acid to *para*-coumaric acid (ρ-coumaric acid). In some grasses, ρ-coumaric acid is produced by deamination of tyrosine under the tyrosine ammonia-lyase (TAL) activity of PAL (Neish, 1961; Camm & Towers, 1977; Rösler *et al.*, 1997), but usually it is synthesised from the cinnamic acid precursor by the action of a cytochrome P450. The enzyme that mediates this reaction is the prominent cytochrome P450, *trans*-cinnamic acid 4-hydroxylase (CA4H; E.C. 1.14.13.11) which hydroxylates the 4 position of the aromatic ring. The product of this reaction, ρ-coumaric acid, is then activated to its CoA thioester by 4-coumarate:CoA ligase (4CL; E.C. 6.2.1.12) and shunted into one of the branched pathways leading to the production of either cell wall constituents (lignin), pigments (flavonoids), ultraviolet light protectants (coumarins, flavonoids, furanocoumarins) or plant defence compounds (coumarins, isoflavonoids, furanocoumarins; Werck-Reichhart, 1995b). The enzyme CA4H appears highly specific for it's substrate, however, when CA4H from *H. tuberosus* was expressed in yeast, demethylation of the herbicide chlorotoluron was shown to occur with low efficiency (Werck-Reichhart, 1995a). Transgenic tobacco plants with reduced levels of CA4H activity indicate that the syringyl unit (S) : guaiacyl unit (G) ratios are decreased in a way
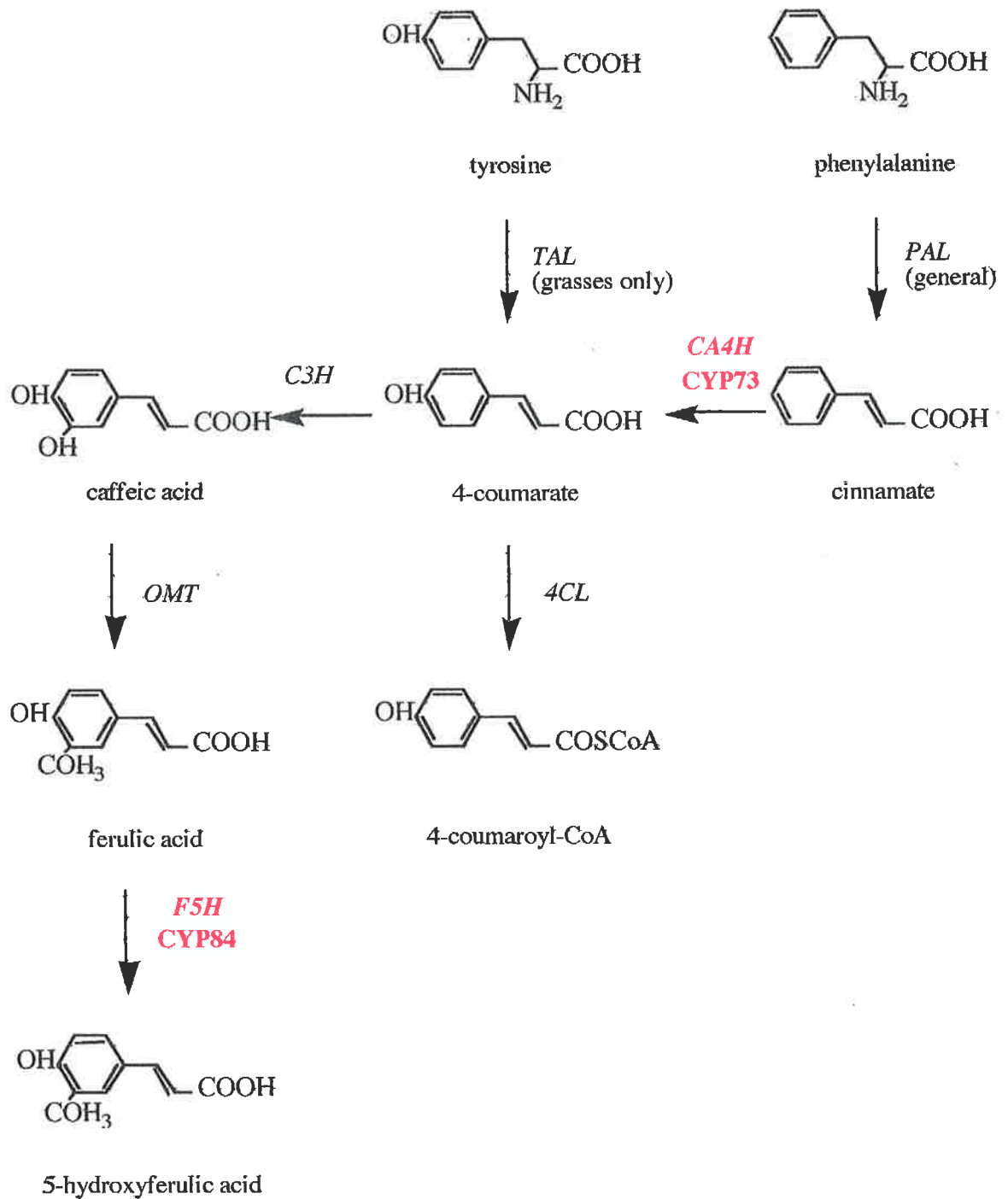
Figure 1.6. The general phenylpropanoid biosynthetic pathway showing the hydroxylation of cinnamic acid and ferulic acid. *TAL*: tyrosine ammonia-lyase; *PAL*: phenylalanine ammonia-lyase; *CA4H*: cinnamic acid 4-hydroxylase (CYP73); *4CL*: 4-coumarate:CoA ligase; *C3H*: *p*-coumarate-3-hydroxylase; *OMT*: *O*-methyltransferase; *F5H*: ferulate 5-hydroxylase (CYP84). (Ruegger *et al.*, 1999).

not consistant with the accepted pathway of monolignol biosynthesis (Sewalt *et al.*, 1997). An approximate 2-fold reduction in Klason lignin polymer (as a percent of dry matter) is observed with both antisense expression or sense suppression of CA4H. This may suggest that down-regulation of CA4H may lead to feed-forward effects on later downstream enzymes or that the CA4H may be organised into more than one complex or metabolic channel (Sewalt *et al.*, 1997). These results indicate the feasibility of reduced lignin content for forage improvement with targeted changes in lignin monomer composition (Sewalt *et al.*, 1997).

CA4H was first described in *P. sativum* seedlings (Russell & Conn, 1967; Russell, 1971) but since then, cDNA sequences coding for CA4H have been isolated from many sources including *P. americana* (Bozak *et al.*, 1990), *H. tuberosus* (Teutsch *et al.*, 1993), *Phaseolus aureus* L., formerly *Vigna radiata* L. (mung bean; Mizutani *et al.*, 1993a; 1993b), *Medicago sativa* L. (alfalfa; Fahrendorf & Dixon, 1993), *Petroselinum hortense* Hoffm. (parsley; Logemann *et al.*, 1995), *Catharanthus roseus* (L.) G. Don (Madagascar periwinkle; Hotze *et al.*, 1995), *Zinnea elegans* Jacq. (Ye, 1996), and *A. thaliana* (Bell-Lelong *et al.*, 1997). All CA4H proteins sequenced to date share more than 85% amino acid sequence identity, but show less than 30% overall sequence identity to any other P450 sequence reported so far. They have, therefore, been classed in a separate cytochrome P450 family, CYP73 (Nebert *et al.*, 1991). One exception is the CA4H (CYP73A15) isolated and identified from *Phaselous vulgaris* L. (French bean) which shares only 58 to 66% sequence similarity to the other CA4H sequences (Nedelkina *et al.*, 1999).

A second cytochrome P450-mediated reaction has also been identified in the lignin biosynthetic pathway. The enzyme ferulate 5-hydroxylase (F5H; no E.C. number assigned) catalyses the irreversible hydroxylation of ferulate to 5-hydroxyferulate (Figure 1.6) in the pathway that diverts ferulic acid away from guaiacyl lignin biosynthesis and

toward sinapic acid and syringyl lignin (Shimada *et al.*, 1973; Grand, 1984). F5H has been implicated in the differences of lignin composition between angiosperms and gymnosperms (Whetten & Sederoff, 1995; Meyer *et al.*, 1998). Evidence for this arises from an identified mutation in the F5H gene (*fah-1*) of *A. thaliana* in which mutant plants show a lack of sinapate derived residues in lignin resembling a gymnosperm-type lignin composed of guaiacyl units (G) derived from coniferyl alcohol (Chapple *et al.*, 1992; Ruegger *et al.*, 1999). Overexpression of F5H in *Arabidopsis*, under the control of the CA4H promoter, generates a lignin almost entirely comprised of syringylpropane units (Meyer *et al.*, 1998). The gene encoding F5H (CYP84) has recently been cloned from *A. thaliana* (Meyer *et al.*, 1996).

Many reactions in the anthocyanin biosynthetic branch of the phenylpropanoid pathway are catalysed by cytochromes P450. Flower pigmentation is largely controlled by the β-hydroxylation of the substrate (2*S*)-naringenin (reviewed in Forkmann, 1991). Evidence available indicates that at least four different cytochromes P450 are involved in catalysing the 3'- and the 3',5'-hydroxylation of this compound (Stotz *et al.*, 1985). Two isoforms of the enzyme, flavonoid 3',5'-hydroxylase, have been isolated from *Petunia hybrida* Vilm. (petunia) with cDNA and amino acid sequences showing 93% and 94% homology, respectively (Holton *et al.*, 1993). These enzymes belong to the cytochrome P450 family, CYP75 (Holton & Cornish, 1995). Flavonoid 3'-hydroxylase has been identified at the biochemical level in a number of plants including *Z. mays* (Larson & Bussard, 1986) but most recently was sequenced in *P. hybrida* and assigned as CYP75B2 (Brugliera *et al.*, 1999).

1.3.3 Terpene Biosynthesis

The terpenes (isoprenoids) are a structurally diverse group of natural compounds with over 30,000 members identified to date (J.D. Connolly & R.A. Hill, 1991, cited in McGarvey & Croteau, 1995; Chappell, 1995). These compounds include an array of hormones (gibberellins, abscisic acid), photosynthetic pigments (carotenoids, phytol), plant attractants (ie: for pollinators) and defence compounds (ie: herbivore repellents, phytoalexins), electron carriers (ubiquinone, plastoquinone), and structural membrane components (phytosterols; McGarvey & Crouteau, 1995). Many cytochrome P450 monooxygenases have already been implicated in the terpenoid pathway and, with such a large class of compounds, many more of these enzymes are likely to be discovered.

The cytochromes P450 catalysing the hydroxylation of the monoterpene precursor, limonene, to essential oils in glandular trichomes (epidermal glands) is well documented for a variety of commercial mint species (family Lamiaceae). Limonene hydroxylase is highly specific for its substrate (-)-4S-limonene but exhibits strict regioselectivity for the position of the hydroxylation on the ρ-menthane ring (Figure 1.7). Hydroxylation of (-)-limonene in *Mentha piperita* L. (peppermint) by CYP71D13 (E.C. 1.14.13.47) is at C-3, leading to the formation of (-)-*trans*-isopiperitenol, in *Mentha spicata* L. (spearmint) at C-6 by CYP71D18 (E.C. 1.14.13.48) and produces (-)-*trans*-carveol, while in *Perilla frutescens* L. (perilla) (-)-perillyl aldehyde is produced following C-7 hydroxylation (E.C. 1.14.13.49; Karp *et al.*, 1990; Lupien *et al.*, 1999). Menthone, carvone and perillyl aldehyde have been shown to rarely co-occur in essential oils (Karp *et al.*, 1990). Differences in the specificity for the hydroxylation of limonene and the fact that enzyme activities differed in sensitivity to inhibition to *N*-substituted imidazoles was evidence that the cytochrome P450 enzymes involved were separate proteins (Karp *et al.*, 1989).
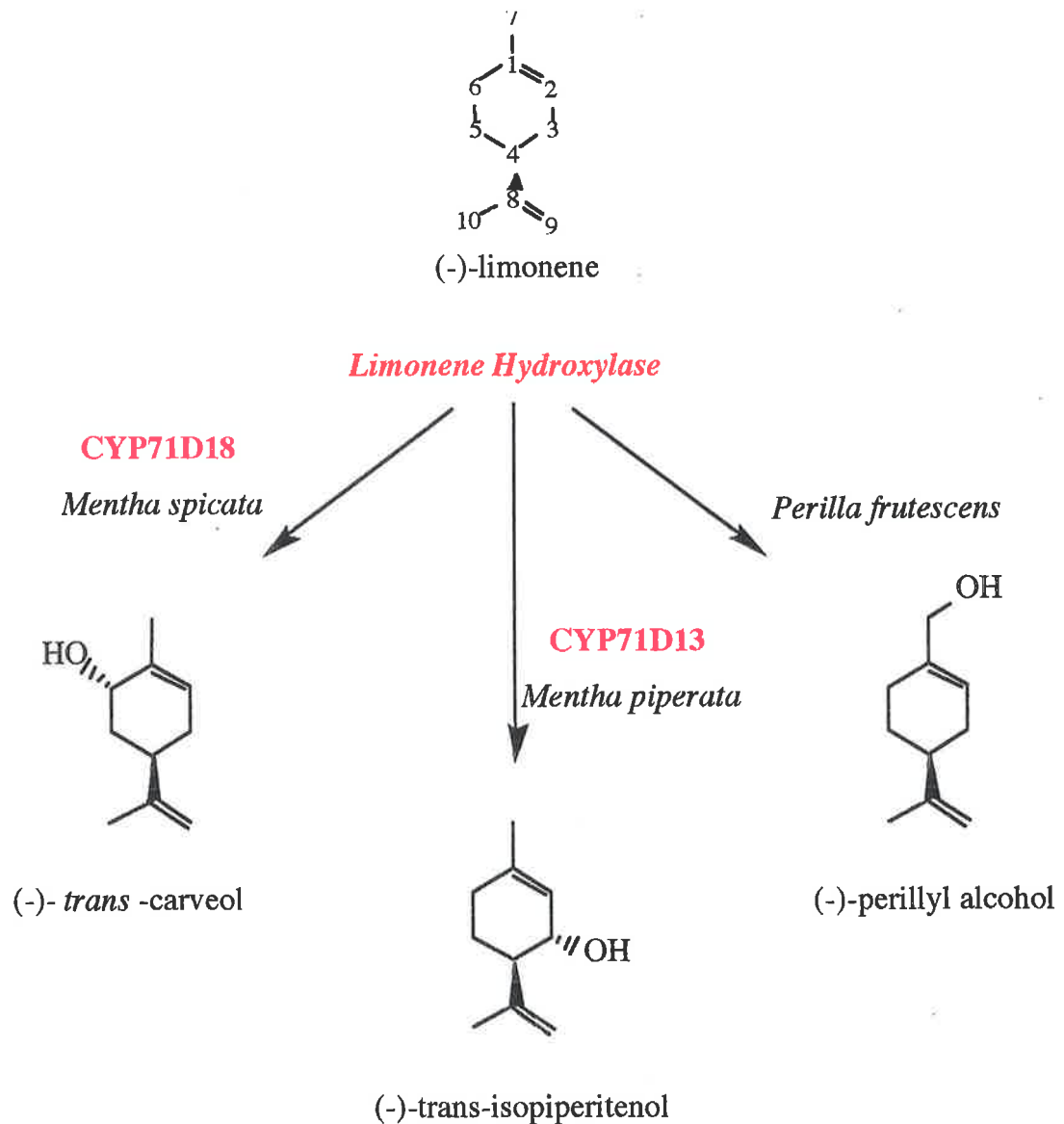
Figure 1.7 Limonene biosynthesis in mint species (*M. piperata*, *M. spicata* and *P. frutescens*) mediated by the cytochrome P450 monooxygenase, limonene hydroxylase (Lupien *et al.*, 1999).

6-Hydroxylation in spearmint was sensitive to miconazole (1-[2,4-dichloro-b-([2,4-dichlorobenzyl]oxy)phenethyl]imidazole), while C-3 and C-6 hydroxylations in peppermint and spearmint were highly sensitive to clotrimazole (1-[chloro-a,a-diphenylbenzyl]imidazole) and 7-hydroxylation in perilla was only slightly inhibited (Karp *et al.*, 1990). The recent cloning of C-3 and C-6 hydroxylases show that the proteins exhibit only 70% identity and 85% similarity when compared to each other despite these enzymes performing a very similar function (Lupien *et al.*, 1999).

Biochemical analysis of a mutant of Scotch spearmint (*Mentha* x *gracilis* Sole.) has shown this mutant produces essential oils containing principally C-3 hydroxylated (-)-limonene, typical of peppermint, rather than the C-6 hydroxylation product characteristic of spearmint (Croteau *et al.*, 1991). A second feature of this mutant strain is the appearance of an epoxidase activity at C1-2 to produce 3-keto-1,2-oxides, not previously reported in either native peppermint or spearmint species. It is believed that the same altered enzyme in this mutant strain catalyses both the C-3 hydroxylation and epoxidation reactions (Croteau *et al.*, 1991).

The 10-hydroxylation of the acyclic monoterpene alcohols, geraniol and its *cis*-isomer, nerol, have long been known to be mediated by the cytochrome P450 geraniol/nerol 10-hydroxylase (Figure 1.8; Meehan & Coscia, 1973; Madyastha *et al.*, 1976; 1977). Recently, the protein has been purified from *C. roseus* (Meijer *et al.*, 1993a), however, attempts to isolate a cDNA clone for molecular analysis have so far failed to conclusively identify the enzyme (Meijer *et al.*, 1993b; Vetter *et al.*, 1992; Clark *et al.*, 1997). Although many potential cytochrome P450 isolates have been purified (CYP71A5, CYP71A6 and two CYP72s) their lack of expression in yeast has failed to elucidate their physiological substrates or their metabolic role in plants (Meijer *et al.*, 1993b; Vetter *et al.*, 1992;

Geraniol

10-Hydroxygeraniol

Nerol
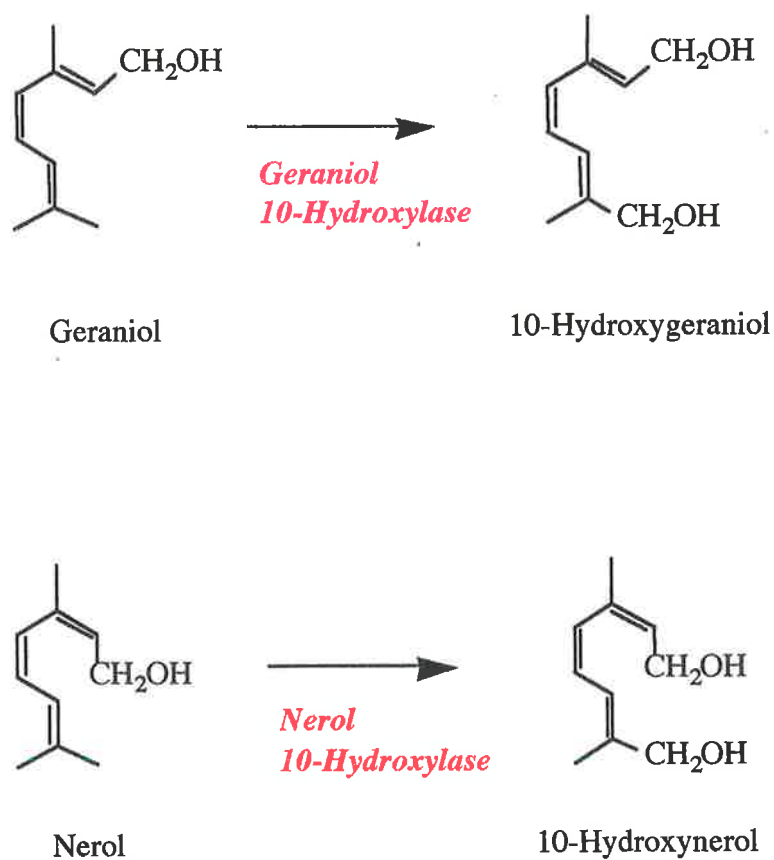
10-Hydroxynerol

Figure 1.8   Geraniol and nerol biosynthesis involving the cytochrome P450 monooxygenases geraniol 10-hydroxylase and nerol 10-hydroxylase, respectively (Madyastha *et al.*, 1976).

Mangold *et al.*, 1994; Clark *et al.*, 1997). Interestingly, CY71A1, isolated from *P. americana* mesocarp, shown to metabolise *p*-chloro-*N*-methylaniline (pCMA), also metabolises nerol and geraniol (O'Keefe & Leto, 1989; Hallahan *et al.*, 1992). Unlike *C. roseus* and *Nepetea racemosa* Lam. (catmint), which specifically hydroxylate nerol and geraniol at C-10, CYP71A1 expressed in yeast microsomes, introduces oxygen across the two available double bonds to produce 2,3- and 6,7-epoxides (Hallahan *et al.*, 1994). It is unlikely that nerol and geraniol are physiological substrates of CYP71A1 as neither of these substrates or corresponding metabolites have been detected in extracts of ripe *P. americana* (J.A. Pickett & D.L. Hallahan, cited in Hallahan *et al.*, 1994). However, strong antibody cross-reactivity indicates that the enzymes present in *N. racemosa* and *P. americana* are closely related (Hallahan *et al.*, 1994).

Two substrate specific, cytochrome P450-dependent monooxygenases have been identified in *Salvia officinalis* L. (common sage). The first enzyme, (+)-sabinene hydroxylase, isolated from the oil glands of sage, converts the substrate (+)-sabinene to (+)-*cis*-sabinol (Karp *et al.*, 1987), while the second P450 enzyme, camphor-6-*exo*-hydroxylase, mediates the oxidation of the monoterpene ketone, (+)-camphor to 6-*exo*-hydroxycamphor (Funk & Croteau, 1993; Funk *et al.*, 1992).

Many stress-induced (biotic and abiotic) compounds synthesized by plants are classified as phytoalexins and often show antimicrobial properties. The biosynthesis of capsidol, the principal phytoalexin of *Capsicum annuum* Linn. (sweet pepper), also produced by *Nicotiana* spp. (tobacco), and the closely related phytoalexin debneyol, from the precursor (+)-5-*epi*-aristolochene are mediated by cytochromes P450 (Threlfall & Whitehead, 1988; Whitehead *et al.*, 1988; 1989). These phytoalexins are produced when the plants are infected with conidia of the fungus *Glomerella cingulata* (Stoneman) Spaulding et Schrenk, tobacco mosaic virus (TMV), tobacco necrosis virus (TNV) or treated with certain

chemicals (Burden *et al.*, 1985; 1986; Watson *et al.*, 1985; Uegaki *et al.*, 1988; Whitehead *et al.*, 1989).

Ipomeamarone 15-hydroxylase is a cytochrome P450 that catalyses the conversion of ipomeamarone to 15-hydroxyipomeamarone and 4-hydroxymyoporone (Fujita *et al.*, 1982). These reactions occur in wounded tissue and/or following infection by fungal pathogens (Fujita *et al.*, 1981; 1982). Isolated from the root tissue of *Ipomoea batatas* Lam. (sweet potato), these furanosesquiterpenoid phytoalexins inhibit spore germination of the fungus *Ceratocystis fimbriata* Ell. and Halst. (Fujita *et al.*, 1982; Schneider & Nakanishi, 1983).

One group of cyclic diterpenes that has received much attention is the gibberellin plant growth hormones, over 50 of which have been structurally identified (Graebe, 1987). The four consecutive steps in the initial stages of gibberellin synthesis, oxidations of kaurene (*ent*-kaur-16-ene), are all mediated by cytochrome P450 monooxygenases (Hasson & West, 1976a; 1976b; Dennis & West, 1967; Murphy & West, 1969). These steps are, *ent*-kaurene to *ent*-kaurenol to *ent*-kaurenal to *ent*-kaurenoic acid to *ent*-a7-hydroxykaurenoic acid (Figure 1.9). The enzyme that mediates three of these steps, from kaurene to kaurenoic acid, has been identified as the multifunctional cytochrome P450, kaurene oxidase (Murphy & West, 1969; Graebe, 1987; Archer *et al.*, 1992), while kaurenoic acid hydroxylase, also a cytochrome P450, mediates the last step in this pathway (Graebe, 1987).

Recently, the *GA3* gene of *Arabidopsis* has been shown to encode *ent*-kaurene oxidase, belonging to the CYP701(A3) family (Helliwell *et al.*, 1998) and direct evidence now verifies that this single gene catalyses the oxidation of all three intermediate steps from

Kaurene Oxidase
CYP701(A3)

ent -kaurene

ent-kaurenol

CH$_2$OH

Kaurene Oxidase

ent -kaurenal

CHO

Kaurene Oxidase

ent-kaurenoic acid

COOH

Kaurenoic Acid
Hydroxylase

ent-7$^\alpha$-hydroxy-
kaurenoic acid

COOH OH
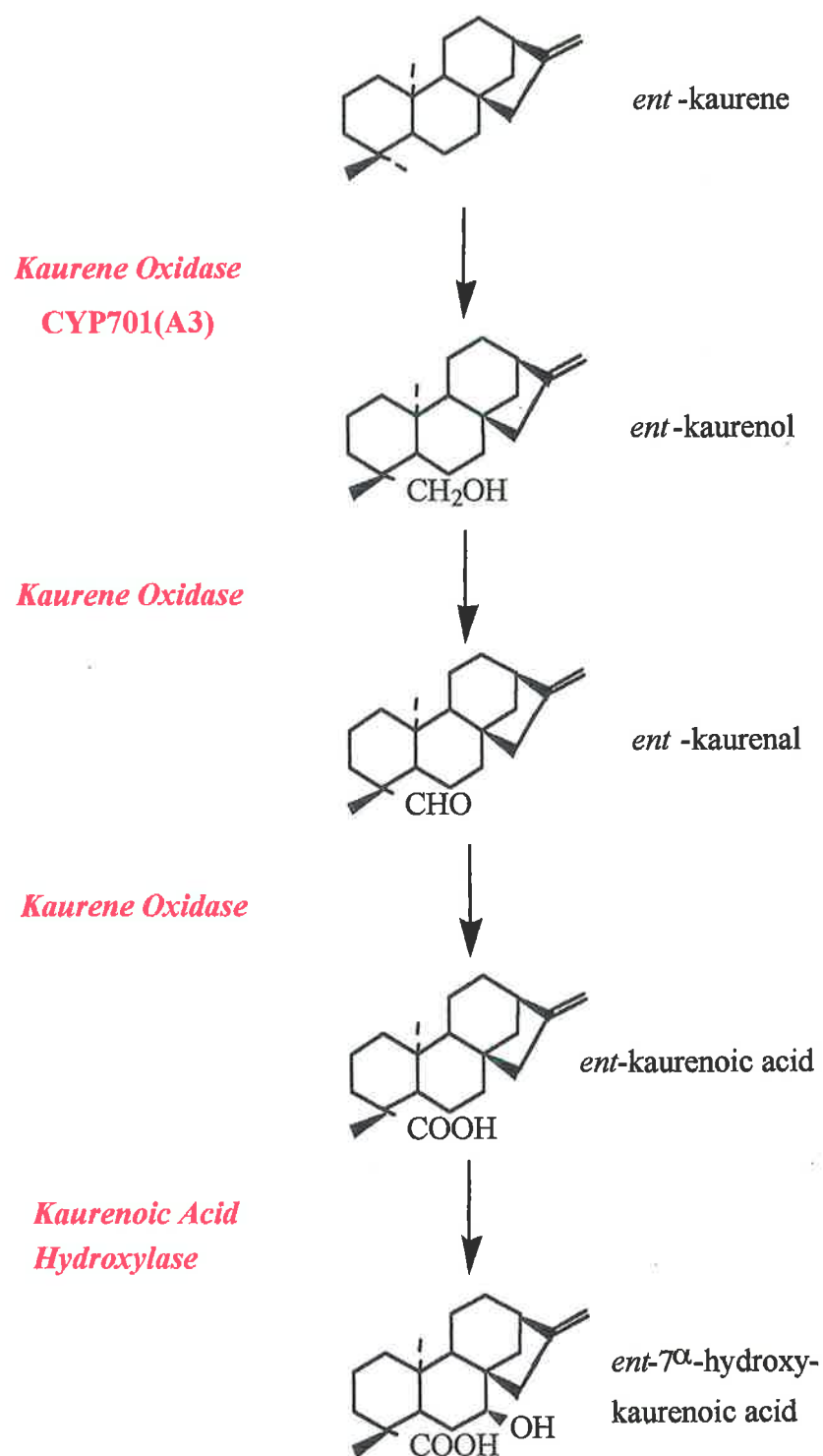
Figure 1.9 Kaurene biosynthesis. All four steps in the early stages in the gibberellin biosynthetic pathway are mediated by cytochrome P450 monooxygenases, kaurene oxidase (CYP701A3) and kaurenoic acid hydroxylase (Graebe, 1987).

ent-kaurene to *ent*-kaurenoic acid (Helliwell *et al.*, 1999). Similarly, a dwarf mutant *P. sativum* strain with a *lh-2* mutation, affecting stem elongation and seed development, was shown to be deficient in *ent*-kaurene oxidase activity (Swain & Reid, 1992; Swain *et al.*, 1993; 1995). However, the gene has not yet been identified. The *Dwarf-3* gene of *Z. mays*, also involved in gibberellin deficiency, although of unknown function, has been identified as a member of the CYP88 family (Winkler & Helentjaris, 1995).

Similar to the kaurene oxidation sequence mentioned above, the synthesis of abietic acid in conifers requires the action of two distinct, stereo-specific cytochrome P450 hydroxylases (Funk & Croteau, 1994).

1.3.4 Induction of Cytochrome P450 Enzyme Activity

Cytochrome P450 studies in plants have been hampered by the low level of the enzyme present and interference from other plant pigments such as flavonoids, xanthophylls, carotenes and chlorophylls. Cytochrome P450 levels are much lower in non-induced (in this instance induction will refer to the elevated activity of cytochrome P450 monooxygenases) plants (0.005 to 0.40 $nmol.mg^{-1}$ microsomal protein) compared to mammals (2 to 3 $nmol.mg^{-1}$ microsomal protein) and their content may vary depending on the age of the plant (Guengerich, 1990). The synthesis of these enzymes is usually specific to certain tissues or organs, developmental stages or plant species. However, in many cases, like the microbial and mammalian monooxygenases, the cytochrome P450 content in plant tissue may be increased significantly in response to the use of various treatments or stimuli (often referred to as P450 inducers). These include selected chemicals (i.e. ethanol, phenobarbitol, manganese), xenobiotics, physical stress (i.e. wounding), exposure to light and ultraviolet (UV) irradiation (Durst, 1976; Benveniste *et al.*, 1977; 1978; Rich & Lamb, 1977; Reichhart *et al.*, 1979; 1980; Batard *et al.*, 1995; Solecka *et al.*, 1999). Aside from developmental regulation and the above mentioned inducing treatments, these

enzymes may be specifically induced as a response to pathogen challenge (bacterial and fungal elicitors; Church & Gilbert, 1984; Schmelzer *et al.*, 1989; Dixon & Paiva, 1995).

Cytochrome P450 levels in both *H. tuberosus* and *Solanum tuberosum* L. (potato) tuber microsomes rise on aging from almost undetectable levels just after wounding to approximately 0.10 nmol.mg^{-1} of protein 16 h later (Benveniste *et al.*, 1977; Rich & Lamb, 1977). Increased contents of both CA4H and NADPH-cytochrome c reductase are observed. CA4H activity has also shown to be stimulated by exposure of plant tissue to light in both *P. sativum* seedlings (Russell, 1971) and *P. hortense* cell suspension cultures (Scheel & Sandermann, 1975; Hahlbrock *et al.*, 1976; Chapell & Hahlbrock, 1984). The addition of a yeast elicitor to *M. sativa* cell suspension cultures resulted in increased levels of CA4H and a range of other enzymes of secondary biosynthesis, particularly those of the phenylpropanoid biosynthetic pathway (Dalkin *et al.*, 1990; Fahrendorf & Dixon, 1993). The principal mode of cytochrome P450 gene regulation for many isozymes is at the level of transcription (Nebert & Gonzalez, 1987; Batard *et al.*, 1997) as evidenced by an increase in mRNA levels.

The inducible effects of xenobiotics are selective at the level of the different redox components and hydroxylase activities between and within plant species. The addition to *H. tuberosus* tissue of 2,4-D (2,4-dichlorophenoxyacetic acid), an artificial hormone used widely as a herbicide, results in selectively elevating the levels of CA4H and fatty acid ω-hydroxylase activity (ω-LAH) but not the in-chain lauric acid hydroxylase (IC-LAH) or (ω-1)-LAH from *T. aestivum* seedlings (Adele *et al.*, 1981; Salaün & Helvig, 1995). In contrast, manganese or phenobarbitol-induction of microsomal preparations favour an increase in IC-LAH over CA4H activity (Reichhart *et al.*, 1980; Adele *et al.*, 1981).

In agricultural systems, certain chemicals, commercially known as safeners or antidotes, can protect crops against the otherwise deleterious effects of certain herbicides. Some of these safeners are known to selectively elevate cytochrome P450 enzymes in certain crop species. Seed applied safeners (such as naphthalic anhydride (NA), dichlormid and cyometrinil) or treatment of plant tissue with chemicals (such as phenobarbitol, clofibrate and ethanol) have been shown to enhance the catalytic activity of cytochrome P450 monooxygenases in some plant species (Reichhart *et al.*, 1979; Salaün *et al.*, 1986; Pinot *et al.*, 1992; Hallahan *et al.*, 1993). Coating *T. aestivum* seed with the safener NA results in a 4.5 fold increase of (ω-1)-LAH and (ω-1)-oleic acid hydroxylase activities and a 1.5 fold increase in cytochrome P450 content, whereas the activity of CA4H is reduced (Zimmerlin *et al.*, 1992; Salaün & Helvig, 1995). NA induction of herbicide metabolism with repression of CA4H activity is also seen in various other grass crops (Zimmerlin *et al.*, 1992; Persans & Schuler, 1995). However, NA is a good inducer of CA4H in *H. tuberosus* which is unexpected as NA is thought to exert a selective action on monocots (Hatzios, 1991). Several lines of evidence, including induction of both cytochrome P450 synthesis and hydroxylase activities following pretreatment of plants with certain compounds, validates the presence of multiple cytochrome P450 enzymes.

1.3.5 Inhibition of Cytochrome P450 Enzyme Activity

In addition to non-specific inhibition by CO, cytochrome P450 activity may be selectively inhibited by various imidazole, pyrimidine and triazole derivatives (Salaün & Helvig, 1995). Irreversible destruction of cytochrome P450 enzymes, in which the active site of the enzyme is destroyed, has been demonstrated using a number of compounds termed 'suicide' substrates. Suicide substrates must first be substrates for the target enzyme, consequently enzyme turnover and irreversible covalent binding of the destructive agent becomes a suicide process thereby inhibiting the enzyme. Many of these mechanism-based inhibitors are structurally similar to endogenous substrates of cytochrome P450

enzymes but include novel structures such as fatty acids containing a terminal acetylene (Salaün & Helvig, 1995; Salaün *et al.*, 1988).

Inhibitor binding may be determined spectrophotometrically by changes in difference spectra, therefore, selective inhibitors may be used to distinguish different types of cytochrome P450 enzymes involved in specific reactions. Selective covalent binding of cytochrome P450 apoproteins with radiolabelled mechanism-based inhibitors may also provide a useful means of purifying plant cytochrome P450 enzymes. While inhibitor studies can often distinguish between single and multiple cytochrome P450 enzymes involved in a given reaction, they do not indicate the number of different enzymes that may contribute to a single activity.

1.4   Metabolism of Xenobiotics

For a herbicide to be effective in severely disrupting normal growth and development of a plant, the accumulation of a critical amount of the active compound at the biochemical target site of action is required. This process is dependent on a number of morphological and physiological factors, such as efficient absorption of the herbicide, translocation within the plant, and arrival at the correct subcellular location in a biologically active form. Consequently, herbicide selectivity is generally assumed to result from differences between and within species and individual plants in those delivery factors that effect the levels of herbicidal compound accumulating at its biochemical target site (Shimabukuro *et al.*, 1982). Apart from the factors mentioned, sequestration, compartmentation, and particularly metabolism may also account for this differential response in plants (Shimabukuro *et al.*, 1982). The metabolism of herbicides to inactive compounds is a key factor in the tolerance to herbicides and consequently selectivity between crops and weeds.

The basis for selectivity of many herbicides is the ability of major crops to efficiently and rapidly detoxify the active, toxic compound while weeds are unable to do so or not at comparable rates. This phenomenon is widely exploited and commercially important in agriculture to control competing weeds with herbicides in crops tolerant to the particular herbicide used.

The occurrence of natural tolerance to herbicides in weed species often occurs. Mechanisms that enable a plant to survive herbicide applications may exist at low gene frequency levels in natural populations never before exposed to chemicals. These natural variants may be selected following herbicide application, which controls susceptible individuals. In the case of crop species, the metabolic capacity to detoxify herbicides must have developed before the introduction of herbicides by man. Plants have only been exposed to herbicides for the last 50 years, therefore, the ability of cytochrome P450 monooxygenases to metabolise these compounds is likely due to the fortuitous binding of xenobiotics into the active site. If pre-existing enzymes, involved in normal secondary plant biosynthesis, have been recruited to metabolise xenobiotics within crop species then this scenario is highly likely to occur within weedy species as well. The more genetic variability in a weed population, the greater the prospect of individuals with similar herbicide resistance characteristics to those that are already present in crop species. The appearance of resistance in populations of the formerly susceptible weedy species *L. rigidum* has been surprisingly rapid. Metabolism-based herbicide resistance has developed in some populations following just 3 to 5 years of herbicide application (Preston & Powles, 1998).

Plants possess biochemical mechanisms capable of the degradation of many xenobiotic compounds. Such mechanisms have the capacity to interact with reactive groups or linkages in the xenobiotic compound that are susceptible to enzymatic or chemical

modification. Thus, certain chemical structures within the xenobiotic may be readily metabolised to an inactive form, while other xenobiotics are relatively resistant to degradation. In some cases, as with proherbicides, the resulting metabolite can be highly toxic to the organism compared to the original starting compound (Cole, 1994). Many degradation products may be formed as many different reactions may take place during the metabolism of a given chemical. The metabolic fate (residues) of xenobiotics in crops destined for human consumption is of great importance for consumers.

The detoxification of xenobiotics often involves conjugation to glutathione or hydroxylation of the molecule, making the pesticide residue more water-soluble and biologically inert (Cole, 1994). The increase in water solubility is important as it decreases the ability of the compound to partition in biological membranes, so restricting its distribution within cells and tissues (Coleman et al., 1997). This may be followed by conjugation to carbohydrates (to form ether glycosides) and subsequent vacuolar sequestration or binding to insoluble cellular constituents, such as lignin (Cole, 1994). Xenobiotic metabolism may be seen to occur as a three-phase process in plants (Shimabukuro et al., 1982; Coleman et al., 1997). Phase I reactions generally detoxify the herbicide or enable the molecule to follow onto Phase II conjugations by creating reactive sites in the herbicide with the addition or exposure of functional groups. Conjugation of herbicides in Phase II usually results in the loss of any phytotoxic activity remaining after Phase I reactions. Phase II deactivates the Phase I-activated metabolites by covalent linkage to endogenous hydrophilic molecules, such as glucose or malonate, to form water-soluble conjugates. The water-soluble conjugates formed in Phase II are exported from the cytosol by membrane-located transport proteins which initiate Phase III metabolism. Phase III serves to produce secondary conjugates or insoluble-bound residues (Baldwin, 1977; Shimabukuro et al., 1982; Cole, 1994). It is assumed that intermediates involved in Phase III metabolism are no longer phytotoxic to the plant. However, not all examples of

metabolism follow this general rule. Some compounds may be directly reacted with natural plant constituents which may act as an irreversible one-step detoxification mechanism. On the other hand, a one-step detoxification of the compound may be temporary and, under the right conditions, the active, toxic parent compound may be reformed. Such is the case with diclofop acid in susceptible plant species (Shimabukuro *et al.*, 1979). This is not considered a genuine detoxification process.

