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Manipulation of defence related lignification in wheat

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES

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MANIPULATION OF DEFENCE RELATED LIGNINIFICATION IN WHEAT

by Christopher John Loades

Lignin is a complex phenolic hetropolymer with an established role in structure, support and defence in higher plants. The chemical structure of lignin is as yet undefined but controlled by an enzymatic pathway leading to three monomeric sub-units. Lignin accumulates in plants in response to pathogen challenge. A scanning densitrometric assay to detect lignin was developed that was non-invasive, quantitative and quick to perform. The assay was used in conjunction with assessments of phytotoxicity, mycotoxity and pathogen resistance to assess the efficacy of potential biochemical inhibitors of the phenylpropanoid pathway in vivo. With this information, tolerances for biochemical inhibition of the phenylpropanoid pathway were obtained. This allowed further investigation of the basis of genetic and metabolic regulation of one form of one enzyme of the pathway, phenylalanine ammonia lyase, in wheat. Evidence of a potential role for endogenous elicitation in the ligninification pathway was also gained by the use of the assay. Elicitation in terms of the hypersensitive response was also investigated during attempts to purify the fungal elicitor Avr2 using the tomato/*Cladosporium fulvum* model; however this work was completed by an alternative genetic screen protocol published elsewhere. Control of ligninificiation and the enzymes that produce the polymer is therefore an essential part of the defence response in wheat. This has important implications for genetic modification of the pathway. It was shown in this study that the phenylpropanoid pathway controls one aspect of resistance in wheat and concludes that care must be taken when manipulating the pathway in plants for increased digestibility or ease of pulping.

In addition, a separate project was undertaken in order to purify an avirulence protein possessed by the *Cladosporium fulvum* fungus. The projects aim was to obtain amino acid sequence(s) of potential interacting proteins that would be used to design primer sequences to provide a genetic sequence of the target avirulence protein Avr2. Although several candidate proteins were obtained and amino acid sequencing attempted; a competing group obtained the genetic sequence of Avr2. The sequence of this clone predicts a protein whose molecular weight and isoelectric point falls within a region of proteins whose isoelectric points and molecular weights show activity in a bioassay for Cf-2 interacting proteins. This data supports the conclusion that the work by Luderer *et al* (2002) defines the genetic sequence of Avr 2.

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"*Nil illegitium carborundum*"

The Machine Gunners

Robert Westall.

Abbreviations

Abbreviation	Description
1.2 Na	1.2. Naptholic Acid
2.1 Na	2.1 Naptholic Acid,
3.2 Na	3.2 Naptholic Acid
4CL	4-coumarate co-enzyme A ligase,
5-HFA	5-hydroxyferulic acid
ABT	1-aminobenzoltriazole
ABTS	2.2-Azino-bis (3-Ethylbenz-Thiazoline-6-Sulfonic acid)
ADG	Average daily growth
AIP	2-amino indan 2-phosphonic acid
AOA	Alpha aminixyacetic acid
AOPP	Alpha aminoxy phenyl propionic acid,
C3H	Cinnamate 3 hydroxylase
C4H	Cinnamate 4 Hydroxylase
CAD	Cinnamoyl alcohol dehydrogenase,
Cald5H	coniferal aldehyde 5-hydroxylase.
cAMP	Cyclic AMP
CCoAOMT	Caffeoyl CoA O-methyltransferase
CCR	Cinnamoyl Co-Enzyme A Reductase
CDPK	Calmodulin-like domain protein kinase
CNCPS	Cornell Net Carbohydrate and Protein system
COMT	Caffeic acid O-methyltransferase
COMT	Caffeoyl O methyltransferase
CQT	Hydroxycinnamoyl CoA:quinate hydroxycinnamoyl transferase
CST	Hydroxycinnamoyl CoA:shikmate hydroxycinnamoyl transferase
CWDE	Cell wall degrading enzyme
DE	Digestible Energy
DNA	Deoxyribose nucleic acid
F5H	Ferulate 5 Hydroxylase
G	Guiaiacyl unit

H	Hydroxyphenyl unit
H_2O	Water
HAMMA	4-hydroxy alpha mecapto-3-methoxycinnamic acid,
HPLC	High pressure liquid chromatography
IDV	Integrated density value
lEF	Isoelectric focusing
IEMT	Isoeugenol O-methyltransferase
LAC	Laccase
MAP	Mitogen associated protein
MDF	Medium Density Fibres
MDCA	3,4 methylene dioxy cinnamic acid
Mr	Molecular weight
n.d.	Not determined
NDF	Neutral Detergent Fibre
NH_2-PAS	N-(O aminophenyl) sulfinamoyltertiobutyl acetate,
OH-PAS	N-(O-hydroxyphenyl) sulfinamoyltertiobutyl acetate,
OMT	O-methyl transferase
PA	Piperonylic acid
PAL	Phenylalanine Ammonia Lyase
PCR	Polymerase chain reaction.
POD	Peroxidase
PPM	Phenylpropanoid metabolism
RNA	Ribonucleic acid
S	Syringyl unit
SAD	Sinapoyl alcohol dehydrogenase
SAR	Systemic aquired resistance
SDW	Sterile distilled water
TAL	Tyrosine ammonia lyase
T-DNA	Transfer deoxyribose nucleic acid
TLC	Thin layer chromatography
Tropolone	2-Hydroxy-2,4,6-cycloheptatrien-1-one
UV-B	Ultra violet

General Introduction :

Manipulation of defence related lignification in wheat.

1.1 The role of lignin in plants

Lignin is the second most abundant biological polymer, second only to cellulose. It is a complex phenolic hetropolymer derived from the activities of the shikimate pathway, general phenylpropanoid pathway (GPPP) and the lignin specific pathway (LSP) (Figure 1.1). The final structure of lignin is so complex that it has been compared to the variation in snowflakes (Hopkins 1999).

Lignin functions in the cell as a matrix polymer that encloses cellulose and other cell wall materials, which renders the cellulose fibrils inaccessible to microbial enzymes. *In situ* lignins perform a variety of useful functions such as secondary reinforcement to the secondary cell wall. Lignin forms a protective barrier of metabolically inert non-repetitive units and is most abundant in the vascular tissues, where its hydrophobicity waterproofs the conducting cells of the xylem and its rigidity strengthens the supporting fibre cells of both the xylem and phloem (Walter 1992). It may also play an important role in defence against pathogen attack (Hawkins *et al*, 1997).

The polymer has a wide significance to man, lignin is a major sink for carbon and accounts for 30 % of the more than 1.4×10^{12} kg of carbon sequestered into plant material (Battle *et al* 2000). The presence of lignin affects pulping processes and lodging because of its role as a mechanical support to cell walls. The digestibilities of forage crops such as alfalfa are also affected, which impacts the livestock industry (Humphreys and Chapple 2002).

1.2 The role of lignin in primary cell wall architecture.

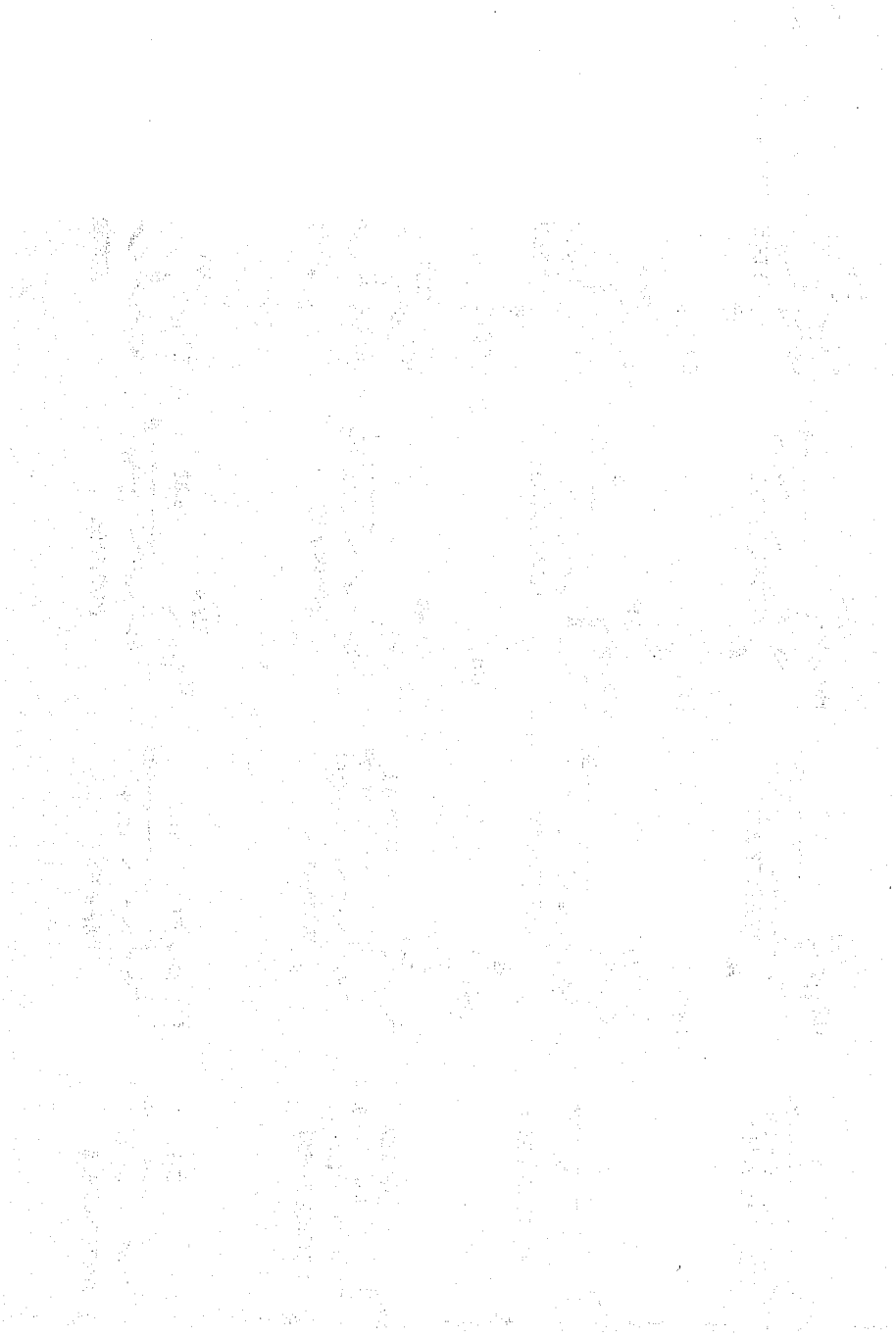
The cell wall of plants is composed of two layers; the primary wall is a thin micrometre thick polymer of glucose monomers called cellulose, which is a long unbranched β-1,4-glucan (Hopkins 1999). Cellulose is grouped into microfibril arrays and embedded in a matrix of noncellulosic polysaccarides chiefly made up of hemicelluloses; this is a highly branched network of sugars and sugar derivatives. The final part of the primary cell wall is the pectic substance characterised by the abundance of galacturonic acid as a major part of this hetrogenous polysaccaride. The secondary cell wall is laid down on the inside of the primary cell wall and is comprised of 45 % cellulose and relative to the primary wall less hemicelluloses and pectic substance. It is on this structure that the cells differentiation is built. Outside the primary cell wall, the middle lamella forms the interface between the primary cell walls of neighbouring cells, the primary component of this space being pectin rich polysaccarides

Lignin is primarily found in the secondary cell wall of plant cells and complexes with cellulose and hemicellulose through the middle lamella to form a matrix. Lignin has been described as the cement of the cell wall matrix (Hopkins 1999). The phenylpropanoid pathway, the enzymes of which control the carbon flux to the production of the hetropolymer, primarily controls the assembly of lignin. Therefore the activation of this pathway controls the lignin deposition and the make up of the polymer.

1.3 The biosynthesis of lignin.

Lignin biosynthesis begins with the deamination of phenylalanine to produce trans cinnamic acid. The hydroxycinnamic acids are then formed by a series of hydroxylation and methylation reactions. The hydroxycinnamic acids are precursors for several other defence related metabolites such as the flavanoids, suberin, the coumarins and salicylic acid. The further routes to lignin biosynthesis were initially thought to proceed via co-enyzyme A ligation to the CoA esters of the acids (Barber and Mitchell 1999). These esters were then reduced by a reductase to the aldehyde form. The final enzyme was then an alcohol dehydrogenase that forms the alcohols of the corresponding acids. This gave the (H) hydroxyphenyl (G) guiaiacyl and (S)

syringyl monomers that form the heterogeneous polymer (Figure 1.1). However, recent research has given greater insight into this pathway.



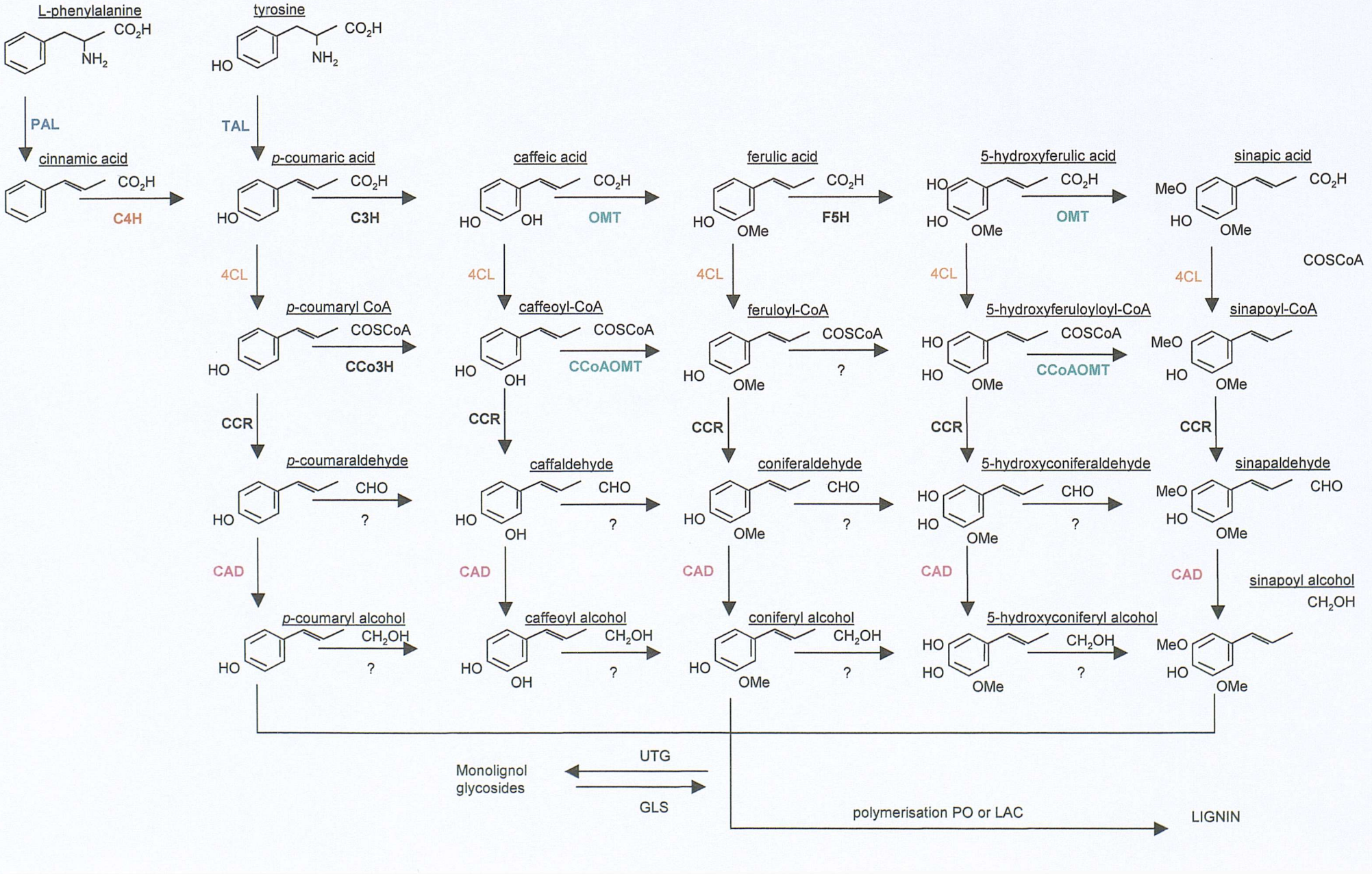

L-phenylalanine
tyrosine
PAL
TAL
cinnamic acid
p-coumaric acid
caffeic acid
ferulic acid
5-hydroxyferulic acid
sinapic acid
C4H
C3H
OMT
F5H
OMT
COSCoA
4CL
4CL
4CL
4CL
4CL
p-coumaryl CoA
caffeoyl-CoA
feruloyl-CoA
5-hydroxyferuloyloyl-CoA
sinapoyl-CoA
CCo3H
CCoAOMT
?
CCoAOMT
CCR
CCR
CCR
CCR
CCR
p-coumaraldehyde
caffaldehyde
coniferaldehyde
5-hydroxyconiferaldehyde
sinapaldehyde
CAD
CAD
CAD
CAD
CAD
sinapoyl alcohol
p-coumaryl alcohol
caffeoyl alcohol
coniferyl alcohol
5-hydroxyconiferyl alcohol
Monolignol glycosides
UTG
GLS
polymerisation PO or LAC
LIGNIN

Figure 1.1 The general phenypropanoid pathway. Offshoots to metabolic pathways are indicated with arrows and capital letters. **PAL**, Phenylalanine Ammonia Lyase; **TAL**, Tyrosine Ammonia Lyase; **C4H**, Cinnamate 4 Hydroxylase; **C3H**, Cinnamate 3 Hydroxylase; **F5H**, Ferulate 5 Hydroxylase; **OMT**, *O* – Methyl Transferase; **COMT**, Caffeic acid / 5-hydroxferulic acid *O*-methyltransferase; **CCoAOMT**, Caffeoyl CoA *O*-methyltransferase; **4CL**, 4 Coumarate Co-Enzyme A Ligase; **CCR**, Cinnamoyl Co-Enzyme A Reductase; **CAD**, Cinnamoyl Alcohol Deydrogenase.

1.4 The "primary" route

It now believed that not all the hydroxycinnamic acids have a role in the production of lignin and the previously described grid structure is not strictly appropriate. The primary route of lignin biosynthesis is now believed to change at the point of production of *p*-coumaric acid. The order of catalysis is now directed through 4-coumarate Co-enzyme A ligase to *p*-coumaryl CoA, which is a part of the flavanoid biosynthetic pathway. At this point it is now believed the order of catalysis does not pass directly through to Cinnamoyl Co-Enzyme A reductase (CCR) to form *p*-coumaraldehyde but continues through two previously unrelated enzymes in this context: **hydroxycinnamoyl CoA:shikimate hydroxycinnamoyltransferase (CST) and** hydroxycinnamoyl CoA:quinate hydroxycinnamoyltransferase (CQT) which form the shikimic acid and quinic acid derivatives of *p*-coumaryl CoA.

The reason for the inclusion of these enzymes is due to the discovery and characterisation of the C3H enzyme. It is a P450 that uses the shikimate and quinate esters of *p*-coumarate as substrates Schoch *et al* (2001). The biochemical characterisation of the candidate C3H enzyme CYP98A3 shows that the enzyme actively converts the 5-*O*-shikimate and 5-*O*-D-quinate esters of *p*-coumaric acid into their corresponding caffeic acid conjugate, thus meaning that *p*-coumaryl shikimate and *p*-coumaryl quinate are important intermediates in the pathway. These data implicate the CoA esters of these acids as very important intermediates in the biosynthesis of lignin (Humpreys and Chapple 2002).

The action of the C3H enzyme hydroxylates the shikimic acid and quinic acid derivatives of *p*-coumaryl CoA and feeds into the co-enzyme A ligation pathway provided by 4CL. This in turn provides a build up of caffeoyl CoA, which is methylated by the action of caffeoyl CoA *O*-methyltransferase (CCoAOMT), which forms feruloyl-CoA.

First indications of the role of the C3H enzyme were provided by experiments with an elicitor inducible form of caffeoyl CoA 3-*O* methyltransferase. This suggested that *p*-coumaryl CoA is converted to feruloyl CoA, providing an alternative route for lignin

biosynthesis. (Pakusch *et al.* (1991), Schmitt *et al* (1991), Ye *et al* (1994), Ye and Varner (1995) Zhong *et al* (1998).

The actions of CCR and CAD then lead directly to the production of guiaiacyl units in lignin through the production of coniferaldehyde and coniferyl alcohol respectively. At this same point Ferulate 5 hydroxlase (F5H) hydrolyses coniferaldehyde and coniferyl alcohol to their 5 hydroxlated derivatives.

Kinetic analysis of the F5H enzyme shows that although F5H does catalyse the 5-hydroxylation of Ferulic Acid, there is a thousand fold greater affinity for coniferaldehyde and coniferyl alcohol (Humpreys *et al* 1999). Recent experiments with recombinant forms of COMT from popular (Li *et al* 2000), *Arabidopsis* (Humpreys *et al* 1999) and alfalfa (Parvathi *et al* 2001) show that COMT has a higher kcat for 5-hydroxyconiferaldehyde than 5-hydroxyferulate. This leads to the positioning of COMT acting after F5H in the conversion of coniferaldehyde and coniferyl alcohol to sinapaldehyde and sinapoyl alcohol. Coniferaldehyde and coniferyl alcohol are therefore further methylated by caffeic acid / 5-hydroxferulic acid *O*-methyltransferase (COMT) to produce sinapaldehyde and sinapoyl alcohol. This forms the syringyl units in lignin through the action of SAD (a dedicated form of alcohol dehydrogenase for sinapaldehyde), or CAD (Figure 1.2).

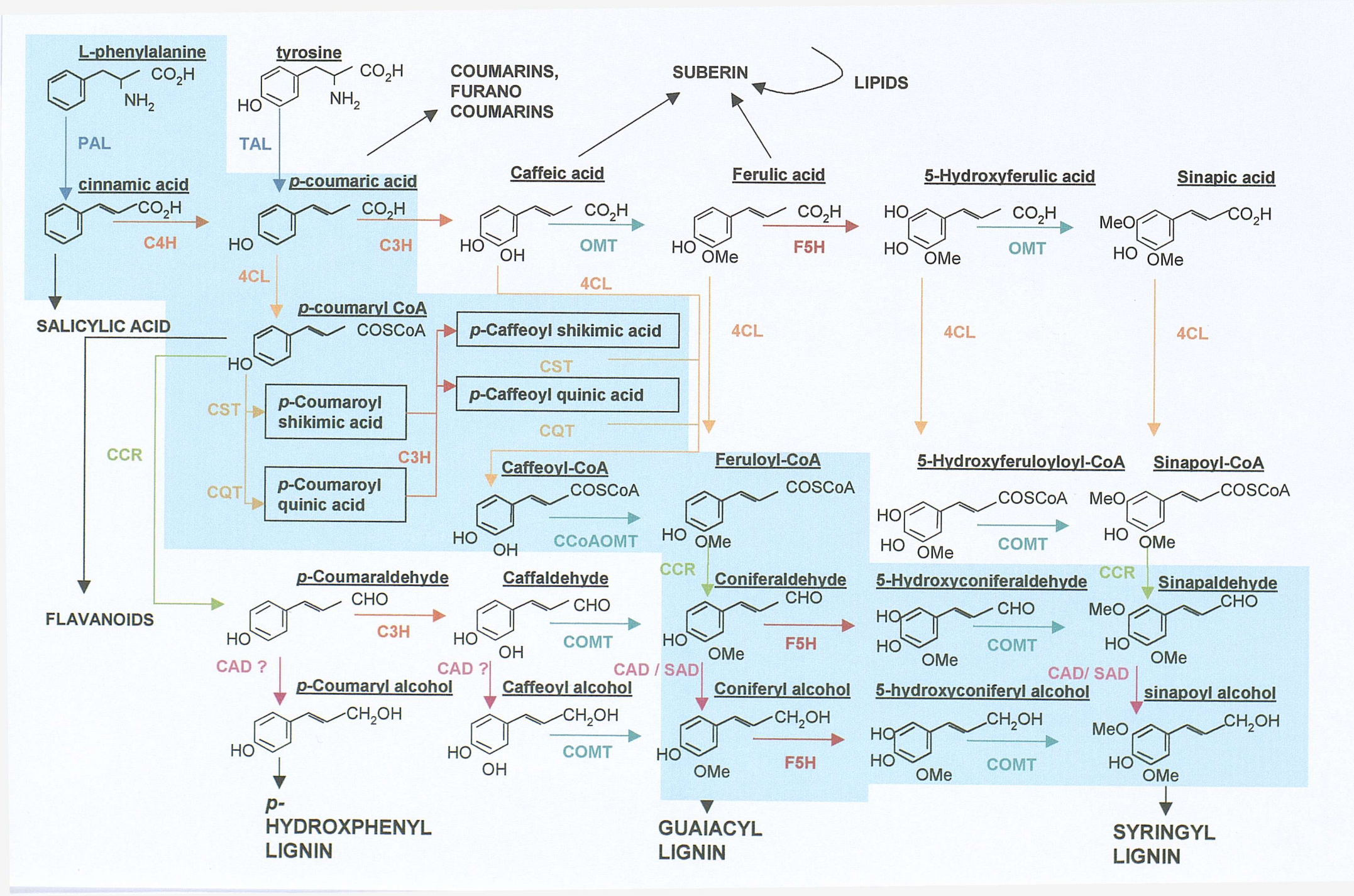

L-phenylalanine
tyrosine
COUMARINS, FURANO COUMARINS
SUBERIN
LIPIDS
PAL
TAL
cinnamic acid
p-coumaric acid
Caffeic acid
Ferulic acid
5-Hydroxyferulic acid
Sinapic acid
C4H
C3H
OMT
F5H
OMT
4CL
4CL
4CL
4CL
4CL
SALICYLIC ACID
p-coumaryl CoA
p-Caffeoyl shikimic acid
CST
p-Caffeoyl quinic acid
CQT
CST
p-Coumaroyl shikimic acid
CQT
p-Coumaroyl quinic acid
C3H
CCR
Caffeoyl-CoA
Feruloyl-CoA
5-Hydroxyferuloyloyl-CoA
Sinapoyl-CoA
CCoAOMT
COMT
CCR
CCR
FLAVANOIDS
p-Coumaraldehyde
Caffaldehyde
Coniferaldehyde
5-Hydroxyconiferaldehyde
Sinapaldehyde
C3H
COMT
F5H
COMT
CAD ?
CAD ?
CAD / SAD
CAD/ SAD
p-Coumaryl alcohol
Caffeoyl alcohol
Coniferyl alcohol
5-hydroxyconiferyl alcohol
sinapoyl alcohol
COMT
F5H
COMT
p-HYDROXPHENYL LIGNIN
GUAIACYL LIGNIN
SYRINGYL LIGNIN

Figure 1.2 The primary route pathway. (Adapted from Humpreys and Chapple 2002). Intermediate compounds thought to form the most likely route to lignin biosynthesis are highlighted in light blue. Metabolic intermediates of the phenylpropanoid pathway are underlined in bold; their reactions are shown in coloured arrows to indicate the enzyme involved. Offshoots to other metabolic pathways are indicated with arrows and capital letters. **PAL**, Phenylalanine Ammonia Lyase; **TAL**, Tyrosine Ammonia Lyase; **C4H**, Cinnamate 4 Hydroxylase; **C3H**, Cinnamate 3 Hydroxylase; **F5H**, Ferulate 5 Hydroxylase; **OMT**, *O* – Methyl Transferase; **COMT**, Caffeic acid / 5-hydroxferulic acid *O*-methyltransferase; **CCoAOMT**, Caffeoyl CoA *O*-methyltransferase; **4CL**, 4 Coumarate Co-Enzyme A Ligase; **CST**, hydroxycinnamoyl CoA:shikimate hydroxycinnamoyltransferase; **CQT**, hydroxycinnamoyl CoA:quinate hydroxycinnamoyltransferase; **CCR**, Cinnamoyl Co-Enzyme A Reductase; **CAD**, Cinnamoyl Alcohol Deydrogenase; **SAD**, Sinapyl alcohol dehydrogenase.

1.5 The enzymes of the phenylpropanoid pathway

1.5.1 Phenylalanine Ammonia Lyase. PAL; EC 4.3.1.5

The PAL enzyme represents the first step of the phenylpropanoid pathway and catalyses the deamination of phenylalanine to cinnamic acid.

The PAL enzyme is generally found as a tetramer and exists in multigene families that have two to fourty different members (depending on the species) that encode PAL subunits in plants. These are well reviewed by Wanner *et al.* (1995).

Considerable divergence is shown between species in the nature of the PAL enzyme. Induced PAL in rice is a single isoform of 84 kD, (Sarma and Sharma 1999) whereas in Chinese cabbage PAL exists in four sub-units of 38 kD (Lim *et al.* 1998). In wheat the tetramer is made up of pairs of two different subunits, which may be identical with the subunits perhaps generated by proteolytic modification (Barber and Mitchell 1997).

A site of phosphorylation of PAL has been identified as Thr 545 in French bean, (Allwood *et al* 1999). The kinase that phosphorylates PAL has also been identified and has a molecular weight of 55 kD, both in *Aabidopsis* and French bean, (Cheng *et al* 2001, Allwood *et al* 2002). It is a calmodulin-like domain protein kinase (CDPK). In-gel assays were also used to show that this kinase and a number of other CDPKs of similar molecular weight showed complex changes in elicitor-treated suspension-cultured cells of French bean. The kinase was activated 10 min after elicitation and stayed activated up to 4 h. This activation was different from MAP kinase and casein kinase assayed in the same extracts. When added 30 min before elicitation, addition of forskolin (an activator of adenylate cyclase that increases the concentration of cAMP) changed the cellular pH. This mimicked the observed transient inactivation of the CDPK five min after elicitation. When elicitor is added the in the presence of forskolin the oxidative burst started. Also forskolin treatment stops the CDPK's activation by an elicitor (Allwood *et al* 2002).

PAL is most often reported in the cytoplasm (Smith *et al* 1994) although there is one report of PAL being localised to the membrane (Halbrock and Grisebach 1979). Organelles such as plastids, microbodies, glyoxysomes and peroxisomes also contain PAL (Hanson and Havir 1981). It was thought that PAL was the single controlling factor in the phenylpropanoid pathway but that idea has been shown to be incorrect (Barber and Mitchell 1999, Humpherys and Chapple 2002).

1.5.2 Tyrosine Ammonia Lyase TAL; EC 4.3.1.5

Tyrosine ammonia lyase (TAL) catalyses the deamination of L-tyrosine to *para*-coumaric acid. Although PAL and TAL are two measurable enzyme activities it is generally believed that the same enzyme catalyses both phenylalanine deamination and tyrosine deamination. The flux of the material by the TAL route is thought to be secondary and most material enters the general phenylpropanoid pathway via PAL. So there remains little data on this entry point into the pathway (Barber and Mitchell 1997).

1.5.3 Cinnamate 4 Hydroxylase C4H; EC 1.14.13.11

This enzyme introduces the *para*-hydroxyl group in coumaric acid (Walter 1992) and is dependent on cytochrome P450. C4H is widely expressed in many *Arabidopsis* tissues, particularly in roots and cells undergoing lignification (Bell-Lelong *et al*, 1997). C4H accumulation is light dependent but is detectable even in dark grown seedlings. Expression of C4H is also coordinated with both PAL and 4CL in response to light, wounding, fungi and elicitors (Mizutani *et al.*, 1997; Batard *et al.*, 1997; Koopmann *et al.*, 1999, Ride and Pearce 1987).

Work on French Bean C4H by Nedelkina *et al.* (1999) has lead to the isolation of cDNAs showing high sequence similarity to plant CYP73A orthologues from other species. This open reading frame, codes for a protein with a predicted molecular weight of 59 kD and an isoelectric point of 8.8.

C4H has been shown to be associated with ER membranes in xylem parenchyma cells and has also been found in Golgi stacks (Smith *et al* 1994).

1.5.4 Coumarate 3 Hydroxylase (C3H)(no EC)

Until recently two enzymes were thought to catalyse the hydroxylation on the 3rd carbon of *para*-coumaric acid and its Co enzyme-A ester. One enzyme was thought to hydroxylate *para*-coumaryl Co-A and termed Caffeoyl Co enzyme A 3 Hydroxylase (CCoA3H) (EC 2.1.1.104). The evidence for the existence of this enzyme was supported by a mutation in *Silene dioica* that prevented the conversion of *para*-coumaryl Co-A to caffeoyl Co-A, blocking the production of anthocyanins (Kamsteeg *et al* 1981) and also by the ability of microsomal fractions to hydroxylate hydroxycinnamyl shikimate esters in parsley, (Heller and Kuhnl, 1985) and hydroxyquinate esters in carrot (Kuhul *et al* 1987) thus fixing the hypothetical location of this enzyme to the microsomes.

The enzyme most involved in 3-hydroxylation has recently been isolated by Schoh *et al* (2001) and has been identified as a cytochrome P450 enzyme. It has been identified as CYP98A3 and was discovered by phylogenetic analysis of the now complete *Arabidopsis thaliana* genome. It is highly expressed in inflourescence stems and wounded tissues. CYP98A3 does not metabolise *para*-coumaric acid or its glucose or CoA esters, *p*-coumaryl aldehyde and *p*-coumaryl alcohol. This is unexpected, as previous thinking preferred a "metabolic grid" model of lignin biosynthesis. The function of CYP98A3 is to convert 5-*O*-shikimate and 5-*O*-quinate esters of *trans*-p-coumaric acid into the corresponding caffeic acid conjugates. Of these derivatives the shikimate ester is converted four times faster than the quinate ester. CYP983A has been localised to the vascular tissues in stem and root.

1.5.5 Ferulate 5 hydroxylase (F5H)(no EC)

Ferulate 5 hydroxylase (F5H) catalyses the hydroxylation of the 5th carbon of cinnamic acid to produce 5-hydroxy cinnamic acid. This is the rate-limiting step in syringyl lignin biosynthesis. There are exceptions to this; in the presence of coniferaldehyde, ferulate 5 hydroxylation does not occur in *Liquidambar styraciflua* (Osakabe *et al* 1999). The Arabidopsis mutant sinl (also known as fahl) is blocked in the conversion of ferulate to 5-hydroxyferulate (ferulate-5-hydroxylase (F5H)); the lignin of the mutant lacks sinapic acid-derived components typical of wild-type lignin, and the mutant lacks sinapyl esters which may play an important role in UV-B resistance (Chapple *et al*, 1992; Landry *et al*, 1995). The ferulate-5-hydroxylase was cloned by T-DNA tagging and was found to be a cytochrome P-450 monooxygenase (Meyer *et al*, 1996). F5H expression has been shown to parallel sinapate ester accumulation in developing siliques and seedlings of *Arabidopsis thalaina* (Ruegger *et al*, 1999).

1.5.6 Coumaryl aldhyde 5 hydroxylase CAld5H

From work by Osakabe *et al* (1999) it has been shown that in *Liquidambar styraciflua* CAld5H also catalyses the step attributed to F5H in some systems. CAld5H has a km value 140 times greater than that of ferulate 5 hydroxylase making it a more likely catalytic partner for this step of the pathway.

1.5.7 Methyl transferases

Methyl transferases are enzymes that add on a methyl group at the 5,3 or 2 positions on the primary aromatic ring. They represent an important step in the pathway as their action is thought to be a major controlling factor in the distribution of H, G and S units. There are two parts of the pathway where methyl transferases operate, firstly in the conversion of the caffeic derivatives (acid, CoA, aldehyde, alcohol). The second place is in the conversion of 5-hydroxyferulic acid (5-HFA) derivatives to sinapoyl derivatives.

1.5.8 Caffeic acid 3-O-methyltransferase C-OMT EC 2.1.1.68

Caffeic acid 3-O-methyltransferase (C-OMT), catalyses the conversion of caffeic acid to ferulic acid. The same enzyme is thought to be responsible for converting caffeyl aldehyde to coniferaldehyde and caffoyl alcohol to coniferal alcohol.
Two COMT classes (I and II) occur in tobacco (*Nicotiana tabacum*) (Maury *et al.* 1999). Expression profiles upon tobacco mosaic virus infection of tobacco leaves revealed a biphasic pattern of induction for COMT II and I. COMTs efficiently methylated hydroxycinnamoyl-CoA esters. COMT I was also active toward 5-hydroxyconiferyl alcohol, indicating that COMT I that catalyses syringyl unit synthesis *in planta* and may operate at the free acid, CoA ester, or alcohol levels. COMT II was found to be highly inducible by infection and also accepted caffeoyl-CoA as a substrate, thus suggesting a role in ferulate derivative deposition in the walls of infected cells. Elicitation has also been found to increase COMT activity in wheat (Maule and Ride, 1976) and in alfalfa (Goweri *et al.* 1991).

There is evidence that just a few amino acid residues could determine O-methyltransferase substrate preference. Wang and Pichersky (1999) demonstrated this using a hybridisation approach on two methyl transferases from *Clarkia breweri* an annual plant from California. When seven crucial amino acids were removed from (Iso)eugenol O-methyltransferase (IEMT) and replaced with seven from caffeic acid O-methyltransferase (COMT) the resultant protein had activity with caffeic acid and hydroxyferulic acid. The converse was true as well making these amino acids very important in the phylogenetic evolution of these enzymes.

Recently, several groups (Oskabe *et al.* 1999, Li *et al.* 2000, Parvathi *et al.* 2001) have reported that the alfalfa *O*-methyltransferase (OMT) has a substrate preference. This preference is for the alcohol and aldehyde precursors in the pathway that contain either the 3 or 5 hydroxyl motif (Caffeate and 5-hydroxyferulate), as opposed to the free acids. The protein exists as a dimer in solution and has a molecular weight of 43 kD (Zubieta *et al* 2001). The crystal structure of OMT in alfalfa shows that 5-hydroxy

precursors are preferred over 3-hydroxy precursors as the position of the alpha hydrogen bond allows its sequestration closer to the catalytic site of the enzyme. In addition the propanoid tails of the precursors are bound in the active site, which shows specificity in the order of aldehyde-alcohol-acid thus explaining substrate preference. It is also reported that the use of the structural data enables them to design mutant plants with point mutations in the COMT enzyme. These mutants show complete losses of caffeate binding.

The divergence of COMT is quite great; examples have been cloned in many species including *Pinus radiata, Habenero chile* and Basil. The brown-midrib (bm3) mutation in maize and sorghum has been shown to be in the caffeic acid 3-O-methyltransferase structural gene responsible for converting caffeate to ferulate (Vignols *et al.* 1995). This enzyme can utilize either caffeic acid or 5-hydroxyferulic acid as substrates (Tsai *et al.* 1995). However, the alfalfa enzyme has a preference for 5-HFA (Inoue *et al.* 1998). Suppression of the caffeic O-methyltransferase gene in the xylem of quaking aspen results in novel phenotypes with mottled or red-brown wood (Tsai *et al.* 1998). A high amount of coniferyl aldehyde residues in the lignin is the origin of the red-brown coloration (Tsai *et al.* 1998).

All of the methyl transferases are S-adenosyl-L-methionine dependent. OMT is found in the xylem adjacent to the cambium, with no activity in the phloem. It appears that OMT could be a key factor that explains differences in lignin composition. The methylation of caffeic acid specifically is only catalysed by OMT from gymnosperms. Whereas angiosperm type OMT preferentially methylates 5-hydroxyferullic acid over caffeic acid. It appears that bamboo OMT methylates both with equal efficiency (Humpherys and Chapple 2002). However it does not necessarily follow that if a plant possess specific OMTs that these are the one controlling factor in the distribution of G, S and H units.

In alfalfa Kersey *et al.* (1999) have localised both OMT and COMT to the cytoplasm of xylem parenchema cells these enzymes have also been found to a lesser extent in the cytoplasm of phloem cells of *Medicago sativa L.* It was found that there was no significant difference in the localisation pattern of these two enzymes, which suggests a role in the lignification of alfalfa stem internodes.

1.5.9 Caffeoyl CoA 3-O-methyltransferase CCoAOMT EC 2.1.1.104

The CCoAOMT of *Vitis vinifera* methylates both caffeoyl- and 5-hydroxyferuloyl-CoA (Busam *et al.* 1997). The CCoAOMT of alfalfa has a preference for caffeoyl-CoA (Inoue *et al.* 1998). CCoAOMT expression has been associated with lignification in several dicot species (Ye *et al.* 1997). In cell-suspension cultures of *V. vinifera* CCoAOMT activity is induced upon fungal elicitation (Busam *et al.* 1997). Treatment with a fungal elicitor, low concentrations of salicylic acid and some other inducers of the systemic acquired resistance (SAR) response, raised the abundance of transcripts of CCoAOMT (Busam *et al.* 1997). Tobacco plants that express antisense CCoAOMT show marked reductions in lignin content and altered lignin composition. In these experiments by Zhong *et al.* (1998); guaiacyl lignin was preferentially reduced, resulting in an increase in the syringyl / guiaiacyl ratio.

When an aspen xylem cDNA library (Bugos *et al.* 1991) was screened with the Zinnia CCoA-OMT cDNA (Ye *et al.* 1994). A novel cDNA (Ptccomt1) was found that encodes a polypeptide of 247 amino acid residues with a predicted Mw of 28 kD. The deduced amino acid sequence is similar to other higher plant CCoA-OMTs including maize and parsley. The Aspen CCoA-OMT polypeptide also has significant similarity to *Homo sapiens* and *Rattus norvegicus* catechol OMT and *Streptomyces mycarofaciens* OMT (Meng and Campbell 1995). Should this similarity hold true for all plant CCoA-OMTs there is a reasonable chance for known inhibitors of catechol OMT in other systems being effective in the plant system.

1.5.10 4-coumarate CoA-ligase 4CL [EC 6.2.1.12]

The ligation of CoA glucose to the acids of the phenylpropanoid pathway is catalysed by 4CL. At least 3 forms of 4CL are found in poplar; all share similar hydroxycinnamic acid substrate specificities (4-coumarate >ferulate > caffeate), all are unable to utilise sinapate (Allina *et al.* 1998). The encoding of the 4CL enzyme is by a multi gene family in poplar (Allina *et al.* 1998). In *Arabidopsis thaliana* however the three forms of 4CL can be classified into two different classes (Ehlting *et al* 1999). Class I 4CL are encoded by the transcripts of At4CL1 and At4CL 2; based on their evolutionary characteristics and expression patterns they are likely to participate in lignin formation. At4CL 3 however, is an independent class of transcript that encodes a protein that has a role in the flavanoid biosynthetic pathway. However with the increased knowledge of the 3-hydroxylation step, it is possible that At4CL3 might be a significant part of the lignification pathway.

Transgenic plants of tobacco in which the activity of 4-coumarate CoA-ligase is very low (due to down-regulation of the endogenous gene(s)) contain a novel lignin in their xylem. The levels of three hydroxycinnamic acids, *para*-coumaric, ferulic, and sinapic, which were bound to the cell walls, were increased. Some of these hydroxycinnamic acids were linked to cell walls via ester and ether linkages (Kajita *et al*, 1997). In *Arabidopsis* antisense suppression of 4CL has resulted in a transgenic plant with a large decrease in guiaiacyl (G) to syringyl (S) lignin units. This suggests that there is an uncharacterized metabolic route to sinapoyl alcohol that is independent of 4CL (Lee *et al*, 1997).

1.5.11 Cinnamyl CoA reductase CCR (EC.1.2.1.44)

CCR is the enzyme that diverts the general phenylpropanoid intermediates toward the accumulation of monolignols by reducing the CoA esters to their aldehyde form.

Two full length cDNAs have been isolated from Maize by Pichon *et al* (1998). These two cDNAs designated ZmCCR1 and ZmCCR2 exhibit 73 % sequence conservation at the nucleotide level for their coding regions and are relatively divergent at their 5'- and 3'-untranslated regions. They both contain a common signature, which is thought to be involved in the catalytic site of CCR. Northern blot analysis indicated that ZmCCR2 was expressed at very low levels in roots whereas ZmCCR1 was widely expressed in different organs. The high level of ZmCCR1 gene expression along the stalk suggests that the corresponding enzyme is probably involved in constitutive lignification.

CCR is thought to be a monomeric protein with molecular weight of 36-40 kD. Recently SelmanHousein *et al* (1999) have cloned a full-length cDNA from sugar cane that encodes for a protein of 40 kD.

Ralph *et al* (1998) found that lignin content was reduced in antisense-CCR tobacco, which displayed a markedly reduced vigor. The lignin contained fewer coniferyl alcohol-derived units and significant levels of tyramine ferulate, Tyramine ferulate is a sink for the anticipated build-up of feruloyl- SCoA, and may be up-regulated in response to a deficit of coniferyl alcohol.

Piquemal *et al* (1998) have transformed tobacco plants with antisense constructs and have examined lignin content and composition in the progeny of primary transformants down-regulated for CCR activity and exhibiting one single T-DNA integration locus. All CCR down-regulated lines displayed common features, such as an orange brown coloration of the xylem cell walls, an increase in the syringyl over guiaiacyl (S/G) ratio, and the presence of unusual cell wall bound phenolics. The less severely depressed lines exhibited a normal phenotype and a very slight reduction of the thioacidolysis yield, which is an indication of the abundance of the beta-O-4 linkages in lignin. The line with the most severely depressed CCR activity exhibited a

strong reduction in lignin content together with altered development (reduced size, abnormal morphology of the leaves, collapsed vessels).

CCRs activation appears dependant on what substrates are available; this depends on the species of plant. Most angiosperms produce sinapoyl Co-A, thus this is the route taken to produce the corresponding aldehydes (Barber and Mitchell 1997).

When tobacco plants that carry an antisense construct for CCR are crossed with those containing an antisense construct for CAD, the result is a major loss of lignin content. Under controlled conditions these plants exhibit normal developmental characteristics, thus giving some hope toward reducing lignin for environmental and biotechnological applications (Chabbenes *et al.* 2001).

1.5.12 Cinnamyl-Alcohol Dehydrogenase CAD EC 1.1.1.195

Cinnamyl alcohol dehydrogenase is the enzyme that catalyses the production of the final primary alcohols at the end of the phenylpropanoid pathway. From this point the alcohols are converted into the lignin polymer.

CAD isoforms have been found in a number of species including wheat, eucalyptus, soybean and bean (Ros Barcello *et al.* 1995). Expression of different isoforms of CAD with different substrate specificities is a potential mechanism for control of lignin heterogeneity (Campbell and Sederoff 1996). CAD has been confirmed to be a dimeric protein of 63-84 kD with sub units of 38-45 kD. Extensive polymorphism has been found in angiosperm CAD. This leads to great difficulty in determining the substrate specificity of CAD.

In wheat Mitchell *et al.* (1994) showed that there are three different forms of CAD (CAD A CAD B and CAD C). Only CAD C was found to be responsive to elicitors. Its preferred substrate was sinapyl alcohol; this correlates well with the deposition of defence related syringyl residues in wheat.

CAD has been found in sub epidermal layers of roots and shoots, xylem elements and phloem fibers and is localised in the cytoplasm (Ros Barcello *et al.* 1995).

1.6 The role of defence related lignification.

1.6.1 The defence response to pathogens

Plants possess many ways to defend themselves against pathogens; these responses may be constitutive or induced. Constitutive structures include the waxy surface of the plant and the cuticle that cover the epidermal cells. These provide a barrier to fungal attachment and penetration. Other structural barriers to pathogen ingress form during the defence responses to pathogens such as cork layers beyond the point of infection, tyloses that form from overgrowths of the protoplast into the xylem and gums that form in intracellular spaces. In particular, an abscission layer often forms in healthy layers adjacent to lignified cells in order to completely isolate or excise the affected area of the plant. In the plant cell the outer layer of the cell wall swells and produces an amorphous, fibrilar material that surrounds and traps bacteria to prevent their multiplication. This material is cellulosic but infused with phenolic substances. Papillae made from callose often are deposited in fungal plant interactions, on the inner side of the cell wall and appear to repair or prevent cellular damage. In some cases a sheath or lignotuber forms around penetrating fungal hyphae and phenolic substances infuse into it (Agrios 1993). The rigid lignin polymer is also believed (through its lack of biodegradability) to be a structural chemical barrier to pathogen ingress (Ride 1983). Other specific constitutive responses include the cytoplasmic defence reaction that occurs in conditions of weakly pathogenic fungal-plant interactions the cytoplasm surrounds the pathogen and enlarges along with the nucleus, this eventually causes disintegration of the fungal mycelium (Reviewed by Hooker 1974). The hypersensitive response involves the death of the cell that the pathogen has ingressed into. When the pathogen contacts the protoplast of the cell, the nucleus moves toward the pathogen and disintegrates leaving a browning discolouration of the cytoplasm. The hypersensitive response destroys all membranes in contact with the pathogen and the cell dies, the pathogen can then no longer proliferate in the plant. This is a very common form of plant defence and is highly effective as the faster the invaded cell dies the more resistant to infection the plant appears (Agrios 1993).

1.6.2 Biochemical inhibitors of pathogens

Some common phenolic compounds notably Caffeic acid (produced by the phenylpropanoid pathway) accumulate at a faster rate after infection in resistant plants. Recently, the anti-microbial potential of several intermediates of the phenylpropanoid pathway has been determined Barber *et al.* (2000).
Some phenolics are linked to sugars such as glucose, when glucosidases, released by some bacteria and fungi are present; the toxic phenolic compound is released and causes damage to the pathogen. Polyphenyloxidases play a role in defence by oxidising phenols to quinones that are often more toxic than the original phenols. In particular peroxidases catalyse the production not only of phenolics but increase the rate of polymerisation of phenols into lignin and lignin like substances.

1.6.3 The synthesis of defence related lignin

Although plants naturally possess lignin and the compounds of the general phenylpropanoid pathway, defence related lignin must be induced or elicited by a challenge by a stimulus of microbiological origin. The compounds that do this are termed elicitors. Examples of these include extracellular fungal proteins, (Ricci *et al* 1989) chitin oligomers (Barber and Ride 1988) and fungi themselves (Ride 1975). They act as the starting point in a range of defence responses in plants. The early events in these responses can include protein phosphorylation, high calcium influxes, hydrogen, potassium and chloride effluxes, plasma membrane depolarisation and activation of NADPH oxidases, which produce active oxygen species. Later events include the elicitation of PAL, C4H, 4CL, CAD and more than likely all the other enzymes of the phenylpropanoid pathway (Somssich and Halbrock 1998).

First indications of the significance of the liginification pathways significance to defence were observed in experiments with *Botrytis cinerea* where autoradiography of the ultrastructure of wounded wheat leaves revealed significant deposits of lignification that prevented ingress of the non host pathogen *B. cinerea* (Maule and Ride 1982). Further experiments revealed that the activation of Cinamate 4 Hydroxylase and Coumarate Co-Enzyme A Ligase were both important in producing the lignification response in response to challenge with *B. cinerea* in wheat (Maule and Ride 1983). Cellular ligninification was also shown to be a major factor in the hypersensitive resistance to stem rust in wheat (Beardmore *et al.* 1983). CAD and its

specific forms e.g. CAD C, which is specific for syringyl units have also been found to be defence related (Barber and Mitchell 1998).

In relation to these later events much work has been done on the localisation and nature of the response. It has been found that cinnamic acids incorportate into cell walls under conditions of elicitation after 24 hours. These acids are esterifed rather than lignified in nature, thus raising the question why. An answer is that the acylation of these acids protects them from the hydrolytic enzymes of pathogenic fungi in the first stages of the interaction. This acylation is a process that is thought to be random throughout the cell wall whereas lignification is perceived to stem from a cell corner using hydroxycinnamic acids as an anchoring site. Acylation is reported to give mechanical rigidity to the cell wall thus giving some protection to the aforementioned oxidative burst generated active oxygen species. In addition, hydroxycinnamic acids are thought to work as scavengers of radicals; this is also postulated as one of the phenomena that help the plant survive the oxidative burst Matern and Grimmig (1993).

Coumaroyl and feruloyl wall esters may also play a role in the defence response. These compounds, under physiological pH conditions change to 4-hydroxybenzaldehyde and vanillin. This is important because these are compounds that have antimycotic potential Matern and Grimmig (1993). The antimicrobial compounds that are actively produced in plants are known as phytoalexins. A great many exist such as pistain in pea and wyerin in bean, however none have been described in wheat to date.