It is now well established that cytochrome P450 enzymes are involved in the metabolism of herbicides, pesticides and other xenobiotics in higher plants (Cole, 1994). Cytochromes P450 exist as multiple isoforms within plant species. Individual isoforms exhibit very narrow substrate specificities, evolved to catalyse specific reactions in the secondary biosynthetic pathways, the nature of which differ dramatically from one plant species to another. Therefore, cytochrome P450 isoforms must differ widely between plant species. It is believed that the cytochromes P450 that function in the detoxification of xenobiotics have a normal physiological function(s) in secondary plant metabolism. Evidence to support a range of substrate specificities by a single cytochrome P450 comes from the example of hydroxylation of the endogenous lauric acid (fatty acid) and the exogenous diclofop-methyl (a herbicide) which is assumed to be by the same cytochrome P450 (Zimmerlin & Durst, 1992). The range of herbicides known to be metabolised suggests that multiple cytochrome P450 isoforms are responsible for the various detoxification reactions that occur within a particular plant species. The basis for selectivity of a herbicide, based on rates of metabolism, may be governed by the affinity of a compound for a cytochrome P450 (ie: certain structural features and/or chemical linkages). Selection pressure, from constant use of herbicides from a similar chemical class, may recruit one or more of a subset of cytochrome P450 enzymes able to use the herbicide as a substrate resulting in a population exhibiting metabolism-based detoxification. Each cytochrome P450 isoform would most likely possess stringent herbicide specificities.

1.4.1 Diclofop-methyl Metabolism

Diclofop-methyl ((*RS*)-2-[4-(2',4'-dichlorophenoxy)phenoxy]-propionic acid) is an aryloxyphenoxypropionate (APP) graminicide used for post-emergence control of annual grass weeds (developed originally for *Avena fatua* L. (wild oat) control) in cereal crops, particularly *T. aestivum* (Friesen *et al.*, 1976; Gorecka *et al.*, 1981). Diclofop-methyl (commercially known as Hoegrass) is applied as a proherbicide (or ester) that is rapidly hydrolysed upon entering the plant (in both susceptible and resistant species) to the active free-acid, diclofop, via carboxylesterase activity (Shimbukuro *et al.*, 1979). The primary mode of action of APP herbicides is the inhibition of ACCase of Gramineae, an essential enzyme in *de novo* fatty acid biosynthesis and located in the stroma of plastids (Burton *et al.*, 1987; Secor & Cséke, 1988; Gronwald, 1991).

Most plant species other than grasses are not affected by APPs as they contain a herbicide-insensitive multisubunit ACCase, whereas grasses contain a multifunctional ACCase which is sensitive (Sasaki *et al.*, 1995). The crops, *T. aestivum* and *H. vulgare* are not affected by diclofop, despite having a sensitive ACCase, as they rapidly detoxify the herbicide via the action of cytochrome P450 monooxygenases (Secor *et al.*, 1989). In this manner, selectivity between resistant crops, *T. aestivum* and *H. vulgare*, and susceptible plants is based on the ability of the former to rapidly metabolise the active free-acid, diclofop, to non-toxic products (Shimabukuro *et al.*, 1979; Donald & Shimabukuro, 1980). Resistant crops, such as *T. aestivum*, further metabolise diclofop acid *in vivo* by irreversible aryl-hydroxylation of the dichlorophenoxy moiety, with subsequent conjugation to glucose to produce non-toxic derivatives (Shimabukuro *et al.*, 1979; Donald & Shimabukuro, 1980; Figure 1.10). Thus, in *T. aestivum*, diclofop is hydroxylated and detoxified before reaching it's target, the ACCase enzyme. Susceptible plants, such as *A. fatua* and *Avena sativa* L. (oats), predominantly convert the diclofop acid into a bound
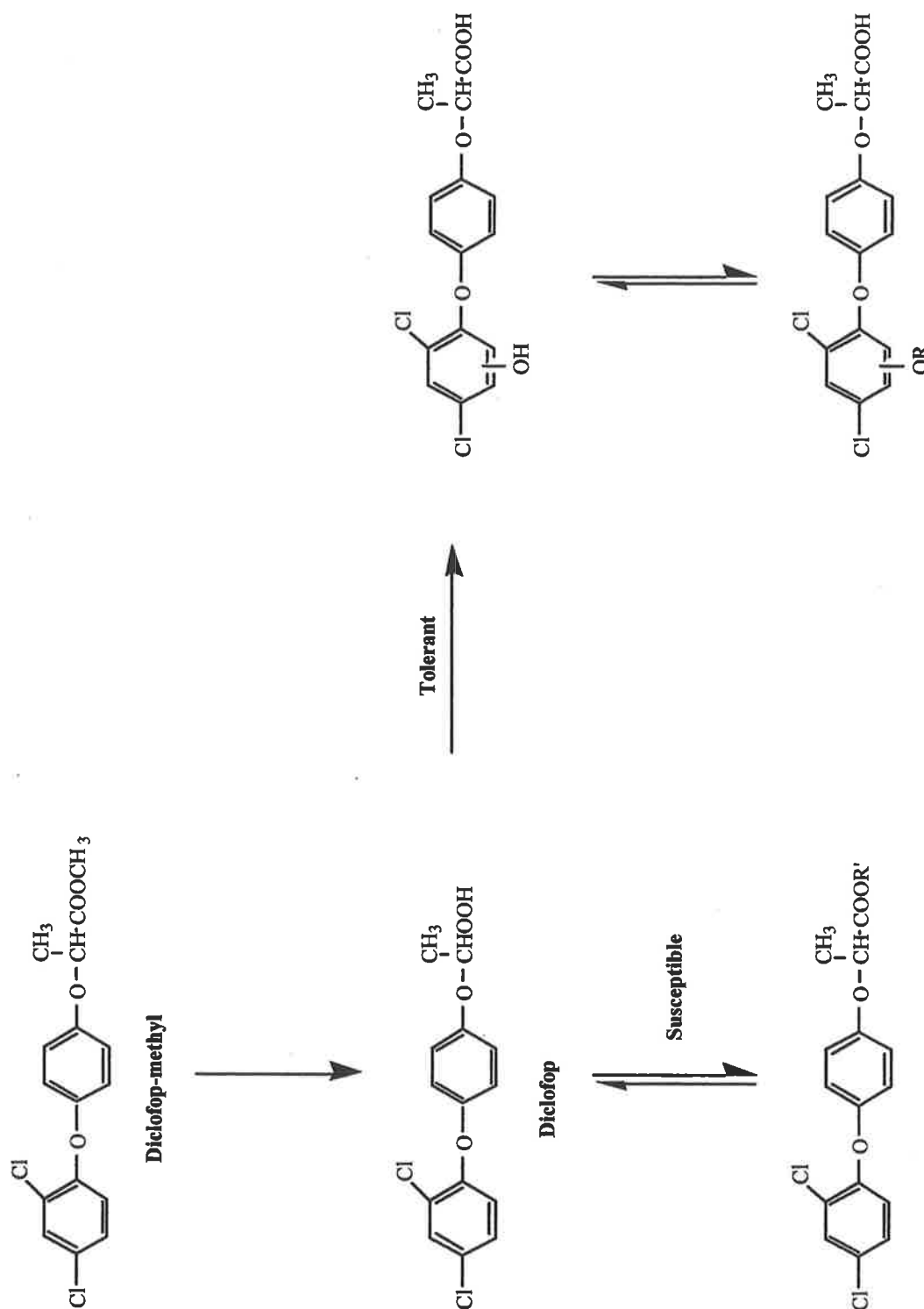
Figure 1.10 The metabolic pathway for the activation of the proherbicide diclofop-methyl and subsequent metabolism of diclofop in susceptible and tolerant plants (Shimabukuro *et al.*, 1979).

glycosyl ester conjugate that may be readily and reversibly hydrolysed (Gorecka *et al.*, 1981; Jacobson & Shimabukuro, 1984).

Aryl-hydroxylation of diclofop in *T. aestivum* is likely to be mediated by a cytochrome P450. This is based on the observation that this reaction can be inhibited by tetcyclasis and 1-aminobenzotriazole (ABT), both established cytochrome P450 inhibitors (Gaillarden *et al.*, 1985; Ortiz de Montellano & Reich, 1986; Cole & Owen, 1987; Rademacher *et al.*, 1987; McFadden *et al.*, 1989). Evidence for inhibition of diclofop metabolism is also seen in the herbicide resistant weed species *Alopercurus mysuroides* Huds. (blackgrass) with ABT (Menendez & De Prado, 1996) and in *A. sterilis* with tetcyclasis (Maneechote *et al.*, 1997). The herbicides, chlorsulfuron, haloxyfop, and primisulfuron also strongly inhibit aryl-hydroxylation of diclofop, whereas 2,4-D, also aryl-hydroxylated in plants (Makeev *et al.*, 1977), does not inhibit metabolism (McFadden *et al.*, 1989). This suggests that different cytochrome P450 monooxygenases are involved in 2,4-D and diclofop hydroxylation. Chemical analogues of diclofop, such as haloxyfop and fluazifop are not metabolised in *T. aestivum* indicating that the P450 monooxygenase responsible for diclofop metabolism is unable to recognise stereospecific alterations to the compound (Zimmerlin & Durst, 1992).

Diclofop hydroxylase activity can be increased 5- to 16-fold by treatment of *T. aestivum* seedling tissue with the chemical safener, NA, ethanol (EtOH) or phenobarbitol (PB; Frear *et al.*, 1991). Zimmerlin & Durst (1990) reported diclofop hydroxylase and general cytochrome P450 levels were increased 16- and 2-fold, respectively, when etiolated *T. aestivum* seedlings were treated for 48 h with 8 mM PB. Pre-treatment of *T. aestivum* seedlings with diclofop or 2,4-D did not correlate with an increase in P450 content (McFadden *et al.*, 1989). An increase in herbicide metabolism is not always correlated with an increase in general cytochrome P450 levels (Frear *et al.*, 1991).

Strong correlations between the cytochrome P450 monooxygenases that metabolise diclofop and lauric acid (ω-1) hydroxylase indicate that they may be encoded by a single gene or closely related isoforms (Zimmerlin & Durst, 1992). Similarities include concomitant induction by the same compounds with comparative kinetics, comparable K_m values for lauric acid and diclofop, and both enzymes are inhibited by the suicide substrate 11-dodecenoic acid (11-DDNA; Zimmerlin & Durst, 1992). Strong parallel inhibition is also seen in both enzymes when incubated with the inhibitors 9-UDYS and 10-UDYS (undec-n-yne-1-sulfonic acid (sodium salt); Forthoffer, 1998). Only purified enzyme studies will elucidate this question.

1.4.2   Chlorsulfuron Metabolism

The sulfonylurea herbicide chlorsulfuron (1-(2-chlorophenylsulfonyl)-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)urea) was developed for the control of dicot weeds and some grasses in cereal crops (Blair & Martin, 1988). The primary mode of action of chlorsulfuron is to inhibit plant growth and cell division by inhibiting the ALS enzyme (also referred to as acetohydroxyacid synthase; AHAS), involved in the biosynthesis of the branched-chain amino acids valine, leucine and isoleucine in plants (Ray, 1982; 1984).

Selectivity in crops is primarily attributed to the rate of metabolism of chlorsulfuron to inactive products in tolerant crops with up to 4000-fold difference in tolerance to the herbicide seen between sensitive and resistant plants (Sweetser *et al.*, 1982; Hageman & Behrens, 1984). In resistant crops, such as *T. aestivum, H. vulgare, A. sativa* and *Secale cereale* L. (rye), chlorsulfuron is rapidly metabolised and so detoxified in leaf tissue (also shown in microsomes) by hydroxylation at the 5-position on the chlorophenyl ring to produce (5-hydroxyphenyl) chlorsulfuron (Frear *et al.*, 1987; Hutchison *et al.*, 1984; Sweetser *et al.*, 1982; Figure 1.11). This product is then rapidly conjugated as a
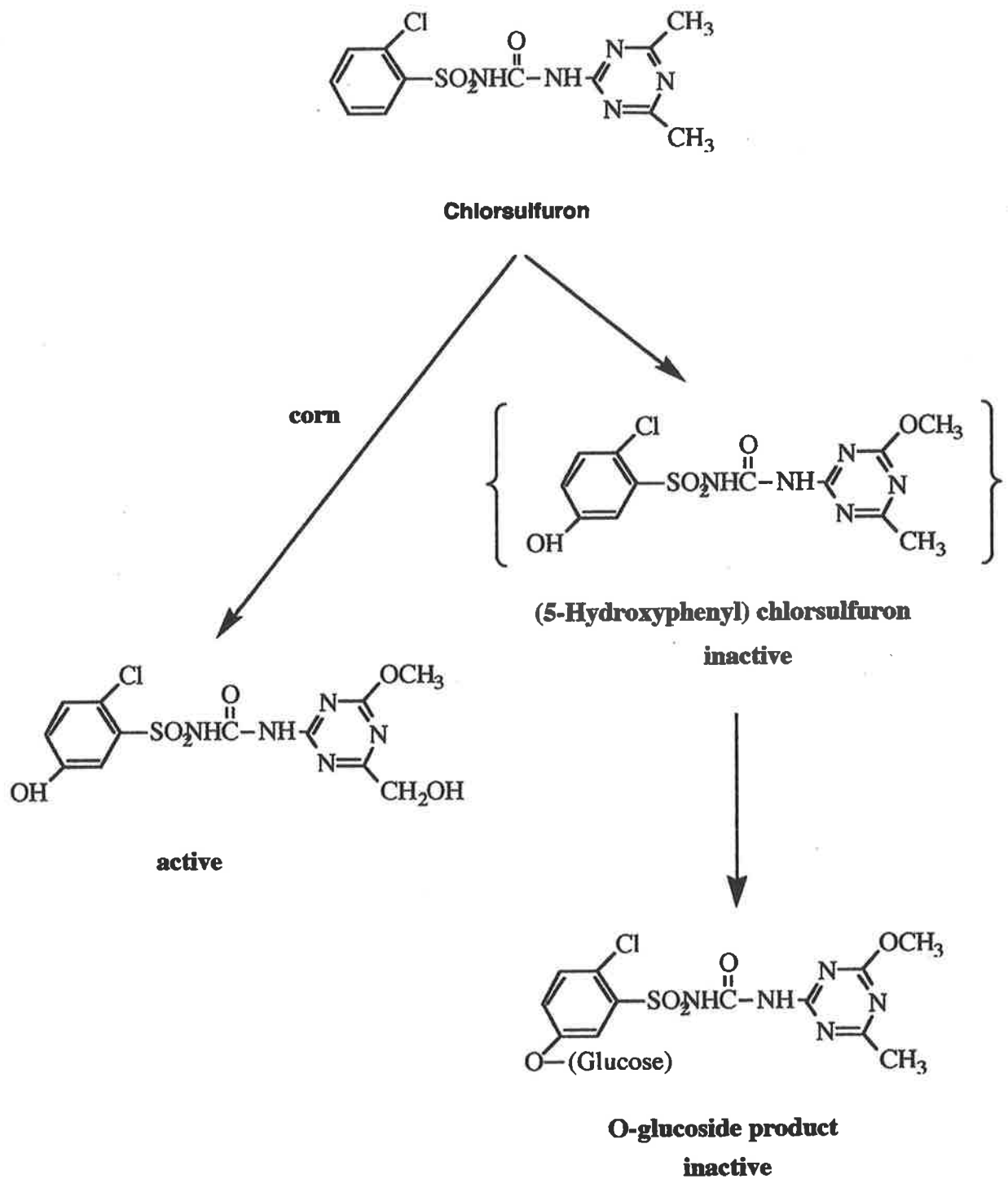
Figure 11. The metabolism of chlorsulfuron in plants (Sweetser, 1985).

polar, inactive *O*-glucoside, formed by the conjugation of a plant carbohydrate moiety with the hydroxylated chlorsulfuron phenyl ring (Sweetser *et al.*, 1982; Frear *et al*, 1991). The *O*-glucoside product is the major metabolite detected in tolerant grasses (Sweetser *et al.*, 1982).

The addition of herbicide safeners have shown to enhance crop tolerance to a number of herbicides applied at otherwise phytotoxic doses. One example demonstrating increased herbicide metabolism due to the addition of a crop safener is evident in *Z. mays*. *Z. mays* has intermediate tolerance to chlorsulfuron due in part to the fact that a second metabolic pathway for chlorsulfuron is present (Figure 1.11). The resulting metabolite from this pathway is not rapidly conjugated and shows considerable herbicidal activity (Sweetser, 1985). However, treatment of *Z. mays* seedlings leaves with NA 16 h prior to herbicide treatment results in a dramatic increase in sulfonylurea herbicide metabolism with the metabolic half life reduced by approximately one third (Chkanikov *et al.*, 1990; Sweetser, 1985). Other commercial crop safeners, DDCA (*N,N*-diallyl-2,2-dichloroacetamide) and cyoxmetrinil also induce a similar increase in the metabolism of chlorsulfuron in *Z. mays* (Sweetser, 1985).

Plant selectivity based on cytochrome P450 metabolism is evident for other sulfonylurea herbicides, including triasulfuron, primisulfuron, prosulfuron, and nicosulfuron, in microsomes of etiolated *Z. mays*, *Sorghum bicolor* (L.) Moench (grain sorghum), *Hordeum vulgare* L. (barley), *A. sativa*, *S. cereale*, *Oryza sativa* L. (rice) and *T. aestivum* seedlings (Fonné-Pfister *et al.*, 1990; Kruez & Fonné-Pfister, 1992; Moreland *et al.*, 1993; Burton *et al.*, 1994; Thalacker *et al.*, 1994; Barrett, 1995; Diehl *et al.*, 1995; Persans & Schuler, 1995; Frear & Swanson, 1996; Hinz & Owen, 1996; Moreland *et al.*, 1996; Hinz *et al.*, 1997). Pre-treatment of seed with the plant safener NA has shown selective increase in cytochrome P450-based metabolism of all these sulfonylurea herbicides (Burton & Maness,

1992; Kruez & Fonné-Pfister, 1992; Moreland *et al.*, 1993; Haack & Balke, 1994; Thalacker *et al.*, 1994; Barrett, 1995; Diehl *et al.*, 1995; Persans & Schuler, 1995; Hinz & Owen, 1996; Hinz *et al.*, 1997).

Differential inhibition studies with the inhibitors tetcyclasis, ABT and piperonyl butoxide (PBO; 4,5-methylendioxy-2-propyl-benzyl-diethylenglycol-butylether; Hodgson, 1985) indicate that more than one isoform of cytochrome P450 monooxygenase is involved in the metabolism of these sulfonylurea herbicides (Frear *et al.*, 1991; Kreuz & Fonné-Pfister, 1992; Moreland *et al.*, 1993; Hinz *et al.*, 1997).

1.4.3 Chlorotoluron Metabolism

The substituted phenylurea herbicide, chlorotoluron (3-(3-chloro-*p*-tolyl)-1,1-dimethylurea), is used for selective graminaceous and dicot weed control in *T. aestivum* and *H. vulgare* (Tysoe, 1974; Ryan *et al.*, 1981). The mode of action of phenylurea herbicides is to bind to the Q_B binding niche of the D1 protein, inhibiting photosystem II (PSII) electron transport and subsequently Q_A oxidation by acting as a non-reducible analogue of plastoquinone (Fuerst & Norman, 1991).

Some plant species are able to readily detoxify chlorotoluron. The different pathways of chlorotoluron metabolism in *T. aestivum* have been well studied. Chlorotoluron can be detoxified by a combination of stepwise (or progressive) oxidative *N*-demethylations and hydroxylations of the ring-methyl group (Gross *et al.*, 1979; Ryan *et al.*, 1981; Cabanne *et al.*, 1985, Fonné-Pfister & Kreuz, 1990). Degradation products are *N*-dealkylated compounds and hydroxylated intermediary metabolites which may slowly undergo further oxidation to produce inactive *O*-glucoside conjugates (Ryan *et al.*, 1981; Gonneau *et al.*, 1988; Figure 1.12). The *N*-monomethyl metabolites are usually less phytotoxic than their
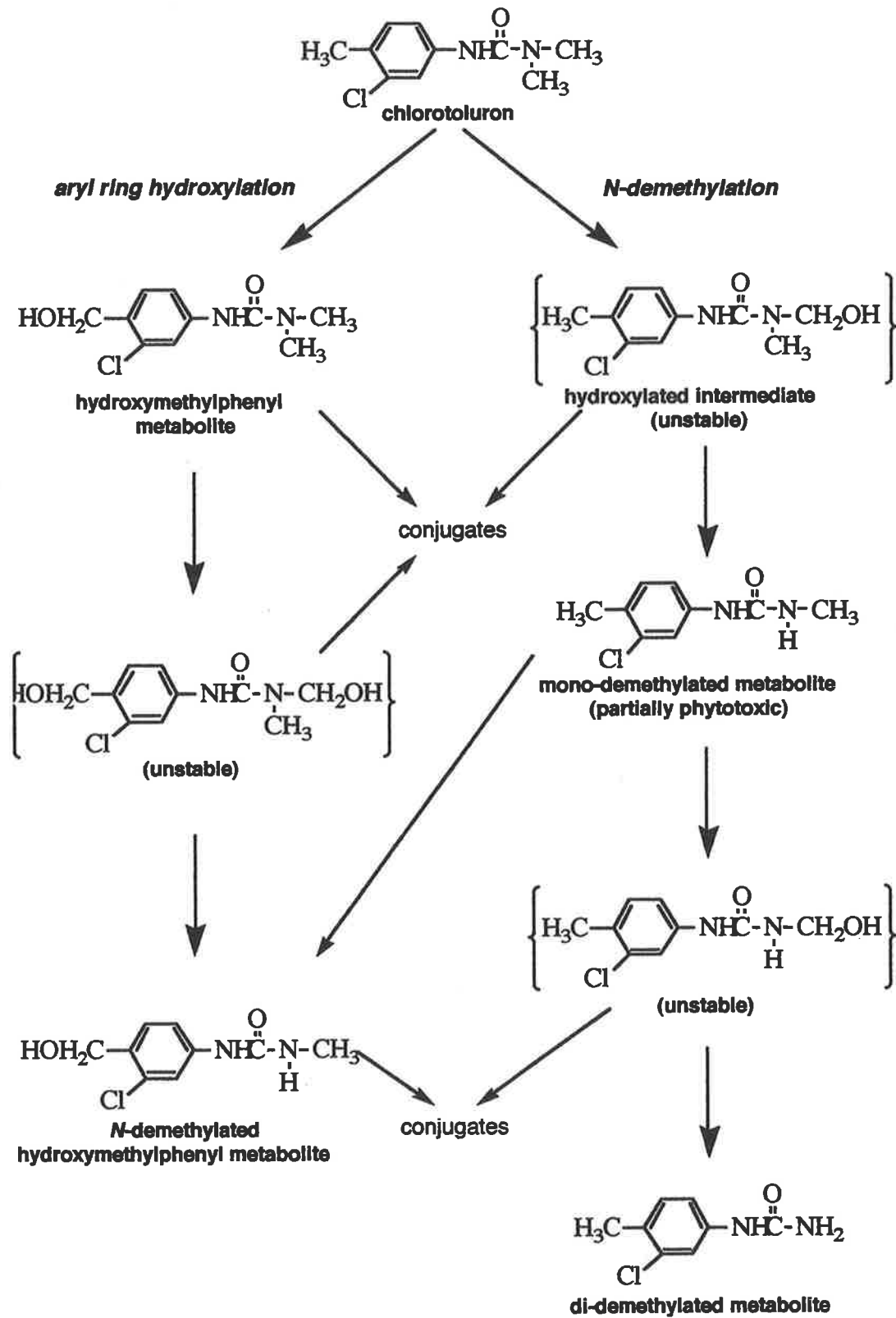
Figure 1.12 The metabolism of chlorotoluron in plants (Hall *et al.*, 1995).

parent compounds and phytotoxicity disappears completely with the concomitant second demethylation.

The tolerance of some *T. aestivum*, *H. vulgare* and *Z. mays* varieties to chlorotoluron is largely attributed to the ability of these species to efficiently perform predominantly ring-methyl hydroxylations, so rapidly detoxifying the herbicide (Gross *et al.*, 1979; Ryan *et al.*, 1981; Ryan & Owen, 1982; Gonneau *et al.*, 1988; Fonné-Pfister & Kreuz, 1990). In contrast, in the susceptible weed species *A. fatua*, *Lolium perenne* L. (perennial ryegrass), and *A. myosuroides*, also in *Gossypium hirsutum* L. (cotton) and *H. tuberosus*, *N*-demethylation activity predominates over ring-methyl oxidation (Ryan *et al.*, 1981; Ryan & Owen, 1982). Detoxification of chlorotoluron in susceptible species favours the formation of the mono-*N*-demethylated metabolite which retains significant phytotoxicity (between 50 and 70% of the parent herbicide; Ryan & Owen, 1982). Therefore, selective control is due to this differential metabolism of the herbicide between susceptible weed and tolerant crop species. However, chlorotoluron-resistant weed species have emerged with mechanisms of resistance due to enhanced metabolism as discussed in section 1.5.

Selectivity of herbicides, based on metabolism, has been shown with other substituted phenylurea herbicides. *N*-demethylation of monuron is a well established method of detoxification in *G. hirsutum* microsomes and has been shown to involve a cytochrome P450 monooxygenase (Frear *et al.*, 1969). A similar case exists for the metabolism of diuron and an analogue of chlorotoluron, CGA 43 057 (1-(3-methyl-4-chlorophenyl)-3,3-dimethylurea). Interestingly, degradation by *N*-demethylation is not the major pathway of chlorotoluron metabolism in *T. aestivum*, rather aryl-hydroxylation is more effective (Gross *et al.*, 1979), indicating that two cytochrome P450 monooxygenases may be involved in the different metabolic pathways of this herbicide group. Positioning of the ring-methyl group of chlorotoluron with respect to susceptibility to metabolic attack is an

important factor in selectivity. CGA 43 057, an analogue of chlorotoluron in which the positions of the ring chlorine and methyl substituents are reversed, is non-selective and metabolised at a much slower rate than that of chlorotoluron (Ryan *et al.*, 1981). Similarly, diuron, although structurally similar to chlorotoluron does not possess a ring-methyl substituent and is non-selective in cereals. *T. aestivum* and *H. vulgare*, although able to metabolise diuron, do so at a much slower rate and via *N*-demethylation (Ryan *et al.*, 1981). In most species studied, *N*-demethylation of substituted phenylurea herbicides is the primary method of metabolism (Ryan *et al.*, 1981).

Inhibitor studies have suggested that a cytochrome P450 monooxygenase is involved in the ring-methyl hydroxylation of chlorotoluron. Gaillardon and coworkers (1985) have shown that addition of ABT, a powerful mechanism-based inactivator of animal cytochromes P450 (Ortiz de Montellano & Mathews, 1981), acts as a synergist of chlorotoluron, thereby preventing production of the non-phytotoxic, aryl-methoxy metabolite. However, *N*-demethylation of chlorotoluron is less sensitive to the addition of ABT (Cabanne *et al.*, 1987; Kemp & Caseley, 1987; Gonneau *et al.*, 1988; Mougin *et al.*, 1990; Hall *et al.*, 1995; Hyde *et al.*, 1996; Menendez & De Prado, 1996). PBO also inhibits chlorotoluron metabolism but is a weaker synergist than that of ABT and does not exert the same level of inhibition (Gaillardon *et al.*, 1985). Cytochrome P450 monooxygenase inhibitors paclobutrazol and tetcyclasis have also been shown to inhibit both ring-methyl hydroxylation and *N*-demethylation of chlorotoluron (Cole & Owen, 1987; Fonné-Pfister & Kreuz, 1990). Isoproturon has exhibited a similar metabolic pathway in *T. aestivum* and *Phalaris minor* Retz (little canaryseed) to that of chlorotoluron (Gaillardon *et al.*, 1985; Singh *et al.*, 1998a; Gläßgen *et al.*, 1999). Addition of inhibitors ABT and PBO have shown results that parallel those for chlorotoluron (Singh *et al*, 1998a; b). Interestingly, isoproturon-resistant biotypes of *P. minor* have shown no cross resistance to chlorotoluron, which implies that different isoforms of cytochrome P450 monooxygenase are involved in

the selective metabolism of these herbicides with a similar mode of action (Singh *et al.*, 1998b).

Mougin and coworkers (1990) have reported an increase in chlorotoluron oxidation and cytochrome P450 levels of 1.6- to 3.3- fold, respectively, from *T. aestivum* cells induced with cyometrinil (a-[(cyanomethoxy)imino]benzeneacetonitrile). This compound has also been shown to increase IC-LAH activity but not that of CA4H. Addition of the safener CGA154281 (4-[dichloroacetyl]-3,4-dihydro-3-methyl-2H-1,4,benzoxazine) to *Z. mays* increases the activity of ring hydroxylation of chlorotoluron by up to 15-fold and the total P450 content rises 2-fold in microsomes prepared from treated plants (Fonné-Pfister & Kreuz, 1990).

1.5   Evidence for Enhanced Cytochrome P450 Activity in *Lolium rigidum*

Many *L. rigidum* populations exhibit resistance to a wide range of herbicides by a variety of mechanisms, which may include reduced herbicide entry, subsequent translocation, target site insensitivity, changes in intracellular compartmentation of herbicides, and enhanced metabolic detoxification (Holtum & Powles, 1991; Powles *et al.*, 1997). The frequency with which resistance arises is dependent on such factors as the initial frequency of resistance genes present in a population, the ease by which resistant mechanisms may evolve, the type and dosage of herbicide used, and the persistence of the herbicide once applied (Tardif & Powles, 1993). The features of *L. rigidum*, including it's abundance, high genetic variability and being and obligate outcrosser, may contribute to rapid responses to any type of selection pressure and the onset of resistance (Gill, 1995). Resistant populations may exhibit more than one mechanism of resistance to a particular herbicide. Some of these multiple mechanisms of resistance may endow cross-resistance

to herbicides of unrelated chemistries that the plant has never previously been exposed to (Heap & Knight, 1986; Tardif & Powles, 1993). Resistance has been observed in *L. rigidum* for almost every herbicide chemistry available for its control.

1.5.1 Target Site Resistance

Resistance to the APP, CHD sulfonylurea, imidazolinine, triazolpyrimidine and pyrimidinyl(thio)ester herbicides has become widespread in *L. rigidum* in Australia (Powles *et al.*, 1996; 1997), with onset of resistance being observed in some populations following just three herbicide treatments (Tardif & Powles, 1993; Tardif *et al.*, 1993; Gill, 1995). Many cases of resistance to these herbicides are due to modifications of the target sites ACCase or ALS. There are many patterns of target site cross-resistance suggesting several possible mutations to these target sites confer resistance (Tardif & Powles, 1993). However, evidence also suggests that a non-target site, enhanced metabolism mechanism of resistance to these herbicides also exists within this species (Christopher *et al.*, 1991; 1992; Preston *et al.*, 1996).

Resistant ACCase has been identified in four *L. rigidum* biotypes (SLR3, SLR31, VLR69 and WLR96). This target site is unaffected by high concentrations of APP and/or CHD herbicides (Tardif & Powles, 1994; Preston *et al.*, 1996; Tardif *et al.*, 1995). Biotypes SLR3 and WLR96 have a form of ACCase highly resistant to APP herbicides but only moderately resistant to CHD herbicides, while a subset of biotype SLR31 (approximately 15% of the population) has a modified ACCase highly resistant to both APP and CHD herbicide groups (Tardif & Powles, 1993; 1994). The remainder of the SLR31 population is resistant to highly resistant to APP herbicides due to enhanced metabolic detoxification but susceptible to CHD herbicides, showing that there are two mechanisms for herbicide resistance present in this population (Preston & Powles, 1998). The possession of an insensitive form of ACCase does not appear to reduce the fitness of the plants in

comparison to susceptible forms (Matthews & Powles, unpublished, cited in Tardif & Powles, 1993). Similarly, resistant ALS has been identified in two *L. rigidum* biotypes (VLR69 and WLR1; Burnet *et al.*, 1994). This target site modification also confers high resistance to sulfonylureas and imidazolinones. The level of herbicide resistance resulting from target site modifications is usually higher than that achieved by increased metabolism.

1.5.2 Enhanced Herbicide Metabolism

Studies to determine the number of putative cytochrome P450 enzymes involved in enhanced metabolism of herbicides in resistant *L. rigidum* biotypes have been undertaken by Preston and coworkers (1996). The addition of synergists at the whole plant level serves to inhibit cytochrome P450 action and has given insight into the involvement of the enzymes in herbicide metabolism. ABT, PBO, and tetcyclasis are broad-range cytochrome P450 inhibitors, while malathion has been shown to synergise sulfonylurea herbicides via competitive inhibition of cytochromes P450 (Kreuz & Fonné-Pfister, 1992). Evidence from studies with the use of these cytochrome P450 inhibitors indicates that more than one putative cytochrome P450 enzyme is involved in herbicide metabolism in resistant *L. rigidum* populations (Preston *et al.*, 1996). Enhanced degradation of herbicides from five chemical groups in four different biotypes (WLR2, VLR69, SLR31 and WLR1) has been observed. At least three different cytochrome P450 enzymes are believed to be involved in herbicide metabolism based on these inhibition studies.

Two *L. rigidum* biotypes WLR2 and VLR69, despite having a susceptible PSII target site, are resistant to the substituted urea and triazine herbicides (Burnet *et al.*, 1991; 1993a; 1993b). Metabolism of these compounds in resistant biotypes occurs at twice the rate of that measured in a susceptible biotype (Burnet *et al.*, 1993a; 1993b). Addition of the synergist ABT, that acts to inhibit chlorotoluron metabolism, causes a decrease in the rate of metabolism of the herbicide so increasing its toxicity to the plant. Significant reductions

in fresh weights have been noted on the addition of ABT and chlorotoluron together compared with the addition of chlorotoluron alone to *L. rigidum* plants (Burnet, 1992). PBO and tetcyclasis have also been shown to synergise chlorotoluron effectively in the biotypes WLR2 and VLR69, whereas malathion is not effective in increasing phytotoxicity of the herbicide to the plant (Burnet *et al.*, 1993a; Christopher *et al.*, 1994; Preston *et al.*, 1996).

Similarly, in biotypes SLR31 and VLR69, enhanced metabolism of the herbicide chlorsulfuron has been noted (Christopher *et al.*, 1991; Burnet *et al.*, 1994). The biotype SLR31 contains a chlorsulfuron-sensitive ALS, as does *T. aestivum*, both relying on increased rates of herbicide metabolism to detoxify chlorsulfuron. Herbicide metabolism in these resistant *L. rigidum* biotypes occurred at twice the rate of that in a susceptible biotype (Christopher *et al.*, 1991). On addition of the organophosphate insecticide malathion, a reduction in the rate of herbicide metabolism occurred (Christopher *et al.*, 1994; Preston *et al.*, 1996). Pretreatment of resistant biotypes with either PBO, ABT or tetcyclasis has shown that these are not effective synergists for chlorsulfuron metabolism (Christopher *et al.*, 1994; Preston *et al.*, 1996).

Enhanced metabolism of diclofop-methyl has been shown to occur at a rate 1.6 fold faster in herbicide-resistant biotypes SLR31 and VLR69 compared to a susceptible biotype (Holtum *et al.*, 1991; Preston & Powles, 1998). Only the synergist ABT (and amitrole in biotype SLR31) was able to inhibit diclofop-methyl metabolism, with PBO or malathion pretreatment had no effect (Preston & Powles, 1994; Preston *et al.*, 1996). Interestingly, the herbicide-resistant biotype SLR31 has two mechanisms for resistance to diclofop-methyl. A small portion of the population (15%) is resistant due to an insensitive target site ACCase while the remaining 85% of the population is resistant to the herbicide due to enhanced metabolism (Tardif & Powles, 1994; Preston & Powles, 1998).

1.6 Objectives of This Study

Previous studies have concluded that one mechanism endowing herbicide-resistance in *L. rigidum* is enhanced herbicide metabolism, mediated by cytochrome P450 monooxygenases. Repeated attempts to prepare microsomes from *L. rigidum* to measure cytochrome P450 levels and activity have failed (S. Powles and C. Preston, personal communication) most likely due to the low abundance, interference of other pigments (such as chlorophyll) and difficulties in extraction of cytochrome P450 enzymes from plants. This has led to the exploration of alternative strategies in an attempt to isolate these enzymes from *L. rigidum*. Objectives of this study were to harness molecular techniques to isolate and identify cytochrome P450 monooxygenases from known herbicide-resistant *L. rigidum* biotypes. Rapid isolation of cytochrome P450 monooxygenases can be now be achieved using degenerate PCR primers. This strategy utilises PCR primers to highly conserved amino acids positioned immediately upstream from the heme-binding motif present in all cytochrome P450 monooxygenases. Potential clones would be further characterised and their possible role in metabolism of herbicides within this species determined. Analysis of corresponding transcript levels, characterised by Northern analyses, would also be used to observe potential induction mechanisms of cytochrome P450 monooxygenases in *L. rigidum* in response to chemical induction. This new information would further our understanding of cytochrome P450 monooxygenase diversity and their role in herbicide metabolism within this species.

Chapter 2

Materials & Methods

Chapter 2

Materials & Methods

2.1 Materials

2.1.1 Plant material

Classification of *Lolium rigidum* Gaudin

Kingdom	Plantae
Division/Phylum	Magnoliophyta (flowering plants)
Class	Liliopsida (monocotyledons)
Sub-class	Commelinidae
Order	Graminales
Family	Gramineae or Poaceae (grasses)
Sub-family	Pooideae
Genus	*Lolium*
Species	*rigidum*

2.1.1.1 Herbicide Susceptible Biotypes

SLR2 Collected from a pasture field near Bordertown in South Australia with no prior herbicide history. The biotype is susceptible to all herbicides known to control *L. rigidum* (Heap & Knight, 1986).

VLR2 Susceptible to all herbicides registered for the control of *L. rigidum* in Australia (C. Preston, personal communication).

VLR1 Collected in Victoria near Bordertown in a pasture field with no prior herbicide history. Susceptible to all herbicides registered for control of *L. rigidum* in Australia (Burnet, 1992).

2.1.1.2 Herbicide Resistant Biotypes

SLR31 Collected from a field near Bordertown in South Australia treated with twelve applications of trifluralin and four applications of diclofop-methyl. The biotype is resistant to aryloxyphenoxypropionate herbicides and cross-resistant to chlorsulfuron. This biotype exhibits resistance to many different herbicides (Heap & Knight, 1986).

VLR69 Collected from a property near Mansfield, Victoria, producing perennial ryegrass seed, treated with 19 applications of diuron, six of chlorsulfuron, five of atrazine, two of diclofop-methyl and three of a mixture of paraquat and diquat over 22 years. Has multiple resistance to at least nine herbicide chemistries with five modes of action (Burnet *et al.*, 1994).

WLR2 Treated for ten years with a mixture of atrazine and amitrole. Resistant to a wide range of triazine, substituted urea, triazinone and aminotriazole herbicides (Burnet *et al.*, 1991).

2.1.2 Synthesis of Synthetic DNA Oligonucleotides

Oligonucleotides were synthesized by GeneWorks Pty. Ltd. (formerly Bresatec Pty. Ltd.), Thebarton, SA, Australia. Mixed bases are represented by the I.U.B. single letter code, where Y = (CT), W = (AT), H = (ACT), D = (AGT), S = (GC), K = (GT) and M = (AC). The letter I represents deoxy inosine.

Oligonucleotide Name	Oligonucleotide Sequence (5' - 3')	Length
Meijer[#]	YWI HTI CCI TTY DSI {IK}{IY}I GGI {IM}{IS}I MG	26bp
oligo d(T)$_{18}$[*]	TTT TTT TTT TTT TTT TTT	18bp
FUBAR	GTA AAA CGA CGG CCA GT	17bp
RUBARB	GGA AAC AGC TAT GAC ATG	17bp
CYPX1	CCT TCA TCT TTG CTG GCA GCT CCC	24bp
CYPX2	GTT CGC TAG CAT CAA TTC CAC GGC	24bp
71-UP	SCC CAT CRT SGG SCA CCT VCA CC	23bp

[#] Meijer oligonucleotide sequence from Meijer *et al.*, 1993b.

[*] Oligonucleotide kindly provided by the Durst laboratory, Strasbourg, France.

2.1.3 Insertion Vectors and *E. coli* Host Strains

2.1.3.1. Insertion Vectors

For cloning double-stranded PCR products, vectors with single 3'-T overhangs at the polyclonal insertion site were used. The vectors used were pGem-T® and pGem-T® Easy (Promega, Madison, WI, USA) and contain an ampicillin resistance (Amp^R) gene for their selection on antibiotic medium. The promoters of the *lacZ* gene in pGem-T® and pGem-T Easy® vectors also provides a means by which "positive" clones (those containing an insert) can be detected by blue/white colour selection when using X-gal and IPTG in the medium.

The Lambda ZAP® II vector (Stratagene, La Jolle, CA, USA) was used to prepare cDNA libraries. This insertion vector allows cDNA fragments, up to 10 kb in size, to be inserted into the multiple cloning site (Appendix A).

2.1.3.2   *E. coli* Host strains

The genes listed signify that the bacterium carries mutated alleles. Genes listed as being present on the F' episome, however, represent the wild-type alleles.

Strain	Genotype
XL1-Blue MRF' (Stratagene)	C*(mcrA)183*, C*(mcrCB-hsdSMR-mrr)173*, *endA1*, *supE44*, *thi-1*, *recA1*, *gyrA96*, *relA1*, *lac*, [F', *proAB*, *lacI*ᵠ*ZCM15*, Tn5, (Kanʳ)].
JM109 (Stratagene)	e14⁻(McrA⁻), *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* (r$_K^-$,m$_K^+$), *relA1*, *supE44*, C*(lac-proAB)*, [F', *traD36*, *proAB*, *lacI*ᵠ*ZCM15*].

2.2   Methods

All experiments were conducted at ambient room temperature (RT) unless otherwise stated.

2.2.1   Preparation of Glassware and Solutions for Molecular Techniques

All solutions were prepared using millipore water (0.04 μSiemens) and sterilized by autoclaving at 121°C, 100 kPa, 20 min. Solutions used for RNA techniques were treated with 0.1% (v/v) diethylpyrocarbonate (DEPC), incubated for a minimum of 12 h at 37°C and autoclaved at 121°C, 100 kPa, 20 min. Buffers and solutions commonly used are listed in Appendix B.

Glassware was treated in Pyroneg detergent (DiverseyLever, Smithfield, NSW, Australia) rinsed in deionized water, dried overnight then sterilized by autoclaving at 121°C, 100 kPa, 20 min. Glassware used for RNA techniques was treated as above, with a final rinse in DEPC-treated millipore water (0.04 μSiemens) and baked for a minimum of 8 h at 160°C.

2.2.2   Seed Germination

L. rigidum seeds were germinated in plastic trays (16.5 x 10.5 x 6.5 cm) containing a 1 cm layer of 0.6% (w/v) agar in an incubator at 19°C with a 12 h light (22 – 42 μmol.m^{-2}.s^{-1} light intensity) and a 12 h dark regime. After six days, seedlings of similar size (approximately 3 cm in height) and vigour were selected at the one-leaf stage for transplanting.

2.2.3   Hydroponic Culture

Seedlings were transplanted into perforated plastic trays (16.5 x 10.5 x 6.5 cm) containing 400 ml black, polypropylene beads to support the seedlings during their growth. Each tray was then placed inside a second tray containing 500 ml nutrient solution (based on that of

Hoagland & Arnon, 1938; Appendix B) such that the roots were immersed in the nutrient solution. The lower tray was covered with aluminium foil, to prevent light reaching the roots. Nutrient solution was topped up to 500 ml with millipore water for two days and, on the third day, with fresh nutrient solution to replenish losses due to transpiration and evaporation. Plants were cultured in a growth room with a 12 h, 19°C day (550 – 650 μmol.m^{-2}.s^{-1} light intensity) and a 12 h, 15°C night regime.

2.2.4 Induction of Herbicide Metabolism

L. rigidum seedlings were treated with chemical inducers of cytochrome P450 monooxygenases, at the two-leaf stage, after five days in hydroponic culture. Following addition of the inducer by subirrigation (inducers were added to the nutrient solution in the lower tray of the hydroponic system), plants were left in the growth room under the same conditions described in Section 2.2.3 for a further 24 h.

2.2.4.1 Ethanol

The chlorsulfuron-resistant biotype, VLR69, was treated with a final concentration of 3% (v/v) EtOH. Fifteen ml of 100% EtOH was added to the 500 ml nutrient solution in the lower tray of the hydroponic culture.

2.2.4.2 2,4-D

The diclofop-methyl-resistant biotype, SLR31, and the susceptible biotype, VLR1, were treated with a final concentration of 500 μM 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma-Aldrich, St. Louis, MI, USA). To 10 ml of millipore water was added 55 mg of 2,4-D and the solution sonicated for 5 min. The 10 ml of 2,4-D suspension was then added to the 500 ml nutrient solution in the lower tray of the hydroponic culture.

2.2.4.3   Clofibrate

The diclofop-methyl-resistant biotype, SLR31, and the susceptible biotype, VLR1, were treated with a final concentration of 500 μM clofibrate (2-(ρ-chlorophenoxy)-2-methyl-propionic acid ethyl ester; Sigma-Aldrich). To the lower tray of the hydroponic culture 53.22 μl of clofibrate (density 1.14 g.ml^{-1}) was added.

2.2.5   Inhibition of Herbicide Metabolism

L. rigidum seedlings were treated with chemical inhibitors of cytochrome P450 monooxygenases, at the two-leaf stage, after five days in hydroponic culture. Following the addition of the inhibitors by subirrigation, plants were left in the growth room under the same conditions described in 2.2.3 for a further 24 h.

2.2.5.1   Suicide Substrates - Compounds 2, 5 and 8

The herbicide-resistant biotype SLR31 and the susceptible biotype VLR1 were treated with 2.5 μM each of the compounds 2, 5 and 8 (Appendix C). Inhibitors were dissolved in 40 ml nutrient solution and 5 ml aliquotted into glass scintillation vials wrapped in aluminium foil to prevent light from reaching the plant roots. Six seedlings per sample were transferred to each vial containing one of the three compounds. The compounds 2, 5 and 8 were synthesised and kindly donated by Dr C. Mioskowski (Strasbourg, France).

2.2.6   Treatment with Herbicides

2.2.6.1   [^{14}C]-Chlorsulfuron

Treatment of plants with [^{14}C]-chlorsulfuron was performed by the cut-shoot technique (Christopher *et al.*, 1991). Plants were removed from hydroponic culture and the roots washed in millipore water to remove any remaining polypropylene beads. Shoots were then excised under water, six seedlings per sample, just above the seed and transferred to an eppendorf tube containing 300 μl of 500 μM [^{14}C]-chlorsulfuron (Dupont, Wilmington,

DE, USA). Twelve seedlings per treatment were harvested after a 6 h uptake period. Shoots were washed in millipore water, blotted dry, weighed and frozen in liquid nitrogen. Samples were stored at -80°C until extracted (Section 2.2.7). Experiments contained two replications and were conducted twice. Data from replicated experiments were pooled prior to analysis with means and standard deviations calculated for each herbicide tested.

2.2.6.2 [^{14}C]-Diclofop-methyl

Plants at the two-leaf stage were treated with a single 1 µl drop of 2.5 mM [^{14}C]-diclofop-methyl solution (4.5 mM [^{14}C]-diclofop methyl (-dichlorophenyl-U-^{14}C, with a specific activity of 849.2 µBq.g^{-1} (Hoechst AG, Frankfurt (Main), Germany), dissolved in 0.5 mM commercial Hoegrass® and 10 µl of the wetting agent Agral-600, a non-ionic surfactant) per plant, placed into the leaf axil. Shoots were harvested (excised just above the seed) 24 h following herbicide treatment, with each replicate consisting of eight plants. Shoots were thoroughly washed in 50 ml 20% (v/v) methanol containing 0.1% (v/v) Triton X-100 to remove unabsorbed radioactivity. Samples were then blotted dry, weighed, frozen in liquid nitrogen and stored at -80°C until extracted (Section 2.2.7). The wash solution of each replicate was kept for determination of [^{14}C] content (Section 2.2.9). Experiments contained four replications and were conducted three times. Data from replicated experiments were pooled prior to analysis with means and standard deviations calculated for each herbicide tested.

2.2.7 Extraction of Metabolites

Frozen plant tissue was homogenised to a fine powder in liquid nitrogen using a cold mortar and pestle, extracted with 3 ml ice-cold 80% (v/v) MeOH and centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was removed and the pellet was again extracted, twice more, with a further 1 ml ice-cold 80% (v/v) MeOH and centrifuged at 12,000 x g for 10 min at 4°C. The pooled extracts were evaporated to dryness *in vacuo* on

a rotary evaporator and resuspended in 120 µl 50% (v/v) MeOH for [^{14}C]-chlorsulfuron samples or 160 µl 80% (v/v) MeOH for [^{14}C]-diclofop-methyl samples. Prior to injection onto HPLC column, samples were briefly centrifuged to remove insoluble material then filtered through a 0.2 µm nylon filter (Lida, Kenosha WI, USA).

2.2.8 HPLC Analysis of Metabolites

[^{14}C]-Radiolabelled metabolites and parent herbicide were quantified and analysed by reverse phase high performance liquid chromatography (HPLC). HPLC was performed with 2 x LC 1100 dual plunger pumps (ICI Instruments, Dingley, Victoria, Australia) utilising WinChrom chromatography software (GBC Scientific Equipment, Dandenong, Victoria, Australia) and equipped with an Goldpak EXSIL 100 column (5 µods; 250 mm x 4.6 mm; Activon, Thornleigh, NSW, Australia). Radioactivity was detected using an in-line radio-chromatography detector (FLO-ONE®\Beta Series A-100; Radiomatic Instruments & Chemical, Canberra - Packard, Tampa, FL, USA) with a solid flow YtSi cell (250 µl; Canberra – Packard , Tampa, FL, USA). Solvents used were 90:9:1 (v/v/v) H$_2$O:acetonitrile:glacial acetic acid (Solvent A) and 99:1 (v/v) acetonitrile:glacial acetic acid (Solvent B). The elution gradient of actonitrile solvents A and B are listed in Table 2.1 for the corresponding herbicides used.

2.2.9 Liquid Scintillation Spectrometry

[^{14}C]-Radioactivity in washes was quantitated by liquid scintillation spectroscopy (LSS) using a water-compatible scintillant, Ultima Gold (Packard) in a LS 5000TD liquid scintillation spectrometer (Beckman, Fullerton, CA, USA).

Herbicide	Diclofop-methyl		Chlorsulfuron	
Flow rate (ml.min^{-1})	1.5		1.5	
	Time	% B	Time	% B
	0	30.0	0	8.0
	10	70.0	26	42.0
	13	100.0	33	100.0
	18	100.0	38	100.0

Table 2.1 The elution gradient of actonitrile solvents used during HPLC for the corresponding herbicides.

2.2.10 Preparation and Analysis of RNA

All solutions for this procedure were treated with 0.1% (v/v) DEPC or prepared with DEPC-treated millipore water and glassware baked at 160°C for a minimum of 8 h (Section 2.2.1). Three different methods to prepare total RNA were undertaken as described below.

2.2.10.1 Total RNA Extraction

2.2.10.1.1 Method 1

Total RNA was isolated from hydroponically cultured *L. rigidum* seedling material following induction with either ethanol, 2,4-D or white light (Section 2.2.4), according to the method in the Practical Workshop in Basic Recombinant DNA Techniques course manual (1997). Specifically, approximately 1.5 g of plant material was ground to a fine powder in liquid nitrogen using a cold mortar and pestle. Four ml RNA extraction buffer

(REB; 100mM Tris-HCl, pH 8.4; 4% (v/v) sarkosyl; 10mM EDTA) was added and homogenised to a slurry. The slurry was transferred to 2 ml eppendorf tubes and centrifuged (3,000 x g, 10 min, 4°C) to pellet solid plant matter in an RC2-B Sorvall centrifuge (Dupont) fitted with an SS-34 rotor. The volume of supernatant was measured and transferred to a 10 ml ultracentrifuge tube (UltraBottle, PC, 16 x 76 mm; Nalgene™, Rochester, NY, USA) with 1 g of caesium chloride (CsCl) added for every 1 ml of supernatant. The tube was gently inverted to ensure complete dissolution of the CsCl crystals. Three ml of a CsCl cushion solution (9.65 g CsCl + TE buffer to 10 ml) was gently layered to the bottom of the tube using a pasteur pipette and the homogenate was centrifuged at 126,000 x g for 18 h at 4°C in a L8-70 ultracentrifuge (Beckman) with a fixed angle TY65 rotor (pre-chilled to 4°C). The upper, sticky green surface layer was removed using clean cotton buds and the supernatant was removed leaving a translucent RNA pellet at the bottom of the tube. The RNA pellet was washed twice with sterile millipore water and dissolved in 500 μl REB. The RNA was transferred to a 1.5 ml eppendorf tube and extracted with 500 μl phenol:chloroform:isoamyl alcohol (50:49:1 v/v/v). The upper aqueous phase was transferred to a fresh tube, with care not to remove any debris from the interface, and the RNA was precipitated with a tenth the volume of 3 M sodium acetate (NaAc, pH 4.8) and twice the volume of 100% EtOH at -20°C, overnight. The following day, RNA was pelleted (27,000 x g, 15 min, 4°C), the supernatant removed and the pellet washed with 500 μl 70% (v/v) EtOH. The pellet was briefly dried under vacuum and resuspended in 30 μl sterile millipore water. The concentration and purity of isolated mRNA was determined by spectrophotometry (Section 2.2.10.3) and native agarose gel electrophoresis (Section 2.2.10.4). Samples were stored at -80°C until used.

2.2.10.1.2   Method 2

Total RNA was isolated from five day old *L. rigidum* seedling material following induction with clofibrate (Section 2.2.4), according to the modified hot-borate method of Wan & Wilkins (1994). Briefly, approximately 1.5 g of plant material was ground to a fine powder in liquid nitrogen using a cold mortar and pestle and transferred to a glass Erlenmeyer flask containing 5 ml.g^{-1} of preheated (80°C) RNA extraction buffer (200 mM Borax, pH 9.0; 30 mM EGTA; 1% (w/v) SDS; 1% (w/v) Na decoxycholate; 2% (w/v) polyvinylpyrrolidine (PVP); 0.5% (v/v) NP-40; 10 mM DTT). The homogenate was transferred to a 30 ml Corex tube containing 0.5 mg.ml^{-1} proteinase K and incubated on a rotary shaker at 100 rpm for 1.5 h at 42°C. The homogenate was adjusted to 160 mM KCl and chilled on ice for 1 h. After centrifugation (12,000 x g, 20 min, 4°C) in a J2-21 centrifuge (Beckman) fitted with a JA-20 rotor, the supernatant was filtered through miracloth (Calbiochem®, La Jolle, CA, USA) and the RNA precipitated overnight in 2M LiCl on ice. Following centrifugation (12,000 x g, 20 min, 4°C) the RNA pellet was washed three times with 5 ml ice-cold 2 M LiCl until the supernatant appeared colourless. The RNA pellet was then suspended in 2 ml 10 mM Tris-HCl, pH 7.5, and clarified by centrifugation (12,000 x g, 10 min, 4°C). The RNA was then precipitated in 200 mM potassium acetate, pH 5.5, for 15 min on ice to remove salt insoluble material. The RNA was again precipitated overnight with 2.5 volumes of 100% EtOH at -20°C. The RNA was pelleted, washed in 70% (v/v) EtOH, briefly air-dried and resuspended in sterile millipore water.

2.2.10.1.3   Method 3

Total RNA was isolated from hydroponically cultured *L. rigidum* seedling material following induction with either clofibrate or 2,4-D (Section 2.2.4), using the ToTally RNA Extraction Kit (Ambion, Austin, TX, USA) according to the manufacturers instructions. Approximately 1.0 g of plant material was ground to a fine powder in liquid nitrogen using

a cold mortar and pestle. Denaturation buffer (5 ml) was added and homogenised to a slurry. The slurry was transferred to 30 ml Oak Ridge centrifuge tubes (Nalgene®, Rochester, NY, USA) and one starting volume of phenol/CHCl3 was added. The tubes were incubated on ice for 15 min, then centrifuged in an RC2-B Sorvall centrifuge fitted with an SS-34 rotor (12,000 x g, 15 min, 4°C) to pellet solid plant matter. The volume of supernatant was measured and transferred to clean 50 ml Oak Ridge tubes. One tenth of the supernatant volume of sodium acetate solution was added and the tubes were gently inverted for 10 s to mix. One starting volume of phenol/CHCl3 solution 2 was added to tubes and vigorously shaken for 1 min. Again, the tubes were incubated on ice for 15 min and centrifuged (12,000 x g, 15 min, 4°C). The upper aqueous phase was transferred to clean 50 ml Oak Ridge tubes, measured, and an equal volume of isopropanol added. After thorough mixing, the tubes were incubated at -20°C for a minimum of 20 min. RNA was pelleted by centrifugation (12,000 x g, 20 min, 4°C). The RNA pellet was washed with 1 ml 70% (v/v) EtOH, briefly air-dried and resuspended in RNase-free water containing 0.1 mM EDTA.

2.2.10.2 Isolation of Poly(A)$^+$ mRNA

Poly(A)$^+$ mRNA was isolated from total RNA, (Section 2.2.10.1) prepared from induced *L. rigidum* seedling material (Section 2.2.4), using Dynabeads® mRNA purification kit (Dynal®, Oslo, Norway). In this system a biotinylated oligo (dT) primer is used to hybridise to the 3' poly(A)$^+$ region of the mRNA. The hybrids coupled with Strepavidin MagneSphere particles are then captured with a magnetic stand. mRNA was eluted from the solid phase by adding ribonuclease-free millipore water. The concentration and purity of isolated mRNA was determined by spectrophotometry (Section 2.2.10.3) and native agarose gel electrophoresis (Section 2.2.10.4). Samples were stored at -80°C until used.

2.2.10.3 Spectrophotometry of RNA

UV absorption spectra were determined using a DMS 100/D5 15 spectrophotometer (Varian Techtron Pty. Ltd., Mulgrave, Victoria, Australia) or a UV-Visible Cintra 10/20/40 Spectrometer (GBC Scientific Equipment Pty. Ltd., Dandenong, Victoria, Australia) with 1 cm-path quartz cuvettes. The concentration of RNA was determined by measurement of absorption at 260 nm (assuming an A_{260} of 1.0 is equal to 40 μg ssRNA.ml^{-1}).

2.2.10.4 Electrophoresis of Total RNA

Total RNA was electrophoresed through horizontal 1.2% (w/v) agarose (Seakem GTG, Sigma-Aldrich)/2.2 M formaldehyde gels using the method of Sambrook *et al.* (1989) with the following modifications. The gel was prepared by dissolving 0.6 g agarose in 40 ml millipore water and allowed to cool slightly before adding 10 ml 5 x MOPS buffer (0.1 M MOPS, pH 7.0; 40 mM NaAc; 5 mM EDTA, pH 8.0), 1.5 ml 37% (v/v) formaldehyde and 1 μl ethidium bromide (EtBr; 10 mg.ml^{-1}), then pouring into the casting tray. The gel was pre-electrophoresed in 1 x MOPS buffer for 30 min at 60 V. To each RNA sample was added 4 μl 5 x MOPS buffer, and 13.5 μl of a solution of deionised formamide:formaldehyde:water (3.5:10:3.5 v/v/v). Prior to loading on the gel samples were heated at 65°C for 10 min, quenched on ice and 1 μl of RNA loading buffer (50% (v/v) glycerol; 1 mM EDTA, pH 8.0; 0.25% (w/v) bromophenol blue; 0.25% (w/v) xylene cyanol FF) was added. The gel was run at 60 V for 30 min, then at 100 V for 2 h until the loading buffer dye had run halfway down the length of the gel.

2.2.10.5 Northern Blot Analysis

Northern blot analysis was carried out as described by Sambrook *et al.* (1989). Following electrophoresis (Section 2.2.10.4), the RNA gel was soaked in two changes of 10 x SSC to remove formaldehyde. The gel was placed on top of two sheets of 3MM chromatography paper (Whatman International Ltd., Maidstone, England; presoaked in 10 x SSC and

connected to a 10 x SSC reservoir below the gel support) and the edges of the gel surrounded with Parafilm® M (American National Can™, Chicago, IL, USA). The gel was then covered with a piece of Hybond™-N⁺ nitrocellulose (Amersham, Buckinghamshire, England) of the same size (presoaked in 10 x SSC) ensuring no air bubbles were trapped between layers. Three sheets of dry Whatman paper were placed on top of the nitrocellulose membrane and a stack of paper towels topped with a small glass sheet for weight. Transfer was allowed to proceed overnight and RNA was fixed to the membrane using a GS Gene Linker™ U.V. Chamber (BioRad, Hercules, CA, USA) and baked at 80°C for 1 h.

2.2.10.6 Preparation of [α-^{32}P]dCTP-labelled DNA Probes

Radiolabelled DNA probes were prepared by random primed incorporation of [α-^{32}P]dCTP. Briefly, 5 µl template DNA (200 ng.µl^{-1}) and 14.8 µl sterile millipore water were mixed and denatured in a boiling waterbath for 5 min, then quenched on ice for 5 min. Added to the tube in order was 5 µl 10 x Klenow buffer, 5 µl of each of dATP, dGTP and dTTP (each 200 µM), 5 µl bovine serum albumin (BSA; 4 µg.ml^{-1}), 0.5 µl DNA polymerase I Klenow fragment (5 u.µl^{-1}; Promega), and 5 µl [α-^{32}P]dCTP. The tube was flick mixed and the contents briefly spun down before incubation overnight at 37°C. Unincorporated [α-^{32}P]dCTP was removed using ProbeQuant™ G-50 micro columns (Pharmacia Biotech, Milwaukee, WI, USA) according to the manufacturers instructions. The resin in the column was resuspended by briefly vortexing, the bottom closure was removed and the column centrifuged for 1 min at 735 x g. The [α-^{32}P]dCTP-labelled probe was transferred onto the centre of the resin bed and again centrifuged for 2 min at 735 x g. The [α-^{32}P]dCTP-labelled probe was denatured for 5 min in a boiling waterbath and quenched immediately on ice for 5 min prior to use.

2.2.10.7 Hybridisation of [α-^{32}P]dCTP-labelled DNA Probes to Total RNA

Nitrocellulose filters (Section 2.2.10.5) were wet in 6 x SSC and placed into a hybridisation bottle. One ml of prehybridisation solution (5 x SSC, 5 x Denhardt's reagent, 100 µg.ml^{-1} denatured herring sperm DNA, 2.5% SDS (w/v), 2 mM EDTA, 0.05 M Na-phosphate buffer) per 10 cm^2 of membrane was added to the bottle and incubated with rotation for 1 h at 65°C. The denatured [α-^{32}P]dCTP-labelled DNA probe (Section 2.2.10.6) was added to the hybridisation bottle and incubation was continued overnight at 42°C with constant rotation.

2.2.10.8 Northern Blot Washing and Autoradiography

The following day the hybridisation solution was removed and the filter washed twice in 2 x SSC, 0.1% (w/v) SDS for 10 min at RT then twice in 0.2 x SSC, 0.1% (w/v) SDS for 30 min at 65°C. Autoradiography was performed with exposure of membranes, hybridised with [α-^{32}P]dCTP-labelled DNA probes, to X-ray film (Kodak Scientific Imaging Film, X-Omat AR; Integrated Sciences, Willoughby, NSW, Australia) between two intensifying screens (X-Omatic regular screens; Eastman Kodak Company, Rochester, NY, USA). X-ray cassettes were stored at -80°C for up to two weeks and developed using automated Curix 60 or CP-1000 film developers (Agfa-Gevaert, N.V., Mortsel, Belgium).

2.2.11 Construction of cDNA Libraries

Complementary DNA (cDNA) libraries were constructed using poly(A)$^+$ RNA isolated from 12 day old seedlings of *L. rigidum*, induced for 24 h with appropriate elicitors (Section 2.2.4). Following cDNA synthesis libraries were constructed in the Lambda ZAP®II vector (Stratagene; Section 2.1.3) according to the manufacturers instructions.

2.2.11.1   Preparation of cDNA for Libraries

cDNA was prepared using the TimeSaver™ cDNA Synthesis kit (Pharmacia Biotech) according to the manufacturers instructions. Briefly, 5 to 6 µg poly(A)$^+$ mRNA (Section 2.2.10.2) and sterile millipore water, to a total volume of 20 µl, was heated at 65°C for 10 min, then quenched on ice. To a tube of first-strand reaction mix was added 1 µl DTT, 1 µl oligo (dT)$_{18}$ primer (250 ng.µl^{-1}) and the heat-denatured RNA. The reaction was gently mixed and incubated at 37°C for 1 h. The first-strand reaction mix was then completely transferred to a tube of second-strand reaction mix and gently mixed. This tube was incubated at 12°C for 30 min then at 22°C for 1 h and finally at 65°C for 10 min. The reaction was extracted with an equal volume of phenol:chloroform (1:1 v/v) and centrifuged (27,000 x g, 1 min) to separate the phases. The upper aqueous layer was transferred onto the centre of the resin bed of a Sepharose® CL-4B gel spun column and centrifuged in a Technospin R centrifuge (Sorvall Instruments, Dupont) at 400 x g. The eluate was collected into a sterile 1.5 ml eppendorf tube ready for the addition of linkers.

2.2.11.2   Addition of Linkers

Still using the TimeSaver™ cDNA Synthesis kit (Section 2.2.11.1), 1 µl of the ATP solution was diluted in 4 µl of sterile millipore water. To the spun column eluate (Section 2.2.11.1) was added 5 to 6 µg EcoRI/NotI adaptor solution, 30 µl PEG buffer, 1 µl of diluted ATP solution and 1 µl of T4 DNA ligase. The reaction was gently mixed, incubated at 16°C for 1 h, then at 65°C for 10 min to denature the DNA ligase, before quenching on ice. To the tube was added 1.5 µl undiluted ATP solution and 1 µl T4 Polynucleotide kinase. After gently mixing the reaction was incubated at 37°C for 30 min and then at 65°C for 10 min. A volume of 140 µl of phenol:chloroform (1:1 v/v) was added, the tube vortexed and centrifuged (27,000 x g, 1 min) to separate the two phases. The upper aqueous layer was transferred onto the centre of the resin bed of a Sepharose®

CL-4B gel spun column, then centrifuged in a Technospin R centrifuge at 400 x g. The eluate was collected into a sterile 1.5 ml eppendorf tube ready to ligate Lambda arms.

2.2.11.3   Ligation into Vector

Lambda ZAP® II prepared arms (Stratagene) were ligated to the prepared cDNA (Section 2.2.11.2). Into a sterile 0.5 µl eppendorf tube was pipetted 1.0 µl Lambda ZAP® II prepared arms (1 µg.µl⁻¹), 1.0 µl cDNA (100 ng.µl⁻¹), 0.5 µl 10 x ligase buffer, 0.5 µl 10 mM rATP (pH 7.5), 0.5 µl T4 DNA ligase and sterile millipore water to a final volume of 5.0 µl. The reaction was incubated overnight at 12°C.

2.2.11.4   Packaging into Lambda Phage

A freeze-thaw extract, from the Gigapack® II packaging extract (Stratagene), was thawed rapidly by hand and the ligation reaction (Section 2.2.11.3) was immediately added. The tube was placed on ice and 15 µl sonic extract was added rapidly. The reaction was incubated at RT for 2 h. At the end of the incubation, 500 µl SM buffer (Appendix B) and 20 µl chloroform were added and gently mixed. A short centrifugation sedimented any debris.

2.2.11.5   Titration of cDNA Library

2.2.11.5.1 Preparation of Host Cells

A single, fresh colony of the bacterial host XL1-Blue MRF' (Stratagene; Section 2.1.3) was used to inoculate 50 ml of LB medium, supplemented with 0.2% (v/v) maltose and 10 mM MgSO$_4$. The culture was incubated overnight at 30°C with constant agitation at 200 rpm. The following day the cells were pelleted for 10 min at 1,000 x g at 4°C and the supernatant carefully removed. The pellet was resuspended in approximately 5 ml 10 mM MgSO$_4$. Prior to use cells were diluted to an OD$_{600}$ of 0.5 with 10 mM MgSO$_4$.

2.2.11.5.2 Preparation of Phage Dilutions & Plating of Library

Appropriate serial dilutions of phage in SM buffer (Appendix B) were prepared. Into 10 ml plastic tubes was pipetted a 200 µl aliquot of freshly prepared XL1-Blue MRF' (OD_{600} = 0.5) and 10 µl of the prepared phage dilutions. The tubes were mixed and incubated in a 37°C waterbath for 10 min. Molten top agarose (cooled to 50°C) was added (3 ml) to each tube, mixed and poured onto pre-warmed 90 mm NZY plates. The plates were gently swirled to ensure an even coverage of the plate surface, allowed to set for 5 min at RT and incubated at 37°C for 12 h or until plaques were visible. The library titre was calculated by counting the number of visible plaques on the plates and multiplying by the appropriate dilution factor to determine the number of plaque forming units per ml ($pfu.ml^{-1}$). Prepared libraries contained approximately 2×10^6 $pfu.ml^{-1}$.

2.2.11.6 Plating of cDNA Libraries for Primary Screening

For each library screening, six plates of 50,000 plaques each (a total of 3×10^5 plaques) were required. To plate the packaged library the appropriate volume of the final 500 µl packaged reaction (Section 2.2.10.4) to obtain 50,000 plaques was mixed with 600 µl aliquots of freshly prepared XL1-Blue MRF' cells (OD_{600} = 0.5 in 10 mM $MgSO_4$) in 10 ml tubes and incubated at 37°C in a waterbath for 15 min. To each tube, 8 ml molten top agarose (cooled to 50°C) was added, mixed and poured onto pre-warmed 140 mm NZY plates. The plates were gently swirled to ensure an even coverage of the plate surface, allowed to set for 5 min at RT and incubated at 37°C for 12 h or until plaques were visible. Plates were then removed from 37°C incubation and transferred to 4°C for a minimum of 1 h to stop plaques from enlarging.

2.2.11.6.1 Transfer of cDNA Library Phage to Nitrocellulose Filters

Circular Hybond-N^+ filters (Amersham) were numbered and asymetrically marked for orientation using a soft (6B) lead pencil. Each filter was placed (marked side downwards)

centrally onto the surface of the top agarose of each of the plates with care not to trap any air bubbles. Transfer was allowed to proceed for 1 min during which time the pencil markings on the filters were copied to the bottom of the plates using a marker pen. Filters were gently removed from the plate surface and transferred (marked side upwards) to Solution I (Appendix B) for 5 min. Care was taken to gently push the filter under the solution using forceps rather than shaking the solution possibly causing the plaques to become fuzzy. Filters were then transferred to Solution II (Appendix B) for 5 min followed by Solution III (Appendix B) for 30 sec. Filters were then placed (marked side upwards) on Whatman paper to dry at RT.

2.2.11.6.2 Hybridisation of [α-^{32}P]dCTP-labelled DNA Probes to cDNA Libraries

Screening of *L. rigidum* cDNA libraries (3 x 10^5 plaques) was conducted at moderate stringency with [α-^{32}P]dCTP-labelled DNA probes (Section 2.2.10.6) in hybridisation bottles. Filters were incubated in prehybridisation solution (5 x SSC, 5 x Denhardt's solution, 2.5% (w/v) SDS, 2 mM EDTA, 0.05 M Na-phosphate buffer, pH 6.0, and 100 µg.ml^{-1} denatured herring sperm DNA), preheated to 65°C and using a volume of 0.2 ml.cm^{-2} filter, for a minimum of 1 h with constant rotation. The denatured [α-^{32}P]dCTP-labelled DNA probe (Section 2.2.10.6) was added to the prehybridisation solution and hybridisation was performed at 65°C overnight with constant rotation. Hybridisations were carried out in a Shake 'n' Stack hybridisation oven (Hybaid Ltd., Teddington, Middlesex, UK).

2.2.11.6.3 cDNA Library Membrane Washing and Autoradiography

Filter washing was carried out with two washes at RT in 2 x SSC, 0.1% (w/v) SDS for 15 min each, one wash at 55°C in 0.2 x SSC, 0.1% (w/v) SDS for 30 min and one wash at 65°C in 0.1 x SSC, 0.1% (w/v) SDS for 30 min. Filters were left to air dry on Whatman paper for 15 min. Autoradiography was performed as described in Section 2.2.10.7.

2.2.11.6.4 Plating of cDNA Libraries for Secondary and Tertiary Screenings

Positive plaques, identified from the primary screening, were selected and isolated for a further round of screening. Plaques were isolated from the appropriate plates using the wide end of a glass pasteur pipette to remove a small plug of agarose from the plate. This plug was transferred to a 1.5 ml eppendorf tube containing 1 ml SM buffer (Appendix B) and 20 μl chloroform. Tubes were left at RT for a minimum of 4 h or overnight at 4°C to allow the phage to diffuse into the buffer. Appropriate dilutions of the isolated phage were prepared and plated onto 90 mm NZY plates (Section 2.2.10.5.2). Transfer to nitrocellulose filters, prehybridisation and hybridisation were conducted as described in 2.2.11.4 and 2.2.11.5. This procedure was repeated for a tertiary round of screening, if required, to allow isolation of single plaque preparations.

2.2.12   Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

2.2.12.1   Reverse Transcription

Conditions for RT-PCR were adapted from the protocol of Meijer *et al.*, 1993b. The following components were added in sequence to a sterile 0.5 μl reaction vial; 3 μg total RNA (Section 2.2.9.1), 2 μl of 250 ng.μl^{-1} oligo d(T)$_{18}$ primer and 9 μl sterile millipore water. The reaction mix was incubated at 70°C for 10 min and quenched on ice for 5 min. To this vial was added, in order; 4 μl 5 x first strand RT buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl$_2$), 2 μl 0.1 M DTT, 0.67 μl 15 mM dNTP's and sterile millipore water to a total volume of 19 μl. The tube was briefly spun down before incubating at 50°C for 2 min. At this stage 1.0 μl of Superscript™ II RNase H⁻ reverse transcriptase (200 units.μl^{-1}; GibcoBRL® Life Technologies Pty. Ltd., Mulgrave, Victoria, Australia) was added, mixed by gently pipetting up and down and the reaction was incubated at 50°C for 50 min. To inactivate the enzyme a 15 min incubation at 70°C followed. On completion of the reverse transcription reaction, 30 μl sterile millipore water was added to the reaction vial to give a final volume of 50 μl.

2.2.12.2 Polymerase Chain Reaction

PCR was carried out in a DNA thermal cycler (PTC-100 MJ Research, Inc., Watertown, MA, USA) using 5 μl diluted, oligo d(T)-primed cDNA as the template (Section 2.2.12.1). Reactions contained 1.5 μl oligo d(T)$_{18}$ primer (250 ng.μl^{-1}), 5 μl Meijer primer (5 μg.μl^{-1}), 5 μl 10 x magnesium-free PCR buffer (100 mM Tris-HCl, pH 9.0, 500 mM KCl and 1.0% Triton® X-100), 0.67 μl 15 mM dNTP's, 3 μl 25 mM MgCl$_2$, 0.2 μl *Taq* DNA polymerase (5 u.μl^{-1}; Promega) and sterile millipore water to a final volume of 50 μl. The reaction mixture was briefly centrifuged then subjected to a 4 min denaturation step at 94°C. Each subsequent cycle included a denaturation step at 94°C for 1 min, an annealing step at 47°C for 2 min and an extension step at 72°C for 3 min. After 30 cycles of amplification the reaction was completed by an extension step at 72°C for 10 min before cooling to 4°C.

2.2.12.3 Agarose Gel Electrophoresis of RT-PCR Products

Electrophoresis of RT-PCR products, for analytical purposes, was carried out in tanks for horizontal submerged gel electrophoresis. Samples were prepared in 1 x DNA loading buffer (0.05% (w/v) BPB; 30% (v/v) glycerol in millipore water) and separated electrophoretically on 2.0% (w/v) agarose Tris-acetate/EDTA (TAE) minigels at a constant 60 V for 2 h in TAE buffer (400 mM Tris base; 200 mM sodium acetate; 100 μM EDTA, pH 7.8). DNA bands were visualised by transillumination with UV light (354 nm) after brief staining of the gel in a solution of 10 μg.ml^{-1} EtBr. A positive photograph of the stained gel was taken using Polaroid land film 667. Sizes of RT-PCR products were determined by comparing the relative mobility with known DNA bands of the molecular weight markers IV or VI (Boehringer Mannheim Australia Pty. Ltd, Castle Hill, NSW, Australia).

2.2.12.4   Purification of RT-PCR Products

Purification of RT-PCR products from reaction components was attained using a number of alternative methods, described below. Quantification of purified RT-PCR products was estimated by agarose gel electrophoresis and comparison with similar sized DNA bands of molecular weight marker VI.

2.2.12.4.1   Method 1 - Wizard® PCR Preps DNA Purification System

To directly purify PCR products from contaminants the Wizard® PCR Preps DNA Purification System (Promega) was used according to the manufacturers instructions. To the entire 50 μl PCR amplification was added 100 μl Direct Purification Buffer and the tube was vortexed to mix. Resin (1 ml) was added, and the mixture again briefly vortexed three times over a 1 min period. For each purification, one Wizard® Minicolumn was prepared. The plunger was removed from a 3 ml disposable syringe and the syringe barrel was attached to the Luer-Lok® extension of a Minicolumn. The resin/DNA mix was pipetted into the syringe barrel, the plunger inserted and the slurry was gently pushed into the Minicolumn. After detaching the syringe from the Minicolumn, the plunger was removed and the syringe and Minicolumn reattached. Two ml of 80% (v/v) isopropanol was pipetted into the syringe barrel and gently pushed into the Minicolumn with the plunger. The Minicolumn was then detached from the syringe, placed into a 1.5 ml eppendorf tube and centrifuged for 2 min at 10,000 x g to dry the resin. The Minicolumn was transferred to a new eppendorf tube and 50 μl sterile millipore water was added. After 1 min the Minicolumn was centrifuged for 20 s at 10,000 x g to elute the DNA fragment.

2.2.12.4.2   Method 2 - Jetsorb Gel Extraction Kit

To purify DNA from agarose gel pieces the Jetsorb Gel Extraction Kit (Genomed Inc., NC, USA) was used according to the manufacturers instructions. For each 100 mg gel piece, 300 μl buffer A1 and 10 μl JETSORB suspension was added. After vortexing, tubes were

incubated at 50°C in a waterbath for 15 min, with gentle mixing every 3 min. Centrifugation for 30 s at 12,000 x *g* was performed to pellet the JETSORB and allow removal of the supernatant. Two washes with 300 µl reconstituted buffer A2 were performed with the supernatant removed following centrifugation as before. The JETSORB pellet was then air dried for 15 min. The pellet was resuspended in 20 µl sterile millipore water, incubated for 5 min at 50°C, centrifuged as before and the supernatant transferred to a clean tube.

2.2.12.4.3   Method 3 - Prep-A-Gene DNA Purification Kit

To purify DNA from agarose gel pieces the nucleic acid purification kit, Prep-A-Gene (Biorad), was used according to the manufacturers instructions. To an agarose piece containing the DNA of interest, three volumes of DNA binding buffer and 15 µl of glass milk was added. A 10 min incubation at room temperature, with occasional gentle flick-mixing, was followed by centrifugation for 30 s at maximum speed in a centrifuge. The supernatant was removed and replaced with 25 times of the added glass milk volume (375 µl) of wash buffer. The glass milk was resuspended, again centrifuged as previously described and the wash discarded. This washing step was repeated a total of three times. A final centrifugation was performed to remove any traces of wash buffer and one pellet volume (15 µl) of elution buffer was added. Incubation in a 37°C water bath for 10 min was followed by centrifugation for 1 min at maximum speed. The eluate was transferred to a fresh tube and the elution was repeated one further time with 10 µl of elution buffer. The eluates were pooled before quantification.

2.2.12.5   Spectrophotometry of DNA

UV absorption spectra were determined using a DMS 100/D5 15 spectrophotometer or a UV-Visible Cintra 10/20/40 spectrometer with 1 cm-path quartz cuvettes. The concentration of DNA was determined by measurement of absorption at 260 nm (assuming an A_{260} of 1.0 is equal to 50 µg dsDNA.ml^{-1}).

2.2.12.6   Cloning of RT-PCR Products

2.2.12.6.1   Ligation Reaction

Double-stranded RT-PCR products were ligated into the pGEM®-T or pGEM®-T Easy vector system (Section 2.1.3; Promega). To calculate the moles of ends of linear vector and insert DNA the following formula was used:

$$2 \times \left\{ \frac{\text{g of DNA}}{\text{\# of bp}} \right\} \times 649 \ \text{Daltons} / \text{bp}$$

A 1:2 and a 1:3 vector:insert ratio (of moles of ends of linear DNA) used in ligation reactions was calculated using the following formula:

$$\left\{ \frac{\text{ng of vector x kb size of insert}}{\text{total kb size of vector}} \right\} \quad \times \quad \left\{ \text{molar ratio of } \frac{\text{insert}}{\text{vector}} \right\} = \text{ng of insert}$$

The DNA ratio of vector to insert used was 1:2 or 1:3. Routine ligation reactions consisted of 0.5 µl 10 x T4 DNA ligase reaction buffer, y µl volume of vector DNA, z µl volume of insert DNA, 0.5 µl 10 mM ATP, 0.5 µl T4 DNA ligase (Boehringer Mannheim) and sterile millipore water to a final volume of 5 µl. The reaction was ligated at 23°C for 15 min, then overnight at 4°C.