1.6.4 Defence vs developmental lignin.

There may be a difference in the forms of lignin that are elicited by microbial attack and those forms deposited during development. Walter (1992) refers to several papers on the nature of lignin induced by defence responses. For instance in radish root cells it has been found that high amounts of syringyl units are found on vessel walls whereas guiaiacyl lignin predominates in the parenchyma. In cucurbits, material that was rich in coumaryl moieties but poor in guiaiacyl units was found in response to infection. In wheat, a higher proportion of 4-coumaryl and syringyl units have been

found in cells infected by fungi. Walter (1992) criticises the available literature, as developmental lignin has not been distinguished from pathogen related "defence" lignin thus making it harder to know if there is really a difference between the two.

Lignin and lignification are clearly important processes in plant defence and further understanding of the nature of this response by careful manipulation of the phenylpropanoid pathway may lead to some insights into the way the response functions in wheat. It is also possible that information gathered in this study may aid the ability of agro-industrial companies to evaluate the best options for lignin manipulation in other plant species.

1.7 Artificial manipulation of lignification

The goal of lignin reduction can also be applied in an agro-industrial context, mainly in two areas, the increase in the digestion of forage crops and the reduction of lignins for kraft pulping methods (paper making). These two goals are not necessarily mutually exclusive however, care must be taken to ensure that transgenic or biochemically modified plants going toward these processes are not altered in their susceptibility to biotic attack.

1.7.1 Forage crops digestion

If lignin is reduced in forage crops such as wheat or grasses, it is thought that the digestibility of the fodder will increase, thus increasing weight gain for the animal in question and less requirement of feed reducing the primary costs of farming, or alternatively allowing more animals to be fed with the same amount of feed.

Lignin interferes with the digestion of cell-wall polysaccharides by acting as a physical barrier to digestive enzymes. Lignification therefore has a direct and often important impact on the digestible energy (DE) value of the forage.

Using chemical composition and in vitro digestibility data from temperate and tropical forages Traxler *et al* (1998) have developed relationships between indices of lignification and forage indigestible Neutral detergent fibre (NDF). Indigestibility was

shown to increase nonlinearly as the lignin concentration of the NDF increased. Differences in estimated indigestible NDF using equations developed for a specific forage class (C-3 and C-4 grasses and legumes) were small and are probably not biologically significant when compared to those estimated from a common equation. Selected equations were compared with the Cornell Net Carbohydrate and Protein System (CNCPS) for the prediction of average daily growth (ADG). Despite some discrepancies in the methods of obtaining lignin measurements it can be shown in this work that lignin and forage crop digestibility are well related.

1.7.2 Paper making

The reduction of lignins for kraft pulping would reduce the amount of environmentally undesirable chlorine containing waste that it is present in the chemical treatments used for eliminating lignin. The market for these types of woods is increasing with the advent of medium-density fibres (MDF) for domestic use.

Hu *et al* (1999) have made some progress with their transgenic studies on aspen down-regulation of 4CL reduces lignin by 45 % and increases cellulose by 15 %, also the lignin esters moved to the cell wall providing some support, thus reducing the effects on hydrostatic pressure due to the loss of lignin.

In work by Lapierre *et al* (1999) the downregulation of COMT led to a non-reduction in lignin but a decrease in the efficiency of kraft pulping, due to a dramatic increased the frequency of guaiacyl units and resistant biphenyl linkages in the lignin present. However a severely depressed line where CAD had been downregulated did not change the frequency of labile ether bonds or guaiacyl units in lignin, it increased the proportion of syringaldehyde and diarylpropane structures and, more importantly with regard to kraft pulping, of free phenolic groups in lignin. This means that free phenolic units were created, this aided lignin solubilization and fragmentation during kraft pulping.

1.8 Manipulation of defence related lignification in wheat

Clearly, the pathway that forms lignin is still a complex and unresolved puzzle. The present trend is to try to manipulate the pathway not only to discover its nature but also for biotechnological goals as outlined above. By manipulating purely defence related lignin it may be possible to add to the understanding of some of the underlying mechanisms that control this particular phenomenon. This may allow some insight into the relative importance of some of the enzymes and metabolites of the pathway in the production of the defence related lignification response in wheat.

Chapter 2 Development of a quantitative assay for induced lignification in wounded wheat leaves.

2.1 Analysis of lignin

The analysis of lignin is extremely difficult due to the high molecular weight, mass, insolubility and multiplicity of the functional groups and bonding patterns occurring in lignin (Ros Barcleo 1997). There are many analytical methods, some histochemical, some extractive, some qualitative and some quantitative.

As lignification is one result of the hypersensitive response to pathogens, it is important to be able to quantitatively analyse the amount, deposition and composition of the polymer. This would allow the quantitation of the level of plant resistance to a pathogen and the nature of the lignification response present.

However, the analysis of lignin has been reviewed all methods have relative advantages and disadvantages associated with the particular technique Boudet *et al* (1995). Some methods are so invasive and destructive that no single method can provide a complete analysis of lignin.

2.2 Histochemical techniques

Specific histochemical tests give information about the localisation and sometimes the composition of the lignin barrier. The chlorine sulphite method, (Campbell 1937) is specific for syringyl groups and has been used to locate *in situ* syringyl groups in defence related lignin in wheat (Ride 1975). This technique is only appropriate for those lignin compositions rich in syringyl moieties, which is a disadvantage for total lignin quantification.

The reaction involving *O*-toluidine blue turns lignified cell walls blue and cellulose cell walls purple (Salisbury and Ross 1992). Toludine is an aromatic amine that also turns glucose residues green; this could lead to some confusion particularly in wounded tissues.

Another histochemical stain that has been used is the phloroglucinol (HCl) test; this test turns lignified cell walls red. Ride and Pearce (1975) found that this test for cinamaldehyde end groups in lignin did not react with papillae haloes or lateral walls in wheat tissues infected with *Botrytis cinerea.*

Other stains include Lignin pink and fast red GG salt (p-Nitrobenzene diazonium tetrafluoroborate) in 0.1 mM phosphate buffer Barber and Ride (1987).

Lignin autofluoreses blue under UV light, this has been used for quantitative purposes. This property allows the variation in lignin depositions to be more closely studied than with invasive methods. It could be that more advanced forms of microscopy could conceivably be used for lignin such as confocal microscopy which may allow the imaging of the build up of lignin in a 3D environment.

More recently Kapat and Dey (2000) have developed a specific detection method for lignin using polyclonal antibodies raised against BSA coupled lignin. This method was found to be highly sensitive and linear in enzyme linked immunosorbant assay (ELISA) within the coating lignin concentration range of 0.01 $\mu g\ ml^{-1}$ to 1 $\mu g\ ml^{-1}$.

2.3 Extractive techniques

Chemical techniques such as nitrobenzene oxidation, permanganate oxidation, acidolysis and thioacidolysis can all be used to determine the monomeric composition of lignins (Ros Barcello 1997).

The Klason lignin process involves vigorous treatment with ethanol, Sodium Dodecyl Sulphate and Sodium hydroxide in ethanol. After these treatments leaves are put into 72 % (v/v) Sulphuric acid solution and refluxed for 4 hours and filtered onto a glass fibre disc. The disc is then washed at 80 ^0C and then dried at 105 ^0C the mass of the solid residue remaining is calculated. This is the amount of lignin per gram fresh weight (Ros Barcello 1997).

The acetyl bromide method consists of the solubilization of lignins with acetyl bromide in glacial acetic acid. This method is only appropriate for brassicas and legumes that have lignified cell walls in tissues that do not contain significant amounts of ester bound cinnamic acids (Ros Barcelo 1997).

Nitrobenzene oxidation is commonly used as a determination for the monomeric composition of lignins; lignified cell walls are treated with nitrobenzene in sodium hydroxide. This releases the lignin monomers as hydroxybenzaldehyde (H), vanillin (G) and syringaldehyde (S). The relative abundance of these monomers can then be quantified by HPLC. The drawback to this method is that cell wall phenolics such as coumaric acid and ferulic acid interfere with the assay as they are oxidised to benzaldehydes. Nitrobenzene oxidation causes shortening of lignin side chains, thus no information on functionality and interconnections can be derived from this method (Ros Barcello 1997).

Thioacidolysis involves solvolysis of lignin in dioxane or ethanethiol, which breaks the structure into thioethylated H, G and S monomers. This method provides information on the core lignin structure and on the nature of C-C and C-O-C bonds. This method does not have the drawback of interference by other cell wall phenolics Ros Barcelo (1997).

2.4 Assay of Barber and Ride

The most quantitative assay for induced lignification in wounded wheat leaves is that of Barber and Ride (1987). The method involves killing and decolourising leaves by boiling in ethanol. Phenolic compounds are then extracted by incubating in 0.5 M NaOH in ethanol. After washing, leaves are stained with *p*-nitrobenzenediazonium tetrafluroborate, blotted and dried. Gel scanning was then used to make a linear trans-section through the leaf wound; this can then be used as a basis of comparison between wounds.

2.5 Aim

The aim of this section is to improve the assay of Barber and Ride (1987) to provide a simpler method of quantifying the relative amount of lignin deposited during the elicitation of a defence response to a non-pathogen.

Materials and Methods

2.6 Chemicals and reagents

Agar No 2	Oxoid
Malt extract agar	Oxoid
Mycological peptone	Oxoid
Silwet	Dr M.S.Dixon, Southampton University
Levington F2 compost	Levington Seeds
Sodium Hydroxide	Fisher Scientific
Ethanol	Fisher Scientific
p-nitrobenzenediazoniumtetrafluoroborate (fast GG)	Sigma Aldrich
Phosophate buffer	Fisher Scientific

Treatment of fungi

2.7 Culture of *Botrytis cinerea*

Botrytis cinerea (Dr C. Jackson Southampton University, UK) was grown on 3.0 % (w/v) malt extract agar with 0.5 % (w/v) mycological peptone and 1.5 % (w/v) agar No 2. The media was autoclaved at 15 psi 115 °C for 10 min. This agar composition facilitated high levels of sporulation (Galloway and Burgess 1952). Spore suspensions were prepared by washing 10-day-old plates of *B. cinerea* in 0.0001 % (v/v) silwet. Spores were washed twice with sterile distilled water by centrifugation for 1 min at 11 200 g. Spore density was adjusted to 1 x 10^6 spores ml^{-1} using a haemocytometer.

Treatment of plants

2.8 Growth of plant material

Seeds of *Triticum aestivum* c.v. Brigadier (Dr R. Stratford, Monsanto, UK) were grown in Levington Professional F2s compost in a plant growth room at 22 °C with lighting providing a 16 h photoperiod. Seeds were sown adjacent to the long side of the seed trays (35 by 22 cm). All experiments were performed on 10 days old primary leaves.

2.9 Bioassay Preparation

Primary leaves were taped horizontally without detaching them from the plants, on a rigid plastic sheet and wiped with a tissue soaked in 70 % (v/v) ethanol to reduce microbial contamination of the surface. The leaves were wounded by compression using a 3 mm diameter metal rod, each leaf receiving five wounds approximately 0.5 cm apart, the first being 1.5 cm from the tip. Drops (10 µL) of distilled water containing the compound in question at 0.2, 1.0, 5.0 mM and in a spore suspension of *Botrytis cinerea* (1×10^6) spores per ml were placed on each wound. Similarly, 10 µl drops of *Botrytis cinerea* suspension alone and distilled water alone were used as positive and negative controls. The treatments were then left for the allotted time for the assay under clear plastic bags. During the course of the treatment, should the leaf drops appear to dry off, the wounds were re-inoculated with 10 µL distilled water per wound (Barber 1987).

2.10 Lignin staining by p-nitrobenzene diazonium tetrafloroborate – Fast GG

Treated leaves were excised and boiled in two washes of 70 % (v/v) ethanol. Unbound phenolic residues were extracted by boiling with 0.5 M NaOH in 70 % (v/v) ethanol for 1 h. The leaves were then washed for 2 h in distilled water and stained with 0.5 % (w/v) p-nitrobenzenediazoniumtetrafluoroborate (fast red GG salt) in 0.1 M pH 7.0 phosphate buffer to produce rings of lignification. The leaves were left in 70 % (v/v) ethanol and dried at room temperature. A digital scan was taken using a video camera of the wheat leaves and a quantitative measurement of the density of black pixels around the wound area taken. Values obtained from *B. cinerea* innoculated leaves was set as 100 % and used as the basis for reduction in lignification using the value obtained for water treated leaves as 0 %.

2.11 The determination of lignin by the scanning densitometric method.

Scanned leaves were visualised with the alpha imager software package (Alpha Image, UK), using set parameters. The filter was set to chemiluminescence, all illumination was switched on, (diaphragm level 16), zoom was set to max, (level 75) and focused to a clear image (level 3). The alpha ease software enhanced the picture (level 4) and the images were stored as a Tagged Image File Format (TIFF).

Leaves were scanned and measured using the alpha imager software package to determine pixel density. Measurements were taken from wounded leaves inoculated with 1 x 10^6 *B. cinerea* spores produced strong rings of lignification (Figure 2.1). This was used as a basis for levels of biochemical inhibition of lignification and comparisons were made between treatments with a repetition of five wounds per leaf and four leaves per treatment.

Method development

2.12 Development of the scanning densitrometric method.

In order to improve the quantification of the lignification response at wound margins it was necessary to modify the assay of Barber and Ride (1987). The original staining method for lignification was retained and the image was captured in grey scale using a video scan (Figure 2.1).

The alpha imager software spot densitometry package was used to draw (toolbar 4) two computer generated concentric circles around the lignin ring image (Figure 2.2). The outer concentric circle (objective) was a measurement that calculated a count of all the pixels in its area and marked them on a scale between 255 (Black) and 0 (white). This gave the integrated density value (IDV) for the area. Similarly, the inner concentric circle (background) calculates the IDV for its area. The outer concentric circle was linked (using the software package) to the inner concentric circle by clicking on the link background option (toolbar 4). This subtracted the count for the inner circle (background) from that of the outer circle; giving the IDV value for the ring produced by lignification at the wound margins.

The software allowed an exact copy to be made of the two concentric circles so that the area analysed for each wound was identical for each measurement. The measured area of the ring was therefore standardised and it was possible to use the IDVs to compare the differences in lignification between wounds by different treatments that may affect lignification.

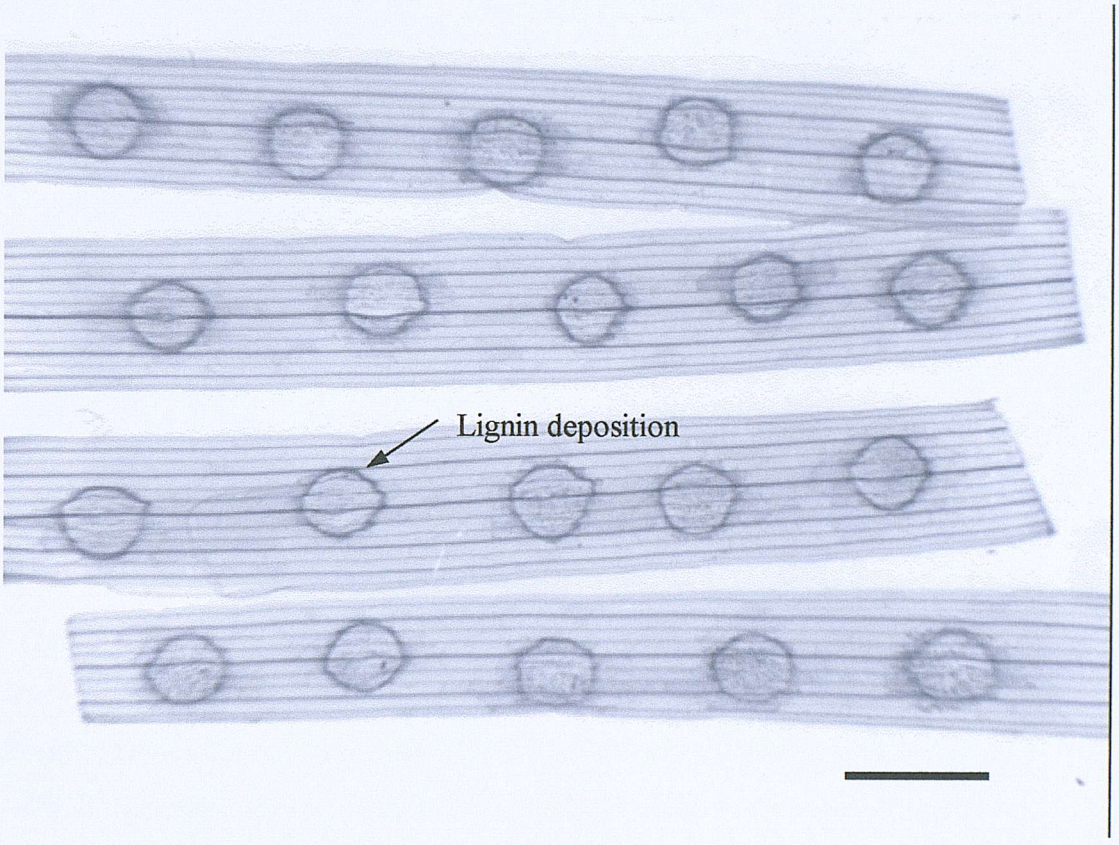


Figure 2.1 Grey scale image of Lignin deposition at wound margins induced by *Botrytis cinerea* on wheat leaves after 48 h. Leaves were stained with fast red GG salt and scanned using the alpha imager software package; Arrow indicates lignin deposition at wound margin. Bar = 5 mm

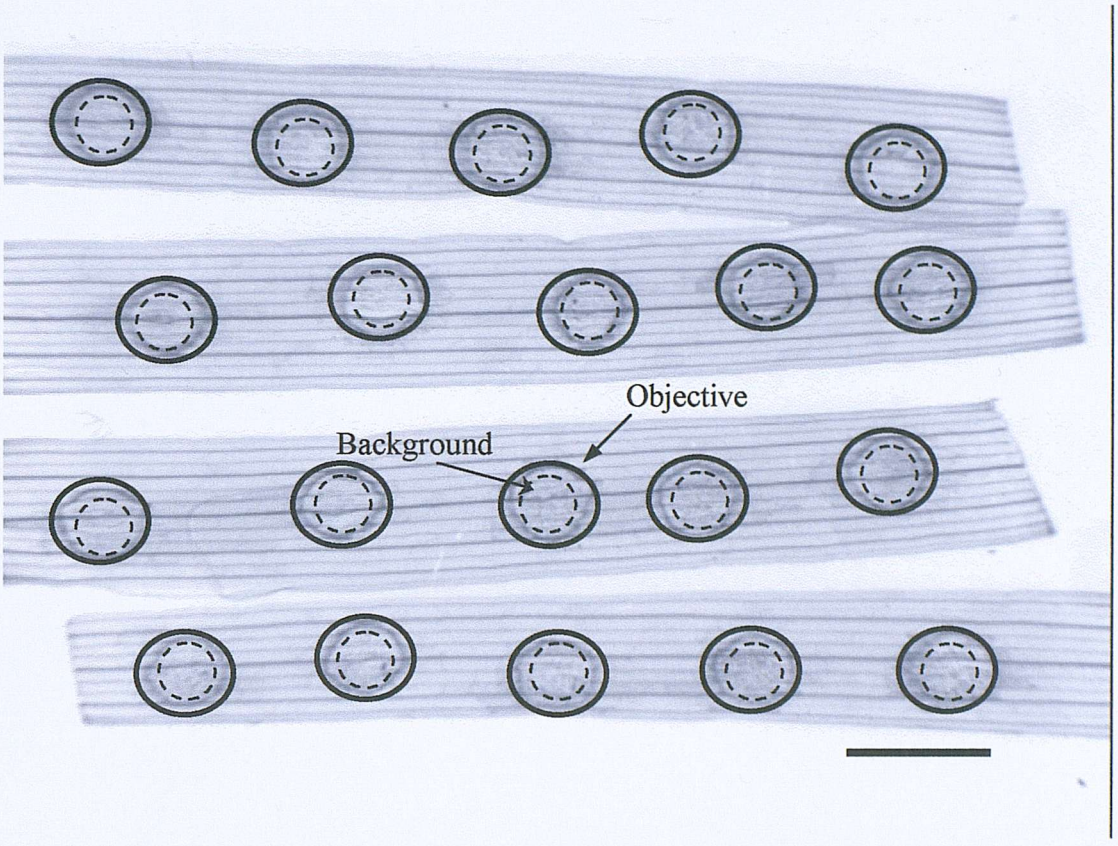


Figure 2.2 Grey scale image of Lignin deposition at wound margins induced by *Botrytis cinerea* on wheat leaves after 48 h; with overlaid software based measurement. Leaves were stained with fast red GG salt and scanned using the alpha imager software package, bar = 5 mm, Objective, outer concentric circle count of pixels; Background, inner concentric circle count of pixels.

2.13 Analysis of lignification at wound margins

2.13.1 Fungal data

Wounding alone can also induce lignification; to account for this a negative control of distilled water was used in place of the fungal spore suspension. To account for wounding the average IDV for distilled water treated wound margins was subtracted from the individual IDV for each *B. cinerea* inoculated wound margins. This gave the IDV (therefore the amount of lignification) induced by *B. cinerea* alone. The average IDV due to *B. cinerea* alone was calculated from 20 wounds and used as the base to calculate the percentage lignification by converting the IDV induced by *B. cinerea* alone into a percentage (Formulae A). Consequently, the average of all of the results is 100 % as it is the maximum lignification induced by *B. cinerea* alone in this system.

A. Fungal data:

Individual IDV(*B. cinerea*) – average IDV (water) = IDV by *B. cinerea* alone

$$\frac{\text{IDV (}B.\ cinerea\text{ alone)}}{\text{average IDV (}B.cinerea\text{ alone)}} \times 100 = \%\ \text{lignification (}B.\ cinerea\text{)}$$

Example analysis

These calculations show the fluctuations in lignification apparent between wound margins on the same leaves. Lignification due to the presence of *B. cinerea* alone at 48 h on leaf (1) varies between 58 and 146 % on five wounds. Lignification due to the presence of *B. cinerea* alone at 48 h on leaf (2) varies between 75 and 181 % on five wounds. Lignification due to the presence of *B. cinerea* alone at 48 h on leaf (3) varies between 23 and 146 % on five wounds. Lignification due to the presence of *B. cinerea* alone at 48 h on leaf 4 varies between 23 and 146 % on five wounds (Table 2.2).

Table 2.1 Sample data set of integrated density values obtained by inoculation of wounded wheat plants with *B.cinerea*: Leaf, *T. aestivum* 10-day-old leaf; Wound, compression wound; Individual IDV, IDV per wound; Average IDV (Water), Average integrated density value for wounded leaves with distilled water; Individual IDV (*B. cinerea* alone), IDV subtracted by average IDV (Water); Percentage lignification (*B. cinerea*), degree of lignification per wound (*B. cinerea* = 100 %).

Leaf	Wound	Individual IDV	Average IDV (water)	Individual IDV (*B. cinerea* alone)	Percentage lignification (*B.cinerea*)
	1	60781	34229.3	26551.7	146
	2	51184	34229.3	16954.7	93
1	3	51184	34229.3	16954.7	93
	4	44786	34229.3	10556.7	58
	5	51184	34229.3	16954.7	93
	6	67179	34229.3	32949.7	181
	7	60781	34229.3	26551.7	146
2	8	60781	34229.3	26551.7	146
	9	47985	34229.3	13755.7	75
	10	47985	34229.3	13755.7	75
	11	60781	34229.3	26551.7	146
	12	51184	34229.3	16954.7	93
3	13	38388	34229.3	4158.7	23
	14	44786	34229.3	10556.7	58
	15	51184	34229.3	16954.7	93
	16	60781	34229.3	26551.7	146
	17	57582	34229.3	23352.7	128
4	18	54383	34229.3	20153.7	111
	19	47985	34229.3	13755.7	75
	20	38388	34229.3	4158.7	23

Average IDV (*B. cinerea*) = 18234.3 Average % lignification (*B. cinerea*) = 100 %

2.13.2 Treatment data

In order to calculate the effect of a treatment on lignification; data from the scanning densitrometric assay were entered into formulae A in place of data obtained from the effect of *B. cinerea* alone (formulae B). When combined with data for the positive control (*B. cinerea* alone) the effect of the treatment on the level of lignification induced by the presence of the fungus was shown. As average IDV (*B. cinerea* alone) is a maximum level of lignification the average of the results obtained for the treatment show a difference equivalent to the level of its effect on lignification.

The data from experiments where treatments were incorporated into the assay (such as the PAL inhibitor AIP; see results Chapter 3) was handled in the exact same manner as outlined above, with the treatment data replacing that of the fungus in the calculation. However, the Average IDV (*B. cinerea* alone) from formulae A was used to calculate percentage lignification. Therefore the result obtained by Average IDV (*B. cinerea* alone) acts as a positive control for the effect of the treatment in question (formulae B). Consequently, the average of the results is dependent on the difference between the treatment and the positive control.

B. Treatment data:

Individual IDV (Treatment) – Average IDV (water) = IDV by treatment alone

$$\frac{\text{IDV (Treatment alone)}}{\text{average IDV (}B.\ cinerea\text{ alone) \{A\}}} \times 100 = \%\ \text{lignification (treatment)}$$

Average (n=20) = percentage lignification

Example analysis

These calculations show the fluctuations in lignification apparent between wound margins on the same leaves. Lignification due to the presence of 0.2 mM AIP and *B. cinerea* alone at 48 h on leaf 1 varies between -30 and -100 % on 5 wounds. Lignification due to the presence of 0.2 mM AIP and *B. cinerea* alone at 48 h on leaf 2 varies between -82 and -12 % on 5 wounds. Lignification due to the presence of *B. cinerea* alone at 48 h on leaf 3 varies between -170 and -47 % on 5 wounds. Lignification due to the presence of *B. cinerea* alone at 48 h on leaf 4 varies between -153 and -30 % on 5 wounds (Table 2.2).

Table 2.2 Sample data set of integrated density values obtained by inoculation of wounded wheat plants with *B.cinerea* in the presence of AIP 0.2 mM: Leaf, *T. aestivum* 10-day-old leaf; Wound, Consecutively numbered wound obtained by compression using a metal stake; Water controls, Average integrated density value for wounded leaves treated with distilled water; IDV-Water, Integrated density value subtracted by average IDV from water controls; Percentage lignification accumulation, Relative contribution to total lignification (*B. cinerea* = 100 %).

Leaf	Wound	Individual IDV	Average IDV (Water)	Individual IDV (*B. cinerea* alone)	Percentage lignification (treatment)
1	1	25592	34229.3	-8637.3	-47
	2	28791	34229.3	-5438.3	-30
	3	22393	34229.3	-11836.3	-65
	4	15995	34229.3	-18234.3	-100
	5	19194	34229.3	-15035.3	-82
	6	31990	34229.3	-2239.3	-12
	7	22393	34229.3	-11836.3	-65
2	8	22393	34229.3	-11836.3	-65
	9	19194	34229.3	-15035.3	-82
	10	19194	34229.3	-15035.3	-82
	11	12796	34229.3	-21433.3	-118
	12	3199	34229.3	-31030.3	-170
3	13	6398	34229.3	-27831.3	-153
	14	9597	34229.3	-24632.3	-135
	15	25592	34229.3	-8637.3	-47
	16	19194	34229.3	-15035.3	-82
	17	6398	34229.3	-27831.3	-153
4	18	12796	34229.3	-21433.3	-118
	19	25592	34229.3	-8637.3	-47
	20	28791	34229.3	-5438.3	-30
Averages				-15355.2	-84.15

2.13.3 Calculation of lignification reduction or accumulation

A final value for the percentage reduction or accumulation in lignification is obtained by subtraction of the average percentage lignification value for the treatment from the *B. cinerea* alone value (Formulae C). To check the statistical significance of the results, a 1 tailed t-test was calculated for the data (Table 2.3). Conversely because of the nature of the calculation if the data produced by the treatment is negative i.e. less than the water controls then the result would be recorded as an over 100 % reduction in defence related lignification.

C. Calculation of lignification reduction or accumulation

% lignification (*B. cinerea*) – % lignification (treatment)

= % accumulation or reduction of lignification

Example analysis

Treatment with *B. cinerea* alone to wounds on wheat plants results in a 100 % response of defence related lignification on wound margins. Treatment with 0.2 mM AIP with *B. cinerea* after 48 h results in an average -84 percent defence related lignification response. Subtracting the result from (100 %- -84 %) results in a 184 % reduction in defence related lignification in the presence of this inhibitor. This is recorded as a greater than 100 % reduction of lignification for simplicity.

2.14 The accumulation of lignin

Induced lignin is known to increase over time; 48 h was picked as an appropriate time to measure lignification due to the strong results shown (Figure 2.1). By using the result at 48 h as 100 % measure of lignification an estimate can be made of when defence related lignification i.e. an amount of lignification above the stimulus induced by wounding alone has accumulated.

The accumulation of lignin in wounded wheat leaves increases in a linear fashion with time (R^2 0.9495), as such it is appropriate to relate values obtained from the scanning densitrometric analysis to time points along the linear regression line shown by use of the equation $y = 2.5856x - 40.868$. The line of accumulation of lignin passes through the origin after 20 h; after this point the lignin synthesised can be interpreted as defence related only, rather than as a response to wounding alone (Figure 2.3).

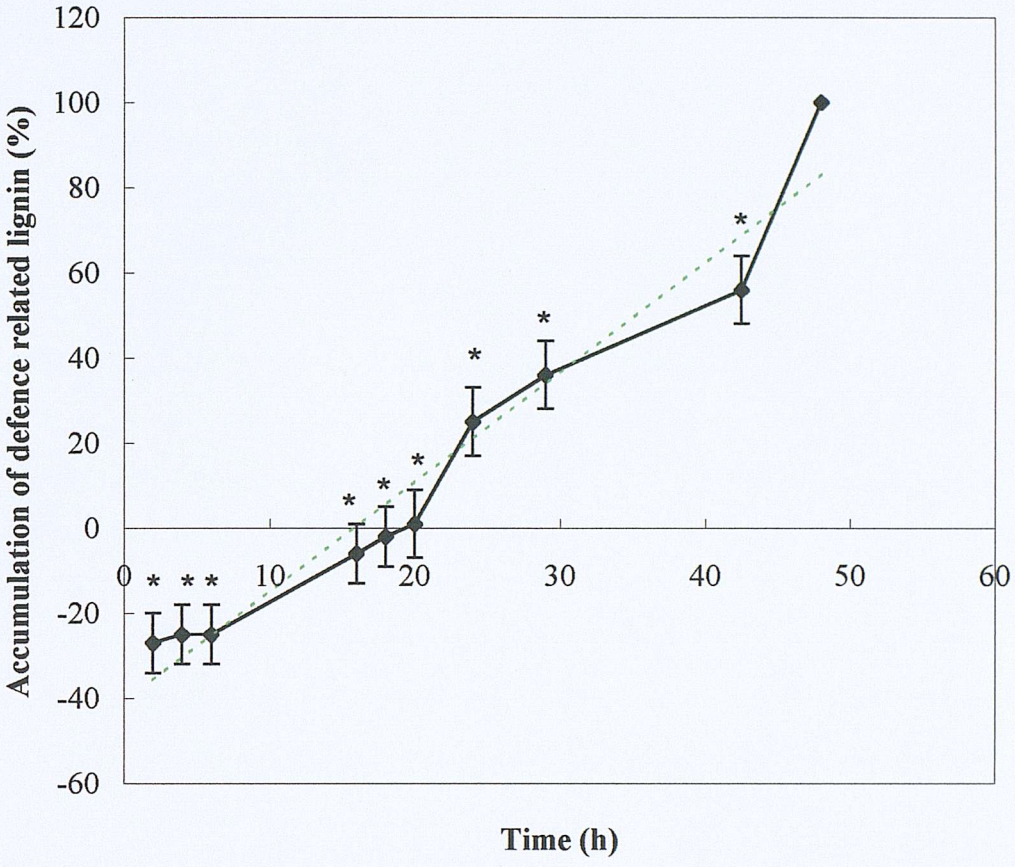


Figure 2.3 Time course of accumulation of lignin due to inoculation with *B. cinerea* in wheat leaves based on that observed at 48 h. Data is expressed as percentage accumulation of lignin based on IDV values for *B. cinerea* at 48 h (100 %) and water alone at 48 h (0 %). Error bars represent standard errors of the mean (n = 20); Green dashed line represents the trend of linear regression (R^2 0.9495); Equation, y = 2.5856x – 40.868; (*), significant difference from lignification at 48 h.

2.15 Discussion

By localising the lignin wound and taking away the background measurement (Figure 2.2) it can be seen that the estimated lignification will only be that which is directly associated with the defence response, i.e. by fungal induction. The gel-scanning assay of Barber and Ride (1987) does not allow for this, being a measure of lignification per leaf. The lignification per wound margin is a more reliable estimate of total lignification and allows higher numbers of replications per treatment. The throughput of analysis is significantly improved; four leaves gave four results in the assay of Barber and Ride (1987) and four leaves give 20 results in this scanning densitrometic assay.

As a specific template can be drawn and measured each time for each treatment, complete reproducibility can be maintained between treatments. Slight variation can still occur as wheat leaves grow differently after germination, perhaps due to intergenic differences.

Another improvement of the assay is in the areas of file storage and of access to information. These can be stored on computer and the software allows the gel scanning data to be directly imported into a spreadsheet for manipulation purposes.

The use of this assay is demonstrated by the accumulation of lignin (Figure 2.3). It is now possible to model how much a non-pathogen directly induces lignin over time post inoculation as only that lignin produced in response to the non-pathogen is measured. The accumulation of lignin shown (Figure 2.3) is the accumulation from two potential stimuli wounding and the presence of a non-pathogenic fungus. However, wounding itself appears to induce a small level of lignification, from this graph it may be as much as 25 %. This correlates with reports of wounding leaves of *Triticum aestivium* induced lignification Ride and Barber (1987).

It is probable that for further validation a technique such as the nitrobenzene oxidation or the thioglycollic acid extraction procedure might be required to completely relate the defence related values to actual lignin composition. As

however the original leaf scanning assay of Barber and Ride (1987) has been validated in this way it is unlikely that the results would differ.

Chapter 3: Biochemical inhibition of the phenylpropanoid pathway enzymes.

3.1 Manipulation of the phenylpropanoid pathway

The phenylpropanoid pathway is clearly a complex and unresolved puzzle, as highlighted in the general introduction. There are three avenues that are employed to manipulate and study phenylpropanoid metabolisms; transgenic manipulation, the study of natural mutations and biochemical inhibition. This study will focus on specific biochemical inhibitors to the enzymes of the phenylpropanoid pathway.

3.2 Occurrence of natural mutation

Within generations of plants deletion or loss of gene base pairs occurs spontaneously. This is sometimes manifested by a change in physiological character.

This is the first advantage manipulating the phenylpropanoid pathway via the process of natural mutation is that all the work required is the characterisation of the phenotype. For natural mutants, containment or biological safety issues are not as pertinent as for GM crops. Natural mutants provide a better model for understanding the phenylpropanoid pathway because the mutation is a permanent alteration that leads to a non-lethal phenotype, this usually means that they are stable. However, mutations seldom occur in the genes required naturally. Exposure to mutation agents such as ultra violet radiation and chemical agents such as psorlen can lead to increased frequency of mutation. Mutants have been characterised in *Arabidopsis*, maize, rice, populus and pine species. Most of the enzymes of the phenylpropanoid pathway have a mutant type in a plant species. Mutation offers a chance to see a change in a biological system that may not yet be fully characterised; this provides important information that may not be easily discovered by other means (Table 3.1). This is particularly true of the mutation of the C3H gene in the phenylpropanoid pathway (Section 1.6).

Enzyme	Species	Enzyme Activity	Lignin content	Lignin composition	Reference
S-adenosylmethionine synthetase	*A. thaliana*	n.d.	22 %	n.d.	Shen *et al* (2002)
PAL	*Oryza sativa*	increased	n.d.	Increased *p*-CA and FA	Nishikubo *et al* (2000)
C3H	*A. thaliana*	n.d.	n.d.	H units only	Franke *et al* (2002)
4CL	*A. thaliana*	decreased	n.d.	n.d.	Stuible *et al* (2000)
COMT	*Zea. Mays*	n.d.	n.d.	H units only	Veremis *et al* (2002)
COMT	*Populous spp*	n.d.	n.d.	H units only	Ralph *et al* (2001)
CAD	*Pinus taeda L.*	Reduced	decreased	Increased coniferaldehyde	Mackay *et al* (2002)
CAD	*Zea. Mays*	Reduced	n.d.	Increased coniferaldehyde	Veremis *et al* (2002)

Table 3.1 Physiological mutants impaired in the phenylpropanoid pathway. Enzyme activity, The level of enzymatic activity relative to wild type; Lignin content, Content of lignin c.f. wild type; Lignin composition, proportions of H, G and S and other units relative to wild type; SAM S-adenosylmethionine synthetase; PAL, phenylalanine ammonia lyase; C3H, Cinnamate 3 Hydroxylase; 4-CL, 4-coumarate Co-A ligase; COMT, Caffeic acid O-methyltransferase; CAD, Cinnamoyl alcohol dehydrogenase. H, hydroxyphenyl lignin unit; *p*-CA, *p*-Coumaric Acid; FA, Ferulic Acid; n.d, not determined.

3.3 Transgenic manipulation

Molecular biology can be used to reduce lignification by various methods (Table 3.1 , 3.2) including anti-sense or sense suppression, transposon mutatgensis, tDNA introgression, siRNA.

The genetic engineering approach involves making a sense or anti-sense construct to the gene encoding one of the enzymes of the lignin biosynthetic pathway. Sense suppression can be a side affect of overexpression of a transgene. The presence of the extra copy of the gene perturbs the normal function of the target overexpressed gene Flavel (1994). This technique has been used successfully to suppress the O-Methyltransferase gene of Aspen (Tsai *et al* 1998). Commonly this technique is also reffered to as Co-supression, following work by Jorgensen *et al* on petunia (Napoli *et al* 1990, Jorgensen *et al* 1996). Anti-sense involves putting in a copy of the gene of interest in the plant that represents the mirror image of the coding strand, so the mRNA produced is not read by the ribosome and hence no protein is produced. Promoter fusion involves joining a gene to a different promoter in order to regulate transcription (Griffiths *et al.*1996). It also is possible that induction of an alternative enzyme to the phenylpropanoid pathway related enzyme may shift the carbon flux away from the production of lignin.

Other methods are more recent in their construction and use and may not be applicable for use in the wheat system. The bacterium *Agrobacterium tumefaciens* can in the course of its life cycle produces a crown gall on susceptible plants. The bacterium possesses the Ti plasmid which contains virulence and T-DNA transfer regions that allow intra-species DNA transfer between *A. tumefaciens* and its host plant. The Ti plasmid has been used in several plant species to integrate non-host DNA randomly into the host chromosome (Griffiths *et al.* 1996).

More recent innovations in transgenic manipulations focus on induction of post-transciptional gene sllencing. This is the silencing of an endogenous gene caused by the introduction of homologous double stranded RNA (dsRNA). The direct introduction of dsRNA is refered to as RNA interference (RNAi). Induction of

smaller nucleotide sequences of 21-23 dsRNA as an interference mechanism is known as small interfering RNAs (siRNA) (Ambion.com 2002).

The main advantage of transgenic manipulation is the specificity of the approach, no indirect effects that are not a direct consequence of the manipulation should occur. Once a sequence of any phenylpropanoid related enzyme is available it is possible to use any of the above methods to manipulate the enzyme or the sequence. Transgenic manipulation is therefore very applicable and allows close comparison of manipulation strategies between species.

However, as the enzymes of the phenylpropanoid pathway differ from species to species in their nature (chapter 1); this difference may hinder any cross species comparison of their down regulation Whetten *et al* (1998). In wheat the molecular biology approach is hampered by the fact that most commercial varieties are hexaploid in nature, making plant regeneration and gene silencing more difficult. Another drawback is that comparisons of genes across species are used to identify genes and therefore only those enzymes broadly similar to those already discovered can be successfully discovered using this method. The transgenic method is costly and difficult to perform, requiring specific techniques for each species, particularly in regeneration of fragile transformed plants. Plants must also be contained and currently are under review for their viability as commercial products. Lignification is normally permanently altered by the transgenic methods means that all development is changed in terms of the change in the phenylpropanoid pathway. The difficulty in transformation of plants often leads to model plant systems being used, although plants like *Nicotiana tabaccum* and *Arabidopsis thaliana* are easy to manipulate it is hard to correlate enzyme effects to the whole of the plant kingdom if only one or two species of plants are used.

Table 3.2 Transgenic plants where enzymes of the General Phenylpropanoid Pathway have been targeted. (Whetten *et al.* 1998). Enzyme activity; The level of enzyme activity relative to wild type (%), Lignin content: percentage of wild type content. Lignin composition: relative to wild type. POD: peroxidase LAC: laccase. n.d.: not determined. S and G: Syringyl and Guiacyl moieties.

Transgenic method	Species	Enzyme activity	Lignin content	Lignin composition
Sense suppression of PAL (heterologous PAL)	*N. tabaccum*	PAL 5–30 %	Decreased 10–80 %	n.d.
Sense suppression of PAL	*N. tabaccum*	PAL decreased	Decreased	Increased
Antisense C4H	*N. tabaccum*	C4H decreased	Decreased	Decreased S:G
Antisense OMT	*N. tabaccum*	OMT 5 %	No effect	Decreased S:G
Sense suppression OMT	*N. tabaccum*	OMT 180 %	No effect	No effect
Antisense OMT	*Populous spp*	OMT 2 %	No effect	Decreased S:G 5-OH-G subunits
Antisense and sense suppression OMT	*Populous spp*	OMT decreased	No effect	Decreased S only
Antisense 4CL	*N. tabaccum*	4CL 8 %	Decreased to 50 %	Decreased G only
Antisense 4CL and sense suppression	*N. tabaccum*	n.d.	n.d.	Decreased G & S

Table 3.3 Transgenic plants where enzymes of the Lignin Specific Pathway have been targeted. (Whetten *et al.* 1998). Enzyme effect: The level of enzyme activity relative to wild type (%). Lignin content: % of wild type content. Lignin composition: relative to wild type. POD: peroxidase LAC: laccase. n.d.: not determined. S and G: Syringyl and Guaiacyl moieties.

Transgenic method	Species	Enzyme activity	Lignin content	Lignin composition
Antisense CCR	*N. tabaccum*	CCR 25 %	Decreased to 75 %	Increased S:G
Sense suppression CCR	*N. tabaccum*	CCR 2 %	n.d.	Decreased S:G
Antisense CAD	*N. tabaccum*	CAD 7 %	No effect	Increased aldehyde
Antisense CAD	*N. tabaccum*	CAD 50 %	No effect	Increased aldehyde
Antisense CAD	*Popolous*	CAD 30–50 %	No effect	Increased aldehyde
Antisense POD	*N. tabaccum*	POD decreased	No effect	n.d.
Over expression POD	*N. tabaccum*	POD increased	Increased to 130 %	n.d. slower growth
Antisense LAC	Tulip poplar	LAC 10 % (preliminary)	n.d.	n.d
Constitutive f5h overexpression	*A. thaliana*	F5H ectopic	No effect	Ectopic S lignin
F5h-C4H promotor fusion	*A. thaliana*	n.d.	n.d.	Almost only S lignin
Introduction of TDC (tryptophan decarboxylase)	*S. tuberosum*	TDC active	Decreased to 60 %	Decreased S:G

3.4 Biochemical Inhibition

Many inhibitors of enzyme activity have been used in the study of phenylpropanoid biosynthesis (Table 3.4). Biochemical inhibitors have been tested on almost every known enzyme in the pathway. Many different inhibitors exist and some have already been documented to have effects across the phenylpropanoid pathway, whereas others are more specific to individual enzymes. Three different strategies are used. Substrate analogues (SA) are the most abundant and mimic the substrate of the enzyme and reduce the availability of the active sites of the enzymes for catalysis. A suicide inhibitor (SI) irreversibly binds to the enzyme preventing the true substrate from binding. Chelators bind the inorganic co-factors required for enzyme function (Table 3.4).

The main advantage of the inhibitor method is speed and broad application range. An inhibitor of PAL in alfalfa for example is likely to have the same effect in wheat. For the same process to work using molecular biology the specific gene(s) must be isolated, cloned, transformed into a vector and antisensed and re-transformed successfully into the target plant which must also survive long enough for experiments to proceed. To make a less severely impaired transgenic plant the whole process must be repeated again. When inhibitors are used the target compound only requires dilution. Inhibitors allow specific rather than continuous manipulation at different plant developmental levels, whereas when a plant is transgenically modified it is always impaired in that function.

One of the main disadvantages of inhibitors comes from their innate lack of specificity *in planta*, which can lead to non-target enzymes being inhibited by the presence of the inhibitor. The presence of the inhibitor itself will also cause changes in the concentrations of solutes in the plant cell and this may also cause non-target effects. Inhibitors are often only soluble in solvents, which damage the *in vivo* plant system. These solvents can often lead to phytotoxic symptoms independent of the inhibitor effect. Equally important are the effects of these compounds on microbial communities, especially in terms of defence related studies.

Table 3.4 Inhibitors of the Phenylpropanoid Pathway: PAL, Phenylalanine ammonia lyase; C4H, Cinnamate 4 Hydroxylase; OMT, O-methyltransferase; 4CL 4-coumarate co-enzyme A ligase; CAD, Cinnamoyl Alcohol Dehydrogenase; SA, Substrate analogue; SI, Suicide Inhibitor; CuCh, Copper Chelator; ZnCh, Zinc Chelator.

Target enzyme	Inhibitor	Mode of Action	Reference
PAL	2-amino indan 2-phosphonic acid. (AIP)	SA	Zon and Amrhein (1992)
PAL	Alpha aminoxy phenyl propionic acid. (AOPP)	SA	Morschbacher *et al* (1990)
PAL	Alpha Aminoxyacetic Acid (AOA)	SA	Ride and Barber (1987)
C4H	1 aminobenzoltriazole (ABT)	SI	Reichart *et al* (1982)
C4H	Piperonylic Acid (PA)	SA	Schalk *et al* (1997)
C4H	1.2. Naptholic Acid (1.2 Na)	SA	Schalk *et al* (1997)
C4H	2.1 Naptholic Acid (2.1 Na)	SA	Schalk *et al* (1997)
C4H	3.2 Naptholic Acid (3.2 Na)	SA	Schalk *et al* (1997)
OMT	2-Hydroxy-2,4,6-cycloheptatrien-1-one (Tropolone)	CuCh	Eshelman *et al* (1997)
4CL	3,4 methylene dioxy cinnamic acid (MDCA)	SA	Funk + Brodelius(1990)
CAD	N-(O-hydroxyphenyl) sulfinamoyltertiobutyl acetate (OH-PAS)	ZnCh + SI	Carver *et al* (1994)
CAD	N-(O-aminophenyl) sulfinamoyltertiobutyl acetate (NH_2-PAS)	ZnCh + SI	Carver *et al* (1994)
CAD	CI	SA	Hall (1998)
CAD	ML19	SA	Hall (1998)
CAD	4-hydroxy alpha mecapto-3-methoxycinnamic acid. (HAMMA)	SA	Hall (1998)
CAD	2.2. dipyridyl	SA	Hall (1998)

Each enzyme of the phenylpropanoid pathway has been studied to a greater or lesser extent by the use of biochemical inhibitors. Generally, the effect of biochemical inhibition is considered only *in vitro* and potential conflicts of specificity are not fully considered. A brief description of the known characters of these inhibitors follows.

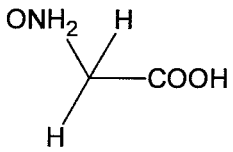
3.5 Inhibition of Phenalanine Ammonia-lyase

PAL converts phenylalanine to *trans*-cinnamic acid. Several compounds have been documented to inhibit PAL. These compounds are alpha aminoxyacetic acid (AOA), 2-iminoindan-2-phosphonic acid (AIP) and alpha-aminooxi-beta-phenylpropionic acid (AOPP) (Figure 3.1). These compounds act as substrate analogues of phenylalanine but, only AOA is currently commercially available (Sigma Aldrich). Sources of both AIP and AOPP have been obtained for this study (Grabber: Personal communication and Amrhein: Personal communication).

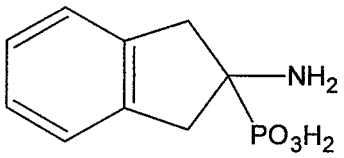
3.6 Inhibiton of Cinnamate 4 hydroxylases

C4H converts cinnamic acid to *para*-coumaric acid. 1-Aminobenzotriazole (ABT) is a suicide inhibitor of C4H (Reichart *et al.* 1982). In the presence of 1 mM ABT, C4H activity was reduced to 3 % of the control enzyme in *Helianthus tuberosus*. Inhibition of C4H by ABT is irreversible or the inhibitor has a much higher affinity for the enzyme than the substrate. It is reasonable to assume toxic effects by ABT on non-target hydroxylases. ABT is commercially available (Sigma Aldrich).

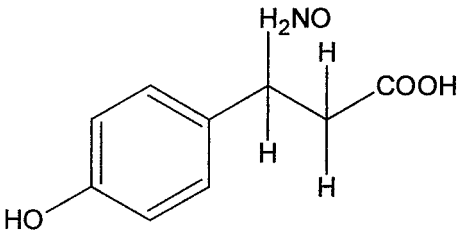
Other mechanism-based inhibitors have been developed from the naptholic acid groups of compounds (Schalk *et al.* 1998, Schalk *et al.* 1997). Piperonylic acid is claimed to be the ideal ligand for C4H *in vitro*. All the naptholic acid derivatives are generic inhibitors of cytochrome P450 hydroxylases of which the phylogenetic family includes C4H. The inhibitors are commercially available (Sigma Aldrich).



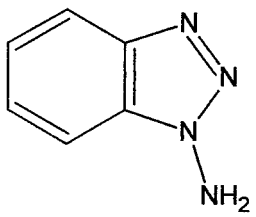
AOA (Sigma)



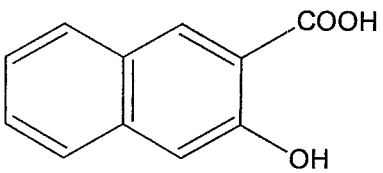
AIP (Amrhein / Grabber)



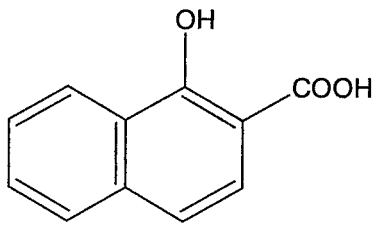
AOPP (Carver)



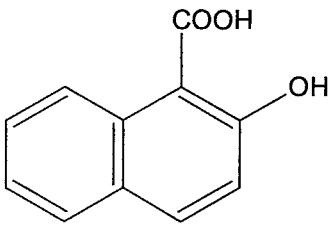
ABT (Sigma)



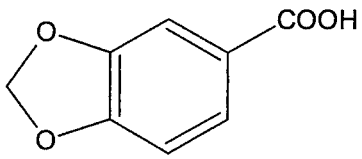
3.2 Naphtholic Acid (Sigma)



2.1 Naphtholic Acid (Sigma)



1.2 Naphtholic Acid (Sigma)



Piperonylic Acid (Sigma)

Figure 3.1 Structures of inhibitors of PAL and C4H

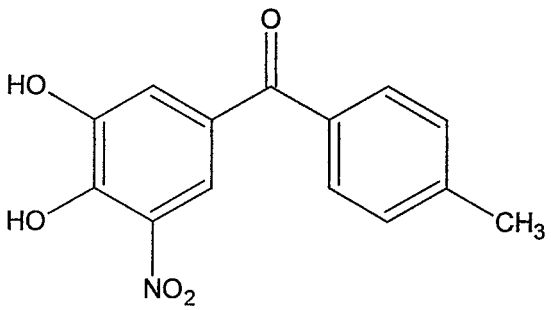
3.7 Inhibition of methyl transferases

The methyl transferases are a diverse group of enzymes that act across the phenyl propanoid pathway to catalyse the addition of methyl groups to the intermediates. The problem with blocking the lignification response at the precursor of methyl group approach is that the ethylene biosynthetic pathway would also be affected. Such effects would make interpretation difficult.