2.2.12.6.2   Transformation Reaction

Recombinant plasmids were transformed into high efficiency JM109 supercompetent cells (Stratagene; Section 2.1.3.2). Competent cells (50 µl) were aliquotted into a pre-chilled capped polystyrene tube, 2.5 µl of the ligation mixture (30 to 50 ng DNA) was added and incubated on ice for 10 min. Cells were heat shocked at 42°C in a waterbath for 45 s and immediately placed back on ice for a further 2 min. Gently, 450 µl of SOC (Appendix B) was added to the cells and the tube was incubated at 37°C, with gentle agitation at 200 rpm for 1 h. Aliquots of 100 µl and 200 µl were plated onto the surface of LB supplemented with ampicillin (Amp; 100 µg.ml^{-1}) plates. As the plasmid vectors contained the *lacZ* promoter, X-gal and IPTG were spread onto the surface of LB + Amp plates prior to the plating of transformed cells. Plates were incubated overnight at 37°C.

2.2.12.6.3 Plasmid DNA Isolation

Single isolated, white colonies were picked from plates and inoculated into 4 ml LB or TB media supplemented with 100 µg.ml^{-1} Amp. Bacteria were grown overnight at 37°C with constant agitation at 200 rpm to late log phase. Plasmid DNA was then isolated by one of the following methods.

Method 1 - Small Scale Alkali Lysis Method

A small scale (mini-prep) alkali lysis procedure was used to isolate DNA to confirm the presence of a plasmid following transformation experiments. This procedure was also used to isolate plasmid DNA for restriction digestion. Bacteria were harvested by centrifugation of 1.5 ml of culture in eppendorf tubes at 6,000 x g for 2 min. The supernatant was removed and the bacterial pellet resuspended in 100 µl Solution I (Appendix B). Tubes were incubated on ice for 10 min and 200 µl freshly prepared Solution II (Appendix B) was added. After gently inverting tubes to mix, they were again placed on ice for 2 min. To each tube was added 150 µl Solution III (Appendix B), the tubes were gently inverted to mix, and placed on ice for 10 min. Precipitated proteins, cellular debris, SDS and chromosomal DNA were removed by centrifugation (20,000 x g, 10 min). The supernatant was transferred to a fresh tube and the plasmid DNA precipitated with the addition of 1 ml 100% EtOH. Following 30 min incubation at -20°C, tubes were centrifuged (20,000 x g, 15 min) to pellet the nucleic acid. The supernatant was removed, the pellet washed with 200 µl 70% (v/v) EtOH and briefly air-dried before resuspension in sterile millipore water. Isolated nucleic acids were stored at -20°C until used.

Method 2 - BRESAspin™ Plasmid Mini Kit

For high yields of pure plasmid DNA required for sequencing, the BRESAspin™ Plasmid Mini Kit (Geneworks) was used according to the manufacturers instructions. In a centrifuge tube, 2 ml overnight culture was centrifuged (20,000 x g, 1 min) to pellet cells. The supernatant was removed and the cells resuspended in 50 µl Resuspension Buffer. To each tube was added 100 µl Lysis Buffer and the tubes gently inverted to mix. Neutralisation Buffer (325 µl) was then added, again the tubes gently inverted to mix contents, followed by centrifugation (20,000 x g, 5 min) to pellet the precipitate. The supernatant was transferred to spin column units and centrifuged (20,000 x g, 30 s). The

flow-through was discarded and the spin columns replaced into the collection tubes. Wash Buffer (300µl) was added to the spin columns and again the tubes were centrifuged (20,000 x *g*, 30 s). The spin columns were transferred to clean collection tubes and the DNA eluted from the column by centrifugation (20,000 x *g*, 30 s) with 50 µl sterile millipore water. Isolated nucleic acid samples were stored at -20°C until used.

2.2.12.6.4 Restriction Enzyme Analysis of Plasmid DNA

Recombinant transformants were screened by restriction analysis of isolated plasmid DNA to excise and so confirm the presence and size of any inserts. Restriction enzymes were obtained from Promega and digests were performed in reaction buffers supplied by the manufacturer. Digests were carried out at 37°C for 2 to 3 h in a waterbath and analysed by agarose gel electrophoresis (Section 2.2.10.4). Those plasmids containing inserts of the appropriate size were prepared for sequencing.

2.2.12.7 Sequencing of RT-PCR Products

Sequencing was performed using PRISM™ Ready Reaction Dye Primer Cycle Sequencing kit (Applied Biosystems, Inc.) with M13-forward and -reverse primers on an automated Applied Biosystems Model 373A DNA sequencing system. DNA sequences of interest were sequenced in duplicate in both forward and reverse directions.

2.2.12.8 Sequence Analysis of RT-PCR Products

DNA and protein sequence comparisons were performed using the BLAST network service from the National Centre for Biotechnology Information (Bethesda, MD, USA).

Chapter 3

Induction of Herbicide Metabolism

Chapter 3

Induction of Herbicide Metabolism

3.1   Introduction

Plants are subjected to many stresses including physical (wounding), biological (pathogenic invasion), and xenobiotic challenge. Perception of these primary signals by plants may trigger rapid defence responses via a series of signal transduction pathways. The main target of these signal transductions is the cell nucleus. Here, transcriptional activation of a large array of genes results in the *de novo* synthesis of numerous proteins and defence mechanisms (Hammond-Kosack & Jones, 1996). Transcriptional activation of genes is a vital part of the plant's defence system against these challenges and includes a variety of genes involved in diverse primary and secondary metabolic pathways. Genes which may be rapidly induced or upregulated include those involved in the shikimate pathway and the general phenylpropanoid pathway, including glutathione transferases and plant cytochrome P450 monooxygenases. This increase in the level of enzyme activity is defined as induction. Enzyme induction, particularly of cytochrome P450 monooxygenases, usually enhances the initial step in detoxification pathways, therefore, under most conditions, induction is a protective mechanism for the plant (Porter & Coon, 1991). Knowledge of these induction mechanisms may provide an insight into strategies used by plants to adapt to an ever changing environment and also an understanding of the biochemical pathways by which cells recognise and respond to certain stimuli. This may lead to the understanding of the mechanisms controlling enzyme levels by which individuals are susceptible to adverse effects of certain compounds or treatments.

As plant cytochrome P450 levels are low in abundance, induction also provides a potential means of enriching specific cytochrome P450 forms, aiding in their isolation and subsequent detailed enzymatic studies (Barrett, 1995). Cytochrome P450 enzyme activities are often highly variable as they may either be expressed constitutively or specifically expressed only at certain developmental stages or in localised tissues and organs within the plant. The generally low levels of cytochrome P450 monooxygenases in most plants may be significantly increased in response to various stimuli or treatments. Induction is usually specific for certain cytochrome P450 monooxygenases and it is likely that each enzyme will be differentially expressed in response to both developmental and environmental cues. In turn these enzymes respond by modulating the rates of biosynthetic pathways and it would be assumed that the level of cytochrome P450 protein would be coordinately regulated with other proteins involved in the same pathway. Light and physical stresses, including mechanical wounding and pathogen invasion (bacterial and fungal antagonists), have been shown to result in elevated cytochrome P450 levels in plants (Hahlbrock *et al.*, 1976; Benveniste *et al.*, 1977; 1978; Rich & Lamb, 1977; Reichhart *et al.*, 1979; 1980; Uritani & Asahi, 1980; Church & Gilbert, 1984; Dixon & Pavia, 1995; Frank *et al.*, 1996). Cytochrome P450 levels may also rise in response to compounds including solvents, divalent ions, polyamines, metal complexing agents, heme ligands, steroids, xenobiotics, paraoxon and flavones (reviewed in Durst, 1991; Bolwell *et al.*, 1994; Batard *et al.*, 1995). In many cases, inducers are also substrates for the induced enzyme, therefore, cytochrome P450 activities remain elevated specifically as needed. Such induction by exogenous compounds and stimuli is most likely an evolutionary adaptation to allow organisms to survive potentially detrimental stresses or aggressions, such as environmental toxicants and herbivory.

It is not clear how inducers specifically activate enzymatic activities, the magnitude, expression, or time course for their action, or how many genes may respond to each type of

stimulus. Chemical inducers may bind directly to a receptor or act indirectly by activating a secondary messenger, which in turn acts to increase transcription levels. Alternately, the efficiency of translation, post-translational modifications (including mRNA stability) protein stabilization and degeneration may also have an effect on transcript expression levels.

In *L. rigidum* four chemical inducers, NA, 2,4-D, PB and EtOH, have been evaluated as potential inducers of herbicide metabolism and also cytochrome P450 monooxygenase transcript expression levels (C. Preston, personal communication). Diclofop-methyl metabolism may be induced up to 2-fold with the addition of 2,4-D. PB is also shown to induce diclofop-methyl metabolism (up to 2-fold) with NA and EtOH showing no significant effect. Alternately, chlorsulfuron metabolism may be induced by PB (2-fold) and EtOH (1.5-fold) with 2,4-D and NA having no significant effect (C. Preston, personal communication).

Induction of herbicide metabolism will be examined in this chapter in two sections. Firstly, in order to understand the molecular mechanisms coordinating and controlling expression of herbicide metabolising cytochrome P450 monooxygenases in *L. rigidum*, seedlings were treated with known chemical inducers to assess their effect on the rate of herbicide metabolism. Plants have the ability to metabolise xenobiotics, including herbicides, in processes analogous to those of drug metabolism in animal species (Shimabukuro *et al.*, 1992). The involvement of cytochrome P450 monooxygenases has been implicated in many cases of herbicide resistance in plants documented to date. Secondly, the effects of these chemical inducers, over time, on the transcript expression levels of two cytochrome P450 monooxygenases in *L. rigidum* were analysed. The addition of cytochrome P450 monooxygenase reaction substrates, such as *trans*-cinnamic acid or geraniol, to *Vigna radiata* L. microsomes have been shown to substantially increase

the concentration of microsomal cytochrome P450 content (Hendry & Jones, 1984). A similar method of induction was also used as a means of enriching specific cytochrome P450 forms in *L. rigidum* to aid in their isolation and subsequent enzymatic studies during this course of study.

3.2   Materials & Methods

3.2.1   Elicitors and Herbicide Metabolism

Two biotypes of *L. rigidum* exhibiting multiple herbicide resistance, designated SLR31 and VLR69, and one herbicide-susceptible biotype, VLR1 (section 2.1.1) were treated with chemical inducers to investigate their effect on the *in vivo* rates of metabolism of herbicides within seedlings. *L. rigidum* seed was germinated and grown in hydroponic culture as described in section 2.2.2 and 2.2.3, respectively. A number of compounds have been implicated in the increased level of herbicide metabolism by cytochrome P450 monooxygenases in plants. These compounds have included ethanol, clofibrate (ethyl-[4-chlorophenoxy]-2-methylpropanoate), herbicide safeners (eg: napthalic anhydride) and the herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D).

The induction properties of compounds were evaluated by comparing the concentration of [^{14}C]-radio-labelled parent herbicide remaining and production of metabolites produced *in vivo* between control and treated *L. rigidum* seedlings over a 24 h fixed time period. Induction was performed as in section 2.2.4 and herbicide treatment of seedlings as in section 2.2.6. Extraction of metabolites, subsequent HPLC analysis and LSS were performed as described in sections 2.2.7, 2.2.8, and 2.2.9, respectively. Experiments conducted contained three replicates. Data from each replicate were pooled prior to analysis with means and standard deviations calculated for each treatment.

3.2.2. Elicitors and Transcript Expression of Cytochrome P450 Monooxygenases

The addition of the elicitors clofibrate and 2,4-D to seedlings of the herbicide-resistant (SLR31) and herbicide-susceptible (VLR1) *L. rigidum* biotypes and their effect on transcript expression levels of cytochrome P450 monooxygenases was also investigated. *L. rigidum* seed was germinated and grown in hydroponic culture as described in section 2.2.2 and 2.2.3, respectively. Induction was performed as in section 2.2.4 using the chemicals, clofibrate and 2,4-D. Total RNA was prepared as in section 2.2.10.1, and electrophoresis, Northern blot analysis, hybridisation, washing and autoradiography performed as in sections 2.2.10.4 to 2.2.10.8.

3.3   Results

3.3.1   Diclofop-methyl Metabolism - Control

Radioactivity elution profiles of RP-HPLC analysis for both the *L. rigidum* susceptible biotype, VLR1, and the resistant biotype, SLR31, were comparable. Both profiles showed three major peaks with retention times of 15.40 min for diclofop-methyl, 14.20 min for diclofop acid and approximately 8 min and 10 min for metabolites (peak A and peak B, respectively). Shimabukuro and coworkers (1979; 1980) assumed that the metabolites in peak A were a mixture of ring-hydroxylated diclofop isomers and those in peak B were aryl-*O*-glucoside isomers. These compounds were not further characterised. Both resistant and susceptible biotypes produced the same pattern of metabolites as evidenced by comparison of HPLC chromatograms (Figure 3.1).

3.3.1.1   Resistant vs Susceptible *L. rigidum* Biotypes

Diclofop-methyl metabolism in 12 day old susceptible, VLR1, and resistant, SLR31, *L. rigidum* seedlings is shown is Figure 3.2. De-esterification of diclofop-methyl to the

Figure 3.1 RP-HPLC elution profiles for diclofop-methyl metabolism in 12 day old control seedlings of the *L. rigidum* herbicide-susceptible biotype, VLR1 (A), and the herbicide-resistant biotype, SLR31 (B).

active diclofop acid, was faster for the susceptible biotype, VLR1, than the resistant biotype, SLR31, in which 1.1-fold more parent herbicide remained after 24 h. Subsequent metabolism of diclofop acid was faster in the herbicide-resistant biotype, SLR31, with 1.3-fold more metabolites formed. The herbicide-susceptible biotype, VLR1, retained 1.3-fold more diclofop acid and less metabolites than the herbicide-resistant biotype, SLR31.

3.3.1.2   The Effect of Clofibrate on Diclofop-methyl Metabolism

The prolonged incubation of *L. rigidum* seedlings with 500 µM clofibrate induced severe necrosis of leaf tissues. Seedlings became shrivelled and yellowed in comparison to untreated seedlings.

The addition of 500 µM clofibrate to the herbicide-susceptible biotype, VLR1, seedlings caused an increase in the metabolism of diclofop-methyl when compared to control (uninduced) seedlings over a 24 h period. Diclofop-methyl levels decreased by 10%, diclofop acid levels decreased by 42% and the production of metabolites increased 1.5-fold. The addition of clofibrate to the herbicide-resistant biotype, SLR31, seedlings also caused an increase in the production of diclofop-methyl metabolites when compared to untreated seedlings over a 24 h period. Diclofop-methyl levels decreased by 30%, diclofop acid levels decreased by 23% and the production of metabolites increased by 1.3-fold (Figure 3.2).

3.3.1.3   The Effect of 2,4-D on Diclofop-methyl Metabolism

The addition of 500 µM 2,4-D to the herbicide-susceptible, VLR1, seedlings resulted in an increase in the level of diclofop-methyl metabolites produced over a 24 h period when compared to control (uninduced) seedlings. Diclofop-methyl levels were 1.1-fold higher, diclofop acid levels decreased by 48% and the production of metabolites increased 1.5-fold.
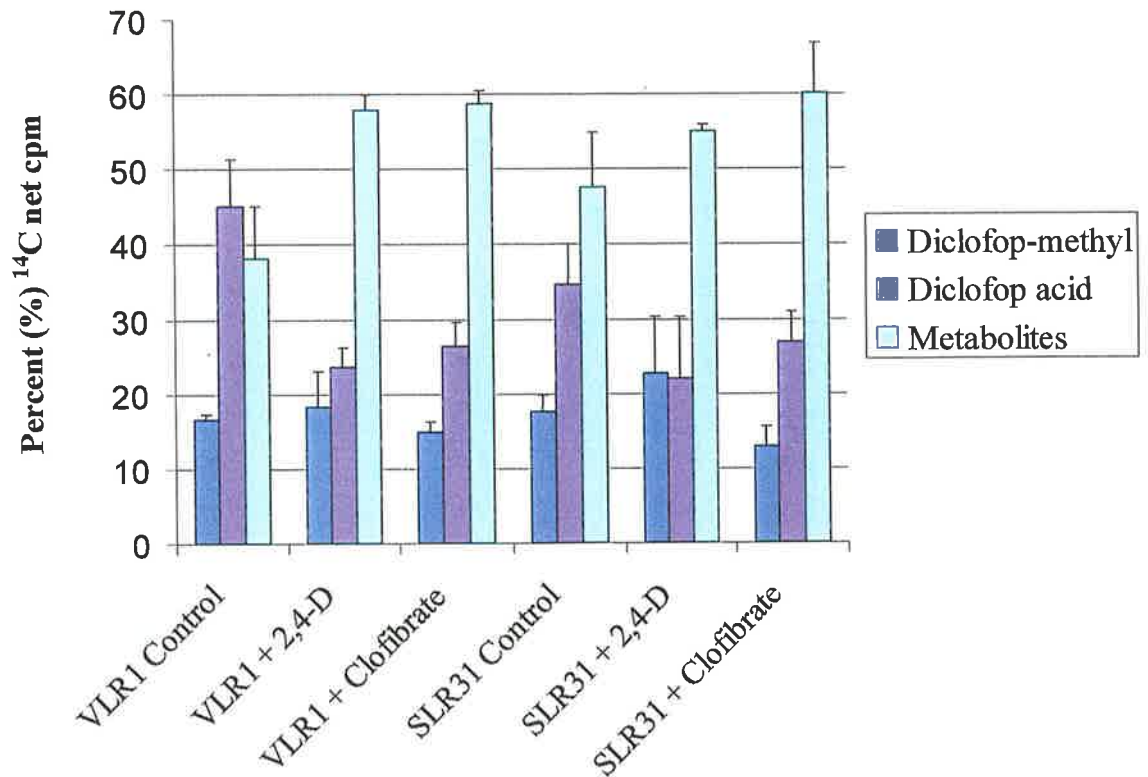
Figure 3.2 The effect of clofibrate and 2,4-D on diclofop-methyl metabolism *in vivo* in seedlings of the *L. rigidum* herbicide-susceptible biotype, VLR1, and the herbicide-resistant biotype, SLR31, over 24h.

The addition of 2,4-D to the herbicide-resistant, SLR31, seedlings resulted in an increase in the level of diclofop methyl metabolites produced over a 24 h period when compared to the control (uninduced) seedlings. Diclofop-methyl levels were 1.3-fold higher, diclofop acid levels decreased by 36% and the production of metabolites increased 1.2-fold (Figure 3.2).

3.3.2 Effect of Ethanol on Chlorsulfuron Metabolism

Absorption of chlorsulfuron, by the cut-shoot technique (Christopher *et al.*, 1991), was similar for both resistant and susceptible biotypes as indicated by the amount of radioactivity recovered. Radioactivity elution profiles of RP-HPLC analysis show three major peaks with retention times of approximately 22 min for chlorsulfuron, 10 min and 12 min for metabolites (peak A, peak B, respectively). According to Sweetser and coworkers (1982) it was assumed that the metabolites in peak A were a mixture of ring-hydroxylated glucose conjugate isomers and those in peak B were *O*-glycosylated derivatives of 5-hydroxyphenyl chlorsulfuron. These compounds were not further characterised. Sweetser and coworkers (1982) have identified the major metabolite of tolerant grasses as the *O*-glycosylated derivative of 5-hydroxyphenyl chlorsulfuron.

Chlorsulfuron metabolism in 12 day old herbicide-resistant, VLR69, *L. rigidum* seedlings is shown in Figure 3.3. Hydroxylation of chlorsulfuron was faster in the EtOH-induced VLR69 seedlings when compared to control seedlings. The same pattern of metabolites was obtained with induced and control seedlings, indicating that an increase in metabolism accounted for the decrease in parent herbicide remaining after 24 h, rather than an alternate metabolic pathway. The addition of 3% EtOH to the herbicide-resistant, VLR69, seedlings resulted in an increase in the level of chlorsulfuron metabolites produced over a 24 h period when compared to the control (uninduced) seedlings. The remaining chlorsulfuron levels were 20% lower and the production of metabolites increased. Overall, an approximate 16% induction of chlorsulfuron metabolism was observed on the addition of 3% EtOH to 12 day
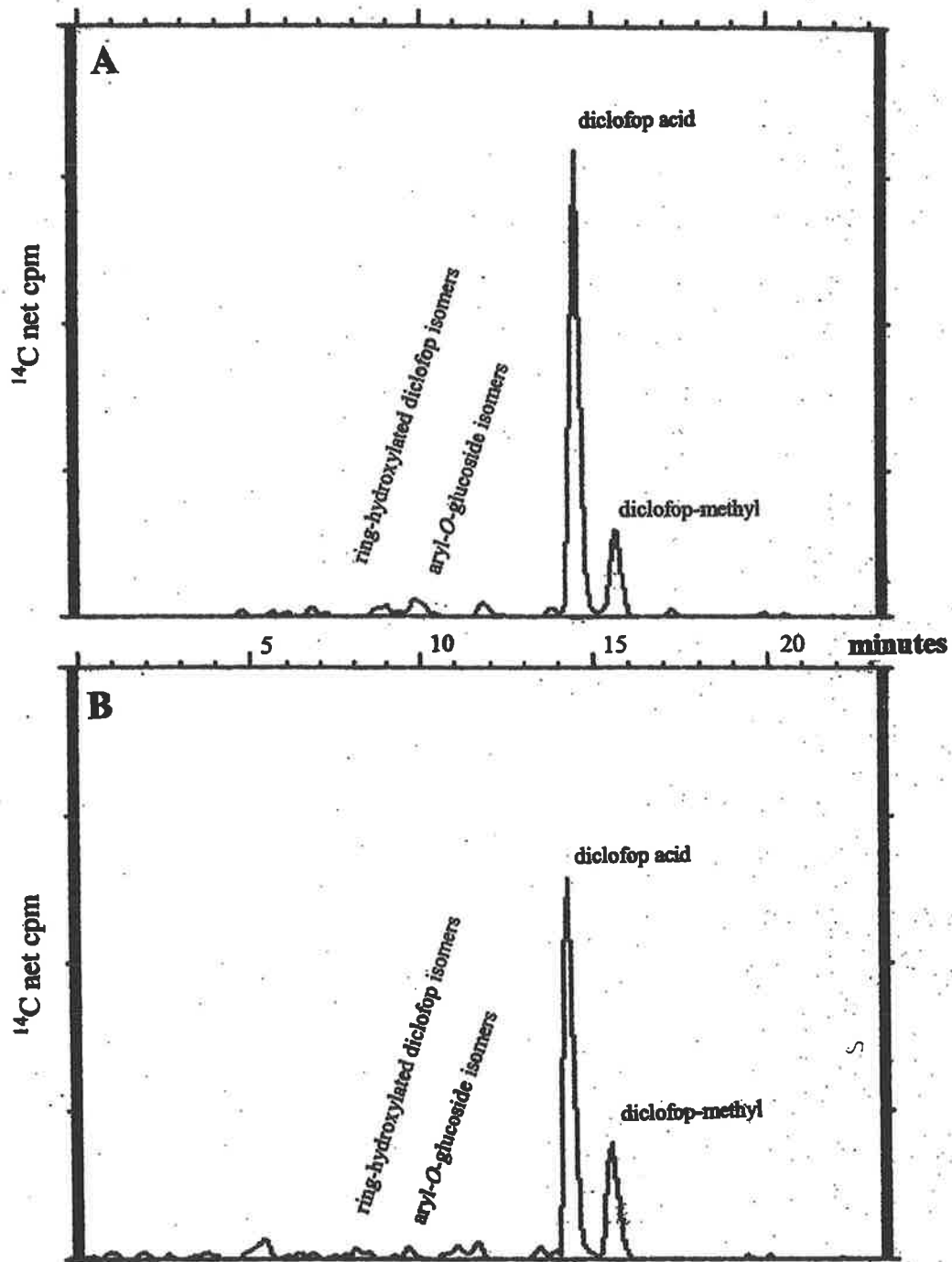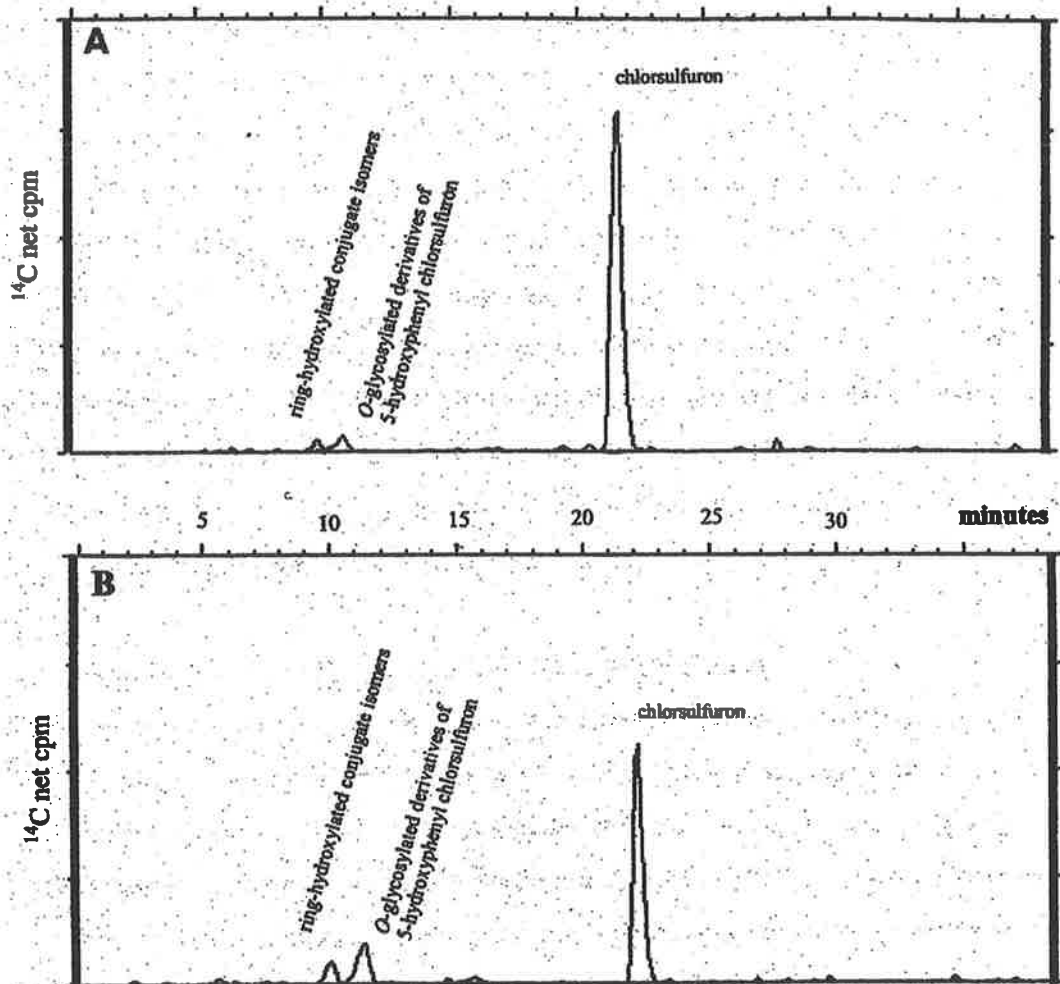
Figure 3.3 RP-HPLC elution profiles for chlorsulfuron metabolism in 12 day old seedlings of the *L. rigidum* herbicide-resistant biotype, VLR69. Control seedlings are shown in A and seedlings induced with 3% EtOH are in B.

old herbicide-resistant, VLR69, seedlings. These results are similar to those shown for herbicide-susceptible, VLR1, and herbicide-resistant, SLR31 and VLR69, biotypes performed by Christopher and coworkers (1991) and Burnet (1992).

3.3.3   Transcript Expression of Cytochrome P450 Monooxygenases

Total RNA was extracted from chemically-induced *L. rigidum* seedlings harvested at a series of time points to determine the transcript expression patterns of cytochrome P450 monooxygenases. Approximately 40 µg of RNA from each time point was loaded onto 1.2% denaturing agarose gels and separated by electrophoresis (Section 2.2.10.5). RNA was transferred to nitrocellulose membranes and hybridised with [^{14}C]-labelled probes of CYP73.301 (CA4H; from *T. aestivum*), LrCYP (EcoR1 fragment of clone A3; isolated during this study from the *L. rigidum*, herbicide-resistant biotype SLR31) and CYP94A1 (from *V. sativa*). High-stringency conditions were used for membrane washes to lower the possibility of cross-hybridisation of other cytochrome P450 monooxygenase sequences present. Relative amounts of RNA loaded onto the gel for each time point were estimated on ethidium bromide stained gels.

3.3.3.1   Northern Blot Analysis of CYP73.301 (*T. aestivum*)

The response of induced and uninduced seedlings of herbicide-resistant and -sensitive biotypes was compared using 2 week old seedlings (with two fully expanded leaves). As shown in Figure 3.4, the CYP73.301 probe hybridised to a band of approximately 1800 nucleotides in length. A low constitutive CYP73.301 transcript level is present in both herbicide-sensitive and -resistant *L. rigidum* seedlings, prior to any treatment with chemical inducers. The addition of 500 µM 2,4-D to the herbicide-susceptible biotype VLR1 (Figure 3.4 A) caused a sharp increase (~3-fold) in the level of CA4H (CYP73.301) transcript as soon as 3 h following addition of 2,4-D to the seedlings. Thereafter, CA4H

Time (h)

0   3   6   12   24   36   48   60

A. VLR1 + 2,4-D

B. VLR1 + clofibrate

C. SLR31 + 2,4-D
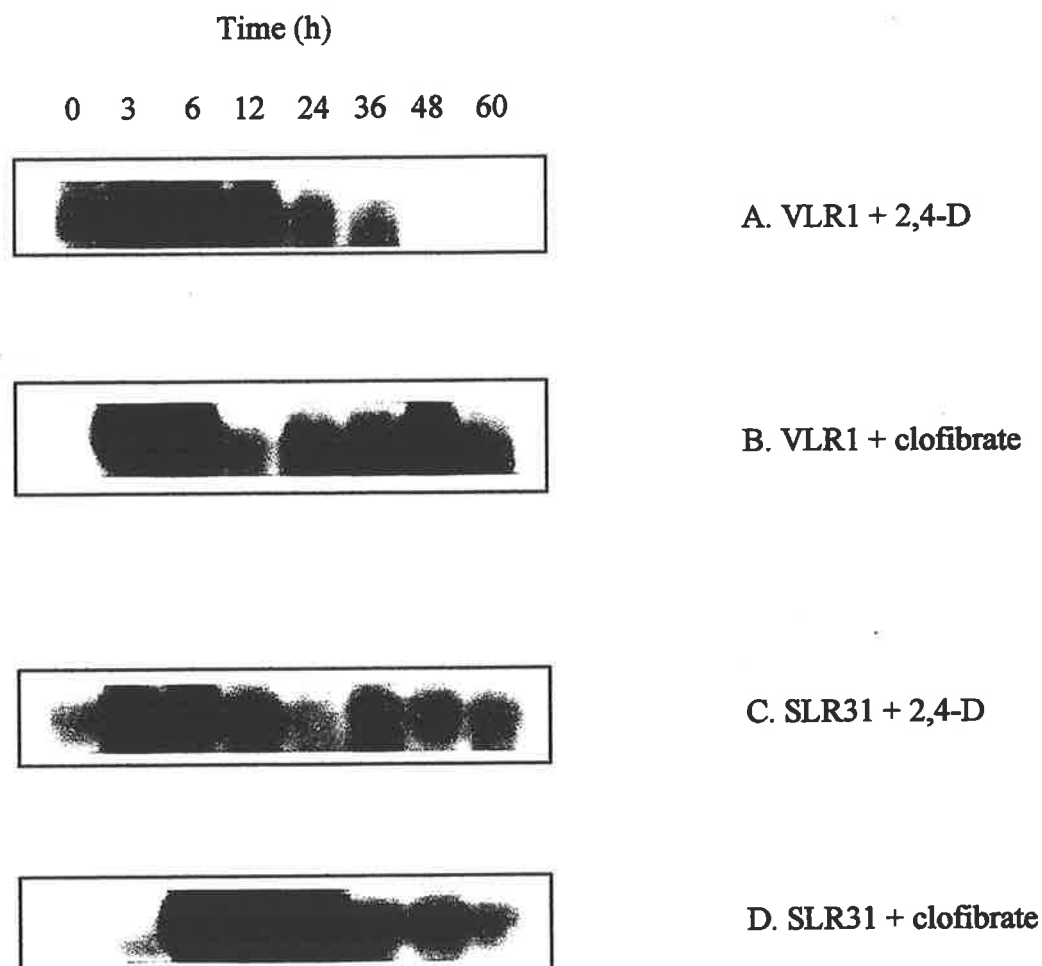
D. SLR31 + clofibrate

Figure 3.4   Northern blot analysis of CA4H expression over 60h in the *L. rigidum* herbicide-resistant biotype SLR31 and -susceptible biotype VLR1 with the addition of the chemical inducers 2,4-D and clofibrate. RNA was hybridised with CA4H isolated from *T. aestivum* (CYP73.301). In each case, 40µg total RNA was loaded into each lane .

transcript levels slowly abate back to a level comparable to that of the control where no 2,4-D had been introduced to seedlings (at time 0 h)

Transcript induction of CYP73.301 (CA4H), with the addition of 500 μM 2,4-D to the herbicide-resistant biotype, SLR31, seedlings (Figure 3.4 C), showed a similar time course expression to that of VLR1, however, the elevation of the CA4H transcript was not as high as seen with VLR1.   A maximum in the CA4H (CYP73.301) transcript expression level was seen at 3 h (~2-fold) after which CA4H levels began to decline to the 24 h time point. A slight increase in CYP73.301 transcript expression (~1.5-fold) was then seen from 36 h to 60 h.

The addition of 500 μM clofibrate to both VLR1 (Figure 3.4 B) and SLR31 (Figure 3.4 D) seedlings caused a similar time course expression of the CYP73.301 (CA4H) transcript to that observed on addition of 2,4-D.   However, the elevation of the CA4H transcript was not as high as seen with the addition of 2,4-D and the maximum level of transcript expression was reached at 6 h rather than at 3 h.   An increase in expressed transcript level of CA4H at 3 h (~3-fold) and a maximum at 6 h (~8-fold) after addition of the clofibrate to VLR1 seedlings was evident. As time progressed, expression of the CA4H transcript declined in *L. rigidum* seedlings after 6 h with a slight increase seen at 48 h (~5-fold). The expression of the CA4H transcript never returned to the equivalent of the basal level at time 0 even 60 h after addition of either 2,4-D or clofibrate to the seedlings.

The biotype SLR31 (Figure 3.4 D) also showed a maximum CA4H transcript expression level at 6 h (~5-fold).   Levels of CA4H transcript did not decline as quickly as that seen in VLR1 but remained elevated as long as 24 h (~4-fold).   Only after 24 h did CA4H transcript levels begin to subside but again not to the equivalent of the basal level at time 0 even 60 h after addition of clofibrate to the SLR31 seedlings.

3.3.3.2   Northern Blot Analysis of LrCYP (*L. rigidum*)

Figure 3.5 shows the response of induced and uninduced seedlings of herbicide resistant and sensitive biotypes using 2 week old seedlings (with two fully expanded leaves).   The probe LrCYP hybridised to a band of approximately 1800 nucleotides in length.   A very low constitutive level of LrCYP transcript was present in both herbicide -sensitive and -resistant *L. rigidum* seedlings prior to any treatment with chemical inducers.   The herbicide-susceptible biotype VLR1 (Figure 3.5 A) showed only a slight increase (~2-fold) in the LrCYP transcript expression 3 h following addition of 500 µM 2,4-D to the seedlings.   This signal remained elevated until 6 h following treatment, after which expression of LrCYP transcript decreased back to the level seen at 0 h.

In the herbicide-resistant biotype, SLR31 (Figure 3.5 C), again only a slight increase in the level of LrCYP transcript was seen at 6 h (~1.5-fold) following addition of 500 µM 2,4-D to the seedlings.   After this time the level of expression declined back to that seen at 0 h.

The addition of 500 µM clofibrate to both VLR1 (Figure 3.5 B) and SLR31 (Figure 3.5 D) seedlings showed a similar time course expression of the LrCYP transcript to that seen with the addition of 2,4-D.   An increase in LrCYP transcript expression at 3 h and 6 h after addition of clofibrate to the seedlings was evident with a return to basal levels of expression seen after 6 h.

Time (h)

0   3   6 12 24 36 48 60

A. VLR1 + 2,4-D

B. VLR1 + clofibrate

C. SLR31 + 2,4-D
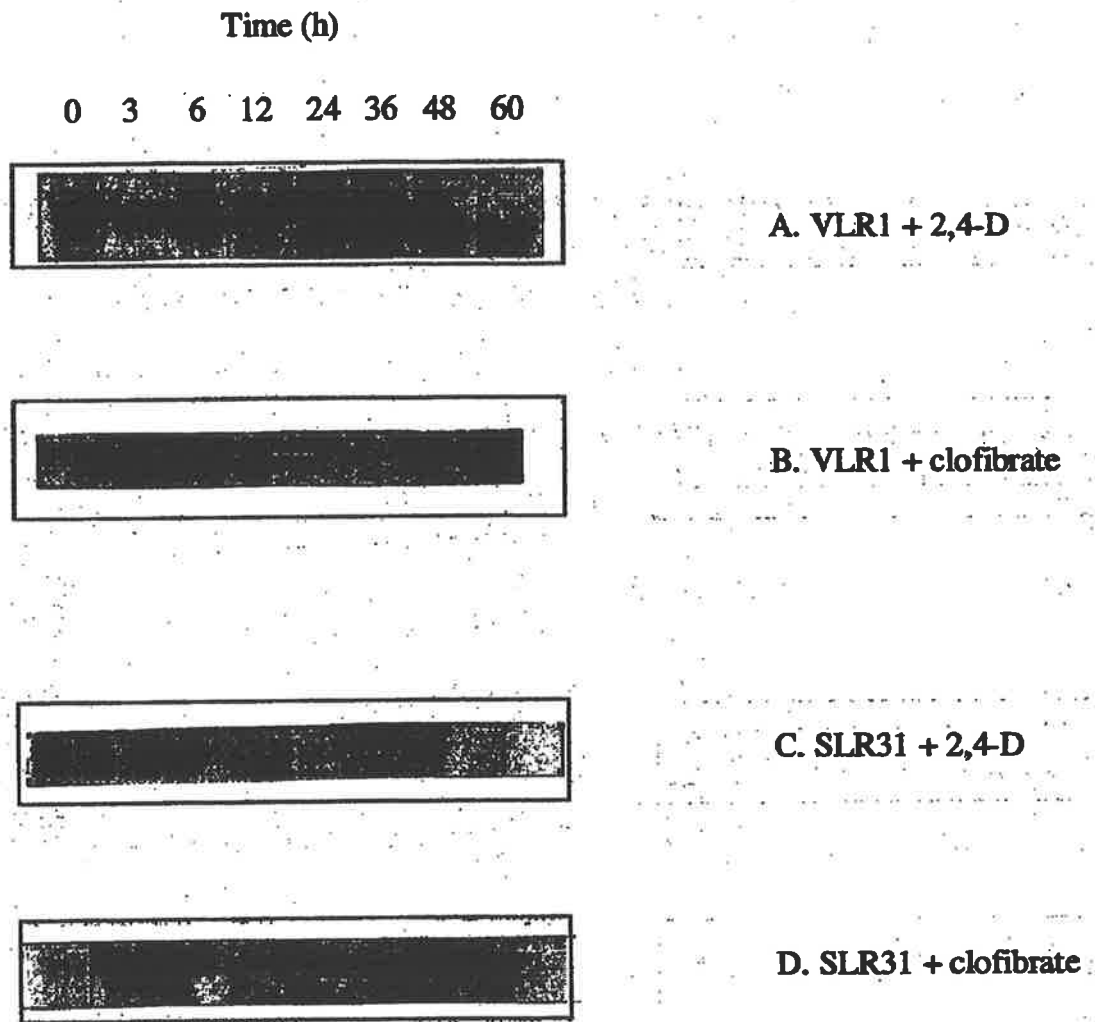
D. SLR31 + clofibrate

Figure 3.5 Northern blot analysis of LrCYP expression over 60h in the *L. rigidum* herbicide

resistant biotype SLR31 and −susceptible biotype VLR1 with the addition of the chemical

inducers 2,4-D and clofibrate. RNA was hybridised with LrCYP isolated from *L. rigidum*. In

each case 40μg total RNA was loaded into each lane.