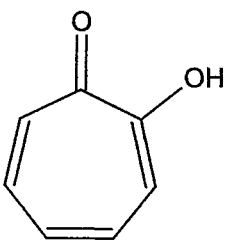
Some examples of cited inhibitors include competitive product inhibition by S-adenosyl-L-homocysteine and probably the most useful inhibitor has only been tested *in vitro*, it is a rabbit anti-O-methyltransferase IgG (Bugos *et al.* 1992). An inhibitor of a catechol-O-methyltransferase called tolcapone is used as a treatment for parkinsons disease in humans (Jorga *et al.* 1999) (Figure 3.2). It may be possible that this compound could be a general inhibitor of O-methyltransferases in plants, but it remains to be tested. Tropolone (Figure 3.2) was the older predecessor of tolcapone and inhibits catechol O-methyltransferases. Catchetol-O-methyl transferases require copper as a co-factor. Tropolone is a chelator of copper ions and as such will impair physiological function of all copper requiring enzymes (Eschelman *et al.* 1997). However tropolone has only been used in humans as an adjunct to Parkinsons disease therapy. It is available (Sigma Aldrich), but has never been tested in plants.

3.8 Inhibition of 4-Coumarate co-enzyme A ligase

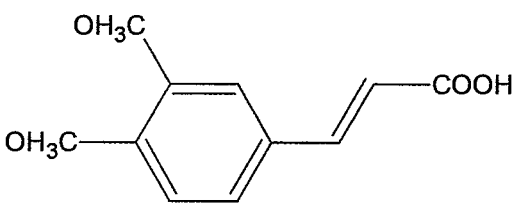
4-Coumarate co-enzyme A ligase (4CL) converts all the derivatives of cinnamic acid to their corresponding CoA ester. Only one inhibitor of 4CL, has been reported in the literature 3,4 (methylenedioxy) cinnamic acid (MCDA) (Fig 3.2). MDCA is commercially available and in addition to competitively inhibiting 4CL also has no effects on the activities of PAL, OMT and CAD in *Vanilla planfolia* (Funk and Brodelius 1990). However, MDCA has a non-competitive inhibition effect on CCR.



Tolcapone (not available)



Tropolone (Sigma)



MDCA (Sigma)

Figure 3.2 Structures of inhibitors of OMT and 4CL The compounds source is denoted by brackets.

3.9 Inhibition of Cinnamoyl Co-Enzyme A reductase

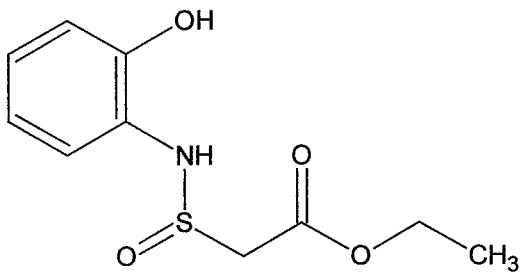
CCR converts some CoA esters to their corresponding aldehydes. MCDA (the inhibitor of 4CL, see above) is a non-competitive inhibitor of CCR (Funk and Brodelus 1990). Similarly, an inhibitor of CAD called NH_2PAS has also been reported to inhibit CCR in vitro (Carver *et al.* 1994). Other potential inhibitors include Co-A, $NADP^+$ and protein-modifying agents specific for lysine and cysteine residues are cited as potential inhibitors, but these are too non-specific to use *in planta* (Goffner *et al.* 1994).

3.10 Inhibition of Cinnamoyl Alcohol Deydrogenase

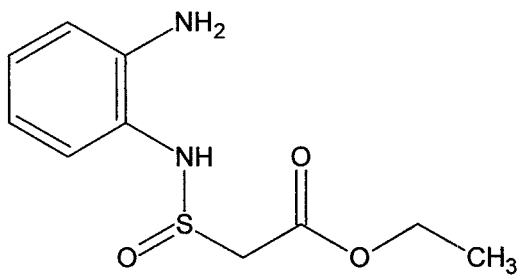
CAD catalyses the production of the alcohols formed from the aldehydes of the phenylpropanoid pathway. Several inhibitors of CAD have been reported some of which are commercially available (Figure 3.3). Zinc is a co-factor for CAD and a number of zinc chelating substrate analogues have been developed as potential CAD inhibitors. OH-PAS and NH_2PAS are both suicide inhibitors of CAD and are also zinc chelators (Carver *et al.* 1994).

2.2. dipyridyl is a non-specific cation chelator that has been shown to reduce defence related lignin in wheat (Riatt 1998).

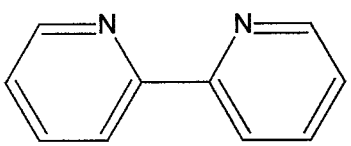
Several novel inhibitors have been designed and produced at Southampton University. These include ML19, a zinc-chelating compound produced from eugenol and two sinapoyl analogues HMMCA and CI (Hall 1998). These compounds have been specifically designed to inhibit CAD *in planta* and have dramatic effects on the activity of CAD *in vitro*.



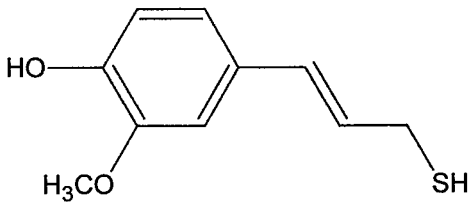
OH-PAS (Carver)



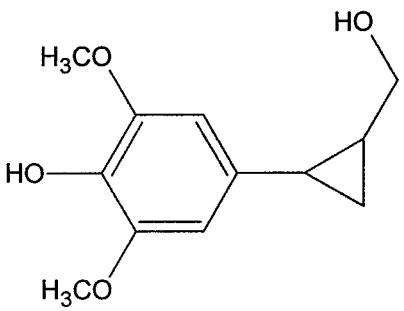
NH_2PAS (Carver)



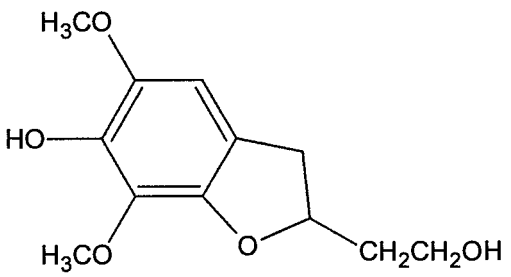
2.2 Dipyridyl (Sigma)



ML19 (Hall)



HMMCA (Hall)



CI (Hall)

Figure 3.3 Structures of inhibitors of CAD. Sources are denoted by brackets.

3.11 Aims

To evaluate inhibitors of phenylpropanoid metabolism as potential tools for the assessment of the role of lignification in plant defence. This will be achieved by testing the efficacy, phyto and mycotoxicity of the compounds and by challenging inhibitor-treated plants with a non-pathogenic fungus.

3.12 Chemicals and reagents

Agar No 2	Oxoid
Aniline blue	Sigma-Aldrich
Chloral hydrate	Sigma-Aldrich
Dimethylformamide	Fisher
Ethanol	Fisher
Glycerine	Sigma-Aldrich
Lactic acid	BDH
Mycological peptone	Oxoid
Malt extract agar	Oxoid
p-Nitrobenzene diazonium tetrafloroborate – Fast GG	Sigma-Aldrich
Phenol	Fisher

3.13 Culture of *Botrytis cinerea*

Botrytis cinerea was grown on 3.0 % (w/v) malt extract agar with 0.5 % (w/v) mycological peptone and 1.5 % (w/v) Agar No 2. The media was autoclaved at 115 °C 121 psi for 10 min (Galloway and Burgess 1952).

3.14 Treatment of plants

Plants were grown, wounded and inoculated as described in Chapter 2.

3.15 Preparation of inhibitors

A specific dissolution protocol was developed for each inhibitor and is presented in the results section. All inhibitor solutions were initially prepared as 5.0 mM and stored at –20 °C. All treatment solutions were subsequently diluted with distilled water from these stocks to the required concentrations.

3.16 Lignin staining by p-nitrobenzene diazonium tetrafloroborate – Fast GG

All staining by this method was carried out in the same manner as that used in chapter 2.

3.17 Phytotoxicity: - Chlorophyll assay

Leaves were wounded and inoculated as per the general bioassay method (Section 2.7). The inhibitors were applied with or without *B. cinerea* (1×10^6) spores ml^{-1}. After 48 h four primary leaves were cut, (32 mm in length) weighed and transferred into 1 ml of Dimethylformaldehyde (DMF). After 72 h at 4 °C the absorbance of the DMF was recorded at 647 and 664 nm. The total amount of chlorophyll was calculated using the following equation (Moran 1982):

$$\frac{(7.04 \times \text{Absorbance 664 nm} + 20.27 \times \text{Absorbance 647 nm})}{\text{Average weight of a wheat leaf} \times 1000} = \text{total chlorophyll } (\mu g\ ml^{-1})$$

$$\frac{\text{Total chlorophyll } \mu g\ ml^{-1}}{1000} = \text{total chlorophyll } (mg\ ml^{-1})$$

Percentage reduction in chlorophyll

$$100 - \left[\frac{\text{total chlorophyll mg.ml}^{-1} \text{ (inhibitor value)}}{\text{total chlorophyll mg.ml}^{-1} \text{ (water control)}} \right] \times 100$$

Results are presented as percentage reductions in chlorophyll for the inhibitor alone and in the presence of *B. cinerea.*

Worked example 2.1 Naptholic acid

	Total chlorophyll mg ml^{-1}
1.0 mM 2.1 Naptholic acid	0.518
Wounded control (water)	1.811

$$100 - \left[\frac{0.518}{1.811} \right] \times 100 = 71\ \% \text{ reduction in total chlorophyll}$$

3.18 Fungal Germination assay

Wounded wheat leaves were inoculated with *B. cinerea* 1 x 10^6 spores ml^{-1} containing inhibitors at 0.2, 1.0 and 5.0 mM.

After 8 h, leaves were stained with alcoholic lactophenol cotton blue; prepared according to Riatt (1998). Alcoholic lactophenol cotton blue was prepared by mixing one part lactophenol cotton blue (10 g phenol, 10 ml glycerine, 10 ml lactic acid, 0.02 g aniline blue and 10 ml distilled water) with two parts 95 % (v/v) ethanol. Slides were rinsed briefly (10 s) in 2.5 M chloral hydrate to clear the stain before mounting in 50 % (v/v) glycerol.

Five conidia chosen at random from each wound were examined under the light microscope at X 10. Germination was defined as when the length of the germinating hyphae exceeded the diameter of the conidia. The calculation of percentage germination is given below.

Reduction in spore germination

$$\text{Reduction (\%)} = 100 - \left[\frac{(a \times 100)}{b} \right]$$

a = Number of germinated conidia

b = Total number of conidia

Worked example OH-PAS 0.2mM

$$\frac{47}{100} \times 100 = 47\ \%$$

$$100 - 47 = 53\ \%$$

3.19 Quantification of resistance.

Four leaves comprising five wounds per leaf were inoculated with spores of *B. cinerea* as described in the general bioassay method and left for 72 h. The leaves were stained with Alcoholic lactophenol cotton blue as described previously and the extent of fungal growth examined by light microscopy between 8 and 72 h. Resistance breaking was judged on two criteria, penetration through the lignin barrier and presence in the healthy tissue at 72 h after inoculation. If these criteria appear to be met at any point along the leaf section it was regarded as a break in resistance.

Results

3.20 Screen of potential inhibitor compounds

To ascertain if the known inhibitors of phenylpropanoid metabolism are effective and non-toxic a screen of these compounds was set up. This study tested four parameters mycotoxicity, phytotoxicity and reduction of lignin. Once tolerance levels had been established on these three criteria the candidate inhibitors were tested for their ability to break resistance to *Botrytis cinerea*. For this *in vivo* study attempts were made to dissolve the inhibitors in a potentially non-toxic solvent, these are outlined for each inhibitor.

Conidia germination (mycotoxicity) was measured at 8 h after inoculation with *Botrytis cinerea* in the presence of the test inhibitor compound. Conidia germination was defined as an extension of the fungal hyphae above the width of the conidia itself. An example of ungerminated and germinated conidia is shown (Figure 3.4 A).

The reduction in total plant chlorophyll (phytotoxicity) by the inhibitor after 48 h was measured in the presence and absence of *Botrytis cinerea* according to Moran (1982).

Reduction in defence related lignin at wound margins (efficacy) by the inhibitor was calculated by the scanning densitometry method (chapter 2).

Only those compounds that at any concentration significantly reduced defence related lignin in the presence of the fungus and were sufficiently non-toxic were tested for the ability to break non-host resistance. An example of the staining shown for fungal hyphae that have extended into healthy tissue from a wound margin in the presence of a PAL inhibitor (5.0 mM AOPP) is shown. The adjacent cartoon illustrates the penetration through the wound margin that is shown in the micrograph (Figure 3.4 B). In addition fungal hyphae that persist in the wound area after 72 h in the presence of a 4-CL inhibitor (1.0 mM MDCA) is shown. The following diagram illustrates the restriction in the wound area that is shown in the micrograph (Figure 3.4 C).

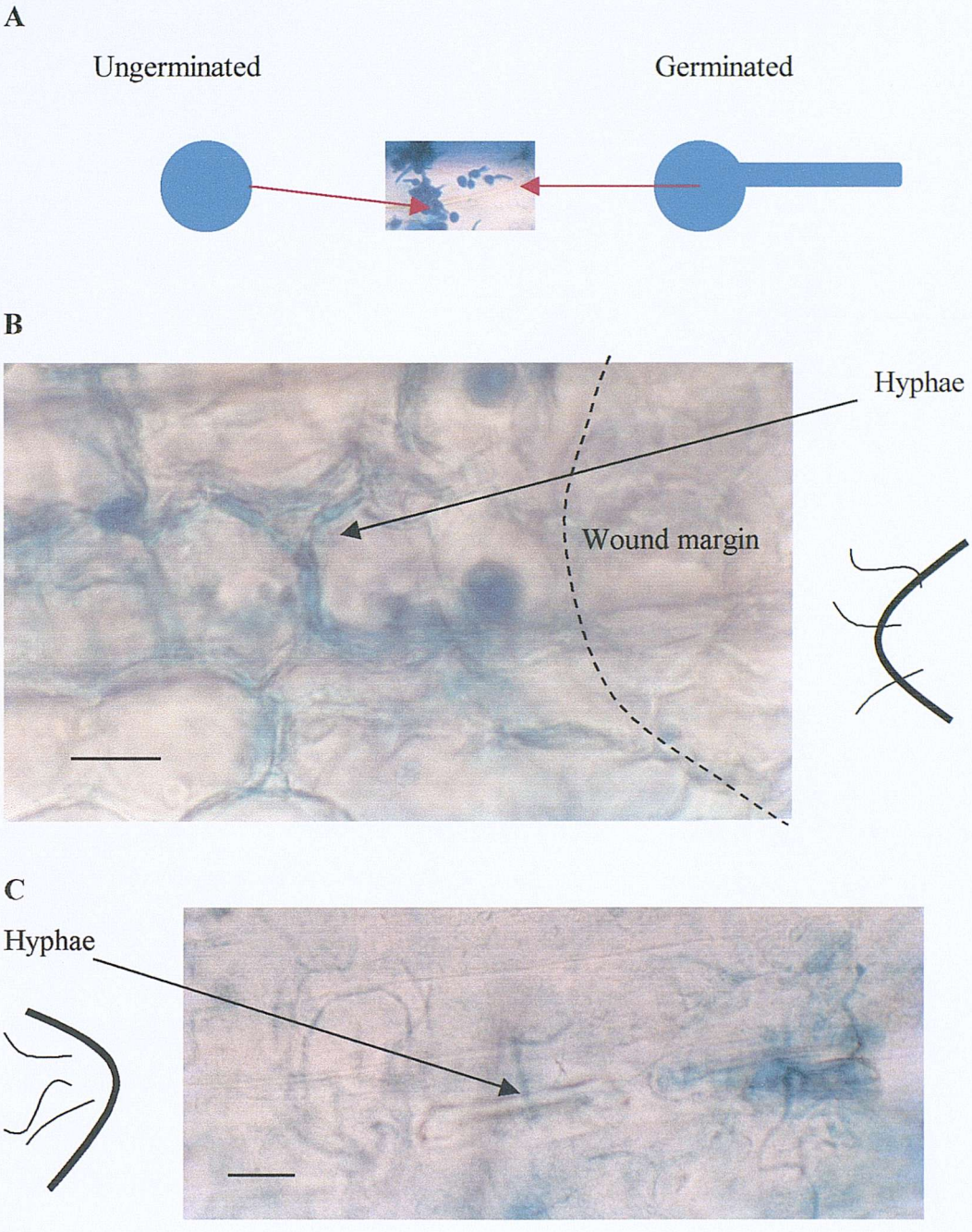


Figure 3.4 Fungal germination and resistance breaking. In all photographs the stain used is alcoholic lactophenol cotton blue: **A**, Comparison of germinated and ungerminated conidida at 8 h, (x 10). Test inhibitor AOA 1.0 mM **B**: Resistance breaking through lignin barrier after 72 h, (x 100). The arrow shows a fungal hyphae penetrating intracellular space. Bar: 2 µM. Test inhibitor: AOPP 5.0 mM. **C**: Restriction in wound after 72 h, photograph at (x 10) The arrow shows a fungal hyphae growing in a compression wound. Bar: 10 µM. Test inhibitor: MDCA 1.0 mM.

3.21 Screening of potential inhibitors of Phenylalanine Ammonia Lyase (PAL)

Three known PAL inhibitors: AOA, AIP and AOPP were screened.

Alpha aminoxyacetic acid (AOA) was dissolved in distilled water. Dilution to 5.0, 1.0 and 0.2 mM was with distilled water. Significantly reduced lignification at wound margins was observed at concentrations of 1.0 mM and 5.0 mM. At the higher concentrations this compound showed some toxicity effects significantly reducing plant chlorophyll levels in the presence of *B. cinerea* and reducing spore germination. At 1.0 mM these toxic effects were not observed and this concentration was deemed appropriate for subsequent resistance studies (Table 3.5).

2-iminoindan-2-phosphonic acid (AIP) was dissolved in distilled water. Dilution to 5.0,1.0 and 0.2 mM was with distilled water. Significantly reduced lignification at wound margins was observed at concentrations of 0.2 mM, 1.0 mM and 5.0 mM. Fungal spore germination is unaffected by the compound. Interestingly, at the highest concentration in the absence of *B. cinerea* there are no toxicity effects. However, in the presence of *B. cinerea* a slight reduction in plant chlorophyll is observed at the highest concentration. The 0.2 mM concentration displays an ideal screen profile for this study (Table 3.5).

Alpha-aminooxi-beta-phenylpropionic acid (AOPP) was dissolved in distilled water and brought to pH 7 using 2.5 mM NaOH. Dilution to 5.0, 1.0 and 0.2 mM was with distilled water. Significantly reduced lignification at wound margins was observed at concentrations of 0.2 mM, 1.0 mM and 5.0 mM. At 5.0 mM this compound showed some toxicity effects significantly reducing plant chlorophyll levels in the presence and absence of *B. cinerea*. Interestingly, at a concentration of 0.2 mM in the presence of the fungus total plant chlorophyll was significantly reduced. At 1.0 mM these toxic effects were not observed and this concentration was deemed appropriate for subsequent resistance studies (Table 3.5).

Table 3.5 Screen of inhibitors of Phenylalanine Ammonia Lyase (PAL) activity. Inhibitiors were screened by microscopy, chlorophyll extraction and the scanning densitometry method. Data is expressed in terms of percentages of spore germination after 8 h (n = 20), chlorophyll reduction after 48 h (n = 4) and lignin reduction after 48 h (n = 20). W = inhibitor alone (wounded control) F = Presence of *B. cinerea*. A * indicates those results that significantly (p = 0.05) reduced the test criteria.

Inhibitor	Dose (mM)	% Reduction in spore germination	% Reduction in total chlorophyll (W)	% Reduction in total chlorophyll (F)	% Reduction in Lignin
AOA	5.0	50	29	51*	>100*
	1.0	19	8	15	73*
	0.2	18	0	30	34
AIP	5.0	1	0	25	>100*
	1.0	0	0	2	>100*
	0.2	0	0	0	>100*
AOPP	5.0	0	43*	37*	>100*
	1.0	0	8	7	>100*
	0.2	0	12	34*	94*

3.22 The significance of PAL in the defence response

Inhibiting PAL with 1.0 mM AOA, leads to no change in the defence response up to 48 h after inoculation. However, resistance appears to break down by 72 h (Table 3.6). The change in the defence response with AOA is surprising as PAL is the first enzyme in the phenylpropanoid pathway, therefore it would be expected that inhibition of PAL would change the defence response earlier.

Inhibiting PAL with 5.0 mM AOPP, leads to a break down in resistance by 72 h post inoculation (Table 3.6, Figure 3.6). It is also interesting to note that a significant amount of chlorophyll is reduced in the presence of *B. cinerea* after 48 h (Table 3.5). Chlorosis is often associated with fungal penetration into plants. However inhibiting PAL with 1.0 mM AOPP did not lead to a break in resistance. As the screen data implicates 1.0 mM as the only concentration that does not induce chlorosis (Table 3.5) it is difficult to directly relate inhibition of PAL by AOPP to the break down of resistance.

Inhibiting PAL with 5.0 mM AIP, leads to a break down in resistance by 72 h post inoculation (Table 3.6). It is interesting to note that some reduction in total plant chlorophyll is observed in the presence *B. cinerea* after 48 h (Table 3.5). At 1.0 mM AIP, resistance appears to break down by 72 h post inoculation (Table 3.6). It was not possible to repeat these experiments in any further detail due to the short supply of the compound.

Table 3.6 Inhibition of resistance by inhibitors of PAL: All observations were of alcoholic lactophenol cotton blue stained 10 day old wheat leaves wounded and inoculated with 1 x 10^6 spores ml^{-1} *B. cinerea* using a Nikon light microscope between x 10, x 40 and x 100 objectives; +, Potential repression of the defence response; –, Defence response is progressing normally. n = 20 wounds observed. n.d. not determined. * photomicrographs included (Figures 3.5,3.6) .

Compound and dose	8 h	24 h	48 h	72 h	Verdict
AOA 1.0 mM	Low germination (-)	Hyphae contact the lignin wall (-)	Breakthrough lignin wall (+)	Growth in tissue (+)	Break in non host resistance
AIP 5.0 mM	n.d.	n.d.	n.d.	Break resistance (+)	Break in non-host resistance.
AIP 1.0 mM	n.d.	n.d.	n.d.	Break resistance (+)	Break in non-host resistance
AOPP 5.0 mM	Low germination (-)	Break out of barrier (+)	Clearing of fungi (-)	Growth in healthy tissue (+)*	Break in non-host resistance
AOPP 1.0 mM	Good germination (-)*	Immature spores (-)	Breakthrough lignin wall (+)	No Growth in healthy tissue (-)	No Break in non-host resistance

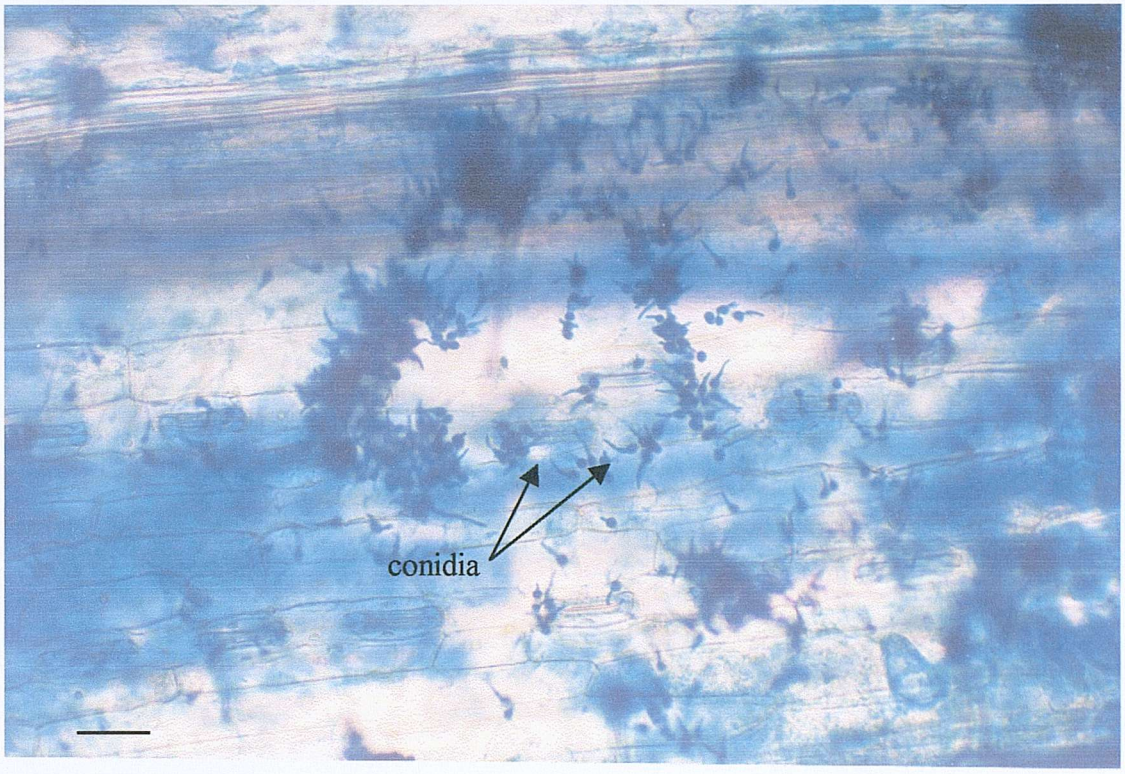


Figure 3.5 Photomicrograph of a wheat leaf surface inoculated with *B.cinerea* in the presence of 1 mM AOPP, 8 h post inoculation. The leaf surface is stained with lactophenol cotton blue. Arrows show conidia germinating on leaf surface, inside a compression wound.

X 100 magnification Bar = 10 μM

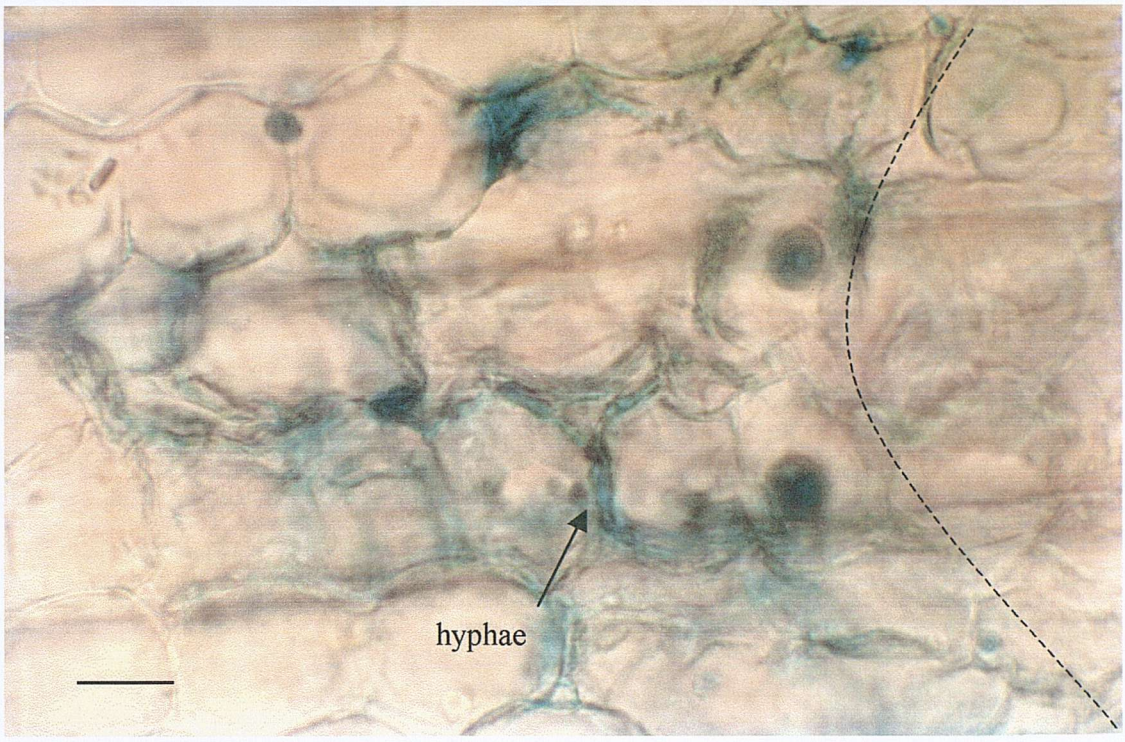


Figure 3.6 Photomicrograph of a wheat leaf surface inoculated with *B.cinerea* in the presence of 5 mM AOPP, 72 h post inoculation. The leaf surface is stained with lactophenol cotton blue. The arrow shows a fungal hyphae pentrating through the mesophyll layer from a compression wound (dashed line).
X 1000 magnification Bar = 1 μM

3.23 Screening of potential inhibitors of Cinnamate 4 Hydroxylase (C4H)

Five known C4H inhibitors: ABT, 3.2 naphthoic Acid, 2.1 naphthoic Acid, 1.2 naphthoic Acid and piperonylic Acid were screened.

1-Aminobenzoletriazole (ABT) was dissolved in distilled water. Dilution to 5.0, 1.0 and 0.2 mM was with distilled water. Significantly reduced lignification at wound margins was observed at concentrations of 1.0 mM and 5.0 mM. At the highest concentration this compound showed some toxicity effects but did not significantly reduce plant chlorophyll levels in the presence of the fungus. At the highest concentration spore germination was affected. At 1.0 mM these toxic effects were not observed except in the presence of *B. cinerea* and this concentration was deemed appropriate for subsequent resistance studies (Table 3.7).

3.2 Naphthoic Acid was dissolved in 100 µl acetone, 9 ml distilled water was added to the solution which was then de-protonated with 2.5, 1.0 and 0.1 mM NaOH to adjust to pH 7.0. Dilution to 5.0, 1.0 and 0.2 mM was with distilled water. Significantly reduced lignification at wound margins was observed only at 5.0 mM. Fungal spore germination was severely impaired at 5.0 mM but unaffected at lower concentrations. In the absence of the fungus only the 5.0 mM concentration induced phytotoxic effects. In the presence of *B. cinerea* all concentrations exhibited phytotoxic effects. Therefore no concentration can be considered for resistance studies (Table 3.7).

2.1 Naphthoic Acid was dissolved in 100 µl acetone, 9 ml distilled water was added to the solution which was then de-protonated with 2.5, 1.0 and 0.1 mM NaOH to adjust to pH 7.0. Dilution to 5.0,1.0 and 0.2 mM was with distilled water. Significantly reduced lignification at wound margins was observed at all concentrations. Fungal spore germination was unaffected at all concentrations. In the absence of *B. cinerea* only the 0.2 mM concentration did not induce phytotoxic effects. In the presence of the fungus all concentrations exhibited phytotoxic effects. Only low concentrations can be considered for resistance studies (Table 3.7).

1.2 Naphthoic Acid was dissolved in 100 µl acetone; 9 ml water was added to the solution, which was then de-protonated with 2.5, 1.0 and 0.1 mM NaOH to adjust to pH 7.0. Dilution to 5.0,1.0 and 0.2 mM was with distilled water. Significantly reduced lignification at wound margins was observed at all concentrations. Fungal spore germination was badly affected at 5.0 mM. In the absence of *B. cinerea* only the 5 mM concentration induced phytotoxic effects. In the presence of *B. cinerea* no concentration significantly reduced total plant chlorophyll. Only 1.0 and 0.2 mM concentrations can be considered for resistance studies (Table 3.7).

Piperonylic Acid was dissolved in 2 ml 0.1 M PO_4 buffer and then de protonated with 2.5, 1.0 and 0.1 mM NaOH to adjust to pH 7.0. Dilution to 5.0,1.0 and 0.2 mM was with distilled water. Lignification at wound margins was not reduced at all concentrations. Fungal spore germination was badly affected at all concentrations. In the absence of *B. cinerea* all concentrations induced phytotoxic effects. In the presence of *B. cinerea* all concentrations reduced total plant chlorophyll. No concentration of this compound can be considered for resistance studies (Table 3.7).

Table 3.7 Screen of inhibitors of Cinnamate 4 hydroxylase (C4H) activity. Inhibitiors were screened by microscopy, chlorophyll extraction and the scanning densitrometric method. Data is expressed in terms of percentages of spore germination after 8 h (n = 20), chlorophyll reduction after 48 h (n = 4) and lignin reduction after 48 h (n = 20). W = inhibitor alone (wounded control) F = Presence of *B. cinerea*. A * indicates those results that significantly (p = 0.05) reduced the test criteria.

Inhibitor	Dose (mM)	% Reduction in spore germination	% Reduction in total chlorophyll (W)	% Reduction in total chlorophyll (F)	% Reduction in Lignin
ABT	5.0	27	14	17	>100*
	1.0	0	0	29	>100*
	0.2	6	0	14	0
3.2	5.0	58	71*	67*	>100*
Naphthoic	1.0	0	7	20*	0
Acid	0.2	0	0	28*	0
2.1	5.0	0	92*	44*	>100*
Naphthoic	1.0	0	71*	22*	100*
Acid	0.2	0	16	32*	100*
1.2	5.0	93	69*	25	>100*
Naphthoic	1.0	0	13	4	>100*
Acid	0.2	0	12	6	>100*
Piperonylic	5.0	31	36	23	0
Acid	1.0	79	21	23	0
	0.2	74	23	36	0

3.24 The significance of C4H in the defence response.

ABT at 1.0 mM causes a break in non-host resistance at 24 h post inoculation. It is interesting to note that some reduction in total plant chlorophyll is observed in the presence *B. cinerea* after 48 h (Table 3.7). By 72 h *B. cinerea* appears to be able to penetrate the stomata. This indicates that the C4H step is very important in terms of defence related lignification (Table 3.8).

In the experiments involving 1.2 Naphthoic acid and 2.1 Naphthoic acid microbial contamination occurred, this could mean a break in non-host resistance with these compounds but these experiments were not repeated.

Table 3.8 Inhibition of resistance by inhibitors of C4H. All observations were of alcoholic lactophenol cotton blue stained 10 day old wheat leaves wounded and inoculated with 1 x 10^6 spores ml^{-1} *B. cinerea* using a Nikon light microscope between x 10, x 40 and x 100 objectives; +, Indicates potential repression of the defence response; –, indicates defence response is progressing normally. For each time point 20 wounds were observed. n.d. not determined.

Compound and dose	8 h	24 h	48 h	72 h	Verdict
ABT 1.0 mM	Germination in healthy tissue (-)	Break in resistance (+)	n.d.	Penetration of stomata (+)	Break in non – host resistance
2.1 Naphthoic Acid 1.0 mM	n.d.	n.d.	n.d.	Growth (-)	Repetition req
1.2 Naphthoic Acid 1.0 mM	n.d.	n.d	n.d.	Growth and some contamination (-)	Repetition req

3.25 Screening of potential inhibitors of O-Methyltransferases (OMT)

One known inhibitor of methyltransferases was screened.

Tropolone was dissolved in water. Dilution to 5.0, 1.0 and 0.2 mM was with distilled water. Significantly reduced lignification at wound margins was observed only at 5.0 mM. Fungal spore germination was reduced at 0.2 mM. There are significant reductions in total chlorophyll at 1.0 and 5.0 mM in both the presence and absence of *B.cinerea*. Therefore this compound was not studied further (Table 3.9).

3.26 The significance of OMT in the defence response

As no inhibitors were obtained that effectively inhibited the defence response, no results can be obtained on this enzyme.

Table 3.9 Screen of the inhibitor of O-methyltransferase (OMT) activity. Data is expressed in terms of percentages of spore germination after 8 h (n = 20), chlorophyll reduction after 48 h (n = 4) and lignin reduction after 48 h (n = 20). W = inhibitor alone (wounded control) F = Presence of *B. cinerea*. A * indicates those results that significantly (p = 0.05) reduced the test criteria.

Inhibitor	Dose (mM)	% Reduction in spore germination	% Reduction in total chlorophyll (W)	% Reduction in total chlorophyll (F)	% Reduction in Lignin
Tropolone	5.0	1	88*	93*	>100
	1.0	4	76*	46*	14
	0.2	75	19	20	14

3.27 Screening of potential inhibitors of 4-coumarate co-enzyme A Ligase (4CL)

One inhibitor of 4CL was screened.

Methylene dioxy cinnamic acid (MDCA) was dissolved in 200 µl acetone and 9 ml water was added. The solution was sonicated for 5 mins @ 60 °C and de-protonated using 2.5 and 0.5 mM NaOH adjust to pH 7.0. Dilution to 5.0, 1.0 and 0.2 mM was with distilled water. Significantly reduced lignification at wound margins was observed at 5.0, 1.0, and 0.2 mM. Interestingly, the lower concentrations only reduced lignification by approximately a half. Fungal spore germination was severely impaired at 5.0 mM but this effect was less apparent at 1.0 and 0.2 mM. In the absence of the fungus no concentration induced phytotoxic effects. In the presence of the fungus only the 5.0 and 1.0 mM concentrations exhibited phytotoxic effects. Therefore this inhibitor merits further study at a 1.0 mM concentration (Table 3.10).

Table 3.10 Screen of the inhibitor of 4-coumarate co-enzyme A Ligase (4CL) activity. Data is expressed in terms of percentages of spore germination after 8 h (n = 20), chlorophyll reduction after 48 h (n = 4) and lignin reduction after 48 h (n = 20). W = inhibitor alone (wounded control) F = Presence of *B. cinerea*. A * indicates those results that significantly (p = 0.05) reduced the test criteria.

Inhibitor	Dose (mM)	% Reduction in spore germination	% Reduction in total chlorophyll (W)	% Reduction in total chlorophyll (F)	% Reduction in Lignin
MDCA	5.0	95	15	27*	>100*
	1.0	20	16	24*	46*
	0.2	0	0	12	48*

3.28 Significance of 4CL in the defence response.

Application of MDCA caused no visible break in non-host resistance at both the 1.0 and 0.2 mM level (Table 3.11). It is interesting to note that in the presence of the fungus total plant chlorophyll was significantly reduced at 1.0 mM but not at 0.2 mM (Table 3.10).

Table 3.11 Inhibition of resistance by inhibitors of 4CL: All observations were of alcoholic lactophenol cotton blue stained 10 day old wheat leaves wounded and inoculated with 1 x 10^6 spores ml^{-1} *B. cinerea* using a Nikon light microscope between x 10, x 40 and x 100 objectives; +, Indicates potential repression of the defence response; – , indicates defence response is progressing normally. n = 20 wounds observed. * Illustrated by photomicrograph (Figure 3.7).

Compound and dose	8 h	24 h	48 h	72 h	Verdict
MDCA 1.0 mM	Germination	Could be breaking resistance (+)	No break (-)	Growth in wound (-)*	Does not break non-host resistance
MDCA 0.2 mM	Germination	No change in lignin barrier (-)	Break (+)	No Growth (-)	Repetition req

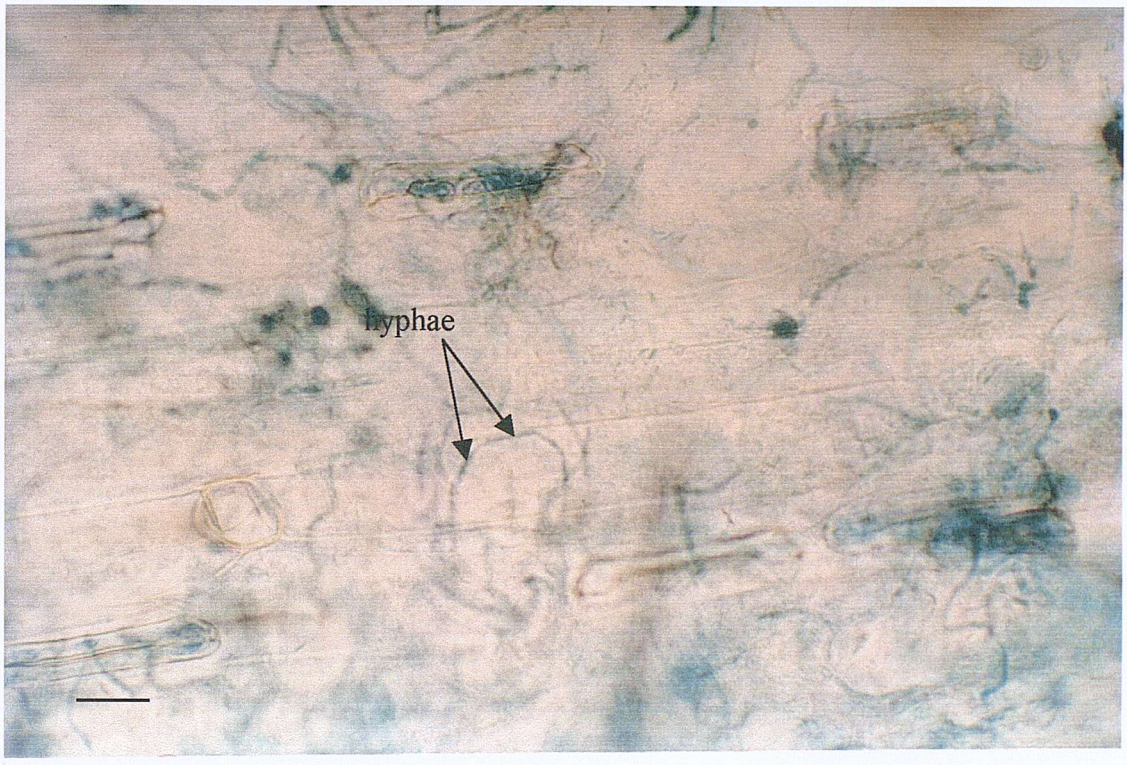


Figure 3.7 Photomicrograph of a wheat leaf surface inoculated with *B. cinerea* in the presence of 1 mM MDCA, 72 h post inoculation. The leaf surface is stained with lactophenol cotton blue. The arrow shows a fungal hyphae growing in a compression wound. X 10 magnification Bar = 10 μM.

3.29 Screening of potential inhibitors of Cinnamoyl Alcohol Dehydrogenase (CAD)

Six CAD inhibitors were screened.

OH-PAS, was dissolved in distilled water and diluted to 5.0, 1.0 and 0.2 mM concentrations. Significantly reduced lignification at wound margins was observed at all concentrations. Spore germination was reduced greatly at all concentrations. There were significant phytotoxic effects at all concentrations in the presence and absence of *B. cinerea.* The only possible concentration for further study may be 0.2 mM (Table 3.12).

NH_2PAS was dissolved in distilled water and diluted to 5.0, 1.0 and 0.2 mM concentrations. Significantly reduced lignification at wound margins was observed at all concentrations. Spore germination was reduced greatly at 5.0 and 1.0 mM concentrations. There were significant phytotoxic effects at 5.0 and 1.0 mM in the absence of *B. cinerea.* In the presence of *B. cinerea* total plant chlorophyll was only significantly reduced at 0.2 mM. The only possible concentration for further study may be 0.2 mM (Table 3.12).

2.2 Dipyridyl dissolves in water after sonnication at 50 °C for 10 min.
Significant reduction in lignification at wound margins was observed at 5.0 and 1.0 mM concentrations, but at these concentrations spore germination is heavily reduced. Total plant chlorophyll in the presence and absence of *B. cinerea* is not significantly affected. Due to the mycotoxic effects, this inhibitor is will not be considered further (Table 3.12).

ML19 was received as a 5 mM stock (Hall 1998). Significant reduction in lignification at wound margins was observed at 5.0 mM. Spore germination is only slightly affected at 5.0 and 1.0 mM. Total plant chlorophyll in the presence and absence of *B. cinerea* is not significantly affected. As only the 5.0 mM concentration reduces lignification this will be used for further study (Table 3.12).

4-hydroxy alpha mecapto-3-methoxycinnamic acid (HMMCA), dissolved in distilled water by boiling with gentle agitation. No concentration significantly inhibited the lignification response. Spore germination was only affected at 5.0 mM. There were no significant reductions in total chlorophyll any concentration in the presence or absence of *B. cinerea*. As this compound does not reduce lignification it is not appropriate for further study (Table 3.12).

CI was as a 5.0 mM stock (Hall 1998). Significant reduction in lignification at wound margins was observed at 5.0, 1.0 and 0.2 mM. Spore germination is only affected at 5.0 mM. Total plant chlorophyll in the absence of *B. cinerea* is not significantly reduced. In the presence of *B. cinerea* total plant chlorophyll is significantly reduced. The 1.0 and 0.2 mM concentration will be used for further study (Table 3.12).

Table 3.12 Screen of the inhibitors of cinnamoyl alcohol dehydrogenase (CAD) activity. Data is expressed in terms of percentages of spore germination after 8 h (n = 20), chlorophyll reduction after 48 h (n = 4) and lignin reduction after 48 h (n = 20). W = inhibitor alone (wounded control) F = Presence of *B.cinerea*. A * indicates those results that significantly (p = 0.05) reduced the test criteria.

Inhibitor	Dose (mM)	% Reduction in spore germination	% Reduction in total chlorophyll (W)	% Reduction in total chlorophyll (F)	% Reduction in Lignin
OH-PAS	5	100	66*	31*	>100*
	1	100	60*	44*	81*
	0.2	53	28*	19*	100*
NH_2PAS	5	100	32*	7	>100*
	1	93	42*	35	>100*
	0.2	12	18	43*	100*
2.2 Dipyridyl	5	100	6	22	>100*
	1	100	21	6	99*
	0.2	22	26	11	30
ML19	5	17	0	25	>100*
	1	23	14	33	0
	0.2	0	26	24	0
HMMCA	5	44	0	20	0
	1	0	0	17	0
	0.2	0	0	14	0
CI	5	88	0	1	72*
	1	0	0	21	65*
	0.2	0	0	22*	61*

3.30 The significance of CAD in the defence response

OH-PAS at 0.2 mM shows a break in non-host resistance after 72 h (Table 3.13). However, at all concentrations total plant chlorophyll was significantly reduced in both presence and absence of *B. cinerea* (Table 3.12).

Treatments with 1.0 mM NH_2PAS and 5.0 mM 2.2 dipyridyl did not break resistance; fungal spore germination in these experiments was not recorded (Table 3.13).

Treatment with ML19 5.0 mM does cause a break in non-host resistance after 72 h the hyphae are present in the healthy tissue of the plant (Table 3.13, Figure 33).

In the resistance experiments with CI growth is observed after 72 h on the healthy tissues with the 0.2 mM concentration rather than the 1.0 mM (Table 3.13). Although the initial tests do not confirm that 1.0 mM CI is mycotoxic, this is one possible explanation for the lack of spores after 72 h in these experiments. Interestingly at 0.2 mM chlorophyll is significantly reduced only in the presence of the fungus (Table 3.12).

Table 3.13 Inhibition of resistance by inhibitors of CAD. All observations were of alcoholic lactophenol cotton blue stained 10 day old wheat leaves wounded and inoculated with 1 x 10^6 spores ml^{-1} *B. cinerea* using a Nikon light microscope between x 10, x 40 and x 100 objectives; +, Indicates potential repression of the defence response; –, indicates defence response is progressing normally. n = 20 wounds observed.

Compound and dose	8h	24h	48h	72h	Verdict
OH PAS 0.2 mM	n.d.	n.d.	n.d.	Break of resistance (+)	Breaks non-host resistance
NH_2PAS 1.0 mM	No growth (-)	n.d.	n.d.	n.d.	No break
2.2 Dipyridyl 5.0 mM	No growth (-)	n.d.	n.d.	No growth	No break
ML19 5.0 mM	n.d.	n.d.	n.d.	Breaks resistance (+)*	Breaks non-host resistance
CI 1.0 mM	No growth (-)	No change in healthy tissue (-)	n.d.	No spores (-)	Does not break non-host resistance
CI 0.2 mM	Growth (-)	Very immature spores (-)	n.d.	Growth on healthy tissues (+)	Breaks non-host resistance

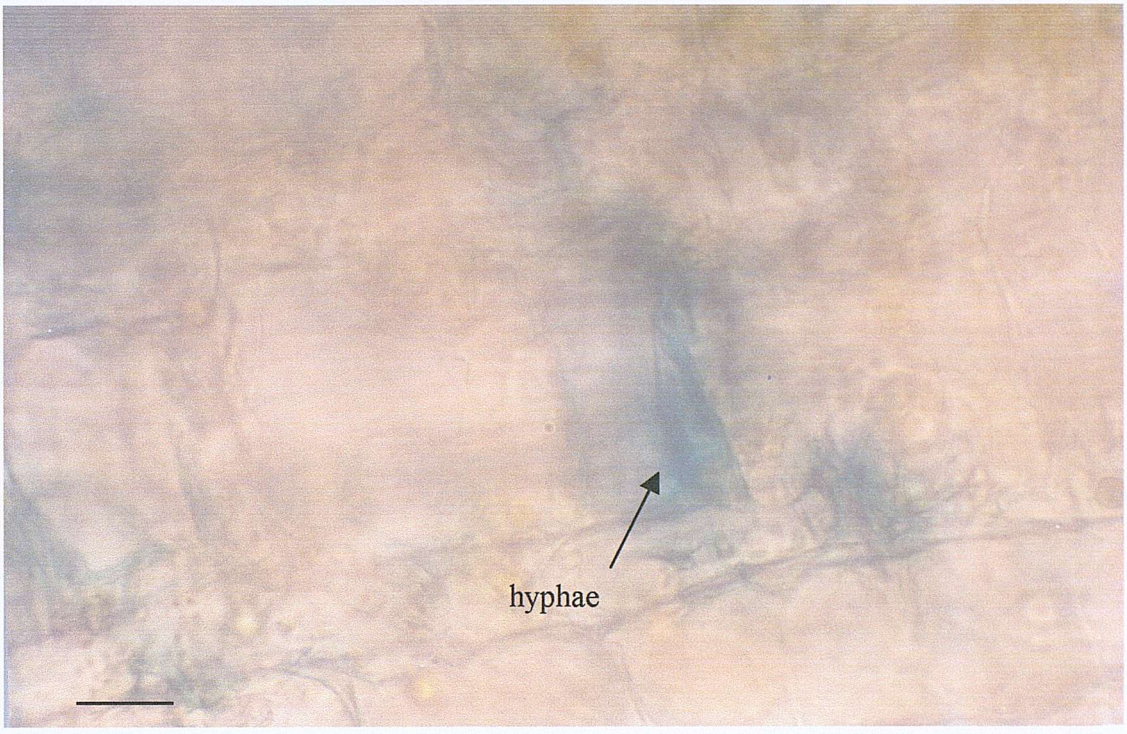


Figure 3.8 Photomicrograph of a wheat leaf surface inoculated with *B. cinerea* in the presence of 5 mM ML19, 72 h post inoculation. The leaf surface is stained with lactophenol cotton blue. Arrows show hyphal structures growing in healthy tissue. X 1000 magnification Bar = 1 μM.

Discussion

Several studies have shown that in general the tested compounds work well to reduce lignification *in vitro* (Table 3.4). The question remained if these inhibitors had any general problems that prohibited their use *in vivo* for agro-industrial purposes or for the study of defence related lignification.

3.31 Potential for PAL manipulation to reduce lignification.

3.31.1 Specificity of the inhibitors of PAL

All the inhibitors of PAL tested here are well-characterised inhibitors of the PAL enzyme.

2-Aminoxyacetate (AOA) was first introduced by Amrhein *et al* (1976), but has been criticised because its lack of specificity. Pyrodoxial phosphate enzymes such as 1-aminocyclopropane-1-carboxylate synthase an enzyme involved in ethylene synthesis (De Laat and Van loon 1981) and aminotransferases (Amrhein *et al* 1976) are affected by AOA. However, this study shows that at 1.0 mM concentration *in vivo* the production of chorophyllus pigments is not affected. The lack of specificity therefore makes AOA alone not a good candidate inhibitor for study of defence related lignification. Studies that make exclusive use of AOA as an inhibitor of PAL may be viewed with some caution.

Recent research highlights AIP as possibly the best inhibitor to study PAL action (Appert *et al* 2003). When compared to AOPP 100 fold less AIP is required to completely inhibit cinnamic acid synthesis (measured by its effect on light induced anthocyanin synthesis) (Zon and Amrhein 1992). However further research in *Vica faba* shows that AIP can slow down the cell cycle progression between G1 to S phase. This is likely to because free phenylalanine is required for normal cell cycle progress. However, it is observed that this difference gradually diminished during the later stages of the cycle (Cvikrova *et al* 2003). Although AIP does have some limitations for long-term study it clearly reduces lignification and is a good tool for study of PAL as a part of defence related lignification.

AOPP was not as effective as AIP in reducing defence related lignification.
Only the highest (5.0 mM) concentration of AOPP causes a break in resistance, at these levels the reduction in total chlorophyllus pigments is quite high, and this non specific inhibition may mask the true effect of the inhibitor on the PAL enzyme. No further data is available on the side effects of AOPP.

Clearly, as would be assumed, the first step of the production of lignin is very important in the final outcome or progression of the response. The flux induced by the presence of the non-host response however is so great that unless PAL is very significantly inhibited, then the non-host response will occur as normal. It will be interesting to see more evidence for the mechanism of action of AIP later in this series.

3.31.2 Significance of PAL to plant defence in wheat

The deamination of phenylalanine to *trans*-cinnamic acid makes PAL an important molecule in plant defence (Appert *et al* 2003) as trans cinnamic acid leads to several other defence related metabolic pathways.

This study shows that in all the PAL inhibitors can provide some information on the defence response but are best viewed as a suite of inhibitors rather than individuals.

Recent studies on barley with the wheat pathogen *Erysiphe graminis* using inhibitors show that penetration of the fungus is increased in the presence of the inhibitors AIP, AOA and AOPP (Arkawa *et al* 1997). A criticism of this work is that *E. graminis* is a pathogen of graminacae and thus it is harder to quantify the susceptibility produced to all potential pathogens when PAL is inhibited. Although this supports the work here the same limitations may well exist in barley as in wheat and the use of the suite of inhibitors is important to show that PAL inhibition is the caustive factor in loss of the defence response.

Another similar study shows that 1 mM AOPP treated wheat plants did not show increased penetration efficiency by *Blumeria graminis f.sp. tritici* (Stadnik and Buchenauer 2000). Again the same criticism is that a direct pathogen of wheat is not the best choice to model a break in constitutive resistance of wheat. The work here

shows that albeit with some induced phytotoxicity, resistance to *B. cinerea* is decreased after inhibition of the PAL enzyme.

3.31.3 Potential for manipulation of lignin by inhibiting PAL

As PAL is an important branch point for many pathways, attempts have already been made to manipulate it for commercial purposes. The production of taxol (Brincat *et al* 2002), the control of abscission of citrus fruits (Kostenyuk *et al* 2002) and the control of browning of lettuce (Peiser *et al* 1998) have all been successfully manipulated using biochemical PAL inhibitors. Although none of the inhibitors have been approved for food use there is some commercial interest in their application.

With the production of taxol only AOA and AOPP were considered and differing effects were observed in that AOA decreased taxol production and AOPP slightly enhanced and then had no effect at 1 and 10 μM respectively. The shutting down effect may be due to AOA having effects on other pathways (not necessarily the phenylpropanoid pathway). Interestingly AOPP in this study also has an effect on chlorophyll biosynthesis (phytotoxic effect) at 200 μM and not at 1 mM. A possible explaination could be dissociation of the compound during dilution however, there is no evidence for this.

Recently some new inhibitors of PAL, (AIC, APEP) have been developed Appert *et al* (2003). These were not available at the time of this study but may well be useful tools to further investigate PALs action. A recommendation would be to subject them to similar bioassay tests to further define the effects *in vivo*.

3.31.4 The study of plant defence with the inhibitors of PAL.

The combination of AOA as inhibitor of the PAL enzyme at a 1.0 mM concentration may be used to study PAL or the phenylpropanoid pathway but results should be considered in terms of other inhibitors. Only if other inhibitors also show affects in a similar fashion should results with AOA be taken to be the effect of direct inhibition of the PAL enzyme.

The combination of AIP as inhibitor and PAL as enzyme at 1.0 mM can be used to study PAL or the phenylpropanoid pathway. As PAL is a major step in the pathway and resistance to non-pathogens is directly affected this inhibitor may be a useful tool to study the metabolic background in which PAL functions.

Some evidence may also be gained on PAL function with AOPP. However the likelihood of studies continuing with this compound is reduced due to its lack of availability at this time, as with many compounds a source is hard to secure. Recent work however has cited Genosys Biotechnologies Inc, Cambridge UK (Stadnik and Buchenauer 2000) as a possible source, or relied on synthesis by the laboratory itself Appert *et al.* (2003).

The best strategy is to use AIP as the major component of any further study on the PAL enzyme or genes. Data from AOA and AOPP may well be of use *in vitro* but their use *in vivo* should be viewed with some caution.

PAL is clearly an important enzyme in the wheat defence response as inhibition of PAL by three inhibitors at non-toxic concentrations leads to a break in resistance accompanied by a significant reduction in lignification and total plant chlorophyll.

3.32 Potential for C4H manipulation to inhibit lignification

3.32.1 Specificity of the inhibitors of C4H

1-Aminobenzyltriazole (ABT); is an inhibitor of hydroxylation reactions that may be considered too unspecific due to its probable effects on non-target hydroxylases (Reichart *et al* 1982). This study shows at 5.0 mM concentration and 1.0 mM concentration, 17 and 29 % of total chlorophylls are reduced respectively.

The naptholic acid and piperonylic acid compounds are cytochrome p450 inhibitors, C4H is believed to be a p450 dependent hydroxylase (Schalk *et al.* 1998). Piperonylic acid was the most effective compound *in vitro* but causes some chlorosis *in vivo*. Lignification was not affected at any concentration by Piperonylic acid. The degree of difficulty of dissolution of the compound may explain its lack of efficacy. However Piperonylic acid has been shown to be effective as an inhibitor of C4H at 10 μM, measured by the accumulation of salicyclic acid (Schoch *et al.* 2001).

Although being less potent inhibitors of p450 hydroxylases the compounds 2.1 and 1.2 Naptholic acid did cause some phytotoxicity. These inhibitors could only be used at low concentrations.

3.32.2 Significance of C4H to plant defence in wheat

With ABT lignification is reduced significantly and resistance can be seen to be broken at 1.0 mM concentration. This is consistent with the results of Riatt (1998) who provided some preliminary data on this inhibitor.

The lack specificity of the inhibition by the naptholic acid compounds *in vivo* due to their phytotoxicity makes the significance of C4H to plant defence is hard to quantify. The resistance breaking experiments for these compounds were not successful and required repetition.

C4H may be significant to plant defence but the inhibition caused by the compounds tested here cannot show this due to their lack of specificity.

3.32.3 Potential for manipulation of lignification by inhibiting C4H

Several new inhibitors of C4H have been developed these are substrate analogues of C4H (Schoch *et al.* 2001). However several reactions do involve hydroxylation via p450s and it is likely that substrate analogues of one hydroxylase substrate may cause damaging effects on other plant metabolic pathways. These substrates were not available at the time of the experiments and were not considered for this study.

3.32.4 The study of plant defence with inhibitors of C4H

The inhibitors tested here fall short of the criteria required for a good inhibitor of C4H, as they are too phytotoxic. The newly developed inhibitors (Schoh *et al.* 2001) may have greater specifity and could provide a better model to understand the significiance of lignification.

3.33 Potential for Methyltransferase manipulation to inhibit lignification

3.33.1 Specificity of the inhibitors of OMT

The OMT described by Funk and Brodelius (1990) in phenylpropanoid metabolism is a catechol *O*– methyltransferase. The catetchol *O*-methyltransferase inhibitor Tropolone had not been previously tested in plants. This chemical was hoped to have enough similarity to plant caffeoyl *O*-methyltransferase reactions to reduce lignification. In this study, the presence of tropolone does reduce defence related lignification but at the expense of the loss of chlorophyllus pigments and at lower concentrations causes reduction in spore germination. One possible explaination is the requirement of the metabolism of *B. cinerea* using methyl-transfer enzymes. The plant toxicity may come from the interaction between tropolone and copper (Eschelman *et al* 1997). Although some potential compounds for inhibiting OMT exist, at present no further progress can be made at present by manipulating the phenylpropanoid pathway by inhibiting OMT.

OMT may be significant step in plant defence in wheat, this study is unable to expand on the known evidence. Considering its position in the phenylpropanoid pathway the potential for manipulating lignification via OMT is great but no potent inhibitors exist of this compound.

3.34 Inhibitors of 4-coumarate co-enzyme A Ligase

3.34.1 Specificity of the inhibitors of 4CL.

Only one potential inhibitor has been characterised for this particular step in the pathway. However Funk and Brodelius (1990) note that there is some effect on the CCR enzyme as well. This inhibitor has great potential as it reduced lignification by almost exactly half. It is possible therefore that the lignin specific pathway of PPM could be isolated and further study could be made of uncharacterised routes into the pathway as if lignification is reduced by half, this half must have come from some other source. If the lignins present in MDCA treated leaves are sinapoyl in nature it could be good evidence for a pathway in wheat. The maule test Chapple *et al.* (1992), Ilyama *et al.* (1988) is postulated to preferentially bind sinapoyl units. This is because of the creation of methoxy-o-quinone structures in the reaction that produce a purple-red colour. This reaction is probably only specific to the "sinapoyl" end of the pathway. This represents one way of quantifying this potential difference.

Another method would be nitrobenzene oxidation which transforms the H,G and S units into substrates which can be visualised by chromatography and quantified. Obviously the nature of the lignins produced with this particular inhibitor present would be very interesting to ascertain.

3.34.2 Significance of 4CL to plant defence in wheat.

This study cannot conclusively show a break in non-host resistance because 4CL is inhibited. The method of examining leaves for breaking resistance by looking at the change in the lignin barrier or for presence in the leaf itself is time consuming and not a very robust assay. Other studies that examine lignification as a defence response look at penetration efficiency of the fungus but this requires a fungus that penetrates and is therefore pathogenic to the plant. No other work has attempted to correlate purely defence related lignification and the inhibition of 4CL.

3.34.3 Potential for manipulation of lignin by inhibiting 4CL.

As inhibition of 4CL by MDCA does not break resistance to non-host fungi, this step could therefore be a safer target for genetic manipulation or the development of new inhibitor compounds on this step. The recent development of the aspen mutants by Chiang *et al.* (2002) and the reduction of the lignification thereby and its use for paper-making makes this the ideal step for further research into the mechanism of inhibition.

3.34.4 The study of plant defence with the inhibitor of 4CL.

Use of MDCA at 0.2 mM to inhibit 4CL has potential for further investigation of the phenylpropanoid pathway, it would be hoped that further studies could design more specific inhibitors that could truly test the inhibition of 4CL alone.

3.35 Inhibitors of Cinnamoyl Alcohol Co-enzyme A Reductase

There are no direct inhibitors of this step of metabolism, however it is clearly a target of researchers, (Grabber, personal communication). The best inhibition comes from NH_2PAS and MDCA, it is unclear therefore the best method of inhibition of this compound and could be a target for further studies of potential inhibitors using the bioassay method.

Although hydroxycinammic acid is mentioned as an inhibitor of CCR in popular (Oskabe *et al.* 1999), it is currently unavailable. No direct data has been obtained on the nature of CCR to plant defence in wheat by biochemical inhibition. Therefore its potential or significance to the manipulation of lignification in wheat will not be considered.

3.35 Inhibitors of Cinnamoyl Alcohol Dehydrogenase

3.35.1 Specificity of the inhibitors of CAD

HMMCA appears to augment rather than reduce lignification, this compound was designed at Southampton University. However HMMCA does show effects *in vitro* on CAD activity and a preference for CAD A (Hall 1998). A possible explaination for the effect on lignification could be its structural similarity to the other units of lignification. This may lead to its direct incorporation into lignin itself. A possible method for investigating this would be to use an extractive technique such as nitrobenzene oxidation. This may lead to changes in an HPLC elution profile of the lignin monomers. Previous work has shown a 70 % reduction in lignification measured by the Klasson technique, however no effect on lignification was shown in this study. One possible explanation could be dissociation of the compound over time in storage; re-synthesis of this compound would be required to test this.

2.2. Dipyridyl has been shown by to inhibit CAD *in vitro* Hall (1998). However *in vivo* this compound has mycotoxic effects and was not investigated further. This may be due to its broad spectrum of activity as a chelator of Zinc, which is presumed also to be the cause of phytotoxic effects (Hall 1998).

NH_2PAS has been shown to inhibit CAD and CCR *in vitro* by Carver *et al.* (1996). However in this study only at the lowest concentration (0.2 mM) NH_2PAS shows any potential to break resistance, even so total chlorophylls are reduced significantly heavily. This compound is probably not a good choice for the study of the defence response.

OH PAS has been shown to inhibit CAD by Carver *et al.* (1996), although being a more effective inhibitor of CAD. At the lowest concentration (0.2 mM) total chlorophylls are slightly reduced making it not ideal for the study of lignification.

ML19 is a suicide inhibitor of CAD (Hall 1998). Only the highest (5.0 mM) concentration seems to be effective at reducing lignification. At 0.2 mM ML19 has been shown to have a preference for CAD C but only shows a 30 % inhibition of

CAD. Possibly a tighter dilution series could lead to more accurate inhibition data. However, at the 5.0 mM concentration the criteria for a good inhibitor are met.

CI (Coniferal thiol) is also an inhibitor developed by Hall (1998), again being specifically designed to inhibit CAD. In contrast to ML19, CI does not completely inhibit lignification. There are three isoforms of CAD in wheat (Mitchell *et al.* 1996) and CAD C is inducible. Previous work shows that CI preferentially inhibits CAD isoforms A and B over CAD C but that only 40-50 % of the activity is reduced. It is hypothesised that the oxygen group of CI may form a stable anion during the dehydrogenase reaction of CAD, which may chelate zinc, the co-factor of CAD.

3.35.2 Significance of CAD to plant defence

Resistance breaking experiments with ML19 and CI do detect a break in non-host resistance. Both ML19 and CI have been shown to be inhibitors of CAD *in vitro* with a preference for CAD C (Hall 1998) this study gives some evidence that they are good candidates for study of the defence response. Taken together these experiments give evidence that *in vivo* CAD induction is a vital step for the progression of the defence related lignification and the preservation of non-host resistance.

Interestingly, application of CI breaks non-host resistance only at the 0.2 mM concentration. As the phyotoxicity results and lignification inhibition levels are so similar for both the 1.0 mM and 0.2 mM concentration this is surprising. One possible explanation could be a threshold inhibition of the CAD isoforms, the plant may synthesise more CAD in response to the inhibition by CI, and therefore in the end the non-host fungus cannot break resistance. This remains to be tested.

3.35.3 Potential for manipulation of lignin by inhibiting CAD

CAD is four enzymes downstream of the start of the phenylpropanoid pathway. The potential for manipulation is therefore less as the derivatives that feed to CAD are already part of a complex pathway.

CAD C in wheat has been shown to be inducible defence related interactions (Mitchell *et al.* 1996), therefore it is not the best candidate to manipulate for lignification, as treatments that effect CAD are shown to cause a non-pathogen to have greater penetration in plant cells.

3.35.4 The study of plant defence with the inhibitors of CAD.

Two of the potential CAD inhibitors provide a potential novel approach to managing plant defence. Some intermediates of the phenylpropanoid pathway have been shown to be mycotoxic Barber *et al.* (2000). Analogues of the phenylpropanoid pathway that are not only mycotoxic such as 2.2 Dipyridyl but augment lignification such as HMMCA may provide some protection against potential pathogens. Potentially these compounds could be phytoalexin like chemicals.

The compounds ML19, CI and OH-PAS all show at some concentration an ability to reduce plant defence for the germination of a non-host fungus, these compounds therefore are useful for manipulation of lignification through inhibition of CAD.

Possibly as with the PAL inhibitors the best data would be obtained by use of the suite of inhibitors rather than a single inhibitor. To confirm the specificity of the novel CAD inhibitors assays of their effect against the enzymes of the phenylpropanoid pathway must be performed.

3.36 Conclusions

Botrytis cinerea is an obligate biotroph that will grow well on necrotised tissue. Therefore any inhibitor that produces phytotoxicity defined as loss of 25 % of the chlorophyllus pigments could lead to a false positive break in resistance. This is a weakness of the study as is the lack of a quantitative assay to measure the breaking of resistance. One possible improvement would be to add a positive control of a fungus that is known to affect wheat such as *Puccina graminis* to allow actual comparison of penetration efficiency. More sophisticated microscope techniques such as electron or confocal microscopy may also provide improved data on the maintenance of resistance.

In terms of useage of these inhibitor compounds in an industrial context, it is clear that much more work needs to be done, however the only steps of inhibition not clearly shown to lead to a break in resistance are the 4CL, OMT and CCR steps. This is chiefly due to the lack of availability of appropriate specific inhibitor compounds.

Only 4-CL can be judged as possibly being a good target for transgenic inhibition strategies, it is very important that non-host elements are taken into account when choosing a strategy for manipulating lignin. Indeed the 4CL reduced apsen lines of the Chiang group show a comparable level of lignin reduction as shown in this study. If these values hold true for the safe manipulation of lignin by the transgenic method then there is great hope for both the forage crop and paper milling industries.

It is clear that both the PAL and CAD steps are very important in determining the non-host resistance outcome. The C4H step may also be important. However conclusive data cannot be drawn from this study. More inhibitors however have recently been developed and with access to the sequences for the C3H enzyme perhaps more specific inhibitors can be designed.

It is too simple to conclude that reduction in lignification can directly lead to non-pathogen colonisation. Factors such as pathogen strain and whether it has recently infected a plant, humidity and light may well have effects on pathogencity of the pathogen. In the end it is a myriad of defence responses that control accessibility and

no one response at a particular time or space can be said to be the overriding factor in the determination of the compatible or incompatible host outcome for every species. So it is prudent to say that in fact pathogens in general only infect when both biotic and abiotic conditions are heavily weighted in their favour and that changing one of those conditions may not necessarily influence the outcome.

3.37 Further work

An interesting avenue would be the evaluation of the units of lignin (hydroxyphenyl, guaiacyl and syringyl) present in the inhibited tissues. This could yield useful information on the flux of metabolites through the system.

An assay of PAL/CAD in the presence of MDCA may show that in the case of CAD that it can work preferentially on sinapic acid.

Another useful technique would be to use nitrobenzene oxidation to see if it can shed any light on the nature of lignin units left after breaking resistance. This may also yield useful information on some of the contaminated resistance experiments.

Chapter 4: Regulation of gene expression and role of PAL in defensive lignification in wheat.

4.1 Regulation and role of PAL in the defence response in wheat

The PAL enzyme represents the first step of the phenylpropanoid pathway and catalyses the deamination of phenylalanine to cinnamic acid. The process continues with hydroxylation, methyl transfer, ligation and dehydrogenation reactions to produce lignification (see chapter 1). The knowledge of which intermediates of the lignification pathway affect PAL is therefore important and may allow a greater understanding of what parts of the defence response are significant.

4.2 PAL expression and the defence response

PAL expression is accepted as a marker of the defence response. In monocots such as barley PAL expression is induced in response to the development of primary germ tubes and appressoria of *Blumeria graminis* a fungal pathogen of barley (Boyd *et al.* 1994).

PAL expression is also correlated to harpin (a bacterial elicitor secreted by a plant pathogens) and to hydrogen peroxide (a component of the hypersensitive response) in *Arabidopsis thaliana* making its expression an essential component of the defence response (Deskian *et al.* 1998).

In wheat, three PAL genes have been reported (Liao *et al* 1996; Snowden and Gardner 1993) and challenge by the wheat pathogen *Puccinia graminis* f.sp. *tritici* is known to induce Wpal1 (Li *et al.* 2001). A recent study has isolated a clone of PAL from elicitor treated wheat undergoing defensive lignification Hall (1998).

Winter wheat has already been used as a model system to study PAL. Under controlled conditions of growth, hardening and dehardening of winter wheat, PAL expression was measured in wheat plants resistant to snow mould. (Gaudet *et al.* 2000). PAL expression was found to be low or absent in the autumn, reached high levels by midwinter and decreased during the spring. PAL transcripts were weakly expressed in unhardened tissues, strongly up-regulated following hardening and down regulated under dehardening conditions. It is clear that environmental conditions play an important role in the transcriptional regulation of PAL. This is

important in the seasonal susceptibility of wheat plants to fungal invasion, however this study was carried out using a non defence related PAL.
No study as yet can correlate defence related expression of a PAL gene in wheat to any specific time of induction of the defence response as a purely defence related PAL in wheat is yet to be fully characterised.

4.3 PAL activity and the defence response

Clearly if expression of PAL is a factor in the defence response it is likely that the activity of the PAL enzyme produced by the gene is also an important component. The timing of the activation of PAL may be a critical component of the defence pathway in wheat. In the interaction between the coffee plant and orange rust fungus, PAL is activated. The activation has two peaks of activity at two and five days after inoculation of the fungus. The 1st peak coincides with accumulation of phenolic compounds and with the beginning of cell death. The 2nd peak is related to later accumulation of phenols and lignification of the host cell wall. Interestingly although PAL activity was stimulated in susceptible plants, the delay in the host response allowed fungal growth and sporulation (Silva *et al.* 2002).

One piece of evidence that points to PAL having a significant role in the defence response has been shown using poplar suspension cultures. Eleven fungal phytotoxins postulated as pathogenicity factors that help circumvent plant defence responses were shown to reduce elicitor induced increases in PAL activity. Critically, two toxins appeared to have little effect on cell growth but were able to suppress PAL activity by 40-50 % (Vurro and Ellis 1997). If fungal populations possess phytotoxins that reduce PAL activity by a large amount it is likely be a critical component of the defence response in wheat.

In conclusion, if PAL activity is delayed or reduced fungal penetration may be more likely to succeed. The catalysis of phenylalanine to cinnamic acid is therefore an important part of the defence response.

4.4 Role of intermediates in the regulation of PAL

The role of the metabolic intermediates of the phenylpropanoid pathway is important to understand in the defence response in wheat. It is known that expression and or activity of PAL increases as part of the defence or stress response. As acids and aldehydes and alcohols are all formed continuously during plant development, levels may exist that can stimulate or reduce PAL activity and expression.

Supporting evidence for this regulation in wheat is found in alfalfa suspension cultures. Where it is proposed that PAL transcript levels are regulated by endogenous phenylpropanoid intermediates. This regulation may come about because of the microsomal location of the C4H enzyme (Orr *et al.* 1993).

4.5 Biochemical inhibitors and PAL

The aforementioned inhibitors AOA, AIP and AOPP have already been tested for their ability to reduce defence related lignification at wound margins (Chapter 3). By inhibiting PAL, the effect on PAL gene(s) may be that of up or down regulation. Interestingly, the inhibitor AIP although being a potent reducer of PAL activity has been shown to reduce the level of the PAL promoter in *Arabidopsis thaliana* c.v. Columbia (Mauch-mani and Slusarenko 1996).

The previously tested inhibitors of the phenylpropanoid pathway that are not PAL specific (chapter 3), have not yet been fully evaluated for their effects on activity and expression of PAL. As some authors report effects by inhibitors on different parts of the pathway it is reasonable to assume that some inhibitors may affect PAL activity or expression. These effects should be quantified in order to give a clearer idea of the specificity of the inhibitors.

4.7 Aims

To investigate the effect of phenylpropanoid intermediates and known inhibitors on PAL regulation in wheat.

4.8 Chemicals and reagents

Tryptone	Oxoid
Yeast Extract	Oxoid
Agar No 2	Oxiod
Sodium Chloride	Fisher Scientific
Sodium Hydroxide	Fisher Scientific
Glycerol	Fisher Scientific
Ampicillin	Sigma Aldrich
Tris-HCl	Sigma Aldrich
Ethylene diaminotetracetic acid (EDTA)	Sigma Aldrich
Glucose	Sigma Aldrich
Sodium Dodecyl Sulphate	BDH
Sodium Acetate	Sigma Aldrich
Phenol	Fisher
Chloroform	Fisher
Chloroform / Isoamyl Alcohol solution	Sigma Aldrich
Ethanol	Fisher
Sequence Mix	Applied Biosystems
Bromophenol Blue	Sigma Aldrich
Buffer H	Applied Biosystems
M13 Primer Forward and Reverse	Applied Biosystems
Tris-Acetate	Sigma Aldrich
Ethidium Bromide	Sigma Aldrich
Sodium Iodide	Sigma Aldrich
λ DNA	Bio 101
NEW WASH	Bio 101
Glassmilk	Bio 101
Hybond-N	Pharmacia Biotech
Lithium Chloride	Sigma Aldrich
[α-^{32}P] dCTP Rediprime	Pharmacia Biotech
Formamide	Sigma Aldrich

Sodium Citrate	Sigma Aldrich
X-ray film Kodak BioMax MS	Pharmacia Biotech
3-(*N*-morpholino)propanesulfonic acid	Sigma Aldrich
Formaldehyde	Sigma Aldrich

4.9 Growth and storage of bacteria

Bacterial cultures were grown in Luria-Bertani (LB) medium (10 g tryptone, 5 g yeast extract, 10 g $NaCl_2$, in 1 L distilled water, pH 7.0 adjusted with 0.1 M NaOH, autoclaved). Addition of 15 g agar l^{-1} to LB prior to autoclaving produced solid media. Cultures were grown for 14-18 h at 37 °C in liquid medium. Glycerol stocks were produced by addition of 0.3 ml 50 % (v/v) glycerol to 0.7 ml of bacterial culture; storage was at –80 °C.

4.10 Preparation of transformation-competent *E. coli* for electroporation

A culture of *E. coli* strain DH5α cells (a gift of Dr A. McCormac, Southampton University) was grown to log phase (OD 600nm = 0.4; 4 h growth at 37 °C) in LB medium. The culture was chilled on ice and cells pelleted by centrifugation (3,000 g for 5 min). The pellet was washed three times with ice-cold 10 % glycerol. The final pellet was re-suspended in $1/10^{th}$ original culture volume. Aliquots of 80 µL were used for transformation with plasmid.

4.11 Vectors

The pBluescript SK (-) phagemid carried the gPAL insert (Hall 1998). It has a universal primer compatible origin of replication and an ampicillin resistance gene for antibiotic selection of the plasmid vector. The plasmid pBG35 contained the insert for the 18S transcript (Goldsbrough and Cullis 1981).

4.12 Transformation of *Escherichia coli* cells using electroporation

An 80 µL aliquot was thawed on ice and 5 µL of approximately 5 ng ml^{-1} gPAL cDNA clone (Hall 1998) was added to 0.2 cm cuvette and electroporated in a Bio-Rad Gene pulser (2.5 Kv 0.2 cm^{-1}, 25 µF and 400 ohms). After the pulse, the cells were re-suspended in 500 µL LB broth, and incubated at 37 °C for 0.5-1 h before plating out onto LB agar plates containing 100 mg L^{-1} ampicillin.

4.13 Plasmid DNA mini-preps

A transformed colony was aseptically transferred from antibiotic selection media to 20 ml of liquid LB medium containing 100 mg l^{-1} ampicillin and shaken at 20 r.p.m overnight. *E. coli* cells were recovered by centrifugation at 1000 g for 5 min. The resulting pellet was re-suspended in 400 µl (100 µl 5 ml^{-1}) culture ice-cold cell re-suspension solution (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM glucose) and incubated for 5 min at room temperature. To lyse cells; 800 µL cell lysis solution was added (0.2 N NaOH, 1 % SDS) and incubated for 5 min. To neutralise the lysate; 600 µl of 3.0 M-sodium acetate solution (pH 4.8) was added, the solution shaken by hand for 10 s and left on ice for 15-30 min. The resulting supernatant was dispensed into aliquots of 0.5 ml in fresh sterile eppendorf tubes and extracted twice via centrifugation in the presence of (1:1) phenol:choloroform.
The upper aqueous phase of 500 µL was subjected to 500 µL chloroform isoamyl alcohol solution, vortexed for 10 s and centrifuged at 11,200 g for 5 min. The upper 0.5 ml of the extract was washed in 1 ml 95 % (v/v) ethanol and centrifuged at 11 200 g for 5 min. The pellet was dried for 30 min and resuspended in 50 µl SDW.

4.14 Polymerase Chain Reaction (PCR) sequencing

The gPAL plasmid was verifed by sequencing. Plasmid DNA (200 ng) was mixed with 4 µL water and added to 4 µL (Applied Biosystems) sequence mix. To this 1.6 pM of forward or reverse M13 primer (1 µL stock) was added. After PCR at 96, 60, 50 and 14 °C stages and 25 cycles the sample was ½ diluted with SDW. This was added to 50 µL 95 % (v/v) ethanol and 2 µL 3.0 M sodium acetate pH 4.8. The solution was incubated at room temperature for 30 min and centrifuged at 11,200 g. The pellet was washed two times with 250 µL 70 % (v/v) ethanol and vortexed for 10 s and centrifuged at 11,200 g for 3 min. The ethanol was removed and the pellet was dried at 94 °C on a heating block for 1-2 min. Samples were analysed on an AB1377 sequencer.

4.15 Plasmid digestion and electrophoresis.

The program "Remap" (NCBI 2001) was used to identify potential restriction enzyme sites in the gPAL sequence. Combination of this data and the choice of adapter ligands used for the synthesis of gPAL (Hall 1998) lead to the choice of *Eco* RI, as an appropriate enzyme to cut the plasmid.

For each sample 3 μL 10 X buffer H (*Eco* RI) was used in conjunction with 1 μL enzyme, 5 μL gPAL cDNA, this was made up to 30 μL with distilled water and incubated overnight at 37 °C. After incubation 3 μL bromophenol blue dye (50 mM EDTA, 0.2 % (w/v) SDS, 50 % (v/v) glycerol and 0.05 % (w/v) bromophenol blue) was added and the sample was mixed and loaded into a well on a TAE buffer (0.4 M Tris-acetate, 0.1 M EDTA pH 7.0) 0.8 % (w/v) agarose and 500 μg L^{-1} ethidium bromide gel. Electrophoresis was performed at 120 V for 30-60 min.

4.16 Removal of DNA gel consituents

After exposure to Ultra Violet (UV) light the gel fragment containing the 1.9 kb insert was excised and frozen at –20 °C. Gel size was determined by weight 0.1 g = 100 μL. Sodium iodide 6 M, 600 μL (3 volumes) was added and the sample was incubated at 50 °C for 2 min. "Glassmilk" 5 μL (Bio 101) was added, mixed by gentle agitation and incubated at room temperature for 5 min. The mixture was centrifuged at 11,200 g, the precipitate retained and 500 μL NEW WASH (Bio 101) was added. The precipitate was re-suspended and washed three times with the NEW WASH solution. The supernatant was discarded and the DNA eluted by addition of 20 μL SDW, with mixing and centrifugation at 11 200 g. A sample of the supernatant was electrophoresed on a TAE buffer gel as above, against a λ DNA marker (1 μL λ DNA, 10 μL SDW, 1 μL ethidium bromide).

4.17 Electrophoresis of RNA in a denaturing gel system

Gels were made up by melting 1-1.5 g agarose in 10 ml 10 X MOPS buffer (0.2 M 3-(*N*-morpholino)propanesulfonic acid pH 7.0, 0.5 M sodium acetate, 0.01 M EDTA) and 75 ml SDW. After dissolution 40 % (v/v) formaldehyde was added and gels poured into a gel tank. Electrophoresis was carried out in 1 X MOPS buffer and at 40-80 V. Samples were prepared by incubating 12 µL RNA, 25 µL Formamide, 5 µL 10 X MOPS buffer and 8 µL Formaldehyde at 65 °C for 5 min.

4.18 RNA gel blot analysis.

Total RNA was extracted from 10-day-old wheat leaves. Wheat leaves were wounded five times with a rounded metal stake and treated with 10 µL per wound of either 5 mg ml^{-1} chitin (section 4.19) or distilled water. The leaves were covered and left for 16 h (22°C 16 h day) and cut to 32 mm strips encompassing the five wounds. Unwounded leaves were cut at the same time as wounded leaves.

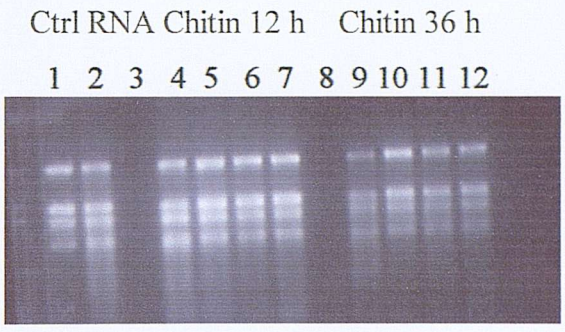
An excised leaf was frozen in 1.5 ml liquid N_2 and ground in 800 µL extraction buffer (phenol: 0.1 M LiCl, 0.1 M Tris-HCl pH 8.0, 10 mM EDTA and 1 % (w/v) (SDS) (1:1, v/v). RNA was extracted by partioning with 400 µL chloroform and precipitated in an equal volume of 4 M LiCl, followed by precipitation with (100 %) ethanol. Total RNA (5 µg per lane) was heat deatured (65 °C for 10 min) and separated in the presence of 50 % (w/v) formamide, separated on a denaturing 1.3 % agarose gel and blotted onto Hybond-N (Amersham Pharmacia Biotech, UK) overnight at room temperature according to Sambrook *et al* (1989). Equal loadings of RNA were calculated via ethidium bromide staining of samples before loading and confirmed by hybridisation of stripped blots with an 18s rDNA probe (section 4.17). Prehybridisation and hybridisation was at 42 °C in the presence of 50 % formamide. Washing was completed to a final stringency of 0.2 X SSC + 0.1 % (w/v) SDS at 42 °C. (20 X SSC, 175.3g NaCl, 88.2 g sodium citrate pH to ~7.0 with 10 M NaOH Adjust volume to 1L). The gPAL DNA probe was obtained by digestion of the the cDNA clone (Hall 1998) with *Eco* R1 and labelled with [α-^{32}P] dCTP using the Rediprime (Alpha Pharmacia Biotech) system. Blots were exposed onto X-ray film (Kodak BioMax MS, Amersham Pharmacia Biotech, UK) and densitrometric scans were performed using a digital imaging system (Alpha

Innotech Corp., San Leandro, CA, USA) and the AlphEase software package. Hybridisation was performed separately for the time course blots and the metabolite or inhibitor blots.

4.19 RNA quantitiation

Extracted RNA samples were prepared by incubating 2 µL RNA, 5 µL Formamide, 1 µL MOPS buffer and 2 µL Formaldehyde at 65 °C in the presence of 0.2 µL Ethidium Bromide for 5 min. "Mini" gel electrophoresis was carried out as above and differences were compared by eye to a sample with a known volume of RNA (5 µg ml^{-1}). The 5 µg ml^{-1} sample was quantified from a comparison with RNA obtained from *Arabidopsis* seedlings that had been quantified at 260 nm to 5 µg ml^{-1} (McCormac personal communication). Dilutions were then prepared with RNAse free water equalise RNA volumes to account for differences in extraction.

For example, duplicate standards of RNA of 5 µg ml^{-1} were compared with RNA extracted from chitin treated wheat plants at 12 and 36 h. For the final adjustment the brightest of the four 36 h treatment RNAs had 2 µl water extra added to the mix (12 µl max) before loading into wells in preparation for electrophoresis (Table 4.1, Figure 4.1).



Total RNA

Figure 4.1 Ethidium bromide labelled RNA from wheat leaves. RNA was extracted from wheat leaves using the phenol: chloroform method. Samples were prepared in a formaldehyde, formamide and MOPS buffer in the presence of ethidium bromide lanes 1 and 2, 5 μg ml^{-1} RNA; lanes 4-7, RNA extracted from chitin treated wheat leaves at 12 h; lanes 9-12, RNA extracted from chitin treated wheat leaves extracted at 36 h.

Lane	Description	Volume used to equalise RNA for Northern analysis
1	5 μg ml^{-1} of known quantified RNA (McCormac)	10 μL
2	5 μg ml^{-1} of known quantified RNA (McCormac)	10 μL
3	Empty	
4	Chitin treated Wheat leaf RNA extracted at 12 h	10 μL
5	Chitin treated Wheat leaf RNA extracted at 12 h	10 μL
6	Chitin treated Wheat leaf RNA extracted at 12 h	10 μL
7	Chitin treated Wheat leaf RNA extracted at 12 h	10 μL
8	Empty	
9	Chitin treated Wheat leaf RNA extracted at 36 h	12 μL
10	Chitin treated Wheat leaf RNA extracted at 36 h	10 μL
11	Chitin treated Wheat leaf RNA extracted at 36 h	12 μL
12	Chitin treated Wheat leaf RNA extracted at 36 h	12 μL

Table 4.1 Dilutions prepared from ethidium bromide stained gels to equalise RNA loading; Lane, Lane on gel (Figure 4.1); Description, concentration of RNA or plant treatment before RNA quantitiation; Volume used to equalise RNA for Northern analysis, based on 12 μL as maximum RNA and water mix.

4.20 Northern blot analysis

To quantify northern blots the Alpha Imager software (Alpha Innotech) spot densitometry package was used to draw (toolbar 4) a square around a video captured image of the blot (section 2.10). Another square of equal size was fixed to the unhybridised area of the blot and set as background. The IDV (integrated density value) for each areas pixels was calculated on a scale between 255 (Black) and 0 (white). After subtracting the background value the magnitude of gPAL expression could be ascertained. After normalising this value with the value obtained by 18S RNA analysis the fold induction of gPAL from a set time (0 h or unwounded) could be ascertained.

Biochemical methods

4.21 Preparation of Chitin

Chitin was prepared by adapting the protocol of Shimahara and Takiguchi (1988). Chitin suspension was prepared by dissolving 8 g ground chitin in 80 ml concentrated HCl at 0 °C for 12 h with slow agitation. The solution was centrifuged at 500 g for 10 min, the supernatant collected and slowly added to rapidly agitated 50 % pre-cooled ethanol solution and allowed to precipitate for 2 h. The precipitate was collected by centrifugation (500 g 10 min^{-1}) and washed three times with distilled water. A polytron homogeniser (10 mm head) at speed five for 1 min was used to fully disperse the chitin. The precipitate was centrifuged (500 g 10 min^{-1}) and treated with 1 M NaOH for 36 h at 90 °C. The pellet was collected via centrifugation (500 g 10 min^{-1}) and washed extensively with distilled water. Ethanol (95 % v/v) was added and the solution was incubated at 60 °C for 6 h. Reacetylation was achieved by addition of 2 % (v/v) acetic anhydride in methanol and cooling to 0 °C for 2 h. The chitin suspension was then dialysed in two batches of 2 L distilled water overnight at 4 °C. A dry weight analysis was performed by transferring three replicates of 1 ml samples of the solution to aluminium cups and drying at 60 °C for 5 hours. The solution was diluted to 5 mg ml^{-1}, autoclaved (121 °C 15 min) and dispensed into 10 ml fractions stored at -20 °C.

4.22 Assay of PAL activity

The measurement of PAL activity was based on the method of Thorpe and Hall (1992) ^{14}C L-phenylalanine (Amersham) was diluted to 2.5 μ Ci μ mol^{-1} in 100 mM Tris-HCl pH 8.8. A chitin treated wheat leaf was ground in 200 μL Extraction Buffer (100 mM Tris-HCl pH 8.8, 0.05 % (w/v) EDTA, 0.05 % (v/v) Mecaptoethanol). The solution was centrifuged at 11 200 g and 100 μL of the aqueous phase was added to a solution of 200 μL L-phenylalanine solution and 200 μL 2.5 mM phenylpropanoid pathway intermediate or 1.0 mM phenylpropanoid enzyme inhibitor. This solution was incubated at 35 °C in a water bath for 3 h. To stop the reaction 50 μL 50 mM *trans*-cinnamic acid in 0.1 % (w/v) NaOH and 50 μL 50 % (v/v) trichloroacetic acid were added. The solution was mixture shaken by inversion and left for 10 min. Toluene was added (200 μL) and the mixture centrifuged at 1000 g. The "organic" phase of 150 μL was removed and counted in 5 ml scintillation fluid. Counts were performed on a Wallac 1209 liquid scintillation counter, using water as a blank.

Results

4.23 Expression of gPAL

The gPAL clone was sequenced previously from nucleosomic cDNA stocks by Hall (1998). The sequence revealed high similarity to PAL clones of wheat and as it was from genetic stocks from diseased afflicted wheat the clone was referred to as gPAL (Stratford 2002).

Initially, a time course of chitin-stimulated expression of gPAL related RNA was performed. This was achieved by northern analysis of chitin and water treated wheat leaves over a 48 h period. RNA was prepared from 10-day-old wheat plants. Gels (5 μg RNA per lane) were probed under high stringency conditions with gPAL and constitutive 18s RNA (Figure 4.2). This allowed quantification of the normalised levels of gPAL RNA accumulation using a scanning densitrometric package (Alpha Imager).

Northern analysis is presented in terms of fold induction of gPAL normalised against 18s RNA expression (Figure 4.3). Treatment with water has little effect on gPAL induction, expression remains unchanged from that at 0 h throughout the time course (Figure 4.3a). The gPAL related RNA is induced at the highest levels (7 fold) with chitin between 12 and 20 h and this increase is maintained at above 6 fold for 24 h until 36 h where expression begins to decline until reaching 2 fold at 48 h (Figure 4.3a).

As both chitin and water treatment involves wounding, subtraction of the water treated values from the chitin treated results reveals the effect of the chitin treatment alone. The net effect of chitin alone is to induce a 4-fold induction of gPAL by 8 h that increases to a maximum of over 6-fold induction of gPAL expression at 16 h. This induction is maintained at 5-6 fold for a 24 h period until 36 h. Expression then declines to 4 fold by 40 h, which continues to decline to a 2-fold induction by 48 h (Figure 4.3 b).

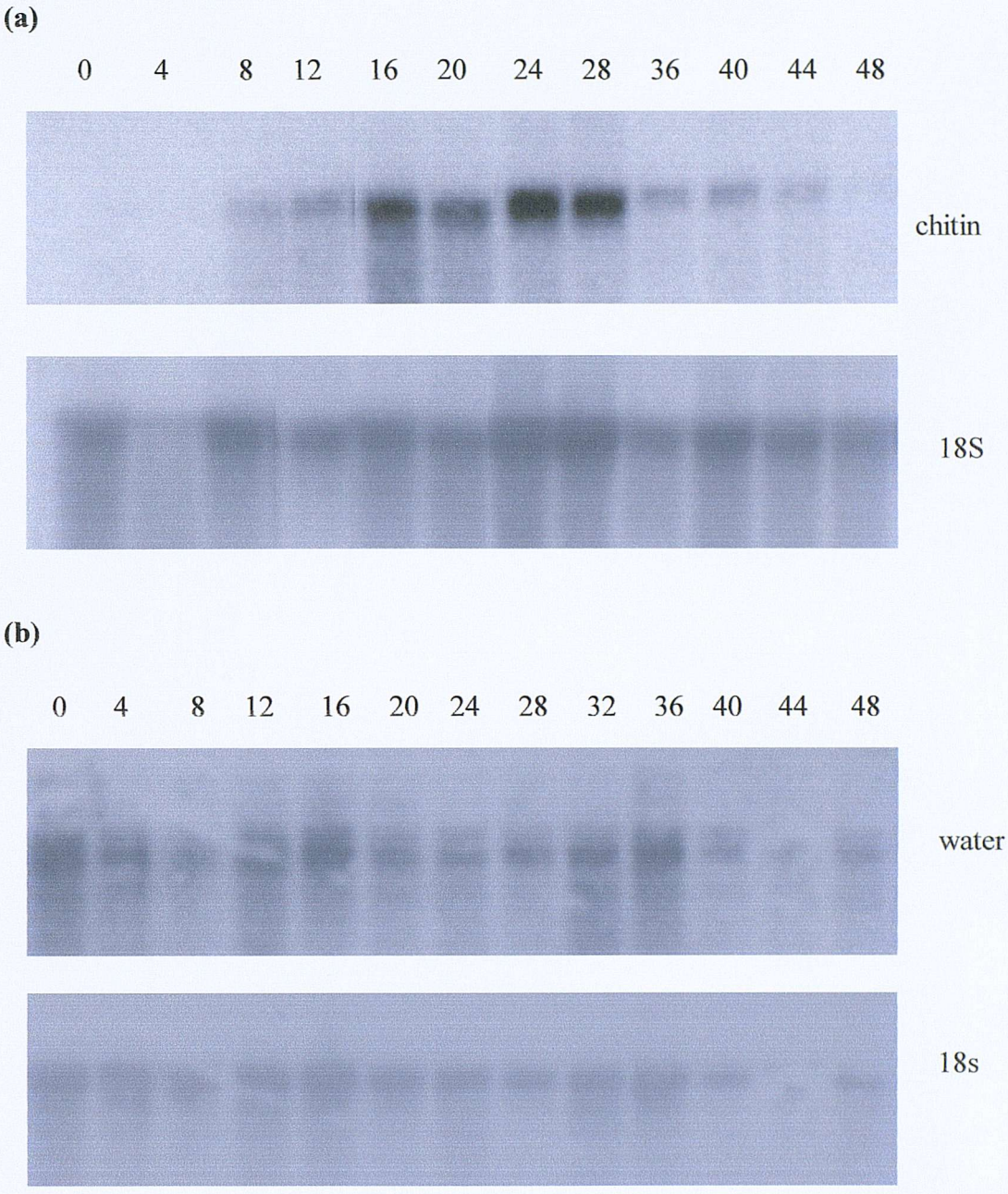


Figure 4.2 Accumulation of gPAL RNA in wheat leaves in response to chitin and water: Northern analysis using gPAL and 18S probes was used to quantify the response of PAL transcript induction over 48 h; (a), accumulation of gPAL RNA in wheat leaves after treatment with 5 mg ml^{-1} chitin; (b), accumulation of gPAL RNA in wheat leaves after treatment with water. In both (a) and (b) the level of the corresponding constitutive 18S RNA is shown. RNA loading (5 µg per lane) was equalised across the gels by reference to ethidium bromide stained mini gels.

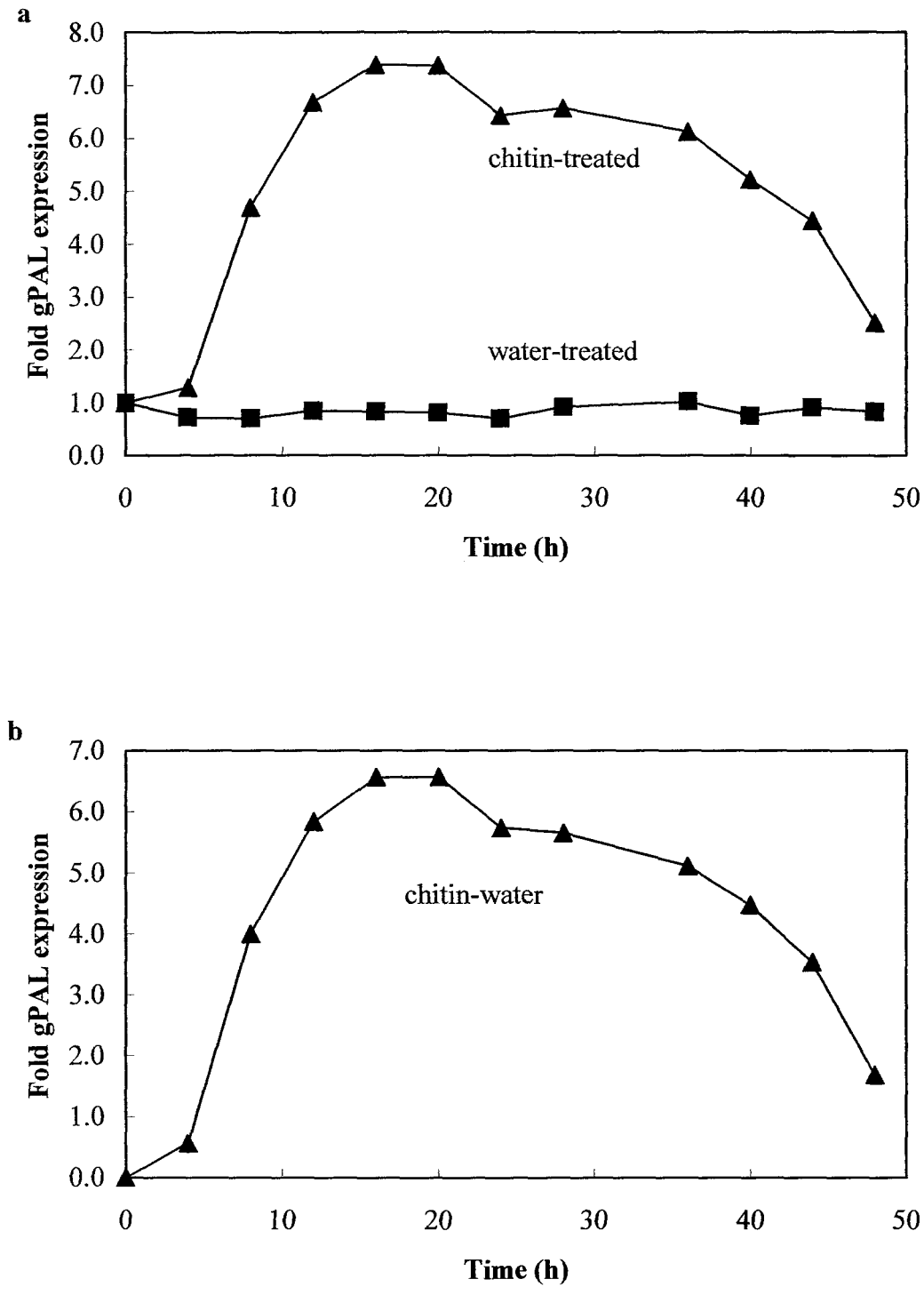


Figure 4.3 Time course of chitin-induced gPAL expression in wheat leaves: Northern analysis was performed (a), Fold induction of gPAL by 5 mg ml^{-1} chitin(▲ and water(■); (b), Net gPAL induction (chitin-water) (▲). RNA was loaded at 5 μg per lane. Gels were electrophorised under high stringency conditions. Scanning densitrometric analysis was used to normalise data by probing blots with a probe specific for 18s RNA (n = 1).

4.24 PAL activity

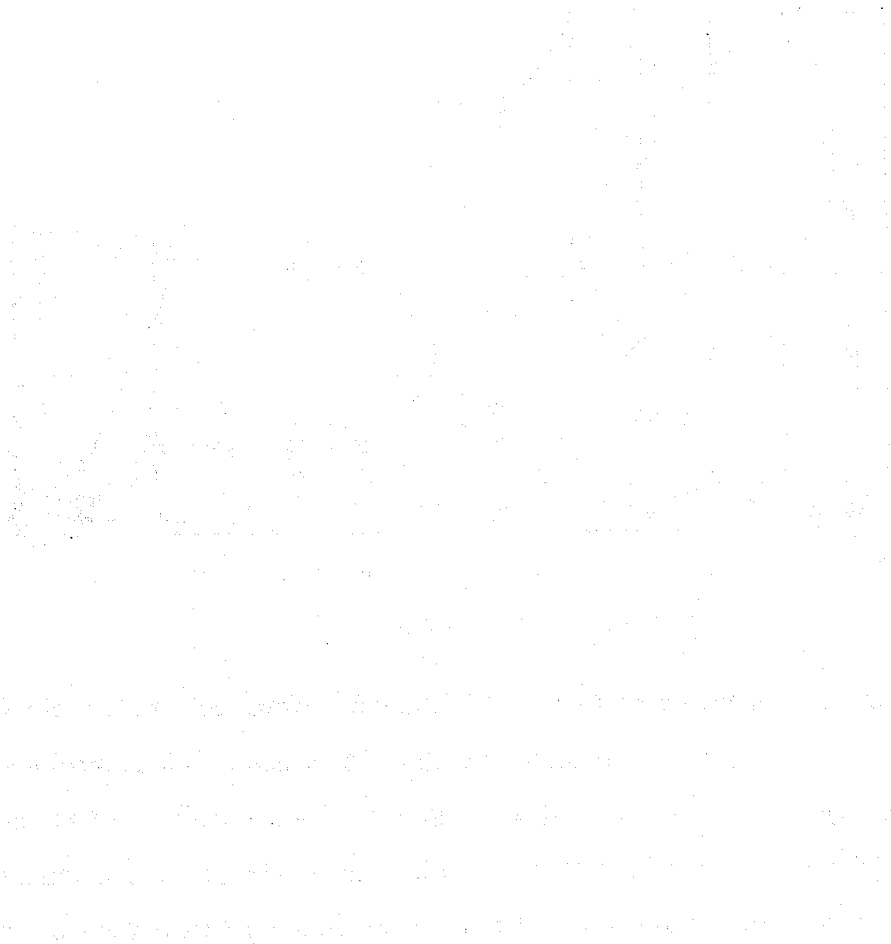
In order to determine how the expression of gPAL related RNA fits into the total activation of PAL in the lignification response, the PAL activity stimulated by chitin was measured using the radiochemical method of Mitchell *et al* (1994) to ascertain the activity of PAL over a 48 h period. Data is expressed in terms of total activity per gram fresh weight and further analysis in terms of activity per wound. Unwounded leaves elicit PAL activity to no greater than two pkat per gram fresh weight; this level is maintained throughout the 48 h period. In wounded leaves treated with water, initial PAL activity is below 5 pkat per gram fresh weight. However at 32 h the activity rises to 8 pkat per gram fresh weight, a 2-fold induction over unwounded leaves. This activity is maintained at 5 pkat per gram fresh weight until 48 h (Figure 4.4).