3.3   Discussion

3.4.1   Chlorsulfuron Metabolism

As reported by Burnet and coworkers (1994), the herbicide-resistant biotype, VLR69, is more than 20 times less sensitive to chlorsulfuron than the herbicide-susceptible biotype, VLR1.   Given that only 4% of the VLR69 population exhibits an insensitive ALS (Burnet, 1992) an alternate mechanism obviously confers resistance to chlorsulfuron in the bulk of the population.   Seedlings of the herbicide-resistant VLR69 population exhibited the capacity to metabolise chlorsulfuron at a faster rate than susceptible VLR1 population. Absorption of chlorsulfuron, by the cut-shoot technique, for both biotypes was similar as indicated by the amount of radioactivity recovered, indicating that enhanced metabolism contributes to the mechanism of resistance in VLR69.   The metabolites observed with VLR69 are similar to those HPLC profiles shown for susceptible, VLR1 and resistant, SLR31, biotypes performed by Christopher and coworkers (1991).

Ethanol-inducible forms of cytochrome P450 monooxygenases are found in rabbits, rats, humans and plants.   On addition of EtOH, an increase in the amount of CYP2E1 protein is seen without the corresponding increase in the mRNA level (Koop *et al.*, 1985).   EtOH treatment of *Z. mays*, *T. aestivum* and *S. bicolor* seedlings has resulted in the increased metabolism of the herbicides prosulfuron (CGA-152005) and metolachlor (Potter *et al.*, 1995; Frear & Swanson, 1996; Moreland *et al.*, 1996).   EtOH-induced etiolated *Z. mays* seedlings showed an increase in mRNA levels of the subfamily CYP81A in both the roots and shoots (Potter *et al.*, 1995).   CA4H is induced following 300 mM EtOH after 72 h and to a lesser extent with isopropanol and methanol (Reichhart *et al.*, 1979).   In this study, the corresponding increase in mRNA transcript expression levels, with the addition of EtOH, was not studied in the chlorsulfuron-resistant biotype, VLR69.

3.4.2 The Effect of Clofibrate on *L. rigidum* Seedlings

Severe necrosis of leaf tissues was observed following the prolonged incubation of *L. rigidum* seedlings with 500 µM clofibrate. Seedlings became shrivelled and yellowed in comparison to untreated seedlings. This has been previously observed in leaves of *P. sativum* (Palma *et al.*, 1991), where necrosis of leaf tissue and alterations in cell ultrastructure, particularly in cell compartmentation with breakage of membranes and disorganisation of chloroplast and mitochondrial structures, was observed following prolonged incubation of leaf tissue with 1 mM clofibrate. These changes may produce cytoplasmic autodegradation with the release of enzymes normally present in vacuoles and an increase in activated oxygen species, particularly hydrogen peroxide (H_2O_2) and O_2^- radicals. The number of both peroxisomes and mitochondria are also seen to increase in *P. sativum* leaves following incubation with 1 mM clofibrate (Palma *et al.*, 1991). A concomitant inhibition of catalase and Mn-superoxide dismutase (Mn-SOD), the main peroxisomal enzymatic defenses against H_2O_2 and O_2^- and an increase in the activity of acyl-CoA oxidase, a key enzyme in fatty acid β-oxidation, is also seen (Palma *et al.*, 1991).

3.3.3 Diclofop-methyl Metabolism

The results presented in this chapter show that pretreatment with 500 µM of either 2,4-D or clofibrate exerts a synergistic effect on the metabolism of the herbicide diclofop-methyl in seedlings of both herbicide-resistant (SLR31) and herbicide-susceptible (VLR1) biotypes of *L. rigidum* over a 24 h period. Without prior treatment the resistant biotype (SLR31) was able to convert the herbicidally-active diclofop acid to polar metabolites 1.3-fold faster than the susceptible biotype (VLR1). The decline in diclofop acid was accompanied by an increase in polar metabolites in both resistant and susceptible biotypes, although to a greater extent in the herbicide-resistant biotype, SLR31. These results are consistent with those previously reported by Preston & Powles (1998) and in resistant biotypes of *Avena sterilis* (Maneechote *et al.*, 1997). Pretreatment of seedlings with 2,4-D demonstrated that

the herbicide-susceptible biotype (VLR1) increased diclofop acid metabolism 1.5-fold while the herbicide-resistant biotype (SLR31) showed a 1.2-fold increase in metabolite production. Clofibrate pretreatment exerted a similar effect to that of 2,4-D with the susceptible biotype (VLR1) metabolising diclofop acid 1.5-fold faster and the resistant biotype (SLR31) showed a 1.3-fold increase in metabolite production. It is interesting to note that the inducers 2,4-D and clofibrate exert a greater effect on the susceptible biotype VLR1 than the resistant biotype SLR31 in the metabolism of diclofop-methyl. The addition of EtOH as an inducer for chlorsulfuron metabolism exerts a greater effect on the resistant biotype VLR69 than seen in the susceptible biotype VLR1.

3.4.4 Transcript Expression of Cytochrome P450 Monooxygenases

Previous evidence indicates that aryl hydroxylation of diclofop-methyl is most likely mediated by a cytochrome P450 monooxygenase (Gaillarden et al., 1985; Ortiz de Montellano & Reich, 1986; Cole & Owen, 1987; Rademacher et al., 1987; McFadden et al., 1989; Menendez & De Prado, 1996; Maneechote et al., 1997). Investigations have shown that both 2,4-D and clofibrate induce the metabolism of diclofop-methyl in resistant and susceptible seedlings of L. rigidum. One aim of this study was to investigate the inducible effect of these two compounds on transcript expression levels of three identified cytochrome P450 monooxygenases in L. rigidum.

The chemical structure of both clofibrate and 2,4-D are similar, both sharing a halogen-substituted phenoxy ring. 2,4-D is a potent, synthetic plant hormone of the auxin family, also an effective, inexpensive herbicide used primarily for post-emergent annual and perennial broadleaf weed control in cereals (Adele et al., 1981). It is believed to mimic the function of the intrinsic auxin, indole-3-acetic acid, on auxin receptors of these plants. High concentrations of auxins are toxic to many dicotyledonous plants but not monocotyledonous plants, which are naturally tolerant to 2,4-D (Adele et al., 1981). 2,4-D

metabolism is relatively rapid in non-dormant plant tissue and undergoes cleavage of the aliphatic side chain, ring hydroxylation and conjugate formation to produce 2,4-dichlorophenol and glycoxylate. The structurally related compound, clofibrate, is a hypolipidemic drug known to induce peroxisome proliferation in mammalian liver (Reddy *et al.*, 1982). This response is characterised by the increased activity of several peroxisomal enzymes involved in ω-oxidation of fatty acids (Sharma *et al.*, 1988). In Leguminoseae, clofibrate selectively induces P450-dependent fatty acid ω-hydroxylase activities (Aoyama *et al.*, 1990). It has been demonstrated in *H. tuberosus*, *Glycine max* L. and *V. sativa* seedlings that both 2,4-D and clofibrate specifically induce the activity of the cytochrome P450 monooxygenase, lauric acid ω-hydroxylase (Salaün *et al.*, 1986).

CA4H is expressed constitutively in *L. rigidum* seedlings. On exposure to either 2,4-D or clofibrate, a sharp increase is observed in transcript levels of this gene as soon as 3 h post-treatment. The increased level of transcipt expression only remained for a short period of time and by 6 h post-treatment, transcript levels began to abate. This rapid rise in transcript expression has also been observed in *P. sativum*, where Persans & Schuler (1996) demonstrated that both CYP73A9 and CYP82 transcripts accumulate to their maximal level 3 h after wounding. In contrast, *H. tuberosus* tubers, aged on 1 mM 2,4-D, show that CA4H transcript is stimulated 85% after 48 h and 200% after 72 h. Cytochrome P450 content is increased by 70% and 55% after 48 h and 72 h of ageing, respectively (Salaün *et al.*, 1986). In *V. sativa* seedlings, incubated on 2 mM clofibrate for 48 h, CA4H transcript expression is stimulated only 1.5-fold whereas the laurate ω-hydroxylase is enhanced 30-fold (Salaün *et al.*, 1986). In *Zinnia elegans*, substantial induction with 1-naphthaleneacetic acid (NAA) and benzyladenine (BA) or onset of lignification resulted in an increase in the transcript level of CA4H at 48 h and 68 h (Ye, 1996). Little or no induction in the level of transcript of this gene was observed before 48 h. Pre-treatment of *T. aestivum* seedlings with either diclofop or 2,4-D has not been seen to correlate with an increase of cytochrome

P450 content (McFadden *et al.*, 1989). Therefore, an increase in herbicide metabolism is not always correlated with an increase in transcript levels of general cytochrome P450 levels (Frear *et al.*, 1991).

The putative cytochrome P450 monooxygenase (LrCYP), isolated and sequenced from *L. rigidum* (see Chapter 5), shows a high degree of sequence similarity to other characterised plant cytochrome P450 monooxygenases belonging to the CYP71C family. Northern blot analysis has shown that transcript expression of LrCYP increases within 3 h of treatment of seedlings with the chemical inducers 2,4-D and clofibrate. The level of transcript expression remained high for up to 6 h, after which levels reduced back to the basal level equivalent to time 0 h when the plants had not been exposed to the inducers.

These findings need to be carefully evaluated. Exogenous inducers may result in marked overexpression of specific enzymes. Inducers have been shown to stimulate some biosynthetic pathways strongly, leading to various secondary metabolites but, in spite of interest in one particular activity, these inducers may have secondary effects that may complicate the analysis of results. For instance, these compounds may activate genes other than their normal targets, or may have pleiotropic effects. It is possible that LrCYP may be involved in the metabolism of herbicides, such as diclofop-methyl, that are detoxified at a faster rate than normal with the addition of the chemical inducers clofibrate or 2,4-D. However, the elevated transcript expression of LrCYP following the addition of these chemical inducers to *L. rigidum* seedlings may also indicate a secondary induction effect and this gene may not be involved in herbicide metabolism at all. A further problem with induction studies in plants is that of feeding relatively water-insoluble organic compounds through the roots. This has been overcome in the past by slicing plant tissue (Reichhart *et al.*, 1979) but the act of mechanical wounding has also been shown to substantially induce certain cytochrome P450 isoforms (Rich & Lamb, 1977). More precise experimentation is

possible once a gene encoding an enzyme involved in the biosynthetic pathway concerned becomes available. Stable transformation using a gene normally present in the host organism can be expected to alter the amount of gene product in the host in a concomitant effect on the rate of the corresponding biosynthetic step. This would enable the actual function of the LrCYP gene to be determined and it's possible role in herbicide metabolism to be evaluated.

The mechanism(s) by which 2,4-D or clofibrate may trigger elevated CA4H and LrCYP trancript expression levels remains to be elucidated. To date, the knowledge of the mechanism of induction by clofibrate in plants is based on evidence obtained with mammals. Clofibrate is a hypolipidemic drug that is known to induce peroxisome proliferation in mammalian liver (Reddy et al., 1982). This response is characterised by the increased activity of several peroxisomal enzymes involved in ω-oxidation of fatty acids (Sharma et al., 1988). In addition, exposure to peroxisome proliferators leads to an increase in hepatic smooth endoplasmic reticulum, mitochondria and the induction of lauric acid ω-hydroxylase activity catalysed by CYP4A1 (Gibson et al., 1982). In rat liver, clofibrate increases the transcription rate of CYP4A1 gene within 1 h, followed by a large increase in the corresponding mRNA, protein and lauric acid hydroxylase activity (Hardwick et al., 1987). Similarly, peroxisome proliferators rapidly increase transcription rates of the genes for fatty acyl-CoA oxidase (ACO) and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase, components of the peroxisomal fatty acid oxidation pathway (Reddy et al., 1986).

It is known that transcriptional induction of the mammalian CYP4A subfamily of enzymes, which generally catalyse the ω-hydroxylation of medium and long chain fatty acids, by peroxisome proliferators or fatty acids is mediated by a peroxisome proliferator-activated α receptor (PPARα; Hardwick et al., 1987; Keller et al., 1993). PPARs are members of

the steroid nuclear hormone receptor family that, in conjunction with retinoid X receptor α (RXRα), regulate transcriptional expression of genes controlling fatty acid synthesis, storage and catabolism, by binding as a heterodimer to the peroxisome proliferator response element (PPRE; Isseman & Green, 1990; Keller *et al.*, 1993; Johnson *et al.*, 1996). These receptors recognise the DNA response sequence AGGTCA and accordingly possess the same P-box amino acid sequence in the first zinc finger of their DNA-binding domain (Wahli & Martinez, 1991). The resulting protein/DNA complex activates transcription of these genes following a variety of stimuli, including peroxisome proliferators and elevated concentrations of endogenous fatty acids (Johnson *et al.*, 1996). Targeted disruption of the mouse PPAR gene has been shown to prevent the induction of CYP4A enzymes and peroxisome proliferation *in vivo* when animals are treated with peroxisome proliferators (Lee *et al.*, 1995). Evidence for the specificity of ligand binding has not been determined. Several isoforms of PPARs have been cloned from animals (Isseman & Green, 1990; Dreyer *et al.*, 1992; Göttlicher *et al.*, 1992; Zhu *et al.*, 1993).

The cytochrome P450 monooxygenases, CYP102 from the prokaryote, *Bacillus megaterium* and CYP52 from the yeast, *Candida maltosa*, have both been shown to be strongly induced by peroxisome proliferators (English *et al.*, 1994; Ohtomo *et al.*, 1996). CYP102 is identified as a fatty acid hydroxylase and exhibits most sequence identity with members of the mammalian CYP4 subfamily. Although a sequence similar to that of the mammalian PPRE has not been found in either system, they do contain direct repeated sequences in the promoter region or operator sequence required for the activation of genes (English *et al.*, 1994; Ohtomo *et al.*, 1996).

The difference in the level of expression of these genes is a result of the promoters that directly regulate the genes. The inducible activity of clofibrate in plants appears analogous to that in mammals, therefore, a receptor, similar to that found to regulate the CYP4A

subfamily of enzymes, may also be present in plants. Common *cis*-acting enhancer-like box elements in the promoters of many of these genes are recognised by distinct DNA-binding, *trans*-acting proteins required to regulate transcription (reviewed in Rushton & Somssich, 1998). Some of these, such as ethylene-responsive element binding protein (EREBP) and WRKY protein, appear unique to plants, whereas others, including bZIP and MYB proteins, have counterparts in animals (Rushton & Somssich, 1998). The GCC Box (AGCCGCC) is found in the promoter regions of many pathogen-responsive genes and a number of proteins that bind to GCC Boxes have been isolated. These proteins have been identified as members of the EREBP family of DNA-binding proteins (Ohme-Takagi & Shinshi, 1995; Büttner & Singh, 1997). WRKY proteins are defined by the presence of the WRKY domain (containing the amino acid sequence WRKYGQK) conserved in all WRKY domains so far analysed (Rushton *et al.*, 1996). W Boxes (TTGACC and TGAC-N$_X$-GTCA) have been identified as binding sites for the WRKY family of DNA-binding proteins, while G Boxes (CACGTG) function during regulation of diverse genes by environmental cues (such as light, UV radiation and wounding). G Boxes are members of the family of ACGT-containing *cis*-acting elements. However, it is still unclear how cytochrome P450 monooxygenase transcript expression in plants is controlled. Until a promoter sequence can be isolated that is directly linked to gene activation we can only speculate on the method of induction by these two chemical inducers (2,4-D and clofibrate), as well as others that cause an increase in cytochrome P450 monooxygenase expression levels.

Alternate mechanisms that can play a fundamental role in the regulation of gene expression in plants and other eukaryotes include the control of mRNA stability. While transcript expression may be a result of increased or decreased transcriptional activity of a given gene, it may also be a result of the gene's mRNA stability. Plants have the ability to recognize and target specific transcripts for rapid decay from among the majority of relatively stable

mRNAs present within cells. The balance between mRNA degradation and synthesis determines the level of mRNAs in cells. Control can be influenced by the basal mRNA decay machinery, sequence-specific decay elements, and regulatory factors that respond to various stimuli. Sequences rich in A/U can contribute to mRNA stability in plants. In mammalian cells, sequences with a high A/U content and multiple AUUUA motifs have been shown to cause mRNA instability when present in the 3′ untranslated regions of several transcripts. An upto 6-fold induction of CYP2E1, on the addition of EtOH or acetone to rats, can be seen with no evidence of an increase in the corresponnding mRNA (Koop et al., 1985). In this instance, induction is not due to an increase in CYP2E1 mRNA or increased mRNA synthesis (Song et al., 1987) but rather degradation of the CYP2E1 transcript is markedly altered (Song et al., 1989). Abolition of the rapid degradation compnent of the CYP2E1 turnover cycle indicates that acetone, also metabolised by CYP2E1, may be a substrate-induced enzyme stabilizer (Song et al., 1989). The mechanism of this stabilization appears to be through ligand-mediated protection from phosphorylation, which otherwise leads to denaturation and degradation of the CYP2E1 protein (Song et al., 1989). Elucidation whether mRNA expression of LrCYP is regulated transcriptionally or post-transcriptionally in this instance is yet to be determined.

The best known agricultural use of chemical inducers of cytochrome P450 monooxygenase activity is found in the use of safeners. Safeners (or antidotes) specifically act to protect crops against the otherwise deleterious effects of herbicides, without affecting weed control efficacy of the herbicide. Increased herbicide tolerance conferred by safeners in crops, either applied to the seed before planting or used as a spray mixture with the herbicide, is exploited in agricultural systems for greater selective weed control. Most safeners are structurally homologous to herbicides and are believed to stimulate enhanced metabolism of herbicides by cytochrome P450 monooxygenases and particularly glutathione

S-transferases (GST; E.C. 2.5.1.18; reviewed in Farago *et al.*, 1994; De Veylder *et al.*, 1997). However, the actual mechanisms for safener-inducible gene expression remain unknown.

Chapter 4

Inhibition of Herbicide
Metabolism
by
Mechanism-based
Inhibitors

Chapter 4

Inhibition of Cytochrome P450 Monooxygenases
by Mechanism-based Inhibitors

4.1   Introduction

It is virtually impossible to find an adequate and reliable solution to herbicide resistance of weed species in the field until the ensuing mechanism(s) of resistance have been understood. Understanding some of the more important processes, particularly those that arise from abnormalities of enzymatic reactions involved, is a crucial part of comprehending the physiological role of enzymes in detoxification of herbicides in plants. Interest in the metabolic processes that render herbicides inactive has led to the evaluation of possible inhibitors that target the potential enzyme(s), in this case, cytochrome P450 monooxygenases, involved in metabolism of xenobiotics in higher plants (Jablonkai & Hulesch, 1996).

Inhibitors are substances that act to decrease the rate of an enzyme-catalysed reaction. Many effective compounds act by inhibiting key enzymatic processes and may be used to help elucidate metabolic pathways by causing accumulation of biosynthetic intermediates and decreasing pathway end product(s). However, a consequence of this may be the disclosure of parallel pathways that perform the same reaction as the inhibited enzyme pathway. Detailed investigations of the binding characteristics of various inhibitors that target the same site as a natural substrate can generate useful information regarding factors governing the binding of that substrate. Enzyme inhibitors may be divided into two main

classes, reversible and irreversible, depending on the manner in which they interact with the target enzyme (Sandler & Smith, 1989). Reversible inhibitors bind to an enzyme in a reversible manner and can be removed to restore full enzyme activity, whereas suicide inhibitors may undergo a structural change during the course of catalysis that means the inhibitor either cannot be removed or is difficult to remove.

4.1.1   Reversible Inhibition

These inhibitors often closely resemble the substrate whose reactions they inhibit and, therefore, may compete for the same binding site on the enzyme (Sandler & Smith, 1989). The enzyme-bound inhibitor then either lacks an appropriate reactive group or is held in an unsuitable position with respect to the catalytic site of the enzyme for a reaction to take place. In either case a dead-end complex is formed and the inhibitor must disassociate from the enzyme and be replaced by substrate before a reaction can occur at that site. The effect of a competitive inhibitor depends on the inhibitor concentration, substrate concentration and relative affinities of both compounds for the enzyme (Sandler & Smith, 1989). In general, if substrate concentration is low, the inhibitor will compete favourably with the substrate for the binding site on the enzyme and the degree of inhibition will be high. However, if substrate concentration is high the inhibitor will be less successful in competing with the substrate for available sites and the degree of inhibition will be less marked.

4.1.2   Irreversible Inhibition

Enzyme-activated irreversible inhibitors are among the most specifically acting chemical compounds (Rando, 1974). These inhibitors are relatively unreactive molecules, some with sufficient structural similarity to the substrate so that they interact with the active site of the target enzyme. Other inhibitors may be structurally different to the substrate and act in a site away from the actual enzyme target site. In the case of cytochrome P450

monooxygenases, the enzyme-activated irreversible inhibitors are converted, at the active site through the catalytic action of the enzyme, to a reactive moiety that can then form a covalent bond with a functional group at the active site to their prosthetic heme and inactivate the target enzyme (Rando, 1974; Abeles & Maycock, 1976). In essence, the specific catalytic mechanism of cytochrome P450 monooxygenases enables them to promote their own destruction during the metabolism of these suicide substrates.

Simple kinetic experiments may demonstrate that a compound may be a potential suicide inhibitor. Loss of enzyme activity must be shown as a time-dependent, first-order process (Abeles & Maycock, 1976). Showing that the loss of enzyme activity at constant inhibitor concentrations is first-order provides evidence that inactivation occurs before the activator is released from the enzyme, a fundamental property of suicide inhibition (Rando, 1974; Ortiz de Montellano & Mico, 1981). Therefore, the specificity of these inhibitors is not only based on their similarity to the substrate, but also on the mechanism of action of the target enzyme and the efficiency and selectivity of inhibitors. Hence their destructive processes, are dependent on their relative affinities for specific cytochrome P450 forms as well as a favourable ratio between rate of inactivation and dissociation constant (Rando, 1974).

For cytochrome P450 monooxygenases, the introduction of double bonds into known substrate molecules can create potential suicide inhibitors (Ortiz de Montellano & Kunze, 1980). Inactivation of plant fatty acid hydroxylases by acetylenic fatty acid analogues with terminal double and triple bonds has been demonstrated (Salaün et al., 1986; 1988; Simon 1988, cited in Durst, 1991; Helvig et al., 1997). IC-LAH, capric acid- and myristic acid-hydroxyalses are all inhibited by 11-dodecynoic acid (11-DDYA; Ortiz de Montellano & Kunze, 1980; Ortiz de Montellano & Reich, 1984; CaJacob & Ortiz de Montellano, 1986; Salaün et al, 1988; Simon, 1988, cited in Durst, 1991; Helvig et al., 1997) containing a

terminal triple acetylenic bond. It is possible that these inhibitors may also react with other enzymes that may have similar active sites and a similar catalytic mechanism. Without further knowledge of these enzymes it would be difficult to find a compound that would discriminate between closely related isoforms.

The effect of three suicide substrates, specifically designed for inhibition of the cytochrome P450 monooxygenase LAH, on the metabolism of diclofop-methyl in the herbicide-resistant *L. rigidum* biotype, SLR31, was examined in this chapter. It was hoped that these suicide substrates would provide the specificity needed to inactivate the potential cytochrome P450 monooxygenase(s) involved in the metabolism of diclofop-methyl within this biotype.

4.2   Materials & Methods

Three established cytochrome P450 monooxygenase suicide substrates were applied to herbicide-resistant *L. rigidum*, biotype SLR31, seedlings. Seedlings were germinated and grown in hydroponic culture as described in 2.2.2 and 2.2.3. The inhibitory properties of these compounds were evaluated by comparing the concentration of parent herbicide remaining and production of metabolites produced between control and treated *L. rigidum* seedlings over a 24 h fixed period. Inhibition treatment of seedlings was performed as in section 2.2.5.1. Extraction of metabolites, subsequent HPLC analysis and LSS were performed as described in sections 2.2.7, 2.2.8 and 2.2.9. Experiments contained two replicates. Data from each replicate were pooled prior to analysis with means and standard deviations calculated for each treatment.

4.3   Results

4.3.1   Diclofop-methyl Metabolism - Control

Radioactivity elution profiles of RP-HPLC analysis for both the *L. rigidum* susceptible biotype, VLR1, and the resistant biotype, SLR31, were comparable. Both profiles showed three major peaks with retention times of 15.40 min for diclofop-methyl, 14.20 min for diclofop acid and approximately 8 and 10 min for metabolites (peak A and peak B, respectively).   Both resistant and susceptible biotypes produced the same pattern of metabolites, based on comparison of HPLC chromatograms (Figure 3.1), indicating that both biotypes contain an equivalent pathway for the metabolism of diclofop-methyl.

4.3.1.1   Resistant vs Susceptible *L. rigidum* Biotypes

Diclofop-methyl metabolism in 12 day old susceptible, VLR1, and resistant, SLR31, *L. rigidum* seedlings is shown is Figure 4.1. De-esterification of diclofop-methyl to the active diclofop acid, was faster in the resistant biotype, SLR31, than the susceptible biotype, VLR1, in which 2-fold more parent herbicide remained after 24 h. Subsequent levels of diclofop acid remained approximately the same in both biotypes. However, the resistant biotype, SLR31, produced 2.4-fold more metabolites than the susceptible biotype, VLR1, over a 24 h period.

4.3.2   Mechanism-based Inhibitors

4.3.2.1   Compound 2

The addition of 2.5 μM compound 2 to susceptible biotype VLR1 seedlings produced a slight decrease in the metabolism of diclofop-methyl when compared to control seedlings over a 24 h period. Diclofop-methyl levels remaining within the seedlings increased by 1.1-fold, diclofop acid levels decreased 1.05-fold and the production of metabolites
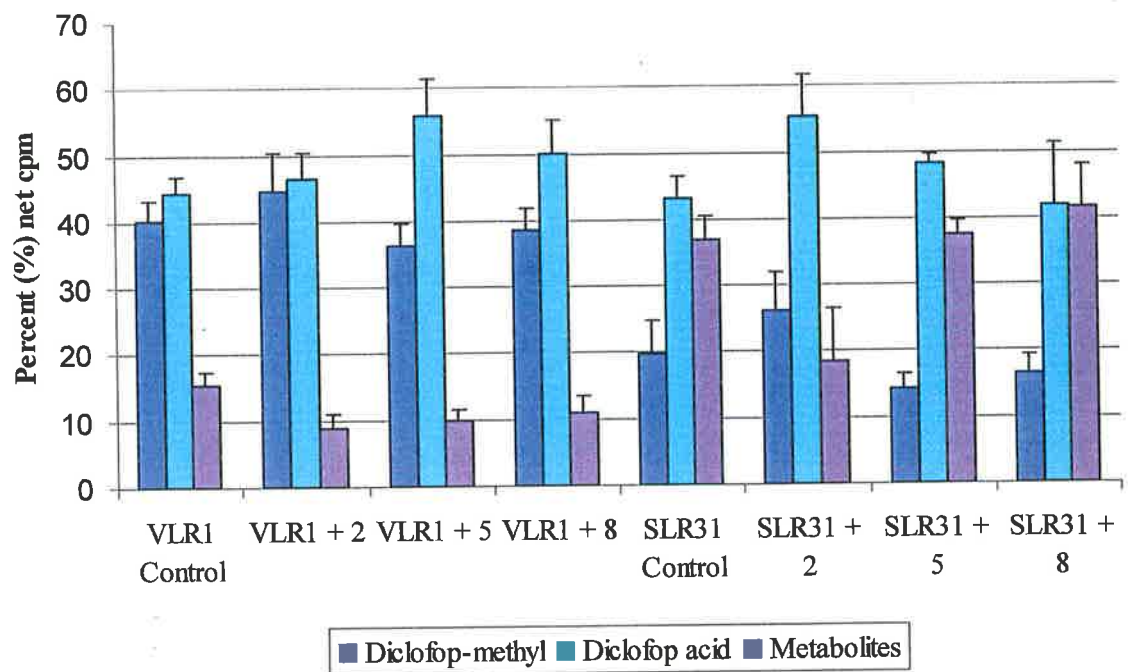
Figure 4.1 The effect of suicide inhibitors 2, 5 and 8 on diclofop-methyl metabolism *in vivo* in seedlings of the *L. rigidum* herbicide-susceptible biotype, VLR1, and the herbicide-resistant biotype, SLR31, over 24 h.

decreased by 50%. The addition of 2.5 μM compound 2 to the resistant biotype, SLR31, seedlings also decreased the production of diclofop-methyl metabolites when compared to untreated seedlings over a 24 h period. Diclofop-methyl levels remaining within the seedlings increased by 1.3-fold, diclofop acid levels increased by 1.3-fold and the production of metabolites decreased by 50% (Figure 4.1).

4.3.2.2   Compound 5

The addition of 2.5 μM compound 5 to the herbicide-susceptible seedlings, VLR1, resulted in a decrease in the level of diclofop methyl metabolites produced over a 24 h period when compared to the control seedlings. Diclofop-methyl levels were 0.1-fold lower, diclofop acid levels increased by 1.25-fold and the production of metabolites decreased 0.35-fold. The addition of 2.5 μM compound 5 to the herbicide-resistant seedlings, SLR31, resulted in diclofop-methyl levels decreasing 0.25-fold, diclofop acid levels increased 1.1-fold, and the production of metabolites remained equivalent to those produced in the control seedlings (Figure 4.1).

4.3.2.3   Compound 8

The addition of 2.5 μM compound 8 to the herbicide-susceptible seedlings, VLR1, resulted in very little change in the level of diclofop-methyl in comparison to the control. Diclofop acid levels increased 1.15-fold, and the production of metabolites decreased by 0.3-fold on comparison to levels in control seedlings. The addition of 2.5 μM compound 8 to the herbicide-resistant seedlings, SLR31, resulted in diclofop-methyl levels decreasing 0.85-fold, diclofop acid levels decreased 0.05-fold, and the production of metabolites increased by 1.1-fold on comparison to levels in control seedlings (Figure 4.1).

4.4   Discussion

The use of specific reversible and irreversible inhibitors plays an important part in the elucidation of the catalytic mechanisms of cytochrome P450 monooxygenases. This may potentially lead to the development of specific and controllable enzyme inhibitors and allow a study of the metabolic consequences of a specific enzyme block within a determined biochemical pathway.

The implication of cytochrome P450 monooxygenases in herbicide metabolism in *L. rigidum* has been previously demonstrated using the known cytochrome P450 inhibitors, ABT, PBO and tetcyclasis. Addition of these compounds 24 h prior to the addition of some herbicides to *L. rigidum* seedlings has shown a decrease in the rate of herbicide metabolism with more parent herbicide detected and less metabolites being produced (Burnet *et al.*, 1993a; Christopher *et al.*, 1994; Preston *et al*, 1996).

Tetcyclasis is a plant growth regulator and a potential inhibitor of many plant cytochrome P450 monooxygenase-mediated reactions. It has been shown to strongly inhibit bentazon, lauric acid, and primisulfuron metabolism in *Z. mays* and diclofop-methyl metabolism in *T. aestivum* (McFadden *et al.*, 1989; 1990; Fonné–Pfister & Kruez, 1990; Fonné-Pfister *et al.*, 1990; Mougin *et al.*, 1990; Moreland *et al.*, 1993). Chlorotoluron metabolism is also inhibited on the addition of tetcyclasis but at varying levels. In *Z. mays*, metabolism is strongly inhibited while in *T. aestivum* inhibition appears to be only moderate (Cabanne *et al.*, 1987; Fonné–Pfister & Kruez, 1990). In *L. rigidum* seedlings, chlorotoluron metabolism is also inhibited by tetcyclasis but diclofop-methyl metabolism is not (Preston *et al.*, 1996).

PBO is a commonly used insecticide synergist and is a strong inhibitor of many plant cytochrome P450 monooxygenase-mediated reactions. It has been demonstrated to strongly inhibit the oxidation of lauric acid and moderately inhibit bentazon metabolism in sorghum microsomes (Moreland *et al.*, 1993). In *T. aestivum*, PBO strongly inhibits triasulfuron metabolism (Frear *et al.*, 1991), moderately inhibits chlorotoluron metabolism (Gaillardon *et al.*, 1985; Mougin *et al.*, 1990), and only slightly inhibits the metabolism of diclofop-methyl (Frear *et al.*, 1991). Chlorotoluron metabolism is also inhibited by the addition of PBO to *L. rigidum* seedlings (Burnet *et al.*, 1993a; Christopher *et al.*, 1994).

ABT has been implicated as a specific inhibitor for CA4H activity in *H. tuberosus* by autocatalytically inactivating the enzyme (Reichhart *et al.*, 1982). However, in *Z. mays*, ABT only moderately inhibits CA4H activity (Moreland et al., 1993). Inhibition of diclofop-methyl metabolism by ABT is moderate in *T. aestivum* (McFadden et al., 1989), while triasulfuron metabolism is only weakly inhibited in *T. aestivum* but moderately inhibited in *Z. mays* (Frear *et al.*, 1991; Moreland *et al.*, 1993), and chlorotoluron metabolism is strongly inhibited in *T. aestivum* (Gaillardon *et al.*, 1985; Cabanne *et al.*, 1987; Mougin *et al.*, 1990). In *L. rigidum* chlorotoluron, diclofop-methyl and simizine metabolism are all strongly inhibited by ABT, whereas chlorsulfuron metabolism is only weakly inhibited (Burnet *et al.*, 1993a; 1993b; Christopher *et al.*, 1994; Preston *et al.*, 1996).

Although ABT, PBO and tetcyclasis have been shown to inhibit known cytochrome P450 monooxygenase functions in both mammalian and plant systems (Ortiz de Montellano & Mathews, 1981; Reichhart *et al.*, 1982; Gaillardon *et al.*, 1985; Cabanne *et al.*, 1987; Canivenc *et al.*, 1989; McFadden *et al.*, 1989; 1990; Jablonkai & Hulesch, 1996; Leah *et al.*, 1997) they are not regarded as specific inhibitors for these enzymes. These chemicals act as broad range inhibitors with varying degrees of specificity on a vast range of

cytochrome P450 monooxygenases in plants. Inhibition of metabolic function, following addition of these chemicals, may indicate that a cytochrome P450 monooxygenase is directly involved in that function or it may indicate that an alternate enzyme in the pathway has been inhibited rather than the target enzyme being studied. Differential inhibitory response patterns between substrates obtained with these known cytochrome P450 monooxygenase indicates that these reactions may be catalysed by different cytochrome P450 isoforms.

The three suicide substrates used in this study were specifically designed for inhibition of the cytochrome P450 monooxygenase LAH (Helvig *et al.*, 1997; Salaün & Benveniste, 1998; Salaün *et al.*, 1986; 1988). Each of the compounds, 2, 5 and 8, has terminal double or triple bond introduced into the substrate molecule to create a potential suicide inhibitor (Appendix C). This technique has been previously demonstrated with the inactivation of plant fatty acid hydroxylases by acetylenic fatty acid analogues with terminal double and triple bonds (Salaün *et al.*, 1986; 1988; Simon 1988, cited in Durst, 1991; Helvig *et al.*, 1997; Forthoffer, 1998). Selectivity of suicide substrates for cytochrome P450 isozymes is determined by protein constraints on the binding and orientation of substrates in their active sites. The compound 9-decanoic acid (10 μM) has beeen demonstrated as an effective, irreversible inhibitor of microsomal IC-LAH (Salaün *et al.*, 1986) with a reduction in LAH activity of 8%. IC-LAH, capric acid- and myristic acid-hydroxyalses are all inhibited upto 22% by 10 μM 11-dodecynoic acid (11-DDYA; Ortiz de Montellano & Kunze, 1980; Ortiz de Montellano & Reich, 1984; CaJacob & Ortiz de Montellano, 1986; Salaün *et al*, 1988; Simon, 1988, cited in Durst, 1991; Helvig *et al.*, 1997) containing a terminal triple acetylenic bond. While 11-dodecenoic acid (10 μM) also inhibits IC-LAH by 65% and CA4H activity by 25% at 100 μM and 75% at 1000 μM (Salaün *et al.*, 1986). IC-LAH from *T. aestivum* has been implicated in the hydroxylation of diclofop-methyl in addition to fatty acid substrates (Zimmerlin & Durst, 1992; Zimmerlin *et al.*, 1992;

Forthoffer, 1998). Therefore, the enzyme that is responsible for diclofop-methyl metabolism in *L. rigidum* may also have involvement in lauric acid hydroxylation.

The results from this study have shown that diclofop-methyl metabolism is inhibited in *L. rigidum* by varying amounts on addition of all three of these suicide substrates. Compounds 2, 5 and 8 all showed similar inhibition profiles in diclofop-methyl metabolism in both herbicide-resistant and -susceptible biotypes of *L. rigidum*. In most cases, the conversion of diclofop-methyl to diclofop acid was not significantly affected with only small decreases in the amount of remaining parent herbicide detected when compared to control seedlings after 24 h. The exception was the addition of compound 2 to the herbicide-resistant biotype, SLR31, where 1.3-fold more parent herbicide was remaining in the seedlings 24 h post-treatment. Partial inhibition of the de-esterification reaction of diclofop-methyl to diclofop acid is seen with compound 2 in this biotype. Compound 2 also demonstrated 50% inhibition of the metabolism of diclofop acid to non-toxic metabolites in 24 h. Although compound 5 and compound 8 also demonstrated inhibition of diclofop acid to corresponding metabolites in the herbicide-susceptible biotype, VLR1, neither of these compounds was as effective as an inhibitor as compound 2. In contrast, compounds 5 and 8, in the herbicide-resistant biotype, SLR31, demonstrated no effect or a slight increase in the metabolism of diclofop acid to subsequent metabolites. These results indicate that more than one enzyme may be involved in the metabolism of diclofop-methyl in the *L. rigidum* biotypes, VLR1 and SLR31. However, it is possible that these inhibitors may also react with other enzymes that may have similar active sites within the plant.

Suicide inhibitors, with chemical structures similar to those used in this experiment, have previously been tested for their ability to inhibit diclofop-methyl and chlorotoluron metabolism in *T. aestivum* seedlings *in vivo*. The compounds undec-9-yne-1-sulfonic acid

(9-UDYS) with similarity to compound 8, and undec-10-yne-1-sulfonic acid (10-UDYS), with similarity to compound 5, significantly inhibited diclofop-methyl metabolism by reducing the formation of intermediate metabolites and conjugates (Forthoffer *et al.*, 2001). Inhibition of diclofop-methyl metabolism by 9-UDYS and 10-UDYS was approximately 75% and 72%, respectively. Only a weak inhibitory effect was seen in chlorotoluron metabolism with these two inhibitors. A third suicide substrate, undecan-1-sulfonic acid (UDSA), with similarity to compound 2 in this study, only had a weak effect on both diclofop-methyl and chlorotoluron metabolism in *T. aestivum* seedlings (Forthoffer *et al.*, 2001). These findings are in contrast to those found in this study, where compound 2 showed the largest inhibitory effect (50%) on diclofop-methyl metabolism in *L. rigidum*.

While inhibitor studies may often distinguish between single and multiple cytochrome P450 monooxygenases involved in a given reaction, in this case diclofop-methyl metabolism, they do not indicate the number of different enzymes that may contribute to a single activity. This study shows that diclofop-methyl metabolism can be inhibited by compounds previously shown to inhibit IC-LAH. Therefore, we can predict that this enzyme may in fact be involved in both diclofop-methyl metabolism and possibly fatty acid hydroxylation, as with IC-LAH in *T. aestivum* (Zimmerlin & Durst, 1992; Zimmerlin *et al.*, 1992). Only isolation of this gene and expression in a suitable vector will verify this point.

Efficient suicidal inactivation by acetylenic compounds is dependent on the position of then acetylenic bond in the inhibitor and the regiochemistry of the cytochrome P450 oxidation. Although these three suicide substrates have not shown complete inactivation of diclofop-methyl metabolism, the development of mechanism-based inhibitors is useful in probing for the physiological significance and importance of cytochrome P450 monooxygenases. In *T. aestivum*, LAH and diclofop-methyl hydroxylase activity are

strongly inhibited by the terminal olefin 11-DDNA (Zimmerlin & Durst, 1992; Zimmerlin *et al.*, 1992). However, it is also apparent in this case that inactivation is not complete. The remaining activity of LAH in *T. aestivum* is approximately 55% (Zimmerlin *et al.*, 1992), comparable to that seen in *L. rigidum* following incubation with compound 2. The use of potential suicide substrates to inhibit cytochrome P450 monooxygenase function, specifically in the area of diclofop-methyl metabolism, would confirm and clarify previous work indicating the involvement of an isoform(s) of cytochrome P450 monooxygenase in the metabolism of this herbicide. In *V. sativa*, substrate analogues containing an internal acetylene were ω-hydroxylated by ω-LAH without significant enzyme inactivation (Helvig *et al.*, 1997). Only the methyl end of substrates is accessible to oxidation by *V. sativa* ω-LAH. Therefore, internal double or triple bonds do not affect regioselectivity only kinetic parameters of the hydroxylase. These inhibitors can also provide information about the active sites of enzymes they interact with. They may elucidate the physiological roles of specific enzymes and may ultimately control specific physiological function or malfunction through the development of synergists or antidotes of xenobiotics that are either detoxified or activated by cytochrome P450 monooxygenases. It is not clear how these inhibitory compounds interact with physiological processes, other than herbicide metabolism, within the plant.