In wounded leaves treated with chitin, PAL activity is significantly increased compared to unwounded plants at 4 h. This significant increase continues to a peak of activity at 12 h of 12 pkat per gram fresh weight, which represents a 4-fold induction over water treated leaves (3 pkat per gram fresh weight) (Figure 4.4). This level is maintained until 48 h where activity drops to 9 pkat per gram fresh weight but remains significantly different from water treated controls (Figure 4.4).

As both chitin and water treatments involve wounding, subtraction of the water-treated values from the chitin-treated results reveals the effect of the chitin treatment alone. Chitin alone induces a significant increase of PAL activity at 4 h. However, at 8 h this is not statistically significant (Figure 4.5). By 12 h chitin induced PAL rises to 65 fkat per wound. Although declining, this significant difference between chitin and water treated leaves is maintained until 36 h after which it is no longer statistically significant. After 36 h the induced PAL activity rises to 50 fkat per wound and from 40 to 44 h declines to 20 fkat per wound but remains statistically significant (Figure 4.5).

When net PAL activities from unwounded and water treated leaves are compared there is no significant difference until 24 h. After this point PAL activity in the water-treated leaves remains significantly different from unwounded leaves until 48

h. The PAL activity in the water treated leaves is highest at 32 h (28 f kats per wound) (Figure 4.6).



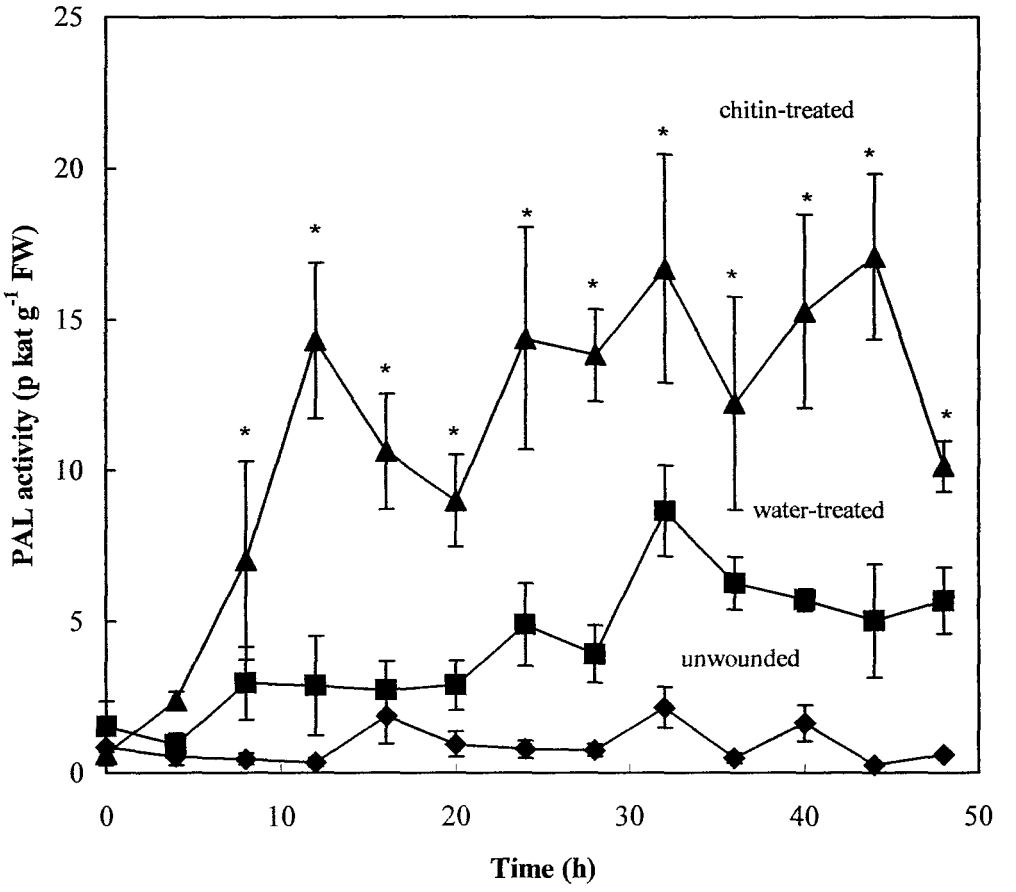


Figure 4.4 Time course of chitin induced PAL activity in wheat leaves: Data was obtained from radiochemical assay of incorporation of ^{14}C from Phenylalanine to *trans*-cinnamic acid: Treatments (▲, 5 mg ml^{-1} chitin; (■ water; (◆), unwounded plants; (*), Significant difference using students t-test ($p = 0.05$, $n > 4$) between chitin induced and unwounded plants. Vertical bars show standard errors.

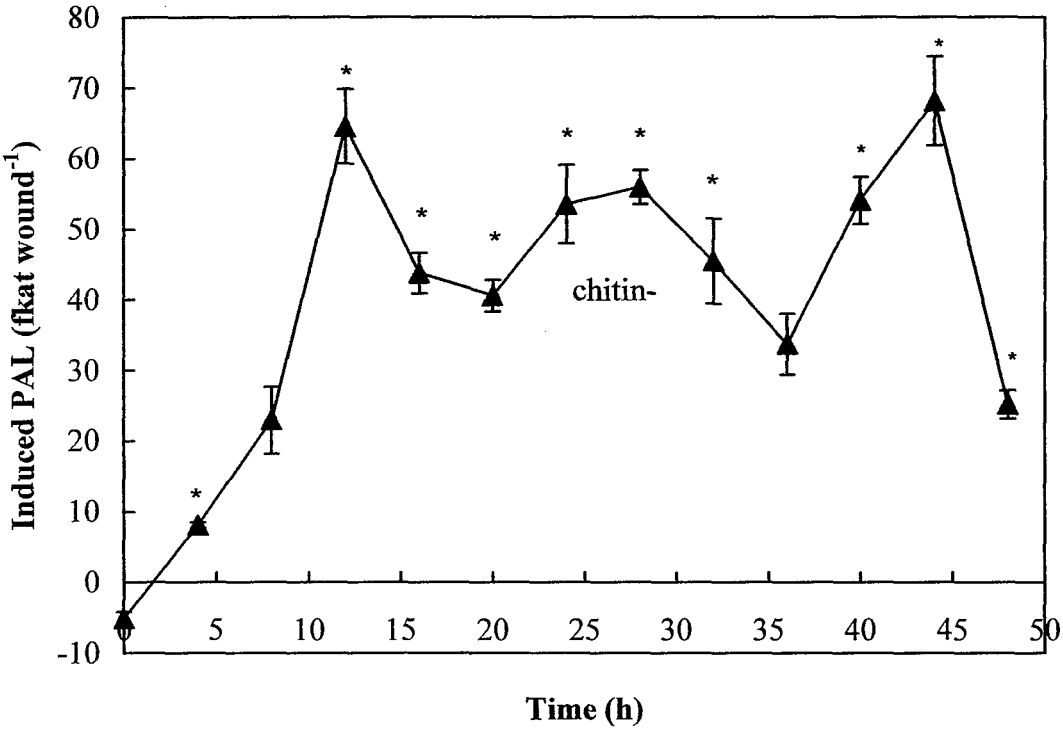


Figure 4.5 Net effect of chitin on PAL activity in wheat leaves: Data was obtained from radiochemical assay of incorporation of ^{14}C from Phenylalanine to *trans*-cinnamic acid: Treatment (▲), 5 mg ml^{-1} chitin. * Significant difference using students t-test ($p = 0.05$, $n => 4$) between chitin and water induced activity. Vertical bars show standard errors.

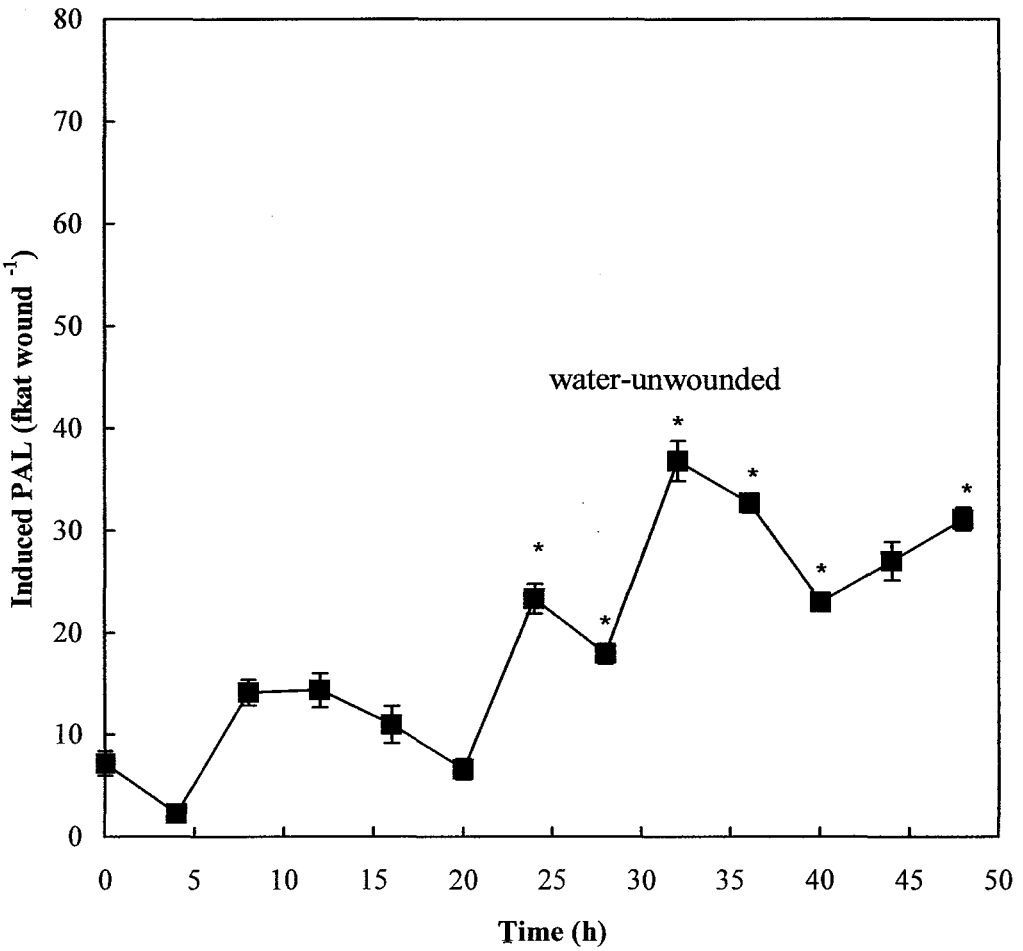


Figure 4.6 Net effect of wounding on PAL activity in wheat leaves. Data was obtained from radiochemical assay of incorporation of ^{14}C from Phenylalanine to *trans*-cinnamic acid: Treatment (■ water. * Significant difference using students t-test ($p = 0.05$, $n > 4$) between water treated and unwounded leaves. Vertical bars show standard errors.

4.25 Effect of the metabolic intermediates of the phenylpropanoid pathway on gPAL expression.

To investigate the regulation of the response to chitin of gPAL by the presence of the intermediates of the phenylpropanoid pathway, RNA was prepared from 10-day-old wheat plants. The plants were treated with a combination of 5 mg ml^{-1} chitin and 1 mM phenylpropanoid pathway intermediate for 16 h. Results are presented in terms of fold gPAL expression induced by chitin. Subtracting results of RNA expression for leaves treated with water alone from those obtained for treatment with chitin and a metabolite or inhibitor revealed the effect of chitin alone. Dividing results of RNA expression for leaves treated with chitin and a metabolite or an inhibitor by those obtained for chitin alone revealed the percentage inhibition of the gPAL transcript by the metabolite or inhibitor.

The presence of 1 mM phenylalanine, 1 mM 5-hydroxyferulic acid and 1 mM sinapic acid with chitin although leading to a greater than 1-fold induction of gPAL; did not give a significant decrease in gPAL expression. The presence of 1 mM Ferulic acid with chitin lead to either a total reduction of all RNA or a problem with loading on the gel, as such no data can be determined for this compounds effect on expression. However, its effect on activity of PAL will be determined later.

The presence of 1 mM *trans*-cinnamic acid with chitin leads to a greater than 2-fold expression of gPAL representing a significant difference in expression from chitin treated, water treated and unwounded leaves (Figure 4.7). The net effect (-water) of *trans*-cinnamic acid is a greater than 1 fold induction of gPAL (Figure 4.8). *trans*-Cinnamic acid increases gPAL expression by a factor of 55 % (Table 4.1).

The presence of 1 mM *para*-Coumaric acid with chitin leads to a greater than 3-fold expression of gPAL (Figure 4.7). The net effect (-water) of *para*-Coumaric acid is a 3-fold induction of gPAL (Figure 4.8). *para*-Coumaric acid increases gPAL expression by a factor of 176 % (n =1) (Figure 4.7).

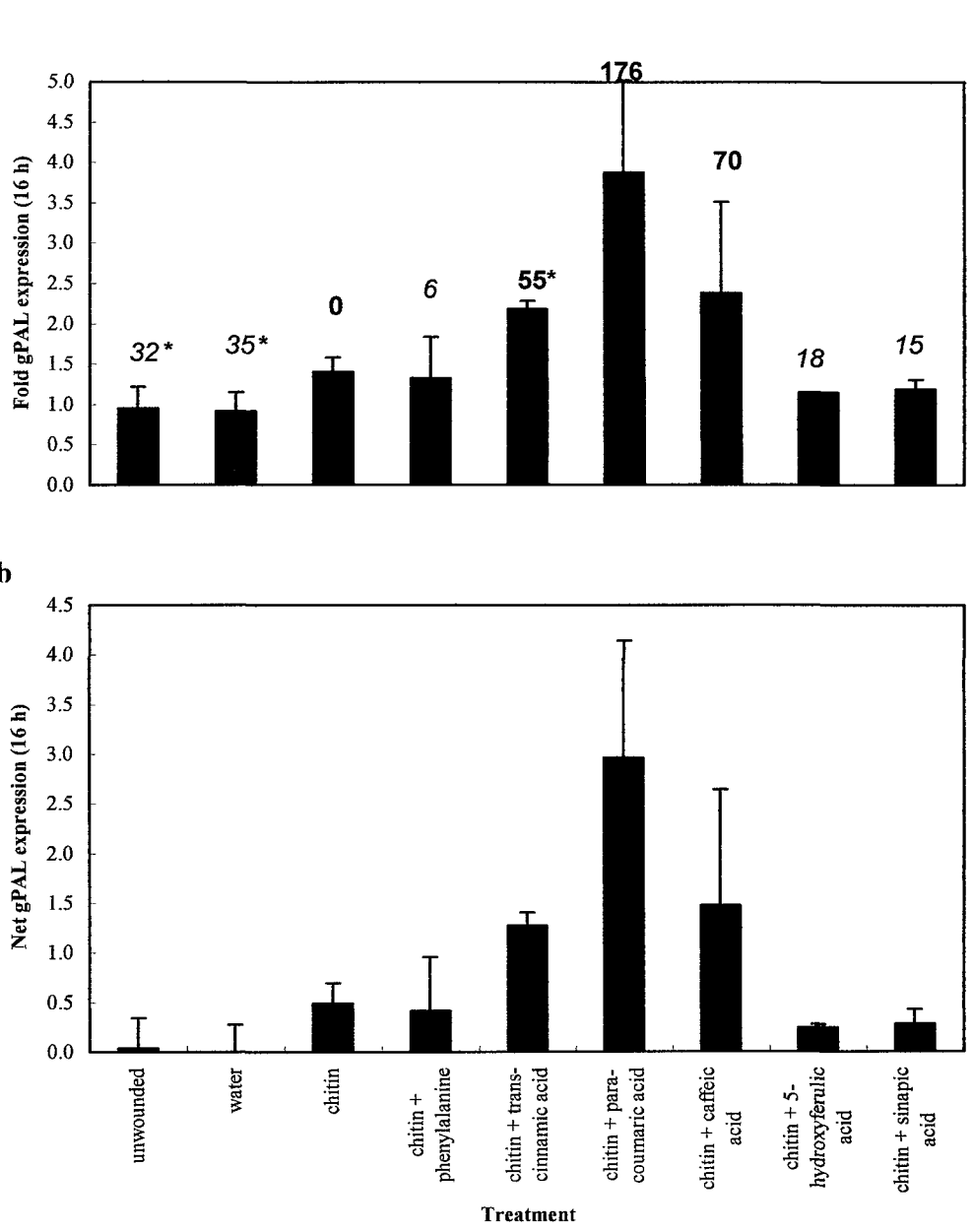


Figure 4.7 Fold gPAL expression in the presence of cinnamic acids of the phenylpropanoid pathway: Data obtained from scanning densitrometric analysis of northern blots. a, Total fold gPAL expression, b net (-water) gPAL expression. Unwounded, untreated leaves Water, wounded leaves treated with distilled water, all other treatments were with 5 mg ml^{-1} chitin. **Increase**, *decrease*; percentage change relative to chitin in gPAL expression under the treatment. * significant difference using students t-test (n = 2, p = 0.05) from unwounded, water and chitin treatments. Vertical bars show the standard error of the mean.

The presence of 1 mM caffeic acid with chitin leads to a greater than 2-fold expression of gPAL (Figure 4.7). The net effect (-water) of caffeic acid is a greater than 1-fold induction of gPAL (Figure 4.7). The presence of caffeic acid increases gPAL expression by 70 % (n = 1) (Figure 4.7).

Not all the hydroxycinnamyl aldehyde and hydroxycinnamoyl alcohol intermediates of the phenylpropanoid pathway were available at this time. Of those tested, *p*-coumarylaldehyde and sinapaldehyde did not significantly decrease the chitin stimulated gPAL expression (Figure 4.8). However, it is likely from the data from one experiment that the presence of coniferal aldehyde does inhibit the chitin stimulated gPAL expression (Figure 4.8). The alcohols of the phenylpropanoid pathway that were tested are all likely to inhibit chitin stimulated gPAL expression from the data obtained from one experiment (Figure 4.8).

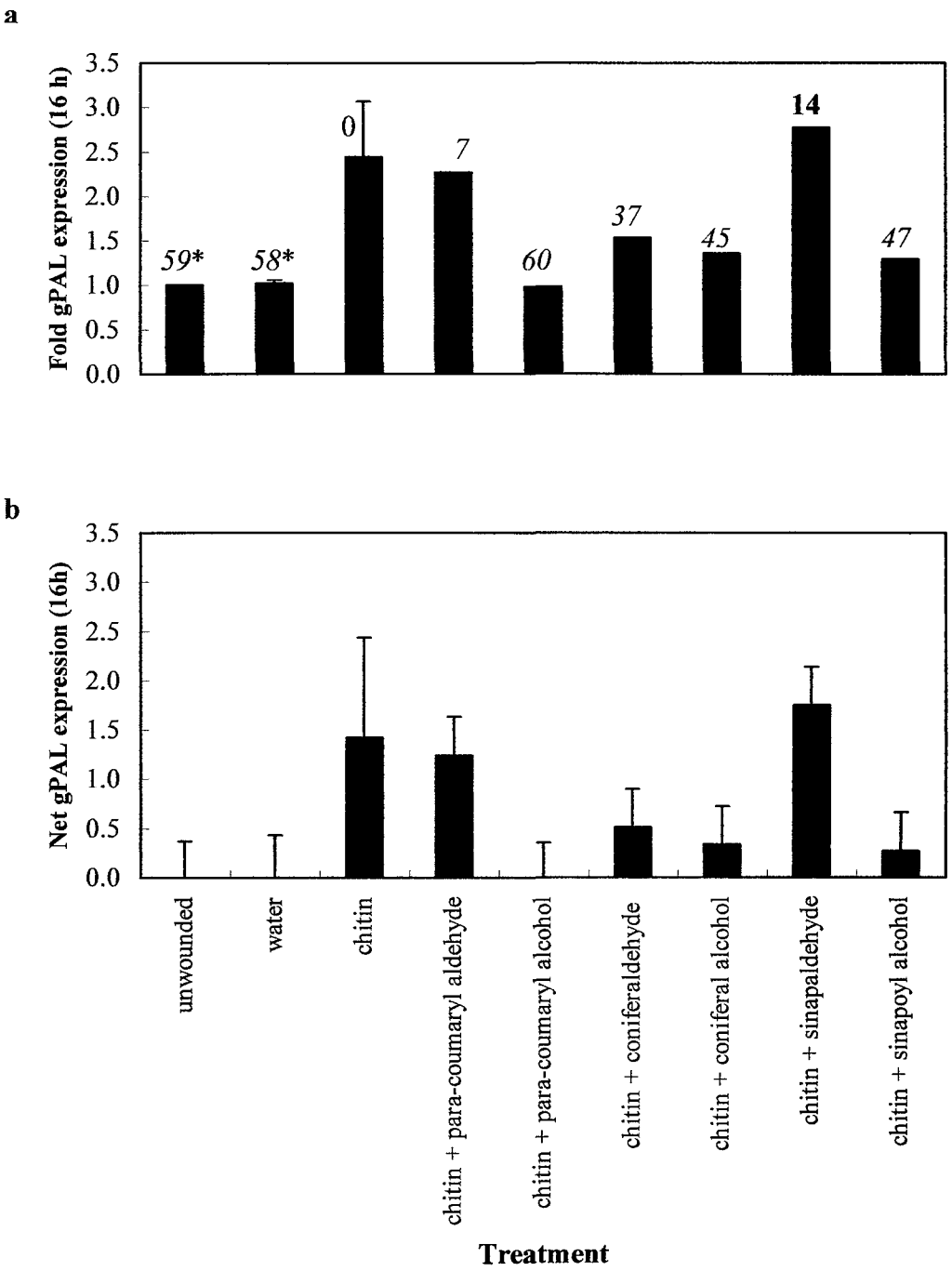


Figure 4.8 Fold gPAL expression in the presence of hydroxycinnamyl aldehydes and hydroxycinnamyl alcohols of the phenylpropanoid pathway: Data obtained from scanning densitrometric analysis of northern blots. a, Total fold gPAL expression, b net (-water) gPAL expression. Unwounded, untreated leaves Water, wounded leaves treated with distilled water, all other treatments were with 5 mg ml^{-1} chitin. **Increase**, *decrease*; percentage change in gPAL expression under the treatment. * Significant difference (n = 2, p = 0.05) from unwounded, water and chitin treatments. Vertical bars show the standard error of the mean.

4.26 PAL activity in the presence of metabolic intermediates of the phenylpropanoid pathway.

To investigate the possibility of feedback inhibition of PAL by phenylpropanoid intermediates, the intermediates were incorporated into the enzyme assay of Mitchell *et al* (1994). Wounded primary leaves of wheat were treated with 5 mg ml^{-1} chitin and harvested after 16 h post inoculation. PAL activity was extracted from leaves in the presence of 2.5 mM phenylpropanoid pathway intermediates. To calculate induced PAL per wound, water control values (p kat $leaf^{-1}$) were subtracted, the result multipled by 1000 (fkat) and divided by the number of wounds (5). The presence of both phenylalanine and *trans*-cinnamic acid inhibited PAL activity significantly as did 5-hydroxy ferulic acid. Coumaric acid and ferulic acid also appeared to inhibit the response to chitin however this is not significant ($p=0.05$). Caffeic and sinapic acid did not inhibit the response at all (Table 4.3).

None of the hydroxycinnamyl aldehydes or hydroxycinnamyl alcohols present in the phenylpropanoid pathway can be shown to significantly inhibit PAL activity. However both the coniferal aldehyde and coniferal alcohol analogues show some inhibition of PAL activity. This inhibiton is comparable to that shown by ferulic acid (Table 4.4).

Table 4.2 Activity of PAL chitin-stimulated in the presence of hydroxycinnamic acids of the phenylpropanoid pathway. The effect of hydroxycinnamic acids on the activity of phenylalanine ammonia lyase (PAL) activity induced by chitin in wounded wheat leaves at 16 h post inoculation. Data is expressed in terms of total activity per gram fresh weight of tissue and as the activity induced per wound by chitin in the presence of the metabolite and as the degree of inhibition of the induction of chitin. All assays were repeated at least eight times. A * indicates those compounds that significantly ($p = 0.05$) inhibited the chitin response.

Plant treatment	Addition to PAL assay (2.5 mM)	Total PAL (pkats g^{-1} FW)	Induced PAL (fkat $wound^{-1}$)	Inhibition (%)
Unwounded	water	1.29	0	
Wounded and water	water	3.46	0	
Wounded and chitin (5 mg ml^{-1})	water	13.7	58.0	
Wounded and chitin	Phenylalanine	4.44	5.60	90*
Wounded and chitin	*trans* Cinnamic acid	3.07	-2.20	104*
Wounded and chitin	*para*-Coumaric acid	9.45	33.9	42
Wounded and chitin	Caffeic acid	15.1	66.0	-14
Wounded and chitin	Ferulic acid	9.45	33.9	41
Wounded and chitin	5-Hydroxyferulic acid	4.51	5.90	90*
Wounded and chitin	Sinapic acid	15.3	66.9	-15

Table 4.3 Activity of PAL in the presence of aldehydes and alcohols of the phenylpropanoid pathway. The effect of various treatments on the induction of phenylalanine ammonia lyase (PAL) activity in wounded wheat leaves at 16 h post inoculation. Data is expressed in terms of total activity per gram fresh weight of tissue and as the activity induced per wound by chitin in the presence of the metabolite and as the degree of inhibition of the induction of chitin. All assays were repeated at least eight times. A * indicates those compounds that significantly (p = 0.05) inhibited the chitin response

Plant treatment	Addition to PAL assay (2.5 mM)	pkats g^{-1} FW	PAL fkat $wound^{-1}$	% Inhibition
Unwounded	Water	1.29	0	
Wounded and water	Water	3.46	0	
Wounded and chitin (5 mg ml^{-1})	Water	13.7	58.0	
Wounded and chitin	*para*-Coumaryl aldehyde	16.8	75.7	-31
Wounded and chitin	Coniferal aldehyde	8.07	26.1	55
Wounded and chitin	Sinapaldehyde	13.5	57.0	2
Wounded and chitin	*para*-Coumaryl alcohol	15.6	68.6	-18
Wounded and chitin	Coniferyl alcohol	9.25	32.8	43
Wounded and chitin	Sinapoyl alcohol	13.6	57.5	1

4.27 Effect of the known inhibitors of the phenylpropanoid pathway on chitin induced gPAL expression.

To investigate the regulation of the response to chitin of gPAL by the inhibitors of the phenylpropanoid pathway, RNA was prepared from 10-day-old wheat plants. The plants were treated with a combination of 5 mg ml^{-1} chitin and 1 mM phenylpropanoid pathway inhibitor. Results are presented in terms of induction of gPAL by chitin after 16 h post inoculation. The PAL inhibitor AOA significantly reduced gPAL expression, whereas AIP had no discernable effect. The hydroxylase inhibitor ABT, the 4CL inhibitor MDCA and the CAD inhibitor OH-PAS all increased gPAL expression. The CAD inhibitors NH_2PAS and ML19 also decreased expression.

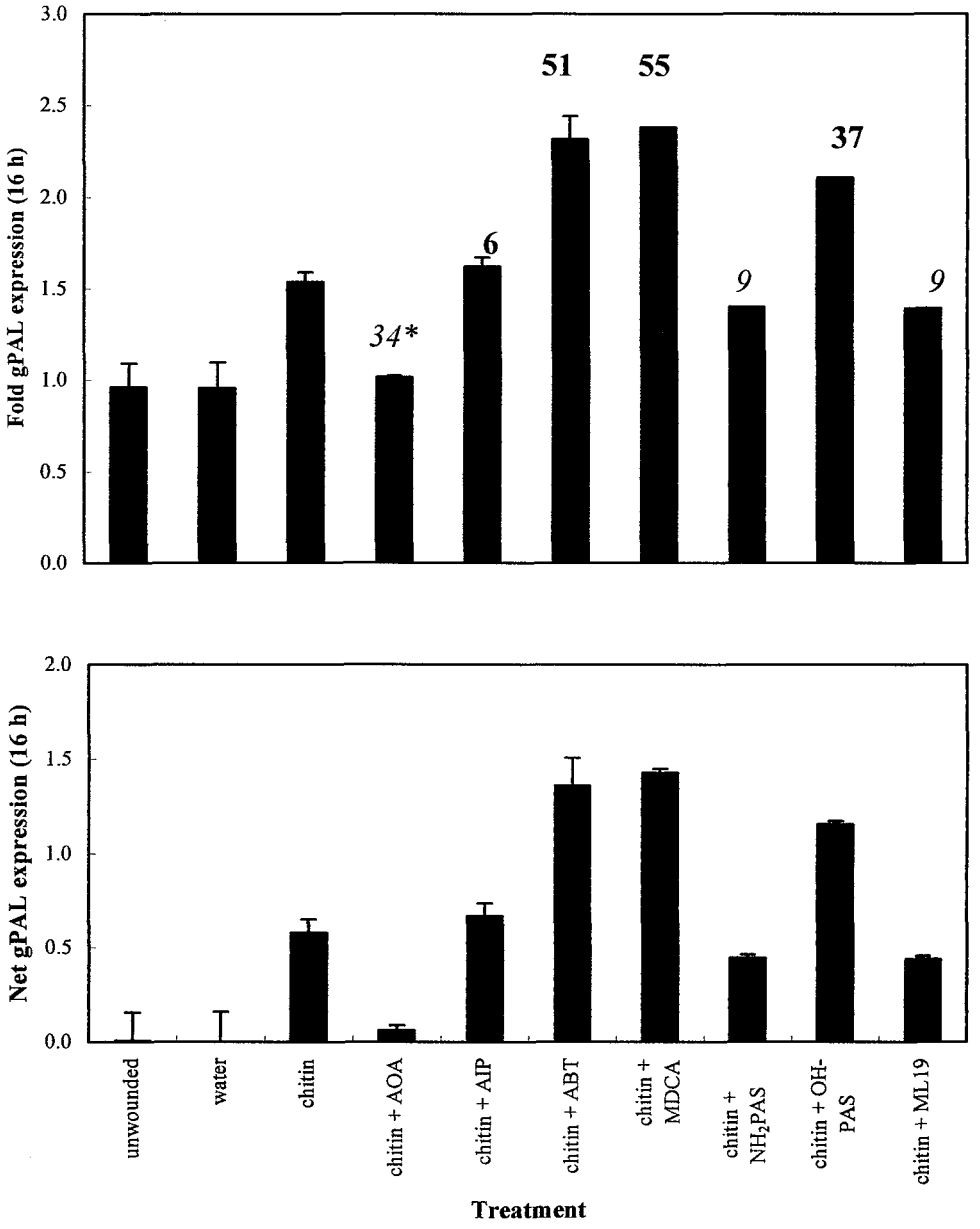


Figure 4.9 Effect of the inhibitors of the phenylpropanoid pathway on chitin induced gPAL expression. Data obtained from scanning densitrometric analysis of northern blots. a, Total fold gPAL expression, b net (-water) gPAL expression. Unwounded, untreated leaves Water, wounded leaves treated with distilled water, all other treatments were with 5 mg ml^{-1} chitin. **Increase**, *decrease*; percentage change in gPAL expression under the treatment. * Significant difference (n = 2, p = 0.05) from chitin alone treatment. Vertical bars show the standard error of the mean.

4.28 PAL activity in the presence of known inhibitors of the phenylpropanoid pathway.

To check the effect and relative efficacy of the known inhibitors of lignification on PAL activity the inhibitors of the phenylpropanoid pathway were incorporated into the enzyme assay of Mitchell *et al* (1994). Wounded primary leaves of wheat were treated with 5 mg ml^{-1} chitin and harvested after 16 h post inoculation. PAL activity was extracted from leaves in the presence of 2.5 mM phenylpropanoid pathway biochemical inhibitor. To calculate induced PAL per wound, water control values (pkat $leaf^{-1}$) were subtracted, the result multipled by 1000 (f kats) and divided by the number of wounds (5). Unsurprisingly both AOA and AIP significantly reduced PAL activity significantly. AIP appears to completely reduce all PAL activity. More surprising was that both the CAD inhibitors NH_2PAS and OH-PAS also significantly reduced PAL activity. None of the other phenyl propanoid pathway inhibitors can be shown to reduce PAL activity.

Table 4.4 Activity of PAL in the presence of known inhibitors of the phenylpropanoid pathway. The effect of various treatments on the induction of phenylalanine ammonia lyase (PAL) activity in wounded wheat leaves at 16 h post inoculation. Data is expressed in terms of total activity per gram fresh weight of tissue and as the activity induced per wound by chitin in the presence of the metabolite and as the degree of inhibition of the induction of chitin. Proposed target a, PAL; b C4H; c MDCA/CCR d CAD. All assays were repeated at least eight times. A * indicates those compounds that significantly (p = 0.05) inhibited the chitin response

Plant treatment	Addition to PAL assay (2.5 mM)	pkats g^{-1} FW	Induced PAL fkat $wound^{-1}$	% Inhibition
Unwounded	Unwounded	1.29	0	
Wounded and water	water	3.46	0	
Wounded and chitin	water	13.70	58.0	
Wounded and chitin	AOA[a]	5.16	09.6	83*
Wounded and chitin	AIP[a]	0.18	-19.1	133*
Wounded and chitin	ABT[b]	17.58	79.9	-38
Wounded and chitin	MDCA[c]	12.29	50.0	14
Wounded and chitin	OH-PAS[d]	2.75	-04.0	107*
Wounded and chitin	NH_2PAS[d]	5.41	11.1	81*
Wounded and chitin	ML19[d]	13.04	54.3	6

Discussion

4.29 The expression of gPAL activity induced by chitin and wounding.

Although the defence related clone of phenylalanine ammonia lyase (gPAL) has been sequenced (Hall 1998), the relationship between the expression of this defence related clone and total PAL activity had not been fully explored. These experiments investigated the consequence of adding a defence response elicitor (chitin) to wheat leaves on the induction of gPAL.

gPAL is induced over 48 h in 10 day old wheat plants wounded and inoculated with chitin (Figure 4.3). In the presence of chitin the pattern of gPALs expression mirrors that of the total PAL activity. The expression of the gPAL gene increases 12 h after wounding and chitin treatment (Figure 4.3); similarly PAL activity increases at 12 h (Figure 4.4). Both the activity and expression patterns are maintained throughout 48 h period. Chitin therefore is a potent elicitor of gPAL activity and expression in 10-day-old wheat plants.

In water-treated wounded 10-day-old wheat plants an increase in PAL activity is observed at 32 h (Figure 4.4). Although this point is not directly measured in the expression data the data shown does not support that gPAL expression is directly responsible for the increases in PAL activity at this point. In addition the net effect of wounding on PAL activity (Figure 4.6) shows that after 24 h through to 48 h PAL activity induced by wounding is significantly different to untreated plants. This is in contrast to reports that wounding does not induce PAL activity in wheat (Peltonen 1998). This may be due to the increased sensitivity of the radiochemical assay used in this study.

As wound induction does not stimulate gPAL it is likely that some other PAL genes are involved in the activation of PAL. At least two PAL genes (Liao *et al* 1996, Snowden and Gardner 1993) are known to exist in wheat and one possible explanation may be that another gene is activated to increase PAL activity. Many mechanisms may underlie PAL expression. In tobacco and populus at least two

different temporally activated PAL genes are expressed and regulate the PAL levels (Moriwaki *et al* 1999) (Thamarus and Furnier 1998). Another possible mechanism of regulation may be increased activation of PAL enzyme via a protein kinase such as the CDPK shown in *Arabidopsis* (Cheng *et al* 2001). Further analysis such as microarray or proteomics techniques may reveal the temporal expression and activation patterns of wheat PAL.

Regulation of PAL therefore is under the control of at least four genes, which contribute to the PAL activity profile. The first described defence related PAL in wheat is activated significantly only by direct elicitor treatment and not by wounding. Wounding itself however causes a small increase in PAL activity but this cannot be attributed to gPAL, or from the literature any other PAL described in wheat to date.

4.30 Specificity of PAL inhibitors

The inhibitors of PAL activity were chosen for two reasons. In the case of the documented PAL activity inhibitors AOPP, AIP and AOA, the effect of the inhibition of the PAL enzyme can be correlated with the effect on the transcription of gPAL in wheat. This gives some insight into the dependence of wheat for gPAL as part of the defence response.

Both PAL inhibitors do reduce PAL activity but only AOA reduces PAL expression (Figure 4.13, 4.14). This is in contrast to work reporting that the PAL promoter in *Arabidopsis* was negatively affected by the presence of AIP (Mauch-Mani and Sluslarenko 1996). Further work with AIP indicates that high levels may be phytotoxic and the competition with phenylalanine by AIP may arrest the cell cycle (Appert *et al* 2001). The inhibitor AOPP was not considered in this study because of its short supply, and its lack of specificity as highlighted in chapter 3. However, AOPP has been reported to increase PAL expression in *Medicago sativa* Ni *et al* (1996), clearly the structures of the known inhibitors and their enzymes are sufficiently different to have divergent effects on expression and activity. A possible explanation for these differences may be genetic variation between wheat and these other species.

The specificity of other phenylpropanoid enzyme inhibitors was investigated for their effect on gPAL expression and PAL activity. This gives some insight into the specificity of the inhibitor for its target enzyme. A facet of this approach is if the inhibition of the target enzyme (e.g. CAD by ML19) is specific enough the effect on PAL activity may be akin to that achieved by mutational analysis of the wheat plant. Mutational analysis is a difficult tool to use in wheat due to the hexaploid nature of the wheat genome (Barber and Mitchell 1999).

The C4H inhibitor ABT increases both gPAL expression and PAL activity in the presence of chitin (Figure 4.11, Table 4.7). This indicates some regulation by the presence of active hydroxylase enzymes of the phenylpropanoid pathway; as C4H is activated during defence related stimulus in *Medicago sativa* (Ni *et al* 1996). In particular the presence of 1 mM cinnamic acid is shown in this study to increase PAL expression indicating a feedback loop. This is some evidence toward the C4H enzyme being sufficiently different from the PAL enzyme to allow discrimination between the two potential substrates, phenylalanine and cinnamic acid.

The 4CL inhibitor MDCA increases expression of PAL but does not have any effect on activity (Figure 4.11, Table 4.7). It is likely therefore that this inhibitor is quite specific for 4CL and the effect of inhibiting 4CL would be to produce less aldehydes and alcohols across the pathway. As defence related lignin is still produced in the presence of MDCA it is likely that some other route is taken to produce lignin. As mentioned below it appears that only the addition of alcohols reduces PAL activity and expression therefore it is logical that physiological loss of these increases PAL expression.

The CAD inhibitors NH_2PAS and OH-PAS both reduce PAL activity. This may be due to a lack of specificity for these two inhibitors. The treatment with OH-PAS has a negligible effect on PAL expression (Figure 4.11, Table 4.7). This effect with OH-PAS mirrors results with the PAL1 promoter in *Arabidopsis* (Mauch-Mani and Sluslarenko 1996). Alternatively NH_2PAS has been shown to show some activity toward CCR (Carver *et al* 1996) this may be why NH_2PAS shows no effect on PAL expression.

The CAD inhibitor ML19 however appears specific, as PAL activity is relatively unaffected. Data with ML19 indicate that if CAD is inhibited so expression of gPAL is decreased (Figure 4.11, Table 4.7). ML19 is specific for the form of CAD known as CAD C, which produces the syringyl-rich defence lignins. Syringyl lignins are the most common in defence related interactions (Agrios 1993).

4.31 Regulation of PAL by the metabolites of the phenylpropanoid pathway.

4.31.1 Evidence for feedback inhibition of PAL

The metabolites of the phenylpropanoid pathway used in this study were chosen primarily because of their availability from commercial companies or had been previously chemically synthesised. The metabolities chosen represented a broad base of the pathway intermediates but were not a complete list.

The main candidates that inhibit PAL activity are phenylalanine, *trans*-cinnamic acid and 5-hydroxyferulic acid which all show significant reductions in PAL activity in wheat. Other possible candidates include ferulic acid, *para*-coumaric acid, coniferal aldehyde and coniferal alcohol.

It was expected that phenylalanine and *trans*-cinnamic acid would feedback inhibit PAL activity, unfortunately one problem with this result is that the extra phenylalanine in the radiochemical assay reduces the specific activity of C^{14} in the assay and consequently a reduction in PAL activity is recorded. Similarly; addition of *trans*-cinnamic acid is the end point of the reaction and therefore C^{14} concentration in the organic phase is decreased. The reduction of PAL activity by *trans*-cinnamic acid is well documented (Reichart 1982).

In this study *para*-coumaric acid decreases elicited gPAL activity. However, in cucumber, feeding *para*-coumaric acid increases the activity of PAL (Politycka 1999). Although these experiments are not necessarily comparable as *para*-coumaric acid was not measured under defence-stress conditions. This is some

evidence for a change in the role of the phenylpropanoid pathway intermediates in plant metabolism in conditions of challenge compared to usual conditions. In wheat resistant to *Fusarium culmorum* head blight increased levels of para coumaric acid are observed (Siranidou *et al* 2002). A possible hypothesis therefore would be that the production of *para* coumaric acid in wheat continues the PAL expression pathway.

In this study caffeic acid increases PAL activity and has no effect on elicited gPAL expression. Caffeic acid has been shown to be inhibitory to PAL activity in sweet potato but not in pea (Sato *et al* 1982). This is some evidence for a change in discrimination between species, but the experiments with sweet potato and pea were not in an elicited system.

In wheat ferulic acid also decreases PAL activity, but not significantly, no data can be presented for expression as no signal was detected on the northern blot (Fig 4.13, 4.14). This result is unexpected as several authors report in sweet potato, pea and soybean that ferulic acid increases PAL activity (Sato *et al* 1982), (Herrig *et al* 2002). However yeast PAL activity is decreased by ferulic acid (Sato *et al* 1982); the isoform nature of PALs in different systems may account for the discrepancy of this substrate. Ferulic acid levels were also found to be unchanged between susceptible and resistant cultivars of wheat in response to *Fusarium culmorum* (Siranidou *et al* 2002).

The only significant inhibitor of PAL activity is 5-hydroxyferulic acid. This may be due to its similarity to *trans*-cinnamic acid, this has not yet been reported in the literature. However as sinapic acid does not appear to induce PAL activity and the general consensus of the role of sinpoyl derivatives is that the path of sinapic acid is independent to that involving PAL, the role of production of 5-hydroxyferulic acid may be as a metabolic block. This is conjecture however and would be best assessed with a dose response of 5-hydroxyferulic acid to determine the kI. The main problem with this result is that the compound was manufactured some years ago and may have changed somewhat over time. The effects shown here therefore may not be true of 5-hydroxyferulic acid (Barber personal communication 2003).

Clearly these results are a first step to understanding the regulation and role of these metabolities in the phenylpropanoid pathway. Confirmation by analysis of mutants of wheat on the effects of the levels of metabolities is required for verification.

The best study would be one that could measure all the levels of all the metabolities concurrently *in vivo* over time in normal development and correlate these with the levels in elicited conditions. In addition, the expression levels and activities of all the defence related enzymes of the phenylpropanoid pathway would have to be measured.

4.31.2 Evidence for feedback repression of PAL

To date there is no other evidence in any plant species to show that aldehydes or alcohols of the phenylpropanoid pathway reduce expression of the defence related forms of PAL.

In wheat, the main candidates that inhibit gPAL expression are coniferaldehyde, *para* coumaryl alcohol, coniferal alcohol and sinapoyl alcohol. Suprisingly, gPAL expression was not affected by *trans*-cinnamic acid as some previously reported PAL genes from alfalfa (Orr *et al* 1993) and bean (Mavandad *et al* 1990) are repressed in the presence of *trans*-cinnamic acid.

When the gene PAL2 is overexpressed in *Nicotiana tabaccum*, the amount of lignin does increase and the flux of carbon appears to move toward the production of chlorogenic acid. This may not be analogous to the defence response shown here in wheat but in *N. tabaccum* 4CL is believed to be the limiting factor. This overexpression of PAL leads to a build up of coumaric acid, which in wheat increases gPAL expression (Howells *et al* 1996).

4.33. Three possible regulatory mechanisms that involve PAL in wheat.

From the evidence found in this study there appears to be three possible modes of regulation of PAL by the phenylpropanoid pathway intermediates and the activity of the PAL enzyme. These are hydroxycinnamic acid de-repression of gPAL, PAL inhibition by methoxy-hydroxycinnamates and repression by hydroxycinnamoyl alcohols of gPAL.

4.33.1 Hydroxycinnamic acid de-repression of gPAL.

In the early stages of stimulation of the phenylpropanoid pathway, the presence of hydroxycinnamic acids, *trans*-cinnamic acid, *para*-coumaric acid and caffeic acid appear to de-repress expression of gPAL. This correlates well with the effects of ABT appearing to increase PAL activity and gPAL expression. As ABT is a broad spectrum cytochrome p450 inhibitor (Sigma) its application would lead to a build up of cinnamic acid.

4.33.2 Methoxy-hydroxycinnamate inhibition of PAL

The second mode of regulation appears to be a substrate based inhibition involving intermediates methoxylated at the three or five carbon position of the phenylpropanoid. The substrates tested here; ferulic acid, coniferaldehyde and coniferal alcohol all appear to inhibit PAL activity by half. Interestingly MDCA the inhibitor of 4CL which leads to the production of coniferal aldehyde (after action of CCR) has no effect on PAL activity but increases PAL expression. This can be taken to be consistent with the theory of one of the acids acting as a metabolic block as activity is slightly decreased, eventually the block would build up. If PAL was examined at a later time point activity would be declining, however this is speculation.

4.33.3 Hydroxycinnamoyl alcohol repression of gPAL.

The final mode of regulation comes from the presence of the alcohol group at the end of the phenylpropanoid pathway. Addition of all three primary alcohols that form the hydroxyphenyl, guiaiacyl and syringyl units (*para*-coumaryl alcohol, coniferyl alcohol and sinapoyl alcohol) results in repression of gPAL. All CAD and CCR inhibitors have no effect (ML19) or heavily inhibit PAL activity. As ML19 is CAD C specific (Hall 1998) this is some evidence that direct control of the final production of the S units of lignin is not under feedback control to PAL, this may be why lignin in wheat is so rich in syringyl units (Ride 1975), this however is speculation. Surprisingly OH-PAS although thought to be a specific CAD inhibitor also affects PAL activity, so its usefulness in this discussion is limited. NH_2PAS has effects on CCR and CAD could cause a build up of intermediates e.g. coniferal aldehyde that depresses PAL activity. It is surprising that the expression is not as markedly decreased as when coniferal aldehyde is added alone, this may be due to a difference in the actual levels of the metabolites in planta during defence being different from those used here. The addition of inhibitors therefore does not back up the asserion that all phenylpropanoids with alcohol groups decrease gPAL expression, the unavailable intermediates caffeoyl alcohol and 5-hydroxyconiferal alcohol when tested may shed some light on this apparent discrepancy.

A scheme for this possible regulation and its adherence to the criteria of the main flux of carbon through the phenylpropanoid pathway (Humpreys and Chapple 2002) is shown below (Figure 4.10).

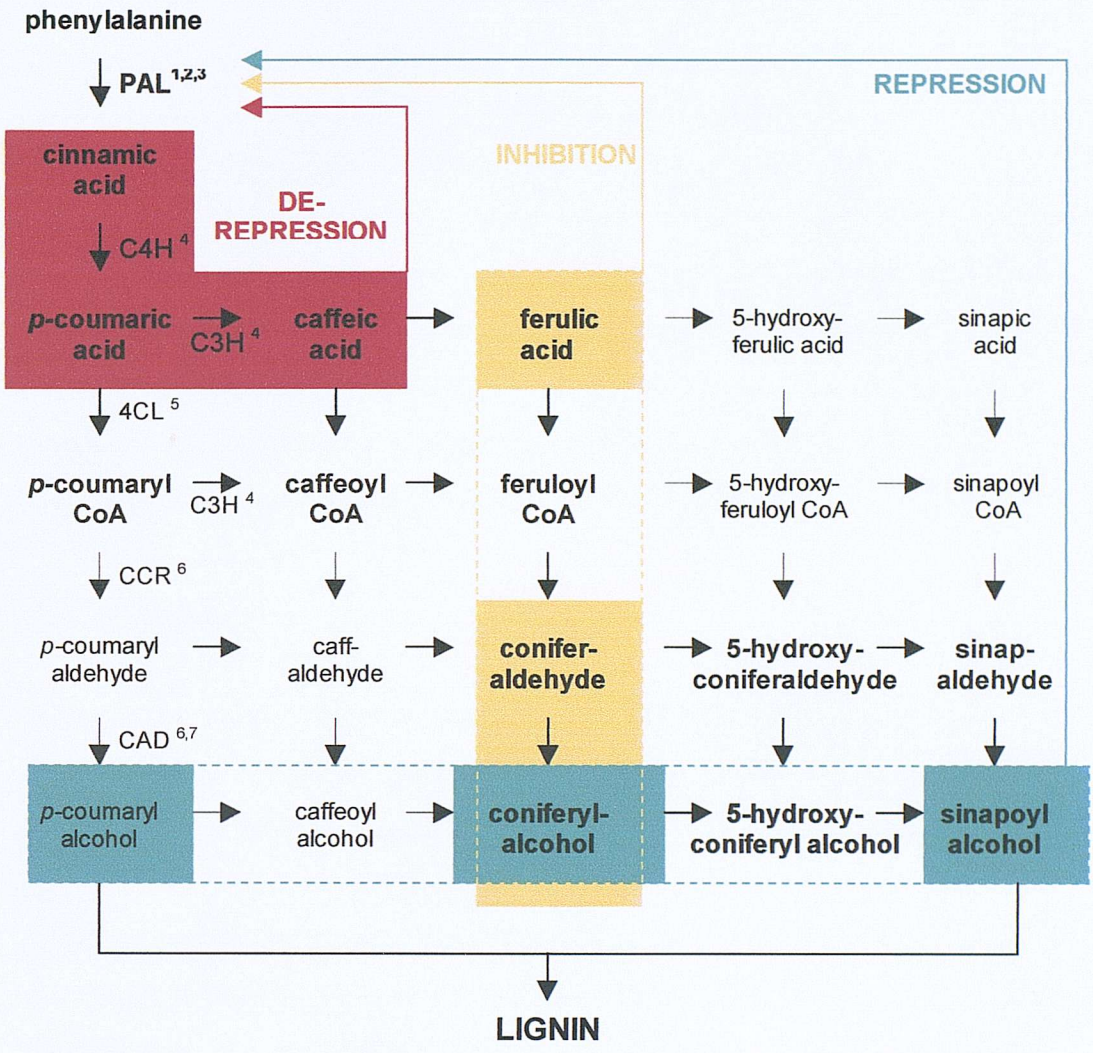


Figure 4.10 The effect of intermediates and biochemical inhibitors of the phenylpropanoid pathway on the regulation of activity and expression of chitin stimulated PAL. Analysis of PAL and gPAL was by spectrophotometric and Northern blot methods respectively. Intermediates denoted bold indicate the major carbon flux through the pathway (Humpreys and Chapple 2002); Red, intermediates involved in de-repression of PAL; blue intermediates with 3 or 5 carbon methoxylation substitution patterns involved with inhibition of PAL; green, intermediates involved in PAL repression; numbers one through seven denote biochemical inhibitors; 1 AIP; 2, AOA; 3, AOPP; 4, ABT; 5, MDCA; 6, NH_2PAS; 7, OH-PAS.

4.34 Further work

To fully understand the changes in PAL activity and expression over time in response to a defence related stimulus in wheat, all the PAL genes must be fully characterised. This allows a full assessment of the role of defence related PAL genes and developmental related PAL genes at times of plant defence.

It is also important to fully explore the levels of all the metabolites in wheat both in defence related (stimulated) conditions and in normal development. The ideal study would be one that could relate all lignin pathway substrate concentrations and enzymes to their sub-cellular compartments. This would further enhance the knowledge of how and where the precursors to the lignin scaffold are controlled.

4.35 Conclusions.

As aforementioned the presence of all alcohols measured reduces expression of gPAL. Taken together these results show some evidence that the presence of the precursors to the hydroxyphenyl, guaiacyl and syringyl units down-regulates the expression of PAL. It is therefore interesting that in the presence of *para*-coumaryl alcohol, activity of PAL is increased whereas expression is decreased of gPAL. The most likely explanation would be the effect of another PAL gene and others have been reported (Liao *et al* 1996, Snowden and Gardner 1993).

Chapter 5: Evidence for endogenous elicitation of the lignification response in wheat

5.1 Defence related endogenous elicitation

Molecules that induce a defence related biological response are termed elicitors. Essentially, two types of elicitors exist exogenous and endogenous. The process of exogenous elicitation in plant defence concerns the outcome of a host pathogen interaction. This may govern the hosts ability to perceive and initiate defence responses against a pathogen via perception of the elicitors the pathogen produces. Elicitors derived from plants are called endogenous or constitutive elicitors and have been reported in many species (Table 5.1). The first so called endogenous elicitor was characterised by Hargreaves and Bailey (1978), in true bean (*Phaesolous vulgaris*). The majority of endogenous elicitors are derived from the plant cell wall and are of a pectic nature. Some exceptions exist; ethylene (Dutta and Briggs 1991) and cutin monomers (Schweizer *et al* 1996) have some endogenous elicitation capability. It is not surprising that some plant-derived signals are also involved in suppressing plant defence. These molecules are known as endogenous suppressors (Table 5.2). The suppressors are diverse in form ranging from single molecules like methyl jasmonate (Andi *et al* 2001) to various oligomers of galacturonic acid (Morshbacher *et al* 1999). In wheat both an elicitor (Hahn *et al* 1981) and a suppressor (Morshbacher *et al* 1999) molecule have been reported.

Species	Elicitor	Origin	Reference
Citrus spp	Ethylene	ACC synthase	Dutta and Briggs (1991)
Acer plantus	Polysaccaride	Cell wall	Hahn *et al* (1981)
Nicotiana tabaccum	Polysaccaride	Cell wall	Hahn *et al* (1981)
Glycine max	Fragment	Cell wall	Hahn *et al* (1981)
Glycine max	Galacturonic acid oligosaccaride	Cell wall	Nothangel *et al* (1983)
Solanum tuberosum	Cutin monomers	Cuticle	Schweizer *et al* (1996)
Lycospersicon esculentum	Pectic polysaccaride	Cell wall	Walker-Simmonds *et al* (1983, 1984)
Dacus carrota	Polygalacturonide / Polypeptide	Cell surface	Kurosaki and Nishi (1984)
Phaseolus vulgaris	Aqueous extract of Bean tissue.	N.D.	Hargreaves and Bailey (1978)
Raphinus spp	Lignification inducing factor	N.D.	Asada and Matsumoto (1987)
Racinus spp	Pectic fragments	Cell wall	Bruce and West (1982)
Triticum aestivum	Polysaccaride	Cell wall	Hahn *et al* (1981)

Table 5.1 Endogenous elicitors in plants. N.D. not determined.

Species	Elicitor	Origin	Reference
Nicotiana tabaccum	Methyl Jasmonate	Jasmonic acid	Andi *et al* (2001)
Pisum sativum	N.D.*	N.D.	Nasu *et al* (1992)
Triticum aestivum	Di and tri oligomer of galacturonic acid	Pectic fractions	Morshbacher *et al* (1999)
Lycospersicon esculentum	N.D.	Inter-cellular fluid	Peever and Higgins (1989)

Table 5.2 Endogenous suppressors in plants. N.D. not determined.* only evidence rather than nature is presented.

It is hypotheised that cutin monomers have a role in recognition of tissue damage, such as is caused by fungal attack (Schweizer *et al* 1996).

5.2.3 Gaseous elicitors

These elicitors are diffusible throughout the whole plant and are thought in general to be involved in longer distance signalling between organs of the plant. The plant hormone ethylene is involved in many aspects of the life cycle of plants, including seed germination, root hair development, root nodulation, flower senescence, abcission and fruit ripening (Johnson and Ecker 1998). Ethylene (Fig 5.1) ultimately functions as a defence response by leading to leaf abscission. In particular ethylene stimulates PAL, β-1,3 glucanase and chitinase (Salisbury and Ross 1990).

5.2.4 Ethylene production.

The biosynthesis of ethylene comes from 1-aminocyclopropane 1-carboxylic acid (ACC). The rate limiting step for its synthesis comes from the the conversion of *S*-Adomet to ACC by the enzyme ACC synthase. The final step in the production of ethylene is the enzyme ACC oxidase which converts ACC to ethylene (Wang *et al* 2002).

5.2.5 Ethylene receptor pathway.

The full extent of the ethylene reception signalling pathway is not fully known. Evidence for the pathway comes from ethylene insensitive mutants (ein) or ethylene resistant mutants (etr) of *Arabidopsis*. Initially ethylene binds to one or more members of a family of membrane localised two-component receptor kinases, ETR1, ETR2, EIN4, ERS1 and ERS2 and inactivates them. This inactivation triggers the switch off of CTR1 (a RAF-like serine/threonine kinase). The inactivation of CTR1 activates the integral membrane protein EIN2 which is known to function downstream of CTR1. This activation activates the gene EIN3. EIN3 is a known transcription factor which in turn activates ethylene responsive genes. Other proteins as yet undefined in function; but possibly also transcription factors

(EIN5 and EIN6) have been shown to act downstream of EIN2 but their function is unknown Gazzaraini and McCourt (2003), Wang *et al* (2002)

5.2.5 Ethylene as a defence response.

Confusingly plants deficient in ethylene biosynthesis can show increased susceptibility or resistance to virulent pathogens. Soyabean mutants with reduced ethylene sensitivity showed increased resistance to *Psedomonas syringae* p.v. *glycinea* and *Phytophythora sojae* but less resistance to *Septoria glycines* and *Rhizoctonia solani* (Hoffman *et al* 1999).

An accepted reason for this apparent discriminatory behaviour of ethylene is the conversion of defence signalling pathways. Other defence related pathways involve compounds such as Jasmonic acid and Salicyclic acid. Jasmonic acid is independently sensed by a pathway involving the gene coi1 in *Arabidopsis* and elicits a range of pathogensis related (PR) proteins. Salcyclic acid increases endogenously with PR gene accumulation after the activation of the hypersensitive response. (Wang *et al* 2002).

Evidence for this conversion comes from experiments particularly with ethylene insensitive mutants (ein2) together with mutants that have defects in the coi1 gene are impaired in the jasmonic acid signal transduction pathway in *Arabidopsis*. In particular the expression of the plant defencin gene PDF.1.2. requires both jasmonic acid and ethylene signalling pathways to be operative (Penninckx *et al* 1996). Also a recent survey of expression levels of many genes to pathogen infection, salicylic acid, jasmonic acid and ethylene shows that although some genes are affected by one signal or another, many respond to two or more defence related signals (Schenk *et al* 2000). Salicylic acid and ethylene signalling pathways are linked together from evidence showing that the expression of the gene PR1 which is consitutively expressed in the presence of SA can only be completely switched off in the npr-1(non-expressor of PR-1) mutation if the ein2 mutation is present as well. This suggests the existence of an ethylene independent pathway the existence of interactions between ethylene and SA dependant signalling through an NPR-1 independent pathway (Wang *et al* 2002).

Clearly these defence related pathways may also be subject to endogenous regulation or suppression.

5.3 Evidence for the physiological significance of the role of endogenous elicitors in plant defence.

Although the general form of an endogenous elicitor is thought to be a hydrolysed part of the cell wall, fungi also use this mechanism for plant perception. In *Solanum tuberosum* L soft rot is caused by *Erwina carotovora*. In potato plants transformed with the gene encoding the isoenzyme of pectate lyase from *E. carotovora* ssp *altroseptica* resistance was enhanced to *E. caratovora*. Compared to untransformed plants soft rot was significantly ($p=0.001$) reduced. The presence of the pectate lyase gene mediates the degradation of plant cell wall pectin into oligogalacturonates that are known to induce plant defence responses. The transformed plants could activate defence responses earlier compared to untransformed plants and although this lead to greater numbers of bacteria on the tubers, the rotting caused was diminished (Wegener 2002).

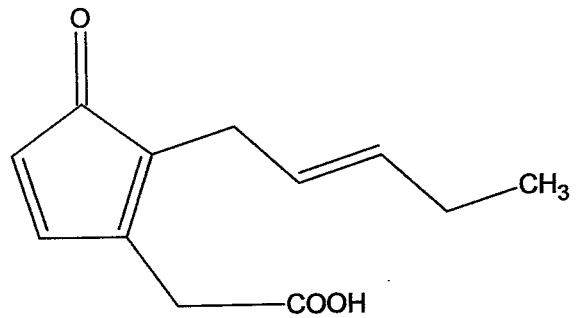
5.4 Forms of endogenous suppressors

Endogenous suppressors appear to constitutively regulate the defence mechanisms when the defence mechanisms are not required. Endogenous suppressors become an important part of the host pathogen interaction when their activity is induced by a pathogenic mechanism. This is usually through the action of CWDE and the suppressors are usually degradation products of the cell wall. An endogenous suppressor has been proposed in this vein in wheat. This was found by extraction of pectic fractions with the use of calcium chelators, pectic enzymes and a recombinant endopolygalactuonidase. Interestingly, only co-injection of the fractions with a glycoproteogalactan elicitor caused suppression of phenylalanine ammonia lyase and peroxidase activities. The activity of the suppressor against PAL and peroxidase activities was correlated with the presence of galacturonic acid and the di and trimer of this oligomer were shown to be the most effective suppressors. The role of this suppressor is unknown but is hypothesised to have some role in the establishment of the host-pathogen interaction (Morshbacher *et al* 1999).

5.5 Gaseous suppressors

It has recently been proposed that some gaseous compounds released during pathogen invasion may retard the defence response. Jasmonate (Figure 5.1) and methyl jasmonate are formed from linoleic acid, which is one of the major fatty acids that make up plant membranes. These compounds are released when plant pathogens degrade plant membranes and act as a long distance signalling molecule involved in such responses such as systemic acquired resistance (Ryals *et al* 1994).

Methyl jasmonate (MeJA) has recently been proposed as an endogenous suppressor in *N. tabaccum* c.v. BY-2. MeJA has been found to inhibit responses mediated by the elicitor harpin such as PAL induction and generation of the oxidative burst. Recent evidence shows that Harpin alone elicits hydrogen peroxide as measured by chemiluminsence of luminol by a relative factor of 50 after 4 h compared to a relative factor of 17 when 20 μM MeJA is added in the presence of harpin in *N. tabacum*. MeJA is thought to function by inducing jasmonate inducible proteins to inactivate *de novo* protein synthesis (Andi *et al* 2001).



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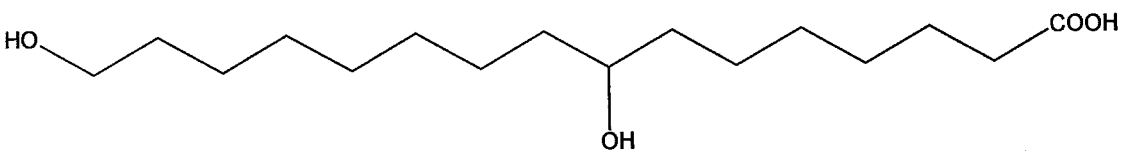

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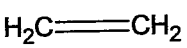


C

Figure 5.1. Structures of endogenous elicitors. A, Jasmonic acid B, 8, 16-Dihydroxypalmitic acid (8, 16-DHPA) C, Ethylene.

5.6 Rationale

To date there is no published evidence that endogenous elicitors are involved in the regulation of defence related lignification in wheat. However, there are some observations that such molecules may exist. Firstly, wounding alone induces a small but detectable lignification response. Secondly, water droplets from water treated wounds induce PAL when injected into healthy leaves. Finally, cell wall degrading enzyme preparations from *Trichoderma spp* are potent elicitors of the lignification response.

5.7 Approach

To ascertain if any there is any evidence for an endogenous elicitor acting in the induction of the lignification response in wheat the following strategy will be employed. Firstly culture filtrates of *T. viride* will be fractionated and used to identify heat-labile elicitors. Secondly, the identified heat-labile elicitors will be incubated with wheat cell walls to determine if any heat stable elicitors are generated. The elicitors will again be detected by the scanning densitometric assay for lignification.

5.8 Aim

The aim is to investigate whether endogenous elicitors operate in the lignification response in wheat.

5.11 Culture of micro-organsims

Trichoderma viride was cultured on 2 % (w/v) Malt Extract Agar (Malt Extract 20 g L^{-1}, Agar 20 g L^{-1}, SDW to 1 L, adjusted to pH 6.5 and autoclaved 121 °C, 20 min, 15 psi). After 7 days growth at 24 °C the fungus was sub cultured into 175 ml 2 % (w/v) Malt Broth (Malt Extract 20 g L^{-1}, SDW to 1 L autoclaved 121 °C, 20 min adjusted to pH 6.5 with 0.1 M HCl) by cutting out 10 plugs using a sterile 8 mm cork borer. After 6 days the culture filtrate was collected through a buchner funnel, using Whatman No 1 filter paper and stored at –20 °C.

5.12 Treatment of plant material.

Details of the wheat leaf bioassay are described in full in chapter 2 (section 2.7). Culture filtrates fractions were applied to wounded wheat leaves at 10 µl per wound. In addition a visual assessment was made on the presence (+) or absence (-) of lignin ring on fast GG stained leaves.

5.13 Wheat cell wall extraction

Triticum aestivum plants were grown for 10 days (16 h photoperiod, 21°C) in Levington F2 soil. Leaves were cut from the stem and ground using a chilled pestle and mortar in 2-fold excess (w/v) 0.1 M sodium phosphate buffer pH 7.0. The solid matter containing the cell walls was collected by centrifugation for 10 min at 2000 g, re-suspended in 0.1 M sodium phosphate buffer pH 7.0 and centrifuged again for 10 min at 2000 g. The cell walls were washed twice in ethanol and twice in acetone, collecting via centrifugation for 10 min at 2000 g.

5.14 Column chromatography

G25 Sephadex beads were packed in a 60 cm column (radius 2.35 cm, volume 1308 ml). Flow rate was set at 1.5 ml min^{-1}. Crude culture filtrate (5 ml) was passed down the column and collected as 50 ml fractions with distilled water as a running solvent. The column was run at 4°C. The bed and void volume was determined by loading a mixture of blue dextran (mw $1x10^6$) and sodium chloride (mw 60). Blue dextran was detected by absorbance at 600 nm, sodium chloride by reaction with $AgNO_3$ to yield a white precipitate of AgCl.

5.15 Protein assay

Protein content was determined by the Bicinchoninic acid(BCA) method (Smith *et al* 1985).

5.16 Dialysis of candidate fractions

Dialysis of 5 ml of candidate fractions took place in visking tubing at 4 °C in 200 ml distilled water over 48 h, changing the water twice. For the second dialysis 1 g wheat cell wall was added and incubation was at 25 °C in 200 ml distilled water for 24 h. The diasilate was collected and frozen at –20 °C and freeze-dried.

5.17 Assay of candidate pectic elicitor fractions

A 1 ml aliquot of candidate fraction was removed and boiled in an eppendorf tube for 5 min. After cooling, drops were inoculated onto wheat leaves as described in chapter 2. Assay was by the scanning densitometric method and a visual assessment.

Results

5.18 Endogenous elicitor activity.

To test if the culture filtrate components have the ability to elicit lignification, preparations of Malt broth 2 %, Cellulase R10 1 mg ml^{-1}, *Trichoderma viride* conidial suspension 10 mg ml^{-1} and culture filtrate were placed in 10 µl droplets on wound lesions of taped down 10-day-old wheat leaves. Droplets were left for 48 h and leaves stained for lignin with fast GG stain. Scanning densitometric analysis was used to compare relative levels of lignification at wound margins.

Accumulation of lignin was calculated from integrated density values generated from examination of images of leaves, using the value achieved by chitin alone as 100 %. None of the components of the culture media could elicit accumulation of lignin to the same degree as 5 mg ml^{-1} chitin. The highest accumulation was observed with *T. viride*. Cellulase R10 and sterile distilled water produced a very similar accumulation, with Malt Extract Agar producing only 50 % of the activity shown by chitin (Figure 5.2).

In order to observe endogenous elicitation a stimulus must be used to faciliate the production of this phenomena. If *Trichoderma viride* produces heat labile elicitors they will be secreted into the mycelial culture. These heat-labile elicitors may be capable of releasing heat stable "endogenous" elicitors from wheat cell walls.

5.19 Identification of elicitor active, heat labile fractions in *T. viride*

In order to separate components of the *T. viride* culture, the filtrate was passed through a sephadex G25 column. Large peaks of protein were observed between the void (231 ml) and bed (833 ml) volumes (fractions 5 and 20). There were also several other peptide peaks that eluted after the bed volume. Peptides did not elute until 380 ml (fraction 9) and peaked at 2 mg ml^{-1} (fractions 9,10 and 11) and then reduced only to increase sharply to 5 mg ml^{-1} (fraction 20) as the column was eluted toward the bed volume (Figure 4.3).

Column fractions were then tested for their ability to elicit lignification in wheat leaves. Both a visual assessment and a quantitative assessment using the scanning densitrometric method of accumulated lignification were used to analyse the lignification data. Fractions 9 to 15 all induced the accumulation of lignin comparing favourably to that induced by chitin at 5 mg ml^{-1}. This was confirmed by visual assessment. Most fractions appeared to elicit lignification, by the scanning densitometric method. The visual assessment however gave only 5 more candidates 22,29,31,33 and 34 (Table 5.3). The elicitor active fractions were re-assayed for their ability to induce lignin after boiling for 1h. This indicated three heat-labile fractions 10,12 and 33 that lost their activity after heat treatment.

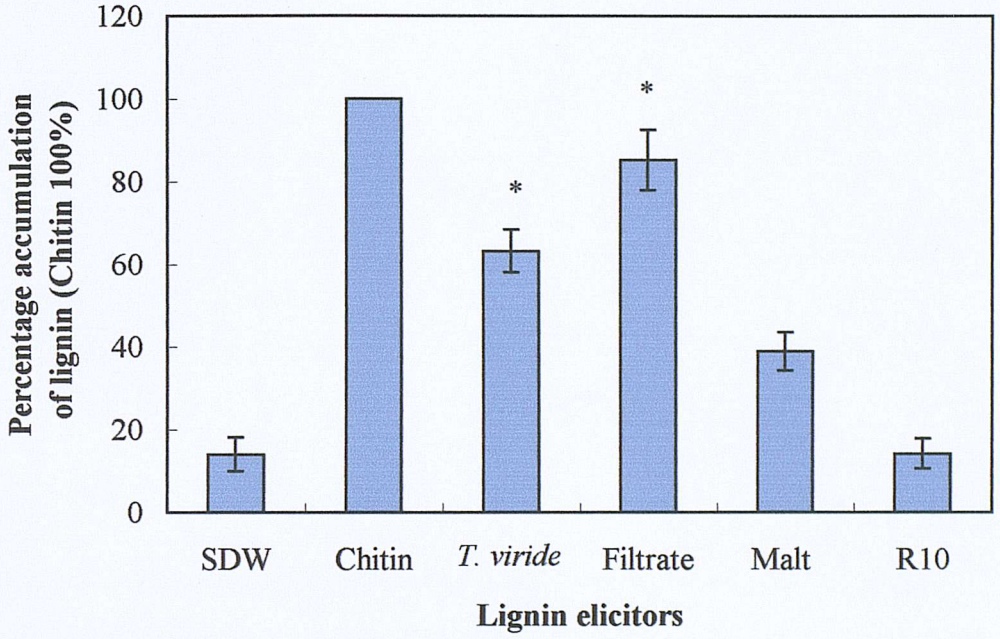


Figure 5.2 Ability of the culture filtrate of *Trichoderma viride* to elicit lignification in wheat; SDW, sterile distilled water; Chitin, Chitin 5 mg ml^{-1}; *T. viride*, *Trichoderma viride* condial suspension 10 mg ml^{-1}; Filtrate, accumulation induced by culture filtrate, Malt, Malt Broth 2 % (w/v); R10, Cellulase R10 1 mg ml^{-1}; Culture filtrate components were analysed for their ability to elicit lignification using the scanning densitrometric assay compared to chitin (Chapter 2); (*), significant difference from water treatment. Vertical bars represent standard errors

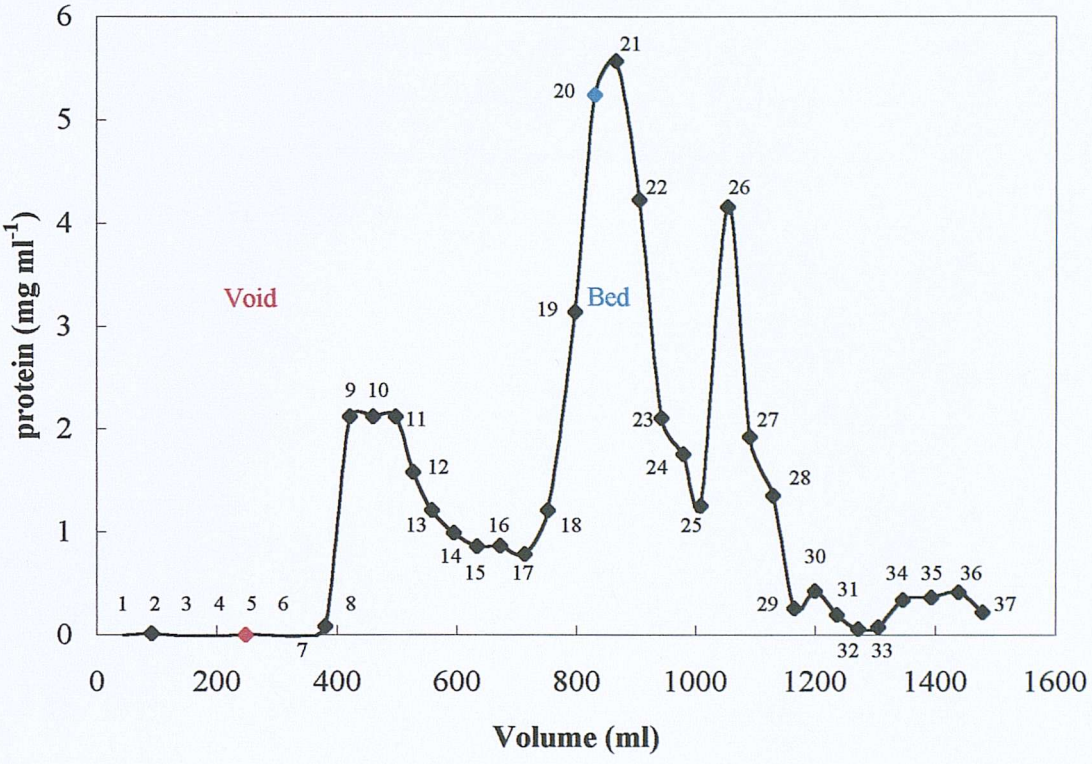


Figure 5.3 Separation of *Trichoderma viride* culture filtrate by Sephadex G25 column. Data is expressed in terms of protein mg ml^{-1} and was determined using the BCA method. ◆ Void volume (231 ml) ◆ Bed volume (833 ml). 1-37 fraction numbers.

Table 5.3 Ability of fractions of *T. viride* culture filtrate to elicit lignification. Wounds were assessed visually after fast GG staining (+/-). Percentage accumulation of lignin, as compared to 5 mg ml^{-1} chitin treated leaves (100 %): Grey shading indicates elicitor active fractions. Dark grey shading indicates elicitor active fractions inactive after boiling. (*) significant difference (p = 0.05 n = 20 per fraction.) from water treatment.

Fraction Number	Unboiled		Boiled	
	Visual assessment of lignin	Percentage accumulation of lignin	Visual assessment after boiling	Percentage accumulation after boiling
9	+	52+/-14*		
10	+	77+/-16*	-	0+/-4
11	+	107+/-16*	+	41+/-9*
12	+	92+/-13*	-	0+/-3
13	+	89+/-15*		
14	+	39+/-14*	+	0+/-4
15	+	45+/-16*	+	0+/-30
16	-	54+/-14*	+	0+/-23
17	-	51+/-14*		
18	-	84+/-15*	-	9+/-9
19	-	47+/-16*		
20	-	54+/-13*		
21	-	42+/-14*		
22	+	91+/-14*	+	0+/-8
23	-	73+/-15*	+	0+/-8
24	-	87+/-17*		
25	-	41+/-15*		
26	-	71+/-14*		
27	-	105+/-15*	+	0+/-5
28	-	100+/-18*	-	0+/-30
29	+	131+/-15*	+	0+/-30
30	-	115+/-15*	+	0+/-30
31	+	107+/-18*	+	0+/-30
32	-	88+/-21*		
33	+	161+/-14*	-	0+/-41
34	+	111+/-16*		
35	-	154+/-16*	-	0+/-55
36	-	103+/-15*		
37	-	100+/-21*		

5.20 Release of heat-stabile elicitors from wheat cell walls

Candidate fractions (10, 12, 33) were incubated with or without wheat cell walls in dialysis bags. The diasilates from these incubations were freeze-dried and assayed for the presence of elicitor activity (Table 5.4). Of the three fractions tested activity was only found in the diasilate of fraction 12 when incubated with wheat cell walls. Boiling of this fraction failed to inactivate elicitor activity.

Table 5.4 Ability of heat labile *Trichoderma viride* fractions to release heat stable elicitors of lignification from wheat cell walls. Wounds were assessed visually after fast GG staining (+/-). The percentage accumulation of lignin as compared to 5 mg ml^{-1} chitin treated leaves (100%). n = 20 per fraction. Brackets indicate standard errors.

Fraction number	+ Wheat cell walls		-Wheat cell walls		Heat treatment + Wheat cell walls	
	Visual assessment	Percentage accumulation	Visual assessment	Percentage accumulation	Visual assessment	Percentage accumulation
10	-	4 +/-10	-	0 +/-10	-	0 +/- 14
12	+	49 +/-15	-	0 +/-10	+	30 +/- 12
33	-	0 +/-15	-	0 +/-10	-	0 +/- 9

5.21 Discussion

Plants perceive fungal pathogens through chemical signals termed elicitors and can initiate host defence responses against these diseases. Some of these signals in are perceived when part of the host cell wall is degraded and are thus termed endogenous elicitors. Many examples of endogenous elicitors exist and are usually pectic cell wall fragments involved in defence related responses. Lignin is a constitutive part of the cell wall and is well established as an important part of the plant defence response to pathogens.

Evidence for an endogenous elicitor mediated lignification comes from a heat labile culture filtrate fraction having the ability to induce wheat cell walls to release a heat stable inducer of lignification. Although only three candidate fractions were examined it is clear that fraction 12 shows some evidence for this activity pattern. The size of the endogenous inducer of lignification in fraction 12 is likely to be less than 10 kD due to the dialysis used. Further separation techniques such as TLC or HPLC could be used to further explore the nature of this endogenous elicitor.

Previous evidence for both endogenous elicitation (Hahn *et al* 1981) and suppression (Morschbacher *et al* 1999) has been shown in wheat, but this has not been linked directly to lignification. A possible role for the endogenous inducer for lignification may not be defence related. A hypothesis is that plants require gain and loss of lignin units in the cell wall during the period of wall loosening during growth. A factor inducing and controlling the production of lignin units that are sequestered into the plant cell wall is not inconceivable. However little is known about this subject (Salisbury and Ross 1989).

In conclusion evidence is presented here of a link in wheat between endogenously induced lignification and wheat cell wall fragments. Although its nature has not yet been fully explored.

Chapter 6: General discussion on Manipulation of defence related phenylpropanoid metabolism in wheat.

6.0 Manipulation of defence related phenylpropanoid metabolism in wheat*

Lignin is a major constituent of the cell plant wall where it provides mechanical strength, waterproofing and plays a role in defence. In wheat defensive lignification occurs at wound margins in response to challenge by potential pathogens (Ride 1975). This response occurs at the correct time and in the correct place and in significant concentration to account for resistance (Ride and Pearce 1982), (Beardmore *et al* 1983). If defensive lignification does indeed contribute towards non-host resistance, then treatments that inhibit its production might be expected to lower resistance. As lignification is dependant on phenylpropanoid metabolism for the supply of hydroxycinnamoyl alcohols, it might be expected that inhibitors of phenylpropanoid metabolism reduce lignification and lower resistance. It is important to assess any treatment that can be used to reduce lignification *in vivo* for its potential toxicity to the plant and to fungal non-pathogens because of the dual role of lignification in defence and mechanical support.

6.1 Assessment of potential compounds as suitable inhibitors of the phenylpropanoid pathway.

The main aim of this part of the study is to quantify the efficacy and toxicity of the inhibitors of the phenylpropanoid pathway. These inhibitors can then be used as tools to further investigate the importance of lignification in wheat in determining resistance.

This study investigates the efficacy, toxicity and defence response reducing capabilities of the current biochemical inhibitors of the phenylpropanoid pathway. This provides a useful basis for comparison of the known literature and allows the comparison of newer inhibitors in a well characterised *in vitro* system, allowing some assessment of the relative importance of the targeted enzyme to the pathway. Furthermore, the quantitative nature of the lignin assay allows comparisons between treatments that can effect lignification to further investigate the nature of the response.

* This section deals with experiments detailed in chapters 1,2,3 and 4 only, chapters 5 and 7 are discussed in their own sections.

Inhibition of enzymes of the phenylpropanoid pathway can lead to a break in resistance to a non-pathogenic fungus at wound margins. This is further evidence for the role of lignification as a defence response in wheat. Furthermore many of the inhibitors such as AOPP have been shown to be toxic toward plants and fungi. This questions their use in both *in vitro* Appert *et al* (2003) and *in vivo* Carver *et al* (1992), Arakawa *et al* (1997) as tools for studying the importance of defence related lignification in higher plant systems.

The main drawback in the use of inhibitors is that they are artificial compounds and as such may have inherent non-specific effects on other plant metabolic pathways. Thus it is highly possible that indirect effects of the inhibitors not accounted for by this study and that are as yet unknown could be the responsible factors for observed breaks in resistance. This has been highlighted in *Arabidopsis thaliana* when using AIP to inhibit PAL where the PAL promoter was downregulated (Mauch-Mani and Sluslarenko 1996). The pleiotropic effects of the PAL inhibitors are also further investigated by Appert *et al* (2003).

A major difficulty of the current work has been assessing the break of resistance due to reduction in lignification for a non-pathogen. Although in some cases (ML19 and AOPP) it is easy to tell via microscopy at the wound margins that resistance has been broken, in others it is less conclusive. Indicators such as penetration efficiency (Morshbacher *et al* 1999) have been used but are not appropriate for assessment with non-pathogens. Perhaps a better comparison could have been made using a wheat pathogen in this system as a positive control. True resistance between plants and pathogens is a multi-variable trait where responses that occur in the correct time and place are thought to be directly responsible for the outcome of the interaction.

Many enzymes of the phenylpropanoid pathway such as CCR or C3H do not have direct inhibitors, further interest and work on the nature of the function and structure of lots of the enzymes needs to be done to achieve this. However in the current post-genomics era the most likely target to manipulate the phenylpropanoid pathway is through transgenic techniques. It may be that combinations of mutation, transgensis and biochemical inhibition may be the most powerful tools to

manipulate lignification for its industrial goals. This is perhaps stlll far in the future.

6.2 PAL as a target for manipulation of the phenylpropanoid pathway.

Phenylalanine ammonia lyase is the first enzyme of the phenylpropanoid pathway. Its role is to convert the amino acid phenylalanine to *trans*-cinnamic acid. This response is a well-characterised part of the defence response in many plant species. The study of PAL in wheat is particularly important as it is used worldwide as a staple crop plant. In wheat, four PAL genes, (Liao *et al.* 1996, Snowden and Gardner 1993, Hall 1998) at least one of which there is good evidence for induction during resistance (gPAL) have been characterised. The inhibitors tested in the first part of the study AOA, AOPP and AIP are the most consistently used to study the phenylpropanoid pathway by affecting PAL (Morsbacher *et al.* 1999, Carver *et al.* 1996).

The aim of this part of the study is to assess what available biochemical inhibitors have on PAL activity and gPAL expression. The PAL inhibitors need to be assessed for their specificity and should just affect PAL activity and not gPAL expression. Other inhibitors targeted to other enzymes in the pathway (e.g. MDCA to 4CL) should also not affect expression. If non-PAL inhibitors affect activity of PAL it might be taken as the same affect as the target enzyme being inactivated during the course of the defence response. This gives some idea of the importance of these enzymes during the response.

The effect of the expression of the defence related gPAL clone is unlikely to account for all of the PAL activity shown, it is likely therefore that in wheat other PALs must be involved in plant defence. Both PAL inhibitors AOA and AIP reduced PAL activity and defence related lignin. However, AOA reduced gPAL expression casting more doubt on its use as a PAL inhibitor. In contrast, the highly effective at reducing liginification phenylpropanoid pathway inhibitor AIP did not affect gPAL expression. The inhibitor of C4H (ABT) increased gPAL expression as did that of 4CL (MDCA) and one of the CAD (OH-PAS) inhibitors. Interestingly,

two of the CAD inhibitors OH-PAS and NH_2PAS both reduced PAL activity, casting some doubt on their specificity.

One problem in studying PAL and lignin using the wheat system is the hexaploid nature of the wheat genome. The gPAL clone was obtained from a lengthy cloning process. To obtain further clones, even some for comparison was outside the remit of this study. The best comparison would be between some of the existing wheat PAL clones.

The major problem with the biochemical inhibition approach is the lack of specificity of these inhibitors, this is criticised in the literature (Blount *et al.* 2000), however by studying the effects more closely in the first section inhibitors such as AIP can be seen to be good candidates for PAL inhibition. However, it is very hard to quantify exactly what effects each enzymes inhibition is having on PAL activity or gPAL expression. The effect of increased gPAL expression with hydroxylase, 4CL and some CAD inhibitors may be indicative of a feedback loop requiring levels of some of the compounds produced by these enzymes.

Another mechanism that may be affected by the presence of biochemical inhibitors may be the phosphorylation state of the PAL enzyme. However the precise nature of the role of phosphorylation of PAL in wheat has yet to be explored. Some evidence exists of regulation of PAL involving phosphorylation in bean (Allwood *et al.* 2002). To fully evaluate the effect of inhibition on the PAL enzyme the protein-protein interactions concerning the level of phosphorylation of the PAL enzyme required for activation to be fully explored in the presence of these inhibitors.

In the future perhaps proteomics or genomics in the form of microarray analysis from elicited wheat plants could shed light on PAL isoforms and relative abundance and potential roles.

6.3 The role of regulation of the phenylpropanoid pathway in the defence response.

Metabolic pathways are regulated by a variety of mechanisms. The expression of genes encoding enzymes that catalyse the pathway influnces the amount of transcripts available to produce the enzymes from the messenger RNA thus the amount of active sites available for catalysis. The catalytic sites are often hidden within the protein based enzyme complex and often phosphorylation or de-phosphorylation of the protein reveals the active site for catalysis. Another control comes from the end product of the catalysis, where the build-up of end product inhibits the further catalysis of substrate. This is normally referred to as end product inhibition and can occur both up and downstream of a specific enzyme reaction. It is also possible that the end product of catalysis influences the expression of genes directly both up and downstream of the specific enzyme, thus providing interlinks between enzymes and genes. This is known as cross-talk.

In the phenylpropanoid pathway the role played by the metabolites of the pathway is not yet fully understood. The aim of the study was to find out if any of the metabolites present in the phenylpropanoid pathway influence the defence related PAL genes or PAL activity.

Activity of wheat PAL stimulated by chitin was inhibited by the presence of phenylalanine, *trans*-cinnamic acid and 5-hydroxy ferulic acid. The activity was increased by the presence of caffeic acid, sinapic acid *para* courmaryl aldehyde and *para* coumaryl alcohol. These elements therefore may also play some role in the defence response.

Expression of gPAL was negligibly decreased by phenylalanine, 5-hydroxy ferulic acid, sinapic acid and *para*-coumaryl aldehyde. Other compounds such as *para*-coumaryl alcohol, coniferal aldehyde, coniferal alcohol and sinapoyl alcohol all heavily decreased gPAL expression. Whereas; gPAL expression was increased by *trans* cinnamic acid, *para* coumaric acid, caffeic acid, and sinapaldehyde. These findings point toward some endogenous regulation of the phenylpropanoid pathway from its metabolites. In contrast to this in pea and in sweet potato PAL is inhibited by cinnamic acid and caffeic acid (Sato *et al.* 1982). However, in wheat cultivars

resistant to *Fusarium culmorum para*-coumaric acid increased in response to challenge by this pathogen (Siranidou *et al.* 2002). In conditions of over-expression of PAL2 in *Nicotiana tabaccum para*-coumaric acid increases indicating that the weakest link in the chain is the hydroxylase step to produce caffeic acid. This correlates well with the observation in this study that ABT (a dedicated hydroxylase inhibitor) breaks resistance to the non-pathogenic fungus *B. cinerea* in wheat.

This may mean a three component regulatory system exists around PAL in the defence response to produce lignin in wheat. The first three intermediates of the pathway may act as de-repressors of gPAL expression, with caffeic acid acting as the first activator of defence related PAL activity. This may be part of an "overdrive" mechanism that helps the plant overcome the first stages of fungal infection. The activity of wheat PAL may be governed by the accumulation of ferulic acid as this is the first substrate that in the phenylpropanoid pathway that decreases PAL activity. The important aspect may be the presence of a methyl group at position three or five on the carbon ring as addition of coniferaldehyde and coniferal alcohol also significantly reduces the activity of PAL. The final part of the regulation comes from the presence of the alcohol group on the phenylpropanoid. All the alcohols tested in this system show a decrease in gPAL expression. This is logical as a shut off to the system (Figure 4.10)

The major problems associated with these findings is whether the physiological concentrations of these metabolites are sufficient enough to play a regulatory role. As aforementioned not all phenylpropanoid pathway and particularly PAL genes have been characterised and the full regulation of the pathway in any plant species is not known. However most of the findings in this study correlate well with the model offered by Humpfreys and Chapple (2002). Some evidence has already been presented of cinnamic acid acting as an endogenous regulator of PAL and operating as a feedback loop in Alfalfa Blount *et al.* (2000). Although *trans* cinnamic acid reduces PAL activity and it increases gPAL expression indicating that the feedback loop extends to the genome and involves defence related PAL.

6.4 Conclusion

This study has revealed that biochemical inhibition of the phenylpropanoid pathway is a valid technique to further understand the nature of the pathway. However, careful consideration must be given to choices of compounds and their effects on plant or fungal toxicity particularly in the case of the study of defence related lignin. The most specific inhibitor is one that affects PAL (AIP) and completely reduces PAL activity. Any new inhibitor compounds need to be assessed for this toxicity as well as their effects on enzyme activity.

With the current knowledge and availability of the substrates of the phenylpropanoid pathway a great deal of information has been gained about the nature of defence related lignification passing through the PAL enzyme. Several inhibitor compounds affect not only PAL activity but also PAL expression. This gives some evidence for endogenous regulation by these enzymes. Further insight might be gained if all the defence and developmental related forms of the enzymes of the phenylpropanoid pathway need to be assessed in stimulated and unstimulated plants. The advent of microarray technology and proteomics may well assist in this aim. The substrates and metabolites of the phenylpropanoid pathway also affect PAL activity and expression. Clearly, the levels of the substrates *in vivo* need to be measured to allow accurate modelling.

Manipulation of the phenylpropanoid pathway has a wider application. Down-regulation of lignin pathway enzymes is a goal in the paper industry. Loss of lignin units may lead to a reduction in chlorinated waste production in the paper making process, which is of environmental benefit. Equally, reducing lignin increases digestibility of forage crops for animal feed which could lead to higher production.

However, a great deal more work is required on the phenylpropanoid pathway before major manipulation changes can be performed to reduce lignin for agro-industrial purposes. Evidence is growing of the nature of the pathway. The goal of understanding how to produce a lignin-reduced plant that is not compromised from one of its important defence mechanisms is a long way off. As more intensive molecular biology techniques improve and the genome of wheat and other

important crop plants is sequenced, perhaps more interest in this topic will be created in the post-genomic era.

Chapter 7

Purification of a fungal elicitor specific for the Cf-2 protein in *Lycospersicon esculentum*

7.1 Pathogenesis and life cycle of *Cladosporium fulvum.*

Cladosporium fulvum is a biotrophic pathogen of the tomato *Lycopersicon spp* and causes leaf mould on susceptible plants. It was first described by Cooke in 1883 and belongs to the Fungi Imperfecti, also known as the Deuteromyctes. The pathogen is relatively economically unimportant as a disease of tomato, only slightly affecting greenhouse grown crops (Joosten and De Witt 1999). *Cladosporium fulvum* colonises only the intracellular spaces of tomato and enters the plant through stomata and is most successful in high humidity environments such as greenhouses. This is due to the increased numbers of open stomata. Infection occurs after spore germination on the leaf surface and germ tubes penetrate through the stomata and grow between the mesophyll and the vascular bundle cells. The germ tubes of *C. fulvum* do not form haustoria but remain in the apoplast until asexual conidophores emerge onto the leaf surface. Nutrient transfer is thought to come about from secretion of proteins in to the apoplastic fluid that induces host cells to leak nutrients into the extracellular fluid, thus establishing a sink relationship. The lack of a sexual stage in the fungi gives rise to a well-conserved genome that is ideal for academic study. Unlike most biotrophic pathogens *Cladosporium fulvum* is easily cultured on ¼ strength potato dextrose agar *in vitro* (Joosten and De Witt 1999).

In a resistant interaction the outcome of an attempted infection is a hypersensitive response (HR). The HR is a localised self-induced cell death that initially prevents a pathogen from gaining entry into the host. This necrosis produces a lesion that is easily identifiable microscopically and is thought to be a key stage in triggering other host cell defence responses. The host plant responds after the elicitation of the hypersensitive response and produces defence related chemicals such as phytoalexins, lignin and hydrogen peroxide (May *et al* 1996). These biochemical changes therefore can be used as a marker for the presence of the HR response.

7.2 Specificity of resistance in the *C. fulvum* / tomato interaction.

Resistance in plant pathogen interactions is thought of in two ways horizontal and vertical. Horizontal or non specific resistance refers to the general ability of plants to resist their pathogens and is controlled in general by multigenic traits. Vertical or specific resistance refers to an interaction involving one or two genes controlling a major step in the host pathogen interaction. A specific form of vertical resistance termed race-cultivar specificity is observed in *C. fulvum* / tomato interactions. *C. fulvum* is classified into race types due to the possession of "avirulence" genes (*Avr*) in the pathogens genome. The nature of avirulence genes is surprising in that they confer the inability to infect to the pathogen. A race of *C. fulvum* that carries all avirulence genes with respect to *L. esculentum spp* is referred to as race 0. If a specific gene is lacked this is reflected in the *C. fulvum* race type. A race 9 *C. fulvum* carries all avirulence genes bar *Avr9* (Lucas 1998)(Table 7.1).

The host tomato plants exhibit varying degrees of ability to defend themselves against *C. fulvum*. Similarly tomato cultivars are classified according to the resistance genes present in their genome. A cultivar of *L. esculentum* with no resistance genes with respect to *C. fulvum* is referred to as a Cf0 plant (moneymaker). If a resistance gene is possessed then this is reflected in the cultivar name. Cf5 tomato plants contain the resistance gene *Cf-5* (Table 7.1).

Table 7.1 Race-cultivar specifity in the *Cladosporium fulvum* / tomato interaction adapted from Lucas (1998): Cf, host resistance gene; A, pathogen avirulence gene (dominant allele); a, pathogen avirulence gene (recessive allele); +, susceptible interaction; -, resistant interaction.

Tomato cultivar (genotype)	*C.fulvum* race (genotype)				
	0 (A2, A5, A9)	2 (a2, A5, A9)	2,5 (a2, a5, A9)	5 (A2, a5, A9)	9 (A2, A5, a9)
Cf0	+	+	+	+	+
Cf2	-	+	+	-	-
Cf5	-	-	+	+	-
Cf9	-	-	-	-	+

7.3 The gene for gene hypothesis

Work on flax and flax rust proposed the gene for gene concept to account for the race-cultivar specificity observed in race-cultivar resistance Flor (1971). This broadly states that for every gene that confers resistance in the host there is a corresponding gene in the pathogen that confers avirulence to the pathogen. This denotes a stepwise evolution of resistance and virulence.

In the gene for gene concept genes for resistance are dominant in a host plant while genes for susceptibility are recessive. In the pathogen the genes for avirulence are dominant and the genes for virulence are recessive. The Avirulence genes (A,a) control the production of a microbial elicitor that can be recognised by a host receptor protein, encoded for by a dominant or recessive resistance gene (R,r). If the gene for this receptor protein is recessive (r) the receptor will not be functional when produced and thus no pathogen recognition occurs. Similarly no pathogen recognition can occur if the pathogen avirulence gene is recessive (a) and the elicitor is not produced. Only when both the dominant characteristics of receptor and elicitor production are expressed then the gene for gene interaction occurs. The plants defence mechanisms are then initiated resulting in an HR that renders the plant resistant to the attacking pathogen (Figure 7.1).

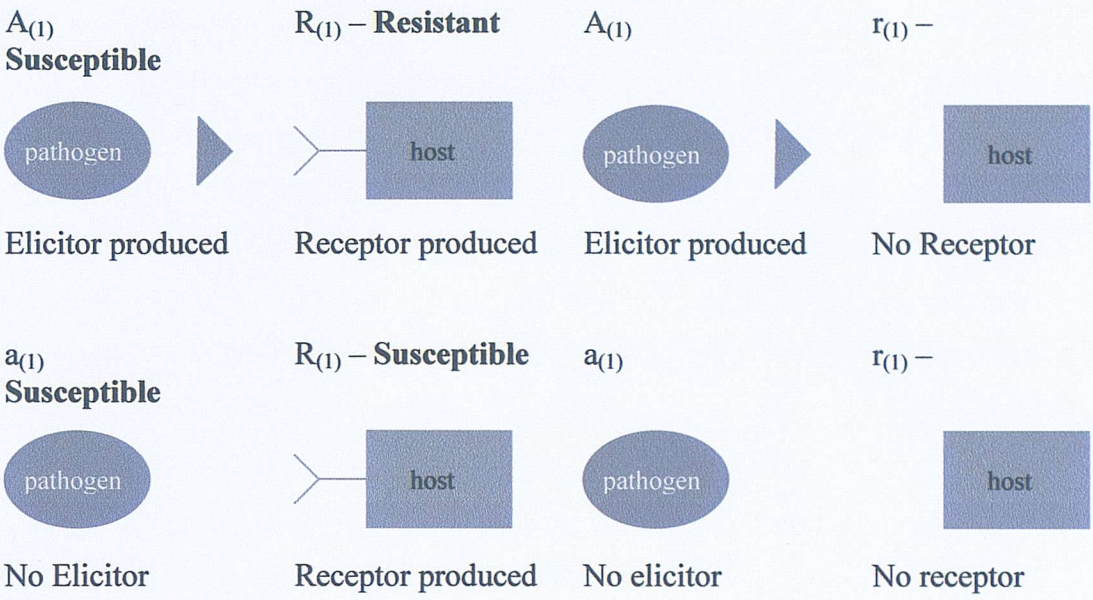


Figure 7.1 The gene for gene hypothesis. (Adapted from Lucas 1998), dominant allele avirulence gene; a, recessive allele avirulence gene; R, dominant allele resistance gene; r, recessive allele resistance gene; $_{(1)}$ designates the gene for gene pair.

7.4 Virulence / Avirulence (*Avr*) genes in *C. fulvum*.

The Avirulence genes that have been fully characterised in *C. fulvum* (*Avr4* and *Avr9*) both encode low molecular weight proteins (AVR4 and AVR9). Both the *Avr4* and *Avr9* genes encode for a pre-pro-protein that includes a signal sequence for extracellular targeting. *Avr4* lacks introns whereas the *Avr9* gene contains one intron of 59 base pairs in the open reading frame. *Avr9* is transcribed only *in planta* (Joosten and De Wit 1999). The expression of *Avr9* is regulated by the nitrogen response factor *Nrf1*. Under conditions of nitrogen starvation without the presence of *Nrf1 Avr9* was no longer induced (Perez Garcia *et al.* 2001). *Avr4* and *Avr9* are true avirulence genes. Transformation of the *Avr4* or *Avr9* genes into *C. fulvum* strains that do not produce the corresponding AVR proteins made the strains avirulent on tomato cultivars containing the corresponding *Cf* resistance genes (Joosten and De Wit 1999).

7.5 Products of Avirulence genes.

Virulence/Avirulence factors are those that determine the outcome of a race-cultivar interaction, specifically these are the products of the avirulence genes. Both the *Avr9* and *Avr4* genes in *C. fulvum* encode a precursor protein of 63 amino acids and 135 amino acids respectively. Both appear to be modified by plant and fungal proteolytic modification. Both proteins contain cysteine residues that are involved in di-sulphide bridges. The mature AVR9 protein is 28 amino acids and the mature AVR4 is 86 amino acids. (Luderer *et al.* 2002). The structure of AVR9 has been determined by ^{1}H NMR analysis. The AVR9 peptide contains three anti parallel strands that form a compact region of β-sheet and two solvent exposed loops, a short one between Phe26 and Leu20 and an extended one from Thr12 to Gln20. There is great structural similarity between AVR9 and serine protease inhibitors, ion channel blockers and growth factors. The greatest similarity is to be found between AVR9 and carboxy peptidase inhibitor. The hydrophobic residues of AVR9 present in the solvent exposed loops are essential for the necrosis inducing activity of the AVR9 peptide. Within this it is thought that Phe21 is one of the essential residues. No complete structural data exists for AVR4 except for the fact that the AVR4 protein has a hydrophobic centre that may be crucial for antigenicity (Joosten and De Wit 1999).

So far no evidence has been shown for direct binding of these proteins to their complementary *Cf* gene product. Recent work shows that there is no evidence of a direct interaction between AVR9 and Cf-9 (Luderer *et al.* 2001).

7.6 Resistance (Cf) genes in *L. esculentum.*

Many Cf resistance genes from tomato have been cloned and localised to a complex resistance locus by use of classical and Restriction Fragment Length Polymorphism (RFLP) analysis. The *Cf-2* and *Cf-5* genes are found on chromosome 6 of *L. esculentum.* Cf-2 comes from two closely linked near identical genes *Cf-2.1* and *Cf-2.2.* Little is more known about the *Cf-5* gene however experiments are continuing Dixon (personal communication).

The genes *Cf-4* and *Cf-9* are found on chromosomes 1 and 10 respectively (Jones *et al.* 1993). It has either been demonstrated or hypothesised that the tomato *Cf-2, Cf-4, Cf-5* and *Cf-9* genes confer resistance to *C. fulvum* by recognition of the complementary *Avr* genes *2, 4, 5* and *9.*

7.7 Cf proteins of *L. esculentum*

Three Cf proteins have been characterised. Cf-9 is a 863 amino acid membrane anchored, extracytoplasmic glycoprotein containing 27 imperfect leucine rich repeats (LRRs) of 24 amino acids in length. The repeats have a consensus sequence of LxxLxxLxxLxLxxNxLxGxIPxx. The LRRs are interrupted by a short region termed a "loop out" domain which divides the LRRs into 23 amino terminal and 4 carboxyl terminal LRRs. As the carboxyl terminal ends with the motif KKxx it is thought the protein is localised to the endoplasmic recticulum Jones and Jones (1997).