Characterisation and identification of cytochrome P450 isoforms in relation to substrate specificity and comparative distribution between species may enable the development and selection of treatments that would be species and isoform specific. Inhibitors could potentially be used to selectively inactivate cytochrome P450 isoforms in critical biosynthetic pathways. For example, inhibition of herbicide metabolic pathways conferring resistance in weedy species would potentially enable preferential destruction of problem species over beneficial crop species.

Chapter 5

Isolation of Cytochrome P450 Monooxygenase from *Lolium rigidum*

Chapter 5

Isolation of Cytochrome P450 Monooxygenase from
Lolium rigidum

5.1   Introduction

Difficulties in the isolation of cytochrome P450 monooxygenases from plant tissues have been encountered in the past. This has been mainly due to low abundance and general instability of these enzymes in plant extracts. Successful purifications have usually been from specialised tissues, such as mesocarp or tubers, where the purified cytochrome P450 monooxygenase was the dominant P450 present in that particular tissue (O'Keefe & Leto, 1989; Gabriac *et al.*, 1991). Isolation strategies for cytochrome P450 monooxygenase genes in *L. rigidum* using techniques such as microsome preparations used by Tijet and coworkers (1999) have proved to be unsuccessful in the past (S. Powles and C. Preston, personal communication). Although CA4H activity in *L. rigidum* microsomes has been measured, herbicide metabolising activity was too low for further analysis or isolation of the cytochrome P450 monooxygenase protein (C. Preston, personal communication). Hallahan and coworkers (1993) noted that levels of spectroscopically-detectable cytochrome P450 levels in membrane preparations from *L. rigidum* were negligible, precluding attempts to purify the protein.

Assays performed with plant microsomes have shown that herbicide-metabolising activities are almost always induced in much larger proportions than the total cytochrome P450 monooxygenase content. This indicates that the detoxification pathways involve P450 isozymes that are relatively minor, and hence difficult to purify compared with

constitutively expressed enzymes (Werck-Reichhart *et al.*, 2000). In the case of *L. rigidum*, a more sensitive means of approach would be required to identify if any such enzymes were present. More recently, the use of molecular biological techniques has aided in the acceleration of discovery and identification of novel cytochrome P450 monooxygenases in plants.

This chapter describes the cloning of cytochrome P450 monooxygenase cDNA clones from *L. rigidum* and possible classification of these genes as compared to other identified cytochrome P450 monooxgenases. Degenerate primers corresponding to highly conserved regions within the heme-binding domain at the 5′-end of all cytochrome P450 monooxygenases were coupled with a non-degenerate primer, complementary to the poly(A)$^+$ tail of RNA transcripts, and reverse transcription-polymerase chain reaction (RT-PCR) was used to amplify potential cytochrome P450 monooxygenase sequences from herbicide-resistant biotypes of *L. rigidum*.

5.2   Materials and Methods

L. rigidum seedlings, germinated and grown in hydroponic culture (Sections 2.2.2 and 2.2.3) and induced by chemical treatment for 24 h (Section 2.2.4), were used to extract total RNA (Section 2.2.10.1) and subsequently to prepare cDNA for RT-PCR and library preparation (Section 2.2.11 and 2.2.12). RT-PCR was performed to isolate partial clones encoding sequences of novel cytochrome P450 species. Following sequence analysis and design of new PCR primers further RT-PCR amplification was performed to obtain larger cDNA clones. Isolated PCR fragments were also used to screen cDNA libraries in an attempt to obtain full-length clones.

5.3 Results

5.3.1 PCR Amplification of a Cytochrome P450 Monooxygenase Fragment from the Herbicide-resistant *L. rigidum* Biotype, SLR31

RT-PCR amplification (Section 2.2.12), using the degenerate Meijer primer (Section 2.1.2) homologous to the highly conserved heme-binding domain at the C-terminus of cytochrome P450 monooxygenases, and oligo $d(T)_{18}$ (Section 2.1.2), was performed on first-strand cDNA prepared from the herbicide-resistant *L. rigidum* biotype, SLR31. Amplified PCR products were separated by agarose gel electrophoresis. PCR products appeared as a diffuse band migrating in the size range of 350 – 450 bp. This 100 bp region was excised from the gel, purified and subcloned into a T-tailed pGem vector (Section 2.1.2). Following antibiotic and colour selection to obtain colonies containing inserts, the plasmids were digested with the restriction enzymes *NcoI* and *PstI* to excise inserts and then separated by agarose gel electrophoresis. Two of the 24 isolated clones contained an insert in the expected size range of 400 – 450 bp. DNA was prepared from these two clones and sequenced using the M13 forward and reverse primers.

Sequence data obtained enabled a prediction of the open reading frame which was subsequently submitted to a Blast search on the GenBank database. Sequence analysis of one independent isolate of the cloned SLR31 PCR products confirmed its identity as a novel cytochrome P450 monooxygenase. The 360 bp fragment encoded 98 amino acid residues from the carboxy-terminal end of the protein and obeyed the consensus **PFGXGXRXCXG** for the highly conserved heme-binding domain in the catalytic site of the Group A plant cytochrome P450 monooxygenases (Durst & O'Keefe, 1995). Figure 5.1 shows the nucleotide and deduced amino acid sequences of the SLR31 cytochrome P450 monooxygenase 3′-end fragment, designated LrCYP. The predicted amino acid

```
                                                                      51
CTG CCG TTT GGG TGG GGG CCG CGG ATC TGT CCA GGC ATA AAC TTC GCG AAT
 L   P   F   G   W   G   P   R   I   C   P   G   I   N   F   A   N
```

```
                                                                      102
ACT GCC GTG GAA TTG ATG CTA GCG AAC CTC GTC TAC CAT TTC GAT TGG GAG
 T   A   V   E   L   M   L   A   N   L   V   Y   H   F   D   W   E
```

```
                                                                      153
CTG CCA GCA AAG ATG AAG GAG GTT GAT ATG AAG GAG GTG TTC AGC TTG TCG
 L   P   A   K   M   K   E   V   D   M   K   E   V   F   S   L   S
```

```
                                                                      204
ATC CGG CGT AAG GAG AAG CTC CTC CTT GTC CCA GTT TCA AGA TCA ATC CCT
 I   R   R   K   E   K   L   L   L   V   P   V   S   R   S   I   P
```

```
                                                                      255
CTC TCC GAT AAG ACA GAA TGA CAA CTA GCC AAC CAC GAA GTA CTA GTA CGA
 L   S   D   K   T   E   *   Q   L   A   N   H   E   V   L   V   R
```

```
                                                                      306
GAT AAG TAC TGT ACT TAC ATT TGG CGT GTT CAA ATT TAA CTA GCC ACT TAT
 D   K   Y   C   T   Y   I   W   R   V   Q   I   *   L   A   T   Y
```

```
                                                                      357
TCG TTC ATA ATG TCA TGA GAA AAT CTG AAT TTT CAA GCA AAA AAA AAA AAA
 A   K   W   G   E   T   G   F   F   C   G   Q   A   K   K   K   K
```

```
AAA
 K
```

Figure 5.1   Nucleotide and deduced amino acid sequences of the putative cytochrome P450 cDNA fragment isolated by RT-PCR of induced *L. rigidum* biotype SLR31. Nucleotides corresponding to the cDNA sequence are shown in uppercase letters with the inferred amino acid sequence indicated below as their single-letter abbreviations. The area underlined indicates the highly conserved heme-binding domain (**PFGXGXRXCXG**) in the catalytic site of the Group A plant cytochrome P450 monooxygenases.

sequence of LrCYP shows high levels of sequence similarity to the identified cytochrome P450 monooxygenase, CYP71C2 (GenBank Accession No. X81829), cDNA sequence from *Z. mays* (Frey *et al.*, 1995).

5.3.2   Sequence Analysis of a *L. rigidum* Cytochrome P450 Monooxygenase

The sequence of the cytochrome P450 fragment, obtained from the herbicide-resistant *L. rigidum* biotype, SLR31, was compared with a number of previously characterised cytochrome P450 monooxygenases belonging to the CYP71 subfamily (Table 1.1). The members of this group each contain the highly conserved FXXGXRXCXG heme-binding motif in their catalytic site near the C-terminus, as do all cytochrome P450 monooxygenases. Figure 5.2 shows details of these regions as well as alignment to the other members of the CYP71C subfamily outlined in Table 1.1.

Sequence similarity of the 3′-end of this gene indicates that it is most likely that LrCYP codes for a cytochrome P450 monooxygenase. The LrCYP 3′-end fragment has over 63% amino acid sequence identity to a member of the CYP71C subfamily.

5.3.3   Screening of cDNA Libraries

The 360bp cloned PCR fragment from SLR31 (LrCYP) was radio-labelled with [α-^{32}P]dCTP and used to screen cDNA libraries constructed from poly(A$^+$) RNA from induced herbicide-resistant biotypes of *L. rigidum* (Section 2.2.11). It was assumed that preparing a cDNA library from plant material grown with cytochrome P450 inducers, known to increase the rate of herbicide metabolism, would potentially increase the level of that cytochrome P450 monooxygenase protein involved in the metabolism of that herbicide within the plant. It was hoped that better representation of these cytochrome P450 monooxygenases would result in these induced libraries than in libraries constructed from non-induced *L. rigidum* plant leaf material.

```
PaCYP71A1    .IPFGAGRRG CPGIAFGISS VEISLANLLY WFNWELPGDL TKE..DLDMS EAVGITVHMK
SmCYP71A2    .LPFGAGRRG CPGSSFAIAV IELALARLVH KFDFALPEGI KPE..DLDMT ETIGITTRRK
SmCYP71A4    .LPFGSGRRG CPGSSFAIAV IELALARLVH KFNFALPKGT KPE..DLDMT ECTGIATRRK
NrCYP71A5    .LPFGAGRRG CPGATFAVAI DELALAKLVH KFDFGLPNGA RME..ELDMS EPSGMTVHKK
NrCYP71A6    .IPFGSGRRG CPGATFAAAI DELALATLVH KFDFKLPNGV RVE..DLDMS EGSGFTIHKK
GmCYP71A10   .IPFGIGRRG CPAMSFGLAS TEYVLANLLY WFNWNMSESG RILMHNIDMS ETNGLTVSKK
ZmCYP71C1    .VPFGAGRRI CAGATFAIAT VEIMLANLIY HFDWEMPAEM ERTGAKVDMS DQFGMTLRRT
ZmCYP71C2    .IPFGSGRRI CPGMNFGFAT MEVMLANLMY HFDWEVPGSG AG....VSME ESFGLTLRRK
ZmCYP71C3    .LPFGSGRRI CPGANFGLAT MEIMLANLMY HFDWEVPNEK EDGCWKVSMD EKFGLMLRRN
ZmCYP71C4    .LAFGSGRRM CPGVHSASAT IEAMLSNLMY RFDWQLPAGM KAE..DVDMT EVFGITVSRK
LrCYP        .LPFGWGPRI CPGINFANTA VELMLANLVY HFDWELPAKM KE....VDMK EVFSLSIRRK
Consensus     FG G R   C          E  L  L     F                   M
```

```
PaCYP71A1    FP........ ........LQ LVAKRHLS*K *LLINPFLCV CDTICFIMYL
SmCYP71A2    LP........ ........LL VVATP..C*E .FFLHIFL.. HLATSVIFFT
SmCYP71A4    SP........ ...:.....LP VVATP..FSG .*LGDFSP.. CL.INQCYQL
NrCYP71A5    SP........ ........LL LLPIPHHAAP .*LI...... .SHRSIIY..
NrCYP71A6    FP........ ........LL VVPTPHACTS .*LLYTIV.. QLYFK*LC..
GmCYP71A10   VP........ ........LH LEPEP..YKT .*
ZmCYP71C1    QK........ ........LY LVPRIPK*VS SSYVAIGNES SSPLLPVALS
ZmCYP71C2    EK........ ........LL LVPRIAS... ....*SSSIR .KLVSC*LYR
ZmCYP71C3    EL........ ........LY LVPRASSYA. .SYCASSSSY PRMHEC*TCG
ZmCYP71C4    EK........ ........LL LVPQAA***. .**Y*CNAIG F*ISNTKGCV
LrCYP        EK........ ........LL LVPVSRSIPL SDKTE*QLAN HEVLVRDKYC
Consensus                       L
```

Figure 5.2 Deduced amino acid sequence of putative cytochrome P450 monooxygenase from the herbicide-resistant *L. rigidum* biotype SLR31 (LrCYP) aligned with 5′-ends of other known cytochrome P450 monooxygenases. Peptide sequences are indicated as their single-letter abbreviations. (Gm = *Glycine max*; Nr = *Nepetea rasemosa*; Zm = *Zea mays*; Pa = *Persea americana*; Sm = *Solanum melongena*; Lr = *Lolium rigidum*).

A primary cDNA library (constructed in Lambda ZAP®II) was plated out on six 11-cm NZY plates to give approximately 50,000 pfu per plate. A total of 3×10^5 pfu were screened to attempt to isolate a novel full-length cytochrome P450 monooxygenase cDNA clone from *L. rigidum*. Membranes were prepared from each plate and hybridised to the LrCYP probe. Moderate stringency washes were done to allow any cytochrome P450 monooxygenases present to be detected. The membranes were exposed to x-ray film at −80°C for two weeks after which a number of plaques gave signals of varying intensity. The most intense of those plaques were selected and picked from the primary plates with the wide end of a sterile pasteur pipette and eluted into one ml of SM buffer. Each plaque isolate was diluted to a phage concentration of 10^{-4} and 100 µl was replated onto 90 mm NZY plates. Again membranes were prepared from each plate and screened with LrCYP.

Sequence similarity is not sufficient to guarantee success in homology probing with cDNA libraries and as none of the primary eluates contained any plaques that hybridised to the LrCYP probe in the secondary screening an alternative PCR-based strategy was undertaken to endeavour to isolate a full-length cytochrome P450 monooxygenase clone.

5.3.4 RT-PCR Strategy to Isolate a Larger Cytochrome P450 Monooxygenase Fragment from the Herbicide-resistant *L. rigidum* Biotype SLR31

Alignments of the members of the CYP71 subfamily were made and a unique primer to the 5′-end of these genes was designed (designated 71-UP). Using cDNA prepared from induced SLR31 *L. rigidum* plant material, the 71-UP and the reverse sequence Meijer primer (designated CYPX1), RT-PCR was performed. A first round of PCR using 71-UP and CYPX1 primers was followed by a nested PCR using primers 71-UP and CYPX2 and a one in 100 dilution of the first round PCR as template. Amplified PCR products were separated by agarose gel electrophoresis. A band migrating at the size of approximately 1300 bp was identified. This PCR product was excised from the gel, purified and

subcloned into a pGem-T Easy vector (Section 2.1.2). Following antibiotic and colour selection to obtain colonies containing inserts, the plasmids were digested with the restriction enzyme *EcoRI* to excise inserts and then separated by agarose gel electrophoresis. DNA was prepared from two of the isolated clones containing an insert in the expected size range of 1300 bp. Both clones were sequenced using the M13 forward and reverse primers.

After prediction of the open reading frame, sequence data was submitted to a Blast search on the GenBank database. Sequence analysis of both isolates confirmed their identities as novel cytochrome P450 monooxygenases. High sequence similarity to members of the CYP71C subfamily indicates that these two clones belong to this subfamily of the Group A plant cytochrome P450 monooxygenases. The full-length sequences of CYP71C1 are 553 amino acids for the deduced protein and 1890 bp for the mRNA. The full-length sequences of CYP71C2 are 536 amino acids for the deduced protein and 1786 bp for the mRNA. Aligned LrCYP sequences showed that these clones were not full-length, missing approximately 70 amino acids from the 5′-end and 30 amino acids from the 3′-end, a total of 100 amino acids (Figure 5.3).

The CYP71C subfamily contains cytochrome P450 monooxygenases isolated from *Z. mays* (Table 1.1). The functions of these genes have been identified: CYP71C1 codes for HBOA synthase, CYP71C2 and CYP71C4 code for indolin-2-one hydroxylase, and CYP71C3 codes for HBOA-*N*-hydroxylase

```
                                                                                              60
ZmCYP71C1   MALEAGYDYL HVAVVQCTPT QAAAVLGVLL LLAIRLAAAA RSATSPKWRK HRLPPTPPGK

                                                                                             150
ZmCYP71C1   MLVQVGAVI- VV-STPQAAE AVLRTHDHVL ASRPRNPVAD IIRYNSTDVA FAPYGVYWRT LPIGHLHLI GSHPHVSFRD LHAKYGHNGL
SLR31(1A3)  MLLRLGAVNT VVVSSPRAAE AVLRTHDHAL ASRPRSTVAD IILYGSSDVG FAPYGDPWRQ LHLV      GSPPHVSFRD LARKYGRD-L
SLR31(3A3)  MLLRLGAVNT VVVSSPRAAE AVLRTHDHAL ASRPRSTVAD IILYGSSDVG FAPYGDPWRQ LHLV      GSLPHVSFRD LARKHGRD-L
SLR31(A2F)  MLLRLGAVNT VVVSSPRAAE AVLRTHDEAL ASRPRSTVAD IILYGSSDIG FAPYGDPWRQ LHLV      GSPPHVSFRD LARKHGRD-L
VLR69(12)   MLLRLGAVNT VVVSSPRAAE AVLRTHDHAL ASRPRSIVAD IILYSSSDIG FAPYGDPWRQ LHLV      GSLPHVSFRD LARKHGRD-L

                                                                                             240
ZmCYP71C1   ARKVNLLSA  KMVFSK-RRE -REEEVRLVV ARIRDAAE-A SPGTAL---- DMTELLGGYA SDFVCRAVLS HRKQGRNKLF RELTETSAAL
SLR31(1A3)  AGKVVTTHML NAKKVHSFRH DREEEVRITV AKIRASATAA SAGTVPATGV DMSEHLDSYA
SLR31(3A3)  ARKVVSTHML NAKKVHSFRH DREEVRITV  AKIRAFAKAG SAGTVPATGV DMSEHLDSYA
SLR31(A2F)  ARKVVTHML  NAKKVHSFRH HREEEVRITV AKIRASATAA SAGTVPATAV DMSEHLDSYA
VLR69(12)   ARKVVTHML  NAKKVHSFRH HREEEVRITV AKIRASATAA SAGTVPATAV DMSEHLDSYA

                                                                                             330
ZmCYP71C1   LGGFNVEDYF PKLADVDLFL RIICAKAKSV SKRWDFNELL SEYALSGGKQ GDHNSEDFVH LLLSLQKDYG LTTDNIKGIL VNMFEAALET

                                                                                             420
ZmCYP71C1   SVLEYSMSEL MNNRHVLAKL QKEVRTA--- ---------- ---------- TPDGRMVMEE DLSRMPYLKA TIKESMRIHP P--FLLPHFS THDCEINGYT
SLR31(3A3)                        QE                               EPQQDMVTEE ELSDMAYLRA TVKETLR?HP PASLLIPHLS IADCEIDGYT
SLR31(A2F)  MNNRQVMAKL QGWRSRRHN  TGTGRGKDRE NQE                    EPRQDVLTEE ELGDMAYLRA TVKETLRQHP PGPLLIPHLS IADCQIDGYM
VLR69(7)                          QGWRSRRHN

                                                                                             510
ZmCYP71C1   IPAGTRVIVN AWALARDPTC WDKAEEFFPE MYGKDIRFV- -PFGAGRRIC AGATFAIATV EIMLANLIYH FDWEMPARME
SLR31(3A3)  IPSGTRLMVN VWALARDPAS WDTPEEFAPE LNGKGNDFEY LPFGSGRRIC PGINFANAAV ELMLAN
SLR31(A2F)                        WDTPEEFAPE LNGKGKDFEY LPFGSGRRIC PGINFANAAV ELMLAN
VLR69(7)    IPAGTRL VN AWALGRDPVS GEMPEEFMPE
LrCYP                                                  LPFGWGPRIC PGINFANTAV ELMLANLVYH FDWELPAKMK

                                                                    546
ZmCYP71C1   RTGAKVSDQF GMTLRRTQKL YLVPRIPKCV SSS*
LrCYP       GVDMKEVFSL SI--RRKEKL LLVPVSRSIP LSDKTE*
```

Figure 5.3   Alignment of the isolated LrCYP sequences from the *L. rigidum* herbicide resistant biotypes, SLR31 and VLR69, and CYP71C1 from *Z. mays*. The LrCYP clones are all missing approximately 70 amino acids from the 5-end and 30 amino acids from the 3-end. Amino acids in red indicate those that are consistent between LrCYP sequences but different from those in the *Z. mays* sequence, while those in blue indicate differences both between LrCYP sequences and the *Z. mays* sequence.

5.3.5 Cloning of Cytochrome P450 Monooxygenases from the Herbicide-resistant *L. rigidum* Biotype, VLR69

The same RT-PCR strategy used to clone the cytochrome P450 monooxygenases from the herbicide-resistant biotype SLR31 was undertaken to clone the same cytochrome P450 from the herbicide-resistant *L. rigidum* biotype, VLR69. RT-PCR amplification (Section 2.2.12), using the primer 71-UP and CYPX1was performed on first-strand cDNA prepared from the herbicide-resistant *L. rigidum* biotype, VLR69. Amplified PCR products were diluted one in 100 and used as starting template for a nested PCR using the primer set 71-UP and CYPX2. The PCR product appeared as a band migrating in the size range of 1300bp. The PCR product was excised from the gel, purified and subcloned into a T-tailed pGem vector (Section 2.1.2). Following antibiotic and colour selection to obtain colonies containing inserts, the plasmids were digested with the restriction enzyme *EcoRI* to excise inserts and then separated by agarose gel electrophoresis. Four of the 20 isolated clones contained an insert in the expected size range. DNA was prepared from these four clones and sequenced using the M13 forward and reverse primers.

After prediction of the open reading frame, sequence data was submitted to a Blast search on the GenBank database. Sequence analysis of isolates confirmed their identities as cytochrome P450 monooxygenases. High sequence similarity to members of the CYP71C subfamily indicates that these four clones belong to this subfamily of the Group A plant cytochrome P450 monooxygenases. Aligned LrCYP sequences showed that these clones were not full-length, also missing approximately 70 amino acids from the 5′-end and 30 amino acids from the 3′-end, a total of 100 amino acids, similar to the SLR31 clones (Figure 5.3).

5.4   Discussion

As a result of the high number of cytochrome P450 enzymes in plants and the low abundance of each protein, molecular techniques are proving to be the method of choice for the rapid identification and isolation of cytochrome P450 monooxygenases.   The technique of RT-PCR was employed with the aim of detecting cytochrome P450 monooxygenases in *L. rigidum*, potentially involved in the metabolism of herbicides within this species.   Putative cytochrome P450 monooxygenases from the herbicide-resistant *L. rigidum* biotypes, SLR31 and VLR69, have been cloned and sequenced.   The high degree of sequence similarity to other characterised plant cytochrome P450 monooxygenases classifies these clones in the subfamily CYP71C of the group A cytochrome P450 monooxygenase superfamily.

The CYP71C cytochrome P450 monooxygenases, listed in Table 1.1, have been characterised and their functions have been elucidated.   None of these cytochrome P450 monooxygenases to date have been implicated in the metabolism of xenobiotics.   Further analysis and characterisation of the *L. rigidum* clones are needed to justify their potential role in the plant.   Whether these enzymes have a role in the metabolism of herbicides is yet to be determined.

The closely identified forms of cytochrome P450 monooxygenase genes isolated from *L. rigidum* may be isozymes (ie: identical function gene products of different alleles) or alternatively different post-translational modifications of the same gene product.   Sequence alignments that are highly identical (>97%) are assumed to be allelic variants of a single locus.   The small differences in sequences are important and even a single base change may have dramatic effects on specificity.   For example, mutagenesis experiments on CYP2C2 have shown that a single amino acid substitution enables this enzyme to accept

progesterone as a substrate when CYP2C2 normally accepts lauric acid hydroxylase (Ramarao & Kemper, 1995). Lindberg & Negishi (1989) have also demonstrated that mutation of three amino acids in the mouse CYP2A5 has been shown to abolish coumarin-7-hydroxylase activity and enhance testosterone-15α-hydroxylase activity. Even the mutation of one of these three amino acids was enough to alter the catalytic specificity of the enzyme (Lindberg & Negishi, 1989).

Another possible explanation for the closely related cytochrome P450 monooxygenase genes isolated is the fact that a population of plants, rather than a single plant, were used to prepare cDNA used in the RT-PCR amplification of potential genes. As a species evolves complexity there is a premium placed on specialised proteins (Nelson, 1999). If these proteins can be useful for a new task that may not have existed before, they will be duplicated and expanded to fulfil these needs. Plant populations generally contain a mixture of genotypes, each of which potentially may exhibit a variety of phenotypes depending on environmental conditions. Genotypes are able to alter physiology or morphology in changing environments and selection pressures will influence the patterns found within a population. Plants have only been exposed to herbicides for the last 50 years, therefore, the initial ability of cytochrome P450 monooxygenases to metabolise these compounds is most likely due to the fortuitous binding of the xenobiotics into the active site. However, adaptive evolution in plant and animal populations is mostly achieved by selection of phenotypes encoded by many genes with small additive effects (Lande, 1983). The P450 polymorphisms present, therefore, may be a mixture of closely related isoforms that have been isolated from this population. They may represent a series of plants that have been exposed to different selection pressures or possibly just a consequence of genetic plasticity of this species. Wide genetic variation, as measured by large variation in isoenzymes within populations, is evident in this species (Matthews, 1994). Therefore, given sufficient time and selection pressure, evolutionary responses

altering resistance traits are inevitable. Further studies to look at the genetic variability of this sub-family of genes in *L. rigidum* will help clarify this issue.

The model plant *A. thaliana* had been predicted to have around 170 cytochrome P450 monooxygenases in 41 families. However, genomic sequencing of *Arabidopsis* is now complete and, after final analysis, there may be as many as 400 cytochrome P450 monooxygenases present (see URL:http://drnelson.utmem.edu/CytochromeP450.html). This large number of cytochrome P450 monooxygenases is likely to have evolved to allow plants to produce a large number of secondary metabolites, many which may require cytochrome P450 monooxygenase hydroxylation. Additionally, with the completion of the *O. sativa* (rice) genome, we will have a much greater understanding of the total numbers of cytochrome P450 monooxygenases in these plant species, however, it will take much longer to determine the specific role of these enzymes. Comparison of the families between the *O. sativa* and *Arabidopsis* genomes show that 32 of 45 families (71%) are present in both species. It appears that most plant cytochrome P450 families existed before the monocot-dicot divergence.

One method to examine the physiological function of these genes is to express the full-length genes in a suitable expression vector such as yeast (*Saccharomyces cerevisiae*) and assay for activity using an array of putative substrates. Proteins generated in this way may yield valuable information regarding the structure and function of these genes. Many of these enzyme reactions are highly specific and, given the perplexing volume of possible secondary metabolites produced by plant tissues, the extent of testing is limited by the number of available substrate analogues and the sensitivity of the analysis. With the physiological substrate unknown it is also impossible to distinguish between an inactive expressed enzyme and poor substrate. Cell or yeast strain, culture media and temperature may also have a dramatic effect on the expression level of cytochrome P450

monooxygenase proteins (Barnes, 1996). In the case of xenobiotic metabolism, there is also the problem that the number of enzymes required for detoxification is unknown. Is a single enzyme sufficient for detoxification of a xenobiotic or are a series of cytochrome P450 monooxygenases required? Elucidation of this question is of great economic importance to the commercial development of transgenic crops that are able to confer resistance to both chemical and biological stresses.

Chapter 6

Final Discussion

Chapter 6

Final Discussion

Since the cloning of the first plant cytochrome P450 monooxygenase in 1990 (Bozak *et al.*, 1990) a large number of plant cytochrome P450 genes have been identified and isolated. These genes are involved in a vast diversity of reactions giving an indication of the broad capacity in which these enzymes function. Cytochrome P450 monooxygenases are typically involved in the synthesis of secondary metabolites, which are chemically diverse and are often involved in plant defence. Many cytochrome P450 monooxygenase genes are highly specific for a particular reaction and are found in a limited number of plant species (eg: limonene hydroxylases, CYP71D13 and CYP71D18; Karp *et al.*, 1990; Lupien *et al.*, 1999) while others appear to be more universal between plants (eg: CA4H, CYP73; Bozak *et al.*, 1990; Teutsch *et al.*, 1993; Hotze *et al.*, 1995; Bell-Lelong *et al.*, 1997).

In the past isolation strategies, relying on the ability to purify functional microsomes, to identify cytochrome P450 monooxygenases from *L. rigidum* have proved unsuccessful (S. Powles, personal communication). Active, herbicide-metabolising cytochrome P450-dependent microsomal oxidases have been very difficult to isolate and those that have been successfully isolated and activity measured have been at levels so low as to be of no substantial value for further analysis (C. Preston, personal communication). Purification of cytochrome P450 monooxygenases has generally been difficult from plants due to their low abundance and instability (Guengerich, 1990). The use of cytochrome P450 inducers and inhibiters on whole plant herbicide metabolism studies in the past have indicated that these enzymes were present in *L. rigidum* and were implicated in the detoxification of herbicides (Christopher *et al.*, 1991; 1994; Burnet, 1992; Burnet *et al*, 1993a; 1994;

Preston *et al.*, 1996). Molecular techniques were successfully used in this study to firstly identify a fragment of a cytochrome P450 monooxygenase and then further isolate and clone larger fragments of genes from the herbicide resistant *L. rigidum* biotypes, SLR31 and VLR69 (Chapter 5).

It was anticipated that the screening of cDNA libraries prepared from chemically-induced *L. rigidum* seedlings would potentially isolate cytochrome P450 monooxygenases from this species involved in herbicide metabolism. However, exhaustive screenings with a number of full-length P450 monooxygenases isolated from other plant species failed to identify any potential P450 clones. Sequence similarity is not sufficient to guarantee success in homology probing and it is often difficult to design PCR-based strategies for the cloning of these genes. Therefore, a PCR-based strategy was employed to isolate a fragment from the 5'-end of a *L. rigidum* cytochrome P450 monooxygenase. This 360 bp fragment was isolated by RT-PCR and showed high sequence similarity to CYP71C2. The fragment, designated LrCYP, obeyed the consensus PFGXGXRXCXG for the highly conserved heme-binding domain in the catalytic site of the group A plant cytochrome P450 monooxygenases (Durst & O'Keefe, 1995). Subsequent, screening of *L. rigidum* cDNA libraries with this LrCYP fragment also failed to identify any larger cytochrome P450 clones.

Again, utilising the RT-PCR technique and newly designed primers to conserved regions of the published CYP71C gene subfamily from *Z. mays* (Frey *et al.*, 1995), further cytochrome P450 clones were isolated from *L. rigidum*. These clones were missing 70 amino acids from the 5'-end and 30 amino acids from the 3'-end, a total of 100 amino acids. All the genes isolated showed high sequence similarity indicating they may be variations or isoforms of the same gene isolated from a population of plants within one biotype of *L. rigidum*. The cDNA initially used in the PCR reactions to amplify these

Although these *L. rigidum* biotypes express herbicide resistance as a whole population it is not to say that all the seedlings are genetically identical within the population. This may account for some of the variation observed in gene sequences isolated. Nevertheless, highly conserved sequence motives can be seen between all these *L. rigidum* genes and contribute to the high level of identity within the gene family. It is possible that these genes may have a similar function within the plant as indicated by the high level of amino acid identity between these genes. However, even a single amino acid change may completely alter the substrate specificity and subsequent function of a particular cytochrome P450 monooxygenase, therefore the protein may exhibit different functions (Lindberg & Negishi, 1989; Ramarao & Kemper, 1995).

In whole plant studies (Chapter 3), hydrolysis of diclofop-methyl to the active diclofop acid occurs faster in herbicide-susceptible biotype, VLR1, than in the herbicide-resistant biotype, SLR31, in which 1.1-fold more parent herbicide remained after 24 h. However, the subsequent conversion of diclofop acid to metabolites was faster in biotype SLR31, with 1.3-fold more metabolites formed after 24 h. The addition of either 500 µM 2,4-D or clofibrate to biotypes VLR1 and SLR31 seedlings resulted in an increase in the level of diclofop-methyl metabolites produced over a 24 h period when compared to control (uninduced) seedlings. In VLR1, induced with 2,4-D, diclofop-methyl levels were 1.1-fold higher, diclofop acid levels decreased by 48% and the production of metabolites increased 1.5-fold. The addition of clofibrate to VLR1 resulted in diclofop-methyl levels decreased by 10%, diclofop acid levels decreased by 42% and the production of metabolites increased by 1.5-fold. In SLR31, induced with 2,4-D, diclofop-methyl levels were 1.3-fold higher, diclofop acid levels decreased by 36% and the production of metabolites increased 1.2-fold. With the addition of clofibrate to SLR31, diclofop-methyl levels decreased by 30%, diclofop acid levels decreased by 23% and the production of metabolites increased by 1.3-fold. These levels of induction by 2,4-D and clofibrate in *L. rigidum* are much

smaller than the 5- to 16-fold increases in diclofop hydroxylase activity seen in *T. aestivum* seedling tissue following treatment with the chemical safener, NA, ethanol (EtOH) or phenobarbitol (PB; Frear *et al.*, 1991). This may indicate that different isoforms of the diclofop-methyl hydroxylase may exist in *L. rigidum* and *T.aestivum*. As mentioned earlier, even a single amino acid change may completely alter the substrate specificity and subsequent function, including the rate of enzymatic activity, of a particular cytochrome P450 monooxygenase. Alternately, a more active promoter or more copies of diclofop hydroxylase may be present in biotype SLR31 than in biotype VLR1. Isolation and functional analysis of the genes involved in diclofop-methyl metabolism in both the herbicide-susceptible and –resistant biotypes of *L. rigidum* need careful evaluation before any firm conclusions can be drawn about these observed differences.

Northern expression analysis of the LrCYP gene isolated during this study (Chapter 3) indicated that this gene is induced in response to addition of 2,4-D or clofibrate. The LrCYP transcript levels increase as soon as 3h following the addition of 2,4-D to the seedling growth medium. Expression of the LrCYP transcript is maximal at 3 h in biotype VLR1 (2-fold increase) and 6 h in biotype SLR31 (1.5-fold increase) following addition of 2,4-D. Addition of clofibrate also increases transcript levels of LrCYP in a similar patter to that seen with 2,4-D.

Expression of CYP73.301 (CA4H, isolated from *T. aestivum*) showed a faster and more severe response in *L. rigidum* with the addition of both 2,4-D and clofibrate. A 3-fold increase in transcript levels was seen as soon as 3 h following 2,4-D or clofibrate induction. This rapid rise in transcript expression has also been observed in *Z.mays*, where Persans & Schuler (1996) demonstrated that both CYP73A9 and CYP82 transcripts accumulate to their maximal level 3 h after wounding.

The observed increases of the LrCYP transcript does not necessarily implicate this gene in metabolism of diclofop-methyl. The resulting increases in transcript levels may indicate a secondary induction effect, possibly even the result of a plant stress response to phytotoxicity on addition of either of these two chemicals. This may certainly be the case for clofibrate which, following prolonged incubation, may cause necrosis of leaf tissue and alterations in cell ultrastructure. Monocotyledonous plants, however, are naturally tolerant to high concentrations of 2,4-D (Adele *et al.*, 1981) and stress responses would be less likely to be observed with this chemical than with clofibate. Zimmerlin & Durst (1990) reported diclofop hydroxylase and general cytochrome P450 levels were increased 16- and 2-fold, respectively, when etiolated *T. aestivum* seedlings were treated for 48 h with 8 mM PB. Whereas pre-treatment of *T. aestivum* seedlings with diclofop or 2,4-D did not correlate with an increase in P450 content (McFadden *et al.*, 1989). An increase in herbicide metabolism is not always correlated with an increase in general cytochrome P450 levels (Frear *et al.*, 1991). Isolation and expression of the LrCYP gene would enable the putative function to be determined and it's possible role in herbicide metabolism to be evaluated.

Inhibition of diclofop-methyl hydroxylase in *L. rigidum,* using specific suicide substrates, designed and synthesised in Strasbourg, France, did not completely inhibit the function of this cytochrome P450 monooxygenase (Chapter 4). The most marked level of inhibition was seen with the addition of compound 2, where 50% inhibition was seen in the production of diclofop-methyl metabolites. A related compound, undecan-1-sulfonic acid (UDSA), with similarity to compound 2 in this study, has been shown to exhibit only a weak effect on both diclofop-methyl and chlorotoluron metabolism in *T. aestivum* seedlings (Forthoffer *et al.*, 2001). The compounds undec-9-yne-1-sulfonic acid (9-UDYS) with similarity to compound 8, and undec-10-yne-1-sulfonic acid (10-UDYS), with similarity to compound 5, significantly inhibited diclofop-methyl metabolism by

reducing the formation of intermediate metabolites and conjugates (Forthoffer *et al.*, 2001). Inhibition of diclofop-methyl metabolism by 9-UDYS and 10-UDYS was approximately 75% and 72%, respectively. These findings are in contrast to those found in this study, where compounds 5 and 8 exhibited only a weak effect on diclofop-methyl metabolism. Selectivity of suicide substrates for cytochrome P450 isozymes is determined by protein constraints on the binding and orientation of substrates in their active sites. As both the herbicide-susceptible and –resistant biotypes exhibited similar levels of inhibition by all three suicide substrates we can assume that the diclofop-hydroxylase gene in each of the *L. rigidum* biotypes are closely related isoforms, having high affinity for the same inhibitory compounds.

These types of enzyme studies potentially open the door for a vast array of beneficial developments. There is increasing interest in developing crop plants to produce compounds of pharmaceutical and industrial importance. Heterologous expression of plant cytochrome P450 monooxygenases, to allow the identification and ultimately, genetically modified expression of these genes, for the production of important compounds is a significant outcome of these studies. Introduction and manipulation of the flavonoid 3′,5′-hydroxylase, involved in flower colour, is already being investigated (Tanaka *et al.*, 1980. Many other plant cytochrome P450 monooxygenases are also potentially useful, such as those genes that contribute to plant fibrosity, pigmentation, aroma, flavour and as sources of pharmaceuticals. Resistance to agrochemicals is also having a significant economic and environmental impact. Transgenic herbicide-tolerant crops are been produced that have a single gene introduced that detoxifies the herbicide (reviewed in Devine & Preston, 2000). Several genes have been transferred to crop species already to create transgenic crops, some of which have been successfully marketed commercially. The cytochrome P450 monooxygenase, CYP1A1, isolated from both human and rat, have been transferred to *N. tabacum* plants. These plants have demonstrated enhanced

metabolism of the herbicide chlorotoluron, predominantly by ring-methyl hydroxylation (Shiota *et al.*, 1996; Inui *et al.*, 1999), as well as resistance to the herbicides atrazine and pyriminobac-methyl. Thus, the expression of CYP1A1 in plants confers cross-resistance to herbicides with different structures and modes of action. The cytochrome P450 monooxygenase, CYP105A1, isolated from the bacterium *Streptomyces griseolus*, has also been transferred into *N. tabacum*. This enzyme has been successfully expressed in chloroplasts where it metabolises a sulfonylurea pro-herbicide, R7402, to a more toxic product (O'Keefe *et al.*, 1994).

One of the intriguing questions is how many cytochrome P450 genes are present in *L. rigidum* that are involved in conferring herbicide resistance? Evidence indicates that there are a number of different isoforms responsible for herbicide metabolism (Preston *et al.*, 1996). However, we still have much to learn regarding this plant and ultimately how we may gain better control of this weed in crops.

Pesticides and herbicides play an important role in the production and storage of our food and fibre supplies. They act to control the large number and variety of pests that attack crops and agricultural commodities involved. Benefits include increased crop yields, better crop quality and improved storage. There are a great number of chemical compounds applied in agricultural systems, most of them highly toxic. It is therefore, important to know their transitory and ultimate fate. In plants, internal compartmentation or apoplastic deposition of metabolites occurs following detoxification. These residues may persist in plant tissues for considerable periods of time and may have toxicological implications for consumers of these tissues. Many of the conjugates formed in phase II are susceptible to cleavage by mammalian digestive enzymes (Sandermann, 1992; Sandermann *et al.*, 1992), which may result in the release of parent compounds or their activated metabolites. Some of these xenobiotic metabolites may be mutagenic to both the

plant and their consumers (Plewa & Wagner, 1993).   Therefore, a thorough understanding of the metabolic fate of xenobiotics in plants and their subsequent bioavailability is of great importance.   This would allow us to elucidate their mode of action, increase the efficacy of their use and provide a basis for residual tolerance.   Also, cytochrome P450 monooxygenases that efficiently metabolise xenobiotics to non-toxic products may potentially be used for remediation of herbicide- or xenobiotic-contaminated soils.