Cf-4 has also been localised to the same complex locus as *Cf-9* on the short arm of chromosome 1. As the Cf-4 carboxyl terminal is almost identical to that of Cf-9 it is thought that the specificity between the two proteins is derived from the amino terminus.

Cf-2 comes from two closely linked near identical genes *Cf-2.1* and *Cf-2.2*. The products of these two genes differ only by three amino acids. The *Cf-2* gene encodes for a protein of 1112 amino acids. The localisation of Cf-2 is currently under investigation and appears to be in the microsomal fraction of the cell (Poole 2003). Cf-2 is composed of 34 LRRs (2-31) and (35-38) which are of 24 amino acids. There are 20 LRRs (6-24) which show an alternating structure of two highly conserved repeats designated type A and B and are reminiscent of the alternating LRRs of porcine ribonuclease inhibitor. The consensus sequence for Type A is EEIGYLRSL(T/N)xL(D/S/G)LSENALNGSIP and Type B has a consensus sequence ASLGNLNNLS(M/H/R)L(Y/F/N)LYNNQLSGSIP. These are arranged $(AB)_3AB_4(AB)_4$ followed by the first 5 residues of a B repeat. The carboxy terminal 360 amino acids of Cf-2 show high homology to the 352 carboxy terminal amino acids of Cf-9, which includes the region that is identical between, *Cf-9* and *Cf-4*. This region of conservation may play a similar and important role in all 3 proteins. It is thought that the loop out domains and the amount of conservation between Cf9 and Cf2 might mean that they interact with similar components Jones and Jones (1997).

7.8 Pathogenicity factors of *C. fulvum.*

Pathogenicity factors are molecules possessed by a pathogen to aid its interaction with the host. *Cladosporium fulvum* possesses several proteins that aid its interaction with tomato. The hydrophobin protein HCf-1 of *C. fulvum* is required for efficient water-mediated dispersal of conidia. Six hydrophobin genes (HCf-1 to -6) have thus far been identified in the tomato pathogen *C. fulvum*. Recently, analysis of the mutant strains that lack HCf-1 revealed that HCf-1 confers hydrophilic character to the conidia and this facilitated the dissemination of conidia on the surface of water droplets. Other hydrophobins, may be involved in the development and germination of conidia (Whiteford and Spanu 2001). The direct interaction that these proteins affect is the initial colonisation of the intracellular space by the water borne conidia of *C. fulvum*.

During the colonisation of a susceptible tomato plant by *C. fulvum* two extracellular proteins called ECP1 and ECP2 are secreted. Of these ECP2 is very important as a

pathogenicity factor, as a deficient strain poorly colonised the leaf tissue and secreted lower amounts of other pathogenesis related proteins (Lauge *et al.* 1997). In contrast, deletion of ECP1 did not affect the colonisation ability of the fungus but reduced the production of *in-planta* produced proteins. Other effects included the quicker accumulation of pathogen related proteins in the plant and quicker induction of leaf desiccation and abscission of leaves. These results suggest that these two proteins play a role in the suppression of the host response. An effect on cytokines, a proteinaeous mediator in the animal immune system has been proposed. A pathogenicity gene has been discovered that complements this system, designated ECP2 (Lauge *et al.* 1998).

Other factors that are present in the interaction in the apoplastic fluid that have an effect on the disease process are Nitrogen, the presence of which has been shown to induce the expression of the *Avr9* gene (Van Den Akerveken *et al.* 1994). A wall bound invertase in *C. fulvum* likely of use in degrading photoassimilates in the interaction has also been discovered Noeldner *et al.* (1994).

7.9 Resistance factors of *L. esculentum*

Resistance factors are required as part of the recognition process of the gene for gene signal. However, although not being the primary part of the complementary gene for gene system, resistance factors may act as mediators of the response that comes about because of the gene for gene interaction. A high affinity binding site (HABS) has been found in the plasma membrane of *L. esculentum* that is required for AVR9 function in lines containing Cf-9. HABS is thought to be a third component that is required for perception of AVR9 by Cf-9, but has yet to be isolated (Luderer *et al.* 2002).

The gene Required for *Cladosporium* Resistance 3 (*Rcr3*) has been shown to be specifically required for *Cf-2* mediated resistance to *C. fulvum*. Two other genes *Cf-5* and *Cf-9* were unaffected by the deletion of the *Rcr3* gene (Dixon *et al* 2000). Recently, the *Rcr3* gene has been cloned and the proteinaceous product purified via an affinity column to a molecular weight of around 38 kd. *Rcr3* is a secreted

cysteine protease that functions upstream of *Cf-2*. The actual role of *Rcr3* in the defence response has yet to be ascertained (Kruger *et al.* 2002).

7.10 Aim

At the time of the study Avr 2 had not been characterised. The aim of this study was to obtain amino acid sequence of Avr 2 to allow subsequent cloning of the gene.

7.11 Approach

In order to purify AVR2 a bioassay for its detection is required. It has been shown that Avr9 induces a burst of hydrogen peroxide (H_2O_2) at a very early stage of the recognition process (Hammond-Kosack *et al.* (1996), May *et al.* 1996). A reasonable assumption is that the AVR2-Cf2 interaction would produce an oxidative burst as; this is a fairly ubiquitous phenomenon in elicitor-receptor interactions (Baker and Orlandi 1995). It is proposed to detect AVR2 by monitoring H_2O_2 production in tomato leaf discs using the chromophore ABTS. Peroxidase in the leaf discs in the presence of H_2O_2 converts colourless ABTS to a coloured compound (radical cation) that can be measured spectrophotometrically.

Using this method of detection for AVR2 standard protein purification techniques will be employed in order to purify AVR2 to homogeneity and allow subsequent amino acid sequencing.

7.12 Plant maintenance

Lycospersicon esculentum cv. Cf2 and Cf0 were a gift of Dr Mark Dixon and were sown on F2 soil in 3 cm pots under greenhouse conditions (16 h photoperiod 25°C). After 10 days post germination, plantlets were transferred to individual pots in M2 soil until they reached a height of 10 cm and were suitable for inoculation. For seed collection plants were maintained in 9 cm pots on M2 soil with a bottom layer of "Grits".

Nicotiana tabaccum c.v. *Petit gerard* and c.v. 2.1D were a gift of Dr Mark Dixon. *Petit Gerard* is a cultivar *Nicotiana tabaccum* (J.D.G Jones unpublished) and carries no resistance genes for *C.fulvum*. 2.1D is a transformant of *Petit gerard* transformed with *Cf-2.1* from *Lycospersicon esculentum* (Dixon unpublished). Seeds of *Nicotana tabacum* c.v. 2.1D and c.v. *Petit gerard*, were sown on Levington F2 compost, by spreading on damp soil and watering in. Plantlets were transferred to 3 cm pots and then further to 9 cm pots containing F2 soil. Sowing times were altered to ensure comparable stages of development of leaves between the two species.

7.13 Fungal culture maintenance

Cladosporium fulvum Race 5 was a gift of Dr P.Seear and Dr M.S.Dixon and was maintained on ¼ strength potato dextrose agar (1 % Potato Dextrose Media and 0.625 % Agar No 2) and sub cultured every 2 weeks.

7.14 Infection of plants with Race 5 *Cladosporium fulvum*

Tomato plants (10 cm high) were placed in an inoculation tent and 100 µl L^{-1} paclobutrazol was added (50 ml per plant) as a soil drench, the apical meristem was also removed. To make the spore suspension a single two-week-old plate of *C. fulvum* was used to 750 ml distilled water, for 15 plants. Plants were inverted and immersed into the spore solution for 10 s ensuring that all leaves were well covered with the spore suspension. The remainder of the solution was drenched over the plants. Infection in general took place when overcast weather conditions prevailed; in order to prevent direct sunlight the plants were shaded.

When plants had visible coverings with powdery spores (generally two weeks after inoculation) the leaves were removed and vacuum infiltrated with distilled water. Saturated leaves were carefully blotted to remove excess surface water. The leaves were rolled into cylinder shape and inserted into the barrel of a 20 ml syringe. The syringe was placed into a 50 ml centrifuge tube and centrifuged at 400 g for 10 min. The apoplastic fluid was collected and stored on ice. The tubes were centrifuged two further times to extract the maximum amount of apoplastic fluid. To ensure protein to ensure protein homogeneity throughout the batch all samples were pooled and then stored at –20 °C in 1 ml aliquots.

7.15 Detection by visual assay of HR due to Avr2 in intact leaves

Tomato plants either containing no resistance genes for the fungus *Cladosporium fulvum* (Cf 0*)* or the resistance gene *Cf-2* were grown for three weeks to reach approximately 15 cm in height. Leaves were injected with 0.1 ml candidate fraction on the underside of a leaf at an interstice between the major and minor veins; until a visibly darkened panel appeared. Primary leaves were not used for this assay.

Plants were grown for 14 days after injection and assessed for signs of chlorosis by visual inspection throughout this period. The visual assay of chlorosis was initially taken on a semi-quantitative three-point scale defined as: (1) slight activity flecking or darkening of the leaf area, (2) beginnings of yellowing or chlorosis, (3) chlorosis. All injections into leaves were at the concentration achieved directly from apoplastic fluid harvest. Digital photographs were taken to record the symptoms.

In later experiments (purification method three), four independent assessors scored leaves on a five-point scale (5 chlorotic to 1 no symptoms). After averaging the data and subtracting values for the Cf 0 (no *Cf-2* gene) control plants, the level of Avr2 in these test samples was expressed as a semi quantitative value ranging from zero to five.

7.16 Biochemical detection of Avr 2 in leaf discs

Prior to the experiment leaf discs (7 mm diameter) were incubated in 70 % (v/v) leaf disc buffer (LDB) (70 % (v/v) 0.2 M acetate buffer pH 5.75, 0.0005 % (v/v) Silwet, 2 % (v/v) 1.0 M $MnCl_2$) and incubated at 25 °C for 18 h in the dark with gentle shaking. Three leaf discs were then transferred into a 24-well assay plate. For each sample tested, six treatments as were set up in triplicate (table 7.2).

The assay plate was incubated in the dark for 2 h with agitation (50 r.p.m) and the level of H_2O_2 in the bathing solution was measured indirectly by measurement of peroxidase activity. Three 100 µl fractions were taken from each well and were transferred to a 96-multiwell plate and incubated with 100 µL of ABTS (2.2-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)) 1.1mg ml^{-1} in 0.3 mM acetic acid, pH 4.4. The plate was read at 410 nm at t = 0 min and t = 20 min and the level of induced H_2O_2 in the last samples was calculated (table 7.3).

From the resulting calibration curve the following equation was derived for transformation of absorbance values obtained to f mol hydrogen peroxide.

$$\frac{\text{Abs } 400\text{nm} - 0.0173}{0.0001} = \text{f mol hydrogen peroxide}$$

Table 7.2 Experimental set up for leaf disc assay; Lane, Assay plate lane; Leaf Disc, plant type; LDB, Leaf disc buffer; Test, candidate fraction, e.g Apoplastic fluid from Cf0 plants infected with Race 5 *C.fulvum*; Control, distilled water.

	Experimental conditions		
Lane	Leaf Disc	LDB	Test (T) or Control (C)
1	Petit Gerard	420µl	T
2	Petit Gerard	420µl	C
3	2,1 D	420µl	T
4	2,1 D	420µl	C
5	No leaf discs	420µl	T
6	No leaf discs	420µl	C

Table 7.3 Calculation of hydrogen peroxide burst by the *Cf*-2 / Avr2 interaction: Plant, *N. tabaccum* c.v. Petit Gerard (PG), (2.1D), or no plant (NP); Treatment; T, Test, (apoplastic fluid); C, Control, (distilled water); Rep: Replicate number; 20, 0, Time of incubation (min); *, average of 3 experiments; 20-0, Subtracting background hydrogen peroxide; Test-Control, Subtraction of elicited cells from non-elicited cells; TC–NP, Subtraction of no disc control. 2.1D – PG: Subtraction of values for plants containing no Cf genes from one containing the *Cf-2* gene.

Hydrogen peroxide (f mol)										
Plant	**Treatment**	**Rep**	**20***	**0***	**20-0**	**T-C**	**TC-NP**	**2.1D – PG**	**Average**	**S.E.**
***N. tabaccum* c.v. Petit Gerard (PG)**	T	1	0.437	0.248	0.189	0.174	0.163	-0.005	0.019	0.01
		2	0.438	0.252	0.185	0.157	0.147	0.020		
		3	0.415	0.223	0.192	0.167	0.161	0.026		
	C	1	0.092	0.076	0.016					
		2	0.130	0.102	0.028					
		3	0.113	0.088	0.025					
***N. tabaccum* c.v. Petit Gerard (2.1 D)**	T	1	0.374	0.186	0.189	0.168	0.158			
		2	0.451	0.262	0.189	0.177	0.167			
		3	0.430	0.222	0.208	0.193	0.188			
	C	1	0.093	0.072	0.021					
		2	0.083	0.071	0.012					
		3	0.085	0.070	0.015					
No plant (NP)	T	1	0.150	0.130	0.020	0.010				
		2	0.174	0.154	0.020	0.010				
		3	0.144	0.128	0.016	0.005				
	C	1	0.076	0.067	0.009					
		2	0.077	0.067	0.010					
		3	0.084	0.073	0.011					

Methods - Protein purification

7.17 Protein estimation by Bicinchoninic acid method

Protein estimation was performed from Smith *et al* (1985), with modifications. Reagent A was comprised as follows: 0.4 % (w/v) NaOH, 1.71 % (w/v) Na_2CO_3, 1 % BCA (Na_2), 0.16 % (w/v) Sodium tartrate 0.95 % (w/v) $NaHCO_3$, with distilled water to volume. Reagent B comprised 4 % (w/v) $CuSO_4$. Prior to the assay the standard working reagent (SWR) was prepared by mixing 50 volumes reagent A with one volume reagent B. Test samples (10 µL) were incubated with 200 µL SWR in multi-well plates and left overnight at 20 °C. The absorbance at 570 nm was then read on a multi-well test plate reader (Dynatech Mr 5000). The standard used was 100 mg L^{-1} Bovine Serum Albumin.

7.19 Gel filtration

A 90 x 2 cm column was prepared by adding 28 g of Bio Rad P6 gel to 250 ml of water and poured according to the manufacturer's instructions. The resulting column separated between 6000-1000 Daltons. Blue dextran and copper chloride were used to find the void and bed volumes of the column. The column was run at 20 ml per hour with fractions of 5 ml collected every 15 min with an automatic collector (Foxy instruments). Protein estimation was taken via the BCA method on 20 µL of each tube. Fractions were pooled and frozen overnight at –80 °C. Fractions were dried on a freeze drier, and re-suspended in 1 ml of distilled water and used in the visual or biochemical assay, at the appropriate dilution.

7.20 Rotofor protein purification – Preparative Isoelectric focusing.

Preparative isoelectric focusing took place using a Rotofor cell (Bio-Rad). The cell was pre-focused two times with distilled water at four watts power prior to injection of apoplastic fluid. Apoplastic fluid (30 ml) was mixed with 15 ml buffer A (α-Amino-Caproic Acid) and 15 ml buffer B (Bis-Tris) to produce a pH gradient of 8.8-9.3. The cell was run at 12 W constant power for 4 h to produce an even separation. The anode and cathode buffers were per the manufacturers instructions. A Bio Rad peristaltic pump provided cooling. After focusing a vacuum pump was used to draw the sample out of the cell and into test tubes where the pH of the fractions was measured on a Corning 245 pH meter.

Methods - Gel electrophoresis.

7.21 Isoelectric Gel focusing

Ampholine PAGE gel plates were used at pH range 3-9.5. Standards of a range of isoelectric points were obtained from Sigma-Aldrich. The standards used were: Amyloglucosidase, pI 3.3; Glucose oxidase, pI 4.2; Trypsin inhibitor, pI 4.6; Beta lactoglobulin, pI 5.1; Carbonic anhydrase II, pI 5.9; Carbonic anhydrase I; pI 6.6 and Trypsinogen; pI 9.3.

Apoplastic fluids harvested from Cf-0 plants infected by Race 5 *C. fulvum* and normal healthy Cf-0 plants were used as a control. Protein solutions were applied in 10 µL volumes onto applicator wicks and laid in lanes. The gel was cooled to 10 °C with an ice cooled water pump (Bio Rad) and run at 1 500 V 25 mA and 15 W for 3 h as per the manufacturers instructions. All fixation, staining, anode and cathode solutions were from the manufactures booklet Pharmacia (1990).

7.22 SDS page electrophoresis

SDS page electrophoresis was carried out using the protean II gel tank system, 30:0.8 acyrlamide: bis acrylamide was used to make gels of 15 % total acrylamide in the gel. The buffers used were reservoir: 0.025 M Tris, 0.192 M glycine pH 8.3. Resolving: 0.375 M Tris-HCl, pH 8.8 and Stacking: 0.125 M Tris-HCl pH 6.8. Cooling was provided as aforementioned.

7.23 Tris-Tricine gel electrophoresis

Tris tricine gel electrophoresis allows resolution of proteins between 1 and 100 kD Schagger and von Jagow (1987). A mix of 29:1 acrylamide: bis-acrylamide was used to produce a 15.8 % acrylamide gel to resolve the proteins. Ultra low molecular weight standards were obtained from Sigma and ranged from 1 kD to 26.6 kD and contained: Triose phosphate isomerase (26.6 kD), horse heart myoglobin (17 kD), bovine β-lactalbumin (14.2 kD), bovine aprotinin (6.5 kD), bovine insulin chain B (3.5 kD), and bradykinin (1.1kD). High molecular weight

standards ranged from 6 kD to 65 kD and consisted of Bovine serum albumin (66 kD), Ovalbumin (45 kD), Glyceraldehyde-3-phosphate dehydrogenase (36 kD), Carbonic anhydrase (29 kD), Typsinogen (24 kD) Trypsin inhibitor (20 kD) and ■-Lactalbumin (14.2 kD). Cathode buffer was made to a final buffer concentration of 0.1 M Tris-Cl, 0.1 M Tricine and 0.1 % SDS. Anode buffer concentration was 0.2 M Tris-Cl pH 8.9. Samples were prepared in Tricine Sample Buffer. (TSB: 1 ml 1 M Tris-Cl pH 6.8 (see SDS page method) 2.4 ml (3 g) glycerol, 0.8 g SDS, 2 mg Coomassie blue G-250 and 0.31g DTT made up to 10 ml. A three mm gel containing 15 wells was prepared, measuring 160 mm by 160 mm with a 30 mm stack and hence a 160 by 120 mm resolving gel.

The freeze-dried protein sample (10 µL) was boiled with the TSB in a total volume of 30 µL for 5 min. Samples were loaded through the cathode solution, into the wells. Electrophoresis (Protean II gel tank system (Bio-Rad)) was at 30 V for 1 h (to stack the gel) and then 150 V for 7 h, or until the dye front had reached the bottom of the resolving gel. The apparatus was cooled to 4 °C with a peristaltic pump.

7.24 Purification of a specific proteins by Isoelectric focusing.

An IEF gel was used in order to gel purify a protein from a fraction. Electrophoresis was carried out as per above. The fraction was added in 10 µL aliquots to the wicks and separated on the gel. Loading positions were omitted to aid in continuous separation. A few lanes of the gel were excised and stained using aforementioned methods, then realigned to show the position of the candidate protein. The candidate band was then excised and prepared for western blotting.

7.25 Mini Gel electrophoresis for Protein transfer

This was carried out using a mini protean II apparatus (Bio Rad). Each gel was 0.75 cm thick and comprised a 5 cm deep resolving gel (12.5 % acrylamide pH 8.8) and a 2 cm deep stacking gel (4.5 % pH 6.8) The resolving gel was prepared by mixing 7 ml water, 8 ml acrylamide monomer (30 % (v/v) pre-mix Acrylogel; BDH, Poole, UK), 5 ml 1.5 M Tris, pH 8.8, 100µL 10 % SDS, 20 µL TEMED and 100 µL 10 % (w/v) ammonium persulphate. The stacking gel was prepared similarly using 5.8 ml water, 1.5 ml monomer, 2.5 ml 0.5 M Tris pH 6.8, 50 µL 10 % (w/v) SDS, 10 µL

TEMED and 50 µL 10 % (w/v) ammonium persulphate. Electrode buffer contained 25 mM Tris, 3.5 mM SDS and 0.2 M glycine. Gels were run at a constant 200 V for 45 min.

7.26 Western blotting

The electrophoretic transfer of the SDS mini gel to a nitrocellulose membrane was carried out in a Bio-Rad Mini-Protean II transblot apparatus, according to the manufacters instructions. Low molecular weight markers as aforementioned (Sigma) were used to assess approximate molecular weight of the transferred product. Gels, membranes and filter papers were all soaked in ice-cold transfer buffer (20 % (v/v) methanol containing 20 mM Tris and 0.15 M glycine) at 4 °C for 30 min prior to blotting. The gel was blotted at 30 V for 2 h and the resulting blot stained with 2 % (w/v) amido black in 90 % (v/v) methanol 10 % glacial acetic acid to visualise the protein. The blot was washed in once in 90 % (v/v) methanol 10 % glacial acetic acid and again in distilled water.

7.27 Reduction of sulphydrylls on western blot

After western blotted protein band excision, 1.0 ml, 10 mM dithiothreitol (DTT) in 0.1 M ammonium bicarbonate was added to the protein blot. The blot was incubated for 30 min at 56 °C to reduce the protein. After reduction the DTT was removed and replaced with 1.0 ml 55 mM iodoacetamide in 0.1 M ammonium Bicarbonate and incubated in the dark for 20 min at room temperature. The iodoacetamide solution was removed and 1 ml 0.1 M ammonium bicarbonate was added to alkylate the blot for 15 min. The blot was then air-dried and sent for sequencing.

Gel staining methods

7.28 IEF gels

After electrophoresis gels were stained with 0.2 % (w/v) coomassie brilliant blue R-250 in 20 % (w/v) methanol, 0.5 % (w/v) acetic acid for 20 min (without fixing), and destained using 30 % (w/v) methanol until the protein bands became visible.

7.29 Silver staining of IEF gels

The gel was fixed in 20 % (w/v) TCA for 30 min. The gel was then sensitised in a solution of 0.125 % (w/v) Glutaraldehyde, 0.2 % (w/v) Sodium Thiosulphate, 6.8 % (w/v) sodium acetate and 30 % (v/v) ethanol for 30 min. The gel was washed was three times in distilled water, for five min per wash. The gel was then exposed to a 0.25 % (w/v) silver nitrate solution with 0.15 % (v/v) formaldehyde. After 20 min the gel was washed two times for 1 min with distilled water. Developing of the gel took place in 2.5 % (w/v) sodium carbonate with 0.0074 % (v/v) formaldehyde until clear bands could be seen. The developing reaction was stopped by addition of 1.46 % (w/v) EDTA-Na_2 and washed three more times for 5 min in distilled water. Gels were preserved in a solution of 8.7 % (w/v) glycerol.

7.30 Staining method for Tris tricine gels

The gel was washed for 5-10 min in distilled water to decrease the background staining and fixed in freshly prepared 5 % (w/v) glutaraldehyde for 1 h. Washing was three times with distilled water for 5 min per wash. Staining was in 0.025 % (w/v) coomassie brilliant blue G in 10 % (v/v) acetic acid for 1 h. De-staining took place in 10 % (v/v) acetic acid overnight with several changes of de-staining solution, until bands were easily visible. The advantage of this method was that it prevented the lower molecular weight peptides from diffusing out of the gel.

7.31 Development of the leaf assay

In order to find the biological activity of an elicitor of the hypersensitive response induced via the Cf-2 protein (Avr2) a specific assay was required that could detect this element in apoplastic fluid. Two methods were attempted. The first method involved a direct injection of apoplastic fluid into tomato plants containing the gene Cf-2. Tomato plants containing the gene Cf-2 exhibit a chlorotic response to injection of neat apoplastic fluid from *C. fulvum* Race 5 infected plants. This response is absent in isogenic plants without the Cf-2 gene (Cf-0) (Figure 7.2). This characteristic chlorosis formed the basis for the leaf assay. The assay involves direct injection of separated fractions of apoplastic fluids into leaves of the two tomato varieties and scoring on a three-point scale (1) slight activity flecking or darkening of the leaf area, (2) beginnings of yellowing or chlorosis, (3) chlorosis. This assay also served throughout the experimental program as an indicator of Avr2 presence in batches of apoplastic fluid.

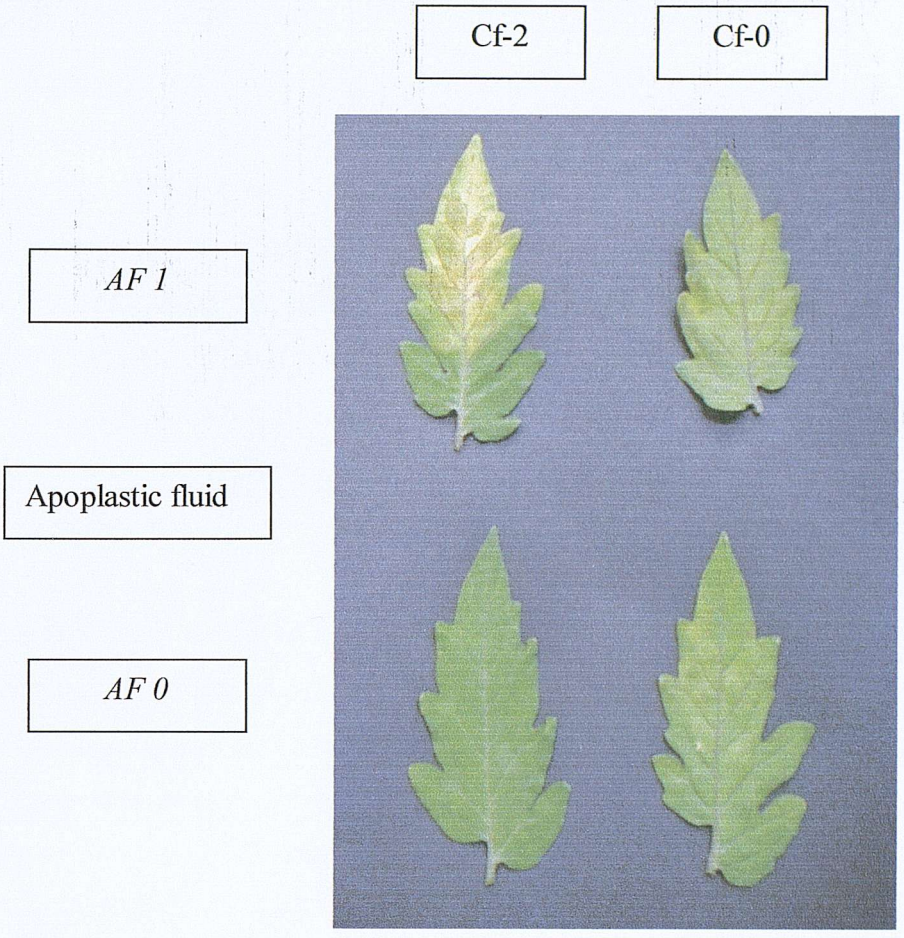


Figure 7.2 Hypersensitive response induced by apoplastic fluids from infected and healthy Cf2 plants. *AF 1* apoplastic fluid from Cf0 plants infected with race 5 *C. fulvum*. *AF 0* apoplastic fluid collected from healthy Cf0 plants. Cf2 and Cf0 plants carrying the *Cf2* gene or no resistance gene respectively.

7.32 Development of the hydrogen peroxide bioassay.

The second method for detection of Avr2 involved biochemical detection of the oxidative burst produced, in separated apoplastic fluids. The assay was based on the premise that the direct interaction of Avr2 and Cf-2 would produce a detectable oxidative burst in a similar fashion to the Avr9 – Cf-9 protein interaction. To control for intergenic differences and to assure the assay detected only the oxidative burst produced by the Avr2 – Cf-2 interaction; two cultivars of *Nicotiana tabaccum* were used. *N. tabaccum* c.v. Petit Gerard carried no Cf genes whereas the gene *Cf-2.1* from tomato had been transformed into the cultivar *N. tabaccum* 2.1 D (Dixon unpublished).

In order to detect Avr2 activity, hydrogen peroxide evolution was assessed indirectly by measuring peroxidase activity. The assay involves the conversion of the chromogen ABTS is to radical cation measured by its absorbance at 400 nm, pH 4.4 (Shindler *et al* 1976). In this hydrogen peroxide limited enzyme assay peroxidase activity is proportional to hydrogen peroxide production. This principle was adapted to work on a multiwell plate to produce a small volume activity assay for directly interacting proteins that produce hydrogen peroxide.

$$\text{fmol hydrogen peroxide} = \frac{\text{Absorbance 400 nm} - 0.0173}{0.0001}$$

To calculate activity, the equation above was calculated by use of dilutions of hydrogen peroxide appropriate to preliminary absorbance readings from neat apoplastic fluids in the assay (Figure 7.3).

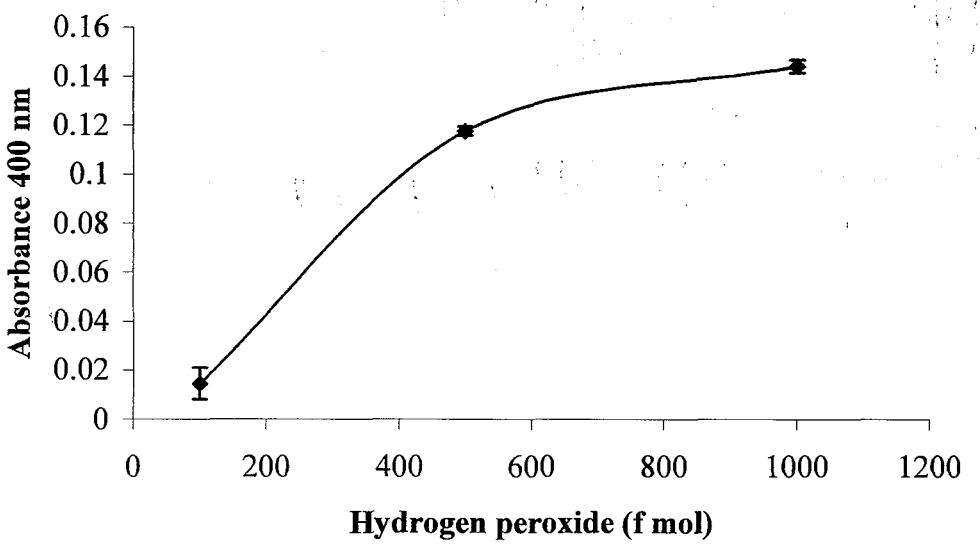


Figure 7.3 Calibration assay for hydrogen peroxide. Absorbance was measured on a multiwell plate reader at 400nm and fmol dilutions of hydrogen peroxide were added in place of apoplastic fluid. Error bars represent standard error of nine experiments.

7.33 Strategies to improve the hydrogen peroxide assay

In the presence of neat apoplastic fluids a significant difference in hydrogen peroxide production could not be measured between the two plant varieties. Several factors were tested that could have aided the assay. Senescent tissue was shown to stop the assay from producing any detectable hydrogen peroxide. Wounded leaf discs from each variety were found to produce some non-specific hydrogen peroxide. In order to eliminate these factors leaf discs from visually healthy plants were pre-incubated for 18 h in leaf disc buffer before using them for the measurement of hydrogen peroxide. However, pre-incubating the leaf discs in glass vials at pH 5.75 also had a detrimental effect (Figure 7.4). All of these measures did not result in a detectable oxidative burst. However it was thought that pure Avr2 interacting with Cf-2 would result in a detectable oxidative burst once any factors suppressing the interaction already present in the apoplastic fluid were eliminated by separation. This observation does not support the hypothesis that the direct interaction of Avr2 and Cf-2 produces detectable hydrogen peroxide.

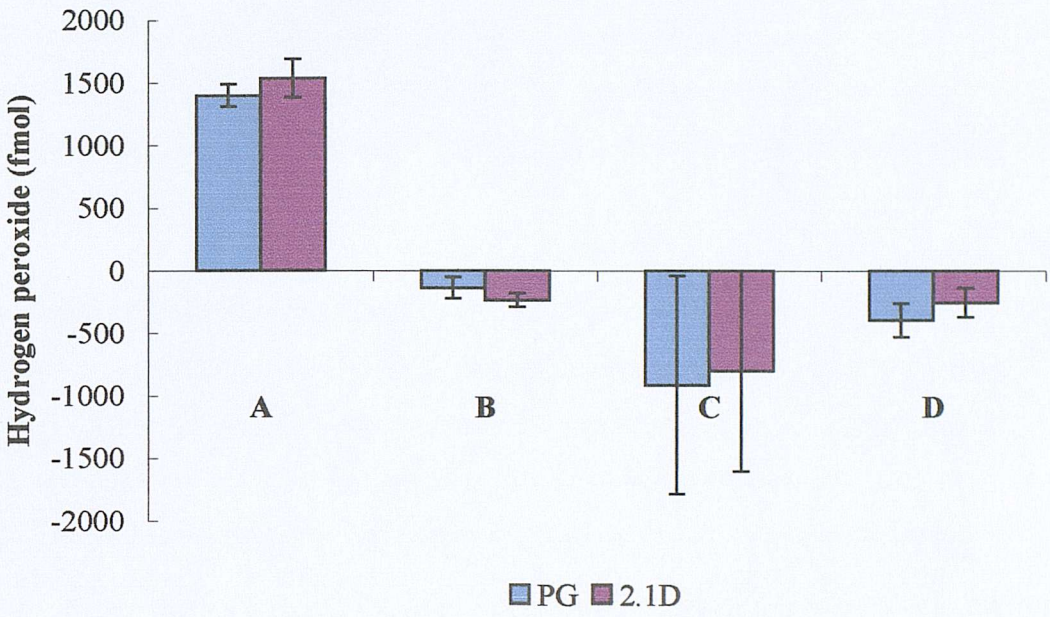


Figure 7.4 Strategies to improve the hydrogen peroxide bioassay: Absorbance was measured on a multi-well plate reader, all experiments used neat apoplastic fluids from Race 5 *C. fulvum* infected Cf-0 plants; A, original assay; B, presence of senescent leaf tissue in assay; C, using pH 5.75 buffer; D, pre-incubating leaf tissue and using glass vials. Error bars represent the standard error of three experiments.

7.34 Purification method 1

In order to obtain DNA sequence of the Avr2 protein, the method of protein purification was employed to yield candidate proteins of which the amino acid sequence would be determined. Eventually, this information would be used to generate degenerate oligonucleotide sequences capable of amplifying Avr2 related DNA fragments via a PCR method.

The apoplastic fluid harvested from Cf-0 plants infected with race 5 *Cladosporium fulvum* was applied to a biogel P6 column (Bio Rad). Four major peaks of protein eluted from the column between the void and the bed volumes. Seven fractions (A-F) of around 30 ml were dialysed and freeze dried and assayed for production of hydrogen peroxide in the leaf disc assay and chlorotic lesions in the leaf assay (Figure 7.5).

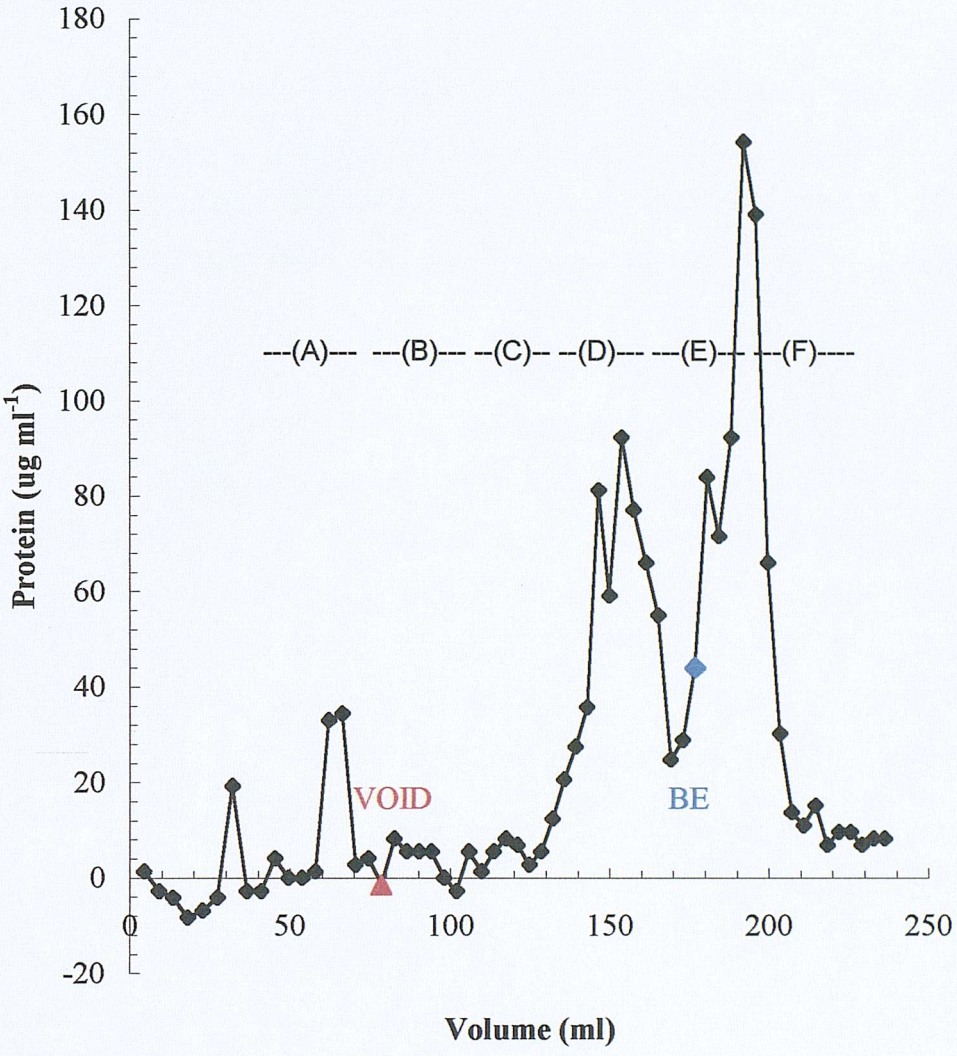


Figure 7.5 Separation of apoplastic fluids by gel filtration on biogel P6.

▲ Void volume ◆ Bed volume. Protein determined by BCA method; --(A)--, Fraction A (41 - 78.5 ml); --(B)--, Fraction B (82.6-105.9 ml); --(C)-- Fraction C (109.7-135.8 ml); --(D)--, Fraction D (139.4-169.3 ml), --(E)--, Fraction E (173.1-203.4 ml) --(F)--, Fraction F (207.2-236.3 ml).

7.35 Analysis of Avr2 activity in fractions

The visual assay of chlorosis was taken on a semi-quantitative three-point scale (Figure 7.6). This was defined as: (1) slight activity flecking or darkening of the leaf area, (2) beginnings of yellowing or chlorosis, (3) chlorosis. All injections into leaves were at the concentration achieved directly from apoplastic fluid harvest.

Injection of water did not induce a chlorotic lesion with the leaf assay. Injection of neat apoplastic fluid induced a yellow chlorotic lesion (3) with the leaf assay. Fraction A (41 - 78.5 ml) was active (2) by the leaf assay. Fraction B (82.6-105.9 ml) was inactive (1) by the leaf assay. Fraction C (109.7-135.8 ml) was inactive (1) by the leaf assay. Fraction D (139.4-169.3 ml) was inactive (1) by the leaf assay. Fraction E (173.1-203.4 ml) was inactive (1) by the leaf assay. Fraction F (207.2-236.3 ml) was active (3) by the leaf assay (Figure 7.6). The leaf disc assay was also performed on all pooled fractions (A-F) from the column however; no significant results were achieved (data not shown).

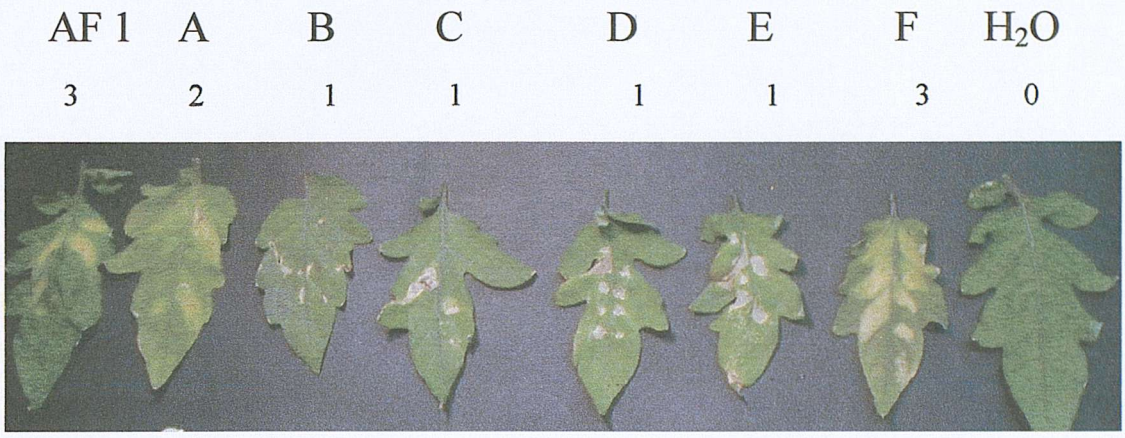


Figure 7.6 Chlorosis development in Cf-2 leaves injected with column fractions. AF, Apoplastic fluid from Cf-0 plants infected with Race 5 *C. fulvum*; H_2O, Water control; A-F, fractions from gel filtration column. Numbers indicate activity value of chlorosis (3 chlorotic, 2 turning yellow, 1 other symptoms).

7.36 Isoelectric focusing of separated column fractions

In order to separate candidate proteins to a greater degree, fractions from Bio Gel P6 separation were analysed by isoelectric focusing. (IEF). Apoplastic fluids from Race 5 infected Cf-0 plants produced 18 resolvable proteins ranging from pI 3.9 to pI 10.1. Apoplastic fluids obtained from healthy Cf-0 plants produced 11 resolvable proteins ranging from pI 4.1 to pI 10.1. A 50:50 (v/v) mixture of the neat apoplastic fluids produced eight resolvable proteins ranging from pI 3.9 to 9.9. Analysis by IEF of fraction A revealed 10 proteins ranging from pI 4.2 to 10.2. Analysis by IEF of fraction B revealed 12 proteins ranging from pI 4.3 to 10.3. Analysis by IEF of fraction C revealed 15 proteins ranging from pI 3.9 to 10.2. Analysis by IEF of fraction D revealed 13 proteins ranging from pI 4.0 to 10.2. Analysis by IEF of fraction E revealed 13 proteins ranging from pI 4.2 to 9.6. Analysis by IEF of fraction F revealed 12 proteins ranging from pI 4.0 to 9.2 (Figure 7.7).

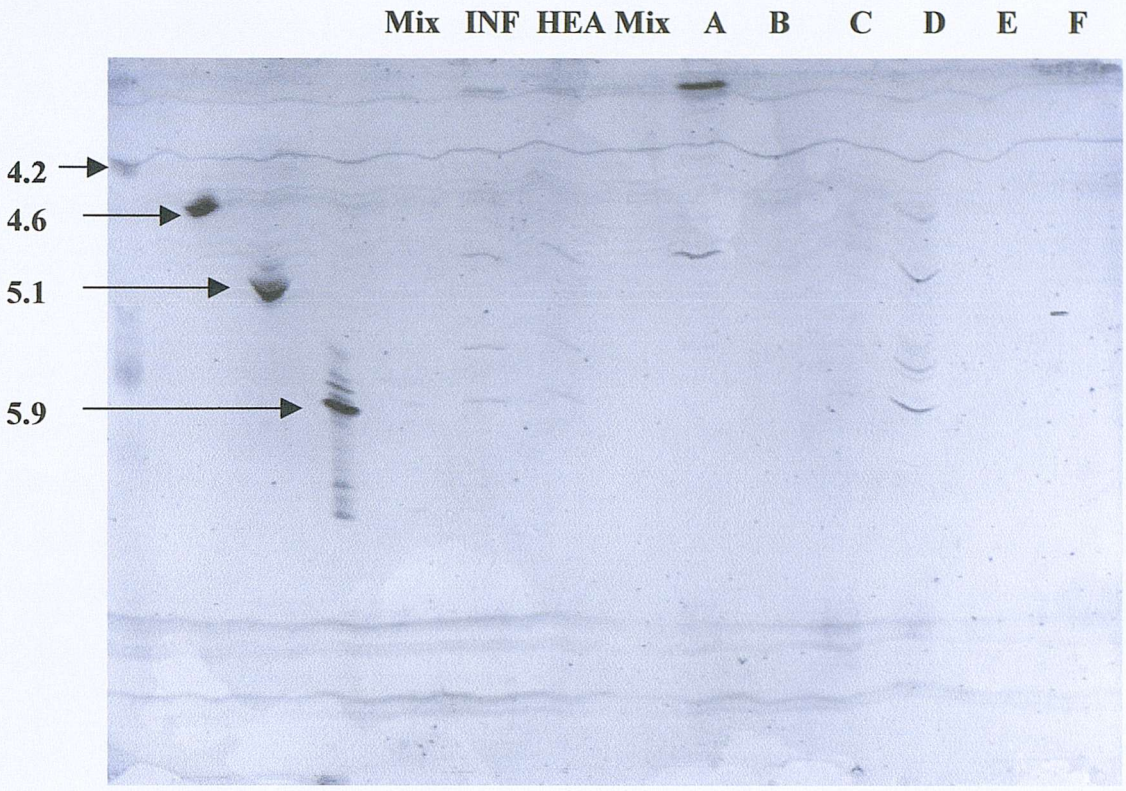


Figure 7.7 Isoelectric focusing gel of fractions from gel filtration column: 4.2 - 5.9, migration of standard proteins; Mix, 50:50 (v/v) A mix of apoplastic fluids harvested from Cf-0 plants that are infected with Race 5 *C. fulvum* and healthy Cf-0 plants; INF, Apoplastic fluid from Cf-0 plants infected with Race 5 *C. fulvum*; HEA, Apoplastic fluid from healthy Cf-0 plants; A-F, Pooled fractions from gel filtration column.

7.37 Candiadate proteins for Avr2 from purification method 1.

Six resolvable candidate proteins were present exclusively in apoplastic fluids harvested from Race 5 infected Cf0 plants but absent from apoplastic fluids obtained from healthy Cf0 plants. Of these, one appears in the active fraction A (pI 6.1).

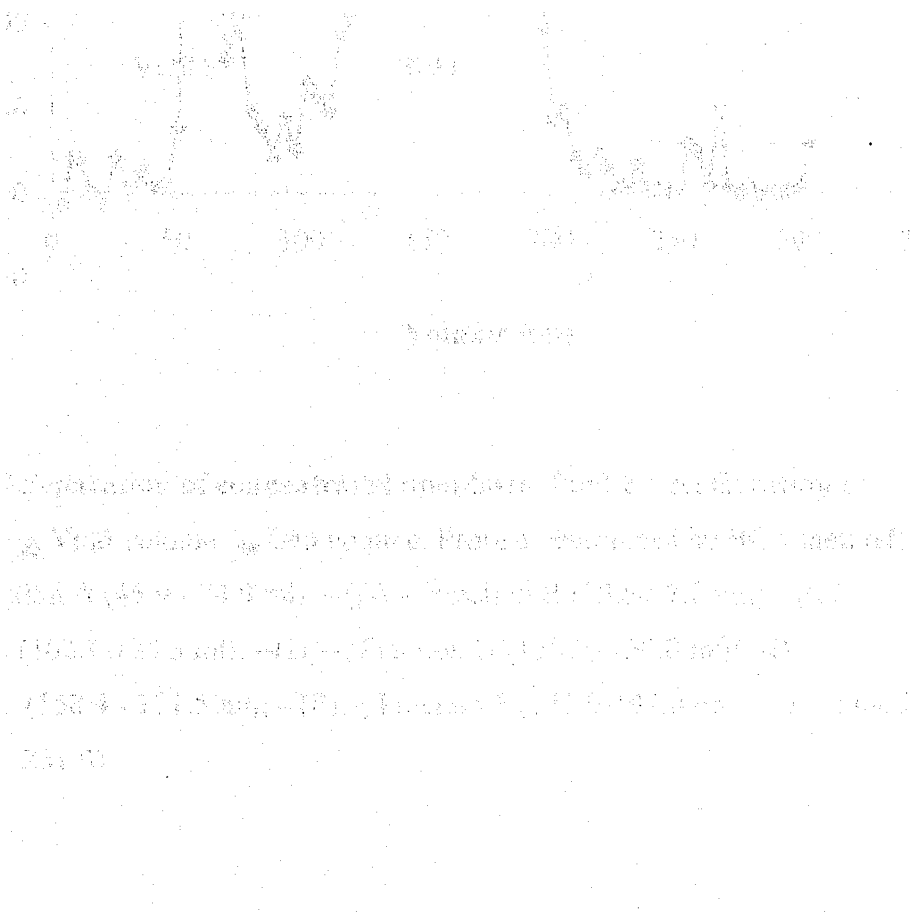
The IEF also reveals 23 resolvable proteins that are not resolved in lanes electrophoriesed with Race 5 or Cf0 apoplastic fluid. These proteins are likely to be present in low (unresolvable) amounts in neat apoplastic fluids from the Race 5 infected or healthy Cf0 plants and therefore are also potential candidates for Avr2. Of these, three proteins appear in the active fraction F (pI, 4.2, 4.7, 8.7) and two proteins appear in active fraction A (pI, 4.2, 5.7) (Table 7.4).

Table 7.4 Isoelectric points of resolved proteins from IEF. Fractions were tested by direct injection into Cf-2 and Cf-0 plants: (A-F), fractions from gel filtration column; **Red**, present only in apoplastic fluid from plants infected with Race 5 *C.fulvum*; **Green**, present in healthy apoplastic fluids. *Orange*, protein not resolved in lanes electrophoriesed with apoplastic fluid from Race 5 infected or healthy Cf-0 plants; tolerance, +/– 0.1 pH units; (*), candidate proteins for Avr2.

Apoplastic fluid from Race 5	Healthy from Cf0	Mix of fluids	A	B	C	D	E	F
3.9	3.9	3.9	*4.2**		3.9	4.0		4.0
4.1	4.1		4.3	4.3	4.0	4.3	*4.2*	*4.2**
4.4	4.3		4.4	4.4	4.3	4.4	4.6	4.3
4.8		4.8		*4.7*	*4.6*	**4.9**	**4.9**	4.4
5		5	5.1		**5.0**	**5.0**		*4.7**
5.3		5.3		*5.2*	*5.2*	5.5	*5.2*	5
5.4	5.4		5.5	5.5		5.8		5.4
6	5.9		*5.7**	*5.7*		5.9	5.9	5.6
6		6	**6.1***		*6.4*	*6.3*	*6.2*	
6.8	6.6	6.8		*7*	6.8		6.6	6.9
7.5		7.5			*7.1*	*7.1*	*7*	
8.2	8.1			8.2	8.2	8.3	8.1	8.1
8.5	8.4		8.4	8.5	8.4		8.3	
9	9.0				*8.8*	8.9	*8.8*	*8.7**
9.3	9.3		9.2	*9.6*	9.4		9.4	9.2
9.9		9.9		**9.9**	**9.9**		*9.6*	
10.1	10.1		10.2	10.3	10.2	10.2		

7.38 Purification method 2.

As resolution of the proteins lead to some difficulty in the quantification of the proteins character, for the second purification method the apoplastic fluid was concentrated 10 fold to improve resolution. The Bio Gel P6 column was again run with apoplastic fluid obtained from Cf-0 tomato plants infected with Race 5 *C. fulvum*. Three major peaks of protein eluted from the column between the void and the bed volumes. Eight fractions (A-G) of around 30 ml were dialysed and freeze dried and assayed for chlorotic lesions in the leaf assay and production of hydrogen peroxide (Figure 7.8).