Many biotypes of *L. rigidum* already display extensive herbicide resistance to a wide range of chemicals with different modes of action.   Although herbicides with different mechanisms of action are needed for rotation in resistant management strategies, few are likely to be available in the near future.   Cost, social and environmental concerns have slowed down the development and registration of herbicides and so increased the cost of controlling weed species.   Neither cultural practices nor herbicides alone can solve the problem of weeds in the field.   Integrated management strategies, with more reliable information on which to base weed control decisions will be required to eradicate weeds in crops.   Studies of weed resistance to herbicides will assist in improving weed management and will allow examination of evolutionary processes.   Identification of potential methods to inhibit the function of herbicide detoxifying cytochrome P450 monooxygenases in weed species may allow novel methods of weed control to be developed and integrated into crop management practices.

References

References

A

Abeles RH, and Maycock AL. (1976). Suicide enzyme inactivators. *Accounts of Chemical Research* **9**, 313 – 319.

Adele P, Reichhart D, Salaün J-P, Benveniste I and Durst F. (1981). Induction of cytochrome P-450 and monooxygenase activity by 2,4-dichlorophenoxyacetic acid in higher plant tissue. *Plant Science Letters* **22**, 39 - 46.

Aoyama T, Hardwick JP, Imaoka S, Funae Y, Gelboin HV, ad Gonzalez FJ. Clofibrate-inducible rat hepatic P450s IVA1 and IVA3 catalyze the ω– and (ω-1)-hydroxylation of fatty acids and the ω–hydroxylation of prostaglandins E_1 ad $F_{2\alpha}$. *Journal of Lipid Research* **1990**, 1477 – 1482.

Archer C, Ashman PJ, Hedden P, Bowyer JR, and Bramley PM. (1992). Purification of ent-kaurene oxidase from *Gibberella fujikuroi* and *Cucurbita maxima*. *Biochemical Society Transactions* **20**, 218s.

B

Baldwin BC. (1977). Xenobiotic metabolism in plants. In *Drug metabolism – from microbe to man*. (D.V. Parke and R.L. Smith, eds). pp. 191 – 217. Taylor and Francis Ltd., London.

Barnes HJ. (1996). Maximising expression of eukaryotic cytochrome P450s in *Escherichia coli*. In *Methods in Enzymology. Cytochrome P450* (EF Johnson, and MR. Waterman, eds.). Vol. 272. pp 3 – 14. Academic Press, New York.

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

Batard Y, Schalk M, Pierrel M-A, Zimmerlin A, Durst F, and Werck-Reichhart D. (1997). Regulation of the cinnamate 4-hydroxylase (CYP73A1) in Jerusalem artichoke tubers in response to wounding and chemical treatments. *Plant Physiology* **113**, 951 – 959.

Barrett M. (1995). Metabolism of herbicides by cytochrome P450 in corn. *Drug Metabolism and Drug Interactions* **12**, 299 – 315.

Bell-Lelong DA, Cusumano JC, Meyer K, and Chapple C. (1997). Cinnamate-4-hydroxylase expression in Arabidopsis. *Plant Physiology* **113**, 729 – 738.

Bendall D, and Hill R. (1956). Cytochrome components in the spadix of *Arum maculatum*. *New Phytologist* **55**, 206 - 212.

Benveniste I, Salaün, J-P, and Durst F. (1977). Wounding induced cinnamic acid hydroxylase in Jerusalem artichoke tuber. *Phytochemistry* **16**, 69 - 73.

Benveniste I, Salaün, J-P, and Durst F. (1978). Phytochrome-mediated regulation of a monooxygenase hydroxylating cinnamic acid in etiolated pea seedlings. *Phytochemistry* **17**, 359 - 363.

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

Benveniste I, Gabriac B, and Durst F. (1986). Purification and characterization of the NADPH-cytochrome P450 (cytochrome c) reductase from higher plant microsomal fraction. *Biochemical Journal* **235**, 365 - 373.

Benveniste I, Lesot A, Hasenfratz MP, Kochs G, and Durst F. (1991). Multiple forms of NADPH-cytochrome P450 reductase in higher plants. *Biochemical and Biophysical Research Communications* **177**, 105 - 112.

Blair A, and Martin TD. (1988). A review of the activity, fate and mode of action of sulfonylurea herbicides. *Pesticide Science* **22**, 195 - 219.

Blée E, and Joyard J. (1996). Envelope membranes from spinach chloroplasts are a site of metabolism of fatty acid hydroperoxides. *Plant Physiology* **110**, 445 – 454.

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

Bozak KR, Yu H, Sirevåg R, and Christoffersen RE. (1990). Sequence analysis of ripening-related cytochrome P-450 cDNAs from avocado fruit. *Proceedings of the National Academy of Science, U.S.A.* **87**, 3904 - 3908.

Brugliera F, Barri-Rewell G, Holton TA, and Mason JG. (1999). Isolation and characterization of a flavonoid 3'-hydroxylase cDNA clone corresponding to the Ht1 locus of *Petunia hybrida*. *The Plant Journal* **19**, 441 – 451.

Burden RS, Rowell PM, Bailey JA, Loeffler RST, Kemp MS, and Brown CA. (1985). Debneyol, a fungicidal sesquiterpene from TNV infected *Nicotiana debneyi*. *Phytochemistry* **24**, 2191 – 2194.

Burden RS, Loeffler RST, Rowell PM, Bailey JA, and Kemp MS. (1986). Cyclodebneyol, a fungitoxic sesquiterpene from TNV infected *Nicotiana debneyi*. *Phytochemistry* **25**, 1607 – 1608.

Burnet MW. (1992). Mechanisms of herbicide resistance in *Lolium rigidum*. PhD Thesis, The University of Adelaide, South Australia, Australia.

Burnet M, Hildebrand OB, Holtum JAM, and Powles SB. (1991). Amitrole, triazine, substituted urea, and metribuzin resistance in a biotype of rigid ryegrass (*Lolium rigidum*). *Weed Science* **39**, 317 - 323.

Burnet M, Loveys BR, Holtum JAM, and Powles SB. (1993a). A mechanism of chlorotoluron resistance in *Lolium rigidum*. *Planta* **190**, 182 - 189.

Burnet M, Loveys BR, Holtum JAM, and Powles SB. (1993b). Increased detoxification is a mechanism of simazine resistance in *Lolium rigidum*. *Pesticide Biochemistry and Physiology* **46**, 207 - 218.

Burnet M, Christopher JT, Holtum JAM, and Powles SB. (1994). Identification of two mechanisms of sulfonylurea resistance within one population of rigid ryegrass (*Lolium rigidum*) using a selective germination medium. *Weed Science* **42**, 468 - 473.

Burton JD, and Maness, EP. (1992). Constitutive and inducible bentazon hydroxylation in shattercane (*Sorghum bicolor*) and johnsongrass (*S. halapense*). *Pesticide Biochemistry and Physiology* **44**, 40 – 49.

Burton J, Gronwald JW, Somers DA, Connelly JA, Gengenbach BG, and Wyse DL. (1987). Inhibition of plant acetyl-coenzyme A carboxylase by the herbicides sethoxydim and haloxyfop. *Biochemical and Biophysical Research Communications* **148**, 1039 - 1044.

Burton JD, Maness EP, Monks DW, and Robinson DK. (1994). Sulfonylurea selectivity and safener activity in 'Landmark' and 'Merit' sweet corn. *Pesticide Biochemistry and Physiology* **48**, 163 – 172.

Büttner M, and Singh KB. (1997). *Arabidopsis thaliana* ethylene-responsive element binding protein (AtEBP), an ethylene-inducible, GCC box DNA-binding protein interacts with an ocs element binding protein. *Proceedings of the National Academy of Science, U.S.A.* **94**, 5961 – 5966.

C

Cabanne F, Gaillardon P, and Scalla R. (1985). Phytotoxicity and metabolism of chlorotoluron in two wheat varieties. *Pesticide Biochemistry and Physiology* **23**, 212 - 220.

Cabanne F, Huby D, Gaillardon P, Scalla R, and Durst F. (1987). Effect of the cytochrome P450 inactivator 1-aminobenzotriazole on the metabolism of chlorotoluron and isoproturon in wheat. *Pesticide Biochemistry and Physiology* **28**, 371 - 380.

CaJacob CA, and Ortiz de Montellano PR. (1986). Mechanism-based *in vivo* inactivation of lauric aid hydroxylases. *Biochemistry* **25**, 4705 - 4711.

Camm E, and Towers GH. (1977). Phenylalanine ammonia lyase. *Progress in Phytochemistry* **4**, 169 - 188.

Canivenc M-C, Cagnac B, Cabanne F, and Scalla R. (1989). Induced changes of chlorotoluron metabolism in wheat cell suspension cultures. *Plant Physiology and Biochemistry* **27**, 193 - 201.

Casida J, and Lykken L. (1969). Metabolism of organic pesticide chemicals in higher plants. *Annual Review of Plant Physiology* **20**, 607 - 636.

Chappell J. (1995). The biochemistry and molecular biology of isoprenoid metabolism. *Plant Physiology* **107**, 1 – 6.

Chappell J, and Hahlbrock K. (1984). Transcription of plant defense genes in response to UV light or fungal elicitor. *Nature* **311**, 76 - 78.

Chapple CC, Vogt T, Ellis BE, and Somerville CR. (1992). An Arabidopsis mutant defective in the general phenylpropanoid pathway. *The Plant Cell* **4**, 1413 - 1424.

Chkanikov DI, Makeev AM, Nazarova TA, and Ustimenko NV. (1990). Effect of the antidote 1,8-naphthalic anhydride on phytotoxicity and the rate of metabolism of 2,4-dichlorophenoxyacetaic acid and chlorsulfuron in plants. 207 – 212.

Christopher J, Powles SB, Liljegren DR, and Holtum JA. (1991). Cross-resistance to herbicides in annual ryegrass (*Lolium rigidum*): II. Chlorsulfuron resistance involves a wheat-like detoxification system. *Plant Physiology* **95**, 1036 - 1043.

Christopher J, Powles SB, and Holtum JA. (1992). Resistance to acetolactate synthase inhibiting herbicides in annual ryegrass (*Lolium rigidum*) involves at least two mechanisms. *Plant Physiology* **100**, 1909 - 1913.

Christopher J, Preston C, and Powles SB. (1994). Malathion antagonizes metabolism-based chlorsulfuron resistance in *Lolium rigidum*. *Pesticide Biochemistry and Physiology* **49**, 172 - 182.

Church GM, and Gilbert W. (1984). Genomic sequencing. *Proceedings of the National Academy of Science, U.S.A.* **81**, 1991 - 1995.

Clark IM, Forde BG, and Hallahan DL. (1997). Spatially distinct expression of two new cytochrome P450s in leaves of *Nepeta racemosa*: identification of a trichome-specific isoform. *Plant Molecular Biology* **33**, 875 - 885.

Cole D. (1994). Detoxification and activation of agrochemicals in plants. *Pesticide Science* **42**, 209 - 222.

Cole D, and Owen WJ. (1987). Influence of monooxygenase inhibitors on the metabolism of the herbicides chlorotoluron and metolachor in cell suspension cultures. *Plant Science* **50**, 13 - 20.

Coleman JO, Blake-Kalff MM, and Davis TG. (1997). Detoxification of xenobiotics by plants: chemical modification and vacuolar compartmentation. *Trends in Plant Science* **2**, 144 – 151.

Coon MJ. (1983). Cytochrome P-450: a multifaceted catalyst. *Transactions of the New York Academy of Sciences* **41**, 1 - 8.

Creelman RA, and Mullet JE. (1997). Biosynthesis and action of jasmonates in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**, 355 – 381.

Croteau R, Karp F, Wagschal KC, Satterwhite DM, Hyatt DC, Skotland CB. (1991). Biochemical characterization of a spearmint mutant that resembles peppermint in monoterpene content. *Plant Physiology* **96**, 744 - 752.

D

Dalkin K, Edwards R, Edington B, and Dixon RA. (1990). Stress response in alfalfa (*Medicago sativa* L.). I. Induction of phenylpropanoid biosynthesis and hydrolytic enzymes in elicitor-treated cell suspension cultures. *Plant Physiology* **92**, 440 - 446.

Dawson JH, and Eble KS. (1986). Cytochrome P-450: Heme iron coordination, structure and mechanisms of action. *Advances in Inorganic and Bioinorganic Mechanisms* **4**, 1 - 64.

Dawson JH, Andersson LA, and Sono M. (1982). Spectroscopic investigations of ferric cytochrome P-450-CAM ligand complexes. *The Journal of Biological Chemistry* **257**, 3606 - 3617.

Dennis D, and West CA. (1967). Biosynthesis of gibberellins. III. The conversion of (-)-kaurene to (-)-kauren-19-oic acid in endosperm of *Echinocystis marcrocarpa* Greene. *The Journal of Biological Chemistry* **242**, 3293 - 3300.

De Veylder L, Montagu M, and Inzé D. (1997). Herbicide safener-induced gene expression in *Arabidopsis thaliana*. *Plant and Cell Physiology* **38**, 568 - 577.

Devine MD, and Preston C. (2000). The molecular basis of herbicide resistance. In: *Herbicides and Their Mode of Action* (AH Cobb, and RC Kirkwood, eds). pp 72 - 104. Sheffield Academic Press.

Diehl KE, Stoller EW, and Barrett M. (1995). *In vivo* and *in vitro* inhibition of nicosulfuron metabolism by terbufos metabolites in maize. *Pesticide Biochemistry and Physiology* **51**, 137 - 149.

Dixon R, and Paiva NL. (1995). Stress-induced phenylpropanoid metabolism. *The Plant Cell* **7**, 1085 - 1097.

Donald W, and Shimabukuro RH. (1980). Selectivity of diclofop-methyl between wheat and wild oat: growth and herbicide metabolism. *Physiol Plant* **49**, 459 - 464.

Dreyer C, Krey G, Keller H, Givel F, Helftenbein G, and Wahli W. (1992). Control of the peroxisome β–oxidation pathway by a novel family of nuclear hormone receptors. *Cell* **68**, 879 – 887.

Durst F. (1976). The correlation of phenylalanine ammonia-lyase and cinnamic acid hydroxylase activity changes in Jerusalem artichoke tuber tissues. *Planta* **132**, 221 - 227.

Durst F. (1991) Biochemistry and physiology of plant cytochrome P-450. In *Frontiers in Biotransformation*. (K. Ruckpaul and H. Rein, eds.), pp. 191 - 232. Taylor & Francis, London, UK.

Durst F, Benveniste I, Salaün J-P, and Werck-Reichhart D. (1992). Function, mechanism and regulation of cytochrome P-450 enzymes in plants. *Biochemical Society Transactions* **20**, 353 - 357.

Durst F, and Nelson DR. (1995). Diversity and evolution of plant P450 and P450-reductases. *Drug Metabolism and Drug Interactions* **12**, 189 - 206.

Durst F, and O'Keefe DP. (1995). Cytochromes P450: An overview. *Drug Metabolism and Drug Interactions* **12**, 171 – 187.

E

English N, Hughes V, and Wolf R. (1994). Common pathways of cytochrome P450 gene regulation by peroxisome proliferators and barbiturates in *Bacillus megaterium* ATCC14581. *The Journal of Biological Chemistry* **269**, 26836 – 26841.

Espelie K, Davis RW, and Kolattukudy PE. (1980). Composition, ultrastructure and function of the cutin- and suberin-containing layers in the leaf, fruit peel, juice-sac and inner seed coat of grapefruit (*Citrus paradisi* Macfed). *Planta* **149**, 498 - 511.

Estabrook R, Hildebrandt AG, Baron J, Netter KJ, and Leibman K. (1971). A new spectral intermediate associated with cytochrome P-450 function in liver microsomes. *Biochemical and Biophysical Research Communications* **42**, 132 - 139.

F

Fahrendorf T, and Dixon RA. (1993). Stress responses in alfalfa (*Medicago sativa* L.) XVIII: molecular cloning and expression of the elicitor-inducible cinnamic acid 4-hydroxylase cytochrome P450. *Archives of Biochemistry and Biophysics* **305**, 509 - 515.

Farago S, Brunold C, and Kreuz K. (1994). Herbicide safeners and glutathione metabolism. *Physiologia Planatarum* **91**, 537 – 542.

Farmer EE, Johnson RR, and Ryan CA. (1992). Regulation of expression of proteinase inhibitor genes by methyl jasmonate and jasmonic acid. *Plant Physiology* **98**, 995 - 1002.

Fonné-Pfister R, and Kreuz K. (1990). Ring-methyl hydroxylation of chlorotoluron by an inducible cytochrome P450-dependent enzyme from maize. *Phytochemistry* **29**, 2793 – 2796.

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

Forkmann G. (1991). Flavonoids as flower pigments: The formation of the natural spectrum ad its extension by genetic engineering. *Plant Breeding* **106**, 1 – 26.

Forthoffer N. (1999).   Charactérisation et marquage chimique d'un cytochrome P450 responsable de la tolerance à l'herbicide diclofop chez le blé. PhD dissertation. Strasbourg, France.

Forthoffer N, Helvig C, Dillon N, Benveniste I, Zimmerlin A, Tardif F, and Salaün J-P. (2001). Induction and inactivation of a cytochrome P450 confering herbicide resistance in wheat seedlings. *European Journal of Drug Metabolism and Pharmacokinetics* **26**, 9 – 16.

Frank MR, Deyneka JM, and Schuler MA. (1996). Cloning of wound-induced cytochrome P450 monooxygenases expressed in pea. *Plant Physiology* **110**, 1035 – 1046.

Frear DS, Swanson HR, and Tanaka FS. (1969). *N*-demethylation of substituted 3-(phenyl)-1-methylureas, isolation and characterization of a microsomal mixed function oxidase from cotton. *Phytochemistry* **8**, 2157 - 2169.

Frear DS, Swanson HR, and Mansager ER.. (1987). 1,8-Naphthalic anhydride/auxin protection against chlorsulfuron inhibition of corn seedling growth. In *Pesticide Science and Biotechnology* (G. Greenhalgh, and TR Roberts, eds.). pp. 499 –503, Blackwell Scientific Publications, London, England.

Frear DS, Swanson HR, and Thalacker FW. (1991). Induced microsomal oxidation of diclofop, triasulfuron, chlosulfuron, and linuron in wheat. *Pesticide Biochemistry and Physiology.* **41**, 274 - 287.

Frear DS, and Swanson HR. (1996). Cytochrome P450-dependent hydroxylation of prosulfuron (CGA 152005) by wheat seedling microsomes. *Journal of Agricultural and Food Chemistry* **44**, 3658 - 3664.

Frey M, Kleim R, Saedler H, and Gierl, A. (1995). Expression of a cytochrome P450 gene family in maize. *Molecular and General Genetics* **246**, 100 –109.

Friesen H, O'Sullivan PA, and Vanden Born WH. (1976). HOE 23408, a new selective herbicide for wild oats and green foxtail in wheat and barley. *Canadian Journal of Plant Science* **56**, 567 - 578.

Fuerst E, and Norman MA. (1991). Interactions of herbicides with photosynthetic electron transport. *Weed Science* **39**, 458 - 464.

Fujita M, Ôba K, and Uritani I. (1981). Ipomeamarone 15-hydroxylase from cut-injured and *Ceratocystis fimbriata*-infected sweet potato root tissues. *Agricultural Biological Chemistry* **45**, 1911 - 1913.

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

Funk C, and Croteau R. (1993). Induction and characterization of a cytochrome P450-dependent camphor hydroxylase in tissue cultures of common sage (*Salvia officinalis*). *Plant Physiology* **101**, 1231 - 1237.

Funk C, and Croteau R. (1994). Diterpenoid resin acid biosynthesis in conifers: characterization of two cytochrome P450-dependent monooxygenases and an aldehyde dehydrogenase involved in abietic acid biosynthesis. *Archives of Biochemistry and Biophysics* **308**, 258 - 266.

Funk C, Koepp AE, and Croteau R. (1992). Catabolism of camphor in tissue cultures and leaf disks of common sage (*Salvia officinalis*). *Archives of Biochemistry and Biophysics* **294**, 306 - 313.

G

Gabriac B, Werck-Reichhart D, Teutsch H, and Durst F. (1991). Purification and immunocharacterization of a plant cytochrome P450: the cinnamic acid 4-hydroxylase. *Archives of Biochemistry and Biophysics* **288**, 302 – 309.

Gaillardon P, Cabanne F, Scalla R, and Durst F. (1985). Effect of mixed function oxidase inhibitors on the toxicity of chlorotoluron and isoproturon in wheat. *Weed Research* **25**, 397 - 402.

Gibson GG, Orton TC, and Tamburini PP. (1982). Cytochrome P450 induction by clofibrate. *Biochemical Journal* **203**, 161 - 168.

Gill GS. (1995). Development of herbicide resistance in annual ryegrass populations (*Lolium rigidum* Gaud.) in the cropping belt of Western Australia. *Australian Journal of Experimental Agriculture* **35**, 67 - 72.

Gläβgen WE, Komoβa D, Bohnenkämper O, haas M, Hertkorn N, May RG, Szymczak W, and Sandermann H. (1999). Metabolism of the herbicide isoproturon in wheat and soybean cell suspension cultures. *Pesticide Biochemistry and Physiology* **63**, 97 – 113.

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

Gorecka K, Shimabukuro RH, and Walsh WC. (1981). Aryl hydroxylation: A selective mechanism for the herbicides, diclofop-methyl and clofop-isobutyl, in gramineous species. *Physiologica plantarum* **53**, 55 - 63.

Göttlicher M, Widmark E, Li Q, and Gustafsson J-A. (1992). Fatty acids activate a chimera of the clofibric acid-activated receptor and the glucocorticoid receptor. *Proceedings of the National Academy of Science, U.S.A.* **89**, 4653 – 4657.

Graebe JE. (1987). Gibberellin biosynthesis and control. *Annual Review of Plant Physiology* **38**, 419 - 465.

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

Gronwald J. (1991). Lipid biosynthesis inhibitors. *Weed Science* **39**, 435 - 449.

Gross D, Laanio T, Dupuis G, and Esser HO. (1979). The metabolic behavior of chlorotoluron in wheat and soil. *Pesticide Biochemistry and Physiology* **10**, 49 - 59.

Guengerich FP. (1990). Enzymatic oxidation of xenobiotic chemicals. *CRC Critical Reviews in Biochemistry and Molecular Biology* **25**, 97 – 153.

Guengerich FP. (1991). Reactions and significance of cytochrome P-450 enzymes. *The Journal of Biological Chemistry* **266**, 10019 - 10022.

Gundlach H, Mäller MJ, Kutchan TM, and Zenk MH. (1992). Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. *Proceedings of the National Academy of Science, U.S.A.* **89**, 2389 - 2393.

Gunsalus I, Meeks JR, Lipscomb JD, Debrunner PG, and Münck E. (1974). *Molecular Mechanisms of Oxygen Activation.* (O. Hayaishi, ed). pp 559 – 613. Academic Press, New York.

H

Haake AE, and Balke NE. (1994). Enhancement of microsomal bentazon 6-hydroxylase and cinnamic acid 4-hydroxylase activities from grain sorghum shoots. *Pesticide Biochemistry and Physiology* **50**, 92 – 105.

Hageman LH, and Behrens R. (1984). Basis for response differences of two broadleaf weeds to chlorsulfuron. *Weed Science* **32**, 162 - 167.

Hahlbrock K, Knobloch K-H, Kreuzaler F, Rowell J, Potts M, and Wellman E. (1976). Coordinated induction and subsequent activity changes of two groups of metabolically interrelated enzymes. *European Journal of Biochemistry* **61**, 199 - 206.

Hall LM, Holtum JAM, and Powles SB. (1994). Mechanisms responsible for cross resistance and multiple resistance. In *Herbicide Resistance in Plants: Biology and Biochemistry* (S.B. Powles, and J.A.M Holtum, eds.). pp. 243 – 261. Lewis Publishers, Boca Raton, FL.

Hall LM, Moss SR, and Powles SB. (1995). Mechanism of resistance to chlorotoluron in two biotypes of the grass weed *Alopecurus myosuroides* **53**, 180 – 192.

Hallahan DL, Nugent JHA, Hallahan BJ, Dawson GW, Smiley DW, West JM, and Wallsgrove RM. (1992). Interactions of avocado (*Persea americana*) cytochrome P-450 with monoterpenoids. *Plant Physiology* **98**, 1290 - 1297.

Hallahan DL, Cheriton AK, Hyde R, Clark I, and Forde BG. (1993). Plant cytochrome P-450 and agricultural biotechnology. *Biochemical Society Transactions* **21**, 1068 - 1073.

Hallahan DL, Lau S-MC, Harder PA, Smiley DWM, Dawson GW, Pickett JA, Christoffersen RE, and O'Keefe DP. (1994). Cytochrome P-450-catalysed monoterpenoid oxidation in catmint (*Nepeta racemosa*) and avocado (*Persea americana*); evidence for related enzymes with different activities. *Biochimica et Biophysica Acta* **1201**, 94 - 100.

Hammond-Kosack KE, and Jones JD. (1996). Resistance gene-dependent plant defence responses. *Plant Cell* **8**, 1773 – 1791.

Hardwick JP, Song BJ, Huberman E, and Gonzalez FJ. (1987). Isolation, complimentary DNA sequence, and regulation of rat hepatic lauric acid ω-hydroxylase (cytochrome P-450$_{LA\omega}$): identification of a new cytochrome P450 gene family. *The Journal of Biological Chemistry* **262**, 801 – 810.

Hasson E, and West CA. (1976a). Properties of the system for the mixed function oxidation of kaurene and kaurene derivatives in microsomes of the immature seed of *Marah macrocarpus*. *Plant Physiology* **58**, 473 - 478.

Hasson E, and West CA. (1976b). Properties of the system for the mixed function oxidation of kaurene and kaurene derivatives in microsomes of the immature seed of *Marah macrocarpus*. *Plant Physiology* **58**, 479 - 484.

Hatzios KK. (1991). An overview of the mechanisms of action of herbicide safeners. *Z. Naturforsch* **46c**, 819.

Hayaishi O, Katagiri M, and Rothberg S. (1955). Mechanism of the pyrocatechase reaction. *Journal of the American Chemical Society* **77**, 5450 - 5451.

Hayaishi O, Rothberg S, and Mehler AH. (1956). *Abstracts of the 130th Meeting of the American Chemical Society* p. 53c.

Hayaishi, O. (1974). In *Molecular Mechanisms of Oxygen Activation*. (O. Hayaishi, ed.) pp. 1 - 28. Academic Press, New York.

Heap J, and Knight R. (1982). A population of ryegrass tolerant to the herbicide diclofop-methyl. *The Journal of the Australian Institute of Agricultural Science* **12**, 156 - 157.

Heap I, and Knight R. (1986). The occurrence of herbicide cross-resistance among populations of annual ryegrass (*Lolium rigidum*) resistant to diclofop-methyl. *Australian Journal of Agricultural Research* **37**, 149 - 156.

Helliwell CA, Sheldon CC, Olive MR, Walker AR, Zeevaart JAD, Peacock WJ, and Dennis ES. (1998). Cloning of the *Arabidopsis ent*-kaurene oxidase gene *GA3*. *Proceedings of the National Academy of Science, U.S.A* **95**, 9019 - 9024.

Helliwell CA, Poole A, Peacock WJ, and Dennis ES. (1999). *Arabidopsis ent*-kaurene oxidase catalyzes three steps of gibberellin biosynthesis. *Plant Physiology* **119**, 507 - 510.

Helvig C, Alayrac C, Mioskowski C, Koop D, Poullain D, Durst F, and Salaün J-P. (1997). Suicide inactivation of cytochrome P450 by midchain and terminal acetylenes. *The Journal of Biological Chemistry* **272**, 414 - 421.

Hendry GA, and Jones OT. (1984). Induction of cytochrome P-450 in intact mung beans. *New Phytologist* **96**, 153 – 159.

Herrmann KM. (1995a). The shikimate pathway: Early steps in the biosynthesis of aromatic compounds. *The Plant Cell* **7**, 907 - 919.

Herrmann KM. (1995b). The shikimate pathway as an entry to aromatic secondary metabolism. *Plant Physiology* **107**, 7 - 12.

Higashi K, Ikeuchi K, Obara M, Hirano H, Gotoh S, and Koga Y. (1985). Purification of a single major form of microsomal cytochrome P-450 from tulip bulbs (*Tulipa gesneriana* L.). *Agricultural and Biological Chemistry* **49**, 2399 - 2405.

Hinz JR, and Owen MD. (1996). Nicosulfuron and primisulfuron selectivity in corn (*Zea mays*) and two annual grasses. *Weed Science* **44**, 219 – 223.

Hinz JR, Owen MD, and Barrett M. (1997). Nicosulfuron, primisulfuron, and bentazon hydroxylation by corn (*Zea mays*), woolly cupgrass (*Eriochloa villosa*), and shattercane (*Sorghum bicolor*) cytochrome P-450. *Weed Science* **45**, 474 – 480.

Hoagland DR, and Arnon DI. (1938). The water-culture method for growing plants without soil. *Californian Agricultural Experimental Station Circulation* **347**, 1 – 32.

Hodgson E. (1985). *Microsomal mono-oxygenases*. Pergamon Press, New York.

Holton TA, and Cornish EC. (1995). Genetics and biochemistry of anthocyanin biosynthesis. *The Plant Cell* **7**, 1071 - 1083.

Holton TA, Brugliera F, Lester DR, Tanaka Y, Hyland CD, Menting JGT, Lu C-Y, Farcy E, Stevenson TW, and Cornish EC. (1993). Cloning and expression of cytochrome P450 genes controlling flower colour. *Nature (London)* **366**, 276 – 279.

Holtum JA, and Powles SB. (1991). Annual ryegrass: An abundance of resistance, a plethora of mechanisms. *Brighton Crop Protection Conference - Weeds* 1071 - 1078.

Holtum J, Matthews JM, Liljegren DR, Häusler RE, and Powles SB. (1991). Cross resistance to herbicides in annual ryegrass (*Lolium rigidum*). III. On the mechanism of resistance to diclofop-methyl. *Plant Physiology* **97**, 1026 - 1034.

Hotze M, Schröder G, and Schröder J. (1995). Cinnamate 4-hydroxylase from *Catharanthus roseus* and a strategy for the functional expression of plant cytochrome P_{450} protein as translational fusions with P_{450} reductase in *Escherichia coli*. *FEBS Letters* **374**, 345 – 350.

Hutchison J, Shapiro R, and Sweetser PB. (1984). Metabolism of chlorsulfuron by tolerant broadleaves. *Pesticide Biochemistry and Physiology* **22**, 243 - 247.

Hyde RJ, Hallahan DL, and Bowyer JR. (1996). Chlorotoluron metabolism in leaves of resistant and susceptible biotypes of the grass weed *Alopecurus myosuroides*. *Pesticide Science* **47**, 185 – 190.

I

Ichihara K, Yamakawa I, Kusunose E, and Kusunose M. (1979). Fatty acid ω- and (ω-1)-hydroxylation in rabbit intestinal mucosa microsomes. *Journal of Biochemistry* **86**, 139 - 146.

Inui H, Ueyama Y, Shiota N, Ahkawa Y, and Ohkawa H. (1999). Herbicide metabolism and cross-tolerance in transgenic potato plants expressing human CYP1A1. *Pesticide Biochemistry and Physiology* **64**, 33 – 46.

Ishimura Y, Ullrich V, and Peterson JA. (1971). Oxygenated cytochrome P-450 and its possible role in enzymic hydroxylation. *Biochemical and Biophysical Research Communications* **42**, 140 - 146.

Isseman I, and Green S. (1990). Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature (London)* **347**, 645 – 650.

J

Jablonkai I, and Hulesch A. (1996). Cytochrome P450 levels of monocot and dicot weeds and influence of herbicides, safeners and P450 inhibitors on enzyme content. *Proceedings of the Second International Weed Control Congress* 789 – 794.

Jacobson A, and Shimabukuro RH. (1984). Metabolism of diclofop-methyl in root-treated wheat and oat seedlings. *Journal of Agricultural and Food Chemistry* **32**, 742 – 746.

Johnson EF, Palmer CN, Griffin KJ, and Hsu M-H. (1996). Role of the peroxisome proliferator-activated receptor in cytochrome P450 4A gene regulation. *FASEB Journal* **10**, 1241 – 1248.

K

Karp F, Harris JL, and Croteau R. (1987). Metabolism of monoterpenes: Demonstration of the hydroxylation of (+)-sabinene to (+)-*cis*-sabinol by an enzyme preparation from sage (*Salvia officinalis*) leaves. *Archives of Biochemistry and Biophysics* **256**, 179 - 193.

Karp F, Mihaliak CA, Harris JL, and Croteau R. (1989). Monoterpene biosynthesis: Specificity of the hydroxylations of (-)-limonene by enzyme preparations from peppermint (*Mentha piperita*), spearmint (*Mentha spicata*), and perilla (*Perilla frutescens*) leaves. *Archives of Biochemistry and Biophysics* **276**, 219 - 226.

Karp F, Mihaliak CA, Harris JL, and Croteau R. (1990). Monoterpene biosynthesis: specificity of the hydroxylations of (-)-limonene by enzyme preparations from peppermint (*Mentha piperita*), spearmint (*Mentha spicata*), and Perilla (*Perilla frutescens*) leaves. *Archives of Biochemistry and Biophysics* **276**, 219 - 226.

Keller H, Dreyer C, Medin J, Mahfoudi A, Ozato K, and Wahli W. (1993). Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. *Proceedings of the National Academy of Science, U.S.A.* **90**, 2160 – 2164.

Kemp MS, and Caseley JC. (1987). Synergistic effects of 1-aminobenzotriazole on the phytotocity of chlorotoluron and isoproturon in a resistant population of black-grass (*Alopecurus myosuroides*). *Proceedings of 1987 British Crop Protection Conference – Weeds* **3**, 895 – 899.

Kjellbom P, Larsson C, Askerlund P, Schelin C, and Widell S. (1985). Cytochrome P-450/420 in plant plasma membranes: A possible component of the blue-light-reducible flavoprotein-cytochrome complex. *Photochemistry Photobiology* **42**, 779 - 783.

Kolattukudy P. (1980). Biopolyester membranes of plants: cutin and suberin. *Science* **208**, 990 - 1000.

Kolattukudy P. (1981). Structure, biosynthesis, and biodegradation of cutin and suberin. *Annual Review of Plant Physiology* **32**, 539 - 567.

Koop DR, Crump BL, Nordblom GD, and Coon MJ. (1985). Immunochemical evidence for induction of the alcohol-oxidizing cytochrome P-450 of rabbit liver microsomes by diverse agents: Ethanol, imidazole, trichloro-ethylene, acetone, pyrazole, and isoniazid. *Proceedings of the National Academy of Science, U.S.A.* **82**, 4065 – 4069.

Kreutz K, and Fonné-Pfister R. (1992). Herbicide-insecticide interactions in maize: malathion inhibits cytochrome P450-dependent primisulfuron metabolism. *Pesticide Biochemistry and Physiology* **42**, 232 - 240.

Kumaki K, Sato M, Kon H, and Nebert DW. (1978). Correlation of type I, type II, and reverse type I difference spectra with absolute changes in spin state of hepatic microsomal cytochrome P-450 iron from five mammalian species. *The Journal of Biological Chemistry* **253**, 1048 – 1058.

L

Lande R. (1983). The response to selection on major and minor mutations affecting a metrical trait. *Heredity* **50**, 47 – 65.

Larson RL, and Bussard JB. (1986). Microsomal flavonoid 3'-monooxygenase from maize seedlings. *Plant Physiology* **80**, 483 – 486.

Lau S-M, Harder PA, and O'Keefe DP. (1993). Low carbon monoxide affinity allene oxide synthase is the predominant cytochrome P450 in many plant tissues. *Biochemistry* **32**, 1945 - 1950.

Laudert D, Pfannschmidt U, Lottspeich F, Holländer-Czytko H, and Weiler, EW. (1996). Cloning, molecular and functional characterization of *Arabidopsis thaliana* allene oxide synthase (CYP74), the first enzyme of the octadecanoid pathway to jasmonates. *Plant Molecular Biology* **31**, 323 - 335.

Leah JM, Caseley JC, Riches CR, and Valverde BE. (1997). Effect of mono-oxygenase inhibitors on uptake, metabolism and phytotoxicity of propanil in resistant biotypes of Jungle-rice, *Echinochloa colona*. *Pesticide Science* **49**, 141 – 147.

Lee SS-T, Pineau T, Drago J, Lee EJ, Owens JW, Kroetz DL, Fernandez-Salguero PM, Westphal H, and Gonzalez FJ. (1995). Targeted disruption of the α isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. *Molecular and Cellular Biology* **15**, 3012 – 3022.

Lindberg RLP, and Negishi M. (1989). Modulation of specificity and activity in mammalian cytochrome-P-450. *Methods in Enzymology* **202**, 741-752.

Logemann E, Parniske M, and Hahlbrook K. (1995). Modes of expression and common structural features of the complete phenylalanine ammonia-lyase gene family in parsley. *Proceedings of the National Academy of Science, U.S.A.* **92**, 5905 – 5909.

Lupien S, Karp F, Wildung M, and Croteau R. (1999). Regiospecific cytochrome P450 limonene hydroxylases from mint (*Mentha*) species: cDNA isolation, characterization, and functional expression of (-)-4*S*-limonene-3-hydroxylase and (-)-4*S*-limonene-6-hydroxylase. *Archives of Biochemistry and Biophysics* **368**, 181 - 192.

M

Madyastha K, Meehan TD, and Coscia CJ. (1976). Characterization of a cytochrome P-450 dependent monoterpene hydroxylase from a higher plant *Vinca rosea*. *Biochemistry* **15**, 1097 - 1102.

Madyastha KM, Ridgway JE, Dwyer JG, and Coscia CJ. (1977). Subcellular localisation of a cytochrome P-450-dependent monooxygenase in vesicles of the higher plant *Catharanthus roseus*. *Journal of Cell Biology* **72**, 302 - 313.

Makeev AM, Makoviechuk AY, and Chkanikov DI. (1977). Microsomal hydroxylation of 2,4-dichlorophenoxyacetic acid herbicide in plants, cucumbers and peas. *Doklady Bot. Soc. Sci.* **233**, 36 - 38.

Maneechote C, Preston C, and Powles SB. (1997). A diclofop-methyl-resistant *Avena sterilis* biotype with a herbicide-resistant acetyl-coenzyme A carboxylase and enhanced metabolism of diclofop-methyl. *Pesticide Science* **49**, 105 – 114.

Mangold U, Eichel J, Batschauer A, Lanz T, Kaiser T, Spangenberg G, Werck-Reichhart D, and Schröder J. (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 thaliana*. *Plant Science* **96**, 129 – 136.

Martin E, and Morton RK. (1957). Haem pigments of cytoplasmic particles from non-photosynthetic plant tissues. *Biochemical Journal* **65**, 404 - 413.

Mason HS. (1957). Mechanisms of oxygen metabolism. *Science* **125**, 1185 - 1188.

Mason HS. (1965). Oxidases. *Annual Review of Biochemistry* **34**, 595 - 634.

Mason HS, Fowlks WL, and Peterson E. (1955). Mechanisms of oxygen metabolism. *Journal of the American Chemical Society* **79**, 2914 - 2915.

Matthews JM. (1994) Management of herbicide resistant weed populations. In *Herbicide Resistance in Plants: Biology and Biochemistry* (S.B. Powles, and J.A.M. Holtum, eds.). pp. 317 - 335. Lewis Publishers, Boca Raton, FL.

Matzke K, and Riederer M. (1991). A comparative study into the chemical constitution of cutins and suberins from *Picea abies* (L.) Karst, *Quercus robur* L., and *Fagus sylvatica* L. *Planta* **185**, 233 – 245.

Mazur BJ and Fulco SC. (1989). The development of herbicide resistant crops mechanisms and agronomic aspects of herbicide resistance. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**, 441 – 470.

McFadden J, Frear DS, and Mansager ER. (1989). Aryl hydroxylation of diclofop by a cytochrome P450-dependent monooxygenase from wheat. *Pesticide Biochemistry And Physiology* **34**, 92 - 100.

McFadden JJ, Gronwald JW, and Eberlein CV. (1990). *In vitro* hydroxylation of bentazon by microsomes from naphthalic anhydride-treated corn shoots. *Biochemical and Biophysical Research Communications* **168**, 206 – 213.

McGarvey DJ, and Croteau R. (1995). Terpenoid metabolism. *The Plant Cell* **7**, 1015 - 1026.

Meehan T, and Coscia CJ. (1973). Hydroxylation of geraniol and nerol by a monooxygenase from *Vinca rosea*. *Biochemical and Biophysical Research Communications* **53**, 1043 - 1048.