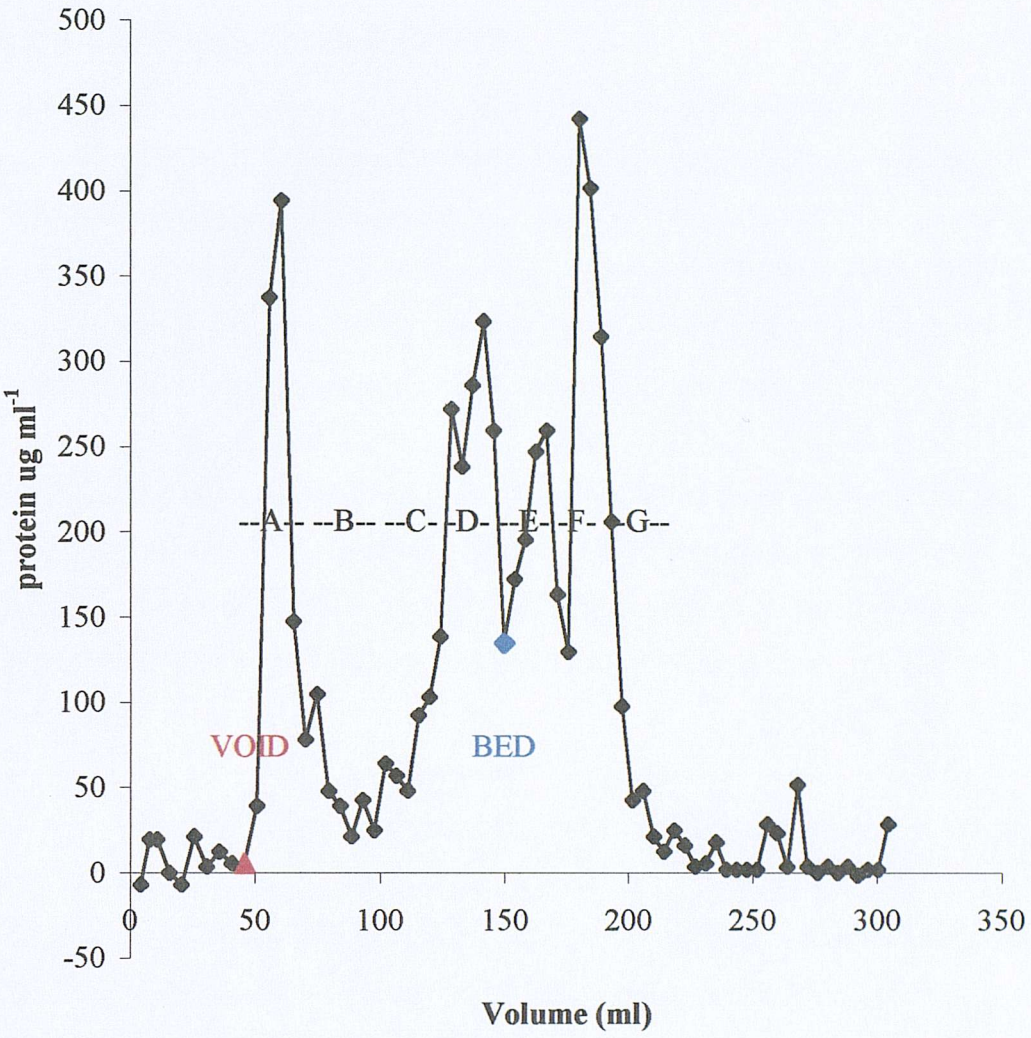


Figure 7.8 Separation of concentrated apoplastic fluid by gel filtration on biogel P6. ▲ Void volume. ◆ Bed volume. Protein determined by BCA method; --(A)--, Fraction A (45.9 - 74.9 ml); --(B)--, Fraction B (79.5-97.8 ml); --(C)--, Fraction C (102.1-124.3 ml); --(D)--, Fraction D (128.0 -150.0 ml); --(E)--, Fraction E (158.4 - 171.5 ml); --(F)--, Fraction F (175.0-197.0 ml); --G--, Fraction G (201.0 – 231.0).

7.39 Analysis of Avr2 activity in fractions

The visual assay of chlorosis was taken on a semi-quantitative three-point scale (Figure 7.9). This was defined as: (1) slight activity flecking or darkening of the leaf area, (2) beginnings of yellowing or chlorosis, (3) chlorosis. All injections into leaves were at five times the concentration achieved directly from apoplastic fluid harvest. Preliminary experiments showed that injection of apoplastic fluid at 10 times the harvested concentration was lethal (data not shown).

Injection of water did not induce a chlorotic lesion with the leaf assay until 13 days after injection and only on Cf0 plants. Injection of five times apoplastic fluid from infected plants induced a yellow chlorotic lesion (3) only on Cf2 plants. Injection of apoplastic fluid from healthy plants did not induce any chlorosis in Cf2 or Cf0 after 13 days.

Fraction A (45.9 – 74.9 ml) showed activity only in Cf0 plants by the leaf assay and is not a candidate fraction for Avr2. Fraction B (79.5-97.8 ml) showed activity only in Cf2 plants and is therefore a strong candidate for the presence of Avr2. Fraction C (102.1-124.3 ml) showed more activity in Cf2 plants than Cf0 plants and is therefore a weak candidate active fraction for the presence of Avr2. Fraction D (128.0-150.0 ml) showed less activity in Cf2 plants than Cf0 and is therefore not a candidate active fraction for the presence of Avr2. Fraction E (158.4-171.5 ml) showed no activity in either plant and is therefore not a candidate active fraction for the presence of Avr2. Fraction F (175.0-197.0 ml) was only active in Cf0 plants is unlikely to contain Avr2. Fraction G (201.0 – 231.0 ml) showed more activity in Cf2 than Cf0 plants and is a weak candidate fraction for Avr2.

The leaf disc assay was also performed on all pooled fractions from the column however; no significant results were achieved (data not shown).

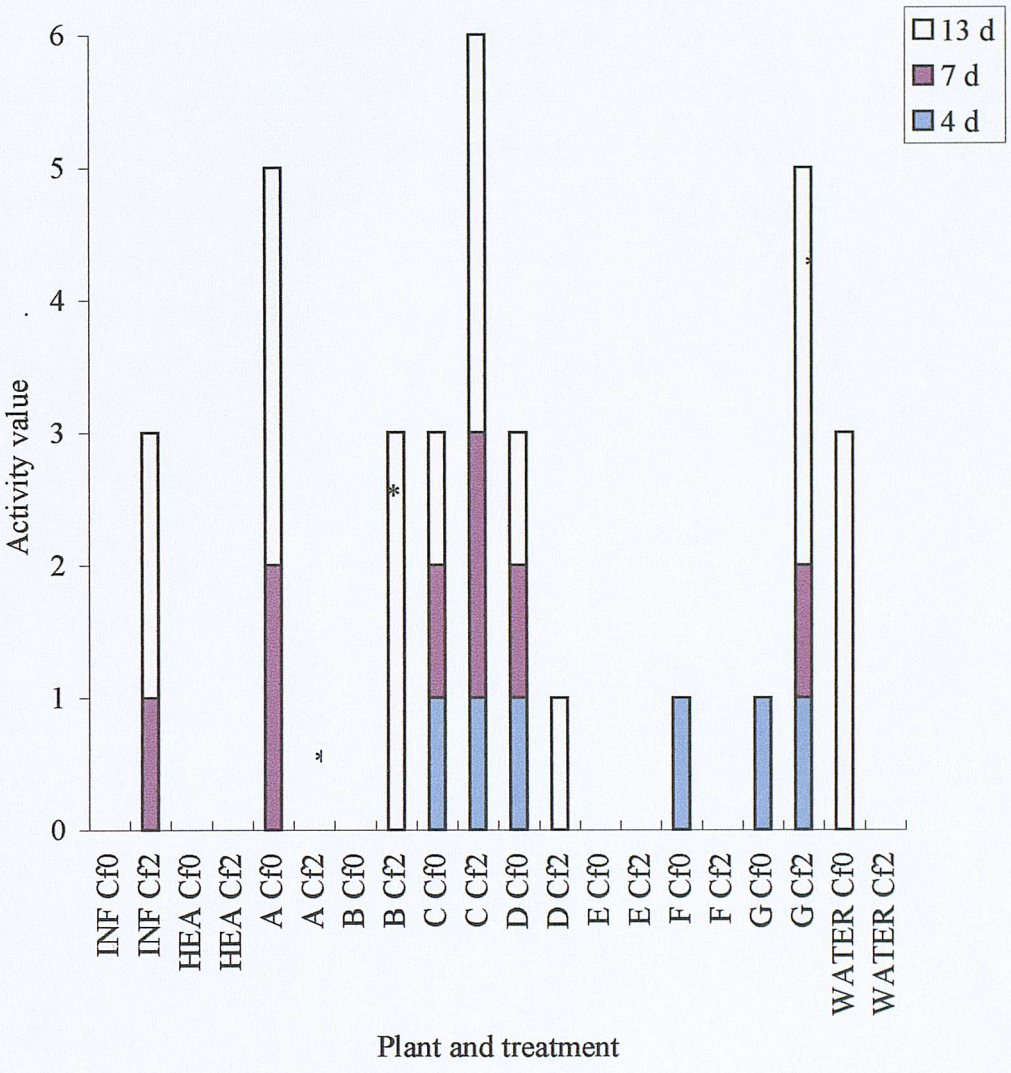


Figure 7.9 Activity of fractions from Bio Gel P6 column in Cf0 and Cf2 tomato plants at 5 X concentration: Plants with (Cf2) and without (Cf0) the *Cf-2* gene were injected with water, apoplastic fluids from infected (INF), healthy (HEA) and separated fractions (A-G) of apoplastic fluids; 4 d, 7 d, 13 d, days after injection. Activity was measured by degree of yellowing (chlorosis) on a three point scale;(*) , candidate active fraction.

7.40 Isoelectric focusing of separated column fractions

In order to separate candidate proteins to a greater degree, fractions from Bio Gel P6 separation were analysed by IEF. A 10 fold concentrated mix of Apoplastic fluids from Race 5 infected Cf0 plants produced seven resolvable proteins ranging from pI 4.6 to 6.4. Apoplastic fluids obtained from healthy plants produced four resolvable proteins ranging from pI 5.0 to 6.6 (Table 7.5). Analysis by IEF of fraction A revealed 14 proteins ranging from pI 3.6 to 9.1. Analysis by IEF of fraction B revealed nine proteins ranging from pI 4.4 to 6.4. Analysis by IEF of fraction C revealed seven proteins ranging from pI 4.4 to 8.8. Analysis by IEF of fraction D revealed seven proteins ranging from pI 4.7 to 5.9. No other fraction produced any resolvable proteins.

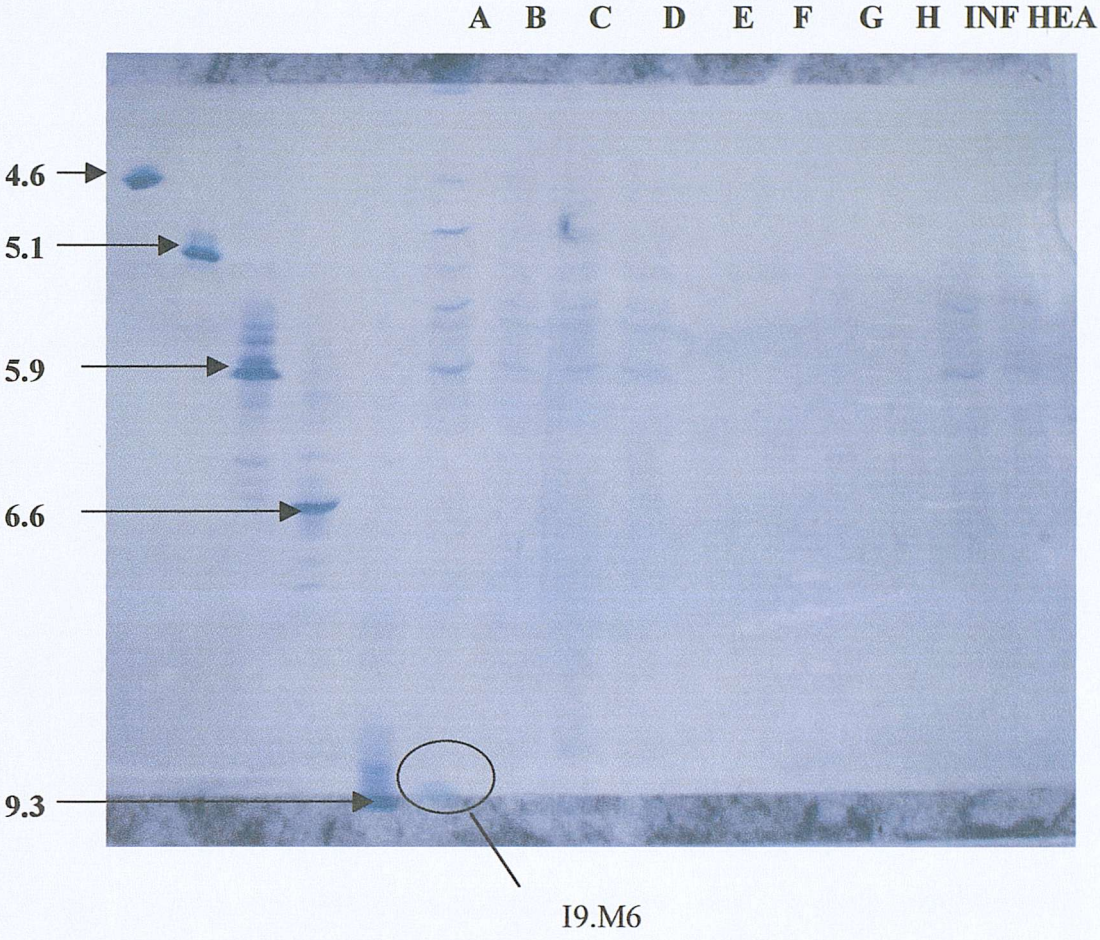


Figure 7.10 Isoelectric focusing gel of fractions from gel filtration column. The gel was stained with Coomaisse blue for proteins: 4.6 – 9.3, migration of standard proteins; INF, Apoplastic fluid from Race 5 *C.fulvum* infected plants; HEA, Apoplastic fluid from healthy Cf-0 plants. I9.M6: A candidate protein that has a molecular weight of around 6 (present in fraction A and an isoelectric point of around 9).

7.41 Candidate proteins for Avr2 from purification method two.

Six resolvable candidate proteins were present exclusively in apoplastic fluids harvested from Race 5 infected Cf0 plants but absent from apoplastic fluids obtained from healthy Cf0 plants. Of these, two appear in the active fraction B (pI 5.4,6.4), and two appear in the active fraction C (pI 5.4, 6.4) no proteins were resolved in active fraction G.

The IEF also reveals 12 resolvable proteins that are not resolved in lanes electrophoresed with Race 5 or Cf0 apoplastic fluid. These proteins are likely to be present in low (unresolvable) amounts in neat apoplastic fluids and are potential candidates for Avr2. Of these, two proteins appear in the active fraction B (pI, 4.4 and 4.8) and three appear in active fraction C (pI, 4.4, 4.8 and 8.8).

Another interesting protein band is at pI 9 in fraction A. The staining on this band is heavier than any other band in the gel. In view of the increase in concentration in this protein purification method may also be considered as a potential candidate for Avr2. In view of its elution on the column, the molecular weight of this band is approximately 6 kD. It was decided to further investigate the character of this band by trying to obtain the amino acid sequence of this protein.

Table 7.5 Isoelectric points of resolved proteins from IEF (2). Fractions were tested by direct injection into Cf-2 and Cf-0 plants: A-G, Fractions from gel filtration column; **Red**, Present only in apoplastic fluid from plants infected with Race 5 *C.fulvum*; **Green**, Present in apoplastic fluid from healthy plants; *Orange*, protein not resolved in lanes electrophoriesed with apoplastic fluid from Race 5 infected or healthy Cf-0 plants; tolerance, +/– 0.1 pH units; (*), candidate proteins for Avr2.

Apoplastic fluid from Race 5 infected plants	Apoplastic fluid from healthy plants	A	B	C	D	E-G
		3.6				No Proteins
		3.9				
		4.1				
		4.2				
		4.3	*4.4**	*4.4**		
4.6		4.5	4.5		4.7	
		4.7		4.7	4.7	
		4.8	*4.8**	*4.8**	4.9	
	5	5	5.1			
5.3	5.3	5.2	5.2		5.2	
5.4			5.4*			
5.5		5.5		5.5*	5.5	
5.8	5.8	5.6	5.6		5.6	
		5.9	5.9	5.9	5.9	
6.3						
6.4			6.4*	6.4*		
	6.6					
		9.1		*8.8**		

7.42 Localisation of candidate protein.

During the course of attempting to use purification method two, information was gained that the correct molecular weight of the Avr2 protein was around 6 kD, and the isoelectric point (pI) of this protein was around nine (Luderer (2000)). A single discreet band of this approximate molecular weight and isoelectric point was observed in fraction A. This band is a good candidate for the Avr2 protein and was designated I9.M6. In order to establish if any other resolvable bands of protein were present around the probable molecular weight region of 6 kD and isoelectric point of nine, a new gel was prepared. Fractions B and G, exhibited the greatest activity in the leaf assay and were subject to electrophoresis again. The new electrophoresis gel was stained with $AgNO_3$, which can detect to protein μg of proteins.

When the new gel was stained with $AgNO_3$, 24 proteins were revealed in fraction A between pI 3.3 and 8.9. However, none of these proteins appeared to co-localise with the candidate I9.M6. However it is possible that any of the newly detected proteins in fraction A could be natively bound to I9.M6 and may impair its biological function.

In fraction B four proteins were resolved between pI 4.7 and 5.8, fraction G revealed no proteins. Both of these fractions were therefore discounted as possible sources of native Avr2 protein (Figure 7.11).

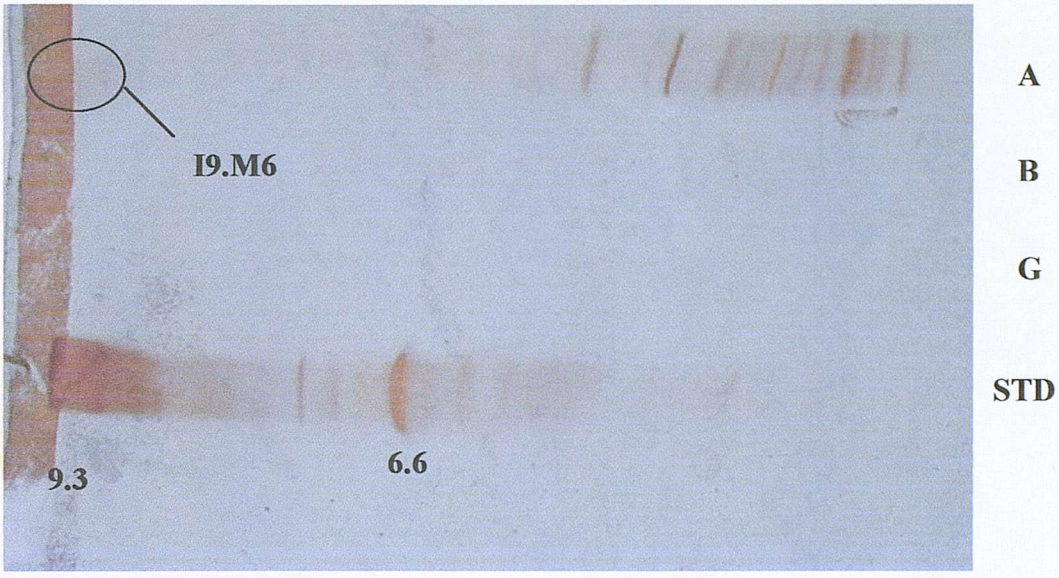
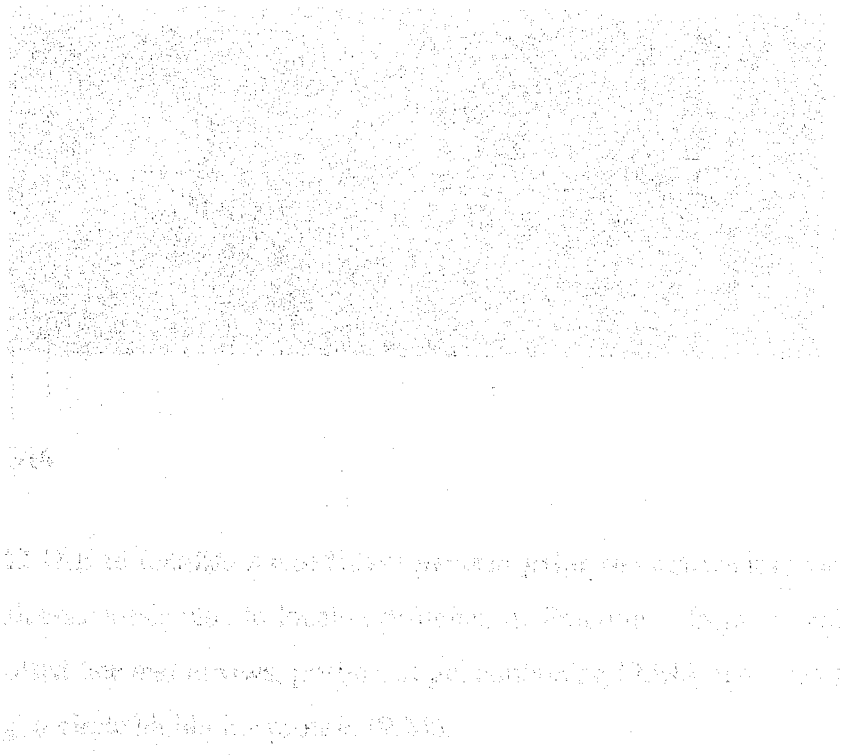


Figure 7.11 Isoelectric focusing gel of fractions from gel filtration column. Gel was stained with silver nitrate. STD 6.6- 9.3: migration of standard proteins. A-G: Fractions from gel filtration column. I9.M6: A candidate protein that has a molecular weight of around 6 (present in fraction A and an isoelectric point of around 9).

7.43 Gel purification for sequencing.

In order to affirm I9.M6 as Avr2 it was decided to attempt to obtain amino acid sequence of this protein. In order to achieve this, the protein band had to be isolated in high amounts. As no discrete bands were observed close to I9.M6 in the previous gel an electrophoresis gel was run entirely with fraction A to gel purify the candidate protein. The portion of the IEF gel containing fraction A was excised and freeze-dried (Figure 7.12).



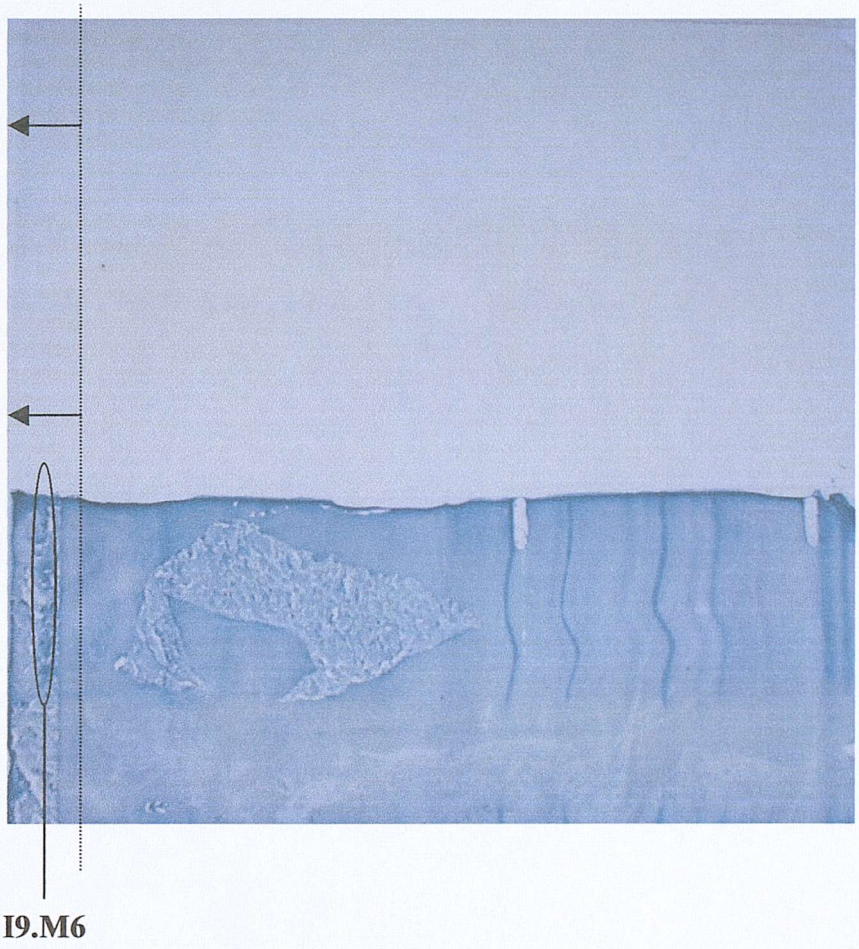
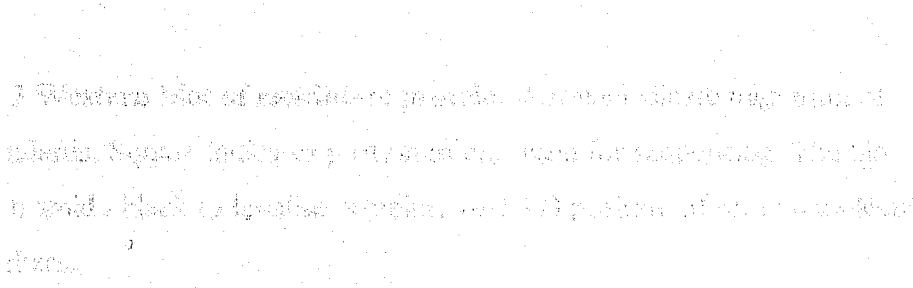


Figure 7.12 IEF to localise a candidate protein prior to sequencing. Gel is stained with coomassie blue to localise proteins: A, Fraction A from bio gel P6 column; dotted line and arrows, portion of gel containing I9.M6, removed for sequencing; a circle highlights protein I9.M6.

7.44 Western blotting for candiate protein

The candidate protein fragment (I9.M6) was freeze-dried to a 30 µl aliquot and subjected to SDS page electrophoresis. The protein was transferred via western blotting to a nitrocellulose membrane from which sequencing could be performed. A large band of protein was observed at around 6 kD, giving confirmation of the molecular weight of I9.M6. Other transferred bands were observed and may be due to very high molecular weight and higher pI proteins (i.e. those greater than 9.3). The portion of the membrane containing the highest amount of the protein present at 6 kD was excised and amino acid sequencing performed (Figure 7.13).



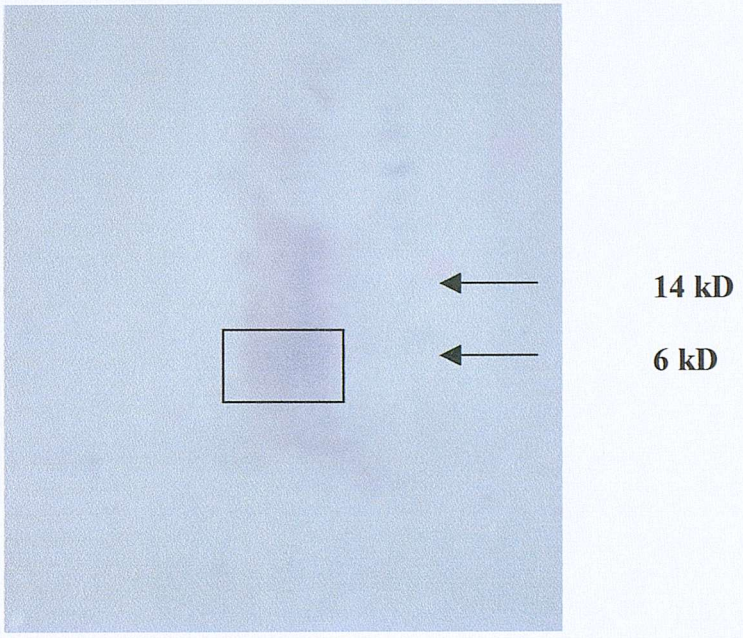


Figure 7.13 Western blot of candidate protein. Arrows indicate migration of protein standards. Square indicates portion of blot used for sequencing. The blot stained with amido black to localise proteins. 14-6 kD position of protein molecular weight markers.

7.45 Result of sequencing

The sequencing of I9.M6 failed, the protein was blocked at the N-terminus, attempts to cleave the sequence by cleaving at methione residues also failed. Therefore this protein cannot be confirmed as Avr2.

7.46 Purification method 3 Preparative Isoelectric focusing

As activity of Avr2 had already been localised to around pI of 9 and very few resolvable proteins were present around that isoelectric point, preparative IEF was used to provide concentrated small volume samples without the need for prior concentration. The rotofor required 30 ml of apoplastic fluid per run.

Separation of apoplastic fluids from the race 5 *C. fulvum*-Cf-0 tomato interaction using preparative isolelectric focusing was between pH two and 12. The highest resolution occurred for 16 fractions between pH six and pH 10. The major peak of protein elution detected by the BCA assay was below pI eight (fraction 11). Protein remained under 0.1 mg ml^{-1} until fraction 20, however a slight peak is observed at fraction 17 (pI 9.4) (Figure 7.14).

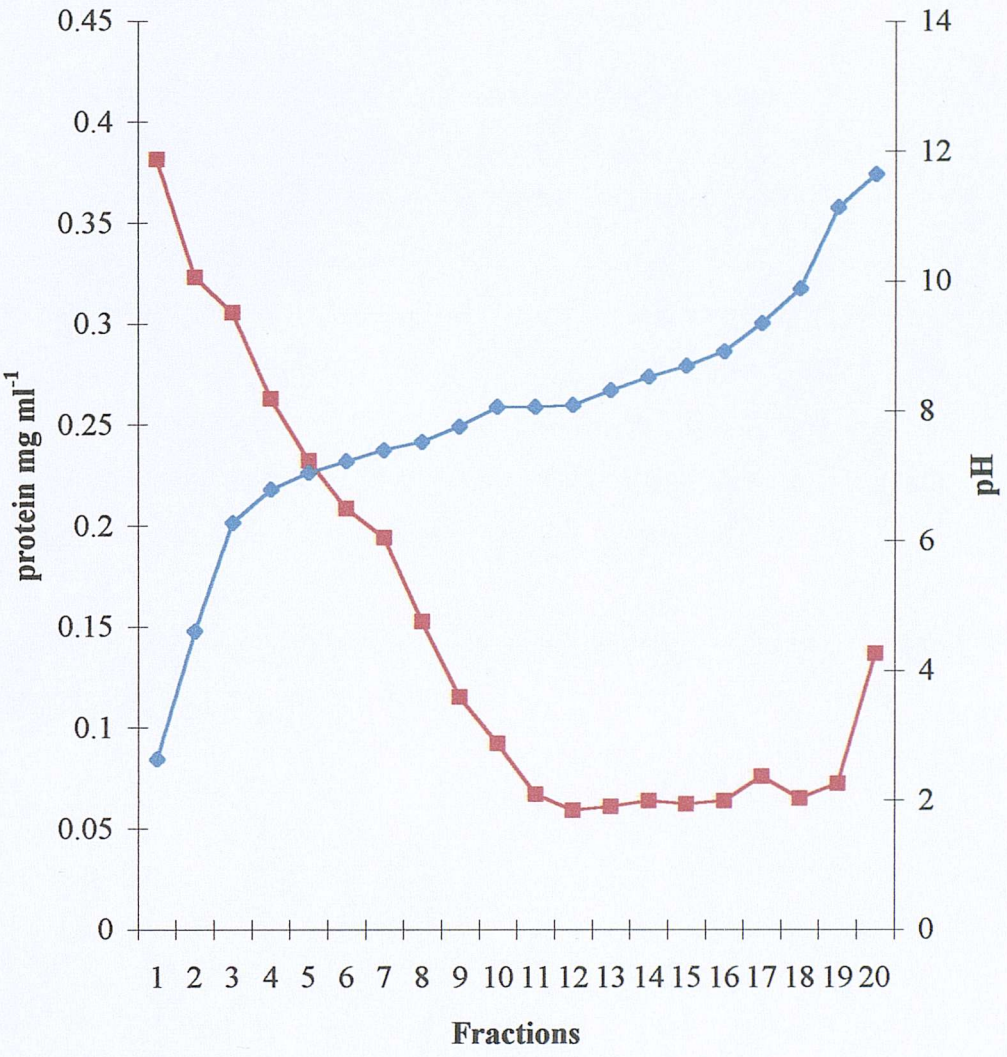


Figure 7.14 Separation by Preparative IEF of apoplastic fluids by Rotofor Bio Rad: ■, protein mg ml^{-1} measured by BCA method; ◆, pH using a pH meter (Corning 245). Fractions of 1.5 ml were collected.

7.47 Analysis of Avr2 activity in fractions

Each fraction was separately tested for activity in the leaf assay. In order to prevent bias, four different observers ranked the degree of chlorosis on a five-point scale (5 highest – 1 lowest). The test was carried out independently on Cf2 and Cf0 plants injected with candidate fractions (10-20) in the range pI 8 to10. The average values of activity for Cf0 plants were subtracted from those obtained for Cf2 plants to give the net activity value, which was divided by the number of observers. Net activity appeared to be clustered around pH 8.5 to 10. Another peak of activity was observed at pH 6.75 to 4.5. This peak was not investigated further due to the resolution of the fractions between pI 6.75 and 4 (Figure 7.15).

Fractions were also tested in the hydrogen peroxide assay, however; no significant results were produced (data not shown).

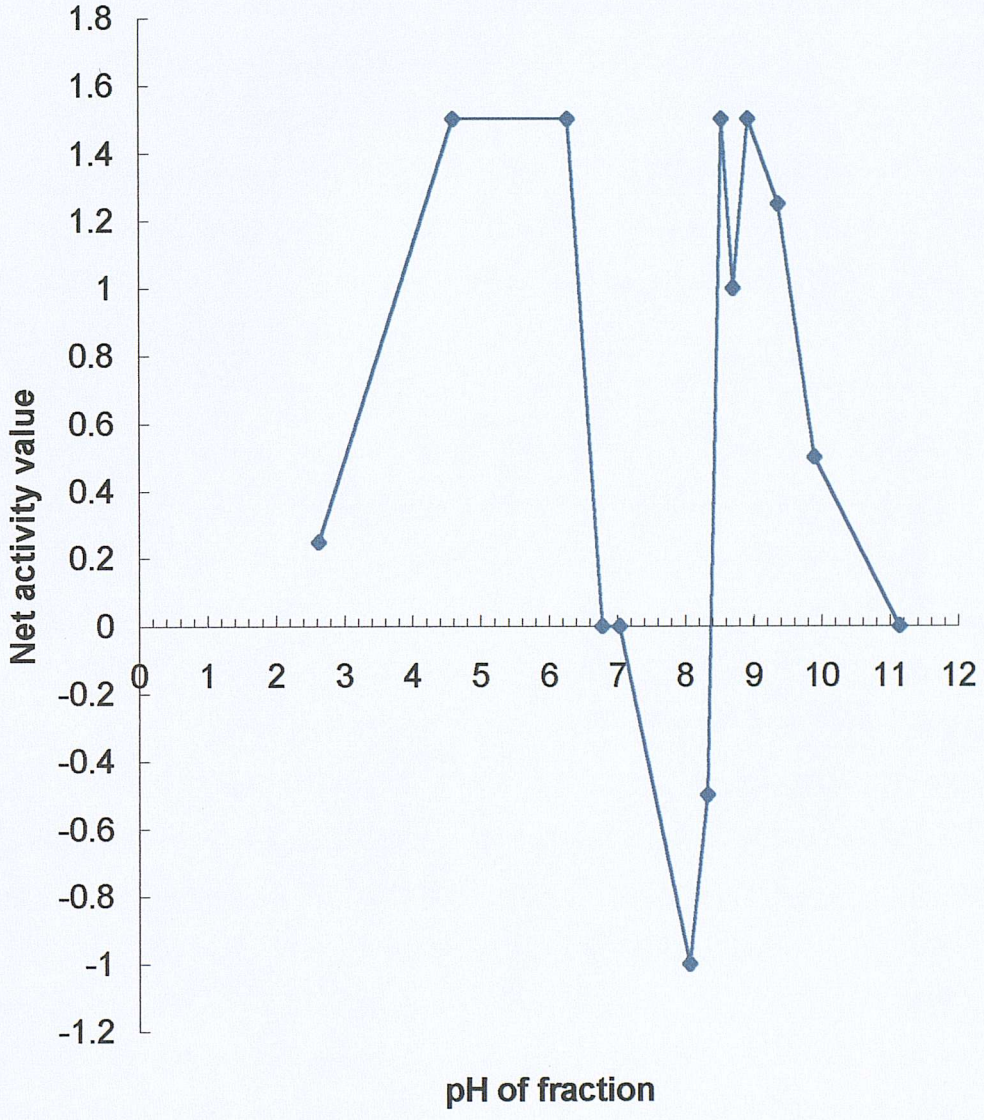


Figure 7.15 Activity of fractions from rotofor focusing via bioassay: Activity value, calculated on a scale (1-5) and averaged between 5 operators; pH, measured by pH meter.

7.48 Tris tricine Gel electrophoresis

In order to resolve proteins below 10 kD tris-tricine gel electrophoresis was used in order to further separate the candidate active fractions (Schagger and Von Jaggow 1998). The gene Avr2 predicts a 78 amino acids protein (Luderer 2002), this refers to native protein of 6 kD that should be resolved in a strong band in an active fraction at an isoelectric point of nine. Standards between 66 and 6 kD and 26 and 1 kD were run in order to find apparrent molecular weights of candidate proteins on gels in active fractions (Figure 7.16).

On all gels proteins were stained with co-maissie blue. No proteins were detected in a pI 11.7 fraction. Five proteins between 56.3 and 9.6 kD were detected in a fraction of pI 11.1. Two proteins between 13.4 and 5.3 kD were detected in a fraction of pI 9.9. Seven proteins between 58.3 and 4.3 kD were detected in a fraction of pI 9.4. Three proteins between 13.4 and 5.3 kD were detected in a fraction of pI 8.9. Eight proteins between 56.1 and 4.3 were detected in a fraction of pI 8.7. Five proteins between 33.7 and 5.3 kD were detected in a fraction of pI 8.5. Six proteins between 56.1 and 6.8 kD were detected in a fraction of pI 8.3. Five proteins between 34.6 and 2.3 kD were detected in a fraction of pI 8.1. Six proteins between 60.2 and 12.2 kD were detected in a fraction of pI 8.1. Five proteins between 57.2 and 26.7 kD were detected in a fraction of pI 8.0. Ten proteins between 57.2 and 7.0 kD were detected in a fraction of pI 7.8. Eleven proteins between 41.4 and 2.4 kD were detected in a fraction of pI 7.5. Ten proteins between 57.2 and 7.0 kD were detected in a fraction of pI 7.4. Twelve proteins between 40.2 and 2.6 kD were detected in a fraction of pI 7.2. Ten proteins between 56.3 and 7.3 kD were detected in a fraction of pI 7.0. Nine proteins between 40.2 and 2.6 kD were detected in a fraction of pI 6.8. Eight proteins between 41.8 and 7.0 kD were detected in a fraction of pI 6.3. Seven proteins between 40.2 and 10.5 kD were detected in a fraction of pI 4.6. Ten proteins between 58.3 and 7.3 kD were detected in a fraction of pI 2.6. In total 139 proteins were detected ranging from 58.1 kD to 2.3 kD (Figure 7.16).

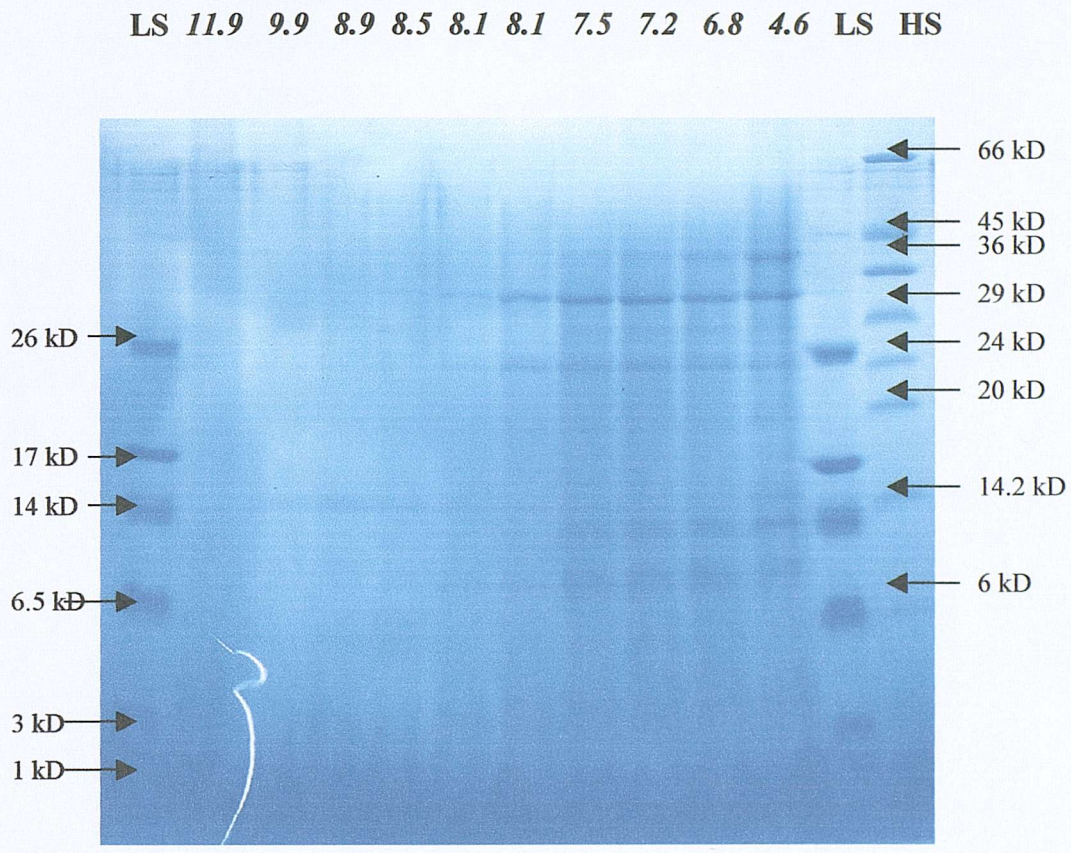


Figure 7.16 Tris-tricine gel of fractions from preparative isoelectric focusing; the gel was stained with Coomaisse blue for proteins, **LS**, low molecular weight standards 26-1 kD; ***11.7-4.6***, isoelectric point of fraction. **HS**, high molecular weight standards. Arrows indicate molecular weights of standard proteins.

7.49 Correlation of activity with candidate protein isoelectric points and molecular weights.

Activity from the leaf assay toward Cf-2 was related to apparent molecular weight and isoelectric point of the candidate proteins. The criteria for a candidate active protein was that its apparent molecular weight band encompassed the entirety of the active fractions between either pI 4.6 to 6.3 or pI 8.5 to 9.4. If the apparent molecular weight band of the protein crossed into an inactive fraction it was not considered a candidate Cf-2 active protein band (Figure 7.17).

The two-dimensional separation combined with the activity data reveals 13 candidate bands that may be linked to nine separate proteins. Six candidate bands lay at pI 9.4 at 58.3 kD, 53.9 kD, 41.8 kD, 26.7 kD, 16.4 kD and 6.8 kD. One candidate band lies at pI 8.9 at 6.8 kD. Three candidate bands lay at pI 6.3 at 41.0, 26.2 and 6.8 kD. One candidate band lies at pI 6.3 at 21.6 kD. Two candidate bands lay at pI 4.6, at 20.0 kD and 12.8 kD (Figure 7.17).

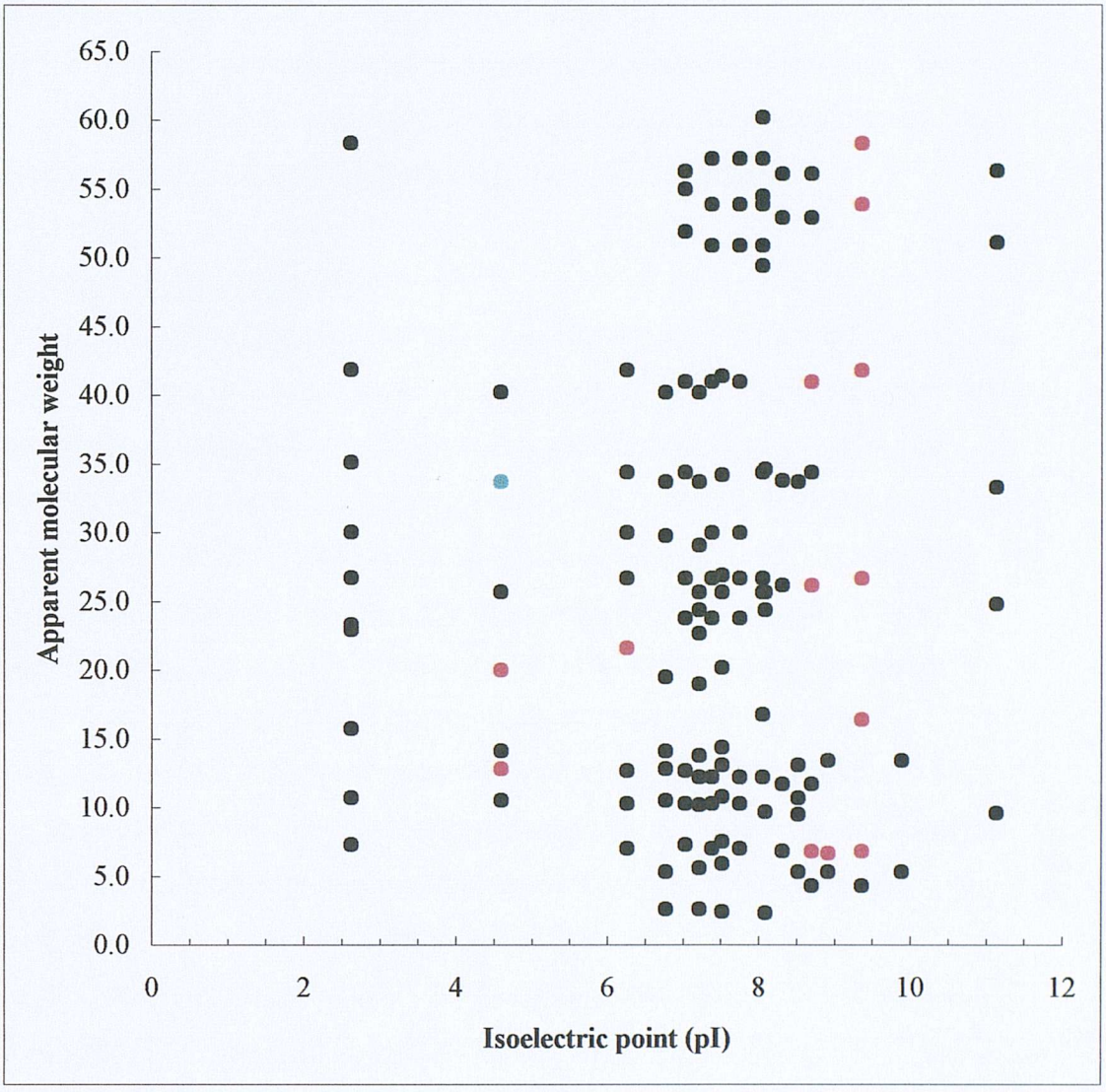


Figure 7.17 Molecular weight estimation and activity of proteins separated by preparative isoelectric focusing: Proteins were electrophesised on tris-tricine gels and detected by staining with Coomassie brilliant blue. , inactive protein in Cf-2 interaction; , candidate active protein , Avr2 from Luderer *et al* (2002).

7.50 Discussion

The main aim of this study was to provide information on the protein character of Avr2 or possible interacting proteins in order to facilitate cloning of the Avr2 gene by a PCR method. This study however only provides some complementary data about the nature of resistance mediated by the Cf-2 interaction.

7.51 Avr2 is already purified

Cloning of the *Avr2* gene has now been completed Luderer *et al* (2002). A screen of a cDNA library from a *C. fulvum* strain avirulent on Cf2 plants was performed using a potato virus X expression vector system. This gave two isolates that reproducibly induced an HR upon *Agrobacterium tumefaciens*-mediated expression in Cf2 plants. The *Avr2* ORF predicts a cysteine-rich protein of 78 amino acids, with a predicted signal peptide of 20 amino acids (Accession no AJ421629). The predicted molecular weight of this protein is 33 kD with an isoelectric point of 4.75. This is in contrast to information received previously. However, by examination of the data shown in this study some similarities in activity patterns can be drawn. Avr2 related activity is shown in this study between pI 4.6 and pI 6.3. Also a protein can be resolved in the active fraction at pI 4.6 that has a molecular weight of 33.7 kD (Figure 7.17). However, Luderer *et al* (2002) state that antibodies could not be raised against a synthetic peptide derived from Avr2 and therefore the molecular weight of Avr2 from a western blot could not be confirmed. It may be that using the isoelectric focusing method could allow the production of native Avr2 protein. This would allow more efficient binding studies. Additionally the protein resolved at 33.7 kD in this study is also resolved in fractions without activity. It is unlikely however that any protein can exist between pI 4.7 and pI 6.3, these may be separate proteins, so this may not hold true.

In the first purification method (section 7.15) a protein is resolved at pI 4.6 that is separate from other fractions in the apoplastic fluid and is high in its abundance. Similar proteins are also present on this gel at pI 4.5 but they are resolved in inactive fractions.

7.53 A method for Avr2 purification

The preferred method for successful purification of Avr2 from apoplastic fluid would be to run a rotofor cell between pI 4 and 5 approximately and separate fractions using gel electrophoresis designed to run between 25 and 40 kD. From this study perhaps some proteins would co-localise with Avr2 and make it difficult to discern the mature protein.

7.54 Lack of function of hydrogen peroxide assay

The simplest explanation for why hydrogen peroxide inducing activity could not be detected is that RCR3 is not present in the system, although an alternative explaination may be that not enough hydrogen peroxide is detectable by the assay system used. The basis of the assay possibly is sound and it would be useful to try a different detection system such as Luminol as used by Hammond-Kosak *et al* (1989).

7.55 How does Avr2 compare with other Avrs

Cladosporium fulvum and its interaction with tomato closely resemble the gene for gene model. The implication of a complementary gene interaction implies a complementary protein interaction. Although this seems to be true for both the Avr4 Cf-4 interaction (Joosten *et al* 1997) and the Avr9 Cf-9 interaction (Piedras *et al* 1998) it may be that the interaction between Cf-2 and *C. fulvum* is far more complex. Already implicated in the interaction is the plant gene RCR3, (Dixon *et al* 2002) a gene absolutely required for mediation of resistance through Cf-2.

7.54 Possible dual activity in the Avr 2:Cf-2 interaction

An explanation for the two points activity in the Avr2 : Cf-2 interaction shown in this study could be endogenous activity. Two main studies support this Peever and Higgins (1997) show that endogenous elicitation activity exists between the *C. fulvum* and Tomato interaction. RCR3 is completely required for absolutely required for mediation of resistance through Cf-2 (Dixon *et al* 2002). One hypothesis that encompasses all the known elements in the equation is that RCR3 itself can activate the Cf-2 gene, Avr2 therefore only is a factor in releasing RCR3 and perhaps interacts with RCR3 in such a way that induces a conformational

change in the protein which allows it to become delocalised and cause the elicitation effects mediated by Cf-2.

This would mean that one element of the equation is clearly RCR3 and one element is Avr2 with RCR3 in a bound or initiated state. This may explain some of the difficulties observed trying to separate activities between RCR3 and Avr2 and why activity is not recoverable. This however is supposition.

Further experiments involving RCR3 being injected with and without the presence of Avr2 would confirm RCR3 as an endogenous elicitor.

To understand the role of these elements more clearly, studies such as binding and X-ray crystallography could be undertaken to model these proteins structures and attempt to understand the nature of the confirmational changes that may play some role in the outcome of the interaction.

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