Meijer AH, De Waal A, and Verpoorte R. (1993a). Purification of the cytochrome P450 enzyme geraniol 10-hydroxylase from cell cultures of *Catharanthus roseus*. *Journal of Chromatography* **635**, 237 - 249.

Meijer AH, Souer E, Verpoorte R, and Hoge JHC. (1993b). Isolation of cytochrome P-450 cDNA clones from the higher plant *Catheranthus roseus* by a PCR strategy. *Plant Molecular Biology* **22**, 379 - 383.

Menendez J, and De Prado R. (1996). Diclofop-methyl cross-resistance in a chlorotoluron-resistant biotype of *Alopecurus myosuroides*. *Pesticide Biochemistry and Physiology* **56**, 123 – 133.

Meyer K, Cusumano JC, Somerville CR, Chapple CC. (1996). Ferulate-5-hydroxylase from *Arabidopsis thaliana* defines a new family of cytochrome P450-dependent monooxygenases. *Proceedings of the National Academy of Science, U.S.A.* **93**, 6869 – 6874.

Meyer K, Shirley AM, Cusumano JC, Bell-Lelong DA, and Chapple CC. (1998). Lignin monomer composition is determined by the expression of a cytochrome P450-dependent monooxygenases in *Arabidopsis*. *Proceedings of the National Academy of Science, U.S.A.* **95**, 6619 - 6623.

Miura Y, and Fulco AJ. (1975). ω-1, ω-2 and ω-3 hydroxylation of long-chain fatty acids, amides and alcohols by a soluble enzyme system from *Bacillus megaterium*. *Biochimica et Biophysica Acta* **388**, 305 - 317.

Mizutani M, Ward E, DiMaio J, Ohta D, Ryals J, And Sato R. (1993a). Molecular cloning and sequencing of a cDNA encoding mung bean cytochrome P450 (P450C4H) possessing cinnamate 4-hydroxylase activity. *Biochemical and Biophysical Research Communications* **190**, 875 - 880.

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

Monaghan N. (1980). The biology and control of *Lolium rigidum* as a weed of wheat. *Weed Research* **20**, 117 - 121.

Moreland DE, Corbin FT, and McFarland JE. (1993). Oxidation of multiple substrates by corn shoot microsomes. *Pesticide Biochemistry and Physiology* **47**, 206 – 214.

Moreland DE, Fleischmann TJ, Corbin FT, and McFarland JE. (1996). Differential metabolism of the sulfonylurea herbicide prosulfuron (CGA-152005) by plant microsomes. *Z. Naturforsch* **51C**,

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

Murakami K, and Mason HS. (1967). An electron spin resonance study of microsomal Fe_χ. *The Journal of Biological Chemistry* **242**, 1102 - 1110.

Murphy PJ, and West CA. (1969). The role of mixed function oxidases in kaurene metabolism in *Echinocystis macrocarpa* Greene endosperm. *Archives of Biochemistry and Biophysics* **133**, 395 - 407.

N

Nebert DW, and Gonzalez FJ. (1987). P450 genes: Structure, evolution and regulation. *Annual Review of Biochemistry* **56**, 945 - 993.

Nebert DW, Nelson DR, Coon MJ, Estabrook RW, Feyereisen R, Fujii-Kuriyama Y, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Loper JC, Sato R, Waterman MR, and Waxman DJ. (1991). The P450 superfamily: Update on new sequences, gene mapping and recommended nomenclature. *DNA and Cell Biology* **10**, 1 - 14.

Nedelkina S, Jupe SC, Blee KA, Schalk M, Werck-Reichhart D, and Bolwell GP. (1999). Novel characteristics and regulation of a divergent cinnamate 4-hydroxylase (CYP73A15) from french bean: engineering expression in yeast. *Plant Molecular Biology* **39**, 1079 – 1090.

Neish A. (1961). Formation of *m*- and ρ-coumaric acids by enzymatic desamination of the corresponding isomers of tyrosine. *Phytochemistry* **1**, 1 - 24.

Nelson DR, Kamataki T, Waxman DJ, Guengerich FP, Estabrook RW, Feyereisen R, Gonzalez FJ, Coon MJ, Gunsalus IC, Gotoh O, Okuda K, and Nebert DW. (1993). The P450 superfamily: Update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA and Cell Biology* **12**, 1 - 51.

Nelson DR. (1999). Cytochrome P450 and the individuality of species. *Archives of Biochemistry and Biophysics* **369**, 1 – 10.

Nicholson RL, and Hammerschmidt R. (1992). Phenolic compounds and their role in disease resistance. *Annual Review of Phytopathology* **30**, 369 - 389.

O

Ohme-Takagi M, and Shinshi H. (1995). Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. *The Plant Cell* **7**, 173 – 182.

Ohtomo R, Kobayashi K, Muraoka S-i, Ohkuma M, Ohta A, and Takagi M. (1996). Peroxisome proliferators activate cytochrome P450 genes in an alkane-assimilating yeast, *Candida maltosa*. *Biochemical and Biophysical Research Communications* **222**, 790 – 793.

O'Keefe D, and Leto KJ. (1989). Cytochrome P450 from the mesocarp of avocado (*Persea americana*). *Plant Physiology* **89**, 1141 - 1149.

O'Keefe D, Tepperman JM, Dean C, Leto KJ, Erbes DL, and Odell JT. (1994). Plant expression of a bacterial cytochrome P450 that catalyses activation of a sulfonylurea pro-herbicide. *Plant Physiology* **105**, 473 – 482.

Omura T. (1993). In *Cytochrome P450* (T. Omura, Y. Ishimura and Fujii-Kuriyama, eds). 2nd ed. VCH, Weiheim.

Omura T, and Sato R. (1962). A new cytochrome in liver microsomes. *The Journal of Biological Chemistry* **237**, 1375 - 1376.

Omura T, and Sato R. (1963). Fractional solubilization of haemoproteins and partial purification of carbon monoxide-binding cytochrome from liver microsomes. *Biochimica et Biophysica Acta* **71**, 224 - 226.

Omura T, and Sato R. (1964a). The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *The Journal of Biological Chemistry* **239**, 2370 - 2378.

Omura T, and Sato R. (1964b). The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification and properties. *The Journal of Biological Chemistry* **239**, 2379 - 2385.

Ortiz de Montellano PR, and Kunze KL. (1980). Self-catalyzed inactivation of hepatic cytochrome P-450 by ethynyl substrates. *The Journal of Biological Chemistry* **255**, 5578 – 5585.

Ortiz de Montellano PR, and Mathews JM. (1981). Autocatalytic alkylation of the cytochrome P-450 prosthetic haem group by 1-aminobenzotriazole. *Biochemistry Journal* **195**, 761 - 764.

Ortiz de Montellano PR, and Mico BA. (1981). Destruction of cytochrome P-450 by allylisopropylacetamide is a suicidal process. *Archives of Biochemistry and Biophysics* **206**, 43 – 50.

Ortiz de Montellano PR, and Reich NO. (1984). Specific inactivation of hepatic faty acid hydroxylases by acetylenic fatty acids. *The Journal of Biological Chemistry* **259**, 4136 – 4141.

Ortiz de Montellano PR, and Reich NO. (1986) Inhibition of cytochrome P-450 enzymes. In *Cytochrome P-450: Structure, Mechanism and Biochemistry*. (P. Ortiz de Montellano, ed), pp. 273 - 314. Plenum Press, New York.

P

Palma JM, Garrido M, Rodríguez-García MI, and del Río LA. (1991). Peroxisome proliferation and oxidative stress mediated by activated oxygen species in plant peroxisomes. *Archives of Biochemistry and Biophysics* **287**, 68 – 74.

Pan Z, Durst F, Werck-Reichhart D, Gardner HW, Camara B, Cornish K, and Backhaus RA. (1995). The major protein of guayule rubber particles is a cytochrome P450. *The Journal of Biological Chemistry* **270**, 8487 - 8494.

Persans MW, and Schuler MA. (1995). Differential induction of cytochrome P450-mediated triasulfuron metabolism by naphthalic anhydride and triasulfuron. *Plant Physiology* **109**, 1483 – 1490.

Peterson J, Ullrich V and Hildebrandt AG. (1971). Metyrapone interaction with *Pseudomonas putida* cytochrome P-450. *Archives of Biochemistry and Biophysics* **145**, 531 - 542.

Pinot F, Salan J-P, Bosch H, Lesot A, Mioskowski C, and Durst F. (1992). ω-Hydroxylation of Z9-octadecenoic, Z9,10-epoxystearic and 9,10-dihydroxystearic acids by microsomal cytochrome P450 systems from *Vicia sativa. Biochemical and Biophysical Research Communications* **184**, 183 - 193.

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

Pinot F, Alayrac C, Mioskowski C, Durst F, and Salaün J-P. (1994). New cytochrome P-450-dependent reactions from wheat – terminal and sub-terminal hydroxylation of oleic acid by microsomes from naphthalic acid anhydride and phenobarbitol induced wheat seedlings. *Biochemical and Biophysical Research Communications* **198**, 795 – 803.

Plewa MJ, and Wagner ED. (1993) Activation of promutations by green plants. *Annual Review of Genetics* **27**, 93 – 113.

Porter TD, and Coon MJ. (1991). Multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms. *The Journal of Biological Chemistry* **266**, 13469 – 13472.

Potter S, Moreland DE, Kreuz K, and Ward E. (1995). Induction of cytochrome P450 genes by ethanol in maize. *Drug Metabolism and Drug Interactions* **12**, 317 – 327.

Powles SB, and Matthews JM. (1991). Multiple herbicide resistance in annual ryegrass (*Lolium rigidum*): A driving force for the adoption of integrated weed management. In *Resistance '91: Achievements and Developments in Combating Pesticide Resistance* (I. Denholm, A.L. Devonshire and D.W. Hollomon, eds.). pp. 75 – 97, Elselvier Applied Science , London.

Powles SB, Preston C, Bryan IB, and Jutsum AR. (1997). Herbicide resistance: Impact and management. *Advances in Agronomy* **58**, 57 - 93.

Practical Workshop in Basic Recombinant DNA Techniques: Applications to Plant Breeding (1997). Compiled by Peter Langridge, Angelo Karakousis and Jan Nield.   Centre for Cereal Biotechnology, University of Adelaide, Glen Osmond SA 5064.

Preston C, and Powles SB. (1998). Amitrole inhibits diclofop metabolism and synergises diclofop-methyl in a diclofop-methyl-resistant biotype of *Lolium rigidum*. *Pesticide Biochemistry and Physiology* **62**, 179 – 189.

Preston C, Tardif FJ, Christopher JT, and Powles SB. (1996). Multiple resistance to dissimilar herbicide chemistries in a biotype of *Lolium rigidum* due to enhanced activity of several herbicide degrading enzymes. *Pesticide Biochemistry and Physiology* **54**, 123 - 134.

R

Rademacher W, Fritsch H, Graebe JE, Sauter H, and Jung J. (1987). Tetcyclasis and triazole-type plant growth retardants: their influence on the biosynthesis of gibberellins and other metabolic processes. *Pesticide Science* **21**, 241 - 252.

Ramarao M, and Kemper B. (1995). Substitution at residue 473 confers progesterone 21-hydroxylase cytochrome P450 2C2. *Molecular Pharmacology* **48**, 417 – 424.

Rando RR. (1974). Chemistry and ezymology of k_{cat} inhibitors. *Science* **185**, 320 – 324.

Ray T. (1982). The mode of action of chlorsulfuron: A new herbicide for cereals. *Pesticide Biochemistry and Physiology* **17**, 10 - 17.

Ray T. (1984). Site of action of chlorsulfuron: Inhibition of valine and isoleucine synthesis in plants. *Plant Physiology* **75**, 827 - 831.

Reddy JK, Warren JR, Reddy MK, and Lalwani ND. (1982). Hepatic and renal effects of peroxisome proliferators: Biological implications. *Annals of the New York Academy of Sciences* **386**, 81 – 110.

Reddy JK, Goel SK, Nemali MR, Carrino JJ, Laffler TG, Reddy MK, Sperbeck SJ, Osumi T, Hashimoto T, Lalwani ND, and Rao MS. (1986). Transcriptional regulation of peroxisomal fatty acyl-CoA oxidase and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase in rat liver by peroxisome proliferators. *Proceedings of the National Academy of Science, U.S.A.* **83**, 1747 – 1751.

Reichhart D, Salaün J-P, Benveniste I, and Durst F. (1979). Induction by manganese, ethanol, phenobarbitol, and herbicides of microsomal cytochrome P-450 in higher plant tissues. *Archives of Biochemistry and Biophysics* **196**, 301 - 303.

Reichhart D, Salaün J-P, Benveniste I, and Durst F. (1980). Time course induction of cytochrome P450, NADPH-cytochrome c reductase, and cinnamic acid 4-hydroxylase by manganese, ethanol, phenobarbitol and herbicides in higher plant tissues. *Plant Physiology* **66**, 600 - 604.

Reichhart D, Simon A, Durst F, Mathews JM and Ortiz de Montellano PR. (1982). Autocatalytic inactivation of plant cytochrome P-450 enzymes: Selective inactivation of cinnamic acid 4-hydroxylase from *Helianthus tuberosus* by 1-aminobenzotriazole. *Archives of Biochemistry and Biophysics* **216**, 522 – 529.

Rich P, and Lamb CJ. (1977). Biophysical and enzymological studies upon the interaction of *trans*-cinnamic acid with higher plant microsomal cytochromes P-450. *European Journal of Biochemistry* **72**, 353 - 360.

Rösler J, Krekel F, Amrhein N, and Schmid J. (1997). Maize phenylalanine ammonia-lyase has tyrosine ammonia-lyase activity. *Plant Physiology* **113**, 175 – 179.

Ruegger M, Meyer K, Cusumano JC, and Chapple. (1999). Regulation of ferulate-5-hydroxylase expression in *Arabidopsis* in the context of sinapate ester biosynthesis. *Plant Physiology* **119**, 101 – 110.

Rushton PJ, Torres JT, Parniske M, Wernert P, Hahlbrock K, and Somssich IE. (1996). Interaction of elicitor-induced DNA binding proteins with elicitor response elements in the promoters of parsley PR1 genes. *EMBO Journal* **15**, 5690 – 5700.

Rushton PJ, and Somssich IE. (1998). Transcriptional control of plant genes responsive to pathogens. *Current Opinion in Plant Biology* **1**, 311 – 315.

Russell DW. (1971). The metabolism of aromatic compounds in higher plants. X. properties of the cinnamic acid 4-hydroxylase of pea seedlings and some aspects of its metabolic and developmental control. *The Journal of Biological Chemistry* **246**, 3870 - 3878.

Russell DW, and Conn EE. (1967). The cinnamic acid 4-hydroxylase of pea seedlings. *Archives of Biochemistry and Biophysics* **122**, 256 - 258.

Ryan P, and Owen WJ. (1982). The mechanism of selectivity of chlorotoluron between cereals and grass weeds. *Proceedings of the 1982 British Crop Protection Conference - Weeds* 317 - 324.

Ryan PJ, Gross D, Owen WJ, Laanio TL. (1981). The metabolism of chlorotoluron, diuron and CGA 43 057 in tolerant and susceptible plants. *Pesticide Biochemistry and Physiology* **16**, 213 - 221.

S

Salaün J-P and Benveniste I. (1998). On the role of cytochrome P450-dependent fatty acid hydroxylases from plant. *Recent Research & Development in Lipid Research* **2**, 249 – 262.

Salaün J-P, and Helvig C. (1995). Cytochrome P450-dependent oxidation of fatty acids. *Drug Metabolism and Drug Interactions* **12**, 261 - 283.

Salaün J-P, Benveniste I, Reichhart D, and Durst F. (1978). A microsomal (cytochrome P-450)-linked lauric-acid-monooxygenase from aged Jerusalem-artichoke-tuber tissues. *European Journal of Biochemistry* **90**, 155 - 159.

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

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

Salaün J-P, Simon A, and Durst F. (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, Simon A, Durst F, Reich NO, and Ortiz de Montellano PR. (1988). Differential inactivation of a plant lauric acid ω- and in-chain-hydroxylases by terminally unsaturated fatty acids. *Archives of Biochemistry and Biophysics* **260**, 540 – 545.

Sambrook J, Fritsch EF, and Manniatis T. (1989). *Molecular Cloning: A Laboratory Manual.* 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sandermann H., Jr. (1992). Plant metabolism of xenobiotics. *Trends in Biochemical Sciences* **17**, 82 – 84.

Sandermann H., Jr. (1994). Higher plant metabolism of xenobiotics: the 'Green Liver' concept. *Pharmacogenetics* **4**, 225-241.

Sandermann H., Jr., Musick TJ, Aschbacher PW. (1992). Animal bioavailability of a 3,4-dichloroaniline lignin metabolite fraction from wheat. *Journal of Agriculture and Food Chemistry* **40**, 2001 – 2007.

Sandler M, and Smith HJ. (1989). Introduction to the use of enzyme inhibitors as drugs. In *Design of Enzyme Inhibitors as Drugs* (M. Sandler and H.J. Smith, eds.). pp 1 – 18. University Press, Oxford.

Sasaki Y, Konishi T, and Nagano Y. (1995). The compartmentation of acetyl-CoA carboxylase in plants. *Plant Physiology* **108**, 445 -

Schmelzer, E., Krüger-Lebus S, and Hahlbrock K. (1989). Temporal and spatial patterns of gene expression around sites of attempted fungal infection in parsley leaves. *The Plant Cell.* **1**, 993 - 1001.

Schneider J, and Nakanishi K. (1983). A new class of sweet potato phytoalexins. *Journal of the Chemical Society - Chemical Communications* 353 - 355.

Scheel D, and Sandermann Jr H. (1975). On the mechanism of light induction of plant microsomal cinnamic acid 4-hydroxylase. *Planta* **124**, 211 – 214.

Schuler M. (1996). Plant cytochrome P450 monooxygenases. *Critical Reviews in Plant Sciences* **15**, 235 - 284.

Secor J, and Cséke C. (1988). Inhibition of acetyl-CoA carboxylase activity by haloxyfop and tralkoxydim. *Plant Physiology* **86**, 10 - 12.

Secor J, Cséke C, and Owen WJ. (1989). The discovery of the selective inhibition of acetyl coenzyme A carboxylase activity by two classes of graminicides. *Proceedings of the British Crop Protection Conference – Weeds* **3**, 145 – 154.

Sewalt VJ, Ni W, Blount JW, Jung HG, Masoud SA, Howles PA, Lamb C, and Dixon RA. (1997). Reduced lignin content and altered lignin composition in transgenic tobacco down-regulated in expression of L-phenylalanine ammonia-lyase or cinnamate 4-hydroxylase. *Plant Physiology* **115**, 41 - 50.

Sharma R, Lake BG, and Gibson GG. (1988). Co-induction of microsomal cytochrome P-450 and the peroxisomal fatty acid β–oxidation pathway in the rat by clofibrate and di-(2-ethylhexyl)phthalate. *Biochemical Pharmacology* **37**, 1203 – 1206.

Shimabukuro R, Walsh WC, and Hoerauf RA. (1979). Metabolism and selectivity of diclofop-methyl in wild oat and wheat. *Journal of Agricultural and Food Chemistry* **27**, 615 - 623.

Shimabukuro R, Lamoureux GL and Frear DS. (1982) Pesticide metabolism in plants: Reactions and mechanisms. In *Biodegradation of Pesticide* (F. Matsumura and C.R. Krishna Murti., eds.), pp. 21 - 66. Plenum Press, New York.

Shimada M, Kuroda H, and Higuchi T. (1973). Evidence for the formation of methoxyl groups of ferulic and sinapic acids in *Bambusa* by the same *O*-methyltransferase. *Phytochemistry* **12**, 2873 - 2875.

Shiota H, Inui H, and Ohkawa H, (1996). Metabolism of the herbicide chlorotoluron in transgenic tobacco plants expressing the fused enzyme between rat cytochrome P4501A1 and yeast NADPH-cytochrome P450 oxidoreductase. *Pesticide Biochemistry and Physiology* **54**, 190 - 198.

Singh S, Kirkwood RC, and Marshall G. (1998a). Effect of ABT on the activity and rate of degradation of isoproturon in susceptible and resistant biotypes of *Phalaris minor* and in wheat. *Pesticide Science* **53**, 123 - 132.

Singh S, Kirkwood RC, and Marshall G. (1998b). Effect of the monooxygenase inhibitor piperonyl butoxide on the herbicidal activity and metabolism of isoproturon in herbicide resistant and susceptible biotypes of *Phalaris minor* and wheat. *Pesticide Biochemistry and Physiology* **59**, 143 - 153.

Solecka D, Boudet A-M, and Kacperska A. (1999). Phenylpropanoid and anthocyanin changes in low temperature-treated winter oilseed rape leaves. *Plant Physiology and Biochemistry* **37**, 491 - 496.

Soliday CL, and Kolattukudy PE. (1977). Biosynthesis of cutin. ω-Hydroxylation of fatty acids by a microsomal preparation from germinating *Vicia faba*. *Plant Physiology* **59**, 1116 - 1121.

Soliday CL, and Kolattukudy PE. (1978). Midchain hydroxylation of 16-hydroxypalmitic acid by the endoplasmic reticulum fraction from germinating *Vicia faba*. *Archives of Biochemistry and Biophysics* **188**, 338 – 347.

Song B-J, Matsunaga T, Hardwick JP, Park SS, Veech RL, Yang CS, Gelboin HV, and Gonzalez FJ. (1987). Stabilization of cytochrome P450j messenger ribonucleic acid in the diabetic rat *Molecular Endocrinology* **1**, 542 – 547.

Song B-J, Veech RL, Park SS, Gelboin HV, and Gonzalez FJ. (1989). Induction of rat hepatic *N*-Nitrosodimethylamine demethylase by acetone is due to protein stabilization. *The Journal of Biological Chemistry* **264**, 3568 – 3572.

Song W-C, and Brash AR. (1991). Purification of an allene oxide synthase and identification of the enzyme as a cytochrome P-450. *Science* **253**, 781 - 784.

Song W-C, Baertschi SW, Boeglin WE, Harris TM, and Brash AR. (1993). Formation of epoxyalcohols by a purified allene oxide synthase. *The Journal of Biological Chemistry* **268**, 6293 - 6298.

Stotz G, de Vlaming P, Wiering H, Schram AW, and Forkmann. (1985). Genetic and biochemical studies on flavonoid 3'-hydroxylation in flowers of *Petunia hybrida*. *Theoretical and Applied Genetics* **70**, 300 -.

Swain SM, and Reid JB. (1992). Internode length in *Pisum*; a new allele at the *Lh* locus. *Phsiologica Planta* **86**, 124 – 130.

Swain SM, Reid JB, and Ross JJ. (1993). Seed development in *Pisum*. The *lh^i* allele reduces gibberellin levels in developing seeds, and increases seed abortion. *Planta* **191**, 482 – 488.

Swain SM, Ross JJ, Kamiya Y and Reid JB. (1995). Gibberellin and seed development. *Plant Cell Physiology* **36**, S110.

Sweetser PB. (1985). Safening of sulfonylurea herbicides to cereal crops: mode of herbicide antidote action. *British Crop Protection Conference – Weeds* **3**, 1147 – 1154.

Sweetser P, Schow GS, and Hutchison JM. (1982). Metabolism of chlorsulfuron by plants, biological basis for selectivity of a new herbicide for cereals. *Pesticide Biochemistry and Physiology* **17**, 18 - 23.

T

Tanaka Y, Tsuda S, and Kusumi T. (1998). Metabolic engineering to modify flower colour. *Plant and Cell Physiology* **39**, 1119 - 126.

Tardif FJ, and Powles SB. (1993) Target site-based resistance to herbicides inhibiting acetyl-coA carboxylase. *Proceedings Brighton Crop Protection Conference - Weeds* 533 - 540, British Crop Protection Council, Surrey, UK.

Tardif FJ, and Powles SB. (1994). Herbicide multiple resistance in a *Lolium rigidum* biotype is endowed by multiple mechanisms: isolation of a subset with resistant acetyl-Co-A carboxylase. *Physiologica Plantarum* **91**, 488 - 494.

Tardif FJ, Holtum JAM, and Powles SB. (1993). Occurrence of a herbicide-resistant acetyl-coenzyme A carboxylase mutant in annual ryegrass (*Lolium rigidum*) selected by sethoxydim. *Planta* **190**, 176 - 181.

Tardif FJ, Preston C, and Powles SB. (1997). Mechanisms of multiple herbicide resistance in *Lolium rigidum*. In *Weed and Crop Resistance to Herbicides* (R. de Prado, J. Jorrín, and L. García-Torres, eds). pp 117 – 124. Kluwer Academic Publishers, Dordrecht, The Netherlands

Terrell, E. (1968). A taxonomic revision of the genus *Lolium*. *Technical Bulletin 1392, United States Department of Agriculture*. 1 - 57.

Teutsch HG, Hasenfratz MP, Lesot A, Stoltz C, Garnier J-M, Jeltsch J-M, Durst F, and Werck-Reichhart D. (1993). Isolation and sequence of a cDNA encoding the Jerusalem artichoke cinnamate hydroxylase, a major plant cytochrome P450 involved in the general phenylpropanoid pathway. *Proceedings of the National Academy of Science, U.S.A.* **90**, 4102 - 4106.

Thalacker FW, Swanson HR, and Frear DS. (1994). Characterization, purification, and reconstitution of an inducible cytochrome P450-dependent triasulfuron hydroxylase from wheat. *Pesticide Biochemistry and Physiology* **49**, 209 – 223.

Threlfall D, and Whitehead IM. (1988). Co-ordinated inhibition of squalene synthetase and induction of enzymes of sesquiterpenoid phytoalexin biosynthesis in cultures of *Nicotiana tabacum*. *Phytochemistry* **27**, 2567 - 2580.

Tijet N, Helvig C, Pinot F, Le Bouquin R, Lesot A, Durst F, Salaün J-P, and Benveniste I.. (1998). Functional expression in yeast and characterization of a clofibrate-inducible plant cytochrome P-450 (CYP94A1) involved in cutin monomers synthesis. *Biochemical Journal* **332**, 583 – 589.

Tsai R, Yu CA, Gunsalus IC, Peisach J, Blumberg W, Orme-Johnson WH, and Beinert H. (1970). Spin-state changes in cytochrome P-450$_{cam}$ on binding of specific substrates. *Proceedings of the National Academy of Sciences, U.S.A.* **66**, 1157 - 1163.

Tysoe R. (1974). The control of *Avena fatua* in winter sown cereals with chlorotoluron. *Proceedings of the 12th British Weed Control Conference*, 41.

U

Uegaki R, Kubo S, and Fujimori T. (1988). Stress compounds in the leaves of *Nicotiana undulata* induced by TMV inoculation. *Phytochemistry* **27**, 365 – 368.

Ullrich V, Cohen B, Cooper DY, and Estabrook RW. (1968) Reactions of hemoprotein P-450. In *Structure and Function of Cytochromes*. (K. Okunuki, M.D. Kamen, and I. Sekuzu, eds), pp. 649 - 655. University of Tokyo Press, Tokyo.

Uritani I, and Asahi T. (1980). Respiration and related metabolic activity in wounded and infected tissues. In *The Biochemistry of Plants*, Vol. 2. (D. Davies, ed), pp.463 – 485. Academic Press, New York.

V

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

Vick BA, and Zimmerman DC. (1984). Biosynthesis of jasmonic acid by several plant species. *Plant Physiology* **75**, 458 - 461.

Vick BA, and Zimmerman DC. (1987). Pathways of fatty acid hydroperoxide metabolism in spinach leaf chloroplasts. *Plant Physiology* **85**, 1073 - 1078.

W

Wahli W, and Martinez E. (1991). Superfamily of steroid nuclear receptors: positive and negative regulators of gene expression. *The FASEB Journal* **5**, 2243 – 2249.

Walton T, and Kolattukudy PE. (1972). Enzymatic conversion of 16-hydroxypalmitic acid into 10,16-hydroxypalmitic acid in *Vicia faba* epidermal extracts. *Biochemical and Biophysical Research Communications* **46**, 16 - 21.

Wan C-Y, and Wilkins TA. (1994). A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L.). *Analytical Biochemistry* **223**, 7 – 12.

Watson DG, Rycroft DS, Freer IM, and Brooks CJW. (1985). Sesquiterpenoid phytoalexins from suspended callus cultures of *Nicotiana tabacum*. *Phytochemistry* **24**, 2195 – 2200.

Weissbart D, Salaün J-P, Durst F, Pflieger P, and Mioskowski C. (1992). Regioselectivity of a plant lauric acid omega hydroxylase. Omega hydroxylation of *cis* and *trans* unsaturated lauric acid analogs and epoxygenation of the terminal olefin by plant cytochrome P-450. *Biochimica et Biophysica Acta* **1124**, 135 - 142.

Werck-Reichhart D. (1995a). Herbicide metabolism and selectivity: Role of cytochrome P450. *Brighton Crop Protection Conference - Weeds* 813 - 822.

Werck-Reichhart D. (1995b). Cytochromes P450 in phenylpropanoid metabolism. *Drug Metabolism and Drug Interactions* **12**, 221 - 243.

Werck-Reichhart D. (2000). Cytochromes P450 for engineering herbicide tolerance. *Trends in Plant Science* **5**, 1360- 1385.

West CA. (1980). Hydroxylases, monooxygenases and cytochrome P-450. In *The Biochemistry of Plants* (P. Stumpf, and E.E. Conn, eds). Vol 2. pp. 317 – 364. Academic Press, NY.

Whetten R, and Sederoff RO. (1995). Lignin biosynthesis. *The Plant Cell* **7**, 1001 - 1013.

White RE, and Coon MJ. (1982). Heme ligand replacement reactions of cytochrome P-450. Characterisation of the bonding atom of the axial ligand *trans* to thiolate as oxygen. *The Journal of Biological Chemistry* **257**, 3073 - 3083.

Whitehead I, Ewing DF, and Threlfall DR. (1988). Sesquiterpenoids related to the phytoalexin debneyol from elicited cell suspension cultures of *Nicotiana tabacum*. *Phytochemistry* **28**, 775 - 779.

Whitehead IM, Threlfall DR, and Ewing DF. (1989). 5-*Epi*-aristolochene is a common precursor of the sesquiterpenoid phytoalexins capsidol and debneyol. *Phytochemistry* **28**, 775 – 779.

Winkler RG, and Helentjaris T. (1995). The maize *Dwarf3* gene encodes a cytochrome P450-mediated early step in gibberellin biosynthesis. *The Plant Cell* **7**, 1307 - 1317.

Y

Ye Z-H. (1996). Expression patterns of the cinnamic acid 4-hydroxylase gene during lignification in *Zinnia elegans*. *Plant Science* **121**, 131 – 141.

Young O, and Beevers H. (1976). Mixed function oxidases from germinating castor bean endosperm. *Phytochemistry* **15**, 379 - 385.

Z

Zhu Y, Alvares K, Huang Q, Rao MS, and Reddy JK. (1993). Cloning of a new member of the peroxisome proliferator-activated receptor gene family from mouse liver. *The Journal of Biological Chemistry* **268**, 26817 – 26820.

Zimmerlin A, and Durst F. (1990). Xenobiotic metabolism in plants: Aryl hydroxylation of diclofop by a cytochrome P-450 enzyme from wheat. *Phytochemistry* **29**, 1729 - 1732.

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

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

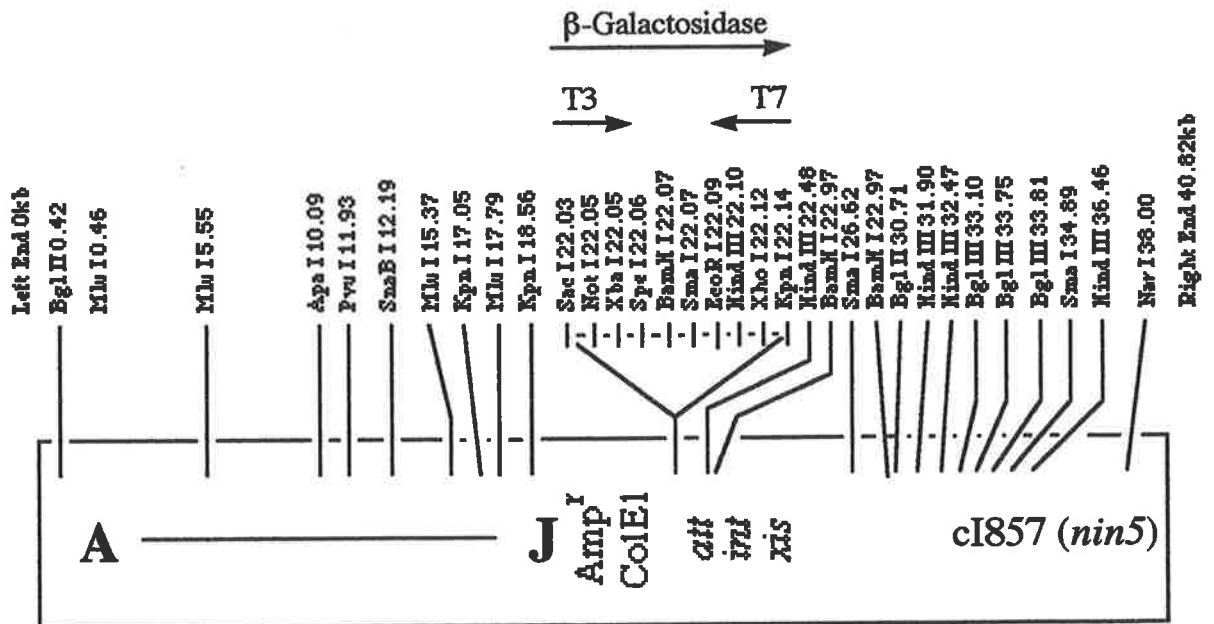Zimmerman DC, and Vick BA. (1970). Hydroperoxide isomerase. *Plant Physiology* **46**, 445 - 453.

Appendices

Appendix A

Lambda Zap® II Insertion Vector

40,820bp

Appendix B

Common Buffers & Solutions

1x Alkaline Electrophoresis Buffer

NaOH	50 N
EDTA, pH 8.0	1m M

6x Alkaline Gel Loading Buffer

NaOH	300 mN
EDTA, pH 8.0	6 mM
Ficoll, Type 400	18% (w/v)
Bromocresol green	0.15% (w/v)
Xylene cyanol FF	0.25%

Alkaline Lysis Solution I

Glucose	50 mM
EDTA	10 mM
Trizma base	25 mM
pH 8.0	

Denhardt's Reagent

Ficoll, Type 400	1% (w/v)
(approx. molecular weight 400,000	
Polyvinylpyrrolidone	1% (w/v)
(average molecular weight 360,000	
Bovine serum albumin	1% (w/v)
(BSA, Fraction V)	

Stored at -20°C

Hoagland's Nutrient Solution

KH_2PO_4	0.5 mM
K_2SO_4	0.4 mM
$MgSO_4.7H_2O$	1 mM
$Ca(NO_3)_2.4H_2O$	1.67 mM
KNO_3	1.67 mM
$FeSO_4.7H_2O$	72 μM
Na_2EDTA	64 μM
$Ma_2MoO_4.2H_2O$	0.25 μM
$CuSO_4.5H_2O$	0.16 μM
$ZnSO_4.7H_2O$	0.38 μM
$MnCl_2.4H_2O$	4.6 μM
$HsBO_3$	23 μM
$CaSO_4.2H_2O$	0.8 mM

HPLC - Solution A

H$_2$O	90% (v/v)
acetonitrile	9% (v/v)
glacial acetic acid	1% (v/v)

HPLC - Solution B

acetonitrile	99% (v/v)
glacial acetic acid	1% (v/v)

Luria Bertani Broth (LB media)

Tryptone	1% (w/v)
Yeast Extract	0.5% (w/v)
NaCl	1% (w/v)

pH 7.0 using HCl

LB Agar Plates

add 1.5% (w/v) of bactoagar per litre LB media

5x MOPS Buffer

MOPS (pH 7.0)	0.1 M
sodium acetate	40 mM
EDTA (pH 8.0)	5.0 mM

NZY Broth

NaCl	0.5% (w/v)
$MgSO_4.7H_2O$	0.2% (w/v)
Yeast extract	0.5% (w/v)
Caesin hydrolysate	1% (w/v)

pH 7.5 using NaOH

NZY Agar

add 1.5% (w/v) of Difco agar per litre of NZY Broth

Prehybridisation Buffer

SSC	5x
Denhardt's Reagent	5x
single stranded herring sperm DNA	$100 \mu g.ml^{-1}$
SDS	2.5% (v/v)
EDTA, pH 8.0	2.0 mM
Na Phosphate buffer, pH 6.0	0.05 M

REB

Tris-HCl, pH 8.4	100 mM
Sarkosyl	4% (v/v)
EDTA, pH 8.0	10 mM

0.1M NaPhosphate Buffer, pH 6.0

Na_2HPO_4	12ml 1.0 M
NaH_2PO_4	88ml 1.0 M

make up to 1 litre

SOC Media

bacto-tryptone	2% (w/v)
yeast extract	0.5% (w/v)
NaCl	0.05% (w/v)
KCl	2.5 mM
glucose	0.35% (v/v)

Solution I (for library lifts)

NaOH	0.5 N
NaCl	1.5 M

Solution II (for library lifts)

NaCl	1.5 M
Tris-HCl, pH 7.2	0.5 M
EDTA, pH 8.0	2 mM

Solution III (for library lifts)

Tris-HCl, pH 7.6	0.2 M
SSC	2x

20x SSC

NaCl	3.0 M
Tri-Na-citrate.2H$_2$O	0.3 M

pH 7.0 using NaOH

50x TAE

Trisma Base	2.0 M
NaAc	1.0 M
EDTA	0.05 M

pH 7.8 using glacial acetic acid

Top Agarose

0.7% (w/v) agarose in NZY Broth

Appendix C

Chemical structure of suicide substrates numbers 2,5 and 8. Designed and synthesised by Dr C. Mioskowski, le laboratoire de Synthèse Bio-Organique de Strasbourg, France.

$SO_3\ Na$ Compound No.2

$SO_3\ Na$ Compound No.5

$SO_3\ Na$ Compound No.8

Appendix D

List of Amino Acids

A	(ala)	alanine
C	(cys)	cysteine
D	(asp)	aspartic acid
E	(glu)	glutamic acid
F	(phe)	phenylalanine
G	(gly)	glycine
H	(his)	histidine
I	(ile)	isoleucine
K	(lys)	lysine
L	(leu)	leucine
M	(met)	methionine
N	(asn)	asparagine
P	(pro)	proline
Q	(gln)	glutamine
R	(arg)	arginine
S	(ser)	serine
T	(thr)	threonine
V	(val)	valine
W	(trp)	tryptophane
Y	(tyr)	tyrosine
*		stop

Appendix E

List of Herbicides

Common Name	IUPAC Name
Amitrole	1H-1,2,4-triazol-3-ylamine
Atrazine	6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine
Bentazone	3-isopropyl-1H-2,1,3-benzothiadiazin-4(3H)-one 2,2-dioxide
CGA 43 057	1-[3-methyl-4-chlorophenyl]-3,3-dimethylurea
Chlorsulfuron	1-(2-chlorophenylsulfonyl)-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)urea
Chlorotoluron	3-(3-chloro-p-tolyl)-1,1-dimethylurea
Diclofop-methyl	(RS)-2-[4-(2,4-dichlorophenoxy)phenoxy]-propionic acid
Diquat dibromide	1,1'-ethylene-2,2'-bipyridyldiylium dibromide
Fluazifop-butyl	(RS)-2-[4-(5-trifluoromethyl-2-pyridyloxy)phenoxy]propanoate
Haloxyfop	(RS)-2-[4-(3-chloro-5-trifluoromethyl-2-pyridyloxy)phenoxy]-propionic acid
Isoproturon	3-(4-isopropylphenyl)-1,1-dimethylurea
Metolachlor	2-chloro-6'-ethyl-N-(2-methoxy-1-methylethyl)aceto-o-toluidide
Nicosulfuron	2(4,6-dimethoxypyrimidin-2-ylcarbamoylsulfamoyl)-N,N-dimethylnicotinamide
Monuron	3-(4-chlorophenyl)-1,1-dimethylurea
Paraquat dichloride	1,1'-dimethyl-4,4'-bipyridinium dichloride
Primisulfuron-methyl	methyl-2-[4,6-bis(difluoromethoxy)pyrimidin-2-ylcarbamoylsulfamoyl]benzoate
Prosulfuron	1-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)-3-[2-(3,3,3-trifluoropropyl)phenylsulfonyl]urea
Triasulfuron	1-[2-(2-chloroethoxy)phenylsulfonyl]-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)urea
Simazine	6-chloro-N^2,N^4-diethyl-1,3,5-triazine-2,4-diamine